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

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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.

Keywords: heavy metals; metal salts; bacteria survival; antibiotic resistance gene persistence; soil bacterial communities

1. 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; Urra, Alkorta and Garbisu 2019; Chojnacka, Moustakas and Witek-Krowiak 2020). 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 (Munir, Wong and Xagoraraki 2011; Udikovic-Kolic *et al.* 2014; Chen and Xia 2017; Liu *et al.* 2017; McKinney *et al.* 2018; Murray *et al.* 2019). 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 (Malik and Aleem 2011; Amador *et al.* 2015; 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 (Wang *et al.* 2014; Abd-Elwahed 2018; He *et al.* 2020).

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 (Tchounwou *et al.* 2012; Ali, Khan and Ilahi 2019). 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 (Mantovi *et al.* 2003; Berenguer *et al.* 2008; Xiong *et al.* 2010; Donner *et al.* 2012; Guo *et al.* 2018; Qian *et al.* 2018). 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; MacKie *et al.*, 2012; La Torre *et al.*, 2018; Lamichhane *et al.*, 2018). 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 (Ju *et al.*, 2004; Nielsen, 2012; Dwivedi and Srivastva, 2014). 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 (Poulsen 1998; Mantovi *et al.* 2003; 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 (Luo and Rimmer 1995; Korthals *et al.* 2000; 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;

Yazdankhah, Rudi and Bernhoft 2014; Becerra-Castro *et al.* 2015; Dickinson *et al.* 2019). 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 cooper 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 (Verraes *et al.* 2013; Rather *et al.* 2017).

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* (Heuer, Schmitt and Smalla 2011; Marti *et al.* 2013; Udikovic-Kolic *et al.* 2014; Faissal *et al.* 2017; Li *et al.* 2017b; Zhao *et al.* 2017; Wang *et al.* 2019). 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 (Negreanu *et al.* 2012; Gatica and Cytryn 2013; Marano *et al.* 2019). 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

(Seiler and Berendonk 2012; Dickinson *et al.* 2019; 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 harbour 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 (Poulsen 1998; Mantovi *et al.* 2003; Tóth *et al.* 2016; Sonoda *et al.* 2019; Zwolak *et al.* 2019) and have recognized impacts on the microbiota (Dumestre *et al.* 1999; Kunito *et al.* 2001; Jacquiod *et al.* 2018; Song *et al.* 2018; Dickinson *et al.* 2019). 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 (Fajardo *et al*., 2008; Breidenstein *et al*., 2011). 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

153 sequencing to infer if metal soil amendments triggered changes that could explain the persistence
154 of *Pseudomonas aeruginosa* (*bla*$_{VIM+}$).

155

156 2. Materials and methods

157 2.1. Soil samples and microcosms assays

158 Assays were conducted in microcosms with soil collected from an agricultural greenhouse, located
159 in Vila do Conde, Northern Portugal (41°25' N; 8°45' W) and that adopts Good Agricultural and
160 Environmental Practices. This greenhouse soil has been characterized as being a sandy soil, with
161 pH (H_2O) of 7.5, organic matter of 1.8%, electric conductivity of 0.32 dS m^{-1}, a concentration of
162 soluble cations (Ca, Mg, Na, K) of 717 mg kg^{-1} and of metals (Cr, Cu, Ni, Pb, Zn) totalizing 98.5 mg
163 kg^{-1} and 25 mg kg^{-1} of nitrate (Becerra-Castro *et al*., 2017). Soil composite samples were collected
164 between windrows from the same greenhouse in three different occasions; the soil collected in
165 April 2017 (tomato crop) was used for microcosm assay named M1; the soil collected in March
166 2018 (not cultivated) was used for microcosm assays M2 and M3, which were carried out in two
167 independent dates; and the soil collected in June 2018 (lettuce crop) was used for microcosm
168 assay M4 (Table 1). The sampling dates and the period between collection and experiment start
169 are reported in Table 1. Sampling procedures and other soil characteristics were previously
170 described (Becerra-Castro *et al.* 2017). Microcosm assays were established by weighing 300 g of
171 soil (wet weight), spiked with a solution of copper and zinc salts, sulfate or nitrate (Sigma Aldrich
172 ®), to reach a final concentration of 20 mM (equivalent to 830 mg kg^{-1} of copper, 850 mg kg^{-1} of
173 zinc, 1250 mg kg^{-1} of sulfate and 1600 mg kg^{-1} of nitrate). Molar units were used to allow the
174 comparison of metal molecules available when using different salts. As a control, a corresponding
175 soil aliquot was spiked with the same volume of sterile distilled water. After vigorous stirring,
176 control and metal spiked soils (M2, M3, M4) were aged for one month in the dark at room
177 temperature.

178 Amended and non-amended (control) soil microcosms were inoculated with a suspension of the
179 carbapenem-resistant *P. aeruginosa* strain H1FC49 isolated from hospital effluent (Vaz-Moreira *et*
180 *al.*, 2016). This strain is resistant to carbapenems due to the presence of the metallo β-lactamase
181 gene bla_{VIM}, known to be inserted in the variable region of a class 1 integron (Vaz-Moreira *et al.*,
182 2016). *P. aeruginosa* strain H1FC49 is resistant to carbapenems, penicillins, cephalosporins,
183 fluoroquinolones, sulfonamides, and aminoglycosides, as determined based on the disc diffusion
184 method (Vaz-Moreira et al., 2016). In addition, the genome analysis, based on the annotation
185 against the Resistance Gene Identifier (RGI) of the Comprehensive Antibiotic Resistance
186 Database (CARD) (Alcock *et al*. 2020), revealed the presence of the antibiotic resistance genes
187 sulfonamide resistant dihydropteroate synthase (*sul1*), aminoglycoside 3'-phosphotransferase
188 (*APH(3')-IIb)*, and chloramphenicol acetyltransferase (*catB7*).

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

202

203 2.2. 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.

2.3. 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 *intl1*, 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*, *intl1* 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 *intl1* 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 ºC for 10 minutes followed by 40 cycles at 95 ºC for 15 seconds, 55 ºC for 30 seconds, 72 ºC for 30 seconds. 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 Limit of Detection (LOD), and below the Limit of Quantification (LOQ) (Fortunato *et al.* 2018).

2.4. 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.

2.5. 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.

3. 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 3.1), or (b) in soil microcosms amended with copper and zinc as sulfate salts, or (c) as nitrate salts (section 3.2), as well as the (d) effect of these metals and salts in the soil microbiota (section 3.3).

3.1. 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 1). Consistently, similar results were observed when the fate of *P. aeruginosa* was assessed based on the abundance of gene *ecf* (orange boxplots, Figure 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 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 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 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).

3.2. 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 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 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

309 further statistical analysis. The different soil samples used in the microcosms presented similar
310 total bacteria, total cultivable bacteria and pseudomonads loads (Table 1).

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

316 The results over time of the targeted biomarkers (colonies on cetrimide agar and the genes *ecf*,
317 bla_{VIM}, and *intI1*) were not totally reproducible in the three microcosms sets (Figure 2), suggesting
318 some stochasticity in the system, which may be due to slight variations on the physico-chemical
319 and biological parameters of the soil samples, collected over a period of 2 years. However, there
320 were common patterns of variation for the three distinct conditions tested. The C/C_0 ratio of the
321 abundance of typical *P. aeruginosa* colonies on cetrimide agar (green colonies) showed a sharp
322 decrease in the first week of incubation independently of the condition. In the absence of metal
323 amendment and in the presence of sulfate salts a decrease to a C/C0 ratio of ~0.8 was observed
324 (Figure 2A, 2B, 2C). In the presence of nitrate salts, the decrease was higher, reaching C/C0 ratios
325 of ~0.7 in the same period (Figure 2B, 2C). The C/C0 values of colony forming units continued to
326 decrease until time t30, reaching values between 0.64-0.68 in the three conditions at t30 (Figure
327 2C). The decrease observed with nitrate salts was always significantly higher in comparison to the
328 non-amended control (Figure 2B and 2C) or amended with sulfate assays ($p < 0.05$) (grey
329 boxplots, Figure 2). From the initial 7.9 log-CFU g^{-1} soil dry weight, the load of *P. aeruginosa*
330 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
331 log-CFU g^{-1} soil dry weight in sulfate-amended and non-amended soil, respectively (M4, Figure
332 2C). The measurement of the *P. aeruginosa* H1FC49 genes showed a distinct pattern of variation.
333 The abundance of the gene *ecf* (orange boxplots, Figure 2) had a mild decrease after the first
334 week (C/C0 ~0.95 in non-metal; 0.85-0.95 in sulfate salts or nitrate salts), was fairly stable after the
335 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 2C). The abundance of this gene, independently of the metal and salt amendment, decreased from a range of 6.9 to 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 2A) than in the assays M3 and M4 (C/C0 ~0.9) (Figure 2B, 2C). 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 7.4-7.7 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 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 to 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

363 mild and when noticed, contributed to reducing and not selecting for exogenous bacteria and
364 genes.

365

366 3.3. Effect of copper and zinc amendment on the composition and structure of the soil bacterial
367 communities

368 The microbial community of microcosms M3 and M4 was examined at t0, t14, and t30 aiming to
369 assess if metal amendments could impact the composition or structure of the communities and to
370 infer if these could be related to the fate of *P. aeruginosa* H1FC49. M3 and M4 soil samples were
371 obtained in distinct sampling events, a fact that can be reflected in the slight differences in the
372 community structure (Figure 3). The effect of metals and salts on the community composition was
373 analysed through the comparison of microcosms M3 and M4 samples at t0, t14, and t30 (M4)
374 produced with soil that was aged for one month with copper and zinc sulfate, copper and zinc
375 nitrate, or synthetic wastewater as a control (Figure 4). The inoculum did not produce a significant
376 effect on the community according to PERMANOVA analyses (Table S2). As hypothesized, metals
377 were associated with significant changes in the bacterial community structure. The relative
378 abundance of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* was significantly higher
379 ($p<0.05$) in assays with metal amendment than in non-amended ones (Figure 4A), mainly due to
380 the higher percentage values observed in members of the classes *Alpha*- (from 15.0-19.0% to
381 20.8-22.5%) and *Gammaproteobacteria* (from 10.0-12.0% to 12.4-14.3%), *Actinobacteria* (from
382 2.7-7.0% to 6.0-7.6%) and *Thermoleophilia* (from 1.0-1.6% to 2.3-2.6%), and *Bacilli* (from 3.5-8.0%
383 to 6.7-8.7%) and *Clostridia* (from 0.6-1.0% to 1.0-1.6%), respectively (Figure 4B). Also, the
384 hypothesis that the metals salts form, sulfate or nitrate, could be associated with distinct patterns of
385 variation in the bacterial community structure was confirmed. The relative abundance of phyla
386 *Chloroflexi*, *Planctomycetes, Patescibacteria*, and *Latescibacteria* was higher in nitrate salts than in
387 sulfate salts amended soil. The opposite was observed for phyla *Bacteroidetes* and *Proteobacteria*,
388 with lower relative abundance in nitrate salts than in sulfate salts amended soil. This could be
389 explained because the orders of the latter two phyla, respectively *Cytophagales* and

390 *Chitinophagales*, and *Betaproteobacteriales* and *Shingomonadales*, presented increased relative
391 abundance in soil amended with sulfate salts. In contrast, the unc. *Alphaproteobacteria* (phylum
392 *Proteobacteria*), *Ardenticatenales* (phylum *Chloroflexi*)*, Planctomycetacia* (phylum
393 *Planctomycetes*), and the *Saccharimonadales* (phylum *Patescibacteria*) had higher relative
394 abundance in soil amended with nitrate salts (Figure 4C).

395 Consistent with these variations on the bacteria community structure, it was observed that metal-
396 amended soil samples, compared with the non-amended controls presented lower richness
397 (number of ASVs and Chao indexes) and diversity (Fisher index) indices (Table S1). Considering
398 the effects of salts, it was observed that sulfate had a higher impact on the reduction of these
399 indices than nitrate, significantly higher in this case. The bacterial community patterns of the three
400 types of microcosm (non-amended, nitrate copper and zinc or sulfate copper and zinc) at three
401 incubation times were inspected based on beta diversity analyses, reported as a PCoA biplot
402 (Figure 5). Axes 1 and 2 explained ~60% of the variation, with segregation of samples per metal
403 amendment condition, and inside these groups per soil sampling campaign (M3 and M4), with the
404 incubation time (t0, t14, and t30) demonstrating minimal influence. The samples from soil amended
405 with sulfate or nitrate salts present a different microbial community if compared with the non-
406 amended soil ($q < 0.01$, F = 856 and 11.614 respectively, PERMANOVA results, Table S2). Metal
407 salts had a minor influence on the separation of the groups ($q < 0.01$, F 5.790, PERMANOVA
408 results, Table S2), probably due to the lower number of sulfate-amended microcosm for which it
409 was possible to examine the community. The increased relative abundance of members of the
410 orders *Rhizobiales*, *Xanthomonadales, Sphigomonadales* of the phylum *Proteobacteria*, and
411 *Bacillales* of the phylum *Firmicutes* in metal-amended microcosms, observed in Figure 4C, seemed
412 to be the major driver for the organization of the biplot (Figure 5).

413

414 **4. Discussion**

415 Copper and zinc, although not considered alarming pollutants, may reach high concentrations in
416 the environment (Klimek 2012; Tóth *et al.* 2016; Lamichhane *et al.* 2018). 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) (Mengel *et al.* 2001; Alloway 2008; 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 harboured 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; Li *et al.*, 2017; Jacquiod *et al.*, 2018), 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 (Trevors 1986; Lazdins *et al.* 2020). 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

maintain 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 °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; Hu *et al.* 2016; Dickinson *et al.* 2019), 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) (Wuana and Okieimen 2011; Nielsen 2012; Tóth *et al.* 2016; Lamichhane *et al.* 2018). 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; Li *et al.* 2017a; Jacquiod *et al.* 2018). 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 (van Elsas *et al.* 2007, 2012).

5. 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 emphasises the extreme vulnerability of soil to different modes of contamination, even when sustainable agriculture practices are in place.

Acknowledgments

This work is part of a project that has received funding from the European Union's Horizon 2020, under the Innovative Training Networks (ITN-ETN) programme Marie Skłodowska-Curie grant (ANSWER - ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions) agreement No 675530. The ANSWER project was hosted by CBQF, and LEPABE through FCT projects UID/Multi/50016/2019, and UIDB/00511/2020, respectively.

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796

Table 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.

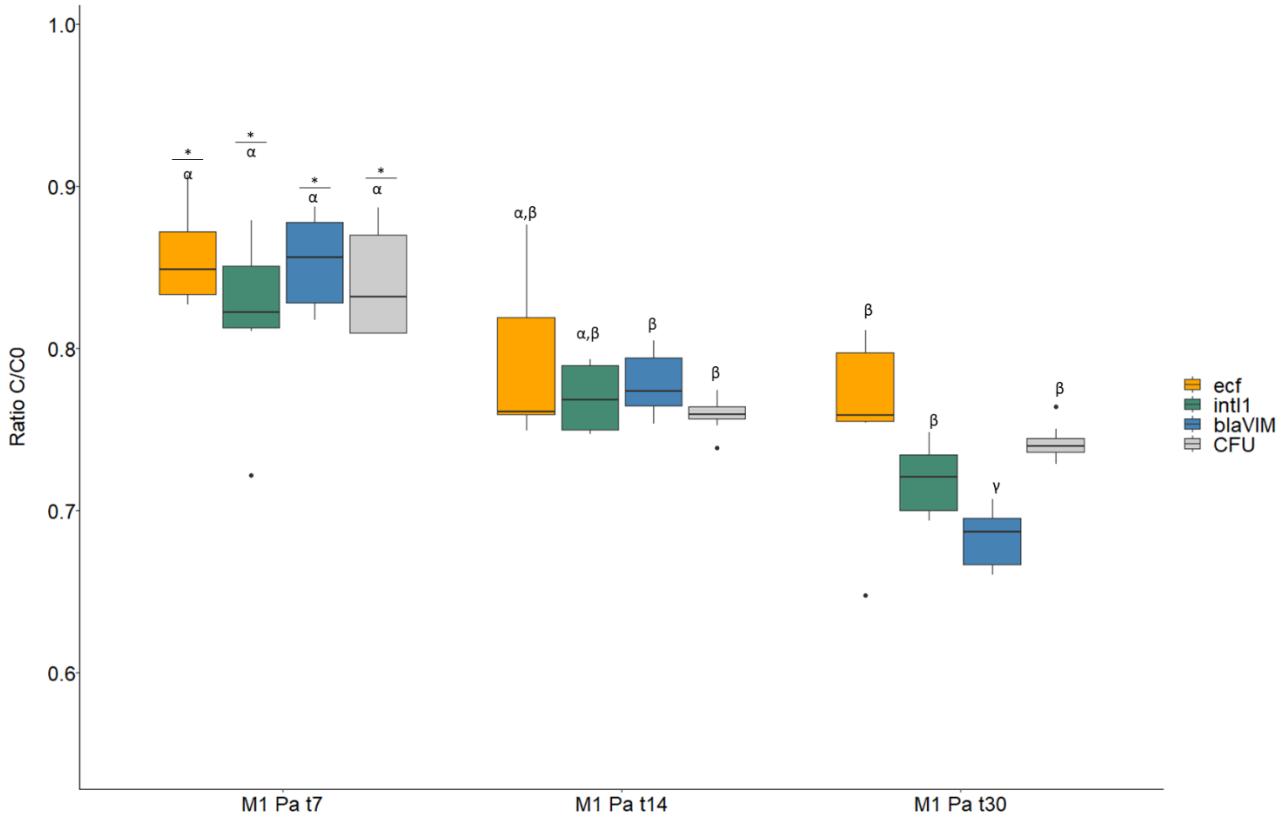


Figure 1. Variation (C/C0) of the biomarkers of *Pseudomonas aeruginosa* H1FC49 - cetrimide agar green colonies, housekeeping *ecf* and acquired genes *bla*$_{VIM}$ and *intl1*, examined in non-metal amended microcosms (M1).

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), *intl1* (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).

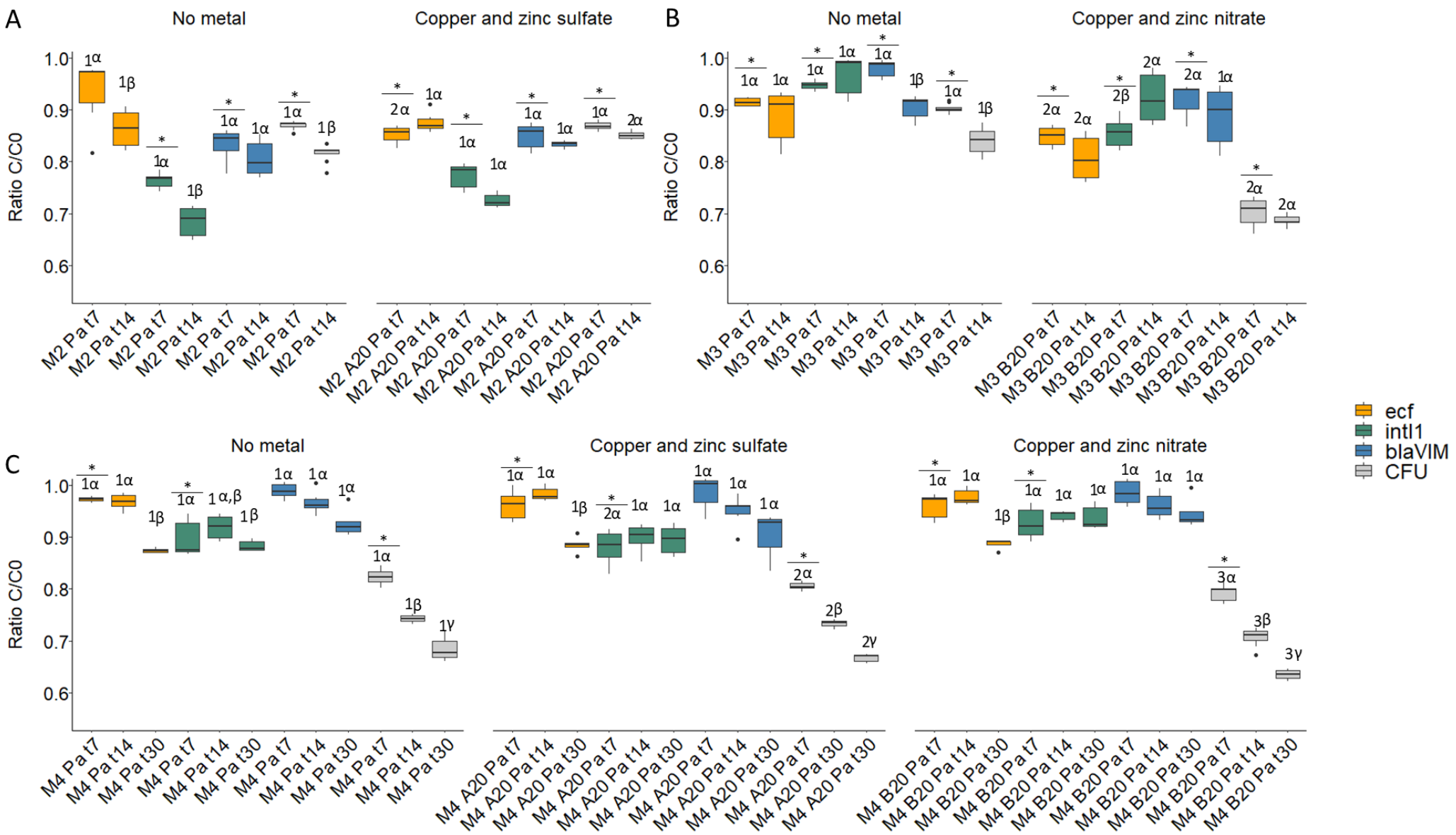

A
No metal
Copper and zinc sulfate
B
No metal
Copper and zinc nitrate
C
No metal
Copper and zinc sulfate
Copper and zinc nitrate
Ratio C/C0
ecf
intI1
blaVIM
CFU

Figure2. Variation (C/C0) of the biomarkers of *Pseudomonas aeruginosa* H1FC49 - 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).

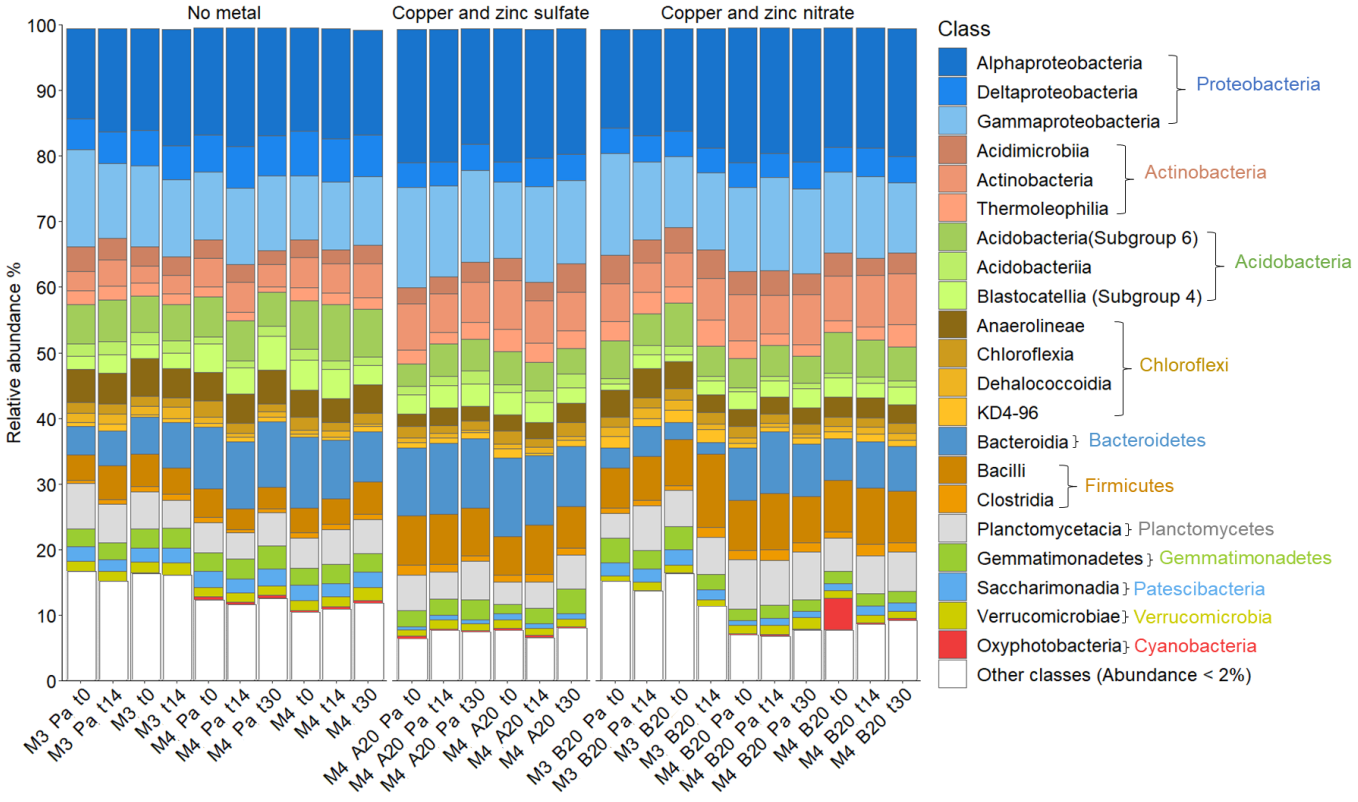
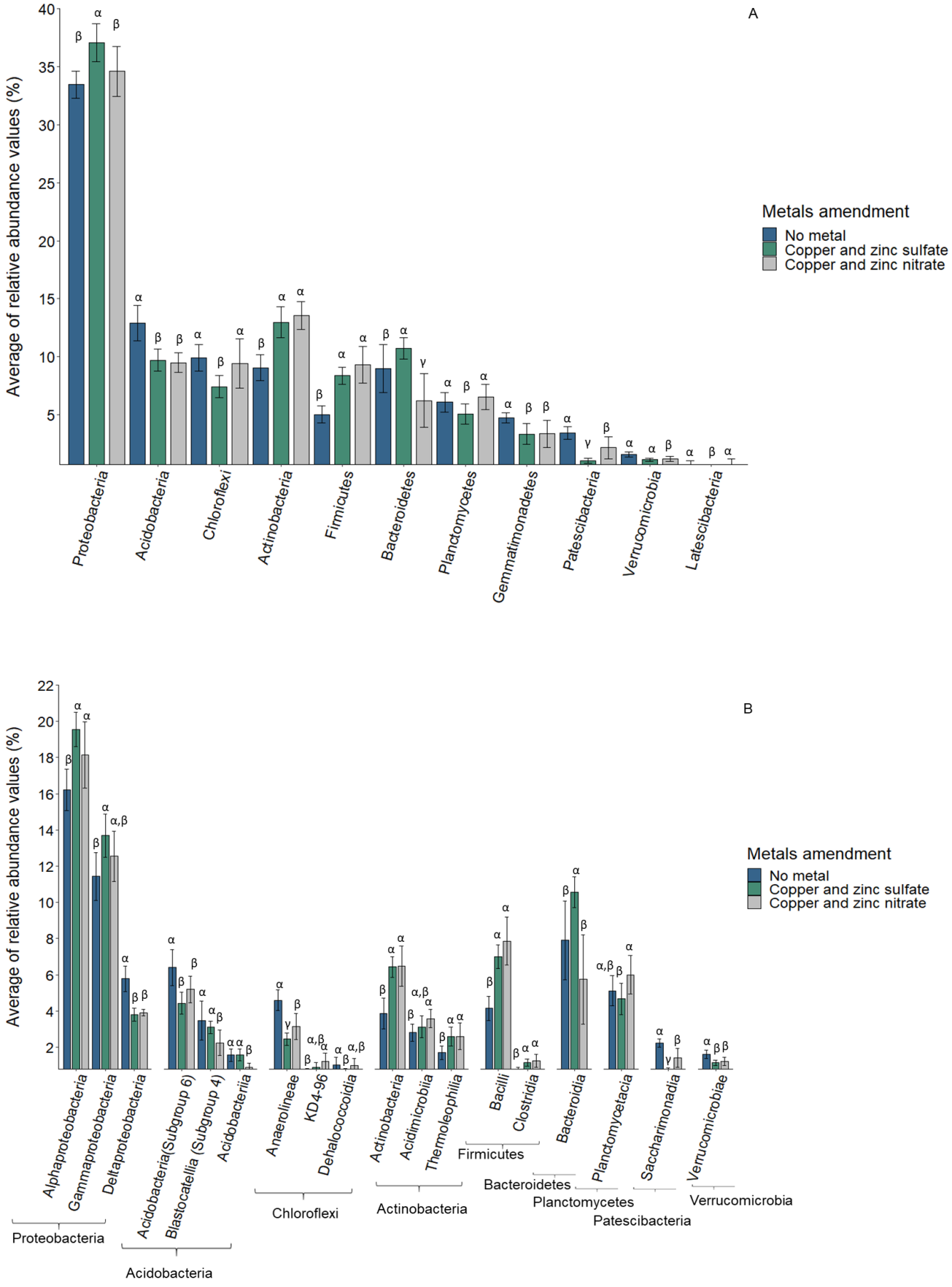


Figure 3. **Relative abundance of classes and phyla in non-amended or amended with copper and zinc, as sulfate or nitrate salts in microcosm assays sampled over time (0 to 30 days)**. The barplot reports the relative abundance as percentage of most abundant phyla (>2%) present in the samples. The sum of the relative abundance of the most abundant classes in each column was calculated in a range of 83-92%.


A
Average of relative abundance values (%)
Metals amendment
No metal
Copper and zinc sulfate
Copper and zinc nitrate
Proteobacteria
Acidobacteria
Chloroflexi
Actinobacteria
Firmicutes
Bacteroidetes
Planctomycetes
Gemmatimonadetes
Patescibacteria
Verrucomicrobia
Latescibacteria
B
Average of relative abundance values (%)
Metals amendment
No metal
Copper and zinc sulfate
Copper and zinc nitrate
Alphaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Acidobacteria(Subgroup 6)
Blastocatellia (Subgroup 4)
Acidobacteriia
Anaerolineae
KD4-96
Dehalococcoidia
Actinobacteria
Acidimicrobiia
Thermoleophilia
Bacilli
Clostridia
Bacteroidia
Planctomycetacia
Saccharimonadia
Verrucomicrobiae
Proteobacteria
Acidobacteria
Chloroflexi
Actinobacteria
Firmicutes
Bacteroidetes
Planctomycetes
Patescibacteria
Verrucomicrobia

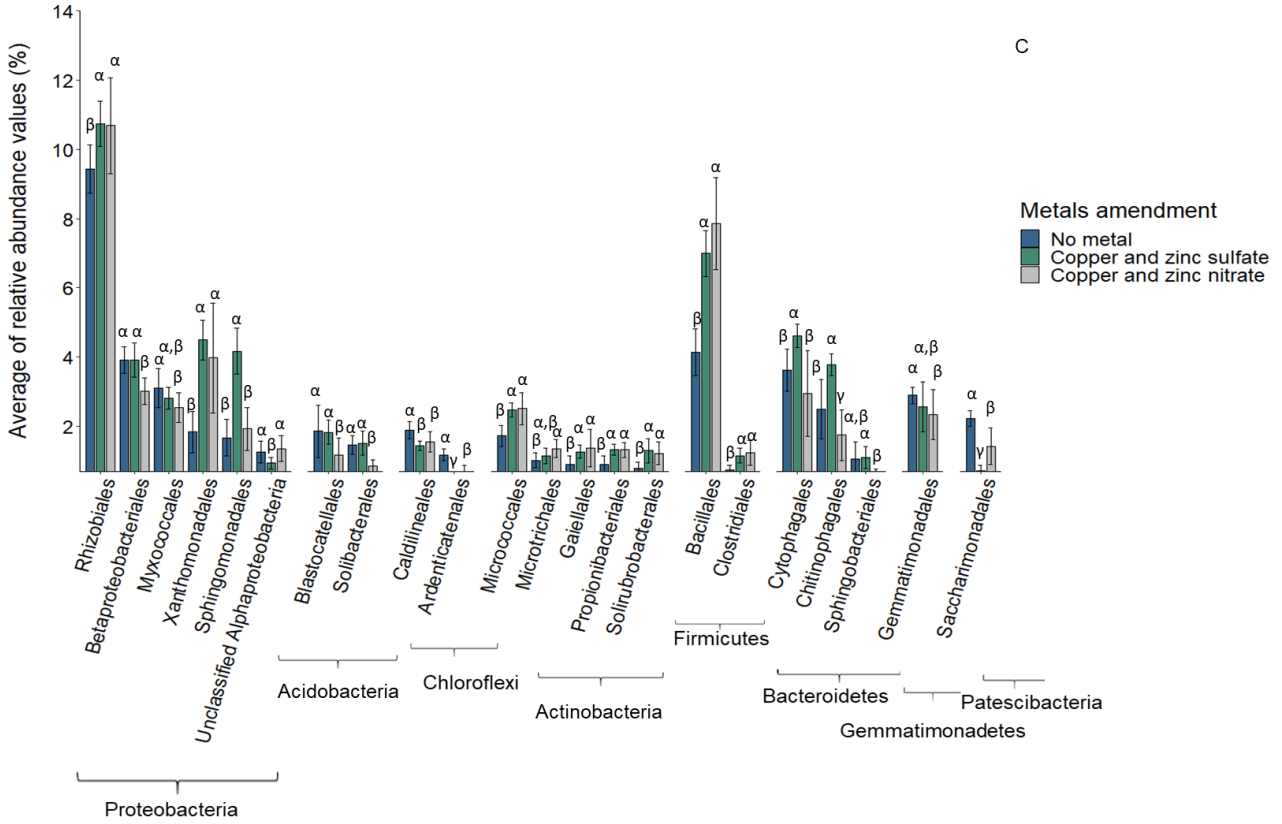


Figure 4. Average relative abundance of most abundant Phyla, Class and Order (>2% relative abundance) with significative variances in the samples analysed, grouped per soil metals salts amendment. The barplot reports the mean relative abundance of the most abundant Phyla (A), Classes (B) and Orders (C) 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.

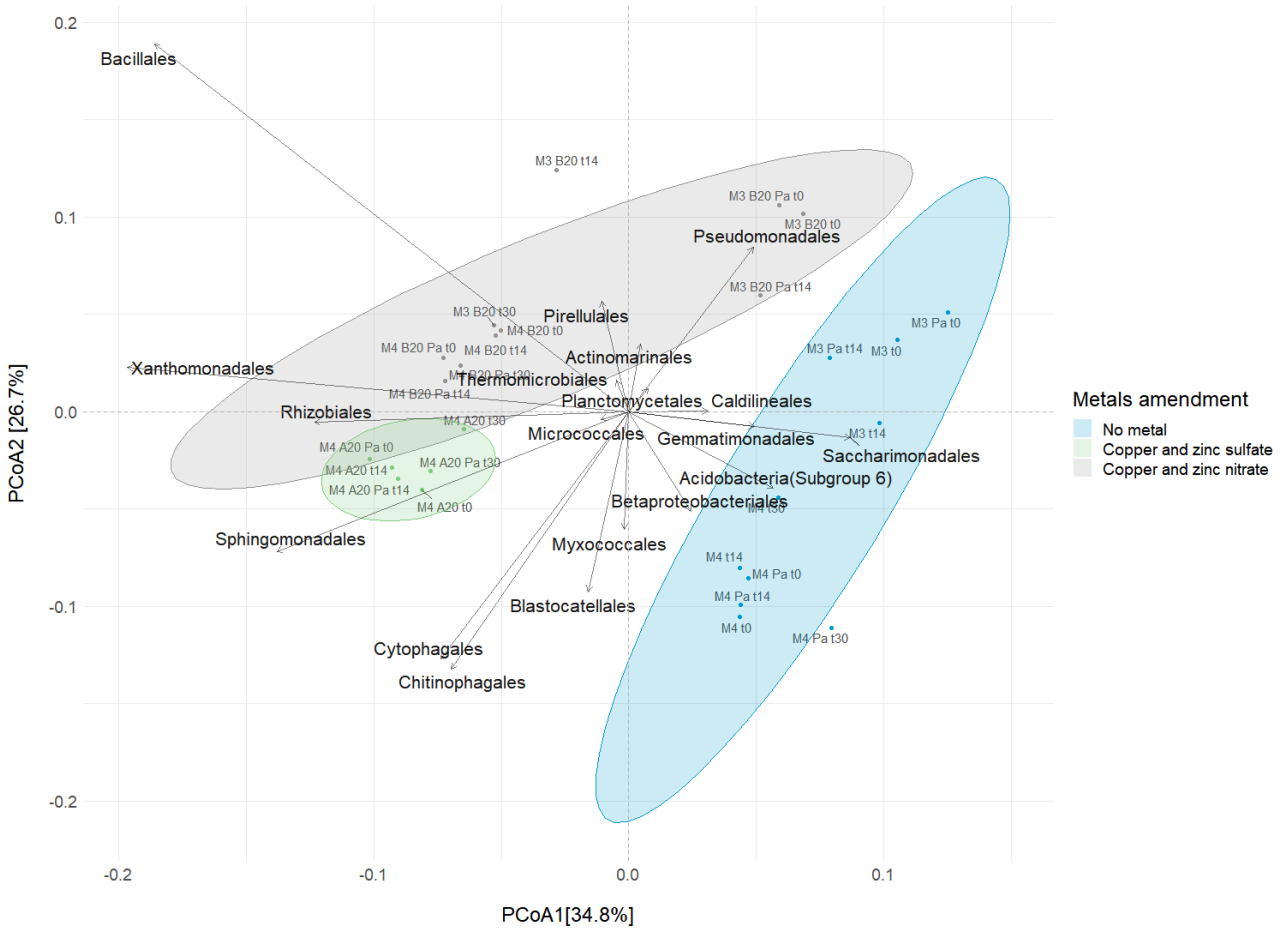


Figure 5. **Biplot of beta-diversity distance metrics (Weighted Unifrac distance) of bacterial communities (at Order level) in soil amended or non-amended with sulfate or nitrate zinc and copper metal salts**. The plot reports the distribution of the samples depending on the soil metal amendment (blue, 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 samples distances. The contribution is quantified with the length of each arrow.