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5 Sensitive response of sediment-grown *Myriophyllum spicatum* L. to
6 arsenic pollution under different CO₂ availability

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9 Elisabeth M. Gross ^{1,2,*}, Andréina Nuttens ^{1,2}, Darya Paroshin ^{1,2} & Andreas Hussner ³

10

11 ¹ Université de Lorraine, LIEC UMR 7360 CNRS, Rue Général Delestraint, Bâtiment IBISE,
12 F-57070 Metz, France.

13 ² LTER-”Zone Atelier Moselle”, LIEC UMR 7360 CNRS, Rue Général Delestraint, Bâtiment
14 IBISE, F-57070 Metz, France.

15 ³ Institute of Botany, Heinrich-Heine-Universität Düsseldorf, Geb. 26.13.O2, Universitätsstr.
16 1, D-40225 Düsseldorf, Germany

17 * Corresponding author: gross5@univ-lorraine.fr; Tel.: +33 3 87 37 86 26; Fax +33 3 87 37

18 85 12

19 **Abstract**

20 Aquatic plants may face resource constraints or anthropogenic pollution, and effects might be
21 heightened under multiple stress conditions. We investigated if arsenate effects on
22 *Myriophyllum spicatum* L. would be stronger under CO₂ limitation and low phosphorus
23 availability. In a factorial design, we exposed sediment-grown plants to either CO₂ (high
24 carbon or HC) or bicarbonate (low carbon or LC) and four levels of arsenate. We observed
25 strong effects of arsenate exposure on growth, biomass allocation (leaf, stem and root mass
26 fractions), pigments and phenolic compounds. CO₂ availability strongly affected the content
27 in phenolic compounds and a few other response variables, yet overall effects were less
28 pronounced than expected. Strong interactive effects of CO₂ availability and arsenic
29 concentration were only observed for carotenoids, the carotenoid/chlorophyll ratio and
30 phenolic compounds in leaves. Only the carbon content declined with increasing arsenic
31 concentration, otherwise leaf elemental content and stoichiometry were not affected by
32 arsenic or CO₂ availability, suggesting that plants strived to maintain leaf functions. The
33 observed effects on biomass allocation and plant quality, specifically elemental stoichiometry
34 and phenolic compounds content of *M. spicatum* not only show direct changes in plant
35 performance but suggest also indirect effects on ecological interactions such as competition or
36 herbivory.

37

38 **Keywords:** Submerged macrophyte, Trace metal, Element pollution, OECD test guideline,
39 aquatic ecotoxicology, CO₂-based growth and physiology

40

41 **Introduction**

42 Multiple stress scenarios are gaining focus both in ecology and ecotoxicology as we are
43 facing effects at the global, regional and local scale, strongly enhanced by anthropogenic
44 activities (Meybeck 2004; Segner et al. 2014). Since the response to a combination of
45 stressors might be specific for a certain type or group of organisms (Segner et al. 2014), we
46 need to understand the reaction of key organisms, such as those used in environmental risk
47 assessment. The rooted submerged macrophyte *M. spicatum* is now used as OECD
48 (Organisation for Economic Co-operation and Development) test organism because it allows
49 testing for both sediment and water exposure to anthropogenic pollutants (OECD 2014).

50 While normalized test systems in ecotoxicology strive for optimal growth (see details
51 on the OECD TG 239 for sediment grown *M. spicatum* below in materials and methods),
52 resource availability are known to affect plant growth and physiology (Hussner & Jahns
53 2015), and might thus also be involved in the response to pollution stress. Submerged
54 macrophytes, such as *M. spicatum*, can take up nutrients via roots or shoots, but root uptake is
55 the primary pathway (Grace & Wetzel 1978; Smith & Barko 1990). While excess or limiting
56 nutrient amounts can be a constraint for submerged macrophytes, the availability of CO₂ can
57 even be more restraining, as CO₂ diffusion is much slower in stagnant water, and the
58 availability depends on the pH and total dissolved inorganic carbon (DIC) availability
59 (Hussner & Jahns 2015). Although the growth of *M. spicatum* is enhanced in alkaline lakes
60 (Smith & Barko 1990), CO₂ compensation points are lower than those for HCO₃⁻ (Grace &
61 Wetzel 1978).

62 The genus *Myriophyllum* is known to accumulate trace metal elements, but this does
63 not exclude negative effects on plant growth and physiology (e.g., Harguinteguy et al. 2015;
64 Robinson et al. 2006). Arsenic is a trace metal element often found in aquatic systems

65 resulting from geochemical or anthropogenic processes. The New Zealand *M. propinquum* in
66 the Taupo Volcanic area hyperaccumulated 2101 mg [As] per kg dry mass (Robinson et al.
67 2006), and the North American *M. sibiricum* Kom. (syn. *M. exalbescence* Fernald) from a
68 lake polluted by mine tailings accumulated more than 140 mg kg⁻¹ [As] in its dry mass
69 (Dushenko et al. 1995). In freshwaters, arsenic occurs mainly as arsenate under oxygenic or
70 arsenite under anoxic conditions (Rahman et al. 2012), and the adsorption of arsenic to
71 sediments is influenced by metal oxides but also by carbonate and bicarbonate concentrations
72 (Smedley & Kinniburgh 2002).

73 Various environmental factors influence the uptake of arsenic by plants. Plants take up
74 arsenate via the phosphate uptake transporter (Finnegan & Chen 2012). In laboratory
75 experiments, high phosphate availability reduced the arsenate uptake by *Lemna minor*
76 (Mkandawire & Dudel 2012) and rice (Geng et al. 2005). However, no relationship between
77 *in-situ* phosphate availability and arsenic uptake was observed for a range of submerged and
78 emergent macrophytes in Canadian lakes (Dushenko et al. 1995). Under alkaline pH, the fern
79 *Pteris vittata* contained less arsenic than under neutral pH, but bicarbonate concentration in
80 natural ranges (up to 2 mM) showed little effect on arsenic uptake by the plant (Guo et al.
81 2012). Yet, eutrophic conditions reduced the arsenate uptake by the congeneric *M.*
82 *alterniflorum*, a species adapted to oligo-mesotrophic conditions (Krayem et al. 2016). The
83 effect of alkalinity, and thus pH and CO₂ availability, on the uptake of arsenic has, as far as
84 we know, not yet been tested with submerged freshwater plants. In this context we performed
85 our study, knowing that *M. spicatum* prefers meso- to eutrophic and alkaline environments.

86 Environmental stressors and resource constraints can have a wide range of effects in
87 plants. They may not only affect growth, but also morphological traits such as biomass
88 allocation to leaves, shoots and roots or leaf dry matter content (LDMC), changes that may
89 explain plant performance at the inter- and intraspecific level (Elger & Willby 2003; Fornoff

90 & Gross 2014; Hussner & Jahns 2015; Pérez-Harguindeguy et al. 2013) or physiological
91 traits, such as pigment composition, changes in elemental stoichiometry (i.e. carbon, nitrogen
92 and phosphorus content), and the production of defensive substances such as phenolic
93 compounds (Fornoff & Gross 2014; Gross 2003; Hussner et al. 2015; Hussner & Jahns 2015).
94 Phenolic compounds are widely involved in plant responses to abiotic and biotic stressors
95 (Dixon & Pavia 1995), and *M. spicatum* is a species well known for its high content in
96 bioactive polyphenols acting against competitors and herbivores (Fornoff & Gross 2014; Leu
97 et al. 2002) but also varying depending on resource availability (Gross 2003). High CO₂
98 availability increased the production of phenolic compounds in terrestrial plants (Hartley et al.
99 2000), and trace metal elements affected the content of phenolic compounds (Lavid et al.
100 2001), possibly because of oxidative stress responses (Waśkiewicz et al. 2014).

101 Since the availability of CO₂ is a major constraint for submerged macrophytes, and
102 because of the potential impact of pH, bicarbonate and nutrient availability on the effect of
103 arsenic on aquatic plants, we used a factorial design experiment to test the effects of arsenate
104 under different CO₂ availability and at low phosphorus availability on *M. spicatum*. Our
105 hypotheses were that 1) environmentally relevant arsenic concentrations would negatively
106 affect growth and physiological response variables (i.e. “endpoints” *sensu* ecotoxicological
107 test protocols, e.g., OECD 2014) in *M. spicatum*, 2) effects would be enhanced at high pH
108 reflecting low CO₂ availability, and 3) changes in morphological and physiological plant traits
109 of *M. spicatum* reflect specific responses towards a certain combination of stressors. We
110 included growth, pigment content, plant stoichiometry, and the content in phenolic
111 compounds as response variables to see if these “endpoints” are useful for predicting potential
112 ecological and ecosystem effects caused by multiple environmental stressors.

113

114 **Material and Methods**

115 Cultivation of plants and experimental set-up

116 We performed our experiment in a plant-water-sediment test system modified after the
117 currently accepted new OECD test guideline for the rooted aquatic plant *M. spicatum* (OECD
118 2014). This test system uses 2 L glass beakers, a sediment mixed from sand, kaolin, white
119 peat and calcium carbonate (74/20/5/1% on a weight basis), amended with rather high
120 concentrations of nutrients (each 200 mg of ammonium chloride and sodium phosphate per kg
121 of dry sediment), and a water column based on the so called Smart & Barko medium (Smart
122 & Barko 1985), a mineral solution rich in calcium chloride, magnesium sulphate, potassium-
123 and sodium bicarbonate, but free of nitrogen and phosphorus. In a typical set-up, three shoots
124 of *M. spicatum* per beaker grow without any aeration for 14 days. For our purposes, we
125 modified container size, shoot density, nutrient provision and CO₂ availability.

126 Approximately 4 cm long apical shoot sections of *M. spicatum* from axenic cultures
127 maintained in the laboratory of EMG (Gross 2003) were adapted during two weeks to Smart
128 & Barko medium (Smart & Barko 1985) supplemented initially with 143 μM N-NO₃ and 2.6
129 μM P-PO₄ as potassium salts, and after one week with half the dose of N and P. This resulted
130 in a molar N:P ratio of 55:1, as we intended to apply a slight phosphorus limitation (Güsewell
131 & Koerselman 2002) to enhance effects of arsenate. We applied the nutrients only to the
132 water column, and not in the sediment to avoid direct competition with arsenate. For the
133 sediment-based pre-culture lasting 5 days, shoots were planted in artificial sediment modified
134 slightly from the OECD 239 test protocol (OECD 2014), omitting CaCO₃ and nutrient
135 additions, and only using 75% sand, 20% kaolin 5% white peat. The water column consisted
136 of Smart & Barko (1985) solution amended with N and P as described above.

137 In the experiment, we applied 0, 1.44, 7.2 and 36 mg [As] in the form of $\text{Na}_2\text{HAsO}_4 \times$
138 $7 \text{ H}_2\text{O}$ per kg dry sediment, mixed as aqueous solution with the wetted sediment to reach 20%
139 water content. An equivalent of 200 g dry sediment was added to square plastic pots (250 ml;
140 $11 \times 7 \times 3 \text{ cm}$; $w \times d \times h$), and one each was then placed in a 3-L plastic container ($15 \times 11 \times$
141 19 cm ; $w \times d \times h$). The total volume of medium added per container was 2.5 L.

142 We set up 32 containers, 16 dedicated for each of the two different DIC conditions
143 (HC / high CO_2 availability or LC / low CO_2 availability/ dominance of HCO_3^- treatment),
144 and four replicates for each [As] concentration (4 levels including control) and carbon
145 condition. Half of the containers were filled with the standard Smart & Barko solution at 0.85
146 mM DIC (LC treatment), the other half with only 0.45 mM DIC (HC treatment) but amended
147 with CO_2 aeration from a CO_2 tank (50 l; NE45 – 99.995%) via needle valves to yield final
148 DIC concentrations of 0.8 – 0.9 mM. The LC treatments were only bubbled with air to allow
149 gas exchange with the atmosphere. The addition of CO_2 to the HC treatments shifted the total
150 DIC concentration to 0.8 – 0.9 mM (Hussner & Jahns 2015). Thus, different proportions of
151 CO_2 and HCO_3^- were available in the HC and LC treatments, as quantified by standard
152 methods (see detailed description in Hussner & Jahns, 2015, and yielding pH values of $6.8 \pm$
153 0.2 and 8.2 ± 0.2 in the HC and LC treatments, respectively). All containers were placed in a
154 water bath at $22 \pm 2^\circ\text{C}$ and $120 (\pm 15\%) \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ illumination by OSRAM
155 SubstiTUBE LED light tubes (ST8-HV4-170-840) at a 16/8 h L/D photoperiod.

156 Each pot was planted with two shoots of *M. spicatum*. Shoots were selected from the
157 pre-cultures to have a shoot length between 6 and 14 cm ($9.0 \pm 1.8 \text{ cm}$, mean $\pm 1 \text{ SD}$) and
158 well developed roots of approximately 1 to 2 cm length. Because shoots differed much in
159 length, we decided to use each one larger shoot (A; 8.7 – 14 cm) and one smaller shoot (B; 6
160 – 11 cm) per container. Before planting, each shoot was carefully blotted dry to take the initial
161 fresh weight ($0.19 \pm 0.06 \text{ g}$; equivalent to $0.014 \pm 0.004 \text{ g}$ dry mass based on dry-to-fresh

162 mass calibration of 10 control shoots) before planting. Preliminary experiments had shown
163 that carefully blotting-to-dry would not harm plant development. Care was taken to distribute
164 shoots evenly among all containers. No difference in initial total length or fresh mass for the
165 different treatments and containers existed (1-way ANOVA, $F < 1.22$; $P > 0.33$).

166

167 Maintenance and end of experiment

168 The experiment was run for 14 days, and pH and water level of all containers was
169 controlled and eventually adjusted daily, or once every two days during the weekend. We
170 occasionally measured oxygen content around midday, and found all containers at or above
171 100% saturation, indicating ongoing photosynthetic activity of the plants. At the end of the
172 experiment, we carefully removed the two shoots per container, and measured main shoot
173 length, root length and eventual side shoot length, as well as total fresh mass. Each shoot was
174 photographed to document its appearance. Shoot A was dissected into root, shoot and leaf
175 biomass, each fraction was placed individually into pre-weighted tubes to determine fresh
176 mass, and shock frozen in liquid nitrogen. Shoot B was left intact and whole plants were shock
177 frozen using liquid nitrogen. All frozen samples were freeze-dried for 24 h, providing
178 individual total shoot or root, stem, and leaf dry mass. From leaves, we calculated for shoot A
179 the respective leaf mass fractions ($LMF = \text{leaf dry mass}/\text{total dry mass}$ or $LMF-a = \text{leaf dry}$
180 $\text{mass}/\text{leaf}+\text{stem dry mass}$; the latter to account for aboveground biomass), as well as stem and
181 root mass fractions ($SMF = \text{stem dry mass}/\text{total dry mass}$; $RMF = \text{root dry mass}/\text{total dry}$
182 mass). For all samples, we calculated also the respective dry matter content (DMC) for total
183 shoots or plant organs (leaf, stem and root dry matter content as LDMC, SDMC and RDMC,
184 respectively) based on the respective ratios between dry and fresh mass.

185 Relative growth rates were calculated for fresh and dry mass (RGR-fm, RGR-dm) and
186 length (RGR-L) based on the equation $RGR = (\ln W_f - \ln W_i)/d$ with W_f the end value, W_i the
187 initial value and d the time in days.

188

189 Chemical analyses

190 Inorganic nutrients in the water column (N-NO₃ and P-PO₄) were measured at the end
191 of the experiment using ion chromatography (ICS-1100, Thermo-Fisher Scientific) and
192 spectrophotometry following standard procedures (AFNOR 1990). Arsenic in the sediment
193 was analysed after mineralization in 35 % ultrapure HNO₃ for 24 h. Arsenic in the
194 mineralized sediment samples and the water samples were then quantified by atomic
195 absorption spectroscopy (AAS; Perkin-Elmer, AAnalyst 100).

196 Dry biomass samples were ground to a fine powder using a Fritsch pulverisette 23 ball
197 mill before use in chemical analyses. All sample mass data are approximate values, the exact
198 mass for each sample was determined with a Sartorius MC2 ultra-microbalance at a precision
199 of 10 µg. Elemental content for carbon (C), nitrogen (N) and phosphorus (P) were determined
200 in leaves from plant A. The content in C and N were determined based on analyses of
201 subsamples of 1 mg encapsulated in tin containers in a Carlo-ERBA Na 2100 CE elemental
202 analyser. P was determined according to AFNOR (1990) using alkaline digestion (1 N NaOH
203 and 0.45 M Na₂S₂O₈) of 1 mg samples for 2 h at 120 °C and 1 bar followed by
204 spectrophotometric quantification in an automated analyser (Konelab 420-XTi, Thermo
205 Fischer Scientific).

206 Chlorophyll, anthocyanins and total phenolic contents were analysed based on
207 methods described in Fornoff & Gross (2014). All measurements were made in a Varian Cary
208 50 spectrophotometer. Chlorophyll was extracted from 0.5 mg samples extracted in 1 ml

209 acetone/TRIS–hydrochloric acid-buffer (0.1 M), 8/2 (v/v); pH 7.8, for 2 h at 16 °C. The
210 absorbance was measured at 470, 664 and 647 nm to calculate the carotenoid, the chlorophyll
211 *a* and *b* content per extracted biomass based on the equations provided by Lichtenthaler &
212 Buschmann (2001). Anthocyanin was extracted with acidified methanol
213 (methanol/water/hydrochloric acid 37 %, 80/20/1 [v/v/v]) from 1 mg samples in 1 ml solvent
214 for 2 h at 16 °C. The absorbance at 532 and 653 nm was used to calculate the anthocyanin
215 content (cyanidin, the major anthocyanin found in *Myriophyllum* spp. (Reznik & Neuhausel
216 1959) based on the formula developed from Murray & Hackett (1991): Anthocyanin [mg/g] =
217 $((A_{532 \text{ nm}}) - (0.24 * A_{653 \text{ nm}})) / (30 / 287.24 * m)$; with A = absorbance at the respective
218 wave length and m the exact dry mass of the sample extracted in one ml solvent. Total
219 phenolic compounds (TPC) were extracted in aqueous acetone (50/50 [v/v]; 1 mg sample in 1
220 ml solvent) followed by the Folin–Ciocalteu assay with tannic acid (no. 403040, batch
221 06817CJ; Sigma-Aldrich) as standard, following methods described in Fornoff & Gross
222 (2014).

223

224 Statistical analyses

225 Data were analysed using R Studio (2015). Homoscedasticity and normality were
226 verified respectively by the Levene and Shapiro–Wilk tests, and if needed, data were log or
227 1/x transformed to ensure normality and equal variance. Two-way ANOVAs with carbon and
228 arsenic condition as fixed factors were calculated for all response variables using a probability
229 threshold of $p \leq 0.05$, followed by Tukey post-hoc tests in case of significant differences
230 among treatments. A Principal component analysis (PCA) was performed on the full set of
231 endpoints except global DMC to visualize differences according to the experimental
232 conditions using FactoMineR (Lê et al. 2008). The contribution of each variable (endpoint)

233 for the different axes (i.e. the quality of representation of endpoints upon axes) was computed
234 and called column inertia.

235

236 **Results**

237 Sediment and water column

238 The pH in the high and low CO₂ treatments stayed constant at the values provided in
239 Material and Methods due to the continuous aeration (LC treatment) and CO₂ aeration (HC
240 treatment). Oxygen concentrations remained near or slightly above 100%, also due to the
241 aeration-induced turbulence. No visual algal growth in the water column was observed, only
242 occasionally we found some algae growing on the sediment, but not on the plants. Reference
243 plants which were visibly free from epiphytes are documented in Supplementary Figure A1.
244 Plants had absorbed about 36 % of the added N-NO₃ and 22 % of the added P-PO₄ (data not
245 shown).

246 At the end of the experiment, we typically recovered 75-150 % of the initially applied
247 arsenic in the containers, when combining concentrations found in sediment and water (Table
248 1). Part of the arsenic had leached into the water column, and this effect increased with
249 increasing initial sediment arsenic concentrations. Controls exhibited low concentrations of
250 arsenic, possibly leaching from background levels in sediments. In the low arsenic treatment
251 (1.44 ppm) we found about 50 % higher concentrations than intended, possibly caused by
252 sediment background levels. While the arsenic concentrations for the levels 7.2 and 36 ppm in
253 the HC treatments were close to the intended values with 110 and 100 % measured
254 concentrations, they were significantly lower in the LC treatments, with only 85 and 76 %.
255 Thus, recovery of arsenic was significantly lower in the LC treatments (Tukey post-hoc test, p
256 < 0.05).

257

258 Plant responses

259 CO₂ availability and arsenic concentrations affected growth, plant traits, and plant
260 physiology of *M. spicatum*. In general, arsenic effects were pronounced at 7.2 and 36 ppm
261 [As] in sediment, while high or low CO₂ availability affected only a few response variables,
262 and almost no interactive effects between factors were observed (Table 2, referring also to all
263 response variables outlined in detail below).

264

265 A – Effects on the growth of shoots A and B

266 The growth of *M. spicatum* based on of length, fresh and dry weight was strongly
267 influenced by arsenic, while low CO₂ availability only significantly reduced length-based
268 growth, with marginal effects on biomass-based growth parameters (RGR-L, RGR-fm and
269 RGR-dm). The dry matter content (DMC) increased by about 50% at arsenic concentrations
270 of 7.2 and 36 ppm, resulting in somewhat weaker effects on dry mass-related growth (Fig. 1
271 A-C).

272

273 B – Effects on biomass allocation of shoot A

274 Arsenic and to a lesser extend CO₂ availability affected the biomass allocation to
275 leaves, stems and roots in *M. spicatum* (Fig. 2). This resulted in differences in the respective
276 mass fractions of leaves (LMF), stems (SMF) or roots (RMF). With increasing arsenic
277 concentrations, RMF declined while stems got thicker resulting in a higher SMF. Under low
278 CO₂ availability, the allocation to leaves increased while that to stems decreased. For leaves,
279 we calculated two indices: One dividing leaf dry mass by total biomass (LMF), and one using

280 only the aboveground biomass (LMF-a). Since root biomass declined strongly with increasing
281 arsenic, the effect at the whole plant biomass on LMF was rather weak, and no differences
282 between treatments were found. When roots were omitted in the calculation, the LMF-a
283 declined with increasing arsenic concentrations, and that this effect was enhanced in LC
284 treatments. At the two highest arsenic concentrations, the dry matter content (DMC) increased
285 for all plant organs in shoot A (leaves - LDMC, stems - SDMC and roots - RDMC) while
286 high CO₂ availability only increased the RDMC.

287

288 C – Effects on pigment and phenolic compound content in leaves of shoot A

289 Effects on pigments in leaves are shown in Fig. 3A-D. Total chlorophyll content
290 declined with increasing arsenic concentration, but the chlorophyll a/b ratio did not differ
291 between treatments. A strong interaction between arsenic concentration and CO₂ availability
292 was observed for the carotenoid concentration, which specifically declined at high CO₂
293 availability and higher arsenic concentrations. Subsequently, also the ratio of carotenoids to
294 total chlorophyll exhibited a strong interaction effect between arsenic concentration and CO₂
295 availability.

296 Anthocyanins (Fig. 4A) declined at low CO₂ availability and higher arsenic
297 concentrations in leaves, but we observed no arsenic-induced differences at high CO₂
298 availability. The content in total phenolic compounds (TPC; Fig. 4B) in leaves A was strongly
299 affected by CO₂ availability and arsenic concentration, but with no clear pattern, thus
300 resulting in strong interactive effects. While high CO₂ availability increased the TPC in
301 leaves, we observed no consistent trend with increasing arsenic concentrations. Only the
302 leaves from the LC treatment had higher TPC at higher arsenic concentrations.

303

304 D – Effects on elemental stoichiometry in leaves from shoot A (Fig. 5)

305 Both factors, arsenic concentration and CO₂ availability, had only slight effects on the
306 elemental composition of carbon, nitrogen and phosphorus in leaves. With higher arsenic
307 concentrations, the C content declined in leaves, irrespective of CO₂ availability. Only
308 marginal effects were observed for nitrogen and phosphorus. Leaves in the LC treatment at
309 higher arsenic concentrations had a slightly lower N content, and the phosphorus content was
310 not affected by arsenic and only marginally reduced by low CO₂ availability. Overall leaf
311 stoichiometry was not affected, as neither CO₂ availability nor arsenic concentration affected
312 the C:N, C:P or N:P molar ratio.

313

314 E – Overall effects on plant performance (Fig. 6)

315 The principal component analysis (PCA) on the whole set of endpoints and for the
316 different treatment combinations showed distinct differences between treatment
317 combinations. Axes 1 and 2 explained 37.2 and 15.7 % of the variance. The axes 3 and 4
318 added another 9.9 and 7.8 % to explain in total more than 70 % of the total variance.
319 Differences between low (0 and 1.44 ppm) or high (7.2 and 36 ppm) levels of [As] in
320 sediment were visible along axis 1, while less pronounced differences based on CO₂
321 availability were related to axis 2. The correlation circle (Fig. 6A) shows the significant
322 correlations for fresh mass based growth (RGR-fm), root mass fraction (RMF) and
323 chlorophyll content (chl *a*, chl *a+b*), and in opposite direction stem mass fraction (SMF) and
324 the dry matter content of all plant organs, leaves, roots and stems (LDMC, RDMC, SDMC)
325 with axis 1. Axis 2 is mainly explained by phosphorus content (P) and the resulting C:P molar
326 ratio, and to a lesser extent by the N:P molar ratio and pigment ratios (car/chl, chl *a/b*).

327 Detailed correlation coefficients and associated P-values can be found in the Supplementary
328 Material, Table A1.

329

330 **Discussion**

331 In a 14-day exposure to arsenate in combination with high or low CO₂ availability we
332 observed treatment-specific effects on the growth, biomass allocation and plant metabolism in
333 the rooted, submerged macrophyte *M. spicatum*. In the following we will discuss our initial
334 hypotheses in detail.

335

336 Hypothesis 1: Environmentally relevant arsenic concentrations negatively affect growth and
337 physiological response variables in *M. spicatum*

338 Most selected endpoints measured in *M. spicatum* showed the effect of arsenic applied
339 to the sediment. Negative effects on growth and physiology started at a nominal concentration
340 of 7.2 ppm arsenic applied to the sediments. This is a rather low concentration, and in the
341 range of “normal”, natural values found in lake or river sediments (Smedley & Kinniburgh
342 2002). However, the observed effects might have been higher in our experiment because the
343 applied arsenate is highly soluble, and diffused rapidly from the sediment to the overlying
344 water column, as our measurements show (Table 1). Also under natural conditions, sediments
345 and rocks release arsenic to the water column. Robinson et al. (2006) observed water
346 concentrations up to 180 µg l⁻¹ in the Taupo Volcanic Area, but in areas with no geochemical
347 or anthropogenic sources for arsenic, concentrations are mostly below 10 µg l⁻¹ (Smedley &
348 Kinniburgh 2002).

349 We could not assess the fate of arsenic accumulated in plant tissues or plant uptake as
350 we only had limited biomass available (50 to 150 mg per replicate, depending on treatment

351 and used for all chemical analyses). Nevertheless, the negative effects already at 7.2 and 36
352 ppm nominal sediment concentrations suggest that at least part of the arsenic had been taken
353 up by the plants, either from sediment or water. Laboratory studies showed growth reductions
354 in *M. propinquum* from the Taupo Volcanic Zone rich in arsenic at about 170 to 290 $\mu\text{g l}^{-1}$
355 (Robinson et al. 2006). In our study, first effects occurred at measured final concentrations of
356 approximately 300 $\mu\text{g l}^{-1}$ in water and 3.25 ppm in sediment originally spiked with 7.2 ppm
357 [As]. No effects were observed in the control and the low arsenic treatment, although they
358 contained some arsenic in water and sediment.

359 A remarkable finding is the strong increase in the dry matter content (DMC) of the
360 whole shoots or in the different plant organs (leaves - LDMC, stems - SDMC, roots - RDMC)
361 at higher arsenic concentrations. This indicates that plant physiology was altered, as plants
362 had reduced water content and/or changed some metabolites or components, such as increased
363 allocation to storage carbohydrates and starch (Hussner & Jahns 2015). Exposure to arsenic
364 also increased starch content in *Ceratophyllum demersum* due to negative effects on
365 photosynthesis (Mishra et al. 2014), thus it is likely that the observed increase in DMC here is
366 caused by starch accumulation.

367 Plant leaves, stems, roots, and tissue biomass ratios also changed following exposure
368 to arsenic. Despite significant amounts of arsenic leaching into the water column, we
369 observed a strong reduction in root mass, both absolute and relative to total biomass (root
370 mass fraction / RMF), from above 15 % to around 5 % in high arsenic treatments. RMF
371 provides comparable results to the frequently used root/shoot ratio, with the difference that
372 the latter divides the root biomass only by the aboveground biomass, thus in our case the
373 root/shoot ratio in the controls and low arsenic treatments would be approximately 20 %, a
374 value comparable to that reported by Hussner & Jahns (2015).

375 The roots of arsenic exposed plants were thicker and darker than those found in
376 controls (Fig. A1). This is a well described phenomenon for macrophytes growing in metal-
377 polluted sediments. The darkening results in part from iron plaques formed on the roots.
378 Many trace metal elements, including arsenic, bind to iron plaques on macrophyte roots
379 (Taggart et al. 2009), and the magnitude of iron plaque formation on roots influences the
380 accumulation of trace metal elements in aquatic plants (Tripathi et al. 2014). Stem appearance
381 did not differ visually (Fig. A1), but the biomass allocation to stems (SMF) rose sharply with
382 higher arsenic exposure, increasing from 25 % up to 40 % of the total biomass. We assume
383 that plants used the stems to store arsenic and avoid negative effects on leaves.

384 The decline in chlorophyll *a* and *b* in leaves of plants exposed to higher arsenic
385 concentrations is not unexpected, and has been observed in other plants exposed to arsenic or
386 other trace metal elements. For example, negative effects on photosynthetic pigments have
387 been observed in the floating fern *Azolla filiculoides* exposed to 60 $\mu\text{g l}^{-1}$ As(V) (Sánchez-
388 Viveros et al. 2011). Overall we can confirm our first hypothesis that arsenic exposure at
389 environmentally realistic concentrations caused strong effects on different growth and
390 physiology related parameters in *M. spicatum*.

391

392 Hypothesis 2: Effects of arsenic exposure are enhanced at high pH and low CO₂ availability

393 Low CO₂ availability caused much less, and usually less strong effects on the growth
394 and physiology of *M. spicatum*, and only a few interactive effects between CO₂ availability
395 and arsenic exposure could be observed. High bicarbonate concentration in the medium
396 together with a high pH had affected arsenic uptake in the fern *Pteris vittata* (Guo et al.
397 2012). Yet, we observed only little differences between the two carbon treatments for arsenic
398 induced effects, and thus assume that the applied higher pH or lower CO₂ availability,

399 resulting in a higher bicarbonate concentration of the medium, might not have affected the
400 bioavailability, or negative effects of arsenic on *M. spicatum*. Only length growth was
401 significantly affected and mass-based growth rates declined only marginally. A much stronger
402 effect of CO₂ availability was observed on biomass allocation to leaves, stems and roots. We
403 found an increase in RDMC in the high CO₂ treatments, while Hussner & Jahns (2015) found
404 an increase in LDMC at high CO₂ availability. Possibly the light intensity in our experiment
405 was too low, as the effects were also reduced or not visible in the low light treatments of
406 Hussner & Jahns (2015). Leaf biomass increased (LMF-a and LMF) in the low CO₂
407 treatments, and this can be seen in accompanying photographs (Fig. A1). We explicitly
408 calculated both the LMF based on total biomass and that based only on aboveground biomass
409 (LMF-a). While the former is commonly used as plant trait (Pérez-Harguindeguy et al. 2013),
410 the strong changes in root biomass resulted in a higher significance when considering only the
411 aboveground biomass for the calculation (LMF-a; Table 2). Our results indicate that plants in
412 the low CO₂ treatments invested more in leaf biomass to improve the diffusional dissolved
413 inorganic carbon uptake (Eusebio Malheiro et al. 2013).

414 Except for a few cases, low CO₂ availability has not enhanced negative effects of
415 arsenic exposure on *M. spicatum*. This might be due to the less severe carbon limitation in our
416 set-up that provided still some aeration and kept the pH at about 8.2. If the higher pH of 9 as
417 observed in the new OECD TG 239 (OECD 214) would have stronger effects needs to be
418 shown. Yet, effects with the fern *Pteris vittata* (Guo et al. 2012) were also only visible at
419 rather high bicarbonate levels, and not in environmentally relevant ranges as used in our
420 experiment. We thus cannot confirm our second hypothesis.

421

422 Hypothesis 3: Changes in morphological and physiological traits of *M. spicatum* reflect
423 specific responses towards a certain combination of stressors.

424 Although we could not confirm our second hypothesis, we think that our extended
425 analysis of additional parameters provides a good picture of specific responses of *M. spicatum*
426 to certain stressors. To achieve this we used morphological plant traits, such as the dry matter
427 content (DMC) and mass fractions for leaves, stems and roots (LMF, SMF, RMF), or
428 physiological traits such as the elemental composition and stoichiometry and the content in
429 phenolic compounds. The principal component analysis shows (Fig. 6B) that for the two
430 higher arsenic concentrations CO₂ availability did make a difference in the overall plant
431 response, based on individual endpoints, or response variables.

432 Only arsenic increased the DMC in *M. spicatum*, but an increase in DMC has several
433 implications. From an ecological perspective, plants with a higher LDMC are less palatable to
434 herbivores (Elger & Willby 2003) and *M. spicatum* even increases the DMC of apical tissues
435 when attacked by herbivores (Fornoff & Gross 2014). With respect to common endpoints
436 used in ecotoxicology, an increased DMC can mask effects on dry mass based yield or
437 relative growth rate calculations. Our results show that effects based on dry mass (RGR-dm)
438 were weaker than those based on fresh mass or total shoot length (RGR-fm; RGR-L). Arsenic
439 is not the only pollutant causing this effect, as the herbicides metsulfuron-methyl (Cedergreen
440 et al. 2004) and mesosulfuron-methyl previously showed no effects on dry-mass based growth
441 estimations in *M. spicatum*, due to increases in DMC as we observed recently (Nuttens et al.
442 2016). From an ecotoxicological perspective, it seems thus prudent to select several growth
443 endpoints to avoid the masking effect of increases in DMC on dry mass related results.

444 Changes in the proportion of leaf, stem and root biomass have ecological and
445 ecotoxicological implications. From an autecological perspective, stronger stems can provide

446 more stability and resistance to mechanical forces, but if this is at the expense of leaf biomass,
447 plants will have less photosynthetic tissue and thus risk slower growth rates. The new test
448 guideline for *M. spicatum* growing in a water-sediment set-up (OECD 2014) requires only
449 evaluation of root development. Though it seems unlikely that all plant traits used here could
450 be included for most normalized testing, the selection of additional endpoints would be quite
451 useful for understanding processes at the plant level and implications in an ecological context.

452 We found significant effects of both CO₂ availability and arsenic, and interaction
453 effects on levels of phenolic compounds in *M. spicatum*, suggesting that this endpoint is
454 reacting strongly influenced by environmental conditions. A decline in phenolic compounds
455 was found in *A. filiculoides* exposed to arsenic (Sánchez-Viveros et al. 2011), yet we
456 observed either no effect on TPC (HC for leaves) or an increase (LC for leaves exposed to 7.2
457 or 36 ppm [As]). Anthocyanin increased in *Lemna* exposed to water containing arsenic (Leao
458 et al. 2014), but we observed a decline in anthocyanins. Phenolic compounds are ubiquitous
459 in terrestrial and aquatic plants, but the relative content and composition can vary strongly
460 depending on environmental conditions, and in response to a wide range of abiotic and biotic
461 stressors (Dixon & Pavia 1995; Gross & Bakker 2012). We think that phenolic compounds
462 are interesting indicators for environmental stressors acting on *M. spicatum* and other plants,
463 and propose to have a more detailed look how they are modified e.g. by trace metal elements
464 in combination with environmental conditions, and how this affects plant performance and
465 biotic interactions.

466 Effects of CO₂ availability and arsenic on elemental composition and plant
467 stoichiometry were relatively low in *M. spicatum* leaves. We expected that increased arsenic
468 concentration would reduce the uptake of phosphate, as had been observed for *Lemna*
469 (Mkandawire & Dudel 2012), but this effect was not found in leaves. The nitrogen content
470 was slightly affected by arsenic (P = 0.066), and the significant lower carbon content due to

471 arsenic might be related to a lower photosynthetic activity (Mishra et al. 2014) and the lower
472 chlorophyll content. After two weeks exposure, we observed no effects on leaf stoichiometry.
473 Apparently, N:P molar ratios are relatively robust, as Li et al. (2015) reported similar values
474 along a range of water depths for three common submerged macrophytes. Also other authors
475 report a relative homeostasis of the N:P ratio in aquatic plants irrespective of environmental
476 nitrogen or phosphorus concentrations (Demars & Edwards 2007; Güsewell & Koerselman
477 2002). However, severe carbon stress due to very low CO₂ (and HCO₃⁻) availability as it
478 occurs in the standard set-up of the *M. spicatum* sediment-water OECD test system (OECD
479 2014) where no aeration occurs and resulting pH values exceed 9 (EMG, pers. observ.), might
480 modify plant stoichiometry. Nitrogen content in several *Myriophyllum* and other submerged
481 macrophyte species was reduced under high CO₂ availability, with *M. spicatum* exhibiting the
482 lowest effects (Hussner et al., in press).

483 The principal component analysis revealed links between the different endpoints
484 chosen in our study. All different treatment combinations were rather well separated along
485 axes 1 and 2. The separation of treatments along axis 1 was influenced by differences in
486 arsenic concentration, separating between the two low (0 and 1.44 ppm [As]) and the two
487 high [7.2 and 36 ppm [As]) concentrations. Differences based on CO₂ availability showed
488 along axis 2, but only for the two highest arsenic concentrations. Along axis 1, a high growth
489 rate based on fresh mass corresponded to a high chlorophyll content, a high root mass and
490 aboveground leaf mass fraction (RMF, LMF-a), and was opposed to higher stem mass
491 fraction (SMF) and increases in all dry matter related endpoints (LDMC, RDMC, SDMC).
492 We thus consider endpoints pointing to the left in the circle of correlation (Fig. 6B) reflecting
493 active growth and development, while those pointing to the right as endpoints showing
494 impaired growth. Interestingly, although we had not found many significant effects of CO₂
495 availability on individual endpoints, treatments for one given arsenic level differed between

496 high and low CO₂ availability (HC, LC), with the strongest effect for 7.2 ppm [As] in
497 sediment. Thus, low CO₂ availability had not aggravated the impact of arsenic, but exerted
498 different effects on the plants. These were a slight reduction in phosphorus content in leaves,
499 modifications in the content of phenolic compounds (TPC) and the ratio of carotenoids to
500 chlorophyll (car/chl), the latter two possible indicators of oxidative stress (Wańkiewicz et al.
501 2014).

502

503 **Conclusions**

504 This laboratory experiment combined realistic CO₂ availability scenarios with presumed
505 environmentally relevant arsenic exposure settings. Despite the high leaching of arsenic from
506 the sediment to the water column, the effective concentrations for *M. spicatum* were in the
507 range that can be found in areas with natural or anthropogenic high exposure to arsenic via
508 sediment or water. We can thus partly confirm our first hypothesis that negative effects on
509 growth and physiology can be observed at environmentally relevant arsenic concentrations.
510 Our second hypothesis that low CO₂ availability would aggravate the effects of arsenic could
511 only partly be verified, but results might be different for a longer, chronic exposure or under
512 more severe CO₂ stress and pH values above 9, as caused by the complete absence of aeration
513 in the new OECD test guideline for *M. spicatum* (OECD 2014). With respect to our third
514 hypothesis, we think that our experiment shows the complex response of *M. spicatum* to
515 combined stressors. The combination of different endpoints allowed a rather clear distinction
516 of treatment combinations. We suggest investigators consider whether a broader range of
517 response variables can help identifying responses to stress-induced changes. Additional
518 response variables may include plant traits such as biomass allocation, dry matter content,
519 stoichiometry and the content in secondary metabolites in addition to general “endpoints”

520 based on growth and pigment content. These additional endpoints might be useful in
521 predicting the plant performance in an ecological context, as for example increased leaf
522 biomass would result in better photosynthetic performance, or increased content in phenolic
523 compounds could explain the plant's capacity to be defended against competitors or
524 herbivores. Such effects on the “plant quality”, i.e. the dry matter content, phenolic content or
525 elemental stoichiometry, have already been shown to affect herbivory (Fornoff & Gross
526 2014), and possibly other biotic interactions as allelopathy.

527

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538

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661 **Table 1:** Recovery of arsenic at the end of the experiment in the different treatments. Data represent means ± 1 SE; n = 4.

Treatment	Nominal sediment As concentration [mg kg ⁻¹]	Recovery		% of total recovery found in		% recovery of initially applied [As]
		Sediment [mg kg ⁻¹]	Water [μg l ⁻¹]	Sediment	Water	
HC	0	0.88 ± 0.14	2.75 ± 0.75	96.3	3.7	
	1.44	1.45 ± 0.12	50.50 ± 5.85	69.9	30.1	145.0 ± 6.6
	7.20	3.60 ± 0.43	344.75 ± 25.96	45.6	54.4	110.0 ± 7.4
	36.00	8.67 ± 0.60	2196.75 ± 226.01	24.5	75.5	100.4 ± 8.0
LC	0	0.64 ± 0.79	16.50 ± 3.43	44.4	55.6	
	1.44	1.63 ± 0.16	52.0 ± 6.76	71.5	28.5	158.4 ± 10.4
	7.20	2.89 ± 0.15	260.50 ± 25.01	47.4	52.6	85.4 ± 4.1
	36.00	7.20 ± 0.67	1618.75 ± 344.10	28.1	71.9	76.2 ± 12.6

662

663

664 **Table 2:** 2-way ANOVA results based on the fixed factors carbon and arsenic. Shown are the
 665 effect of CO₂ availability and arsenic pollution on different response variables measured in
 666 *Myriophyllum spicatum*. Results are based a) on growth data for both shoots per microcosm,
 667 b) on plant traits and metabolism of leaves from shoot A and c) on the stoichiometry of the
 668 whole shoot B. Degrees of freedom: df = 1 for carbon, df = 3 for arsenic; df = 3 for carbon x
 669 arsenic. * log transformed data. ** 1/x transformed data. Bold numbers indicate significant
 670 results (P < 0.05). Marginally significant results (P < 0.1) are marked in italics.

671 RGR – Relative growth rate based on dm – dry mass, fm – fresh mass or L – length. DMC –
 672 Dry matter content. LMF and LMF-a – leaf mass fraction based on total biomass or
 673 aboveground biomass. SMF, RMF – stem and root mass fractions. LDMC, SDMC and
 674 RDMC – leaf, stem and root dry matter content. Chl – chlorophyll. Car – carotenoids. TPC –
 675 total phenolic compounds. C – carbon. N – nitrogen. P – phosphorus.

	Carbon		Arsenic		Carbon x Arsenic	
	F	P	F	P	F	P
A) Growth rate and dry matter content of both shoots						
RGR-dm	3.494	<i>0.074</i>	5.366	0.006	0.607	0.617
RGR-fm	3.235	<i>0.085</i>	42.95	< 0.001	1.683	0.197
RGR-L	7.277	0.013	15.590	< 0.001	2.365	<i>0.096</i>
DMC	0.015	0.902	27.831	< 0.001	1.779	0.178
B1) Plant traits of shoot A						
LMF**	5.415	0.029	3.530	0.030	0.396	0.757
LMF-a	13.335	0.001	13.249	< 0.001	1.278	0.304
SMF	12.329	0.002	32.794	< 0.001	1.065	0.382
RMF	0.309	0.583	36.651	< 0.001	0.242	0.866
LDMC	0.026	0.874	17.030	< 0.001	2.281	0.105
SDMC	0.512	0.481	48.158	< 0.001	3.358	0.036
RDMC*	24.906	< 0.001	49.897	< 0.001	1.478	0.246
B2) Pigments in leaves of shoot A						
Chl <i>a</i>	1.064	0.313	10.755	< 0.001	2.206	0.114
Chl <i>b</i>	3.375	<i>0.079</i>	9.630	< 0.001	1.870	0.162
Chl <i>a & b</i>	1.768	0.196	10.744	< 0.001	2.109	0.126
Chl <i>a/b</i> ratio	3.643	<i>0.068</i>	0.341	0.796	1.535	0.231
Car	3.462	<i>0.075</i>	2.718	<i>0.067</i>	9.629	< 0.001
Car/chl ratio	20.639	< 0.001	6.572	0.002	9.489	< 0.001
Anthocyanins	1.977	0.173	4.991	0.008	2.782	<i>0.063</i>
TPC	576.87	< 0.001	43.03	< 0.001	28.16	< 0.001
C1) Plant stoichiometry of leaves from shoot A						
C	0.934	0.346	6.610	0.002	0.997	0.411
N	1.258	0.273	2.730	<i>0.066</i>	1.784	0.177
P	3.093	<i>0.091</i>	0.825	0.493	0.407	0.749
CN	1.284	0.268	1.652	0.204	1.680	0.198
CP	2.080	0.162	1.146	0.351	0.441	0.726
NP	0.919	0.347	1.554	0.226	0.233	0.872
C2) Plant stoichiometry of shoot B						
C	5.848	0.024	0.573	0.638	7.242	0.001
N	0.164	0.689	5.518	0.005	2.302	0.101
P	2.837	0.105	3.808	0.023	2.988	<i>0.051</i>
CN	0.013	0.910	6.366	0.003	1.040	0.393
CP	6.196	0.020	4.722	0.010	2.510	<i>0.083</i>
NP	5.343	0.030	1.053	0.387	0.787	0.513

676

677

678 **Figure legends**

679 Fig. 1. Effect of arsenic and CO₂ availability (HC – high CO₂; LC – low CO₂) on the relative
680 growth rate of *Myriophyllum spicatum* based on dry mass (RGR-dm; A) or total shoot length
681 (RGR-L; B) and the dry matter content (DMC; C). Results are based on data for both shoots
682 A and B used per replicate, and represent means ± 1 SE from n = 4. Results from the Tukey
683 post-hoc tests following the 2-way ANOVA are shown as small letter code next to each
684 treatment. If no letters are shown for a response variable, treatments were not different to each
685 other. Note that y-axes do not start with zero but are adapted to the data range.

686 Fig. 2. Effect of arsenic and CO₂ availability (HC – high CO₂; LC – low CO₂) on plant traits,
687 here the biomass allocation to leaves, stems and roots (LMF, SMF, RMF). The proportion of
688 leaves on the total aboveground biomass is shown as LMF-a. Results are based on data for
689 shoots A, and represent means ± 1 SE from n = 4. Results from the Tukey post-hoc tests
690 following the 2-way ANOVA are shown as small letter code next to each treatment. If no
691 letters are shown for a response variable, treatments were not different to each other. Note that
692 y-axes do not start with zero but are adapted to the data range.

693 Fig. 3. Effect of arsenic and CO₂ availability (HC – high CO₂; LC – low CO₂) on leaf
694 pigments and relevant pigment ratios. A – Total chlorophyll content. B – Carotenoid content.
695 C – Chlorophyll a/b (chl a/b) ratio. D – Carotenoid/total chlorophyll ratio (Car/chl ratio).
696 Results are based on data for leaves from shoot A, and represent means ± 1 SE from n = 4.
697 Results from the Tukey post-hoc tests following the 2-way ANOVA are shown as small letter
698 code next to each treatment. If no letters are shown for a response variable, treatments were
699 not different to each other. Note that y-axes do not start with zero but are adapted to the data
700 range.

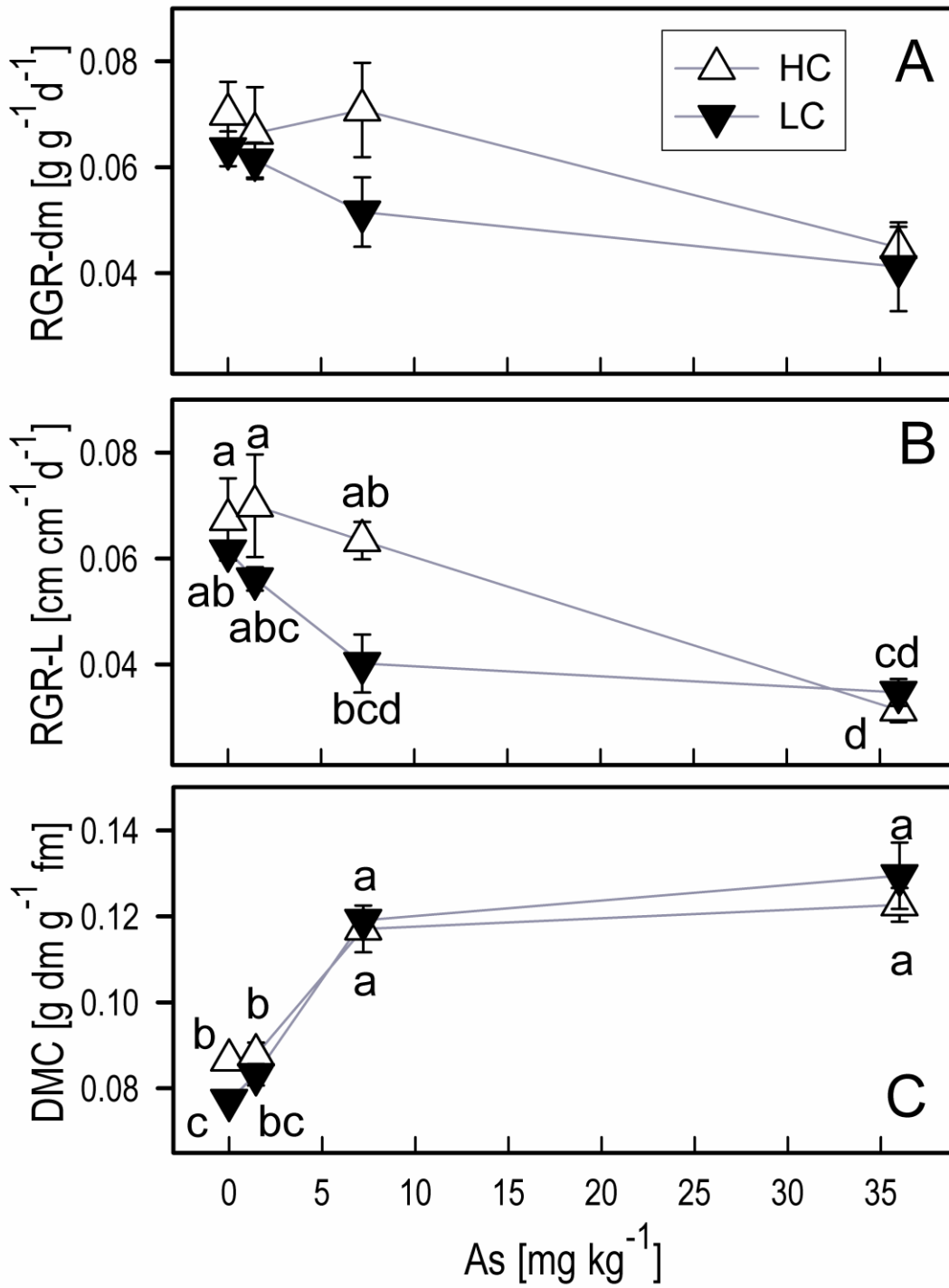
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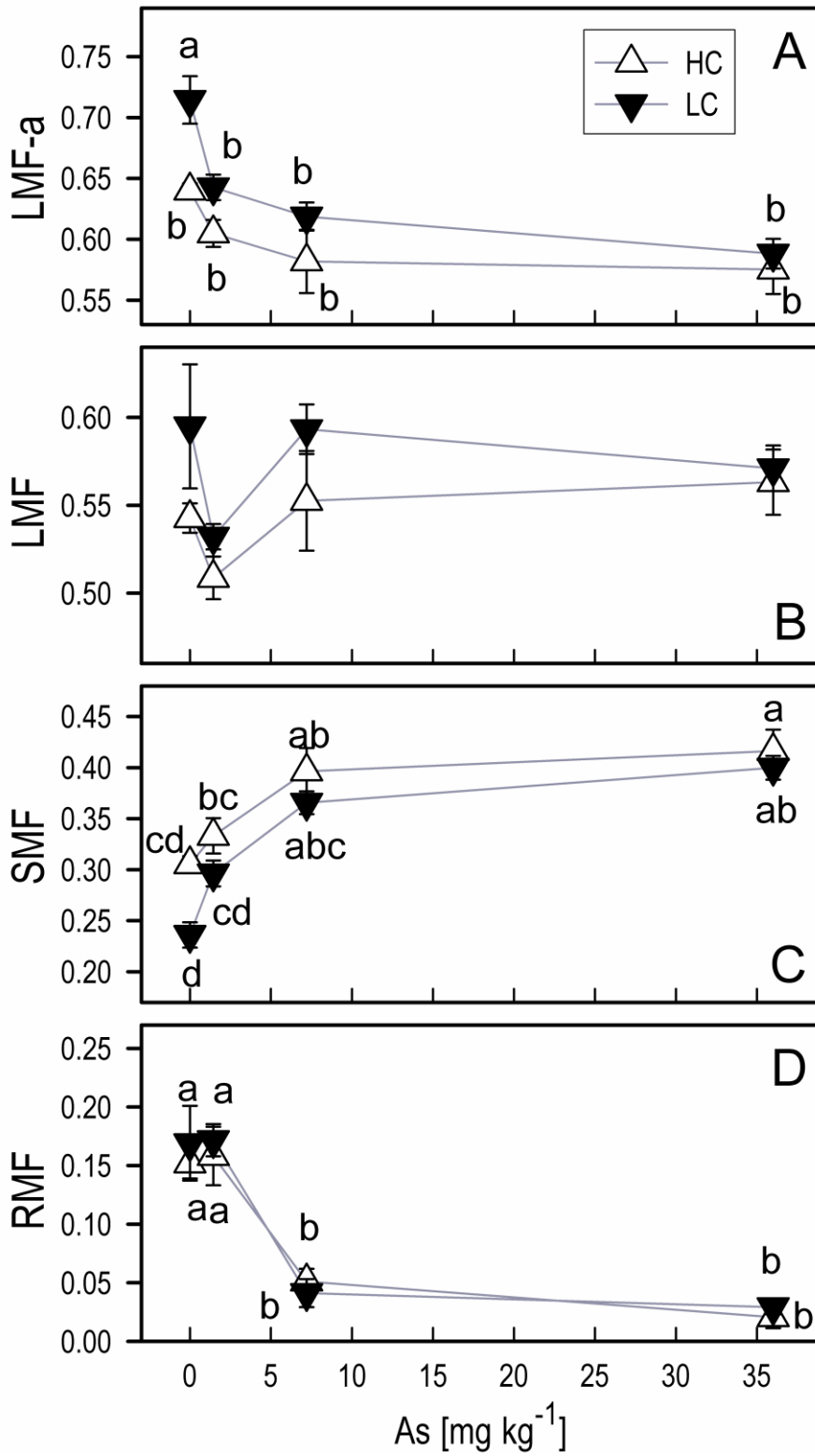
702 Fig. 4. Effect of arsenic and CO₂ availability (HC – high CO₂; LC – low CO₂) on leaf
703 phenolic compounds, here content in anthocyanins (A) and total phenolic compounds (TPC;
704 B). Results are based on data for leaves from shoot A, and represent means ±1 SE from n = 4.
705 Results from the Tukey post-hoc tests following the 2-way ANOVA are shown as small letter
706 code next to each treatment. If no letters are shown for a response variable, treatments were
707 not different to each other. Note that y-axes do not start with zero but are adapted to the data
708 range.

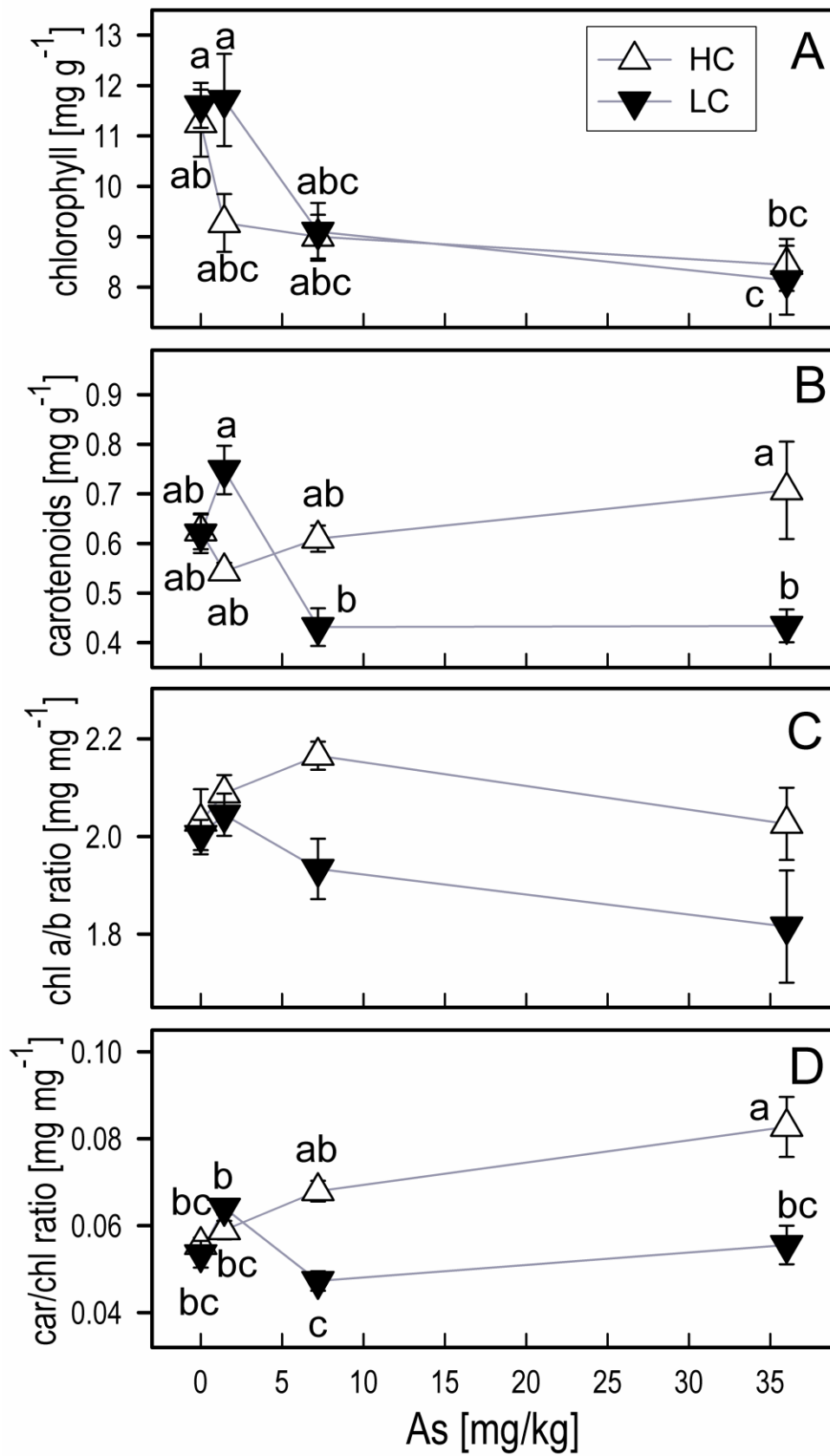
709
710 Fig. 5. Effect of arsenic and CO₂ availability (HC – high CO₂; LC – low CO₂) on elemental
711 composition, (panels A - C: Content in carbon – C; nitrogen – N; phosphorus – P) and
712 resulting molar ratios (leaf stoichiometry; molar ratios of panels D – F: C:N, C:P and N:P).
713 Results are based on data for leaves from shoots A and represent means ±1 SE from n = 4.
714 Results from the Tukey post-hoc tests following the 2-way ANOVA are shown as small letter
715 code next to each treatment. If no letters are shown for a response variable, treatments were
716 not different to each other. Note that y-axes do not start with zero but are adapted to the data
717 range.

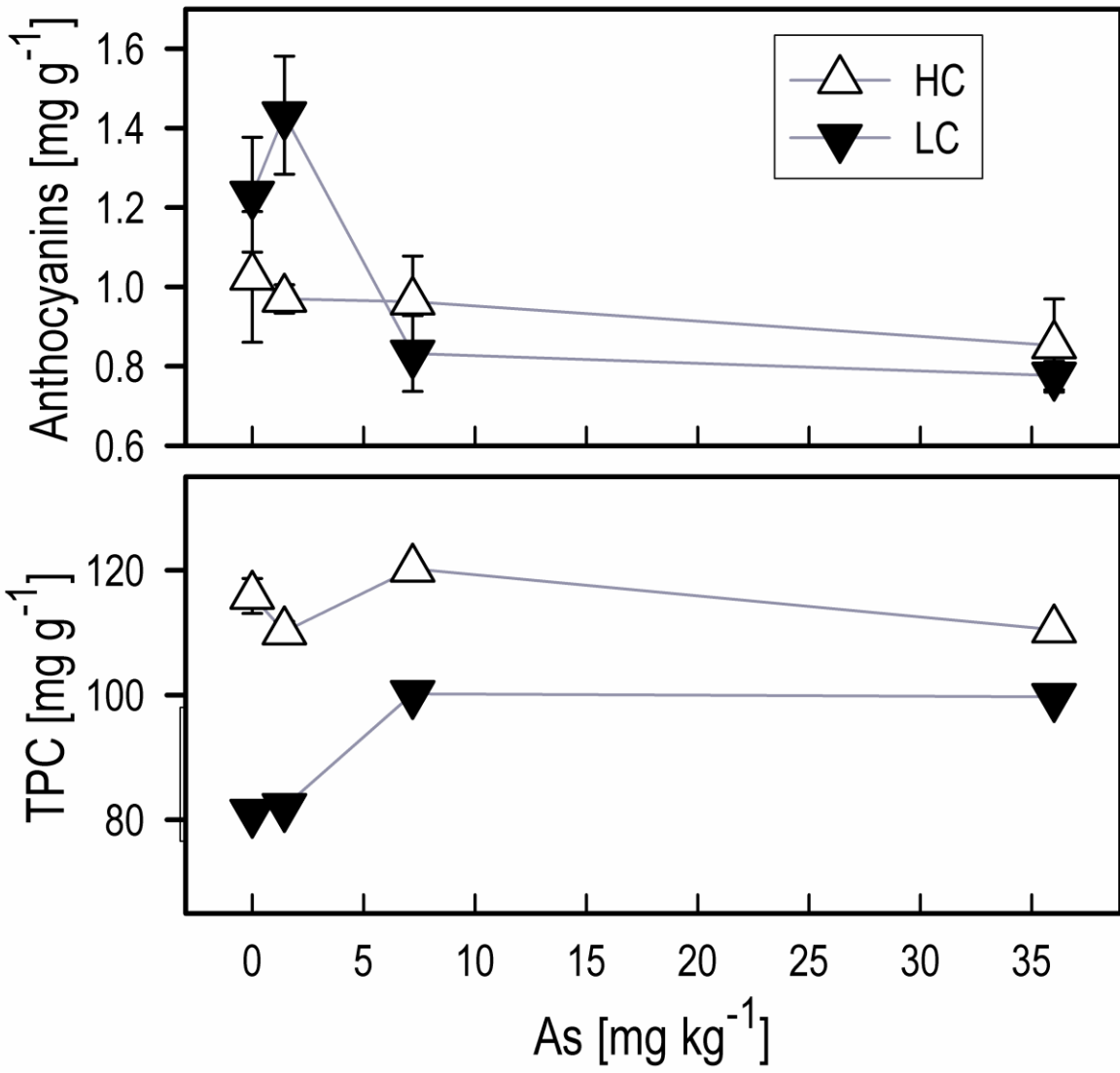
718
719 Fig. 6. Principal component analysis of the response of *Myriophyllum spicatum* to increasing
720 concentrations of arsenate in sediment (0, 1.44, 7 and 36 mg kg⁻¹), cultivated either in high or
721 low CO₂ availability (HC, LC), and after 14 days of exposure. A) Correlation circle showing
722 vectors for all measured endpoints (except DMC of whole shoots) as shown in Table 2 (see
723 list of abbreviations, aty - anthocyanins). B) PCA for all different treatment combinations.
724 Axes 1 and 2 explained 37.2 and 15.7 % of the total inertia.

725





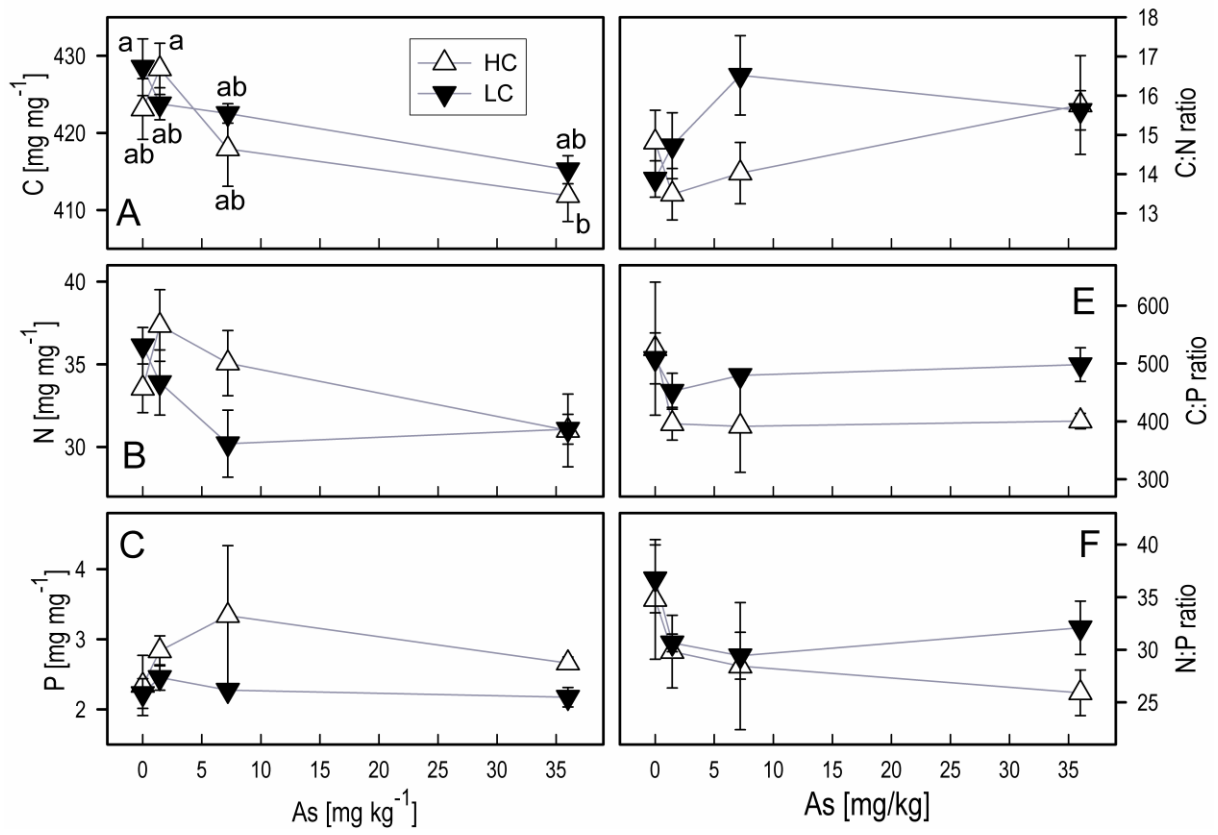




734

735

736 Fig. 5



737

738

