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► To cite this version:

Simon Pouil, Nikki Jones, John Smith, Shovon Mandal, Natalie Griffiths, et al.. Comparing trace element bioaccumulation and depuration in snails and mayfly nymphs at a coal ash-contaminated site. *Environmental Toxicology and Chemistry*, 2020, 39 (12), pp.2437-2449. 10.1002/etc.4857. hal-03163614

HAL Id: hal-03163614

<https://hal.science/hal-03163614>

Submitted on 5 Sep 2023

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1 **Comparing trace element bioaccumulation and depuration in snails and mayfly nymphs**
2 **at a coal-ash contaminated site**

3
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11 **Acknowledgments**

12 This research was sponsored by the Tennessee Valley Authority (TVA) and performed by Oak
13 Ridge National Laboratory (ORNL). ORNL is managed by UT-Battelle, LLC, for the U.S.
14 Department of Energy under contract DE-AC05-00OR22725. Special thanks to Tyler Baker,
15 Susan Malone, John Justice, and Amber Stojak for help with sample collections. Thanks to Neil
16 Carriker, Michelle Cagley, and Suzy Walls for discussions and support, and to Jesse Morris,
17 Allison Fortner, Trent Jett, and Michael Jones for lab and field support.

18
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27
28 **Data Accessibility statement**

29 Data generated or analyzed during the present study are available from the corresponding author
30 on request.

31 **Running head:** Trace elements in mayfly and snail after coal ash spill

32

33 **Abstract**

34 We examined the bioaccumulation of essential (Cu, Fe, Se, and Zn) and non-essential (As and
35 Hg) trace elements in two aquatic invertebrate species (adult snails and mayfly nymphs) with
36 different feeding habits at the site of a coal ash spill. Differences in food web pathway, exposure
37 concentrations, and biological processing affected bioaccumulation patterns in these species.
38 Mayflies had higher body burdens, but snails had higher retention of most elements studied.

39

40 **Keywords:** Aquatic invertebrates, Bioaccumulation, *Hexagenia bilineata*, Metal, *Pleurocera*
41 *canaliculatum*

42

INTRODUCTION

43 Ecological risk assessments at contaminated sites often require bioaccumulation data to evaluate
44 site-specific contaminant exposure or to derive parameters to calibrate bioaccumulation and risk
45 models (Suter et al. 2000). Benthic macroinvertebrates are the most commonly used organisms
46 for biomonitoring studies in freshwater ecosystems. In addition to being sensitive indicators of
47 water quality, benthic macroinvertebrates perform important ecosystem functions in aquatic
48 systems and serve as a food source for fish and other consumers, making them a critical link in
49 elemental and energetic cycling within the aquatic ecosystem and between aquatic and terrestrial
50 systems (Covich et al. 1999). Because food chain pathways can be important in the trophic
51 transfer of bioaccumulative contaminants (Cabana and Ramussen 1994; Kidd et al. 1999), the
52 structure of the invertebrate community can affect how effectively these contaminants are
53 transferred from the base of aquatic food webs to fish and higher trophic level consumers (Wong
54 et al. 1997; Johnson et al. 2015).

55 Although toxicity standards and bioaccumulation models often consider individual contaminants,
56 most environmental spills consist of contaminant mixtures whose constituents can interact in
57 complex ways, potentially affecting the toxicity and bioavailability of each of the contaminants
58 in the mixture (Connon et al. 2012). Coal ash, the residual from coal combustion, has been the
59 focus of regulatory actions and ecological risk assessments due to a number of recent
60 environmental spills (Lemly and Skorupa 2012; Mathews et al. 2014; Lemly 2015). Coal ash
61 contains elevated concentrations of various trace elements that can adversely affect aquatic
62 ecosystems. Many coal ash-associated trace elements are essential micronutrients at low
63 concentrations (e.g., Zn, Se, Cu, Fe), but become toxic at elevated concentrations. Others (e.g.,
64 Hg, As) are toxic, having no known biological function.

65 Bioaccumulation of many trace elements in aquatic organisms is largely a function of dietary
66 exposure while dissolved concentrations are poor predictors of their bioaccumulation and toxic
67 effects (Stewart et al. 2004). For these trace elements, assimilation efficiencies are often the
68 most relevant parameters to quantify when assessing bioaccumulation in aquatic food chains
69 (Wang and Fisher 1999). Trace element assimilation efficiencies can be effectively measured in
70 laboratory studies using simple pulse-chase methods with radiolabeled food (Wang and Fisher
71 1999). In field studies, however, trace element body burdens in aquatic animals are often
72 measured even though body burdens typically include gut contents in the analysis. In benthic
73 invertebrates collected from contaminated sites, gut contents can often contain sediments which
74 can comprise a large proportion of the animal's body weight and can have elevated trace element
75 concentrations. While body burdens measured in this way can be useful in assessing exposure,
76 risk can potentially be overestimated for trace elements that are not efficiently assimilated.

77 Here we evaluate the bioaccumulation of essential and non-essential trace elements in two
78 invertebrate species with different feeding habits, the mayfly nymph (*Hexagenia bilineata*) and
79 the adult silty horn snail (*Pleurocera canaliculatum*) at the site of the Tennessee Valley
80 Authority's coal ash spill in Kingston, Tennessee, USA. In order to assess the effects of the coal
81 ash spill on trace element bioaccumulation, we collected sediment, periphyton, and biota from
82 sites both up- and down-stream of the coal ash spill, in years two and three after the event and
83 allowed a subset of animals to purge their gut contents before analysis to compare assimilation
84 among essential (i.e., Cu, Fe, Se, and Zn) and non-essential (i.e., As and Hg) trace elements,
85 animals, and sites. Because the Kingston spill was by far the largest release of coal ash in U.S.
86 history, there is much concern over the ecological effects of the spill, both locally and nationally
87 (Lemly and Skorupa 2012; Mathews et al. 2014; Pracheil et al. 2016). Our results are relevant to

88 evaluating risks at the Kingston spill site, but also to the fundamental understanding of metal
89 bioaccumulation and assimilation in different aquatic food web pathways.

90

91 **METHODS**

92 *Sampling sites*

93 The Tennessee Valley Authority's Kingston Fossil Plant (KIF) is located in East Tennessee near
94 the convergence of the Emory and Clinch Rivers (Figure 1). In December 2008, an earthen dike
95 in an ash settling pond failed, releasing ash both up- and down-stream of the KIF in the Emory
96 and Clinch Rivers (TVA 2009). The ash settling pond that failed in 2008 was located on the
97 northeast end of the plant at approximately Emory River mile 2.5 (ERM2.5). Ash was released
98 near ERM2.5 and traces were found upstream nearly to ERM6.0 and as far downstream as
99 Tennessee River mile 564, 11 miles downstream of ERM2.5 (TVA 2009). Samples were
100 collected at four ash-affected sites, ERM2.5 and 1.0 and Clinch River mile 3.5 (CRM3.5) and
101 CRM1.5, and two reference sites, ERM6.0 and CRM6.0. For this study, reference sites represent
102 locations where organisms were not exposed to ash from the KIF spill. This study took place
103 soon after the spill, just after major dredging activities occurred that removed 2.7 million m³ of
104 ash and sediment from the Emory River spill site. However, 400,000 m³ of ash were estimated to
105 remain in the Emory and Clinch Rivers in the vicinity of the ash spill (Walls et al. 2015).

106

107 *Target species*

108 We studied two organisms with differing feeding habits: the nymph stage of the burrowing
109 mayfly *Hexagenia bilineata* and the adult silty horn snail *Pleurocera canaliculatum*. Burrowing

110 mayflies in the genus *Hexagenia* (Ephemeroidea) are found throughout much of the eastern
111 United States, primarily in larger rivers and reservoirs. *Hexagenia* nymphs create shallow U-
112 shaped burrows by excavating highly adhesive soft mud (clay and silt) (Wallace and Merritt
113 1980; Brittain 1982) and typically spend 1-2 years in the sediment before emerging (Steingraeber
114 and Wiener 1995).

115 *P. canaliculatum* is a freshwater snail whose range extends throughout eastern North America,
116 where it is generally found in larger river systems. It has an elongated, dextrally coiled shell and
117 a conical spire. Pleurocerid snails are longer-lived than mayflies (2-10 years, (Brown et al.
118 2008); therefore, accumulation of contaminants can occur over a much longer period.

119

120 *Field procedures*

121 *Biota collection*

122 Mayfly nymphs were collected from each site in spring 2010 and 2011. Three samples were
123 collected from each site along three transects perpendicular to the river channel. Each sample
124 was a composite of nymphs collected at each transect, with approximately 120 nymphs to be
125 depurated prior to analysis and 80 to be analyzed without depurating. A Peterson dredge was
126 used to complete ≥ 10 drops per transect. Each drop was collected and emptied into a 1-mm
127 nylon-mesh sieve pan and rinsed with water from the site. *Hexagenia* nymphs were removed
128 from the sieve with stainless steel forceps and placed into a 1-L I-Chem jar (either new or
129 previously used and acid-washed) with water from the site. The jars were placed on ice for
130 transport back to the laboratory.

131 Snails were collected by hand from submerged rocks, logs, and sediment in shallow water along
132 the shoreline in summer 2010 and 2011. Three samples were collected from each site from three
133 transects 50-250 m apart. Sixty snails were gathered from each transect; 35 for depuration prior
134 to analysis and 25 for analysis without depuration. Upon collection, snails were transferred to
135 clean 1-L I-Chem jars filled with water from the site and placed on ice for transport back to the
136 laboratory.

137

138 *Sediment and periphyton collections*

139 Sediment and periphyton samples were collected as part of other studies at the Kingston site
140 (Walls et al. 2015), so while many samples exist, in our analysis we only include samples that
141 correspond to the same sample transects and sampling seasons as the biota samples. For this
142 reason, sample sizes and collection methods varied slightly between sites as described below.

143 Sediment samples (n = 2-6 per site) were collected from all sites in spring 2010 and 2011.
144 Samples were collected from the top 15 cm of the sediment surface by either Ponar grabs or box
145 cores, homogenized using disposable plastic laboratory scoops, and then stored in glass jars. One
146 field duplicate sample was collected for every 20 samples.

147 Periphyton (n = 1-6 per site) was collected in summer 2011 using surface periphytometers (three
148 per site) at all sites except ERM1.0 and 6.0. Periphytometers were constructed of four ceramic
149 tiles suspended from a float and anchored in the river by a cinder block. The tiles were allowed
150 to colonize for three to four weeks prior to collection. Periphyton was collected using plastic
151 scrapers to composite the growth from all 12 tiles from a site into one sample. Due to technical
152 constraints, for ERM1.0 and 6.0, periphyton was collected by scrubbing large, flat rocks with

153 acid-washed stiff plastic brushes. Rocks were rinsed with water from the site and the sample was
154 then filtered through a 500- μm sieve to exclude large particles and invertebrates. Samples were
155 placed in clean glass sample jars and stored frozen until shipped to an analytical lab for analysis.

156

157 *Biota sample processing*

158 Upon arrival at the laboratory, mayfly nymphs and snails from the field were kept in 1-L I-Chem
159 jars in a shallow water bath to allow acclimation to within 5 °C of laboratory temperatures. Once
160 acclimation was complete, mayfly nymphs were triple rinsed in distilled water to remove any
161 clinging sediment and detritus, while snails were scrubbed with a small, stiff, acid-washed nylon
162 brush to remove any periphyton and sediment particles from the shells, then triple rinsed with
163 distilled water. A subset of mayfly nymphs and snails was then immediately placed into clean,
164 pre-labeled glass containers and stored in a freezer until further processing (non-depurated
165 samples). The other subset of animals was kept for depuration.

166 For depuration, animals from a given site were placed in 0.74- to 1.2-L acid-washed Pyrex
167 crystallizing dishes ($n = \sim 10\text{-}15$ individual snails or nymphs per dish) that were partially filled
168 with distilled water. The dishes then were placed in a shallow flow-through water bath
169 maintained at a constant temperature (20-23 °C). Every 12 h during the depuration process, the
170 dishes were emptied and rinsed with distilled water, then fresh distilled water was added.
171 Mayfly nymphs were purged for 48 h, while snails were purged for 72 h, to, at least, cover the
172 depuration time from previous studies performed on mayfly and freshwater snail species (Brooke
173 et al. 1996; Croteau et al. 2007). At the end of depuration, animals were counted, weighed, and
174 stored in I-Chem bottles until further processing. All snail samples were thawed, the soft tissue
175 was removed with stainless steel forceps after cutting the shell along the spire with small

176 stainless-steel cutters, and the tissue was then weighed. All depurated and non-depurated mayfly
177 nymph and snail samples were freeze-dried, re-weighed, and stored in a freezer until they were
178 shipped to an analytical lab for analysis.

179

180 *Trace element analysis*

181 All samples were shipped to PACE Analytical Services (Green Bay, WI) for trace element
182 analysis. Aliquots of all samples were placed in drying ovens (103 °C) for a minimum of 8 h to
183 determine percentage moisture. One aliquot from each sample was analyzed for Hg with EPA
184 Method 7473 in a DMA 80 automatic mercury analyzer. A second aliquot was microwave
185 digested in nitric acid per EPA Method 3052 (biota) or SW-846 method 3051A (sediment)
186 before analysis for As, Cu, Fe, Se, and Zn with EPA Method 6020A using Inductively Coupled
187 Plasma Mass Spectrometry (ICP-MS). All quality assurance procedures (e.g., blanks, matrix
188 spikes, etc.) were as specified in each analytical method. All trace element results were reported
189 on a dry weight basis.

190

191 *Data analysis*

192 Biota and their respective food matrices were collected during the same time of year (i.e., spring
193 for mayfly nymphs and sediment and summer for periphyton and snails) to allow for a direct
194 comparison between biota and their respective food source. For each matrix (i.e., mayfly, snail,
195 periphyton, and sediment), trace element concentrations from samples collected in 2010 and
196 2011 were compared using Wilcoxon rank-sum non-parametric tests. Since no significant

197 difference was found in the data collected in 2010 vs 2011, data from both sampling years were
198 combined in all analyses.

199 To study the biomagnification potential of the studied trace elements, the biomagnification
200 factors (BMFs) were calculated as the ratio between the average concentration of each trace
201 element (expressed as ppm dry weight [DW]) in the biota (i.e., mayfly nymphs and adult snails),
202 before and after depuration period, and the average concentration of each trace element
203 (expressed as ppm DW) in their respective food resources (i.e., sediment and periphyton). A
204 $BMF > 1$ suggests biomagnification (Franklin 2016).

205 The time-dependent trace element concentration in mayfly nymphs and snails during depuration
206 was determined by the procedure described previously by (Choi et al. 2006). The differential
207 equation during depuration is given by:

$$208 \quad q = q_0 e^{-\lambda t} \quad (1)$$

209 where, q_0 is the initial trace element concentration in mayfly nymphs or snails before depuration,
210 q is the trace element concentration after depuration, t is the time for depuration, and λ is trace
211 element removal rate (d^{-1}). Using Eq. (1), the removal rate will be:

$$212 \quad \lambda = (1/t) \ln (q_0/q) \quad (2)$$

213 A positive removal rate suggests the elimination of unassimilated trace elements from the tissues
214 while a negative removal rate indicates that assimilation is continuing when organisms are kept
215 in distilled water.

216 A combination of multivariate and univariate statistical analysis methods was used to analyze the
217 data. Multivariate analysis was used to visualize site and subgroup (e.g., matrix, ash-affected and
218 reference sites) trends, and to identify the trace elements that contributed most strongly to the

219 observed trends. Principal Component Analysis (PCA) was run on \log_{10} transformed data to
220 visualize interactions in element concentrations among sites and sample matrices and identify the
221 elements contributing to those trends. Eigenvalues were used to determine the number of
222 principal components and only the principal components with eigenvalues >1 were adopted.
223 Univariate analyses were then used to evaluate the magnitude of differences of the individual
224 trace elements among factors. For each trace element, differences in concentration between the
225 two non-depurated animals (i.e., mayfly and snail) and the two food matrices and among all the
226 sites were assessed using a Scheirer–Ray–Hare non-parametric test (Dytham 2011). An
227 interaction term was included in the model. The same procedure was used to test variability in
228 mean trace element removal rate for different trace elements and sites followed by Dunn’s test.
229 For each species and each element, differences in (1) concentrations between depurated and non-
230 depurated individuals and (2) removal rates were assessed using non-parametric Dunn tests.
231 The level of significance for statistical analyses was set to $\alpha = 0.05$. All statistical analyses were
232 performed in R v. 3.6.3 (R Development Core Team 2020).

233

234 **RESULTS AND DISCUSSION**

235 *Trace element concentrations in biota and food resources*

236 Trace element concentrations varied among the six studied elements (i.e., As, Cu, Fe, Hg, Se,
237 and Zn), among sample matrices (i.e., mayfly, periphyton, sediment, and snail), and among sites
238 (i.e., reference or ash-affected). PCA was applied on the element concentrations in periphyton,
239 sediment, and non-depurated animals at all sampling sites (Figure 2A). The first two principal
240 components (PC) explained ~68% of the variation in the data set. The first principal component

241 (PC1), driven by differences in concentrations of Se and Zn, explained 45% of the variance and
242 discriminated the biota, especially the adult snails and the food resources (i.e., periphyton and
243 sediment). PC2 explained 23% of the variation in the dataset and was largely driven by
244 differences in Fe and mainly discriminated mayfly nymphs and sediment, collected in the
245 reference and ash-affected sites. Applying the same PCA in depurated animals (Figure 2B) for
246 the corresponding sites showed slight differences in the relationship between the trace element
247 concentrations in the depurated biota and their respective food matrices compared to Figure 2A.
248 Indeed, biota were better discriminated in this PCA mainly due to their As concentrations (up to
249 54.5 ppm, Table 1). Differences in depurated and non-depurated mayflies and snails are shown
250 in Figure 2C where PC1 (explaining 36% of the variance) led to discrimination of depurated and
251 non-depurated organisms, especially mayflies, mainly based on As, Cu, and Zn concentrations.
252 Overall, Figure 2 highlights that taking depuration into account can affect the interpretation of
253 the relationships between trace element concentrations in biota and their food matrices. These
254 findings also illustrate variability in depuration between biotic species and among trace elements
255 (Rainbow 2002), suggesting that laboratory depuration studies may be necessary to help interpret
256 field-based measurements.

257 In the biota, our results revealed that trace element concentrations in organisms differed between
258 species and among sites, but the effects of these variables differed depending on the trace
259 element studied (Figure 3). Trace element concentrations in invertebrates observed in the present
260 study were compared to the fish tissues criteria available for Se (i.e. 8.5 ppm, EPA 2016) and
261 MeHg (i.e. 0.3 ppm, EPA 2001) as indicated in Figure 3. Total Hg is often used as a proxy for
262 MeHg in fish fillets where MeHg makes up >90% total Hg, while the proportion of MeHg in
263 invertebrates is likely much lower (Mason 2000). For As and Hg, the main contaminants of

264 concern in coal ash, concentrations were not significantly different between non-depurated
265 mayfly nymphs and non-depurated snails ($H = 5.26-7.71$, $p = 0.053-0.154$), but concentrations of
266 these elements in the biota were significantly different among sampling locations ($H = 56.15-$
267 71.69 , $p < 0.001$). We found that concentrations of As were higher in biota, especially in mayfly
268 nymphs, collected from ash-affected sites than the ones collected from reference sites. For Hg,
269 differences among sites were mainly driven by the high concentrations observed in the biota
270 collected at CRM6.0 (0.68-1.2 ppm and 0.25-0.42 ppm for mayflies and snails, respectively;
271 Table 1). Concentrations of Cu, Fe, and Se were both significantly different between non-
272 depurated mayfly nymphs and non-depurated snails ($H = 56.51-94.69$, $p < 0.001$) and among
273 sampling locations ($H = 22.90-71.69$, $p \leq 0.046$) with the highest concentrations observed in the
274 ash-affected sites (Figure 3). Concentrations of Zn in biota were similar among sampling
275 locations ($H = 7.15$, $p = 0.210$) but were species dependent ($H = 65.37$, $p < 0.001$) with higher
276 concentrations of Zn in mayfly nymphs than snails (137-209 ppm and 89-236 ppm for mayflies
277 and snail, respectively; Table 1 and Figure 3).

278 Trace element concentrations were also different in sediment vs. periphyton and among sampling
279 locations, but again, these responses varied by trace element (Figure 3). Indeed, while As and Fe
280 concentrations varied among sampling locations ($H = 17.35-21.61$, $p \leq 0.004$), there were no
281 statistical differences between sediment and periphyton ($H = 1.16$, $p = 0.281$). For Se, the sample
282 matrix affected the concentrations ($H = 19.11$, $p < 0.001$) with higher concentrations of these
283 elements in periphyton (1.8-13.6 ppm) than in sediment (0.6-3.5 ppm). Both sample matrix and
284 sampling location played a role in the concentrations of Cu, Hg, and Zn ($H = 4.70-16.90$, $p \leq$
285 0.030 and $H = 15.95-27.54$, $p < 0.001$; for matrix and sampling locations respectively) with
286 higher concentrations of these elements at ash-affected sites (except for Hg, where the highest

287 concentrations were observed in the reference site CRM6.0 at 2.9-4.1 ppm and 0.4-0.7 ppm for
288 periphyton and sediment, respectively) than at reference sites and higher concentrations in
289 periphyton (0.1-4.1 ppm) than sediment (≤ 1.1 ppm, Table 1 and Figure 3).

290 It is not surprising that sediment and periphyton concentrations of most of the trace elements in
291 this study were higher at ash-affected sites (i.e., CRM1.5, CRM3.5, ERM1.0, and ERM2.5) than
292 at reference sites (i.e., CRM6.0 and ERM6.0). Indeed, many of the trace elements associated
293 with coal ash are particle reactive and tend to be associated with sediments or are readily taken
294 up by algae (IAEA 2004). As opposed to the other elements, sediment Hg concentrations were
295 more related to watershed than proximity to the coal ash spill. Sediment Hg concentrations were
296 highest in the Clinch River, even at the reference site CRM6.0. While this sampling location
297 served as a reference for coal ash exposure, this site is downstream of industrial facilities which
298 have released large quantities of Hg into the environment since the 1950's (Brooks and
299 Southworth 2011) explaining the high concentrations of Hg measured in sediments.

300 Overall, most trace element concentrations in periphyton and sediment followed the same trends
301 longitudinally, with an increase in trace elements concentrations from ERM1.0 to CRM1.5
302 (Table 1 and Figure 3). However, it is notable that concentrations of As, Fe, Se, and to a lesser
303 extent, Zn were higher in periphyton collected at the spill site (ERM2.5) than at downstream
304 sites. For sediment, our findings were similar to Stojak et al. (2015) who found As and Se
305 concentrations in sediments were strongly correlated with the percent of ash closest to the spill
306 site ($r = 0.89$ and 0.75 , for As and Se, respectively).

307 Food web structure strongly influences trophic uptake by organisms (Cardwell et al. 2013), and
308 trophic pathways can be a primary source of trace elements accumulating in aquatic
309 invertebrates. Thus, the differences seen in bioaccumulation patterns between mayfly nymphs

310 and snails were likely due to differences in feeding habits. While mayfly nymphs incidentally
311 ingest large quantities of sediment, gaining nourishment from the organic matter and detritus
312 contained within (Wallace and Merritt 1980; Brittain 1982), snails preferentially scrape algae
313 and detritus off rocks and other surfaces (Dillon and Davis 1991). In general, trace element
314 concentrations were higher in periphyton than in sediments at both ash-affected and reference
315 sites. However, although concentrations of Fe and Zn were significantly higher in periphyton
316 than in sediment, concentrations of these elements remained higher in sediment-ingesting mayfly
317 nymphs than in snails (Figure 3), suggesting that diet alone does not explain the differences
318 observed in the concentration of trace elements in the studied aquatic invertebrates.

319

320 *Effects of depuration on trace metal concentrations*

321 Trace element bioaccumulation among macroinvertebrate taxa also frequently varies due to
322 differences in exposure, uptake rates from solution and diet, as well as efflux rates. Whether an
323 aquatic invertebrate accumulates an essential or non-essential element, and whether that element
324 biomagnifies or is regulated can also be element- and species-specific and may be related to the
325 toxicity of the trace elements (Rainbow 2002). Indeed, if the concentration of essential elements
326 in food is limiting, aquatic invertebrates would accumulate necessary levels that may exceed that
327 in their diet. In contrast, the bioaccumulation of elements with no known biological role will
328 depend on the environmental concentrations and the ability of the organisms to excrete those
329 elements.

330 Previous studies have found that the gut content in invertebrates can account for up to 65% of the
331 total body burden for many trace elements (Hare et al. 1989). We found significant differences
332 between concentrations of all the elements in depurated and non-depurated mayfly nymphs ($Z =$

333 2.66-7.30, $p \leq 0.008$), except Se ($Z = 1.32$, $p = 0.185$), while effects of depuration were more
334 modest in snails (Figure 4). In snails, only concentrations of Cu and Fe differed between
335 depurated and non-depurated individuals ($Z = 2.73$ - 6.24 , $p \leq 0.006$). Calculations of removal
336 rates revealed differences between mayfly nymphs and snails for all the studied elements ($Z =$
337 2.41 - 7.07 , $p < 0.001$). Se concentrations were higher in mayfly nymphs (2.4-11.9 ppm) than in
338 sediment samples (0.6-3.5 ppm, Table 1), and there were no significant differences between
339 concentrations in depurated vs. non-depurated animals, suggesting high assimilation of this
340 element. Such results are in accordance with literature, where assimilation of Se of the studied
341 aquatic invertebrates, mostly from marine environments, is usually high (up to 90%; Chapman et
342 al. 2010).

343 **Biomagnification factors (BMFs)** estimated for mayfly nymphs and snails before and after
344 depuration suggest biomagnification potential in mayflies for Se and Zn at all sites (ash-affected
345 or reference) with BMF values ranging from 2.68 to 8.94 and 1.76 to 8.85 for Se and Zn,
346 respectively (Table 2). Overall, BMF values were intermediate for As, Cu, and Hg with maximal
347 values of 6.27 remaining site-dependent while BMFs were low for Fe (≤ 0.88 , Table 2)
348 regardless of site. In snails, BMFs were >1 only for Cu (i.e. 1.48-10.12) at all sites except
349 CRM1.5. Changes in BMF values related to depuration were only observed for mayflies
350 especially for As and Fe (Table 2). Estimated BMF values are in accordance with findings from
351 other studies. For examples, (Opfer et al. 2011) found Cd and Zn concentrations in *Hexagenia*
352 mayflies were on average approximately 4 and 2 times greater, respectively, than sediment level
353 while field BMF values for Cu in the aquatic snail *Cipangopaludina chinensis* and periphyton
354 estimated from (Cui et al. 2012) were ranging from 0.23 to 8.22. In our study, for most of the
355 investigated trace elements except As and Cu, BMF values were similar between sites suggesting

356 that the observed biomagnification potential does not seem to be related to coal ash spill.
357 Overall, these results suggest that mayflies, components of the diet of many species including
358 fish and birds, are potential vectors of contaminant transfer in freshwater ecosystems (Covich et
359 al. 1999). Our results show bioconcentration at the base of aquatic food webs, but there has been
360 no evidence so far of biomagnification at higher trophic levels of coal ash associated trace
361 elements at the site of the Kingston coal ash spill as highlighted by previous studies in fish
362 (Mathews et al. 2014; Pracheil et al. 2016).

363

364 *Trace element removal rates in biota*

365 Overall, our results suggest that retention of most of the studied elements was greater in snails
366 than in mayfly nymphs suggesting a higher assimilation. The removal rates of As, Hg, Se, and
367 Zn remained close to zero in snails (Figure 4). Previous studies have shown that aquatic snails
368 may have much higher assimilation efficiencies for many trace elements, both essential and non-
369 essential, than other invertebrates (e.g. Croteau et al. 2007). Feeding physiology of the animals
370 and cellular partitioning and biochemical composition of food particles can all affect chemical
371 assimilation (Wang and Fisher 1999). Gut transit time is one of the physiological factors known
372 to influence assimilation of trace elements in invertebrates. Thus, we assume that the long-coiled
373 intestines of snails may lead to longer gut passage times than mayfly nymphs and potentially to
374 higher assimilation rates in snails. Herbivorous snails are surprisingly efficient in assimilating
375 trace elements from their food sources and may be an important reason as to why they are strong
376 trace element bioaccumulators independent of potential toxicity. Interestingly, at the same time,
377 we found that mayfly nymphs were able to efficiently depurate the two non-essential studied
378 trace elements (i.e., As and Hg, Figure 4). In addition to biological factors, geochemical factors

379 including speciation and desorption can greatly affect trace element assimilation, and this is
380 particularly true for As and Hg. The gut pH, by affecting both speciation and desorption, may
381 have affected assimilation of these elements in the studied species. Nevertheless, studies
382 performed on the aquatic snail *Pomacea canaliculata* (Godoy et al. 2013) and the mayfly
383 *Ephemera danica* (Austin and Baker 1988) suggested that gut pH is close to neutral in these
384 species. Thus, we assumed that differences in As and Hg speciation and/or desorption among the
385 ingested food matrix (i.e., periphyton and sediment) likely explained the greater ability of mayfly
386 nymphs to depurate these non-essential elements. Further investigations regarding As and Hg
387 depuration mechanisms are needed to support this assumption.

388 Some of the differences observed between mayfly nymphs and snails can also be explained by
389 the biological roles of certain essential trace elements. For example, while concentrations of
390 most trace elements were higher in mayfly nymphs than snails, Cu concentrations were higher in
391 snails than in mayfly nymphs ($H = 70.29$, $p < 0.001$) with removal rates tending to be lower in
392 snails. Such differences were likely due to the presence of the Cu-containing respiratory protein
393 hemocyanin which floats freely in the hemolymph of gastropods to deliver oxygen to tissues
394 (Lieb et al. 2010) while hemocyanin has been lost in mayflies (Burmester 2015).

395

396 *Concluding remarks*

397 Because there have been several coal ash spills in the years following the KIF coal ash spill
398 (Hesterberg et al. 2016), there has been much concern over the ecological effects of coal ash,
399 both locally and nationally (EPA 2015, 2016). The present study examined the fundamental
400 differences in the bioaccumulation and retention of essential and non-essential elements in two
401 invertebrate species with different feeding habits in the years immediately following the spill.

402 Our results are relevant to evaluating risks at ash-affected sites, but also to the fundamental
403 understanding of trace element dynamics in aquatic food web pathways. Aquatic snails and
404 mayflies are important to the diets of various fish and bird species. These benthic invertebrates
405 are therefore key components in the trophic transfer of contaminants in freshwater food webs
406 (Covich et al. 1999).

407 While our work showed that concentrations of the coal-ash associated contaminants As and Se
408 were higher in all the sample matrices, including snails and mayflies at ash-affected sites than at
409 the reference sites, major remediation activities removed much of the spilled ash from the site in
410 2010-2011, and as a result, trace element concentrations in mayfly nymphs were shown to
411 decrease in the four years following the spill (Smith et al. 2016). Overall, concentrations of trace
412 elements associated with coal ash measured in mayfly nymphs in our study remained similar to
413 the ones reported in mayflies in other sites (Cid et al. 2010; Opfer et al. 2011; Fletcher et al.
414 2014, 2017).

415 It is always complex to address the risks associated with trace elements on aquatic biota based
416 solely on their concentrations because many factors can significantly affect trace element
417 bioavailability and toxicity, and therefore the consequences on the invertebrate populations (e.g.
418 (Mathews et al. 2020)). However, it is interesting to note that Mebane et al. (2020), showed that
419 mayflies were species sensitive to trace elements and that their distribution could be strongly
420 influenced by the concentrations of trace elements such as Cd, Cu, Ni and Zn, alone or in
421 mixture. Smith (2003) already demonstrated that the discharge of fly ash in an East Tennessee
422 stream had a lasting negative impact on communities of mayflies affecting both their abundance
423 and taxonomic richness.

424 Interestingly, we found differences in trace element concentrations between mayfly nymphs and
425 adult snails, at least partially due to their respective food resources but, more importantly, we
426 found removal rates of all trace elements studied were very low in snails while they exhibit high
427 accumulations of them. Such findings suggest that *P. canaliculatum* can be used as an
428 environmental archive (Gärdenfors et al. 1988) while *H. bilineata* nymphs, may be good
429 candidates for biomonitoring recovery of metal-contaminated sites because of the high rate of
430 metal removal in these animals. Because many of the contaminants considered in this study are
431 accumulated through food chain exposure, it may take several years to see significant increases
432 in the concentrations of these elements in higher trophic-level organisms. Future work should
433 consider the longer-term bioaccumulation trends of these elements at the Kingston site.

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579 **Figure captions**

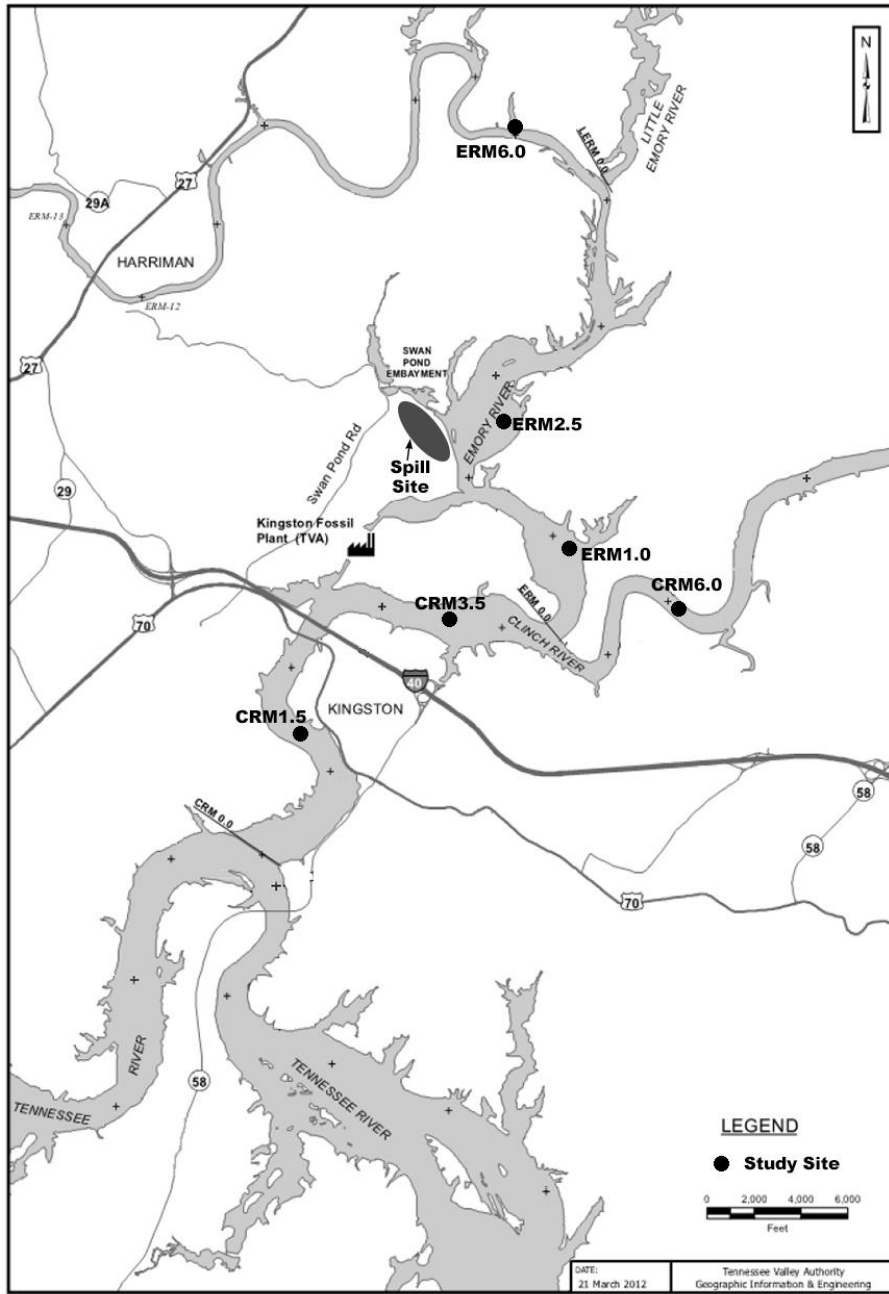
580

581 Figure 1. Map of sampling sites on the Emory (ER) and Clinch (CR) Rivers in East Tennessee,
582 USA. Sampling sites are labeled with miles upstream from the river mouth. For example,
583 ERM2.5 refers to the sampling site located 2.5 miles upstream from the Emory River's
584 confluence with the Clinch River. The location of the coal ash spill is shown as an oval in the
585 middle of the map.

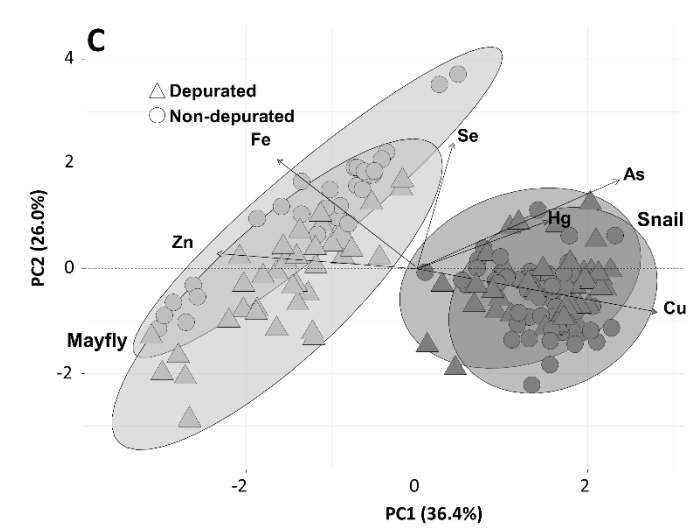
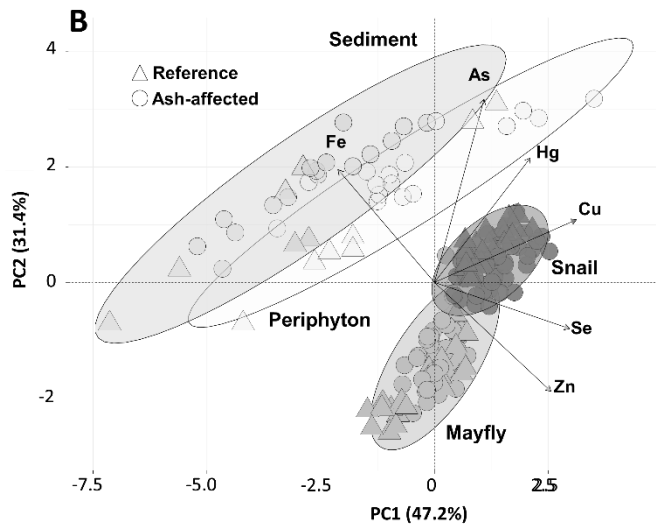
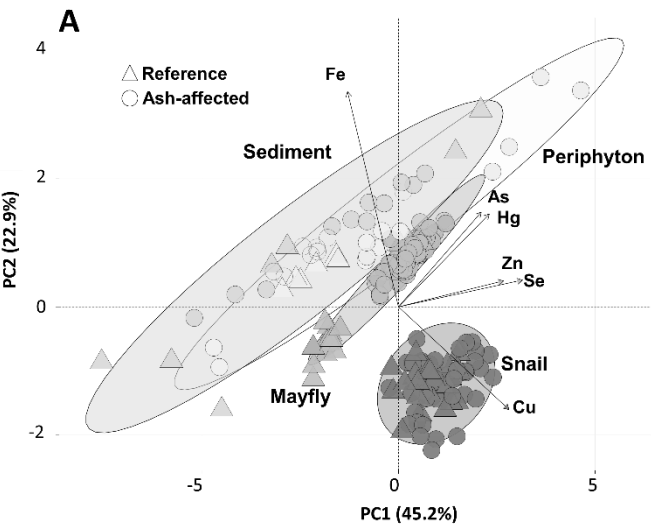
586 Figure 2. Principal Component Analysis (PCA) performed on concentration data of the six
587 studied elements (As, Cu, Fe, Hg, Se, and Zn) in food resources (i.e., periphyton and sediment)
588 in (A) non-depurated biota or (B) after depuration sampled at four locations exposed to the coal
589 ash spill (CRM1.5, CRM3.5, ERM1.0, and ERM2.5) and two reference locations not affected by
590 the spill (CRM6.0 and ERM6.0). The last PCA (C) was performed only with trace element data
591 in biota (i.e., depurated and non-depurated). Each data point represents one sample.

592 Figure 3. Concentrations of the six studied elements (As, Cu, Fe, Hg, Se, and Zn) in depurated
593 (white dots) and non-depurated (black dots) mayfly nymphs (*Hexagenia bilineata*) and adult
594 snails (*Pleurocera canaliculatum*) collected at four locations exposed to the coal ash spill (grey
595 bars; CRM1.5, CRM3.5, ERM1.0, and ERM2.5) and two reference locations not affected by the
596 spill (white bars; CRM6.0 and ERM6.0) in relation to the six elements in their respective food
597 resources (i.e., sediment [left panels] and periphyton [right panels]). Data from the four ash-
598 affected sites are presented in order of distance from the spill (from left to right). Data are means
599 \pm SE (n = 1-7). Dashed lines are National Recommended Aquatic Life Criteria in fish tissues for
600 MeHg and Se (EPA 2001, 2016). Note that the y-axes are not on a linear scale.

601 Figure 4. Removal rates of the six studied elements (As, Cu, Fe, Hg, Se, and Zn) in mayfly
602 nymphs (*Hexagenia bilineata*) and adult snails (*Pleurocera canaliculatum*) collected at four
603 locations exposed to the coal ash spill (grey bars; CRM1.5 and 3.5, ERM1.0 and 2.5) and two
604 reference locations not affected by the spill (white bars; CRM6.0 and ERM6.0) after 48 h
605 (mayfly) or 72 h (snail) of depuration in clean water in the laboratory. A positive removal rate
606 suggests unassimilated trace elements are eliminated while a negative removal rate suggests that
607 assimilation of trace elements is continuing. Data from the four ash-affected sites are presented
608 in order of distance from the spill (from left to right). Data are means \pm SE (n = 3).

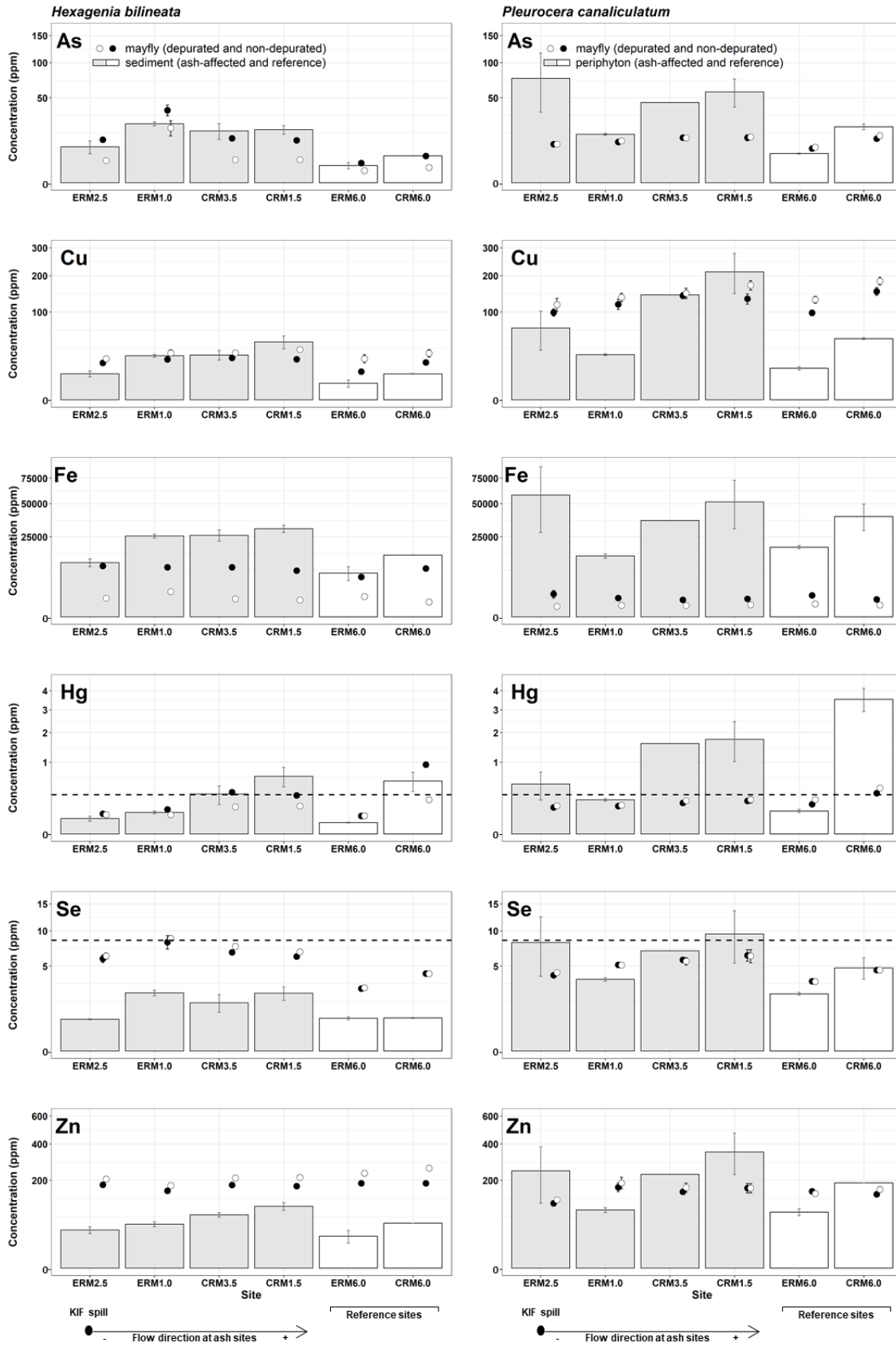


609 Figure

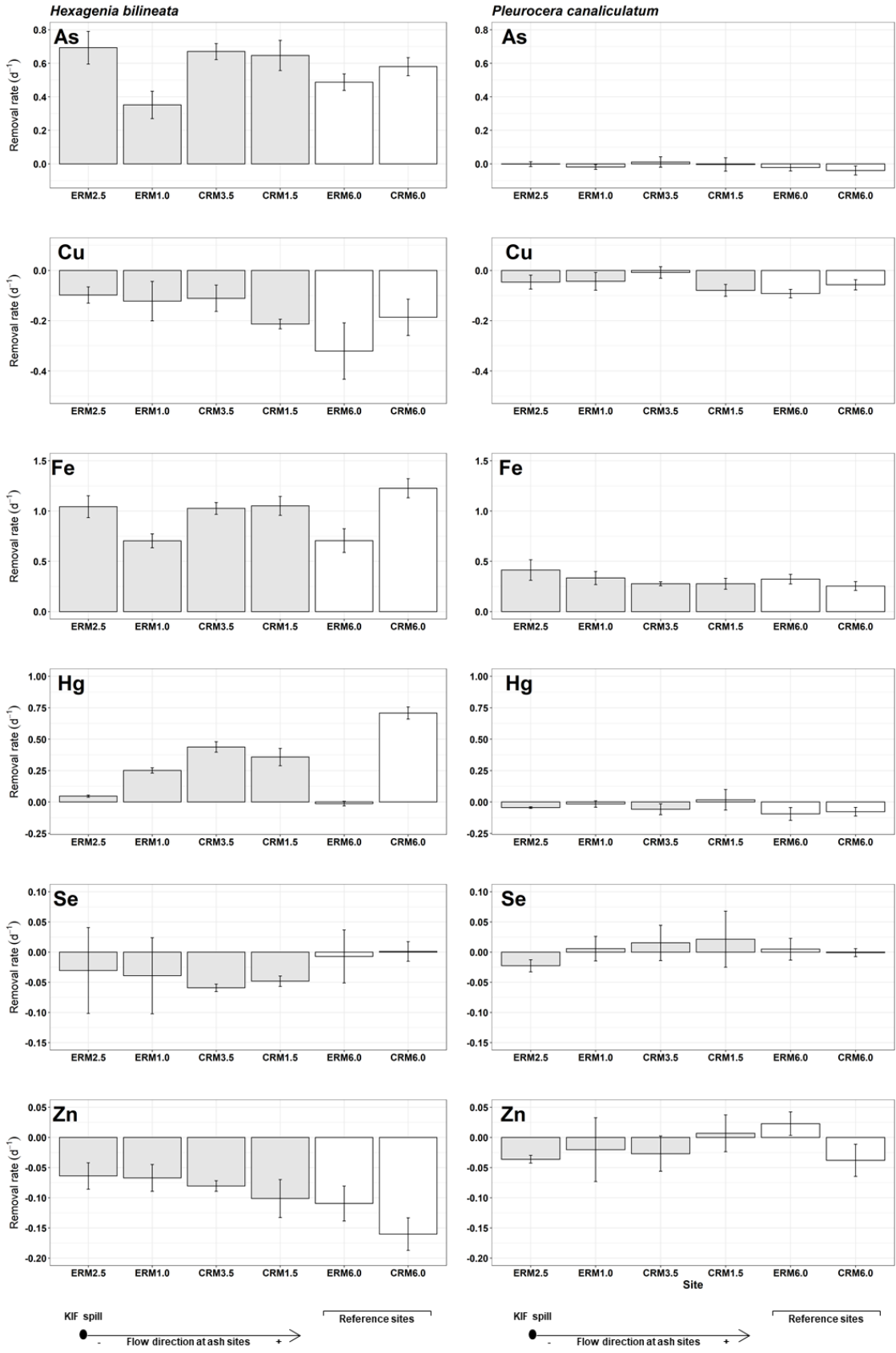


610 **Figure**

2



611 **Figure 3**



612 **Figure 4**

613 Table 1. Summary of the trace elements concentrations (ppm of dry weight [DW]) measured in the sample matrices (i.e., mayfly
614 nymphs, periphyton, sediment, and snails) for the different sites (i.e., reference or ash-affected) in Clinch and Emory rivers.

Element	Sample	Clinch River sites								
		CRM3.5			CRM1.5			CRM6.0 (reference)		
		N	Mean ± SE	Min-Max	N	Mean ± SE	Min-Max	N	Mean ± SE	Min-Max
As	Mayfly non-depurated	6	13.6 ± 0.6	12.0-16.4	6	12.5 ± 0.6	10.9-14.6	6	5.0 ± 0.1	4.6-5.4
	Mayfly depurated	6	3.7 ± 0.4	2.5-5.3	6	3.7 ± 0.6	1.9-5.0	6	1.6 ± 0.2	1.2-2.2
	Sediment	5	18.6 ± 5.6	8.3-36.8	4	19.5 ± 3.1	10.9-25.3	2	5.0 ± 0.1	4.9-5.1
	Snail non-depurated	6	14.0 ± 0.8	11.3-16.9	6	14.0 ± 1.9	8.9-20.1	6	13.4 ± 0.8	11.0-16.4
	Snail depurated	6	13.9 ± 1.3	10.0-17.8	6	14.5 ± 1.8	9.4-19.8	6	15.4 ± 1.2	10.8-18.1
	Periphyton	1	44.4	-	2	57.1 ± 17.4	39.7-74.5	2	21.6 ± 2.2	19.4-23.9
Cu	Mayfly non-depurated	6	22.8 ± 1.2	19.3-26.6	6	21.3 ± 0.8	18.6-23.4	6	18.1 ± 0.4	16.7-19.3
	Mayfly depurated	6	28.6 ± 1.7	23.4-35.1	6	32.8 ± 1.2	27.7-36.3	6	28.1 ± 4.4	17.1-40.4
	Sediment	5	25.9 ± 5.5	14.1-44.7	4	43.3 ± 9.5	21.0-62.4	2	8.7 ± 0.2	8.5-9.0
	Snail non-depurated	6	141.2 ± 8.8	117-170	6	132.4 ± 13.3	95.5-169	6	152.7 ± 10.8	122-197
	Snail depurated	6	147.5 ± 14.2	102-196	6	170.7 ± 14.1	131-217	6	183.2 ± 11.9	150-226
	Periphyton	1	143.1	-	2	212.6 ± 65.5	147.1-278.2	2	48.5 ± 1.5	46.9-50
Fe	Mayfly non-depurated	6	9562 ± 166	8970-10100	6	8298 ± 331	7180-9540	6	9097 ± 414	7820-10900
	Mayfly depurated	6	1271 ± 156	908-1900	6	1108 ± 213	536-1990	6	858 ± 159	412-1400
	Sediment	5	25920 ± 3570	15700-35300	4	30250 ± 2483	26000-37000	2	14850 ± 150	14700-15000
	Snail non-depurated	6	1120 ± 211	483-2000	6	1264 ± 143	858-1810	6	1195 ± 162	666-1700
	Snail depurated	6	496 ± 49	379-680	6	568 ± 70	360-790	6	546 ± 27	439-614
	Periphyton	1	36111	-	2	51446 ± 21281	30165-72727	2	39268 ± 10391	28878-49659
Hg	Mayfly non-depurated	6	0.34 ± 0.03	0.26-0.43	6	0.29 ± 0.01	0.26-0.33	6	0.94 ± 0.08	0.68-1.20
	Mayfly depurated	6	0.14 ± 0.01	0.12-0.20	6	0.15 ± 0.03	0.10-0.28	6	0.23 ± 0.01	0.18-0.28
	Sediment	5	0.31 ± 0.14	0.05-0.84	4	0.65 ± 0.22	0.17-1.10	2	0.55 ± 0.20	0.35-0.74
	Snail non-depurated	6	0.19 ± 0.03	0.10-0.31	6	0.21 ± 0.01	0.17-0.26	6	0.32 ± 0.03	0.25-0.42
	Snail depurated	6	0.22 ± 0.02	0.14-0.26	6	0.23 ± 0.04	0.07-0.37	6	0.41 ± 0.04	0.30-0.54
	Periphyton	1	1.59	-	2	1.74 ± 0.72	1.02-2.46	2	3.53 ± 0.61	2.92-4.14
Se	Mayfly non-depurated	6	6.8 ± 0.2	5.9-7.4	6	6.2 ± 0.2	5.6-6.6	6	4.2 ± 0.2	3.6-4.6
	Mayfly depurated	6	7.6 ± 0.2	7.2-8.3	6	6.8 ± 0.2	6.1-7.4	6	4.1 ± 0.1	4.0-4.5
	Sediment	5	1.6 ± 0.6	0.7-3.5	4	2.3 ± 0.5	0.7-3.0	2	0.8 ± 0.0	0.7-0.8
	Snail non-depurated	6	5.8 ± 0.4	4.7-6.8	6	6.4 ± 0.8	4.3-8.5	6	4.5 ± 0.2	4.1-5.3
	Snail depurated	6	5.6 ± 0.5	4.3-7.1	6	6.3 ± 0.9	3.7-9.2	6	4.6 ± 0.1	4.3-4.9
	Periphyton	1	6.9	-	2	9.5 ± 4.1	5.4-13.6	2	4.8 ± 1.2	3.6-6.0
Zn	Mayfly non-depurated	6	180 ± 2	172-187	6	174 ± 7	153-194	6	187 ± 4	168-197
	Mayfly depurated	6	211 ± 5	197-227	6	214 ± 8	184-238	6	260 ± 14	228-324
	Periphyton	1	228	-	2	350 ± 123	227-473	2	188 ± 0	188-189
	Sediment	5	74 ± 6	55-89	4	99 ± 12	73-126	2	52 ± 0	52-53
	Snail non-depurated	6	151 ± 7	132-171	6	166 ± 19	121-236	6	141 ± 5	134-163
	Snail depurated	6	169 ± 19	115-227	6	166 ± 20	126-246	6	160 ± 12	132-213

615 Table 1. Continued

Element	Sample	Emory River sites								
		ERM2.5			ERM1.0			ERM6.0 (reference)		
		N	Mean ± SE	Min-Max	N	Mean ± SE	Min-Max	N	Mean ± SE	Min-Max
As	Mayfly non-depurated	6	12.9 ± 1.3	7.8-17.6	6	36.4 ± 5.4	21.3-54.5	6	2.7 ± 0.2	2.2-3.2
	Mayfly depurated	6	3.4 ± 0.6	2.1-6.2	6	20.8 ± 5.7	9.3-41.6	6	1.0 ± 0.1	0.9-1.3
	Periphyton	2	75.4 ± 40.9	34.5-116.3	6	16.2 ± 0.6	14.5-18.4	6	5.9 ± 0.2	5.1-6.7
	Sediment	5	8.9 ± 3.1	3.9-20.0	6	24.0 ± 1.5	21.0-30.6	3	2.0 ± 0.7	0.7-3.2
	Snail non-depurated	6	10.3 ± 0.8	8.4-13.6	6	11.5 ± 0.7	9.0-13.9	6	8.1 ± 0.8	5.8-10.6
	Snail depurated	6	10.3 ± 0.7	8.2-13.0	6	12.2 ± 0.7	10.0-14.7	6	8.7 ± 0.5	7.0-10.4
Cu	Mayfly non-depurated	6	17.7 ± 0.6	15.5-19.4	6	21.3 ± 0.5	19.5-22.8	6	10.4 ± 0.2	9.5-10.9
	Mayfly depurated	6	21.9 ± 2.0	16.6-29.0	6	28.5 ± 3.9	17.7-41.3	6	22.0 ± 4.4	11.3-33.1
	Periphyton	2	66.9 ± 35.1	31.8-102.0	6	26.5 ± 1.0	23.6-29.8	6	12.9 ± 1.2	10.3-17.0
	Sediment	5	8.8 ± 2.0	4.4-14.7	6	25.2 ± 1.6	20.6-32.5	3	3.5 ± 1.5	0.7-5.8
	Snail non-depurated	6	99.1 ± 8.2	64.4-121.0	6	118.7 ± 12.7	85-175	6	98.3 ± 6.5	80.8-127.0
	Snail depurated	6	117.6 ± 15.8	77.5-184.0	6	136.3 ± 11.2	103-170	6	130.3 ± 9.1	107-167
Fe	Mayfly non-depurated	6	10070 ± 591	7730-11400	6	9583 ± 535	8070-11600	6	6208 ± 656	4490-8430
	Mayfly depurated	6	1353 ± 244	728-2130	6	2468 ± 341	1580-3430	6	1600 ± 272	810-2640
	Periphyton	2	57809 ± 29946	27864-87755	6	14432 ± 1019	11446-17766	6	18924 ± 840	16265-21562
	Sediment	5	11452 ± 1595	6990-15200	6	25483 ± 1286	20000-28800	3	7477 ± 2360	2880-10700
	Snail non-depurated	6	2036 ± 683	514-4620	6	1381 ± 276	416-2120	6	1812 ± 328	851-3090
	Snail depurated	6	434 ± 36	349-546	6	508 ± 89	258-776	6	660 ± 62	462-894
Hg	Mayfly non-depurated	6	0.08 ± 0.00	0.07-0.09	6	0.12 ± 0.01	0.10-0.14	6	0.06 ± 0.00	0.06-0.07
	Mayfly depurated	6	0.07 ± 0.00	0.07-0.08	6	0.07 ± 0.00	0.06-0.09	6	0.06 ± 0.00	0.06-0.07
	Periphyton	2	0.48 ± 0.26	0.23-0.74	6	0.23 ± 0.02	0.18-0.29	6	0.10 ± 0.01	0.07-0.16
	Sediment	5	0.05 ± 0.01	0.02-0.10	6	0.09 ± 0.01	0.07-0.14	3	0.03 ± 0.00	0.02-0.03
	Snail non-depurated	6	0.14 ± 0.02	0.07-0.20	6	0.15 ± 0.02	0.10-0.19	6	0.17 ± 0.03	0.09-0.26
	Snail depurated	6	0.16 ± 0.02	0.12-0.20	6	0.16 ± 0.02	0.09-0.22	6	0.23 ± 0.02	0.15-0.32
Se	Mayfly non-depurated	6	5.9 ± 0.5	4.7-7.2	6	8.2 ± 1.0	6.3-11.9	6	2.7 ± 0.1	2.4-3.2
	Mayfly depurated	6	6.3 ± 0.4	4.8-7.8	6	8.8 ± 0.4	6.9-9.8	6	2.8 ± 0.2	2.2-3.3
	Periphyton	2	8.2 ± 4.3	3.9-12.4	6	3.5 ± 0.2	3.0-4.1	6	2.3 ± 0.1	1.8-2.5
	Sediment	5	0.7 ± 0.0	0.6-0.8	6	2.3 ± 0.2	1.9-3.3	3	0.7 ± 0.1	0.6-0.9
	Snail non-depurated	6	4.0 ± 0.2	3.3-4.5	6	5.2 ± 0.2	4.7-5.7	6	3.4 ± 0.3	2.5-4.1
	Snail depurated	6	4.3 ± 0.1	3.9-4.8	6	5.1 ± 0.3	4.1-6.1	6	3.3 ± 0.2	2.9-3.9
Zn	Mayfly non-depurated	6	180 ± 5	162-200	6	155 ± 5	137-168	6	18 ± 7	167-209
	Mayfly depurated	6	206 ± 9	181-229	6	177 ± 4	165-189	6	233 ± 8	215-255
	Periphyton	2	246 ± 136	110-382	6	88 ± 7	70-114	6	81 ± 10	53-118
	Sediment	5	38 ± 7	24-61	6	50 ± 6	37-69	3	26 ± 10	12-47
	Snail non-depurated	6	109 ± 8	89-140	6	170 ± 18	122-225	6	153 ± 4	144-166
	Snail depurated	6	121 ± 2	112-126	6	189 ± 26	107-292	6	144 ± 9	125-171

616 Table 2. Biomagnification factor factors (BMFs) calculated as the ratio between the average
 617 concentrations of each trace element (expressed as ppm DW) in the biota (i.e., mayfly nymphs
 618 and adult snails) and the average concentration of each trace element (expressed as ppm DW) in
 619 their respective food resources (i.e., sediment and periphyton). Estimations were done in both
 620 non-depurated (ND) and depurated (D) organisms. BMFs > 1 are in bold.

Element	ERM2.5		ERM1.0		CRM3.5		CRM1.5		ERM6.0		CRM6.0	
Mayfly (non-depurated – depurated)/Sediment												
	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D
As	1.44	0.38	1.52	0.87	0.73	0.20	0.64	0.19	1.32	0.50	0.99	0.32
Cu	2.01	2.48	0.84	1.13	0.88	1.10	0.49	0.79	2.95	6.27	2.08	3.22
Fe	0.88	0.12	0.38	0.10	0.37	0.05	0.27	0.04	0.83	0.21	0.61	0.06
Hg	1.72	1.57	1.31	0.8	1.10	0.46	0.45	0.23	2.52	2.56	1.72	0.42
Se	8.46	8.94	3.51	3.76	4.19	4.72	2.68	2.96	3.63	3.72	5.47	5.45
Zn	4.80	5.48	3.11	3.55	2.43	2.86	1.76	2.16	7.10	8.85	3.58	4.96
Snail (non-depurated – depurated)/Periphyton												
	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D
As	0.14	0.14	0.71	0.75	0.32	0.31	0.25	0.25	1.39	1.49	0.62	0.71
Cu	1.48	1.76	4.49	5.15	0.99	1.03	0.62	0.80	7.63	10.12	3.15	3.78
Fe	0.04	0.01	0.10	0.04	0.03	0.01	0.02	0.01	0.10	0.03	0.03	0.01
Hg	0.28	0.32	0.67	0.70	0.12	0.14	0.12	0.13	1.69	2.25	0.09	0.12
Se	0.49	0.52	1.45	1.44	0.83	0.80	0.67	0.66	1.49	1.47	0.94	0.95
Zn	0.44	0.49	1.94	2.16	0.66	0.74	0.48	0.47	1.89	1.78	0.75	0.85

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