Bioaccumulation and chronic toxicity of arsenic and zinc in the aquatic oligochaetes *Branchiura sowerbyi* and *Tubifex tubifex* (Annelida, Clitellata)

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Abstract

Oligochaetes feed on bulk sediment and penetrate the sediment through the construction of burrows, making them especially vulnerable to sediment metal contamination. However, the few oligochaete species that have been tested to date are almost exclusively temperate test species. Although the warmwater adapted species *Branchiura sowerbyi* has been indicated as a promising candidate for tropical sediment toxicity testing, few (especially chronic) studies have been conducted so far to confirm this. Therefore, the aim of the present study was to evaluate the bioaccumulation and chronic 28d lethal and sublethal toxicity of arsenic (As) and zinc (Zn) to both the warmwater-adapted *B. sowerbyi* and the coldwater-adapted oligochaete *Tubifex tubifex* for comparison. Arsenic was more toxic to both oligochaete species than Zn. Inter- and intra-species variability in toxicity values of the two test species and other benthic invertebrates was within an order of magnitude. However, *B. sowerbyi* was the most sensitive species to As even for sediment concentration (EC_{50}: 36.6 ± 2.1 µg/g and 147.1 ± 21.7 µg/g, for *B. sowerbyi* and *T. tubifex*, respectively) and for tissue concentration (ER_{50}: 9.2 ± 0.9 µg/g and 887.0 ± 35.0 µg/g, for *B. sowerbyi* and *T. tubifex*, respectively). Finally, the Tissue Residue-effects Approach (TRA) using Effective Tissue Residues appears to be a promising way forward in advancing in this since it considers internal body concentrations.

Keywords: Ecotoxicology; freshwater macroinvertebrates; metals; sediment-spiked toxicity test; tropics

1. INTRODUCTION

Most aquatic oligochaetes are detritivores, deposit-feeding benthic invertebrates that occupy a variety of microhabitats in the sediments (Rodriguez and Reynoldson, 2011). Tubificid and lumbriculid oligochaete taxa feed on bulk sediment and burrow into the sediment through the construction of galleries that may extent into anoxic layers (Hamburger et al., 2000). Therefore, these organisms are exposed to chemicals via several uptake routes, including direct contact with contaminated sediment particles by ingestion, and by integumentary absorption via porewater and overlying water (OECD, 2008). Since oligochaetes are also easy to maintain under laboratory conditions, standardized protocols for laboratory ecotoxicological testing were developed over the last fifteen years (e.g. ASTM, 2005; OECD, 2007, 2008). However, only a few oligochaete species have been used so far in sediment toxicity and bioaccumulation studies (Rodriguez and Reynoldson, 2011; Méndez-Fernández et al., 2017a). Toxicity test guidelines and research efforts during the last decades of the 20th century focused mostly on the cold water-adapted oligochaetes *Lumbriculus variegatus* (Müller) and *Tubifex tubifex* (Müller). However, in the first decade of the 21st century, toxicity testing guidelines also included protocols for the warm water-adapted species *Branchiura sowerbyi* Beddard (OECD, 2007, 2008) which are thus used in tropical sediment toxicity testing (Lobo and Alves, 2011; Lobo and Espíndola, 2014; Lobo et al., 2016). The limited number of ecotoxicity studies conducted with this species are mostly acute water-only tests, and only a very limited number of chronic toxicity and bioaccumulation studies have been conducted so far (Lobo and Espíndola, 2014; Lobo et al., 2016). This is surprising given that *B. sowerbyi* appears particularly promising to assess the bioaccumulation potential of substances due to its relatively high individual biomass (OECD, 2008; Lobo and Alves, 2011).

Deposit-feeding oligochaete worms play an important role in the bioturbation of freshwater ecosystems through their burrowing activity (Nogaro et al., 2009), which affects the transport of pollutants from the sediment to the water column, and vice versa (Karickhoff and Morris, 1985; Ciutat et al., 2005). Oligochaetes may also serve as prey for other aquatic organisms such as

benthivorous insect larvae, fish and birds (Rodriguez and Reynoldson, 2011; Horváth et al., 2012), thus eventually allowing for biomagnification of toxicants to higher trophic levels (Egeler et al., 2001).

Metals and metalloids (henceforth, jointly referred to as metals) bioaccumulation is a complex and dynamic time-dependent process, and its interpretation may be complicated because some are essential for the metabolism of the organism and their uptake can be actively regulated (Adams et al., 2011; McCarty et al., 2011). In general, the effective dose of a pollutant to the receptor organism comes from multiple routes, such as ingestion through diet and/or sediment, in addition to water (ingestion + uptake through respiratory organs and skin), so that the internal dose in field organisms (tissue residues) is additive across exposure routes (Sappington et al., 2011). Regarding all its complexity (mineral and organic particles, microorganisms, and interstitial water), sediment can be the main source of metal bioaccumulation in benthic organisms (Méndez-Fernández et al., 2014, 2017a), especially through ingestion as an uptake route (Camusso et al., 2012).

In the last two decades, the ‘‘Tissue Residue-effects Approach’’ (TRA: Meador et al., 2011) has been developed to assess the toxic responses using metal tissue residue as a dose metric. According to TRA, a toxic effect is assumed to occur when the excess of metal is present internally in a form that is metabolically available and bound to sites where it can also disrupt biological and biochemical processes (Rainbow, 2002; Adams et al., 2011; McCarty et al., 2011). However, a simple relationship between metal bioaccumulation and toxicity generally does not exist, and factors such as nature of the metals (e.g., essential versus non-essential), bioavailability, or taxa behaviour and detoxification processes, all can contribute to the final observed toxic effects. The suitability of the TRA approach for metals has been disputed since the assumption of proportionality in concentrations among compartments (i.e., exposure *vs*. whole body) is often violated (Adams et al., 2011). However, recent studies have demonstrated the relationships between metal tissue residues in target organisms and measurable toxic effects using both laboratory and

field organisms (Meador et al., 2011; Méndez-Fernández et al., 2013, 2017b; Goulet and Thompson, 2018).

The first aim of the present work was to study the chronic effects of arsenic (As) and zinc (Zn) sediment contamination on the survival, growth and reproduction of the cold water-adapted oligochaete *T. tubifex* and the warm water-adapted species *B. sowerbyi*. The second aim was to assess the chronic risk of As and Zn present in sediments on the basis of their bioaccumulation by both species, through the TRA approach. The selection of As and Zn was motivated by their relevance, both in terms of occurrence and ecotoxicity, in river sediments affected by mining activities in several study areas previously studied by the research teams. The ecotoxicity of these metals has also previously been evaluated by the authors in the same target species, *B. sowerbyi* and *T. tubifex,* but only under acute exposure in water-only tests and under subchronic exposure (14d) to spiked sediments (Lobo et al., 2016). This first study indicated that prolonged exposure to these metals could lead to sublethal effects to these oligochaetes (autotomy of the posterior body parts, abnormal behavior and appearance). Therefore, the present study was initiated as a follow-up study to evaluate the bioaccumulation and a wider range of sublethal effects (growth, reproduction) after a chronic (28d) exposure of the test species to these metals.

2. MATERIALS AND METHODS

2.1. Culture of test organisms and test sediments

The test organisms were obtained from existing in-house cultures at the laboratories where the tests were conducted. The culture of *T. tubifex* had been maintained for over 25 years at the Animal Ecotoxicology and Biodiversity laboratory (University of the Basque Country, UPV/EHU, Bilbao, Spain). This culture was initiated with individuals collected from a mountain stream of the Gorbeia Natural Park (northern Spain). Animal cultures were maintained in complete darkness, at 22 ± 1 ºC, in 2.2-L plastic containers containing a 3-cm sediment layer (natural sediment from an

uncontaminated pond in the mountains of Álava, Iturbatz, Spain, grain particle size < 0.25 mm; organic matter: 3.1 ± 0.5%) and dechlorinated tap water (see below, section 2.2), and were organized in worm cohorts of about 100 juveniles. More details about the culture are provided in Méndez-Fernandez et al. (2013).

The culture of *B. sowerbyi* was maintained at the Center for Water Resources and Applied Ecology (University of São Paulo, São Carlos, São Paulo State, Brazil) at 25 ± 1ºC, in the dark, with moderate aeration. Each 3.5-L culture recipient contained a sediment layer of approximately 5 cm derived from the waterbody alongside a spring in Brotas (São Paulo State, Brazil; grain particle size < 0.25mm; organic matter: 1.7%), reconstituted water (pH = 7.0; EC = 100 µS/cm; total hardness = 40 mg/L as $CaCO_3$), and 80 young worms. More details on the culture of this species are described in Lobo et al. (2016).

The sediments used for the toxicity tests were obtained from sites other than those used in the cultures to assure that they had comparable and low organic matter levels. The test sediments from the Barrundia (Spain) and the Perdizes (Brazil) streams that were used had similar granulometric characteristics: both are sandy (grain particle size < 0.25mm), with 4% silt-clay in Barrundia and 6% fines in Perdizes, had low organic content (1.7% in Barrundia and 0.8% in Perdizes) and had low concentrations of As (2.7 ± 1.4 mg/kg and 5.0 ± 0.7 mg/kg for Barrundia and Perdizes, respectively) and Zn (27 ± 28 mg/kg and 56 ± 25 mg/kg for Barrundia and Perdizes, respectively) (on a dry weight basis). For more details about the characteristics of both sediments, refer to Lobo et al. (2016).

2.2. Experimental design of the chronic toxicity tests

The toxicity tests with *T. tubifex* and *B. sowerbyi* were conducted at the laboratory of Animal Ecotoxicology and Biodiversity of the University of the Basque Country (Spain) and at the Center for Water Resources and Applied Ecology (CRHEA) of the University of São Paulo (Brazil), respectively. The test design was based on the OECD (2007) and ASTM (2005) guidelines. Test

chambers consisted of 250-mL glass beakers, containing 100-mL test sediment and 100 mL water. The water column consisted of dechlorinated tapwater in the *T. tubifex* test with the following physico-chemical conditions: pH = 6.8 ± 0.16, electrical conductivity = 279 ± 3.1 µS/cm, total hardness = 127 mg $CaCO_3$/L. Reconstituted water was used in the *B. sowerbyi* test: pH = 7.4 ± 0.02; electrical conductivity = 129 ± 2.0 µS/cm; total hardness = 40 mg$CaCO_3$/L). The entire 28-d test period was conducted in darkness, under a constant temperature of 22 ± 1 °C (*T. tubifex*) and 25 ± 1 °C (*B. sowerbyi*). It attempted to respect the characteristics of tropical (for *B. sowerbyi*) and temperate (for *T. tubifex*) environmental, and, because of that, some differences at the tests conditions can be observed.

The sediment metal spiking followed the EPS (1995) guideline, with modifications reported in Méndez-Fernández et al. (2013), and the full method used is fully described in Lobo et al. (2016). The salts dissodium arsenate ($HAsNa_2O_4 \cdot 7H_2O$; 98% of purity) and zinc sulfate ($ZnSO_4 \cdot 7H_2O$; 99% of purity) were used in the sediment spiking procedure for As and Zn, respectively. It was added Tetramin® fish food (approximate amount of 80mg per chamber test) as complementary food source during this process. After the one-week equilibration phase in the spiking procedure, as recommended by OECD (2008), the sediment (100 mL) was added to each of the corresponding treatment replicates and topped with 100-mL overlying water. After gently aerating the water column for 48 hours (at test temperature in darkness) to allow sediment and porewater partitioning equilibrium, four worms were added to each test vessel to start the bioassay. Prior to the transfer from the cultures to the test vessels, worms were kept in dilution water for 5h to empty their gut content (Martinez-Madrid et al., 1999), after which their wet weight was measured. Separately, wet-to-dry weight ratios were determined at the beginning of the experiment from 30 worms randomly selected from the cultures of each species, which were 0.107 ± 0.01 for *T. tubifex* and 0.152 ± 0.08 for *B. sowerbyi*. All worms used each test were obtained from the same culture batch (6-7 week old). The weight of the worms at test initiation was 0.97 ± 0.17 mg dw for *T. tubifex* and 3.45 ± 0.63 mg dw for *B. sowerbyi* (values averaged for all tests).

In each bioassay, five sediment metal concentrations were tested (Table 1), with six replicates for each test concentration. Two replicates were used for chemical analysis: one at the beginning and another at the end of the experiments (see section 2.4), while the remaining four replicates were used for biological determinations (toxicity and bioaccumulation; see section 2.3). Test concentrations were chosen based on previously data obtained from 14-d water-sediment tests with the same metals and species (Lobo et al., 2016), and they are expressed as an average value between the initial and final measurements (Table 1; supplementary data S1). Every test also included a control series with non-spiked sediments, the worms and with the same number of replicates (n = 6) as part of the quality assurance/control procedures.

2.3. Endpoints

The chronic bioassays included lethal (survival percentage, SUR) and sublethal (reproduction and growth) endpoints. For reproduction, the following endpoints were measured: number of total cocoons, TCC; number of empty cocoons, ECC; cocoon biomass, CCB (only for *T. tubifex*); number of eggs per cocoon, EgC (only for *B. sowerbyi*); and number of total young, TYG. Regarding growth, the somatic growth rate (SGR) and total growth rate (TGR) were measured (see below). The biomass was always expressed and analyzed on a dry-weight basis. At the end of the 28-d exposure period, the sediment from the test beakers was washed through a 0.50-mm mesh sieve to separate adults and cocoons from the sediment, followed by a 0.25-mm mesh sieve to extract the juveniles. After the completion of the test, the number of dead and living adult worms were counted, the surviving specimens of *T. tubifex* were purged for 5h (Martinez-Madrid et al., 1999), frozen in liquid nitrogen and subsequently stored at -20 °C. Afterwards, the worms were freeze-dried overnight to a constant weight. Somatic weight for *B. sowerbyi* adults was calculated for surviving adults, after purging for five hours and dried at 60°C, for 48h. Cocoons of *T. tubifex* were relatively small (ca. 1 mm diameter) and usually contained many eggs, hampering the quantification of the number of eggs per cocoon (EgC) without breaking the cocoons. Therefore, the

total biomass of the cocoons containing eggs (CCB) was determined for this species placing the cocoons containing eggs from each replicate on preweighted glass microfibre filters (Whatmann® 2.5-cm diameter), which had been dried at 60°C, for 48h. Somatic weight of adults and cocoons were determined on a Sartorius® M3P Electrobalance (accuracy limit: 1 µg) for *T. tubifex*, and on a Mettler AE240 analytical balance (accuracy limit: 1 µg) for adults of *B. sowerbyi.* As compared to *T. tubifex*, the cocoons of *B. sowerbyi* were larger (ca. 3 mm diameter) and transparent, so the number of eggs per cocoon (EgC) could be determined. The juveniles obtained from sieving sediment through a 0.25 mm mesh sieve were fixed with formalin (4%) and stored for posterior quantification on a stereomicroscope (magnification 100x).

The calculation of the growth parameters was done using the following formulas:

$$\text{Somatic daily growth rate (SGR)} = ((\text{Ln}W_2 - \text{Ln}W_1)100)/t$$

$$\text{Total daily growth rate (TGR)} = (\text{Ln}(W_2 + \text{CCB}) - \text{Ln}W_1)100/t$$

Where: W_1 and W_2 are the initial and final biomass (expressed on a dry weight basis), respectively, t = test duration (i.e. 28 days), and CCB is the total cocoon biomass.

Dissolved oxygen and pH of both tests were measured twice a week, and the other physical variables were measured at the beginning and the end of the experiments. In the *B. sowerbyi* test, we used a Yellow Springs YSI-55 probe to measure the dissolved oxygen in the overlaying water, a Micronal B374 electrode for pH, an Orion M145 for electric conductivity, and the spectrophotometry method described in APHA (1995) for ammonia. In the tests conducted with *T. tubifex,* the same parameters were measured with a Thermo Scientific Orion 5-Star Plus multi-parameter meter. In addition, the biota-sediment bioaccumulation factor (BAF) for each metal was calculated as the ratio between the mean worm tissue residues of the metal measured at the end of the toxicity test and the corresponding mean concentration (between initial and final) in the sediment (Egeler et al., 2001).

2.4. Metal analysis

Sediment and porewater concentrations were analyzed individually by separating the porewater from the sediment through centrifugation of 50-mL sediment (4500 rpm, 30 min). Subsequently, porewater was filtered through a 0.45-µm filter (Whatmann®) before chemical analyses, as described below. The solid sediment fraction was dried at ambient temperature and sieved through a 63-µm mesh, before acid digestion. At the end of the toxicity tests, surviving adult worms were purged, freeze-dried and weighed, and then worms were digested at room temperature with nitric acid (70%, Baker Instra-Analyzed) and H_2O_2 (30%, R.P. Normapur Prolabo) at a 10:1 v:v ratio (Clements, 1994). A control series (3 replicates) containing only acid and H_2O_2, and no worms, was also included as a blank in every analytical batch. Internal standards for metal tissue residues (Mussel tissue, NIST 1643e, USA), water (TMDA 52.3) and sediment (Buffalo River sediment, RM8704, NIST, USA) were also included as reference materials.

Chemical analyses of arsenic and zinc in the overlaying and porewater of the *T. tubifex* test were made by Inductively Coupled Plasma-Atomic Emission Spectrometers (ICP-AES; Limits of Quantification, LOQ= 0.05 mg As/L and 0.1 mg Zn/L) and by Inductively Coupled Plasma Mass Spectrometry (ICP-MS; LOQ= 0.3 µg As/L and 5 µg Zn/L) on the SGIker from the UPV/EHU. Acid digestion of the sediment was performed according to the USEPA 3052 (USEPA, 1996) and UNE-EN 13656 (UNE, 2003) procedures (9 mL HNO_3 65 % and 4 mL HF were added to 0.2 g of sediment). Sediment and tissue metal concentrations in the *T. tubifex* tests were measured at the SOSPROCAN unit (University of Cantabria, Spain) by ICP-MS (LOQ= 0.3 µg As/L and 5 µg Zn/L). Analytical recovery rates were 87% for Zn in the Buffalo sediment (no reference data for As); recovery rates for As and Zn tissue concentrations were 85% and 103%, respectively.

Analytical quantification of the metal concentrations in porewater, worms and sediment from the *B. sowerbyi* tests was performed at the Poços de Caldas laboratory (LAPOC) of the National Nuclear Energy Commission (CNEN) in Brazil. Water samples were analyzed by Hydride Generation Atomic Absorption Spectroscopy (HG-ASS) for As (LOQ = 0.02 µg/L) and Flame Atomic Absorption Spectrometry (F-AAS) for Zn (LOQ = 6 µg/L); recoveries from reference water

(NIST 1643e) were 107% for As and 103% for Zn. Sediment was digested following USEPA 3052 (USEPA, 1996), and analyses performed though HG-AAS for arsenic and F-AAS for zinc; the recovery rate attained for the Buffalo river standard sediment (RM8704, NIST, USA) was 90% for zinc (no reference data for As).

2.5. Statistical analysis

Statistical analyses were conducted using mean sediment concentrations (i.e., the mean of the concentrations at the start and end of the tests, supplementary data S1). Survival was analyzed with the Fischer's Exact test. The Shapiro-Wilk's test was conducted to test for normality of the data distribution. Subsequently, statistical differences between treatments and controls were evaluated by ANOVA followed by the Dunnett´s *t* test for normal-distributed data or the Kruskal-Wallis test followed by the Dunn's test for data that did not pass the Shapiro-Wilk's test for normality. These statistical analyses were conducted using the free R software (R Core Team, 2013) and extension package *multicomp* (Hothorn et al., 2008). The significance level for rejection of the null hypothesis was 0.05.

Median lethal concentrations (mortality; $LC_{50,}$) and median effect concentrations (growth or reproduction; $EC_{50,}$), as well as median Lethal and Effect body Residues (LR_{50}, ER_{50}) for As and Zn were estimated from non-linear regression models, using the free R software (R core team, 2013) in combination with the extension package *drc* (Ritz and Streibeig, 2005). The best fitted model (among the 14 non-linear dose–response regression models tested) was selected using Akaike's Information Criterion (AIC) and its validation was based on graphical assessment (Burnham and Anderson, 2002; Zuur et al., 2007) and the results of the goodness-of-fit (assessed by R^2) and the lack-of-fit (*p* value, with 0.05 of significance) tests, both included in the drc package (Ritz and Streibeig, 2005). The LC and EC values were calculate based on the metal' concentration in the sediment (instead of porewater), once ingestion is the principal route of exposition of these species (Camusso et al., 2012; Mendez-Fernández et al. 2014, 2017a).

3. RESULTS

3.1. Quality Assurance and Quality Control

No statistically significant differences in physical-chemical variables were found within each treatment (i.e., between the replicates of the same treatment) along the toxicity tests, comparing data form day 1 and day 28. Dissolved oxygen and pH in the water column in the As tests were: 7.7 ± 0.39 mg/L and 8.2 ± 0.18 in the *T. tubifex* test and 6.6 ± 0.55 mg/L and 7.8 ± 0.26 in the *B. sowerbyi* test; corresponding values for the Zn tests were: 7.73 ± 0.24 mg/L and 8.26 ± 0.21 (*T. tubifex*) and 6.98 ± 0.30 mg/L and 7.65 ± 0.28 (*B. sowerbyi*). The coefficients of variation (CV%) were lower than 10%, except for ammonia, that had a concentration of 6.2 mg/L in the water column corresponding to the 97 µg As/g treatment in the *T. tubifex* test on the first day and dropped to zero at the end of the experiment (mean values for ammonia in As and Zn tests respectively: 2.45 ± 2.63 mg/L and 1.42 ± 1.68 for *T. Tubifex* and 0.21 ± 0.1 mg/L and 0.37 ± 0.27 mg/L for *B. sowerbyi*). Despite this, test conditions appeared to be adequate in the *T. tubifex* toxicity test as confirmed by the control treatments performance, without mortality or sublethal effects (Table 1).

3.2. Chronic toxicity

At high As concentrations, the survival of both species was reduced (Table 1), resulting in a LC_{50} of 189 ± 41 µg As/g and 102.87 ± 27.16 µg As/g for *T. tubifex* and *B. sowerbyi*, respectively (Table 2, supplementary data S2). The reproduction of *T. tubifex* was impaired ($p < 0.05$) at 179.50 µg As/g, as evidenced by the number of empty cocoons (ECC) and, consequently, by the number of juveniles (TYG; Table 1). It is also interesting to point out that one juvenile with a bifid posterior region was observed at 55 µg As/g. *B. sowerbyi* did not reproduce at all in the test evaluating As (Table 1), even in the control group, so it was not possible to assess the possible effects of As exposure on the reproduction of this species. However, the inhibition in growth (SGR) was significant ($p < 0.001$) for the worms exposed to concentrations greater than 42 µg As/g.

305 The presence of Zn in the sediment did not have any significant adverse effects on any of
306 the lethal or sublethal endpoints evaluated for either species at the concentrations tested (Table 1).
307 However, a tendency toward an increase in reproduction and growth was observed for several
308 endpoints at concentrations below 253 µg Zn/g for *B. sowerbyi* and below 1801 µg Zn/g for *T.*
309 *tubifex*. Individuals of *T. tubifex* exposed to concentrations of 725 and 1204 µg Zn/g appeared to
310 produce more cocoons with a greater biomass than in other treatments or the control, although this
311 was not statistically significant.

312 **Table 1**. As and Zn lethal and sublethal endpoints (mean ± SD) observed at the 28d-chronic bioassays in spiked sediments, with *B. sowerbyi* and *T.*
313 *tubifex*.
314

Metal	SED	SUR	TCC	ECC	EgC	CCB	TYG	SGR	TGR
B. sowerbyi									
As	2.66 (c)	100	0	0	-	-	0	0.016 ± 0.005	-
	15.52	100	0	0	-	-	0	0.015 ± 0.003	-
	21.42	100	0	0	-	-	0	0.014 ± 0.002	-
	30.74	100	0	0	-	-	0	0.011 ± 0.002	-
	42.20	100	0	0	-	-	0	0.005 ± 0.001***	-
	108.00	35.0 ± 19.1***	0	0	-	-	0	-0.001 ± 0.002***	-
Zn	34.40 (c)	100	3.7 ± 4.3	0.00	0.8 ± 0.5	-	0.00	0.017 ± 0.003	-
	82.20	100	5.7 ± 7.5	0.5 ± 0.6	1.3 ± 0.4	-	0.2 ± 0.5	0.024 ± 0.006	-
	103.40	100	8.5 ± 9.1	0.5 ± 1.0	1.2 ± 0.2	-	0.2 ± 0.5	0.022 ± 0.003	-
	156.40	100	9.7 ± 8.7	1.2 ± 1.2	1.2 ± 0.3	-	0.5 ± 0.6	0.022 ± 0.002	-
	242.20	100	12.2 ± 10.1	1.7 ± 2.1	1.4 ± 0.3	-	0.7 ± 1.0	0.022 ± 0.006	-
	253.40	100	7.7 ± 7.6	1.0 ± 1.4	1.2 ± 0.2	-	0.7 ± 1.0	0.022 ± 0.005	-
T. tubifex									
As	8.38 (c)	100	40.8 ± 3.3	25.5 ± 1.3	-	1.0 ± 0.2	198.5 ± 54.0	0.009 ± 0.006	0.030 ± 0.005
	17.55	100	43.5 ± 6.9	25.5 ± 6.5	-	0.9 ± 0.2	189.2 ± 69.4	0.008 ± 0.002	0.028 ± 0.003
	27.55	100	39.3 ± 6.0	24.5 ± 4.4	-	0.9 ± 0.2	174.5 ± 57.7	0.009 ± 0.001	0.029 ± 0.003
	54.85	100	37.3 ± 15.0	22.3 ± 10.2	-	0.8 ± 0.3	180.0 ± 119.3	0.004 ± 0.013	0.021 ± 0.006
	97.30	100	36.3 ± 6.9	21.3 ± 6.8	-	0.8 ± 0.3	184.7 ± 66.0	0.005 ± 0.003	0.024 ± 0.008
	179.50	68.8 ± 23.9**	28.5 ± 12.1	9.5 ± 6.2*	-	0.6 ± 0.2	45.5 ± 57.1*	0.006 ± 0.006	0.021 ± 0.003
Zn	177.30 (c)	100	37.5 ± 3.0	22.0 ± 1.4	-	0.7 ± 0.1	153.3 ± 16.0	0.009 ± 0.005	0.026 ± 0.005
	329.70	100	34.8 ± 5.3	19.3 ± 2.6	-	0.7 ± 0.3	174.3 ± 19.2	0.010 ± 0.010	0.026 ± 0.005
	463.50	93.8 ± 12.5	38.5 ± 4.2	23.0 ± 3.2	-	0.9 ± 0.1	193.0 ± 79.9	0.007 ± 0.008	0.028 ± 0.005
	724.90	100	40.5 ± 4.1	22.0 ± 5.5	-	1.1 ± 0.3*	198.3 ± 43.9	0.013 ± 0.005	0.036 ± 0.001
	1203.50	100	39.5 ± 3.9	22.0 ± 2.9	-	1.2 ± 0.1**	209.5 ± 74.4	0.016 ± 0.008	0.039 ± 0.008*
	1805.50	100	32.9 ± 3.9	17.0 ± 2.9	-	0.9 ± 0.1	183.0 ± 53.4	0.004 ± 0.006	0.023 ± 0.004

315 *Abbreviations: SED* real sediment concentration (µg/g dw); *SUR* percentage of survival; *TCC* total number of cocoons; *ECC* number of empty cocoons; *EgC* number of
316 eggs per cocoon; *CCB* average biomass of total cocoons (mg); *TYG* total of youngs; *SGR* somatic growth rate (day^{-1}); *TGR* Total growth rate (day^{-1}); Significant
317 difference from the control (c): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
318

Table 2. Lethal (LC) and Effect (EC) Concentrations values of the best-fitted models, based on sediment concentration, for *B. sowerbyi* and *T. tubifex* after 28d-chronic bioassays with As and Zn.

Species	Metal	Endpoint	Best fitted model	LC_{10}/EC_{10} ± SE (µg/g)	LC_{50}/EC_{50} ± SE (µg/g)
B. sowerbyi	As	SUR	LL.2	86.54 ± 103.03	102.87 ± 27.16
		SGR	W1.3	22.13 ± 4.46	36.61 ± 2.14
		TCC, ECC, EgC, TYG	-	*nd*	*nd*
	Zn	All	-	*nd*	>253.40
T. tubifex	As	SUR	LL.2	163.58 ± 60.50	189.15 ± 40.55
		TCC	LN.3	96.10 ± 35.90	254.52 ± 81.34
		ECC	W2.3	87.03 ± 15.01	149.35 ± 17.50
		TYG	LN.3	104.27 ± 31.81	147.06 ± 21.73
		CCB, SGR, TGR	-	*nd*	>179.50
	Zn	All	-	*nd*	>1805.52

SUR percentage of survival; *TCC* Total number of cocoons; *ECC* number of empty cocoons; *EgC* number of eggs per cocoon; *CCB* average biomass of total cocoons; *TYG* total of youngs; *SGR* somatic growth rate (day^{-1}); *TGR* Total growth rate (day^{-1}). For the models: *LL.2* Log-logistic with 2 parameters; *LN.3* Log-normal with 3 parameters; *W1.3, W2.3* Weibull with 3 parameters; *nd* not determined due the lack of effects.

3.3. Bioaccumulation and Tissue Residue Approach

The tissue concentration of As and Zn was positively related to the metal concentrations in the sediment, showing dose-dependent functions (Fig. 1). The highest metal body concentrations recorded were as follows: 937 ± 219 µg As/g and 1818 ± 368 µg Zn/g for *T. tubifex* and 14 ± 2.1 µg As/g and 207 ± 73 µg Zn/g for *B. sowerbyi* (Table 3). In *T. tubifex*, As uptake gradually increased with increasing As sediment concentration, without clearly stabilizing within the range of concentrations in the test (Fig. 1). In *B. sowerbyi*, however, As uptake increased rapidly at lower test concentrations to attain a steady value (no increase in tissue residue concentration between 42 to 108 µg As/g exposure in sediment; Fig. 1). In the case of Zn, *T. tubifex* revealed internal regulation up to about 1000 µg Zn/g sediment, while in *B. sowerby,* the model showed a poor adjustment (R^2= 0.39) and no regulation of the Zn uptake could be noted (Fig. 1). The As-BAF was high in *T. tubifex,* up to 8.0 at intermediate sediment concentrations; contrarily, the As-BAF in *B. sowerbyi* was below 1, attaining a maximum of 0.34 (Table 3).

The median As-Lethal Body Residue (LBR_{50}) value of *T. tubifex* was 1,002 ± 55 µg/g, whereas As- LBR_{50} of *B. sowerbyi* was more than 50 times lower (17 ± 2.1 µg/g) (supplementary data S3). Fig. 2 show the relationship between the reproduction and growth parameters with the As tissue residues. The estimated Effect Residue (ER_{50}) values for *B. sowerbyi* calculated for growth were about half the LBR_{50} value, while in *T. tubifex* both parameters had comparable values. Zinc did not have adverse effects at any of the concentrations tested for either species, thus it was not possible to calculate the LC_{50}, EC_{50}, LBR_{50} and ER_{50} values.

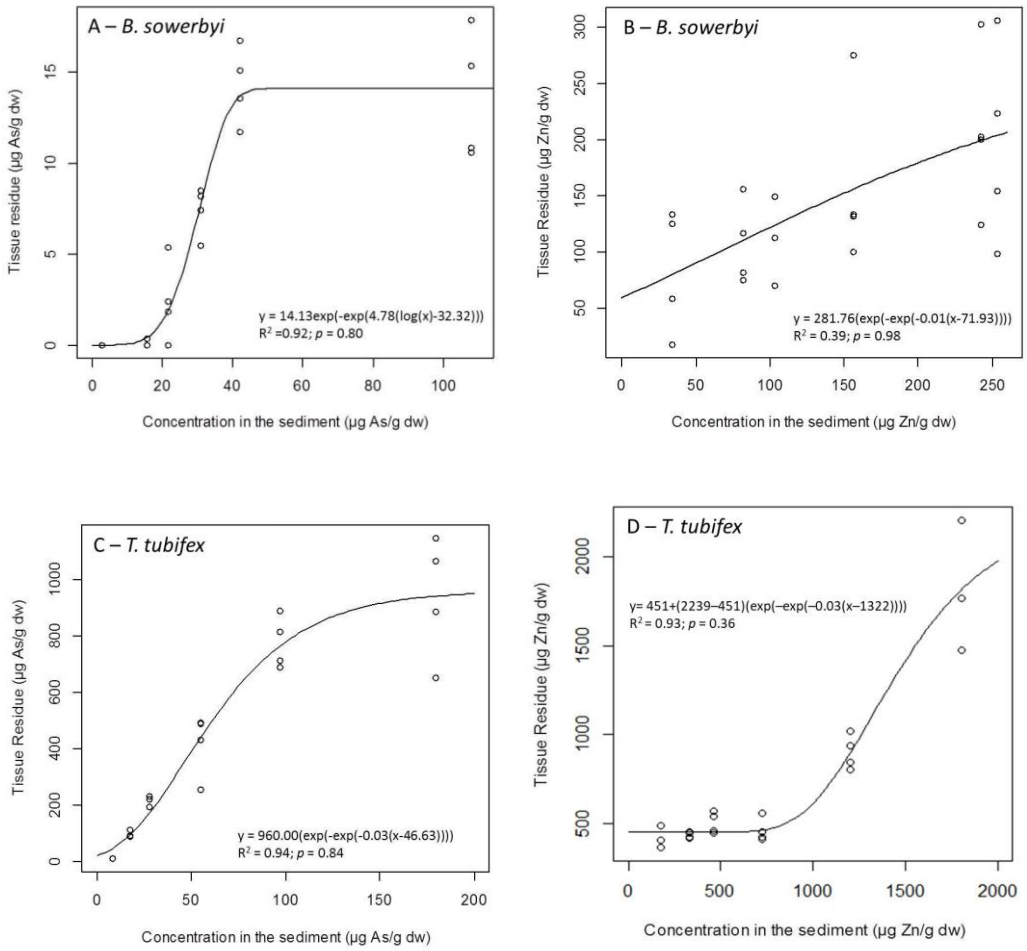

Fig. 1. Relation between the metal concentration in the sediment and the tissue-residue concentrations of the test species: **A**. As - Weibull model with 3 parameters; **B** Zn - Gompertz model with 3 parameters **C.** As - Gompertz model with 3 parameters; **D**. Zn - Gompertz model with 4 parameters.

Table 3. Tissue Residue (TR) (mean ± SD) measured at each sediment dose of As and Zn (mean values from the beginning and the end of the test) at the 28d-chronic bioassays with *B. sowerbyi* and *T. tubifex* and their respective bioaccumulation factors (BAF = tissue residue/sediment concentration).

	Arsenic			Zinc		
Specie	**Sediment (µg/g dw)**	**Tissue residue (µg/g dw)**	**BAF**	**Sediment (µg/g dw)**	**Tissue residue (µg/g dw)**	**BAF**
B. sowerbyi	2.66 (c)	0.00	0.00	34.40 (c)	83.18 ± 55.45	2.46
	15.52	0.09 ± 0.18	0.01	82.20	106.19 ± 37.25	1.31
	21.42	2.40 ± 2.23	0.11	103.40	119.98 ± 37.75	1.16
	30.74	7.38 ± 1.36***	0.24	156.40	160.01 ± 78.42	1.02
	42.20	14.30 ± 2.14***	0.34	242.20	207.24 ± 73.28	0.86
	108.00	13.69 ± 3.55***	0.11	253.40	195.34 ± 89.83	0.77
T. tubifex	8.38 (c)	8.99 ± 0.67	1.07	177.30 (c)	415.04 ± 51.34	2.34
	17.55	95.72 ± 11.14	5.45	329.70	434.70 ± 16.97	1.32
	27.55	213.96 ± 20.05	7.77	463.50	502.34 ± 61.04	1.08
	54.85	414.89 ± 110.96***	7.56	724.90	461.42 ± 66.64	0.64
	97.30	775.29 ± 92.14***	7.96	1203.50	900.87 ± 96.35	0.75
	179.50	937.40 ± 219.35***	5.22	1805.50	1818.12 ± 367.85**	1.01

Significant difference from the control (c): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

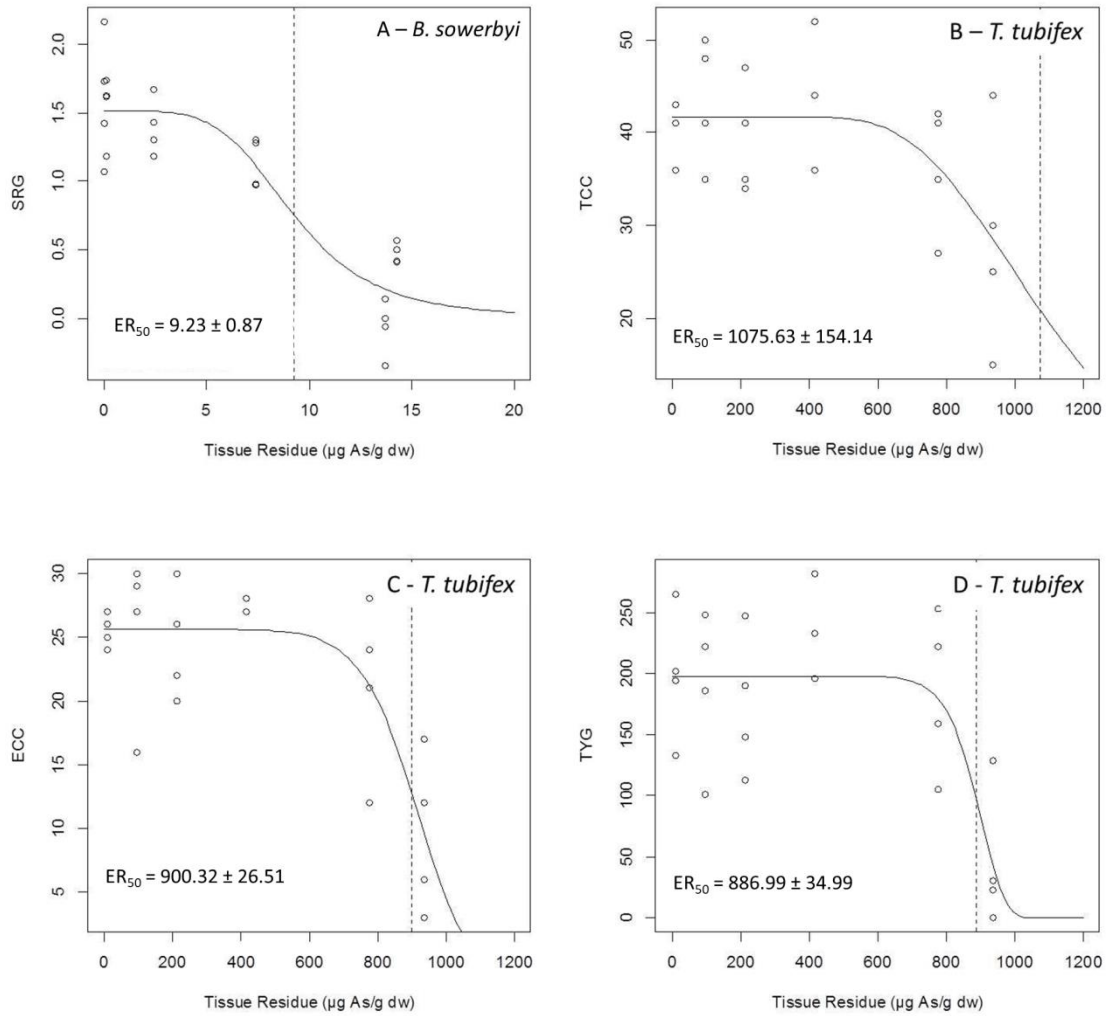

Fig. 2. (**A**) Relation between the Somatic Growth Rate (SGR) and the tissue residue concentration of As in *B. sowerbyi;* (**B**) relation between the Total of Cocoons (TCC), (**C**) Empty Cocoons (ECC) and (**D**) Total of Young (TYG) and the Tissue Residue of As in *T. tubifex*, and the vertical dashed line represents the ER_{50} calculated from the corresponding models (**A**) Log-logistic with 3 parameters; (**B**) Log-normal with 3 parameters and (**C** and **D**) Weibull-1 with 3 parameters.

4. DISCUSSION

Bioassays assessing the toxicity of sediments contaminated with As or Zn in tropical regions are still incipient, which makes it difficult to compare our results. However, several authors have reported As toxicity data from laboratory experiments using various mostly temperate benthic organisms (Martinez et al, 2006; Liber et al, 2011; Lobo et al. 2016). From the literature review conducted for toxicity endpoints in benthic taxa (Table 4), it may be deducted that the overall order of As sensitivity (in decreasing order of sensitivity) was: *B. sowerbyi* > *T. tubifex* = *Chironomus*

373 *tentans* > *Chironomus dilutes* > *Hyalella azteca*. Given the higher toxicity of As to *B. sowerbyi*, it
374 may be questioned whether the sensitivity of these temperate species are representative for tropical
375 benthic organisms. On the other hand, despite the differences in the experimental design including
376 test duration and, hence, metal speciation in the data listed in Table 4, all the As-LC_{50} and EC_{50} for
377 both oligochaetes species are generally within one order of magnitude. Subsequently, the
378 uncertainty factors that are usually applied to temperate toxicity data (factor 100 to acute and factor
379 10 to chronic data; EFSA, 2015) may suffice to protect tropical species. Regarding Zn, no statistical
380 adverse effects were denoted at any of the test concentrations evaluated in the present study on both
381 species (Table 1). Based on the subchronic exposure evaluated in our previous study (Lobo et al.,
382 2016), *B. sowerbyi* was approximately three times more sensitive than *T. tubifex*.
383
384 **Table 4**. Literature review of LC_{50} and EC_{50} for As and Zn for sediment exposure in other benthic organisms.
385 Values showed as mean ± sd or mean (95% CL).

Species	Endpoint	Value (µg/g dw)	Reference
Arsenic			
Hyalella azteca	10d-EC_{50} (growth)	> 462	Liber et al. (2011)
	10d-LC_{50}	532 (495-557)	Liber et al. (2011)
Chironomus dilutus	10d- EC_{50} (growth)	342 (317-362)	Liber et al. (2011)
	10d-LC_{50}	642 (561-736)	Liber et al. (2011)
Chironomus tentans	50d-clear effects on growth and development[a]	130	Martinez et al. (2006)
T. tubifex	14d-LC_{50}	251 ± 47	Lobo et al. (2016)
	28d-EC_{50} (growth)	> 179.50	This study
	28d-EC_{50} (reproduction)	147.06 ± 21.73	This study
	28d-LC_{50}	189.15 ± 40.55	This study
B. sowerbyi	28d-EC_{50} (growth)	36.61 ± 2.14	This study
	28d-LC_{50}	102.87 ± 27.16	This study
Zinc			
B. sowerbyi	14d-LC_{50}	280 ± 2.3	Lobo et al. (2016)
	28d-NEC (survival)	2023.5 (1806.0 – 2173.0)	Ducrot et al. (2010)
	28d-NEC (growth)	1021.0 (737.8 – 1254.0)	Ducrot et al. (2010)
T. tubifex	14d-LC_{50}	>679.0	Lobo et al. (2016)
	14d-EC_{50} (autotomy)	635 ± 25	Lobo et al. (2016)

386 [a] - Statistical significance and endpoints not provided

No reproduction for As and very low reproduction levels for Zn occurred in the *B. sowerbyi* tests, which was probably due to a low level of maturity of the test worms. In a previous study on the reproductive cycle of this species under laboratory conditions the time to reach sexual maturity was established at 41 ± 7 days (Lobo and Alves, 2011). Since animals used in the present study were 6-7 weeks of age, reproduction was therefore anticipated, although a longer time to first reproduction for this species has also been reported (i.e., 57-62 days: Ducrot et al., 2007). Therefore, the age of the test worms should be better evaluated in order to optimize the reproduction in the sediment bioassays with the species.

In the case of Zn, Lobo et al. (2016) calculated a 14-d LC_{50} of 280 ± 2.3 μg Zn/g for *B. sowerbyi*, therefore, it was expected to find effective concentrations within the range of concentrations studied, but the essential nature of this element might be the cause of the lack of sublethal effects. In the present study, no significant deleterious effects on *B. sowerbyi* reproduction were observed even at the highest tested sediment concentration of 253 μg Zn/g. Only significant increases in cocoon biomass (CCB) and growth rates (TGR) were measured for *T. tubifex* at sediment concentrations of 725 and 1204 μg Zn/g. This increase in growth and reproduction at low to intermediate sediment concentrations for *T. tubifex* may suggests a hormetic response of the oligochaete to these Zn concentrations. Hormesis is a physiological stimulatory effect when compared to control levels (Calabrese, 2008). In the literature, similar responses have been reported for the essential metal Cu in *T. tubifex* after sediment exposures (Méndez-Fernández et al., 2013). In other study with spiked natural sediments Ducrot et al., (2010) found that Zn also did not cause: i) significant mortality to *B. sowerbyi* adults, even at high concentrations (3,317 μg Zn/g); ii) deleterious effects in juveniles at concentrations below 1,819 μg/g (28d bioassays); or iii) effects on reproduction at concentrations lower than 1,651 μg Zn g^{-1}.

According to Marchese et al. (2008), metal tissue concentrations of organisms reflect the bioavailability of these compounds in the environment. However, whether the metal of concern is

413 an essential or non-essential metal should be considered, since uptake of essential metals can be
414 actively regulated (Adams et al., 2011). This Zn regulation can be noted for *T. tubifex*, which
415 showed a constant tissue residue up to 1000 µg/g (Fig. 1). *B. sowerbyi* exposed to both metals
416 through spiked sediment hardly accumulated the metals in their bodies (Tables 3 and 5). For
417 example, BAF values decreased from 2.5 in controls to around 1 in the lower Zn treatments to 0.77
418 in the highest Zn sediment concentration (Table 4). This could be due to the regulation of essential
419 metals like Zn. Arsenic also was hardly accumulated in *B. sowerbyi*, with a maximum BAF of 0.34
420 (Table 2) and a maximum average tissue concentration of 14.3 µg As/g (Table 5). In contrast, As
421 was bioaccumulated in *T. tubifex* following sediment As exposure, with the BAF increasing from
422 1.1 in controls to 5 to 8 in the As treatments (Table 3). A similar model of bioaccumulation has been
423 described for other non-essential toxic metals. Méndez-Fernández et al. (2013), for example,
424 observed a significant increase in Cd tissues residues of *T. tubifex* exposed to increased Cd sediment
425 concentrations, with BAF values up to 42. Similarly, Alves et al. (2016) also reported a positive
426 correlation between bioaccumulation and the As test concentration by the terrestrial oligochaete
427 *Eisenia andrei* in a 28d-exposure test, with *E. andrei* body As concentrations of up to 60 times the
428 As concentration in artificial soil and 3.6 times of that in natural soil. From the literature data
429 revised for bioaccumulation of As and Zn in benthic organisms (Table 5), *T. tubifex* showed the
430 highest tissue concentration for As and Zn among all the species listed. It's also interesting to notice
431 that only Goulet and Thompson (2018) and the present study made experiments with spiked
432 sediment to test the bioaccumulation of these metals, highlighting the scarcity of this kind of data.
433 As indicated by their BAF values, *T. tubifex* accumulated more As than *B. sowerbyi*, this
434 might be due to (1) the more efficient mechanisms of elimination of As from body of *B. sowerbyi*,
435 compared with *T. tubifex*, at the expense of a large energy expenditure reflected in the growth rate
436 (Table 1), probably due to the autotomy of the posterior body fragment as part of this process
437 (Paris-Palacios et al., 2010); or due to (2) a better detoxification process by *T. tubifex,* that allows
438 this species to withstand high concentration of this metal at its body without adverse effects, as

439 observed by Goulet and Thompson (2008) for the amphipod *H. azteca*. This is an important issue
440 related to risk assessment for other species through trophic transfer, and these two hypotheses
441 should be tested in future works. For instance, the tissue residue concentrations of *B. sowerbyi* was
442 lower than in *T. tubifex* in present study but also than those observed for *L. variegatus* by Winger et
443 al. (2000) and Camusso et al. (2012), even though *B. sowerbyi* was exposed to concentrations three
444 times higher than *L. variegatus* (Table 5). Thus, further research is needed to understand the
445 underlying mechanisms for their high or low sensitivity of these two deposit-feeders in ecological
446 toxicity assessments of metals, both in temperate and tropical environments.

447

448 **Table 5.** Some bioaccumulation data of arsenic and zinc from the sediment available in the literature.

449

Taxon	Test type (range of concentration µg g^{-1} dw)	Range of Tissue concentration (µg g^{-1} dw)	Reference
Arsenic			
H. azteca	10-d exposure to spiked sediment (5 – 324)	0.7 – 7.1	Goulet and Thompson (2018)
L. variegatus	28-d exposure to dredge spoil sediments (6.6 – 32.8)	4.1 – 26.7	Winger et al. (2000)
L. variegatus	28-d exposure to field collected sediments (170 - 186)	3.6 – 362 .0	Lyytikainen et al. (2001)
L. variegatus	28-d exposure to field collected sediments (1.3 – 32.0)	2.1 – 30.0	Camusso et al. (2012)
T. tubifex	28-d exposure to potentially toxic and toxic sediments (6.9 - 5321)	11.2 -2165.2	Mendez-Fernandez et al. (2015)
T. tubifex	28-d exposure to spiked sediment (8.4 – 179.5)	9.0 – 937.4	Present study
B. sowerbyi	28-d exposure to spiked sediment (15.5 – 108.0)	0.1 – 14.3	Present study
Zinc			
L. variegatus	28-d exposure to dredge spoil sediments (61 - 126)	430 – 956	Winger et al. (2000)
L. variegatus	28-d exposure to field collected sediments (36.5 – 26,152)	163.5 – 375.7	Camusso et al. (2012)
Tubifex sp	Field worms from sites polluted by sewage and industrial wastes (300 – 2055)	60.2 – 166.6	Singh et al. (2007)
T. tubifex	28-d exposure to sediments from sites with elevated metal concentration (30.1 – 1728.6)	297.5 – 514.5	Gills et al. (2006)
T. tubifex	28-d exposure to potentially toxic and Toxic sediments (9.9 - 266)	117.0 – 2497.5	Méndez-Fernández et al. (2015)
T. tubifex	28-d exposure to sediment spiked (177.3 – 1805.5)	415.04 – 1818.12	Present study
B. sowerbyi	28-d exposure to sediment spiked (34.4 – 253.4)	83.18 – 207.24	Present study

450
451

452 The LC_{50} values greatly vary among and within studies, even when considering the same
453 species (Tables 2 and 4). However, the tissue residue approach (TRA) provides reliable results
454 relating metal bioaccumulation to measurable effects at population level, as described by several

455 authors (Meador, et al., 2011; Penttinen et al., 2011). Using the TRA, we calculated the ERs for As
456 based on the causal relationships between tissue residues and sublethal responses (growth and
457 reproduction), providing data for future development of criteria to protect freshwater communities
458 from As pollution. The lack of available Zn toxicity data for benthic invertebrates exposed to spiked
459 sediment reinforces the need for future studies with this metal.
460
461 **5. CONCLUSIONS**
462 Particle-feeding oligochaetes are exposed to chemicals via various uptake routes, i.e.
463 through contact with, and ingestion of, contaminated sediment particles, besides tegumentary
464 diffusion via porewater and overlying water. Oligochaetes have therefore attracted increasing
465 attention as test organisms for sediment quality assessments in both the scientific and Regulatory
466 fields. This study demonstrated that both species are susceptible to metal exposure through the
467 sediment. Although derived and published toxicity LC50 and EC50 values of As and Zn were
468 generally within an order of magnitude, sensitivity of benthic organisms appear to vary largely
469 between and within studies and test species. The ‘‘Tissue Residue-effects approach’’ (TRA) appears
470 to be a promising way to overcome these differences and to go forward ecotoxicological
471 assessments which include metal bioavailability and elimination processes.
472 This present paper and a previous paper by the authors (Lobo et al., 2016) confirm *B.*
473 *sowerbyi* as a sensitive representative and a logistically suitable test species for tropical sediment
474 toxicity assessments. Basic research is still needed to better understand the reproductive aspects, the
475 underlying mechanisms of observed differences in metal availability, detoxification and efflux
476 mechanisms in this species. In addition, a test guideline should be developed for this species that
477 could be based on the methodology applied by the authors in this and our previous study.
478
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489

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Bioaccumulation and chronic toxicity of arsenic and zinc in the aquatic oligochaetes *Branchiura sowerbyi* and *Tubifex tubifex* (Annelida, Clitellata)

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Abstract

Oligochaetes feed on bulk sediment and penetrate the sediment through the construction of burrows, making them especially vulnerable to sediment metal contamination. However, the few oligochaete species that have been tested to date are almost exclusively temperate test species. Although the warmwater adapted species *Branchiura sowerbyi* has been indicated as a promising candidate for tropical sediment toxicity testing, few (especially chronic) studies have been conducted so far to confirm this. Therefore, the aim of the present study was to evaluate the bioaccumulation and chronic 28d lethal and sublethal toxicity of arsenic (As) and zinc (Zn) to both the warmwater-adapted *B. sowerbyi* and the coldwater-adapted oligochaete *Tubifex tubifex* for comparison. Arsenic was more toxic to both oligochaete species than Zn. Inter- and intra-species variability in toxicity values of the two test species and other benthic invertebrates was within an order of magnitude. However, *B. sowerbyi* was the most sensitive species to As even for sediment concentration (EC_{50}: 36.6 ± 2.1 µg/g and 147.1 ± 21.7 µg/g, for *B. sowerbyi* and *T. tubifex*, respectively) and for tissue concentration (ER_{50}: 9.2 ± 0.9 µg/g and 887.0 ± 35.0 µg/g, for *B. sowerbyi* and *T. tubifex*, respectively). Finally, the Tissue Residue-effects Approach (TRA) using Effective Tissue Residues appears to be a promising way forward in advancing in this since it considers internal body concentrations.

Keywords: Ecotoxicology; freshwater macroinvertebrates; metals; sediment-spiked toxicity test; tropics

1. INTRODUCTION

Most aquatic oligochaetes are detritivores, deposit-feeding benthic invertebrates that occupy a variety of microhabitats in the sediments (Rodriguez and Reynoldson, 2011). Tubificid and lumbriculid oligochaete taxa feed on bulk sediment and burrow into the sediment through the construction of galleries that may extent into anoxic layers (Hamburger et al., 2000). Therefore, these organisms are exposed to chemicals via several uptake routes, including direct contact with contaminated sediment particles by ingestion, and by integumentary absorption via porewater and overlying water (OECD, 2008). Since oligochaetes are also easy to maintain under laboratory conditions, standardized protocols for laboratory ecotoxicological testing were developed over the last fifteen years (e.g. ASTM, 2005; OECD, 2007, 2008). However, only a few oligochaete species have been used so far in sediment toxicity and bioaccumulation studies (Rodriguez and Reynoldson, 2011; Méndez-Fernández et al., 2017a). Toxicity test guidelines and research efforts during the last decades of the 20th century focused mostly on the cold water-adapted oligochaetes *Lumbriculus variegatus* (Müller) and *Tubifex tubifex* (Müller). However, in the first decade of the 21st century, toxicity testing guidelines also included protocols for the warm water-adapted species *Branchiura sowerbyi* Beddard (OECD, 2007, 2008) which are thus used in tropical sediment toxicity testing (Lobo and Alves, 2011; Lobo and Espíndola, 2014; Lobo et al., 2016). The limited number of ecotoxicity studies conducted with this species are mostly acute water-only tests, and only a very limited number of chronic toxicity and bioaccumulation studies have been conducted so far (Lobo and Espíndola, 2014; Lobo et al., 2016). This is surprising given that *B. sowerbyi* appears particularly promising to assess the bioaccumulation potential of substances due to its relatively high individual biomass (OECD, 2008; Lobo and Alves, 2011).

Deposit-feeding oligochaete worms play an important role in the bioturbation of freshwater ecosystems through their burrowing activity (Nogaro et al., 2009), which affects the transport of pollutants from the sediment to the water column, and vice versa (Karickhoff and Morris, 1985; Ciutat et al., 2005). Oligochaetes may also serve as prey for other aquatic organisms such as

benthivorous insect larvae, fish and birds (Rodriguez and Reynoldson, 2011; Horváth et al., 2012), thus eventually allowing for biomagnification of toxicants to higher trophic levels (Egeler et al., 2001).

Metals and metalloids (henceforth, jointly referred to as metals) bioaccumulation is a complex and dynamic time-dependent process, and its interpretation may be complicated because some are essential for the metabolism of the organism and their uptake can be actively regulated (Adams et al., 2011; McCarty et al., 2011). In general, the effective dose of a pollutant to the receptor organism comes from multiple routes, such as ingestion through diet and/or sediment, in addition to water (ingestion + uptake through respiratory organs and skin), so that the internal dose in field organisms (tissue residues) is additive across exposure routes (Sappington et al., 2011). Regarding all its complexity (mineral and organic particles, microorganisms, and interstitial water), sediment can be the main source of metal bioaccumulation in benthic organisms (Méndez-Fernández et al., 2014, 2017a), especially through ingestion as an uptake route (Camusso et al., 2012).

In the last two decades, the ‘‘Tissue Residue-effects Approach’’ (TRA: Meador et al., 2011) has been developed to assess the toxic responses using metal tissue residue as a dose metric. According to TRA, a toxic effect is assumed to occur when the excess of metal is present internally in a form that is metabolically available and bound to sites where it can also disrupt biological and biochemical processes (Rainbow, 2002; Adams et al., 2011; McCarty et al., 2011). However, a simple relationship between metal bioaccumulation and toxicity generally does not exist, and factors such as nature of the metals (e.g., essential versus non-essential), bioavailability, or taxa behaviour and detoxification processes, all can contribute to the final observed toxic effects. The suitability of the TRA approach for metals has been disputed since the assumption of proportionality in concentrations among compartments (i.e., exposure *vs*. whole body) is often violated (Adams et al., 2011). However, recent studies have demonstrated the relationships between metal tissue residues in target organisms and measurable toxic effects using both laboratory and

96 field organisms (Meador et al., 2011; Méndez-Fernández et al., 2013, 2017b; Goulet and
97 Thompson, 2018).

98 The first aim of the present work was to study the chronic effects of arsenic (As) and zinc
99 (Zn) sediment contamination on the survival, growth and reproduction of the cold water-adapted
100 oligochaete *T. tubifex* and the warm water-adapted species *B. sowerbyi*. The second aim was to
101 assess the chronic risk of As and Zn present in sediments on the basis of their bioaccumulation by
102 both species, through the TRA approach. The selection of As and Zn was motivated by their
103 relevance, both in terms of occurrence and ecotoxicity, in river sediments affected by mining
104 activities in several study areas previously studied by the research teams. The ecotoxicity of these
105 metals has also previously been evaluated by the authors in the same target species, *B. sowerbyi* and
106 *T. tubifex,* but only under acute exposure in water-only tests and under subchronic exposure (14d) to
107 spiked sediments (Lobo et al., 2016). This first study indicated that prolonged exposure to these
108 metals could lead to sublethal effects to these oligochaetes (autotomy of the posterior body parts,
109 abnormal behavior and appearance). Therefore, the present study was initiated as a follow-up study
110 to evaluate the bioaccumulation and a wider range of sublethal effects (growth, reproduction) after a
111 chronic (28d) exposure of the test species to these metals.
112
113 **2. MATERIALS AND METHODS**
114
115 **2.1. Culture of test organisms and test sediments**
116 The test organisms were obtained from existing in-house cultures at the laboratories where
117 the tests were conducted. The culture of *T. tubifex* had been maintained for over 25 years at the
118 Animal Ecotoxicology and Biodiversity laboratory (University of the Basque Country, UPV/EHU,
119 Bilbao, Spain). This culture was initiated with individuals collected from a mountain stream of the
120 Gorbeia Natural Park (northern Spain). Animal cultures were maintained in complete darkness, at
121 22 ± 1 ºC, in 2.2-L plastic containers containing a 3-cm sediment layer (natural sediment from an

uncontaminated pond in the mountains of Álava, Iturbatz, Spain, grain particle size < 0.25 mm; organic matter: 3.1 ± 0.5%) and dechlorinated tap water (see below, section 2.2), and were organized in worm cohorts of about 100 juveniles. More details about the culture are provided in Méndez-Fernandez et al. (2013).

The culture of *B. sowerbyi* was maintained at the Center for Water Resources and Applied Ecology (University of São Paulo, São Carlos, São Paulo State, Brazil) at 25 ± 1ºC, in the dark, with moderate aeration. Each 3.5-L culture recipient contained a sediment layer of approximately 5 cm derived from the waterbody alongside a spring in Brotas (São Paulo State, Brazil; grain particle size < 0.25mm; organic matter: 1.7%), reconstituted water (pH = 7.0; EC = 100 µS/cm; total hardness = 40 mg/L as $CaCO_3$), and 80 young worms. More details on the culture of this species are described in Lobo et al. (2016).

The sediments used for the toxicity tests were obtained from sites other than those used in the cultures to assure that they had comparable and low organic matter levels. The test sediments from the Barrundia (Spain) and the Perdizes (Brazil) streams that were used had similar granulometric characteristics: both are sandy (grain particle size < 0.25mm), with 4% silt-clay in Barrundia and 6% fines in Perdizes, had low organic content (1.7% in Barrundia and 0.8% in Perdizes) and had low concentrations of As (2.7 ± 1.4 mg/kg and 5.0 ± 0.7 mg/kg for Barrundia and Perdizes, respectively) and Zn (27 ± 28 mg/kg and 56 ± 25 mg/kg for Barrundia and Perdizes, respectively) (on a dry weight basis). For more details about the characteristics of both sediments, refer to Lobo et al. (2016).

2.2. Experimental design of the chronic toxicity tests

The toxicity tests with *T. tubifex* and *B. sowerbyi* were conducted at the laboratory of Animal Ecotoxicology and Biodiversity of the University of the Basque Country (Spain) and at the Center for Water Resources and Applied Ecology (CRHEA) of the University of São Paulo (Brazil), respectively. The test design was based on the OECD (2007) and ASTM (2005) guidelines. Test

148 chambers consisted of 250-mL glass beakers, containing 100-mL test sediment and 100 mL water.
149 The water column consisted of dechlorinated tapwater in the *T. tubifex* test with the following
150 physico-chemical conditions: pH = 6.8 ± 0.16, electrical conductivity = 279 ± 3.1 µS/cm, total
151 hardness = 127 mg $CaCO_3$/L. Reconstituted water was used in the *B. sowerbyi* test: pH = 7.4 ±
152 0.02; electrical conductivity = 129 ± 2.0 µS/cm; total hardness = 40 mg$CaCO_3$/L). The entire 28-d
153 test period was conducted in darkness, under a constant temperature of 22 ± 1 °C (*T. tubifex*) and 25
154 ± 1 °C (*B. sowerbyi*). It attempted to respect the characteristics of tropical (for *B. sowerbyi*) and
155 temperate (for *T. tubifex*) environmental, and, because of that, some differences at the tests
156 conditions can be observed.
157 The sediment metal spiking followed the EPS (1995) guideline, with modifications
158 reported in Méndez-Fernández et al. (2013), and the full method used is fully described in Lobo et
159 al. (2016). The salts dissodium arsenate ($HAsNa_2O_4 \cdot 7H_2O$; 98% of purity) and zinc sulfate
160 ($ZnSO_4 \cdot 7H_2O$; 99% of purity) were used in the sediment spiking procedure for As and Zn,
161 respectively. It was added Tetramin® fish food (approximate amount of 80mg per chamber test) as
162 complementary food source during this process. After the one-week equilibration phase in the
163 spiking procedure, as recommended by OECD (2008), the sediment (100 mL) was added to each of
164 the corresponding treatment replicates and topped with 100-mL overlying water. After gently
165 aerating the water column for 48 hours (at test temperature in darkness) to allow sediment and
166 porewater partitioning equilibrium, four worms were added to each test vessel to start the bioassay.
167 Prior to the transfer from the cultures to the test vessels, worms were kept in dilution water for 5h to
168 empty their gut content (Martinez-Madrid et al., 1999), after which their wet weight was measured.
169 Separately, wet-to-dry weight ratios were determined at the beginning of the experiment from 30
170 worms randomly selected from the cultures of each species, which were 0.107 ± 0.01 for *T. tubifex*
171 and 0.152 ± 0.08 for *B. sowerbyi*. All worms used each test were obtained from the same culture
172 batch (6-7 week old). The weight of the worms at test initiation was 0.97 ± 0.17 mg dw for *T.*
173 *tubifex* and 3.45 ± 0.63 mg dw for *B. sowerbyi* (values averaged for all tests).

In each bioassay, five sediment metal concentrations were tested (Table 1), with six replicates for each test concentration. Two replicates were used for chemical analysis: one at the beginning and another at the end of the experiments (see section 2.4), while the remaining four replicates were used for biological determinations (toxicity and bioaccumulation; see section 2.3). Test concentrations were chosen based on previously data obtained from 14-d water-sediment tests with the same metals and species (Lobo et al., 2016), and they are expressed as an average value between the initial and final measurements (Table 1; supplementary data S1). Every test also included a control series with non-spiked sediments, the worms and with the same number of replicates (n = 6) as part of the quality assurance/control procedures.

2.3. Endpoints

The chronic bioassays included lethal (survival percentage, SUR) and sublethal (reproduction and growth) endpoints. For reproduction, the following endpoints were measured: number of total cocoons, TCC; number of empty cocoons, ECC; cocoon biomass, CCB (only for *T. tubifex*); number of eggs per cocoon, EgC (only for *B. sowerbyi*); and number of total young, TYG. Regarding growth, the somatic growth rate (SGR) and total growth rate (TGR) were measured (see below). The biomass was always expressed and analyzed on a dry-weight basis. At the end of the 28-d exposure period, the sediment from the test beakers was washed through a 0.50-mm mesh sieve to separate adults and cocoons from the sediment, followed by a 0.25-mm mesh sieve to extract the juveniles. After the completion of the test, the number of dead and living adult worms were counted, the surviving specimens of *T. tubifex* were purged for 5h (Martinez-Madrid et al., 1999), frozen in liquid nitrogen and subsequently stored at -20 °C. Afterwards, the worms were freeze-dried overnight to a constant weight. Somatic weight for *B. sowerbyi* adults was calculated for surviving adults, after purging for five hours and dried at 60°C, for 48h. Cocoons of *T. tubifex* were relatively small (ca. 1 mm diameter) and usually contained many eggs, hampering the quantification of the number of eggs per cocoon (EgC) without breaking the cocoons. Therefore, the

total biomass of the cocoons containing eggs (CCB) was determined for this species placing the cocoons containing eggs from each replicate on preweighted glass microfibre filters (Whatmann® 2.5-cm diameter), which had been dried at 60°C, for 48h. Somatic weight of adults and cocoons were determined on a Sartorius® M3P Electrobalance (accuracy limit: 1 µg) for *T. tubifex*, and on a Mettler AE240 analytical balance (accuracy limit: 1 µg) for adults of *B. sowerbyi.* As compared to *T. tubifex*, the cocoons of *B. sowerbyi* were larger (ca. 3 mm diameter) and transparent, so the number of eggs per cocoon (EgC) could be determined. The juveniles obtained from sieving sediment through a 0.25 mm mesh sieve were fixed with formalin (4%) and stored for posterior quantification on a stereomicroscope (magnification 100x).

The calculation of the growth parameters was done using the following formulas:

$$\text{Somatic daily growth rate (SGR)} = ((\text{Ln}W_2 - \text{Ln}W_1)100)/t$$

$$\text{Total daily growth rate (TGR)} = (\text{Ln}(W_2 + \text{CCB}) - \text{Ln}W_1)100/t$$

Where: W_1 and W_2 are the initial and final biomass (expressed on a dry weight basis), respectively, t = test duration (i.e. 28 days), and CCB is the total cocoon biomass.

Dissolved oxygen and pH of both tests were measured twice a week, and the other physical variables were measured at the beginning and the end of the experiments. In the *B. sowerbyi* test, we used a Yellow Springs YSI-55 probe to measure the dissolved oxygen in the overlaying water, a Micronal B374 electrode for pH, an Orion M145 for electric conductivity, and the spectrophotometry method described in APHA (1995) for ammonia. In the tests conducted with *T. tubifex,* the same parameters were measured with a Thermo Scientific Orion 5-Star Plus multi-parameter meter. In addition, the biota-sediment bioaccumulation factor (BAF) for each metal was calculated as the ratio between the mean worm tissue residues of the metal measured at the end of the toxicity test and the corresponding mean concentration (between initial and final) in the sediment (Egeler et al., 2001).

2.4. Metal analysis

Sediment and porewater concentrations were analyzed individually by separating the porewater from the sediment through centrifugation of 50-mL sediment (4500 rpm, 30 min). Subsequently, porewater was filtered through a 0.45-µm filter (Whatmann®) before chemical analyses, as described below. The solid sediment fraction was dried at ambient temperature and sieved through a 63-µm mesh, before acid digestion. At the end of the toxicity tests, surviving adult worms were purged, freeze-dried and weighed, and then worms were digested at room temperature with nitric acid (70%, Baker Instra-Analyzed) and H_2O_2 (30%, R.P. Normapur Prolabo) at a 10:1 v:v ratio (Clements, 1994). A control series (3 replicates) containing only acid and H_2O_2, and no worms, was also included as a blank in every analytical batch. Internal standards for metal tissue residues (Mussel tissue, NIST 1643e, USA), water (TMDA 52.3) and sediment (Buffalo River sediment, RM8704, NIST, USA) were also included as reference materials.

Chemical analyses of arsenic and zinc in the overlaying and porewater of the *T. tubifex* test were made by Inductively Coupled Plasma-Atomic Emission Spectrometers (ICP-AES; Limits of Quantification, LOQ= 0.05 mg As/L and 0.1 mg Zn/L) and by Inductively Coupled Plasma Mass Spectrometry (ICP-MS; LOQ= 0.3 µg As/L and 5 µg Zn/L) on the SGIker from the UPV/EHU. Acid digestion of the sediment was performed according to the USEPA 3052 (USEPA, 1996) and UNE-EN 13656 (UNE, 2003) procedures (9 mL HNO_3 65 % and 4 mL HF were added to 0.2 g of sediment). Sediment and tissue metal concentrations in the *T. tubifex* tests were measured at the SOSPROCAN unit (University of Cantabria, Spain) by ICP-MS (LOQ= 0.3 µg As/L and 5 µg Zn/L). Analytical recovery rates were 87% for Zn in the Buffalo sediment (no reference data for As); recovery rates for As and Zn tissue concentrations were 85% and 103%, respectively.

Analytical quantification of the metal concentrations in porewater, worms and sediment from the *B. sowerbyi* tests was performed at the Poços de Caldas laboratory (LAPOC) of the National Nuclear Energy Commission (CNEN) in Brazil. Water samples were analyzed by Hydride Generation Atomic Absorption Spectroscopy (HG-ASS) for As (LOQ = 0.02 µg/L) and Flame Atomic Absorption Spectrometry (F-AAS) for Zn (LOQ = 6 µg/L); recoveries from reference water

252 (NIST 1643e) were 107% for As and 103% for Zn. Sediment was digested following USEPA 3052
253 (USEPA, 1996), and analyses performed though HG-AAS for arsenic and F-AAS for zinc; the
254 recovery rate attained for the Buffalo river standard sediment (RM8704, NIST, USA) was 90% for
255 zinc (no reference data for As).
256
257 **2.5. Statistical analysis**
258 Statistical analyses were conducted using mean sediment concentrations (i.e., the mean of
259 the concentrations at the start and end of the tests, supplementary data S1). Survival was analyzed
260 with the Fischer's Exact test. The Shapiro-Wilk's test was conducted to test for normality of the
261 data distribution. Subsequently, statistical differences between treatments and controls were
262 evaluated by ANOVA followed by the Dunnett´s *t* test for normal-distributed data or the Kruskal-
263 Wallis test followed by the Dunn's test for data that did not pass the Shapiro-Wilk's test for
264 normality. These statistical analyses were conducted using the free R software (R Core Team, 2013)
265 and extension package *multicomp* (Hothorn et al., 2008). The significance level for rejection of the
266 null hypothesis was 0.05.
267 Median lethal concentrations (mortality; $LC_{50,}$) and median effect concentrations (growth
268 or reproduction; $EC_{50,}$), as well as median Lethal and Effect body Residues (LR_{50}, ER_{50}) for As and
269 Zn were estimated from non-linear regression models, using the free R software (R core team,
270 2013) in combination with the extension package *drc* (Ritz and Streibeig, 2005). The best fitted
271 model (among the 14 non-linear dose–response regression models tested) was selected using
272 Akaike's Information Criterion (AIC) and its validation was based on graphical assessment
273 (Burnham and Anderson, 2002; Zuur et al., 2007) and the results of the goodness-of-fit (assessed by
274 R^2) and the lack-of-fit (*p* value, with 0.05 of significance) tests, both included in the drc package
275 (Ritz and Streibeig, 2005). The LC and EC values were calculate based on the metal' concentration
276 in the sediment (instead of porewater), once ingestion is the principal route of exposition of these
277 species (Camusso et al., 2012; Mendez-Fernández et al. 2014, 2017a).

3. RESULTS

3.1. Quality Assurance and Quality Control

No statistically significant differences in physical-chemical variables were found within each treatment (i.e., between the replicates of the same treatment) along the toxicity tests, comparing data form day 1 and day 28. Dissolved oxygen and pH in the water column in the As tests were: 7.7 ± 0.39 mg/L and 8.2 ± 0.18 in the *T. tubifex* test and 6.6 ± 0.55 mg/L and 7.8 ± 0.26 in the *B. sowerbyi* test; corresponding values for the Zn tests were: 7.73 ± 0.24 mg/L and 8.26 ± 0.21 (*T. tubifex*) and 6.98 ± 0.30 mg/L and 7.65 ± 0.28 (*B. sowerbyi*). The coefficients of variation (CV%) were lower than 10%, except for ammonia, that had a concentration of 6.2 mg/L in the water column corresponding to the 97 µg As/g treatment in the *T. tubifex* test on the first day and dropped to zero at the end of the experiment (mean values for ammonia in As and Zn tests respectively: 2.45 ± 2.63 mg/L and 1.42 ± 1.68 for *T. Tubifex* and 0.21 ± 0.1 mg/L and 0.37 ± 0.27 mg/L for *B. sowerbyi*). Despite this, test conditions appeared to be adequate in the *T. tubifex* toxicity test as confirmed by the control treatments performance, without mortality or sublethal effects (Table 1).

3.2. Chronic toxicity

At high As concentrations, the survival of both species was reduced (Table 1), resulting in a LC_{50} of 189 ± 41 µg As/g and 102.87 ± 27.16 µg As/g for *T. tubifex* and *B. sowerbyi*, respectively (Table 2, supplementary data S2). The reproduction of *T. tubifex* was impaired ($p < 0.05$) at 179.50 µg As/g, as evidenced by the number of empty cocoons (ECC) and, consequently, by the number of juveniles (TYG; Table 1). It is also interesting to point out that one juvenile with a bifid posterior region was observed at 55 µg As/g. *B. sowerbyi* did not reproduce at all in the test evaluating As (Table 1), even in the control group, so it was not possible to assess the possible effects of As exposure on the reproduction of this species. However, the inhibition in growth (SGR) was significant ($p < 0.001$) for the worms exposed to concentrations greater than 42 µg As/g.

305 The presence of Zn in the sediment did not have any significant adverse effects on any of
306 the lethal or sublethal endpoints evaluated for either species at the concentrations tested (Table 1).
307 However, a tendency toward an increase in reproduction and growth was observed for several
308 endpoints at concentrations below 253 µg Zn/g for *B. sowerbyi* and below 1801 µg Zn/g for *T.*
309 *tubifex*. Individuals of *T. tubifex* exposed to concentrations of 725 and 1204 µg Zn/g appeared to
310 produce more cocoons with a greater biomass than in other treatments or the control, although this
311 was not statistically significant.

312 **Table 1**. As and Zn lethal and sublethal endpoints (mean ± SD) observed at the 28d-chronic bioassays in spiked sediments, with *B. sowerbyi* and *T.*
313 *tubifex*.
314

Metal	SED	SUR	TCC	ECC	EgC	CCB	TYG	SGR	TGR
B. sowerbyi									
As	2.66 (c)	100	0	0	-	-	0	0.016 ± 0.005	-
	15.52	100	0	0	-	-	0	0.015 ± 0.003	-
	21.42	100	0	0	-	-	0	0.014 ± 0.002	-
	30.74	100	0	0	-	-	0	0.011 ± 0.002	-
	42.20	100	0	0	-	-	0	0.005 ± 0.001***	-
	108.00	35.0 ± 19.1***	0	0	-	-	0	-0.001 ± 0.002***	-
Zn	34.40 (c)	100	3.7 ± 4.3	0.00	0.8 ± 0.5	-	0.00	0.017 ± 0.003	-
	82.20	100	5.7 ± 7.5	0.5 ± 0.6	1.3 ± 0.4	-	0.2 ± 0.5	0.024 ± 0.006	-
	103.40	100	8.5 ± 9.1	0.5 ± 1.0	1.2 ± 0.2	-	0.2 ± 0.5	0.022 ± 0.003	-
	156.40	100	9.7 ± 8.7	1.2 ± 1.2	1.2 ± 0.3	-	0.5 ± 0.6	0.022 ± 0.002	-
	242.20	100	12.2 ± 10.1	1.7 ± 2.1	1.4 ± 0.3	-	0.7 ± 1.0	0.022 ± 0.006	-
	253.40	100	7.7 ± 7.6	1.0 ± 1.4	1.2 ± 0.2	-	0.7 ± 1.0	0.022 ± 0.005	-
T. tubifex									
As	8.38 (c)	100	40.8 ± 3.3	25.5 ± 1.3	-	1.0 ± 0.2	198.5 ± 54.0	0.009 ± 0.006	0.030 ± 0.005
	17.55	100	43.5 ± 6.9	25.5 ± 6.5	-	0.9 ± 0.2	189.2 ± 69.4	0.008 ± 0.002	0.028 ± 0.003
	27.55	100	39.3 ± 6.0	24.5 ± 4.4	-	0.9 ± 0.2	174.5 ± 57.7	0.009 ± 0.001	0.029 ± 0.003
	54.85	100	37.3 ± 15.0	22.3 ± 10.2	-	0.8 ± 0.3	180.0 ± 119.3	0.004 ± 0.013	0.021 ± 0.006
	97.30	100	36.3 ± 6.9	21.3 ± 6.8	-	0.8 ± 0.3	184.7 ± 66.0	0.005 ± 0.003	0.024 ± 0.008
	179.50	68.8 ± 23.9**	28.5 ± 12.1	9.5 ± 6.2*	-	0.6 ± 0.2	45.5 ± 57.1*	0.006 ± 0.006	0.021 ± 0.003
Zn	177.30 (c)	100	37.5 ± 3.0	22.0 ± 1.4	-	0.7 ± 0.1	153.3 ± 16.0	0.009 ± 0.005	0.026 ± 0.005
	329.70	100	34.8 ± 5.3	19.3 ± 2.6	-	0.7 ± 0.3	174.3 ± 19.2	0.010 ± 0.010	0.026 ± 0.005
	463.50	93.8 ± 12.5	38.5 ± 4.2	23.0 ± 3.2	-	0.9 ± 0.1	193.0 ± 79.9	0.007 ± 0.008	0.028 ± 0.005
	724.90	100	40.5 ± 4.1	22.0 ± 5.5	-	1.1 ± 0.3*	198.3 ± 43.9	0.013 ± 0.005	0.036 ± 0.001
	1203.50	100	39.5 ± 3.9	22.0 ± 2.9	-	1.2 ± 0.1**	209.5 ± 74.4	0.016 ± 0.008	0.039 ± 0.008*
	1805.50	100	32.9 ± 3.9	17.0 ± 2.9	-	0.9 ± 0.1	183.0 ± 53.4	0.004 ± 0.006	0.023 ± 0.004

315 *Abbreviations: SED* real sediment concentration (µg/g dw); *SUR* percentage of survival; *TCC* total number of cocoons; *ECC* number of empty cocoons; *EgC* number of
316 eggs per cocoon; *CCB* average biomass of total cocoons (mg); *TYG* total of youngs; *SGR* somatic growth rate (day^{-1}); *TGR* Total growth rate (day^{-1}); Significant
317 difference from the control (c): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
318

Table 2. Lethal (LC) and Effect (EC) Concentrations values of the best-fitted models, based on sediment concentration, for *B. sowerbyi* and *T. tubifex* after 28d-chronic bioassays with As and Zn.

Species	Metal	Endpoint	Best fitted model	LC_{10}/EC_{10} ± SE (µg/g)	LC_{50}/EC_{50} ± SE (µg/g)
B. sowerbyi	As	SUR	LL.2	86.54 ± 103.03	102.87 ± 27.16
		SGR	W1.3	22.13 ± 4.46	36.61 ± 2.14
		TCC, ECC, EgC, TYG	-	*nd*	*nd*
	Zn	All	-	*nd*	>253.40
T. tubifex	As	SUR	LL.2	163.58 ± 60.50	189.15 ± 40.55
		TCC	LN.3	96.10 ± 35.90	254.52 ± 81.34
		ECC	W2.3	87.03 ± 15.01	149.35 ± 17.50
		TYG	LN.3	104.27 ± 31.81	147.06 ± 21.73
		CCB, SGR, TGR	-	*nd*	>179.50
	Zn	All	-	*nd*	>1805.52

SUR percentage of survival; *TCC* Total number of cocoons; *ECC* number of empty cocoons; *EgC* number of eggs per cocoon; *CCB* average biomass of total cocoons; *TYG* total of youngs; *SGR* somatic growth rate (day^{-1}); *TGR* Total growth rate (day^{-1}). For the models: *LL.2* Log-logistic with 2 parameters; *LN.3* Log-normal with 3 parameters; *W1.3, W2.3* Weibull with 3 parameters; *nd* not determined due the lack of effects.

3.3. Bioaccumulation and Tissue Residue Approach

The tissue concentration of As and Zn was positively related to the metal concentrations in the sediment, showing dose-dependent functions (Fig. 1). The highest metal body concentrations recorded were as follows: 937 ± 219 µg As/g and 1818 ± 368 µg Zn/g for *T. tubifex* and 14 ± 2.1 µg As/g and 207 ± 73 µg Zn/g for *B. sowerbyi* (Table 3). In *T. tubifex*, As uptake gradually increased with increasing As sediment concentration, without clearly stabilizing within the range of concentrations in the test (Fig. 1). In *B. sowerbyi*, however, As uptake increased rapidly at lower test concentrations to attain a steady value (no increase in tissue residue concentration between 42 to 108 µg As/g exposure in sediment; Fig. 1). In the case of Zn, *T. tubifex* revealed internal regulation up to about 1000 µg Zn/g sediment, while in *B. sowerby,* the model showed a poor adjustment (R^2= 0.39) and no regulation of the Zn uptake could be noted (Fig. 1). The As-BAF was high in *T. tubifex,* up to 8.0 at intermediate sediment concentrations; contrarily, the As-BAF in *B. sowerbyi* was below 1, attaining a maximum of 0.34 (Table 3).

The median As-Lethal Body Residue (LBR_{50}) value of *T. tubifex* was 1,002 ± 55 µg/g, whereas As- LBR_{50} of *B. sowerbyi* was more than 50 times lower (17 ± 2.1 µg/g) (supplementary data S3). Fig. 2 show the relationship between the reproduction and growth parameters with the As tissue residues. The estimated Effect Residue (ER_{50}) values for *B. sowerbyi* calculated for growth were about half the LBR_{50} value, while in *T. tubifex* both parameters had comparable values. Zinc did not have adverse effects at any of the concentrations tested for either species, thus it was not possible to calculate the LC_{50}, EC_{50}, LBR_{50} and ER_{50} values.

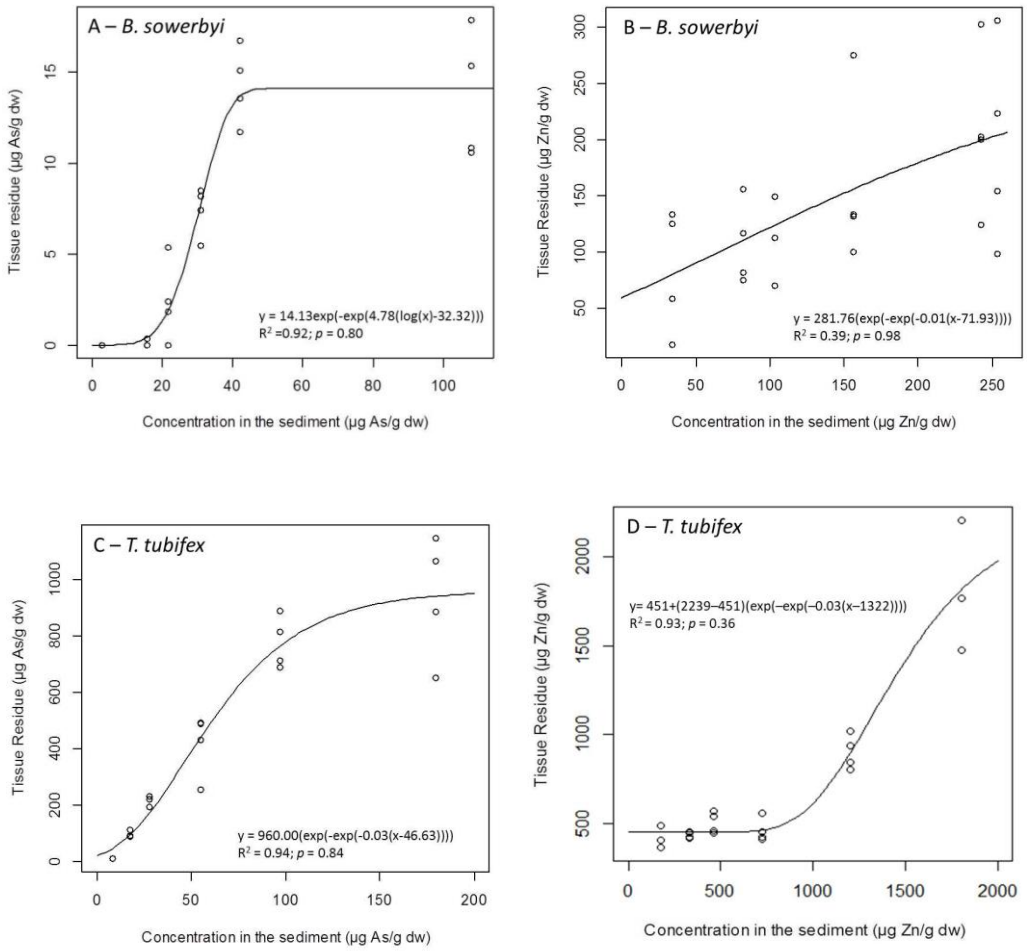

Fig. 1. Relation between the metal concentration in the sediment and the tissue-residue concentrations of the test species: **A**. As - Weibull model with 3 parameters; **B** Zn - Gompertz model with 3 parameters **C.** As - Gompertz model with 3 parameters; **D**. Zn - Gompertz model with 4 parameters.

Table 3. Tissue Residue (TR) (mean ± SD) measured at each sediment dose of As and Zn (mean values from the beginning and the end of the test) at the 28d-chronic bioassays with *B. sowerbyi* and *T. tubifex* and their respective bioaccumulation factors (BAF = tissue residue/sediment concentration).

	Arsenic			Zinc		
Specie	**Sediment (µg/g dw)**	**Tissue residue (µg/g dw)**	**BAF**	**Sediment (µg/g dw)**	**Tissue residue (µg/g dw)**	**BAF**
B. sowerbyi	2.66 (c)	0.00	0.00	34.40 (c)	83.18 ± 55.45	2.46
	15.52	0.09 ± 0.18	0.01	82.20	106.19 ± 37.25	1.31
	21.42	2.40 ± 2.23	0.11	103.40	119.98 ± 37.75	1.16
	30.74	7.38 ± 1.36***	0.24	156.40	160.01 ± 78.42	1.02
	42.20	14.30 ± 2.14***	0.34	242.20	207.24 ± 73.28	0.86
	108.00	13.69 ± 3.55***	0.11	253.40	195.34 ± 89.83	0.77
T. tubifex	8.38 (c)	8.99 ± 0.67	1.07	177.30 (c)	415.04 ± 51.34	2.34
	17.55	95.72 ± 11.14	5.45	329.70	434.70 ± 16.97	1.32
	27.55	213.96 ± 20.05	7.77	463.50	502.34 ± 61.04	1.08
	54.85	414.89 ± 110.96***	7.56	724.90	461.42 ± 66.64	0.64
	97.30	775.29 ± 92.14***	7.96	1203.50	900.87 ± 96.35	0.75
	179.50	937.40 ± 219.35***	5.22	1805.50	1818.12 ± 367.85**	1.01

Significant difference from the control (c): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

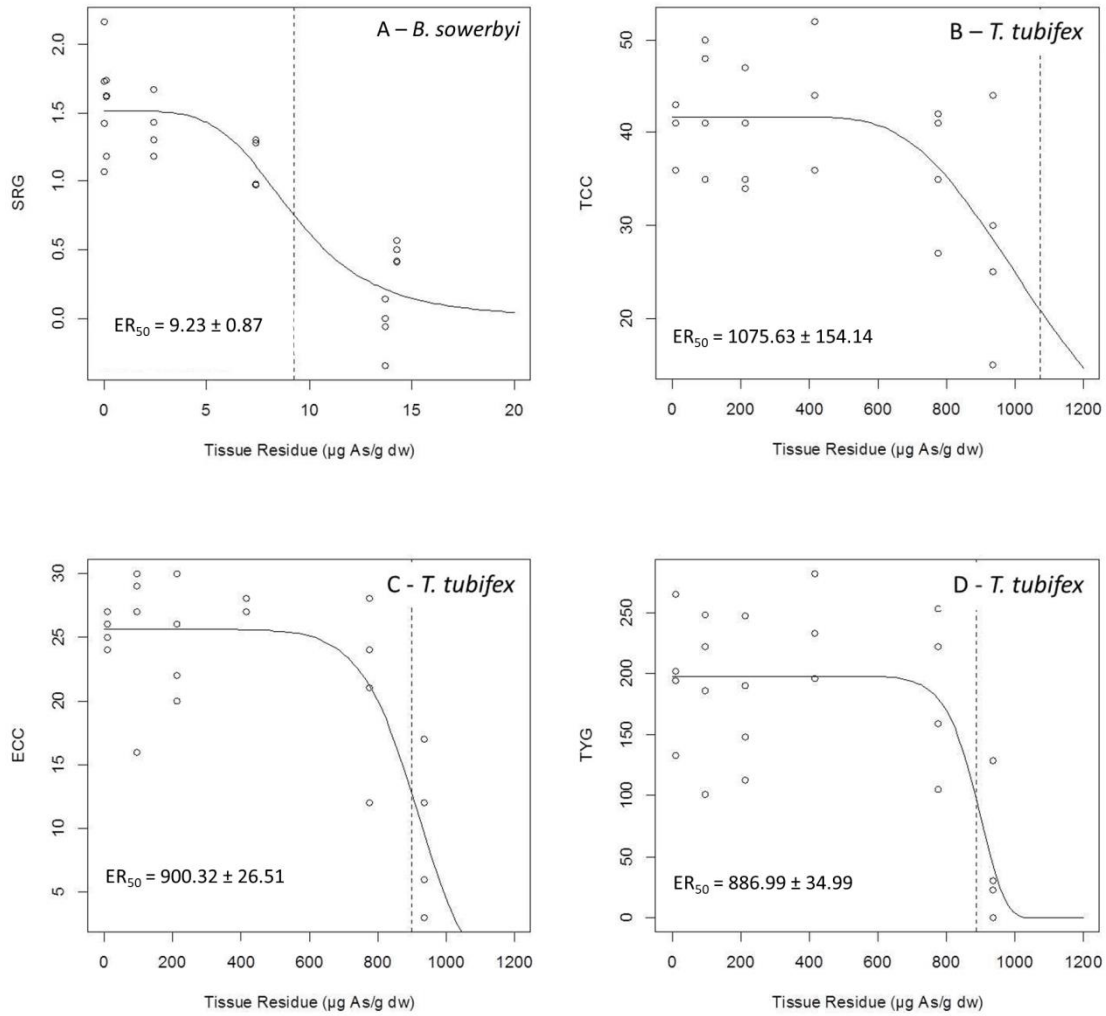

Fig. 2. (**A**) Relation between the Somatic Growth Rate (SGR) and the tissue residue concentration of As in *B. sowerbyi;* (**B**) relation between the Total of Cocoons (TCC), (**C**) Empty Cocoons (ECC) and (**D**) Total of Young (TYG) and the Tissue Residue of As in *T. tubifex*, and the vertical dashed line represents the ER_{50} calculated from the corresponding models (**A**) Log-logistic with 3 parameters; (**B**) Log-normal with 3 parameters and (**C** and **D**) Weibull-1 with 3 parameters.

4. DISCUSSION

Bioassays assessing the toxicity of sediments contaminated with As or Zn in tropical regions are still incipient, which makes it difficult to compare our results. However, several authors have reported As toxicity data from laboratory experiments using various mostly temperate benthic organisms (Martinez et al, 2006; Liber et al, 2011; Lobo et al. 2016). From the literature review conducted for toxicity endpoints in benthic taxa (Table 4), it may be deducted that the overall order of As sensitivity (in decreasing order of sensitivity) was: *B. sowerbyi* > *T. tubifex* = *Chironomus*

373 *tentans* > *Chironomus dilutes* > *Hyalella azteca*. Given the higher toxicity of As to *B. sowerbyi*, it
374 may be questioned whether the sensitivity of these temperate species are representative for tropical
375 benthic organisms. On the other hand, despite the differences in the experimental design including
376 test duration and, hence, metal speciation in the data listed in Table 4, all the As-LC_{50} and EC_{50} for
377 both oligochaetes species are generally within one order of magnitude. Subsequently, the
378 uncertainty factors that are usually applied to temperate toxicity data (factor 100 to acute and factor
379 10 to chronic data; EFSA, 2015) may suffice to protect tropical species. Regarding Zn, no statistical
380 adverse effects were denoted at any of the test concentrations evaluated in the present study on both
381 species (Table 1). Based on the subchronic exposure evaluated in our previous study (Lobo et al.,
382 2016), *B. sowerbyi* was approximately three times more sensitive than *T. tubifex*.
383
384 **Table 4**. Literature review of LC_{50} and EC_{50} for As and Zn for sediment exposure in other benthic organisms.
385 Values showed as mean ± sd or mean (95% CL).

Species	Endpoint	Value (µg/g dw)	Reference
Arsenic			
Hyalella azteca	10d-EC_{50} (growth)	> 462	Liber et al. (2011)
	10d-LC_{50}	532 (495-557)	Liber et al. (2011)
Chironomus dilutus	10d- EC_{50} (growth)	342 (317-362)	Liber et al. (2011)
	10d-LC_{50}	642 (561-736)	Liber et al. (2011)
Chironomus tentans	50d-clear effects on growth and development[a]	130	Martinez et al. (2006)
T. tubifex	14d-LC_{50}	251 ± 47	Lobo et al. (2016)
	28d-EC_{50} (growth)	> 179.50	This study
	28d-EC_{50} (reproduction)	147.06 ± 21.73	This study
	28d-LC_{50}	189.15 ± 40.55	This study
B. sowerbyi	28d-EC_{50} (growth)	36.61 ± 2.14	This study
	28d-LC_{50}	102.87 ± 27.16	This study
Zinc			
B. sowerbyi	14d-LC_{50}	280 ± 2.3	Lobo et al. (2016)
	28d-NEC (survival)	2023.5 (1806.0 – 2173.0)	Ducrot et al. (2010)
	28d-NEC (growth)	1021.0 (737.8 – 1254.0)	Ducrot et al. (2010)
T. tubifex	14d-LC_{50}	>679.0	Lobo et al. (2016)
	14d-EC_{50} (autotomy)	635 ± 25	Lobo et al. (2016)

386 [a] - Statistical significance and endpoints not provided

No reproduction for As and very low reproduction levels for Zn occurred in the *B. sowerbyi* tests, which was probably due to a low level of maturity of the test worms. In a previous study on the reproductive cycle of this species under laboratory conditions the time to reach sexual maturity was established at 41 ± 7 days (Lobo and Alves, 2011). Since animals used in the present study were 6-7 weeks of age, reproduction was therefore anticipated, although a longer time to first reproduction for this species has also been reported (i.e., 57-62 days: Ducrot et al., 2007). Therefore, the age of the test worms should be better evaluated in order to optimize the reproduction in the sediment bioassays with the species.

In the case of Zn, Lobo et al. (2016) calculated a 14-d LC_{50} of 280 ± 2.3 μg Zn/g for *B. sowerbyi*, therefore, it was expected to find effective concentrations within the range of concentrations studied, but the essential nature of this element might be the cause of the lack of sublethal effects. In the present study, no significant deleterious effects on *B. sowerbyi* reproduction were observed even at the highest tested sediment concentration of 253 μg Zn/g. Only significant increases in cocoon biomass (CCB) and growth rates (TGR) were measured for *T. tubifex* at sediment concentrations of 725 and 1204 μg Zn/g. This increase in growth and reproduction at low to intermediate sediment concentrations for *T. tubifex* may suggests a hormetic response of the oligochaete to these Zn concentrations. Hormesis is a physiological stimulatory effect when compared to control levels (Calabrese, 2008). In the literature, similar responses have been reported for the essential metal Cu in *T. tubifex* after sediment exposures (Méndez-Fernández et al., 2013). In other study with spiked natural sediments Ducrot et al., (2010) found that Zn also did not cause: i) significant mortality to *B. sowerbyi* adults, even at high concentrations (3,317 μg Zn/g); ii) deleterious effects in juveniles at concentrations below 1,819 μg/g (28d bioassays); or iii) effects on reproduction at concentrations lower than 1,651 μg Zn g^{-1}.

According to Marchese et al. (2008), metal tissue concentrations of organisms reflect the bioavailability of these compounds in the environment. However, whether the metal of concern is

413 an essential or non-essential metal should be considered, since uptake of essential metals can be
414 actively regulated (Adams et al., 2011). This Zn regulation can be noted for *T. tubifex*, which
415 showed a constant tissue residue up to 1000 µg/g (Fig. 1). *B. sowerbyi* exposed to both metals
416 through spiked sediment hardly accumulated the metals in their bodies (Tables 3 and 5). For
417 example, BAF values decreased from 2.5 in controls to around 1 in the lower Zn treatments to 0.77
418 in the highest Zn sediment concentration (Table 4). This could be due to the regulation of essential
419 metals like Zn. Arsenic also was hardly accumulated in *B. sowerbyi*, with a maximum BAF of 0.34
420 (Table 2) and a maximum average tissue concentration of 14.3 µg As/g (Table 5). In contrast, As
421 was bioaccumulated in *T. tubifex* following sediment As exposure, with the BAF increasing from
422 1.1 in controls to 5 to 8 in the As treatments (Table 3). A similar model of bioaccumulation has been
423 described for other non-essential toxic metals. Méndez-Fernández et al. (2013), for example,
424 observed a significant increase in Cd tissues residues of *T. tubifex* exposed to increased Cd sediment
425 concentrations, with BAF values up to 42. Similarly, Alves et al. (2016) also reported a positive
426 correlation between bioaccumulation and the As test concentration by the terrestrial oligochaete
427 *Eisenia andrei* in a 28d-exposure test, with *E. andrei* body As concentrations of up to 60 times the
428 As concentration in artificial soil and 3.6 times of that in natural soil. From the literature data
429 revised for bioaccumulation of As and Zn in benthic organisms (Table 5), *T. tubifex* showed the
430 highest tissue concentration for As and Zn among all the species listed. It's also interesting to notice
431 that only Goulet and Thompson (2018) and the present study made experiments with spiked
432 sediment to test the bioaccumulation of these metals, highlighting the scarcity of this kind of data.
433 As indicated by their BAF values, *T. tubifex* accumulated more As than *B. sowerbyi*, this
434 might be due to (1) the more efficient mechanisms of elimination of As from body of *B. sowerbyi*,
435 compared with *T. tubifex*, at the expense of a large energy expenditure reflected in the growth rate
436 (Table 1), probably due to the autotomy of the posterior body fragment as part of this process
437 (Paris-Palacios et al., 2010); or due to (2) a better detoxification process by *T. tubifex,* that allows
438 this species to withstand high concentration of this metal at its body without adverse effects, as

439 observed by Goulet and Thompson (2008) for the amphipod *H. azteca*. This is an important issue
440 related to risk assessment for other species through trophic transfer, and these two hypotheses
441 should be tested in future works. For instance, the tissue residue concentrations of *B. sowerbyi* was
442 lower than in *T. tubifex* in present study but also than those observed for *L. variegatus* by Winger et
443 al. (2000) and Camusso et al. (2012), even though *B. sowerbyi* was exposed to concentrations three
444 times higher than *L. variegatus* (Table 5). Thus, further research is needed to understand the
445 underlying mechanisms for their high or low sensitivity of these two deposit-feeders in ecological
446 toxicity assessments of metals, both in temperate and tropical environments.

447

448 **Table 5.** Some bioaccumulation data of arsenic and zinc from the sediment available in the literature.

449

Taxon	Test type (range of concentration µg g^{-1} dw)	Range of Tissue concentration (µg g^{-1} dw)	Reference
Arsenic			
H. azteca	10-d exposure to spiked sediment (5 – 324)	0.7 – 7.1	Goulet and Thompson (2018)
L. variegatus	28-d exposure to dredge spoil sediments (6.6 – 32.8)	4.1 – 26.7	Winger et al. (2000)
L. variegatus	28-d exposure to field collected sediments (170 - 186)	3.6 – 362 .0	Lyytikainen et al. (2001)
L. variegatus	28-d exposure to field collected sediments (1.3 – 32.0)	2.1 – 30.0	Camusso et al. (2012)
T. tubifex	28-d exposure to potentially toxic and toxic sediments (6.9 - 5321)	11.2 -2165.2	Mendez-Fernandez et al. (2015)
T. tubifex	28-d exposure to spiked sediment (8.4 – 179.5)	9.0 – 937.4	Present study
B. sowerbyi	28-d exposure to spiked sediment (15.5 – 108.0)	0.1 – 14.3	Present study
Zinc			
L. variegatus	28-d exposure to dredge spoil sediments (61 - 126)	430 – 956	Winger et al. (2000)
L. variegatus	28-d exposure to field collected sediments (36.5 – 26,152)	163.5 – 375.7	Camusso et al. (2012)
Tubifex sp	Field worms from sites polluted by sewage and industrial wastes (300 – 2055)	60.2 – 166.6	Singh et al. (2007)
T. tubifex	28-d exposure to sediments from sites with elevated metal concentration (30.1 – 1728.6)	297.5 – 514.5	Gills et al. (2006)
T. tubifex	28-d exposure to potentially toxic and Toxic sediments (9.9 - 266)	117.0 – 2497.5	Méndez-Fernández et al. (2015)
T. tubifex	28-d exposure to sediment spiked (177.3 – 1805.5)	415.04 – 1818.12	Present study
B. sowerbyi	28-d exposure to sediment spiked (34.4 – 253.4)	83.18 – 207.24	Present study

450
451

452 The LC_{50} values greatly vary among and within studies, even when considering the same
453 species (Tables 2 and 4). However, the tissue residue approach (TRA) provides reliable results
454 relating metal bioaccumulation to measurable effects at population level, as described by several

455 authors (Meador, et al., 2011; Penttinen et al., 2011). Using the TRA, we calculated the ERs for As
456 based on the causal relationships between tissue residues and sublethal responses (growth and
457 reproduction), providing data for future development of criteria to protect freshwater communities
458 from As pollution. The lack of available Zn toxicity data for benthic invertebrates exposed to spiked
459 sediment reinforces the need for future studies with this metal.
460
461 **5. CONCLUSIONS**
462 Particle-feeding oligochaetes are exposed to chemicals via various uptake routes, i.e.
463 through contact with, and ingestion of, contaminated sediment particles, besides tegumentary
464 diffusion via porewater and overlying water. Oligochaetes have therefore attracted increasing
465 attention as test organisms for sediment quality assessments in both the scientific and Regulatory
466 fields. This study demonstrated that both species are susceptible to metal exposure through the
467 sediment. Although derived and published toxicity LC50 and EC50 values of As and Zn were
468 generally within an order of magnitude, sensitivity of benthic organisms appear to vary largely
469 between and within studies and test species. The ‘‘Tissue Residue-effects approach’’ (TRA) appears
470 to be a promising way to overcome these differences and to go forward ecotoxicological
471 assessments which include metal bioavailability and elimination processes.
472 This present paper and a previous paper by the authors (Lobo et al., 2016) confirm *B.*
473 *sowerbyi* as a sensitive representative and a logistically suitable test species for tropical sediment
474 toxicity assessments. Basic research is still needed to better understand the reproductive aspects, the
475 underlying mechanisms of observed differences in metal availability, detoxification and efflux
476 mechanisms in this species. In addition, a test guideline should be developed for this species that
477 could be based on the methodology applied by the authors in this and our previous study.
478
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489

Conflict of Interest:

The authors declare that they have no conflict of interest.

493 **7. REFERENCES**

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