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**Profiling metal contamination from ultramafic sediments to biota along the Albanian shoreline of Lake Ohrid (Albania/Macedonia)**

Laetitia Minguéz<sup>a\*</sup>, Elisabeth M. Gross<sup>a</sup>, Davide A.L. Vignati<sup>a</sup>, Ana Romero Freire<sup>a</sup>, Estelle Camizuli<sup>b</sup>, Frédéric Gimbert<sup>b</sup>, Céline Caillet<sup>c</sup>, Sandrine Pain-Devin<sup>a</sup>, Simon Devin<sup>a</sup>, François Guérol<sup>a</sup>, Laure Giambérini<sup>a</sup>

<sup>a</sup> Université de Lorraine, CNRS, LIEC, F-57000 Metz, France

<sup>b</sup> UMR CNRS 6249 Chrono-Environnement, University of Bourgogne Franche-Comté, 16 route de Gray, 25030, Besançon Cedex, France

<sup>c</sup> Université de Lorraine, CNRS, LIEC, F-54000 Nancy, France

\*Corresponding author: Tel.: +33 (0)372 748927; fax: +33 (0)372 748974

*Email address:* laetitia.minguez@univ-lorraine.fr (L. Minguéz).

## **Abstract**

Ultramafic sediments exhibit high levels of geogenically-derived and potentially toxic metals, with Ni, Cr and Co often exceeding benchmark values. As yet, a holistic understanding of the bioavailability, mobility, potential ecotoxicity and trophic transfer of trace elements in both benthic and pelagic food chains in aquatic ultramafic environments (UME) is lacking. We investigated potential environmental health issues due to metal contamination by jointly implementing chemical, ecological and toxicological tools, along the Albanian shoreline of Lake Ohrid. It is an aquatic system of worldwide importance, representative of temperate UME with a legacy of Ni and Cr contamination from mining activities. Levels of Ni, Cr, Cd, Cu, Co, Fe, Mn and Zn were determined in waters, sediments and native biota. The potential environmental mobility of sediment-bound elements was further assessed using CaCl<sub>2</sub>, EDTA and acetic acid extractions. Whole-sediment ecotoxicity tests were also carried out using ostracods and chironomids, according to standardized procedures. Despite Ni and Cr concentrations above the sediment quality guidelines for probable effect levels, we did not observe ecotoxic effects in laboratory tests. However, these elements were bioavailable to native organisms under field conditions, especially to benthic primary producers in direct contact with sediments (up to 139 mg Cr kg<sup>-1</sup> and 785 mg Ni kg<sup>-1</sup>). Although biomagnification was not observed, further investigations of metal translocation, metabolism and elemental trophic transfer along benthic food webs appears to be a general research priority in the management of temperate UME. The present study shows that proper management of temperate UME requires not only the integration of data from different lines of evidence, but also laboratory vs. field approaches to understand the subtler, long-term effects of increased elemental body burdens in native organisms.

*Keywords: Heavy metals, trophic transfer, Freshwater organisms, Ancient lake, Serpentine ecotoxicology*

## 1. Introduction

Ultramafic environments (UME) present unique challenges for environmental risk assessors and managers. These ecosystems are naturally characterized by high levels of potentially toxic elements (e.g., Ni, Cr, Co) and challenging edaphic conditions such as low Ca:Mg ratios and low overall concentrations of macronutrients (Bani et al, 2014; Marescotti et al, 2019). UME are found worldwide in ophiolite belts along tectonic plate margins (Circum-Pacific margin and Mediterranean Sea) and are frequent in Europe (Gwenzi, 2020). In particular, ultramafic substrates cover 11–15% of the total Albanian surface according to different estimates (Lekaj et al, 2019; Nicolas et al, 1999). Due to their natural characteristics, elemental concentrations measured in UME can exceed environmental quality standards or guidelines by orders of magnitude, which may trigger risk assessment and management programs for protecting environmental and human health. Nickel concentrations in ultramafic soils can reach 10,000 mg kg<sup>-1</sup> compared with a global average concentration of only about 16 mg kg<sup>-1</sup>. Chromium concentrations up to 30,000 mg kg<sup>-1</sup> have been found in soil of New Caledonia (Hseu et al, 2018). High levels of the human carcinogenic Cr(VI), exceeding the WHO drinking water quality guideline of 50 µg L<sup>-1</sup>, have also been reported in UME surface and groundwater as reviewed by Gwenzi (2020). Finally, UME are often mined for Ni and Cr ores, which can exacerbate the potential risks linked to the geogenically high elemental levels during extraction and leave a legacy of contaminated waste heaps and sediments in the absence of appropriate decommissioning procedures. When using conceptual approaches such as DPSIR frameworks (Driver, Pressure, State Change, Impact, Response – Gari et al, 2015) in UME management, it is therefore important to gather information to distinguish Pressures, State Changes and Impacts associated with natural Drivers (i.e., basin geology) from those originating from anthropogenic causes (e.g., changes in land use and mining). While the origin of trace elements is immaterial in relation to their ecotoxic potential, management Responses will obviously differ depending

on their actual Drivers. Proper assessment of environmental and human health risks in UME actually requires combined information on elemental chemistry, ecotoxicity, bioaccumulation and trophic transfer of contaminants to avoid unnecessary interventions or leaving actual environmental risks unmanaged (Burton, 2018).

Most studies in UME have been concerned with geochemistry, terrestrial plant ecology and the ability of terrestrial plants to hyperaccumulate metals for remediation or agromining purposes (Bani et al, 2013; Vithanage et al, 2019, Gwenzi, 2020). However, none of the existing studies seems to have simultaneously examined bioavailability, mobility, potential ecotoxicity and trophic transfer of trace elements in both benthic and pelagic food chains in a given UME; although benthic and pelagic habitats are strictly coupled (Baustian et al, 2014; Vadeboncoeur et al, 2002).

In the present study, we examine the elemental mobility, ecotoxicity and trophic transfer of sediment-bound trace elements at five sites along the Albanian shoreline of Lake Ohrid; a major European diversity hotspot and representative temperate UME with a legacy of sedimentary Ni and Cr contamination (Malaj et al, 2012, Lorenschat et al, 2014). Measured concentrations of Ni and Cr in Albanian soils reach around 3,000 mg kg<sup>-1</sup> and 2,400 mg kg<sup>-1</sup>, respectively (Bani et al, 2013, 2014) and sedimentary concentrations of up to 1600 mg kg<sup>-1</sup> and 700 mg kg<sup>-1</sup> have been reported (Malaj et al 2012). Because trace metals from historical mining sites can still be bioavailable to organisms years after decommissioning (Camizuli et al, 2018), metal contamination of Lake Ohrid remains an important local issue and its study can provide a reference point for other UME in Albania or, more in general, in temperate European areas. Besides Ni and Cr., we also determined levels of Cd, Cu, Co, Fe, Mn and Zn, considering their possible potential to act as additional stressors or to bioaccumulate/biomagnify in local food webs. The potential environmental mobility of sediment-bound elements was assessed using various partial extraction procedures, while two sediment-contact bioassays with the ostracod

*Heterocypris incongruens* and the midge larva *Chironomus riparius* were performed to study the actual ecotoxicological potential of the same sediment. To the best of our knowledge, no data about ultramafic whole-sediment ecotoxicity tests are available in the literature. Native organisms from Lake Ohrid were also sampled to quantify their total element burden as an integrated indicator of metal bioavailability, bioaccumulation and trophic transfer under *in situ* conditions.

## **2. Materials and Methods**

### **2.1. Study area**

We selected five sites along the Albanian shoreline of Lake Ohrid based on their expected levels of metal contamination following past mining activities and catchment mineralogy (Malaj et al, 2012). The first site, at the south end of the Lake in an urbanized area, was considered as control site with possibly low metal levels due to the geology of its catchment (fluvial/lacustrine sediment) (Pogradec or “Pog” in the following, N 40°54'03.62" E 20°42'08.36"). Three sites were located along the ultramafic shoreline of the lake: one in the vicinity of a Fe-Ni dump site at Memëlisht (“Mem”, N 40°55'53.53" E 20°38'28.60"), another near a creek mouth at Pojskë (“Poj”, N 40°59'59.05" E 20°38'07.61"), and a third close to the mouth of a creek flowing near an ancient mine extracting Cr, at Piskupat (“Pis”, N 41°01'51.71" E 20°38'06.63”). The last study site, at Lin (“Lin”, N 41°03'03.55" E 20°38'45.15") was chosen as a slightly contaminated site since its catchment was calcareous and no high metallic concentrations were expected in water and sediment.

### **2.2. Sampling and characterization of waters and sediments**

In early September 2016, we collected surface waters and sediments in each sampling site. Surface water samples were collected and preserved in acid-washed polyethylene containers. Samples were either directly acidified by adding 1% (v/v) concentrated ultrapure nitric acid for

later analyses of total metal contents, or first filtered through syringe Millex filters (PVDF; 0.45 µm nominal pore size) and then acidified as above for later measurements of dissolved metal concentrations. A fraction of each water sample was filtered through pre-combusted GF/F filters (Whatman), and was not acidified to quantify dissolved organic carbon. All samples were stored at 4°C pending analysis. Water temperature, dissolved oxygen, conductivity and pH were measured *in situ* with WTW probes.

At each site, the upper 10 cm of sediment were collected at three points about a meter apart, using a stainless-steel shovel and well mixed to obtain one composite sample, representative of each site. Samples were placed in polyethylene containers and stored at 4°C until further processing. Representative subsamples of each sediment were dried at 65°C for 72h and sieved to <2 mm fraction. Pseudo-total metal concentrations were determined by digesting sediments in aqua regia. The potential availability of sediment-bound metals was further evaluated using four widely applied procedures: (1) the mobile amounts of metals through 0.01 M CaCl<sub>2</sub> extraction (Houba et al, 1990); (2) the potentially phytoavailable fraction through 0.05 M EDTA extraction (Ure et al, 1993); (3) the mobilizable pool by treating subsamples with 0.11 M acetic acid (Quevauviller et al, 1997); and (4) the amorphous Fe, Mn and Al oxides through the 0.2 M acid ammonium oxalate extraction in the dark (Schwertmann, 1964). The above chemical procedures have been validated by a certified reference material BCR® 701 (European Commission) helping to monitor potential pitfalls that may occur during extractions. Particle size distribution was determined by laser diffraction granulometry (SympaTEC Helos BF granulometer) on a smaller fraction further sieved to <1 mm and expressed as dry-weight (DW) percentage. Sediment total organic carbon (TOC) was determined by loss on ignition at 550°C (APHA, 2005). Details on the different analytical procedures are given in Supporting Information (SI Methods).

### **2.3. Chronic Bioassays**

We performed two chronic bioassays to assess the potential toxicity of sampled sediments, using two sediment-dwelling species. First, the ostracodTox test, using neonates (<52h old) of the benthic crustacean *Heterocypris incongruens* (Ramdohr, 1808), consists in putting the ostracods in contact with a sediment for 6 days (MicroBioTest, 2001) and in measuring their survival and length at the end of the exposure period, as a proxy of growth. The second bioassay was the 28-day *Chironomus riparius* test following the OECD guideline 218 (2004). Survival and growth (mass and length) of larvae after 10 days and the emergence of adults after 28 days were measured (for more details, see SI Methods). As the amount of sediment from Lin was limited, only sediments from Pog, Mem, Poj and Pis (i.e. the control sediment and the three sediments under ultramafic influence) were tested for their ecotoxicity to chironomids.

#### **2.4. Aquatic biota sampling**

Biota samples (n = 257) were collected at the same time as waters and sediments to quantify *in situ* bioaccumulation by organisms from different taxa and feeding guilds and define their trophic level (Table S1): (i) Benthic and pelagic primary producers: The periphytic biofilm was collected by brushing rock surfaces with a medium bristled toothbrush. Large organisms were removed by filtration through an 80- $\mu$ m mesh nylon filter. Specimens of the submerged aquatic plant *Myriophyllum spicatum* L. (Haloragaceae) were collected at two-meter depth by snorkeling. In the lab, plants were separated into leaves, stems and belowground parts (roots and rhizomes). The latter were not analyzed in this study since they are not accessible for grazers. Phytoplankton was sampled offshore during daylight by two vertical tows, from 15 m depth to the surface using a net of 34- $\mu$ m mesh size, then filtered through an 80- $\mu$ m mesh nylon filter to remove larger organisms. (ii) Benthic and pelagic consumers: Benthic invertebrates were hand-collected in the littoral zone and included amphipods (gammarids), bivalves (*Unio tumidus* and *Dreissena carinata*, synonym of *D. stankovici* and *D. presbensis*) and gastropods (*Radix relicta*). After collection, organisms were rinsed with ultrapure water. A batch of



individuals of each taxon was depurated for 48h to verify the possible contribution of gut content to metal accumulation in the sampled organisms. Another batch was not depurated since predators are exposed to both digestive lumen contents and prey tissues. A third batch of gut-purged invertebrates was taken for stable isotope analyses (see section 2.6). All organisms were finally frozen in liquid nitrogen. Bivalves and gastropods were removed from their shells before snap-freezing in liquid nitrogen. Zooplankton was sampled offshore during daylight by two vertical tows, from 15 m depth to the surface using a net of 83- $\mu$ m mesh size. (iii) Fish species: At least three individuals of five fish species were obtained from fishermen: *Alburnus albidus* (size range: 10.9–11.6 cm), *Barbus meridionalis petenyi* (9.2–9.9 cm), *Leuciscus cephalus albus* (10.2–13.3 cm), *Rutilus ohridanus* (7.7–9.3 cm) and *Salmo letnica* (20–25 cm), the last two species being endemic of this lake. Muscles were dissected, snap frozen in liquid nitrogen and stored at -80°C until further processing. All biological samples were freeze-dried before metal analyses. Not every organism was found at each site (Table S1).

## **2.5. Metal analysis**

Aqueous concentrations of Cd, Cr, Cu, Mn, Ni were determined by graphite furnace atomic absorption spectrophotometry (VARIAN Spectraa 800 with ZEEMAN correction), while Fe and Zn were determined by flame atomic absorption spectrophotometry (FAAS) (Perkin-Elmer Aanalyst 100). Surface water SPS-SW1 (Batch 120) was used as quality control material. In sediments and biota, Cd, Co, Cr, Cu, Fe, Mn, Ni, and Zn were quantified by FAAS after mineralization. Sediments (500 mg) were digested in 19 mL aqua regia (HNO<sub>3</sub>/HCl ratio 1:3) and biota samples in 10 or 19 mL 69% HNO<sub>3</sub> (according to the available sample mass: <100 mg or >100 mg, respectively) for 48h at 60°C. All reagents and solvents used for metal analysis were analytical grade. Certified reference material TORT-2 (National Research Council of Canada) was submitted to the same digestion procedure during each analytical run. Percent recovery for elements in certified reference material were as follows: Cd (103%), Cu (100%),

Co (86%), Cr (102%), Fe (98%), Mn (97%), Ni (61%), and Zn (100%). Data were not corrected for percent recovery.

## **2.6. Stable isotopes and trophic level calculations**

Nitrogen isotopes were used to estimate the trophic positions of consumers and carbon isotopes to estimate the carbon flow to consumers in food webs. Carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) stable isotopes were analyzed on all sampled species at the Service d'Analyse des Roches et des Minéraux (SARM), CRPG (Nancy, France) using a GasBench/isotope ratio mass spectrometer (Thermo Mat 253). Plankton and periphyton were filtered on site using pre-combusted GF/F filters (Whatman) to provide at least 1 mg dry weight of sample on each filter. Filters were treated with 1 M HCl and dried overnight at 50°C to remove inorganic carbon. Dried leaves of aquatic plants, whole tissues of depurated invertebrates, and muscles of fishes were ground to a fine powder using a ball mill (Retsch MM400). Approximately 2 mg of each biological sample were weighed into tin capsules and analyzed. Isotopic ratios were calculated using the following equation:

$\delta X (\text{‰}) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$ , where  $X = {}^{13}\text{C}$  or  ${}^{15}\text{N}$ , and  $R$  = corresponding ratio of  ${}^{13}\text{C}/{}^{12}\text{C}$  or  ${}^{15}\text{N}/{}^{14}\text{N}$ . Samples were not delipidated, but since lipid content can impact  $\delta^{13}\text{C}$ , C/N ratios were measured. Eight international standards of known  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were used (NBS 22, IAEA CH3, IAEA CH6, IAEA CH7, USGS 24, IAEA N1, USGS 25 and Urea IVA). Analytical precision was  $\pm 0.1 \text{‰}$ .

## **2.7. Data handling and statistics**

All statistical analyses were performed with R version 3.6.1 (R Core Team 2019), using the RStudio interface (version 1.0.153).

### **2.7.1. Sediment quality and toxic effects**

The Sediment Quality Index (SQI) developed by the Canadian Council of Ministers of the Environment (CCME, 2002) and modified later by Marvin et al (2004), was chosen to describe

the overall quality of the sediments. The index score was computed for each sample using the probable effect concentrations (PEC) guideline values (Table 1), developed by MacDonald et al (2000). Sediments were classified in five categories according to their SQIs: poor when  $0 \leq \text{SQI} < 45$ , marginal when  $45 \leq \text{SQI} < 60$ , fair when  $60 \leq \text{SQI} < 80$ , good when  $80 \leq \text{SQI} < 95$ , excellent when  $95 \leq \text{SQI} \leq 100$  (Marvin et al, 2004). Correlation coefficients between sediment parameters were determined using the Spearman's rank correlation test. Linear regressions between selected variables (see section 3.2 for details) were derived using the least square method.

Differences in survival of *C. riparius* larvae after 10 days of exposure were tested using a generalized linear mixed-effects model (glmer R package). The relationship between emergence rates and sediments was assessed using a non-linear mixed effect regression model (nlme R package). For both models, the sediment was integrated as fixed effect and the beaker as random effect. Pairwise comparisons were done using a likelihood-ratio test. When a significant relationship was found between emergence rate and sediment, pairwise Wald t-test of the estimated coefficients were carried out between treatments by re-ordering them in nlme models using the *relevel* R function (stats R package). The effect of sediment on the growth of ostracods and chironomids was tested using ANOVA, followed by post-hoc pairwise comparisons using Tukey's HSD test.

### **2.7.2. Metal profile of native biota**

Principal component analysis (PCA) was used to summarize the variations in the bioaccumulated concentrations of metals, and to facilitate the comparison between sites and organisms. PCA analysis was performed with the FactoMineR package (Lê et al, 2008) and data were visualized using the factoextra package (Factoextra, 2018). Data did not satisfy the assumptions of normality and variance homogeneity, thus significant differences between metal contents in biota were tested using the Kruskal-Wallis ANOVA and the non-parametric Mann-

Whitney test for paired comparisons corrected for multiple comparisons using the Benjamini-Hochberg procedure. For primary producer and fish data with low sample sizes, we calculated bootstrapped 95% confident intervals (CIs) of the difference between group medians (BCa method, R = 1000). When the 95% CIs of the difference in medians excluded zero, we concluded there was a statistically significant difference in median values between groups.

### 2.7.3. Trophic transfer

Corrections of  $\delta^{13}\text{C}$  values were performed when the C/N ratio exceeded 3.5. A normalization was applied following the equation of Post et al (2007):  $\delta^{13}\text{C}_{\text{lipid corrected}} (\text{‰}) = \delta^{13}\text{C} - 3.32 + 0.99 \times \text{C/N}$ . Since the C/N ratio is not a good predictor of lipid content in aquatic macrophytes, normalization was performed based on the carbon content (Post et al, 2007), following the equation:  $\delta^{13}\text{C}_{\text{lipid corrected}} (\text{‰}) = \delta^{13}\text{C} - 3.09 + 0.09 \times \%C$ . The bivalves *Dreissena carinata* (Dc) were assumed to occupy a second trophic level (TL = 2.0) as primary herbivores. Trophic levels of other organisms were determined using the following equation:  $TL = [(\delta^{15}\text{N}_{\text{org}} - \delta^{15}\text{N}_{\text{Dc}})/3.4] + 2$ , where  $\delta^{15}\text{N}_{\text{org}}$  is the  $\delta^{15}\text{N}$  signature in a given organism,  $\delta^{15}\text{N}_{\text{Dc}}$  is the mean  $\delta^{15}\text{N}$  value ( $\pm$  sd) for *D. carinata* ( $3.60 \pm 0.16\text{‰}$ ), and 3.4 is the trophic enrichment factor for  $\delta^{15}\text{N}$  (Post, 2002). The quality of linear regressions between log[metals] and TL was assessed by the squared coefficient of correlation ( $R^2$ ), Fisher's criterion (F) and the p-value. The slopes were used to calculate trophic magnification factors (TMF), as  $\text{TMF} = 10^{\text{slope}}$ . Trophic magnification and dilution are defined, respectively, by TMF above and below 1.

## 3. Results and Discussion

### 3.1. Metal profile of waters

The oligohumic (DOC  $<4 \text{ mg L}^{-1}$ ) and soft waters (conductivity  $<300 \mu\text{S cm}^{-1}$ , Ca  $<30 \text{ mg L}^{-1}$ ) of Lake Ohrid displayed metal contamination below or in the same range as those found in other

lakes worldwide (for reviews Chen et al, 2018; Sibal and Espino, 2018). All studied metals were also below threshold concentrations defining a potential environmental hazard condition (EQS) ([dataset] Minguez et al, 2021). Even for the three metals (Cu, Ni and Zn) for which bioavailable concentrations can be estimated using “user-friendly” biotic ligand models ([www.bio-met.net](http://www.bio-met.net)), no environmental risk is predicted. One should note that all the “user-friendly” biotic ligand models free-available to estimate bioavailable concentrations of Cu, Ni and Zn and the associated environmental risk at a specific site, reach their limit for those ultramafic aquatic ecosystems characterized by high pH (above 8.5-8.7), rather low hardness and low DOC. All these models have been validated for certain pH, Ca and DOC ranges, and the pH measured in all study sites are equal to or above the upper limits of the validated pH ranges in the models (pH ranges: 6-8.5 for Cu, 6.5-8.7 for Ni and 6-8 for Zn), meaning that results should be interpreted with caution.

It is essential to confirm this non-exceeding of the EQS by a monitoring at different seasons, thus integrating episodes of precipitation. Lake Ohrid, has a prevailing Mediterranean climate with enhanced precipitation relative to the rest of southern Europe. It is especially supplied with water from surface run-off via creeks and rivers flowing through ultramafic soils (Sinopoli et al, 2019). Rain events could largely determine metal loadings into the lake.

### **3.2. Metal profile of sediments**

Sediments were sandy and contained low amounts of organic matter and low (Pog and Mem) to intermediate (Poj and Pis) calcium carbonate. Only sediment from Lin was rich in  $\text{CaCO}_3$  due to the limestone geology of its shoreline ([dataset] Minguez et al, 2021). High concentrations of Cr, Ni, Fe and Mn were found at Mem, Poj and Pis, (Table 1). The maximum concentrations of Cr ( $345 \text{ mg kg}^{-1}$ ) and Ni ( $554 \text{ mg kg}^{-1}$ ) were found at Pis and Mem, respectively; i.e. the closest sites to former Cr or Ni mines. Mining of Ni and Cr was in the form

of Fe alloys (Malaj et al, 2012), which accounts for the presence of high Fe concentrations, up to  $50 \text{ g kg}^{-1}$  at Pis, in these sediments. Significant positive correlations ( $r_s$  between 0.88 and 1) between the concentrations of Co, Cr, Fe and Mn (Figure 1) confirmed the co-occurrence of these metals, as already observed by Malaj et al (2012). Metal concentrations in sediments were generally lower than those reported by Malaj et al (2012), especially for Ni (10-fold) and Cr (1.5-fold) at Pis and Poj. Further studies are required to determine if such differences simply reflect a patchy distribution of contaminated sediments or decreasing trends in Cr and Ni levels. The sediment quality index (SQI) classified sediments from Mem, Poj and Pis as of poor and marginal qualities ( $SQI_{\text{Mem}}= 37.64$ ,  $SQI_{\text{Poj}}= 56.34$ ,  $SQI_{\text{Pis}}= 51.96$ ). The concentrations quantified in those sites were also higher than or in the same range as those reported in the literature for freshwater sediments considered as severely polluted (e.g. in Tigris River, Varol, 2011; in several Chinese lakes, Xu et al, 2017). Site Lin, not directly linked with any source of pollution (creeks or ore dump sites), displayed an enrichment in Cr and Ni concentrations ( $43$  and  $75 \text{ mg kg}^{-1}$ ) compared to Pog. In this area, Vogel et al (2010) have reported the occurrence of strong wind-driven surface currents, which redistribute total suspended solids and thus metals in the lake. These currents may explain this enrichment at Lin, lowering sediment status from excellent to good quality ( $SQI_{\text{Pog}}=100$ ,  $SQI_{\text{Lin}}=80.42$ ).

Correlations with Fe would indicate that the high concentrations of Cr and Ni likely originate from natural weathering of ferronickel and chromite minerals found in ultramafic soils (Malaj et al, 2012). For our data, such a correlation exists for Cr, but not for Ni (Figure 1). Single extraction procedures showed that Ni was generally more mobile than Cr, although the exchangeable fraction was  $\leq 0.12\%$  of the total for both elements (Table 1). Sediment extraction with acetic acid (AA) suggested that 3 to 25% of Ni could be associated with carbonates; while the corresponding figures for Cr did not exceed 1.6%. In the case of Ni, acetic acid extractable concentrations and EDTA extractable levels (1–16% of the total) showed similar patterns

among sampling sites, but the correlation between the two variables was not significant ( $r_s = 0.898$ ;  $p = 0.102$ ). On the other hand, EDTA extractable Cr (0.15–1% of the total) correlated well with amorphous Mn oxides as estimated by ammonium oxalate extraction ( $r_s = 0.982$ ,  $p = 0.018$ ). In both cases, no correlations were observed between partial and pseudo-total metal concentrations. These results are in agreement with those of Malaj et al (2012) although the comparison must take into account methodological differences in the extraction procedures. Overall, Ni and Cr appeared strongly associated with different mineral phases that remain to be better identified. However, partial extraction procedures poorly mimic the actual processes occurring in the digestive apparatuses of particle-ingesting organisms. Thus, additional information on the actual ecotoxicological and bioaccumulation potential of these elements must be directly obtained from other lines of evidence (e.g. assessment of ecotoxicological risk and bioaccumulation).

### **3.3. Ecotoxicity screening of sediments**

Ostracods exposed to lake sediments did not show any mortality or growth reduction compared to the non-toxic reference sediment (Figure S1A). The 10-d survival of *C. riparius* ranged from  $93 \pm 8\%$  to  $98 \pm 3\%$ , with no significant differences among samples (Figure S1B). Concerning the growth (in mass and length) of midge larvae (Figure 2A) and their emergence (Figure 2B, Table S2), only the sediment from Pog displayed a pattern significantly different from the reference sediment (sand), with the highest growth after 10 days. The sex ratio of adults was on average 0.52 at the end of experiment. Although 3 out of the 5 studied lake sediments displayed metal concentrations exceeding quality guidelines (de Deckere et al., 2011; MacDonald et al., 2000; Vangheluwe et al, 2013), the low amounts of organic matter, the high concentrations of Fe- and Mn-oxides and the low concentrations of metals in the water-soluble fraction imply that Ni and associated metals were not available and toxic for ostracods and chironomids.

As metals in lake sediments were not readily available, it is therefore unlikely that the sediment contamination by metals led to the observed variations in the growth and emergence of *C. riparius* larvae. The sandy matrix (reference) leading to similar results as field contaminated sediments also supports this assertion. The low (but significant) delay in growth (Fig. 2A) and emergence (Fig. 2B) may be explained by the absence of organic matter (as a source of food and energy for larvae) in the reference sand sediment. The quantity and/or quality of organic matter in the sediment is probably a key variable explaining the results. Further data analyses or experiments to measure bioaccumulation and the subcellular fate of metals in chironomid tissues would also provide information to better understand these differences (Gimbert et al., 2016). Two final caveats conclude this critical evaluation of these ecotoxicological results. Laboratory experiments with whole sediment cannot account for metal body-burdens originating from (freshly deposited) suspended particulate matter; an exposure route that can strongly contribute to elemental accumulation in chironomids and other organisms (Ferrari et al, 2019). Finally, chironomids may be rather insensitive to elemental contamination and can tolerate metal burdens high enough to eliminate more sensitive taxa from the benthic community occurring at a given site (Bervoets et al, 2016).

#### **3.4. Metal profile of *in situ* biota**

Metal concentrations in organisms result from prolonged exposure, making it difficult to link them with ambient aqueous concentrations at the time of sampling (Ravera et al 2003). Thus, we focused on comparing concentrations measured in benthic organisms sampled in at least 3 sites to those in sediments that are more representative of exposure levels over time. Cadmium was not detected in biota and will not be discussed in this section. Concentrations in organisms were better correlated to pseudo-total concentrations in sediments (i.e. aqua regia extraction) than to concentrations found in the different sediment fractions (Figure 3). While EDTA is often considered as the best extractant for phytoavailable metals, Co, Cr and Ni concentrations



accumulated by the macrophyte *M. spicatum* were better correlated to concentrations in the acetic acid fraction. Under nutrient-limited conditions, such as those encountered in ultramafic environments (Bani et al, 2014; Marescotti et al, 2019), aquatic macrophytes are known to release organic acids from their roots to increase nutrient availabilities, especially by dissolving carbonate complexes (Gunnison and Barko, 1988; Xing et al, 2018). Experimental studies following macronutrient nutrition of macrophytes demonstrated that the uptake pattern of both essential and non-essential elements was similar, from the sediment by the roots followed by a translocation to above-ground tissues (Barko and Smart, 1986; Jackson et al, 1994).

The PCA synthesizes results of the metal contents accumulated by primary producers (phytoplankton, *Myriophyllum spicatum* and periphytic biofilm) and consumers (zooplankton, *Dreissena carinata*, *Unio tumidus*, *Radix relicta* and *Gammarus* sp.) from benthic-pelagic food webs (Figure 4). The first two axes explain 80.2% of the total variance in the data matrix. Factor 1 explains 53.2% of the total variance and is characterized by measured concentrations of Cr, Ni, Fe and Co in organisms (positive loads: higher concentrations bioaccumulated). This axis discriminates benthic primary producers (Biofilm and *M. spicatum*) from the other organisms. Factor 2 explains 27.0% of the total variance and is defined by concentrations of Zn and Cu in consumers. This axis distinguishes zooplankton and *U. tumidus* from the other organisms.

The highest concentrations of Cr and Ni were found in benthic primary producers from the three most polluted sites (Mem, Poj and Pis) (Figure 4, Figure S2). Maximum levels of Cr and Ni in biofilms were 95 mg kg<sup>-1</sup> and 785 mg kg<sup>-1</sup>, respectively; while the corresponding concentrations for *M. spicatum* reached 115 mg kg<sup>-1</sup> and 461 mg kg<sup>-1</sup>. Several submerged macrophyte species, such as *Myriophyllum* spp., and biofilms are known to exhibit high accumulation capability for metals (Cardwell et al, 2002; Geng et al, 2019; Harguinteguy et al, 2015; Xing et al, 2013). Rooted species can extract metals either from the sediment via their roots or from the surrounding water via their shoots. Others (e.g. *Ceratophyllum demersum* and *Vallisneria*

*natans*) can even hyperaccumulate metals such as Cr (Xing et al, 2013). In Lake Ohrid, the threshold for hyperaccumulation of Cr and Ni was not reached (i.e. 1,000 mg kg<sup>-1</sup>) (Reeves and Baker, 2000). Metals accumulated in stems and leaves might ultimately be transferred to herbivores when plant tissues are consumed. Similarly, metals could be released during macrophyte senescence through leaching, mineralization and microbial degradation, and thus become available to detritus feeders (Weis and Weis 2004). The different patterns of metal concentrations between macrophytes and biofilm suggest that the latter have a higher ability to accumulate metals compared to *M. spicatum*. However, we cannot exclude the fact that biofilms also contained some resuspended particles from sediments or trapped sinking suspended particles. A separation of the living (algae, bacteria, meiofauna) and dead fractions (suspended particles) of a biofilm is not possible without loss of biotic material, and is also unlikely to happen during consumption of biofilm by most grazers, such as snails.

Composite samples of phytoplankton showed lower metal contents (e.g. ranges for Cr: 5-38 mg kg<sup>-1</sup> and Ni: 10-116 mg kg<sup>-1</sup>) compared to benthic primary producers. Several factors influence the accumulation of metals in phytoplankton such as the productivity of the water body, the physicochemical properties of the water, the composition of phytoplankton and the season (Mazej et al, 2010). In oligotrophic lakes, benthic primary production dominates, and since benthic primary producers are more effective at sequestering nutrients, they regulate the availability of sediment nutrients to phytoplankton (Vadeboncoeur et al, 2002). Metals, some of which are essential for plants, may follow the same process, resulting in higher metal concentrations in benthic primary producers than in pelagic ones.

Consumers accumulated less metals than primary producers (negative coordinates in the first PCA-axis, Figure 4). The 48h-depuration period did not significantly affect measured metal concentrations, except for non-depurated gammarids from Mem that showed 32 and 50% higher values for Fe and Ni compared to the corresponding depurated specimen (Figure S3). This

suggests that in general the gut contents did not significantly contribute to the concentrations measured in consumers. Significant interspecific differences were found, even between taxonomically similar species (Figure 4, Figure S4). The highest concentrations of Ni and Cr in invertebrates were found at Mem, Poj and Pis, similar to the pattern found for primary producers. Bivalve mollusks have been commonly used to assess the levels of contamination, since these organisms provide a time-integrated measure of metal bioavailability (Rzymiski et al, 2014). In the present study, metal concentrations differed between the two studied bivalve species. Cr and Zn concentrations in *U. tumidus* were 4-6 times higher than those found in *D. carinata*, whereas Ni, Co and Cu concentrations were almost 2 times lower in *U. tumidus* than in *D. carinata*. These findings are in agreement with those of Rzymiski et al (2014), who observed that *U. tumidus* was able to accumulate high Cr contents and, suggested using this species as a bioindicator of Cr contamination. However, metal accumulation in bivalves strongly depends on biotic and abiotic parameters, making interspecific comparison between species difficult. Accumulation can be a function of age, size, feeding activity, physiology of organisms and the physicochemistry of the surrounding environment (Boening, 1999). *Dreissena carinata* were smaller than *U. tumidus* and the habitat of *D. carinata* is related to the water-column whereas sediments define the habitat of *U. tumidus*.

Gastropods, *R. relictta*, were the highest accumulators of metals (17 mg Cr kg<sup>-1</sup> at Poj and 252 mg Ni kg<sup>-1</sup> dry weight at Mem, respectively). Unlike bivalves, which are filter-feeders of phytoplankton displaying low metal concentrations, the feeding habit of gastropods involves grazing of epiphytes, periphyton and aquatic macrophytes, which accumulated the highest concentrations of metals among all studied organisms (Figure S2, S4). On the other hand, gammarids were characterized by higher Cu concentrations and zooplankton by higher contents of Cu and Zn compared with other studied organisms. Cu and Zn are essential elements and are regulated by physiological mechanisms in most organisms. In crustaceans, these two metals are

required for the functioning of both enzymatic (e.g. carbonic anhydrase a Zn-containing protein) and non-enzymatic proteins (e.g. proteins Cu-associated with respiratory processes such as the respiratory pigment hemocyanin) (White and Rainbow, 1985). Estimations of the amount of Cu and Zn needed for these protein activities yield values of 83 mg Cu kg<sup>-1</sup> and 68 mg Zn kg<sup>-1</sup> dry weight (Depledge, 1989). Observed concentrations in *Gammarus* spp. were close to the metabolic requirements for both metals, thus they did not show abnormal metal contents. However, measured contents for zooplankton are greater than the estimated metabolic requirements. Possible explanations include the fact that they feed on phytoplankton, which was also rich in Zn and Cu, and zooplankton may be slow to metabolize or excrete these metals. Knowledge of metal concentrations in fish is important for both conservation purposes and management of the human consumption of fish (Papagiannis et al, 2004). In Lake Ohrid, fishes displayed even lower concentrations in their muscles than primary consumers, with no strong differences between species in non-essential element concentrations (Figure 5). These results are in agreement with the concentrations below detection limits observed by Malaj et al (2012) on *S. letnica* and *Salmo ohridanus* in Lake Ohrid. One reason for this low accumulation in fishes could be their continuous migration to uncontaminated parts of the Lake (Malaj et al, 2012), such as the pelagic zone where organisms (phytoplankton and zooplankton) showed low concentrations. It should also be noted that these concentrations were measured in muscles and not on the whole organism as for the invertebrates. Muscles are known to display lower capacity of contaminant accumulation compared to other tissues such as the liver and intestine. Knowledge of fish behaviors in this area and a better understanding of their detoxifying metabolism integrating other tissues are necessary to draw conclusions about this biotic compartment. The consumption of these edible parts would not present a health risk. Estimated daily intake of metals considering an adult of 70kg with a mean consumption of 8 g per day

(FAO, 2015) would be exposed to concentrations much lower than tolerable daily intake defined by the World Health Organization.

### **3.5. Transfer of metals along food webs**

Mean values of stable  $\delta^{15}\text{N}$  isotope signatures ranged from 2.6 to 11.8‰ (without considering the enrichment factor) (Figure S5). Four trophic levels were identified, with primary producers representing the bottom trophic level in studied food webs (lowest values of  $\delta^{15}\text{N}$ ). All metals analyzed in this study showed a decrease of concentrations with increasing trophic level, except Zn which showed no variations in concentrations between trophic levels (Figure 6, Figure S6). These regressions resulted in a trophic magnification factor (TMF) of 0.16 for Cr (slope =  $-0.79 \pm 0.09$ ,  $R^2 = 0.52$ ,  $F = 68.41$ ,  $p < 0.001$ ), 0.07 for Ni (slope =  $-1.15 \pm 0.09$ ,  $R^2 = 0.69$ ,  $F = 138.2$ ,  $p < 0.001$ ), and 0.37 for Cu (slope =  $-0.43 \pm 0.08$ ,  $R^2 = 0.29$ ,  $F = 26.67$ ,  $p < 0.001$ ). The TMF for the other metals (Co, Fe and Mn) were around 0.2. Our results are consistent with the concept of trophic dilution, defined by a decrease in contaminant concentration as trophic level increases (Nordberg et al, 2009), and agree with previous studies reporting that these metals generally do not biomagnify in food chains (Cardwell et al, 2013; Cui et al, 2011; Ward et al, 2012). However, Zn biomagnification may occur where ambient Zn concentrations are deficient or less than optimal (Cardwell et al, 2013; Ward et al, 2012). For Zn, which showed different patterns of trophic transfer, the assessment of subcellular bioaccumulation could provide insight into Zn handling strategies by organisms from Lake Ohrid (Rosabal et al, 2014). It is noteworthy that even if biomagnification is unlikely when food chains are studied as a whole, the transfer of metals from a food source to its consumer (i.e. trophic transfer or “biotransference” as used by the following authors) can occur (Barwick and Maher, 2003), as observed here between phytoplankton and zooplankton (for Cu, Cr, Ni and Zn) or *D. carinata* (for Ni). Several cases of trophic transfer were reported in aquatic ecosystems between primary

producer species and their invertebrate consumers, especially for Cu and Zn (Barwick and Maher, 2003; Jara-Marini et al, 2009; Wang, 2002).

#### **4. Conclusion**

Sediments from the Albanian shoreline of Lake Ohrid can still exhibit Ni and Cr concentrations above quality guidelines for probable effect levels. However, the present study provides first time evidence that such concentrations do not elicit direct ecotoxic effects on model ostracods and chironomids in standardized laboratory tests. On the other hand, both elements appear to be bioavailable to indigenous organisms under field conditions, especially to benthic primary producers in direct contact with sediments. Based on the available results, the following general recommendations can be formulated to improve the current understanding of pressures and impacts in the management of aquatic ecosystems located in temperate UME.

Although biomagnification was not observed in the present study, further investigations of elemental trophic transfer along benthic food webs appear as a general research priority. This includes accumulation in organisms directly feeding on primary producers, but also transfer from detritivorous invertebrates to predators such as Odonata that could not be sampled in the present study. In the case of primary producers, *M. spicatum* appears as a valuable tool to better understand the potential adverse effects of Ni and Cr because it offers the possibility of combining sediment contact tests in the laboratory with analysis of specimens collected in situ for a single biological model. Overall, the present study shows that proper management of (anthropogenically impacted) aquatic UME requires not only the integration of data from different lines of evidence (geochemistry, ecology and ecotoxicology), but also laboratory vs. field approaches to understand the subtler, long-term effects of increased elemental body burdens in indigenous organisms.

### **Data availability**

The data about physicochemical parameters in waters and sediments discussed in this study are available through the online database ORDaR (OTELo Research Data Repository) at <https://doi.org/10.24396/ORDAR-67> (Minguez et al, 2021).

### **Credit author statement**

**L. Minguez:** Conceptualization, Methodology, Resources, Investigation, Validation, Formal analysis, Writing original draft, Visualization, Supervision. **E.M. Gross and D.A.L. Vignati:** Conceptualization, Methodology, Resources, Investigation, Validation, Writing original draft. **A. Romero Freire:** Investigation, Writing original draft. **E. Camizuli:** Formal analysis, Writing original draft. **F. Gimbert:** Resources, Investigation, Validation, Formal analysis, Writing original draft. **C. Caillet:** Investigation, Writing original draft. **S. Pain-Devin:** Writing original draft. **S. Devin and F. Guéroid:** Conceptualization, Writing original draft. **L. Giambérini:** Conceptualization, Methodology, Resources, Investigation, Validation, Writing original draft, Project administration, Supervision, Funding acquisition.

### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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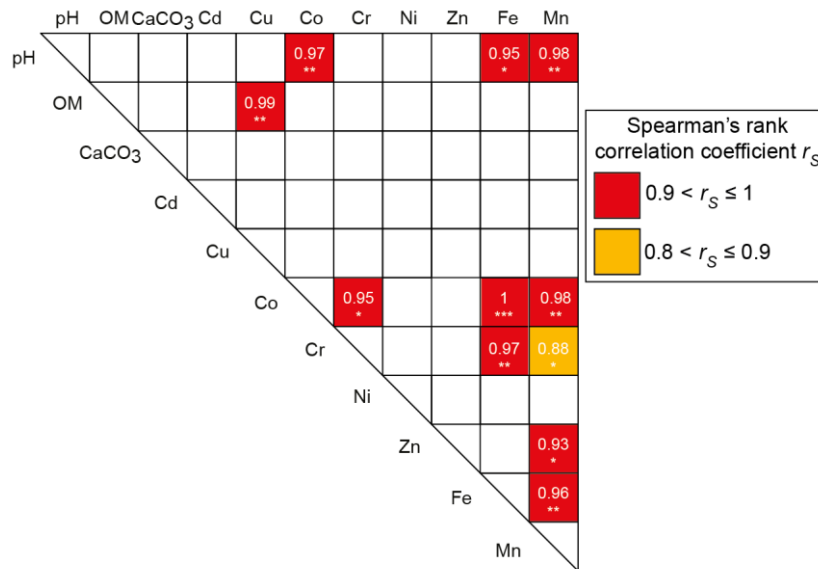
## Table and Figures

**Table 1.** Trace element concentrations measured in sediments from sites Pog, Mem, Poj and Pis using pseudo-total and partial extraction procedures (see text for details). All values are in mg kg<sup>-1</sup> dry weight.

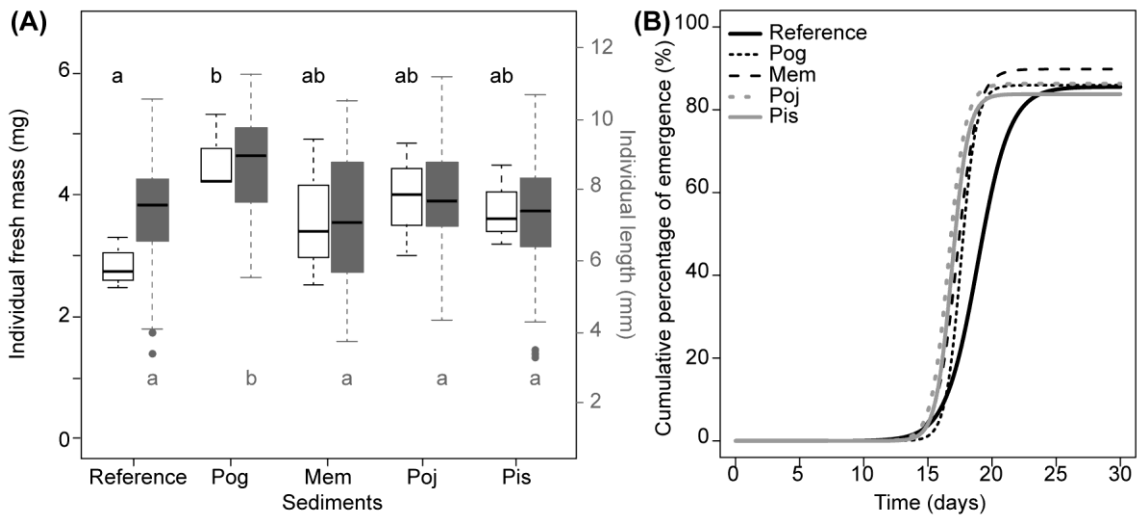
	Cu	Co	Cr	Fe	Mn	Ni	Zn
PEC <sup>a</sup>	149	NA	111	NA	NA	48.6	459
<b><u>Pog:</u></b>							
Pseudo-total	5	4.2	17.6	7067	241	23.2	18.7
CaCl <sub>2</sub>	0.04	0	0	0	0.127	0.009	1.46
EDTA	0.93	0.166	0.176	74.6	81.7	0.491	1.28
Acetic acid	0.862	0.889	0.277	52.1	127	1.66	3.77
Oxalic acid	-	-	-	1943	81.1	-	-
<b><u>Mem:</u></b>							
Pseudo-total	6.2	24.3	137	18282	283	<b>554</b>	21.5
CaCl <sub>2</sub>	0.022	0.001	0	0.019	0.189	0.153	1.84
EDTA	0.953	1.21	0.384	112	40.1	4.95	0.728
Acetic acid	0.348	4.76	0.994	112	103	17.3	1.42
Oxalic acid	-	-	-	5256	117	-	-
<b><u>Poj:</u></b>							
Pseudo-total	16.4	93.8	<b>245</b>	45955	873	<b>100</b>	64.2
CaCl <sub>2</sub>	0.004	0	0.05	0	0.12	0.062	0.054
EDTA	2.82	2.72	0.949	125	87.2	16.2	2.2
Acetic acid	0	1.41	0.206	0	129	25.2	0.516
Oxalic acid	-	-	-	6243	430.3	-	-
<b><u>Pis:</u></b>							
Pseudo-total	11.5	92.2	<b>345</b>	49915	742	<b>112</b>	35.5
CaCl <sub>2</sub>	0.017	0	0.032	0	0.261	0.137	0.034
EDTA	1.12	1.42	0.54	73	49.7	4.67	1.03
Acetic acid	0	0.91	0.112	0	70.4	14	1.78
Oxalic acid	-	-	-	7226	199	-	-

<sup>a</sup> PEC: levels above which harmful effects on sediment-dwelling organisms are expected to occur, concentrations above guidelines are in bold. NA: Non-available

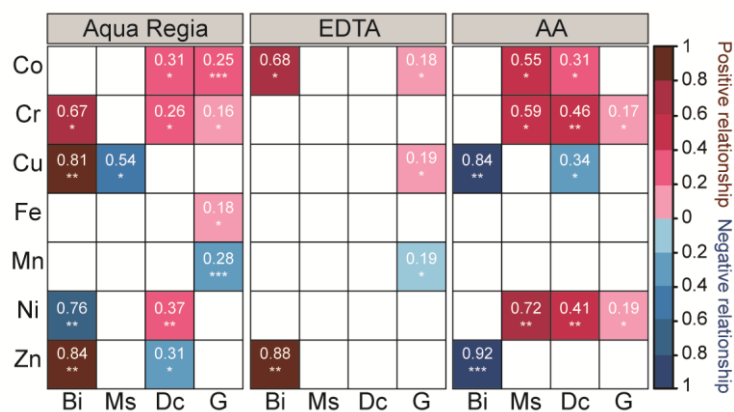




**Figure 1.** Matrix of spearman correlations between pseudo-total metal concentrations in sediments and properties of sediments. \*\*\* for  $p < 0.001$ , \*\* for  $p < 0.01$ , \* for  $p < 0.05$ . Empty boxes indicate the absence of significant correlations.

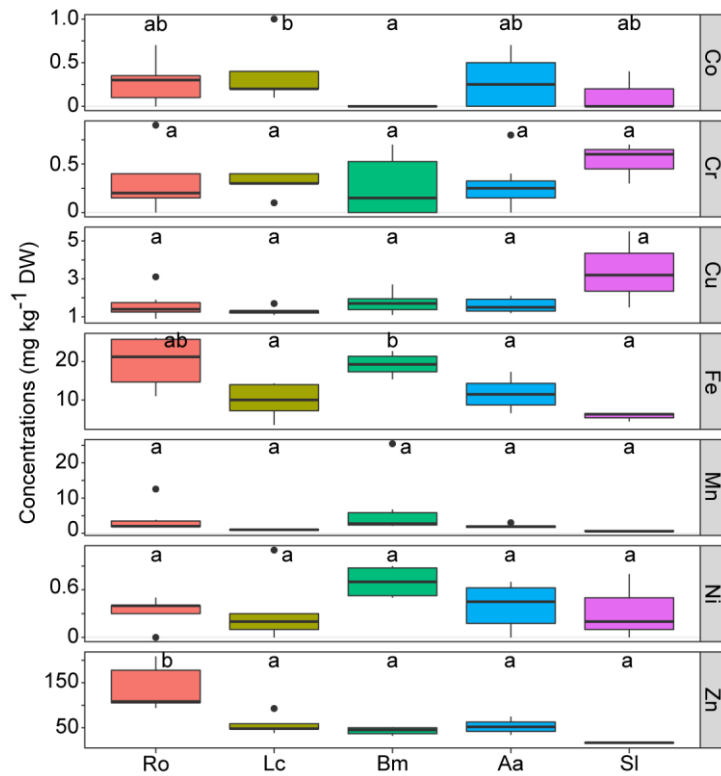


**Figure 2.** Ecotoxicity assessment of lake sediments on chironomids. A reference non-toxic sediment (sand) was also tested. (A) Individual fresh mass ( $n=3$ , white boxes) and length ( $n=60$ , grey boxes) of *C. riparius*. Box plots show the median values, quartiles (“hinges”), extremes (“whiskers”) and outliers (dots). Different letters indicate significant differences. (B) Logistic modelling of the time course of the cumulative percentage of *C. riparius* emergence for the different treatments.

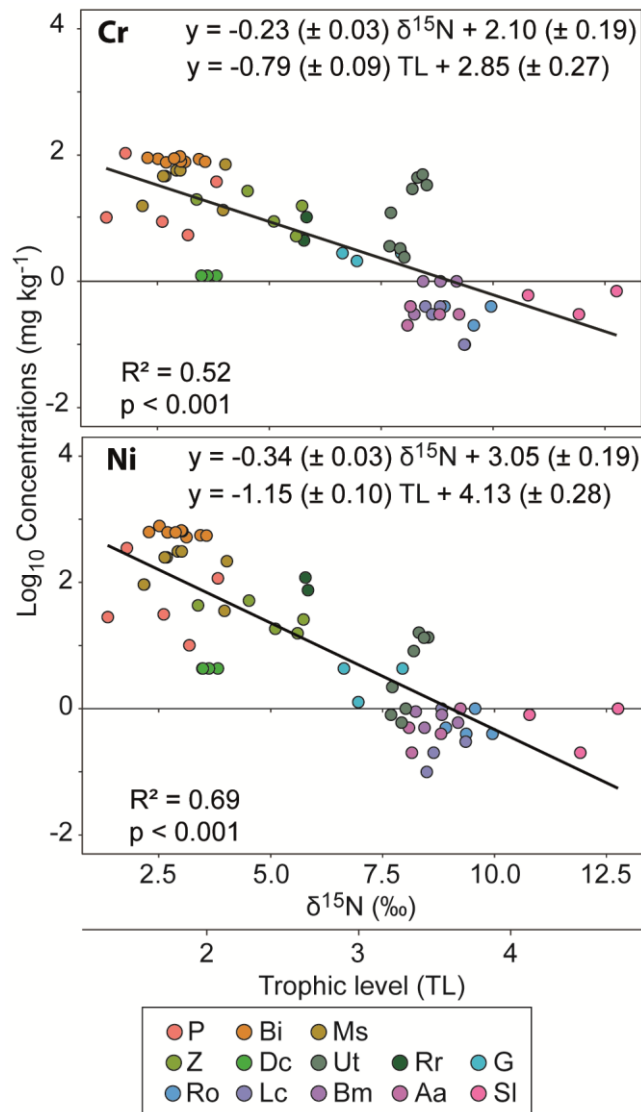


**Figure 3.** Linear correlation coefficients ( $r_s$ ) between pseudo-total metal concentrations in sediments (aqua regia extraction), concentrations after EDTA or acetic acid (AA) extractions and concentrations in organisms. \*\*\* for  $p < 0.001$ , \*\* for  $p < 0.01$ , \* for  $p < 0.05$ . Different colors are used according to the direction of the relationship (right scale). Legend: periphytic biofilm (Bi), *M. spicatum* (Ms), *D. carinata* (Dc), *Gammarus* sp. (G).





**Figure 5.** Boxplot of metal concentrations ( $\text{mg kg}^{-1}$  dry weight) bioaccumulated in fish muscles. Fish species are *R. ohridanus* (Ro), *L. cephalus albus* (Lc), *B. meridionalis petenyi* (Bm), *A. albidus* (Aa) and *S. letnica* (Sl). Box plots show the median values, quartiles (“hinges”), extremes (“whiskers”) and outliers (dots). Different lowercase letters indicate significant differences in metal concentrations in a given organ between fish species.



**Figure 6.** Logarithm of Cr and Ni concentrations ( $\text{mg kg}^{-1}$  dry weight) as a function of  $\delta^{15}\text{N}$  values and trophic level (TL). See legends of Fig. 4 and 5 for species codes.