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1 Identification of threshold body burdens of metals for the protection of the aquatic ecological status
2 using two benthic invertebrates.

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4 Lieven Bervoets, Maarten De Jonge, Ronny Blust

5 Department of Biology, Systemic Physiological and Ecotoxicological Research group, University of
6 Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

7 Email contact: lieven.bervoets@uantwerpen.be

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Abstract

In this study accumulated concentrations of As, Cd, Cr, Cu, Ni, Pb and Zn in two benthic invertebrate taxa, *Chironomus sp.* and Tubificidae are related to ecological responses expressed as changes in macro invertebrate communities. In addition critical body burdens were estimated above which ecological quality was always lower than a certain threshold. Data from existing studies on bioaccumulation of the metals in both taxa were combined with different biological water quality indices. For all metal-endpoint combinations threshold values could be calculated above which ecological quality was always low. Safe threshold body burdens could be estimated for both species for all metals although the data set was more extended for *Chironomus sp.* with estimated threshold values being 65, 3.2., 10, 57, 6.5, 73 and 490 µg/g dw for As, Cd, Cr, Cu, Ni, Pb and Zn.

This study demonstrated that metal accumulation in resistant species such as chironomids and tubificid worms have the potential to be used as predictors of ecological effects in aquatic ecosystems. However, the estimated threshold values have to be validated and supported by more lines of evidence before they can be used by regulators.

24

Key words : metal accumulation ; biological water quality ; critical body burdens ; benthic invertebrates

26

Capsule Abstract

Maximal body burdens of metals in two invertebrate species could be estimated above which ecological quality was always low.

1. Introduction

Monitoring of micro pollutants in aquatic ecosystems is mainly based on measurements in the environment, i.e. in the water column, suspended matter and/or sediment (e.g. Kördel et al., 2013; Loos et al., 2008; Roots and Roose, 2013). However, these measurements only reflect the momentary pollution status and do not take into account possible differences in bioavailability, affected by abiotic factors such as pH, water hardness and temperature and by biotic factors such as feeding habits (Luoma, 1983; Tessier et al., 1984; Bervoets et al., 1997; Clearwater et al., 2002). As a consequence, current water or sediment quality criteria for micro pollutants are not necessarily adequate and well related to effects on aquatic communities observed in the real world (Bervoets et al., 2005; De Jonge et al., 2012; Van Ael et al., 2014). Direct measurement of pollutants in biota could tackle these problems. For this, indicator species are needed which are tolerant of pollution and can accumulate high levels of micro contaminants in their tissues. Moreover, these tissue levels should be related to changes in ecological relevant end-points such as the structure of aquatic communities. Recent studies have demonstrated that accumulated micro-pollutant levels in some aquatic invertebrates (e.g. Luoma et al., 2010; Rainbow et al., 2012; De Jonge et al., 2012; 2013) can be potentially used to predict ecological effects of micro contamination on aquatic communities and to estimate safe pollutant levels. As stated by Rainbow et al. (2012) risk assessments should put a greater emphasis on ‘lateral risk assessment’ by integrating biological and ecological principles from field observations. Several countries worldwide use biotic indices based on the macro invertebrate composition to evaluate the general status of the ecological quality of surface waters (e.g. Metcalfe, 1989; Abbasi and Abbasi, 2011; Nichols and Dyer, 2013). Within the aquatic macro invertebrates especially Ephemeroptera, Plecoptera and certain families of Trichoptera, the so-called EPT-taxa proved to be sensitive to micro pollutants and more specifically metals (Beasley and Kneale, 2003; De Jonge et al., 2008; Rainbow et al., 2012). In order to comply with the guidelines of the European Water Framework Directive (WFD) many countries moved from biotic indices, which are based only on the presence and

sensitivity of macro invertebrate taxa, towards a multimetric index that provides a more complete description of the community (Böhmer et al., 2004; Rosenberg et al., 2004; Solimini et al., 2008; Teixeira et al., 2009; Mondy et al., 2012). In Flanders (Belgium) until about 4 years ago the Belgian Biotic Index (BBI; De Pauw and Vanhooren, 1983) was used to evaluate the biological water quality based on macro invertebrates. The BBI combines the diversity of a sample based on certain taxonomic groups with the tolerance to pollution of individual taxa into one score, ranging from 0 (very bad) to 10 (very good). Since 2010 the Multimetric Macroinvertebrate Index Flanders (MMIF) developed by Gabriels et al. (2010) is used by the Flemish Environment Agency (FEA). The MMIF combines 5 metrics including taxa richness, number of EPT-taxa, number of sensitive taxa (exclusive EPT-taxa); Shannon-Weiner diversity and mean tolerance score. The resulting MMIF-score will change from 0 to 1, with score 0 representing a very low score and score 1 a very high ecological quality.

Rainbow et al. (2012), who related metal accumulation in hydropsychid (Trichoptera) caddisfly larvae to mayfly abundance to estimate threshold values for metals, recommend in their paper that this approach should be tested in other metal-contaminated freshwater rivers in the world. In Flanders however, even under uncontaminated conditions hydropsychid larvae are not very abundant (from absent to < 1 % of taxa; www.vmm.be/geoview). In addition, the number of mayfly species is rather limited under undisturbed conditions (Lock and Goethals, 2011). This is probably due to the fact that almost all water courses have mainly soft sediments as substratum. Therefore we had to consider other suitable species or taxa. Good candidates are larvae of the non-biting midge (Chironomidae) and oligochaeta worms of the family of the tubificidae. Both taxa are sediment dwelling organisms and good accumulators of metals and tolerate low levels of oxygen, making them suitable as biomonitors in a broad range of water qualities (e.g. Bervoets et al., 1997; Chapman, 2001; Di Veroli et al., 2014). In addition, a good relationship was found between cadmium body burdens in Chironomidae and ecological endpoints on a restricted data set by De Jonge et al. (2012). Instead of mayfly abundance the MMIF-index and the number of EPT-taxa seem good endpoints to assess the

ecological quality of freshwaters with a soft substratum (Beasley and Kneale, 2003; De Jonge et al., 2008; Rainbow et al., 2012).

The aim of this study is therefore to investigate the possible relationships between accumulated metals in *Chironomus sp.* and/or Tubificidae and ecological endpoints expressed as the BBI, the MMIF, the total number of taxa, the number of EPT-taxa and the relative abundance of Ephemeroptera. Furthermore, we also investigated the possibility to define thresholds of accumulated metals which can be considered as critical tissue concentrations to protect the aquatic ecosystem.

2. Materials and Methods

2.1. Metal concentrations in Chironomids and Tubificidae.

Data on metal body burdens in *Chironomus sp.* (Chironomidaen Diptera) and Tubificidae (Oligochaeta) were derived from several sampling campaigns between 1990 and 2010. All sites were situated in Flanders with most of them in the Campine region in the catchments of the Grote and Kleine Nete (Scheldt basin) or the Meuse River basin. Data on the metal body burdens were partially published elsewhere (Bervoets et al., 1994; 1997; 2004; De Jonge et al., 2009; 2010; 2012) and partially unpublished).

In general the sampling procedure was similar at all sites. Invertebrate samples were taken with a pond net (500 mm mesh, 200-300 mm frame and 500 mm bag depth) fitted to a 1.5 m handle. From all sites invertebrates that were selected for metal analysis were identified as *Chironomus sp.* gr. thummi and/or Tubificidae if present in sufficient numbers. Concerning *Chironomus sp.* only fourth instar larvae were collected. Between 5 and 20 individuals were pooled per taxon per site. The collected invertebrates were depurated by placing them for 24 h in artificial OECD (Organization of Economic Cooperation and Development) water (2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 500 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 771 mM NaHCO_3 and 77.1 mM KCl). The samples were dried for at least 24 h at 60 °C in polypropylene 14 ml tubes. Subsequently, the biological material was digested with concentrated ultrapure nitric acid (69% HNO_3) in a microwave (Blust et al., 1988) and stored until analysis for maximally one

month. Metals were analyzed with inductively coupled plasma mass spectrometer (ICP-MS) from different types and brands. For the already published data see for details on the type of instruments and quality control in the papers mentioned above. For the unpublished data, metals were analyzed using a high resolution ICP-MS (HR-ICP-MS; Thermo Scientific, Finnigan Element 2, Waltham, MA, USA) or a Q-ICP-MS (Varian Ultra Mass 700, Australia). Analytical quality was assured by measuring blanks and certified reference material (mussel tissue CRM 278) from the Community Bureau of Reference (European Union, Brussels). Recoveries were always within 10% of the certified values. Depending on the study different metals were analyzed. However, at all selected sites Cd, Cu, Pb and Zn were analyzed and at most sites also As, Cr and Ni.

2.2. Biological quality data

In total 88 sites were sampled but although most sites were sampled only once, some sites were repeatedly sampled between 1990 and 2009 (2 to 7 times), resulting in a total of 156 samples from which either *Chironomus*, Tubificidae or both were analysed on metals.

From about 97 samples all macro-invertebrates were collected from the net samples and identified. As a result we could calculate the Belgian Biotic Index (BBI) according to De Pauw and Vanhooren (1983), and the MMIF according to Gabriels et al. (2010). For an additional 59 samples we were able to retrieve the ecological data from the monitoring network of the FEA (www.vmm.be/geoview).

Only assessments of biotic data collected by the FEA that were within 30 days before or after our sampling were included in the dataset unless drastic changes in the water courses took place after our collection, e.g. due to removal of discharges or dredging of the river. In the latter case these data were excluded from the analysis. In addition to the BBI and the MMIF it was possible to assess the total number of taxa, the number of EPT taxa and the relative abundance of Ephemeroptera (mayfly larvae) at most sites. Combining the data on the tissue residues of metals with own biological assessment or by coupling to the VMM database resulted in a maximum (depending on the metal considered) of 137 samples for *Chironomus* and 62 samples for Tubificidae for which body burdens could be related

134 to ecological endpoints. From all samples information on BBI and total number of taxa was available,
135 whereas from 122, 132 and 127 samples information on respectively MMIF, EPT taxa and
136 Ephemeroptera abundance was obtained.

137

138 *2.3. Physical and Chemical Analysis*

139 From each site, water chemistry variables that largely affect macro invertebrate communities were
140 available including conductivity, pH, oxygen content, ammonium and orthophosphate. In addition,
141 data on dissolved metal concentrations were available from about half of the sites. These variables,
142 were either measured by ourselves according to the methods described in Bervoets et al. (1994; 1997;
143 2004) and De Jonge et al. (2009; 2010; 2012) or were measured by the FEA and retrieved from their
144 website (www.vmm.be/geoview).

145

146 *2.4. Statistical analysis*

147 The individual accumulated metal concentrations are related to the different ecological endpoints
148 applying scatterplots. The aim was to estimate thresholds of accumulated metals above which good
149 ecological quality was never reached. These thresholds are 6 for the BBI and 0.60 for the MMIF
150 (Gabriels et al., 2010). Based on undisturbed sites in Flanders the thresholds for total number of taxa,
151 total EPT taxa and Ephemeroptera abundance can be put on 16, 4 and 0.15 respectively
152 (www.vmm.be/geoview).

153 Because other factors than metals might affect the ecological responses as well (e.g. general water
154 characteristics, food availability, habitat quality), a linear relationships was not expected between
155 accumulated metals and community responses. Therefore quantile regression analysis was applied.
156 With this type of regression analysis it was possible to estimate the 90th quantile of ecological
157 responses as a function of an environmental stressor and thus to relate to only the maximum ecological
158 responses and to compensate for the un modelled factors such as the general water quality variables
159 or other contaminants (Crane et al., 2007; Linton et al., 2007). The 90th quantile regression models

160 ($\tau = 0.9$) were constructed using the quantreg package of the statistical software R (Koenker, 2005).
161 Based on the significant constructed quantile regression models critical accumulated metal
162 concentrations representing a certain threshold of the biotic indices could be calculated (De Jonge et al.,
163 2013).
164 However, only for a few combinations of accumulated metal and ecological endpoints significant
165 quantile regression models could be constructed. Therefore in addition, the concentration thresholds
166 were calculated as the 95th percentile of the concentrations measured in organisms present at sites
167 with a good ecological score. In order to exclude or minimize the effect of general water
168 characteristics on the ecological quality the same calculation was done on only those sites where the
169 quality standards, with respect to the water characteristics, had been met.
170 Nonparametric Wilcoxon signed rank tests were applied to test for significant differences in metal
171 concentrations between *Chironomus sp.* and Tubificidae. Spearman rank correlations were applied
172 to examine correlations between metal concentrations in both taxa and among the different ecological
173 endpoints and to investigate relationships between water characteristics and ecological endpoints and
174 between accumulated concentrations and ecological endpoints. When carrying out multiple
175 comparisons α was adjusted using a Bonferroni correction (Sokal and Rohlf, 1981) Median values
176 are presented in the tables.
177

178 3. Results

179 3.1 Water chemistry and invertebrate body burdens.

180 For all of the water chemistry variables measured in the period between 1990 and 2009 a broad range
181 was observed (table 1), some sites having a very low oxygen levels and/or very high orthophosphate
182 and ammonium levels and/or high conductivity.

183 The metal body burdens also varied substantially among different samples and for most measured
184 metals very high maximal concentrations were measured in both *Chironomus* and Tubificidae (table
185 2). From about 51 samples both *Chironomus sp.* and Tubificidae collected at the same moment were
186 analysed on metals (always on Cd, Cu and Zn and in most cases also on the other metals). With a
187 Wilcoxon signed rank tests differences in accumulation were tested between both taxa. Only for Cd
188 and As were body burdens significantly different with higher concentrations measured in Tubificidae
189 (As: $Z=3.401$, $p < 0.001$; Cd: $Z= 2.581$, $p=0.01$).

190 For both chironomid larvae and tubificid worms a spearman rank correlation analysis was performed
191 among the accumulated metals and between the accumulated metals and the water chemistry
192 variables. Only few significant correlations were found with R-values ranging from 0.29 to 0.71. In
193 some cases even negative R-values were found (see table SI.1.)

194 In addition a spearman rank correlation analysis was performed for each individual metal between
195 *Chironomus* and Tubificidae body burdens. For all metals rather weak, although significant, ,
196 correlations were found between concentrations in both taxa with R-values ranging from 0.36 to 0.78
197 (As: $R=0.50$, $p=0.002$; Cd: $R=0.78$, $p < 0.001$; Cr: $R=0.72$, $p < 0.001$; Cu: $R=0.36$; $p=0.009$; Ni:
198 $R=0.51$, $p=0.001$; Pb: $R=0.59$, $p < 0.001$; Zn: $R=0.61$, $p < 0.001$).

199

200 3.2 Biological water quality and relationship with body residues in invertebrates

201 As shown in table 3, the BBI ranged from 1 to 10 and the MMIF from 0 to 0.85 whereas the number
202 of taxa ranged from 1 to 31, EPT taxa from 0 to 9 and relative abundance of Ephemeroptera from 0
203 to 0.28, covering the whole range from very low to very high ecological quality.

204 The five ecological endpoints were positively inter-correlated as shown in table 4 with R-values
205 ranging from 0.55 to 0.86. When relating the water chemistry variables to the ecological endpoints
206 weak significant positive correlations were found for conductivity, oxygen content and ammonium
207 concentration although R values were never exceeding 0.38. No significant linear relationships were
208 observed between body burdens and ecological endpoints for none of the metals. In some cases
209 however, significant negative relationships representing maximum (90th quantile) MMIF as a function
210 of accumulated metals in *Chironomus sp.* were observed applying quantile regression analysis (table
211 5). Concerning the other ecological endpoints some significant relationships were found as well (table
212 SI.1). No significant relationships however, were found with accumulated concentrations in
213 Tubificidae

214 Figures 1 and 2 present the relationship between the individual metal concentrations in *Chironomus*
215 *sp.* and Tubificidae, respectively, plotted against the MMIF. The relationships with the other
216 ecological endpoints are reported in figures SI.1 and SI.2 for *Chironomus sp.* and Tubificidae
217 respectively. In those cases where a significant quantile regression could be derived this was plotted
218 on the graphs. Applying these quantile regression models, critical body burdens could be calculated
219 corresponding to good ecological quality (table 6). In the case of the MMIF, it was not possible to
220 calculate the accumulated metal concentrations corresponding to a MMIF of 0.6, since the quantile
221 regression line was situated below 0.6 (see figure 1). However, also in the cases where no significant
222 quantile regression could be calculated it was possible to estimate body residues for the individual
223 metals, above which ecological endpoints were always below a certain threshold. This was done by
224 calculating the 95 percentile value of the accumulated concentrations in organisms collected at sites
225 with good ecological quality. The critical body residues obtained from these thresholds are
226 summarised in table 6.

227 The most sensitive ecological endpoints proved in most cases to be the MMIF and the number of
228 EPT-taxa. In some cases large differences were observed between the two taxa with generally higher
229 thresholds for Tubificidae. Due to a restricted number of samples it was not always possible to obtain

critical body residues for Tubificidae. Lowest thresholds for *Chironomus* were (in $\mu\text{g/g dw}$): 65 (As); 3.2 (Cd); 10 (Cr); 57 (Cu); 6.5 (Ni); 73 (Pb); 490 (Zn). For Tubificidae lowest threshold concentrations were (in $\mu\text{g/g dw}$): 85 (As); 28 (Cd); 24 (Cr); 71 (Cu); 8.4 (Ni); 79 (Pb); 930 (Zn). In the case of arsenic in Tubificidae nor *Chironomus* sp. critical body residues could be calculated for MMIF since the maximal MMIF was 0.55. In addition, for some other metals in Tubificidae the range in ecological endpoints was much narrower compared to the dataset in *Chironomus* sp. Since other factor than metals could affect the ecological endpoints the critical body concentrations were also calculated on a reduced data set where all samples were excluded if the water quality standards were violated (table 1). This resulted in a data-set of 104 samples for *Chironomus* sp. and 48 for Tubificidae. The calculated threshold values were very comparable to the ones calculated on the whole data sets (Table SI.2).

243 4. Discussion

244 In the present study a unique dataset was built on metal accumulation in two benthic invertebrate
245 taxa, i.e. *Chironomus sp.* and Tubificidae in combination with ecological data. Some other studies
246 applied invertebrate body residues as indicators for ecological quality of surface waters (e.g. Luoma
247 et al., 2010; Adams et al., 2011; Rainbow et al., 2012; De Jonge et al., 2012; 2013). However, in most
248 of those studies the data set was rather limited, whereas in the present study data from 140 samples
249 have been used. Metal concentrations in both taxa varied a lot and maximal values were among the
250 highest ever reported in literature. Van Ael et al. (2014) related concentrations to the MMIF by
251 combining the chemical with the biological databases of the Flemish Environment Agency
252 (www.vmm.be/geoview). Although this combination led to paired data of 10,118 measurements
253 belonging to 1189 sampling locations they could not estimate clear threshold values for most of the
254 metals. This is probably due to differences in bioavailability of the metals at different sites (Rainbow
255 and Luoma, 2011). Relating accumulated levels to ecological endpoints is much more relevant
256 because it integrates the bioavailability of the metals at the study sites (Luoma et al., 2010; Rainbow
257 et al., 2012). In the present study we were able to estimate critical body residues of two pollutant-
258 resistant species that could be related to minimal ecological requirements and that potentially can be
259 used to predict ecological effects of individual metals. Lowest estimated critical body residues were
260 found for the MMIF and the number of EPT taxa, suggesting that these endpoints are the most
261 sensitive ones. The BBI, total number of taxa and Ephemeroptera abundance appeared less sensitive
262 in most cases although also thresholds, though higher in most cases, could be estimated. It is not
263 surprisingly that MMIF was more sensitive compared to the BBI since not only a multimetric
264 approach, combining 5 different metrics, is followed but also the type of water course is taken into
265 account, diminishing effects of natural factors on the index (Gabriels et al., 2010). Rainbow et al.
266 (2012) did find clear threshold levels of metals accumulated in Hydropsichidae larvae (Trichoptera)
267 related to Ephemeroptera abundance. In our study however, Ephemeroptera abundance proved less
268 sensitive. This might be due to the fact that in Flanders the most abundant Ephemeroptera family in

269 running water are the Baetidae (Lock and Goethals, 2011) which are the most metal-tolerant family
270 within the Ephemeroptera (Clements et al., 2000; Rainbow et al., 2012). The most metal-sensitive
271 families, i.e. Heptageniidae and Ephemerellidae are rare in Flanders because they need fast running
272 water and a rather stony substrate (Lock and Goethals, 2011), whereas most water courses in Flanders
273 are slow flowing and have soft substrates (Gabriels et al., 2010).

274 No significant linear relationships were found between accumulated concentrations and ecological
275 endpoints for both investigated taxa and for none of the measured metals. It was clear from the scatter
276 plots that even at low individual metal concentrations, sometimes low to very low ecological scores
277 were observed. This is probably due to the fact that at low individual metal concentrations other
278 factors such as low oxygen content, high levels of nutrients and/or other pollutants or habitat
279 degradation might have been responsible for lower ecological quality. Similar observations were
280 made by Bervoets et al. (2005) when relating accumulated metals in the liver of gudgeon (*Gobio*
281 *gobio*) to the Index of Biotic Integrity.

282 Despite the absence of significant relationships in many cases, it was possible to estimate threshold
283 values in accumulated metal concentrations above which ecological quality was never high. In the
284 present study for each individual metal at high accumulated concentrations the ecological quality
285 scores were always low. Although the selection of the ‘good ecological quality’ levels could seem
286 arbitrary, these are the limits put forwarded by the Flemish Government and calibrated in a European
287 context (Gabriels et al., 2010) or based on reference sites (www.vmm.be/geoview). For some metals
288 in Chironomus (As) and for most metals in Tubificid worms, sites with a good ecological quality
289 were rather scanty. In other words at the sites with good ecological quality only rarely arsenic was
290 measured in Chironomus and rarely Tubificid worms could be found. Especially for those situations,
291 the estimated values should be considered with caution. General water characteristics such as pH,
292 conductivity, oxygen level and nutrients might affect the community structure as well and since high
293 metal levels in surface waters are often correlated to high nutrient levels and low oxygen levels, the
294 threshold values might be affected by those general factors rather than or in addition to the metals.

295 Therefore we did the same analysis on the data set without the sites where the quality standards of the
296 general water quality variables were violated. This however, resulted in only marginal differences in
297 threshold values, indicating that the low ecological values at high accumulated metal concentrations
298 indeed reflect the effects of the metals.

299 In the present study both investigated taxa, chironomids and Tubificid worms proved applicable as
300 indicator species although threshold concentrations in Tubificidae generally were higher than in
301 *Chironomus sp.* However, we did not take into account effect of season on the body burdens. The
302 organisms were sampled at different times of the year mostly between March and October but at
303 several sites also in the winter months. It is likely that sampling in different seasons will affect the
304 threshold values since temperature is an important ecological and physiological factor affecting
305 uptake, excretion and accumulation of micro pollutants (Bervoets et al., 1996; Noyes et al., 2009).
306 Further research is needed to investigate the effect of season on relationships between accumulated
307 levels and ecological endpoints.

308 No other studies could be found where concentrations in chironomids or tubificid worms were
309 related to community responses with the exception of the study by De Jonge et al. (2012) but these
310 data were included in the present study. Critical body burdens are therefore compared below with
311 concentrations found in other organisms that were considered as safe values for the ecological quality.
312 For arsenic the lowest calculated critical body burdens were 65 and 85 µg/g dw for *Chironomus sp.*
313 and Tubificidae respectively. These concentrations are in the same order of magnitude as found by
314 Rainbow et al (2012). These authors found that sensitive mayflies were limited when As
315 concentration in *Hydropsyche siltalai* exceeded 85 µg/g dw. For cadmium the lowest estimated
316 critical body burdens were 3.2 and 28 µg/g dw for *Chironomus sp.* and Tubificidae respectively.
317 Concerning chromium lowest estimated critical body burdens were 10 and 24 µg/g dw for
318 *Chironomus sp.* and Tubificidae respectively. However, for Tubificidae only sufficient paired data with
319 BBI were available. No field studies were found that could estimate critical body burdens for Cd nor
320 for Cr in aquatic invertebrates in relation to community responses. Lowest critical copper burdens

321 found were 57 µg/g for *Chironomus sp.* and 71 µg/g dw for Tubificidae. Rainbow et al. (2012) found
322 a critical body burden for copper of 170 µg/g dw in *Hydropsyche siltalai* resulting in a decreased
323 mayfly abundance, Luoma et al. (2010) found that sensitive mayflies did not survive at sites where
324 accumulated copper in Hydropsychid larvae approach 100 µg/g dw and that dramatic community
325 degradation, as measured by EPT taxa, was observed at accumulated concentrations of 200 µg/g dw.
326 Comparable results were found by De Jonge et al. (2013) who found that accumulated copper in
327 *Rhitrogena sp.* of 165 µg/g resulted in a reduction of the O/E (Observed/Expected) taxa with 20%
328 and 349 µg/g dw in a 50% reduction. At a copper body concentration of 636 µg/g dw in *Rhitrogena*
329 *sp.* a total disappearance of invertebrate taxa was predicted. The lowest observed critical body
330 burdens for Ni in the present study were 6.5 and 8.4 µg/g dw for *Chironomus sp.* and Tubificidae
331 respectively. As for Cd and Cr no threshold values could be found in literature. Lead threshold values
332 found in the present study were 73 and 79 µg/g dw for respectively *Chironomus sp.* and Tubificidae.
333 Much higher critical body residues were found by De Jonge et al. (2013) who found 1635 and 3146
334 µg/g dw in respectively Simuliidae and *Leuctra sp.* resulting in a 20% decrease in the BMWP index.
335 On the other hand Rainbow et al. 2012 observed a decrease in diversity of Ephemeroptera larvae at
336 Pb body concentrations of 441 µg/g dw in Hydropsychidae, being much lower than found by De
337 Jonge et al. (2013) but still about five times higher than in the present study. Lowest zinc critical body
338 burdens in the present study were 490 and 930 µg/g dw for *Chironomus sp.* and Tubificidae
339 respectively. De Jonge et al. (2013) were able to calculate critical threshold values for four different
340 taxa of invertebrates ranging from 968 (in Simuliidae) to 8369 µg/g dw (*Rhitrogena sp.*). Sola et al.
341 2004 observed a 50% decrease in macroinvertebrate diversity and density at a body concentration of
342 3210 µg/g dw in *Hydropsyche sp.* and Rainbow et al. 2012 observed a decrease in Ephemeroptera
343 abundance at a Zn concentration of 636 µg/g dw in Hydropsychidae .
344 From these comparisons with literature it is clear that great differences exist among different species
345 and that calculated threshold values that are predictive of ecological effects are species or taxon

specific. Therefore the calculation of the critical body burdens applies only to the investigated species or taxon and extrapolation to other taxa should be avoided (De Jonge et al., 2013).

Although other factors than the investigated metals will affect the community structure of the macro invertebrates, the calculated critical body residues in this study seemed not affected by these confounding factors since omitting all sites from the data sets that didn't meet the quality criteria for the general water characteristics resulted in almost identical values. Since for some accumulated metals (As) in *Chironomus sp.* and for most metals in Tubificid worms only few sites with a good ecological quality were included, the estimated critical body burdens should be considered with caution. Although correlations among accumulated levels were rather weak, co-occurrence of pollutants may influence the estimated values, making a validation study on the individual estimated values needed. As a consequence, the estimated critical body residues are indicative and in order to be applicable by regulators they should be supported by other lines of evidence.

In the present study 5 different ecological endpoints were used all reflecting the status of the invertebrate community. Lowest critical body residues were obtained in most cases for the MMIF and the EPT taxa, followed by the total number of taxa. Several studies showed that invertebrate species, or taxa, richness is a sensitive indicator of metal pollution (Hickey and Clements, 1998; Clements 2004), whereas other studies found that abundance or population densities of Mayflies were more sensitive towards metal pollution (Clements et al., 2000; Clark and Clements, 2006). Luoma et al. (2010) and Rainbow et al. (2012) used Ephemeroptera densities as an ecological endpoint in relation to accumulated metals in Hydropsychid larvae to estimate critical body residues. In the present study in all but one case (accumulated Cr in chironomid larvae), however, the Ephemeroptera abundance was the least sensitive endpoint although clear threshold values of accumulated metals could be estimated, i.e. for all metals mayfly abundance was always low at high accumulated concentrations. The chosen limit of 0.15 or Ephemeroptera abundance was rather arbitrary but under undisturbed circumstances in Flemish rivers Ephemeroptera represent between 15 and 40% of the macro invertebrate community (www.vmm.be/geoview). De Jonge et al. (2013) related accumulated

372 concentrations to the taxonomic composition applying the RIVPACS bioassessment system, which
373 compares the expected composition of the invertebrate community, based on a set of abiotic and
374 geomorphological characteristics with the measured one (Clarke et al., 2003). Although with this
375 approach the natural variation in taxa composition is taken into account, only absence/presence of
376 families is considered. As mentioned before EPT-taxa have been proved to be sensitive to metals
377 (Beasley and Kneale, 2003; De Jonge et al., 2008; Rainbow et al., 2012), which was confirmed in the
378 present study where relating accumulated levels to either MMIF or EPT-taxa resulted in the lowest
379 body burdens.

380 In the present study the MMIF, was applied that also takes into account the differences in water type
381 but in addition combines different response variables of the invertebrate community, including taxa
382 richness, number of EPT taxa, number of sensitive taxa, Shannon –Wiener diversity index and mean
383 tolerance score. As a result it seemed most logic to relate the accumulated concentrations to response
384 of the MMIF, especially since the FEA applies the MMIF for biological water quality assessment.

385

386 5. Conclusion

387 The present study added to the growing body of evidence that accumulated metal concentrations,
388 expressed as critical body burdens, in relative resistant macroinvertebrates can be used as predictors
389 of ecological effects of metals in aquatic ecosystems. Even if other factors than metals affect the
390 community composition, it is still possible to estimate safe levels for individual metals when a large
391 data set is available.

392 Both *Chironomus sp.* and Tubificidae proved to be suitable invertebrate taxa for the calculation of
393 critical body burdens although differences between taxa have to be taken into account. However,
394 more research is needed on the effect of temperature or season on the obtained safe body levels. In
395 addition, differences in exposure (mainly water or mainly sediment) might affect the results. As a
396 consequence the found threshold values should be validated in other river systems.

397

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401

402 Appendix A. Supplementary data

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578 Figure Legends

579

580 Figure 1. Scatterplots of the MMIF as a function of the accumulated metals in *Chironomus sp.* The
581 dashed line indicates the threshold of a MMIF value of 0.6. For Cr, Cu, Ni and Zn significant 90th
582 percentile quantile regressions could be calculated

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584

585 Figure 2. Scatterplots of the MMIF as a function of the accumulated metals in Tubificidae. The
586 dashed line indicates the threshold of a MMIF value of 0.6.

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592 Fig 1

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595 Fig 2

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598 Table 1. Median and range of some general water characteristics

	minimum	maximum	Median	Quality Standards
pH	4.9	8.2	7.2	6.5-9.0 ^a
O ₂ (mg/l)	1.4	12.7	7.5	5.5-9.5 ^a
Conductivity (μS/cm)	46	5395	603	< 1000 ^b
PO ₄ ³⁻ P (mg/l)	0.01	14.3	0.91	< 0.30 ^b
NH ₄ ⁺ -N (mg/l)	0.01	3.77	0.10	< 5.0 ^b

599 ^a USEPA: US Environmental Protection Agency (US Environmental Protection Agency, 1999); ^b
600 Flemish Quality Criteria (Flemish Government, 2000)
601

602 Table 2. Metal concentration in *Chironomus sp.* and Tubificidae (µg/g dry weight) sampled at the
603 different locations in Flanders.

604

		<i>Chironomus sp.</i>	Tubificidae
As	Min. Max. Median N	0.05 151 9.0 80	0.10 246 24.5 40
Cd	Min. Max. Median N	0.05 113 3.2 137	0.06 2600 9.2 62
Cr	Min. Max. Median N	0.10 205 5.7 108	0.10 223 4.5 36
Cu	Min. Max. Median N	6.8 707 33 137	4.7 1490 38 62
Ni	Min. Max. Median N	0.05 321 4.5 114	0.15 105 4.4 40
Pb	Min. Max. Median N	0.10 1430 10 137	1.31 1260 22 56
Zn	Min. Max. Median N	30 3310 297 137	72 11600 529 62

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614 Table 3. Median and range of the ecological endpoints

	minimum	maximum	Median
BBI	0	10	5
MMIF	0	0.85	0.30
# taxa	1	31	10
# EPT taxa	0	9	0
Relative Eph. abund	0	0.36	0.00

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618

619 Table 4. Spearman Rank correlation among the different ecological endpoints.

	BBI	MMIF	# taxa	# EPT taxa
MMIF	0.818 N=133			
# taxa	0.857 N=150	0.873 N=133		
# EPT taxa	0.654 N=145	0.808 N=133	0.669 N=145	
Eph. abund	0.552 N=139	0.628 N=128	0.556 N=139	0.729 N=139

620

621 R-values; p always < 0.001; N: number of samples

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625 Table 5. Results of the 90th quantile regression analysis based on body burdens in *Chironomus sp.*
 626 α : toxicity coefficient of the metal on the observed MMIF

627

metal	α	SE	Intercept	SE	t-value
As	ns				
Cd	ns				
Cr	-0.0013	0.00056	0.56	0.0035	-2.23*
Cu	-0.00054	0.00013	0.58	0.046	-4.15***
Ni	-0.0016	0.00069	0.56	0.043	-2.29*
Pb	ns				
Zn	-0.0001	0.00005	0.62	0.061	-2.06*

628 Ns: not significant

629 With * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

630

631 Table 6. Estimated critical body burdens for the different metals for both *Chironomus* and Tubificidae in µg/g
632 dry weight.

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	As		Cd		Cr		Cu		Ni		Pb		Zn	
	Chi	Tub	Chi	Tub	Chi	Tub	Chi	Tub	Chi	Tub	Chi	Tub	Chi	Tub
BBI (6) QR	65 ns	85 ns	17 38	71 ns	33 ns	24 ns	96 300	120 ns	30 ns	14 ns	88 ns	98 ns	850 2300	1360 ⁶³⁷ ns ⁶³⁸
MMIF (0.60) QR	na na	na na	3.2 ns	41 ns	36 na	na na	57 na	71 ns	25 na	8.4 ns	73 ns	81 ns	490 230	1400 ⁶³⁹ ns ⁶⁴⁰
# taxa (16) QR	90 ns	89 ns	13 27	28 ns	37 69	26 ns	83 220	110 ns	27 ns	16 ns	79 ns	79 ns	730 ns	1200 ⁶⁴¹ ns
# EPT taxa (4) QR	130 ns	92 ns	8.5 ns	34 ns	13 ns	na na	92 na	76 ns	6.5 ns	na na	78 ns	na na	1200 ns	930 ⁶⁴² ns ⁶⁴³
Rel. Ephem. Abund. (0.15) QR	82 ns	93 ns	85 ns	47 ns	10 ns	na na	130 ns	130 ns	17 ns	na na	120 ns	87 ns	2600 ns	1800 ⁶⁴⁴ ns ⁶⁴⁵

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648 Chi: *Chironomus* sp.; Tub: Tubificidae. QR: values are calculated from the quantile regression analysis.; ns: not significant;

649 na: insufficient data to calculate