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Distribution of microbial abundance in long-term copper contaminated soils from Topolnitsa-Pirdop valley, Southern Bulgaria

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Abstract

This study presents the distribution of bacterial and fungal abundances in long-term copper (Cu) contaminated soils in Topolnitsa-Pirdop valley – a highly industrialized zone with a number of mines and processing plants for copper and other non-ferrous metals. The bacterial (16S rRNA gene copies) and fungal (ITS rRNA gene copies) were estimated using quantitative PCR technique in five topsoils, differently Cu contaminated (ranging from 28.05 to 198.9 mg kg^{-1}). Bacterial abundance varied in a range of 1.68 × 10^{11} to 3.24 × 10^{11}16S rRNA genes, whereas fungi amounted from 1.95 × 10^{8} to 6.71 × 10^{8} ITS rRNA genes. Fungal and bacterial abundances were significantly (fungi) and insignificantly (bacteria) influenced by Cu contamination. The fungal/bacterial ratio related negatively with soil Cu, which shifted microbial communities' structure towards bacterial dominance. Since the ratio between bacteria and fungi are vital in explaining many soil functions, the calculated changes in this ratio indicated deterioration in soil quality, being of primary importance for plant production.

Keywords

ITS rRNA gene, microbial abundance, qPCR, soil contamination, 16S rRNA gene

Introduction

Heavy metals (HMs) are natural constituents of the environment, but intensive use for human purposes has altered their geochemical cycles and biochemical balance. The extraction and processing of mineral raw materials are one of the main sources of soil and water pollution with HMs and other toxic elements. In this respect, of particular interest are the open-pit mines and their generated waste materials, which cause serious environmental damage and significant changes to the landscapes of the impacted regions (Dabeva et al. 2012). Prolonged exposures to HMs in high concentrations are harmful to microorganisms, plants and animals. Furthermore, HMs can be absorbed by food crops, and entering into the food chain has proven to be a potential health hazard for plants and humans (Huang et al. 2018; Ali et al. 2019; Afonne and Ifediba 2020; Hasan et al. 2020). Microorganisms are highly diverse and ubiquitous in soil ecosystems and participate in a variety of key ecosystem functions such as nutrient cycling, structuring of soil aggregates and biomass production. A stable microbial community contributes essentially to stabilizing soil structure and maintaining soil ecosystem services (Bissett et al. 2013). Studies have shown that microorganisms can significantly promote the circulation of soil nutrients, maintain soil fertility, and improve crop health (Fierer 2017). The negative impacts of HMs on soil microbial communities have been reported in several studies. HMs have been shown to be harmful to soil microorganisms and may lead to significant changes in microbial diversity (Chodak et al. 2013; Zampieri et al. 2016), richness (Cui et al. 2018), abundance (Deng et al. 2015), metabolic activity (Chen et al. 2014; Hong et al. 2015; Zampieri et al. 2016; Fajardo et al. 2019) and structure of the communities (Cui et al. 2018; Feng et al. 2018; Zhang et al. 2018).

Copper is one of the most common soil contaminants (Griffiths and Philippot 2013) among heavy metals. Soils represent the largest sink of Cu, being released to the environment by anthropogenic activities, such as sewage irrigation, mining activities, municipal waste disposal, and intensive use of pesticides and herbicides (Smith 2009; Zhuang et al. 2009; Ballabio et al. 2018). A moderate level of Cu is regarded as a micronutrient and it is essential for microorganisms to carry out their metabolic activities (Giller et al. 2009). However, excessive inputs of Cu to soil ecosystems could persist for a long time after their introduction and cause toxic effects on soil microorganisms (Giller et al. 2009). Some authors found that Cu contamination had significant effects on bacterial abundance and diversity (Li et al. 2015), as well as fungal diversity (Zhang et al. 2022). Therefore, understanding the responses of soil microbial communities to Cu contamination is essential to counteract its negative effect on ecosystem functions and services.

The aim of this study was to assess the distribution of bacterial and fungal abundances in Cu long-term contaminated soils from the valley of Topolnitsa-Pirdop and its relationship with the local soil properties.

Materials and methods

Study area and soil sampling

Topolnitsa River and its tributaries (Medetska, Zlatishka, Pirdopska and Bunovska Rivers) were influenced by the ore industry, agriculture activities, and domestic wastewater discharge, which led to the deterioration of its ecological status according to the River Basin Management Plan (2016–2021) (East Aegean River Basin Directorate. MoEW). Three large metal mining and metal processing enterprises are located in this region- "Elatsite Med", "Dundee Precious Metals" and "Aurubis". Additionally, in the area, the open-pit "Medet" is located, and although the mine was closed in 1992, it is considered the main source of pollution in this area.

The study area is located in the Topolnitsa-Pirdop valley (Fig. 1). Topsoil samples (0–20 cm) with different Cu concentrations (ranging from 28.05 to 198.9 mg kg^{-1}) were collected in May 2021. Five sampling sites have been selected from a monitoring map (Kancheva et al. 2018), considering the sources of Cu pollution as follows: S1 – located in the valley of Medetska River at 710 m from the "Medet" mine tailing (42°39'12.9"N, 24°09'04.2"E), S2 – located in the valley of Topolnitsa River (after Zlatishka River) at 250 m from the "Medet" mine tailing (42°39'31.8"N, 24°08'26.6"E), S3 – located in the valley of Pirdopska River at 750 m from the "Medet" mine tailing (42°39'48.2"N, 24°08'11.3E), S4–located in the valley of Bunovska River at 2200 m from the mine tailings of "Elatsite-Med" A.D. (42°36'47.5"N, 24°00'44.8"E), and S5–located in the valley of Topolnitsa River-next to Muhovo village at 25–30 km straight from S4 and S1 (42°24'45.5"N, 23°59'54.4"E). Samples were taken in three replicates per site. All sampled soils were classified as Fluvisols (FAO 2015).

Soil physicochemical properties and copper concentration

Soil pH was potentiometrically measured in 0.1 M $CaCl_2$ soil suspension according to ISO 10390:2005. The soil organic carbon (SOC) was determined by sulfochromic oxidation according to BSS ISO 14235:2002. Nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen were determined according to Keeney and Nelson (1982). Inorganic phosphate (P_2O_5) was determined by the method of Olsen (1982). Water content (WtC) was calculated after oven drying (105 °C). The concentration of Cu was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) according to ISO 22036:2008.

DNA extraction

The genomic DNA was extracted from 0.5 g soil, using the E.Z.N.A soil DNA kit (Omega Bio-tek, USA) according to the manufacturer`s recommended protocol. The quality and quantity of the extracted DNA were subsequently assessed by Qubit 4 fluorometer (Invitrogen) and 1% agarose gel electrophoresis.

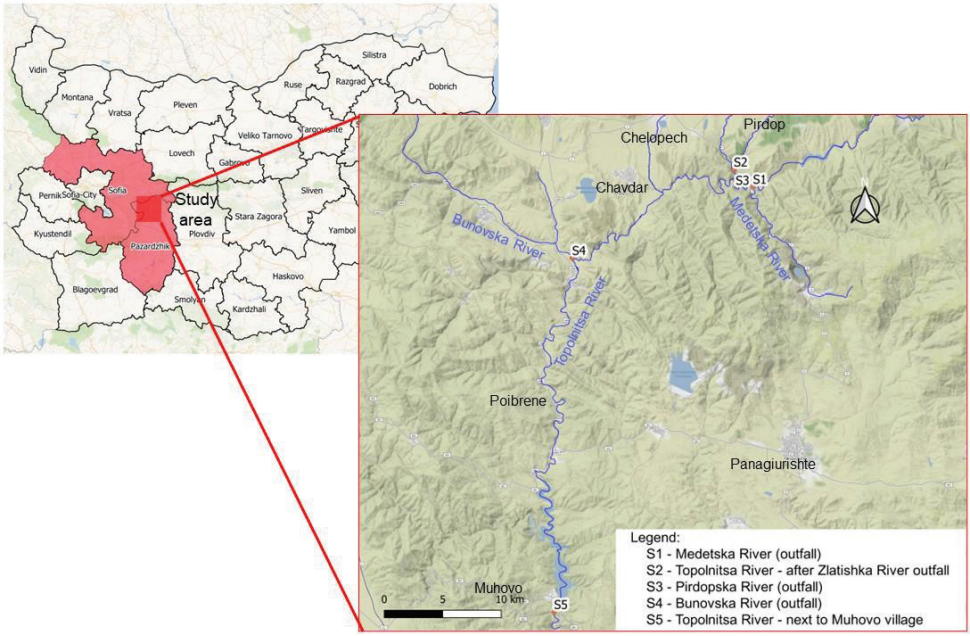

Figure 1. Map of the study area and sampling sites.

Real-time Q-PCR

Q-PCR analyses were performed using iTaqTM Universal SYBRGreen Supermix (BioRad) on Rotor Gene 6000 (Corbett Life Science) according to the manufacturer's protocols. Bacterial abundance was estimated using 0.4 μM of primers Eub338f (5'-ACTCCTACGGGAGGCAGCAG-3') and Eub518r (5'-ATTACCGCGGCTGCTGG-3') (Aleksova et al. 2020). The three-step thermal program was used at 95 °C for 5 min,45 cycles of denaturing at 95 °C for 10 s, annealing at 61 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. The plasmid DNA from the bacterial standard of Uncultured α – *proteobacterium* clone BuhD-104 (FM877535.1) was used to generate a linear calibration curve of the threshold cycle versus the gene copy numbers of four-point serial decimal dilution – 10^3, 10^5, 10^6 and 10^8. All measurements were run in triplicates. Data were expressed as gene copy numbers per gram of dry soil, and the specificity of amplicons was confirmed by melting curve and agarose gel electrophoresis. Amplification efficiency was estimated to be 109% (R^2=0.99). The results were processed via ROTOR-GENE Q SERIES, Software version 2.3.1.

The fungal abundance was estimated using 0.4 μM primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The following thermal program was used: 95 °C for 5 min, 45 cycles of 95 °C for 30 s, annealing 55 °C for 30 s, 72 °C for 60 s, and final extension at 72 °C for 10 min.

A four-point serial decimal dilution of a plasmid DNA of Uncultured *Basidiomycota* clone LS_Az0_D1_31 (MT785782.1) was used as a standard to generate a linear calibration curve of the threshold cycle versus a number of gene copies ranging from 10^2 to 10^5. All measurements were run in triplicates. Data were expressed as gene copy numbers per gram of dry soil. Amplification efficiency was estimated to be 92% (R^2=0.99). The results were analysed via ROTOR-GENE Q SERIES, Software version 2.3.1.

The relative ratio of fungi/bacteria was calculated as the ratio of the fungal gene copy numbers to bacterial gene copy numbers (Fierer et al. 2005).

Statistical analysis

Pearson correlation analysis was used to find the relationships between soil metrics. Hierarchical clustering (Algorithm: UPGMA; Similarity index: Bray-Curtis) was conducted to evaluate the distance between soil physical environments. Non-metric multidimensional scale (nMDS) ordination was applied to assess the similarity between abundance-weighted microbial communities. Statistical analyses were performed using the software PAST ($p<0.05$).

Results

Soil environmental variables

The values of studied soil properties are shown in Table 1. The soil pH was moderately acidic – pH 5.57 (S1 and S2) to 5.76 (S4). SOC ranged from 1.22 g kg^{-1} (S3) to 2.67 g kg^{-1} (S2). Concentrations of inorganic nitrate (except S3) and ammonium (except S2 and S4) ions and inorganic phosphates were relatively equally distributed among the soils.

Cu concentrations varied among the soils as follows: the concentrations in S1 and S3 exceeded the maximum permissible concentration (MPC) of 80 mg kg^{-1} under Bulgarian Regulation 3/2008 (2008) (http://eea.governement.bg/bg/legislation/soil). The most contaminated soils are located near the tailing of mine "Medet" in the valleys of the Medetska and Pirdopska Rivers. In the other soils, S2, S5 and S4, the concentrations of Cu were under or around MPC. In this regard, S5 can be assumed as a reference site.

Table 1. Soil physicochemical properties and Cu concentrations in the region of Topolnitsa River and its tributaries.

Soil sample	Soil physicochemical properties						
	pH	WtC (%)	SOC g kg^{-1}	NO_3-N mg kg^{-1}	NH_4-N mg kg^{-1}	P_2O_5 mg kg^{-1}	Cu mg kg^{-1}
S1	5.57	13.33	2.56	11.44	7.82	2.93	198.90
S3	5.60	11.67	1.22	0.65	8.32	2.37	110.00
S4	5.76	12.33	2.16	8.84	5.74	3.40	82.40
S2	5.57	12.33	2.67	11.12	3.07	3.47	67.40
S5	5.61	10.00	2.05	†ND	10.74	2.78	28.50

†ND – No data.

Pearson correlation analysis indicated a linear relationship (-0.61; p=0.015) between the level of soil Cu contamination and the distance to the source of pollution.

A cluster analysis of soil abiotic factors was conducted to visualize the similarity among the tested soils (Fig. 2).

Two main clusters were demonstrated: cluster I containing soil S5 with the lowest Cu concentration and cluster II, consisting of other soils sub-clustered according to the Cu threshold concentration of 100 mg kg^{-1}. This pattern of soil clustering identified the major role of Cu in structuring soil environments.

Soil bacterial abundance

Bacterial abundance varied in a relatively narrow range. The highest number was observed in S4 (3.24×10^{11}), and the lowest one in S5 (1.68×10^{11}) (Fig. 3). Pearson correlation analysis did not indicate a significant correlation (0.31; p=0.26) between bacterial abundance and soil Cu concentrations.

Soil fungal abundance

The highest fungal abundance was detected in uncontaminated soil S5 (6.71×10^{8}) and the lowest one in S3 (1.95×10^{8}) (Fig. 4). The fungal gene copy number variance among the sampling soils was higher (Coef. variance – 40.48) than that of bacterial gene copy number (Coef. variance – 25.07), indicating possible resistance of bacteria to Cu toxicity.

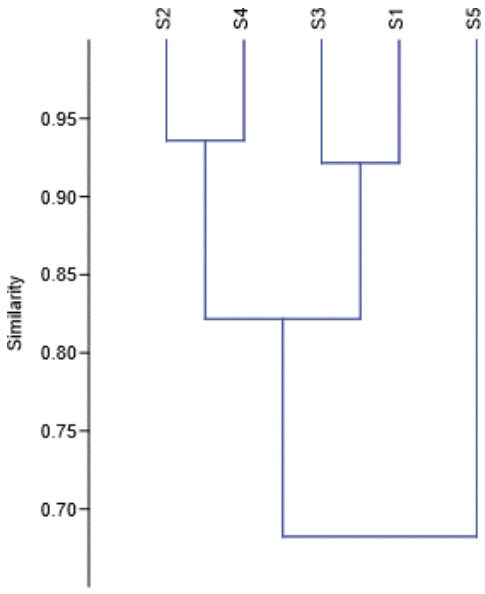

Figure 2. Cluster analysis of soil physical environments.

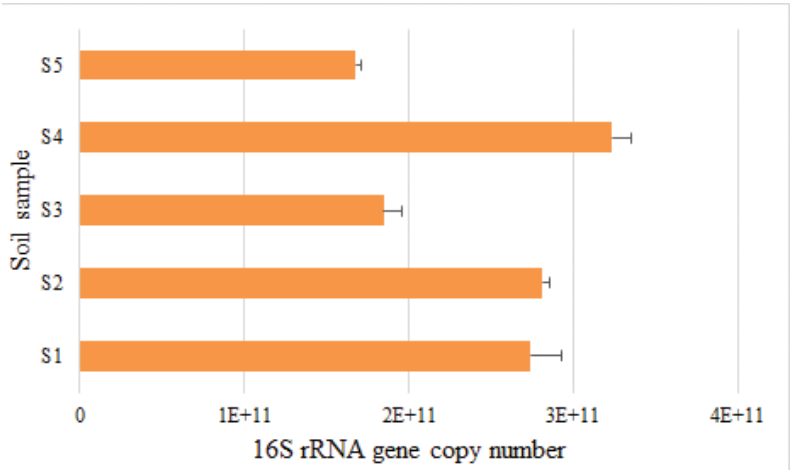

Figure 3. A bacterial abundance of 16S rRNA gene. Error bars are the standard deviations of the mean for the three replicates.

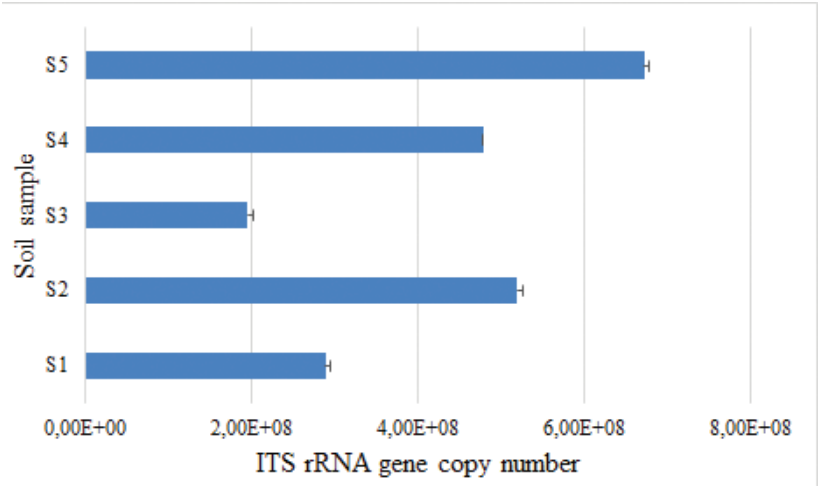

Figure 4. An abundance of ITS rRNA gene. Error bars are the standard deviations of the mean for the three replicates.

Pearson correlation analysis showed a significant negative correlation (-0.76; p=0.001) between fungal abundance and soil Cu contamination.

The highest relative fungal to bacterial abundance was observed in the uncontaminated soil S5 (3.98×10^{-3}), and the lowest ratio was recorded in the highly contaminated soil S3 (6.80×10^{-4}) (Fig. 5). Fungal to bacterial ratio related negatively with soil Cu (-0.66; p=0.007), probably as a result of the higher Cu impact on the fungal abundance.

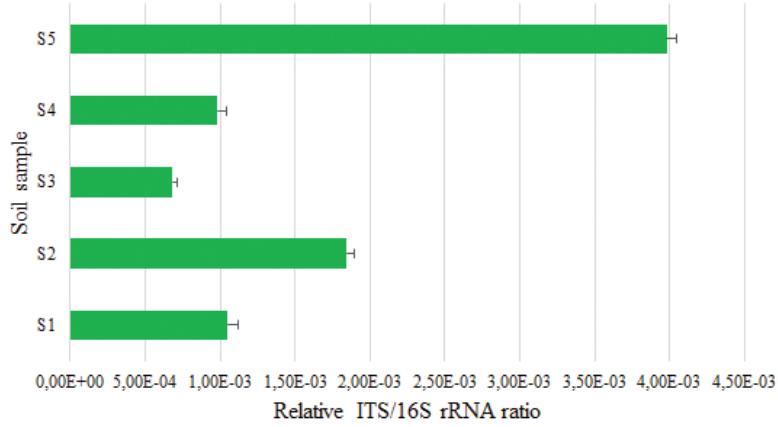

Figure 5. Relative fungal: bacterial ratio in the studied soils. Error bars are the standard deviations of the mean for the three replicates.

Relationships between microbial abundance, soil properties and Cu concentrations

Statistical non-metric multidimensional scaling (nMDS) was performed on patterns of fungal and bacterial abundances to assess soil similarities with respect to their microbiology (Fig. 6).

Plots separated the soils into four distinct abundance-weighted microbial communities The soils S2 and S4 grouped closely, indicating similar microbial abundances. The other soils were placed individually both away from the other and from the group S2 and S4. This distribution was probably due to differences in microbial abundance-weighted communities. Our results showed that the important factors influencing soil microbial abundance were soil Cu concentration and water content in S1, and soil organic carbon and phosphates concentrations in S2 and S4 (Fig. 6).

Discussion

The present study was focused on the distribution of microbial abundance as a response to the influence of Cu contamination in the area of Topolnitsa-Pirdop valley – a highly industrialized zone with a number of mines and processing plants for Cu and other non-ferrous metals.

The soil concentrations of the main contaminant Cu varied from 28.5 mg kg^{-1} to 189.9 mg kg^{-1}, being above MPC in three (S1, S3, and S4) of five tested soils. The mode of clustering highlighted the significant effects of Cu on soil environments, grouping soils in clusters or sub-clusters depending on the level of contamination. Additionally, soil pH (acidic) may influence soil microbial communities by primarily suppressing the bacterial diversity (Fierer and Jackson 2006) and increasing the mobility/toxicity (Wang et al. 2022) of HMs, including Cu. We expected also a high manifestation of Cu toxicity, taking into account the low levels of SOC (Chen et al. 2014).

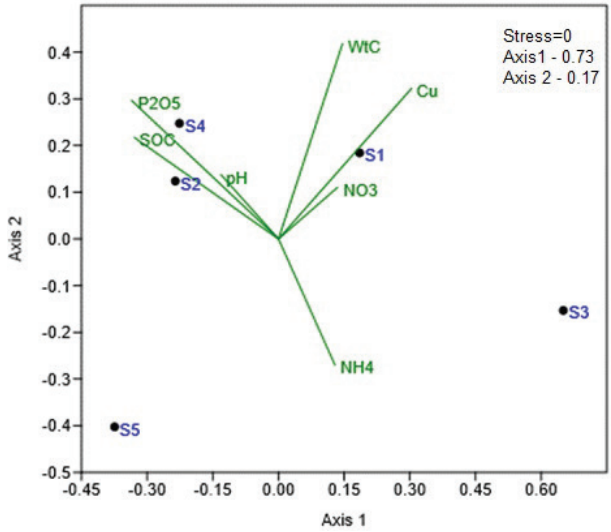

Figure 6. Microbial abundances in soils of Topolnitsa-Pirdop valley as indicated by non-metric multi-dimensional scaling plots.

The results showed high bacterial abundance in soils, ranging from 1.68×10^{11} to 3.24×10^{11} 16S rRNA gene copy numbers, being one-fold higher compared to the abundances estimated in our previous studies on mining activities in the area, where the soil Cu concentrations ranged from 53 mg kg^{-1} to 860 mg kg^{-1} (Aleksova et al. 2020; Palov et al. 2020; Nikolova et al. 2022). We suggested that this difference resulted from the ranges of soil Cu concentrations, but also microbial abundances might be edaphic- and climatic-dependent. In this respect, our results confirmed the findings of Yin et al. (2015), that long-term contamination with high Cu concentrations can decrease soil bacterial abundance. In our study, Pearson correlation analysis showed a lack of significant correlation between bacterial abundance and soil Cu concentration, probably due to the relatively low levels and long history of soil contamination. Hence, we could assume that bacteria developed mechanisms for adaptation to the newly created soil environments, and relatively low Cu concentrations can even stimulate their proliferation (positive correlation between Cu and relative bacterial abundance) (Zhao et al. 2019).

Similar to other authors (Chen et al. 2014), we found that the fungal abundance was sensitive to heavy metal contamination stress in the opposite to bacteria, proved by the significant and negative relationship between fungi and Cu. We suggested that the different sensitivity of bacteria and fungi to Cu resulted from their patterns of microbial- and HM- distributions among soil fractions. Several authors reported that bacteria dominated in silt and clay fractions, whereas fungi inhabited mainly the coarse sand fraction (Kandeler et al. 2000; Sessitsch et al. 2001; Poll et al. 2003). Using the Nemerow pollution index in their study, Chen et al. (2014) concluded that HMs (Cu, Pb, Zn) and their bioavailable forms were distributed in the order: coarse sand > clay > silt > fine sand. The similar mode of fungi and Cu distribution among soil fractions might be interpreted as a prerequisite for the higher Cu toxicity compared to bacteria.

The higher exerted Cu toxicity on fungi reflected also on the fungal/bacterial ratio, which decreased with increasing soil Cu contamination. According to some authors (Rajapaksha et al. 2004; Chen et al. 2014), the fungal/bacterial ratio is a sensitive indicator for soil health and its decrease in Cu-contaminated soils is a sign of deteriorated soil quality. Chen et al. (2014) reported a similar pattern of decreasing fungal/bacterial ratios under HM contamination in their study.

Although microbial communities were differently abundance-weighted (except that of S2 and S4) according to the ordination procedure nMDS, it demonstrated that only the highest soil Cu concentration (189.9 mg kg^{-1}) influenced significantly soil microbial communities (S1). The abundances of soil microorganisms from the other sampling sites were under the influence of local soil abiotic factors.

Conclusion

The present study showed that microbial abundance, especially fungal, was significantly affected by long-term Cu contamination of Fluvisols. The Cu shifted microbial communities' structure towards bacteria, suggesting that in this case, bacteria could be better at developing Cu resistance than fungi. Further studies should be implemented to clarify microbial functional responses to Cu.

Acknowledgements

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