CATÓLICA
FACULTY OF BIOTECHNOLOGY

PORTO

MEASUREMENT OF THE IMPACT OF ANTIBIOTIC RESISTANCE DISCHARGE IN WASTEWATER AND IN SOIL: ECOLOGICAL ASPECTS

Thesis submitted to Universidade Católica Portuguesa to attain the degree of Ph.D. in Biotechnology,
with specialization in Microbiology

Gianuario Fortunato

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Gianuario Fortunato

Supervisor: **Célia Maria Manaia Rodrigues**

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July, 2022

To Dr. Cristina Becerra-Castro, to my family and friends, companions of this beautiful journey

Abstract

Antibiotic resistance represents a serious threat to human health and a relevant environmental contaminant. Antibiotic-resistant bacteria (ARB) and harboured antibiotic resistance genes (ARGs) were described in different settings, mainly in clinical contexts, but also in wastewater treatment plants or agricultural soil. In the environment, the general pollution context may favour the persistence or proliferation of ARB due to multiple genetic characteristics. Monitoring of ARGs and ARB in the environment plays an important role in unveiling sources and paths of dissemination. To address these issues this thesis explored three topics based on antibiotic resistant Pseudomonas aeruginosa and on soil: i) the biases that may be imposed by the high limits of quantification of ARGs in soil; ii) the survival of an exogenous ubiquitous bacterial strain (bla_{VIM}+ *P. aeruginosa*) in soil and the possible effects of metal salts; and iii) the inferred interplay between phylogeny and accessory genome in distinct genotypes of carbapenem resistance (bla_{VIM}+ or bla_{NDM}+ *P. aeruginosa*).

The first work (chapter 3) aimed to assess the limit of quantification (LOQ) for ARGs (*vanA*, *qnrS*, bla_{TEM}, bla_{OXA}, bla_{IMP}, bla_{VIM}) in soil, based on the hypothesis that low doses of ARB and ARGs in soil are not quantifiable with current qPCR techniques, mainly due to DNA extraction procedures. To determine the LOQ, microcosms (10 g of agricultural soil, potting soil, sand, fallow soil and compost) were spiked with wastewater isolated ARB doses ranging from 10^2 to 10^7 CFU/g of dry soil. These spiked ARB harboured respectively the vancomycin resistance gene *vanA* (*E. faecalis*), quinolone resistance gene *qnrS* (*Escherichia coli*), and β-lactam resistance genes bla_{TEM} (*E. coli*), bla_{OXA} (*E. coli, Acinetobacter johnsonii*), bla_{IMP} (*A. johnsonii*), bla_{VIM} (*P. aeruginosa*) based on which the LOQs were determined. The microcosms were sampled to enumerate bacterial colony forming units (CFU), and extract DNA for ARGs quantification by qPCR. The LOQ was determined to be 10^4 copies of ARG per g of dry soil, independently of the soil type. Below this limit, it was not possible to quantify ARGs even when the respective host ARB could be cultivated. The results support the hypothesis that LOQ values are relatively high and may suggest the absence of ARGs in situations in which these may represent a threat.

The second work (chapter 4) aimed at assessing the metal impact on the survival of a hospital effluent bla_{VIM}+ *P. aeruginosa* and on the soil microbial community's diversity. Microcosms were prepared with agricultural soil non-amended or amended with copper and zinc sulfate or nitrate aged for one month, and spiked with known doses of bla_{VIM}+ *P. aeruginosa*. The ARB survival was monitored based on CFU enumeration and quantification of selected genes - extracytoplasmic function sigma factor, *ecf*, Verona Integron–encoded Metallo-β-Lactamase, bla_{VIM}, class 1 integron-integrase gene, *intI1*, over 30 days. In addition, the microbial community composition (V3-V4 16S rRNA gene amplicon sequencing) was analysed. Over this period, the *P. aeruginosa* content (CFUs/g dry soil) and ecf and bla_{VIM} (copy number/g dry soil) decreased in all the tested conditions, but was still quantifiable after 30 days. This confirms the ARB persistence in soil along the 30 days, excluding the hypothesis of ARG loss during this period. Microbiome analysis revealed a clear influence of metals in the bacterial community diversity, independent of the metal type and salts nature. This study permitted to conclude that the metal amendment affects the soil microbial quality

but has a negligible impact on the exogenous bacteria survival. These results highlight the importance of considering microbial interaction and characteristics, such as metal tolerance, in the assessment of ARB persistence in the environment.

In chapter 5 the genomes of bla_{VIM-2}+ or bla_{NDM-1}+ *P. aeruginosa* strains were compared. The ARG bla_{VIM-2} is mostly observed in *Pseudomonas* species, while bla_{NDM-1} is distributed among distinct genera and orders. The work focused on phylogenetic distribution and genomic features of a dataset of 116 bla_{VIM-2}+ and 27 bla_{NDM-1}+ genomes, from 38 countries. The selected genomes were annotated and the core sequence multilocus sequence typing (MLST) were determined. The bla_{VIM-2}+ and bla_{NDM-1}+ genomes were analysed using a comparative genome approach to assess the core and accessory genes, later used to determine the bacteria antibiotic resistance and functional profile. To describe the bla_{VIM-2} and bla_{NDM-1} genomic environment, the flanking regions were annotated through sequence comparison. The phylogenetic and geographic distribution analyses suggested a worldwide distribution of the strains belonging to several STs with cases of endemism. The bla_{VIM-2} + and bla_{NDM-1} + accessory genomes presented different antibiotic resistance and functional profiles, regardless the majority of the ARGs and proteins families were shared. Interestingly, the copresence of bla_{VIM-2} and bla_{NDM-1} and other carbapenems resistance genes in different genomes was observed. The genomic environments of the two ARGs were different, being bla_{VIM-2} associated with distinct transposons structures (Tn21, Tn402-like mostly) and bla_{NDM-1} to different elements (ISAba125 and ble_{MBL} and IS91). This work emphasized the importance of considering different approaches to tackle the spread of carbapenem resistant bacteria evaluating the phylogeny, and geographical distribution but mostly the genomic characteristics of the strains.

Our works aimed to indicate possible weaknesses to be improved in antibiotic resistance monitoring and highlight the ARB phylogenetic role and genetic characteristics favouring the ARGs spread. In particular, the experimental work evidences the possible survival of ARB in soil, mostly in extremely polluted conditions. Moreover, the survival and presence of these ARB in soil could avoid the quantification by molecular biology methods due to their high LOQ. The study of the ARB genomic characteristics may be useful to prevent the adaptation to environments and to find additional biomarkers for their monitoring.

Keywords: Antibiotic resistance, *Pseudomonas aeruginosa*, Microbiome analysis, Wastewater reuse

Resumo

A resistência a antibióticos representa uma séria ameaça para a saúde humana e um contaminante ambiental relevante. As bactérias resistentes a antibióticos (ARB) e os genes de resistência a antibióticos (ARGs) foram descritos em diferentes contextos, principalmente em casos clínicos, mas também em estações de tratamento de águas residuais ou solos agrícolas. No ambiente, o contexto geral da poluição pode favorecer a persistência ou a proliferação de ARB devido a múltiplas características genéticas. A monitorização de ARGs e de ARB no ambiente pode ter um papel importante na identificação de fontes e vias de disseminação. Para abordar estas questões, esta tese explorou três temas baseados em *Pseudomonas aeruginosa* resistente a antibióticos e em solo: i) as limitações que podem existir devido aos elevados limites de quantificação de ARGs no solo; ii) a sobrevivência de uma estirpe bacteriana exógena ubíqua (bla_{VIM}+ *P. aeruginosa*) no solo e os possíveis efeitos de sais de metais; e iii) a relação que poderá existir entre a filogenia e o genoma acessório em diferentes genótipos de resistência a carbapenemos (bla_{VIM}+ ou bla_{NDM}+ *P. aeruginosa*).

O primeiro trabalho (capitulo 3) visou avaliar o limite de quantificação (LOQ) de ARGs (*vanA*, *qnrS*, bla_{TEM}, bla_{OXA}, bla_{IMP}, bla_{VIM}) em solo, baseado na hipótese de que doses baixas de ARB e ARGs no solo não são quantificáveis com as atuais técnicas de qPCR, principalmente devido aos procedimentos de extração de DNA. Para determinar o LOQ, microcosmos (10 g de solo agrícola, substrato, areia, solo pousio e composto) foram inoculados com ARB isoladas de águas residuais em doses que variam de 10^2 a 10^7 CFU/g de solo seco. As ARB usadas como inóculo continham respetivamente o gene de resistência a vancomicina *vanA* (*E. faecalis*), o gene de resistência a quinolonas *qnrS* (*Escherichia coli*), e os genes de resistência a β-lactâmicos bla_{TEM} (*E. coli*), bla_{OXA} (*E. coli, Acinetobacter johnsonii*), bla_{IMP} (*A. johnsonii*), bla_{VIM} (*P. aeruginosa*) com base nos quail os LOQs foram determinados. Os microcosmos foram amostrados para enumerar unidades de formação de colónias bacterianas (CFU), e extrair DNA para quantificação de ARGs por qPCR. O LOQ foi determinado como sendo de 10^4 cópias de ARG por g de solo seco, independentemente do tipo de solo. Abaixo deste limite, não foi possível quantificar os ARGs mesmo quando o respetivo hospedeiro ARB foi detetado por cultivo. Os resultados apoiam a hipótese de que os valores de LOQ são relativamente elevados e podem sugerir a ausência de ARGs em situações em que estes possam representar um perigo.

O segundo trabalho (capítulo 4) visou avaliar o impacto de metais na sobrevivência de um isolado de *P. aeruginosa* proveniente de efluente hospitalar bla_{VIM}+ e na diversidade da comunidade microbiana do solo. Microcosmos foram preparados com solo agrícola ou neste suplementado com sulfato ou nitrato de cobre e zinco envelhecidos durante um mês, e inoculados com doses conhecidas de *P. aeruginosa* bla_{VIM}+. A sobrevivência da ARB foi monitorizada com base na enumeração das CFU e quantificação de genes selecionados - extracytoplasmic function sigma factor, *ecf*, Verona Integron–encoded Metallo-β-Lactamase, bla_{VIM}, class 1 integron-integrase gene, *intI1*, ao longo de 30 dias. Além disso, foi analisada a composição da comunidade microbiana (sequenciação de amplicão V3-V4 do gene 16S rRNA). Durante este período, a abundância *de P.*

aeruginosa (CFUs/g solo seco) e ecf e *bla*$_{VIM-2}$ (número de cópia/g de solo seco) diminuiu em todas as condições testadas, mas foi ainda assim quantificável após 30 dias. Estes resultados confirmam a persistência da ARB no solo ao longo dos 30 dias, excluindo a hipótese de perda do ARG durante este período. A análise do microbioma revelou uma clara influência dos metais na diversidade da comunidade bacteriana, independentemente do tipo de metal ou sal. Este estudo permitiu concluir que a suplementação com metais afeta a qualidade microbiana do solo, mas tem um impacto negligenciável na sobrevivência das bactérias exógenas. Estes resultados destacam a importância de considerar a interação e características microbianas, como a tolerância a metais, na avaliação da persistência da ARB no ambiente.

No capítulo 5 foram comparados os genomas de estirpes de *P. aeruginosa bla*$_{VIM-2}$+ ou *bla*$_{NDM-1}$+. O ARG *bla*$_{VIM-2}$ é observado principalmente em espécies de *Pseudomonas*, enquanto *bla*$_{NDM-1}$ é distribuído entre géneros e ordens distintos. O trabalho centrou-se na distribuição filogenética e características genómicas de um conjunto de 116 genomas *bla*$_{VIM-2}$+ e 27 genomas *bla*$_{NDM-1}$+, de 38 países. Os genomas selecionados foram anotados e as sequências do core genome multilocus sequence type (MLST) foram determinadas. Os genomas *bla*$_{VIM-2}$+ e *bla*$_{NDM-1}$+ foram analisados usando uma abordagem comparativa do genoma para avaliar os genes do genoma *core* e acessório, mais tarde usados para determinar a resistência a antibióticos e o perfil funcional das bactérias. Para descrever o ambiente genómico de *bla*$_{VIM-2}$ e *bla*$_{NDM-1}$, as regiões onde se integravam foram anotadas através da comparação de sequências. As análises da distribuição filogenética e geográfica sugeriram uma distribuição mundial das estirpes pertencentes aos vários STs com casos de endemismo. Os genomas acessórios de *bla*$_{VIM-2}$+ e *bla*$_{NDM-1}$+ apresentaram diferentes perfis de resistência a antibióticos e funcionais, independentemente da maioria dos ARGs e das famílias de proteínas terem sido partilhados. Curiosamente, a presença simultânea de *bla*$_{VIM-2}$ e *bla*$_{NDM-1}$ e outros genes de resistência a carbapenemos foi observada em diferentes genomas. Os ambientes genómicos dos dois ARGs eram distintos, sendo *bla*$_{VIM-2}$ associado a diferentes transposões (Tn21, Tn402-like na sua maioria) e *bla*$_{NDM-1}$ a diferentes sequências de inserção (ISAba125 e *ble*$_{MBL}$ e IS91). Este trabalho enfatizou a importância de considerar diferentes abordagens para combater a propagação de bactérias resistentes a carbapenemos que avalim a filogenia, e a distribuição geográfica, mas principalmente as características genómicas das estirpes.

Os nossos trabalhos visavam indicar possíveis limitações a serem melhoradas na monitorização da resistência ao antibióticos e destacar o papel da filogenia das ARB e das características genéticas que favorecem a propagação dos ARGs. Em particular, o trabalho experimental evidencia a possível sobrevivência de ARB no solo, principalmente em condições de elevada poluição. Além disso, a sobrevivência e a presença destas ARB no solo pode não ser possível de quantificar por métodos de biologia molecular devido ao seu elevado LOQ. O estudo das características genómicas das ARB pode ser útil para prevenir a adaptação aos ambientes e para encontrar mais biomarcadores para a sua monitorização.

Keywords: Resistência a antibióticos, *Pseudomonas aeruginosa*, Analise da comunidade microbiana, Tratamento de águas residuais

Acknowledgements

The presented PhD thesis represents one of the most important achievements of my scientific life. During these years I faced several difficulties that improved me as a researcher but mostly a person. I corrected some weaknesses and learned that in life we can achieve great goals by working focused, with a free and organized mindset. I am really grateful for this opportunity and proud of my PhD journey.

If I achieved these important goals, I have to thank several people fundamental for my progress and for my PhD work.

I would like to thank the Universidade Católica Portuguesa as the institution giving me the possibility and all the facilities needed to complete my PhD.

The first person I would like to thank is my supervisor professor Célia Manaia for trusting in my skills selecting me as a PhD student and for being over these years a guide in my work and personal improvements. Moreover, I would like to thank my co-supervisors prof. Olga Nunes for being a precious and wise help for my PhD work. A special thanks go to Dr Ivone Vaz-Moreira for being more than a supervisor, for being a friend supporting me and for understanding my moments of bad mood; thanks to Dr Cristina Becerra-Castro, my unforgettable supervisor and friend that left us too early but I always feel her presence, her support and "reprimands" fundamental to achieve this PhD, I will never forget you and I am dedicating to you this success.

I am grateful to all my work colleagues, part of the Lab. Manaia group, Pompeyo, Gonçalo, Michael, Diana, Carlos, Telma, Catarina, Joana, Jaqueline, Nazareno, Ana, Inês, João, Reginaldo, Daniel and all the students for sharing a great moment of work and friendship these years.

I would like to thank you for all the help people from Católica human resources, especially Cláudia Cabral for the great help and all the workers at the university from all the professors, laboratory assistants, security, food, cleaning, and financial staff to take care of us in all our needs.

During these years at Católica I make a lot of friends and I would like to thank all, especially Cláudia and Sérgio for our never-ending working days and all the laughs, support and good time spent together.

Another big group I would like to thank are my dear friends from my "gabinete", also known as "amigos dos Mateus": Ana Luiza, Marta, Nádia, Tatiana, Sofia Pereira, Sofia Salsinha, Rosa, Inês. We shared more than working time together, literally, we lived a great adventure together, sharing all our care and friendship, as colleagues, as friends and this will last forever.

I think I missed someone from this group ?! of course not!! I have a special thanks to Catarina (João and Bernardo), Helena and Joana (and Nuninho). You are more than my friends, we create an unbreakable connection that will always join us, forever. Thank you for the friendship, for a great

time, for this deep feeling, and for being a second family here. All in different ways help me to achieve this PhD and to improve me as a person, Thank you “maravilhosas”.

I would like to thank the football group, friends of several games and even more lough. In particular, I would like to thank Miguel, Eze and Pedro, o capitão, a wise friend of this group.

During these years I met a lot of friends and flatmates and to them go my gratefulness for the great times. In particular, a big thanks go to Carla, Cândida (and Duarte), Clara and Francisco, we shared in our house the lockdown, took care of each other, create a strong friendship and became a family. You will be forever my Portuguese family. I would like to thank also my flatmate Inês, Bruna, Guilherme, Luca, Stefania, Rita, Rafael, Marcela, Cátia, Daniela for being day-by-day friends, and family, for helping me and understanding me, we shared unforgettable moments and you will be forever in my mind.

The PhD gave me the possibility to meet a lot of people and in particular two of them become a fundamental part of my life. Francesco and Valentina, thank you for all, for being my friends, for being my supporters and make me feel at home even at km of distance. In particular, Francesco thank you for your brotherhood.

I think now is the moment to thank my family, my great supporters my father, my mother and my sister. They never stop to believe in me, encouraging me even in the toughest moments, if I succeed is because of your support.

Finally, I would like to thank all the ANSWER group, from the managers, the professors and in particular the ESR, we did a great job and an unforgettable journey together. In particular, thank my friends Ioannis, Vasiliki and Big Jack for being friends more than colleagues.

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List of abbreviations

ARB – Antibiotic resistant bacteria

ARGs – Antibiotic resistance genes

CFU – Colony forming units

HGT – Horizontal gene transfer

ICEs – Integrative conjugative elements

In - integrons

IR – Inverted repeat

IS - insertion sequences

MIC – Minimum inhibitory concentration

MGE – Mobile genetic element

MLST – Multilocus sequence typing

LOQ – Limit of quantification

qPCR – Quantitative polymerase chain reaction

ST – Sequence Type

Tn - transposons

WWTP – Wastewater treatment plant

Publications

The content of this thesis is partially published in:

Fortunato, G., Vaz-Moreira, I., Becerra-Castro, C., Nunes, O.C., Manaia, C.M., 2018. A rationale for the high limits of quantification of antibiotic resistance genes in soil. Environmental Pollution 243, 1696–1703. https://doi.org/10.1016/j.envpol.2018.09.128

Fortunato, G., Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2021. Effect of copper and zinc as sulfate or nitrate salts on soil microbiome dynamics and *bla*-positive *Pseudomonas aeruginosa* survival. Journal of Hazardous Materials 415, 125631. https://doi.org/10.1016/j.jhazmat.2021.125631

Fortunato, G., Vaz-Moreira, I., Gajic, I., Manaia, C.M. Insight into phylogenomic bias of bla_{VIM-2} or bla_{NDM-1} dissemination amongst carbapenem resistant *Pseudomonas aeruginosa*. Submitted for publication.

Introductory note

The present doctoral thesis was designed and carried out as part of the framework of the Innovative Training Networks (ITN) programme Marie Skłodowska-Curie grant, through the project ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions, ANSWER (http://www.answer-itn.eu/), funded by the European Union's Horizon 2020 research and innovation programme. As Early Stage Researcher (ESR), during the three years contract I was involved in scientific and divulgation projects allowing to accomplish the doctoral program at Universidade Católica Portuguesa. The ANSWER project was designed to tackle the antibiotic resistance dissemination in agricultural and food chain through wastewater reuse, aiming to provide possible guidance for a safe reuse. As ESR1 out of 15 hired, my contribution was focused on assessing the potential impact of wastewater reuse in the ARGs and ARB in the environment. The present work was carried out mainly at Universidade Católica Portuguesa in Porto while three secondments scheduled in the project were carried for two months in each location. The first secondment was done at University of Cyprus, Cyprus, in Prof. Despo Fatta-Kassinos group aiming to collect soil samples to evaluate the ARGs and ARB presence in real fields irrigated with treated wastewater. During the following secondment carried at The Agriculture Research Organisation of Israel - The Volcani Center, Israel, in Dr. Eddie Cytryn group I was involved in a project to test different soil conditions to settle ARB survival assay in arid soil. In the last secondment at Technische Universität Dresden, Germany, in Prof. Thomas Berendonk group I analysed the samples using qPCR methods. During these secondments period I had the possibility to work in different groups, collaborating with my coleagues ESRs and other researches, learning new working methods and improving my networking skills. The constant project achievements were evaluated in meetings organized by the project managers each six-months. Moreover, the project organized training events for the ESRs to improve our technical and networking skills.

1. Introduction

1.1. Antibiotic resistance in natural environments

Antibiotic resistance is defined according to the medical microbiology and bacterial infection therapy requirements. It refers to the capacity of a bacterial strain to survive in the presence of an antibiotic used at therapeutical concentrations (Davison et al., 2000; Martinez, 2014). Although this may be a property intrinsic to some bacterial groups, being designated intrinsic resistance, it may emerge in others, being designated as acquired resistance. Acquired antibiotic resistance refers to the modification of a bacterial strain, that was once susceptible, to resistant, and this process may be due to gene mutation, or the acquisition of genes through HGT (Martinez and Baquero, 2000; Partridge et al., 2018; Woodford and Ellington, 2007). Most of the discussions about the spread of antibiotic resistance and the associated human health risks have been focused on the acquired type (Hosein et al., 2002; Read and Woods, 2014; Ventola, 2015). In nature, specifically in water and soil, antibiotic resistance is frequent, and it is mostly intrinsic. This is relevant in terms of antibiotic resistance ecology as it may create charity mechanisms that may favour the acquisition of resistance by some community members (Bottery et al., 2020; Walsh and Duffy, 2013). Moreover, some of the genes that are intrinsic in some species may become acquired in others, as was reported for the genes bla_{CTX-M} and *qnrA* that originated in *Kluyvera* and *Shewanella*, reported also in other human pathogens as *Acinetobacter* spp. or *Pseudomonas* spp. (Ali et al., 2018; Farmer et al., 1981; Poirel et al., 2005b; Potron et al., 2011). The intensification of shotgun metagenomics studies has led to the analysis of the whole set of ARGs in a sample, irrespective of their intrinsic or acquired character, normally designated as resistome (de Abreu et al., 2021; Mullany, 2014). Because most of the environmental bacteria are non-culturable, shotgun metagenomic approaches contributed to changing the paradigm of microbial diversity studies in complex matrices as it is the soil. These studies have highlighted how the soil resistome may be an important source of known ARGs, irrespective of their intrinsic nature in the native microbial communities (Forsberg et al., 2012; Graham et al., 2016).

1.1.1 Some notes about the soil resistome

Soil is a living material composed of minerals, organic matter, and living organisms embedded in gas and water. Soil-living organisms, particularly bacteria, fungi, and archaea, represent an impressive fraction of the Earth's biodiversity (McClellan, 2020; Needelman, 2013; Voroney, 2007). Competition, communication, regulation, and intercellular interactions are examples of key functions of the soil microbiota (Dessaux et al., 2011; Hibbing et al., 2010; Witzany, 2011). Some of these involve not only the production of metabolites such as antibiotics, but also the development of functions that, depending on the genetic, cellular or community context may confer antimicrobial resistance phenotypes (Allen et al., 2010; Laskaris et al., 2010; Martínez, 2012). For example, soil bacteria and

fungi able to produce a wide variety of compounds with antimicrobial activity have been reported and some of these are in the origin of drugs used nowadays for the treatment of bacterial infections (Chandra and Kumar, 2017; Rehman et al., 2020). In parallel, soil bacteria also hold a broad array of antibiotic resistance determinants (Armalytė et al., 2019; Cytryn, 2013; D'Costa, 2006; Nesme and Simonet, 2015; Willms et al., 2019). Genes presumably conferring resistance to antibiotics were described in pristine or minimally human-impacted soils such as the remote Antarctica lands (Van Goethem et al., 2018). This is supported by the observation that 30.000 years old permafrost soils contained genes sharing high sequence identity with clinically relevant genes conferring resistance to a broad spectrum of antibiotics (D'Costa et al., 2011). These studies suggest that soil microorganisms evolved a variety of resistance mechanisms to survive in the presence of antibiotics and highlight the origin of the clinically relevant ARGs observed nowadays. The role of environmental bacteria, in particular from soil as reservoirs of ARGs has been widely discussed (D'Costa et al., 2007; Sanderson et al., 2016). For example, the soil resistome holds a broad variety of ARGs related to others reported in clinical settings conferring resistance to different antibiotics such as sulfonamides, tetracyclines, or penicillins (e.g. *tet*-, *sul*- and *bla*$_{TEM}$- like genes) (Zhuang et al., 2021). The use of antibiotics in human medicine and animal prophylaxis and treatment led to the accumulation of antibiotic residues in the environment, which are supposed to have the potential to exert selective pressures with the consequent increase of antibiotic resistance (Ma et al., 2020; Martin et al., 2015; Ventola, 2015). However, antibiotics are not the only potential selective pressures. Other environmental contaminants, such as metals or pesticides have been also mentioned as possible selectors associated with resistance acquisition and dissemination (Baker-Austin et al., 2006; Curutiu et al., 2017; Dickinson et al., 2019; Ramakrishnan et al., 2019). These substances, naturally present or associated with human activities, may favour the evolution or selection of antibiotic and metal resistance genes in the same organisms (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). The external factors that may enhance the dissemination of antibiotic resistance are complex, interconnected, and still poorly understood, mainly in habitats where the microbiome is complex and diversified, such as soils (Cytryn, 2013). This is an area in which further knowledge is needed to leverage the capacity to control the dissemination of antibiotic resistance.

1.1.2 Spread of antibiotic resistance in soil

In soil, the large and diverse microbiome and the existence of naturally antibiotic resistant bacteria (ARB) may create favourable conditions for the proliferation of some antibiotic resistant lineages or for the dissemination of resistance genes, for example of human commensal or pathogenic bacteria (Jiang et al., 2017). The dissemination of genes may occur through HGT (Sun et al., 2019). Conjugation is supposed to be a major mechanism of HGT involved in ARGs acquisition in soil, involving mainly extrachromosomal elements like plasmids, through pili that establish the contact between cells (Hall et al., 2016; Pukall et al., 2006). Other HGT mechanisms, such as transformation, through which free DNA can be acquired by bacteria, and transduction mediated by bacteriophages may also occur (Burmeister, 2015; Frosini et al., 2020; Gabashvili et al., 2020; Thomas and Nielsen,

2005; von Wintersdorff et al., 2016). These types of intercellular genetic recombination are complemented by intracellular rearrangements in which genetic elements may be integrated into the chromosome or plasmids. Examples of these genetic elements are integrons or transposons, recognized for their role in the mobilization of genes between the chromosome and extrachromosomal elements, in both natural and clinical environments (Aminov, 2011; Zhuang et al., 2021). Class 1 integrons, the integron type most discussed in the literature due to its association with clinical ARGs, have received particular attention as a proxy of anthropogenic pollution (Gillings et al., 2014). These integrons have a variable region where genes, often encoding antibiotic resistance or other, are inserted through site-specific recombination, catalysed by an integrase encoded by the *intI1* gene (Mazel, 2006). Class 1 integrons have been observed over a broad range of phylogenetic groups, but it is in members of the phylum *Pseudomonadota*, in particular in the classes *Gammaproteobacteria* and *Betaproteobacteria* that frequently thrive in both clinical and natural environments (Betteridge et al., 2011; Ghaly et al., 2017; Gillings et al., 2015; Liao and Chen, 2018; Zhang et al., 2018). The conserved region of class 1 integrons includes *sul1* and *qacE* genes (encoding for sulfonamide and quaternary ammonium compound resistance, respectively) (Chaturvedi et al., 2021; Gillings et al., 2008; Jechalke et al., 2013b). The variable region is frequently empty, mainly in bacteria under low selective pressure conditions. However, in clinical bacterial isolates, even after they are released into the environment, that variable region harbours acquired genes frequently associated with aminoglycoside resistance, and also with last-resort antibiotics, where they are transported and expressed (Gillings et al., 2008; Partridge et al., 2018, 2009). Despite the common reference of class 1 integrons as a proxy for anthropogenic impacts and the use of the integrase gene *intI1* as its biomarker, their presence has been reported in environments under mild human impacts, like soil and rhizosphere, yielding their high sequence variety (Gillings et al., 2008; Jechalke et al., 2014). However, integrons may have different origins. Indeed, nonclinical-related intI1 presents a different sequence from clinical, suggesting the possible environmental origin and later spread to clinical pathogens (Gillings et al., 2008). Practices such as manuring and irrigation may promote the dissemination of integrons in soil, creating a favourable environment for the spread of antibiotic resistance (Guron et al., 2019; Lima et al., 2020). Although integrons alone cannot promote intercellular dissemination of antibiotic resistance, they may be part of the dissemination processes known to occur in the environment. This transfer was demonstrated, for instance, in soil microcosms where donor cells were observed to transfer reporter plasmids through conjugation to soil native bacteria (Fan et al., 2019; Pukall et al., 2006). These approaches have been used also to demonstrate the transformation in vitro of members of different genera (*Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Agrobacterium*) inoculated in soil amended with cell-lysates or free DNA (Demanèche et al., 2001; Graham and Istock, 1979; Nielsen et al., 2000; Sikorski et al., 1998). Transformation requires the natural competence of the cells, i.e. the capacity to uptake exogenous DNA, which has been described in members of many species of different phyla (*Bacillota*, *Pseudomonadota* or *Actinomycetota*) (Johnsborg et al., 2007). The transformation has been reported as being influenced by external factors such as the integration of biofilm structures or response to pollutants (Bae et al., 2014; Domingues et al., 2012; Winter et al., 2021). Indeed, abiotic variables

such as the abundance and type of nutrients, temperature, pH, or other, and biological factors, such as microbial density and diversity may affect the rate and stability of antibiotic resistance acquisition events (Aminov, 2011). For example, gene transfer in soil has been considered more likely to occur under a high nutritional load, as can be found in manured soil or in the rhizosphere zone (Elsas and Bailey, 2006). These evidence suggests that soils enriched in nutrients, or chemically or physically disturbed, once exposed to ARB and genes of human or animal origin, may represent an ecosystem highly favourable for the dissemination of resistance. If these soils are used for the production of vegetables to be consumed raw, the eventual transmission to humans is a risk that should not be neglected (Scaccia et al., 2021).

1.1.3 Antibiotic resistance in soil and the link with the clinical environment

The occurrence of antibiotic resistance in the environment is nowadays of major concern due to the dissemination of bacteria and genes as environmental contaminants (Koch et al., 2021; Sanderson et al., 2016). Despite the wide and intense research efforts worldwide on antibiotic resistance, not much is known about the origins of the thousands of ARGs, reported nowadays as being of clinical relevance (Alcock et al., 2020; Bortolaia et al., 2020). Among the potential environmental reservoirs of acquired antibiotic resistance, the soil is a strong candidate (Lee et al., 2018; Nesme and Simonet, 2015; Riesenfeld et al., 2004). Soil holds a rich and diverse microbiome capable of competing and lessening the impacts of exogenous bacteria, including antibiotic resistant (Becerra-Castro et al., 2015). Nevertheless, when ARB settle in the soil ecosystem they can survive, accumulate and spread ARGs. This is likely to occur mainly when the exogenous ARB are closely related, phylogenetically and physiologically, to the native bacteria. Depending on their natural properties and history and current use, agricultural soils, those more subjected to external impacts, are reported to hold a microbiome mostly dominated by the phyla *Pseudomonadota* (~ 30-57%), *Actinomycetota* (~ 7.9-31%) and *Acidobacteriota* (~ 3.9-10.7) (Pershina et al., 2015; Sengupta and Dick, 2015; Tan et al., 2020). The microbial community composition is affected by soil characteristics, such as pH, temperature, moisture, plant diversity, nutrient availability, and soil carbon content decreasing or increasing the relative abundance of the different microbial groups (Castro et al., 2010; Cong et al., 2015; Leff et al., 2015; Trivedi et al., 2016). In soil and rhizosphere, depending on the plant species present, the microbiome may include ARB and human pathogens of genera such as *Pseudomonas, Acinetobacter*, or *Staphylococcus* (Berg et al., 2005; Sah and Singh, 2016). Human activities, like fertilization, manuring, and irrigation with water of inadequate quality, may affect the soil microbiome shifting the microbial communities (Li et al., 2017; Lopatto et al., 2019). In particular, it has been argued that these practices may introduce ARB or ARGs into the soil (Chen et al., 2019; Marti et al., 2013; Smith et al., 2019; Udikovic-Kolic et al., 2014). In parallel, manuring and irrigation with water of inadequate quality may introduce antibiotics, inorganic compounds, or other contaminants in soils (Guo et al., 2018; Xue et al., 2021). These contaminants have the potential to create a reservoir of antibiotic resistance and favourable conditions for the spread of antibiotic resistance in soil

(Fahrenfeld et al., 2013; Udikovic-Kolic et al., 2014). The transfer of antibiotic resistance from soil to humans is not convincingly documented, nevertheless, such a possibility should not be neglected. Contamination through a food source may increase the possibility that antibiotic resistance reaches humans, through the colonization of the gut microbial community (Chang et al., 2015). Ubiquitous bacteria that are abundant in soil are phylogenetically closely related to bacteria thriving in human-related environments (Grenni et al., 2018). This is a strong argument to regard the barriers between soil and human environments fragile in what refers to the transport of bacteria between both ecosystems. Indeed, bacteria of the genera *Pseudomonas*, *Acinetobacter,* or *Bacillus* belonging to *Pseudomonadota* and *Bacillota* phyla, are widely reported in healthcare units around the world, being simultaneously detected in soils (Barrie et al., 1994; Dohmae et al., 2008; Rampelotto et al., 2019). Although different species and strains may prefer distinct environments, this suggests that they can exchange the habitat when the conditions are favourable. It is of note that microbiome analysis carried out in hospital's environment (medical equipment, intensive care units, and workstation surfaces) revealed the presence of *Pseudomonadota* and *Bacillota* phyla (22-67% and 10-46%) as the most prevalent phyla (Chen et al., 2017; Poza et al., 2012). The contemporary presence of pathogens in soil and hospital environment warns about possible interaction or spread of soil-born bacteria to nosocomial facilities.

1.2. Soil contamination and antibiotic resistance: invasion and stimulation

1.2.1 Discharge of antibiotic-resistant bacteria in soil from anthropogenic activities

Soil contamination may be attributed to different practices and uses. Agriculture, in particular fertilization and nutrient exhaustion, may disturb soil properties (Bai et al., 2018; Wu et al., 2020). The use of animal manure and sewage treatment sludges for fertilization or treated wastewater for irrigation are important sources of ARB or ARGs in soils (Lima et al., 2020; Wang et al., 2014; Wu et al., 2020). Besides a possible organic pollution source, agricultural practices like irrigation with reclaimed water could be a response to the increasing water scarcity (Garcia and Pargament, 2015; Reznik et al., 2019; Shevah, 2014). Water scarcity is a worldwide issue, increasing over the last century and becoming a challenge to provide water with the required quality for agriculture and human consumption (J. Liu et al., 2017; Mancosu et al., 2015; Mancuso et al., 2020). Possible solutions include the use of renewable water sources that may tackle water scarcity and desertification even needing further and sensible improvement (Mancuso et al., 2020; Tortajada, 2020). Recently, the European Commission implemented regulations, aiming to improve the sustainable reuse of water. Aspects considered in these regulatory guidelines include the control of physical, chemical, and biological pollutants, among which antibiotic resistance is not considered (Helmecke et al., 2020; The European Commission, 2020). Wastewater treatment plants are designed to reduce organic matter, nutrients, pathogens, and other contaminants from sewage,

although have been regarded as insufficiently efficient to produce safe water, including in what refers to antibiotic resistance (Englande et al., 2015; Hong et al., 2018; Rizzo et al., 2013; Rodriguez-Mozaz et al., 2020). Advanced treatments like membrane filtration ensure the reduction of pollutants in water, respecting the parameters reported by legislation (Madsen, 2014; Nqombolo et al., 2018; Zielińska and Galik, 2017). Nevertheless, wastewater treatment plants are recognized sources of antibiotic resistance and human pathogens, including antibiotic resistant, like those of the genera *Acinetobacter*, *Enterobacter*, *Pseudomonas,* among others, and of a wide range of ARGs against all antibiotic classes, spanning from the first drug generations to last-resort antibiotics (Amador et al., 2015; Jäger et al., 2018; Narciso-da-Rocha et al., 2018; Pärnänen et al., 2019; Vaz-Moreira et al., 2016; Volkmann et al., 2004; Zhang et al., 2009). After wastewater treatment, most of the ARB and ARGs present in the treatment plants are successfully eliminated (Jäger et al., 2018; Zhu et al., 2021). However, while the number of gene copies decrease after treatment, the relative abundance is not always reduced (Pärnänen et al., 2019). For instance, the relative abundance of genes highly prevalent in the environment like *sul1* and the integrase encoding genes *intl1* may not decrease after treatment (Narciso-da-Rocha et al., 2018). These genes once discharged in wastewater may spread and/or accumulate in the environment, including in soil, when unintentionally, or in water reuse agriculture irrigation is implemented. Inconsistent results are reported in the literature regarding the risks of reclaimed water irrigation or sludge or manure amendments. This fact may be explained based on the diversity of the type of soil, the type of ARGs, or even the methodology and sampling sites used in the different studies (Gatica and Cytryn, 2013; Negreanu et al., 2012; Wang et al., 2014). Nevertheless, it is assumed as a real risk, associated with irrigation with reclaimed wastewater and other practices such as soil fertilization with manure or wastewater biological treatment sludges (Udikovic-Kolic et al., 2014; Zalewska et al., 2021). Widely used in agriculture, manuring process are highly beneficial for soil fertilization, increasing the nutritional content (Zhang et al., 2020). However, manure often is impacted by the fact that antibiotics are used in livestock for infection prevention or growth promotion (forbidden in Europe), hence with risk of an increased load of ARB (He et al., 2020; Heuer et al., 2011; Lima et al., 2020; McKinney et al., 2018; Williams-Nguyen et al., 2016) and also of high contents of antibiotics and/or metals (Marti et al., 2013; Udikovic-Kolic et al., 2014). Extended periods of manuring have been described as responsible for the increasing abundance of antibiotic resistance in soil (Marti et al., 2014; McKinney et al., 2018). For example, ARGs related to sulfonamide resistance (*sul1* or *sul2*) have been detected in manure and manure amended soil, along with other resistance genes of different families (e.g. tetracycline, β-lactam, or aminoglycoside) in doses ranging 5 to 10 log-units copies/g of wet soil (Macedo et al., 2020; Xu et al., 2020; Y. J. Zhang et al., 2019). Some studies show the simultaneous increase of *sul1* and the gene *intl1* (Lopatto et al., 2019; McKinney et al., 2018b; Wu et al., 2020), while others reported an increase in occurrence and mobilization of the plasmids (in particular IncP-1 plasmid group) in soil (Götz and Smalla, 1997; Heuer et al., 2012). The increase in the abundance of antibiotic resistance and plasmids raise particular concerns about the mobilization and the spread of antibiotic resistance in manured soil (Jechalke et al., 2014). These effects can be aggravated if combined with the also reported impacts on the soil microbial diversity (Riber et al., 2014; Ye et al., 2021). The continuous influence of human

activities in soil poses a serious question on the creation of antibiotic resistance reservoirs. Monitoring and determining the effective survival of ARB discharged in soil is crucial to assessing the real anthropogenic impacts.

1.2.2 Soil characteristics affecting antibiotic resistance survival

Soil microbial communities play an important role in the survival of exogenous bacteria. The microbial diversity is probably a major barrier (Van Elsas et al., 2012), as soils with reduced microbial diversity are permissive to the colonization by exogenous bacteria (Matos et al., 2005). Soil microbial diversity may be threatened by contaminants that result from human activities, including antibiotics or heavy metals (Fajardo et al., 2019; Huang et al., 2021; Jacquiod et al., 2018a; Song et al., 2018). Pollution was observed to be correlated with a microbiota shift and an increase in the antibiotic resistance occurrence in soil (Baker-Austin et al., 2006; Berg et al., 2005). The occurrence of antibiotics in soil has been reported to determine a decrease in *Pseudomonadota* and *Bacillota*, while did not affect or contributed to the increase the relative abundance of other phyla like *Actinomycetota* and *Acidobacteriota* (Cleary et al., 2016; Semedo et al., 2018). The shift of the soil microbial community has been described to be directly correlated with a variation in the ARGs distribution (Qian et al., 2021). Monitoring the microbial communities can contribute to a better understanding of the antibiotic resistance invasion and residence in soil. The degree of contamination is dependent on the pollution loads that are discharged or accumulated in the soil, and also on the capacity of the soil to attenuate the impacts, characteristics that vary with pedological and geographic conditions (FAO and UNEP, 2021). Biotic, abiotic stressors and geography have been reported as variables that may affect the survival of antibiotic-resistant bacteria in soil (Yan et al., 2021). Climate conditions affect the soil characteristics and microbial activity, mostly due to temperature and moisture. Temperature is an important variable that affects soil microbial community composition and also antibiotic resistance occurrence (Bárcenas-Moreno et al., 2009; Frindte et al., 2019). High temperatures (>20 °C) have been reported to cause a decrease in soil microbial communities diversity, and also a reduction in the abundance of antibiotic resistance and *intI1* genes (Dunivin and Shade, 2018). Soil moisture is impacting the microbial communities, although its effect on antibiotic resistance is still unclear (Borowik and Wyszkowska, 2016). Other soil characteristics, like salinity, were described to be correlated to a decrease of the antibiotic resistance in soil and a concurrent decrease of *Actinomycetota* relative abundance (Tan et al., 2019). Soil texture, is also an important variable as it influences nutrient retention, moisture, pH, and other characteristics that may affect the microbial communities and supposedly the degree of permissiveness for antibiotic resistance invasion (Armalytė et al., 2019; Jechalke et al., 2013). These arguments suggest that soil characteristics and microbial community composition may be good predictors of the risks of antibiotic resistance dissemination in soils.

1.2.3 Role of contaminants in antibiotic resistance dissemination in soil

Major soil contaminants include organic and inorganic substances that may affect the physicochemical properties (Ashraf et al., 2014; Hussain and Keçili, 2020; Sethi and Gupta, 2020). Pharmaceutical compounds, where antibiotics are included, may reach the soil via industrial or urban waste and have the potential to produce important and persistent impacts (Devesh and Dayaram, 2015; Gworek et al., 2021). Antibiotics can be emitted by urban wastewater effluents, animal (meat and fish) farming practices, or even crop agriculture, and have been considered relevant soil contaminants (Felis et al., 2020; Williams-Nguyen et al., 2016). Antibiotics mostly reported in soils due to human activities are sulfonamides, tetracycline, fluoroquinolones, and β-lactams, in concentrations ranging from 0 to 2000 mg kg^{-1} (Grenni et al., 2018). The contamination of soil with antibiotics has been reported to affect the microbiota while promoting the increase of ARB (Xu et al., 2021). Knapp et al. (2010) described an increase of tetracycline-related ARGs in contemporaneous soils when compared to soils archived since 1940, a fact the authors attributed to the "antibiotics era", which intensified after the second half of the 40s. In the soil analysed, besides the natural antibiotic resistance, genes conferring resistance to synthetic antibiotics were detected, suggesting the adaptation to new drugs (Walsh and Duffy, 2013). In summary, antibiotics may be discharged with ARB and/or may favour their subsistence, hence may influence their occurrence and persistence in soil, a role that may be also played by other contaminants like metals (Pal et al., 2017; Salam, 2020).

Metals like Cu, Cd, Ni, and Zn have been reported to affect the microbial communities and antibiotic resistance in soil (Chen et al., 2014; Hu et al., 2016; Huang et al., 2021; Song et al., 2018). In particular, a high concentration of Cu or Zn (> 200 mg kg^{-1} of soil) is reported to have the potential to affect the bacterial communities composition and diversity (Kelly *et al.*, 2003; Moffett *et al.*, 2003; Wakelin *et al.*, 2010; Song *et al.*, 2018). In metal contaminated soils, some taxonomic groups like members of the phyla *Pseudomonadota* and *Bacillota* were reported to be dominant, with increased abundance when compared to others (*Bacteroidota*) (Li *et al.*, 2017; Jacquiod *et al.*, 2018). Besides an impact on the microbial communities composition, heavy metals have been correlated with the occurrence of ARGs in soil (Berg et al., 2010; Hu et al., 2017; Ji et al., 2012; Wang et al., 2021; Zhang et al., 2018). For example, Cu and Zn were reported to promote an increase in the abundance of ARGs (e.g. tetracycline and sulphonamide resistance genes) present in contaminated fields (Berg et al., 2005; Ji et al., 2012; Knapp et al., 2017). The simultaneous presence of heavy metals and antibiotics in soil has been suggested as generating favourable conditions for the co-evolution or co-selection of metals-antibiotic resistance (Baker-Austin et al., 2006; Dickinson et al., 2019; Mazhar et al., 2021; Wang et al., 2021; Wang et al., 2020; Zhao et al., 2019).

Also, other contaminants, such as quaternary ammonium compounds, emitted by different types of pollution sources, can influence the occurrence of antibiotic resistance through integrons that also hold a gene that increase the tolerance to quaternary ammonium compounds (Buffet-Bataillon et al., 2012; Mulder et al., 2017; Zhang et al., 2015). Other contaminants, like pesticides, which use is

regulated in agriculture, were observed to be positively correlated with the increase of antibiotic resistance in soil (Carpio et al., 2020; Pose-Juan et al., 2017; Silva et al., 2019; Silva et al., 2018). These observations show how complex can be the prediction of the fate of antibiotic resistance discharged in soil, unintentionally or due to fertilization or irrigation practices.

1.2.4 Bacterial characteristics favouring the dissemination of antibiotic resistance in soils

Microorganisms and microbial communities have mechanisms to overcome external threats (Nesme and Simonet, 2015; Wang et al., 2020). For example, efflux pumps are mechanisms for extruding a broad range of inorganic molecules from bacterial cells (Alvarez-Ortega et al., 2010; Blanco et al., 2016). Organized in protein complexes, the efflux systems are membrane channels capable of conferring antibiotic resistance through a pumping mechanism that expels antibiotics from the cell (Blanco et al., 2016; Du et al., 2018; Sun et al., 2014). Widely distributed in bacteria, efflux pumps proteins are expressed by genes operons induced by effectors or constitutively expressed (Sun et al., 2014). The efflux pumps function is not limited to the reduction of antibiotics inside the cells, they are also involved in the extrusion of other substances like metals, solvents, or detergents (Alvarez-Ortega et al., 2010; Ebbensgaard et al., 2020), being possibly key features for bacterial survival in soil. Biofilm formation is another mechanism reported in bacteria to overcome stress in soil and promote survival through cell protection (Cai et al., 2019; Wu et al., 2019). Biofilms are matrices of bacteria surrounded by extracellular polymeric substances representing the physical scaffold (Flemming et al., 2016). The biofilm formations guarantee an enhanced survival rate for the bacteria compared to free-living cells due to metabolic advantages (Wu et al., 2019). Also, biofilms may enhance the survival of soil bacteria in presence of contaminant metals or high salinity (Bybin et al., 2021; Yin et al., 2019). Soil biofilms have been suggested as antibiotic resistance niches favouring the spread of ARGs through bacterial conjugation (Balcázar et al., 2015; Wu et al., 2021). Microbial communities organized in biofilm structures may have an increased capacity to face pollution in soil and survive in hostile conditions (Koechler et al., 2015; Lee et al., 2014; Yin et al., 2019). These strategies may allow the survival of metal and antibiotic co-resistance in polluted soils.

1.3. Culture versus genomic insights to explore soil microbiota

Human activities have the potential to strongly influence the assembly of microbial communities, specifically affecting their composition and structure (Flandroy et al., 2018; Gupta et al., 2017; Lenart-Boroń and Boroń, 2014; Zhang and Xu, 2008). Understanding the dynamics and composition of microbial communities in soil may be useful to decipher and preserve complex environments. Culture-dependent and independent methods have been applied, often in combination, in the study of microorganisms characteristics, distribution, and diversity in different environments and matrices

(Jin et al., 2011; Li et al., 2019). Culture-dependent methods are used to enumerate, isolate and characterize a wide, although limited, diversity of microorganisms (Davis, 2014; Ifeanyi, et al., 2014). Improvements in culture-dependent methods included the use of selective media and enrichment growth conditions that favour specific groups of bacteria (Erkmen, 2021; Vandeplassche et al., 2017). Culture-dependent methods are highly valuable, but the incapacity to culture most environmental microorganisms and the technical and labour efforts required, have motivated the use of alternatives (Alain and Querellou, 2009; Ellis, 2003). The major limitation of culture-dependent methods to unveil bacterial diversity resides in the low percentage of culturable bacteria, commonly referred to be 1%, but strongly dependent on the environment, and conditions used, among others (Kralik and Ricchi, 2017; Steen et al., 2019; VanGuilder et al., 2008; Vaz-Moreira et al., 2011; Wong et al., 2015). To study the unculturable fraction of microbial communities molecular techniques are indispensable and permit not only the assessment of the diversity but also the profiling or quantification of genes (Gupta, 2019). Molecular biology techniques like polymerase chain reaction (PCR) and the derivate quantitative PCR (qPCR) provide quantitative data based on genetic information (Deepak et al., 2007; Frey, 2003; Gupta, 2019; Mullis et al., 1986). PCR is mainly used for the gene detection and/or quantification of bacterial DNA or genomic material extracted from a matrix analysed (Bej et al., 1991; Ghatak et al., 2013; Yeates et al., 1998).

Quantitative PCR, using fluorescent dyes or probes, allows monitoring the targeted genes in real-time, representing a gold standard in quantitative microbiology. Based on a standard curve of known concentrations and the determination of amplification cycles needed to detect an amplicon, it is possible to estimate the abundance of a target gene in a test sample (Adams, 2020; Kralik and Ricchi, 2017). The qPCR technique is commonly used in the quantification of genes harboured by bacteria like antibiotic resistance and housekeeping genes (Colinon et al., 2013; Galazzo et al., 2020; Ogier et al., 2019). However, the potential presence of qPCR inhibitors, present in complex matrices like soil or due to the DNA extraction process, may occur and it is a bias that needs to be addressed when qPCR is used to quantify genes in environmental samples (Combs et al., 2015; Luby et al., 2016; Sidstedt et al., 2015; Watson and Blackwell, 2000). Inhibition may be overcome through DNA extract dilution and inhibition effect quantification (Acharya et al., 2017; Wang et al., 2017). The digital droplet PCR (ddPCR), a recent upgrade of the qPCR, does not require standard curves and present reduced limits of quantification (Cavé et al., 2016; Hindson et al., 2011, 2013). The use of ddPCR is promising for massive monitoring processes (Manoj, 2014).

To investigate the microbial diversity in soil, the microbiome analysis based on 16S rRNA gene sequence analysis represents a simplified and suitable method (Sergaki et al., 2018). In this method, the 16S rRNA gene, highly conserved and present in all prokaryotes, is amplified and based on its sequence, amplicons with high sequence identity (>97%) are classified as operational taxonomic units (OTU), whose identification is made based on the query of taxonomy databases like SILVA or Greengenes (Balvočiūtė and Huson, 2017; Buermans and den Dunnen, 2014; Ju and Zhang, 2015; Quast et al., 2013). Despite its convenience and popularity, the assessment of the bacterial community diversity based on the 16S rRNA gene sequencing has low taxonomic resolution and

important biases related to unequal 16S rRNA gene sequence amplification among taxa or poor resolution among close phylogenetic neighbours (Bharti and Grimm, 2021; Fanning et al., 2017; Straub et al., 2020). Other techniques based on DNA sequence analyses allow the characterization of the metagenome that relies on the assembly, alignment, and comparison to public databases of the whole genetic information of a microbiome, to infer gene and function diversity (Chauhan, 2019; Lam et al., 2015). Different pipelines are available for microbiome analyses that involve some standard steps such as, removing barcodes, denoising, checking the sequence quality, and assigning the taxonomic units (examples are tools like QIIME2, Bioconductor, DADA2) and metagenome analyses that include checking the sequence quality, cleaning the reads and profile taxonomically and functionally (using tools like Kraken, CLARK, MEGAHIT, metaSPAdes, Prokka, metaGeneMark) (Liu et al., 2021). The selection of distinct methods, pipelines, and query databases may lead to divergent estimations of the microbiome and metagenome composition and structure (Marizzoni et al., 2020; Siegwald et al., 2017). In addition to the approaches mentioned, other analytical methods, like metatranscriptomics, metaproteomics, or data exploitation, like metagenome-assembled genomes (MAGs) illustrate interesting complements used in microbiome studies. Metatranscriptomics provides a functional profile, based on genes being expressed, through the analysis of cDNA (reversely transcribed from mRNA) high throughput sequencing (Aguiar-Pulido et al., 2016; Shakya et al., 2019). The deduced amino acid sequences annotated using different databases (e.g. KEGG or EggNOG) of metagenomic or metatranscriptomic analyses help to profile the functional characteristics of the microbial community (Aguiar-Pulido et al., 2016; Leimena et al., 2013; Niu et al., 2018). A more comprehensive study is represented by MAGs analysis, giving an overview of whole genomes that can be retrieved from the whole microbial community metagenome (Meziti et al., 2021). The described methods, even presenting biases and limitations, offer renewed opportunities to investigate microbial diversity and function considering the phylogeny and external conditions.

1.4. *Pseudomonas aeruginosa*: ubiquity and antibiotic resistance

P. aeruginosa is a Gram-negative ubiquitous bacterial species, able to adapt to different environments including water, soil, and the clinical environment worldwide (Frimmersdorf et al., 2010; Moradali et al., 2017). The wide distribution of *P. aeruginosa* is due to the presence of different adaptative mechanisms like quorum sensing, biofilm formation, metal resistance, high-affinity transport, virulence, and antibiotic resistance (Abdelbary et al., 2019; Mohanty et al., 2021; Teitzel and Parsek, 2003; Vanderwoude et al., 2020; Wylie and Worobec, 1993). Often found in natural and anthropogenic water environments, the occurrence of *P. aeruginosa* in wastewater and their persistence after treatment can contribute to its distribution in downstream systems (Mena and Gerba, 2009; Slekovec et al., 2012). Naturally present in the soil, *P. aeruginosa* has been recovered

from agricultural fields as well as from vegetable products and cultivated plants (Green et al., 1974; Ruiz-Roldán et al., 2021). Moreover, strains infecting humans in clinical facilities were described as being able to successfully infect plants of agricultural interest (Schroth et al., 2018). In health care facilities, *P. aeruginosa*, as an opportunistic pathogen, is responsible for life-threatening infections, particularly severe in patients with compromised immune defences and specific pathologies such as cystic fibrosis or extensive burns (Lyczak et al., 2000). Infections caused by *P. aeruginosa* may be challenging to treat due to the intrinsic and acquired capacity to resist antibiotics of different classes (Azam and Khan, 2019; Lister et al., 2009; López-Causapé et al., 2018). The intrinsic antibiotic resistance, corresponding to the innate capacity to maintain cell activity and viability in the presence of antibiotics, is represented in *Pseudomonas* by efflux pump systems, reduced outer membrane permeability or drug inactivation (Blair et al., 2015; Breidenstein et al., 2011). In particular, efflux pump systems, mostly belonging to the resistance-nodulation-division (RND) family, were recognized as key factors in the *Pseudomonas* spp. antibiotic resistance (Li and Nikaido, 2009; Zahedi bialvaei et al., 2021). Within the RND family, the multidrug efflux (Mex) proteins are commonly involved in the antibiotic resistance being *MexAB-OprM* responsible for the efflux of quinolones and β-lactams, *MexCD-OprJ* of β-lactams, *MexEF-OprN* of quinolones and *MexXY-OprM* of aminoglycosides (Masuda et al., 2000; Pang et al., 2019; Poole, 2001). The overexpression of *mex* genes is also possible due to mutational events that lead to increased minimum inhibitory concentrations (MIC) for a wide range of antibiotics commonly used in medicine (Baum et al., 2009; López-Causapé et al., 2018; Shigemura et al., 2015). Similarly, the expression of outer membrane porins (Opr) represents an important mechanism of intrinsic antibiotic resistance in *P. aeruginosa,* preventing antibiotic penetration into the cells (Pang et al., 2019). The overexpression of the gene *oprH,* regulated by *PhoP-PhoQ* that may be associated with Mg^{2+} starvation, was observed to be correlated with the resistance to polymyxin B (Macfarlane et al., 1999). However, also the downregulation or the absence of an *opr* gene (*oprD*) was observed to increase the MIC of β-lactam antibiotics (Li et al., 2012). Another intrinsic antibiotic resistance mechanism is represented by the production of antibiotic degrading enzymes (Alvarez-Ortega et al., 2011). *P. aeruginosa* is known to produce enzymes that are directly or indirectly involved in the disruption of β-lactams and aminoglycosides, preventing their antimicrobial activity (Wolter and Lister, 2013; Zhao and Hu, 2010). For example, a*mpC* genes typically located on the chromosome encode cephalosporinases, which mediate the resistance to a broad variety of β-lactams antibiotics (Jacoby, 2009; Tamma et al., 2019). The known metabolic versatility of *P. aeruginosa* is also illustrated by adaptive resistance processes, through which efficient gene expression regulation responds to external stimuli (Sandoval-Motta and Aldana, 2016). A good example is the intensification of biofilm formation after antibiotic exposure reported in different bacteria, including *P. aeruginosa,* which leads to an increase in the MIC for antibiotics of distinct classes (Ciofu and Tolker-Nielsen, 2019; Taylor et al., 2014).

Gene mutation has been also described as a driver for broadening antibiotic resistance in *P. aeruginosa* (López-Causapé et al., 2018). Point mutations in the *gyrA* and *parC* genes, determine a decreased affinity of quinolones with DNA gyrase and Type II topoisomerase proteins (Van Nguyen et al., 2018; Yonezawa et al., 1995). Besides the possible effects on drug targets, as occurs in DNA

gyrases, the mutation of regulatory genes like *parRS* or *amgS* and *nalC* or *nalD*, have been reported to determine the overexpression of *MexXY* and *MexAB*, respectively, leading to aminoglycoside and β-lactams resistance in *P. aeruginosa* (Guénard et al., 2014; Llanes et al., 2004; Sadeghifard et al., 2012; Yan et al., 2019). However, the acquired resistome of *P. aeruginosa* is mainly associated with the transfer of ARGs from or to other microorganisms (Breidenstein et al., 2011). *P. aeruginosa* can acquire ARGs through HGT, involving conjugation, transduction, and transformation (Lerminiaux and Cameron, 2019; Nolan et al., 2020; Wintersdorff et al., 2016). In particular, the transfer of ARGs related to β-lactams, aminoglycosides, and sulfonamides to *P. aeruginosa* through HGT, has been reported, often as part of the same mobile genetic element (Poirel et al., 2001a; Rada et al., 2021). Among these genes, the carbapenemase encoding bla_{VIM}, bla_{IMP} or bla_{NDM}, can be of particular concern as they confer resistance to a valuable therapeutic antibiotic class and are associated with outbreaks in the last years (Queenan and Bush, 2007; Tavoschi et al., 2020).

1.4.1 Carbapenemases and the respective encoding genes

Carbapenems are β-lactam antibiotics, targeting the penicillin-binding protein (PBP), presenting the broadest spectrum of action and higher resistance to β-lactamases compared to other β-lactam antibiotics (Papp-Wallace et al., 2011). Widely used in clinical treatments, β-lactams are actively hydrolysed by different β-lactamases including penicillinase or oxacillinases, able to disrupt the β-lactam ring but not effective in hydrolysing the carbapenems ring presenting a carbon atom instead of a sulfur in position 1 (Figure 1.1) (Papp-Wallace et al., 2011). Carbapenems are largely used to treat infections of Gram-negative bacteria like *P. aeruginosa*, *Klebsiella pneumoniae* or *Acinetobacter baumannii*, which evolved or acquired carbapenemases encoding genes (Zhanel et al., 2007).

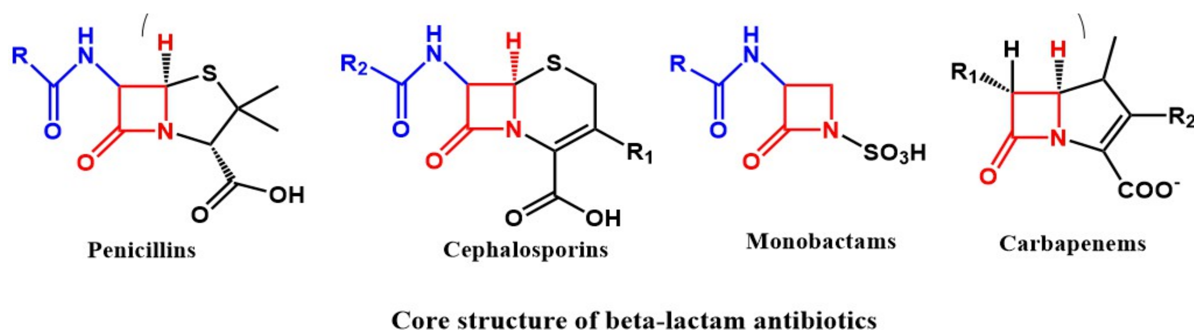

Figure 1.1 Beta-lactams molecular structures.(source: http://ringbio.com/press-release/introduction-of-beta-lactams-antibiotics)

Carbapenemases are serine- or metallo-enzymes capable of hydrolysing the double cyclic core of different extended-spectrum β-lactams antibiotics including imipenem and meropenem, widely used for clinical treatment (Queenan and Bush, 2007; Zhanel et al., 2007). The carbapenemases enzymes have been classified into three main classes (class A, B and D) and further subclasses were defined based on sequence homology (Ambler, 1980; Palzkill, 2013; Rasmussen and Bush, 1997). Classes A and D carbapenemases present a serine residue in the active site while class B are metallo-β-lactamases including a zinc atom in the active site (Frère et al., 2005; Queenan and Bush, 2007). The carbapenemases in Gram-positive and Gram-negative bacteria are encoded by a group of genes that can be located in plasmids or in chromosomes. In Gram-positive bacteria, carbapenem resistance is mild due to the low affinity to penicillin-binding protein2 (PBP2) encoded by genes such as the *mecA* often found in *Staphylococcus aureus* (Gajdács, 2019). Carbapenemases encoding genes are widely diffused in opportunistic pathogens, most of which may harbour ARGs from different classes as shown in Table 1.1.

The class A carbapenemases, actively hydrolysing penicillins, cephalosporins and carbapenems like monobactam, imipenem and meropenem, were described in different opportunistic pathogenic bacteria (Walther-Rasmussen and Høiby, 2007). These carbapenemases are encoded by different ARGs having located in chromosomes or plasmids. For instance, bla_{KPC} and bla_{GES} are often included in MGEs harboured by opportunistic pathogens *K. pneumoniae* and *P. aeruginosa*, indicating their possible mobilization (Chen et al., 2016; Du et al., 2021; Firoozeh et al., 2016; Poirel et al., 2001b; Valdemir et al., 2017). Class D carbapenemases, also known as oxacillinases, hydrolyse isoxazolyl penicillins and are less effective in cephalosporine hydrolyzation compared to the other classes (Walther-Rasmussen and Høiby, 2007). The oxacillinase enzymes are encoded by genes bla_{OXA}, a large family of genes including the bla_{OXA-10} family responsible for extended-spectrum β-lactams resistance, originally identified in *P. aeruginosa* and the carbapenemase encoding genes bla_{OXA-23} and bla_{OXA-58} described in different opportunistic pathogens (Table 1.1) (Evans and Amyes, 2014; Verma et al., 2011). Among the carbapenem resistance genes, the ARGs belonging to class B coding for enzymes with an extended spectrum of resistance to carbapenems, cephalosporins and penicillins (Queenan and Bush, 2007). Sub-categorized in B1 and B2 due to the zinc atom position, the class B carbapenemases encoding genes are worldwide diffused and actively spread between microorganisms (Walsh, 2005; Walsh et al., 2005). Of particular concern, the genes bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{GIM} and bla_{SIM} are diffused across different countries and opportunistic pathogens like *P. aeruginosa, K. pneumoniae* and *A. baumannii* (Table 1.1) (Codjoe and Donkor, 2018; Diene and Rolain, 2014; Queenan and Bush, 2007; Yoon and Jeong, 2021). The majority of the carbapenemases encoding genes have been observed to contribute to the wide and spreadable resistome of *P. aeruginosa* (Yoon and Jeong, 2021).

Since the first detection in 1991 in a *P. aeruginosa* plasmid, the bla_{IMP} gene that encodes imipenem hydrolysing enzymes has diffused worldwide in different microorganisms (Arakawa et al., 1995; Hanson et al., 2006; Riccio et al., 2000; Watanabe et al., 1991). Similarly, the bla_{VIM} gene, included in a class-I integron, was primarily described in a *P. aeruginosa* and has been subsequently observed

in MGEs like plasmids favouring its worldwide diffusion (Botelho et al., 2017a; Lauretti et al., 1999; Tato et al., 2010; Walsh, 2005). An emerging class B carbapenemase encoding gene, bla_{NDM-1}, firstly isolated in *K. pneumoniae* was reported in different countries associated to MGEs and in different microorganisms like *P. aeruginosa* (Carattoli et al., 2013; Dortet et al., 2014; Jovcic et al., 2011; Yong et al., 2009; Yoon and Jeong, 2021).

Harbouring carbapenemases encoding genes of class A (bla_{GES} and bla_{KPC}), class B (bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{GIM} and bla_{SIM}) and class D (several bla_{OXA} genes) in the chromosome or plasmids, *P. aeruginosa* is a crucial organism in the evolution and dissemination of these ARGs (Botelho et al., 2018c; Tato et al., 2010; Yoon and Jeong, 2021). In addition, *P. aeruginosa* may co-harbour different carbapenemase encoding genes like bla_{GES}, bla_{NDM} and bla_{VIM}, often in the same genomic environment, posing a serious concern for infection treatments and ARGs spread (Pacheco et al., 2019; Paul et al., 2016, 2015; Rada et al., 2021). A genomic approach and extensive analysis of carbapenems resistance in *P. aeruginosa* may represent a valuable contribution to better understanding the ecology and dissemination of these genes.

1.4.2 Mobile genetic elements (MGEs) associated with ARGs in P. aeruginosa

The gene mobilization through HGT contributes to the evolution of *P. aeruginosa*, being mostly associated with virulence and ARGs acquisition and spread (Freschi et al., 2019; Frost et al., 2005; Qiu et al., 2009; Valot et al., 2014). Intracellular gene mobilization promotes gene integration in the genome through insertion sequences (IS), transposons (Tn), or integrons (In). Intercellular mobilization consists of the exchange of genetic material between cells and can be mediated by MGEs like plasmids and integrative conjugative elements (ICEs). Transposons (Tn) are genetic elements encoding a transposase allowing the mobilization of this element in the genome (Muñoz-López and García-Pérez, 2010). Insertion sequences (IS) are short DNA sequences, often included in transposons, promoting the mobilization inside a genome or between genomes through a recombination event (Chandler and Siguier, 2013). Transposons may include an integron (In) and genes encoding an integrase (e.g. *intI*) that promote the integration of genes in an integron-associated recombination site (*attI*) (Boucher et al., 2007; Cambray et al., 2010; Partridge et al., 2000). Transposons can be found both in bacterial chromosomes or in plasmids, circular DNA molecules transferable between bacteria (Babakhani and Oloomi, 2018; Clark et al., 2019). In bacterial chromosomes, transposons can be included in a mobile environment, the integrative conjugative elements (ICEs) (Botelho et al., 2018c; Johnson and Grossman, 2015). Both plasmids and ICEs are mobile elements, in some cases self-transferable, requiring the presence of a relaxase protein that nicks the DNA in a specific site (origin of transfer, oriT) resolving the double-strand and starting the transfer (Smillie et al., 2010a; Waldor, 2010). Conjugative plasmids and ICEs mobilization involve direct physical contact between the cells and a consequent transfer of these genetic elements (Smillie et al., 2010a; Waldor, 2010). In *P. aeruginosa* ICEs mostly belong to the families ICE_{Tn4371},

$ICE_{pKLC102}$ and ICE_{clc}, the last described as involved in carbapenems resistance genes mobilization (Botelho et al., 2018c; Botelho and Schulenburg, 2021). Plasmids of the incompatibility groups IncH, incF, and incP-type (often observed in *Enterobacteriaceae*) represent the most commonly described plasmids carrying ARGs in *P. aeruginosa* (Carattoli, 2013; Rozwandowicz et al., 2018). In particular, IncP group plasmids, frequently harboured by *P. aeruginosa,* were described to include carbapenemase genes like bla_{VIM}, bla_{IMP} or bla_{SIM} (Botelho et al., 2019, 2017b). In recent years, novel large plasmids, mostly non-conjugative, carrying ARGs and specific carbapenems resistance genes like bla_{VIM}, bla_{IMP} or bla_{OXA} were described in *P. aeruginosa* (Botelho et al., 2017b, 2017a; Pilato et al., 2019; San Millan et al., 2015) (Table 1.1). Most of the ARGs transferred by IncP group plasmids or ICE_{clc} and ICE_{Tn4371} were often described as being associated with MGEs like transposons or integrons (Botelho et al., 2018a; Heuer et al., 2012; Popowska and Krawczyk-Balska, 2013; Toleman and Walsh, 2011). One of the main transposon families responsible for ARGs mobilization in *P. aeruginosa* is the Tn*3*, typically flanked by inverted repeats (IR) and containing a transposase *TnpA,* which promotes the transposition of the element often including β-lactam resistance genes (Table 1.1) (Gömez-Lus, 1998; Nicolas et al., 2017, 2015; Pacheco et al., 2019). Also part of Tn*3* family, Tn*21* transposons are characterized by the presence mercury resistance operon (*mer* genes) and often are observed to include carbapenem resistance genes (Table 1.1) (Essa et al., 2003; Liebert et al., 1999; Schmidt et al., 1988; Zee et al., 2018). Another transposon part of Tn*3-family* associated with carbapenem resistance is the Tn*402-like*, characterized by a Tni module (*TniA* transposase, *TniB, TniQ,* and *TniR*) (Betteridge et al., 2011; Gillings et al., 2009; Marchiaro et al., 2010). In *P. aeruginosa,* transposon Tn*402-like* and Tn*21* are among the elements most frequently associated with the ARGs dissemination, particularly carbapenem and aminoglycosides resistance genes (Partridge et al., 2018; Pilato et al., 2019). Composite transposons (e.g. Tn*6* family, with flanked insertion sequences as the IS-*6 like* family), associated with ARGs mobilization in *P. aeruginosa* often include integrons incorporating as passenger genes β-lactams, aminoglycoside, or sulfonamide resistance genes (Coyne et al., 2010; Tseng et al., 2007). Insertion sequences described in *P. aeruginosa*, identified as IS*Pa*, were described as flanking transposons or genomic environments including carbapenemase resistance genes (Table 1.1) (Botelho et al., 2017a; Janvier et al., 2013; Llanes et al., 2004). Class-I integron may include carbapenem resistance genes and be captured by transposons as Tn*21* and Tn*402-like*, as well as flanked by IS*6-like* (IS*6100*) insertion sequences, often associated with the spread of ARGs like bla_{VIM} and bla_{NDM} (Gillings et al., 2015, 2009; Marchiaro et al., 2010; Partridge et al., 2001; Stalder et al., 2012; Suzuki et al., 2019; Targant et al., 2010). In summary, the mobilization of ARGs involves different and diverse mechanisms promoting the enrichment of bacterial genomes with novel resistance features and the occurrence of multidrug resistance phenotypes. To properly face the worsening antibiotic resistance problem, particular attention should be given to MGEs associated with ARGs (Table 1.2) as a key factor in their dissemination in different environments.

Table 1.1 Carbapenem resistance genes reported in Gram-negative opportunistic pathogens.

In table is reported the ARGs prevalence (%) in the analysed gram-negative bacteria retrieved from CARD database. Moreover, are reported the bibliographic reference to works reporting isolates harbouring the ARGs of interest.

Carbapenem resistance genes	Prevalence (%)				**References**
	P. aeruginosa	*K. pneumoniae*	*E. coli*	*A. baumannii*	
Class-A					
bla_{GES}	*3.1*	*3.5*	*<1*	*<1*	(Firoozeh et al., 2016; Kirtikliene et al., 2021; Poirel et al., 2001b; Ramadan et al., 2018)
bla_{KPC}	*16.3*	*32.5*	*<1*	*<1*	(Du et al., 2021; Hazen et al., 2018; Pacheco et al., 2019; Shealy et al., 2020)
$bla_{CTX\text{-}M}$	*<1*	*18.2*	*16.8*	*3.1*	(Cantón et al., 2012; Eskandari-Nasab et al., 2018; Khalilzadegan et al., 2016; Ramadan et al., 2018)
bla_{SHV}	*72.9*	*<1*	*<1*	1.8	(Hammond et al., 2005; Peymani et al., 2017; Pishtiwan and Khadija, 2019)
Class-B					
bla_{SIM}	*<1*	*<1*	*<1*	*<1*	(Emara et al., 2020; Lee et al., 2005; Lü et al., 2019)
bla_{VIM}	*16.7*	*2.6*	*<1*	*<1*	(Botelho et al., 2018b; Kirtikliene et al., 2021; Murugan et al., 2019; Psichogiou et al., 2008)
bla_{IMP}	*4.5*	*1.4*	*<1*	*<1*	(Abe et al., 2020; Hashemi et al., 2017; Kirtikliene et al., 2021; Stoesser et al., 2016)
bla_{GIM}	*<1*	*0*	*0*	*0*	(Castanheira et al., 2004)
bla_{NDM}	*12.5*	*8.9*	*2.6*	*8.9*	(Janvier et al., 2013; Kirtikliene et al., 2021; Sun et al., 2019; Xiang et al., 2020)
Class-D					
$bla_{OXA\text{-}23}$	*<1*	*<1*	*<1*	*48.6*	(Das et al., 2020; Kirtikliene et al., 2021; Paul et al., 2017; Ramadan et al., 2018)
$bla_{OXA\text{-}58}$	*0*	*0*	*<1*	1.4	(Poirel et al., 2005a)
$bla_{OXA\text{-}10}$	*13.8*	*1.1*	*<1*	*<1*	(Kotsakis et al., 2019; Odumosu et al., 2016)

Table 1.2 Main MGEs associated with carbapenem resistance in *P. aeruginosa.*

In the table are reported the principal MGEs associated to β-lactamases encoding genes retrieved from NCBI database. For each MGEs are reported genetic information and bibliography references of some isolated strains harbouring these elements including ARGs.

MGEs	N° of strains (in NCBI database) harbouring the MGEs	Mobilization mechanism	Genomic location	Mobilization protein	β-lactamases encoding genes	reference
Plasmids						
IncP-2	301	Intercellular	Plasmid	Tra proteins	bla_{IMP}, bla_{VIM}, bla_{SIM}	(Botelho et al., 2017b; Zhang et al., 2021)
IncP-6	6				bla_{KPC}	(Dai et al., 2016)
p-NOR 2000	4				bla_{VIM}	(Bonnin et al., 2013)
pjB12	2				bla_{VIM}	(Botelho et al., 2017a)
pBM413	2				bla_{IMP}, bla_{OXA}	(Liu et al., 2018)
pAMBL1	1			MOB proteins	bla_{VIM}	(San Millan et al., 2015)
pAMBL2	1				bla_{VIM}	
pMOS94	1				bla_{VIM}	(Pilato et al., 2019)
ICEs						
$ICE_{pKLC102}$	11	Intercellular	Chromosome	Tra proteins	*None*	Klockgether et al., 2004)
ICE_{Tn4371}	8				bla_{SPM}, bla_{NDM}, bla_{KPC}	(Botelho et al., 2018c; Fonseca et al., 2015)
ICE_{clc}	8				bla_{VIM}, bla_{GES}, bla_{DIM}, bla_{IMP}	(Botelho et al., 2018c)
ICE_{Tn6417}	1			rlx protein	bla_{GES}, bla_{CARB}	(Yu et al., 2021)
Transposons						
Tn3	340		Chromosome or plasmid	TnpA	bla_{VIM}, bla_{KPC}	(Botelho et al., 2017b; Galetti et al., 2016)
Tn402-like	271			TnpA	bla_{VIM}, bla_{OXA}	(Marchiaro et al., 2010)
Tn21	216			TniA	bla_{VIM}	(Samuelsen et al., 2010; Shi et al., 2018)
Tn1403	*31*		Chromosome	TnpA	bla_{PER}, bla_{CARB}, bla_{OXA},	(Yu et al., 2021)
Tn501	11		Chromosome or plasmid		bla_{VIM}	(Perez et al., 2014)
Tn6061	11		Chromosome		bla_{VEB}, bla_{OXA}	(Coyne et al., 2010)
Tn*4401b*	*7*				bla_{KPC}	(Abril et al., 2019)
Tn6060	*2*				bla_{VIM}	(Chowdhury et al., 2009)

Tn2521	*2*	Intracellular	Plasmid	TniA	*bla*$_{SPE}$	(Partridge et al., 2002)
Tn6001	*1*			TnpA	*bla*$_{VIM}$	(Tseng et al., 2007)
Tn6352	*1*			TniA	*bla*$_{VIM}$	(Botelho et al., 2017a)

Insertion sequences

IS6100	*205*	Intracellular	chromosome or plasmid	IS6100	*bla*$_{VEB}$, *bla*$_{OXA}$, *bla*$_{VIM}$	(Zee et al., 2018)
ISPa7	*124*		chromosome	ISPa7	*bla*$_{NDM}$	(Janvier et al., 2013)
ISPa17	*9*		plasmid	IsPa17	*bla*$_{VIM}$	(Botelho et al., 2017a)
ISPa23	*1*		chromosome	ISPa23	*bla*$_{PER}$	(Llanes et al., 2006)
ISPa24	*1*		chromosome	ISPa24	*bla*$_{PER}$	

Integron

Class-1-integron	*1727*	Intracellular	chromosome or plasmid	*intI1*	*bla*$_{VIM}$	(Botelho et al., 2018a; Pilato et al., 2019)

1.5. Hypotheses and objectives

Human activities like agricultural irrigation with treated wastewater may contribute to enriching ARGs in soils, whose persistence, mainly if harboured by ubiquitous bacteria, may be enhanced by specific physicochemical conditions. However, the current literature suggests that even if present, antibiotic resistance may be unnoticed in soils, due to the low levels at which they may persist. These facts established the background to design the thesis hypotheses. It was hypothesized that:

1) due to matrix effects, DNA extraction limitations, and qPCR analysis, the limits of quantification of ARGs might impede the reliable assessment of impacts of discharges in soil.

2) an antibiotic resistant strain of the ubiquitous species *P. aeruginosa* harbouring the carbapenemase encoding gene bla_{VIM-2} and isolated from wastewater might be able to survive in soil, and metals might favour that process.

3) carbapenem-resistant *P. aeruginosa* due to the gene bla_{VIM-2}, common in this species and not in others, and bla_{NDM-1}, observed in diverse lineages of *Gammaproteobacteria*, would exhibit distinct molecular epidemiologic and geographic patterns, suggesting that the first may be more fitted to environmental settings than the latter.

To test the hypotheses, the work was organized aiming to:

a) assess the limit of quantification for ARGs in soil microcosms assays under controlled conditions;

b) infer about the influence of external factors (e.g. metals salts) on the survival of a bla_{VIM-2}+ *P. aeruginosa* strain in soil, using microcosms assays incubated up to 30 days;

c) compare the *P. aeruginosa* genome characteristics of bla_{VIM-2}+ and bla_{NDM-1}+ strains, mainly regarding functional categories present in the accessory genome and the mobile genetic elements associated with those genes.

2 Thesis roadmap

Antibiotic resistance is a global problem with important implications on human health, with causes that span from socio-economic factors to environmental pollution. ARB are not limited to nosocomial contexts, being described in several natural environments, such as soil or water. In soil, mostly when used for agricultural production, antibiotic resistance is a combination of natural and acquired properties, the latter due to human impacts, like soil fertilization and irrigation. Soil pollution and degradation may create physicochemical conditions, such as metals and salinity accumulation, that may influence the survival and the occurrence of antibiotic resistance in soil. The survival of bacteria under these conditions, the technical limitations that we may face to assess antibiotic resistance contamination in soils, and the interplay between bacterial genome and epidemiologic distribution were the drivers to design the experimental work, centred on carbapenem-resistant *Pseudomonas aeruginosa*.

Chapter 3 aimed to determine the limits of quantification of ARGs in soil samples based on qPCR, the most commonly used method for absolute quantifications. Soil microcosms were inoculated with known amounts of bacteria - *Escherichia coli* strain A1FCC2 harbouring the *qnrS* and bla_{TEM} genes, *E. coli* strain A2FCC14 harbouring bla_{OXA}, bla_{TEM} and *intI1, E. faecalis* H1EV10 *vanA*, *A. johnsonii* H1PC5 bla_{IMP}, bla_{OXA} and *intI1, P. aeruginosa* H1FC49 harbouring bla_{VIM-2} and *intI1*. The inoculum consisted of a bacterial suspension, whose density was confirmed based on cultivation, added and homogenized in soil microcosms (in triplicate) to obtain colony-forming units between 10^7 and 10^2 CFU/g of dry soil. Each microcosm was sacrificed for DNA extraction and qPCR analyses. Two DNA extraction methods were compared. The plotting of inoculated bacteria versus quantified ARGs revealed the limits of quantification, ranging 1.1×10^4 and 1.4×10^5 gene copies/g of dry soil, and which were not reverted by the use of higher soil amounts for DNA extraction. The reasons for the high quantification limits are mainly due to DNA extraction, rather than the qPCR process and may explain the controversial reports in the literature about the impacts of manure or wastewater on soils. This study was published as “A rationale for the high limits of quantification of ARGs in soil” (Fortunato et al., 2018).

In Chapter 4, the strain of *P. aeruginosa* H1FC49 harbouring the bla_{VIM-2} gene, isolated from hospital effluent (Vaz-Moreira et al., 2016), was used to test the survival and carbapenemase encoding gene maintenance in soil, in the absence or presence of 20 mM copper and zinc as sulfate or nitrate salt forms. The use of the two types of anion aimed to test if besides the metal, also the salt could influence the potential selective effect. The results evidenced the capacity of that strain to survive up to 30 days in soil, with significant decreases over the first days of incubation. The observed decreases were suggested to be due to cellular decay as were proportional to the gene bla_{VIM-2} and the *P. aeruginosa* housekeeping gene extracytoplasmic function sigma factor (*ecf*). Metals and the respective salts were observed to produce mild effects on *P. aeruginosa* H1FC49 survival and bla_{VIM-2} persistence, although had impacts on the microbial community composition. A high metal

amendment (20 mM) affected the microbiome distribution after a long period (30 days) of exposure, being some phyla more abundant (*Pseudomonadota*, *Actinomycetota* and *Bacillota*) than in the control. In particular, members of the orders *Bacillales*, *Xanthomonadales*, *Rhizobiales* and *Sphingomonadales* showed a higher relative abundance in metal amended soil than in non-amended soil. Instead, members of the orders *Caldilineales* and *Saccharimonadales* presented a lower relative abundance in metal amended soil than in non-amended. This study was published as “Effect of copper and zinc as sulfate or nitrate salts on soil microbiome dynamics and *bla*-positive *Pseudomonas aeruginosa* survival” (Fortunato et al., 2021).

Chapter 5 was dedicated to the study of molecular epidemiology of carbapenem-resistant *P. aeruginosa*. The genomes of five *P. aeruginosa* strains harbouring the carbapenem-resistance genes bla_{VIM-2} (strain H1FC49, hospital effluent) and bla_{NDM} (strains NDM1, NDM2, NDM3, NDM4, clinical isolates kindly donated by dr. Gajic, University of Belgrade) were sequenced using short-read (Illumina Miseq, STAB VIDA, Lda) and long-read sequencing methods (Oxford Nanopore MinION). These strains were integrated in a comprehensive analysis of 115 bla_{VIM-2}+ and 23 bla_{NDM-1}+ *P. aeruginosa* genomes, collected from NCBI pathogen database. The core and accessory genome of both bla_{VIM-2}+ and bla_{NDM-1}+ *P. aeruginosa* groups served to assess phylogenetic diversity and infer functional traits. The distribution of the phylogenetic groups suggested that bla_{VIM-2} and bla_{NDM-1} are disseminated by distinct lineages and geographies, differing also on some functional features of the accessory genome. It is suggested that clonal dissemination may be an important mechanism of dissemination of those strains. The core and accessory genomes proteins of bla_{VIM-2}+ and bla_{NDM-1}+ were functionally categorized using EggNOG database and statistically compared. The analysis showed a protein abundance similar in bla_{NDM-1}+ than in bla_{VIM-2}+ core genome while some differences were observed in the accessory genome. The bla_{VIM}+ strains presented a statistically significant higher abundance of proteins involved in metabolic pathways, while virulence, conjugation, and secretion systems related proteins were observed in higher number in bla_{NDM-1}+ accessory genomes. The ARGs annotated in the core genome of bla_{NDM-1}+ than in bla_{VIM-2}+ strains and the ones observed in other *P. aeruginosa* available at the NCBI database were identical. In addition, the accessory genome of bla_{NDM-1}+ and bla_{VIM-2}+ strains had common ARGs, mostly aminoglycosides resistance genes, besides other unique genes in each group and also differing from the genomes available at CARD. Interestingly, the co-occurrence in the same strain of carbapenem resistance genes (bla_{GES} and bla_{KPC}) other than bla_{VIM-2} and bla_{NDM-1} was observed. The gene bla_{VIM-2} was in most cases integrated with MGEs structures like Tn21, Tn402-like and IS6100 and bla_{NDM-1} flanked by *ISAba125*-ble_{MBL} or IS91. The bla_{VIM-2} or bla_{NDM-1} genomic environment represents a fundamental tool to monitor the spread of carbapenem resistance genes. This study is submitted for publication as “Insight into the phylogenomic bias of bla_{VIM-2} or bla_{NDM-1} dissemination amongst carbapenem resistant *Pseudomonas aeruginosa*” (Fortunato et al. submitted).

3 A rationale for the high limits of quantification of antibiotic resistance genes in soil

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	Planning	**Experimental work**			**Data analysis**	**Writing**
		Microcosms preparation and sampling	qPCR and CFU enumerat ion	Statistical analysis and graphical visualization		
Gianuario Fortunato	X	X	X	X	X	X
Ivone Vaz-Moreira	X				X	X
Cristina Becerra-Castro	X	X	X	X		
Olga C. Nunes	X				X	X
Celia M. Manaia	X				X	X

3.1 Abstract

The determination of values of abundance of antibiotic resistance genes (ARGs) per mass of soil is extremely useful to assess the potential impacts of relevant sources of antibiotic resistance, such as irrigation with treated wastewater or manure application. Culture-independent methods and, in particular, quantitative PCR (qPCR), have been regarded as suitable approaches for such a purpose. However, it is arguable if these methods are sensitive enough to measure ARGs abundance at levels that may represent a risk for environmental and human health. This study aimed at demonstrating the range of values of ARGs quantification that can be expected based on currently used procedures of DNA extraction and qPCR analyses. The demonstration was based on the use of soil samples spiked with known amounts of wastewater antibiotic resistant bacteria (ARB) (*Enterococcus faecalis*, *Escherichia coli*, *Acinetobacter johnsonii*, or *Pseudomonas aeruginosa*), harbouring known ARGs, and also on the calculation of expected values determined based on qPCR.

The limits of quantification (LOQ) of the ARGs (*vanA, qnrS,* bla_{TEM}, bla_{OXA}, bla_{IMP}, bla_{VIM}) were observed to be approximately 4 log-units per gram of soil dry weight, irrespective of the type of soil tested. These values were close to the theoretical LOQ values calculated based on currently used DNA extraction methods and qPCR procedures. The observed LOQ values can be considered extremely high to perform an accurate assessment of the impacts of ARGs discharges in soils. A key message is that ARGs accumulation will be noticeable only at very high doses. The assessment of the impacts of ARGs discharges in soils, of associated risks of propagation and potential transmission to humans, must take into consideration this type of evidence, and avoid the simplistic assumption that no detection corresponds to risk absence.

3.2 Introduction

The wide dissemination of antibiotic resistant bacteria (ARB) and their genes (ARGs) as environmental contaminants, often released with treated wastewater, is considered a serious problem by the scientific community and public health authorities, for which control measures are urgently needed (Berendonk et al., 2015; Bürgmann et al., 2018; Hong et al., 2018). Water stress, due to scarcity or to deterioration of the quality of the natural freshwater resources, is a major driving force for water reuse, and inevitably, raises concerns about the associated risks of contamination of soils and of the human food chain with ARGs (Becerra-Castro et al., 2015; Negreanu et al., 2012). For these reasons, the reliable and sensitive quantification of ARGs in environmental samples exposed to human impacts is the basis of any reliable risk assessment framework.

The development of quantitative polymerase chain reaction (qPCR) remarkably improved the study of antibiotic resistance, allowing the quantification of ARGs, first in clinical samples (Espy et al., 2006), extended later on to environmental samples (Schwartz et al., 2003). The quantification of

ARGs opened new perspectives to study the antibiotic resistance distribution and spread in the environment, mainly because it overcomes the dependence on culture dependent methods to measure the occurrence of antibiotic resistance in a given site (Rizzo et al., 2013). In spite of its potential, qPCR has, as any other method, limitations sometimes related to the technique in itself, others due to the specificities of the matrix to be characterized. One of such limitations may be related with the sensitivity, critical to assess the impact of the discharge of contaminant ARGs in the environment, for instance in soils receiving reused water. The crucial question here is if the qPCR limits of quantification (LOQ) can be considered adequate to assess the risks associated with some ARGs (Christou et al., 2017; Manaia, 2017). The ARGs LOQ values are not expected to result only from the qPCR process. Instead they are expected to result from a combination of factors, such as the complexity of the environmental samples, the DNA extraction process, the limitation to concentrate DNA extracts, and/or the occurrence of PCR inhibitors (Combs et al., 2015; A.-D. Li et al., 2017; Luby et al., 2016; Schrader et al., 2012; Sidstedt et al., 2015; Watson and Blackwell, 2000). In this study we aimed at determining the lowest range of abundance of selected ARGs and *intI1* gene that might be quantified in a soil or related matrices, using state-of-the-art procedures, namely qPCR. The possible reasons and implications of the LOQ values observed are discussed.

3.3 Materials and Methods

In order to assess the LOQ and LOD of ARGs and related genes, soil samples were spiked with known amounts of ARB (10^7-10^2 CFU/g of soil) harboring known ARGs. Samples were collected immediately after ARB inoculation and cultivable bacteria were enumerated on different culture media, DNA was extracted and ARGs and other genes were measured based on qPCR.

3.3.1 Soil, ARB and ARG

Soil samples used in this study were collected as composite samples, from a greenhouse agricultural soil, located in Vila do Conde, Northern Portugal. Sampling procedures and soil and related matrices characteristics were reported before (Becerra-Castro et al., 2017). To determine the soil dry weight, samples of soil (1 g) were weighed before (wet soil) and after drying (dry soil) by incubation at 120 °C, until no weight variation was observed, which corresponded to ~2 days.

Five ARB strains harboring ARGs or other genetic elements of interest were selected for this study (Table 3.1). Specifically, were analyzed: i) the *intI1* gene, encoding the class 1 integrons integrase, which is abundant in soil and considered a proxy for human impacts of antibiotic resistance (Gillings et al., 2008); ii) the genes, *bla*$_{TEM}$*, bla*$_{OXA}$ *and qnrS*, which are common in wastewater habitats (Narciso-Da-Rocha et al., 2014; Szczepanowski et al., 2009); and iii) *bla*$_{IMP}$, *bla*$_{VIM}$ and *vanA*, which,

although reported in wastewaters, are yet not so common in the environment, being more associated with clinical samples (Vaz-Moreira et al., 2016; Yang et al., 2012).

For each strain, a calibration curve between the optical density at 610 nm (OD_{610}) and the number of colonies forming units *per* mL (CFUs/mL) was determined. Briefly, from an initial bacterial suspension with an OD_{610} = 1, serial dilutions with a dilution factor of 2 were prepared, for which the optical density at 610 nm and the number of CFUs *per* mL on Plate Count Agar (PCA) were determined. The curves of CFU/mL in function of the OD_{610} allowed the preparation of the bacterial suspensions with the adequate density to spike the soil slurries.

3.3.2 Soil slurries

Ten grams of well-homogenized soil were spiked with 3 mL of a bacterial suspension prepared in synthetic wastewater (Sousa et al., 2017) in 50 mL plastic tubes. Three different assays were settled, A1 and A2 with greenhouse soil Three different assays were settled, A1 and A2 with greenhouse soil collected in July 2014 and A3 with the same soil collected in March 2016. A1 was spiked with *Escherichia coli* strain A1FCC2; A2, spiked with *E. coli* strain A2FCC14 and *Enterococcus faecalis* strain H1EV10; and A3, spiked with *Pseudomonas aeruginosa* strain H1FC49 and *Acinetobacter johnsonii* H1PC5 (Table 3.1).

The bacterial suspensions were prepared from overnight cultures grown on PCA, using an OD_{610} corresponding to a bacterial density of 10^8 CFU/mL. The suspensions were then further diluted in order to reach an abundance in the initial bulk of soil of 10^7-10^2 CFU/g of wet soil. The spiked soils were thoroughly 1 mixed and homogenized. In parallel, for each assay a soil sample supplemented with 3 mL of synthetic wastewater was used as a control (non-spiked control). Assays were prepared and analyzed in triplicate.

3.3.3 Quantification of ARB

For bacteria enumeration, one gram of soil was sampled from each soil slurry, suspended in 9 mL of hexametaphosphate sterile solution 1% (w/v), and serially diluted in sterile saline solution (0.85% (w/v) NaCl). Bacterial counts were made on selective culture media based on the membrane filtration method, with cellulose nitrate membranes (0.22 µm pore size, 47 mm diameter; Albet). The species E. coli, *A. johnsonii*, and *P. aeruginosa* were enumerated on mFC agar (fecal coliform agar, Difco BD), where they produce blue, pink or orange colonies, respectively. In addition, the species *P. aeruginosa* was also enumerated on Cetrimide agar (Pronadisa) with nalidixic acid (Sigma-Aldrich), where it selectively grows and forms green colonies. *Ent. faecalis* was enumerated on m-

Enterococcus agar (Difco BD). Cultures were incubated at 30 °C for 48 h (mFC and m-Enterococcus agar) or 72 h (Cetrimide agar).

3.3.4 DNA extraction and quantitative PCR

Total DNA was extracted, in triplicate, from 0.25 g of soil using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc.) according to the manufacturer instructions. DNA concentration was measured with the Qubit^{TM} fluorometer (ThermoFisher Scientific). The selected genes were quantified using the conditions described in Table S3.2. For the assay A1 the genes *qnrS* and *bla*$_{TEM}$ were quantified; for the assay A2 the genes *intI1*, *bla*$_{TEM}$, *bla*$_{OXA}$ and *vanA*; and for the assay A3 the genes *intI1*, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA}$ (Table 3.1). For each assay, three independent DNA extracts were analyzed, using the Standard

Curve method as described in Brankatschk et al. (2012) in a StepOne^{TM} RealTime PCR System (Life Technologies, Carlsbad). The quality criteria for acceptable qPCR determinations considered the possibility of interpolation to the calibration curve, the correct melting temperature of the amplicon, and absence of multiple amplification peaks or shoulders (Rocha et al., 2018). The DNA concentration to use in the quantification assays was adjusted to avoid an excess of target DNA in comparison to the primer. The lowest gene copy number obtained for each calibration curve is indicated in Table 3.2. The observation of amplification at Ct values below the lowest limit of the calibration curve, fitting the expected melting temperature were considered as being above the Limit of Detection (LOD), and below the LOQ (Table 3.2).

Table 3.1 Bacterial strains used in this study.

Assay	Strain	Source	ARGs quantified	Reference
A1	*Escherichia coli* A1FCC2	Raw wastewater	*qnrS*; *bla*$_{TEM}$	(Varela et al., 2015)
A2	*Escherichia coli* A2FCC14		*bla*$_{OXA}$; *bla*$_{TEM}$; *intI1*	
	Enterococcus faecalis H1EV10	Hospital wastewater	*vanA*	
A3	*Acinetobacter johnsonii* H1PC5		*bla*$_{IMP}$; *bla*$_{OXA}$; *intI1*	(Vaz-Moreira et al., 2016)
	Pseudomonas aeruginosa H1FC49		*bla*$_{VIM}$; *intI1*	
A4-A7	*Pseudomonas aeruginosa* H1FC49		*bla*$_{VIM}$; *intI1*	(Vaz-Moreira et al., 2016)

3.3.5 *Influence of the soil sample mass on the observed LOQ and LOD*

In an attempt to lower the copy number of genes quantifiable in the greenhouse soil slurry samples, i.e. the observed LOQ, a comparison between the PowerSoil (Mo Bio) and the FastDNA™ soil kit (MP Biomedicals) was performed. The FastDNA soil kit allows extraction from 10 g of soil, with the recommendation to resuspend in a final volume of 5 mL. The PowerSoil kit recommends the extraction from 0.25 g of soil and resuspension in a final volume of 100 µL. While the recommended proportion soil:final suspension volume is similar in both systems (1:0.5 or 1:0.4), it differs considerably in the initial amount of soil (10 g or 0.25 g), with FastDNA allowing the extraction from larger amounts of soil.

To evaluate the influence of the initial DNA extract concentration, the FastDNA soil extracts were concentrated in an attempt to lower the observed LOQ. Hence, 1 mL of FastDNA extract was concentrated using the SpeedVac concentrator (ThermoFisher Scientific) following the manufacturer instructions and re-suspended in 100 µL of ultrapure water. To compare both extraction methods and the implications of DNA concentration, namely on the PCR inhibition, the 16S rRNA gene was measured.

3.3.6 *Effect of the type of soil or related matrix on the observed LOQ and LOD*

In order to assess if the results were influenced by the type of soil or related matrix, assays were conducted with: A4) beach sand collected in the Northern region of Portugal; A5) a thermal compost produced from urban wastewater sludge currently used in gardening and agriculture; A6) commercial potting soil obtained in the retail market; and A7) a fallow soil collected in the Centre of Portugal. The physicochemical properties of these samples are shown in Table S3.1. Using the procedure described above, the test samples were inoculated with 10^5-10^3 CFU/g of soil/matrix of *Pseudomonas aeruginosa* strain H1FC49 (Table 3.1). The genes monitored to determine the observed LOQ and LOD values were the *16S rRNA*, bla_{VIM} and *intI1*, as described above.

3.3.7 *Statistical analyses*

The bacterial counts and gene copy number were expressed per gram of soil dry weight and compared using analysis of variance (ANOVA) and the post-hoc Tukey test, with a significance level (P) of 0.05. Statistical analyses were performed using SPSS 24.0, SPSS Inc., Chicago, IL.

Table 3.2 LOQ and LOD observed for the analyzed genes.

LOQ and LOD values observed for the analyzed genes per gram of soil/matrix dry weight and comparison with the lowest values that could be quantified (theoretical LOQ). *LOQ, minimum quantification value (per gram dry soil) that could be interpolated in the calibration curve; **LOD, quantification values below the lowest value of the calibration curve, but with amplicons with the correct melting temperature.

Gene	Assay	Lowest value of gene copy number in the calibration curve		Observed LOQ*	Observed LOD**
		In a qPCR reaction	Correspondence *per* gram of dry weight of soil (Theoretical LOQ)§		
16S rRNA	A1	385	8.8×10^5	n.d.	n.d.
	A2		9.9×10^5		
	A3		1.0×10^6		
	A4		1.0×10^6		
	A5		1.8×10^6		
	A6		1.8×10^6		
	A7		1.0×10^6		
intI1	A2	54	1.4×10^4	n.d.	n.d.
	A3		1.4×10^4		
	A4		9.8×10^3		
	A5		1.0×10^4		

	A6		1.0×10^4	2.0×10^4	1.7×10^4
	A7		9.8×10^3	n.d.	n.d.
qnrS	A1	54	1.2×10^4	1.9×10^4	5.2×10^3
vanA	A2	54	1.4×10^4	4.7×10^4	4.4×10^3
bla$_{TEM}$	A1	54	1.2×10^4	1.3×10^4	5.3×10^3
	A2		1.4×10^4	6.3×10^4	8.5×10^3
bla$_{OXA}$	A2	64	1.5×10^4	1.4×10^5	1.2×10^4
	A3		1.7×10^4	8.3×10^4	1.1×10^4
la$_{IMP}$	A3	10	2.7×10^3	1.2×10^4	8.1×10^2
bla$_{VIM}$	A3		5.9×10^3	2.3×10^4	$<6.9 \times 10^{3*}$
	A4		5.8×10^3	3.3×10^4	$<1.6 \times 10^{3*}$
	A5		1.0×10^4	3.6×10^4	$<1.6 \times 10^{3*}$
	A6		1.0×10^4	1.2×10^4	1.2×10^4
	A7		5.8×10^3	1.1×10^4	1.1×10^4

n.d., not determined; x, for the same lowest value of gene copy number in the calibration curve the correspondence per gram of dry weight of soil may vary, according to the humidity content of each sample. *double peaks, one with the correct melting temperature, suggest that LOD is lower than the corresponding Ct value.

Assays: A1) greenhouse soil spiked with *E. coli* strain A1FCC2; A2) greenhouse soil spiked with *E. coli* strain A2FCC14 and *Ent. faecalis* strain H1EV10; A3) greenhouse soil spiked with *P. aeruginosa* strain H1FC49 and *A. johnsonii* strain H1PC5; A4) sand spiked with *P. aeruginosa* strain H1FC49; A5) compost spiked with *P. aeruginosa* strain H1FC49; A6) potting soil spiked with *P. aeruginosa* strain H1FC49; and A7) fallow soil spiked with *P. aeruginosa* strain H1FC49.

3.4 Results and Discussion

ARB and ARGs are important environmental contaminants, whose growing accumulation and spread in the environment is a matter of concern (Manaia, 2017; Pruden et al., 2006; Vaz-Moreira et al., 2014). Indeed, these contaminants may not only persist in the environment but also self-replicate and proliferate. Self-replication is one, although not the unique, reason why extremely low doses of ARB or ARGs in a given environment (e.g. soil) may represent a high direct or indirect risk for human health. One possible source of soils contamination with ARB and ARGs is the water reuse in irrigation (Becerra-Castro et al., 2015; Christou et al., 2017; Negreanu et al., 2012; Wang et al., 2014). However, an overview of the literature suggests contradictory conclusions, with the observation that ARGs abundance increases in soils associated with water reuse practices being reported in parallel with no noticeable effects (Gatica and Cytryn, 2013; Negreanu et al., 2012; Wang et al., 2014). Whilst it is not the aim of this paper the discussion of possible reasons for such an apparent inconsistency, the background information gives space for a critical discussion about methodological aspects that may explain the apparently contradictory findings. This was indeed the aim of this paper: determine the abundance of ARGs that can be quantified in soils or related matrices based on commonly used DNA extraction procedures and qPCR. The final goal of studies like this is to bring a critical look about ARGs quantification in soils and stimulate the discussion, which is out of the scope of this paper, if ARGs that are not detected by this technique may, in fact, being accumulated in soil at values exceeding those considered safe levels.

3.4.1 Quantification of ARB

Reference bacteria were spiked in greenhouse soil at a density of 7 to 2 log units *per* gram of wet soil as an approach to assess the LOQ and LOD that could be achieved for the different genes under analyses. Not surprisingly, the enumeration of bacteria in soil corresponded to the expected values, with a linear variation between the amount of spiked bacteria and the CFU counts in soil (Figures 3.1 A-C). The only exception was a slight deviation observed for *P. aeruginosa* or *A. johnsonii* in the samples with 2 log-units inoculum, for which a higher CFU counts than expected could be due to the existence of autochthonous bacteria of these genera in soil (Figure 3.1 C). Indeed, the nonspiked soil was observed to contain 2 log-units CFU/g with morphologies that, although lacking the typical morphologies considered for the analyzed strains of *P. aeruginosa* or *A. johnsonii* might, in the spiked samples, be confounded with the inoculants (data not shown).

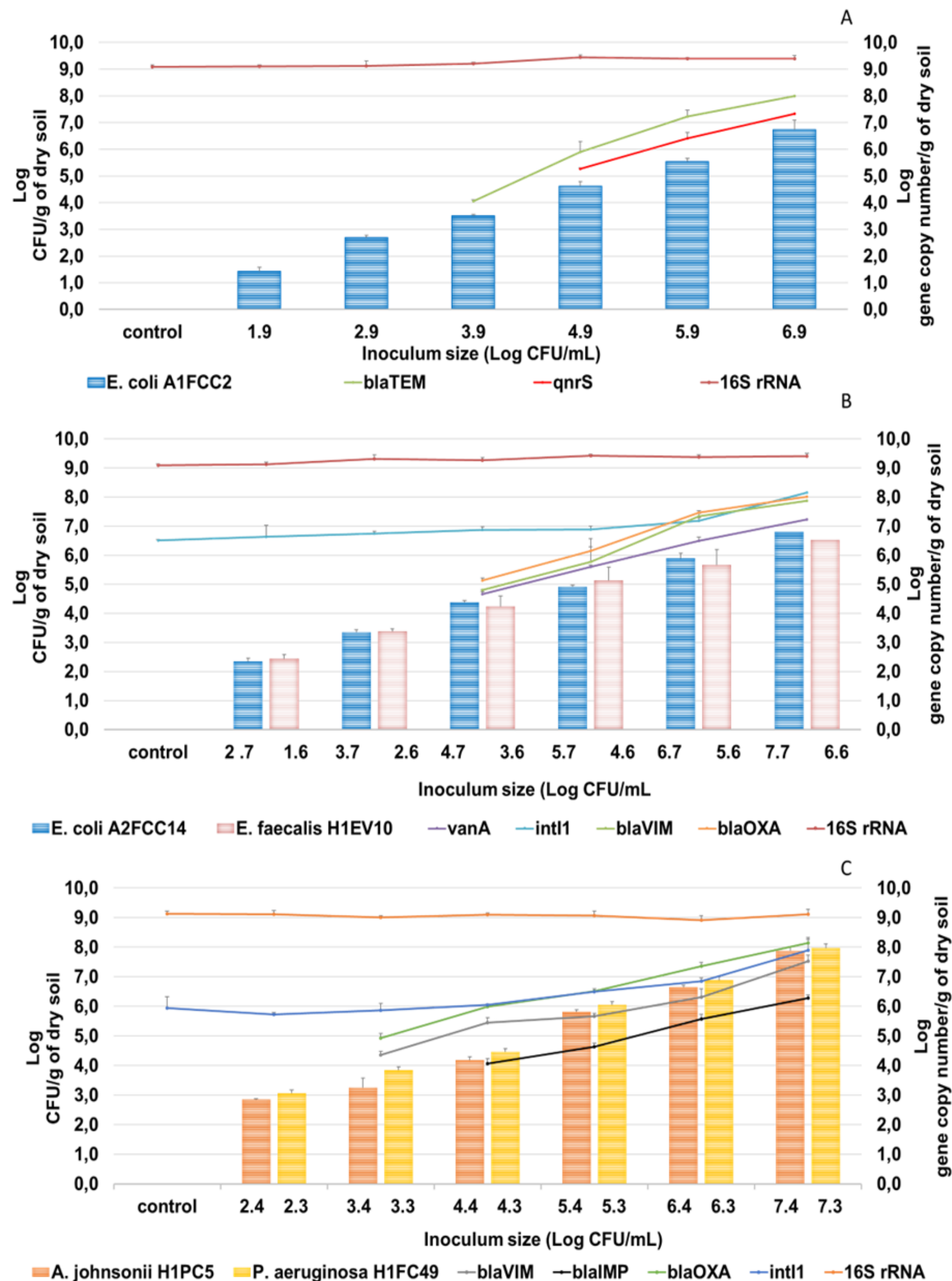

Figure 3.2 CFU and genes quantification for the presented assays

Colony forming units (CFU) enumeration of spiked ARB (columns) per gram of dry weight of soil and corresponding qPCR quantification of ARG expressed in copies per gram of weight dry soil. Control refers to soil spiked with synthetic wastewater. A) spiked with *E. coli* strain A1FCC2; B) spiked with *E. coli* strain A2FCC14 and *Ent. faecalis* strain H1EV10; and C) spiked with *P. aeruginosa* strain H1FC49 and *A. johnsonii* strain H1PC5.

3.4.2 *Quantification of ARGs and other genes*

In the greenhouse soil assays (A1-A3), irrespective of the amount of inoculum used, the copy number of the 16S rRNA gene was approximately 9 log-units/g of dry soil, even in non-spiked controls (Figures 3.1A-C). As could be expected, the supplementation of soil with bacteria at densities at least 2 log-units lower than the original soil was not noticeable based on the qPCR quantifications. The same observation is valid for the *intI1* gene, whose presence in non-inoculated soil at densities of 6-7 log-units/g dry soil led to a narrow margin to measure exogenous inputs of this gene (Figure 3.1B and C; Table 3.2). Although not related to the problem of the high LOQ values, the existence of a relatively high background is another drawback when the impact of exogenous ARGs sources are to be measured. This problem has been discussed before by other authors (Gatica and Cytryn, 2013).

None of the ARGs quantified in this study were detected in non-spiked greenhouse soil control samples (Figure 3.1). This fact allowed the determination of the observed LOQ and LOD values for each gene, in some cases hosted by different bacterial strains. For soil slurries with inoculum size (bacterial density) ≥ ~3.5-4.5 log CFUs/g dry soil, the increase of the inoculum size in one log-unit corresponded to an increase of one log-unit in the gene copy number quantified for each gene (Figure 3.1A-C). Such achievement is in agreement with the adequate efficiency values of the qPCR assays (Table S3.2). However, for soil slurries with inoculum size below ~3.5-4.5 log CFUs/g dry soil, the analyzed ARGs were below the lowest value of the calibration curve, and, therefore, could not be quantified with accuracy. Consequently, in assays A1-A3 the observed LOQ values ranged, for all analyzed ARGs 4-5 log-units *per* gram of dry soil (Figure 3.1A-C, Table 3.2). With these assays we could, thus, conclude that, in average, to achieve a reliable qPCR quantification of a given ARG in the soil, the correspondent ARB should have at least a density of 10^4 CFUs/g dry soil. While quantification should be made based on the interpolation to a calibration curve and the LOQ value depends on the amplitude of this curve, qPCR detection may be made based on the analyses of the correct melting temperature of the amplicons. Using this rationale, the limits of detection (LOD) were determined to range 2-4 log-units of ARGs *per* gram of soil (Table 3.2), corresponding to ARB densities of 2-4 log CFUs/g dry soil.

3.4.3 *Limit of quantification in different matrices*

To assess the influence of the matrix in the observed LOQ and LOD values, distinct samples were tested to quantify genes harbored by *P. aeruginosa* H1FC49 (bla_{VIM} and *intI1*, Table 3.1). The four matrices tested differed in physicochemical properties, mostly texture and organic matter content, in particular humic substances content (Table S3.1), which is known to affect the efficiency of DNA extraction (Sidstedt et al., 2015). In this set of assays (A4-A7), the enumeration of the ARB (*P. aeruginosa*) in the different spiked samples corresponded also to the expected values (Figure 3.2).

With the exception of the sand sample (Figure 3.2A), with a density of 7 log units/g dry soil for the 16S rRNA gene, the other samples presented a density of this gene of 8 log-units/g dry soil (Figure 3.2B-D). The *intI1* gene was quantifiable in all samples, with the exception of the non-spiked potting soil control (Figure 3.2C). The observed LOQ for the ARG bla_{VIM} was determined to be 4 log copies/g of dry soil/matrix for the different matrices tested (Figure 3.2, Table 3.2), similar to what was observed for the greenhouse agriculture soil (Figure 3.1, Table 3.2). These results confirm that independently of the soil matrix, the observed LOQ of ARGs is 4 log copies per gram of dry weight sample.

3.4.4 Influence of the sample mass and DNA concentration on the qPCR LOQ

Total DNA extractions using PowerSoil or FastDNA led to similar DNA concentrations, although different amounts (Table 3.3). The comparison of the 16S rRNA gene quantification in samples extracted with both methods suggest that the effect of qPCR inhibition was not observed in PowerSoil DNA extracts in contrast with the FastDNA extracts. Indeed, only after DNA dilution, the 16S rRNA gene quantification observed for the FastDNA extracts was similar to that obtained with the PowerSoil DNA extracts. It is suggested that the FastDNA kit, although allowing the recovery of a higher quantity of DNA, did not support the preparation of DNA solutions with an higher concentration than PowerSoil given the potential risks of qPCR inhibition (Table 3.3).

Table 3.3 Comparison of DNA extraction kits efficiency in gene quantification

Comparison of 16S rRNA gene quantification in DNA extracts (E) obtained with FastDNA™ soil kit (MP Biomedicals) or PowerSoil kit (Mo Bio).

DNA extraction method	Amount of soil and extract volume	DNA extract	DNA concentration (mg/mL)	Log (gene copy number/g soil dry weight)
FastDNA™ soil kit (MP biomedicals)	5 g soil 5 mL extract (E) 10x concentrated (10 C)	E	11.8	7.4 ± 0.20[a]
		10 C	110.7	7.8 ± 0.05[b]
		10 C + 10 D	11.1	8.6 ± 0.03[c]
PowerSoil (Mo Bio)	0.25 g soil 100 mL extract (E) 10x diluted (10 D)	E	16.5	8.8 ± 0.07[c]
		10 D	1.7	8.8 ± 0.13[c]

E, DNA extract; 10 C, DNA extract concentrated 10; 10 D, DNA extract diluted 10.

a,b,c - statistically different ($p < 0.05$) using ANOVA.

3.4.5 Theoretical vs experimental LOQs and implications on ARGs risk assessment

The results obtained in this study showed that the ARGs LOQ values, irrespective of the solid matrix analyzed and of the gene or of the gene host introduced in the soil, was of approximately 10^4 gene copies *per* g of dry weight of solid matrix, corresponding to an ARB density of ~ 10^4 CFUs/g dry solid matrix, and to a ratio of approximately 10^{-3} - 10^{-5} of ARG copy number *per* total 16S rRNA gene copy number, with the exception of the sand sample where a ratio of 10^{-1} was found. An additional tenfold dilution of the bacterial inoculum did not support the quantification of any of the analyzed ARGs. The question raised was, then, if this was a practical limitation due to operational conditions or if, otherwise, it was intrinsic to the methodological constraints. Based on the average water content of a soil sample, the DNA extraction procedure and the volume of DNA extract used in the qPCR reaction, it is expected a quantification, per qPCR reaction of approximately 2-3 log-units below the real number of copies of ARGs in the sample. For example, considering the greenhouse agriculture wet soil containing approximately 20% humidity, the use of 0.25 g of wet soil for DNA extraction and of 2 µL of DNA extract in each qPCR reaction, a value of 4×10^4 copies of a ARG can be measured in a qPCR reaction when analyzing a soil sample containing 10^7 gene copies *per* gram of wet soil (Table 3.4, bold numbers). Therefore, from the real abundance of an ARG in the soil to what can be measured there is a reduction by a factor of 4×10^{-3}, only due to DNA extraction and qPCR reaction. This means that in a soil containing one thousand ARG copies *per* gram of soil wet weight, which can be meaningful in terms of human health, one would expect to be able to amplify 4 ARG copies by PCR (Table 3.4), which is not a realistic expectation with the most commonly used real-time PCR protocols. Using qPCR procedures, like those of the present study, where the lowest value of gene copy number in the calibration curve was around 20-60, the theoretical and observed LOQ values were shown to be in agreement for most of the ARGs and all the matrices analyzed (Table 3.2). These results suggest that methodological improvements are required to achieve lower LOQ values. The extraction of DNA from a larger soil mass could be a positive contribution. However, the extraction and concentration of qPCR inhibitors is a problem to overcome, as discussed above (Table 3.3). Another helpful modification may be the use of a larger volume of DNA extract per qPCR reaction, which can increase the LOQ in about 1 log unit. However, this procedure has the potential to increase inhibition, either due to the excess of DNA that can inhibit PCR or due to the increasing amount of inhibitors in the extract. Droplet digital PCR (ddPCR) has been proposed as a good alternative to overcome limitations of qPCR. In average, ddPCR can reduce in about 10 times the LOQ of ARGs in comparison to qPCR (Cavé et al., 2016). Nevertheless, a major constraint that results from DNA extraction from soil and the avoidance of PCR inhibitors seems to represent a bottleneck still to solve.

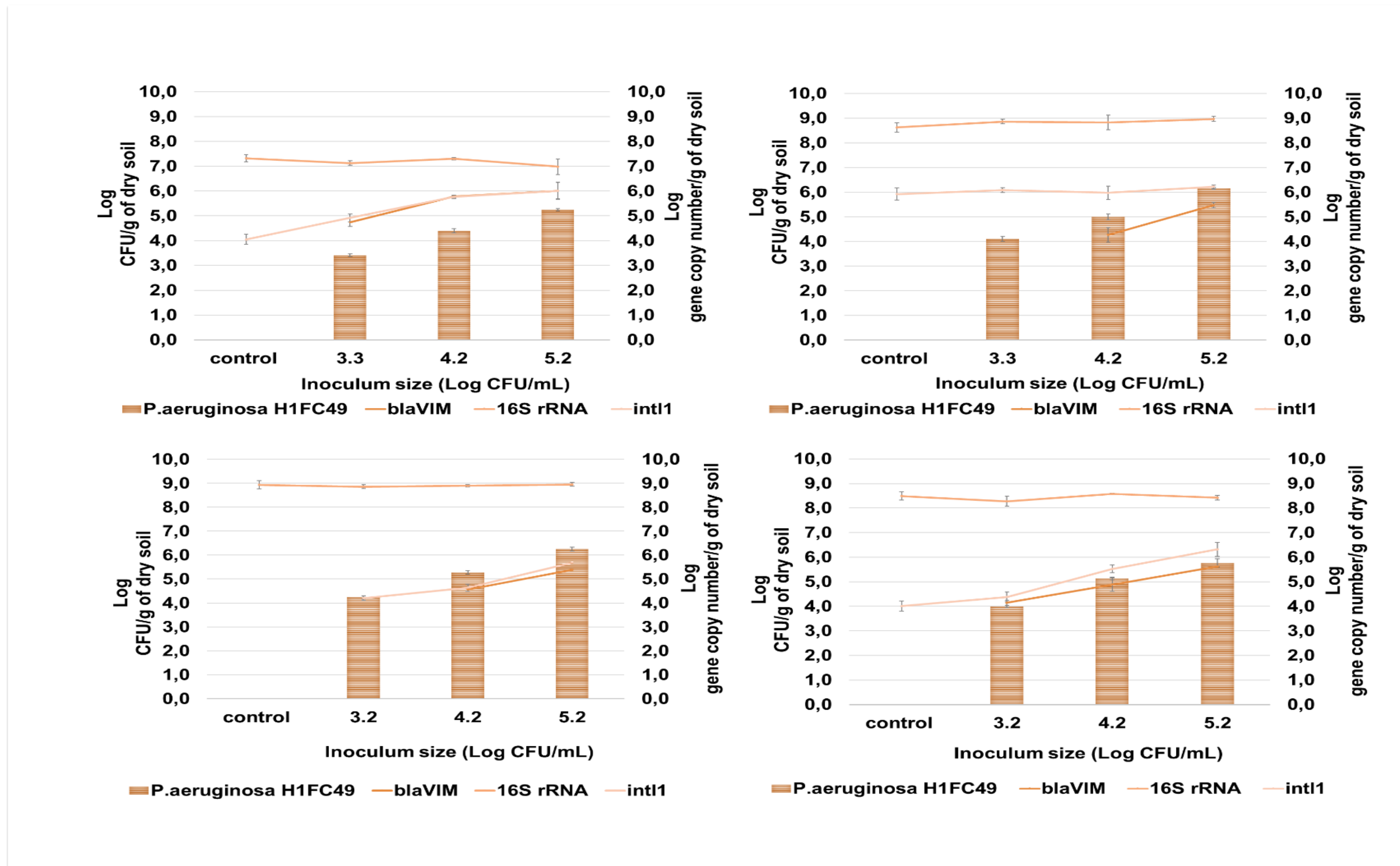

Figure 3.3 CFU and gene quantification in different soil matrices

Colony forming units (CFU) enumeration of spiked ARB (columns) per gram of dry weight of soil and corresponding qPCR quantification of ARG expressed in copies per gram of weight dry soil/matrix, for A) sand; B) compost; C) potting soil; and D) fallow soil.

Table 3.4 Estimation of gene copy number in qPCR quantification

Estimation of the gene copy number in a qPCR reaction using DNA extracts obtained from 0.25 g of wet soils with different densities of a given gene. It is assumed that i) the final DNA extracts are suspended in 100 mL; ii) DNA extraction has an efficiency of 100%; iii) 20% of soil mass is water; iv) each qPCR reaction uses 2 mL of DNA solution.

Gene copies per gram of wet soil	Mass of wet soil used (g)	Humidity percentage (%)	Volume of DNA extract (mL)	Volume of DNA used in the	Gene copy number expected to be quantified in a qPCR (mL) qPCR reaction
1×10^7	0.25	20	100	2	4×10^4
1×10^6					4×10^3
1×10^5					4×10^2
1×10^4					4×10^1
1×10^3					4×10^0

The discussion about the risks of contamination of the human food chain with ARB and ARGs due to intended or inadvertent water reuse has been intensified over the last years. In this discussion, one of the central issues is the technical capability to detect and quantify ARB and ARGs that may accumulate or proliferate in soil, representing a threat to humans and animals. It is recognized that it is very difficult to propose a threshold for the maximum admissible emission values of ARB and ARGs, for instance by wastewater treatment plants. However, it is consensual that extremely low doses of some ARB, namely those harboring last generation ARGs such as those encoding carbapenem or colistin resistance, may represent high risks for humans and the environment. The ranking of risks and the concerns raised by the presence of specific ARGs cannot be made out of a context. It is important to take into consideration the soil history and properties as well as the land use and end users. In this way, when such evaluations are to be made, it is important to consider that qPCR, the current methodology used to quantify ARGs, may fail to quantify potentially hazardous biological contaminants, whose abundance is below 10^4 CFU *per* g of soil wet weight.

3.5 Conclusions

The key message of this article is clear – the results of ARGs quantification in soils using state-of-the-art procedures need to be interpreted with caution. We demonstrated that with commonly used qPCR procedures it is not possible to quantify ARGs in solid matrices, independently of the type, where they are at or below an abundance of one thousand copies *per* gram of soil dry weight. Moreover, we demonstrated that these results were not due to practical biases, as theoretical and observed LOQ values were in the same range. Values below the LOQ may represent an overwhelming amount of ARB and ARGs, mainly if one considers the high ecological fitness of bacteria, and high capability to reproduce in the environment.

Supplementary information

Chapter 3: A rationale for the high limits of quantification of antibiotic resistance genes in soil

Published:

Authors:Fortunato, G., Vaz-Moreira, I., Becerra-Castro, C., Nunes, O.C., Manaia, C.M.

Year: 2018

Title: “A rationale for the high limits of quantification of antibiotic resistance genes in soil”.

Journal: Environmental Pollution 243, 1696–1703. https://doi.org/10.1016/j.envpol.2018.09.128

Table S3.1 Physicochemical characteristics of the different solid matrices.

Characteristics	Greenhouse soil*	Sand	Compost	Potting soil	Fallow soil
Soil texture	sand	sand	n.a.	n.a.	loamy-sand
pH_{H20}	7.5	7.3	4.7	4.9	7.2
pH_{KCl}	7.1	6.7	3.7	4.2	6.7
OM (%)	1.8	0.13	86.82	72.51	3.41
Humic substances (g Kg^{-1})	4.3	5.4	236.9	483.3	30.7
EC (dS m^{-1})	0.32	0.08	2.05	1.67	0.12
SAR	6.0	0.15	0.46	0.58	0.17
N content (mg Kg^{-1})					
$N\text{-}NH_4$	5	1	223	525	6
$N\text{-}NO_3$	25	<0.1	315	346	14
Soluble cations (mg Kg^{-1})					
Ca	127	726	11621	3348	1024
Mg	40	118	3572	1183	174
Na	300	16	223	154	22
K	250	14	465	343	24
P	17	1	1018	19	12

* data from Becerra-Castro et al. (2017);OM, organic matter; EC, electrical conductivity; SAR, sodium absorption rate; n.a., not available.

Table S3.2 Primers and qPCR conditions and parameters.

Target Gene	Primers	Primers Reference	qPCR standard	qPCR conditions	Efficiency (%)	Ct value limit
16S rRNA	1114F (CGGCAACGAGCGCAACCC) 1275R (CCATTGTAGCACGTGTGTAGCC)	(Denman and McSweeney, 2006)	*E. coli* ATCC 25922	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s (35 cycles) Other: 1a	100	18
intI1	intILC5_fw (GATCGGTCGAATGCGTGT) intIILC1_rv (GCCTTGATGTTACCCGAGAG)	(Goldstein et al., 2001)	pNORM clone (Rocha et al., 2018)	95 °C for 10 min (1 cycle) 95 °C for 15 s, 60 °C for 30 s (40 cycles) Other: 3a	94	28
qnrS	qnrSrtF11 (GACGTGCTAACTTGCGTGAT) qnrSrtR11 (TGGCATTGTTGGAAACTTG)	(Marti and Balcázar, 2013)		95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2c	95	27
vanA	vanA3FP (CTGTGAGGTCGGTTGTGCG) vanA3RP (TTTGGTCCACCTCGCCA)	(Volkmann et al., 2004)		95 °C for 5 min (1 cycle) 95 °C for 3 s, 60 °C for 30 s (40 cycles) Other: 2a	98	32
bla_{TEM}	blaTEM-F (TTCCTGTTTTTGCTCACCCAG) blaTEM-R (CTCAAGGATCTTACCGCTGTTG)	(Bibbal et al., 2007)		95 °C for 10 min (1 cycle) 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s (40 cycles) Other: 2a	96	31
bla_{OXA}	OXA1B14_fw (CACTTACAGGAAACTTGGGGTCG) blaOXA1_rv (AGTGTGTTTAGAATGGTGATC)	(Ahammad et al., 2014)	*bla_{OXA}* clone (from *E. coli* A2FCC14)	95 °C for 5 min (1 cycle) 95 °C for 15 s, 60 °C for 1 min (40 cycles) Other: 2c	95	29
bla_{IMP}	IMPgen-F1 (GAATAG(A/G)(A/G)TGGCTTAA(C/T)TCTC) IMPgen-R1 (CCAAAC(C/T)ACTA(G/C)GTTATC)	(Mendes et al., 2007)	*bla_{IMP}* clone *(from A. johnsonii* H1PC5)	95 °C for 10 min (1 cycle) 95 °C for 15 s, 55 °C for 30 s and 72 °C for 10 s (35 cycles) Other: 1b	95	29
blaVIM	VIM-fw (GTACGCATCACCGTCGACAC) VIMspec2-re (AGACGGGACGTACACAACTAAG)	(Bisiklis et al., 2007)	*bla_{VIM}* clone (from *P. aeruginosa* H1FC49)	95 °C for 10 min (1 cycle) 95 °C for 30 s, 60 °C for 1 min (40 cycles) Other: 2a	99	31

qPCR conditions: 1) KAPA SYBR® FAST ABI Prism® qPCR Master Mix; 2) SYBR® Select Master Mix; 3) Power SYBR® Master Mix; a) 200 nM of primer; b) 400 nM of primer; c) 600 nM of primer.

4 Effect of copper and zinc as sulfate or nitrate salts on soil microbiome dynamics and *bla*$_{VIM}$-positive *Pseudomonas aeruginosa* survival

Authors: Gianuario Fortunato, Ivone Vaz-Moreira, Olga C. Nunes, Celia M. Manaia

	Planning	**Experimental work**				**Data analysis**	**Writing**
		Microcosms preparation and sampling	qPCR and CFU enumeration	Microbial community analysis	Statistical analysis and graphical visualization		
Gianuario Fortunato	X	X	X	X	X	X	X
Ivone Vaz-Moreira	X			X	X	X	X
Olga C. Nunes	X					X	X
Celia M. Manaia	X					X	X

4.1 Abstract

The exposure of soil to metals and to antibiotic resistant bacteria may lead to the progressive deterioration of soil quality. The persistence of antibiotic resistant bacteria or antibiotic resistance genes in soil can be influenced by the microbial community or by soil amendments with metal salts. This work assessed the effect of soil amendment with copper and zinc, as sulfate or nitrate salts, on the fate of a carbapenem-resistant (bla_{VIM}^{+}) hospital effluent isolate of *Pseudomonas aeruginosa* (strain H1FC49) and on the variations of the microbial community composition. Microcosms with soil aged or not with copper and zinc salts (20 mM), and inoculated with *P. aeruginosa* H1FC49 were monitored at 0, 7, 14 and/or 30 days, for community composition (16S rRNA gene amplicon) and strain H1FC49 persistence. Data on culturable *P. aeruginosa*, quantitative PCR of the housekeeping gene *ecf*, and the presumably acquired genes bla_{VIM}^{+} and integrase (*intI1*), and community composition were interpreted based on descriptive statistics and multivariate analysis. *P. aeruginosa* and the presumably acquired genes, were quantifiable in soil for up to one month, in both metal-amended and non-amended soil. Metal amendments were associated with a significant decrease of bacterial community diversity and richness. The persistence of *P. aeruginosa* and acquired genes in soils, combined with the adverse effect of metals on the bacterial community, highlight the vulnerability of soil to both types of exogenous contamination.

4.2 Introduction

Soil is a living environment that hosts 25% of the world biodiversity and holds a complex and rich microbial community (Bach et al., 2020; Guerra et al., 2020). Given the long time required for soil formation it can be considered a non-renewable resource, subjected to threats of different types such as erosion, loss of organic matter, salinization or contamination, with implications on the ecosystems health and human wellbeing (Brevik et al., 2020; Prăvălie et al., 2021). Soil contamination may result from unintended human actions and diffuse pollution sources, such as industrial effluent discharges, stormwater, among others (Barałkiewicz et al., 2014; Zwolak et al., 2019). In addition, it can also result from unsustainable practices that are associated with intensive conventional agriculture where synthetic substances are used as fertilizers or pesticides, among others (Aktar et al., 2009; Sebilo et al., 2013; Silva et al., 2019). This scenario, as well as the drought threat imposed by the continually approaching climate change, has been calling for a shift towards sustainable practices, in particular in agriculture, where organic fertilization and water reuse for irrigation can become the rule (Becerra-Castro et al., 2015; K. Chojnacka et al., 2020; Urra et al., 2019). However, these practices may bring new risks. Organic fertilization with animal manure or sludge may reduce the application of synthetic fertilizers in soils, with important benefits for the environment (Chojnacka et al., 2020). However, this practice may represent a source of antibiotic resistant bacteria and antibiotic resistance genes, introducing these biological contaminants in soils (Chen and Xia, 2017; P. Liu et al., 2017; McKinney

et al., 2018; Munir and Xagoraraki, 2011; Murray et al., 2019; Udikovic-Kolic et al., 2014). Antibiotic resistant bacteria and antibiotic resistance genes are also known to be present in treated wastewater and, therefore, while this means irrigation may supply nutrients and contribute to protecting water resources, it may also enrich the soil in those biological contaminants (Amador et al., 2015; Malik and Aleem, 2011; Manaia et al., 2018). Unlike chemical contaminants, once spread in the environment antibiotic resistant bacteria can proliferate, as long as favourable conditions are met, even if for long periods of time they persisted without noticeable growth (Abd-Elwahed, 2018; He et al., 2020; Wang et al., 2014).

Like antibiotic resistant bacteria, metals are also highly persistent environmental contaminants. Although these contaminants may suffer transformation, they are non-degradable, a fact that leads to an unavoidable accumulation in the environment (Ali et al., 2019; Tchounwou et al., 2012). As above mentioned for antibiotic resistant bacteria and antibiotic resistance genes, also metals can be supplied and transferred to soil by manure, sludge or treated wastewater (Berenguer et al., 2008; Donner et al., 2012; Guo et al., 2018; Mantovi et al., 2003; Qian et al., 2018; Xiong et al., 2010). Moreover, the use of metal salts in agriculture or livestock is not incompatible with organic agriculture practices (The Council of the European Union, 2010).

Copper-based treatments are widely used in agriculture due to phytosanitary versatility and low costs (Lamichhane et al., 2018). For example, copper sulfate is an antimicrobial agent commonly used to prevent crop phytopathogenic activity in vineyards (Flores-Vélez et al., 1996; La Torre et al., 2018; Lamichhane et al., 2018; MacKie et al., 2012). However, the intensive application of copper sulfate, which is allowed in organic farming, promotes the accumulation of copper in the soil, representing a non-negligible pollution source (Komárek et al., 2010; Melendez et al., 2020). Copper application in crops, besides the fungicide activity, may affect other non-target organisms in the environment (Flemming and Trevors, 1989; Michaud and Grant, 2003; Yang et al., 2011) and even became a threat to humans health (Mathew et al., 2015; Rehman et al., 2019). Another important metal commonly found in agriculture supplements is zinc, with synthetic fertilizers and plant supplements, rich in zinc sulfate and nitrate salts, acting as potential soil contamination sources (Dwivedi and Srivastva, 2014; Ju et al., 2004; Nielsen, 2012). Widely utilized to support plant growth, zinc overuse may lead to accumulation in soil and crops (Broadley et al., 2007; Nielsen, 2012). At high concentrations (in a range of 55–400 mg kg^{-1}), zinc can be phytotoxic and affect the soil bacterial communities (Chaney, 1993; Long et al., 2003; Moffett et al., 2003). Due to the wide and often simultaneous use, copper and zinc may co-occur and co-accumulate in the agricultural soil (Mantovi et al., 2003; Poulsen, 1998; Sonoda et al., 2019). As expected, the combination of metals, such as copper and zinc, is described to enhance the toxic effect on plants, soil multicellular organisms and microbial community activity (Korthals et al., 2000; Luo and Rimmer, 1995; Song et al., 2018). In addition, metals like copper and zinc are directly correlated with antibiotic resistance in the environment (Baker-Austin et al., 2006; Becerra-Castro et al., 2015; Dickinson et al., 2019; Yazdankhah et al., 2014). For example, Wang et al. (2019) described a positive correlation between the copper and zinc bioavailable in soils and the abundance of the antibiotic resistance genes *ermC*

and *qnrS*. In summary, antibiotic resistant bacteria and metals such as copper and zinc may be supplied by the same type of source, become important soil contaminants and produce different types of soil microbiota disturbance. This scenario meets the One Health concept that considers the holistic protection and health promotion of the environment, animals and humans. Accordingly, the environmental contamination endangers not only the ecosystems, but also human health, for example, through the food-web contamination (Rather et al., 2017; Verraes et al., 2013).

The contamination of soils with antibiotic resistant bacteria or with antibiotic resistance genes due to wastewater irrigation or manure application is described by some studies, mainly when long-term application is used. Manure application was associated with an increase of bacteria abundance in soil, mainly antibiotic resistant bacteria, as well as an increase in the abundance of antibiotic resistance genes (e. g. *ermB*, *ermC*, *qnrS*, *sul1*, *sul2*, *tet*-type and beta-lactamase genes) and genes related with mobile genetic elements, as *intI1* (Faissal et al., 2017; Guo et al., 2017; Heuer et al., 2011b; Marti et al., 2013b; Udikovic-Kolic et al., 2014; Wang et al., 2021; Zhao et al., 2017). Also, accumulation of antibiotic resistant bacteria and antibiotic resistance genes in soil irrigated with treated wastewater is reported (Chen et al., 2014; Wang et al., 2014). In contrast, some other studies have shown that resistant bacteria that enter the soils from the treated wastewater are not able to compete or survive in the soil environment and, hence, do not significantly contribute to the accumulation of antibiotic resistance genes in soils (Gatica and Cytryn, 2013; Marano et al., 2019; Negreanu et al., 2012). However, it is important to critically analyse these results as the high limits of quantification of antibiotic resistance genes in soil (estimated to be 4 log-units genes copy number per g of soil, by traditional real-time PCR), may be responsible for the apparently contradictory findings reported in different publications (Fortunato et al., 2018).

The association between metals and antibiotic resistance has been suggested in different studies, being the genetic linkage the supposed most effective mechanism of co-selection between both (Dickinson et al., 2019; Seiler and Berendonk, 2012; Zhao et al., 2019). In addition, other mechanisms may be involved, in particular, due to the effect of metals on microbial community disturbance, creating the opportunity for fast growing bacteria, as is the case of human and animal commensal bacteria prone to harbor antibiotic resistance genes, proliferate (Dickinson et al., 2019).

The combined effects of soil contamination with metals and antibiotic resistant bacteria and antibiotic resistance genes motivated the current study. The complexity of the topic required a feasible experimental design based on the use of model metals and antibiotic resistant bacteria. The selected metals were cooper and zinc, which, in spite of being not ranked among the most critical environmental contaminants, may reach high concentrations in agriculture soil (Mantovi et al., 2003; Poulsen, 1998; Sonoda et al., 2019; Tóth et al., 2016; Zwolak et al., 2019) and have recognized impacts on the microbiota (Dickinson et al., 2019; Dumestre et al., 1999; Jacquiod et al., 2018b; Kunito et al., 2001; Song et al., 2018). These metals can be supplied in different salt forms. This information motivated the simultaneous testing of both metals and in different salts forms, in order to differentiate between what might be the effect of the metal and of the associated anion. The selected antibiotic resistant bacteria was *Pseudomonas aeruginosa* because it is an important opportunistic

pathogen with recognized ubiquity due to its extraordinary adaptive capacity (Moradali et al., 2017). Besides a rich pool of genetic determinants that confer intrinsic tolerance to a wide array of metals, biocides and antibiotics, members of this species are also important reservoirs of acquired antibiotic resistance genes (Breidenstein et al., 2011; Fajardo et al., 2008). For the study, it was selected a carbapenem-resistant (*bla*$_{VIM}$+) strain, *P. aeruginosa* H1FC49, whose history includes isolation from untreated hospital effluent and a multidrug resistance profile, underling its adaptive capacity (Vaz-Moreira et al., 2016).

The experimental design was settled to test the hypothesis that the persistence of *P. aeruginosa* strain H1FC49 or of the *bla*$_{VIM}$ gene could be affected by: i) the presence of the metals copper and zinc, and that ii) the salts in which these metals are supplied, nitrate or sulfate, could produce distinct effects. Concurrently bacterial communities were compared based on the 16S rRNA gene amplicon sequencing to infer if metal soil amendments triggered changes that could explain the persistence of *Pseudomonas aeruginosa* (*bla*$_{VIM}$+).

4.3 Materials and methods

4.3.1 Soil samples and microcosms assays

Assays were conducted in microcosms with soil collected from an agricultural greenhouse, located in Vila do Conde, Northern Portugal (41°25′ N; 8°45′ W) and that adopts Good Agricultural and Environmental Practices. This greenhouse soil has been characterized as being a sandy soil, with pH (H_2O) of 7.5, organic matter of 1.8%, electric conductivity of 0.32 dS m^{-1}, a concentration of soluble cations (Ca, Mg, Na, K) of 717 mg kg^{-1} and of metals (Cr, Cu, Ni, Pb, Zn) totalizing 98.5 mg kg^{-1} and 25 mg kg^{-1} of nitrate (Becerra-Castro et al., 2017). Soil composite samples were collected between windrows from the same greenhouse in three different occasions; the soil collected in April 2017 (tomato crop) was used for microcosm assay named M1; the soil collected in March 2018 (not cultivated) was used for microcosm assays M2 and M3, which were carried out in two independent dates; and the soil collected in June 2018 (lettuce crop) was used for microcosm assay M4 (Table 4.1). The sampling dates and the period between collection and experiment start are reported in Table 4.1. Sampling procedures and other soil characteristics were previously described (Becerra-Castro et al., 2017). Microcosm assays were established by weighing 300 g of soil (wet weight), spiked with a solution of copper and zinc salts, sulfate or nitrate (Sigma Aldrich ®), to reach a final concentration of 20 mM (equivalent to 830 mg kg^{-1} of copper, 850 mg kg^{-1} of zinc, 1250 mg kg^{-1} of sulfate and 1600 mg kg^{-1} of nitrate). Molar units were used to allow the comparison of metal molecules available when using different salts. As a control, a corresponding soil aliquot was spiked with the same volume of sterile distilled water. After vigorous stirring, control and metal spiked soils (M2, M3, M4) were aged for one month in the dark at room temperature.

Amended and non-amended (control) soil microcosms were inoculated with a suspension of the carbapenem-resistant *P. aeruginosa* strain H1FC49 isolated from hospital effluent (Vaz-Moreira et al., 2016). This strain is resistant to carbapenems due to the presence of the metallo β-lactamase gene *bla*$_{VIM}$, known to be inserted in the variable region of a class 1 integron (Vaz-Moreira et al., 2016). *P. aeruginosa* strain H1FC49 is resistant to carbapenems, penicillins, cephalosporins, fluoroquinolones, sulfonamides, and aminoglycosides, as determined based on the disc diffusion method (Vaz-Moreira et al., 2016). In addition, the genome analysis, based on the annotation against the Resistance Gene Identifier (RGI) of the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020), revealed the presence of the antibiotic resistance genes sulfonamide resistant dihydropteroate synthase (*sul1*), aminoglycoside 3'-phosphotransferase (*aph(3')-IIb*, and chloramphenicol acetyltransferase (*catB7*).

Four soil microcosm sets (M1–M4) were prepared in triplicate for each condition and sampling time (metal amendment and *P. aeruginosa* inoculation and respective controls) in 50 mL tubes containing 10 g of soil (Table 4.1). Metal-amended and control microcosms were inoculated with 3 mL of a suspension of a fresh culture of *P. aeruginosa* H1FC49, prepared in synthetic wastewater as previously described by Fortunato et al. (2018), to reach a final density of 10^7 CFU g^{-1} of soil dry weight. Non-inoculated (spiked with synthetic wastewater) metal-amended and control microcosms were processed in parallel and incubated at 25 °C (Table 4.1). Microcosm replicates were sacrificed at each sampling time (0, 7, 14 days, in M1–M4 and 30 days in M1 and M4), for bacterial enumeration and DNA extraction for further analyses. Microcosm assays were designated as M1–M4, corresponding to four independent assay sets (Table 4.1). Metal amendment (copper and zinc) at a final concentration of 20 mM were labeled as "A20" for sulfate salts and as "B20" for nitrate salts. *P. aeruginosa* inoculation was labeled as "Pa". Controls, without amendment were labeled with "C". The incubation time was labeled as "t" (0, 7, 14, 30 days).

4.3.2 Bacteria enumeration

Culturable bacteria were enumerated in microcosms, by suspending 1 g of soil in 10 mL hexametaphosphate 1% (w/v), to facilitate the suspension of soil particles, with serial dilutions in sterile saline solution (0.85% (w/v), NaCl). From each dilution, 20 µL were dropped on the surface of Plate Count Agar (PCA, Sigma Aldrich) and Cetrimide agar supplemented with nalidixic acid (1.5%) (Sigma Aldrich) plates (Miles et al., 1938). Cultures were incubated at 30 °C for 48 h, with total heterotrophs being registered as those enumerated on PCA and *P. aeruginosa* as those forming green colonies on Cetrimide agar plus nalidixic acid.

Table 4.1 Microcosms assays composition and sampling.

MICROCOSM					SOIL SAMPLES INFORMATION			
Assay	**Sample labels***	**Copper and Zinc salts**	***Pseudomonas aeruginosa* (Pa)**	**Sampling (days)**	**Sampling date →Experiment start**	**Total Bacterial load** (16S rRNA log-gene copies g^{-1} soil dry weight)	**Cultivable bacteria load** (log-CFU g^{-1} soil dry weight)	**Pseudomonads load** (log-CFU g^{-1} soil dry weight)
M1	M1 Pa	None	*Yes*	0, 7,14, 30	April → June 2017			
	M1 C	None	*No*	0, 7, 14, 30		8.8	n.d.	4.5
M2	M2 A_{20} Pa	Sulfate (20 mM)	*Yes*	0, 7, 14	March → June 2018			
	M2 A_{20}	Sulfate (20 mM)	*No*	0, 7, 14				
	M2 Pa	None	*Yes*	0, 7, 14				
	M2 C	None	*No*	0, 7, 14		8.1	6.6	<3.8
M3	M3 B_{20} Pa	Nitrate (20 mM)	*Yes*	0, 7, 14	March → October 2018			
	M3 B_{20}	Nitrate (20 mM)	*No*	0, 7, 14				
	M3 Pa	None	*Yes*	0, 7, 14				
	M3 C	None	*No*	0, 7, 14		8.1	6.6	<3.8
M4	M4 A_{20} Pa	Sulfate (20 mM)	*Yes*	0, 7, 14, 30	April → May 2019			
	M4 B_{20} Pa	Nitrate (20 mM)	*Yes*	0, 7, 14, 30				
	M4 A_{20}	Sulfate (20 mM)	*No*	0, 7, 14, 30				
	M4 B_{20}	Nitrate (20 mM)	*No*	0, 7, 14, 30				
	M4 Pa	None	*Yes*	0, 7, 14, 30				
	M4 C	None	*No*	0, 7, 14, 30		8.6	6.5	3.5

*Pa, *Pseudomonas aeruginosa*; A, sulfate; B, nitrate; C, control (non-inoculated with Pa and non-amended with metals); n.d., not determined.

4.3.3 DNA extraction and quantitative PCR

DNA was extracted in triplicate from each microcosm from 0.25 g of soil, using the DNeasy PowerSoil kit (Qiagen) following the manufacturer's instructions. The DNA concentration was determined with the Qubit® HS DNA kit (Life Technologies Corporation). The genes *ecf*, a *P. aeruginosa* marker encoding the RNA polymerase sigma-70 factor, *bla*$_{VIM}$, encoding the metallo β-lactamase VIM-2, and *intI1*, encoding the class 1 integron integrase, as well as the 16S rRNA gene were quantified by quantitative PCR. The 16S rRNA gene was quantified to estimate the total bacterial load, *ecf* to estimate the abundance of *P. aeruginosa*, *intI1* as a proxy for mobile resistome, and *bla*$_{VIM}$ to assess the persistence of this carbapenem resistance gene in soil. The genes 16S rRNA, *bla*$_{VIM}$, and *intI1* were quantified following the conditions previously described (Fortunato et al., 2018). The gene *ecf* was quantified using the primers ECF5 – AAGCGTTCGTCCTGCACAA and ECF2– TCATCCTTCGCCTCCCTG (Colinon et al., 2013) with an initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s. Real-time quantitative PCR used a StepOne Real-Time PCR System (Life Technologies, Carlsbad), and the Standard Curve method (Brankatschk et al., 2012). All quantifications adopted the same quality criteria: the possibility of interpolation to the calibration curves, the correct melting temperature of the amplicon, and the absence of multiple peaks or shoulders (Rocha et al., 2018). The amplification at Ct values below the lowest concentration of the calibration curve, at the expected melting temperature, were considered as being above the LOD, and below the LOQ (Fortunato et al., 2018).

4.3.4 Soil microbial community analysis

For the bacterial community analysis the microcosms M3 (t0 and t14) and M4 (t0, t14, and t30) were selected because were those representing distinct soil collection events that permitted the comparison of the metals and salts effects. The amplicon sequencing analysis targeted the V3/V4 hypervariable region of the 16S rRNA gene of triplicate DNA pools, using paired-end Illumina Miseq (STAB VIDA, Lda). As a quality control, sequences shorter than 300 bp or with a quality score lower than 25 were eliminated. The good quality reads were analysed and processed using Quantitative Insights Into Microbial Ecology (QIIME2) (version 2019.7; http://qiime2.org/) (Bolyen et al., 2019). Sequences were filtered, merged and, chimeric reads removed by the DADA2 software package enclosed in QIIME2 (Bolyen et al., 2019). Taxonomy was assigned to the amplicon sequence variants (ASVs), using the ARB SILVA taxonomic database version 132 (Yilmaz et al., 2014). In addition, the ASVs' relative abundance, the alpha diversity indexes (reported in Table S1), and the beta diversity metrics were calculated for each sample using QIIME2. The relative abundance of the most abundant (> 2%) bacterial groups at phylum, class, and order levels was represented as barplot graphics using the R package Phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2016). The beta

diversity metrics was imported to R using the package QIIME2R (https://github.com/jbisanz/qiime2R) and plotted as biplot PCoA using the package ggplot2.

4.3.5 Statistical analyses

The relative abundance of bacterial groups at phylum, class, and order levels were analysed using the statistical software STAMP v2.1.3 (Parks et al., 2014). One-way analysis of variance ANOVA (post hoc Tuckey HSD and Bonferroni) was applied to define the statistical differences (*p*-value 0.05) among the alpha diversity indexes measured in the different soil conditions. Differences in beta diversity were quantified using the permutational multivariate analysis of variance (PERMANOVA), with 999 permutations. The significance was determined by Benjamini/Hochberg FDR p-value adjustment for pairwise comparisons (*q-value* < 0.05). The CFUs and genes quantified were normalized, as the ratio of the *log* value measured at each sampling time versus the *log* value measured at the time zero. Parametric analysis as ANOVA with post hoc Bonferroni and Tuckey HSD and non-parametric analysis (Wilcoxon test) were used to define statistical differences (*p*-value 0.05) among the CFUs and genes quantified in the different soil conditions, over the incubation period.

4.4 Results

This study comprises four major topics, (a) the survival of *P. aeruginosa* H1FC49 and the persistence of the gene *bla*$_{VIM}$ in non- amended soil microcosms (Section 4.3.1), or (b) in soil microcosms amended with copper and zinc as sulfate salts, or (c) as nitrate salts (Section 4.3.2), as well as the (d) effect of these metals and salts in the soil microbiota (Section 4.3.3).

4.4.1 Survival of P. aeruginosa H1FC49 bla$_{VIM}$*+ in non-amended soil microcosms*

The initial load of *P. aeruginosa of* 7.9 log-CFU g^{-1} soil dry weight decreased to 5.9–6.0 log-CFU g^{-1} soil dry weight at t14 and maintained the same value at t30. The C/C_0 ratio, which expresses the abundance at a given time in comparison to time zero, indicates that the abundance of the typical *P. aeruginosa* colonies on cetrimide agar (green colonies) in non-amended and non-aged soil (M1 microcosms) decreased significantly in the first week of incubation (C/C0 ~ 0.85) and continued to decrease up to 14 days of incubation ($p < 0.05$). From day 14 on, no significant variation in the abundance of *P. aeruginosa* colonies was observed (grey boxplots, Figure 4.1). Consistently, similar results were observed when the fate of *P. aeruginosa* was assessed based on the abundance of

gene *ecf* (orange boxplots, Figure 4.1). The abundance of this gene decreased from 7.6 log-gene copy number g^{-1} soil dry weight at t0 to 5.7 log-gene copies g^{-1} soil dry weight at t30. This same pattern was observed for *intI1* gene, whose abundance decreased from 8.0 log-gene copies g^{-1} soil dry weight at t0 to 5.8 log-gene copies g^{-1} soil dry weight at t30 (green boxplots, Figure 4.1). The slightly higher abundance *intI1* gene observed at t0 might be due to its occurrence in indigenous soil microbiota. The carbapenem resistance gene bla_{VIM} (blue boxplots, Figure 4.1) presented a pattern of variation identical to *ecf* and *intI1*, in the first weeks, with C/C0 significantly decreasing to ~ 0.85 in the first week and to 0.78 in the second, but took off in the period t14–t30 when C/C0 reached 0.68, the lowest value observed. This variation corresponded to a significant abundance decrease of the bla_{VIM} gene ($p < 0.05$) from 7.8 log-gene copies g^{-1} soil dry weight at t0 to 5.3 log-gene copies g^{-1} soil dry weight at t30. These variations were not accompanied by a significant decrease in the abundance of soil microbiota, as was indicated by the maintenance of the abundance of the 16S rRNA gene, with values ranging 8.5–8.9 log-gene copies g^{-1} soil dry weight, observed over the 30 days incubation period (data not shown). In addition, the non-inoculated soil used for M1 assays presented 4.5 log-CFU g^{-1} soil dry weight on cetrimide agar supplemented with nalidixic acid (Table 4.1), and the *ecf* and bla_{VIM} genes quantifications were below the limits of detection (3.97 and 4.01 log-gene copies g^{-1} soil dry weight, respectively).

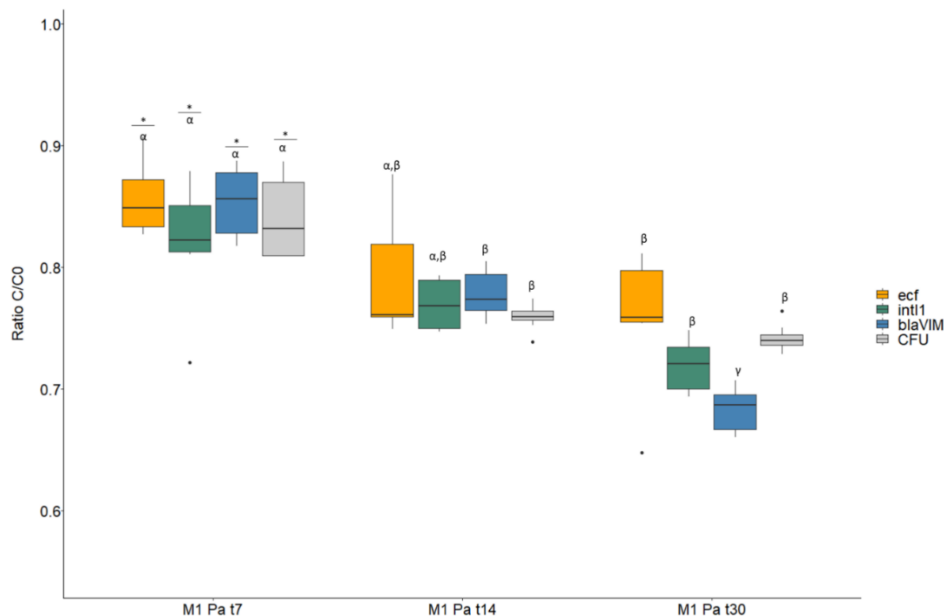

Figure 4.1 Variation (C/C0) of the biomarkers of *Pseudomonas aeruginosa* H1FC49 and harboured genes

Cetrimide agar green colonies, housekeeping ecf and acquired genes blaVIM and intI1, examined in non-metal amended microcosms (M1).
C/C0 - quantification at time (C) *per* time 0 (C0). From right to left, C7/C0, C14/C0 and C30/C0 for 7, 14 and 30 days of incubation, respectively, of the genes *ecf* (orange), *intI1* (green), bla_{VIM} (blue), and colony forming units (CFU) (grey).
The statistical analysis was conducted using the non-parametric analysis Wilcoxon test. The significative (p< 0.05) variation for the genes quantification and CFU enumeration along the time are reported as α,β,γ. The difference between the C/C0 measured at time 0 and time7 for the CFU and genes is reported as *

The abundance of the 16S rRNA gene was constant over time (8.5-8.9 log-gene copies g^{-1} soil dry weight) (data not shown).

4.4.2 Survival of P. aeruginosa H1FC49 bla$_{VIM}$*+ in copper and zinc amended soil microcosms*

Soils samples were aged with metals, or synthetic wastewater in the case of the controls, for one month before the inoculation. In M2 it was tested the effect of copper and zinc sulfate and in M3 the equivalent with nitrate salt. The soil used in these microcosms was collected in the same date although M2 and M3 were set up in independent dates (Table 4.1). As an additional control of this variable, i.e. the same soil tested in different occasions, the soil used in M4 was collected in a different occasion. In contrast to M2/M3, in M4 the effects of sulfate and of nitrate were assessed in parallel (Table 4.1). In all microcosms, M2-M4, controls with soil samples that were not aged or supplemented with metals at any moment were analysed simultaneously. This design supported further statistical analysis. The different soil samples used in the microcosms presented similar total bacteria, total cultivable bacteria and pseudomonads loads (Table 4.1).

For microcosms M2–M4, total heterotrophs counts presented minor and non-significant variations over time (from t0 to t14 or t30), being enumerated during the whole period in the same range of CFU g^{-1} (log values range: 6.0–6.8 CFU g^{-1} soil dry weight) independently of the metal addition and salt type, sulfate or nitrate. Also, the abundance of the 16S rRNA gene presented small variations, with values in the order of 8–9 log-gene copies g^{-1} soil dry weight.

The results over time of the targeted biomarkers (colonies on cetrimide agar and the genes *ecf*, *bla*$_{VIM}$, and *intI1*) were not totally reproducible in the three microcosms sets (Figure 4.2), suggesting some stochasticity in the system, which may be due to slight variations on the physico-chemical and biological parameters of the soil samples, collected over a period of 2 years. However, there were common patterns of variation for the three distinct conditions tested. The C/C_0 ratio of the abundance of typical *P. aeruginosa* colonies on cetrimide agar (green colonies) showed a sharp decrease in the first week of incubation independently of the condition. In the absence of metal amendment and in the presence of sulfate salts a decrease to a C/C0 ratio of ~ 0.8 was observed (Figures 4.2A–C). In the presence of nitrate salts, the decrease was higher, reaching C/C0 ratios of ~ 0.7 in the same period (Figures 4.2B, C). The C/C0 values of colony forming units continued to decrease until time t30, reaching values between 0.64 and 0.68 in the three conditions at t30 (Figure 4.2C). The decrease observed with nitrate salts was always significantly higher in comparison to the non-amended control (Figures 4.2B and C) or amended with sulfate assays ($p < 0.05$) (grey boxplots, Figure4. 2).

From the initial 7.9 log-CFU g^{-1} soil dry weight, the load of *P. aeruginosa* decreased to 5.0 log-CFU g^{-1} soil dry weight at t30 in the nitrate- amended soil and to 5.2 and 5.5 log-CFU g^{-1} soil dry weight in sulfate-amended and non-amended soil, respectively (M4, Figure 4.2C). The measurement of the

P. aeruginosa H1FC49 genes showed a distinct pattern of variation. The abundance of the gene *ecf* (orange boxplots, Figure 4.2) had a mild decrease after the first week (C/C0 ~ 0.95 in non- metal; 0.85–0.95 in sulfate salts or nitrate salts), was fairly stable after the second week and had a significant decrease between day 14 and day 30 (M4) reaching C/C0 values of 0.85–0.89 in all the conditions (orange boxplots, Figure 4.2C). The abundance of this gene, independently of the metal and salt amendment, decreased from a range of 6.9–7.2 log-gene copy number g^{-1} soil dry weight at t0 to 6.2 log-gene copies g^{-1} soil dry weight at t30 (M4). For the *intI1* gene, it was also observed a significant decrease in the first week of incubation, independently of the condition. This decrease was more notorious in the assay M2 (C/C0 ~ 0.75) (green boxplots, Figure 4.2A) than in the assays M3 and M4 (C/C0 ~ 0.9) (Figures 4.2B, C). However, this effect could not be attributed to the presence of sulfate, since in the assay M4 the decrease of *intI1* gene showed a similar pattern in both salts, nitrate and sulfate, with abundance decreasing from 7.4 to 7.7 log-gene copies g^{-1} soil dry weight at t0 to 6.8–6.9 log- gene copies g^{-1} soil dry weight at t30. The gene *bla*$_{VIM}$ presented a significant decrease in the first 7 days, mainly in assays M2 and M3 with ratios C/C0 of 0.83–0.85 in M2 and 0.92–0.98 in M3, being stable until t14, while in M4 assay the decrease was not statistically significant over 30 days (Figure 4.2C). In these assays (M4), the *bla*$_{VIM}$ abundance decreased from a range of 6.8–6.9 log-gene copies g^{-1} soil dry weight at t0 to a range of 6.3–6.4 log-gene copies g^{-1} soil dry weight at t30.

In summary, the results observed for *P. aeruginosa* H1FC49 culture suggested the loss of viability in soil. However, it must be emphasized that in spite of the reduction, after 30 days of incubation at least 60% of the cells were viable in soil, irrespective of the metal amendment. The loss of culture viability was not accompanied by identical losses of the two biomarkers of *P. aeruginosa* H1FC49, the housekeeping gene *ecf* and the acquired gene *bla*$_{VIM}$. The analyses of these two genes revealed that, in general, both had higher persistence in soil than viable cultures and that both shared identical patterns of variation. The identical behaviour of *ecf* and *bla*$_{VIM}$ genes overtime suggests that *bla*$_{VIM}$ curing (e.g. loss due to excision) is not taking place in any condition. In general, it was observed that the most important variations on *P. aeruginosa* H1FC49 abundance, either measured based on viable cultures or biomarker genes, occurred during the first week, probably corresponding to an adaptation process. The M4 assay showed that irrespective of the conditions, for each biomarker similar values were reached at t30, suggesting that the exogenous bacteria and its genetic elements reached stability. In general, the metal amendment effects were mild and when noticed, contributed to reducing and not selecting for exogenous bacteria and genes.

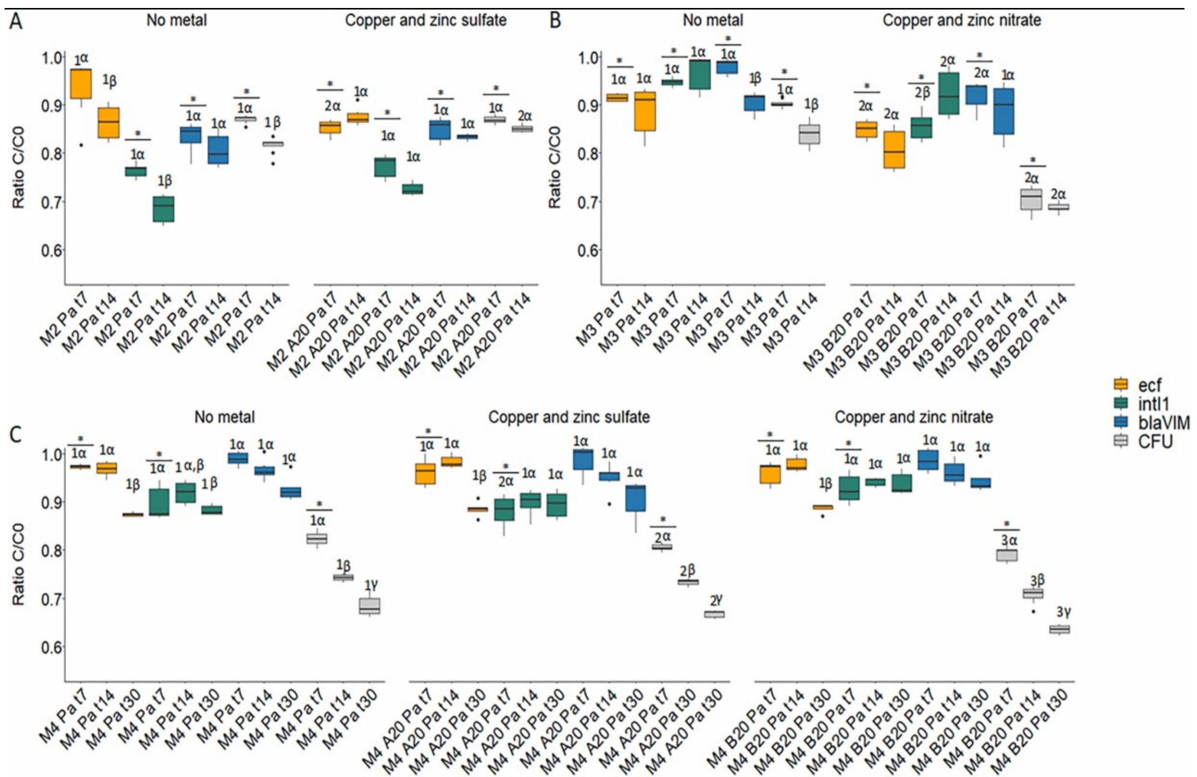

Figure 4.2 Variation (C/C0) of the biomarkers of *Pseudomonas aeruginosa* H1FC49 and harboured genes in soil amended and non-amended with metals

Cetrimide agar green colonies, housekeeping *ecf* and acquired genes bla_{VIM} and *intI1*, examined in non-metal amended microcosms (M2, M3, M4) and amended with copper and zinc sulfate (M2, M4) or nitrate (M3, M4).

A) microcosms M2; B) microcosms M3; C) microcosms M4. C/C0 – quantification at time (C) *per* time 0 (C0). From right to left, C7/C0, C14/C0 and C30/C0 for 7, 14 and 30 days of incubation, respectively, of the genes *ecf* (orange), *intI1* (green), bla_{VIM} (blue), and colony forming units (CFU) (grey). The statistical analysis was conducted using the non-parametric analysis Wilcoxon test. The significative ($p < 0.05$) variation for the genes quantification and CFU enumeration along the time are reported as α, β, γ. The variation of the genes at the same sampling time but in different soil conditions were reported using the index: 1, 2, 3. The difference between the C/C0 measured at time 0 and time 7 for the CFU and genes is reported as *. The total heterotrophs, counted on PCA, ranged log magnitude of 6 CFU g^{-1} soil dry weight (data not shown). The 16S rRNA gene was quantified in all the samples in a constant range of 8–9 log-gene copies g^{-1} soil dry weight (data not shown).

4.4.3 Effect of copper and zinc amendment on the composition and structure of the soil bacterial communities

The microbial community of microcosms M3 and M4 was examined at t0, t14, and t30 aiming to assess if metal amendments could impact the composition or structure of the communities and to infer if these could be related to the fate of *P. aeruginosa* H1FC49. M3 and M4 soil samples were obtained in distinct sampling events, a fact that can be reflected in the slight differences in the community structure (Figure 4.3). The effect of metals and salts on the community composition was analysed through the comparison of microcosms M3 and M4 samples at t0, t14, and t30 (M4) produced with soil that was aged for one month with copper and zinc sulfate, copper and zinc nitrate, or synthetic wastewater as a control (Figure 4.4). The inoculum did not produce a significant effect on the community according to PERMANOVA analyses (Table S4.2). As hypothesized, metals were associated with significant changes in the bacterial community structure. The relative abundance of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* was significantly higher ($p < 0.05$) in assays with metal amendment than in non-amended ones (Figure 4.4A), mainly due to the higher percentage values observed in members of the classes *Alpha*-(from 15.0–19.0% to 20.8–22.5%) and *Gammaproteobacteria* (from 10.0–12.0% to 12.4–14.3%), *Actinobacteria* (from 2.7–7.0% to 6.0–7.6%) and *Thermoleophilia* (from 1.0–1.6% to 2.3–2.6%), and *Bacilli* (from 3.5% to 8.0% to 6.7–8.7%) and *Clostridia* (from 0.6–1.0% to 1.0–1.6%), respectively (Figure 4.4B). Also, the hypothesis that the metals salts form, sulfate or nitrate, could be associated with distinct patterns of variation in the bacterial community structure was confirmed. The relative abundance of phyla *Chloroflexi*, *Planctomycetes, Patescibacteria*, and *Latescibacteria* was higher in nitrate salts than in sulfate salts amended soil. The opposite was observed for phyla *Bacteroidetes* and *Proteobacteria*, with lower relative abundance in nitrate salts than in sulfate salts amended soil. This could be explained because the orders of the latter two phyla, respectively *Cytophagales* and *Chitinophagales*, and *Betaproteobacteriales* and *Sphingomonadales*, presented increased relative abundance in soil amended with sulfate salts. In contrast, the unc. *Alphaproteobacteria* (phylum *Proteobacteria*), *Ardenticatenales* (phylum *Chloroflexi*), *Planctomycetacia* (phylum *Planctomycetes*), and the *Saccharimonadales* (phylum *Patescibacteria*) had higher relative abundance in soil amended with nitrate salts (Figure 4.4C). Consistent with these variations on the bacteria community structure, it was observed that metal-amended soil samples, compared with the non-amended controls presented lower richness (number of ASVs and Chao indexes) and diversity (Fisher index) indices (Table S4.1). Considering the effects of salts, it was observed that sulfate had a higher impact on the reduction of these indices than nitrate, significantly higher in this case. The bacterial community patterns of the three types of microcosm (non-amended, nitrate copper and zinc or sulfate copper and zinc) at three incubation times were inspected based on beta diversity analyses, reported as a PCoA biplot (Figure 4.5). Axes 1 and 2 explained ~ 60% of the variation, with segregation of samples per metal amendment condition, and inside these groups per soil sampling campaign (M3 and M4), with the incubation time (t0, t14, and t30) demonstrating minimal influence. The samples from soil amended with sulfate or nitrate salts present a different microbial community if compared with the

non-amended soil ($q < 0.01$, F = 856 and 11.614 respectively, PERMANOVA results, Table S4.2). Metal salts had a minor influence on the separation of the groups ($q < 0.01$, F 5.790, PERMANOVA results, Table S4.2), probably due to the lower number of sulfate- amended microcosm for which it was possible to examine the community. The increased relative abundance of members of the orders *Rhizobiales*, *Xanthomonadales, Sphigomonadales* of the phylum *Proteobacteria*, and *Bacillales* of the phylum *Firmicutes* in metal-amended microcosms, observed in Figure 4.4C, seemed to be the major driver for the organization of the biplot (Figure 4.5).

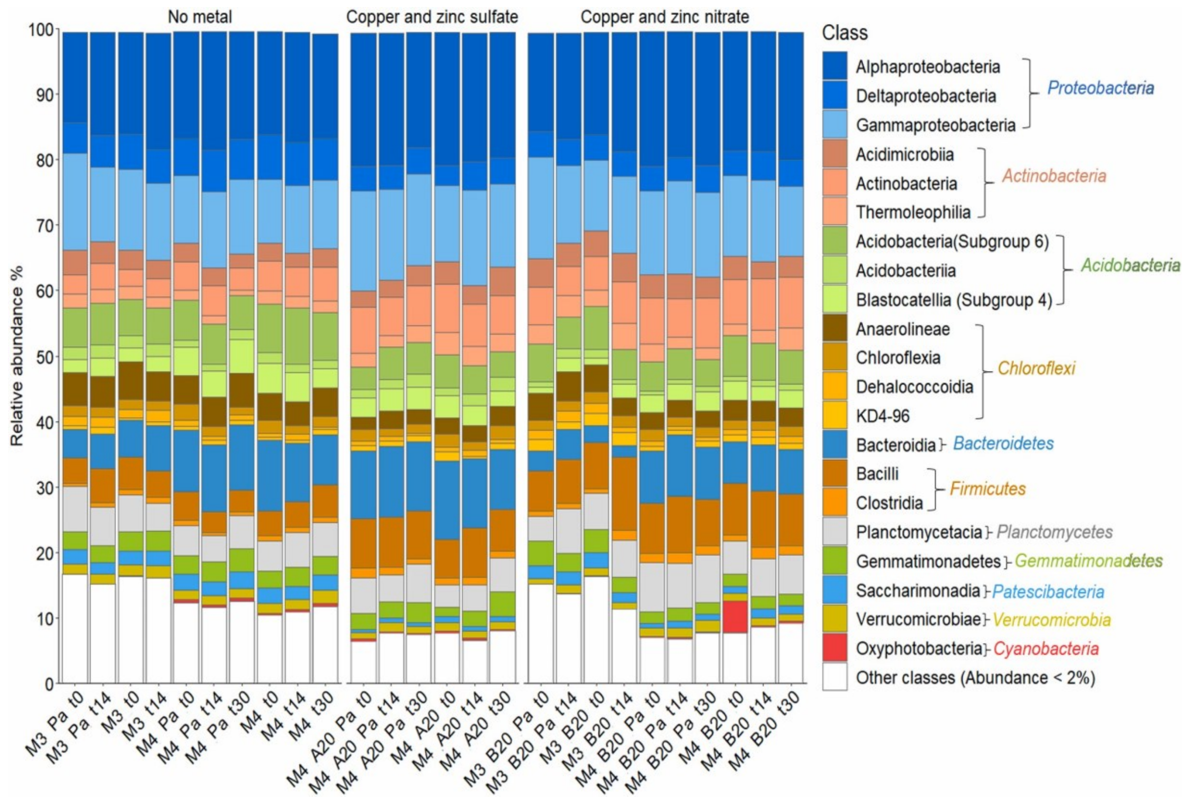

Figure 4.3 Soil microbiome relative abundance at phylum level

Relative abundance of classes and phyla in non-amended or amended with copper and zinc, as sulfate or nitrate salts in microcosm assays sampled over time (0–30 days). The barplot reports the relative abundance as percentage of most abundant phyla (> 2%) present in the samples. The sum of the relative abundance of the most abundant classes in each column was calculated in a range of 83–92%.

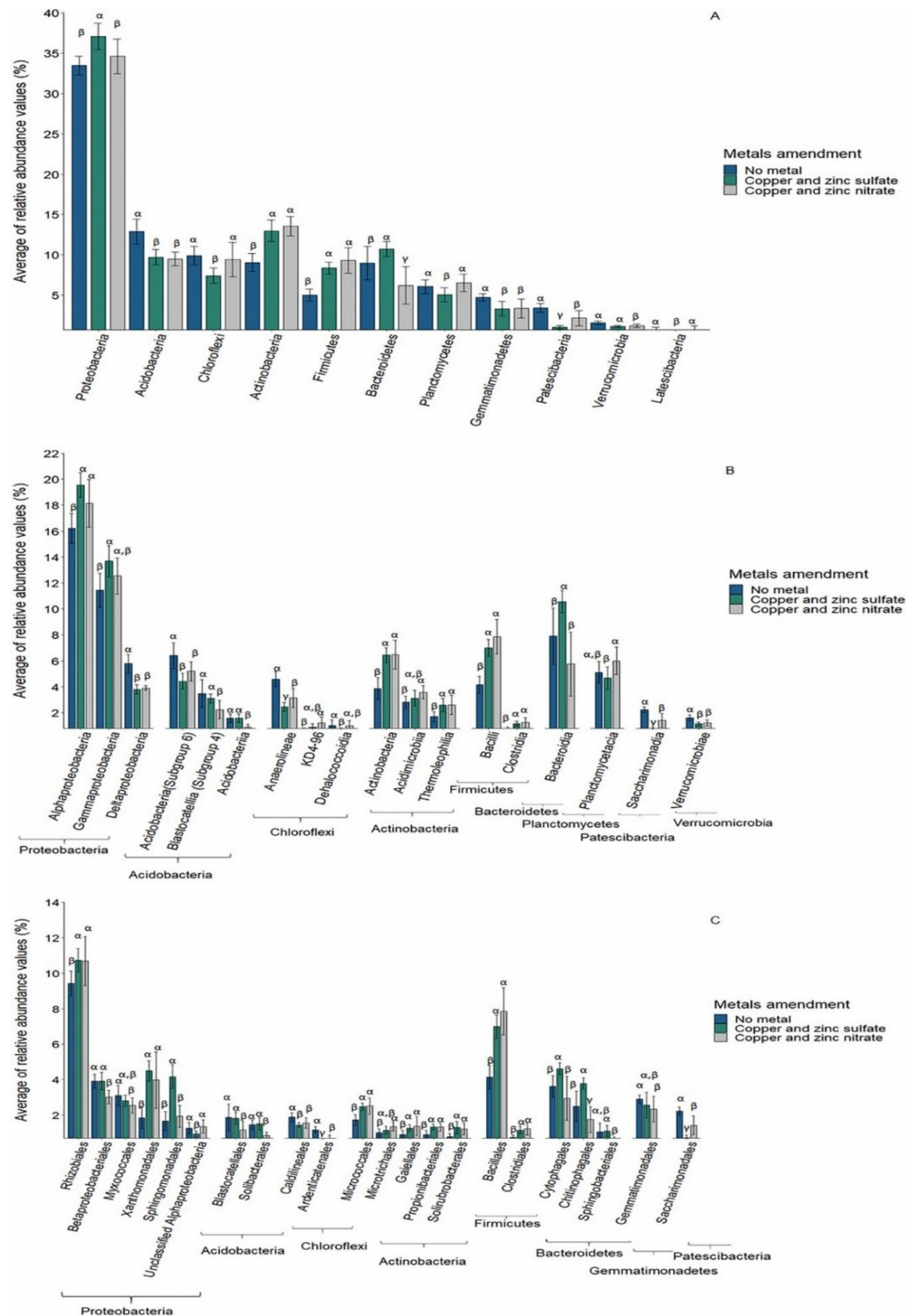

Figure 4.4 Average relative abundance of most abundant phylum, class and order (> 2% relative abundance) with significative variances in the samples analysed, grouped per soil metals salts amendment.

The barplot reports the mean relative abundance of the most abundant Phyla (A), Classes (B) and Orders (C) present in the samples. The blue bar refers to soil microcosms non-amended, the green bar to soil amended with copper and zinc sulfate at final concentration 20 mM and the grey bar to soil amended with copper and zinc nitrate at final concentration 20 mM. The significant difference (p-value 0.05) between no metal or amendment with sulfate or nitrate metal salts are reported in figure as α, β, γ. The reported data was grouped by metal amendment, including the data from the sampling times 0, 7, 14, and 30. The soil, before the inoculation with bacteria, was aged for one month with the metals solutions or synthetic wastewater. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

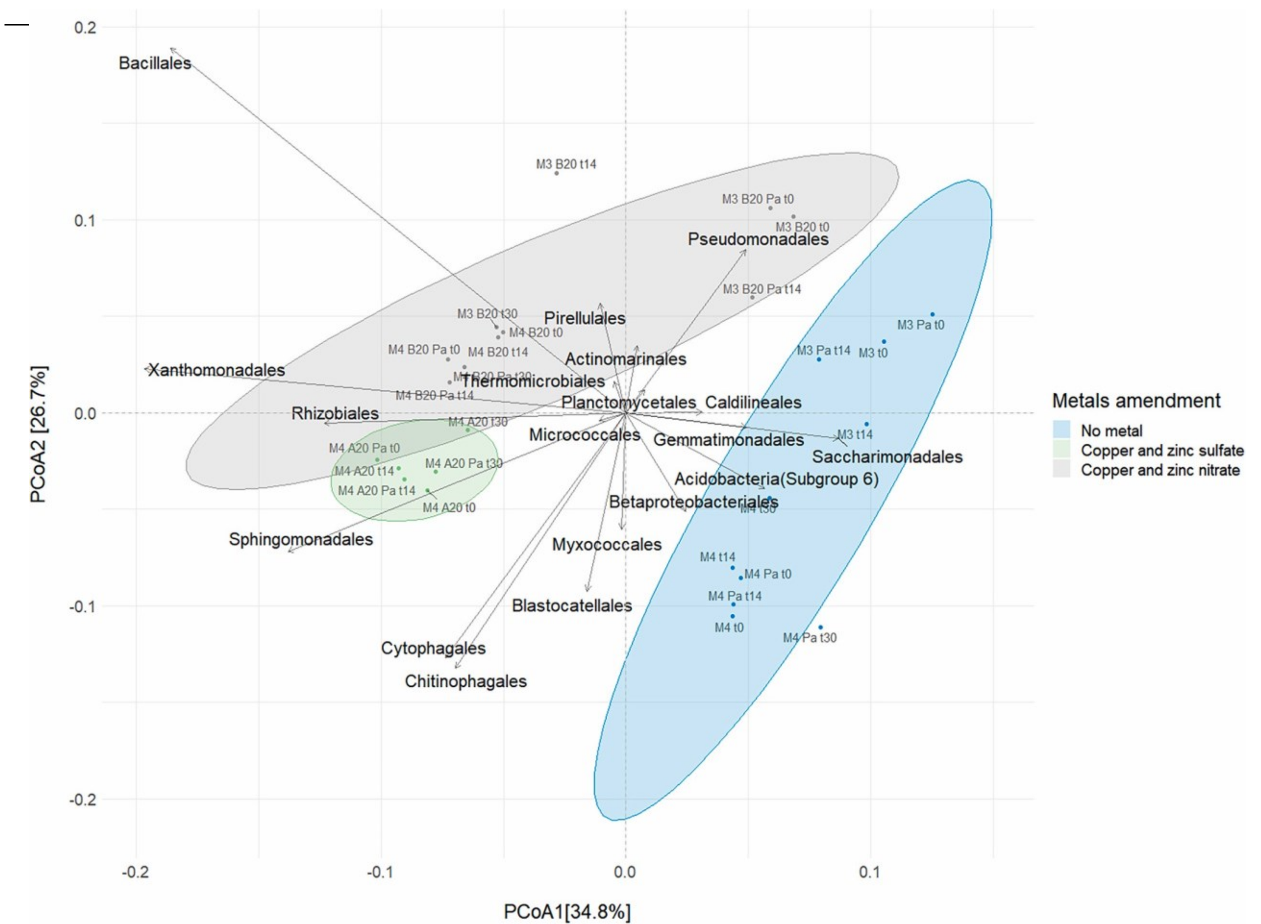

Figure 4.5 Biplot of beta-diversity distance metrics (Weighted Unifrac distance) of bacterial communities (at Order level) in soil amended or non-amended with sulfate or nitrate zinc and copper metal salts.

The plot reports the distribution of the samples depending on the soil metal amendment (blue, non-amended; green, amended with copper and zinc sulfate; grey, amended with copper and zinc nitrate). Reported in the plot the orders contributing the most to the sample distances. The contribution is quantified with the length of each arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

4.5 Discussion

Copper and zinc, although not considered alarming pollutants, may reach high concentrations in the environment (Klimek, 2012; Lamichhane et al., 2018; Tóth et al., 2016). Given the high probability of co-occurrence of these metals in agricultural soils (Tóth et al., 2016), the effect of their mixture was herein assessed. Indeed, the effects of mixtures of pollutants are expected to be more informative when tested in complex systems, as is the case of soil microcosms, whereas the use of single elements are more adequate to assess specific cellular/culture responses. In this study it was a used a concentration of metals exceeding (roughly 4–8 times higher) the levels reported as acceptable for a non-polluted soil (100 mg kg^{-1} for copper and 200 mg kg^{-1} for zinc) (Alloway, 2008; Mengel et al., 2001; Tóth et al., 2016). The use of extreme conditions was considered strategic to avoid the observation of effects that, affected by stochasticity, might be unreproducible. The bacterial surrogate selected for this study was a carbapenem-resistant *P. aeruginosa* strain H1FC49 isolated from untreated hospital effluent that harbored the carbapenemase *bla$_{VIM}$* gene. The work hypotheses were 1) that the exogenous bacteria at an initial density in the same order of magnitude as the heterotrophic bacteria in soil would decay, eventually to levels below the detection limit, 2) that the acquired resistance gene (*bla$_{VIM}$*) would be lost earlier than its host, due to gene excision, 3) that metals could act as selective agents, as it has been reported (Gillan et al., 2015; Jacquiod et al., 2018b; Li et al., 2017), 4) that metals salts could influence the decay process, and 5) that metals amendment effects would imply changes in the bacterial community with implication on the fate of the exogenous *P. aeruginosa* H1FC49.

Regarding the decay of exogenous *P. aeruginosa* H1FC49 it was observed a reduction, mainly of cultivability and in a much lower extent of the respective genes, suggesting that even if cells lose viability, their DNA is still integer. Indeed, the reductions observed were never above 40% for culturable bacteria or above 35% for genes, and, when observed, was after 30 days of incubation, it was possible to detect most of the inoculum added. These observations are in line with previous studies that have shown that after cell death, amplifiable extracellular DNA can persist in soils for weeks to years (Levy-Booth et al., 2007; Nielsen et al., 2007; Pietramellara et al., 2009). The possibility of curing, meaning the loss of acquired genes, in this case *bla$_{VIM}$*, was hypothesized as it has been reported in the literature (Lazdins et al., 2020; Trevors, 1986). Even if it took place in the first week of incubation in some of the assays, the effect vanished in the following incubation period. These results suggest that *bla$_{VIM}$* is stable in the *P. aeruginosa* H1FC49 genome or at least maintains integrity in extracellular genetic material. The fact that most important variations were observed after 7 days of incubation may suggest some adaptative processes. The adaptation of exogenous bacteria in soil has been reported (Soda et al., 1998) and may explain the results obtained. Soda et al. (1998) inoculated soil microcosms with exogenous bacteria, observing in 7 days of incubation at a defined temperature (25 $^{\circ}$C) a rapid but not total decrease of the bacteria spiked.

According to literature, metals can exert an important effect as selective agents for exogenous bacteria and antibiotic resistance genes (Berg et al., 2010; Dickinson et al., 2019; Hu et al., 2016),

and copper and zinc are very well known agents used to prevent microbial growth (Grass et al., 2011; Vincent et al., 2016). However, in spite the high concentrations tested, metals had a weak antimicrobial effect in the surrogate *P. aeruginosa* H1FC49. These results are elucidative if we consider that it was used a high dose of metals (20 mM, equivalent to 830 mg kg^{-1} of copper, 850 mg kg^{-1} of zinc, 1250 mg kg^{-1} of sulfate, and 1600 mg kg^{-1} of nitrate), comparatively to the levels of metals commonly found in soil (up to 100 mg kg^{-1} of copper and up to 200 mg kg^{-1} of zinc), in the range of values that are only observed for contaminated soils (up to 1500 mg kg^{-1} for copper and up to 5000 mg kg^{-1} for zinc) (Lamichhane et al., 2018; Nielsen, 2012; Tóth et al., 2016; Wuana and Okieimen, 2011). The fact that sulfate is known as an antimicrobial agent and nitrate is reported as fertilizer (Sebilo et al., 2013) motivated the analysis of the effects of these metal salts. However, it was observed a higher decrease of *P. aeruginosa* H1FC49 in nitrate amended soil than in sulfate amended or non-amended soil, mainly in what concerns cultivability. This can be attributed to the effect of nitrate per se or to the fact that this anion had a double concentration than sulfate (respectively Cu/ZnSO_4 and Cu/Zn $(NO_3)_2$).

The analysis of the bacterial community had two major aims, the assessment of the impact of metals/salts in soil, and the evaluation if hypothetical changes in the bacterial community composition and structure, due to metals, could be related with the fate of *P. aeruginosa*. The observation that *P. aeruginosa* and the respective genes persistence was not significantly affected by metals, lowered the original expectations of finding a significant correlation between the community composition and the exogenous inoculum. However, the assessment of the impacts on the bacterial community was still a major objective that could be addressed by the analysis of M3 and M4, with soil collected in different occasions that was aged with metals and could be compared with the respective controls. As expected, the bacterial community of the assays M3 and M4, in the same conditions, showed very similar patterns, and supported reliable inferences about the impact of metals/ salts in soils.

Metals amendment produced effects on the richness, diversity and structure of the bacterial communities, a finding that comes in line with previous studies (Gillan et al., 2015; Jacquiod et al., 2018b; Li et al., 2017). In those studies, it is reported that exposure to heavy metals affect the structure of the sediments/soil microbial communities, although do not significantly affect the bacterial diversity. After a long-term metal contamination of river sediments, Gillan et al. (2015) reported a significant, although subtle, increase in the relative abundance of *Pseudomonas* (+0.4%), *Thiobacillus* (+0.36%), and *Acidovorax* (+0.48%), and decrease of *Leptothrix* (− 0.4%). The authors concluded that metal amendment was associated with the increase in the relative abundance of those groups that might have occurred at the expenses of some minor groups (< 1%), whose relevance for soil quality and resilience or effect on the survival of exogenous bacteria is difficult to predict (Gillan et al., 2015). In the current study, a parallel between *P. aeruginosa* H1FC49 fate and the bacterial community composition can be inferred from a faster decay of the exogenous strain over the first week, which is coincident with the increase in the relative abundance of members of the orders *Rhizobiales, Xanthomonadales, Sphigomonadales*, and *Bacillales*. This observation may suggest competition between native bacteria of those orders and the exogenous strain H1FC49.

Indeed, competition is regarded as a major mechanism for the elimination of exogenous bacteria (Hibbing et al., 2010). These observations highlight the importance of the quality of soil to prevent the invasion by exogenous bacteria, as has been argued (Elsas et al., 2012; Van Elsas et al., 2007).

4.6 Conclusion

This study showed that a carbapenem-resistant *P. aeruginosa* strain isolated from hospital effluent was able to persist in soil up to 30 days, while the carbapenemase encoding gene bla_{VIM} was also still quantifiable after that period. The presence of 20 mM of zinc and copper sulfate or nitrate (830 mg kg^{-1} of copper, 850 mg kg^{-1} of zinc, 1250 mg kg^{-1} of sulfate or 1600 mg kg^{-1} of nitrate mg kg^{-1}), a concentration that simulates an extreme contamination situation, did not lead to an apparent selective advantage of *P. aeruginosa*. However, it is also impressive that this strain was mainly insensitive to these metals and salts that are sometimes used as biocides. This strain was also not affected by the disturbance of the microbial community caused by the metal salts. The disturbance observed included the reduction of the richness and diversity of the bacterial community, with a shift of groups such as members of the orders *Bacillales, Xanthomonadales, Rhizobiales* and *Sphingomonadales* at expenses of groups such as *Caldilineales* and *Saccharimonadales*. These results highlight the risks posed by the spread of antibiotic resistant bacteria in soils, with serious implications under a One-Health vision. The study also emphasizes the extreme vulnerability of soil to different modes of contamination, even when sustainable agriculture practices are in place.

Supplementary information

Chapter 4: Effect of copper and zinc as sulfate or nitrate salts on soil microbiome dynamics and *bla*$_{VIM}$-positive *Pseudomonas aeruginosa* survival

Published:

Authors: Fortunato, G., Vaz-Moreira, I., Nunes, O.C., Manaia, C.M.

Year: 2021.

Title: “Effect of copper and zinc as sulfate or nitrate salts on soil microbiome dynamics and *bla*-positive *Pseudomonas aeruginosa* survival”.

Journal: Journal of Hazardous Materials 415, 125631. https://doi.org/10.1016/j.jhazmat.2021.125631

Table S4.1 Alpha diversity indexes measured in the analyzed samples.

For all the microcosms analyzed were calculated the different alpha diversity indexes. To compare the index calculated in the different soil conditions, the samples were grouped using the metal amended or bacterial inoculum as variables. The comparison was statistically evaluated using analysis of variance ANOVA and Wilcoxon paired test. The significant difference (p-value 0.05) between the groups analyzed are reported in figure as α,β,γ.

Samples	Metal amendment	Bacterial Inoculum	Observed ASVs	Chao1 index	Shannon	Simpson	Fisher	Dominance
M3 C t0	None	None	1413^{α}	1.419^{α}	6.975^{α}	0.9988^{α}	2.913^{α}	0.0011^{α}
M4 C t0			1541^{α}	1.549^{α}	7.061^{α}	0.9989^{α}	3.250^{α}	0.0010^{α}
M3 C t14			1621^{α}	1.630^{α}	7.119^{α}	0.9990^{α}	3.465^{α}	0.0009^{α}
M4 C t14			1439^{α}	1.444^{α}	7.005^{α}	0.9989^{α}	2.981^{α}	0.0010^{α}
M4 C t30			1464^{α}	1.467^{α}	7.010^{α}	0.9988^{α}	3.046^{α}	0.0010^{α}
M3 Pa t0	None	*P. aeruginosa*	1637^{α}	1.640^{α}	7.076^{α}	0.9987^{α}	3.508^{α}	0.0012^{α}
M4 Pa t0			1897^{α}	1.912^{α}	7.271^{α}	0.9991^{α}	4.235^{α}	0.0008^{α}
M3 Pa t14			1724^{α}	1.744^{α}	7.187^{α}	0.9990^{α}	3.747^{α}	0.0008^{α}
M4 Pa t14			1539^{α}	1.546^{α}	7.043^{α}	0.9989^{α}	3.244^{α}	0.0010^{α}
M4 Pa t30			1493^{α}	1.497^{α}	7.014^{α}	0.9988^{α}	3.122^{α}	0.0011^{α}
M4 A20 t0	Cu^{2+} and Zn^{2+} sulfate	none	1126^{β}	1.132^{β}	6.729^{β}	0.9985^{β}	2.194^{β}	0.0014^{β}
M4 A20 t14			1122^{β}	1.125^{β}	6.720^{β}	0.9985^{β}	2.184^{β}	0.0014^{β}
M4 A20 t30			1118^{β}	1.119^{β}	6.728^{β}	0.9985^{β}	2.175^{β}	0.0014^{β}
M4 A20 Pa t0	Cu^{2+} and Zn^{2+} sulfate	*P. aeruginosa*	1028^{β}	1.028^{β}	6.645^{β}	0.9983^{β}	1.960^{β}	0.0015^{β}
M4 A20 Pa t14			1005^{β}	1.005^{β}	6.619^{β}	0.9983^{β}	1.906^{β}	0.0016^{β}
M4 A20 Pa t30			1265^{β}	1.268^{β}	6.844^{β}	0.9986^{β}	2.536^{β}	0.0013^{β}
M3 B20 t0	Cu^{2+} and Zn^{2+} nitrate	none	1368^{γ}	1.375^{γ}	6.932^{β}	0.9988^{β}	2.797^{γ}	0.0011^{β}
M4 B20 t0			1216^{γ}	1.218^{γ}	6.779^{β}	0.9984^{β}	2.414^{γ}	0.0014^{β}

M3 B20 t14			1215$^{\gamma}$	1.215$^{\gamma}$	6.790$^{\beta}$	0.9985$^{\beta}$	2.412$^{\gamma}$	0.0014$^{\beta}$
M4 B20 t14			1146$^{\gamma}$	1.147$^{\gamma}$	6.748$^{\beta}$	0.9985$^{\beta}$	2.242$^{\gamma}$	0.0014$^{\beta}$
M4 B20 t30			1259$^{\gamma}$	1.260$^{\gamma}$	6.844$^{\beta}$	0.9986$^{\beta}$	2.521$^{\gamma}$	0.0013$^{\beta}$
M3 B20 Pa t0	Cu^{2+} and Zn^{2+} nitrate	*P. aeruginosa*	1197$^{\gamma}$	1.200$^{\gamma}$	6.705$^{\beta}$	0.9980$^{\beta}$	2.367$^{\gamma}$	0.0019$^{\beta}$
M4 B20 Pa t0			1202$^{\gamma}$	1.203$^{\gamma}$	6.818$^{\beta}$	0.9986$^{\beta}$	2.379$^{\gamma}$	0.0013$^{\beta}$
M3 B20 Pa t14			1382$^{\gamma}$	1.385$^{\gamma}$	6.964$^{\beta}$	0.9988$^{\beta}$	2.833$^{\gamma}$	0.0011$^{\beta}$
M4 B20 Pa t14			1336$^{\gamma}$	1.341$^{\gamma}$	6.881$^{\beta}$	0.9987$^{\beta}$	2.715$^{\gamma}$	0.0012$^{\beta}$
M4 B20 Pa t30			1206$^{\gamma}$	1.209$^{\gamma}$	6.759$^{\beta}$	0.9985$^{\beta}$	2.389$^{\gamma}$	0.0014$^{\beta}$

Table S4.2 Pairwise PERMANOVA applied on beta diversity metrics grouped on different variables.

The beta diversity results calculated for the assay M3 and M4 were statistically analysed using pairwise PERMANOVA (999 permutations). The data was grouped using the variables (in order): the metal amendment of the soil, the sampling times, and the inoculation with *P. aeruginosa* H1FC49.

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Soil metals amendment					
Nitrate	Sulfate	16	5.790	0.001	0.001
Nitrate	None	20	856	0.001	0.001
Sulfate	None	16	11.614	0.001	0.001
Sampling times					
t0	t14	20	0.5567	0.787	0.787
t0	t30	16	0.8624	0.487	0.787
t14	t30	16	0.6457	0.667	0.787
***P. aeruginosa* H1FC49 inoculation**					
P. aeruginosa	none	26	0.5503	0.793	0.793

5 Insight into phylogenomic bias of *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ dissemination amongst carbapenem resistant *Pseudomonas aeruginosa*

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	Planning	**Experimental work**			**Data analysis**	**Writing**
		Bacterial isolation and sequencing	Phylogenetic and comparative genomic analysis	Statistical analysis and graphical visualization		
Gianuario Fortunato	x	x	x	x	x	x
Ivone Vaz-Moreira	x	x		x	x	x
Ina Gajic		x				x
Celia M. Manaia	x				x	x

5.1 Abstract

Pseudomonas aeruginosa are ubiquitous opportunistic pathogens, harboring intrinsic and acquired multidrug resistance phenotypes. Carbapenem resistance has been increasing in this species, due to different types of acquired genes. In this study, we hypothesised that the spread of carbapenem-resistance among *P. aeruginosa* is influenced by phylogenomic features, being distinct for different genes. To test this hypothesis, we compared the genomes of *P. aeruginosa* harbouring bla_{VIM-2} or bla_{NDM-1} genes. The gene bla_{VIM-2} was selected because, although frequent, it is almost restricted to this species and bla_{NDM-1} due to its wide interspecies distribution. A group of genomes harbouring the genes bla_{VIM-2} (n=116) or bla_{NDM-1} (n=27), available in the GenBank, was characterized based on core phylogenomic analysis, functional categories in the accessory genome and mobile genetic elements flanking the selected genes. Most bla_{VIM-2} gene hosts belonged to multilocus sequence type (ST) ST233 (n=27/116) and ST111 (n=32/116) and were reported in Europe (n=75/116). The bla_{NDM-1} gene hosts were distributed by different ST (ST38, ST773, ST235, ST357, ST654), mainly from Asia (n=13/27). Significant differences in protein/enzyme prevalence (per number of genomes) between bla_{VIM-2}+ or bla_{NDM-1}+ accessory genomes were observed. The bla_{VIM-2} gene was frequently inserted in Tn402-like and Tn21 transposons family and rarely in IS6100, while bla_{NDM-1} gene was preferentially flanked by *ISAba125* and ble_{MBL} genes or associated with IS91 insertion sequence. The hypothesis that carbapenem resistance gene acquisition is not random among phylogenomic lineages was confirmed, suggesting the importance of phylogeny in the dissemination of antibiotic resistance genes.

5.2 Introduction

Pseudomonas aeruginosa is one of the 12 priority pathogens listed by the World Health Organization (WHO) (Pelegrin et al., 2021; World Health Organization, 2019). International surveillance programs (CDC - Centers for Disease Control and Prevention, 2019; European Centre for Disease Prevention and Control, 2018; WHO, 2020) show the high rates of *P. aeruginosa* antibiotic resistance against frontline antibiotics, particularly beta-lactams and carbapenems. High carbapenem resistance prevalence has been reported in clinical *P. aeruginosa* worldwide - Europe (8 to 58% of the isolates), South and East Asia (8 to 36% of the isolates) and North America (10 to 31% of the isolates) (“The Center for Disease Dynamics Economics & Policy. ResistanceMap: Antibiotic resistance. 2018. ,” 2021; WHO, 2020).

With genome sizes ranging 5.5 to 7.0 million base pairs (Freschi et al., 2019), *P. aeruginosa* has the potential to adapt to changing environments and successfully compete in complex microbial communities(Stover et al., 2000). A recent study that analysed 1 311 genomes of *P. aeruginosa*

showed that among the 54 272 genes identified, only about 1% represented the core, while about 50% were unique genes (Freschi et al., 2019). According to Freschi et al.(Freschi et al., 2019), the core genome was dominated by functions related to RNA processing, chromatin structure, cell division and partitioning, metabolism and transport of amino acids, nucleotides, coenzymes and lipids, transcription, and translation. In contrast, drug resistance, secretion systems and other functions mainly related to survival in changing environments have been frequently observed in the accessory genome (Freschi et al., 2019; Mosquera-Rendón et al., 2016). Core features and the dynamic gene acquisition revealed by the pangenome analyses support the ubiquitous character of the species (Abril et al., 2019; Nolan et al., 2020), thriving across distinct natural environments (soil, water, plants) and animal or human bodies, including clinical settings (Frimmersdorf et al., 2010; Mena and Gerba, 2009).

The first reports of acquired carbapenems resistance were in *Enterobacteriaceae,* and later in other *Gammaproteobacteria*, including *P. aeruginosa* (Codjoe and Donkor, 2018; Cui et al., 2019; Nordmann et al., 2012). The gene encoding the Verona integron-encoded metallo-beta-lactamase (VIM) was reported for the first time in *P. aeruginosa* in 1997, isolated from a Hospital inpatient in Verona (Italy), as an integron-borne gene (Lauretti et al., 1999). To date, at least 76 *bla*$_{VIM}$ variants have been reported, mostly in *P. aeruginosa*, with occasional reports in *P. putida* and *Enterobacteriaceae* (Alcock et al., 2020; Matsumura et al., 2017; NCBI, 2021). The variant *bla*$_{VIM-2}$ is the most frequent in *P. aeruginosa*, reported in more than 4% of chromosomes and whole-genome sequences (WGS) available at Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020; NCBI, 2021). Whereas *bla*$_{VIM}$ can be considered a gene originally dispersed by *P. aeruginosa*, the gene encoding the New Delhi metallo-beta-lactamase (NDM-1), another class B beta-lactamase, is widely disseminated through other bacterial orders, mainly *Enterobacterales*. The *bla*$_{NDM}$ gene was reported for the first time in Sweden in a hospital patient who travelled from India, diagnosed with a multidrug resistant *Klebsiella pneumoniae* infection (Yong et al., 2009). The first report of *bla*$_{NDM}$ in *P. aeruginosa* resulted from routine screening of carbapenemases-producing Gram-negative bacteria in the Military Medical Academy in Belgrade, Serbia, in 2010 (Jovcic et al., 2011). Currently, *bla*$_{NDM-1}$ is reported in about 3.7% of the chromosomes and 0.7% of the whole genome sequences of *P. aeruginosa* available at NCBI and CARD (Alcock et al., 2020; NCBI, 2021)

Given the liability of *P. aeruginosa* for antibiotic resistance acquisition, and the distinct patterns of dissemination of the genes *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$, these were considered interesting models to investigate possible phylogenomic drivers of resistance acquisition hypothetically reflected on the accessory genome and geographic distribution. Given the importance of geographic distribution, we used genomes available in public databases.

5.3 Materials and Methods

5.3.1 P. aeruginosa genomes selection

P. aeruginosa genomes containing the genes bla_{VIM-2} (n=272) or bla_{NDM-1} (n=40) (Supplementary Table S5.1), available at NCBI Pathogen Detection isolates browser database (https://www.ncbi.nlm.nih.gov/pathogens/isolates/; accessed on 17.11.2020), were collected for this study. Five additional genomes that at that time were not available at NCBI, one containing the bla_{VIM-2} (Vaz-Moreira et al., 2016), and four containing the bla_{NDM-1} gene (Gajic et al., in preparation), were also included (assembly accession numbers: GCA_020404715.1, GCA_022559565.1, GCA_020404785.1, GCA_020404825.1, and GCA_020404705.1) (Supplementary Table S5.1). Presumable duplicated genome sequences were eliminated whenever were deposited by the same authors/institution, had the same Multi Locus Sequence Type (ST), determined according to the MLSTFinder (https://cge.cbs.dtu.dk/services/MLST/) (Larsen et al., 2012) and the same sample origin. In these cases, was selected the one with the lowest number of contigs and highest genome coverage. After this data trimming, a total of 116 bla_{VIM-2}+ and 27 bla_{NDM-1}+ genomes were considered for the study (Supplementary Table S1, highlighted in light blue). Different DNA sequencing technologies were used (Supplementary Table S5.1).

5.3.2 Genome analysis

The genomes were annotated using RASTtk pipeline (Brettin et al., 2015), which output was used as input to the Roary: pan-genome pipeline (Page et al., 2015). The Roary pipeline used default settings, allowing the paralogs to split into clusters, and performing a core alignment with MAFFT (Katoh and Standley, 2013). The sequence identity threshold was settled by default at 95% and the core-genome was settled for genes shared by 99% of the genomes (Supplementary Table S5.2). The bla_{VIM-2}+ or bla_{NDM-1}+ strains genomes were first used to create a wgMLST scheme, including the common genes based on the default BLAST score ratio value of 0.6. From the wgMLST scheme was created the cgMLST, based on genes common to all the analysed genomes with sequence identity values of 99%, using chewBBACA tool (Silva et al., 2018). The cgMLST scheme was then represented as a minimum spanning tree using the software Grapetree (Zhou et al., 2018).

5.3.3 Functional annotation of the accessory genome

The accessory genome, defined as genes shared by < 95% of the analysed genomes, for each set of bla_{VIM-2}+ and bla_{NDM-1}+ genomes were annotated based on functional categories. Functional

annotation of deduced aminoacid sequences used the EggNOG database (Huerta-Cepas et al., 2019) based on the parameters 95% sequence coverage and 100% sequence identity, providing a Cluster of Orthologous Groups (COG) assignment for each protein-coding gene. For each functional category were identified (based on similar protein functions in UniProt (https://www.uniprot.org/) (Bateman, 2019)) subcategories of the most represented protein families. The respective relative abundance over the total proteins annotated in each category was expressed as proteins percentage values and graphically represented using the ggplot2 R package (Wickham, 2016). Kolmogorov-Smirnov means comparison was used to analyse the difference in protein distribution in *bla*$_{VIM-2}$+ or *bla*$_{NDM-1}$+ strains functional annotations using SPSS 26.0 (IBM SPSS Inc., Chicago, IL).

5.3.4 Other antibiotic resistance genes

The core- and accessory- *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes were screened for antibiotic resistance genes, using the CARD Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) (Alcock et al., 2020). The prevalence of the identified genes in *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes was represented graphically as a barplot and Venn diagram using the R packages ggplot2 and Venn.diagram (Chen and Boutros, 2011). Moreover, the antibiotic resistance gene prevalence in the analysed genomes was compared with the data available for all *P. aeruginosa* available at CARD databases, based on NCBI deposited genomes (accessed on November 2021).

5.3.5 bla$_{VIM-2}$ *and bla*$_{NDM-1}$ *genetic environment and transposons comparative analysis*

The flanking regions of the *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ genes were analysed for 80 genomes. For 55 *bla*$_{VIM-2}$+ and 8 *bla*$_{NDM-1}$+ genomes, the targeted genes were in short contigs (800 to 3000 bp), hindering a proper genetic environment analysis. Insertion sequences and mobile genetic elements were annotated using the IS finder (https://isfinder.biotoul.fr/blast.php) (Siguier et al., 2006). Transposons were identified by alignment against reference transposons described in the literature using UGENE MAFFT (Katoh and Standley, 2013; Okonechnikov et al., 2012) and manually annotated using NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The structure of the transposons associated with *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ genes was depicted using the R package gggenes (available at https://github.com/wilkox/gggenes).

5.4 Results

Most of the 116 bla_{VIM-2}+ and 27 bla_{NDM-1}+ non-repetitive genomes examined were of clinical origin, with only 7 bla_{VIM-2}+ and 1 bla_{NDM-1}+ of environmental sources (e.g. home and hospital environments, animals associated, hospital effluent, or other unidentified sources) (Supplementary Table S5.1). The bla_{VIM-2}+ genomes were from strains isolated in Europe (n=64), Asia (n=17), South America (n=22), Africa and the Middle East (n=5), North America (n=7), and one of unknown origin. The bla_{NDM-1}+ genomes were from strains isolated in Asia (n=11), Europe (n=9), North America (n=3), South America (n=1), Africa (n=1), and two of unknown origin (Supplementary Table S5.1).

5.4.1 Phylogenetic diversity and genome features

The analysis of the whole gene set among bla_{VIM-2}+ genomes revealed 33 527 genes (3 142 present in ≥ 99%, 1 157 in 95-99%, 4 276 in 15-94%, and 24 952 in <15% of the strains). In the bla_{NDM-1}+ genomes were identified 14 080 genes (4 599 present in ≥ 99%, 468 in 95-99%, 2 990 in 15-94%, and 6 023 in <15% of the strains) (Supplementary Table S5.2). The phylogenomic relationships among bla_{VIM-2}+ and bla_{NDM-1}^{+} genomes were assessed based on the core genome MLST (cgMLST) and Pasteur MLST, being the identified sequence types (STs) instrumental in the analysis of the results. As expected, the cgMLST-based phylogenetic analysis distributed the genomes according to the MLST types. Phylogenomic analysis suggested a close relationship among specific STs, for instance, between ST111 and ST233, ST175 and ST179, ST235 and ST357, or ST823, ST308 and ST773 (Figure 5.1). Despite the wide geographic distribution of the predominant phylogenetic lineages, some clades were mostly populated by genomes of some regions. For instance, ST823, ST308 and ST773 in Asiatic countries, or ST244, ST175, ST179, ST17 in European countries (mainly Portugal, Spain and Poland) (Supplementary Figure S5.1), which may suggest some degree of endemism. However, the sample size and the fact that most genomes were of clinical isolates, with only 8 (7 bla_{VIM-2}+ and 1 bla_{NDM-1}+) from environmental origin scattered by different STs - ST111 (n=2), ST175, ST179, ST233, ST386, and ST823, and ST308 (bla_{NDM-1}+) (Supplementary Table S5.1, Figure 5.1), limits this interpretation. Identical core genomes (100%) were observed only for six pairs of genomes (ST111, ST244, ST233, ST823, ST175, ST235), most of the times of the same country except in the case of ST235.The diversity of STs was proportional to the number of bla_{VIM-2}+ and bla_{NDM-1+} genomes. The bla_{VIM-2}+ genomes were distributed by 29 STs, being ST111 (n=32, from Europe, South America, and USA), ST233 (n=28, from Europe, Africa, South America, Asia, and USA), ST235 (n=14, from Europe, Asia, and USA), and ST823 (n=8, from Asia/Oceania and USA), the predominant (Figure 5.1A; Supplementary Figure S5.1 and Table S5.1).

The bla_{NDM-1}^{+} genomes were distributed by 9 STs, mainly in lineages closely related to ST823, most of which were represented by ST308/ST773 (n=5, from Asia, and n=5, from Europe, Asia, USA, and unknown origin, respectively) and ST235/ST357 (n=4, from Europe, and n=4, from Asia, and Africa, respectively). Other bla_{NDM-1}^{+} genomes were observed in lineages closely related to ST111, specifically ST654 (n=4, from Europe, North America, and South America) and ST233 (n=2, from

Myanmar and an unspecified location) (Figure 5.1; Supplementary Figure S5.1 and Table S5.1). Unique STs harbouring the *bla*$_{NDM\text{-}1}^{+}$ gene (ST316, ST1023, or ST234) were related closely related to the same lineages ST823 and ST111.

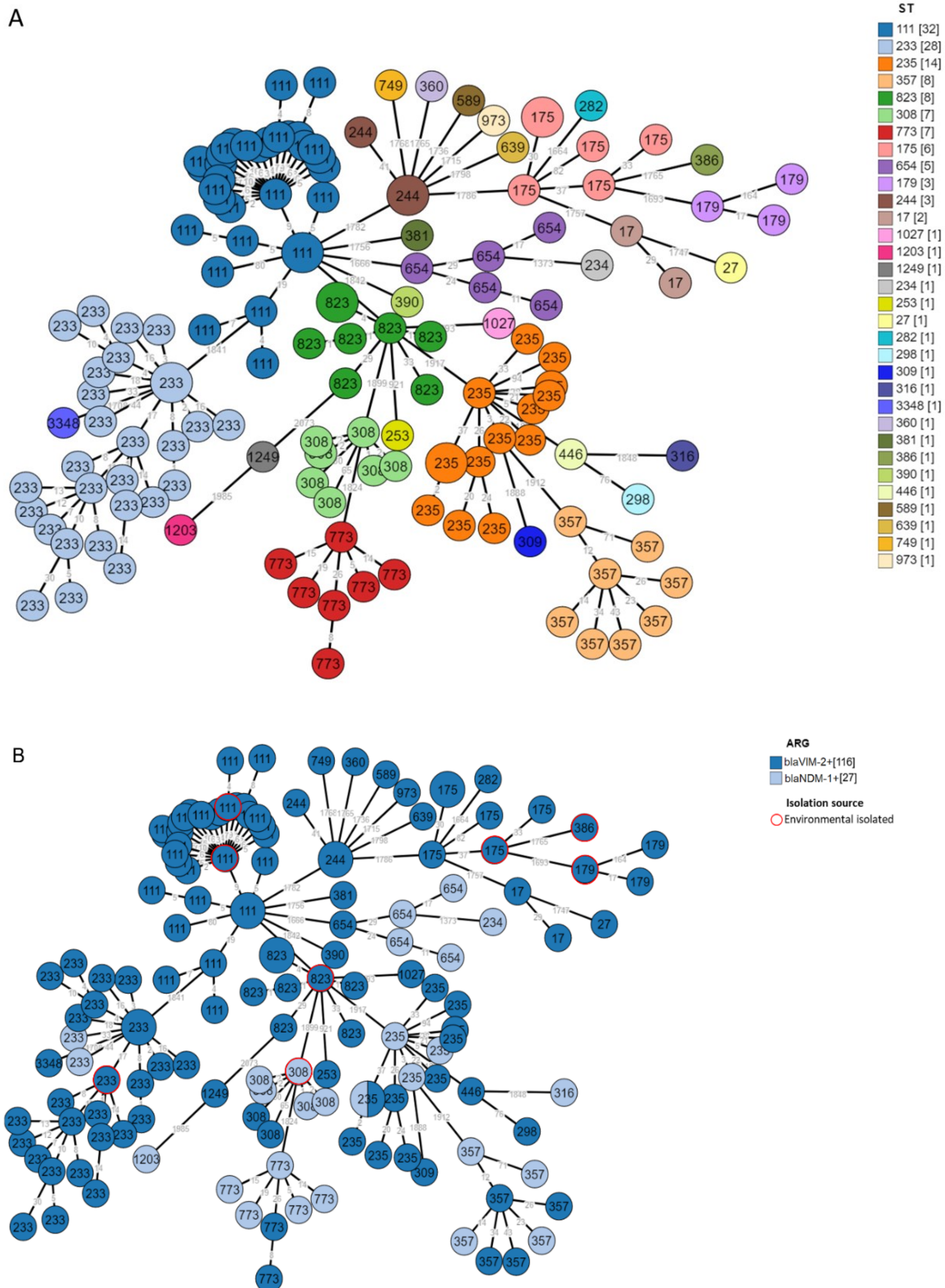

Figure 5.1 Phylogenetic distribution of *bla*$_{VIM\text{-}2}$+ and *bla*$_{NDM\text{-}1}$+ strains.

A) Minimum spanning tree based on cgMLST, reporting the phylogenetic distribution of *bla*$_{VIM\text{-}2}$+ and *bla*$_{NDM\text{-}1}$+ strains identified with the ST (different colors). B) Minimum spanning tree based on cgMLST, reporting the phylogenetic distribution of *bla*$_{VIM\text{-}2}$+ and *bla*$_{NDM\text{-}1}$+ strains identified by ST

numbers in the nodes and colored by ARGs harbored. The isolation source is indicated by a red circle around the nodes referring to environmental isolated strains.

The branches label refers to the number of locus differing between the strains reported.

5.4.2 Functional annotation of core and accessory genome

Core and accessory genomes retrieved from comparative genomics analysis were functionally annotated using orthologs databases with 99% of identity. The core-genome functional analysis revealed negligible differences in the type of gene abundance between bla_{VIM-2}+ and bla_{NDM-1}+ genomes (data not shown). Based on the strict parameters used (95% coverage, 100% identity), the proteins annotated in accessory genomes were 3040 for bla_{VIM-2}+ and 775 for bla_{NDM-1}+. For each functional category were identified (based on similar protein functions) subcategories of the most represented protein families (Figure 5.2). Aiming to assess if the accessory genome was distinct in both groups of genomes, the prevalence of major proteins (proteins per number of genomes) included in different functional subcategories was compared. Proteins involved in transport (e.g. carbohydrate transport, extracellular transport), metabolism (e.g. permeases, dehydrogenases, or ATPases) and transcriptional regulators were observed to be significantly (p-*value* < 0.05) more prevalent among bla_{VIM-2}+ than bla_{NDM-1}+ genomes. Protein subcategories like coenzyme catalysis, conjugation, virulence, ABC transporters, membrane-binding protein and pilus, secretion related proteins were observed significantly (p-*value* < 0.05) more abundant in bla_{NDM-1}+ than in bla_{VIM-2}+ genomes.

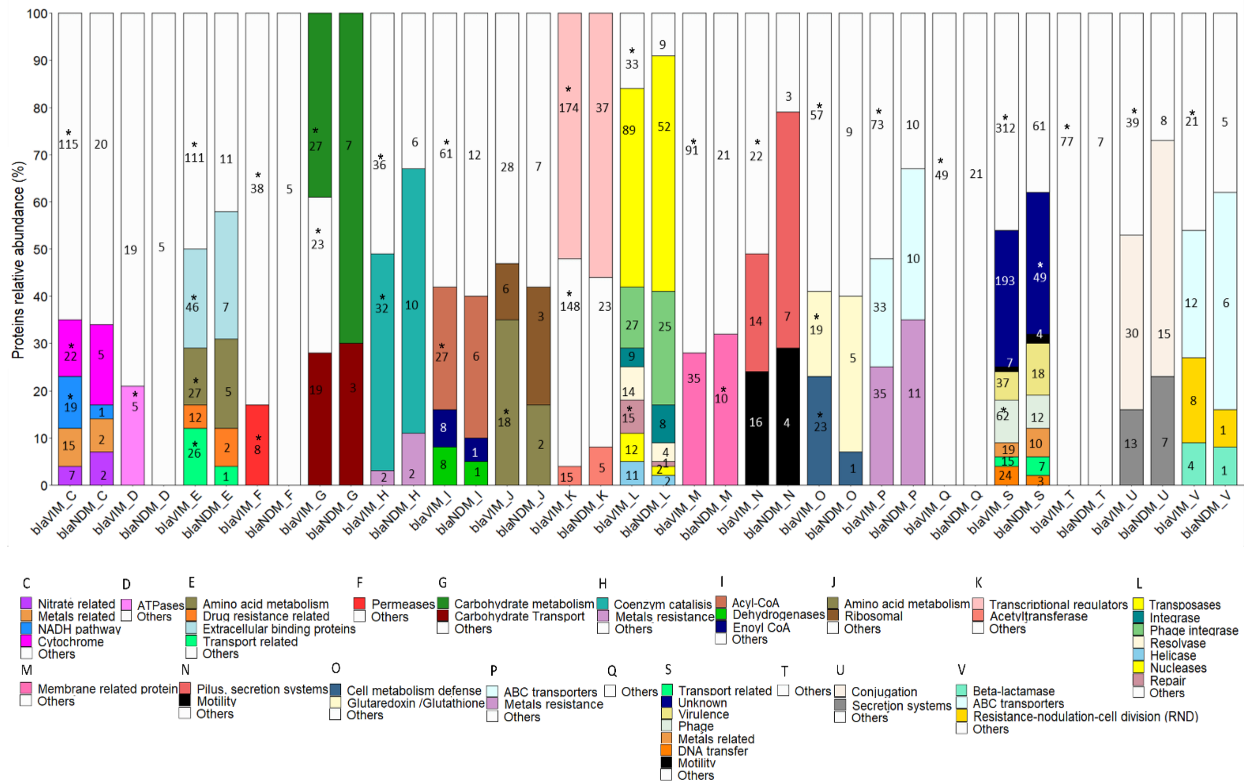

Figure 5.2 Functional analysis of bla_{VIM-2}+ and bla_{NDM-1}+ genomes.

Barplot with the functional annotation of proteins relative abundance retrieved from the accessory genomes of bla_{VIM-2}+ and bla_{NDM-1}+ strains. The bars refer to the functional categories: C. Energy production and conversion, D. Cell cycle control and mitosis, E. Amino Acid metabolism and transport, F. Nucleotide metabolism and transport, G. Carbohydrate metabolism and transport, H. Coenzyme metabolism, I. Lipid metabolism, J. Translation, K. Transcription, L. Replication and repair, M. Cell wall/membrane/envelop biogenesis, N. Cell motility, O. Post-translational modification, protein turnover, chaperone functions, P. Inorganic ion transport and metabolism, Q. Secondary Structure, S. Function Unknown, T. Signal Transduction, U. Intracellular trafficking and secretion, and V. Defense mechanisms.

In the figure are reported the number of proteins in each sub-category and indicated with an * the statistical differences (p-value < 0.05) between bla_{VIM-2}+ and bla_{NDM-1}+ functional sub-categories analysed.

5.4.3 Other antibiotic resistance genes in P. aeruginosa bla_{VIM-2}+ and bla_{NDM-1}+

The core-genome of both *P. aeruginosa* bla_{VIM-2}+ and bla_{NDM-1}+ groups revealed the presence of other antibiotic resistance mechanisms, with efflux pumps representing 85% and 76% of the resistance genes analysed, respectively. The other respective 15% and 24% encoded beta-lactamases and other mechanisms (Supplementary Table S5.3). The identified efflux pump genes were mainly part of *Opr*, *Opm*, *Mex* and *Mux* operons, representing 56% in the bla_{VIM-2}+ and 54% in the bla_{NDM-1}+ of the total antibiotic resistance genes. The prevalence of the genes encoding the efflux pumps observed in bla_{VIM-2}+ and bla_{NDM-1}+ genomes was not different from that reported in *P. aeruginosa* genomes available in the public database NCBI (n°=7813), >90% (Supplementary Table S5.3). The β-lactamase encoding genes observed in most/all the bla_{VIM-2}+ and bla_{NDM}+ genomes were represented by bla_{PDC-3} and $bla_{OXA-850}$, while these genes are reported respectively in 26 and 32% of the *P. aeruginosa* isolates, available at NCBI.

Regarding the accessory genome, the bla_{VIM-2}+ and bla_{NDM-1}+ genomes shared ~ 53% of the antibiotic resistance genes (Figure 5.3). These were mostly related to aminoglycoside resistance (47%), with β-lactams resistance representing 6% of the genes (Figure 5.3). The prevalence of antibiotic resistance genes observed in the bla_{VIM-2}+ and bla_{NDM-1}+ genomes was compared with that of 7813 *P. aeruginosa* genomes available in the CARD database. In general, the bla_{VIM-2}+ and bla_{NDM-1}+ genomes presented a higher prevalence of antibiotic resistance genes than the average for the species (Supplementary Table S5.3). The clearest examples of these were the gene *aac*(3)-I (aminoglycoside resistance) present in 77% of the bla_{VIM-2}+ genomes and *aph*(3')-III/IV/VI/VII (aminoglycoside resistance), *cat* and floR (chloramphenicol resistance), present in 63-74% of the bla_{NDM-1}+ genomes, in comparison with < 1% of the average of all *P. aeruginosa* genomes (Supplementary Table S5.3).

Some bla_{VIM-2}+ and bla_{NDM-1}+ genomes harboured other carbapenemase encoding genes. In bla_{VIM-2}+ genomes were observed the bla_{IMP} or bla_{KPC} in two ST111 genomes and in a distinct genomic environment of bla_{VIM-2}. In two ST654 bla_{NDM-1}+ genomes was detected the bla_{GES} gene integrated into the same mobile genetic element of bla_{NDM-1}.

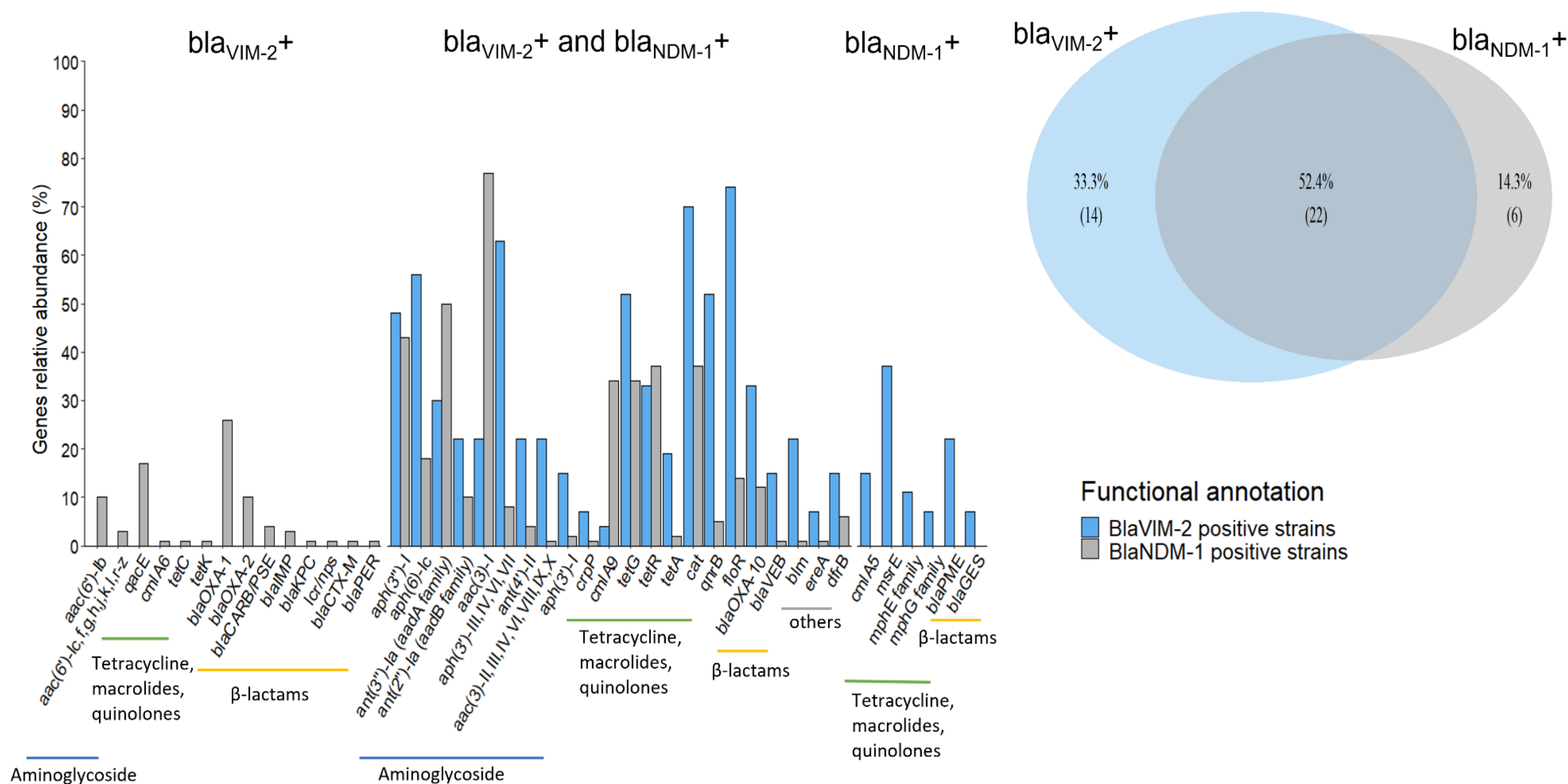

Figure 5.3 Antibiotic resistance profile analysis of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ accessory genomes.

The barplot represents the antibiotic resistance genes carried by *bla*$_{VIM-2}$+ (grey bars), *bla*$_{NDM-1}$+ (blue bars) genomes, and shared between both. The bars correspond to the genes relative abundance considering the total number of strains analyzed (116 *bla*$_{VIM-2}$+ and 27 *bla*$_{NDM-1}$+). The antibiotic resistance genes are reported and grouped in the barplot sections from left to right based on the antibiotic class (aminoglycosides, tetracycline, macrolides and quinolones, beta-lactams, and others). The overview of the antibiotic resistance genes in *bla*$_{VIM-2}$+ (grey circle) and *bla*$_{NDM-1}$+ (blue circle) are reported in the Venn diagram.

5.4.4 *Transposons and mobile genetic elements associated with bla_{VIM-2} and bla_{NDM-1} genes*

The genetic environment of the genes *bla_{VIM-2}* and *bla_{NDM-1}* inserted in contigs with >3000 bp was analysed. This analysis unveiled five main genomic environments, three including *bla_{VIM-2}* or *bla_{NDM-1}*, and two identified only in *bla_{NDM-1}^{+}* genomes. In the group of 61 *bla_{VIM-2}^{+}* genomes that gene was included in Tn21 transposon structures (n=17), Tn402-like transposons (n=23), or in insertion sequences IS6100 (n=19), while seven could not be identified (e.g. related to Tn3 family). In the group of 27 *bla_{NDM-1}^{+}* genomes that gene was flanked by ISAba125 and *ble_{MBL}* (n=8) and IS91 (n=19), 8 of which included also an IS6100 sequence (Figure 5.4). The most commonly observed structure including *bla_{NDM-1}* was represented by IS91 which flanked the gene on both sides (Figure 5.4B). The Tn21 depicted in Figure 4, besides the *mer* operon, transposase (tniA) and resolvase, includes an integron containing *bla_{VIM-2}* or *bla_{NDM-1}* and other antibiotic resistance genes, mostly aminoglycosides resistance genes. A particular Tn21 structure (Figure 5.4C) was observed in a *bla_{NDM-1}*+ genome (GCA_020404785.1, whose genome was sequenced for this study) where that gene was flanked by ISAba125 and *ble_{MBL}* inserted upstream to an integron containing another carbapenemase resistance gene, *bla_{GES}*. The Tn402-like presented a structure similar to the Tn21 in transposase and resolvase, including an integron containing *bla_{VIM-2}* or *bla_{NDM-1}* and other antibiotic resistance genes (Figure 5.4). As observed for Tn21, also the Tn402-like was described including *bla_{NDM-1}* flanked by ISAba125 and *ble_{MBL}* as the unique ARG. The complete transposons Tn21 and Tn402-like were associated with *bla_{NDM-1}* only in 9 of the 27 genomes. Finally, the insertion sequence IS6100 was observed upstream or downstream of integrons including *bla_{VIM-2}* (Figure 5.4) or the *bla_{NDM-1}* genetic environment. The same genetic environment was observed in *bla_{VIM-2}*+ and *bla_{NDM-1}*+ genomes, sometimes empty or including other antibiotic resistance genes. The only exception was ISAba125 and IS91 associated only with *bla_{NDM-1}* (Figure 5.4).

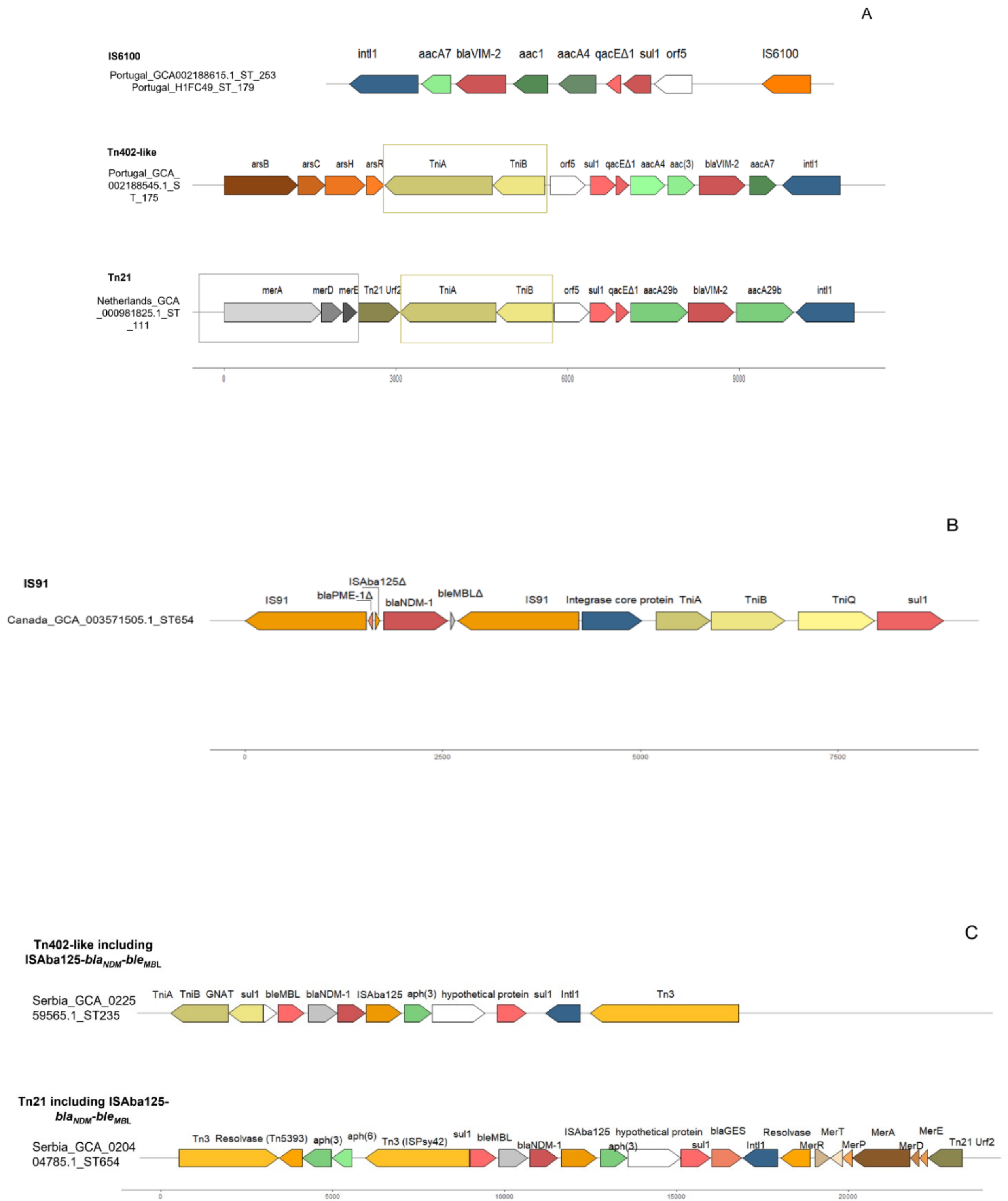
A
IS6100
Portugal_GCA002188615.1_ST_253
Portugal_H1FC49_ST_179
intI1
aacA7 blaVIM-2
aac1
aacA4 qacEΔ1 sul1 orf5
IS6100
Tn402-like
Portugal_GCA_002188545.1_ST_175
arsB arsC arsH arsR TniA TniB orf5 sul1 qacEΔ1 aacA4 aac(3) blaVIM-2 aacA7 intI1
Tn21
Netherlands_GCA_000981825.1_ST_111
merA merD merE Tn21 Urf2 TniA TniB orf5 sul1 qacEΔ1 aacA29b blaVIM-2 aacA29b intI1
0 3000 6000 9000
B
IS91
Canada_GCA_003571505.1_ST654
ISAba125Δ
IS91 blaPME-1Δ blaNDM-1 bleMBLΔ IS91 Integrase core protein TniA TniB TniQ sul1
0 2500 5000 7500
C
Tn402-like including ISAba125-blaNDM-bleMBL
Serbia_GCA_022559565.1_ST235
TniA TniB GNAT sul1 bleMBL blaNDM-1 ISAba125 aph(3) hypothetical protein sul1 IntI1 Tn3
Tn21 including ISAba125-blaNDM-bleMBL
Serbia_GCA_020404785.1_ST654
Tn3 Resolvase (Tn5393) aph(3) aph(6) Tn3 (ISPsy42) sul1 bleMBL blaNDM-1 ISAba125 hypothetical protein aph(3) sul1 blaGES IntI1 Resolvase MerR MerT MerP MerA MerD MerE Tn21 Urf2
0 5000 10000 15000 20000

Figure 5.4 Transposons organization and genetic environment of bla_{VIM-2} or bla_{NDM-1} in the analyzed *P. aeruginosa* genomes.

In the figures are reported the most representative transposons organization and genomic environments of bla_{VIM-2}or bla_{NDM-1} harbored by the analyzed *P. aeruginosa* strains. The gene bla_{VIM-2} was described as associated principally with insertion sequences IS6100, Tn402-like and Tn21 transposons (A). The gene bla_{NDM-1} was described as associated with the insertion sequence IS91 (B) and Tn21 and Tn402-like transposons including the ISAba125-bla_{NDM}-ble_{MBL} (C). In all the transposons and genetic environments represented, the aminoglycosides resistance genes are colored in green shape, the insertion sequences in orange, antibiotic resistance genes in red, Tni modules in beige, mercury resistance genes in brown, integrase (*intl1*) in blue and other genes in white. The transposons sequences annotation was curated manually using BLAST databases and similar transposons were aligned using MAFFT. The distribution of MGEs analysed in bla_{VIM-2} + and bla_{NDM-1} + strains are described in Supplementary Table S5.5.

The class 1 integron including the $bla_{VIM,}$ associated with IS6100 (A), was described in a plasmid (Botelho et al., 2017b) and chromosome (our study) with 100% of sequence identity.

The Tn402 and Tn21 transposons family harboring bla_{VIM} reported in (A) were previously published (Botelho et al., 2017b; Zee et al., 2018) and selected as examples.

5.5 Discussion

P. aeruginosa is a ubiquitous multidrug resistant pathogen distributed worldwide (Laborda et al., 2021; Moradali et al., 2017). Despite the large number (>3000) of sequence types deposited in the public database PubMLST (Jolley et al., 2018), beta-lactamase-producing *P. aeruginosa* belongs mostly to ST235, ST111 and ST233 (del Barrio-Tofiño et al., 2020; Kocsis et al., 2021). *P. aeruginosa bla*$_{NDM-1}$+ has been frequently identified as ST235, and also as ST233, ST111, or ST654 (Janvier et al., 2013; Kocsis et al., 2021; Loconsole et al., 2020). Our results based on cgMLST confirm this phylogenetic distribution and evidence of the wider presence of *bla*$_{NDM-1}$ across a broader phylogenetic amplitude than *bla*$_{VIM-2}$. An interesting result was the observation that some lineages may prevail in distinct geographic origins, like the lineage ST823/ST308/ST73 in Asiatic countries or the lineage ST244/ST175/ST179/ST17 in Portugal, Spain and Poland. ST179, although not widely distributed (Botelho et al., 2018b; Hishinuma et al., 2020; Moloney et al., 2020) should be considered as part of the international high-risk clones (Wang et al., 2021). The rich accessory genome of *P. aeruginosa* has been associated with ubiquitous and worldwide distribution and adaptation to different environments (Lee et al., 2020; Sawa et al., 2020). Genes involved in metal or antibiotic resistance, biofilm formation and virulence present in the accessory genome of *P. aeruginosa* can probably explain the ability to survive under challenging conditions and environments (Mathee et al., 2008; Moradali et al., 2017; Teitzel and Parsek, 2003; Wolfgang et al., 2003). The functional analysis of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ accessory genomes showed a similar presence of protein involved in metals tolerance, antibiotic resistance and MGEs. In particular, it was observed the presence of proteins involved in the resistance to metal like copper (*cop* operon) or mercury (*mer* operon), being these operons described in genomes of bacteria adapted to metal contaminated environments (Mathema et al., 2011; Osborn et al., 1997; Rensing and Grass, 2003; Shafeeq et al., 2011). Other proteins observed in a similar amount in *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ accessory genome were represented by mobile genetic elements like transposases, integrases and phage integrases. The consistent presence of mobile genetic elements in *P. aeruginosa* accessory genomes highlights the important role they may have in gene sharing and consequent genome improvement (Espinosa-Camacho et al., 2021; Kung et al., 2010; Rodríguez-Beltrán et al., 2021; Virolle et al., 2020). Virulence factors were instead observed to be more prevalent among *bla*$_{NDM-1}$+ than *bla*$_{VIM-2}$+ accessory genomes, as well as conjugation and secretion systems related proteins. Secretion systems play an important role both in virulence and conjugation (Goessweiner-Mohr et al., 2013; Juhas et al., 2008; Trokter and Waksman, 2018). The protein abundance in accessory genomes reflects substantial differences between both groups of genomes analysed. In particular, the higher presence of secretion systems, virulence factors and conjugation elements in *bla*$_{NDM-1}$+ may suggest the strong influence of horizontal gene transfer processes and also its clinical relevance.

The mobilization of antibiotic resistance genes enriches the bacterial resistome, sometimes enhancing functions and mechanisms available in the intrinsic resistome (López-Causapé et al., 2018; Partridge et al., 2018). The genes *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ are examples of such accessory genes

that can increase the bacterial ability to survive in clinical treatments (Pacheco et al., 2019; Paul et al., 2016; Quinones-Falconi et al., 2010). In *P. aeruginosa*, the intrinsic resistance is often due to efflux pumps systems, often with multidrug resistance potential (Alvarez-Ortega et al., 2011; López-Causapé et al., 2018; Sultan et al., 2018). Most of the antibiotic resistance genes detected in the accessory genome of bla_{VIM-2}+ and bla_{NDM-1}+ genomes were associated with aminoglycoside resistance, previously described as included in integron structures where other genes may be also transported, explaining the multidrug resistance phenotype (Botelho et al., 2018b; Marchiaro et al., 2010; Poirel et al., 2001a). It was curious to note that in a few cases, more than one carbapenemase resistance gene was present, suggesting some epistatic or duplicated function, with bla_{VIM-2} combined with bla_{IMP} (n=2) or bla_{KPC} (n=1) and bla_{NDM} with bla_{GES} (n=2). The co-existence of different carbapenemase resistance genes in ubiquitous bacteria like *P. aeruginosa* is emerging as a serious threat (Ellappan et al., 2018). The cases of multiple carbapenemase resistance genes may deserve a deep analysis, mostly defining their genomic environment, to avoid the spread of these lineages.

The mobilization of carbapenemase resistance genes among *P. aeruginosa* can use different mechanisms (Botelho et al., 2018c; Yoon and Jeong, 2021). Transposons are mobile genetic elements commonly involved in antibiotic resistance gene mobilization, being Tn21 and Tn402-like often associated with carbapenemase resistance genes, such as bla_{VIM}. These findings were confirmed in this study. Instead, bla_{NDM-1} has been described mostly flanked by insertion sequences IS91 or ISAba125 (Janvier et al., 2013; Poirel et al., 2011). Interestingly, in genomes of isolates from Serbia, the bla_{NDM-1} flanked by *ISAba125* and ble_{MBL} was included in Tn21 or Tn402-like transposons (Figure 5.4). The Tn21, in particular, was observed to include bla_{NDM-1} flanked by *ISAba125* and ble_{MBL} and another integron containing bla_{GES}. The study of adaptative characteristics of bacteria and the monitoring of the high-risk clones, as well as the mobilization of carbapenem resistance genes, are valuable approaches to controlling the propagation of multidrug-resistant *P. aeruginosa*.

The mobile genetic elements observed to be associated with the genes bla_{VIM-2} and bla_{NDM-1} in this study are present in about 3-4% of the *P. aeruginosa* genomes and have a wide distribution over distinct species (Supplementary Table S5.4). Besides *P. aeruginosa,* the same genetic mobile elements were observed in *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii* and *Acinetobacter baumannii. K. pneumoniae* and *E. coli* were the species with the highest share of the elements Tn21 (19% and 23%), Tn402-like (22% and 20%, respectively) and IS6100 (28% and 26%, respectively). Although these two species shared also the highest proportion of IS91 (22% and 29%, respectively) or ISAba125 (19% and 27%, respectively), *P. aeruginosa* shared 19% and 15% of each and *A. baumannii* 15% of ISAba125 (Supplementary Table S5.4). These observations show that the mobile genetic elements observed to carry the genes bla_{VIM-2} or bla_{NDM-1} are spread over distinct phylogenetic groups, confirming *Enterobacteriaceae* as major reservoirs of transposons and insertion sequences responsible for antibiotic resistance acquisition (Potter et al., 2016). These results also confirm the widest phylogenetic distribution of IS91 and ISAba125, observed in this study to be associated with the bla_{NDM-1} gene (Janvier et al., 2013; Poirel et al., 2011). The bla_{VIM-2} or bla_{NDM-}

1 genomic environment represents a fundamental case study to monitor the spread of carbapenems resistance genes and the occurrence of reservoirs.

Studies like this highlight the relevance of phylogenomic lineages on the dissemination of antibiotic resistance determinants, as a result of ecology, virulence and biogeography.

Supplementary information

Submitted:

Authors: Fortunato, G., Vaz-Moreira, I., Gajic, I., Manaia, C.M.

Title: "Insight into phylogenomic bias of *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ dissemination amongst carbapenem resistant *Pseudomonas aeruginosa*".

Submitted for publication.

Table S5.1 List of *P. aeruginosa* genomes harbouring the genes *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ available in the NCBI Pathogen Detection isolates browser (https://www.ncbi.nlm.nih.gov/pathogens/isolates/; accessed on 17.11.2020).

In grey are indicated the genomes that were used for the analysis. The strains indicated in bold were sequenced during this study.

Table_S1.xlsx_

Table S5.2 Number of genes distributed by the core and accessory genomes of bla_{VIM-2} (n=116) and bla_{NDM-1} (n=27) positive strains.

	bla_{VIM-2}+ strains (n=116)	**bla_{NDM-1}+ strains (n=27)**
Core genes (99% ≤ strains ≤ 100%)	3142	4599
Soft core genes (95% ≤ strains < 99%)	1157	468
Shell genes (15% ≤strains < 95%)	4276	2990
Cloud genes (0% ≤ strains < 15%)	24952	6023
Total genes (0% ≤ strains ≤ 100%)	33527	14080

Table S5.3 Antibiotic resistance genes (ARGs) detected in the core and accessory genomes.

Comparison of prevalence values with *Pseudomonas aeruginosa* whole genome sequences (WGS), available at NCBI (accessed on November 2021), and on the bla_{VIM-2} and bla_{NDM-1} positive strains under study.

Class	ARG	% of NCBI WGS (n=7813)	% of bla_{VIM-2}+ strains (n=116)	% of bla_{NDM-1}+ strains (n=27)
Accessory genomes ARGs				
Aminoglycosides	*aph*(3")-I	4.8	43.0	48.0
	aph(6)-Ic	0.2	18.0	56.0
	ant(3")-Ia (*aadA* family)	1.8	50.0	30.0
	ant(2")-Ia (*aadB* family)	4.3	10.0	22.0
	aac(3)-I	0	77.0	22.0
	aph(3')-III/IV/VI/VII	0.9	8.0	63.0
	aac(6')-Ib	0.02	10.0	0
	ant(4')-II	0.2	4.0	22.0
	aac(6')-Ic,f,g,h,j,k,l,r-z	0.02	3.0	0
	aac(3)-II,III,IV,VI,VIII,IX,X	0.1	1.0	22.0
	aph(3')-I	0.9	2.0	15.0
Beta-lactamases	bla_{OXA-1}	0.4	26.0	0
	bla_{OXA-2}	2.1	10.0	0
	bla_{OXA-10}	2.0	12.0	33.0
	$bla_{CARB/PSE}$	1.6	4.0	0
	bla_{IMP}	0.1	3.0	0
	bla_{KPC}	0.2	1.0	0
	lcr/nps	0.2	1.0	0
	bla_{CTX-M}	0.1	1.0	0
	bla_{PER}	0.4	1.0	0
	bla_{VEB}	0.1	1.0	15.0
	bla_{PME}	0	0	22.0
	bla_{GES}	0.6	0	7.0
Tetracycline	*tet*A	0.9	2.0	19.0
	*tet*C	0.7	1.0	0
	*tet*G	0.03	34.0	52.0
	*tet*K	0	1.0	0
	tetR	0.02	37.0	33.0
Macrolides	*msr*E	0.5	0	37.0
	*mph*E family	0	0	11.0
	*mph*G family	0.05	0	7.0
	ereA	0.09	1.0	7.0
Quinolones	*crpP*	16.2	1.0	7.0
	qnrB	0.02	5.0	52.0
Phenicol	*cmlA5*	1.6	0	15.0
	cmlA6	0.9	1.0	0
	cmlA9	3	34.0	4.0
	cat	0.9	37.0	70.0

	floR	0.3	14.0	74.0
Trimethoprim	*dfrB*	1.3	6.0	15.0
Quaternary ammonium compounds	*qacE*	1.3	17.0	0
Bleomycin	*blm*	0.4	1.0	22.0
Core genome ARGs				
Aminoglycosides	aph(3')-IIb	95	100	100
Peptide antibiotics	*arnA*	96	100	100
	basS	96	100	100
	cprR	98	100	100
	cprS	98	100	100
Fosfomycin	*fosA*	96	100	100
Bicyclomycin	*bcr-1*	96	90	100
Peptide antibiotic, diaminopyrimidine, sulfonamide, penam, phenicol, cephalosporin, aminocoumarin, carbapenem, macrolide, fluoroquinolone, monobactam, tetracycline, penem, cephamycin, aminoglycoside	*mexA*	97	100	100
	mexB	97	100	100
	mexC	97	98	100
	mexD	95	98	100
	mexE	96	100	100
	mexF	72	100	100
	mexR	97	100	100
	mexV	97	100	100
	mexW	97	100	100
	muxA	97	100	100
	muxB	97	100	100
	muxC	97	100	100
	opmB	97	100	100
	opmD	92	100	100
	Type A NfxB	97	100	100
	rsmA	98	100	100
	oprJ	96	100	100
Disinfecting agents and intercalating dyes, fluoroquinolone, tetracycline, acridine dye	*mexG*	95	89	100
	mexH	95	100	100
	mexI	94	77	100
Tetracycline, macrolide, triclosan	*mexJ*	97	100	100
	mexK	98	100	100
	mexL	97	100	100
	oprM	96	100	100
	oprN	96	100	100
	opmH	97	100	100
	triA	95	100	100
	triB	97	100	100
	triC	97	100	100
	yajC	98	100	100
Disinfecting agents and intercalating dyes, diaminopyrimidine, acridine dye, macrolide, tetracycline,	*mexM*	95	100	100
	mexN	97	100	100
	mexP	98	100	100
	mexQ	95	100	100
	mexS	96	100	100
	mexT	96	100	100
	mexY	80	100	100

phenicol, carbapenem, cephalosporin, cephamycin, penam	*mexZ*	90	100	100
	nalC	95	100	100
	nalD	97	100	100
	opmE	96	100	100
	parR	98	100	100
	parS	98	100	100
	pmpM	98	100	100
Beta-lactamases	$bla_{OXA-850}$	32	97	100
	bla_{PDC-3}	26	100	100

Table S5.4 Description of transposon Tn21 and Tn402 and insertion sequences IS6100, ISAba125 and IS91, harbored by the analysed *P. aeruginosa* genomes

Description of transposon Tn21 and Tn402 and insertion sequences IS6100, ISAba125 and IS91, harbored by the analysed *P. aeruginosa* genomes, reporting quantitative information, the association with ARGs and the distribution among the strains. Moreover are reported the distribution of the MGE in *P. aeruginosa, E.coli, K. pneumoniae, C. freundii and A. baumannii* expressed as % of MGEs in the total observed in the species analyzed and in the total species harboring the analyzed MGE. The search was carried in the NCBI database using a sequence of the backbone genes of the MGEs then filtered using as parameters the sequence coverage 80 to 100% and identity 80 to 100.

MGEs search in the bacterial species (NCBI database)	in *P. aeruginosa* (6351 assemblies)	in *K. pneumoniae* (12363 assemblies)	in *E. coli* (26849 assemblies)	in *C. freundii* (569 assemblies)	in *A. baumannii* (5776 assemblies)	total MGEs retrieved (relative to bacteria analysed)
Total **Tn21** retrieved from NCBI database	216	753	894	111	75	3880
% of genomes including **Tn21** in each species reported	3%	6%	3%	20%	1%	
% of each species harboring **Tn21** over the total Tn21 database	6%	19%	23%	3%	2%	
Total **Tn402-like** retrieved from NCBI database	271	944	831	75	83	4228
% of genomes including **Tn402-like** in each species reported	4%	8%	3%	13%	1%	
% of each species harboring **Tn402-like** over the total Tn402-like database	6%	22%	20%	2%	2%	

Total **IS6100** retrieved from NCBI database	205	1036	972	72	52	3726
% of genomes including **IS6100** in each species reported	3%	8%	4%	13%	1%	
% of each species harboring **IS6100** over the total IS6100 database	6%	28%	26%	2%	1%	
Total **ISAba125**-like retrieved from NCBI database	235	312	438	26	235	1616
% of genomes including **ISAba125** in each species reported	4%	3%	2%	5%	4%	
% of each species harboring **ISAba125** over the total ISAba125 database	15%	19%	27%	2%	15%	
Total **IS91** retrieved from NCBI database	17	22	30	2	0	102
% of genomes including **IS91** in each species reported	0.3%	0.2%	0.1%	0.4%	0%	
% of each species harboring **IS91** over the total IS91 database	17%	22%	29%	2%	0%	

Table S5.5 Distribution of MGEs analysed in bla_{VIM-2} + and bla_{NDM-1} + strains.

The Tn21, Tn402-like, IS6100, ISAba125 and IS91 were identified using BLAST database and enumerated in each genome. For each of the MGEs was analysed the genomic environment, distinguishing the empty elements and the ones including ARGs. The MGEs prevalence was reported in percentage considering the total number of elements enumerated. In some cases, Tn21 and Tn402-like, were identified multiple copies of the same MGE.

Transposon family	Composition	MGEs harbored by bla_{VIM-2}+ strains	MGEs characteristics	Strains presenting MGEs	MGEs harbored by bla_{NDM-1}+ strains	MGEs characteristics	Strains presenting MGEs
Tn21 family	Mer-operon/ Tni-module/ integron(possible) /transposase	122 on 116 (106%), multiple copies in some	89 empty (73%) 17 include blaVIM (14%) 11 include ARGs like sul2, CARB, OXA (1%) 5 include intI1 (0.5%)	79 strains harbor Tn21 47 strains co-harbor Tn21 and IS6100 28 strains co-harbour Tn21 and Tn402-like	21 on 27 strains (77%)	19 empty (90%) 1 includes NDM (0.47%) 1 includes ARGs (0.47%)	18 harbor only Tn21 12 strains harbor Tn21 and IS6100 12 strains harbor Tn21 and Tn402-like 8 strains includes ISAba125 and Tn21 5 co-harbor Tn21 and IS91
Tn402-like	Tni module/ integron	76 on 116(66%), multiple copies in some	50 empty (66%) 23 include VIM(30%)	54 strains harbor Tn402-like transposons 28 strains co-harbour Tn21 and Tn402-like 25 strains co-	16 on 27 strains (59%)	12 empty (75%) 3 include NDM (18%) 1 include ARGs (0.06%)	14 harbor only Tn402-like 12 strains harbour Tn21 and Tn402-like 9 strains co-harbor Tn402-like and IS6100; 5 co-harbor Tn402-like and IS91

				harbor IS6100 and Tn402-like			
IS6100	IS6100/integron or transposon	80 on 116 (69%), multiple copies in some	38 empty (48%) 19 includes VIM(23%) 15 includes ARGs (19%)	62 strains harbor IS6100 47 strains co-harbor Tn21 and IS6100 25 strains co-harbor IS6100 and Tn402-like	25 on 27 strains (92%)	20 empty (59%) 5 include NDM (15%) 4 close to Tn21	9 strains co-harbor Tn402-like and IS6100 8 strains co-harbor IS6100 and IS91 8 strains include ISAba125 and IS6100
ISAba125	ISAba125-blaNDM-blm	0 on 116 (0%),	0 on 117(0%)	0 strains harbor ISAba125	8 on 27 strains (30%)	8 include NDM (30%)	8 strains include ISAba125 and IS6100 8 strains include ISAba125 and Tn21 4 strains include ISAba125 and Tn402-like; = coharbor ISAba125 and IS91
IS91	IS91-blaNDM-IS91	0 on 116(0%),	0 on 117(0%)	0 strains harbor IS91	19 on 27 strains (70%)	19 include NDM (70%)	5 co-harbor Tn21 and IS91 5 co-harbor Tn402-like and IS91 3 harbor Tn402-like

							and IS6100 = coharbor IS91 and ISAba125

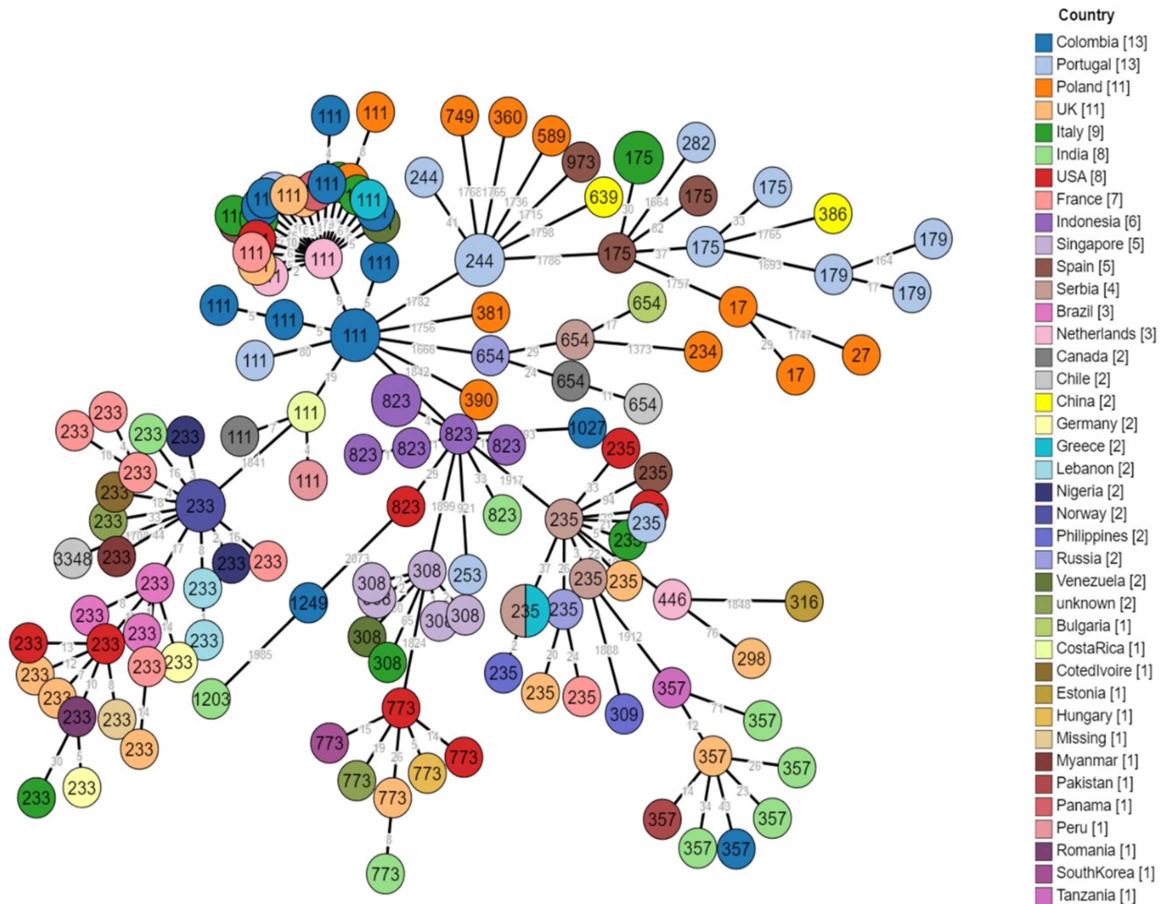

Figure S5.1 Phylogenetic and geographic distribution of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ strains, based on cgMLST.

Minimum spanning tree based on cgMLST, reporting the phylogenetic distribution of *bla*$_{VIM-2}$+ and *bla*NDM-1+ strains identified by ST numbers in the nodes and colored by the geographical locations.

6 General Discussion and Conclusion

The survival of ARB and the possible ARGs transfer to other microorganisms may contribute to the dissemination of antibiotic resistance reservoirs (Cytryn, 2013; Forsberg et al., 2012; Heuer et al., 2011). Even if in low abundance, the presence of ARB and ARGs in treated effluents raises concerns about the possible persistence in soil, if further reused in irrigation (Becerra-Castro et al., 2015; Kampouris et al., 2022). Monitoring the antibiotic resistance occurrence in different environmental compartments, and especially in soil, can represent an important step in preventing the spread of emerging ARGs (Sanderson et al., 2016; Singer et al., 2016; Thanner et al., 2016). As mentioned, human activities are responsible for the discharge of pollutants, including ARB, into the environment. Wastewater treatment plants (WWTPs) represent an important recipient of municipal and/or hospital wastewater (Guo et al., 2017; Rizzo et al., 2013; Rozman et al., 2020). Indeed, several authors report the presence in WWTPs of opportunistic pathogenic bacteria (like *P. aeruginosa, K. pneumoniae, Acinetobacter* spp. *or Staphylococcus* spp.) and ARGs conferring resistance to a broad variety of antibiotics like tetracycline, sulfonamides or β-lactams and carbapenems (Cacace et al., 2019; Hong et al., 2018; Pärnänen et al., 2019; Zhang et al., 2020). WWTPs technologies, designed to reduce physicochemical contaminants present in water, decrease both the ARB and ARGs load although not completely (e.g. the fecal coliforms removal ranges between 82.7 to 95.99%) (Aghalari et al., 2020; Barrios-Hernández et al., 2020). In this regard, the literature reports an increase of ARGs and ARB in soil irrigated with reclaimed water while others do not observe a significant increase of it (Gatica and Cytryn, 2013; Negreanu et al., 2012; Slobodiuk et al., 2021; Wang et al., 2014).

Hence, the monitoring and quantification of ARB and ARGs in soil represent a valuable approach to tackling the antibiotic resistance spread from treated wastewater and improving the actual unclear findings. The qPCR method, in this perspective, allows quantifying soil ARGs, harboured by the ARB, accurately and in a short time (Jalali et al., 2017; Ricchi et al., 2017; Sivaganesan et al., 2010). However, this quantification may be limited due to bias in the DNA extraction methods or the presence of inhibitors (like humic acids) naturally present in the matrix (Sidstedt et al., 2015; Watson and Blackwell, 2000) or due to the intrinsic quantification limits of the method. These limitations may lead to possible underestimation of the ARG presence or contrasting results depending on the number of genes present in the soil analysed. Using microcosms assays it was possible to assess the limit of quantification for ARGs (*bla_{VIM}, bla_{IMP}, vanA, bla_{TEM}, bla_{OXA} and qnrS*), observed as corresponding to 4 log-units of ARGs copies per g of dry soil, corresponding to ~10^4 colony forming units (CFU) per gram of dry soil (Chapter 3). Above this LOQ it was not possible to have a reliable quantification of ARGs even in microcosms inoculated with bacteria (10^3 or 10^2 CFU per g of dry soil). The LOQ must be taken into account and should be always reported when ARGs are monitored, in complex matrices such as soil.

Soil contamination due to anthropogenic activities is affecting the physicochemical characteristics and microbial communities composition (Bååth, 1989; Hu et al., 2016; Hu et al., 2021; Jacquiod et

al., 2018a; Wuana and Okieimen, 2011). Among the contaminants observed to affect the soil microbial communities are the heavy metals, like copper and zinc, that may reach elevated concentrations (respectively in concentrations >100 and >200 mg Kg^{-1} of soil) (Hu et al., 2016; Kunito et al., 2001; Moffett et al., 2003; Wakelin et al., 2010). Since metals are non-degradable, the discharge in soils by human activities (mining, farming, or industry) leads to an accumulation, with the possibility of transformation of oxidation state or complexation with other chemical substances, with the risks of creating hazardous pollution for most of the living microorganisms (Gillan et al., 2015; Li et al., 2019; Lipoth and Schoenau, 2007). The contemporary discharge of ARB and pollutants like heavy metals may raise concerns about the favourable conditions for antibiotic resistance proliferation, as discussed before. Our results (Chapter 4) emphasize the hazardous role of heavy metals on bacterial communities and showed the survival of a ubiquitous ARB like *P. aeruginosa* in metal-polluted (copper and zinc sulfate or nitrate at 20 mM) microcosms soil for long periods (30 days). Observing the survival of the *P. aeruginosa* in soil amended with high metal concentration we suggest considering the hazardous role of ARB adapting and persisting in these environments. In this sense, the source of ARB should be considered and monitored as hotspots for the ARB dissemination in environments.

Carbapenem resistance genes, described in both environment and clinical settings, represent worrisome health threats conferring resistance to antibiotics like ertapenem, imipenem, or meropenem, considered as last chances therapy (Aslam et al., 2020; Meletis et al., 2014). The distribution of carbapenemases in the environment is influenced by anthropogenic activities and geographical location (Mills and Lee, 2019). Carbapenem resistance gene reservoirs were identified in water supplies like wastewater, or river but also in agricultural soil and even wildlife and companion animals (Abraham et al., 2014; Adegoke et al., 2020; Oliveira et al., 2021). When these genes are harboured by highly fitted bacteria, the risks are increased. An example of such a host is *P. aeruginosa* represented by bacteria with a versatile lifestyle and genome with a singular capacity for adaptation to different conditions (Kung et al., 2010; Moradali et al., 2017). *P. aeruginosa* genome presents genes encoding for proteins involved in the transport of metabolites, virulence, antibiotic efflux, motility, and environmental sensing (Kung et al., 2010; Silby et al., 2011). Moreover, *P. aeruginosa* has been described as being tolerant to different metals (such as copper, zinc, arsenic or cadmium), a feature that may facilitate the survival in polluted environments (Elguindi et al., 2009; Perron et al., 2004; Raja and Selvam, 2012; Teitzel et al., 2006). Besides the intrinsic resistance to carbapenems conferred by *ampC* gene, other genes have been described in members of this species, such as bla_{OXA}, bla_{VIM}, bla_{IMP}, bla_{KPC}, or bla_{NDM} (Alexander et al., 2020; Graham et al., 2016; Pellegrini et al., 2009; Queenan and Bush, 2007). These ARGs are often associated with MGEs such as transposons, integrons, conjugative plasmids or ICEs, favouring its spread (Botelho et al., 2018c; Che et al., 2021; Smillie et al., 2010b; Toleman and Walsh, 2011). In particular, transposons such as Tn21 or Tn402-like represent a crucial MGE for carbapenem resistance gene spread and acquisition, being described in association with bla_{VIM}, bla_{NDM}, bla_{IMP} or bla_{CTX-M} in different species (e.g. *P. aeruginosa*, *Proteus mirabilis*, *Achromobacter xylosoxidans*, *E. coli* or *K. pneumoniae*) (Chen et al., 2014; Dong et al., 2019; Novais et al., 2006; Tato et al., 2010a; Toleman et al., 2003). Our

work (Chapter 5) confirmed the association between Tn21 and Tn402-like and the gene bla_{VIM-2}, and described the integration of bla_{NDM-1} flanked by IsAba125 and ble_{MBL} in these transposons. The transposons harboured by bla_{VIM-2}+ or bla_{NDM-1}+ *P. aeruginosa* strains also included other ARGs, principally aminoglycoside resistance genes and, in few cases, carbapenem resistance genes. The acquired genes, encoding for enzymes disrupting antibiotics, improve consistently the resistance strategies favouring the occurrence of multidrug resistance phenotypes (Glen and Lamont, 2021; Rice, 2012). The presence of different ARGs and in particular carbapenem resistance genes raises concerns about the spread of these multidrug resistant bacteria resistant to different antibiotic treatments (Khalifa et al., 2020; Pacheco et al., 2019; Papa et al., 2018; Valdemir et al., 2017). In chapter 5 it was highlighted the copresence of bla_{VIM-2} or bla_{NDM-1} with other carbapenem resistance genes like bla_{GES}, bla_{KPC} in the same strain (assemblies accession numbers: GCA_013413775.1, GCA_020404785.1, GCA_013413775.1). Moreover, in the case of bla_{NDM} and bla_{GES}, the ARGs were described as included in the same transposon, promoting the possible ARGs mobilization. The different distribution of carbapenem resistance genes and in particular of bla_{VIM-2} could be attributed to the more recent identification of the bla_{NDM-1} gene and so fewer works dedicated rather than to a lower gene spread. Monitoring the carbapenem resistance genes in the environment could contribute to avoid the diffusion of resistance against this last-resort class of antibiotic. In addition, a better understanding of the role of the phylogenomic lineages and accessory genome traits on the dissemination of emerging ARGs may contribute to improving the tracking and control of these phenotypes in the environment and risks of transmission to humans.

Antibiotic resistance is a wide and complex problem observed in every human-related environment and represents a serious health threat. Tackle the ARGs spread and the occurrence of novel multidrug resistant ARB should be considered a priority in the antibiotic resistance race. A comprehensive approach analysing ARB phenotypic and genotypic characteristics could provide valuable and detailed results to understand and prevent antibiotic resistance spread.

7 Future Work

- An interesting future study would consist on tracking ARGs and MGEs in order to assess which type of biomarker can be more reliable and have more predictive capacity. Mobile elements like transposons commonly associated with ARGs may represent an additional marker to study antibiotic resistance. Between the MGEs, plasmids play an important role in ARGs spread. To optimize the methodology to recover and genetically analyse the plasmids may improve the actual knowledge about the antibiotic resistance diffusion. Moreover, a database including crossed information about ARGs and MGEs associated could greatly improve the monitoring of antibiotic resistance spread.
- To further investigate the survival of ARB in metal-polluted environments. Real agricultural fields contaminated with metals could be analysed to assess the microbiome composition and the antibiotic resistance presence using genetic and molecular biology techniques. A comprehensive study about the antimicrobial presence in pristine, contaminated and agricultural soil may provide important information about the different ARB and ARGs distribution. Monitoring and assessing the antibiotic resistance distribution in soil, mostly if affected by anthropogenic activities, should be considered as a priority in an antibiotic resistance crisis.
- A crucial point in the antibiotic resistance spread is the direct transfer of ARGs between different bacteria. ARGs like bla_{VIM}, even if integron borne, or bla_{NDM} are often included in MGEs, favouring the spread between different microorganisms. Laboratory assays could be settled to assess the ARGs mobilization, plasmids harbouring ARGs or monitoring the acquire of genes in selective pressure assays. ARB and competent bacteria could be exposed to different concentrations of antibiotic or metals to induce the ARGs transfer.

Annexes

Annex I. Scientific communication and training activities carried during the ANSWER project.

Project meetings and summer schools

- ANSWER Summer school and Training course on "Mechanisms and processes involved in the crops uptake". 13 – 23 June 2016, Spanish National Research Council (CSIC), Barcelona, Spain.
  Summer school focused on wastewater treatment methodologies and reuse for crops irrigation. This meeting was the first ANSWER project meeting where allt the ESRs presents their research project.

- "Microcontaminants in the aquatic water cycle – wastewater reuse – the Cypriot/Israeli experience". 5 - 10 March 2017, Nireas – International Water Research Center, University of Cyprus, The Agriculture Research Organization of Israel – The Volcani Centre (ARO), The Hebrew University of Jerusalem (HUJI), Tel Aviv, Israel.
  The training event includes the visit to wastewater facilities and the analysis of wastewater reuse practice in Israel/Cyprus.

- "Wastewater microbiota and the effects of treatment processes". 5 – 7 July 2017, Escola Superior de Biotecnologia (ESB) of Universidade Católica Portuguesa (UCP), Porto, Portugal.
  This summer school includes lectures about wastewater treatment methods, antibiotic resistance study and entrepreneurship ("Fostering entrepreneurship - from business models to clients"). During this course was, moreover, write a with paper published as review.

- "Microbiology in wastewater treatment; Design criteria for wastewater treatment plants; Horizontal resistance gene transfer in soil". 23 – 27 April 2018, Technische Universität Wien (TU-Wien) Vienna, Austria.
  This training event was focused on the current wastewater treatment technologies available in Austria. During the event was possible to have an introductive lecture of Microbiome analysis using QIIME pipeline.

- "Environmental and human health risk assessment of antibiotics". 18 – 21 June 2018, KWR Watercycle Research Institute (KWR) Utrecht, The Netherland.
  During this training event were addressed issues related to human and environmental risk of antibiotic resistance dissemination. As part of this training was organized a meeting about the wastewater reuse policy

Outreach activities:

- Visit to a high school. 20 January 2017, Colégio Luso-Internacional do Porto (CLIP) Porto, Portugal. One-hour lecture entitled "Antibiotic Resistance: Environmental and public health problem" at the Oporto International school to the 11th grade students

- Radio talk organized by the Universty of Cyprus, NIREAS at University of Cyprus radio 29 September 2017, Nicosia, Cyprus.

- The Cyprus "Researchers' Night 2017", event organized by University of Cyprus to share scientific knowledge with non-scientific attendance. 29 September 2017, Nicosia, Cyprus.

- Café scientifique about the antibiotic resistance issues. 27 January 2018, Dresden, Germany

- Visit to end user at Braunschweig water treatment plant 2 February 2018, Braunschweig,Germany

Scientific publication outside of the Ph.D. thesis:

- Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I. C., Zammit, I., Kampouris, I., Vaz-Moreira, I., & Nunes, O. C. 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ Int, 115: 312-324. doi.org/10.1016/j.envint.2018.03.044

Conference attendance and poster presentation:

- Poster presentation "Effect of metals contamination in the survival of antibiotic-resistant bacteria and their genes in soil" **Gianuario Fortunato,** I. Vaz-Moreira, O. C. Nunes, C. M. Manaia at Halting Antimicrobial Resistance Dissemination in Aquatic Environments (HEARD2018). 16th – 19th, September 2018, Ascona, Switzerland

- Poster presentation "High limits of quantification of antibiotic resistance genes may mask the impacts of water reuse in soils" **Gianuario Fortunato,** I. Vaz-Moreira, Cristina Becerra-Castro, O. C. Nunes, C. M. Manaia at Challenges and Solutions related to Xenobiotics and Antimicrobial Resistance in the Framework of Urban Wastewater Reuse: Towards a Blue Circle Society (XENOWAC II), 10th – 12th October, Limassol, Cyprus

- Poster presentation "Fate of Pseudomonas aeruginosa and bla_{VIM} in soil under selective pressure by copper and zinc" **Gianuario Fortunato,** I. Vaz-Moreira, O. C. Nunes, C. M. Manaia at Federation european microbiological societies FEMS conference. 7th-11th July 2019, Glasgow, Scotland.

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- Poster presentation "Pan-genome and pan-resistome analysis of bla_{VIM-2}+ or bla_{NDM-1}+ carbapenem resistant *Pseudomonas aeruginosa*" **Gianuario Fortunato,** I. Vaz-Moreira, C. M. Manaia at Microbiotec 21 webconference, 23rd-26th November 2021, Lisboa, Portugal.

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