A multi-metal risk assessment strategy for natural freshwater ecosystems based on the additive inhibitory free metal ion concentration index☆

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ABSTRACT

Scientifically sound risk assessment strategies and derivations of environmental quality standards for metals present in freshwater environments are currently hampered by insufficient chronic toxicity data collected from natural ecosystems, as well as inadequate information on metal speciation. Thus, the aim of the present study was to evaluate the impact of freshwater containing multiple metals (Cd, Cr, Cu, Ni, Pb and Zn) on the chronic toxicity (72h) to the alga *Pseudokirchneriella subcapitata* and compare the observed toxicity results to the total and free metal concentration of the samples. Based on the information obtained herein, an additive inhibitory free multi-metal ion concentration index, calculated as the sum of the equivalent toxicities to the free metal ion concentration of each sample, was developed. The proposed index was well correlated to the observed chronic toxicity results, indicating that the concentration addition, when expressed as the free-ion activity, can be considered a reliable indicator for the evaluation of ecological risk assessments for natural waters containing multiple metals.

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1. Introduction

Metal contamination in aquatic systems has been a subject of great concern for many years due to the abundance, persistence and toxicity of many metals, as well as their ability to bio-accumulate in the food chain and impact human welfare. In natural waters, a small portion of dissolved metals may exist as free metal ions because metals form stable complexes with a large variety of inorganic and organic ligands, which influence its bioavailability, toxicity and mobility. Therefore, the total metal concentration of a water sample is insufficient when interpreting the reactivity of the metal in biological or environmental processes (Mota and Correia dos Santos, 1995). Because metal speciation plays a key role in determining the potential fate and toxicity of a given metal, all available approaches for predicting metal toxicity consistently incorporate geochemical speciation.

Organisms in natural aquatic systems are typically exposed to a mixture of different metals. Although aquatic toxicity tests have been conducted using mixtures of various metals for several decades (Meyer et al., 2015), clear patterns that could be used to predict toxicity have not yet been identified. In many of these studies, acute exposures were examined; therefore, acclimation of the organism or metal tolerance development was not considered (Campbell and Fortin, 2013). Because few studies have been performed under conditions that mimic the complexity of natural waters, these results may not directly or completely translate into natural systems (Campbell and Fortin, 2013).

Authorities such as the US Environmental Protection Agency (EPA), World Health Organization (WHO) and European Environmental Agency (EEB) have developed and set recommended thresholds for water quality, including those related to metal toxicity guidelines for monitoring metal contamination (European Union, 2008; Fairbrother et al., 2007; US-EPA, 2009; WHO, 2011). Although organisms in natural aquatic systems are usually exposed to a mixture of metals instead of a single metal, most legislation continues to be based on the concentration of only one metal,

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except for laws enacted in Australia and New Zealand (Meyer et al., 2015).

Chronic exposure is more common in natural waters than acute exposure. Thus, water quality guidelines should be designed to protect against chronic toxicity to minimize the negative effects in aquatic ecosystems and water resources. Until now, sufficient data have not yet been presented to enable the meaningful conclusions on long term, chronic exposure to multiple metals, which hinders regulatory decisions. Thus, additional chronic toxicity tests must be performed, especially when various metals are present in natural systems, to obtain more robust data. The pursuit of this objective will certainly contribute to the development of efficient and economic science-based risk assessment tools for future water regulatory demands.

Recently, the Metal Mixture Modelling Evaluation (MMME) project, which was commissioned by several organizations related to mining and metal activities, performed a comparative analysis of bioavailability-based tools available for the evaluation of metal mixtures in natural waters (Van Genderen et al., 2015). The results of the MMME project indicated that available models (*Biotic Ligand Model*, BLM, and *Windermere Humic Aqueous Model using the toxicity function*, WHAM-FTox) were generally able to fit additive (or nearly additive) toxicity data (Farley et al., 2015). It was suggested that future efforts should address the lack of chronic toxicity data based on metal mixtures in natural ecosystems, further specifying that the following key research points should be considered in the interpretation of biological effects: (i) parameters such as pH, dissolved organic carbon content and major cation and anion content should be measured and reported; (ii) metal concentrations should follow the concentrations found in natural systems; and (iii) a single binding site is oftentimes a sufficient framework for predicting organism responses (Van Genderen et al., 2015).

From a regulatory point of view, independent of the nuances of metal interactions that can directly affect biological activity, additional tests are urgently needed to confirm additive toxicity at a specific site. These results will enable the development of efficient and economic science-based risk-assessment approaches to predict metal mixture toxicity to aquatic organisms. Thus, our motivation for performing the present research was to evaluate the impact of metal-contaminated freshwater samples, collected from impacted metal mining sites, wherein metals were the sole or dominant contaminants, on the chronic toxicity (72h) of the alga *Pseudokirchneriella subcapitata*. Therefore, in the present work, the collected samples were chemically characterized to determine the main water quality parameters and speciation of individual metals. The chronic toxicity was evaluated based on a standard 72-h chronic algal assay, preconized by OECD (OECD, 2006), and the results were analysed considering the total and free metal concentrations of the sample. An additive inhibitory free multi-metal ion concentration index was proposed, and the reliability of the new model for assessing the ecological risk of waters containing multiple metals was determined.

2. Material and methods

2.1. Study area

Two streams affected by local deactivated mining sites in central Portugal were selected. The sites were located in low to very low density urban areas, where other sources of contamination (besides metals) were negligible, as is described in the *Supplementary Information*. The exact location and the coordinates of the sampling sites are presented in Fig. S1 and Table S1, respectively.

2.2. Surface and pore water sampling

Pore waters (PW) were collected below the surface of the sediment using a 250-ml plastic syringe. At each station, various samples were collected and combined in a sealed plastic bag. After being transported to the lab, the samples were centrifuged for 15 min at $450 \times g$. Subsequently, the supernatant was collected and filtered through a 0.45-μm Millipore membrane filter.

Surface waters (SW) were collected and transported to the laboratory, where they were filtered through a 0.45-μm Millipore membrane filter.

Every sample (SW and PW) from each station was divided into two parts, and one was frozen for later use in the toxicity tests. Suprapure nitric acid was added to the other portion to decrease the pH to approximately 2, and the samples were stored at 4 °C in high density polyethylene bottles until analysis.

2.3. Physico-chemical analytical procedures

To characterize the stream samples, the pH, redox potential (Eh), total metals (Fe, Cu, Ni, Zn, Pb, Cr and Cd), total inorganic ligand content (chlorides, sulphates, ammonium and nitrates), hardness ($CaCO_3$) and total organic (TOC) and inorganic carbon (InorgC) concentration were determined as described in the *Supplementary Information* section.

2.4. Toxicity study

The toxicity of surface and pore water samples was evaluated using the green alga *Pseudokirchneriella subcapitata* and the standard 72-h chronic algal assay. OECD medium (Table S2) was employed, and the assay was conducted in a 96-well microplate, as described in the *Supplementary Information* section. The OECD medium contained a small amount of ethylenediaminetetraacetic acid (EDTA) (2.69×10^{-7} mol/l). At this concentration, almost all EDTA was used to prevent the precipitation of iron in the OECD medium, indicating that the chelation of metal ions was minimized (OECD, 2006).

2.5. Metal speciation modelling

To evaluate the metal speciation, specific software was used (Visual MINTEQ, KTH, Sweden; Stockholm humic acid model was used to estimate metal binding by organic matter). For all surface and pore water samples, the chemical speciation of the dissolved metals, which was defined as the distribution of the free metal ion, $[M^{n+}]$, total inorganic labile fraction (this fraction represented the sum of free metal ions and all inorganic metal complexes) and total organic complexed fraction, was calculated taking into account the total metal (Cd, Cu, Pb and Zn) concentrations and other physico-chemical parameters (pH, total inorganic ligand content and total dissolved organic carbon content) of the samples. Because the inhibition of algal growth does not occur in the control samples, the maximum variation in the pH after 72 h of algal growth was 0.5 units, and the maximum variation in the pH of surface and pore water samples was presumed to be less than 0.5 units. Hence, the pH was considered constant throughout all of the experiments.

2.6. Total toxic unit model

The total toxic unit (TTU) model was calculated, as described by Lynch et al. (2016), using the following equations:

$$TTU = \sum TU_i$$

and

$$TU_i = \frac{\left[Metal_{free,i}\right]}{^{72h}IC_{50,i}}$$

where TU_i is the toxic unit (TU) of the metal; $[Metal_{free,i}]$ is the concentration of free metal i in the sample; and $^{72h}IC_{50,i}$ is the concentration of metal i that resulted in the inhibition of 50% of the algal growth compared to the positive control (algal cells not exposed to the toxicant).

2.7. Additive inhibitory metal concentration index

The additive inhibitory metal concentration index (AIMCi) was calculated by adding the IC values for the various metals in each sample, including both total and free metal ions, according to the following equation:

$$AIMCi = \sum IC_{metal}$$

where IC_{metal} is the 72h-IC (10, 25, 50, 75 or 90) closest to the metal concentration (total or free) of the sample.

2.8. Statistical analysis

Multivariate analyses were conducted using Principal Component Analyses (PCA) in MatLab software (MathWorks, Natick, MA, USA), coupled with the PLS ToolBox for MAtLab from Eigenvector Research Inc. (version 5.5, Engeinvector, Wenatchee, USA). The metal concentrations and toxicity were tested for dimension reduction using the sampling site as the dependent/labelling variable. Eigenvalues greater than 1 were used to define the number of components. Subsequent analyses were carried out considering the inhibitory concentrations (72h-IC values) (see below, section 3.3) and Criterion Continuous Concentrations (CCCs) described in the EPA's National Recommended Water Quality Criteria (US-EPA, 2009).

3. Results and discussion

3.1. Physico-chemical characterization

A summary of the physicochemical parameters measured in the Coval da Mó and Pintor streams are presented in Table S3. A brief discussion of the physico-chemical characteristics of both streams can also be found in the *Supplementary Information* section.

3.2. Evaluation of the metal pollution

According to EPA recommendations (US-EPA, 2009), water quality can be evaluated based on estimates of the CCC, which is the highest concentration of a pollutant in the surface water to which an aquatic community can be indefinitely exposed without effect.

In the Pintor basin, the total copper and zinc concentrations (Table 1) were mostly above the CCC limits for fresh water (Table S4) (US-EPA, 2009), except, for zinc, in samples SWPt0, SWPt4, PWPt4, PWPt5 and, for copper, in samples SWPt4, SWPt5, PWPt1 and PWPt5. For lead, similar results were observed, and approximately half of the samples possessed values that exceeded the CCC, including samples SWPt1, SWPt3 and SWPt4 and PWPt0, PWPt1 and PWPt4. The concentration of cadmium exceeded the CCC in all surface and pore waters (Table 1).

In the Coval da Mó basin, the concentrations of cadmium, copper and zinc were typically lower than the CCC, except for cadmium in samples SWCM1, SWCM3, PWCM0, PWCM1 and PWCM3, copper in samples SWCM2 and PWCM6 and zinc in samples SWCM1, PWCM1 and PWCM3. Lead had the most samples with concentrations above the recommended threshold, and only samples SWCM2, SWCM5 to SWCM7 and PWCM7 showed lead contents below the CCC.

Finally, for all of the samples, the total nickel and chromium concentrations were significantly lower than the 72h-IC10 and 72h-IC25 values (13 µg L^{-1} and 100 µg L^{-1}, respectively; data not shown).

Zinc was observed in larger quantities in both basins, particularly in the Pintor samples, although zinc concentrations decreased at the Ul river (SWPt4 and SWPt5, see Fig. S1). In the Coval da Mó basin, lead was the second most common metal present in the water, reflecting the adjacent mine and mineral content in the region; however, this effect was diluted downstream, especially upon reaching the Caima river (samples SWCM5 to SWCM7). Samples from the Pintor showed lower lead contents and, instead, possessed more copper than Coval da Mó samples. Nickel, followed by cadmium and then chromium had the lowest loadings of all water samples (data not shown for nickel and chromium).

3.3. Algae chronic toxicity

The chlorophyta (green alga) *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) has been used in standard methods developed by international agencies (such as: ASTM, APHA, OECD, ISO and USEPA) as a reliable indicator of toxicity (Janssen and Heijerick, 2003) due to its ecological relevance and higher sensitivity than invertebrates, fish and other standard test organisms to a wide range of hazardous substances, including metals (Geis et al., 2000; Rojíčková and Maršálek, 1999). Thus, in the present work, *Pseudokirchneriella subcapitata* was selected to evaluate the toxicity of the environmental samples.

The registered toxicity of surface water samples was higher in the Pintor River than the Coval da Mó River (Table 2). At Coval da Mó stations, the toxicity clearly decreased in the downstream direction, and low toxicity values were detected in stations downstream of SWCM3. On the contrary, upstream (SWCM0 and SWCM1) stations (Fig. S1) showed the highest toxicities. In the Pintor River, the toxicity was consistently high ($\geq$74% of algal growth inhibition), except at station SWPt5, where an algal growth inhibition of 44% was observed.

The toxicity values for the pore water samples followed a different pattern: all of the Coval da Mó samples, except for PWCM1 and PWCM3, showed little to no toxicity. In the Pintor River, the first four stations (PWPt0 to PWPt3) presented high toxicities ($\geq$70% of algal growth inhibition), while the remaining two samples did not show any toxicity (Table 2).

To assess the toxic effects of individual metals on *P. subcapitata*, the algal cells were exposed to Cd(II), Cu(II), Cr(VI), Ni(II), Pb(II) and Zn(II), and the 72-h IC values were determined (Table 3; data not shown for Cr and Ni). The metal concentration that inhibited 50% of the algal growth after 72 h (72-h IC$_{50}$) was compared to those described in the literature (Table S5) and briefly discussed in the *Supplementary Information*.

3.4. Metal speciation and algal toxicity

For each surface and pore water sample, the resulting metal speciation pattern of each metal is summarized in Table 1. Generally, the free metal ion concentration contributes significantly to the total dissolved zinc concentration, and to a lesser extent, the Cu

Table 1
Concentration of total, free and inorganic and organic fractions of metals ($\mu g\ L^{-1}$).

Sample	Cu				Zn				Pb				Cd			
	Total	Free	Inorg	Org	Total	Free	Inorg	Org	Total	Free	Inorg	Org	Total	Free	Inorg	Org
SWCM0	≤0.57	n.d.	n.d.	n.d.	33.6	33.1	33.3	0.20	13.9	12.5	13.0	0.94	0.23	0.22	0.22	0.01
SWCM1	≤0.57	n.d.	n.d.	n.d.	235	231	233	1.41	22.2	18.4	20.6	1.62	1.67	1.60	1.65	0.02
SWCM2	18.2	11.5	15.5	2.71	34.9	33.8	34.5	0.32	≤1.13	n.d.	n.d.	n.d.	≤0.07	n.d.	n.d.	n.d.
SWCM3	≤0.57	n.d.	n.d.	n.d.	86.6	81.5	85.6	1.00	11.4	4.00	9.52	1.88	0.65	0.61	0.64	0.01
SWCM4	2.54	0.93	2.06	0.48	41.3	39.2	40.8	0.53	6.62	2.81	5.45	1.19	≤0.07	n.d.	n.d.	n.d.
SWCM5	≤0.57	n.d.	n.d.	n.d.	38.7	36.6	38.1	0.61	≤1.13	n.d.	n.d.	n.d.	≤0.07	n.d.	n.d.	n.d.
SWCM6	≤0.57	n.d.	n.d.	n.d.	64.1	58.0	60.1	4.02	≤1.13	n.d.	n.d.	n.d.	≤0.07	n.d.	n.d.	n.d.
SWCM7	≤0.57	n.d.	n.d.	n.d.	65.7	58.9	61.8	3.93	≤1.13	n.d.	n.d.	n.d.	≤0.07	n.d.	n.d.	n.d.
SWPt0	15.0	9.49	12.4	2.60	83.8	80.9	82.3	1.46	2.34	1.46	1.93	0.41	0.64	0.61	0.63	0.01
SWPt1	39.4	31.9	36.2	3.27	360	348	357	2.93	7.35	5.70	6.69	0.66	3.22	3.01	3.19	0.03
SWPt2	29.0	23.6	26.6	2.41	345	334	343	2.53	≤1.13	n.d.	n.d.	n.d.	2.91	2.73	2.89	0.02
SWPt3	18.8	13.1	15.8	2.98	307	294	303	4.26	3.56	2.41	3.00	0.57	2.82	2.62	2.77	0.04
SWPt4	4.01	2.54	3.50	0.51	105	101	103	1.03	4.38	2.75	3.81	0.57	0.95	0.89	0.94	0.01
SWPt5	8.45	4.50	6.82	1.64	124	118	121	2.40	≤1.13	n.d.	n.d.	n.d.	0.65	0.60	0.64	0.01
PWCM0	≤0.57	n.d.	n.d.	n.d.	28.4	4.84	6.15	22.2	50.1	0.68	0.73	49.3	0.38	0.06	0.06	0.32
PWCM1	≤0.57	n.d.	n.d.	n.d.	375	336	350	25.7	52.4	21.6	26.7	25.6	3.57	3.14	3.32	0.26
PWCM2	≤0.57	n.d.	n.d.	n.d.	18.1	14.4	15.8	2.29	4.55	0.90	1.07	3.49	≤0.07	n.d.	n.d.	n.d.
PWCM3	≤0.57	n.d.	n.d.	n.d.	222	196	208	13.6	33.7	7.28	20.6	13.1	1.99	1.76	1.87	0.12
PWCM4	2.29	0.01	0.06	2.23	44.4	25.0	26.8	17.6	14.4	0.25	0.84	13.6	≤0.07	n.d.	n.d.	n.d.
PWCM5	2.59	0.01	0.02	2.58	38.7	11.8	12.4	26.3	14.2	0.09	0.22	14.0	≤0.07	n.d.	n.d.	n.d.
PWCM6	9.84	0.12	0.22	9.61	27.1	11.9	12.2	14.9	8.28	0.21	0.35	7.93	≤0.07	n.d.	n.d.	n.d.
PWCM7	3.91	1.89	2.24	1.67	66.0	61.6	62.4	3.39	≤1.13	n.d.	n.d.	n.d.	≤0.07	n.d.	n.d.	n.d.
PWPt0	14.9	3.08	3.18	11.8	191	146	148	42.3	17.8	3.57	3.79	14.0	1.92	1.43	1.48	0.44
PWPt1	7.49	2.67	2.92	4.58	175	151	153	22.6	3.19	1.03	1.17	2.02	1.36	1.14	1.18	0.17
PWPt2	30.1	14.0	16.1	14.0	300	274	282	17.8	1.17	0.51	0.61	0.56	1.88	1.65	1.76	0.11
PWPt3	19.9	9.76	13.7	6.18	248	232	238	10.4	2.26	1.05	1.52	0.73	0.59	0.54	0.57	0.02
PWPt4	22.1	3.43	4.88	17.3	39.2	29.2	30.0	9.20	12.9	1.92	2.70	10.2	1.27	0.91	0.97	0.30
PWPt5	8.48	1.21	2.84	5.64	28.8	23.2	24.3	4.48	2.26	0.36	0.81	1.44	0.35	0.28	0.30	0.06

SW - surface water; PW - pore water; Total-total metal concentration; Free-free metal concentration; Inorg − free metal plus metals complexed with inorganic ligands, i.e. labile metal complexes; Org - metal complexed with organic compounds; n.d. - not determined.

Table 2
Toxicity of surface and pore water samples evaluated using a 72-h chronic algal growth assay.

Sample reference	Growth inhibition (% of control)	
	Surface water	Pore water
Coval da Mó stream		
CM0	90	0
CM1	99	100
CM2	28	9
CM3	70	100
CM4	0	0
CM5	26	0
CM6	0	0
CM7	17	2
Pintor stream		
Pt0	83	72
Pt1	96	76
Pt2	92	70
Pt3	74	85
Pt4	81	0
Pt5	44	0

and Pb concentration. The calculated speciation pattern showed large variations in the free metal (Cu, Pb and Zn) ion concentration for both surface and pore waters.

Since the toxicity of the algal was tested in the samples mixed with the OECD medium (for further details see section Materials and Methods-toxicity study of the *Supplementary Information*), metal speciation calculated in the original samples was compared with those calculated with OCDE medium (Table S6). This table shows that no noteworthy modifications of the free metal (Cd, Cu, Pb and Zn) ion concentrations occurs between the original samples and the samples with OCDE medium as a consequence of the addition of EDTA and others component salts available in the OECD medium (Table S2). Based on these facts, all discussion below was performed based on the metal speciation calculated in the original samples (Table 1).

The free metal ion activity reflects the chemical reactivity, bioavailability and toxicity of the metal (Max Ferguson, 1986). Thus, assuming that the free ion activity was well correlated with the metal toxicity to algae, the integrated comparative analysis

Table 3
Toxicity of metals in *P. subcapitata* evaluated via algal growth-inhibition assay.

Metal	72h-IC ($\mu g\ L^{-1}$)[a]				
	10	25	50	75	90
Cd(II)	14 (11–17)	25 (16–34)	36 (30–42)	49 (43–55)	71 (69–73)
Cu(II)	11 (10–12)	18 (11–25)	29 (16–42)	34 (21–47)	41 (29–53)
Pb(II)	0.7 (0.3–1.1)	3 (1–5)	4 (2–6)	n.d.	n.d.
Zn(II)	n.d.	34 (25–43)	91 (47–135)	240 (164–316)	546 (522–570)

The mean values and respective standard deviations were obtained from three to five independent experiments performed in quintuplicate ($n = 15$–25).
n.d. - not determined.
[a] IC - inhibitory concentration: 72h-IC$_{10}$, 72h-IC$_{25}$, 72h-IC$_{50}$, 72h-IC$_{75}$ and 72h-IC$_{90}$ values represent the total metal concentration that caused the inhibition of 10, 25, 50, 75 and 90% of algal growth, respectively, compared to the positive control (algal cells not exposed to the toxicant).

between the toxicity of surface and pore water samples (Table 2), the free metal ion fractions calculated for all samples (Table 1) and the 72-h IC values of the algae (Table 3) provided the following observations:

i. For most samples (Coval da Mó: SWCM1, SWCM3, PWCM1 and PWCM3; Pintor: for all samples for which toxicity was detected), the free zinc ion was an important contributor ($\geq$72h-IC$_{50}$) to the toxicity profiles, and the observed correlation between the free zinc concentration and toxicity was predictable. Although sample SWCM0 had a lower concentration of zinc, a high toxicity value was recorded. However, high concentrations of lead were also found in this sample (>72h-IC$_{50}$), which may explain the toxicity. Samples PWCM4, PWCM7, PWPt4 and PWPt5 showed free zinc concentrations near the 72h-IC$_{25}$ (or above for PWCM7) and were considered exceptions because toxicity was not detected.

ii. Lead also contributed (free metal concentrations > 72h-IC$_{50}$ value, as shown in the integrated analysis of Tables 1 and 3) to the toxicity of several samples (SWCM0, SWCM1, SWCM3, SWPt1, PWCM1 and PWCM3).

iii. For most samples, free copper was present at low concentrations and was expected to have little or no impact on the toxicity. Samples SWCM2, SWPt0, SWPt1, SWPt2, SWPt3 and PWPt2 were the exception to this observation; however, in these samples, other metals were also present in high quantities, preventing further analysis with the present data.

Similar conclusions were attained when TTU model was used (Fig. S2).

3.5. Multivariate analysis: toxicity versus total or free metal concentrations

For a deeper understanding of the effect of the total metal concentration of surface and pore water samples on the chronic toxicity of *P. subcapitata*, principal component analysis (PCA) was applied to the data. First, all surface and pore water data were used, which returned into a separation of surface and pore waters. Therefore, further analyses were carried out individually. Nickel and chromium were also discarded, as the total metal concentration of all samples were below the 72h-IC$_{10}$ and 72h-IC$_{25}$ values, respectively (data not shown). The statistical data for the PCA analysis is provided in the *Supplementary Information* section (Table S7).

In short, the components that primarily contributed to the variation in PCA1 were Cd and Zn for all of the samples and toxicity (algal growth inhibition) for pore water samples. In PCA2, the highest contribution to the variation was Pb, which presented a strong positive correlation for surface water samples and a negative correlation for pore water samples. The toxicity provided a higher contribution to component 1 for pore water samples (positive) and a substantially higher but negative contribution to component 2 for pore water *versus* free metal concentration, which correlates with the free Pb concentration and anti-correlates with the free copper concentration (Table S7).

From the analysis of the distribution of the stations in Fig. 1, we can compare the differences regarding the total and free metal ion concentrations. For example, from Fig. 1a (total metal in surface waters) to Fig. 1b (free metal in surface waters), it is possible to see that there is a decrease in the number of groups, with groups becoming more concentrated and well defined, especially those corresponding to stations with less toxicity. Groups C, D and E in Fig. 1a rearranged into two groups in Fig. 1b: one corresponding to

intermediate or low toxicity (group D′) and the other to high toxicity (group C′) (Table 2). Moreover, group B′, consisting of samples SWCM0 and SWCM1, was more homogenous and showed high toxicity values ($\geq$90%) compared to group C. The incipient complexation between organic matter and metals (mainly copper and zinc) was primarily responsible for the toxicity of samples in group A (Fig. 1a), which explains the similar total and free metal concentration profiles (Table 1) and the maintenance of the group in the same position (Fig. 1b). However, rearrangements, such as those of station SWCM3, were mainly due to changes in the metal concentration profiles of the samples. For example, for SWCM3, the toxicity was underestimated compared to the total concentration of lead because significant lead complexation by organic matter (Table 1) reduced the free metal concentration and resulted in a lower toxicity (Table 2). For pore water samples, in opposition to Fig. 1c, where four major classes were clearly observed, the new data set distribution of the stations resulted in three major classes (A′, B′ and C′) (Fig. 1d). The rearrangement of groups C and D (Fig. 1c) into the well-defined group C′ (Fig. 1d) was due to the complexation of lead and zinc by organic matter, which significantly reduced the bioavailable metal fractions and explains the low (<10%) chronic toxicity. As shown in Fig. 1, the correlation between free metal concentration and toxicity appeared to be more linear than that of the total metal and toxicity, and this difference should be considered when applying toxicity models. The present results are consistent with most conclusions and models, which indicate that the speciation of metals is critical to assessing the toxicity in a given medium. Moreover, metal speciation is incorporated into many models, such as the BLM (Farley and Meyer, 2015).

## 3.6. Proposal of an additive inhibitory free metal ion concentration index

For each sample, assuming that the toxicity was related to the additive inhibitory effect of the metal, a correlation may exist between the toxicity and additive inhibitory metal concentration (AIMCi) index. Hence, this correlation was subsequently evaluated.

The AIMCi index was calculated as described in section 2.7. Briefly, for each sample, the free or total concentration of each metal (Table 1) was compared to various IC (IC10, IC25, IC50, IC75 and IC90) metal concentrations determined for each metal (Table 3). For example, in sample SWCM2, the total concentration of copper, zinc and lead was 18.2, 34.9 and <1.13 μg L^{-1} (Table 1), respectively, corresponding to IC values on the magnitude of 72h-IC$_{25}$ for copper and zinc and ~72h-IC$_{10}$, for lead (Table 3); thus, for this sample, the AIMCi was equal to IC$_{60}$. For the same sample, the free concentration of copper and zinc were 11.5 and 33.8 μg L^{-1}, respectively (Table 1), which corresponded to the 72h-IC$_{10}$ value for copper and the 72h-IC$_{25}$ value for zinc (Table 3). In this case, the AIMCi was equal to IC$_{35}$.

After calculating the AIMCi for each sample and both cases (total and free metal concentrations), the chronic toxicity (72 h) results obtained for surface and pore water samples were compared to both AIMCi values (Fig. 2). A good, almost direct correlation ($r^2 = 0.80$; slope = 0.921) between the toxicity and the additive inhibitory free metal concentration index was observed, contrary to the correlation between the toxicity and additive inhibitory total metal concentration index ($r^2 = 0.42$). The data analysis performed above demonstrated that the chronic toxicity observed in *P. subcapitata* and the free metal ion concentrations were correlated, indicating that the total metal concentration is not, by itself, satisfactory to explain the observed toxicity. The toxicity behaviour detected at several stations was better explained using the additive effect of the free metal concentrations than the individual
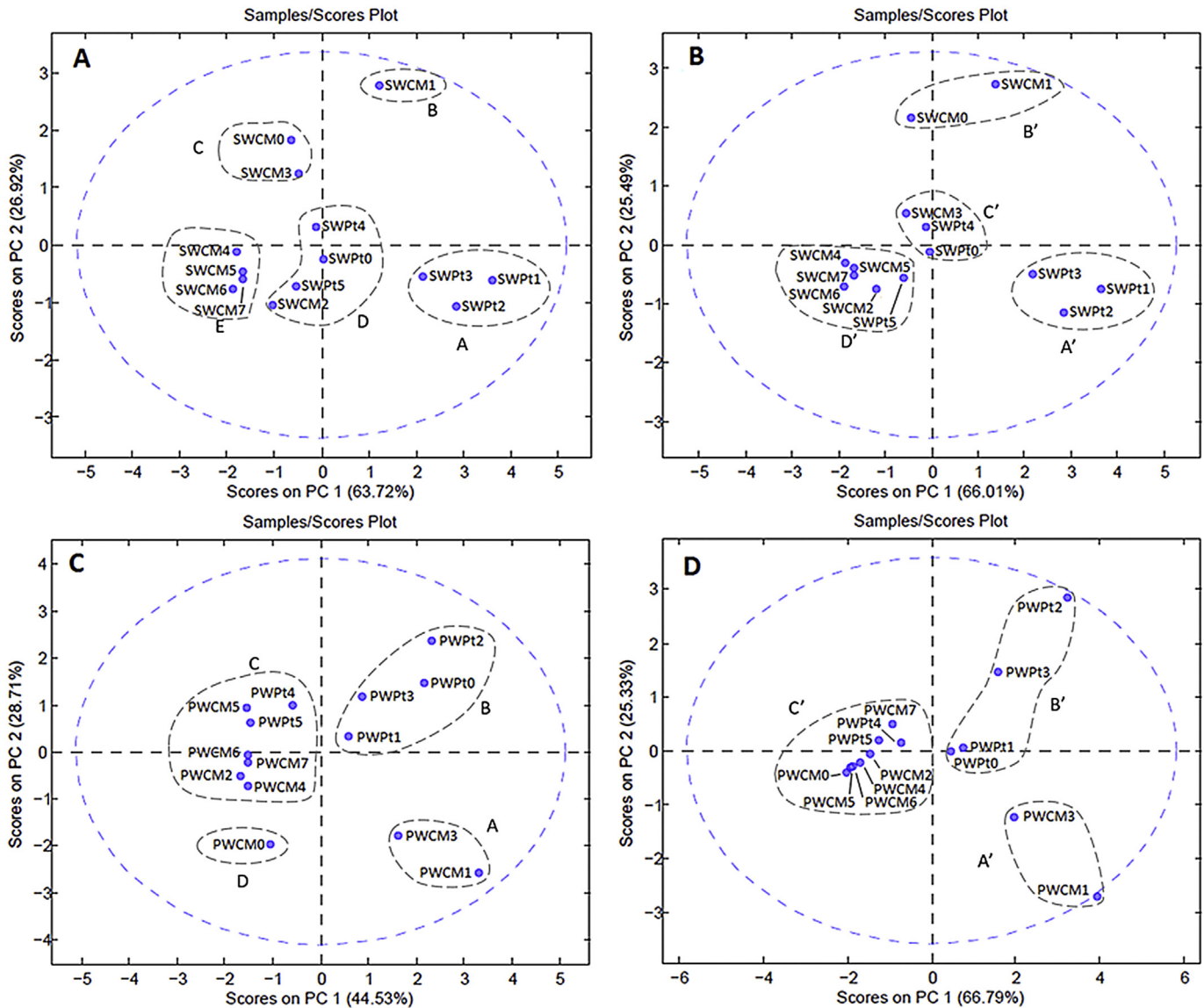
Fig. 1. PCA biplots. A - Surface water *versus* total metal concentration. B − Surface water *versus* free metal concentration. C − Pore water *versus* total metal concentration. D- Pore water *versus* free metal concentration.
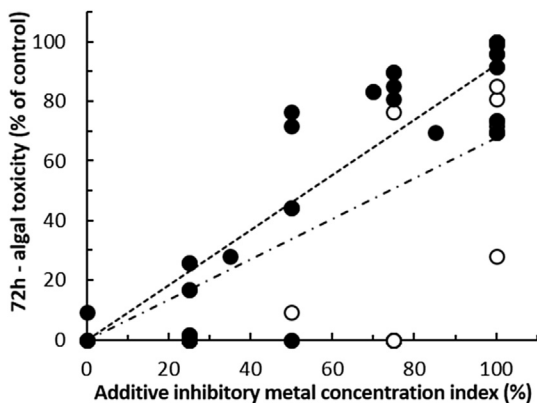
Fig. 2. Chronic toxicity (72 h) results evaluated using the alga *P. subcapitata versus* the additive inhibitory metal concentration indexes. AIMCi calculated using the free metal concentration (closed circles; y = 0.92x; R^2 = 0.80). AIMCi calculated using the total metal concentration (open circles; y = 0.68x; R^2 = 0.42).

toxicities. For example, at SWPt0, none of the metal concentrations exceeded the respective IC_{50}, yet an 83% inhibition rate was obtained. In this case, the AIMCi predicted an inhibition rate of 70%, which was similar to the observed toxicity. Moreover, at SWCM3, all of the metal concentrations did not exceed the IC_{50}, except for Pb. The inhibition of algal growth was 70%, which was similar to the value predicted by AIMCi (75%). These results were in agreement with those of a recent study, which showed that the toxicity of Ni, Cu and Cd to *Lemna minor* was less than the additive toxicity (overestimated toxicity) when expressed as the total metal concentration but was additive when expressed as the free ion concentrations (the bioavailable fraction) (Gopalapillai and Hale, 2015).

Regarding the surface and pore water toxicity results obtained in the present work, the pore water toxicity was better correlated with the free metal concentration than the surface water toxicity due to the high organic matter content of the pore water (Table S3), which could complex with metals present at high concentrations (Table 1), as observed in the following samples: (i) lead in samples PWCM0 to PWCM6 and PWPt0, PWPt1 and PWPt4; (ii) copper in

samples PWPt0 and PWPt4; and (iii) zinc in sample PWCM0. Pore water is the main exposure pathway for many benthic organisms (Chapman et al., 2002) and is a major entry point of metals into the food chain, which highlights its relevance in metal monitoring in water systems.

The application of the TTU model to assess the toxicity of our samples presented several limitations. For example, SWPT4 and SWPT5 displayed similar TTU values (1.92 and 1.54, respectively) (Fig. S2) but completely different toxicity results (81% and 44% of algal growth inhibition, respectively) (Table 2). The model failed because it assumed that the toxicity and pollutant concentration were linearly related. Limitations to the fit of toxicity data to the metal concentration using the TTU approach have been reported in other studies (Nys et al., 2016; Rogers, 2002). Nevertheless, TTU models can provide a quick indication of the main contributors to the toxicity for a specific site, as demonstrated by de Castro-Català et al. (2016). The AIMCi index proposed herein provides more accurate results, as shown in Fig. 2, because a more thorough analysis of the toxicity behaviour of each metal and its bioavailability is taken into consideration before assessing the final toxicity of the sample. In addition, these results are easier to understand, do not depend on specific ligand models and are directly related to the toxicity.

4. Conclusions

The present study provided evidence that the chronic toxic effects (72 h) observed in *P. subcapitata* due to exposure to fresh water samples contaminated with multiple metals were better correlated with the additive free metal ion concentration than with the total metal concentration.

The results described herein demonstrated that the toxicity of fresh water containing multiple metals (Cd and/or Cu and/or Pb and/or Zn) to *P. subcapitata* was additive when expressed as the free metal ion concentration, especially when different toxicity parameters (such as IC_{10}, IC_{25}, IC_{50} and IC_{75}) were employed. These data support the idea that concentration addition based on the free metal ion can be a reliable tool for evaluating the ecological risk of natural waters containing multiple metals and can be leveraged to influence the development of future regulatory targets.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.01.053.

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