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Reference:

Méndez-Fernández Leire, de Jonge Maarten, Bervoets Lieven.- *Influences of sediment geochemistry on metal accumulation rates and toxicity in the aquatic oligochaete Tubifex tubifex*

Aquatic toxicology - ISSN 0166-445X - 157(2014), p. 109-119

DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2014.10.009

Influences of sediment geochemistry on metal accumulation rates and toxicity in the aquatic oligochaete *Tubifex tubifex*

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Highlights

Biodynamic model in *T. tubifex*: sediment ingestion predicts metal bioaccumulation.

Metal bioaccumulation and toxicity in *T. tubifex* was time-dependant.

AVS did not play a major role in influencing toxicity or metal bioaccumulation.

Autotomy actively promoted during the exposure period in polluted sediments.

ABSTRACT

Metal bioaccumulation and toxicity in the aquatic oligochaete *Tubifex tubifex* exposed to three metal-contaminated field-sediments was studied in order to assess whether sediment-geochemistry (AVS, TOC) plays a major role in influencing these parameters, and to assess if the biodynamic concept can be used to explain observed effects in *T. tubifex* tissue residues and/or toxicity. An active autotomy promotion was observed in three studied sediments at different time points and reproduction impairment could be inferred in *T. tubifex* exposed to two of the tested sites after 28-d. The present study showed that sediment metal concentration and tissue residues followed significant regression models for 4 essential metals (Cu, Co, Ni and Zn) and one non-essential metal (Pb). Organic content normalization for As also showed a significant relationship with As tissue residue. Porewater was also revealed to be an important source of metal uptake for essential metals (e.g. Cu, Ni and Zn) and for As, but AVS content was not relevant for metal uptake in *T. tubifex* in studied sediments. Under the biodynamic concept, it was shown that Influx rate from food (I_F, sediment ingestion) in *T. tubifex*, in a range of sediment geochemistry, was able to predict metal bioaccumulation, especially of the essential metals Cu, Ni and Zn, and for the non-essential metal Pb. Additionally, I_F appeared to be a better predictor for metal bioaccumulation in *T. tubifex* compared to sediment geochemistry normalization.

Keywords: Metals; bioaccumulation; sediment geochemistry; biodynamic model; oligochaeta

1. INTRODUCTION

Sediments are essential, integral and dynamic components of aquatic ecosystems (Salomons and Brils, 2004). Contaminants are mainly associated with the fine particulate material (both organic and mineral) and sediments can act as a reservoir and source of contamination to the water column and aquatic biota (Burton, 2002). Metals can occur in different chemical forms, and their bioavailability in sediments is difficult to determine because it may depend on several factors: such as their speciation (e.g., dissolved, sulfide/organic carbon-bound metals); the sediment-water partitioning relationships; the organism physiology (e.g., uptake rates and assimilation efficiency, excretion); feeding behavior (e.g., particle-size selection) and other behavior (e.g., burrowing activity) (Luoma and Rainbow, 2005; Simpson, 2005; Simpson and Batley, 2007). Thus, metal toxicity depends on the relative contribution of each exposure route, on the metal concentration in each compartment (i.e., overlying water, porewater, and sediment), the relative importance of each compartment for the individual organism biology, and upon duration of exposure (Adams et al., 2011; Simpson and Batley, 2007).

A factor that has been proposed to be important in controlling metal bioavailability in anoxic sediments is the amount of acid volatile sulfides (AVS). Metals associated with AVS are called simultaneously extracted metals (SEM). SEM is generally defined as the sum of molar concentrations of toxicologically important, cationic metals (Cu, Pb, Cd, Zn, Ni, and also Cr and Ag) which are extracted together with AVS. From this, Di Toro et al. (1990) formulated the SEM-AVS model for estimating metal toxicity from contaminated sediments. This model predicts that when AVS concentrations in sediments, on a molar basis, exceed SEM concentrations (SEM_{Me} - AVS < 0), all metals will be bound to sulfides and the sediment porewater is considered to be nontoxic. In contrast, when the sediment contains an excess of SEM (SEM_{Me} - AVS > 0), metals will be released into the porewater and become potentially toxic to the aquatic life. Organic matter can also bind non-sulfide bound trace metals, thus preventing them from entering the dissolved phase (Mahony et al., 1996). Based on this, [SEM-AVS]/TOC has been proposed as a measure of bioavailable metal (Di Toro et al., 2005). The [SEM-AVS]/TOC concept assumes that there is no metal toxicity caused by transformations of the sulfide and organic matter bound metal in the gut of sediment-ingesting organisms or via exposure to contaminated food (Meyer et al., 2005). Although the SEM-AVS model has been validated for both acute and chronic toxicity (Di Toro et al., 1990; Casas and Crecelius, 1994; Hare et al., 1994; Pesch et al., 1995), significant evidence has been gained that benthic invertebrates can accumulate metals in large amounts, even when SEM_{Me} - AVS < 0 (Lee B.G. et al., 2000a, b; Lee J.S, et al. 2001; Hare et al., 2001; De Jonge et al., 2009, 2010).

But the presence of accumulation of a metal in an organism is not an adverse biological effect in and of itself; only the biological responses induced by the presence of the metal(s) are potential adverse effects (Rand, 1995). A key factor in understanding observed bioaccumulated metals in aquatic organisms is based on understanding biodynamics of metal bioaccumulation processes; the biodynamic concept provides a framework to explain how and why trace elements bioaccumulation differs among metals, species, and environments (Luoma and Rainbow, 2005). Toxicity is then expected to occur when the rate of metal uptake summed across all sources (solution and diet) exceeds the combined rates of efflux and detoxification of metal into metabolically inert forms (Rainbow 2002, Luoma and Rainbow, 2005, 2008).

To our knowledge, the influences of sediment geochemistry (e.g. AVS, organic matter) on accumulation rates and resulting chronic toxicity from field-collected sediments are poorly documented. For that purpose, we used the model organisms *Tubifex tubifex* (Müller 1774), an aquatic oligochaete commonly used in sediment toxicity assessment (ASTM, 2005). Therefore, the general aim of this study is 1) to evaluate metal bioaccumulation and toxicity in *T. tubifex* exposed to three metal-contaminated field-sediments; 2) to assess whether sediment-geochemistry (AVS, TOC) plays a major role in influencing these parameters in *T. tubifex*; and 3) to assesses if the biodynamic concept can be used to explain observed effects in *T. tubifex* tissue residues and/or toxicity.

2. MATERIAL AND METHODS

2.1. Study area and sediment sampling

The study area included 3 sites from the Nete/Scheldt basin (Flanders, Belgium): Scheppelijke nete (208591UTMX, 210830UTMY), Kneutersloop (185353UTMX, 208963UTMY), and Molse nete (201657UTMX, 207709UTMY). These sites were selected based on previously conducted studies (Bervoets et al., 1997, 2004; Bervoets and Blust, 2003; De Jonge et al., 2009, 2010), in which high sediment metal concentrations have been measured. Historic metal pollution dating back to the nineteenth century has left numerous areas in Flanders, the northern part of Belgium, contaminated with high concentrations of zinc, cadmium, copper, nickel, lead and arsenic. One of these areas is situated in the north-eastern part (i.e. Noorderkempen). The contamination in this area originated from three zinc smelters that discharged metals to the environment using very polluting pyrometallurgic zinc refining procedures (Groenendijk et al., 1999). Despite a stop of direct emission to the environment since the early nineties of last century, soils and sediments still contain high metal concentrations and can act as possible sources of metal pollution for the aquatic environment in this area (De Jonge et al., 2008).

Sediment samples were taken in the field, from the upper 10 cm layer with a spade, for the realization of the sediment bioassays and for metal concentrations analysis. A subsample was taken for measurements of sediment AVS concentrations, as well as total organic content (TOC) and particle size distribution estimation. Sediments were stored in a cool chamber at 4ºC for maximum period of 3 weeks before the experiments were conducted.

2.2. Experimental design

Sediment used for the worm cultures and as control in the toxicity test was obtained from a large pool, filled with groundwater, at Iturbatz (556682X, 4740877Y, 30T) in Entzia Mountains (Álava, Spain), named Special Area of Conservation by Natura 2000 network (Habitat Directive 92/43/EEC). Sediment was also collected with a spade from the upper 10 cm layer. The aquatic oligochaete worm *Tubifex tubifex* was used as study organism. A healthy stock of the aquatic oligochaete *T. tubifex* was provided by the Animal Ecotoxicity and Biodiversity laboratory from the University of Basque Country (UPV/EHU). Individual culture batches were initiated with a cohort of 100–150 young worms, and reached sexual maturity in six to seven weeks. A supplement of finely ground 1g Tetramin® fish food was added at the beginning of each culture as a nutritional complement.

Tubifex tubifex toxicity experiments were conducted following the methods proposed by Reynoldson et al. (1991) and ASTM (2005). Sediment used for the bioassay was sieved through a 500-µm mesh to remove indigenous species so they do not interfere in experiment results (Reynoldson et al., 1995). *T. tubifex* worms were exposed for 3, 10 and 28 days. Four sexually mature worms with a well developed clitellum were included in each replicate. These worms were in their first reproductive cycle and had similar age (7 to 8 weeks). Four replicates were prepared per tested sediment and were used for biological determinations at the different exposure times (metal bioaccumulation and toxicity endpoints). For the 28 d experiment, one extra replicate per tested sediment was included for sediment chemical measurements at the end of the experiment. Each replicate contained 100 ml of sediment, 80 mg of supplementary food (Tetramin®) to minimize inter-sediment differences due to nutritional quality and quantity, and 100 ml of overlying reconstituted water (ISO 6341-1982), in a 250 ml glass beaker. These beakers were placed into an incubator at 22 ± 1ºC, in the dark and gently aerated. Beakers were prepared 24 h prior to the beginning of the experiment to allow the sediment to settle.

Studied endpoints at 3 d, 10 d and 28 d were: survival (% SUR), autotomy (% AUT) and worm biomass (WB, mg). Autotomy is the loss of segments in the hind part of the body in oligochaetes due to a local constriction of the circular muscles, which can be seen

macroscopically (Kaster, 1979). In this study, autotomy was calculated as proposed by Meller et al. (1998), who calculated autotomy as the ratio of dead plus autotomized to the total exposed animals (constrictions were not considered autotomy). After 28 d exposure, it was considered as a chronic bioassay, so reproduction endpoints were also measured (No. of Total Cocoons: TCC; No. of Total Cocoons per Adult: CCAD; No. of Empty Cocoons: ECC; percentage of Hatched cocoons: % HATCH; No. of Total Young: TYG; and number of Total Young per Adult: YGAD). Working procedures are detailed in a previous publication (ASTM, 2005; Maestre et al., 2007) and test conditions are summarized in Appendix A, Table A.1.

At the end of the experiment, worms were allowed to empty their guts by placing them for 5 h in clean reconstituted freshwater, and then worms were stored at -20 ºC (for tissue residue) until the samples could be prepared. Worm dry weight was determined in an electrobalance (Sartorius M3P, D.L = 1 µg) after drying in an oven at 60 ºC for at least 48 h. For tissue residue analysis, worms were digested with ultrapure 69% HNO_3 and 30% H_2O_2 in a Hotblock and diluted to a final volume of 7 ml with Mili-Q water. Samples were stored at -20º C until analysis.

During the experiments, dissolved oxygen, pH and T (Hach HQ30d Multi-Parameter kit) were measured twice per week in the overlying water (while aeration was visually checked every working day).

2.3. Sediment characterization

Sediment and water samples were taken in order to measure metal concentrations, as well as for characterization of sediment´s AVS concentrations, TOC percentage and Particle size distribution. Water and sediment samples were frozen at -20 ºC until analysis were conducted.

AVS were extracted from wet sediment using the modified diffusion method of Leonard et al. (1996); the extracted amount of sulfides was measured with an ORION 96-16 ion-selective sulphur electrode (Ionplus, Beverly, MA, USA). Afterwards the wet/dry ratio of the sediment sample was determined in order to convert the measured sulfide value to dry weight. The remaining SEM fraction was stored at 4º C until metal analysis could be conducted. Total Organic Carbon content (TOC%) was determined following the method proposed by Bryan et al. (1985) through the loss-on-ignition method (mineralised at 450 ºC, for 6 h, in a Mufla oven). Ashes were then rewetted with distilled water and brought to constant weight at 60 ºC (USEPA, 1990). Particle size distribution was determined via laser diffraction (Malvern Mastersizer S., Worcestershire, UK, Queralt et al., 1999).

The total metal content was measured from the whole sediment (at sampling day) or from 500 µm sieved fraction (at experiments) by drying the sediments at 60 ºC for 24 h and adding a mixture of HNO_3 (69%) and HCl (37%) (1:3, v/v); subsequently, samples were transferred to Teflon® bombs and digested in a microwave oven (ETHOS 900 Microwave Labstation, Milestone, Italy) (Tessier et al., 1984). After digestion, samples were filtered through a 0.20 µm cellulose acetate filter (Schleider and Schuell MicroScience GmbH, Dassel, Germany), and diluted with ultrapure water (MilliQ®, Bedford, MA, USA) up to 50 ml and stored until metal analysis.

2.4. Metal analysis

A total of 7 metals (Cd, Cu, Co, Cr, Ni, Pb and Zn) and one metalloid (As) were measured. Here after, in order to simplify, we will use the word "metals" to refer to these eight elements. Filtered samples were analyzed by ICP-MS (Varian UltraMass 700, Victoria, Australia; Detection Limit, DL= 0.1 µg l^{-1}). When data were below DL they were substituted by ½ of DL for data analysis (only occurred in the case of Cd for days 0 to 10). Every batch of samples included 3 blanks. All batches included 3 replicate samples from reference material (BCR-142R river sediment or ERM-CE278 mussel tissue) for quality control. Sediment recovery rates in reference sediment were 64.8% for Cd, 51.9% for Cu, 49.9% for Co, 47.0% for Ni, 55.1% for Pb and 56.2% for Zn. Certified values were not available for As and Cr. In all cases Tissue Reference Material recovery rates were within the uncertainty range of the certified values for As, Cd, Cr, Cu, Pb and Zn (91.2-111.8% recovery rates). Certified values were not available for Co and Ni.

Prior to the introduction of the worms to the experimental units, a pool of worms was obtained from the culture and stored for metal tissue residue analysis (4 replicates, 4 worms per replicate).

2.5. Data processing and statistical analysis

Mortality and autotomy frequencies were compared with the control using Fisher's Exact test. For the sublethal endpoints (at 28 d) and metal tissue residues (over time exposure) one-way ANOVA test followed by posthoc Dunnett's t-test, Tukey´s t-test or t-test with Bonferroni's adjustment to compare differences with controls was used. Otherwise, Kruskal-Wallis nonparametric test followed by Mann-Whitney U test was applied. Additionally, two-way ANOVA was conducted to examine the effect on metal bioaccumulation in *T. tubifex* at different exposure times (3, 10 and 28 days) and the different studied sediments (Control, Scheppelijke nete, Kneutersloop and Molse nete). Tissue residue data were examined for detection of single outliers using Dixon´s Q-test (Newman, 1995).

Regression models over time and metal tissue residues were run, and estimated time-dependent accumulation rates from those significant regressions where compared using custom made R script (http://www.r-project.org/) based on Zar (1996) multiple slope comparison Tukey´s test. Regression models were also applied between porewater (PW), sediment total metal concentration (SED_{Me}), sediment TOC% normalization (Sed_{Me}/TOC), sediment SEM-AVS content (SEM_{Me}-AVS) and sediment SEM-AVS content TOC% normalization ([SEM_{Me}-AVS]/TOC) with metal tissue residues after 28 d exposure. To determine the relation between metal bioaccumulation and autotomy/worm biomass at different exposure times Spearman´s rank correlation was used. Additionally, median effective residues (ERs) were estimated using R software and the extension package *drc* (Ritz and Streibeig 2005). Model selection was carried out using Akaike´s information criterion (AIC) and model validation was based on graphical assessment (Burnham and Anderson, 2002; Zuur et al., 2007); see Méndez-Fernández et al. (2013) for detailed procedure.

The biodynamic concept includes the application of bioenergetic-based kinetic bioaccumulation models (Casado-Martínez et al., 2009, 2010; Kalman et al., 2014; Luoma and Rainbow, 2005). Those are based on the knowledge that organisms can accumulate metals from both water and food. Cammusso et al. (2012) showed that in *Lumbriculus variegatus* (sediment-dwelling oligochaete) when one route prevails over the others, the models can be simplified into simple equations. Here, metal uptake from these two sources were treated separately, explained in a simple way considering unidirectional Influx rates from water (I_w) and unidirectional Influx rates from food (I_F):

$$I_w = K_u * C_W \quad [1]$$

$$I_F = IR * AE * C_F \quad [2]$$

where K_u is the uptake rate from solution, C_W is the concentration of metal in solution, IR is the ingestion rate, AE is the assimilation Efficiency (%) and C_F is the concentration of metal in food (sediment ingestion in the case of *T. tubifex*). In the present study I_w has been tested using porewater metal concentrations (PW).

AE% and IR are bioenergetic terms that need to be determined in *T. tubifex* in order to determine I_F. But it has to be taken into account that *T. tubifex* exhibits two levels of selectivity in its feeding behavior (Rodriguez et al., 2001): 1) Selection is based on particle size, avoiding the ingestion of sand particles; 2) Selection of particles is associated with organic material, within the fin (silt-clay) fraction of the sediment. Additionally, the following assumptions were also considered: 1) Egestion rates are a good approximation to the ingestion rates (Cammen, 1980; Martinez-Madrid et al., 1999); 2) a reduction in sediment ingestion may occur due to

avoidance behavior of the worms as a way of minimizing the exposure to toxicants (Martinez-Madrid et al., 1999). Thus, IR were calculated from the egestion rates data reported in Martinez-Madrid et al. (1999), for a normalized worm weight of 1.5 mg and applying a mean faecal density of 4.05 mg/mm^3 (from data reported in Rodriguez et al., 2001). From this data source, two levels of IR were obtained, being of 0.372 mg mg^{-1} h^{-1} or 8.931 mg mg^{-1} d^{-1} when non toxic sediments are considered (IR_N; n=16) and of 0.157 mg mg^{-1} h^{-1} or 3.762 mg mg^{-1} d^{-1} when toxic sediments are considered (IR_T; n=4). AE% was obtained from data reported by Brinkhurst and Austin (1979), who reported that in *T. tubifex* AE% overall mean value was of 4.1%. Thus, two levels of IR were used (IR_N and IR_T) with the aim of testing the ability of I_F of predicting metal bioaccumulation, and secondly to test the relevance of sediment avoidance in toxic sediments affecting I_F (I_{F1}= IR_N * AE * SED_{Me} vs I_{F2}= IR_T * AE * SED_{Me}). The ability of I_F to predict metal bioaccumulation was tested through regression models. Additionally, sediment geochemistry variables ([SEM-AVS], TOC%) were used to assess their ability to predict I_F using regression models.

Statistical analyses were done with SPSS 19 (2011) and R software. The significance level for all test acceptance was $\alpha = 0.05$.

3. RESULTS

3.1 Sediment characteristics

Sediment and water characteristics at the day of sampling are provided in Table A.2. Among studied sediments, KN showed the highest conductivity value (2058 µS cm^{-1}) and also the lowest oxygen levels (5.63 mg O_2 l^{-1}) (Table A.2); whereas SN showed slightly acidic conditions (pH= 6.64). Metal concentrations in the sediments were above established background levels of trace metals in Flemish Rivers by De Cooman et al. (1998) and above Environmental Quality Standards in Flanders (Flemish Government, 2012). AVS content was highest in sediment from MN (194.5 µmol g^{-1} dw). Scheppelijke nete and KN had low TOC%, whereas MN had moderate TOC% (5.44%); three sediments were considered to be sandy. Control sediments had low values for all the measured variables (except a higher silt and clay content).

Sediment total metal concentrations (SED_{Me}) remained stable from the beginning to the end of the experiment (Table 1), with coefficient variation percentages < 25%. SEM concentrations varied > 25%: for Cr and Ni in Control and SN; As, Cu and Zn in KN; and for all metals in MN (Table 1). However, metal concentrations in SN and KN during the experiments were lower than measured levels at the sampling day (Table A.2). Coefficient of variation of AVS content was around 100% in all sediments. Indeed, AVS increased during storage period in SN and MN

(although experiments were conducted within 3 weeks after sediment collection), but decreased by the end of the toxicity test (compare Table A.2 and Table 1). Kneutersloop AVS content decreased since sampling moment towards the end of the experiment. Moreover, by the end of the bioassay, all the sediment had lower values of AVS, which could be related to water column oxygenation during the experiment.

3.2 Toxicity effects

During the completion of the chronic tests, overall oxygen average concentration was 8.0 ± 1.3 mg O_2 l^{-1} and pH 8.53 ± 0.22. In the individual test sediments, oxygen average concentration was 7.90 ± 1.20 in SN, 7.90 ± 1.30 in KN and 7.57 ± 1.35 mg O_2 l^{-1} in MN, and pH 8.13 ± 0.19 in SN, 8.38 ± 0.31 in KN and 7.89 ± 0.39 in MN. Acceptability criteria for chronic tests in controls (n=4) were achieved for mortality (< 10%), No. of Total Cocoon (coefficient of variation, CV< 25%) and No. of Total Young (CV< 50%) (ASTM, 2005; Maestre et al., 2007).

3.2.1. Behaviour and other Observations

The tubificid worms exhibited a normal behaviour in controls: with the head downwards the sediment and the posterior part of the body projecting upwards in water waving in a regular rhythm; also a high amount of faecal pellets could be seen in the surface, and the burrowing activity was high. In the tested sediments a weak burrowing activity was observed but no faecal pellets could be distinguished since the sediment surface layer (0-0.5 cm) was mainly desegregated. Visual observations revealed that worms were on the sediment surface with scarce movements (sediment avoidance) at different time points during the experiments, e.g. from day 7 on MN. In addition, worms exhibited autotomy in different degrees, and in KN and MN some worms had constrictions at the clitellum region. In MN worms also had sediment particles attached to the chaetae at the end of the experiment, which may be related to a higher mucus production.

3.2.2. Toxicity endpoints

In none of the tested sediments significant mortality ($p > 0.05$) was observed. Autotomy significantly increased compared to the control after 10-d exposure in MN (81.3% AUT; $p < 0.001$) (Fig.1a), and after 28-d exposure in KN (25% AUT; $p < 0.05$). In control sediment and SN, worms increased their weight (individual worm biomass) in a linear manner until day 10 (Fig.1b), and then worm weight decreased to approximately initial values (presumably due to laying of cocoons). In contrast, worms from MN maintained the same weight from day 3 to day 28, showing a significant lower biomass compared to the control after 10-d exposure ($p < 0.05$). On the other hand, worms reached a significantly higher biomass than control at day 28 in KN sediment ($p < 0.05$), although autotomy was also occurring in worms of this treatment. With

respect to reproduction endpoints, the number of TCC and CCAD were not significantly reduced compared to the control, whereas HATCH % was significantly affected in SN sediment (Table 2). Regarding TYG and YGAD, both SN and MN sediments had significant reductions compared to the control treatment, indicating that alterations in embryological development and/or post-developmental stages were occurring.

3.3 Metal Tissue Residues

Metal tissue residues in most sediments were generally significantly higher ($p < 0.05$, Table 3) after 28 d exposure than tissue residues at the beginning of the experiment (day 0). However, increasing metal tissue residues were not always found during time exposure and a decrease in metal tissue residues after 3 or 10 day exposure was observed at some sites (Table 3). For instance, at SN a significant decrease ($p < 0.05$) occurred at day 10 for all metals; Cd and Pb in MN decreased significantly ($p < 0.05$) after day 3 and then maintained constant; and Pb and Zn in KN decreased significantly ($p < 0.05$) after day 10.

Cd, Co, Cu and Pb showed a significant effect of exposure time, between different tested sediments and also the interaction between time and studied sediment was found to be significant for these metals (Two-way ANOVA, Table A.3). For As, Ni and Zn, exposure time showed a significant effect, as well as studied sediment, but the interaction between time and sediment was not found to be significant. In the case of Cr, only a significant time effect was observed and a significant interaction between time and sediment, but not a significant effect was found for sediment alone.

For As, Co, Ni and Zn significant linear regressions were obtained ($p < 0.05$) with increasing metal tissue residues in worms over time, whereas for Cd and Cu exponential models showed best fit to the data (Table A.4). For Cr and Pb no significant regressions were found ($p > 0.05$). High coefficient of determination where obtained in KN for As (R^2= 0.81), Co (R^2= 0.86), Cu (R^2= 0.91) and Zn (R^2= 0.81), whereas for other metal-sediment combinations coefficient of determination varied from low to moderate (R^2= 0.34–0.76). From these data, accumulation rates in different test sediment were estimated with highest rates for Zn, followed by As, and lower but similar for Co and Ni. Individual accumulation rates from each of the sediment were compared, and it was observed that the rate of accumulation was significantly higher ($p < 0.05$) for As, Co and Ni in KN, and Zn in MN (Fig.2). For Cu and Cd it was not possible to study significant differences between accumulation rates, but obtained values were similar for both metals in KN sediment.

If we analyzed all possible metal uptakes routes for *T. tubifex* (Table 4), it is observed that PW shows the highest coefficient of determination for As (R^2 = 0.74) and for the essential metal Ni (R^2 = 0.84), whereas PW contributed in the same amount than Sed_{Me} in the case of other essential metals, with varying coefficient of determination (Cu R^2 = 0.91; Co R^2 = 0.25; Zn R^2 = 0.52). Metal tissue concentration in *T. tubifex* showed a positive relation with sediment-bound metal concentration (Sed_{Me}) or sediment TOC% normalization (Sed_{Me}/TOC). Exponential models showed best fit between Sed_{Me} and metal tissue residue data, with high coefficient of determination for Cu (R^2= 0.89) and moderate for Ni (R^2= 0.71), Pb (R^2= 0.58) and Zn (R^2= 0.50). For Co a simple linear regression model was found to explain data best, although the coefficient of determination was low (R^2= 0.28). For As tissue concentration a moderate significant regression was only found with Sed_{Me}/TOC (R^2= 0.58). Coefficient of determination was improved only in the case of Co (R^2= 0.58) when taking into consideration Sed_{Me}/TOC. Finally, only in the case of Pb and Co sediment uptake routes, Sed_{Me} or Sed_{Me}/TOC respectively, improved the R^2 compared with PW. In the case of Cd and Cr no significant regressions were found.

Contrarily, individual metal [SEM_{Me}-AVS] content showed moderate significant positive regressions only for Cu tissue residues (R^2= 0.37), and this relation was slightly improved when taking into consideration [SEM_{Me}-AVS]/TOC, with a R^2= 0.48. In the case of Zn tissue concentration, a significant moderate negative regression was obtained for [SEM_{Me}-AVS] (R^2=0.54). In this case, a negative slope was obtained, with higher metal tissue concentration in *T. tubifex* when [SEM_{Me}-AVS] < 0, and supposedly less bioavailable.

It was observed that As, Cd and Cr bioaccumulation was not significant in either of tested IF conditions (Table 4). Cu and Ni were explained best by both I_F conditions, with very high coefficient of determinations (R^2), above 0.91 for Cu and 0.80 for Ni. For Zn and Pb moderate coefficient determinations were obtained (0.45–0.58) with either both I_{F1} and I_{F2}. In the case of Co, R^2 was improved from low to moderate when taking into account sediment avoidance (I_{F2}). Regression models using sediment geochemistry variables were used to assess their ability to predict I_F; only the non essential metal Cd resulted to be related to I_{F1}, showing a negative regression with SEM-AVS_{Cd} content (R^2= 0.98, $p < 0.05$, n= 4). No significant regressions were found between organic content and tested IF conditions.

3.4. Relating toxicity, metal bioaccumulation and sediment geochemistry

Autotomy was best correlated with Ni (r= 0.802), As (r= 0.791) and Cd (r= 0.765), and moderate correlations were obtained for Co (r= 0.541), Zn (r= 0.632) and Cu (r= 0.688) tissue residues (Table A.5). Cr and Pb did not show significant correlations with AUT. Worm biomass

was only moderately correlated with Co (r= 0.491), Cr (r= -0.557) and Pb (r= -0.610); no significant correlations were found for other metals (As, Cd, Cu, Ni, and Zn).

The relations between toxicity endpoints at 28-d and different studied variables (sediment geochemistry, influx rates and tissue residues) were analyzed through regression models (Table 5). Reproduction endpoints (young production) and/or *T. tubifex* biomass (WB) showed positive significant regressions with Cu, Co and/or Ni sediment metal concentration, influx rate from food, porewater and tissue residues ($p < 0.05$). However, coefficients of variation varied from low to moderate (R^2= 0.25–0.68) in most cases. As and Cd tissue residues also showed positive regressions with WB, as well as As and Pb porewater concentrations with toxicity endpoints; however, As and Cd were highly co-correlated with both Cu and Ni porewater and tissue residues (Pearson´s correlation $r > 0.99$, data not shown). On the contrary, As, Cd, Pb and Zn sediment concentrations and influx rates from food showed significant negative regressions with toxicity endpoints, i.e., young production (R^2= 0.32–0.76, $p < 0.05$). SEM-AVS_{Me} for Cd, Cu, Cr, Ni and Pb showed positive significant regressions with reproduction endpoints, R^2= 0.66-0.68 ($p < 0.05$), and only for Zn a moderate negative significant relationship was found between SEM-AVS_{Me} and reproduction endpoints (R^2=0.31–0.42, $p < 0.05$). Similarly, only Zn porewater concentration showed a negative significant regression with young production (R^2=0.56–0.59, $p < 0.05$), but in the case of Cr, Pb and Zn tissue residues negative significant regressions were obtained with other reproduction endpoints and/or worm biomass (R^2=0.30–0.50, $p < 0.05$).

Effective Residues (ER) at 28-d using dose-response models for reproduction endpoints were only possible to derive for Zn tissue residues. No. of Total Young (TYG) was the most sensitive endpoint for Zn exposure in *T. tubifex* with an ER_{50} of 7.40 ± 3.88 µmol g^{-1} dw (Fig. 3). Zinc ER_{20} in reproduction were of 22.57 ± 10.70 µmol g^{-1} dw for No. of Total Cocoon (TCC) and of 5.55 ± 4.25 µmol g^{-1} dw for TYG (Fig. 3). Non significant dose-response models were found for the other metals.

DISCUSSION

Total metal concentrations in the sediment were high compared to natural Flemish background levels (De Cooman et al., 1998) and EQS in Flanders (Flemish Government, 2012). The present study showed that there was a general time-dependent metal bioaccumulation, however increasing tissue residues were not always found at longer time exposure (28-d). Indeed, a decrease in metal tissue residues after 3 or 10 days exposure was observed at some sites.

Sediment total metal concentration, and for some metals porewater, were found to be a better predictor for tissue residues, whereas sediment geochemistry (e.g. SEM-AVS content) not always improved the regression models. However, changes in AVS content measured in the experiments may have consequences for metal bioaccumulation and toxicity to other macroinvertebrate species; i.e., De Jonge et al. (2012) showed that elevated oxygen concentrations in overlaying surface can directly enhance metal accumulation and toxicity in the invertebrates *Asellus aquaticus* (epibenthic) and *Daphnia magna* (pelagic).

Weak relationships were found for accumulated Cu and Zn with SEM-AVS content, but no significant relationships were found for the other metals, suggesting that SEM-AVS content is not relevant for metal uptake in *T. tubifex* in studied sediments. These observations were also made by De Jonge et al. (2009, 2010, 2011), who found that an excess of AVS did not turn to be an important factor determining metal bioaccumulation in field-collected benthic invertebrates (*Chironomus* gr. *thummi* and *Tubifex* sp.). These results may be explained by the feeding behaviour and food preferences of *T. tubifex*, an infaunal species that feeds on the fine-sediment fraction (< 63 µm) (Rodriguez et al., 2001), and its entire life cycle takes place in sediment (Reynoldson et al., 1994). The importance of the ingestion of sediment as a major route of metal bioaccumulation has been demonstrated for tubificids oligochaetes, both for *T. tubifex* (Steen Redeker et al., 2004) and for *L. variegatus* (Camusso et al., 2012). Under the biodynamic model framework, using Influx rate from food (I_F) in *T. tubifex* in a range of sediment geochemistry characteristics confirmed previous observations of these authors, showing that I_F from sediment ingestion was able to predict metal bioaccumulation, especially of the essential metals Cu, Ni and Zn, and for the non-essential metal Pb. Additionally, I_F appeared to be a better predictor for metal accumulation in *T. tubifex* compared to sediment geochemistry normalization. In future studies, in order to apply the model more accurately, we must carefully consider including I_F with varying parameters of ingestion rate (IR) and assimilation efficiency (AE%) in *T. tubifex*, that would allow to capture site-specific conditions. In Cardwell et al. (2013) both IR and AE% were obtained from bibliography and varied across the same species, and these data were successfully applied in the biodynamic model. However, within studied organisms not a detrivore was included, that would allow comparisons with the present study. Therefore, we think that the present study using *T. tubifex*, which feeds on the fine sediment fraction, with each own and probably different microorganism community, adds value for future detailed studies on metal bioaccumulation under the biodynamic framework.

During the experiments *T. tubifex* exhibited in different degrees sediment avoidance, reduction of burrowing activity and production of fecal pellets. We included a lower ingestion rate to assess the importance of sediment avoidance in toxic sediments, such as MN (I_{F2}), but this only

improved the model for Co bioaccumulation. These results may be explained by the fact that sediment avoidance prevented metal bioaccumulation for other metals via dietary uptake (but not via PW as shown), or alternatively, that sediment avoidance is of little importance for metal uptake in the present study. However, behavioral responses, such as sediment avoidance, is both a realistic and ecologically relevant response in tubificid worms, and proposed by different authors as an escape response from polluted sediments (Gerhardt, 2007; Meller et al., 1998; Méndez-Fernández et al., 2013). Mucus production was also observed in worms exposed to MN, where a significant decreases of Cd and Pb tissue residues where observed after day 3 (and maintained constant during the rest of the experiment). The production of a mucus/metal complex has been interpreted as a barrier to metal uptake by different authors, e.g., in tubificids after exposure to Pb and Zn (Whitley, 1968), in *T. tubifex* after exposure to Cd in 96 h water-only (Bouchè et al., 2000), and in *T. tubifex* after chronic exposure (28-d) to Cu-spiked sediment (Mendez-Fernández et al., 2013). Using non-destructive toxicity endpoints, such as behavioral responses, could provide relevant ecological information on sediment pollution, but standardization and calibration of the methods needs to be done in order to be used in future toxicity studies.

Autotomy has been proposed by several authors as a mechanism to detoxify metals and organic compounds in aquatic oligochaetes (Lagauzère et al., 2009; Lucan-Bouchè et al., 1999a; Paris-Palacios et al., 2010; Vidal and Horne, 2003). In the present study, autotomy was promoted during the exposure period in all studied sediments, with the highest frequency in MN (81.3%, day 10) and KN (25%, day 28). Autotomy frequency was also significantly correlated with increasing As, Cd, Cu, Co, Ni and Zn tissue residues. The regeneration of a functional posterior end (i.e., when defecation is possible) occurs 7 days after autotomy (Bouchè et al., 2003), so it is plausible that it may have occurred at several times during the experiments without noticing it. Anyway, autotomy has been shown to be an early response indicator of sediment toxicity for *T. tubifex,* which supports other author´s findings of using autotomy as a useful tool for monitoring exposure and effects of metal contamination in aquatic oligochaetes (Lagauzère et al., 2009; Lucan-Bouchè et al., 1999b; Meller et al., 1998; Méndez-Fernández et al., 2013).

Impairment in reproduction and/or growth was observed in *T. tubifex* exposed to SN and MN sediments, but not at KN. But, reductions in worm biomass may be also explained by the high autotomy percentage detected in MN at day 10. Offspring production (TYG or YGAD) was the most sensitive endpoint, as has been previously reported by other authors for *T. tubifex* (Gillis et al., 2002; Maestre et al., 2007; Méndez-Fernández et al., 2013; Reynoldson et al., 1991; Vecchi et al., 1999). According to Reynoldson and Day (1998) criteria for determining toxicity in near shore sediment of the Great lakes regarding young production in *T. tubifex* are: non-toxic: > 9.9

YGAD, potentially toxic: 9.8–0.8 YGAD, and toxic: < 0.8 YGAD. Based on these criteria, SN and MN would be classified as potentially toxic regarding young production.

Maximum Cu and Zn tissue residues measured in the present study (3.49 and 18.05 µmol g^{-1} dw, respectively) were close to maximun reported values for field-collected *T. tubifex* 3.40 µmol Cu g^{-1} dw and 9.02 µmol Zn g^{-1} dw where moderate to severe sediment pollution has been observed (De Jonge et al., 2010). Gillis et al. (2006) reported up to 35.59 µmol Zn g^{-1} dw in field-collected oligochaetes (pool of different species) in highly polluted sediments. But for the other metals, maximum measured tissue residues were bellow reported values for field collected tubificids (data not shown), and were not related with reproduction impairment using dose-response models. Contrarily, Zn ERs were successfully obtained from reductions in reproductive endpoints (5.55–7.40 µmol Zn g^{-1} dw for TYG, 25.74 µmol Zn g^{-1} dw for TCC). These values are similar to those reported by other authors relating tissue Zn thresholds with ecotoxicologically relevant endpoints in *T. tubifex* and other aquatic macroinvertebrates: Méndez-Fernández et al. (2014) reported tissue concentrations of 33.31 and 42.10 µmol Zn g^{-1} dw (ER_{50} for TCC and SUR, respectively) in *T. tubifex* exposed to mine sediments; De Jonge et al. (2013) estimated Zn concentrations in aquatic insects associated with a 20% decrease in taxonomic completeness to be 14.8 µmol g^{-1} dw for Simulidae, 15.8 µmol g^{-1} dw for Perlodidae and 27.5 µmol g^{-1} dw for *Leuctra* sp; and Schmidt et al. (2011) obtained that 4.08 µmol g^{-1} dw in *Rhithrogena* spp. and 9.70 µmol g^{-1} dw for *Drunella* spp. resulted in 20% reduction in macroinvertebrate population density. However, the essential nature of zinc for all living organisms and additive interactions with other metals (Cd, Cu, Pb and Ni), complicates the assessment of this element in toxicity studies associated to bioaccumulation (Eisler, 2000). Recently, De Jonge et al. (2013) have demonstrated a stronger decrease in taxonomic completeness related to lower Zn critical body burdens in *Rhitrogena* sp. in presence of higher Cu bioavailability than taken Zn exposure alone. Thus, the study of metal mixture toxicity is a field that requires more attention in future studies. In addition, it is known that the relationship between sediment geochemistry and metal accumulation by aquatic invertebrates varies between taxonomic groups and is highly dependent on feeding behavior and ecology (De Jonge et al., 2010). Therefore, future sound ecological risk assessment of metals requires the study of various organisms with different positions in the aquatic trophic web and with different metal exposure routes.

CONCLUSIONS

Metal bioaccumulation and toxicity in *T. tubifex* exposed to metal-contaminated field-sediments was dependent on time exposure and detoxification capability, such as autotomy, and was

influenced by total sediment metal concentration and organic content normalization. However AVS content did not play a major role in influencing toxicity or metal bioaccumulation. For the essential metals (Cu, Ni and Zn) and As, porewater was also a relevant metal uptake route. Under the biodynamic framework, the measure of the Influx rate (I_F) from food (sediment ingestion) in *T. tubifex* showed that it was a better predictor of metal bioaccumulation and toxicity rather than sediment geochemistry, especially for the essential metals Cu, Ni and Zn, and for the non-essential metal Pb.

ACKNOWLEDGEMENTS

Leire Méndez-Fernández was assisted in this work by a doctoral grant from the Basque Government. Maarten De Jonge is funded by a post-doctoral research grant of the Research Foundation Flanders (FWO). We gratefully acknowledge Steven Joosen for accurate metal analyses. Our special thanks go to Dr. Rodriguez and Dr. Martínez-Madrid for their useful comments in *T. tubifex* physiology and for providing culture worms. We thank P. Markaide for his valuable contribution on the custom made R script. We are also gratefully acknowledged to the anonymous reviewers that help improving this manuscript with useful comments and suggestions.

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LEGEND OF FIGURES

Fig.1. a) Autotomy percentage and **b)** Worm individual biomass (mg dw) at each time point for control and tested sediments. Symbols: ○: Control; ■: Scheppelijke nete; ▲:Kneutersloop; ◆: Molse nete. Significant differences between tested sediments and respective control at each time point are marked: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig.2. Linear regression models for metal bioaccumulation in *T. tubifex* worms over time (days 0, 3, 10 and 28). Symbols: ○: Control (n=4); ■: Scheppelijke nete (n=4); ▲:Kneutersloop (n=4); ◆: Molse nete (n=4). Different letters represent significant differences between slopes of linear regressions.

Fig.3. Dose-response model for Zn tissue residues and reproduction endpoints after 28 day chronic bioassays. a) TCC followed a 3 parameters Weibull type 1 model; b) TYG followed a 3 parameters Weibull type 2 model. Dotted line = Effective Residue that causes 20% reduction in the endpoint (ER_{20}); Dashed line = Effective Residue that causes 50% reduction in the endpoint (ER_{50}). Abbreviations: TCC: number of Total Cocoons; TYG: number of Total Young.

TABLES CAPTION

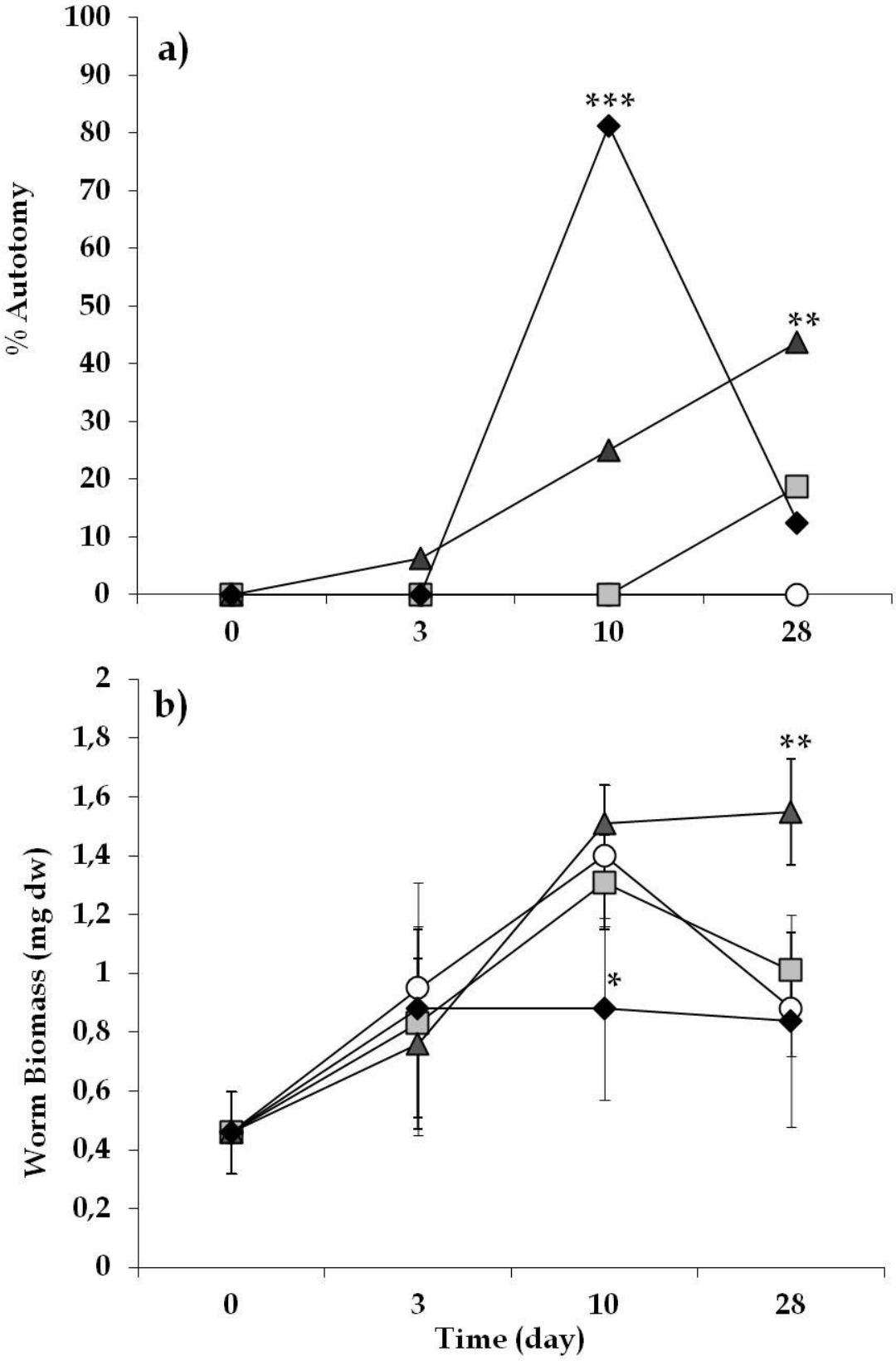
Table 1. General sediment characteristics in time dependant exposure experiment. Values above Flemish Environmental Quality Standards are indicated in bold (Flemish Government, 2012). Abbreviations: SN= Scheppelijke nete, KN= Kneutersloop; MN= Molse nete; SED_{Me}= Total sediment metal concentration (µmol g^{-1} dw); SEM= Simultaneously extracted metal concentration (µmol g^{-1} dw); PW= Porewater metal concentration (µmol l^{-1}); TOC= Total Organic Carbon percentage; AVS= Acid Volatile Sulfides (µmol g^{-1} dw): DL: Detection limit. For SED_{Me} and SEM the range at beginning (0d) and the end (28d) is indicated, with coefficient variation percentage in parenthesis. For PW only data measurements at 28d are indicated.

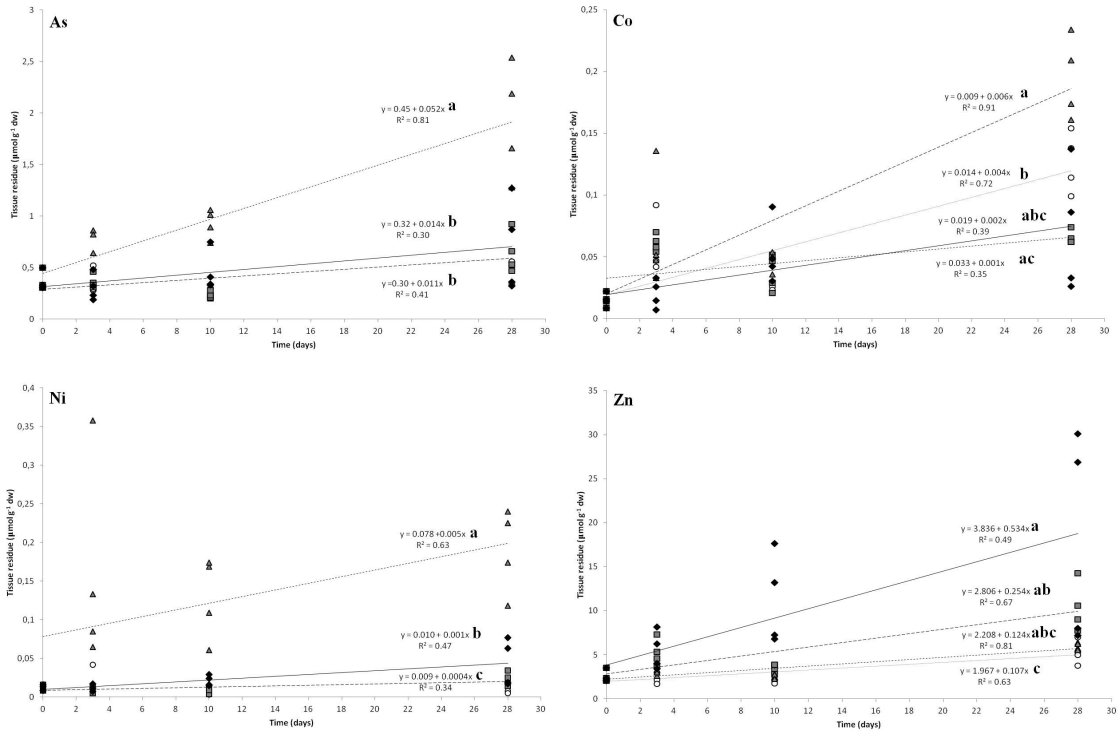
Table 2. Measured reproduction endpoints after 28d exposure in studied sediments. Abbreviations: SN= Scheppelijke nete, KN= Kneutersloop; MN= Molse nete; TCC= No. of Total Cocoons; CCAD= No. of cocoon per adult; ECC= No. of Empty Cocoons; % HATCH= Hatch percentage; TYG= No. of Total Young; YGAD= No. of young per adult. Significant differences between tested sediments and respective control are marked: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

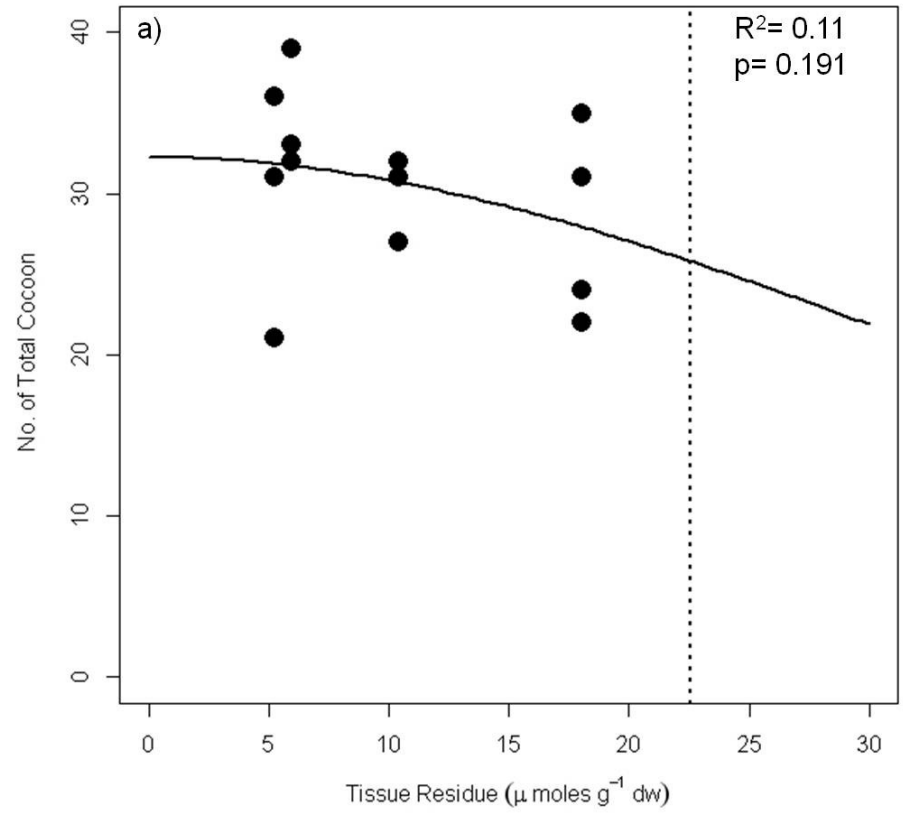
Table 3. Metal Tissue Residues for each sediment and at each time point. Data in µmol g^{-1} dw. Abbreviations: SN= Scheppelijke nete, KN= Kneutersloop; MN= Molse nete. Different letters represent significant differences ($p < 0.05$) over time for each sediment.

Table 4. Regression models for the prediction of metal bioaccumulation due to all possible uptake routes in *Tubifex tubifex*. Abbreviations: PW: pore water; Sed_{Me}: total metal concentration in the sediment; Sed_{Me} /TOC: total metal concentration in sediment normalized for Total Organic Content; [SEM_{Me}-AVS]/TOC: individual metal molar differences between SEM and AVS normalized for organic matter content; TR: Tissue residue (bioaccumulation). Influx rate from sediment (I_F), evaluated at two levels (I_{F1} takes all sediments into account while I_{F2} accounts for sediment avoidance in MN toxic sediment). Only significant regression models are presented. No significant regression models were found for Cd and Cr.

Table 5. Coefficient of determination (R^2) of the significant regression models for the prediction of individual metal toxicity in *Tubifex tubifex* due to all possible variables. Abbreviations: Sed_{Me}: total metal concentration in the sediment; [SEM_{Me}-AVS]: individual metal molar differences between SEM and AVS; PW: pore water; I_F: Influx rate from sediment (evaluated at two levels; I_{F1} takes all sediments into account while I_{F2} accounts for sediment avoidance in MN toxic sediment); TR: Tissue residue (bioaccumulation); Endpoints as in Table 2. Arrows indicates positive or negative regression model for the combination of each endpoint-variable.


a)
% Autotomy
100
90
80
70
60
50
40
30
20
10
0
***
**
0
3
10
28
b)
Worm Biomass (mg dw)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
**
*
0
3
10
28
Time (day)


As
y = 0.45 + 0.052x a
R² = 0.81
y = 0.32 + 0.014x b
R² = 0.30
y =0.30 + 0.011x b
R² = 0.41
Tissue residue (µmol g⁻¹ dw)
Time (days)
Co
y = 0.009 + 0.006x a
R² = 0.91
y = 0.014 + 0.004x b
R² = 0.72
y = 0.019 + 0.002x abc
R² = 0.39
y = 0.033 + 0.001x ac
R² = 0.35
Tissue residue (µmol g⁻¹ dw)
Time (days)
Ni
y = 0.078 +0.005x a
R² = 0.63
y = 0.010 + 0.001x b
R² = 0.47
y = 0.009 + 0.0004x c
R² = 0.34
Tissue residue (µmol g⁻¹ dw)
Time (days)
Zn
y = 3.836 + 0.534x a
R² = 0.49
y = 2.806 + 0.254x ab
R² = 0.67
y = 2.208 + 0.124x abc
R² = 0.81
y = 1.967 + 0.107x c
R² = 0.63
Tissue residue (µmol g⁻¹ dw)
Time (days)


a)
R^2= 0.11
p= 0.191
No. of Total Cocoon
0
10
20
30
40
0
5
10
15
20
25
30
Tissue Residue (μ moles g^{-1} dw)

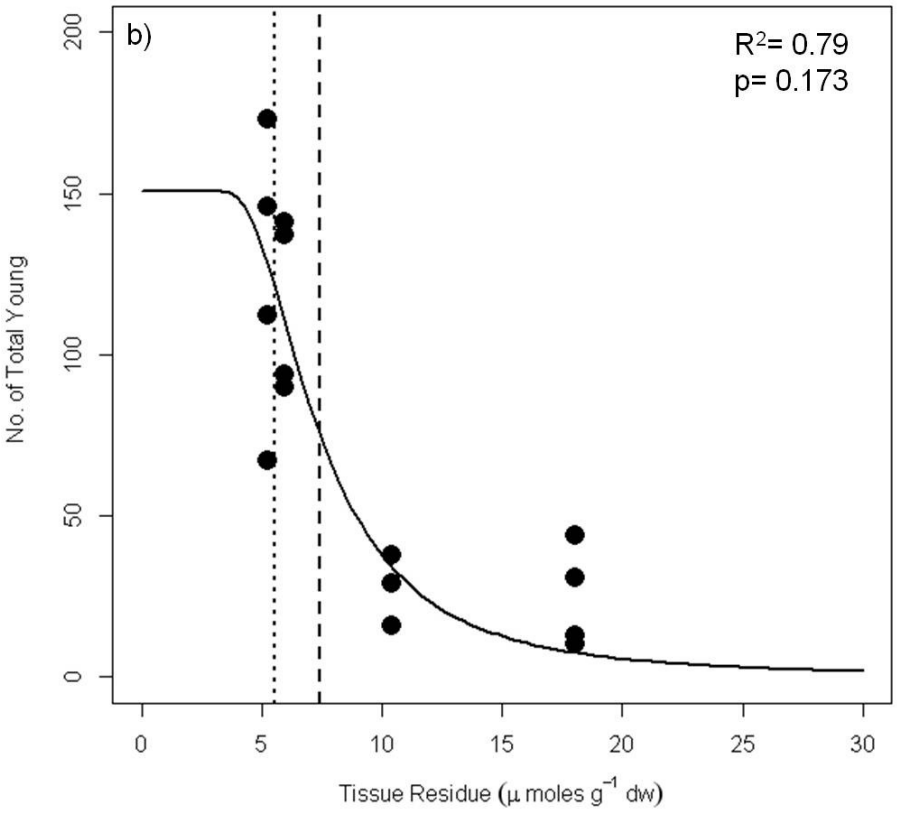

b)
R^2= 0.79
p= 0.173
No. of Total Young
0
50
100
150
200
0
5
10
15
20
25
30
Tissue Residue (μ moles g^{-1} dw)

Table A.1. *Tubifex tubifex* Time Dependant Exposure test conditions. Abbreviations: % SUR= Survival percentage; % AUT= Autotomy percentage; TCC= No. of Total Cocoons; CCAD= No. of cocoon per adult; ECC= No. of Empty Cocoons; % HATCH= hatch percentage; TYG= No. of Total Young; YGAD= No. of young per adult; WB= Worm individual Biomass (mg); % CV= coefficient of variation percentage.

Parameter	**Conditions**
Test type:	Whole-sediment toxicity test without renewal of overlying water
Temperature:	22 ± 1.5 ^{o}C
Light:	None
Sediment:	100 ml sieved through 500 µm mesh size
Overlying water:	100 ml reconstituted water (ISO 6341-1982)
Age of organisms:	Adult mature worms (first reproductive cycle, 7-8 weeks old)
Number of	4
Number of	4 + 1 replicate for Chemicals analysis at 28d exposure
Feeding:	Trout flakes (80 mg per beaker at the beginning of the test)
Aeration:	Yes
Test duration:	3d, 10d & 28 d
Overlying water control	Dissolved oxygen, temperature, pH
Endpoints:	% SUR, % AUT, TCC, CCAD, ECC, %HATCH, TYG, YGAD &
Test acceptability:	In controls: ≥90% mean survival, <25% CV for production of total cocoons, <50% CV for production of total young

Table A.2. Sediment and water characteristics measured at sampling day. Abbreviations: SN= Scheppelijke nete, KN= Kneutersloop; MN= Molse nete; FBC: Flemish Background Criteria (De Cooman et al., 1998); EQS: Environmental Quality Standards in Flanders (Flemish Government, 2012); TOC%: Total Organic Carbon Percentage; AVS: Acid Volatile Sulfides; n.m: not measured. Metal concentrations expressed as µmol g^{-1} dw. Values in bold are above EQS.

	Control	SN	KN	MN	FBC	EQS
Sediment Characteristics						
As	0.06	**0.41**	**0.57**	**0.60**	0.28	0.25
Cd	0.004	**0.21**	**0.05**	**0.29**	0.004	0.01
Co	0.06	0.07	0.57	0.14	-	-
Cr	**0.26**	0.10	0.14	**0.31**	0.25	1.19
Cu	0.04	**0.28**	**14.10**	**0.42**	0.14	0.31
Ni	0.18	0.11	**5.35**	0.19	0.15	0.27
Pb	0.04	**0.21**	**0.24**	**0.32**	0.10	0.19
Zn	0.15	**51.18**	**2.60**	**69.57**	1.76	2.25
TOC %	3.1	1.19	0.69	5.44	-	-
Clay %	28.6*	1.67	0.85	0.95	-	-
AVS (µmol g^{-1} dw)	n.m	179.6	54.9	194.5	-	-
Water Characteristics						
O_2 (mg l^{-1})	9.0	9.6	5.6	8.8		
O_2 %	92.4	82.9	54.1	76.9		
T (°C)	10.1	8.9	13.5	9.6		
pH	8.0	6.6	7.39	7.2		
Conductivity (µS cm^{-1})	425.8	447	2058	421		

* Expressed as Silt & Clay percentage

Table A.3. Summary of Two-Way ANOVA between time dependant exposure and studied sediment for each metal.

Factors	DF	SS	MS	F	p
As					
Time	2	0.883	0.441	22.102	7.08×10^{-7}
Sed	3	1.892	0.63	31.562	6.13×10^{-10}
Time * Sed	6	0.217	0.036	1.815	0.125
Residuals	34	0.679	0.019		
Cd					
Time	2	3.533	1.766	20.786	1.12×10^{-6}
Sed	3	3.861	1.287	15.141	1.75×10^{-6}
Time * Sed	6	4.61	0.768	9.04	5.49×10^{-6}
Residuals	35	2.975	0.081		
Co					
Time	2	1.539	0.769	21.553	7.92×10^{-7}
Sed	3	0.731	0.243	6.827	0.0009
Time * Sed	6	0.975	0.162	4.549	0.0016
Residuals	35	1.25	0.035		
Cr					
Time	2	0.751	0.375	5.654	0.007
Sed	3	0.127	0.042	0.637	0.596
Time * Sed	6	1.403	0.233	3.517	0.008
Residuals	34	2.26	0.066		
Cu					
Time	2	1.416	0.708	27.061	9.3×10^{-8}
Sed	3	8.315	2.771	105.93	2.2×10^{-16}
Time * Sed	6	1.605	0.267	10.224	1.83×10^{-6}
Residuals	34	0.889	0.026		
Ni					
Time	2	1.013	0.506	8.493	0.0009
Sed	3	11.032	3.677	61.647	4.76×10^{-4}
Time * Sed	6	0.773	0.128	2.161	0.07
Residuals	35	2.087	0.059		
Pb					
Time	2	0.796	0.398	8.716	0.0008
Sed	3	3.214	1.071	23.738	1.69×10^{-8}
Time * Sed	6	1.289	0.214	4.702	0.0013
Residuals	35	1.599	0.045		
Zn					
Time	2	1.198	0.599	27.228	8.72×10^{-8}
Sed	3	1.721	0.573	26.08	6.11×10^{-9}
Time * Sed	6	0.251	0.041	1.904	0.108
Residuals	35	0.748	0.022		

Table A.4. Accumulation rate (Accum. rate, d^{-1}) from fitted regression models for the relationship between metal bioaccumulation in *T. tubifex* over time exposed to tested sediments. Best fitted significant

model in each case is presented. Abbreviations: SN= Scheppelijke nete, KN= Kneutersloop; MN= Molse nete; N.S= Non Significant regression.

Metal	Sediment	Accum. rate	Model	F, p-value	R^2
As	Control	-	-	N.S	-
	SN	0.011	Linear	$F_{1,14}$= 9.724, p= 0.008	0.41
	KN	0.052	Linear	$F_{1,14}$= 55.992, p= 0.000	0.81
	MN	0.014	Linear	$F_{1,14}$= 6.051, p= 0.028	0.3
Cd	Control	-	-	N.S	-
	SN	-	-	N.S	-
	KN	0.121	Exponential	$F_{1,14}$= 41.534, p= 0.000	0.76
	MN	0.045	Exponential	$F_{1,14}$= 5.562, p= 0.033	0.28
Cu	Control	-	-	N.S	-
	SN	-	-	N.S	-
	KN	0.110	Exponential	$F_{1,14}$= 81.905, p= 0.000	0.86
	MN	-	-	N.S	-
Co	Control	0.004	Linear	$F_{1,14}$= 32.802, p= 0.000	0.72
	SN	0.001	Linear	$F_{1,14}$= 7.551, p= 0.016	0.35
	KN	0.006	Linear	$F_{1,14}$= 128.792, p= 0.000	0.91
	MN	0.002	Linear	$F_{1,14}$= 8.969, p= 0.010	0.39
Ni	Control	-	-	N.S	-
	SN	0.0001	Linear	$F_{1,14}$= 7.186, p= 0.018	0.34
	KN	0.005	Linear	$F_{1,14}$= 22.558, p= 0.000	0.63
	MN	0.001	Linear	$F_{1,14}$= 12.568, p= 0.003	0.47
Zn	Control	0.107	Linear	$F_{1,14}$= 21.737, p= 0.000	0.63
	SN	0.254	Linear	$F_{1,14}$= 28.935, p= 0.000	0.67
	KN	0.124	Linear	$F_{1,14}$= 54.439, p= 0.000	0.81
	MN	0.534	Linear	$F_{1,14}$= 13.367, p= 0.003	0.49

Table A.5. Spearmans´s Correlation coefficient (r) Autotomy and worm biomass as dependant variables and mean metal tissue residues, measured for all time points and all sediments (n=16), as independent variables.

Variable/ Metal	Spearman´s rank cor.
Autotomy	
As	r= 0.791, p= 0.000
Cd	r= 0.765, p= 0.001
Co	r= 0.541, p= 0.030
Cu	r= 0.688, p= 0.003
Ni	r= 0.802, p= 0.000
Zn	r= 0.632, p= 0.009
Worm Biomass	
Co	r= 0.491, p= 0.053
Cr	r= -0.557, p= 0.025
Pb	r= -0.610, p= 0.012