

EVOLUTION OF ANTIBIOTIC RESISTANCE ALONG THE TREATED WASTEWATER-SOIL-PLANT CONTINUUM OVER TWO SEASONS OF CROP IRRIGATION

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ABSTRACT

1. Introduction

The reuse of treated wastewater (TWW) for crop irrigation is a solution to reduce the pressure on freshwater resources (Ait-Mouheb et al., 2020). Even if this practice is subject to regulations, it still poses a health threat as it may carry antibiotics, antibiotic resistant genes (ARG), antibiotic resistant bacteria and pathogens, not considered in regulations (European Parliament, Council of the European Union, 2020). This practice could thus lead to antibiotic resistance (ATBR) dissemination in the environment. Numerous studies have investigated the effect of TWW quality on ATBR dissemination in soils (Gatica and Cytryn, 2013; Della-Negra et al., 2024). However, relatively few studies have taken into account the role of plants in the mitigation/accumulation of ATBR or the transfer of ATBR markers within plants.

The aim of this study was to measure the effect of three water qualities on the fate of ATBR in the soil, the rhizosphere and within the roots of leek and lettuce, over two seasons of crop irrigation. Through these experiments, the following questions were addressed (i) What is the effect of water quality on the evolution of ATBR in soils and plants? (ii) What is the effect of the plants on the spatial distribution of ATBR (barrier effect or transfer)? (iii) Does the nature of the plants (leek vs lettuce) have an effect on the above questions? (iv) How do the ATBR markers evolve between the first and second crop irrigation seasons?

2. Material and Methods

Experiments were conducted in a greenhouse located at the wastewater treatment plant at Murviel-Les-Montpellier, in microcosms (large bins 1 m² x 0.6 m depth) containing a loamy clay soil and either Batavia lettuces (four lettuces per bin) or leek plants (16 leeks per bin). Over two years, 2 irrigation cycles were conducted (June-September 2018 and May-August 2019). Lettuces and leeks were irrigated using 3 different water qualities: tapwater (TP), treated wastewater (TWW) and raw wastewater (RW). The wastewater was treated by stabilisation ponds and three successive lagoons. The plant has a nominal capacity of 1,500 Inhabitant Equivalent. Surface drippers were positioned at each lettuce and leek plant: each delivering a nominal flow rate of 2 L.h⁻¹. A similar experimental approach was already applied successfully and is described in (Moulia et al., 2023).

Soil, rhizosphere and root samples were sampled over the two cycles of irrigation at different time point to extract DNA and perform qPCR analyses. Genes related to antibiotic resistance (macrolide resistance: *ermB*, mobile genetic element: *intl1* and sulfonamide resistance: *sul1*) and the V3 region from 16S rDNA were thus quantified. 16S rDNA sequencing was also carried out on all samples.

3. Results and Discussion

First, the results obtained on the soil seemed to indicate that the presence and nature of the plant (leek or lettuce) had no influence on the ARGs studied here, nor on the abundance of 16S rDNA. However, the structure of the soil bacterial communities was marked by a slight increase in Acidobacteria (subgroup 6) in the presence of leek. This observation has already been made in other studies, which suggests that the local conditions established

by the leek roots could have a slight impact on the pH of the soil, to which Acidobacteria are highly sensitive (Rocha et al., 2013). The dynamics of 16S rDNA in the soils followed an overall decrease during cycle 1 of irrigation and the latent phase before cycle 2 (Figure 1a, in the soil irrigated by TP). It seems that the soil used in 2018 had not reached a state of equilibrium. It was only during cycle 2 that a significant increase in 16S rDNA was observed in soils irrigated with RW or TWW, which could be explained by a greater supply of nutrients, thereby promoting the growth of bacterial communities.

While the dynamics of the *sull* gene was correlated with that of 16S rDNA (Figure 1c, $R^2 = 0.7$), the evolution of the *intl1* gene seemed to be related to the direct addition of RW or TWW (Figure 1b). An increase in the abundance of this gene was observed just after the addition of water followed by a decrease during the latent phase (return to the initial level). The increase in this indicator is often observed with the addition of RW/TWW and is a proxy for horizontal transfer of resistance genes, favoured by the presence of resistant bacteria in these waters but also by the effects of selection pressure exerted by the residual presence of contaminants (Gillings et al., 2015).

Unlike the others, the *ermB* gene was not observed in the soil irrigated with TP and its abundance increased continuously all along the cycle 1 and 2 before decreasing at the end of cycle 2 (Figure 1d). This gene, which encodes for macrolide resistance, could be maintained due to the soil persistence of macrolides (present in RW/TWW), exerting thus a constant selection pressure on the soil microbial community (Schlüsener and Bester, 2006). At the end of cycle 2, 3 significantly different levels could be observed for all the genes quantified here, and corresponding to the water quality level (TP<TWW<RW). There also appeared to be a drop in resistance at the end of cycle 2, particularly with TWW, suggesting resilience/adaptation of the soil ecosystem (Jaunatre et al., 2013) and underlying the importance of treatment in reducing nutrient, microbial and contaminant supplies to the soil.

Even if the soil bacterial community structure did not evolve a lot during the study, statistical analyses highlighted the emergence/selection of specific taxa in soil irrigated with RW and TWW. Indeed, we observe a significant increase of Rhodocyclaceae family in soil irrigated with RW and TWW, emphasized during the cycle 2, and a significant increase in *Ignavibacterium* and *Thiobacillus* specifically in soils irrigated with TWW (Figure 1e-f). According to the literature, these taxa are associated with high levels of organic matter, can degrade organic pollutants, can be selected in the presence of antibiotics and carry ARGs (Wang et al., 2020; Wu et al., 2024). Here, it is highly likely that water quality affects the physico-chemical properties of soils and contaminant levels, providing specific niches for the development of these species.

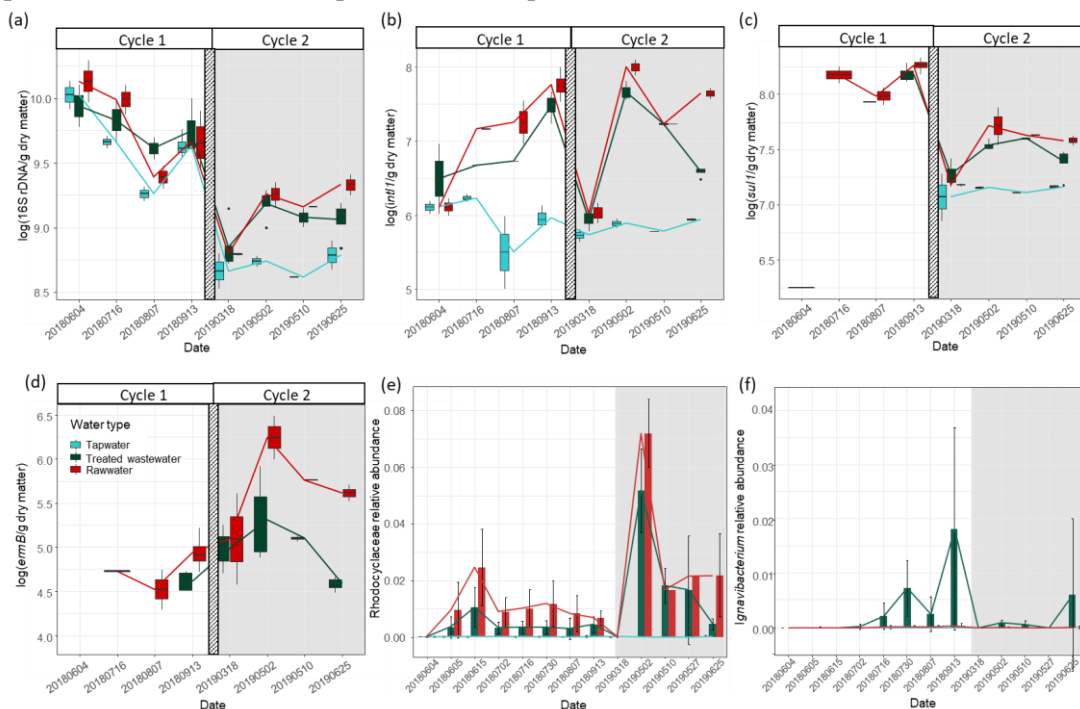


Figure 1. Temporal dynamic of (a) 16S rDNA, (b) *intl1* gene, (c) *sull* gene and (d) *ermB* gene in soils irrigated with tapwater, treated wastewater or raw water over two cycles of irrigation. Temporal dynamic of the relative abundance of (e) Rhodocyclaceae family and (f) *Ignavibacterium* in soils irrigated with tapwater, treated wastewater or raw water over two cycles of irrigation. Average from four biological replicates were displayed here.

In the rhizosphere and roots, distinctive bacterial communities were observed between leek and lettuce (Figure 2a). The root microbiome is different from one plant to another linked to the plant physiology, structure of the root and root/soil interactions. here, the rhizospheric soil may be influenced by the root microbiome and the root functioning (Zolti et al., 2019).

However, the type of plant did not have an effect of antibiotic resistance gene abundance in this study. Irrigation water type might exceed the effect of soil or plant type on root/rhizosphere resistome (Zolti et al., 2019).

Despite the different bacterial community structure, ARG abundance in rhizosphere was of the same order of magnitude of that found in soils. In roots, the abundance of ARGs was lower (Figure 2b, up to 1 log lower for *int11* or *sull1*, *ermB* gene was not detected in roots). However, at the end of cycle 2, a higher ARG abundance was observed in roots when RW was used by comparison to TP. This increase could be due to the presence of Rhizobiales and Gammaproteobacteria in roots, which are copiotrophic bacteria, known to be hosts of endophytic ARGs and prone to disseminate ARGs via horizontal gene transfer (Gao et al., 2023; Zolti et al., 2019). Contrary to other studies, roots seemed to act as a buffer/barrier effect here, mitigating ARG dissemination.

Among the previous indicator taxa identified in soils with RW or TWW, a slight selection effect was also observed in roots irrigated with RW or TWW but to a lesser extent compared to soil or rhizosphere, suggesting once again a buffer effect of roots. However, specific taxa were identified in roots and were attributed to *Rhizorhapis*, increasing when RW was used (Figure 2c) and *Tahibacter* increasing when both TWW and RW were used (Figure 2d). These two bacteria were included in Proteobacteria phyla known to increase in root environment irrigated with TWW (Zolti et al., 2019). *Rhizorhapis* known to cause corky root disease in lettuce is generally favored when exposed to high concentration of nitrogen, which is the case with RW (van Bruggen et al., 2015). Its prevalence could explain the presence of damaged leaves on RW-irrigated lettuces.

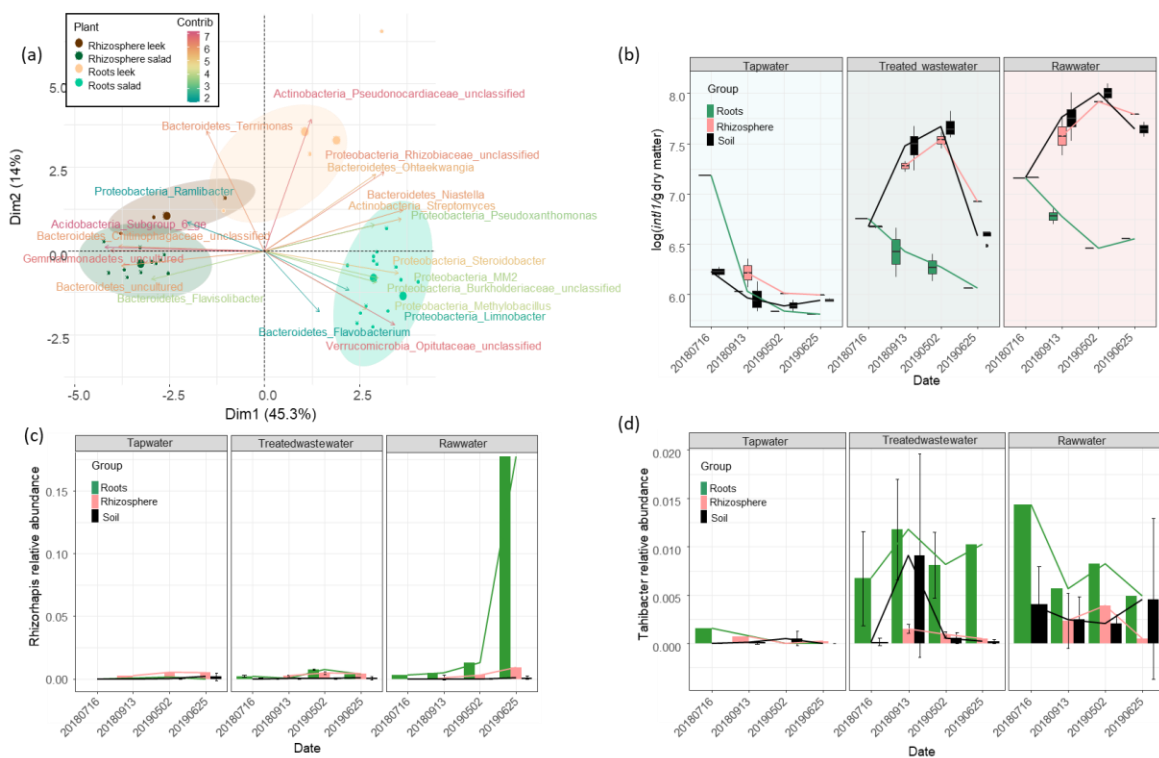


Figure 2. (a) Principal coordinate analysis of the bacterial community structures in leek and lettuce rhizospheres and roots and the contribution of the 20 most abundant genera. (b) Temporal dynamic of *int11* gene in soil, rhizosphere and roots of lettuce. Temporal dynamic of the relative abundance of (c) *Rhizorhapis* and (d) *Tahibacter* in soil, rhizosphere and roots of lettuce irrigated with tapwater, treated wastewater or raw water over two cycles of irrigation.

4. Conclusions

In conclusion, the use of TWW (and RW) for fields irrigation seemed to lead to an increase of the ATBR indicators in soils, rhizosphere and roots associated with an increase in the relative abundance of some Firmicutes

and Proteobacteria specific to soil/rhizosphere and roots. If plant types influenced microbial community structure in root and rhizosphere, it seemed that it did not impact ARG abundance and bacterial indicators in soils. ARG and bacterial indicator increases were associated with TWW and RW irrigation and returned to initial thresholds between the irrigation cycles, suggesting a transient phenomenon associated with soil resilience (Frenk et al., 2015). Plants and more specifically roots seemed to act as a barrier to ATBR indicators, mitigating ARGs increases. However, irrigation with RW promoted the development of *Rhizorhapis* in roots after two irrigation seasons of lettuce which had deleterious effect on lettuce quality. Further studies are needed to assess whether the root buffer effect on ATBR is due to a physical limitation (limited transfer of antibiotics/resistant bacteria) and/or to endophytic bacteria mitigating the transfer of antibiotic resistant bacteria.

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