A multibiomarker approach for evaluating environmental contamination : common carp (**Cyprinus carpio**) transplanted along a gradient of metal pollution

A Multibiomarker Approach for Evaluating Environmental Contamination: Common Carp Transplanted Along a Gradient of Metal Pollution

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Abstract:

Environmental monitoring and risk assessment approaches which include a more holistic view on the effects of pollutants on biota are increasingly sought by regulators and policy makers. The present study aimed to evaluate the suitability of multiple biomarkers for applications in active biomonitoring programmes. Caged carp juveniles (*Cyprinus carpio*) were transplanted along a known Cd and Zn pollution gradient. After 7 weeks of exposure, metal (Cu, Cd and Zn) accumulation in gill and liver tissue and effect biomarkers (growth, condition factor (CF), hepatosomatic index (HSI), oxygen consumption, swimming capacity, Na^+/K^+-ATPase activity (NKA) and metallothionein (MT) levels) were compared.

Up to 10- fold higher cadmium concentrations were measured in the gills of the fish at the most polluted locations compared to the laboratory control fish. Similarly, cadmium concentrations in liver tissues of field-exposed fish were significantly higher than those measured in laboratory control fish. Cu and Zn concentrations in the gills were not significantly different between field-exposed and control organisms, whereas higher levels in liver tissues were measured in carps deployed in some locations. Effects on liver

MT levels were up to 10 times greater for organisms exposed to the field, whereas no clear effect of the metal exposure on NKA in the gill tissue was observed. A decrease in muscle glycogen stores was observed for all organisms deployed in the field, while liver glycogen levels decreased only in fish exposed to two of the 5 sites compared to the laboratory control fish. Additionally, significant drops in liver protein- and lipid stores were observed. No effect on oxygen consumption rates and swimming capacity was observed. The CF and HSI of caged fish reflected the pollution gradient in the river and considerable loss of weight was observed for fish transplanted in the most polluted site.

Keywords: metal accumulation, cadmium, copper, zinc, biomarkers

1. Introduction

Human activities of the past centuries have left a legacy of metal pollution in both terrestrial and aquatic ecosystems (De Vleeschouwer et al., 2007). Due to this historical metal pollution, metal concentrations in sediments and surface waters are still exceeding natural levels and may pose a threat to aquatic ecosystems health (Fu et al., 2016; Golovanova, 2008; Meena et al., 2018). Currently, regulators and policy makers attempt to mitigate pollution related issues via legislative tools such as the water framework directive 2000/60/EC (Commission, 2000), with the objective of reaching a better ecological status of all water bodies.

This requires monitoring programmes which evaluate the chemical and biological quality of water bodies. Conventional monitoring approaches relying on the periodic collection of water and sediment samples provide only a snapshot of the degree of contamination occurring in aquatic environments, neither do they represent the bioavailable fraction of the pollutants (Kördel et al., 2013). Passive biomonitoring is an alternative approach which provides an integrative measure of bioavailable pollutants in water and sediment. However, life-history traits, seasonal factors, and the presence and mobility of species may make it difficult to implement this type of monitoring to all sites of interest. In contrast, active biomonitoring has shown to overcome these limitations (Bervoets et al., 2004a; Ji et al., 2010; Oikari, 2006). With this

approach, a particular species can be transplanted from a reference site or a culture to the locations of interest. Further, the biotic parameters (e.g. size, age, gender) are controlled, and the experiment can easily be replicated in the field without the availability of the test organism being a limitation (Besse et al., 2012).

Previous research has proven the efficacy of common carps (*Cyprinus carpio*) as a sentinel species in active biomonitoring campaigns (Bervoets et al., 2009; Reynders et al., 2008; Schoenaers et al., 2016). Common carp is a bottom-dwelling species, and is therefore likely to be directly exposed to metals in the sediment by ingestion of particles, or through the consumption of contaminated benthic invertebrates (Bury et al., 2003). Additionally, fish are also exposed to dissolved metal ions in the water column which can lead to disruptive effects on the gill tissue. A well-studied target site for metals (Cu, Cd) is the enzyme Na^+/K^+-ATPase. This transmembrane pomp located in the basolateral membrane of gill epithelial cells is responsible for the flow of sodium ions from the cell to the plasma in exchange for potassium ions. Active binding of certain metals with Na^+/K^+-ATPase does decrease the cells' potential to maintain osmotic balance (De Boeck et al., 2001; Mcgeer et al., 2000; Vassallo et al., 2011).

To counteract increasing cellular metal concentrations, organisms can rely on protective proteins such as metallothioneins. These low-molecular weight proteins regulate the cellular concentration of essential metals by making metal-protein bounds, and as such making the metals biologically inactive. In case of an increase in metal influx, the production of MTs is upregulated, and the cell is protected from deleterious reactions caused by reactive oxygen species. Hence, metallothioneins are also known to bind non-essential metals i.e. cadmium, they play an important protective role. (De Boeck et al., 2003; De Smet et al., 2001; Roesijadi, 1996).

When general defence mechanisms fail, serious physiological implications for the fish may occur. It has been reported that growth and the main energy stores (glycogen, lipids, protein) of fish can be affected by a wide range of pollutants (Golovanova, 2008). Glycogen stores are the most accessible energy provision and are generally addressed before lipids or proteins (Goertzen et al., 2011; Melvin, 2016). Consequently, fast drops in liver- and muscle glycogen stores are often observed as a result of increased energy requirements in (sub)acute metal exposure experiments (Cicik and Engin, 2005; Hallare et al., 2005). Implications of an

altered energy budget can be observed on a higher level by evaluating endpoints including oxygen consumption and swimming capacity, condition factor and hepatosomatic index. Swimming capacity is considered a good proxy to assess the overall health status of fish after exposure to environmental stressors (Kolok, 2001). The oxygen consumption of metal exposed fish can be altered in two ways. High concentrations of some metals damage the gill epithelium to such an extent that the gas exchange capacity is affected towards lethal levels. On the other hand, even sublethal exposures can cause an increase in metabolic rate which will be reflected as an increased oxygen consumption of the animals. The effect size is not always that strong, but a large body of research reports significant differences in oxygen consumption between organisms exposed to metal contaminated and control waters (Couture and Kumar, 2003; Pistole et al., 2008).

The goal of the present study was to evaluate the suitability of common carp (*Cyprinus carpio*) as a sentinel species for active biomonitoring. A field study was conducted in a stream characterised by historical metal contamination which originated from a decommissioned industrial site. Previous studies have shown that Cu, Cd,Zn concentrations were significantly higher close to the polluted area, and decreased further downstream (Bervoets et al., 2013, 2009, 2004b). After the field exposure, bioaccumulation in gill and liver tissues was assessed. Biochemical, physiological, and organismal parameters were tested for their robustness in discriminating the different exposure regimes to which the fish were subjected to.

2. Materials and Methods

2.1.Study area

Based on information from the Flemish Environmental Agency (www.vmm.be/geoview) and own studies, the rivers 'Scheppelijke Nete' and 'Molse Nete' were selected as field exposure sites (Bervoets et al., 2013). The geographical location of the 5 monitoring points selected for this study are plotted on Figure 1. One reference location (Ref) was chosen as the field-control site, upstream of a highly polluted ditch debouching

into the Scheppelijke Nete. The monitoring points L1-L4 are located further downstream of the channel, following the decreasing pollution gradient (Table 1).

2.2.Test animals and exposure conditions

In total 56 juvenile common carps with an average weight of 20.6 ± 6.91 gram were purchased from an accredited breeding centre in the Netherlands (Wageningen University). The fish were acclimatised to dechlorinated and softened tap water (Ca: 79.3 mg/L Mg: 7.4 mg/L, Na: 27.8 mg/L) for four weeks in 200 L plastic tanks, equipped with an active biofilter and foreseen of continuous aeration (O_2: 6.9-7.4 mg/L). The fish received a diet of Hikari Staple mini pellets (proteins: 38%, fat: 5%, cellulose: 3.3%, ash:11.6% and phosphor: 1.2%), at a regime of 2% of their body weight. Concentrations of NH_3/NH_4^+, NO_2^-, NO_3^- and pH were maintained at <0.1 mg/l, <0.03 mg/l, <25 mg/l and 7.4-7.6 respectively by renewing approximately 30% of the rearing water twice per week.

For the actual field exposure study, groups of eight fish were distributed among the five selected field locations. They were transplanted for seven weeks in submerged plastic cages (pond baskets: 60 cm x 40 cm x 40 cm) with a mesh size of 2 x 4 mm and were firmly attached to the riverbed. On a weekly basis, all cages were checked, and cleaned if necessary. One group of eight fish was kept in the lab as and external control group. Fin-clipping of the pelvic, pectoral, dorsal and caudal fin was applied to make the animals of each group distinguishable from each other. General water parameters oxygen, conductivity and pH were measured with a field meter (HQ3d*flexi*, HACH). After seven weeks of field exposure, the plastic cages with fish were randomly collected and brought back to the lab in site water.

2.3.Oxygen consumption and swimming speed

After 7 weeks, individual fish were pre-acclimated overnight in Blazka-type swimming respirometers (volume: 3.9 L, outside dimensions: 50 cm (length) x 11 cm (diameter)) filled with dechlorinated and softened tap water (15°C) for 12 hours. The current speed was set at 5 cm/s and the respirometers were

placed in a recirculation system containing approximately 180 L of dechlorinated and softened tap water. At the start of oxygen consumption measurements, water circulation through the respirometers was cut off, air bubbles were removed and one oxygen electrode (WTW OxiCal-SL) was inserted in each respirometer. Dissolved oxygen concentrations in the water were recorded until oxygen levels had dropped below 70% of the initial value. The final oxygen consumption was calculated as µmol O_2 consumed per gram body weight (BW) per hour with the following formula (De Boeck et al., 2006; Sinha et al., 2012):

$$MO_2 = (O_{2i} - O_{2f}) \times V \times 1000 \times \frac{1}{O_{2MW}} \times \frac{1}{BW} \times T \quad \textit{(Eq.1)}$$

with O_{2i} = initial O_2-concentration (mg/L),

O_{2f} = final O_2-concentration (mg/L),

V = chamber volume (L),

O_{2MW} = molecular weight of O_2

BW = Body weight

T = Temperature (°C)

Subsequently, swimming capacity of the fish was assessed within the Blazka type swimming respirometers. The initial speed of 5 cm/s stimulated the fish to start swimming. Gradually, the current speed was increased with 5 cm/s every 20 minutes until the moment the fish were swept against the rear end of the respirometer. When this occurred, the current speed was briefly reduced to allow the fish to start swimming again. The second time the fish are swept downstream they are considered fatigued and the critical swimming speed (U_{crit}) could be calculated according to equation 2 (Brett, 1964):

$$U_{crit} = \frac{U_i + \left[U_{ij} \times \frac{T_i}{T_{ij}}\right]}{FL} \text{ (Eq. 2)}$$

147 *with U_i = highest velocity maintained during the whole interval,*

148 *U_{ij} = velocity increment,*

149 *T_i = time elapsed at fatigue velocity*

150 *T_{ij} = time interval.*

151 *FL = Fork length (cm)*

152

153

154 ## 2.4.Growth and condition

155 The fish were then euthanized with an overdose (1g/L) of neutralised ethyl-3 aminobenzoate
156 methanesulfonic acid (MS222, Sigma). Fork length (L), body weight (BW) and liver weight (LW) were taken
157 of each fish, and used to calculate the condition factor (CF) and hepathosomatic index (HSI) according to
158 the following formulas:

159 $$CF = \frac{BW}{L^3} \times 100 \text{ (Eq.3)}$$

160 $$IISI\ (\%) - \frac{LW}{BW} \times 100 \text{ (Eq. 4)}$$

161 Additionally, the weight gain (WG) was calculated based on the weight taken right before (IW) and after the
162 exposure period (FW):

163 $$WG\ (\%) = \frac{FW - IW}{IW} \times 100 \text{ (Eq. 5)}$$

164

165 ## 2.5.Biochemical analyses

166 Subsamples of approximately 100 mg liver and muscle tissue were analyzed for protein content by
167 Bradford's method (Bradford, 1976), glycogen content by using the Anthrone reagent (Roe and Dailey,
168 1966) and lipid content was measured following Bligh and Dyer, 1959. The energetic equivalents of the

169 protein, carbohydrate and lipid fraction were calculated with respect to the enthalpy of combustion of each
170 type of macromolecule; 24.0 kJ/g, 17.5 kJ/g and 39.5 kJ/g respectively (Gnaiger, 1983).
171 Approximately 190 mg of liver tissue was dissected and stored at -80°C until analysed for MT concentration.
172 The MT determination was executed according to the procedure of Klein et al., 1990, which allows the
173 quantification of total MT in the tissue. The main features of the procedure are that oxidized MT is converted
174 into native MT with 2-mercaptoethanol as a reducing agent and Zn^{2+} as a metal donor. Subsequently, MT is
175 quantified via Cd saturation. High molecular weight Cd-binding compounds are denatured with acetonitrile,
176 Cu bound to MT is removed with ammonium tetrathiomolybdate and excessive tetrathiomolybdate, and its
177 Cu complexes are removed with DEAE-Sephacel (Sigma, St. Louis, MO, USA). Then, apothionein is
178 saturated with ^{109}Cd-labeled $CdCl_2$ solution (Amersham Pharmacia Biotech, Buckinghamshire, England:
179 50ppm of 37MBq/mg Cd in 0.1M HCl), and excessive Cd is bound to Chelex 100 (Bio-Rad, Munich,
180 Germany). The precipitate was removed by centrifugation and the supernatant counted for 1 min in a
181 gamma counter (Minaxi g, Canberra Packard, Frankfurt, Germany). The MT concentration was calculated
182 assuming a molar ratio of Cd/MT of 7 (De Boeck et al., 2003).
183 Na^+/K^+-ATPase (NKA) activity were measured in crude gill homogenates using previously published
184 methods (McCormick, 1993), as modified by Nawata et al. (2007). Tissues were homogenized in ice cooled
185 SEID-EGTA buffer solution (150 mM sucrose; 10 mM EGTA; 50 mM imidazole with 0.1% sodium
186 deoxycholate) and centrifuged (2 min at 12 000 rpm, 4°C). Duplicate homogenates of 10 µL were pipetted
187 into 96-well microplates in four series. A first series of homogenate was mixed with 200 µl assay solution A
188 (400 U lactate dehydrogenase; 500 U pyruvate kinase; 2.8mM phosphoenolpyruvate; 3.5 mM ATP; 0.22
189 mM NADH; 50 mM imidazole) and a second series with 200 µL assay solution B (mixture assay A with 10.5
190 mM ouabain). The enzyme activities were measured kinetically at 30 s intervals for 30 min at wavelength of
191 340 nm with a spectrophotometer (Synergy Mx, Biotek Instruments Inc., Vermont, USA). Protein
192 concentrations were measured with Bradford reagent and BSA standards (Sigma). Calculation was
193 performed with a standard curve of ADP (Adenosine diphosphate). ATPase activities were calculated by
194 subtracting oxidation rate in the absence and the presence of ouabain.

2.6.Metal analysis

Cadmium, zinc and copper concentration was measured in the gill- and liver tissue of all fish. Therefore, between 50 and 100 mg fresh weight of each tissue was transferred to 14 ml polypropylene tubes and accurately weighed. Hereafter samples were dried in an oven (IP60, LTE Scientific) at 60°C for minimal 48 hours. Then, ultrapure nitric acid (67-69%, Fisher Scientific) was added, and the samples were left to digest for 12 hours at room temperature. In a last step, microwave (Samsung) assisted tissue destruction was performed to completely dissolve the entire tissue (Blust, 1988). The metal analysis was performed by inductive coupled mass spectrometry (ICP-MS, Varian UltraMass 700, Australia). For quality control, blanks and certified reference material were included and processed in the same way as the samples. As reference material Codd muscle (BCR422; IRMM, Geel, Belgium) was used and recoveries were 90, 92 and 99 % for respectively Cu, Cd and Zn. All metal concentration in the tissues are expressed on a dry weight basis (µg/g dw). Besides the individual metal accumulation, a measure for total metal accumulation was calculated. Therefore the measured metal concentrations in the tissue was corrected for the concentration of that metal in the respective tissue of fish from the reference site. As such the toxic unit (TU_t) of the respective tissues could be calculated according to Equation 6 (Bervoets et al., 2005). In this case, the fish which remained in the lab were taken as the control group in order to calculate the concentration ratios for each metal.

$$\mathbf{TUt} = \frac{\left[\sum_i \left(\frac{C_{ij}}{C_{ctr}}\right)\right]}{N} \quad \text{(Eq.6)}$$

With C_{ij}= tissue concentration of metal i in fish from site j,

C_{ir} = tissue concentration of metal i in fish from the control site

N = number of metals included to calculate the TUt

2.7.Statistics

Data analysis was executed with the open source software R (version 3.4.0), and the graphs were made with the package *ggplot2* (Wickham, 2009). Normal distribution of the dataset was tested with the Shapiro-Wilks test, while homogeneity of the variances was evaluated with Levene's test. Parametric datasets were analysed with a one-way ANOVA, followed by Tukey HSD as a post-hoc test. Non-parametric data was further processed with a Kruskal-Wallis test and a Dunn test with Bonferroni adjusted p-values for pairwise comparisons. The multiple linear regression analysis was performed with the package *relaimpo* (Grömping, 2006).

3. Results

3.1. Water parameters

The five field locations were all within the same range regarding the abiotic parameters pH and conductivity (Table 2). The dissolved oxygen level at L4 was much higher compared to the other sites but it should be noted that these data result only from one single measurement.

3.2.Metal accumulation

All transplanted fish survived the exposure period and could be used for analysis in the laboratory. The median concentrations of Cu, Cd, and Zn measured in the gill tissue of the carps are shown in figure 2a. An exposure-dependent difference in Cd accumulation was observed in the gill tissue of the fish caged at the 5 field locations (Figure 2a). The levels were up to 10 times higher in the sites close to the pollution source (L1 and L2), compared to the samples from the laboratory and reference site, respectively; 1.20 ± 0.07 µg/g DW (L1) and 1.28 ± 0.07 µg/g DW (L2) versus 0.12 ± 0.01 DW (LAB) and 0.10 ± 0.00 µg/g DW (Ref). The fish at sites L3 and L4 accumulated low levels of Cd during the 7 weeks of field exposure, but the concentrations were not statistically different from the laboratory control fish or reference site. The Cu and

Zn content in the gills were within the same range for all groups and were characterized by more variation within gill samples from the same location.

In general, the field exposed fish did accumulate more Cd, Cu and Zn in their livers than the carp which remained in the lab (Figure 2b). The highest average Cd, Cu and Zn concentrations were measured respectively at L3 (1.57 ± 0.2 µg/g DW), L3 (72.6 ± 3.42 µg/g DW) and L2 (519 ± 43.5 µg/g DW). Significantly higher Cd concentrations were found in the fish liver from sites L1, L2, L3, L4 compared to the laboratory control group, but not compared to the reference group. The livers of the carp exposed to the water at the reference location accumulated approximately twice as much Cd as the fish from the lab, and only slightly less than the fish transplanted downstream of the pollution source. Cu levels in the liver of field exposed fish did all increase within the same range and up to seven times higher values were measured in these samples compared to the laboratory control group. The difference was statistically significant for all groups, except for the samples from L4. The average Zn content in the liver of the carp in the lab was 151 ± µg/g DW. A three-fold increase in Zn content was observed in the field exposed groups at location L2 and L3. Surprisingly the fish at L1, the closest to the pollution source, accumulated lower levels of Zn than the fish at L2 and L3, and the levels were not significantly higher than those observed in the fish from the lab or reference site.

The toxic units, as a measure for total metal accumulation compared to the non-exposed laboratory group, indicated an exposure to much higher metal concentrations at site L1 and L2 compared to the three other field locations (Figure 3). However, this was only observed for the gill samples, with significantly higher TU_t at L1 and L2 in compared to the fish transplanted at L4 and the reference site. The toxic units associated with the liver samples did not reflect the same differences in metal exposure among the field sites and showed a higher degree of variation within the 5 locations.

3.3.Biochemical endpoints

3.3.1. Energy stores

The energy budget of the fish was determined in both liver- and muscle samples. The energy stores in the liver were clearly affected by the 7 weeks of field exposure (Figure 4a). The glycogen levels were significantly lower at site L1 (28.4 ± 5.73 µg/mg) and L3 (29.8 ± 3.19 µg/mg) compared to the levels found in the fish held in the laboratory (60.4 ± 4.62 µg/mg). Among the fish exposed in the field, only a significant lower glycogen content was observed in the livers of the fish at L3 compared to the reference site (70.3 ± 8.90 µg/mg). The lipid and protein stores in the livers of also followed the pollution gradient, with significantly lower levels found in the fish caged at the two most polluted sites L1 and L2 when compared to the laboratory control group. Regarding the lipid content there was a respective decrease of 53.7% (L1) and 36.9 % (L2), and the protein levels decreased with 62.3 % (L1) and 42.6 % (L2) compared to levels measured in the control fish from the lab. The effect on the overall energy budget of the livers is also clearly visible as all the fish exposed to the highest degree of pollution were characterised by a significant lower energetic capacity; 2.0 kJ/g (L1) and 3.3 kJ/g (L2) compared to the laboratory control group: 5.5 kJ/g.

The energy stores in the muscle tissue were also different among caged groups in the field, although not to the same extend as the livers (Figure 4b). The glycogen levels were all suppressed in all the caged fish, with significant decreases of 79%, 77%, 80%, 73% at L1, L2, L3 and L4 respectively. However, no significant differences were observed in the lipid stores, nor between lab and transplanted groups and neither among the caged groups. In comparison to the lab control group, the fish caged in the field were also not characterised by a decrease in protein content of the muscles. Between the exposed groups 27% and 30% lower protein contents were measured in the muscles of the L3 and L4 cage compared to the fish caged at L2. This pattern was also reflected in the total energy budget of the muscle tissue, with significant lower values found for the fish from L3 and L4 compared to the fish of L2.

The multiple linear regression analysis of all three metals together indicated a significant correlation between the liver Cd and the glycogen stores (Table 4). The model explains 33% of the variation in the

289 dataset and cadmium has the highest relative importance (55%) of all three metals. Significant correlations
290 between the gill Cd levels and the glycogen and lipid stores in the liver were also observed. Individually
291 these Cd levels have a relative importance of 94% and 93% in the model regarding the liver glycogen and
292 lipid stores respectively. 61% of the variation in the muscle glycogen stores is explained by the metal
293 content in the liver, but only for Cu a significant correlation was found. The Cd levels in the gills were also
294 significantly correlated with the measured glycogen and protein content of the muscle tissue and had a
295 relative importance of 89 and 60% respectively.
296

297 3.3.2. Metallothionein levels and Na^+/K^+ ATPase activity

298 In general, lower metallothionein levels were measured in the liver samples of the control fish which
299 remained in the laboratory (Figure 5a). The highest liver MT levels were observed in the fish transplanted at
300 L1, with concentrations reaching up to 8.4 nmol per gram of tissue. The hepatic cells of the lab control group
301 had significantly lower MT levels (0.44 ± 0.08 nmol/g), compared to the fish which were exposed to the
302 water at the L1, L2 and L3. Further, lower MT levels are seen at those sites far from and upstream of the
303 pollution source, in L4 and Ref respectively. The measured concentrations of the three metals in the liver
304 tissue explained 51% of the variation of the observed MT levels in a multiple regression model. However,
305 only the Cu content was significantly correlated with the dependent variable (Table 4).
306 No clear trend in Na^+/K^+-ATPase activity can be distinguished, neither among the field exposed groups, nor
307 between the lab and field groups (Figure 5b). The fish transplanted at L2 did show a higher average activity
308 (14.80 ± 3.92 µmol/mg*h) compared to the other groups (7.65 ± 0.64 µmol/mg*h). Also, no significant
309 correlation was found between the accumulated metals in the gill tissue and the corresponding Na^+/K^+
310 ATPase activity (Table 4).
311

312 3.4.Physiological endpoints

313 The two non-invasive fitness parameters; critical swimming speed and oxygen consumption rate (Table 3),
314 were not robust enough to discriminate groups according to different metal exposures in the field. The
315 average critical swimming speed of the carp of all groups ranged between 2.71 ± 0.22 body lengths/s and
316 3.62 ± 0.19 body lengths/s, with a lot of variation among the individual fish within the treatment groups (up to
317 21%). There was a lower oxygen consumption of the fish which were caged at L2 (4.38 ± 0.38 µmol
318 O_2/gram*h) compared to the lab-held fish (8.23 ± 1.13 µmol O_2/gram*h), but not compared to the fish from
319 the reference site (4.71 ± 1.13 µmol O_2/gram*h). In general, this parameter also showed a lot of variation
320 among individual fish within groups (up to 63%). Neither the swimming speed, nor the oxygen consumption
321 was affected by the increased metal exposure at site L1.
322

323 ## 3.5.Organismal endpoints

324 The three parameters; condition factor, hepatosomatic index and weight loss (%) were measured as a proxy
325 for the overall health status of the fish after 7 weeks of exposure (Figure 6).
326 In general, the calculated condition factors (Figure 6a) follow a trend that corresponds to the pollution
327 gradient within the river, where the groups transplanted close to the pollution source are characterised by an
328 overall lower CF. Compared to the unexposed fish from the laboratory (2.13 ± 0.03), the CF of the carp
329 which resided in the cages at L1 (1.74 ± 0.06) and L2 (1.78 ± 0.04) was significantly lower.
330 The hepatosomatic index was less affected by the exposure conditions, with more similar indices among the
331 fish kept at different locations (Figure 6b). Nevertheless, a correlation between the HSI and the degree of
332 pollution can be seen when only the field exposed groups are considered. However, a significant higher HSI
333 was found for the fish caged at L1 compared to the fish from reference site ($p = 0.01$). The fish kept in the
334 lab were characterised by an average HSI of 2.13 ± 0.1%, which was not significantly different from the fish
335 caged at the polluted locations, but was significantly higher than the HSI of the fish for the reference site.
336 The fish of the different groups were not significantly different in weight, but the fish from both L1 (0.33 ±

337 0.05g) and the laboratory control fish (0.34 ± 0.02g) had a relatively large liver compared the small liver of
338 the fish at the reference site (0.24 ± 0.02g).
339 An overall weight loss (WL) was observed for all fish which were caged in the field for 7 weeks (Figure 6c).
340 A negative trend in body weight evolution can be observed in function of the distance from the pollution
341 source. The decrease in body weight was the strongest for those fish from site L1 (-20.5 ± 16.5 %), which
342 was significantly lower than the fish kept at the reference site.
343 The three parameters CF, HSI and WL did correlate with the metal concentrations measured in the gill
344 tissue. Significant correlations were found for all three endpoints with the gill Cd levels and also a significant
345 effect of copper on the HSI was observed (Table 4). 47% of the variation in CF is explained by metal load of
346 all three metals together, where a relative importance of 76% can be attributed to the Cd levels in the gills.
347

348 4. Discussion

349 The observed Cd levels in the gill tissue did comply with the pre-existing knowledge of the environmental
350 contamination; higher levels close to the pollution source (L1 and L2) and decreasing levels further
351 downstream (L3 and L4) and upstream of the pollution source (Ref) (Bervoets et al., 2013). The liver Cd
352 levels did differ less between high- and low polluted locations. This suggests that the main route of uptake
353 was via the water. Additionally, it is possible that Cd reaching the liver is more efficiently being removed
354 from the body and/or redistributed to other tissues e.g. kidneys (Reynders et al., 2006).
355 A caging study conducted in 2006 in the same river reported accumulated Zn levels of up to 500 µg/g DW
356 and 2000 µg/g DW in the liver and gill tissue respectively (Reynders et al., 2008). The highest average Zn
357 level measured after this study was 519 ± 43 µg/g DW and 1273 ± 130 µg/g DW (L2) in the liver and gill
358 tissue respectively. Whether there is a significant decrease in bioavailable Zn levels in the river cannot be
359 confirmed. It should be noted that the former caging experiment was only conducted for 5 instead of 7
360 weeks, which suggest that our fish accumulated less zinc per day, at least in the gills.

In a lab experiment where adult common carps were exposed to 50 µg Cu/L, all copper levels in the gill tissue increased up to 30 µg/g after 1 month of exposure. Moreover, unexposed fish had slightly higher gill Cu concentrations (5 µg/g) compared to what we measured in our field exposed fish (2.49-2.93 µg/g) (Eyckmans et al., 2012). However, the latter experiment reports lower liver Cu levels following an exposure period of 1 month (till 100 µg/g DW), whereas in the present study the average gill Cu reached much higher concentrations (126-205 µg/g DW). These findings suggest that the diet was the main route of Cu exposure rather than the river water.

The mode of internal handling of metals depends on the type of metal, but also interspecies differences have been identified (ČelechOvská and SvObOdOvá, 2007; De Boeck et al., 2004). Literature data suggests that accumulation of Cd in the liver tissue tends to reach much higher levels than in the gill tissue (De Smet et al., 2001). A similar trend was clearly visible at the locations L3, L4 and Ref, with a respective 3.5, 4.0 and 10.6-fold higher Cd concentration in the liver samples than gill samples. Surprisingly, this pattern was not reflected in the fish transplanted at the 2 most polluted sites, L1 and L2. The gill Cd levels were also much higher at those sites, which led to smaller differences in Cd content between liver and gill.

Regarding the accumulation of Zn as a consequence of waterborne exposure Hattink et al., 2006 observed higher levels of Zn in the gills compared to the liver tissue. The same was seen in this field exposure scenario, with 2-3 fold higher levels of Zn measured in the gills compared to the livers.

High basal Cu concentrations in the liver tissue are to be expected due to the important metabolic role of in this organ. The data from the lab control group (4 times more copper in the liver vs gills), and other laboratory studies confirm this statement (De Boeck et al., 2003). The Cu_{liver}/Cu_{gill} ratio in the caged fish, however, reached values of 22-27 and is a clear effect of the field exposure.

The strong decrease in muscle glycogen observed here corresponds to a study where carps were exposed to sublethal concentrations of cadmium (Cicik and Engin, 2005). However, the muscle glycogen stores at the polluted sites (L1-L4) decreased up to 80% and 65% of the values measured in the laboratory control fish and fish at reference site respectively, whereas Cicik and Engin (2015) reported decreases of 29% only.

The overall decrease in liver lipid stores of the fish transplanted at the most polluted sites (L1 and L2) can

387 be explained by an increased utilisation of triglycerides as a direct consequence of the Cd exposure
388 (Bervoets et al., 2009; Pierron et al., 2007). The absence of clear effects on the muscle lipid content
389 suggests that there was still enough food available and only the glycogen stores needed to be addressed to
390 accommodate with the energy requirement (Liew et al., 2012). Lastly, the protein stores of the fish caged at
391 L1 and L2 was slightly supressed in the liver tissue in comparison with the laboratory animals. Protein
392 catabolism would be very harmful for overall health of fish and is therefore only to be expected in case of
393 emergency e.g. long-term starvation (Baumgarner and Cooper, 2012; Shrivastava et al., 2017).
394 Apart from the effect of the metal exposure on the energy budget, food availability is another important
395 factor that could have affected the observed responses on the energy budget. The macroinvertebrate
396 community at the locations was studied concurrently for a master thesis and overall invertebrate
397 abundances were rather low within this river, but no obvious differences among the locations were found
398 (Epede et al., 2014). Although this suggests that the fish from different cages have been subjected to similar
399 circumstances in terms of food availability, we cannot rule out that limited food supply had an effect on the
400 energy stores of the fish at all locations.
401 Since the liver stores energy as glycogen, fat and protein molecules, it is expected that healthier fish
402 possess larger livers, and are therefore characterized by a higher hepatosomatic index. The HSI however
403 did not follow the trend in any of the storage molecules or the total energetic capacity of the liver. In other
404 studies researchers state that higher HSIs are a proxy for metal pollution, since exposure to increased metal
405 concentrations requires a higher detoxification capacity of the liver (Ozmen et al., 2006; Sanchez et al.,
406 2007). The latter argumentation might be more valid for the present study since the HSI follows the metal
407 pollution gradient in the river with the lowest value for the fish at the reference site. As mentioned above, the
408 low HSI of the latter group is mainly a consequence of the smaller livers of the fish caged at the reference
409 site.
410 The measured MT levels in this study were lower compared to the concentrations measured in in resident
411 roach, perch and gudgeon populations along the same pollution gradient (Bervoets et al., 2013).
412 Additionally, the latter study reported only a significant correlation of MT levels with the accumulated zinc in

413 the liver tissue for all three species, whereas in the present study only liver Cu levels did significantly
414 correlate with the increased MT concentrations. In an experiment where common carp was exposed to 1 µM
415 of Cu^{2+}, the accumulated copper in the liver was approximately 37 µg/g DW and corresponding MT
416 concentrations reached 30 nmol/g. Our carp, however, had nearly the double amount of copper in their
417 livers (72 µg/g), but MT levels remained below 4 nmol/g) (De Boeck et al., 2003).
418 Oxygen consumption as a proxy for metabolic activity in fish is, though contested, widely applied in
419 experimental research (Nelson, 2016). De Boeck et al. (1995) observed a decreased oxygen consumption
420 immediately after the waterborne copper exposure but noticed a recovery further onwards in the experiment.
421 Likewise, the transplanted carp might have had the time to acclimatise to the high metal load in the water
422 which could explain why no differences in oxygen consumption among the field exposed groups were
423 observed.
424 In a study where brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*) were exposed to
425 waterborne cadmium, a 31% and 38% reduction in swimming performances was seen respectively
426 (Beaumont et al., 1995; Cunningham and Mcgeer, 2016) while another study found an initial 48%, 31% and
427 13% reduction in swimming capacity for rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus*
428 *carpio*), and gibel carp (*Carassius auratus gibelio*) exposed to copper respectively (De Boeck et al., 2006).
429 However, the critical swimming speed of the fish in the present study was not affected by an increase in
430 metal exposure. Although the glycogen in the muscles was depleted after 7 weeks of exposure, it seems
431 that the muscle still contained enough energy to minimise the effect on the U_{crit}. Additionally, it is also
432 possible that the high natural variation in swimming performance among individual fish did mask the effect
433 of the metal exposure (Kolok, 2001).
434 A decrease of the condition factor is often observed in field studies on fish populations inhabiting metal
435 polluted environments (Cazenave et al., 2014; Levesque et al., 2003). In contrast to the study of Reynders
436 et al. (2007), the condition factor was now significantly correlated with the accumulated Cd, Cu and Zn in
437 both the liver and gill tissue but was the strongest for the cadmium in the gills ($R^2 = 0.44$, $p < 0.001$). The
438 observed weight loss of 15-20% corresponds to a previous study with caged common carp in the same river

(Bervoets et al., 2009). However, as mentioned before the aspect of food availability can also be partially responsible for this observation.

5. Conclusion

The historical pollution gradient present in the Scheppelijke- and Molse Nete was clearly reflected by the accumulated Cd in the gill tissue of the caged common carp but no exposure effect on gill Cu and Zn levels was observed. However, the livers of the field exposed fish did accumulate higher levels of all three metals compared to the laboratory control group. The liver MT levels were also clearly affected by metal exposure in the field, with higher concentrations measured in the fish exposed to the most polluted sites. Likewise, the glycogen content in liver and muscle tissue was found to be a robust biochemical biomarker of exposure. The lipid and protein stores were also affected, but only in the liver tissue of the fish. At the physiological level, neither the oxygen consumption nor the critical swimming speed was influenced by the field exposure. Further, a clear effect of pollution was observed on the overall condition factor of the fishes that were caged in the field. We conclude that active biomonitoring using caged organisms can provide biologically relevant information on environmental pollution and can be applied for a wide range of purposes i.e. regular monitoring campaigns, risk assessment, remediation projects etc.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

References

Baumgarner, B.L., Cooper, B.R., 2012. Evaluation of a tandem gas chromatography/time-of-flight mass spectrometry metabolomics platform as a single method to investigate the effect of starvation on whole-animal metabolism in rainbow trout (Oncorhynchus mykiss). J. Exp. Biol. https://doi.org/10.1242/jeb.059873

Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Exposure of brown trout, Salmo trutta, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. Aquat. Toxicol. 33, 45–63.

Bervoets, L., Campenhout, K. Van, Reynders, H., Knapen, D., 2009. Bioaccumulation of micropollutants and biomarker responses in caged carp (Cyprinus carpio). Ecotoxicol. Environ. Saf. 72, 720–728. https://doi.org/10.1016/j.ecoenv.2008.10.008

Bervoets, L., Knapen, D., De Jonge, M., Van Campenhout, K., Blust, R., 2013. Differential Hepatic Metal and Metallothionein Levels in Three Feral Fish Species along a Metal Pollution Gradient. PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0060805

Bervoets, L., Meregalli, G., De Cooman, W., Goddeeris, B., Blust, R., 2004a. Caged midge larvae (Chironomus riparius) for the assessment of metal bioaccumulation from sediments in situ. Environ. Toxicol. Chem. 23, 443–454. https://doi.org/10.1897/03-1

Bervoets, L., Voets, J., Chu, S., Covaci, A., Schepens, P., Blust, R., 2004b. Comparison of accumulation of micropollutants between indigenous and transplanted zebra mussels (Dreissena polymorpha). Environ. Toxicol. Chem. 23, 1973. https://doi.org/10.1897/03-365

Bervoets, L., Voets, J., Covaci, A., Chu, S., Qadah, D., Smolders, R., Schepens, P., Blust, R., 2005. Use of

transplanted zebra mussels (Dreissena polymorpha) to assess the bioavailability of microcontaminants in flemish surface waters. Environ. Sci. Technol. 39, 1492–1505. https://doi.org/10.1021/es049048t

Besse, J.-P., Geffard, O., Coquery, M., 2012. Relevance and applicability of active biomonitoring in continental waters under the Water Framework Directive. https://doi.org/10.1016/j.trac.2012.04.004

Bligh, E.G., Dyer, W.J., 1959. A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37, 911–917.

Blust, R., 1988. Evaluation of microwave heating digestion and graphite furnace atomic absorption spectrometry with continuum source background correction for the determination of iron, copper and cadmium in brine shrimp. J. Anal. At. Spectrom. 3. https://doi.org/10.1039/JA9880300387

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72, 248–254.

Brett, J.R., 1964. The Respiratory Metabolism and Swimming Performance of Young Sockeye Salmon. J. Fish. Res. Board Canada 21, 1183–1226.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206, 11–23. https://doi.org/10.1242/jeb.00068

Cazenave, J., Bacchetta, C., Rossi, A., Ale, A., Campana, M., Parma, M.J., 2014. Deleterious effects of wastewater on the health status of fish: A field caging study. Ecol. Indic. 38, 104–112. https://doi.org/10.1016/j.ecolind.2013.10.029

ČelechOvská, O., SvObOdOvá, Z., 2007. Distribution of Metals in Tissues of the Common Carp (Cyprinus carpio L.). Acta Vet Brno 76, 93–100. https://doi.org/10.2754/avb200776S8S093

Cicik, B., Engin, K., 2005. The Effects of Cadmium on Levels of Glucose in Serum and Glycogen Reserves in the Liver and Muscle Tissues of Cyprinus carpio (L., 1758). Turkish J. Vet. Anim. Sci. 29, 113–117.

Commission, E., 2000. Water Framework Directive - Integrated River Basin Management for Europe.

509 Couture, P., Kumar, P.R., 2003. Impairment of metabolic capacities in copper and cadmium contaminated
510 wild yellow perch (Perca flavescens). Aquat. Toxicol. 64, 107–120. https://doi.org/10.1016/S0166-
511 445X(03)00028-6

512 Cunningham, J.L., Mcgeer, J.C., 2016. The effects of chronic cadmium exposure on repeat swimming
513 performance and anaerobic metabolism in brown trout (Salmo trutta) and lake whitefish (Coregonus
514 clupeaformis). Aquat. Toxicol. 173, 9–18. https://doi.org/10.1016/j.aquatox.2015.12.003

515 De Boeck, G., Meeus, W., De Coen, W., Blust, R., 2004. Tissue-specific Cu bioaccumulation patterns and
516 differences in sensitivity to waterborne Cu in three freshwater fish : rainbow trout (Oncorhynchus
517 mykiss), common carp (Cyprinus carpio), and gibel carp (Carassius auratus gibelio). Aquat. Toxicol.
518 70, 179–188. https://doi.org/10.1016/j.aquatox.2004.07.001

519 De Boeck, G., Ngo, T.T.H., Van Campenhout, K., Blust, R., 2003. Differential metallothionein induction
520 patterns in three freshwater fish during sublethal copper exposure. Aquat. Toxicol. 65, 413–424.
521 https://doi.org/10.1016/S0166-445X(03)00178-4

522 De Boeck, G., van der Ven, K., Hattink, J., Blust, R., 2006. Swimming performance and energy metabolism
523 of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat.
524 Toxicol. 80, 92–100. https://doi.org/10.1016/j.aquatox.2006.07.017

525 De Boeck, G., Vlaeminck, A., Balm, P.H., Lock, R. a, De Wachter, B., Blust, R., 2001. Morphological and
526 metabolic changes in common carp, Cyprinus carpio, during short-term copper exposure: interactions
527 between Cu2+ and plasma cortisol elevation. Environ. Toxicol. Chem. 20, 374–81.
528 https://doi.org/https://doi.org/10.1002/etc.5620200219

529 De Smet, H., De Wachter, B., Lobinski, R., Blust, R., 2001. Dynamics of (Cd,Zn)-metallothioneins in gills,
530 liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquat. Toxicol. 52, 269–
531 281. https://doi.org/10.1016/S0166-445X(00)00136-3

De Vleeschouwer, F., Gérard, L., Goormaghtigh, C., Mattielli, N., Le Roux, G., Fagel, N., 2007. Atmospheric lead and heavy metal pollution records from a Belgian peat bog spanning the last two millenia: Human impact on a regional to global scale. Sci. Total Environ. 377, 282–295. https://doi.org/10.1016/j.scitotenv.2007.02.017

Epede, M.N., De Jonge, M., Bervoets, L., 2014. Using Caged Zebra Mussels (Dreissena polymorpha) for the assesment of ecological effects of metal pollution.

Eyckmans, M., Blust, R., De Boeck, G., 2012. Subcellular differences in handling Cu excess in three freshwater fish species contributes greatly to their differences in sensitivity to Cu. Aquat. Toxicol. 118–119, 97–107. https://doi.org/10.1016/j.aquatox.2012.03.019

Fu, Z., Wu, F., Chen, L., Xu, B., Feng, C., Bai, Y., Liao, H., Sun, S., Giesy, J.P., Guo, W., 2016. Copper and zinc, but not other priority toxic metals, pose risks to native aquatic species in a large urban lake in Eastern China. Environ. Pollut. 219, 1069–1076. https://doi.org/10.1016/J.ENVPOL.2016.09.007

Gnaiger, E., 1983. Calculation of Energetic and Biochemical Equivalents of Respiratory Oxygen Consumption., in: Forstner, H., Gnaiger, E. (Eds.), Polarographic Oxygen Sensors. Springer, Berlin, Heidelberg. https://doi.org/https://doi.org/10.1007/978-3-642-81863-9_30

Goertzen, M.M., Driessnack, M.K., Janz, D.M., Weber, L.P., 2011. Swimming performance and energy homeostasis in juvenile laboratory raised fathead minnow (Pimephales promelas) exposed to uranium mill effluent. Comp. Biochem. Physiol. Part C 154, 420–426. https://doi.org/10.1016/j.cbpc.2011.07.012

Golovanova, I.L., 2008. Effects of Heavy Metals on the Physiological and Biochemical Status of Fishes and Aquatic Invertebrates. Inl. Water Biol. 1, 93–101. https://doi.org/10.1134/S1995082908010148

Grömping, U., 2006. Relative Importance for Linear Regression in R: The Package relaimpo, JSS Journal of Statistical Software. https://doi.org/10.18637/jss.v017.i01

Hallare, A. V, Schirling, M., Luckenbach, T., Ko¨hlerko¨hler, H.-R., Triebskorn, R., 2005. Combined effects of

555 temperature and cadmium on developmental parameters and biomarker responses in zebrafish (Danio
556 rerio) embryos. J. Therm. Biol. 30, 7–17. https://doi.org/10.1016/j.jtherbio.2004.06.002

557 Hattink, J., De Boeck, G., Blust, R., 2006. Toxicity, accumulation, and retention of zinc by carp under
558 normoxic and hypoxic conditions. Environ. Toxicol. Chem. 25, 87–96. https://doi.org/10.1897/04-
559 355R.1

560 Ji, Y., Lu, G., Wang, C., Song, W., Wu, H., 2010. Fish transplantation and stress-related biomarkers as
561 useful tools for assessing water quality. J. Environ. Sci. 22, 1826–1832. https://doi.org/10.1016/S1001-
562 0742(09)60326-0

563 Klein, D., Bartsch, R., Summer, K.H., 1990. Quantitation of Cu-containing metallothionein by a Cd-saturation
564 method. Anal. Biochem. 189, 35–39. https://doi.org/10.1016/0003-2697(90)90040-G

565 Kolok, A.S., 2001. Sublethal Identification of Susceptible Individuals: Using Swim Performance to Identify
566 Susceptible Fish while Keeping them Alive. Ecotoxicology 10, 205–209.

567 Kördel, W., Garelick, H., Gawlik, B.M., Kandile, N.G., Peijnenburg, W.J.G.M., Rüdel, H., 2013. Substance-
568 related environmental monitoring strategies regarding soil, groundwater and surface water — an
569 overview. Environ. Sci. Technol. 20, 2810–2827. https://doi.org/10.1007/s11356-013-1531-2

570 Levesque, H.M., Dorval, J., Hontela, A., 2003. Hormonal, Morphological, and Physiological Responses of
571 Yellow Perch (Perca flavescens) to Chronic Environmental Metal Exposures. J. Toxicol. Environ.
572 Health 66, 657–676.

573 Liew, H.J., Kumar, S.A., Mauro, N., Diricx, M., Blust, R., De Boeck, G., 2012. Fasting goldfish, Carassius
574 auratus, and common carp, Cyprinus carpio, use different metabolic strategies when swimming. Comp.
575 Biochem. Physiol. Part A 163, 327–335. https://doi.org/10.1016/j.cbpa.2012.07.012

576 Mcgeer, J.C., Szebedinszky, C., Mcdonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure
577 to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat.

578 Toxicol. 50, 231–243.

579 Meena, R.A.A., Sathishkumar, P., Ameen, F., Yusoff, A.R.M., Gu, F.L., 2018. Heavy metal pollution in
580 immobile and mobile components of lentic ecosystems—a review. Environ. Sci. Pollut. Res. 25, 4134–
581 4148. https://doi.org/10.1007/s11356-017-0966-2

582 Melvin, S.D., 2016. Short-term exposure to municipal wastewater influences energy, growth, and swimming
583 performance in juvenile Empire Gudgeons (Hypseleotris compressa). Aquat. Toxicol. 170, 271–278.
584 https://doi.org/10.1016/j.aquatox.2015.06.003

585 Nelson, J.A., 2016. Oxygen consumption rate v. rate of energy utilization of fishes: a comparison and brief
586 history of the two measurements. J. Fish Biol. 88, 10–25. https://doi.org/10.1111/jfb.12824

587 Oikari, A., 2006. Caging techniques for field exposures of fish to chemical contaminants. Aquat. Toxicol. 78,
588 370–381. https://doi.org/10.1016/j.aquatox.2006.03.010

589 Ozmen, M., Ng Rd, A.G., Zehra Kucukbay, F., Elif, A.R., Ler, G., 2006. Monitoring the Effects of Water
590 Pollution on Cyprinus carpio in Karakaya Dam Lake, Turkey. Ecotoxicology 15, 157–169.
591 https://doi.org/10.1007/s10646-005-0045-1

592 Pierron, F., Baudrimont, M., Bossy, A., Bourdineaud, J.-P., Ethes, D., Elie, P., Massabuau, J.-C., 2007.
593 Impairment of lipid storage by cadmium in the European eel (Anguilla anguilla). Aquat. Toxicol. 81,
594 304–311. https://doi.org/10.1016/j.aquatox.2006.12.014

595 Pistole, D.H., Peles, J.D., Taylor, K., 2008. Influence of metal concentrations, percent salinity, and length of
596 exposure on the metabolic rate of fathead minnows (Pimephales promelas). Comp. Biochem. Physiol.
597 Part C 148, 48–52. https://doi.org/10.1016/j.cbpc.2008.03.004

598 Reynders, H., Bervoets, L., Gelders, M., De Coen, W.M., Blust, R., 2008. Accumulation and effects of
599 metals in caged carp and resident roach along a metal pollution gradient. Sci. Total Environ. 391, 82–
600 95. https://doi.org/10.1016/j.scitotenv.2007.10.056

601 Reynders, H., Van Campenhout, K., Bervoets, L., De Coen, W.M., Blust, R., 2006. Dynamics of cadmium
602 accumulation and effects in common carp (Cyprinus carpio) during simultaneous exposure to water
603 and food (Tubifex tubifex). Environ. Toxicol. Chem. 25, 1558–1567. https://doi.org/10.1897/05-239R.1

604 Roe, J.H., Dailey, R.E., 1966. Determination of Glycogen with the Anthrone Reagent. Anal. Biochem. 15,
605 245–250. https://doi.org/10.1016/0003-2697(66)90028-5

606 Roesijadi, G., 1996. Metallothionein and its role in toxic metal regulation. Comp. Biochem. Physiol. - C
607 Pharmacol. Toxicol. Endocrinol. 113, 117–123. https://doi.org/10.1016/0742-8413(95)02077-2

608 Sanchez, W., Sé, A., Ae, A.-A., Palluel, O., Ditche, J.-M., Porcher, J.-M., 2007. Preliminary investigation of
609 multi-biomarker responses in three-spined stickleback (Gasterosteus aculeatus L.) sampled in
610 contaminated streams. Ecotoxicology 16, 279–287. https://doi.org/10.1007/s10646-006-0131-z

611 Schoenaers, S., Vergauwen, L., Hagenaars, A., Vanhaecke, L., AbdElgawad, H., Asard, H., Covaci, A.,
612 Bervoets, L., Knapen, D., 2016. Prioritization of contaminated watercourses using an integrated
613 biomarker approach in caged carp. Water Res. 99, 129–139.
614 https://doi.org/10.1016/j.watres.2016.04.059

615 Shrivastava, J., Sinha, A.K., Cannaerts, S., Blust, R., De Boeck, G., 2017. Temporal assessment of
616 metabolic rate, ammonia dynamics and ion-status in common carp during fasting: A promising
617 approach for optimizing fasting episode prior to fish transportation. Aquaculture 481, 218–228.
618 https://doi.org/10.1016/j.aquaculture.2017.09.008

619 Sinha, A.K., Liew, H.J., Diricx, M., Blust, R., De Boeck, G., 2012. The interactive effects of ammonia
620 exposure, nutritional status and exercise on metabolic and physiological responses in gold fish
621 (Carassius auratus L.). Aquat. Toxicol. 109, 33–46. https://doi.org/10.1016/j.aquatox.2011.11.002

622 Vassallo, D. V, Almenara, C.C.P., Gilson, &, Broseghini-Filho, B., Ariane, &, Teixeira, C., Chaves, D., Da
623 Silva, F., Angeli, J.K., Padilha, A.S., 2011. Preliminary Studies of Acute Cadmium Administration

624 Effects on the Calcium-Activated Potassium (SKCa and BKCa) Channels and Na + /K + -ATPase
625 Activity in Isolated Aortic Rings of Rats. https://doi.org/10.1007/s12011-017-1150-8
626 Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
627