Universitat Politècnica de Catalunya

Influence of agricultural practices on the Microbiome and the Antibiotic Resistance Gene complement in soils, plants, and crops

by

Francisco Diogo de Almeida Cerqueira

Department of Agri-Food Engineering and Biotechnology

Doctoral Programme Agri-Food Technology and Biotechnology

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Supervisor: Dr. Benjamín Piña i Capó (IDAEA-CSIC)
Co-supervisor: Prof. Josep Maria Bayona i Térmens (IDAEA-CSIC)
Advisor: Dr. Nuria Cañameras i Riba (UPC)

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La lutte elle-même vers les sommets suffit à remplir un cœur d'homme. Il faut imaginer Sisyphe heureux.

Albert Camus, Le Mythe de Sisyphe

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Abstract

Antibiotic (AB) Resistance is an increasing global problem for public health, with multi-resistant bacteria persisting and spreading not only in clinical settings but also in the environment. The Wellcome Trust estimated 10 million deaths worldwide by 2050 due to AB resistant pathogenic bacteria. One of the possible vectors of this spread is the use and re-use of waste water (WW), manure and sludge from WW treatment plants (WWTPs) in different agriculture practices (irrigation, soil amendment). These practices are becoming widespread worldwide, especially in regions with scarcity of water. WWTPs are known as AB resistance hotspots, whereas soil amendment by organic fertilization is known to increase AB resistance in the soil microbiomes. Hence, there is a growing concern about the possible transmission of AB resistance from agricultural soils to crops and, ultimately, to the microbiomes of consumers' guts.

Studies about the uptake of AB Resistant Bacteria (ARB) and Antibiotic Resistant Genes (ARGs) have been mainly limited to soil and root studies. Therefore, research regarding the spread of ARBs and ARGs into the environment and their transmission into the food chain is of the utmost importance. Currently, there are no studies addressing the impact of irrigation water quality and soil amendment in the ARGs and microbiomes along the soil-plant-food continuum. In this Thesis, the latter issue was assessed in commercial fields producing vegetable crops. This strategy allowed us to analyze AB resistance elements in foodstuffs actually reaching the consumers, and to be able to predict the potential risk for consumers, due to the entering of ARGs into the food-chain. Moreover, this Thesis tackles the alterations in soil, plant and crops bacterial communities due to several agricultural practices, and the association of taxa with the ARGs and *intI*1, an anthropogenic pollution marker. To achieve this goal, five agricultural fields located in the area of Barcelona (NE Spain) were selected, under different irrigation regimes and soil amendment. The selected crops were lettuce, tomatoes, and broad beans, as they represent vegetables with different edible parts, commonly eaten either raw or cooked.

The Thesis is divided into six chapters. Chapter I describes the state of the art of the studies of ABR in food and agricultural fields and presents the objectives of the PhD project. Chapter II and III assess the distribution of ARGs and microbiomes along the soil-plant continuum in *Lycopersicum esculentum* and *Vicia faba* in peri-urban and rural agricultural fields under different irrigation regimes and soil amendments. Our data show that field practices influenced

the distribution of ARGs and *intI*1 along the soil-plant continuum in both cases, although the impact on soil and plant microbiomes differed between the two crops. In tomatoes, few abundant bacterial families discriminated the different agricultural fields, including Pseudomonadaceae and Enterobacteriaceae. The predominance of Pseudomonadaceae correlated positively with the levels of *intI*1, bla_{TEM}, bla_{OXA-58} and *sul*1 detected in the fruits. In contrast, microbiomes from broad bean plant organs (beans, leaves, roots) were dominated by Rhizobiales, whose predominance inversely correlated with *intI*1 and ARGs abundances. We concluded that Rhizobiales limits the translocation of ARGs into the crops, as well as the colonization of roots and plant parts by other bacteria. Chapter IV integrates the data from the distribution and abundance of the ARGs in different model crops (*Lactuca sativa* L. cv. Batavia, *Vicia faba* L., *Lycopersicon esculentum* Mill). The crops were grown in three peri-urban and two rural agricultural fields. ARGs were detected in all plant compartments, with highest loads in roots and lowest in fruits or beans. ARGs profiles within the crops reflect the soil ARG composition. The statistical analyses also showed positive correlations between certain soil taxa and ARG profiles. The crop type revealed to be determinant for both ARG distribution and microbiome composition. Irrigation water appeared to have a limited influence, suggesting that the soil amendment practices were determinants on ARG distribution and on their abundance in edible crop parts. Chapter V presents a general discussion of the results showed previously, followed in Chapter VI including the main conclusions of the Thesis.

Resumen

La resistencia bacteriana a los antibióticos (AB) es un problema mundial cada vez mayor para la salud pública, con bacterias multirresistentes que persisten y se propagan no solo en entornos clínicos sino también en el medio ambiente. Wellcome Trust estimó en 10 millones las muertes en todo el mundo hasta 2050 debidas a bacterias patógenas resistentes a AB. Uno de los posibles vectores de esta propagación es el uso y la reutilización de aguas residuales (AR) y de estiércol y lodos de plantas depuradoras (EDAR) en diferentes prácticas agrícolas (riego, enmienda del suelo). Estas prácticas se están generalizando en todo el mundo, especialmente en regiones con escasez de agua. Las EDAR se conocen como puntos calientes de generación de resistencia a los AB, mientras que la enmienda del suelo mediante fertilización orgánica aumenta la resistencia a los AB en los microbiomas del suelo. Por lo tanto, existe una creciente preocupación por la posible transmisión de la resistencia AB de los suelos agrícolas y a los cultivos y, en última instancia, a los microbiomas del intestino de los consumidores.

Los estudios sobre la absorción de bacterias resistentes a AB (ARB) y genes resistentes a antibióticos (ARG) se han limitado principalmente a estudios de suelo y raíces. Por lo tanto, la investigación sobre la propagación de ARB y ARG en el medio ambiente y su transmisión a la cadena alimentaria es de suma importancia. Actualmente, no hay estudios que aborden el impacto de la calidad del agua de riego y la enmienda del suelo en los ARG y los microbiomas a lo largo del continuo suelo-planta-alimento. Este tema se ha evaluado en esta Tesis partiendo de parcelas comerciales de productores de hortalizas para consumo humano. Esta estrategia nos permitió analizar los elementos de resistencia a los AB en los alimentos que llegan a los consumidores, y comprender el riesgo que plantea su consumo debido a la entrada de ARG en la cadena alimentaria. Además, esta Tesis aborda las alteraciones en las comunidades bacterianas del suelo, de las plantas y de los cultivos comerciales debidas a estas prácticas agrícolas, así como la asociación de diferentes taxones bacterianos con los ARG e *intI*1, un marcador de contaminación antropogénico. Para lograr esto, se seleccionaron cinco zonas producticvas ubicadas en el área de Barcelona (NE España), bajo diferentes regímenes de riego y enmienda del suelo. Los cultivos seleccionados fueron lechugas, tomates y habas, ya que representan vegetales con diferentes partes comestibles, que comúnmente se comen tanto crudos como cocidos.

La Tesis se divide en seis capítulos. El Capítulo I describe el conocimiento actual sobre la presencia de ARB en campos agrícolas y productos alimentarios y presenta los objetivos del proyecto de tesis doctoral. Los capítulos II y III evalúan la distribución de ARG y microbiomas a lo largo del continuo suelo-planta en *Lycopersicum esculentum* y *Vicia faba*, cultivadas en campos agrícolas periurbanos y rurales bajo diferentes regímenes de riego y manejo agronómico. Nuestros datos muestran que las prácticas de campo influyeron en la distribución de ARG e *intI*1 a lo largo del continuo suelo-planta en ambos casos, aunque el impacto en el suelo y los microbiomas de las plantas difirió entre los dos cultivos. En los tomates, pocas familias bacterianas abundantes discriminaron los diferentes campos agrícolas, incluidas Pseudomonadaceae y Enterobacteriaceae. El predominio de Pseudomonadacea se correlacionó positivamente con los niveles de *intI*1, bla_{TEM}, bla_{OXA-58} y *sul*1 detectados en las frutas. En contraste, los microbiomas de los órganos de la planta de haba (habas, hojas, raíces) estuvieron dominados por Rhizobiales, cuyo predominio se correlacionó inversamente con las abundancias *intI*1 y ARG. Llegamos a la conclusión de que Rhizobiales limita la translocación de ARG en los cultivos, así como la colonización de raíces y partes de plantas por otras bacterias. El Capítulo IV integra los datos de la distribución y abundancia de los ARG en diferentes cultivos modelo (*Lactuca sativa* L. cv. Batavia, *Vicia faba* L., *Lycopersicon esculentum* Mill). Los cultivos se obtuvieron de tres parcelas agrícolas periurbanas y dos rurales. Los resultados obtenidos de los diferentes suelos y cultivos mostraron que se detectaron ARG en todos los compartimentos de las plantas, con cargas más altas de ARG en las raíces y más bajas en frutas o habas. Los perfiles de ARG dentro de los cultivos reflejan la composición de los del suelo, y los análisis estadísticos también mostraron correlaciones positivas entre ciertos taxones bacterianos del suelo y los perfiles de ARG. El tipo de cultivo se reveló como factor determinante tanto para la distribución de ARG como para la composición del microbioma. El agua de riego parece tener una influencia limitada, lo que sugiere que las prácticas de enmienda del suelo fueron determinantes en la distribución de ARG y en su abundancia en las partes comestibles del cultivo. El Capítulo V presenta una discusión general de los resultados obtenidos anteriormente, seguidos del Capítulo VI que incluyen las principales conclusiones de la Tesis Doctoral.

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Acronyms List

AB	Antibiotic
ARB	Antibiotic-Resistant Bacteria
ARG	Antibiotic Resistance Genes
CEC	Contaminants of Emerging Concern
Ct	Cycle Threshold
eDNA	Extracellular DNA
HGT	Horizontal Gene Transfer
MIC	Minimum Inhibitory Concentration
MGE	Mobile Genetic Element
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Discriminant Analysis
qPCR	Quantitative Real-Time Polymerase Chain Reaction
Ta	Annealing Temperature
Tm	Melting Temperature
TWW	Treated Wastewater
WHO	World Health Organisation
WW	Wastewater
WWTP	Wastewater Treatment Plant

Motivation and Thesis outline

Motivation

Antibiotic Resistance is an increasing problem all over the world. Many reports confirmed the persistence and spreading of AB multi-resistant bacterial strains not only in clinical settings but also in the environment, a phenomenon that has a potential to become a future global crisis. The “golden era” of AB development, when many new ABs were discovered in matter of years, is long gone, further contributing to fuel the crisis of antimicrobial resistance. The actual scarcity of new ABs is due both to the low probability of pharmaceutical industry making a substantial return from the investment, and to the financial costs of their development to fulfill current regulatory requirements. The misuse of ABs either for human or veterinary uses accentuates the AR problem.

Studies across clinical settings and environment and agriculture/livestock sectors to understand the spread of ARB and ARGs in the food chain requires a multidisciplinary effort. The MSCA-ITN "Antibiotics and mobile resistance elements in wastewater reuse applications: risks and innovative solutions: ANSWER” aims to apply state-of-the-art knowledge in the fields of Chemistry, Biology, and Civil Engineering to study the spread of ABs, ARGs, and ARBs from the WWTPs to the edible part of crops, along with the development of new technologies to remove more efficiently those agents from the WWTP effluents.

The topic and goals of this study are very relevant both scientifically and socially. There is an urgent need to increase our understanding about the potential dissemination of ARG in the environment, and little is known yet of whether farming practices increase ARG in edible parts. The uptake of these ARBs and ARGs occur through the food chain, when humans later consume these plants. Over the past few years, great advances have been done, to develop high-throughput sequencing methods with increased outputs of data per instrument run at lower costs. These techniques allowed the study of microbial ecology to new levels, by a combination of meta-barcoding sequencing (Dormontt et al., 2018; Hebert et al., 2003) and large-scale metagenomics (Handelsman, 2004; Tringe, 2005). Meta-barcoding consists in using specific DNA sequences for identification of the different organisms present in complex samples (Dormontt et al., 2018). Metagenomics goes one step farther, aiming to know the habitat-specific gene fingerprints of sampled environments, by sequencing the total DNA in collected samples (Tringe, 2005). The

innovative combination of molecular biology methods with next-generation sequencing technologies allows the study of the microbiomes and how they modulate the ARGs prevalence and persistence in the environment. We wanted to understand the influence of the use of treated wastewater (TWW) and of other agricultural practices in the uptake of ARGs along the soil-plant continuum. The final aim is to characterize the interactions between agricultural practices, water quality, and food safety.

Thesis outline

Chapter I provides the state-of-the-art knowledge related to the antibiotic resistance spread in the environment, and its risks for the food-chain. The study characterizes the sources of ABs, ARGs and ARBs, including agricultural fields, the influence of certain agricultural practices in the microbiomes and ARGs, and ARBs transmission along the soil-plant continuum. Moreover, we explore the relationship between Horizontal Gene Transfer (HGT), bacterial Ecology and Phylogeny. Finally, this Chapter includes the hypotheses and the objectives of the Thesis.

Chapter II describes the distribution of several clinically relevant ARGs (*sul*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *mecA*, *qnrS1*, *tet*M, $bla_{OXA\text{-}58}$), *intI*1 and 16s rDNA in *L. esculentum* peri-urban and rural agricultural fields. Samples were collected to have a survey of the soil plant continuum (soil, roots, leaves, fruits). Bacterial communities were analysed by next-generation sequencing, and the potential relationships were studied by bioinformatics and statistical analysis.
This Chapter is based on the paper: Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Benjamin Piña. (2019). Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices. Science of the Total Enviroment, 652, 660-670.

Chapter III studies the distribution of selected ARGs (*sul1*, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *mecA*, *qnrS1*, *tet*M, $bla_{OXA\text{-}58}$), and *intI,* and their association with specific taxa along the soil plant continuum in legume plants (*V. faba* L.) grown under different agricultural practices. In addition, it includes the classification of the agricultural regimes by applying a machine learning model.
This Chapter is based on the paper: Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Goffe Elsinga, Luc M. Hornstra, Benjamin Piña. (2019). Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (*V. faba* L.) grown under different watering regimes. Environmental Research, 170, 16-25.

Chapter IV presents an integrative study of the distribution and abundance of ARGs in different vegetables (*V. faba* L., *L. sativa* L. cv. Batavia, *L. esculentum* Mill. cv. Bodar) grown in four several peri-urban and two rural agricultural fields, and the risks that agricultural practices may have in the incorporation of ARGs and ARBs into the food-chain. This Chapter aimed to produce useful information for policy makers to set

regulations, as it shows the associations between the type of crop, agricultural practices, water quality, and food safety.

This chapter is based on the paper: Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Thomas U. Berendonk, Goffe Elsinga, Luc M. Hornstra, Benjamin Piña. (2019). Antibiotic Resistant Gene distribution in agricultural fields and crops. A soil-to-food analysis. Environmental Research, 177, 108608.

Chapter V and **Chapter VI**, include a general discussion and a final conclusion from all the research performed and published throughout this Ph.D. Thesis.

Chapter I: Introduction

1.1. AB resistance world-wide

Antibiotics have helped to save innumerable lives, and helped to increase life expectancy, since diseases such as diphtheria, pneumonia, typhoid fever, plaque, tuberculosis, typhus, syphilis, were a major cause of death until their use become globally widespread (Piddock, 2012; https://www.cdc.gov/nchs/fastats/life-expectancy.htm).

Penicillin was discovered by Sir Alexander Fleming in 1928 and became the first AB medically used (Piddock, 2012). It started to be prescribed to treat infections in the 1940s, and just in the 1950s the first signs of resistance to the penicillin treatments started to arise. Over the decades, new AB were discovered, such as vancomycin, tetracyclines, etc., but soon bacteria started to resist treatments with the newly discovered ABs as well.

AB resistance is becoming a global threat in the near future, due to multi-resistant bacteria becoming widespread. Cases of bacterial strains resistant to most or even all known ABs (also known as pan-drug resistance) have been identified for different pathogenic species, like *Neisseria gonorrhoeae* (Suay-García and Pérez-Gracia, 2018), *Klebsiella pneumonia*e (Krapp et al., 2018), and *Pseudomonas aeruginosa* (Fernandes et al., 2016). The number of deaths due to the AB resistance is increasing, with recent estimations of 23,000 and 25,000 annual deaths caused by ARB in the United States of America and in the European Union, respectively (https://www.cdc.gov/drugresistance/, Piddock, 2012). The numbers of deaths are predicted to keep raising and there is an estimate of 10 million deaths annually by 2050 (O'Neill, 2016).

The complex relationship between environmental and human settings and their interactions, show the potential of serious public health problem due to ARB and ARGs spread (https://www.who.int/antimicrobial-resistance/en/). The high concentrations of ABs used to treat infections and the subsequent high selective pressure (Almagor et al., 2018; Oz et al., 2014) made the clinical settings prone to fast emergence of resistant and multi-resistant bacteria. ABs are over-used outside controlled clinical settings, possibly leading to an increased selective pressure also for ARBs resistance reservoirs such as

WWTPs and soils (Lüneberg et al., 2018; Michael et al., 2013). They This may lead to incorporation of ARB and ARGs in the food-chain (Berendonk et al., 2015).

1.2. Resistance Mechanisms

Resistance to ABs is an ancient, natural occurring phenomenon (D'Costa et al., 2011; Peterson and Kaur, 2018). Production of ABs results from processes of co-evolution. Bacteria compete with their neighbours in their habitat for the limited space and resources. It represents one of many strategies to gain competitive advantage over competitors (Hibbing et al., 2010).

Two classic examples of inter-species competition are the scramble competition and the contest competition. The former involves the fast usage of a limiting resource (Nicholson, 1954; Rozpędowska et al., 2011). Contest competition refers to the direct removal of other competitors (Nicholson, 1954). ABs production relates to the latter strategy. The spectrum of the ABs synthesized by a bacterial species is related to their specialization. A species capable of thriving in a wide variety of habitats may synthesize either multiple ABs or broad-spectrum ABs, therefore, enabling it to deal with a diverse group of competitors. *Streptomyces* spp. of the Actinobacteria Phylum are a good example (de Lima Procópio et al., 2012). In contrast, species adapted to a specific environment would probably only target a specific competitor (Hibbing et al., 2010).

Antibiotic resistance can be classified as either intrinsic or acquired, depending on whether or not the mechanism involves a genetic change. Intrinsic resistance refers to a generalizable trait that does not change regardless of antibiotic selective pressure (Culyba et al., 2015). By contrast, the acquired resistance develops when a new trait is expressed, often because of a genetic change that has been selected due to antibiotic exposure (van Hoek et al., 2011).

Several mechanisms are known to give resistance to ABs. The resistance may come by affecting the ABs molecules, modifying them, or inactivating/degrading them (Munita and Arias, 2016). The latter is illustrated by the action of β-lactamases, which hydrolyse the amide bond of the β-lactam ring (Munita and Arias, 2016), which is essential for β-lactam ABs. This is the mechanism of action TEM (Bush, 2013), OXA (Evans and Amyes, 2014) and CTX (Bonnet, 2004) β-lactamases. Resistance is also gained by altering the

permeability of the Gram negative outer membrane (Delcour, 2009). It can be accomplished by changes in the sequences of the expressed porins, or in their expression levels (Munita and Arias, 2016; Nikaido, 2003).

Efflux-pumps provide a way to extrude ABs from the cell cytoplasm (Du et al., 2018). They can be very specific for a given type of substrate, like the Tet efflux pumps (Kumar and Varela, 2012), or to have a wide variety of substrates to act on. There are five main families (Du et al., 2018) of unspecific target efflux pumps: the major facilitator superfamily (MFS) (Pao et al., 1998), the small multidrug resistance family (SMR) (Bay et al., 2008), the resistance-nodulation-cell-division family (RND) (Tseng et al., 1999), the ATP-binding cassette family (ABC) (Fath and Kolter, 1993), and the multidrug and toxic compound extrusion family (MATE) (Pallen, 1999). They are present in gram-positive and gram-negative bacteria, except the RND family, which is only present in gram-negative bacteria, and they typically confer multi-drug resistance.

Bacteria can protect, change, replace, or bypass of the target sites to make certain ABs inefficient. For example, the *tet*M gene encodes a protein that competes with tetracycline for the same ribosomal space and it alters the geometry of the binding site (Li et al., 2013). The QnrS1 protein competes with quinolones for the DNA binding site of the DNA gyrase and topoisomerase IV. Quinolone targets are DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive ones (Rodríguez-Martínez et al., 2011). By binding to the DNA it decreases the opportunities of the quinolones to form and stabilize the gyrase-cleaved DNA-quinolone complex (Rodríguez-Martínez et al., 2011). The replacement or bypass of the target site happens when the bacteria synthetises new targets that accomplish similar functions to the AB intended target, but that are not affected by the AB. Resistance to methicillin in *Staphylococcus aureus* is conferred by the acquisition of the *mec*A gene, which codifies the Penicillin Binding Protein PBP2a. This protein has low affinity for all β-lactam ABs, including penicillin, cephalosporins, and carbapenem. It has the same function of the PBPs produced by *S. aureus,* carrying the transpeptidation and transglycosylation of the peptidoglycan (Chambers and DeLeo, 2009).

1.3. ARGs transmission

1.3.1. Vertical Gene Transfer

The transmission of bacterial genes generally occurs either by vertical gene transfer or by HGT. The vertical gene transfer consists in transmission of genetic material from the parent to the daughter bacterial cell (Lawrence, 2004).

1.3.2. Horizontal Gene Transfer

HGT is the process of acquiring new genetic material without this vertical flow of the genetic material relationship. There are three basic forms of HGT: conjugation, in which conjugative plasmids are exchanged between bacteria; transformation, in which free DNA in the environment is uptaken by bacteria; and transduction, in which viruses act as a vectors for HGT (Thomas and Nielsen, 2005). Genes acquired through HGT generally have negative effects for the host bacteria, as they can destabilize the genome. However, they may also confer selective advantage to the bacteria provided the environmental condition are appropriate, and therefore they may spread in the bacterial communities. A good example of this is the contribution of the presence of ABs in soil and the spread of ARGs by HGT.

1.3.2.1. Conjugation

Conjugation occurs when there is direct cell-cell contact between two bacteria to transfer genes through conjugative plasmids (Ryan et al., 2004). The conjugative process involves a donor and a recipient cell. The donor bacteria needs to possess the conjugative plasmids (Norman et al., 2009) and specific structures. There are three types of such structures, the F-like pili (mainly IncF plasmids), the P-like pili (IncP, -N, and -W), or type IV secretion apparatus (Hong et al., 2017). In Gram positive bacteria, conjugation occurs either through type IV secretion systems (in the case of single strand DNA) or by the TraB-dependent spreading mechanism, identified in *Streptomyces* spp., where double-stranded DNA translocation is performed by a single protein during cell division (Goessweiner-Mohr et al., 2017; Sepulveda et al., 2011; Thoma and Muth, 2012).

1.3.2.2. Transformation

Transformation is a process where DNA fragments are taken up by bacteria from the environment, coming usually from the lysis of other bacteria. The uptake of DNA, its integration and its expression requires the bacteria to be in a regulated physiological state of competence. DNA uptake requires protein components also utilized in type IV pilus formation, twitching mobility, and the type II secretory system (Zaneveld et al., 2008). The fact that transformation is widely spread among diverse taxonomic groups of bacteria indicates that it has an important function in the environment (Thomas and Nielsen, 2005). DNA, including ARGs, may persist in the environment for weeks or even months in soil after its release from dead cells (Dong et al., 2019; Pietramellara et al., 2009). Most competent bacteria can uptake both chromosomal and plasmid DNA, although plasmidic DNA requires linearization prior to its integration into the host chromosome (Thomas and Nielsen, 2005). These mechanisms are important contributors to the spread of ARGs in the environment (Dong et al., 2019; Thomas and Nielsen, 2005).

1.3.2.3. Transduction

Transduction occurs when bacteria-specific viruses (called bacteriophages or, simply, phages) transfer DNA between two bacteria, therefore acting as DNA vectors. Phages gain entry to their microbial host cell by binding to extracellular receptors on the cell surface. The origin of DNA obtained through transduction largely depends on the host range of the phage (Andam et al., 2015), which may cover from very narrow to rather broad taxonomical groups (Ma et al., 2014; Smith et al., 2013). Interestingly, the phage life cycle may have an extra impact on HGT, as lytic bacteriophages may release a considerable quantity of plasmid and chromosomal DNA after provoking bacterial lysis. This increases the free DNA in the environment and therefore increases the chances for transformation to occur (Keen et al., 2017).

1.3.3. MGEs

Mobile genetic elements (MGEs) are segments of DNA that encode enzymes and other proteins that mediate the movement of DNA from a bacterial cell to another. Integration of new genetic information into bacteria constitutes a large functional leap that allows

fast adaptation to new environments or to stressful conditions. Plasmids and transposons are relevant members of the class of MGEs. Integrons may be associated with plasmids or transposons (Stokes et al., 2006) and facilitate the transmission of several genes. As they usually encompass gene cassettes codifying for different ARGs, they contribute to the spread of AB multi-resistance (Labbate et al., 2009). In some cases, they also encode genes conferring heavy metal resistance, which may drive co-selection of ABR and heavy metal resistance (Seiler and Berendonk, 2012).

1.3.3.1. Plasmids

Plasmids are collections of functional genetic modules organized into a stable, self-replicating entity. While plasmids usually do not contain genes required for essential cellular functions, they usually encompass genes that give bacteria phenotypic advantages in response to selective pressures, such as ARGs or genes conferring resistance to heavy metals (Li et al., 2015; Seiler and Berendonk, 2012). Plasmids are found in three domains of life (Shintani et al., 2015), although the vast majority of them (93.5 %) are present in Bacteria (Fig. 1.1).

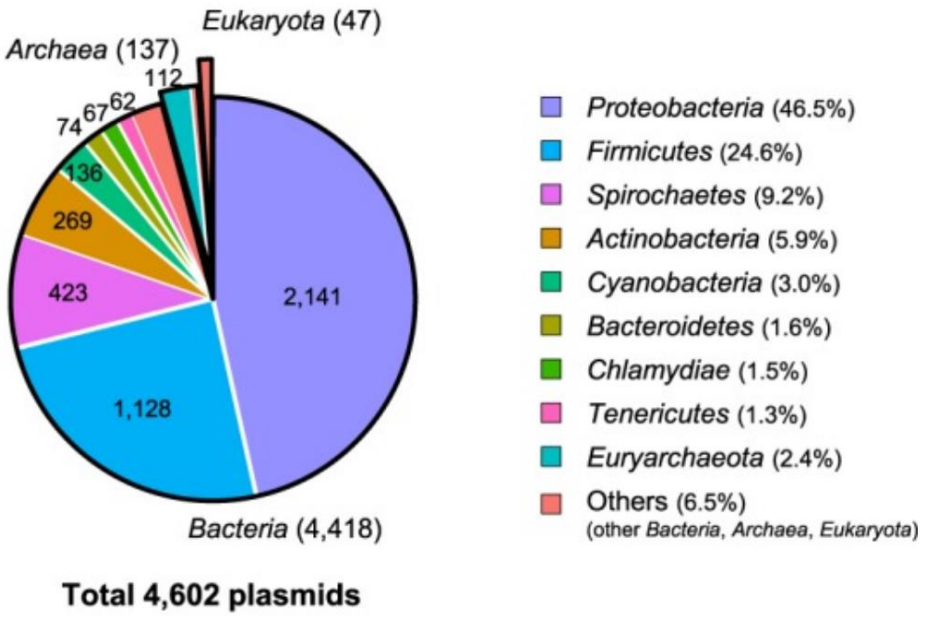


Figure 1. 1. The taxonomic distribution of fully sequenced plasmids (Shintani et al., 2015).

Plasmid may be classified according to their function, their capability for conjugation, or by incompatibility groups (Shintani et al., 2015). The term Incompatibility (Inc) refers to

the fact that two plasmids belonging to the same Inc group cannot be stably present in the same bacterial cell (Popowska and Krawczyk-Balska, 2013). Incompatibility results from two plasmids possessing replicons with the same specificity for Rep protein or controlling elements (Thomas, 2014). Therefore, related plasmids are usually incompatible, and non-related plasmids tend to be compatible, with exceptions. Related plasmids can coexist in a bacterial cell due to sequence differences in regulatory RNA, whereas non-similar plasmids that share a common ancestor can show incompatibility, such as the IncP-1 replicons (Thomas, 2014).

The IncP-1 plasmids contain mosaic structures that originated from several parental plasmids lines that have evolved and adapted different in phylogenetically distant bacterial species (Norberg et al., 2011). Therefore, IncP-1 are pervasive and have a broad host range (Sen et al., 2011) due to their highly efficient conjugative transfer in Gram-negative bacteria (Popowska and Krawczyk-Balska, 2013), including *Escherichia coli*, *Pseudomonas* spp., *Klebsiella aerogenes* (Popowska and Krawczyk-Balska, 2013; Shintani et al., 2010; Thomas, 2000). Bacteria carrying IncP-1 have been isolated from soils under anthropogenic pressure (Heuer et al., 2012; Popowska and Krawczyk-Balska, 2013). IncP-1 plasmids are potential AB resistance vectors, and they may carry *intI*1, *sul*1, *tet*, bla_{OXA} and bla_{TEM}, just to mention genetic elements relevant for this Thesis (Heuer et al., 2012; Sen et al., 2013, 2011; Szczepanowski et al., 2011).

1.3.3.2. Integrons

Integrons are genetic elements that contain a site-specific recombination system able to integrate, express and exchange specific DNA elements, called gene cassettes (Fig. 1.2). The complete integron is not considered to be a mobile element as such, as it lacks functions for self-mobility, whereas the gene cassettes present in integrons are considered as mobile (Domingues et al., 2012).

1.3.3.3. Class 1 integrons

Integrons are divided into different classes. The classification system is based on the amino acid sequences of IntI integrases, with some of them associated to mobile genetic elements (e.g. Class 1, 2 and 3), while others are associated to the bacterial chromosome (e.g. Class 4) (Deng et al., 2015).

Class 1 integrons are widely reported in pathogens from clinical settings, carrying gene cassettes associated with antibiotic resistance, and can be found in transposons and plasmids, allowing their dissemination through HGT. Integrons have been recently detected in microbial species from various environments, including wastewater, river water, and soil, suggesting that the dissemination of integrons may be a general problem of environmental pollution and public health (Gillings et al., 2015).

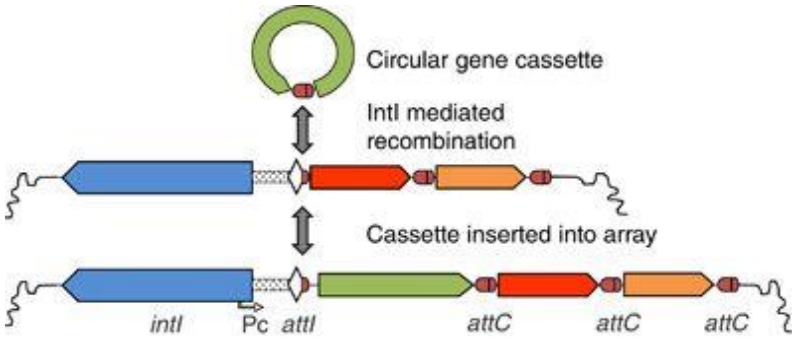


Figure 1. 2. Integron structure and function. Integrons consist of a gene for an integron-integrase (*intI*) that catalyses recombination between the *attC* site of circular gene cassettes and the attendant integron recombination site, *attI* (Gillings et al., 2015).

1.3.4. ARGs and MGE associated gene selection

This Ph.D. Thesis is focused on the distribution of ARGs and microbiomes along the soil-plant continuum. Seven ARGs (*bla*$_{TEM}$, *bla*$_{CTX-M-32}$, *bla*$_{OXA-58}$, *mec*A, *qnrS*1, *sul*1, *tet*M) were selected based on their relevance in the assessment of the antibiotic resistance status in environmental settings, on their clinical relevance (described below), and association with MGEs (Berendonk et al., 2015). In addition, we chose the integron class 1 a proxy for anthropogenic impact and because of its association with HGT activity (Gillings et al., 2015). These are the main characteristics of these selected, AB resistance related sequences:

- The *bla*$_{TEM}$ genes encode for TEM β-lactamases (Muhammad et al., 2014). *bla*$_{TEM}$ genes have a high prevalence and distribution in the environment (Lachmayr et al., 2009). They require just few specific single nucleotide polymorphisms (SNPs) to turn from specific to extended-spectrum β-lactamase (ESBL) genes (Muhammad et al., 2014).

- *bla*$_{CTX\text{-}M\text{-}32}$ encodes an extended-spectrum β-lactamase (ESBL) found mostly in the Enterobacteriaceae family members, including *E. coli.* (Cartelle et al., 2004; Mugnaioli et al., 2006).

- *bla*$_{OXA\text{-}58}$ confers resistance to carbapenems, which are considered drugs of last resort in *Acinetobacter* species. Several of those species are responsible for nosocomial infections (Bertini et al., 2007).

- *mec*A confers resistance to methicilin, and it is present in *Staphylococcus* species MRSA (Methicilin resistant *Staphylococcus aureus*), also causing nosocomial infections (Wielders et al., 2002).

- *qnr*S1 confers resistance to quinolones and it is found in Enterobacteriaceae clinical isolates (Tamang et al., 2008).

- *tet*M (tetracycline resistance) and *sul1* (Sulfonamide resistance) have been shown to co-occur in plasmids derived from environmental samples with anthropogenic impact, sometimes linked to *intI1*. (Gillings et al., 2015, 2008; Hu et al., 2008; Nonaka et al., 2012).

- *intI1* encodes the site-specific recombinase *IntI1*, responsible for the insertion and excision of exogenous gene cassettes at the integron-class 1 associated recombination site *attI1* (Mendes et al., 2007).

1.4. ARGs quantification

Molecular biology has as wide range of applications in environmental studies. PCR (Bartlett and Stirling, 2003) is widely used to detect the presence of target genes present in soil, water, etc. This allows the detection of bacteria of interest, or even or with further processing study microbial communities (bacteria, fungi). qPCR (Higuchi et al., 1993) is a variant of the PCR method that allows detection and quantification of target genes with a high sensitivity and precision (Muniesa et al., 2014).

1.4.1. PCR

The PCR reaction allows the amplification of specific DNA sequences from a DNA sample. It requires the template DNA, primers, nucleotides (Adenine, Guanine, Cytosine and Thymine), and a heat-stable DNA polymerase, usually the Taq polymerase (Chien et al., 1976). The primers are short nucleic acid sequences complementary to the target DNA. They are required to bind to the 3' end of the target DNA for the Taq polymerase to start incorporating the nucleotides to synthesize a complementary strand (Garibyan and Avashia, 2013).

The PCR reactions are carried in a thermocycler, a programmable system able to perform the polymerase chain reaction. It works by creating repeated thermal cycles, by increasing and lowering temperature to allow DNA amplification in three steps (Fig. 1.3). The first step is named denaturation. The solution with DNA template and reagents is heated above the melting point of the double strand DNA of the target sequence, allowing the two strands of DNA to separate one each other. Secondly, the temperature is lowered slightly below the primers T_m to allow the primers to bind to the target DNA sequences, in a step named annealing. At the third step, the temperature is raised, which allows the Taq polymerase to extend the primers by adding nucleotides to the complementary DNA strand (Garibyan and Avashia, 2013).

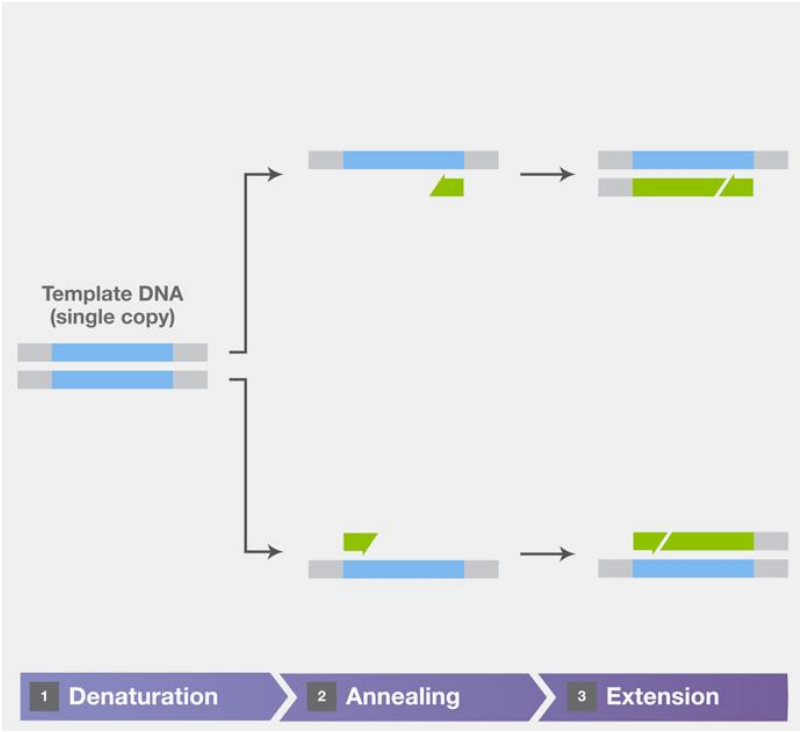


Figure 1.3. The three steps of each PCR cycle. **(1)** Denaturation, which results in the separation of the DNA strands due to the temperature rise. **(2)** Annealing, reflecting a lowering of temperature

allowing the primers to bind to the targeted sequences. **(3)** The final step, extension happens with the bidding of DNA polymerase and synthesis of a new DNA strand (https://www.thermofisher.com).

1.4.2. qPCR

The qPCR technique serves for quantification of target sequence by measuring the amount of amplified product in real time. This is achieved by either using a fluorescent dye, Sybr Green I (Zipper, 2004) or by the use of fluorophore-containing DNA probes such as TaqMan (Holland et al., 1991). The methods used in this Thesis were based on Sybr Green technology. This dye binds to all double–stranded DNA and the binding is monitored by measuring the increase in fluorescence throughout reiterative qPCR reactions (Fig. 1.4 B). The qPCR reaction has four phases. The first phase, named ground or lag phase, corresponds to the first amplifications, during which the amount of fluorescence produced is too low to be detected. The second, exponential phase, occurs once the amplification of the target sequence reached a number of copies that make the subsequent doublings detectable as increases in the fluorescence of the solution. The amount of amplicons, and therefore, of the recorded fluorescence, doubles every cycle when the efficiency is 100% (equation 1.4). Thirdly, as the reagents are being consumed and their concentrations start to run low, the reaction slows down and linear phase is reached. Lastly, a plateau is reached, where there is no more amplification as the reagents run out (Fig. 1.4) (Page and Stromberg, 2011).

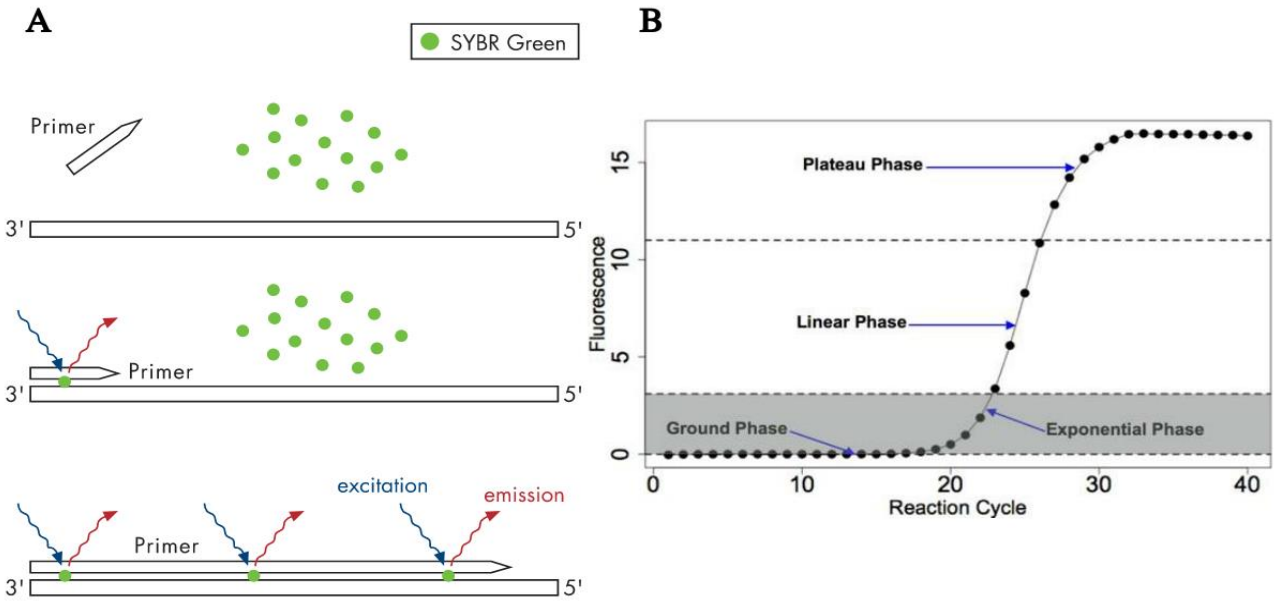


Figure 1.4. (A) Mechanism of Sybr Green I fluorescence emission by binding to double-stranded DNA. **(B)** The four qPCR phases in a linear view: plotting the number of cycles (x-axis) to the

emitted fluorescence (y-axis) in real time. The dashed line separating the grey and white backgrounds represents the Ct (Page and Stromberg, 2011; Rutledge, 2003).

1.4.2.1 Gene quantification

Relative quantification is performed by comparing the copy numbers of target amplicon with a housekeeping gene (positive control), and it is commonly used for studies of gene expression (Čikoš et al., 2007). Absolute quantification of a target is measured by the standard curve method (Rutledge, 2003), and it was the method used in this Thesis

1.4.2.2. Absolute Quantification by the standard curve method

The standard curve provides information about the 10-fold serial dilution prepared with the standard. In each dilution is possible to calculate the copy numbers of the standard, by measuring its concentration and knowing beforehand its length in base pairs. This is shown in formula (1.1):

$$(1.1) \quad \frac{X\ ng\mu l^{-1}}{standard\ (bp) \times 660\ gmole^{-1}} \times 6.0022 \times 10^{23}\ molecules\ mole^{-1} = Y\ copies\mu l^{-1}$$

The serial dilutions should cover the complete range of expected number of DNA copies of the unknown samples. The standard curve is specific to each qPCR assay; therefore, samples must run in parallel. The Ct value of the samples is compared with the standard curve to determine their copy numbers.

The Ct value may be obtained by two methods, the fit-points and the Second Derivative Maximum calculation (Luu-The et al., 2005). The latter method was used in this Thesis. It identifies the turning point when the fluorescence turns sharply upward during the exponential phase (Fig. 1.4 B), which corresponds to a maximum on the second derivative of the fluorescence values (Luu-The et al., 2005). This method is applied automatically and calculated by the PCR machine without the user interference. The obtained Ct values (Fig. 1.5 A) are then plotted as function of the copy numbers calculated with the serial dilution curve (Fig. 1.5 B).

The standard curve allows to evaluate the performance of the qPCR assay (Fig. 1.5 B) using several parameters. Ct values are inversely proportional to the logarithm of DNA concentration, and the efficiency of the reaction is calculated from the slope between the

target concentration and Ct values. The efficiency of the qPCR (equations 1.2 and 1.3) should be between 90–110%, corresponding to a slope ranging from −3.6 to −3.3, meaning that Ct values of the standards 10-fold serial dilution should be 3.3 cycles apart -i.e., $\log_2(10)$. This means there is a 2-fold change in DNA concentration between two cycles when the efficiency is 1 (100%), as showed in the formula 1.4, in which *Cn* is copy number at cycle *n* and *Ci* the initial copy number.

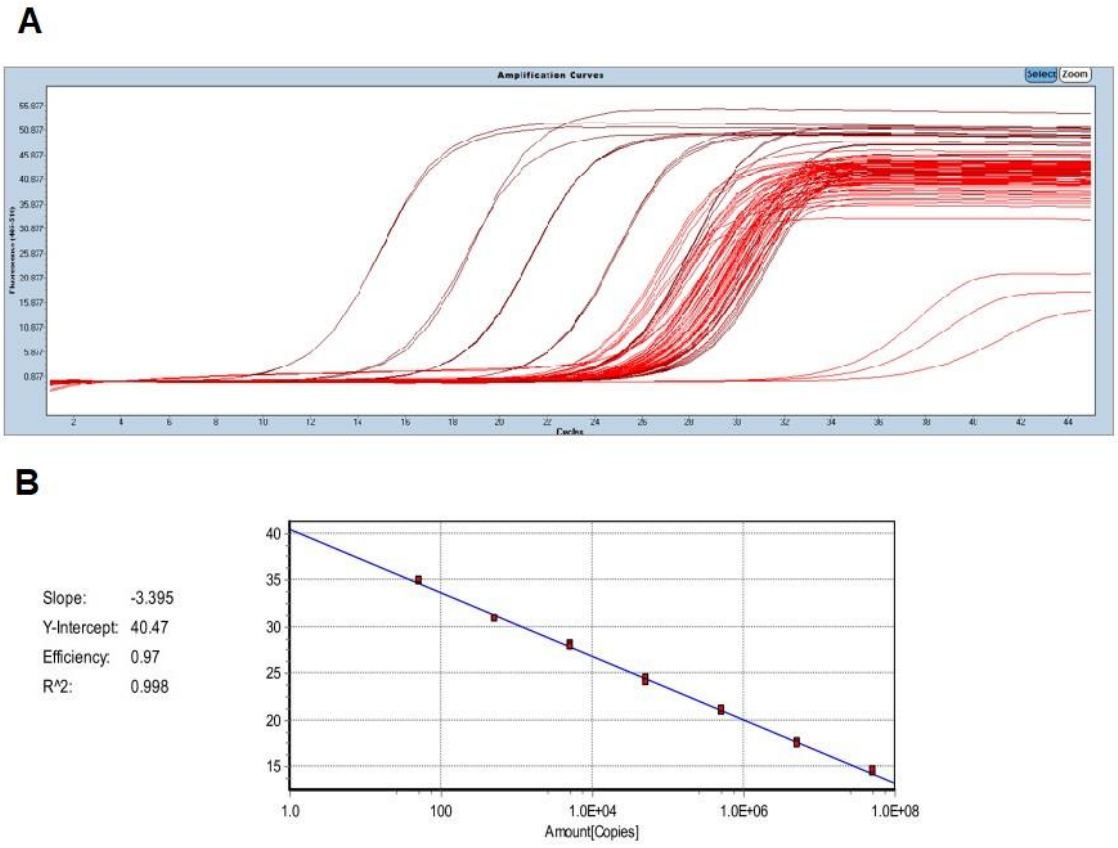


Figure 1.5. (A) Amplification curves with the number of qPCR cycles on the x-axis and y-axis the fluorescence levels. The brown amplification lines correspond to the standard serial dilutions, which will be used for the standard curve. The standard curve allows to calculate the number of DNA copies of the samples (red curves). **(B)** Example of a standard curve, with 7 dilution points. The x-axis contains the number of DNA copies and the y-axis the Ct value (Yan et al., 2011).

$$(1.2) \quad Efficiency = 10^{-\frac{1}{slope}}$$

$$(1.3) \quad Efficiency\ (\%) = (Efficiency - 1) \times 100\%$$

$$(1.4) \quad C_n = C_i(1 + Efficiency)^n \Rightarrow C_i \times 2^n$$

The correlation coefficient (R^2) value (Fig. 1.5 B) is obtained after a linear regression analysis on the measured Ct values. It shows how well the regression line fits the data points, plus the scattering of the standard technical replicates. The correlation coefficient should be above 0.98 (Broeders et al., 2014).

The reproducibility of technical replicates should also be taken into account. The maximum allowed standard deviation in Ct value between the qPCR replicates should be approximately 0.35, corresponding to 0.5 cycles. In the work showed in this Thesis the maximum standard deviation accepted was 0.3. Low values are important since a small contribution of the technical variance means that most variance will be due to the biological variability.

1.4.2.3. Melting curve analysis

As Sybr Green I only binds to double-stranded DNA, the fluorescence levels maybe plotted as a function of the temperature. As soon as the temperature rises, the double-stranded DNA start to dissociate leading to a decrease in fluorescence levels. The T_m corresponds to the inflection point of the melting curve (Broeders et al., 2014). The melting curve is essential to detect dimers or unspecific amplifications, and can be used to differentiate amplicons separated by less than 2 ^oC. This is possible as Tm depends on the G+C/A+T ratio of the amplicon (Ririe et al., 1997).

1.5. HGT, Ecology and Phylogeny

1.5.1. Ecology

HGT between different species has been recognized as a common and major evolutionary process (Reitner and Thiel, 2011) whose impact in soil resistomes is still under investigation (Zhaxybayeva and Doolittle, 2011). HGT frequency and effects are dependent on both Ecology and Phylogeny. The analysis of the potential transfer of genes between human bacteria and bacteria from different environments, such as soil, livestock, and others, showed an approximately threefold rate of HGT between bacteria of the same environment than from different environments (Fig. 1.6), irrespectively of their phylogenetic distance (Smillie et al., 2011).

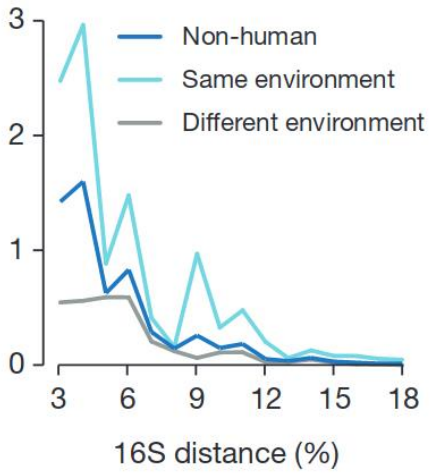


Figure 1.6. HGT frequency is plotted as a function of the phylogenetic divergence between species of environmental bacteria (Smillie et al., 2011).

Bacteria from environmental and human-associated microbiomes differ in their core resistomes (Fig. 1.7). Some ARGs that define those environments provide resistance to β-lactams and tetracyclines by different mechanisms. Despite soil and human gut microbiota have functionally distinct ARGs, there are no significant differences in the number of distinct ARGs between them when grouped by functional AB resistance (Gibson et al., 2015). Therefore, Ecology defines different core resistomes, including human-, water- and soil-associated resistomes (Fig. 1.7). Ecology is then a barrier as donor and recipient bacteria have to be in the same niche for HGT to occur (Schmidt and Hensel, 2004). The conclusion is that Ecology, that is, the co-occurrence of donor and acceptor bacteria in a same environmental compartment, is more important than phylogeny for HGT.

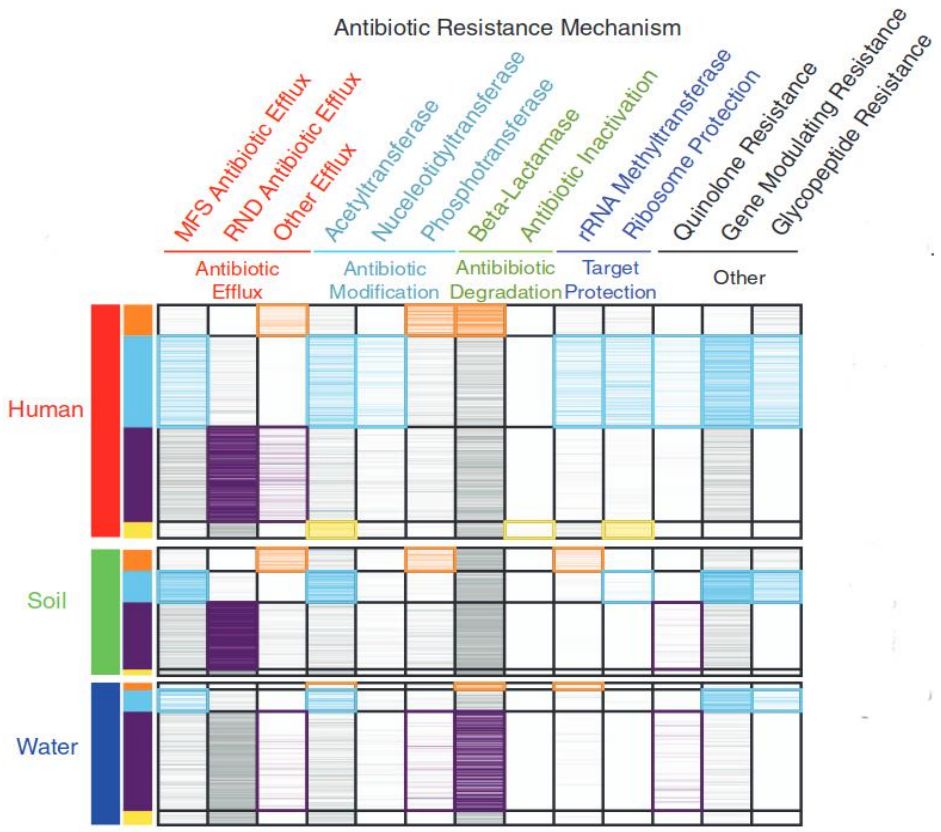


Figure 1.7. Phylogeny and habitats of 6179 sequenced bacterial genomes. Colored if a particular AR mechanism is significantly enriched within a particular phyla or habitat (Gibson et al., 2015).

1.5.2. Persistence of AB resistance

Many resistance genes persist for long periods in the absence of antibiotics. The factors that affect the maintenance of ABs resistance have a different weight according to the system type (Andersson and Hughes, 2011). In an open system in which external interactions and bacterial migrations occur (e.g. hospitals, farms), AB resistance will either persist or reverse, according the dilution of the ARB present in that same system. This is dependent on the influx and outflow of non-resistant bacteria from the system, compared to the number of the system resistant bacteria. Thus, if the proportion of non-resistant bacteria in arriving to the system is higher than the resident community, the incoming bacteria will reduce the resistance frequency. Conversely, the fitness cost of resistance has a major role determining ARBs persistence in systems with low bacteria migration or closed systems. Therefore, if the fitness cost of keeping the AB resistance is low, or if there are compensatory mechanisms, the ARB survival and spreading is not affected and the AB resistance will persist (Andersson and Hughes, 2011).

The fitness cost may be counter-balanced by several mechanisms. Bacterial co-selection with heavy-metals and AB resistance (Baker-Austin et al., 2006) has been studied in contaminated water and soils (Baker-Austin et al., 2006; Nguyen et al., 2019; Seiler and Berendonk, 2012), likely due to the presence genes codifying both resistances in a same MGE (Chapman, 2003).

Evolution of the host (Millan et al., 2014), plasmid (Harrison et al., 2015), or both (Dahlberg and Chao, 2003; De Gelder et al., 2008; Loftie-Eaton et al., 2016) also decreases the fitness cost of carrying AB resistance (e.g. in plasmids). This happens due to compensatory mutations in the plasmid, bacterial chromosome or a combination of both. It leads to an interaction shift between plasmid and hosts proteins/enzymes (Yano et al., 2016). For example, this has been observed in mutations of the replication initiation gene (*trfA*1). Carrying plasmids with the mutated gene have a lower fitness cost to the bacteria with the non-mutated gene. This happens due to lower affinity of the TrfA1 protein to the DNA helicase DnaB. The mutated TrfA1 cannot activate DnaB at the plasmid origin of replication (*ori*V) without the host DnaA gene, as both helicases are involved in chromosomal replication. Two models were used to explain the improvement in fitness cost: the titration model, in which the non-mutated trfA1 causes a reduced concentration of free DnaB, and the inhibition model, which postulates the formation of a TrfA1-DnaB complex that inhibits chromosomal DNA replication (Loftie-Eaton et al., 2017; Yano et al., 2016). Nevertheless, the reason DnaB-TrfA1 interactions have a negative effect on host fitness, and the underlying mechanisms that explain the compensatory mutations are still poorly understood (Millan et al., 2014; Yano et al., 2016).

Several factors are critical for the non-conjugative plasmids statibility and persistence, including the genetic makeup of the hosts, the proportion of bacteria carrying the plasmid with compensatory mutations, and the rate at which they appear in the microbiome before antibiotic exposure. Therefore, the combination of selective pressure, even in rare events, and compensatory mutations may overcome the fitness costs of non-conjugative plasmids (Millan et al., 2014).

In the case of conjugative plasmids, even with high fitness costs their stability and persistence, may not be affected as long high conjugation rates are present in a population. High conjugation compensates for fitness cost and plasmid segregational loss in the absence of the ABs selective pressure. The fate of a conjugative plasmid in a

sub-population may not be affected by the presence of other plasmids, if for instance they belong to other incompatibility groups. The main barrier would be the Ecological relations between the sub-populations carrying the different plasmids, reflected in the microbiome composition (Lopatkin et al., 2017).

1.5.3. Phylogeny

Conjugation between different Phyla may happen more often than previously thought, (Klümper et al., 2015), meaning that broad-host range plasmids can be hosted by diverse types of bacteria in complex communities. These plasmids can be received by bacteria distantly related to the donor, along with genes associated to transposons and class 1 integrons, such as the referred IncP-1 plasmids between gram-negative bacteria (Klümper et al., 2015; Sen et al., 2011). Transfers between gram-negative and positive bacteria may also happen, when fueled by selective pressure (Jiang et al., 2017; Klümper et al., 2015).

Inter-Phyla HGT with conjugative plasmids between Proteobacteria and Actinobacteria may happen quite often in soil (Klümper et al., 2015). It has been hypothesized that Actinobacteria, known to synthesize different bacterial antibiotics may be the source of transmission to Proteobacteria, and that they may represent both old and recent HGT events (Fig. 1.8) (Jiang et al., 2017).

As stated previously, bacteria need to be in the same environment for high rates of HGT to be able to counteract the fitness cost of maintaining plasmids. In an open system, this physical barrier maybe be broken by the inflow and outflow of bacteria. Incoming bacterial species may not be able to outcompete and colonize the Ecological niche (Gatica and Cytryn, 2013), but, if they persist in the recipient environment, intra-taxon genetic exchange will occur more often than inter-taxon. In this case, Phylogeny will be the main influencer of the global HGT rate (Y. Hu et al., 2016; Schmidt and Hensel, 2004). This is related to host range of the conjugative plasmids (Klümper et al., 2015), as some of them show a narrow host range (e.g. IncF, IncH, and IncI) while others have a wider range (e.g. IncN, IncP, and IncW) (Suzuki et al., 2010). Therefore, Phylogeny acts as a complementary barrier to Ecology, as clusters of ARGs associated with MGEs were limited by the bacterial lineages. For instance, *sul*1, *sul*2, *str*A, and *str*B tend to transferred in Proteobacteria, while *tet*M, *tet*O, and *erm*B are transferred preferentially among Firmicutes species (Y. Hu et al., 2016).

Streptomyces ARG proteins				Proteobacteria proteins	
ARG	Antibiotic	Resistance mechanism	Sequence identy	Protein ID	The host strain is isolated from
cml_e	Chloramphenicol	Efflux	63% →	WP_005297378.1 (*cmx*)	Patients
lmrA	Lincomycin	Efflux	50% →	WP_038989331.1 (*lmrA*)	Farm animals
aph33ia	Streptomycin	Inactivating enzyme	51% →	WP_031942890.1 (*aph(3")*)	Patients
otrb	Tetracycline	Efflux	39% →	WP_048022769.1	Cabbage (98% to WP_0.64384801.1 from clinical isolates of *Klebsiella pneumoniae*)[a]
cata5	Chloramphenicol	Inactivating enzyme	56% →	WP_053238935.1	Soil
aph33ia	Streptomycin	Inactivating enzyme	51% →	WP_037160408.1	Populus root
tet	Tetracycline	Target protection	48% →	WP_046110059.1	Soil
rph	Rifamycin	Inactivating enzyme	68% →	WP_014395981.1	Soil
pur8	Puromycin	Efflux	48% →	WP_043284319.1	Soil
pac	Puromycin	Inactivating enzyme	47% →	WP_046974149.1	Entomopathogenic nematodes
tcma	Tetracenomycin_c	Efflux	35% →	EFG83002.1	Environmental
facT	Factumycin	Efflux	43% →	WP_045683650.1	Marine plant
sul1	Sulfonamide	Target replacement	← 95%	ALJ92876.1 (*sul1*)	Patients

Figure 1.8. Selected pairs of proteins that might be connected via inter-phylum HGT are summarized here, with the proposed transfer direction indicated by an arrow. Protein sequence identity relationships between all experimentally validated *Streptomyces* ARG proteins and their most similar homologies in Proteobacteria (Jiang et al., 2017).

1.6. Microbiomes

1.6.1. Soil microbiomes

Soils are the most complex and heterogeneous ecosystems on earth, probably containing more unravelled biodiversity than any other genetic reservoir of the biosphere (Fitter, 2005). Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes are the predominant phyla in soils from different environments (Gibson et al., 2015), but the final microbiome composition depends on several biotic and abiotic factors. It modulates several biogeochemical processes, including nitrogen fixation (*Rhizobium* spp., *Bradyrhizobium,* spp., *Azotobacter* spp., etc), nitrification (*Nitrosomonas* spp*.,* *Nitrococcus* spp*.,* etc) and denitrification (*Flavobacterium* spp., *Bacillus* spp.) (Fierer, 2017). It has been estimated that each gram of soil contains about 10,000 different bacterial species (Torsvik and Øvreås, 2002), although the vast majority of the soil biodiversity remains uncharacterised (Banwart et al., 2019).

1.6.2 Root microbiome assemblage

1.6.2.1. Soil type and properties

Soil properties influence the formation of bacterial communities in the roots (Lundberg et al., 2012)(Fierer, 2017), including pH, O_2 concentration, redox status, and soil nutrients (Xue et al., 2018). Roots also affect soil properties, in a distance-dependent manner (Costa et al., 2006; Dotaniya and Meena, 2015). The portion of soil adjacent to the roots, which presents different physical and chemical properties from the bulk soil due to roots' influence, is referred to as the rhizosphere, whereas the surface of plant roots is denominated rhizoplane (Fageria and Moreira, 2011). Soil, rhizosphere, and rhizoplane form a continuum, in which the soil type influences the microbiomes in the rhizosphere, rhizoplane, and, finally, the bacterial population inside of the root, the root endosphere (inner tissues) (Berg et al., 2014). In this scheme, the bulk soil acts as reservoir of bacteria that can be recruited to colonize the roots. In general, it is assumed that the rhizhospheric community is shaped by the plant, based on the reservoir present in the soil, and that there is a mutual interaction between the rhizosphere and the plant genome (Costa et al., 2006; Schreiter et al., 2014; Tan et al., 2003).

1.6.2.2. Rhizosphere Effect

The rhizosphere forms a narrow zone of soil (~ 2mm) surrounding the roots, and it shows a microbial community structure different from the bulk soil. This difference is strongly dependent on the distance to the roots (Costa et al., 2006; Dotaniya and Meena, 2015; Smalla et al., 2001), with a decrease on bacterial diversity and increase in dominance, on what is named the “rhizosphere effect” (Dotaniya and Meena, 2015). The plant contributes to this “rhizosphere effect” by producing root exudates that contain small-molecular weight metabolites, amino acids, enzymes, mucilage, and cell lysates. The root exudates may represent a substantial proportion of the net carbon assimilation of the plant, from less than 10% to as much as 44%, for nutrient-stressed plants (Fig. 1.9) (Bais et al., 2006). This output of carbon-rich material significantly contributes to the composition and properties of the rhizospheric bacteria, acting as signaling molecules, attractants, stimulants, inhibitors or repellents (Baetz and Martinoia, 2014; Haichar et al., 2008). The exudation patterns may change over time, as plants can select a subset of microbes at different stages of development, presumably for specific functions.

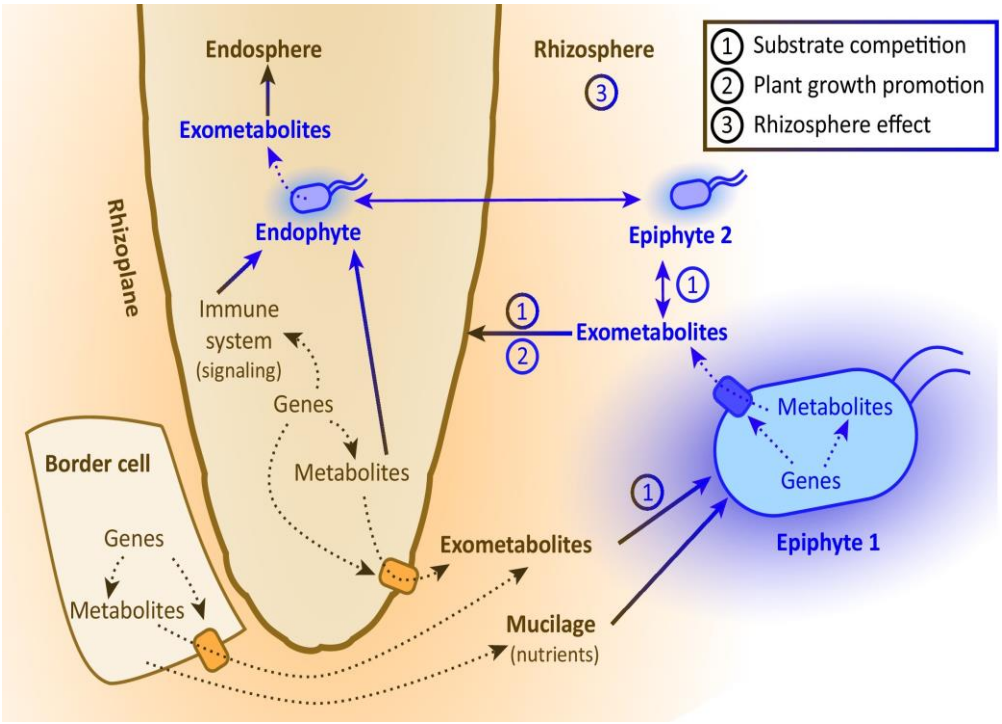


Figure 1.9. Plant-soil bacteria feedback. The interaction between the plant's roots and the microorganisms creates the rhizosphere effect (Chaparro et al., 2014).

1.6.2.3. Plant Host and Genotype

The assemblage of the plant roots' microbiome is complex and affected by various factors including the plant genotype (Reinhold-Hurek et al., 2015; Zolti et al., 2019) (Fig. 1.10). The host plant has important role in the bacterial community composition, affecting the active bacterial community in the soil. This effect, which decreases with the distance to the root, varies according the plant species (cucumber, tomato, maize, wheat). Gene expression profiles in both bacteria and roots' cells reflect the requirements to colonize the roots (Yu and Hochholdinger, 2018) and to the selection of appropriate bacterial species, including motility and chemotaxis, polysaccharide degradation, various two-component systems, multiple secretion systems, and lipopolysaccharide biosynthesis (Ofek et al., 2014).

The richness of bacteria in the root compartments decreases to approximately half of the OTUs of the bulk soil (Ofek et al., 2014). This "funnel effect" implies that only a fraction of the bacterial pool present in the bulk soil colonizes the rhizosphere, and that, from this community, only another fraction colonizes the rhizoplane and roots' endosphere. Due to the factors mentioned previously, the microbiome of root endosphere is usually distinct

from the rhizosphere and rhizoplane bacterial populations. This is due to the phenotypic traits required to colonize the plant, as root surface attachment and colonization are necessary steps for root colonization (Dörr et al., 1998; Reinhold-Hurek et al., 2006).

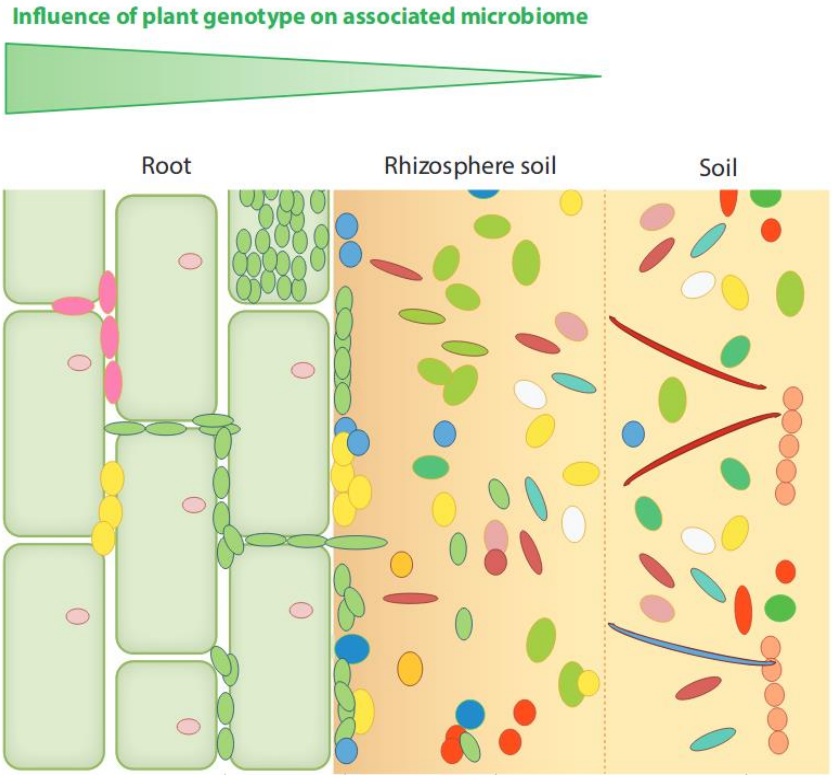


Figure 1.10. The effects of the plant genotype according the distance to the roots (Reinhold-Hurek et al., 2015).

1.7. Microbial Ecology Methods

The advent of Molecular Biology, invention of PCR, and, more recently, DNA sequencing, broaden the ways how Ecosystems could be studied (Fox et al., 1977; Gupta et al., 1983; Hugenholtz et al., 1998; Staden, 1979). It became possible to focus on microbial communities, thanks to the development of specific genetic markers. Among them, ribosomal DNA is widely used for Phylogenetic studies, and allowed (Woese, 1990) to define the three domains of life: Bacteria, Archea, Eukarya.

Bacteria and Archaea have a 50S large ribosomal subunit that contains two rRNA species, the 5S and 23S rRNAs. The Prokaryote 30S small ribosomal sub-unit contains a single 16S rRNA. rRNAs evolve more slowly than protein encoding genes, and are particularly important for the phylogenetic analysis of distantly related species. The 16S rDNA is about 1550 base-pairs and it is constituted by 9 hypervariable regions (Fig. 1.11),

V1 to V9. Particular phylogenetic groups have unique oligonucleotide sequences in the variable regions, surrounded by conserved regions, thus allowing for the design of “universal“ primers. The “universal” primers allow DNA amplification of conserved regions and hyper-variable regions and the subsequent identification of bacteria (Amit Roy, 2014).

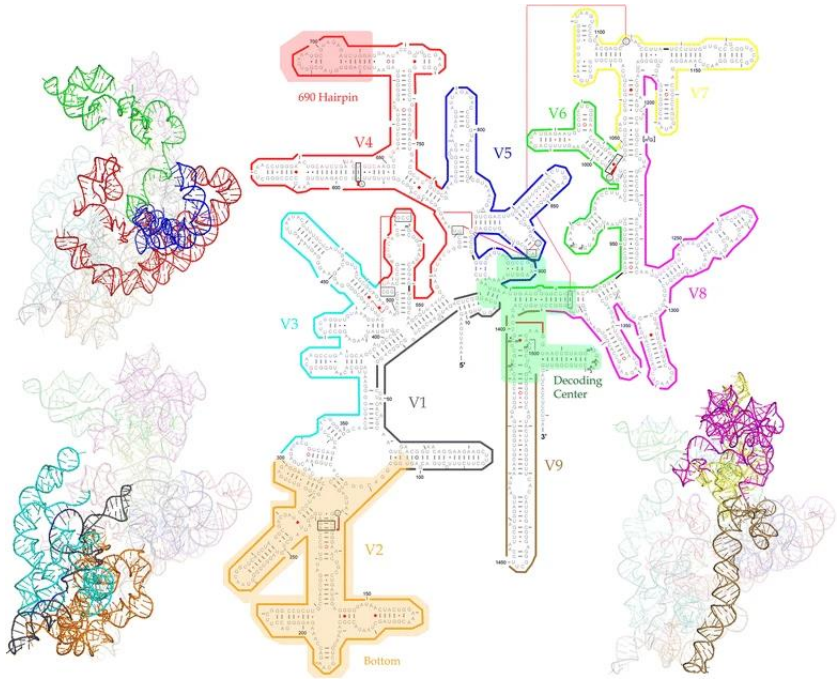


Figure 1.11. Schematic representation of the hyper-variable regions of the 16S rDNA gene, from V1 to V9 (Yang et al., 2016).

The utilization of 16S rDNA has its shortcomings, particularly concerning sensitivity and resolution. Short amplified sequences from next-generation sequencing results in a narrow choice of sets of primers for certain hypervariable regions. The short size of these sequences, added to the fact many bacteria may share the same sequences, leads to the uneven amplification of certain taxa, with their posterior erroneous taxonomy attribution, under- or over- estimation in their abundance, and a consequent reduction in sensitivity of the system (Wang and Qian, 2009; Yang et al., 2016). Connected to the sensitivity is the correlation of different Phylogenetic groups to different hypervariable regions (Yang et al., 2016), wich represents an extra bias. Several genetic markers have been proposed over the years as alternative or complement to 16S rDNA, such as *rpo*B (Case et al., 2007; Vos et al., 2012) *or gyr*B (Peeters and Willems, 2011; Wang et al., 2007).

1.7.1. Methodology for 16S rDNA amplicon sequencing

An outline of the procedure for 16S rDNA sequencing is shown in Fig. 1.12. Isolated DNA from biological material is subjected to targeted enrichment to amplify the 16S rDNA regions of interest, producing amplicon libraries enriched in the targeted sequences. These libraries are then subjected to two additional PCRs. The purpose of the first PCR is to add unpaired nucleotides at 5' of both sense and anti-sense sequences. The subsequent PCR will add a specific sequence of nucleotides (multiplexing indexes) per sample, allowing either single (Fig. 1.12 A) or double indexation (Fig. 1.12 B). Afterwards the samples are pooled and a single NGS run is performed.

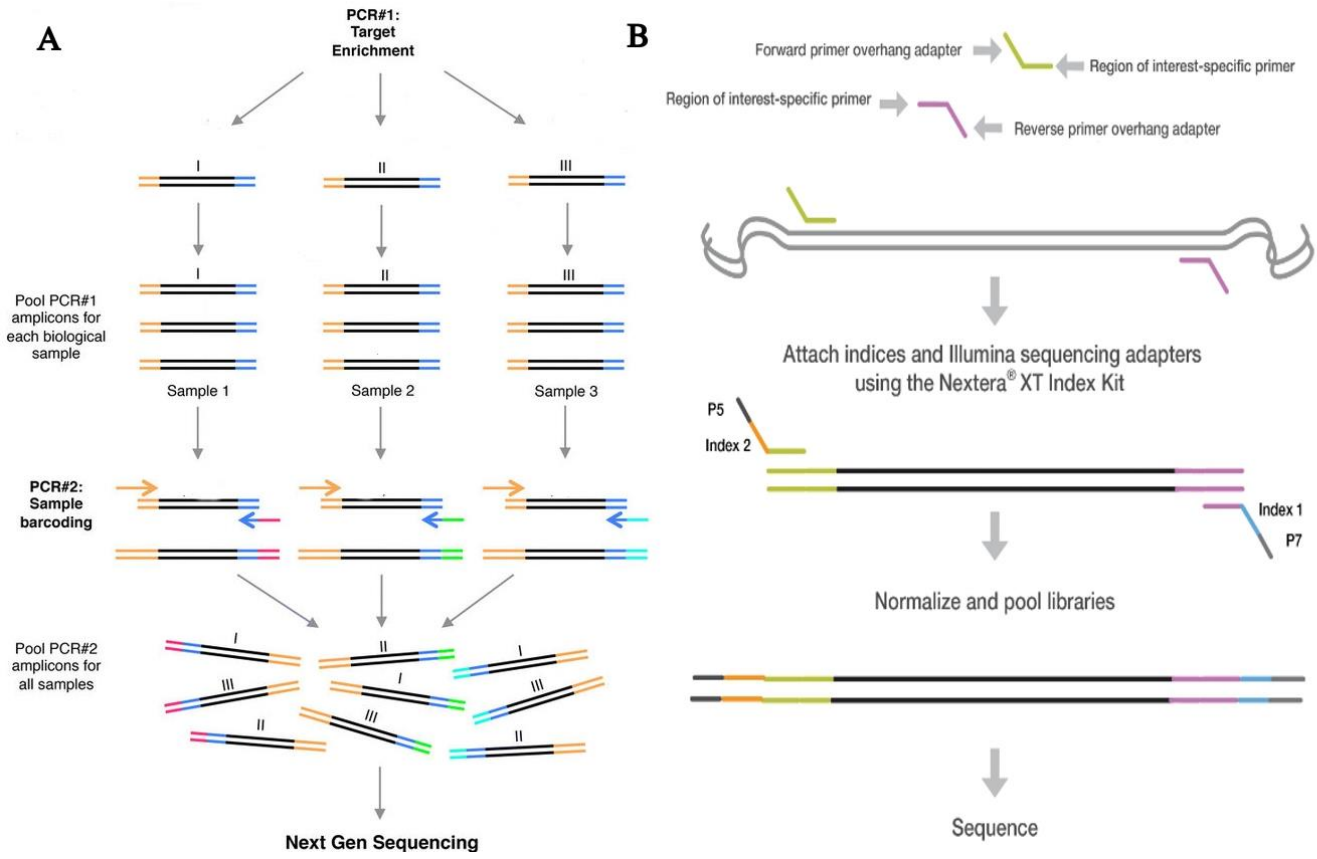


Figure 1.12. Steps for preparing the 16S rDNA library allowing pooling of different samples, in two sequential PCRs. Firstly, to add the overhangs to the target sequences. Secondly, to add the DNA barcodes to allow sample multiplexing. **(A)** 16S rDNA library preparation using Illumina Universal Adapters® (Bernstein et al., 2015) and **(B)** using Illumina Nextera XT® indexes (https://www.illumina.com/).

1.7.3. Ecology Measurements

The basis to study microbial communities is their diversity, a concept that was developed in the mid-20th century. The denominations of α-, β- , and γ-diversity were introduced by Robert Harding Whittaker (Whittaker, 1960), but only the first two are used for microbiome studies. The concept of α-diversity in Ecology is related to richness and/or evenness of species on a local site, whereas β-diversity is broadly defined as the variation in the identities of species between two sites (Anderson et al., 2011).

The presented work in this Thesis is mainly focused on β-diversity, as it is more relevant to compare microbiome compositions, either in longitudinal or cross-sectional studies, and to understand the impact of stressors on the microbiomes (agricultural practices). There are dozens of developed equations/algorithms for α- and β-diversity (Anderson et al., 2011; Hill et al., 2003; Koh, 2018; Wilson and Shmida, 1984). However, it will be only presented the most commonly α-diversity measurements used and the relevant β-diversity measurements for this Thesis.

1.7.4. α-diversity

Diversity describes both richness, the number of different classes measured (species, OTUs, etc) and evenness, the distribution of individuals among classes, and both parameters have to be taken into account by diversity descriptors (Hill et al., 2003). The different α-diversity metrics are classified into non-phylogenetic and phylogenetic metrics. The non-phylogenetic measures are constructed based solely on microbial abundance information, while the phylogenetic metrics further utilize information from phylogenetic trees (Koh, 2018). Non-phylogenetic measures are more common and have been used since mid-20th century. Three of the most known metrics are the Shannon (Shannon, 1948), the Simpson (Simpson, 1949; Hurlbert, 1971), and the Pielou index (Pielou, 1966).

The Shannon index (*H'*) is calculated by the equation 1.5, in which is p_i stands for relative abundance value of species *i* and *S* is the number of species.

$$H' = \sum_{i=1}^{S} p_i \ln(p_i) \quad (1.5)$$

The evenness are relative measures expressing the degree of evenness and inequality in a given community, derived from species richness and diversity (Jost, 2010). Pielou's eveness index (1.6) is derived from the Shannon's diversity index, by dividing it by the natural logarithm of the number of classes (*S*).

$$J = \frac{\sum_{j=1}^{S} p_i \ln(p_i)}{\ln(S)} \quad (1.6)$$

The Simpson index (1.7) (*D*) access diversity by measuring dominance, gives more weight to the most common species. In this equation *S* is the number of classes, n_i is the number of bacteria observed from the ^{i}th class and *N* is the total number of individuals observed in the sample.

$$D = \frac{\sum_{I=0}^{S} n_i(n_i-1)}{N(N-1)} \quad (1.7)$$

The Simpson's index D_s (1.8) is a derivative of Simpson index (*I*) that is normally used to calculate the probability of two random selected individuals belong to two different species. It serves as an evenness measure.

$$D_s = 1 - \frac{\sum_{I=0}^{S} n_i(n_i-1)}{N(N-1)} \quad (1.8)$$

1.7.5. β-diversity

α-diversity does not give values for species turnover, gradients, or variation in bacterial communities (Anderson et al., 2011). β-diversity measurements approach either turnover, or variation of species within a given population, or between two populations. In microbial ecology, β-diversity is derived from multivariate measures based on pairwise comparisons among samples. (Anderson et al., 2011). This approach explores the relationships between the communities' composition and environmental factors, and variation comparison among *a priori* stablished groups (Fig. 1.13) (Anderson et al., 2011). Many other approaches may be applied according the research objective. This

may include study of partition variation in communities' composition by specific factors, environmental variables, or hierarchical scales. It can be used as well for comparisons of effect sizes of factors (Anderson et al., 2011).

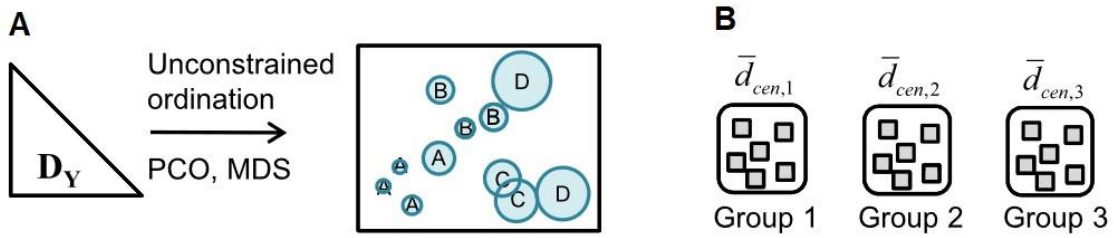


Figure 1.13. Multivariate measures applied for the present work. **(A)** exploration of the relationships between the community composition and factors. **(B)** variation comparison among *a priori* groups (Anderson et al., 2011).

Two algorithms are mainly used to analyse dissimilarities in microbiome β-diversity, the Bray-Curtis dissimilarity index (Bray and Curtis, 1957), and the UniFrac metrics (Lozupone and Knight, 2005; Lozupone et al., 2007). Both of them calculate pair-wise sample comparisons generating samples dissimilarity matrixes. The matrixes are then used for permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), which allows the analysis of microbiomes composition variation between *a priori* defined groups.

1.7.5.1. Bray-Curtis

The Bray-Curtis dissimilarity is a non-phylogenetic β-diversity measure and depends on the number of a species counts that are unique to any of both communities in relation to the sum of abundances in both communities (Baselga, 2013). It excludes joint absences of species in the compared communities (Anderson et al., 2011). Abundant species have more weight in the coefficient than rare species, as the latter add very little to the value of the coefficient.

The Bray-Curtis dissimilarity (1.9) takes in consideration X_{Ai} and X_{Bi}, representing the abundance of species *i* on the communities A and B. This will result in a value between 0 and 1.

$$(1.9) \quad d_{BC} = \frac{\sum_{i=1}^{S} |X_{Ai} - X_{Bi}|}{\sum_{i=1}^{S} (X_{Ai} + X_{Bi})}$$

Two completely equal communities, having equal species abundances will present a value of 0, whereas different communities will have a value of 1. This would mean that both communities have no species in common and that the abundance of species in one community is balanced by the abundance of species in the other community. Bray-Curtis dissimilarity is an example of a classical β-diversity that does not take into consideration evolutionary relationships between species present in the same population, butonly their abundances (Lozupone and Knight, 2005).

1.7.5.2. UniFrac dissimilarities

The UniFrac metrics were developed to test differences between microbiomes, including phylogenetic information (Lozupone and Knight, 2005). There are two methods to calculate UniFrac dissimilarities, the unweighted and weighted. They were created to avoid the shortcomings of the non-phylogenic β-diversity measures, like Bray-Curtis dissimilarity. In UniFrac, phylogenetic distances are taken into consideration, measured as the length of the branches from phylogenetic trees constructed for each community, an information usually extracted from 16S rDNA sequencing data (Lozupone and Knight, 2005; Lozupone et al., 2007).

1.7.5.3. Unweighted Unifrac (UniFrac value)

The UniFrac value (*u*) (1.10) is calculated by using n as the total number of branches in the phylogenetic tree, and being bi the length of branch *i*. *Ai* and *Bi* are the numbers of sequences that descend from branch *i* in communities *A* and *B*, respectively. A_T, B_T are the total numbers of sequences in those communities.

$$u = \sum_i^n b_i \times \left| \frac{A_i}{A_T} - \frac{B_i}{B_T} \right| \quad (1.10)$$

1.7.5.4. Weighted Unifrac

Weighted UniFrac is used to count how many sequences from each lineage are present, as well as detect changes in which taxa are present (Lozupone et al., 2007). It quantifies the compositional dissimilarity between two different communities based on the sequences (OTUs) counts as well as the phylogenetic dissimilarity. It is calculated by dividing the u coefficient from formula (1.10) by the scaling factor *D* (1.11). Where d_j is

the distance of the sequence j from the root. *Aj*, *Bj* represent the number of occurrence of sequence *j* in communities *A* and *B*, respectively. A_T and B_T are the total numbers of sequences from communities *A* and *B* (1.12).

$$D = \sum_j^n d_j \times \left(\frac{A_j}{A_T} - \frac{B_j}{B_T}\right) \quad (1.11)$$

$$wUniFrac = \frac{\sum_i^n b_i \times \left|\frac{A_i}{A_T} - \frac{B_i}{B_T}\right|}{\sum_j^n d_j \times \left(\frac{A_j}{A_T} - \frac{B_j}{B_T}\right)} \quad (1.12)$$

1.8. Water scarcity and water reuse

Quality fresh water for agriculture is becoming an increasingly scarce resource due to climate change effects and to the escalating competition from other water-using sectors. TWW can constitute an alternative source of water, capable to preserve socio-economic development, vital ecosystems, and security in food production. This is especially true for arid and semi-arid regions where water shortage is a severe problem. Although TWW reuse for irrigation has gained an acceptance as an economic alternative that could substitute nutrient needs and water requirements of crop plants, TWW may contain undesirable chemical constituents and pathogens that pose negative environmental and health impacts (Piña et al., 2018).

1.8.2. WWTPs and TWW

WWTP treatments usually consist in two or three distinct stages (Michael et al., 2013) (Fig. 1.14). The primary treatment uses a physical unit to remove large objects, and a grit chamber to remove small stones or sand from the raw influent. This physical screening is followed by sedimentation tanks, to remove suspended solids still present in the WW, allowing the formation of a primary sludge. The effluent from the sedimentation tanks then goes through the secondary treatment, in which biodegradable organic matter, including organic matter, nitrogen (N) and phosphorus (P), and suspended solids are removed to a great extent using activated sludges.

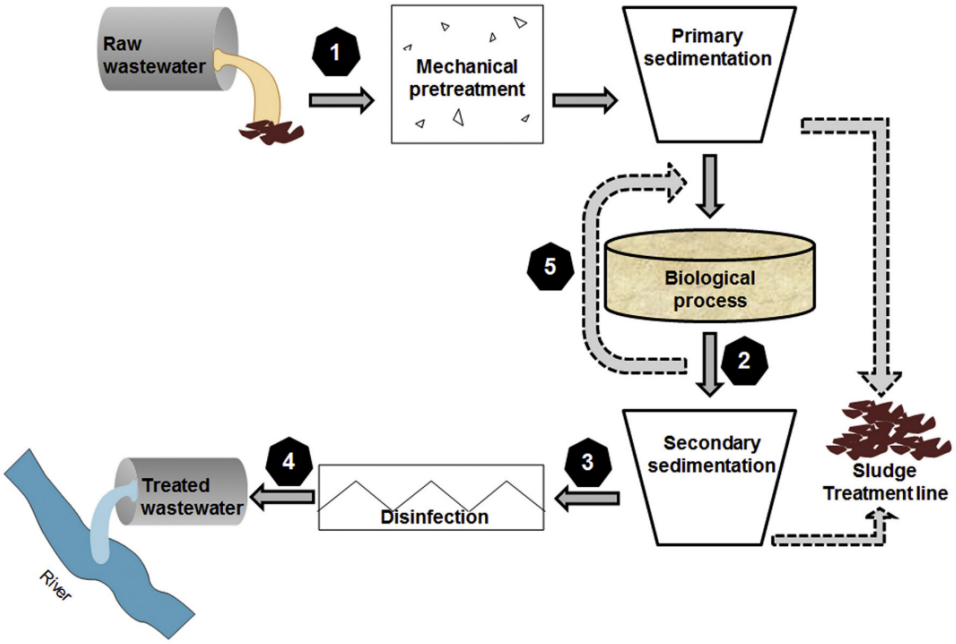


Figure 1.14. Typical WWTPs processes in a conventional activated sludge treatment plant. Desinfection (Step 4) is optional, only applied if WW is going to be reused (Manaia et al., 2018).

The activated sludge step consists in the utilization of aerobic bacteria and other microorganisms, as protozoa, that form flocs in aerobic tanks. Next, WW flows to sedimentation tanks for WW clarification, henceforth originating the secondary sludge. Both primary and secondary sludge may be used as fertilizers, after composting or stabilization (Kirchmann et al., 2017; Yoshida et al., 2015) with certain limitations regarding the content of heavy metals (EC Council Directive 86/278). Finally, a tertiary treatment may be employed to disinfect the WW, but this step is not required nor frequently employed in WWTPs. These processes mainly include media filtration (e.g., granular activated carbon/sand/gravel) and disinfection (e.g., chlorination, UV irradiation, and ozonation), and it may include a combination of individual treatment processes. (Manaia et al., 2018; Michael et al., 2013).

1.8.3 Removal Efficiencies of ABs

Wastewater treatment plants (WWTPs) have been identified as “hot spots” of ABs, ARB, ARGs (Burch et al., 2019; Rizzo et al., 2013), and as a main source of them in downstream environments. WWTPs fail to completely remove antibiotics, antibiotic resistant bacteria (ARB), and ARGs in most cases (Manaia et al., 2018; Michael et al., 2013). Removal efficiency varies markedly under different environmental conditions and

as a function of chemical and physical properties of the compounds (Burch et al., 2019) as shown in Table 1.1.

Table 1.1. Removal efficiencies with secondary treatment plant. Negative removal efficiency values refer to increases, rather than decreases, of the concentration of a given analyte, which can happen due to several factors, including operational parameters, chemical analytical procedures, deconjugation of conjugated metabolite, and other unique WW characteristics (Burch et al., 2019).

ABs	Removal efficiency (%)	Country/region of research	References
Sulfamethoxazole	−36 to 50	Canada; China; India; Switzerland; Taiwan; USA	(Blair et al., 2015; Gobel et al., 2007; Karthikeyan and Meyer, 2006; Lin et al., 2009, 2009; Prabhasankar et al., 2016; Xu et al., 2007)
Sulfamethazine	−5 to 20	Canada; Spain; USA	(Blair et al., 2015; Jelic et al., 2011; Miao et al., 2004; Prabhasankar et al., 2016; Xu et al., 2007)
Tetracycline	−88 to 22	Canada; China; Japan; Taiwan	(Ghosh et al., 2009; Gulkowska et al., 2008; Li and Zhang, 2011; Lin et al., 2009; Miao et al., 2004)
Trimethoprim	-53 to 90	Australia; India; Spain; Switzerland; Taiwan; USA	(Batt et al., 2007; Gobel et al., 2007; Jelic et al., 2011; Lin et al., 2009; Prabhasankar et al., 2016; Watkinson et al., 2007)
Ciprofloxacin	-89 to 30	Germany; USA; Canada; Switzerland	(Blair et al., 2015; Golet et al., 2003; Karthikeyan and Meyer, 2006; Lindberg et al., 2005)

Norfloxacin	-165 to 20	Switzerland; China; Canada	(Golet et al., 2003; Lindberg et al., 2005; Miao et al., 2004; Xu et al., 2007)
Clarithromycin	-73	Switzerland; USA	(Blair et al., 2015; Giger et al., 2003)
Ofloxacin	40	Canada; China; Switzerland; Taiwan	(De la Cruz et al., 2012; Jia et al., 2012; Lin et al., 2009; Miao et al., 2004; Peng et al., 2006; Xu et al., 2007)
Erythromycin	80	China; India; Germany; Spain; Taiwan; UK	(Lin et al., 2009; Miao et al., 2004; Prabhasankar et al., 2016; Roberts and Thomas, 2006; Suarez et al., 2010; Ternes et al., 2007; Xu et al., 2007)

The efficiency of different tertiary treatments for ABs removal are based mainly on bench-scale studies, as the available data in full scale WTTPs is still limited. Tertiary treatments are not mandatory in most countries, and are expensive to use at a full scale (Burch et al., 2019). Table 1.2 shows results from some of these studies.

Table 1.2. Removal after the tertiary treatment varied from chlorination, sand filtration, UV radiation (Burch et al., 2019).

Antibiotics	**Tertiary removal (%)**	**Country**	**References**
Sulfamethoxazole	51-100	China, Swizterland, Japan	(Alajmi, 2014; De la Cruz et al., 2012; Kim et al., 2009; Li and Zhang, 2011)
Ciprofloxacin	48-88	China, Switzerland	(De la Cruz et al., 2012; Giger et al., 2003; Golet et al., 2003; Li and Zhang, 2011)
Norfloxacin	50-92	China, Switzerland	(Golet et al., 2003; Li and Zhang, 2011)
Tetracycline	39	China	(Li and Zhang, 2011)

Trimethoprim	7-100	China, Switzerland, Japan	(De la Cruz et al., 2012; Kim et al., 2009; Li and Zhang, 2011)
Ofloxacin	65-100	China, Japan, Switzerland	(De la Cruz et al., 2012; Kim et al., 2009; Peng et al., 2006)
Erythromycin	43	China	(Li and Zhang, 2011)

1.8.3. Removal efficiencies of ARB and ARGs

WWTPs are considered “hotspots” for ARGs dissemination as well as for propagation of clinically relevant ARB, depending on the origin of the influent: housing areas, industrial or hospitals. For example, hospital WW show higher abundance of multi-resistant *P. aeruginosa,* which can disseminate into receiving waters and ultimately reaching irrigated soils (Czekalski et al., 2012). Several studies indicate that clinically relevant bacteria (*E.coli*), MGE, and integrons can survive most WWTP processes (Table 1.3) (Fekadu et al., 2019; Gatica et al., 2016; Michael et al., 2013). Bacteria may enter a state of dormancy due to the stress, re-covering when the stressors disappear (Becerra-Castro et al., 2016; Moreira et al., 2018; Sousa et al., 2017). In addition, some disinfectants, as peracetic acid chlorine, or elevated temperatures, may even have a positive selective effect on ARGs (Di Cesare et al., 2016; Novo et al., 2013).

Table 1.3. Antibiotic resistance in WWTPs after biological process (Rizzo et al., 2013).

WWTP	Biological process	ARB	Resistance to antibiotics	Reference
UWTP (150,000 habitants, 11,000 m^3/day) in Beni Mellal (Morocco) receives sewage of essentially domestic origin.	Activated sludge	Faecal coliforms	111 strains isolated: overall resistance (resistance to at least one antibiotic) was 72.1%. The antibiotic resistance of FC was found to be comparable between raw (71.0%) and treated sewage (77.8%). The higher levels of antibiotic resistance were towards streptomycin (54.0%), ampicillin (42.3%), amoxicillin (42.3%) and amoxicillin–clavulanic acid (31.5%).	(Fars et al., 2005)

WWTP	Biological process	ARB	Resistance to antibiotics	Reference
UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.	Activated sludge	*Enterococcus hirae*, *Enterococcus faecium* and *Enterococcus faecalis*	Tetracycline (31/33%), gentamicin (50/50%), erythromycin (33/23%), ciprofloxacin (9/25%), amoxicillin (0/3%), sulfamethoxazole/trimethoprim (0/1%) and no resistance to vancomycin	(Da Silva et al., 2006)
14 UWTPs in Portugal	N.A.	Enterococci	983 isolates: multidrug resistance (49.4%), rifampicin (51.5%), tetracycline (34.6%), erythromycin (24.8%), nitrofurantoin (22.5%), ciprofloxacin (14%), ampicillin (3.3), vancomycin (0.6%)	(Martins da Costa et al., 2006)
Gdansk–Wschod' UWTP (700,000 PE) in Northern Poland, mainly domestic ww, only limited industrial (5%) and undisinfected hospital (0.17%) ww	Activated sludge process working in modified UCT (University of Cape Town) type system	Enterococci	199 isolates: nitrofuration (53%), erythromycin (44%), ciprofloxacin (29%), tetracycline (20%).	(Łuczkiewicz et al., 2010)
		Escherichia	153 isolates: ampicillin (34%), piperacillin (24%), tetracycline (23%), levofloxacin (10/15%), Trimethoprim/Sulphamethoxazole (11%), ciprofloxacin (10%)	
UWTP (72,000 inhabitants) in Ireland under the effect of ww from four hospitals.	Activated sludge	*Escherichia*	Ampicillin (24.5/12.5%), streptomycin (16.5/0%), sulfamethoxazole (11.1/12.5%), tetracycline (12.4/39%), ciprofloxacin (7.15/0%), cefoxitin (0.11/2.6%)	(Galvin et al., 2010)
UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.; 400 mg COD/L, 250 mg BOD/L	Activated sludge	*Escherichia*	Tetracycline (32.1/36.8%), amoxicillin (28/34.8%), sulfamethoxazole/trimethoprim (22.2/22.5%), cephalothin (10.5/20.5%), ciprofloxacin (2.5/9.7%)	(Da Silva et al., 2006)
		Shigella	Tetracycline (0/25%), amoxicillin (20/12.5%), sulfamethoxazole/trimethoprim (0/12.5%), cephalothin (12/0%), ciprofloxacin (0/6.3%)	

WWTP	Biological process	ARB	Resistance to antibiotics	Reference
		Klebsiella	Tetracycline (5/13%), amoxicillin (94.7/95.7%), sulfamethoxazole/trimethoprim (0/8.7%), cephalothin (0/5.9%), ciprofloxacin (0/4.4%)	
2 large-scale Danish UWTPs (240,000 and 500,000 inhabitants)	Activated sludge for N and P removal	*Acinetobacter*	442 isolates: ampicillin (27/22.3%), gentamicin (7.2/5.8%), tetracycline (11.5/12.9%), multidrug resistance (n.d./0.3%)	(Guardabassi et al., 2002)
UWTP (210,700 PE) in Ann Arbor, Michigan, USA, mostly domestic ww, only limited industrial and untreated hospital ww	Activated sludge for N and P removal (+ ferric chloride P removal)	*Acinetobacter*	366 isolates: trimethoprim (92.2/100%), rifampin (63.1/77.5%), chloramphenicol (25.2/35%), amoxicillin/clavulanic (8.7/20%), sulfisoxazole (8.7/22.5%), ciprofloxacin (4.9/11.3%),	(Zhang et al., 2009)

1.8.4. Sources of contamination for downstream environments

WWTPs rank among the most important reservoirs of antibiotic resistance in urban environments. Their fairly stable pH and temperature favour a remarkable diversity of microorganisms present in these peculiar habitats, leading to the release of high amounts of bacteria into the environment, many of which of animal (e.g. pets or small husbandry or animal farms) or human origin (Berendonk et al., 2015; Manaia et al., 2018; Rizzo et al., 2013). Many of these bacteria harbour acquired ARGs and are potential carriers for the dissemination of these genes in the environmental microbiome (Berendonk et al., 2015; Manaia et al., 2018; Pruden, 2014). Therefore, their contribution to the emergence of resistance in pathogenic bacteria cannot be neglected (Manaia et al., 2018).

1.9. Downstream Environments

1.9.1. Fate of ARGs in soils and crops/ impacts of agricultural practices

Human activity, in particular agricultural practices such as the application of manure and sewage sludge to crop fields, can significantly alter the composition of the so-called resistome, the sum of ARGs and of their precursors present in a particular microbiome (Wright, 2007) (Fig. 1.15). Several studies have shown that these practices increase the presence in soil of ARGs that are usually found in pathogenic bacteria, thereby contributing to the mix of human and environmental resistomes (Banwart et al., 2019).

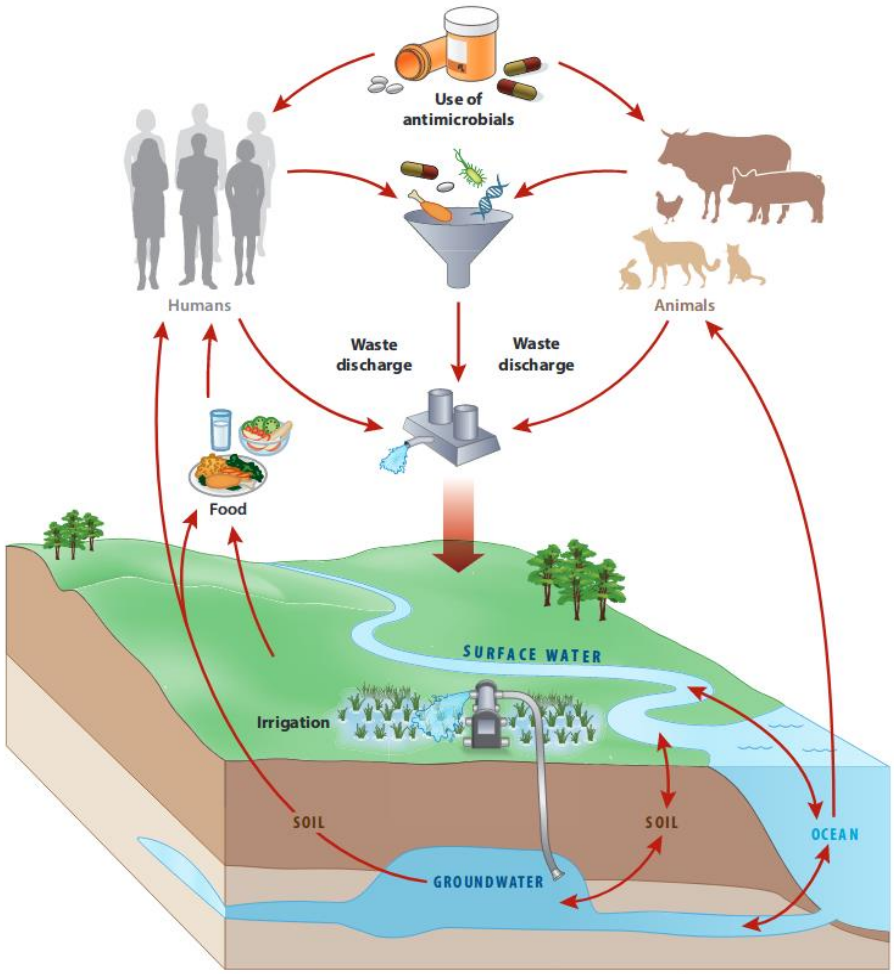


Figure 1.15. Network of ARGs between environmental and human settings and its connections to the soil environment (Banwart et al., 2019).

1.9.2. Irrigation water impact

1.9.2.1. ABs uptake by crops

The use of TWW for irrigation has the potential to transfer heavy metals (Khan et al., 2008), ABs and other CECs (Conkle et al., 2008 ; Christou et al., 2019), and pathogens (Manaia et al., 2018) to soils, crops, and, finally, foodstuffs. This potential risk increases when TWW are directly or indirectly reused for agricultural irrigation, a practice becoming more and more frequent due to the actual water scarcity in many countries (Piña et al., 2018). ABs used in human or livestock healthcare become excreted and ultimately discharged into the aquatic environment, either as the primary products or as metabolites, in some cases with similar or even higher biological activities than the parental compound (Marx et al., 2015). Once ABs reach the soil, their degradation depends on soil physicochemical properties and microbial activity, so they may exert a continuous selective pressure over soil microbiomes for long periods (Kümmerer, 2009; Pan and Chu, 2016; Segura et al., 2009). Plants may uptake ABs via their roots, and they can be found in different plant parts (root, fruit, leaves). Plant tissues may therefore accumulate sub-inhibitory concentrations of different ABs, such as tetracycline, streptomycin, and ciprofloxacin (Ahmed et al., 2015; Gullberg et al., 2011).

The AB uptake capability of different plants varies due to several factors, including the soil properties, plant evapotranspiration potential and its net irrigation requirements (Christou et al., 2019; Trapp, 2015). Plants grown in a hot and dry environment have increased rates of evapotranspiration compared to those grown in milder environmental conditions (Christou et al., 2019). Fruit vegetables (cucumber, tomato, pepper, beans) have a lower ability for uptake of soil-borne pollutants, including AB, compared to leafy and root vegetables (Christou et al., 2019) (Fig. 1.16). The uptaken pollutants become ultimately translocated to leaves and fruits (Christou et al., 2019; Goldstein et al., 2014).

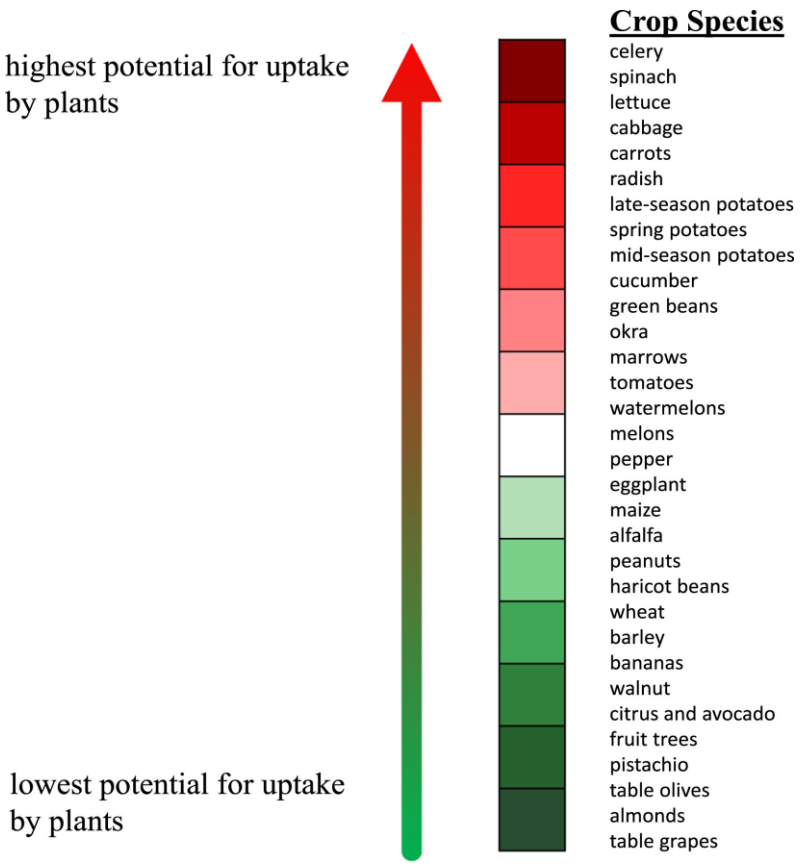


Figure 1.16. Potential of several crops regarding CECs uptake (including ABs). The highest potential for uptake is indicated with dark red; the lowest potential with dark green (Christou et al., 2019).

1.9.3. Soil amendment impact

1.9.3.1. Manure

The application of manure for amending crop soils may cause a series of environmental problems, among them the spread of ARGs is considered as a serious concern (Heuer and Smalla, 2007; Jechalke et al., 2014; Liu et al., 2017; Marti et al., 2013; Riber et al., 2014; Udikovic-Kolic et al., 2014). Large amounts of in-feed ABs that cannot be absorbed by livestock are excreted through feces or urine, forming part of the manure and, if used for soil amendment and fertilization, leading to an elevated level of residual antibiotics in arable soil (Christou et al., 2019; Liu et al., 2017). In addition, animal manure has been proved to be an important reservoir of bacteria carrying ARGs and MGEs, whose relative abundances in soil are mutually correlated (Heuer and Smalla, 2007; Wang et al., 2015; Xiang et al., 2018; Zhu et al., 2013). The high abundance of MGEs facilitates a higher rate of gene transfer between different bacterial species by HGT. This is a main factor

driving the alteration of soil resistome by manure application (Stalder et al., 2012; Tamang et al., 2008), as ARBs in the manure may be introduced into soil and the ARGs they carried could be horizontally transferred to native soil bacteria and/or human pathogens present in soils, like *Salmonella* spp., Shigella spp., and *E. coli* (Ding et al., 2014; Forsberg et al., 2012; Gatica and Cytryn, 2013; Wang et al., 2017).

1.9.3.2. Chemical fertilizers

Use of chemical fertilizers is known to have a high influence on the soil bacterial community, particularly on bacterial species related to the nitrogen cycle. Nitrogen fixing bacteria (Berthrong et al., 2014), ammonia-oxidizing bacteria (Ai et al., 2013), and denitrifiers (Tang et al., 2010; Zhou et al., 2015) are affected by chemical fertilization. Long time application (decades) of chemical fertilization is known to alter soil bacterial populations (Zhou et al., 2015), likely due to changes in soil properties. Fertilization significantly increased total Nitrogen, Phosphorus, NO_3^-, NH_4^+, and decreased soil pH. From these parameters, soil pH and NO_3^- concentration are the most important factors affecting the composition of the bacterial communities. A high α-diversity in the microbial community is negatively correlated with NO_3^- concentration and positively correlated with pH. The application of inorganic chemical fertilizers caused the increase of the relative abundances of Actinobacteria, Proteobacteria, Saccharibacteria, and Verrucomicrobia, among other taxa (Zhou et al., 2015).

1.9.3.3. ARBs and ARGs uptake in crops

ARB and ARGs are found naturally in soil, where they likely originated (Gibson et al., 2015). In turn, plants hold a large microbial diversity either adsorbed onto the surface, or inside the vascular system, forming communities designated as phyllosphere and endosphere, respectively. Soil is assumed to be the major source of endophytes, the entrance of which can be considered as a dynamic interaction between the plant root system, the rhizosphere, and soil microbiomes (Gibson et al., 2015; Reinhold-Hurek et al., 2015). Practices that have the potential to alter the soil microbiome can be regarded as a source of contamination first for the plant phyllosphere and endosphere and, ultimately, for the human and animal food chain, in the case of agriculture exploitation. Irrigation with TWW and soil amendment with manure are two agricultural practices that may cause such alterations in soil, plant, and food microbiomes.

In summary, there is a growing concern that the currently increasing presence of ABs in the environment may affect soil and plant microbiomes and that this may lead to the increase of antibiotic resistance of bacteria present in the human and livestock food chains, from soils and harvested vegetables up to the human consumers (Chen et al., 2016; H.-W. Hu et al., 2016; Marti et al., 2013; Westhoff et al., 2017). As the fitness costs of the resistance to antibiotics are apparently null or very low, concentrations as low as 1/10 the minimum inhibitory concentration (MIC) for susceptible strains may still favour antibiotic resistant strains over sensitive ones in food microbiomes (Westhoff et al., 2017).

1.10. Hypotheses

The project was performed at the Institute of environmental research and Water Studies (IDAEA), under the supervision of Dr. Benjamin Piña and Dr. Josep Bayona. It focuses in soil and plant-associated microbiology, with emphasis on the impact of ABs present in TWW used for crops' irrigation, the distribution of ARGs and their modulation, and the microbiomes dynamics correlated to TWW use and soil amendment. Therefore, there were two initial hypotheses.

Hypothesis I: agricultural practices affect the abundance of ARGs along the soil-plant continuum.

Hypothesis II: agricultural practices cause a shift in the bacterial communities along the soil-plant continuum.

These hypotheses were tested using culture-independent methods. They included qPCR for the quantification of ARGs and next-generation sequencing methods along with bioinformatics approaches to analyze the DNA sequences, assessing the microbiomes composition, diversity, and possible shifts along the soil-plant continuum.

The project was divided in three main objectives:

- Quantify selected ARGs in soil, rhizospheric soil, roots, leaves, and fruits or beans (seeds) in different crops from real commercial fields

- Detect and quantify proxies for anthropogenic impact and HGT on crop resistomes and microbiomes

- Assess changes of soil and endophytic microbiomes related to the quality of irrigation water using commonly eaten plants

Hence, as part of the MSA-ITN project, this project aimed to promote safe TWW reuse and agricultural practices, by generating relevant data based on validated scientific information.

Chapter II: Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices

This chapter is based on the article:

Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Benjamin Piña. (2019). Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial *Lycopersicon esculentum* fields under different agricultural practices. Science of the total Enviroment, 652, 660-670.

While the presence of ARGs in agricultural soils and products has been firmly established, their distribution among the different plant parts and the contribution of agricultural practices, including irrigation with reclaimed water, have not been adequately addressed yet. To this end, we analysed the levels of seven ARGs (*sul*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *mec*A, *qnrS*1, *tet*M, $bla_{OXA\text{-}58}$), plus the integrase gene *intI*1, in soils, roots, leaves, and fruits from two commercial tomato fields irrigated with either unpolluted groundwater or from a channel impacted by TWW, using culture-independent, qPCR methods. ARGs and *intI*1 sequences were found in leaves and fruits at levels representing from 1 to 10% of those found in roots or soil. The relative of *intI*1 sequences correlated with *tet*M, bla_{TEM}, and *sul*1 levels, suggesting a high horizontal mobility potential for these ARGs. High-throughput 16S rDNA sequencing revealed microbiome differences both between sample types (soil plus roots versus leaves plus fruits) and sampling zones, and a correlation between the prevalence of Pseudomonadaceae and the levels of different ARGs, particularly in fruits and leaves. We concluded that both microbiome composition and ARGs levels in plants parts, including fruits, were likely influenced by agricultural practices.

2.1. Introduction

The occurrence of pathogenic bacterial strains increasingly recalcitrant to medical treatment with the existing ABs is becoming a worldwide concern (World Health Organization, 2014). AR is associated to increased hospitalization and mortality rates of humans and with AR-resistant zoonotics, two serious concerns for the health and welfare of both humans and animals (Berendonk et al., 2015; Koch et al., 2017). Moreover, the estimate number of deaths caused annually by ARB in the United States of America and in the European Union are calculated as 23,000 and 25,000, respectively (https://www.cdc.gov/drugresistance/;Freire-Moran et al., 2011).

The onset of ARB, and of its worse manifestation, multi-antibiotic-resistant microbes, is linked to the ever-increasing presence of ABs in the environment, largely related to human activities (Chen et al., 2016; Han et al., 2016; H.W. Hu et al., 2016; Koch et al., 2017). ABs reach the environment mainly from excretion of treated humans and livestock, whose wastes contain either non-metabolized ABs or their active (or activable) metabolites (Marx et al., 2015). As WWTPs can only partially remove ABs, ARB, or ARGs (Le-Minh et al., 2010; Michael et al., 2013), they have been identified as relevant contributors to their total environmental loads. This potential risk increases when TWWs are directly or indirectly reused for agricultural irrigation, a practice becoming more and more frequent due to the actual water scarcity in many food production regions in the Mediterranean and other areas (Piña et al., 2018). Once ABs reach the soil, their degradation depends on soil physicochemical properties and microbial activity, so they may exert a continuous selective pressure over soil microbiomes for long periods (Kümmerer, 2009; Pan and Chu, 2016; Segura et al., 2009). Plants may uptake ABs via their roots, and they are found in different plant parts (root, fruit, leaves), which may accumulate sub-inhibitory concentrations of different ABs, like tetracycline, streptomycin, and ciprofloxacin (Ahmed et al., 2015; Gullberg et al., 2011). As fitness costs of the resistance to ABs are apparently null or very low (Westhoff et al., 2017), concentrations as low as 1/10 the MIC for susceptible strains may still favor AB resistant strains over sensitive ones in food microbiomes (Westhoff et al., 2017). Therefore, and due to all this anthropogenic pressure, soils may turn into hotspots for ARGs.

ARB and ARGs are naturally found in microbial soil communities even in unpolluted and/or isolated areas (Allen et al., 2010; Bhullar et al., 2012). However, there is a growing

concern that the currently increasing presence of ABs in the environment may affect soil and plant microbiomes and that this may lead to the increase of AR of bacteria present in the human and livestock food chains from soils and harvested vegetables up to the human consumers (Chen et al., 2016; H.-W. Hu et al., 2016; Y. Hu et al., 2016; Marti et al., 2013; Westhoff et al., 2017). This potential risk is increased as many AB-resistant genetic elements may transfer from essentially innocuous soil bacterial species to potentially pathogenic ones by mechanisms of HGT, thanks to their association to MGEs (Berendonk et al., 2015). The relevance of the presence of ARB and ARGs in food on the global impact of ARB in human health is currently unknown, but it is one of the major uncertainty factors for the use of TWW and other potential sources of ABs, ARB and ARGs for agricultural purposes (Becerra-Castro et al., 2015; Freire-Moran et al., 2011).

The present study intends to investigate the possible influence of agricultural practices in the microbiome of plants and fruits grown for human consumption. To this end, we selected two currently operative agricultural tomato fields, one indirectly irrigated with TWW and a second one devoted to organic farming, irrigated with groundwater. Seven ARGs of clinical relevance (*sul*1, bla_{TEM}, $bla_{\text{CTX-M-32}}$, *mec*A, *qnrS*1, *tet*M, $bla_{\text{OXA-58}}$), were quantified by culture-independent, qPCR methods used in samples from soil, roots, leaves and fruits (Berendonk et al., 2015; Bertini et al., 2007; Hembach et al., 2017; Tamang et al., 2008; Wielders et al., 2002). In addition, the integrase gene *intI*1 was added to the study as a diagnostic for potentially HGT activity and a marker for anthropogenic pollution (Gillings et al., 2015). In parallel, the composition of the bacterial communities present in the different samples was analyzed using high throughput 16S rDNA sequencing methods. The objective was to assess the effect of using TWW for agricultural irrigation on the occurrence and distribution of bacterial communities and of ARGs in soil and the edible parts of the plants.

2.2 Materials and Methods

2.1 Site description and sample collection

Tomato crops (*Lycopersicon esculentum* Mill. cv. Bodar) were collected in commercially operated fields in the delta of the Llobregat River (Catalonia, Spain, Fig. 2.1. The Zone 1 (Z1) was located in the Littoral Mountains west of Llobregat delta, away from most human impact, irrigated with groundwater and/or rainwater, and fertilized with horse manure. In contrast, zone Z2, in the Llobregat low valley, receives water from the Infanta

Channel, with a considerable contribution (up to 92%) of TWW from 10 different WWTP effluents that discharge into the Rubí Creek. Chemical fertilizers were applied when needed. Soil chemical analysis were performed before cultivation and have been reporter elsewhere (Margenat et al., 2017; note that Z1 and Z2 correspond to plots P1 and P3 in the Margenat paper). They show similar level of fertility to grow vegetables ($N\text{-}NO_3 > 2$ mg L^{-1}; $P > 15$ mg L^{-1}; $K^+ > 180$ mg L^{-1}), similar eletrical conductivity (2.2–2.8 dS m^{-1}) and pH (7.6–7.8). Soil texture was sandy loam for both sites (Margenat et al., 2017). Conversely, the water used for irrigation in both sites did vary in chemical properties, as channel water (Z2) presented higher conductivity and ammonium nitrogen content than groundwater, as well as higher phosphorous, solid content and nitrates, and similar pH (Margenat et al., 2017), Supplementary Table S1). Samples of bulk soil, roots, leaves, and tomato fruits, were collected from 4 plants in each plot in September of 2016, when fruits reached their commercial size (32 samples in total).

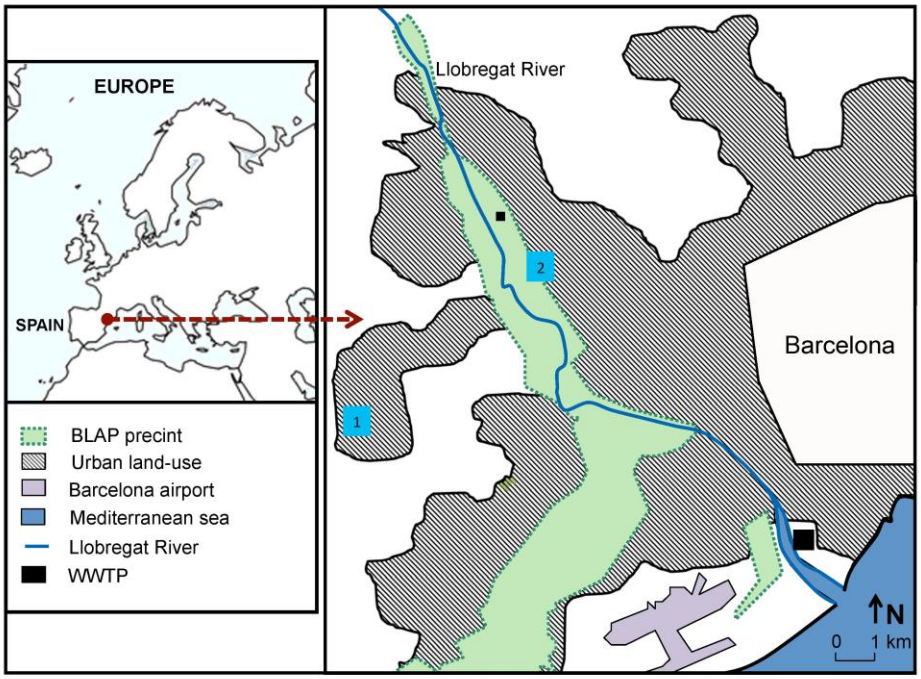


Figure 2.1. Map of the sampling fields. Tomato plants and soil were collected in zone 1 (Z1) and zone 2 (Z2).

2.2.2. DNA extraction

Preliminary procedures for DNA extraction were performed for the plant parts as described (Bodenhausen et al., 2013; Qvit-Raz et al., 2008), with slight modifications.

Roots were vortexed for 5 s for pre-washing with 20 mL of sterilized phosphate buffered saline (PBS) (NaCl 0.138 M; KCl - 0.0027 M); (pH 7.4, at 25 °C), transferred to a new 50 mL tube with 20 mL of sterilized PBS, and vortexed for 1 min, to remove contaminating bacteria from the soil. Tomato fruits were washed in sterilized distilled water. Leaves and roots were sterilized by washing in 70% ethanol for 1 min, rinsing with sterilized distilled water, washed again for 1 min in ethanol 70%, and rinsed with sterilized distilled water twice. To check the efficacy of the sterilization, the water utilized to rinse the samples the last time, was plated in LB agar media for three days in an incubator at 28 °C to evidence lack of contaminating bacteria.

Preprocessed roots (5 g), leaves (6–12 g), and tomato fruits (one piece, 60–350 g) were macerated with mortar and pestle. During leaves maceration, 20 mL of PBS buffer was added. Macerated samples were then transferred to 50 mL tubes through a 100 µm mesh nylon Cell strainer (Corning® Cell Strainer), centrifuged at 4500 rpm for 15 min, and the pellets proceeded for DNA extraction.

DNA from soil samples (250 mg) and from the pellets of the different plant parts was extracted using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc.), to a final elution volume of 100 µL. The concentration and the quality of the DNA were tested using a NanoDrop Spectrophotometer 8000 (Thermofisher Scientific, Inc). Extracted DNA samples were stored at −20 °C.

2.2.3. Detection and quantification of ARGs copy numbers

Primers for *intI*1, *sul*1 (sulfonamide resistant dihydropteroate synthase), *tet*M (A family of proteins known to bind to the 30S ribosomal subunit), *qnrS*1 (protein family that protects DNA gyrase and topoisomerase IV), *mec*A (penicillin binding protein PBP 2a), bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ (class A β- lactamases), $bla_{OXA\text{-}58}$ (class D β-lactamase), and 16S rDNA sequences are listed in Supplementary Table S2 (Bradford, 2001; Chen et al., 2014; Donhofer et al., 2012; Frank et al., 2007; Tamang et al., 2008). Dynamo ColorFlash SYBR Green (Thermo Scientific, Inc.) chemistry was used for *mec*A, *tet*M, and $bla_{OXA\text{-}58}$ qPCR quantifications; all the other ARGs were quantified with LightCycler 480 SYBR Green I Master (A F. Hoffmann–La Roche AG, Inc). Two thermal cycling conditions were employed. For *sul*1, *intI*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *qnrS*1, and 16S rDNA amplification: 95 °C for 10 min as activation step, 45 extension cycles (15 s at 95 °C, 1 min at the selected Ta, Supplementary Table S2). For *tet*M, *mec*A, and $bla_{OXA\text{-}58}$ amplification: 7 min at 95 °C as activation step, 40 amplification cycles (10 s at 95 °C, 30 s at the selected Ta,

Supplementary Table S2). All qPCR reactions were performed in a Lightcycler 480 II (A F. Hoffmann–La Roche AG, Inc). Melting curves were obtained to confirm amplification specificity. Reactions were conducted in 20 µL volumes on 96-well plates. Optimal primer concentrations were 0.15 µM for *tet*M, *mec*A, and bla_{OXA-58} primers and 0.3 µM for *sul*1, *intI*1, bla_{TEM}, $bla_{CTX-M-32}$, *qnrS*1, 16S rDNA primers. All qPCR assays were run as technical duplicates. A fixed dilution of raw DNA extract was used. The quality criteria used was an $R^2 > 0.99$, the slope of the standard curve should be between −3.1 and −3.4, the accepted efficiency of the reactions was ranging from 90% to 110%.

2.2.4. Data analysis for ARGs and *intI*1

The analysis of the ARGs profile and abundance was performed in the R environment (version 3.4.0;http://www.r-project.org/). Due to the amplification of chloroplastidial and mitochondrial DNA, bacterial 16S rDNA copy numbers were calculated by assessing the proportion of reads attributed to chloroplasts and mitochondria, using the dada2 (Callahan et al., 2016) and the phyloseq package in R (McMurdie and Holmes, 2013). After the sequencing of the 16S rDNA amplicons, quality control and taxonomy assignment, it was known the number of sequences attributed to bacteria, chloroplasts and mitochondria in each sample. The proportion of the bacterial sequences was later used as a rule to calculate the proportion of the 16S rDNA copy numbers from the qPCR reactions in the samples. Heatmaps and hierarchical clustering were performed using the function heatmap2, from the R package gplots (https://CRAN.R-project.org/package=gplots). Principal Component Analysis (PCA) was performed with the function principal, from the package psych (https://cran.r-project.org/web/packages/psych/index.html). The distribution of genetic elements across different sample types and sampling zones was analyzed by the Analysis of Variance (ANOVA) plus Tukey's B post-hoc correction for multiple tests, using logarithmic values to meet normality requirements (Levene's test) with the multcomp R package (Hothorn et al., 2008). Significance levels were set at $p \leq 0.05$. Partial Least Squares-Discriminant Analysis (PLS-DA) was performed using the function plsda from the R package mixOmics (https://CRAN.R-project.org/package=mixOmics).

2.2.5. 16S rDNA library preparation and sequencing

A pooled library of 16S rDNA amplicons from the 32 samples was prepared from the soil and plant DNA samples using 331F- and 518R-primers. PCR protocols were adapted

from (Bernstein et al., 2015), with the preparation involving two PCR steps. The first step added overhangs to the 16S rDNA amplicons, using the following thermal cycling conditions: 1 min at 98 °C for activation followed by 10 cycles of amplification (30 s at 94 °C, 1 min at 60 °C for 30 s, 1 min at 72 °C), plus 10 min at 72 °C for completing the extension reaction. The second PCR step added the Illumina universal sequence barcodes under the following thermal cycling conditions: 98 °C for 1 min followed by 10 amplification cycles (30 s at 94 °C, 1 min touchdown step from 68 °C to 56 °C, plus a final 1 min at 72 °C), and the final extension step as above (Bouwmeester et al., 2016). The composed, tagged sample was mass sequenced at the Centre for Genomic Regulation (Barcelona, Spain) (http://www.crg.eu/), using an Illumina MiSeq sequencer (300 bp, Multiplex sequencing). Quality control of the sequences was done using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and TrimGalore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequences were quality trimmed with TrimGalore! for a minimum Q score of 30. The taxonomic assignment of OTUs was performed using the dada2 package with SILVA database v128 in the R environment, fixing a 97% sequence similarity as a threshold. Raw sequences were deposited at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the accession number SRP133154.

2.2.6. Analysis of bacterial communities and relationship with ARGs

The analysis of microbial communities was performed with R environment, with the packages phyloseq, VennDiagram (https://cran.r-project.org/package=VennDiagram), vegan (https://cran.r-project.org/package=vegan), and mixOmics. OTUs abundances were log transformed for analysis. PLS-DAs were used to maximize the separation between classes of factors (sample types, sampling zones), and to understand which variable OTUs carry group-separating information. Plastid DNA sequences were excluded from the analyses. To increase the depth of the sequence analysis, reads from the four replicates from each site and sample type were added. Correlations between genetic element abundances (in copies per g of sample) and OTU prevalence (relative abundances, fraction of total reads per sample) were analyzed using the non-parametric Spearman's test plus the false discovery ratio (FDR) correction for multiple tests, also in R, and setting $p \leq 0.05$ as significance level. The non-parametric test was preferred given the different distributions of the two sets of data. OTUs were grouped at the taxonomic family level.

2.3. Results

2.3.1. ARGs quantification and distribution

Bacterial 16S rDNA, *intI*1, bla_{TEM}, and *tet*M were detected in all samples analyzed, whereas bla_{OXA-58} and *mec*A were only found in 53% and 38% of samples, respectively (Table 2.1). Bacterial 16S sequences, considered as a proxy for bacterial abundance, were found at counts from 2.9×10^8 to 8500 copies g^{-1} of sample, whereas *intI*1, a measure of MGEs, was found at approximately at 1000 times lower levels (2.9×10^5 to 3 copies g^{-1} of sample, Table 2.1). Among ARGs, abundance levels varied in several orders of magnitude, being bla_{TEM} the most prevalent one (1.2×10^7 to 340 copies g^{-1} of sample, Table 2.1). All other ARGs were found at much lower levels in fruits, if not below the limit of detection (Table 2.1, Fig. 2.2). The abundance of bla_{TEM} clearly exceeded the levels of *intI*1 by a factor of 100 or even more, whereas the rest of ARGs were found at counts generally below *intI*1 levels. Thus, the data suggests that bla_{TEM} is widespread in the bacterial population, and not necessarily linked to MGEs or, at least, not to *intI*1.

Table 2.1. Quantitative results for ARG/*intI*1/Bacterial 16S abundance in all samples (copies/g of sample).

	Prevalence	Median[a]	Range
Bacterial 16S	100%	2.1×10^6	(2.9×10^8–8500)
bla_{TEM}	100%	4.0×10^4	(1.2×10^7–340)
***intI*1**	100%	280	(2.9×10^5–3)
***sul*1**	94%	440	(5.0×10^5–<LOQ)
***tet*M**	88%	420	(5.3×10^4–<LOQ)
$bla_{CTX-M-32}$	63%	420	(1.2×10^7–<LOQ)
bla_{OXA-58}	53%	19	(2600–<LOQ)
***mec*A**	28%	240	(7000–<LOQ)
***qnrS*1**	25%	2	(1200–<LOQ)

a) Excluding values below limits of quantification (LOQ).

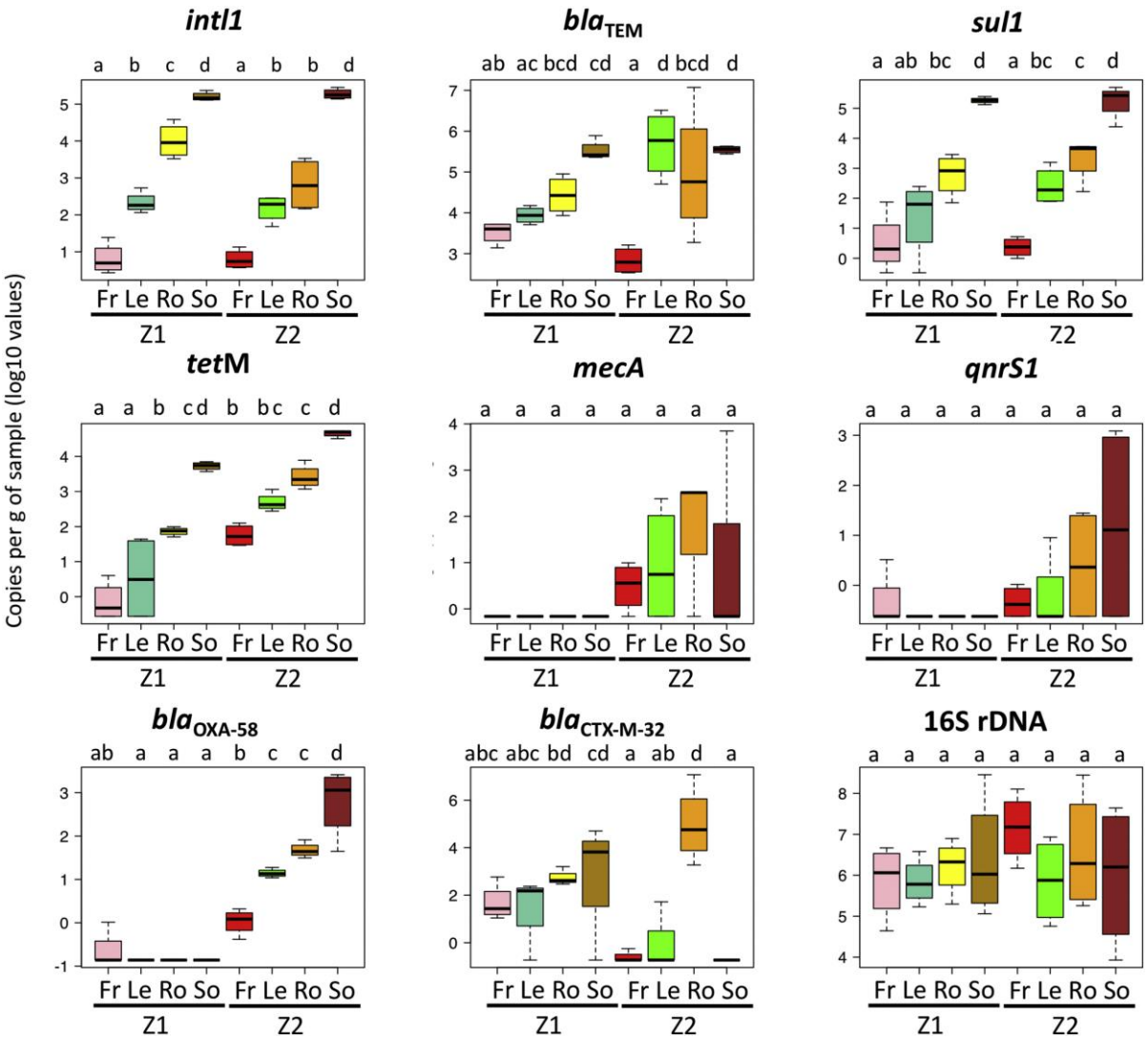


Figure 2.2. Relative abundances of the different genetic elements in all samples. Data are expressed as copies of each sequence per g of tissue (log_{10} values). Fruit, Leaf, Root, and Soil samples are labelled as Fr, Le, Ro, and So, respectively and color-coded depending on the zone: pink, cyan, yellow and brown for Z1 and red, green, orange, and deep red for Z2. Lowercase letters *a* to *d* indicate statistically different groups of data ($p < 0.05$, Tukey HSD test).

The distribution of the different genetic elements among sampling zones and plant parts is summarized in Fig. 2.2 Surprisingly, 16S gene abundance values were relatively similar in soil and plant parts in both zones, and they differed very little between zones (see letters at the top of the graphs, representing statistically different value distributions). In contrast, levels of *intI*1, *tet*M, *sul*1, bla_{TEM}, and bla_{OXA-58} (the latter only for Z2) showed a clear gradient from a maximum in soil samples to significantly lower levels in fruits (Fig. 2.2). These results indicate that endophytic bacteria were essentially as abundant as soil bacteria, although they presented a considerable lower level of MGEs (20–22,000 times less, Fig. 2.2, mean the log scale). ARGs show very different distributions, from being more or less uniformly distributed among samples (considering value dispersion), like bla_{TEM}, *mec*A, or *qnrS*1, to showing significant differences between zones (*tet*M) and/or between soil and

plant parts (*tet*M, *sul*1, bla_{OXA-58} in Z2, Fig. 2.1). Note that $bla_{CTX-M-32}$ showed a unique distribution pattern, as its abundance in soil was higher in Z1 than in Z2 (Fig. 2.1).

The mutual correlation between genetic element abundances, zones and sample types was further analyzed by PCA (Fig. 2.3). The first PCA component, PC1, explaining 39% of the total variance, clearly separated sample types (Fig. 2.3 A), whereas the second component PC2 (21% total variance) grossly separated samples from Z1 and Z2 (circles and triangles in Fig. 2.3 A). Loading plots (arrows and genetic element names in Fig. 2.3) indicate that ARGs and *intI*1 were associated to soil and root samples. Bacterial 16S gene abundance showed a strong contribution only to PC2, and did not show any particular association to any defined group of sample types (Fig. 2.2). Fig. 2.2 B indicates the correlations between the tested genetic elements, showing the strong positive correlations between *intI*1 and the ARGs bla_{TEM} ($r = 0.80$), *tet*M ($r = 0.84$), and *sul*1 ($r = 0.89$), and much weaker correlations with the rest of ARGs. Note the essentially nil correlation between bacterial 16S abundance and the prevalence of either *intI*1 or the different ARGs, except for a rather weak correlation with *qnrS*1, suggesting that these correlations do not simply reflect variations in bacterial abundance among the different zones and sample types.

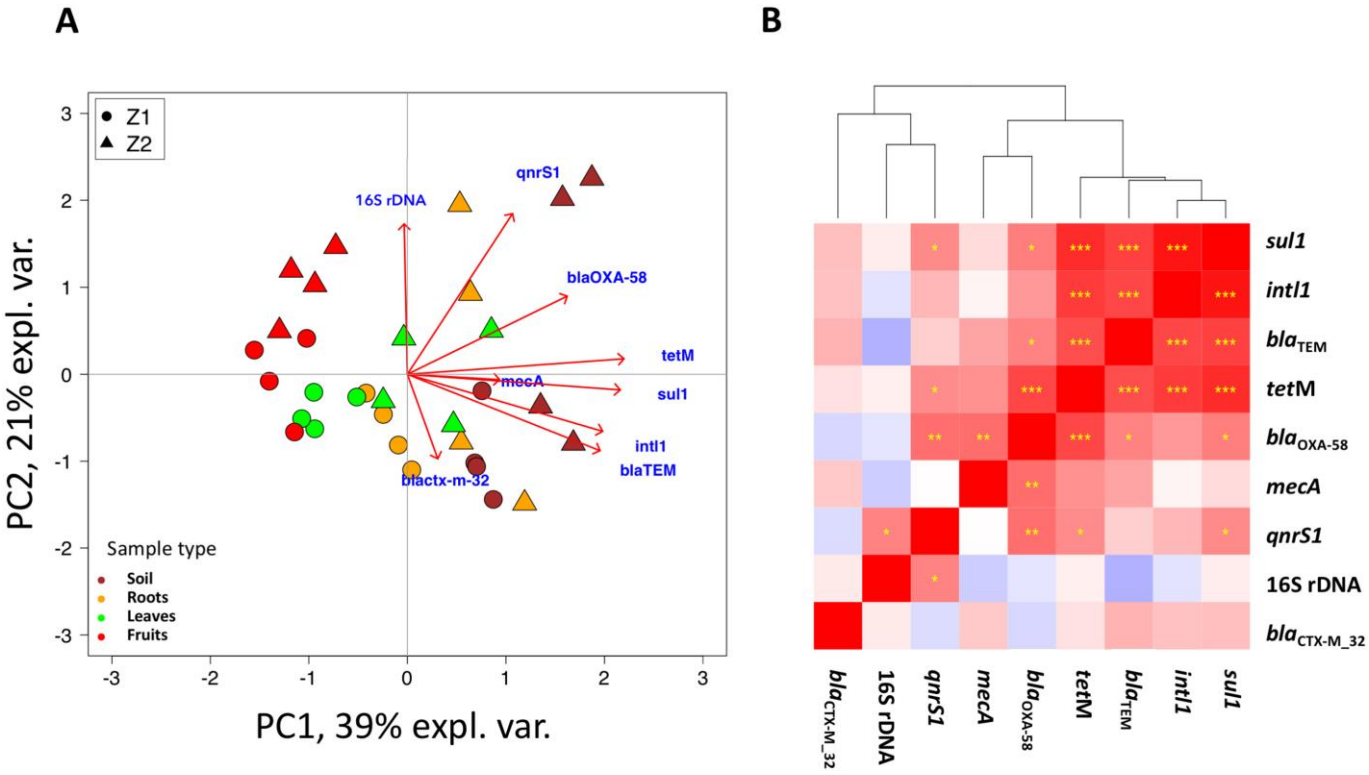


Figure 2.3. Analysis of the distribution of genetic elements among sample types and sampling zones. **(A)** Graphic representation of PCA results. Sample scores are represented as circles (Z1) or triangles (Z2). Red, green, orange and brown symbols correspond to Fruit, Leaf, Root and Soil samples, respectively. Gene element loadings are represented by the element name in blue and a red arrow. The plot corresponds to the

two first components, the percentage of variance explained by each one is indicated. **(B)** Correlation maps between the different genetic elements. Cell colors from red to blue indicate positive and negative Pearson correlations, respectively, white corresponds to no correlation. Asterisks indicate significant correlations (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, False Discover Ratio correction).

2.3.2. Microbiome Analysis

A total of 4895 OTUs were detected from a total of 7,124,468 readings in 32 samples with a median sequencing depth of 230,348.5 reads per sample after quality control (range 70,176 to 300,977). One out of three OTUs (1644) were common to Z1 and Z2 zones, whereas the remaining two- thirds were almost equally distributed between both sampling zones (Supplementary Fig. S2.1 A). Only 5% of OTUs (248 OTUs) were only found in the soil samples, a similar fraction as the number OTUs found in all sample types (Supplementary Fig. S2.1 B). Leaves and fruits shared 37.6% of their OTUs, whereas only 27% of OTUs found in roots were also found in soils (Supplementary Fig. S2.1 C–E). As many OTUs showed relatively low number of occurrences in many of the samples, we opted to group them at the taxonomic family level for all further analyses. Fig. 2.4 shows the relative abundances of the 20 most prevalent OTU families for all samples. Note that only seven families (Pseudomonadaceae*,* Moraxellaceae*,* Flavobacteriaceae*,* Rhodospirillaceae*,* Comamonadaceae*,* Enterobacteriaceae*, and* Oxalobacteraceae) represent >10% of the total reads in at least one sample (Fig. 2.3)

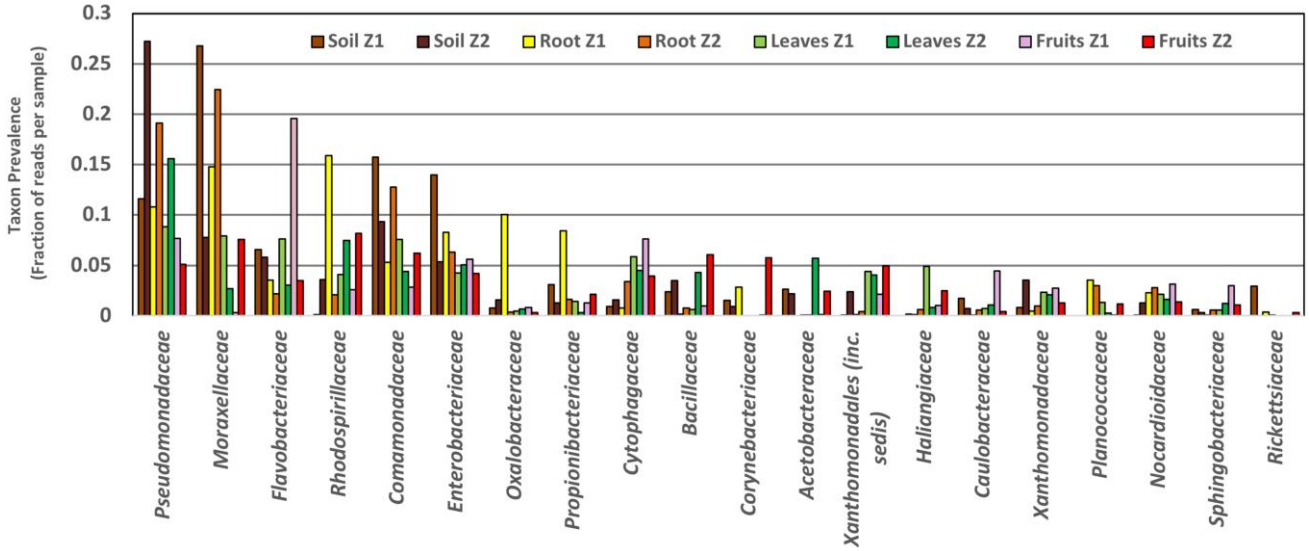


Figure 2.4. Relative abundance of the 20 most prevalent OTUs families. Values represent the fraction of reads corresponding to a given family for the total reads obtained from each sample type and zone; plastid sequences were excluded from the analysis. Zone and sample types were color-coded, as indicated at the top of the graph.

Hierachical clustering of samples based on their relative abundance in OTUs families reveals two major clusters of samples, one including roots and soil samples and a second one including leaves and fruits, whereas the distinction between zones appeared less clear (Fig. 2.5, dendrogram on the left). These data can be interpreted as a colonization of roots by soil bacteria, as well as indicating the existence of a common microbiome for the aerial parts of the plants.

Predictive PLS-DA analyses were used to investigate the individual contribution of different bacterial families to discriminate between Z1 and Z2 microbiomes, on one side, and between aerial (fruits + leaves) and underground bacterial populations (soil + roots), on the other. Both models explained >50% of the OTUs abundance variations (Fig. 2.6). The first model (Fig. 2.6 A) shows that only four families with high prevalence (Comamonadaceae*,* Enterobacteriaceae*,* Moraxellaceae*,* and Pseudomonadaceae), numbers 14, 20, 38, and 52 in the orange area in Fig. 2.6 A, (note the relative size of the circles) were associated to underground samples, whereas aerial plant parts associated to a large number of less prevalent OTU families (Fig. 2.6 A, small grey circles). In contrast, both high and low abundance OTU families contributed to differentiate between Z1 and Z2 samples in the second model (Fig. 2.6 B). Z1 samples were associated to high proportions of Enterobacteriaceae and Flavobacteriaceae (numbers 20 and 23 in Fig. 2.6 B), whereas the largest OTU group related to Z2 samples was the Pseudomonadacea family (number 52 in Fig. 2.6 B).

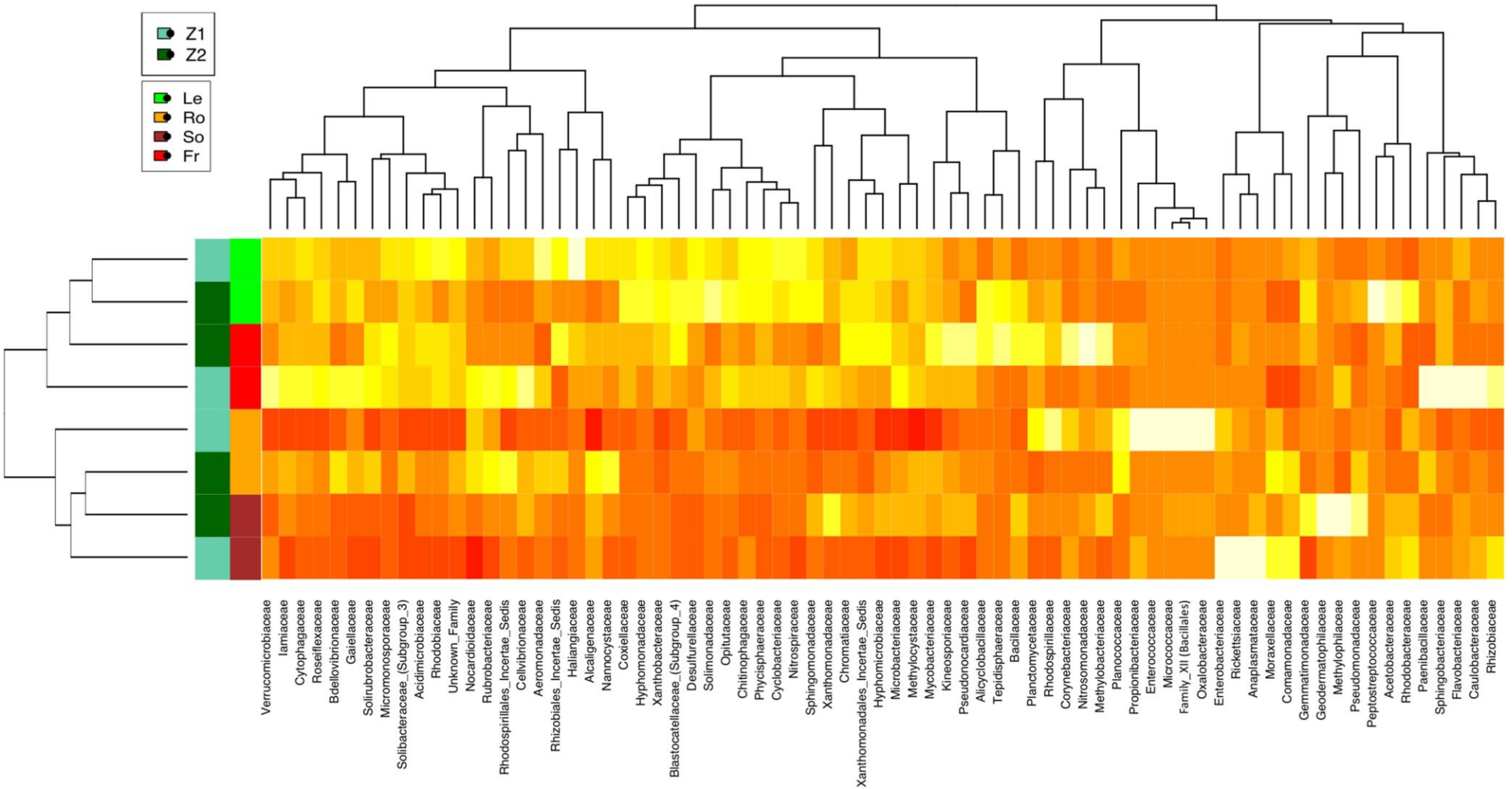
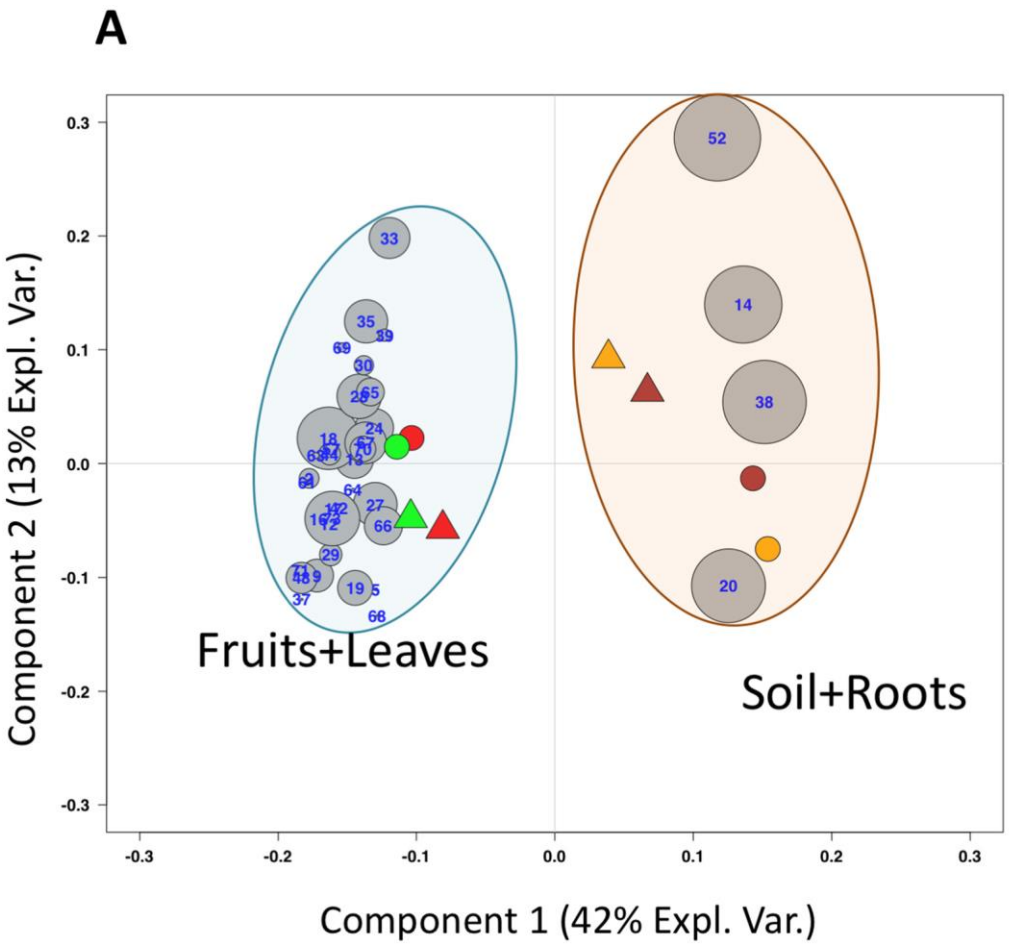


Figure 2.5. Hierarchical clustering of samples by microbiome composition. Values correspond to relative abundances in OTUs grouped by taxonomic families. Only OTUs representing >0.1% of the total abundance were included in the analysis. The separation between Zones and sample types is marked by the bars on the left of the map, plant parts (leftmost bar) labeled as in Fig. 2.1, and Z1 and Z2 samples colored in light and dark green, respectively (rightmost bar). Cell colors indicate relative abundances for each family in each sample, red corresponding to the minimum and red to the maximum value ("heatmap colors")


A
Component 2 (13% Expl. Var.)
Component 1 (42% Expl. Var.)
Fruits+Leaves
Soil+Roots
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
52
14
38
20
33
35
39
69
30
65
24
18
27
66
29
19
5
68
37

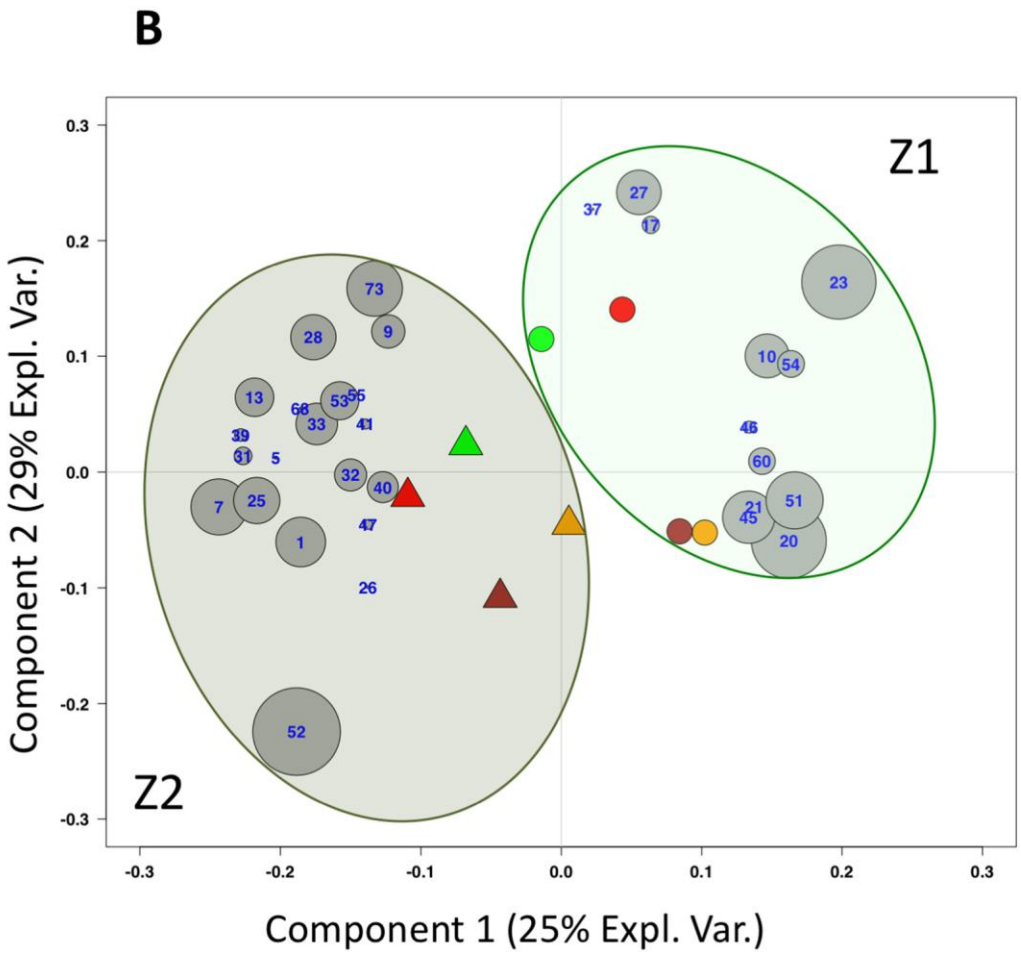
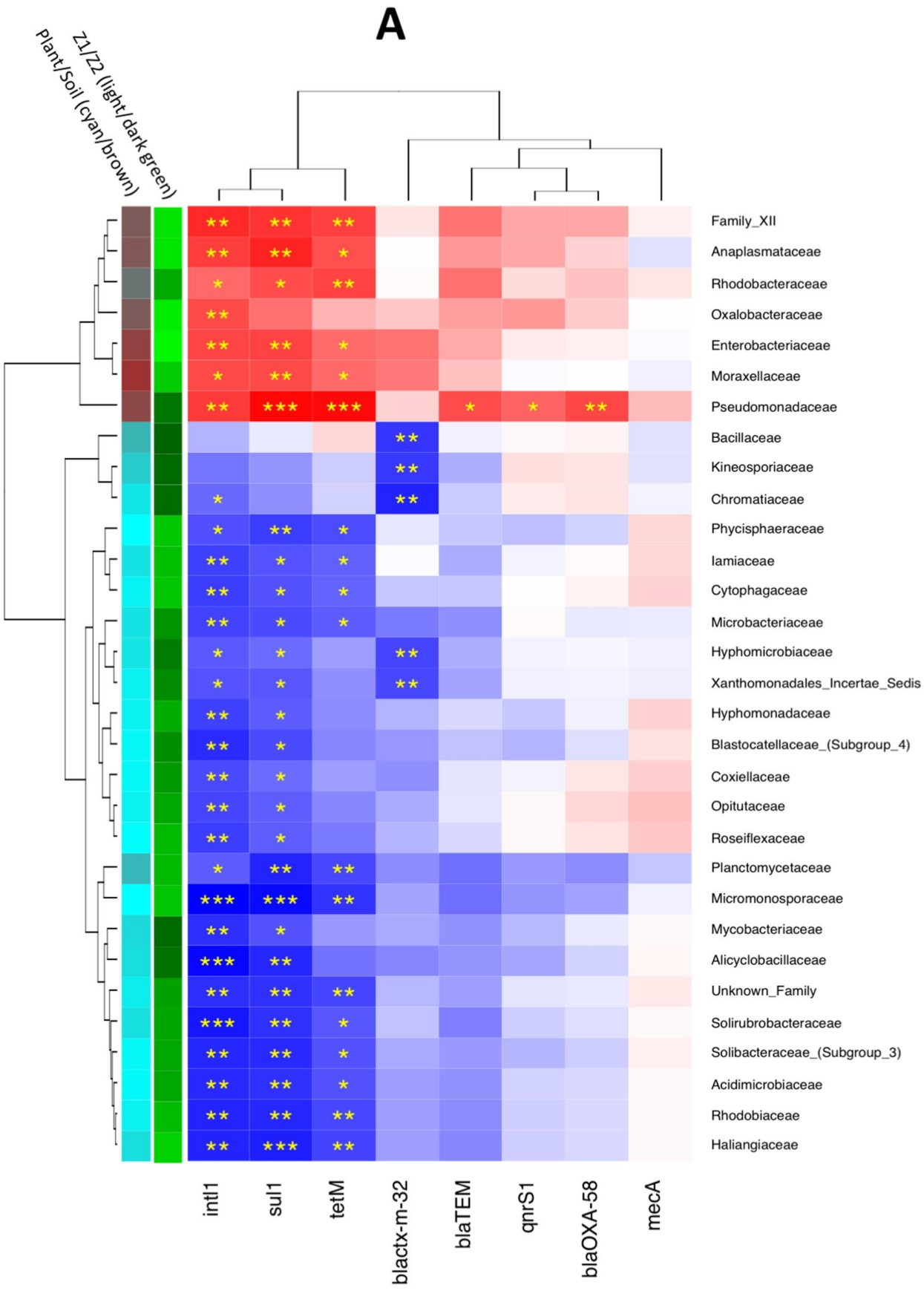


Figure 2.6. Graphic representation of PLS-DA results. The two bi-plots (loadings and scores) correspond to PLS-DA models based on **(A)** Z1 versus Z2 sampling zones and **(B)** aerial plant parts (fruits, leaves) versus underground samples (soil, roots). Radius of the grey circles represent values of OTU families' relative abundance in log scale. Family names are substituted by numbers as follows: 1*:* Acetobacteraceae*;* 2*:* Acidimicrobiaceae*;* 3*:* Aeromonadaceae*;* 4*:* Alcaligenaceae*;* 5*:* Alicyclobacillaceae*;* 6*:* Anaplasmataceae*;* 7*:* Bacillaceae*;* 8*:* Bdellovibrionaceae*;* 9*:* Blastocatellaceae_*(*Subgroup_4*);* 10*:* Caulobacteraceae*;* 11*:* Cellvibrionaceae*;* 12*:* Chitinophagaceae*;* 13*:* Chromatiaceae*;* 14*:* Comamonadaceae*;* 15*:* Corynebacteriaceae*;* 16*:* Coxiellaceae*;* 17*:* Cyclobacteriaceae*;* 18*:* Cytophagaceae*;* 19*:* Desulfurellaceae*;* 20*:* Enterobacteriaceae*;* 21*:* Enterococcaceae*;* 22*:* Family_XII *(*Bacillales*);* 23*:* Flavobacteriaceae*;* 24*:* Gaiellaceae*;* 25*:* Gemmatimonadaceae*;* 26*:* Geodermatophilaceae*;* 27*:* Haliangiaceae*;* 28*:* Hyphomicrobiaceae*;* 29*:* Hyphomonadaceae*;* 30*:* Iamiaceae*;* 31*:* Kineosporiaceae*;* 32*:* Methylobacteriaceae*;* 33*:* Methylocystaceae*;* 34*:* Methylophilaceae*;* 35*:* Microbacteriaceae*;* 36*:* Micrococcaceae*;* 37*:* Micromonosporaceae*;* 38*:* Moraxellaceae*;* 39*:* Mycobacteriaceae*;* 40*:* Nannocystaceae*;* 41*:* Nitrosomonadaceae*;* 42*:* Nitrospiraceae*;* 43*:* Nocardioidaceae*;* 44*:* Opitutaceae*;* 45*:* Oxalobacteraceae*;* 46*:* Paenibacillaceae*;* 47*:* Peptostreptococcaceae*;* 48*:* Phycisphaeraceae*;* 49*:* Planctomycetaceae*;* 50*:* Planococcaceae*;* 51*:* Propionibacteriaceae*;* 52*:* Pseudomonadaceae*;* 53*:* Pseudonocardiaceae*;* 54*:* Rhizobiaceae*;* 55*:* Rhizobiales_Incertae_Sedis*;* 56*:* Rhodobacteraceae*;* 57*:* Rhodobiaceae*;* 58*:* Rhodospirillaceae*;* 59*:* Rhodospirillales_Incertae_Sedis*;* 60*:* Rickettsiaceae*;* 61*:* Roseiflexaceae*;* 62*:* Rubrobacteriaceae*;* 63*:* Solibacteraceae_*(*Subgroup_3*);* 64*:* Solimonadaceae*;* 65*:* Solirubrobacteraceae*;* 66*:* Sphingobacteriaceae*;* 67*:* Sphingomonadaceae*;* 68*:* Tepidisphaeraceae*;* 69*:* Unknown_Family*;* 70*:* Verrucomicrobiaceae*;* 71*:* Xanthobacteraceae*;* 72*:* Xanthomonadaceae*;* 73*:* Xanthomonadales_Incertae_Sedis.

2.3.3. Analysis of the influence of the plant microbiome on ARG loads

The mutual correlations between OTU abundance and ARG prevalence values in plant and soil samples is shown in the correlation map in Fig. 2.7 A. Only OTU families with at least one significant correlation with at least one ARG or *intI*1 are represented. Hierachical clustering defined a subset of genetic elements (*intI*1, *sul*1, and *tet*M, see also Fig. 2.5) with strong positive correlations with a small subset of mostly abundant bacterial families (Pseudomonadaceae, Moraxellaceae, Enterobacteriaceae, red cells in Fig. 2.7 A). Another subset of three ARGs (bla_{TEM}, bla_{OXA-58}, and *qnrS*1) showed weaker positive correlations only with Pseudomonadaceae (Fig. 2.7 A), whereas many bacterial families showed negative correlations with the presence of the same genetic elements (Fig. 2.7 A, blue cells). Bacterial families showing strong positive correlations with the measured genetic elements appeared associated to soil/roots samples in the PLS-DA shown in Fig. 2.6 A, whereas essentially all families with negative correlations associated to fruit/leaves samples in the same analysis (brown and cyan sectors in the left sidebar in Fig. 2.7 A, respectively). Both positive and negatively-correlated bacterial family clusters include families associated with Z1 and Z2 in the PLS-DA from Fig. 2.6 B (light and dark green sectors in the right sidebar in Fig. 2.7 A, respectively), whereas Pseudomonadaceae was the only Z2-associated bacterial family showing strong positive correlations with ARGs (Fig. 2.7 A).


A
Z1/Z2 (light/dark green)
Plant/Soil (cyan/brown)
Family_XII
Anaplasmataceae
Rhodobacteraceae
Oxalobacteraceae
Enterobacteriaceae
Moraxellaceae
Pseudomonadaceae
Bacillaceae
Kineosporiaceae
Chromatiaceae
Phycisphaeraceae
Iamiaceae
Cytophagaceae
Microbacteriaceae
Hyphomicrobiaceae
Xanthomonadales_Incertae_Sedis
Hyphomonadaceae
Blastocatellaceae_(Subgroup_4)
Coxiellaceae
Opitutaceae
Roseiflexaceae
Planctomycetaceae
Micromonosporaceae
Mycobacteriaceae
Alicyclobacillaceae
Unknown_Family
Solirubrobacteraceae
Solibacteraceae_(Subgroup_3)
Acidimicrobiaceae
Rhodobiaceae
Haliangiaceae
intI1
sul1
tetM
blactx-m-32
blaTEM
qnrS1
blaOXA-58
mecA

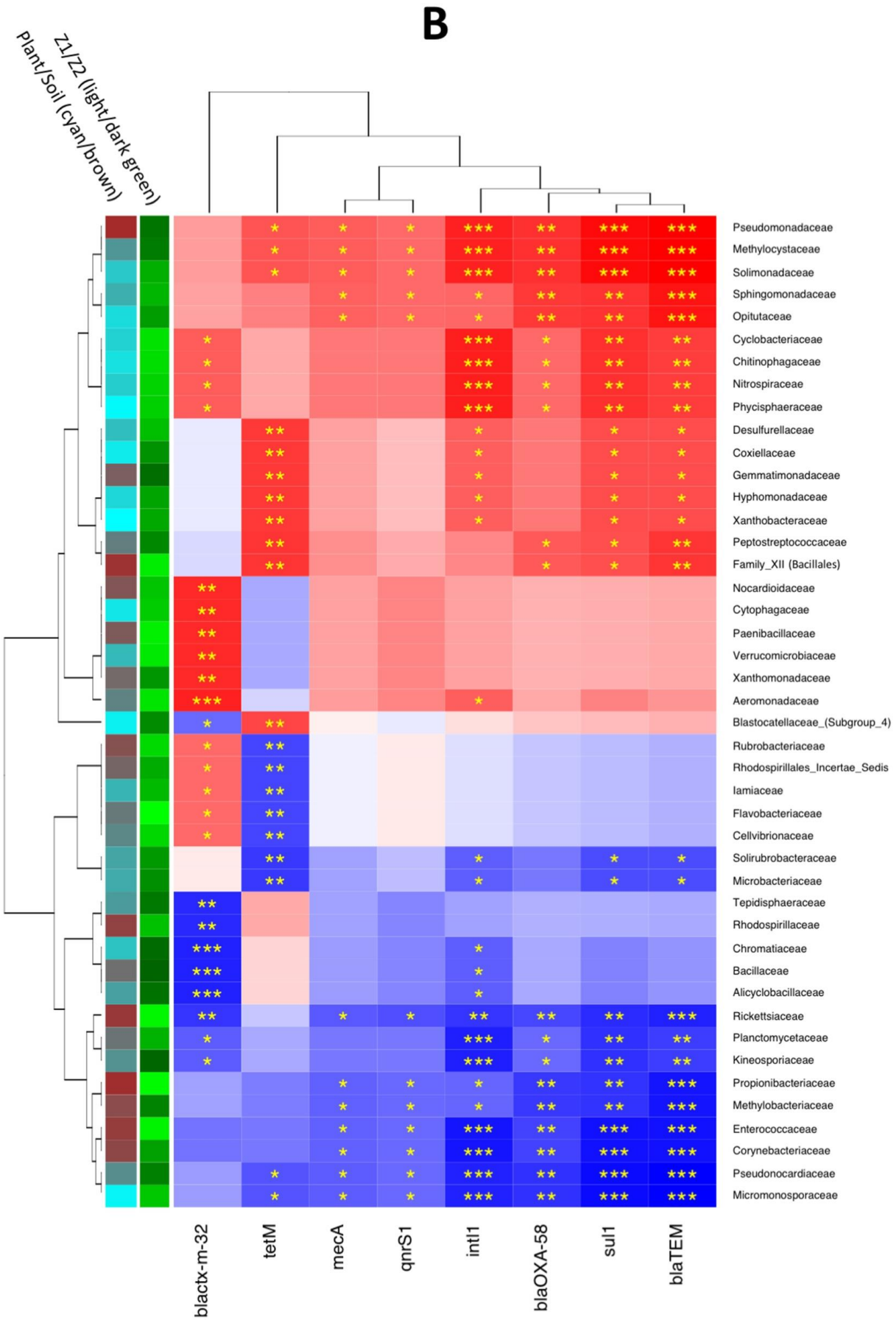

B
Z1/Z2 (light/dark green)
Plant/Soil (cyan/brown)
Pseudomonadaceae
Methylocystaceae
Solimonadaceae
Sphingomonadaceae
Opitutaceae
Cyclobacteriaceae
Chitinophagaceae
Nitrospiraceae
Phycisphaeraceae
Desulfurellaceae
Coxiellaceae
Gemmatimonadaceae
Hyphomonadaceae
Xanthobacteraceae
Peptostreptococcaceae
Family_XII (Bacillales)
Nocardioidaceae
Cytophagaceae
Paenibacillaceae
Verrucomicrobiaceae
Xanthomonadaceae
Aeromonadaceae
Blastocatellaceae_(Subgroup_4)
Rubrobacteriaceae
Rhodospirillales_Incertae_Sedis
Iamiaceae
Flavobacteriaceae
Cellvibrionaceae
Solirubrobacteraceae
Microbacteriaceae
Tepidisphaeraceae
Rhodospirillaceae
Chromatiaceae
Bacillaceae
Alicyclobacillaceae
Rickettsiaceae
Planctomycetaceae
Kineosporiaceae
Propionibacteriaceae
Methylobacteriaceae
Enterococcaceae
Corynebacteriaceae
Pseudonocardiaceae
Micromonosporaceae
blactx-m-32
tetM
mecA
qnrS1
intI1
blaOXA-58
sul1
blaTEM

Figure 2.7. Correlation between genetic element abundance and microbiome compositions. Heatmaps represent Spearman correlation matrices between the abundance of genetic elements (represented as copies per bacterial 16S rDNA copy in each sample) and prevalence of OTUs families, as relative proportion of the total sequences found in each zone and plant part, for the whole dataset **(A)** or when considering only fruit and leaf samples **(B)**. Red and blue squares represent positive and negative correlations, respectively. One, two, or three asterisks indicate *p*-values lower than 0.05, 0.01, and 0.001, respectively (Spearman correlation, False Discovery Rate correction). Dendrograms correspond to a Euclidian hierarchical clustering of OTUs and genetic elements. Vertical sidebars represent PLS-DA loadings (Fig. 2.6, first component). The left sidebar corresponds to the plan part discrimination model (Fig. 2.6 A); cyan and brown sectors indicate OTU families associated to aerial parts and to underground samples, respectively. The right sidebar corresponds to the zone discrimination model (Fig. 2.6 B); lighter green sectors indicate OTUs families strongly associated to Z1, dark green sectors corresponded to OTUs families predominantly associated to Z2.

A similar correlation analysis using only data from fruits and leaves' samples showed quite different correlations between genetic elements and bacterial families (Fig. 2.7 B). In this case, a subset of four genetic elements (*intI*1, bla_{TEM}, $bla_{\text{OXA-58}}$, and *sul*1) showed strong positive correlations with a cluster of nine bacterial families, including again Pseudomonadaceae as the most abundant taxon (Fig. 2.7 B). Note that, except for the Pseudomonadaceae family, the rest of bacterial families included in this cluster scored as “fruit + leaves”-associated taxa in the PLS-DA from Fig. 2.6 A (cyan sectors in the left sidebar in Fig. 2.7 B), and almost equally divided between zone Z1 and Z2-associated taxa (light and dark green sectors in the right sidebar in Fig. 2.7 B). Conversely, most OTU families showing negative correlations with the same genetic elements scored as “soil+roots”-associated taxa (brown sectors in the left sidebar in Fig. 2.7 B), again, with no clear prevalence for Z1 or Z2-associated taxa.

The rest of ARGs not strongly linked to *intI*1 showed somewhat weaker correlation with different OTU families, except for $bla_{\text{CTX-M-32}}$, which showed a strong correlation with a relatively minor family, Aeromonadaceae (Fig. 2.7 B), and weaker, but significant direct associations with another cluster of five bacterial families. In this case, most of these families were associated to Z1 (light green sectors in in the right sidebar in Fig. 2.7 B). The situation was reversed for taxa showing negative correlations with $bla_{\text{CTX-M-32}}$, most of them associated to Z2 (dark green sectors in the middle sidebar in Fig. 2.7 B). The correlations between *tet*M loads and specific OTU families were somewhat weaker, with positive correlations with taxa associated to Z2 and to plant parts, and negative ones with families associated to Z1 and soil (Fig. 2.7 B). The two remaining ARGs (*mec*A and *qnrS*1) showed no convincing correlation with any OTU taxon (Fig. 2.7 B). Some of the significant correlations between genetic elements and the relative abundance of selected bacterial families are shown as binary plots in Supplementary Fig. S2.2.

2.4. Discussion

Plant-associated microbiomes are rapidly becoming a major topic of interest for the scientific community, due to the recent realization of its complexity, prevalence, and fundamental roles for plant physiology, mainly thanks to the implementation of culture-independent quantitative methods (Berg et al., 2014; Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Ruppel et al., 2006; Ryan et al., 2008). These studies demonstrate that endophytic bacteria actively participate in many plant functions, in some cases actively promoting plant growth and health (Hardoim et al., 2008).

Our finding that the abundance of bacterial 16S sequences in the different plant parts is of the same order of magnitude that the corresponding values in soils (between 10^4 and 10^8 copies per gram of tissue) is consistent with previous estimates using comparable methodologies (Ruppel et al., 2006). Bacteria in plants can be associated to three major compartments, rhizosphere, endosphere and phyllosphere. Rhizosphere is considered as the portion of soil whose microbial community is directly influenced by plant metabolism. Endosphere includes inner plant tissues, whereas the phyllosphere is composed by microbial cells able to colonize plant surfaces (reviewed in Andreote et al., 2014). These different habitats are differentially represented in our samples, as root samples are likely influenced by the rhizospheric microbiome, whereas leaves and fruits include both endospheric and phyllospheric bacteria. We choose not to actively eliminate epiphytic bacteria from fruit samples, as these bacteria are likely to remain into the final commercial product up to the end consumers. While endophytes are part of the normal and healthy inner environment for plants, it is clear that they can include both human pathogens and can act as vectors of spreading ARGs and other potentially dangerous genetic elements (van Overbeek et al., 2014; Zhu et al., 2017). This is particularly relevant when crops are in contact with human and animal farm residues, either by watering using TWW or by manure fertilization (Becerra-Castro et al., 2015; Ben et al., 2017; Han et al., 2016; Marti et al., 2013; Piña et al., 2018; Westhoff et al., 2017). We found a higher prevalence of *intI*1 in soil and roots than in aerial parts of plant, with very little differences between sampling zones, indicating that their abundance was not influenced by the different agricultural practices in Z1 (virtually organic farming irrigated with groundwater) and in Z2 (regular farming irrigated with TWW). We also found a close correlation between the abundance of *intI*1 and *tet*M, *sul*1, and bla_{TEM} in leaf and fruit samples. The association between the presence of *sul* genes in environmental samples

and human activity has been repeatedly observed (Muziasari et al., 2014; Pruden et al., 2012), as well as the correlation between *intI*1, *sul*1 and *tet* genes in reclaimed water (Gillings et al., 2015). We interpret our data as an indication for a connection between *intI*1 and the presence of multi-resistant bacteria in the bacterial communities in the sampled agricultural fields.

Our analysis of the bacterial 16S sequences indicates that microbiomes in the aerial plant parts were more complex and enriched in low-abundance OTUs than soil or root microbiomes, which showed a clear prevalence of a restricted number of families. Although it is difficult to establish a direct correlation with the present data, it is tempting to relate the different taxon distribution in these prevalent taxa in soils and roots from zones Z1 and Z2 (Enterobacteriacea and Flavobacteriaceae versus Pseudomonadaceae, respectively) to the different regimes of fertilization and irrigation for both zones (horse manure and ground/rainwater for Z1, chemical fertilizer and TWW for Z2).

We observed strong positive and negative correlations between the prevalence of *intI*1 and different ARGs in the different samples and the respective abundance of different OTUs. Using the whole dataset, positive correlations corresponded to soil-associated bacterial families, and they include some of the most abundant ones, like Enterobacteriacea and Pseudomonadaceae. In fact, the abundance of Pseudomonadaceae appears as a major determinant of the presence of at least four out of the eight analyzed genetic elements. We interpret the absence of significant correlations with the rest of the genetic elements to their more or less even distribution among bacterial families. This is particularly true for bla_{TEM}, the most prevalent ARG found in the whole dataset and whose prevalence exceeded that of *intI*1, for example. When a similar analysis was performed only with plant- associated samples (fruits and leaves), the picture was rather different. On one side, both soil- and plant-associated bacterial families showed direct correlations with at least three sets of genetic elements. The most prevalent group, formed by *intI*1, bla_{TEM}, $bla_{\text{OXA-58}}$, and *sul*1, correlated to the presence of mainly plant-associated families, with the exception of Pseudomonadaceae. This suggests that these ARGs may be associated with specific endophytic taxonomic groups. Two more ARGs, *sul*1 and *tet*M, also showed significant, and unrelated, correlations with specific taxa, both soil- and plant-associated ones. Many of these taxa belong to the Beta- and Gammaproteobacteria classes reported to harbor plasmids, found to be vectors for several ARGs in WW environments (Vaz-Moreira et al., 2014;

Zhang et al., 2011). Resistance to different class of ABs may be potentially encoded in the same MGE. For instance, plasmids encompassing different ARGs have been detected in environmental samples with anthropogenic impact, namely, marine aquaculture (Muziasari et al., 2014; Nonaka et al., 2012). In addition, the association between *intI*1 and *sul* genes has been repeatedly observed (Hu et al., 2008; Pruden et al., 2012), as well as the correlation between *intI*1, *sul*1 and *tet* genes in reclaimed water (Gillings et al., 2015). Therefore, the results suggest that at least these ARGs may be present in the same MGE in our samples, favoring the presence of multi-resistant bacteria.

2.5. Conclusions

Our results show a complex interrelationship between soil and plant microbiomes, ARGs prevalence, and farming activities. A subset of ARGs, and particularly bla_{TEM} and *sul*1, seems to be relatively unaffected by the use of TWW for irrigation, whereas *tet*M, *mec*A, and $bla_{\text{OXA-58}}$ showed higher levels in Zone 2 than in Zone 1. Similar selective effects are found when analyzing the soil and plant microbiomes: only a defined subset of taxa appear to be affected by agricultural practices, particularly Pseudomonadaceae and Enterobacteriaceae in soils. While our data indicate that the microbiome structure and ARGs content in edible parts were modulated by the soil microbiome, only 14% of detected OTUs were shared by aerial and underground samples.

Soil composition, aeration, and fertilization regime may also influence microbiomes and ARG contents. While the results on ARG and microbiome composition represents a further support on the role of agricultural practices in ARG dissemination, further research using carefully controlled conditions is required to determine the relevance of the quality of the irrigation water and of other agricultural practices in the observed spread of ARGs worldwide.

2.6. Supporting Information

Supplementary Table S2.1. Minimum, maximum and average levels of quality parameters measured in the waters used for sampling fields' irrigation (Margenat et al., 2017).

	Z1 (n=5)	**Z2 (n=10)**
Conductivity (µS cm^{-1})	(968–1211) 1049	(1490–2148) 1944
NH_4^+–N(mg L^{-1})	(0.002–0.167) 0.05	(3–47) 14
Nitrates (mg L^{-1})	(2.8–4.6) 3.9	(3.4–7.4) 5.4
Total Phosphorous (mg L^{-1})	(0.03–3.0) 0.6	(0.6–2.5) 1.5
Total suspended solids (mg L^{-1})	(10–55) 21	(14–94) 46
pH	(7.5–8.6) 8.1	(7.7–8.1) 7.9

Supplementary Table S2.2. Primers utilized for detection and quantification of ARGs in soil and plant parts.

Target gene	**Primers**	**Sequence (5'-> 3')**	**Amplicon size (bp)**	**Tm(°C)**	**Reference**
***intI*1**	intI1LC5	GATCGGTCGAATGCGTGT	196	60	Barraud et al, 2010
	intI1LC1	GCCTTGATGTTACCCGAGAG			
16s rDNA	331F	TCCTACGGGAGGCAGCAGT	195	60	Bräuer et al., 2011
	518R	ATTACCGCGGCTGCTGG			
***bla*$_{TEM}$**	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113	60	Di Cesare et al., 2016
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG			
***bla*$_{CTX-M-32}$**	ctx-m-32-FW	CGTCACGCTGTTGTTAGGAA	156	60	Hembach et al., 2017
	ctx-m-32-R	CGCTCATCAGCACGATAAAG			
***bla*$_{OXA-58}$**	OXA58F	GCAATTGCCTTTTAAACCTGA	152	63	Laht et al., 2014
	OXA58R	CTGCCTTTTCAACAAAACCC			
***mec*A**	mecAF	AAAAAGATGGCAAAGATATTCAA	185	63	Szczepanowski et al., 2009
	mecAR	TTCTTCGTTACTCATGCCATACA			
***qnrS*1**	qnrSrtF11	GACGTGCTAACTTGCGTGAT	118	60	Marti & Balcázar, 2013
	qnrSrtR11	TGGCATTGTTGGAAACTTG			
***sul*1**	sul1-FW	CGCACCGGAAACATCGCTGCAC	162	60	Pei et al., 2006
	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
***tet*M**	tetMF	GCAATTCTACTGATTTCTGC	186	60	Tamminen & Karkman, 2011

	tetMR	CTGTTTGATTACAATTTCCGC

Barraud, O., Baclet, M. C., Denis, F., & Ploy, M. C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *Journal of Antimicrobial Chemotherapy*, *65*(8), 1642–1645. https://doi.org/10.1093/jac/dkq167

Bräuer, S. L., Adams, C., Kranzler, K., Murphy, D., Xu, M., Zuber, P., … Tebo, B. M., 2011. Culturable Rhodobacter and Shewanella species are abundant in estuarine turbidity maxima of the Columbia River. *Environmental Microbiology*, *13*(3), 589–603. https://doi.org/10.1111/j.1462-2920.2010.02360.x

Di Cesare, A., Losasso, C., Barco, L., Eckert, E. M., Conficoni, D., Sarasini, G., Ricci, A.,2016. Diverse distribution of Toxin-Antitoxin II systems in Salmonella enterica serovars. *Scientific Reports*, *6*, 1–9. https://doi.org/10.1038/srep28759

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., & Schwartz, T.,2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology*, *8*(JUL), 1–11. https://doi.org/10.3389/fmicb.2017.01282

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., & Kisand, V.,2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS ONE*, *9*(8), 1–8. https://doi.org/10.1371/journal.pone.0103705

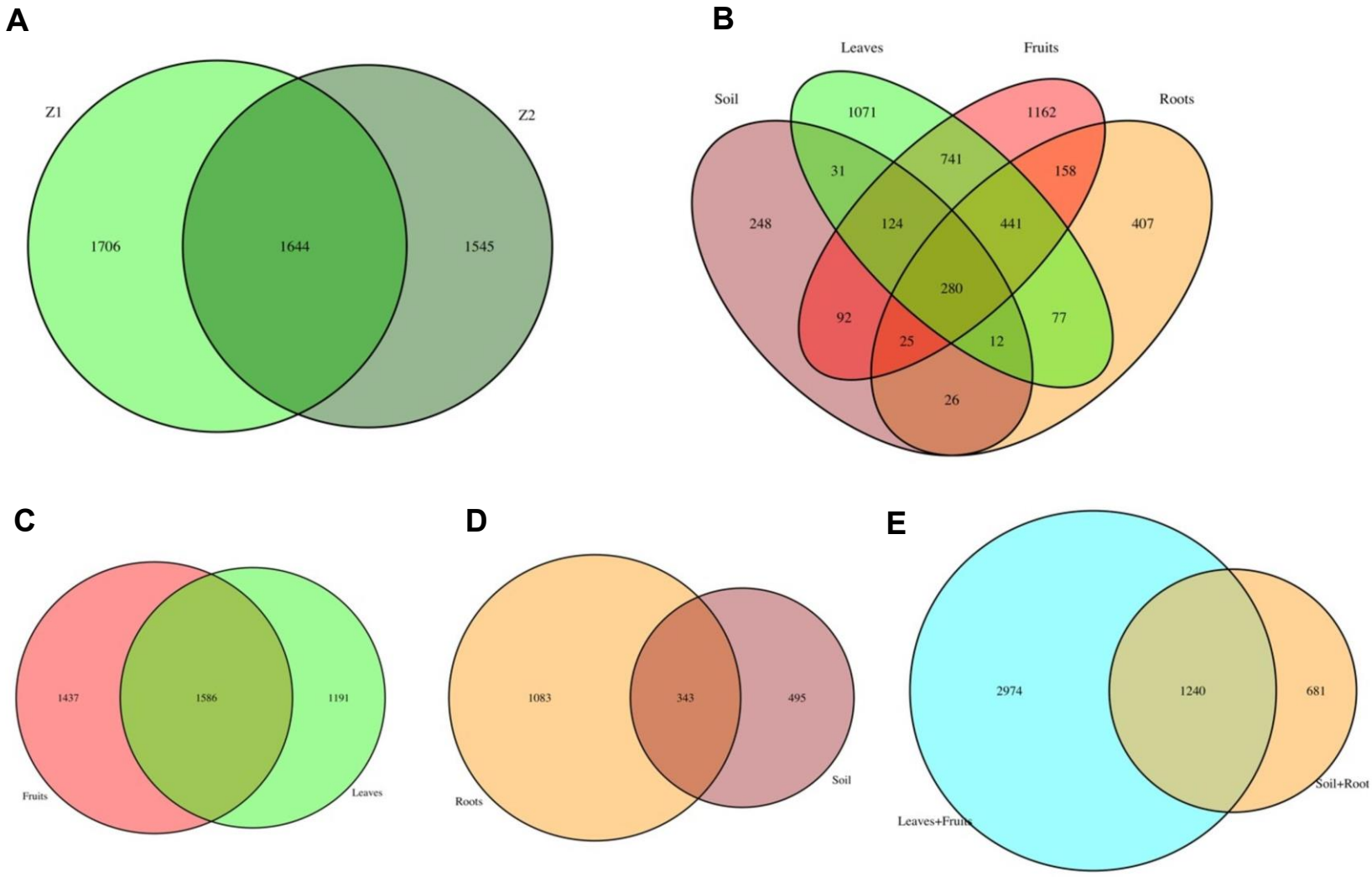
Margenat, A., Matamoros, V., Díez, S., Cañameras, N., Comas, J., Bayona, J.M., 2017. Occurrence of chemical contaminants in peri-urban agricultural irrigation waters and assessment of their phytotoxicity and crop productivity. Sci. Total Environ. 599–600, 1140–1148. https://doi.org/10.1016/j.scitotenv.2017.05.025

Marti, E., & Balcázar, J. L.,2013. Real-time PCR assays for quantification of qnr genes in environmental water samples and chicken feces. *Applied and Environmental Microbiology*, *79*(5), 1743–1745. https://doi.org/10.1128/AEM.03409-12

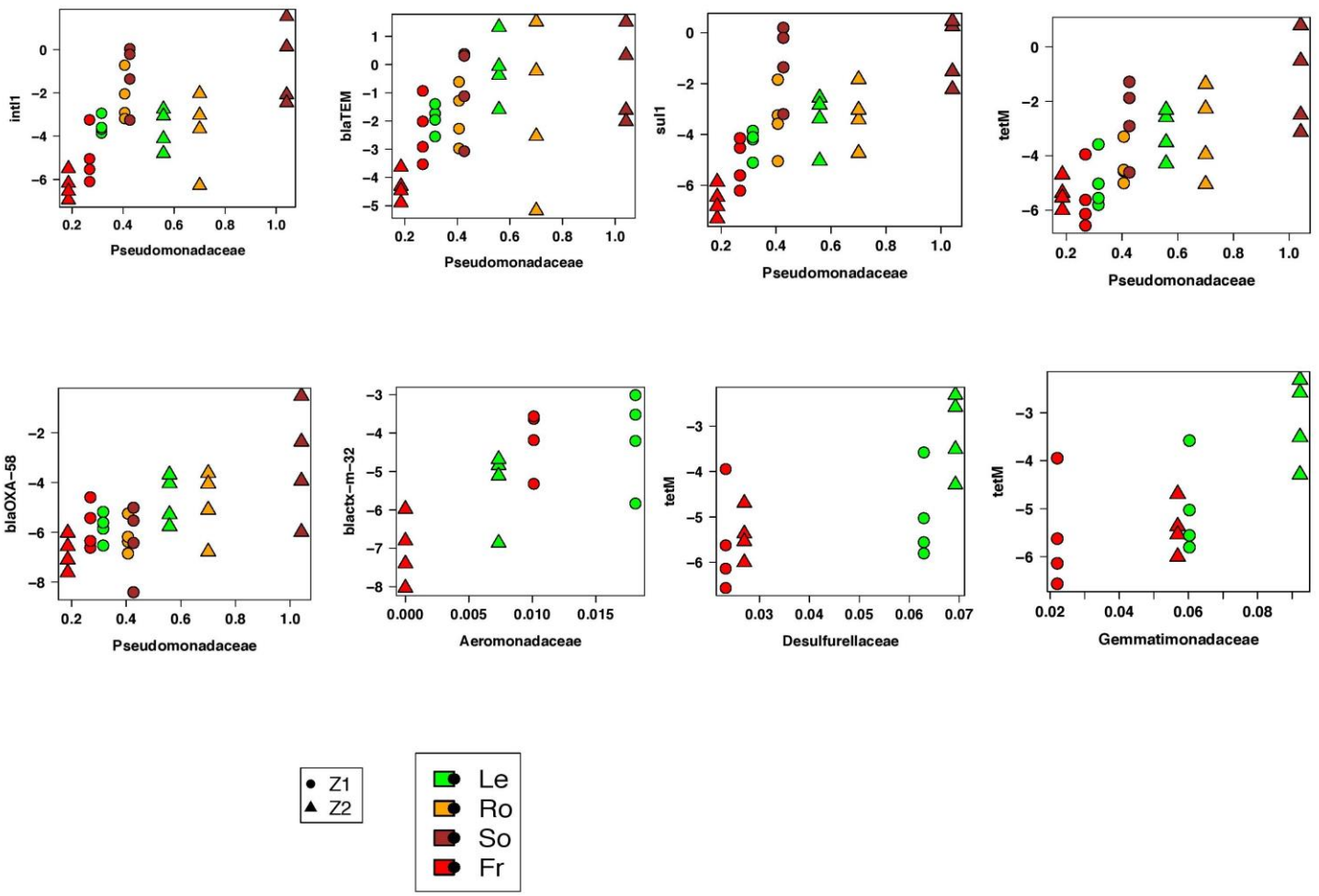
Pei, R., Kim, S. C., Carlson, K. H., & Pruden, A.,2006. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research*, *40*(12), 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., … Schlüter, A.,2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, *155*(7), 2306–2319. https://doi.org/10.1099/mic.0.028233-0

Tamminen, M., & Karkman, A.,2011. Tetracycline Resistance Genes Persist at Aquaculture Farms in the Absence of Selection Pressure Tetracycline Resistance Genes Persist at Aquaculture Farms in the Absence of Selection Pressure, *45*(November 2015), 386–391. https://doi.org/10.1021/es102725n



Supplementary Figure S2.1. Venn's diagrams of the distribution of unique and shared OTUs per sampling zone **(A)**, sample type **(B)**, Fruits vs Leaves **(C)**, Root vs Soil **(D)**, and Leaves+Fruits vs Soil+Roots **(E)**.



Supplementary Figure S2.2. Sample are represented as circles (Z1) or triangles (Z2). Red, green, orange and brown symbols correspond to Fruit, Leaf, Root and Soil samples, respectively. Correlations between the relative abundance of different OTUs families and genetic elements. Gene element levels (Y axes) are represented as log values of the number of copies per g of sample; OTU family abundances are given as fraction of the total reads for each sample. Sample are represented as circles (Z1) or triangles (Z2). Red, green, orange and brown symbols correspond to Fruit, Leaf, Root and Soil samples, respectively.

Chapter III: Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (*Vicia faba* L.) grown under different watering regimes

This chapter is based on the article:

Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Goffe Elsinga, Luc M. Hornstra, Benjamin Piña. (2019). Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (*Vicia faba* L.) grown under different watering regimes. Environmental Research, 170, 16-25.

Social concern has raised during the last years due to the development of AR hotspots in different environmental compartments, including the edible parts of crops. To assess the influence of the water quality used for watering, we collected samples from soil, roots, leaves and beans from the legume plant *V. faba* (broad beans) in three agricultural peri-urban plots (Barcelona, NE Spain), irrigated with either groundwater, river water, or reclaimed water. ARGs *sul*1, *tet*M, *qnrS*1, $bla_{\text{CTX-M-32}}$, $bla_{\text{OXA-58}}$, *mec*A, and bla_{TEM} were qPCR, along with 16S rDNA and *intI*1 sequences, as proxies for bacterial abundance and integron prevalence, respectively. Microbiome composition of all samples were analyzed by high-throughput DNA sequencing. Results show a gradient of bacterial species diversity and of ARG prevalence from highly diverse soil samples to microbially-poor beans and leaves, in which Rhizobiales essentially displaced all other groups, and that presented very small loads of ARGs and integron sequences. The data suggest that the microbiome and the associated resistome were likely influenced by agricultural practices and water quality, and that future irrigation water legal standards should consider the specific Physiology of the different crop plants.

3.1. Introduction

AR hotspots occur in essentially all environmental compartments subjected to anthropogenic pressure, such as WWTPs, pharmaceutical manufacturing plant effluents, and aquaculture facilities (Michael et al., 2013). While the reuse of polluted water from these sites represents a number of benefits, and that major advances have been made with respect to producing safe treated effluents, there are a number of issues that have to be solved before using reclaimed water in a general basis. Currently operating WWTPs fail to completely remove ABs, ARBs, and ARGs (Le-Minh et al., 2010; Manaia et al., 2018; Michael et al., 2013). Therefore, the use of reclaimed water in agriculture may favor the dissemination of ABs, ARB, and ARGs in the environment (Ben et al., 2017).

The proliferation of ARB and ARGs is frequently associated with MGEs, being a relevant example gene cassettes present in integrons (Domingues et al., 2012). Class 1 integrons are widely reported in pathogens from clinical settings, carrying gene cassettes associated with antibiotic resistance, and can be found in plasmids or associated with transposons, allowing their dissemination through HGT (Gillings et al., 2008; Labuschagne et al., 2008; Li et al., 2006; Lind et al., 2006; Mendes et al., 2007). Integrons have been recently detected in microbial species from many different environments, including WW (Tennstedt et al., 2003), river water (Mukherjee and Chakraborty, 2006), and soil (Agerso and Sandvang, 2005). This suggests that the dissemination of integrons may be a general problem of environmental pollution and public health (Stokes et al., 2006).

HGT frequency is determined by two complementary main barriers: Phylogeny and Ecology. Considering antibiotic exposure as a source of selection, ecology provides a physical barrier, as donor and recipient bacteria have to be in the same niche for HGT to occur. Once this physical barrier has been broken, phylogeny determines the HGT network of ARGs and other transposable elements, as intra-taxon genetic exchange occurs more often than inter-taxon (Y. Hu et al., 2016). Microbial communities in natural environments harbor ARB and ARGs, even in unpolluted and/or isolated areas (Allen et al., 2010; Bhullar et al., 2012). Selective pressure on these environments, as it may occur by the incorporation of antibiotics from different farming practices, may increase the abundance of ARB in soil microbiomes (Chen et al., 2016; Han et al., 2016; Hartmann et al., 2015; Heuer et al., 2011; Y. Hu et al., 2016). This effect may in turn lead to the subsequent increase of antibiotic resistance levels on soils and in harvested vegetables

(Han et al., 2016; Marti et al., 2013; Westhoff et al., 2017), which may ultimately result in an impact in human health (Becerra-Castro et al., 2015). The estimate number of deaths caused annually by ARB in the United States of America and in the European Union, calculated as 23,000 and 25,000, respectively (https://www.cdc.gov/drugresistance/, Freire-Moran et al., 2011), but the relevance of the presence of ARB and ARGs in food in these figures is currently unknown.

In this work we surveyed seven ARGs of environmental and clinical relevance (*sul*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *mec*A, *qnrS*1, *tet*M, and $bla_{OXA\text{-}58}$) together with the integrase *intI*1, diagnostic for potentially HGT activity and marker of anthropogenic pollution (Berendonk et al., 2015; Bertini et al., 2007; Gillings et al., 2015; Hembach et al., 2017; Tamang et al., 2008; Wielders et al., 2002). Both integrons and ARGs have been found associated to MGEs in crop fields (Berendonk et al., 2015), and in edible vegetables (Hu et al., 2008; Nonaka et al., 2012; Wang et al., 2015). In a previous study, we found a gradient of microbial species and ARB-related genetic elements along the soil-plant-fruit continuum in commercial tomato crops grown under different water regimes, and found a correlation between the presence of ARGs in fruits and the soil and plant microbiome composition, which are, in turn, under the influence of agricultural practices (Cerqueira et al., 2019b). Here we present a comparable study using a very popular legume crop, the broad bean *V. faba*, which, contrary to tomatoes, is consumed generally (but not always) cooked, once removed from the pod. The study was carried out in three farm fields, two of them indirectly irrigated with TWW, and a rural organic farming plot irrigated with groundwater, all located at less than 30 km from Barcelona (Spain). Our main goal is generating a predictive model allowing the classification of the sampling zones as control or polluted areas, incorporating to it as many different types of crops and different water qualities as possible, always within the current EU regulation for crop irrigation and soil amendment procedures.

3.2. Materials and Methods

3.2.1. Site description

Broad beans crops were collected in agricultural fields in the delta of the Llobregat River (Catalonia, Spain, Fig. 3.1). The reference zone (Z1) was located in a field near Cabrera de Mar, northeast of the Barcelona metropolitan area, away from most human impact, irrigated with groundwater, and fertilized with local pigeon manure. In contrast, zone Z2,

in the Llobregat low valley, receives water from the Infanta Channel, which is mainly fed by TWW from 10 different WWTP effluents that discharge into the Rubí Creek, representing about 92% of the total water flow Z3 was irrigated with river water also impacted by WWTPs (see map as Fig. 3.1), but in this case TWW represented less than 18% of the water flow (Marcé et al., 2012). Z2 and Z3 applied chemical fertilizers when needed. Broad beans plants were collected in April of 2017, when they reached their commercial size. The samples included bulk soil, roots, leaves, and beans, removed from the pod. Plants were collected in a systematic random sampling scheme, in which each collection site was divided into five equal sections. One plant (roots, leaves, and broad beans) and a soil sample were collected per each sampling site (Z1, Z2, Z3) and section (five sections per site), 60 samples in total. Samples were collected into sterile plastic containers, and, within few hours, transported on ice to the laboratory, where they were processed and total DNA was extracted.

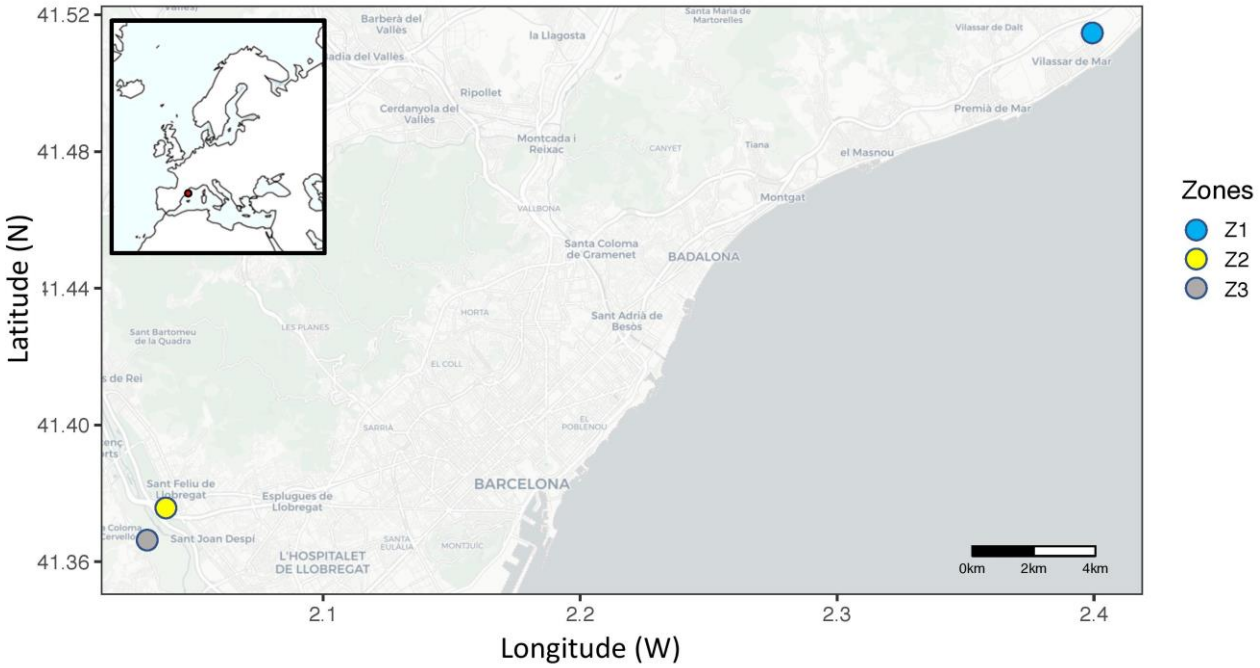


Figure 3.1. Geographic location of the sampling sites.

3.2.2. DNA extraction

Preliminary procedures for DNA extraction were performed as described (Bodenhausen et al., 2013; Qvit-Raz et al., 2008) with slight modifications. Broad beans were washed in sterilized distilled water. Roots and leaves samples in addition had their surfaces sterilized by washing in 70% ethanol for 1 min, rinsing with sterilized distilled water for

1 min, washed again in ethanol 70%, and finally rinsed with sterilized distilled water twice. Samples from the water used to the last rinse were plated in LB agar media for three days in an incubator at 28 °C to evidence lack of contaminating bacteria. Leaves, roots, and broad beans seeds were macerated with mortar and pestle, adding 20 mL of PBS in the case of leaves, and transferred to 50 mL tubes, through a 100 µm nylon Cell strainer (Corning® Cell Strainer). The samples were centrifuged in an Eppendorff Centrifuge 5804 R (Eppendorf, AG) at 4500 rpm for 15 min. The supernatants were discarded, and the pellets (about 250 mg) processed for DNA extraction using the Qiagen DNeasy PowerSoil kit (Qiagen, Inc., Valencia, CA), to a final elution volume of 100 µL. The concentration and the quality of the DNA were tested using a NanoDrop Spectrophotometer 8000 (Thermofisher Scientific, Inc, Waltham, MA). Extracted DNA samples were stored at − 20 °C until further use.

3.2.3. Detection and quantification of ARGs copy numbers

Primers for *intI*1*, sul*1, *tet*M*, qnrS*1*, mecA*, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$*,* $bla_{OXA\text{-}58}$, and bacterial 16S rDNA sequences are listed in Supplementary Table S3.1 (Bradford, 2001; Chen et al., 2014; Donhofer et al., 2012; Frank et al., 2007; Mugnaioli et al., 2006; Tamang et al., 2008). Dynamo ColorFlash SYBR Green (Thermofisher Scientific, Inc.) chemistry was used for *mec*A, and $bla_{OXA\text{-}58}$ qPCR quantification; all the other ARGs were quantified with SYBR green (A F. Hoffmann–La Roche AG, Inc, Switzerland) in a Lightcycler 480 (A F. Hoffmann–La Roche AG, Inc). Thermal cycling conditions differed for both technologies: 95 °C for 10 min, 45 cycles at 95 °C for 15 s and Ta for 1 min for the first one, and 95 °C for 7 min, 40 cycles at 95 °C for 10 s and Ta for 30 s for the second one. All qPCR reactions were performed in a Lightcycler 480 II (A F. Hoffmann–LaRoche AG, Inc). Melting curves were obtained to confirm amplification specificity. Optimized primer concentrations were 0.15 µM for *tet*M, *mec*A, and $bla_{OXA\text{-}58}$ primers, and 0.3 µM for *sul*1, *intI*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *qnrS*1, and 16S rDNA primers; qPCR reactions were performed by duplicates, using a fixed dilution of raw DNA extract. The quality criteria used was an $R^2 > 0.99$ and an accepted efficiency of reactions from 90% to 110%.

3.2.4. Standards and normalization methods

Four plasmid vectors were used as standards for absolute quantification of qPCR amplicons. For 16S rDNA gene, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *sul*1, *qnrS*1, *intI*1, purified DNA

samples from pNORM1 plasmid (Gat et al., 2017), were used as quantification standards (Supplementary Table S3.1). For *tet*M, bla_{OXA-58} and *mec*A, purified DNA samples from three different pUC19 plasmids were used as standards (Laht et al., 2014; Szczepanowski et al., 2009; Tamminen et al., 2011). (Supplementary Table ST3.1). Standard curves (Ct per log copy number) for quantification of all genes were obtained for each run, using 10-fold serial dilutions of the corresponding standards. Gene copy number of standards was determined via the plasmid/genomic DNA concentration using the NanoDrop Spectrophotometer 8000, applying the DNA copy number and the Dilution Calculator algorithm (https://www.thermofisher.com/us). Three technical replicates were run for the standard curves; complete reaction mixes plus nuclease free water, but no template, were used as negative control qPCR reactions. ARG concentration in samples was calculated using the standard curve equation and the measured Ct value. The quality control of raw Ct values for standard curve and unknown samples was performed before the analysis. The limit of quantification (LOQ) was defined as the lowest point on the linear part of the standard curve: 100 gene copies per reaction for ARGs and *intI*1 and 1000 gene copies per reaction for 16S rDNA. Copy numbers of all ARGs and *intI*1 were normalized to gram of sample (gene copies g^{-1}).

3.2.5. Data analysis for ARGs and *intI*1

The analysis of the ARGs profile and abundance was performed in the R environment (version 3.4.0; http://www.r-project.org/). The ARGs quantified by qPCR were normalized by gram of sample and log transformed. PCA was performed with the function *principal*, from the package psych (https://cran.rproject.org/web/packages/psych/index.html). One-way and two-way ANOVA tests, Tukey'B post-hoc analysis, Pearson's correlations, and fdr (false discovery ratio) correction for multiple tests were also performed with the psych package. Due to the amplification of chloroplastidial and mitochondrial DNA, bacterial 16S rDNA copy numbers were calculated by assessing the proportion of reads attributed to chloroplasts and mitochondria after sequencing the corresponding 16s rDNA amplicons using dada2 (Callahan et al., 2016, p. 2) and phyloseq packages in R (McMurdie and Holmes, 2013).

3.2.6. 16S rDNA library preparation and sequencing

We prepared of a pooled library of 16S rDNA amplicons from 60 soil and broad beans samples' DNA, using primers 331F-TCCTACGGGAGGCAGCAGT and 518R-ATTACCGCGGCTGCTGG (Supplementary Table S3.1). Illumina Nextera XT Index Kit v2 - Index 1 (i7) and Nextera XT Index Kit v2 - Index 2 (i5) adapters were used to tag the samples. A two-step PCR protocol was used to prepare samples. The first step added overhangs to the 16 s rDNA amplicons, using the following thermal cycling conditions: 98 °C for 1 min, 8 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. The second PCR step added the Illumina Nextera XT adapters under the following thermal cycling conditions: 95 °C for 3 min, 8 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The composed, tagged sample was mass sequenced at the KWR Watercycle Research Institute (Nieuwegein, Netherlands) (https://www.kwrwater.nl/), with high-throughput sequencing, using an Illumina MiSeq. Quality control of the sequences was done using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Illumina Nextera adapters were removed and the sequences were quality trimmed for a minimum Phred quality score ($Q \geq 30$) with Trim Galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). R environment was used for all the downstream analysis of the sequences, specifically, the dada2 package. Sequences shorter than 160 bp were discarded. The sequences were dereplicated and PCR chimeras were checked and filtered out. The taxonomic assignment of OTUs was done with SILVA database v128 for dada2 package. Sequences were assigned to OTUs based on a 97% similarity. The sequences classified as chloroplasts and mitochondria were removed. Raw sequences were deposited at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the accession number SRP151778.

3.2.7. Analysis of microbiomes

The analysis of microbial communities was performed with R environment (version 3.4.0), with the packages phyloseq, vegan (https://cran.rproject.org/package=vegan), VennDiagram (https://cran.rproject.org/package=VennDiagram), caret (https://cran.r-project.org/package=caret), and mixOmics (https://cran.r-project.org/package=mixOmics). OTUs abundances were log transformed for β-diversity analysis.

Microbiomes variation between samples were visualized by a NMDS using Weighted-UniFrac dissimilarity (Lozupone et al., 2007), with the functions *ordinate* of the Phyloseq package. The function *adonis* from Vegan package to measure effect size and significances on β-diversity. The *betadisper* function was utilized to test multivariate homogeneity of group variances. Taxa with a prevalence lower than 3% were excluded from the β-diversity analyses, in order to study the "core" microbiomes.

Differences in OTU distribution between reference and TWW-impacted sampling zones were explored using the supervised machine learning classification technique Random Forest (Breiman, 2001). The data was partitioned in train and test set (70% and 30%, respectively) randomly splits it according the grouping classes. Absolute abundance values were centered and scaled. Accuracy was the metric chosen for model evaluation. For re-sampling a 5-fold cross validation repeated 3 times was chosen. The model was fitted over different tuning parameters, using the function *train*. Variable importance was measured by the Median Accuracy Decrease. The model created with the training data was utilized to predict the sampling fields on the test data according the OTUs abundances. A confusion matrix was created to evaluate the predictions.

3.3. Results

3.3.1. ARG abundance and distribution

Bacterial 16S rDNA and *bla*$_{TEM}$ DNA sequences were detected in all samples analyzed. Whereas *intI1*, a marker for anthropogenic pollution, was found in 43% of the samples (Table 3.1). Bacterial 16S sequences, considered as a proxy for bacterial abundance, were found at counts from 140 to more than 5×10^7 copies g^{-1} of sample. The integron *intI1* sequence was found at approximately 70 times lower levels even at its maximum amounts, at around 7.5×10^5 copies g^{-1} of sample (Table 3.1). Among ARGs, abundance levels varied in several orders of magnitude, being *bla*$_{TEM}$ the most abundant one by far, with counts ranging from 30 to 4×10^5 copies g^{-1} of sample (Table 3.1). All other studied ARGs were found at much lower levels or below the limit of detection (Table 3.1); *mec*A levels were below the limit of quantification in all samples (not shown). Despite having a prevalence of only 43%, the maximum counts of *intI*1 surpasses the levels of the rest of ARGs in samples where it was present, except for *bla*$_{TEM}$. Thus, the data suggest that at least *bla*$_{TEM}$ is widespread in the bacterial population, and not necessarily linked to MGEs or, at least, not to *intI*1 (Table 3.1).

The distribution of genetic element abundances in the different sample types and zones was further investigated by two-way ANOVA ("Zone" times "Sample type", Table 3.1). The analysis shows a very strong influence of sample type (soil vs. root endophytes vs. leaves vs. beans) for all genetic elements, and for bacterial 16S abundance, whereas the factor "Zone" was only relevant for the most prevalent ARGs (*bla*$_{TEM}$, *sul*1*, qnrS*1) and *intI*1 (Table 3.1). The interaction between the two factors was non-significant or very weak in all cases (Table 3.1). These different distribution patterns are shown in Fig. 3.2, which includes results from a one-way ANOVA/Tukey's B post hoc test across the different zones. Note the decrease in bacterial abundances from soils (10^6–10^8 copies g^{-1} of sample) to roots and leaves (10^4–10^6 copies/g of sample) and to beans (10^4–10^5 copies g^{-1} of sample), as well as the essentially lack of *intI*1 and ARG sequences in beans and leaves (except for some Z1 samples). In fact, *bla*$_{TEM}$ was the only ARG present in beans and leaves at least moderate levels (some 100 copies g^{-1} of sample). This genetic element showed, in addition, a defined distribution by zones, being more prevalent in samples from Z3 than in those from Z1 or Z2. This differential distribution was observed for all sample types, from soils to beans, suggesting that it the soil determined the final loads in *bla*$_{TEM}$ in the plant (Fig. 3.2). A similar pattern was observed for *qnrS*1, although the levels in bean samples in this case were too low for any meaningful statistical analysis (Fig. 3.2). Finally, *intI*1 was more abundant in soil and root samples from Z1 than from Z2 or Z3, and too scarce in leaves and beans for statistical analyses (Fig. 3.2). ARGs *tet*M and *bla*$_{OXA-58}$ were only found in soil samples and at low abundances in all cases (100 copies g^{-1} of sample or less, Fig. 3.2).

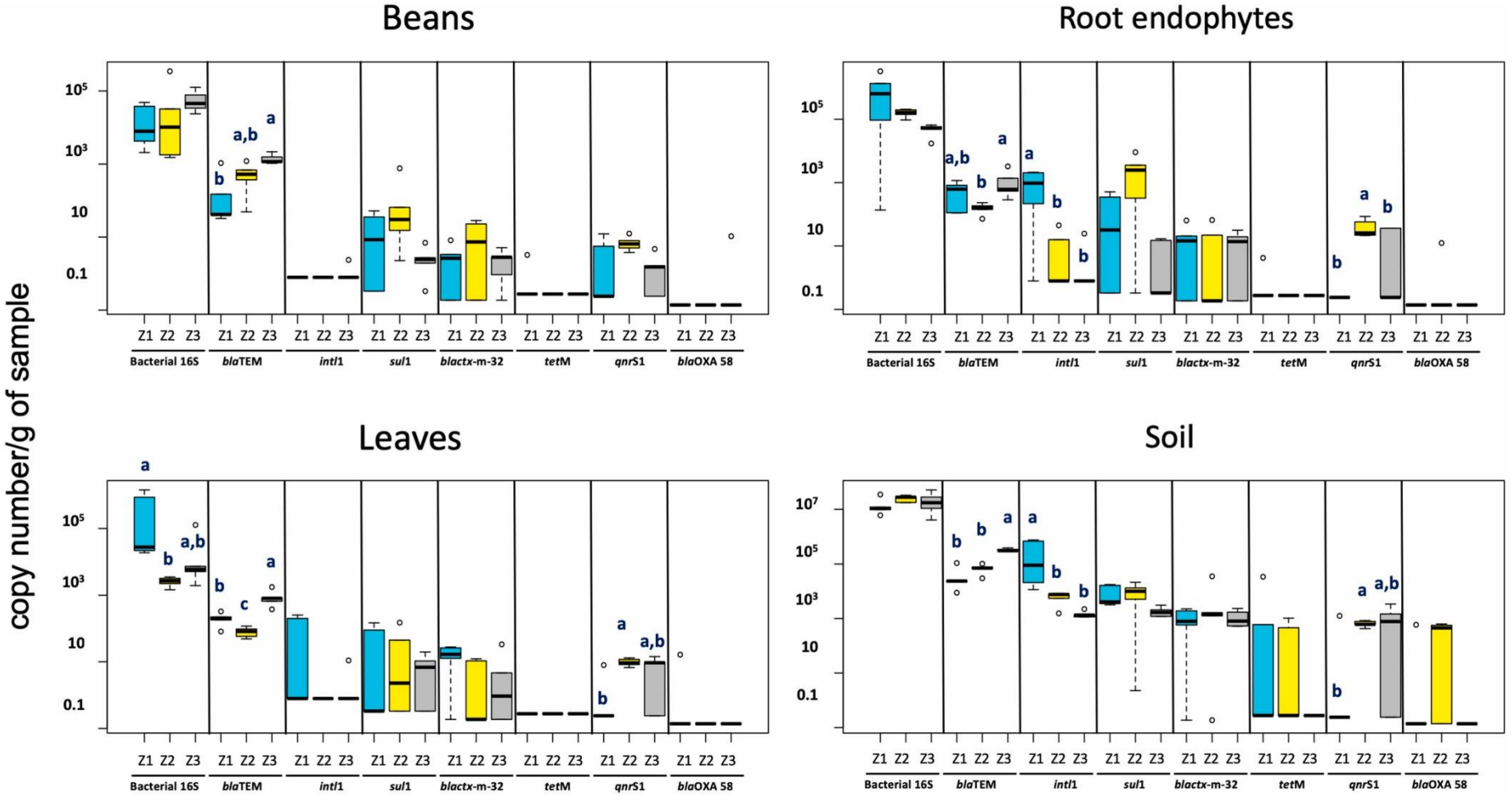


Figure 3.2. Absolute abundances of the different genetic elements in all samples, separated by sample types (Beans, Leaves, Roots, and Soil). Data are expressed as copies of each sequence per g of tissue, and code colored by zones as in Fig. 3.1: cyan, yellow and gray correspond to Zones 1, 2, and 3, respectively. Lowercase letters a to c, when corresponding) indicate statistically different groups of data ($p < 0.05$, ANOVA+Tukey HSD test); absence of letters indicates no statistical differences between zones. Sample points not fitting the normal distribution (outliers) are represented by empty dots

Table 3.1. Genetic element distribution among different sample types and sampling zone.

	Aggregate results (copies/g of sample)			F Fisher's Test (tow-way ANOVA, "Zone"x "Sample Type")[a]		
	Prevalence	Mean	Range	Zone	Sample Type	Interaction
16S rDNA	100 %	$5.3x10^6$	$14\text{-}5.2x10^7$	0.86	59.68 ***	2.76
*intI*1	43 %	$2.7x10^5$	$LOQ\text{-}7.5x10^5$	15.97***	97.51 ***	2.97
bla_{TEM}	100 %	$3.6x10^4$	$30\text{-}3.9x10^5$	32.98 ***	180.08 ***	3.02
*sul*1	73 %	2000	$LOQ\text{-}2.1x10^4$	3.62 *	19.77 ***	1.29
$bla_{CTX\text{-}M\text{-}32}$	67 %	860	$LOQ\text{-}3.5x10^4$	0.09	15.11 ***	0.49
*qnrS*1	58 %	180	LOQ-3400	16.52 ***	5.88 **	1.10
$bla_{OXA\text{-}58}$	12 %	38	LOQ-630	2.06	3.50 *	2.39
*tet*M	10 %	600	LOQ-600	1.81	4.34 **	1.11
Degrees of Freedom				2	3	6

a) F Fisher's Test. *, $p \leq 0.05$; **, $p \leq 0.01$;***,$p \leq 0.001$; n=60

LOQ, limit of quantification

3.3.2. Microbiome analysis

A total of 839 OTUs were annotated from a total of 3,114,367 readings, in 60 samples with a median sequencing depth of 57,617 (range 1,883 to 89,098) reads per sample after quality control. The presence of chloroplastidial and mitochondrial sequences was only marginal in most soil samples, but became predominant in roots, leaves and particularly beans samples, in which the presence of actual bacterial sequences was only marginal, and even absent in some leaves' samples and in most beans' samples (Supplementary Fig. 3.2). In fact, none of the Zone 3 beans' samples showed significant numbers of bacterial reads (not shown). The distribution of OTUs among the different samples, taxonomically grouped at the order level, is shown in the graph in Fig. 3.3. The graph shows a rich bacterial diversity in soil samples, whereas plant samples showed a clear prevalence of Rhizobiales' sequences, which started at root endophyte microbiomes to become clearly predominant in leaves' and beans' microbiomes (red sectors in Fig 3.3 note that there was no data for Z3 beans). Therefore, we concluded that Rhizobiales essentially displaced most of the other bacterial groups in the endophytic microbiome, occasionally mixed with other groups (Micrococcales in leaves

from Z2, Enterobacteriales in leaves from Z3, and several minor groups in beans from Zone 1, Fig. 3.3).

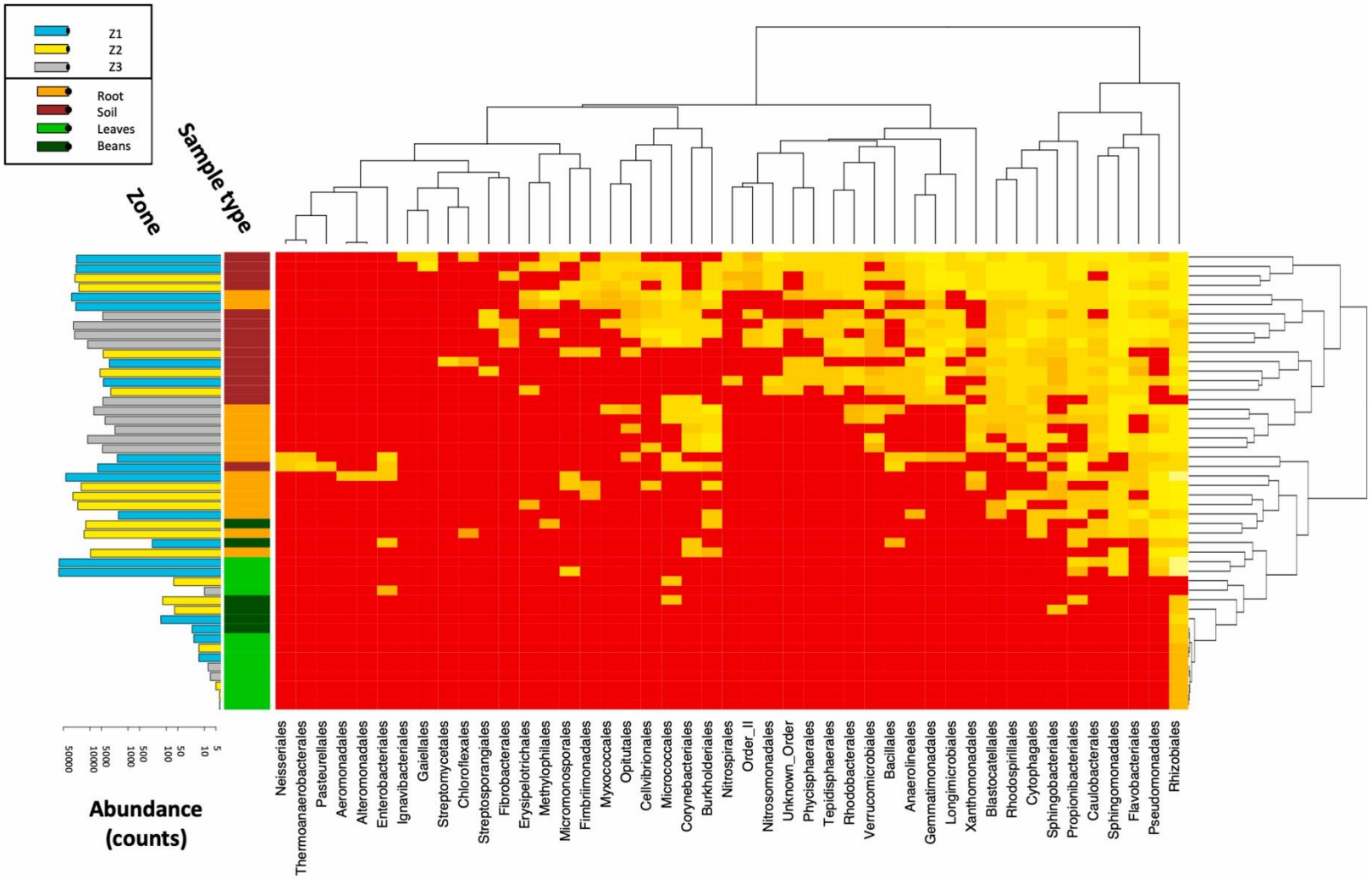


Figure 3.3. Hierarchical clustering of samples grouped at the order taxonomic level. The graph shows the log-transformed of and sample. OTUs representing more than 0.1% of the total abundance were included. The left vertical side bar indicates sample types, as brown, orange, light green and dark green represent soil, root endophytes, leaves and beans, respectively. The left-most bar graph shows the total number of reads included (plastid sequences were excluded), in logarithmic annotation. Colors indicate sampling zones, coded as in Fig. 3.1. Cell colors in red indicates the lowest values (absence of the sequences, transformed to 0.1 to allow log transformation) and white the highest one.

Variations among the different soil and endophytic microbiomes were analyzed by β-diversity and Random Forest classification analyses. The NDMS plot in Fig. 3.4 A (stress=0.06), based on Weighted UniFrac dissimilarity index, revealed a much higher dissimilarity for the OTUs from soil and roots (brown and orange symbols in Fig. 3.4A than for those from leaves and beans (light and dark green symbols). The contribution of the sampling zone on data dispersion was tested by the *adonis* algorithm, which detected significant differences in soil microbiome composition between sampling zones, but not for roots' or leaves' microbiomes (Table 3.2). We interpret these results as the demonstration that both sampling zones and sample types determine the microbiome composition, and that the geographical effect was only significant for soils. Fig. 3.4B shows the different distributions of eight representative orders among soil samples from the different sampling zones; the complete results from the ANOVA+Tukey's B analyses are shown in the Supplementary Table S3.2.

Table 3.2. Results from weighted UniFrac analysis (*adonis*).

Partition (Sample type)	F model	R^2	*p*-values
Soil	4.11	0.411	0.003
Roots	1.51	0.201	0.116
Leaves	2.25	0.272	0.121

To explore the effects of agricultural practices (including irrigation water) on the microbiome we build a Random Forest classification model in which all samples from Z1 (irrigated with groundwater) were confronted with all samples from Z2 and Z3 (both irrigated with TWW-fed effluents) combined. The Random Forest classifier validated the model, correctly grouping the different samples with an accuracy of 88.9% (Supplementary Table S3.3). The random forest model also assigned a variable importance score to each OTU by estimating the Mean Decrease in Accuracy. The highest scores (i.e., the highest importance for classification) corresponded to OTUs classified in the orders Sphingomonadales, followed by Rhizobiales, Proprionibacteriales, Pseudomonadales, Caulobacterales and Enterobacteriales (See Supplementary Fig. S3.2). We thus concluded that the prevalence of these orders in the microbiomes was significantly different in Z1 than in Z2 and Z3, although the exact driver or drivers behind these differences could not be determined at present.

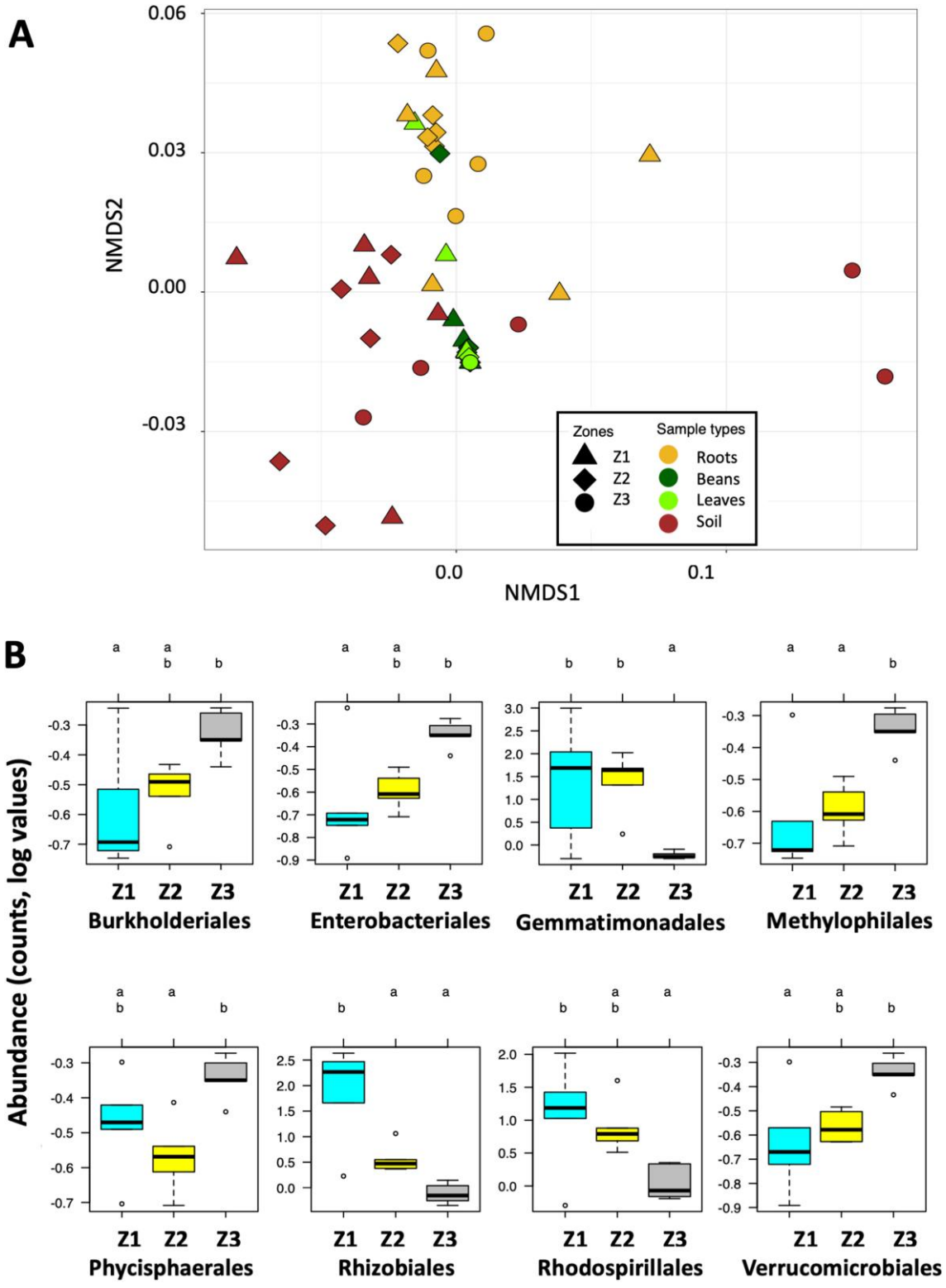


Figure 3.4. Analysis of OTU distribution, grouped at the taxonomic order level. **(A)** NMDS plot for all samples, labelled by sample type as in Fig. 3.3, and by sampling zone (triangles, diamonds and circles represent samples from Z1, Z2 and Z3, respectively). **(B)** Taxon distribution in soil samples from the different sampling zones. Graphs present absolute abundances (read counts) for different taxonomical orders in soil samples from Z1, Z2, and Z3 (labelled as in Fig. 3.2, Fig 3.3). Boxes indicate the first to 3rd quartile interval, whiskers correspond to the 95% distribution interval, and the thick line indicates the distribution mean. Low-case letters at the top of the graphs indicate statistically different groups of data ($p < 0.05$, ANOVA+Tukey HSD test).

3.3.3. Correlations between microbiome composition and ARGs abundance

The correlations between OTU abundance (relative abundances at the order level) and genetic element prevalence was analyzed for all studied samples (Fig. 3.5). Only 16 orders showed significant correlation (Pearson's correlation, fdr correction) with at least one genetic element. Conversely, only *intI*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$ and *sul*1 prevalences correlated with the relative abundance of one or more bacterial orders, plus the total amount of bacterial 16S rDNA gene (Fig. 3.5). All observed correlations were positive, except for the order Rhizobiales, whose relative abundance showed negative correlations with all five genetic elements (Fig 3.5). Complete results from the analysis is shown in Supplementary Table S3.4; Fig. 3.5 shows some of the most significant correlations, with added information about sampling zone and sample type.

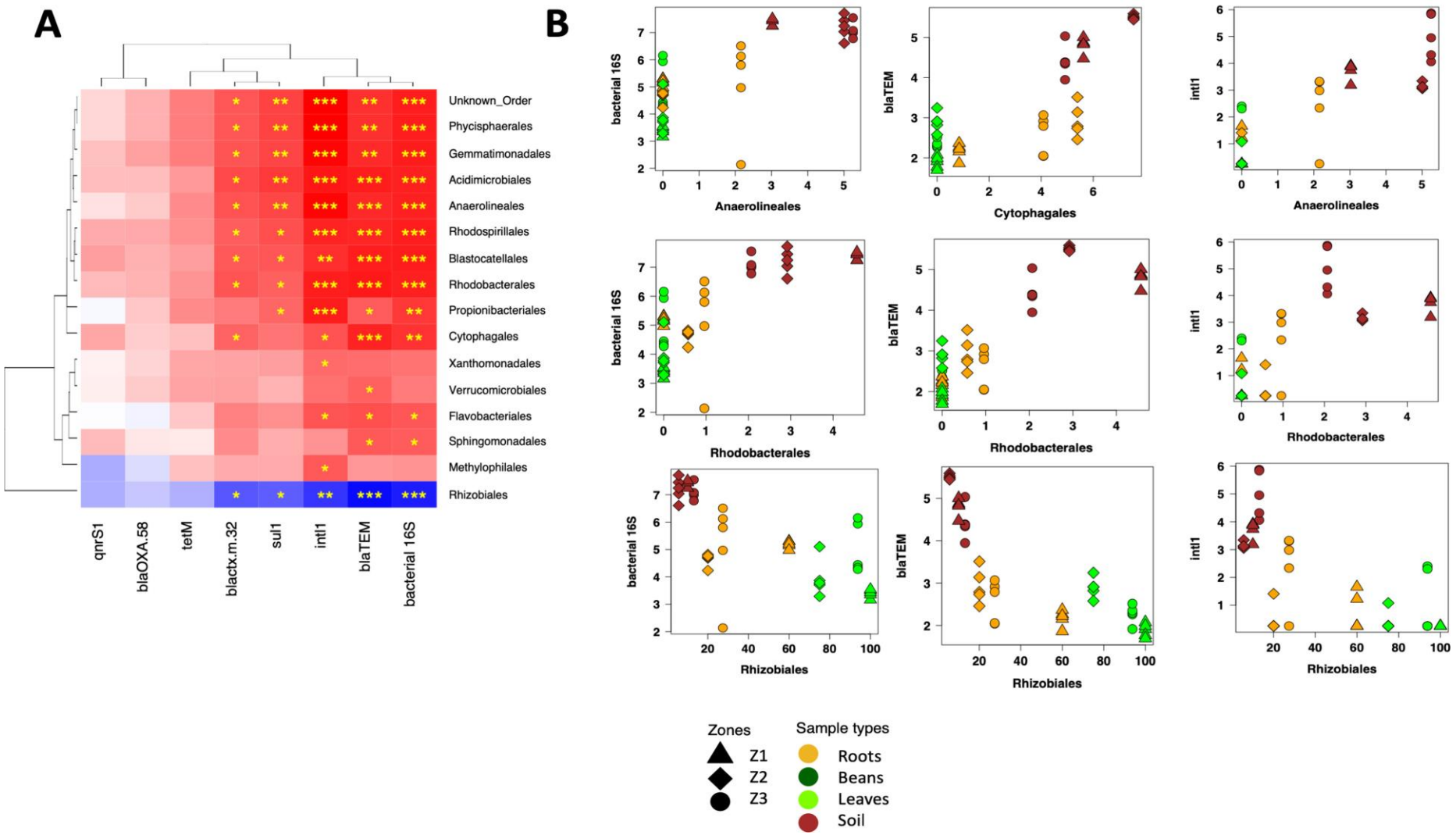


Figure 3.5. Correlation between genetic element abundance (in copies per g of sample) and relative proportion of different taxonomical order in all samples. **(A)** Heatmap with hierarchical clustering. Red and blue cells indicate positive and negative correlations, respectively, asterisks indicate significant correlations (Pearson's + fdr correction, one, two or three asterisks represent p-values under 0.05, 0.01 and 0.001, respectively) only orders with at least one significant correlation were included. **(B)** Individual biplots showing different examples of significant correlations between genetic elements and relative abundances of some bacterial orders. Samples are shown individually, with colors representing the different sample types, and symbols indicating sampling zones, as in Fig. 3.4.

3.4. Discussion

The study of plant-associated microbiomes is gaining a lot of interest in the scientific community for two apparently disparate reasons. First, recent studies using of culture-independent quantitative methods demonstrated the amazing complexity of plant microbiomes, as well as their complex interaction with many physiological functions of the plant (Andreote et al., 2014; Berg et al., 2014; Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Ruppel et al., 2006; Ryan et al., 2008). Secondly, there is the suspicion that plant may become contaminated with toxicants or potentially pathogenic bacteria or bacterial genetic elements and thus become a risk for consumers, particularly linked to the reuse of WW for irrigations (Becerra-Castro et al., 2015; Ben et al., 2017; Han et al., 2016; Marti et al., 2013; Piña et al., 2018; van Overbeek et al., 2014; Westhoff et al., 2017; Zhu et al., 2017).

Our results showed that the bacterial abundance in both roots and leaves (and, presumably, in most parts of *V. faba* plants) is around 1–10 % of the levels found in soils, a figure that is consistent with previous estimations using comparable methodologies for *Brassica oleacea* (Ruppel et al., 2006). These levels drop another 10-fold, at least, in beans. In addition, and with the exception of bla_{TEM}, most of the studied genetic elements (*intI*1, ARGs) were only found in significant amounts in soil and root endophytes. These low values in *intI*1 and ARGs appear to correlate with the predominance of Rhizobiales in all plant compartments, likely a specific feature of legume plants. In fact, we have data from non-leguminous cultivars showing a radically different distribution of both endophytes and antibiotic resistance-related genetic elements (Cerqueira et al., 2019b).

We found a higher prevalence of *intI*1 in soil and roots than in the aerial parts of plant, with significant differences between sampling zones. For example, *intI*1 was completely absent from beans' samples and showed very low representation in leaves, which nevertheless contained low, but significant levels of bla_{TEM} or, in the latter, *qnrS*1 sequences. These results are in line with the fact that the presence of integrase genes, like *intI*1, have been associated to a higher potential for dissemination of antibiotic resistance in both clinical samples and different environmental compartments (Domingues et al., 2012). Therefore, our results may suggest that the consumption of beans represents a low human health risk in terms of exposition to antibiotic resistance gens. Furthermore, *intI*1 was found at 5–10 fold higher levels in the roots and soils from the field irrigated with groundwater (Z1) compared to those irrigated with river water impacted by TWW (Z2, Z3). While this may reflect the influence of agricultural practices

(manure fertilization in the field irrigated with groundwater) in the abundance of ARGs and related genetic elements, as previously suggested (Cerqueira et al., 2019b; Piña et al., 2018). It is interesting to remark that Z1 was irrigated with groundwater, so the results indicated that other factors, both geographic and operational (manure application), may be at least equally important for the interpretation of *intI*1 abundance.

In addition to *intI*1, only bla_{TEM} and *qnrS*1 abundances showed clear differences between sampling zones. In the case of *qnrS*1, this variability reflects its virtual absence in Z1 samples, whereas bla_{TEM} showed a very consistent distribution pattern for all sample parts, in which the highest prevalence corresponded to Z3 samples. The abundance of bla_{TEM} was similar for all plant parts, around 100–1000 copies per g of sample, and clearly higher than the levels on *intI*1 in most cases. Then, the results suggest that the endophytic bla_{TEM} appears to be independent from the presence of integrons, at least to *intI*1. They also are at variance with the commonly assumed link between the presence of *intI*1 and the presence of anthropogenic-driven abundance of ARGs. The high abundance of bla_{TEM} in Z3 is consistent with the observation that this ARG is prevalent in sediments impacted by WWTPs (O'Flaherty and Cummins, 2017). Z3 is the only sampling site 92% irrigated with TWW, in comparison with the 18% of TWW for Z2 and essentially nil for Z1.

Different recent studies showed the influence of using TWW, or water with strong TWW contribution, in soil and roots' microbiome from non-legume plants, like *L. esculentum* or *L. sativa* (Cerqueira et al., 2019b; Zolti et al., 2019). Our results showing that only a small part of the OTUs present appeared as responsible for differentiating between fields with different irrigation regimes, may be due to the characteristics of the plant host, as previously suggested (Lundberg et al., 2012; Ofek et al., 2014; Ofek-Lalzar et al., 2014). In our case, it is particularly remarkable the prevalence of Rhizobiales species (mainly, *Rhizobium* spp.) in plant parts' microbiome, up to 100% of bacterial sequences from many leaves' and beans' samples. The presence of Rhizobiales in roots from legume plants is expected*, as Rhizobium* is known to colonize legume roots as a symbiont, where it fixes nitrogen in specific structures called nodules (Ferguson et al., 2019). However, as nitrogen fixation in legumes does not occur outside roots' nodules, their presence (and even predominance) in aerial parts is somewhat surprising. A previous study (Zgadzaj et al., 2016) found that the enrichment of symbiosis-linked root bacteria imposes distinctive rhizosphere, root, and nodule bacterial communities, affects the soil microbiome. Our results are consistent with this study, suggesting that this influence extends to the microbiome of surrounding soil, with relatively high levels of Rhizobiales,

and that it propagates to the rest of the plant, outside the nodule-root system, and eventually becoming the predominant, if not unique, endobacterial group. The role of this presence, that may be assimilated to commensalism, in plant physiology it is unclear. Despite nitrogen fixation cannot occur outside the root nodules, as a rule, bacteria of *Rhizobium* genus may have other functions that benefit the host plants, such as phosphate solubilization or phytohormone synthesis from absicic acid to auxins, among others (Alori et al., 2017; Ferguson and Mathesius, 2014). Our data suggests that the prevalence of Rhizobacteria associates to a very low level both of other bacterial types and of ARGs, which may have relevant meaning for both plant (as it may well allow the plant to fend off potentially pathogenic bacteria) and for consumer health. In fact, the extremely low levels of ARGs and of non-Rhizobial bacterial groups in beans, in contrast with the corresponding high levels in soils, suggests that the requirements for water quality may be lower for legume crops than for than other cultivars in terms of microbial food safety. TWW irrigation is currently limited for certain crops in different countries, like lettuces and other leafy food products in Cyprus (Kalavrouziotis et al., 2015). Whereas the main rationale for these limitations is the way they are consumed (i.e., raw versus cooked), our results suggest that the Physiology of the crop plant should be considered to set water standard qualities for irrigation of different edible crops (Freire-Moran et al., 2011; Marcé et al., 2012).

3.5. Supporting Information

Supplementary Table S3.1. Primers utilized for detection and quantification of ARGs in soil and plant parts.

Target gene	Primers	Sequence (5'-> 3')	Amplicon size (bp)	Tm(°C)	Reference
***intI*1**	intI1LC5	GATCGGTCGAATGCGTGT	196	60	Barraud et al, 2010
	intI1LC1	GCCTTGATGTTACCCGAGAG			
16s rDNA	331F	TCCTACGGGAGGCAGCAGT	195	60	Bräuer et al., 2011
	518R	ATTACCGCGGCTGCTGG			
***bla*$_{TEM}$**	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113	60	Di Cesare et al., 2016
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG			
***bla*$_{CTX-M-32}$**	ctx-m-32-FW	CGTCACGCTGTTGTTAGGAA	156	60	Hembach et al., 2017
	ctx-m-32-R	CGCTCATCAGCACGATAAAG			
***bla*$_{OXA-58}$**	OXA58F	GCAATTGCCTTTTAAACCTGA	152	63	Laht et al., 2014
	OXA58R	CTGCCTTTTCAACAAAACCC			
***mec*A**	mecAF	AAAAAGATGGCAAAGATATTCAA	185	63	Szczepanowski et al., 2009
	mecAR	TTCTTCGTTACTCATGCCATACA			
***qnrS*1**	qnrSrtF11	GACGTGCTAACTTGCGTGAT	118	60	Marti & Balcázar, 2013
	qnrSrtR11	TGGCATTGTTGGAAACTTG			
***sul*1**	sul1-FW	CGCACCGGAAACATCGCTGCAC	162	60	Pei et al., 2006
	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
***tet*M**	tetMF	GCAATTCTACTGATTTCTGC	186	60	Tamminen & Karkman, 2011
	tetMR	CTGTTTGATTACAATTTCCGC			

Barraud, O., Baclet, M. C., Denis, F., & Ploy, M. C.,2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *Journal of Antimicrobial Chemotherapy*, *65*(8), 1642–1645. https://doi.org/10.1093/jac/dkq167

Bräuer, S. L., Adams, C., Kranzler, K., Murphy, D., Xu, M., Zuber, P., Tebo, B. M., 2011. Culturable Rhodobacter and Shewanella species are abundant in estuarine turbidity maxima of the Columbia River. *Environmental Microbiology*, *13*(3), 589–603. https://doi.org/10.1111/j.1462-2920.2010.02360.x

Di Cesare, A., Losasso, C., Barco, L., Eckert, E. M., Conficoni, D., Sarasini, G., … Ricci, A., 2016. Diverse distribution of Toxin-Antitoxin II systems in Salmonella enterica serovars. *Scientific Reports*, *6*, 1–9. https://doi.org/10.1038/srep28759

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., & Schwartz, T., 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology*, *8*(JUL), 1–11. https://doi.org/10.3389/fmicb.2017.01282

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., & Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS ONE*, *9*(8), 1–8. https://doi.org/10.1371/journal.pone.0103705

Marti, E., & Balcázar, J. L., 2013. Real-time PCR assays for quantification of qnr genes in

environmental water samples and chicken feces. *Applied and Environmental Microbiology*, *79*(5), 1743–1745. https://doi.org/10.1128/AEM.03409-12

Pei, R., Kim, S. C., Carlson, K. H., & Pruden, A., 2006. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research*, *40*(12), 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., Schlüter, A.,2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, *155*(7), 2306–2319. https://doi.org/10.1099/mic.0.028233-0

Tamminen, M., & Karkman, A.,2011. Tetracycline Resistance Genes Persist at Aquaculture Farms in the Absence of Selection Pressure, *45*(November 2015), 386–391. https://doi.org/10.1021/es102725n

Supplementary Table S3.2. Bacterial orders distribution among soil samples from the different sampling zones (Z1-Z3).

Genera	Z1	Z2	Z3	Z1	Z2	Z3	*p*-value
Methylophilales	-0.62	-0.59	-0.34	b	b	a	0.0025
Rhizobiales	1.85	0.57	-0.12	a	b	b	0.0027
Verrucomicrobiales	-0.63	-0.56	-0.34	b	ab	a	0.0027
Enterobacteriales	-0.66	-0.59	-0.35	b	ab	a	0.0029
Gemmatimonadales	1.36	1.38	-0.22	a	a	b	0.0060
Unknown_Order	-0.53	-0.55	-0.34	a	a	a	0.0093
Rhodospirillales	1.07	0.89	0.05	a	ab	b	0.0143
Pseudomonadales	-0.36	-0.58	-0.26	a	a	a	0.0147
Phycisphaerales	-0.48	-0.57	-0.34	ab	b	a	0.0153
Anaerolineales	-0.08	-0.45	-0.20	a	a	a	0.0175
Acidimicrobiales	-0.40	-0.55	-0.33	a	a	a	0.0207
Burkholderiales	-0.58	-0.53	-0.33	b	ab	a	0.0236
Sphingomonadales	1.01	3.21	2.50	a	a	a	0.0278
Rhodobacterales	-0.54	-0.31	-0.27	a	a	a	0.1358
Flavobacteriales	-0.10	-0.27	1.87	a	a	a	0.1784

Propionibacteriales	1.02	0.55	-0.04	a	a	a	0.2775
Cytophagales	-0.26	-0.19	-0.06	a	a	a	0.3337
Blastocatellales	-0.34	-0.01	-0.25	a	a	a	0.3833
Caulobacterales	-0.34	-0.42	-0.30	a	a	a	0.3986
Xanthomonadales	-0.39	-0.42	-0.34	a	a	a	0.5

a) Log transformation of absolute abundance (counts) in soil samples (average values)
b) Letters indicate statistically different distribution groups.

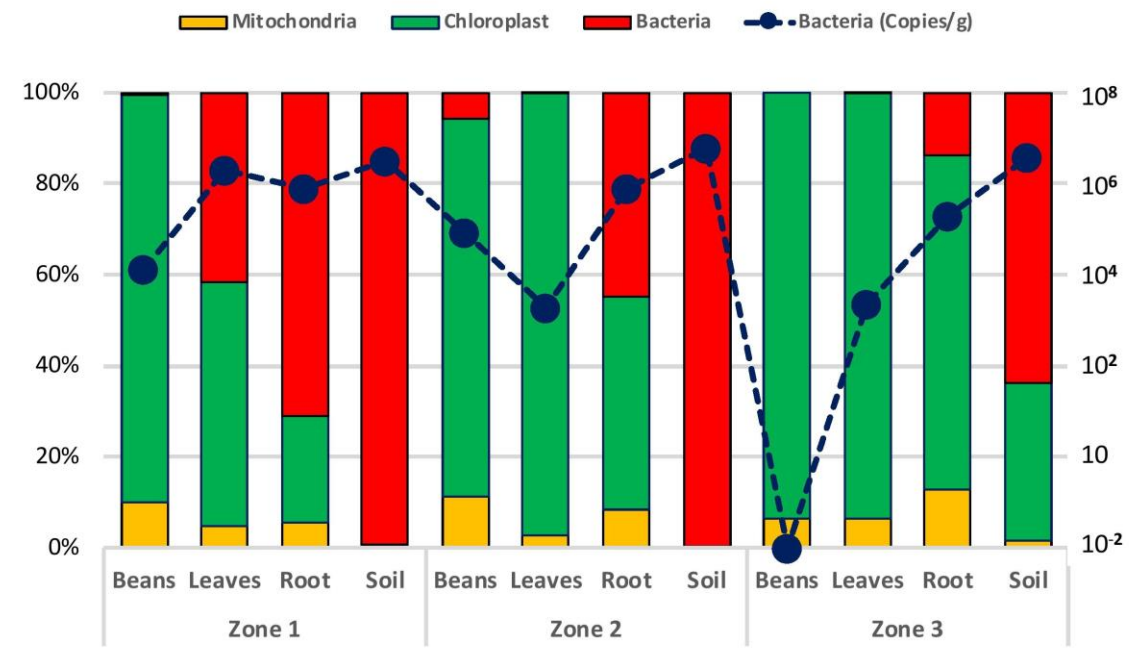
Supplementary Table S3.3. Detailed results of the Random Forest classifier model predictions based on the OTUs abundances.

Accuracy	0.8889
95% Confidence Interval	0.6529-0.9862
No information Rate	0.6667
***p*-value**	0.032
Kappa	0.727
Mcnemar's Test p-value	0.47950
Precision	1
Recall	0.666
F1	0.8
Prevalence	0.333
Detection Rate	0.222
Detection Prevalence	0.222
Balanced Accuracy	0.833

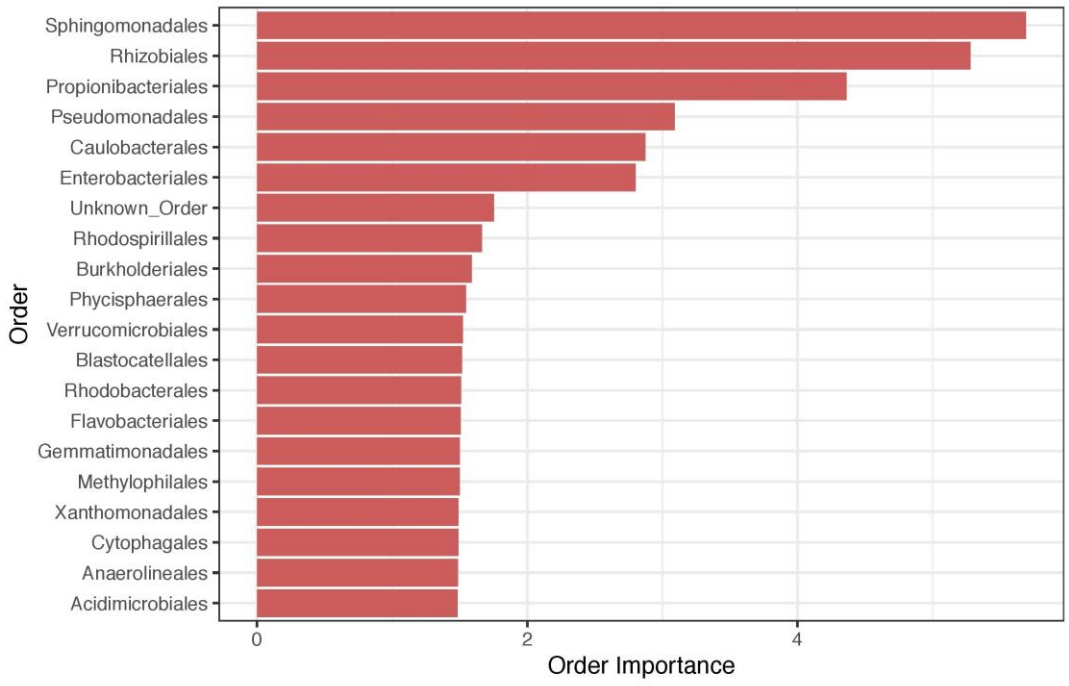
Supplementary Table S3.4. Pearson's correlations[a] between OTUs orders prevalence and abundance of different genetic elements (all samples, only orders with at least one significant correlations.

	bacterial 16S	*bla*$_{TEM}$	*intI*1	*sul*1	*bla*$_{CTM-M-32}$	*tet*M	*qnrS*1	*bla*$_{OXA-58}$
Acidimicrobiales	0.78 ***	0.79 ***	0.82 ***	0.69 **	0.64 *	0.36	0.23	0.23
Anaerolineales	0.78 ***	0.75 ***	0.88 ***	0.65 **	0.61 *	0.40	0.10	0.19
Blastocatellales	0.80 ***	0.79 ***	0.74 **	0.59 *	0.58 *	0.34	0.34	0.28
Cytophagales	0.72 **	0.77 ***	0.61 *	0.48	0.54 *	0.22	0.31	0.17
Flavobacteriales	0.59 *	0.62 *	0.60 *	0.39	0.44	0.19	0.00	-0.04
Gemmatimonadales	0.80 ***	0.74 **	0.88 ***	0.66 **	0.60 *	0.45	0.22	0.33
Methylophilales	0.39	0.36	0.58 *	0.30	0.29	0.22	-0.29	-0.13
Phycisphaerales	0.79 ***	0.73 **	0.90 ***	0.67 **	0.59 *	0.46	0.14	0.28
Propionibacteriales	0.70 **	0.57 *	0.80 ***	0.54 *	0.46	0.41	-0.02	0.20
Rhizobiales	-0.79 ***	-0.86 ***	-0.71 **	-0.56 *	-0.60 *	-0.28	-0.28	-0.20
Rhodobacterales	0.78 ***	0.81 ***	0.79 ***	0.56 *	0.60 *	0.37	0.24	0.26
Rhodospirillales	0.79 ***	0.76 ***	0.78 ***	0.62 *	0.56 *	0.40	0.29	0.31
Sphingomonadales	0.57 *	0.58 *	0.38	0.28	0.40	0.08	0.24	0.08
Unknown_Order	0.79 ***	0.73 **	0.90 ***	0.67 **	0.59 *	0.46	0.14	0.28
Verrucomicrobiales	0.46	0.55 *	0.50	0.26	0.33	0.32	0.06	0.18
Xanthomonadales	0.49	0.51	0.52 *	0.34	0.32	0.31	0.05	0.15

a) Pearson Correlations. *,$p \leq 0.05$; **, $p \leq 0.01$,***,$p \leq 0.001$; n=60



Supplementary Figure S3.1. Results from the sequencing data, separated by sampling zone and sample type. The vertical bars correspond to the relative proportion of mitochondrial (yellow), chloroplast (green) and bacterial (red) sequences for each group of samples (left axis); dots indicate the average amount of bacterial 16S, in copies per g of sample corresponding to each group and calculated by qPCR combined with 16S amplicon sequencing (right axis, note the log scale). Note the lack of bacterial sequences in bean samples from Z3.



Supplementary Figure S3.2. Importance of the top 20 orders for sample discrimination as calculated by the random forest analysis.

Chapter IV: Antibiotic Resistance Gene distribution in agricultural fields and crops. A soil-to-food analysis

This chapter is based on the paper:

Francisco Cerqueira, Víctor Matamoros, Josep Bayona, Thomas U. Berendonk, Goffe Elsinga, Luc M. Hornstra, Benjamin Piña. (2019). Antibiotic Resistant Gene distribution in agricultural fields and crops. A soil-to-food analysis. Environmental Research, 177, 108608.

Despite the social concern about the generalization of antibiotic resistance hotspots worldwide, very little is known about the contribution of different potential sources to the global risk. Here we present a quantitative analysis of the distribution of ARGs in soil, rhizospheric soil, roots, leaves and beans in tomato, lettuce and broad beans crops (165 samples in total), grown in nine commercial plots distributed in four geographical zones in the vicinity of Barcelona (North East Spain). We also analyzed five soil samples from a nearby forest, with no record of agricultural activities. DNA samples were analyzed for their content in the ARGs *sul*1, *tet*M, *qnrS*1, $bla_{\text{CTX-M-32}}$, $bla_{\text{OXA-58}}$, *mec*A, and bla_{TEM}, plus the integron *intI*1, using qPCR methods. In addition, soil microbiomes from the different plots were analyzed by amplicon-targeted 16S rRNA gene sequencing. Our data show a decreasing gradient of ARG loads from soil to fruits and beans, the latter showing only from 0.1 to 0.01% of the abundance values in soil. The type of crop was the main determinant for both ARG distribution and microbiome composition among the different plots, with minor contributions of geographic location and irrigation water source. We propose that soil amendment and/or fertilization, more than irrigation water, are the main drivers of ARG loads on the edible parts of the crop, and that they should therefore be specifically controlled.

4.1 Introduction

Environmental contamination with ABs, ARBs, and ARGs is becoming a global health concern (Berendonk et al., 2015), largely due to human activities (Chen et al., 2016; Han et al., 2016; Y. Hu et al., 2016; Koch et al., 2017). In agricultural fields the impact may come from the wide utilization of chemical fertilizers, or animal manure on soils (Liu et al., 2017), as well as from water used for irrigation (Han et al., 2016). According to the last UN Global Environmental Outlook, "Human illnesses and deaths due to antibiotic- and antimicrobial-resistant infections are increasing rapidly and are projected to become a main cause of death worldwide by 2050" (UNO, 2019). The same report classified ARB and ARGs as main pollutants for water sources, and call for the application of effective policies for its control in water bodies worldwide (UNO, 2019). For this reason, it is of maximal importance to evaluate the loads on ARB and ARGs in water supplies and to derive workable risk analyses from their measured prevalence and capacity to incorporate into the human food chain.

Soil has been regarded as a rich source of ABs and ARGs, deriving from both natural and anthropogenic processes. Anthropogenic pressure may come from ABs used in healthcare or livestock that are discharged into the aquatic environment via WWTPs. ABs, like other organic pollutants, are only partially removed by current WWTP technologies (Hartmann et al., 2015; Le-Minh et al., 2010; Michael et al., 2013). In addition, WWTPs have been pointed out as potential hotspots for ARBs and ARGs selection and for their release into the environment (Manaia et al., 2018; Pärnänen et al., 2019).

Soil amendment with animal manure is another potential source of ABs, ARBs, and ARGs in soil, as ABs are overused to treat animal diseases and promote growth (Martin et al., 2015). Applications of manure have been shown to increase the abundance of ARBs and ARGs in soil (Chen et al., 2016; McKinney et al., 2018; Udikovic-Kolic et al., 2014; Xiong et al., 2015). In addition, most ABs are excreted by treated animals in active or activable forms (Heuer et al., 2011; Ji et al., 2012; Negreanu et al., 2012), making the manure a major source of ABs that can provide a selective advantage for resistant bacteria. Moreover, vegetables can uptake pollutants (including ABs), the concentration of which may be enhanced by the addition of manure or other biosolids (Christou et al., 2019; Kumar et al., 2005; Sabourin et al., 2012). As it happens for all CECs, the capability AB for uptake depends on several biotic and abiotic factors, and varies among the

different types of crop. Fruit vegetables and cereal crops showed a lower ability for uptake of CECs, compared to leafy and root vegetables. Therefore, consumption of vegetables represents a way of exposure of bacteria carrying ARGs and pathogens (Berger et al., 2010; van Hoek et al., 2015).

The presence of ABs in the soil may exert selective pressure over soil microbiomes for long periods, eventually altering their composition (Heuer et al., 2011; Kümmerer, 2009; Pan and Chu, 2016; Segura et al., 2009; Xiong et al., 2015). The selective pressure on ARB in the soil occurs at sub-inhibitory concentrations of ABs (Andersson and Hughes, 2011), and it promotes the transfer of genes among bacteria, the so-called HGT (Berendonk et al., 2015). Conversely, reversion of AB resistance may occur slowly or not happen at all, due to compensatory mutations, low (or no) fitness cost of AB resistance mutations, and/or co-selection between resistance markers, among other factors (Andersson and Hughes, 2011).

The capacity of alteration of soil microbial communities and ARG loads is not exclusive of organic fertilizers. Nitrogen or phosphorous chemical fertilizers may alter soil microbial communities by changing soil properties, among other mechanisms (Fierer et al., 2012; Li et al., 2014; Liu et al., 2017; Zhou et al., 2015). The observed effects of this type of fertilization in the soil resistome might be indirect, as ARG content is dependent on the microbiome composition (Forsberg et al., 2014). For example, Proteobacteria, Actinobacteria, and Bacteroidetes, which are known to increase their prevalence in inorganically fertilized soils (Fierer et al., 2012; Zhou et al., 2015), show high HGT rates and therefore they may contribute to the spread of ARGs in the environment (Gibson et al., 2015; Hu et al., 2016). In addition, Proteobacteria usually carry broad-host range plasmids that promote HGT in agricultural fields (Heuer et al., 2011), whereas Actinobacteria appears to be one of the major sources of soil-borne ARGs (D'Costa et al., 2011; Jiang et al., 2017).

In this and previous works, we found evidence than ARGs can reach edible parts of commercial crops, and that their presence and concentrations depends upon farming practices (Cerqueira et al., 2019a, 2019b; Manaia et al., 2018). In this work, we quantified their loads in the different compartments (soil, rhizospheric soil, roots, leaves, and fruits) of the soil-plant continuum for three different crops in different plots and geographic areas in the surroundings of Barcelona. These plots differ on their irrigation water sources, from essentially clean wells to reclaimed water, as well as on agricultural practices, from relatively artisanal to semi-industrial ones. We also analyzed soil samples from a nearby

forest, with no record of agricultural activities. We investigated the influence of all these different factors for the spread of seven ARGs of clinical relevance (*sul*1, bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$, *mec*A, *qnrS*1, *tet*M, $bla_{OXA\text{-}58}$), and of the integrase gene *intI*1, considered as a proxy for anthropogenic impact (Gillings et al., 2015), in soils and in plants, including their edible parts. We also checked the composition of the soil microbiomes, to analyze its correlation with ARG composition. The final objective is to evaluate the impact of the different agricultural practices for the risk associated to the presence of ARG in foodstuffs, identifying the leading factors determining their loads in the different crops.

4.2. Materials and methods

4.2.1. Site description and sample collection

Lettuces (*Lactuca sativa* L. cv. Batavia), tomatoes (*Lycopersicon esculentum* Mill) and broad beans (*Vicia faba* L.) crops were collected in fields in the delta of the Llobregat River (Catalonia, Spain, Fig. 1). Plots in Z1 (lettuces and tomatoes) were located in the littoral mountains west of Llobregat Delta, irrigated with groundwater and/or rainwater, and fertilized with horse manure. Zones Z2 (lettuces and broad beans), Z3 (all three crops), and Z4 (broad beans) were located in the Llobregat low valley, and they were irrigated with water from irrigation channels, and chemically fertilized when needed. Previous studies reported that these channels showed significant contributions of Barcelona metropolitan area. This plot was irrigated with groundwater, and fertilized with local pigeon manure. Forest samples were collected in an area close to Z1, away from most anthropogenic impacts, and with no records of agricultural activities (See map in Fig.1). General quality parameters of the irrigation waters were measured (conductivity, NH_4^+–N, nitrates, total phosphorus, total suspended solids, and pH), with the exception of Z5 (see supplementary table ST1). NH_4^+–N, NO_3-N were measured with Hach Lange, and Total Phosphorus cell tests (LCK 03, 304, 339, and 349) on a spectrophotometer (Hach Lange DR 1900 Portable Spectrophotometer). Measurements of water pH and conductivity were taken using Hach Lange sensors (Margenat et al., 2017). Lettuces were planted in Z1, Z2, Z3, and Z4, while broad beans were planted in three zones (Z2, Z3, and Z5), and tomatoes were planted in two zones (Z1, and Z3). The vegetables were harvested when they reached their commercial size, in a systematic random sampling scheme, with the fields being divided in equal parts, according the number of samples collected in each field (Margenat et al., 2019). Lettuces were collected in May-June of 2016 (3 plants, 12 samples per field, 36 samples in total), tomato sampling campaign took place in September 2016 (4 plants, 20 samples per field, 40 samples in total), and

broad beans plants were collected in March 2017 (5 plants, 25 samples per field, 75 samples in total). Roots, leaves from all crops, tomato's fruits, and beans were separated in the field and placed into sterile plastic containers. Bulk soil samples were collected near each plant, at a depth of 10 cm. Within few hours, samples were transported on ice to the laboratory, where they were processed and total DNA was extracted.

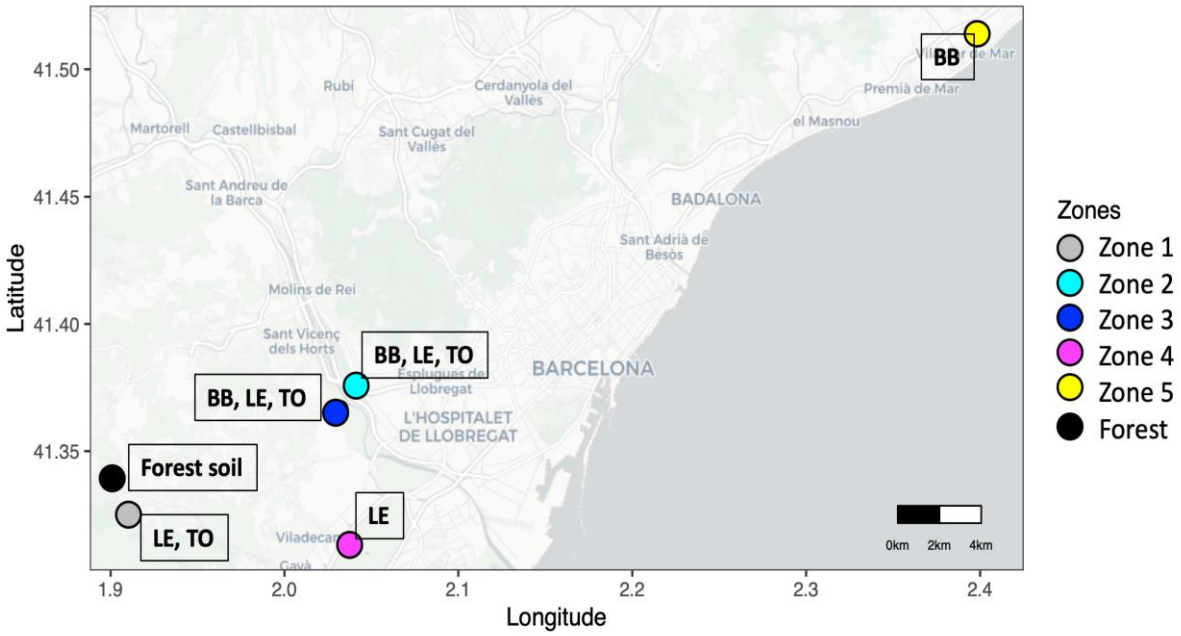


Figure 4.1. Map of the sampling fields. Upper case letters indicate the type of crops sampled in each zone: BB, broad beans, LE, Lettuce, TO, tomato.

4.2.2. DNA extraction and analyses

DNA extraction of tomato fruits and broad beans samples were performed as described previously (Cerqueira et al., 2019a; Cerqueira et al., 2019b), with slight modifications (Bodenhausen et al., 2013; Wang et al., 2017). Rhizospheric soil from all roots' crops was extracted as described (Bodenhausen et al., 2013; Wang et al., 2017), with slight modifications. Roots were vortexed for 5s for pre-washing with 20 mL of sterilized phosphate buffered saline (PBS, 0.138 M NaCl, 0.0027 M KCl, pH 7.4), transferred to a new 50 mL tube with 20 mL of sterilized PBS, and vortexed for 1 min, to extract the rhizospheric soil. The samples were centrifuged at 4500 rpm for 15 min, and the pellets proceeded for DNA extraction. DNA extraction of soil, roots and lettuce leaves was performed as described (Cerqueira et al., 2019a; Cerqueira et al., 2019b), with slight modifications. After removing the rhizospheric soil, leaves were washed in sterilized distilled water. To check the efficacy of the roots sterilization, the water utilized for the

last rinse was plated in LB agar media for three days in an incubator at 28 °C to evidence lack of contaminating bacteria. Pre-processed roots and lettuce leaves were macerated with mortar and pestle, upon addition of 20 mL of PBS buffer. Macerated samples were then transferred to 50 mL tubes through a 100 µm mesh nylon Cell strainer (Corning ® Cell Strainer). Both macerated leaves and roots were centrifuged at 4500 rpm for 15 min, and the pellets proceeded for DNA extraction. DNA from soil samples (250 mg) and from the pellets of the different plant parts was extracted using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc.), to a final elution volume of 100 µL. The concentration and the quality of the DNA were tested using a NanoDrop Spectrophotometer 8000 (Thermofisher Scientific, Inc). Extracted DNA samples were stored at -20 °C.

4.2.3. Quantification of ARGs and *intI*1 sequences by qPCR

The ARGs and *intI*1 from lettuce samples were quantified by qPCR as previously described (Cerqueira et al., 2019a).The ARGs quantified by qPCR were normalized by gram of sample.

4.2.4. 16S rDNA library preparation and sequencing

The protocols used for 16S rDNA library preparation and sequencing of the tomatoes and broad beans campaigns were described previously (Cerqueira et al., 2019a, 2019b). The pooled library of 16S rDNA amplicons from the soil and lettuce samples was prepared as described (Cerqueira et al., 2019b). Raw sequences from lettuces field campaign were deposited at the NCBI BioProject archive (https://www.ncbi.nlm.nih.gov/bioproject/) under the accession number PRJNA516199.

4.2.5. Statistical analyses

Statistical tests were performed in the R environment (version 3.4.2; http://www.r-project.org/). PCAs were performed using the principal function from the psych R package (https://cran.rproject.org/web/packages/psych/index.html), were one-way ANOVA and performed using the function heatmap2, from the gplots R package (https://CRAN.R-project.org/package=gplots). Soil microbiomes' β-diversity analysis was performed with R environment (version 3.4.2), with the packages phyloseq (McMurdie and Holmes, 2013) and vegan (https://cran.rproject.org/package=vegan).

The OTUs were pre-processed, by removing chloroplastidial and mitochondrial reads. OTU counts were $\log_{10}$ transformed before a NMDS with the Bray–Curtis dissimilarity index (Bray and Curtis, 1957). Effects of crop type and sampling field in soil microbiomes composition were analyzed using the *adonis* test from the vegan R package. Correlations between bacteria composition and AB resistance were analyzed by Spearman's correlations between OTUs counts grouped at the order level and genetic elements (ARGs and *intI*1) in gene copies g^{-1}. The false positive discovery rate (FDR) correction was set at $p<0.05$ (Benjamini and Hochberg, 1995), using the psych R package.

4.3. Results

4.3.1. ARG abundance and distribution

ARG sequences were detected in all samples. The most prevalent gene was *bla*$_{TEM}$, which was detected in all analyzed samples, whereas *sul*1 and *intI*1 were detected in 85% and 78% of cases, respectively. On the contrary, *bla*$_{OXA-58}$ and *mec*A were found only sporadically in 18% and 8% of the samples, respectively. The different compartments showed similar prevalence for all genes, except for fruits and beans (Table 4.1).

Table 4.1. Prevalence of different genetic elements along the soil-plant continuum

	intI1	*bla*$_{TEM}$	*sul1*	*qnrS1*	*bla*$_{CTX-M-32}$	*tetM*	*bla*$_{OXA-58}$	*mecA*
All	78%	100%	85%	61%	58%	34%	18%	8%
Soil	98%	100%	100%	58%	73%	60%	20%	3%
Rizhospheric Soil	94%	100%	83%	66%	49%	26%	17%	11%
Roots	77%	100%	83%	54%	57%	26%	14%	9%
Leaves	66%	100%	77%	63%	51%	17%	14%	6%
Fruits (Tomato)	100%	100%	88%	38%	63%	75%	63%	38%
Beans (Broad beans)	7%	100%	80%	67%	67%	7%	7%	0%

Note that these two categories refer to a single species (tomatoes and broad beans, respectively), whereas the figures for rest of compartments include data from all crops. Despite the similar prevalence of the analyzed genes in the different compartments, their

relative concentrations differed in several orders of magnitude. The highest ARG and *intI*1 loads generally corresponded to soil samples, whereas the lowest values were found in leaves, beans and fruits (Fig. 4.2). This trend was more evident for the more prevalent genes, whereas low-prevalence ARGs showed more stochastic distributions (Fig. 4.2).

The distribution of ARG and *intI*1 gene across plots and plant parts is displayed in the heatmap in Figure 4.3. Hierarchical clustering of samples defined three main clusters of samples. Cluster A includes samples with the lowest loads in ARGs or *intI*1, represented by orange and red sectors in the heatmap. Grey cells correspond to values below LOD (no PCR product obtained). This cluster includes all samples from the broad beans plant (beans, leaves, and roots), and leaves and fruits from tomato samples (see the upper and middle column bars at the bottom of the heatmap). In contrast, cluster B, which corresponds to samples with the highest ARG and *intI*1 loads (yellow sectors in the heatmap), includes most soil and rhizospheric soil samples from all three crops. Finally, cluster C, showing intermediate ARG and *intI*1 loads, includes essentially all samples from lettuce plots, except soils, and the remaining tomato and broad beans samples, mostly from roots, soil and rhizospheric soil (see column bar colors at the bottom of the heatmap, upper and middle bars.

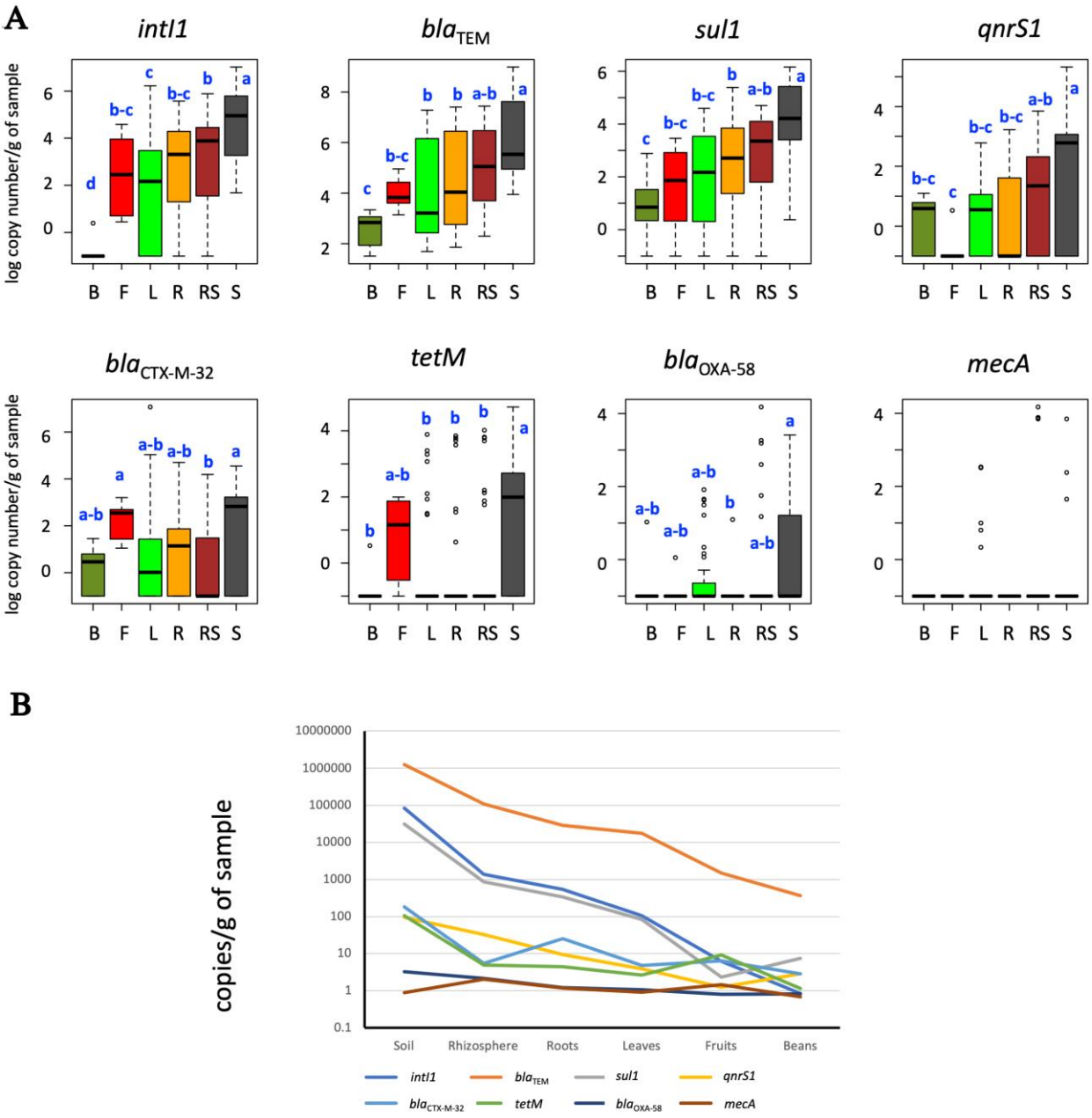


Figure 4.2. Distribution of the different genetic elements along the soil-plant continuum. **(A)** Relative abundances in beans (B), fruits (tomatoes, F), leaves (L), roots (R), rhizospheric soil (RS), and soil (S) samples. Data are expressed as copies of each sequence per g of tissue (log_{10} values), note that the two last categories refer to a single species (tomatoes and broad beans, respectively), whereas the figures for rest of compartments include data from all crops. Boxes include values from the 1st to the 3rd quartiles, the thick horizontal black line indicates the median, and whiskers refer to 95% total distribution, except outliers (empty dots). Lowercase letters a to c indicate statistically different groups of data (p<0.05, Tukey HSD test). **(B)** Relative abundances abundance of ARGs and *intI*1 along the soil plant continuum.

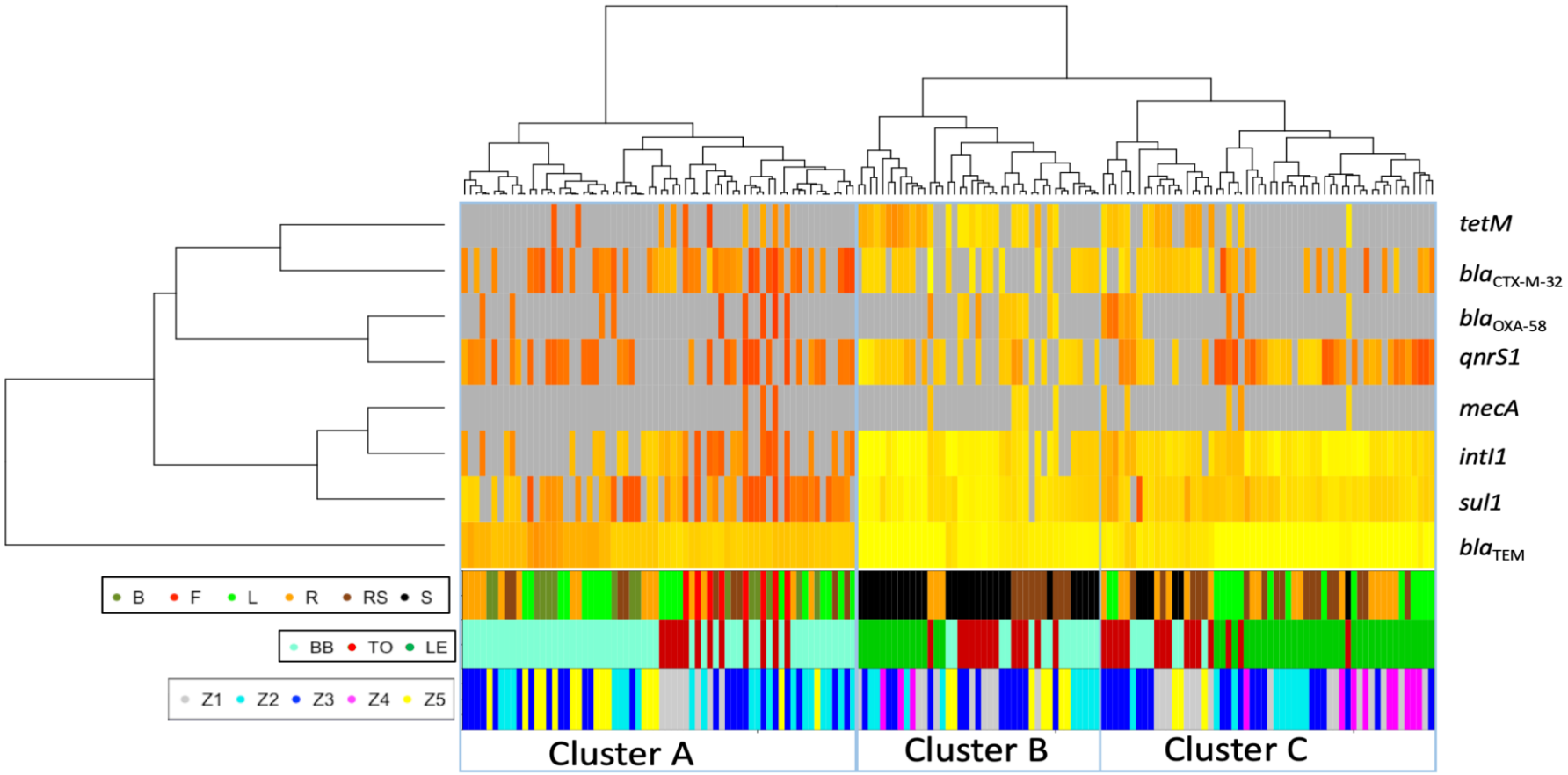


Figure 4.3. Distribution of the different genetic elements in all samples. The heatmap represents the abundances of different genetic elements. Values are color coded from red (least) to yellow/white (most), grey sectors correspond to values under the limit of detection. The three column bars at the bottom indicate sample characteristics, classified by type of sample (top bar) type of crop (central bar), or geographical zone (bottom bar). The three main cluster of samples, as identified by hierarchical clustering, are indicated at the bottom of the figure. A letter-coded version of the same figure is included as supplementary Figure SF1

4.3.2. Analysis of geographical and plot distribution of ARG loads

Soil ARG abundance values showed variations of several orders of magnitude (Fig. 4.4 A). The three most prevalent sequences (*intI*1, bla_{TEM} , and *sul*1, see Table 4.1) were found at their highest values in all four lettuce plots, whereas their values were significantly lower in broad beans' plots (Fig. 4.4 A, statistical analysis in Supplementary Table S4.3). The pattern was much more complex for the less-prevalent genes. Values for *qnrS*1 abundance showed a similar trend as bla_{TEM}, whereas *mec*A and bla_{OXA-58} appeared only sporadically, limited to one and three plots, respectively (Fig. 4.4 A). With almost no exception, the forest soil sample showed the lowest levels for all ARG and *intI*1, including the absence of the three minor ARGs, *tet*M, bla_{OXA-58}, and *mec*A (Fig. 4.4). The correlations between the different soil samples can be observed in the PCA in Figure 4.4 B. The dual loadings'/scores' plot shows the clustering of samples by the type of culture, more than by geographic zone, as well as the very similar distribution of *intI*1 and *sul*1 on one side, and bla_{OXA-58} and *mec*A on the other (Fig. 4.4 B). It also shows the similarity of soil samples from broad beans' plots with the forest soil, and the tight clustering of all samples from lettuce plots, irrespectively their geographic location. The clustering of samples by the type of crop was also observed when ARG and *intI*1 abundance in plant parts (including rhizospheric soil) were analyzed by PCA (Fig. 4.4 C). In this case, the different score values reflected the gradient of ARG and *intI*1 loads from rhizospheric soil to fruit and beans (see the color codes in the figure), but the samples clustered in a very similar way as the soil samples. In both cases, PC (principal component) 1 reflected the loads of the most prevalent ARG and *intI*1, whereas PC2 separated samples with different loads of the minor ARGs (note the opposite sense of PC2 in the two plots in Figures 4.4 B and C). *tet*M abundances in rhizospheric soils, roots and leaves can be explained almost solely (more than 50% explained variance) by the corresponding abundances in the soils of the respective plots (Supplementary Figure S4.1, Table 4.2). The correlations were weaker, although still significant, for *sul*1, bla_{OXA-58}, and *mec*A, and essentially non-significant for *qnrS*1 and $bla_{CTX-M-32}$ (Supplementary Figure S4.1). The corresponding graphs are shown in Figure 5 B. Note that the correlations combine values from all crops for all plots and zones.

Table 4.2. Pearson's correlations between ARG loads in soil and in different plant parts

	intl1		bla_{TEM}		*sul1*		*qnrS1*		$bla_{CTX\text{-}M\text{-}32}$	*tetM*		$bla_{OXA\text{-}58}$		*mecA*	
Beans (Broad beans)	-0.26		0.51		-0.08		0.21		-0.06	-0.16		-0.16		N/A	
Fruit (Tomato)	0.05		0.24		0.32		-0.22		0.4	0.92	**	0.48		0.13	
Leaves	0.77	***	0.85	***	0.5	**	0.3		0.22	0.65	***	0.54	**	-0.04	
Roots	0.8	***	0.88	***	0.49	**	0.39	*	-0.3	0.71	***	0.73	***	0.56	**
Rhizospheric Soil	0.56	**	0.76	***	0.63	***	0.23		0.25	0.73	***	0.6	**	0.47	**

Pearson's *p* values: *, p<0.05; **, p<0.01; ***, p<0.001

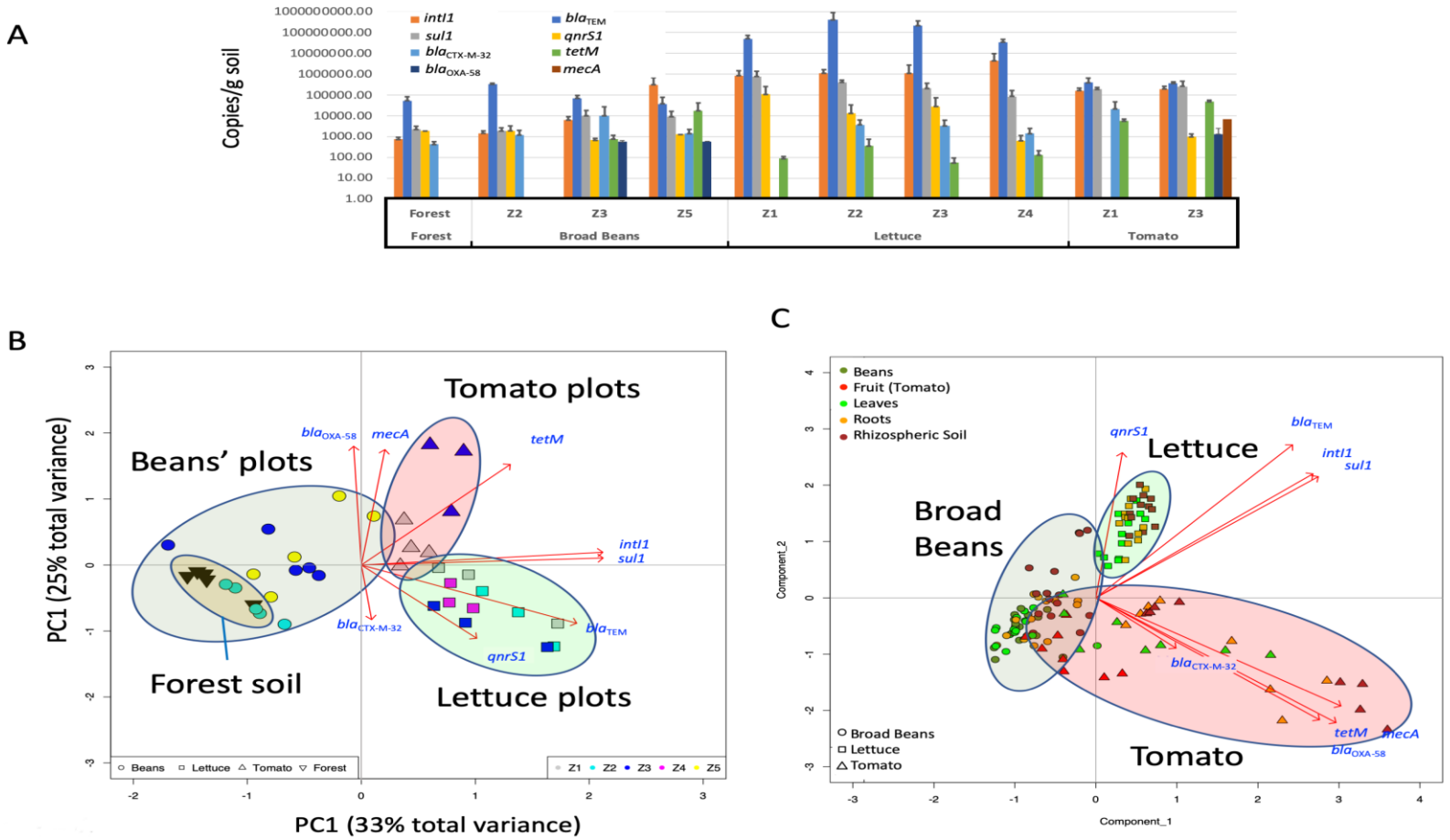


Figure 4.4. Analysis of geographical and plot distribution of ARG and intI1 loads. (A) Distribution of the different genetic elements in soil samples, color-coded as indicated in the inset. Values indicate the average values of all soil samples for each plot, distributed by geographical zones and represented as copies per g of soil. Whiskers represent standard deviations. (B) PCA of abundance values for all genetic elements in soil samples. The plot combines a score plot for each sample (symbols) and a loadings' plot for the different genetic elements, indicated by red arrows and gene names. Loading values were scaled to match the limits of the score plot). Symbol shapes indicate the type of crop harvested in each plot (beans, lettuce, or tomatoes) or uncultured, forest samples, represented as circles, squares, triangles and inverted triangles, respectively. Symbol colors indicate geographical zones, color-coded as in Figure (C) PCA analysis of abundance values for all genetic elements in plant samples, including rhizospheric soil. As in panel B, the graph combines a score plot (symbols) and a loadings' plot (arrows and gene names). Symbol shapes indicate the type of crop, as in panel B, whereas symbol colors indicate the different plant parts, as in Figure 2. Color-shadowed areas group samples according the type of crop, as in panel B.

4.3.3. Soil microbiome β-diversity and ARG distribution

Statistics of the three sequencing runs included in this paper (35 samples in total) are shown in the Supplementary Table S4.2. PERMANOVA analysis showed that the distribution of OTUs is soil microbiomes were strongly influenced by the type of crop, whereas the effect of the geographical origin was not significant (Table 4.3). This result can be visualized in the NMDS ordination plot in Figure 5, based on the Bray-Curtis dissimilarity, which groups by crop (shadowed polygons in Fig. 4.5) and not by geographic origin. Therefore, the analysis of soil microbiome composition indicates its strong dependence on the type of vegetable and a weak or nil influence from the geographical region. The correlation between microbiome composition and ARGs abundance in soil samples was tested by correlation analyses (Fig. 4.6). There were strong correlations between the abundance of the most prevalent genetic elements in soil samples (*intI*1, bla_{TEM}, *sul*1, and *qnrS*1) and the relative proportion of specific phylogenetic groups (Figure 6, note that microbiome data is grouped at the taxonomic order levels, and only order with a total number of counts above the global mean were considered). It is particularly relevant the apparent link between *intI*1 and *sul*1 abundance and the prevalence of Pseudomonadales and Enterobacteriales in the soil microbiome (Fig. 4.6). It is also interesting the correlation between the abundance of *tet*M and the prevalence of the relatively rare group of Burkholderiales (Fig. 4.6, the barplot on the left indicates the total numbers of reads for each taxonomic order). These three orders include known human and animal pathogens, like *Pseudomonas* spp., *Salmonella* spp, *Shigella* spp. or *Burkholdelia cepacia* (Mahenthiralingam and Vandamme, 2005). Some relevant correlations between ARG abundances and taxon prevalence are shown in Supplementary Figure S4.2.

Table 4.3. *adonis* significance test for vegetable- and zone-group differences[a]

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)
Vegetable	2	1.8784	0.93919	6.804	0.30222	0.001
Zones	4	0.472	0.11799	0.8548	0.07594	0.639
Residuals	28	3.865	0.13804		0.62185	
Total	34	6.2153			1	

a) adonis (formula = A3 ~ vegetable + zones, method = "bray")

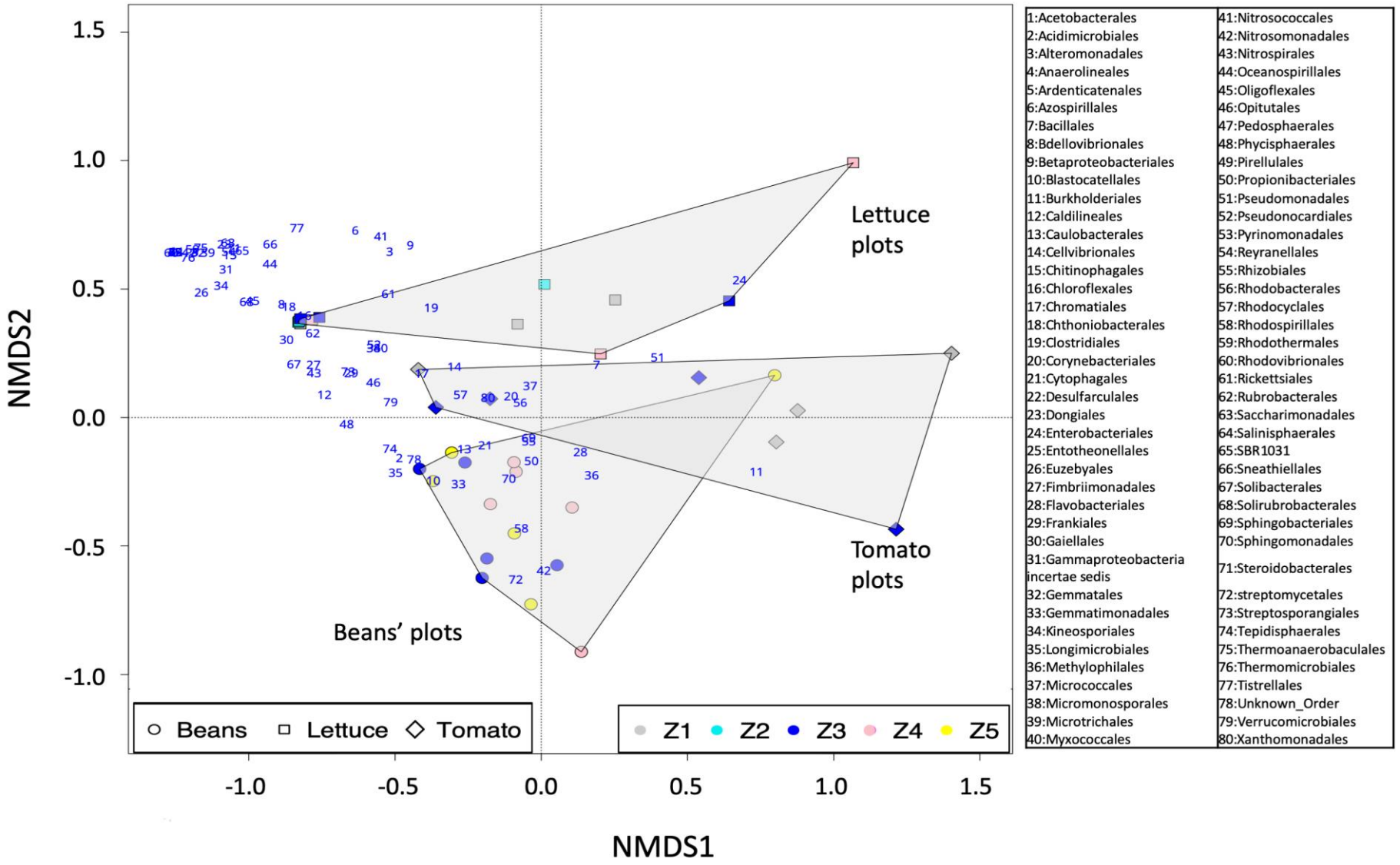


Figure 4.5. NMDS plot for β-diversity analysis, based on the Bray-Curtis dissimilarity. The data correspond to OTU abundances, log transformed. Color labels represent the sampling zones and the shapes represent the type of crop. Numbers in blue represent OTUs grouped at the order level. Order names are substituted by numbers as indicated in the legend on the right. Polygons delimitate the area containing all samples from each type of crops

4.4. Discussion

Our data shows a consistent trend on the distribution of the most abundant ARGs throughout the soil-plant continuum. ARG loads decreased steadily from the soil to the fruit, resulting in a 100- to 1000-fold dilution for most studied ARGs. The data also show that the concentration and composition of plant edible parts was strongly dependent on the soil ARGs. In our samples, the type of crop appeared as the most important factor to predict the ARG loads in crops' edible parts, superseding the geographical distribution or the quality of the water used for irrigation. A similar conclusion was reached in a parallel study analyzing pollutant content in a very similar set of samples of lettuce, tomato, cauliflower, and beans from the same geographical areas studied here (Margenat et al., 2019, 2017). We found similar loads of *sul*1, bla_{TEM}, and *intI*1 copies in equivalent compartments for samples from all plots, including the forest soil. These sequences may be therefore regarded as belonging to the core resistome, present in essentially all analyzed soils (and therefore, all plants) of our experimental zone, including forest soils that have not been cultivated in at least a century. In contrast, less-abundant ARGs, like *tet*M, *mec*A, and bla_{OXA-58} were only detected in particular plots. We consider the presence of these less-frequent ARGs as indicators of anthropogenic water/soil pollution, and, therefore, they should be monitored in future quality standards regulations for reclaimed water to be used for agricultural purposes.

We are unsure about which parameters determine differences in ARG and *intI*1 loads between plots in very close geographical proximity and, likely, using the same irrigation water. Our previous results showed different microbiome compositions influenced the profile and abundance of the ARGs present, and, therefore, they shape the soil resistome (Cerqueira et al., 2019a, 2019b; Forsberg et al., 2014). Geography and spatial distance have been found to be less important shaping soil microbiomes than other local conditions, like pH, moisture, organic carbon, nitrogen availability, and even the type of crop (Burns et al., 2015; Fierer, 2017). These parameters can be modulated by agricultural practices (Chen et al., 2016; Fierer et al., 2012; Forsberg et al., 2014). For example, studies on long-term applications of chemical fertilizers showed relevant an increase in the relative abundance of Proteobacteria and Actinobacteria in the soil microbiome (Zhou et al., 2015). These two Phyla have been linked to high HGT activity and ARG presence in soils, respectively (Gibson et al., 2015; Jiang et al., 2017). In comparison, organic fertilization seems to have a stronger impact in soil ARGs loads, as they may increase the load of ARGs in soil and vegetables (Rahube et al., 2016) or cause the appearance of unique ARGs that are not present in vegetables grown in non-

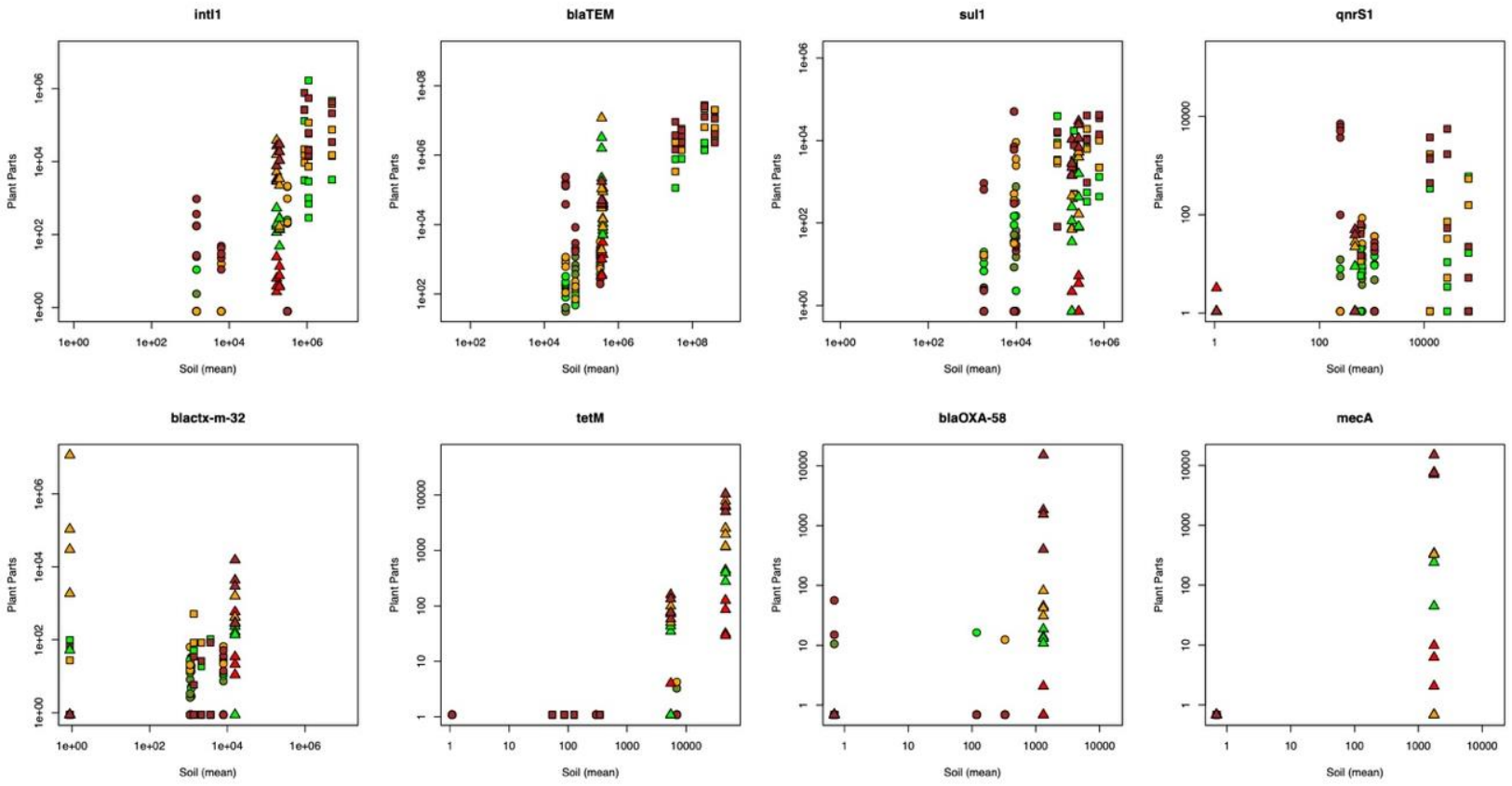
amended soil (Marti et al., 2013). Use fertilizers of fecal origin may represent an additional risk, as they are particularly enriched in ARBs and pathogen associated bacteria, like *Clostridium* spp., among others (Scott et al., 2018). This increase on ARGs and other relevant sequences in manure- or sewage-amended soil is apparently permanent, as it remains significant.

Compared to organic and fecal-related amendments, the impact in soil and plant microbiomes of the use of TWW appears to be milder or negligible (Christou et al., 2017a; Gatica and Cytryn, 2013), even after long, multi-decade periods of wastewater irrigation (Jechalke et al., 2014). Our results agree with these studies, which also show a weak or no influence of the quality of the water used for irrigation, from clean groundwater wells to heavily TWW-impacted channels, in the abundance of ARGs in plants and soils. Unfortunately, our experimental setup, which uses real commercial plots and crops intended for public consumption, does not allow a precise tracking of the practices (amendment, fertilization, irrigation, treatments) for each plot. However, it is likely that similar crops are treated in similar ways, at least between relatively close areas with similar weather and agricultural traditions. In any case, it is clear that the nature of the edible plant part does have an influence of the final loads on ARG and *intI*1 that eventually reach the consumer. Lettuces tend to show higher ARG loads than tomatoes or beans, if only because the edible part is a leaf, rather than a fruit or a bean. On the other side, beans typically show low ARG and *intI*1 loads, as the colonization of the plant and of the surrounding soil by Rhizobiales appeared to limit ARG loadings in the plant (Cerqueira et al., 2019a). Therefore, regulations for limiting the presence of ARGs in irrigation waters (and likely, amendments and fertilizers) should vary according the characteristics of each crop. A similar conclusion was reached when considering pollution of reuse waters for irrigation, although in this case the capacity of evapo-transpiration appeared to be one of the most critical parameters (Christou et al., 2019; Piña et al., 2018).

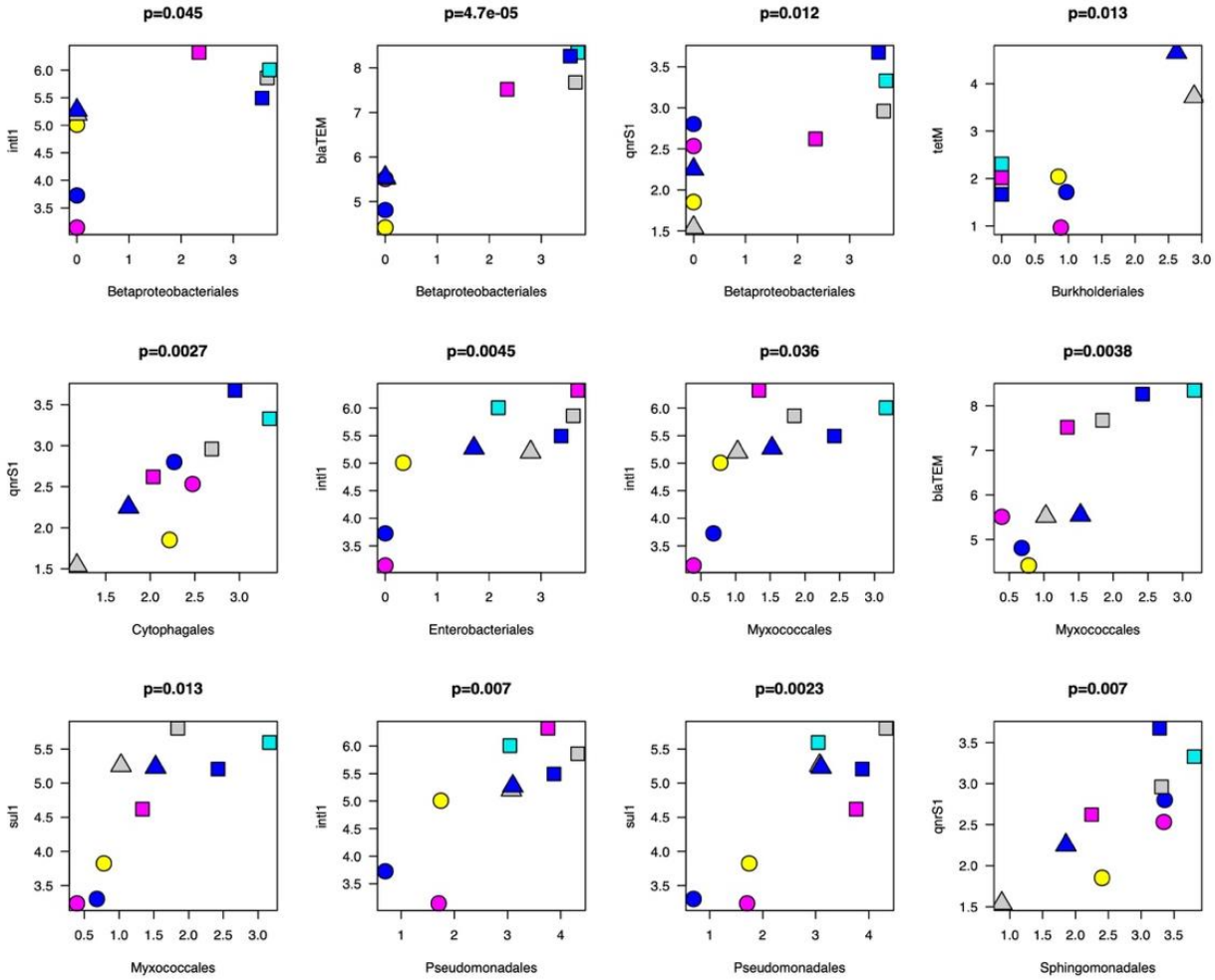
Taken together, our results underscore the need for regulating the presence of ARGs in agricultural soils, as they appear as the major drivers of the load of ARGs that ultimately will reach the human food chain. The fact that we found a similar correlation between soil and plant ARGs in different crops intended for human consumption suggest that this may be a general feature, not only limited to our particular case. Another aspect arguing for this control is the presence of core resistomes, AB resistance elements that have been incorporated into the soil (and, consequently, into the plant) microbiome and that, therefore cannot be easily counter selected or eliminated from the food chain. Therefore,

all regulatory efforts should be focused in avoiding new resistance elements to become prevalent (i.e., part of the core microbiome) and therefore essentially unavoidable in the human and animal food chain. Even from our limited ARG subset, we found at least three of them related to resistance to tetracyclines, methicillin, or carbapenems. Finally, our results suggest that the quality of water used for irrigation, at least within the standards set in our area of study, has a limited influence in the total soil (and plant) resistome. Instead, we propose the use of biosolids for soil amendment and/or fertilization as likely the major determinant of the presence of ARGs and ARB in crops, a hypothesis that is supported by the existing bibliography (Marti et al., 2013; Rahube et al., 2016) and that should be taken into account in further regulations on the use of these agricultural practice

4.5. Supporting information



Supplementary Figure S4.1. Correlations between ARG abundance in the different plant parts (Y-axis) and in the soil samples from the same plots (X-axis). Values are represented as copies per gram of sample. Soil abundances represent averages from four (Tomatoes and beans plots) or five (lettuces plots) samples, taken in the vicinity of the sampled plants. Circles, squares and triangles represent data from beans, lettuces and tomatoes, respectively; brown, orange, green, dark green and red symbols correspond to rhizospheric soil, roots, leaves, beans and tomatoes, respectively.



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Supplementary Figure S4.2. Correlations between the prevalence of different bacterial groups (at the taxonomic order level, X-axis, log values of total counts) and the abundance of different ARGs or *intI*1 (Y-axis, copies per gram of sample) in soil samples. Data are represented as averaged values form all samples from each plot, log-transformed. Circles, squares and triangles represent data from beans, lettuces and tomatoes, respectively. Grey, cyan, blue, magenta and yellow symbols correspond to samples from Z1, Z2, Z3, Z4, and Z5, respectively. Pearson's correlation *p*-values are represented at the top of each graph.

Supplementary Table S4.1. Minimum, maximum and average levels of quality parameters measured in the waters used for sampling fields' irrigation (Margenat et al., 2017).

	Z1	Z2	Z3	Z4
Conductivity (µS cm^{-1})	(968–1211) 1049	(1490–2148) 1944	(1255–1707) 1482	(1272–2370) 1663
NH_4^+–N(mg L^{-1})	(0.002–0.167) 0.05	(3–47) 14	(0.1–0.6) 0.2	(0.1–12.8) 4.2
Nitrates (mg L^{-1})	(2.8–4.6) 3.9	(3.4–7.4) 5.4	(1.5–2.5) 2.0	(4–175) 55
Total Phosphorous (mg L^{-1})	(0.03–3.0) 0.6	(0.6–2.5) 1.5	(0.2–0.7) 0.3	(0.6–6.2) 2.5
Total suspended solids (mg L^{-1})	(10–55) 21	(14–94) 46	(13–90) 63	(2–40) 18
pH	(7.5–8.6) 8.1	(7.7–8.1) 7.9	(6.7–8.6) 8.1	(6.8–7.8) 7.4

A. Margenat, V. Matamoros, S. Díez, N. Cañameras, J. Comas, J.M. Bayona. Occurrence of chemical contaminants in peri-urban agricultural irrigation waters and assessment of their phytotoxicity and crop productivity. Sci. Total Environ., 599–600 (2017), pp. 1140-1148

Supplementary Table S4.2. Statistics of soil sample microbiome sequencing.

	Number of OTUs	Median Sequencing Depth	Minimum number of sequences in a sample	Maximum number of Sequences in a Sample
Lettuces	4165	243,095	141,607	301,036
Tomato	840	168,507	119,344	202,409
Broad Beans	588	19,047	3,607	56,524

Supplementary Table S4.3. Statistical analysis of ARG and *intI*1 abundances in soil samples from the three crops analyzed in this work.

	Copies per g of soil (1)					
Gene	**Broad Beans**		**Lettuce**		**Tomato**		***p* value(2)**
*intI*1	$1.06x10^5$	b	$1.85x10^6$	a	$1.80x10^5$	b	0.002
bla_{TEM}	$1.44Ex10^5$	b	$1.75x10^8$	a	$3.69x10^5$	b	$3.56x10^{-05}$
*sul*1	6898	b	$3.68x10^5$	a	$2.25x10^5$	a	$9.46x10^{-06}$
*qnrS*1	676	a	$2.79x10^4$	a	239		0.067
$bla_{CTX-M-32}$	3403		1802		7943		0.757
*tet*M	2410	b	153	b	$2.59x10^4$	a	0.001
bla_{OXA-58}	149		1		657		NA
*mec*A	1		1		879		NA

(1) Average values. Small-case letters indicate statistically different subsets of data (ANOVA+Tukey's B)
(2) ANOVA, square-root transformed values. NA indicates that the structure of data was not adequate for the test

Chapter V: General Discussion

The soil is the habitat for thousands of different bacteria, with up to 10^{10} bacteria and $5x10^4$ species per gram of soil (Raynaud and Nunan, 2014). It may also be a hotspot for HGT and AB resistance development and spread (Hu et al., 2016; Jiang et al., 2017). Agricultural soils are extremely impacted due to anthropogenic pressure, stimulating higher rates of HGT and AB resistance through exposure to chemical and/or biotic stressors, including ARBs and ARGs, compared to undisturbed soils.

Pollutants reach soils as a consequence of different agricultural practices, like using WW for irrigation or amendment of soils with manure, biosolids, or chemical fertilization (Berthrong et al., 2014; Manaia et al., 2018; Marti et al., 2013; Michael et al., 2013; Rizzo et al., 2013; Sabourin et al., 2012; Zhou et al., 2015). While WW reuse is a popular method in recent years, it generates the problem of the possible contamination of soils and produce by ARBs and ARGs, a potential risk that has not been fully characterized yet.

WWTPs are probable hotspots for AB resistance dissemination, since they do not remove completly AB, ARBs and ARGs (Manaia et al., 2018; Michael et al., 2013; Rizzo et al., 2013). Though, the characterization of WTTPs as an AB resistance hotspot is still under debate in the scientific community. The microbiomes present at each stage of the treatments is different and adapted to the local conditions. Nevertheless, the recipient river's microbiomes are affected by the incoming WWTPs, and consequently the resistomes. They ressemble a mixture of the raw wastewater sources, recycled activated sludge, and upstream river (Quintela-Baluja, 2019). WWTPs have a stable core resistome, with less than 10 % of the ARGs shared with other environments (Munck et al., 2015). Therefore, the HGT potential is possibly lower than previously tought. In any case, WTTPs are a reservoir of a wide range of ARGs.

The problem even increases when animal waste is used as amendment, as ABs are excreted in active or activable forms, therefore increasing ARBs and ARGs levels in WW from WWTPs (Heuer et al., 2011; Ji et al., 2012; Negreanu et al., 2012; Manaia et al., 2018). AB resistance selection also occurs among gastrointestinal bacteria, which are also excreted in manure used for soil amendment (Chen et al., 2016; McKinney et al., 2018; Udikovic-Kolic et al., 2014). Subsequently, applications of manure have been shown to increase the abundance of ARBs and ARGs in soil (Marti et al., 2013; McKinney

et al., 2018; Scott et al., 2018; Udikovic-Kolic et al., 2014), and to impact the resident soil microbiomes (Udikovic-Kolic et al., 2014).

Development of AR hotspots in different environmental settings is a matter of growing social and scientific concern. Nevertheless, studies considering the fate of the ARGs along the soil-plant continuum and the risk of accumulation in the crops' edible parts may pose to humans are still limited. Furthermore, most of the current studies conducted in real fields only reported effects on single compartment types. There are only a handful of mesocosms studies approaching the ARGs distribution over several compartments (Chen et al., 2018; Zhang et al., 2019; Zhu et al., 2017), studying both epyphitic and endophytic bacteria. In the Mediterranean Basin, research on this subject has been mostly done in countries with frequent droughts (Israel and Cyprus) and encompassed the effect of TWW irrigation on ABs, microbiomes and/or ARG load in downstream environments (Christou et al., 2017b; Gatica et al., 2016; Gatica and Cytryn, 2013; Goldstein et al., 2014; Malchi et al., 2014; Marano et al., 2019; Negreanu et al., 2012).

Studies on AB resistance can be performed using culture-dependent methods allowing enumeration of known pathogens, as well as the effects of ABs or other chemical compounds in their frequency and properties. However, most existent bacteria are not culturable, and this applies to around 99% of environmental bacteria (Amann et al., 1995). Therefore, determination of MIC with culturable bacteria are not really representative of the soil microbiomes and, subsequently, the environmental AB concentrations able to trigger selective pressure on real soil populations cannot be assessed. These bacterial communities can only be studied through DNA high-throughput sequencing techniques and bioinformatic approaches. These are the methods of reference when studying the effects of stressors, including ABs, on environmental bacteria.

This Thesis evaluates microbiome modulation, ARG translocation and their co-occurrence with anthropogenic markers in different types of crops under different agricultural practices (Gillings et al., 2015). The presence of ARGs and *intI*1 and their association with bacteria within the soil-plant-continuum was analyzed in four farm plots located in the peri-urban area of Barcelona (NE Spain), plus two rural farm plots far away from the peri-urban area, and a forest soil site, considered as a pristine area (Fig. 4.1). These studies are presented in the Thesis as three chapters referring to ARGs, *intI*1, and microbiomes.

Chapter II and Chapter III show the analyses of the *L. esculentum* and *V. faba* crops, respectively. Analysis of β-diversity of *L. esculentum* bacterial communities showed no dissimilarities among the different plots (*adonis* test $p>0.05$) in any sample type. Nevertheless, PLS-DA analyses revealed that the taxa at the Family level was affected by agricultural practices, showing that certain bacterial families discriminate the plots by the agricultural practices applied on them. Namely, abundant families of the Proteobacteria Phylum (Pseudomonadaceae, Enterobactereaceae, Flavobactereaceae). Pseudomonadaceae were associated with soil and roots from peri-urban fields, whereas Enterobacteriaceae and Flavobacteriaceae were more abundant rural fields. Rhizobiaceae family also contributed to the differentiation among the two categories of fields. Previous studies showed that Proteobacteria tend to increase in fertilized soils (Fierer et al., 2012; Ndayegamiye and Côté, 1989; Udikovic-Kolic et al., 2014; Zhou et al., 2015) or in those irrigated with TWW (Broszat et al., 2014). In addition, our results showed that most of the ARGs and *intI*1 were only found in significant amounts in soil and root endophytes, with the exception of bla_{TEM}. Samples from the peri-urban field showed significantly higher abundance of *tet*M, bla_{OXA-58}, and *mec*A, the latter not being detected in samples from the rural site. Pseudomonadaceae was the only bacterial family associated to periurban fields and showing strong positive correlations with ARGs in soil and roots. In addition, its abundance also correlated positively to *intI*1, bla_{TEM}, bla_{OXA-58}, and *sul*1 in fruits.

Chapter III shows the effect of the agricultural practices in the ARG and bacterial profiles in *V. faba* fields. In this case, from the most prevalent ARGs, as bla_{TEM}, *sul*1, *qnrS*1, only bla_{TEM} showed a higher abundance in the edible parts from vegetables from the peri-urban fields. In regard to bacteria profiles, taxa at the order level were used to make a Random Forest classification model, which was able to predict samples' origin with an accuracy of 88.9% (Supplementary Table S3.2). Orders Rhizobiales, Pseudomonadales, Enterobacteriales, and Burkholderiales (Supplementary Fig. S3.1) were among the most important bacteria for the classification. All of them belong to the Proteobacteria Phylum, known to have species potentiality pathogenic (Mahenthiralingam and Vandamme, 2005; Rizzatti et al., 2017). One of the most important taxons to differentiate the rural from the peri-urban plots was the Rhizobiales order (Supplementary Fig. S3.2).

Characteristically, *V. faba* crops showed fairly uniform microbiomes independently on the plant tissue and geographic location, despite that soil microbiome composition differed between sampling fields (Table 3.2). Rhizobiales were significantly more abundant in

rural than in peri-urban sites, whereas Enterobacteriales and Burkholderiales showed the opposite distribution (Fig. 3.4). Rhizobiales contributed to the soil microbiomes β-diversity dissimilarity between soils, and their distribution suggest that their predominance limits the colonization of the roots by other bacteria. It may prevent or impair the translocation of ARGs from soils to the palt parts, as the prevalence of Rhizobiales correlated negatively with *bla*$_{TEM}$, *sul*1, *bla*$_{CTX-M-32}$, and *intI*1. This result is consistent with a previous study, which found that the enrichment of Rhizobiales may lead to a distinctive soil microbiome (Zgadzaj et al., 2016). Nonetheless, different phyla may harbor ARGs, as the resistome compostion is associated to the microbiome composition. Therefore, different ARGs and integrons, may be harbored by different bacterial lineages in soil (Forsberg et al., 2014), as Phylogeny is a barrier for HGT and certain ARGs are transferred preferentially among certain taxa (Y. Hu et al., 2016). Therefore, as Rhizobiales were negatively correlated, they possibly did not carry *bla*$_{TEM}$, *sul*1, *bla*$_{CTX-M-32}$, and *intI*1, which were associated with other bacteria.

The impact of agricultural practices in Chapter II and III at the soil level was quantitatively different. Unlike tomato plots, in *V. faba* fields showed a shift in the soil microbiomes by β-diversity analysis, depending on the agricultural regime. The ARGs distribution between *L. esculentum* and *V. faba* differed as well, being less abundant in *V. faba* samples, in which *mec*A was below LOQ in all of the samples. In addition, *V. faba* neighboring plots (Chapter III Z2 and Z3, Fig. 3.1) with similar irrigation and amendment had different soil ARGs profiles; *bla*$_{TEM}$ was significantly higher in Z2 and *tet*M and *bla*$_{OXA-58}$ were present there as well.

Data from three sampling campaigns (*L. sativa*, *V. faba*, *L. esculentum*) and pristine soil (as background) was processed and analyzed in Chapter IV, as well as soil from a forest with no agricultural use over the last century. The use of a “background” soil permitted to inquire if the ARGs profiles were in certain aspects non-concordant. In Chapter II significant differences were detected between fields in the low prevalent ARGs, whereas in in Chapter III differences were detected in the most prevalent ones. The selected ARGs were researched to inquire which would be part of a “core” resistome in the soils, and the ones associated with anthropogenic pressure. Therefore, data integration from all sampling campaigns allowed a better insight of the environment state (ARGs and microbiomes), and mitigate any possible batch-effects.

The abundance of ARGs in the collected soil samples (Fig. 4.2 A) revealed that forest

samples had the lowest levels for all ARG and *intI*1, whereas *mec*A, bla_{OXA-58}, and *tet*M were below LOQ. PCA analyses provided information of the distribution of the ARGs in the soils, identifying three main groups, corresponding to lettuces, broad beans and tomatoes plots. The forest soils were very similar in terms of ARG content to the broad beans plots (Fig 4.2 B). However, the analysis did not differentiate sampling fields by their geographical origin or agricultural practices (rural vs peri-urban). Despite the gradient of ARGs and *intI*1, with a decreasing abundance from rhizospheric soil to fruit and beans, plant samples clustered in a very similar way as the soil samples, revealing that ARGs potential uptake in plant tissues also varied between crop types (Fig. 4.2 C). ARG abundances varied several orders of magnitude between plots and crop types. Four genes were found abundantly in most soils, including in the forest soil, bla_{TEM} ($\geq 10^4$ copies g^{-1} sample), *sul*1 ($\geq 10^3$ copies g^{-1} sample), *qnrS*1 (mostly $\geq 10^3$ copies g^{-1} sample), and *intI*1 ($\geq 10^3$ copies g^{-1} sample).

The type of crop and the soil microbiome composition appeared as the two most important factors to predict the ARG loads in crops edible parts. Microbiome composition and ARGs were deeply intertwined in all compartments. The positive correlation of *intI*1 and *sul*1 and Pseudomonadales and Enterobacteriales in the soil microbiome was a confirmation of the results obtained in Chapters II and III. The data suggest that certain bacterial taxa, including pathogen associated bacteria, which originate in the soil, tend to be associated with ARGs and *intI*1 as an anthropogenic pollution proxy (Gillings et al., 2015, 2008). The differences in soil microbiomes' composition were significant (β-diversity). Plant-soil feedback mechanisms are known to exist, and the effect of the plants' genotype/crops (Chaparro et al., 2014; Ofek et al., 2014; Zolti et al., 2019) and the distance to roots dictates their influence in soil microbiomes (Reinhold-Hurek et al., 2015). Consequently, the feed-back mechanism also impacts the endophyte bacteria. Agricultural practices may disturb this equilibrium, shifting compositions of microbiomes and ARGs, as discussed in Chapter IV. ABs uptake from the soil also varies according the vegetable (Ahmed et al., 2015; Christou et al., 2019; Tadić et al., 2019), possibly impacting endophytes and ARGs presence in crops. This uptake is likely to be higher in leafy vegetables, such as lettuces, than in tomatoes or broad beans crops, due to several factors including higher evapotranspiration rate, and net irrigation requirements (Christou et al., 2019). Previous studies conducted in the same geographical area showed potential of uptake of chemical contaminants varied according the vegetable tested (Margenat et al., 2019, 2017).

Nonetheless, prevalent ARGs, as $bla_{TEM,}$ *sul*1, *qnrS*1, appeared as part of the soil core

resistome, as they were present even in pristine soil (forest). The statistical models performed on each crop revealed Enterobacteriales and Pseudomonadales (abundant in both environmental and clinical settings) showed different relative abundances between rural and peri-urban fields. They appeared positively associated to *intI*1, bla_{TEM}, *sul*1, suggesting a link between anthropogenic pollution and the spread of potential multi-resistant pathogens, which are possible reservoirs of the core resistome. The resistance to at least to β-lactams and tetracyclines is a matter of concern, as they represent two of the most widely used classes of ABs in the clinical settings (Gibson et al., 2015). Therefore, there is a risk of transfer to consumers, despite that soil and human associated microbiomes are widely different, and that they carry different ARGs (Gibson et al., 2015), as those ARGs are a part of the mobile resistome (Y. Hu et al., 2016). Despite the ecological barrier, and that HGT rates are relatively low between bacteria from different environments (Smillie et al., 2011), the rate of HGT increases as the 16S rDNA distance decreases, even if bacteria are from different origins. For example, Burkholderiales, known to be pathogen associated bacteria (Mahenthiralingam and Vandamme, 2005), showed positive correlation with *tet*M, whose abundance in plant tissues can be mostly explained by their abundance in soil. This may represent a likely path for transmission of tetracycline resistance from soil to food.

Culture-independent methods such as qPCR, cannot discriminate between intracellular or extracellular DNA (eDNA), though progresses have recently been made (Nagler et al., 2018b). Therefore, part of DNA quantified in our assay might possibly be extracellular. eDNA adsorption to the soil matrix is primarily influenced by soil characteristics such as concentration of humic substances, ions, and soil pH (Levy-Booth et al., 2007), which may be altered by agricultural practices. Soil acidification caused by chemical fertilization (Goulding, 2016; Qiao et al., 2018) promotes adsorption of eDNA into soil particles and therefore its stability and persistence. eDNA also stimulates the occurrence and stabilization of biofilms (Mulcahy et al., 2008; Nagler et al., 2018a), which by themselves promote HGT. In any case, our study does not allow any estimation of the relevance of eDNA abundance in our results, although it is likely that intra- and extracellular DNA should shown a similar qualitative composition, as one originates from the other.

AB resistance is ancient and natural occurring phenomenon (D'Costa et al., 2006; 2011), widespread in the environment, even in undisturbed soils. In Antartic soils, multi-drug efflux pumps and β-lactam resistance mechanism were present (Van Goethem et al., 2018). Therefore, measures to fight the dissemination of AB resistance in the environment should be focused on other ARGs that are not integrated yet in the core

resistomes, such as *tet*M, *mec*A and *bla*$_{OXA-58}$. Their presence can be used as indicator of anthropogenic water and soil pollution, and therefore should be monitored in future quality standards regulations for reclaimed water to be used for agricultural purposes. The natural ecological barriers (soil and roots) coupled with optimal administration of reclaimed wastewater policies, significantly impair the effect of the irrigation water in ARGs spread in downstream environments and their translocation to plant edible parts. Very likely, soil amendment has a much higher impact than the irrigation water, directly or indirectly, affecting soil properties and, consequently, the respective microbiomes, and the plant-soil feedback (Reinhold-Hurek et al., 2015).

Several studies reported WW irrigation have non-significant or limited effect on ARGs abundance and prevalence, and on soil microbiomes even after long, multi-decade periods of usage (Christou et al., 2019; Gatica and Cytryn, 2013; Jechalke et al., 2015). The results in this Thesis point to a similar limited effect of WW on the ARGs loads and ARBs presence in the soil and crops, in nearby fields, with similar irrigation regimes. Soil amendment was probably sufficient to significantly impact on the crop's ARGs even if this effect was indirect, by shifting the soil local properties, and consequently affecting soil microbiomes and resistomes. The ABs arriving in the receiving soils via manure or TWW, may last up to thousands of days, such as fluoroquinolones (Cycoń et al., 2019; Walters et al., 2010). ABs exposure may cause a continuous selective pressure over soil microbiomes for long periods (Kümmerer, 2009; Pan and Chu, 2016; Segura et al., 2009). Also, may significantly alter the soil processes, e.g. nitrification and/or denitrification rates (Toth et al., 2011). Sulfonamides may block iron reduction in soil for extended periods (Toth et al., 2011). Therefore, even low concentrations may change the activities and the process of microbiomes in the soil, and affect their diversity as well due the complex relations in the microbiomes, as changes in functional groups may affect others groups (Wagg et al., 2014; Zolti et al., 2019).

The plant-soil feedback-mechanism can be altered as well. The magnitude of the effects varies according to the crops ABs uptake capabilities (Christou et al., 2019), as showed by the ARGs loads present (decreasing order) in *L. sativa*, *L. esculetum*, and *V. faba* samples. Therefore, guidelines for agricultural practices either irrigation water or soil amendment should take in consideration the type of crop. *"Everything is everywhere*: but *the environment selects"* (Becking, 1934, 1931), an unifying concept in Microbial Ecology is a good summary of the results. Legislative efforts have to be directed to prevent the agricultural environment from selectively favouring the growth and spread of ARBs and ARGs that could ultimately colonize the human microbiomes.

The Hypothesis validation generated for this Thesis are described in this chapter.

Hypothesis I: Agricultural practices impact ARGs distribution along the soil-plant-continuum

This hypothesis is validated. The soil is a reservoir of ARGs. The results provided in the Thesis indicate that the levels of ARGs in soils were significantly affected by the agricultural practices. As soils act as ARG reservoirs, the ARG soil profile determines the ARGs that can be translocated into the plants.

Hypothesis II: Agricultural practices cause a shift in the bacterial communities along the soil-plant continuum

This Hypothesis is validated. Different clades of Proteobacteria, including Pseudomonadales, Enterobacteriales, and Burkholderiales, known to be potential multi-resistant pathogens, were abundant in soils and their relative abundance in the fields was afected by agricultural practices. More importantly, Pseudomonales were also present in the edible parts. The results suggest a strong influence of the type of crop in the soil microbiome, as well as the mutual correlation between soil and endophytic microbiomes.

Chapter VI: Conclusions

The main conclusions from the Thesis are described below.

1. Abundant Proteobacteria families could discriminate between rural and periurban *L. esculetum* fields. This shows that bacteria that are pathogen associated bacteria may be more or less abundant in soil and vegetables due to different agricultural practices.
2. Pathogen associated bacteria with potential for multi-resistance phenotypes were detected in the *L. esculetum* fruits. Pseudomonadaceae was detected in tomatoes fruits from the peri-urban field.
3. The *L. esculetum* peri-urban field was characterized by the presence of less-prevalent ARGs, whereas differences between *V. faba* plots were driven by the most prevalent ARGs (bla_{TEM}, *qnrS*1) and *intI*1. The only differences detected in the beans refered to bla_{TEM} levels.
4. The abundance of Proteobacteria (pathogen associated bacteria) was indicative of the field type (rural vs peri-urban), with a high degree of accuracy in *V. faba* fields.
5. Rhizobiales were the main dominant endobacteria from the roots to the edible parts of *V. faba* plants. The results suggest that Rhizobiales are occupying a niche, making more difficult for ARBs to colonize the plants. They appear to impair the translocation of ARGs into the plant and edible parts, independently of the soil ARGs and *intI*1 abundances.
6. The abundances of ARGs decreased from the soil to the edible parts, independently of the crop type. Despite the similar trends of distribution along the soil-plant-continuum, the absolute values varied by several fold among crop types.
7. Three sampling campaigns revealed that the soil microbiomes β-diversity is significantly different, grouped by the type of crop. The soil microbiomes composition depended on the crop rather than on the farming practices or location (peri-urban and rural agriculture).
8. The plants ARGs were strongly dependent on soil ARGs abundance and profile. ARGs composition and even the entire resistome composition was dependent on

the bacterial community composition. The microbiome is affected by the soil properties and vegetable, which are modulated by agricultural practices.

9. All soils, including the forest soil, presented *bla*$_{TEM}$, *sul*1 and *qnrS*1. This suggests that these ARGs are incorporated into the local soil resistomes. Therefore, low prevalent genes from clinical origin should be targeted, as they are not incorporated in the soil/crops core microbiomes and resistomes, and they can be used as markers for anthropogenic pressure/pollution.
10. Pseudomonales and Enterobacteriales abundances have potential as a descriptor of the agricultural fields status. On a global level, those taxa tend to be positively associated with *intI*1, bla$_{TEM}$, and *sul*1, revealing a link between anthropogenic pollution and potential multi-resistant pathogens.
11. The Thesis results show the focus of regulation should be on soil amendment practices, vegetables physiology, rather than only focusing in the irrigation water quality.

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