Does intraspecific variability matter in ecological risk assessment? Investigation of genotypic variations in three macrophyte species exposed to copper

Eva Roubeau Dumont, Camille Larue, Sophie Lorber, Hervé Gryta, Elise Billoir, Elisabeth Maria Gross, Arnaud Elger

Does intraspecific variability matter in ecotoxicological risk assessment? Investigation of genotypic variations in three macrophyte species exposed to copper

Eva Roubeau Dumont[1], Camille Larue[1], Sophie Lorber[2] Hervé Gryta[3], Elise Billoir[4], Elisabeth Maria Gross[4] & Arnaud Elger[1]

[1] ECOLAB, Université de Toulouse, CNRS, INPT, UPS, France

[2] Toxalim, Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France

[3] Laboratoire Evolution & Diversité Biologique (EDB UMR 5174), Université de Toulouse, CNRS, IRD, UPS, 118 route de Narbonne, Bat 4R1, 31062 Toulouse, France

[4] LIEC, Université de Lorraine, CNRS, UMR 7360, Metz, Lorraine, France

Corresponding author: Eva Roubeau Dumont, eva.roubeaudumont@ensat.fr
EcoLab, Campus INPT-ENSAT. Avenue de l'Agrobiopole – BP 32607 - 31326 Castanet Tolosan Cedex, France

ABSTRACT

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

Keywords: Genotype, copper toxicity, freshwater macrophyte, interspecific variation, intraspecific variation

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1 Species and chemicals studied

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

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

Copper sulfate from Merck KGaA (CAS number 7758-98-7, Darmstadt, Germany) was prepared in ultrapure water at a concentration of 1 g/L Cu^{2+}, and diluted in the different media.

2.2 Genetic characterization of strains by ISSR

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

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

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

2.3 Effective Cu concentrations

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

2.4 Growth experiments

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

2.4.1 Lemna minor

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

2.4.2 Myriophyllum spicatum and Ceratophyllum demersum

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

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

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

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

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

2.6 Calculations and statistics

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

$$RGR_{i-j} = (\ln(Nj) - \ln(Ni))/t$$

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

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

$$\%Ir = \left(\frac{\overline{RGR}c - RGRt}{RGRc}\right) * 100$$

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

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

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

3. Results

3.1 Effective concentrations in the exposure media

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

3.2 Intraspecific variations

3.2.1 Lemna minor

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

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

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

3.2.2 Myriophyllum spicatum

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

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

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

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

3.2.3 Ceratophyllum demersum

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

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

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

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

3.3 Relative importance of intraspecific vs. interspecific variations

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

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

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

4. Discussion

4.1 Endpoint sensitivity

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

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

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

4.2 Intraspecific variation

All three species showed statistically significant differences in sensitivity among genotypes, depending on the endpoint considered. The high variability within genotypes among replicates, especially for *C. demersum* and *M. spicatum* in growth-related endpoints, slightly interfered with the significance of the results, and resulted in EC_{50} values with high standard deviations. However, the robustness of the concentration-response approach managed to highlight differences in sensitivity among genotypes.

Traits showing significant differences in productivity among genotypes in absence of contamination always resulted in significant differences in sensitivity to Cu. Furthermore, genotypes which were the most performing were also the most sensitive to contamination, exhibiting the lowest EC_{50} values, as it was demonstrated in other studies at an interspecific level (Cedergreen *et al*., 2004; Coutris *et al.*, 2011). Indeed, actively growing individuals will be more impacted when facing a chemical stress, both as they are more exposed to contamination via a rapid uptake of elements, and as they preferentially allocate their resources to biomass production and/or elongation, than to stress defense processes such as antioxidant balance (Delmail *et al.*, 2010).

It was interesting to notice that for *L. minor*, the difference in sensitivity of F_v:F_m among genotypes did not confer any growth advantage in terms of sensitivity to the genotype that had a more tolerant F_v:F_m. On the contrary, *M. spicatum* did not show significant growth difference in absence of contamination, but Cu stress highlighted significant differences in sensitivity, as demonstrated by the different EC_{50} values in RGR_{length} among genotypes. This suggests that genetic variations among those genotypes might influence their response to chemicals, and therefore their susceptibility and their resilience capacity. Genetic diversity within ecosystems may enhance their resilience to abiotic factors, as well as their productivity (Reusch and Hughes, 2006; Sgrò, Lowe and Hoffmann, 2011; Sjöqvist and Kremp, 2016).

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

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

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

4.3 Interspecific variation in Cu sensitivity

Overall, interspecific variation was more important than intraspecific variation. Indeed, total variation in EC_{50} values among genotypes of the various species was explained by interspecific variation at 77% for RGR_{fm} and 99% for F_v:F_m, although *M. spicatum* had no EC_{50} value for the last endpoint. Based on RGR_{fm}, *C. demersum* was the most sensitive species, and *M. spicatum* was the most tolerant once EC_{50} values were averaged among genotypes. The duckweed *L. minor* was in the middle of the sensitivity range covered by the three species, however our EC_{50} values were lower than those found in literature. Khellaf and Zerdaoui (2010) have found an EC_{50} of 0.47 mg/L for Cu on *L. minor* on RGR_{fronds} against 0.25 mg/L Cu in our study; however the pH used in their experiment was lower (6.1) and the duration was over four days.

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

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

Finally, only *M. spicatum* showed a significantly high range of EC_{50} values for both RGR values among genotypes. It might require further investigations to assess the importance of genotypic variability in its sensitivity to chemicals, and whether or not this variability should be taken into account in risk assessment during lab tests.

5. Conclusion

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

6. Acknowledgments

This research was funded by the EC2CO program from the National Institute of Sciences of the Universe (CNRS/INSU), granted to the VIRMA project, and by the French Ministry of research and higher education through a Doctoral Fellowship awarded to ER. We thank David Baqué

(R&D Engineer in ICP-OES and ICP-MS analysis techniques) for his support for ICP-AES metal analyses, as well as Maëlle Beriou for technical assistance on *C. demersum* experiments. HG is member of the EDB laboratory that is supported by the French Laboratory of Excellence project TULIP (ANR-10-LABX-41:ANR-11-IDEX-0002-02) and by an Investissement d'Avenir grant of the Agence Nationale de la Recherche (CEBA : ANR-10-LABX-25-01).

7. References

Andresen, E. *et al.* (2016) 'Cadmium toxicity investigated at the physiological and biophysical levels under environmentally relevant conditions using the aquatic model plant *Ceratophyllum demersum*', *New Phytologist*, 210(4), pp. 1244–1258. doi: 10.1111/nph.13840.

Barrett, S. C. H., Eckert, C. G. and Husband, B. C. (1993) 'Evolutionary processes in aquatic plant populations', *Aquatic Botany*, 44(2–3), pp. 105–145. doi: 10.1016/0304-3770(93)90068-8.

Bornette, G. and Puijalon, S. (2011) 'Response of aquatic plants to abiotic factors: A review', *Aquatic Sciences*, 73(1), pp. 1–14. doi: 10.1007/s00027-010-0162-7.

Bradshaw, A. D. (2006) 'Unravelling phenotypic plasticity – why should we bother?', *New Phytologist*, 170(4), pp. 644–648. doi: 10.1111/j.1469-8137.2006.01758.x.

Brown, A. R. *et al.* (2009) 'Genetic variation, inbreeding and chemical exposure--combined effects in wildlife and critical considerations for ecotoxicology', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1534), pp. 3377–3390. doi: 10.1098/rstb.2009.0126.

Cao, Q. J., Mei, F. F. and Wang, L. (2017) 'Population genetic structure in six sympatric and widespread aquatic plants inhabiting diverse lake environments in China', *Ecology and Evolution*, 7(15), pp. 5713–5723. doi: 10.1002/ece3.3141.

Cardwell, R. D. *et al.* (2013) 'Do Cd, Cu, Ni, Pb, and Zn Biomagnify in Aquatic Ecosystems?', *Reviews of Environmental Contamination and Toxicology*, 226, pp. 101–122. doi: 10.1007/978-1-4614-6898-1.

Carriconde, F. *et al.* (2008) 'High sexual reproduction and limited contemporary dispersal in the ectomycorrhizal fungus *Tricholoma scalpturatum*: New insights from population genetics

and spatial autocorrelation analysis', *Molecular Ecology*, 17(20), pp. 4433–4445. doi: 10.1111/j.1365-294X.2008.03924.x.

Cedergreen, N., Streibig, J. C. and Spliid, N. H. (2004) 'Sensitivity of aquatic plants to the herbicide metsulfuron-methyl', *Ecotoxicology and Environmental Safety*, 57, pp. 153–161. doi: 10.1016/S0147-6513(02)00145-8.

Choi, C. J., Berges, J. A. and Young, E. B. (2012) 'Rapid effects of diverse toxic water pollutants on chlorophyll a fluorescence: Variable responses among freshwater microalgae', *Water Research*. Elsevier Ltd, 46(8), pp. 2615–2626. doi: 10.1016/j.watres.2012.02.027.

Coutris, C. *et al.* (2011) 'Can we predict community-wide effects of herbicides from toxicity tests on macrophyte species?', *Aquatic Toxicology*, 101, pp. 49–56. doi: 10.1016/j.aquatox.2010.08.017.

Dalton, R. L. *et al.* (2013) 'Comparing the sensitivity of geographically distinct *Lemna minor* populations to atrazine', *Ecotoxicology*, 22, pp. 718–730. doi: 10.1007/s10646-013-1064-y.

Delmail, D. *et al.* (2010) 'Differential responses of *Myriophyllum alterniflorum DC* (Haloragaceae) organs to copper: Physiological and developmental approaches', *Hydrobiologia*, 664(1), pp. 95–105. doi: 10.1007/s10750-010-0589-9.

Didierjean, L. *et al.* (2002) 'Engineering Herbicide Metabolism in Tobacco and Arabidopsis with CYP76B1, a Cytochrome P450 Enzyme from Jerusalem Artichoke', *Plant physiology*, 130, pp. 179–189. doi: 10.1104/pp.005801.

EFSA (2008) 'Commission regulation (EC) no 889/2008 of 5 September 2008 laying down detailed rules for the implementation of council regulation (EC) no 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and co', *Official Journal of the European Union*, L 250(834), pp. 1–84.

Ehlers, B. K., Damgard, C. F. and Laroche, F. (2016) 'Intraspecific genetic variation and species co-existence in plant communities', *Biology Letters*, 12(1), p. 20150853. doi: 10.1098/rsbl.2015.0853.

Fones, H. N. and Preston, G. M. (2013) 'Trade-offs between metal hyperaccumulation and induced disease resistance in metal hyperaccumulator plants', *Plant Pathology*, 62(S1), pp. 63–71. doi: 10.1111/ppa.12171.

Gallagher, D. L., Johnston, K. M. and Dietrich, A. M. (2001) 'Fate and transport of copper-

based crop protectants in plasticulture runoff and the impact of sedimentation as a best management practice', *Water Research*, 35(12), pp. 2984–2994. doi: 10.1016/S0043-1354(00)00594-7.

Grativol, C. *et al.* (2012) 'Genetic and epigenetic regulation of stress responses in natural plant populations', *Biochimica et Biophysica Acta*, 1819(Plant gene regulation in response to abiotic stress. ∗), pp. 176–185. doi: 10.1016/j.bbagrm.2011.08.010.

Grenier, S., Barre, P. and Litrico, I. (2016) 'Phenotypic Plasticity and Selection: Nonexclusive Mechanisms of Adaptation', *Scientifica*. Hindawi Publishing Corporation, 2016, p. 9. doi: 10.1155/2016/7021701.

Gross, E. M., Meyer, H. and Schilling, G. (1996) 'Release and ecological impact of algicidal hydrolysable polyphenols in *Myriophyllum spicatum*', *Phytochemistry*, 41(1), pp. 133–138. doi: 10.1016/0031-9422(95)00598-6.

Hantula, J., Dusabenyagasani, M. and Hamelin, R. C. (1996) 'Random amplified microsatellites (RAMS) — a novel method for characterizing genetic variation within fungi', *European Journal of Forest Pathology*, 26, pp. 159–166. doi: 10.1111/j.1439-0329.1996.tb00720.x.

Hering, D. *et al.* (2010) 'The European Water Framework Directive at the age of 10: A critical review of the achievements with recommendations for the future', *Science of the Total Environment*. Elsevier B.V., 408(19), pp. 4007–4019. doi: 10.1016/j.scitotenv.2010.05.031.

Honnay, O. *et al.* (2010) 'Patterns of population genetic diversity in riparian and aquatic plant species along rivers', *Journal of Biogeography*, 37(9), pp. 1730–1739. doi: 10.1111/j.1365-2699.2010.02331.x.

Huffman, D. L. and O'Halloran, T. V. (2001) 'Function, structure, and mechanism of intracellular copper trafficking proteins.', *Annual review biochemistry*, 70, pp. 677–701.

Huot, B. *et al.* (2014) 'Growth-defense tradeoffs in plants: A balancing act to optimize fitness', *Molecular Plant*. The Authors. All rights reserved., 7(8), pp. 1267–1287. doi: 10.1093/mp/ssu049.

Jiao, W. *et al.* (2012) 'Environmental risks of trace elements associated with long-term phosphate fertilizers applications: A review', *Environmental Pollution*. Elsevier Ltd, 168, pp. 44–53. doi: 10.1016/j.envpol.2012.03.052.

Khellaf, N. and Zerdaoui, M. (2010) 'Growth, photosynthesis and respiratory response to

copper in *Lemna minor* : a potential use of duckweed in biomonitoring', *J. Environ. Health. Sci. Eng*, 7(2), pp. 299–306.

Knauert, S. *et al.* (2010) 'Phytotoxicity of atrazine, isoproturon, and diuron to submersed macrophytes in outdoor mesocosms', *Environmental Pollution*. doi: 10.1016/j.envpol.2009.07.023.

Kupper, H., Kupper, F. and Spiller, M. (1996) 'Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants', *Journal of Experimental Botany*, 47(295), pp. 259–266.

Loreau, M. and Hector, A. (2001) 'Partitioning selection and complementary in biodiversity experiments', *Nature*, 412(July), pp. 72–76. doi: 10.1038/35083573.

Macinnis-Ng, C. M. O. and Ralph, P. J. (2003) 'Short-term response and recovery of *Zostera capricorni* photosynthesis after herbicide exposure', *Aquatic Botany*, 76(1), pp. 1–15. doi: 10.1016/S0304-3770(03)00014-7.

Maxwell, K. and Johnson, G. N. (2000) 'Chlorophyll fluorescence - a practical guide', *Journal of Experimental Botany*, 51(345), pp. 659–668.

Moss, B. (2008) 'Water pollution by agriculture', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1491), pp. 659–666. doi: 10.1098/rstb.2007.2176.

Murchie, E. H. and Lawson, T. (2013) 'Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications', *Journal of Experimental Botany*, 64(13), pp. 3983–3998. doi: 10.1093/jxb/ert208.

Nei, M. and Li, W.-H. (1979) 'Mathematical model for studying genetic variation in terms of restriction endonucleases.', *Proceedings of the National Academy of Sciences of the United States of America*, 76(10), pp. 5269–5273. doi: 10.1073/pnas.76.10.5269.

Nõges, P. *et al.* (2016) 'Quantified biotic and abiotic responses to multiple stress in freshwater, marine and ground waters', *Science of the Total Environment*, 540, pp. 43–52. doi: 10.1016/j.scitotenv.2015.06.045.

OECD (2006) 'OECD 221 Guidelines for testing of chemicals : Lemna sp. Growth Inhibition Test'.

OECD (2014) 'OECD TG 239 Guidelines for the testing of chemicals water-sediment

596 *Myriophyllum spicatum* toxicity test INTRODUCTION'.

597 Pádua, M. *et al.* (2010) 'Effects of copper on the photosynthesis of intact chloroplasts:
598 Interaction with manganese', *Physiologia Plantarum*, 138(3), pp. 301–311. doi:
599 10.1111/j.1399-3054.2009.01335.x.

600 Peng, H. *et al.* (2012) 'Differences in copper accumulation and copper stress between eight
601 populations of *Haumaniastrum katangense*', *Environmental and Experimental Botany*, 79, pp.
602 58–65. doi: 10.1016/j.envexpbot.2011.12.015.

603 Pollux, B. J. A. *et al.* (2009) 'Gene flow and genetic structure of the aquatic macrophyte
604 *Sparganium emersum* in a linear unidirectional river', *Freshwater Biology*, 54(1), pp. 64–76.
605 doi: 10.1111/j.1365-2427.2008.02100.x.

606 Printz, B. *et al.* (2016) 'Copper Trafficking in Plants and Its Implication on Cell Wall
607 Dynamics', *Frontiers in Plant Science*, 7(May), pp. 1–16. doi: 10.3389/fpls.2016.00601.

608 Reusch, T. B. H. and Hughes, a R. (2006) 'The Emerging Role of Genetic Diversity for
609 Ecosystem Functioning : Estuarine Macrophytes as Models', *Estuaries and Coasts*, 29(1), pp.
610 159–164. doi: 10.1007/BF02784707.

611 Ribolzi, O. *et al.* (2002) 'Speciation and origin of particulate copper in runoff water from a
612 Mediterranean vineyard catchment', *Environmental Pollution*, 117(2), pp. 261–271. doi:
613 10.1016/S0269-7491(01)00274-3.

614 Ritz, C. *et al.* (2015) 'Dose-response analysis using R', *PLoS ONE*, 10(12), pp. 1–13. doi:
615 10.1371/journal.pone.0146021.

616 Santamaría, L. (2002) 'Why are most aquatic plants widely distributed? Dispersal, clonal
617 growth and small-scale heterogeneity in a stressful environment', *Acta Oecologica*, 23(3), pp.
618 137–154. doi: 10.1016/S1146-609X(02)01146-3.

619 Sgrò, C. M., Lowe, A. J. and Hoffmann, A. A. (2011) 'Building evolutionary resilience for
620 conserving biodiversity under climate change', *Evolutionary Applications*, 4(2), pp. 326–337.
621 doi: 10.1111/j.1752-4571.2010.00157.x.

622 Del Signore, A. *et al.* (2016) 'Development and application of the SSD approach in scientific
623 case studies for ecological risk assessment', *Environmental Toxicology and Chemistry*, 35(9),
624 pp. 2149–2161. doi: 10.1002/etc.3474.

Silander, J. A. (1985) ‘The genetic basis of the ecolgical amplitude of Spartina patens. II. Variance and correlation analysis’, *Evolution*, 39(5), pp. 1034–1052.

Sjöqvist, C. O. and Kremp, A. (2016) ‘Genetic diversity affects ecological performance and stress response of marine diatom populations’, *The ISME Journal*, 10(11), pp. 2755–2766. doi: 10.1038/ismej.2016.44.

Sree, K. S. *et al.* (2015) ‘Natural variance in salt tolerance and induction of starch accumulation in duckweeds’, *Planta*, 241(6), pp. 1395–1404. doi: 10.1007/s00425-015-2264-x.

Steam, S. C. (2012) ‘The Evolutionary significance of phenotypic plasticity’, *BioScience*, 39(7), pp. 436–445.

Tlili, A. *et al.* (2016) ‘Pollution-induced community tolerance (PICT): towards an ecologically relevant risk assessment of chemicals in aquatic systems’, *Freshwater Biology*, 61(12), pp. 2141–2151. doi: 10.1111/fwb.12558.

Triest, L. *et al.* (2010) ‘Genetic differentiation of submerged plant populations and taxa between habitats’, *Hydrobiologia*, 656(1), pp. 15–27. doi: 10.1007/s10750-010-0434-1.

Vasseur, L. and Aarssen, L. W. (1992) ‘Phenotypic plasticity in *Lemna minor* (Lemnaceae)’, *Plant Systematics and Evolution*, 180, pp. 205–219. doi: 10.1007/BF00941151.

Vitasse, Y. *et al.* (2010) ‘Quantifying phenological plasticity to temperature in two temperate tree species’, *Functional Ecology*, 24(6), pp. 1211–1218. doi: 10.1111/j.1365-2435.2010.01748.x.

Voulvoulis, N., Arpon, K. D. and Giakoumis, T. (2017) ‘The EU Water Framework Directive: From great expectations to problems with implementation’, *Science of the Total Environment*. The Authors, 575, pp. 358–366. doi: 10.1016/j.scitotenv.2016.09.228.

Weyl, P. S. R. and Coetzee, J. A. (2016) ‘Morphological variations in southern African populations of *Myriophyllum spicatum*: Phenotypic plasticity or local adaptation?’, *South African Journal of Botany*. South African Association of Botanists, 103, pp. 241–246. doi: 10.1016/j.sajb.2015.07.016.

Wu, Z. *et al.* (2016) ‘Influence of geography and environment on patterns of genetic differentiation in a widespread submerged macrophyte, Eurasian watermilfoil (*Myriophyllum spicatum L.*, Haloragaceae)’, *Ecology and Evolution*, 6(2), pp. 460–468. doi: 10.1002/ece3.1882.

Xue, H. *et al.* (2012) ‘Genetic diversity and geographic differentiation analysis of duckweed using inter-simple sequence repeat markers’, *Molecular Biology Reports*, 39(1), pp. 547–554. doi: 10.1007/s11033-011-0769-3.

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

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

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

Species	***L. minor***		***M. spicatum***		***C. demersum***	
EC_{50} Endpoints	**RGR_{fm}** **$RGRf_{ronds}$**	**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

683 **Table 3**: Half maximal effective concentrations (EC_{50}, 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 EC_{50} values in %, calculated within species
688 (based on averaged EC_{50} values per genotype and endpoint). Interspecific variability was
689 assessed from the R^2 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*.

691

Species	Genotypes	EC_{50} values RGR_{fm}	EC_{50} values RGR_{fronds} /RGR_{length}	EC_{50} values F_v:F_m
L. minor (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**
M. spicatum (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
C. demersum (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**

692

Legends

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

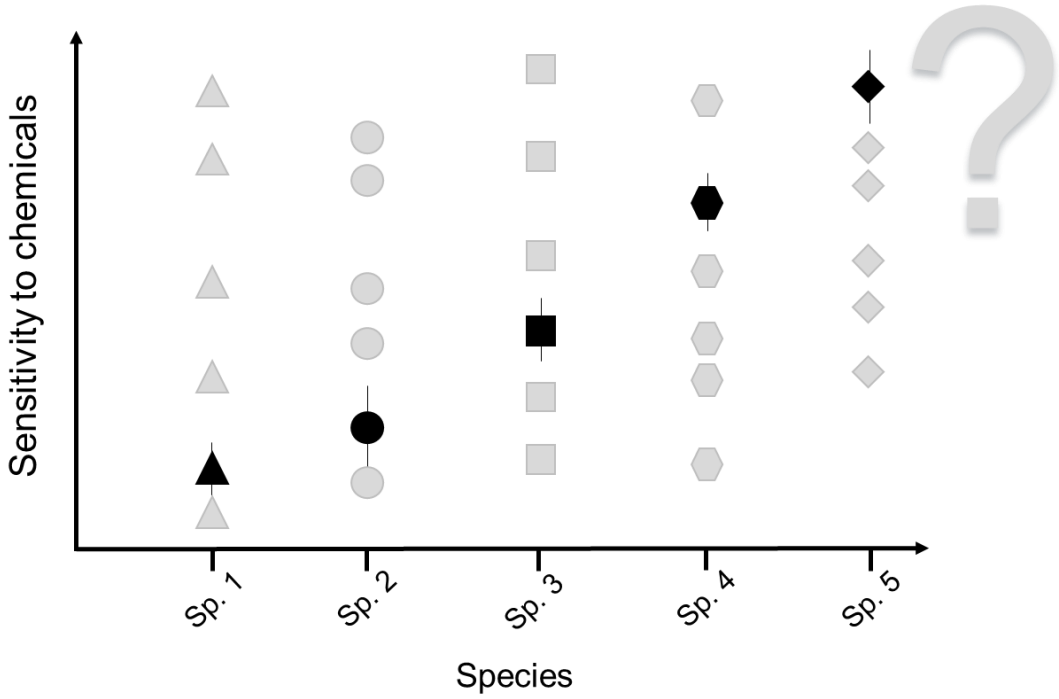
Figure 2: Concentration-response curves for three genotypes of *L. minor* exposed to copper, with relative growth rates (RGR) based on fresh mass (**A**) and frond number (**B**) after 7 days of exposure, and (**C**) F_v:F_m after 96h. Curves were fitted with non-linear log-logistic models with 4 parameters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

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

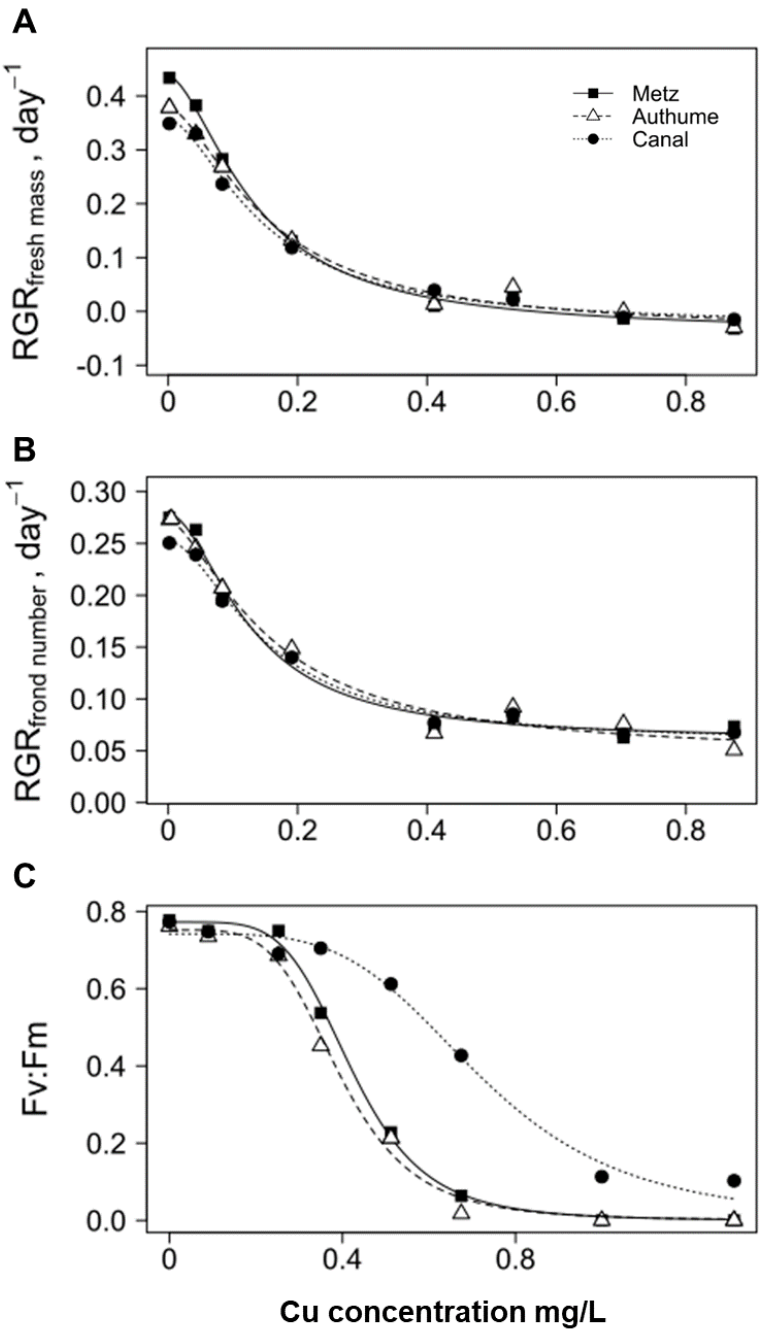
Figure 4: Concentration-response curves for two to three genotypes of *C. demersum* exposed to copper, with relative growth rates (RGR) based on fresh mass (**A**) and shoot length (**B**) after 14 days of exposure, and F_v:F_m (**C**) after 96h. Curves were fitted with non-linear log-logistic models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

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

Fig.1

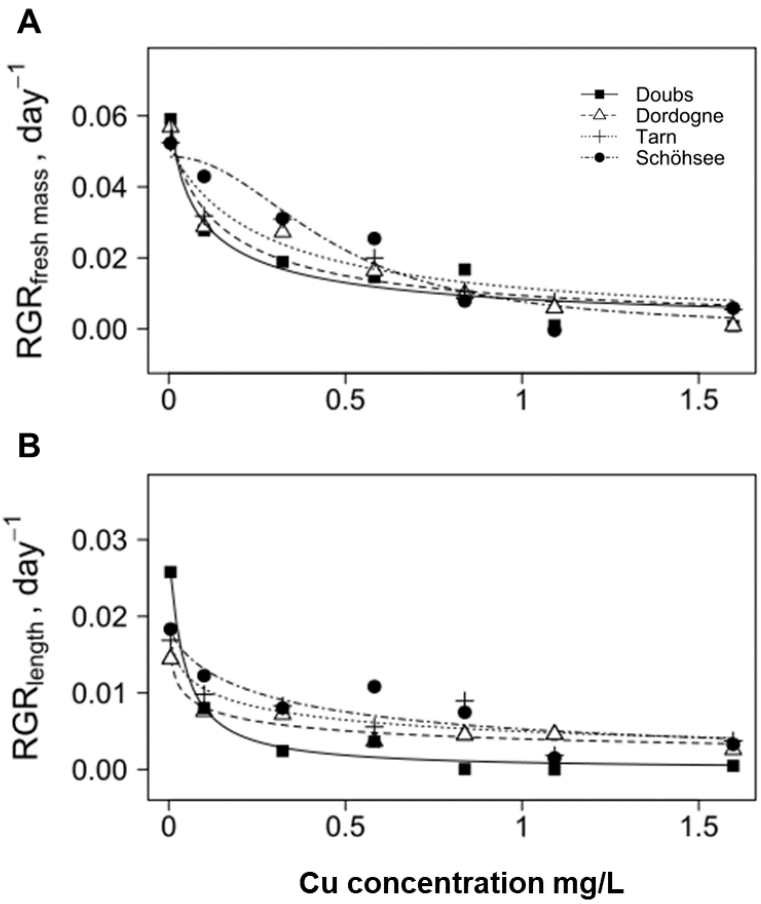


732 **Fig. 2**


A
B
C
Metz
Authume
Canal
RGR fresh mass , day−1
RGR frond number , day−1
Fv:Fm
Cu concentration mg/L

733

734 **Fig. 3**

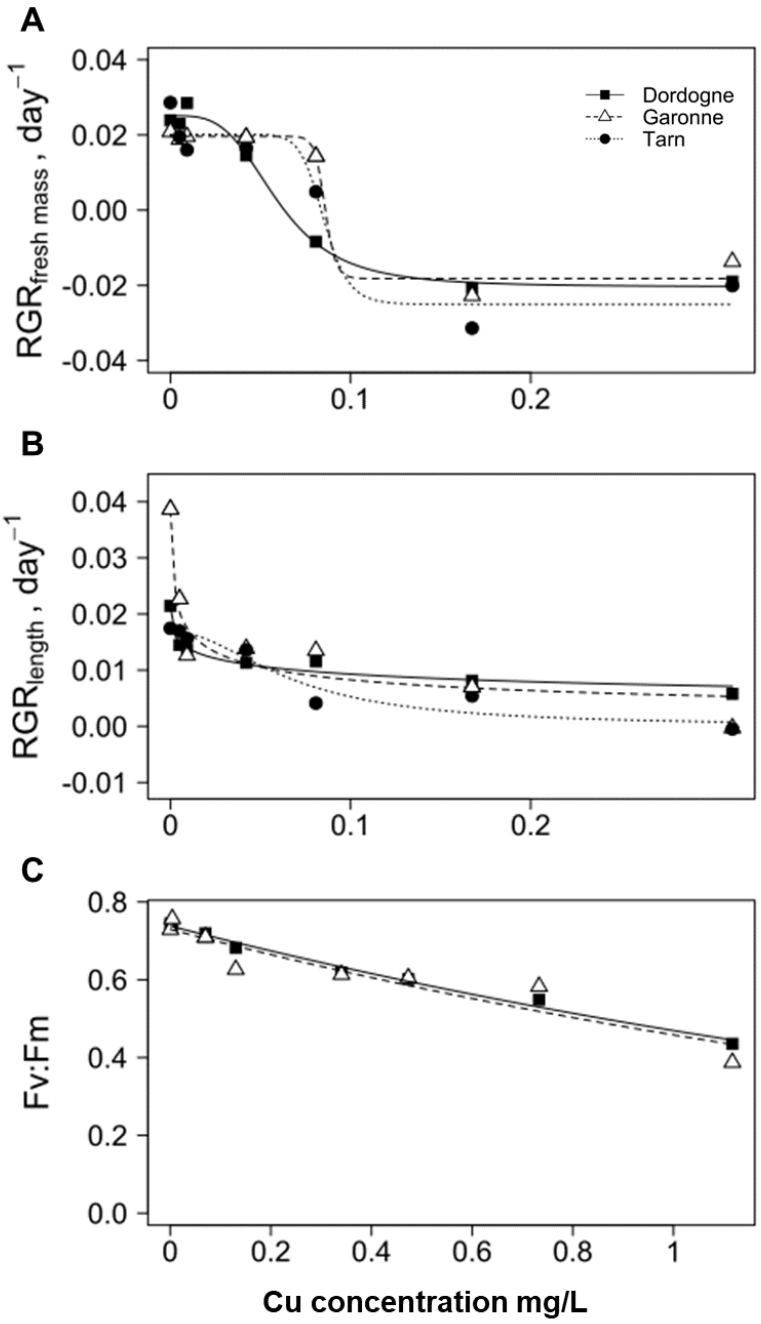


735

736

737

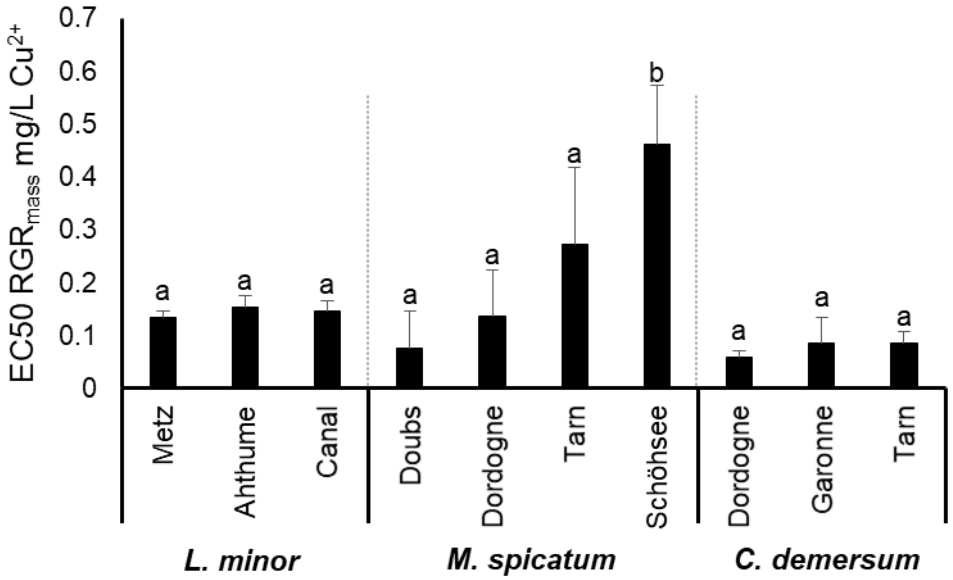
738 **Fig. 4**



739

740

Fig. 5


EC50 RGR$_{mass}$ mg/L Cu^{2+}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
a
a
b
a
a
a
Metz
Ahthume
Canal
Doubs
Dordogne
Tarn
Schöhsee
Dordogne
Garonne
Tarn
L. minor
M. spicatum
C. demersum

SUPPLEMENTARY DATA

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

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

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

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.

		Myriophyllum spicatum			Ceratophyllum demersum			Lemna minor		
Primers*	Sequences ** (5'-3')	T_A	No. scored fragments	No. polymorphic fragments	T_A	No. scored fragments	No. polymorphic fragments	T_A	No. scored fragments	No. polymorphic 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$(CGA)_5$	-	-	-	-	-	-	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.

796

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