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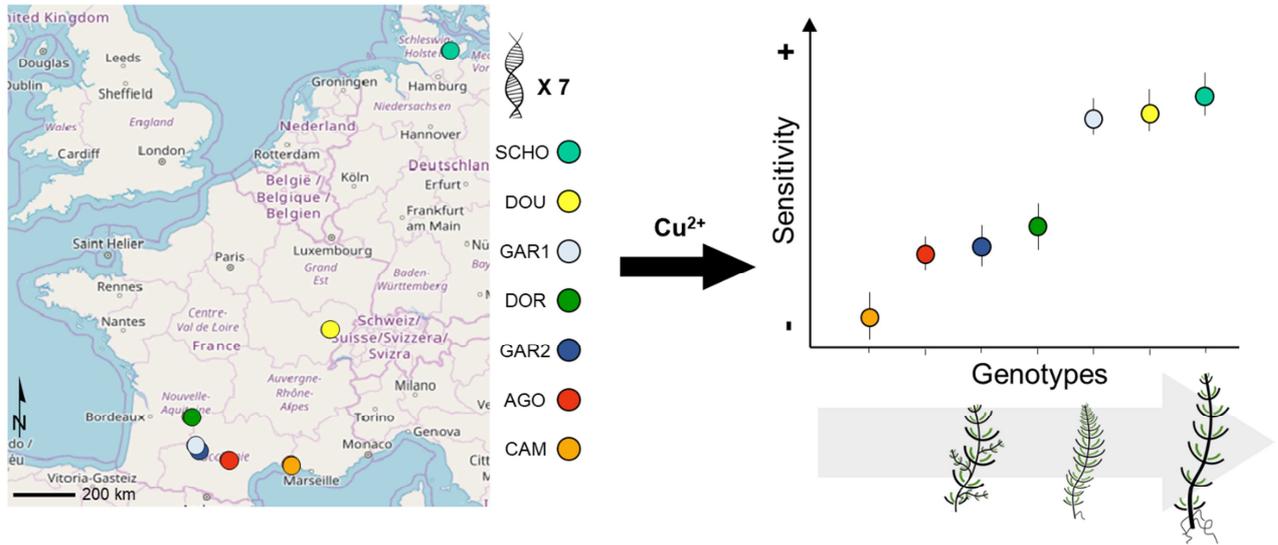
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## Graphical abstract



# Genotypes of the aquatic plant *Myriophyllum spicatum* with different growth strategies show contrasting sensitivities to copper contamination

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## 1 ABSTRACT

2 Genotypic variability has been considered for years as a key attribute in species adaptation to  
3 new environments. It has been extensively studied in a context of chemical resistance, but  
4 remains poorly studied in response to chemical exposure in a context of global change. As  
5 aquatic ecosystems are particularly affected by environmental changes, we aimed to study how  
6 genotypic variability could inflect the sensitivity of aquatic plants to chemicals. Seven genotypes  
7 of *Myriophyllum spicatum* were exposed to three copper concentrations at 0, 0.15 and 0.5 mg/L.  
8 The sensitivity of the different genotypes was assessed through several endpoints such as relative  
9 growth rate (RGR) and morphological traits, as well as physiological markers, such as plant  
10 biomacromolecular composition. Our results showed that genotypes exhibited significant  
11 differences in their life-history traits in absence of chemical contamination. Some trait syndromes

12 were observed, and three growth strategies were identified: (1) biomass production and main  
13 shoot elongation, (2) dry matter storage with denser whorls to promote resource conservation and  
14 (3) lateral shoot production. An up to eightfold difference in sensitivity for growth-related  
15 endpoints was observed among genotypes. Differences in sensitivity were partly attributed to  
16 morphological life-history traits. Our results confirm that genotypic variability can significantly  
17 affect *M. spicatum* sensitivity to Cu, and may influence the outcomes of laboratory testing based  
18 on the study of one single genotype. We recommend including genotypic variation as an  
19 assessment factor in ecological risk assessment and to study this source of variability more in  
20 depth as a possible driver of ecosystem resilience.

21

## 22 **1. Introduction**

23 Intraspecific variability has been investigated over the years as a key attribute in the  
24 adaptation and evolution of species (Bradshaw 1965; Matesanz et al. 2010; Oliver et al. 2015). It  
25 is acknowledged to play an important role in species adjustment to short or long term  
26 environmental shifts. Phenotypic plasticity is a response to short-term shifts, *i.e.* the ability of one  
27 genotype to produce several phenotypes depending on its environment (Sultan 2000; Whitman  
28 and Agrawal 2009). Adaptation to long term environmental changes occur *via* genotypic  
29 variation, *i.e.* the evolution of the genetic code due to somatic mutations, selection pressures and  
30 gene flow (Harris et al. 1992; Cao et al. 2017). Genotypic variation can sometimes confer  
31 specific life-history traits differing among populations (Reusch and Hughes 2006; Weyl and  
32 Coetzee 2016; Tóth et al. 2017). Recently, it has been shown that genotypic variation strongly  
33 influences the resilience of ecosystems after disturbances (Oliver et al. 2015; Ehlers et al. 2016;

34 Timpane-Padgham et al. 2017). Indeed, a broad gene pool will be more likely to provide the  
35 proper response to an environmental shift, such as changes in nutrient load, heat waves or  
36 chemical contamination (Reynolds et al. 2012). Conversely, a population exhibiting a low gene  
37 pool and not able to cope with a given environmental pressure may disappear from the  
38 ecosystem, whose dynamics and functions may drift due to the change in its species composition  
39 (Timpane-Padgham et al. 2017). As such, genotypic variation is considered as a driver of  
40 ecosystem functioning, since it will affect population resistance and resilience in variable  
41 environments (Woodward et al. 2010; Oliver et al. 2015; Timpane-Padgham et al. 2017).  
42 Differences in the sensitivity to various stressors among populations might increase over time due  
43 to their adaptation to contrasting environments through selective pressures (Brown et al. 2009;  
44 Dalton et al. 2013; Esteves et al. 2017).

45 Changes in environmental conditions are more likely to occur with human activities which  
46 inevitably impact ecosystems, notably via chemical contamination due to runoffs, wastewaters  
47 and atmospheric depositions (Ehlers et al. 2008; Oliver et al. 2015). In order to limit human  
48 impact on ecosystems, several policies have been implemented over the years, such as the E.U.  
49 Water Framework Directive, the E.U. REACH regulation, the U.S. Endangered Species Act or  
50 the U.S. Clean Water Act (Bouwma et al. 2018; Rouillard et al. 2018). Those policies are based  
51 on ecological risk assessment (ERA) which allows to assess potential impacts of stressors on  
52 ecosystems as a result of human activities. It first started in the 1970s and has evolved since,  
53 along with environmental policies and global awareness of human impact on the environment  
54 (Shea and Thorsen 2012; Bouwma et al. 2018). The realism and complexity of ERA increase as  
55 scientific breakthrough highlights different processes which may inflect the current approaches  
56 and their outcomes (Johnson and Sumpter 2016). It is difficult to assess properly via laboratory

57 testing the impact of a given molecule on its environment as many inherent and external factors  
58 will strongly influence the toxicity of chemical compounds, or the sensitivity of organisms.  
59 Among those factors, genotypic variation is currently not taken into account in ERA approaches.

60 Very few studies have looked into the impact of genetic variation in aquatic species  
61 sensitivity to chemicals. This is of concern, as this sensitivity might significantly vary among  
62 genotypes due to contrasting morphological or physiological traits, and ultimately among  
63 populations, depending on selection pressures having filtered out unfitted genotypes (Brown et al.  
64 2009; Dalton et al. 2013). Among those studies, one has investigated the resistance development  
65 in *Hydrilla verticillata* L.f. populations exposed to chemicals for weed control (Arias et al. 2005).  
66 Another has investigated the difference in sensitivity to pesticides in populations of  
67 *Myriophyllum spicatum* L. and hybrid populations with *M. sibiricum* kom. with resistant  
68 genotypes (Thum et al. 2012). More recently, we looked into the importance of genotypic  
69 variation of three aquatic macrophyte species exposed to Cu and found significant differences in  
70 sensitivity within species (Roubeau Dumont et al. 2019a).

71 To address the question of genotypic variation and its implication in species sensitivity to  
72 chemicals, we examined whether contrasting sensitivities to copper (Cu) exposure can be found  
73 across seven genotypes of *M. spicatum* potentially differing in their life-history traits. Copper is  
74 an essential trace element, which is involved in several metabolic pathways, such as reactive  
75 oxygen metabolism, photosynthesis and respiration (Thomas et al. 2013; Peñarrubia et al. 2015).  
76 It is also harmful beyond a species-specific physiological threshold, due to excessive reactive  
77 oxygen species (ROS) production which triggers cellular damages (Fidalgo et al. 2013; Costa et  
78 al. 2018). For example, Cu concentrations in leaves of terrestrial plants range between 5 to 20  $\mu\text{g}$   
79  $\text{g}^{-1}$  dry mass (Yruela 2009), and the toxicity threshold in leaves of crop species is generally above

80 20-30  $\mu\text{g g}^{-1}$  dry mass (Marschner and Marschner 2012). Copper is widely used in agriculture as  
81 fungicide, and is also released by industries and mining activities; thus high Cu concentrations  
82 can subsequently be found in top soils and aquatic ecosystems (Willis and Bishop 2016; Ballabio  
83 et al. 2018). According to a recent study, Cu concentration in European top soils is on average  
84 16.0  $\text{mg kg}^{-1}$ , and going up to 91.3  $\text{mg kg}^{-1}$  on average in French vineyards (Ballabio et al. 2018).  
85 *M. spicatum* originates from Eurasia, but is present now as invasive species in North America and  
86 South Africa (Weyl and Coetzee 2016). It is used as a model species for rooted aquatic plants in  
87 freshwater ecosystems and is the subject of two standardized protocols in ERA since 2014  
88 (OECD tests n°238 and n°239, OECD 2014a, b). Its genotypic variation and phenotypic  
89 plasticity have been widely documented regarding different populations and their genetic  
90 structure, or their plasticity toward environmental variations (Barko and Smart 1981; Hussner et  
91 al. 2009; Li et al. 2010; Cao et al. 2012; Weyl and Coetzee 2016; Tóth et al. 2017). In this study,  
92 we exposed seven genotypes of *M. spicatum* to three Cu concentrations and (1) assessed their  
93 difference in their life-history traits, (2) studied their difference in sensitivity through  
94 physiological and morphological endpoints and (3) linked their differences in life-history traits  
95 with their sensitivity.

96

## 97 **2. Material and methods**

### 98 **A. Growth and Cu exposure**

99 Six strains of *M. spicatum* (Haloragaceae) were randomly harvested in natural freshwater  
100 rivers in France between 2013 and 2016, and one strain of *M. spicatum* was regrown from an  
101 axenic culture established from material collected in Germany in 1990 (**Table 1, Figure S1**).  
102 Each strain was collected from a different waterbody, in order to increase the probability to

103 harvest different genotypes. All stock cultures were started from a single plant shoot to establish  
 104 clonal strains. Each strain was grown outdoor in a 210 L tank filled with tap water and quartz  
 105 sediment enriched with Osmocote® (granulated fertilizer, NPK 16-09-10, Hortensia, KB) during  
 106 at least six months prior to exposure experiments. For genotype distinction, inter simple sequence  
 107 repeat (ISSR) method was used (see section 2.B for further details). Prior to Cu exposure, each  
 108 shoot was cut to a length of 6 cm and rinsed in tap water before an acclimatization of 5 days in  
 109 Smart & Barko medium pH  $7.0 \pm 0.1$  (Smart and Barko 1981). Experimental units (15 cm height  
 110 and 9 cm diameter) contained 500 mL media with 50 mL of quartz sediment (0.5 - 2mm,  
 111 Castorama, France) enriched with 66.6 mg Osmocote®. For exposure, one apex was placed in  
 112 each experimental unit during 10 days, with 1 cm of the shoot placed in sediment for root  
 113 development. Temperature was set at  $23.0 \pm 0.1$  °C, and light intensity at  $153.0 \pm 2.5$   $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$   
 114 provided by fluorescent tubes (Grolux®, Sylvania Inc., Danvers, MA, USA), with a 14h/10h  
 115 day/night cycle. Three Cu concentrations were used: 0, 0.15 and 0.5 mg/L  $\text{Cu}^{2+}$  (*i.e.* 0, 2.38 and  
 116 7.94 mmol/L  $\text{Cu}^{2+}$ ), with five biological replicates per concentration.

117 **Table 1.** GPS coordinates and general characteristics of the French (F) and German (D) sites  
 118 from which the 7 genotypes of *Myriophyllum spicatum* were harvested between 2013 and 2016,  
 119 and 1991 for “SCH”.

Names	Streams	Waterbody	GPS coordinates	Eutrophication status	Land use
DOU	Doubs (F)	River	47.23153, 6.02252	Mesotrophic	Urban
DOR	Dordogne (F)	River cut-off channel	44.84584, 0.90596	Mesotrophic	Urban and agricultural
SCH	Schöhsee (D)	Lake	54.16624, 10.44114	Mesotrophic	Rural and agricultural
CAM	Camargue (F)	Drainage ditch	43.51133, 4.60584	Eutrophic	Agricultural
GAR1	Garonne (F)	River	44.02281, 1.07797	Eutrophic	Agricultural
GAR2	Garonne (F)	River	43.94622, 1.1711	Eutrophic	Agricultural
AGO	Agout (F)	River	43.6395, 2.31875	Mesotrophic	Natural parc

120 Copper sulfate was purchased from Sigma (Analytical grade, purity > 99%, CAS number  
121 7758-99-8, Sigma, Saint-Quentin-Fallavier, France). A concentrated solution of 1 g/L Cu<sup>2+</sup> was  
122 prepared in ultrapure water, and diluted in the different media before pH adjustments.

### 123 *B. Distinction of genotypes*

124 Inter simple sequence repeat (ISSR) molecular typing method (Zietkiewicz et al. 1994; Gupta  
125 et al. 1994) was used to verify that clonal strains corresponded to different genotypes. ISSR  
126 typing was conducted with 13 primers (**Table S1**) previously used with *M. spicatum* and other  
127 organisms (for details, see Roubeau Dumont et al. 2019a). DNA was extracted and purified with  
128 the WIZARD Genomic DNA Purification kit (Lyon, Promega France) from about 100 mg of  
129 fragments of apex plant samples stored at -20°C in Nuclei Lysis Solution (Lyon, Promega  
130 France) until DNA extraction. ISSR fragments amplification procedure, banding pattern analysis  
131 and calculation of genetic distances among strains were performed as in Carriconde et al. (2008)  
132 with adaptations detailed in supplementary material and in Roubeau Dumont et al. (2019a).

### 133 *C. Copper concentration in water samples*

134 Copper concentrations in the media were calculated by sampling water from experimental  
135 units at the beginning and at the end of Cu exposure, in order to assess effective concentrations at  
136 the beginning of exposure, and calculate time-averaged concentrations. Samples were acidified  
137 twice with one drop of 0.37M HNO<sub>3</sub>, prior to cold storage and before measurements using  
138 inductively coupled plasma with mass spectrometer (ICP-MS, 7500ce, Agilent, California, USA).  
139 Three technical replicates were realized for each vessel, with control samples and re-calibration  
140 every 12 measurements to ensure quality (reference material: SLRS-6 and EPOND waters,  
141 provided by the National Research Council of Canada and EPOND, Switzerland, respectively)

142 with a residual standard deviation of 4.02%. Effective Cu concentrations in the media were on  
143 average  $96.7 \pm 1.9$  % of nominal concentrations at the beginning of exposure. Time-averaged  
144 concentrations were  $54.7 \pm 0.6$  % and  $50.9 \pm 1.4$  % for 0.15 mg/L and 0.5 mg/L Cu respectively.

#### 145 *D. Life-history traits*

##### 146 1) Growth-related and morphological traits

147 The number of roots, lateral shoots, as well as the number of whorls over 5 cm from the  
148 bottom of the shoot, were recorded on the first and the last day of Cu exposure for each  
149 experimental unit. Stem width was measured 5 cm away from the shoot tip, using a digital sliding  
150 caliper. Shoot length based on main shoot and lateral shoots was measured at the beginning and  
151 at the end of exposure to calculate the Relative Elongation Rate (RER), based either on total  
152 shoot length (RER) or on main shoot length (RERms). Fresh mass was measured at the same time  
153 after having gently dried the plants with blotting paper, to calculate the relative growth rate  
154 (RGR). Samples were oven-dried at 70 °C during 72 h before weighting to measure the Dry  
155 Matter Content (DMC).

156 RGR was calculated for each experimental unit as follows:

$$RGR_{i-j} = (\ln(N_j) - \ln(N_i)) / t$$

157 where  $RGR_{i-j}$  is the relative growth rate from time  $i$  to  $j$ ,  $N_i$  and  $N_j$  are the endpoint (frond  
158 number, fresh mass) in the test or control vessel at time  $i$  and  $j$ , respectively, and  $t$  is the time  
159 period from  $i$  to  $j$ . RER and RERms were calculated following the same formula, replacing the  
160 fresh mass by the length of the main shoot (RERms) or total length (RER).

161 Dry matter content (DMC) in % was calculated as:

$$\%DMC = \left( \frac{100 \times DM}{FM} \right)$$

162 where FM is the fresh mass of plant samples, DM is their corresponding dry mass.

## 163 2) Biomacromolecule composition

164 Fourier Transform InfraRed spectroscopy (FTIR) was used to probe molecular vibrations in  
165 plant samples and thus gain information on their biochemical composition (lipids, carbohydrates,  
166 proteins). Samples were analysed using a FTIR microscope in attenuated total reflection (ATR)  
167 mode (Thermo Nicolet NEXUS 470 ESR, ThermoFisher™, Massachusetts, USA) over the  
168 frequency range of 4000 – 400 cm<sup>-1</sup> with a spectral resolution of 4 cm<sup>-1</sup>. One spectrum was an  
169 average of 64 scans per sample. Each powdered plant was placed on the sample plate and three  
170 independent technical replicates for each sample (5 biological replicates per treatment) were  
171 acquired. OMNIC software was used to export experimental spectra after Attenuated Total  
172 Reflectance (ATR) correction (OMNIC™ FTIR Software, ThermoFisher™, Massachusetts,  
173 USA). FTIR data treatment was performed using the Orange software (Demšar et al. 2013).  
174 Briefly, data were pre-processed which implies selection of the region of interest (including most  
175 of the variance among samples), vector normalization and smoothing by Savitzky-Golay filter.  
176 Using the second derivative, a principal component analysis (PCA) was carried out for graphical  
177 display (n=45). The components permitting to explain at least 70% of the variance were used to  
178 perform a subsequent linear discriminant analysis (LDA). This approach permitted to plot the  
179 samples and detect differences among groups of samples. When a difference was detected, a  
180 logistic regression was applied to the pre-processed data to identify wavenumbers contributing to  
181 the difference detected among groups by the PCLDA.

## 182 *E. Endpoints assessing Cu sensitivity*

183 1) Growth inhibition

184 The inhibition percentage of RGR was calculated for each treated plant to assess the  
 185 sensitivity of genotypes to Cu exposure regardless of their growth performance in absence of  
 186 chemical contamination, following the formula:

$$\%Ir = \left( \frac{\overline{RGRc} - RGR_t}{\overline{RGRc}} \right) \times 100$$

187 where %*Ir* is the inhibition percentage of the specific growth rate for a given individual plant  
 188 from the treatment group,  $\overline{RGRc}$ , is the average value for RGR in the control group and  $RGR_t$  is  
 189 the RGR value of the individual treated plant. Similar calculations were performed for both RER  
 190 and RERms.

191 2) Copper concentration in plants

192 Copper concentration in shoot at the end of Cu exposure was measured after acid digestion of  
 193 dry plant material, which occurred in two steps. First, plant material (at least 20 mg per sample)  
 194 was heated at 94°C during 30 minutes in a 1:1 ratio of deionized water: HNO<sub>3</sub> (65%), then  
 195 allowed to return to room temperature. Second, the samples were brought to a 2:1 ratio of HNO<sub>3</sub>  
 196 (65%):H<sub>2</sub>O<sub>2</sub> (30%) and heated at 94°C during 2 hours. Once the temperature cooled down to  
 197 room temperature, samples were filtered with a 0.45 µm filter and diluted to obtain a final HNO<sub>3</sub>  
 198 concentration of 2%. Samples were measured using ICP-MS with helium gas (7500ce, Agilent,  
 199 California, USA), with three technical replicates per sample. Quality control was achieved with  
 200 the use of control (only chemicals to test their purity) and reference material (white cabbage,  
 201 certified reference material BCR® – 679, NIVA17 and EPOND waters, provided by the  
 202 Norwegian Institute for Water Research and EPOND, Switzerland, respectively). Quality

203 assurance was ensured with a full recalibration every 12 measurements (residual standard  
204 deviation of 2.34%).

#### 205 *F. Statistical analyses*

206 Results were analysed using the R studio software (R Core Team (2016) V 3.3.1) to assess  
207 the effects of genotypes on plant traits with or without Cu. Homoscedasticity was tested using  
208 Bartlett test. Data normality was assessed with Shapiro-Wilk test on ANOVA residuals, with log-  
209 transformation when normality assumption was not met with raw data. In control plants, one-way  
210 ANOVAs were performed to assess the effect of genotypes on life-history traits. The relationship  
211 among the different life-history traits was assessed through PCA (8 variables,  $n=35$ ) using the  
212 FactoMineR package (Lê et al. 2008). The interactive effect of genotype and Cu concentration  
213 during exposure was assessed through two-way ANOVAs on results showing normal  
214 distribution, with or without log transformation. Tukey HSD post-hoc tests were used to identify  
215 significant differences among Cu concentrations and among genotypes. Generalized linear  
216 models (GLMs) with gamma distribution were used to assess interactions in dataset showing no  
217 normality despite log-transformation. The link between life-history traits and Cu response was  
218 determined with co-inertia analysis, with the RV coefficient indicating the degree of co-structure  
219 between the two matrices (MASS package, Venables and Ripley 2003). The differences in plant  
220 responses among genotypes and Cu concentrations were analysed with linear discriminant  
221 analysis (LDA,  $n=105$ , ade4 package, Dray and Dufour 2007). The significance of co-inertia and  
222 discriminant analyses were assessed with Monte-Carlo tests with 1000 repetitions. A summary of  
223 the analyses performed on the different endpoints can be found in Table S2.

224

### 225 **3. Results**

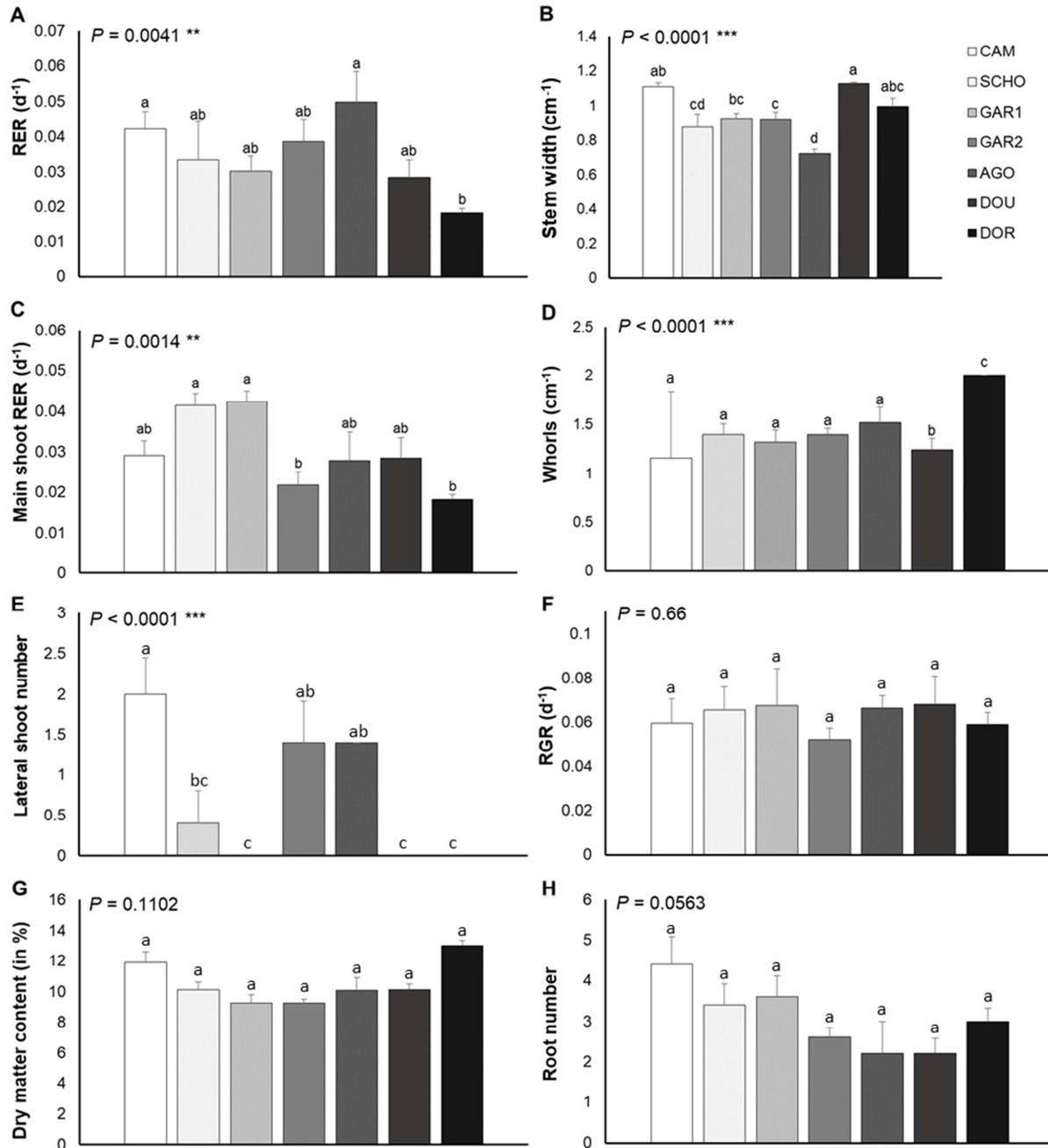
226 A. Genetic differentiation of the 7 genotypes

227 ISSR amplifications performed with the 7 *M. spicatum* studied strains yielded a total of 88  
228 clear and reproducible fragments of which 39 were polymorphic. Comparisons of resulting ISSR  
229 profiles and analyses among strain genetic relationships showed that each clonal strain  
230 genetically differed from the others, and that strains thus corresponded to different genotypes  
231 (**Figure S2**).

232 B. Differences in life-history traits among genotypes

233 In absence of Cu exposure, the studied genotypes showed differences in their life-history  
234 traits, with 5 of the 8 traits considered being significantly different among genotypes (**Figures**  
235 **1A, B, C, D and E**). Some genotypes were significantly more efficient than others regarding  
236 RER (1-way ANOVA,  $F_{6,28} = 4.156$ ,  $P = 0.0041$ ), with the “AGO” genotype having a RER of  
237  $0.049 \text{ d}^{-1}$ , compared to  $0.018 \text{ d}^{-1}$  for the “DOR” genotype (**Figure 1A**). Stem width exhibited  
238 significant differences among genotypes (1-way ANOVA,  $F_{6,28} = 11.55$ ,  $P < 0.0001$ ), with the  
239 genotypes “CAM”, “DOR” and “DOU” exhibiting a stem diameter  $> 1 \text{ mm}$ , against  $< 0.8 \text{ mm}$  for  
240 “AGO” (**Figure 1B**). Main shoot elongation differed significantly among genotypes as well (1-  
241 way ANOVA,  $F_{6,28} = 4.987$ ,  $P = 0.0014$ ), with “GAR1” having a RERms of  $0.042 \text{ d}^{-1}$ , against  
242  $0.018 \text{ d}^{-1}$  for the “DOR” genotype (**Figure 1C**). The density of whorls also significantly differed  
243 from one genotype to another, ranging from 1.2 whorls/cm (DOU) to 2.0 whorls/cm (“DOR”) (1-  
244 way ANOVA,  $F_{6,28} = 47.97$ ,  $P < 0.0001$ , **Figure 1D**). The number of lateral shoots varied among  
245 genotypes, some producing none (1-way ANOVA,  $F_{6,28} = 7.268$ ,  $P < 0.0001$ , **Figure 1E**).  
246 Overall, the “DOR” genotype exhibited the lowest elongation rate but the highest whorl density,  
247 whereas the “GAR1” showed the highest main shoot elongation rate but produced no lateral

248 shoot. RGR, DMC and root number, did not significantly vary among genotypes (**Figure 1F, G**  
 249 and **H**, respectively).



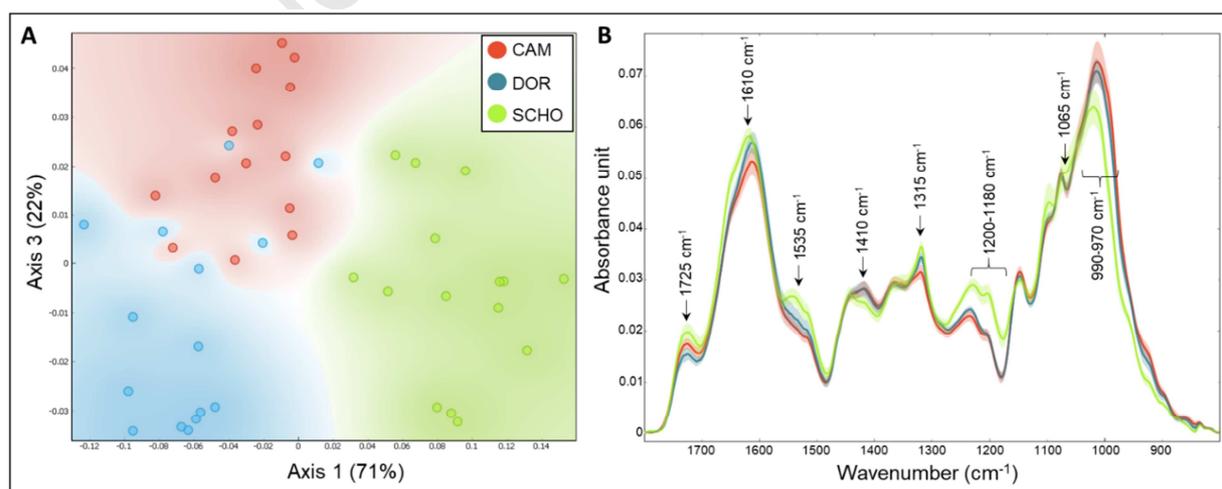
250 **Figure 1.** Life-history traits measured on 7 genotypes of *Myriophyllum spicatum* in absence of  
 251 copper contamination. The names of the different genotypes are listed in (B) where “CAM” =  
 252 Camargue, “SCHO” = Schöhsee, “GAR1/2” = Garonne, “AGO” = Agout, “DOU” = Doubs,  
 253 “DOR” = Dordogne, and their geographic origin can be found in Figure S1. Relative Elongation

254 Rate (RER) based on total shoot length (A), Stem width (B), main shoot elongation (RERms)  
255 (C), Whorls density (D), lateral shoot number (E), Relative Growth Rate (RGR) based on fresh  
256 mass (F), Dry Matter Content (DMC) (G) and root number (H) were measured. 1-way ANOVA  
257 P-values for genotype effects are provided (HSD Tukey test),  $n = 5$ .

258

259 Three genotypes with contrasting life-history traits, “SCHO” (high RERms), “DOR” (high  
260 whorls/cm and DMC) and “CAM” (high LS and Stem width), were used to assess differences in  
261 biomacromolecule composition through FTIR. Significant differences were found in their  
262 functional groups (Figure 2). The PCLDA highlighted differences between “SCHO” genotype  
263 and the two others mainly along the first axis, with the first 3 dimensions explaining 93% of the  
264 total dataset inertia. Axis 1 and 3 were chosen for graphical display to highlight differences  
265 among genotypes (Figure 2A). Logistic regression for spectrum analysis fitted the data with an  
266 Area Under Curve (AUC) value of 0.992, and further analysis in the spectra showed 8 main  
267 peaks with significant differences among genotypes in the fingerprint region (1800-800  $\text{cm}^{-1}$ ,  
268 Figure 2B). An inverse pattern was observed between “SCHO” and “CAM” genotypes, with  
269 either “SCHO” exhibiting the highest peaks and “CAM” the lowest, or the opposite. The first  
270 peak around 1725  $\text{cm}^{-1}$  corresponds to C=O bonds from carboxylic acids and esters from various  
271 polysaccharides and fatty acids (Dokken et al. 2005; Ibrahim et al. 2015). The second peak, from  
272 1650-1610  $\text{cm}^{-1}$ , was attributed to C=O stretching bonds from carbonyl, C=C of aromatic ring  
273 vibrations (Duarte et al. 2005; Qu et al. 2013; Regvar et al. 2013) and C=O stretching from  
274 proteins (Regvar et al. 2013; Bind et al. 2018). This peak slightly shifted toward higher  
275 wavenumbers for “SCHO” genotype compared to the 2 others, with a peak area of 3.13, against  
276 2.97 and 2.81 for “DOR” and “CAM”, respectively. The same pattern was observed for the broad  
277 peak around 1535  $\text{cm}^{-1}$  which was the highest for “SCHO”, and was attributed to proteins (amide  
278 II bands) through N and N-H vibrations, and aromatic systems through C=C vibrations (Duarte et

279 al. 2005; Regvar et al. 2013). The peak around  $1410\text{ cm}^{-1}$  was the lowest for “SCHO” and was  
280 assigned to amine groups from proteins (Dokken et al. 2005), phenolic O-H compounds (Qu et al.  
281 2013; Ibrahim et al. 2015), various polysaccharides from cellulosic compounds and O-H bending  
282 vibrations from carboxylic acid groups (Regvar et al. 2013). The sharp peak around  $1315\text{ cm}^{-1}$   
283 was the highest for “SCHO” and was identified as C-O, C-H and C-N stretching vibrations  
284 (Regvar et al. 2013; Ibrahim et al. 2015) in polysaccharides, mainly from cellulosic compounds.  
285 The broad peak at  $1200\text{-}1180\text{ cm}^{-1}$  was assigned to C-O stretching vibrations from alcohol and  
286 esters, proteins (amide III) mainly from polysaccharides in cellulosic compounds (Bonetta et al.  
287 2002). It was the highest for “SCHO” genotype, with a peak area of 0.70, against 0.46 for the two  
288 others. The peak at  $1065\text{ cm}^{-1}$  was assigned to S=O stretching bonds from sulfoxide, C=O from  
289 polysaccharides and esters (Regvar et al. 2013). Finally, the peak at  $990\text{-}970\text{ cm}^{-1}$  was the lowest  
290 for “SCHO” (peak area of 3.31 against 3.89 and 4.1 for “DOR” and “CAM”, respectively) and  
291 assigned to alkene (Arun et al. 2016), alcoholic and C-O stretching vibrations (Qu et al. 2013), as  
292 well as polysaccharides such as cellulose, hemicellulose and starch compounds (Regvar et al.  
293 2013).



295 **Figure 2.** PCLDA analysis of the Fourier-transformed infrared spectra from 3 genotypes of  
 296 *Myriophyllum spicatum*, “CAM” = Camargue, “DOR” = Dordogne and “SCHO” = Schöhsee,  
 297 exhibiting differences in life-history traits (A), with significant differences among genotypes  
 298 identified by a logistic regression marked with black arrows on FTIR spectra (B),  $n = 45$ .

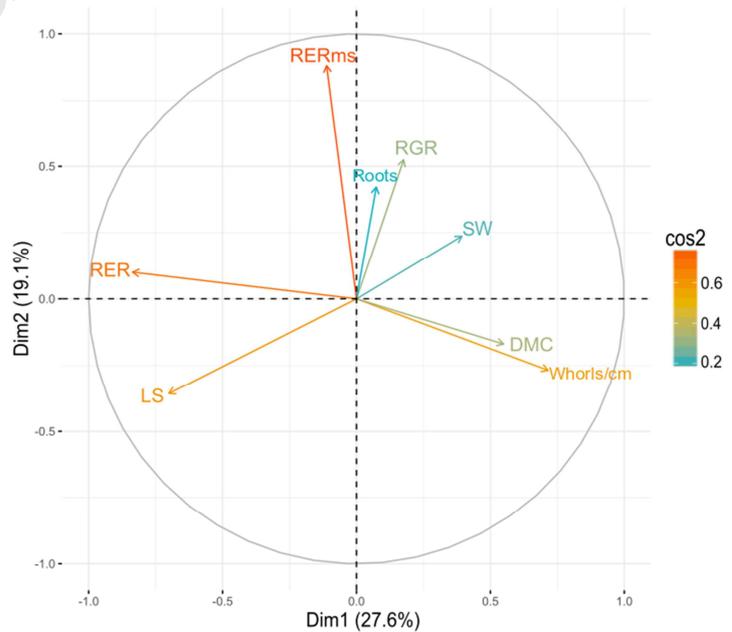
299

### 300 C. Relationships among life-history traits

301 The relationships among life-history traits were assessed through PCA on control plants and  
 302 several trends were observable (**Figure 3**). Three main clusters were represented: Stem width was  
 303 correlated to DMC and whorls/cm, and anti-correlated to Lateral shoot and RER, which were  
 304 again positively correlated. RGR was clustered with RERms and root number, although the last  
 305 one was poorly represented in the factorial map. RERms was the main contributor to the first  
 306 axis, whereas RER was the main contributor of the second axis.

307

308 **Figure 3.** Principal Component  
 309 Analysis performed on 7 life-history  
 310 traits for 7 genotypes of *Myriophyllum*  
 311 *spicatum* in absence of Cu  
 312 contamination. Relationships between  
 313 the 8 variables Relative Growth Rate  
 314 (RGR), Relative Elongation Rate  
 315 (RER), RER based on main shoot  
 316 (RERms), whorl number per cm  
 317 (whorls\_cm), Dry Matter Content  
 318 (DMC), root number (Roots) as well as  
 319 lateral shoot number (LS) and Stem  
 320 width (SW) were assessed. Color  
 321 gradient represents the goodness of  
 322 representation of each variable in the  
 323 factorial map defined by axes 1 and 2,  $n$   
 324 = 35.



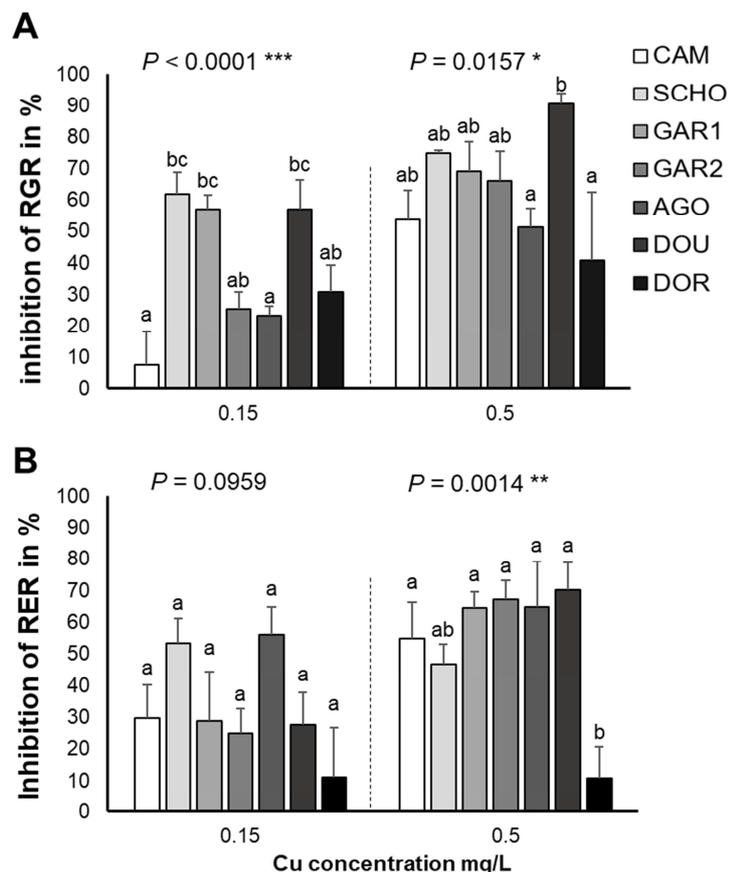
325

326 *D. Copper impact on M. spicatum: general patterns and intraspecific variation*

327 Copper exposure negatively affected all genotypes with a concentration-dependent effect on  
 328 growth related endpoints, and sensitivity to Cu significantly differed among genotypes (**Figure**  
 329 **4**). Sensitivity at 0.15 mg/L Cu varied up to 8 times for RGR, with biomass production inhibited  
 330 from 7 % for “CAM” to 62 % for “SCHO”. At 0.5 mg/L, the range of inhibition slightly  
 331 decreased, and growth was inhibited from 40 % for “DOR” to 90 % for “DOU” (**Figure 4A**).  
 332 RER showed significant variations at 0.5 mg/L Cu with differences in sensitivity up to 7 times  
 333 (**Figure 4B**). Elongation inhibition ranged from 11 % for “DOR” to 70 % for “DOU” at 0.5  
 334 mg/L. Generally, the “DOU” genotype was the most impacted genotype at high Cu  
 335 concentration, and “DOR” the least impacted, while this genotype exhibited the lowest RER  
 336 without Cu exposure. RERms was affected by Cu the same way as RER, and its sensitivity was  
 337 similar, with at 0.5 mg/L “DOR” being the least impacted, and “DOU” being seven-fold more  
 338 impacted (supplementary data **S3**).

339

340 **Figure 4:** Copper sensitivity of  
 341 7 genotypes of *Myriophyllum*  
 342 *spicatum*, assessed through the  
 343 inhibition of their Relative Growth  
 344 Rate (RGR) (**A**) and Relative  
 345 Elongation Rate based on all  
 346 shoots (RER) (**B**) during a 10-day  
 347 exposure. The names of the  
 348 different genotypes are listed in  
 349 (**A**), where “CAM” = Camargue,  
 350 “SCHO” = Schöhsee, “GAR1/2” =  
 351 Garonne, “AGO” = Agout,  
 352 “DOU” = Doubs, “DOR” =  
 353 Dordogne, and their geographic  
 354 origin can be found in Figure **S1**.



355 ANOVA P-values for genotype effects are provided; genotypes with same letters for a given  
356 combination endpoint x Cu concentration are not significantly different in terms of copper  
357 sensitivity (HSD Tukey test),  $n = 5$  for each combination.

358

359 Significant differences in Cu accumulation in plant tissues were found at 0.5 mg/L Cu, which  
360 ranged from 2.68 mg Cu/g DW for “GAR1” to 5.18 mg Cu/g DW for “SCHO”, which  
361 accumulated on average 1.5-fold more Cu than other genotypes (supplementary data **S4**). The  
362 increase in Cu concentration in plants was tightly correlated with Cu in the exposure media ( $r^2$  of  
363 0.96, Supplementary data **S4**).

364 The LDA revealed that Cu, Genotypes and their interactions significantly influenced life-  
365 history traits (Monte-Carlo test,  $P = 0.001$ , **Figure 5**, 43.7% of inertia explained). Copper  
366 concentrations expressed 14 % of inertia, whereas Genotypes expressed 18.8 % of inertia. The  
367 interactions between Cu concentrations and Genotypes explained 10.9 % of inertia, and were  
368 consistent with the results in **Fig. 4** showing that there was a significant difference in Cu  
369 sensitivity among genotypes. The link between the life-history traits of genotypes and their  
370 sensitivity to Cu revealed that the studied life-history traits explained 63.2 % of the response  
371 variables; however, it was not statistically significant according to the Monte-Carlo test (Monte-  
372 Carlo,  $P = 0.1189$ ).

373

#### 374 **4. Discussion**

##### 375 *A. Intraspecific trait variability and trait syndromes*

376 Our results show that the different genotypes of *M. spicatum* significantly differed in their  
377 morphology traits, their growth abilities and their biomacromolecule composition. Whorl density,

378 stem width, lateral shoot production, as well as total shoot and main shoot elongation greatly  
379 varied among genotypes when no chemical stress was applied. It has been acknowledged in  
380 numerous studies that plant individuals can exhibit contrasting morphologies depending on their  
381 environment, both through phenotypic plasticity and genotypic variation (Wersal and Madsen  
382 2013; Arshid and Wani 2013; Ganie et al. 2015; Weyl and Coetzee 2016; Grenier et al. 2016;  
383 Tóth et al. 2017, Roubeau Dumont et al. 2019b). In our case, the plants were grown during at  
384 least 6 months in a similar environment prior to the experiment, and genotype morphologies  
385 never converged, indicating that genotypic variation was the main source of variation. Five of the  
386 seven genotypes were harvested from different streams, which were not connected, increasing the  
387 probability to get clonal strains corresponding to different genotypes, due to genetic  
388 heterogeneity among populations (Harris et al. 1992; Chen et al. 2009; Cao et al. 2017). The  
389 ISSR analysis confirmed that clonal strains were indeed different genotypes, even for the two  
390 Garonne strains.

391 Biomacromolecule composition significantly differed among genotypes, especially for  
392 “SCHO” and “CAM” which exhibited opposite peak intensities. Spectrum analyses showed that  
393 “SCHO” genotype had a relatively higher content of fatty acid compounds, proteins (amide III)  
394 and cellulosic compounds, compared to the 2 other genotypes, suggesting a higher investment in  
395 growth processes (Bashline et al. 2014). In contrast, the 2 other genotypes, especially “CAM”  
396 exhibited higher contents in phenolic compounds, hemicellulose and starch compounds, which  
397 have been shown to vary in other species (Howard et al. 2003; Mpofu et al. 2006). This suggests  
398 differences in cell wall structure and starch storage, hence in resource conservation (Elger and  
399 Willby 2003), although further experiments are required to confirm these hypotheses.

400 Several trends among life-history traits were observed as traits syndromes (*e.g.* link between  
401 DMC and whorl density), and different growth strategies were drawn from these observations. A  
402 morphology with (1) numerous, dense whorls, likely promoting nutrient absorption and possibly  
403 light, (2) an architecture with a longer internode which allows to be closer to the light source  
404 through lateral shoot production to form a canopy or (3) an elongation of the main shoot to  
405 increase both light harvesting and nutrient absorption (Liu et al. 2017).

#### 406 *B. Variations in Cu sensitivity*

407 Strong differences in sensitivity to Cu were observed among genotypes, with up to eightfold  
408 difference in the inhibition range of growth-related endpoints. Those differences decreased along  
409 with an increasing Cu concentration, suggesting that at 0.5 mg/L Cu all the genotypes could no  
410 longer cope with this stress. The differences in sensitivity to Cu were partly (62%) attributed to  
411 the variations in life-history traits among genotypes, although the co-inertia analysis was not  
412 significant, most likely due to the low number of genotypes considered. Biomacromolecule  
413 composition and metabolic traits, such as antioxidant compounds and enzymatic activities, may  
414 better explain the differences in Cu sensitivity (Singh et al. 2016). Indeed, visual observations  
415 showed a difference in pigment composition among genotypes (in supplementary data **S5**), which  
416 has been previously proved to influence photosynthetic efficiency, antioxidant balance and metal  
417 interactions (Sivaci et al. 2007; Zvezdanović and Marković 2009; Delmail et al. 2010; Brewer  
418 2011 Thomas et al. 2016; Borisova et al. 2017). The biomacromolecule composition analysis also  
419 revealed some significant variations in phenolic compounds, which may be directly linked to a  
420 difference in sensitivity among genotypes (Gross et al. 1996; Gross 2001; Das and Roychoudhury  
421 2014). Further investigations with more focus on specific metabolic changes would be required to  
422 identify properly the origin of the differences in chemical response among genotypes

423 (Pflugmacher et al. 1997; Kanoun-Boulé et al. 2009; Fidalgo et al. 2013; Thomas et al. 2013).  
424 Epigenetic mechanisms are also involved in stress response in plants, although few studies have  
425 investigated their impact in aquatic plants as a way to cope with abiotic stressors (Regier et al.  
426 2013; Gamain et al. 2017). Our results highlight the need to go further into the mechanisms  
427 explaining aquatic plant responses and adaptation to environmental factors, including chemical  
428 stressors.

#### 429 *C. Implications of intraspecific variation for environmental risk assessment*

430 The genotypes used in these experiments were coming from relatively similar aquatic  
431 environments in terms of nutrient loads, climate and environmental pollution, thus decreasing the  
432 probability to harvest very different genotypes undergoing strong selection pressures (Weyl and  
433 Coetzee 2016). Our results have shown that the genotypic variation detected in *M. spicatum* may  
434 influence the outcomes of toxicity laboratory tests, with up to eightfold growth inhibition  
435 differences among genotypes. This implies that depending on the genotype used in the laboratory,  
436 the threshold value modeled from single-species laboratory assays may not be protective enough  
437 to ensure the absence of harmful effects on living organisms in this compartment. Hence, a given  
438 genotype might not be representative of the species sensitivity, and might mislead the following  
439 steps of ERA (Clark et al. 1999; Johnson and Sumpter 2016; Roubeau Dumont et al. 2019a). It is  
440 therefore crucial to take into account the different sources of variation that may influence the  
441 outcomes of laboratory testing in order to ensure their reliability.

#### 442 *D. Ecological implications of intraspecific variation*

443 Intraspecific variability has been recognized for decades as a promoter of diversity and as a  
444 condition for coexistence of species within communities (Bolnick et al. 2011). According to

445 Albert *et al.* (2011), intraspecific variability should be taken into account in small-scale studies  
446 like single-species and mesocosm approaches, as it is higher than interspecific variability.  
447 Linking ecosystems and species relies on approaches that consider how species traits affect  
448 ecosystem processes (Bolnick *et al.* 2011). Cianciaruso *et al.* (2009) have shown that there was  
449 no relationship between plant species richness and functional diversity, as intraspecific variability  
450 in functional traits was very high. They concluded that assessing intraspecific variation would  
451 provide better predictions in species extinction consequences for ecosystem processes. Several  
452 studies have investigated phenotypic plasticity. For instance, Kremp *et al.* (2012) highlighted its  
453 importance in diatom response to climate change and thus in ecosystem resilience. Similarly,  
454 Wolf *et al.* (2018) studied arctic diatoms which adapted to climate change through plastic  
455 responses, and showed very high variability in their sensitivity. Roubeau Dumont *et al.* (2019b)  
456 showed that environmental fluctuations could significantly influence aquatic plant ability to cope  
457 with chemical exposure. Very few studies have focused on the role of genotypic variation when it  
458 comes to chemical exposure (Dalton *et al.* 2013; Roubeau Dumont *et al.* 2019a). It is critical to  
459 do so in the current context, in which environmental pollution increases and threatens  
460 ecosystems, to assess how such variation may influence species sensitivity and ecosystem  
461 resilience (Oliver *et al.* 2015). Our results have shed light on the importance of genotypic  
462 variation in the sensitivity of *M. spicatum* to Cu, demonstrating that individuals can exhibit  
463 significantly different sensitivities, depending on their traits. Although we did not measure the  
464 genetic variation within populations, our results give insights on the increased resilience potential  
465 that a broad genetic diversity could provide to an ecosystem exposed to chemicals (Oliver *et al.*  
466 2015).

467

468 **5. Conclusion**

469 We assessed the importance of genotypic variation in the intraspecific variability of *M.*  
470 *spicatum* sensitivity to Cu, and showed up to eightfold difference in sensitivity among genotypes.  
471 ERA is an always evolving process along with scientific discoveries, in order to increase the  
472 relevance of environmental regulations. As such, we highlighted that genotypic variation is a  
473 source of variation in species sensitivity to chemicals, and we showed that life-history traits are  
474 partly linked to these differences in sensitivity. We call for further studies to be conducted to  
475 understand the underlying mechanisms, with a focus on physiological endpoints and epigenetic  
476 changes.

477

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486

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Genotypic variability has the potential to influence species sensitivity to chemicals

*M. spicatum* exhibits different growth strategies depending on the genotypes

Up to eightfold differences in Cu sensitivity were found among the genotypes tested

Variations in chemical sensitivity are linked to differences in life-history traits

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Arnaud Elger, Eva Roubeau Dumont and Camille Larue conceived and designed the experiments. Eva Roubeau Dumont performed the experiments, Hervé Gryta performed the ISSR analyses, Hiram Castillo Michel helped to perform FTIR analyses and data treatment along with Clarisse Liné. David Baqué helped to perform ICP-MS analyses. Eva Roubeau-Dumont, Arnaud Elger, Camille Larue, Elisabeth Maria Gross together wrote the paper.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: