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**DEVELOPMENT OF SOIL QUALITY INDICES BASED
ON BIOLOGICAL INDICATORS TO ASSESS CHEMICAL
SOIL DEGRADATION BY METAL(LOID)S IN SOILS
NEAR TO A COPPER SMELTER**

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“DEVELOPMENT OF SOIL QUALITY INDICES BASED ON BIOLOGICAL INDICATORS TO ASSESS CHEMICAL SOIL DEGRADATION BY METAL(LOID)S IN SOILS NEAR TO A COPPER SMELTER”

Esta tesis fue realizada bajo la supervisión del Profesor Guía de Tesis, Dr. Pablo Enrique Cornejo Rivas, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y ha sido aprobada por los miembros de la comisión examinadora.

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A mi familia y la memoria de mis padres

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Summary and thesis outline

Soil degradation by contamination with metals and metalloids [metal(loid)s] represents a widespread environmental threat mainly associated with industrial processes such as mining and thermoelectric activities. High metal(loid) contents can negatively affect biological processes that take place in soil, which can disturb ecosystem services that soil provides. Despite this, permissible levels of metal(loid)s in soil are usually based on total contents that do not represent potential toxicity, by which monitoring procedures that reflect biological availability and toxicity are necessary to evaluate environmental impacts by metal(loid)s on soils. In this context, soil quality assessment considers some ecologic topics such as soil multifunctionality, resistant, resilience and ecosystem services, which can be useful to evaluate disturbed soils such as metal(loid) contaminated soils. For this, soil biological properties such as enzyme activities (EAs) are good soil quality indicators due to their direct connection to functions related to C, N, P and S cycles and their sensitivity to metal(loid) contamination. Nevertheless, the development of soil quality indices based on biological indicators for metal(loid) contaminated soils has been scarcely studied.

In Chile, Cu mining is the main economic activity, which has negatively affected natural resources and inhabitants. For instance, in the Puchuncaví Valley, which is located in the V Region of Chile, are situated thermoelectrical plants, oil refineries and the “Ventanas” Cu smelter, which during the last five decades have generated a strong negative impact on soils and urban areas that surround it. Puchuncaví Valley was one of the first areas of Chile declared by the government as saturated with pollutants. Currently, this location has large areas with low plant cover and organic matter, extreme erosion, and soil with high-to-extreme metal(loid) contents. Despite this, the industrial impact on the functioning of microbial communities of soil from the Puchuncaví Valley has not been tackled, especially through the development of soil quality indices (SQIs), which represent a non-studied field in other Chilean locations according to literature.

This Doctoral Thesis aimed to develop SQIs based on biological indicators to assess the chemical degradation of soils by metal(loid)s. In Chapter I, a general introduction is displayed, showing the hypothesis and aims of this Doctoral Thesis. Chapter II provides an overview of the development of SQIs based on EAs in metal(loid) contaminated soils, and evaluates the effects of individual metal(loid)s and their combinations on the activities of the most common enzymes involved in the C, N, P and

S cycles. In this chapter, we showed that the main EAs used as indicators in metal(loid) contaminated soils are dehydrogenase (DHA), arylsulfatase (ARY), urease (UA), acid phosphatase (Pacid), alkaline phosphatase (Palk), and catalase (CAT). These EAs were sensitive to metal(loid) contamination, with DHA and ARY as the most sensitive indicators. In Chapter III, a meta-analysis found the activities of seven enzymes to decrease in response to soil metal(loid) contamination. Here, metal(loid) contamination linearly reduced the activities of all enzymes in the following order: ARY > DHA > β -glucosidase (Gluc) > UA > Pacid > alkaline phosphatase (Palk) > catalase (CAT). The activities of two endoenzymes: ARY (partly as exoenzyme) and DHA were the most negatively affected, which was two times greater than of exoenzymes. This reflects the much stronger impact of metal(loid)s on living microorganisms and their endoenzymes than on exoenzymes stabilised on clay minerals and organic matter. Additionally, increasing clay content weakened the negative effects of metal(loid) contamination on EAs, which also decreased with soil depth. EAs involved in the cycling of C and S were more affected by metal(loid)s than the enzymes associated with the cycling of N and P. Consequently, metal(loid) contamination may alter the stoichiometry of C, N, P and S released by enzymatic decomposition of organic compounds that consequently affect microbial community structure and activity.

Enzyme activities do not show all soil microbial functionality, by which they should be complemented with soil biological properties to get a fuller understanding of metal(loid) contamination on soils. In this sense, Chapter IV evaluates the alteration of soil EAs and soil microbial functional diversity by the community level physiological profile (CLPP, MicroRespTM) over time with Cu/As contamination. This allowed selecting the most reliable biological and biochemical indicators of Cu/As contamination. Results showed reduced EAs over time with increasing Cu and Cu/As levels. The most Cu-sensitive EAs were DHA, Pacid, and ARY. At the same time, arginine ammonification (AA) 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 UA the only EA negatively affected by As. On the other hand, MicroRespTM showed reduced carbon (C) substrate utilisation with increasing Cu levels, indicating a microbial community shift in C acquisition. These results support the use of EAs to assess the

environmental impact of metal(loid)s, being also the first assessment of EAs and the use of CLPP (MicroResp™) to study the environmental impact in Cu/As contaminated soils.

In Chapter V, we show the study of the effect of metal(loid) contamination on soil physic-chemical properties and microbial functionality, as well as the detection of suitable indicators of soil quality in metal(loid) contaminated soils at field conditions. EAs of DHA, ARY, Gluc, UA, and AA were negatively affected by increasing metal(loid) contamination; however, organic matter counterbalanced the negative contamination effects of metal(loid)s on EAs. DHA and AA showed significant ED₅₀ values with bioavailable Cu and total As content. Community-level physiological profile (CLPP) showed lower and different profiles for the utilisation of all applied C substrates, discriminating between some metal(loid) contamination degrees. Thus, some EAs and CLPP were suitable indicators of metal(loid) contamination in soils from the Puchuncaví Valley. This is the first assessment of EAs and CLPP (MicroResp™) to evaluate the environmental impact of metal(loid) contamination in soils of Chile.

In Chapter VI, some soil quality indices (SQIs) were calculated with previous results to assess the chemical degradation by metal(loid)s on soils. Five SQI approaches were here tested to compare their sensitivity and relationship with metal(loid) contamination. Biological soil indicators, especially CLPP, explained the most variability proportion in metal(loid) contaminated soils. The Multifunctionality assessment and the Area_{SQI} were suitable SQIs based on their higher discriminant power and relationship with metal(loid) contamination compared to other SQI approaches. Both SQIs were successfully validated with data from literature, with decreasing soil quality at higher metal(loid) contaminated soils. However, the Multifunctionality assessment showed a better response and fast and easy calculation. Also, SQIs here developed should be applied based on methodological aspects beyond specific indicators here selected. This study was limited to explain the variability due to metal(loid) contamination in the Puchuncaví Valley, by which other studies should assess the *in situ* variability to select the most appropriate soil quality indicators. This study represents the first approach of soil quality assessment, especially with SQI development, in metal(loid) contaminated soils in Chile, which can be used to monitor metal(loid) contamination or soil recovery. Finally, all the results here obtained are discussed comprehensively in Chapter VII of this Doctoral Thesis, and the most important conclusions and perspectives are presented.

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CHAPTER I. *General introduction*

1.1. General Introduction

Metal(loid)s [heavy metals and metalloids] occur naturally in soils mainly from weathering of parental material at trace levels ($<1000 \text{ mg kg}^{-1}$; Alloway, 2013), which are rarely found at toxic levels (Pierzynski et al., 2000). However, some anthropogenic activities, especially mining and smelting, have concentrated large metal(loid) amounts in soils (Chandrasekaran et al., 2015). Serious environmental and human health threats can arise when high bioavailable contents of metal(loid)s exist close to urban and agricultural areas, with regions of India and Mexico providing examples of such cases (Flores-Magdaleno, 2011; Kumar & Anbazhagan, 2018).

In soils, some metal(loid)s are required by living organisms at low quantities (Chibuike and Obiora, 2014) for several biochemical reactions (Kavamura and Esposito, 2010). Nevertheless, high metal(loid) contents lead to toxicity symptoms to microorganisms and plants (Oves et al., 2016; Prasad et al., 2004). Specifically, metal(loid)s are toxic to microorganisms due to they bind with more affinity to thiol-containing groups and oxygen sites of proteins than essential elements (Poole and Gadd, 1989). Also, metal(loid)s can displace essential elements from their binding sites in biomolecules or through ligand interactions (Bruins et al., 2000). In this context, elevated soil metal(loid) contents can result in alterations to the conformational structure of nucleic acids and proteins. It can also interfere with oxidative phosphorylation and osmotic balance (Poole and Gadd, 1989). Therefore, high metal(loid) contents can reduce soil microbial biomass along with genetic and functional diversity (Abdu et al., 2017; Gans et al., 2005; Kandeler et al., 1996), and enzyme activity (Bhattacharyya et al., 2008). Thus, metal(loid) contamination can negatively affect the soil and ecosystem functioning.

Based on the above, monitoring procedures that reflect biological functioning and metal(loid) toxicity are necessary to evaluate environmental impacts and success of remediation technologies applied to contaminated soils (Korkina and Vorobeichik, 2016). In this context, the soil quality concept can be a useful approach to assess the chemical soil degradation by metal(loid)s in soils since the soil quality objective has recently focused on the multifunctionality and resilience of soil along with the ecosystem services it provides (Bünemann et al., 2018). For this, suitable soil quality indicators are needed to explain the most variability proportion of soil as ecosystem. In this context, enzyme activities (EAs) can be useful indicators of soil quality (Bastida et al., 2008); however, results related to the impact of metal(loid)s on EAs are sometimes ambiguous

(Ciarkowska, 2015). Moreover, most recent studies have focused on the effects of only four metal(loid)s (Cd, Pb, Cu, and Zn) with other such as Ni and Cr studied less frequently and V, Co, As, Hg, Ag, Mn, and Se rarely studied (Stazi et al., 2015). Additionally, despite results showing the inhibition of soil enzymes with metal(loid) contamination (D'Ascoli et al., 2006), enzymes are influenced by other soil properties such as pH (Dick, 2011), clay content (Tietjen and Wetzel, 2003), humus fraction, and organic matter (Dick, 1997). Furthermore, the toxicity mechanism depends on the ionic form of the metal(loid) and its chemical speciation (Nies, 1999; Turpeinem, 2002). Some metal(loid)s have affinity for carboxyl and amine functional groups that are part of active sites of some enzymes. Thus, the exact effect on EAs ultimately depends on the amino acid composition of the active site of each enzyme (Geiger et al., 1998). Consequently, EAs can respond to metal(loid)s in different ways depending on soil conditions and perturbation. On the other hand, soil enzymes do not reflect all aspects of soil microbial activity and function, by which it is necessary to look at other approaches such as soil microbial respiration, biomass, and functional diversity, to gain a fuller understanding of the impacts of metal(loid)s on soil microbial communities.

In Chile, Cu mining is the main economic activity that represents about a third of global Cu production (Cornejo et al., 2008), being known its negative effects on the environment by metal(loid) discharges (González and Ite, 1992). Mining activity in Chile is concentrated in the North and Central regions (MMA, 2011), finding other contaminating activities such as thermoelectric, smelting, and cement production (MMA, 2011). According to De Gregori et al. (2003), important levels of Cu and As have been found in some locations of these Chilean regions, which represent an important issue to consider due to their environmental impact. An example of this is the Puchuncaví Valley, which is located in the V Region of Chile, Valparaiso Province. In this area are located numerous thermoelectrical plants, oil refineries and the Ventanas Cu smelter (CODELCO), which have generated a strong impact in many cities and towns that surround it (González et al., 2014). Puchuncaví Valley was one of the first areas of Chile declared by the government as saturated with pollutants (Decrete No. 346, Dec. 1993). Currently, this region has large areas affected by industrial activities, showing low plant cover, extreme erosion, and soil with high-to-extreme metal(loid) contents (De Gregori et al., 2003; González and Ite, 1992; Neaman et al., 2009). Despite this, the industrial impact on the functioning of microbial communities of soil from the Puchuncaví Valley

has not been tackled, especially through the SQI development, which has not also been performed in other Chilean areas.

Based on the antecedents mentioned above, we proposed the following hypotheses and objectives.

1.2. Hypotheses

- 1) Biological properties, such as enzyme activities and microbial functional diversity, will explain most of the variability proportion in metal(loid) contaminated soils, due to their high sensitivity against perturbations.
- 2) Metal(loid) contaminated soils will show a decreasing quality reflected by developed soil quality indices (SQIs), due to reduced values and negative alteration of biological properties with high metal(loid) contents, which will allow differentiating between metal(loid) soil contamination ranges.

1.3. General objective

To develop soil quality indices based on biological and biochemical properties to assess chemical degradation of soils by metal(loid)s.

1.4 Specific objectives

- 1) To evaluate the alteration of enzyme activities and their relationship with soil microbial functional diversity under Cu/As contaminated soils for selecting the most accurate soil enzyme indicators.
- 2) To study metal(loid) contamination effect on biological and physic-chemical properties of soils at field conditions.
- 3) To assess global variability proportion of biological and physic-chemical soil properties in metal(loid) contaminated soils and measure their quality through the development of soil quality indices.
- 4) To validate soil quality indices from a conceptual perspective.

CHAPTER II. *“Soil quality indices for metal(loid) contamination: An enzymatic perspective”*

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Soil quality indices for metal(loid) contamination: An enzymatic perspective

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Running title: Soil quality indices for metal(loid) contamination: enzymatic outlook

Abstract

Soil degradation by metal and metalloid [metal(loid)] contamination represents a widespread environmental threat. Most studies investigating soil metal(loid) contamination have disregarded the soil quality concept. Enzyme activities (EAs) are good soil quality indicators due to their direct connection to functions related to C, N, P and S cycles and their sensitivity to metal(loid) contamination. This review 1) provides an overview of the development of soil quality indices (SQIs) based on EAs in metal(loid) contaminated soils, and 2) evaluates the effects of individual metal(loid)s and their combinations on the activities of the most common enzymes involved in the C, N, P and S cycles. Four intracellular mechanisms dominate the inhibition of EAs by metal(loid)s: i) transcription inhibition, ii) protein denaturation, iii) cell division inhibition, iv) cell membrane disruption. These mechanisms can also be further exacerbated in soil by non-specific exoenzyme inhibition. The main EAs used as indicators for metal(loid) contaminated soils are dehydrogenase (DHA), arylsulfatase (ARY), urease (UA), acid phosphatase (Pacid), alkaline phosphatase (Palk), and catalase (CAT). These enzymes are sensitive to metal(loid) contamination, with DHA and ARY being the most sensitive indicators (62% and 56% inhibition, respectively, when averaged over all metal(loid) contamination levels). Other enzymes are inhibited by 32% (UA), 23% (Palk), 18% (Pacid), and 18% (CAT). We suggest principles for the development of SQIs considering biotic soil functions via the use of EAs. This review is the first presenting SQIs based on EAs for metal(loid) contaminated soils, and represents the first quantitative analysis of metal(loid) effects on EAs.

Keywords

Enzyme activities; Soil biochemical properties; Heavy metals; Potentially toxic elements; Pollution.

2.1. Introduction

High contents of metals and metalloids [hereafter referred to as “metal(loid)s”] in soil can adversely affect soil quality and alter the activity of microbial communities (Bruins et al., 2000; Gohre and Paskowski, 2006; Rathnayake et al., 2010). A range of indexes has been developed to assess soil quality, most of which have been proposed for agricultural soils, with the soil quality related primarily to crop yield at the expense of all other soil functions.

Aside from crop yield, enzyme activities (EAs) can be used as both soil quality indicators in numerical indexes (SQIs) and as a measure of soil metal(loid) contamination. Determination of soil EAs reflects processes directly related to the biological functioning of soil and is a relatively fast and cost-efficient form of analysis. Such uses of EAs can often be complicated by the inherent variability of EAs in response to other physicochemical soil properties (Bastida et al., 2008; Trasar-Cepeda et al., 2000). Additionally, there are very few reviews devoted to the effect of metal(loid)s on EAs, and a quantitative approach has never been undertaken. In line with these potential assets and limitations of the use of EAs as indicators of SQIs, this review provides 1) an overview of the development of SQIs based on EAs in metal(loid) contaminated soils, and 2) a quantitative evaluation of the effects of individual metal(loid)s and their combinations on the activities of the most common enzymes involved in the C, N, P and S cycles.

2.1.1. Metal(loid) contamination of soil and its distribution

Metals such as copper (Cu), cadmium (Cd), and lead (Pb), and metalloids such as arsenic (As), are naturally occurring elements present in soils primarily due to the weathering of parental materials (Alloway, 2013). Usually, background contents of these metal(loid)s in non-contaminated soils are at trace levels and therefore not sufficient to adversely affect the biological functions of microorganisms, plants, and animals (Pierzynski et al., 2000). However, global economic development, and associated industrial and agricultural activities, have resulted in soil metal(loid) contents reaching dangerous levels in many locations across the world. The anthropogenic enrichment of metal(loid)s in soils creates severe environmental pollution (Chao et al., 2014) and associated human health risks of global relevance (Figure 1).

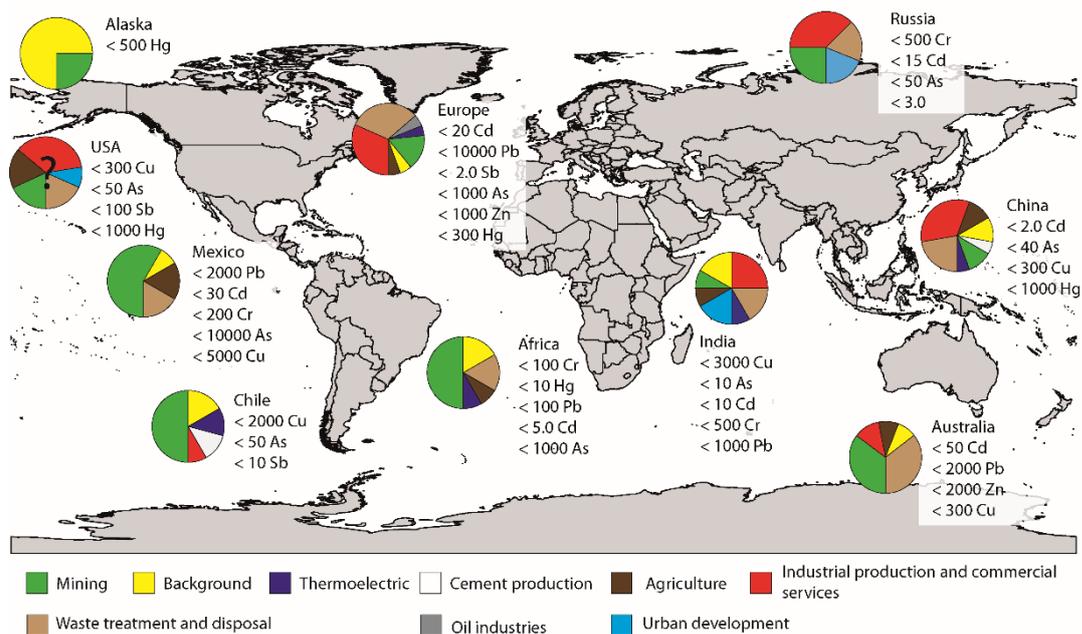


Figure 1. Overview map of metal(loid) contaminated soil regions at the continental level and their main sources (presented in colour). Values associated with each pie chart represent the total metal(loid) concentration (mg kg⁻¹ soil) in contaminated areas. Total metal(loid) concentrations were taken from papers or official reports. The proportion of the metal(loid) sources is based on reports from environmental agencies or information from scientific literature (the data of USA were not available). The references are listed in Supporting Information.

In Europe, the most polluted regions for Hg, Pb, Sb and Cd include southwestern Germany, southern Poland, southern Spain, and northern Italy (Gil et al., 2018; Suschka, 1993; Yewale et al., 2017). In Africa, As concentrations in drinking water are beyond the maximum acceptable limit (0.01 mg/L) in many countries including Botswana, Burkina Faso and Cameroon (World Health Organization, 2014). Arsenic contamination is also prevalent in Mexico, where several cities have As concentrations in drinking water beyond the maximum permissible limit of 25 µg/L (Hurtado-Jiménez and Gardea-Torresdey, 2006). In the central-north region of Chile, high contents of Cu, As and Sb [among other metal(loid)s] in soils have been caused by mining activities and erosion of As-rich epithermal ores (De Gregori et al., 2003; González et al., 2014; Oyarzun et al., 2004). In the same context, high soil metal(loid) contents have been found at several sites in Asia and Australia (Yewale et al., 2017). Serious environmental and human health

threats can arise when high bioavailable contents of metal(oids) exist in close proximity to urban and agricultural areas, with regions of Chile, India and Mexico providing examples of such cases (Cornejo et al., 2008; Flores-Magdaleno, 2011; Kumar and Anbazhagan, 2018).

It is therefore clear that metal(loid) contaminated soils are found in many regions worldwide (Figure 1) and are primarily driven by anthropogenic activities (Chibuike and Obiora, 2014), which include the rapid expansion of industrial areas, mine tailings, metal-rich waste disposal, and wastewater irrigation (Khan et al., 2008; Oves et al., 2016; Wuana & Okieimen, 2011). The release of metal(loid)s into the environment through human activities and their accumulation in soils can be dangerous to the ecosystems as well as to their inhabitants (Nouri et al., 2009), and may also alter the microbial processes that mediate the mobilisation of essential plant nutrients. Soil metal(loid) pollution is thus a global environmental issue (Chandrasekaran et al., 2015) and we refer the reader to other reviews for further specifics of metal(loid) contamination in various regions (Mahar et al., 2016; Yewale et al., 2017).

2.1.2. Effects of metal(loid)s on microorganisms

Metal(loid)s are toxic to microorganisms due to two primary mechanisms: 1) metal(loid)s bind with more affinity to thiol-containing groups and oxygen sites of proteins than essential elements (Poole and Gadd, 1989), and 2) the displacement of essential elements from their binding sites in biomolecules or through ligand interactions (Bruins et al., 2000). Other mechanisms resulting in metal(loid) toxicity to microorganisms are mainly cascading effects of these two. The exact toxicity mechanism is dependent upon the metal(loid) type, its ionic form and chemical speciation (Table 1). For instance, Hg^{2+} , Cd^{2+} , and Ag^{1+} tend to bind to $-\text{SH}$ groups, which inhibit the activity of sensitive enzymes (Nies, 1999; Turpeinem, 2002), whilst other metal(loid)s have affinity for carboxyl and amine functional groups that are a part of active sites of some enzymes.

Inhibition of EAs as a result of elevated metal(loid) contents can occur in several ways (D'Ascoli et al., 2006): i) masking of catalytical active groups; ii) denaturing protein conformation, or iii) competing with metal cofactors that are needed to form enzyme-substrate complexes (Gianfreda and Bollag, 1996). Additionally, metal(loid)s can inhibit enzyme production by depressing the expression of RNA (Kapoor et al., 2015).

Table 1. The main toxic mechanisms of metal(loid)s on microbial cells.

Metal(loid)	Dominant mechanisms	References
Pb	<ul style="list-style-type: none"> • Pb ions replace other bivalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations such as Na^+, and hinder cell metabolism. • The imbalance between the production of free radicals and the generation of antioxidants (oxidative stress). 	Jaishankar et al. (2014)
Cd	<ul style="list-style-type: none"> • Cd binds to cysteine, glutamate, histidine, and aspartate ligands, which lead to Fe deficiency. Mechanisms of Cd toxicity are not widely understood. 	Castagnetto et al. (2002) Patrick (2003)
Cu	<ul style="list-style-type: none"> • Cu displaces essential metals and denatures proteins. • Redox cycling between Cu^{2+} and Cu^{1+} catalyse the production of reactive hydroxyl radicals, which damage lipids, proteins and nucleic acids. • Cu^{2+} has a specific affinity for DNA and disorders its helical structure by crosslinking within and between strands. 	Borkow & Gabbay (2005)
As	<ul style="list-style-type: none"> • As mainly affects sulfhydryl groups in cells, causing malfunctioning of cell respiration, cell enzymes, and mitosis. • Arsenate (AsO_4^{3-}) interferes with phosphorylation and energy production due to its similarity with PO_4^{3-}. 	Gordon & Quastel (1948)
Hg	<ul style="list-style-type: none"> • Hg^{2+} interferes with the mitochondrial activity and induces oxidative stress by triggering the generation of reactive oxidative species. 	Messer et al. (2005)
Cr	<ul style="list-style-type: none"> • Detention of cells in the G-2 phase of the cell cycle and inhibition of respiration and photosynthesis. Cr(VI) induces alterations in the cytoskeleton which may be involved in motility loss. • Inhibition of oxygen uptake and induction of mutations. • Chromate (CrO_4^{2-}) also specifically targets the mitochondria of microorganisms. 	Bassi & Donini (1984) Cervantes et al. (2001) Henderson (1989)
Co	<ul style="list-style-type: none"> • Cobalt ions induce respiratory deficiency, as well as spindle inhibition and chromosomal anomalies. 	Komczynski et al. (1963) Lindegren et al. (1958)

Elevated soil metal(loid) contents can also result in alterations to the conformational structure of nucleic acids and proteins and can interfere with oxidative phosphorylation and osmotic balance (Poole and Gadd, 1989) – both contributing to the toxicity of these elements to microorganisms. Therefore, metal(loid) toxicity can cause:

1) protein denaturation; 2) inhibition of cell division; 3) cell membrane disruption; 4) inhibition of EAs; and 5) transcription and translation inhibition (Uslu & Hanay, 2017; Figure 2). Together these act to reduce microbial biomass along with genetic and functional diversity (Abdu et al., 2017; Gans et al., 2005; Kandeler et al., 1996), and EAs (Bhattacharyya et al., 2008). This review hereafter focuses upon on the effects of metal(loid)s on EAs, for which the five mechanisms of toxicity outlined above are of particular importance (Figure 2).

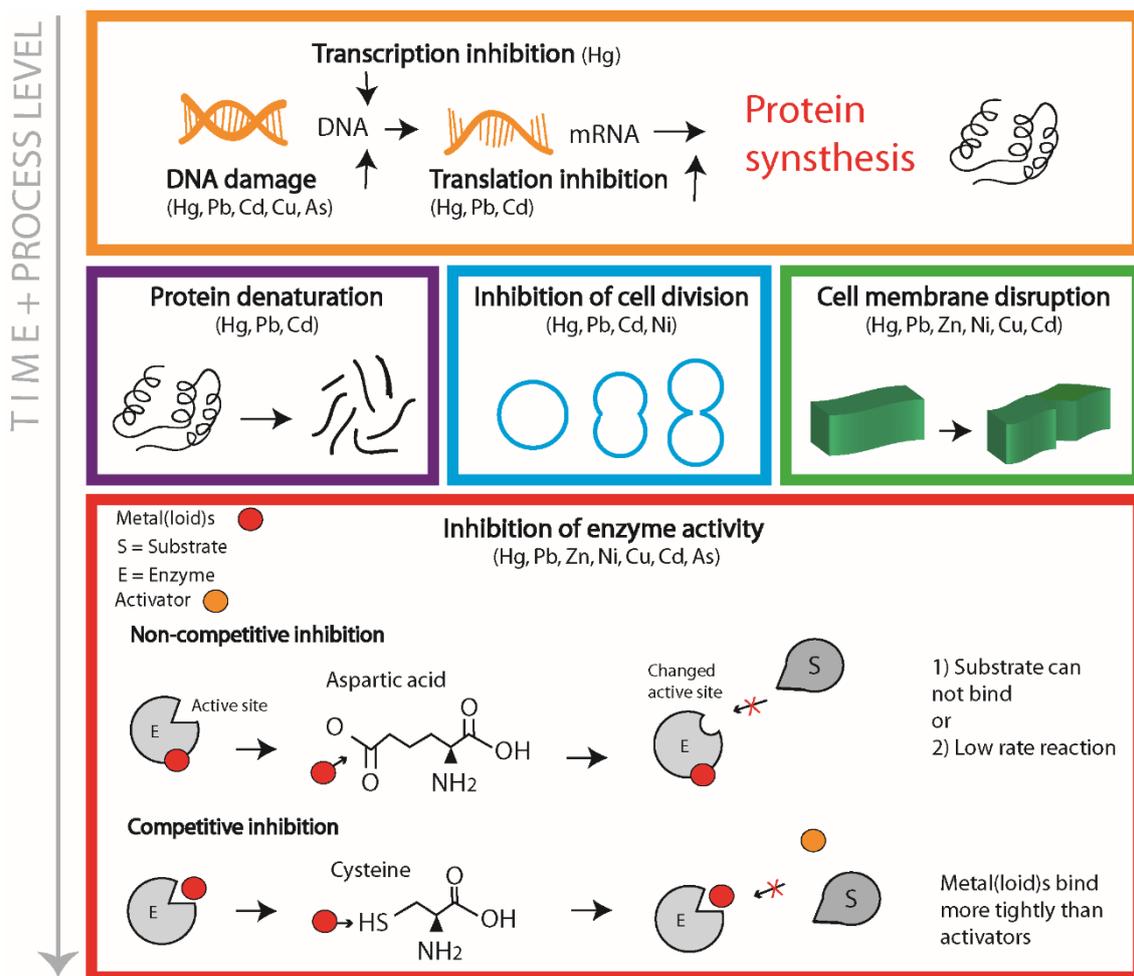


Figure 2. Generalized schema of the main toxic effects of metal(loid)s on microorganisms by process levels and time, with focus on enzyme activity (adapted from Abdu et al. 2017 and Turpeinem, 2002). The toxic effects of metal(loid)s have 3 process levels: genetic level (top), cell level (middle), and biochemical level (bottom), and 5 main mechanisms (presented here with colour frames). The TIME reflects the time necessary for the toxic metal(loid)s effects. The inhibition of enzyme activity is ongoing inside and outside (exoenzymes) of the living cells, whilst the other four mechanisms are ongoing inside the cells.

In most countries the permitted levels of metal(loid)s in soil are based on their total contents, which do not necessarily reflect their biological availability or potential toxicity - both essential for assessing environmental impacts (Stazi et al., 2015). The bioavailability of a given metal(loid) is a function of its content, chemical form, the nature of metal(loid) binding to soil particles, soil pH, clay and organic matter content, and other physicochemical factors (Marabottini et al., 2013). Furthermore, the bioavailability also depends on the ability of living organisms within the soil to take up chemicals from the surrounding environment (Bolan et al., 2014). In the context of metal(loid) contamination, bioavailability refers to the biologically available fraction of the total pool that can interact with organisms and their biochemical processes (Vangronsveld and Cunningham, 1998).

2.1.3. Soil quality indexes

Soil quality has been defined by The Soil Science Society of America (1995) as “*the capacity of a specific soil to work within the boundaries of natural ecosystems or under management to sustain vegetation and animal productivity, keep or improve the water and air quality, and support the health and living of humans*”. In the last decade, the soil quality objective has focused on the multifunctionality and resilience of soil along with the ecosystem services it provides (Bünemann et al., 2018). Bastida et al. (2008) reviewed literature by “soil quality” and “index”, which provided few SQIs that allowed for an integrated and objective measurement of soil quality. Nevertheless, the number of studies that use biological properties to measure soil quality has increased in recent years (Paz-Ferreiro and Fu, 2016), most of which focus upon agricultural soils. Thus, biological properties such as soil respiration, microbial biomass, EAs, and soil fauna can provide useful soil quality indicators (Bastida et al., 2008; Paz-Ferreiro and Fu, 2016). Most SQIs that use biological soil properties have been developed and used for agricultural soils. Thus, the soil’s potential for buffering contaminants, or the capability of soil microbial communities to detoxicate organic toxicants, are not usually considered in SQIs for agricultural soils. To a lesser extent, SQIs have also been developed for soil degradation by pollutants, organic contaminants, deforestation, and desertification, while studies concerning SQIs for metal(loid) contamination are very scarce.

Nearly all of the indices that use biological soil properties were constructed using soil EAs because they are early indicators of changes in biological and biochemical functions (Bastida et al., 2008; Trasar-Cepeda et al., 1998; Trasar-Cepeda et al., 2000). Therefore, EAs can identify the impacts of factors that affect soil fertility, such as metal(loid) contamination. Despite the widespread use of EAs as SQIs, Trasar-Cepeda et al. (2000) concluded that some EAs have a limited ability to show the effects of soil degradation from hydrocarbons and tanning/landfill effluents due to their inability to discriminate between soil degradation events and prior soil degradation. Further complexity is illustrated in the work of Riah et al. (2014), who reported that some EAs were inhibited, unaffected, and activated by pesticides. Thus, the current application of EAs as soil quality indicators has some shortcomings (Nannipieri et al., 2012; Trasar-Cepeda et al., 2000), which include: 1) their variable behaviour and relationship with other soil factors, such as organic matter content/quality, clay content, pH, climatic conditions, salt type and content, specific vegetation, and land management (Bünemann et al., 2018); 2) current enzyme assays measure the potential activity (at optimum conditions) instead of the real enzyme activity; and 3) accurate differentiation between intra- and extra-cellular enzyme activity cannot be achieved (Nannipieri et al., 2012). Also, most EAs represent very specific functions (e.g. nutrient and carbon cycling), and soil quality assessment should ideally be performed based on an integrated perspective including soil physicochemical and other biological properties.

Consequently, the aim of subsequent sections is 1) to provide an overview of the SQIs that include EAs, with particular emphasis on metal(loid) contaminated soils, and 2) to evaluate the effects of individual metal(loid)s and their combinations on the activities of the most common enzymes involved in the C, N, P and S cycles.

2.2. Soil quality in metal(loid) contaminated soils

The use of the traditional “soil quality” (SQ) concept for metal(loid) contamination is a relatively novel perspective. Ideally, SQ assessment in metal(loid) contaminated soils should account for a variety of ecological functions as opposed to the soil’s capability to maintain crop growth alone. Thus, the “soil quality index” – a quantitative value of SQ – in metal(loid) contaminated soils could also be considered as a degradation index such as the “microbiological degradation index” of Bastida et al. (2006) that was developed to evaluate soil desertification.

The number of publications about “soil quality” has increased in recent years. However, studies including both “soil quality” and “heavy metals” are about 15 times fewer (Figure 3a), which reflects how SQI development is seldom applied to cases of metal(loid) contamination (Figure 3b). Also, many studies related to SQ do not develop numerical indexes, which should be the primary goal of the SQ assessment.

Most studies using the SQ concept are focused on agricultural soils and are not developed for metal(loid) contamination (Table 2). Further, most studies concerning SQIs do not reflect an integrated and objective approach considering physical, chemical and biological soil functions. More specifically, most of these SQIs do not include any evaluation of the microbial community, which is an important component for assessing soil functions. This is relevant since some microbial groups are very sensitive to soil changes and perturbations, such as gram-positive bacteria in metal(loid) contaminated soils (Turpeinen et al., 2004). Also, some microbial groups can be “keystones” that sustain the community structure and its functionality (Banerjee et al., 2018).

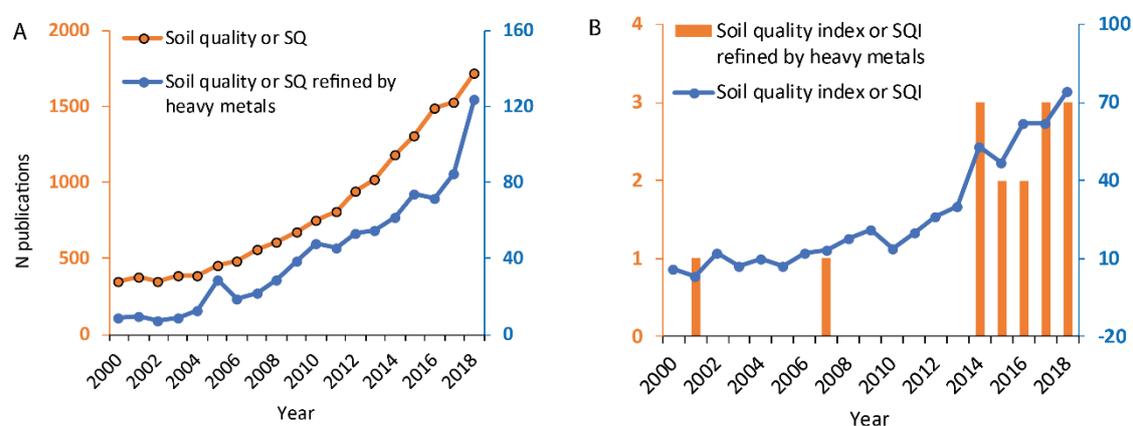


Figure 3. Number of publications (A) using “soil quality” or “SQ” refined by “heavy metal*” words in the title, abstract or keywords according to Web of Science for the period 2000-2018; (B) using “soil quality index” or “SQI” and refined by “heavy metal*” in the topic, according to Web of Science for the period 2000- 2018. The term “heavy metal” was used due to it is being the most common term used in literature.

The SQIs for metal(loid) contamination that have either been suggested or applied (Table 3) have various advantages and shortcomings, which are related to methods for selecting indicators, statistical analysis, index development, validation, and applicability.

In general, these indices are easy, fast, and cheap to determine, with the latter being the main advantage compared to costs of molecular high throughput methods. Of particular importance for metal(loid)s is that the SQIs are sensitive enough to discriminate between contaminated and non-contaminated soils. However, these SQIs can be subject to a range of limitations that include: 1) arbitrary criteria to select indicators and microbial groups, statistical analysis methods, and index development methods; 2) absence of other soil properties (Paz-Ferreiro and Fu, 2016) such as total and bioavailable metal(loid) contents; 3) various methods for assessing the same biological indicator (e. g., classical colourimetric vs fluorometric methods for assessing EAs); and 4) SQIs are made on few or very limited numbers of soils without validation with other soils. All these shortcomings can contribute to limited applicability (Bastida et al., 2008), and should be taken into account when determining SQIs for metal(loid) contaminated soils.

Table 2. Proposed soil quality indices (SQIs) that include soil EAs. Only SQIs or models which included soil EAs are shown along with the additional indicators used for each study (Phy: Physical parameters; Che: Chemical parameters; Bio: general biological parameters; MC: microbial community parameters).

Author	Treatments:	Method of indicators selection	Enzymatic activities	Phy	Che	Bio	Fau	MC
Agricultural soils								
García-Ruiz et al. (2008)	Organic vs neighboring conventional management	A priori selection (personal criteria)	Pacid, Palk, β -Gluc, ARY, DHA	-	-	+	-	-
Mijangos et al. (2010)	Soil under liming effect	A priori selection (personal criteria)	DHA, UA, β -Gluc, Palk, ARY	-	-	-	-	-
Sharma et al. (2011)	Soils under cotton and green gram systems	PCA	DHA	-	+	+	-	-
Paz-Ferreiro et al. (2012)	Organic soils with biochar, unpyrolyzed sewage sludge and control	A priori selection (personal criteria)	DHA, β -Gluc, Pacid, ARY	-	-	-	-	-
Morugán-Coronado et al. (2013)	Treated wastewater soils	Multiple regression analysis (TOC)	Pacid, β -Gluc	+	+	-	-	-
Sharma et al. (2013)	Soils under rainfed pearl millet-mung bean system	PCA	DHA	-	+	-	-	-

Continuation Table 2.

Shahid et al. (2013)	Soils under rice under wet and dry seasons and varying levels of chemical fertilizer and manure applications	PCA	DHA	+ + - - -
Sharma et al. (2014)	Soils under three cropping systems	PCA and multiple regression analysis	DHA	+ + - - -
Basak et al. (2016)	Soils under long-term cultivation of rice-potato-sesame cropping systems	Discriminate analysis based on Factor analysis	DHA, β -Gluc	+ + + - -
Obriot et al. (2016)	Soils under mineral fertilization or repeated application of exogenous organic matter	PCA and correlation analysis	β -Gluc, UA, ARYL, Palk, LAC	- - - - -
Raiesi & Kabiri (2016)	Soils under tillage practices	Factor analysis	Palk, UA, CAT	- - - - -
Sharma et al. (2016)	Soils under restorative soil management practices	A priori selection (personal criteria)	Pacid, Palk, ARY, DHA, UA	- + + - -
Sofi et al. (2016)	Soils under different crop cafeterias	PCA and correlation analysis	FDA	- + - - -
Sarma et al. (2017)	Soils under combined application of hardwood biochar and inorganic fertilizers	PCA	Pacid	- + - - -
Non-agricultural soils				
Reclamation or restoration				
Dawson et al. (2007)	Remediation treatments	Sensitivity and robustness	DHA	- + + + -
Mukhopadhyay et al. (2014)	Reclamation success in reclaimed mine soil	PCA	DHA	+ + + - -
Mukhopadhyay et al. (2016)	Reclamation success in reclaimed mine soil	PCA	DHA	+ + - - -
Degradation				
Armas et al. (2007)	Degradation in Andisols and Aridisols	Multiple regression analysis (TOC)	Cellulase	- + + - -
Zornoza et al. (2007)	Degradation in forests degraded soils	Multiple regression analysis (TOC/N)	Pacid, UA, β -Gluc	+ + + - -
Zornoza et al. (2008)	Degraded soils	Multiple regression analysis (TOC)	Pacid, β -Gluc	+ + - - -
Li et al. (2013)	Human disturbed soils and land use	PCA	UA, PROT	- + + - -

Continuation Table 2.

Gao et al. (2013)	Oil-contaminated saline soils	A priori selection (personal criteria)	DHA, CAT, PPO, LIP	-	+	-	-	-
Raiesi (2017)	Rangeland conversion and degradation	Factor analysis	ARY	+	+	-	-	-
Metal(loid) contaminated								
Chaer et al. (2009)	Simulated chemically stressed (Cu addition or pH alteration) soils	Multiple regression analysis (TOC/N)	PA	-	-	+	-	-
Blecker et al. (2012)*	Disturbed (mineralization) and undisturbed systems	Principal component analysis	Palk, AP-asa, FDA, ARY	+	+	+	-	+
Wyszowska et al. (2012)	Cd, Cu and Zn polluted soils	A priori selection (personal criteria)	DHA, CAT, UA, β -Gluc, Pacid, Palk, ARY	+	+	-	-	-
Burges et al. (2015)	Microcosm study with natural grassland soils contaminated with Cd, Pb, Cu, and Zn	A priori selection (personal criteria)	β -Gluc, Pacid	-	-	+	-	+

(+) represents the parameters which were used together with the EAs in a SQI. (-) represents the kind of parameters which were not used together with the enzymatic activities in a SQI. PA: phosphatase (determined without buffer addition); Pacid: acid phosphatase; Palk: alkaline phosphatase; FDA: fluorescein diacetate hydrolysis; AMY: amylase; INV: invertase; UA: urease; β -Gluc: β -glucosidase; ARYL: arylamidase; LAC: laccase; ARY: arylsulfatase; DHA: dehydrogenase; CAT: catalase; PPO: polyphenol oxidase; LIP: lipase; PROT: proteinase; TOC: organic carbon; N: total nitrogen; PCA: principal component analysis. Web of Science and Scopus databases were used for the search using the following criteria in both sources: Title (Soil quality indices) OR (Soil quality index), Refined by Topic (Soil enzymes) and Time period (2007-2018). Also google scholar was used with the same key words. * It is assumed that the principal issue is heavy metals, but authors did not focus solely on measuring heavy metal content.

Some of the SQIs for metal(loid) contamination presented in Table 3 included other degradation sources in addition to metal(loid)s, such as oil contamination and pH changes, which is a limitation considering their associated effects on microbial communities and EAs. Thus, the heterogeneity of available SQI studies makes direct comparison difficult. However, Puglisi et al. (2006) compared and validated their Alteration Index 3 (AI3) with results from published papers related to agricultural soils, metal(loid) contamination (Leirós et al., 1999) and bioremediation monitoring. This was a useful approach since the AI3 was able to discriminate controls from altered soils by increasing metal(loid) contents under controlled conditions. Therefore, although SQI validation is a difficult task considering the limitations of SQIs outlined above, Puglisi et al. (2006) showed a useful validation alternative.

Table 3. SQIs that include EAs for metal(loid) contaminated soils.

SQI	Indicators	Metal(loid)	Contamination source	Equation	Applied on	Advantages	Shortcomings	Reference
Geometric mean (GMea)	Pacid Palk β-Gluc ARY UA	Cd Cu Zn	Accidental rupture of pyritic slurry	$GMea = (Pacid \times Palk \times \beta-Gluc \times ARY \times UA)^{1/5}$	Metal(loid) contaminated, remediated, and non-contaminated soils	- Easy, fast, economic determination - Simple analysis - Good sensitivity	- Arbitrary criteria for selecting indicators, it was not based on statistical analysis. - Difficult applicability in other conditions due to specific characteristics of contamination. - Non-consideration of other soil indicators, specially metal(loid)s, in the index. - Absence of thresholds.	Hinojosa et al. (2004)

Continuation Table 3.

Alteration index (AI3)	β - Gluc Pacid UA	NA	Water contaminated with oil, metal(loid), and other toxic substances	$AI3 = 7.87 \beta$ - Gluc - 8.22 Pacid - 0.49 UA	Agricultural soils irrigated with contaminated water	- Easy, fast, economic determination - Indicators selected with statistical criteria - Simple analysis - Theoretically validated - Good sensitivity	- Non-consideration of other soil indicators. - Limited number of indicators. - Non-consideration of other soil indicators, especially metal(loid)s, in the index. - Absence of thresholds. - Results appeared to be more related to organic matter effects due to agricultural management than to metal(loid) contamination.	Puglisi et al. (2006)
C_p/C_m	CBM PA SOC	Cu	Simulated contamination and pH alteration	$C_p/C_m =$ $(17.2/C_m)^{0.435}$ $(PA/C_m)^{0.276} \times$ $(MBC/C_m)^{0.289}$	Chemical stress by Cu addition and pH alteration	- Consideration of other soil indicators - Indicators selected with statistical criteria - Good sensitivity - Simple analysis	- Limited number of indicators - Non-consideration of metal(loid) measurements - No thresholds - Limited applicability in other conditions	Chaer et al. (2009)

Continuation Table 3.

BA ₂₁	DHA CAT Pacid Palk UA β-Gluc ARY	Cd Cu Zn	Artificial metal(loid) contamination	BA ₂₁ = DHA x CAT x Pacid x Palk x UA x β- Gluc x ARY	Plant experiment set upon a loamy- sand and sandy- loam soil that had been contaminated with Cd, Cu, and Zn	- Easy, fast, economic determination - Simple analysis -Good sensitivity	- Non- consideration of other soil indicators, especially metal(loid)s, in the index. - Arbitrary criteria for selecting indicators, it was not based on statistical analysis. - Absence of thresholds. - Difficult applicability in other conditions.	Wyszkowska et al. (2012)
SQI (Battle mountain)	DTPA- Zn DTPA- Ca DTPA- Cu DTPA- S DTPA- Pb Tota-N ARY	Zn Cu Pb	Last Cu, Co, and Au mining	Non- equation. Weighted indicator values were added to make the index	Disturbed vs undisturbed	- Consideration of other soil indicators - Indicators selected with statistical criteria - Good sensitivity - Simple analysis	- Limited number of biological indicators. - Non-final numerical calculation of an index.	Blecker et al. (2012)

Continuation Table 3.

Functional diversity indices*	ARY, Pacid, PROT, UA, β -Gluc, INV	Zn Galvanized pylons	WM, GMea, $(\prod_{i=1}^n y_i)^{1/n}$	$\sum_{i=1}^n w_i \cdot y_i$	Zn contaminated soils under galvanized pylons	- Consideration of other soil indicators such as bioavailable metal(loid) concentration.	- Some functional diversity indices were not sensitive.	Lessard et al. (2014)
Weighted mean (WM) GMea Shannon Simpson Gini			Shannon, $-\sum_{i=1}^n p_i \log_2 p_i$					
			Simpson, $\frac{1}{\sum_{i=1}^n p_i^2}$					
			Gini, $\frac{2}{n} \sum_{i=1}^n q_i - (\frac{n+1}{n})$					
			See the study for further details					
SQI	Pacid, β -Gluc, PNM, BGA, FGA, F:B	Cd, Pb, Cu, Zn	Microcosm study with grassland soil artificially contaminated	$SQI = 10^{\log_m \frac{\sum_{i=1}^n \log m - \log n_i }{n}}$	Individual and multicontaminated soils vs control	- Good sensitivity	- Non-consideration of metal(loid) measurements	Burges et al. (2015)
						- Consideration of other soil indicators	- Arbitrary criteria for selecting indicators, it was not based on statistical analysis.	
						- Simple analysis	- Absence of thresholds.	

NA= non-published information. Web of Science and Scopus databases were used for the search using the follow criteria in both sources: "soil quality", "index", "heavy metals", "enzyme activity". Also google scholar was used with the same key words. PNM = potentially mineralizable nitrogen. BGA= bacterial gene abundance. FGA= fungal gene abundance. F:B= ratio of fungal to bacterial gene abundance. *Data from additional searches.

Most of the SQIs presented in Table 3 do not include measurements of metal(loid) contents, which are necessary due to the absence of thresholds for EAs in soils. The functional diversity indices performed by Lessard et al. (2014) was the only index that included bioavailable metal(loid) contents, yielding concentration-response curves between enzyme activities and soluble Zn contents. Thus, future research should consider both total and bioavailable metal(loid) contents in contaminated and non-contaminated soils developed on the same parent materials (Blecker et al., 2013). In this sense, critical

or internal effect metal(loid) content for EAs should define SQI thresholds. Furthermore, other soil physicochemical properties such as organic matter, pH, and texture can strongly affect soil microbiota and enzyme activities (Blecker et al., 2013; Niemeyer et al., 2012), and should therefore also be accounted for assessing SQ in metal(loid) contaminated soils. Lastly, non-contaminated soils, or controls, should be considered when developing SQIs to obtain a representative condition that can be compared to contaminated soils. Finally, the direction for developing SQIs in metal(loid) contaminated soils should be established.

2.3. Soil enzyme activities as soil quality indicators for metal(loid) contaminated soils

EAs are important SQ indicators, reflecting various functions related to biogeochemical cycles (Table 4), and being sensitive to soil changes and perturbations. There is a clear theoretical basis that explains the inhibition of soil enzymes due to metal(loid)s (Figure 2).

The effects of metal(loid)s on EAs are also based on physicochemical parameters. For instance, extracellular enzymes can be immobilized in enzyme-clay-humus complexes (Figure 4a), which results in more resistance to thermal and proteolytic degradation (Burns, 1986; Nannipieri et al., 1978; Tietjen & Wetzel, 2003). Furthermore, organic substances react with metal(loid)s, forming metal-humate complexes and immobilising them (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999). Metal(loid)s can also interact with clay surfaces by sorption (Bolan et al., 2014; Harter and Naidu, 1995) and therefore might compete with enzymes to form metal-humus-clay complexes, which would act to promote enzyme degradation by temperature and proteolytic attacks (Figure 4b). Thus, the mentioned soil physicochemical properties should be considered where possible to discriminate their effects from metal(loid) stress.

Analysis of EAs in soils with high metal(loid) contents requires careful consideration because the use of buffers changes the soil pH, and the resulting modified H^+ or OH^- concentrations can alter the metal(loid) availability (Lessard et al., 2013). More specifically, buffer application can increase metal(loid) availability from metal(loid)s stabilised in organo-mineral complexes (Figure 4c), Al/Fe oxides, and carbonates (Figure 4d). Therefore, Nannipieri et al. (2017) recommend using both water and buffers in

enzyme assays to enable better conclusions related to specific soil properties. An additional strategy is to mimic in situ soil pH by using solutions adjusted to the pH of the assayed soil (German et al., 2011).

Table 4. Ecological functions of the main soil enzymes related to biogeochemical cycles of C, N, P and S (Blagodatskaya & Kuzyakov, 2008; Dick, 1997; Dick, 2011; Skujiņš & Burns, 1976).

Enzyme	Functions
Dehydrogenase	Related to the total oxidative activity of soil microorganisms.
Glucose oxidase	Glucose oxidation.
Catalase, peroxidase	Releases oxygen from hydrogen peroxide.
Polyphenol oxidase	Oxidizes phenolic compounds; is involved in litter and soil organic matter ageing.
Amylase	Hydrolyses starch from maltose.
Cellulase	Responsible for intracellular hydrolysis of the 1,4-β-D-cellulose bonds, which is an important component of wood and plant fiber.
Xylanase	Hydrolyses the 1,3-β-D-xylan bond from xylan, which is a polysaccharide found in cellulose.
β y α-glucosidase	Releases glucose from cellulose hydrolysis.
β y α-galactosidase	Hydrolyses melibiose and lactose.
N-Acetyl-β-Glucosidase	Hydrolyses the terminal nonreducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides. This activity leads to the ultimate release of NAG residues from chitin.
Phosphatase (mono and diester)	Releases HPO_4^{2-} from organic matter.
Inorganic pyrophosphatase	Catalyses the hydrolysis of pyrophosphate to orthophosphate.
Trimetaphosphatase	Participates in the hydrolysis of trimetaphosphate, which is not sorbed in soils.

Continuation Table 4.

	Arylsulfatase	Catalyses the hydrolysis of ester-bond S by fission of the O-S linkage. Releases available SO_4^{2-} to plants from organic matter.
Sulfur	Cystathionine lyase (γ -lyase and β -lyase)	Cleaves cysteine to form thiocysteine, NH_4^+ , and pyruvate; desulfurates cysteine.
	Rhodanase	Catalyses the formation of thiocyanate and sulfite from thiosulfate and cyanide.
	Urease	Breaks urea's C-N bond.
Nitrogen	Protease	Hydrolyses proteins, releasing amino acids.
	Peptidase	Hydrolyses peptides releasing amino acids.
	L-asparaginase, L-glutaminase	Catalyses the hydrolysis of L-asparagine to produce L-aspartic acid and NH_4^+ .
	Deaminase	Involved in purine metabolism. Breakdown of adenosine turnover of nucleic acids.

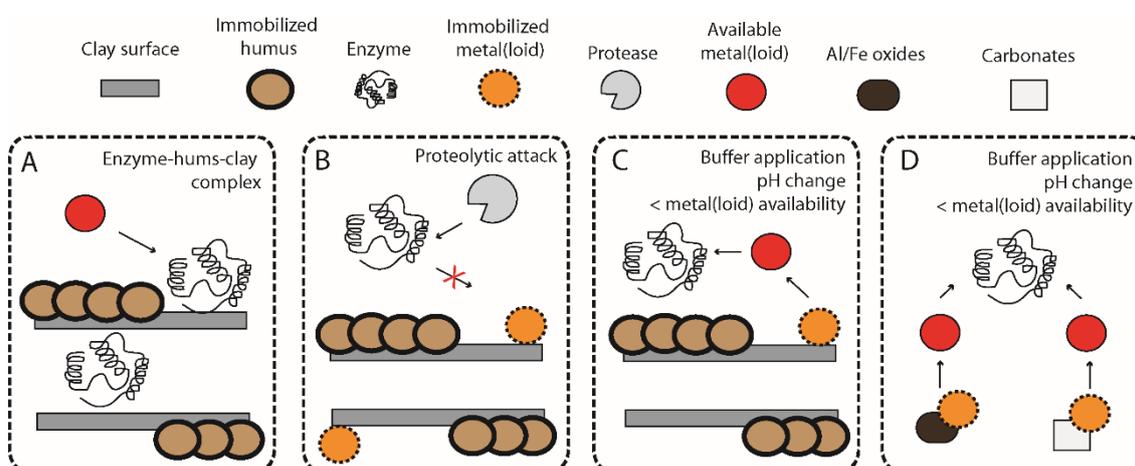


Figure 4. Main interactions between metal(loid)s and enzymes, organic matter, clay minerals, Al/Fe (sesqui)oxides, and carbonates. A) metal(loid) interactions leading to enzyme stabilization on organo-mineral clay complexes; B) metal(loid) competition with enzymes by stabilization on organo-mineral complexes, which lead to proteolytic attacks on free enzymes; C and D) in analytical methods for measuring enzyme activities: buffer application might change soil pH and increase metal(loid) availability, which can negatively affect enzymes.

The activities of intracellular enzymes (only present inside the cell; e.g. dehydrogenase) are considered better indicators under short-term metal(loid) contamination on active microbial communities, while that of extracellular enzymes (e.g. phosphatases) are better indicators for long-term metal(loid) contamination. However, for enzymes that exhibit both intra- and extra-cellular functions, no methods allow simple disentangling of how their own activities are affected. Nevertheless, there are some methodological alternatives such as measuring EAs after soil incubation with CHCl_3 (Schimel et al., 2017), which gives a better approach for measuring extracellular enzymes. Most soil functions are related to extracellular enzymes and their association with biogeochemical cycles since these enzymes generally remain active in soils for between two weeks and one month, but can persist for several months in mineral soils (Schimel et al., 2017).

The specific enzymes that could be considered as good SQIs for metal(loid) contaminated soils – meaning they are especially sensitive to metal(loid) contamination – are not well defined. However, this reviews finds the most common EAs measured in metal(loid) contaminated soils are dehydrogenase (DHA), urease (UA), acid phosphatase (Pacid), alkaline phosphatase (Palk), catalase (CAT), and arylsulfatase (ARY), followed by the lesser use of protease, cellulase, β -glucosidase, N-acetyl- β -glucosidase, asparaginase, phosphatase (without buffer), nitrate reductase, polyphenol oxidase, and amylase (Figure 5).

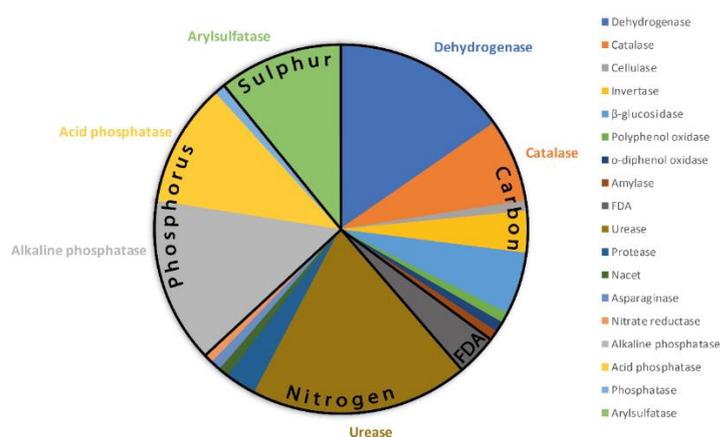


Figure 5. Activities of the main enzymes used in studies related to soil metal(loid) contamination. The enzymes of C, N, P and S cycles are labeled in individual segments with bold lines (data of the figure is available in Table S2 available in Aponte et al., 2020b). FDA = fluorescein diacetate hydrolysis. Nacet = N-acetyl- β -glucosidase.

2.4. Effects of metal(loid)s on enzyme activities

DHA, Pacid, Palk, CAT and ARY are all sensitive to metal(loid) soil contamination, and their sensitivity depends on the metal(loid) content. Therefore, data for the inhibition intensity of EAs (in % of uncontaminated control soil) available from the literature were evaluated for three metal(loid) contamination levels in laboratory experiments and field studies (Figure 6 and Figure S1 available in Aponte et al., 2020b). These metal(loid) contamination levels were chosen according to that used by many authors (Friedlová, 2010; Mikanova, 2006; Šmejkalová et al., 2003; Stazi et al., 2017) and were suggested for this study as follows: 1) “Very low” and “Low” = Low; 2) “Moderate” and “Medium” = Medium; and 3) “High” and “Source of contamination” = High (details are shown in Figure S1 available in Aponte et al., 2020b). Contamination levels and threshold values are related to ecological and/or health risk.

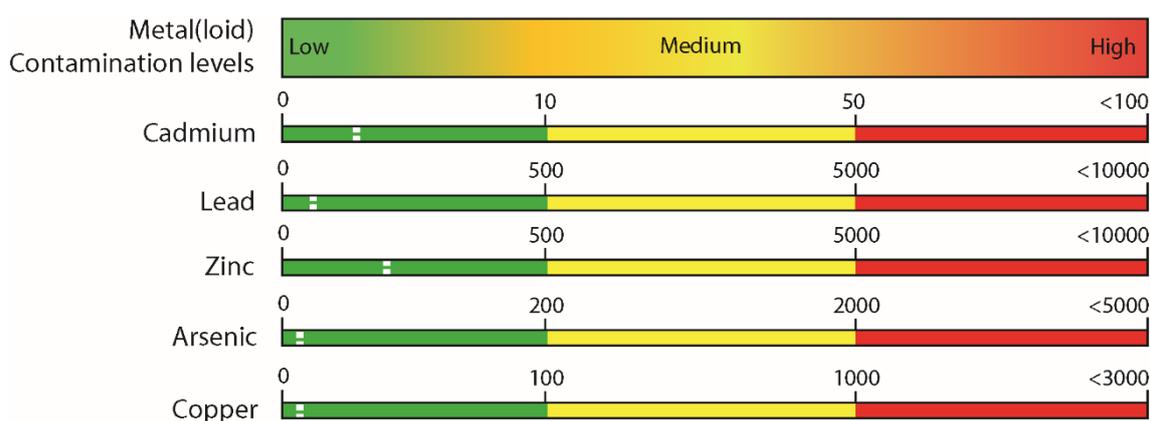


Figure 6. Total content of various metal(loid)s (mg kg^{-1}) in soil corresponding to three contamination levels: Low, Medium and High. The dotted white line on the left of each scale represents the threshold value related to ecological risks or health (Ministry of the Environment, 2007). The three levels were defined taking into account levels used in most publications (Friedlová, 2010; Mikanova, 2006; Šmejkalová et al., 2003; Stazi et al., 2017).

Data collection of EAs in metal(loid) contaminated soils was based on the following criteria for available literature: articles should have at least two levels of metal(loid) contamination: 1) a non-contaminated reference soil and the enzyme activity within it; 2) metal(loid) contaminated soil with similar physicochemical properties to the non-contaminated soil and its associated enzyme activity. The final dataset was

comprised of 31 studies. Note that some of the studies contained more than one metal(loid) contamination form or level: with various metal(loid)s or with increasing metal(loid) contents. Data for each metal(loid) effect on individual enzymes were scarce. Also, EAs from reference and contaminated soils varied in all studies. Therefore, the enzyme inhibition percentage was calculated with Equation 1:

$$\text{Enzyme inhibition (\%)} = \frac{(\text{Ref} - \text{Contam})}{\text{Ref}} \times 100 \quad \text{Eq. 1}$$

Where *Ref* is the value corresponding to the enzyme activity in non-contaminated reference soil; and *Contam* is the value of each enzyme activity in contaminated soil. The enzyme inhibition results were classified by one of the three metal(loid) contamination levels (Figure 6) to get an average for each group. As the number of values was different for each enzyme and contamination level, the overall weighted mean enzyme inhibition percentage (WM) was calculated with Equation 2:

$$\text{WM} = \frac{(\text{Low}_N \times \text{Low}_{\text{mean}}) + (\text{Medium}_N \times \text{Medium}_{\text{mean}}) + (\text{High}_N \times \text{High}_{\text{mean}})}{(\text{Low}_N + \text{Medium}_N + \text{High}_N)} \quad \text{Eq. 2}$$

Where, *N* is the number of values, and *mean* is the arithmetic mean of the enzyme inhibition percentage, both per each metal(loid) contamination level.

Results from the available data indicate that DHA is the most sensitive indicator for soils contaminated with metal(loid)s, with a weighted mean inhibition of 62% (Figure 7). The inhibition of DHA increased with rising metal(loid) contamination (corresponding in some studies to proximity to contamination source), reaching an inhibition of 82% in high metal(loid) contamination. Generally, DHA activity reflects the active portion of microbial biomass (Nannipieri et al., 1990) and overall microbial activity (Stevenson, 1959); therefore, it is a suitable indicator of intracellular enzyme activity. Most of the DHA studies (and it is common for all other enzymes) are related to the combined effect of Cd, Pb, and Zn contamination. Few studies show the isolated inhibition of DHA by Cd (> 10 mg kg⁻¹), Pb (> 50 mg kg⁻¹), As (> 15 mg kg⁻¹) and Cr (> 0.05 mg kg⁻¹) (Table S2 available in Aponte et al., 2020b). Thus, the effect of each metal(loid) on DHA has not been widely investigated.

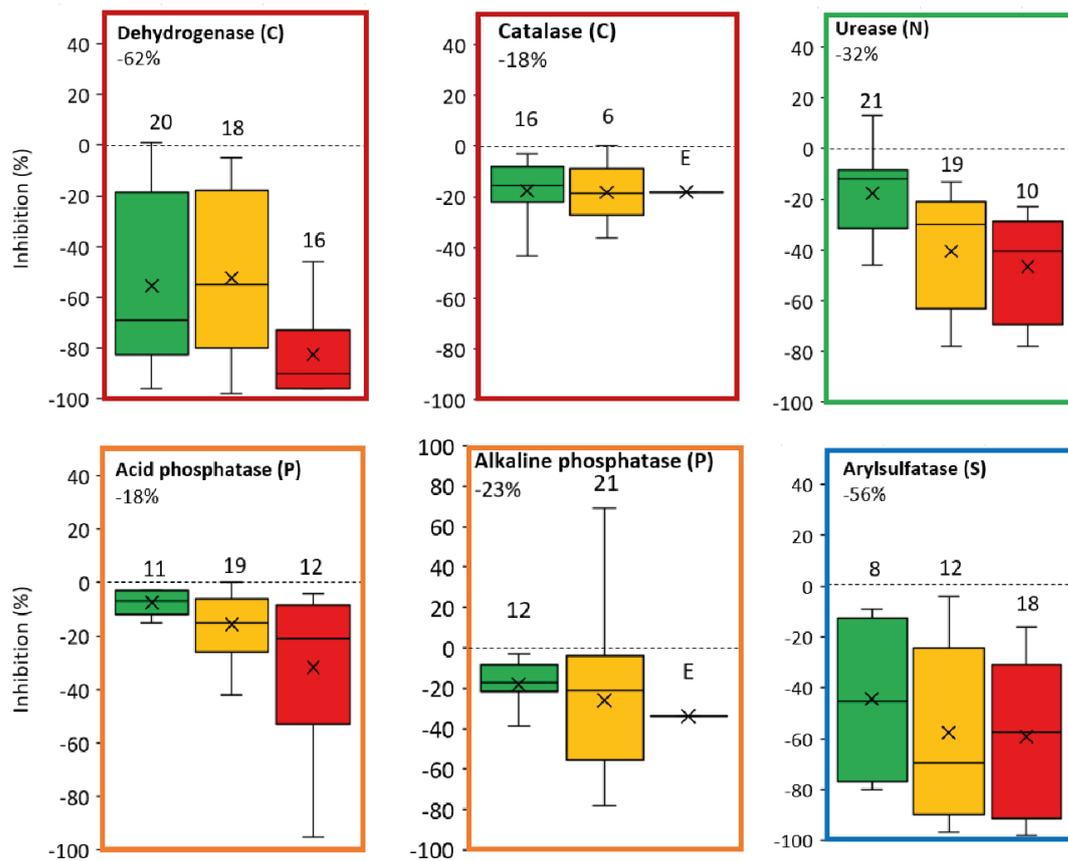


Figure 7. Enzyme activities depending on the three levels of metal(loid) contamination (Figure 6): Low (green), Medium (yellow) and High (red). Inhibition percentage was calculated by Equation 1. The percentage value at the top is the weighted overall effect and standard error for all contamination levels (Eq. 2). Color borders and letters in parenthesis are related to enzymes and the biogeochemical cycles they are involved in: C = carbon (red), N = nitrogen (green), P = phosphorus (orange), S = sulphur (blue). Crosses inside each box show the weighted mean inhibition. The horizontal line inside the box is the median value. Lower and upper whiskers represent the minimum and maximum values, respectively. Lower and upper limits of the box are lower and upper quartile, respectively. Outliers were not presented. The number on each bar is the number of sampling points (independent observations). Letter E on bars indicates that this value was extrapolated considering the other two metal(loid) contamination levels. The Y scale is not unified for a better comparison of the effects on individual enzymes. The effects are considered significantly different if the whisker does not cross the 0-line (dotted horizontal line).

Some studies have reported DHA activation with increasing metal(loid) contents (Epelde et al., 2015), which was driven by high soil organic carbon content. In this sense,

D'Ascoli et al. (2006) suggested that soil organic matter might have counterbalanced the negative effect of metal(loid)s on microbial activity. Additionally, Niemeyer et al. (2012) concluded that changes in physicochemical soil properties (soil organic carbon, pH, and nutrient availability) might have led to alteration of habitat functions for soil microbiota and hence affected the processes at play in Pb contaminated soils. On the other hand, it should be noted that Cu can interfere with the analytical procedure of DHA activity since Cu^{2+} affects the absorbance of the DHA reaction products and leads to their reduction. Thus, soils with high Cu concentrations in solution, or that have received Cu-contaminated amendments, are susceptible to underestimation of DHA activity (Chander and Brookes, 1993). Therefore, calibration curves with increasing Cu content in soil are necessary to avoid DHA underestimations.

ARY was inhibited by 54% on average in metal(loid) contaminated soils (Figure 7). The activity of ARY decreased with rising metal(loid) contamination (from 44% to 59% in low and high levels of metal(loid) contamination, respectively). ARY is understood to be a good indirect indicator of soil fungal biomass and activity since this enzyme catalyses the hydrolysis of sulfate aromatic esters that are constituents of fungal cell walls (M. C. Moscatelli et al., 2005). As seen for DHA, most studies used ARY as an indicator for combined Cd, Pb, Cu, and Zn contamination. Studies that evaluate individual metal(loid) effects on ARY are consequently scarce but show consistent inhibition by Cd ($> 20 \text{ mg kg}^{-1}$), Pb ($> 40 \text{ mg kg}^{-1}$), and Cu ($> 50 \text{ mg kg}^{-1}$) (Table S3 available in Aponte et al., 2020b). Few studies on the effect of As on ARY activity showed inhibition at high As contents ($> 250 \text{ mg kg}^{-1}$ soil). Because metal(loid) effects on EAs depend on the amino acid composition and the protein sequence (Geiger et al., 1998), we hypothesise that ARY activity will be inhibited by Cu, Pb, Zn, and Cd due to their interactions with glycine, aspartic acid, and histidine in the ARY active site (Boltes et al., 2001).

UA activity was inhibited by metal(loid) contamination by 35% on average, and this inhibition increased from low (18%) to high (47%) metal(loid) contamination (Figure 7). This enzyme is responsible for the urea hydrolysis to CO_2 and NH_4^+ through the breaking of C-N bonds (Kandeler, Poll, Frankenberger, & Tabatabai, 2011). UA activity has been used as an indicator of soils contaminated with several metal(loid) contaminants. Consequently, there are joint negative effects of Cd, Pb, Cu, and Zn on UA activity. Several studies show the inhibition of UA due to Cd ($> 1.5 \text{ mg kg}^{-1}$ soil), Pb ($> 40 \text{ mg kg}^{-1}$

¹ soil), Cu (> 50 mg kg⁻¹ soil), and As (> 10 mg kg⁻¹ soil) (Table S4 available in Aponte et al., 2020b). It is understood that metal(loid) ions interact with cysteine residues in the UA active site, especially with thiol groups. Further, metal(loid)s interact with the oxygen atom in the urea molecule and stop the urea interactions with Ni atoms in the UA active site, which also inhibits the catalytic activity of UA (Mazzei et al., 2018). An exception was found by Karaca et al. (2002) where UA was activated in Cd contaminated soils (50 mg Cd kg⁻¹) after 112 incubation days, which can be related to high organic matter content (25%) such as mentioned before for DHA.

Pacid and Palk were inhibited by metal(loid) contamination by 18% and 26% on average, respectively (Figure 7). Pacid inhibition increased from low (8%) to high (32%) metal(loid) contamination levels. Palk was inhibited by 26% in medium and 34% at high metal(loid) contamination, though, the inhibition for the “high” level was extrapolated due to scarce data. Some exceptions, however, do not show any effects of metal(loid)s on the activities of both phosphatases. Phosphatase enzymes are involved in P mineralisation from organic to inorganic forms; thus, they are important components in the biogeochemical P cycle (Speir and Ross, 1978). Similar to other enzymes, most studies used phosphatases as indicators on soils contaminated with Cd, Pb, and Zn. Several studies that evaluated the effects of individual metal(loid)s showed the inhibition of phosphatases at high contents of Cd (> 100 mg kg⁻¹), Pb (> 1000 mg kg⁻¹), and As (> 1000 mg kg⁻¹) (Table S5, S6 available in Aponte et al., 2020b). These metal(loid)s might interact with histidine, serine, and arginine residues in the phosphatase active site (Guija et al., 2013; Ullah and Dischinger, 1993) and hence inhibit its activity.

CAT activity was inhibited by 18% on average (Figure 7) and was not sensitive to rising metal(loid) contamination. This enzyme is involved in the disproportionation of H₂O₂ (Prosser et al., 2011). Despite a small number of publications that used CAT as an indicator, they show a clear negative effect of Cd (> 1.5 mg kg⁻¹), Pb (> 150 mg kg⁻¹), and Cr (> 0.05 mg kg⁻¹) on this enzyme’s activity (Table S7 available in Aponte et al., 2020b). These metal(loid)s can interact with the CAT active site because of the presence of active functional groups such as residues of arginine, histidine, and tyrosine (Jeoung et al., 2007; Mashhadi et al., 2016; Melik-Adamyan et al., 2001). Also, direct adverse effects of metal(loid)s on microorganisms (Table 1; Figure 2) can inhibit the protein synthesis.

Considering the quantitative estimates of enzyme inhibitions by metal(loids) presented here, DHA and ARY can be considered as the most sensitive and, thus, adequate indicators for metal(loid) contaminated soils. Both DHA and ARY are well related to the overall microbial activity and biomass, which suggests a stronger negative effect on microbial activity and thus protein synthesis instead of direct effects on enzyme activities. This agrees with Karaca et al. (2010) who concluded that intracellular enzymes of active microorganisms (e.g. dehydrogenase) are more vulnerable to metal-related short-term changes than extracellular enzymes. In this context, several studies have reported decreasing in total culturable numbers of nitrogen-fixers and heterotrophic bacteria (Oliveira and Pampulha, 2006), fungi biomass (Stazi et al., 2017; Wang et al., 2011), taxonomic diversity of microbes (Feng et al., 2018), and bacteria transcriptional activity (Kapoor et al., 2015) in metal(loid) contaminated soils. Pacid, Palk, CAT, and UA were less sensitive to metal(loid) contamination, with an average inhibition of less than 30%, which is related to higher resistance of extracellular enzymes due to interactions with organo-mineral complexes. Therefore, the inhibition trend corresponding to their sensitivity to metal(loid) contamination followed the order: DHA > ARY > UA > Palk > Pacid > CAT, which is similar to sensitivity found by Wyszowska et al. (2006a). This suggests that the impact of metal(loid) contamination on microbial communities is more substantial than the direct inhibition of enzymes.

2.5. Future perspectives and conclusions

SQIs based on EAs are scarce for metal(loid) contaminated soils, and most of these SQIs do not reflect the wide-ranging soil functions. Even so, there are useful approaches such as the index developed by Blecker et al. (2012), and the alteration index of Puglisi et al. (2006, 2005). At present it is not possible to conclude which of the developed SQIs are the most suitable for evaluating metal(loid) contaminated soils because of the absence of SQI comparisons on the same soils. For instance, although the AI3 proposed by Puglisi et al. (2006) was tested with data from different soil types, including agricultural soils, it has not been experimentally validated in metal(loid) contaminated soils. Nevertheless, Puglisi et al. (2006) showed a useful alternative for validating SQIs.

The most useful suggestions about the SQI development for metal(loid) contaminated soils are: 1) A SQI should consider several sensitive biological properties (e.g. basal respiration, metabolic community profile, and microbial biomass) in addition

to EAs instead of only one or a couple of them; 2) The selection of SQ indicators should be based on statistical analysis (e.g. principal component analysis, correlations, and regressions); 3) It should account for the physicochemical soil properties especially the total and available content of metal(loid)s, which can yield critical metal(loid) values for EAs and thus be used to propose thresholds for SQIs; 4) The SQI development should be performed through a general and standardised method for indicator measurements (e.g. classic colourimetric vs fluorometric methods for enzyme assays); 5) It is necessary to include non-contaminated or reference soils; and 6) The SQI validation on similar metal(loid) contaminated soils should be performed, and SQIs should be validated with data from published studies. These criteria will strongly improve the validation and comparisons between the studies, and, therefore, will make possible the future application of SQIs based on EAs to metal(loid) contaminated soils.

It is important to stress that the inclusion of non-contaminated soils is necessary to develop SQIs due to the absence of the thresholds of EAs under metal(loid) contamination. Thus, a baseline or reference value for EAs in soils not contaminated with metal(loid)s is required. For this reason, it is still not possible to associate enzyme activity with metal(loid) contamination levels. In this context, experimentally defined critical metal(loid) levels are needed for EAs under several conditions that also consider soil mineral matrix characteristics. This would establish a baseline of EAs associated with various metal(loid) contamination levels while also accounting for biotic and abiotic factors, especially for field studies where organic matter can have stronger influences on microbiota and enzyme activities than metal(loid) contamination.

Furthermore, the classical enzyme assays for metal(loid) contaminated soils need to use both water and buffers to avoid additional metal(loid) input and their effects in the presence of buffer (H^+ or OH^- ions, Nannipieri et al., 2017). Also, calibration curves should be done on the same soil with metal(loid) content to evaluate possible chemical interactions between available metal(loid) and enzyme products.

Most of studies reviewed here were done on soils contaminated with Cd, Pb, Zn, and Cu, and there are nearly no studies that examine the effects of the very high metal(loid) contents. Therefore, more studies about the effects of metal(loid)s on EAs are needed that consider their high natural content in some regions, especially in relation to less studied metal(loid)s such as As, Cr, and Sb.

In conclusion, EAs are useful indicators of metal(loid) contamination due to their fast response and sensitivity. However, such enzyme indicator studies should be complemented with additional physicochemical and biological indicators. EAs also reflect soil functions, and further advantages of their use as an indicator are the low costs of analysis and fast measurement times. The activity of dehydrogenase, arylsulfatase, urease, alkaline and acid phosphatase, and catalase are the most frequently used indicators under metal(loid) contamination. Dehydrogenase and arylsulfatase are the most sensitive indicators for metal(loid) contaminated soils; therefore, these enzymes can be considered the most suitable SQI indicators. Because indicators should: 1) be sensitive and 2) reflect various soil functions, other enzymes should also be accounted for to reflect functions related to C, P, N and S cycles. From the most widely used EAs, acid phosphatase, urease, and arylsulfatase can be good indicators related to P, N, and S cycles, respectively. Dehydrogenase is a good indicator associated to the C cycle in metal(loid) contaminated soils. Alkaline phosphatase and catalase should not be considered as SQ indicators for metal(loid) contaminated soils since inhibition of its activity was very low at each metal(loid) contamination level. We conclude that SQIs based on EAs can reflect the effects of metal(loid) contamination on soil functions related to the C, N, P and S cycles, which represents an opportunity to improve the monitoring of soil degradation and ecosystem functioning.

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CHAPTER III. “*Meta-analysis of heavy metals effects on soil enzyme activities*”

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Meta-analysis of heavy metal effects on soil enzyme activities

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Abstract

Enzyme activities (EAs) respond to contamination in several ways depending on the chemical form and content of heavy metals and metalloids (HMs) and their interactions with various soil properties. A systematic and mechanistic understanding of EA responses to HM contamination in soil is necessary for predicting the consequences for nutrient availability and the cycling of carbon (C), nitrogen (N), phosphorus (P) and sulphur (S). In this study, a meta-analysis based on 671 observations found the activities of seven enzymes to decrease in response to soil contamination with Pb, Zn, Cd, Cu and As. HM contamination linearly reduced the activities of all enzymes in the following order: arylsulfatase > dehydrogenase > β -glucosidase > urease > acid phosphatase > alkaline phosphatase > catalase. The activities of two endoenzymes: arylsulfatase (partly as exoenzyme) and dehydrogenase were reduced by 72% and 64%, respectively. These reductions were two times greater than of exoenzymes: β -glucosidase, urease, acid phosphatase, alkaline phosphatase and catalase (partly endoenzyme). This reflects the much stronger impact of HMs on living microorganisms and their endoenzymes than on extracellular enzymes stabilized on clay minerals and organic matter. Increasing clay content weakened the negative effects of HM contamination on EAs. All negative effects of HMs on EAs decreased with soil depth because HMs remain mainly in the topsoil. EAs involved in the cycling of C and S were more affected by HMs than the enzymes associated with the cycling of N and P. Consequently, HM contamination may alter the stoichiometry of C, N, P and S released by enzymatic decomposition of organic compounds that consequently affect microbial community structure and activity.

Keywords

Metal(loid)s, Soil pollution, Toxic elements, Microbial activity, Biogeochemical cycles

3.1. Introduction

Heavy metals and metalloids (HMs) are naturally present in soils mainly from weathering of parent materials (Alloway, 2013). Usually, these natural occurrences of HMs in soils are at trace contents and therefore not toxic for microorganisms, plants, animals, and humans (Pierzynski et al., 2000). However, anthropogenic activities such as mining and other heavy industry can contaminate soils with HMs, which in some cases can result in severe environmental pollution (Chao et al., 2014) and human health risks (Chibuike and Obiora, 2014). The risks of HM accumulation in soils are particularly great when mining and industrial activities occur near to urban and/or agricultural areas (Huang et al., 2019; Nouri et al., 2009), with cases in Chile, China, India, and Mexico providing examples of large areas that have been seriously affected in this way (Cornejo et al., 2008; Flores-Magdaleno, 2011; kumar and Anbazhagan, 2018; Wang et al., 2020).

Enzyme activities (EAs) are good indicators of soil quality and health because of their sensitivity to HM contamination and direct relation with soil functions related to C, N, P, and S cycles (Aponte et al., 2020b). According to their localization and functioning in soil, there are three groups of enzymes: 1) intracellular enzymes: catalysing reactions within living microbial or root cells, 2) extracellular enzymes (or exoenzymes): released by roots and microorganisms outside of living cells to catalyse the decomposition of large molecules or biopolymers, and 3) enzymes located outside of cells that were released by death, killing or damage of microbial and root cells, which can be immobilised in organo-mineral complexes (Burns, 1982; Kuzyakov and Mason-Jones, 2018; Maire et al., 2013). HM toxicity depends on enzyme location since extracellular immobilised enzymes are more resistant to denaturation and proteolytic attack (Karaca et al., 2010). However, HM toxicity occurs through two primary mechanisms: 1) binding to thiol-containing groups and oxygen (Poole and Gadd, 1989) and, 2) displacement of essential elements from their native binding site (Bruins et al., 2000). The toxicity mechanism depends on the ionic form of the HM and its chemical speciation (Nies, 1999; Turpeinem, 2002). Some HMs have affinity for carboxyl and amine functional groups that are part of active sites of some enzymes. Thus, the exact effect on EAs ultimately depends on the amino acid composition of the active site of each enzyme (Geiger et al., 1998). Additionally, HMs can also inhibit enzyme production by depressing the RNA expression (Kapoor et al., 2015) in microbial cells.

D'Ascoli et al. (2006) reported that HMs inhibit EAs in several ways: i) masking catalytically active groups, ii) denaturing the protein conformation, or iii) competing with HM ions that are needed to form enzyme-substrate complexes (Deng and Tabatabai, 1995; Gianfreda and Bollag, 1996). Aside from the potential effects of HMs, EAs are also affected by soil properties such as pH (Dick, 2011), clay content (Burns, 1986; Nannipieri et al., 1978; Tietjen and Wetzel, 2003), soil organic matter (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999) and nutrient availability (Liang et al., 2014). In this manner, soil physicochemical properties can also alter HM toxicity since: 1) organic substances react with HMs forming organo-mineral complexes and 2) HMs can interact with clay mineral surfaces and compete with enzymes to also form metal-organo-mineral complexes, which might accelerate enzyme degradation by a reduction in the enzyme protection against temperature and proteolysis (Burns, 1986).

Aside from soil properties, methodological aspects can also affect the measured EAs since enzymatic assays use various chemicals and conditions during incubation and analysis. For example, the use of buffers in enzymatic assays can result in changes to the availability of HMs and their toxicity (Nannipieri et al., 2017). Further, the use of HM salts in bioassays can result in misinterpretation of HM effects on EAs due to salt toxicity instead of HM influence.

Currently, at least 500 enzymes are known to have critical roles in the cycling of C, N, P, and S in soil (Gianfreda and Ruggiero, 2006), with some hydrolases, transferases, oxidoreductases, and lipases related to these cycles being widely studied (Aponte et al., 2020b; Dick, 1997; Skujiņš and Burns, 1976). In HM contaminated soils, the most commonly studied EA assays are those of dehydrogenase (DHA), arylsulfatase (ARY), urease (UA), acid (Pacid) and alkaline phosphatases (Palk), and catalase (CAT) (Aponte et al., 2020b). Other EAs such as β -glucosidase (Gluc), invertase, and some proteases have also been widely studied, but their responses to HM contamination are still scarcely reported (Aponte et al., 2020b; Dick, 1997; Sinsabaugh, 2010). Most studies have reported enzyme inactivation by HMs via reactions with sulfhydryl groups for most of the mentioned EAs (except DHA; Eivazi and Tabatabai, 1990; Frankenberger and Tabatabai, 1991), which are involved in the catalytically active sites of enzymes and the protein structure (Al-Khafaji and Tabatabai, 1979; Karaca et al., 2010; Tabatabai, 1977). However, specific interactions between HMs and other amino acidic residues in active sites of different enzymes have been scarcely discussed, especially from a quantitative and systematic perspective.

Previous meta-analyses have evaluated the response of EAs to global change (Xiao et al., 2018), tillage (Zuber and Villamil, 2016), and climate change (Chen et al., 2018). However, the response of EAs to HM contamination in soil has not been tackled, despite enzymes regulating the cycling of all nutrients for plants and microorganisms. In this context, meta-analysis would allow the statistical generalisation of EA responses to HM contamination. Additionally, such meta-analysis is necessary to estimate changes in nutrient cycling, soil quality, and soil recovery processes in HM contaminated soils.

Here, we hypothesise that endoenzymes involved in C and S cycles are more sensitive to HM contamination than exoenzymes related to N and P cycles. This is based on the dependence of endoenzymes on microbial metabolism and the stabilization of exoenzymes by organo-mineral complexes, which increase exoenzyme resistance to perturbations including deactivation by HMs. Thus, C and S cycling would be expected to be more retarded than P and N cycles when subject to HM contamination, which might result in stronger C and S limitations to microorganisms in HM contaminated soils. More specifically, we carried out a meta-analysis to evaluate: 1) the effects of soil HM contamination on EAs involved in C, N, P, and S cycles; and 2) the influence of soil properties on EAs in HM contaminated soils; and 3) the influence of experimental factors on the measurement of EAs affected by HM contamination. The magnitude and direction of HM effects on EAs were considered, taking into account that enzymes can be 1) inhibited, 2) non-affected, or 3) activated by HMs.

3.2. Material and methods

3.2.1. Data collection

Results for EAs of dehydrogenase (DHA), arylsulfatase (ARY), urease (UA), acid (Pacid) and alkaline phosphatases (Palk), β -glucosidase (Gluc), and catalase (CAT) were extracted from studies regarding HM contaminated soils. Other EAs such as invertase and some proteases were not included in the analysis due to scarce data. Data collection was made using the Thomas Reuters Web of Science and Elsevier's Scopus. Keywords used for the search included “soil*”, “enzyme*”, “enzyme activities”, “enzymatic activities”, and “microbial activity” (Figure S1 available in Aponte et al., 2020c). This searching resulted in 553.008 titles. This first list was refined by the topics “heavy metal*”, “pollution”, and “contamination”, which resulted in 405 articles. The searching was

further refined by the kind of document (only articles), resulting in 374 scientific articles. From these articles, the final selection was made manually by selecting the papers that addressed HM contamination and met criteria relating to quality (mentioned below) and data availability. The reference lists of all the selected papers were also checked for further potential articles. In total, this search protocol resulted in the selection of 46 research articles. Only studies that presented the means, number of replicates, and standard deviations (or standard errors) of the results along with the descriptions of the controls and HM treatments in tables, figures, or text were considered. When some of this information was not shown, the corresponding author of the article was contacted in attempt to obtain the relevant data. The data extraction from figures was made using the PlotDigitizer software (<http://plotdigitizer.sourceforge.net/>).

From this selection eight influential factors on soil EAs were obtained: 1) total and available HM contents (Pb, Zn, Cd, Cu and As), 2) soil pH, 3) soil texture (total clay and sand particle size fractions), 4) soil organic C content, 5) experiment type (field or laboratory), 6) contamination type (individual or multicontaminated), 7) salt type (for bioassays), and 8) soil depth.

The following quality criteria were set for each paper: 1) At least two levels of HM contamination: a) Control: EAs in non-contaminated soil; and b) Treatment: EAs in HM contaminated soil of the same origin and properties. Note that some of the studies contained more than one contaminated condition; 2) Studies should contain the sampling depth and associated soil property information including pH, and total contents of clay, sand, and organic carbon; 3) studies that included a control condition (e.g. non-contaminated soils), or reference sites should have HM content statistically different to the treatment condition (contaminated soils); 4) in cases of studies with several incubation periods, the longest incubation duration was taken; 5) in studies with factorial designs, only significant treatments focused on the HM contamination effects were considered, avoiding interactions with other factors; 6) when a study did not have an experimental control or uncontaminated reference sample, the treatment or site with statistically lower HM content was included as non-contaminated soil as long as its physicochemical properties were similar to those of the contaminated soils; 7) when studies included the effects of individual and grouped HM contaminants, both cases were considered.

For the analysis, data were organised for direct comparison between the Control and Treatment, which was considered as an Observation. Several observations per study were obtained for each enzyme depending on the number of HM levels/treatments. For instance, a study with one control and three contamination levels results in three observations in the following order: 1) Control vs Level 1; 2) Control vs Level 2, and 3) Control vs Level 3. The same approach was applied to studies with different spatial points. Only EAs whose data came from at least five publications were analysed. We obtained a total of 671 observations, which were distributed per enzyme as follows: 171 for UA, 143 for DHA, 107 for CAT, 103 for Pacid, 56 for ARY, 67 for Palk, and 24 for Gluc.

3.2.2. Data analysis

The effects of HMs on EAs were evaluated by the decrease or increase in EA (%), relative to the control or reference condition. In this context, positive values indicate EA increases, while negative values indicate decreases. To estimate individual effects on EAs, we used the log response ratio (LRR) (Hedges et al., 1999).

$$LRR = \ln (Y_{contaminated}/Y_{non-contaminated}) \quad (\text{Eq. 1})$$

where “Y” represents the arithmetic mean values of the EA in the contaminated (treatment) and non-contaminated (control or reference) soils. The variance (v_i) of LRR was calculated as:

$$v_i = \frac{SD_{contaminated}^2}{n_{contaminated} * Y_{contaminated}^2} + \frac{SD_{non-contaminated}^2}{n_{non-contaminated} * Y_{non-contaminated}^2} \quad (\text{Eq. 2})$$

where “SD²” represents the standard deviations for contaminated and non-contaminated soils, and “n” is the number of replicates (sample size) for each condition.

The LRR was back-transformed to percentage change to ease interpretation in figures, as:

$$Percentage (\%) = [\exp(ES) - 1] \times 100 \quad (\text{Eq. 3})$$

Due to our focus on evaluating responses of the various EAs under different HM contamination levels as opposed to a global effect, a heterogeneity test was not carried out (Mergensen et al., 2013). Instead, we evaluated differences among responses of the seven EAs tested using the omnibus test of moderators (QM test). After checking differences among the enzymes, we performed separate meta-analyses for each one.

Meta-analyses are frequently weighted by the inverse variance of the response ratio in each study (Gurevitch et al., 2001); but variance information was not available for several papers. Thus, we used a subset of studies reporting variances to estimate the average within-study variances as an arithmetic mean of the available within-study variances for each outcome variable, and used this obtained value to approximate within-study variances for the remaining effect sizes which had that information missing (Moreno-Mateos et al., 2017).

Bootstrapping tests with 999 iterations were used to generate the 95% confidence interval (CI) (Hedges et al., 1999). To address potential moderators, meta-regression analysis was applied for those continuous factors. We explored the potential moderating role of total and available HM contents, pH, organic carbon, and the clay and sand content, considering only observations with available data ($n > 7$). In these analyses, HM outliers were not considered. In some cases, we established categories for the type of experiment, type of salt used (for bioassays), and HM contamination levels.

Publication bias was checked by visual examination of the funnel plots for each EA (Zuber and Villamil, 2016). All EAs except Gluc and CAT showed balanced funnel plots (Figure S2 available in Aponte et al., 2020c). All analyses were made in the R Language and Environment for Statistical Computing version 3.3.1 using the “metafor” package (Viechtbauer, 2010).

3.3. Results

3.3.1. General impacts of heavy metals and metalloids on enzyme activities

The activities of all enzymes studied strongly decreased under HM contamination relative to baseline values in non-contaminated soil (Figure 1), as evidenced by negative LRRs. Activities of ARY and DHA were the most sensitive to HM contamination, showing 72% and 64% reductions, respectively. Activities of Gluc and UA showed an intermediate

decrease of 45% and 40%, followed by Pacid with a 29% decrease. The least sensitive enzymes were Palk and CAT (Figure 1).

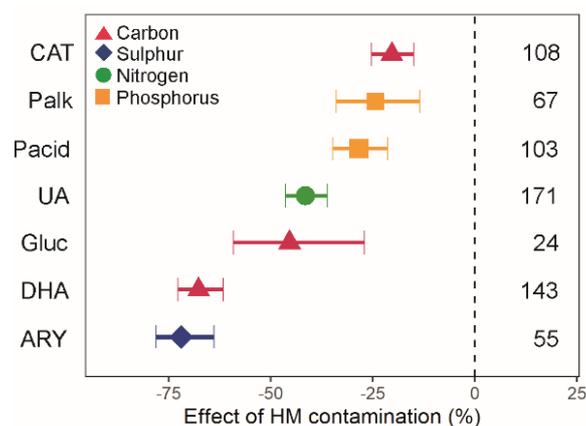


Figure 1. Overall effects of heavy metal (HM) contaminated soils on the activity of seven enzymes. The numbers on the right represent the number of observations per enzyme activity (EA). The negative percentage corresponds to the decrease of EAs by HM contamination compared to uncontaminated control soil (zero line). The effects are significant if whiskers (confidence intervals) do not overlap the zero line. Note that the presented responses of EAs covers means over various HMs and with varying contents. ARY = arylsulfatase; DHA = dehydrogenase; UA = urease; Pacid = acid phosphatase; Palk = alkaline phosphatase; CAT = catalase; Gluc = β -glucosidase. Red symbols denote enzymes associated with C cycling, green for N cycling, yellow for P cycling, and blue for S cycling.

3.3.2. Effects of contamination levels on individual enzyme activities

Four HM contamination levels were chosen according to that used by many authors (Friedlová, 2010; Mikanova, 2006; Šmejkalová et al., 2003; Stazi et al., 2017) and were termed as follows: 1) “Very low” and “Low” = Low; 2) “Moderate ” and “Medium ” = Medium; 3) “High ” and “Source of contamination ” = High; 4) Extreme. These contamination levels are summarised in Figure 2 along with estimates of threshold values, which represent concentrations associated with ecological and/or health risks. Considering the similar general toxicity mechanisms for some HMs in living cells, these contamination levels can also account for a joint HM effect.

The activity of most of the enzymes decreased in response to increasing total and available HM content (Figure 3). DHA was the most sensitive, with the activity decreasing from 41% (Low) to 80% (Extreme). UA was the only enzyme with significant difference between “Low”, two intermediate contamination levels (“Medium” and “High”), and “Extreme”. Palk was only inhibited at “Low” and “Medium” HM levels. Gluc and CAT did not show consistent responses with the increasing HM contamination levels (Figure 3).

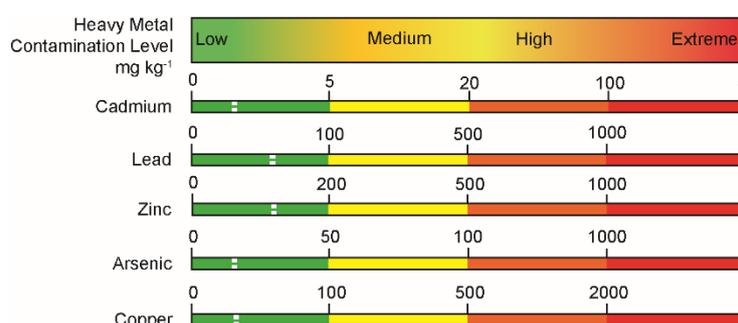


Figure 2. Classification of the total contents (mg kg⁻¹) of various HMs in soil into four contamination levels for health risks: Low, Medium, High and Extreme. The white dotted white line on the left of each scale represents the threshold value related to ecological and/or health risks (Ministry of the Environment, 2007). The four levels were adapted from those used in many studies (e.g. Aponte et al., 2020; Friedlová, 2010; Mikanova, 2006; Šmejkalová et al., 2003; Stazi et al., 2017) based on general toxicity mechanisms of HMs in living cells.

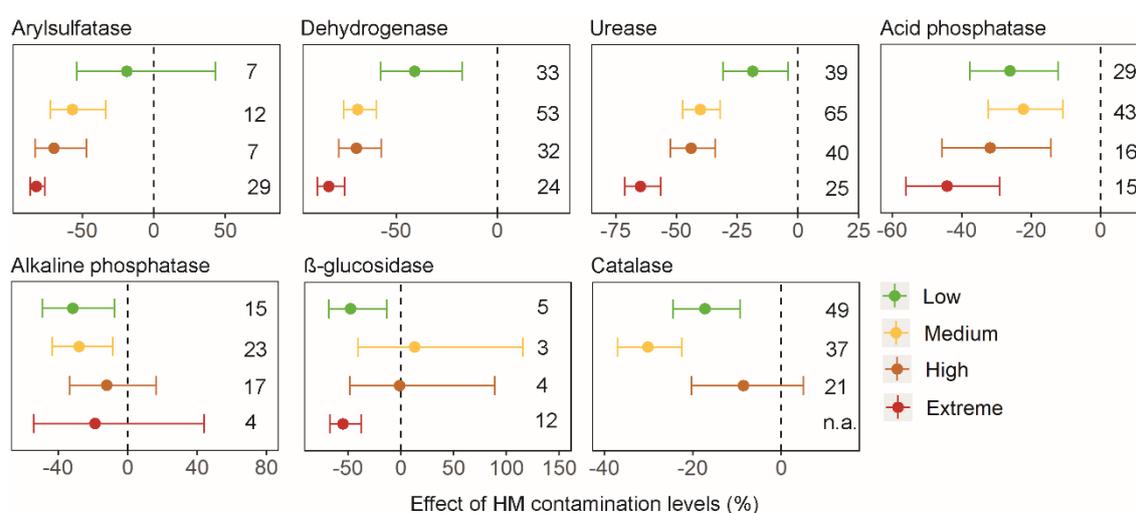


Figure 3. Effect of four HM contamination levels (Figure 2) on activities of seven enzymes. Number on the right represents the number of observations per enzyme and

contamination level. The effects are significant if whiskers (confidence intervals, CI) do not overlap the zero line. n.a. = no available data. Note that large CIs are mainly associated with cases that had a low number of observations.

3.3.3. Specific effects of individual heavy metals on individual enzyme activities

Meta-regressions allowed for evaluation of the relationships between individual HM contamination levels and EAs. Activities of ARY and UA were diminished by increasing the total contents of Pb, Zn, Cd, and Cu (Figure 4). DHA was only decreased by increasing As total content (Figure 4). In correspondence with the results displayed in Figure 1, ARY and DHA activities had the strongest negative slope with the increasing HM contents. Pacid and Palk were not included in this analysis because HM data did not comprise contrasting contents.

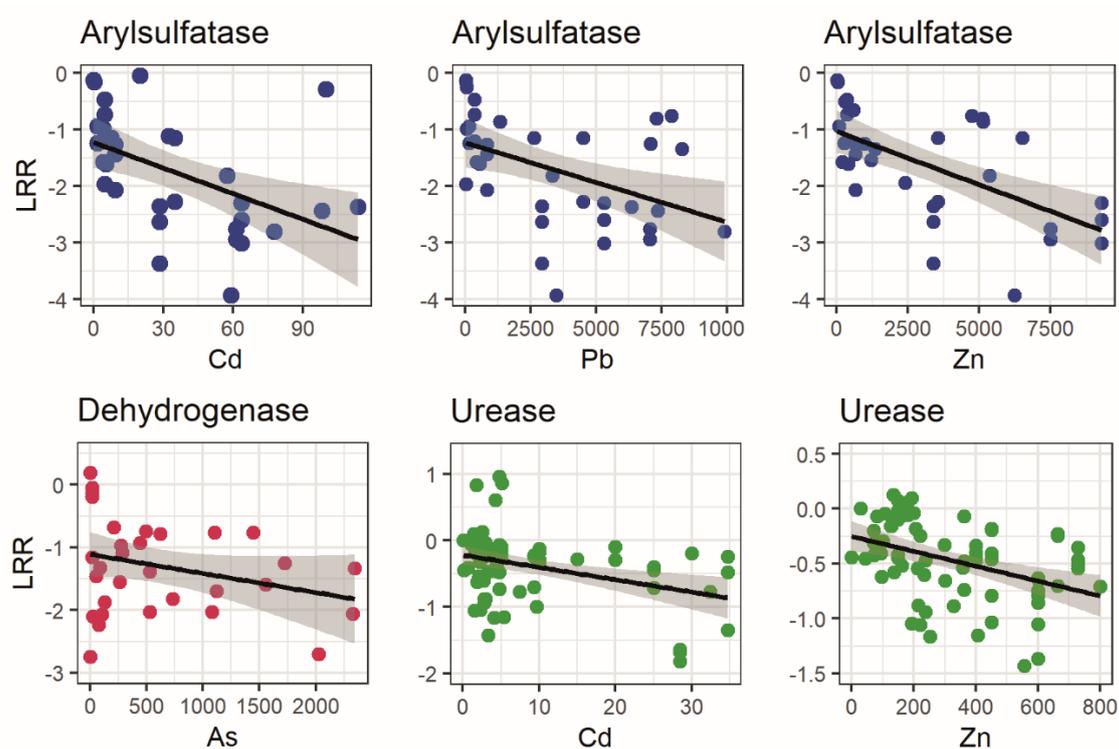


Figure 4. Meta-regression between the total heavy metal (HM) content and log response ratios (LRR) of enzyme activities (EAs). Total HM contents are presented in mg kg^{-1} . Only significant regressions ($p < 0.05$; Table S1 available in available in Aponte et al., 2020c) between EAs and HMs are shown. The enzyme-HM combinations without significant effects are not presented.

Available HM contents in soil also decreased EAs (Figure 5). DHA activity was only diminished by increasing available As (Figure 5). Similarly, activities of UA and CAT were linearly decreased by increasing contents of available Zn and Cu (Figure 5). Contrarily, Pacid activity increased with increasing contents of available of Cd and Cu contents (Figure 5).

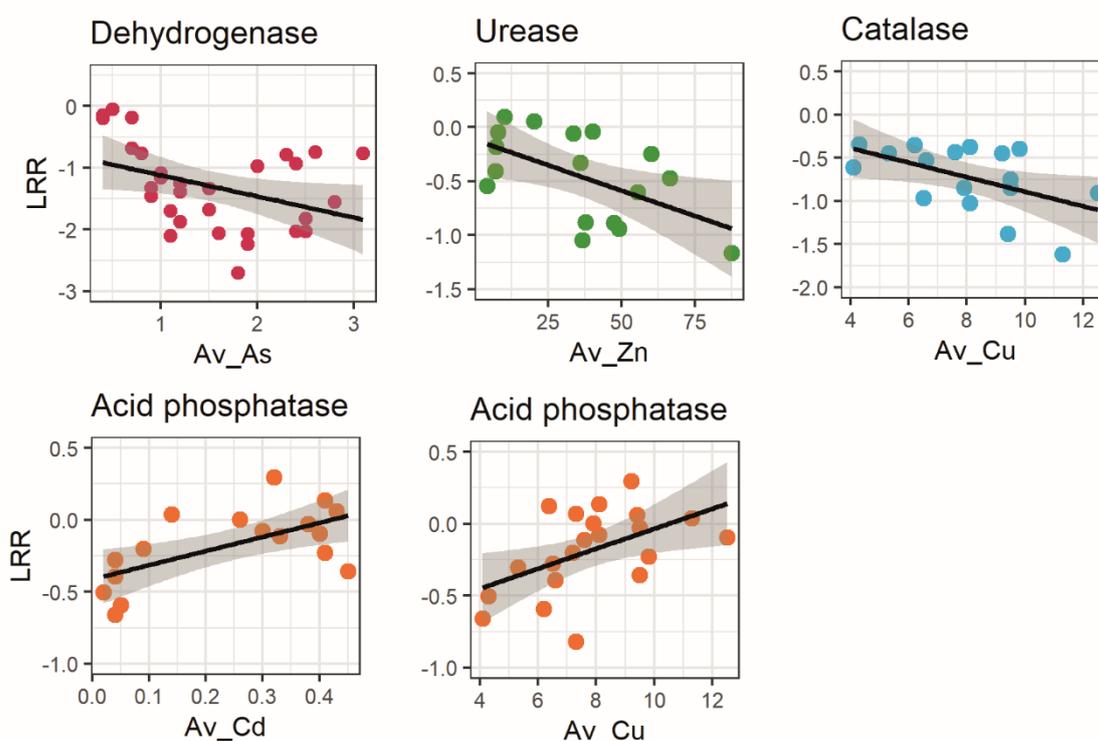


Figure 5. Meta-regression between available heavy metal (HM) contents and the log response ratio (LLR) for enzyme activities (EAs). Available HM content is presented in mg kg^{-1} . Only significant regressions ($p < 0.05$; Table S1 available in Aponte et al., 2020c) between HMs and the LRRs of the EAs are shown.

3.3.4. Effects of soil properties on the responses of enzyme activities

All EAs were affected by soil sand and/or clay content (Figure 6A and B). Therefore, the soil texture was the most important factor moderating the negative effects of HM contamination on the activities of the most enzymes. The HM effect on the activities of Pacid, Gluc and ARY decreased with the clay content (Figure 6A). Correspondingly, DHA, Pacid, Palk, Gluc, and UA linearly decreased with increasing sand content (Figure 6B). Of note is that the increase of activities with increasing clay content, or decrease with increasing sand content, have similar slopes for some enzymes (Figure 6). Further

to clay and sand content, ARY increased with increasing pH and total organic C (Figure S3 available in Aponte et al., 2020c).

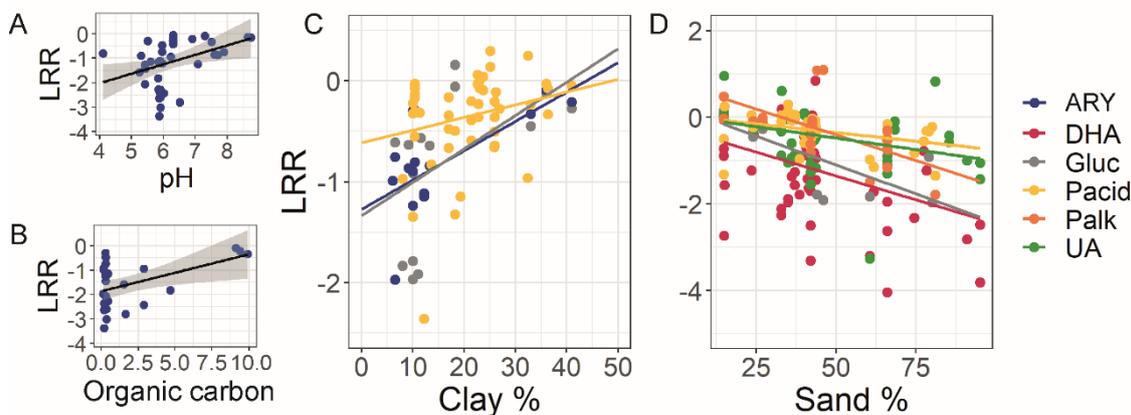


Figure 6. Meta-regression between the log response ratio (LRR) of A) enzyme activities (EAs) versus clay content, and B) EAs versus sand content. Only significant regressions ($p < 0.05$; Table S2 available in Aponte et al., 2020c) between soil texture parameters and the LRRs of each EA are shown. ARY = arylsulfatase; DHA = dehydrogenase; UA = urease; Pacid = acid phosphatase; Palk = alkaline phosphatase; CAT = catalase; Gluc = β -glucosidase.

3.3.5. Effects of experimental factors on enzyme activities

Comparing the experiments under field and controlled (e.g. laboratory) conditions, the activities of ARY and Gluc were only reduced in field experiments (Figure S4 available in Aponte et al., 2020c). Contrarily, Palk only decreased in laboratory experiments. The activities of DHA, UA, Pacid, and CAT were suppressed identically in laboratory and field experiments.

The HM salt type applied in laboratory experiments showed specific effects on EAs, with exception to Gluc activity. There is a clear difference in the use of some HM salts for artificial contamination (spiked soils) studies (Figure 7). Chloride and acetate salts of HMs are more widely used to study EAs than nitrate and sulphate salts. Chloride and sulphate salts decreased the activity of DHA, Pacid, Palk, and UA (Figure 7). Nitrate and the mixture of sulphate plus nitrate salts (dual contamination) did not affect EAs at all (Figure 7).

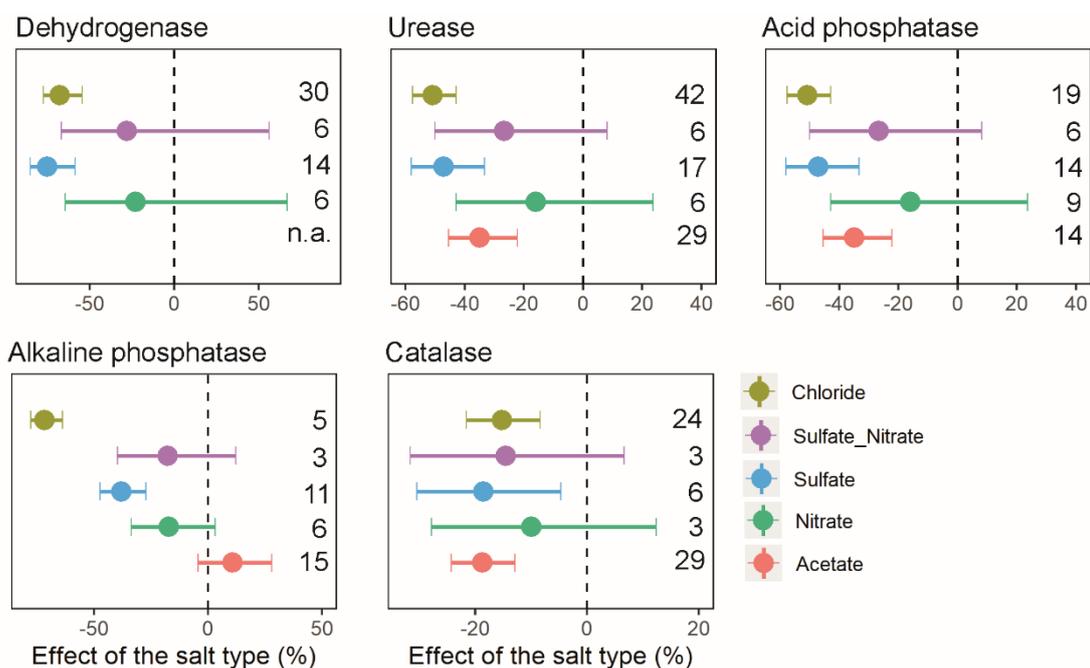


Figure 7. Effects of heavy metal (HM) contamination on five enzyme activities (EAs) depending on the salt type used in laboratory experiments (spike studies). The number on the right represents the number of observations. The effects are significant if whiskers (confidence intervals, CI) do not overlap the zero line. Note that these results show the effect of various HMs together, and that the large CIs are mainly associated with cases that have low numbers of observations.

The contamination type (CT) only showed different trends for the activity of ARY and Palk. HMs only diminished ARY activity in multicontaminated soils (Figure S5 available in Aponte et al., 2020c). Contrarily, Palk activity was reduced by individual HM contamination. Activities of DHA, Palk, and CAT increased with soil sampling depth (SD), showing a positive linear relation (Figure 8). Thus, the HM effects on EAs decreased with depth (LRR closer to zero), showing the strongest negative effect of HMs in the topsoil.

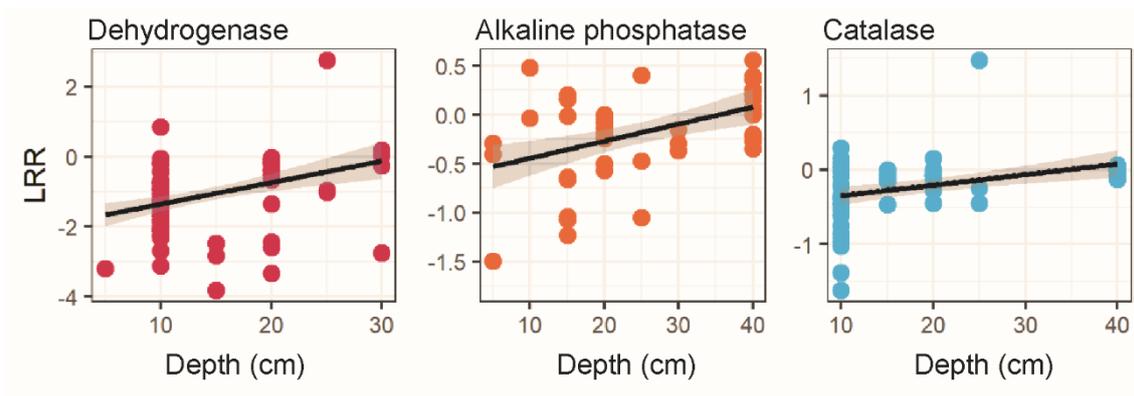


Figure 8. Meta-regression between the log response ratio (LRR) of heavy metal effects on activities of three enzymes depending on soil depth. Only significant regressions ($p < 0.05$; Table S3 available in Aponte et al., 2020c) between soil depth and the LRR of EAs are shown.

3.4. Discussion

3.4.1. Impacts of heavy metal contamination on enzyme activities

Our global meta-analysis shows that metal(loid) contamination decreased the activities of all enzymes studied, and these decreases were enzyme specific. The activities of ARY and DHA were the most affected because they mainly depend on active microbial biomass since: 1) ARY catalyses the hydrolysis of sulphate aromatic esters of fungal cell walls (Moscatelli et al., 2005) and it is mainly produced by certain bacteria (Beil et al., 1995; Ueki et al., 1995). Because the intracellular activity of ARY is more than ~70% compared to its extracellular activity (Dick and Burns, 2011; Klose et al., 1999), we interpret ARY mainly as an endoenzyme; and 2) DHA only reflects the intracellular activity of active microorganisms (Nannipieri et al., 1990) transferring hydrogen from organic substrates to inorganic acceptors (L. H. Zhang et al., 2010; N. Zhang et al., 2010). According to our hypothesis, ARY and DHA are more sensitive to HM contaminated soils because they are mainly endoenzymes and their activities are directly dependent on microbial metabolism. Increasing HM contents inside microbial cells cause protein denaturation of endoenzymes, interactions with amino acidic residues in the catalytically active sites of enzymes, and/or reactions with enzyme substrate complexes, which result in enzyme activity reduction. It is also worth considering that both ARY and DHA are strongly correlated with the microbial activity and soil organic matter content (Awad et al., 2018; Liang et al., 2012; Nivellet al., 2016). Thus, the mechanisms involved in the

decreasing of DHA and ARY activity by HMs are based on the associated toxicity of these elements to microorganisms mainly due to: 1) HMs binding with greater affinity than essential elements (Poole and Gadd, 1989) and 2) the displacement of essential metals from their native binding site or through ligand interactions (Bruins et al., 2000).

The increasing of both total and available As contents reduced DHA activity (Figure 4), which reflects the negative effects of As on active microbial cells by: 1) affecting sulfhydryl groups in proteins, which causes malfunctioning of cell respiration, cell enzymes, and mitosis; and 2) arsenate (AsO_4^{3-}) interfering with phosphorylation and energy production because of its chemical similarity to PO_4^{3-} (Gordon and Quastel, 1948). Other HMs, such as Cd, Pb, and Zn, did not influence DHA, possibly due to most of the associated data focusing on low and medium levels of HM contamination.

Arylsulfatase (ARY) can be linked to intra and extracellular activity (Klose et al., 1999; Tabatabai and Bremner, 1970a, 1970b). ARY activity decreases with increasing HM contamination because of 1) HMs masking catalytically active groups; 2) the denaturing of protein conformation, or 3) HMs competing with metal ions that are needed to form enzyme-substrate complexes (D'Ascoli et al., 2006; Gianfreda and Bollag, 1996). In this sense, the intensity of the hydrolysis of sulfate esters can be inhibited by Cu, Pb, Zn, and Cd because of the reaction with -SH groups (Al-Khafaji and Tabatabai, 1979) and their interactions with glycine, aspartic acid, and histidine in the ARY active site (Boltes et al., 2001). This aligns with the findings of this meta-analysis, whereby ARY activity was inhibited by increasing total contents of Cd, Pb, and Zn.

β -Glucosidase (Gluc) was the third most sensitive enzyme to the overall level of HM contamination. Gluc regulates one of the primary energy sources to microorganisms since it catalyses the cellulose hydrolysis that results in two glucose molecules (Alef and Nannipieri, 1995). This inhibition of Gluc activity is caused by the HMs either forming complexes with the substrate, combining with the protein-active groups of the enzymes, or reacting with the enzyme-substrate complex (Eivazi and Tabatabai, 1990). Gluc is understood to be sensitive to HM contamination, but its response to increasing HM contents is not well known due to scarce data. This lack of data likely contributed to the absence of statistically significant regressions for Gluc activity as a function of available or total HM contents.

Urease (UA) was the fourth most sensitive enzyme to overall HM contamination (Figure 3). The UA activity decreased with HM content because of the changes of chemical conformation mainly due to coordination reactions. Based on Lewis's hard and soft acids and base theory (HSAB), the active sites in UA, such as thiol or imidazolyl groups, are preferentially coordinated when HM contents are low (Yan et al., 2013). When assessed across individual HMs, UA was only negatively moderated by total and available Zn concentrations, explained by the interactions of Zn with cysteine residues in the UA active site (Mazzei et al., 2018).

Acid phosphatase (Pacid) activity increased as a function of low available contents of Cd and Cu (Figure 5). Pacid was the only enzyme that increased in activity due to HM contamination. Although Cd and Cu can interact with amino acidic residues in the phosphatase active site, and hence inhibit its activity, the positive effects of some HMs to Pacid and Palk can be related to 1) a stimulatory effect of very low HM contents on microbiota, 2) the positive effect of some physicochemical properties such as pH, organic matter, and clay content, or 3) the absence of available phosphorus that enhances the phosphatases releasing. Thus, Pacid and Palk were not very sensitive to HM contamination (Figure 1), by which a stimulatory effect on microbiota could have occurred since most of Pacid and Palk data showed moderate acidity, clay contents of >20%, and organic matter contents of >40%.

Catalase (CAT), which is involved in the disproportionation of (Prosser et al., 2011), was the least sensitive enzyme to HM contamination. This enzyme is primarily reported as intracellular (Trasar-Cepeda et al., 1999); however, this enzyme has also been suggested as an exoenzyme, which can be more stable than intracellular CAT due to its sorption on organo-mineral complexes (Bello et al., 2013; Calamai et al., 2000). We hypothesise that this is because: 1) CAT is involved in H₂O₂ detoxication processes (Duan et al., 2018) and HM contamination frequently results in oxidative stress, which acts to increase H₂O₂ production and hence stimulate CAT activity; 2) CAT immobilisation on organo-mineral surfaces increase the enzyme stability, which results in less sensitivity to HM contamination.

3.4.2. Effects of soil properties on the response of enzymes to heavy metal contamination

The effects of HM contamination on EAs can be mediated by soil pH (Dick, 2011), clay content and composition (Burns, 1986; Nannipieri et al., 1978; Tietjen and Wetzel, 2003), and organic matter content (Dick, 1997; Nannipieri, 1994; Tang et al., 2020). Since extracellular enzymes can be immobilised in organo-mineral-clay complexes, they are more resistant to thermal and proteolytic degradation compared to intracellular enzymes (Burns, 1986; Nannipieri et al., 1978; Tietjen and Wetzel, 2003). However, organo-mineral complexes can also interact with HMs, lessening the effects of contamination on EAs (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999). HMs can interact with clay surfaces by sorption (Bolan et al., 2014; Harter and Naidu, 1995), and so HMs might compete with enzymes to form metal-organo-mineral complexes, which lead to enzyme deactivation because free (non-stabilized) enzymes are more susceptible to temperature and proteolytic attacks (Aponte et al., 2020b; Burns et al., 2013).

The HM effects on ARY activity decreased (LRR closer to zero) at higher pH and lower TOC (Figure S3 available in Aponte et al., 2020c). The optimum pH range for ARY is between 7 and 10 (Beil et al., 1995; Ueki et al., 1995), and the ARY activity increases upon increasing soil pH from 4 to 7 by liming (Ekenler and Tabatabai, 2003), which is a similar trend as in our meta-regression (Figure S3 Aponte et al., 2020c). Acidification of the soil may additionally intensify the HM effect (Wyszkowska et al., 2016). The increasing pH leads to the decreasing bioavailability of Cu, Cd, Zn, and Pb in soil (Xian and In Shokohifard, 1989), which results in lower HM toxicity for microorganisms and reduced inhibition of EAs.

Increasing clay content decreased the negative HM effect on the activities of ARY, Gluc, and Pacid (Figure 6). Similarly, HM effects on some enzyme activities (DHA, Gluc, Pacid, Palk, UA) increased with higher sand content. Therefore, soil texture is an important moderator of the effects of HM contamination on EAs since 1) extracellular enzymes can be immobilised on clay mineral surfaces (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999), which retard temperature denaturation and proteolytic attacks (Burns et al., 2013), 2) HM toxicity decreases with clay content because of HM sorption and complexation reactions with clay minerals (Bolan et al., 2014), which reduces HM bioavailability and toxicity to EAs, and 3) organic matter content is related to clay content in many soils via the formation of organo-mineral

complexes (Cotrufo et al., 2019), and hence related to higher microbial biomass resulting in more enzyme production (Tabatabai and Bremner, 1970b).

3.4.3. Effects of experimental factors on enzyme responses to heavy metal contamination

Previous results confirm the strong influence of some physicochemical soil properties on EAs. Still, they can also be moderated by some experimental factors such as the soil depth in field experiments or the HM salt type used in laboratory studies. Furthermore, the contamination type (individual HMs vs dual or multi-contaminated soils) moderated the HM effect on ARY activity in HM contaminated soils. Cumulative or even synergistic negative effects (Khan et al., 2007) are found to be strong for ARY activity in soils multi-contaminated with Pb, Cd, and Zn (Figure S5 available in Aponte et al., 2020c).

The experiment type (field or laboratory) was a significant moderator only for ARY activity in field studies, while HM contamination reduced Palk activity only in laboratory experiments (Figure S4 available in Aponte et al., 2020c). These results in both cases (ARY and Palk) are attributed to experimental conditions since field studies of ARY activity are generally characterised by “High” HM contents and the presence of several HMs that can have synergetic effects. Furthermore, the duration of the HM contamination in the field is commonly much longer than in laboratory studies, and consequently, microbial communities have longer to adapt to both HM contamination and the concomitant salt effect. Contrarily to ARY, Palk activity was only reduced by HMs in laboratory experiments, where HM contents ranged from “Medium” to “High” levels, while field experiment mostly showed “Low” HM levels. Additionally, other experimental factors such as the salt type and soil depth, which also depend on the experiment type (laboratory and field), can influence the effects of HMs on EAs.

The effects of HMs of EAs in laboratory experiments depend on the salt type (Carine et al., 2009; Karaca et al., 2010; Yang et al., 2006). The HM effects on all EAs were influenced by the salt type (at least by one salt; Figure 7). Salts decreased EAs in the following order: chloride > sulphate > acetate > nitrate. Chloride ions act as competitive or non-competitive inhibitors of EAs, which can disturb the structure of the enzyme active sites (Naki, 1981). Sulphate or nitrate salts supply N and S to microorganisms that supports enzyme production (Karaca et al., 2010), which is partly

similar to acetate supply for C acquisition. Therefore, it is necessary to consider the salt type in laboratory experiments. In this sense, the use of chloride salts reflects both HM and chloride effects, which may mask the precise impact of HMs on EAs. On the other hand, sulphate and nitrate salts provide nutrients for microorganisms, and so, the negative effect of HMs on EAs can be weakened. For this, a well-designed spiking process and suitable control treatments are needed to ensure HM interaction with the soil matrix and to avoid the initial effect of sulphate and nitrate salts (see example in Ginocchio et al., 2006).

Another moderator of the HM effects on EAs is soil depth. DHA, Palk, and CAT showed lower HM inhibition of EAs with increasing depth. This result is similar to that found in a meta-analysis of tillage effects on EAs (Zuber and Villamil, 2016). In our meta-analysis, the negative effect of HMs on EAs is greatest in the topsoil, and weakens with depth. As the HM input occurs primarily at the soil surface, the most active HM forms will be adsorbed in the topsoil by clays and organic matter, with less HMs reaching the subsoil.

3.4.4. Consequences of heavy metal contamination for C, N, P, and S cycling in soil

The results of the meta-analysis showed clear negative effects of HM contamination on all EAs, which has consequences for C, N, P and S and cycling in soil (Figure 9). Of all enzymes, ARY and DHA have the greatest activity decreases in response to HM contamination. DHA is related to the C cycle since it transfers hydrogen from organic compounds to inorganic acceptors (L. H. Zhang et al., 2010; N. Zhang et al., 2010) in living microbial cells. More broadly, dehydrogenases are the main representative of the oxidoreductase enzyme class (Gu et al., 2009). In contrast to DHA, ARY hydrolyses organic sulphate esters (Tabatabai and Bremner, 1970a) and releases available sulphate from organic matter, and so, regulates the S availability to plants and microorganisms (Li and Sarah, 2003). Therefore, given the susceptibility of ARY and DHA activities to HM contamination, microbial processes involved in C and S cycles might be retarded by HM contamination, resulting in potential C and S limitation for microorganisms. This is especially relevant given the background of worldwide S depletion in soils caused by continuous S removal from harvests, the absence of acid rains, and low or absent S fertilization (Lucheta and Lambais, 2012). Furthermore, HM contamination may slow down C cycle related processes in various intensities since Gluc and CAT activities were

less affected by HMs than DHA (Figure 9). This suggests that the total oxidative activity of soil microorganisms (reflected by DHA activity) is more strongly inhibited than cellulose hydrolysis (Gluc activity) and the releasing of oxygen from hydrogen peroxide (CAT).

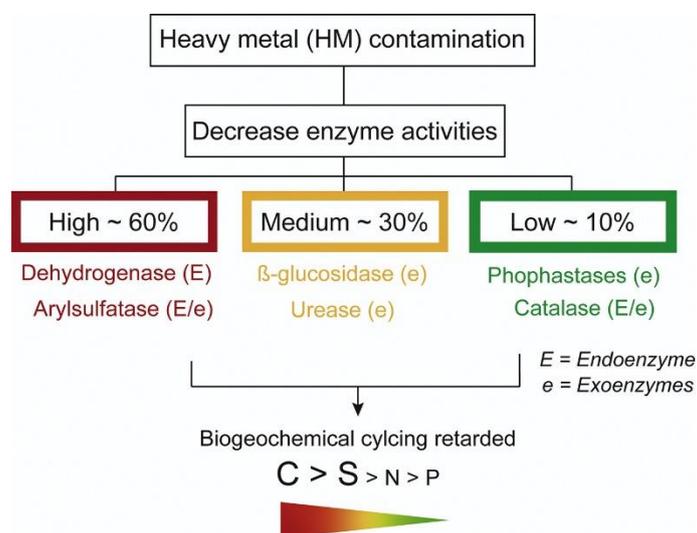


Figure 9. Conceptual diagram summarizing the results of the heavy metal (HM) contamination effects on enzyme activities (EAs).

Concerning N and P cycles, the activity of UA and Pacid were inhibited by HM contamination, which can result in the slower N and P cycling, respectively. Since UA activity was reduced by 36% compared to 21% for Pacid activity, HM contamination could result in a lower N/P ratio in the available pool, causing stronger N limitation for plants and microorganisms. In this context, it can be suggested that HM contamination might alter the stoichiometry of nutrients due to the contrasting ways EAs associated with C, N, P and S cycles are affected. This alteration to EAs is important since EA ratios reflect the equilibria between the litter, soil organic matter, and the elemental composition of microbial biomass in soil. Also, nutrient stoichiometry is related to the efficiency of the microbial nutrient acquisition and growth (Sinsabaugh et al., 2009). Because of the smaller reduction of Pacid activity compared to UA and especially to ARY and DHA, less N and especially P fertilization may be necessary, whilst S input may be useful to counterbalance the HM induced nutrient limitation in soil.

In evaluating the HM effects on EAs, it must be considered that EA assays showed reaction rate (V_{max}) values obtained under controlled conditions with optimal

temperature, pH, and excess of substrate artificially added (Dick, 2011; Kandeler, 2015). Thus, the V_{max} from traditional enzyme assays does not reflect the real process rates in situ under field conditions. Nevertheless, there is a clear link between the V_{max} and the process rates at the real substrate concentrations under soil conditions: Assuming that the enzymes remain the same (no isoenzymes will be produced by HM contamination) then the Michaelis constant (K_m) remains the same. Consequently, the decreased V_{max} (e.g. by HM contamination) by maintaining K_m will slow down the whole Michaelis-Menten curve, shifting it down and to the right. Consequently, the reaction rates at low substrate concentration (as is common in soils) will decrease. The only assumption here is the absence of isoenzyme production by HM contamination. Since most microorganisms are not adapted to HM contaminated soils, they do not necessarily have specific molecular mechanisms for isoenzyme production under anthropogenic contamination.

3.5. Conclusions

This meta-analysis showed a clear decrease in the activities of all enzymes in response to HM contamination. The most sensitive EAs to soil HM contamination (according to their decreasing activities) were ARY (endo- and exoenzyme) and DHA (exoenzyme) followed by Gluc, UA, and Pacid (all exoenzymes). Consequently, active microorganisms and their production of endoenzymes (including DHA and the intracellular ARY activity) are more stressed by HM contamination than biochemical processes governed by exoenzymes (such as Gluc, phosphatases, and UA). Increasing HM contents linearly decreases EAs, which was evident especially for ARY with Cd, Pb, and Zn.

The effects of HM contamination on EAs are influenced by soil physicochemical properties. The negative effects of HMs on ARY were moderated by increased soil pH and organic carbon content. The activities of ARY, Gluc, and Pacid increased with clay content under HM contamination, while all EAs decreased with increasing sand content. Thus, physicochemical soil properties such as pH, organic matter, and texture can strongly change the effects of HM contamination on EAs. Experimental factors such as experiment type (field or laboratory), contamination type (individual or multicontaminated soils), and salt type used for bioassays also influenced the effects of HM contamination on EAs, with stronger effects in the topsoil compared to

the subsoil. Chloride salts of HMs negatively affected the EAs more so than sulphate, nitrate, or acetate HM sources. Since chloride ions act as an EA inhibitor, the chloride salts of all HMs have stronger negative effects on EAs compared to sulphates and nitrates. Consequently, the correct selection of concomitant anions is crucial for spiking studies.

This meta-analysis suggests that C and S cycling might be more negatively affected (retarded) by HM contamination than N and P cycling. The individual steps of biogeochemical cycles are not equally slowed down by HM contamination since the activities of DHA, Gluc, and CAT decreased by varying magnitudes. To confirm this statement, more EAs for each biogeochemical cycle should be evaluated. Nevertheless, we hypothesise stronger retardation of C and S cycling compared to N and P cycling, which could be accounted for with decreased N and P fertilization on cultivated land. Because HM contamination is a global environmental issue, stoichiometric ratios between C, N, P and S as well as their limitation for plants and microorganisms can be predicted based on the EAs response. Finally, the results of this meta-analysis suggest that EAs, especially DHA and ARY, are good indicators for soil health and quality, chemical degradation and restoration processes, as well as ecosystem functioning in HM contaminated soils.

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CHAPTER IV. *“Alteration of enzyme activities and functional diversity of a soil contaminated with copper and arsenic”*

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

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Abstract

Copper (Cu) mining has to address a critical environmental issue related to the disposal of heavy metals and metalloids (metal(loid)s). 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 metal(loid)s, being also the first assessment of EAs and the use of CLPP (MicroResp™) to study the environmental impact in Cu/As contaminated soils.

Keywords

Biochemical properties, heavy metals, metal(loid)s, toxic elements, physiological profile, ecotoxicology.

4.1. Introduction

Soils contaminated with heavy metals and metalloids (metal(loid)s) 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 metal(loid)s 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 metal(loid)s 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 metal(loid) 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 metal(loid) 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 metal(loid)s 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 metal(loid)s such as Ni and Cr studied less frequently and V, Co, As, Hg, Ag, Mn, and Se rarely studied (Stazi et al., 2015). Additionally, despite results showing the inhibition of soil enzymes with metal(loid) 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 metal(loid)s 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 metal(loid) 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 and it is necessary to look at other approaches to gain a fuller understanding of the impacts of metal(loid)s 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, the aim of this study was to compare the impacts of Cu/As contamination on EAs and soil microbial functional diversity.

4.2. Materials and methods

4.2.1. 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 physico-chemically characterized (Table 1) as described below. This soil was spiked with Cu (CuSO₄·5H₂O; grade: ACS, ISO, Reag. pH. Eur; Merck, Chile) and As (HAsNa₂O₄; ≥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 Figure S1 available in Aponte et al. (2020a).

Table 1. Soil composite sample characterization.

pH	5.55
EC ($\mu\text{S cm}^{-1}$)	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.

4.2.2. Spiking process

The spiking process was made according to Ginocchio et al. (2006) to simulate 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 three litres, 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 (Figure S2 available in Aponte et al., 2020a). 500 g of each soil was placed in triplicate plastic pots for each treatment (Figure S1 available in Aponte et al., 2020a) and kept under greenhouse conditions ($25\pm 3^\circ\text{C}$ day $15\pm 3^\circ\text{C}$ 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 metal(loid) contamination as described above.

4.2.3. 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).

4.2.4. Chemical analysis.

Chemical measured variables in experimental samples (Table S1 available in Aponte et al., 2020a) were: 1) pH and EC; 2) moisture content; and 3) Cu availability by DTPA extraction and absorption atomic measurement. DTPA extraction was used since chelating agents (e. g. DTPA and EDTA) are more effective in removing soluble metal(loid)-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 hours. 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 pre-treated to reduce As(V) (absorbance from P) (Dhar et al., 2004). The colorimetric assessment was performed by a microplate spectrophotometer (Epoch, BioTek).

4.2.5. 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 & 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 & 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 & Nannipieri (1995) where *p*-nitrophenol is released after incubation with *p*-nitrophenyl-sulfate. 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 (ED₅₀) was calculated to quantify the effects of Cu and As on EAs. ED₅₀ shows the concentration of metal(loid)s at which the enzyme activity is reduced to 50% of its uninhibited value (Tejada et al., 2008). ED₅₀ was calculated according to Knezevic et al. (2007) using the package “drc” in R statistic version 3.5.1.

4.2.6. Microbial community-level physiological profile (CLPP).

CLPP was measured using the MicroRespTM soil respiration system (MicroRespTM, James Hutton Ltd, Aberdeen, UK) according to Campbell et al. (2003). MicroRespTM 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 BiologTM 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 CO₂ 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).

4.2.7. Statistical analysis.

Results were analyzed for each metal(loid) 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 assumptions, an ANOVA was applied for knowing the effect of each metal(loid) 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. MicroRespTM 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.

4.3. Results

4.3.1. Enzyme activities

Results showed consistent results in the effects of Cu and Cu/As on all studied EAs (Figure 1) except for FDA (Figure S3 available in Aponte et al., 2020a). 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 metal(loid) levels.

Responses of individual enzymes varied according to metal, concentration and sampling time.

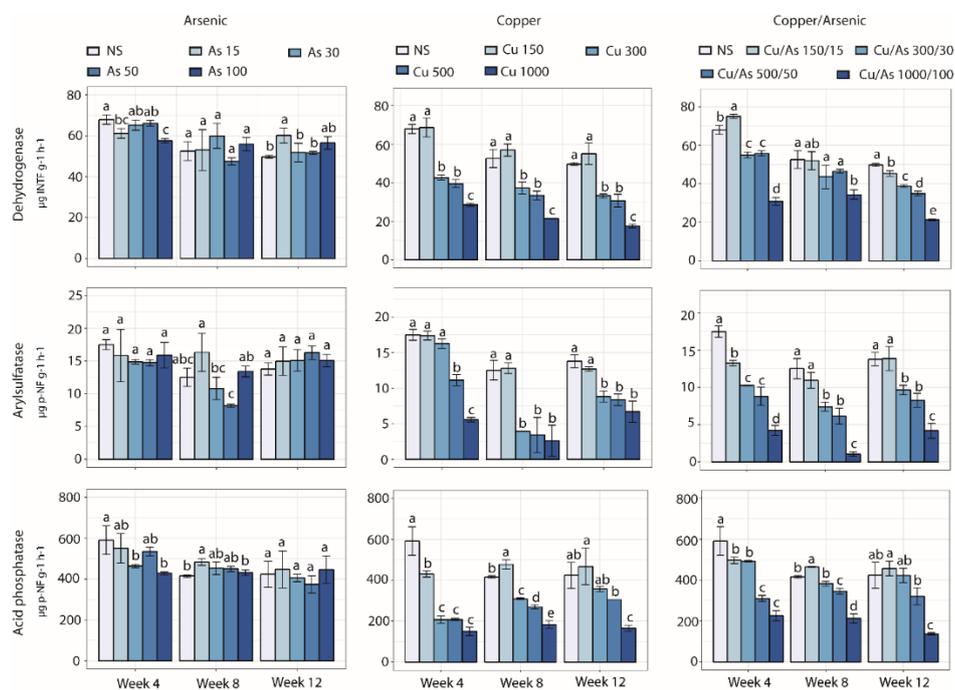


Figure 1. Activity of dehydrogenase, arylsulfatase, and acid phosphatase for copper, arsenic, and copper/arsenic contamination per each sampling time. Different letters show significant differences according to Tukey HSD ($P < 0.05$). Copper and arsenic levels represent spiking content in mg kg^{-1}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

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 (Figure 1). Arsenic negatively affected DHA only on As 15 and As 100 compared to NS at Week 4 (Figure 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 (Figure 1). These trends agree with the negative correlation between available Cu and DHA (Figure 2), where available Cu showed the highest negative relation with this EA.

Cu negatively affected to ARY at every sampling time (Figure 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 (Figure 1). Arsenic only affected ARY at Week 8 but this trend was highly variable (Figure 1). Dual contamination negatively affected to ARY in each sampling time (Figure 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 ($r = -0.84$, Figure 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 15 %. At Week 12, Pacid only showed negative effects for Cu 500 and Cu 1000 (Figure 1). Arsenic contamination only affected to Pacid in As 30 and As 100 levels at Week 4, which was absent for other sampling times (Figure 1). Dual contamination negatively affected to Pacid in a trend similar to Cu treatment (Figure 1). Correlation between Cu and Pacid was negative for all the experiments (Figure 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 (Figure 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 (Figure 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 with Cu availability in every experimental time (Figure 2).

Copper only negatively affected to UA at Week 4 (Figure 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 ($r = -0.29$, Figure 2). Additionally, UA was negatively correlated with available As(V) at Week 4 and Week 12 (Figure 2); however, its correlation strength was low ($R^2 = -0.34$ and -0.37 , respectively).

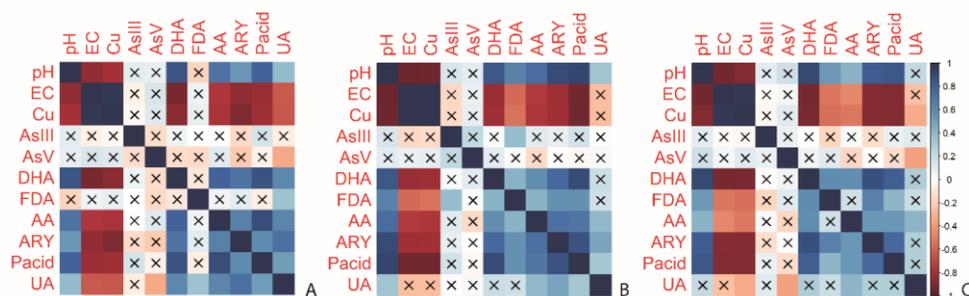


Figure 2. Correlation between enzyme activities and physicochemical properties (complete correlation plots are shown in supplementary material available in Aponte et al. (2020a) for Week 4 (A), Week 8 (B), and Week 12 (C). Crossed boxed represent non-significant correlations. Red boxes show negative 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.

Linear simple regressions showed significant relationships between all enzymes (excluding FDA) and available Cu (Table 2). AA and UA 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 in Aponte et al., 2020a). Available As(III) was not analysed as too many non-detectable values were obtained, however it was considered in correlation test by adding zeros in “non-detectable” cases.

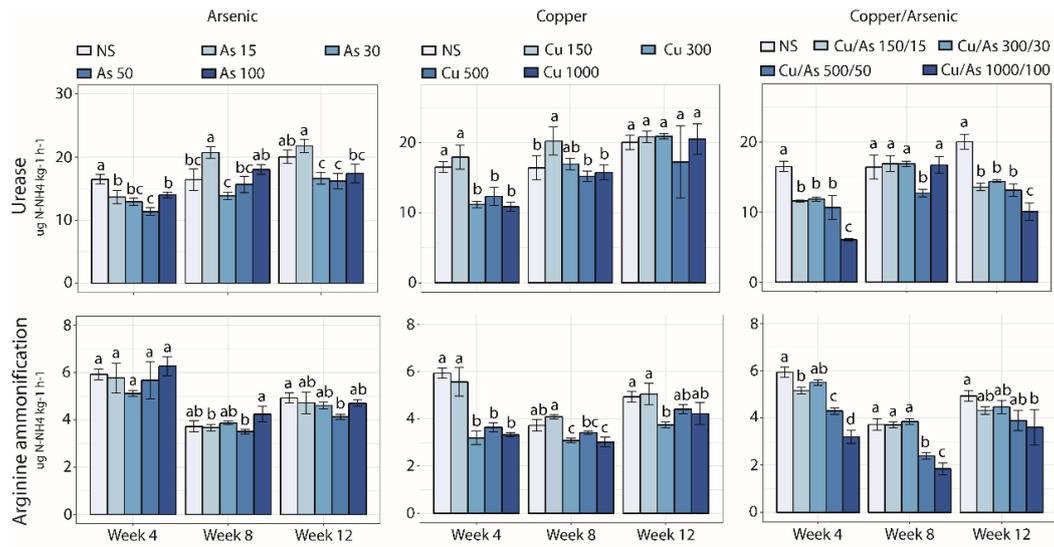


Figure 3. Activity of urease and arginine ammonification for copper, arsenic, and copper/arsenic contamination per each sampling time. Different letters show significant differences according to Tukey HSD ($P < 0.05$). Copper and arsenic levels represent spiking content in mg kg^{-1} . NS = non-spiked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

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: $\text{ARY} < \text{DHA} < \text{Pacid}$, which evidence a reduction of 50% of their uninhibited values at 128, 223, and $376 \text{ mg Cu kg}^{-1}$, respectively.

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} .

4.3.2. Community-level physiological profile (CLPP)

Carbon substrates were clearly affected by Cu and Cu/As treatments for all experimental times (Table S2 available in Aponte et al., 2020a), which allowed the CLPP discrimination of treatment levels (Figure 4). Almost all the individual C substrates were negatively affected by increasing Cu and Cu/As concentrations (especially at Week 4, Figure S4 and S6 available in Aponte et al., 2020a). 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 (Figure 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 available in Aponte et al., 2020a). Under As contamination, only AKGA, CIT, and MAL were negatively affected at Week 4 (Figure S5 available in Aponte et al., 2020a). Nevertheless, at Week 8 and 12, the negative effect of As, especially at As 30 and As 50, increased for almost all C sources (Figure S5 available in Aponte et al., 2020a). This last trend corresponds with negative correlations between available As(III) and the utilization of specific C sources at Week 8 (Figure S7 available in Aponte et al., 2020a).

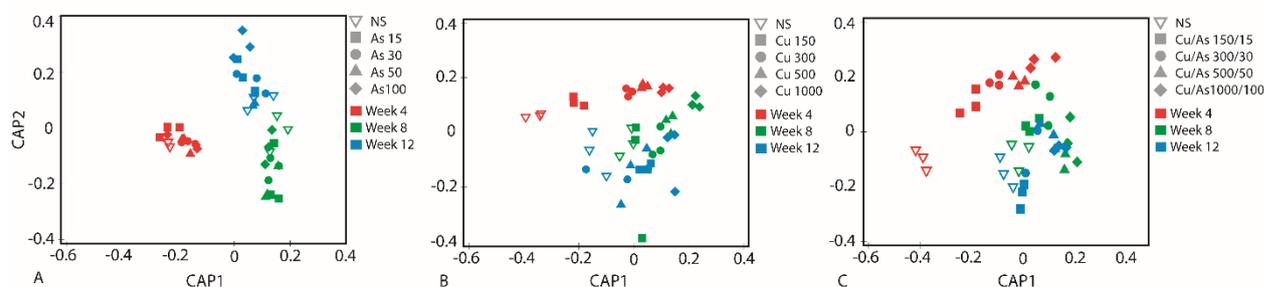


Figure 4. Canonical analysis of principal coordinates (CAP) of community level physiological profile (CLPP) for As (A), Cu (B) and Cu/As (C) treatments by each experimental time. Cu and As levels represent spiking content in mg kg^{-1} . NS = non-spiked soils.

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 (Figure 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 (Figure S7 available in Aponte et al., 2020a). 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 (Figure 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 (Figure 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.

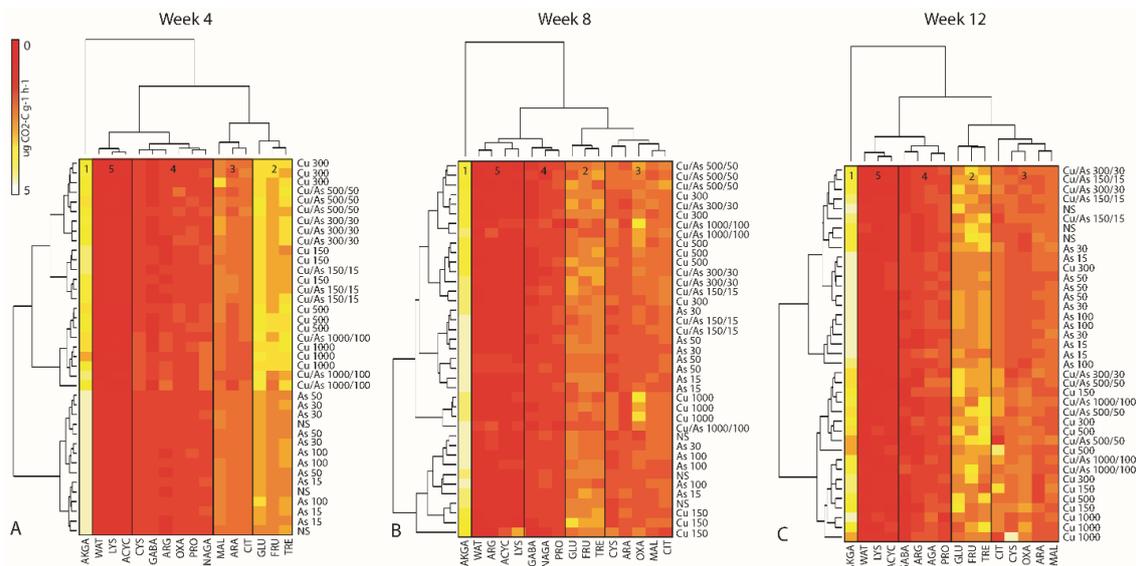


Figure 5. Heatmaps and cluster of the community level physiological profile (CLPP) by each experimental time: A) Week 4, B) Week 8, and C) Week 12. Black boxes show groups of C source utilization. The number inside the box represents the C utilization order from 1 (more used) to 5 (less used). Red represents 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 -amino butyric acid, GLU= glucose, LYS= lysine, MAL= malic acid, NAGA= N-acetyl glucosamine, OXA= oxalic acid, PRO= protocatechuic acid, TRE= trehalose, WAT= water.

4.4. 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 (L. H. 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; Wyszowska et al., 2006b). 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 ED₅₀ 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 crosslinking 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 colour 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 NH₄⁺ 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 metal(loid)

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 CO₂ and NH₃ 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 metal(loid) 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. metal(loid)s 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 ED₅₀ for urease was in the range reported by Doelman and Haanstra (1986). It is important to note that studied As concentrations are considerably lower than Cu concentrations, by which As did not affect most of biological properties here studied. Thus, Cu/As treatments showed only a Cu effect, especially since only As treatments did not affect biological properties (except for UA). 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 here evaluated. Thus, metal(loid) 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 catalyses 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 (M. C. Moscatelli et al., 2005). This last EA has been found as a good indicator of metal(loid) 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 ED₅₀ 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 metal(loid)s 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 with 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 MicroRespTM enabling the assessment of catabolic activity in the microbial community (Stazi et al., 2017). This method has been compared to the BiologTM using soils treated with sludge enriched with metal(loid)s (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 metal(loid) 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 to 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 decreasing from Week 8 to Week 12, which might be related to decreasing of microbial biomass and its 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 of microbial population groups across experimental times in Cd/Pb contaminated soils, which was associated to metal toxicity and unavailability of nutrients the whole incubation period.

4.5. 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 ED₅₀ 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 (MicroRespTM) as indicators of metal(loid) contamination. These results represent the first approach to CLPP under Cu/As contamination using MicroRespTM system. Thus, future researches should tackle the effect of individual and multiple metal(loid)s -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-MicroRespTM), which is necessary to be performed in laboratory experiments and field conditions.

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CHAPTER V. *“Enzyme activities and functional diversity in metal(loid) contaminated soils near to a copper smelter”*

Paper to be submitted to Environmental Pollution, manuscript in preparation.

Enzyme activities and functional diversity in metal(loid) contaminated soils near to a copper smelter

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Abstract

Because deleterious effects on living organisms, metal(loid)s have gained global attention, being their monitoring in the environment an important task. Soil biological properties, especially enzyme activities (EAs), are good indicators of metal(loid) contamination due to their high sensitivity, fast response, and low-cost assessment. The aims of this research were: 1) to study the effect of metal(loid) contamination on soil physic-chemical properties and microbial functionality, and 2) to detect suitable indicators of soil quality in metal(loid) contaminated soils. Soil composite samples were randomly taken at 2 to 10 Km respect with a industrial complex mainly associated to metal(loid) contamination. The Integrated Pollution Index (IPI) and the contamination degree (C_d) were calculated. EAs of dehydrogenase (DHA), arylsulfatase (ARY), β -glucosidase, urease, and arginine ammonification (AA) were studied including their ecological dose (ED_{50}) with respect to the bioavailable Cu and total As contents. Also, the community level physiological profile (CLPP) was evaluated with MicroRespTM, with the assessment of the functional diversity and catabolic evenness. All EAs were negatively affected by increasing metal(loid) contamination; however, organic matter masked the contamination negative effects of metal(loid)s on EAs. DHA and AA showed significant ED_{50} values with bioavailable Cu and total As content. CLPP showed lower and different profiles for the utilisation of all applied carbon substrates, discriminating between some metal(loid) contamination degrees. Thus, some EAs and CLPP, were suitable indicators of metal(loid) contamination in soils. This is the first assessment of EAs and CLPP (MicroRespTM) to evaluate the environmental impact of metal(loid) contamination in soils of Chile.

Keywords

Heavy metals; soil biological properties; MicroResp; ED_{50} ; Environmental pollution.

5.1. Introduction

High contents of metals and metalloids [hereafter referred to as “metal(loid)s”] in soil can adversely affect soil quality or health and the activity of microbial communities (Bruins et al., 2000; Gohre and Paskowski, 2006; Rathnayake et al., 2010). Enzyme activities (EAs) can be used as both soil quality indicators in numerical indexes (SQIs) and as a measure of soil metal(loid) contamination. Determination of soil EAs reflects processes related to the biological functioning of soil and is a relatively fast and cost-efficient form of analysis. Such uses of EAs can often be complicated by inherent variability in response to other physic-chemical soil properties (Bastida et al., 2008; Trasar-Cepeda et al., 2000). Additionally, most recent studies have focused on the effects of only four metal(loid)s (Cd, Pb, Cu, and Zn), with other such as Ni and Cr being less frequently studied and V, Co, As, Hg, Ag, Mn, and Se rarely studied (Stazi et al., 2015). Also, despite several researches show the inhibition of soil enzymes with metal(loid) contamination (D’Ascoli et al., 2006), enzymes are influenced by pH (Dick, 2011), clay content (Burns, 1986; Nannipieri et al., 1978; Tietjen and Wetzel, 2003), humus fraction and organic matter (Dick, 1997; Medina et al., 2017; Pascual et al., 1999), among others. Thus, EAs can response in several ways, especially at field conditions, by which their study in metal(loid) contaminated soils should be performed considering the majority of potential variation sources.

Soil enzymes do not reflect all aspects of soil microbial activity and function, being necessary to look at other approaches to gain a fuller understanding of the impacts of metal(loid)s on microbial communities (Aponte et al., 2020a). Thus, community level physiological profile (CLPP) that reflects the utilisation of carbon (C) substrates may complements enzyme responses providing further insights into soil microbial functional diversity and community structure (Aponte et al., 2020a). In this sense, enzyme hydrolysis focuses on the breakdown of complex organic polymers, which not necessarily leads to the complete mineralisation of substrates, but can also lead to anabolic pathways for biosynthetic processes, polymerisation, and condensation (e.g. humification, interaction with mineral colloids). Conversely, CLPP-MicroRespTM measures the complete mineralisation of simple and complex organic compounds to CO₂, which represents the final step of decomposition process. Therefore, based on the above a comprehensive assessment of microbial functional diversity can be provided by the

integration of both techniques. For this reason, they can be considered complementary components of microbial functional diversity (Moscatelli et al., 2018).

Niemeyer et al. (2012) found that the ecological parameters based on biological properties indicated a clear distinction between sites inside and outside of a Pb smelter area, indicating an ecological risk to soil ecosystems even 17 years after cessation of smelting activities. In this study, authors reported increasing inhibition of EAs at nearer areas to smelting zones. Similarly, Šmejkalová et al. (2003) found increasing inhibition of EAs in function to the shortest distance to alluvial soils contaminated by repeated floods with sediments from a lead processing factory. Contrarily, Bartkowiak et al. (2016) reported the activation of some EAs in locations near to anthropogenic contaminated soils with metal(loid)s compared to farther locations. In Chile, industrial activities have strongly affected natural and agricultural ecosystems in the Puchuncaví Valley mainly due to mining activities, which resulted in high metal(loid) contaminated soils (De Gregori et al., 2003; González et al., 2014; Salmanighabeshi et al., 2015). However, the industrial impact on the functioning of microbial communities of soil from the Puchuncaví Valley has not been tackled. Aponte et al. (2020a) artificially contaminated a soil from the Puchuncaví Valley in a laboratory experiment, which resulted in clear negative effects of Cu and As contamination on EAs and CLPP. Nevertheless, considering all mentioned above it is necessary the study of metal(loid) contamination on field conditions. Therefore, this work aims 1) to study the effect of metal(loid) contamination on soil physic-chemical properties and microbial functionality, and 2) to detect suitable indicators of soil quality in metal(loid) contaminated soils, which might be included in the future development of numerical indexes.

5.2. Materials and methods

5.2.1. Study site and field sampling

The study site covered a range of metal(loid) contaminated soils in the Puchuncaví Valley, located in the Valparaíso Region, Chile. In this area are located numerous thermoelectrical plants, oil refineries and “Las Ventanas” Cu smelter (CODELCO), which during the last five decades have generated a strong impact in many cities and towns that surround it (González et al., 2014). Puchuncaví Valley was one of the first areas of Chile declared by the government as saturated with pollutants (Decrete No. 346,

Dec. 1993). Currently, Puchuncaví Valley has large areas affected with low plant cover, extreme erosion, and soils with high-to-extreme metal(loid) contents (De Gregori et al., 2003; González & Ite, 1992; Neaman et al., 2009). The soils at the study site are classified as Entisols (Salmanighabeshi et al., 2015). The topsoil is sandy loam with low nutrient bioavailability and limited water holding capacity (Ginocchio, 2000; Neaman et al., 2009). Also, soil organic matter ranges from 1.0 to 1.8%, and soil pH varies between 4.6 and 5.5 (Ginocchio, 2000; Neaman et al., 2009).

Some studies have reported increasing contents of metal(loid)s, especially Cu and As, in soils near to the industrial area, which decreases with larger distances from the industrial complex (De Gregori et al., 2003; González et al., 2014). However, there is high land heterogeneity with metal(loid) contents in soil concerning to results reported by González et al. (2014). Additionally, industrial activities have emitted high amounts of sulfur dioxide (SO₂), which is dispersed by wind and deposited in soils and has acidified soils, increasing metal(loid) bioavailability, with negative effects on soil, which resulted in low plant cover, biodiversity, and severe erosion (De Gregori et al., 2003; González et al., 2014). Therefore, for this study, a spatial buffer or influence zone was performed respect to the industrial complex “Las Ventanas” with intervals of 2 Km, which resulted in six categories that range from 2 Km to 12 Km in the Puchuncaví Valley (Figure 1). The area corresponding to 12 Km was not considered due to big differences in soil physic-chemical properties and poor accessibility. The spatial analysis was performed in the QGIS software version 3.12.3. Distances were established considering the assumption of the increasing of soil metal(loid) contents in function to the industrial complex according to De Gregori et al. (2003) and González et al. (2004). Six composite topsoil samples (0-5 cm) were randomly taken at each studied distance (Figure 1) with available access near to ways. Soils with vegetation were avoided. Therefore, a total of 30 soil samples were taken, which were stored in thermic bags with gel pack coolers previously frozen. In the laboratory, each soil sample was sieved through 2 mm, homogenised, cleaned of smaller roots by handpicking, and finally portioned for the following analysis: 1) EAs (stored at 4°C); 2) CLPP-MicroRespTM (stored at 4°C); 3) physic-chemical properties (air-dried). EAs were firstly measured followed by the CLPP-MicroRespTM.

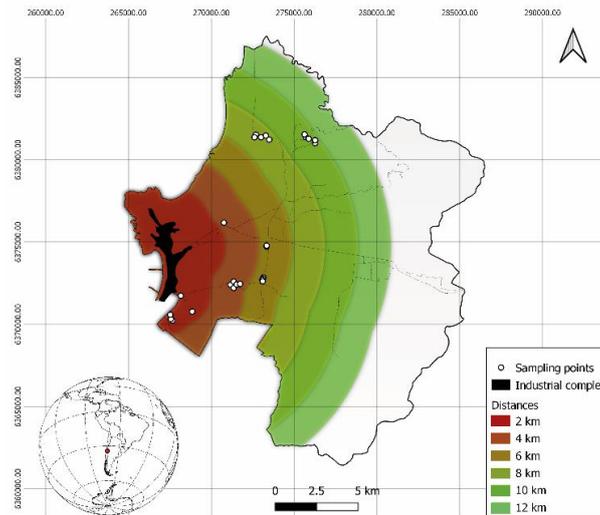


Figure 1. Map of the site study (Puchuncaví Valley) with established distances and the location of soil samples according to a spatial buffer.

5.2.2. Soil physic-chemical analysis

Chemical measured properties in soil samples were: 1) pH and electrical conductivity according to Sadzawka et al. (2006) by the use of a pH electrode and a conductivity meter in a soil-deionized water suspension; 2) 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 nitrogen by elemental analysis; 5) bioavailable phosphorus 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) total and bioavailable contents of Cu, Zn, Pb, and As (water extraction) by atomic absorption measurement (Lindsay and Norvell, 1978). DTPA extraction was used (except for As) since chelating agents (e.g. DTPA and EDTA) are more effective in removing soluble metal(loid)-organic complexes that are potentially bioavailable (Bolan et al., 2014). The colorimetric assessments were performed in a microplate spectrophotometer (Epoch, BioTek).

5.2.3. Metal(loid) contamination indices

Contamination factor (C_f), contamination degree (C_d), and the integrated pollution index (IPI) were calculated. The contamination factor (C_f) is calculated in Eq. (1):

$$C_f = C_n(\text{sample}) / C_n(\text{background}) \text{ (Eq. 1)}$$

Where C_n is the content of a particular metal(loid) in a contaminated soil (“sample”) and a reference non-contaminated value (“background”). Thus, each studied metal(loid) will have an associated C_f value inside each soil sample.

The Contamination degree (C_d) is defined as the sum of the individual contamination factors in Eq. (2):

$$C_d = \sum C_f \text{ (Eq. 2)}$$

The integrated pollution index (IPI) is defined as the average of the contamination factors computed for each sample (Yaylali-Abanuz, 2011). IPI values are divided into four categories (Wei and Yang, 2010): Low pollution level ($IPI \leq 1$); Moderate pollution level ($1 < IPI \leq 2$); High pollution level ($2 < IPI \leq 5$) and extremely high pollution level ($IPI > 5$).

5.2.4. Soil enzyme activities

Biological measured variables were the following: 1) dehydrogenase activity 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) acid phosphatase by *p*-nitrophenol released after soil incubation with *p*-nitrophenyl-phosphate (Tabatabai, 1994); 3) urease by ammonium determination after soil incubation with urea as substrate (Kandeler and Gerber, 1988); 4) arginine ammonification by ammonium released from the soil after incubation using arginine as substrate (Kandeler and Gerber, 1988); 5) β -glucosidase by *p*-nitrophenol released after soil incubation with *p*-nitrophenyl- β -D-glucopyranoside (Alef and Nannipieri, 1995); and 6) arylsulfatase according to Alef & Nannipieri (1995) where *p*-nitrophenol is released after incubation with *p*-nitrophenyl-sulphate. Enzyme activities were promptly measured after sampling using a microplate spectrophotometer (Epoch BioTek) except for the acid phosphatase, which was measured in a UV-V spectrophotometer (Helios Alfa) for avoiding reading mistakes due to the presence of precipitates.

5.2.5. Community level physiological profile (CLPP), MicroResp.

Community level physiological profile (CLPP) was measured using the MicroRespTM soil respiration system (MicroRespTM, Macaulay Scientific Consulting Ltd, Aberdeen, UK) according to Campbell et al. (2003). This method is based on a microrespirometry system that combines the advantages of BiologTM and Substrate Induced Respiration (SIR) using the whole soil that is incubated with some C sources, which are ecologically relevant to soil (Campbell et al., 2003). The emission of CO₂ by soil microorganisms was estimated using a colorimetric method before and after six hours of incubation at 25° C. The colorimetric assessment was performed by a microplate spectrophotometer (Epoch, BioTek).

5.2.6. Biological indices

Microbial functional diversity was assessed calculating the Shannon diversity index (Kennedy and Smith, 1995) corresponding to the entropy concept defined by: $H' = - \sum p_i * \ln p_i$ (Shannon, 1948), where p_i is the ratio of the respiration rate of every single C-substrate to the sum of all substrates. Shannon diversity index is related to the entropy of a system and when applied as a measure of microbial functions entropy, may express the heterogeneity of soil organic substrates bioavailability and microbial processes (Marinari et al., 2013). Catabolic evenness (E'MR) was calculated by: $\exp(H) / S$, where H is the Shannon diversity index and S the ratio of the respiration rate of every single C-substrate to the sum of all substrates from MicroRespTM. These biological indexes were calculated with the package "BiodiversityR" in the software R statistic. The ecological dose (ED₅₀) was calculated to quantify the effect of Cu and As on enzyme activities. ED₅₀ was calculated according to Knezevic et al. (2007) using the package "drc" in R statistic that calculates arbitrary effective dose values based on a fit model.

5.2.7. Statistical analysis

Results were analysed using the distance from the "Las Ventanas" smelter as fixed factor since contamination levels (C_d and IPI) do not result in balanced categories. Firstly, Shapiro-Wilks and Levene tests were applied for evaluating normality and

homoscedasticity assumptions, respectively. An ANOVA with Tukey HSD as *post hoc* was applied for normal and homogeneous variables. Contrarily, a Kruskal-Wallis (KW) test with “Holm” *p-value* adjustment was applied in cases where assumptions were not met for variables. A Spearman correlation test was applied between all variables. MicroResp™ results were analysed using a principal coordinate analysis (PCO) and PERMANOVA. Also, a principal component analysis (PCA) was performed to reduce the dimensionality and detect the variables that explain the most variability proportion. All the analyses were performed in R statistic v. 3.5.1.

5.3. Results

5.3.1. Physic-chemical properties

Different values of some soil physic-chemical properties with the distance from the industrial complex “Las Ventanas” were registered (Table 1). The most noticeable differences by the industrial influence zones were observed for the total organic carbon (TOC), bioavailable phosphorus (AP), and clay/sand content. For instance, TOC and Clay's contents were higher at farther distance to the industrial complex, where AP and Sand's contents increased (Table 1).

Table 1. Means and standard deviations of soil physic-chemical properties by distance respect to the industrial complex “Las Ventanas” in some soils from the Puchuncaví Valley (n= 6).

	2 Km		4 Km		6 Km		8 Km		10 Km	
pH	5.20	± 0.87 ^a	4.74	± 1.00 ^a	4.96	± 0.18 ^a	5.12	± 0.46 ^a	5.93	± 0.98 ^a
CE (S cm ⁻¹)	86.3	± 6.5 ^a	324.8	± 493.4 ^a	96.0	± 42.7 ^a	123.8	± 37.5 ^a	165.3	± 65.5 ^a
TN %	0.16	± 0.03 ^{ab}	0.13	± 0.07 ^b	0.20	± 0.04 ^{ab}	0.20	± 0.07 ^a	0.16	± 0.05 ^{ab}
TOC %*	0.61	± 0.23 ^b	0.57	± 0.50 ^b	1.57	± 0.66 ^a	1.62	± 0.89 ^a	1.18	± 0.50 ^{ab}
AP (mg kg ⁻¹)	7.75	± 2.05 ^a	4.55	± 2.82 ^{ab}	5.33	± 3.23 ^{ab}	2.76	± 0.36 ^b	2.51	± 0.16 ^b
Clay %	5.0	± 2.2 ^b	5.4	± 6.0 ^b	7.1	± 2.5 ^b	18.3	± 5.2 ^a	17.0	± 12.7 ^a
Sand %	84.3	± 4.8 ^a	65.0	± 14.2 ^{bc}	78.6	± 9.5 ^{ab}	61.8	± 8.8 ^c	58.8	± 9.0 ^c
Texture	Loamy Sand		Sandy Loam		Sandy Loam		Sandy Loam		Sandy Loam	

CE=electrical conductivity; TN= total nitrogen; TOC= total organic carbon; AP= bioavailable phosphorus. Different letters show significant differences according to Tukey HSD (P < 0.05) and Kruskal-Wallis* (P < 0.05). Texture represents the average of the area or distance.

Total metal(loid) contents (except for As) increased in areas located from 8 to 2 Km respect to the industrial area (Table 2), especially Cu that increased 767.3% from 10

Km to 2 Km. Similarly, bioavailable metal(loid) contents increased at nearer industrial locations (except for Zn_{DTPA}). In this context, Contamination Degree (C_d) and The Integrated Pollution Index (IPI) showed Moderate and High metal(loid) contamination at 2 Km of the industrial influence, respectively. This contamination level decreased at 4, 6, and 8 Km with Moderate contamination for IPI, which reached Low contamination at 10 Km of the industrial influence (Table 2). C_d showed low contamination from 8 Km to 10 Km. In this sense, the industrial influenced areas are clearly affected by increasing metal(loid) contamination and poor soil texture and organic matter at closest distances to the industrial complex “Las Ventanas”. Additionally, it is remarkable that there was not a non-contaminated area according to metal(loid) contents according to C_d and IPI values for soil samples taken from 2 Km to 10 Km. Therefore, we confirm the increasing metal(loid) contamination in function of closer distances respect to the industrial complex, which is later used for evaluating the metal(loid) contamination on soil biological properties.

Table 2. Means and standard deviations of metal(loid) contents in soils from the Puchuncaví Valley by distance respect to the industrial complex “Ventanas” (n= 6).

	2 Km		4 Km		6 Km		8 Km		10 Km	
Cu*	643.6	± 433.8 ^a	276.0	± 92.7 ^a	259.5	± 107.9 ^a	245.1	± 75.3 ^{ab}	64.5	± 33.1 ^b
Zn	112.8	± 56.6 ^a	69.1	± 35.8 ^a	71.3	± 30.0 ^a	50.2	± 21.5 ^a	79.7	± 40.2 ^a
As*	69.5	± 63.7 ^a	24.1	± 22.0 ^{ab}	20.5	± 6.9 ^{ab}	5.7	± 6.5 ^{ab}	0.6	± 1.1 ^b
Pb*	168.4	± 102.4 ^{ab}	286.1	± 214.6 ^a	278.7	± 245.0 ^a	48.7	± 14.7 ^{ab}	50.4	± 20.2 ^b
Cu _{DTPA}	186.5	± 76.9 ^a	153.4	± 64.4 ^a	120.8	± 59.6 ^a	61.6	± 43.8 ^{ab}	24.9	± 8.0 ^b
Zn _{DTPA}	4.6	± 4.0 ^a	4.5	± 4.5 ^a	4.8	± 4.7 ^a	2.8	± 0.9 ^{ab}	1.0	± 0.4 ^b
As _{H₂O} *	0.056	± 0.03 ^a	0.016	± 0.03 ^{ab}	0.012	± 0.01 ^{ab}	0.011	± 0.01 ^{ab}	0.008	± 0.00 ^b
Pb _{DTPA} *	6.09	± 9.11 ^a	0.00	± 0.00 ^a	1.28	± 1.24 ^a	0.76	± 1.18 ^a	0.46	± 0.82 ^a
Cd _{DTPA} *	0.09	± 0.11 ^{ab}	0.16	± 0.11 ^a	0.03	± 0.02 ^{ab}	0.04	± 0.03 ^{ab}	0.02	± 0.01 ^b
C _d	Moderate (13.0)		Moderate (8.7)		Moderate (8.3)		Low (7.0)		Low (2.8)	
IPI	High (3.2)		Moderate (2.2)		Moderate (2.1)		Moderate (1.8)		Low (0.7)	

Different letters show significant differences according to Tukey HSD test (P < 0.05) and Kruskal-Wallis* (P < 0.05). Total and bioavailable metal(loid) contents are shown in mg kg⁻¹.

5.3.2. Enzyme activities

All EA decreased in function to closer distances to the industrial complex, which mostly resulted in the following discrimination: 2 Km = 4 Km < 6 Km = 8 Km = 10 Km (Figure 2). The most sensitive EA was arginine ammonification (AA), which showed the

following trend: 2 Km = 4 Km ≤ 6 Km ≤ 8Km = 10 Km. Nevertheless, AA was inhibited from ≈ 45 to 67% (6 Km and 2 Km, respectively) compared to its activity at 8 and 10 Km. This inhibition rate was less than other EA such as dehydrogenase (DHA), arylsulfatase (ARY), and acid phosphatase (Pacid), which reached a decrease of almost 90 and 85% at 2 Km of the industrial influence zone compared to 8 and 10 Km of distance, respectively. β-glucosidase (Gluc) and urease (UA) showed a maximum inhibition rate of 81.5 and 84.5%, respectively, in the first 2 Km compared to 8 Km of distance to the industrial area.

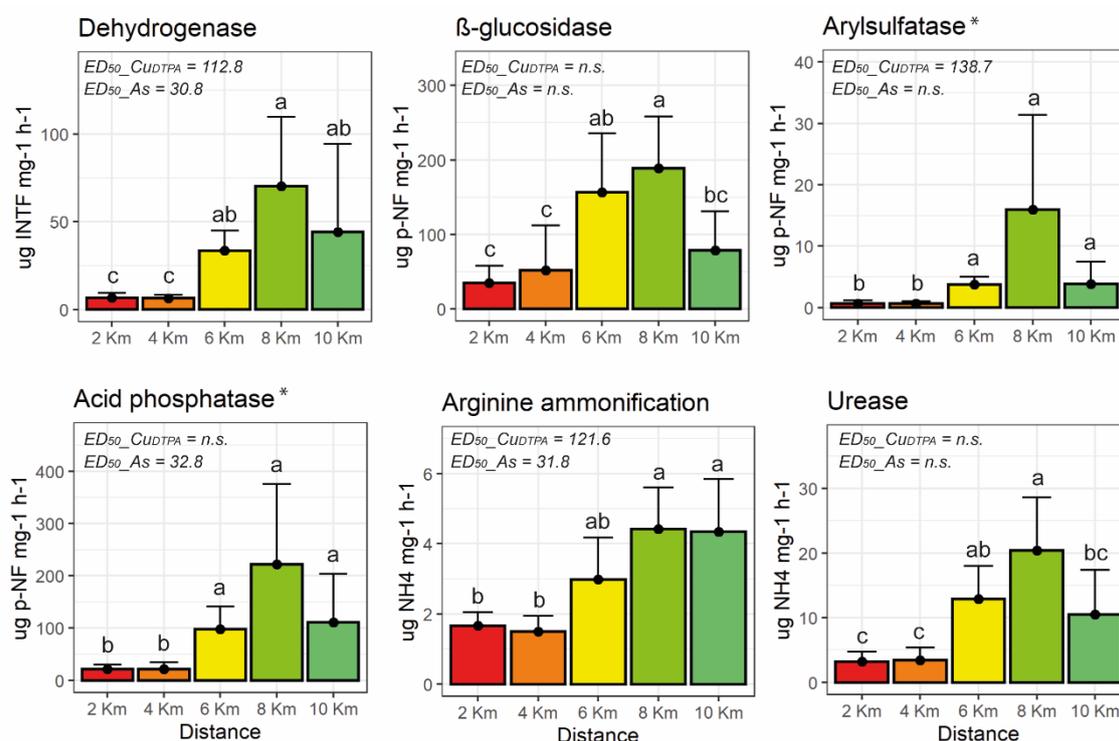


Figure 2. Dehydrogenase, β-glucosidase, arylsulfatase, acid phosphatase, arginine ammonification (AA), and urease activities in soils at increasing distances with respect to the industrial complex “Las Ventanas” in the Puchuncaví Valley, central Chile. Different letters showed significant differences according to the Tukey HSD test ($p < 0.05$; only for AA) and Kruskal-Wallis* ($P < 0.05$). ED_{50} values for Cu_{DTPA} and enzyme activities are also shown.

It is noticeable the strong and positive correlation between all EAs, TOC, and TN (Figure 3), especially for Gluc that showed the same TOC trend by studied distances. AA was the only enzyme activity negatively correlated with total and bioavailable contents of some metal(loid)s (Figure 3). Additionally, ED_{50} based for Cu_{DTPA} was only significant

for DHA, ARG, and AA with values approximated to 100 mg Cu_{DTPA} kg⁻¹ (Figure 2). There were not significant ED₅₀ models between bioavailable contents of Cd, Zn, Pb, and As for enzyme activities. Nevertheless, DHA, Pacid, and AA showed ED₅₀ values \approx 30 mg As kg⁻¹ (Figure 2).

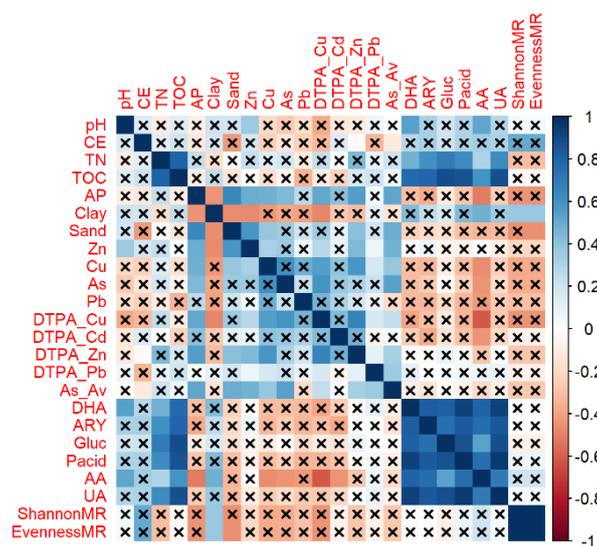


Figure 3. Correlation matrix between physic-chemical and biological soil properties (except for detailed CLPP results). Red boxes show negative correlation, while blue boxes show positive correlations. Non-crossed boxes show significant correlations ($P < 0.05$) according to Spearman rank test. Full correlation matrix is shown in Figure S1.

5.3.3. Community level physiological profile (CLPP)

CLPP was affected by metal(loid) contamination showing decreasing microbial respiration for all applied C sources in function of the nearest distance to the industrial area (Figure 4). Although almost all C substrate utilisation was positively correlated with TOC and TN (Figure S1), most of the C substrates were strong and negatively correlated with total and bioavailable Cu contents (Figure S1). In this sense, C substrate utilisation showed the following overall cluster: 2 Km = 4 Km < 6 Km = 8 Km < 10 Km (Figure 4), which is similar to results of enzyme activities (Figure 1). Additionally, principal coordinate analysis (PCO) showed significant discrimination of studied areas ($P < 0.05$, PERMANOVA), which implies a shift in the C substrate utilisation by microbial communities associated to metal(loid) contamination. Only microbial utilisation of oxalic acid (OXA), N-acetyl glucosamine (NAGA), and arginine (ARG) were negatively related

to Cd_{DTPA}. On the other hand, C substrate utilisation was separated in five groups as follows: 1) α -ketoglutaric acid (AKGA) and citric acid (CIT); 2) glucose (GLU), fructose (FRU), trehalose (TRE) and arabinose (ARA); 3) OXA and malic acid (MAL); 4) ARG, α -cyclodextrin (ACYC), γ -amino butyric acid (GABA), lysine (LYS), and water (WAT); and 5) cysteine (CYS), protocatechuic acid (PRO), NAGA (Figure 4). It is noticeable the higher utilisation of AKGA, CIT, MAL, and OXA only in the farthest and low contaminated locations (10 Km).

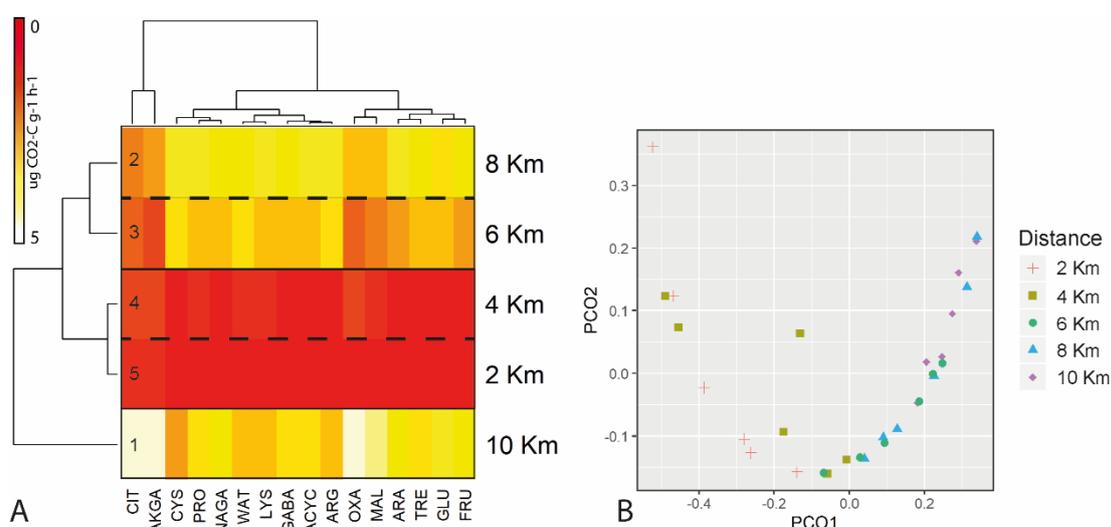


Figure 4. Community level physiological profile (CLPP) by A) heatmap scaled by each carbon source utilization and B) principal coordinate analysis. Red represents low microbial respiration. ACYC= α -cyclodextrin, AKGA= α -ketoglutaric acid, ARA= L-arabinose, ARG= L-arginine, CIT= citric acid, FRU=fructose, GABA= γ -amino butyric acid, GLU= glucose, LYS= lysine, MAL= malic acid, NAGA= N-acetyl glucosamine, OXA= oxalic acid, PRO= protocatechuic acid, TRE= trehalose, WAT= water.

5.3.4. Biological indices

Increasing metal(loid) contamination slightly affected the Shannon-MicroRespTM index (H'MR), and Evenness-MicroRespTM (E'MR), especially in the most contrasting conditions (2 Km vs 8 or 10 Km; Figure S2). H'MR and E'MR decreased 7.4 and 16.8% (non-significant), respectively, in areas near the industrial complex compared to farther locations (10 Km), which showed lower H'MR and E'MR in function of metal(loid) contamination or distances to the industrial complex.

5.3.5. Multivariate analysis

Principal component analysis (PCA) showed clear discrimination of industrial influenced areas similar to PCO and PERMANOVA (Figure 5A), which resulted in the following order: 2 Km = 4 Km \neq 6 Km = 8 Km \neq 10 Km. PCA also showed higher positive eigenvalues of metal(loid) contents, C_d , and IPI with locations at 2 and 4 Km (PC2), which suggest the positive correlation and increasing of these chemical properties in the most metal(loid) contaminated areas. In this context, PC1 explained the most variability proportion, being positively associated with all biological properties (enzymes and CLPP), especially for areas located at 6 and 8 Km of distance from the industrial complex. However, some C substrates (CIT, AKGA, and OXA) were more positively correlated in locations at 10 Km from the industrial influence [less metal(loid) contaminated soil], which suggest the lower use of these C substrates with increasing metal(loid) contents. On the other hand, considering a determination coefficient ≥ 0.6 for reducing the dimensionality, only PC1 comprised all variables with higher weights. Thus, variables that better explained the overall variability were: 1) all C substrates used by CLPP (except for AKGA, OXA, and CIT); 2) enzyme activities (except for ARY); and 3) TOC (Figure 5B). Thus, the most variability proportion was explained by biological soil properties and TOC.

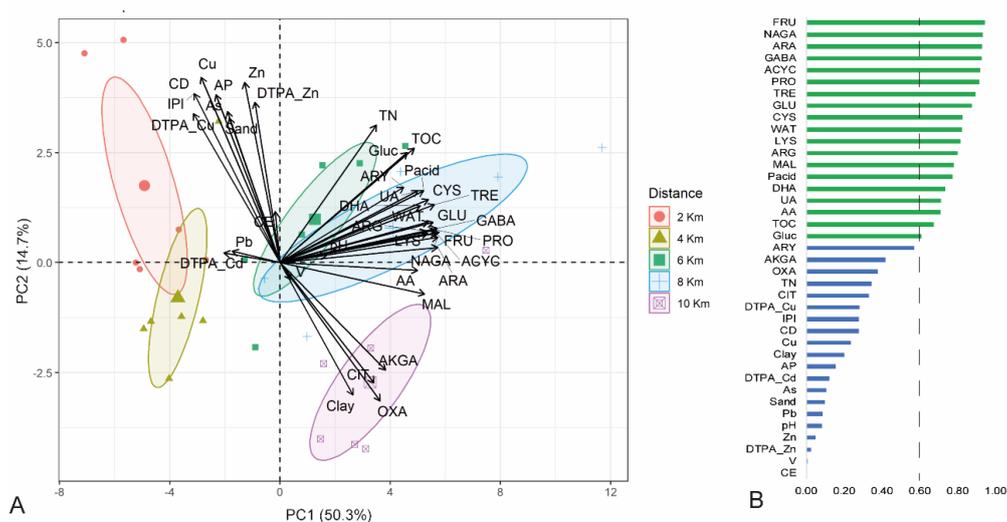


Figure 5. Principal component analysis (PCA) (A) and determination coefficients (R^2) from PC1 of response variables (B). Green bars of R^2 show the most representative response variables.

5.4. Discussion

Physic-chemical properties of studied soils were similar to reported by other authors in the same study site (De Gregori et al., 2003; González et al., 2014; Salmanighabeshi et al., 2015). The strongest differences in studied soils were evidenced for TOC and sand/clay contents, which was also reported by De Gregori et al. (2003) in similar locations from Puchuncaví Valley, being this trend also evidenced in several metal(loid) contaminated soils from industrial activities in other countries (Bartkowiak et al., 2016; Niemeyer et al., 2012; Stazi et al., 2017). The changes on TOC, clay, and sand contents in industrial influenced areas can be related to long periods of metal(loid) contamination that resulted in scarce vegetation and strongly exposed and eroded soils, especially in the closest areas to the industrial complex. IPI values here obtained are lower than reported by Salmanighabeshi et al. (2015), which reached values of $IPI \geq 20$ in the Puchuncaví Valley. Nevertheless, the contamination factor calculated by Salmanighabeshi et al. (2015) was based on the upper crust elemental contents of metal(loid)s (Hans Wedepohl, 1995) instead of pre-industrial background metal(loid) contents (Hakanson, 1980) as here performed.

Enzyme activities decreased in nearer industrial areas compared with farther locations, which has been reported in other studies (Niemeyer et al., 2012; Stazi et al., 2017). DHA, ARY, and AA were the most reliable indicators, which agree with previous review, meta-analysis, and experimental approaches where DHA and ARY were the most inhibited enzymes in metal(loid) contaminated soils (Aponte et al., 2020a, 2020b, 2020c). For instance, a previous laboratory experiment reported to DHA, ARY, and Pacid as the most accurate indicators in a soil that was taken at 8 Km of the industrial complex “Las Ventanas” (one of the same locations here studied), showing negative relationships with Cu_{DTPA} (Aponte et al., 2020a). In this last experiment, metal(loid) content was the only variation source, avoiding effects of organic matter changes. However, in the present study, TOC strongly influenced all EAs more than metal(loid) contents (except for AA). Therefore, soil organic matter was able to counterbalance the negative metal(loid) effects on microbial community in contaminated soils. Similarly, D’Ascoli et al. (2006) studied the effects of chromium (Cr) and Cu on EAs and found negative correlations between DHA, ARY, and Pacid with Cr fractions. However, soil organic matter was able to mask the Cu effect on EAs. Wang et al. (2018) reported an inhibitory response of As on Pacid, which was mostly affected by the soil organic matter and the cation exchange capacity.

In this context, organic substances can react with metal(loid)s forming metal-humate complexes and immobilizing them, which promotes metal(loid) stabilization and lower toxicity (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999; Medina et al., 2017). On the other hand, considering long-term SO₂ emissions, high sulfate contents might be found in soils, which can mask negative metal(loid) effects on ARY since it can be less synthesised by high sulfate contents.

Clay and sand contents were also important physic soil properties that considerably influenced EAs in metal(loid) contaminated soils. In a previous global meta-analysis (Aponte et al., 2020c), similar relations were found between all EA and texture with increasing activities at higher clay contents under metal(loid) contamination. In this context, soil texture is also an important moderator of the metal(loid) contamination effect on EAs and microorganisms since exoenzymes can be immobilized in clay surfaces (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999), avoiding temperature denaturation and proteolytic attack (Burns et al., 2013). Additionally, metal(loid)s can interact with clay surfaces by sorption and complexation reactions (Bolan et al., 2014), which reduces the metal(loid) bioavailability and also the negative effects on EAs across time [metal(loid) ageing] (Lock and Janssen, 2003). For instance, As can be adsorbed on some clay surfaces, involving outer-sphere surface complexation and strong specific ion adsorption (Mohapatra et al., 2007), which can be accentuated by long-term periods as occur in the Puchuncaví Valley. Metal oxides can strongly bind metal(loid)s through specific sorption, coprecipitation, and by forming inner-sphere complexes (Harter and Naidu, 1995). For instance, arsenate can form inner-sphere surface complexes with hydrous ferric oxides, playing an important role in the retention of As anions (Bolan et al., 2014).

Based on the above, Cu can be adsorbed on clay surfaces (Medina et al., 2017), but it can be strongly adsorbed in organic matter (Sodré et al., 2019) and Fe/Mn oxides. The Cu²⁺ form stable complexes with carboxylic, amine, and thiol groups, and to a lesser extent with phenolics in organic acids (Alloway, 2013; Harter and Naidu, 1995). Despite, Cu²⁺ is strongly adsorbed by Mn oxides > organic matter > Fe oxides > clay minerals. Metal(loid)s can also interact with clay surfaces by sorption (Bolan et al., 2014; Harter and Naidu, 1995) and therefore might compete with enzymes to form metal-humus-clay complexes, which would act to promote enzyme degradation by temperature and proteolytic attacks (Aponte et al., 2020b). Thus, the metal(loid) effect on EAs is mainly

altered by clay content (Burns, 1986; Nannipieri, Ceccanti, Cervelli, & Sequi, 1978; Tietjen & Wetzel, 2003) and soil organic matter (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999).

ED₅₀ value for DHA (112.8) agree with Fernández-Calviño et al. (2010), who reported that DHA changes on Cu contaminated soils occurred since 150-200 mg total Cu kg⁻¹. ED₅₀ value for ARY (138.7) was similar to ED₅₀=128 reported by Aponte et al. (2020a) in a laboratory experiment. In the case of AA, ED₅₀=121.6 was in accordance with DHA and ARY. No ED₅₀ comparative values are bioavailable for the As total content and DHA, AA, and Pacid. Our results suggest a negative effect of metal(loid) contamination on the overall microbial activity and biomass due to: 1) DHA represents the intracellular activity of microbiota (Nannipieri et al., 1990); 2) ARY catalyses the hydrolysis of sulfate aromatic esters that are constituents of fungal cell (Bandick and Dick, 1999; Moscatelli et al., 2005); 3) AA is an intracellular process that consists of NH₄⁺ releasing from N-compounds, which are used as C and N sources (Alef and Kleiner, 1987); and 4) strong positive correlations with all C substrate utilisation. In this sense, concerning DHA, ARY, and AA, Cu negatively affects to microorganisms due to: 1) the displacement of essential metals and denaturation of proteins, 2) redox cycling between Cu⁺² and Cu⁺¹ that catalyse the production of reactive hydroxyl radicals producing damage in lipids, proteins and DNA, and 3) Cu⁺² has a specific affinity for DNA and disorders its helical structure by crosslinking within and between strands (Borkow and Gabbay, 2005). Similarly, As negatively affect to DHA, AA and Pacid due to: 1) As mainly affects sulfhydryl groups in cells, causing malfunctioning of cell respiration, cell enzymes, and mitosis (Gordon and Quastel, 1948; Shen et al., 2013); 2) Arsenate interferes with phosphorylation and energy production due to its similarity with PO₄⁻³ (Gordon and Quastel, 1948; Shen et al., 2013).

Community level physiological profile (CLPP) provides information on the functioning of soil microorganisms involved in the C cycle (Hinojosa et al., 2010) with MicroRespTM enabling the assessment of catabolic activity in the microbial community (Stazi et al., 2017). In this study, multivariate analysis of C substrate utilisation was an effective method to differentiate between industrial influenced areas with different metal(loid) contamination levels. More recalcitrant C substrates were less affected by metal(loid) contamination, which suggests that some specialised microorganisms were non-affected. This trend is similar to results reported by Kenarova et al. (2014), Stazi et

al. (2017), and Aponte et al. (2020a). In this sense, the soil microbial community used C substrates in the following order: carbohydrates and carboxylic acids > phenolic acids > polymeric compounds. Nevertheless, a shift in the use of AKGA, CIT, MAL and OXA from farthest locations (10 Km) compared to other areas was observed. This reflects changes in the microbial catabolic activity and functional diversity possibly associated to changes in community structure influenced by the bioavailability and turnover of these compounds, especially taking into account the increasing TOC and TN in farther locations. This could be associated to the functional diversity (H'MR) and catabolic evenness (E'MR) slight decreases in the most industrial influenced areas. In this sense, microbial communities of the most metal(loid) contaminated soils are less functionally diverse since they are not able to break down a broad range of organic molecules at similar rates as microbial communities from low metal(loid) contaminated soils (Degens, 2000). Additionally, other soil properties such as TOC and clay content might offer protection to microbial communities possibly reducing the effect of stress or disturbances on catabolic evenness (Degens et al., 2001).

Considering the above, there are useful highlights for a better selection of soil quality indicators and later indices development. Current soil quality assessment looks for evaluating ecological aspects such as multi-functionality, ecosystem services, resistance and resilience, which are studied by high-throughput methods and novel indicators (Bünemann et al., 2018). In this sense, biological properties are key elements of soil and ecosystem functioning. For instance, DHA is related to C cycle since it transfers hydrogen from organic compounds to inorganic acceptors (Zhang et al., 2010) in living microbial cells. Also, dehydrogenases are the main representative enzymes of the oxidoreductase class in soil (Gu et al., 2009). On the other hand, ARY is important in the S cycling since ester sulfates represent of 30-50% of the total organic S in soils (Klose et al., 2015; Li and Sarah, 2003), and AA is well related to N cycling. Therefore, selected enzyme activities, CLPP-MicroResp and important soil physic-chemical properties such as bioavailable contents of Cu and As, TOC, and clay content could be considered as good soil quality indicators in relation to soil functioning.

5.5. Conclusion

There is a negative effect of industrial activities on physic-chemical and biological soil properties in the Puchuncaví Valley, which is evidenced by a strong decrease of total

organic carbon, total nitrogen, clay content, enzyme activities, and microbial functional diversity at nearer industrial areas. In this sense, enzyme activities were suitable indicators of metal(loid) contamination according to distances from the industrial complex “Las Ventanas” and contamination levels according to The Integrated Pollution Index. Nevertheless, total organic carbon was able to counterbalance the metal(loid) effect on enzyme activities. Only dehydrogenase, arylsulfatase, and arginine ammonification showed significant ED₅₀ responses with Cu_{DTPA} and were negatively correlated with Cu_{DTPA} and Cd_{DTPA}. Similarly, DHA, AA and Pacid showed significant ED₅₀ responses with total As. From these enzyme activities, arginine ammonification was the only biological property more related to Cu_{DTPA} than total organic carbon. On the other hand, the community level physiological profile (CLPP-MicroRespTM) was negatively affected at nearer industrial areas, which was related to lower utilisation of all applied carbon substrates. Therefore, a shift in the carbon substrate utilisation was observed in function to metal(loid) contamination, being carboxylic acid less used in more contaminated soils compared with low contaminated soils. In this sense, stress conditions in closest industrial areas resulted in the decreasing of catabolic capacity and functional diversity of soil microbial communities.

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CHAPTER VI. *“Development of novel soil quality indices based on biological properties to assess chemical soil degradation by metal(loid)s”*

Paper to be submitted to Ecological Indicators, manuscript in preparation.

Development of novel soil quality indices based on biological properties to assess chemical soil degradation by metal(loid)s

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Abstract

In recent years, soil quality assessment takes into account some ecologic topics such as soil multifunctionality, resistant, resilience and ecosystem services, which can be a useful approach to evaluate disturbed soils such as metal(loid) contaminated soils in Chile. Despite this, soil quality based on biological properties for metal(loid) contaminated soils has been scarcely studied. This work aims to develop soil quality indices (SQIs) to assess the chemical degradation by metal(loid)s in soils. Metal(loid) contamination effect was evaluated in soils at increasing distances respect to an industrial complex and copper smelter. A principal component analysis showed some enzyme activities and functional diversity explained the most variability proportion, which were selected to develop soil quality indices. Five SQIs approaches were here tested to compare their sensitivity and relationship with metal(loid) contamination. Biological soil properties explained the most variability proportion in metal(loid) contaminated soils. Multifunctionality assessment and the $Area_{SQI}$ were suitable SQIs based on their higher discriminant power and relationship with metal(loid) contamination compared to other SQI approaches. Both SQIs were successfully validated with data from literature, with decreasing soil quality values at higher metal(loid) contaminated soils. The $Area_{SQI}$ was an attractive approach due to its visualisation and easy interpretation, which can be useful for decisionmakers. However, the Multifunctionality assessment showed better sensitivity response, and fast and easy calculation. This study represents the first approach of soil quality assessment, especially with SQI development, in metal(loid) contaminated soils in Chile, which can be used to monitor metal(loid) contamination or soil recovery.

Keywords

Soil health; Heavy metal; Contamination; Indicators; Soil biological properties.

6.1. Introduction

Soil quality has been defined by The Soil Science Society of America (1995) as “*the capacity of a specific soil to work within the boundaries of natural ecosystems or under management to sustain vegetation and animal productivity, keep or improve the water and air quality, and support the health and living of humans*”. There are an increasing number of studies that use biological properties to measure soil quality in recent years (Paz-Ferreiro and Fu, 2016), most of which focus upon the study of soil respiration, microbial biomass, and enzyme activities (Bastida et al., 2008; Paz-Ferreiro and Fu, 2016). However, the use of the traditional “soil quality” (SQ) concept for contamination by metals and metalloids [metal(loid)s] is a relatively novel perspective (Aponte et al., 2020b). Ideally, SQ assessment in metal(loid) contaminated soils should account for a variety of ecological functions as opposed to the soil’s capability to maintain crop growth alone. Thus, the soil quality index (SQI) in metal(loid) contaminated soils could also be considered as a degradation index such as the “microbiological degradation index” of Bastida et al. (2006b) that was developed to evaluate soil desertification. The SQIs for metal(loid) contamination, that have either been suggested or applied, have various advantages and shortcomings, which are related to methods for selecting indicators, statistical analysis, index development, validation and applicability (Aponte et al., 2020b). For instance, some SQIs are based on totally arbitrary criteria to select indicators and microbial groups, as well as do not consider other soil properties (Paz-Ferreiro and Fu, 2016) such as total and bioavailable metal(loid) contents, among others. All these shortcomings can contribute to limited applicability (Bastida et al., 2008), and should be taken into account when determining SQIs for metal(loid) contaminated soils.

To measure SQ, the most traditional analytical approach is building a minimum data set (MDS) that comprises the most important indicators based on their contribution to explain variability, which can be performed by multivariate analysis (Mukhopadhyay et al., 2016; Puglisi et al., 2006; Sharma et al., 2011). Also, selected indicators can be scored depending on the idea that “*more is better*” and “*less is better*” proposed by Glover et al. (2000). For example, in metal(loid) contaminated soils, the metal(loid) bioavailability would be a “*less is better*” indicator, whereas microbial biomass and activity, and functional diversity, would be “*more is better*” indicators. After weighing and scoring, several calculation methods have been applied to assess SQI in degraded soils such as the alteration index (AI3) proposed by Puglisi et al. (2006), the

microbiological degradation index proposed by Bastida et al. (2006b), the geometric mean (Hinojosa et al., 2004), among others. These indices exclusively included enzyme activities without considering other soil properties such as organic carbon, pH, and metal(loid) content (total and bioavailable), which can strongly influence to soil biological properties (Aponte et al., 2020c; D'Ascoli et al., 2006; Niemeyer et al., 2012). Also, various calculation methods might result in different discriminant power for the same condition, which has not been tested, especially in metal(loid) contaminated soils.

In the last decade, the SQ objective has focused on the multifunctionality and resilience of soil along with the ecosystem services it provides (Bünemann et al., 2018). Soil multifunctionality has been assessed with microbial diversity data due to the functional role of several microorganisms, but this has not been performed based on soil biological functional properties (e.g. enzyme activities and functional diversity). Additionally, there are no studies about multifunctionality in metal(loid) contaminated soils (according to Scopus: multifunctionality AND soil* AND heavy metal*). Kuzyakov et al. (2020) recently proposed a new approach to evaluate soil health or quality, sensitivity, and resistance to degradation, which is an attractive method based on the comparison of the area on a radar chart that is produced by some soil properties contributing to the SQI. Kuzyakov et al. (2020) tested this SQI in two chronosequences of recovery of agricultural soils after abandonment and natural succession under deciduous forest. Nevertheless, this SQI has not been applied in metal(loid) contaminated soils. In Chile, it is well known the negatives effects of industrial activities on environment mainly by metal(loid) discharges, which is most evident in the Puchuncaví Valley (Cornejo et al., 2008; González et al., 2014; Salmanighabeshi et al., 2015). Despite this, the industrial impact on the functioning of microbial communities of soil from the Puchuncaví Valley has not been tackled, especially through the SQI development, which has not also been performed in other Chilean areas according to literature. Therefore, this work aims to develop SQIs to assess the chemical degradation by metal(loid)s in soil from the Puchuncaví Valley. For this, traditional and novel SQI methods were here tested and compared in metal(loid) contaminated soils from the Puchuncaví Valley, and the most suitable SQIs were validated with data from literature.

6.2. Materials and methods

Soil sampling and analytical measurements were presented in Chapter V, by which the current section shows the most important points related to new numerical results here described. Briefly, the study site covered a range of metal(loid) contaminated soils in the Puchuncaví Valley, Valparaíso Region, Chile. Six composite topsoil samples (0-5 cm) were randomly taken at each studied distance, avoiding soils with vegetation. A total of 30 soil samples were taken. Soil physic-chemical and biological properties were measured as described in Chapter V.

6.2.1. Soil quality indices (SQI) development

Due to SQIs performed in metal(loid) contaminated soils have several shortcomings related to methodological processes (Aponte et al., 2020b), this study compares some of the most commonly used methods and novel approaches for developing SQIs. Also, a theoretical validation of the best SQIs here developed was performed with data from literature.

The first SQI was based on the degradation assessment. A principal component analysis (PCA) was used to select appropriate indicators and their weighting factor according to Mukhopadhyay et al. (2016). For this, PCs with eigenvalue ≥ 1 that explained at least 5% of the variation of the data were considered (Sharma et al., 2005). In each PC, only variables with high factor loadings were retained for indexing. The high factor loadings were defined as values within 10% of the highest factor loading (Andrews et al., 2002). Due to absolute values of some parameters are bigger than others, a normalisation was performed to avoid underestimation of the importance of some variables. Therefore, values of selected indicators were normalised from 0 to 1. Selected indicators functioned on the “more is better” and “less is better” basis (Glover et al., 2000), for example, higher TOC (“more is better”) improves soil physicochemical properties, which enhance biological activity and thus soil quality and less degradation. For this, a sigmoidal equation with an asymptote tending to 1 and another tending to 0 (Eq. 1) was applied as follows:

$$y = a/(1 + (x + x_0)^b) \quad \text{Eq. 1}$$

where a represents the maximum score of the function ($a=1$), x is the indicator value, x_0 is the mean of the value of each indicator, and b is the value of the slope of the equation. This slope was -2.5 for the “more is better” curve and 2.5 for the “less is better” curve to obtain a sigmoidal curve tending to 1 for all chosen indicators. The normalised values were weighted since all indicators do not have the same importance in soil quality or degradation. In this study, the correction factor corresponds to the PC loading (w) of each indicator which better explained it, multiplying this for y to finally calculate the SQI as follows (Eq. 2):

$$SQI = \sum_{i=1}^n w_i y_i \quad \text{Eq. 2}$$

The statistical method for selecting SQI indicators is the basis for all SQI evaluated in the current study. On the other hand, due to this study aims to develop SQI based on biological properties, physic-chemical properties that were not correlated to biological properties (according Spearman correlation) were avoided. Additionally, considering results from our previous review and meta-analysis (Aponte et al., 2020a, b), the “expert criteria” was applied by selecting some key soil properties based on their role in soil biological functioning under metal(loid) degradation. These additional variables were also standardised and weighted as described below. For these SQI, the degradation perspective proposed by Bastida et al. (2006) was applied, by which higher values represent more soil degradation.

In the geometric mean (GM) assessment, only “more is better” indicators were considered. GM was calculated as: $GM = (\text{pH} * \text{TOC} * \text{Clay} * \text{DHA} * \text{ARY} * \text{Pacid} * \text{AA} * \text{AWCD})^{1/8}$, where indicators represent their absolute values. On the other hand, due to current goals for developing SQI are focused on the assessment of multifunctionality, ecosystem services, resistance, and resilience (Bünemann et al., 2018), in this study the Multifunctionality was calculated since soil microbial biological properties here studied represent several soil functions, as well as, physic-chemical properties are involved in soil functioning. For this, the equation proposed by Rodríguez-Loinaz et al. (2015) (Eq. 3) was applied as follows:

$$Multifunctionality = \sum_{i=1}^{11} \frac{\text{Observed value}_i - \text{Low value}_i}{\text{Target}_i - \text{Low value}_i} \quad \text{Eq. 3}$$

where *Observed value* and *Low value* are the absolute and minimum values of indicators, respectively. *Target* represents the maximum value for “more is better” indicators and the minimum value for the “less is better” indicators.

Currently, Kyzkaykov et al. (2020) proposed the $Area_{SQI}$, which is a very simple SQI based on the areas of a radar chart, representing a soil condition by each area. For this method, indicators were firstly standardised (Eq. 4):

$$stP_i = P_{deg}/P_{nat} \quad \text{Eq. 4} \quad Area_{SQI} = 0.5 \times \sum_i^n stP_i^2 + \sin\left(\frac{2 \times \pi}{n}\right) \quad \text{Eq. 5}$$

where P_{deg} and P_{nat} represent average values of indicators from degraded (contaminated) and natural conditions (here as low contaminated soils). For “less is better” indicators, Eq. 4 was inverted (P_{nat}/P_{deg}). Standardised values (stP_i) were used for the calculation of the $Area_{SQI}$ (Eq. 5), where n is the number of indicators used, and $\pi = 3.14$. Finally, the ratio between $Area_{contaminated}/Area_{non\ contaminated}$ is calculated to reflect the overall intensity of degradation. Values comprise from 0 (more degradation) to 1 (less degradation).

The last SQI here tested was the Alteration Index proposed by Puglisi et al. (2006), which was calculated as follows:

$$IA3 = 7.78 \times \beta - glucosidase - 8.22 \times alkaline\ phosphatase - 0.49 \times urease \quad \text{Eq. 6}$$

where, absolute values of enzyme activities were used. Numbers represent the product of a discriminant canonical function, which are proposed as constants by Puglisi et al. (2006). In this study, acid phosphatase was used instead alkaline phosphatase since our previous meta-analysis showed a similar trend of both enzymes by metal(loid) contamination. This is the only SQI theoretical validated in literature at the date.

6.2.2. *Theoretical validation*

The developed SQIs were theoretically validated with data from literature, which is based on the calculation of SQI with the most suitable selected methods. For this, databases from Aponte et al. (2020c, 2020b) were used since they collected a wide number of studies about the metal(loid) effect on biological properties, especially on enzyme activities. From these databases, only field studies were considered, as well as studies that evaluated as minimum three of the enzyme activities selected in the current study. Due to there is not any published research that evaluated the metal(loid) effect on the same biological properties here assessed, this validation was mainly based on the method application beyond the comparison of the same indicators, which is not possible. Therefore, the validation was performed with the indicators from each research that coincided with indicators selected in this study.

6.2.3. *Statistical analysis*

Results from SQI were analysed using the distance from “Las Ventanas” smelter as fixed factor. An ANOVA with Tukey HSD (post hoc) was applied for normal and homogeneous variables. A Kruskal-Wallis (KW) test with “Holm” *p-value* adjustment was applied when assumptions were not met for SQI. A Spearman correlation test was applied between all variables. All the analyses were performed in R statistic v. 3.5.1.

6.3. Results

Absolute results for physic-chemical and biological properties were presented in Chapter V, by which current results are focused on only new numerical analysis. Briefly. Previous results showed that some soil physic-chemical properties changed with the distance to the industrial complex “Las Ventanas”, which was mainly reflected by higher total organic carbon (TOC), total nitrogen (TN), clay content, and lesser metal(loid) contents in farther locations. Additionally, contamination degree (Cd) and the integrated pollution index (IPI) showed “Considerable” and “High” metal(loid) contamination at 2 Km of the industrial influence, respectively, which decreased with distance. Additionally, soil enzyme activities and functional diversity decreased in the most industrial influenced

locations, which was related to increasing TOC, clay content, and decreasing metal(loid) bioavailability.

6.3.1. Selection of soil quality indicators

Soil physic-chemical and biological properties here studied were suitable indicators of the soil degradation and quality by the metal(loid) contamination. In this context, multivariate analysis showed a separation of almost all distances studied (Figure 1), especially when comparing 2 Km with 8 Km and 10 Km, which represent high vs low contamination. Results showed that 72.8% of the total variance was explained by four principal components (PCs) (Table 1). The first four PCs with eigenvalues > 1.0 were considered for the development of SQIs. Soil biological properties explained the most variability proportion in the PC1 followed by some physic-chemical properties such as pH, EC, Pb, Zn, and Zn_{DTPA} in other PCs (Table 1). However, these physic-chemical properties were not included because they were not correlated with any biological properties (Chapter V). In this context, TOC, clay content, Cu_{DTPA}, Cd_{DTPA}, and AS_{H2O} were considered since they were closely correlated with biological properties (Chapter V).

The most important variable was the average well colour development (AWCD), with the maximum R² in the PC1 (Table 1). All enzyme activities, except for Gluc and ARY, were selected by their contribution in the PCA. Nevertheless, ARY was finally considered due to their good response to metal(loid) contamination (Aponte et al., 2020a, 2020b), being this decision based on the “*expert criteria*”. On the other hand, to avoid redundancy, UA activity was not included due to their very similar response and role to AA, which was the most sensitive indicator of metal(loid) increasing contamination in the current study location. These selection criteria are discussed below. Selected indicators for the SQI development were: AWCD, DHA, AA, ARY, Pacid, TOC, Clay, Cu_{DTPA}, Cd_{DTPA}, and AS_{H2O}. Mentioned indicators explain 66% of the overall variability proportion.

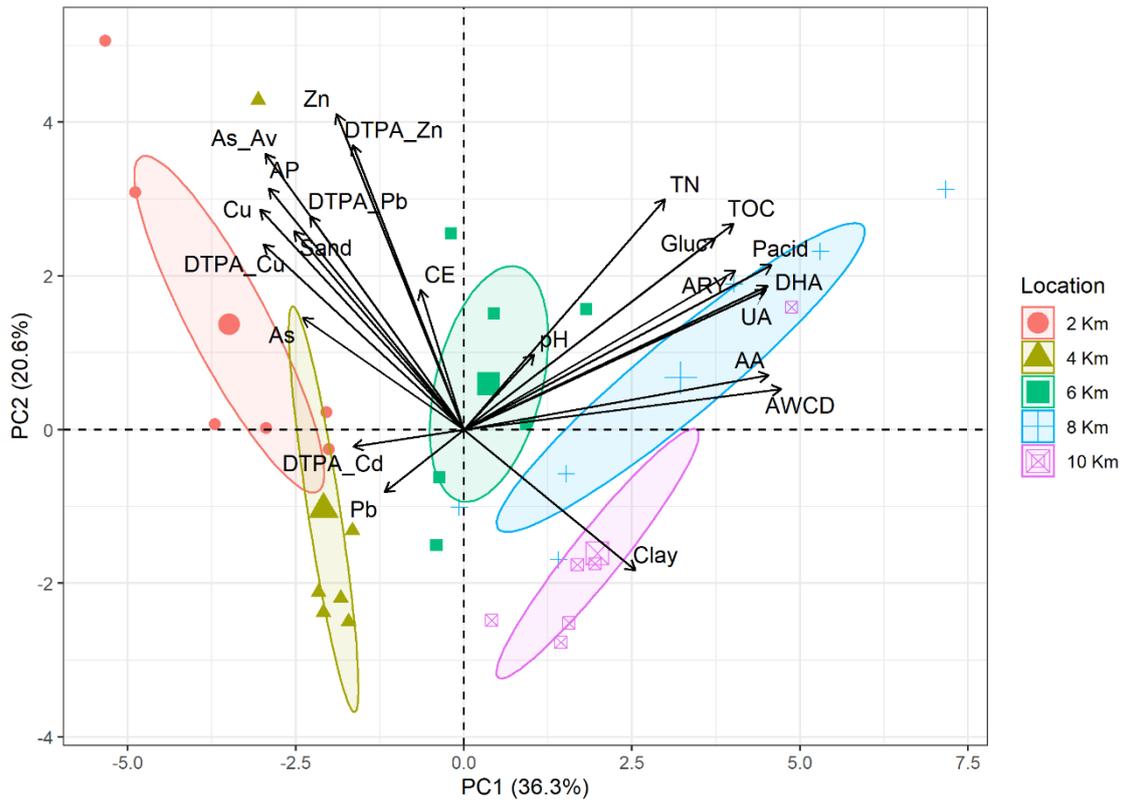


Figure 1. Principal component analysis (PCA) for metal(loid) contaminated soils.

Table 1. Results of principal component analysis for metal(loid) contaminated soils

Principal components	PC1	PC2	PC3	PC4
Eigenvalues	8.34	4.74	2.06	1.60
Variance (%)	36.27	20.63	8.98	6.96
Cumulative variance (%)	36.27	56.90	65.88	72.85
Eigenvectors				
pH	0.20	0.19	0.82	0.13
CE	-0.12	0.35	-0.21	-0.61
TN	0.57	0.57	-0.48	0.04
TOC	0.76	0.51	-0.27	-0.03
AP	-0.55	0.60	-0.12	-0.20
Clay	0.49	-0.35	0.26	-0.34
Sand	-0.48	0.49	0.18	0.10
Zn	-0.36	0.78	0.41	-0.04
Cu	-0.58	0.55	0.18	0.32
As	-0.46	0.28	-0.26	-0.14
Pb	-0.22	-0.16	-0.03	0.61
DTPA_Cu	-0.57	0.46	-0.34	0.25
DTPA_Cd	-0.31	-0.04	-0.24	0.44
DTPA_Zn	-0.31	0.70	0.00	-0.23
DTPA_Pb	-0.44	0.53	0.40	0.20
As_Av	-0.56	0.68	0.11	-0.26
DHA	0.86	0.36	0.11	0.14
ARY	0.77	0.39	-0.02	0.14
Gluc	0.71	0.47	-0.29	0.09
Pacid	0.87	0.41	0.00	0.13
AA	0.86	0.14	0.35	-0.03
UA	0.85	0.34	-0.13	0.20
AWCD	0.90	0.10	0.26	-0.17

Bold and **underlined** are values within 10% of the highest factor loading.

6.3.2. Soil quality indices in metal(loid) contaminated soils.

Soil quality indices here developed showed different performance according to their discriminant power in metal(loid) contaminated soils (Figure 2). The alteration index (AI3) showed a slight decreasing of soil alteration (degradation) with longer distances (lower contamination); however, this index did not discriminate between any contamination level. The Degradation index (DI) showed decreasing degradation from 6 to 10 Km, discriminating only between 2 and 4 Km with 6, 8, and 10 Km. Similarly, the Geometric mean (GM) showed increasing SQ (less degradation) only for 8 and 10 Km. On the other hand, the Multifunctionality assessment showed the best discrimination according to the metal(loid) contamination, showing the following order: 2 Km = 4 Km < 6 Km < 8 Km = 10 Km. Also, despite field heterogeneity, variability was reduced with the calculation method of Multifunctionality, by which outliers were absent.

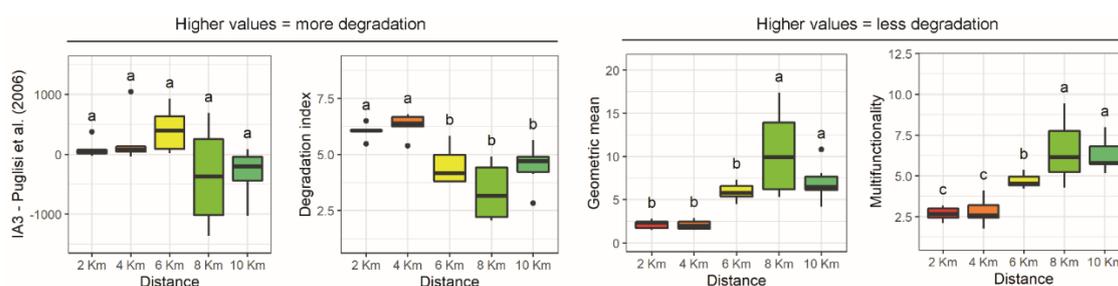


Figure 2. Soil quality indices developed for metal(loid) contaminated soils in the Puchuncaví Valley. IA3 = alteration index (Puglisi et al., 2006). The IA3 and Degradation index show soil degradation with increasing values. Geometric mean and Multifunctionality indices show less soil degradation with higher values.

The $Area_{SQI}$ decreased with higher metal(loid) contamination in soils (Figure 3); however, this was only possible by comparing 8 and 10 Km independently. Specifically, soils from both locations showed similar metal(loid) contamination levels and quality (Figure 2), but they strongly differed in ARY, TOC, Cu_{DTPA} , and Cd_{DTPA} , which resulted in non-clear diagrams for the $Area_{SQI}$ (Figure S1). Therefore, two independent analysis were performed considering both 8 and 10 Km as low-contaminated soils (here referred as reference soils). For instance, when 10 Km was considered as reference, data from 8 Km was included in the analysis. On the other hand, it is noticeable the higher values for TOC and Cd_{DTPA} ($Area_{SQI} > 1.00$) in soils from 6 Km compared to 10 Km and 8 Km,

respectively, which occurred by higher TOC and lower Cd_{DTPA} in these soils. Despite this, the area ratios were able to distinguish between all metal(loid) contamination levels.

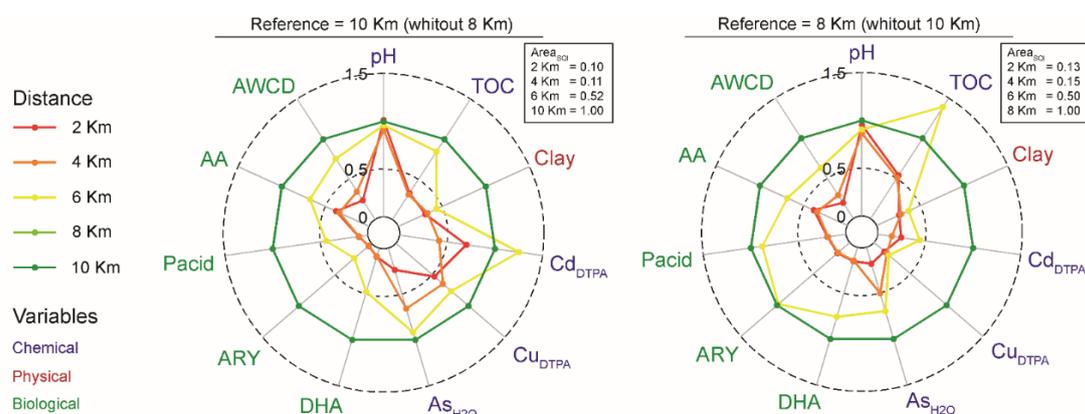


Figure 3. Soil quality index area (Area_{SQI}) considering soils from 8 Km (left) and 10 Km (right) as references independently. For both cases, the soil properties considered are: pH; TOC (total organic carbon); clay content; bioavailable contents of Cd, Cu, and As; the activity of DHA (dehydrogenase), ARY (arylsulfatase), Pacid (acid phosphatase); arginine ammonification (AA); AWCD (average well colour development from MicroResp). Absolute values of mentioned soil properties are shown in Chapter V.

6.3.3. Theoretical validation of SQIs

Taking into account mentioned results, Multifunctionality and Area_{SQI} methods were selected to be validated with data from literature. Table 2 shows the variables used in some studies about metal(loid) contamination, which were utilized to validate the selected SQI methods. Although databases contained more studies, only few met the requirements of using at least three of the biological properties here studied, and to be field experiments. Most of studies shared the use of DHA, ARY, Pacid, UA, and pH with the current research. Very few studies reported AWCD results in metal(loid) contaminated soils, and no studies were found that used AA in the same context. Also, studies generally show total metal(loid) contents, by which bioavailable metal(loid) data were scarce, and it was absent for Cu_{DTPA}.

Table 2. Biological and physic-chemical properties of published papers regarding metal(loid) contaminated soils. More studies are continuously revised to be included in this validation

Reference	Soil properties used in the current study											
	DHA	ARY	Pacid	AA	UA	AWCD	ph	TOC	Clay	Cd _{DTPA}	Cu _{DTPA}	As _{H2O}
Mikanova et al. (2006)	X	X			X							
Bhattacharyya et al. (2008)		X	X		X		X	X	X			X
Friedlova (2010)	X	X			X		X	X				
Hinojosa et al. (2004)	X	X	X		X		X		X			
Epelde et al. (2015)	X	X	X		X	X	X		X	X		
Lebrun et al. (2012)	X		X		X							
Blecker et al. (2013)		X	X			X	X	X				

Where enzymes measured in the published studies correspond with those measured in the present study, it is indicated as X.

Both methods were successfully validated since they were able to discriminate between metal(loid) contaminated and reference soils in most of studies (Table 3), showing clear linear trends in most of cases. In this context, all studies successfully showed decreasing multifunctionality with increasing metal(loid) contamination. Also, the Areas_{SQI} showed similar good results (Table 3), except for data from Epelde et al. (2015) where contaminated soils displayed higher areas (quality) than reference soils. Details of study conditions are mentioned in Discussion.

Table 3. Application of multifunctionality and Areas_{SQI} methods to data set from some published papers.

Reference	Contamination	Multifunctionality	Ratio Areas _{SQI}
Mikanova et al., (2006)	Unpolluted	3.00	1.00
	Medium pollution	0.48	0.06
	Moderate pollution	0.49	0.07
	High pollution	0.05	0.02
	High pollution	0.08	0.02
Bhattacharyya et al., (2008)	Canning (Reference)	5.86	1.00
	Deganga (Low contamination)	7.32	0.72
	Bariapur (Medium contamination)	7.65	0.68
	Chakdah (Moderate contamination)	7.78	0.56
Friedlova (2010)	No contamination	4.19	1.00
	Low contamination	3.04	0.46
	Medium contamination	1.26	0.10
	Moderate contamination	1.39	0.27
	High contamination	0.83	0.16
	Contamination source	1.95	0.25

Continuation Table 3.

Hinojosa et al., (2004)	Non-polluted	5.44	1.00
	Reclaimed	2.89	0.39
	Polluted	0.00	0.07
Epelde et al., (2015)	Low contamination	4.93	1.00
	Medium contamination	3.92	2.29
	High contamination	2.74	1.63
Lebrun et al., (2012)	Grassland-reference	2.00	1.00
	Grassland-contaminated	1.00	0.33
	Agricultural soil-reference	2.00	1.00
	Agricultural soil-contaminated	1.00	0.50
Blecker et al. (2013)	Castle Peak: Unmineralized (Reference)	4.00	1.00
	Castle Peak: Tailings (Contaminated)	1.00	0.10
	Masonic: Unmineralized (Reference)	5.00	1.00
	Masonic: Tailings (Contaminated)	0.00	0.09
	Battle Mountain: Unmineralized (Reference)	5.00	1.00
	Battle Mountain: Tailings (Contaminated)	0.00	0.02

6.4. Discussion

Biological properties explained the most variability proportion, which is similar to reported in the Chapter V and other studies (see Aponte et al., 2020b for more references). The most contribution of AWCD was expected since all C source utilisation were negatively affected by increasing metal(loid) contamination, by which AWCD is a sensitive, practical, and easy to manage indicator for the SQI development. ARY was arbitrary selected since it has been recognised as one of the most sensitive enzyme activities of metal(loid) contamination in soils (Aponte et al., 2020b). ARY generally decreases with abrupt changes that do not always follow a linear trend, which might be the reason by which this enzyme did not represent high variability proportion. In this context, the “expert criteria” or arbitrary selection was also applied for TOC, clay content, Cu_{DTPA} , Cd_{DTPA} , and As_{H_2O} . Regarding this soil physic-chemical properties, they were selected by its correlation with biological properties. In this sense, the role of TOC and clay contents in counteracting metal(loid) contamination was evident in previous results for the Puchuncaví Valley (Chapter V), which was associated to the enzyme stabilisation on organo-mineral surfaces (Burns, 1986; Nannipieri et al., 1978; Tietjen and Wetzel, 2003), and also the metal(loid) ageing and stabilisation by the interaction with clay and organic matter, respectively, reducing its bioavailability and toxicity (Bolan et al., 2014). Similarly, Cu_{DTPA} , Cd_{DTPA} , and As_{H_2O} were previously found to be the most contaminant

contributors in the studied location (Chapter V). Therefore, arbitrary selection only refers to the inclusion of indicators without a statistical basis (here referred to the PCA), which was managed due to previous comprehensive results.

Several methods were applied to develop SQIs in metal(loid) contaminated soils from the Puchuncaví Valley. The Alteration index 3 (AI3) proposed by Puglisi et al. (2006) showed non clear results and no discrimination between contamination levels. This result was expected due to: 1) the use of specific constant coefficients that do not consider variability of other studies, and 2) the use of Gluc and UA, which were not sensitive indicators in the current study. Therefore, the AI3 was not a suitable SQI for metal(loid) contamination in the study location. The Degradation Index was able to discriminate between 2 and 4 Km with 6, 8, and 10 Km. This index included indicators based on PCA and the “expert criteria”, by which it considered *in situ* variability that resulted in a better discrimination. The GM showed similar discrimination respect to the degradation index; however, this index can be calculated only with “more is better” or “less is better” indicators, but both can not be considered. For instance, Hinojosa et al. (2004) calculated three independent GM for 1) enzyme activities, 2) metal(loid) contents, and 3) other biological properties for metal(loid) contaminated soils, showing good discrimination between contaminated and non-contaminated soils with the GM based on enzyme activities (alkaline phosphatase, Pacid, ARY, UA, and Gluc). Therefore, all these SQIs were not considered as suitable methods to assess chemical degradation and soil quality in metal(loid) contaminated soils from the Puchucanví Valley.

The most suitable SQI to assess chemical degradation and quality of metal(loid) contaminated soils were the Multifunctionality and $Area_{SQI}$. The average response of all measured ecosystem functions is known as ecosystem functionality (Wagg et al., 2014). In soils, Multifunctionality has been calculated with taxonomic diversity data of soil microorganisms, assuming that microbial groups are involved in different ecological processes (Delgado-Baquerizo et al., 2016; van der Plas et al., 2016; Wagg et al., 2014). Delgado-Baquerizo et al. (2016) reported empirical evidence that show that any loss in microbial diversity will likely reduce the soil Multifunctionality. Biological properties used in this study are closely related to organic substrate decomposition and C, N, S, and P cycling, by which the Multifunctionality assessment showed a direct measure of ecological functions in metal(loid) contaminated soils. Therefore, our results suggest that metal(loid) contamination might change the stoichiometry of C, N, P and S released by

enzymatic decomposition of organic compounds that consequently affect microbial community structure and activity, and decouple biogeochemical cycling of C, N, P and S in soil (Aponte et al., 2020c). On the other hand, this index is practical and easy to use, and it could integrate functional and taxonomic data. Thus, the main advantages of the Multifunctionality assessment index are: 1) data is standardized, by which the index can be used with their original units (conversions are not needed); 2) calculations are easy and practical; 3) functional and taxonomic data can be integrated; 4) its calculation allows statistical inference.

The $Area_{SQI}$ was a suitable, practical, and easy method for assessing the chemical degradation and soil quality in metal(loid) contaminated soils. Various traditional SQIs have been suggested to evaluate specific groups of soil functions (e.g. the alteration index 3 proposed by Puglisi et al., 2006), but the comparison is impossible because they are based on a combination of specific soil properties (Kuzyakov et al., 2020). Due to most of soil biological properties decrease with metal(loid) contamination, it is simple to calculate their standardised values based on undisturbed soil and then to compare the relative decrease in the parameters in degraded soils. Similar to Multifunctionality, it is irrelevant the parameter units because all indicators are standardised with their undisturbed values. Also, this approach provides a useful visualisation and estimation of the decrease of individual parameters.

Kuzyakov et al. (2020) related the $Area_{SQI}$ with the soil organic carbon (SOC) to get an approach of a universal and master reference soil property. Specifically, if a soil property decreases proportionally to the SOC decrease, it has the same sensitivity and resistant as the SOC. If the changes are faster than SOC, the property is sensitive, or if slower, the property is resistant to the degradation compared to SOC. Thus, sensitivity and resistance of any soil parameters can easily be evaluated based on the comparison of its relative decrease to the decrease of SOC content. Therefore, soil biological properties here assessed can be considered as sensitive, and physic-chemical properties are resistant to metal(loid) contamination. Both approaches, the $Area_{SQI}$ and sensitivity-resistance can be well used not only for basic and applied research questions, but also are attractive for decisionmakers, as they provide simple and unified options to evaluate the land-use practices and measures against soil degradation, and so, the SQ or health. Both Multifunctionality and $Area_{SQI}$ are limited by the indicator selection methods, which can

result in a totally arbitrary process. To avoid this, multivariate analysis (as PCA here performed) can be a useful method to correctly applied both SQIs.

The Multifunctionality and the AreaSQI were successfully validated. However, the AreaSQI showed higher quality in contaminated than non-contaminated soils in data from (Epelde et al., 2015), which occurred due to DHA, pH, and Clay content were considerably lower in non-contaminated soils. Thus, these variables had a stronger contribution in the final AreaSQI result than ARY, Pacid, UA, AWCD and Cd_{DTPA}, which gradually decreased (except for Cd_{DTPA} that increased) with metal(loid) contamination, but displayed lower differences than DHA, pH, and Clay content. Although this different contribution of soil properties, the Multifunctionality assessment showed linear results according to the metal(loid) contamination in the data reported by Epelde et al. (2015), which is attributed to the more elaborated standardization method. Therefore, despite both methods were suitable approaches to assess chemical degradation by metal(loid)s and SQ, we propose the Multifunctionality assessment as a more appropriate method due to their high discriminant power, easy and fast determination, and its close relation with ecological functions that can take place in soils. On the other hand, the AreaSQI can be more appropriate to decisionmakers or to monitoring recovering of degraded soils. It is important to consider the use of statistical methods to select soil indicators, and to include reference soil for both methods since universal application of any SQI is still no possible due to the absence of thresholds. Finally, it is necessary to consider the limited applicability of both SQIs due to the explanation of variability of a particular degraded soil. Therefore, we recommend the methodological adoption instead of the use of the specific indicators here selected.

6.5. Conclusions

Biological soil properties, especially the overall microbial catabolic activity from MicroRespTM, explained the most variability proportion in metal(loid) contaminated soil from the Puchuncaví Valley. Multifunctionality assessment and the AreaSQI were suitable SQIs due to their higher discriminant power and relation with metal(loid) contamination compared to other SQI approaches. Therefore, both hypotheses proposed in this Doctoral Thesis were met. Both SQIs were successfully validated with data from literature, with decreasing soil quality values at higher metal(loid) contaminated soils. An exception was only found for the AreaSQI, where the most contaminated soils showed higher quality

values in data from a particular paper. This method was attractive due to its visualisation and easy interpretation, by which it can be useful for decision makers. However, the Multifunctionality assessment was considered as the most appropriate methodological approach to calculate soil quality in metal(loid) contaminated soils mainly due to its good sensitivity response, and fast and easy calculation. Also, the multifunctionality was a more analytical method, which can also integrate functional and taxonomical data. The application of SQIs here developed should consider the *in situ* variability explanation of each location or soils, by which, methods of development SQIs here presented can be recommended beyond the use of the indicators here selected. Finally, this study represents the first approach of soil quality assessment, especially with SQI development, in metal(loid) contaminated soils in Chile, which can be used by other researchers and policy makers in the Puchuncaví Valley to monitor metal(loid) contamination or soil recovery.

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Chapter VII. *“General discussion and concluding remarks”*

7.1. General discussion

7.1.1. Enzyme activities in metal(loid) contaminated soils

Inhibition of enzyme activities (EAs) by metal(loid) contamination can occur in several ways (D'Ascoli et al., 2006): i) masking of active functional groups; ii) denaturing protein conformation, or iii) competing with metal cofactors which are needed to form enzyme-substrate complexes (Gianfreda and Bollag, 1996). Also, metal(loid)s can inhibit the enzyme synthesis by decreasing the expression of RNA (Kapoor et al., 2015). Regarding high Cu contents in soils, its effect on EAs is 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 catalyse 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 helicoidal structure by crosslinking 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). Similarly, As negative effects on EAs are related to: 1) affecting thiol groups in proteins, which causes malfunctioning of cell respiration, cell enzymes, and mitosis; and 2) arsenate (AsO_4^{3-}) interfering with phosphorylation and energy production because of its chemical similarity to PO_4^{3-} (Gordon and Quastel, 1948).

It should be considered that the exact metal(loid) effect on EAs partly depends on the amino acid composition of the active site of each enzyme (Geiger et al., 1998). Nevertheless, the active site is a highly conserved region in most enzymes, by which many studies suggest most interactions between HMs and thiol groups of several EAs (Khan et al., 2007). In this context, Hemida et al. (1997) reported a stronger urease inhibition compared to amidase, which was explained by citing the different functional groups at the active sites of both enzymes. In cellulases, cellulose-binding domains contain tryptophan residues, which can be complexed by Cu, resulting in its inhibition (Karaca et al., 2010). Also, the shape of the active site in cellulase is mainly provided by glycine and cysteine between disulfide bonds. Thus, HMs can also interact with aminoacidic residues involved in the maintaining of the structure and shape of proteins, by which EA inhibition can be associated with competitive (temporary) and non-competitive inhibition.

Our results showed dehydrogenase (DHA) and arylsulfatase (ARY) as the most reliable indicators in metal(loid) contaminated soils compare to other EAs here studied. The mechanisms involved in the decreasing of DHA and ARY (both related to active microbiota) by metal(loid)s are 1) metal(loid)s bind with greater affinity than essential elements (Poole and Gadd, 1989) and 2) the displacement of essential metals from their original binding site or through ligand interactions (Bruins et al., 2000). ARY can be related to intra and extracellular activity (Klose et al., 1999; Tabatabai and Bremner, 1970a, 1970b). Therefore, ARY activity can also decrease by metal(loid) contamination because of the inhibited hydrolysis of sulfate esters by metal(loid)s due to the reaction with thiol groups (Al-Khafaji and Tabatabai, 1979) and possibly by their interactions with glycine, aspartic acid, and histidine in the ARY active site (Boltes et al., 2001). It is important to consider that high sulfate contents might also inhibit ARY in soils, which can occur in soils affected by SO₂ industrial emissions, and laboratory experiments when metal(loid) spiking was performed with sulfate metal sources, both cases here studied.

Regarding other EAs (mostly exoenzymes), the enzyme inactivation is possibly due to metal(loid) reactions with thiol groups (Eivazi and Tabatabai, 1990; Frankenberger and Tabatabai, 1991), which are involved in the catalytically active sites of enzymes and the protein structure (Al-Khafaji and Tabatabai, 1979; Karaca et al., 2010; Tabatabai, 1977). For instance, metal(loid) ions can interact with cysteine residues in the urease (UA) active site, especially with thiol groups (Yan et al., 2013). Thus, our results reflect the stronger impact of metal(loid)s on living microorganisms and their endoenzymes (DHA and ARY) than on extracellular EAs as was hypothesized by Karaca et al. (2010). This was also supported by the decreasing catabolic activity in higher metal(loid) contaminated soils. However, inhibition of endoenzymes by metal(loid) contamination depends on the type of endoenzyme. For example, our results consistently showed stronger inhibition of DHA, followed by Gluc and CAT. This suggests that the total oxidative activity of soil microorganisms (mostly reflected by DHA) can be more strongly inhibited than cellulose hydrolysis (partly reflected by Gluc) and the releasing of oxygen from hydrogen peroxide (CAT). We hypothesised that CAT was the less inhibited EA partly because it is involved in H₂O₂ detoxication processes (Duan et al., 2018) and metal(loid) contamination frequently results in oxidative stress, which acts to increase H₂O₂ production and hence stimulate CAT activity.

The less sensitivity of exoenzymes can be related to the enzyme immobilisation in organo-mineral complexes, which might result in more resistance to thermal and proteolytic degradation (Burns, 1986; Nannipieri et al., 1978; Tietjen & Wetzel, 2003). In this sense, metal(loid) effect on EAs was counterbalanced by the total organic carbon (TOC) and clay content, especially at field conditions where long-term contamination can promote metal(loid) ageing (Lock and Janssen, 2003). For instance, As can be adsorbed on some clay surfaces, sometimes involving outer-sphere surface complexation and strong specific ion adsorption (Mohapatra et al., 2007), which can be accentuated by long-term periods as occur in the Puchuncaví Valley. Similarly, Cu can be adsorbed on clay surfaces (Medina et al., 2017), but it can be strongly adsorbed in organic matter (Sodré et al., 2019). Additionally, metal oxides can strongly bind metal(loid)s through specific sorption, coprecipitation, and by forming inner-sphere complexes. For instance, arsenate can form inner-sphere surface complexes with hydrous ferric oxides, playing an important role in the retention of As anions (Bolan et al., 2014). On the other hand, organic substances can react with metal(loid)s forming metal-humate complexes and immobilising them, promoting metal(loid) stabilisation and lower toxicity (Dick, 1997; Nannipieri, 1994; Pascual et al., 1999; Medina et al., 2017). Nevertheless, the strength of metal-organic bonds are variable, depending on the functional group, the type of bond formed, and the coordinating ability of metal ion (Harter and Naidu, 1995), which is important in the retention/mobilisation of metal(loid)s.

Based on the above, organic matter supplies C and nutrients to soil microorganisms, which might increase EAs, especially DHA, ARY, and AA since they are related to the active microbiota. In this sense, possible significant effects of dissolved organic carbon (DOC) on soil biological biomass and activity can be found. In this sense, DOC includes amino acids, sugars, and organic acids that can be used by active microorganisms in soils, promoting their activity, but also DOC can interact with metal ions (Harter and Naidu, 1995). For instance, high DOC content can promote the formation of soluble Cu complexes, as well as DOC, can increase As mobility by competing for sorption sites such as those on Fe oxides (Bolan et al., 2014). Therefore, in the Puchuncaví Valley, the interaction between organic matter and TOC, clay content, and metal(loid)s (Cu and As) determined the response of soil microbial activity to metal(loid) contamination. Nevertheless, other soil physic-chemical properties and landscape differences might also be moderating the metal(loid) effect on soil biological activity.

7.1.2. Metal(loid) contamination effect on soil functional diversity

Metal(loid) contamination influences the soil microbial catabolic activity and functional diversity possibly associated with changes in community structure and the bioavailability and turnover of these elements. Based on our results, metal(loid) contamination decreased soil C microbial utilisation. Also, more recalcitrant C substrates were less affected by metal(loid) contamination, which suggests that some specialised microorganisms were non-affected. In this sense, and considering low sensitivity of some enzyme activities, it is expected that microbial communities develop adaptative mechanisms in soils from the Puchuncaví Valley due to long-term metal(loid) contamination. In this sense, several mechanisms of metal(loid) resistance in microorganisms have been described such as (Bruins et al., 2000): 1) metal exclusion by permeability barrier; 2) active transport of the metal away from the cell/organism; 3) intracellular sequestration of the metal by protein binding; 4) extracellular sequestration; 5) enzymatic detoxification of the metal to a less toxic form; 6) reduction in metal sensitivity of cellular targets. For instance, bacteria can reduce metal ions that can result in the synthesis of non-toxic metallic nanoparticles after metal(loid) interaction with intracellular proteins and organic molecules or by interaction with the cell wall (Iravani and Varma, 2020).

Additionally, the reduced catabolic capability of microorganisms may reflect stress conditions due to Cu and As contamination but also due to declining bioavailability of organic matter and nutrients. In this sense, microbial communities of highly metal(loid) contaminated soils are less functionally diverse since they are not able to break down a broad range of organic molecules at similar rates as microbial communities from soils with a low metal(loid) contamination (Degens, 2000). Additionally, other soil properties such as TOC and clay content might offer protection to microbial communities, possibly reducing the effect of stress or disturbances on catabolic evenness (Degens et al., 2001).

7.1.3. Soil quality indices development in metal(loid) contaminated soils

Soil biological properties (EAs and CLPP) better explained the experimental variability and were suitable indicators of metal(loid) contamination in the Puchuncaví Valley. These were expected results considering metal(loid) effects on EAs described above, which was closely related to the active microbiota of soil. Due to soil quality concept is

currently tackled from an ecological perspective (Bünemann et al., 2018), soil quality indices (SQIs) here developed included soil biological properties and the most important soil physic-chemical properties related to metal(loid) contamination. Therefore, TOC, clay content and bioavailable contents of some metal(loid)s were included as indicators due to their influence on EAs and CLPP. However, it is needed to recognize the potential effect of other soil physic-chemical properties on biological activity, especially taking into account the particular soil degradation dynamic in the Puchuncaví Valley, which not exclusively involves metal(loid) effects. In this sense, microbial biomass and microbial alpha and beta diversity could show whether soil microorganisms are stress by metal(loid) contamination. Also, diversity can display interactions between microbiota and improve the Multifunctionality assessment. On the other hand, organic matter fractionation, metal(loid) fractionation, DOC, As speciation, cation exchange capacity, redox potential and aggregate stability can be potentially considered to improve the variability explanation in the Puchuncaví Valley, especially by their influence on microbial communities (Fierer, 2017).

Two SQIs showed reliable discrimination of metal(loid) contaminated soils. In this sense, the Multifunctionality assessment and the $Area_{SQI}$ are calculated with more elaborated standardisation of data considering *in situ* variability. For these reasons, these SQIs resulted in more accurate discrimination. However, both at the local scale and worldwide, there are not antecedents of the assessment of Multifunctionality and $Area_{SQI}$ with the biological properties here studied in metal(loid) contaminated soils. Theoretical validation showed useful results with the Multifunctionality as the most reliable SQI in metal(loid) contaminated soils. Despite this, the application of SQIs here developed should consider the *in situ* variability explanation of each location or soils, by which, methods of development SQIs here presented can be recommended beyond the use of the indicators here selected.

7.2. Concluding remarks

In this Doctoral Thesis, the response of some enzyme activities (EAs) to metal(loid) contamination in soils was assessed by theoretical and experimental approaches. Firstly, our review and meta-analysis showed arylsulfatase (ARY) and dehydrogenase (DHA) as the most sensitive indicators to metal(loid) contamination compared to β -glucosidase (Gluc), urease (UA), acid phosphatase (Pacid), alkaline phosphatase (Palk), and catalase (CAT). Consequently, active microorganisms and their production of endoenzymes (DHA and partly ARY) are more stressed by metal(loid) contamination than biochemical processes governed by exoenzymes. Additionally, some soil physic-chemical properties and experimental factors moderated the effects of metal(loid) contamination on EAs, with stronger effects in the topsoil compared to the subsoil. Therefore, literature analysis suggested DHA and ARY as suitable indicators of metal(loid) contamination in soils. Similarly, an experimental assessment showed that the most suitable indicators of Cu contamination were ARY, DHA, and Pacid due to: 1) they were significantly inhibited during all experimental times, 2) showed consistent negative correlations with bioavailable Cu, 3) were the best predictors of bioavailable Cu, and 4) showed low-to-medium ED₅₀ values. Thus, our results confirm the negative effect of Cu and As on microbial functionality represented by EAs (especially by DHA and ARY) and changes in the community level physiological profile (CLPP, MicroRespTM). This suggests the utilisation of the most sensitive EAs (DHA, ARY, and Pacid) and CLPP as indicators of metal(loid) contamination. Also, these results represent the first approach to CLPP under Cu/As contamination using MicroRespTM system.

At field conditions, specifically in soils from the Puchuncaví Valley, a negative effect of industrial activities on soil physic-chemical and biological properties was found, which was evidenced by a strong decrease of TOC, total nitrogen, clay content, EAs, and CLPP at nearer industrial areas. In this sense, EAs were suitable indicators of metal(loid) contamination according to studied distances from the industrial complex and contamination levels. In these soils, TOC and clay content were apparently able to counterbalance the metal(loid) effect on EAs. Only DHA, ARY, and arginine ammonification (AA) showed significant ED₅₀ responses with Cu_{DTPA} and were negatively correlated with Cu_{DTPA} and Cd_{DTPA}. Similarly, DHA, AA and Pacid showed significant ED₅₀ responses with total As. From these EAs, AA was the only biological property mostly related to Cu_{DTPA} compared to TOC. On the other hand, the CLPP was

negatively affected at nearer industrial areas, which was related to lower utilisation of all applied C substrates, especially on labile substrates. Therefore, a shift in the C utilisation was observed in function to metal(loid) contamination, being carboxylic acid less used in more contaminated soils compared with low contaminated soils. Multivariate analysis showed that soil biological properties explained the most variability proportion due to their sensitivity to soil degradation, especially to changes in bioavailable metal(loid) contents, TOC, and clay. This suggests the utilisation of these EAs and CLPP as soil quality indicators in metal(loid) contaminated soils. Also, stress conditions in the closest industrial areas resulted in the decreasing of catabolic capacity and functional diversity because of soil degradation.

From different soil quality indices (SQIs) approaches here developed, the Multifunctionality assessment and the $Area_{SQI}$ were the most suitable SQIs due to their higher discriminant power and relation with metal(loid) contamination. However, indicators here selected can be complemented with other soil physic-chemical and biological indicators related to soil degradation in the Puchuncaví Valley, which can better explain the studied variability. Despite this, both hypotheses proposed in this Doctoral Thesis were met. Both SQIs were successfully validated with data from literature, with decreasing soil quality values at higher metal(loid) contaminated soils. An exception was only found for the $Area_{SQI}$, where the most contaminated soils showed higher quality values in data from a particular study. This method was attractive due to its visualisation and easy interpretation, by which it can be useful for decision-makers. However, the Multifunctionality assessment was considered as the most appropriate methodological approach to calculate soil quality in metal(loid) contaminated soils mainly due to its good sensitivity response, and fast and easy calculation. Also, the Multifunctionality was a more analytical method, which can also integrate functional and taxonomical data. Nevertheless, it is needed to consider that SQIs here developed only explained a portion of the local variability in the Puchuncaví, which can be a vastly different case compared to other metal(loid) contaminated soils due to the particular long-term soil degradation. Therefore, the applicability of these SQIs should be focused on the methodology instead of specifically selected indicators since the variability of each location can be explained by other indicators not considered here. This study represents the first approach of soil quality assessment including SQI development in metal(loid) contaminated soils from Chile, which can be used as a starting point by other researchers

and policymakers working in the Puchuncaví Valley to monitor metal(loid) contamination or soil recovery, but also can be adapted to be used in different places with presence of soils contaminated with metal(loid)s.

Future researches should tackle the effect of individual and multiple metal(loid)s -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-MicroRespTM and alpha/beta taxonomical diversity), which is necessary to be performed in laboratory experiments and field conditions. Also, in the Puchuncaví Valley, future studies should account other indicators that can better explain soil degradation beyond the metal(loid) effects in the Puchuncaví Valley, which allow to consider the effect of extreme erosion, metal(loid) ageing, differences in soil types and landscape, and potentially high sulfate contents in soil. On the other hand, this Doctoral Thesis suggests that C and S cycling might be more negatively affected (retarded) by metal(loid) contamination than N and P cycling, with individual steps of biogeochemical cycles being not equally slowed down by metal(loid) contamination. Thus, to confirm this statement, more EAs for each biogeochemical cycle should be evaluated, which can relate stoichiometric ratios between C, N, P and S to predict nutrient limitation for plants and microorganisms based on the EAs response.

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Supplementary data

Chapter V

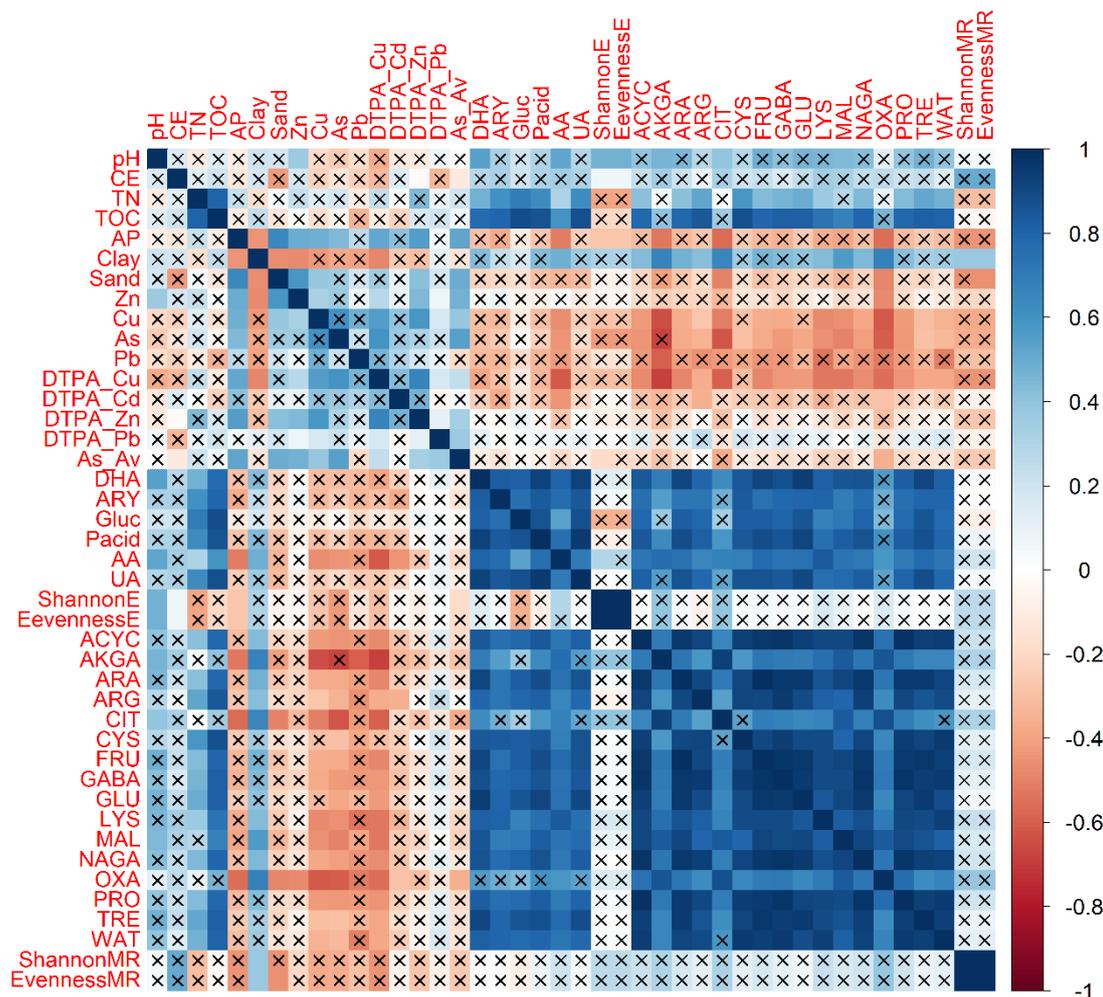


Figure S1. Full correlation matrix between physico-chemical and biological soil properties. Red boxes show negative correlation, while blue boxes show positive correlations. Non-crossed boxes show significant correlations ($P < 0.05$) according to Spearman rank test. CE= electrical conductivity; TN=total nitrogen; TOC=total organic carbon; AP=available phosphorus; CD= contamination degree; IPI= Integrated Pollution Index; DHA=dehydrogenase; Gluc= β -glucosidase; ARY=arylsulfatase; AA=arginine ammonification; UA=urease; Pacid=acid phosphatase; ACYC= α -cyclodextrin; AKGA= α -ketoglutaric acid; ARA= L-arabinose; ARG= L-arginine; CIT= citric acid; FRU=fructose; GABA= γ -amino butyric acid; GLU= glucose; LYS= lysine; MAL= malic acid; NAGA= N-acetyl glucosamine; OXA= oxalic acid; PRO= protocatechuic acid; TRE= trehalose; WAT= water; ShannonMR=Shannon-MicroResp; EvennessMR=Evenness-MicroResp.

Chapter VI

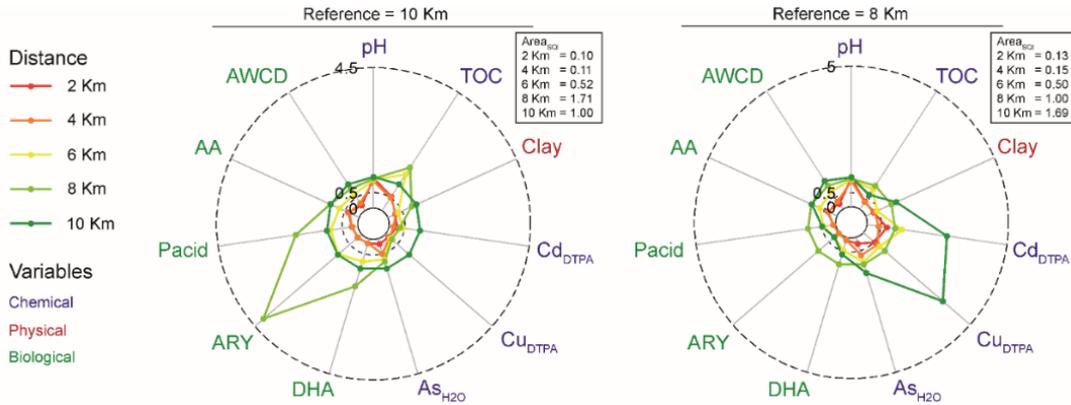


Figure S1. Soil quality index area ($Area_{SQI}$) considering soils from 8 Km (left) and 10 Km (right) as references, including both for the two cases. For both cases, the soil properties considered are: pH; TOC (total organic carbon); clay content; available contents of Cd, Cu, and As; the activity of DHA (dehydrogenase), ARY (arylsulfatase), Pacid (acid phosphatase); arginine ammonification (AA); AWCD (average well colour development from MicroResp). Absolute values of mentioned soil properties are shown in Advance 3.

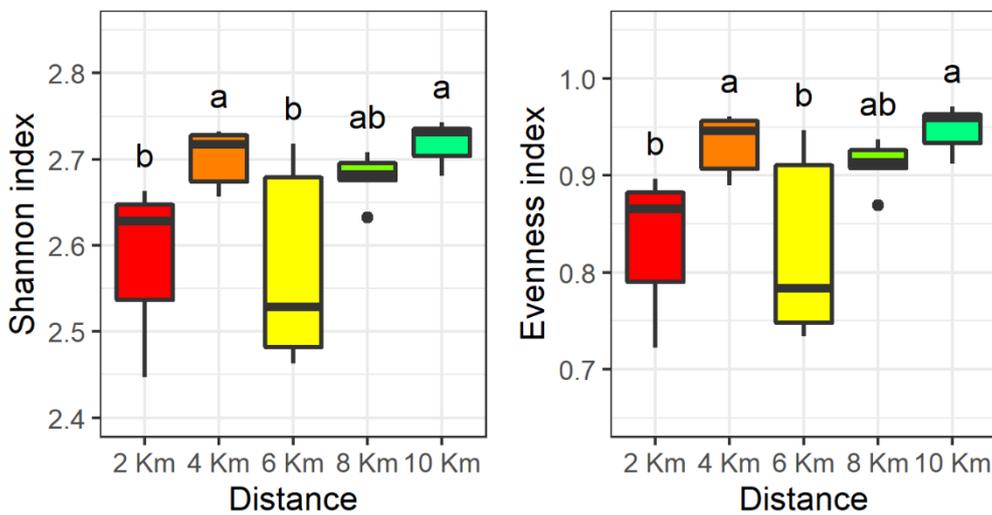


Figure S2. Shannon and evenness indices based on the community level physiological profile (CLPP).