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# Sediment toxicity and bioaccumulation assessment in abandoned Cu and Hg mining areas of the Nalón River basin (Spain)

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## Abstract

Sediment toxicity and metal bioaccumulation were assessed in sites affected by historical Cu and Hg mining activities in the Nalón River basin, Asturias, Spain. Toxicity assessment of stream sediments was based on a 28-d oligochaete *Tubifex tubifex* sediment bioassay, which allowed the classification of sites into three levels of toxicity: 11 sites classified as Non-Toxic (including Cu mine sites), 3 sites as Potentially Toxic, and 7 sites as Toxic (all located in Hg mine districts). Highest levels of As, Cr, Hg, Pb and Zn in *T. tubifex* were measured at sites affected by Hg mining, and the highest Cu levels in tissues at Cu mining sites. Chronic toxicity responses were best explained by As and Hg sediment concentrations, and by As, Pb and Zn tissue residues. Residue levels of As, Hg, Zn and Pb were successfully used to predict sediment chronic toxicity and estimate Effective Tissue Residues (ERs).

**Keywords**

Tissue residues; Mines; Arsenic; Copper; Mercury

# Introduction

Assessing river sediment quality requires an integrated approach based on several lines of evidence, including sediment chemistry, chronic toxicity and field benthic communities. Several authors have included additional or alternative measures, such as bioaccumulation (Burton et al. 2002; Chapman and McDonald 2005; Grapentine et al. 2002), Critical Body Residues (CBRs, Gust and Fleeger 2005; Rosen and Lotufo 2005), biomarkers (Hollert et al. 2002; Riba et al. 2004), or habitat alterations (Maestre et al. 2009). Incorporating data on tissue residues provides evidence not only on the bioavailability of chemicals, but also on their potential for biomagnification (Krantzberg et al. 2000). Most current sediment quality assessment procedures compare conditions at the study sites to the expected conditions derived from reference sites. This procedure is known as the Reference Condition Approach (RCA: Reynoldson et al. 1997), which is in agreement with the European Water Framework Directive (WFD: EC 2000) for quality assessment of water bodies. Sediment and biota have been recently recognized as suitable matrices to monitor long-term changes in water quality of European water bodies (EC 2010; Carère et al. 2012), but in practice, environmental quality standards for these compartments have been developed only by some State Members.

Metal mining activities represent an environmental problem for freshwater ecosystems (Luoma et al. 2010; Solá et al. 2004). Once the mining activity has stopped, the abandoned mine sites usually continue to be sources of pollution to water bodies. Asturias (northern Spain) has historically been a rich metal mining area. After the Law of Mines from 1825, more than 800 sites with mining activity were registered in Asturias, and the period between 1950 and 1975 represented the highest level of mining activity (Rodríguez-Terente et al. 2006). In the Nalón River basin (Asturias), two main mining industries were active until the early 1970s: Texeo Cu mines (Riosa district) and Hg mines (Mieres, Pola de Lena and Somiedo districts). Texeo mines were the most important source of Cu in NW Spain since Roman times, and were exploited from Bronze Age (3810-4090 BP: De Blas 1996, 2009) until the last century. Hg mining also has a long history in Asturias, and extraction of cinnabar dates back to the Roman period (about 2000 BP) (Rodríguez-Terente et al. 2006). Since the closure of the mines, spoil heaps have not received any type of treatment to avoid mobilization of pollutants, except for the El Terronal site (Mieres), where most of the wastes were isolated in an *in situ* security landfill in 2002. However, no maintenance has been performed since then (Loredo et al. 2010).

Aquatic oligochaetes have been used in metal sediment toxicity and/or bioaccumulation assessment in both laboratory (e.g., Bouché et al. 2000; De Jonge et al. 2012; Maestre et al. 2007; Steen-Redeker et al. 2004) and field exposures (De Jonge et al. 2010; Protano et al.

2014). The study of sediment toxicity and bioaccumulation in Environmental Risk Assessment (ERA) using aquatic oligochaete worms was highlighted by Chapman (2001), and more recently reviewed by Rodriguez and Reynoldson (2011). In the present study, we assess sediment toxicity and metal bioaccumulation at several sites affected by historical mining activities in the Nalón River basin (Asturias, Spain), using the aquatic oligochaete *Tubifex tubifex* (Müller). The present study also evaluates the utility of metal tissue residues in *T. tubifex* to predict chronic toxicity effects due to the exposure to metal polluted sediments.

# Materials and Methods

## Study area

Twenty-five sites were studied in the Nalón River basin during September 2010 and 2011 (three sites were sampled twice: N6, N11 and N15, Table 1). Four reference sites were located in the study area (N1r, N2r, N18r and N22r), belonging to the Water Surveillance networks in Spain (Cantabrian Hydrographical Confederation, CHC). Among the study sites, 15 were located in mining districts: 6 in Cu mine area (Riosa: N3-N8: Fig. 1 a, b Appendix A) and 9 in Hg mine areas (Pola de Lena: N9-N13; Mieres: N14-N17: Fig. 1 b, c Appendix A). Two of these sites were located upstream any mining or industrial areas (sites N7 and N12) to complete information provided by reference sites on background metal levels in the study area.

## Sediment sampling and characterization

Sediment sampling was conducted under a low flow regime, in September of 2010-2011, when most of the fine-grained suspended sediments become deposited on the river bed (Mudroch and Azcue 1995), and when worst conditions for toxicity and bioaccumulation for biota are expected to occur (AQEM 2002). At each site, a composite sample of sediment was taken with a stainless steel spade from the upper 5–10 cm layer of fine sediment, settled along about 25-m reach of the river bank. The sediment was sieved in the field through 500-µm mesh size to eliminate coarse particles and indigenous fauna (Reynoldson et al. 1995). Samples were taken to the laboratory on ice and stored at 4ºC in the dark, during a maximum period of 6 months (as recommended by Reynoldson et al. 1991). Sediment subsamples for metal concentration analyses were air-dried and sieved through a 63-µm mesh. Particle size distribution of unsieved sediment was expressed as dry weight percentage according to Udden-Wenworth scale (Teruggi 1982). Sediment TOC% was determined through the loss-on-ignition method, after calcination at 450ºC, for 6 h, in a muffle furnace (Bryan et al. 1985; USEPA 1990). Several water variables also were measured *in situ*: conductivity portable device (Orion 3-Star, ThermoScientific), and dissolved oxygen, pH and temperature with a multiparameter portable device (Orion 5-star, ThermoScientific) (Table 1 Appendix A).

## Chronic toxicity and metal bioaccumulation

The 28-d *T. tubifex* sediment bioassay was developed as a standardized chronic bioassay by Reynoldson et al. (1991), and later published by ASTM (2005) for sediment toxicity assessment, and by the OECD (2008) for testing of chemicals for bioaccumulation. In the present study, chronic bioassays included survival (%), reproduction (number of Total Cocoons: TCC; number of Empty Cocoons: ECC; and number of Total Young: TYG), and growth endpoints (Total Growth Rate: TGR; Maestre et al. 2007). Twice per week, we measured dissolved oxygen (Orion 5-star), pH (pH-meter Crison 2001), and total ammonia (Nessler method, Hach model DR2000 spectrophotometer) in the overlying water, while aeration was visually checked daily (Mon-Fri). For a detailed description of *T. tubifex* culture, see Méndez-Fernández et al. (2013).

Adult worms surviving by the end of the 28-d sediment bioassays were used for metal tissue residue analysis. A total of 21 samples were analyzed for metal tissue residues, because exposure at 4 sites (N9, N10, N11b and N15b) resulted in 100% mortality. Five laboratory replicates were examined per site, except for N11a and N15a where a pool of surviving worms was used to get enough biomass for metal tissue analyses. Worms were purged in dechlorinated tap water for 5 h. Measuring egestion rates in *T. tubifex*, a gut-clearing period of 4 h was recommended by Martinez-Madrid et al. (1999), while based in gut transit of cationic metals in the oligochaete *Lumbriculus variegatus*, a 6 h period was proposed by Dawson et al. (2003). Then, worms were digested for one week in trace element-free nitric acid (70% Baker Instra-Analyzed) and afterwards for 24 h with $H_2O_2$ (30% R.P. Normapur Prolabo) in a ratio 10:1, at room temperature (Clements 1994). Samples were stored at -20ºC until metal analysis was completed.

## Metal analysis

A total of 7 metals (Cd, Cu, Cr, Hg, Ni, Pb and Zn) and one metalloid (As) were measured at SOSPROCAN Unit (University of Cantabria, Spain). Acid digestion of sediment samples followed EPA 3052 and UNE-EN 13656:2003 procedures (9 ml $HNO_3$ 65 % and 4 ml HF were added to a 0.2 g sediment). For Hg analysis, $AuCl_3$ was added after acid digestion for Hg preservation (EPA method 6020A). Digested sediment samples were measured by ICP-MS (7500ce, Agilent Technologies), and detection limits were 0.07 µg $l^{-1}$ As, 0.01 µg $l^{-1}$ Cd, 0.10 µg $l^{-1}$ Cu, 0.02 µg $l^{-1}$ Cr, 0.03 µg $l^{-1}$ Hg, 0.06 µg $l^{-1}$ Ni, 0.01 µg $l^{-1}$ Pb, 0.03 µg $l^{-1}$ Zn. All batches included Buffalo River sediment as reference material (RM8704, USA) for quality control and recovery rates (82.5-104.4%) were within certified values

Metal tissue residues were also measured by ICP-MS, and detection limits were 0.002 µg $l^{-1}$ As, 0.001 µg $l^{-1}$ Cd, 0.025 µg $l^{-1}$ Cu, 0.009 µg $l^{-1}$ Cr, 0.001 µg $l^{-1}$ Hg, 0.008 µg $l^{-1}$ Ni, 0.009 µg $l^{-1}$ Pb, 0.002 µg $l^{-1}$ Zn. Every batch of tissue samples included 3 blanks and 3 replicates of a certified reference material (Mussel Tissue ERM-CE278, Belgium). Tissue reference material recovery rates (80.4-106.3%) were within the certified values for Cd, Cr, Cu, Pb and Zn; except for As (140.1%). No reference values were available for Hg and Ni, but their concentration showed small variations between different batches of reference material (Hg= 0.20 ± 0.04 µg/g dw; Ni = 0.94 ± 0.17 µg/g dw, n= 18). All measurements are expressed in molar mass, related with the worm body mass, in a dry weight basis.

**Statistical analyses**

Sites were first classified based on *a priori* known anthropological pressures on river systems. A total of 4 pressure groups were indentified: (a) absence of disturbance (CHC Reference sites); (b) undetermined/unknown pressures or weak hydromorphological alterations; (c) Cu mining sites; and (d) Hg mining sites.

Metal concentration in sediment and tissue was assessed using non parametric tests: Kruskal-Wallis followed by multiple comparisons with Dunn´s test (Zar 1996). The validity of pressure groups was assessed by ANOSIM procedure (Clarke 1993). Principal Component Analysis (PCA) combined with Varimax rotation examined dominant patterns of intercorrelation among sediment variables (previously transformed and standardised). Data analyses were conducted in IBM® SPSS (2011) and PRIMER 6 (Clarke and Gorley 2006) softwares.

Reference and test sites were included in the same data matrix and sediment toxicity evaluated through nMDS, using Euclidean distance (PRIMER 6). Reference condition for toxicity assessment was established from a database of 58 reference sites in Northern Spain (Rodriguez et al. 2011; Méndez-Fernández 2013), including 4 additional sites from the present study area (N1r, N2r, N18r and N22r). Sediment toxicity assessment in the Nalón River basin was performed site by site in the multivariate space of reference sites using probability ellipses of 80% and 95%, following the procedure described in detail by Rodriguez et al. (2011). Test sites were assessed as Non-Toxic (NT) when placed within the 80% probability ellipse and thus considered similar to the reference condition; sites were assessed as Potentially Toxic (PT) when placed within 95% and 80% probability ellipses; finally, those sites placed outside 95% probability ellipse were assessed as Toxic (T), thus being interpreted as different from reference condition.

Linking multivariate biotic with abiotic matrices was performed through BEST procedure and the correlation between the two matrices was evaluated through Spearman´s rank correlation, for 999 permutations and 10 restarts. The “best” match between a subset of selected environmental variables and the biotic matrix was examined with RELATE test (PRIMER 6).

Non linear dose-response regression models were applied to toxicity and tissue residues, and median lethal and effective residues (LRs and ERs: Meador et al. 2011) were estimated using R software and the extension package *drc* (Ritz and Streibeig 2005). Model selection was carried out using custom made R script based on Akaike´s information criterion (AIC) and model validation was based on graphical assessment. Potential outliers in the regression models were identified and excluded through the analysis of standardized and Studentised residuals (Zuur et al. 2007). Goodness-of-fit was assessed by $R^2$ and the Neill´s lack-of-fit test for no-replicates included in the *drc* package (Ritz and Streibeig 2005).

# Results

**Sediment metal concentration**

Metals showed maximum concentration at N16 (5320.9 µg As $g^{-1}$, 186.9 µg Ni $g^{-1}$, 265.6 µg Zn $g^{-1}$), N15 (312.5 µg Hg $g^{-1}$, 44.9 µg Pb $g^{-1}$), N8 (1.76 µg Cd $g^{-1}$), N3 (115.2 µg Cu $g^{-1}$), and N7 (102.0 µg Cr $g^{-1}$) (Table 1). In the absence of Sediment Quality Guidelines in Spain (den Besten et al. 2003), sediment metal concentrations were evaluated using TEC (Threshold Effect Concentration) and PEC (Probable Effect Concentration) values proposed by MacDonald et al. (2000) for North American freshwater sediments. Sediment metal concentration in the mine districts of Asturias showed moderate to high levels. PEC value was exceeded in 28%–48% of study sites for Ni, Hg and As; TEC value was exceeded for Cd at 84% of study sites, followed by Cr (56%), Cu (40%), Ni (36%) and As (28%) (Fig. 1). Pb and Zn never exceeded PEC values, and only in 1 or 2 sites, respectively, had values above TEC. Interestingly enough, some reference sites from the Nalón River basin (N2r, N18r and N22r), as well as other sites from tributaries not altered by mining works (N7 and N12), showed As and/or Hg sediment concentrations over TEC and, occasionally, even PEC values. This fact reveals that background geological levels in the study area may naturally contribute to higher metal levels in sediments.

Significant differences in As, Cu, Cr, Hg and Ni sediment concentration (Dunn´s test: $p < 0.05$) were obtained comparing site groups subject to different anthropogenic pressure types (Fig. 2). As, Cr, Hg and Ni concentration measured in Hg mines were significantly higher than in other pressure types; Cu concentration in Cu mines was also significantly higher than in undetermined pressure sites. On the contrary, no differences were detected for Cd, Pb and Zn sediment concentration, regarding pressure types (Dunn´s test: $p > 0.05$).

Sediment characteristics (As, Cd, Cu, Cr, Hg, Ni, Pb, Zn, TOC and SC fraction) were analyzed through multivariate analysis, and confirmed that site clustering (Euclidean distance) due to metal sediment concentration was at least in part related to mining activity (ANOSIM Global R = 0.411, p = 0.001). ANOSIM analysis indicated that reference sites were significantly different from Cu mines (R = 0.433, p = 0.043) and Hg mines (R = 0.548, p = 0.001), and that undetermined pressure sites showed significant differences with Hg mines (R = 0.700, p = 0.001). No differences were found between other pressure groups.

PCA analysis was run with As, Cu, Cr, Hg, Ni, Zn, TOC% and silt-clay (SC) fraction. PCA after Varimax rotation defined 2 first principal components (PCs) with eigenvalues > 1 (Kaiser criterion), that explained the 80.5 % of the accumulated variance (PC1: 51.0%, PC2: 29.5%). PC1 was strongly correlated with Cu, Cr, Ni and Zn concentrations and SC fraction (loadings > 0.80), and PC2 was strongly correlated with As and Hg (loadings > 0.80) (Table 2 Appendix A). Thus, PC1 defined a gradient from unpolluted reference sites towards polluted mining sites; and PC2 readily distinguishes Hg mining sites, with higher As and Hg metal concentration, from other sites (Fig. 2 Appendix A).

**Toxicity assessment**

Results from chronic bioassays are reported in Table 2. Site toxicity classification using probability ellipses in the nMDS multivariate space of the reference sites database (n = 58) resulted in 11 sites classified as Non-Toxic (NT) (including Cu mine sites), 3 sites as Potentially Toxic (PT) (N5, N19 and N21) and 7 sites as Toxic (T) (N9, N10, N11a, N11b, N15a, N15b and N16). All Toxic sites were located at Hg mine districts, with high levels of As, Hg and Cd in sediments. Grouping of study sites in the Nalón River basin based on toxicity classification produced, as expected, a high Global R value of 0.843 (p = 0.001). However, site grouping based on general anthropogenic pressures did not explain the observed toxicity (ANOSIM Global R = 0.037, p = 0.272), suggesting that toxicity responses were not always attributable to pressures related to mining activities. nMDS analysis based on toxicity data (Fig. 2) showed accurately the dissimilarities between study sites (stress = 0.02), with T sites (all from Hg mines) placed opposite to reference sites. Reference and NT sites showed high values in all bioassay endpoints, whereas T sites showed marked reductions in all studied endpoints (Table 2). Significant differences on sediment toxicity was found between T sites from Hg mining areas and Reference sites (ANOSIM R = 0.775, p = 0.003), between T sites and NT sites (R =

0.923, $p = 0.001$); as well as between PT and NT sites ($R = 0.847$, $p = 0.003$) and PT and T sites ($R = 0.659$, $p = 0.008$). Results in the Nalón River basin also indicated non-significant differences between Reference sites and NT sites ($R = 0.175$, $p = 0.173$) or PT sites ($R = 0.278$, $p = 0.143$).

In sites N19 and N21, assessed as PT, impairment in both ECC and TYG was observed (Table 2), which may be an indication of embryogenesis alterations and/or young mortality after hatching. However, it is noteworthy that the classification of site N5 (from Cu Mines district) as PT is due to high reproduction values (Table 2), much higher than those found in most reference sites in our data base of Northern Spain.

**Metal tissue residues**

The highest Cu (3.75 µmol $g^{-1}$ dw) tissue residues in bioassay worms were measured at a Cu mining site (N6a), whereas the highest As (28.92 µmol $g^{-1}$ dw), Cr (0.28 µmol $g^{-1}$ dw), Hg (104.29 nmol $g^{-1}$ dw), Pb (46.43 nmol $g^{-1}$ dw) and Zn (38.21 µmol $g^{-1}$ dw) tissue residues were measured at a Hg mining site (N11a) (Table 3). Interestingly, two reference sites showed the highest Cd and Ni tissue residues (36.15 nmol Cd $g^{-1}$ dw: N18r; 0.26 µmol Ni $g^{-1}$ dw: N22r), and none of the reference sites in the Nalón River basin showed the lowest metal tissue residues.

Comparison of *T. tubifex* metal tissue residues between T, PT, NT and Reference site groups showed significant differences only for As when comparing PT and T sites (Dunn´s test: $p < 0.05$). Multivariate analysis of metal bioaccumulation data showed low differences between those 4 toxicity groups (ANOSIM Global $R = 0.335$ $p = 0.011$). Significant differences were only found between NT and T site groups ($R = 0.662$, $p = 0.011$), whereas differences between Reference and T sites were not significant ($R = 0.148$, $p = 0.257$), probably due to relatively high metal tissue residues found at two Reference sites (N18r, N22r, see Table 3).

Spearman correlation values revealed that As and Hg in sediment were moderately correlated ($\rho = 0.57$–$0.73$, absolute values) with nMDS site ordination based on toxicity (TOX-SED) and tissue residues (TR-SED) (Table 3 Appendix A). Correlations between metal tissue residues and nMDS site ordination based on toxicity (TOX-TR) showed moderate values for As, Hg, Pb and Zn ($\rho = 0.58$–$0.74$, absolute values). Metals identified by this approach (As, Hg, Pb and Zn) were tested using RELATE procedure, resulting that pair-wise correlations of metal sediment concentration, chronic toxicity and tissue residues resemblance matrices were significant ($p = 0.001$) (Table 3 Appendix A). Toxicity data matrix was best explained by As and Hg sediment concentration (BEST, $\rho = 0.614$), whereas the subset of As, Pb and Zn tissue residues accounted for toxicity (BEST, ρ = 0.739). Tissue residues data matrix was best explained by As, Cu, Hg and Zn sediment metal concentrations (BEST, ρ = 0.588).

Toxicity endpoints values and As, Hg, Pb and Zn tissue residues were fitted against several non-linear dose-response regression models, and $LR_{50/20}$ or $ER_{50/20}$ estimated for each combination of metal residue and toxicity endpoint (Fig. 3). $LR_{20}$ and $LR_{50}$ were estimated from a log-logistic model and were 3.41 and 15.90 µmol $g^{-1}$ dw for As, and 14.79 and 42.10 µmol $g^{-1}$ dw for Zn, respectively. Reproduction $ER_{20}$ and $ER_{50}$ values were estimated from Weibull models of Total No. of Cocoons (TCC) for As: 2.48 and 10.79 µmol $g^{-1}$ dw, Zn: 9.56 and 32.31 µmol $g^{-1}$ dw, and Pb: 0.031 and 0.032 µmol $g^{-1}$ dw; as well as Total No. of Young (TYG) for Hg: 0.034 and 0.067 µmol $g^{-1}$ dw. Other metal tissue residue *vs*. toxicity endpoint relationships were not significantly fitted by either model.

## Discussion

Forty years after mining activities have ceased, sediment metal concentrations of the Nalón River basin remain high to very high in the Cu and Hg mining districts, respectively. These results are in agreement with the several studies on soil and surface water contamination reported by Loredo et al. (2006 and 2010). But it is noteworthy that we have usually found lower metal levels in the river sediments than those measured several years ago in the same areas (N9, N10, N11, N13 and N16), with the exception of N16, where As was about 250 times higher than values reported by Loredo et al. (2005). Studies conducted in Hg and Cu mine districts in Asturias suggest that variations in sediment metal concentrations may be severely influenced by climate of the region, e.g. precipitation as a key factor for As leaching (Loredo et al. 2007, 2010).

The Environmental Quality Standards (EQS) directive (EC, 2008) was an important improvement of long-term water quality monitoring at the European level, pointing towards the use of sediments and biota as matrices for assessment of priority substances under the WFD (EC 2000), with emphasis on Cd and Hg. Some European countries have developed independent Sediment Quality Guidelines (SQGs), as in Flemish basins (De Cooman et al. 1999) or in The Netherlands (Crommentuijn et al. 1997), but the absence of SQGs in Spain limits the development of a sound ERA and water quality protection plans. Unfortunately, the only mandatory requirement by the European directives (2000, 2008) for sediment and biota quality is that contamination levels should not increase. This is clearly insufficient for the objective of attaining Good ecological status in rivers subject to historical high contamination.

Maximum As, Cu, Hg and Zn tissue residues in our laboratory study were above those previously reported for sediment-dwelling annelids in the field (Table 4), although most field data have not been obtained from mining sites. When comparing laboratory tissue residues with values from field-collected aquatic annelids in the literature (Table 4), As and Hg bioaccumulated at Toxic sites showed higher values, while Cd had always lower values. For essential metals, such as Cu and Ni, mean concentrations were relatively constant in the present study, independent of sediment toxicity classification, and were within the range of concentrations reported in the literature. In the case of Zn, mean concentration was not only higher at Toxic sites, but also higher than most values reported for field aquatic annelids. Cr showed less variation from field to laboratory studies, and in all cases Cr tissue residues in aquatic annelids were below 1 µmol g$^{-1}$ dw.

Adverse effects of As on some aquatic organisms are expected to occur at tissue concentrations between 0.17 and 0.67 µmol g$^{-1}$ dw (Eisler 2000). Threshold values based on field ecological effects were higher; for instance, Rainbow et al. (2012) reported 1.13 µmol As g$^{-1}$ dw in *Hydropsyche siltalai* related to mayfly population impairment, a value close to the ER$_{20}$ for *T. tubifex* reproduction (2.48 µmol g$^{-1}$ dw) in the present study. For Hg, the proposed criteria for the protection of freshwater species is about 0.150 µmol g$^{-1}$dw (Eisler 2000). However, tissue residues as low as 0.067 µmol Hg g$^{-1}$ dw were related to 50% reduction in total young production (TYG) in the present study. Nevertheless, at Toxic sites from Hg mines, high As and Hg tissue residues were measured, suggesting that the combination of both As and Hg, as well as other metals, are likely responsible for the observed toxicity impairments.

Lead is a known accumulative metabolic poison, and existing data suggest that it may have adverse effects on organisms (Eisler 2000). However, no protection criteria based on tissue residues for freshwater invertebrates are known by the authors. Rainbow et al. (2012) reported benthic community alterations in metal-rich streams when Pb tissue concentration in *Hydropsyche siltalai* exceeded 1.45 µmol g$^{-1}$ dw, but laboratory effective tissue residues (ER$_{20/50}$) in the present study were below that value (0.03 µmol g$^{-1}$ Pb dw). Regarding Zn, ER$_{50}$ for reproduction (TCC) and LR$_{50}$ in present study were 33.31 and 42.10 µmol Zn g$^{-1}$ dw, respectively. Similar threshold values were reported for Zn tissue concentration in Simulidae (14.8–30.3 µmol g$^{-1}$ dw) and *Leuctra* sp (27.5–58.6 µmol g$^{-1}$ dw) (De Jonge et al. 2013), or *Hydropsyche* spp. (18.6–49.1 µmol g$^{-1}$ dw) (Solá et al. 2004) related to field ecological effects on macroinvertebrate fauna. In the present study, laboratory Pb and Zn tissue residues appear to be related to sediment toxicity. However, Zn is an essential metal for all living organisms, which complicates the toxicity assessment of this element with respect to bioaccumulation (Eisler 2000), and estimated Zn-ER values should be taken with caution.

Data reported in the literature suggest that Cr, Cd, Ni, and Cu tissue residues in the present study are not likely responsible of causing the observed toxicity effects. Méndez-Fernández et al. (2013) calculated a Cr-$CBR_{50}$ (Critical Body Residue) for reproduction in *T. tubifex* of 0.65 µmol Cr $g^{-1}$ dw, a value 2.3 times higher than the maximum tissue residues measured in the present study. Regarding Cd, metal tissue residues in *T. tubifex* exposed to the Nalón River sediments were 3–4 orders of magnitude lower than the reproduction $CBR_{50}$ values reported for the same species in Cd-spiked sediment bioassays (Méndez-Fernández et al. 2013: 13.57–29.54 µmol Cd $g^{-1}$ dw; Gillis et al. 2002: 30.38–32.18 µmol Cd $g^{-1}$ dw). With respect to Ni, Borgmann et al. (2001) found that Ni-ERs for growth and survival in *Hyalella azteca* varied between 0.12 to 0.19 µmol Ni $g^{-1}$ dw (4–10 week sediment exposure), whereas worms exposed to the Nalón River Toxic sites showed a lower tissue concentration (0.07 ± 0.03 µmol Ni $g^{-1}$ dw). Cu critical tissue concentrations reported from other studies were usually higher than maximum tissue residues measured in present study; for instance, reproduction $CBR_{50}$ values ranged 3.88–4.47 µmol $g^{-1}$ dw for *T. tubifex* in laboratory Cu-spiked sediment bioassays (Méndez-Fernández et al. 2013), and $CBR_{50}$ values estimated in relation to field benthic community alterations were 5.5 µmol Cu $g^{-1}$ dw in *Rhithrogena* sp. (De Jonge et al. 2013) and 2.68 µmol Cu $g^{-1}$ dw in *Hydropsyche siltalai* (Rainbow et al. 2012). These data can explain in part the classification of sites affected by Cu mining works (N3-N7) as Non-Toxic, and may support the statement that historical Cu mining activity in Asturias represents a moderate and local environmental problem (< 1 km from mine facilities, Loredo et al. 2007).

Some differences between field and laboratory data can possibly be discussed in terms of metal bioavailability that may change through the processing of the sediment samples (i.e. sieving). Nevertheless, unsieved sediment has been demonstrated to cause “false positives” in sediment bioassays (Reynoldson et al. 1994) and sediment sieving was preferred over heating, freezing or drying to remove competing or predating resident invertebrates (Day et al. 1995). One of the most important factors controlling metal availability in anoxic sediments has been the amount of acid volatile sulfides (AVS). However, in the studied area AVS probably were not of concern, since the water column was well mixed and oxygenated. Moreover, De Jonge et al. (2009, 2010, 2011) found that an excess of AVS did not turn to be an important factor determining metal bioaccumulation in field-collected benthic invertebrates (*Chironomus* gr. *thummi* and *Tubifex* sp.). However, De Jonge et al. (2012) showed that elevated oxygen concentrations in overlaying surface can directly enhance metal accumulation and toxicity in some invertebrates (namely, *Asellus aquaticus* and *Daphnia magna*), which could also explain some differences between laboratory and field data. Variability may also be expected from different populations or genetic strains of the same species (Reynoldson et al. 1996; Sturmbauer et al. 1999).

Finally, although toxicity and bioaccumulation in the field cannot be readily implied from laboratory studies, results from the present study show that polluted sediments in Hg mining areas entail a high risk that programs that ignore sediment ecotoxicity and bioaccumulation, such as the European Water Framework Directive, fail to meet their own objectives of attaining Good ecological status (Byrne et al. 2012) due to sediment pollution.

## Conclusions

Sediments downstream of Hg mines showed impairment of survival and reproduction in *T. tubifex* bioassays, related to sediment metal pollution and As and Hg bioaccumulation. This fact provides information on metal bioavailability and evidence of metal transfer from the sediment to the food web. Results suggest the existence of an important environmental problem in the study area where there is a long history of mining activities, and demand effective remediation plans to reduce runoff and other sources of metal pollution that contaminate the river sediments below abandoned Hg mine facilities. Comprehensive and long-term studies on sediment toxicity, bioaccumulation and field community alterations are necessary for a sound environmental risk assessment of water courses in the mining districts of Asturias.

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## FIGURE LEGENDS

**Fig. 1** Boxplots comparing sediment metal concentration (µg $g^{-1}$ dw), in 25 study sites attending to anthropogenic pressure groups: Cu mines, n=6; Hg mines, n=11; Reference sites, n=4; Undetermined pressures, n=4. Box is built with 25 and 75 percentiles, and show inside the median marked by a bold line. For each metal, their respective TEC value (dotted line) and when necessary the PEC value (dashed line), are indicated. Piecharts show the proportion of test sites in the study area above PEC (dark grey), above TEC (grey) and below TEC (light grey). Open circles indicate sites with extreme data values (over 1.5 times the interquartile range of the data). Significant differences using Dunn´s test are marked as: * $p< 0.05$; ** $p< 0.01$.

**Fig. 2** Spatial ordination by nMDS for the 25 study sites based on chronic toxiciy resemblance matrix. Each site is marked by a symbol corresponding to four categories after sediment toxicity risk classification (REF: Reference, NT: Non-Toxic, PT: Potentially Toxic, T: Toxic), using 5 endpoints from *T. tubifex* chronic bioassay (survival, No. of total cocoons, No. of empty cocoons, No. of total young and total growth rate).

**Fig. 3** LR and ER values estimated from the best fitted models, on a tissue residue basis, after 28-d chronic bioassays for As, Zn, Pb and Hg (µmol $g^{-1}$ dw). Dashed line represents $LR_{50}$ or $ER_{50}$, and dotted line represents $LR_{20}$ or $ER_{20}$. Abbreviations: SUR: % Survival; TCC: No. of Total Cocoons; TYG: No. of Total Young. For model descriptions: LL.2: 2 parameter log-logistic models; W1.3, W1.4: Weibull type 1 model with 3 and 4 parameters; W2.3: Weibull type 2 model with 3 parameters (*dcr* package, Ritz and Strebeig, 2005). Goodness-of-fit was assessed by $R^2$ and Neill´s lack-of-fit test for no replicates (p-value) included in the *drc* package (Ritz and Streibeig, 2005). Outliers are represented by a grey square symbol.

Figure 1
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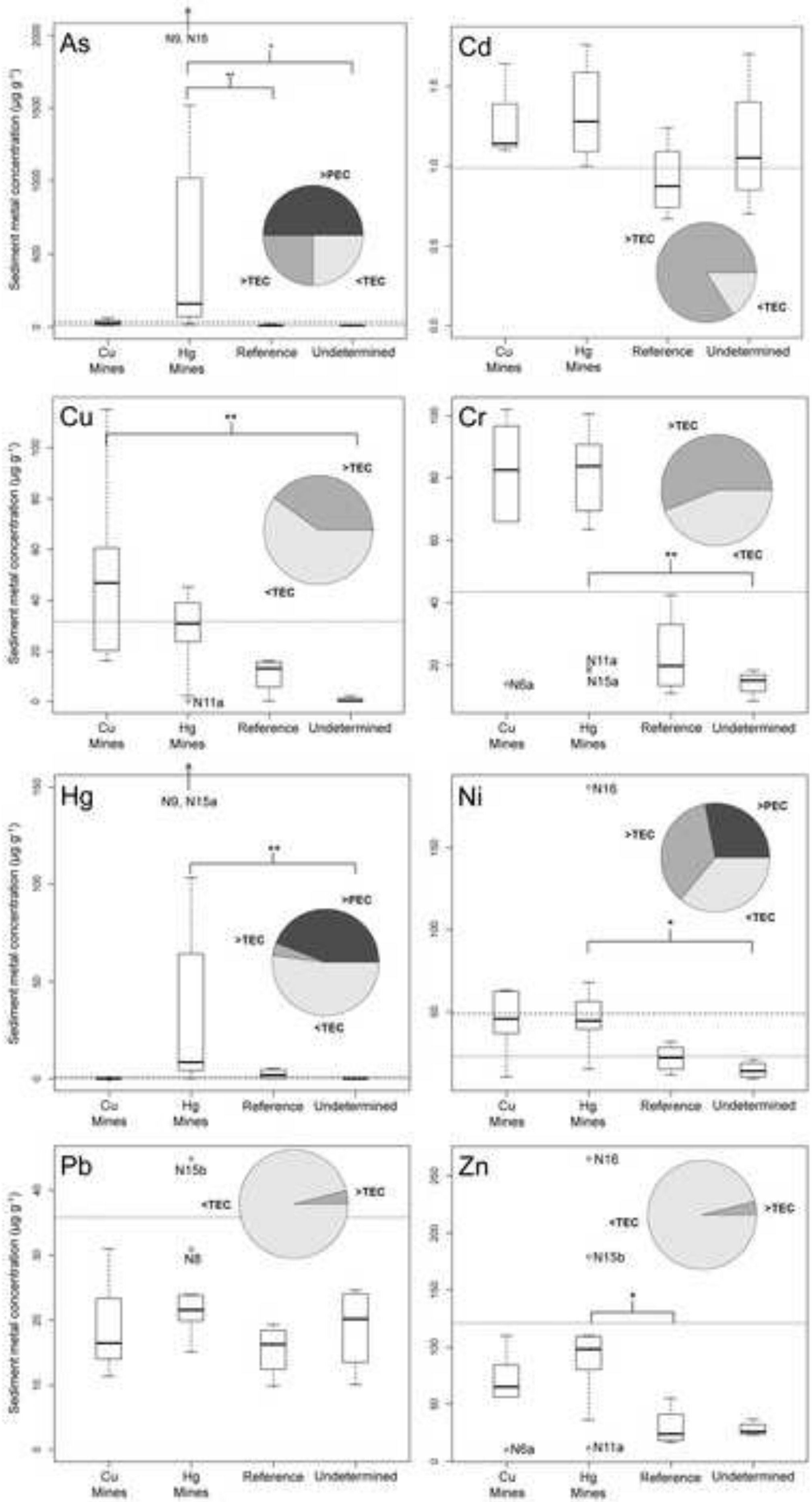

Figure 2

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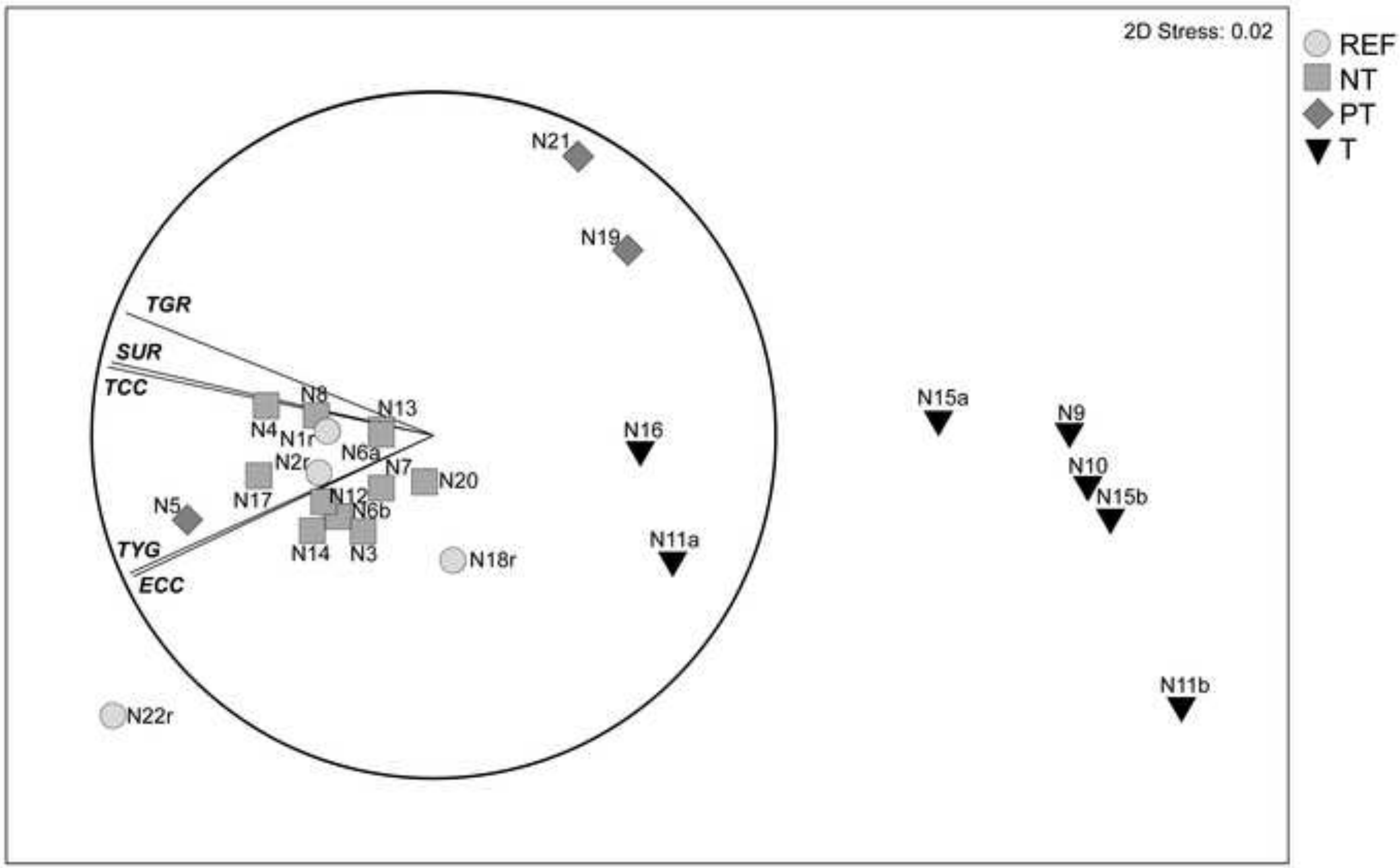

Figure 3

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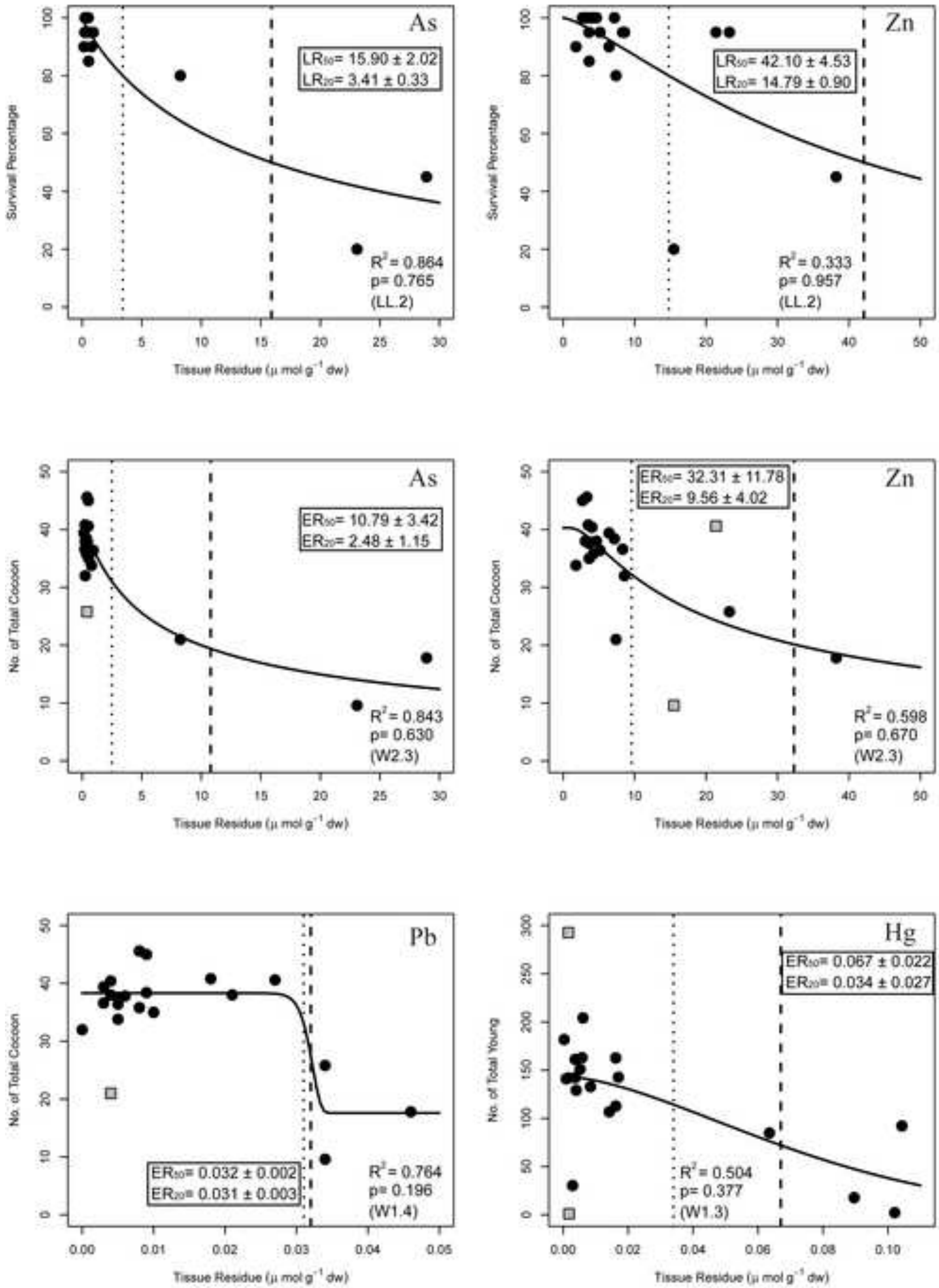

**Table 1.** Study sampling sites in Nalón River Basin, with identification code (ID.), river basin, municipality, UTM coordinates, altitude (Alt., in meters), and sampling year. Abbreviations: a,b: site sampled in different years; r: reference sites defined by Water Authorities; Sites marked by the superscript CHC are within the Surveillance network of the Cantabric Hydrographical Confederation. Sediment metals concentration expressed as µg g$^{-1}$ dw. Sediment Quality Guidelines (SQG) from MacDonald et al. (2000): TEC = Threshold Effect Concentration; PEC = Probable Effect Concentration. In bold marked the concentrations exceeding the TEC; in bold and underlined those exceeding the PEC).

| ID. | River basin | Municipality | UTM-X | UTM-Y | Alt. | Year | Pressure | As | Cd | Cu | Cr | Hg | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **N1r$^{CHC}$** | Genestaza | Tineo | 227060 | 4795590 | 286 | 2011 | Reference | 7.13 | 0.81 | 14.9 | 23.6 | 0.10 | 18.7 | 9.81 | 27.6 |
| **N2r$^{CHC}$** | Villabre | Yermes y Tameza | 246779 | 4794814 | 562 | 2011 | Reference | **16.8** | 0.67 | 16.1 | 42.4 | 0.09 | **31.5** | 15.0 | 55.4 |
| **N3** | Llamo | Riosa | 265801 | 4785959 | 471 | 2011 | Copper mines | **64.0** | **1.14** | **115** | **96.4** | 0.14 | **63.1** | 18.1 | 84.6 |
| **N4** | La Reguera | Riosa | 265613 | 4785980 | 531 | 2011 | Copper mines | **33.7** | **1.39** | **46.2** | **96.7** | 0.13 | **52.3** | 31.0 | 110 |
| **N5** | Llamo | Riosa | 265839 | 4787692 | 411 | 2011 | Copper mines | **15.7** | **1.14** | 20.1 | **68.4** | 0.15 | **36.5** | 14.0 | 60.5 |
| **N6a** | Llamo | Riosa | 265907 | 4789095 | 353 | 2010 | Copper mines | **10.3** | **1.10** | **16.2** | 13.9 | <D.L. | 10.3 | 11.3 | 9.90 |
| **N6b** | Llamo | Riosa | 265907 | 4789095 | 339 | 2011 | Copper mines | **23.6** | **1.12** | **60.7** | **65.8** | 0.13 | **39.4** | 14.8 | 70.0 |
| **N7** | Reguero de Code | Riosa | 263985 | 4788906 | 617 | 2011 | Copper mines | **46.9** | **1.64** | **47.5** | **102** | 0.14 | **62.4** | 23.3 | 56.6 |
| **N8 $^{CHC}$** | Llamo-Riosa | Morcin | 267410 | 4794605 | 214 | 2011 | Copper mines | **20.5** | **1.76** | **43.5** | **75.5** | 0.11 | **39.8** | 30.9 | 99.4 |
| **N9** | Reguero de La Soterraña | Pola de Lena | 269000 | 4786198 | 593 | 2011 | Mercury mines | **3091** | **1.00** | **33.4** | **87.1** | **213** | **60.1** | 19.7 | 98.4 |
| **N10** | Muñón | Pola de Lena | 269032 | 4783932 | 385 | 2011 | Mercury mines | **521** | **1.12** | 25.3 | **91.3** | **15.5** | **42.4** | 20.3 | 90.2 |
| **N11a** | Muñón | Pola de Lena | 269096 | 4783838 | 343 | 2010 | Mercury mines | **110** | **1.60** | <D.L. | 19.0 | **25.1** | **15.1** | 16.5 | 11.6 |
| **N11b** | Muñon | Pola de Lena | 269135 | 4783836 | 251 | 2011 | Mercury mines | **479** | **1.05** | 23.8 | **90.0** | **8.68** | **44.4** | 20.1 | 85.1 |
| **N12** | Rubial | Pola de Lena | 268269 | 4785640 | 454 | 2011 | Mercury mines | **29.6** | **1.57** | 23.6 | **82.8** | **0.58** | **39.3** | 21.6 | 75.3 |
| **N13** | Brañalemosa | Pola de Lena | 267849 | 4783599 | 485 | 2011 | Mercury mines | **38.0** | **1.06** | **34.4** | **100** | **4.72** | **52.4** | 23.1 | 109 |
| **N14** | San Tirso | Mieres | 274859 | 4794530 | 569 | 2011 | Mercury mines | **103** | **1.34** | 30.7 | **83.7** | **5.99** | **46.9** | 23.9 | 110 |
| **N15a** | San Tirso | Mieres | 274516 | 4794073 | 203 | 2010 | Mercury mines | **157** | **1.20** | 2.30 | 18.1 | **312** | 16.5 | 23.8 | 36.5 |
| **N15b** | San Tirso | Mieres | 274434 | 4793952 | 207 | 2011 | Mercury mines | **1519** | **1.72** | **43.9** | **93.7** | **103** | **67.7** | **44.9** | **180** |
| **N16** | Morgao | Mieres | 274863 | 4793401 | 221 | 2011 | Mercury mines | **5321** | **1.28** | **45.2** | **63.4** | **4.17** | **186** | 15.1 | **266** |
| **N17 $^{CHC}$** | San Juan | Mieres | 277082 | 47933111 | 275 | 2010 | Undetermined | 9.30 | 0.70 | < D.L. | 8.40 | <D.L. | 9.00 | 10.1 | 23.4 |
| **N18r$^{CHC}$** | Turón | Mieres | 282671 | 4788130 | 436 | 2010 | Reference | 7.43 | 0.94 | <D.L. | 10.9 | **4.13** | 11.3 | 19.3 | 20.5 |
| **N19 $^{CHC}$** | Nalón | Laviana | 291654 | 4792027 | 281 | 2010 | Undetermined | 9.30 | **1.70** | <D.L. | 15.4 | <D.L. | 20.6 | 24.6 | 27.9 |
| **N20 $^{CHC}$** | Villoria | Laviana | 292218 | 4789475 | 317 | 2010 | Undetermined | 8.90 | **1.10** | 2.20 | 18.3 | <D.L. | 16.0 | 23.5 | 36.6 |
| **N21 $^{CHC}$** | Raigoso | Ribota | 294192 | 4789307 | 239 | 2010 | Undetermined | 6.90 | **1.00** | <D.L. | 14.8 | <D.L. | 11.6 | 16.8 | 24.8 |
| **N22r$^{CHC}$** | Alba | Sobrescobio | 298990 | 4784776 | 474 | 2010 | Reference | **12.9** | **1.24** | 11.4 | 15.8 | **5.29** | **25.1** | 17.5 | 16.5 |
| | | | | | | | ***SQG*** | | | | | | | | |
| | | | | | | | ***TEC*** | *9.79* | *0.99* | *31.6* | *43.4* | *0.18* | *22.7* | *35.8* | *121* |
| | | | | | | | ***PEC*** | *33* | *4.98* | *149* | *111* | *1.06* | *48.6* | *128* | *459* |

Table 2
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**Table 2**. Endpoints values from the 28-d sediment toxicity test with *Tubifex tubifex*, for the 25 study sites. Toxicity classification of test sediments using 80 and 95% probability ellipses, in a reference condition multivariate space (see text). Mean values (± sd) of the endpoints for each group of study sites is included. Abbreviations: SUR: Survival %; TCC= No. of Total Cocoons; ECC= No. of Empty Cocoons; TYG: No. of Total Young; TGR= Total Growth Rate ($d^{-1}$); n.d.= not determined.

| ID. | SUR | TCC | ECC | TYG | TGR ($d^{-1}$) | TOXICITY CLASIFICATION |
|---|---|---|---|---|---|---|
| **N1r** | 100 ± 0 | 38.0 ± 4.8 | 14.4 ± 4.3 | 141.0 ± 60.4 | 0.036 ± 0.003 | **Reference** |
| **N2r** | 95 ± 11.2 | 37.6 ± 3.6 | 17.8 ± 2.8 | 142.0 ± 47.6 | 0.035 ± 0.005 | **Reference** |
| **N3** | 90 ± 22.4 | 33.8 ± 6.3 | 19.6 ± 2.4 | 142.2 ± 47.6 | 0.016 ± 0.003 | **Non toxic** |
| **N4** | 100 ± 0 | 45.0 ± 1.6 | 16.4 ± 5.5 | 132.8 ± 75.2 | 0.050 ± 0.005 | **Non Toxic** |
| **N5** | 100 ± 0 | 45.6 ± 2.7 | 21.6 ± 5.3 | 204.2 ± 47.8 | 0.040 ± 0.002 | **Potentially Toxic** |
| **N6a** | 100 ± 0 | 38.4 ± 1.9 | 13.6 ± 3.4 | 129.2 ± 33.9 | 0.016 ± 0.005 | **Non toxic** |
| **N6b** | 85 ± 22.4 | 35.0 ± 9.7 | 18.0 ± 7.5 | 163.0 ± 67.7 | 0.029 ± 0.006 | **Non toxic** |
| **N7** | 100 ± 0 | 38.0 ± 3.6 | 18.8 ± 2.2 | 106.8 ± 17.8 | 0.015 ± 0.003 | **Non toxic** |
| **N8** | 95 ± 0 | 41.2 ± 1.9 | 20.6 ± 3.8 | 188.2 ± 38.5 | 0.040 ± 0.003 | **Non toxic** |
| **N9** | 0 | 0 | 0 | 0 | n.d* | **Toxic** |
| **N10** | 0 | 2.2 ± 1.1 | 0 | 0 | n.d* | **Toxic** |
| **N11a** | 45 ± 11.2 | 17.8 ± 4.8 | 12.2 ± 5.2 | 92.2 ± 31.0 | -0.011 ± 0.007 | **Toxic** |
| **N11b** | 0 | 4.4 ± 2.1 | 2.2 ± 1.6 | 0.2 ± 0.4 | n.d* | **Toxic** |
| **N12** | 100 ± 0 | 35.8 ± 3.7 | 17.4 ± 4.0 | 162.6 ± 40.4 | 0.022 ± 0.007 | **Non toxic** |
| **N13** | 100 ± 0 | 37.8 ± 8.8 | 14.4 ± 4.3 | 112.8 ± 60.9 | 0.025 ± 0.004 | **Non toxic** |
| **N14** | 95 ± 11.2 | 36.4 ± 1.9 | 20.0 ± 1.6 | 161.2 ± 18.6 | 0.021 ± 0.006 | **Non toxic** |
| **N15a** | 20 ± 20.9 | 9.6 ± 2.3 | 1.8 ± 1.1 | 2.2 ± 2.9 | -0.017 ± 0.006 | **Toxic** |
| **N15b** | 0 | 1.8 ± 1.3 | 1.0 ± 0.7 | 0.6 ± 0.5 | n.d* | **Toxic** |
| **N16** | 80 ± 20.9 | 21.0 ± 3.5 | 13.2 ± 4.1 | 30.4 ± 16.1 | -0.010 ± 0.004 | **Toxic** |
| **N17** | 100 ± 0 | 40.4 ± 1.9 | 17.2 ± 3.6 | 181.8 ± 44.4 | 0.041 ± 0.004 | **Non toxic** |
| **N18r** | 95 ± 11.2 | 25.8 ± 8.7 | 15.8 ± 5.6 | 142.6 ± 44.6 | -0.005 ± 0.006 | **Reference** |
| **N19** | 95 ± 11.2 | 32.0 ± 2.3 | 2.8 ± 1.3 | 17.8 ± 9.0 | 0.002 ± 0.005 | **Potentially Toxic** |
| **N20** | 95 ± 11.2 | 36.6 ± 5.9 | 19.2 ± 5.9 | 84.8 ± 42.2 | 0.011 ± 0.009 | **Non toxic** |
| **N21** | 90 ± 22.4 | 39.4 ± 4.4 | 1.0 ± 0.7 | 1.0 ± 1.4 | 0.029 ± 0.004 | **Potentially Toxic** |
| **N22r** | 95 ± 11.2 | 40.6 ± 3.4 | 26.0 ± 3.4 | 292.6 ± 59.1 | 0.018 ± 0.009 | **Reference** |
| **Groups** | | | | | | |
| **Reference** | 96.3 ± 2.5 | 35.5 ± 6.6 | 18.5 ± 5.2 | 179.6 ± 75.4 | 0.021 ± 0.019 | |
| **Nont Toxic** | 96.4 ± 5.0 | 38.0 ± 3.2 | 17.7 ± 2.2 | 142.3 ± 32.5 | 0.026 ± 0.013 | |
| **Potentially Toxic** | 95.0 ± 6.8 | 39.0 ± 6.8 | 8.5 ± 11.4 | 74.3 ± 112.8 | 0.024 ± 0.020 | |
| **Toxic** | 20.7 ± 8.3 | 8.1 ± 8.3 | 4.3 ± 5.8 | 17.9 ± 34.6 | -0.047 ± 0.042 | |

*For multivariate analyses, TGR values where 100% mortality occurred were estimated by a logarithmic regression model between total cocoon biomass and TGR data, from bioassay control batches ($R^2$ = 0.73, p < 0.001, n = 40): N9= -0.013 $d^{-1}$, N10= -0.038 $d^{-1}$, N11b= -0.056 $d^{-1}$, N15b= -0.070 $d^{-1}$.

Table 3
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**Table 3.** *Tubifex tubifex* metal tissue residues (mean ± sd), after 28-d exposure to Nalón River sediments. Data are reported in μmol $g^{-1}$ dw tissue for As, Cu, Cr, Ni and Zn, and in nmol $g^{-1}$ dw tissue for Cd, Hg and Pb. Abbreviations: D.L.= detection limit; min= minimum value; max= maximum value.

| ID. | As | Cd | Cu | Cr | Hg | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|
| **N1r** | 0.25 ± 0.01 | 0.21 ± 0.06 | 0.27 ± 0.01 | 0.01 ± 0.001 | 0.86 ± 0.13 | 0.02 ± 0.03 | 3.76 ± 0.95 | 3.10 ± 0.38 |
| **N2r** | 0.30 ± 0.09 | 0.34 ± 0.14 | 0.17 ± 0.04 | 0.01 ± 0.003 | 1.58 ± 0.90 | 0.04 ± 0.01 | 5.04 ± 1.11 | 3.61 ± 0.23 |
| **N3** | 0.78 ± 0.11 | 0.56 ± 0.12 | 0.36 ± 0.07 | 0.002 ± 0.001 | 3.67 ± 0.64 | 0.01 ± 0.0004 | 5.13 ± 0.42 | 1.79 ± 0.33 |
| **N4** | 0.51 ± 0.04 | 0.47 ± 0.11 | 0.23 ± 0.03 | 0.01 ± 0.005 | 8.38 ± 1.37 | 0.03 ± 0.01 | 9.13 ± 1.37 | 2.70 ± 0.11 |
| **N5** | 0.40 ± 0.07 | 0.78 ± 0.15 | 0.34 ± 0.06 | 0.01 ± 0.005 | 6.12 ± 1.31 | 0.05 ± 0.05 | 7.77 ± 2.38 | 3.31 ± 0.37 |
| **N6a** | 0.36 ± 0.07 | 19.7 ± 5.02 | 3.75 ± 0.51 | 0.02 ± 0.003 | 4.04 ± 0.46 | 0.03 ± 0.03 | 9.36 ± 1.29 | 7.17 ± 0.40 |
| **N6b** | 0.54 ± 0.09 | 0.83 ± 0.20 | 0.40 ± 0.08 | 0.01 ± 0.004 | 5.82 ± 0.72 | 0.07 ± 0.04 | 10.35 ± 2.18 | 3.63 ± 0.16 |
| **N7** | 0.32 ± 0.05 | 25.5 ± 8.38 | 0.75 ± 0.19 | 0.01 ± 0.005 | 14.2 ± 2.28 | 0.10 ± 0.03 | 21.37 ± 5.66 | 4.66 ± 0.62 |
| **N8** | 0.24 ± 0.04 | 0.33 ± 0.10 | 0.53 ± 0.47 | 0.01 ± 0.005 | 5.22 ± 0.82 | 0.08 ± 0.04 | 18.12 ± 6.25 | 3.51 ± 0.47 |
| **N11a** | 28.9 | 31.2 | 1.08 | 0.28 | 104 | 0.08 | 46.4 | 38.2 |
| **N12** | 0.35 ± 0.03 | 1.70 ± 0.18 | 0.45 ± 0.19 | 0.004 ± 0.002 | 16.2 ± 6.81 | 0.12 ± 0.03 | 7.85 ± 3.38 | 4.22 ± 0.41 |
| **N13** | 0.44 ± 0.04 | 1.09 ± 0.51 | 0.21 ± 0.04 | 0.003 ± 0.002 | 16.2 ± 12.0 | 0.12 ± 0.02 | 5.94 ± 1.93 | 3.87 ± 0.17 |
| **N14** | 0.93 ± 0.16 | 0.73 ± 0.56 | 0.26 ± 0.09 | 0.01 ± 0.002 | 3.71 ± 1.28 | 0.07 ± 0.03 | 4.88 ± 3.24 | 5.15 ± 0.71 |
| **N15a** | 23.1 | 10.6 | 0.42 | 0.03 | 102 | 0.03 | 33.8 | 15.5 |
| **N16** | 8.24 ± 1.05 | 0.95 ± 0.23 | 0.23 ± 0.06 | 0.02 ± 0.01 | 2.93 ± 1.69 | 0.09 ± 0.07 | 3.57 ± 2.91 | 7.39 ± 0.69 |
| **N17** | 0.31 ± 0.05 | 0.11 ± 0.08 | 0.04 ± 0.01 | 0.01 ± 0.001 | 0.30 ± 0.12 | 0.03 ± 0.01 | 3.69 ± 1.80 | 4.02 ± 0.43 |
| **N18r** | 0.40 ± 0.11 | 36.1 ± 8.47 | 1.20 ± 0.28 | 0.09 ± 0.04 | 17.0 ± 1.98 | 0.09 ± 0.05 | 33.6 ± 33.6 | 23.3 ± 2.45 |
| **N19** | 0.26 ± 0.02 | 17.6 ± 4.52 | 0.88 ± 0.13 | 0.04 ± 0.03 | 89.6 ± 24.5 | 0.06 ± 0.02 | <D.L. | 8.58 ± 1.35 |
| **N20** | 0.22 ± 0.02 | 6.31 ± 3.29 | 0.88 ± 0.07 | 0.04 ± 0.05 | 63.5 ± 11.5 | 0.04 ± 0.01 | <D.L. | 8.32 ± 0.50 |
| **N21** | 0.15 ± 0.01 | 4.46 ± 4.79 | 0.19 ± 0.08 | 0.01 ± 0.01 | 1.82 ± 0.61 | 0.02 ± 0.01 | 3.05 ± 0.87 | 6.42 ± 0.29 |
| **N22r** | 0.51 ± 0.11 | 32.6 ± 16.7 | 1.37 ± 0.30 | 0.17 ± 0.11 | 16.2 ± 2.06 | 0.26 ± 0.13 | 26.9 ± 4.77 | 21.4 ± 2.29 |
| **min** | 0.15 | 0.11 | 0.04 | 0.002 | 0.30 | 0.01 | 3.05 | 1.79 |
| **max** | 28.9 | 36.1 | 3.75 | 0.28 | 104 | 0.26 | 46.43 | 38.2 |

Table 4
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**Table 4**. Tissue residue data (in µmol $g^{-1}$ dw, except for Hg in nmol $mg^{-1}$ dw) for the eight elements (As, Cd, Cu, Cr, Hg, Ni, Pb, Zn) in marine and freshwater sediment-dwelling annelids, field collected or from sediment bioassays (exposure days within parenthesis). .

| Organisms | As | Cd | Cu | Cr | Hg | Ni | Pb | Zn | References (exposure) |
|---|---|---|---|---|---|---|---|---|---|
| ***A. marina*** | 0.17–1.63 | | 0.05–1.97 | | | | | 0.66 – 2.16 | Casado-Martinez et al., 2010 (field) |
| | 1.04 | | 0.73 | | | | | 1.71 | Casado-Martinez et al., 2010 (10d) |
| | 1.86 | | 1.54 | | | | | 1.70 | Casado-Martinez et al., 2010 (30d) |
| | 1.32 | | | | | | | | Casado-Martinez et al., 2012 (60d) |
| | 1.63 | | | | | | | | Casado-Martinez et al., 2012 (8d) |
| ***L. hoffmeistieri*** | | 3–15 | | | | | | | Klerks and Bartolomew, 1991 (28d) |
| ***L. variegatus*** | 4.83 | | 0.82 | | | | | | Lyytikäinen et al., 2001 (28d) |
| ***L. variegatus*** | 5.60–26.7 | 0.002–0.004 | 0.28–1.25 | 0.00–0.06 | 0.05–0.35 | 0.03 | 0.016–0.032 | 6.58–14.62 | Winger et al., 2000 (28d) |
| ***L. variegatus*** | 0.23 | 0.013 | 0.87 | 0.60 | | | 0.23 | 11.30 | De Jonge et al., 2012 (54d) |
| ***L. variegatus*** | 0.003–0.37 | 0.015–0.166 | 0.10–2.36 | | | 0.04–0.36 | 0.012 | 0.092 | Camusso et al., 2012 (field) |
| ***Oligochaetes*** | | 0.002–0.32 | 0.31–1.19 | | | 0.10–0.53 | 0.05–0.10 | 3.78–35.59 | Gillis et al., 2006 (field) |
| ***Oligochaetes*** | | | | | | 0.55 | | | Eisler, 2000 (field) |
| ***Tubificids*** | | | | | | | 0.077–1.771 | | Eisler, 2000 (field) |
| ***Tubificids*** | | | 0.50–0.85 | | | | 0.086–0.103 | | Hernández et al., 1988 (field) |
| ***Tubificids*** | | 0.003–0.006 | | | 1.00–5.98 | | 0.039–0.111 | | Kaiser et al., 1989 (field) |
| ***Tubificids*** | | 0.009 | 3.11 | | | | | 0.10 | Say and Giani, 1981 (field) |
| ***Tubifex* sp.** | | 0.021–0.064 | 0.46–1.71 | | | | 0.072–0.162 | 0.92–2.55 | Singh et al., 2007 (field) |
| ***Tubifex* sp.** | 0.27–4.22 | 0.0006–1.23 | 0.14–3.18 | 0.06–0.24 | | 0.01–0.17 | 0.015–0.718 | 2.60–9.02 | De Jonge et al., 2010 (field) |
| ***T. tubifex*** | | | 1.57–3.40 | | | | 0.09–0.91 | 3.52–7.87 | Gillis et al., 2006 (28d) |
| ***T. tubifex*** | | 0.001–60.4 | 0.24–6.17 | 0.01–0.50 | | | | | Méndez-Fernández et al., 2013 (28d) |
| ***T. tubifex*** | **0.15–28.9** | **0.0001–0.036** | **0.04–3.75** | **0.002–0.28** | **0.30–104** | **0.01–0.26** | **0.003–0.046** | **1.79–38.2** | **Present study** |
| | 0.37 ± 0.12 | 0.017 ± 0.020 | 0.75 ± 0.62 | 0.07 ± 0.08 | 5.26 ± 7.85 | 0.10 ± 0.11 | 0.017 ± 0.015 | 12.8 ± 11.0 | Reference sites (n=4) |
| | 0.45 ± 0.22 | 0.005 ± 0.009 | 0.72 ± 1.07 | 0.01 ± 0.01 | 12.8 ± 17.7 | 0.06 ± 0.04 | 0.009 ± 0.006 | 4.46 ± 1.87 | Non Toxic sites (n=11) |
| | 0.27 ± 0.12 | 0.008 ± 0.009 | 0.47 ± 0.36 | 0.02 ± 0.02 | 32.5 ± 49.5 | 0.04 ± 0.02 | 0.004 ± 0.004 | 6.10 ± 2.65 | Potentially Toxic sites (n=3) |
| | 20.1 ± 10.7 | 0.014 ± 0.015 | 0.58 ± 0.45 | 0.11 ± 0.15 | 69.8 ± 557 | 0.07 ± 0.03 | 0.028 ± 0.002 | 20.3 ± 16.0 | Toxic sites (n=3) |

# APPENDIX A

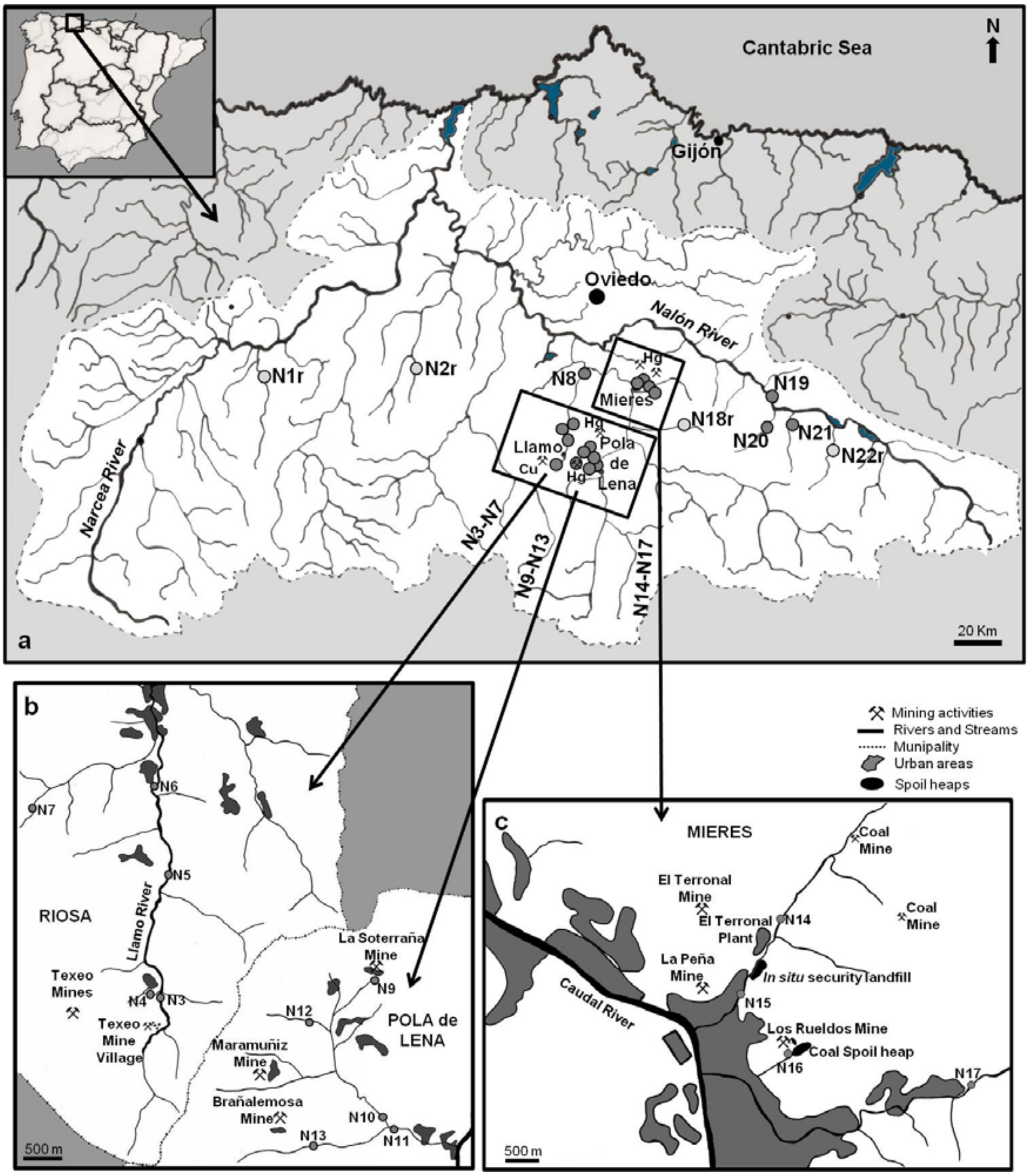


**Fig. 1** Map of study area in Nalón River basin (a) with detailed map of mining areas in Riosa and Pola de Lena (b) and Mieres (c). N1r, N2r, N18r and N22r are reference sites from Water Authorities surveillance nets.

**Table 1** River water and sediment physical-chemical characteristics. Abbreviations: ID.= Site identification code; $O_2$%= Oxygen saturation percentage; [$O_2$] = Oxygen concentration (mg $l^{-1}$); T = temperature (ºC); EC = Electrical Conductivity (µS $cm^{-1}$); Sal = Salinity (ppt); TOC% = Total Organic Content percentage; G% = Gravel percentage, S% = Sand percentage, SC% = Silt & Clay percentage; n.m =not measured.

| ID. | Water | | | | | | Sediment | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **$O_2$ %** | **[$O_2$]** | **T** | **pH** | **EC** | **Sal.** | **TOC %** | **G %** | **S %** | **SC %** |
| N1r | 83.4 | 8.3 | 15 | 7.7 | 144 | 0.1 | 1.6 ± 0.1 | 8.1 | 88.0 | 3.9 |
| N2r | 101.1 | 10.71 | 10.6 | 8.1 | 330 | 0.2 | 1.8 ± 0.0 | 8.8 | 88.6 | 2.6 |
| N3 | 94.3 | 9.7 | 11.4 | 8.2 | 288.6 | 0.1 | 2.4 ± 0.1 | 3.9 | 83.4 | 12.7 |
| N4 | 93.3 | 9.3 | 12.9 | 8.2 | 342 | 0.2 | 9.7 ± 0.1 | 0.3 | 83.7 | 16.0 |
| N5 | 89.0 | 8.6 | 14.1 | 8.3 | 331 | 0.2 | 4.1 ± 0.1 | 13.8 | 69.1 | 17.1 |
| N6a | 98.0 | 9.4 | 15.6 | 8.5 | 312 | 0.1 | 0.9 ± 0.1 | 3.7 | 92.4 | 3.9 |
| N6b | 84.7 | 8.4 | 14.7 | 8.3 | 310 | 0.1 | 2.6 ± 0.2 | 0.6 | 83.1 | 16.3 |
| N7 | 93.3 | 10.1 | 9.5 | 8.3 | 302 | 0.1 | 0.7 ± 0.7 | 2.4 | 82.5 | 15.1 |
| N8 | 88.7 | 8.8 | 15.1 | 8.2 | 437 | 0.2 | 1.5 ± 0.0 | 7.2 | 88.2 | 4.6 |
| N9 | 93.8 | 8.5 | 15.5 | 8.1 | 2030 | 1.0 | 6.8 ± 0.4 | 6.9 | 85.9 | 7.2 |
| N10 | 92.6 | 9.4 | 13.8 | 8.2 | 432 | 0.2 | 2.7 ± 0.0 | 1.8 | 77.1 | 21.1 |
| N11a | 94.0 | 9.4 | 14.1 | 8.5 | 383 | 0.2 | 1.4 ± 0.0 | 4.3 | 93.4 | 2.3 |
| N11b | 94.1 | 9.3 | 14.4 | 8.3 | 403 | 0.2 | 2.1 ± 0.1 | 10.2 | 80.3 | 9.5 |
| N12 | 93.8 | 9.09 | 14.8 | 8.2 | 406 | 0.2 | 1.3 ± 0.1 | 0.9 | 87.0 | 12.1 |
| N13 | 94.0 | 9.4 | 14 | 8.2 | 327 | 0.2 | 2.9 ± 0.1 | 5.5 | 87.9 | 6.6 |
| N14 | 97.7 | 10.13 | 13.7 | 8.2 | 1259 | 0.6 | 3.9 ± 0.1 | 2.3 | 80.7 | 17.0 |
| N15a | 117.0 | 11.2 | 17.3 | 8.4 | 1201 | 0.6 | 3.2 ± 0.1 | 12.3 | 84.2 | 3.5 |
| N15b | 101.5 | 10.3 | 14.1 | 8.4 | 1143 | 0.6 | 4.3 ± 0.3 | 5.6 | 84.9 | 9.5 |
| N16 | 93.6 | 9.6 | 14.3 | 8.3 | 1373 | 0.7 | 6.3 ± 0.4 | 24.5 | 69.3 | 6.2 |
| N17 | 95.0 | 9.7 | 13.5 | 8.6 | 1144 | 0.6 | 3.0 ± 0.2 | 40.6 | 55.5 | 3.9 |
| N18r | 98.0 | 9.4 | 15.1 | 8.6 | 485 | 0.2 | 1.7 ± 0.0 | 0.6 | 97.6 | 1.8 |
| N19 | 95.0 | 8.8 | 18.5 | 8.5 | 216 | 0.1 | 1.0 ± 0.0 | 4.6 | 94.5 | 0.9 |
| N20 | 80.0 | 7.8 | 16.8 | 8.0 | 373 | 0.2 | 1.6 ± 0.1 | 2.5 | 96.5 | 1.0 |
| N21 | 100.0 | 9.5 | 16.2 | 8.3 | 353 | 0.2 | 0.8 ± 0.1 | 10.7 | 88.0 | 1.3 |
| N22r | 118.0 | 11.3 | 17.5 | 8.4 | 205 | 0.1 | 0.8 ± 0.1 | 4.2 | 94.4 | 1.4 |

**Table 2** Variable loadings on each principal components (PC) (bivariate correlations between the observed variables and the first two PCs). Abbreviations: TOC% = Total Organic Content percentage; SC% = Silt & Clay percentage.

| Variables | PC1 | PC2 |
|---|---|---|
| **As** | 0.37 | **0.87** |
| **Cu** | **0.90** | 0.09 |
| **Cr** | 0.93 | 0.24 |
| **Hg** | -0.02 | **0.92** |
| **Ni** | **0.84** | 0.41 |
| **Zn** | **0.82** | 0.41 |
| **TOC%** | 0.44 | 0.59 |
| **SC%** | **0.84** | 0.09 |

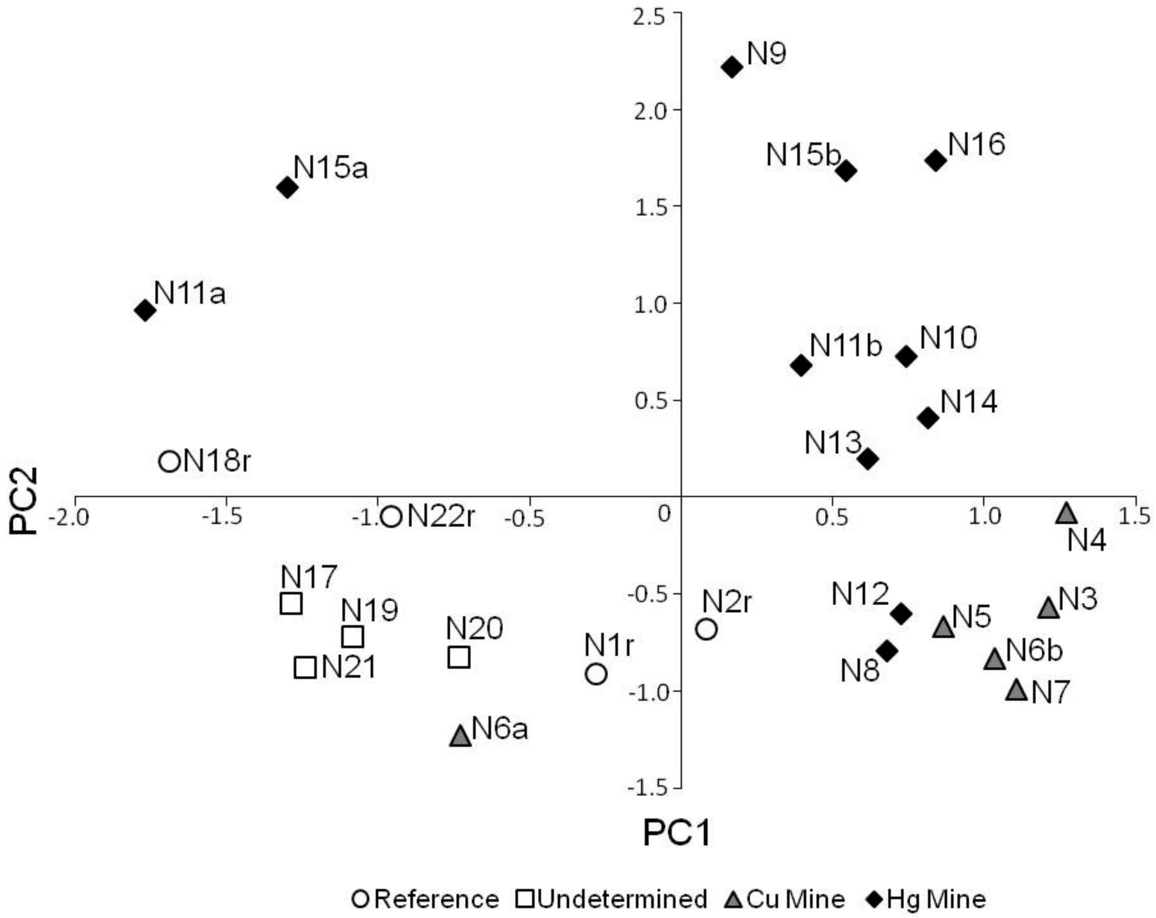


**Fig.2** PCA ordination after Varimax rotation of 25 sites in the Nalon River basin. Each site is marked by a symbol corresponding to four different anthropogenic pressure types.

**Table 3** Spearman´s rank correlation values (ρ) between nMDS axes (MDS1 and MDS2) and metal levels in sediment and tissue residues, used as vectors. RELATE and BEST procedures are also indicated for each matrix combination. Abbreviations: TOX: toxicity data matrix; SED: sediment metal concentration data matrix; TR: Tissue Residue data matrix. *Significant differences: p = 0.001.

| **Metals** | **Toxicity MDS (vectors:SED)** | | **ToxicityMDS (vectors: TRs)** | | **Tissue Residues MDS (vectors: SED)** | |
|---|---|---|---|---|---|---|
| | **MDS1** | **MDS2** | **MDS1** | **MDS2** | **MDS1** | **MDS2** |
| As | **0.73** | -0.21 | **-0.74** | -0.45 | 0.14 | **-0.57** |
| Cd | 0.12 | 0.02 | -0.47 | -0.25 | 0.42 | 0.19 |
| Cu | -0.02 | -0.31 | -0.19 | -0.31 | -0.33 | -0.19 |
| Cr | 0.19 | -0.19 | -0.41 | -0.38 | -0.35 | -0.23 |
| Hg | **0.67** | -0.37 | **-0.62** | -0.11 | **0.61** | **-0.61** |
| Ni | 0.19 | -0.18 | 0.12 | -0.47 | -0.23 | -0.23 |
| Pb | 0.34 | 0.04 | -0.05 | **-0.62** | 0.20 | 0.13 |
| Zn | 0.26 | -0.04 | **-0.58** | -0.43 | -0.48 | -0.31 |

| PAIRWISE MATRICES CORRELATION | **TOX-SED** | **TOX-TR** | **TR-SED** |
|---|---|---|---|
| RELATE (As, Hg, Pb, Zn) | ρ = 0.403* | ρ = 0.661* | ρ = 0.613* |
| BEST | As, Hg (ρ = 0.614) | As, Pb, Zn (ρ = 0.739) | As, Cu, Hg, Zn (ρ = 0.588) |