Baseline tissue concentrations of metal in aquatic oligochaetes: Field and laboratory approaches

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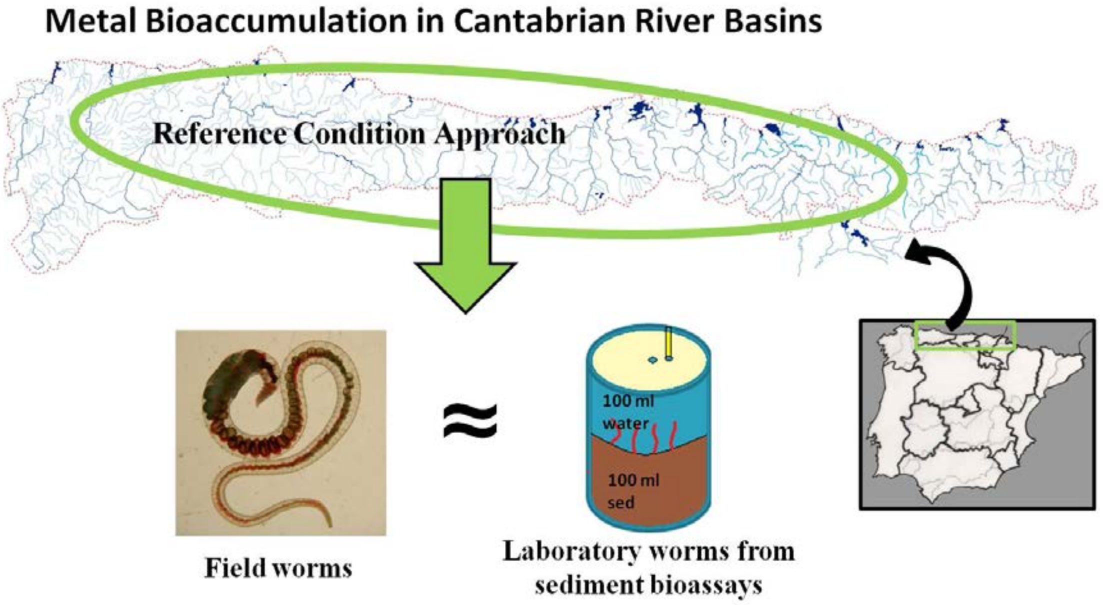
Abstract

Metal tissue residue evaluation in benthic macroinvertebrates is an important component of an integrated approach to ecological risk assessment of metals and metalloids in the Nalón River basin (North Spain), where historic mining activities took place. The purpose of this study was to know the baseline tissue concentration of 7 metals (Cd, Cu, Cr, Hg, Ni, Pb, and Zn) and one metalloid (As) in aquatic oligochaetes, sediment burrower organisms, representative of the collector-gatherer functional feeding group in the macroinvertebrate community. Metal concentration was measured in sediment and field aquatic oligochaetes at several reference (minimally disturbed) sites of the Nalón River basin, selected following Water Framework Directive criteria. Metal tissue residues were measured separately in field microdriles and lumbricids and compared with tissue concentrations measured in the aquatic oligochaete *Tubifex tubifex* exposed to reference sediments from the Nalón and other Cantabrian River basins in 28-d chronic laboratory bioassays. Metal tissue residues in bioassay organisms attained usually higher levels than in field worms, in special for As, Cu, Hg and Zn, although metal levels were within the same order of magnitude. The baseline values for metals were calculated from 90th percentile (P90) values in field aquatic oligochaetes (microdriles and lumbricids). The P90 for Hg, As and Zn could efficiently discriminate Toxic and Non-Toxic sites, while baseline values calculated for the other metals deserve further research due either to the low range of values found in the present study, or to the regulation of the metal body concentration, as in the case of Cu.

***Keywords**:* reference condition approach, metal bioaccumulation, microdriles, lumbricids, bioassays



Capsule: Differences between field and laboratory metal bioaccumulation in aquatic oligochaetes exposed to the sediments from reference unpolluted sites were low for most metals (2-5 times difference), and up to 10 times higher for Hg in laboratory worms.

Highlights

High levels of As and Hg were measured in reference sediments of the Nalón R. basin

Baseline metal levels in oligochaetes are in the range reported in the literature

Metal tissue residues in field and laboratory oligochaetes are comparable

The use of metal tissue baseline values in biomonitoring is encouraged

1. Introduction

The development of chemical-specific Environmental Quality Standards (EQS) within the Water Framework Directive (WFD: EC, 2000) has become an integral component in the assessment and management of contaminants in aquatic ecosystems (EC, 2013). In the case of metals, the European guidelines are based on the *stand still* principle, i.e., priority substances in the sediments or biota should not significantly increase their concentration in the long-term (Article 4 in the WFD: EC, 2000; Article 3 in the EQS Directive: EC, 2013). However, this approach can be inadequate for the protection of aquatic biota because, in many sites, the current tissue concentration can be hazardous to wildlife. Therefore, there is a need for the development of tissue-based EQS for metals (Meador et al. 2014), and the risk assessment in Europe might consider the natural baseline (or reference) concentrations in the biota (EC, 2013).

Metal bioaccumulation is an adaptive and space and time-dependent process (Adams et al., 2011), and benthic organisms have evolved across a wide range of natural sediment metal concentrations; therefore, tissue concentrations can vary over several orders of magnitude between geographic areas (see Chapman et al., 1999 and references therein). Consequently, in regions with diverse lithology, it is important to establish the background tissue metal levels as baseline concentrations, identify atypical levels in the biota and correctly assess the alterations due to metal bioaccumulation. The identification of atypical metal concentrations, in particular aquatic organisms, has great potential for the development of tissue metal guidelines if correlated with alterations in the biota (Rainbow and Luoma, 2011). On the other hand, tissue levels in benthic taxa vary depending on their membership in different functional feeding groups (Goodyear and McNeill, 1999; De Jonge et al., 2013). In the present study, aquatic oligochaetes were selected as representatives of sediment-dweller, collector macroinvertebrates. This functional group can be significant in metal risk assessment due, first, to the high capacity of sediment to hold metals (Reynoldson and Day, 1993; Burton and MacPherson, 1995; Simpson and Batley, 2016) and, second, to the fact that the benthic route can be the main uptake route for the bioaccumulation of some metals in aquatic communities (Simpson, 2005; Camusso et al., 2012; Méndez-Fernández et al., 2014).

The core of the classification system for the ecological status of water bodies in the European WFD is based on a comparison of biological metrics in a particular study site with a defined reference condition with the risk assessment resulting from the degree of deviation of a metric from a given reference value (Reference Condition Approach, RCA: Reynoldson and Wright, 2000; Bailey et al., 2004). In Europe, the process of establishing a reference condition relies on the use of minimally disturbed streams and rivers in a defined ecoregion (Stoddard et al., 2006) characterized by type-specific, chemical and hydromorphological conditions. According to the provisions of the WFD, there are different methodologies for the development of tissue EQS (EC, 2011) varying with the use of laboratory toxicity data (deterministic and probabilistic approaches) or field-based data. In this regard, a comparison between field and laboratory approaches appears necessary due to the differences in exposure conditions, species studied, and possible acclimation or genetic resistance processes in different populations (see Chapman 1985a, b; Klerks and Levinton, 1989; Vidal and Horne 2003 a, b). In any case, integrative science entails the knowledge of both field and laboratory data on metal bioaccumulation because together they can provide not only an overall perspective of chemical bioavailability but also information on cause-effect relationships.

In the Cantabrian region (north Spain), lithology can be associated with high levels of different metals, which explains the existence of historical mining activities (Águeda-Villar and Salvador-González, 2008; Ordoñez et al., 2008). Sediment pollution, toxicity and/or metal bioaccumulation at several sites affected by mining works in the Nalón River basin (Asturias, North Spain) were previously reported (Loredo et al., 2006, 2010; Méndez-Fernández et al., 2015). This study is part of a larger research effort that includes several representative taxa from different functional feeding groups of macroinvertebrates to assess the metal bioaccumulation in rivers affected by metal mines in north Spain. Oligochaetes are infaunal, detritus-feeder macroinvertebrates which are exposed to contaminants through the body wall and the digestive epithelia, and their entire life cycle occurs in the sediment. Aquatic oligochaetes are widely distributed (Martin et al., 2008), and some species have a large data base as bioindicators of toxicity and bioaccumulation (e.g., Bervoets et al., 2016; De Jonge et al., 2010, 2012; Méndez-Fernández et al., 2015; Rodriguez and Reynoldson, 2011).

The main objective of this study was to establish baseline metal tissue levels for field aquatic oligochaetes at reference sites as a tool for future environmental risk assessment in the Cantabrian River basins. Furthermore, we aimed to evaluate the reliability of baseline metal tissue levels derived from field aquatic oligochaetes to assess metal tissue levels measured in laboratory chronic bioassays with oligochaetes exposed to polluted and unpolluted sediments and classified by the chronic toxicity effects (Mendez-Fernandez et al. 2015).

2. Materials and methods

2.1. Study area

A total of 27 sites (3 sites sampled in two occasions), including the main river axis and tributaries, were studied in the Cantabrian region, north Spain, and they flow south to north through steep valleys. Most rivers are fast flowing and of short length due to the proximity of their mountainous origin to the sea, and their substrate is generally coarse as a consequence of large hydrological variations (Pardo et al., 2014). Two sets of data were considered here: a sampling survey conducted in 2008-2011 in four Cantabrian river basins (Miera, Nansa, Nalón and Saja-Besaya) to study sediment toxicity and metal bioaccumulation in the oligochaete worm *Tubifex tubifex* through 28-d chronic bioassays, and a second sampling survey in 2014-2015 where field aquatic oligochaete worms were collected at the Nalón R. basin to study metal bioaccumulation (Table 1). In the chronic bioassays, 4 reference sites belonged to the Nalón R. basin (Méndez-Fernández et al., 2015), and 8 were located in other Cantabrian R. basins (Miera, Nansa and Saja-Besaya). In the survey conducted in 2014-2015, a total of 15 reference sites were studied, all in the Nalón R. basin. Eleven study sites were validated as reference sites by the Cantabrian Hydrographical Confederation according to the criteria outlined in the WFD and described in Pardo et al. (2012). This complemented with an assessment of the macroinvertebrate community using both the multimetric index METI (Multimétrico Específico del TIpo, Pardo et al., 2010) and NORTI (Northern Spain Indicators system, Pardo et al., 2014) in use in the area to classify the ecological status. For some sites, data on anthropological pressures were not available (NO2259, N12, R2 and R4), and validation was performed using aerial images, (GIS databases), field visits and expert judgment (I. Pardo).

2.2. Field oligochaete sampling

Field oligochaetes were sampled using a multi-habitat scheme, often in transects, during summer (September 2014 and July 2015). The collected worms were separated for metal analyses into 2 groups: lumbricids and microdriles. When possible, 3 replicates per taxon were collected; otherwise, individuals were pooled to obtain sufficient biomass for the metal analysis for a given site (only a pooled sample in 3 sites for both microdriles and lumbricids).

2.3. *Tubifex tubifex* chronic sediment bioassay

In the 2008-2011 surveys, a composite sediment sample was taken at 12 sites during low water in September-October from the upper 5–10 cm layer of fine sediment to conduct laboratory toxicity bioassays. A sample of 1-L sediment was obtained after fresh-sieving in the field through 500-µm mesh size to eliminate coarse particles and indigenous fauna before conducting the chronic bioassays with the aquatic microdrile oligochaete *T. tubifex* (Reynoldson et al., 1994). Sediment bioassays were run in the laboratory of Animal Ecotoxicity and Biodiversity at the University of the Basque Country (UPV/EHU, Spain). The detailed methods for the 28-d sediment chronic bioassay with *T. tubifex* are based on ASTM (2005) and described by Méndez-Fernández et al. (2015). Briefly, twenty-four hours prior the bioassay beginning, all sediments were sieved through 500 µm mesh, mixed with the overlying water, and distributed into five replicates. Each replicate contained 100 ml sediment, 80 mg supplementary food (Tetramin®) to minimize inter-sediment differences due to nutritional quality and quantity, and 100 ml overlying, dechlorinated tap water in a 250 ml glass beaker. Four mature worms were placed into each beaker; they were in their first reproductive cycle, had a similar size and age (6-8 week old), and were individually weighed in a Sartorius M3P electrobalance (DL = 1 µg) after 5 h gut purging in dechlorinated tap water. Bioassays were run at 22 ± 1ºC, in the dark, and gently aerated, during 28 days. At the end of the bioassay measured endpoints were: survival (%), reproduction (number of Total Cocoons; number of Empty Cocoons; and number of Total Young), and growth endpoints (Total Growth Rate; see Maestre et al. 2007).

2.4. Sediment characterization and metal analysis

From the composite sediment sample collected for the bioassays, subsamples were taken for chemical analyses of the metal concentration, particle size distribution and organic content (see methods in Méndez-Fernández et al., 2015). Seven metals (Cd, Cu, Cr, Hg, Ni, Pb and Zn) and one metalloid (As) were measured in the 63-µm sediment fraction after air-drying (hereafter, we will use the word "metals" to refer to these eight elements). The sediments were acid digested, and chemical analyses from the 2008-2011 survey were performed at the SOSPROCAN Unit at the University of Cantabria (Santander, Spain), following EPA 3052 and UNE-EN 13656:2003 procedures and USEPA method 6020A for Hg (detailed in Méndez-Fernández et al., 2015); the Limits of Quantification (LOQ) 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, and 0.03 µg l^{-1} Zn. In the 2014-15 survey, sediments were digested following EPA 3051 at the UPV/EHU by the Technical Services of SGIker and analyzed by ICP-MS (Agilent 7700X); the LOQ were 0.001 µg l^{-1} As, 0.01 µg l^{-1} Cd, 0.1 µg l^{-1} Cu, 0.06 µg l^{-1} Cr, 0.02 µg l^{-1} Hg, 0.10 µg l^{-1} Ni, 0.05 µg l^{-1} Pb, and 0.05 µg l^{-1} Zn.

All analytical batches included 3 replicates of Buffalo River sediment (RM8704, USA) (as well as Sewage Sludge-3, CRM 031-040, UK in the 2014-15 survey) as reference materials for quality control. The recovery rates for all metals were within certified values (80-118 %). The metal sediment concentrations were reported on a dry weight basis (µg g^{-1} dw).

2.5. Metal analysis in aquatic oligochaetes

Field aquatic oligochaetes (1-3 replicates) (2014-2015 survey) were placed in 15-ml tubes with river water and stored on ice during transport at 4°C (up to 10 h) until they were processed. Worms (1-10 specimens were selected for microdriles, 1-2 lumbricids) were cleaned from sediment particles under a dissecting microscope and frozen at -20°C. The worms may have partially purged their guts before being frozen. In the laboratory, worms were freeze-dried, weighed on an electrobalance (Sartorius M3P, detection limit, DL = 1 µg), and acid digested in 15-ml polypropylene conic tubes as follows: 500 µl of nitric acid (70 % Baker Instra-Analyzed) at room temperature for 1 week; afterwards, 50 µl of H_2O_2 (30 % R.P. Merck Suprapur) was added for 24 h. The digested samples were stored at -20°C until analytical measurements were conducted. The concentrations of 8 metals (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) were analyzed by ICP-MS (Agilent 7700X) by the Technical Services of SGIker. The LOQ were 0.010 µg l^{-1} As, 0.010 µg l^{-1} Cd, 0.097 µg l^{-1} Cu, 0.049 µg l^{-1} Cr, 0.031 µg l^{-1} Hg, 0.171 µg l^{-1} Ni, 0.079 µg l^{-}

^{1} Pb and 0.443 μg l^{-1} Zn. For the statistical data analysis, data below the LOQ were substituted by ½ LOQ. Every batch of samples included blanks (no organisms) and Mussel Tissue Standard Reference Material (NIST 2976) for quality control. The recovery rates were within certified values for all metals (80-115 %).

Worms (adult *T. tubifex*) from the chronic sediment bioassays (5 replicates) were purged (5 h), frozen with liquid nitrogen, freeze-dried, weighed, and stored as explained for field samples. The metal tissue analysis of the bioassay worms was performed by the SOSPROCAN Unit (University of Cantabria, Santander, Spain). The digestion method and LOQ are detailed in Méndez-Fernández et al., (2015). Every batch of samples included blanks and certified reference material (Mussel Tissue ERM-CE278, Belgium), and the recovery rates were within the certified values for Cd, Cr, Cu, Pb and Zn (80.4 - 106.3 %) but not for As (140.1 %). No reference values were available for Hg and Ni, but analyses showed low variation (Hg = 0.20 ± 0.04 and Ni = 0.94 ± 0.17 μg g^{-1} dw, n = 18).

In all cases (field and bioassay worms), metal tissue concentrations were corrected for background interference by subtracting mean blank values and reported in a dry weight basis (μg g^{-1} dw).

2.6. Data processing and statistical analysis

The differences in metal tissue residues measured in aquatic oligochaetes were assessed by the non-parametric Kruskal-Wallis test followed by multiple pairwise comparisons with Dunn´s test. Pearson´s correlation was used to evaluate metal bioavailability between the metal concentration in the sediments and the body tissue concentration (log-transformed data). Multivariate analyses were performed to test the differences in metal bioaccumulation in field microdriles, field lumbricids and bioassay *T. tubifex*. The differences between groups was assessed by an ANOSIM procedure (Clarke, 1993). A Principal Coordinate Analysis (PCoA) examined the dominant patterns of intercorrelation among metal concentrations in different macroinvertebrate taxa (previously transformed and standardized). The 90th percentile (P90) values for metal concentrations are presented as plausible baseline values (Adams et al., 2011). All data analyses were conducted in IBM® SPSS 22 (2013), R v3.1.0 and PRIMER 6 (Clarke and Gorley, 2006) software.

3. Results

3.1. Sediment physical and chemical characteristics

The studied reference sediments had low organic content (0.4-2.3 % TOC), and they were predominantly sandy (overall mean composition of sand was 97 %: 24.4 % fine and very fine sands, 21.1 % medium, and 51.6 % coarse and very coarse sands) and only 3 % silt-clay. In the absence of Sediment Quality Guidelines (SQGs) in Spain, the metal (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) concentrations were evaluated using the TEC (Threshold Effect Concentration) and PEC (Probable Effect Concentration) values proposed by MacDonald et al. (2000) for North American freshwater sediments. In the Nalón R. basin, the metal concentration in the sediment can attain moderate to high levels for several metals according to these guidelines (Table 1). Regarding the metal TEC value, it was exceeded at 79 % of the sites for As, at 63 % for Ni, at 32 % for Hg, and in only a few instances for Cu, Cr and Zn. The Hg and Ni-PEC values were exceeded at 16 % and 21 % of the sites, respectively, while the As-PEC value was exceeded at only one site. For Cd and Pb, the TEC (only one site for Cd) or PEC values were never exceeded. In other Cantabrian river basins (Miera, Nansa and Saja-Besaya), the levels of metal in the reference sediments never exceeded the PEC values, and at only two sites was the Hg-TEC level exceeded by less than twofold (Table 1).

3.2. Metal tissue concentration in aquatic oligochaetes

During the field surveys in 2014-2015, aquatic oligochaetes (both microdriles and lumbricids) were found at all sites except at site N12; only one of the groups was present in NAL049 and NAL011. The mean individual biomass sampled at each reference site was 15.7 ± 6.6 mg dw for lumbricids (n=13) and 0.7 ± 0.5 mg dw for microdriles (n=13). Lumbricids were identified as *Eiseniella tetraedra* Savigny at 88 % of the sites. Aquatic microdriles were usually represented by the species *Stylodrilus heringianus* Claparède (Lumbriculidae) identified as mature specimens at 32 % of the sites, and most likely the same species was present as immature at an additional 32 % of the sites.

A summary description of the tissue metal concentrations in the field-collected lumbricids and microdriles and in the bioassay *T. tubifex* is reported in Table 2. A comparison between the field microdriles and lumbricids from the Nalón R. basin showed minor differences between the median metal tissue concentrations, i.e., 1.4 to 2.3 times higher in microdriles than in lumbricids (except for As). The metal tissue residues in the bioassay *T. tubifex* exposed to reference sediments attained higher maximum values than in field oligochaetes for As, Cu, Hg, Ni and Zn (i.e., 38.2 µg As g^{-1}, 87.0 µg Cu g^{-1}, 3.42 µg Hg g^{-1}, 21.5 µg Ni g^{-1}, 1520 µg Zn g^{-1}); however, the opposite occurred for Cd, Cr and Pb, metals that were present at relatively low levels in the sediments (Table 1). The differences in bioassay *T. tubifex* when exposed to reference sediments from the Nalón R. *vs* other Cantabrian river basins were of less than one order of magnitude (median tissue concentration 1.7-6.2 times higher when exposed to the Nalón R. sediments for As, Cd, Cu, and Zn with similar values for Cr and Ni but 0.3 times lower for Pb). For comparisons of the metal concentrations measured in bioassays with field-collected worms, we pooled the data from the *T. tubifex* bioassay (n= 12, except for Hg n= 4). The median tissue residues in laboratory *T. tubifex* were 2.4 to 5 times higher for As, Cu and Hg than in field microdrile oligochaetes, 3.3 and 9.9 times higher for Cu and Hg in lumbricids, respectively, and within the same range for the other metals.

The metal concentration in the sediments and in *T. tubifex* from the bioassays were negatively correlated for Cr (r= -0.76) and Zn (r= -0.69) (n=12), whereas the relationships were positive for Hg (r= 0.98, n=4) and Pb (r= 0.59, n=12). In field oligochaetes, no significant correlations with sediment metal concentrations were found for microdriles and only for As (r= 0.59, n=13) in the case of lumbricids.

Multivariate analyses of the metal concentrations in sediment and tissue levels in the field and the laboratory oligochaetes were used to study the ordination patterns and differences between groups (Hg was not included due to the lack of data in *T. tubifex* bioassays with several Cantabrian sediments). The PCoA of the tissue metal concentrations of field and worm bioassays defined two principal components (Fig. 1) that explained 71.7 % of the accumulated variance (PCo1=43.0 %; PCo2=28.7 %). PCo1 defined a gradient in worm tissue residues, where the laboratory *T. tubifex* represented the whole range of values, attaining higher metal levels for As, Cu, Ni and Zn in some bioassay worms (Pearson's correlation with PCo1: $r \geq 0.60$) (Table S1, Supplementary material). The PCo2 component defined a gradient in tissue residues from higher metal concentrations in microdriles to lower values in *T. tubifex*, attaining higher levels of Cd, Cr and Pb in microdriles (Pearson's correlation with PCo2: $r \geq 0.60$) (Table S1, Supplementary material).

The differences between three *a priori* defined oligochaete groups (bioassay *T. tubifex*, field microdriles and field lumbricids) in the multivariate space were not very high but significant (ANOSIM Global R=0.441, p= 0.001). The differences were mainly due to the higher metal tissue levels in the bioassay *T. tubifex* than in field oligochaetes (bioassay worms *vs* lumbricids: R=0.547, p=0.001; bioassay worms *vs* microdriles: R=0.502, p=0.001), while the low R value for field lumbricids *vs* microdriles (R=0.292, p=0.001) implied little differences between both groups. Therefore, we calculated the 90th percentile (P90) of the data distribution from the pooled database of metal tissue concentration measured in the field-collected aquatic oligochaetes (microdriles and lumbricids) for use as an interim metal tissue baseline concentration for oligochaete worms. The P90 values were as follows: 12.3 µg As g^{-1}, 5.98 µg Cd g^{-1}, 14.3 µg Cu g^{-1}, 9.2 µg Cr g^{-1}, 0.48 µg Hg g^{-1}, 6.9 µg Ni g^{-1}, 33.6 µg Pb g^{-1} and 381.7 µg Zn g^{-1} (Table 3).

3.3. Metal baseline in aquatic oligochaetes and relation to sediment ecotoxicity

The results from the chronic *T. tubifex* bioassays exposed to reference, unpolluted sites from the Cantabrian river basins in north Spain are reported in Table 4. New data from Cantabrian rivers do not significantly modify previous reference values reported in Rodriguez et al. (2011) regarding survival and reproduction endpoints; however young production is generally higher and growth rates are lower in the Cantabrian R. basins. The results derived from bioassays with reference Cantabrian sediments were validated using the methodology described in Rodriguez et al. (2011): the benthic community was assessed as Good or Very Good, the survival in sediment bioassays was greater than 50 %, and the toxicity data in at least 3 (out of 5) endpoints was greater than the 5^{th} percentile of the total data distribution. The whole reference data for north Spain (n=58) are shown in the Table 4.

In a previous publication, 17 sediments from the Nalón R. basin were classified as Non-Toxic, Potentially Toxic and Toxic using a reference condition approach (n= 58 reference sites) and probability ellipses (based on survival, reproduction and growth endpoints) (Méndez-Fernández et al., 2015). The metal body concentration data in *T. tubifex* for each of these toxicity categories are represented in boxplots (Fig. 2) and compared with the P90 values calculated as a baseline for each of the metals studied in field oligochaetes in the Cantabrian region. In the Nalón R. basin, median worm tissue levels at Toxic sites exceeded 3 to 141 times the baseline concentrations for As, Hg and Zn; however, the baseline values for Cd, Cr, Ni and Pb were not exceeded. For Cu, no differences were observed between tissue residues at each toxicity category; however, in all cases they were slightly higher than baseline values.

4. Discussion

The interpretation of metal body concentrations is not straightforward because aquatic organisms have evolved strategies to regulate metal bioaccumulation within a range of concentrations in the sediment, without adverse biological effects (Chapman, 2008; Chapman et al., 1999; Meador, 2006, 2015; Rainbow, 2002). In the same way, the acclimation and resistance of organisms to different levels of metals present in the environment can be responsible for higher metal tissue levels as reported in the literature for organisms exposed to concentrations within the same range of values, a phenomenon that is documented in aquatic oligochaetes (Cd: Klerks and Levinton, 1989; Klerks and Bartholomew, 1991; Hg: Vidal and Horne, 2003b). Using the SQGs derived for North American freshwater sediments (MacDonald et al., 2000), metal concentrations in sediments in the study area, especially in the Nalón R. basin, can be naturally high for As, Cu, Hg, Ni and Zn; thus field worms from this area can be expected to have higher tissue concentrations for these metals. However, the expected differences between the metal body concentration in field worms from the Nalón R. basin and other Cantabrian basins or between field *vs* bioassay worms differed by less than one order of magnitude. Moreover, the baseline values (P90) calculated from data reported in the literature are similar to those measured in our study region for Hg, Pb and Zn; however, they were 3-4 times higher for As, Cd, Cu and Ni (Table 3 and Table S2, Supplementary Material).

On the other hand, threshold body concentrations, that is, body concentrations that do not cause significant adverse effects in the organisms, have been reported in the literature for aquatic oligochaetes and freshwater macroinvertebrate communities, and they reflect statistical relationships to biological variables (survival, reproduction, etc.) in bioassays, or ecological impacts on macroinvertebrate communities. Baseline and threshold values are not directly comparable, and it is generally expected that baseline body concentrations are lower, because they reflect background levels derived from natural concentrations in the field organisms (due to the basin lithology and characteristics of the water). Baseline values calculated from field worms in present study are up to 7 times lower than the Critical Body Residues (CBR) calculated for aquatic oligochaetes in Flanders (Bervoets et al., 2016) (Table 3). In the laboratory, chronic *T.* t*ubifex* bioassays with sediments from the Nalón R. basin (Méndez-Fernández et al., 2015) As, Hg and Zn-CBR_{20} were up to 15 times higher, except Pb-CBR_{20}, which was 5 times lower than the baseline value calculated in the present study. There is very limited information on Critical Tissue Level (CTL) guidelines for the protection of aquatic life. Metal guidelines from the Oregon Department of Environmental Quality (DEQ, 2007) are of the same order of magnitude (2 to 5 times higher or lower, see Table 3) than the baseline values of the present study in the case of As, Cd and Hg; however, it was 28 times lower for Pb. Therefore, in future studies estimates of the CBR values based on alterations of the field biota will be compared with the baseline values presented here.

The last question that we have addressed is the following: do metals bioaccumulate above baseline values in toxic sediments? The metal tissue levels measured from the bioassay exposed to sediments from the Nalón R. basin, previously assessed as Non-Toxic, Potentially Toxic and Toxic in a separate study (Mendez-Fernandez et al., 2015), clearly identified a bioaccumulation risk for As, Hg and Zn that was above baseline tissue levels in Toxic sediments. Other metals were in all cases well below the baseline levels calculated in the region; i.e., the tissue levels of Cd, Cr and Pb, though showing a clear increase from Non-Toxic to Toxic sediments, did not appear to be responsible for the observed chronic toxicity. This observation agrees with the interpretation that As and Hg were the metals in the sediments of maximum concern related to the observed chronic toxicity (Méndez-Fernández et al., 2015). Baseline levels thus provide an accurate basis for predicting sediment toxicity in the case of the tissue levels of As, Hg and Zn in the bioassay worms. In the case of Cu, no differences were observed in tissue residues between the toxicity categories, which suggests that the essential nature of Cu results in active regulation (excretion, storage in cells and tissues, or metabolic immobilization) without causing toxicity to the organisms when exposed at high concentrations (Chapman, 2008; Meador et al., 2011).

The mismatch observed in metal bioaccumulation between field and laboratory organisms can usually be explained by differences in bioavailability. This factor can be advocated to explain the higher metal tissue concentration range in the *T. tubifex* bioassay than in field worms. Mechanical manipulation of the sediments (i.e., sieving) or physical and chemical variables (e.g., Acid Volatile Sulfides-AVS, dissolved oxygen, and temperature) can influence metal bioavailability (Chapman, 2008; Simpson and Batley, 2007). However, unsieved sediment has been demonstrated to cause "false positives" in sediment bioassays (Reynoldson et al. 1994), and sieving is usually necessary to eliminate indigenous fauna before performing a sediment bioassay. With regard to AVS content, it did not seem to play a major role in influencing toxicity or metal bioaccumulation in experiments with *T. tubifex* (Méndez-Fernández et al., 2014) or field-collected tubificids (De Jonge et al., 2009, 2010, 2011). Moreover, in the studied reference sites AVS was not of concern because in most Cantabrian rivers the water column is well mixed and oxygenated, and the sites fulfilled the reference criteria on dissolved oxygen (Pardo et al., 2012) to prevent anoxia in the sediments. Water temperature can play a direct role in the physiology of aquatic organisms and, in particular, in the uptake, excretion and accumulation of metals (Bervoets et al., 1996; Sokolova and Lannig 2008); in field conditions, the water temperature is much lower (11.2-18.3°C) than in *T. tubifex* laboratory bioassays (22-23°C). Yet, these differences did not result in important changes in tissue concentrations which were less than one order of magnitude. However, effective tissue concentrations could change under pollution scenarios in combination with increasing water temperature, water flow decline and a decrease in dissolved oxygen, as expected under the current global climate change (Verberk et al., 2016) because minor increases in field temperature can enhance metal toxicity (Heugens et al., 2006).

As far as we know, the data reported in the present study are the first to use a set of reference sites to establish baseline metal values in benthic organisms in compliance with the European WFD. These data can be relevant for the development of European, national or regional benchmarks for metal bioaccumulation in biota of benthic target organisms as stated by the European normative (EC, 2013). Baseline concentrations can be a trigger point in environmental risk assessment because biota concentrations higher than baseline values might indicate the requirement for biomonitoring programs (EC, 2013; Real Decreto 817/2015). This approach is applicable to habitats where contamination by metals constitute the main factor explaining the ecotoxicological alterations in aquatic communities (Awrahman et al., 2016; Rainbow and Luoma, 2011), and it will be addressed by the authors in a separate contribution.

Finally, our results suggest the potential for derived baseline values of metals in field oligochaetes to assess the environmental risk due to bioaccumulation and develop tissue-based guidelines. However, the presence of metal mixtures can also cause antagonistic or synergic effects and be responsible for unexpected results in sediment toxicity (De Jonge et al., 2012), enhanced bioaccumulation (De Jonge et al., 2013; Norwood et al. 2007) or field alterations (Awrahman et al., 2016), which suggest the need to develop new models that help refine the estimates of tissue-based guidelines for a sound environmental risk assessment (Meador et al., 2014; Sappington et al., 2011).

5. Conclusions

High background concentrations of As, Cu, Hg, Ni and Zn can occur naturally in reference (unpolluted) sediments, especially in river catchments where historical mining activities exist. Regardless of the lithology of the area, the tissue concentrations of metal in field aquatic oligochaetes in Nalón R. were within the same order of magnitude as in other Cantabrian river basins as well as within the range reported worldwide for aquatic oligochaetes in unpolluted sites. This suggests that the baseline values derived in the Cantabrian region for aquatic oligochaetes can also be a valuable monitoring tool for aquatic oligochaetes in other geographic areas. Although worms exposed to reference sediments in chronic sediment bioassays usually have higher tissue concentrations of As, Cu, Hg, Ni and Zn, the differences in tissue levels of metal between field and laboratory bioassays with natural sediments were less than one order of magnitude. This enables the use of both types of data for developing future guidelines for aquatic biota. The tissue levels of metal in aquatic lumbricids and microdrile oligochaetes are comparable, which favors the use of different oligochaete taxa or a pool of them, in environmental assessments for metal bioaccumulation as representative of sediment-dweller macroinvertebrates. The calculation of threshold metal concentrations for macroinvertebrates in Europe still requires additional data on the relationships of metal bioaccumulation and the effects in both laboratory bioassays and field populations representative of the different functional groups of the benthic community.

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Figures

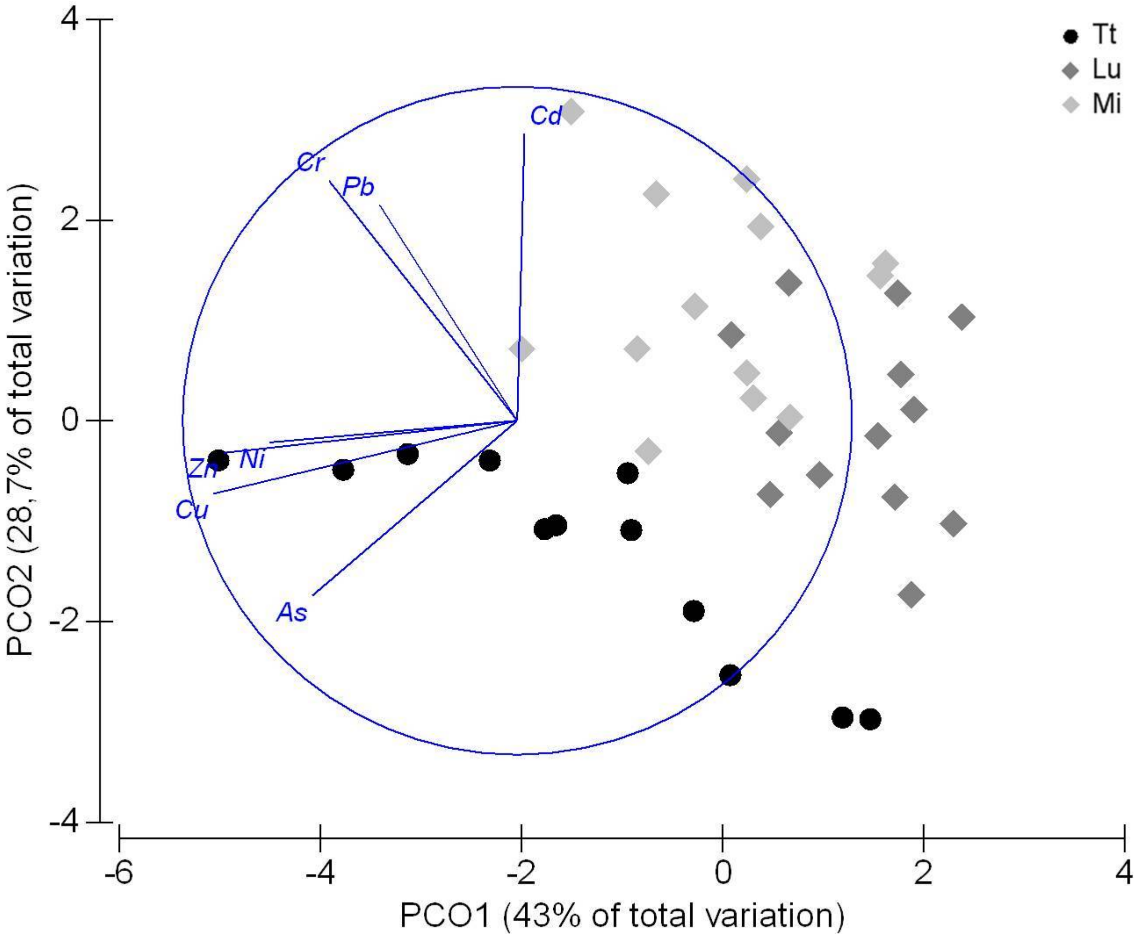

Fig.1. Spatial ordination by PCoA for the 3 studied oligochaete groups (Tt: *Tubifex tubifex*; Lu: Lumbricids; Mi: Microdriles) based on seven metal (As, Cd, Cu, Cr, Ni, Pb and Zn) resemblance matrix.

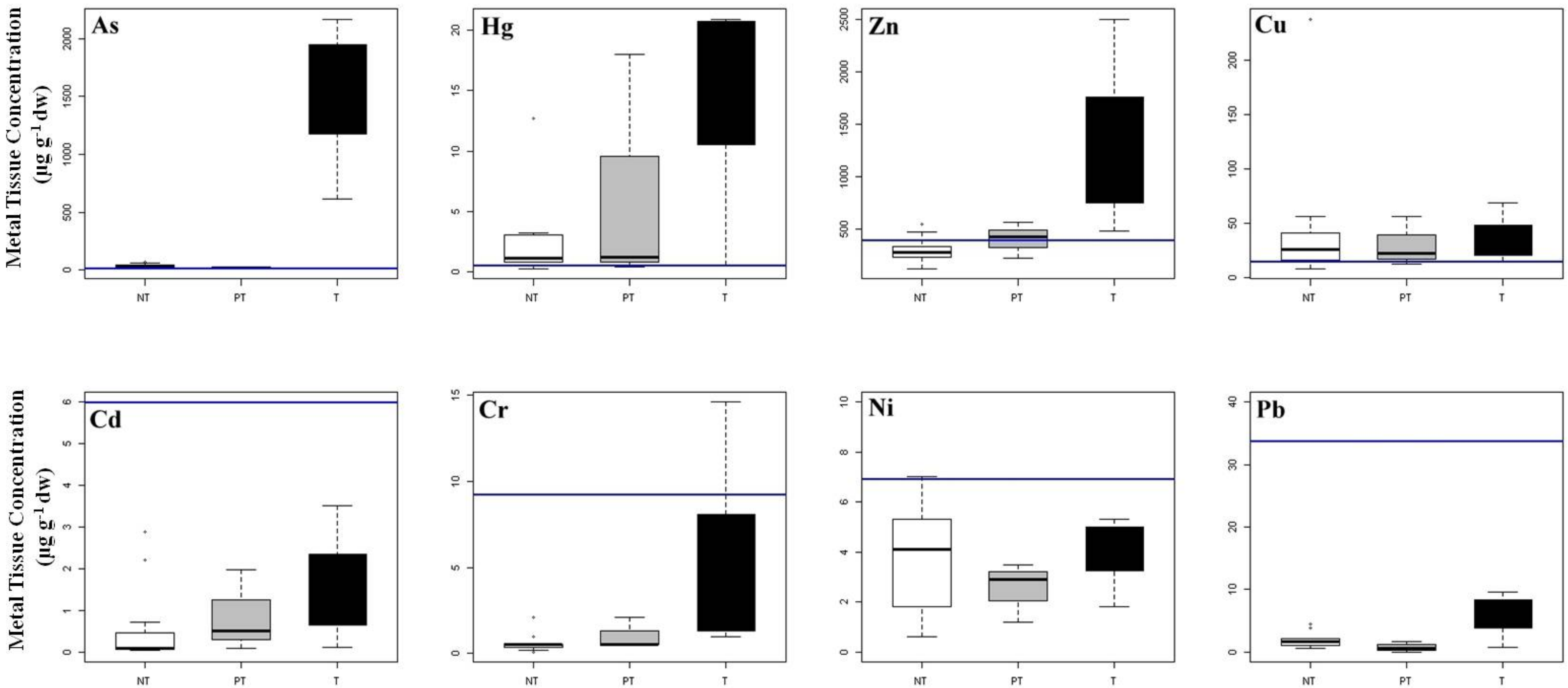

Fig. 2. Boxplots comparing tissue metal concentration (µg g^{-1} dw) in *T. tubifex* after toxicity classification (NT: Non-Toxic, white color, n= 11; PT: Potentially Toxic, grey color, n= 3; T: Toxic, black color, n= 3). Box is built with 25 and 75 percentiles, and show inside the median marked by a bold line. For each metal, their respective baseline value, P90, for field oligochaetes (blue line) is indicated. Open circles indicate sites with extreme data values (over 1.5 times the interquartile range of the data).

Table 1. Sediment metal concentration (μg g^{-1}) at reference sites from several basins in the Cantabrian region. Site codes correspond to those given by the Cantabrian Hydrographical Confederation; except for N12, NO2259, R2 and R4 that were included in the study after expert judgment (see in the text). Sediment Quality Guidelines 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. Abbreviations: B, sediments used for laboratory chronic bioassays with *Tubifex tubifex*; F, Field collected sediments from sites were field worms were collected.

Site	Basin	As	Cd	Cu	Cr	Hg	Ni	Pb	Zn	Year	B/F
MIE002	Miera	4.0	0.14	7.1	9.7	0.02	10.4	12.7	51.6	2008	B
LA001	Nansa	5.3	0.08	8.9	13.2	**0.34**	14.3	19.6	44.9	2008	B
NAN001	Nansa	7.5	0.11	6.7	12.5	0.04	13.1	18.4	47.8	2008	B
NAN002	Nansa	9.0	0.12	8.6	14.7	0.04	16.4	19.9	60.1	2008	B
SB002	Saja-Besaya	**9.9**	0.45	20.1	26.0	0.10	21.0	8.7	29.1	2008	B
SB003	Saja-Besaya	6.8	<D.L.	10.3	9.5	**0.29**	16.3	19.9	42.8	2008	B
SB022	Saja-Besaya	5.5	<D.L.	7.6	9.0	0.11	13.8	17.6	38.6	2008	B
SB017	Saja-Besaya	4.7	0.08	7.5	14.0	0.05	12.7	16.7	38.3	2008	B
NAL011*	Nalón	7.4	0.94	<D.L.	10.9	**4.13**	11.3	19.3	20.5	2011	B
NAL043*	Nalón	7.1	0.81	14.9	23.7	0.10	18.7	9.8	27.6	2011	B
NAL047*	Nalón	**16.8**	0.67	16.1	42.4	0.09	**31.5**	15.0	55.4	2011	B
NAL060*	Nalón	**12.9**	**1.24**	11.4	15.8	**5.29**	**25.1**	17.5	16.5	2011	B
N12	Nalón	**16.1**	0.19	21.8	23.1	**2.92**	**34.8**	20.8	94.8	2014	F
NAL009	Nalón	**17.9**	0.16	21.0	30.2	0.07	**32.6**	25.4	80.8	2014	F
NAL011	Nalón	**15.5**	0.37	**33.9**	35.6	**0.28**	**47.0**	35.7	**135**	2014	F
NAL029	Nalón	**10.1**	0.19	10.5	**105.7**	**0.25**	**60.7**	25.5	58.0	2015	F
NAL031	Nalón	**89.2**	0.94	25.4	**82.4**	0.15	**53.1**	23.7	111	2015	F
NAL038	Nalón	**14.3**	0.33	13.3	**77.0**	**0.19**	**49.8**	22.0	79.0	2015	F
NAL042	Nalón	**14.9**	0.29	25.8	**85.5**	**0.28**	**59.9**	16.4	100	2015	F
NAL043	Nalón	8.1	0.18	17.6	20.5	0.07	16.7	22.3	50.9	2014	F
NAL047	Nalón	**10.3**	0.23	16.8	29.9	0.06	**27.3**	19.2	68.7	2014	F
NAL049	Nalón	**18.5**	0.53	**37.1**	30.5	0.16	**37.0**	27.1	**125**	2014	F
NAL050	Nalón	**12.5**	0.22	20.7	19.2	0.09	**24.1**	20.6	82.8	2014	F
NAL055	Nalón	**16.6**	0.31	**34.4**	38.3	0.16	**39.7**	35.0	**145**	2014	F
NO2259	Nalón	9.7	0.23	18.2	23.7	**0.20**	**33.0**	18.6	83.0	2015	F
R2	Nalón	**12.9**	0.17	27.7	22.5	**0.20**	**37.6**	18.5	101	2015	F
R4	Nalón	**10.7**	0.34	12.9	21.7	0.04	**26.5**	14.0	79.2	2014	F
	TEC	9.8	0.99	31.6	43.4	0.18	22.7	35.8	121		
	PEC	33.0	4.98	149	111	1.06	48.6	128	459		

*Sites corresponding with codes N18r, N1r, N2r and N22r, respectively, in Méndez-Fernández et al 2015.

Table 2. Metal tissue concentrations (μg g^{-1}) in field-collected microdriles and lumbricids in reference sites of the Nalón River basin, and in *T. tubifex* exposed to reference sediments in laboratory bioassays from Nalón R. and other Cantabrian river basins. Abbreviations: sd= standard deviation; min=minimum; max= maximum.

Oligochaetes		**As**	**Cd**	**Cu**	**Cr**	**Hg**	**Ni**	**Pb**	**Zn**
Field Lumbricidae	mean	7.4	2.21	8.7	2.7	0.22	3.3	6.7	188
Nalón R.	sd	3.8	2.02	1.5	1.1	0.11	1.3	6.2	78.0
(n=13)*	median	7.6	1.72	8.5	2.6	0.18	3.0	4.2	182
	min	2.6	0.50	6.8	1.3	0.06	1.7	0.1	85.4
	max	15.8	7.93	12.1	5.2	0.48	6.1	22.0	420
Field Microdriles	mean	8.0	3.70	11.6	6.7	0.37	5.5	20.1	277
Nalón R.	sd	3.8	1.86	5.0	4.1	0.12	2.6	24.1	79.5
(n=13)*	median	6.7	3.58	11.4	6.0	0.37	5.6	7.9	257
	min	3.3	1.61	5.2	2.2	0.11	2.2	1.6	174
	max	14.9	7.16	23.6	17.2	0.62	11.5	77.5	413
Bioassay *T. tubifex*	mean	27.3	1.95	47.8	3.6	1.79	6.0	3.6	839
Nalón R.	sd	8.6	2.22	39.4	4.0	1.79	6.4	3.1	718
(n=4)	median	26.2	1.85	46.7	2.6	1.79	3.8	3.3	817
	min	18.7	0.02	10.8	0.5	0.17	1.2	0.8	202
	max	38.2	4.06	87.0	8.8	3.42	15.3	7.0	1520
Bioassay *T. tubifex*	mean	16.3	0.36	26.8	3.1	n.m.	8.9	14.4	371
Other	sd	8.7	0.21	10.8	1.4	n.m.	5.8	9.2	92.1
Cantabrian rivers	median	14.6	0.30	27.5	3.1	n.m.	7.3	12.7	342
(n=8)	min	7.8	0.10	8.6	0.7	n.m.	4.0	1.6	263
	max	36.6	0.80	44.6	5.4	n.m.	21.5	28.6	519

*Lumbricids were absent in N12 and NAL049, while microdriles were absent in N12 and NAL011.

Table 3. Field baseline tissue concentration (P90) in present study compared with data reported in the literature for field oligochaetes from unpolluted or reference sites. Critical Body Residues (CBR) from field data in [1]Bervoets et al. (2016) and from bioassays in [2]Mendez-Fernandez et al. (2015). Critical Tissue Level (CTL) for the protection of aquatic life from the Oregon Department of Environmental Quality (2007).

Taxa	***Parameters***	***As***	***Cd***	***Cu***	***Cr***	***Hg***	***Ni***	***Pb***	***Zn***
Field oligochaetes in the Nalón R.	**P90**	12.3	5.98	14.3	9.20	0.48	6.90	33.6	381
Oligochaete data in the literature from field worms or bioassays *	**Median**	18.4	1.28	17.7	-	0.21	4.30	15.1	83.5
	P90	50.9	19.7	44.0	-	0.47	23.5	33.8	514.1
	Range	1.10 - 58.5	0.02 - 36.0	5.80 -54.0	.	0.01 - 0.50	1.80 - 31.1	3.30 - 36.8	4.60 -583
	n	4	6	5	-	5	4	9	7
Oligochaete thresholds	**[1]CBR**	85.0	28.0	71.0	24.0	-	8.40	79.0	930
	[2]CBR_{20}	185.8	-	-		6.82		6.42	625
Aquatic life threshold	**CTL**	66.0	1.50	-	-	0.88	-	1.20	-

* See Table S2 in Supplementary Material

Table 4. Variability in the endpoints in *T. tubifex* chronic bioassay from the reference sites studied in the Cantabrian river Basins (Miera, Nansa, Saja-Besaya and Nalón River Basin) and compared with previously reported endpoint variability in reference sites in north Spain (Modified from Rodriguez et al., 2011). Validation of the data resulted in a database of 58 reference sites in north Spain. Abbreviations: SUR: Survival %; TCC= No. of Total Cocoons; ECC= No. of Empty Cocoons; TYG: No. of Total Young; TGR= Total Growth Rate (d^{-1}).

Basin		**SURV**	**TCC**	**ECC**	**TYG**	**TGR**
Cantabrian	mean	92.9	35.8	17.9	157	0.017
n= 12	sd	6.20	5.10	4.20	50.8	0.014
Present study	min	80.0	25.8	13.2	92.2	-0.007
	max	100	42.6	26.0	292	0.036
North Spain	mean	94.2	34.9	11.7	100	0.034
N= 46	sd	8.20	5.70	4.40	63.8	0.012
Rodriguez et al., 2011	min	65.0	21.2	2.00	5.20	0.004
	max	100	45.6	20.0	258	0.061
Total north Spain	mean	93.9	35.1	13.0	112	0.030
n= 58	sd	7.80	5.60	5.00	65.2	0.014
	min	65.0	21.2	2.00	5.20	-0.007
	max	100	45.6	26.0	292	0.061

SUPPLEMENTARY MATERIAL

Table S1. Pearson´s rank correlation between PCoA axes and metal levels in the tissues used as vectors.

Metals	PCo1	PCo2
As	**-0,61**	-0,52
Cd	0,02	**0,86**
Cu	**-0,91**	-0,22
Cr	-0,56	**0,72**
Ni	**-0,74**	-0,07
Pb	-0,41	**0,65**
Zn	**-0,88**	-0,10

Tabla S2. Metal tissue concentrations (μg g^{-1}) in oligochaetes from unpolluted or reference sites from the literature data. When a pool of data are reported in the original source only minimum and maximum values are included in the table, otherwise mean values are showed.

Organisms	As	Cd	Cu	Hg	Ni	Pb	Zn	References
Annelids	-	-	-	0.3-0.5	-	-	-	Eisler, 2000*
Oligochaetes	1.1-59	0.02-0.56	-	0.005-0.055	-	3.46-36.8	-	Protano et al., 2014
	-	3.37-36.0	19.7-54.0	-	5.87-31.1	10.3-20.7	4.56-35.6	Gillis et al., 2006
Tubificids	-	-	9.5-15.7	0.11-0.43	1.8-2.7	5.0-5.1	65.1-83.5	Chapman et al., 1980
	-	-	-	-	-	-	583	Say & Giani, 1981
	-	-	-	-	-	16.0	-	Eisler, 2000*
T. tubifex	3.7-33	0.1-2.0	5.8-34.0	-	-	3.30-33.0	158-468	Sanger & Pusko, 1991

*Assuming 90% water content