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Variabilité intraspécifique de la sensibilité des macrophytes aquatiques à la contamination chimique : l'exemple du cuivre.

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ABSTRACT

Intraspecific variability plays a pivotal role in short and long term responses of species to environmental fluctuations. This variability, expressed through different traits of individuals, can potentially influence species sensitivity to chemical contamination. This intraspecific variability is currently not taken into account in ecotoxicological risk assessment, whereas it can mislead its results. To examine this hypothesis, the importance of intraspecific variability in the response to copper (Cu) was quantified in controlled conditions for three aquatic macrophyte species, *Lemna minor*, *Myriophyllum spicatum* and *Ceratophyllum demersum*. Variations among genotypes of each of these 3 species were compared to interspecific variability. Results have highlighted a significant genotypic variability, whose importance depends on the species considered. Indeed, *L. minor* demonstrated a low variability, contrarily to *M. spicatum* whose variability in growth inhibition by Cu was higher than interspecific differences. In order to specify the extent and the mechanisms of genotypic variability in *M. spicatum*, other experiments involving measurements of life-history traits have been conducted on 7 genotypes exposed to Cu. Results showed that some genotypes were up to eightfold more sensitive to Cu than others (at concentrations ranging between 0.15 and 0.5 mg/L). These differences in sensitivity were partly explained by the traits measured, but physiological or transcriptomic endpoints may explain more precisely the source of these differences in sensitivity. Finally, 3 experiments with fluctuations in nutrient concentrations, light intensity and Cu pre-exposure have demonstrated that phenotypic plasticity plays an important role in *L. minor* sensitivity to Cu. Indeed, the weakening of individuals, as a result of unfavorable environmental conditions, can lead to a two-fold increase in sensitivity to Cu. All these results demonstrated that intraspecific variability, whether it comes from genotypic variations or is linked to phenotypic plasticity, was in general lower than interspecific variability for the species and endpoints studied. However, its extent can vary depending on the species. It can therefore significantly influence aquatic macrophyte sensitivity to chemical contamination, and it would be relevant to account for it in ecotoxicological risk assessment.

Keywords: Copper, ecotoxicological risk assessment, aquatic macrophyte, intraspecific variability, genotypic variation, phenotypic plasticity

RESUME

La variabilité intraspécifique fait partie intégrante de la réponse à court et à long terme des organismes vivants aux fluctuations environnementales. Cette variabilité, exprimée au travers de différents traits des individus, peut potentiellement influencer la sensibilité des espèces à une contamination chimique. La variabilité intraspécifique n'est pas, à l'heure actuelle, prise en compte en évaluation des risques écotoxicologiques, alors même qu'elle pourrait en biaiser les résultats. Pour examiner cette hypothèse, l'importance de la variabilité intraspécifique dans la réponse au cuivre (Cu) a été quantifiée en conditions contrôlées pour trois espèces de macrophytes aquatiques, *Lemna minor, Myriophyllum spicatum* et *Ceratophyllum demersum*. Les variations entre génotypes de chacune de ces 3 espèces ont été comparées à la variabilité interspécifique. Les résultats ont mis en évidence une variabilité génotypique significative, dont l'importance dépend de l'espèce considérée. En effet, *L. minor* a montré une faible variabilité, au contraire de *M. spicatum* dont la variabilité de l'inhibition de croissance par le Cu est supérieure aux différences interspécifiques. Afin de préciser l'étendue et les mécanismes de la variabilité génotypique chez *M. spicatum*, d'autres expériences impliquant des mesures de traits d'histoire de vie ont été réalisées sur 7 génotypes exposés au Cu. Les résultats ont montré que certains génotypes étaient jusqu'à 8 fois plus sensibles au Cu à des concentrations allant de 0.15 à 0.5 mg/L). Ces différences de sensibilité sont en partie expliquées par les traits mesurés, mais des mesures physiologiques et/ou des approches en transcriptomique devraient pouvoir expliquer de façon plus consistante la source de ces différences de sensibilité. Enfin, 3 expériences faisant varier respectivement la teneur en nutriments, l'intensité lumineuse et la préexposition au Cu, ont démontré que la plasticité phénotypique joue un rôle majeur dans la sensibilité au Cu chez *L. minor*. En effet, l'affaiblissement des individus, résultant des conditions environnementales défavorables, peut conduire au doublement de la sensibilité de *L. minor* au Cu. L'ensemble des résultats obtenus montre donc que la variabilité intraspécifique, qu'elle soit d'origine génotypique ou liée à la plasticité phénotypique, demeure en règle générale inférieure à la variabilité interspécifique concernant les traits et les espèces étudiés. Cependant, son importance varie selon l'espèce considérée. Elle peut donc influer significativement sur la sensibilité des macrophytes aquatiques à la contamination chimique, et gagnerait donc à être prise en compte dans le cadre de l'évaluation des risques écotoxicologiques.

Mots clés : Cuivre, évaluation des risques écotoxicologiques, macrophyte aquatique, variabilité intraspécifique, variation génotypique, plasticité phénotypique

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INTRODUCTION

(Version française résumée)

1. Impacts de l'homme sur l'environnement

Durant les deux derniers siècles, la révolution industrielle a mené à une forte augmentation de la population humaine (**Figure 1.1**). Pour répondre à ses besoins toujours croissants, des changements, notamment dans les pratiques agricoles, ont été mis en place, et ont mené à la révolution verte. Ainsi, ces cinquante dernières années, la population a plus que doublé, et la production céréalière a triplé, avec seulement 30 % d'augmentation de terres cultivées (Pingali 2012). La croissance de la population humaine, ainsi que la modification des modes de vie, ont mené à l'augmentation des besoins pour les ressources, l'énergie, la nourriture, le logement, les terres cultivables. Réponde à ces besoins demeure un challenge à l'heure actuelle (Goulding et al. 2008; Mozner 2013; García-Mier et al. 2013). De plus, cela a mené à l'augmentation des déchets sous-produits. Jusqu'à maintenant, la croissance humaine a été exponentielle, et son impact sur les écosystèmes a suivi.

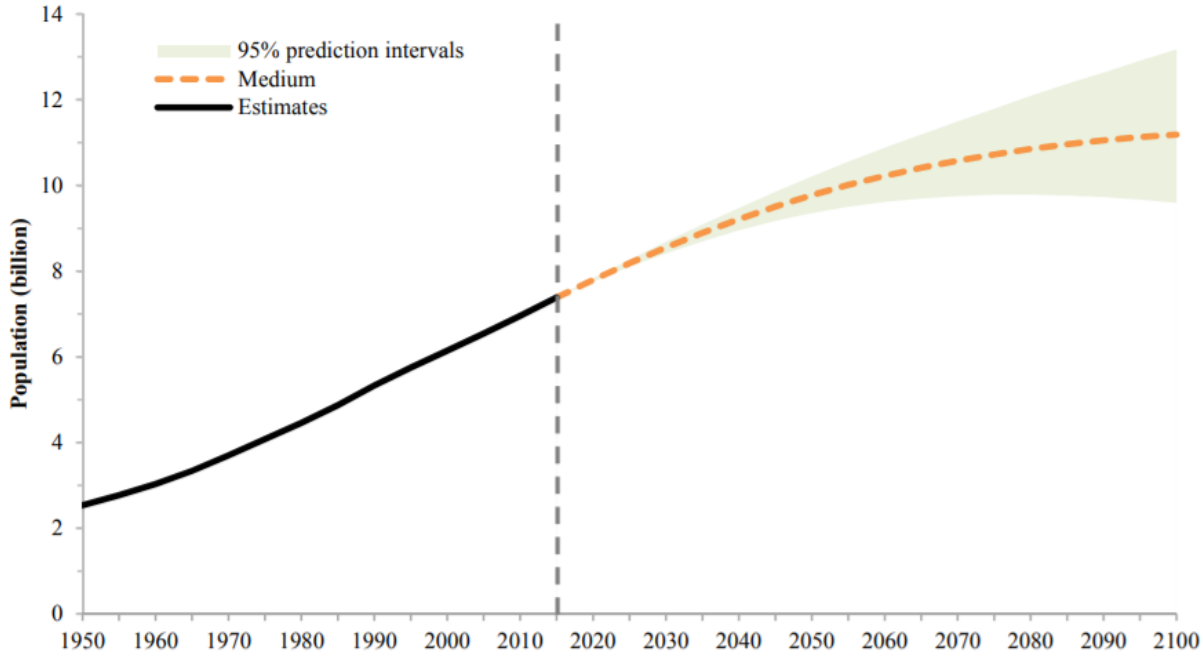


Figure 1.1. Evolution de la population mondiale entre 1950 et 2100. Source: Secrétariat des Nations Unies, prévision de la population mondiale, révisions de 2017 (United Nations 2017).

Les activités humaines constituent une menace majeure pour la biodiversité de la planète, ainsi que pour la santé des écosystèmes (Tilman and Lehman 1987; Dubois et al. 2018; Dodds et al. 2013). Entre autre, ces effets nocifs sur l'environnement sont liés à un changement d'utilisation des terres (urbanisation, industrie minière), qui va perturber et morceler les écosystèmes présents. Ces activités produisent également une multitude de polluants tels que

des hydrocarbures aromatiques polycycliques (HAP), des éléments traces métalliques (ETM), des nanoparticules, des hormones ou encore des microplastiques, avec de nombreuses voies d'entrée dans les écosystèmes.

2. L'environnement aquatique, réceptacle ultime de la contamination chimique

Les eaux douces occupent seulement 0.8 % de la surface planétaire, mais sont l'habitat d'environ 6 % des espèces existantes (Woodward et al. 2010).

En raison de différents processus, tels que l'érosion des sols, la volatilisation atmosphérique et la redéposition des polluants en suspension, les écosystèmes aquatiques sont le réceptacle final de la contamination chimique (**Figure 2.1**, Ærtebjerg et al. 2003; Woodward et al. 2010). Selon Dodds et al. (2013), les impacts anthropiques sur les écosystèmes d'eau douce sont globaux. Ils peuvent entre autres altérer le flux d'écoulement, causer des invasions biologiques, des altérations thermiques, causer des extinctions biologiques ou encore des contaminations chimiques et ainsi menacer l'équilibre fragile de ces écosystèmes, et donc à plus ou moins long terme mettre en péril les services écosystémiques rendus. Les services écosystémiques sont définis comme les bénéfices que les humains retirent des écosystèmes, tels que la production d'oxygène, de biomasse (bois, nourriture pour l'homme ou pour les animaux d'élevage), ou encore l'activité des pollinisateurs pour les cultures (Seppelt et al. 2011). Enfin, les humains utilisent également une portion substantielle de cette ressource en eau douce, que ce soit pour leur survie ou leurs activités domestiques et industrielles (Dodds et al. 2013).

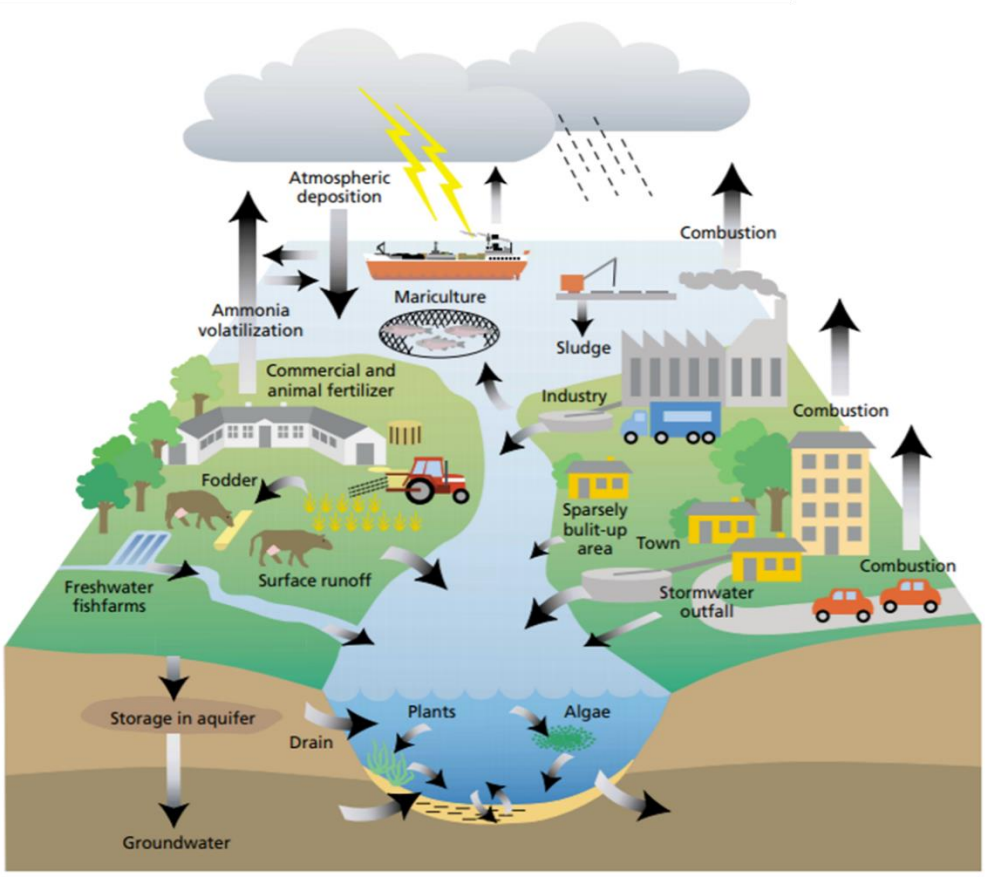


Figure 2.1. Voies d'entrée de la contamination chimique dans les environnements aquatiques (Ærtebjerg et al. 2003).

3. Ecotoxicologie et évaluation des risques écotoxicologiques

A. Prise de conscience et développement de l'écotoxicologie

Certains événements ont contribué à la prise de conscience globale de l'effet néfaste que nos activités pouvaient avoir sur les écosystèmes. Entre autres, l'explosion de la première bombe atomique dans le désert du Nouveau-Mexique en 1945, qui marque l'aboutissement du projet Manhattan et la contamination des écosystèmes par des composés radioactifs. Un autre exemple fut la guerre du Vietnam, qualifiée de guerre écologique car elle détruisit durablement des écosystèmes au moyen d'herbicides de synthèse (Neilands 1970; Prăvălie 2014). Certaines publications ont également eu un retentissement très important, tels que le livre 'Silent Spring' de Rachel Carson, paru en 1962, qui a démontré que l'arme atomique n'était pas la seule à menacer de détruire la vie, et que les pesticides pouvaient à long terme conduire à des résultats similaires. Ce livre a notamment mené à la création des premières lois environnementales aux Etats-Unis et à la formation de l'agence de protection environnementale (US-EPA). En réponse

à cette prise de conscience croissante, un nouveau champ disciplinaire s'est développé dans les années 1970, l'écotoxicologie, qui vise à étudier la toxicité des activités humaines sur l'environnement (Truhaut 1977).

B. Politiques environnementales

Au niveau politique, afin de limiter l'impact de l'homme sur l'environnement, plusieurs mesures ont été mises en place, à la fois pour déterminer la toxicité de molécules manufacturées par l'homme, et pour évaluer et diminuer la pollution potentielle émise par certaines pratiques agricoles et/ou industrielles. Notamment, la Directive Cadre sur l'Eau européenne a été implémentée en octobre 2000, afin de limiter l'impact des activités humaines sur les écosystèmes aquatiques, et augmenter leur qualité (European Commission 2000). Par ailleurs, le règlement REACH (Enregistrement, Evaluation, Autorisation, Restrictions des Substances Chimiques règlement n°1907/2006) a été mis en place en 2007 afin d'évaluer la toxicité des produits chimiques présents sur le marché, ou nouvellement créés. Pour ce faire, des outils spécifiques ont donc été développés faisant l'objet de protocoles standardisés (pour plus de détails: Chapitre 1.3) se focalisant généralement sur des unités taxonomiques et/ou niveaux trophiques différents. L'évaluation des risques écotoxicologiques permet ainsi une approche intégrative, notamment au travers de tests en laboratoire sur des espèces modèles, pour déterminer la toxicité potentielle sur un écosystème donné des produits chimiques présent sur le marché.

C. Les macrophytes, un modèle biologique aquatique pertinent

De par leur place dans les écosystèmes aquatiques en tant que producteurs primaires, leurs implications dans les cycles biogéochimiques ainsi que leur sensibilité aux paramètres environnementaux, les macrophytes sont des organismes très pertinents pour évaluer l'impact potentiel de molécules sur les écosystèmes. En effet, ces organismes chlorophylliens visibles à l'œil nu sont pour la plupart sessiles, et sont de ce fait, utilisés en tant que bioindicateurs de l'état de santé des écosystèmes aquatiques. En d'autres termes, ils attestent de la qualité physico-chimique d'un écosystème de par leur présence, leur diversité ainsi que leur réponse métabolique. Ils sont également utilisés en biosurveillance car l'analyse de leurs tissus reflète souvent le degré de contamination de leur environnement (Haury *et al.*, 2001; Ferrat, Pergent-Martini et Roméo, 2003). Si leur capacité à accumuler les polluants est un atout pour utiliser

ces organismes en tant que sentinelles au sein des écosystèmes, c'est également la raison pour laquelle les macrophytes sont parmi les premiers organismes impactés par les contaminations d'origine anthropique.

Ces organismes sont faciles à manipuler en laboratoire, et sont aujourd'hui incontournables dans les tests de toxicité en laboratoire. De ce fait plusieurs protocoles standardisés ont été mis au point sur les macrophytes par l'Organisation de Coopération et de Développement Economique, ou OCDE (OECD 2006, 2014a, 2014b).

D. La variabilité intraspécifique en évaluation des risques écotoxicologiques

L'impact des polluants organiques comme des ETM sur les macrophytes aquatiques a été démontré dans diverses publications (Pflugmacher et al. 1997; Samecka-Cymerman and Kempers 2004; Knauert et al. 2010; Ladislas et al. 2012). Afin d'améliorer continuellement les démarches d'évaluation des risques écotoxicologiques, leur pertinence et leur transposition *in situ*, de nombreuses études essaient de rendre compte des facteurs qui ne sont pas encore pris en compte dans ces approches (Belanger et al. 2017; Maltby et al. 2005; Forbes and Calow 2002; Pathiratne and Kroon 2016). C'est dans ce contexte que s'inscrit ce travail de thèse, qui vise à étudier l'importance de la variabilité intraspécifique de la réponse des macrophytes aquatiques face à une contamination chimique.

La variabilité intraspécifique peut être définie comme la variabilité observable entre des individus d'une même espèce. Cette variabilité est le fruit de différences génétiques entre ces individus, et de l'influence de l'environnement sur l'expression de leur patrimoine génétique. Elle est considérée comme une étape clé dans l'évolution des espèces et leur adaptation à un nouvel environnement, et les différents mécanismes impliqués sont expliqués plus en détails dans le chapitre I.4. Cette variabilité intraspécifique n'est pas prise en compte en évaluation des risques écotoxicologiques à l'heure actuelle, et elle peut potentiellement impacter de façon significative les résultats des tests en laboratoire (Chapitre **I.3**). En effet, beaucoup d'études font part de l'influence des facteurs environnementaux, tels que le pH ou la teneur en nutriments, sur la morphologie et la physiologie des plantes aquatiques (Puijalon et al. 2008; Vasseur and Aarssen 1992; Gratani 2014). D'autres études font état de l'importance de la diversité génétique au sein des macrophytes aquatiques, et des différences morphologiques et physiologiques entre populations qui peuvent en résulter (Eckert et al. 2008; Pollux et al. 2007; Othman et al. 2007). Cependant, à l'heure actuelle, très peu d'études ont cherché à déterminer

l'importance de la variabilité intraspécifique dans la réponse des plantes aquatiques aux contaminations chimiques. Cette question est pourtant très pertinente, si l'on considère l'importance de la pollution des écosystèmes par les activités humaines.

Ce projet de thèse a pour but de pallier ce manque de connaissances, et de déterminer l'impact que la variabilité intraspécifique pourrait avoir sur les procédures d'évaluation des risques écotoxicologiques telles que nous les connaissons.

Dans cette optique, nous avons cherché à déterminer l'importance de cette variabilité intraspécifique chez des plantes aquatiques exposées à un élément trace métallique, le cuivre.

Cette thèse s'articule autour de trois questions principales:

1) Quelle est l'étendue de la variabilité intraspécifique dans la réponse des plantes aquatiques à la contamination chimique ? J'ai cherché à répondre à cette question pour trois espèces dans le chapitre **III**.
2) Cette variabilité intraspécifique chez les plantes aquatiques est-elle expliquée par leur variabilité génotypique ? C'est ce que j'analyse dans le chapitre **IV**, en me focalisant sur une espèce de plante aquatique, le myriophylle en épis (*Myriophyllum spicatum*).
3) La plasticité phénotypique peut-elle moduler la réponse des plantes aquatiques aux contaminations chimiques ? Les résultats que j'ai obtenus pour répondre à cette question, concernant une autre espèce, la lentille d'eau (*Lemna minor*), sont présentés dans le chapitre **V**.

CHAPTER I

INTRODUCTION

1. Aquatic macrophytes

A. Definition and evolutionary history

Aquatic macrophytes refer to large photosynthetic organisms visible to the naked eye, and adapted to partial or total life in aquatic habitats. They are represented in several plant clades, the main ones being macroalgae (Chlorophyta and Charophyta, or green algae, Xanthophyta, or yellow-green algae, Rhodophyta, or red algae, Cyanobacteria, or blue-green algae, and Phaeophyta, or brown algae), mosses (Bryophyta), ferns (Pteridophyta) and seed-bearing plants (Spermatophyta) (Haury et al. 2001; Chambers et al. 2008). Vascular macrophytes are found among ferns and seed-bearing plants.

In the early Paleozoic (541 to 251 million years ago), ancestral marine plants colonized land, giving rise to the evolution of vascular plants (Chambers et al. 2008). As angiosperms diversified and thrived in terrestrial habitats, some species came back to aquatic environments (freshwater and marine), and became aquatic. The transition back to an aquatic life has been achieved by only 3 % of the approximately 350,000 angiosperm species (Cook 1999). According to the same study, probably 252 events of independent colonization have occurred, with at least seven reversion events in ferns, and 204-245 reversion events in angiosperms.

B. General traits of vascular aquatic macrophytes

Reproductive traits and other life-history traits of aquatic angiosperms are tightly associated with their growth form (*e.g.* root disappearance, free-floating), as they represent different degrees of aquatic life adaptation, and are convergent among aquatic angiosperms (Thomaz et al. 2008). Angiosperms is the main group representing vascular aquatic macrophytes. They can be divided in five main life forms (**Figure 1.1**):

- Emergent (also known as helophyte), with plants being rooted in the sediments with above parts extending into the air, such as *Typha* species,
- Floating-leaved, with plants rooted in the sediments with leaves floating at the water surface, such as *Nymphaea* species,
- Rooted submerged, with plants that are rooted into the sediments and are completely submerged, such as *Myriophyllum* species,
- Free-submerged, with plants non rooted to sediments, and floating freely in the water column, such as *Ceratophyllum* species,

- Free-floating, with plants floating at the water surface without being rooted to the sediments, such as *Lemna* species.

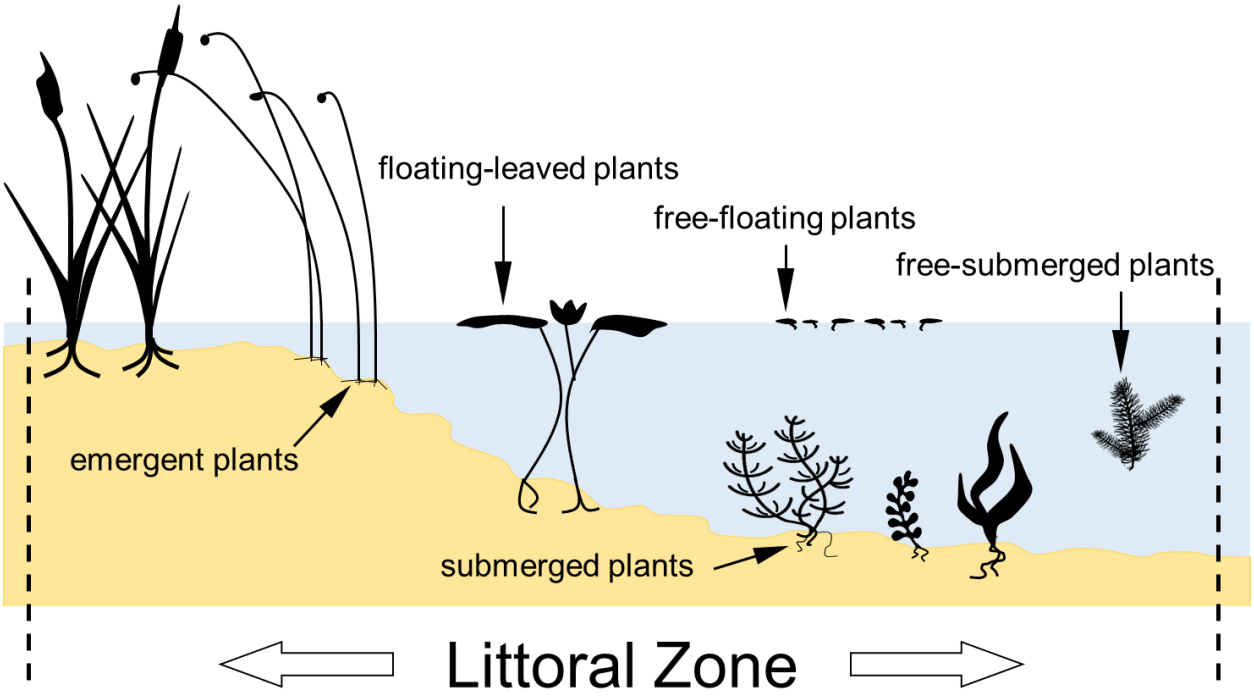


Figure 1.1. Zonation of the different vascular aquatic plants, depending on their life history traits: emergent plants, floating-leaved plants, submerged plants, free-submerged plants and free-floating plants.

As a consequence of this return to aquatic life, many physiological and morphological adaptations occurred in aquatic angiosperms, in order to cope with limited CO_2 (*e.g.* use of bicarbonates) and reduced light and oxygen availability (Chambers et al. 2008). For instance, they have large leaf surface, often highly dissected, to increase surface area (*e.g. Ceratophyllum demersum*, *Myriophyllum spicatum*) in order to enhance light, carbon and nutrient uptake through an increased surface contact with the environment (Bornette and Puijalon 2009). They have a thin cuticle, and also show a high concentration of chloroplasts near the leaf surface to cope with the decreased light availability in water. They are usually poorly lignified, as water preserves plants from gravitational stress, and they are characterized by the presence of aerenchyma, a plant tissue which forms spaces or air channels in the leaves, stems and roots, and increases oxygen flux from shoots to roots. Macrophytes growing in shallow water can overcome aqueous inorganic carbon limitations for photosynthesis through the absorption of atmospheric CO_2 with aerial or floating leaves.

Their dispersal partly relies on water drift, thus on seed buoyancy and on plant ability to break themselves up and regrow from broken fragments, and partly on anemochory and zoochory (*e.g.* by birds or fish). Some species can reproduce under water, relying on underwater transport of pollen, such as *Ceratophyllum demersum*.

Some traits found in submerged species, such as aerial pollination, aquatic pollination and presence of stomata, are interpreted only under an evolutionary perspective.

C. Habitat diversity

Aquatic macrophytes colonize a wide variety of aquatic habitats, from tiny temporary ponds to thermal springs (*e.g. Najas tequefolia*) passing by waterfalls (*e.g. Podostemaceae* family). They are also found in rivers, lakes, lagoons and reservoirs (Thomaz et al. 2008). According to Chambers *et al.* (2008), the diversity of vascular macrophytes is the highest in the Neotropics (984 species), intermediate in the Orient, Nearctic and Afrotropics (664, 644 and 614 species, respectively), lower in the Palearctic and Australasia (497 and 439 species, respectively), and even lower in the Pacific region and Oceanic islands (108 species). Only very few species have been found in the Antarctica, all confined to sub-Antarctic freshwater habitats.

Free-floating and tall species with floating leaves, or forming a canopy just below the water surface, are often the most competitive species for light resource, and dominate when sufficient nutrients are available in the water column, while rooted species are dominant in lotic ecosystems (Bornette and Puijalon, 2009).

D. Ecological services

Aquatic macrophytes are involved in the structure and functioning of aquatic ecosystems. They influence **nutrient cycles** through the transfer of chemical elements from sediments to water, by both active and passive processes, both during their growth phase and during their senescence and decomposition (Magela et al. 2010). Nutrients (phosphorus and nitrogen) and dissolved organic carbon released by aquatic plants are quickly used by micro-algae and bacteria which are free-living or attached to macrophyte surfaces (Sand-Jensen and Borum 1991). They also impact nutrient cycling through the retention of solids (detritus) and nutrients, by their submerged roots and leaves through protection against wave actions (Madsen et al. 2001). Thus, they **protect sediments and riverine soils** from erosion, and can inflect **water**

flow if they form dense canopies. They also influence underwater light availability, hence they interfere with photosynthesis of other organisms.

Aquatic macrophytes have been characterized as an important **food resource** for aquatic organisms, both through dead organic matter for detritivorous organisms, and for living organisms through grazing (Magela et al. 2010). The influence of macrophyte species on populations and communities has been widely studied for a variety of organisms. They foster species diversity, as they are **substrate** for several species of algae and bacteria and can provide shelter for periphyton (Van Donk and Van de Bund 2002), micro- and macroinvertebrates (Schramm and Jirka 1989; Ferreiro et al. 2010; Kouamé et al. 2011), but also interact with fish species (Theel et al. 2008; Schultz and Dibble 2012) and waterbirds (Klaassen and Nolet 2007; Guadagnin et al. 2009; Laguna et al. 2016). To draw a general picture, Scheffer (2004) illustrated the role of aquatic macrophytes as a luxuriant forest full of biodiversity.

Last but not least, some macrophyte species (*e.g*. rice) are widely cultivated for **human consumption** and represent a major food source for many populations.

However, several of the worst **invasive weeds** are aquatic macrophytes, *e.g*., *Myriophyllum spicatum* in North America, *Eichhornia crassipes* in China, *Hydrilla verticillata* in the US, *Ludwigia grandiflora* in Southern Europe (Olden and Tamayo, 2014; Wang *et al.*, 2016; Zhu *et al.*, 2017).

E. Role of macrophytes as bioindicators and biomonitors in aquatic ecosystems

A **bioindicator** is defined as an organism (or a part of an organism) or a community of organisms, that provides qualitative information on the environment, whereas a **biomonitor** is an organism or a community of organisms that provides quantitative information of environmental status (Markert et a. 2003). Some species are also considered as '**sentinel**', as these species accumulate and concentrate pollutants from their surroundings and the analysis of their tissues provides an estimate of the environmentally available concentrations of pollutants (Gerhardt 2011).

The role of **macrophytes** as bioindicator and for biomonitoring has been extensively studied over the years. As primary producers, and due both to their involvement in aquatic ecosystem functioning and their sensitivity to environmental modifications, they are an ideal tool to assess ecosystem health. For instance, Pereira et al. (2012) have demonstrated that

macrophyte communities were relevant bioindicators of limnological conditions of lakes in southern Brazil, as species richness and growth-forms varied depending on nutrients, pH and dissolved oxygen. Furthermore, it was often demonstrated that submerged macrophyte community and diversity respond to changes in the nutrient concentrations of their environment (Kohler and Schneider 2003; Lukács et al. 2009).

To go further, several methodologies based on macrophyte composition, diversity and abundance, have been developed to assess the ecological status of freshwater ecosystems, as tools for the Water Framework Directive (see chapter I.3). For instance, the LEAFPACS method uses macrophyte composition to define ecological quality of rivers and lakes (Willby, Pitt, and Phillips 2012; Penning et al. 2008). Other methods exist, such as the Trophic Index of Macrophytes (TIM) and the Macrophyte Biological Index for Rivers (IBMR) in running waters, or the Macrophyte Index (MI) and the Ecological State Macrophyte Index (ESMI) in lakes (Kohler and Schneider 2003; Fabris et al. 2009; J. Haury et al. 2006; Ciecierska and Kolada 2014).

Many studies focused on the assessment of **chemical pollution** by macrophytes. For instance, Ladislas et al. (2012) have demonstrated that aquatic plants were relevant to assess metal pollution in ecosystems, as plant concentration indicated cumulative effects of environmental pollution from water and sediment. Khellaf and Zerdaoui (2010) have shown that *Lemna minor* was highly relevant in biomonitoring program of copper contamination. Ferrat *et al*., (2003) have suggested that seagrasses showed an early response to environmental pollution, and are thus good bioindicators. Likewise, several species of macrophytes were successfully used in Russia to evaluate trace element contamination of water bodies (Kurilenko and Osmolovskaya 2006). Aquatic macrophytes are therefore highly relevant to assess the toxicity of given molecules, or the impact of agricultural practices through runoffs of crop soils, as well as wastewater treatment quality, among others.

The ability of some species to take up trace elements, as well as to thrive in highly eutrophic waters (*i.e.* rich in ammonia and phosphorus) has led to the development of **depollution practices**, such as phytoremediation to remove pollutants from sediments and water, or for wastewater treatment (Nirmal Kumar et al. 2008; Dosnon-Olette et al. 2011; Nair and Kani, 2016; Newete and Byrne, 2016). The use of macrophytes in phytoremediation of copper has been extensively studied as this metal is broadly found in aquatic ecosystems due to multiple uses, and because its excess is known to cause damages to aquatic organisms (Ha et al. 2009;

Mokhtar et al. 2011; Basile et al. 2012; Sood et al. 2012; Üçüncü et al. 2013; Putra et al. 2015; Costa et al. 2018).

2. Copper fate and toxicity in the environment

A. Generalities

Copper occurs naturally in the Earth crust and topsoils, with concentrations around 24 to 68 mg kg^{-1} and below 30 mg kg^{-1}, respectively (Karczewska et al. 2015).

In the **industry**, Cu is broadly used for its conductive properties. In Europe, according to the European Copper Institute, 50 % of the Cu produced is used in electricity industry, 25 % is used for construction, 10 % for mechanic and thermal exchanges, and 5 % for vehicle manufacturing. Worldwide, Asia is the main user of Cu and use it primarily in construction (**Figure 2.1**).

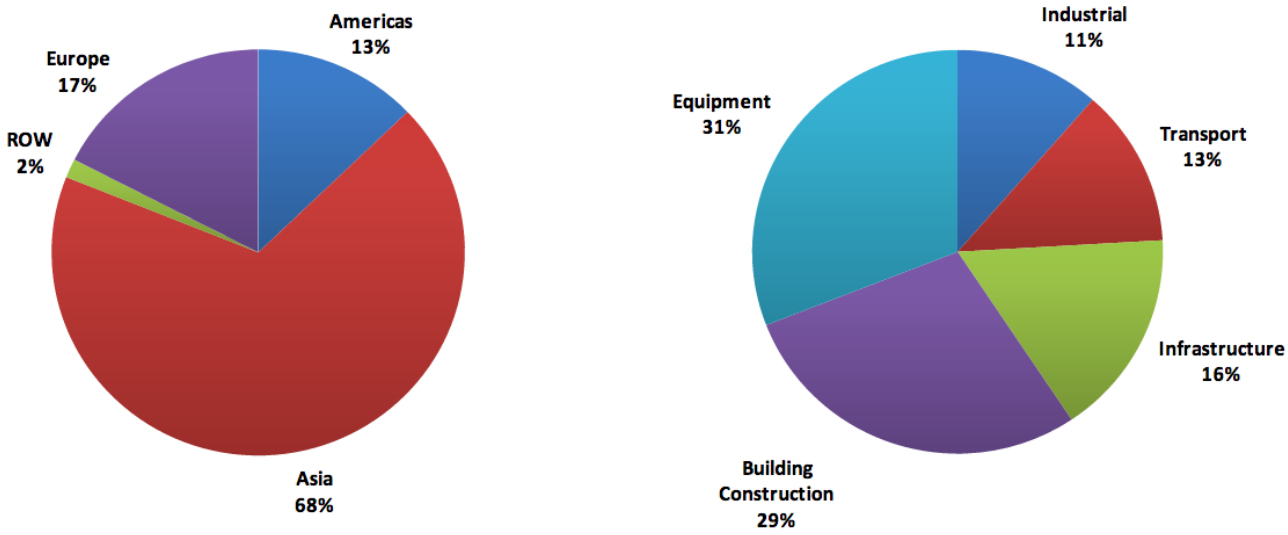


Figure 2.1. Major uses of copper: usage by region and end use sector, 2016. Graphic from the International Copper Study Group, ICSG [http://www.icsg.org/]. ROW: rest of the world.

Copper is also broadly used in **agriculture**, both as fertilizer and as biocide (Borkow and Gabbay 2005; Fan et al. 2011; Ochoa-Herrera et al. 2011; Rajasekaran et al. 2016). Copper-based biocidal compounds such as Cu hydroxide, Cu oxychloride and Cu chelates, have been widely used in prevention of microbial diseases. The use of Cu as fungicide has been generalized in agriculture since the late 19th century (Alloway 2013). In viticulture, Cu-based

fungicides are used at typical application doses of 2 to 4 kg Cu ha^{-1} $year^{-1}$, and soil concentration sometimes surpass concentration range tolerable for most cultivated crops (Komárek et al. 2010, Alloway, 2013).

B. Copper dissemination in the environment

The wide use of Cu in past decades for anthropogenic activities has led to Cu residues **accumulation in soils** and in surrounding ecosystems, especially in **aquatic ecosystems** through multiple entry points, such as lixiviation (soluble matter) and leaching (solid matter) processes (Heijerick et al. 2006; Schuler et al. 2008).

Recently, a study from Ballabio et al. (2018), has assessed Cu concentrations in European **topsoils**, using 21 682 samples from the LUCAS topsoil survey (**Figure 2.2**). They highlighted that among land uses, vineyards have the highest Cu concentration with on average 49.3 mg kg^{-1} Cu, and olive grove as well as fruit tree crops also had high Cu concentrations in topsoil with on average 33.5 mg kg^{-1} and 27.3 mg kg^{-1}, respectively. The highest Cu concentration in Europe was found in French vineyards, with on average 91.3 mg kg^{-1} Cu, with almost half of the samples having values above 100 mg kg^{-1}. Indeed, viticulture is a very important agricultural sector in the Mediterranean region, and 60 % of the global wine production originates from just France, Italy and Spain (Hall and Richard 2000).

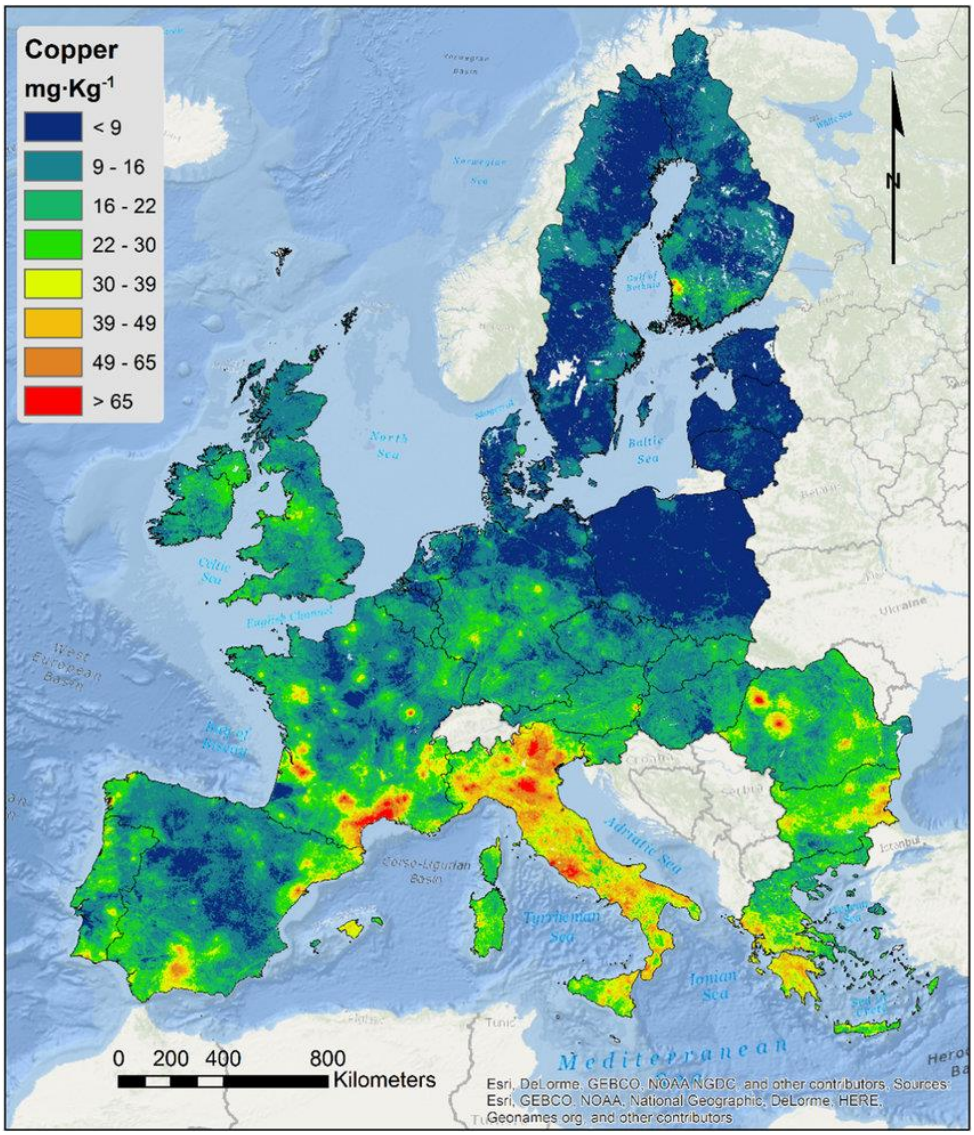


Figure 2.2. Copper distribution in European topsoils: an assessment based on LUCAS soil survey from 2017, and produced by Gallagher et al. 2018. The resulting map shows quite high Cu concentrations in areas typically devoted to wine production, especially in France and northern Italy.

Ultimately, Cu contained in soils can reach **aquatic ecosystems**. Many studies have highlighted the problems triggered by Cu concentrations in runoff from agricultural systems and mining sites (Karczewska et al. 2015; Knabb et al. 2016). It was demonstrated by Gallagher et al. (2001) that although only 1% of Cu was found to leave crop fields, it was enough to cause high Cu concentrations in runoff waters, with on average 2102 ± 433 µg L^{-1} of total Cu, and 189 ± 139 µg L^{-1} of dissolved Cu. They showed that Cu concentrations in groundwater samples were also high, with an average of 312 ± 198 µg L^{-1} of total Cu, and 216 ± 99 µg L^{-1} of dissolved Cu. Other sources of Cu for aquatic systems include wood preservative treatment,

iron and steel production, waste incineration, coal combustion, non-ferrous metal mining, oil and gasoline combustion, and phosphate fertilizer manufacturing (Willis and Bishop 2016). Direct applications of Cu in aquatic systems also account for approximately 13% of Cu contamination, as it is used as biocide to manage noxious algae and invasive weeds. After a pesticide application, Cu will quickly partitions to suspended matter and algae, and more than 90% will be transferred to sediments within 2 days.

C. Copper in living organisms

Copper is an essential redox-active transition metal which is found in all **living organisms**, from bacteria to fungi, passing by mammals and plants (Festa and Thiele 2013). It is required in small amounts (5-20 µg g^{-1}) by living organisms for respiration, carbohydrate metabolism and the functioning of more than 30 enzymes (Solomon 2009; Yruela 2009). For instance, Cu acts as a cofactor in Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino-oxidase, laccase and polyphenol oxidase. It also plays an **essential role** at the cellular level, in signaling of transcription and protein trafficking machinery, as well as in iron mobilization.

In **plants**, Cu plays even more important roles as it is a co-factor for several enzymes involved in photosynthesis, as the most abundant Cu protein is plastocyanin, a protein involved in the electron flow transfer (Droppa and Horváth 1990; Yruela 2009; Printz et al. 2016). Several Cu-dependent proteins are also unique to plants, such as transporters like Copper transporters (Ctr) family involved in Cu acquisition in roots or Heavy metal ATPases (HMA), which are responsible for the export and uptake of Cu (Huffman and O'Halloran 2001; Hötzer et al. 2012).

Under physiological conditions, it exists in two forms, one oxidized (Cu^{2+}), which has affinity for thiol and thioether groups (*e.g.* cysteine or methionine), and one reduced (Cu^{+}), which has affinity for oxygen or imidazole nitrogen groups (*e.g.* aspartic acid). As such, it is involved in a wide spectrum of physiological processes, from structural changes to biochemical reactions, because it can exist in multiple oxidation states *in vivo* (Yruela 2009). Those redox properties that make Cu an essential element also contribute to its inherent **toxicity**. Indeed, Cu is considered to be one of the most toxic metal in aquatic ecosystems (Solomon 2009).

Copper internal concentrations that exceed 20-30 µg g^{-1} can be toxic for organisms, although the threshold is species-dependent (Bradl 2005; Gomes et al. 2012; Marschner and

Marschner 2012). Several **toxicity mechanisms** have been demonstrated throughout the past decades. At high concentration, Cu will trigger the production of reactive oxygen species (ROS) through the Fenton and Haber-weiss reaction (Shahid et al. 2014; Printz et al. 2016):

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$$

This reaction is the oxygen transfer mediated by certain metals in the presence of hydrogen peroxyde, and the reaction generates hydroxyl radical ($OH^{\cdot}$), a highly toxic ROS (Candeias and Wardman 1996). Reactive oxygen species, such as hydroxyl radicals, can cause damage to DNA (DNA adducts), lipid peroxidation, and protein denaturation when the natural antioxidant balance is overwhelmed (Halliwell and Gutteridge 1984; Festa and Thiele 2013; Cadet and Wagner 2013). Another toxicity mechanism is Cu competition with essential metals for binding and uptake, triggering metabolism disturbances (Stauber and Davies 2000). It can therefore lead to non-specific binding of metals, resulting in the blocking of biologically essential functional groups of molecules (Janssen et al. 2003). Furthermore, for photosynthetic organisms, Cu^{2+} will compete with manganese ions in stroma proteins, resulting in the total inhibition of chloroplast photosynthesis (Pádua et al. 2010).

To illustrate **aquatic biota sensitivity to copper**, fish and crustaceans are 10 to 100 times more sensitive to the toxic effects of Cu than mammals, and algae are 1 000 times more sensitive than mammals (Förstner and Wittmann 1981; Solomon 2009; Wright and Welbourn 2002). Microorganism sensitivity to Cu has also been broadly documented for the past 30 years, with Lethal Concentrations 50% (LC_{50}) ranging from 3 to 47 µg/L Cu in different algae species (Trevors and Cotter 1990; Cervantes and Gutierrezcorona 1994; Kunito et al. 1999; Dupont et al. 2011; Ochoa-Herrera et al. 2011). Toxic effects were demonstrated as well as on invertebrates, such as Daphnia species, with LC_{50} values ranging between 34 and 80 µg/L (Campana et al. 2012; Hunting et al. 2013; James et al. 2008; Casares et al. 2012; Crémazy et al. 2016). Noxious effects have been demonstrated on different fish species, with LC_{50} ranging between 35 µg/L to 2.8 mg/L Cu. Gill and gut are commonly considered to be the first targets for metal uptake and toxicity (waterborne and dietary exposure), and Cu sensitivity appears to greatly vary among fish species and exposure pathway, *i.e.* dietary Cu or waterborne Cu (Allinson et al. 2000).

Cu toxicity has been extensively investigated on many **macrophyte** species with different life-history traits. It was studied on *Ceratophyllum demersum* (Devi and Prasad 1998; Thomas et al. 2013), *Lemna minor* (Razinger et al. 2007; Khellaf and Zerdaoui 2010; Basile et al. 2012),

Spirodela polyrrhiza (Xing et Huang 2009), *Myriophyllum spicatum* (Samecka-Cymerman and Kempers 2004; Li et al. 2010; Yan and Xue 2013), *Elodea canadensis* (Mal et al. 2002), *Hydrilla verticillata* (Gupta et al. 1996), and *Potamogeton pectinatus* (Samecka-Cymerman and Kempers 2004; Costa et al. 2018), among others. The sensitivity is highly species-dependent in vascular aquatic plants, starting with a very high Cu toxicity for *C. demersum*, showing toxicity signs from 4.7 ng/L Cu, passing by *L. minor*, demonstrating an LC_{50} of 0.47 mg/L, and to *M. spicatum*, showing an LC_{50} of 1.54 mg/L Cu.

As plants are sessile organisms, they have developed several ways to cope with excess Cu; intracellular copper level is regulated by metallochaperones, and by phytochelatins (Pang et al. 2013). Those mechanisms developed by plants to cope with Cu, in addition with the activation of regulation pathways, come with a cost, as energy is partially allocated in those paths, and not only in growth. Thus several studies have observed a decrease in growth upon Cu exposure, resulting from energy allocation in stress response and coping mechanisms (Khellaf and Zardoui 2010; Roussel et al. 2007; Huffman and O'Halloran 2001; Thomas et al. 2013; Török et al. 2015).

Several **water parameters** will influence Cu toxicity, rendering hard to properly assess its potential impact if conditions are changing rapidly. A study from Meyer et al. (1999) showed that water quality (such as pH, hardness and alkalinity) strongly influenced sensitivity of several organisms to Cu.

3. Policies and methodologies in ecotoxicological risk assessment

A. History of environmental regulations

The raise of concern that anthropic activities may be harmful to the environment triggered the implementation of regulations. This increase of public awareness began in the 60s', with Rachel Carsons' book, 'Silent spring' written in 1962, explaining how the use of synthetic pesticides is harmful to wildlife and to the environment (Carson, 1962). This book ignited the first environmental policies in **the US**, notably the Wilderness act in 1964 (Crocco et al. 2016). In the early 1970s', the first regulation in the US to preserve the environment from industrialization was implemented, known as the National Environmental Policy Act (NEPA, US EPA, 1969). Further legislations were implemented following the NEPA, such as the Clean Air Act in 1970, and the amendment of the Federal Pollution Control Act in 1948, becoming

the Clean Water Act in 1972 (United States Federal Law 2002; US Environmental Protection Agency 1997).

In **Europe**, the Paris Summit meeting of the European Economic Community (EEC) in October 1972 drew the first action program for environmental protection, which was adopted in July 1973, and signed the beginning of EU's environmental policies (European Union 1972). The first United Nation conference on the environment took place in Stockholm in 1972. After this conference, the European Community adopted its first Environmental Action Program (EAP, from 1973 to 1976), which allowed to determine the principles and the priorities that would guide its policies in the future. By the end of 1992, the European environmental law contained 196 Directives and 40 regulations (Markus-Johansson et al. 2008). In October 2000, the EU Water Framework Directive (WFD) was adopted to be an operational tool to set the objectives for **water protection** for the future (European Commission 2000). Although water legislation in Europe started in 1975 with standards for rivers and lakes used for drinking water, and has set binding quality for drinking water in 1980, it was only in 1991 that water pollution by agricultural runoffs and wastewater was accounted for into regulations (European Commission Website).

To go further into **chemical control**, a regulation on Registration, Evaluation, Authorization and Restriction of Chemicals, also called "**REACH**", came into force in 2007 and replaced the former legislation framework on chemicals (European Commission 2007). The main reason for implementing this regulation was that many substances in various amounts were manufactured and placed on the European market for many years with insufficient information about their harmfulness toward humans and environment. This regulation aims to protect the environment and humans by controlling the type and the amount of chemicals authorized on the European market, and follows the idea "No data no market", as it expects industries to provide safety information on the substances (Regulation No. 1488/94). Nowadays, new substances need to be registered immediately before being placed in the EU market (Directive 93/67/EEC).

The registration of chemicals, which is carried out on all newly notified substances and on priority existing chemicals, is made through a **risk assessment process**. Ecotoxicological risk assessment is the approach used to assess the impact of a given molecule on ecosystems, through the study of its toxicity on non-target organisms (Shea and Thorsen 2012).

For instance, due to its impact on aquatic biota, several regulations have been implemented throughout the years to limit **Cu** impact on ecosystems. Europe approved but regulated Cu-

based compounds in organic farming, particularly for potato, grape, tomato and apple production systems, and the authorization has been renewed in 2018 for bactericide and fungicide uses. It is, along with sulfur, the only mineral product allowed in organic agriculture for vineyard in Europe, with up to 6 kg/ha/year, averaged over 5 years (regulation N° 889/2008, EFSA, 2008). No regulation in conventional agriculture has been implemented in Europe, although a European regulation sets the Cu concentration limit at 150 mg/kg in soils. In 1998, the directive 98/83/EC has limited Cu to a maximal concentration of 2 mg/L in drinking water, however no limit has been set groundwater and surface water concentrations, despite the harmful effects demonstrated on aquatic environments.

B. Introduction to ecotoxicological risk assessment

In a context of increasing chemical production, **risk assessment** has been defined at the Earth Summit in Rio de Janeiro as "a scientific process which identifies, characterizes and quantifies the potential adverse effects on human health or ecosystems of defined exposures to a chemical substance or mixture or to a chemically hazardous process or situation". The risk is defined by the European Commission as "the combination of the probability of occurrence of a hazard generating harm in a given scenario, and the severity of that harm" notably depending on the vulnerability of the system considered (European Commission 2015). Risk assessment is used in a wide range of professions and academic subjects.

In that framework, **Ecotoxicological Risk Assessment** (ERA) is the scientific process which allows to determine the nature and the likelihood of toxic effects of chemicals on the environment, through exposure and effect evaluations (Brunström and Halldin 2000; Dimitra G. et al. 2005; Suanon et al. 2018). Ecotoxicological Risk Assessment has emerged as an important part of **environmental protection programs**. It has first started in the 1970s, when it was adapted from health risk assessment to environmental health risk assessment, along with the first environmental regulations. Although similarities exist between the two approaches, ERA is more complex due to the inherent complexity of ecosystems. The EPA and others have issued guidelines in the 1990s, which present a basic framework for conducting ERAs. This framework still persists today, although it has evolved with scientific discoveries and has been complexified through step addition, to increase reliability of the process (Hansen 2007; Hunka et al. 2015; Johnson and Sumpter 2016).

ERA deals with changes caused by humans that may alter ecological systems, such as lakes, rivers, forests, and others. When a new chemical is introduced in an environment, such as the spread of pesticides, it is necessary to assess the changes that will be triggered on species in the area. The approach may be very local, such as wastewater treatment plant site, or regional, such as Virginia coast or the Great Barrier Reef. The risk may be global, such as global warming or global distillation (also known as grasshopper effect), and may involve particular species which are likely to be exposed to the changes, or involve an ecosystem with all its biotic and abiotic components (SETAC 1997).

C. Ecotoxicological risk assessment in Europe

In Europe, the standard approach in ERA includes 4 different phases (Manuilova, 2003; Leeuwen and Vermeire, 2007; ECHA, 2016, **Figure 3.1**):

1) The first step is the **hazard identification**. The effects of concern that a chemical has an inherent capacity to cause are identified. The hazard classification of the substance according to the Global Harmonised System (GHS) is established or reviewed.

2) The second step is the **dose-response assessment**. The relationship between dose or level of exposure of the substance and the severity of the effect are estimated, and the predicted no-effect concentration (PNEC) is developed, for at least three taxonomic/trophic levels. The PNEC is the threshold concentration which must not be exceeded in order to avoid deleterious effects on the environment. The PNEC is derived by applying appropriate assessment factors (AF), which are used to extrapolate from laboratory single-species toxicity test data to multi-species ecosystem effects, following the EU-Technical Guidance Document (TGD). It also takes into account the extrapolation from acute to chronic exposure and the variability among experimental data. If the number of available data and their adequacy increase, the AF will decrease. PNECs are derived from the most sensitive species tested.

3) The third step is the **exposure assessment**, which is an estimation of the concentration to which environmental compartments are or may be exposed. The sources, emission routes and degradation pathways of the chemical are determined by using environmental monitoring data, or by modelling exposure in a hypothetical standard environment. For REACH, the model from the European Union System for the Evaluation of Substances

(EUSES) is employed (ECHA, 2016). It was developed for the quantitative assessment of the risks posed by existing and new chemical substances to the environment. EUSES can work with very limited data sets (Brandes et al, 1996). The Predicted Environmental Concentrations (PECs) are derived for each environmental compartment, and are usually modelled due to the lack of monitoring data.

4) The last step is the **risk characterization**, which is the estimation of the severity and the incidence of the effects likely to occur in an environmental compartment, due to actual or predicted exposure to a given chemical. The Risk Characterization Ratio (RC), is calculated thanks to the PEC and the PNEC, as RCR= PEC/PNEC, for a given compartment. The RCRs take into account populations, exposure routes, time scales, and environmental and human impacts. The RCR needs to be below 1, if not, further refinements (such as the generation of toxicity data to reduce the AF) must be performed to ensure an RCR < 1. The RCR is based on worst-case assumptions on sensitivity and exposure, assuming the presence of the most sensitive species.

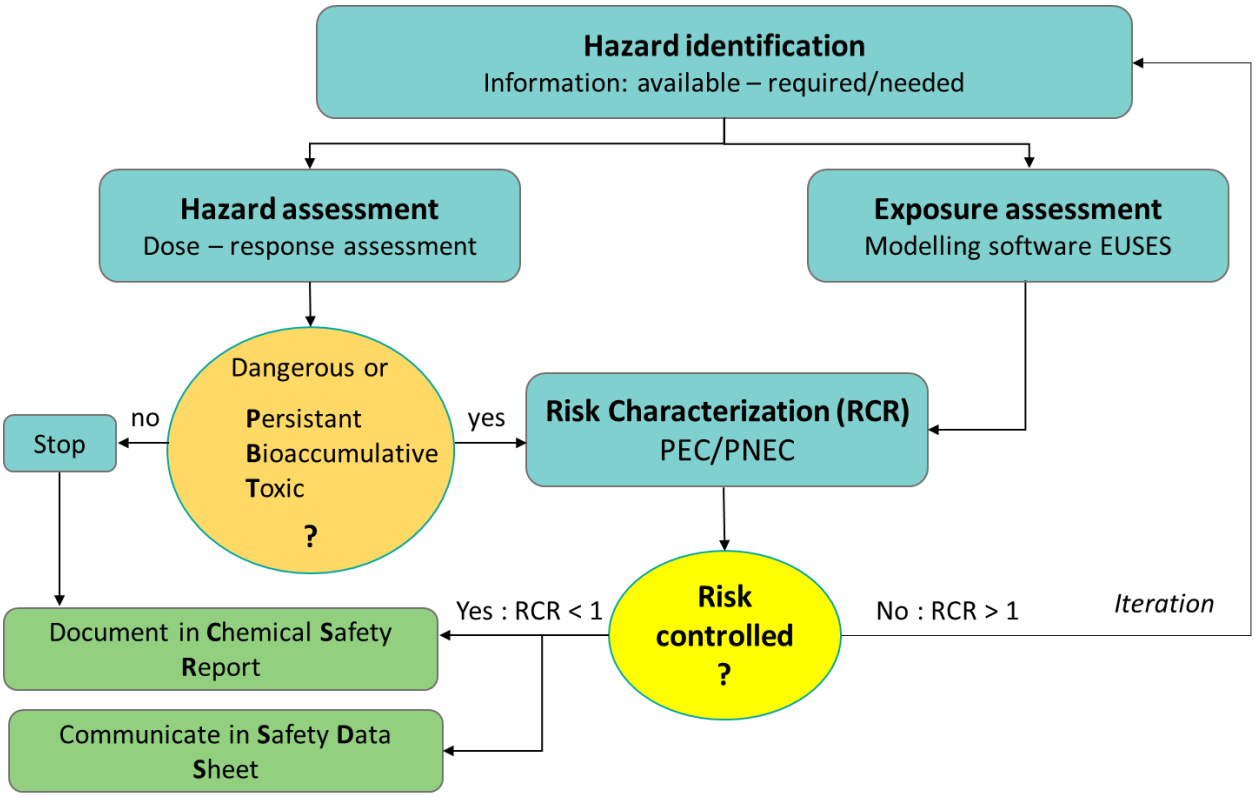


Figure 3.1. Schematic presentation of the four steps in EU ecotoxicological risk assessment: (1) Hazard identification, (2) hazard assessment, (3) exposure assessment and (4) risk characterization. The iteration of the process depends on the toxicity of the product and its probable environmental concentration. Adapted from the European Environment Agency (2016).

As it is not possible to test all chemicals on all species, along with the numerous variables that can inflect the outcomes of laboratory testing, ERA is an iterative process. As conditions change and new information is available during the study, the assessment has to be revised in light of the new information, and improved where needed.

D. Ecotoxicological risk assessment in other countries

Outside Europe, ERA approaches slightly vary, and they are mostly derived from the model proposed by the US. This approach includes 3 phases: **problem formulation**, **analysis** through existing or potential exposure characterization, and **risk characterization** through assessment of exposure effect and toxicity. Notably, in the US, the risk assessment is made through an assessment endpoint, meaning that an "integrative" endpoint which is judged important to protect, and is defined in phase 1. Furthermore, there is a clear separation between the risk manager, *i.e*. the changes that need to be implemented following the risk assessment results, and the risk assessor, the persons realizing the risk assessment. In Europe, risk posed by chemical to the environment is assessed for all environmental compartments, and there is no clear separation between the role of risk manager and risk assessor (Manuilova 2003).

E. Tiered approach in ecotoxicological risk assessment

ERA nearly always follows a tiered approach in order to balance required details and efforts to obtain them (EFSA PPR Panel, 2013). However in US, the USEPA does not explicitly provide a tiered approach, and leave the decision to the risk assessors. The tiered approach consists in increasingly detailed assessment of exposure and effects, and ends up with the determination of a safe concentration for the environment. Each tier is an extended evaluation of the previous one (**Figure 3.2**, Leeuwen and Vermeire, 2007; EFSA PPR Panel, 2013). Furthermore, depending on the RCR value within each tier, an iteration within the tier is performed to refine the RCR.

The assessment often starts with conservative assumptions in order to be resource efficient, and uses the PNEC values from the most sensitive species used in laboratory tests. This is based primarily on the precautionary principle, which was described by Forbes and Calow (2002) as: 'applying controls to chemicals in advance of scientific understanding if there is a presumption that harm will be caused.' Therefore in principle, safely passing the first tier guarantees passing all the superior tiers. If the risk is not controlled, these conservative assumptions are replaced

with less conservative assumptions, and if possible with measured data, to increase the realism of the approach. The tiers are described below:

1) The first tier is laboratory experiments (bioassays) conducted on the most sensitive species from the standard laboratory species (core species), to acquire acute and chronic toxicity data depending on the dose (PNEC predictions), on at least 3 species.

2) The second tier is growth chamber and glasshouse experiments, which are both acute and chronic lab tests performed with additional species to represent a sample of the community that needs to be protected. It takes into account the species interactions, along with the indirect effects. An extrapolation is performed to properly represent the sensitivity of all species in the community, through species sensitivity distribution (SSD) methods performed on at least 6 species (described below). The approach can also be enhanced to better address the risks of time-variable exposures, through toxicokinetic (TK) and toxicodynamic (TD) models.

3) The third tier relies on constructed model ecosystems, with experiments at population and community levels with a range of trophic levels. It can simulate environmentally realistic exposure regimes.

4) Finally the fourth tier is a mix of field studies and landscape modelling, through the monitoring of long term and large scale impact assessment. It refines the realism both in terms of exposure and of ecological relevance of the species community.

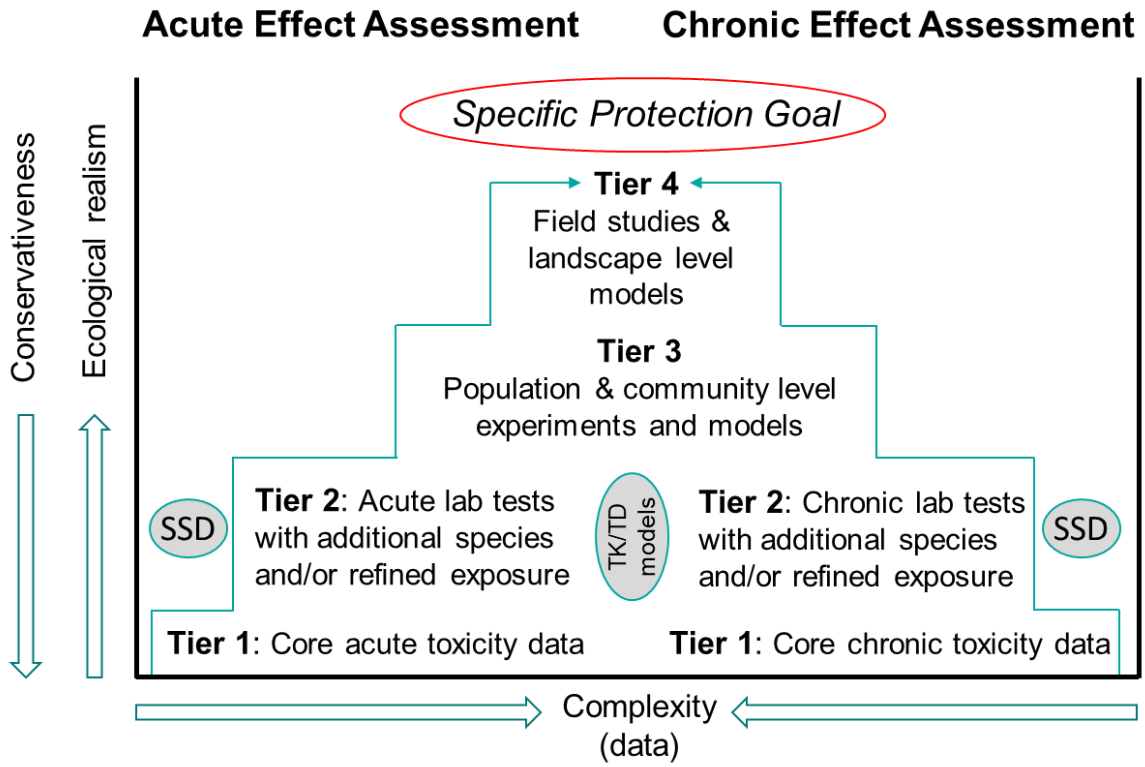


Figure 3.2. Schematic presentation of the four tiers of ecotoxicological risk assessment, with acute (left part) and chronic (right part) effect assessment. Adapted from EFSA PPR Panel, (2013).

F. Toxicity data used in ecotoxicological risk assessment

During tier 1 and tier 2, laboratory bioassays are performed to assess the effect of a given molecule on a species, depending on its concentrations. Predicted No Effect Concentration (**PNEC**) or No Observed Effect Concentration (NOEC) are extracted from those laboratory tests, which are often realized on at least **3 species**. A PNEC value from the most sensitive species is used for derivation by an AF. The use of the NOEC and PNEC has been controversial for the past decade: firstly, because the concept is based on a wrong interpretation of the statistical output (no statistically significant effect does not mean no effect). Secondly, because those values are strongly dependent on the experimental setup and design, and their derivation relies on assessment factors (Fox 2008; Warne and Van Dam 2008; Delignette-Muller et al. 2011; D. R. Fox et al. 2012; Belanger et al. 2017).

Laboratory testing can also produce concentration - response (or effect) curves for different endpoints. Each curve can be summarized by a single value, such as the Effective Concentration

(EC) at which x % of the effect is observed, also known as the EC_x value. To estimate EC values, a model is fitted to the concentration – effect curves, usually log-logistic with 3 or 4 parameters, and the EC values are calculated as a model parameter. A 4 parameters log-logistic is presented below (Ritz 2010): $f(x) = \frac{d-c}{1+\left(\frac{x}{e}\right)^b} + c$

where f is the measured endpoint, x is the concentration, c is the asymptotic value of the endpoint when the concentration grows to infitiny, d the value of the endpoint at 0 concentration, b is a shape parameter and e is the concentration at 50% effect (EC_{50}).

The problem in the whole methodology is that it assesses toxicity thresholds only on few species, as it is impossible to test all the chemical compounds on all species. Those toxicity thresholds are based on observations of effects of a chemical on growth, survival and reproduction. In order to be more ecologically relevant and to extrapolate those results to a community or an ecosystem, several approaches have been developed, such as the SSD.

G. Species Sensitivity Distribution (SSD): a tool for ecotoxicological risk assessment

Nowadays the SSD is routinely used in tier 1 and tier 2 of the ERA process (Del Signore et al. 2016). The SSD method has a significant influence on national and international decision making regarding assessment of chemical exposure to ecosystems (Belanger et al. 2017). The formal adoption of SSDs for the derivation of environmental thresholds dates back to 1985 in the U.S. and 1989 in Europe (Stephan et al. 1985; Van Straalen and Denneman 1989).

It is a process which aims to compare the sensitivity of several species, in order to determine a threshold concentration for which the chemical harms less than 5 % of the species tested (Newman et al. 2000; Del Signore et al. 2016; Pathiratne and Kroon 2016).

The principle of **SSD** is to select at least **6 species** (to ensure the robustness of the model) to carry out bioassay experiments, to estimate their tolerance through EC values, usually EC_{50} or LC_{50} (lethal concentration), and to fit a distribution model to those values which describes the sensitivity of the species pool, assimilated to a community (**Figure 3.3**). The sensitivity of this community is then estimated by the Hazardous Concentration for 5 % of the species (HC_5), also known as the benchmark, which is used as a threshold concentration at which 95 % of the species should not be affected. However, this concentration is not considered as conservative, and several AF are applied.

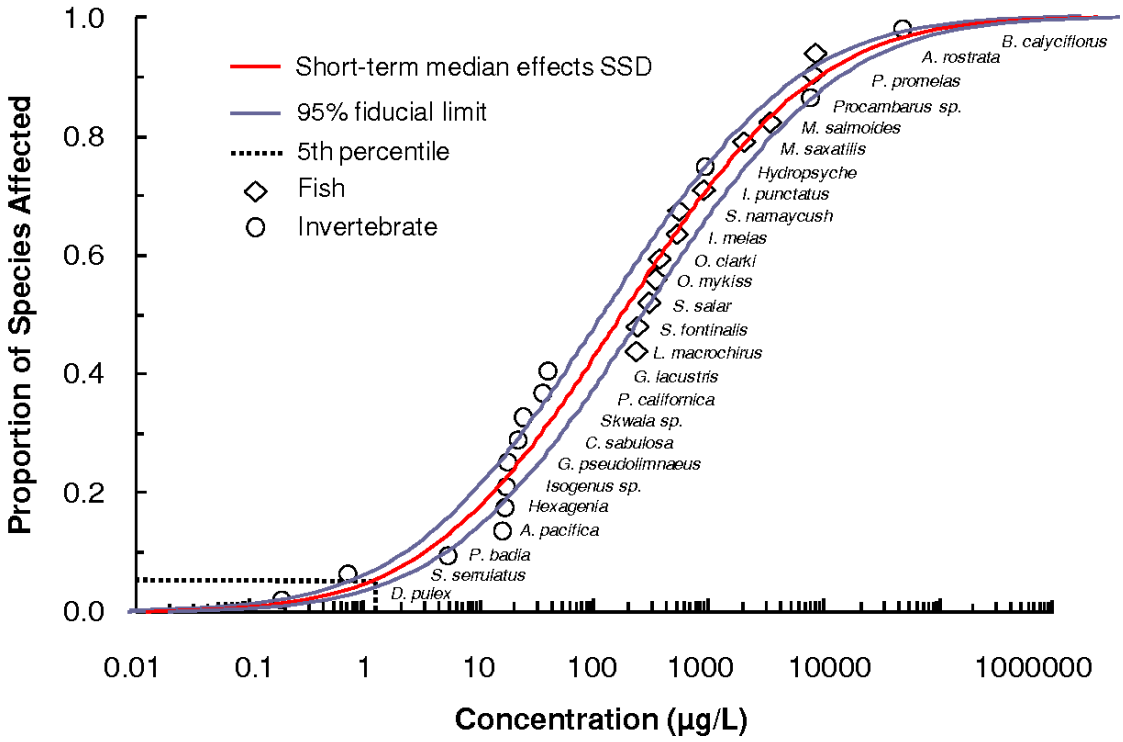


Figure 3.3. SSD representing the toxicity of trichlorfon in freshwater based on short-term LC_{50} and EC_{50} values for 26 aquatic species versus the proportion of species affected. The dashed line in black represent the HC_5 (Canadian Council of Ministers of the Environment, 2012).

It was discussed in the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC 2014) that extrapolation approaches based on SSDs to derive toxicity threshold concentrations, should provide a more relevant assessment of risks than PNEC derivation using generic factors applied to single-species bioassay data (Hanson and Solomon 2002; D. Fox 2008; D. R. Fox et al. 2012; Belanger et al. 2017).

H. Limits of ecotoxicological risk assessment and pitfalls of the SSD approach

Usually, the ERA process is hampered by four types of uncertainty: the lack of information, the measurement uncertainties (low statistical power, inappropriateness of measurements…), the observation conditions (spatio-temporal variability in environmental factors, species sensitivity, differences between natural and laboratory conditions…), and the inadequacies of models (lack of knowledge concerning underlying mechanisms, failure to consider multiple stressors, instability of parameter estimates…).

General limits of ERA:

1) **Statistical power and uncertainties** in PNEC derivations need to be identified. As explained previously by Fox et al. (2008; 2012), the interpretation of the statistical output is

wrong, and an absence of significant difference does not mean no effect, especially knowing that PNECs are based on one single endpoint.

2) The **bioavailable concentrations** should be properly assessed. Indeed, Morselli *et al.*, (2015) have demonstrated that emission, environmental and biomass dynamics caused up to 4.5 times variations in exposure levels. They highlighted the need to identify environmental and ecological conditions in which risk is expected to be the highest.

3) The relevance of **parameters** chosen in laboratory testing for each species. Indeed, ERA aims to extrapolate bioassay results to population level. It takes into account effects on survival, growth and reproduction parameters, but does not consider the fact that effects might be only visible at the metabolism, behavioral or genetic levels, depending on the species. Only monitoring of diversity and abundance approaches can say, over time, if the current approaches are truly protecting the environment from chemicals.

4) The **baseline** of a given ecosystem, or its variation, needs to be quantified before taking management measures. Johnson and Sumpter (2016) took as an example the Swiss initiative to improve many of their sewage treatment plants (STP) in the hope to decrease the chemical release in rivers and increase fish biodiversity. However, it has been demonstrated that fish decline was more closely associated with kidney diseases and declining habitat than by sewage effluent exposure, and that fish populations greatly varied from one year to another. Therefore, investments to improve STPs may not enhance fish populations.

Current limit of SSD approaches:

1) Species relevance in laboratory testing during SSD approaches has to be addressed. Indeed, the concept of **keystone** species is not taken into account in SSD approaches. All species are weighted equally, assuming that the loss of any species will be equally important to the ecosystem. However, keystone or other functionally important species may not be protected by the HC_5 concentration, and would therefore inflect the entire ecosystem dynamics and impact the other species (Forbes and Calow 2002).

2) **Pulsed contaminations** are not taken into account, and SSDs are restricted to constant concentration scenarios (Maltby et al. 2005). This is of concern, as chronic exposure occur very often in ecosystems. Only time-averaged concentrations may partially circumvent the problem, but does not assess the possible weakening of individuals.

3) Although it aims to assess a community sensitivity, it does not take into account **species interactions**, such as predation or competition, as observations are based on single-species

bioassays. However species interactions may significantly inflect the harmfulness of chemicals, as it can impact preys or predators thus indirectly impact other species they interact with. To remedy this issue, it was proposed to validate HC_5 with mesocosms and real ecosystems (Belanger et al. 2017).

4) **Intraspecific variation**, *i.e.* variability within species, is not taken into account during the laboratory testing. It is of concern, as SSD aims to compare the sensitivity between species, and assumes that interspecific variation is higher than intraspecific variation. Laboratory bioassays assume that the harvested individuals from a given species are representative of their entire species sensitivity. These tests do not take into account that some populations or some individuals may have different sensitivities due to environmental factors, selective pressure, gene pool and local adaptation, among others. Extrapolating the sensitivity of an entire species from few individuals can therefore be misleading, as intraspecific variation could be very high; the results of a given laboratory toxicity-test might thus arise from a sampling effect. As such, comparison of sensitivity among species may be distorted, and the determination of a HC_5 may misrepresent the real sensitivity of a given community, and therefore the impact of the chemical on a given ecosystem.

4. Ecological importance of intraspecific variability

A. What is intraspecific variability?

Intraspecific variability is defined as the differences that occur between different individuals from a same species. Variations can be recognized through various characteristics, such as morphology, development, biochemical or physiological properties, but also through genetic differences (*i.e.* differences in the complete set of genes). The term **genotype** is used to describe variations in genetic makeup among individuals, whereas the term **phenotype** is used to describe the observable traits of an individual.

All species, terrestrial or aquatic, demonstrate intraspecific variability from a genotypic and a phenotypic point of view: for instance, all mammals are genetically distinct from each other (excepted homozygous twins), and a person will be physically different from another because their genetic makeup will be different, this is called **genetic variation**. It is due to differences in gene versions, as all members of the same species have the same genes, but these can exhibit different forms, called alleles. Heterozygosity is when an organism has two different versions/forms of alleles for one gene. The different alleles can cause variations in phenotypes,

such as eye color or blood group, but can also provide resistance to environmental stressors. For instance, it has been widely studied for crop plants in agriculture, and to understand the development of herbicide resistance in weeds, such as *Azolla* and *Hydrilla* species (Mitra 2001; Moody et al. 2008). **Genetic variation** is inherited, transferred from parents to offspring. It is considered that heterozygosity increases genetic variation, as there is more genetic material available (Amos and Harwood 1998).

In addition to its gene makeup, an individual may exhibit different phenotypes in its lifespan as a response to environmental changes that will inflect gene expression. The ability of an individual to produce different phenotypes in response to environmental variations is called **phenotypic plasticity** (Bradshaw 1965). For instance, Himalayan rabbits exhibit changes in melanization depending on temperature, and their fur turns black when temperatures drop below 25 degrees (West-Eberhard 2003). Gotham and Song (2013) have demonstrated that two grasshopper species exhibited different morphologies and colors depending on crowding. Plants can also exhibit different phenotypes depending on light intensity, nutrient availability, mechanical constraint, among others (Robe and Griffiths 2000; Pigliucci and Kolodynska 2002; Sultan 2003).

B. Genetic variations

Genetic variations (or variability) can be caused by multiple processes. Changes may occur due to mutations with an error in the DNA replication that will cause structural changes in a gene. Mutations are considered to be the only source of new alleles in a population. In plants, genotypic variation can be expressed through several traits, such as root morphology (O'Toole and Bland 1987), photosynthetic capacity (Flood et al. 2011), leaf anatomy (Olsen et al. 2013), and phenology (Chuine et al. 2000).

1) Processes that inflect genetic variation

The main process which hampers genetic variability is **natural selection**. As a consequence of natural selection, the frequency of favorable alleles increases over several generations, while that of unfavorable ones tends to decrease (Grenier et al. 2016). As a result of this process, differences in reproductive efficiencies will be found, also called fitness, among genotypes under a given set of environmental conditions. Natural selection will tend to decrease genetic diversity within a population, as all genotypes will converge: it is called directional selection. If a strong selective pressure is applied, only individuals able to survive and reproduce will

remain, decreasing the gene pool of the population. It follows the natural selection theory developed by Darwin, which states that individuals who are best adapted to live in an area will survive and reproduce, whereas the others will disappear. However, within a population, there is a certain degree of genetic variation, which may or may not make an individual more adapted to its environment, or to changes in its environment. It has been demonstrated that selection pressures on different populations will increase genetic variation among populations, and decrease genetic variation within population (Fraser et al. 2014).

The second process modifying genetic variability is **genetic drift**, which is due to random changes in allele frequencies occurring from generation to generation, due to a finite population size. Its effects are strongest in small populations, where alleles poorly represented face a greater chance of being lost (Fujisawa et al. 2014). Genetic drift continues until the involved allele is either lost or is the only one present at a particular gene locus in a population. Indeed, if the number of individuals in a population is small, the gene pool in the next generation will demonstrate reduced variation. Genetic drift is particularly common after a population bottleneck, when a significant number of individuals in a population die, or is prevented from breeding, as it results in a strong decrease in the size of the population and of its gene pool (Grenier et al. 2016). Bottlenecks often arise as a result of habitat fragmentation. It can also result in a genetic differentiation from the original population, and if the new population is genetically isolated, in its speciation. This has led to the hypothesis that genetic drift plays a role in the evolution of new species, as they adapt to their new environment without any exchange with other populations.

Both mutations and **gene flow** increase genetic variability. Gene flow is the exchange of genetic information among populations, through migration of individuals or long distance transport of pollen in the case of plants. Gene flow increases when populations are connected, without geographical barriers between them. Nowadays, human activities considerably influence gene flow due to transports and connecting roads, which can increase gene flows for species associated to man-modified habitats. At the opposite, landscape fragmentation can reduce gene flow of separate populations, especially in plants (Aguilar et al. 2008; Chaput-bardy 2008; Abbasi et al. 2016).

2) Genetic variability in plant species evolution

Genetic variability is very important in **species evolution**. Indeed, a population having a broad gene pool will more likely adapt to environmental changes. Individuals separated from their original population may form a new species due to speciation, combining both geographic isolation and gene flux isolation. For instance, Martínez-Garrido et al. (2016) have demonstrated with genetic analyses that processes like speciation and hybridization within the genus *Ruppia* resulted in new species. It has been shown by Barker et al. (2018) that genotypic variation in tree traits (*e.g*. growth and phenology) shapes other organism community, highlighting the importance of genotypic variation in ecosystems. This corroborates the finding made by Whitlock et al. (2010), and Zytynska et al. (2011), who demonstrated that genotypic variation in plants plays an important role in community structure.

C. Phenotypic plasticity

1) Process

The plastic traits of individuals are modified, without modifying the genetic diversity of the populations, as the modifications are not heritable. These changes occur in the lifespan of individuals, and can influence their fitness, as well as be the target of natural selection (Fusco and Minelli 2010).

The relationship between environmental factors and traits is called the **reaction norm.** This term was first introduced by Woltereck in 1909. The norm of reaction is a curve that relates, for a given genotype, the contribution of environmental variation to observed phenotypic variation (Debat and David, 2001, **Figure 4.1**). For instance, the shape of the curve may be flat across environments if the trait is not subject to phenotypic plasticity (**Figure 4.1A**). By opposition, a plastic trait will demonstrate some variation between two environmental sets (**Figure 4.1B**) and genotypes can sometimes exhibit contrasted plasticity for a given trait (**Figure 4.1C)**. Although it was first applied to morphological traits, it has been widely demonstrated that a broad range of traits can demonstrate phenotypic plasticity (Woltereck 1909; Schlichting and Pigliucci 1998). Indeed, organisms can also alter their biochemistry, behavior, physiology, and life history, as a response to environmental changes. Those alterations can include heat shock reaction, learning and imprinting, environmentally induced transcription and translation, and general stress responses.

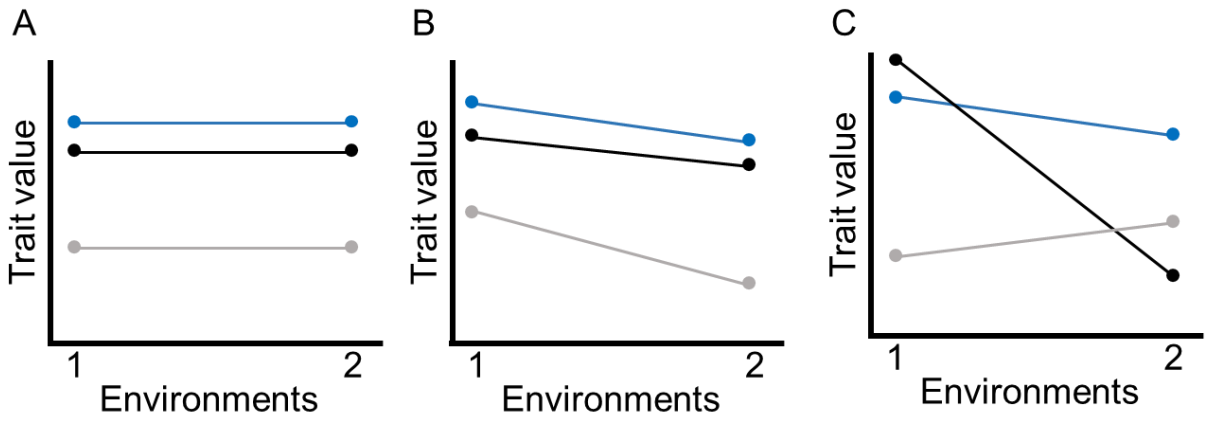


Figure 4.1. Reaction norms of a given trait of three genotypes (different color lines in each graph) under two environments, with (**A**) absence of phenotypic plasticity, (**B**) presence of phenotypic plasticity and (**C**) presence of phenotypic plasticity, and genotype-environment (G*E) interaction.

As explained by Whitman and Agrawal (2009), virtually all phenotypic traits are the result from underlying biochemical and physiological processes, thus phenotypic plasticity results from altered physiology through several mechanisms, such as epigenetic, transcriptional and post-translational regulations (**Figure 4.2**).

Plasticity can be characterized as active or passive, because there might be differences in the way these two types of phenotypic plasticity affect the ecological success of individuals and populations (Kurashige and Callahan 2007; Whitman and Agrawal 2009; Forsman 2015). **Active plasticity** is considered as anticipatory, and reflects modifications of developmental pathways and regulatory genes. A plant or animal with a plastic trait will receive a cue from the environment that will determine the subsequent value of the trait. Organisms can evolve mechanisms to sense and adjust to respond to certain cues that predict environmental changes (Whitman and Agrawal 2009). Cues tend to be non-harmful stimuli, such as predator-released chemicals, or photoperiod, and the magnitude of the phenotypic response induced by the cue is not obviously correlated with the strength of the environmental signal. **Passive plasticity** is considered when the environment directly acts on the expression of the trait, and phenotypic changes are often proportional to environmental differences. Direct environmental stimuli are often harmful, such as an increase in temperature, or a toxin (Whitman and Agrawal 2009). An example of the reaction chain triggered by environmental cue or signal is depicted in **Figure 4.2**, with in panel (**a**) the reaction chain, in panel (**b**) the reaction norms induced both by genotype and environment and in (**c**).the physiological outcomes of the reaction.

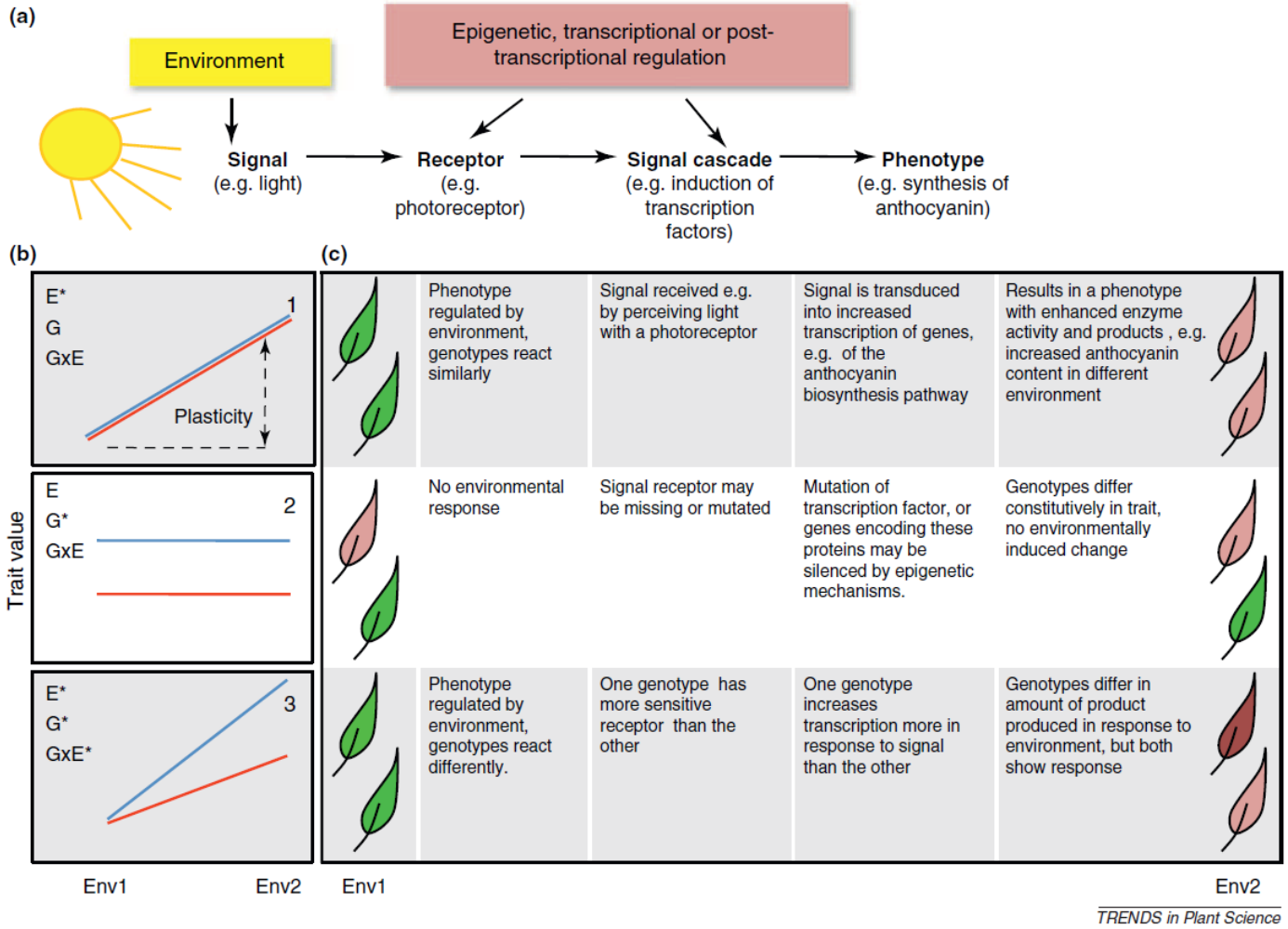


Figure 4.2. Phenotypic plasticity in the production of leaf anthocyanins as a defensive mechanism in response to an excess of light or temperature or to osmotic extremes. (a) Molecular mechanisms involved in plastic response, which translate an environmental signal (excess light in this case) into a phenotype. (b) Responses graphically presented as reaction norms. Here, the blue and red lines indicate the reaction norms of two different genotypes responding to a change from a low light environment (Env1) to a high light one (Env2). The extent of phenotypic change in response to an environmental signal is its phenotypic plasticity. Asterisks in the panels denote the significant effects of environment (E) or genotype (G), and an interaction between both (G × E). (c) Examples of the mechanisms underlying the cases depicted in panels 1–3 are given separately for each point in the signal pathway. The leaves on the left and right represent the phenotypes in Env1 and Env2, respectively. Figure from Nicotra *et al.*, 2010.

2) Role of phenotypic plasticity in plant adaptation

Phenotypic plasticity was first considered as a nuisance in **evolutionary biology**, at the time of the discovery of Mendel's laws on heredity. At that time, biologists considered environmental effects as a problem hampering natural and artificial selection of a given trait

(Falconer, 1952). In the 1980s, it was finally considered as quantity of interest in our understanding of how organisms interact with their environment (Debat and David 2001; Massimo Pigliucci 2005). According to Forsman (2015), research on phenotypic plasticity has grown exponentially, passing from < 10 papers before 1983 to nearly 1300 papers in 2013.

The role of phenotypic plasticity in plants, as an alternative strategy to genetic differentiation in response to environmental variations, was first reviewed by Bradshaw (1965). Many questions are asked to unravel the role of phenotypic plasticity mechanisms in adaptation, and its influence in individual, population and species diversity.

Plasticity is considered as adaptive if it increases an organism's fitness under a given environment, compared to organisms that are not plastic (Liefting et al. 2009). Plasticity can also be non-adaptive and maladaptive. A non-adaptive phenotypic plastic response is when environmental response is passive, and a maladaptive response is when a new environment induces a phenotype which is further away from the optimal phenotype in a given environmental set (Bradshaw 1965; Ghalambor et al. 2007).

Phenotypic plasticity can respond to **natural selection**, and suggests that adaptive plasticity occurs in natural populations (Massimo Pigliucci 2005). Current literature broadly demonstrates gene-by-environment interactions (G×E, genetic variation for plasticity) in organisms (West-Eberhard 1989; Debat and David 2001; Pigliucci and Kolodynska 2002; Pigliucci 2006; Fusco and Minelli 2010; Grenier et al. 2016).

Furthermore, recent studies suggest that phenotypic plasticity can compete with species composition in their effects in environment functioning (Crutsinger et al. 2008; Martin and Blossey 2013; Jackrel et al. 2016;). For instance, Jackrel and Morton (2018) have shown that herbivory resistance demonstrated by some tree species, which is mediated by environmental factors, decreases leaf litter decomposition in streams, and thus strongly alters the carbon source in those aquatic ecosystems.

3) Phenotypic plasticity in aquatic plants

Many studies have demonstrated that phenotypic plasticity is common in **aquatic plants** as a response to environmental fluctuations, such as nutrient availability, flooding conditions (mechanical and hypoxic constraints), water depth, light intensity, and others. Those environmental changes then trigger modifications in their morphology, reproductive traits or their composition and so on.

For instance, Yang et al. (2004) have shown that both *Myriophyllum spicatum* and *Potamogeton maackianus* allocated their biomass differently depending on flooding constraints. The same observation was made by Arshid and Wani (2013) and Cao et al. (2012), that *M. spicatum* demonstrates a plastic response to flooding and nutrient levels both for biomass allocation and for clonal architecture. *Phragmites australis* acclimatizes to water depth through resource allocation in stem weight and length (Vretare et al. 2001).

Olesen and Madsen (2000) have demonstrated that photosynthesis in *Elodea canadensis* and *Callitriche cophocarpa* adjust to temperature and carbon availability to promote growth. Puijalon et al. (2008) have shown adaptive and maladaptive phenotypic plasticity in four aquatic plant species in response to mechanical stress. *Sagittaria latifolia*, known as duck-potatoe, shows different phenotypic plasticity responses to nutrient availability between monoecious and dioecious plants (Dorken and Barrett 2004). Vasseur et al. (1992;1994) demonstrated that *Lemna minor* showed a high plasticity in response to short term environmental variations, and that the degree of phenotypic plasticity varied depending on the genotype, highlighting the influence of genotypic variation on phenotypic plasticity potential.

Furthermore, phenotypic plasticity can play a role in species repartition. Indeed, it has been highlighted by Ganie et al. (2015) that phenotypic plasticity was the cause of the successful spread of *Potamogeton* genus in the Kashmir Himalaya, with ten species demonstrating differences in their morphological and reproductive traits depending on environmental conditions. This phenotypic plasticity may inflect ecosystem dynamics, as aquatic plants play pivotal role in ecosystem functioning.

D. Implications of intraspecific variability in species evolution and ecosystem resilience

1) Intraspecific variability in aquatic plant evolution

Although we still hear sometimes that plasticity and genetic adaptation are opposite processes in adaptation, it has been widely acknowledged that plasticity is a property of the genotype, and that the two mechanisms are non-exclusive and tightly linked to one another (Grenier et al. 2016). The relationship between genotype and phenotype is complex, as they are both part of species evolution. The survival of populations in environments showing spatial and temporal fluctuations goes through shifts in genetic composition or individual phenotype. Phenotypic plasticity within species, and across both time and space, has broad implications both for communities and ecosystem functioning, such as energy flux among trophic levels

(Jackrel and Morton 2018). Plants especially are considered as highly plastic organisms, because they are sessile organisms, incapable of movement (Bradshaw 1965). Indeed, because animals possess locomotory mechanisms and complex behavioral responses, they are less prone to plasticity. They can evade unsatisfactory environments, and select others which are more suitable for them (Waddington 1957).

The process of selection takes more or less time depending on the genetic pool of each population, and their ability to survive. Each population will have a genetic pool, with organisms having different abilities to produce a plastic response, depending on organism's DNA. Selection pressure will occur on phenotypic plasticity as well, as phenotypic plasticity can inflect the fitness of organisms, depending of their adaptive potential to the given environmental set.

For instance, in an aquatic environment, light intensity could drastically decrease during a short time. Aquatic plants will have to quickly adjust to survive in this environment. Some individuals will produce high chlorophyll concentrations as a fixed trait, and will survive without having to adjust to this new environment. Some individuals will adjust to this environment through phenotypic plasticity and produce more chlorophylls to cope with the decrease in available light for photosynthesis. The plastic response may be adaptive if the cost is not at the expense of growth, and will result in individual's survival, and the given trait can even be fixed over time (*i.e*. assimilation, Waddington, 1953). The response may be maladaptive and result in their death and disappearance, if the production costs are too high, or if chlorophyll production is not high enough to cope with light decrease. Either way, individuals able to produce a proper response to the environmental pressure will remain if the cost is not too high, whereas those unable to produce the proper response will disappear. Both genetic diversity and plasticity will shape the response, and the cost of plasticity will strongly inflect the resulting diversity of the population over time (**Figure 4.3**).

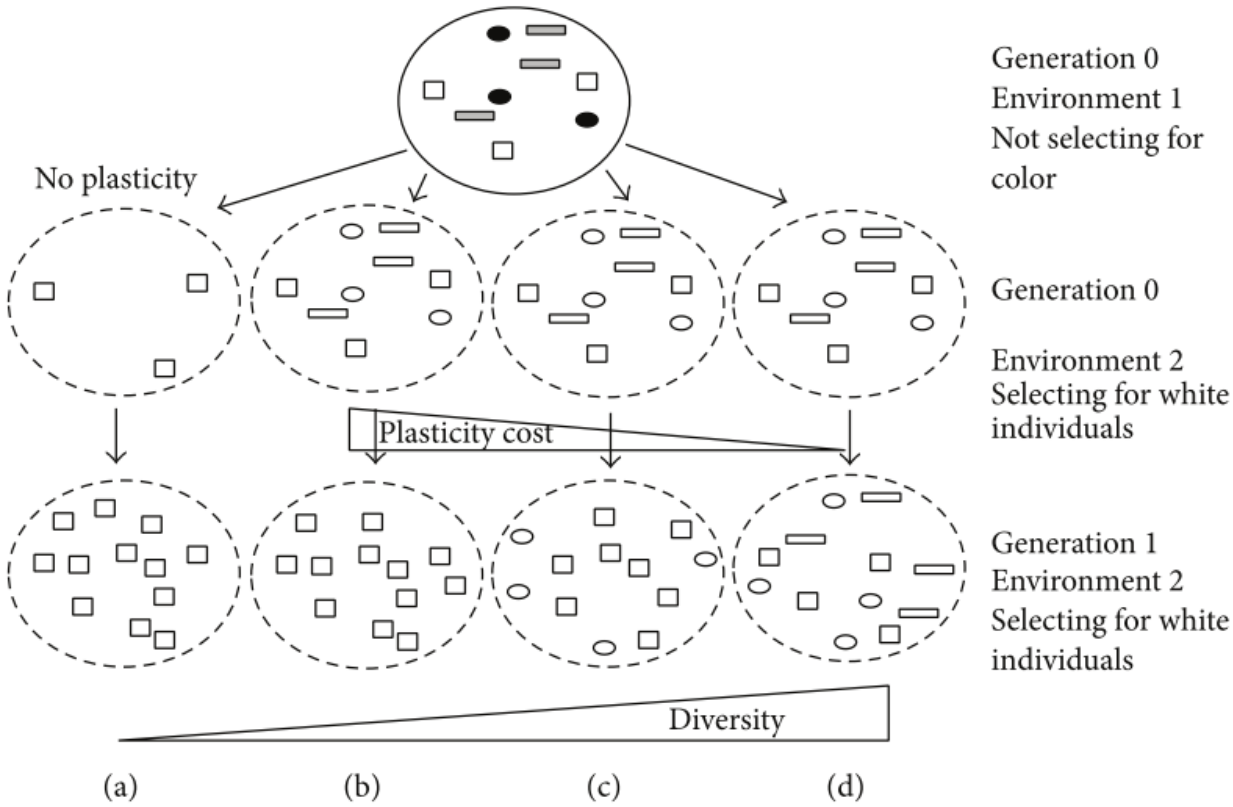


Figure 4.3. Impact of selection and plasticity on genetic diversity, according to the cost of plasticity. Genetic diversity is represented by different forms, and different phenotypes are represented by different colors. The population size is kept constant. (a) Without plasticity, showing a strong decline of genetic diversity, (b) with equal plasticity among genotypes and a very strong cost of plasticity resulting in a strong decline of genetic diversity; (c) with equal plasticity among genotypes and a medium cost of plasticity for the circles and strong cost for the rectangles resulting in a low decline of genetic diversity, and (d) with equal plasticity among genotypes and no cost of plasticity, resulting in the maintenance of genetic diversity. Figure from Grenier et al. 2016.

Several studies have been focused on intraspecific variation as a strategy for aquatic plants to spread and adapt in different ecosystems, whether it results from genotypic variation or phenotypic plasticity (Riis et al. 2010; Ganie et al. 2015; Weyl and Coetzee 2016). Furthermore, it has been broadly demonstrated that aquatic plants show geographic patterns and intraspecific variations among climatic regions (Garbey et al. 2004; Arshid and Wani 2013; Wu et al. 2016; Hu et al. 2017; King et al. 2017; Reynolds et al. 2017).

In a changing environment, it is essential to properly assess macrophyte species ability to adjust and thrive under new conditions. However, the study of combined environmental fluctuations and chemical stress has been poorly investigated so far on aquatic plants, even though it is widely acknowledged that aquatic ecosystems are particularly impacted both by

environmental fluctuations and chemical loads (Meyer et al. 1999; Howarth 1991; Woodward et al. 2010; Angeler et al. 2014).

2) The role of the intraspecific variability in ecosystem resilience

Although there is increasing interest for the ecological effects of intraspecific variation, the importance of such effects compared with **species effects** (*e.g*. ecological services) is not well resolved, and recent studies demonstrated that ecological effects of species was partly caused by intraspecific variation (Fussmann et al. 2007; Read et al. 2016). A meta-analysis from Des Roches et al. (2017) has shown that intraspecific variation effects are often comparable to species effects in ecosystems, and stronger when it comes to indirect interactions that may alter community composition. This finding corroborates the results from Reusch and Hughes (2006), that effects and mechanisms of genotypic and species diversity are analogous. This highlights the importance of intraspecific variability in ecosystem functioning, and its potential implication in ecosystem resilience.

Resilience is usually defined as the capacity of an ecosystem to absorb disturbance without shifting self-organized processes, structures and losing function and services (Holling 1973; Carpenter et al. 2001; Oliver et al. 2015). According to Côté and Darling (2010), the concept encompasses two separate processes: **resistance**, which is the magnitude of disturbance that causes a change in structure and functions, and **recovery**, which is the speed of return to the original structure and functions (Tilman and Downing 1994; Holling 1996).

Species composition and its stability are very important for ecosystem functioning, and are often considered as the target for conservation, as ecosystem functions can suffer from a species disappearance if this one has important functional roles. However, it is the ecosystem functions, rather than species composition, that need to be resilient to maintain ecosystem services.

As explained before, intraspecific variation plays a very important part in adaptive capacity of species toward environmental changes. Several studies have found that intraspecific variation in macrophytes enhances aquatic ecosystem resilience, because intraspecific variation in their life-traits diminishes recovery time after a disturbance (Oliver et al. 2015; Jackrel and Morton 2018). For instance, Reynolds, McGlathery and Waycott (2012) have demonstrated through recovery experiments that a higher genetic diversity in *Zostera marina* allowed ecosystems to recover faster, as they provided more ecosystem services (*e.g*. invertebrate habitat, increased

primary productivity, and nutrient retention). Furthermore, some studies have observed that the adaptive capacity of ecosystems, notably via the phenotypic plasticity or genotypic variability of species, favours ecosystem resilience to climate change (Bernhardt and Leslie 2013; Gibbin et al. 2017). For instance, Reusch et al. (2005) have demonstrated that genotypic diversity of the seagrass *Zostera marina* replaced the role of species diversity in a species-poor coastal ecosystem, and buffered against extreme climatic events.

E. Intraspecific variation in risk assessment approaches

Although intraspecific variation has been studied as a way to cope with environmental fluctuations and to contribute to species adaptation, almost no study has focused on the importance of intraspecific variation in the sensitivity of macrophytes to chemicals.

We explained before that plants can acclimatize to short term environmental fluctuations through phenotypic plasticity, and at a longer time scale, through genetic fixation of efficient traits. In a context of environmental pollution, species sensitivity may be influenced by those adaptive capacities. Furthermore, intraspecific variation is not currently taken into account in ERA, as explained previously in part III. Laboratory testing usually assesses the sensitivity of one population of individuals, or of one clonal population, to a given chemical. However, the population sensitivity may not reflect the species sensitivity across different populations, and in different environments (**Figure 4.4**). As such, the threshold concentrations determined may be over- or under-protective for aquatic ecosystems, if the results obtained in laboratory testing are only the result of a sampling effect, and not representative of the entire species sensitivity.

Several studies have demonstrated that environmental factors can strongly affect trace element uptake by aquatic plants (Fritioff et al. 2005; Verma and Suthar 2015), as well as their sensitivity to chemicals (Gupta et al. 1996; Leblebici and Aksoy 2011; Nuttens and Gross 2017). Dalton et al. (2013) have shown that geographically distinct populations of *Lemna minor* demonstrated different sensitivities to atrazine, with some populations being twice as sensitive as others. This is so far the only study which has investigated the possible impact of genotypic variation in chemical sensitivity of aquatic plants.

However, in terrestrial plants, the importance of genetic variability has been widely studied through the adaptation of plants exposed to herbicides. Indeed, the increasing occurrence of widespread herbicide resistance in weeds has been widely investigated over the years, as the use of herbicides still increases (Caseley et al. 1991; Kandasamy et al. 2002). According to

Schütte et al. (2017), in 2016 a total of 249 weed species (and sometimes several genotypes per species) resistant to various herbicides have been recorded, occupying hundreds of thousands of fields worldwide. The resistance genes can spread by hybridization between related weed species (Green 2014; WSSA Herbicide-Resistant Weeds Committee 1990).

In that context, it is imperative to assess the importance of intraspecific variation, its mechanisms and its impact on the sensitivity of aquatic plants to chemical stress, as aquatic ecosystems are the final receptacle of chemical contamination.

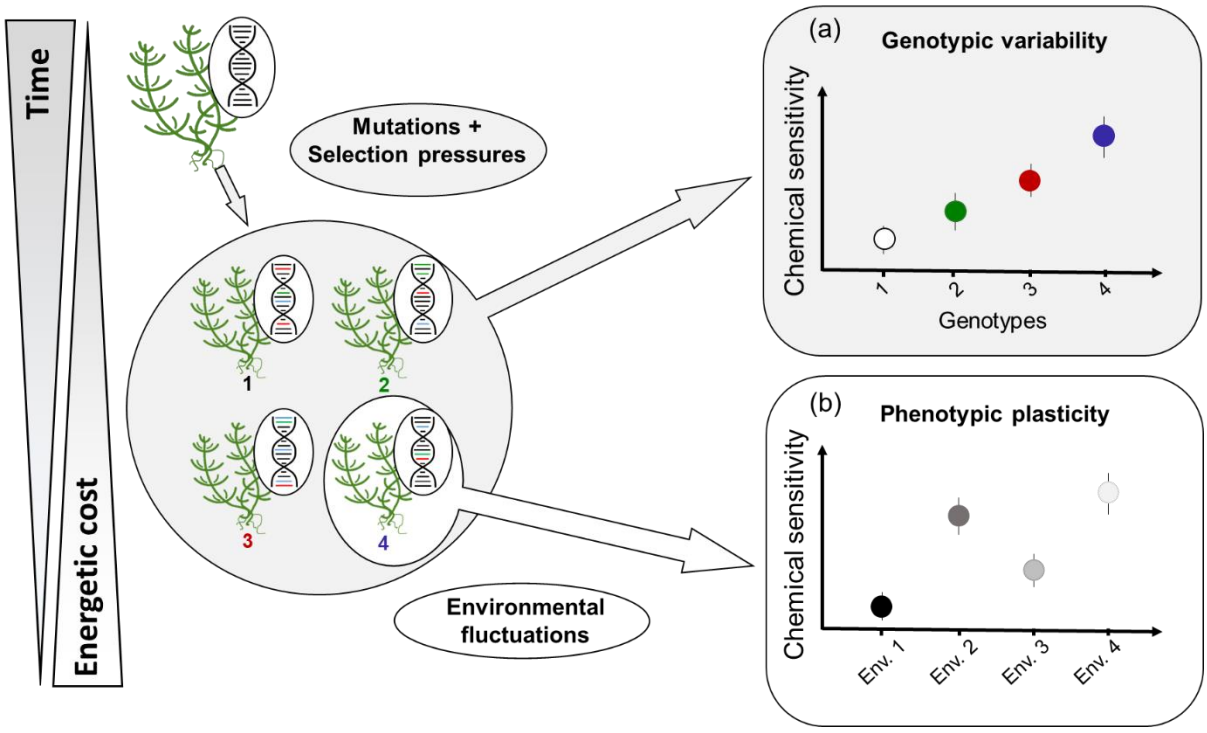


Figure 4.4. Graphical scheme representing the potential impact of intraspecific variation in chemical sensitivity, with (**a**) genotypic variability among four genotypes of aquatic plants and (**b**) phenotypic plasticity in the chemical sensitivity of one genotype across four environmental sets. Genotypic variability takes time, as it occur through several generations, whereas phenotypic plasticity occur during an individual lifespan. Phenotypic plasticity is a costlier process, as it require sensors for environmental cues, and constant adjustments, compared to a fixed trait which will not vary across environmental ranges.

5. Thesis outline

Overall, few studies have assessed the importance of intraspecific variation in species sensitivity to chemicals. As explained in part 1 of this chapter, aquatic plants play a pivotal role in ecosystems, are sensitive to chemical contaminations, and are particularly subject to intraspecific variation due to their absence of motility. Furthermore, these organisms are also model species in ERA, and are extensively used in toxicity laboratory testing. Copper is considered as a model contaminant, as it is environmentally relevant and its effects on the environment have been broadly studied, as explained in part 2 of this chapter.

In order to cope with the current lack of knowledge on the subject, I aimed to investigate the importance of intraspecific variations in macrophyte sensitivity to Cu.

To do so, after describing the materials and methods used along the experiments (chapter **II**), I addressed three main questions, which are presented below and summarized in **Figure 5.1**;

(1) What is the relative importance of intraspecific *vs.* interspecific variations in the chemical sensitivity of macrophytes?

I first aimed to compare intraspecific and interspecific variation in terms of chemical sensitivity, across the following species: *Myriophyllum spicatum*, *Lemna minor* and *Ceratophyllum demersum*. The study is presented in chapter **III**.

(2) How important is genotypic variation in the intraspecific variability of macrophyte sensitivity to chemicals?

Therefore, the importance of genotypic variability in the response of *Myriophyllum spicatum* to Cu exposure was investigated, through the study of seven genotypes. The results are described in chapter **IV**.

(3) How important is phenotypic plasticity in the intraspecific variability of macrophyte sensitivity to chemicals?

The importance of phenotypic plasticity in the sensitivity of *Lemna minor* exposed to Cu was thus investigated across three experiments and two distinct environments for each experiment. The study is presented in chapter **V**.

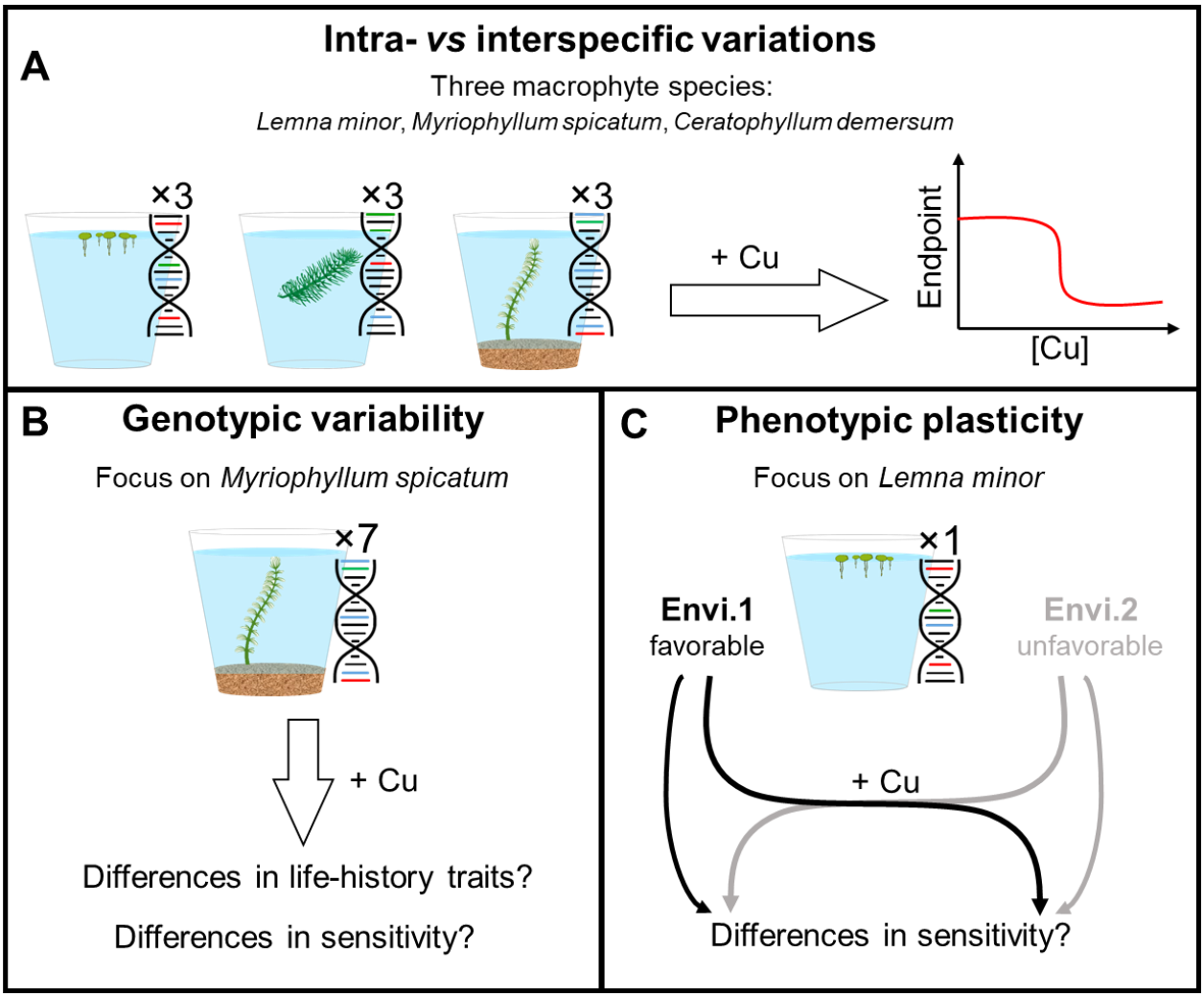


Figure 5.1. Graphical scheme of the questions addressed during my PhD project. Importance of intraspecific compared with interspecific variations in three macrophytes species exposed to copper (Cu), with several genotypes per species (**A**). Underlying mechanisms of intraspecific variation with (**B**) importance of genotypic variability in the sensitivity of seven genotypes of Myriophyllum spicatum exposed to Cu and (**C**) importance of phenotypic plasticity in the sensitivity of *Lemna minor* exposed to Cu under favorable and unfavorable environments (Envi.).

CHAPTER II

MATERIAL AND METHODS

1. Model species

Three species with different life-history traits were selected to study interspecific variability, and the importance of intraspecific variability: *Lemna minor*, *Ceratophyllum demersum and Myriophyllum spicatum*. These species are complementary from each other, as they thrive in the different compartments of an aquatic ecosystem: the water surface, the water column and the sediment/water column. As such, they are representative of the different life forms that can be found across aquatic plants, and are commonly grown if the same water body. These species have also been selected due to their wide repartition area and their use in standardized ecotoxicological tests for two of them.

A. *Lemna minor*

1) Morphology

Lemna minor L., or "duckweed", is a free floating species living at the water-atmosphere interface. It is composed of a rosette of one to twelve "fronds" (resulting from the contraction of stems and leaves in a simplified photosynthetic structure), each of these having a single root which can be several centimeters long (**Figure 1.1A**, **B**). The fronds are oval, 1 to 8 mm long and 0.6 to 6 mm wide, with a developed aerenchyma (i.e. small air gaps between their parenchymatous cells) to allow their flotation. When the plant grows older and produces more fronds, these are split to separate individuals. Flowers are rarely produced (1 to 5% of the fronds), and are about 1 mm in diameter, with a cup shape (Landolt and Kandeler 1987).

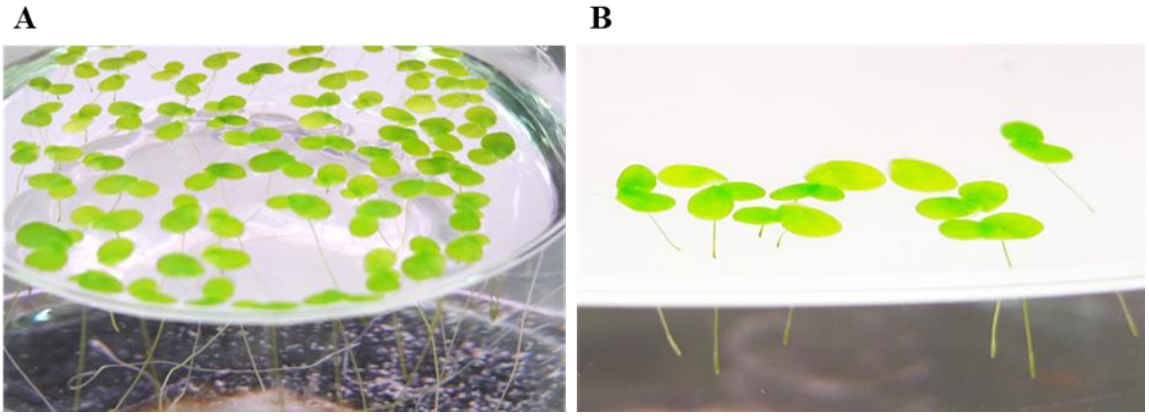


Figure 1.1. (**A**) *Lemna minor* in an axenic culture inside an Erlenmeyer flask, (**B**) close-up picture of *L. minor* controls during Cu exposure. Both pictures were taken in a growth chamber.

2) Distribution and ecology

Lemna minor is a monocotyledon species which belongs to the *Araceae* family, and is able to perform both sexual and clonal reproduction, the last being prevalent. *L. minor* is a fast-growing species which is broadly used in biomonitoring of aquatic environments (Gopalapillai et al. 2014; Szczerbińska and Gałczyńska 2015). Its growth is optimal at pH between 6.5 and 8, and from mesotrophic to eutrophic water (Amoros et al. 2000; Melzer, 1999). Growth stops when the temperature drops below 6°C, but otherwise occurs between 6 to 33°C. *L. minor* can be grown in completely mineral medium, and can be cultivated under axenic conditions (Landolt and Kandeler 1987). This species is easy to cultivate in laboratory conditions; its rapid vegetative reproduction allows the production of genetically uniform clones, and makes them valuable in research.

L. minor has a wide distribution area due to easy dispersion through wind, human transports and animals (**Figure 1.2**). It is found from northern Scandinavia to New Zealand, and therefore lives in a very broad ecological range with different environmental conditions, in freshwater ponds, channels or slow moving streams (Hillman 1961; Landolt and Kandeler 1987).

This species also shows high potential for phytoremediation, due to its very high uptake capacity of different metals such as Pb, Zn, Cu and As (Razinger et al. 2007; Dosnon-Olette et al. 2011; Basile et al. 2012). Its uptake efficiency of phosphorous and ammonia in water makes this species a precious tool for wastewater management (Gürtekin and Şekerdağ 2008). Furthermore, *L. minor* provides food and habitat for numerous species, and plays important roles in ecosystem dynamics. A specific standardized protocol for risk assessment use *L. minor* as a model species since 2006 (OECD protocol n°221, Khellaf & Zerdaoui, 2010; Leblebici & Aksoy, 2011).

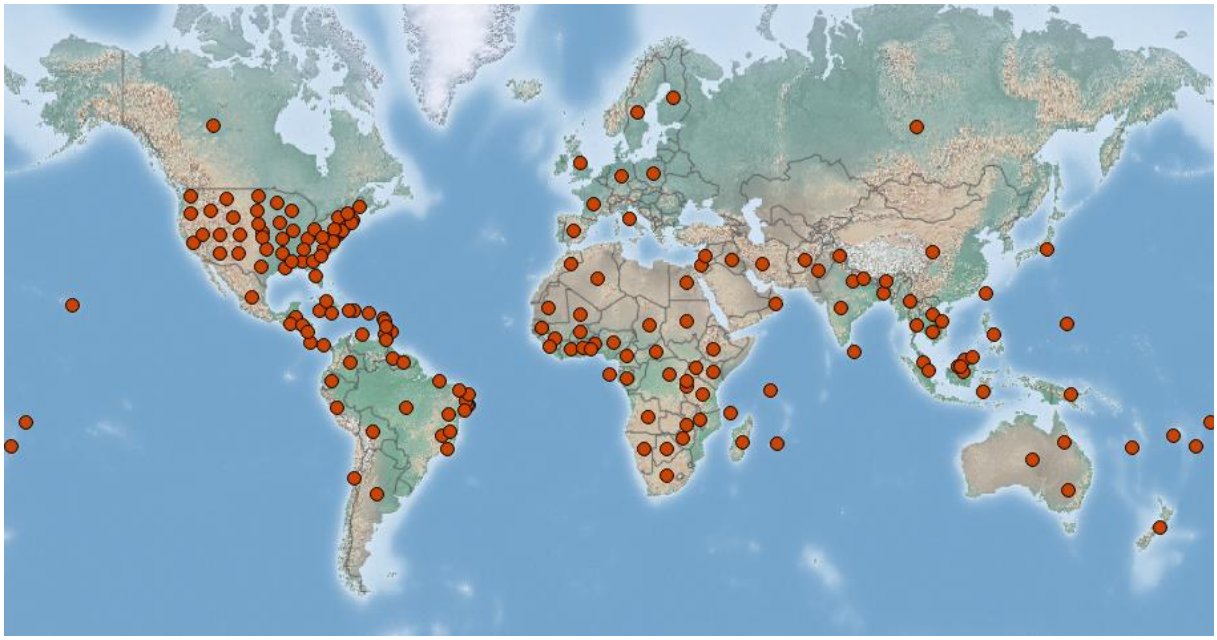


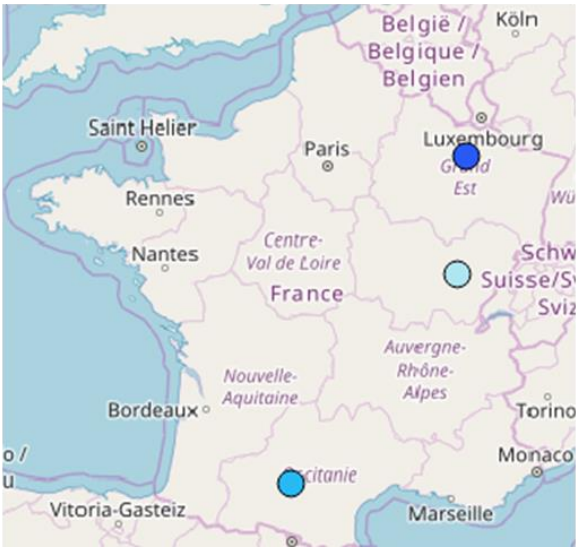
Figure 1.2. Distribution map of *Lemna minor* across the globe. Red dots are the countries/states where the species has been reported (CABI, Centre for Agriculture and Biosciences International, 2011, [www.cabi.org/isc/]).

3) Laboratory cultivation and global maintenance

Three clonal strains were harvested in three different locations across France (**Figure 1.3**). Genetic characterization was performed to ensure that the clonal strains had different genotypes (see section 7 for further details).

From each location, one single frond was placed under axenic conditions through calcium hypochlorite treatment, 1% during 3 minutes ($CaO(Cl)_2$, purchased from Sigma Aldrich). Each stock culture was started from a single frond, and was grown in a specific medium at pH 5.8. A new stock culture was started every 3 weeks for each clonal strain, with 8 to 12 fronds from the older stock culture, in the medium described in **Table 1.1**.

A



B

Genotype	GPS coordinates
Authume	47.12256, 5.5056
Canal du midi	43.56515, 1.47148
Metz	49.03357, 5.72106

Figure 1.3. (**A**) Geographic origin of the different clonal strains of *L. minor* used for experiments and (**B**) GPS location of the harvesting sites.

During experiments, the Steinberg medium was used in accordance with OECD protocol n°221, with modifications (OECD 2006). Notably, a pH of 6.5 instead of 5.5 and an azote/phosphate ratio of 20:1 (38 mg/L KH_2PO_4 and 5 mg/L K_2HPO_4) to decrease algae proliferation, and an Fe-EDTA solution at a 1:1 ratio, (**Table 1.2** for nutrient concentrations).

Table 1.1. Composition of the medium used for stock cultivation of *L. minor* under axenic conditions in a growth chamber, maintained at a pH of 5.8.

Macroelements	**Molecular weight**	**mg/L**
KNO_3	101.1	60.66
NH_4NO_3	80.04	10.88
K_2HPO_4	174.4	7.308
KH_2PO_4	136.08	16.2
$MgSO_4, 7H_2O$	246.47	36.95
$NaHCO_3$	84.007	63
Fe EDTA	55.845	0.5
$Ca(NO_3)_2, 4H_2O$	236.15	150
$CaCl_2, 2H_2O$	147.02	0.72
Oligoelements	**Molecular weight**	**µg/L**
$MnSO_4, 2H_2O$	169.01	70
$CuSO_4, 5H_2O$	249.69	1
$ZnSO_4, 7H_2O$	287.55	22
H_2SeO_3	128.98	1.6
$Na_2MoO_4, 2H_2O$	241.95	24

Table 1.2. Steinberg medium used for *Lemna minor* experiments, modified from the OECD protocol, with a pH of 6.5.

Macroelements	**Molecular weight**	**mg/L**
KNO_3	101.12	350
$Ca(NO_3)_2$ $4H_2O$	236.12	295
KH_2PO_4	136.09	38
K_2HPO_4	174.18	5
$MgSO_4$ $7H_2O$	246.37	100
Microelements	**Molecular weight**	**µg/L**
H_3BO_3	61.83	120
$ZnSO_4$ $7H_2O$	287.43	180
Na_2MoO_4 $2H_2O$	241.92	44
$MnCl_2$ $4H_2O$	197.84	180
Fe EDTA	55.845	160

B. *Myriophyllum spicatum*

1) Morphology

Myriophyllum spicatum L., or "Eurasian watermilfoil", is a submerged rooted dicotyledonous plant which belongs to the *Haloragaceae* family. It is rooted in sediments, and grows in the water column. It has thin stems, which can appear green, brown, or red-pink (**Figure 1.4A**, **B**, C). It can grow up to 3 meters in length, and stems become thinner when they grow further from the main stem (Aiken et al. 1979). There are four leaves of 1.5-4 cm long, feather-like, whorled around the stems, with 14 or more uniform leaflets on each leaf. *M. spicatum* is a perennial plant that flowers twice a year, in mid-June and July-August, and the flowering is followed by auto-fragmentation, easing its dispersion (Nichols 1975; Madsen and Smith 1997). This species is able to take up nutrients both through the leaves and the roots, although the root absorption is preferential (Barko and Smart 1981). The inflorescence rises 5 to 10 cm above the surface of the water from the terminal spike, with both male and female flowers on the same inflorescence (Aiken et al. 1979). *M. spicatum* produces a high quantity of secondary metabolites, like phenolic compounds such as tannins. Among them, the tellimagradin II is well known to be the source of *M. spicatum* allelopathy, as it is repellant for most herbivores (Gross 2001). Furthermore, it has been demonstrated that tellimagrandin II

provide a competitive power, as it shows a seasonal variation in accordance with the developmental peak of the species (Gross 2001; Gross and Jüttner 2003).

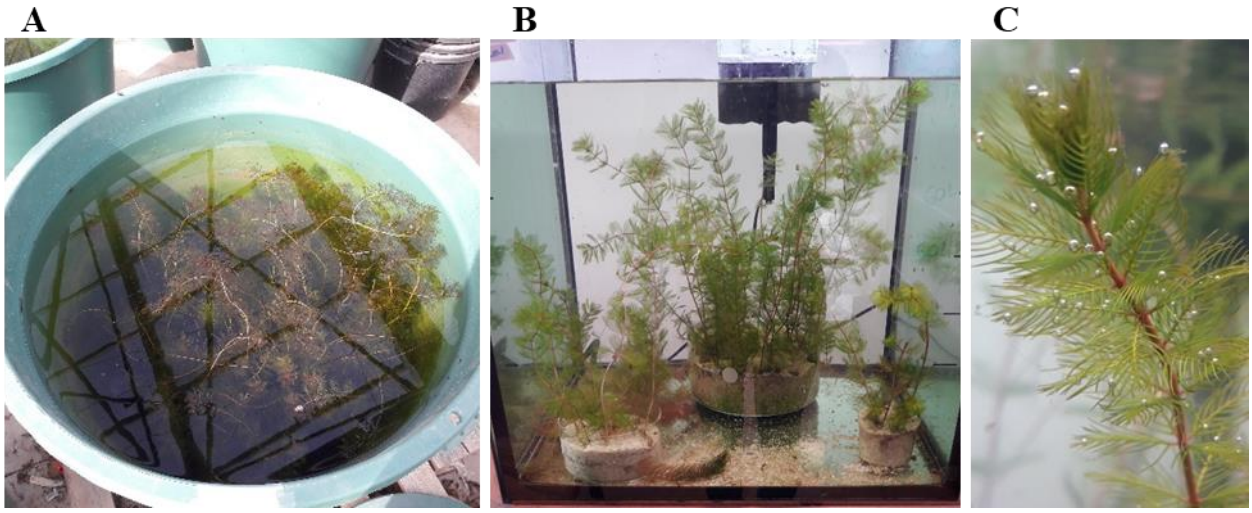


Figure 1.4. (**A**) Picture of stock culture of *Myriophyllum spicatum* in a 220 L outdoor tank, (**B**, **C**) pictures of *M. spicatum* in another stock culture in the growth chamber in an 80 L aquarium.

2) Distribution and ecology

Myriophyllum spicatum L. originates from Asia, Europe and northern Africa (**Figure 1.5**). Nowadays, this species is considered invasive in South Africa and in Northern America due to its high competitiveness (Weyl and Coetzee 2016). Indeed, *M. spicatum* can easily spread through clonal reproduction with fragment dispersal, and also through sexual reproduction with seed dispersion through water and animal transport, and grows quickly to form canopies (Gross 2001). It has been demonstrated that seed production is more important in eutrophic than in mesotrophic waters, and contributes to the expansion of populations (Madsen and Boylen 1989; Wani and Arshid 2013).

This species is found worldwide, in pH ranking from 5.4 to 11, and is tolerant to a wide range of water quality. It prefers hard water (alkaline water) systems with high dissolved inorganic carbon, and usually grows in mesotrophic and eutrophic waters (Barko 1990; Melzer 1999; Amoros et al. 2000; CABI 2011). It can be found at depths of one to ten meters in lakes, ponds, shallow reservoirs and low energy areas of rivers and streams (Amoros et al. 2000). It grows well in areas that have experienced disturbances such as intense plant management, or abundant motorboat use (Aiken et al. 1979). It is considered as a pioneer species, as it is among the first species to colonize ecosystems after a disturbance. It also rapidly colonizes polluted

waters which are usually unsuitable for other species (Yan and Xue 2013). Furthermore, *M. spicatum* is resistant to herbivory, as it is rich in tannins which are repellant to generalist herbivorists, and it provides an advantage in ecosystems with high fish and bird densities (Iason et al. 2012).

Due to its ability to take up metals and other pollutants from waters, *M. spicatum* is used both for phytoremediation and biomonitoring purposes (Sivaci and Sökmen 2004; Keskinkan et al. 2004; Yan and Xue 2013). It also has a high ecological importance, as *M. spicatum* is used as substrate for periphyton and as shelter and forage for other organisms. Furthermore, it has key functions in biogeochemical cycles through the translocation of nutrients from sediments, organic carbon production, and the uptake of phosphorus and ammonia, thus improving water quality (Bornette and Puijalon 2011).

As this species is representative of rooted submerged aquatic plants species, and has a broad ecological range, two OECD protocols have been implemented in 2014 for ecotoxicological risk assessment; one non-rooted protocol under axenic conditions with a medium containing sucrose (OECD test n°238) and one rooted test with sediments but no sugar addition in water (OECD test n°239).

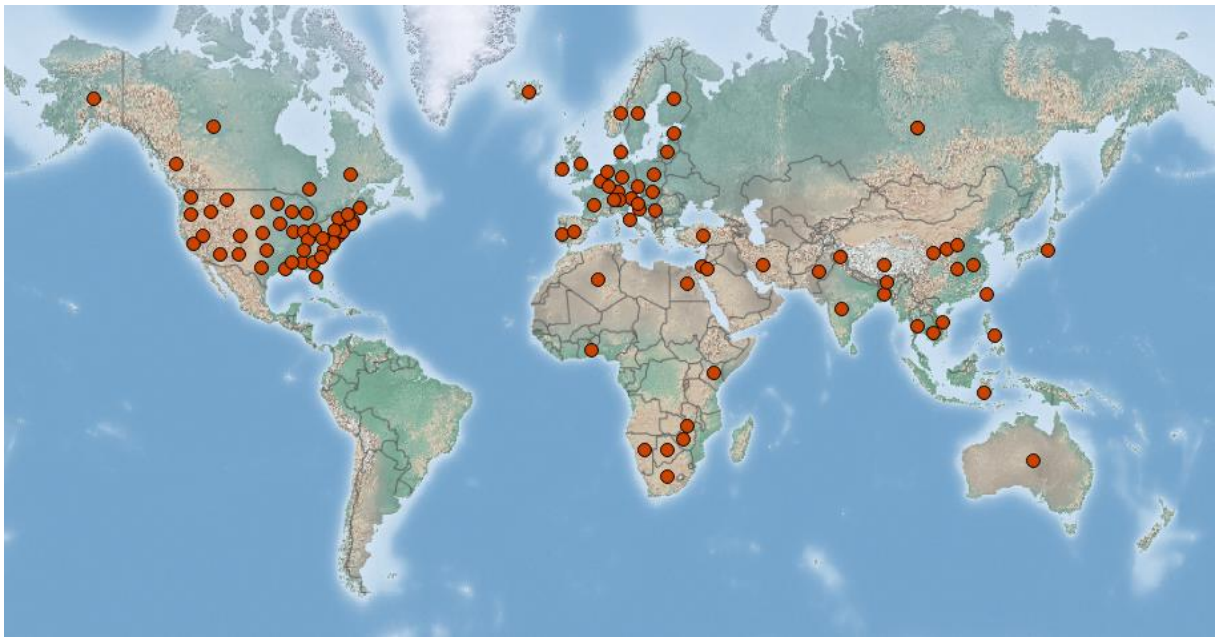


Figure 1.5. Distribution map of *Myriophyllum spicatum* across the globe. Red dots are the countries/states where the species has been reported (CABI, Centre for Agriculture and Biosciences International, 2011, [www.cabi.org/isc/]).

3) Laboratory cultivation and global maintenance

Eight clonal strains were harvested in the field, between 2011 and 2015, from various watersheds in France and Germany (**Figure 1.6**). Within a given watershed, the clonal strains were harvested from different rivers upstream of their confluence to increase the chance to sample genetically different plants. Genetic characterization was performed to ensure that the clonal strains had indeed different genotypes (see section 7 for further details).

A

B

	Genotype	GPS coordinates
●	**Tarn**	43.89067, 1.50656
●	**Doubs**	47.23153, 6.02252
●	**Dordogne**	44.84584, 0.90596
●	**Schöhsee**	54.16624, 10.44114
●	**Camargue**	43.51133, 4.60584
●	**Garonne - Bourret**	43.94622, 1.1711
●	**Garonne – St Aignan**	44.02281, 1.07797
●	**Agout**	43.6395, 2.31875

Figure 1.6. (**A**) Geographic origin of the different clonal strains of *Myriophyllum spicatum* used for experiments and (**B**) GPS coordinates of the harvesting sites.

Each stock culture was started from one stem fragment, placed in 220 L tank with 5 L of quartz sediments mixed with 1.33 g Osmocote® (granulated fertilizers with slow release, NPK: 16-8-12, KB) per liter of sediment. Sediments were changed twice a year, and inflorescences were cut every week during flowering period to avoid hybridization among genotypes. Snails (*Radix* sp. and *Physa* sp.) were added to the containers for algae regulation. These stock cultures were also kept in 80 L aquariums with similar sediment conditions.

In order to ensure optimum conditions with maximal growth during exposure experiments, different media were tested (Steinberg, Smart and Barko, Hoagland, Andrew), with or without sediment, and with different pH (from 5 to 8), bicarbonate sources and sediment levels when sediment was present (OECD 2006, 2014b; Hoagland and Arnon 1950). At first, media with

high N/P concentrations were used, as they were optimized for aquatic plant growth without algae proliferation. Our preliminary results demonstrated that a richer media, bicarbonate supplementation and alkaline pH increased growth of *M. spicatum*, but strongly enhanced algae growth as well in our experimental containers. This proved to be noxious for *M. spicatum* during long term exposure (over a week), and made it difficult to distinguish between algae and pollutant effects at low concentrations. We finally selected Smart and Barko media, with a pH 6.5 instead of 7.8, and with 50mL quartz sediment containing Osmocote® per experimental unit, as the best compromise for growth of *M. spicatum* without having copper precipitation and algae proliferation as the nutrients were mainly in sediments (OECD, 2014, **Table 1.3**, **Figure 1.7**).

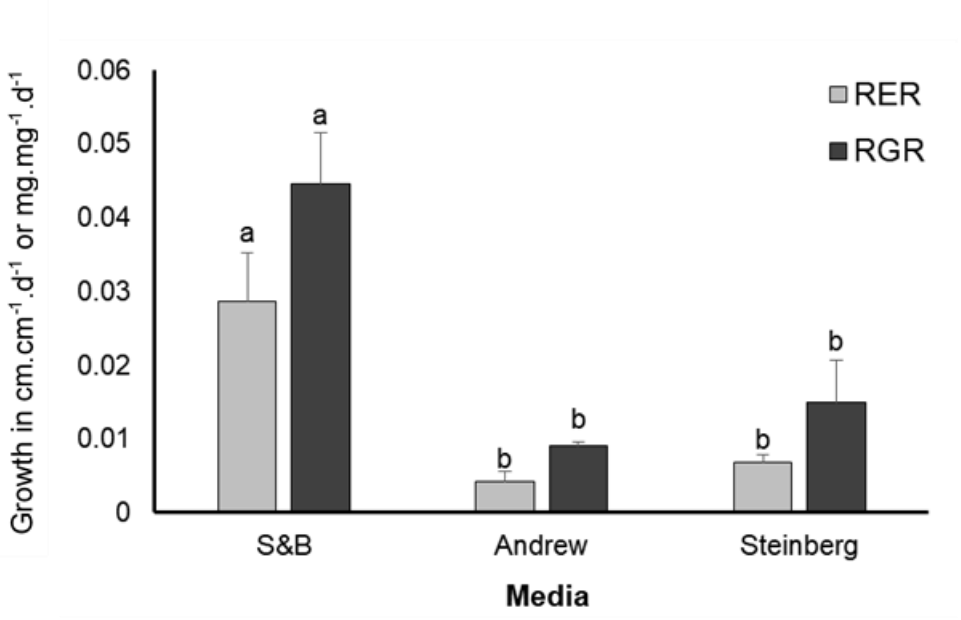


Figure 1.7. Growth media for *Myriophyllum spicatum* tested with sediments mixed with Osmocote® over 10 days. S&B: Smart and Barko media pH6.5, Andrew media pH6.5 and Steinberg media pH6.5. RER stands for Relative Elongation Rate in $cm.cm^{-1}.d^{-1}$, and RGR for Relative Growth Rate in $mg.mg^{-1}.d^{-1}$.

Exposure experiments were adapted from OECD protocols and adjusted to fit with non-axenic conditions and with the intrinsic properties of the different clonal strains. Indeed, the growth of the different strains was not always meeting the requirements of the OECD protocols, notably concerning the doubling time for length during exposure. Furthermore, OECD protocols required to have three shoots per replicate, but this requirement could not be reached

due to the high amount of biomass required for the experiments with multiple genotypes (Paragraph 32 of OECD protocol N°239).

Table 1.3. Medium and sediment used for *Myriophyllum spicatum*: Smart and Barko medium according to OECD protocol n°239 on the left, and Osmocote® (NPK: 16-8-12) composition per g, with 66.6 mg of Osmocote® per experimental unit.

Smart & Barko			**Osmocote® NPK: 16-8-12**		
	Molecular weight g/L	mg/L		Molecular weight g/L	mg/g Osmocote ®
$CaCl_2$ $2H_2O$	147.01	91.7	NO^{3-}	62.005	71
$MgSO_4$ $7H_2O$	246.47	69	NH_4	18.039	89
$NaHCO_3$	84.007	58.4	P_2O_5	283.886	157
$KHCO_3$	100.12	15.4	K_2O	94.2	100
			MgO	40.304	20
			Bo	26.809	0.1
			Fe	55.845	4
			Cu	65.546	4
			Mn	54.938	1
			Mo	95.94	0.1
			Zn	65.38	0.3

C. Ceratophyllum demersum

1) Morphology

Ceratophyllum demersum L., or "hornwort", is a submerged rootless macrophyte belonging to the *Ceratophyllaceae* family. It has stems that reach lengths up to 3 meters, with numerous side shoots. Leaves are produced in whorls of 6 to 8, they are forked into thread-like segments that are edged with spiny teeth (**Figure 1.8A**, **B**). Leaves can be up to 4 cm long, and are stiff and rough due to carbonate inclusions (Sheldon 1987). The shoot color can be yellow to clear brown, and the leaves are green. Roots are lacking. This is a perennial plant, and during autumn it forms hibernacula, which are modified buds consisting of a short main axis, and tightly clustered dark green leaves, containing starch. It remains dormant until spring, when environmental conditions are favorable for growth (Sculthorpe 1967). In temperate regions, the release of hibernacula from the layer of detritus at the bottom of the lake is essential for dispersal, as water temperature is generally too low for flowering and for seed development. Flowering occurs in warmer areas, in Papua New Guinea for example, where seeds are commonly found (Osborne and Polunin 1986). It is monoecious, with separate male and female

flowers produced on the same plant. Sexual reproduction happens underwater, with hydrophilous pollen transport. Vegetative buds are formed in the axil of leaves. Stems break easily, and the pieces continue to grow separately, allowing a very competitive clonal reproduction (Godfrey and Wooten 1981).

C. demersum has allelopathic capacities, and secretes sulfur compounds that inhibit the growth of phytoplankton, including cyanobacteria (Gross et al. 2003).

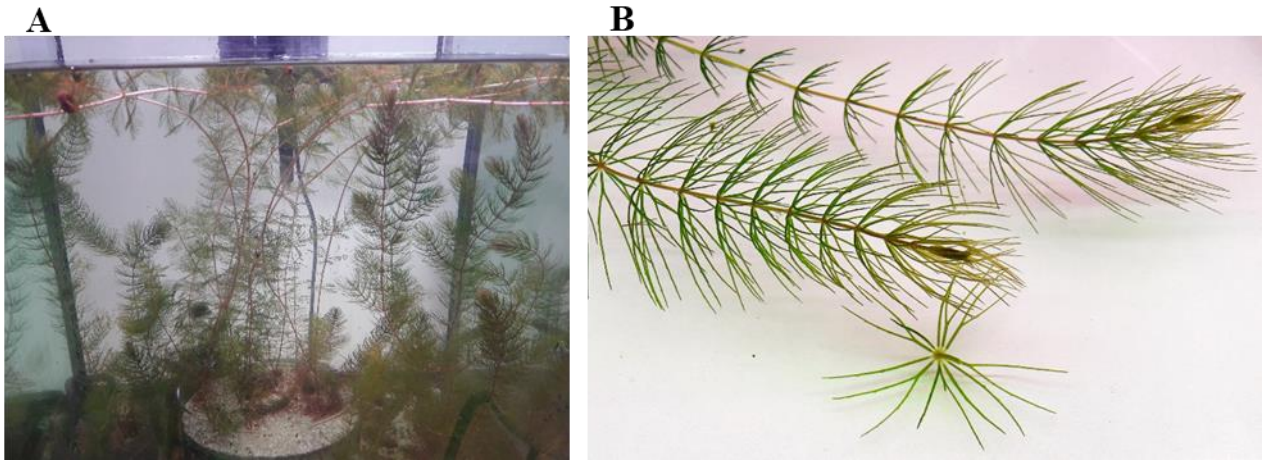


Figure 1.8. (**A**) Stock culture of *Ceratophyllum demersum* in an 80 L aquarium in a growth chamber with *M. spicatum*, (**B**) close-up picture of *C. demersum* shoots.

2) Distribution and ecology

Ceratophyllum position within the phylogeny of angiosperms has been controversial for some time; the first phylogenetical analysis placed *C. demersum* in a sister group of angiosperms. The Angiosperm Phylogeny Group (APG IV) and more recent studies using more data sets but with low support values, have placed *Ceratophyllum* close to the eudicots (Iwamoto et al. 2015; Chase et al. 2016).

This species can perform both sexual and clonal reproduction, and is therefore easily spread. Is has a wide ecological tolerance and a fast growth, and is therefore considered as an ubiquitous species. *C. demersum* grows in hard waters, in moderately to highly eutrophic lakes, slow-moving water streams and ditches (CABI 2011). It thrives under various environmental conditions with high nitrogen and phosphorus concentrations, at temperatures between 18°C to 26°C, and a pH ranging from 6 to 8. In natural environments, the growth starts in March and ends in November, during the time for which the environmental conditions (*e.g.* temperature, light intensity and photoperiod) are favorable. Under experimental conditions, plants undergo

a dormant stage as well, reducing the growth period even under constant environment (Best 1977, 1979).

This species is cosmopolitan, being found on every continent, Antartica excepted. It has a weed status in Tasmania, and is on list the of unwanted organisms in New Zealand (MPI) as it could unbalance aquatic ecosystems (De Winton et al. 2009, **Figure 1.9**). It is used as a bioindicator and for phytoremediation of metals (Ostroumov and Shestakova 2009; Zuccarini and Kampuš 2011). Like other macrophyte species, *C. demersum* plays important roles in ecosystem dynamics, due to both direct effects, through interactions with other organisms, and to indirect effects on organisms, through biogeochemical cycles of nutrients and impact on water quality (Kurilenko and Osmolovskaya 2006; Dhote 2007; Magela et al. 2010).

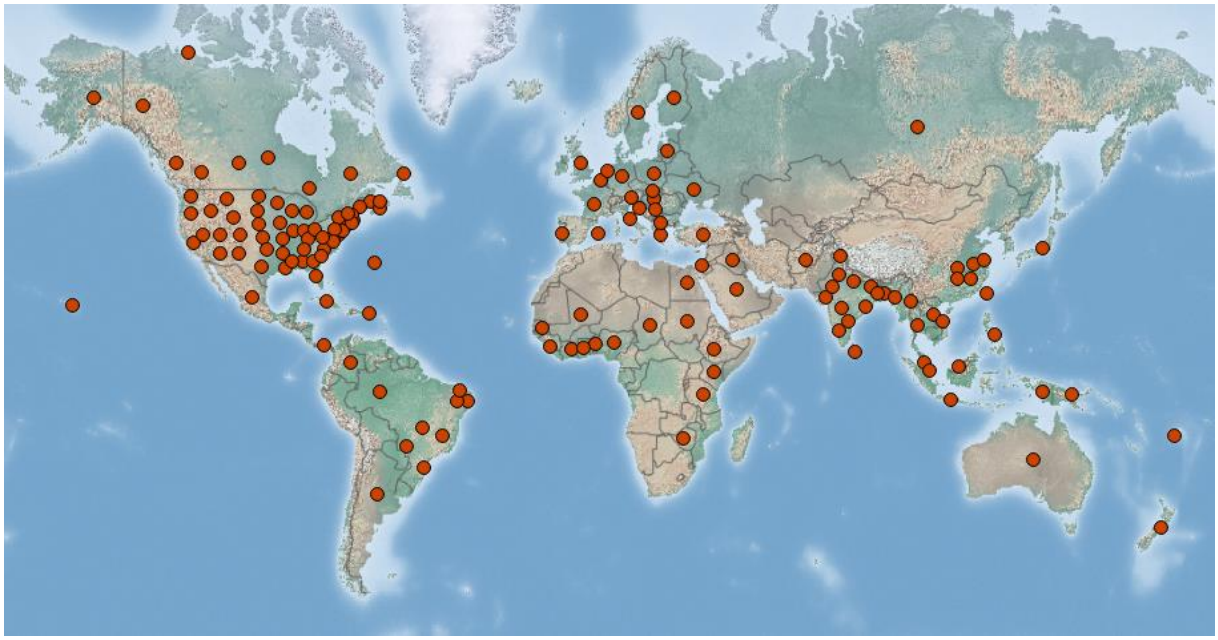


Figure 1.9. Distribution map of *Ceratophyllum demersum* across the world. Red dots indicate the countries/states where the species has been reported (CABI, Centre for Agriculture and Biosciences International, 2011, [www.cabi.org/isc/]).

3) Laboratory cultivation and global maintenance

Three distinct genotypes were harvested in the field, between 2011 and 2015, from distinct populations in France (**Figure 1.10**). When harvested in two rivers within a same watershed, the clonal strains were taken upstream of the confluence to increase the chance to sample genetically different plants. Genetic characterization was performed to ensure that the clonal strains had indeed different genotypes (see section 7 for further details).

A

B

	Genotype	GPS coordinates
●	**Tarn**	44.11785, 1.15908
●	**Garonne**	44.01804, 1.07639
●	**Dordogne**	44.83811, 0.73947

Figure 1.10. (**A**) Geographic origin of the different clonal strains of *Ceratophyllum demersum* and (**B**) GPS coordinates of the harvesting sites.

Each stock culture was started from one individual stem, placed in 220 L tanks with *M. spicatum*. Nutrient fertilization was brought through the 5 L of quartz sediments mixed with 1.33 g Osmocote® (granulated fertilizers with slow release, NPK: 16-8-12, KB) per liter of sediment. Sediments were changed twice a year. These stock cultures were also kept in 80 L aquariums with *M. spicatum* genotypes. Aquatic gastropods (*Radix* sp. and *Physa* sp.) were added to the tanks for algae regulation.

In order to determine which culture medium was optimal to obtain maximal growth during exposure of *C. demersum*, several tests were conducted with different media at pH6.5 to decrease algae proliferation: Steinberg $^1/_2$ strength, Steinberg $^1/_5$ strength (OECD 2006), Combo medium (Kilham *et al.* 1998), Hoagland full strength, Hoagland $^1/_5$ and Hoagland $^1/_{10}$ (Hoagland and Arnon 1950). Although no significant differences were found for the relative elongation rate (RER) due to high variation among replicates, the Steinberg $^1/_2$ was selected as it demonstrated in average the highest RGR and RER compared to the other tested media (**Figure 1.11**), see **Table 1.4** for medium composition.

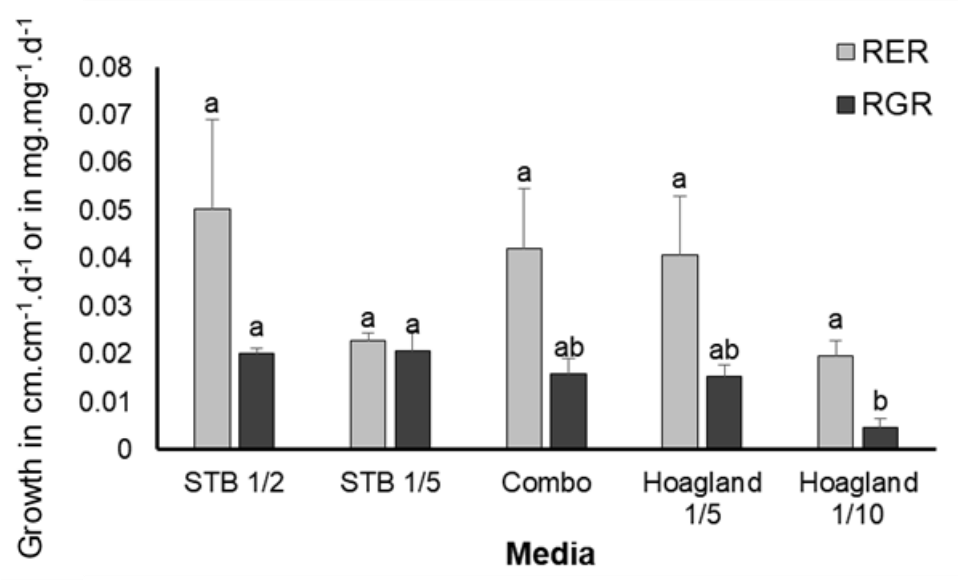


Figure 1.11. Relative Elongation Rate (RER) based on length in $cm.cm^{-1}.d^{-1}$, and Relative Growth Rate (RGR) based on fresh in $mg.mg^{-1}.d^{-1}$ of *Ceratophyllum demersum* growing in different media during 7 days. Stb: Steinberg, Hoag.: Hoagland.

Table 1.4. Steinberg half strength composition used for experiments on *Ceratophyllum demersum.*

Macroelements	**Molecular weight**	**mg/L**
KNO_3	101.12	175
$Ca(NO_3)_2$ $4H_2O$	236.12	147.5
KH_2PO_4	136.09	19
K_2HPO_4	174.18	2.5
$MgSO_4$ $7H_2O$	246.37	50
Microelements	**Molecular weight**	**µg/L**
H_3BO_3	61.83	60
$ZnSO_4$ $7H_2O$	287.43	90
Na_2MoO_4 $2H_2O$	241.92	22
$MnCl_2$ $4H_2O$	197.84	90
Fe EDTA	55.845	80

2. Copper exposure and experimental designs

A. <u>Growth chamber parameters</u>

Each genotype was cultivated in a growth chamber at 20°C ± 0.1, with a light:dark photoperiod of 14h:10h. Photosynthetic photon flux density was maintained at approximately 95 to 120 µmol m^{-2} s^{-1} photosynthetic photon flux density, provided by fluorescent lamp tubes (Philips TL5 HO 39W "day light", and Sylvania T5 Grolux 39W "plant growth").

B. Effective Cu concentrations in water samples

Effective concentrations of ionic copper in water samples were measured through inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis (Thermo-Electron IRIS Intrepid II XLD, see section 3.b for further details). Measurements were performed with three technical replicates per biological replicate. Tubes of 15 mL were filled with 7 to 10 mL of samples, and two steps of acidification were performed in order to ensure Cu dissolution: one drop of 60% HNO_3 was added per sample before being stored in a cold chamber, and one drop before being analyzed with ICP-AES. Samples were filtrated with a 0.45 µm cellulose membrane before the first acidification step.

Monitoring of ionic Cu concentrations performed in preliminary experiments on the three species over 24 hours and 7 days allowed to determine the drop of Cu over time (likely due to plant absorption and/or adsorption), depending on the concentration and the species. It was determined that for *M. spicatum* and *C. demersum* species, Cu concentration in the media decreased by 20% after 4 hours, and the drop was stabilized after 24 hours. The renewal of the media did not achieved a balanced concentration between the media and the plants, therefore the drop was the same after the change of media.

C. Intraspecific and interspecific variations

To determine the relative importance of intraspecific compared to interspecific variations, several experiments were carried out to obtain the effective concentrations at which 50% of the effect is observed (EC_{50} values) for at least 3 genotypes among the 3 species.

For each species, at least two experiments were performed to obtain EC_{50} values for maximum quantum yield of photosystem II (F_v:F_m) and for growth-related endpoints (RGRs). Indeed, in preliminary experiments the F_v:F_m showed high variation among replicates after a week of exposure, which strongly influenced the EC_{50} values, that showed too much variation to distinguish a genotype effect. On the contrary, due to varying growth rates depending on the life-traits of each species, growth experiments were realized for at least a week; it was therefore necessary to perform two different experiments. The exposure duration for F_v:F_m was set at 96 h for all species. Growth experiments were set at 7 days of exposure for *L. minor*, 12 days of exposure for *M. spicatum* and 14 days of exposure for *C. demersum*, as their growth rate is

lower than that of *L. minor*. For *M. spicatum* and *C. demersum*, the medium was renewed in the middle of the experiment (6 or 7 days), as preliminary experiments showed the high ab/adsorption of Cu by those 2 species, which strongly decreased the Cu in the medium. Exposures were therefore semi-static. Prior to exposure, every species was acclimatized during 5 days.

Copper exposures were realized in 0.5 L plastic glasses, filled with specific medium for each species as determined either by OECD protocols, or by preliminary experiments. For *C. demersum* and *M. spicatum*, each experimental unit was composed of one single shoot which was cut at 6 cm at the beginning of acclimatization. *L. minor* was exposed in Steinberg medium at pH 6.5 (**Figure 2.1A**), *C. demersum* was exposed in half-strength Steinberg at pH 6.5 (**Figure 2.1B**). Finally, *M. spicatum* was exposed in Smart & Barko medium at pH 6.5 with 50 mL of quartz sediment mixed with 66.6 mg Osmocote® (**Figure 2.1C**).

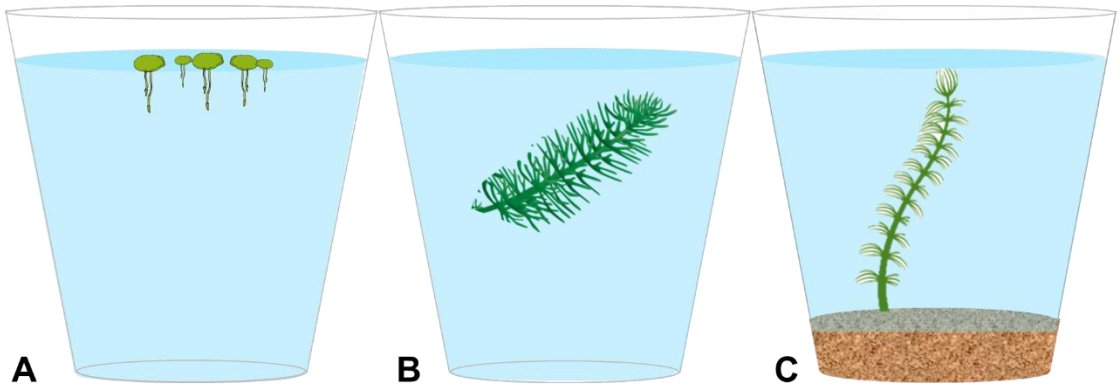


Figure 2.1. Exposure conditions to ionic Cu for the three species (**A**) *Lemna minor* in Steinberg medium, (**B**) *Ceratophyllum demersum* in half strength Steinberg, (**C**) M*yriophyllum spicatum* in Smart & Barko medium with sediment.

D. Genotypic variability

The genotypic variability of *M. spicatum* and its importance in Cu sensitivity was investigated through two exposure experiments conducted on 7 genotypes. These exposures were realized in 0.6 L plastic glasses filled with 0.5 L of Smart and Barko medium at pH 6.5 with 50 mL of quartz sediment mixed with Osmocote® (66.6 mg for 50 mL of sediments, *e.g.* per experimental unit). Prior to exposure, plants were acclimatized in 30 L aquariums during 5 days, rooted in quartz sediment mixed with Osmocote® in the same quantity as for exposure. Each experimental unit was composed of one single shoot which was cut at 6 cm at the beginning of acclimatization.

E. Phenotypic plasticity

To assess the impact of environmental variations on *L. minor* sensitivity to Cu, a crossed experimental design was realized to avoid any confounding effect of environmental conditions prior to the experiments, and also to distinguish between the effects of average environmental conditions and temporal change regime (**Figure 2.2**). The experiments were carried out on *L. minor*, as little variation among replicates was observed during previous experiments, which was a prerequisite to obtain accurate estimations of average effects of the different environmental conditions without increasing too much the number of replicates. There was also less limitation in obtaining biomass for experiments with this species, whatever the season, than for the two other species. The influence of light intensity, nutrient concentrations and Cu pre-exposure was investigated through three distinct experiments. A first phase of acclimatization during 14 days was realized at two levels of environmental conditions (*e.g.* environment 1, rich in nutrients, and environment 2, poor in nutrients). A second phase of Cu exposure was realized during 7 days, both in the same environment as during acclimatization, and in the different environment to trigger an environmental variation (**Figure 2.2**).

These experiments were realized in erlenmeyer flasks to ease experimentation for shading effect and limit water evaporation throughout the exposure.

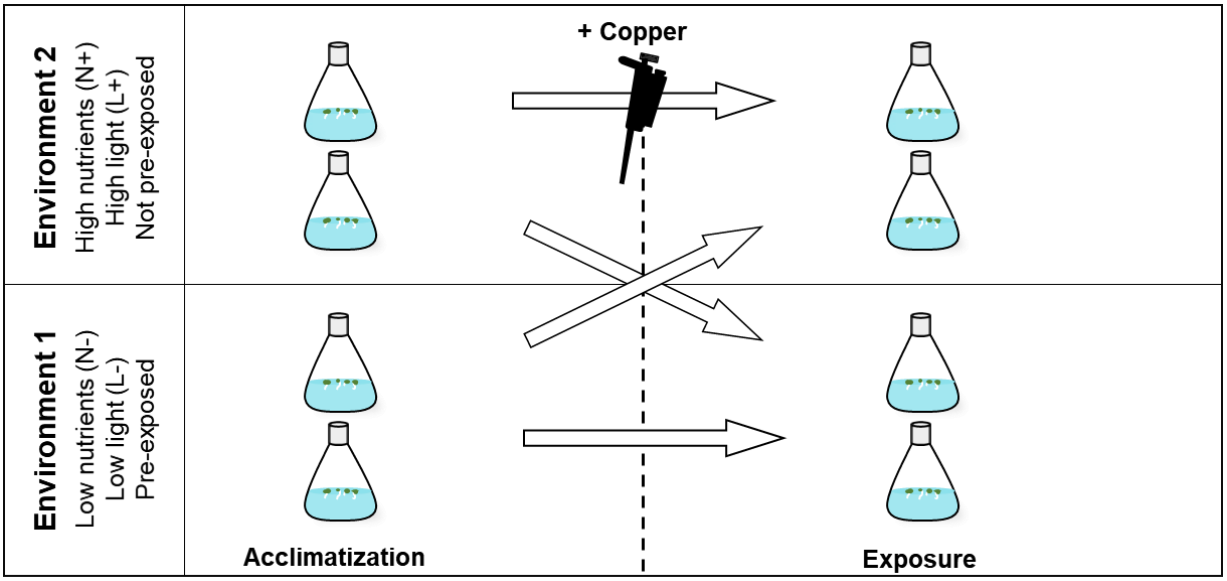


Figure 2.2. Experimental design to highlight the phenotypic plasticity in the response of *Lemna minor* exposed to Cu, with an acclimatization phase (14 days) and exposure phase (7 days).

3. Plant responses to copper

A. *Growth related endpoints*

1) Relative growth rates

Relative growth rates (RGR) based on fresh mass or frond number were calculated with the OECD formula (protocols n°221, 238, 239) as follows:

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

where RGR_{i-j} is the relative growth rate from time i to j, N_i is the endpoint (fresh weight, frond number) in the test or control vessel at time i, N_j is the same variable in the test or control vessel at time j, and t is the time period from i to j.

Fresh masses of *M. spicatum* and *C. demersum* at the beginning of exposure were assessed through the weighting of each shoot after being gently dried on a blotting paper.

Fresh mass of *L. minor* at the beginning of exposure was estimated by weighting at least 15 different bunches of individuals (between 9 to 14 fronds) which were not used afterwards in the experiments, due to the destructiveness of the measurement on *L. minor* (breaking of the roots). The mass at the end of exposure was measured by weighting all the individuals within one experimental unit, with the same balance used for the first weighting.

2) Relative elongation rate

The relative elongation rates (RER) were calculated following the same formula as for the RGRs:

$$RER_{i-j} = (\ln(Lj) - \ln(Li))/t$$

where RER_{i-j} is the relative elongation rate from time i to j, L_i is the length in the test or control vessel at time i, L_j is the same variable in the test or control vessel at time j, and t is the time period from i to j.

The length of *M. spicatum* and *C. demersum* were measured from the beginning of the shoot to the tip of the apex. Length was measured at the beginning of acclimatization when shoots were cut at 6 cm length, at the beginning of exposure, and at the end of exposure.

B. <u>Maximal Quantum Yield of PSII (F_v:F_m)</u>

1) Principle

The measurement of maximal quantum yield of photosystem II (F_v:F_m) lies on the principle of chlorophyll fluorescence. Light energy (photons) is absorbed by the chlorophyll pigments located in the chloroplasts of photosynthetic cells. It can undergo three different fates: chemical energy produced by photosynthesis, excess light energy that can be dissipated as heat, or excess light energy that is re-emitted as light (chlorophyll fluorescence). From the total absorbed light, 1 to 2% is turned into chlorophyll fluorescence, which has a longer wavelength than the absorbed light (Maxwell and Johnson 2000).

The F_v:F_m is the maximal ability of the plant to absorb light energy and to convert it into chemical energy. It is measured by using the Kautksy effect discovered in the early 60s (Maxwell and Johnson 2000; Murchie and Lawson 2013). When photosynthetic material is transferred from the dark to the light, the yield of chlorophyll fluorescence increases during around 1s. It is explained by the reduction of electron acceptors (*e.g.* plastoquinone Q_A) in the photosynthetic pathway downstream of photosystem II (PSII); Q_A is not able to accept another electron unless the first is passed to a subsequent electron carrier (Q_B) (**Figure 3.1A**). During that time, the reaction center is considered as 'closed', and when too many reaction centers are 'closed' this leads to a decrease in photochemistry efficiency, and to an increase in fluorescence (**Figure 3.1B**).

When a leaf is transferred from the darkness to the light, the PSII centers are closed (or saturated) progressively due to massive inflow of photons on the chlorophylls. It is resulting in an increase in chlorophyll fluorescence for a second, then a decrease in the next few minutes.

The F_v:F_m is the quantum yield (maximal efficiency) of PSII, when all the PSII centers are opened. It is given by the following equation:

$$Fv{:}\,Fm = \frac{Fm - Fo}{Fm} = \phi\ \mathrm{PSII}/qP$$

where Fm is the maximal fluorescence, Fo is the basal fluorescence, ϕPSII is the efficiency of PSII chemistry, and qP is an indication of the proportion of open PSII centers.

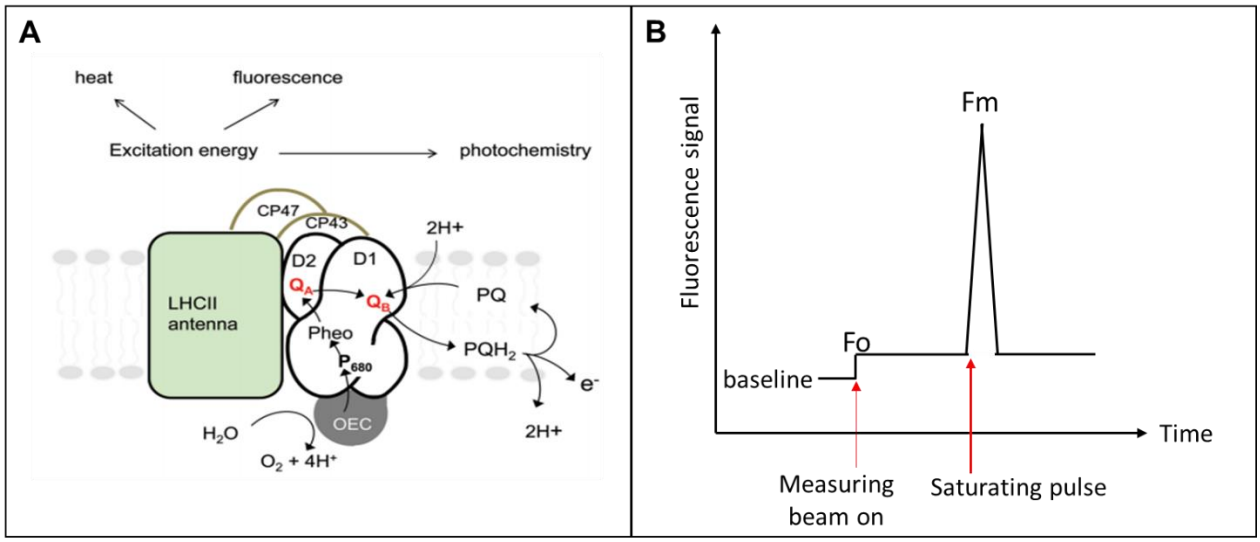


Figure 3.1. A simplified description of the steps occurring in PSII explaining the main parameters in fluorescence analysis. (**A**) A schematic figure showing electron transport within the PSII reaction centre complex. Light energy is absorbed by chlorophyll within the light-harvesting complex, and can be dissipated either via photochemistry, by heat (non-photochemical quenching), or through fluorescence emission. The competition between these processes allows us to resolve the efficiency of PSII. LHC= Light Harvesting Complex, CP = Chlorophyll-proteins, Pheo = Pheophytin, Q_A and Q_B = Quinones A and B, PQ = Plastoquinone, OEC = Oxygen-Evolving Complex. (**B**) Fluorescence trace made on dark-adapted leaf material, showing the formation of F_o and F_m. The measuring beam excites chlorophyll, but its energy is not of a sufficient intensity to induce electron transport through PSII, giving the Fo, corresponding to the minimal level of fluorescence. At this state, the reaction centres are said to be open. Then a saturating pulse of light will result in the formation of the Fm, which is the formation of the maximum possible level of fluorescence, as this pulse closes the reaction centres. (Adapted from Murchie & Lawson, 2013).

2) Measurement settings

The F_v:F_m measurement is considered to be non-invasive and non-destructive. Measurements were made using an underwater fluorometer Diving-Pam (Heinz Walz GmbH, Germany). All measurements were performed in the darkness, after a 30 minute acclimatization into a dark chamber. A special halogen green lamp without actinic light allowed viewing in the dark without interfering with chlorophylls and disturbing the measurements (Sylvania, PAR38 E27 80W).

The basic settings of the Diving-Pam, namely measuring light intensity (50: MEAS-INT) and amplification factor (49: GAIN) were set to 8 and 2 at the beginning of all experiments (over a scale of 0 to 12). When the plant fluorescence was too high and saturated the sensor, the intensity of measuring light was lowered to 4.

At the end of experiments, changes in the Diving-Pam parameters (increase in intensity of measuring light and amplification factor up to 11 over 12) were made when plants were too chlorotic to emit sufficient signal for the light sensor.

C. Cu concentrations in plant samples

1) Mineralization of plant samples

Concentrations in plant samples were measured after rinsing each shoot in 3 baths of deionized water in order to remove copper at the surface. Plant samples were dried during 48 h at 70°C, and mineralized using a DigiPrep (Block Digestion Systems, SCP Science). A first acid digestion step was performed with a ratio of 1:1 HNO_3 65%: deionized H_2O during 30 min at 94°C. After the first heating step, samples were cooled down at room temperature then brought to a 2:1 ratio of HNO_3 65%:H_2O_2 30%, then heated at 94°C during 2 h. Finally, samples were returned to room temperature before being diluted to a final concentration of 2% HNO_3, then filtered through a 0.45 µm membrane.

2) Copper measurement using inductively coupled plasma - atomic emission spectroscopy (ICP-AES)

Principle

Inductively coupled plasma - atomic emission spectroscopy, or ICP-AES, is based on the interaction of a plasma torch and an atomic emission spectrophotometer, and is used for the determination of elementary chemical composition of a sample. This technique allows the quantification of elements due to the ionization of those elements through a plasma flame supplied by electric currents, themselves produced by electromagnetic induction.

The sample is first transformed into a cloud of very thin droplets through a nebulizer, then ionized through an argon flame at 8 000 K, which thermally excites the outer-shell electrons of the elements (**Figure 3.2**). The return to the ground state of excited electrons is accompanied by the emission of photons (light energy) with an energy (wavelength) characteristic of the element. As the sample contains a mixture of different elements, several light wavelengths are emitted simultaneously. The light is then dispersed by a grating in the spectrometer using a photochromator (in our case, a monochromator), separating the different element emissions and

directing them to a dedicated photomultiplier tube detector. The concentration is proportional to the light intensity. The electronic signal is then converted into concentrations by a computer, using calibration solutions.

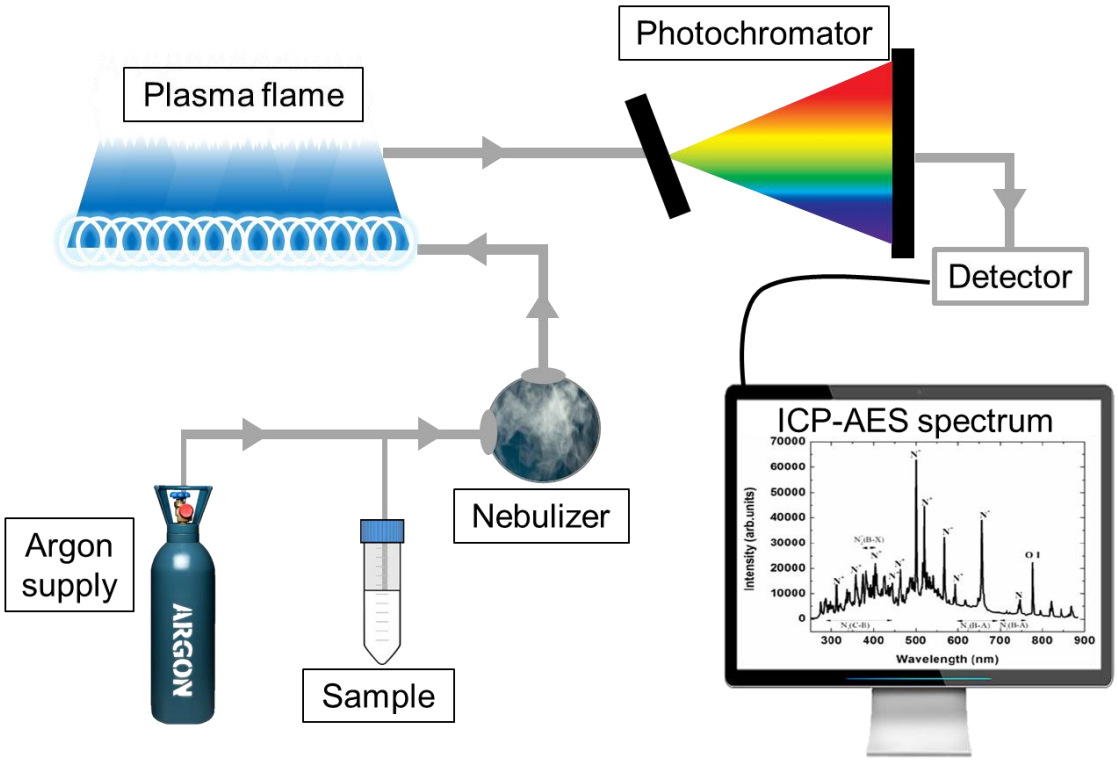


Figure 3.2. Sample analysis using ICP-AES method with the different steps, from the nebulization of the sample to its ionization in the plasma flame, and the separation of the wavelength spectrum and its detection by the detector, ending with concentration determination by the computer.

Analyses

Quality standards were measured by the machine every 60 samples to ensure the accuracy of the measurements, and a rinse step with 5% HNO_3 was performed automatically between each sample.

D. Biomacromolecule analyses using FTIR spectroscopy

1) Principle

Fourier Transform Infrared (FTIR) spectrometers are widely used in chemical industry, polymer science, and others. This technique probes the interactions between infrared radiations and matter (in our case, a solid sample). It can be analyzed in three ways, by measuring

absorption, emission and reflection from the sample. For our analysis, we used emission spectroscopy, which measures the emitted infrared wavelength by the sample. FTIR relies on the fact that most molecules absorb light in the infra-red region of the electromagnetic spectrum (Bacsik, Mink, and Keresztury 2004). This absorption corresponds to the bonds present in the molecule; each bond has a specific frequency of vibration, thus indicates the presence of different functional groups and chemical bonds in the sample.

2) Analyses

Analyses were performed using a Thermo Nicolet NEXUS 470 FTIR, with a 30,000-200 cm^{-1} diamond and a spectral resolution of 4 cm^{-1}. The frequency range are measured as wave numbers, over the range of 5000 – 400 cm^{-1}. Each sample was measured 3 times, with one background measure before each sample measure. For each measure, 64 scans were made with a laser frequency of 15.798 cm^{-1}. Finally, data were processed backward by a computer, to infer what the absorption is at each wavenumber. Results were analyzed with Orange software.

E. Genetic differentiation using Inter Simple Sequence Repeats

1) Principle

Analyses on Inter Simple Sequence Repeats (ISSR) were performed to ensure that the different clonal strains used throughout our experiments had different genotypes (Pradeep Reddy, Sarla, and Siddiq 2002). These analyses were performed by Hervé Gryta, through a collaboration with the Evolution and Biological Diversity research unit (EDB, Paul Sabatier University).

ISSRs are DNA fragments which are flanked by microsatellite sequences. They are short DNA pattern (2-5 nucleotides long) repeated multiple times (e.g. GCGCGCGC). These fragments are amplified through PCR (Polymerase Chain Reaction), by using microsatellite core sequence as a primer, with few nucleotides used as an anchor into the non-repeat adjacent region (Ng and Tan 2015) (**Figure 3.3**).

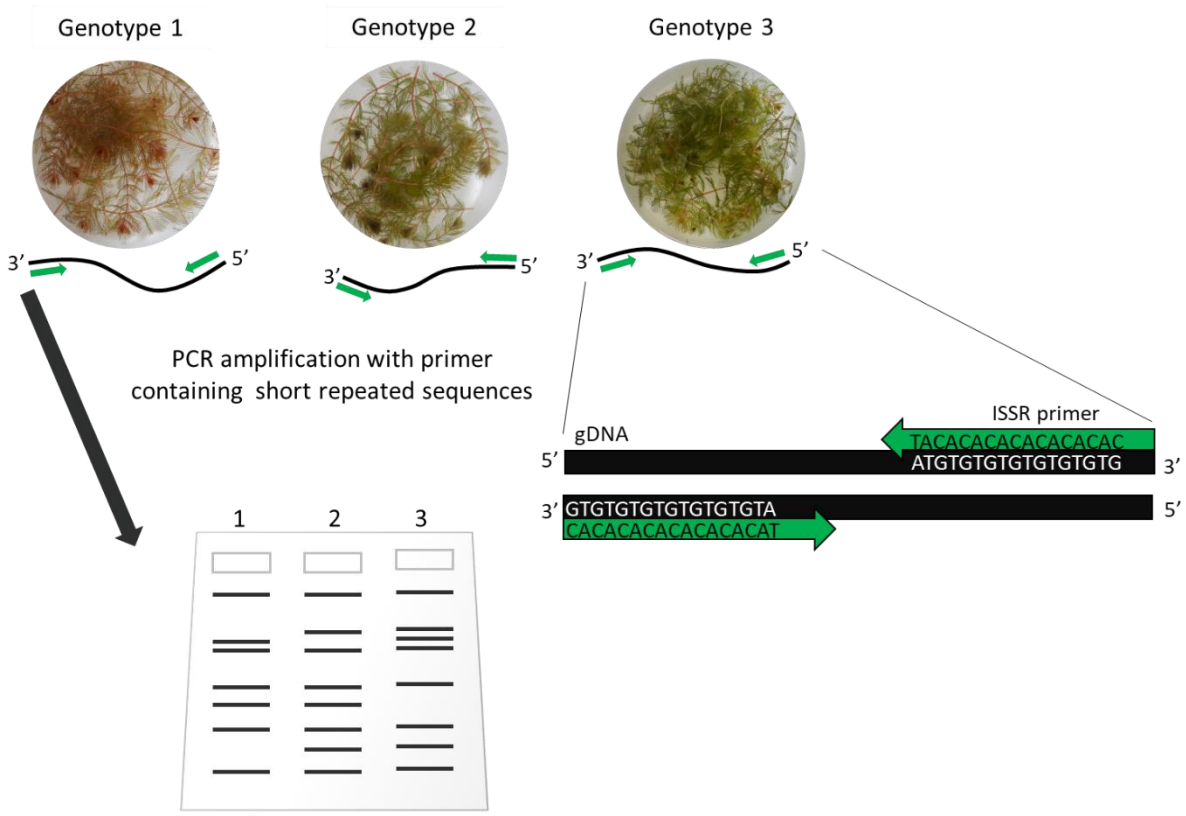


Figure 3.3. Genotypic differentiation with PCR amplification using an ISSR primer on three genotypes of *M. spicatum*. First, DNA is extracted from fresh plant material, and amplified through a PCR step using ISSR primers. Finally, amplified fragments were separated through electrophoresis.

2) Analysis

Plant samples were collected and stored at -20°C in Nuclei Lysis Solution (Promega) until DNA extraction. DNA was extracted and purified from about 100 mg of sample fragments using the WIZARD Genomic DNA Purification kit (Promega) and following the procedure described in Carriconde et al. (2008).

Twenty-two ISSR primers were tested with the three species studied. These primers included primers previously used with different *Lemna* species (UBC811 to UBC861, Xue et al. 2012), with *Ceratophyllum demersum* (ISSR5 to ISSR12, Triest *et al.*, 2010) and with other organisms (RP1 to RP7 and R1 to R6, Hantula et al. 1996 ; Liang et al. 2005 ; Carriconde et al. 2008). Out of these primers, 13, 8 and 20 were selected for their ability to produce clear patterns and polymorphic bands for *Myriophyllum spicatum*, *Ceratophyllum demersum* and *Lemna minor*, respectively.

ISSR amplifications were carried out with 1X 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 thermal cycler (Eppendorf) 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 considered primer , 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. Only clear and well-separated ISSR fragments were retained and scored as present (1) or absent (0). For each plant species, resulting patterns were compared to discriminate the samples.

Finally, in order to estimate genetic relationships among samples within each species, a matrix of pairwise genetic distance was constructed by calculating for all pair of samples the Sørensen–Dice dissimilarity index $GD = 1 - 2n_{XY}/(n_X + n_Y)$ where $2n_{XY}$ is the number of fragments shared by two samples X and Y, and n_X and n_Y are the numbers of present fragments in sample X and in sample Y respectively. Cluster analyses based on UPGMA (Unweighted pair group method with arithmetic mean) were performed with *GD* matrices and dendrograms were constructed to visualize genetic relationships among samples of each species. Computation of *GD* matrices and of UPGMA clusters were done with FAMD 1.30 software (Schülter et al. 2006; http://www.famd.me.uk/famd.html) and dendrograms were edited with MEGA 7 (Kumar et al. 2016).

CHAPTER III

Importance of intraspecific variation on macrophyte sensitivity to chemicals

1. Does intraspecific variability inflect macrophyte sensitivity to copper?

Effects of copper on aquatic macrophytes have been studied for a long time (see chapter **I.2**). Although studies have compared the sensitivity to Cu and the ability to uptake this metal for numerous species, none has considered possible differences in terms of sensitivity within species. The only study having focused on the importance of intraspecific variation, more specifically genotypic variation, in the sensitivity of aquatic plants to chemicals, is that of Dalton et al. (2013). They studied the impact of atrazine on different strains of *L. minor*, and found significant differences in sensitivity among strains, some being twice more sensitive than others. This may have implications in ecotoxicological risk assessment, as current approaches aim to compare the differences of sensitivity among species (*i.e*. SSD) but do not assess how intraspecific variability may influence their outcomes (see chapter **I.3**).

In this chapter, the extent of genotypic variability was studied in three aquatic macrophyte species (*Lemna minor*, *Ceratophyllum demersum* and *Myriophyllum spicatum)*. I assessed whether or not this has implications in risk assessment approaches. To do so, Cu toxicity was measured on growth related endpoints (based on biomass production and shoot elongation or frond number) and light harvesting ratio (maximal quantum yield of PSII). From the modelling of concentration-response curves for these different endpoints, EC_{50} values for each genotype were extracted and compared, to assess the relative importance of intraspecific and interspecific variations in the sensitivity of the aquatic macrophytes studied to Cu contamination.

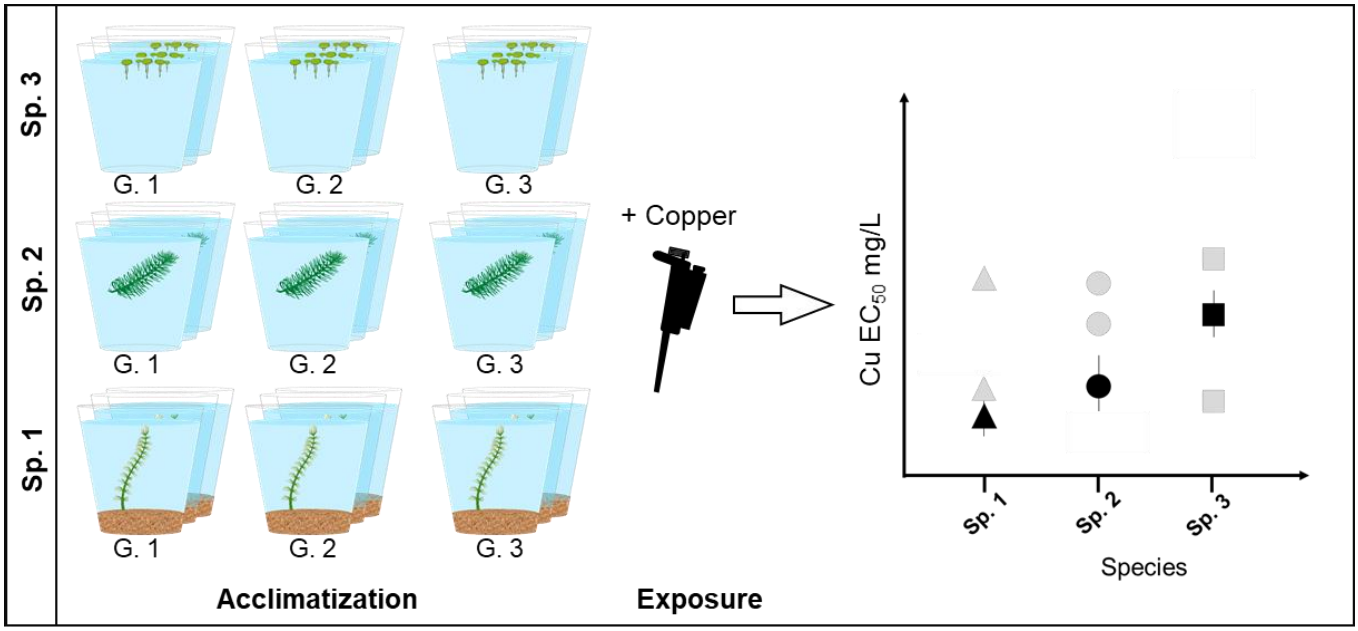


Figure 1.1. Summary of the experimental design to assess the influence of intraspecific variability, more specifically genotypic variation, in three aquatic macrophyte species sensitivity to copper contamination. At least three genotypes of *Lemna minor*, *Ceratophyllum demersum* and *Myriophyllum spicatum* were exposed to Cu.

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

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1. 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

2. 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 extended 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 2.1**). 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; B. K. 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, 2014b). 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.

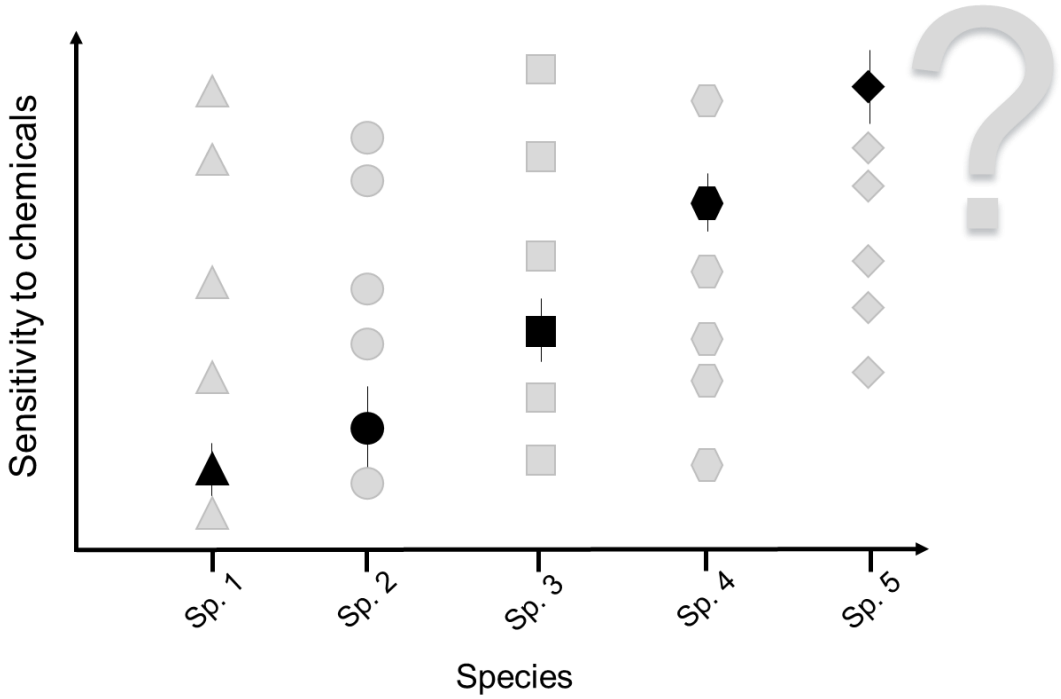

Figure 2.1. Sensitivity to chemicals for five hypothetic species determined using individuals from a single population per species (in black). In this kind of approach, the real variability of the species response to contamination is ignored, and interspecific differences which are highlighted here may 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).

3. Materials and methods

A. Studied species and chemicals

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 et al. (1996) (**Table 3.1** for geographic origin of strains).

Table 3.1. 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

Each strain of *M. spicatum* and *C. demersum* were grown in 210 L outdoor containers with quartz sediments enriched with Osmocote® for at least six months before experiments were conducted. *L. minor* populations were grown under axenic conditions in the lab, and were placed under non-axenic environment one month prior to the experiments (**Table 3.2**).

Inter simple sequence repeat (ISSR) molecular typing method was used to verify that clonal strains corresponded to different genotypes (more details in section 4).

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.

Table 3.2. 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 weight (RGR_{fw}), 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_{fw} $RGRf_{ronds}$	F_v:F_m	RGR_{fw} RGR_{length}	F_v:F_m	RGR_{fw} 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

B. Genetic differentiation of strains by ISSR

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 studied species (Triest et al. 2010; Cao et al. 2017; Xue et al. 2012) or with other organisms (Carriconde et al. 2008; Hantula et al. 1996), 13, 9 and 20 were selected for their ability to produce clear banding patterns and polymorphic bands with studied 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.

C. Effective Cu concentration

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).

D. Growth experiments

Prior to exposure, plants were acclimatized during 5 days under the same environmental conditions as during exposure (**Table 3.2**). 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.

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 weight 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 weight measurements to calculate RGR based on biomass production. Three genotypes were tested.

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 weight 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.

E. 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.

F. 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-j} is the relative growth rate from time i to j, N_i and N_j are the endpoint (frond number, fresh weight 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 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 significant differences among Cu concentrations and genotypes.. 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 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.

4. Results

A. 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.

B. Intraspecific variations in plant sensitivity to copper

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 performing slightly less well 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 4.1A**). 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 4.1**). 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 4.1B**). 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 4.1**).

The F_v:F_m showed stronger variations among genotypes, and a pattern of resistance was observable for the "Canal" genotype (**Figure 4.1C**). 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 4.1**).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.

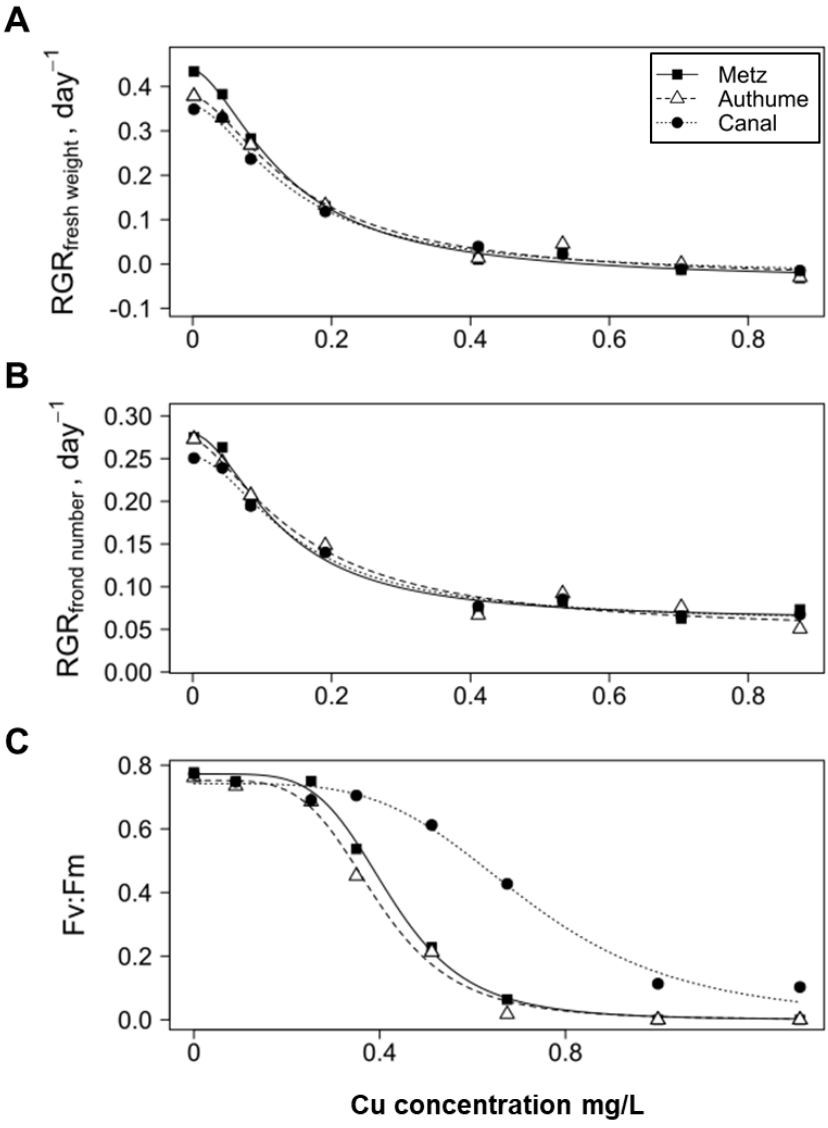

Figure 4.1. Concentration-response curves for three genotypes of *L. minor* exposed to copper, with relative growth rates (RGR) based on fresh weight (**A**) and frond number (**B**) after 7 days of exposure, and (**C**) F_v:F_m after 96h. Curves were fitted with non-linear log-logistic models with 4 parameters (**A** and **B**) and 3 parameters (**C**).

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 4.2B). 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 4.2). 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 4.1**,

Figure 4.2). 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 4.1**,

Figure 4.2A).

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. 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*.

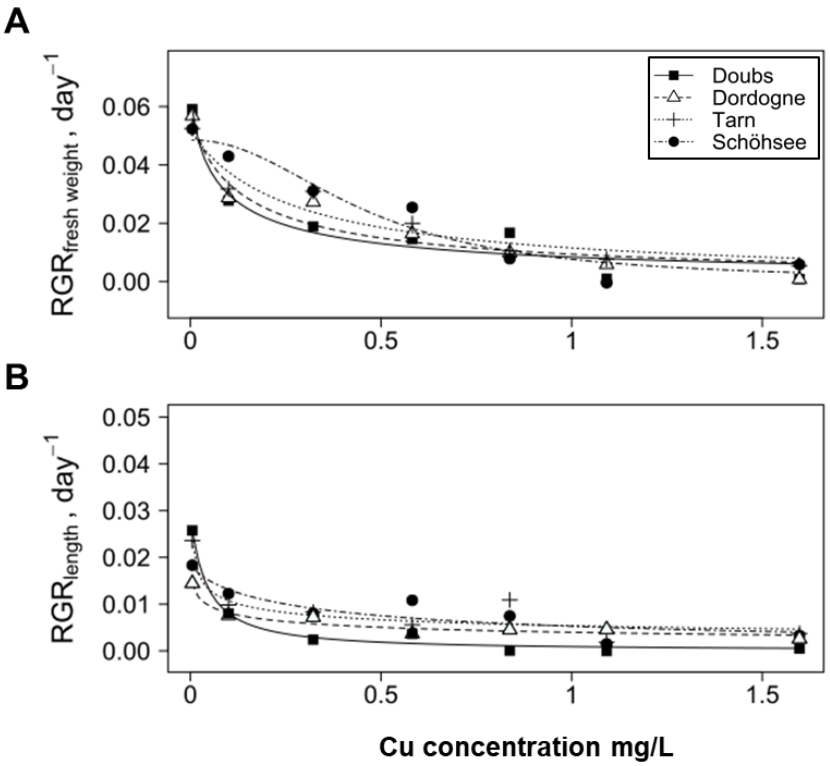

Figure 4.2. Concentration-response curves for four genotypes of *M. spicatum* exposed to copper, relative growth rates (RGR) based on fresh weight (**A**) and shoot length (**B**) after 12 days of exposure. Curves were fitted with non-linear log-logistic models with 3 parameters.

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 4.3A**). 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 4.3B**). 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 (**Figure 4.3A**, **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 4.3C**). 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.

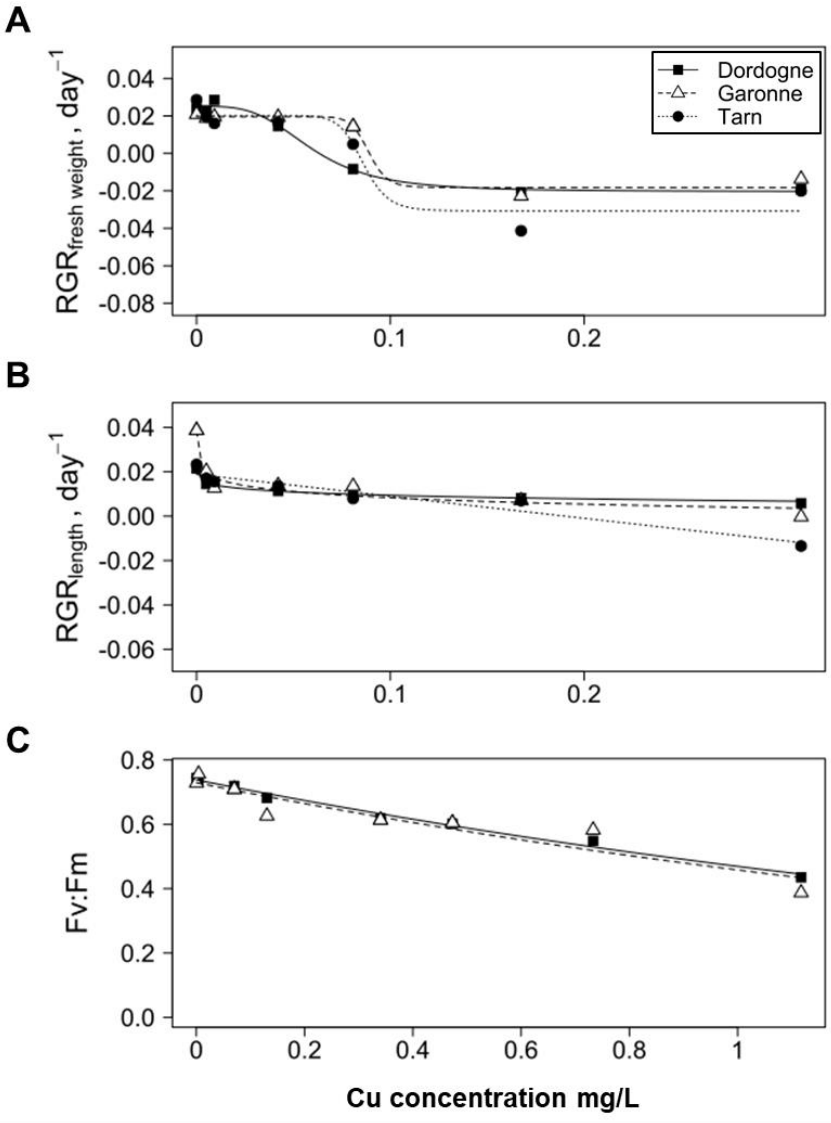

Figure 4.3. Concentration-response curves for two to three genotypes of *C. demersum* exposed to copper, with relative growth rates (RGR) based on fresh weight (**A**) and shoot length (**B**) after 14 days of exposure, and F_v:F_m (**C**) after 96h. Curves were fitted with non-linear log-logistic models with 4 parameters for growth related endpoints (**A**, **B**) and exponential decay models with 2 parameters for F_v:F_m (**C**).

C. <u>Relative importance of intraspecific vs. interspecific variations</u>

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 4.1**). 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.

Table 4.1. Half maximal effective concentrations (EC_{50}, mean ± SD) for different genotypes of three macrophyte species: *L minor*, *M. spicatum* and *C. demersum* exposed to Cu. Maximal Quantum Yield of PSII (F_v:F_m) experiment lasted for 96 h. Growth experiments (relative growth rates, RGR) lasted for 7, 12 and 14 days for *L. minor*, *M. spicatum* and *C. demersum* respectively. CV: coefficient of variation among EC_{50} values in %, calculated within species (based on averaged EC_{50} values per genotype and endpoint). Interspecific variability was assessed from the R^2 value from ANOVA. * For RGR_{length}, interspecific variability was only compared between *C. demersum* and *M. spicatum*, as this endpoint was not used for *L. minor*.

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**

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 4.4**). 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.

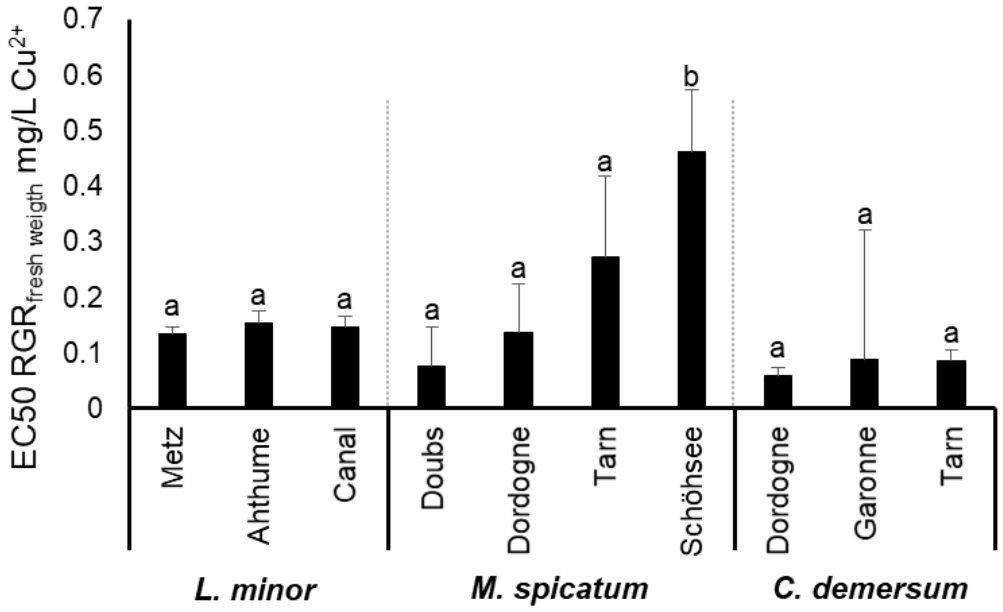

Figure 4.4. EC_{50} values for Relative Growth Rates based on fresh mass of three species, *L. minor*, *M. spicatum* and *C.* demersum exposed to copper. From three to four genotypes of each species were exposed during 7 days (L. minor), 12 days (M. spicatum) or 14 days (C. demersum) to concentrations 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.

5. Discussion

A. 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 experiments have 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 *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).

B. Intraspecific variation

Lemna minor and *M. spicatum*, but not *C. demersum*, showed statistically significant differences of sensitivity among genotypes, depending on the endpoint considered.

The high variability within genotypes among replicates, especially for *C. demersum* and *M. spicatum*, affected the significance of the results. ANOVAs sometimes failed to highlight differences in sensitivity among genotypes, whereas a trend was visually observable for the two species. Indeed, for *M. spicatum*, the shape of the concentration-response curves differed among genotypes for both RGRs despite no significant interaction between Cu and Genotype, whereas it was visually observable that "Doubs" genotype was more sensitive and "Schöhsee" was more resistant to Cu.

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. Furthermore, traits showing significant differences in sensitivity to Cu among genotypes (RGR_{fw}) also showed a genotype effect for control plants, although EC_{50} values were not always significantly different. It is consistent with the fact that based on life-history traits (*e.g.* RGRs) some genotypes are more efficient than others under normal conditions, but respond identically as the less-competitive genotypes when facing a chemical stress. On the contrary, *M. spicatum* did not show significant genotypic effect based on life-history traits in absence of contamination, but Cu stress highlighted significant differences in sensitivity, as demonstrated by the different EC_{50} values. 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 et al. 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 different sensitivity to chemicals (Cao et al. 2017). Indeed, contamination will trigger a strong selection pressure on populations and only individuals able to thrive under chemical stress will be selected. Individuals with increased resistance and/or copping capacities to contamination will 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 selection pressure was applied in the environments in which the genotypes were harvested from, and therefore no structuration was found in term of sensitivity to contamination. It has been well documented that plant adaptation to environmental pressures (metal resistance, pathogen resistance…) is a costly process which decreases fitness when the pressure considered is removed, so these strategy are only selected under stressful conditions (Huot et al. 2014).

C. Interspecific variation in Cu sensitivity

Overall, interspecific variation was more important than intraspecific variation. Indeed, total variation in EC_{50} values among species was explained by interspecific variation at 77%

for RGR_{fw} and 99% for $F_v:F_m$, although *M. spicatum* had no EC_{50} value for the last endpoint. Based on RGR_{fw}, *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 et al. 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). Anyway, the number of genotypes used per species and per population do not allow to assess for the relative importance of geographic distance in 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; Z. 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 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.

6. 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 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-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.

7. Acknowledgments

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8. Supplementary data

Supplemental material 1. 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).

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

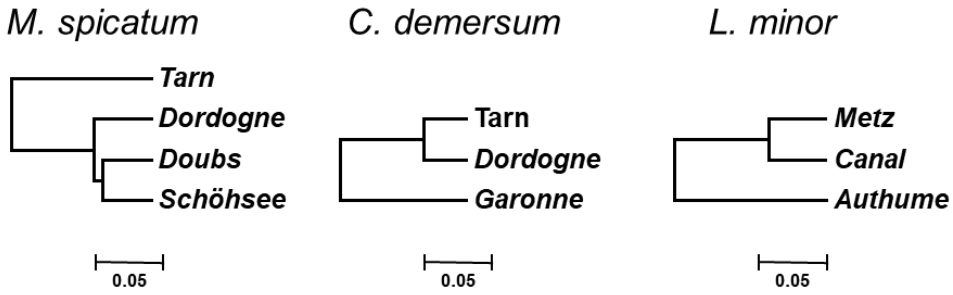
		Myriophyllum spicatum			*Ceratophyllum demersum*			*Lemna minor*		
Primers*	Sequences** (5'-3')	T_A	No. scored fragments	No. polymorphic fragments	T_A	No. scored fragments	No. polymorphic fragments	T_A	No. scored fragments	No. polymorphic fragments
ISSR 5	$(CA)_8$ GT	46°C	7	4	46°C	7	2	50°C	6	4
ISSR 8	$(CA)_7$ ATCC	46°C	4	1	46°C	7	3	50°C	4	2
ISSR 9	$(CA)_7$ GTCT	46°C	7	3	46°C	6	2	50°C	5	3
ISSR 12	GGTC$(AC)_7$	-	-	-	53°C	5	1	53°C	7	2
UBC 811	$(GA)_8$ C	53°C	8	3	52°C	6	3	-		
UBC 827	$(AC)_8$ G	-	-	-	52°C	4	2	53°C	9	4
UBC 845	$(CT)_8$ GG	-	-	-	-	-	-	53°C	8	5

UBC 849	$(GT)_8$ CA	53°C	6	2	-	-	-	53°C	8	4
UBC 855	$(AC)_8$ CT	53°C	7	5	52°C	4	2	53°C	6	4
UBC 856	$(AC)_8$ CA	53°C	6	2	-	-	-	53°C	6	3
UBC 857	$(AC)_8$ TG	-	-	-	-	-	-	53°C	11	6
UBC 861	$(ACC)_6$	-	-	-	-	-	-	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*

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

**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 T.

Figure 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).



CHAPTER IV

Influence of genotypic variability on *Myriophyllum spicatum* exposed to chemicals

1. Does genotypic variability of *M. spicatum* affect its sensitivity to copper?

Genotypic variability has been considered as an important characteristic for species adaptation to new environments (see chapter **I.4**). Several studies have investigated how genetic variability may inflect species adaptation to climate change, and how geographical distribution may shape the genetic structure of populations. However, no study has examined how genotypic variability may affect species sensitivity to chemical contamination.

Assessing the importance of intraspecific variability in response to chemicals is highly important, given the current pollution of ecosystems. Furthermore, it should give insight on how species adjust and adapt to cope with chemical stressors, and if resistance or sensitivity to these stressors can be connected with specific life-history traits.

Based on the results of the previous chapter, I aimed to assess the influence of genotypic variability in the sensitivity to Cu for 7 genotypes of *Myriophyllum spicatum*. This species is used in standardized protocols in ecotoxicological risk assessment, and significant variations among genotypes were demonstrated in previous experiments. There is therefore a need to better evaluate the extent of *M. spicatum* genotypic variation, to identify more contrasted strains in terms of Cu sensitivity. In parallel, this would be particularly relevant to characterize the different genotypes studied in terms of their life-history traits, which may allow the definition of some trait syndromes possibly related to the sensitivity of *M. spicatum* to chemical contamination.

Figure 1.1. Geographic origin of the 7 genotypes of *M. spicatum* used to assess the influence of genotypic variability in chemical sensitivity.

Different genotypes of the aquatic plant *Myriophyllum spicatum* show contrasting sensitivities to copper contamination

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2. Abstract

Genotypic variability has been considered for years as a key attribute in species adaptation to new environments. Extended research on mechanisms of chemical resistance has been conducted for agriculture and weed management purposes. Genotypic variability notably influences the ability of plants to cope with environmental changes in a context of global warming. Aquatic ecosystems are particularly impacted by these environmental changes, and aquatic plants play pivotal role in these ecosystems. Although effects of chemicals and environmental changes have been studied on these organisms, no study has focused on how this variability triggered by environmental changes could inflect the sensitivity of aquatic plants to chemicals. In this study, we assessed the importance of genotypic variability in copper (Cu) sensitivity for seven genotypes of *Myriophyllum spicatum*, a species used in standardized protocols for ecotoxicological risk assessment. Three Cu concentrations were used, 0, 0.15 and 0.5 mg/L. Through various endpoints such as lateral shoot and root production, dry matter content, relative growth rate (RGR), relative elongation rate of shoots (RERall), main shoot elongation (RERmain), internode length, bio-macromolecular composition, as well as Cu content, differences in sensitivity were assessed among these genotypes. Our results showed strong variation in sensitivity depending on the genotype, with up to eightfold difference in sensitivity for RGR at low Cu concentration (0.15 mg/L), and sevenfold difference in RER at high Cu concentration (0.5 mg/L). Genotypes exhibited significant differences in their life-history traits in the absence of chemical contamination, and co-inertia analysis revealed that these life-history traits explained 62% of the total variation in sensitivity to Cu. Indeed, some

life-history trait syndromes were observed: total shoot elongation and lateral shoot production were correlated positively, with internode length and negatively with whorls production and DMC. Main shoot elongation was mainly correlated with RGR. Some genotypes thus demonstrated contrasting strategies, either producing lateral shoots and having a high global elongation rate to promote light harvesting, or producing denser whorls with higher DMC to promote nutrient absorption and conservation. Our results confirm that genotypic variability can significantly inflect the outcomes of laboratory testing, and thus should be studied more in depth.

Keywords: Genotypic variability, aquatic macrophyte, copper, ecotoxicological risk assessment, life-history traits

3. Introduction

Intraspecific variability has been investigated over the years as an important attribute in the adaptation and evolution of species (Bradshaw 1965; Matesanz et al. 2010; Oliver et al. 2015). It has been acknowledged to play an important role in species adjustment to short term environmental shifts, *via* **phenotypic plasticity**, *i.e.* the ability of one genotype to produce several phenotypes depending on its environment (Sultan 1995, 2000; Whitman and Agrawal 2009), and to long term environmental changes, *via* **genotypic variation**, *i.e.* the evolution of the genetic code due to somatic mutations, selection pressures and gene fluxes (Harris et al. 1992; Cao et al. 2017). Genotypic variation can sometimes confer specific life-history traits differing among genotypes and populations (Harris et al. 1992; Hughes and Stachowicz 2004; Reusch and Hughes 2006; Weyl and Coetzee 2016; Tóth et al. 2017). Recently, it has been demonstrated that intraspecific variability, more specifically genotypic variation, strongly inflects the **resilience** of ecosystems after a disturbance (Reusch and Hughes 2006; Ehlers et al. 2016; Oliver et al. 2015; Timpane-Padgham et al. 2017). Indeed, a broad gene pool will be more likely to provide the proper response to an environmental shift, such as changes in nutrient load, heat waves or chemical contaminations (Reynolds et al. 2012). Conversely, a population exhibiting a low gene pool and not able to cope with a given environmental pressure may disappear from the ecosystem, whose dynamics and functions may drift due to the change in its species composition (Timpane-Padgham et al. 2017). As such, genotypic variation is considered as a driver of **ecosystem functioning**, as it will inflect population resistance and resilience

under various environments (Woodward et al. 2010; Oliver et al. 2015; Timpane-Padgham et al. 2017).

Differences in the sensitivity to various stressors among populations might increase over time due to their adaptation to contrasting environments through selective pressures (Dalton et al. 2013; Esteves et al. 2017; Brown et al. 2009). Changes in environmental conditions are more likely to occur with **human activities**, which inevitably impact ecosystems, notably via chemical contamination due to runoffs, wastewaters and atmospheric deposition (Ehlers et al. 2008; Oliver et al. 2015). In order to limit human impact on ecosystems, several **policies** have been implemented over years, such as the E.U. Water Framework Directive, the E.U. REACH regulation, the U.S. Endangered Species Act or the U.S. Clean Water Act (Bouwma et al. 2018; Rouillard et al. 2018). Those policies are based on **ecotoxicological risk assessment** (ERA) which allows to assess ecosystem health and chemical harmfulness.

It first started in the 1970s and has evolved since, along with environmental policies and global awareness of human impact on the environment (Shea and Thorsen 2012; Bouwma et al. 2018). The realism and complexity of ERA increase as scientific breakthrough highlights different processes which may inflect the current approaches and their outcomes (Johnson and Sumpter 2016). It is difficult to properly assess in laboratory testing the impact of a given molecule on its environment, as many factors *in situ* will strongly influence the toxicity of molecules, or the sensitivity of organisms. Among those factors, genotypic variation, which might have a significant impact on results, is not taken into account in ERA approaches

Very few studies have looked into the impact of genetic variability in aquatic species sensitivity to chemicals. This is of concern, as this sensitivity might significantly vary among genotypes due to contrasting morphological or physiological traits, and ultimately among populations, depending on selection pressures having filtered out unfitted genotypes (Dalton et al. 2013; Brown et al. 2009). Among those studies, one has looked into the difference in sensitivity to pesticides between *Myriophyllum spicatum* populations and hybrid populations (Thum et al. 2012), an another one in resistance development of *Hydricilla verticillata* in populations exposed to chemicals for weed control (Arias et al. 2005). Indeed, occurrence of resistant weeds increases with the use of pesticides, which still rise over the years, resulting in ecosystem disturbances.

In this study, we examined whether contrasting sensitivities to copper (Cu) exposure can be found across seven genotypes of *M. spicatum* differing in their life-history traits. Copper is

an essential trace element which is involved in several metabolic pathways, such as reactive oxygen metabolism, photosynthesis and respiration (Hötzer et al. 2012; Thomas et al. 2013; Peñarrubia et al. 2015). It is also harmful beyond a certain physiological threshold, which is species-specific, due to excessive reactive oxygen species (ROS) production which trigger cellular damages (Razinger et al. 2007; Fidalgo et al. 2013; Costa et al. 2018). For example, Cu concentrations in leaves of terrestrial plants range between 5 to 20 µg g^{-1} dry mass (Yruela 2009), and the toxicity threshold in leaves of crop species is generally above 20 - 30 µg g^{-1} dry mass (Marschner and Marschner 2012). Copper is widely used in agriculture as fungicide, and is also released by industries and mining activities; thus high Cu concentrations can subsequently be found in top soils and aquatic ecosystems (Willis and Bishop 2016; Ballabio et al. 2018). According to a recent study, Cu concentration in European top soils is on average 16.0 mg kg^{-1} in all soils combined, and 49.3 mg kg^{-1} in vineyards, going up to 91.3 mg kg^{-1} on average in French vineyards (Ballabio et al. 2018).

Because of its environmental relevance and the extensive documentation on its effects on aquatic plants, Cu was used in this study to assess the importance of genotypic variation of *M. spicatum* in its sensitivity to chemical contamination (Thomas et al. 2013; Yan and Xue 2013). This species is spread worldwide and considered as a model species for rooted aquatic plants in freshwater ecosystems. As such, it is the subject of two standardized protocols in ERA since 2014 (OECD tests n°238 and n°239). Its genotypic variation and phenotypic plasticity have been widely documented regarding different populations and their genetic structure, or their plasticity toward environmental variations (Barko and Smart 1981; Hussner et al. 2009; Cao et al. 2012; Li et al. 2010; Weyl and Coetzee 2016; Tóth et al. 2017).

4. Material and methods

A. Growth and copper exposure

Seven genotypes of *M. spicatum* were randomly harvested in natural freshwater rivers in France and Germany between 2013 and 2016, and one strain of *M. spicatum* was regrown from an axenic culture established from material collected in Germany in 1990 (**Table 4.1**). Each genotype was grown in a 210 L tank with quartz sediment enriched with Osmocote ® (granulated fertilizer, Hortensia, KB) during at least six months prior to exposure experiments. For genotype differentiation, inter simple sequence repeat (ISSR) method was used on polymorphic fragments, see section 2.2 for further details.

Prior to Cu exposure, each shoot was cut to a length of 6 cm and rinsed in tap water before an acclimatization of 5 days in Smart & Barko medium pH 7.0 ± 0.1. Experimental units contained 500 mL media with 50 mL of quartz sediment enriched with 66.6 mg Osmocote ®. For exposure, one apex was placed in each experimental unit during 10 days, with 1 cm of the shoot placed in sediment for root development. Three Cu concentrations were used: 0, 0.15 and 0.5 mg/L Cu^{2+}. Copper sulfate was purchased from Sigma (CAS number 7758-98-7, Saint Quentin Fallavier, France). A concentrated solution of 1 g/L Cu^{2+} was prepared in ultrapure water, and diluted in the different media before pH adjustments.

Table 4.1. GPS coordinates of the French (Fr.) and German (Ge.) sites from which the seven genotypes of *Myriophyllum spicatum* were harvested between 2013 and 2016.

Names	Streams	Stations	GPS coordinates
DOU	Doubs (Fr.)	Gare d'eau	47.23153, 6.02252
DOR	Dordogne (Fr.)	Scierie	44.84584, 0.90596
SCH	Schöhsee (Ge.)	Plön	54.16624, 10.44114
CAM	Camargue (Fr.)	Badon	43.51133, 4.60584
GAR1	Garonne (Fr.)	St Aignan	44.02281, 1.07797
GAR2	Garonne (Fr.)	Bourret	43.94622, 1.1711
AGO	Agout (Fr.)	Burlat	43.6395, 2.31875

B. Distinction of genotypes

Genotypes were distinguished, and genetic distances among genotypes were calculated, using inter simple sequence repeats (ISSRs, 40). Plant samples were collected and stored at -20°C in Nuclei Lysis Solution (Promega) until DNA extraction. DNA was extracted and purified from about 100 mg of sample fragments by using the WIZARD Genomic DNA Purification kit (Promega) and following the procedure described in Carriconde et al. (2008).

C. Copper concentration in water samples

Copper concentrations in the media were calculated by sampling water from experimental units at the beginning and at the end of Cu exposure, in order to assess effective concentrations. Samples were measured after acidification using inductively coupled plasma with mass spectrometer (ICP-MS, Agilent 7500ce). Effective Cu concentrations in the media were in average at 96.7 ± 1.9 % at the beginning of exposure, and averaged concentrations were at 54.7 ± 0.6 % and 50.9 ± 1.4 % of nominal concentration for 0.15 mg/L and 0.5 mg/L Cu respectively.

D. Life-history traits

1) Growth-related and morphological traits

The number of roots, lateral shoots, as well as the number of whorls over 5 cm from the bottom of the shoot, were recorded on the first and the last day of Cu exposure for each experimental unit. Shoot length based on total shoot length and lateral shoots was measured at the beginning and at the end of exposure to calculate the Relative elongation rate (RERall), and main shoot length was assessed as well, to calculate the Relative elongation rate of the main shoot (RERmain). Fresh mass was measured at the same time after having gently dried the plants with blotting paper, to calculate the relative growth rate (RGR).

RGR, RERall and RERmai were calculated for each experimental unit as follow:

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

where RGR_{i-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.

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

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

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

Root number and lateral shoots (LS) were counted at the beginning and at the end of exposure, and whorl number per cm was calculated by measuring the number of whorls on 5 cm of shoot, at the beginning and at the end of exposure.

E. Biomacromolecule composition

Fourier Transform InfraRed spectroscopy (FTIR) was used to probe molecular vibrations in plant samples and thus gain information on their biochemical composition (lipids, carbohydrates, proteins). Samples were analyzed with a microscope FTIR (Thermo Nicolet NEXUS 470, ESRF) over the range of 4000 – 400 cm^{-1} with a spectral resolution of 4 cm^{-1}. One spectrum is an average of 64 scans per sample. Each powdered plant was placed on the

sample plate and three independent technical replicates for each sample (5 biological replicates per treatment) were acquired. The full experimental setup for FTIR acquisition is given in supplementary information. OMNIC software was used to export experimental spectra (Thermo Scientific™ OMNIC™ FTIR Software). FTIR data treatment was performed using the Orange software (Demšar et al. 2013). Briefly, data were pre-processed which implies selection of the region of interest (including most of the variance among samples), vector normalization and smoothing by Savitzky-Golay filter. Using the second derivative, a principal component analysis (PCA) was carried out and the components permitting to explain at least 70% of the variance were used to perform a subsequent linear discriminant analysis (LDA). This approach permitted to plot the samples and detect differences among groups of samples. In case a difference was detected, a logistic regression was applied to the pre-processed data to identify wavenumbers contributing to the difference detected between groups thanks to the PCLDA.

F. Endpoints assessing Cu sensitivity

1) Growth inhibition

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

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

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

2) Copper concentration in plants

Copper concentration in each shoot at the end of Cu exposure was measured after acid digestion of plant material, which occurred in two steps. First, plant material was heated at 94°C during 30 minutes in a 1:1 ratio of deionized water: HNO_3 (65%), then allowed to return to room temperature. Then the samples were brought to a 2:1 ratio of HNO_3 (65%):H_2O_2 and heated at 94°C during 2 hours. Once the temperature cooled down to room temperature, samples

were filtered with a 0.45 µm filter and diluted to obtain a final HNO_3 concentration of 2%. Samples were measured using ICP-MS (Agilent 7500ce).

G. Statistical analyses

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. Two-way ANOVAs were performed on results showing normal distribution, with or without log transformation, to assess the interactive effects of genotype and Cu concentrations during exposure. Tukey HSD post-hoc tests were used to identify significant differences among Cu concentrations and among genotypes. Generalized linear models (GLMs) with gamma distribution were used to assess interactions in dataset showing no normality despite log-transformation. The correlation among traits was assessed through principal component analysis (PCA) using FactoMineR package (Lê, Josse, and Husson 2008). The link between life-history traits and Cu responses was determined with co-inertia analysis, with the RV coefficient indicating the degree of correlation between the two matrices (MASS package, 42). The differences in plant response among genotypes and Cu concentrations were analyzed with linear discriminant analysis (LDA) (ade4 package,48) and plotted using the FactoMineR package. The significance of co-inertia and discriminant analyses were assessed with Monte-Carlo tests on the sum of eigenvalues, with 1000 repetitions.

5. Results

A. Genetic differentiation of the seven genotypes

In total, 22 primers were tested, and 84 fragments were scored. Among those, 35 were polymorphic. The primer N°20 was selected for its ability to produce clear banding patterns and polymorphic bands. Analyzes showed that all the clonal strains differed from each other, and thus corresponded to different genotypes (supplementary material S2).

B. Differences in life-history traits among genotypes

In absence of Cu exposure, the studied genotypes showed differences in their life-history traits, with 4 of the 7 traits considered being significantly different among genotypes (**Figure 5.1**). Some genotypes were significantly more efficient than others regarding RERall, with the "AGO" genotype having a RERall of 0.049 cm/d, compared to 0.018 cm/d for the "DOR" genotype (**Figure 5.1A**). The main shoot elongation demonstrated significant differences among genotypes as well, with "GAR1" having a RERmain of 0.042 cm/d, against 0.018 cm/d for the "DOR" genotype (**Figure 5.1C**). The number of whorls significantly differed from one genotype to another as well, with the "DOR" genotype having on average more whorls per unit length (*i.e.* a shorter internode length) than the others, with 2.0 whorls/cm compared to 1.3 for all the other genotypes (**Figure 5.1D**). The number of lateral shoots also varied among genotypes, with three genotypes which did not produce any lateral shoot ("GAR", "DOU", "DOR") compared to the others which produced from one to two lateral shoots during the experiment (**Figure 5.1E**). RGR, DMC, as well as root number, did not significantly vary among genotypes (**Figure 5.1F**, **G** and **H**, respectively)

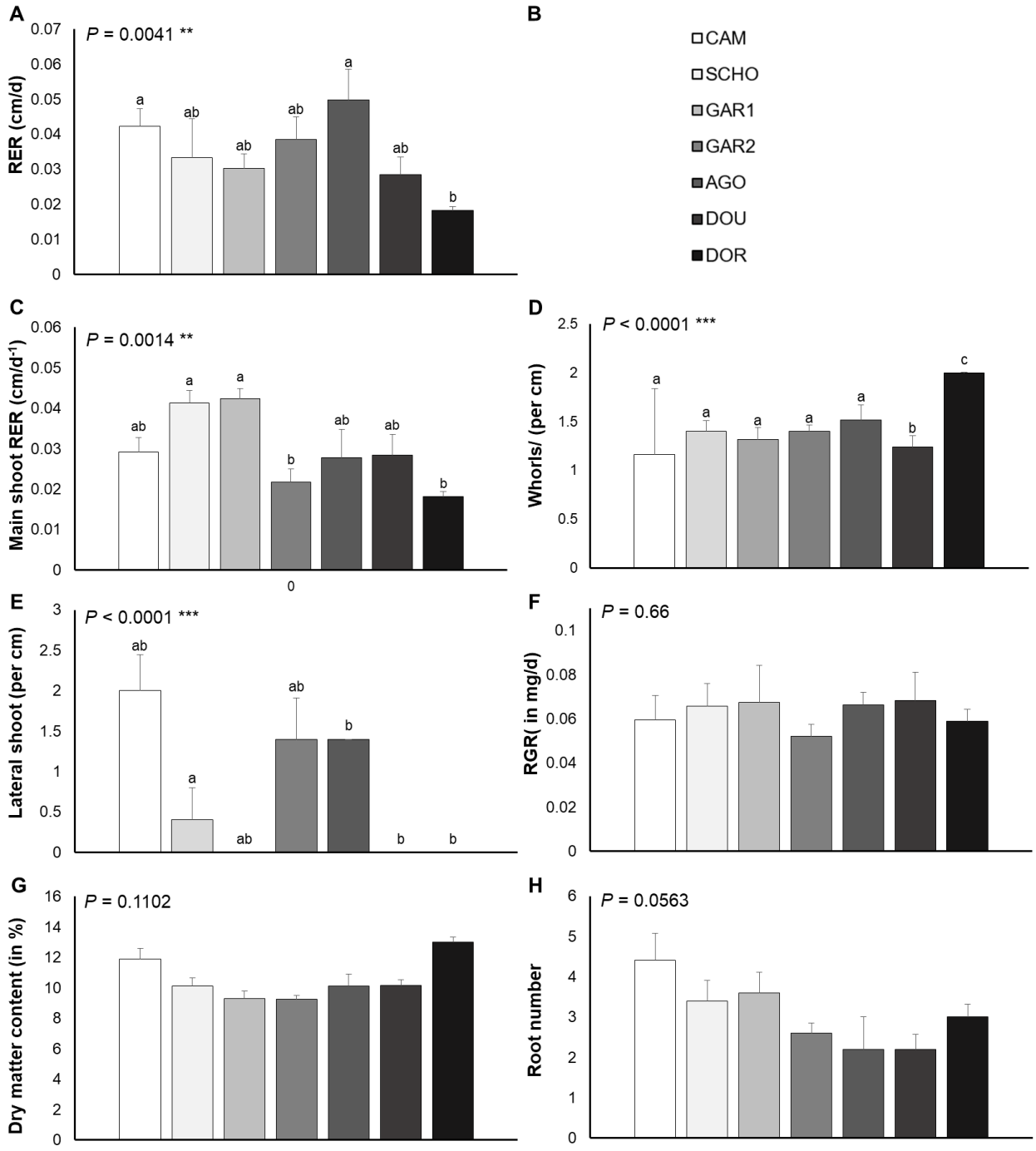

Figure 5.1. Life-history traits measured on 7 genotypes of *Myriophyllum spicatum* in absence of copper contamination. RERall, Relative elongation rate based on total shoots length (**A**), Main shoot elongation (**C**), whorl number (**D**), lateral shoot development (**E**), relative growth rate based on fresh mass (**F**), dry matter content (**G**) and root number (**H**) were measured. The legend with color code is displayed in (**B**).

According to the FTIR analysis, bio-macromolecule composition significantly differed among genotypes, although two genotypes, "AGO" and "GAR1" were not analyzed due to lack

of biomass. In **Figure 5.2**, it is clearly visible that "SCHO' and "GAR2" were clustered aside from the 3 other genotypes, suggesting that they were quite different regarding their bio-macromolecule composition.

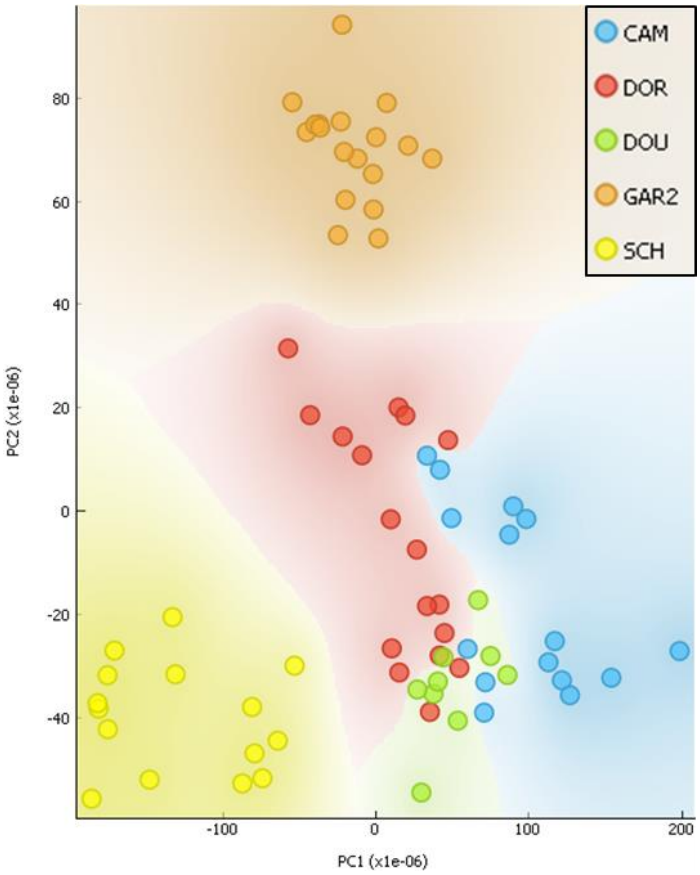

Figure 5.2. Principal component analysis conducted on wavelength numbers from FTIR analysis, performed on five genotypes of *Myriophyllum spicatum.*

C. <u>Relationship among life-history traits</u>

The relationships among life-history traits were assessed through PCA on control plants (**Figure 5.3**). It showed that DMC and whorls/cm were correlated, and both were anti-correlated to RER and lateral shoot number. Expectedly, RERall was relatively correlated with lateral shoot number. RGR was clustered with RERmain and Root number, although the former was poorly represented in the factorial plan. RERall was the main contributor to the second axis, whereas RERmain was the main contributor of the first axis.

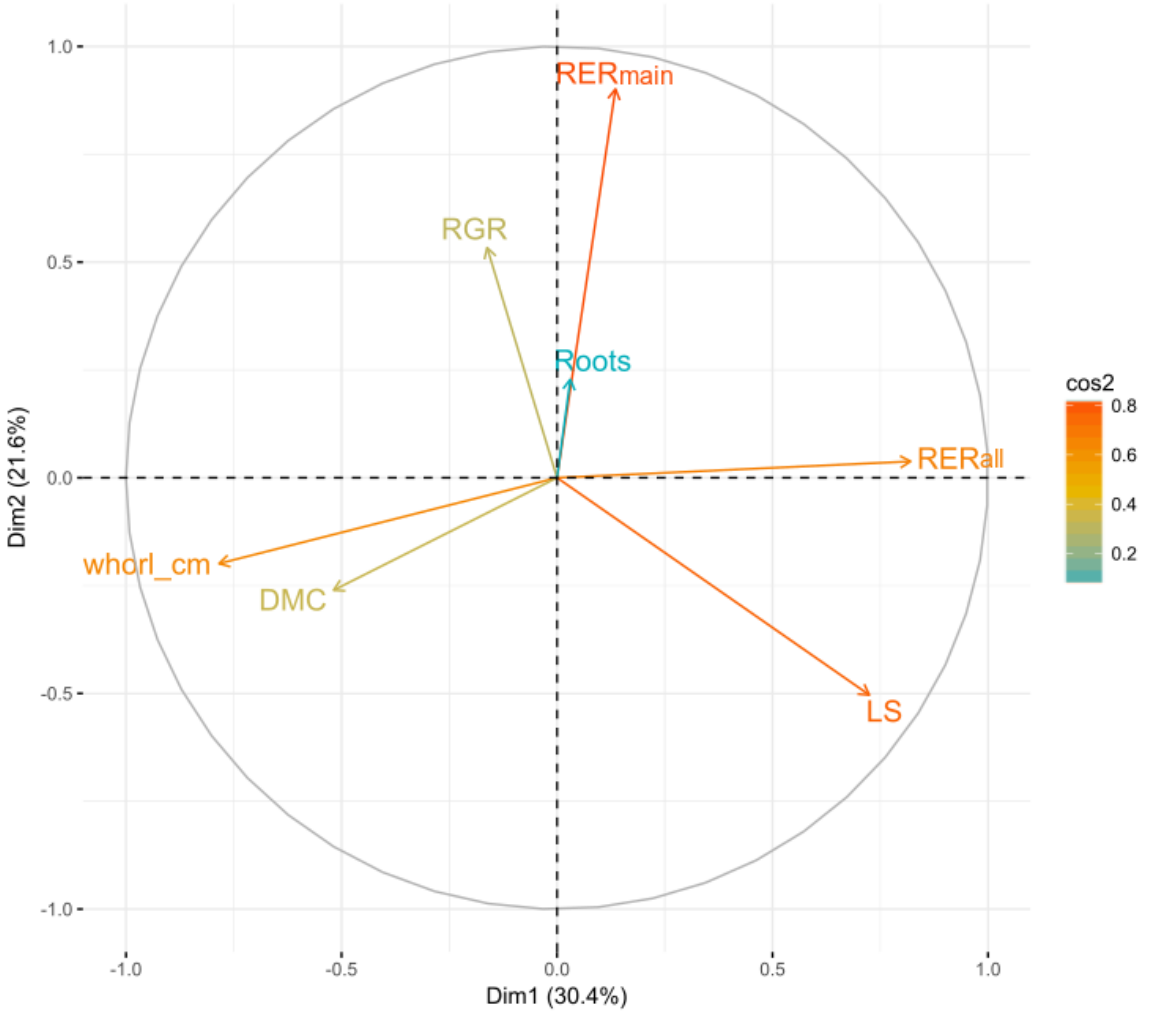

Figure 5.3. Principal Component Analysis (PCA) performed on 7 life-history traits for 7 genotypes of *Myriophyllum spicatum*. Relationship between Relative Growth Rate (RGR), Relative Elongation Rate based on total shoot length (RERall); RER based on main shoot elongation (RERmain), whorl number per cm (whorls_cm), Dry Matter Content (DMC), root number (Roots) as well as lateral shoot number (LS) is assessed. Color gradient represents the goodness of representation of each variable in the factorial plan defined by axes 1 and 2.

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

Copper exposure negatively affected all genotypes, with a dose-dependent effect on growth related endpoints, and sensitivity to Cu significantly differed among genotypes (**Figure 5.4**). Variation in sensitivity at low Cu varied up to 8 times for RGR depending on the concentration, with biomass production inhibited from 7 % for "CAM" to 62 % for "SCHO" at 0.15 mg/L. At 0.5 mg/L Cu, the range of inhibition slightly differed as the Cu stress increased, and growth production was inhibited from 40 % for "DOR" to 90 % for "DOU" (**Figure 5.4A**). RERall demonstrated slightly less variation among genotypes, with differences in sensitivity up to 7

fold depending on the Cu concentration (**Figure 5.4B**). Shoots elongation inhibition ranged from 10.9 % for "DOR" to 56.1 % for "AGO" at 0.15 mg/L, and from 10.8 % for "DOR" to 70.1 % for "DOU" at 0.5 mg/L. Generally, the "DOU" genotype was the most impacted at high Cu concentration, and "DOR" the least impacted.

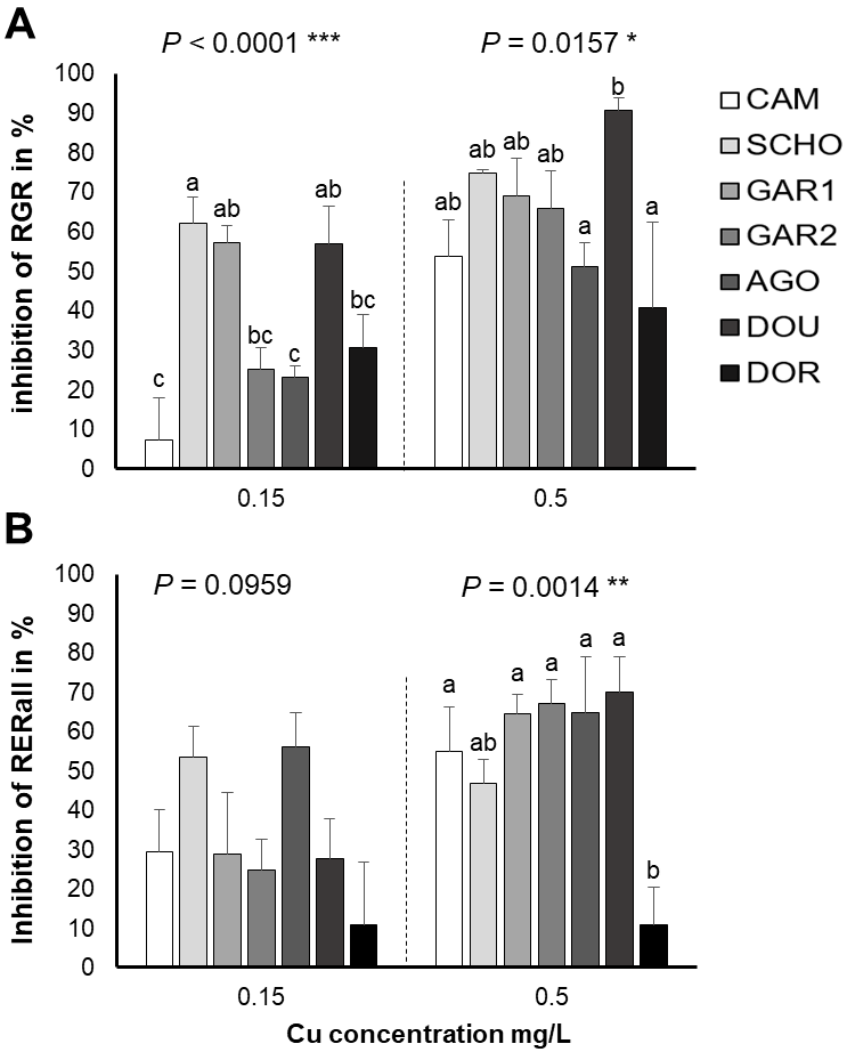

Figure 5.4: Copper sensitivity of 7 genotypes of *Myriophyllum spicatum*, assessed through the inhibition of their Relative Growth Rates (RGR) (**A**) and Relative Elongation Rates based on total shoots elongation (RERall) (**B**) during a 10-day exposure. ANOVA P-values for genotype effects are provided; genotypes with same letters for a given combination endpoint x Cu concentration are not significantly different in terms of copper sensitivity (HSD Tukey test).

RERmain was identically affected by Cu as RERall (assessed from both the main shoot and the lateral ones), and its sensitivity was similar, with at 0.5 mg/L "DOR" being the least impacted, and "DOU" the most sensitive (2-way ANOVA, $F_{6, 84}$ = 6,16, P<0.0001). Root development was significantly affected by Cu, and significantly differed among genotypes, with a decrease in root production associated to the increase in Cu concentration (2-way

ANOVA, $F_{6, 84} = 8.94$, $P<0.0001$). Indeed, root production greatly differed among genotypes, with the "CAM" being the least impacted, with inhibition in root production of 22.7 % at 0.5 mg/L, against the "DOU" genotype which was the most impacted, with a complete inhibition (100 %) of root production at the same Cu concentration. In contrast, lateral shoot production, DMC, and the number of whorls per cm were not significantly affected by Cu.

At the end of exposure, the different genotypes accumulated between 0.39 for "GAR1" and 0.84 mg Cu/g DW for "DOU" when exposed at 0.15 mg/L Cu, and between 2.68 for "GAR1" and 5.18 mg Cu/g DW for "SCHO" when exposed at 0.5 mg/L Cu. Significant differences were found among genotypes exposed at 0.5 mg/L, with the "SCHO" genotype accumulating more Cu than others (supplementary data **S3**). No pattern was found between Cu concentration in plants and the inhibition of growth-related endpoints, with the "DOR", being the least impacted genotype and having a Cu concentration of 3.64 mg Cu/g DW, and the "DOU", the most sensitive genotype, having 3.62 mg Cu/g DW at 0.5 mg/L Cu. Furthermore, Cu accumulation was not directly linked with growth capacities of the different genotypes, as the genotypes with the fastest growth production and elongation ("AGO", "DOU") were not those accumulating the most Cu. The increase in Cu concentration in plants was tightly correlated with Cu in the exposure media, as expected, with a r^2 of 0.96.

Based on discriminant analysis, a Monte-Carlo test showed that Cu and Genotype had a highly significant effect on the response variables (Monte-Carlo test, $P = 0.001$), and 43.7 % of the total inertia came from the differences between genotypes and between Cu concentrations (**Figure 5.5**). Indeed, 18.8 % were explained by genotypes, 14.0 % by Cu concentrations, and 10.9 % by the interactions between genotypes and Cu concentrations. Graphically, a strong separation among Cu concentrations was observed (**Figure 5.5**, right panel). Indeed, plants exposed to 0.5 mg/L Cu were horizontally opposed to the two other concentrations and clustered on the right side of the vertical axis toward the Cu concentration variable, whereas the two other concentrations were on the left side of the vertical axis. Furthermore, these two concentrations were themselves separated vertically, with the non-exposed plants below the horizontal axis and strongly correlated to the growth-related endpoints, and the plants exposed at 0.15 mg/L being anti-correlated with growth variables, "CAM:0.15" excepted. Both RERall and Whorls/cm were anti-correlated to other life-history traits, and RERall was correlated with plants exposed at 0.15 mg/L. The discriminant analysis revealed that RGR and other growth parameters (**Figure 5.5**, left panel) were associated with the absence of Cu in the media (right panel), which is consistent with previous results, showing than Cu decreased growth.

Furthermore, Cu concentration in the plant was correlated with the high Cu concentration in water (0.5 mg/L), which is consistent with an increase in Cu accumulation in plants along with an increase of Cu in water.

The link between the life-history traits of genotypes and their sensitivity to Cu, examined via co-inertia analysis, revealed that the studied life-history traits explained 62.4 % of the response variables. However, according to the Monte-Carlo test, it was only marginally significant (Monte-Carlo test, $P = 0.09$).

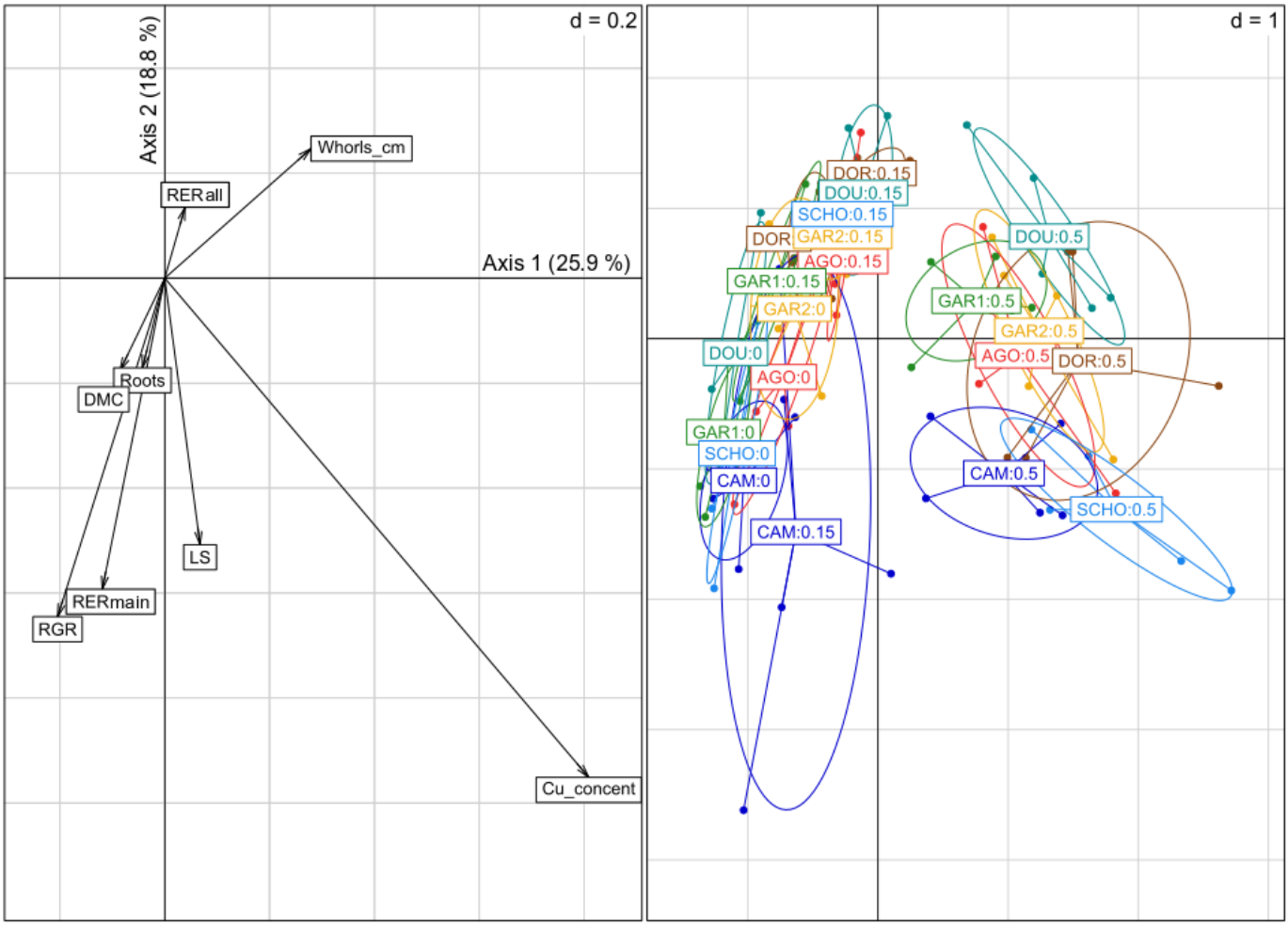

Figure 5.5: Linear discriminant analysis on 7 genotypes of *Myriophyllum spicatum* exposed to Cu during 10 days. The left panel displays the canonical weights of the different variables, with the number of whorls/cm, the number of lateral shoots (LS), Cu plant concentration, dry matter content (DMC), RGR (Relative Growth Rate based on fresh mass), RERall (Relative Elongation Rate, based on total shoots length) and RERmain (RER based on main shoot elongation). The percentages correspond to the inertia of each axis. The right panel displays the scores of the experimental units. These were grouped with ellipses and labelled after the combination genotype x Cu concentration.

6. Discussion

A. Intraspecific trait variability and trait syndromes

Our results showed that the different genotypes of *M. spicatum* significantly differed in their morphology traits and their growth abilities. Indeed, whorl number per length unit, lateral shoot production as well as shoot elongation (both for main and lateral shoots) greatly varied among genotypes when no chemical stress was applied. It has been acknowledged before that different plant individuals can exhibit contrasting morphologies depending on their environment, both through phenotypic plasticity and genotypic variation (Madsen 2013; Arshid and Wani 2013; Ganie et al. 2015; Weyl and Coetzee 2016; Grenier et al. 2016; Tóth et al. 2017). In our case, the plants were grown during at least 6 month in a similar environment prior to the experiment, and genotype morphologies never converged, indicating that genotypic variation would be the main source of the observed differences. Five of the seven genotypes were harvested from different populations in different streams which were not connected (Garonne genotypes excepted, see supplementary data, Figure S1), increasing the probability to get clonal strains with different genotypes, due to genetic heterogeneity among populations (Harris et al. 1992; Chen et al. 2009; Cao et al. 2017). The ISSR analysis confirmed that clonal strains were different genotypes, even for the Garonne individuals (see supplementary data, Figure **S2**).

The PCA showed that RERall and lateral shoot production were positively correlated, and were anti-correlated with DMC and Whorls/cm (which were strongly positively correlated). RERmain was not correlated to these variables, but clustered with RGR. This first suggests that two strategies exist to promote elongation, with either resource allocation in lateral shoot production, or in main shoot elongation. Secondly, this suggests a contrast between actively elongating plants with lateral shoots, long internodes and often a low DMC (*e.g.* "CAM" and "AGO" genotypes), and plants with lower elongation and internode length, with a higher DMC (*e.g.* "DOR"). This can result (1) in numerous, dense whorls, likely promoting nutrient absorption and possibly also light if water is clear enough, (2) an architecture with low tissue density which allows to be closer to the light source through lateral shoot production to form a canopy or (3) a high growth rate associated with a preferential elongation of the main shoot, with traits otherwise intermediate between the two other cases. This possibly illustrates different growth strategies, whose fitness will depend on environmental conditions.

RERall and lateral shoot production were negatively correlated to DMC, suggesting that elongation was mainly promoted by cell elongation, and/or by the production of tissue with high water content and thin cell walls. RGR and DMC were not correlated, which was unexpected, as DMC or close correlates such as specific leaf area (SLA), are considered as good indicators of the resource allocation of plants to growth processes (Westoby 1998; Elger and Willby 2003).

FTIR analysis revealed that some genotypes were different in terms of composition, although those differences do not appear to be correlated with a sensitivity or resistance pattern. Indeed, "SCHO" and "GAR2" genotypes were not more sensitive or resistant, and these are the two genotypes which appear to greatly differ from others. However, further analyses will be performed to complement the results, to assess more precisely *M. spicatum* bio-macromolecule content; the two genotypes currently missing will also be added to the current analysis.

B. Variations in copper sensitivity

Strong differences in sensitivity to Cu were observed among genotypes, as shown by the broad range of inhibition of growth-related endpoints. We demonstrated that genotypes differed in terms of morphology, and those differences were expected to be the cause of the differences in sensitivity to Cu. Further analysis revealed that the results of the analysis were explained slightly more by genotypes than by copper concentrations, supporting the fact that genotypes play an important part in species sensitivity to chemicals. However, according to the co-inertia analysis, 62% of the results were explained by the life-history traits considered in this study. However, the co-structure was not significant, suggesting that some physiological and metabolic traits may better explain differences in copper sensitivity (Singh et al. 2016).

For instance, different leaf color among genotypes were visually observed (supplementary data **S4**), and pigment content might change from one genotype to another, impacting photosynthetic efficiency, antioxidant properties and metal interactions. Indeed, it has been widely demonstrated that plant pigments can chelate metallic compounds, and have antioxidant properties (Zvezdanović and Marković 2009; Brewer 2011). Some genotypes had very red shoots, and were apparently less sensitive to algae proliferation, which could be correlated with phenolic compound production, such as tellimagrandin II, which is an allelopathic compound known to be present in *M. spicatum* (Gross 2001; Gross et al. 1996). Polyphenols are also antioxidant compounds and metal chelators, and their production could provide an advantage

in terms of stress response mechanisms, as they are ROS scavengers (Das and Roychoudhury 2014). However, a study has demonstrated that phenolic compounds act as pro-oxidant in presence of Cu^{2+}, thus increasing ROS production (Iwasaki et al. 2011). Stress tolerance is also mediated by changes in proline production and enzymatic responses, which will be mainly triggered when plants are exposed to stressful conditions (Pflugmacher et al. 1997; Kanoun-Boulé et al. 2009; Fidalgo et al. 2013; Thomas et al. 2013). Some individuals may have higher basal level of production of these compounds, and may therefore be more tolerant. To go further, it has been widely demonstrated that preferential gene expression and post-translational modifications are involved in stress response pathways in plants, but very few studies have investigated their impact in aquatic plants as a way to cope with abiotic stressors (Regier et al. 2013; Gamain et al. 2017). This highlights the need to go further into the mechanisms explaining aquatic plant responses and adaptation to environmental factors, including chemical stressors.

The genotypes used in these experiments were coming from relatively similar aquatic environments in terms of nutrient loads, climate and environmental pollution, thus decreasing the probability to harvest very different genotypes undergoing strong selection pressures. To complement the current dataset and better understand the sources of variability in sensitivity among genotypes, we aim to harvest genotypes originating from more contrasted environments, and to study additional physiological traits in further experiments, listed in **Table 6.1**. Furthermore, some transcriptomic studies will be performed on two contrasted genotypes to assess genetic differences (*i.e*. preferential translation) that may explain different sensitivities to Cu.

Table 6.1: Life-history traits that will be studied in further experiments to help understanding the mechanisms of variations in the sensitivity of *Myriophyllum spicatum* to copper.

Life-traits	**Role**
F_v :F_m	Photosynthesis
Pigment composition	Phytosynthesis & ROS scavenging
C, N, P content	Elemental composition
Phenolic compounds	Allelopathy & ROS scavenging, Biomass conservation
SLA	Photosynthesis, nutrient absorption
Shoot diameter & resistance	Biomass conservation

C. *Implications of intraspecific variation for ecotoxicological risk assessment*

The genotypic variation detected in *M. spicatum* might inflect the outcomes of laboratory tests, as our results have shown that the sensitivity may vary from one genotype to another, with up to fourfold growth inhibition differences among genotypes. This implies that depending on the genotype used in the laboratory, the benchmark values derived from those toxicity tests for an ecosystem compartment may not be protective enough to ensure the absence of harmful effects on organisms living in this compartment. Therefore, a given genotype might not be representative of the species sensitivity, and therefore might mislead the following steps of ecotoxicological risk assessment (Clark et al. 1999; Johnson and Sumpter 2016). Indeed, hazard characterization with lab assays, from which benchmark values and guidelines are derived from, is one of the first steps of ERA. Two main methodologies are used to determine a benchmark value; first, the Predicted No Effect Concentration (PNEC) of the most sensitive species used during lab assays (with a minimal of 3 species tested), which is derived with assessment factors. The second method is the use of Hazardous Concentration 5% (HC_5), which is extracted from Species Sensitivity Distribution (SSD) method, which compares the sensitivity of at least 6 species, and extracts the concentration which will harm 5% of the species tested. If the sensitivity of a given species in those tests is only the fruit of a sampling effect, and is not representative of the entire population sensitivity, then the first step of risk assessment may be impaired, as well as subsequent ones.

It is therefore crucial to properly assess species sensitivity to chemicals, by taking into account the different sources of variation that may influence the outcomes of laboratory testing, in order to insure their reliability.

D. *Ecological implications of intraspecific variation*

Intraspecific variability has been recognized for decades as a promoter of diversity and as a condition for coexistence (Bolnick et al. 2011). Recent work in trait-based community ecology has shed light on the need to integrate intraspecific variability in community ecology studies (Violle et al. 2012; Isaac et al. 2017).

According to Albert et al. (2011), interspecific variability is relatively larger than intraspecific variability at large scale, whereas intraspecific variability gains in importance as the scale of study decreases. As such, intraspecific variability should be taken into account,

especially in small studies, as it could inflect ecological studies outcomes. Furthermore, linking ecosystems and species relies on approaches that consider how species traits affect ecosystem processes (Bolnick et al. 2011). Cianciaruso et al. (2009) have studied the importance of intraspecific variation in functional diversity of plant communities, and they demonstrated that there was no relationship between species richness and functional diversity, as intraspecific variability in functional traits was very high. They concluded that intraspecific variation should allow a better understanding of processes linking individuals and ecosystems, and would also provide better predictions in species extinction consequences for ecosystem processes. Indeed, intraspecific variability in life-history traits (from allelopathy to growth) could strongly inflect the structure and dynamic of assemblages of organisms that co-occur within a local place and time (Violle et al. 2012). For instance, Wolf et al. (2018) have investigated the importance of intraspecific variability in Artic diatoms in the adaptation to climate change, and showed very high variability in their sensitivity due to plastic responses. Similar finding was demonstrated by Kremp et al. (2012); they assessed the importance of intraspecific variability, more specifically phenotypic plasticity, in diatoms response to climate change and highlighted its importance in ecosystem resilience.

However, very few studies have looked into the role of genotypic variation in species adaptation to a changing environment, and even less when it comes to sensitivity to chemical contamination (Dalton et al. 2013). To fill these gaps, the role of intraspecific variability in species sensitivity, ecosystem resilience and recovery needs to be properly assessed (Oliver et al. 2015). Our results have shed light on the importance of genotypic variation in the sensitivity of *M. spicatum* to Cu, demonstrating that individuals can exhibit significantly different sensitivities, depending on their traits. Although we did not measure the genetic variability within populations, our results give insights on the resilience potential that a broad genetic diversity could provide to an ecosystem, if genotypes exhibit differences in sensitivity to stressors and in trait syndromes. Indeed, individuals with traits conferring reduced sensitivity or high coping capacities will confer higher resistance to ecosystem functions (Oliver et al. 2015).

7. Conclusion

We assessed the importance of genotypic variation in the intraspecific variability of *M. spicatum* sensitivity to Cu, and demonstrated impact differences up to fourfold among

genotypes regarding growth related endpoints. ERA is an always evolving process, along with scientific discoveries, in order to increase environmental quality, and reduce as much as possible the impact of chemicals on non-target organisms. As such, we bring to knowledge that genotypic variability is a source of variation in species sensitivity to chemicals, and that further studies should be conducted to properly understand the mechanisms involved. We demonstrated that morphological endpoints do not completely explain these differences in sensitivity, and we highlight that further studies should be focused on physiological endpoints and genetic changes (*e.g.* alternative splicing, preferential translation) to assess the mechanisms of such variation. If those further studies confirm that genotypic variation is correlated with specific traits, it might allow to integrate such variability in ERA approaches, thus enhancing their robustness.

8. Acknowledgments

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9. Supplementary data

Figure S1. Geographic origin of the seven genotypes of *M. spicatum*.

Figure S2. UPGMA cluster analysis based on ISSR data showing genetic relationships among samples of *Myriophyllum spicatum*. The scale refers to genetic distance based on the primer n°20.

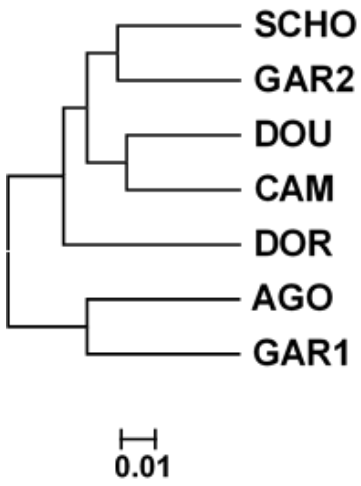


Figure S3. Copper concentration in mg Cu per grams of dry weight in seven genotypes of *Myriophyllum spicatum* exposed to three Cu concentration during 10 days. N=5.

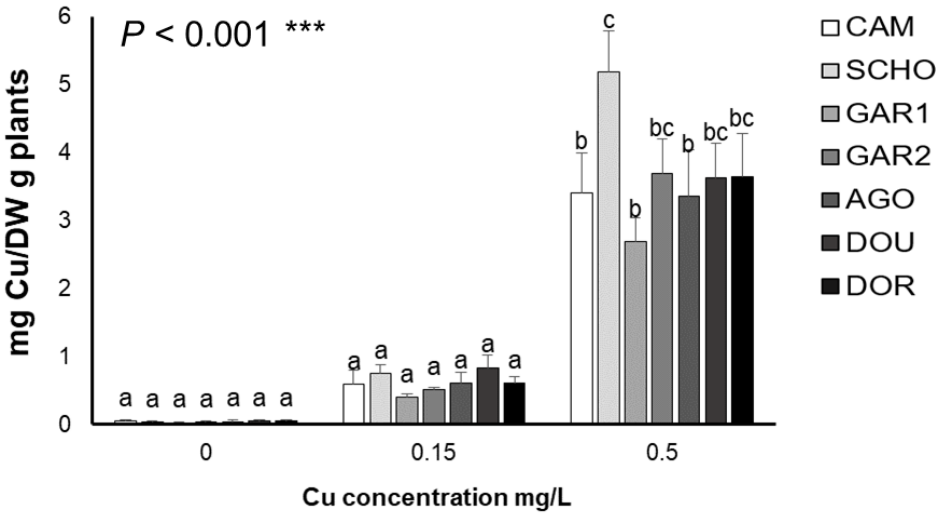
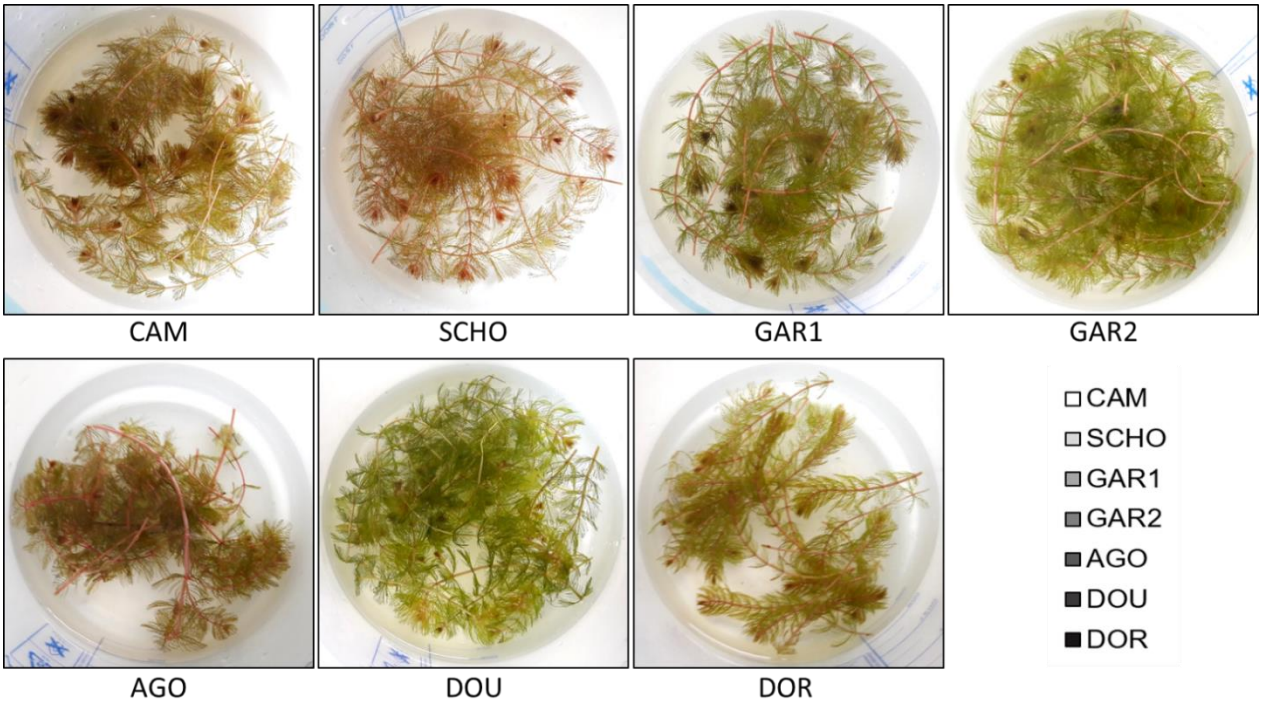


Figure S4. Pictures of the shoots of the seven genotypes exposed during 10 days to copper. Genotypes demonstrate different leaf and shoot colors. Colors match with the color code used in the different figures.



CHAPTER V

Influence of phenotypic plasticity on macrophyte sensitivity to chemicals

1. Does phenotypic plasticity inflect the sensitivity of *Lemna minor* to copper?

Phenotypic plasticity has long been considered by evolutionary ecologists as a nuisance for organisms, hampering the selection of favorable traits in a given environment (see chapter **I.4**). However it was finally recognized as a way to cope with short-term environmental fluctuations. Many studies have notably highlighted the importance of phenotypic plasticity in the adjustment to environmental changes in a context of global warming. Some studies have also investigated how changes in nutrient loads or pH may affect trace elements uptake by aquatic plants in a purpose of phytoremediation. However, no study has directly linked phenotypic plasticity with chemical exposure, and its influence on species sensitivity. This is of concerns, as human activities trigger both environmental fluctuations and ecosystems modification through chemical contamination.

As phenotypic plasticity plays an important role in the adaptive response of species to environmental changes, it is relevant to assess if this process can influence species sensitivity to chemicals.

Thus, in this chapter, I aimed to assess whether or not phenotypic plasticity plays a role in aquatic macrophyte response to Cu exposure. *Lemna minor* was used as a model species, since previous results (chapter **III**) have shown that *L. minor* does not exhibit broad variations in sensitivity among genotypes, and may thus rely more on phenotypic variability to cope with environmental changes.

Environmental variations mediate duckweed (*Lemna minor* L.*)* sensitivity to copper exposure through phenotypic plasticity

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2. Abstract

Environmentally mediated sensitivity of *Lemna minor* to copper (Cu) was evaluated for the first time in three experiments: the effects of two levels of nutrient concentration, light irradiance or Cu pre-exposure were tested. Various Cu concentrations ranging from 0 to 0.25 mg/L were used to assess the sensitivity of *L. minor* to this metal, using one common strain previously acclimatized to two different levels of light intensity, nutrient enrichment and Cu pre-exposure. Our results showed a phenotypic plastic response of the relative growth rates based on frond number and fresh mass production, and maximum quantum yield of photosystem II (F_v:F_m). Growth was affected by the three environmental conditions both prior and during Cu exposure, whereas F_v:F_m was mostly affected during Cu exposure. Copper significantly influenced all the parameters measured in the three experiments. Environmental conditions significantly modified *L. minor* sensitivity to Cu in all experiments, with up to twofold difference depending on the treatment. Growth rate was the parameter the most impacted. Our study revealed for the first time the existence of phenotypic plasticity in *L. minor* sensitivity to chemical contamination, and implies that environmental context need to be taken into account for a relevant risk assessment.

Keywords: *Lemna minor*, copper, phenotypic plasticity, ecotoxicological risk assessment

3. Introduction

Aquatic macrophytes – photosynthetic organisms that can be seen to the naked eye – play a pivotal role in aquatic ecosystems. They have a wide range of life history traits, as they can be free-floating at the water surface, emergent, or submersed in the water column, and are found across various environmental and ecological conditions (Chambers et al. 2008; Thomaz et al. 2008). They provide shelter for other species, and also improve water quality through their involvement in biogeochemical cycles (Onaindia et al. 2005; Bornette & Puijalon 2011; Coutris et al. 2011). As primary producers, they are also the first step of the trophic chain (Chambers et al. 2008; Li et al. 2010; Bornette & Puijalon 2011). Their direct response to changes of their biotic and abiotic environments has the potential to unbalance the whole aquatic ecosystem functioning and food web (Bornette and Puijalon 2011).

Some aquatic macrophyte species are used as bioindicators and for chemical risk assessment in aquatic environment because they have a wide geographical distribution and are sensitive to various environmental parameters and to anthropogenic chemicals (Ferrat et al. 2003; Onaindia et al. 2005; Rai 2009). Among these species, *Lemna minor* was the first macrophyte species to be included in OECD guidelines, as it is a free-floating fast-growing species easily grown in the laboratory (Test N°221, OECD 2006). *L. minor* is composed of photosynthetic fronds grouped by one to twelve, which form new individuals once they are separated. This species is distributed worldwide from northern Scandinavia to southern New Zealand, which is an essential quality for bioindicator species. It thrives under varying environmental conditions, and shows therefore a high potential for phenotypic plasticity (Vasseur and Aarssen 1992).

Phenotypic plasticity, which is the ability for a similar genotype to produce different observable characteristics in different environments, is more likely observed in fast-growing species with clonal reproduction and with a wide geographic range, such as *L. minor*. (Barrett, Eckert, and Husband 1993). Plasticity has received growing interest over the past decades because it can increase the ability of species to survive and adjust to the short-term environmental fluctuations that are more frequent with climate change and agriculture intensification (Bradshaw 1965; Wells and Pigliucci 2000; Matesanz et al. 2010; Vitasse et al. 2010; Eissa and Zaki 2011; Woodward et al. 2016). For instance, it has been shown that several clonal strains of *L. minor* respond by changes at both morphological and biochemical levels to rapid environmental fluctuations (Vasseur, Aarssen, and Lefebvre 1994). Going et al. (2008) have found that *Nasturtium officinal*, another macrophyte species, harbors some morphological plasticity in leaf area to adjust to low light availability. Likewise, *Myriophyllum spicatum* is

characterized by plastic biomass allocation and clonal architecture depending on flooding conditions (Yang et al. 2004; Arshid and Wani 2013). There is growing evidence for the potential for phenotypic plasticity to play a role in the adaptation of aquatic macrophytes to environmental fluctuation (Sultan 1995; van Kleunen and Fischer 2005; Ghalambor et al. 2007; Matesanz et al. 2010).

Plasticity starts to be well acknowledged in aquatic plants, however the importance of abiotic factors on sensitivity to chemicals has been rarely investigated (McLay, 1976; Gupta et al. 1996; Li *et al.*, 2010, Nuttens and Gross, 2016). The effects of chemicals on aquatic macrophytes have been extensively studied, as well as the effect of environmental fluctuations. But very few studies have jointly considered environmental variation and chemical contamination (Fairchild, Ruessler, and Ron 1998; Fritioff et al. 2005; Knauer et al. 2006; Li et al. 2010; Coutris et al. 2011; Boxall et al. 2013; Verma and Suthar 2015). To the best of our knowledge, none has investigated the involvement of phenotypic plasticity in the coping process of chemical stress by aquatic macrophytes.

In order to assess properly the impact of pollutants on aquatic plants, we need to understand how environmental factors can inflect their sensitivity and their response to pollution, and therefore both the resistance and resilience of a given ecosystem to such pressure. In chemical risk assessment, toxicity assays are performed following very clear and reproducible guidelines (*e.g.* OECD protocols). The hypothesis that a given species could be more or less sensitive to one pollutant depending on environmental conditions remains to be tested. This has implications because results obtained in toxicity assays may not reflect what is found in the natural environment.

In this study, we investigated the phenotypic plasticity of *L. minor* sensitivity to Cu contamination by measuring the impact of environmental fluctuations (light intensity, nutrient concentration and Cu pre-exposure). Copper is an environmentally relevant contaminant due to its broad use in both agriculture and industries, and its impact on aquatic biota is well studied. At high concentration, Cu becomes toxic to living organisms, leading to reactive oxygen species (ROS) production and disruption of photosynthesis in chlorophyllous organisms, and therefore to chlorosis (Razinger et al. 2007; Wei Xing, Wenmin Huang 2009; Li et al. 2010; Thomas et al. 2013; Üçüncü et al. 2013). Growth related endpoints (Relative Growth Rates) and photosynthesis endpoint (maximum quantum yield of photosystem II, or F_v:F_m), which are acknowledged to be fitness related traits (Molina-Montenegro et al. 2013; Younginger et al. 2017), were used to quantify *L. minor* response to Cu and environmental factors.

4. Material and methods

A. Experimental design

Three independent experiments were conducted with one clonal strain of *L. minor*, from Avillers-Sainte-Croix, France (49°02'01''N, 5°43'16''E). In order to avoid genetic variation, stock culture was established from one single frond. It was then kept under axenic conditions in 250 mL erlenmeyer flasks containing Steinberg medium at pH 6.5. Environmental conditions in the growth chamber were 23.0 ± 0.1 °C inside experimental units with a 14h/10h day/night period and a light intensity of 96.1 ± 2.3 µmol m^{-2} s^{-1}. All three experiments were conducted with non-axenic Steinberg medium, modified according to table 1 for nutrient and Cu pre-exposure experiments, with a pH of 6.5 ± 0.1.

For each experiment, *L. minor* individuals were first acclimatized for two weeks in a given set of conditions (either "favorable", *e.g.* high light irradiance or "unfavorable", *e.g.* with a low light irradiance), and then exposed for one week to various Cu concentrations under environmental conditions, similar or contrasting with those from the acclimatization period (**Table 4.1**). This set-up allowed to avoid any confounding effect of environmental conditions prior to the experiments and also to distinguish between the effects of average environmental conditions and temporal change regime (*i.e.* changing from "favorable" to "unfavorable", or *vice-versa*, *vs*. stable conditions for the whole experiment, see **Figure 4.1**).

Copper sulfate was purchased from Sigma (CAS number 7758-98-7, Saint Quentin Fallavier, France) and a concentrated solution of 1 g/L $CuSO_4$ was prepared in ultrapure water, and diluted in the different media before pH adjustments.

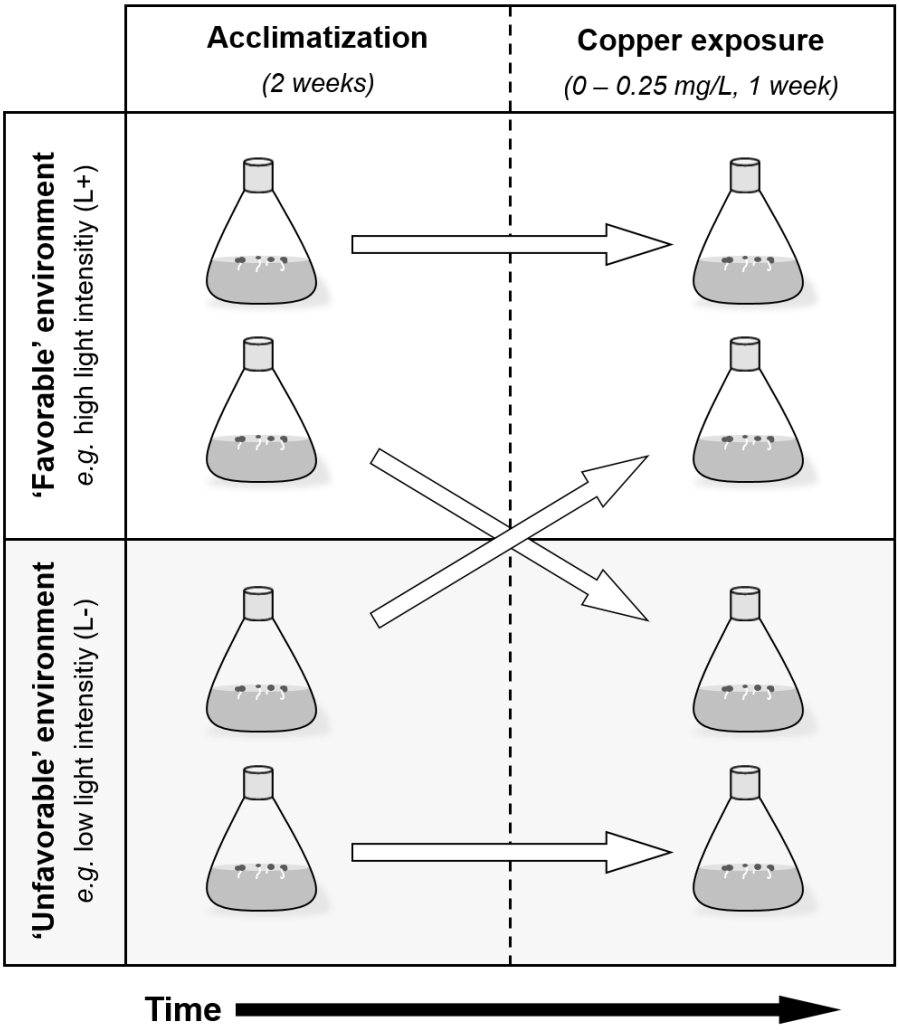

Figure 4.1. The crossed experimental design used in our study, with an acclimatization and a copper exposure phase. One environmental factor (light intensity, nutrient level or copper pre-exposure) varied per experiment, with 'favorable' and 'unfavorable' conditions for plant growth. For example, light intensity was either the one routinely used on the stock cultures, and considered as 'favorable' (L+), or lowered using a shading mesh, and considered as 'unfavorable' (L-) for plant growth. The various combinations of conditions during acclimatization and Cu exposure allowed to avoid any confounding effect of environmental conditions prior to the experiment, and to distinguish between the effects of average environmental conditions and of temporal change regime.

Three water samples per Cu concentration were taken at the beginning of Cu exposure, in order to assess effective concentrations in the media. The samples were measured using inductively coupled plasma with optical emission spectrometry (ICP-OES, Thermo Electron, IRIS INTREPID II XLD). Effective Cu concentrations in the experimental units were on average at 97.0 ± 2.1 % of nominal concentrations at the beginning of experiments. According

to previous measurements conducted in similar conditions, Cu concentration remained > 80% of nominal concentrations after 7 days of exposure.

All experiments were conducted following OECD TG 221 guideline, with some deviations in environmental parameters as described below.

Influence of light intensity

Low light irradiance, which corresponded to a light intensity of 21.7 ± 0.8 µmol m^{-2} s^{-1} of photosynthetic active radiation, was considered as an "unfavorable condition". High light irradiance was considered as a more "favorable condition", with a light intensity of 96.1 ± 2.3 µmol m^{-2} s^{-1}. Copper concentrations tested were 0, 0.05 and 0.25 mg/L (4 replicates per combination of Cu concentration × light irradiance during acclimatization × light irradiance during exposure).

Influence of nutrient concentration

Low nutrient levels, that corresponded to low nitrate and phosphate concentrations (6.5 mg/L KNO_3, 0.66mg/L KH_2PO_4 and 86.9 µg/L K_2HPO_4), were considered as an "unfavorable condition". High nutrient levels were considered as a more "favorable condition" with high nitrate and phosphate concentrations (350 mg/L KNO_3, 38 mg/L KH_2PO_4, and 5 mg/L K_2HPO_4). Copper concentrations tested were 0, 0.1 and 0.25 mg/L (5 replicates per combination of Cu concentration × nutrient concentration during acclimatization × nutrient concentration during exposure).

Influence of Cu pre-exposure

Two sets of environmental conditions were used during the two-week acclimatization: pre-exposure to 0.05 mg/L Cu (*i.e.* "unfavorable" condition) and no pre-exposure to Cu (*i.e.* "favorable" condition). Copper concentrations used during subsequent exposure were 0, 0.015 and 0.20 mg/L (6 replicates per combination of Cu pre-exposure conditions × Cu concentration during subsequent exposure).

Table 4.1. Summary of environmental conditions for the three experiments, during acclimatization and exposure: time. L: light intensity, N: nutrients (KNO_3, KH_2PO_4 and K_2HPO_4), +/- symbols are used for "favorable" (+) and "unfavorable" (-) conditions, and "P-E" for pre-exposed to Cu.

Experiments	Copper during exposure mg/L	Acclimatization and exposure conditions
Nutrients n = 5	0, 0.1, 0.25	**N +** 350 mg/L KNO_3 38 mg/L KH_2PO_4 5 mg/L K_2HPO_4 **N -** 6.5 mg/L KNO_3 0.66mg/L KH_2PO_4 86.9 µg/L K_2HPO_4
Light intensity n = 4	0, 0.05, 0.25	**L +** 96.1 ± 2.3 µmol m^{-2} s^{-1} **L -** 21.7 ± 0.8 µmol m^{-2} s^{-1}
Copper pre-exposure n = 6	0, 0.05, 0.25	**Not P-E** 0 mg/L Cu **P-E** 0.05 mg/L Cu

B. Endpoints

The number of fronds was recorded on the first and the last day of Cu exposure for each experimental unit. Fresh mass per frond was estimated from stock cultures at the beginning of Cu exposure under each environmental set of conditions. For this purpose, six weighings of 9 to 14 randomly chosen fronds were realized for each acclimatization condition, and averaged. These average values were used to assess total biomass in each flask at the beginning of Cu exposure (direct weighting was avoided, as this often breaks roots, and is thus stressful to the plants). Fronds within each flask were counted and weighted at the end of experiments to assess their fresh mass, then oven dried at 70°C until constant weight to assess their dry mass. Relative growth rates (RGR) based on fresh mass or frond number were calculated for each experimental unit (*i.e.* erlenmeyer flask) as follows:

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

where RGR_{i-j} is the relative growth rate from time i to j, N_i is the endpoint (frond number or fresh mass) in the test or control flask at time i, N_j is the same variable in the test or control flask at time j, and t is the time period from i to j.

The inhibition percentage of RGR was also calculated for the experimental units in the different treatment groups to assess both effects of environmental conditions and Cu exposure, following the formula:

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

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

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

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

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

Maximum quantum yield of photosystem II (F_v:F_m), which is the maximal ability of the plant to harvest light, calculated by using the Kautsky effect (Maxwell and Johnson 2000; Murchie and Lawson 2013), was measured using an underwater fluorometer Diving-Pam (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. At the beginning of the experiment, fifteen randomly-chosen *L. minor* bunches of three-four fronds per environmental condition were dark acclimatized during 30 minutes to ensure the opening of reaction centers. At the end of the experiment, three measurements were similarly taken from each experimental unit, with changes in the Diving-Pam parameters (increase in intensity of measuring light and/or amplification factor) when plants were too chlorotic to emit sufficient signal for accurate measurement of F_v:F_m.

C. Statistical analysis

Normality was assessed by a Shapiro-test on ANOVA residuals. Log-transformation of raw data was conducted when the normality assumption was not met. Homoscedasticity was tested by using a Bartlett test. Three-way ANOVAs were performed to assess the interactive effects of acclimatization conditions, exposure conditions and Cu concentrations during exposure. Tukey HSD post-hoc tests were used to identify significant differences between the various combinations of experimental treatments. Generalized Linear Model with Gamma distribution were performed to assess interactions in dataset showing no normality despite log-

transformation. The fit of the models were assessed using the pseudo-R^2 and Akaike Information Criterion (AIC). Statistical analyses were conducted using R studio software (R Core Team (2016) V 3.3.1).

5. Results

All the endpoints, except dry matter content in two experiments, were affected by environmental conditions. We also found that sensitivity to Cu was modified as a result of environmental variations (**Table 5.1**).

A. Light variation

1) Quantum yield of PSII

The results suggest that light intensity had an impact on *L. minor* F_v:F_m and its sensitivity to Cu, which is supported by a three-way interaction between Cu concentration and light intensities during both acclimatization and exposure (P = 0.006, see **Table 5.1**). Copper negatively affected F_v:F_m (P < 0.0001), and the effect was more pronounced when environmental conditions were favorable. Plants exposed to low light intensity throughout the experiment were less affected by Cu than in other treatments, especially at high Cu (0.25 mg/L), with 4.8% of F_v:F_m inhibition, against 11.4% for high light (**Figure 5.1a**). It was visually observed that fronds from controls exposed to low light irradiance were thicker and darker.

2) Relative growth rates

Growth rates were significantly impacted by light intensity during Cu exposure (P < 0.0001 for both RGRs) but not during the acclimatization phase (**Figure 5.1b**, **Table 5.1**). Therefore, only data with steady environmental conditions between acclimatization and Cu exposure were kept for subsequent statistical analysis. Unfavorable treatment (L-) strongly decreased growth of *L. minor*, with a $RGR_{fresh\ mass}$ in the controls of 0.126 d^{-1} under low light intensity, against 0.342 d^{-1} at high light intensity.

Copper negatively affected growth, and its impact was driven by light intensity, as illustrated by a significant two-way interaction for both RGRs (P < 0.001) between Cu and light

intensity during Cu exposure (**Table 5.1**). Under low light intensity, low Cu dose (0.05 mg/L) did not affect growth, whereas plants under high light had a $RGR_{fresh\ mass}$ inhibited by 24.2% (**Figure 5.1b**). However, the highest Cu concentration (0.25 mg/L) inhibited the $RGR_{fresh\ mass}$ by 99% under low light intensity, against 72% under high light intensity.

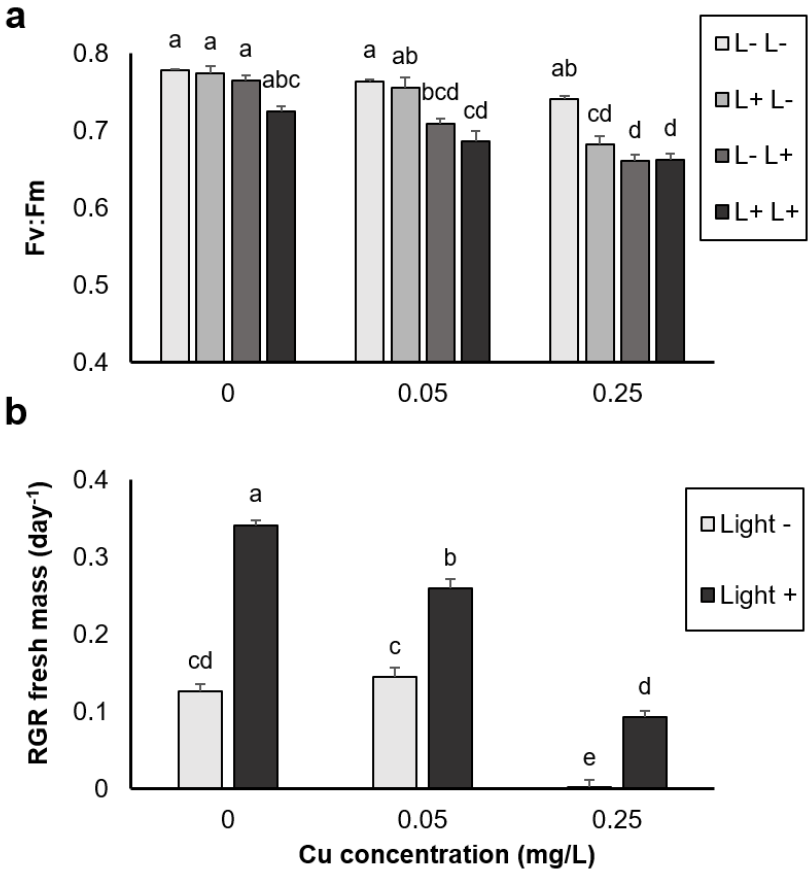

Figure 5.1. (**a**) Maximum quantum yield (F_v:F_m) of *L. minor* under favorable" (L+) and "unfavorable" (L-) light conditions during the phase of acclimatization (first letter in the legend) and the phase of Cu exposure (second letter in the legend). (**b**) Relative growth rate (RGR) based on biomass production under "favorable" (- / L+) and "unfavorable" (- / L-) light conditions during Cu exposure at 0, 0.05 and 0.25 mg/L. Only steady conditions are shown in figure **1b**, as no significant effect of acclimatization was found. Significant differences among treatments and Cu concentrations are labelled with different letters from a to d, error bars correspond to standard errors.

Table 5.1. Summary table of the P-values and significance levels for factorial ANOVAs and GLM (in italic) testing the effects of the independent variables copper concentration (Cu), environmental conditions during acclimatization(Acc.) and during copper exposure (Expo.), and their interactions (Cu*Acc, Cu*Expo, Cu*Acc*Expo). df res.: residual degrees of freedom; df: treatment degrees of freedom; F: Fisher calculated values. DMC: Dry matter content, fm: fresh mass. Stars highlight significant *P*-values.

Experiment	Endpoint	df res.	P-value Cu	P-value Acclim.	P-value Expo.	P-value interactions
Light variations	$F_v:F_m$	47	< 0.0001 *	0.001 *	< 0.0001 *	Cu*Acc*Expo 0.006 *
	df , F values		2, 49.54	1, 12.369	1, 60.549	2, 5.791
	RGR_{frond}	36	< 0.0001 *	0.0550	< 0.0001 *	Cu*Expo : 0.0007 *
	df , F values		2, 42.515	1, 3.933	1, 126.836	2, 8.912
	RGR_{fm}	36	< 0.0001 *	0.0751	< 0.0001 *	Cu*Expo < 0.0001 *
	df , F values		2, 206.709	1, 3.360	1, 306.107	2, 22.252
	DMC	40	< 0.0001 *	0.2428	0.0012 *	0.3672
	df , F values		1, 21.212	1, 1.406	1, 12.153	1, 0.832
Nutrient variations	$F_v:F_m$	48	< 0.0001 *	0.0075 *	0.0003 *	Cu*Acc : 0.0126 *
	df , F values		2, 304.187	1, 7.999	1, 15.303	2, 4.795 Cu*Expo : 0.0059 * 2, 8.718
	RGR_{frond}	59	< 0.0001 *	< 0.0001 *	0.0049 *	Cu*Expo : 0.0003 *
	df , F values		2, 286.978	1, 22.534	1, 8.723	2, 9.635
	RGR_{fm}	48	< 0.0001 *	< 0.0001 *	< 0.0001 *	Cu*Acc*Expo : 0.0072 *
	df , F values		2, 509.128	1, 38.429	1, 40.055	2, 5.474
	DMC	24	< 0.0001 *	0.0694	0.322	0.899
	df , F values		2, 30.193	1, 0.069	1, 0.322	2, 0.246
Copper pre-exposure	$F_v:F_m$	18	< 0.0001 *	0.0234 *		0.8169
	df , F values		2, 159.218	1, 6.136		2, 0.204
	RGR_{frond}	30	< 0.0001 *	0.5365		Acc*Expo : 0.0057 *
	df , F values		2, 308.967	1, 0.391		2, 6.176
	RGR_{fm}	30	< 0.0001 *	0.0833		Acc*Expo : 0.0005 *
	df , F values		2, 542.423	1, 3.209		2, 9.82
	DMC	30	0.0004 *	0.0522		Acc*Cu : 0.0012 *
	df , F values		2, 10.119	1, 4.085		2, 8.516

B. Variation in nutrient concentrations

1) Quantum yield of PSII

Nutrient concentrations significantly affected the sensitivity of $F_v:F_m$ to Cu (**Figure 5.2a**), and it was supported by an interaction between Cu concentrations and nutrient richness, both during acclimatization and Cu exposure (P = 0.0126 and P = 0.0059, respectively, **Table 5.1**).

Copper significantly reduced F_v:F_m at all tested concentrations ($P < 0.0001$), but this effect varied with the level of nutrient concentrations: plants under low nutrient levels were less impacted than plants growing under high nutrient levels. Indeed, when *L. minor* was exposed to 0.1 mg/L Cu, plants growing on rich media during Cu exposure were slightly more impacted than plants growing in poor media (inhibition of 7.4% against 2%, **Figure 5.2a**). At 0.25 mg/L, differences in sensitivity associated with different levels of nutrient concentrations covered a range of inhibition values from 9 to 19%, and plants acclimatized under low nutrient level were less inhibited than plants acclimatized under high nutrient level.

2) Relative growth rates

Growth sensitivity to Cu was mediated by variations in nutrient concentrations, as shown by the three-way interaction between Cu concentration, nutrient levels during acclimatization and exposure, that affected $RGR_{fresh\ mass}$ ($P = 0.0072$, **Table 5.1**). Growth was impacted by nutrient levels: $RGR_{fresh\ mass}$ for control plants passing from a rich medium during acclimatization to a poor medium during exposure was <0.300 d^{-1}, against >0.350 d^{-1} for control plants passing from poor to rich nutrient media (**Figure 5.2b**). Furthermore, plants acclimatized in condition of high nutrient level had the lowest $RGR_{fresh\ mass}$ (0.304 d^{-1}) but the highest RGR_{frond} (0.305 d^{-1}, data not shown), whereas plants acclimatized in condition of low nutrient concentration had the highest $RGR_{fresh\ mass}$ (0.361 d^{-1}) and the lowest RGR_{frond} (0.290 d^{-1}).

Copper significantly decreased the two types of RGR measurements ($P < 0.0001$) for both concentrations. At 0.1 mg/L Cu, a slight decrease of RGRs was observed, following the same pattern as for controls: plants acclimatized under low nutrient level had a higher $RGR_{fresh\ mass}$ and a lower $RGR_{frond\ number}$ compared to plants acclimatized under high nutrient level. Copper impact was more pronounced at 0.25 mg/L Cu, in which case the $RGR_{fresh\ mass}$ inhibition ranged from 56% for plants passing from poor to rich nutrient concentration, to 83% for plants under low nutrient concentration throughout the experiment.

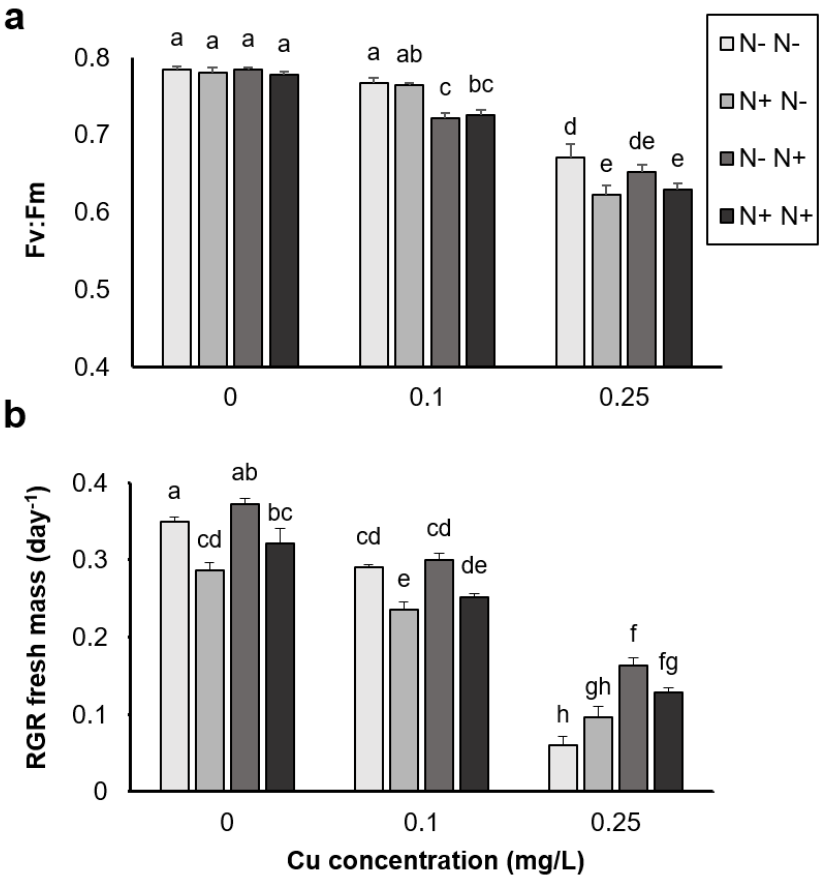

Figure 5.2. (**a**) Maximum quantum yield (F_v:F_m) and (**b**) relative growth rate (RGR) of *L. minor* under "favorable" (N+) and "unfavorable" (N-) nutrient concentrations (KNO_3 and KH_2PO_4) during the phase of acclimatization (first letter in the legend) and the phase of Cu exposure (second letter in the legend), at 0, 0.1 and 0.25 mg/L. Significant differences among treatments and Cu concentrations are labelled with different letters from a to h, error bars correspond to standard errors.

C. <u>Effect of copper pre-exposure</u>

1) Quantum yield of PSII

According to our results, Cu exposure decreased the F_v:F_m at all concentrations ($P < 0.0001$), with a concentration-related effect (**Figure 5.3a**). Cu pre-exposure had a significant impact on F_v:F_m ($P=0.0234$), with an increased inhibition for pre-exposed plants compared to those not-pre exposed. Indeed, the F_v:F_m of pre-exposed plants was inhibited from 13.3% at 0.015mg/L Cu to 19.6% at 0.2 mg/L Cu, against 6.7% and 14.3% for not pre-exposed plants, respectively. However, no significant interaction was found between acclimatization and exposure (**Table 5.1**).

2) Relative growth rates

As observed for F_v:F_m, relative growth rates of *L. minor* were significantly influenced by Cu ($P < 0.0001$, **Table 5.1**). However, interactions between conditions during acclimatization and Cu exposure were found for both types of RGR measurements ($P < 0.01$). Control plants were not significantly affected by acclimatization, with similar growth rates between plants pre-exposed or not (**Figure 5.3b**). Copper exposure impacted growth at all concentrations ($P < 0.0001$), and significant differences depending on acclimatization conditions were observed at low Cu concentration (0.015 mg/L). Indeed, pre-exposed plants were more impacted by Cu, with a $RGR_{fresh\ mass}$ inhibited by 55.8%, against 34.8% for plants not pre-exposed. At 0.2 mg/L Cu, no significant difference was observed between acclimatization conditions, but growth was strongly impaired for both treatments, with $RGR_{fresh\ mass} < 0\ d^{-1}$ on average, due to partial plant decomposition (**Figure 5.3b**).

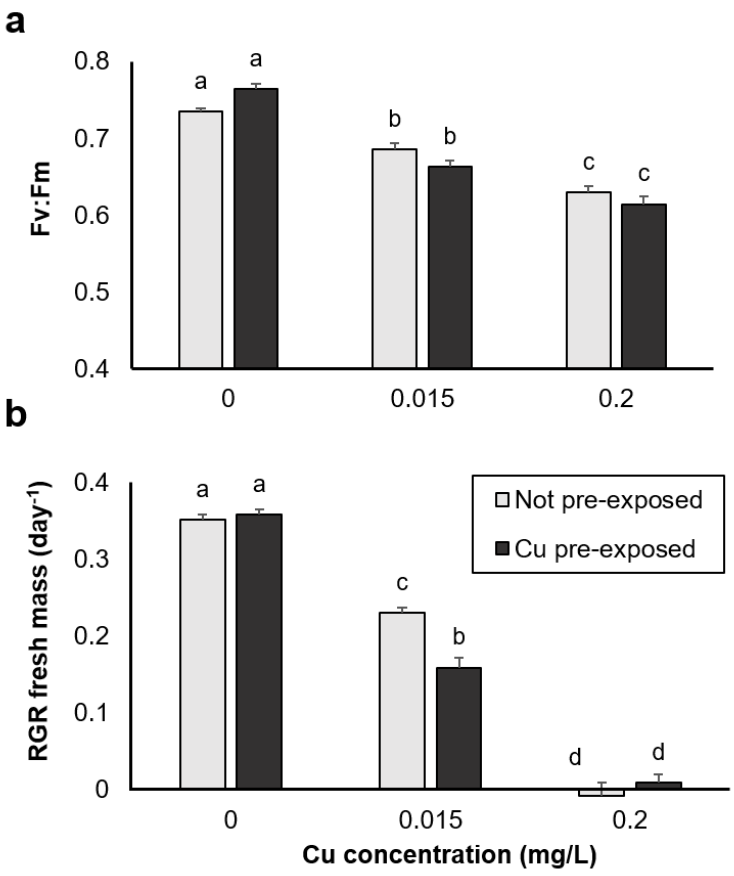

Figure 5.3. (**a**) Maximum quantum yield (F_v:F_m), and (**b)** relative growth rate (RGR) of *L. minor* pre-exposed to 0.05 mg/L Cu or not pre-exposed before Cu exposure at 0, 0.015 and 0.2 mg/L. Significant differences among treatments and concentrations are labelled with different letters from a to d, error bars correspond to standard errors.

6. Discussion

A. Phenotypic plasticity of L. minor exposed to copper

Our results showed that the relative growth rate and photosynthetic efficiency of *L. minor* were affected by exposure to Cu after one week of exposure, and that this response was altered by changes in light irradiance, nutrient concentration, and Cu pre-exposure. They revealed that the influence of environmental changes on *L. minor* sensitivity to Cu was not negligible, with up to a twofold difference in growth inhibition induced by a same Cu concentration under contrasted environments (nutrient variation and pre-exposure experiments). Limitation of resources (nutrients, light) decreased growth, as well as Cu exposure, with a concentration-related effect. Combination of resource limitation and Cu exposure triggered a cumulative environmental stress, which decreased growth even more, but probably limited Cu intake by the plant (**Figure 5.1b**, **Figure 5.2b**). As a result, the F_v:F_m ratio that reflects the light-harvesting efficiency of plants was less impacted by Cu under "unfavorable" conditions than under "favorable" environmental conditions, in contrast to what was observed for growth (**Figure 5.1a**, **Figure 5.2a**).

It has been widely acknowledged that resource limitation decreases growth of aquatic plants because they depend on their environment to convert light energy and produce biomass (Barko and Smart 1981; Hussner et al. 2009; Bornette and Puijalon 2011; Cao et al. 2012). It has also been well documented that Cu is toxic and has a negative impact on both growth and photosynthetic capacity of aquatic plants beyond a physiological threshold, and that this threshold concentration is species-dependent (Razinger et al. 2007; Wei Xing, Wenmin Huang 2009; Khellaf and Zerdaoui 2010; Thomas et al. 2016). Our findings corroborate on *L. minor* the demonstration that nutrient enrichment can increase tolerance of aquatic plants to metals, as previously observed on *S. polyrhiza* and *M. spicatum* (Leblebici and Aksoy 2011; Nuttens and Gross 2017).

According to our results, the F_v:F_m of the plant was not affected by either nutrient availability or light limitation, suggesting an adjustment of plants to ensure maximum photosynthesis, as demonstrated by previous studies (Evans 1989; Eichelmann et al. 2005; Going et al. 2008; Gratani 2014). At low concentration, Cu had a strong impact on the F_v:F_m of actively growing plants (*i.e.* without resources limitation), but this impact was not found on plants for which growth was decreased by resource limitation (**Figure 5.1**, **Figure 5.2** and **Figure 5.3**). However, at high Cu concentration, plants under resource limitation or pre-

exposed to Cu were the most impacted, suggesting that the level of cumulative environmental stress was too high to be copped with (Razinger et al. 2007; Bornette and Puijalon 2011; Thomas et al. 2013, 2016).

B. Ecological and ecotoxicological implications

Environmental changes can occur during the life of an individual, and as demonstrated by our results, these changes might inflect the ability of aquatic plants to respond to chemical exposure. It is widely acknowledged that environmental conditions affect growth and fitness in plants, and *L. minor* is no exception to this rule (Vasseur et al. 1992). Such examples of phenotypic plasticity are common in plants (Barko and Smart 1981; Olesen and Madsen 2000; Hussner et al. 2009; Bornette and Puijalon 2011; Xie and Yu 2011; Cao et al. 2012; Madsen 2013). *L. minor* is a species found worldwide in very different environments that shows high plasticity in its response to abiotic factors. Its high plasticity might be the key to its thrive under various environments (Ghalambor et al. 2007).

Our study is the first to document phenotypic plasticity in sensitivity to a chemical contamination of the environment in *L. minor*. Our finding that plants pre-exposed at low Cu concentration were more sensitive to further Cu contamination highlights the possible role of diffuse pollution in the weakening of populations facing mildly polluted environments. It was explained by Vitasse et al. (2010) that environmental variations might strongly affect the vigor of a species. This change could therefore influence its sensitivity to chemicals. This has implications for ecosystem assembly under chronic and acute contaminations.

According to our results, phenotypic plasticity has the potential to affect the relevance of *L. minor* as a model species in chemical risk assessment. The well-defined environmental conditions in standardized ecotoxicological tests is nevertheless expected to avoid the interference of environmental factors in the sensitivity of a given species.

Our findings imply that no deviation from standardized protocols in terms of environmental conditions during toxicity tests and chemical risk assessment evaluations (*e.g.* OECD guidelines) can be accepted. Otherwise, changes in abiotic conditions may significantly inflect the results of toxicity assessment, as illustrated by our results. Furthermore, caution should be taken when comparing results from different studies. In-between experiment differences in nutrient availability, even for a similar type of medium, and/or light intensity, or photoperiod,

have indeed the potential to modify the sensitivity of a species exposed to chemical stress. As a consequence, the potential for running meta-analyses and endpoint comparisons between studies may be limited if these did not use the same environmental settings, or did not account for possible variations induced by contrasted environmental conditions. Finally, it is important to acknowledge that the sensitivity observed during laboratory experiments is for one specific environmental setup, and is not necessarily representative of field conditions.

7. Conclusion

Improving our understanding of phenotypic plasticity is primordial to properly assess the impact of anthropic activities on ecosystems, and their resilience capacity. From the present study, we conclude that phenotypic plasticity is of major importance in the ability of *L. minor* to cope with chemicals in a changing environment. The strong influence of environmental conditions on Cu sensitivity of *L. minor* emphasizes the importance of strict guidelines in standardized protocols. It also highlights that laboratory results cannot be faithfully transposed to what is found in a natural environment and that further work should be done to evaluate the extent of phenotypic plasticity as a way to respond to chemicals.

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CHAPTER VI

GENERAL DISCUSSION

Intraspecific variability plays an important role in species adaptation, and numerous studies have demonstrated the importance of this variability in aquatic plants, as a response to both short and long-term environmental changes (Reusch and Hughes, 2006; Richards et al. 2006; Mitchell and Bakker, 2014, chapter **I.4**). The role of this intraspecific variability in the response of organisms to chemicals remains poorly studied, despite the high occurrence of this contamination in different ecosystems, notably aquatic ones (Woodward et al. 2010; Friberg et al. 2011).

My PhD had for purpose to cope with the lack of knowledge about this intraspecific variability, in particular its implications in organism sensitivity to chemicals, but also its implications in ecotoxicological risk assessment.

This work is part of an original approach, as it bridges two topics very related, and yet poorly studied together: ecology and ecotoxicology. It also combines a multi-scale approach, as it starts with a plurispecific level down to an intra-individual level, with the study of physiological biomarkers. This PhD project has been organized in three main parts. The first part (**1**) had for purpose to identify the importance of intraspecific variability in the response of three aquatic macrophyte species to chemicals. Secondly (**2**) I aimed to explore more in depth the importance of genotypic variability in the sensitivity of *Myriophyllum spicatum* exposed to copper. Finally, in part (**3**) I determined the implication of phenotypic plasticity in the response of *Lemna minor* exposed to copper.

1. The importance of intraspecific variability of aquatic macrophytes in the response to chemical contamination

The main objective of this PhD was to determine the relative importance of intraspecific variability in the sensitivity of aquatic macrophytes to chemicals, compared to interspecific variability.

Current approaches in ecotoxicological risk assessment, such as Species Sensitivity Distribution (SSD), aim to compare differences in the sensitivity of several species facing a pollutant, to determine its toxic potential in a given ecosystem (Del Signore et al. 2016). From this potential toxicity, established through different laboratory tests, a threshold concentration (or benchmark) is determined, expected to protect 95% of the species within a community (Pathiratne and Kroon 2016).

One of the problems of this approach is the assumption that individuals tested in laboratory are representative of their species in terms of chemical sensitivity (Forbes and Calow 2002). However, this can be asked whether the results obtained for a given species could be influenced by sampling hazards, and thus not properly represent entire species sensitivity.

Indeed, the individuals tested are often collected at one given place, or raised in laboratory conditions, sometime coming from isogenic strains (*i.e.* individuals share the same gene pool) to decrease the variability of results (Festing and Altman 2002). One of the consequences would be to over- or under-estimate the toxicity of the molecule tested, and to establish an irrelevant threshold concentration, and in the worst case scenario, a non-protective concentration for ecosystems.

Results in the chapter **III** demonstrated that genotypic variability can strongly influence species sensitivity to copper (Cu) contamination. The importance of this source of intraspecific variability appears to be **species-dependent**: the duckweed (*Lemna minor*) did not demonstrate a strong variability in its response to copper exposure among different genotypes in a given environmental set. Indeed, only the F_v:F_m sensitivity significantly varied among genotypes with up to 35% of variation, without conferring any significant change in terms of growth sensitivity among the same genotypes. On the other hand, a strong genotypic variability was observed for the water milfoil (*Myriophyllum spicatum*) in its sensitivity to copper exposure, based on growth related endpoints. Indeed, the sensitivity of growth related endpoints varied up to 72% for biomass production. Those differences were sometimes as high as interspecific variations, depending on the considered endpoint, highlighting the importance of accounting for intraspecific variability in SSD approaches.

Furthermore, **species choice** in those ecotoxicological risk assessment approaches is critical, as some species have a fundamental role in ecosystem functioning (called keystone species) and an impact on those species may disturb the entire ecosystem (Forbes and Calow 2002; Maltby et al. 2005; Connon et al. 2012). For instance, the duckweed is generally not considered as a keystone species, but is used as a model species in ecotoxicological risk assessment, because it is an ubiquist species, sensitive to chemicals and easy to use in laboratory assays. The water milfoil is more complicated to use in laboratory assays as it demonstrates a higher variation in its response to chemicals, but plays a very important part in ecosystem services, notably due to its implication in biogeochemical cycles (Sanchez et al. 2007). Its life history-traits, its representativity of submerged aquatic plants species as well as its structuring role in ecosystems, make this species very relevant in risk assessment (Mohr et al. 2013).

Those results, in addition to confirm that intraspecific variability can inflect the outcomes of laboratory testing and that it should be taken into account in risk assessment approaches, highlight the fact that the choice of the **endpoint observed** to represent species sensitivity is crucial. Indeed, a parameter such as growth based on biomass production can be less impacted, for example, than organ development, seed production or even the behavior of an individual (Connon et al. 2012; Horemans et al. 2016). The choice of the specific parameters should thus represent as best as possible the individual's fitness, in order to offer a relevant measure able to inform of the potential impact on ecological functions ensured by this species (Forbes and Calow 2002; Del Signore et al. 2016; Belanger et al. 2017). This is especially highlighted by the results on *M. spicatum*, which demonstrated a maximal quantum yield of PSII (F_v:F_m) poorly impacted by Cu exposure; thus, although often used in plant ecotoxicology, as it is a quick and non-destructive measure of stress, this was irrelevant to assess the harmful impact of Cu on *M. spicatum*.

2. Genotypic variability in the sensitivity of Myriophyllum *spicatum* to chemicals

One of the secondary objectives of my PhD work was to determine the importance of genotypic variability in an aquatic macrophyte species, and its implication in the response to chemical contamination, by considering a greater number of genotypes than in chapter **III**. Following the results of this chapter, this research was focused on the water milfoil, *M. spicatum*, which demonstrated a broad intraspecific variability in its sensitivity to copper. Several studies have demonstrated that water milfoil shows a broad genetic diversity, and can exhibit different life-history traits depending on its gene pool (Miller 2001; Wu et al. 2016; Tóth et al. 2017; Cao et al. 2017). However, no study has studied whether or not this genotypic variability could influence aquatic macrophytes species sensitivity to chemical contamination, excepted on *L. minor* (Dalton et al. 2013).

Results explained in chapter **IV** have shown that *M. spicatum* demonstrate a different sensitivity to Cu depending on the genotype, studied under steady environment. Indeed, some genotypes were 5 times more sensitive to Cu at low concentration (0.15 mg/L), and 7 times more sensitive at high concentration (0.5 mg/L) than others for some growth parameters. This difference in sensitivity can be partially explained by differences in terms of **life-history traits**

among those genotypes, such as dry matter content, internode length, root and lateral shoot production. Furthermore, some trait syndromes can be observed, *e.g*. correlations between them. For instance, elongation rate was correlated with lateral shoot production, and anti-correlated with whorl production. Indeed, it would appear that plant allocate energy either in shoot elongation and ramification, or in a high whorl density / short internode. This relationship is notably illustrated by the "DOR" genotype, with a higher density of whorls than other genotypes, but showing the lowest elongation, also being the least impacted by Cu exposure. This demonstrates that Cu impact is higher on genotypes demonstrating the most active growth. Furthermore, the genotypes with higher whorl densities / lower elongation rates also tended to have higher dry matter contents and higher root productions (some traits typical of stress-tolerant strategists; Grime, 2001) which is consistent with the hypothesis of a trade-off between biomass conservation and growth processes reflected by trait syndromes (Wilson et al. 1999; Elger and Willby 2003). This was notably demonstrated by "CAM" and "DOR" genotypes, which had those trait syndromes, and were less impacted than other genotypes. Despite the observed correlations among life-history traits, coinertia analysis realized in chapter **IV** was not significant, and 42% of the differences in sensitivity among genotypes remain to be explained.

Indeed, observations were focused on life-history traits related to the morphology of the different genotypes, as it has been done on previous studies (Mohr et al. 2013; Cao et al. 2012). However I have not investigated yet the **physiology** of those different genotypes. Results thus suggest that traits explaining those differences in sensitivity may be more physiological, such as detoxification enzyme production, antioxidant balance and chelators (Pascal-Lorber et al. 2004; Yadav 2010). Those mechanisms are directly dependent on the gene pool of individuals, therefore changes in DNA may confer inherent sensitivity or resistance, and transcriptomic approaches might answer the question as well (Saminathan et al. 2015; Wu et al. 2017). Further studies, described in the perspectives, will be performed to complete the current dataset.

3. Implication of phenotypic plasticity in the response of aquatic plants to chemical stress

If it has been demonstrated that genotypic variability plays an important part in species adaptation to new environments, numerous studies showed the importance of phenotypic plasticity in the same context (chapter **I.4**, Bradshaw, 1965; Sultan, 1995; Pigliucci, 2005).

However, the influence of environmental fluctuations in the sensitivity of organisms exposed to contamination remains poorly studied in aquatic plants (Leblebici and Aksoy 2011; Nuttens and Gross 2017), and no study directly connect phenotypic plasticity and response to chemicals. In that context,

I focused on the duckweed *Lemna minor* to study how phenotypic plasticity may impact its sensitivity to Cu. Indeed, previous results shown in chapter **III** demonstrate a low variability among replicates for this species, whose ease of multiplication in lab conditions is also favorable to its use in experimental designs crossing several factors. This species is therefore an ideal model to highlight response patterns to combined effects of environmental fluctuations and chemical stress.

The results demonstrated that environmental changes highly impact Cu sensitivity of *L. minor*, inducing in some cases a multiple stress, resulting from the combination of both environmental stress (such as nutrient or light limitation) and chemical stress. The decrease of light intensity or nutrient concentration does not appear to induce visible damage to the plant, but has a direct impact on its growth, which is strongly reduced due to the lack of resources. Low light intensity reduced plant growth by more than 50 % compared to high light treatment, but the cumulative effect of both low light and Cu stress were only visible at high Cu concentration (0.25 mg/L), where almost no growth was observed. Nutrient variation was less impacting for *L. minor* growth, with only slight differences in growth among nutrient treatments in absence of Cu contamination. Furthermore, the only cumulative effect was observed with plants under low nutrient concentrations during Cu exposure at 0.25 mg/L, with plants being up to twice more sensitive to Cu than plants under rich nutrient concentrations.

Furthermore, the changing environmental conditions (*i.e.* favorable to unfavorable, and vice versa) appears to impact plant growth, while the maximal quantum yield of PSII is not strongly impacted. This appears to depend on the tested chemical, as other studies have shown that PSII was more sensitive than growth parameters (Geoffroy et al. 2004; Park et al. 2017). Copper exposure had a higher toxicity on individuals actively growing, thus on individuals in favorable environment with unlimited resources for their growth (such as high light intensity, or rich concentration in nutrients). Indeed, individuals growing faster may be more exposed to chemicals, as they will take up nutrients in the medium to produce organic matter. Those results underlined at an intraspecific level what had already been described in aquatic plants at an interspecific level, *i.e.* species with higher growth rates being more sensitive to chemical contaminants (Cedergreen et al. 2004; Coutris et al. 2011). Copper pre-exposure had a

deleterious effect on growth, although no difference was observed between pre-exposed plants and not pre-exposed plants at high Cu concentration (0.2 mg/L), suggesting that this concentration was too high and overwhelmed antioxidant balance. However, pre-exposure significantly weakened the plants subsequently exposed to a low Cu concentration (0.015 mg/L), increasing Cu impact compared to the plants not pre-exposed. This finding address the question of progressive weakening of organisms in a context of chronic exposure, and on their ability to cope with a future stress.

Growth-related endpoints and photosystem status were used as stress biomarkers, as they were considered more relevant indicators of *L. minor* fitness, compared to physiological endpoints such as antioxidant balance (Razinger et al. 2007). However, environmental fluctuations may have direct effects on plant physiology, which would be visible through *e.g.* its carbon content, proline concentration and pigment composition (J. Wu et al. 2017; Hayat et al. 2012; Brewer 2011). Those changes may also be the result of plasticity, however such variations in the chemical composition of the plant would not be enough by themselves to highlight a change in plant fitness.

Those results highlight the importance to study how environmental fluctuations can inflect at short term the ability of individuals to cope with a chemical stress, such as an exposure to trace elements or pesticides.

4. Implications for ecotoxicological risk assessment

Several implications can be drawn from the presented results. The first, is that intraspecific variation can be **compared** with interspecific variation in terms of importance, depending on the species considered. This could therefore have an impact on SSD approaches, depending on the species used in laboratory testing, and great care should be taken during data extrapolation. Those results are consistent with other studies in terrestrial ecosystems which showed that intraspecific variability in plant life-trait can sometimes be as high as interspecific variability, although it was not in presence of chemical contamination (Jiang et al. 2016; Kichenin et al. 2013; Cécile Hélène Albert et al. 2010; Bastias et al. 2017).

Secondly, the mechanisms underneath intraspecific variations appear to be **species-dependent**; although not enough genotypes were studied to draw definitive conclusions, it appears that phenotypic plasticity may be the main mechanism under intraspecific variations of

L. minor, whereas genotypic variability appears to have a strong influence on *M. spicatum* variations. Although intraspecific variation mechanisms were not investigated on *C. demersum*, it appears to demonstrate more genotypic variability than the duckweed, and thus may be closer from *M. spicatum*. This could be correlated with their life form, which is more related than with the duckweed. Indeed, several studies have highlighted that life forms of aquatic macrophytes significantly influenced their sensitivity to environmental factors (Schneider et al. 2018).

Thirdly, results showed that **endpoints** could be highly variable in their sensitivity to chemicals, and even growth related endpoints can vary from each other, depending on the parameter they are based on, such as biomass production, frond number or shoot elongation (Bergtold and Dohmeny 2011; Horemans et al. 2016). Literature and databases do not always specify which endpoint is considered for calculations of EC_{50} values based on growth, and this could inflect meta-analysis if growth parameters do not respond the same way, as it was pointed out in previous chapters.

Finally, to enhance the **reliability** of ecotoxicological risk assessment, it would be useful to determine how model species used in laboratory assays, and/or keystone species, respond to chemical stress under the influence of environmental changes at both short and long term. The variation in sensitivity to chemicals could be then modeled, and taken into account during the calculation of benchmark concentrations. It could contribute to increase the realism of such approaches, thus facilitate their transposition *in situ*. It could also allow a better prediction of toxic effects of given molecules on specific biological compartments or life-history traits.

5. Limitations

In all scientific research, no experimental design nor experiment can be perfectly controlled, and some limitations are encountered. During my PhD, I have faced several shortcomings that have to be acknowledged in order to interpret my results as best as possible.

Firstly, the choice of the **model contaminant**. As described in Chapter I, Cu is particularly environmentally relevant. It is an essential trace element which already has metabolic pathways. Numerous chelators and transporters exist, and likely vary from one individual to another, thus increasing the inter-individual heterogeneity in the endpoints measured. Cu speciation is well known to be directly dependent of **pH**, **nutrient concentrations** and dissolved organic

compounds (**DOC**). Indeed, a decreased pH increases the solubility of Cu and thus its bioavailability, while nutrients and DOC interact with it, and thus reduce its bioavailability. pH in the exposure medium will evolve depending on plant photosynthesis, and plant biomass in each experimental unit will influence both pH and DOC. As pH was not measured for all experiments (Chapters III and V), it is not possible to evaluate which fraction of Cu was bioavailable for the plants, and thus to relate it to Cu phytotoxicity. Furthermore, **different media** with different nutrient concentrations were used for the different species in chapter III, and some plant species may produce more DOC than others, due to allelopathy which could further modify Cu speciation. The easiest way to assess which part of Cu in the media was really interacting with the plant, and thus impacting it, is to measure Cu uptake in the plant. This allows to measure, regardless of the factors quoted above, the fraction that caused the effects. Due to the cost, it was only realized for chapter IV and the 7 genotypes of *M. spicatum*.

Secondly, our experiments revealed some variations among replicates, the importance of which depending on the species considered. *Lemna minor* did not demonstrate much variation, whereas *Ceratophyllum demersum* and *Myriophyllum spicatum* exhibited higher variations. It is consistent with the fact that those two species have more complex growth forms than *Lemna minor*, which has a very fast clonal reproduction and a simplified morphology. One way to cope with the high variation would be to increase the number of replicates to **improve the accuracy of statistical estimates**. Indeed, in OECD protocols for *M. spicatum*, at least 4 replicates with 16 shoots in total (4 shoots per experimental unit, 4 experimental units) for control plants and 3 replicates with 12 shoots in total for each chemical concentration are used. This experimental design allows to cope with high variations among replicates and within concentrations, but requires a lot of biomass for a single experiment with a single species to be tested. In our situation, it was not possible to implement, either due to the number of combinations species × genotype (Chapter III) or the number of genotypes (Chapter IV).

CONCLUSION & PERSPECTIVES

This PhD work aimed to build a bridge between ecology and ecotoxicology, in order to provide more realistic tools for ecotoxicological risk assessment and new insights on population biology and ecosystem functioning in a context of environmental pollution. I demonstrated that intraspecific variation can play a significant role in species response to pollutants, and therefore in ecosystem resilience to anthropogenic disturbances (Wolf et al. 2018; Reusch et al. 2005).

The results showed that mechanisms underneath intraspecific variability, *i.e.* genotypic variability and phenotypic plasticity, are species-dependent and can play a role in species adjustment to environmental pressures. Indeed, I demonstrated that phenotypic plasticity has an important part in *Lemna minor* acclimatization to environmental changes, and can significantly inflect its sensitivity to chemicals. Furthermore, results demonstrated that *Myriophyllum spicatum* exhibits a high genotypic variability which modulates its response to copper, and that this variability depends on the observed endpoint. This highlights the need to properly understand the mechanisms underneath genotype variations. Several studies have underlined that **intraspecific variation** plays an important role in ecosystem resilience facing climate change, especially via experiments on diatoms, which are a very good model to investigate community assembly rules (Kremp et al. 2012; Sjöqvist and Kremp 2016; Zuo et al. 2017; Esteves et al. 2017; Wolf et al. 2018). It would therefore be relevant to study intraspecific variability and its mechanisms in a context of acclimatization and adaptation to environmental pollution, through the study of different life forms sensitivity, harvested in contrasted sites. Furthermore, proper assessment of intraspecific variability on model species used in risk assessment should allow to integrate this variability in current approaches, such as SSD, making those more realistic and representative of natural environments (Del Signore et al. 2016).

Further experiments will be performed to assess **mechanisms** underneath intraspecific variability of *Myriophyllum spicatum*, more specifically genotypic variability, across physiological traits. Those experiments would investigate antioxidant balance, photosynthesis efficiency, and biochemical composition including pigment composition, as these parameters can vary among genotypes and potentially provide advantages to cope with stressors. A transcriptomic approach would also be developed. Indeed, metabolic pathways and chemical composition are driven by the genetic code of individuals, thus study the transcripome would therefore be highly relevant to investigate the source of the variations observed. It would allow us to take the measure of differences in transcription for a broad range of metabolic pathways, especially antioxidant balance, and thus to better understand the consequences of genotypic variation. For this purpose, two genotypes with contrasting responses to Cu would be

considered in the transcriptomic study. This should provide insights, along with the genome sequencing, on genetic differences that can be found between a resistant and a sensitive genotype, and on the importance of epigenetic as a response mechanism to abiotic stressors.

One of the next steps would be to assess intraspecific variation among different populations of *Myriophyllum spicatum* across a **contamination gradient**, with harvesting sites highly contaminated and other pristine. This would allow to see if resistance or sensitivity patterns can be correlated with chemical contamination of the harvesting site, and with life-history traits of individuals within the different populations. It would also be very interesting to look across an **environmental gradient**, with for instance contrasting environmental conditions (regardless of the possible contamination) from one harvesting site to another, involving different selection pressures (such as water flow). Indeed, several studies have highlighted genetic patterns among geographically distinct populations. This approach should allow to understand and predict more efficiently the sensitivity and future behavior of those species depending on environmental constrains, whether they are climatic and/or chemical. It could also give insights on resistance mechanisms developed by some species, whether it is due to plastic traits that became fixed, or if some specific traits provide competitive or coping advantages in unfavorable environments.

Finally, in a context of ecotoxicological risk assessment, it would be relevant to study how **environmental fluctuations** may inflect species sensitivity to chemicals. We demonstrated that *Lemna minor* is a plastic organism and that environmental fluctuations inflect its sensitivity to copper. It would be relevant to go further into the study of the extent of phenotypic plasticity in aquatic plants and other organisms, and its potential impact on species sensitivity to contamination. For instance, several studies have demonstrated that *Myriophyllum spicatum* shows phenotypic plasticity, however no study have looked into implications for its sensitivity to chemicals (Cao, et al. 2012; Sri et al. 2013). It is therefore important to properly assess phenotypic plasticity, and determine if it is a parameter of concern in risk assessment approaches. If so, it could be integrated in the assessment factors that are used to derive benchmark values, and would allow a more integrative approach, that accounts for those uncertainties.

CONCLUSION & PERSPECTIVES
(Français)

Ce travail de thèse a eu pour but de réaliser un pont entre l'écologie et l'écotoxicologie, afin de fournir des outils plus réalistes pour les approches d'évaluation des risques écotoxicologiques, et de nouvelles connaissances sur le fonctionnement des écosystèmes dans un contexte de pollution environnementale. J'ai démontré que la variabilité intraspécifique peut jouer un rôle important dans la réponse des espèces aux polluants, et donc dans la résilience des écosystèmes exposés aux rejets anthropiques (Wolf et al; 2018; Reusch et al. 2005).

Les résultats ont montré que les mécanismes sous-jacent de la variabilité intraspécifique, *i.e.* la variabilité génotypique et la plasticité phénotypique, est espèce-dépendante et peut influencer l'ajustement des espèces aux pressions environnementales. En effet, les résultats ont démontré que la plasticité phénotypique joue un rôle important dans l'ajustement de la lentille d'eau aux changements environnementaux, et peut impacter de façon significative sa sensibilité aux substances chimiques. De plus, j'ai démontré que *Myriophyllum spicatum* possède une forte variabilité génotypique qui module sa réponse au cuivre, et que cette variabilité dépend du paramètre observé. Cela surligne le besoin de comprendre les mécanismes sous-jacent de la variabilité génotypique. Plusieurs études ont souligné que la **variation intraspécifique** joue un rôle important dans la résilience des écosystèmes exposés au changement climatique, surtout au travers d'expériences menées sur les diatomées, qui sont un très bon modèle pour étudier l'assemblage des communautés (Kremp et al. 2012; Sjöqvist and Kremp 2016; Zuo et al. 2017; Esteves et al. 2017; Wolf et al. 2018). Il serait donc pertinent d'étudier la variabilité intraspécifique et ses mécanismes dans un contexte d'acclimatation et d'adaptation à la pollution environnementale. De plus, l'évaluation de la variabilité intraspécifique chez les espèces modèles utilisées en évaluation des risques devrait permettre d'intégrer cette variabilité dans les approches actuelles, comme les SSDs, les rendant plus réalistes et représentatives des environnements naturels (Del Signore et al. 2016).

De prochaines expériences seront réalisées dans l'intention d'étudier les **mécanismes** expliquant la variabilité intraspécifique chez *Myriophyllum spicatum*, plus précisément la variabilité génotypique, à travers l'étude de traits physiologiques. Elles auront pour but notamment d'investiguer l'équilibre antioxydant, l'efficacité photosynthétique et la composition biochimique, incluant la composition pigmentaire, comme ces paramètres peuvent varier entre les génotypes, et potentiellement fournir des avantages dans la résistance aux polluants. Une approche transcriptomique sera également mise en place. En effet, le métabolisme et la composition chimique sont conduits par le code génétique des individus, et l'étude du transcriptome pourrait donc être hautement pertinente pour étudier la source de ces

variations. Cette approche permettrait de mesurer la différence de transcription pour un large panel de voies métaboliques, notamment l'équilibre antioxydant, et donc améliorer notre compréhension des conséquences de la variation génotypique. Pour ce faire, deux génotypes avec des réponses contrastées au Cu seraient étudiés pour l'approche transcriptomique. Cela donnera des indices, avec le séquençage du génome, sur les différences génétiques qui peuvent être trouvées entre un génotype résistant et un génotype sensible, et sur l'importance de l'épigénétique en tant que mécanisme de réponse aux stress abiotiques.

L'une des étapes suivantes serait de déterminer l'étendue de la variation intraspécifique au travers de populations différentes de myriophylle en épis, le long d'un **gradient de contamination**, avec des sites de prélèvement très contaminés et d'autres non impactés par la contamination. Cela devrait permettre de voir si un schéma de résistance ou de sensibilité peut être corrélé à la contamination chimique du site de prélèvement, et aux traits d'histoire de vie des individus au sein des différentes populations. Il serait également très intéressant de regarder le long d'un **gradient environnemental** (indépendamment de la contamination chimique éventuelle), par exemple des conditions environnementales contrastées d'un site à l'autre, impliquant des pressions de sélection différentes (telles que le flux de l'eau). En effet, plusieurs études ont mis en évidence une structuration génétique entre des populations géographiquement distinctes. Cette approche devrait permettre de comprendre et de prédire plus efficacement la sensibilité et le comportement futur de ces espèces, en fonction des contraintes environnementales, que celles-ci soient climatiques et/ou chimiques. Cela pourrait également donner des indices quant aux mécanismes de la résistance développée par certaines espèces, si cela est dû à des traits plastiques devenant fixés, ou s'il s'agit de traits spécifiques conférant des avantages compétitifs ou de résistance dans des environnement défavorables.

Finalement, dans un contexte d'évaluation des risques écotoxicologiques, il serait pertinent d'étudier comment les **fluctuations environnementales** pourraient influer sur la sensibilité des espèces à la contamination chimique. J'ai démontré que la lentille d'eau est un organisme plastique, et que les fluctuations environnementales influencent sa sensibilité au cuivre. Il serait pertinent d'aller plus loin dans l'étude de l'étendue de la plasticité phénotypique chez les plantes aquatiques et chez d'autres organismes, et de son impact potentiel dans la sensibilité des espèces à la contamination. Par exemple, plusieurs études ont démontré que le myriophylle en épis est une espèce plastique, cependant aucune étude ne s'est intéressée à l'implication de cette plasticité dans la sensibilité de l'espèce (Cao et al. 2012; Sri, Atapaththu, and Asaeda 2013). Il est donc important d'étudier l'étendue de cette plasticité phénotypique, et de déterminer si ce

paramètre est important dans les approches d'évaluation des risques. Le cas échéant, cette variabilité pourrait être intégrée dans les facteurs d'évaluation qui sont utilisés pour dériver les valeurs seuils, protectrices des écosystèmes, et pourrait donc permettre une approche plus intégrative qui réduirait les incertitudes.

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Abstract

Intraspecific variability plays a pivotal role in short and long term responses of species to environmental fluctuations. This variability, expressed through different traits of individuals, can potentially influence species sensitivity to chemical contamination. This intraspecific variability is currently not taken into account in ecotoxicological risk assessment, whereas it can mislead its results. To examine this hypothesis, the importance of intraspecific variability in the response to copper (Cu) was quantified in controlled conditions for three aquatic macrophyte species, *Lemna minor*, *Myriophyllum spicatum* and *Ceratophyllum demersum*. Variations among genotypes of each of these 3 species were compared to interspecific variability. Results have highlighted a significant genotypic variability, whose importance depends on the species considered. Indeed, *L. minor* demonstrated a low variability, contrarily to *M. spicatum* whose variability in growth inhibition by Cu was higher than interspecific differences. In order to specify the extent and the mechanisms of genotypic variability in *M. spicatum*, other experiments involving measurements of life-history traits have been conducted on 7 genotypes exposed to Cu. Results showed that some genotypes were up to eightfold more sensitive to Cu than others (at concentrations ranging between 0.15 and 0.5 mg/L). These differences in sensitivity were partly explained by the traits measured, but physiological or transcriptomic endpoints may explain more precisely the source of these differences in sensitivity. Finally, 3 experiments with fluctuations in nutrient concentrations, light intensity and Cu pre-exposure have demonstrated that phenotypic plasticity plays an important role in *L. minor* sensitivity to Cu. Indeed, the weakening of individuals, as a result of unfavorable environmental conditions, can lead to a two-fold increase in sensitivity to Cu. All these results demonstrated that intraspecific variability, whether it comes from genotypic variations or is linked to phenotypic plasticity, was in general lower than interspecific variability for the species and endpoints studied. However, its extent can vary depending on the species. It can therefore significantly influence aquatic macrophyte sensitivity to chemical contamination, and it would be relevant to account for it in ecotoxicological risk assessment.

Keywords: Copper, ecotoxicological risk assessment, aquatic macrophyte, intraspecific variability, genotypic variation, phenotypic plasticity

Résumé

La variabilité intraspécifique fait partie intégrante de la réponse à court et à long terme des organismes vivants aux fluctuations environnementales. Cette variabilité, exprimée au travers de différents traits des individus, peut potentiellement influencer la sensibilité des espèces à une contamination chimique. La variabilité intraspécifique n'est pas, à l'heure actuelle, prise en compte en évaluation des risques écotoxicologiques, alors même qu'elle pourrait en biaiser les résultats. Pour examiner cette hypothèse, l'importance de la variabilité intraspécifique dans la réponse au cuivre (Cu) a été quantifiée en conditions contrôlées pour trois espèces de macrophytes aquatiques, *Lemna minor*, *Myriophyllum spicatum* et *Ceratophyllum demersum*. Les variations entre génotypes de chacune de ces 3 espèces ont été comparées à la variabilité interspécifique. Les résultats ont mis en évidence une variabilité génotypique significative, dont l'importance dépend de l'espèce considérée. En effet, *L. minor* a montré une faible variabilité, au contraire de *M. spicatum* dont la variabilité de l'inhibition de croissance par le Cu est supérieure aux différences interspécifiques. Afin de préciser l'étendue et les mécanismes de la variabilité génotypique chez *M. spicatum*, d'autres expériences impliquant des mesures de traits d'histoire de vie ont été réalisées sur 7 génotypes exposés au Cu. Les résultats ont montré que certains génotypes étaient jusqu'à 8 fois plus sensibles au Cu à des concentrations allant de 0.15 à 0.5 mg/L). Ces différences de sensibilité sont en partie expliquées par les traits mesurés, mais des mesures physiologiques et/ou des approches en transcriptomique devraient pouvoir expliquer de façon plus consistante la source de ces différences de sensibilité. Enfin, 3 expériences faisant varier respectivement la teneur en nutriments, l'intensité lumineuse et la préexposition au Cu, ont démontré que la plasticité phénotypique joue un rôle majeur dans la sensibilité au Cu chez *L. minor*. En effet, l'affaiblissement des individus, résultant des conditions environnementales défavorables, peut conduire au doublement de la sensibilité de *L. minor* au Cu. L'ensemble des résultats obtenus montre donc que la variabilité intraspécifique, qu'elle soit d'origine génotypique ou liée à la plasticité phénotypique, demeure en règle générale inférieure à la variabilité interspécifique concernant les traits et les espèces étudiés. Cependant, son importance varie selon l'espèce considérée. Elle peut donc influer significativement sur la sensibilité des macrophytes aquatiques à la contamination chimique, et gagnerait donc à être prise en compte dans le cadre de l'évaluation des risques écotoxicologiques.

Mots clés : Cuivre, évaluation des risques écotoxicologiques, macrophyte aquatique, variabilité intraspécifique, variation génotypique, plasticité phénotypique