# Chromium bioavailability in aquatic systems impacted by tannery wastewaters. Part 2: New insights from laboratory and in situ testing with Chironomus riparius Meigen (Diptera, Chironomidae)

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# Chromium bioavailability in aquatic systems impacted by tannery wastewaters. Part 2: new insights from laboratory and *in situ* testing with *Chironomus riparius* Meigen (Diptera, Chironomidae)

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**Abstract**

Chromium is widely used as a tanning agent and can become a contaminant of concern in aquatic ecosystems receiving discharges from industrial or artisanal tanning activities. In a companion study, we showed that Cr discharged by tanneries was bioavailable to indigenous chironomids with accumulation via sediment ingestion likely to represent the predominant exposure route. However, Cr accumulation by chironomids did not directly reflect the degree of sediment contamination and the potential adverse effects of Cr accumulation on chironomids were not evaluated. In the present study, chironomids were exposed to homogenised, field-collected sediments in the laboratory and to intact sediments in situ using a customised caging system. Chromium concentrations were assessed in sediments, exposed larvae of laboratory-reared *Chironomus riparius* and overlying waters of in situ cages. Experimental results of Cr bioaccumulation were compared with expected Cr body burden in chironomids calculated using biodynamic modelling. Our data provided strong support to the hypothesis that Cr bioaccumulation in the field is specifically controlled by the deposition of contaminated suspended particulate matter (SPM) containing a pool of Cr readily bioavailable to surface deposit feeders. Considering freshly deposited SPM as an additional route of exposure for surface deposit feeders leads to a good agreement between the modelling and experimental results. Additionally, a Cr body burden of about 77 µg $g^{-1}$ d.w was identified as a tentative threshold above which effects on the growth of *C. riparius* may appear. While both laboratory and in situ experiments provide evidence for the availability of Cr in aquatic system impacted by tannery wastewaters, standard laboratory exposure conditions may miss additional exposure routes in the field and underestimate possible adverse effects on benthic organisms.

**Keywords.** Chironomids; Bioaccumulation; Suspended matter; Body residue; Sediment; Reservoir

## 1. Introduction

Bioaccumulation and toxicity of trace elements strongly depend on their speciation and on the actual exposure conditions experienced by target organisms; e.g. continuous *vs.* discontinuous inputs, contaminant uptake via different exposure routes (water vs. food), biological traits (e.g. feeding habit, respiration, growth, reproduction) of the species of interest (Fairbrother et al., 2007). Standardized laboratory experiments cannot account for all these factors although their importance is well-recognized and their predictive ability can be improved by a careful characterization of the actual exposure conditions in the selected laboratory settings (Simpson and Batley, 2007). Similarly, pulse and chase experiments using radiolabelled elements allow the study of the relative importance of different exposure routes, notably water exposure vs. food ingestion, in a variety of organisms (Baumann and Fisher, 2011; Wang et al., 1997), but cannot reproduce the complex array of environmental factors that influences bioavailability and toxicity in situ. To address these problems, the scientific community has engaged in actively developing tools to narrow the gap between laboratory and field studies. At present, microcosm and in situ techniques are sufficiently mature and versatile to be of use as supporting studies in tiered risk assessment procedures (Burton Jr et al., 2012; Crane et al., 2007; Ferrari and Faburé, 2017; Ferrari et al., 2014). An appropriate integration between laboratory and in situ approaches can help to better understand the bioavailability and toxicity of specific contaminants in ecosystems impacted by complex mixtures of contaminants such as tannery effluents (Vignati et al., 2007).

Adverse biological effects on aquatic organism in tannery contaminated systems are well documented (Khwaja et al., 2001; Koukal et al., 2004; Leghouchi et al., 2009). However, tannery effluents have a very complex composition and specifically ascribing the observed effects to Cr, typically used as a tanning agent in its trivalent form, remains difficult. Understanding of Cr bioavailability and toxicity in the field is further complicated by the

possible simultaneous presence of trivalent and hexavalent Cr forms that are characterized by different environmental mobility and toxicity (Vignati et al., this issue). On the other hand, Michailova et al. (2011) showed a genotoxic response in chironomids growing in a tannery-impacted system (the Dunajec river, southern Poland), where Cr and Cd were the only trace elements of concern. Although genotoxic and cancerogenic effects are mostly attributed to Cr(VI) exposure ((De Flora et al., 1990), genotoxic effects of Cr(III) organic compounds were also observed, along with changes in DNA and protein levels, in the yeast *Saccharomyces cerevisiae* (Chatterjee and Luo, 2010). The number of studies documenting a high toxicity of Cr(III) in standard laboratory settings is also increasing (Aharchaou et al., 2018; Bencheikh-Latmani et al., 2007; Kováčik et al., 2015; Ponti et al., 2014; Vignati et al., 2010), suggesting that Cr(III) is less harmless than admitted by the current scientific consensus. Significant polytene chromosome aberrations were found in *Chironomus riparius* exposed to Cr(III) contaminated sediments in laboratory conditions (Michailova et al., 2001).

In part 1 of the present study (Vignati et al., this issue), we showed that Cr discharged by tanneries is bioavailable to chironomids, but that the accumulation patterns do not simply reflect the contamination in abiotic matrices. By combining laboratory tests and in situ studies with caged, transplanted larvae of the model species *Chironomus riparius* Meigen (Diptera, Chironomidae), we specifically tested: a) if bioaccumulation in the field can be controlled by the ingestion of freshly-deposited contaminated SPM containing a pool of Cr readily bioavailable to surface deposit feeders such as chironomids and, b) at which level Cr accumulation may have adverse effects on the growth of *C. riparius*.

## 2. Material and Methods

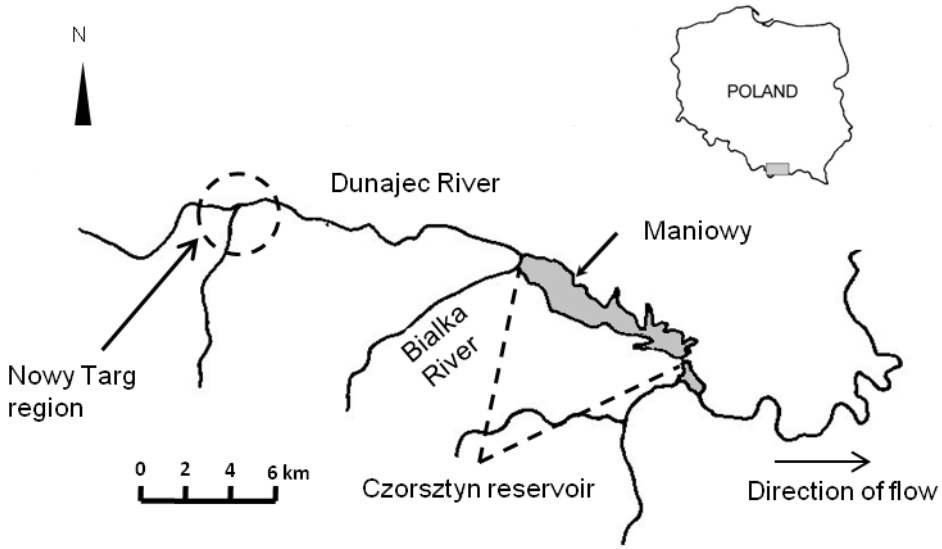
### *2.1 Model organisms and study area*

125 The non-biting midge larvae *Chironomus riparius* were used as model species for all
126 experiments. This species is commonly used for ecotoxicological purposes, can be reared in
127 laboratory conditions and has easily measurable and well modelled life-cycle traits.
128 *Chironomus riparius* has been considered as a model organism in several internationally
129 validated guidelines for assessing the toxicity of chemicals and for evaluating the quality of
130 natural sediments (see Ferrari et al. 2017 and references therein).

131 Larvae used in the tests originated from a culture maintained in our laboratory. The culture
132 was started from egg masses kindly provided by Dr. J. Garric (Irstea, formerly Cemagref,
133 Lyon, France). The methods for maintaining the culture and preparing the organisms for
134 experiments are detailed in Ferrari et al. (2014). For this study, two kinds of experiments were
135 conducted, either by starting with young second instar larvae (7-day laboratory tests) or by
136 starting with young fourth instar larvae (4-day laboratory and in situ tests). The larval stages
137 were determined by checking the length of the larvae and by controlling on few specimens the
138 head capsule width. Each set of experiment was designed to allow assessment of
139 bioaccumulation of Cr, but the toxicity of the whole sediment on chironomids (growth and
140 survival) was recorded at the same time. The chronological steps for implementing the tests
141 are depicted in Figure S1.

142 In situ exposure and sample collection for the concomitant laboratory experiments were
143 carried out in the Czorsztyn reservoir (Southern Poland, Fig. 1) which receives the tannery
144 contaminated wastewaters of the Dunajec river (about 300 small tanneries in the Nowy Targ
145 region) and of other smaller creeks. The in situ exposure/sampling site was located in
146 Maniowy Bay (Fig. 1) which is locally fed by a small creek collecting the effluent from a
147 rural wastewater treatment plant (Fig. 1). Tanneries are also present in the region of Maniowy,
148 although only some of them confer their wastes to the wastewater treatment plant. More

149 details on Cr contamination in the Czorsztyn reservoir can be found elsewhere (Dominik et
150 al., 2007; Szalinska et al., 2010) and in part 1 of this study (Vignati et al., this issue).
151




152
153 Figure 1. Study area (Czorsztyn reservoir; southern Poland) and location of the Maniowy Bay area where in situ
154 exposures performed and samples collected for laboratory experiments. The Nowy Targ region, where the many
155 artisanal tanneries are active, is also indicated.
156
157 *2.2 In situ bioaccumulation*
158 In situ exposure of *C. riparius* was carried out with a customized Sediment Water Interface
159 Study System (S-WISS1.1) suitable for exposing surface deposit feeders to intact sediments
160 for periods up to 4 days and allowing for food delivery to caged organisms during this period.
161 Construction details and functioning were described in detail in Ferrari et al. (2014). Briefly,
162 each exposure chamber consisted in a transparent polyvinyl chloride tube surmounted by a
163 customized corer-head to deliver the chironomids, ensure water exchange between the

exposure chambers and the surrounding waters and suspensions (150 µm Nitex mesh) and deliver the required food doses.

Twelve S-WISS1.1 were deployed at Maniowy (water depth approx. 3 m) by scuba divers. In addition, twelve control cages consisting in 1-L high-density polyethylene (HDPE), large-mouth bottles containing clean sand and partly covered with 150 µm Nitex mesh (Ferrari et al., 2014) to ensure water exchange were deployed in the vicinity of the S-WISS1.1 devices. Food delivery in the control cages was also ensured by adopting the design described in Ferrari et al. (2014). At t=0, 20 individuals of *C. riparius* ($4^{th}$ instar larvae) were introduced into each exposure system (i.e. S-WISS1.1 and control cages). Food doses (1 mg Tetramin® per individual) were delivered immediately after deployment and then every 24 hours until the end of the exposure period (4 days). At t=24, 48, 72, and 96 hours, three exposure chambers and three control cages were randomly recovered.

Upon arrival at the laboratory, each S-WISS1.1 was opened and an aliquot of the overlying water was sampled, filtered at 1.2 µm, acidified to 1% v/v with concentrated (65 %) Suprapur $HNO_3$ and stored in a polypropylene (PP) container at 4°C pending Cr analysis. After siphoning-off the remaining water, an aliquot of the top layer of sediments (0–0.5 cm) was gently scraped with a plastic spatula, passed through a 1 mm sieve to remove debris, and possibly chironomids, and stored in a PP container at –22 °C pending Cr analysis.

The sediment from the surface layer was chosen because it represents the most relevant exposure zone for surface deposit-feeders such as *C. riparius* (Rasmussen, 1984). The whole sediment core was then sieved (500 µm) to recover surviving chironomids. No indigenous chironomids were found in the cores. Recovered organisms were rinsed with site water and placed for 5 min in trays filled with ultrapure water (MilliQ water, Millipore), followed by 10 min in 1 mM EDTA, and additional 5 min in ultrapure water. After gently blotting with paper towels, each larva was measured using a digital image analysis system and organisms were

189 pooled and conditioned for Cr analysis (see Ferrari et al., 2014 for more details). Individual
190 length (n=20) and initial Cr body residue of unexposed larvae (2 pools of 30 individuals) were
191 determined at the start of each experiment.
192
193 *2.3 Laboratory bioaccumulation studies*
194 Laboratory experiments were set up using both field-collected sediments from Maniowy
195 and clean sand. Maniowy sediments were collected with an Ekman grab during the
196 deployment of the in situ test systems and stored at 4 °C. Upon return to the laboratory, the
197 overlying water was decanted and sediment were sieved at 1mm and homogenised on the day
198 before the start of the tests. Sediments (clean sand for controls) were transferred into 1-L
199 HDPE bottles analogous to those used for the control cages in the field experiment and
200 covered with filtered (1.2 µm) Lake Geneva water at a minimum ratio of 1:4. The following
201 day, 20 individuals of *C. riparius* were introduced into each exposure bottle and fed daily as
202 the chironomids exposed in situ. Young second instar larvae were used for performing a 7-day
203 bioaccumulation tests, while young fourth instar larvae were used for a 4-day tests.
204 All exposures were performed in triplicate at 21 °C under a 16:8-h light-dark photoperiod
205 and light intensity of 500 lux. Air was continuously bubbled into each beaker during the test
206 taking care to avoid sediment resuspension. The water level was controlled daily in each
207 beaker and adjusted with deionised water if necessary. Larvae were fed daily ad libitum with
208 0.6 mg of commercial food (Tetramin®) per individual (Péry et al., 2002).
209 For the 4-day bioaccumulation tests, organisms were retrieved and handled for length
210 measurement (i.e. each individual) and Cr content (i.e. pool of 20 individuals) as in situ
211 exposed ones at day 1, 2, 3 and 4. For the 7-day exposure tests, organisms were retrieved and
212 handled in the same way only at the end of the test. For each exposure condition, the surface
213 layer sediments (or sand) were sampled for total Cr analysis.

214

215 *2.4 Chemical analyses*

216 Chromium concentration in sediments and chironomids were assayed using the same
217 methodologies described in Vignati et al. (this issue). Total Cr concentrations in overlying
218 waters (filtered at 1.2 µm) in S-WISS1.1 cages were determined by ICP-MS, while Cr(VI)
219 levels were determined by catalytic adsorptive stripping voltammetry (Bobrowski et al.,
220 2012). Accumulation by chironomids was corrected for gut sediment content according to
221 (Hare et al., 1989) assuming that ingested sediments represented 6% of the total weight of
222 individual organisms (see Ferrari et al., 2014). For quality control procedures, reference
223 materials LKSD-4 (Lynch, 1990) and WQB3 (NRC, Canada) were used for sediments,
224 TORT-2 (NRC, Canada) for biological material and SLRS3 (NRC, Canada) and 1643e
225 (NIST) for waters. Reference material 1643e was analyzed following a 10-fold dilution.

226

227 *2.5 Modelling of bioaccumulation*

228 According to the biodynamic bioaccumulation modelling — DYMBAM (Baumann and
229 Fisher, 2011; Wang et al., 1997), accumulation of a contaminant by living organism can be
230 described by the following formula:

231

232 $$C_{biota} = \frac{k_u \cdot C_w}{k_{ew} + g} + \frac{AE \cdot IR \cdot C_s}{k_{es} + g} \quad (1)$$

233

234 where $C_{biota}$ is the concentration accumulated by organisms (in µg $g^{-1}$), $k_u$ is the uptake rate
235 constant from the dissolved phase (in L $g^{-1}$ $d^{-1}$), $C_w$ is the concentration in the water phase
236 (filtered water, in µg $L^{-1}$), $k_{ew}$ and $k_{es}$ are the efflux rate constants following uptake from the
237 dissolved phase and from food (sediments in the case of deposit feeders; both in $d^{-1}$), *AE* is the

assimilation efficiency (in % total content) from food (i.e., sediment), *IR* is the food ingestion rate (in g $g^{-1}$ $d^{-1}$), $C_s$ is the concentration in sediments (in µg $g^{-1}$) and *g* is the growth rate constant (in $d^{-1}$).

Although model parameters were not available for *C. riparius*, first-order estimates of expected Cr accumulation were obtained based on the following considerations. Accumulation from the aqueous phase was neglected considering that sediments should be the dominant route of contaminant uptake for chironomids (Dabrin et al., 2012; De Jonge et al., 2010; Martin et al., 2008). An ingestion rate of 0.325 g $g^{-1}$ $d^{-1}$ estimated for *C. riparius* at 10 °C (Bervoets et al., 2003) was adopted and corrected for the field/laboratory temperature of this study (21 °C) as described in the supporting information. A value of 0.1%, determined using the biomimetic approach described in the companion paper (Vignati et al., this issue), was used for the AE of Cr from Maniowy sediments. Estimation of the organisms' growth rate constant (*g*) was performed by fitting an exponential curve to the plot of weight of individual chironomids' larvae vs. time (Fig. S2). Finally, the $k_{es}$ constant was neglected considering that literature values for Cr egestion by various organisms (Baumann and Fisher, 2011; Roditi and Fisher, 1999; Wang et al., 1997) did not exceed 0.019 $d^{-1}$ and thus were 4 to 8-fold lower than the growth rates calculated from our experimental data (Fig. S2). The conclusions of the present study would not have been altered by considering $k_{es}$ in model calculations (see section 4.1) and this approach was preferred to the arbitrary choice of parameters derived for organisms other than *C. riparius*.

*2.6 Statistics*

Data were analyzed using Student's t-test, analysis of variance (one-way ANOVA followed by post-hoc Tukey's HSD test) and linear regression. All data were checked for normality and homogeneity of variance by the Shapiro-Wilk test and the Bartlett test,

263 respectively. If conditions for ANOVA were not fulfilled, the data were log-transformed and
264 reanalysed. For all statistical tests, the significance level was set at 0.05 and calculations were
265 performed using the software package R (v 2.9.0).
266
267 **3. Results**
268 *3.1. Quality control*
269 Figures of merit and overall accuracy of Cr analysis in aqueous matrices were analogous to
270 those reported in Vignati et al. (this issue). Percentage Cr recovery from LKSD-4 was about
271 120% when based on the concentrated $HNO_3$/concentrated HCl extractable fraction (21±2 µg
272 $g^{-1}$) and 75% when based on total Cr content determined by X-ray diffraction (33±6 µg $g^{-1}$;
273 n=2 in both cases). For WQB3, measured Cr concentrations were 96±8 % (n=5) of the
274 indicative value of 119 µg $g^{-1}$. Triplicate analyses of sediment subsamples from selected cages
275 agreed to 10% or better. Chromium recovery from TORT-2 (0.77±0.15 µg $g^{-1}$) was 96±3 %
276 (n=3). Due to the insufficient biological material, no replicate analyses of exposed
277 chironomids were possible. However, the small standard deviation on the three replicates of
278 TORT-2 material suggests an acceptable precision in the digestion of the biological material.
279
280 *3.2 Bioaccumulation under in situ and laboratory conditions*
281 Chromium concentrations in sediments recovered inside the S-WISS1.1 units were
282 homogeneous among the cages and across the four days of exposure (325±35 µg $g^{-1}$, n=11).
283 The Cr content of homogenised sediments from Maniowy used for the laboratory exposures
284 was uniform (150±14 µg $g^{-1}$ d.w., n=12), albeit about half of that measured in the sediment
285 recovered from in situ cages. Chromium levels in sand used for control exposures were
286 1.2±0.5 µg $g^{-1}$ (n=12) in the field and 0.8±0.1 µg $g^{-1}$ (n=12) in the laboratory (Fig. S3).

Bioaccumulation kinetic patterns in chironomids exposed to Maniowy sediments were similar for laboratory and in situ exposures, with a marked increase over the first day followed by a plateau in accumulated Cr levels. Daily and overall variability were however higher for organisms exposed in situ (Fig. 2).

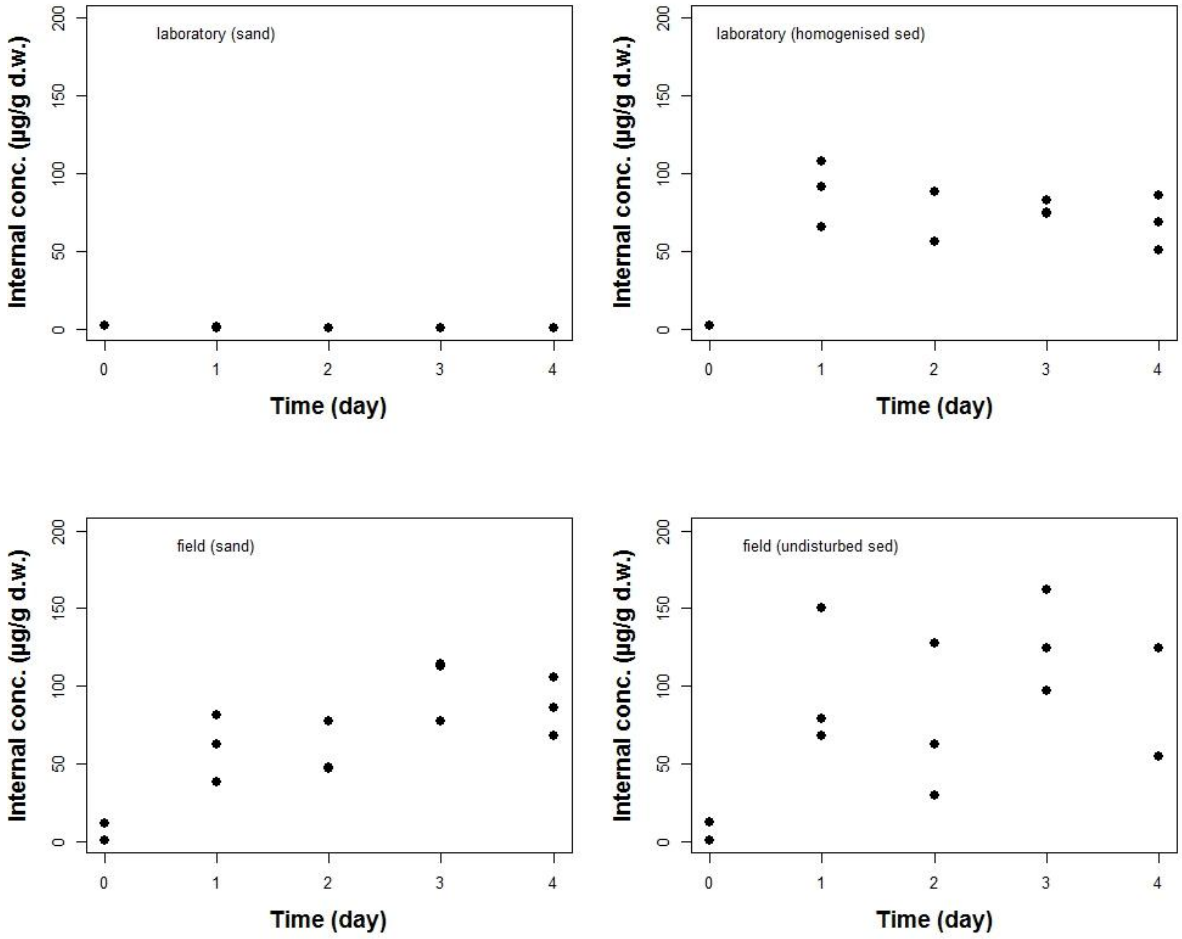



Figure 2. Cr accumulation vs. time in *Chironomus riparius* larvae exposed to sand and sediment in experiments performed in situ (Maniowy Bay, June 2007) and in the laboratory using sand and field collected sediment. In all experiments, each point represents the measure of Cr internal concentration obtained for the pooled organisms retrieved in each exposure system. Concentrations in chironomids were gut corrected according to Hare et al. (1989).

298 At plateau (days 2–4), chironomids exposed in the laboratory to homogenised sediments
299 from Maniowy accumulated an average Cr concentration of 78±16 µg $g^{-1}$ (n=12), comparable
300 to the concentration of 98±43 µg $g^{-1}$ (n=11) measured in chironomids exposed in situ to
301 undisturbed field sediments using the S-WISS1.1 system. At the opposite, bioaccumulation
302 kinetic patterns in chironomids exposed to clean sand showed a marked difference between
303 the laboratory and in situ exposures. Indeed, no increase in Cr concentration was observed
304 during laboratory exposures with control chironomids accumulating less than 1 µg $g^{-1}$ (Fig.
305 2). On the contrary, chironomids exposed in cages filled with clean sand but placed on the
306 reservoir bottom close to the S-WISS1.1 devices accumulated an average of 77±25 µg Cr $g^{-1}$
307 (n=12). This value is comparable to the concentrations obtained for chironomids exposed to
308 the Maniowy sediments, whether in laboratory experiments or after in situ exposures (Fig. 2).
309 Results obtained by exposing 4$^{th}$ instar larvae in situ or in the laboratory also compared
310 favourably with those of 2$^{nd}$ instar larvae exposed for 7 days to homogenised sediments in the
311 laboratory (Fig. 3). Indeed, three days after the start of any type of exposure protocol using
312 young 4$^{th}$ instar larvae (see sections 2.2 and 2.3), all larvae were 9 days old after hatching
313 (Fig. S1). Thus, 4$^{th}$ instar larvae exposed over 3 days to sediments in situ or in the laboratory
314 were as old as 4$^{th}$ instar larvae remaining at the end of the 7-day bioaccumulation test in the
315 laboratory which was started with young 2$^{nd}$ instar larvae (Fig. S1).
316 For 3 days exposures of 4$^{th}$ instar larvae, Cr concentrations in organisms exposed to
317 sediment and sand (i.e., controls) were 128±33 µg $g^{-1}$ vs. 101±21 µg $g^{-1}$ for in situ
318 experiments and 77±5 µg $g^{-1}$ vs. 0.42±0.07 µg $g^{-1}$ for laboratory experiments (Fig. 3). A
319 statistically significant difference in Cr accumulation (Tukey HDS test on log-transformed
320 data, $p<0.05$) existed between controls and exposed chironomids only in laboratory
321 experiments. Despite the variability in the Cr content of sediment substrates (Fig. S3), Cr
322 accumulation was not significantly different between organisms caged in the field (control

and exposed) and organisms exposed to homogenised Maniowy sediments in the laboratory (Tukey HDS test on log-transformed data, $p>0.05$). For 7 days experiments started using 2nd instar larvae, measured Cr accumulation at day 7 was 66±8 µg $g^{-1}$ and 0.67±0.17 µg $g^{-1}$ in organisms exposed to sediments and control sand, respectively. Chromium accumulation in 2nd instar larvae exposed to Maniowy sediment for 7 days was significantly lower than in 4th instar larvae exposed for 3 days to undisturbed field sediments (Tukey HDS test on log-transformed data, $p<0.05$), but did not show significant differences with other experimental conditions (Fig. 3).

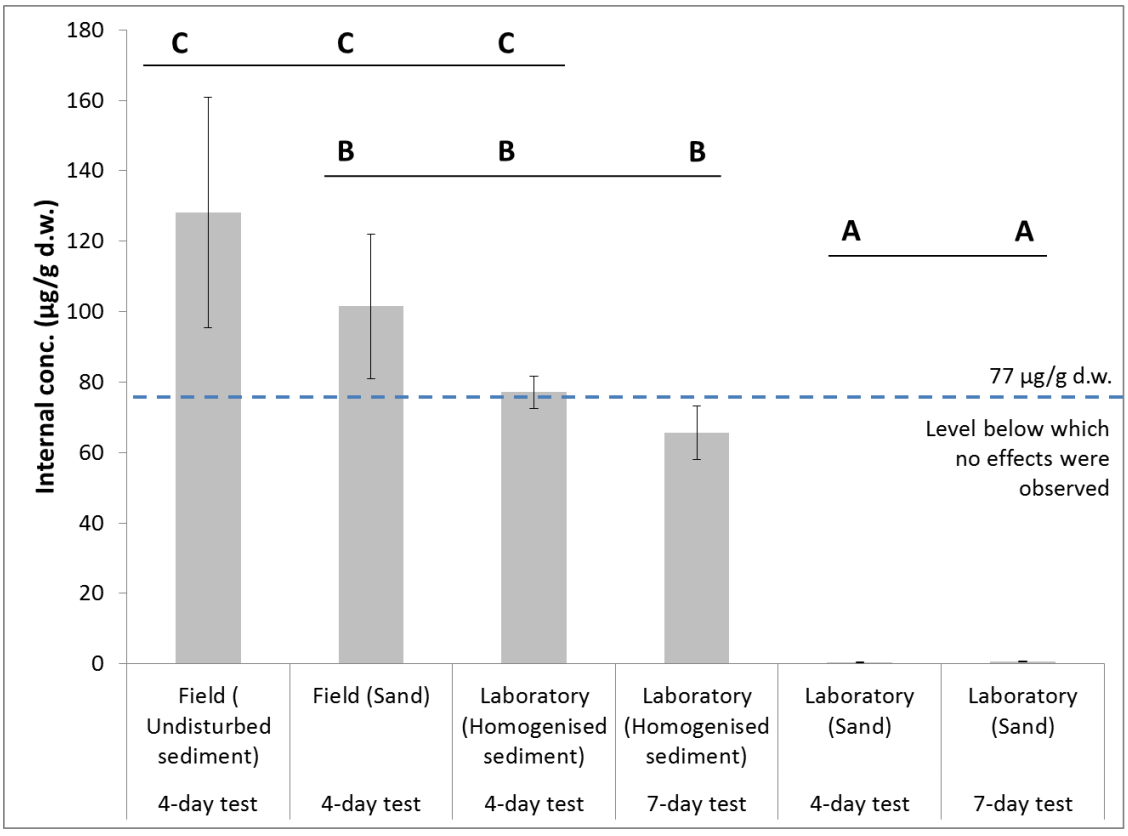



Figure 3. Cr accumulation at day 3 in *Chironomus riparius* larvae exposed to sand and sediment in experiments performed in situ (Maniowy Bay, June 2007) and in the laboratory using field collected material. The 4-day tests were initiated with young fourth instar larvae, while the 7-day tests were initiated with young second instar larvae. Error bars represent 1 standard deviation (n=3). Internal concentrations that do not share the same letter

are significantly different (ANOVA followed by post-hoc Tukey's HSD test, $p<0.05$). The dotted line symbolizes the level below which no effects were observed by considering the results obtained for growth rates (see Fig. S2).

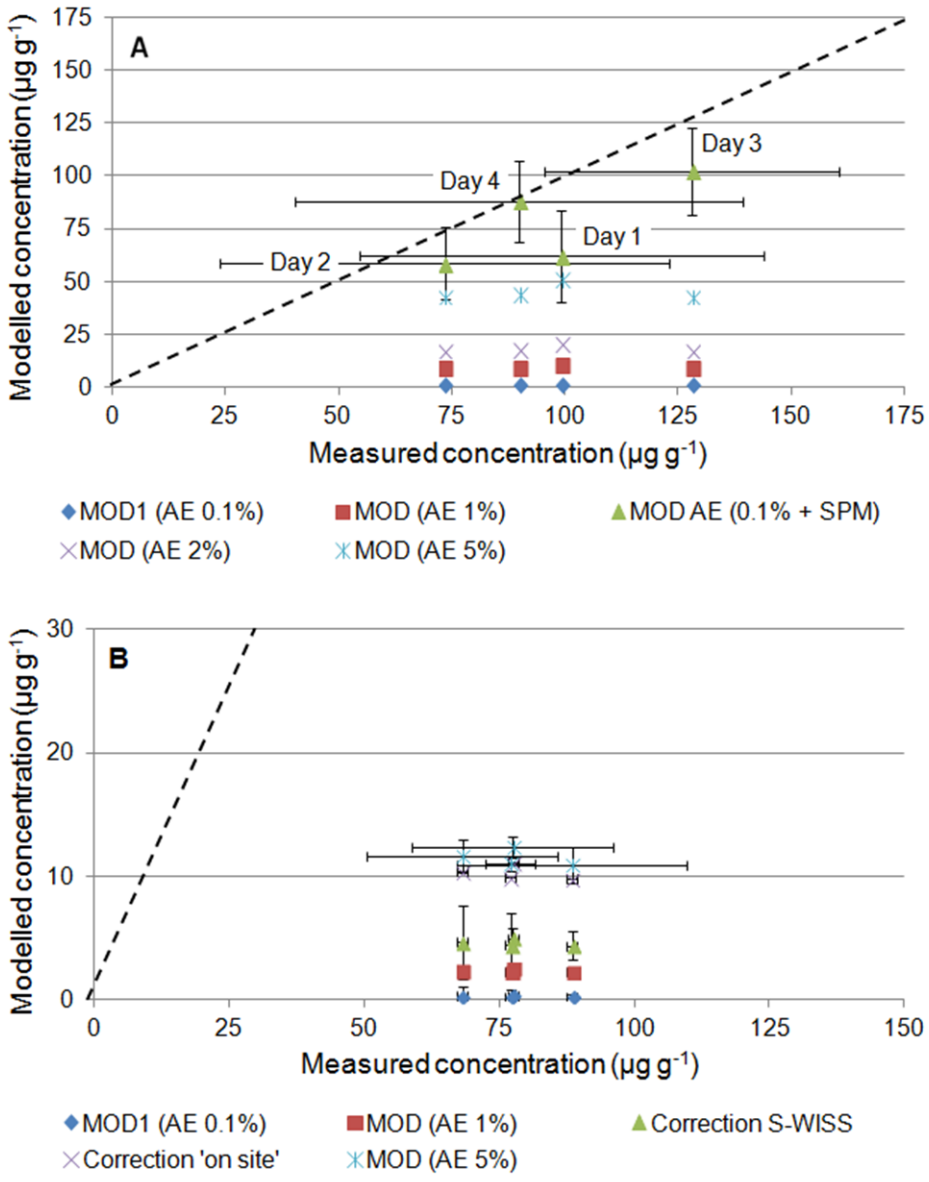
### *3.3. Modelling chromium bioaccumulation via sediment ingestion*

The modelled Cr accumulation via sediment ingestion by *C. riparius* exposed to Maniowy sediments did not exceed 1 µg $g^{-1}$ for neither in situ nor laboratory exposures. These values were 1% (or less) of measured ones and strongly underestimated the actual Cr burden in both scenarios (Fig. 4a, 4b). Because the biomimetic approach used to derive the AE of 0.1% employed in model calculation (see section 2.5) may overlook other factors resulting in higher assimilation efficiencies (Vignati et al., this issue), additional model calculations were performed using AE values of 1, 2 and 5%. These AEs were reported in studies on Cr uptake by various organisms and from different substrates including sediments and algae (Baumann and Fisher, 2011; Roditi and Fisher, 1999; Wang et al., 1997). Even for the maximum AE value of 5%, modelled concentrations were 40–60% lower than the measured ones (Figure 4a, b).

Chironomids exposed to clean sand in laboratory experiments did not show any appreciable Cr bioaccumulation suggesting that neither the ingestion of sand substrate (in field controls) nor the added Tetramin® food (all experimental conditions) contributed to the observed Cr burdens. Measured Cr concentration in laboratory control chironomids actually decreased from 0.9±0.5 µg $g^{-1}$ at day 1 to 0.4±0.06 µg $g^{-1}$ at day 4 (data not shown). At t=0, control chironomids had a Cr content of about 2 µg $g^{-1}$, which was diluted by the ingestion of 'clean' food and by growth. On the other hand, measured concentration in field control organisms were comparable to those of organisms exposed to Maniowy sediments (Fig. 3). As it will be discussed in detail in section 4.1, we surmise that accumulation from freshly

363 deposited SPM was responsible for the Cr burden measured in the field control chironomids
364 and also contributed the majority of accumulated Cr to organisms exposed in situ to
365 unperturbed sediments.

366




367

368 Figure 4. Comparison of measured vs. modelled Cr concentrations in *Chironomus riparius* exposed to
369 undisturbed Maniowy sediments in situ (S-WISS1.1 cages – panel A) and to homogenised Maniowy sediments
370 in the laboratory (panel B). All values are in µg $g^{-1}$. MOD, modelled accumulation of Cr obtained using different
371 values for assimilation efficiency (AE) in formula 1; SPM, Cr accumulation in field control organisms (assumed
372 to originate from ingestion of freshly deposited Suspended Particulate Matter — see text for details); Correction

373 'on site' and 'S-WISS', modelled accumulation using formula 1 and corrected for changes in bioavailability due
374 to sediment manipulation according to Ferrari et al. (2014). The dashed lines show the theoretical 1:1
375 correspondence between modelled and measured concentrations.
376
377 *3.4 Effects on chironomids*
378 Survival of chironomids in laboratory controls was almost complete (19.6±0.7 individuals;
379 n=12) over the 4 days of exposure and only slightly reduced (18.1±2.2 individuals, n=12) for
380 exposure to Maniowy sediments in the laboratory. Average survival in field controls was
381 16.3±3.7 individuals (n=12); significantly higher than in organisms directly caged on
382 sediments in situ (9.8±2.7 individuals, n=11). In this last case, no temporal trend in survival
383 was observed.
384 The length of alive chironomids increased linearly and significantly ($R^2$>0.84, p<0.0001)
385 over time during the 4-day exposure both in the laboratory and in the field (Fig S2). In the
386 laboratory, the growth rate of the chironomids exposed to sand was 0.1499±0.0054 cm $d^{-1}$; not
387 significantly different from the growth rate of 0.1421±0.0096 cm $d^{-1}$ for the chironomids
388 exposed to homogenised Maniowy sediments (ANOVA, p>0.05; Fig. S2). In the field, the
389 growth rate of the chironomids exposed to sand (0.1273±0.0065 cm $d^{-1}$) was significantly
390 lower than the value obtained for control chironomids in the laboratory (ANOVA, p=0.016;
391 Fig. S2). Growth rate further and significantly decreased to 0.092±0.0114 cm $d^{-1}$ when the
392 chironomids were exposed to the undisturbed sediments in situ using S-WISS1.1 (ANOVA,
393 p=0.011; Fig. S2).
394 Concerning the 7-day bioaccumulation test, no significant effect was observed between
395 controls and exposed chironomids. The lowest number of alive chironomids per beaker (n=3)
396 was 19 and 18 in controls and exposed organisms, respectively. The mean length at the end of

exposure period was 1.01 ± 0.02 cm in controls and 0.99±0.05 cm in chironomids exposed to the homogenised Maniowy sediment (t-test, $p>0.05$).

## 4. Discussion

### *4.1. Importance of SPM as an additional exposure route to contaminants in the field*

The comparable Cr body burden in chironomids exposed in situ to undisturbed field sediments and to clean sand (Figs. 2 and 3) suggests the presence of an additional source of Cr for the organisms exposed in the field. The lower accumulation of Cr in chironomids exposed in the laboratory to homogenised sediments from Maniowy also supports the hypothesis that one (or more) additional source of Cr exists in the field. A possible contribution of Tetramin® to Cr bioaccumulation can be ruled out by the absence of Cr accumulation in control laboratory chironomids. We surmise that freshly deposited SPM entering the exposure cages can act as an important source of Cr to surface deposit feeders exposed in the field at the site of Maniowy. Besides the strong Cr accumulation by control chironomids exposed in situ, several observations collectively form a basis for evidence to support this hypothesis as detailed in the next paragraphs.

The lower accumulation by laboratory-exposed chironomids likely reflects the lower Cr concentration in homogenised sediments used for laboratory experiments compared with undisturbed field sediments (Fig. S3). The differences between homogenised and undisturbed (surface) sediments may be explained by the vertical decrease of Cr contamination in Maniowy sediments (Vignati et al., this issue). However, Ferrari et al. (2014) showed that, for sediments collected from the same site in Lake Geneva, Cr bioaccumulation was about 2- to 3-fold higher for chironomids exposed to homogenised sediments in the laboratory (sediment Cr content 25–30 μg $g^{-1}$) than to undisturbed field sediments using S-WISS1.1 type cages (28–32 μg $g^{-1}$ of Cr) or to homogenised sediments placed back in the field (27–32 μg $g^{-1}$ of

422 Cr). In the study of Ferrari et al. (2014), temperature differences between laboratory (21 ºC)
423 and field (15 ºC) alone could explain the observed differences. Sediment manipulation is also
424 known to trigger a series of reactions that increase the bioavailability of sediment-bound
425 contaminants (Vandegehuchte et al., 2013); in disagreement with our observations. Overall,
426 these previous observations suggest that the higher accumulation of Cr in chironomids
427 exposed in the field to undisturbed Maniowy sediments requires a supplementary source of
428 bioavailable Cr.

429 As observed in previous research on metal accumulation by chironomids (Dabrin et al.,
430 2012; De Jonge et al., 2010; Martin et al., 2008), uptake of aqueous Cr species from overlying
431 water and pore water is unlikely to be the main responsible for the observed bioaccumulation
432 patterns in chironomids. In laboratory experiments, homogenized Maniowy sediments were
433 covered with filtered water from Lake Geneva; which has a low Cr content (Edder et al.,
434 2008; Kottelat, 2008) and should not contribute much to Cr accumulation. The occurrence of
435 higher Cr body burdens in field control chironomids compared with organisms exposed to
436 Maniowy sediments in the laboratory can therefore be considered indicative of Cr uptake
437 from the overlying waters of Maniowy. This pattern was actually observed after 3 and 4 days
438 of exposure (Figs. 2 and 3). However, differences in accumulated Cr concentrations were only
439 30% of the total body burdens, confirming the need for another source of Cr to explain the
440 observed bioaccumulation in field controls. Furthermore, field control chironomids were
441 exposed on a substrate of (initially) clean sand so that Cr uptake from pore water is unlikely to
442 have contributed markedly to the elemental burden of these organisms.

443 Similar conclusions can be obtained by examining the total filterable Cr concentrations
444 measured in overlying waters inside S-WISS1.1 cages (Table S1). These concentrations
445 showed some variability between days 1+2 (3.9±1.4 µg $L^{-1}$; n=6) and 3+4 (35±29 µg $L^{-1}$;
446 n=6), but Cr accumulation by chironomids was not directly proportional to filterable Cr

concentration inside the cages (Table S1). DGT measurements over 89 hours (slightly shorter than the 96 hours of chironomid exposure for logistic reasons) did not show any marked change in labile Cr in overlying or interstitial waters either (Fig. S4). The concentration of Cr(VI) measured in overlying waters of cages recovered on days 3 and 4 (Table S1) was 0.59±0.321 µg $L^{-1}$ (n=6, range: 0.076–1.04 µg $L^{-1}$) and comparable to the levels measured in pore waters recovered from sediment cores sampled in Maniowy (Vignati et al., this issue). Otherwise stated, caging did not markedly change Cr(VI) concentrations (which could have had consequences on Cr bioavailability) and DGT-labile Cr, a proxy for bioavailable trivalent Cr, remained constant during chironomids' exposure. Changes in pH, conductivity and oxygen content within the S-WISS1.1 cages are also too limited (Ferrari et al., 2014) to result in significant increases of Cr oxidation from field sediments. Considering the egestion rate of Cr assimilated via the dietary pathway (i.e., the $k_{es}$ parameter in formula 1) would have further reduced the modelled Cr body burden. Overall, Cr uptake from freshly deposited SPM remains the hypothesis best supported by the available data to explain a) the observed Cr accumulation in field controls and b) the higher Cr accumulation by chironomids exposed to Maniowy sediment in situ compared with laboratory exposures to homogenised sediment.

*4.2. A unifying framework for modelling, experimental results and field observations*

The hypothesis that ingestion of freshly deposited SPM (section 4.1) represents a significant route of Cr accumulation for chironomids exposed in situ allows to reconcile modelling calculations and experimental results to a great extent. Summing accumulation via SPM (i.e., the experimental values measured for field control cages) to the quantity of Cr accumulated from sediment ingestion (using an AE of 0.1% as determined by the biomimetic approach – see section 2.5) markedly improves the agreement between modelled and measured bioaccumulation for organisms exposed to Maniowy sediments in situ (Fig. 4a).

With this procedure, modelled concentrations remain within 20% of measured ones for 2, 3 and 4 days of exposure, again supporting the hypothesis that SPM is a major route of exposure in field conditions. The ± 20% differences are within the possible contribution of Cr uptake via the aqueous route; as in indicated by the comparison between laboratory exposure to Maniowy sediments and in situ control cages (see section 4.1).

On the other hand, a contribution of freshly deposited SPM to Cr accumulation cannot be invoked to reduce the discrepancies between modelled and experimental Cr concentrations for chironomids exposed to Maniowy sediments in the laboratory (Fig. 4b). Accumulation via aqueous exposure, if any, would not lead to a better agreement between modelled and experimental results either (see sections 3.3 and 4.1). It is however possible that sediment manipulation during the setup of laboratory exposure may increase Cr bioavailability for chironomids. In the case of the Vidy Bay (a polluted bay in Lake Geneva, Switzerland/France), Ferrari et al. (2014) reported that Cr bioaccumulation by *C. riparius* in the laboratory was about twice than in specimen of the same species exposed in the field (S-WISS1.1 cages). Chromium accumulation in the laboratory was even 4–5 times higher than in organisms exposed on site in cages containing the same homogenised field sediments used in the laboratory. However, including a correction factor of 2 (in situ vs. laboratory) or 4.5 (on site vs. laboratory) into modelled bioaccumulation values for chironomids exposed to Maniowy sediment in the laboratory does not improve the agreement between observed and predicted results to any appreciable extent (Fig. 4b).

Another factor that may increase Cr bioavailability in laboratory exposure following sediment manipulation is the association of Cr with Fe/Mn oxides in Maniowy sediments (Vignati et al., this issue). However, even assuming a Cr assimilation efficiency of 5% for chironomids exposed to Maniowy sediments in the laboratory, modelled results still remain about 15% of the measured accumulation at day 4 (Fig. 4b). One last option is that the

development of an active Mn redox cycle at the sediment-water interface might have led to the formation of highly bioavailable Cr(VI) via oxidation of Cr(III) in the laboratory exposure beakers (Landrot et al., 2012). While the available experimental data do not allow to test this hypothesis, it appears that chromium bioaccumulation in the laboratory occurred to a different extent, and possibly via different routes, compared with real-field situations. Otherwise stated, the use of homogenised sediment to test for contaminant bioavailability may change the relative importance of the various exposure routes and lead to results not directly applicable in the field (Ferrari et al., 2014; Simpson and Batley, 2007; Wang et al., 2004).

It is also instructive to examine how well Cr accumulation by caged specimen of *C. riparius* can mimic Cr accumulation by indigenous chironomids. As already seen, consideration of Cr uptake via ingestion of freshly deposit SPM is necessary to reconcile DYMBAM calculations and experimentally measured accumulation for specimen of *C. riparius* exposed in situ to Maniowy sediment (Fig. 4a). However, even after accounting for the SPM route, the average accumulation of Cr by *C. riparius* inside S-WISS1.1 cages (98±23 µg $g^{-1}$; arithmetic mean ± one standard deviation for 4 days of exposure, n=11) remains about 3 times lower than accumulation by indigenous chironomids recovered from Maniowy sediments during the same period (315 µg $g^{-1}$ for the pool of chironomids sampling in June 2007; see Vignati et al., this issue). This difference may originate from individual indigenous chironomids weighing, on average, about 3.5 times as much as S-WISS1.1 exposed specimen on day 4 (1.43 vs. 0.388 mg). Larger individuals would ingest larger sediment quantities to meet their nutritional needs, while simultaneously accumulating more Cr from contaminated sediments. Furthermore, ingestion rate is inversely related to the organic matter content of a given sediment. Contrary to caged *C. riparius*, indigenous chironomids could not rely on Tetramin® as an additional source of Cr-free carbon; which could again have led to higher ingestion rates and, eventually, higher Cr body burdens. These considerations equally apply to

522 the ingestion of freshly deposited SPM and do not invalidate the hypothesis of SPM as the
523 main Cr source to chironomids. Even if not performed in the same experimental conditions, it
524 is interesting to note that Bervoets et al. (2004) found a very good agreement in Cr internal
525 concentrations between caged and resident larvae, especially in the most contaminated sites.
526
527 *4.3. Effect of chromium on the growth of chironomids*
528 Individuals of *C. riparius* exposed to homogenised Maniowy sediments in the laboratory
529 for 4 or 7 days did not exhibit higher mortality or reduced growth rate compared with control
530 chironomids (section 3.4 and Fig. S2), indicating that Maniowy sediments were not toxic in
531 standardized conditions. The absence of effects in laboratory exposures may be linked with
532 sediment homogenisation. The Cr content of Maniowy sediments rapidly decreases below 5–6
533 cm (Vignati et al., this issue) because the Czorsztyn reservoir was flooded in 1997 and a thin
534 layer of Cr-contaminated sediments overlies a relatively compact layer of former soil with
535 low Cr content.
536 On the other hand, exposure to undisturbed sediments in the field resulted in reduced
537 survival and growth rate compared with laboratory and field controls as well as with
538 homogenised Maniowy sediments tested in the laboratory (section 3.4 and Fig. S2). During
539 the in situ exposure period, water temperature in the field was around 21 ºC and hence similar
540 to the optimal temperature used for laboratory experiments. Moreover, chironomids have been
541 daily fed ad libitum in all laboratory and in situ tests, thus preventing the impact of limiting
542 food availability during in situ exposure. The differences between field controls and field
543 exposed chironomids are therefore indicative of some kind of adverse effect of Maniowy
544 sediments. Furthermore, the difference between field and laboratory controls also points to
545 adverse effect linked to the presence of freshly deposited SPM which appears as an important
546 route of exposure to Cr for chironomids during in situ experiments.

When considering the quantity of Cr accumulated by specimen of *C. riparius* in relation to the growth results, it can be assumed that no effect on growth can be observed up to a total concentration of 77 µg $g^{-1}$ (Fig. 3 and Fig. S2). This concentration could correspond to a Critical Body Residue (CBR). The CBR is the concentration of a chemical bioaccumulated in an organism above which ecotoxicity effects appear (McCarty and MacKay, 1993). The tentative CBR value of 77 µg $g^{-1}$ agrees with the data of Méndez-Fernández et al. (2013) who determined a CBR of approx. 34 µg $g^{-1}$ for *Tubifex tubifex* in tests with natural sediments spiked with $K_2Cr_2O_7$. Upon entering living cells, Cr(VI) is rapidly converted to Cr(III) (Viti et al., 2014), so that a comparison of CBR for the two redox forms of Cr remains meaningful.

Recent studies have demonstrated that metal body burdens in relative resistant invertebrates such as chironomids can be used as predictors of ecological effects of metals on aquatic ecosystems. Bervoets et al. (2016) have linked threshold body burdens of metals in fourth instar larvae of indigenous *Chironomus* sp. to effects on resident macroinvertebrate communities. Depending on the descriptor chosen for the structure of the macroinvertebrate community, the threshold values ranged from 10 to 69 µg $g^{-1}$ d.w. of Cr accumulated in chironomids. It is therefore possible that Cr contamination at Maniowy adversely affects sensitive taxa of macroinvertebrates. Furthermore, the tentative CBR value obtained for *C. riparius* in the present study suggests that Cr levels above 77 µg $g^{-1}$ d.w. can also affect relatively pollution-tolerant organisms such as chironomids themselves. These findings are not at odds with the thresholds proposed by Bervoets et al. (2016) because laboratory-reared organisms are likely more sensitive than adapted local indigenous species.

However, the bioaccumulation results obtained in this study showed a large variability (Fig. 2) which hampers a clear definition of the actual CBR for Cr in *C. riparius*. Other studies also suggest that metals have toxic effects only when their uptake rate exceeds their combined rates of efflux and detoxification (Casado-Martinez et al., 2010) or the fraction of

572 metabolically available metal increases (Rainbow, 2007). Knowledge of the subcellular fate
573 of metals is therefore required to better understand the physiological processes underlying
574 their bioaccumulation and toxicity. Such methods have been developed for larvae of
575 chironomids (Gimbert et al., 2016; Péry et al., 2008) and will have to be considered to further
576 clarify the metabolically available concentration of Cr that can affect the development of
577 chironomids at different stages in real-field situations.
578
579 **5. Conclusions**
580 Comparable levels of Cr accumulation by specimen of *Chironomus riparius* exposed to
581 undisturbed field sediments in situ and to clean sand on-site suggest that ingestion of freshly
582 deposited SPM can be the main route of Cr accumulation by surface deposit feeders in real
583 field conditions. This additional route of exposure is not accounted for in standardized
584 laboratory experiments or, as yet, by biodynamic bioaccumulation modelling, so that both
585 approaches will lead to an underestimation of Cr accumulation compared with field
586 conditions. Changes in the bioavailability of sediment-bound Cr also seem to occur during
587 sediment sampling and handling, with the correction of such experimental artefacts being far
588 from straightforward. While both laboratory and in situ experiments provide evidence for the
589 availability of Cr in aquatic system impacted by tannery wastewaters, in situ investigations
590 appear necessary to obtain an adequate understanding of Cr bioavailability under real
591 environmental conditions.
592 A tentative threshold of Cr accumulation for effects on the growth of *C. riparius* is
593 proposed at 77 µg $g^{-1}$ d.w. Further studies using toxicokinetics approaches and subcellular
594 fractionation methods are however required to refine the actual concentration of Cr linked to
595 the biologically active sites and leading to adverse effects in these benthic organisms.
596 Extrapolation from the model organism *C. riparius* to other species of indigenous

chironomids also represents a future challenge to better link laboratory and in situ results with real field situations.

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# Supporting information

**Chromium bioavailability in aquatic systems impacted by tannery wastewaters. Part 2: new insights from laboratory and *in situ* testing with *Chironomus riparius* Meigen (Diptera, Chrinomidae)**

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**Correction of sediment ingestion rate (IR) for temperature**

Temperature correction for sediment ingestion rates was calculated according to the formula:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)} \qquad \text{(A)}$$

where: $Q_{10}$ (dimensionless) is the rate of increase of a reaction for a 10 degrees temperature increase (in °C or K), $R_1$ and $R_2$ are the reaction rates at $T_1$ and $T_2$, respectively (with $T_1 < T_2$). Web-based calculator (http://www.physiologyweb.com/calculators/q10_calculator.html ) was used to estimated temperature-adjusted sediment ingestion rates entering an arbitrary $Q_{10}$ value of 2 in formula (A) (see below for the rationale behind this choice). Other input values for formula A were the reference temperature of 10°C used by Bervoets et al. (2003), the corresponding IR value of 0.325 g g-1 d-1 for C. riparius and the measured field and laboratory temperature of 21°C for our study. A temperature corrected value of IR equal to 0.697 g g-1 day-1 resulted from this calculations.

The Q10 factor of 2 was selected based on (Croteau et al., 2002; Gresens, 2001) who studied the effect of temperature on Cd accumulation and sediment ingestion and digestion. It is important to note that uncertainties exist as to the influence of temperature on sediment ingestion rates and contaminant accumulation by chironomids In particular, the relationship between temperature and sediment ingestion rate is not necessarily a monotonous one (Gresens, 2001) and its determination would require experimental approaches outside the scope of the present work.

**Table S1.** Concentration of total Cr measured in overlying waters recovered inside each of the three S-WISS cages on days 1, 2, 3, and 4; concentrations of hexavalent Cr in the same waters; and concentration of total Cr in the chironomids exposed within each cage.

| Day | Replicate | Water | | Chironomids |
|---|---|---|---|---|
| | | Total Cr ($\mu g\ L^{-1}$) | Cr(VI) ($\mu g\ L^{-1}$) | Total Cr[a] ($\mu g\ g^{-1}$) d.w. |
| 1 | 1 | 3.42 | n.a. | 68.1 |
| 1 | 2 | 6.31 | n.a. | 150 |
| 1 | 3 | 4.31 | n.a. | 79.2 |
| 2 | 1 | 2.48 | n.a. | 29.9 |
| 2 | 2 | 2.87 | n.a. | 63.0 |
| 2 | 3 | 3.93 | n.a. | 127 |
| 3 | 1 | 93.5 | 0.57 | 125 |
| 3 | 2 | 26.1 | 0.61 | 162 |
| 3 | 3 | 21.8 | 0.46 | 97.3 |
| 4 | 1 | 23.8 | 1.04 | 54.9 |
| 4 | 2 | 29.3 | 0.08 | n.a. |
| 4 | 3 | 18.7 | 0.76 | 125 |

a) Concentrations corrected for sediment content in the gut (see main text for details)

**Figure S1.**

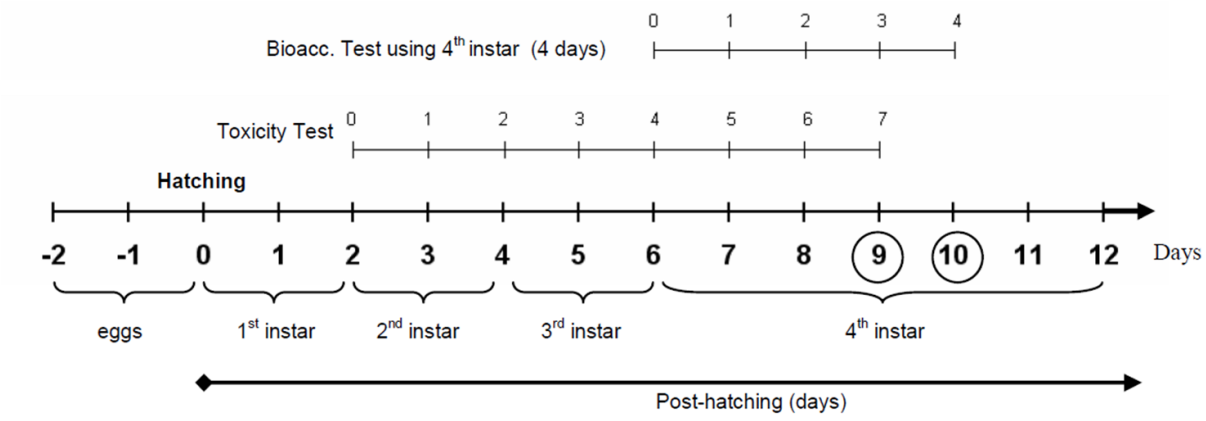


**Figure S1.** Chronological step for implementing the bioaccumulation and toxicity tests with the non-biting midge larvae *Chironomus riparius*. For the present study, experiments were started either with young second instar larvae (7 day laboratory tests) or with young fourth instar larvae (4 day laboratory and in situ tests). Numbers correspond to the post-hatching days needed to reach the end of the corresponding test. Note that 2nd instar larvae used in 7-day toxicity test and 4th instar larvae used in bioaccumulation tests will have the same post-hatching age at day 9.

**Figure S2.**

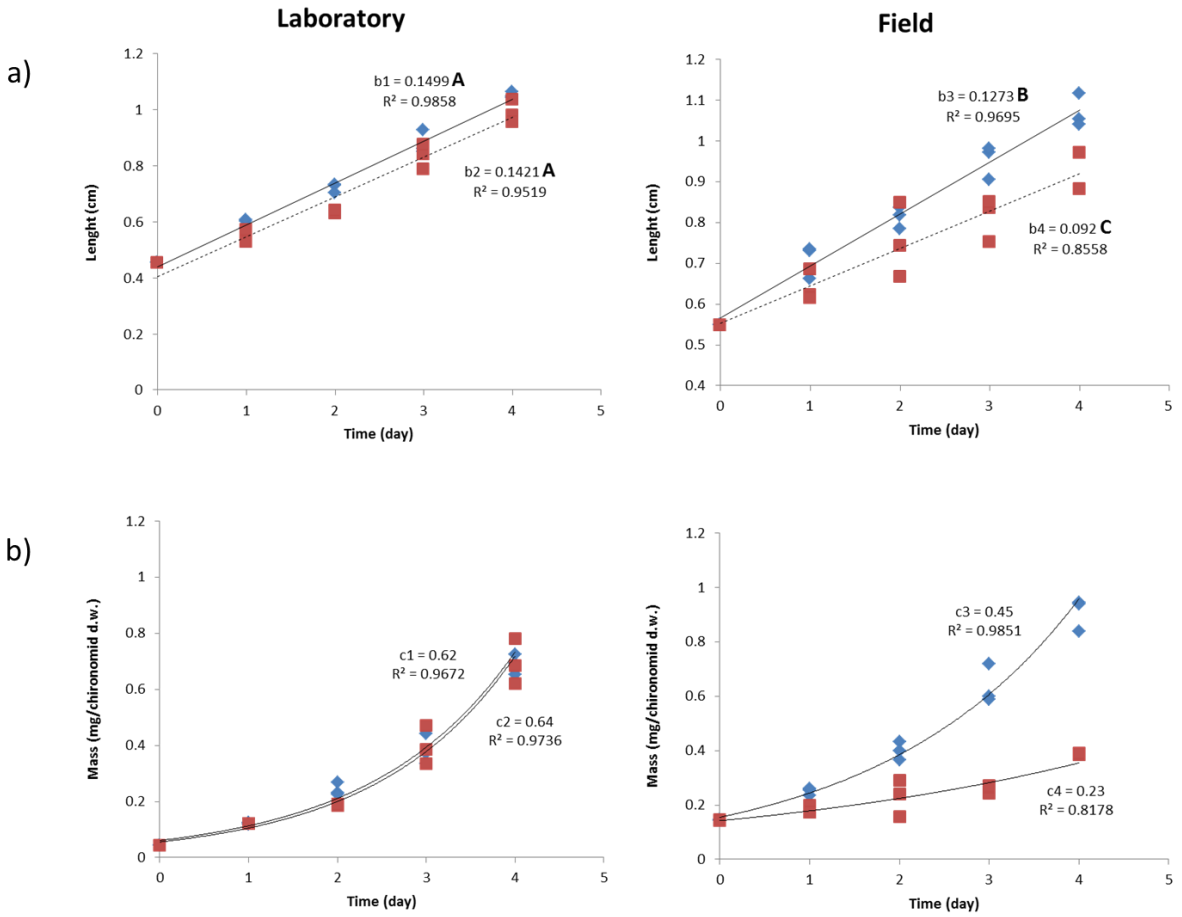


**Figure S2**. Evolution of the length (a) and weight (b) of 4$^{th}$ instar *Chironomus riparius* larvae as a function of time in bioaccumulation experiments performed in the field (Maniowy Bay, June 2007) and in the laboratory using field collected material. Blue diamonds correspond to the measured responses of the chironomids exposed to the sand, while the red squares to the measured responses of those exposed to Maniowy sediments. In figures (a), each point represents the average of the lengths obtained by individually measuring live specimen recovered daily from each exposure batch. The lines represent linear regressions and parameters b1, b2, b3 and b4 their slopes (means ± 1 s.e., in cm $d^{-1}$) defined as the representative growth rates (g) for the different conditions. Slopes that do not share the same capital letter letter are significantly different (ANOVA, $p<0.05$). In the figures (b), each point represents the weight of the pooled live chironomids. The lines represent exponential regressions and parameters c1, c2, c3 and c4 their slopes (means ± 1 s.e., in $d^{-1}$). $R^2$ and p indicate the goodness-of-fit and the statistical significance of each fitted regression.

**Figure S3.**

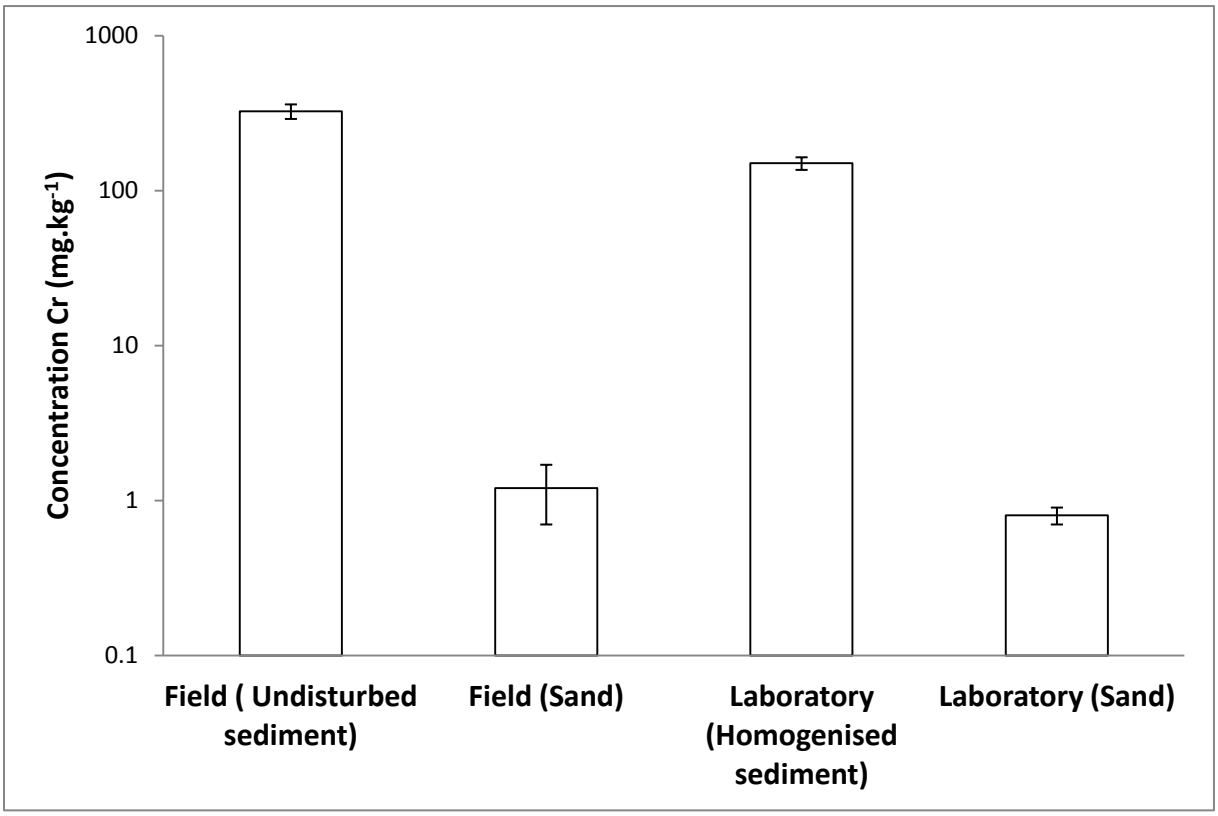


**Figure S3.** Total Cr concentrations (mg $kg^{-1}$ d.w.) in sediments used in bioaccumulation experiments performed in situ (Maniowy Bay, June 2007) and in the laboratory using field collected material. Data are the arithmetic mean ± one standard deviation (n=12) of Cr concentration in each system of exposure retrieved during the 4 days of exposure *in situ* or in the laboratory. Note the log scale on the vertical axis.

**Figure S4**

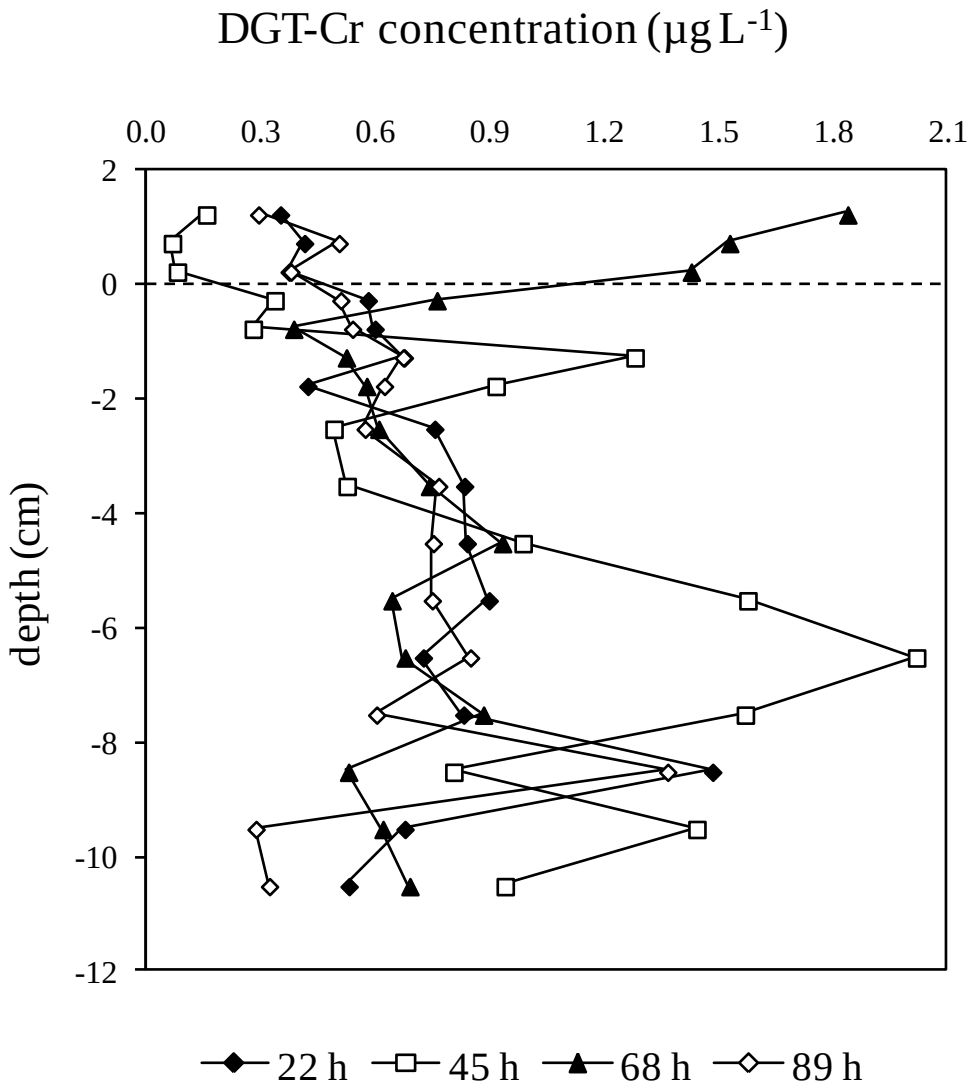


**Figure S4.** Temporal and vertical variability of DGT-labile Cr concentrations in interstitial (negative depth values; in cm) and overlying (positive depth values; in cm) at Maniowy bay in June 2007. Four different DGT probes were exposed at the same time and recovered after 22, 45, 68 and 89 hours of exposures. Exposure location was close to the cages for chironomids and is assumed to be representative of Cr behaviour within the cages.