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1 Does intraspecific variability matter in ecotoxicological risk assessment?

2 Investigation of genotypic variations in three macrophyte species exposed to 3 copper

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15 ABSTRACT

16 To limit anthropogenic impact on ecosystems, regulations have been implemented along with
17 global awareness that human activities were harmful to the environment. Ecotoxicological risk
18 assessment is the main process which allows to assess the toxicity potential of contaminants,
19 through different steps of laboratory testing. This process evolves along with scientific
20 knowledge, to better predict the impact on an ecosystem. In this paper we address the
21 importance of intraspecific variability as a potential source of error in the laboratory evaluation
22 of harmfulness of pollutants. To answer this question, three aquatic macrophyte species with
23 different life-history traits were chosen to cover the main life-forms found in aquatic
24 ecosystems, *Lemna minor* and *Myriophyllum spicatum*, two OECD model species, and
25 *Ceratophyllum demersum*. For each species, three or four genotypes were exposed to 7-8 copper
26 concentrations. To assess species sensitivity, growth-related endpoints such as Relative Growth
27 Rate (RGR), based either on biomass production or on length/frond production, and chlorophyll
28 fluorescence $F_v:F_m$, were measured. For each endpoint, EC_{50} was calculated. Our results
29 showed that all endpoints were affected by Cu exposure, $F_v:F_m$ of *M. spicatum* excepted, and
30 significant differences were found among genotypes in terms of Cu sensitivity. *L. minor*
31 sensitivity to Cu significantly varied for $F_v:F_m$, which showed up to 35 % of variation in EC_{50}
32 values among genotypes. Significant differences in EC_{50} values were found for RGR based on
33 length for *M. spicatum*, with up to 72% of variation. Finally, *C. demersum* demonstrated
34 significant sensitivity differences among genotypes with up to 78 % variation for EC_{50} based
35 on length. Overall, interspecific variation was higher than intraspecific variation, and explained
36 77% of the variation found among genotypes for RGR based on biomass, and 99% of the
37 variation found for $F_v:F_m$. Our results highlight that depending on the endpoint, sensitivity can
38 vary greatly within a species, and not all endpoints should be considered relevant in risk
39 assessment.

40 **Keywords:** Genotype, copper toxicity, freshwater macrophyte, interspecific variation,
41 intraspecific variation

42 **1. Introduction**

43 Over the past decades, the increase of global population has led to an intensification of
44 agricultural practices. To sustain a sufficient yield, many fertilizers and pesticides have been
45 used. The extensive use of these chemicals triggers the progressive contamination of
46 environment. Aquatic ecosystems are the final receivers of these contaminations, through
47 different processes such as atmospheric deposition, runoff and soil leaching (Moss, 2008;
48 Knauert *et al.*, 2010).

49 Organisms within these environments can therefore be exposed to many pollutants
50 (Gallagher, Johnston and Dietrich, 2001; Ribolzi *et al.*, 2002). Some organic chemicals can be
51 degraded by biotic or abiotic processes, some can be modified and become even more harmful
52 through metabolization by living organisms and accumulated. Metals can also accumulate in
53 ecosystems, in particular in sediments, and can be further transferred into the food chain with
54 possible biomagnification (Cardwell *et al.*, 2013; Andresen *et al.*, 2016). This process can lead
55 to the imbalance of aquatic ecosystems through the disruption of food webs, which are essential
56 for biogeochemical cycles (Nøges *et al.*, 2016).

57 To limit environmental contaminations and increase waterbody quality, several regulations
58 have been implemented worldwide (*e.g.* REACH, the European Water Framework Directive,
59 Hering *et al.*, 2010; Voulvoulis, Arpon and Giakoumis, 2017). These regulations aim to
60 decrease the impact of chemicals, by controlling the quantity used and their toxicity through
61 risk assessment evaluations before giving a marketing authorization. Therefore, new threshold
62 concentrations and land management have been enacted in several countries to limit waterbody
63 contamination by pesticides and fertilizers. For instance, copper (Cu) concentration in organic
64 agriculture was limited in Europe with concentrations up to 6 kg/ha/year, averaged over 5 years
65 (regulation N° 889/2008, EFSA, 2008). Indeed, Cu is broadly used as a fertilizer and a biocide,
66 and have a dose-dependent toxicity on living organisms (Jiao *et al.*, 2012; Peng *et al.*, 2012).

67 To properly assess the potential impact of chemicals on the environment, new approaches
68 have been implemented in ecotoxicological risk assessment to determine the impact of target
69 molecules on aquatic biota. Among these approaches, Sensitivity Species Distribution (SSD)
70 aims to compare the sensitivity of several species, which allows to determine a threshold
71 concentration at which less than 5% of the species may be impacted (Del Signore *et al.*, 2016).
72 Species used for risk assessment are usually subject to standardized toxicity tests (such as
73 OECD protocols), to ensure the reproducibility of the results. These species are often

74 ubiquitous, with a very wide repartition area and with a generalist strategy. Among the model
75 species used for ecotoxicological risk assessment in aquatic environment, macrophytes are very
76 important, as they play a fundamental role in aquatic ecosystems due to their involvement in
77 biogeochemical cycles and their interactions with other organisms (Bornette and Puijalón 2011;
78 Coutris et al. 2011). As such, pollution effects on aquatic macrophytes has the potential to
79 strongly alter ecosystem structure and functioning (Bornette and Puijalón 2011).

80 However, species found across the globe could show variations in sensitivity among
81 populations. Indeed, populations growing under different environmental conditions (*e.g.*
82 pristine *vs.* polluted waters) can present genetic differentiation (Santamaría, 2002). Toxicity
83 tests in ecotoxicological risk assessment usually use one clonal strain per species and per
84 experiment, assuming that one strain is representative of the entire species. If these tests can
85 potentially be used to rank various species in terms of sensitivity to chemicals, such a ranking
86 may be biased by the sensitivity of given strains, and may result more from a sampling effect
87 than from real differences among species (Figure 1). Obviously, the greater the intraspecific
88 variation in sensitivity to chemicals, the higher is the risk of biased conclusions.

89 Intraspecific variation can be explained by two processes. The first is phenotypic plasticity,
90 which is the ability of one genotype to produce several phenotypes depending on its
91 environment (Vasseur and Aarssen, 1992; Barrett, Eckert and Husband, 1993). The second is
92 genotypic variation, which is the result of mutations over several generations and their selection
93 by biotic and abiotic pressures in a given environment, or by other processes such as genetic
94 drift (Silander, 1985; Ehlers, Damgard and Laroche, 2016). Some authors suggested that
95 intraspecific variation could increase ecosystem productivity and resilience when exposed to
96 disturbance (Loreau and Hector, 2001; Reusch and Hughes, 2006). However, intraspecific
97 variation, especially in aquatic plants, has so far been poorly investigated, particularly when it
98 comes to the sensitivity to contamination (Weyl and Coetzee, 2016). The few existing studies
99 have highlighted some differences in terms of sensitivity among strains of a same species, but
100 the importance of intraspecific variation was never compared to interspecific variation (Dalton
101 *et al.*, 2013; Sree *et al.*, 2015). Therefore, the extent of intraspecific variation needs to be studied
102 to properly understand the impact of chemicals on aquatic environments, and how genotypic
103 variability may inflect risk assessment results.

104 To address this question, we have performed toxicity tests on three different species of aquatic
105 macrophytes with different life-history traits. For each of the three species, several clonal strains
106 from different populations were tested. We chose the lesser duckweed (*Lemna minor* L.), which

107 is free floating at the water surface, the Eurasian watermilfoil (*Myriophyllum spicatum* L.),
108 which is rooted and submerged in the water column, and the common hornwort (*Ceratophyllum*
109 *demersum* L.), which is submerged but has no root, and can be attached to the sediment or freely
110 sustained in the water column. The use of the two first species in chemical risk assessment is
111 standardized in OECD protocols, n°221 for *L. minor* and n°238-239 for *M. spicatum* (OECD,
112 2006, 2014). Copper (Cu) was used as a model contaminant, as it is broadly used in industry
113 and agriculture, and therefore found at high concentrations in some aquatic environments.

114

115 **2. Materials and methods**

116 ***2.1 Species and chemicals studied***

117 Three species (*L. minor*, *M. spicatum*, *C. demersum*) with three to four distinct clonal strains
118 were randomly harvested from 2013 to 2016 in natural freshwater rivers in France and one
119 strain of *M. spicatum* was regrown from an axenic culture established from material collected
120 in Germany in 1990, following the protocol of Gross, Meyer and Schilling, (1996) (Table 1 for
121 geographic origin of strains).

122 Each strain of *M. spicatum* and *C. demersum* was grown in 210 L outdoor containers with quartz
123 sediments enriched with Osmocote® for at least six months before experiments were
124 conducted. *L. minor* strains were grown under axenic conditions in the lab, and were placed
125 under non-axenic environment one month prior to the experiments (Table 2).

126 Copper sulfate from Merck KGaA (CAS number 7758-98-7, Darmstadt, Germany) was
127 prepared in ultrapure water at a concentration of 1 g/L Cu²⁺, and diluted in the different media.

128

129 ***2.2 Genetic characterization of strains by ISSR***

130 Inter simple sequence repeat (ISSR) molecular typing method was used to verify that clonal
131 strains corresponded to different genotypes.

132 DNA was extracted and purified from about 100 mg of plant fragments by using the WIZARD
133 Genomic DNA Purification kit (Promega) and following the procedure described in Carriconde
134 *et al.*, (2008). Out twenty-two ISSR primers (Table S1) previously used with the three species
135 studied (Triest *et al.*, 2010; Xue *et al.*, 2012; Cao, Mei and Wang, 2017) or with other organisms

136 (Hantula, Dusabenyagasani and Hamelin, 1996; Carriconde *et al.*, 2008), 13, 9 and 20 were
137 selected for their ability to produce clear banding patterns and polymorphic bands with the
138 strains of *M. spicatum*, *C. demersum* and *L. minor*, respectively (Table S1). ISSR amplification
139 procedure, banding patterns analysis and calculation of genetic distances among strains were
140 modified and adapted from Carriconde *et al.*, (2008), and are detailed in Supplemental Material
141 I.

142

143 ***2.3 Effective Cu concentrations***

144 Three Cu concentrations (the lowest, intermediate and highest) were sampled at the beginning
145 of Cu exposure, to assess effective concentrations in the media. These were measured using
146 inductively coupled plasma with optical emission spectrometry (ICP-OES, Thermo Electron,
147 IRIS INTREPID II XLD).

148

149 ***2.4 Growth experiments***

150 Prior to exposure, plants were acclimatized during 5 days under the same environmental
151 conditions as during exposure (Table 1). Different media were used for each species as they
152 had different life-history traits to ensure maximal growth. Media were adapted from OECD
153 protocols for the two model species, *L. minor* and *M. spicatum*. Exposure times differed among
154 species according to their growth rates under control conditions.

155 ***2.4.1 Lemna minor***

156 Each experimental unit was composed of a plastic glass of 500 mL, containing 300 mL of
157 Steinberg medium at pH 6.5 ± 0.1 and between ten to fourteen fronds of *L. minor*. The exposure
158 phase lasted seven days, and eight Cu concentrations were tested, from 0 to 1.25 mg/L Cu. The
159 number of fronds was counted at the beginning and at the end of the exposure to calculate the
160 relative growth rate (RGR) based on frond number (section 3.f for formula). Fresh mass per
161 frond at the beginning of exposure was estimated by weighting different bunches of fronds from
162 the different clonal strains. At the end of the exposure phase and for each experimental unit,
163 plants were placed on blotting paper to be dried softly before fresh mass measurements to
164 calculate RGR based on biomass production. Three genotypes were tested.

165

166 2.4.2 *Myriophyllum spicatum* and *Ceratophyllum demersum*

167 Each apical shoot was cut at a length of 6 cm before the one week acclimatization in medium
168 Smart & Barko pH 6.5 ± 0.1 , with 400 mL medium per experimental unit containing 50 mL of
169 quartz sediments enriched with 66.6 mg Osmocote[®] (granulated slow-release fertilizers, NPK:
170 16-8-12, KB) for *M. spicatum*, and in half strength Steinberg medium at pH 6.5 ± 0.1 for *C.*
171 *demersum*. For exposure, one apical shoot was placed in each experimental unit with quartz
172 sediments during 12 days for *M. spicatum* and 14 days for *C. demersum*, with renewal of the
173 medium at day 6 or day 7, respectively. Seven copper concentrations were used, ranging from
174 0 to 2 mg/L for *M. spicatum* and from 0 to 0.5 mg/L for *C. demersum*. Length was measured at
175 the beginning and at the end of exposure to calculate the RGR based on shoot length, and fresh
176 mass was recorded at the same time after having placed the plants on blotting paper, to calculate
177 the RGR based on biomass production. Three genotypes of *C. demersum* and four genotypes
178 of *M. spicatum* were used.

179

180 2.5 Maximum quantum yield of photosystem II ($F_v:F_m$) experiments

181 $F_v:F_m$ ratio, which is the maximal ability of the plant to harvest light, calculated by using the
182 Kautsky effect, was measured (Maxwell and Johnson, 2000; Murchie and Lawson, 2013).
183 Measures were conducted using a Diving-PAM fluorometer (Heinz Walz GmbH, Germany).
184 The basic settings of the Diving-PAM, namely intensity of measuring light (50: MEAS-INT)
185 and amplification factor (49: GAIN) were set to 8 and 2, respectively. An exposure period of
186 96 h was used, and Cu concentrations were higher than in growth experiment to obtain sufficient
187 inhibition. For each species, $F_v:F_m$ measurements were taken before and after Cu exposure, in
188 a dark chamber, 30 minutes after dark acclimatization of the plant to ensure that all reaction
189 centers were opened for new photons. The same media as those used for growth experiments
190 were used, except for *M. spicatum*, which had no sediment (presumably not necessary for the
191 short duration of the experiment). Each species was acclimatized during three days under
192 similar environmental conditions as used during exposure, and shoots of *M. spicatum* and *C.*
193 *demersum* were cut at 6 cm length at the beginning of acclimatization. Three genotypes of *L.*
194 *minor* were tested, four genotypes of *M. spicatum*, and two genotypes of *C. demersum* due to
195 the lack of available biomass. At the end of the experiments, the DIVING-PAM parameters
196 were adjusted (increase in intensity of measuring light and amplification factor, up to 11 over
197 12) when plants were too chlorotic to emit sufficient signal for accurate measurement of $F_v:F_m$.

198 Eight concentrations of Cu (0 – 2 mg/L) were used for *L. minor*. Four replicates containing ten
199 to fourteen fronds were used for each concentration. $F_v:F_m$ was measured at the beginning of
200 the experiment on fifteen randomly-chosen *L. minor* bunches of three-four fronds within each
201 clonal strain. Three measurements per experimental unit were taken at the end of the
202 experiment.

203 Seven concentrations of Cu ranging from 0 to 35 mg/L were used for *M. spicatum*. Eight
204 concentrations of Cu ranging from 0 to 2 mg/L were used for *C. demersum*. For these two
205 species, five replicates containing one apical shoot each were used per concentration.

206

207 **2.6 Calculations and statistics**

208 Relative growth rates based on biomass production, frond number, or shoot length were
209 calculated for each experimental unit as follows:

$$210 \quad RGR_{i-j} = (\ln(N_j) - \ln(N_i))/t$$

211 where RGR_{i-j} is the relative growth rate from time i to j , N_i and N_j are the endpoint (frond
212 number, fresh mass or length) in the test or control vessel at time i and j , respectively, and t is
213 the time period from i to j .

214 The inhibition percentage of RGR was also calculated on each experimental unit, to assess the
215 sensitivity of genotypes to Cu exposure regardless of their growth performance, following the
216 formula:

$$217 \quad \%Ir = \left(\frac{\overline{RGR}_c - RGR_t}{\overline{RGR}_c} \right) * 100$$

218 where $\%Ir$ is the inhibition percentage of the average specific growth rate, \overline{RGR}_c is the mean
219 value for RGR in the control and RGR_t is an individual value for RGR in the treatment group.

220 Results were analyzed using the R studio software (R Core Team (2016) V 3.3.1).
221 Homoscedasticity was tested using Bartlett test. Data normality was tested with Shapiro test on
222 ANOVA residuals, with log-transformation when normality assumption was not met with raw
223 data. One-way ANOVAs were performed on results showing normal and homoscedastic
224 distribution, with or without log transformation, to assess the differences among genotypes for
225 control vessels. Tukey HSD post-hoc tests were used to identify which genotypes differed from
226 others in absence of contamination. Non-linear log-logistic models with 3 or 4 parameters were
227 used to calculate the half maximal effective concentration (EC_{50}), or exponential decay model

228 for the $F_v:F_m$ experiment of *C. demersum* species, using the `drm()` function from the `drc` R
229 package (Ritz *et al.*, 2015). Coefficients of variation among genotype EC_{50} values were
230 calculated by dividing standard deviation by mean. Comparison of non-linear models among
231 genotypes within species were performed using Akaike information criterion (AIC), through
232 the comparison of models with or without the genotype considered as factor. The best model
233 was selected as the one with the lowest AIC value, and models were considered different when
234 a difference of at least 2 in AIC values was observed. Interspecific variability in EC_{50} (in %)
235 was assessed using the R^2 obtained from one-way ANOVA testing the species effect on EC_{50}
236 values collected for all genotypes during the experiments.

237

238 **3. Results**

239 ***3.1 Effective concentrations in the exposure media***

240 At the beginning of the experiments, effective concentrations varied between 98.9 % and 99.3
241 % of nominal concentration for *L. minor* between 94.4 % and 105.5 % for *M. spicatum*, and
242 between 97.9 % and 112.0 % for *C. demersum*. At the end of exposures, effective concentrations
243 were measured, and time-averaged concentrations were calculated using effectives
244 concentrations at the beginning and at the end of exposure, as well as at media renewal. Time-
245 averaged concentrations were used for the result analysis. In average on both experiments, the
246 time-averaged concentrations were 77.4 % of nominal concentrations for *L. minor*, 69.5 % and
247 74.1 % for *M. spicatum* and *C. demersum*, respectively.

248

249 ***3.2 Intraspecific variations***

250 ***3.2.1 Lemna minor***

251 Without Cu exposure, differences among genotypes were found for RGR_{fm} , showing that some
252 genotypes were more efficient than others in terms of biomass production, with RGR_{fm} ranging
253 from 0.349 d^{-1} for the “Canal” genotype to 0.434 d^{-1} for the “Metz” genotype (1-way ANOVA,
254 $F_{2,15} = 5.12$, $P = 0.0327$). Similar observation was realized for $F_v:F_m$, with the “Authume”
255 genotype being slightly less performing than other genotypes regarding light harvesting (1-way
256 ANOVA, $F_{2,9} = 9.003$, $P = 0.0027$).

257 Based on growth parameters, Cu exposure did not highlight a strong difference in sensitivity or
258 resistance patterns among genotypes, although biomass production significantly differed
259 among genotypes, with the “Canal” genotype being inhibited by 4.2 % at low Cu concentration
260 (0.05 mg/L), against 16.2 % for the two other genotypes. At higher Cu concentration (0.5 mg/L)
261 differences in sensitivity were less observable, with RGR_{fm} being inhibited from 88.4 % to 98.0
262 % (Figure 2A). Confirming those results, EC_{50} values for RGR_{fm} ranged from 0.133 to 0.154
263 mg/L Cu, and showed 7.14% of variation among genotypes (Table 3). The genotype effect on
264 Cu sensitivity was significant according to the concentration-response model, exhibiting an
265 AIC of -508.9, against -499.4 for the model without genotype effect. The RGR_{fronds} varied as
266 well, although differences were not significant (Figure 2B). At 0.5 mg/L it was inhibited by
267 67.7 % for “Canal” genotype, and by 75.37 % for “Authume”, and EC_{50} values ranged from
268 0.127 to 0.157 mg/L Cu, showing 10.9 % of variation among genotypes (Table 3).

269 The $F_v:F_m$ showed stronger variations among genotypes, and a pattern of resistance was
270 observable for the “Canal” genotype (Figure 2C). Indeed, at 0.5 mg/L, the $F_v:F_m$ was inhibited
271 by 8% for the “Canal” genotype, and by 40% for the “Authume” genotype. The pattern was
272 even more contrasted at 1 mg/L Cu, with $F_v:F_m$ being inhibited by 44.73 % for the “Canal”
273 genotype, and by 97.67 % for the “Authume” genotype. Those results are consistent with the
274 EC_{50} values ranging from 0.39 to 0.72 mg/L Cu, and showing 35% of variation among
275 genotypes (Table 3). The genotype effect on Cu sensitivity was significant according to the
276 concentration-response model, showing an AIC of -161.6, against -97.9 for the model without
277 genotype effect. However, these differences were apparently not linked to differences in the
278 sensitivity to Cu in terms of RGR, as the “Canal” genotype did not show a higher tolerance in
279 terms of growth compared to the other genotypes.

280 3.2.2 *Myriophyllum spicatum*

281 No significant difference among genotypes was found for growth-related endpoints in absence
282 of contamination, however a trend was observed with the “Doubs” genotype, which appeared
283 to grow the fastest, especially in length, with a RGR_{length} of 0.0258 d^{-1} against 0.0187 d^{-1} on
284 average for the others (Figure 3B). The $F_v:F_m$ was slightly different among controls, varying
285 from 0.71 for “Doubs” to 0.76 for “Tarn” (1-way ANOVA, $F_{3,16} = 9.356$, $P < 0.001$), and was
286 thus not correlated with growth trends found among genotypes.

287 Copper exposure revealed strong variations in sensitivity within and among genotypes for
288 growth related endpoints (Figure 3). However, those variations were only significantly different

289 for RGR_{length} , which concentration-response model exhibited an AIC of -1095.2, against -
290 1075.3 for the model without genotype effect. The “Schöhsee” genotype was the most resistant
291 genotype to Cu. For instance, at 0.1 mg/L Cu the RGR_{length} was inhibited by 33.1 % for
292 “Schöhsee”, and by 58.3 % for the other genotypes. Furthermore, EC_{50} ranged from 0.042
293 mg/L Cu for “Dordogne”, which was the most sensitive genotype, to 0.296 mg/L Cu for
294 “Schöhsee” genotype. A variation coefficient of 93.8 % was found among the EC_{50} values of
295 those genotypes, highlighting the broad range of sensitivity found among those genotypes for
296 this endpoint (Table 3, Figure 3B). Although no difference in sensitivity was significant, the
297 RGR_{fm} exhibited variations among genotypes and some trends were observed. For instance, at
298 0.1 mg/L Cu the “Schöhsee” was inhibited by 17.9 %, and the “Doubs” by 52.9 %. Accordingly,
299 EC_{50} values varied from 0.077 for “Doubs” which was the most sensitive, to 0.46 mg/L Cu for
300 “Schöhsee” genotype which was the most resistant. EC_{50} values showed a coefficient of
301 variation of 72%, although a high standard deviation was observed for those EC_{50} values,
302 partially explained by the high variability among replicates (Table 3, Figure 3A).

303 Contrasting with the growth-related endpoints, $F_v:F_m$ was not much impacted by Cu exposure,
304 and a decrease by 50% of this ratio was not reached, even with a Cu concentration up to 35
305 mg/L (Figure 3C). Therefore, no concentration-response curve was produced and no EC_{50} value
306 could be calculated. No difference in sensitivity was identified among genotypes, as this
307 endpoint was obviously insensitive to Cu exposure in the case of *M. spicatum*.

308

309 3.2.3 *Ceratophyllum demersum*

310 No significant difference was observed among genotypes both in their $F_v:F_m$ and biomass
311 production in absence of Cu exposure, although some variations were observed for RGR_{fm} ,
312 ranging from 0.019 d⁻¹ for “Garonne” to 0.029 d⁻¹ for “Tarn” genotype (Figure 4A). However,
313 significant differences were observed in their elongation rate, ranging from 0.017 d⁻¹ for “Tarn”
314 to 0.037 d⁻¹ for “Garonne” genotype (Figure 4B). This showed an inverse relationship between
315 RGR_{fm} and RGR_{length} , as the most productive genotype in terms of biomass exhibited the lowest
316 elongation rate.

317 All endpoints were impacted by Cu exposure, and significant differences in sensitivity were
318 highlighted among genotypes despite the high variation among replicates demonstrated for
319 growth-related endpoints (Figures 4A, B, and C). For instance, at 0.1 mg/L Cu, RGR_{fm} was
320 inhibited by 31.2 % to 82.9 % for “Garonne” and by “Tarn” genotypes, respectively. At the

321 same Cu concentration, the RGR_{length} was inhibited by 46.1 % for “Dordogne”, up to 76.3 %
322 for “Tarn” genotype. EC_{50} values varied among genotypes, from 0.06 to 0.086 mg/L Cu for
323 RGR_{fm} and showed a coefficient of variation of 19 %. The genotype effect in Cu sensitivity of
324 biomass production was confirmed by the concentration-response model, which exhibited an
325 AIC value of -547.9, against -515.5 for the model without genotype effect. For RGR_{length} , EC_{50}
326 varied from 0.006 to 0.067 mg/L Cu, and exhibited a coefficient of variation of 75.9 %. The
327 genotype effect in Cu sensitivity for RGR_{length} was confirmed by the most negative AIC value
328 for the response-model with genotype effect (-661.8, against -653.9).

329 The $F_v:F_m$ was not impacted enough by Cu exposure to reach a decrease of 50% of the signal;
330 at 2 mg/L, this endpoint was inhibited by 41.5 % for “Dordogne” genotype and by 46.8 % for
331 “Garonne” genotype (Figure 4C). The EC_{50} values were predicted by the model to be between
332 2.15 and 2.2 mg/L depending on the genotype, showing a low variation coefficient of 1.9 %.
333 This highlights that, as for *M. spicatum*, this endpoint only responds to very high Cu
334 concentration for this species and do not appear relevant as an exposure biomarker.

335

336 ***3.3 Relative importance of intraspecific vs. interspecific variations***

337 Interspecific variability was the main source of variation among species as indicated by a
338 comparison of the EC_{50} values obtained for the various genotypes of each species (Table 3).
339 Indeed, 78.3 % and 99% of the variation in EC_{50} values for RGR_{fm} and $F_v:F_m$, were due to
340 interspecific variability, respectively. EC_{50} values based on RGR_{length} were only compared
341 among *C. demersum* and *M. spicatum* as this endpoint was not used for *L. minor*, and 66 % of
342 the variability was explained by interspecific differences. RGR_{length} was three times more
343 sensitive to Cu for *C. demersum* than for *M. spicatum*, however up to tenfold differences in
344 sensitivity were observed among genotypes. Furthermore, this endpoint demonstrated the most
345 variability among genotypes for both species compared to the other endpoints.

346 For RGR_{fm} , *C. demersum* was the most sensitive species to Cu, with an average EC_{50} value of
347 0.077 ± 0.01 mg/L Cu, against 0.144 ± 0.001 and 0.237 ± 0.09 mg/L Cu for *L. minor* and *M.*
348 *spicatum*, respectively (Figure 5). For $F_v:F_m$, *L. minor* was the most sensitive species with an
349 average EC_{50} value of 0.513 ± 0.1 mg/L Cu, against 2.18 ± 0.03 for *C. demersum*, and no
350 calculated EC_{50} for *M. spicatum*, as no significant inhibition of this endpoint could be observed
351 during the experiment. The comparison among species showed that high variation occurred
352 depending on the endpoint considered. For instance, EC_{50} values for RGR_{length} of *M. spicatum*

353 and *C. demersum* demonstrated a coefficient of variation above 90 and 75 %, against 72 % and
354 19 % for RGR_{fm} , respectively. It suggests that shoot elongation is more subject to variations
355 among genotypes than biomass production, or even light harvesting capacities.

356

357 **4. Discussion**

358 **4.1 Endpoint sensitivity**

359 Species sensitivity to Cu was strongly linked to the endpoints considered, and $F_v:F_m$ was
360 the least sensitive for all species. This suggests that $F_v:F_m$ is not relevant to reveal Cu
361 contamination of aquatic environments for these species, and that growth-related endpoints
362 would be more consistent to use in the case of biomonitoring, as they are more sensitive.
363 However, several studies have shown for different aquatic plant species that $F_v:F_m$ was relevant
364 for very short term exposure to pesticides (few hours), but showed some recovery over time
365 (Macinnis-Ng and Ralph, 2003; Choi, Berges and Young, 2012). The fact that $F_v:F_m$ was not
366 relevant to reveal the sensitivity of *M. spicatum* highlights the importance of selecting proper
367 endpoints for each species. One mechanism which might explain the $F_v:F_m$ signal of *M.*
368 *spicatum* at so high concentrations, and despite a brownish appearance of plants, would be the
369 replacement of Mg^{2+} ions by Cu^{2+} ions in chlorophyll, resulting in a fluorescent signal even if
370 the plant was dead (Pádua *et al.*, 2010). However, no further experiment has been conducted to
371 explore this mechanism, but it could be a further step in the understanding of Cu toxicity on *M.*
372 *spicatum*.

373 The high variability in growth among replicates for *M. spicatum* and *C. demersum* exposed
374 to Cu might be explained by the fact that fragments were not completely identical at the start
375 of the experiment, despite using the same length. The morphology between fragments showed
376 more variation for these species *e.g.* in stem thickness and capacity to elongate than *L. minor*
377 individuals, which have a completely different growth form with floating leaves. Another
378 explanation would be that Cu is an essential element for living organisms. It is the element for
379 which most chelators are found at natural state in cell cytosol, and as such, it already has
380 metabolic pathways and transporters with regulation paths (Huffman and O'Halloran, 2001;
381 Printz *et al.*, 2016). All these elements increase the possibility for variation among individuals
382 and replicates, as numerous pathways to regulate Cu exists at the cellular level, and may vary
383 from one shoot to another. Furthermore, even among clonal individuals some variations can be

384 observed, due to alternative splicing, post-translational modifications or preferential gene
385 expression (Grativol *et al.*, 2012).

386

387 ***4.2 Intraspecific variation***

388 All three species showed statistically significant differences in sensitivity among genotypes,
389 depending on the endpoint considered. The high variability within genotypes among replicates,
390 especially for *C. demersum* and *M. spicatum* in growth-related endpoints, slightly interfered
391 with the significance of the results, and resulted in EC₅₀ values with high standard deviations.
392 However, the robustness of the concentration-response approach managed to highlight
393 differences in sensitivity among genotypes.

394 Traits showing significant differences in productivity among genotypes in absence of
395 contamination always resulted in significant differences in sensitivity to Cu. Furthermore,
396 genotypes which were the most performing were also the most sensitive to contamination,
397 exhibiting the lowest EC₅₀ values, as it was demonstrated in other studies at an interspecific
398 level (Cedergreen *et al.*, 2004; Coutris *et al.*, 2011). Indeed, actively growing individuals will
399 be more impacted when facing a chemical stress, both as they are more exposed to
400 contamination via a rapid uptake of elements, and as they preferentially allocate their resources
401 to biomass production and/or elongation, than to stress defense processes such as antioxidant
402 balance (Delmail *et al.*, 2010).

403 It was interesting to notice that for *L. minor*, the difference in sensitivity of F_v:F_m among
404 genotypes did not confer any growth advantage in terms of sensitivity to the genotype that had
405 a more tolerant F_v:F_m. On the contrary, *M. spicatum* did not show significant growth difference
406 in absence of contamination, but Cu stress highlighted significant differences in sensitivity, as
407 demonstrated by the different EC₅₀ values in RGR_{length} among genotypes. This suggests that
408 genetic variations among those genotypes might influence their response to chemicals, and
409 therefore their susceptibility and their resilience capacity. Genetic diversity within ecosystems
410 may enhance their resilience to abiotic factors, as well as their productivity (Reusch and
411 Hughes, 2006; Sgrò, Lowe and Hoffmann, 2011; Sjöqvist and Kremp, 2016).

412 The fact that genotypes were coming from relatively similar environments in terms of
413 temperature, light, eutrophication levels and water flows, with no highly contaminated nor
414 pristine environments, decreased the probability to harvest a genotype with a contrasted

415 sensitivity to chemicals (Cao, Mei and Wang, 2017). Indeed, diffuse contamination in an
416 ecosystem may trigger a selection pressure on populations, and only individuals able to thrive
417 under this contamination would remain. Individuals with increased resistance or adaptive
418 plasticity to contamination would progressively be selected due to the chemical pressure
419 (Brown *et al.*, 2009). This is depicted by the pollution-induced-tolerance concept, or PICT,
420 which evaluates the selection pressure applied by chemicals on natural populations (Tlili *et al.*,
421 2016).

422 In our case, it could partially explain the low difference in sensitivity among genotypes,
423 except for *M. spicatum*. Here, we can assume that no strong difference in selection pressure
424 was applied in the environments from which the genotypes were harvested, and therefore no
425 structuration was found in term of sensitivity to contamination, reducing the probability to
426 collect resistant strains. It has been well documented that plant resistance to environmental
427 pressures (metals, pathogens...) is a costly process which may decrease fitness when the
428 pressure considered is removed, and thus is only maintained successfully under stressful
429 conditions (Huot *et al.*, 2014).

430

431 ***4.3 Interspecific variation in Cu sensitivity***

432 Overall, interspecific variation was more important than intraspecific variation. Indeed,
433 total variation in EC₅₀ values among genotypes of the various species was explained by
434 interspecific variation at 77% for RGR_{fm} and 99% for F_v:F_m, although *M. spicatum* had no EC₅₀
435 value for the last endpoint. Based on RGR_{fm}, *C. demersum* was the most sensitive species, and
436 *M. spicatum* was the most tolerant once EC₅₀ values were averaged among genotypes. The
437 duckweed *L. minor* was in the middle of the sensitivity range covered by the three species,
438 however our EC₅₀ values were lower than those found in literature. Khellaf and Zerdaoui (2010)
439 have found an EC₅₀ of 0.47 mg/L for Cu on *L. minor* on RGR_{fronds} against 0.25 mg/L Cu in our
440 study; however the pH used in their experiment was lower (6.1) and the duration was over four
441 days.

442 These three species are found across the globe, which denotes a certain ability to tolerate
443 and adjust to a wide range of environments (Grenier, Barre and Litrico, 2016). In this study,
444 whatever the species, no evidence of a relation between intergenotype genetic distance and
445 geographic distance of their origin was found (ISSR analyses, supplementary data). However,
446 the number of genotypes used per species and per population, as well as the number of

447 polymorphic ISSR primers, do not allow to assess the relative importance of geographic
448 distance in the genetic structure. Several studies have investigated the importance of geographic
449 distance in shaping the genetic structure of populations, and have demonstrated contrasting
450 results depending on the species (Pollux *et al.*, 2009; Honnay *et al.*, 2010; Wu *et al.*, 2016).
451 Phenotypic plasticity could play an important role in this tolerance to abiotic factors (including
452 chemical stress) and has been widely investigated as a response to environmental variations
453 (Bradshaw, 2006; Vitasse *et al.*, 2010; Steam, 2012).

454 Finally, only *M. spicatum* showed a significantly high range of EC₅₀ values for both RGR
455 values among genotypes. It might require further investigations to assess the importance of
456 genotypic variability in its sensitivity to chemicals, and whether or not this variability should
457 be taken into account in risk assessment during lab tests.

458 **5. Conclusion**

459 In this study, we assessed the importance of intraspecific variation in the sensitivity of
460 aquatic macrophytes to chemicals. We focused on genotypic variation, which is one source of
461 intraspecific variability. Our results demonstrated that despite some differences in sensitivity
462 among genotypes within species, interspecific variation remained much higher than
463 intraspecific variation. SSD approaches, as well as OECD standardized protocols, are thus not
464 questioned by our results. As the species studied can be found across a broad range of
465 environmental conditions, phenotypic plasticity, which occurs during the life time of an
466 individual, may thus play a more important part in intraspecific variation than genotypic
467 variation. However, supplementary investigations, on more genotypes, are required to assess
468 variability in the sensitivity of *M. spicatum* to chemicals. Indeed, further studies have
469 demonstrated that this species shows broad variations in its life-history traits and genetic shape
470 among populations. Furthermore, it has been demonstrated that environmental conditions (*e.g.*
471 light, nutrients) strongly affect macrophyte phenotypes, and should therefore be considered as
472 a potential source of variation in sensitivity.

473

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483

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658

659

660 **Table 1:** GPS coordinates of collecting sites for the different genotypes within species used in
661 copper exposure experiments.

662

Species	Genotypes	GPS coordinates
<i>Lemna minor</i>	Authume	47.12256, 5.50560
	Canal	43.56515, 1.47148
	Metz	49.02943, 5.71536
<i>Myriophyllum spicatum</i>	Tarn	43.89067, 1.50656
	Doubs	47.23153, 6.02252
	Dordogne	44.84584, 0.90596
	Schöhsee	54.16624, 10.44114
<i>Ceratophyllum demersum</i>	Tarn	44.11785, 1.15908
	Garonne	44.01804, 1.07639
	Dordogne	44.83811, 0.73947

663

664

665 **Table 2:** Environmental conditions and experimental design of copper exposure experiments
 666 conducted on three different species (*L. minor*, *M. spicatum*, *C. demersum*). RGR: Relative
 667 Growth Rate, based on fresh mass (RGR_{fm}), frond number (RGR_{fronds}) or length (RGR_{length});
 668 $F_v:F_m$: maximum quantum yield of PSII, n = number of replicates. S&B: Smart and Barko
 669 medium, Stb: Steinberg medium, Sed. + Osm: Sediments + Osmocote®, for growth experiment
 670 with *M. spicatum*, 50mL of quartz sediments were enriched with 66.6 mg Osmocote®, NPK
 671 16-8-12, KB. Light intensity was measured at the bottom of the water column for *M. spicatum*
 672 and *C. demersum*.

673

674

Species	<i>L. minor</i>		<i>M. spicatum</i>		<i>C. demersum</i>	
EC ₅₀ Endpoints	RGR _{fm} RGR _{fronds}	F _v :F _m	RGR _{fm} RGR _{length}	F _v :F _m	RGR _{fm} RGR _{length}	F _v :F _m
Copper	0 - 1.25 mg/L n=6	0 - 2 mg/L n=4	0 - 2 mg/L n=5	0 - 35 mg/L n=5	0 - 0.5 mg/L n=5	0 - 2 mg/L n=5
Exposure time	7 days	96 h	12 days	96h	14 days	96h
Experimental conditions	23.0 ± 0.1°C Stb pH 6.5 ± 0.1 105.4 ± 9.3 μE	23.0 ± 0.1°C Stb pH 6.5 ± 0.1 121.4 ± 2.3 μE	23.0 ± 0.1°C S & B Sed. + Osm. pH 6.5 ± 0.1 98.3 ± 1.7 μE	23.0 ± 0.1°C S & B pH 6.5 ± 0.1 98.7 ± 2.1 μE	23.0 ± 0.1°C Stb ½ pH 6.5 ± 0.1 94.7 ± 1.3 μE	23.0 ± 0.1°C Stb ½ pH 6.5 ± 0.1 97.0 ± 2.0 μE

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683 **Table 3:** Half maximal effective concentrations (EC₅₀, mean ± SD) for different genotypes of
684 three macrophyte species: *L. minor*, *M. spicatum* and *C. demersum* exposed to Cu. Maximal
685 Quantum Yield of PSII (F_v:F_m) experiment lasted for 96 h. Growth experiments (relative growth
686 rates, RGR) lasted for 7, 12 and 14 days for *L. minor*, *M. spicatum* and *C. demersum*
687 respectively. CV: coefficient of variation among EC₅₀ values in %, calculated within species
688 (based on averaged EC₅₀ values per genotype and endpoint). Interspecific variability was
689 assessed from the R² value from ANOVA. * For RGR_{length}, interspecific variability was only
690 compared between *C. demersum* and *M. spicatum*, as this endpoint was not used for *L. minor*.

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Species	Genotypes	EC ₅₀ values RGR _{fm}	EC ₅₀ values RGR _{fronds} /RGR _{length}	EC ₅₀ values F _v :F _m
<i>L. minor</i> (n = 4 for RGR, n = 6 for F _v :F _m)	Metz	0.133 ± 0.01	0.127 ± 0.02	0.423 ± 0.02
	Doubs	0.154 ± 0.02	0.157 ± 0.03	0.394 ± 0.02
	Canal	0.146 ± 0.02	0.151 ± 0.02	0.72 ± 0.04
	Average	0.144 ± 0.01	0.145 ± 0.01	0.513 ± 0.1
	EC ₅₀ CV %	7.1	10.9	35.2
<i>M. spicatum</i> (n = 5)	Schöhsee	0.46 ± 0.11	0.296 ± 0.23	NA
	Doubs	0.077 ± 0.07	0.042 ± 0.03	NA
	Tarn	0.271 ± 0.15	0.132 ± 0.25	NA
	Dordogne	0.137 ± 0.09	0.043 ± 0.19	NA
	Average	0.237 ± 0.09	0.128 ± 0.06	NA
EC ₅₀ CV %	72.0	93.8	NA	
<i>C. demersum</i> (n = 5)	Dordogne	0.059 ± 0.01	0.051 ± 0.06	2.21 ± 0.28
	Garonne	0.086 ± 0.05	0.006 ± 0.004	2.15 ± 0.27
	Tarn	0.085 ± 0.02	0.067 ± 0.03	NA
	Average	0.077 ± 0.01	0.042 ± 0.02	2.18 ± 0.03
	EC ₅₀ CV %	19.4	76.0	1.9
% of interspecific variability		78.3	66.0*	99.8

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693 **Legends**

694 **Figure 1:** Sensitivity to chemicals for five hypothetical species determined using individuals from
695 a single population per species (in black). In this kind of approach, the real variability of the
696 species response to contamination is ignored, and interspecific differences which are
697 highlighted here may be spurious and result from a “sampling effect” (*i.e.* these differences may
698 be related more to the sensitivities of the populations sampled than to intrinsic characteristics
699 of the species).

700 **Figure 2:** Concentration-response curves for three genotypes of *L. minor* exposed to copper,
701 with relative growth rates (RGR) based on fresh mass (**A**) and frond number (**B**) after 7 days of
702 exposure, and (**C**) $F_v:F_m$ after 96h. Curves were fitted with non-linear log-logistic models with
703 4 parameters (**A** and **B**) and 3 parameters (**C**).

704 **Figure 3:** Concentration-response curves for four genotypes of *M. spicatum* exposed to copper,
705 relative growth rates (RGR) based on fresh mass (**A**) and shoot length (**B**) after 12 days of
706 exposure. Curves were fitted with non-linear log-logistic models with 3 parameters.

707 **Figure 4:** Concentration-response curves for two to three genotypes of *C. demersum* exposed
708 to copper, with relative growth rates (RGR) based on fresh mass (**A**) and shoot length (**B**) after
709 14 days of exposure, and $F_v:F_m$ (**C**) after 96h. Curves were fitted with non-linear log-logistic
710 models with 4 parameters for growth related endpoints (**A**, **B**) and exponential decay models
711 with 2 parameters for $F_v:F_m$ (**C**).

712 **Figure 5:** EC_{50} values for Relative Growth Rates based on fresh mass of three species, *L. minor*,
713 *M. spicatum* and *C. demersum* exposed to copper. From three to four genotypes of each species
714 were exposed during 7 days (*L. minor*), 12 days (*M. spicatum*) or 14 days (*C. demersum*) to
715 concentrations from 0 to 1.25 mg/L, 0 to 2 mg/L and 0 to 0.5 mg/L Cu, respectively. Same
716 letters within a given species indicate genotypes whose EC_{50} values do not differ significantly.

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719 **Fig.1**

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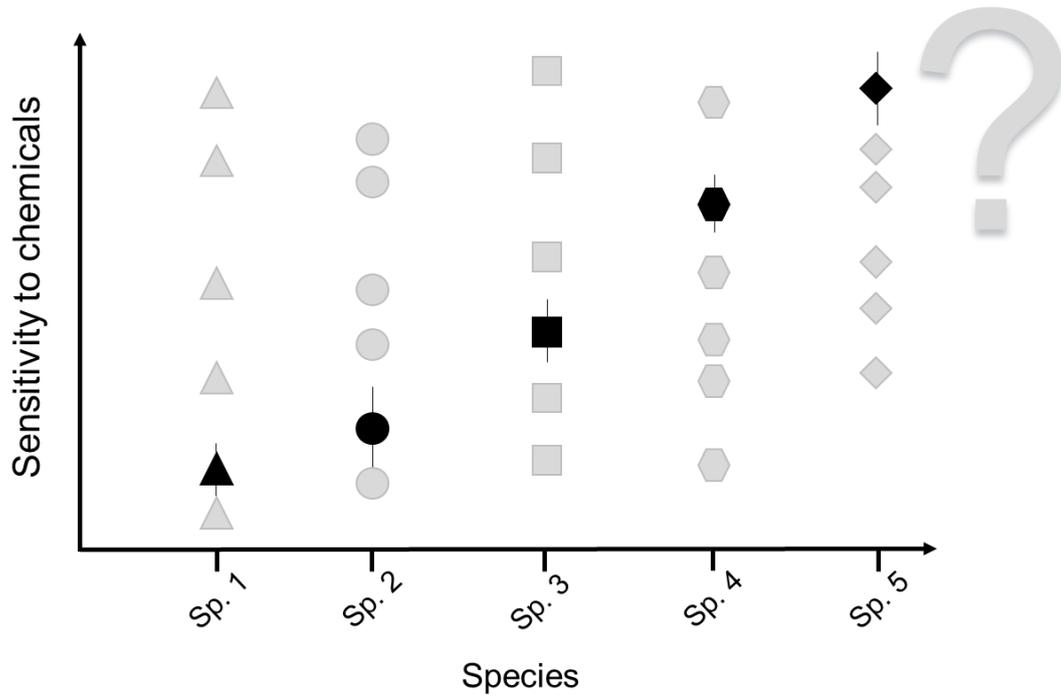
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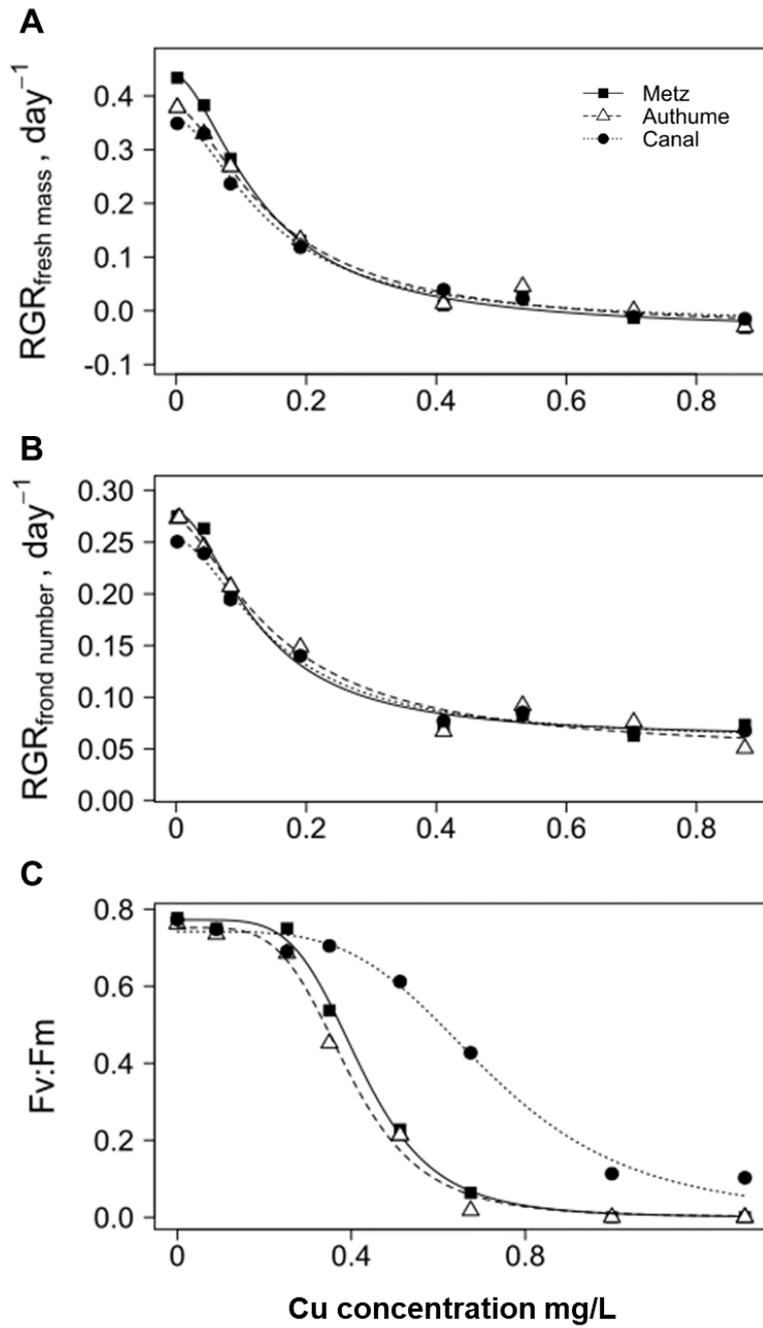
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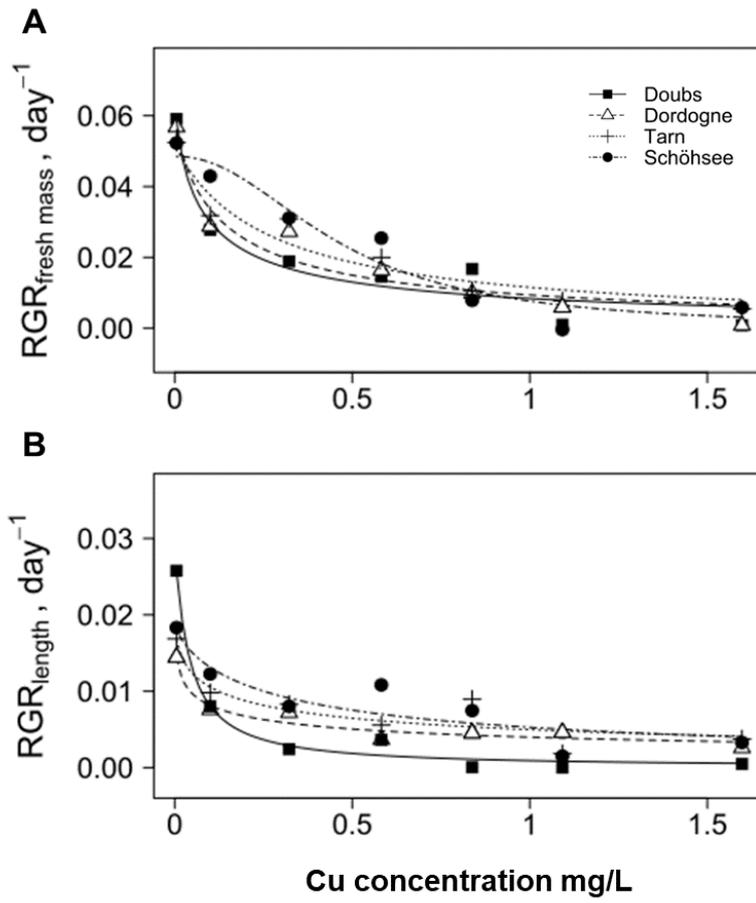
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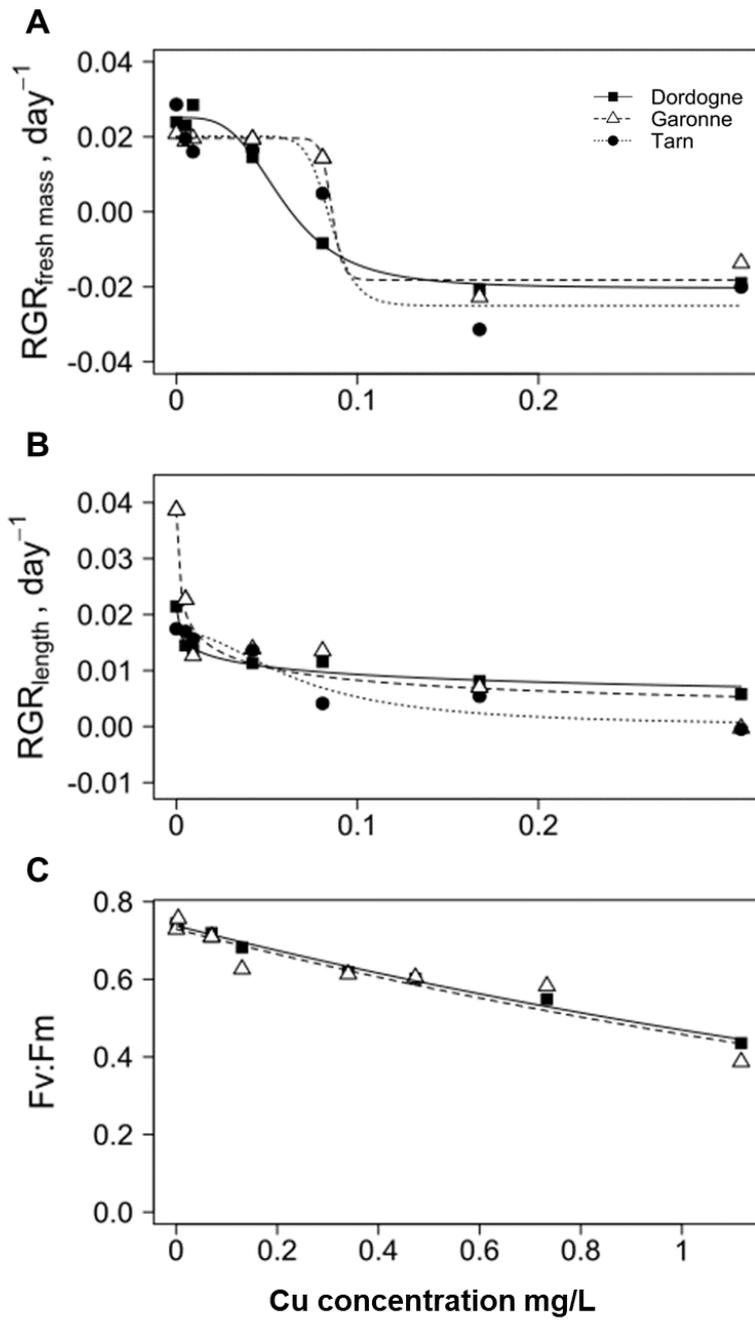
734 **Fig. 3**



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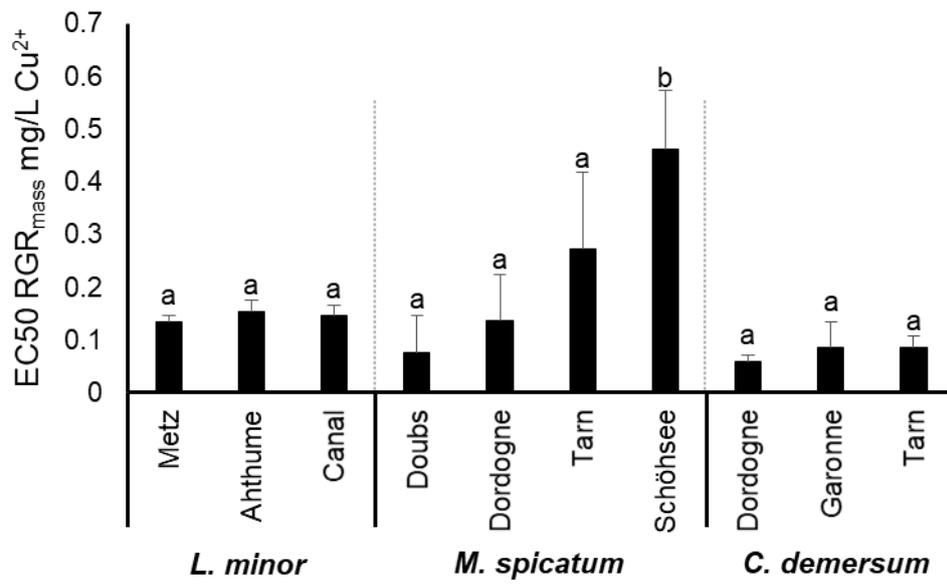
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741 **Fig. 5**



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756 SUPPLEMENTARY DATA

757 **Supplemental material I.** Details of ISSR amplification procedure, of banding pattern analysis
758 and of genetic distance calculation.

759 ISSR amplifications were carried out in a final volume of 25 µl containing 1X of GoTaq green
760 buffer (Promega), 0.2 mM of each dNTP, 1 µM of primer, 0.25 U of GoTaq G2 Hot Start
761 polymerase (Promega) and 10 ng of template DNA. Reactions were performed in a
762 MasterCycler Pro S (Eppendorf) thermal cycler with an initial denaturation step of 3 min at
763 95°C, followed by 37 cycles of 55 s at 95°C, 1 min at annealing temperature required for the
764 primer (Table S1) and 3 min at 72°C, and a final extension step of 10 min at 72°C. A negative
765 control without DNA was included in each run. Amplified fragments were separated by
766 electrophoresis in 0.5X TAE buffer on 1.4% agarose gel including ClearSightDNA
767 (Euromedex) to reveal ISSR banding patterns. Images of patterns were then captured under UV
768 light. The reproducibility of ISSR patterns was assessed by repeating twice the amplifications
769 for each primer and, also, by comparing patterns obtained with two independent DNA
770 extractions of the samples.

771 For each plant species, resulting ISSR patterns were compared to discriminate the strains. In
772 order to estimate genetic relationships among strains within each species, clear and well-
773 separated ISSR fragments were retained and scored as present (1) or absent (0). A matrix of
774 pairwise genetic distance was constructed by calculating for all pairs of samples the DICE
775 dissimilarity index $GD = 1 - 2n_{XY}/(n_X + n_Y)$ where $2n_{XY}$ is the number of fragments shared
776 by two strains X and Y, and n_X and n_Y are the numbers of present fragments in strain X and in
777 strain Y respectively. Cluster analyses based on UPGMA were performed with GD matrices
778 and dendrograms were constructed to visualize genetic differences among strains of each
779 species. Computation of GD matrices and of UPGMA clusters were done with FAMD 1.30
780 software (Schülter *et al.*, 2006, *Molecular Ecology Notes*, 6, pp. 569-572;
781 <http://www.famd.me.uk/famd.html>) and dendrograms were edited with MEGA 7 (Kumar *et al.*,
782 2016, *Molecular Biology and Evolution*, 33, pp. 1870-1874).

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787 **Table S1** Primers used to amplify ISSR fragments in each species, annealing temperatures (T_A),
 788 number of scored fragments and number of polymorphic fragments.

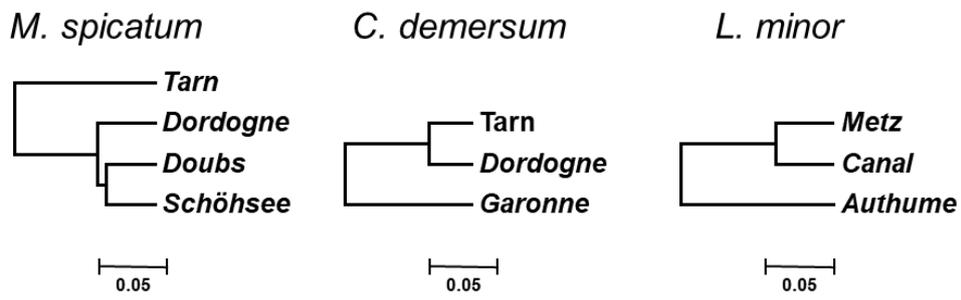
Primers*	Sequences ** (5'-3')	Myriophyllum spicatum			Ceratophyllum demersum			Lemna minor		
		T _A	No. scored fragment s	No. polymorph ic fragments	T _A	No. scored fragment s	No. polymorph ic fragments	T _A	No. scored fragment s	No. polymorph ic fragments
ISSR5	(CA) ₈ GT	46°C	7	4	46°C	7	2	50°C	6	4
ISSR8	(CA) ₇ ATCC	46°C	4	1	46°C	7	3	50°C	4	2
ISSR9	(CA) ₇ GTCT	46°C	7	3	46°C	6	2	50°C	5	3
ISSR12	GGTC(AC)) ₇	-	-	-	53°C	5	1	53°C	7	2
UBC811	(GA) ₈ C	53°C	8	3	52°C	6	3	-	-	-
UBC827	(AC) ₈ G	-	-	-	52°C	4	2	53°C	9	4
UBC845	(CT) ₈ GG	-	-	-	-	-	-	53°C	8	5
UBC849	(GT) ₈ CA	53°C	6	2	-	-	-	53°C	8	4
UBC855	(AC) ₈ CT	53°C	7	5	52°C	4	2	53°C	6	4
UBC856	(AC) ₈ CA	53°C	6	2	-	-	-	53°C	6	3
UBC857	(AC) ₈ TG	-	-	-	-	-	-	53°C	11	6
UBC861	(ACC) ₆	-	-	-	-	-	-	53°C	7	4
R1	DHB(CG A) ₅	-	-	-	-	-	-	53°C	7	2
R2	DDB(CCA) ₅	53°C	8	2	-	-	-	53°C	10	1
R3	BDB(ACA) ₅	53°C	5	1	-	-	-	53°C	6	2
R5	(CCA) ₅ S	53°C	5	2	50°C	4	1	53°C	6	1
R6	(ACA) ₅ S	53°C	8	3	-	-	-	53°C	11	4
RP1	(AC) ₈ YT	-	-	-	53°C	4	1	53°C	13	7
RP2	(CA) ₆ RY	-	-	-	-	-	-	53°C	7	2
RP5	(CTC) ₄ RC	-	-	-	-	-	-	53°C	10	5
RP6	(GTG) ₃ GC	53°C	8	3	-	-	-	-	-	-
RP7	(CAC) ₄ RC	53°C	5	4	-	-	-	53°C	9	1
All			84	35		47	17		156	66

789 * References for primers : ISSR5 to ISSR12 : Triest *et al.* (2010) ; UBC811 to UBC861 :
 790 Primers designed by the University of British Columbia Biotechnology Laboratory (Canada)
 791 and used by Xue *et al.* (2012) with *Lemna* and by Cao *et al.* (2017) with *Myriophyllum* and
 792 *Ceratophyllum* ; R1 to R3 : Hantula *et al.* (1996) ; R5 and R6 : Carriconde *et al.* (2008) ; RP1
 793 to RP7 : Liang *et al.* (2005).

794 **With B = T, C or G ; D = A, T or G ; H = A, C or T ; R = A or G ; S = C or G and Y = C or
 795 T.

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797 **Fig. S1.** UPGMA cluster analysis based on ISSR data showing genetic relationships among
798 strains of *Myriophyllum spicatum*, *Ceratophyllum demersum* and *Lemna minor*. The scale refers
799 to genetic distances (Nei and Li, 1979).



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