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Short-term effect of reclaimed wastewater quality gradient on soil microbiome during irrigation

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Keywords

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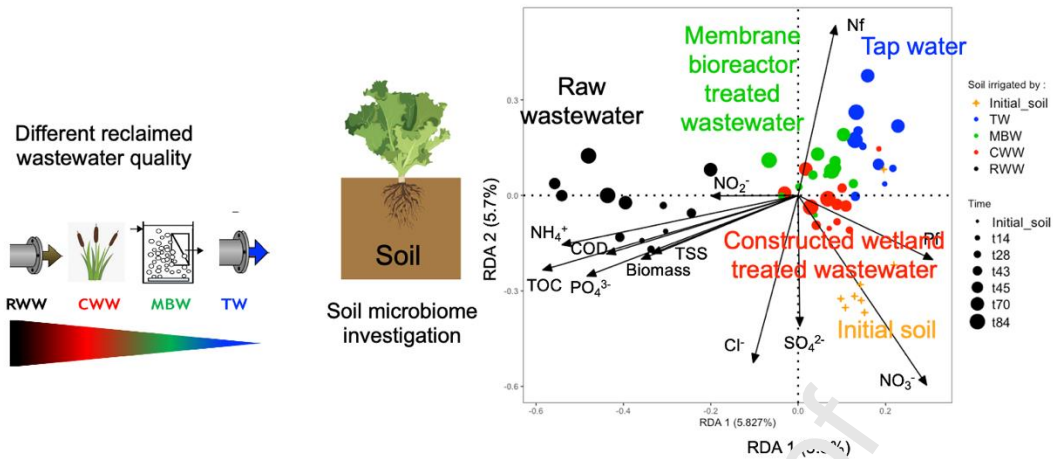
Highlights

- Impact of a reclaimed wastewater quality gradient on soil bacterial communities.
- Only raw wastewater increased the bacterial biomass in irrigated soil.
- Water quality and load shaped the microbial community structures and composition.
- Water quality influenced the abundance of nitrogen-transforming bacteria.

Abstract

To investigate the effect of wastewater (WW) treatment on soil bacterial communities, water of different quality was used to irrigate eight lettuces per tank: raw municipal wastewater (RWW), WW treated with an aerated constructed wetland (CWW) and WW treated with a membrane bioreactor (MBW), and tap water (TW). The physicochemical and microbiological characteristics (quality indicators) of these water types were characterized, and the water and soil bacterial communities were monitored by quantitative PCR (qPCR) and 16S rRNA gene sequencing. Despite marked differences in microbial load and diversity of waters, soil communities remained remarkably stable after irrigation. Microbial biomass was increased only in soils irrigated with RWW. At the end of the irrigation period (day 84), soil and water shared a large fraction of their bacterial communities, from 43% to 70%, depending on the water quality, indicating a transfer of bacterial communities from water to soil. Overall, the relative abundance of *Proteobacteria* and *Acidobacteria* was increased and that of *Actinobacteria* was decreased in soils irrigated with MBW, CWW and even more with RWW. Multivariate ordination clearly separated soils in three groups: soils irrigated with the cleanest water (TW), with treated WW (MBW and CWW), and with untreated WW (RWW). Nitrifying, denitrifying, and nitrogen-fixing bacteria were quantified by qPCR targeting *amoA*, *narG*, and *nifH*, respectively. Nitrifying bacteria were the most affected by the water quality, as indicated by *amoA* copy number increase in RWW-irrigated soil and decrease in CWW-irrigated soil. Overall, the abundance of all three genes was positively influenced by RWW treatment. In conclusion, the 84 days of irrigation influenced the soil microbial communities, and the impact depended on the quality of the used water.

Graphical abstract



1 Introduction

Some arid and semi-arid regions have experienced severe water stress, while the number of regions reporting water scarcity has increased worldwide since the early 2000s (López-Serrano et al., 2021). Water scarcity is the result of several factors, including the heterogeneous distribution of resource and the increase of the world population and consequently of food and service demands (Becerra-Castro et al., 2015). Therefore, it is important to consider wastewater reuse solutions in agriculture to meet these needs. Irrigation with raw or treated wastewater has become an increasingly common practice, particularly in Jordan, Israel, Spain, Italy and the United States of America (Partyka and Bond, 2022). In France, less than 1% of treated wastewater is used for irrigation, compared with 8% in Italy and 14% in Spain (Ait-Mouheb et al., 2020; Jones et al., 2021).

Treated or raw wastewater can have beneficial effects on crop development, such as addition of organic and inorganic matter (Ait-Mouheb et al., 2022; Cosenza et al., 2022; Lahlou et al., 2022). However, in too high quantities, wastewater negatively affects the soil physicochemical characteristics and microbial life (Ibrahimi et al., 2022). Nutrients and

organic compounds (e.g. organic matter, nitrogen, phosphorus, and undesirable salts) present in effluents are the main drivers of the soil physical and biological balance (Jaramillo and Restrepo, 2017; Radini et al., 2023). Studies on the soil microbial community structure, biomass, and activities have increased in recent years. Several authors have reported that the microbial biomass is increased following the use of raw and treated wastewater for irrigation due to the input of organic matter and nutrients (Guedes et al., 2022; Mkhinini et al., 2020). The direct consequences of nutrient and organic matter supply are the stimulation and development of microbial communities involved in nutrient and organic matter metabolic processes (Alegria Terrazas et al., 2022; Zhang et al., 2022). This multiplication of microorganisms implies positive effects, but it can also have detrimental effects on the soil structure. For instance, increased microbial activity promotes the development of biofilms and may lead to soil clogging, and consequently to loss of hydraulic conductivity and reduced nutrient circulation (Leonel and Tonetti, 2021; Nie et al., 2011). Besides the microbial biomass changes, metabolic processes related to nutrients, such as carbon, nitrogen, phosphorus and sulfur, also may increase, suggesting an increase in the related enzymatic activities (Mkhinini et al., 2020). However, some authors reported an inhibition in microbial activity (Leonel and Tonetti, 2021; Sparling et al., 1999) that could be attributed to several contaminants.

The findings of studies on changes in microbial diversity and richness of soils irrigated with treated wastewater are contradictory (Dang et al., 2019; Ibekwe et al., 2018; Lüneberg et al., 2018; Obayomi et al., 2020a). Ibekwe et al., 2018 showed that compared with synthetic fresh water, treated wastewater (flood irrigation) did not have a negative impact on microbial diversity and richness in soils without plant crops. Most studies highlighted a net impact on microbial communities for different soil crop types (corn, wheat or cucumis) after the irrigation period (Dang et al., 2019; Lüneberg et al., 2018; Obayomi et al., 2020a).

Specifically, these effects occur at different phylogenetic scales and they might affect the microbial community functional traits, showing the complexity of such changes (Dang et al., 2019; Farhadkhani et al., 2018; Lüneberg et al., 2018; Obayomi et al., 2020a, 2020b). Reclaimed wastewaters appear to favor microorganisms involved in nitrogen transformation (Ibekwe et al., 2018; Lüneberg et al., 2018). The changes in the general structure of these communities are directly or indirectly related to the wastewater type (treated or raw) used for irrigation, and therefore to its physicochemical and microbiological characteristics (e.g. presence of nitrogen compounds or microbial concentration) (Farhadkhani et al., 2018; García-Orenes et al., 2015; Obayomi et al., 2020a, 2019).

Although the effect on soil bacterial communities has been already studied, it is important to better understand how structural and functional bacterial communities react to irrigation with reclaimed wastewater of different quality. Therefore, in this study, we used reclaimed wastewater with a quality gradient (from untreated wastewater to drinkable water) to assess the effect (i) on the soil bacterial diversity and richness and (ii) on the bacterial biomass associated with the nitrogen cycle. We hypothesized that when soil experiences pulse disturbances, the effect on the microbial community structure will be different depending on the quality of the irrigation water used, by promoting the activity of autochthonous microorganisms and/or by introducing allochthonous microorganisms. To meet our objectives and to test our hypothesis, we determined the microbial communities by 16S sequencing and targeted quantitative PCR (qPCR) (bacterial genes involved in the nitrogen cycle). The study lasted 84 days (i.e., two lettuce crop cycles) and soils were irrigated (drip irrigation system) using reclaimed wastewater with a quality gradient. This allowed us to describe the microbial community changes in soils and their functional potential.

2 Materials and Methods

2.1 Experimental set-up

The study was carried out in an experimental greenhouse at the Murviel-Les-Montpellier wastewater treatment plant (43.605034° N, 3.757292° E), France, in the Mediterranean basin. The wastewater treatment plant included the usual pretreatment (screening and grit removal) followed by a biological treatment with constructed wetland (CW: forced aeration filters with reeds), supplemented by phosphorus treatment with ferric chloride and extensive treatment in a lagoon. This wastewater treatment plant has a nominal capacity of 3000 inhabitant equivalents. Moreover, the facility is equipped with a pilot-scale membrane bioreactor for experimental requirements. This includes a physical barrier (with an ultrafiltration module) and a biological treatment of organic matter with nitrification (biological sludge composed of autotrophic microorganisms) without anoxic phase (i.e., no denitrification). Thus, wastewater that underwent four different treatment levels was available for our irrigation test: raw wastewater (PWW, after screening and grit removal), constructed wetland water (CWW), membrane bioreactor water (MBW), and tap water (TW). To follow pathogenic bacteria indicator (*E. coli* and *Enterococci*) removal in water samples Idexx quantification (Colilert18 et Enterolert, IDEXX Laboratories, Westbrook, ME) was performed once a week (n = 10). The mean removal of *E. coli* was 4.52 ± 0.67 and 5.21 ± 1.65 log MPN.100 mL⁻¹ for CWW and MBW respectively. And the mean removal of *Enterococci* was 4.46 ± 0.88 and 5.15 ± 1.45 log MPN.100 mL⁻¹ for CWW and MBW respectively.

The soil used was composed of loamy clay soils (24.5% clay, 32% fine silt, 13.7% coarse silt, 10.6% fine sand, and 19.2% coarse sand). Eight cultivation tanks (0.95x1.10 m (1 m²) area and 0.60 m depth (total volume: 0.63 m³)) were filled to grow Batavia lettuces (*Lactuca sativa*; 8 lettuces per tank) (Ait-Mouheb et al., 2022) that were irrigated for 43 and 39 days for cycle 1 and 2 respectively (from 13th April to 5th July 2021). The acclimatization

phase was carried out by growing phacelia (*Phacelia tanacetifolia*) to induce the soil microbial activity before the study. Self-regulating drippers were positioned at each lettuce plant and each dripper delivered an average water flow of 2 L.h⁻¹ (for an input of P = 2 bar). The flow rate of each dripper was monitored throughout the experiment. Soil moisture was controlled with several probes (EC5 and ECH20 10HS environment probes, group METER). Lettuces were irrigated twice per week during the first lettuce crop cycle and three times per week during the second cycle. The total water volume received by lettuces in each tank was 172 L.m² spread over the 84 days of the study.

The theoretical nutrient requirements for the growth of lettuces are as follows 9 gN, 1.65 gP, 20.3 gK per m² (Baseline Fig. 1). Then, fertilizers were added to the tanks where the soil did not cover the needs of the plants. During the first cycle, nitrogen fertilization was provided only for the tanks receiving tap water: around 13 g of nitrogen per tank. During the second cycle, the tanks receiving TW and CWW water were chemically fertilized with phosphorus (3 g and 4.75 g of phosphorus for TW and CWW respectively for each tank) and potassium (4.4 g for CWW for each tank). In both lettuce crop cycles, lettuce growth was similar in the tanks irrigated with MBW, CWW or RWW (data not shown). Conversely, lettuce growth was reduced in the tanks irrigated with TW (data not shown) even after fertilizer addition.

2.2 Water and soil physicochemical characteristics

The following physicochemical characteristics were assessed once per week in each water type (total n=12 samples/water type): total suspended solids (TSS), chemical oxygen demand (COD), electrical conductivity, pH, nitrogen compound concentration (nitrate: N-NO₃⁻, nitrite: N-NO₂⁻, and ammonia: N-NH₄⁺), phosphorus compound concentration (phosphate: P-PO₄³⁻), and salt concentrations (Cl⁻, Ca²⁺, Mg²⁺, K⁺ and Na⁺). The concentrations of nitrogen

and phosphorus compounds and COD were measured using a spectrophotometer and HACH reagents (DR1900, Hach Company, Loveland, CO, USA). pH and conductivity were determined using two probes (TetraCon 925 and Sentix 940, WTW, Wilhelm, Germany). Macronutrient concentrations were determined by ion chromatography (Dionex Thermo Fisher ICS 1000, Waltham, MA USA). TSS were determined using filters (Whatman® glass microfiber filters, GF/C) and standard methods. Total organic carbon (TOC) was measured after filtration (0.22 µm filter) with a TOC analyzer (TOC-V_{CSN}, Shimadzu Corporation, Japan). Cumulative loads were calculated according to the mean concentration of each parameter multiplied by the irrigation volume (n=8 samples/water type).

The soil sample used either for physicochemical parameters analysis or for microbial community analyses was taken thanks to a sterile metallic spatula allowing to collect a 7 cm depth soil. This soil was a composite sample of 4 samplings taken approximately 10 cm from the lettuce plant in the dripper humidified zone. To determine soil nutrient concentration changes that could affect the soil microbial communities, several parameters were monitored: pH, conductivity, N-NO₃⁻ (NF ISO 13373), N-NH₄⁺ (NF ISO 13878), Na⁺ (NF X 31-108), Cl⁻, phosphorus (NF ISO 11263), K⁺ (NF X 31-108), Ca²⁺ (NF X 31-108), Mg²⁺ (NF X 31-108) and organic matter percentage (NF ISO 14235). pH and conductivity were monitored once per week. Briefly, 2 g of soil were diluted in deionized water and shaken for 30 mins, and then pH and conductivity (NF ISO 11265) were measured with pH probes (TetraCon 925 and Sentix 940, WTW, Wilhelm, Germany) after water filtration (1.2 µm filters). The other elements were analyzed by an external laboratory according to the International Organization for Standardization (see above) (Celesta-lab, Manguio, France).

2.3 Microbial communities of water and soil samples

The microbial community diversity was analyzed in each water type. The different water types were sampled once every two weeks during the irrigation period at the irrigation network outlet. Water samples were filtered (0.2 µm Supor 200 PES Membrane Disc Filter, Pall Corporation) and stored at -20°C until DNA extraction. Genomic DNA was extracted using the PowerWater DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Composite soil samples were obtained by mixing four surface samples (0-7 cm) according to previous studies which have shown that surface soil hosts the more diverse and active bacterial communities (Eilers et al., 2012). Each composite soil sample was made of soil selected from the root network of four lettuce plants in each tank. Approximately 0.5 g of each composite soil sample was stored at -20°C until DNA extraction. Genomic DNA was extracted using the FastDNA SPIN kit (MP Biomedicals) following the manufacturer's instructions.

The water and soil genomic DNA quantity and purity were verified by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). DNA samples were stored at -20°C before sequencing.

2.3.1 Illumina sequencing

The V4 and V5 variable regions of the 16S rRNA gene were amplified using the primers 515U (5' -GTGYCAGCMGCCGCGGTA -3') and 928U (5' -CCCGYCAATTCMTTTRAGT -3') (Wang and Qian, 2009). Adapters were added during the second amplification for sample multiplexing. The resulting products were purified and loaded into the Illumina MiSeq cartridge for sequencing (paired end reads with a read length of 300 bp) according to the manufacturer's recommendations (v3 chemistry). Sequencing and

preparation of the data library were performed by the Genotoul Lifescience Network Genome and Transcriptome Core Facility, Toulouse, France (get.genotoul.fr). Mothur (version 1.39.5) (Schloss et al., 2009) was used to pair reads, filter and cluster sequences. Uchime was used to remove chimera. Sequences that appeared less than three times in the whole dataset were removed. Sequences were aligned using the SILVA database SSURef NR99 version 128 (Schloss et al., 2009). Sequences with 97% of similarity were sorted into operational taxonomic units (OTUs). Sequences were submitted to GenBank with the accession number: (*in progress*).

2.3.2 Bacterial DNA quantification by qPCR

Total bacterial DNA and bacterial genes implicated in the nitrogen cycle were quantified by qPCR with the primers listed in Table S1.

2.4 Statistical analysis

All graphical representations and statistical analyses were performed with R (R Core Team, 2022, version 4.2.2), using the packages ggplot2 (Wickham, 2016), vegan (Oksanen et al., 2007), microeco (Liu et al., 2021) and phyloseq (McMurdie and Holmes, 2012). Sequences were analyzed with the phyloseq and microeco packages. The Chao1 richness and the Shannon alpha diversity indices were calculated. Data were compared with ANOVA followed by post hoc tests. Differences in microbial community structures among samples were tested by one-way perMANOVA based on Bray-Curtis distances. The OTUs that contributed most to differentiate microbial community structures were tested with a similarity percentage (SIMPER) analysis (Clarke, 1993) that was performed by comparing irrigated soils at day 70 and 84. The percentages of shared bacteria were determined and visualized with Venn diagrams using the weighted abundances of all sequences calculated with relative

abundances and 16S rDNA gene copy abundances for each sample. Nitrogen cycle-related bacteria were selected for weighted abundance analysis and visualization according to Nelson et al., 2016.

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3 Results

3.1 Impact of reclaimed water quality on soil

Supplementary Table S2 lists the results (mean values) of the physicochemical characterization (quality indicators) of the four different irrigation water types (RWW, CWW, MBW, and TW) and Fig. 1 shows the cumulative loads brought into the soil by irrigation. Three groups of components could be identified: (i) components feeding the soil life: biomass, organic matter (TOC, COD, and TSS), (ii) nutrients (total nitrogen, phosphorus and potassium), and (iii) salts (Na^+ , Ca^{2+} , Mg^{2+} , and Cl^-). The following trends in their concentration in water and soil were observed (i) for components feeding the soil life: $\text{RWW} > \text{CWW} > \text{MBW} > \text{TW}$; (ii) for nutrients: $\text{RWW} > \text{MBW} > \text{CWW} > \text{TW}$; and (iii) for salts $\text{RWW} = \text{CWW} = \text{MBW} > \text{TW}$.

As expected, TSS concentrations were significantly different among water types ($p < 0.05$). The mean concentration was $41 \pm 9 \text{ mg.L}^{-1}$ in RWW, 57 mg.L^{-1} in CWW, and 0 mg.L^{-1} in MBW and TW. COD concentrations showed the same trend: $620 \pm 203 \text{ mg(O}_2\text{).L}^{-1}$ in RWW and 41 ± 17 and $89 \pm 60 \text{ mg(C}_2\text{).L}^{-1}$ in CWW and MBW, respectively ($p < 0.05$). For nitrogen compounds, the concentrations were significantly different among water types. In RWW, nitrogen was mainly in N-NH_4^+ form ($54 \pm 7 \text{ mg.L}^{-1}$) with very low N-NO_3^- ($1.4 \pm 2.4 \text{ mg.L}^{-1}$) and N-NO_2^- ($0.4 \pm 0.9 \text{ mg.L}^{-1}$) concentrations. In CWW, N-NH_4^+ was almost completely oxidized to N-NO_3^- ($21 \pm 9 \text{ mg.L}^{-1}$) with low N-NH_4^+ ($2.4 \pm 2.8 \text{ mg.L}^{-1}$) and N-NO_2^- ($0.8 \pm 0.8 \text{ mg.L}^{-1}$) concentrations. In MBW, the concentrations of the different nitrogen forms differed depending on the membrane bioreactor operation time (Fig. S1): N-NH_4^+ was the prevalent form in the first step (from day 1 to day 28), N-NO_2^- in the second step (from day 28 to day 45), and then N-NO_3^- (from day 45 to the study end).

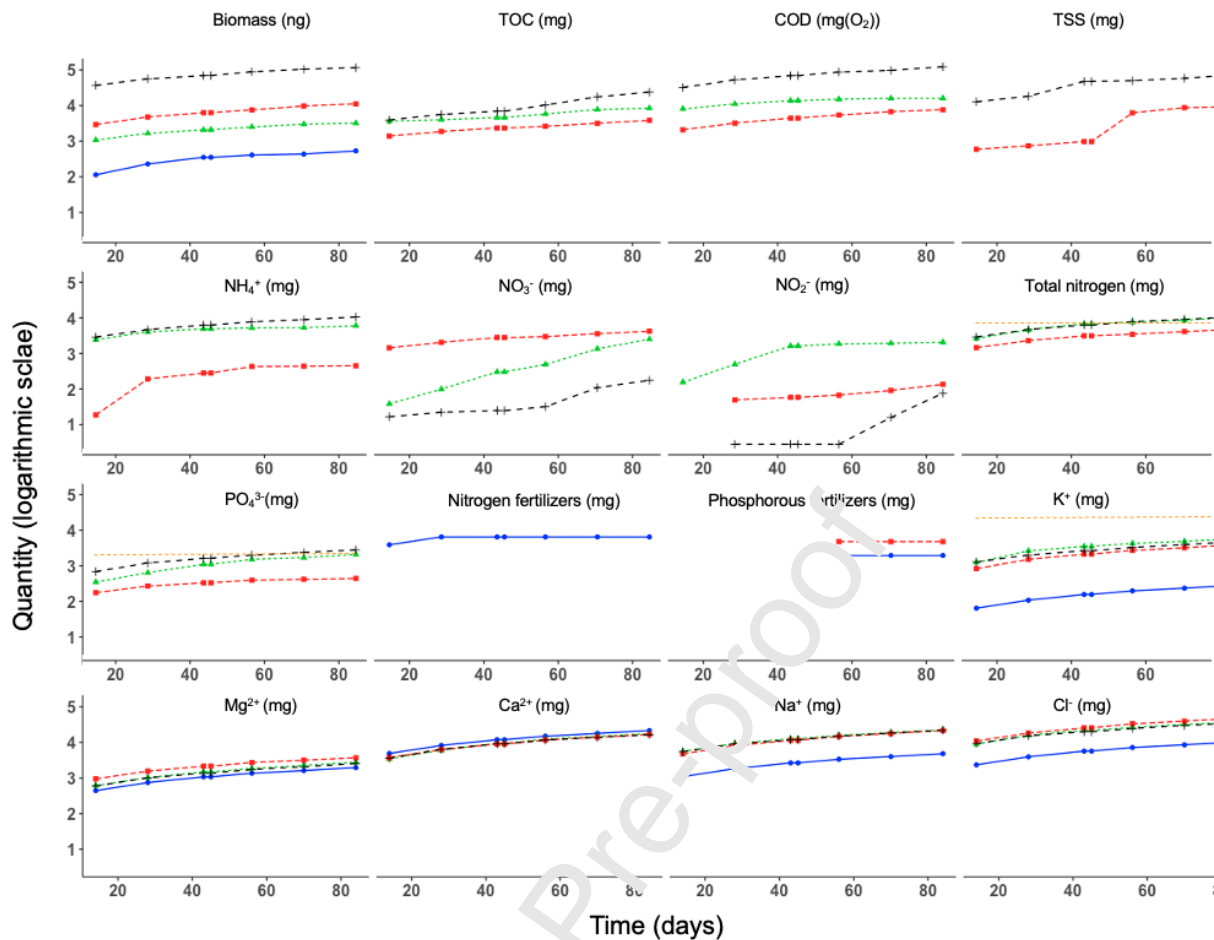


Fig. 1: Temporal changes in the cumulative loads (logarithmic scale) brought by irrigation water for biomass (ng), nutrients (mg), organic matter (mg for TOC and $\text{mg}(\text{O}_2)$ for COD), and salts (mg). TW (blue), MBW (green), CWW (red), and RWW (black). Data not displayed corresponds to 0. Nitrogen and phosphorus fertilizers are the chemical fertilizers provided. Orange dashed line show the baseline quantity of fertilizers needed for one lettuce growth cycle per m^2 .

3.2 Soil response to reclaimed water supplies

The soil physicochemical characters were monitored over time to evaluate their effect on the soil microbial community structures. The results are shown in Table 1. No significant difference was found for pH, organic matter, K_2O and Ca among all irrigated soil samples during the study period. The most drastic change was observed for N-NO_3^- concentrations in all soil types (Table 1) and for Na_2O between the TW-irrigated soil and the other soils.

Electrical conductivity also was higher in MBW-, CWW- and RWW-irrigated soils than in TW-irrigated soils at the study end. The soil electrical conductivity increased from the

beginning to the end of the first crop cycle: from 78 ± 20 to $184 \pm 64 \mu\text{S.cm}^{-1}$ (TW), from 90 ± 40 to $150 \pm 10 \mu\text{S.cm}^{-1}$ (MBW), from 100 ± 16 to $133 \pm 27 \mu\text{S.cm}^{-1}$ (CWW), and from 100 ± 4 to $156 \pm 2 \mu\text{S.cm}^{-1}$ (RWW). It then decreased between the end of the first crop cycle and the end of the second crop cycle in all soils. N-NH_4^+ concentration increased in all soils from the beginning to the study end. At the end of the study (day 84), N-NO_3^- concentration was increased by 7-, 7.5-, 2- and 2.5-fold in soils irrigated with TW, MBW, CWW, and RWW, respectively.

3.3 Temporal changes in bacterial biomass

The total allochthonous bacterial biomass that entered the soil was quantified by determining 16S gene copy number in the samples collected during the study (Fig. 2a). First, analysis of the different water types showed that the mean 16S gene copy numbers (i.e. microbial concentration) were significantly different ($p < 0.05$) among water types: $9.5 \pm 0.3 \log 16\text{S gene copies.mL}^{-1}$ for RWW (indicating high microbial concentration), 7.5 ± 0.4 and $7.4 \pm 2 \log 16\text{S gene copies.mL}^{-1}$ for MBW and CWW respectively (average concentration), and $5.7 \pm 0.5 \log 16\text{S gene copies.mL}^{-1}$ for TW (small concentration). During the irrigation period, no significant increase in 16S gene copies was observed in soils irrigated with TW, CWW or MBW (Fig. 2c) ($p > 0.05$) even after two lettuce crop cycles (Fig. 2b). Conversely, copy number increased from $7.7 \pm 0.1 \log$ (t0) to $8.3 \pm 0.1 \log$ gene copies.g⁻¹ (t84) in RWW-irrigated soil ($p < 0.05$). Although bacterial concentration was significantly higher in RWW-irrigated soils compared with the other soils ($p < 0.05$), it is still difficult to determine whether this change was caused by the microbial concentration in the water or the higher organic matter input.

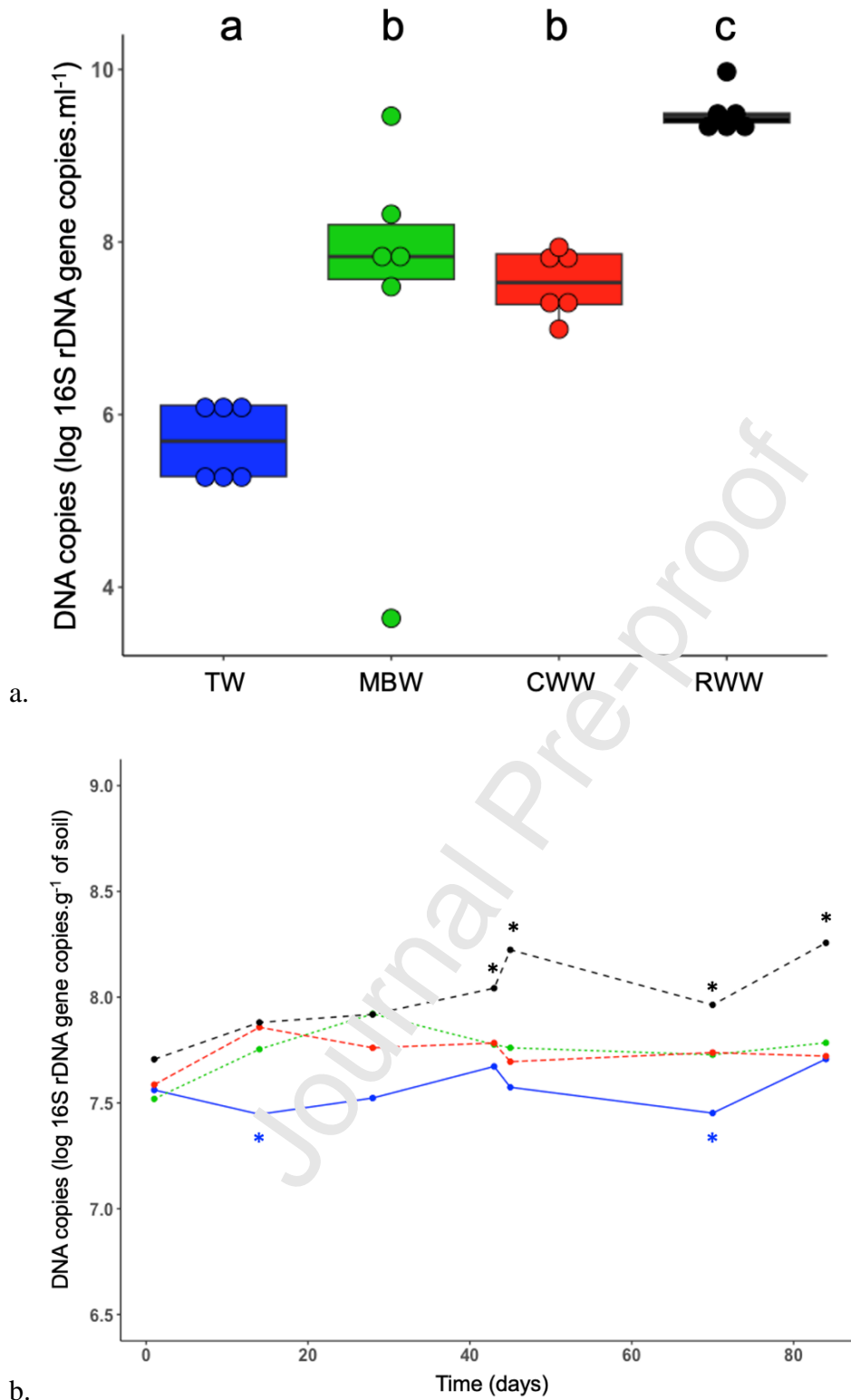


Fig. 2: Mean 16S rDNA gene copy number per mL of water or g of soil in water (a) and in soil (b) samples (n=6 for water and n=2 for soil samples). Letters and asterisks indicate significant differences between groups (one-way ANOVA). Blue: TW, green: MBW, red: CWW, and black: RWW.

3.4 Soil and water bacterial community diversity and richness

The Shannon diversity and Chao1 richness indices were calculated for soil and water samples during the two lettuce crop cycles (Fig. 3). The four irrigation water types displayed significantly different Shannon diversity index values, underlining differences in water quality (Table S2). During the lettuce crop cycle 1, this index was higher in RWW than in the other water types. During cycle 2, it was higher for CWW and RWW than for MBW and TW. Moreover, during the irrigation period, the Shannon diversity index increased only in RWW-irrigated soil (from 5 ± 0.3 at study start to 5.6 ± 0.2 at the study end), but not in the soils irrigated with the other water types (TW, MBW and CWW).

During the first lettuce crop cycle, the Chao1 index value (richness) was higher in CWW and RWW samples than in MBW and TW samples (1471 ± 151 and 967 ± 57 vs 112 ± 71 and 521 ± 135 , respectively). During the second cycle, the Chao1 index varied in all samples: 815 ± 100 , 873 ± 172 , 408 ± 143 and 328 ± 47 for TW, MBW, CWW and RWW, respectively ($p < 0.05$). A clear gradient among irrigation water types was observed, especially during cycle 2. Bacterial richness increased in RWW-irrigated soils throughout the irrigation period, from 1730 ± 49 to 2208 ± 90 (crop cycle 1) and to 2140 ± 211 (crop cycle 2) and also in CWW- and MBW-irrigated soils (from 1625 ± 11 to 2059 ± 124 and from 1769 ± 126 to 2080 ± 120 respectively). During the first crop cycle, the Chao1 index was significantly different between RWW- and TW-irrigated soils ($p < 0.05$). Conversely, no significant difference was observed among irrigated soils in cycle 2 ($p > 0.05$).

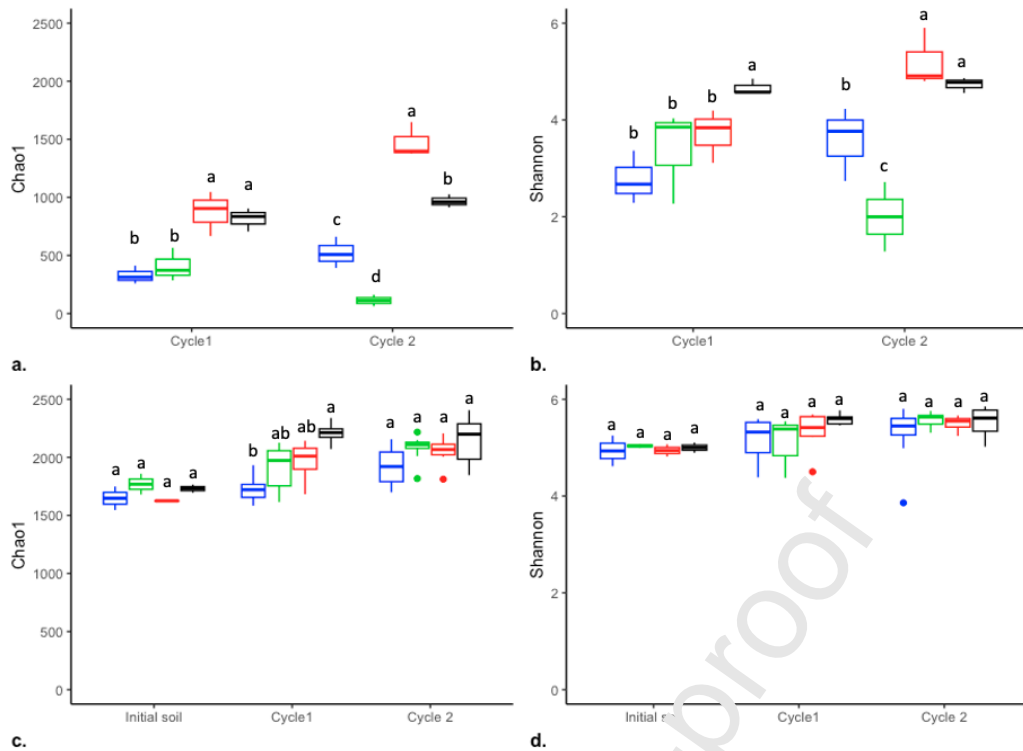


Fig. 3: Boxplots showing species richness (Chao1 index) (a and c) and diversity (Shannon index) (b and d) in irrigation waters (a and b) and soils (c and d) during two lettuce crop cycles. Letters indicate significant differences among groups (one-way ANOVA).

3.5 Shared bacteria between soil and water samples

The percentages of bacteria shared by water samples and soil samples irrigated with that water type were determined over time to assess the survival rate of water-borne bacteria in soil in function of their abundance (Fig. 4). At t0, the lowest mean percentage of shared bacteria ($6.9 \pm 2.5\%$) was between RWW and RWW-irrigated soil. This percentage increased for MBW and MBW-irrigated soil and CWW and CWW-irrigated soil ($49 \pm 27\%$ and $43 \pm 1\%$, respectively) and was highest ($70 \pm 5\%$) for TW and TW-irrigated soil. After 14 days of irrigation, the percentage of shared bacteria increased by 20% and 65% for CWW and RWW and their soils. Conversely, the percentage of shared bacteria did not change for TW and its soil during the whole irrigation period. After only 14 days of irrigation, the bacterial load provided by RWW was sufficient to have similar bacterial communities between water and irrigated soil. For CWW, the percentages of shared bacteria were identical at t0 and t84 ($47 \pm$

0.1%). For MBW, the percentage of shared bacteria decreased during cycle 1, reaching $\sim 30 \pm 2\%$ (the lowest value among all samples), but then increased during cycle 2 to $90 \pm 1\%$ at t70 and $83 \pm 8\%$ at t84.

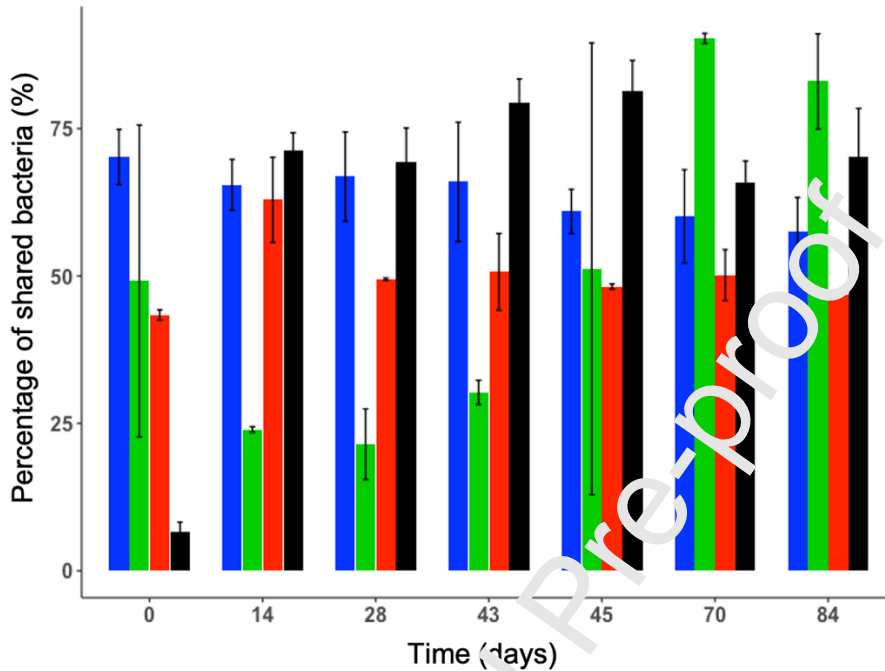


Fig. 4: Percentage of shared bacteria between soils and water according to the weighted abundances over time. TW: blue, MBW: green, CWW: red and RWW: black.

3.6 Influence of the soil and water characteristics on the soil bacterial community structure

Soil bacterial community structures were compared with a redundancy analysis (RDA) using Bray-Curtis distances and grouped by irrigation water type (Fig. 5). The first two axes of the RDA explained 11.5% of the total variance among soil microbial communities (Fig. 5). Then, soil microbial community differences among groups over time were calculated with a perMANOVA. This showed that RWW-irrigated soils were significantly different from the other soils (TW, MBW and CWW) ($p < 0.05$). Moreover, TW-irrigated soils differed significantly from soils irrigated with treated wastewaters (MBW and CWW) ($p < 0.05$). No

statistical difference was observed between soils irrigated with the two treated wastewater types (MBW and CWW).

RDA was also used to study the impact of water and soil physicochemical characteristics on the bacterial community structures (Fig. 5). This analysis was based on the cumulative load over time of the different elements brought by the irrigation waters. Nitrogen compounds (N-NH₄, N-NO₃ and N-NO₂, and ammonium from fertilizers) and organic matter (COD and TOC concentrations) explained most of the bacterial community structure differences. Moreover, nitrogen compounds were the soil characteristic with the strongest influence on the microbial community structures (data not shown).

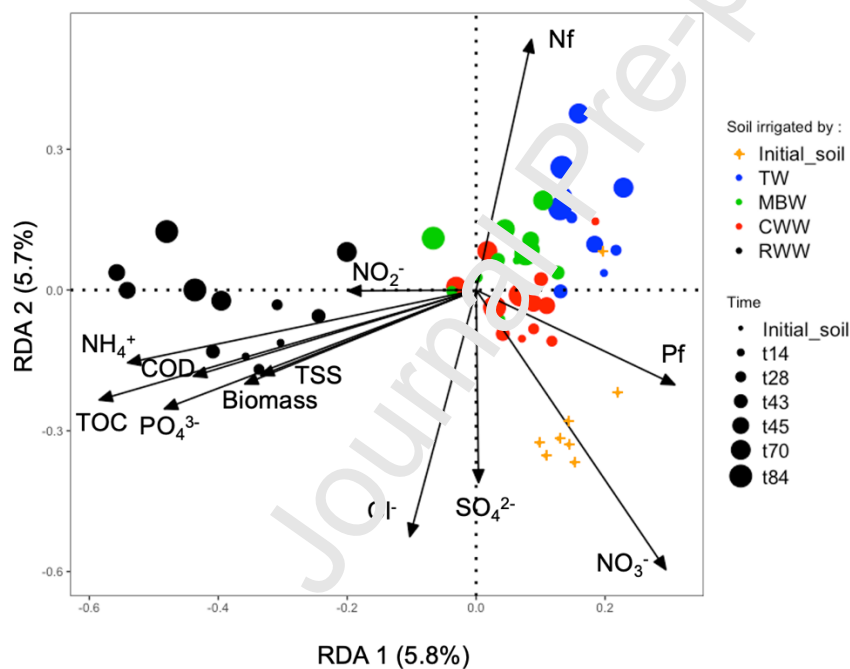


Fig. 5: RDA plot ordination displaying variations in soil bacterial communities. Data points are colored according to the irrigation water type (blue: TW, green: MBW, red: CWW, and black: RWW). The symbol size increases with time. The covariance between soil bacterial communities and water characteristics is indicated by arrows (only significant variables are displayed). Nf and Pf represent the nitrogen and phosphorous provided by the chemical fertilizers.

3.7 Composition of the bacterial community in soil and water samples

The relative abundances of bacterial phyla in soil and water samples were analyzed by 16S rRNA gene sequencing. *Proteobacteria* and *Bacteroidetes* were the most abundant phyla in all water samples (Fig. 6a). These phyla represented 90.0, 92.5, 81.0 and 69.5% of the relative abundance in TW, MBW, CWW and RWW, respectively. Moreover, the microbial community composition of RWW samples was different from that of the other water types. Indeed, the *Firmicutes* phylum was particularly abundant in RWW compared with CWW and MBW (25% vs 1% for both treated wastewater types).

In RWW-irrigated soils, the relative abundances of *Proteobacteria*, *Chloroflexi*, and *Planctomycetes* increased significantly from t0 to t84 (21% to 35.4%; from 3.7% to 7.3%; and from 3.4% to 4.2%) (Fig. 6b). Conversely, the abundance of other phyla decreased over time: *Actinobacteria* (from 17.2% to 10.7%), *Bacteroidetes* (from 29.8% to 20.8%), and *Firmicutes* (from 7.6% to 3.9%). In CWW- and MBW-irrigated soils, results were similar but with smaller variations. Specifically, the relative abundance of some phyla increased: *Proteobacteria* (from 23.5% to 26.1% and from 22.9% to 23.5% in MBW- and CWW-irrigated soils, respectively), *Acidobacteria* (from 6.5% to 9.6% and from 6.3% to 10.2%), *Chloroflexi* (from 3.7% to 6.3% and from 3.9% to 6%), and *Planctomycetes* (from 3.3% to 5.1% and from 3.2% to 5.4%). The abundance of other phyla decreased: *Actinobacteria* (from 16.9% to 10.4% and from 13.1% to 11% in MBW- and CWW-irrigated soils, respectively), *Bacteroidetes* (from 28.6% to 22.4% and from 31.2% to 23.5%), and *Firmicutes* (from 8.6% to 4.9% and from 8.6% to 5.4%).

SIMPER analyses were performed to identify the OTUs that explained most of the differences among soils (data not shown). The relative contribution of each genus did not exceed 3.2%. The relative abundance of the *Flavisolibacter*, *Cytophagaceae_unclassified* and *Lysobacter* genera differed significantly between TW- and RWW-irrigated soils and 58 OTUs

explained 50% of the relative difference. The relative abundance of the *Flavisolibacter*, *Pontibacter* and *Parafilimonas* genera was significantly different in TW- and CWW-irrigated soils and 75 OTUs explained 50% of the relative difference. The relative abundance of the *Flavisolibacter*, *Cyanobacteria (Family I)* and *Salinimicrobium* genera was significantly different between TW- and MBW-irrigated soils and 81 OTUs explained 50% of the relative difference. The relative abundance of the *Flavisolibacter*, *Cytophagaceae_unclassified* and *Ramlibacter* genera differed significantly between CWW- and RWW-irrigated soils and 59 OTUs explained 50% of the relative difference. The relative abundance of *Flavisolibacter*, *Cytophagaceae_unclassified* and *Lysobacter* was significantly different between RWW- and MBW-irrigated soils (like for TW- and RWW-irrigated soils) and 61 OTUs explained 50% of the relative difference. The relative abundance of *Flavisolibacter*, *Salinimicrobium* and *Lysobacter* was significantly different in MBW- and CWW-irrigated soils and 82 OTUs explained 50% of the relative difference. Moreover, the percentage of total OTU differences were higher in all comparisons involving RWW (65%, 60% and 63% for the comparison with TW-, MBW- and CWW-irrigated soils, respectively). The total percentages of OTU differences between soils irrigated with TW and with treated wastewaters (MBW and CWW) were 35% and 36%. The lowest percentage of total OTU differences (31%) was found for MBW- and CWW-irrigated soils.

RDA analyses were performed by comparing two by two the different irrigated soils (Fig. S2). Overall, the genus *Flavisolibacter* was more abundant in TW-irrigated soil and in the initial soil. The genus *Ramlibacter* was more abundant in soils irrigated with RWW, CWW and MBW, and particularly in RWW- and CWW-irrigated soils. The genus *Lysobacter* had one of the highest relative contribution difference in the comparisons involving RWW- with CWW- and MBW-irrigated soils where it was more abundant in RWW irrigated soils. Moreover, the genus *Pontibacter* was among the top fifteen genera involved in the relative

difference contribution for all comparisons. This genus abundance decreased progressively from RWW-, CWW-, MBW- to TW-irrigated soils. In conclusion, *Pontibacter* and *Lysobacter* could be seen as bacterial indicators of irrigation by raw wastewater, whereas *Flavisolibacter* abundance decreased when wastewater (treated or not) was used.

Bacteria with functional traits in microbial ecosystems were part of the top microorganisms involved in explaining the differences among the soils irrigated with the four water types. However, their contribution was lower than that of the previously quoted genera. Specifically, bacteria of the genus *Nitrospira*, of the family *Nitrosomonadaceae* and some Cyanobacteria, known as nitrifying or nitrogen-fixing bacteria, contributed to differentiate the different irrigated soils. Moreover, *Salinimicrobium* are bacteria mostly found in environments with high salinity levels. Bacteria involved in wastewater treatment processes also were found, such as *Comanodaceae_unclassified* (*Lysobacter*) and *Zoogloea*. Opportunistic pathogens, such as *Flavobacterium*, also were detected as well as genera implicated in soil-plant interactions, such as *Lysobacter*, *Cytophagaceae_unclassified*, *Longimicrobiaceae_unclassified* and *Atheribacter*.

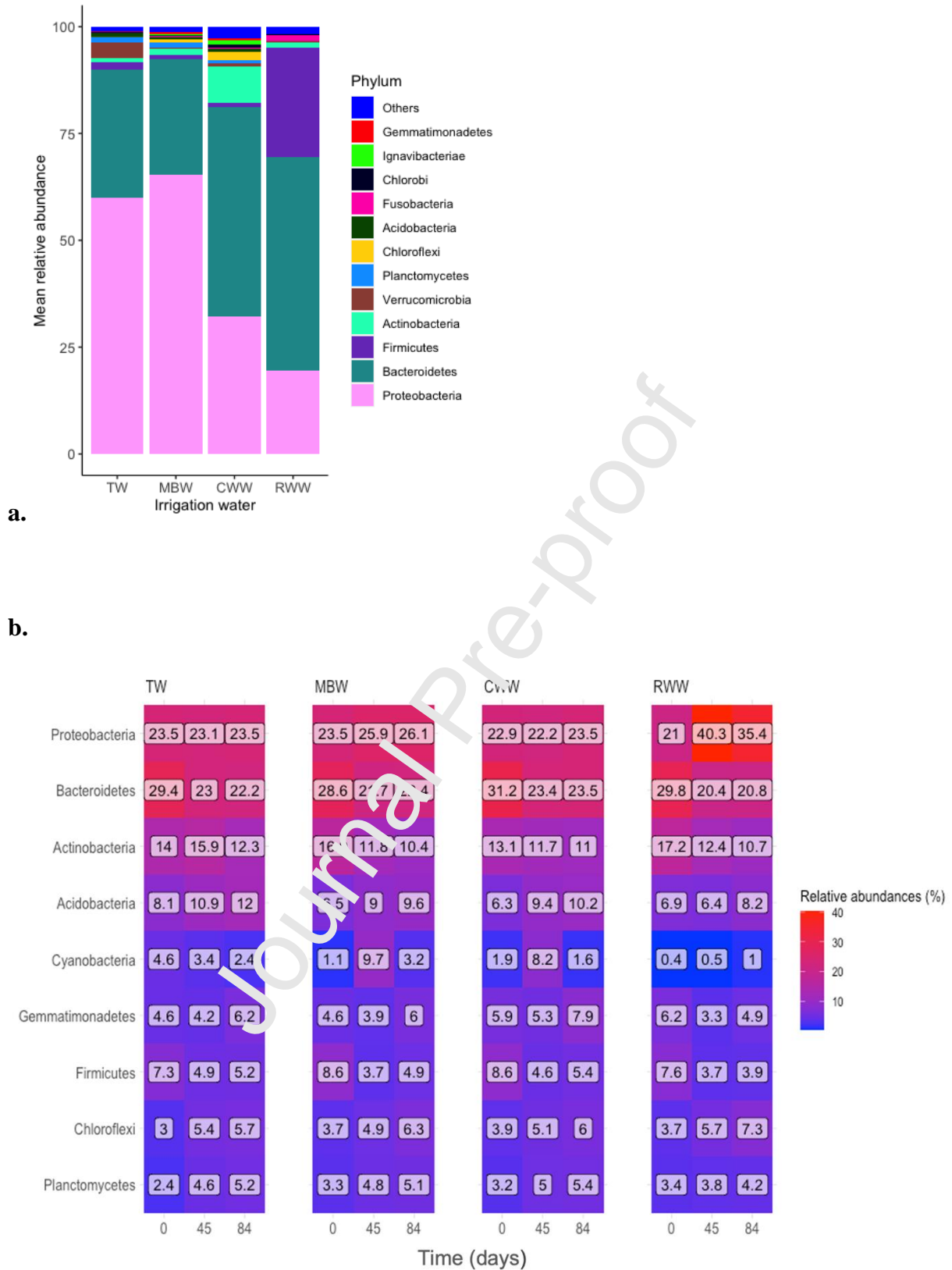


Fig. 6: Mean relative abundance of bacteria in irrigation waters (n=6) (a) and mean relative abundance (n=2) in irrigated soils (b) at the phylum level. Displayed phyla showed a change by 5% of their relative abundance between t0 and t84.

3.8 Changes in the bacterial communities associated with the nitrogen cycle

Three bacterial genes implicated in the nitrogen cycle were quantified to study the functional capacity of microbial communities to influence nitrogen availability for plant growth. Nitrification was targeted by quantifying the *amoA* gene, denitrification by quantifying the *narG* gene, and nitrogen fixation by quantifying the *nifH* gene (Fig. 7). Additional analysis of the weighted abundances were performed for the bacteria implicated in the nitrogen cycle (Fig. S3). No significant variation was found with this analysis. The *amoA* gene copy number (as a marker of nitrifying bacteria) increased in RWW-irrigated soil (from 6.3 ± 0.2 at t0 to 7.1 ± 0.2 log gene copies.g⁻¹ of soil at t84) and to a lesser extent also in MBW-irrigated soils (from 5.9 ± 0.1 to 6.7 ± 0.4 log gene copies.g⁻¹ of soil). Conversely, it strongly decreased in CWW-irrigated soils.

Overall, *narG* gene expression (as a marker of denitrifying bacteria in soils) was higher than that of the *amoA* gene (marker of nitrifying bacteria) and of the *nifH* gene (marker of nitrogen-fixing bacteria) in all irrigated soils. The number of *narG* gene copies was highest in RWW-irrigated soils than in all the other irrigated soils ($p < 0.05$).

The expression of the *nifH* gene (marker of nitrogen-fixing bacteria) did not change significantly in TW-, CWW- and MBW-irrigated soils during the irrigation period ($p > 0.05$). Conversely, at t84, its expression was higher in RWW-irrigated soil than in CWW-, MBW- and TW-irrigated soils (by 10.2%, 9.5% and 11.3%, respectively).

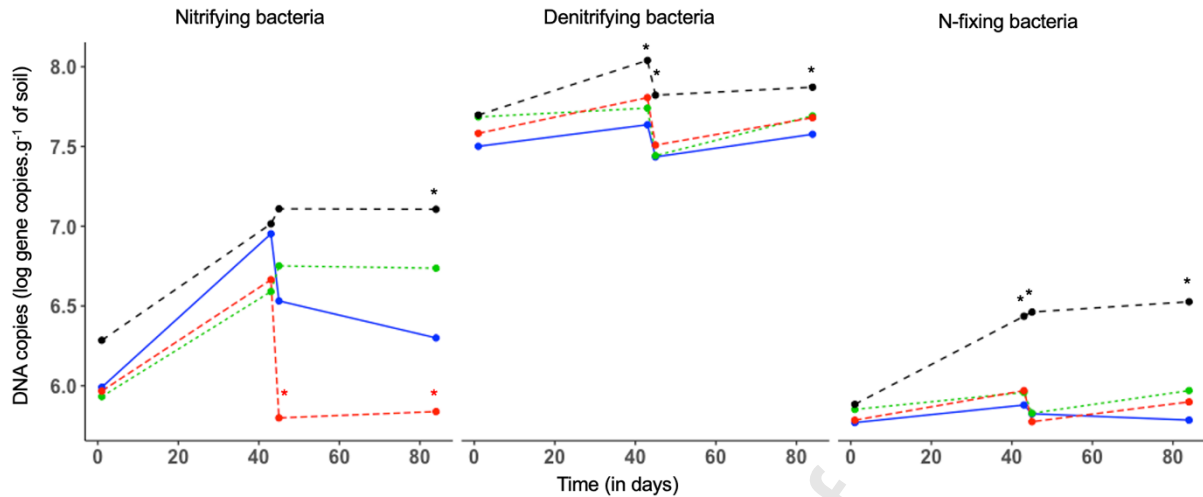


Fig. 7: Weighted abundances of selected genes implicated in the nitrogen cycle: nitrification (*amoA*) (a), denitrification (*narG*) (b), nitrogen fixation (*nifH*) (c). Asterisks indicate significant differences among irrigation water types (one-way ANOVA). Blue: TW-, green: MBW-, red: CWW-, and black: RWW-irrigated soils.

4 Discussion

In this study, the bacterial community structures were monitored during two lettuce crop cycles in which four water types (of different quality) were used for irrigation: raw wastewater (RWW), wastewater treated by planted aerated filters (CWW), wastewater treated by a membrane bioreactor (MBW), and drinking water (TW). During the study period (84 days) water and soil samples were collected to monitor exogenous (water) and autochthonous (soil) bacterial communities. This study focused on the overall structure of bacterial communities to assess their ecology upon exposure to irrigation with reclaimed wastewater of different quality.

The aim of this study was to test a water quality gradient and therefore, the legal requirements concerning the quality of reclaimed wastewater to be used for irrigation were not followed (Fig. 1). The analysis of the quality of the different water types showed differences in some physicochemical characteristics, such as nitrogen and phosphorous

compounds, biomass, salts and physical particles. Other studies on soil bacterial communities have often compared two water qualities (Cirelli et al., 2012; Obayomi et al., 2020a), whereas in this study we used a gradient of water quality.

The physicochemical parameters and bacterial communities were monitored in soils according to the water type used for their irrigation. After a short period of irrigation (84 days), the soil organic matter content did not change significantly. This result was in agreement with some previous studies (Chevremont et al., 2013; Kayikcioglu, 2012), but not with other works that reported an increase or a decrease in organic matter content (Mohammad Rusan et al., 2007; Xu et al., 2010). The increase in organic matter concentration was mainly found in soils after long-term wastewater irrigation. Thus, despite the different organic matter loads of the four water types, these results suggest a high biodegradability or transformation of the organic matter received in the soils. As previously shown in other studies, no pH variation was observed in soils during the irrigation period (Ait-Mouheb et al., 2022; Urbano et al., 2017). The concentration of nitrogen compounds increased over the irrigation period in all irrigated soils. This is not surprising because it is the consequence of the supply of nitrogen in dissolved form in MBW-, CWW-, and RWW-irrigated soils and of fertilizers in TW-irrigated soils. The concentrations of nitrogen compounds in soils increased at the study end compared with baseline. The highest concentration of NH_4^+ was detected in RWW-irrigated soils, but it was lower than that of NO_3^- compounds in all irrigated soils. This is mainly explained by the natural process of nitrification by nitrifying bacteria and the absence of leaching thanks to the drip irrigation system and control of the water needs using moisture sensors. In this study, a loamy clay soil was used because it promotes nutrient retention and allows better nutrient absorption by plants (Ma et al., 2022). Although the need of fertilizers by the plants were not totally covered by the nutrients brought by the irrigation water, nitrogen concentration was higher than expected in all irrigated soils. Our results on

electrical conductivity show little impact of irrigation, in line with the study by Wiel-Shafran et al., 2006. However, the same study showed that long-term reclaimed wastewater irrigation (12 months vs 84 days in the present study) influences the soil electrical conductivity (Wiel-Shafran et al., 2006).

The increase in microbial biomass was monitored through quantification of total DNA and 16S rRNA gene. Except for soils irrigated with RWW, 16S rRNA gene copies did not significantly increase during the study. This is in agreement with two studies showing that the microbial biomass in the soil was not increased after irrigation with treated wastewater (Obayomi et al., 2020a), but only after irrigation with untreated wastewater. High biomass loading in irrigation water had a direct effect on the bacterial concentration in the soil and influenced the soil bacterial composition and could also introduce allochthonous bacteria in soils.

The microbial diversity and richness indices of the irrigated soils increased over the irrigation period (Fig. 3). In line with previous results, our study did not show a graded effect of the treated water quality gradient (loads inputs) on the richness and diversity indices (Leonel et al., 2022). The increase of microbial diversity and richness in soils is a normal process when microbial communities experience stress disturbances brought by the inputs in irrigation waters (Shade et al., 2012). As already shown by Li et al., (2021), the overall soil bacterial community diversity and richness were affected by the supplied water by increasing soil moisture and not particularly by the provided nutrient loads (Table S1, physicochemical characteristics).

The analysis of the OTUs shared between soils and irrigation water types allowed measuring the transfer of bacterial communities from water into the soil (Fig. 4). Surprisingly, soils and waters shared a large fraction of their bacterial communities at the end of the irrigation period, from 43% to 70% of OTUs depending on the water type, indicating the

introduction of allochthonous bacteria in soils, particularly in the case of irrigation with RWW. A previous study already described the development and potential survival of bacterial species originating from water in soil (Mansour et al., 2018). Wastewater or treated wastewater can share many microorganisms, but could also have specific communities (Becerra-Castro et al., 2015). Here, for the first time, the percentage of shared bacteria between soil and water was used as an indicator of the potential increase of allochthonous bacteria in soil.

Differences in microbial community structures at the genus level allowed dividing soils in three groups: soils irrigated with the cleanest water (TW), with treated water (MBW and CWW), and with raw wastewater (RWW) (Fig. 5). These results are in agreement with previous studies showing differences in microbial community structure between soils irrigated with treated or raw wastewater and soils irrigated with tap water (Dang et al., 2019; Ibekwe et al., 2018; Obayomi et al., 2020a). Stone et al., 2023 showed the importance of studying bacterial communities at the genus level to follow their responses to a disturbance and for discriminating among samples. The change in bacterial community structure can be explained by the quick adaptation of the microbial community to the new environmental conditions. In the present study, the relative abundances of the *Acidobacteria*, *Planctomycetes* and *Proteobacteria* phyla were increased whereas those of *Actinobacteria* and *Firmicutes* were decreased (Fig. 6b). Previous studies already highlighted the increase of *Proteobacteria*, *Actinobacteria* and *Acidobacteria* (Obayomi et al., 2020a) and *Bacteroidetes* (Dang et al., 2019; Ibekwe et al., 2018). Moreover, *Pontibacter*, *Flavisolibacter* and *Lysobacter* were the genera that varied most in abundance in soils in function of the water used for irrigation. These are typical bacterial genera found in terrestrial environments (Kim et al., 2021; Liu et al., 2019; Maeng et al., 2021). A previous study also showed the high sensitivity of some families of terrestrial bacteria (e.g. *Cellvibrionaceae*, *Micropepsaceae* and *Burkholderiaceae*)

for differentiating soils irrigated with different water types (Leonel et al., 2022). It has been reported that the first reaction of microbial communities is an adaptation of indigenous microbes (Rillig et al., 2015). Moreover, the SIMPER analysis showed that many bacteria (from 58 to 82 OTUs involved in 50% of relative difference) explained differences among soils irrigated with different water types. In soils irrigated with treated or raw wastewater, microbial composition changes might be due to more complex mechanisms for some specific bacteria. The irrigation effect on soil bacterial communities was higher in RWW-irrigated soils than in MBW- and CWW-irrigated soils by differentiating even more the bacterial communities.

The load of nutrients (i.e., biomass, organic matter and nitrogen species) was the main factor explaining changes in the soil bacterial communities (Fig. 5). Previous studies already showed that the organic matter load is one of the main factors driving modifications in soil bacterial community structures (Martínez-García et al., 2018; Yanardağ et al., 2017). Our results are also consistent with studies showing the influence of nitrogen fertilizers on soil bacterial community structures (Francioli et al., 2016; Ramirez et al., 2012). As previously reported, the Proteobacteria and Firmicutes phyla were positively influenced by the fertilization of agricultural soils (Francioli et al., 2016). Indeed, these phyla include many bacteria involved in the nitrogen cycle (Dincă et al., 2022).

To study more in details some bacterial groups involved in nitrogen recycling (nitrifiers, denitrifiers and nitrogen fixers), key genes (*amoA*, *narG* and *nifH*, respectively) were quantified by qPCR (Fig. 7). The increase in nitrifying bacterial biomass was higher in RWW- than in MBW- and TW-irrigated soils and the concentration of nitrifiers was lower in CWW- irrigated soils. As the *amoA* gene is involved in the transformation of ammonium to nitrite, our results are not surprising due to the concentrations of nitrogen in ammonia form in the different water types. This shows the ability of these microbial groups to increase their

biomass to potentially increase the transformation of these compounds once in the soil. These results are in agreement with previous studies showing that ammonium input to soils increases the abundance of the *amoA* gene (Hellman et al., 2022; Marcos et al., 2021). The increase in denitrifying bacteria in soils irrigated with water with high organic matter load was already shown (Chen et al., 2021; Zhu et al., 2013). It could be explained by the nature of denitrifying bacteria. These heterotrophic bacteria switch to denitrification (using nitrate as electron donors) when oxygen is limited, a condition typically found in flooded soil during irrigation periods. Our results are also in agreement with previous studies showing a significant effect of nutrients on biomass growth linked to nitrogen-fixing bacteria, as indicated by the increase in *nifH* gene in soil (Meyer et al., 2013; Wu et al., 2021).

5 Conclusion

The soil microbial community structure, biodiversity and nitrogen functional traits were monitored during irrigation with different wastewater treatment plant effluent types. The main conclusions are:

- As expected, organic matter, nitrogen compounds and microbial biomass provided in irrigation water (oac) were the parameters that most influenced the structure of bacterial communities in soils.
- The bacterial biomass increased only in soils irrigated with RWW, but not in soils irrigated with treated wastewater (MBW and CWW) or potable water (TW).
- The soil bacterial community structure was progressively altered and soils could be differentiated in three groups in function of the water type: TW, MBW & CWW, and RWW.
- The presence of ammonia nitrogen (RWW, MBW) increased the biomass of nitrifying bacteria compared with CWW and TW.

- The main genera explaining the microbial community structure differences among soils are commonly found in soils, particularly bacteria related to nitrogen transformation.
- Bacteria shared among waters and soils increased only when the water contained high diversity and richness of bacteria (CWW, RWW).

The different quality of the water types used for irrigation influences and drives the soil biodiversity. No negative effect on the diversity and on the functional potential of bacteria related to the nitrogen cycle was observed.

Authors' contribution

VM, NAM, GL, NW, LK and MH conceived the study. VM, LK and VB did the experiments and sample processing with support of NAM, GL, JH, NW and MH. VM analyzed the data with the support of JH and NW. VM wrote the manuscript to which all other authors contributed.

Conflicts of 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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Table 1: Physicochemical characteristics of the soil surface in function of the irrigation water type at study initiation (before lettuce planting; t_0), the end of first lettuce crop cycle (t_{43}), and end of second lettuce crop cycle (t_{84}).

Parameter	Unit	TW			MBW			CWW			RWW		
		t_0	t_{43}	t_{84}	t_0	t_{43}	t_{84}	t_0	t_{43}	t_{84}	t_0	t_{43}	t_{84}
N-NO ₃ ⁻	mg.kg ⁻¹	8 ±	43.7	64.6	17.6	38.8	150.2	45.7	21.2	136.8	47.9	140.6	167.8
	¹	8.2	±	±	±	±	± 12.7	± 6	±	± 115	±	± 91.1	± 72.1
			19.2	41.2	17.7	36.3			12.2		32.3		
N-NH ₄ ⁺	mg.kg ⁻¹	0 ±	0 ± 0	0.3 ±	0 ± 0	0 ± 0	0.85 ±	0 ±	0 ± 0	1.85 ±	0.05	0.05 ±	1.7 ±
	¹	0		0.14			0.64	0		0.92	±	0.07	1.98
										0.07			
P ₂ O ₅ Olsen	µg.kg ⁻¹	32	28 ±	44 ±	41 ±	28 ±	80 ±	35 ±	34 ±	81 ± 5	44 ±	39 ±	53 ±
	¹	±	11	29	4	8	57	3	5		2	13	13
		11											
MO	%	1.2	1 ± 0	1.2 ±	1.3 ±	1.2 ±	1.4 ±	1.2	1.2 ±	1.3 ±	1.3 ±	1.3 ±	1.4 ±
	¹	±		0.1	0	0.1	0.1	±	0.1	0	0.1	0.1	0.2
		0.1						0.1					
pH eau		8.3	8.4 ±	8.4 ±	8.4	8.5 ±	8.3 ±	8.4	8.5 ±	8.1 ±	8.4 ±	8.2 ±	8.3 ±
	¹	±	0	0	0	0.2	0.1	±	0.1	0.1	0.1	0.1	0.1
		0.1						0.1					
Conductivity	µS.cm ⁻¹	78	184	54 ±	90 ±	150	78 ± 8	101	133	77 ± 1	100	156 ±	89 ±
	¹	±	± 64	±	40	± 10		± 16	± 27		± 4	2	10
		20											
Ca total	g.kg ⁻¹	711	373	677	671	742	693 ±	715	508	740 ±	510	657 ±	652 ±
	¹	±	±	± 28	± 17	± 61	8	±	±	9	±	6	76
		75	363					109	236		163		
K ₂ O	µg.kg ⁻¹	180	163	158	161	155	179 ±	183	162	193 ±	188	174 ±	176 ±
	¹	±	± 16	± 12	± 9	± 21	13	± 4	± 12	12	± 15	8	8
		41											
MgO	µg.kg ⁻¹	209	201	226	258	223	300 ±	244	260	385 ±	231	231 ±	233 ±
	¹	± 7	± 24	± 45	± 43	± 48	21	± 37	± 22	40	± 11	6	10
Na ₂ O	mg.kg ⁻¹	104	78 ±	135	348	277	599 ±	341	346	1137	256	478 ±	346 ±
	¹	±	18	± 45	±	± 21	34	± 67	±	± 165	± 70	205	47
		11			156				115				