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Fate and impact of wastewater-borne micropollutants in lettuce and the root-associated bacteria

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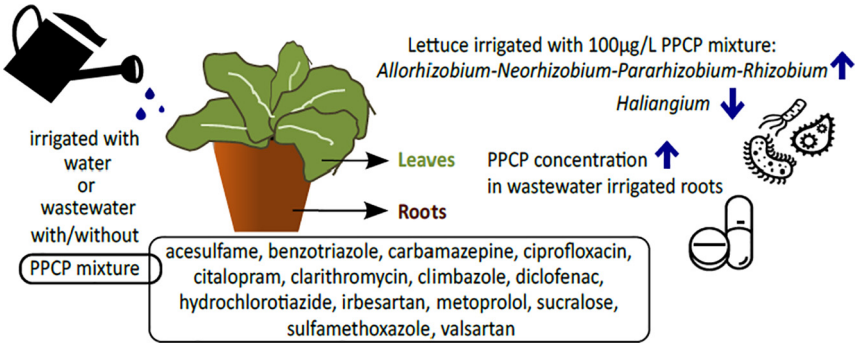
HIGHLIGHTS

- Thirteen out of fourteen spiked PPCPs were detected in the edible part of lettuce.
- Higher PPCP uptake in spiked wastewater- than water irrigated lettuce roots
- Wastewater had an impact on the diversity and composition of root-associated bacteria.
- *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* + *Haliangium* were affected by PPCP.
- *Caulobacter*, *Cellvibrio*, *Hydrogenophaga* and *Rhizobacter* were affected by wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

The reuse of water for agricultural practices becomes progressively more important due to increasing demands for a transition to a circular economy. Treated wastewater can be an alternative option of blue water used for the irrigation of crops but its risks need to be evaluated. This study assesses the uptake and metabolization of pharmaceuticals and personal care products (PPCPs) derived from treated wastewater into lettuce as well as the impact on root-associated bacteria under a realistic and worst-case scenario. Lettuce was grown in a controlled greenhouse and irrigated with water or treated wastewater spiked with and without a mixture of fourteen different PPCPs at 10 µg/L or 100 µg/L. After harvesting the plants, the same soil was reused for a consecutive cultivation campaign to test for the accumulation of PPCPs. Twelve out of fourteen spiked PPCPs were detected in lettuce roots, and thirteen in leaves. In roots, highest concentrations were measured for sucralose, sulfamethoxazole and citalopram, while sucralose, acesulfame and carbamazepine were the highest in leaves. Higher PPCP concentrations were found in lettuce roots irrigated with spiked treated wastewater than in those irrigated with spiked water. The absolute bacterial abundance remained stable over both cultivation campaigns and was not affected by any of the treatments (type of irrigation water (water vs. wastewater) nor concentration of PPCPs). However, the irrigation of lettuce with treated wastewater had a significant effect on the microbial α-diversity indices at the end of the second cultivation campaign, and modified the structure and community composition of root-associated bacteria at the end of both campaigns. Five and fourteen bacterial families were shown to be responsible for the observed changes at the end of the first and second cultivation campaign,

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respectively. Relative abundance of *Haliangium* and the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was significantly affected in response to PCPPs exposure. *Caulobacter*, *Cellvibrio*, *Hydrogenophaga* and *Rhizobacter* were significantly affected in microcosms irrigated with wastewater.

1. Introduction

The key concept of circular economy is to prevent resource scarcity in the context of an increasing demand of resources, the advancing climate change and the loss of resources worldwide. In this regard, circular economy aims at minimizing the total consumption of natural resources by maximizing their reuse (Breure et al., 2018). Nevertheless, the reuse of these resources needs to be safe and their human and environmental risks must be assessed.

The production of food and other biomass is highly dependent on healthy soil and water. Treated wastewater can be a cheap and readily available option to cover blue water demands for irrigation of crops in arid and semi-arid regions. A large body of literature has demonstrated the advantages of the irrigation of crops with treated wastewater (Cirelli et al., 2012; Singh et al., 2012; Vergine et al., 2017). For instance, nutrients introduced by wastewater can reduce the application of agrochemical fertilizers and improve plant growth (Montemurro et al., 2017a; Urbano et al., 2017). Despite numerous benefits of wastewater reuse in agriculture, wastewater-borne microbiological and chemical contaminants can alter soil physicochemical and microbiological properties (Becerra-Castro et al., 2015) with serious consequences on soil health and functioning (Cycon et al., 2016; Wagg et al., 2014).

Among the plethora of contaminants present in treated wastewater, pharmaceuticals and personal care products (PPCPs) are of special concern due to the human health and environmental risks they can pose. To date, there is no uniform regulation available for the risk assessment of complex mixtures of PPCPs (Godoy and Kummrow, 2017) although these micropollutants have been shown to accumulate in soils or plants (Carter et al., 2014; Manasfi et al., 2020), influence plant performance (Bártíková et al., 2016; Bigott et al., 2021a; Chen et al., 2017) and ultimately enter the human food chain in concentrations above the threshold of toxicological concern (Malchi et al., 2014). The most frequently occurring pharmaceuticals in soils are analgesics and anti-inflammatories (especially, nonsteroid anti-inflammatory drugs (NSAID)), antibiotics, cardiovascular pharmaceuticals (β-blockers/diuretics), psychostimulants, hormones, and antiepileptic drugs (Ferrer and Thurman, 2013; Loos et al., 2013; Li, 2014). In this regard, the antiepileptic drug carbamazepine is one of the most abundant PPCPs in wastewater effluents (Zhang et al., 2008) and was furthermore detected together with its metabolites in human urine after the consumption of fresh produce which was irrigated with treated wastewater (Paltiel et al., 2016). Non-nutritive artificial sweeteners are another class of PPCPs commonly used in toothpaste or pharmaceutical formulations. These recalcitrant substances like acesulfame or sucralose were already detected in surface water, tap water, groundwater, seawater and in the atmosphere (Praveena et al., 2019).

The importance of plant-associated bacteria has been recognized (Matilla and Krell, 2018) to notably promote plant growth due to the bacterial production of plant growth regulators (auxins, gibberellins or cytokinins), to improve the nutrient availability for the plant (N fixation, P solubilization or production of siderophores) or to increase the tolerance of plants against various biotic and abiotic stresses (Compant et al., 2005; Goswami and Deka, 2020; Reinhold-Hurek and Hurek, 2011). The plant microbiome can also play a key role in the degradation of PPCPs (Li et al., 2016a, b; Nguyen et al., 2019; Sauvêtre and Schröder, 2015; Syranidou et al., 2018) in addition to the detoxification and metabolization mechanisms in plants (Dordio et al., 2011; Edwards et al., 2011; Huber et al., 2009; Martínez-Piernas et al., 2019; Riemenschneider et al., 2017; Wu et al., 2016). Endophytes isolated from *Phragmites australis* were shown to contribute to the degradation of the anticonvulsant carbamazepine by activating specific metabolic pathways in horseradish hairy root culture (Sauvêtre et al., 2018). Furthermore, bacteria (mainly *Streptomyces*) isolated from roots and rhizomes of *Miscanthus* × *giganteus* plants exhibited removal capacity for sulfamethoxazole and diclofenac (Sauvêtre et al., 2020b). While the exposure to PPCPs can affect the composition and diversity of plant-associated microorganisms, it has been proposed that plants can also impose a selective control on plant-associated microbes favoring the enrichment of specific beneficial bacterial traits within and nearby the plant organs (Hartman and Tringe, 2019; Reinhold-Hurek and Hurek, 2011; Sauvêtre et al., 2020a).

The irrigation of lettuce with a mixture of 8 antibiotics (carbadox, lincomycin, monensin sodium, oxytetracycline, sulfadiazine, sulfamethoxazole, trimethoprim, and tylosin) and 3 other pharmaceuticals (acetaminophen, caffeine, and carbamazepine) was reported to decrease the bacterial alpha diversity in root, shoot and soil samples (Shen et al., 2019). Especially wastewater-borne sulfonamides were shown to reduce the microbial diversity in constructed wetlands planted with either *Cyperus alternifolius*, *Cyperus papyrus* or *Juncus effuse* and to significantly increase putative sulfonamide-degrading methylotrophs (Man et al., 2020). Additionally, the irrigation of lettuce with a mixture of trimethoprim, ofloxacin and sulfamethoxazole antibiotics negatively affected the relative abundance of Rhizobiales in roots (Cerqueira et al., 2020). The effects of treated wastewater on the structure and diversity of plant-associated microbial communities in tomato and lettuce were also studied by Zolti et al. (2019) who reported that 13% of the variation of the rhizoplane bacterial community composition was explained by the type of irrigation water (water vs. wastewater). However, to our best knowledge, until now there are no studies available assessing both the plant accumulation and the impact of complex mixtures of wastewater borne PPCPs on root-associated communities.

This study aimed to investigate the impact of wastewater irrigation on root-associated bacteria and assess the accumulation of 14 PPCPs (acesulfame, benzotriazole, carbamazepine, ciprofloxacin, citalopram, clarithromycin, climbazole, diclofenac, hydrochlorothiazide, irbesartan, metoprolol, sucralose, sulfamethoxazole and valsartan) on lettuce roots and leaves. Lettuce was grown under controlled greenhouse conditions in pots filled with arable soil and irrigated with water or treated wastewater, with or without the addition of a complex mixture of fourteen PPCPs (at 10 μg/L and 100 μg/L each) to study realistic agronomical conditions and the worst-case scenario, respectively. After seven weeks, plants were harvested and the soil was reused for a second cultivation campaign of lettuce. A multiple approach was used to quantify the fourteen targeted PPCPs and their major metabolites in soil and plants by liquid chromatography-high resolution mass spectrometry (LC-HRMS), to assess plant performance and to link those results to the total bacterial community diversity and composition determined by MiSeq sequencing of 16S rRNA amplicons generated from endophytes DNA.

2. Material and methods

2.1. Experimental design

The greenhouse experimental design consisted of 3 L pots containing 2 kg and 1.1 kg dry weight (d.w.) of sieved (4 mm) soil (for the first and the second campaign respectively) per treatment (type of irrigation water (water vs. wastewater) or concentration of PPCPs; five replicates per treatment). Soil pots were pre-incubated for four weeks at 50% of the soil water holding capacity (71.91%). Four-week old lettuce plantlets (*Lactuca sativa* var. Tizian) were transferred into the pots (one lettuce per pot). Lettuce plants were daily irrigated with the same volume of irrigation solution (ca. 30–80 mL per day) and water to adjust the water holding capacity to

70%. The irrigation solutions were: water (W), water spiked with a mixture of 14 compounds at 10 μg/L each (W10), water spiked with a mixture of 14 compounds at 100 μg/L each (W100), wastewater (WW), wastewater spiked with a mixture of 14 compounds at 10 μg/L each (WW10) and wastewater spiked with a mixture of 14 compounds at 100 μg/L each (WW100) (see SM1.1 Soil and wastewater characteristics in Supplementary Materials). The mixture of the compounds was selected based on their frequency of detection and concentration in treated wastewater in addition to including a broad spectrum of PPCPs with various physico-chemical properties (Montemurro et al., 2020). The majority of compounds chosen for the mixture were pharmaceuticals (carbamazepine (antiepileptic drug); ciprofloxacin, clarithromycin and sulfamethoxazole (antibiotics); citalopram (antidepressant); diclofenac (NSAID)); hydrochlorothiazide (diuretics); irbesartan, metoprolol and valsartan (cardiovascular pharmaceuticals)), exceptions were climbazole (antifungal, used as active substance in antidandruff shampoos), benzotriazole (restrainer or anti-fogging agent) as well as acesulfame and sucralose (artificial sweetener). For providing the mixture of compounds, individual solutions of the 14 PPCPs were prepared by dissolving the substances in methanol, ethanol, acetonitrile or water at 10 or 100 μg/L final concentration each. Different concentrations were used to mimic a realistic and the worst-case scenario. The same quantity of water-solvent mixture (with or without the mixture of compounds) was added to all the irrigation solutions (0.2% vol:vol). More details about soil and wastewater characteristics, reference standards and the purity of the solvents are reported in the Supplementary Materials (see SM1.1 Soil and wastewater characteristics and SM1.2 Chemicals), the most relevant physicochemical properties of each compound have been published in Montemurro et al., 2021. Two successive lettuce campaigns planted on the same soil were performed and 3 L and 2.7 L of irrigation solution (water or wastewater with/without PPCPs) were added per pot for the first and second campaign in total. For the second campaign, to overcome nutrient deficiency symptoms, plants were watered four times (once per week) with 60 mL of modified Hoagland ¼ solution (Hoagland and Arnon, 1938). The experiment was carried out in a greenhouse under controlled conditions at 20 °C (±5 °C) with a 16/8 h light/dark period. Soil pots were daily randomized. At the end of each campaign, soil samples and lettuce plants were collected. Soil samples were stored at −20 °C for chemical analysis. Lettuce plants (separated in leaves and roots) were thoroughly washed first with distilled water and then with ethanol to remove soil particles and microorganisms from the root surface but to not destroy DNA of interest (Lundberg et al., 2012). Fresh total plant biomass was weighed. Lettuce leaves and roots were subsequently freeze dried for chemical and DNA based analyses.

2.2. Soil and lettuce chemical analysis

Total nitrogen and carbon in lettuce roots and leaves were estimated from freeze-dried samples ground to a fine powder using a FLASH 2000 CHN Analyzer (Thermo Fisher Scientific).

Analysis of the fourteen compounds and their main metabolites and transformation products in soil and lettuce roots and leaves was performed using a QuEChERS method coupled to LC-HR/MS analysis from soil and lettuce samples irrigated with 100 μg/L spiked water or 100 μg/L spiked wastewater. Information about the extraction protocol of the compounds and their main metabolites, and the chemicals used for extraction and for soil and lettuce chemical analysis can be found in the Supplementary Materials (see SM1.2 Chemicals–SM1.4 Lettuce chemical extraction) and in Montemurro et al. (2020) for the extraction of pharmaceuticals from lettuce leaves and in Manasfi et al. (2022, in preparation) for the extraction from lettuce roots and soil.

After this step, a targeted analysis of the extracts was performed using an integrated SCIEX X500R QTOF system (Sciex, Redwood city, CA, U.S.) with Turbo V™ source and Electrospray Ionization (ESI) operating in positive and negative mode. Full details on chromatography and mass spectrometry parameters are reported elsewhere (Montemurro et al., 2020).

2.3. Quantitative-PCR analysis of 16S rRNA gene expression

DNA was extracted from roots using the DNeasy Plant Mini kit (Qiagen) extraction kit. The concentration of DNA was quantified using the Quant-iT™Pico Green® ds DNA assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and measured on a Tecan Spark® microplate spectrofluorometric reader (Tecan, Männedorf, Switzerland). Total bacterial communities were quantified by a qPCR assay of the 16S rRNA gene using the 335Fc (5′-CADACTCCTACGGGAGGC-3′) as a forward primer and 769Rc (5′-ATCCTGTTTGMTMCCCVCRC-3′) as a reverse primer (Dorn-In et al., 2015). The qPCR assay was carried out on an ABI 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.) with a PCR reaction mixture containing 12.5 μL Power SYBR® Green PCR Master Mix (Life Technologies Ltd., United Kingdom), 0.5 μL of each primer (10 pmol/μL), 0.5 μL of 3% BSA, and 2 μL template DNA (diluted 1:8) added in a final volume of 25 μL. PCR reaction was initiated after 10 min denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and elongation with data acquisition at 72 °C for 30 s. The specificity of the amplified products was confirmed by a melting curve analysis. For quantification, serial dilutions of an external linear standard (source sequence stated in Table S1; obtained from IDT Technologies, San Diego, CA) were used to produce a linear standard curve with an R^2 above 0.99. Additionally, the efficiency of the qPCR was calculated based on the linear standard curve according to the formula $\text{Eff} = [10^{(-1/\text{slope})} - 1]$ (Töwe et al., 2010) and was at 65.51%.

2.4. Library preparation and Illumina sequencing

A library preparation for next generation sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) was performed with the same DNA used for the qPCR analysis. To ensure that no contamination was introduced by the DNA extraction procedure negative controls were introduced using empty extraction tubes and buffers. The sequence specific polymerase chain reaction (PCR) of the 16S rRNA region was performed using the same primer sequences mentioned above with added Illumina adaptors. PCR reactions contained 12.5 μL 2 × NEBNext High Fidelity Master Mix (New England BioLabs Ltd., United Kingdom), 0.5 μL of each primer (10 pmol/μL), 2.5 μL of 3% BSA and 5 ng of template DNA added in a final volume of 25 μL. PCR conditions included an initial denaturation step at 98 °C for 5 min, followed by 28 cycles of denaturation (98 °C; 10 s), annealing (60 °C; 30 s) and elongation (72 °C; 30 s), and afterwards a final elongation step at 72 °C for 5 min. PCR reactions were purified using MagSi-NGS (0.8 × sample volume; Magtivio B.V., Geleen, Netherlands). The absence of primer-dimers was confirmed and the concentration of DNA measured on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies Inc., United States). The Illumina indexing PCR was performed using the Nextera XT Index kit v2 (Illumina Inc.). Therefore, the PCR reaction contained 10 ng of amplicon DNA, 12.5 μL 2 × NEB Next High Fidelity Master Mix, and 2.5 μL of each indexing-primer filled to a final volume of 25 μL. PCR conditions for the indexing PCR were as following: 98 °C for 5 min, eight cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final elongation at 72 °C for 5 min. After a purification and quantification of the indexed amplicons as described above, the libraries were diluted to 4 nM and paired-end sequenced on a MiSeq instrument using the MiSeq Reagent Kit v3 (600 cycles) (Illumina Inc.).

2.5. Sequencing data and statistical analysis

Demultiplexed, sequenced reads were processed with QIIME 2 (v2018.8.01; Caporaso et al., 2010). Paired reads were merged and chimeras removed with the DADA2 plugin (DADA2 R package v1.3.4; (Callahan et al., 2016). Therefore, the N-terminal trimming was adjusted to 15 bp, and the C-terminal trimming of the forward and reverse reads was set to 260 bp and 220 bp based on the quality scores. Amplicon sequence variants (ASVs) were inferred and taxonomically assigned (*P*-confidence ≥ 0.9) as described in Michas et al. (2020). The amplicon sequence

dataset is available in the Sequence Read Archive (SRA) under the accession numbers PRJNA717020: SAMN18475103–SAMN18475166. Details on statistical analyses as well as on the sample ordination by non-metric multidimensional scaling (NMDS) and the PERMANOVA are described in the Supplementary Materials (see SM1.5 Statistical analyses of sequencing data).

3. Results

3.1. Plant performance and accumulation of C and N in roots and leaves

Regardless of the treatment, the fresh weight of lettuce plants collected at the end of the second campaign was significantly lower than that from the first campaign ($p < 0.01$). In both cultivation campaigns, irrigation with treated wastewater (spiked or not) increased plant growth, with significantly higher biomass recorded in lettuce collected at the end of the second cultivation campaign ($p < 0.0001$) (Fig. 1).

In roots, for both campaigns, the total C:N ratio was lower in wastewater-irrigated samples than in water-irrigated samples, although not significant ($p > 0.09$) (Fig. 2A). This trend was due to the significant accumulation of nitrogen during the first campaign ($p = 0.03$) and to the significant decrease in the carbon content in the second campaign ($p = 0.005$) (Table S3).

In the leaves, the C:N ratio remained stable along both cultivation campaigns, and no significant differences were observed in the total nitrogen and carbon content between water and wastewater-irrigated samples ($p = 0.204$) (Fig. 2B).

3.2. Chemical analysis of PPCPs and metabolites

Fourteen spiked chemicals and four major metabolites were analyzed in soil, and in lettuce roots and leaves collected at the end of the second campaign. The eighteen compounds were detected in all analyzed matrices except for 4′-hydroxydiclofenac in soil, ciprofloxacin and valsartan in roots, and diclofenac, 4′-hydroxydiclofenac and 4-nitro-sulfamethoxazole in leaves (Fig. 3, Tables S4, S5 and S6).

The highest concentrations in soils were detected for clarithromycin (218.7 ± 49.0 to 357.0 ± 33.1 ng/g), hydrochlorothiazide (26.6 ± 4.7 to 32.8 ± 2.4 ng/g) and citalopram (5.3 ± 1.1 to 12.5 ± 1.3 ng/g) (see Gallego et al., 2021a and Table S4). In lettuce roots highest concentrations were measured for sucralose (135.3 ± 140.4 to 1335.6 ± 773.0 ng/g), sulfamethoxazole (16.1 ± 13.3 to 628.27 ± 260.0 ng/g) and citalopram (9.8 ± 4.7 to 201.1 ± 109.3 ng/g) (Fig. 3 and Table S5). In leaves, the compounds quantified with the highest concentrations were sucralose (299.3 ± 128.5 to 636.6 ± 196.9), acesulfame (115.0 ± 32.0 to 428.0 ± 184.6) and carbamazepine (83.0 ± 6.9 to 164.2 ± 15.7) (Fig. 3 and Table S6). In contrast, lowest concentrations were observed in soil samples for acesulfame, sulfamethoxazole, metoprolol, diclofenac, and valsartan (from 0.3 ± 0.2 ng/g to below limit of quantification (BLOQ)), in root samples for benzotriazole, diclofenac and carbamazepine epoxide (from 10.2 ± 11.2 ng/g to BLOQ) and in leaves for sulfamethoxazole, valsartan and irbesartan (from 0.8 ± 1.4 ng/g to 0.1 ± 0.2 ng/g).

To compare the accumulation of a given compound between samples collected from soil microcosms irrigated with water or wastewater, the differences [expressed in %] between the concentration of PPCPs in 100 μg/L spiked water and 100 μg/L spiked wastewater were calculated (Fig. S2). The concentration of PPCPs was generally higher in soil irrigated with spiked wastewater than in those irrigated with spiked water but for most of the compounds this trend was not statistically significant ($p > 0.14$). Only the climbazole concentration was significantly increased in soil irrigated with wastewater as compared to those irrigated with water in both campaigns ($p < 0.007$) (accounting up to 322.8 ± 180.3% for the first campaign) while carbamazepine and irbesartan concentrations significantly increased in soil irrigated with wastewater only in the second campaign ($p = 0.001$ and $p < 0.001$, respectively). In roots, the concentration of PPCPs in samples collected from cultures irrigated with spiked wastewater was generally higher or remained at the same concentration as compared to those of cultures irrigated with water. The highest differences were quantified for metoprolol at the end of the first campaign (1333 ± 1674.9%) and citalopram at the end of the second campaign (1036.7 ± 552.4%). Citalopram and irbesartan significantly increased in roots of lettuce plants irrigated with spiked wastewater as compared to those irrigated with spiked water for both campaigns ($p < 0.05$), while acesulfame only increased in the roots collected at the end of the first campaign ($p = 0.02$). Furthermore, the concentration of carbamazepine, climbazole, hydrochlorothiazide, sucralose and sulfamethoxazole increased significantly only in the roots irrigated with spiked wastewater collected at the end of the second campaign ($p < 0.05$). The differences in the concentration of the three metabolites (carbamazepine epoxide, 4′OH-diclofenac and valsartan acid) measured in the roots were significantly higher in roots collected at

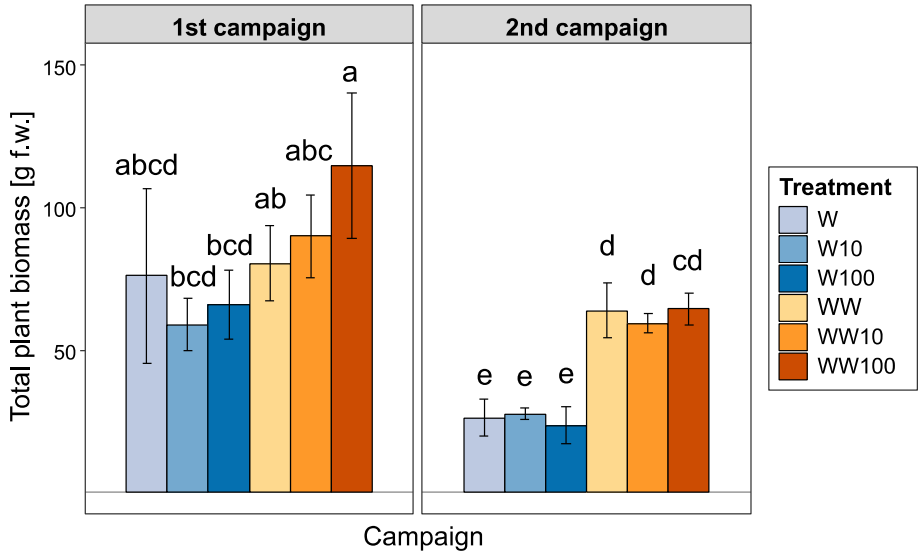

Fig. 1. Total plant biomass [g f.w.] of lettuce plants treated with water (W), 10 μg/L or 100 μg/L spiked water (W10 and W100), wastewater (WW), 10 μg/L or 100 μg/L spiked wastewater (WW10 or WW100) and collected at the end of the first and second cultivation campaign (n = 5). Different letters indicate significant differences ($p < 0.05$) calculated by ANOVA and Tukey's post hoc testing. Standard deviations are indicated by error bars.

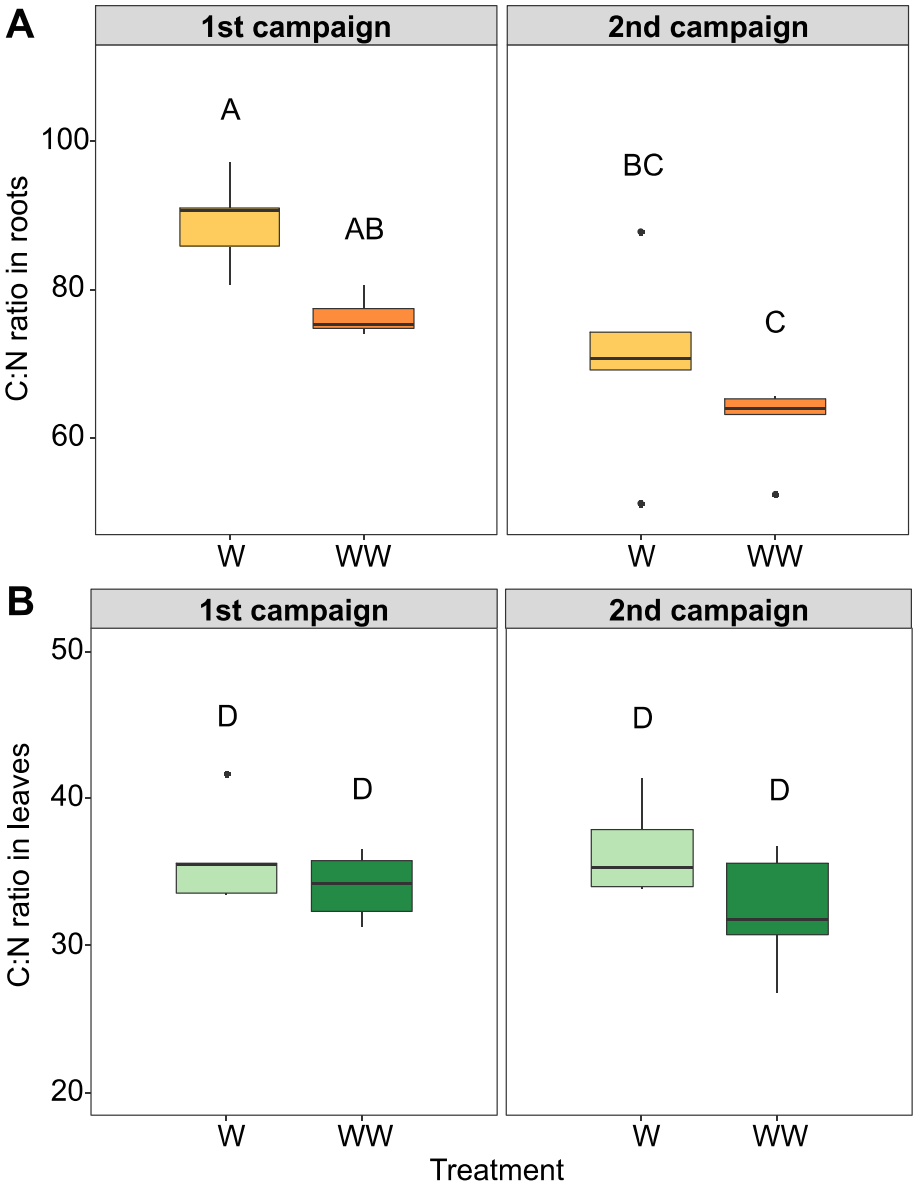

Fig. 2. C:N ratio in lettuce roots (A) and lettuce leaves (B) from planted soil microcosms irrigated with either water (W) and wastewater (WW) collected at the end of the first and second cultivation. Each value is the mean of five replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed. Values indicated by different letters are significantly different.

the end of the second campaign than at the end of the first one. However, it is noteworthy that only the concentration of valsartan acid, one of the three metabolites, significantly increased ($p = 0.006$) in the roots of lettuce irrigated with spiked wastewater than in those irrigated with spiked water.

In leaves, differences ranged from $-67.7 \pm 8.0\%$ for acesulfame in the second campaign to $205.0 \pm 220.1\%$ for sulfamethoxazole in the first campaign. At the end of the first campaign, the concentration of clarithromycin and climbazole was significantly decreased in lettuce irrigated with spiked wastewater as compared to those irrigated with spiked water ($p < 0.02$). At the end of the second campaign, the concentrations of carbamazepine, citalopram, metoprolol and sucralose increased significantly ($p < 0.04$) in the leaves of lettuce irrigated with spiked wastewater as compared to those irrigated with spiked water.

3.3. Bacterial community analyses

3.3.1. Total bacterial abundance

The total bacterial abundance ranged from 2.9×10^7 to 5×10^7 copies of 16S rRNA per g of roots and was not significantly affected by any of the treatments applied (type of irrigation water (water vs. wastewater) nor concentration of PPCPs (10 μg/L or 100 μg/L) ($p > 0.55$) (Fig. S3).

3.3.2. Bacterial diversity

16S rRNA amplicons generated from extracted root DNA were sequenced to calculate a range of bacterial α-diversity indices pertaining richness (Chao1) and evenness (Shannon). At the end of the first campaign, bacterial α-diversity indices seemed to be not affected by any of the treatments (Fig. S4A). In contrast, at the end of the second campaign, Chao1 and Shannon indices were significantly decreased in roots of lettuce irrigated with wastewater ($p = 0.015$ and $p = 0.02$). In addition, a significant increase in the Chao1 index was observed in the roots of lettuce irrigated with 10 μg/L spiked water ($p = 0.028$) as compared to those irrigated with water (Fig. S4B).

Non-metric multidimensional scaling (NMDS) of β-diversity based on Bray-Curtis dissimilarities and PERMANOVA Adonis testing revealed a highly significant difference in the bacterial diversity in roots of lettuce plants irrigated with either water or wastewater no matter of the cultivation campaign ($p = 0.001$) (Fig. 4A, Table 1). For the first campaign, irrigation

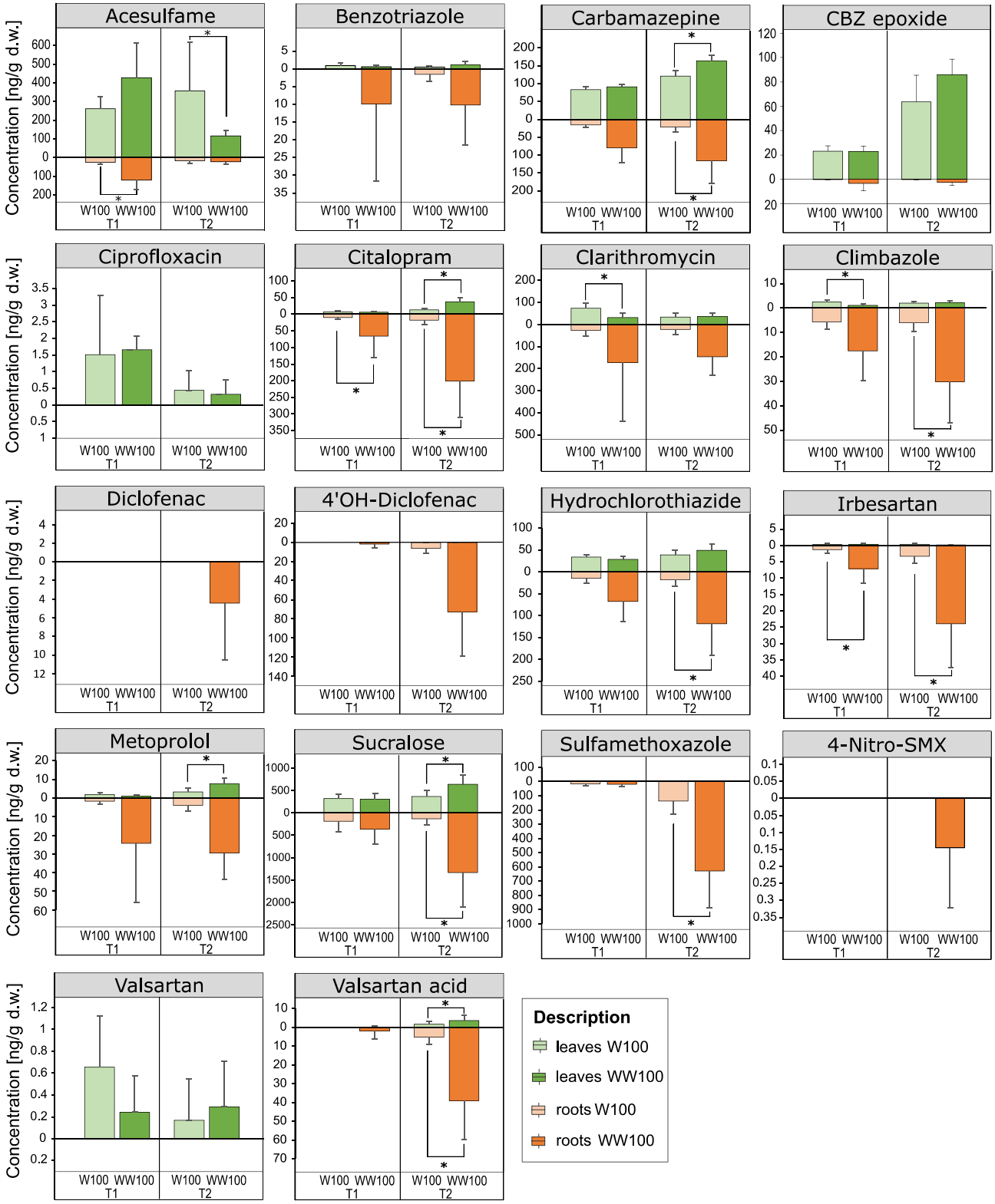

Fig. 3. Concentration [ng/g d.w.] of PPCPs in lettuce roots and leaves, derived from samples irrigated with water 100 μg/L PPCPs (W100) and wastewater 100 μg/L PPCPs (WW100) collected at the end of the first (T1) and second (T2) cultivation campaign. Significant differences between the concentration of PPCPs measured in water compared to wastewater irrigated samples are indicated as "*" for p-value ≤ 0.05. CBZ: carbamazepine; SMX: sulfamethoxazole.

with water or treated wastewater explained 8.5% of the variance in the dataset. The concentration of PPCPs and the interaction between the irrigation type (water vs. wastewater) and the PPCP concentration accounted for 8% and 7.8% but were not statistically significant. For the second campaign, the type of irrigation applied to grow the lettuce plants significantly affected the bacterial community structure ($p = 0.001$) with 19.7% of the variance explained by this factor (Fig. 4B, Table 1). In addition, at the end of the second campaign, both, the PPCP concentration and the interaction between the irrigation type and the PPCP concentration significantly influenced the lettuce-associated bacterial community structure with

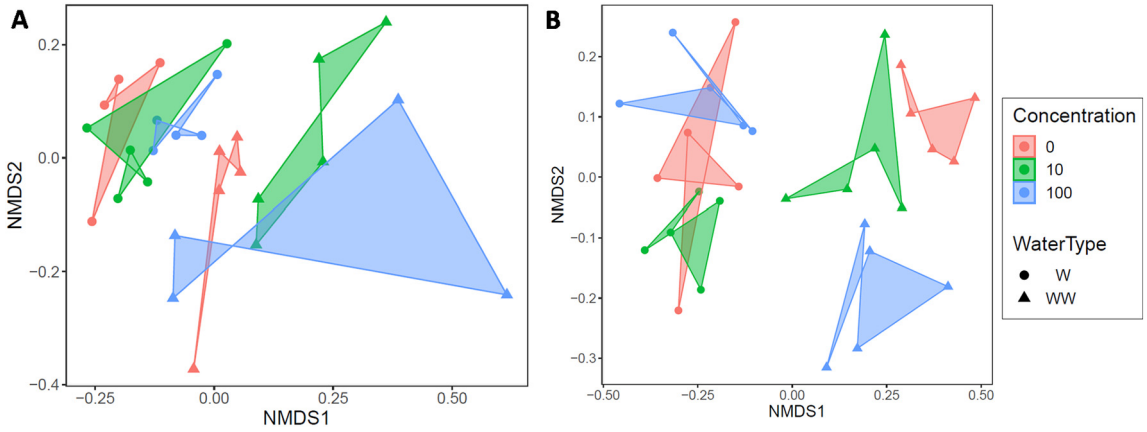

Fig. 4. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of the datasets of the first campaign (A) and the second campaign (B). "W" refers to samples irrigated with water shown as circles; "WW" indicates the wastewater-irrigated samples shown by triangles. Different colors correspond to different concentrations of spiked PPCPS (red: without, green: 10 μg/L, and blue: 100 μg/L PPCPs). For 1st campaign water without PPCPs, and 1st campaign wastewater 100 μg/L, n = 4.

10.2% and 8.1% of the variance explained, respectively. To further confirm the significance of effects detected by PERMANOVA Adonis, a permutation test for homogeneity of multivariate dispersions was performed. All observed significant effects could be clearly explained by the location and not by a multivariate dispersion of the values around the centroid (Table S7).

3.3.3. Bacterial community composition

The bacterial community in the roots of lettuce plants was dominated by six major phyla: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Spirochaetes and Acidobacteria (Fig. S5). Proteobacteria was the prominent one with relative abundance comprised between 77.5% and 88.0%. ASVs related to these six phyla represented up to 99% of all the ASVs. The relative abundance of these major phyla remained constant in all treatments and in both campaigns. However, at lower taxonomic level (families and genera), significant changes between the treatments were observed (Figs. S6 and S7). At the end of the first campaign, the relative abundance of ASVs related to five bacterial families changed significantly in response to the type of irrigation water (Fig. S6A). The relative abundance of the two families *Burkholderiaceae* ($p = 0.0009$) and *Chitinophagaceae* ($p = 0.013$) significantly decreased, whereas the relative abundance of *Enterobacteriaceae* ($p = 0.02$), *Ilumatobacteraceae* ($p = 0.04$) and *Pseudomonadaceae* ($p = 0.02$) significantly increased in roots of lettuce plants irrigated with treated wastewater as compared to water. At the end of the second campaign, fourteen families were significantly affected by the different treatments (Fig. S6B). Among them, four (*Beijerinckiaceae*, *Dongiaceae*, *Haliangiaceae* and *Rhizobiaceae*) were identified to differ between the different PPCP concentrations, while the remaining ones were affected by the type of irrigation water or one by both, the PPCP concentration and the type of irrigation water (*Spirochaetaceae*). At the genus level, twenty-five genera significantly differed between the different irrigation treatments at the end of the second campaign (Fig. S7). Only six genera whose relative abundance was higher than 2% were further analyzed (Fig. 5). The relative abundance of the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was significantly increased in response to the PPCP exposure in the roots of lettuce plants irrigated with water (from a relative abundance of 2.9 ± 0.8% to 5.7% ± 1.5%) and wastewater (2.9 ± 0.4% to 4.5% ± 1.5%) ($p = 0.008$). *Haliangium* was significantly reduced in response to increasing PPCP exposure ($p = 0.004$) in the roots of lettuce plants irrigated with water and wastewater spiked with the highest concentration of PPCPs (100 μg/L each) (Fig. 5A). Four genera were significantly affected by the type of irrigation water (Fig. 5B), where two of those increased (*Cellvibrio and Hydrogenophaga*) and two decreased (*Caulobacter* and *Rhizobacter*) in the roots of lettuce plants irrigated with wastewater. The relative abundance of the genus *Cellvibrio* was twice as high in the roots of lettuce plants irrigated with wastewater (17.2 ± 4.6%) than in those irrigated with water (8.1 ± 2.9%) ($p = 0.002$). Similarly, the relative abundance of *Hydrogenophaga*, a genus belonging to the family *Burkholderiaceae* was five times higher in the roots of lettuce plants irrigated with wastewater (8.24 ± 4.82%) than in those irrigated with water (1.5 ± 1.9%) ($p = 0.004$). In contrast, the relative abundance of the genera *Rhizobacter* ($p = 0.002$) and *Caulobacter* ($p = 0.004$) was found to be significantly lower in the roots of lettuce plants irrigated with wastewater than in those irrigated with water.

Table 1
PERMANOVA adonis analyses of bacterial distribution among samples. Significant differences are indicated as "*" for $0.01 \leq p\text{-value} \leq 0.05$, "**" for $0.001 \leq p\text{-value} \leq 0.01$, and "***" for $p\text{-value} \leq 0.001$.

Dataset	Factor(s)	n	Df	F	R^2	*p*-Value
Campaign T1	WaterType	28	1	2.4693	0.085	0.001***
	PPCP concentration		2	1.1659	0.080	0.108
	Interaction		2	1.1298	0.078	0.159
Campaign T2	WaterType	30	1	7.6339	0.197	0.001***
	PPCP concentration		2	1.9726	0.102	0.004**
	Interaction		2	1.5776	0.081	0.025*

4. Discussion

The uptake and translocation of organic contaminants in plants depend on various factors: the physico-chemical properties of the molecules, the soil/rhizosphere characteristics and the plant composition and physiology (lipid and protein content, biotransformation, sequestration capacities and transpiration rate) (Bigott et al., 2021b). Besides these parameters that account for the passive uptake of micropollutants via diffusion, they can also be translocated via active transporters (Bigott et al., 2021b; Eggen et al., 2011).

As reported in previous studies, the irrigation with treated wastewater had a positive effect on the growth of lettuce plants (Singh et al., 2012; Urbano et al., 2017). This effect was especially visible at the end of the second campaign, when the total plant biomass was significantly higher in treated wastewater-irrigated lettuce plants as compared to water-irrigated ones. No matter the treatment considered, lettuce fresh weight was significantly lower at the end of the second campaign as compared to that of the first campaign possibly because of the fertilization treatment, which did not entirely cover the nutrient depletion due to repeated plant cultivation. Although the C:N ratio, which is a good indicator under different stress and nutrient conditions (Royer et al., 2013; Li et al., 2016a, b) did not change significantly in roots and leaves within the two campaigns, the nitrogen

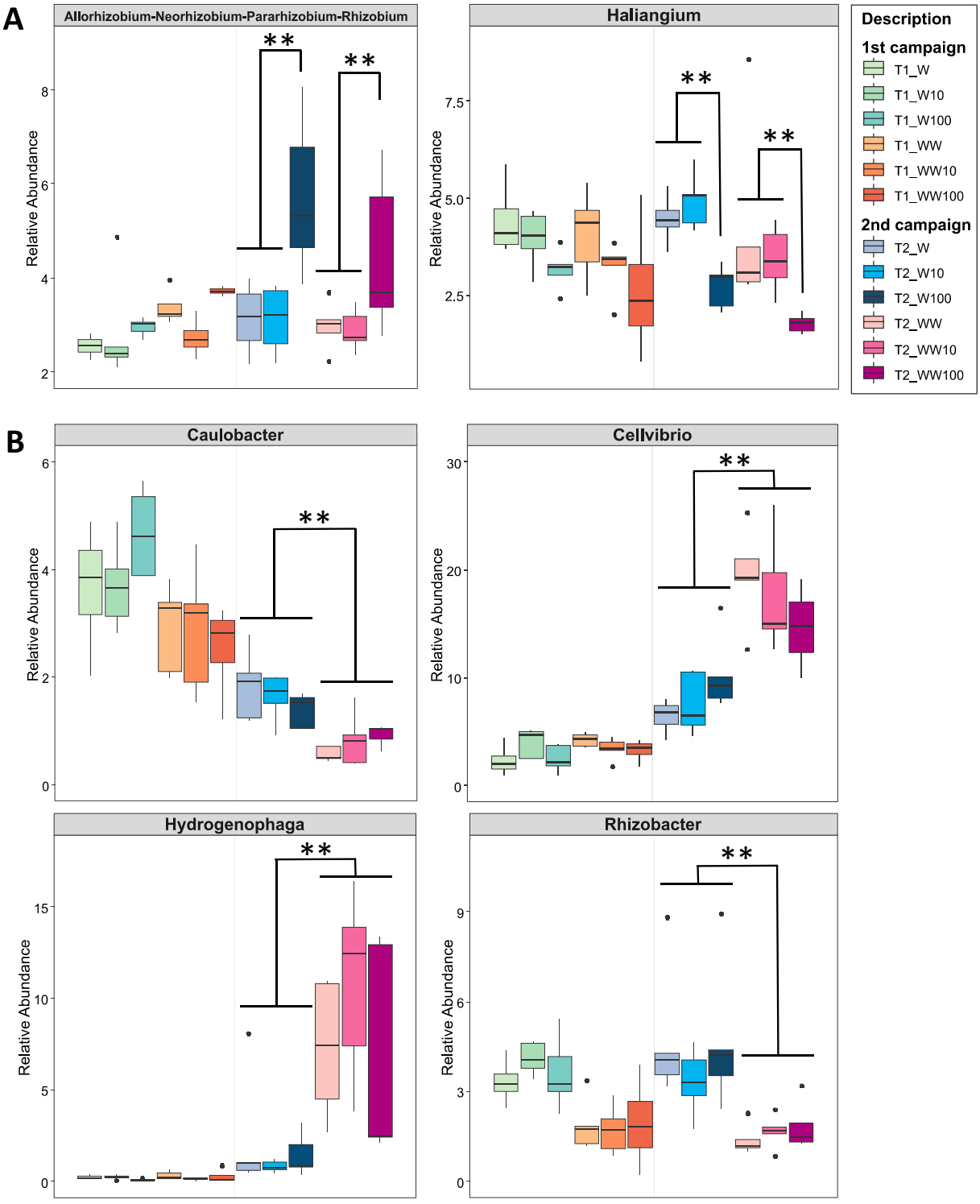

Fig. 5. Relative abundance of significant different genera affected by the PPCP concentration (A) and by the type of irrigation water (B) with relative abundance >2%. "W" refers to samples irrigated with water; "WW" indicates the wastewater-irrigated samples. For 1st campaign water without PPCPs, and 1st campaign wastewater 100 μg/L, n = 4. Significant differences are indicated as "**" for *p*-value ≤0.01.

content in roots significantly increased at the end of the first campaign while the carbon content significantly decreased at the end of the second campaign in wastewater-irrigated samples. Treated wastewater is known to be a good source of nitrogen (Montemurro et al., 2017a) especially in its organic and ammoniacal forms. Our observation is in accordance with Mañas et al. (2009) who reported significant increase of the nitrogen level in lettuce after irrigation with treated wastewater. In addition, fertilization treatment and additional nitrogen input may have led to a plant-induced shift of the microbial community in the root zone as shown in a previous study (Chen et al., 2019; Haichar et al., 2008), which would explain the observed reduction in the carbon content in treated wastewater-irrigated lettuce roots at the end of the second campaign. Chen and co- authors postulate that plants might adjust the quantity and composition of root exudates like organic acids as an adaption strategy to

recruit beneficial plant growth-promoting microbes in the rhizosphere to manage the high N input (Chen et al., 2019). For the synthesis of these organic acids, carbon is essential and might be reduced in the lettuce roots at the end of the second campaign.

Following the irrigation of lettuce with treated wastewater spiked with fourteen PPCPs, twelve PPCPs were detected in roots and thirteen in leaves. The highest concentrations were measured for neutral (sucralose, sulfamethoxazole, carbamazepine and clarithromycin) or positively charged PPCPs (citalopram) mainly in roots but also in leaves. Acesulfame (negatively charged) was also detected in leaves at high concentrations.

Neutral organic compounds at the pH of the experiment (pH 8.2) like benzotriazole, carbamazepine, hydrochlorothiazide, sucralose and sulfamethoxazole have been previously detected and quantified in different plant tissues at relatively high concentrations (Calderón-Preciado et al., 2011; Chuang et al., 2019). Because of its high photodegradation (Hem et al., 2003) and phytotransformation (Castro et al., 2003) benzotriazole was detected in soil, roots and leaves only in very low concentrations. Carbamazepine was detected in high concentrations in leaves, as previously observed by other authors (Montemurro et al., 2017b; Riemenschneider et al., 2017; Shenker et al., 2011). Its intermediate hydrophobicity (log K_{OW} 3.64) and uncharged ionization status may have favored its translocation from roots to the aerial part of the plants by xylem flow (Malchi et al., 2014; Shenker et al., 2011). Interestingly, carbamazepine epoxide, a transformation product (TP) of carbamazepine, was detected in high concentrations in lettuce leaves, which indicates it was metabolized by plants as shown previously (Dordio et al., 2011; Kodešová et al., 2019). In roots, carbamazepine concentrations were similar to those reported by Montemurro et al. (2020) and similar to the concentrations of the hydrochlorothiazide. Lower concentrations of hydrochorothiazide were translocated to the aerial part, in agreement with Manasfi et al. (2020). In the same study, high sucralose uptake and translocation to lettuce leaves was observed because of the structural similarity to sucrose which is an easily transported plant sugar. Indeed, the aquatic plant *Lemna* spp. was shown to assimilate carbon from sucralose (Amy-Sagers et al., 2017). Although not detected in soil, high concentrations of sucralose were observed in lettuce roots and leaves in agreement with Manasfi et al. (2020). Sulfamethoxazole was quantified in higher concentrations in the roots than in the soil. Sulfamethoxazole is generally present as an anionic compound but it also exists in non-ionic form. At the given soil pH, the charge of the substance was estimated to be −0.05 by SPARC (SPARC Performs Automated reasoning) based on the molecule's pK_a and structure. At this charge, sulfamethoxazole can be considered almost neutral which might explain its high accumulation in lettuce roots. In contrast, at pH 7.2 within the plant cytosol sulfamethoxazole has been hypothesized to be trapped as an anionic species in the roots resulting in an absent translocation to the leaves (Chuang et al., 2019). Earlier studies had also detected high accumulation in lettuce roots and reduced translocation to leaves in lettuce, cucumber and tomatoes (Ahmed et al., 2015). Additionally, 4-nitro-sulfamethoxazole, a phototransformation product of sulfamethoxazole (Su et al., 2016) was hardly detected in soil and roots, and it was not detected in leaves.

Positively charged molecules at pH 8.2 were citalopram, clarithromycin, climbazole and metoprolol. Citalopram was found to be taken up in much higher concentrations than metoprolol in lettuce roots, in line with Montemurro et al. (2020). A possible explanation can be that citalopram, clarithromycin and climbazole accumulated in higher concentrations in soil than metoprolol, which could then result in higher plant uptake. The concentrations of citalopram, clarithromycin, climbazole and metoprolol were lower in lettuce leaves as compared to roots. Experiments done by Tian et al. (2019) in lettuce grown in nutrient solution containing clarithromycin showed a high metabolization of clarithromycin in the plant tissue, which may explain our results. Metoprolol was shown to accumulate in carrot and sweet potato roots but not in leaves (Malchi et al., 2014). The high metoprolol concentrations used in our study could explain why in our study it was also detected in leaves.

Negatively charged molecules at pH 8.2 were acesulfame, diclofenac and valsartan. Acesulfame was found at very low concentrations in soil but at higher concentrations in lettuce leaves than in roots, confirming previous studies done in various vegetable species like cabbage, carrot, eggplant, lettuce, parsley, pepper, potato, rucola, tomato and zucchini (Manasfi et al., 2020; Riemenschneider et al., 2016). Diclofenac and valsartan were barely present in soil, lettuce roots and leaves. One possible explanation could be their rapid dissipation in soil (Al-Rajab et al., 2010; Gallego et al., 2021b; Helbling et al., 2010) and rapid metabolization of diclofenac in plants (Bartha et al., 2014; Fu et al., 2017; Huber et al., 2012). Indeed, 4-OH-diclofenac and valsartan acid, which are main TPs of diclofenac and valsartan were barely detected in soil, roots and leaves, which could indicate that subsequent TPs were formed during their transformation.

Ciprofloxacin and irbesartan are zwitterionic molecules at pH 8.2. Ciprofloxacin was not detected in lettuce roots and only at very low concentration in soil and lettuce leaves probably due to its high metabolization by plants with a potential role of root-associated microorganisms (Panja et al., 2019). Irbesartan was detected at very low concentrations in soil and lettuce leaves but at higher levels in roots. The accumulation of irbesartan in plants was firstly reported by Montemurro et al. (2020).

The concentration of eight out of the twelve spiked PPCPs (acesulfame, carbamazepine, citalopram, climbazole, hydrochlorothiazide, irbesartan, sucralose and sulfamethoxazole) significantly increased in lettuce roots irrigated with treated wastewater as compared to plants irrigated with spiked water. Similar results were obtained by Goldstein et al. (2014), who detected a trend of higher concentrations of carbamazepine and lamotrigine in leaves of cucumber plants irrigated with spiked treated wastewater as compared to plants irrigated with spiked water. However, in leaves of tomato plants this trend was only observed for carbamazepine. The concentration of the PPCPs in lettuce roots was higher at the end of the second campaign than at the end of the first campaign (7 vs. 3 significant different PPCPs). On the one hand, one can hypothesize that the metabolization of the PPCPs was slowed down in the plants irrigated with spiked treated wastewater due to the inhibition of detoxification enzymes (Dordio et al., 2011). On the other hand, this trend might result from higher uptake rates of the PPCPs due to interactions between PPCPs and other wastewater borne micro- and macroelements. Papaioannou et al. (2020) identified synergistic interactions between different PPCPs leading to higher PPCP accumulations in beet. However, in another study, the interactions of PPCPs with heavy metals and micro- and macroelements resulted in a decreased uptake of specific PPCPs in beet (i.e. phosphate and metoprolol) (Papaioannou et al., 2019). One exception was the soil nickel concentration, which correlated positively with sulfamethoxazole in beet. Heavy metals like nickel were shown to be significantly enriched in soils irrigated with treated wastewater in previous studies (Mkhinini et al., 2020). Therefore, the higher concentrations of PPCPs (i.e. sulfamethoxazole) detected in treated wastewater-irrigated samples might be partly explained by synergistic effects between the PPCPs or other wastewater borne micro- and macronutrients.

Interestingly, all the PPCPs that significantly increased in wastewater-irrigated samples as compared to water-irrigated samples at the end of the second campaign were either neutral, cationic or zwitterionic compounds. Plants possess a variety of transporters for the uptake and distribution of different structurally diverse substances like secondary compounds (Eggen and Lillo, 2015). Among them, organic cation transporters (OCT) and nitrate and peptide transporters are involved in the sensing and uptake of nitrogen-containing compounds and amino acids in plants (Eggen and Lillo, 2015; Tsay et al., 2007). Given the high nitrogen concentrations in the wastewater, it could be hypothesized that PPCPs were transported together. In this regard, a putative involvement of OCTs for the uptake of the pharmaceutical tramadol by plants was recently postulated by Khalaf et al. (2021).

The effects of the irrigation of lettuce with treated wastewater or water spiked or not with a mixture of PCCPs under a real and worst-case scenario on the community composition and diversity of lettuce-associated bacteria were assessed. The abundance of the total bacterial community did not

change in response to the different irrigation regimes applied. Changes in relative abundance observed in response to the irrigation can be considered as real changes in the community composition and not as loss of entire taxa or of lower absolute abundance within the root-associated bacteria. None of the irrigation regimes had an effect on the bacterial richness and evenness at the end of the first campaign. However, a significant impact was observed in Chao1 and Shannon indices in treated wastewater-irrigated samples at the end of the second campaign. In accordance to our results, Shen and co-workers (Shen et al., 2019) observed a reduced α-diversity in lettuce root, shoot and soil samples after irrigation with a fertilizer solution spiked with a mixture of pharmaceuticals. The significant impacts on the structure of the microbial community were mostly driven by the treated wastewater but also due to the PPCPs. These effects were more pronounced in the second campaign, probably due to the accumulation of PPCPs in the soil (see Gallego et al., 2021a) or due to the depletion in soil nutrients because of repeated culture.

The community composition of lettuce-associated root bacteria was also significantly affected by irrigation with treated wastewater and the concentration of PPCPs. During the first cultivation campaign, the bacterial taxonomic families *Burkholderiaceae* and *Chitinophagaceae* significantly decreased in the treated wastewater-irrigated samples, while the *Enterobacteriaceae*, *Ilumatobacteraceae* and *Pseudomonadaceae* families significantly increased. Members of *Burkholderiaceae*, *Chitinophagaceae*, *Enterobacteriaceae and Pseudomonadaceae* were shown to possess plant growth-promoting activities (Armanhi et al., 2018; Guo et al., 2020; Kuklinsky-Sobral et al., 2004; Ogbo and Okonkwo, 2012; Roquigny et al., 2017). Plant growth-promoting activities can help the plant withstand several biotic and abiotic stresses (Berg, 2009; Goswami and Deka, 2020; Reinhold-Hurek and Hurek, 2011). Additionally, certain plant growth promoting microorganisms can help degrade organic contaminants or enhance plant metabolization capacities (Sauvêtre et al., 2018, 2020b; Shahpoury et al., 2021). The increase in the relative abundance of the family *Ilumatobacteraceae* is consistent with a previous study of Coll and co-workers (Coll et al., 2020), who observed an enrichment of this family in microcosms filled with sediment sampled downstream of a wastewater treatment plant discharge and a mixture of PPCPs. Among the 14 different families that were significantly affected during the second cultivation campaign, members of the *Methylomonaceae* family have been identified as methylotrophs. Methylotrophs, such as *Methylocaldum*, *Methylomonas*, *Methylosinus* and *Methylotenera* have been shown to increase in the rhizosphere of constructed wetlands planted with *C. alternifolius*, *Cyperus papyrus* or *Juncus effuse* and exposed to wastewater containing sulfonamides (Man et al., 2020).

At genus level, the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was increased in response to the exposure to the highest concentration of PPCPs. This agrees with a previous study done on soils contaminated with the plasticizer DEHP (Bai et al., 2020) and with observations from Guo and Chi (2014) in cadmium polluted soil. The plant growth-promoting clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* thus might be resistant against abiotic stress triggered by various contaminants and as a result have an advantage over other competing bacteria. In contrast, the bacterial genus *Haliangium* was decreased in response to the exposure to the highest concentration of PPCPs. The genus *Haliangium* was shown to be sensitive to veterinary antibiotics in plant–soil systems (Uddin et al., 2019), which may explain our findings. At the end of the second campaign, the genera *Cellvibrio* and *Hydrogenophaga* were enriched in the roots of plants irrigated with wastewater. These two genera were described as keystone denitrifiers responsible for nitrogen removal processes in a constructed wetland (Li et al., 2019). In contrast, *Caulobacter* and *Rhizobacter* significantly decreased in the roots of plants irrigated with treated wastewater. The order *Caulobacterales* was shown to decrease with increasing concentrations of nitrate (Hester et al., 2018). In our study, the relatively high ammonium content brought by the wastewater used might have supported the growth of ammonium oxidizers with a subsequent nitrate production, favoring the growth of genera such as *Cellvibrio* and *Hydrogenophaga* but limiting that of the *Caulobacter* and *Rhizobacter*.

5. Conclusions

A multiple approach was used to monitor the soil-plant fate of each element of a complex mixture of PPCPs brought either by water or by treated wastewater into a soil-plant experiment carried out in two successive campaigns and to investigate the effects of different irrigation regimes on root-associated bacteria. Higher uptake rates of PPCPs were found especially in roots of lettuce irrigated with treated wastewater under the tested worst-case scenario of exposure (irrigation with a mixture of 14 PPCPs at 100 μg/L each). Irrigation with treated wastewater had bigger influence on the root-associated bacterial diversity and community composition than the PPCPs, even under the worst-case scenario.

CRediT authorship contribution statement

Yvonne Bigott: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Sara Gallego:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Nicola Montemurro:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Marie-Christine Breuil:** Formal analysis. **Sandra Pérez:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Antonios Michas:** Formal analysis, Data curation. **Fabrice Martin-Laurent:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. **Peter Schröder:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154674.

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