Ecotoxicology and Environmental Safety 192 (2020) 110264

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Alteration of enzyme activities and functional diversity of a soil contaminated with copper and arsenic

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ARTICLE INFO

Keywords:
Biochemical properties
Heavy metals
metal(loid)s
Toxic elements
Physiological profile
Ecotoxicology

ABSTRACT

Copper (Cu) mining has to address a critical environmental issue related to the disposal of heavy metals and metalloids (HMs). Due to their deleterious effects on living organisms, Cu and arsenic (As) have gained global attention, and thus their monitoring in the environment is an important task. The aims of this study were: 1) to evaluate the alteration of soil enzyme activities (EAs) and soil microbial functional diversity with Cu/As contamination, and 2) to select the most reliable biochemical indicators of Cu/As contamination. A twelve-week soil experiment was performed with four increasing levels of Cu, As, and Cu/As from 150/15 to 1000/100 mg Cu/As kg^{-1}. Soil enzyme activities and soil community-level physiological profile (CLPP) using MicroResp™ were measured during the experiment. Results showed reduced EAs over time with increasing Cu and Cu/As levels. The most Cu-sensitive EAs were dehydrogenase, acid phosphatase, and arylsulfatase, while arginine ammonification might be related to the resilience of soil microbial communities due to its increased activity in the last experimental times. There was no consistent response to As contamination with reduced individual EAs at specific sampling times, being urease the only EA negatively affected by As. MicroResp™ showed reduced carbon (C) substrate utilization with increasing Cu levels indicating a community shift in C acquisition. These results support the use of specific EAs to assess the environmental impact of specific HMs, being also the first assessment of EAs and the use of CLPP (MicroResp™) to study the environmental impact in Cu/As contaminated soils.

1. Introduction

Soils contaminated with heavy metals and metalloids (HMs) are found worldwide due to anthropogenic activities (Chibuike and Obiora, 2014); hence this pollution is a global environmental issue (Chandrasekaran et al., 2015). The release of HMs into the environment through human activities has been reported to be dangerous to ecosystems as well as to their inhabitants (de Vries et al., 2013). Permissible levels of HMs in soil are usually based on total concentrations but this does not necessarily represent biological availability or potential toxicity, which are important when assessing environmental impacts in terms of biological effects (Stazi et al., 2015). Thus, monitoring procedures that reflect biological availability and toxicity are necessary in order to evaluate environmental impacts and success of suitable remediation technologies applied to contaminated soils (Korkina and Vorobeichik, 2016). In this context, there are clear effects of HM contamination on soil biota (D'Ascoli et al., 2006; Oves et al., 2016). Nonetheless, it is well-recognized that there are still some limitations in the use of soil biological properties as indicators of HM impacts reflecting variable responses, interactions with other soil factors and difficulties with analytical methods that may produce contradictory results (Bünemann et al., 2018).

Enzyme activities (EAs) can be useful indicators of soil changes and perturbations (Bastida et al., 2008), although results related to the impact of HMs on enzymes are sometimes ambiguous (Ciarkowska, 2015). Moreover, most recent studies have focused on the effects of only four metals (Cd, Pb, Cu, and Zn) with other HMs such as Ni and Cr studied less frequently and V, Co, As, Hg, Ag, Mn, and Se rarely studied

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https://doi.org/10.1016/j.ecoenv.2020.110264
Received 28 October 2019; Received in revised form 22 January 2020; Accepted 29 January 2020
Available online 05 February 2020
0147-6513/

(Stazi et al., 2015). Additionally, despite results showing the inhibition of soil enzymes with HM contamination (D'Ascoli et al., 2006), enzymes are influenced by other soil factors such as pH (Dick, 2011), clay content (Tietjen and Wetzel, 2003), humus fraction and organic matter (Dick, 1997). Thus, EAs can respond to HMs in different ways depending on soil conditions and perturbation. As an example, D'Ascoli et al. (2006) studied the effects of chromium (Cr) and Cu on EAs and found negative correlations between the activities of dehydrogenase, arylsulfatase, and acid phosphatase with Cr fractions. However, soil organic matter was able to mask the Cu effect on EAs.

The most used EAs in studies related to HM contamination are dehydrogenase, urease, alkaline and acid phosphatases, catalase, and arylsulfatase. These enzymes have shown consistent results as indicators; however, they can be affected by analytical errors or soil factors as described above. There is little information about how EAs respond to metal mixtures such as Cu and As combinations in soils; thus, it is important to consider the complex dynamic between Cu/As which can influence bioavailability, toxicity and effects on living organisms. Soil enzymes do not reflect all aspects of soil microbial activity and function, by which it is necessary to look at other approaches to gain a fuller understanding of the impacts of HMs on microbial communities. In this context, community-level physiological profiling (CLPP) reflecting utilization of carbon (C) substrates may complement enzyme responses providing further insights into soil microbial functional diversity and community structure. This study presents the first approach to CLPP under Cu/As contamination using MicroResp™ system. Based on the above, this study aimed to compare the impacts of Cu/As contamination on EAs and soil microbial functional diversity.

2. Materials and methods

2.1. Site description and soil sampling

A soil composite sample (0–20 cm depth) was collected from the Puchuncaví Valley in a site low-impacted by industrial activities, specifically at the "Maintencillo" location (32°40′57.25″ S; 71°25′42.67″ W) where, according to González et al. (2014), the total soil Cu content ranges from 111 to 200 mg kg^{-1}. The soil was 2 mm sieved and physicchemically characterized (Table 1) as described below. This soil was spiked with Cu ($CuSO_4.5H_2O$; grade: ACS, ISO, Reag. pH. Eur; Merck, Chile) and As ($HAsNa_2O_4$; ≥98.0%; Sigma-Aldrich, Chile) based on the existing total content of these elements in the soils to establish the following treatments, with three replicates for each sampling times: 1) Cu; 150, 300, 500, 1000 mg Cu kg^{-1}; 2) As; 15, 30, 50, and 100 mg As kg^{-1}; 3) Cu/As; 150/15, 300/30, 500/50, 1000/100 mg Cu/As kg^{-1} and 4) non-spiked soil (for an overall graphical representation of the experimental design, please see Fig. S1).

Table 1
Soil composite sample characterization.

pH	5.55
EC (μS cm^{-1})	133.27
WHC (%)	41.74
TOC (mg kg^{-1})	17.16
TP (mg kg^{-1})	870.93
AP (mg kg^{-1})	24.13
Cu_{DTPA} (mg kg^{-1})	10.10
As_{Water} (mg kg^{-1})	<0.01
Cu_{Total} (mg kg^{-1})	89.00
As_{Total} (mg kg^{-1})	1.47
Zn_{Total} (mg kg^{-1})	47.00
Se_{Total} (mg kg^{-1})	0.06
Cd_{Total} (mg kg^{-1})	<0.01
Mo_{Total} (mg kg^{-1})	0.01
Pb_{Total} (mg kg^{-1})	2.69
Texture	Sandy Loam

EC = electrical conductivity, WHC = water holding capacity, TOC = total organic carbon, TP = total phosphorus, AP = available phosphorus.

2.2. Spiking process

The spiking process was made according to Ginocchio et al. (2006) to promote Cu and As stabilization with soil matrix. Briefly, 1,7 kg of soil was mixed with the Cu and As sources in a plastic container with a capacity of 3 L, the moisture content was adjusted to 15% of their water holding capacity (WHC) with deionized water. The spiking was carried in three stages, by which the mixtures were: 1) placed in a roller that was manually turned every day for seven days, 2) air-dried for seven days, and 3) rehydrated with deionized water and mixed again for seven days. After the spiking process, a sequential extraction according to Tessier et al. (1979) was performed to evaluate the Cu speciation (Fig. S2). 500 g of each soil was placed in triplicate plastic pots for each treatment (Fig. S1) and kept under greenhouse conditions (25 ± 3 °C day 15 ± 3 °C night, a photoperiod of 16 h light and 8 h dark, and relative humidity of 80–90%). The mixtures were daily hydrated with deionized water to maintain 15% WHC, based on their initial weight. Soil samples were taken and stored in hermetic plastic bags and kept in at 4 °C prior to the measurement of EAs and CLPP. A sample fraction was air-dried for physicochemical analysis. Samples were taken three times at 4 $^{-}$week intervals. Thus, the experimental design is a full factorial split-plot with three levels for Time (4, 8, and 12 weeks) and four levels of each HM contamination as described above.

2.3. Physicochemical characterization of the soil composite sample

The soil characterization was performed by the measurement of the following parameters: 1) pH and electrical conductivity (EC) according to Sadzawka et al. (2006) by the use of a pH electrode and a conductivity meter in a soil-deionized water solution; 2) WHC and moisture content by gravimetric method; 3) total organic carbon by potassium dichromate ($K_2Cr_2O_7$) acid oxidation and colorimetric measure of Cr^{+3} (Heanes, 1984; Sims and Haby, 1971); 4) total phosphorus (P) by acid digestion in a sulfuric-perchloric mixture (Murphy and Riley, 1962) and determination by the blue-molybdate method (Murphy and Riley, 1962); 5) available P by extraction with $NaHCO_3$ at pH 8,5 (Olsen et al., 1954) and determination by the blue-molybdate method (Murphy and Riley, 1962); 6) texture according to Bouyoucos (1962); 7) Cu availability by extraction with DTPA solution and atomic absorption measurement (Lindsay and Norvell, 1978); 8) soluble As fraction by a colorimetric method according to Dhar et al. (2004, see section 2.3); and 9) total content of Cu, As, Zn, Se, Cd, Mo, and Pb by acid digestion (U.S. EPA 3051A) and atomic absorption measurement (UNICAM 969). Colorimetric assessments were performed by a microplate spectrophotometer (Epoch BioTek).

2.4. Chemical analysis

Chemical measured variables in experimental samples (Table S1) were: 1) pH and EC; 2) moisture content; 3) Cu availability by DTPA extraction and atomic absorption measurement. DTPA extraction was used since chelating agents (e. g. DTPA and EDTA) are more effective in removing soluble HM-organic complexes that are potentially bioavailable (Bolan et al., 2014). The soluble As fraction was measured according to Dhar et al. (2004) after extraction with water by shaking for 24 h. In this method, As(V) and phosphate form a complex with reduced molybdate that strongly absorbs in the infrared, which does not occur with As(III). Thus, available As is quantified by the difference in absorbance between a pre-treated sample aliquot (to oxidize As(III) and show the absorbance due to P and As) and another sample aliquot pretreated to reduce As(V) (absorbance from P) (Dhar et al., 2004). The colorimetric assessment was performed by a microplate spectrophotometer (Epoch, BioTek).

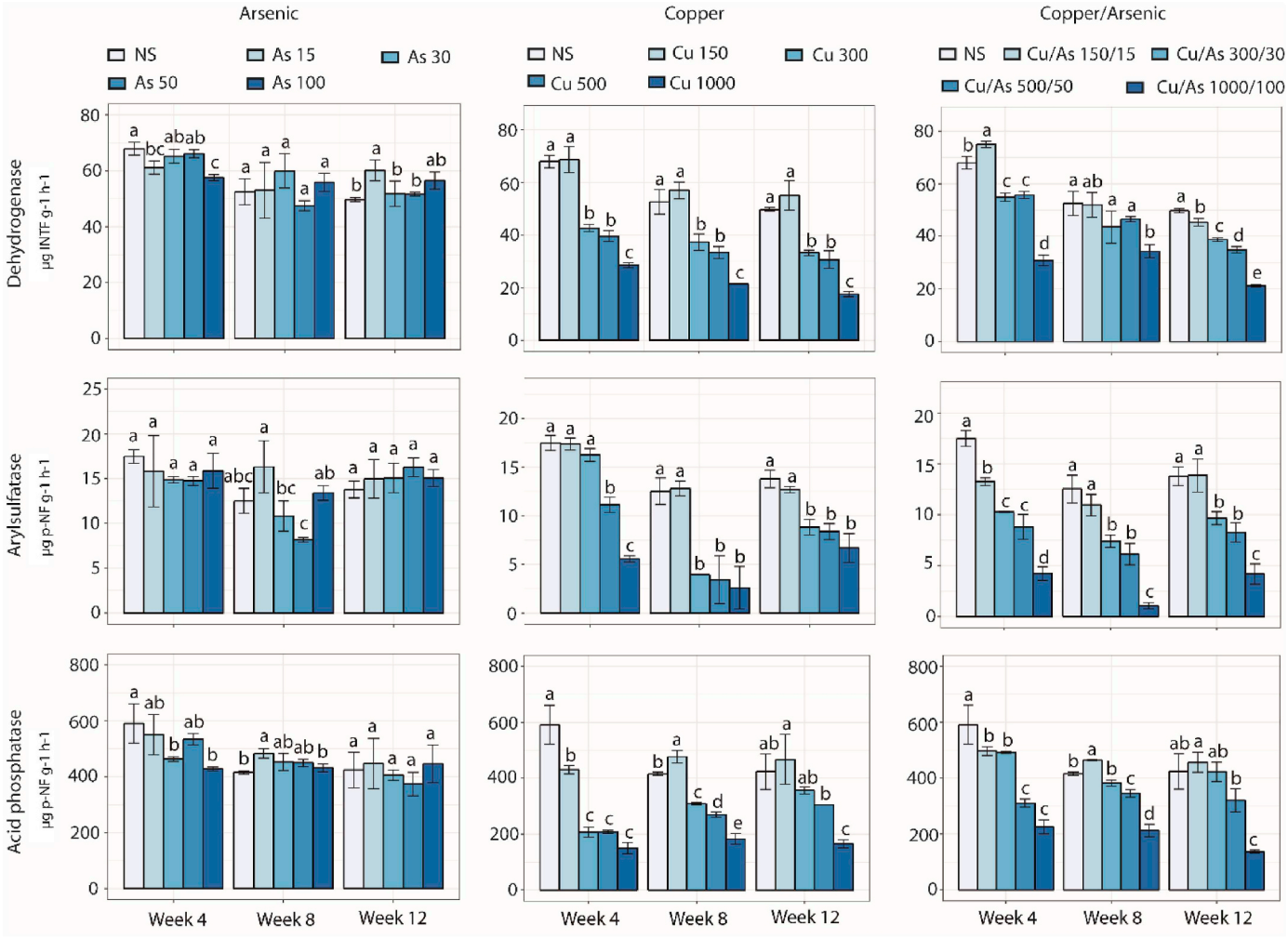

Fig. 1. Activity of dehydrogenase, arylsulfatase, and acid phosphatase for copper, arsenic, and copper/arsenic contamination per each sampling time. Different letters show significant differences according to Tukey HSD ($P < 0.05$). Copper and arsenic levels represent spiking content in mg kg^{-1}. NS = non-spiked soils.

2.5. Enzyme activities

EAs measured were the following: 1) dehydrogenase activity (DHA) by the INT method (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride), measuring the reduced INTF (iodonitrotetrazolium formazan) according to Camiña et al. (1998), 2) fluorescein diacetate (FDA) hydrolysis by the fluorescein produced after soil incubation with FDA (Schnurer and Rosswall, 1982); 3) acid phosphatase (Pacid) by *p*-nitrophenol released after soil incubation with *p*-nitrophenyl-phosphate (Tabatabai, 1994); 4) urease (UA) by ammonium determination (modified Bethelot reaction using salicylate and dichloroisocyanurate) after soil incubation with urea as substrate (Kandeler and Gerber, 1988); 5) arginine ammonification (AA) by ammonium released from the soil after incubation using arginine as substrate (Alef and Kleiner, 1986; Kandeler and Gerber, 1988); and 6) arylsulfatase (ARY) according to Alef and Nannipieri (1995) where *p*-nitrophenol is released after incubation with *p*-nitrophenyl-sulphate. EAs were promptly measured after every sampling time using a microplate spectrophotometer (Epoch BioTek) except for the acid phosphatase, which was measured in a UV-V spectrophotometer (Helios Alfa) to avoid reading errors due to the presence of precipitates.

The ecological dose (ED_{50}) was calculated to quantify the effects of Cu and As on EAs. ED_{50} shows the concentration of HMs at which the enzyme activity is reduced to 50% of its uninhibited value (Tejada et al., 2008). ED_{50} was calculated according to Knezevic et al. (2007) using the package "drc" in R statistic version 3.5.1.

2.6. Microbial community-level physiological profile (CLPP)

CLPP was measured using the MicroResp™ soil respiration system (MicroResp™, James Hutton Ltd, Aberdeen, UK) according to Campbell et al. (2003). MicroResp™ is a rapid and sensitive method for the determination of microbial community functional diversity (Chapman et al., 2007). This method is based on a microrespirometry system that combines the advantages of Biolog™ and Substrate Induced Respiration (SIR) using the whole soil, which is incubated with some C substrates that are ecologically relevant to soil (Campbell et al., 2003). In this study, the C substrates used were the following: α-ketoglutaric acid (AKGA); glucose (GLU); fructose (FRU); trehalose (TRE); malic acid (MAL); citric acid (CIT); L-arabinose (ARA); N-acetyl glucosamine (NAGA); protocatechuic acid (PRO); oxalic acid (OXA); L-arginine (ARG); γ-aminobutyric acid (GABA); cysteine (CYS); α-cyclodextrin (ACYC); lysine (LYS); and water (WAT). The emission of CO_2 by soil microorganisms was estimated using a colorimetric method before and after 6 h of incubation at 25 °C. The colorimetric assessment was performed by a microplate spectrophotometer (Epoch, BioTek).

2.7. Statistical analysis

Results were analyzed for each HM factor; thus final data was split by Cu, As, and Cu/As. Kolmogorov-Smirnoff and Levene tests were applied for evaluating normality and homoscedasticity assumptions, respectively. Since variables met the previously mentioned

Table 2
Linear simple regression between enzyme activities and available copper (Cu-DTPA), and ecological dose (ED_{50}) for each enzyme for all experimental times.

Model	Week 4		Week 8		Week 12	
	R^2	ED_{50}	R^2	ED_{50}	R^2	ED_{50}
Dehydrogenase	0.78 ***	124	0.82 ***	225	0.81 ***	223
Acid phosphatase	0.60 ***	72	0.81 ***	216	0.82 ***	376
Arylsulfatase	0.97 ***	379	0.57 **	140	0.72 ***	128
Arginine ammonification	0.49 **	84	0.46 **	123	0.18	ns
Urease	0.50 **	91	0.21	ns	0.01	ns
FDA	0.08	ns	0.11	ns	0.12	ns

Regressions were only performed with data from individual Cu and As treatments. Significant codes are based on p-values as follows: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1. ED_{50} is expressed in mg Cu kg^{-1}.

assumptions, an ANOVA was applied for knowing the effect of each HM level followed by a HSD Tukey's test in cases where the ANOVA results were significant ($P < 0.05$). A Pearson correlation test was applied between all variables by each sampling time. Simple linear regressions were performed between available Cu and As with EAs. MicroResp™ results were analyzed using canonical analysis of principal coordinates (CAP), PERMANOVA, and heatmap-clusters. The analysis was performed in R statistic version 3.5.1 and PRIMER version 6.1.12.

3. Results

3.1. Enzyme activities

Results showed consistent results in the effects of Cu and Cu/As on all studied EAs (Fig. 1) except for FDA (Fig. S3). FDA results were highly variable for As and with significant effects of Cu and Cu/As only in Week 8, which generally decreased for all treatments at Week 12. Arsenic effects were also highly variable resulted on UA and AA with significant results varying inconsistently over treatments and sampling times (Table 2). Cu and Cu/As resulted in a significant decline in the activity of soil enzymes (DHA, ARY, and Pacid) with increasing HM levels. Responses of individual enzymes varied according to metal, concentration and sampling time.

DHA was negatively affected by increasing Cu contamination for each sampling time, decreasing in the following order: NS = Cu 150 > Cu 300 = Cu 500 > Cu 1000 (Fig. 1). Arsenic negatively affected DHA only on As 15 and As 100 compared to NS at Week 4 (Fig. 1). Cu/As contamination was similar to individual Cu effect at Week 4. Nevertheless, a negative effect was only found on Cu/As 1000:100 at Week 8. At Week 12, all Cu/As levels were different compared to NS and among them, which allowed detecting the following order NS > Cu/As 150:15 > Cu/As 300:30 > Cu/As 500:50 > Cu/As 1000:100 (Fig. 1). These trends agree with the negative correlation between available Cu and DHA (Fig. 2), where available Cu showed the highest negative relation with this EA.

Cu negatively affected to ARY at every sampling time (Fig. 1). At Week 4, ARY resulted in the following trend: NS = Cu 150 = Cu 300 > Cu 500 > Cu 1000. This response was similar for the rest sampling times; however, the negative effect was enhanced since Cu 500 (Fig. 1). Arsenic only affected ARY at Week 8 but this trend was highly variable (Fig. 1). Dual contamination negatively affected to ARY in each sampling time (Fig. 1). At Week 4, ARY decreased from 24% (Cu/As 150:15) to 76% (Cu/As 1000:100), compared to NS. At Week 8 and 12th, the trend was NS = Cu/As 150:15 > Cu/As 300:30 = Cu/As 500:50 > Cu/As 1000:100. Correlation between ARY and copper availability was negative for all experimental times ($r = -0.84$, Fig. 2).

Pacid decreased by Cu addition from 27% (Cu 150) to 75% (Cu 1000) at Week 4, compared to NS. At Week 8, the decreasing trend was similar to Week 4 except for Cu 150 that increased by 15%. At Week 12, Pacid only showed negative effects for Cu 500 and Cu 1000 (Fig. 1). Arsenic contamination only affected to Pacid in As 30 and As 100 levels at Week 4, which was absent for other sampling times (Fig. 1). Dual contamination negatively affected to Pacid in a trend similar to Cu treatment (Fig. 1). Correlation between Cu and Pacid was negative for all the experiments (Fig. 2).

Cu contamination had a negative effect on AA only at Week 4 with the following trend: NS = Cu 150 > Cu 300 = Cu 500 = 1000 (Fig. 3). At Week 8, a negative effect by Cu 300 and Cu 1000 was registered, with a decreasing of 17 and 19% compared to NS. At Week 12, Cu contamination showed a negative effect only in Cu 300. Arsenic supply did not affect to AA (Fig. 3) except for As 50 at Week 12. Dual contamination negatively affected to AA especially at Week 4 when the trend was as follows: NS > Cu/As 150:15 = Cu/As 300:30 > Cu/As 500:50 > Cu/As 1000:100. At Week 8, AA showed absences of differences except to Cu/As 500:50 and Cu/As 1000:100 that decreased 36 and 50%, compared to NS. At Week 12, a negative effect was registered in the highest level of dual contamination, which decreased 27% compared to NS. AA was negatively related to Cu availability in every experimental time (Fig. 2).

Copper only negatively affected to UA at Week 4 (Fig. 3), with the following trend: NS = Cu 150 > Cu 300 = Cu 500 = 1000 similar to AA. Arsenic negatively affected to UA with a decreasing from 17% (As 15) to 15% (As 100) at Week 4, compared to NS. At Week 8, the As effect was not clear, while at Week 12, a negative effect was registered on As 30, As 50, and As 100. Dual contamination negatively affected to UA at Week 4 and Week 12 with the same trend: NS > Cu/As 150:15 = Cu/As 300:30 = Cu/As 500:50 > Cu/As 1000:100. At Week 8, a UA decreasing was only registered in Cu/As 500:50 in relation to NS. Correlation between Cu and UA was negative ($r = -0.29$, Fig. 2). Additionally, UA was negatively correlated with available As(V) at Week 4 and Week 12 (Fig. 2); however, its correlation strength was low ($R^2 = -0.34$ and -0.37, respectively).

Linear simple regressions showed significant relationships between all enzymes (excluding FDA) and available Cu (Table 2). AA and UA

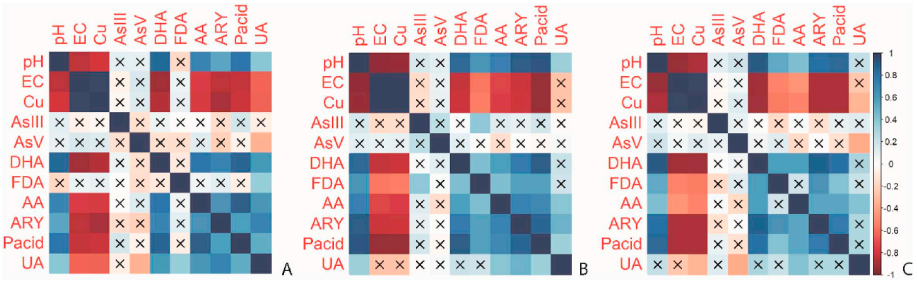

Fig. 2. Correlation between enzyme activities and physicochemical properties (complete correlation plots are shown in supplementary material) for Week 4 (A), Week 8 (B), and Week 12 (C). Crossed boxed represent non-significant correlations. Red boxes show negative correlation, while blue boxes show positive correlations. EC = electrical conductivity; Cu = available Cu (DTPA); AsIII = available As(III) (water extraction); AsV = available As(V) (water extraction; DHA = dehydrogenase; FDA = fluorescein diacetate hydrolysis; AA = arginine ammonification; ARY = arylsulfatase; Pacid = acid phosphatase; UA = urease. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

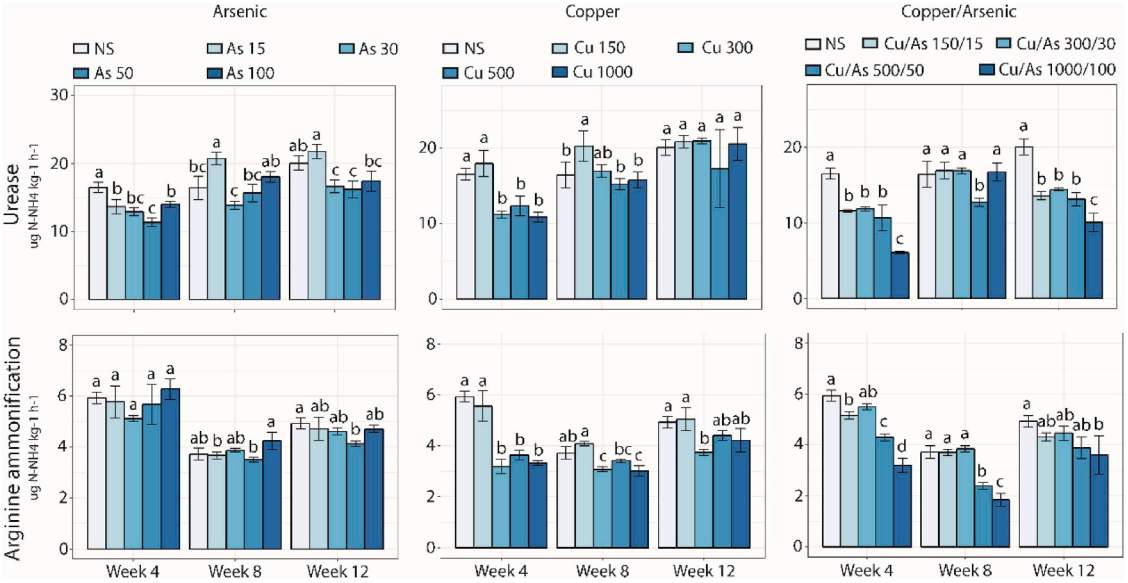

Fig. 3. Activity of urease and arginine ammonification for copper, arsenic, and copper/arsenic contamination per each sampling time. Different letters show significant differences according to Tukey HSD ($P < 0.05$). Copper and arsenic levels represent spiking content in mg kg^{-1}. NS = non-spiked soils.

were significant predictors at Week 4, which was progressively missed across the time. However, UA and AA were not able to predict available Cu concentrations at all experimental times. DHA, Pacid, and ARY were the most suitable enzymes to predict Cu availability across all experimental times with significant higher linear relations ($R^2 > 0.70$). Available As(V) was not significantly predicted by EAs for all experimental times. Only AA showed a significant relation with available As (V) at Week 12 (Table S2). Available As(III) was not analyzed as too many non-detectable values were obtained; however it was considered in correlation test by adding zeros in "non-detectable" cases.

At Week 4, ED_{50} showed significant values for available Cu and all EAs (except for FDA), especially for Pacid, AA, and UA, which were the most sensitive at Week 4 (Table 2). ED_{50} increased across time for DHA, Pacid, AA, and UA, and decreased for ARY. At Week 12, ED_{50} values showed the following trend: ARY < DHA < Pacid, which evidence a reduction of 50% of their uninhibited values at 128, 223, and 376 mg Cu kg^{-1}, respectively.

3.2. Community-level physiological profile (CLPP)

Carbon substrates were clearly affected by Cu and Cu/As treatments for all experimental times (Table S2), which allowed the CLPP discrimination of treatment levels (Fig. 4). Almost all the individual C substrates were negatively affected by increasing Cu and Cu/As concentrations (especially at Week 4, Figs. S4 and S6). However, the Cu individual effect decreased with time. At Week 12, the Cu was not significant in the middle contamination levels (Cu 150, Cu 300, and Cu 500), only showing clear discrimination between NS and Cu 1000 (Fig. 4). Moreover, at this time, dual contamination negatively affected the C substrate utilization more than Cu or As individually as reflected in the effect size in PERMANOVA (Table S2). Under As contamination, only AKGA, CIT, and MAL were negatively affected at Week 4 (Fig. S5). Nevertheless, at Week 8 and 12, the negative effect of As, especially at As 30 and As 50, increased for almost all C sources (Fig. S5). This last trend corresponds with negative correlations between available As(III) and the utilization of specific C sources at Week 8 (Fig. S7).

Heatmap-clusters for Week 4 show that individual C substrate utilization was separated into five groups as follows: 1) AKGA >2) GLU, FRU, and TRE >3) MAL, CIT, and ARA >4) NAGA, PRO, OXA, ARG, GABA, and CYS >5) ACYC, LYS, and WAT (Fig. 5A). In this context, the C sources in groups 1, 2, and 3 were more negatively affected by Cu contamination, showing negative correlation with available Cu and positive correlation with all EAs (Fig. S7). This trend changed at Week 8 with decreasing of utilization of all C sources (specially ARG) and the increasing use of CYS and OXA, which resulted in the following utilization trend: 1) AKGA > 2) GLU, TRE, and FRU > 3) CIT, MAL, OXA, ARA, and CYS > 4) PRO, NAGA, and GABA > and 5) LYS, ACYC, ARG, and WAT (Fig. 5B). The final heatmap-cluster (Week 12) ordered the C substrate utilization in the following trend: 1) AKGA > 2) GLU, FRU, and TRE > 3) CIT, MAL, ARA, OXA, and CYS > 4) PRO, NAGA, ARG, and GABA > 5) LYS, ACYC, and WAT (Fig. 5C). Thus, CYS and OXA were used more than other C substrates in high Cu levels compared to NS and As treatments since Week 8 until Week 12.

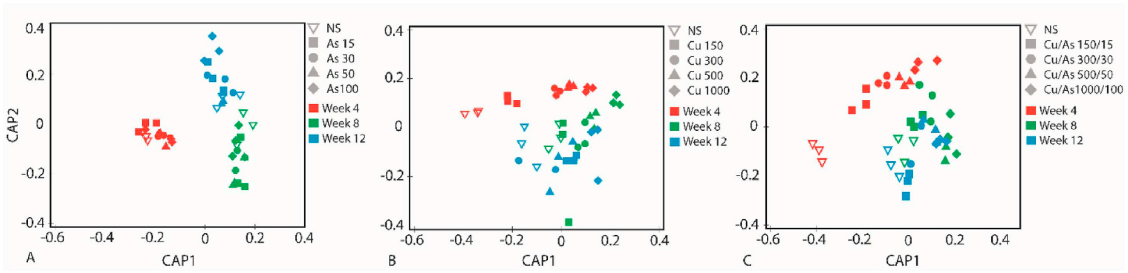

Fig. 4. Canonical analysis of principal coordinates (CAP) of community level physiological profile (CLPP) for As (A), Cu (B) and Cu/As (C) treatments by each experimental time. Cu and As levels represent spiking content in mg kg^{-1}. NS = non-spiked soils.

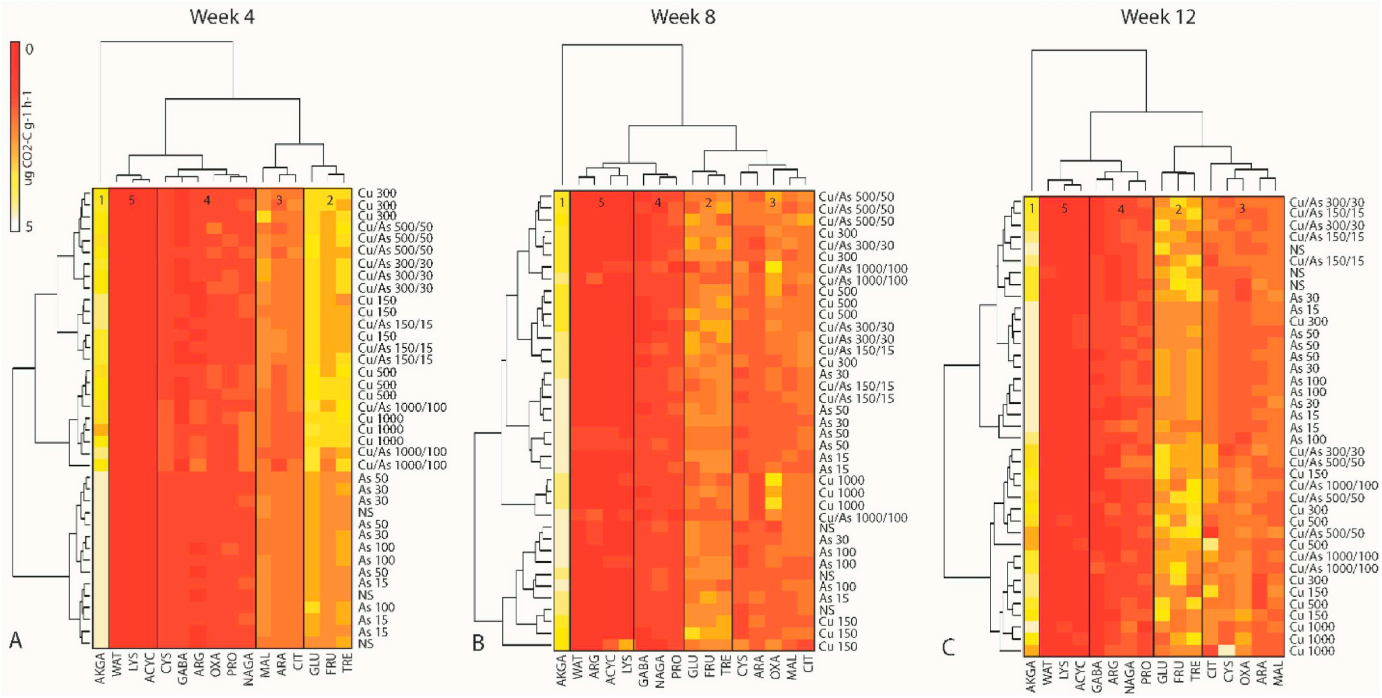

Fig. 5. Heatmaps and cluster of the community-level physiological profile (CLPP) by each experimental time: A) Week 4, B) Week 8, and C) Week 12. Black boxes show groups of C source utilization. The number inside the box represents the C utilization order from 1 (more used) to 5 (less used). Red represents low microbial respiration. White and yellow represent higher microbial respiration. ACYC = α-cyclodextrin, AKGA = α-ketoglutaric acid, ARA = L-arabinose, ARG = L-arginine, CIT = citric acid, FRU = fructose, GABA = γ-aminobutyric acid, GLU = glucose, LYS = lysine, MAL = malic acid, NAGA= N-acetyl glucosamine, OXA = oxalic acid, PRO = protocatechuic acid, TRE = trehalose, WAT = water. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

DHA has been used as a general indicator of soil microbial metabolic activity (Nannipieri et al., 1990) with this enzyme involved in the biological oxidation of organic matter (Zhang et al., 2010). In this study, DHA was inhibited by increasing Cu contamination at every sampling time, which allowed discrimination between most treatment levels. Thus, this enzyme activity can be considered as a good indicator of Cu contamination. These results agree with previous studies that have found a negative Cu effect on DHA (Mikanova, 2006; Wyszkowska et al., 2006). Fernández-Calviño et al. (2010) reported that evident DHA changes on Cu contaminated soil occurred since 150–200 mg Cu kg^{-1}, which is similar to ED_{50} results here shown. This enzyme represents the intracellular activity of microbiota, by which Cu negative effects are related to direct effects on microorganisms based on: 1) the displacement of essential metals and denaturation of proteins (Poole and Gadd, 1989); 2) redox cycling between Cu^{+2} and Cu^{+1} that catalyze the production of reactive hydroxyl radicals producing damage in lipids, proteins and DNA (Borkow and Gabbay, 2005); 3) Cu^{+2} has a specific affinity for DNA and disorders its helical structure by cross-linking within and between strands (Borkow and Gabbay, 2005), and 4) Cu high affinity for thiol and amino radical groups occurring in proteins (Letelier et al., 2005).

An important fact about DHA is that difficulties with analytical methods, relating to formazan color development at high Cu concentration, means past results are inconsistent in relation to high Cu content or Cu-contaminated sewage sludges (Chander and Brookes, 1991), although Chaperon and Sauvé (2007) considered DHA as a successful indicator of Cu toxicity. In this study, additional calibration curves were performed for each Cu level. These curves included soil extracts instead of water, from this test non-apparent differences were detected. Furthermore, DHA results represent the microbial activity response under Cu contamination, which is supported with positive correlations between DHA versus MicroResp™ results.

AA and UA were measured since their activities are related to soil N cycling. AA is an intracellular process that consists of NH_4^+ releasing from N-compounds, which are used as C and N sources. Thus, this ammonification is an indicator of soil microbial activity since plants do not use arginine as C source and animals carry out this process slowly (Alef and Kleiner, 1987). This EA has been scarcely used to assess HM contamination. Here, it was inhibited by Cu contamination; however, this effect was inconsistent with time. Thus, this biochemical property may be a potential indicator of Cu contamination in short timescales after contamination and possibly as an indication of soil microbial resilience to contamination. In this context, Khan et al. (2007) reported enzyme activity declining immediately after Cd and Pb contamination followed by recovery through time possibly due to the adaptation of microorganisms to contamination levels.

UA is responsible for urea hydrolysis to CO_2 and NH_3 breaking C–N bonds (Kandeler et al., 2011). UA responded similarly to AA, showing initial negative effects under Cu contamination that were not consistent through time. Dual contamination showed a greater negative effect suggesting a possible synergistic effect. Thus, this enzyme can only be considered as a “low to medium” sensitive indicator, similar to that reported by Niemeyer et al. (2012) in a Pb smelting area. This enzyme has been studied widely under HM contamination with differing responses from inhibition to activation (Fernández-Calviño et al., 2010; Lorenz et al., 2006; Renella et al., 2004). Furthermore, according to our results, AA and UA are preferable indicators of short-time Cu contamination due to their low discrimination through time. UA was the only EA correlated with available As(V) (Week 4 and 12). These last relations can be explained by As [especially As(III)] availability that was higher at these experimental times. HMs such as As interact with cysteine residues in the UA active site, especially with thiol groups, which inhibit the catalytic activity of this enzyme (Mazzei et al., 2018). Thus, this could be related to higher available As(III) concentration that

reached 3.42 mg As(III) kg^{-1} in As 50 (Week 4). However, available As (III) concentration was not correlated with EAs. The observed ED_{50} for urease was in the range reported by Doelman and Haanstra (1986). It is important to note that, in this study As concentrations are considerably lower than Cu concentrations. Since As-only treatments did not affect biological properties, with the exception of UA, it is evident that Cu/As treatments showed only a Cu effect. In this context, some studies have reported inhibition of enzyme activities by high As concentration in soil (Stazi et al., 2017), which are notably higher than As concentrations evaluated here. Thus, HM concentration used in bioassays is an important factor when dual or multi-contaminated soils are studied.

Phosphatase enzymes are involved in P mineralization from organic to inorganic forms; thus, they are important in P cycling (Speir and Ross, 1978). Pacid was inhibited by Cu and dual contamination. This trend coincides with other studies that have reported negative effect of Cu on this enzyme activity (Fernández-Calviño et al., 2010; Renella et al., 2003; Wang et al., 2007). The same trend was found in ARY. This enzyme catalyzes the hydrolysis of sulphate aromatic esters that are constituents of fungal cell walls, by which it could be used as an indirect indicator of soil fungal biomass (Moscatelli et al., 2005). This last EA has been found as a good indicator of HM contamination since it is sensitive to this perturbation (Mikanova, 2006). Recently, Stazi et al. (2017) found a decrease of 45% in ARY activity in As contaminated soils compared to non-contaminated soils. ARY can be inhibited due to its interaction with glycine, aspartic acid, and histidine in the ARY active site. The ED_{50} for Pacid (376 mg Cu kg^{-1} at Week 12) was similar to results reported by Effron et al. (2004), who found that this enzyme was inhibited by 250 and 500 mg Cu kg^{-1} with a linear response until 2500 mg Cu kg^{-1}. Moreover, Pacid can be negatively affected by HMs such as Cu due to interaction with serine and arginine residues in the phosphatase active site, which inhibits its activity. Both Pacid and ARY can be considered as good indicators of Cu contamination as a representation of soil microbial activity. Thus, these biochemical properties were negatively affected by Cu availability and positively related to C-substrate utilization.

Community-level physiological profiling (CLPP) provides information on the functioning of soil microorganisms involved in the C cycle (Hinojosa et al., 2010) with MicroResp™ enabling the assessment of catabolic activity in the microbial community (Stazi et al., 2017). This method has been compared to the Biolog™ using soils treated with sludge enriched with HMs (Campbell et al., 2003) and demonstrates that a whole-soil method is more rapid and detects C substrate use faster. In this study, multivariate analysis of C substrate utilization was an effective method to differentiate between Cu, As, and dual contamination and treatment levels. More recalcitrant C substrates were less affected by HM contamination, which suggests that some specialized microorganisms were non-affected. This trend is similar to results reported by Kenarova et al. (2014) and Stazi et al. (2017). In this sense, the soil microbial community used C substrates in the following order: carbohydrates > carboxylic acids > phenolic acids > polymeric compounds. However, a shift in the use of CYS and OXA from Week 8–12 was observed for Cu and Cu/As treatments. This reflects changes in the microbial catabolic activity and functional diversity possibly associated to changes in community structure related to the availability and turnover of these compounds. Thus, CYS and OXA were preferentially utilized by microorganisms at higher Cu and Cu/As contamination levels at Week 8 and 12 compared to Week 4. Therefore, reduced catabolic capability of microorganisms may reflect stress conditions due to Cu and As contamination but also due to reducing availability of organic matter and nutrients across time. Thus, a higher energetic demand was produced, which was buffered by the metabolization of a few "secondary" C substrates. This trend agrees with Yang et al. (2015) who found that CYS was preferred compared to ARG in the exponential phase of *Escherichia coli,* which was later balanced (the same use for CYS and ARG) in the stationary phase, as similarly occurred in this study. In this context, an influence of incubation times was also observed. Some enzyme activities and all the C substrate utilization showed a general decrease from Week 8 to Week 12, which might be related to a decrease in microbial biomass and activity due to non-input of organic matter and nutrients as mentioned above. Pan and Yu (2011) reported decreasing microbial populations with increasing Cd/Pb concentration soils, which also decreased with experimental times. Similarly, Khan et al. (2010) evidenced decreasing microbial population groups across experimental times in Cd/Pb contaminated soils, which was associated to metal toxicity and unavailability of nutrients, for the whole incubation period.

5. Conclusions

The most suitable indicators of Cu contamination were arylsulfatase, dehydrogenase, and acid phosphatase activities due to: 1) they were significantly inhibited during all experimental times, 2) showed consistent negative correlations with available Cu, 3) were the best predictors of available Cu, and 4) showed low to medium ED_{50} values. Our results confirm the negative effect of Cu and As on microbial functionality represented by means of the changes in the community level physiological profile (CLPP), which also suggests the utilization of the most sensitive EAs and CLPP (MicroResp™) as indicators of HM contamination. These results represent the first approach to CLPP under Cu/As contamination using MicroResp™ system. Thus, future researches should tackle the effect of individual and multiple HMs -in addition to Pb, Zn, Cd, and Cu-on soil microbial activity through the study of some EAs and other biological properties that show a fuller functioning of soil (e.g. CLPP-MicroResp™), which is necessary to be performed in laboratory experiments and field conditions.

CRediT authorship contribution statement

Humberto Aponte: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Wence Herrera:** Investigation, Data curation, Writing - original draft. **Clare Cameron:** Investigation, Formal analysis, Visualization, Writing - review & editing. **Helaina Black:** Conceptualization, Resources, Writing - review & editing, Supervision. **Sebastian Meier:** Conceptualization, Resources, Writing - review & editing, Supervision. **Jorge Paolini:** Conceptualization, Methodology, Writing - review & editing. **Yasna Tapia:** Resources, Supervision. **Pablo Cornejo:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Acknowledgements

The authors would like to acknowledge the support of the CONICYT 21160049 doctoral scholarship and the FONDECYT 1170264 grant. H. Aponte was supported in The James Hutton Institute by the MECE-UFRO project No. FRO1756. P. Cornejo also thanks CONICYT/FONDAP/15130015 and CONICYT project No. ACM170002. H. Black and C Cameron are supported by the Rural & Environment Science & Analytical Division of the Scottish Government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110264.

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