

1 **Ecotoxicological risk assessment of wastewater irrigation on soil microorganisms: fate**
2 **and impact of wastewater-borne micropollutants in lettuce-soil system**

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15 **Abstract**

16 The implementation of the new Water Reuse regulation in the European Union brings to the
17 forefront the need to evaluate the risks of using wastewater for crop irrigation. Here, a two-
18 tier ecotoxicological risk assessment was performed to evaluate the fate of wastewater-borne
19 micropollutants in soil and their ecotoxicological impact on plants and soil microorganisms.
20 To this end, two successive cultivation campaigns of lettuces were irrigated with wastewater
21 (at agronomical dose (not spiked) and spiked with a mixture of 14 pharmaceuticals at 10 and
22 100 µg/L each) in a controlled greenhouse experiment. Over the two cultivation campaigns,
23 an accumulation of PPCPs was observed in soil microcosms irrigated with wastewater spiked
24 with 100 µg/L of PPCPs with the highest concentrations detected for clarithromycin,
25 hydrochlorothiazide, citalopram, climbazole and carbamazepine. The abundance of bacterial
26 and fungal communities remained stable over the two cultivation campaigns and was not
27 affected by any of the irrigation regimes applied. Similarly, no changes were observed in the
28 abundance of ammonium oxidizing archaea (AOA) and bacteria (AOB), nor in clade A of
29 commamox no matter the cultivation campaign or the irrigation regime considered. Only a
30 slight increase was detected in clade B of commamox bacteria after the second cultivation
31 campaign. Sulfamethoxazole-resistant and -degrading bacteria were not impacted either. The
32 irrigation regimes had only a limited effect on the bacterial evenness. However, in response to
33 wastewater irrigation the structure of soil bacterial community significantly changed the
34 relative abundance of Acidobacteria, Chloroflexi, Verrucomicrobia, Beta-, Gamma- and
35 Deltaproteobacteria. Twenty-eight operational taxonomic units (OTUs) were identified as
36 responsible for the changes observed within the bacterial communities of soils irrigated with
37 wastewater or with water. Interestingly, the relative abundance of these OTUs was similar in
38 soils irrigated with either spiked or non-spiked irrigation solutions. This indicates that under

39 both agronomical and worst-case scenario the mixture of fourteen PPCPs had no effect on soil
40 bacterial community.

41 **Keywords:** microbial ecotoxicology, PhACs, PPCPs, antibiotic resistance, antibiotic
42 degradation, ammonium oxidation

43 **1. Introduction**

44 Water is a finite resource crucial for livestock and agricultural crop production. Agricultural
45 practices alone account for up to 70% of water withdrawals. The effects of global change and
46 water scarcity constitutes a major concern for the agricultural sector, especially in arid and
47 semiarid regions and countries with poor water management practices. Within this context,
48 the use of wastewater can overcome the shortage of freshwater resources for crop irrigation
49 (Garcia and Pargament, 2015; Petrie et al., 2015). Recently, the European Commission
50 launched the Water Reuse regulation that among others, defined the minimum quality
51 requirements of wastewater for crop irrigation (European Commission, 2020). Although the
52 environmental risks caused by water reuse in agriculture are on the top priority,
53 environmental fate of wastewater-borne biotic and chemical contaminants and their possible
54 ecotoxicological effects on soil living organisms and supported ecological functions are still
55 missing.

56 In spite of the important reduction of pollutants in the treatment plants, most of the
57 wastewater discharges remain contaminated with pharmaceutical active compounds (PhACs)
58 and personal care products (PPCPs). Irrigation of crops with wastewater therefore brings a
59 mixture of micropollutants to agricultural soils. In soils, the fate of these contaminants is
60 governed by a series of abiotic and biotic processes interacting together and varying according
61 to the physico-chemical properties of each pollutant and to the edaphoclimatic conditions
62 specific for each soil (Hiller and Šebesta, 2017). While sorption results in the stabilisation of

63 micropollutants in the soil where they persist and accumulate (Tolls, 2001; Xu et al., 2021),
64 abiotic and biotic transformations contribute to their dissipation (Grossberger et al., 2014;
65 Thiele-Bruhn, 2003; Thiele-Bruhn and Peters, 2007). Nevertheless, these transformation are
66 often partial and lead to transformation products that can be more toxic, persistent or
67 bioavailable than the parent compounds from which they originate (Celiz et al., 2009; Pérez
68 and Barceló, 2007). Micropollutants and their transformation products in the soil water can
69 further be transferred to connected water resources *via* leaching or runoff as well as to soil
70 living organisms including plants, macro- and mesofauna (Bigott et al., 2020; Carter et al.,
71 2020; Gallego et al., 2021). Uptake of residues of PhACs and PPCPs by plants can result in
72 the contamination of the food chain in a variety of ways with possible consequences on
73 human health (Aryal and Reinhold, 2011; Kalaji and Rastogi, 2017).

74 Microorganisms are able to degrade a wide range of organic micropollutants including PhACs
75 and PPCPs. In soils microbial degradation of the anti-inflammatory drugs naproxen (Topp et
76 al., 2008), ibuprofen (Girardi et al., 2013) and diclofenac (Facey et al., 2018), the analgesic
77 paracetamol (Li et al., 2014), the antiseptics triclosan and triclocarban (Al-Rajab et al., 2015),
78 and several antibiotics and antifungals (J. Chen et al., 2019; Sabourin et al., 2011) was
79 observed. Degrading microorganisms use PhACs and PPCPs as carbon source or energy to
80 fuel their growth (Moreira et al., 2014). In some cases, their degrading capacities are
81 improved in response to repeated exposure to micropollutants (Hirth et al., 2016). However,
82 other microorganisms can be affected in response to their exposure to these micropollutants.
83 Certain PhACs and PPCPs can be toxic to specific microbial guilds with great repercussion on
84 the accomplishment of key microbial functions with consequences on the numerous
85 ecosystem services supported by soil microorganisms (Cavicchioli et al., 2019; Delgado-
86 Baquerizo et al., 2020; Domeignoz-Horta et al., 2020; Wagg et al., 2014).

87 Despite the important role of soil microorganisms for soil health, studies assessing the
88 ecotoxicological effects of PhACs on soil microorganisms are scarce (Barra Caracciolo et al.,
89 2015; Gallego and Martin-Laurent, 2020). In addition, most of them are focused on the
90 estimation of the effects of single active compounds on the abundance of bacteria and fungi
91 with classical Pasteurian approaches and on enzyme activities (Butler et al., 2011; Cycon et
92 al., 2016; Cycoń et al., 2019; Park et al., 2013; Waller and Kookana, 2009). Only a few
93 studies measured the ecotoxicological effects of PhACs applied alone to agricultural soils
94 using the latest -omic methodologies available (Gallego et al., 2021; Thelusmond et al., 2019,
95 2018, 2016) and to our best knowledge, no studies assess the effects of PhAC mixtures on soil
96 microorganisms.

97 Within this context, this study aims to investigate the impact of crop irrigation with
98 wastewater contaminated with a complex mixture of PPCPs on soil microorganisms. A two-
99 tier experiment to test both agronomical (treated wastewater) and worst-case (treated
100 wastewater spiked with a complex mixture of PPCPs (at 10 µg/L and 100 µg/L each))
101 scenario of exposure was conducted in a greenhouse under controlled conditions over two
102 successive culture cycle of lettuces. The fate of the 14 different pollutants and the presence of
103 transformation products spiked to treated wastewater in soil was determined by liquid
104 chromatography-high resolution mass spectrometry (LC-HRMS). The ecotoxicological
105 effects of the irrigation with treated wastewater spiked or not with a mixture of PPCPs on soil
106 microorganisms were evaluated using an array of approaches including the estimation of the
107 abundances of the total fungal and bacterial communities, component of specific microbial
108 guilds involved in the ammonium oxidation and in the antibiotic resistance and degradation,
109 as well as the bacterial community composition and diversity assessed by 16S rRNA
110 amplicons sequencing.

111

112 **2. Materials and Methods**

113 **2.1 Chemicals**

114 Analytical standards (acesulfame, benzotriazole, carbamazepine, carbamazepine-10,11-
115 epoxide, ciprofloxacin, citalopram, clarithromycin, climbazole, diclofenac, 4'-
116 hydroxydiclofenac, hydrochlorothiazide, irbesartan, metoprolol, sucralose, sulfamethoxazole,
117 4-nitro-sulfamethoxazole, valsartan, valsartan acid were obtained from Sigma Aldrich (St.
118 Louis, MO, U.S.). Isotopically labelled standards for quantitation purposes (acesulfame-d4,
119 benzotriazole-d4, carbamazepine-d10, ciprofloxacin-d8, citalopram-d6, climbazole-d4,
120 diclofenac-13C6, hydrochlorothiazide-d2, irbesartan-d6, metoprolol-d7, sucralose-d6,
121 sulfamethoxazole-d4, valsartan acid-d4, and valsartan-d3) were purchased from Cerilliant
122 (Sigma Aldrich, St. Louis, MO, U.S.) and Toronto Research Chemicals (Toronto, ON,
123 Canada). ¹⁴C-phenyl ring-labelled sulfamethoxazole was purchased from IZOTOP (specific
124 activity 3.0 Mbq·mg). LC-MS grade solvents (acetone, acetonitrile (≥ 99.9%), methanol (≥
125 99.9%), dimethyl sulfoxide (≥ 99.9%), and HPLC water were purchased from Merck
126 (Darmstadt, Germany). All the above-mentioned reference standards were prepared
127 individually in 100% methanol, 100% dimethyl sulfoxide, 100% acetonitrile, or 100% HPLC
128 water according to compounds solubility and stored at -20°C. Their relevant physicochemical
129 properties are reported elsewhere (Montemurro et al., 2021). Commercially available Original
130 QuEChERS extraction salts kit (4 g MgSO₄ + 1 g NaCl) were obtained from BEKOlut GmbH
131 & Co. KG (Hauptstuhl, Germany). Disodium hydrogenphosphate dihydrate
132 (Na₂HPO₄·2H₂O), citric acid monohydrate and anhydrous ethylenediamine tetraacetic acid
133 (EDTA) (≥99%) for preparation of the EDTA-McIlvaine buffer (pH 4) (Montemurro et al.,
134 2021) were obtained from Sigma Aldrich (St. Louis, MO, U.S).

135 **2.2 Soil and wastewater collection and analysis**

136 The soil used in this study was collected from the experimental fields of IRSTEA at
137 Montpellier (Lavalette, France, 43.64682 N, 3.87418 E). Soil was sieved (4mm) and stored at
138 4°C until use. Secondary treated domestic wastewater was collected from the wastewater
139 lagoon at Murviel-les-Montpellier (Hérault, France, 43.605034 N, 3.757292 E) and stored at
140 4°C until use. The wastewater treatment plant operated on the basis of stabilization ponds with
141 three successive lagoons (13,680, 4,784 and 2,700 m³, respectively). It had a nominal capacity
142 of 1,500 Inhabitant Equivalent. The major physico-chemical characteristics of the soil and
143 wastewater are reported in Table 1.

144 **2. 3 Experimental design**

145 A greenhouse experiment consisting in two successive lettuce campaigns planted on the same
146 soil was performed. The experimental set up consisted in 30 soil pots of 3L containing 2 and
147 1.1 kg dwt of soil for the first and second campaign, respectively. Soil water holding capacity
148 was adjusted daily to 50% with deionized water and soil was pre-incubated for four weeks at
149 20°C. Lettuce seeds (*Lactuca sativa* var. Tizian) were germinated in peat soil and plantlets
150 were transferred to pots after 4 weeks (one lettuce per pot). Lettuce plants were then daily
151 irrigated for 7 weeks with the same volume (c.a. 30-80 mL per day) of six different solutions
152 (five replicates per treatment) and with deionized water to adjust the water holding capacity to
153 70%. Soil collected at the end of the first campaign was stored at 4°C for two weeks prior to
154 its use for the second campaign. In total 3 and 2.7 L of solutions were added to each pot for
155 the first and second campaign, respectively. The irrigation solutions were: deionized water
156 (water), deionized water spiked with a mixture of 14 compounds at 10 µg/L (water 10 µg/L),
157 deionized water spiked with a mixture of 14 compounds at 100 µg/L (water 100 µg/L),
158 wastewater (ww), wastewater spiked with a mixture of 14 compounds at 10 µg/L (ww 10
159 µg/L), wastewater spiked with a mixture of 14 compounds at 100 µg/L (ww 100 µg/L). The
160 mixture of compounds was prepared by combining 14 individual solutions of each of them

161 (acesulfame, benzotriazole, carbamazepine, ciprofloxacin, citalopram, clarithromycin,
162 climbazole, diclofenac, hydrochlorotiazide, irbesartan, metropolol, sucralose,
163 sulfamethoxazole and valsartan) dissolved in methanol, ethanol, acetonitrile or water at 10 or
164 100 µg/L final concentration. The selection of the compounds was done in the basis of their
165 frequency of detection and concentration in treated wastewater as well as to include a wide
166 range of compounds with variable physico-chemical properties (Montemurro et al., 2020b).
167 The same quantity of water-solvent mixture (with or without the mixture of compounds) was
168 added to all the irrigation solutions (0.2% v:v). For the second campaign, to overcome
169 nutrient deficiency symptoms, plants were irrigated four times (once per week) with 60 mL of
170 modified Hoagland ¼ solution (Hoagland and Arnon, 1938). This experiment was carried out
171 in a greenhouse under controlled conditions at 20°C (± 5°C) with a 16h photoperiod. Soil pots
172 were daily randomized. At the end of each campaign, lettuce plants and soil samples were
173 collected. Soil samples were stored either at 4°C or -20°C for further chemical and DNA
174 based analyses. Lettuce plants (separated in leaves and roots) were thoroughly washed and
175 weighed.

176 **2.4 Soil chemical analysis**

177 Soil nitrate and ammonium concentrations (NO_3^- and NH_4^+) present in soil were extracted
178 using 25 mL of potassium chloride (KCl) 1M that was added to ca. 5 g fresh soil, shaken
179 vigorously (125 rpm for 1 h at 20°C), filtered and kept frozen until quantification according to
180 ISO standard 14256-2 (ISO 14256-2, 2005). Quantification was performed using a blank in
181 each series by colorimetry in a BPC global 240 photometer (Axflow, Plaisir, France).

182 Soil sample extraction was performed according to Manasfi et al. (2021. in preparation).
183 Briefly, 10 g of air-dried soil sample were added to a 50-mL polypropylene centrifuge tube
184 and 3 mL of acetone were added followed by 50 µL of isotopically labeled compounds
185 mixture (2 µg/mL). The tubes were then vortexed for 2 min at 2500 rpm using a BenchMixer

186 XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US) and left overnight
187 under the hood at room temperature. Next, 8 mL of EDTA-McIlvaine buffer were added to
188 rehydrate the samples and then vortexed and left to stand for one hour prior to the extraction
189 step. When 80% hydration was achieved, 10 mL of acetonitrile was added to the sample and
190 vortexed again. To promote salting out, the QuEChERS original salt kit was emptied into the
191 extraction tube and the resulting mixture was immediately shaken by hand for one minute to
192 avoid salt agglomeration and then vortexed for another minute. Finally, the tube was
193 centrifuged for 10 min at 4000 rpm and 4 °C and 1 mL of the obtained supernatant was
194 evaporated under a gentle nitrogen stream at room temperature to total dryness. Lastly, the
195 samples were reconstituted with 1 mL of water/10% methanol solution and injected for LC-
196 MS/MS analysis. Analysis of pharmaceuticals in samples was based on the high-resolution
197 multiple reaction monitoring (MRMHR) acquisition performed by a SCIEX X500R QTOF
198 system (Sciex, Redwood City, CA, U.S.). All information about MRMHR transitions for each
199 analyte, its corresponding surrogate, optimized parameters, as well as any detailed
200 information regarding LC-MS/MS methodology are described elsewhere (Montemurro et al.,
201 2021, 2020a).

202 **2.5 Mineralization of ¹⁴C- sulfamethoxazole**

203 Sulfamethoxazole mineralization of ¹⁴C-phenyl-ring-labelled sulfamethoxazole was measured
204 by radiorespirometry. ¹⁴C-phenyl-ring-labelled sulfamethoxazole and unlabelled
205 sulfamethoxazole were mixed in water and added to 20 g dwt soil microcosms to a final
206 concentration of 10 mg·kg⁻¹ containing 120.7 bq·g⁻¹. The soil moisture content was adjusted
207 to 70% of the water holding capacity. All soil microcosms were placed in airtight jars
208 containing a plastic vial filled with water to maintain the humidity within the respirometer jar
209 (Soulas, 1993) and incubated at 20°C in the dark. ¹⁴CO₂ was trapped in 5 mL of 0.2 M sodium
210 hydroxide. At each sampling day, the NaOH trap was recovered, mixed with 10 mL

211 scintillation liquid (ACII scintillation fluid, Amersham) and analysed by liquid scintillation
212 counting (LS 6500 Multi-463 Purpose Scintillation Counter, Beckman Coulter, Brea, CA,
213 USA). Sulfamethoxazole mineralization parameters were determined as previously described
214 (Hussain et al., 2013).

215 At the end of the incubation period, ^{14}C mass balance analysis was performed by measuring
216 the amount of extractable (ER) and non-extractable (NER) radioactive residues in the soil
217 samples. To determine the ER fraction, 10 mL of methanol were added to each sample,
218 thoroughly mixed and placed on a rotary shaker at 150 rpm for 24 hours. After centrifugation
219 for 10 min at 6000 x g, the supernatant was recovered, and 5 mL aliquots were mixed with
220 scintillation liquid and measured for radioactivity by liquid scintillation counting. The
221 remaining soil was recovered and entirely dried at ambient temperature. The ^{14}C NER were
222 determined by combustion of 0.5 g of dried packing material under O_2 flow at 900°C for 4
223 min, using a Biological Oxidizer OX-500 (EG&G Instruments, France) as previously
224 described by El-Sebai et al. (2005). Mass balance of ^{14}C -residues was calculated as a
225 percentage of the total amount of ^{14}C -radioactivity retrieved from the different fractions
226 analysed (i.e. $^{14}\text{CO}_2$ residues, ER- and NER- ^{14}C -residues).

227

228 **2.6 Soil DNA extraction and quantification of microbial guilds**

229 Soil DNA was extracted using the DNeasy PowerSoil HTP 96 Kit (Qiagen, Germany). The
230 extracted DNA was quantified by using Quant-iT™ PicoGreen® dsDNA assay kit
231 (Invitrogen, France). Total bacterial and fungal communities were quantified using 16S rRNA
232 and fungal internal transcribed spacer (ITS) region primer-based qPCR assays with 341F
233 CCTACGGGAGGCAGCAG / 534R ATTACCGCGGCTGCTGGCA (López-Gutiérrez et al.,
234 2004; Muyzer et al., 1993) and ITS3 5'-GCATCGATGAAGAACGCAGC-3'/ITS4 5'-
235 TCCTCCGCTTATTGATATGC-3' primers (White et al., 1990), respectively. Bacterial and

236 archaeal ammonia-oxidizers (AOB and AOA, respectively) were quantified targeting *amoA*
237 gene using *amoA1F* GGGGTTTCTACTGGTGGT / *amoA2R*
238 CCCCTCKGSAAAGCCTTCTTC (Leininger et al., 2006) and A23F
239 ATGGTCTGGCTWAGACG / A616R GCCATCCATCTGTATGTCCA (Tourna et al., 2008)
240 primers, whereas quantification of clade A and clade B of commamox was performed
241 targeting *amoA* genes using C1F TAYAAYTGGGTSAAAYTA /C1R
242 ARATCATSGTGCTRTG and C2F TAYTTCTGGACRTTYTA /C2R
243 ARATCCARACDGTGTG primers (Pjevac et al., 2017). The abundance of
244 sulfamethoxazole-resistant bacterial communities was assessed targeting *sul1*, *sul2* and *sulA*
245 genes using *sul1-F* AAATGCTGCGAGTYGGMKCA / *sul1-R*
246 AACMACCAKCCTRCAGTCCG (Wei et al., 2018), *sul2-F*
247 TCCGGTGGAGGCCGGTATCTGG / *sul2-R* CGGGAATGCCATCTGCCTTGAG and
248 *sulA-F* TCTTGAGCAAGCACTCCAGCAG / *sulA-R* TCCAGCCTTAGCAACCACATGG
249 primers respectively (Pei et al., 2006; Wei et al., 2018) whereas the quantification of
250 sulfamethoxazole-degrading bacteria was performed targeting *sadA* gene using *sadA-F*
251 CCGGTACGGATGATGACTCT / *sadA-R* GGGACCATAGGCGTGAGATA primers (Billet
252 et al., 2021).

253 All qPCR assays were carried out in a ViiA7TM thermocycler (Life Technologies, Carlsbad,
254 CA, USA) in a 15- μ l final reaction volume containing 1x Takyon Low Rox SYBR masterMix
255 blue dTTP (Eurogentec, Seraing, Belgium), 1 μ M of each primer (Eurogentec, Seraing,
256 Belgium), 250 ng of T4 gene 32 (MP Biomedicals, Illkirch, France) and 0.5 ng of DNA. The
257 qPCR conditions were 95°C for 3 min for enzyme activation, followed by 40 cycles of
258 denaturation at 95°C for 15 sec, primer annealing at 60°C (for 16S rRNA, *sul1*, *sul2*, *sulA*,
259 *sadA*), 55°C (for ITS, *amoA* for AOA, and AOB) and 52°C (for *amoA* for clade A and B of
260 commamox) for 30 sec and extension at 72°C for 30 sec with data acquisition. After

261 amplification a melting curve stage was performed with 15 sec at 95°C, 1min at 68°C
262 followed by a temperature increase (+0.5°C/sec) to 95°C with data acquisition and 95°C for
263 15 sec. Five independent replicates and three no-template controls (NTC) were used for each
264 real-time PCR assay. Standard curves were obtained using serial dilutions of linearized
265 plasmids containing appropriated cloned targeted genes from bacterial strains or
266 environmental clones. The absence of qPCR inhibitors in DNA extracts was verified by
267 estimating the copy number of the plasmid pGEM-T Easy Vector (Promega, Madison, WI)
268 spiked in known amount with the soil DNA extracts as previously described (Henry et al.,
269 2006)

270 **2.7 Bacterial diversity and composition**

271 The diversity and composition of the bacterial community were determined from MiSeq
272 sequencing of 16S rRNA amplicons. Briefly, a two-step PCR approach targeting the 16S
273 rRNA gene sequence was used. First step PCR was performed using the universal bacterial
274 primers U341_F - 805_R with overhang adapters (forward adapter: TCGTCGGCAGCGTC
275 AGATGTGTATAAGAGACAG, reverse adapter:
276 GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAG). The resulting amplicons were
277 used as template in a second PCR carried out with multiplexed primers containing the
278 universal overhang adaptors and specific barcodes. The first step PCR contained 2 ng of DNA
279 as template, 7.5 µL of 2X Phusion High Fidelity PCR MasterMix (Thermo Scientific,
280 Waltham, MA, USA), 250 ng of T4 gene 32 protein (MP Biomedicals, Santa Ana, CA, USA),
281 0.375 µL of each primer (10 µM) and ultrapure sterile water to a total volume of 15 µL.
282 Thermal conditions were 3 min at 98°C, 25 cycles at 98 C for 30 sec, 55°C for 30 sec and
283 72°C for 30 sec followed by a final extension of 10 min at 72°C. Duplicates of each PCR
284 reaction were pooled and then a 6 µL aliquot was used as template to carry out eight further
285 amplification cycles with the barcoded primers containing the adapters. The second-step PCR

286 was performed using a 384 Nextera XT index kit (Illumina, San Diego, CA, USA) for the
287 addition of multiplexing index-sequences. It was carried out in 30 μ L reaction volumes
288 containing 2.5 μ L sterile water, 15 μ L 2X Phusion HF master mix (Thermo Scientific,
289 Waltham, MA, USA), 250 ng of T4 gp32 (MP Biomedicals, Santa Ana, CA, USA), 3 μ L of
290 each primer (10 μ M) and 6 μ L of the step-one PCR product. The thermal cycling was 98°C
291 for 3 min, followed by eight cycles of 98°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec,
292 with a final extension of 72°C for 10 min. The size of the amplicons was verified by
293 electrophoresis on a 2% agarose gel. PCR products were purified (amplicon library
294 purification, PicoGreen® quantification and pooling) and sequenced (Illumina MiSeq 2
295 x300bp) by Microsynth (Balgach, Switzerland). PCR products were normalized
296 (SequalPrep™ kit), purified (Pippin prep) and sequenced by Microsynth (Switzerland). The
297 sequence data was analyzed using an in-house developed Phyton notebook pipeline and
298 different bioinformatics tools. 16S rRNA sequences were assembled using PEAR software (J.
299 Zhang et al., 2014) with default settings. Further quality checks were conducted using the
300 QIIME 1 pipeline (Caporaso et al., 2010a). Sequences shorter than 380 bp were removed.
301 Reference-based and *de novo* chimera detection, as well as clustering into operational
302 taxonomic units (OTUs) was performed with VSEARCH software (Rognes et al., 2016) using
303 appropriate reference databases (Greengenes' representative set of sequences for 16S rRNA)
304 with a threshold set at 94% identity. Representative sequences for each OTU were aligned
305 using PyNAST (Caporaso et al., 2010b). Phylogenetic trees were constructed using FastTree
306 (Price et al., 2009). Taxonomy was assigned using UCLUST (Edgar, 2010) and the latest
307 released Greengenes database (v.05/2013, McDonald et al., 2012) for 16S rRNA sequences.
308 Sequences were deposited in the GenBank to the sequence read archive (SRA) under the
309 accession number PRJNA630861: SRR12832230-12832289. A range of bacterial α -diversity
310 indices pertaining to richness (Chao1), evenness (Simpson reciprocal) and relatedness (PD

311 whole tree) were calculated based on rarefied tables (14,000 sequences per sample) (Suppl.
312 Fig. S1). In total, after de-multiplexing and removal of low-quality raw sequence reads,
313 amplicon sequencing generated 1,843,505 high quality sequences with an average sequence
314 length of 471.3 ± 1.1 bp. Using a threshold at 94% nucleotide sequence identity, these
315 sequences were grouped in 6,058 different OTUs (Suppl. Table S1). UniFrac distance
316 matrices were also computed to detect changes in the composition of microbial communities.
317 Principal Coordinate Analysis (PCoA) of OTUs on weighted and unweighted Unifrac distance
318 matrices was also performed and plotted. The relative abundance of the different bacterial
319 phyla was also determined using a comparative bar chart.

320 **2.8 Statistical analyses**

321 All statistical analyses were performed in R (<http://www.r-project.org>). The normality of the
322 data and residuals was checked (Shapiro Wilk's test with $p > 0.05$) and the homogeneity of
323 variances was verified (Levene's test with $p > 0.05$). Root square, arcsin and log-
324 transformations of the data were performed when necessary to fulfil the normality and the
325 homogeneity of variances of the dataset. For parametric distributions, ANOVA followed by
326 Tukey's test and t-student were used to determine differences. For non-parametric
327 distributions, data was compared using Kruskal Wallis test followed by pairwise comparisons
328 with Bonferroni correction. Permutational Multivariate Analysis of variance (Permanova) was
329 performed on UniFrac distance matrices using Adonis function from R package "vegan"
330 (Oksanen et al., 2018), while pairwise post-hoc comparisons were detected using "mctoolsr"
331 package (Legendre and Anderson, 1999)

332 Significant differences in OTU abundance between the different treatments were detected
333 using the function "mvabund" from R package. Relative abundance of selected OTUs were
334 then visualized using a heatmap.

335 **3. Results**

336 **3.1 Effects of watering the plant with water and wastewater spiked with fourteen PCPPs** 337 **on plant biomass**

338 At the end of the two campaigns, the biomass of the plants was measured. No matter the
339 irrigation solution applied, the total fresh weight of lettuce plants collected at the end of the
340 second campaign was significantly lower (average weight 44.2 ± 20.0 g) than those of the first
341 campaign (average weight 99.1 ± 30.1 g) ($p < 0.01$) (Suppl. Table S2). In addition, at the end
342 of the second campaign, one can observe that plants irrigated with wastewater (spiked or not)
343 presented significantly higher biomass than those irrigated with water ($p = 0.00000$) (Fig. 1).
344 Concomitantly, a significant NO_3^- depletion ($p < 0.00002$) was observed in the soil no matter
345 the solutions used to water the plants as well as a significant increase in NH_4^+ in soils irrigated
346 with wastewater ($p < 0.02$) (Suppl. Fig. S2). For the two campaigns of production, fresh weight
347 of lettuces obtained in soil irrigated with the solution (either water or wastewater) spiked with
348 PPCPs was not significantly different from their respective water and wastewater control.

349

350 **3.2 Fate of wastewater-borne chemicals in soil**

351 The fourteen compounds and their main transformation products were tracked using LC-
352 HR/MS analyses carried out on QuEChERS extracts of soils irrigated either with $100 \mu\text{g/L}$
353 spiked water or $100 \mu\text{g/L}$ spiked wastewater after two consecutive campaigns. All the spiked
354 compounds were detected in soil extracts (Fig. 2).

355 The highest mean concentrations observed in the different samples analyzed were obtained
356 for clarithromycin (from 219 ± 49 to 357 ± 33 ng/g) and hydrochlorothiazide (from 27 ± 5 to
357 33 ± 2 ng/g) followed by citalopram, climbazole, carbamazepine (from 3 ± 0.3 to 13 ± 1.3
358 ng/g), irbesartan, ciprofloxacin, benzotriazole and sucralose (ranging from 0.2 ± 0.5 to 2 ± 0.2

359 ng/g). The lowest concentrations were obtained for acesulfame, sulfamethoxazole,
360 metoprolol, diclofenac, and valsartan with values ranging from 0.3 ± 0.2 ng/g to below limit
361 of quantification (BLOQ). Carbamazepine epoxide, valsartan acid and 4-
362 nitrosulfamethoxazole, the transformation products of carbamazepine, valsartan and
363 sulfamethoxazole respectively, were detected at low concentrations (from BLOQ to 2.3 ± 0.4
364 ng/g). 4'-hydroxydiclofenac, the transformation product of diclofenac, was not detected. The
365 concentration of spiked compounds and main transformation products was significantly
366 higher in extracts of soil irrigated with both water and wastewater collected at the end of the
367 second campaign than in those collected at the end of the first campaign. However, this trend
368 was not observed for sucralose, which was detected at BLOQ concentrations at the end of the
369 second campaign and for acesulfame, which at the end of the second campaign, slightly
370 decreased in soils irrigated with spiked water and was hardly detected (at BLOQ) in soils
371 irrigated with spiked wastewater. Climbazole ($p < 0.007$), carbamazepine ($p = 0.002$), and
372 irbesartan ($p = 0.0003$) were detected in significantly higher amount in extracts of soils
373 irrigated with wastewater than in those irrigated with water. Contrarily, sucralose, acesulfame
374 (for the first campaign), clarithromycin, hydrochlorothiazide, ciprofloxacin, benzotriazole and
375 sulfamethoxazole (for the second campaign) were detected in slightly lower amount in
376 extracts of soil irrigated with wastewater than in those irrigated with water, but this observed
377 trend was not significant ($p > 0.7$).

378

379 **3.3. Effects of watering the plant with water and wastewater contaminated with PCPPs** 380 **on soil microorganisms**

381 **3.3.1 Effects on the abundance of total bacteria and fungi, ammonia oxidizers, antibiotic** 382 **degradation and resistance.**

383 The effect of watering the plant with water or wastewater spiked or not with PCPPs on the
384 abundance of soil microorganisms was assessed through qPCR assays. The total bacterial
385 community ranged from $1.1 \times 10^5 \pm 1.3 \times 10^4$ to $1.3 \times 10^5 \pm 3.0 \times 10^4$ 16S rRNA sequences
386 per ng of soil DNA. The fungal community ranged from $4.95 \times 10^3 \pm 7.93 \times 10^2$ to 9.20×10^3
387 $\pm 2.52 \times 10^3$ ITS sequences per ng of soil DNA (Suppl. Table S3). The abundances of total
388 fungal and bacterial communities were neither affected by the campaign production nor by the
389 irrigation regimes used to grow the lettuces ($p > 0.07$). The abundance of bacteria involved in
390 the nitrogen cycle was also quantified by qPCR targeting *amoA*. Neither the campaign nor the
391 irrigation regimes changed the proportion of bacterial and archaeal-ammonia oxidizers nor of
392 clade A of commamox bacteria (Suppl. Table S4 and Suppl. Table S5). One can observe that
393 the abundance of the clade B of commamox bacteria increased after the second cultivation
394 campaign no-matter the irrigation regimes considered with significant differences in soil
395 samples irrigated with water and 10 $\mu\text{g/L}$ spiked water as compared to the respective control
396 ($p < 0.007$) (Suppl. Table S5). The proportion of sulfamethoxazole-resistant bacteria, estimated
397 as *sul1*, *sul2* or *sulA* sequence per $10^2/10^4$ 16S rRNA, was similar in all soils no matter the
398 irrigation regime applied and slightly increased but not significantly during the second
399 cultivation campaign (Suppl. Table S6). For both campaigns, no sulfamethoxazole-degrading
400 bacteria could be detected using *sadA* qPCR assay in any of the treatments applied to the soil.
401 Nonetheless, radiorespirometric analysis showed that within 100 days of incubation, the
402 indigenous soil microbial community was able to mineralize in mean $9.2 \pm 0.9\%$ and $8.8 \pm$
403 0.9% of ^{14}C -sulfamethoxazole to $^{14}\text{CO}_2$ for the first and second cultivation campaign,
404 respectively (Suppl. Fig. S3). Modelling the ^{14}C -sulfamethoxazole mineralization kinetics
405 revealed a lag phase (λ) of 12.2 ± 1.4 and $12.6 \pm 1.4\%$ days with a maximum %
406 mineralization (A) of 8.54 ± 0.9 and $7.9 \pm 0.8\%$ ($p = 0.0000000$) and a mineralization rate (μm)
407 of 0.2 ± 0.03 and $0.2 \pm 0.02\%$ day $^{-1}$. Mass balance analysis at the end of the incubation

408 showed that $64.0 \pm 7.6\%$ and $62.01 \pm 3.6\%$ of the initial radioactivity was recovered as $^{14}\text{CO}_2$
409 ($9.2 \pm 0.9\%$ and $8.8 \pm 0.9\%$), ER (0.9 ± 0.2 and $0.9 \pm 0.1\%$) and NER (54.0 ± 7.4 and $52.3 \pm$
410 4.04%) for the first and second cultivation campaign, respectively (Suppl. Table S7).

411

412 **3.3.2 Effects on soil bacterial diversity and composition**

413 The impact of irrigation regime on the diversity and composition of the soil bacterial
414 community was assessed by MiSeq sequencing of 16S rRNA gene. For each irrigation
415 regime, a range of α -diversity indices pertaining richness (Chao1), relatedness (PD whole
416 tree) and evenness (Simpson reciprocal) were calculated using rarefied data (Suppl. Table
417 S8). Statistical analysis showed that none of the irrigation regimes nor the production
418 campaign had an effect on Chao1 and PD whole tree. However, at the end of the second
419 campaign, Simpson reciprocal significantly decreased in soils irrigated with wastewater
420 spiked with $100 \mu\text{g/L}$ of PPCPs ($p=0.04$). β -diversity analyses based on weighted and
421 unweighted Unifrac distance matrices were plotted using PCoA and showed a relatively good
422 reproducibility between replicates from same irrigation regime, which grouped together (Fig.
423 3 and Suppl. Fig. S4). Samples of the first campaign were clearly separated from those of the
424 second campaign along the second axis accounting for 11.3 and 3.3 % of the variance
425 explained in weighted and unweighted Unifrac matrices-based ordinations. Adonis analysis
426 confirmed these observations for both weighted and unweighted PCoA ordinations
427 ($p=0.0001$) (Suppl. Table S9 and Suppl. Table S10). For the first campaign, the soil bacterial
428 community composition from soil irrigated with water clustered together with that of soil
429 irrigated with spiked water ($p>0.139$) and both were clearly separated from those found in
430 soils irrigated with wastewater spiked or not with PPCPs (Fig. 3 and Suppl. Table S9). For the

431 second campaign, a similar trend was observed (Fig. S4) although Permanova analysis
432 showed significant differences between the different irrigation regimes (Suppl. Table S10).

433 Taxonomic analysis led to the identification of 7 major phyla: Proteobacteria, Actinobacteria,
434 Bacteroidetes, Firmicutes, Chloroflexi and Verrucomicrobia (Suppl. Fig. S5). Overall, the
435 relative abundance of major phyla remained constant no-matter the irrigation regime and the
436 production campaign considered. However, for the first campaign, the relative abundance of
437 Verrucomicrobia increased in soils irrigated with 10 $\mu\text{g/L}$ spiked water ($p=0.03$), 10 $\mu\text{g/L}$
438 ($p=0.0002$) and 100 $\mu\text{g/L}$ spiked wastewater ($p=0.003$) as compared to the control irrigated
439 with water. Similarly, the relative abundance of Chloroflexi significantly increased in soils
440 irrigated with 10 $\mu\text{g/L}$ spiked wastewater ($p=0.0003$). For the second campaign, as compared
441 to their respective control, the relative abundances of Chloroflexi and Betaproteobacteria
442 significantly increased in soil irrigated with wastewater ($p=0.02$) and with 100 $\mu\text{g/L}$ spiked
443 wastewater ($p=0.007$), respectively. On the contrary, as compared to control soil samples, the
444 relative abundances of Acidobacteria, Gamma- and Deltaproteobacteria significantly
445 decreased in soil irrigated with 100 $\mu\text{g/L}$ spiked water and wastewater (spiked or not)
446 ($p<0.018$), 100 $\mu\text{g/L}$ spiked water ($p=0.026$) and 100 $\mu\text{g/L}$ spiked wastewater ($p=0.0016$),
447 respectively.

448 To further evaluate the effect of wastewater irrigation on a lower taxonomic level, OTUs
449 represented by at least one sequence per sample and in half of the samples (a total of 2,917
450 OTUs) were analyzed using the mvabund R package. A total of 28 OTUs ($p<0.05$) were
451 selected as responsible for the shifts observed between irrigation regimes (Fig. 4). The
452 majority of these discriminant OTUs were affiliated to the Betaproteobacteria class (a total of
453 21). Two discriminant OTUs affiliated to Deltaproteobacteria (Sorangium and Myxococcales)
454 and the rest affiliated to Chloroflexi (Herpetosiphonales), Gammaproteobacteria
455 (*Pseudomonas*), Actinobacteria (Aeromicrobium), Cytophagia (*Algoriphagus terrigen*) and

456 Alphaproteobacteria (*Rickettsiaceae*). With the exception of three OTUs (two OTUs belonging
457 to the Betaproteobacteria class and one OTU closely related to the Herpetosiphonales), whose
458 relative abundances were lower in wastewater irrigated soils samples than in the control
459 irrigated with water, the majority of the discriminant OTUs were highly represented in soils
460 irrigated with wastewater. Overall, their relative abundances remained constant along the two
461 cultivation campaigns.

462 **4. Discussion**

463 The advantages of treated wastewater reuse in agriculture have been widely reported (Meli et
464 al., 2002; Mohammad Rusan et al., 2007). Nutrients brought by wastewater effluents can
465 reduce the need for supplementary mineral compounds (such as N, P, and K) and increase the
466 concentration of some elements (Ca, B, Fe, Cu, Zn, Mn and Mb), essential for the growth and
467 development of crops (Gatta et al., 2016; Urbano et al., 2017; Vivaldi et al., 2019). In our
468 study, the fresh weight of lettuces irrigated with treated domestic wastewater (spiked with
469 PPCPs or not) was significantly higher than that of lettuces irrigated with clean water (spiked
470 with PPCPs or not) at the end of the second campaign. The high amounts of N-NH₄⁺ (29
471 mg/L) and phosphorous (8 mg/L) supplied by the wastewater could explain the observed
472 difference.

473 Although the supply of mineral nutrients brought by wastewater is valuable to crops,
474 continuous and/or repeated irrigation of crops with wastewater may lead to the accumulation
475 of wastewater-borne micropollutants into the soil. Numerous studies have addressed this
476 environmental key question by monitoring the fate of micro-pollutants in crops irrigated with
477 wastewater. However only a very few have assessed the ecotoxicological effects of PhACs on
478 soil-living organisms, such as microorganisms (Barra Caracciolo et al., 2015; Gallego and
479 Martin-Laurent, 2020). These are generally experiments performed under controlled or
480 laboratory conditions within a limited period of time, considering the effect of contaminants

481 one by one and only a few of them report the fate and the effect of complex mixtures of
482 contaminants in the natural environment or field conditions (Manasfi et al., 2020). In an
483 attempt to evaluate under a worst-case scenario, the fate and ecotoxicological effects of the
484 most relevant wastewater-borne organic contaminants in soil, the accumulation of fourteen
485 chemicals and major transformation products was assessed over two successive lettuce
486 campaigns planted on the same soil in a controlled greenhouse experiment. Water and treated
487 wastewater spiked with a mixture of PPCPs (at 100 µg/L for each compound) were used to
488 mimic the worst-case scenario defined by repeated irrigation of crops with wastewater.
489 Although wastewater generally results in soil alkalisation, the soil pH remained stable
490 during the two campaigns of productions no matter the solution used for watering of the
491 lettuces, (data not shown). This is worth noticing since changes in soil pH have been reported
492 to modify the sorption of several pharmaceuticals active compounds, their bioavailability and
493 consequently their ultimate distribution into the different environmental compartments
494 (Brienza et al., 2020; Hiller and Šebesta, 2017; Y. L. Zhang et al., 2014).

495 Residues of all 14 spiked compounds were detected in soil samples collected after the first
496 and/or second cultivation campaign. The antibiotic clarithromycin, diuretic
497 hydrochlorothiazide, antidepressant citalopram, antifungal clotrimazole and antiepileptic
498 carbamazepine were detected at the highest concentrations ranging from few ng up to several
499 hundred ng per g of soil. These observations are in line with Manasfi et al. (2020), who
500 similarly found the highest concentration for these compounds in a parallel study conducted
501 under realistic agronomical conditions using the same soil and treated wastewater fortified at
502 10 µg/L over five cultivation campaigns. Their relatively high K_d values (Klement et al.,
503 2018; Manasfi et al., 2020; Sibley and Pedersen, 2008) and recalcitrance (Chen et al., 2013;
504 Li et al., 2013; Styriřhave et al., 2011) could account for the observed results. The occurrence
505 of clarithromycin (Corada-Fernández et al., 2015; Dalkmann et al., 2012),

506 hydrochlorothiazide and carbamazepine (Biel-Maeso et al., 2018; Corada-Fernández et al.,
507 2015; Dalkmann et al., 2012; Gibson et al., 2010; Grossberger et al., 2014; Kinney et al.,
508 2006) has been previously documented in soils irrigated with wastewater. However, contrary
509 to our findings, clarithromycin was detected at very low concentrations ($<3\text{ng/g}$), probably as
510 consequence of its enhanced microbial dissipation after years of exposure as reported by Topp
511 et al., 2016. Interestingly, carbamazepine epoxide, a degradation intermediate of
512 carbamazepine was also detected at few ng per g of soil, which evidences the presence of
513 carbamazepine degrading microorganisms in the soil (Golan-Rozen et al., 2015; Kittelmann et
514 al., 1993; Li et al., 2013).

515 A few ng per g of soil of irbesartan, ciprofloxacin and benzotriazole were detected, which is
516 consistent with observations made by Manasfi et al (2020). The angiotensin receptor blocker
517 irbesartan, poorly removed in wastewater treatment plant (Bayer et al., 2014), showed a
518 relatively low sorption to soil (Klement et al., 2018) but is known to be degraded by various
519 soil microorganisms (Frková et al., 2020), which could explain the low concentrations found
520 in our study. The antibiotic ciprofloxacin and the corrosion inhibitor benzotriazole are hardly
521 biodegradable (Girardi et al., 2011; Liu et al., 2011; Walters et al., 2010) but they are known
522 to photodegrade under UV irradiation and simulated solar radiation respectively (Batchu et
523 al., 2014; Hem et al., 2003; Serdechnova et al., 2014), and to be assimilated by plants (Castro
524 et al., 2003; Eggen et al., 2011; LeFevre et al., 2015; Riemenschneider et al., 2016; Sabourin
525 et al., 2012; Zhao et al., 2018), which might explain why they were detected in our
526 experiment at low concentrations. The food additive sucralose was also detected at few ng per
527 g of soil in microcosms irrigated with both spiked water and wastewater, presumably due to
528 its limited biodegradation (Biel-Maeso et al., 2019; Buerge et al., 2011). However, it was
529 hardly detected after the first cultivation campaign, probably because of its high water
530 solubility, lack of sorption to soil ($K_d < 10$) or even plant uptake (Manasfi et al., 2020).

531 Concentrations lower than 1 ng per g of soil were detected for the artificial sweetener
532 acesulfame, the antibiotic sulfamethoxazole and the anti-inflammatory diclofenac. This
533 observation is in agreement with their high mobility (Belton et al., 2020; Drillia et al., 2005;
534 Lin and Gan, 2011; Storck et al., 2016) and fast microbial degradation in soils (Al-Rajab et
535 al., 2010; Buerge et al., 2011; Reis et al., 2014). Additionally, recent studies have shown that
536 they can be transferred from soil to plants (Bartha et al., 2014; González García et al., 2018;
537 Kodešová et al., 2019; Manasfi et al., 2020; Zhang et al., 2017). Diclofenac was only detected
538 in soil microcosms irrigated with spiked wastewater. Given that 4'-hydroxydiclofenac, a
539 biotransformation product from diclofenac (Prior et al., 2010) was never detected, we could
540 hypothesize that the degradation of the diclofenac molecule was complete. Interestingly,
541 mineralization kinetics of ¹⁴C-sulfamethoxazole recorded in soil samples collected at the end
542 of each campaign of production showed that less than 10% of ¹⁴C-sulfamethoxazole initially
543 applied was mineralized by an indigenous soil microbial community and that more than half
544 of the radioactivity was recovered as NER. Additionally, 4-nitro-sulfamethoxazole, a photo-
545 transformation product of sulfamethoxazole (Su et al., 2016) was only detected at BLOQ
546 concentrations, which led us to conclude that contrary to previous studies this compound was
547 hardly transformed and remained adsorbed to soil particles.

548 The anti-hypertensive metoprolol was also found at very low concentrations. Manasfi et al.
549 (2020) using chiral analysis showed that enantiomer profiles did not changed during the time
550 course of the five lettuce growing campaigns as compared to the standard, suggesting that
551 microbial biodegradation did not occur. Given its moderate photostability (Neamțu et al.,
552 2014) and its variable sorption behavior (Kodešová et al., 2015), further studies are needed to
553 elucidate the processes governing its dissipation in soil.

554 While Manasfi et al. (2020) detected the antihypertensive valsartan at concentrations around 2
555 ng per g of soil, in our study the antihypertensive valsartan was always detected in soil

556 microcosms at BLOQ concentrations no matter the production campaign considered.
557 Valsartan acid, its major transformation product was detected at few ng per g of soil in
558 microcosms irrigated with spiked treated wastewater only after the second cultivation
559 campaign. This is in accordance with Gallego et al. (2021) suggesting that valsartan
560 dissipation is accompanied by the formation of valsartan acid.

561 As a general trend, a significant carryover of contaminants was observed over the two
562 cultivation campaigns. The accumulation and persistence of PhACs in soils has been
563 previously reported (Chen et al., 2013; Dalkmann et al., 2012; Williams and McLain, 2012)
564 because of the associated human and environmental risks (Aryal and Reinhold, 2011; Fatta-
565 Kassinos et al., 2011; Pérez et al., 2020). Similar concentrations of spiked PCPPs and
566 metabolites were observed in soil microcosms irrigated with spiked wastewater or water
567 irrigated. For climbazole, carbamazepine and irbesartan significantly higher values were
568 observed in spiked wastewater-irrigated than in spiked water-irrigated microcosms. This
569 difference might be explained by the interaction of these compounds with different
570 components brought by wastewater influencing their fate in soil (Goldstein et al., 2018;
571 Katsoyiannis and Samara, 2007; Müller et al., 2007; Nason et al., 2019). It might also be
572 explained by an inhibitory or toxic effect of other PPCPs brought by wastewater, in addition
573 to the 14 micropollutants spiked, on specific microbial guilds involved in their degradation,
574 which might hinder their elimination and increase their persistence in soil. This hypothesis is
575 supported by the fact that several antibiotics (Cycoń et al., 2019), the non-steroidal anti-
576 inflammatory drugs naproxen and diclofenac (Cycon et al., 2016) and the antiseptic triclosan
577 (Butler et al., 2011; Waller and Kookana, 2009) have previously been shown to induce
578 changes in the abundance and enzymatic activities of soil microorganisms.

579 The ecotoxicological effects of wastewater irrigation on the abundance and the composition
580 of soil microbial communities were assessed. The abundance of bacterial and fungal

581 communities remained stable no-matter the irrigation regime and cultivation campaign
582 considered. This trend was also observed for the abundance of microbial guilds (AOA, AOB
583 and clade A commamox) involved in ammonia oxidation processes. Only the abundance of
584 the clade B commamox bacteria was slightly increased in water and 10 µg/L spiked water
585 irrigated microcosms after the second cultivation campaign. Altogether one can conclude that
586 none of the soil microbial communities targeted in our study were affected in their abundance
587 in response to the nutrients and PhACs brought by spiked wastewater or to the PhACs
588 brought by spiked water used to irrigate the microcosms planted with lettuces. These
589 observations are in agreement with previous studies showing that below 5 µg per g various
590 pharmaceutical and pollutant applied had no effect on the abundance and activities of N-
591 cycling microbial communities (Crouzet et al., 2016; Rosendahl et al., 2012; Wang and
592 Gunsch, 2011). Given these results, it could be hypothesized that none of the irrigation
593 regimes had an effect on the total respiration and nitrification processes. To further test this
594 hypothesis normalized OECD and ISO tests (such as luminescent bacteria tests ISO 11348-
595 3:2007, respiration and nitrification inhibition tests OECD 209 and ISO 9509:2006,
596 respectively) might be applied.

597 Similarly, the abundance of sulfamethoxazole-resistant bacteria remained stable no matter the
598 irrigation regime, probably because this antibiotic was dissipated in soil. Only 9% of ¹⁴C-
599 sulfamethoxazole was mineralized over the incubation period, which is consistent with the
600 fact that *sadA* carrying microorganisms involved in the biodegradation of sulfamethoxazole
601 were not detected. These results indicate that contrary to previous studies (Topp et al., 2016,
602 2013), an enhanced biodegradation of sulfamethoxazole did not occur despite the repeated
603 exposure to this antibiotic. The simultaneous application of a complex mixture of PhACs
604 might have caused a detrimental effect on both sulfamethoxazole-resistant bacteria and

605 sulfamethoxazole-degrading microorganisms, hindering their growth in response to repeated
606 exposure to sulfamethoxazole.

607 α - diversity analyses showed that none of the irrigation regimes had a significant effect on the
608 richness and phylogenetic diversity of the bacterial community. Only a limited effect for soils
609 irrigated with 100 $\mu\text{g/L}$ spiked wastewater on the evenness of the bacterial community was
610 observed. Our findings are in line with other studies (Broszat et al., 2014; Frenk et al., 2014;
611 Ibekwe et al., 2018) but partly in accordance with previous studies performed in agricultural
612 soils receiving swine and dairy manures (Z. Chen et al., 2019) or soil microcosms treated with
613 valsartan, carbamazepine, or tetracycline (Gallego et al., 2021; Thelusmond et al., 2016;
614 Zheng et al., 2020). This discrepancy might be explained by the high doses applied in those
615 studies and shorter incubation times which combined led to the observation of a marked
616 ecotoxicological impact on both diversity and composition of soil bacterial communities.
617 However, the irrigation with wastewater significantly modified the structure of soil bacterial
618 communities, which resulted in changes in its composition. In-depth analysis of the OTUs
619 responsible for the significant differences found between the six irrigation regimes led to the
620 identification of 28 OTUs affiliated to Betaproteobacteria (21), Deltaproteobacteria (2),
621 Chloroflexi (1), Gammaproteobacteria (1), Actinobacteria (1), Cytophagia (1) and
622 Alphaproteobacteria (1). With the exception of two OTUs belonging to Betaproteobacteria
623 and one OTU closely related to Herpetosiphonales, the relative abundance of the rest of them
624 significantly increased in the presence of wastewater, suggesting that those OTUs may benefit
625 from it by using it as carbon source or energy for their growth. Interestingly, similar OTU
626 profiles were found in soils irrigated with either spiked or non-spiked irrigation regimes
627 indicating that in our experimental conditions, the PPCPs had no effect on soil bacterial
628 community.

629 **5. Conclusions**

630 Lab-to-field tiered exposure scenarios are recommended to accurately assess the fate and the
631 assessment of environmental risks for in soil living organisms within the pesticide
632 authorization processes of the active ingredient. This approach was applied in this study to
633 assess the fate and ecotoxicological effects of a mixture of PPCPs frequently found in treated
634 wastewater. This is the first report on the evaluation of the ecotoxicological effects on soil
635 microorganisms of a complex mixture of PPCPs brought by wastewater used to irrigate
636 lettuces. Our results showed that under the worst-case scenario of exposure (irrigation with
637 water or wastewater spiked with a mixture of 14 PPCPs at 100 µg/L each) several PPCPs
638 accumulated in lettuce planted soil. Irrigation of lettuces with wastewater (spiked or not) had
639 limited or no effects on the abundance, diversity and functions of soil microbial populations,
640 but induced significant changes in the structure of the soil bacterial community. PPCPs
641 accumulated in the lettuce planted soil had no effect on soil microorganisms. However, they
642 may transfer from the soil to the plant and enter the food chain.

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650

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Tables & Figures

Ecotoxicological risk assessment of wastewater irrigation on soil microorganisms: fate and impact of wastewater-borne micropollutants in lettuce-soil system

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Table 1. Physico-chemical characteristics of the soil of Lavalette (IRSTEA, Montpellier, France) and treated wastewater of Murviel-les-Montpellier (France) used in this study

| Parameter | Wastewater | Parameter | Soil |
|---|-------------------|---|-------------|
| pH | 7.1 | pH | 8.2 |
| Conductivity ($\mu\text{S}/\text{cm}$) | 1053 | sand/silt/clay (%) | 45/40/15 |
| TSS (mg/L) | 58 | | (loam) |
| VSS (mg/L) | 19.3 | SOM (%) | 3.68 |
| COD (mg O ₂ /L) | 200 | CEC (meq/Kg) | 139 |
| BOD (mg O ₂ /L) | 21 | | |
| TOC (mg/L) | 56.8 | TOC (%) | 2.13 |
| N Kjeldahl (mg/L) | 21 | TN (%) | 0.201 |
| N-NH₄⁺ (mg/L) | 29 | N-NH₄⁺ (mg/Kg) | 4.1 |
| N-NO₃⁻ (mg/L) | <0.22 | N-NO₃⁻ (mg/Kg) | 8.6 |
| Total P (mg/L) | 8.12 | Total P (Kg/L) | 0.57 |
| Soluble P (mg/L) | 7.19 | P₂O₅ (Kg/L) | 0.023 |

TSS: total suspended solids; VSS: volatile suspended solids; COD: chemical oxygen demand; BOD: biochemical oxygen demand; TOC: total organic carbon; SOM: soil organic matter; TN: total nitrogen

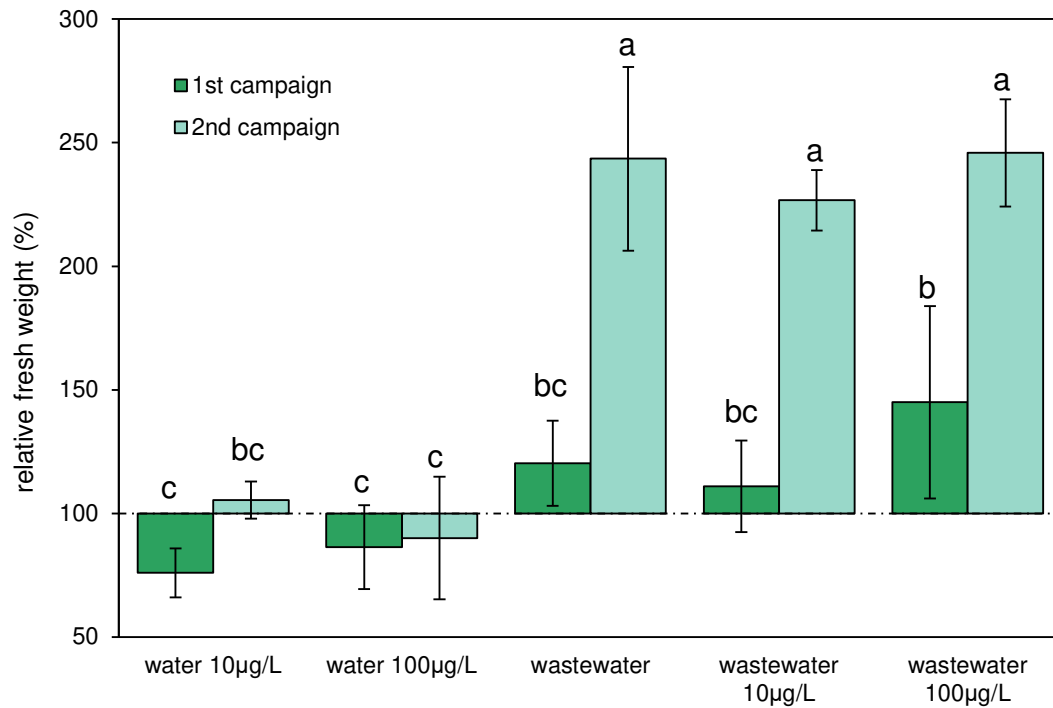


Figure 1. Relative increase or reduction of fresh weight in lettuce plants irrigated with water spiked with a mixture of PPCPs (at 10 µg/L or 100 µg/L each), wastewater or wastewater spiked with a mixture of PPCPs (at 10 µg/L or 100 µg/L each). Measurements were done on plants collected at the end of the first and second cultivation campaign. Each value is the mean of five replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey’s test was performed. Values indicated by different letters are significantly different.

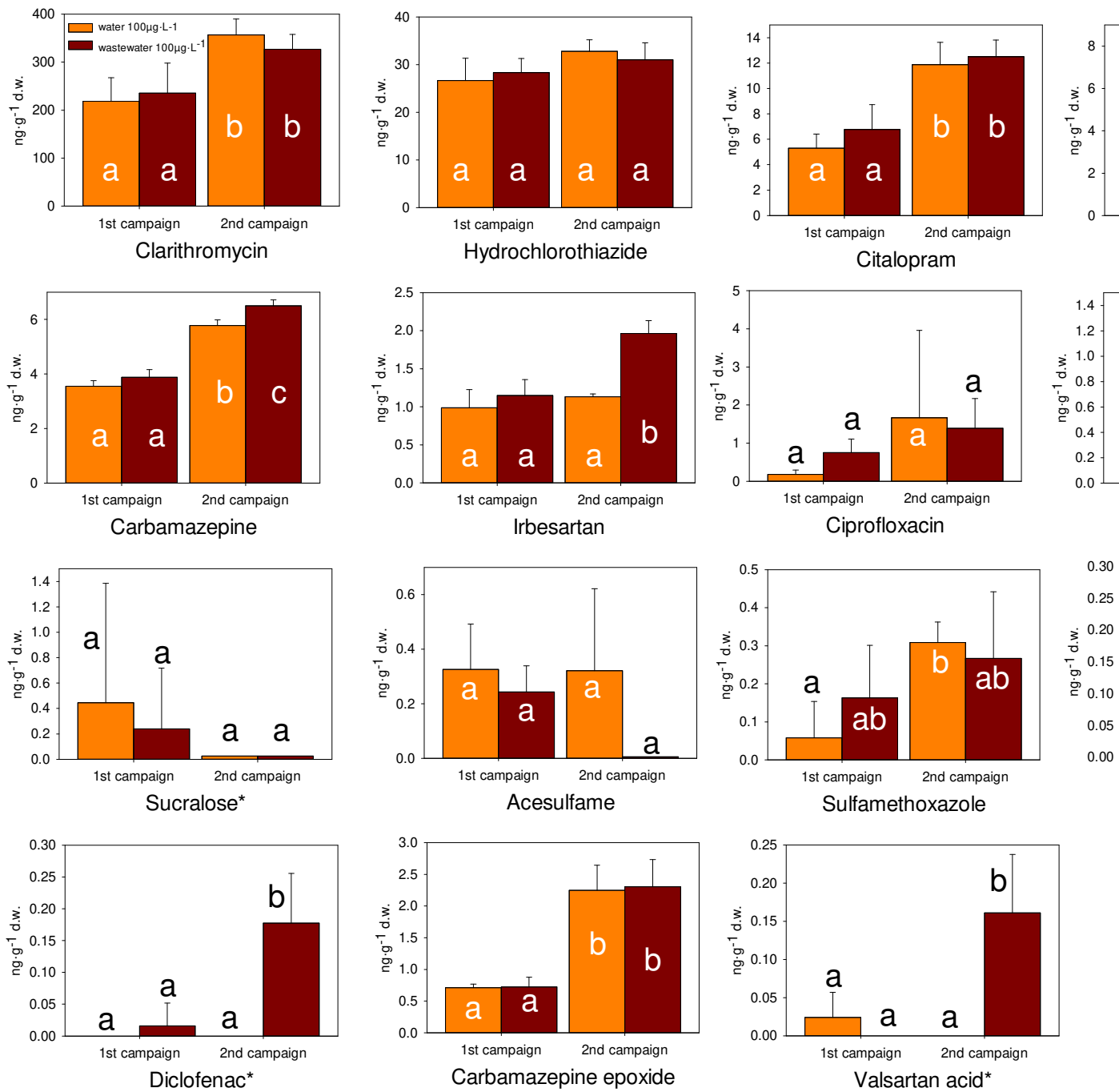


Figure 2. Concentration of the spiked products and major metabolites in soil planted with lettuces irrigated with either water spiked with a PPCPs mixture (at 100 µg/L each) or wastewater spiked (100 µg/L). Measurements were done on soil samples collected at the end of the first and second cultivation campaign. ANOVA followed by Tukey's test and Kruskal

Wallis followed by pairwise comparisons with Bonferroni correction (*) were performed. For benzotriazole and ciprofloxacin ANOVA was performed on log-and root square transformed data. Each value is the mean of five replicates. Standard deviations are indicated by error bars. The MQL (minimum quantification level) [ng g^{-1} d.w.] are ciprofloxacin = 0.03, sucralose = 0.05, acesulfame = 0.01, sulfamethoxazole = 0.03, metoprolol = 0.01, diclofenac = 0.16, valsartan = 0.03, valsartan acid = 0.12 and 4-nitro-sulfamethoxazole = 0.07. For wastewater 100 $\mu\text{g/L}$, 2nd campaign, n=4.

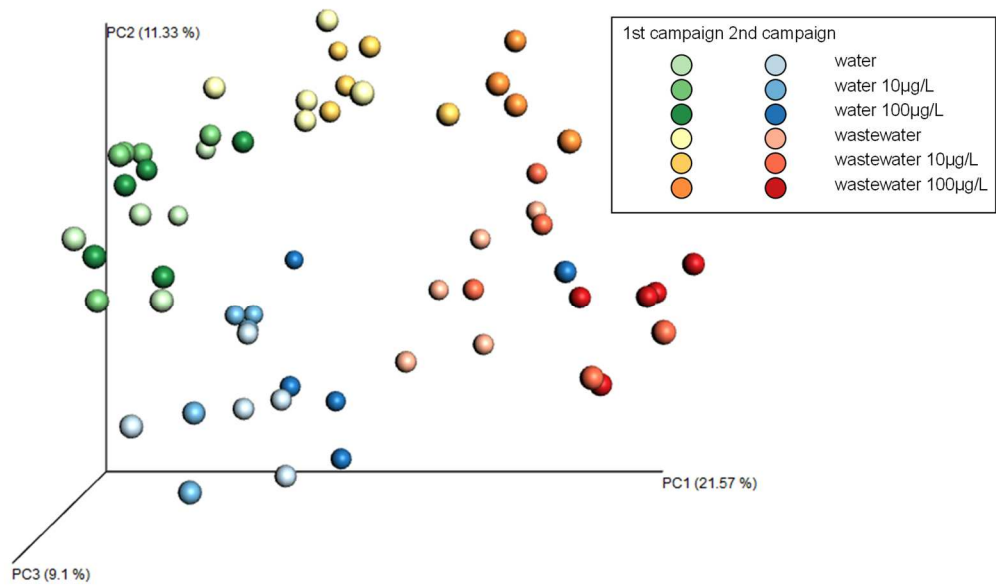


Figure 3. Bacterial β -diversity analysis from soil planted with lettuces irrigated with either water, water spiked with a mixture of PPCPs (at 10 μ g/L or 100 μ g/L each), wastewater or wastewater spiked with a mixture of PPCPs (at 10 μ g/L or 100 μ g/L each). Measurements were done on soil samples collected at the end of the first and second cultivation campaign. The first three axes of the PCoA weighted Unifrac distance matrix of 16S rRNA sequences are shown. The variance explained by each axis is given as a percentage. For each irrigation regime, the five independent replicates were considered except for irrigation with wastewater spiked with 100 μ g/L PPCPs from 1st campaign, which had only four replicates. All replicates of a given irrigation regime are represented by the same color.

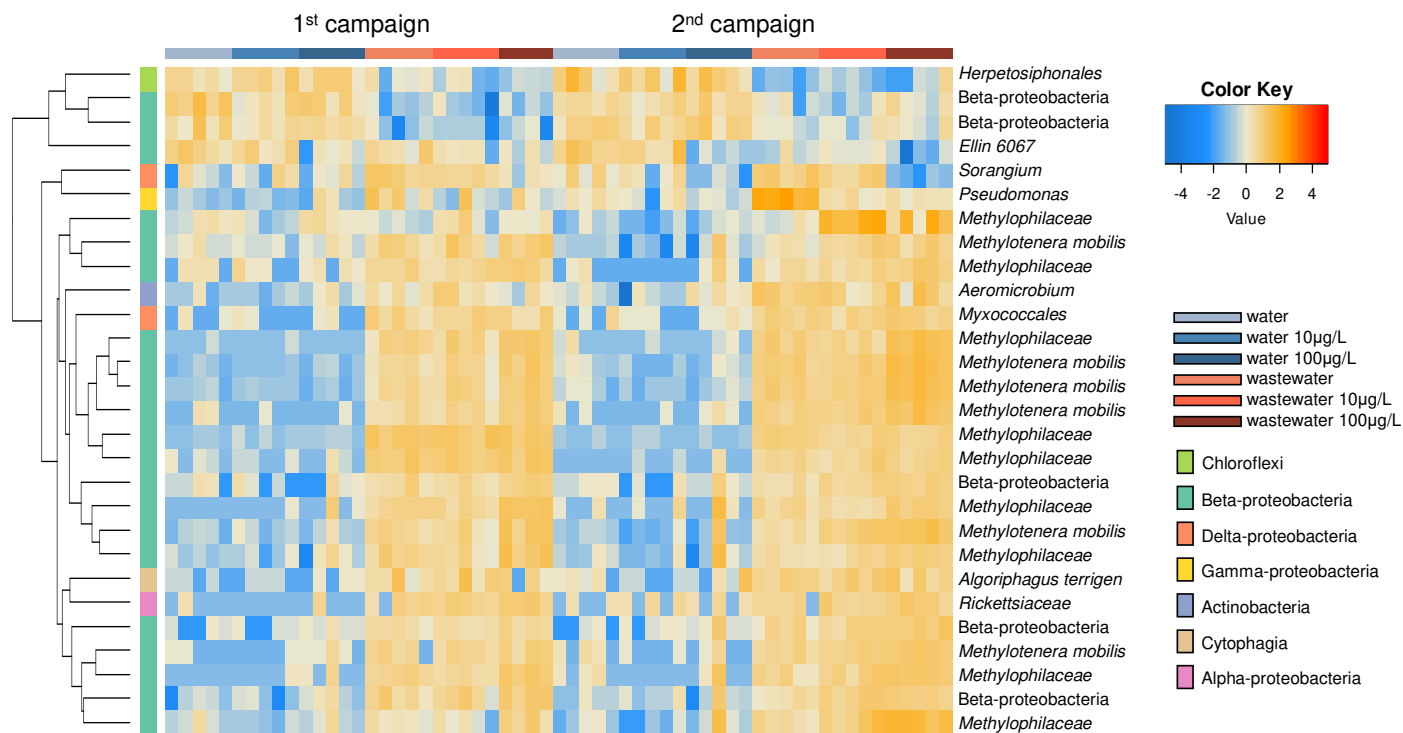
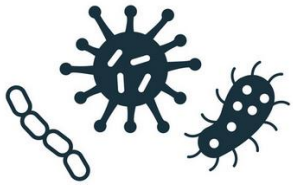


Figure 4. Heatmap plot representing the relative abundances of significant OTUs in soil planted with lettuces irrigated with either water, water spiked with a mixture of PPCPs (at 10 µg/L or 100 µg/L each), wastewater or wastewater spiked with a mixture of PPCPs (at 10 µg/L or 100 µg/L each). Measurements were done on soil samples collected at the end of the first and second cultivation campaign. For wastewater 100 µg/L, 1st campaign, n=4.

Effects on lettuce biomass



Effects on soil microorganisms (qPCR/Mi-Seq/mineralization)



- total bacteria and fungi
- ammonium oxidizing archaea (AOA)
- ammonium oxidizing bacteria (AOB)
- clade A and clade B commamox
- sulfamethoxazole-resistant and degrading bacteria
- bacterial (α and β) diversity and composition



Fate of 14 pollutants in soil (LC-HRMS)

PPCP mixture



acesulfame
benzotriazole
carbamazepine
ciprofloxacin
citalopram
clarithromycin
climbazole
diclofenac
hydrochlorotiazide
irbesartan
metropolol
sucralose
sulfamethoxazole
valsartan

