

Title: Influence of temperature and copper on Oxalobacteraceae in soil enrichments

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Abstract

β -Proteobacteria is one of the most abundant phylum in soils, including autotrophic and heterotrophic ammonium-consumers with relevance in N circulation in soils. The effects of high temperature events and phytosanitary treatments, such as copper amendments, on soil bacterial communities relevant to N-cycling remain to be studied. As an example, South Portugal soils are seasonally exposed to high temperature periods, the temperature at the upper soil layers can reach over 40 °C. Here, we evaluated the dynamics of mesophilic and thermophilic bacteria from a temperate soil, in particular of heterotrophic β -Proteobacteria, regarding the ammonium equilibrium, as a function of temperature and copper treatment. Soil samples were collected from an olive orchard in southern Portugal. Selective enrichments were performed from samples under different conditions of temperature (30 and 50 °C) and copper supplementation (100 and 500 μ M) in order to mime seasonal variations and phytosanitary treatments. Changes in the microbial communities under these conditions were examined by DGGE (Denaturing Gradient Gel Electrophoresis), a molecular fingerprint technique. At moderate temperature -30 °C- either without or with copper addition, dominant members were identified as different strains belonging to genus *Massilia*, a genus of the Oxalobacteraceae (β -Proteobacteria), while at 50 °C, members of the *Brevibacillus* genus, phylum Firmicutes were also represented. Ammonium production during bacterial growth at moderate and high temperatures was not affected by copper addition. Results indicate that both copper and temperature selected specific tolerant bacterial strains with consequences for N-cycling in copper treated orchards.

Keywords: Soil β -Proteobacteria; ammonium; copper-tolerance; N-cycling

INTRODUCTION

Soils are complex biological systems, whose characteristics depend on the interaction of abiotic and biotic factors. The biotic component of soils is greatly ruled by the highly diverse microbial communities and their activities. Between 10^4 - 10^6 different microorganisms and about 10^{10} microbes are estimated per gram of soil [3]. These include soil bacteria with at least 32 phylum-level groups [9]. The dominant phyla are Proteobacteria, Acidobacteria and Actinobacteria. The Proteobacteria usually make up about 39 % of the total soil bacterial communities [9].

Proteobacteria comprises important members for nitrogen and sulfur cycling in soils. The aerobic autotrophic ammonia oxidizing bacteria (AAOB) participate in the nitrification process converting ammonium to nitrite, followed by the conversion of nitrite to nitrate by nitrite oxidizing bacteria (NOB). Ammonia oxidation is considered the limiting step for nitrification in soil ecosystems [1]. AAOB includes the chemolithotrophic β -Proteobacteria genera *Nitrosomonas*, *Nitrospira* and *Nitrosolobus*, and a member of the γ -Proteobacteria (*Nitrosococcus oceanus*) [5]. Heterotrophic ammonia oxidizing bacteria are, for instance, *Pseudomonas* sp. (γ -Proteobacteria), *Ochrobactrum* (α -Proteobacteria) [10] and *Alcaligenes faecalis* (β -Proteobacteria) [18]. A recent analysis reports a higher nitrogen circulation activity for AAOB rich soils, correlated with ammonia-oxidizing activities of AAOB, which are 10^3 to 10^6 times more abundant than those of heterotrophic AOB [13]. However, in organic-rich soils AAOB decrease in number and activity [28], whereas heterotrophic AOB dominate.

Crops are usually treated for prevention of microbial diseases, copper being frequently used as a fungicide [19]. Although copper is an important micronutrient because it is a cofactor for numerous enzymes, its excess causes toxicity, which is associated with the destruction of the iron-sulfur centers of enzymes due to the displacement of iron by copper [12]. Copper treatments can selectively influence the dynamics of soil bacterial communities by modifying the ratio of copper-sensitive to copper-resistant microorganisms. Both Gram-positive and Gram-negative copper-resistant bacteria possess proteins for copper efflux (see e.g. [26]) as a defense mechanism against copper toxicity.

Copper addition to crops is being extensively used, for instance, in grape and olive orchards in South Portugal. These soils are often under thermic stress and the upper soil layers reach over 40 °C during summer. In this study, we assessed the effect of copper and temperature on β -Proteobacteria

dynamics and ammonium release in soil, so that future agricultural models can be conceived regarding the maintenance of the diversity and activity of this soil bacterial phylum.

MATERIALS AND METHODS

Sampling and enrichment cultures

Sampling was carried out in an olive orchard in Alentejo, Southern Portugal (38°29'54.1"N 7°45'38.5"W). The studied soil showed moderate drainage, low erosion and low topsoil content of organic carbon (i.e., 75 %) [14]. This soil has been previously classified as Haplic Luvisol [8]. The analyzed field underwent a phytosanitary treatment with Cuprital® (copper oxychloride) by aspersion on tree crowns. (average air temperature 12 °C, 100 mm precipitation).

Analyzed samples consisted of three adjacent soil cores pooled together which were collected aseptically from topsoil, at 3.5 cm to 7.5 cm depth. Soil temperature was approximately 10°C at the time of collection. Additional samples were collected from areas treated one month (T_0) before and at different periods, six months (T_6) and twenty months (T_{20}), previous to this sampling. Soil was collected either between the tree crop line (EC) or below tree crowns (DC). Physico-chemical parameters were estimated following standard procedures by the Laboratory of Agricultural Chemistry [11].

Six gram aliquots of the composite sample T_{20} -EC were added, under sterile conditions, to 100 ml flasks with 15 ml of NB (Nutrient Broth) (Oxoid) pH 7.0, or to NB supplemented with CuSO_4 , at 100 μM (pH 7.0) and 500 μM (pH 7.0). The cultures were incubated at 30 or 50 °C, with shaking at 180 rpm for 24 hours. Serial dilutions from these enrichments were made in NB; dilutions 10^{-6} and 10^{-8} were plated on NBA (Nutrient Broth with 15 % w/v Agar) at 50° C for 24 hours and colony-forming units/ml for each enrichment were determined.

Measurement of ammonium production

The quantification of the ammonium followed the spectrophotometric method described by Taylor et al. [27]. A reaction mix with 40 μL of sterile non-inoculated medium served as blank. Samples

were analyzed in triplicate. Concentration values were determined using a calibration curve for 0-5 mM NH₄Cl solutions.

DNA extraction and PCR-DGGE

DNA extraction from enrichments was carried out with DNA Isolation kit PowerSoil® (MoBio Laboratories Inc, USA) following the manufacturer's instructions. Fingerprints of the whole bacterial communities were obtained through PCR amplifications using the primers 341F-GC and 518R [15, 16]. A nested-PCR strategy was adopted to obtain β -Proteobacteria fingerprints. After the first amplification, with primers Beta 359F and Beta 682R, the second step of the nested-PCR used the primer pair 518F-GC and Beta 682 R (Table 1). All PCR reactions were performed in a Bio-Rad MyCycler thermocycler.

Denaturing Gradient Gel Electrophoresis (DGGE) was performed following Muyzer et al. [16] in a DGGEK-2401 system coupled to a power supply EPS-300 IIV (CBS Scientific ®, USA). PCR amplified 16S rRNA gene fragments from four bacterial species were used as DGGE migration markers. The fragments were obtained with primers 341F-GC/518R from the DNA of the following bacteria (from top to bottom of a DGGE gel): *Pseudomonas aeruginosa* PAO1, *Escherichia coli* K12 CECT 433, *Paenibacillus* sp. DSM 34, *Streptomyces caviscabies* ATCC 21619. The ImageJ program [23] at NIH (National Institute of Health, USA), was used to evaluate the relative intensity of the distinct bands from ethidium bromide stained gels.

The major DGGE bands were excised from the gels, reamplified, purified, and cloned in pCR2.1-TOPO vector from TOPO TA Cloning Kit (Invitrogen). Inserts were reamplified with GC-tailed forward primers and subjected to a second DGGE analysis together with the original environmental sample for clone selection; the selected clones were sequenced at MacroGen® sequencing services (MacroGen Inc., Korea) Sequences were identified using the Blast tool from NCBI.

Statistic analyses

ANOVA analysis [25] was performed with MedCalc software version 12.3.0. to compare significant differences ($P < 0.05$) among treatments.

RESULTS AND DISCUSSION

Characterization of soil samples

Element contents and other physicochemical characteristics of soil samples are listed in Table 2. A soil can be classified as “high copper content” at 15 ppm [11] and the maxima determined for copper in our samples was about seven fold that level (104.5 ppm). Copper accumulation in the treated soils was deduced from the similarity of copper levels detected in T_6 and T_0 samples.

Characterization of soil enrichments

Ammonium production. Ammonium concentrations were similar (approx. 8 mM)(Fig. 1) for all tested conditions suggesting minimum effects of copper and temperature on final ammonium release by the microbial communities. The viable cell counts at 50 °C were about 1-2 orders of magnitude higher in the enrichments at 50 °C than at 30 °C, without or with copper 100 μ M. Therefore, thermophilic bacteria, including thermophilic copper resistant bacteria, were present at both temperatures and could contribute to ammonium production at different extents in each case. Ammonium concentration was the result of the equilibrium between ammonium production and consumption for each treatment. These results suggest a potential for maintaining ammonium concentrations through N-cycling under mesophilic and thermophilic soil conditions even in the presence of copper at 500 μ M (a value of the same magnitude as the copper value found in T_0 -EC sections). Temperate soils can indeed reach high temperatures, Portillo et al. [20] recorded values over 60 °C in an Andalusian soil. In Évora, average values of 40 °C have been recorded, at 4 cm soil depth, during summer, which supports the potential importance of thermophiles during hot periods and their metabolic relevance under regular periodicity following annual seasons.

Bacterial community composition. Bacterial community fingerprints by DGGE of the enrichments showed a relatively poor influence of copper, at each growth temperature, on these bacterial communities

(Fig. 2). Comparing the enrichments at 30 °C and 50 °C, very different banding patterns were observed suggesting that a higher effect on the microbial community can be induced by temperature than by supplementation with copper (Fig. 2). High concentration of copper (in the 500 µM range) was required to induce significant changes in the thermophilic bacterial community from the studied soils.

Fig 3. shows the fingerprints corresponding to the nested-PCR strategy performed to target the β-Proteobacteria group. Members from β-Proteobacteria belonging to the Oxalobacteraceae Family were identified (Table 3), including genera *Massilia* and *Naxibacter*, which contribute to plant growth promotion as identified in *Massilia* isolates [4]. *Massilia* sp. (1.1. & 3.1) was present in the mesophilic enrichments without or with copper addition, whereas band 2.4., whose sequence was similar to an uncultured *Naxibacter*, was absent in the medium with copper amendment at 500 µM.

While Oxalobacteracea family members were identified in the mesophilic enrichments, *Brevibacillus* sp. from the Firmicutes phylum were the dominant members of the thermophilic enrichments. Mühlhling et al. [15] estimated approximately 86 % of matches for the Beta primers within the β-Proteobacteria group, and only one third of the sequences they obtained for one of the studied locations were from the target group. This was related to the potentially low abundance of Betaproteobacteria in the sample, which might have been also the case in the thermophilic enrichments. Interestingly, bands 4.3. & 6.1, with sequence similar to *Brevibacillus* sp. THG-d53, were dominant in the 50 °C enrichments with no copper and with copper at 500 µM, whereas a band with identical sequence to an uncultured Oxalobacteraceae was dominant at the intermediate copper concentration; this suggests a competitive interaction between *Brevibacillus* and *Massilia*-related Betaproteobacteria (Table 3). Noteworthy, references on thermophiles from Oxalobacteraceae are scarce; nevertheless moderate thermophiles have been reported [6]. Band 6.2 detected in the thermophilic enrichments corresponded to an uncultured Betaproteobacterium.

Recently, it has been suggested that Oxalobacteraceae members are actively implicated in ammonium consumption and denitrification [2] which could occur herein in microoxic zones between soil granules. Major ammonium consumers, i.e. nitrifying bacteria members of AAOB genera *Nitrosomonas* or *Nitrospira*, were not dominant members. This was expected since AAOB generate low biomass yields and are about 0.01 % of total soil bacterial number [13]. Yamamoto et al. [30] found *Nitrosomonas europaea* and *N. eutropha* related AOB at the high-temperature stage (more than 60°C) of cattle manure

composting, and Innerebner et al. [7] reported that the average AAOB abundance in compost-treated soils was two times higher than in mineral-fertilized soils; a high number of *Nitrosomonas* was found in conditions that mimic composts, where peptone (a major component of the broth medium used herein) was included [24]. AAOB population increased in ammonium fertilized versus unfertilized soils, as previously reported [17] at a concentration of 7.5 mM ammonium sulfate, a value similar to the one produced in this study assemblages. Noteworthy, about 1/6 of the sequences of DGGE selected clones for band 2.3. were similar to the 16S rRNA gene sequence of an uncultured *Nitrospira* sp. (GU271371.1) ($3e^{-92}$; 99% identity). Autotrophic AOB have organoheterotrophic growth ability, as reported for *Nitrosomonas* [22] and *Nitrospira* [29] and may co-exist with heterotrophic AOB. Among the latter, members of Firmicutes, β - Proteobacteria and γ - Proteobacteria are included. Their role in nitrification can be significant and should not be neglected. Kouki et al. [10] isolated heterotrophic ammonium oxidizers, which were shown to assimilate nitrogen into the cellular biomass and to oxidize ammonia with efficiencies above 80 %. The isolates belonged to genus *Bacillus*, *Ochrobactrum* and *Bordetella*, among others. Also heterotrophic ammonia-oxidizing *Brevibacillus* were isolated from agricultural soils [13]. Interestingly, *Brevibacillus* genus, as other genera of thermophilic Firmicutes, are hyper-ammonium producers [20, 21], and a model bacterium for thermophiles represented in soil bacterial communities.

The global results from the community fingerprints and from the ammonium quantification suggest that both ammonium producers and heterotrophic ammonium-oxidizer populations were enriched in the assemblages. The presence of these two populations contributed to the equilibrium of ammonium measured in this study. Strain properties and cell status besides other environmental factors certainly influenced regulatory mechanisms aiming to ammonium production and consumption by those bacteria.

Guidelines for olive orchard management

Both ammonium producers and consumers must be present in the soil and in a dynamic equilibrium that benefits plant crops.

The enrichments from this study tried to mimic the use of N-composts that could be added to the crop soil during the summer period, increasing autotrophic and heterotrophic AOB and assuring N-

cycling while maintaining irrigation. During spring, green fertilization and introduction of previous pruning remains would favor nitrifiers' proliferation.

Olive orchards are usually treated with copper before the first rains in autumn and after every period of fog or rain to prevent fungal diseases. Temperature and copper addition interfered with soil bacteria population dynamics, namely with Oxalobacteraceae family, a fundamental family comprising plant growth promoter members, shifting their dominance to members of the Firmicutes Phylum in organic rich soils. Nevertheless, at 30 °C, Oxalobacteraceae members could thrive at levels higher than 15 ppm, these copper-resistant rhizospheric bacteria would be important for lowering copper toxicity in the rhizosphere. Copper treatments should be minimized and should be left to preventive sporadic treatments at end-summer and after large-scale precipitation periods during autumn, followed by monitoring of copper concentration in the soil. The introduction of copper resistant selected Oxalobacteraceae strains after copper treatments may be envisaged as a future strategy to maintain proper N-cycling activity.

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CONFLICTS OF INTEREST. The authors declare they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Similar levels of ammonium were produced during growth of enriched bacterial communities at 30°C and 50°C as well as with (100 µM and 500 µM) and without copper treatment.

Fig. 2. DGGE profiles of the bacterial communities enriched at 30°C and 50°C lacking copper treatment and supplemented with copper at concentrations of 100 µM and 500 µM. 1, 30 °C; 2, 30 °C supplemented with 100 µM Cu; 3, 30 °C supplemented with 500 µM Cu; 4, 50 °C; 5, 50 °C supplemented with 100 µM Cu; 6, 50 °C supplemented with 500 µM Cu. Bars indicate the location of migration markers represented by (from top to bottom): *Pseudomonas aeruginosa*, *Escherichia coli*, *Paenibacillus* sp., *Streptomyces caviscabies*.

Fig. 3. Bacterial community fingerprints by DGGE of the β-Proteobacteria communities enriched at 30 °C and 50 °C with and without copper treatment. 1, 30 °C; 2, 30 °C supplemented with 100 µM Cu; 3, 30 °C supplemented with 500 µM Cu; 4, 50 °C; 5, 50 °C supplemented with 100 µM Cu; 6, 50 °C supplemented with 500 µM Cu. Bars indicate the location of migration markers represented by (from top to bottom): *Pseudomonas aeruginosa*, *Escherichia coli*, *Paenibacillus* sp., *Streptomyces*

caviscabies. The migration of the representative bands identified through sequencing is indicated with arrows.

Primer	Target group	Use	E.coli position	Annealing temperature (°C)	Reference
Beta 359F	β-Proteobacteria	Nested- PCR 1st step	359-378	63	[15]
Beta 682R			682-701		
518F GC	β-Proteobacteria	Nested- PCR 2nd step	518-534	60	[15]
Beta 682R			682-701		
341F GC	Bacteria	DGGE fingerprints	341-357	56	[16]
518R			518-534		

Table 1 Primers used in this study, their target and annealing temperature

Table 2 Comparison of four soil samples based on physico-chemical determinations

Samples	T₀-DC	T₀-EC	T₆-DC	T₂₀-EC
Texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam
pH (H₂O)	6.80	6.60	7.30	7.50
Total Nitrogen (%)	0.076	0.061	0.120	0.041
Nitrate (NO₃) (ppm)	4.50	4.50	7.00	6.50
Potassium (K₂O) (ppm)	470.00	176.00	188.00	106.00
Copper (Cu) (ppm)	104.50	13.90	96.50	3.50
Phosphorus (P₂O₅) (ppm)	104.00	78.00	88.00	42.00

Table 4 Identification of the dominant bands of bacterial community fingerprints from untreated and copper-treated soil enrichments. The closest homologue sequence from blast analysis is shown.

Band number ^a	Closest homologues (accession number)	Similarity (E score; %)
1.1 & 2.5 & 3.1	<i>Massilia varians</i> strain M1303 (KF924221.1)	2e ⁻⁹⁰ ; 100 %
	<i>Massilia</i> sp. THG-HS29 (KF815073.1)	
	Uncultured Oxalobacteraceae bacterium, DGGE band IT_B06 (HF678328.1)	
2.1	Uncultured beta proteobacterium isolate DGGE Band 19A1 (AY648699.1)	3e ⁻⁸⁹ ; 99%
	<i>Massilia aurea</i> strain HME9229 (KF911334.1)	1e ⁻⁸⁸ ; 99%
	<i>Massilia</i> sp. LHG-1BY1 (HF563565.1)	
2.2	Uncultured bacterium clone Nov-control IR3_8 (KF189065.1)	2e ⁻⁹⁰ ; 100 %
	<i>Massilia brevitalea</i> , isolate EB19 (HF566244.1)	
2.3	Uncultured bacterium clone Nov-control IR3_8 (KF189065.1)	9e ⁻⁸⁹ ; 99%
	<i>Massilia brevitalea</i> , isolate EB19 (HF566244.1)	
2.4	Uncultured <i>Naxibacter</i> sp. isolate DGGE gel band KPDSB6 (KC147488.1) Bacterium G2(2009) 1 (GQ398344.1)	3e ⁻⁸⁹ ; 99%
4.1	<i>Brevibacillus</i> sp. THG-d53 (KF999709.1)	3e ⁻⁸⁸ ; 99%
	<i>Brevibacillus panacihumi</i> strain ODB42 (KF835591.1)	
4.2	<i>Brevibacillus limnophilus</i> strain AG-42 (KF817656.1)	3e ⁻⁸⁸ ; 99%
	<i>Brevibacillus</i> sp. 5.5LF 16P (FN666626.1)	
	<i>Brevibacillus limnophilus</i> strain DSM 6472	

(NR_024822.1)		
4.3 & 6.1	<i>Brevibacillus</i> sp. THG-d53 (KF999709.1)	3e ⁻⁷⁸ ; 96%
	<i>Brevibacillus panacihumi</i> strain ODB42 (KF835591.1)	
5.1	Uncultured bacterium clone C-F-17 (AF443582.1)	2e ⁻⁹⁰ ; 100 %
	<i>Massilia varians</i> strain M1303 (KF924221.1)	1e ⁻⁸⁸ ; 99%
	<i>Janthinobacterium</i> sp. E30 AF-15-2 (KC788060.1)	
6.2	Uncultured soil bacterium clone S1P4014 (KF145420.1)	4e ⁻⁸⁷ ; 99%
	Uncultured beta proteobacterium clone M1-064 (F182908.1)	

^a First number in the band nomenclature indicates the gel lane

Figure 1

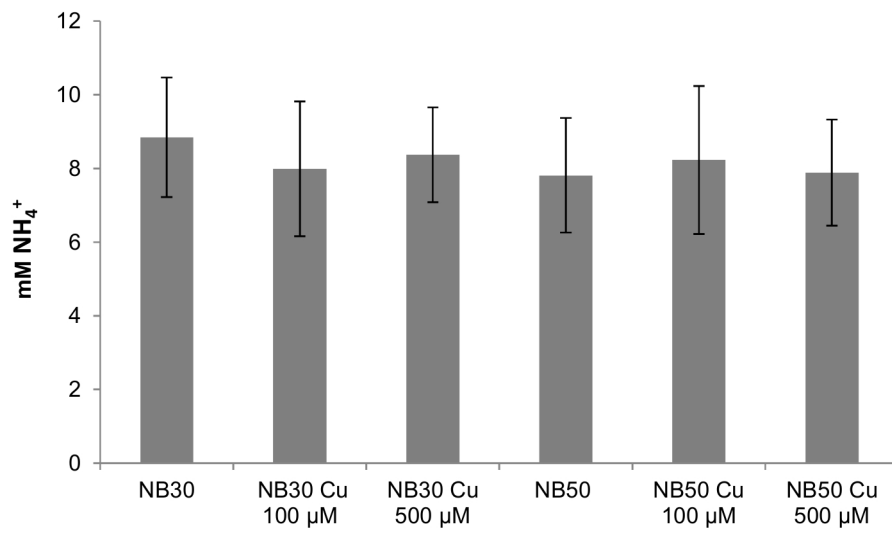


Figure 2

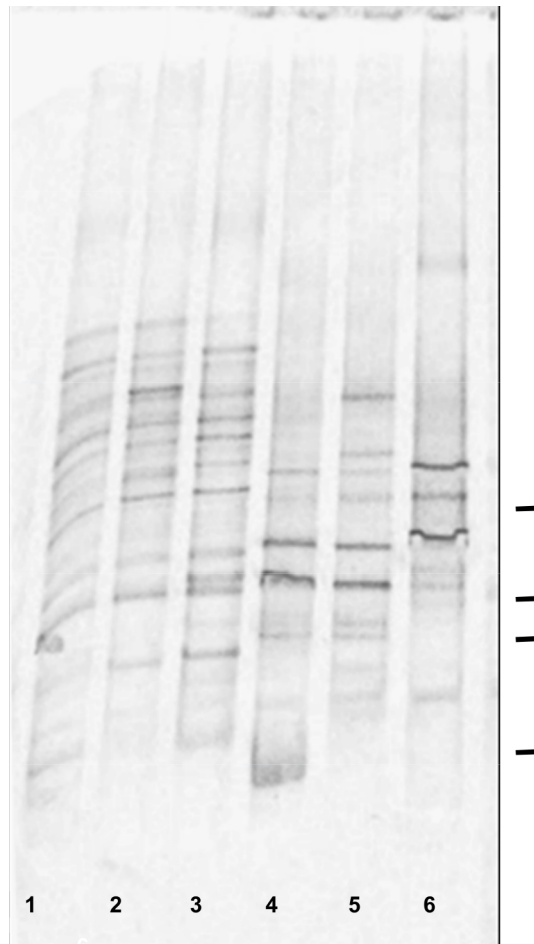


Figure 3

