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Root biomass production in populations of six rooted macrophytes in response to Cu exposure: Intra-specific variability versus constitutive-like tolerance

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Abstract:

Intra-specific variability of root biomass production (RP) of six rooted macrophytes, i.e. *Juncus effusus*, *Phragmites australis*, *Schoenoplectus lacustris*, *Typha latifolia*, *Phalaris arundinacea*, and *Iris pseudacorus* grown from clones, in response to Cu exposure was investigated. Root biomass production varied widely for all these macrophytes in control conditions (0.08 µM) according to the sampling site. Root biomass production of *T. latifolia* and *I. pseudacorus* in the 2.5–25 µM Cu range depended on the sampling location but not on the Cu dose in the growth medium. For *P. australis*, *J. effusus*, *S. lacustris*, and *P. arundinacea*, an intra-specific variability of RP depending on both the sampling location and the Cu-dose was evidenced. This intra-specific variability of RP depending on the sampling location and of Cu-tolerance for these last four species suggests that Cu constitutive tolerance for all rooted macrophytes is not a species-wide trait but it exhibits variability for some species.

Highlights

► Root biomass production varied with the sampling site for six macrophyte species at low Cu exposure. ► Root production of *T. latifolia* and *I. pseudacorus* in the 2.5–25 µM Cu range only depended on the sampling site. ► Other macrophytes displayed an intra-specific variability of root production across the Cu gradient.

Abbreviations

- AMD, Acid Mine Drainage;
- CCA, Chromated Copper Arsenate;
- CW, Constructed wetland;
- HNS, Hoagland Nutrient Solution;
- MetE, methyltetrahydropteroyltriglutamatehomocysteine methyltransferase;
- MT, Metallothionein;
- PC, Phytochelatin;
- RB, Retarding Basin;
- ROL, Radial oxygen loss;
- ROS, Reactive Oxygen Species;
- SAMS, S-adenosylmethionine synthase;
- SOD, Superoxide dismutase;
- TE, Trace Element;
- TIM, Triosephosphate isomerase cytosolic;
- WTP, Wastewater Treatment Plant

Keywords: Copper tolerance ; Phytoremediation ; Root biomass ; Trace element ; Wetland

1.   Introduction

Aquatic ecosystems are used, directly and indirectly, as recipients of potentially toxic effluents and wastes from domestic, agricultural and industrial activities (Demirezen et al., 2007; Peng et al., 2008). Copper is one of the Trace Elements (TE) potentially toxic in excess, which may migrate in dissolved and solid forms from urban areas and (agro)ecosystems to surface waters, groundwater and wetland substrates, and its excess may accumulate in living organisms (Kamal et al., 2004; van der Ent et al., 2013). Copper acts as a cofactor in many processes in plants, e.g. respiration, photosynthesis, scavenging of oxidative stress, perception of ethylene, nitrogen metabolism, molybdenum cofactor synthesis, cell wall remodeling, and response to pathogens (Palmer and Guerinot, 2009; Jung et al. 2012). One abundant protein associated with Cu in the plant cell is the plastocyanin responsible for the electron transfer from the cytochrom $b6f$ complex to the photosystem I (PSI) (Yamasaki et al., 2009; Burkhead et al., 2009). However, at concentrations higher than the cellular Cu homeostasis (5-20 µg Cu g^{-1} DW), Cu induces phytotoxicity symptoms (e.g. biomass reduction, root growth inhibition, bronzing, chlorosis, reduced Fe, Zn and P uptake, chloroplast integrity loss, etc.) (Kopittkke et al., 2010; Marschner, 2011). Excessive free Cu ions can induce the formation of Reactive Oxygen Species (ROS) such as superoxides (O_2^-), hydroxyl radicals (HO•) and hydrogen peroxide (H_2O_2) through Fenton and Haber-Weiss reactions, which can peroxidize lipids and oxidize proteins and guanine (Drazkiewicz et al., 2004; Sharma and Dietz, 2009, Kanoun-Boulé et al., 2009). Detailed molecular mechanisms involved in Cu homeostasis in plants have been reviewed elsewhere (Yruela 2009; Jung et al. 2012).

Natural 'volunteer' wetlands, in particular those associated with mining activities, can improve soil and water quality notably by trapping TE in the rhizosphere (Beining and Otte, 1996; Narhi et al., 2012). Constructed wetlands (CW) have also been used to enhance the quality of contaminated waters for at least two decades (Marchand et al., 2010, Lizama et al., 2011). Rooted macrophytes are key players in both natural and constructed wetlands through radial oxygen loss (ROL) and organic matter production which provide habitats for microorganisms (Cheng et al., 2009; Marchand et al., 2010). Such macrophytes mainly accumulate TE in roots, because of their fibrous system with large contact areas, rhizome tissues (Cardwell et al., 2002; Bonnano and Lo Giudice, 2010; Romero Núñez et al., 2011), and to a lesser extent in stems and leaves (Clemens, 2002; Baldantoni et al., 2004; Bragato et

al., 2006). Root anatomy, through lignin and suberin deposition, sclerenchymatous fibers with thick secondary walls and densely packed cells in the outer layers of cortex, contribute to a tight barrier to ROL in the macrophyte roots and may confer an exclusion ability of TE in such wetland species (Deng et al., 2009). The formation of Fe plaque deposits in the vicinity of wetland plant roots may also strengthen metal accumulation in the rhizosphere (McCabe et al., 2001; Otte et al., 2004). Thus, rooted macrophytes may have been exposed to higher Cu concentrations than most dryland plants over their evolution. Kissoon et al. (2010) reported for example greater Cu concentrations under wetland compared to dryland conditions in the whole plant for *Rumex crispus*. Consequently it is commonly admitted that selection may have shaped a constitutive TE tolerance for macrophytes (Ye et al., 1997a,b, 2003; McCabe et al., 2001; Matthews et al., 2004a,b; 2005; Kissoon et al., 2010), even if for some species such as Phalaris arundinacea such constitutive tolerance was not evidenced (Matthews et al., 2005).

In drylands, a small percentage of plants have the innate capability to develop metal-tolerant populations at TE-contaminated sites (Verkleij et al., 2009; Memon and Schroeder, 2009; Verbruggen et al., 2009). It is argued that when a species establishes on a soil with a too high TE supply, adjustments will take place within the limits of phenotypic plasticity followed by adaptation and evolution of efficiency or tolerance in populations over time (Schat 1999; Pollard et al., 2002; Ernst, 2006; van der Ent et al., 2013). For instance, in some dryland plants, such genetic adaptation may generate distinct populations. Similarly, the presence of rooted macrophytes at TE-contaminated sites has already raised the question of whether these plants may evolve TE tolerance like dryland plants (Deng et al., 2006). In other words, is the TE constitutive tolerance for all rooted macrophytes a species-wide trait or does it still exhibit variability for some species and trace elements? Investigations are needed to provide new insights into choosing plant material in CW, since root biomass in CW determines the system efficiency and promotes its long-term functioning (Marchand et al., 2010). Knowledge is currently lacking on the intra-specific variability of macrophytes in response to TE exposure in wetland communities (Brisson and Chazarenc, 2009; Marchand et al., 2010). Moreover, as suggested by Deng et al. (2006), a full and correct understanding of the nature of TE tolerance in wetland plants should be based on studies of a wide range of populations. In some studies, these populations originate from locations within relatively close proximity to each other, and so could have originated from the same TE-tolerant ancestors (Matthews et al. (2004a). Last, but not least, work must be carried out not only on a single species across a TE gradient but

145 on several macrophyte species simultaneously to really assess the site effect at field scale

146 since each species may react differently when exposed to high TE concentrations.

147

148 Therefore, we investigated the intraspecific variability of root production of macrophyte

149 populations belonging to six species including *Juncus effusus* L., *Phragmites australis* (Cav.)

150 Trin.ex Steud., *Phalaris arundinacea* L., *Typha latifolia* L., *Iris pseudacorus* L., and

151 *Schoenoplectus lacustris* L. These macrophytes were grown under glasshouse conditions in a

152 perlite imbibed growth medium and spiked with increasing Cu concentrations (from 0.08 to

153 25 µM). Macrophyte clones used were sampled at both metal-contaminated and

154 uncontaminated sites in France, Portugal, Italy, Belarus, Spain, and Australia, selected to

155 establish a gradient of Cu contamination in wetlands separated by a large distance, with

156 contrasting edaphic conditions. Selected sites allowed establishing a natural Cu contamination

157 gradient of sampled wetlands separated by a large distance, and with contrasting edaphic

158 conditions. Macrophytes were chosen (1) based on similar studies (i.e. Taylor and Crowder,

159 1984; Ye et al., 2003; Matthews et al., 2004b, 2005; Bonanno and Lo Giudice, 2010) and (2)

160 for their presence at the sampling sites. Altogether, a large number of sampled sites and

161 macrophyte populations allowed assessing whether the TE (Cu)-constitutive tolerance of

162 rooted macrophytes is a species wide trait or if it exhibits variability for some species. As

163 roots are the first plant part exposed to contaminated soils and waters and sensitive to excess

164 Cu, root growth measurement can help to compare plant capacity to withstand Cu

165 contamination and toxicity (Lequeux *et al.* 2010). Consequently it can aid to assess tolerance

166 level and intraspecific variability of rooted macrophyte populations exposed to increasing Cu

167 concentrations. Root growth was considered here in the context of the trait-based approach

168 reflecting resistance and tolerance mechanisms to Cu exposure. The integrative, trait-based

169 option is a relevant tool to understand how organisms face fast changing environmental

170 conditions (Berg and Ellers, 2010).

171

172 **2. Materials and methods**

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174 *2.1. Sites*

6

175

Fourteen wetland sites were investigated between 2009 and 2012 (**Fig. 1, Table 1, Table. S3**). Sampling sites were located in both the Northern hemisphere (i.e. seven in France, one in Spain, one in Portugal, one in Italy, and one in Belarus) and the Southern hemisphere (three in Australia). These sites had wide TE concentration ranges in soils, with total soil Cu (in mg kg^{-1} DW) varying from 2.9 (Kozlovichi, Belarus) to 1750 (Ribeira de Agua Forte, Portugal) and pH ranging from 3 (Gwelup Lake, Australia) to 7.4 (Lagnet, France and Fenice Capanne, Italy) (**Table 1, Fig. S2**). High soil TE concentrations resulted from either the soil geochemical background or industrial and agricultural effluents.

Sites located in France included La Cornubia, Cordon d'Or, Lagnet, Basilique, Jalles d'Eysines, Le Palais sur Vienne and Sanguinet Lake (**Table 1**). The La Cornubia site (Gironde, France) is a CW collecting effluents and runoff from a former Cu sulphate plant, in use for over a century (Basol, 2012). The Cordon d'Or site (Gironde, France) is a natural wetland receiving runoff from an adjacent former wood preservation site, in operation for over a century, where creosote, Cu sulphate, chromated copper arsenate (CCA), and Cu hydroxycarbonates with benzylalkonium chlorides have been successively used (Mench and Bes, 2009; Marchand et al., 2011). The Lagnet and Basilique sites (Gironde, France) are both draining ditches located in the vineyards of Saint-Emilion (Gironde, France), annually treated with Cu sulphate. The Jalle d'Eysines River flows into the Garonne next to Bordeaux (Gironde, France) and receives both contaminated runoffs from industrial, agricultural and residential areas and effluents from two major municipal wastewater treatment plants (WTP) of the Bordeaux suburbs, serving more than 100,000 inhabitants. At Le Palais sur Vienne (Haute-Vienne, France), macrophytes were sampled on the riverbanks of Le Palais creek, a tributary of the Vienne River near Limoges, downstream from a former Cu electro refinery whose runoffs and discharges have occasionally contaminated the river. The riverbank sandy soil of the uncontaminated Sanguinet Lake (Landes, France) has an acid soil pH and its soil TE concentrations are generally lower than the background levels defined by Blum *et al*. (2012).

Non-French sites included Avoca, Gwelup, Argus Street, Fenice Capanne, Touro, Ribeira de Agua Forte and Kozlovichi (**Table 1**). The Avoca and Argus Street sites (Victoria, Australia) are both retarding basins (RB) built by Melbourne Water where stormwater runoff from a drainage catchment is temporarily stored. Due to high urban pressure, these RB are TE-

contaminated (Bourgues et al., 2004; Marshall, 2004). The polymetallic (Zn, Cu, Pb, Fe, and Ag) sulfide deposit of Fenice Capanne (Massa Marittima, Italy) was mined for 25 centuries up until 1985 and the alteration of mine waste materials has generated pollution in superficial waters and sediments (Mascaro et al., 2001). The Touro site (Galicia, NW Spain) is an abandoned opencast mine under restoration, whose tailings mainly consist of oxidized materials such as amphibolites, chalcopyrite, limonite, garnet and mainly Fe and Cu sulfides (Vega et al., 2004; Asencio et al., 2013a, b). Here samples were collected from a CW controlling acid mine drainage (AMD). The Ribeira de Agua Forte (Beja, Portugal) is a tributary stream to the Roxo stream, which receives AMD from the Aljustrel mine, a polymetallic (As, Zn, Cu, and Pb) sulfide deposit of the Iberian pyrite belt (Alvarenga et al., 2008; Candeias et al., 2011). At Gwelup Lake, Australia, high TE concentrations, mainly for As and Zn, result from soil geochemical background. The lake bottom is constituted by mono-sulfidic black ooze (MBO), a concentrated organically derived iron sulfide containing material. In summer, when it dries or saturation is reduced, this produces highly acid conditions (acid sulfate material). Kozlovichi (Belarus) is an uncontaminated wetland, located next a former beer production plant. Physico-chemical parameters and TE concentrations of soils at the Avoca, Gwelup, Argus Street, Fenice Capanne, Touro, and Ribeira de Agua Forte sites were previously reported (**Table 1**). For other sites, three soil samples (0.5 kg fresh weight, FW) were collected with an unpainted steel spade from the 0-25 cm soil layer. Samples were air-dried in the laboratory and sieved (5 mm, nylon mesh) prior to analysis. Total element concentrations and physico-chemical parameters were determined on air-dried soil at the INRA Laboratoire d'Analyses des Sols (LAS, Arras, France) using standard methods (INRA LAS, 2007; **Table 1**).

2.2 Plant sampling, clonal reproduction, and plant exposure to Cu

Emergent and rooted monocot macrophytes, i.e. *P. australis, P. arundinacea, T. latifolia, J. effusus, S. lacustris* and *I. pseudacorus* were mainly collected at the beginning of the growing season in 2009, 2010 and 2011. A few sites were also sampled at the end of the growing season (**Table S3**). These six monocot species were selected because they were the most representative of the sampled wetlands. At each of the fourteen sampling location, 20-30 samples of rhizomes and/or bud-bearing stems were collected in a 1 m² area. In such restricted area rhizomes were supposed to belong to the same clone, as macrophytes tend to

239   spread clonally over short distances (Piquot et al., 1998; Hazelton et al., 2014). A total of 29

240   populations (5 *J. effusus*, 3 *S. lacustris*, 6 *P. arundinacea*, 5 *I. pseudacorus*, 7 *P. australis* and

241   3 *T. latifolia*) were collected. Each population consisted of one clone. Depending on sampled

242   species, populations and sites, plant samples were transported in buckets and as soon as

243   possible placed in water in a greenhouse at the Centre INRA-Bordeaux Aquitaine, Villenave

244   d'Ornon, France. Shortly after collection, rhizomes and/or bud-bearing stems were cut into

245   small pieces (10-20 cm). They were then grown in separate polyethylene containers (volume:

246   60x40x15 cm^3) containing perlite imbibed with a quarter-strength Hoagland nutrient solution

247   (HNS, Hoagland and Arnon, 1950): KNO_3 (1.62 mM), $Ca(NO_3)_2$ (0.69 mM), $NH_4H_2PO_4$

248   (0.25 mM), $MgSO_4$ (0.5 mM), H_3BO_3 (11.53 μM), $MnCl_2$ (2.29 μM), $CuSO_4.5H_2O$ (0.08

249   μM), $(NH_4)6Mo_7O_{24}$ (0.13 μM), $ZnSO_4.7H_2O$ (0.19 μM) and $FeSO_4$ (48.6 μM). Water

250   volume was maintained constant by adding tap water. Water was renewed and nutrients were

251   added every month during the growing season and every two months during winter to avoid

252   anoxia and nutrient depletion in the growth medium. After 6-10 months, in late winter, 24

253   standardized tillers (with equal stem and root size or volume) of each population were isolated

254   from the sprouting rhizomes. These were grown in a new culture medium (imbibed perlite

255   with HNS as described above) in 9x8x9 cm^3 pots for 6-8 weeks, and thereafter, 20 individual

256   plants were selected (with equal stem and root size or volume). At the test beginning (**Table

257   S3**), their roots were stained with activated plant coal (concentration: 1.5 %) according to

258   Schat and Ten Bookum (1992). Thereafter, individuals were transferred into plastic containers

259   (1L) filled with 500 mL of a quarter-strength HNS prepared from ultra-pure water (MilliQ

260   sytem) (Hoagland and Arnon, 1950), and perlite (50 g). The growth medium was spiked with

261   Cu ($CuSO_4.5H_2O$) to achieve five treatments (four replicates treatment^{-1}): 0.08, 2.5, 5, 15, and

262   25 μM Cu (**Table 2**). All plants were randomly placed on a bench in the same greenhouse

263   (day (9-21h) 1911±1232 μM photons m^{-2}s^{-1}, 28±5°C, night (21h-9h) 19±3°C). Nutrient

264   solutions were changed every five days to maintain Cu concentrations and avoid depletion of

265   oxygen and nutrients. According to Kopittke et al. (2010), redox-potential (Eh, WTW

266   Multiline P4 meter, Germany), pH (Hanna instruments, pH 210, combined electrode

267   Ag/AgCl, USA) and Cu^{2+} concentrations (Cupric ion electrode, Fischer Bioblock, USA) were

268   measured in the solution imbibing perlite just after changing the solution and after a 5-day

269   exposure (n=6 for each concentration of the Cu gradient, **Table 2**). No pH adjustment was

270   necessary since it remained roughly constant within each 5-day growth period (**Table 2**). The

271   speciation of metal elements in the bulk solutions was determined using the speciation

272   program MINEQL+4.6 (**Table S4**). Such calculations are based on the total concentration of

273 anions and cations initially present in the culture medium, accounting for the formation

274 constants of the complexes which may be formed at thermodynamic equilibrium. The

275 standard databases included with the software were applied (Schecher and McAvoy, 2003).

276

277 Plants were harvested after a 3-week exposure to Cu. Roots were thoroughly washed with

278 running tap water to remove perlite particles and blotted with a paper towel. White roots, i.e.

279 root biomass formed during Cu exposure, were isolated from coal(black)-stained roots and

280 their FW yield was determined. Roots were oven-dried at 60°C for 48h and then weighed for

281 DW determination.

282

283 *2.3 Statistical analysis*

284 Physico-chemical parameters in the growth medium of the distinct Cu levels were analyzed

285 just after changing the solutions (T0) and after a 5-day exposure (T5). For each parameter and

286 at a given Cu exposure differences between (T0) and (T5) were analyzed using a Student's t-

287 test (**Table 2**). Differences were also analyzed for each parameter across the Cu range (0.08-

288 25 μM Cu) at respectively T0 and T5 using a one-way ANOVA. Root DW yield depending

289 on both Cu concentration in the growth medium (2.5-25 μM) and the sampling site were

290 analyzed for each plant species using an ANCOVA (**Table 4, Fig. 2**). When conditions of

291 homoscedasticity and normality were met, post-hoc Tukey HSD tests were then performed to

292 assess multi-comparison of mean values (**Table 2**). Effect of the sampling site location on

293 root DW biomass production for each plant species in uncontaminated conditions (0.08 μM

294 Cu) was analyzed using a Kruskal-Wallis test (**Table 3**) and a Pairwise Wilcoxon sum ranks

295 test (**S1**). Finally, for each species and within populations, differences in root production

296 between each Cu dose (2.5-25 μM Cu) and the uncontaminated modality (0.08 μM Cu) were

297 assessed using a Pairwise Wilcoxon sum ranks test (**Fig. S1**). All analyses were carried out

298 using R software (version 2.14.1 R foundation for Statistical Computing, Vienna, Austria).

299

300 **3. Results**

301

302 *3.1 Copper concentrations and physico-chemical characteristics of the growth medium*

303

Data at T0: pH and EC at 20 °C did not significantly change across the Cu gradient ($p>0.05$).
Free Cu^{2+} concentrations significantly increased ($p<0.05$) across the Cu gradient (**Table 2**). At
0.08 μM Cu added in the bulk solution, based on speciation calculations, Cu was also present
under both $CuOH^+$ and $CuSO_4$ forms (**Table S4**). Based on the first calculation at 25 μM, Cu
was expected to precipitate as $CuFe_2O_4$. However, such Cu-speciation was unrealistic
according to Tu et al. (2012). After exclusion of the $CuFe_2O_4$ form of the modelling, Cu
would be mainly precipitated as Tenorite (CuO) (**Table S4**).

Data at T5: free Cu^{2+} concentrations were similar across the Cu gradient ($p>0.05$). They did
not significantly vary between T0 and T5 across the 0.08-5 μM Cu gradient ($p>0.05$) (**Table
2**). For 15 and 25 μM Cu, free Cu^{2+} decreased between T0 and T5 ($p<0.05$). Decrease in free
Cu^{2+} ions was relatively higher at high Cu levels than at the low Cu level, leading to similar
values of labile Cu^{2+} pool in the growth medium across the Cu gradient at T5 (**Table 2**). pH
and EC did not significantly change across the Cu gradient. No variation occurred for pH
between T0 and T5 across the whole Cu gradient ($p>0.05$) whereas EC significantly
decreased ($p<0.05$) (**Table 2**). At 0.08 μM Cu, speciation calculations indicated again the
presence of both $CuOH^+$ and $CuSO_4$ forms (**Table S4**). At 25 μM Cu, and after exclusion of
the $CuFe_2O_4$ form in the modelling, Cu would be also mainly under the CuO form.

321

3.2 Crossed effect of sampling site and Cu exposure on root biomass production

323

The lowest mean root biomass production after a 3-week exposure at 0.08 μM Cu were
respectively 30 μg DW for *S. lacustris* and 40 μg DW for *J. effusus*. For convenience, we will
refer to root biomass production as 'RP' throughout the paper. Mean RP of *I. pseudacorus*
was 150 μg DW after three weeks, which was the highest value reported among the six
studied species, followed by *T. latifolia* (120 μg DW) and *P. arundinacea* (116 μg DW) (**Fig.
1, S1**). In the 2.5-25 μM Cu range, RP of *J. effusus* and *S. lacustris* depended on both the total
Cu concentration in the growth medium and the sampling site (**Table 4,** $p<0.001$). Interaction
between both factors was also significant ($p<0.05$). Root biomass production of *P.
arundinacea* depended on both the Cu exposure ($p<0.001$) and the sampling site location
($p<0.001$). Both factors significantly interacted at the 0.001 level (**Table 4**). For *P. australis,*

334   RP across the Cu gradient was influenced by both the sampling site location ($p<0.001$) and

335   the Cu concentration ($p<0.01$). Interaction between both factors was significant at the 0.05

336   level (**Table 4**). Root biomass production of *T. latifolia* and *I. pseudacorus* was only

337   influenced by the sampling site at the 0.05 level ($p<0.001$).

338

339   Root biomass production of *J. effusus, S. lacustris, P. arundinacea, P. australis, I.*

340   *pseudacorus* and *T. latifolia* growing at 0.08 µM Cu significantly differed within species,

341   depending on sampling site locations ($p<0.01$) (**Table 3, Fig. 1, Fig. S1**). In such conditions,

342   the value for *J. effusus* was the lowest for the Argus Street population and the highest for both

343   the La Jalle and Cordon d'Or populations (**Fig. 1, Fig. S1**). Four out of the five populations of

344   *J. effusus* displayed a decreased root production when exposed to 15-25 µM Cu compared to

345   uncontaminated conditions (0.08 µM), except the population Argus Street whose RP

346   remained stable across the whole Cu gradient. For *S. lacustris*, RP was the lowest for the

347   Argus street population exposed to 0.08 µM Cu, and highest for the Gwelup population. The

348   three populations of *S. lacustris* exhibited a significant decrease in RP when exposed to 15-25

349   µM Cu in comparison to uncontaminated conditions. Out of the six populations of *P.*

350   *arundinacea*, both populations from Sanguinet and Le Palais sur Vienne produced the lowest

351   RP when exposed to 0.08 µM Cu, whereas populations from La Jalle, Cordon d'Or and

352   Lagnet displayed the highest one. Populations of *P. arundinacea* from La Jalle, Cordon d'Or

353   and Lagnet produced less root biomass under the 15-25 µM Cu gradient whereas no

354   significant differences in RP were found across the whole Cu gradient for the populations

355   Sanguinet, Le Palais sur Vienne and Cornubia. Root biomass production was similar in

356   uncontaminated conditions for five out of the seven *P. australis* populations, but RP for

357   Lagnet and Fenice Capanne populations was the highest. Except for the Lagnet population,

358   for which RP was lower at 25 µM Cu than at 0.08 µM Cu, no differences were evidenced

359   between populations in terms of RP across the Cu gradient for *P. australis*. The same pattern

360   occurred for *I. pseudacorus* and *T. latifolia,* as no differences in root production was noted

361   between populations across the Cu gradient, except for the *T. latifolia* population from

362   Basilique. This one displayed a lower value when exposed to 5-15 µM Cu compared to

363   uncontaminated conditions. Root biomass production of *T. latifolia* at 0.08 µM however

364   peaked for this population. For *I. pseudacorus*, RP in such conditions was homogeneous for

365   four out of the five populations but was higher for the Sanguinet population (**Fig. 1, Fig. S1**).

366

4. Discussion

4.1. Copper speciation in the growth medium

The six rooted macrophyte species were exposed to increasing total Cu concentration (**Table 2**). Modelling indicated three forms of dissolved Cu in the bulk solution just after 0.08 μM Cu was added, with the decreasing order: Cu^{2+} (75%) > $CuSO_4$ (9%) > $CuOH^+$ (1%). The remaining Cu (15%) may have been immediately adsorbed onto roots and perlite (**S3**). After five days, the ranking order was Cu^{2+} (50%) > $CuOH^+$ (38%) > $CuSO_4$ (6%). A slight pH increase across time would promote the formation of $CuOH^+$ from Cu^{2+} (Brookins, 1988). Free Cu^{2+} may also have been adsorbed onto roots and perlite and/or sorbed in the rhizosphere (notably by microbes and the rhizodeposition). These three processes led to Cu^{2+} decrease in the growth medium after a 5-day exposure. At 25 μM Cu in the bulk solution, based on the speciation calculation, almost all Cu would be precipitated as CuO (Tenorite), and the remaining part under the form Cu^{2+}. After five days, the proportion of CuO slightly increased whilst Cu^{2+} concentrations strongly decreased. Both phenomena may result from Cu trapping onto roots and perlite, as well as Cu sorption in the rhizosphere.

4.2. Inter/intra-specific variability vs. constitutive tolerance to cope with excess Cu

4.2.1 Inter-specific variability of root biomass production across the Cu-exposure gradient

The six investigated macrophytes exhibited an inter-specific variability of RP when grown on imbibed perlite at 0.08 μM (**Table 3**). In such conditions, rhizomatous geophytes *I. pseudacorus*, *T. latifolia* (Raunkiær, 1934), but also the hemicryptophyte *P. arundinacea*, produced the highest root biomass whilst the rhizomatous geophytes *J. effusus* and *S. lacustris* produced the lowest one. The RP of the six macrophytes at 0.08 μM Cu did not depend on the presence/absence of rhizomes. Across the Cu exposure gradient, mean RP value of *J. effusus* gradually decreased from 2.5 μM Cu whilst for *S. lacustris* and *P. arundinacea* it started to decrease from 15 μM Cu (**Fig. 1, Fig. S1**). *Phragmites australis* coped with Cu exposure up to 15 μM Cu, then root production started to decrease at 25 μM Cu. Conversely, mean RP values of the rhizomatous geophytes *T. latifolia* and *I. pseudacorus* remained stable across the

399   Cu gradient. Therefore, an inter-specific variability of Cu-tolerance was evidenced for these

400   six species.

401   The inter-specific variability of Cu-tolerance in our Cu exposure range may be due to

402   differences in (1) the regulation of the expression and stability of Cu transporters of the

403   CTR/COPT family (Jung et al. 2012), (2) PC and MT contents and (3) the number of cation

404   binding sites for the apoplast of macrophyte species. Kopittke et al. (2011) suggested that Cu

405   initially accumulates with Cys, or ligands such as phytochelatins (PC) and metallothioneins

406   (MT) possessing thiol groups, due to its affinity for S, and that once these sites are saturated,

407   Cu then accumulates within the cell wall with polygalacturonic acids in roots of *Vigna*

408   *unguiculata* (L.) Walp. Conversely, another way to cope with Cu exposure may be a

409   limitation of root Cu uptake related to Si supply (Li et al., 2008). The Si deposition in cell

410   walls of the rhizodermis and/or the endodermis may provide additional metal binding sites

411   and reduce their apoplastic bypass flow. Caldelas et al. (2012) showed that Si accumulation in

412   *I.pseudacorus* rhizodermis allowed this plant to cope with Cr exposure. In our culture

413   conditions, Si was likely provided by the perlite. Unequal root biomass production may also

414   generate changes in ROL production and Fe/Mn root-plaque extent in the rhizosphere,

415   contributing to changes in Cu sorption onto the roots between plant species (Batty et al., 2002;

416   Marchand et al., 2010; Wu et al., 2012). Last but not least, Cu-resistant endophytic bacteria

417   can influence aboveground and root DW yield (Sun et al. 2010) and their presence may differ

418   between macrophyte species (Li et al., 2011). Further investigations are needed to elucidate

419   molecular and histological mechanisms underlying the inter-specific variability of macrophyte

420   root growth in response to Cu exposure.

421

422   *4.2.2 Intra-specific variability of root biomass production across the Cu-exposure gradient*

423

424   The six investigated macrophytes exhibited an intra-specific variability of RP when grown in

425   the imbibed perlite at the 0.08 μM Cu exposure (**Table 3**). This agreed with previous findings

426   on macrophytes (Seliskar et al., 2002): ecotypes of *Spartina alterniflora* grown at the same

427   site remained morphologically closer to the ecotypes of their original site than to other

428   ecotypes planted at the same site, their development depending more on genetic variability

429   than on environmental conditions. Here, in the absence of environmental stress, such as high

430   Cu exposure, and despite the acclimation growth period, an intra-specific variability of root

development of rooted macrophytes was found, that may depend on genetic variability among populations.

Within the Cu exposure range, the significant interaction between Cu exposure and the sampling site for *J. effusus, P.arundinacea, P. australis* and *S. lacustris* suggested that the Cu-dose effect on root growth depended on the population origin (**Table 4**). These four species displayed here an intra-specific variability of root production in response to Cu exposure. Such findings agree with Matthews et al. (2004b, 2005) who reported evidences against Zn constitutive-like tolerance in *J. effusus* and *P. arundinacea*. Conversely, the Cu-dose had no significant effect on the root biomass production across populations of *I. pseudacorus* and *T. latifolia*. Root growth of these species was only driven by the sampling location. Thus, *I. pseudacorus* and *T. latifolia* exhibited a Cu constitutive-like tolerance on the 0.08-25 µM Cu range. Such constitutive-like tolerance of *T. latifolia* was reported for Zn, Cd and Pb (Ye et al. 1997b). Regarding *P. australis*, Ye et al. (1997a, 2003) considered that there were insufficient evidences to support the evolution of Cd, Pb, Zn or Cu-tolerant ecotypes in this species, although they found differences in Cu tolerance for various *P. australis* populations grown at seedling stage in the same Cu treatment solution for 3 weeks (**Table 5**)

Populations of *T. latifolia* from TE-contaminated and uncontaminated environments grew equally well in elevated TE conditions (McNaughton et al. 1974). This was confirmed by Taylor and Crowder (1983, 1984) and later by Ye et al. (1997b) for Zn, Pb and Ni tolerance in *T. latifolia*. The innate tolerance to Zn and Pb is reported in *Glyceria fluitans* (McCabe and Otte, 2000, Matthews et al., 2004a). Constitutive Zn tolerance has also been found in *Eriophorum angustifolium* L.*, T. latifolia,* and *P. australis* (Matthews et al., 2004b, 2005). Deng *et al.* (2006) reported no difference in Zn and Pb tolerance in *Alternanthera philoxeroides* (Mart.) Griseb, *Beckmannia syzigachne* (Steud.) Fernald, *Leersia hexandra* Swartz., *Neyraudia reynaudiana* (Kunth) Keng, *Oenanthe javanica* (Bl.) DC, and *Polypogon fugax* Steud between populations from contaminated and uncontaminated sampling sites. Common tolerance mechanisms in the rhizosphere and molecular mechanisms in the plant organs may underlay the TE tolerant phenotype of wetland plants. For instance, metal sorption on the organic matter accumulated in the rhizosphere and on the Fe/Mn root plaque contributes to higher metal accumulation in the macrophyte rhizosphere (McCabe et al., 2001;

15

463   Otte et al., 2004). However, through root exudation and acidification, metals can be mobilized

464   for uptake (Kissoon et al., 2010). Consequently, wetland plants are frequently more exposed

465   to higher metal concentrations than dryland plants: e.g. metal uptake in *Rumex crispus* L. was

466   2.5 times higher under wetland compared to dryland conditions (Kissoon et al., 2010).

467   McCabe et al. (2001) and Otte et al. (2004) hypothesized high metal exposure in wetlands

468   may promote selection for constitutive metal tolerance to cope with realistic TE

469   concentrations in the soil.

470

471   Which molecular mechanisms may underlay the intra-specific variability of Cu-tolerance for

472   the macrophytes cited above? Some might be similar to those for dryland plants. Hego et al.

473   (2014) reported differential responses to Cu excess between populations of *Agrostis capillaris*

474   L.. The Cu-tolerant one did not evolve a specific mechanism in roots, but its Cu resistance

475   would result from the cooperation of various processes in roots including a higher superoxide

476   detoxification, in line with the increase of chloroplastic Cu/Zn-Superoxide dismutase (SOD).

477   The Cu-sensitive one displayed in Cu-stressed roots: (1) alteration of glycolysis with

478   increased production of glycerone-P and methylglyoxal (based on expression of

479   Triosephosphate isomerase cytosolic (TIM) and Fructose bisphosphate aldolase), (2) changes

480   in tubulins and 5-methyltetrahydropteroyltriglutamatehomocysteine methyltransferase (MetE)

481   and S-adenosylmethionine synthase (SAMS) abundances for respectively underpinning the

482   impacts on the cytoskeleton and stimulating the ethylene metabolism, which may reflect a

483   higher Cu-induced senescence, and (3) increase in L-methionine and SAM amounts for

484   facilitating the production of nicotianamine, which complexes Cu, and L-cysteine, which is

485   needed for metallothioneins and GSH production (Hego et al., 2014).

486

487   Based on our findings, selection may have promoted constitutive Cu tolerance, but only for

488   some wetland species. Here, only *I. pseudacorus* and *T. latifolia* exhibited a constitutive-like

489   Cu tolerance for the 0.08-25 µM Cu range. The insignificant effect of the 0.08-25 µM Cu-

490   doses on their RP confirms previous findings (McNaughton et al. 1974, Taylor and Crowder

491   1983, 1984, Ye et al. 1997b, Matthews et al. 2005). However, this so-called innate tolerance

492   might be a buffer effect of the rhizome on a relatively short-term exposure, and should be

493   confirmed with a chronic exposure to excess Cu. The low number of *I.pseudacorus* and *T.*

494   *latifolia* populations investigated here might also explain the lack of intra-specific variability.

495 Further investigations are needed with a wider Cu exposure range and a higher number of
496 populations to find out to what extent these two macrophytes are tolerant to excess Cu.

497

498 The hypothesis of an innate tolerance to Cu must be debated for *J. effusus, S. lacustris, P.*
499 *arundinacea* and *P. australis*. These species displayed an intra-specific variability of root
500 development in response to an increasing Cu-exposure (**Table 4**). As for some Cu-tolerant *A.*
501 *capillaris* populations chronically exposed to excess Cu, it may be partly related to changes in
502 the soluble proteome of Cu-stressed roots (Hego et al, 2014). However, the Cu-exposure at
503 the sampling site and the Cu-tolerance did not correlate when the *J. effusus, S. lacustris, P.*
504 *arundinacea* and *P. australis* populations are grown in imbibed perlite. Other mechanisms
505 such as different nutrient uptake rates might be possible for RP variations in populations
506 (Deng et al., 2006). Here, the sampling site strongly influenced the RP of four out of six
507 macrophyte species across the Cu-exposure gradient. Further investigations are needed to
508 assess which abiotic and biotic parameters drive the molecular mechanisms in their plant parts
509 leading to such differences in RP between distinct populations of macrophytes exposed to
510 excess Cu.

511

512 Here, the intra-specific variability of root development in response to increasing Cu exposure
513 was evidenced for four macrophyte species by working with one genotype represented by 20-
514 30 clones per sampling site. It is likely that Cu-tolerance is a quantitative trait, i.e. polygenic
515 and complex determinism, indeed, the phenotype variability could be explored using classical
516 quantitative genetic equation $P = G + E + (G \times E)$ (Falconer, 1996), with G being the
517 genotype, E the environment and genotype x environment interaction (G x E) occurs when
518 different genotypes respond differently to different environments (Darbeshwar, 2000). Our
519 approach gives some clues about the determinism of P (G, E and G x E), but our sample size
520 impede a robust inference or broader generalization about the G component and consequently
521 to the G*E component. Thus, in a more holistic view, a focus on the G and G x E effects on
522 the root production of rooted macrophytes by considering all the genotypes present at each
523 site would be necessary for assessing to what extent the genetic variation and/or the
524 phenotypic plasticity are also involved in such variability in the context of the process of
525 genetic assimilation. Such insights are useful to select rooted macrophyte populations
526 producing a high root biomass at high Cu exposure and their use in CW treating Cu-
527 contaminated waters.

528

17

Conclusion

This study supports the hypothesis of an inter-specific variability of Cu-tolerance for the six macrophyte species grown in the 0.08-25 µM Cu range. A constitutive-like tolerance to Cu exposure for *I. pseudacorus* and *T. latifolia* was evidenced, but root biomass production depended on the plant sampling site for both species. Such constitutive-like tolerance was not found for *P. australis*, *J. effusus, S. lacustris,* and *P. arundinacea* grown in imbibed perlite. The Cu tolerance depended on the sampling site for these four species supporting an intra-specific variability. Further investigations are needed to assess if - in the context of the process of genetic assimilation – a genetic variability, resulting in phenotypic plasticity for each/some species, at each/some of the sampling site, would be involved in the acquisition of tolerance and/or resistance mechanisms to excess Cu in the case of rooted macrophyte species.

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Table 1 Metal concentrations (mg kg^{-1} DW) and physico-chemical parameters in soils at sampling sites.

sites	geographic coordinates	Country	C	N	Soil pH	CEC	Cu	Zn	Cr	Ni
			g kg^{-1}	g kg^{-1}		cmol kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Cornubia	44°54'26"N;0°32'46"W	France	30.9	1.5	5.7	5.4	205	306	14	7.0
Cordon d'Or	44°43'27"N;0°30'56"W	France	129.0	9.8	6.7	43.3	89.7	176	88	35.2
Lagnet	44°54'54"N;0°08'23"W	France	5.5	0.5	7.4	2.4	27	22.9	14	4.6
Basilique	44°53'59"N;0°06'32"W	France	19.9	1.6	7.1	13.7	71.2	60.5	67.5	31.9
Jalle d'Eysines	44°54'34"N;0°34'56"W	France	28.9	2.6	7.5	26.3	32.7	171.3	79.6	40.2
Le Palais sur Vienne	45°62'31"N;1°19'24"W	France	6.1	0.5	6.5	2.33	21.2	46.9	16.9	6.8
Sanguinet Lake	44°30'20"N;1°08'01"E	France	173	9.1	5.0	13.4	3.3	11.9	5.6	1.5
Avoca**	37°07'56"S;145°01'53"E	Australia	nd	nd	nd	nd	80	1000	28	13
Gwelup	31°52'40"S; 115°47'30"E	Australia	nd	nd	3.0	nd	nd	2.0	12	0.9
Argus Street*	37°47'42"S;145°04'24"E	Australia	nd	nd	nd	nd	94	820	65	37
Fenice Capanne	43°00'39"N;10°55'04"W	Italy	0.9	0.1	7.4	<1	375	720	35.7	29.7
Touro	42°52'34"N;8°20'40"W	Spain	0.5-7.5	0.16-0.7	3.6-4.8	25.7-37.8	200-1200	80-110	100-150	60-70
Ribeira de Agua Forte	37°53'56"N;8°08'12"W	Portugal	nd	nd	3.0	nd	1750	2000	nd	Nd
Kozlovichi	52°06'48"N; 23°37'54"E	Belarus	27.3	2.0	6.2	13.7	2.9	16.1	7.6	3
Background metal concentrations in soils ***							10-40	20-200	10-50	10-50

sites	Co	Pb	Cd	Mo	Mn	Fe	Ca	Al	Mg
	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
Cornubia	3.4	59.7	1.1	0.4	154	8.8	6.7	27.8	1.7
Cordon d'Or	11.1	62.0	0.55	3.1	195	28.3	14.6	57.8	3.3
Lagnet	2.1	15.6	0.07	0.2	139	5.7	9.8	22.6	0.9
Basilique	9.2	24.4	0.17	4	570	22.3	16.1	49.4	3.3
Jalle d'Eysines	19.0	54.9	0.50	1.6	804.7	45.1	6.3	90.4	9.3
Le Palais sur Vienne	4.0	28.5	0.48	0.9	393	11.3	1.17	54.2	2.4
Sanguinet Lake	<1	13.0	0.06	0.2	46.5	5.1	3.1	7.5	0.7
Avoca**	nd	110	1.0	nd	nd	8	nd	nd	nd
Gwelup	nd	nd	nd	nd	8	2.2	nd	3.9	nd
Argus Street*	nd	210	6.0	nd	nd	nd	nd	nd	nd
Fenice Capanne	10.5	66.0	1.03	0.5	3180	38.9	39.3	35.6	5.9
Touro	nd	nd	1.5-2.0	nd	nd	nd	nd	nd	nd
Ribeira de Agua Forte	nd	540	nd	nd	nd	nd	nd	nd	nd
Kozlovichi	<1	7.97	0.05	0.133	72.6	3.5	3.5	9.9	0.7
Background metal concentrations in soils ***	1-10	10-50	0.05-1	0.5-2	300-1000	10-50	nd	nd	nd

(*Marshall, 2004; **Bourgues *et al.*, 2004; ***Blum *et al.*, 2012); nd: not determined

Table 2 Mean comparison of copper concentrations and physico-chemical parameters in growth medium, in the 0.08-25 µM Cu range, at both day 0 and day 5 (n=6).

Total Cu added	Exposure time	pH	EC	Cu^{2+}	Cu^{2+}
(µM)	Days		($\mu S\ cm^{-1}$)	(µM)	($\mu g\ L^{-1}$)
0.08	0	6.6 ± 0.2a	555 ± 34a	0.08± 0.01a	5.8 ± 0.8a
2.5	0	6.9 ± 0.3a	542 ± 25a	0.11± 0.07a	7.1 ± 5.4a
5	0	6.8 ± 0.1a	566 ± 25a	0.30± 0.09b	19.4 ± 6.0b
15	0	6.6 ± 0.2a	562 ± 35a	1.20± 0.20c	74.3 ± 13.1c
25	0	6.6± 0.2a	549 ± 44a	2.80± 0.31d	175.4 ± 52.0d
0.08	5	7.3 ± 0.5a	442 ± 27a, *	0.05 ± 0.05a	3.5± 3.0a
2.5	5	7.3 ± 0.5a	453 ± 22a, *	0.06 ± 0.06a	3.9 ± 4.2a
5	5	7.1 ± 0.7a	458 ± 26a, *	0.16 ± 0.15a	10.4 ± 10.1a
15	5	7.2 ± 0.7a	460 ± 27a, *	0.15 ± 0.12a, *	10.1 ± 8.0 a, *
25	5	7.1± 0.4a	459 ± 34a, *	0.24 ± 0.21a, *	15.4 ± 13.4a, *

In a column and for each exposure time, mean values followed by the same letter did not statistically differ between treatments at the 0.05 level with the Tukey HSD test. Stars (*) indicate for each Cu concentration significant differences between exposure time at the 0.05 level with the Student's t-test.

Table 3 Kruskal-Wallis test for analyzing the effect of sampling site location on root biomass production of six macrophytes in uncontaminated conditions (0.08 µM Cu) (n = 4 ind. $population^{-1}$)

	Root biomass production		
	Df	KW Chi-Squared	*p*
Juncus effusus	4	12.3	**
Schoenoplectus lacustris	4	6.3	**
Phalaris arundinacea	5	16.5	***
Iris pseudacorus	4	9	**
Phragmites australis	6	12.9	**
Typha latifolia	2	6.9	**

Significance levels: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; Df: Degree of freedom, p: p-value, KW Chi-squared: Kruskal-Wallis Chi Square

Table 4 Summary ANCOVA table for analyzing the effects of sampling site location and total Cu concentration in the growth medium (dose, 2.5-25 µM Cu) on root biomass production of six rooted macrophytes. (n = 4 ind. population^{-1} modality^{-1})

		Root biomass production			
		Df	Mean Sq	F value	*p*(>F)
Juncus effusus	site	4	3678	13.5	***
	dose	1	4780	17.6	***
	site*dose	4	947	3.5	*
	residuals	74	272		
Schoenoplectus lacustris	site	2	4412	19.8	***
	dose	1	7132	32.1	***
	site*dose	2	1489	6.7	**
	residuals	45	222		
Phalaris arundinacea	site	5	33726	18.8	***
	dose	1	54393	30.4	***
	site*dose	5	8048	4.5	***
	residuals	104	1792		
Iris pseudacorus	site	4	272976	34.7	***
	dose	1	23618	3.0	.
	site*dose	4	3768	0.5	
	residuals	76	7862		
Phragmites australis	site	6	11740	10.8	***
	dose	1	8970	8.3	**
	site*dose	6	3198	2.9	*
	residuals	109	1084		
Typha latifolia	site	2	62747	14.4	***
	dose	1	4736	1	
	site*dose	2	1357	0.4	
	residuals	39	4341		

*Significance levels: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; Df: Degree of freedom, p: p-value, F: Fisher value, Means sq: Mean of square*

Table 5 Summary of research about intraspecific variability in response to metals developed on the 6 plant species included in this study.

| Species	Reference	Conditions	N sites	Metals	Summary of results
Iris pseudacorus					-
Juncus effusus	Matthews *et al.* (2004b)	Hydroponic experiment	1 high [TE] + 1 low [TE]	Zn	The two populations were tolerant to Zn, but they showed different responses to each of the treatments in survival rates, aerial biomass and Zn concentrations.
Phalaris arundinacea	Matthews *et al.* (2005)	Hydroponic experiment	1 low [TE]	Zn	Plant survival and growth decreased with increasing Zn treatments. Zn tolerance is not constitutive in *P. arundinacea*.
Phragmites australis	Ye *et al.* (1997a)	Hydroponic experiment + Pot experiment	1 high [TE] + 3 low [TE]	Cd, Pb, Zn	All the populations showed the same response (in tolerance and accumulation) to the three metals. Authors suggest that the impact of metals is attenuated in the rhizosphere, so metals do not exert a selective effect on the populations of *P. australis*.
	Ye *et al.* (2003)	Hydroponic experiment	2 high [TE] + 3 low [TE]	Cu	All the populations showed similar growth responses to Cu. However, one of the populations from a site with high [TE] showed significant higher concentrations of Cu in shoots and roots, suggesting a difference in Cu tolerance.
Schoenoplectus lacustris					-
Typha latifolia	Ye *et al.* (1997b)	Analysis of field-collected material + Hydroponic experiment + Pot experiment	3 high [TE] + 1 low [TE]	Cd, Pb, Zn	There were no differences among populations in hydroponics. However, some differences between the populations appeared for metal accumulation in roots when the seedlings were grown on the contaminated substratum. Based on the hydroponics' results, authors conclude that *T. latifolia* shows constitutional tolerance to metals.
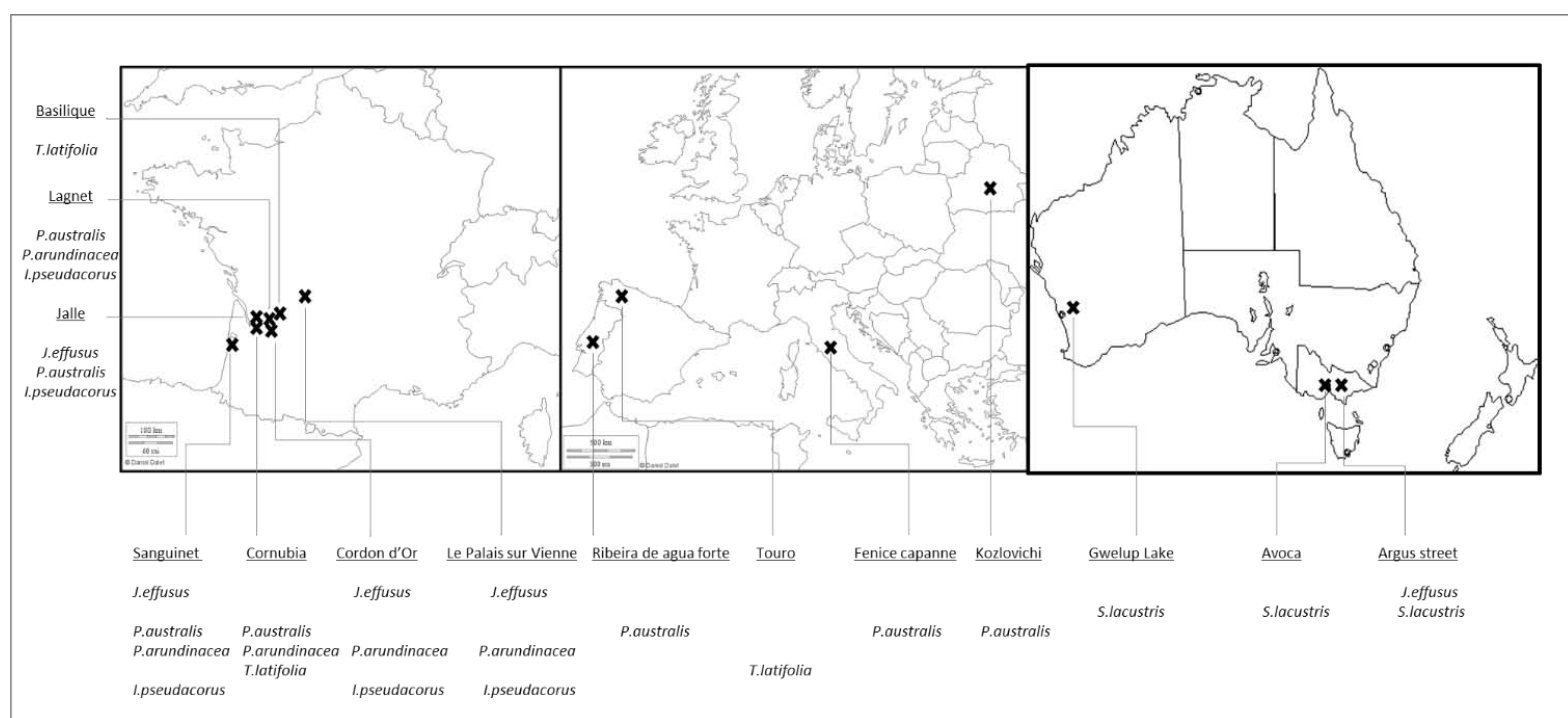
Figure 1 Sample site location map
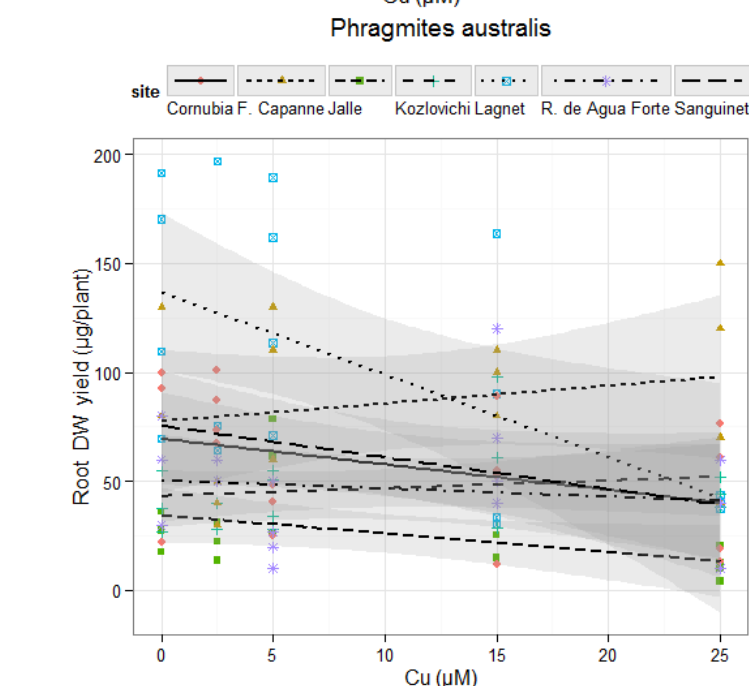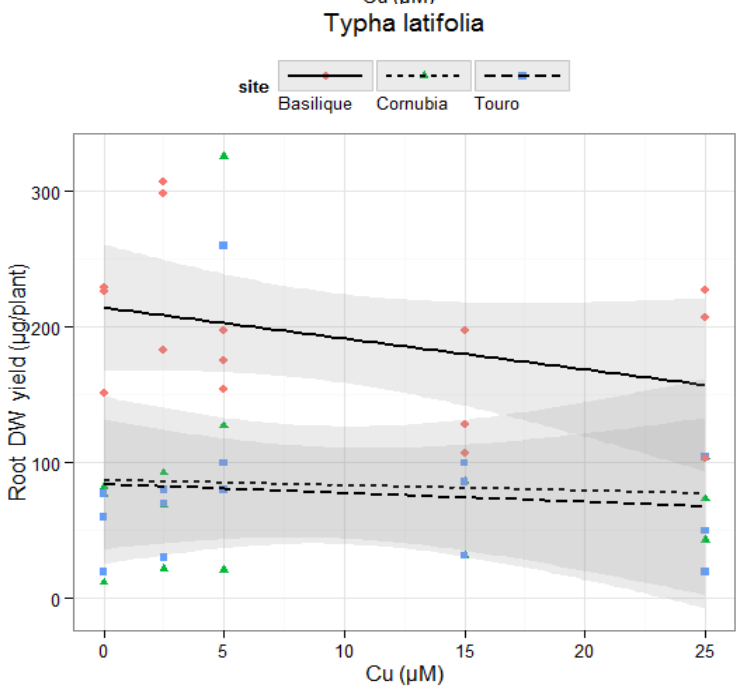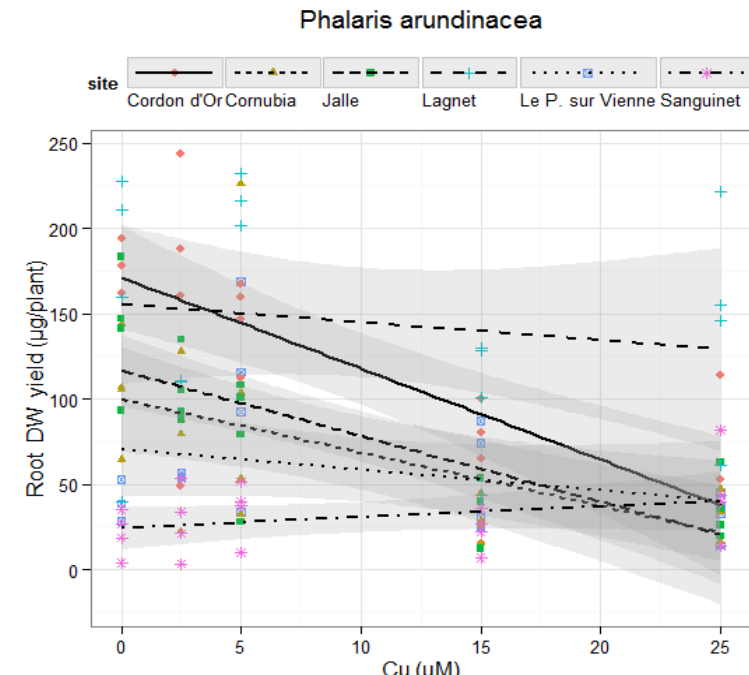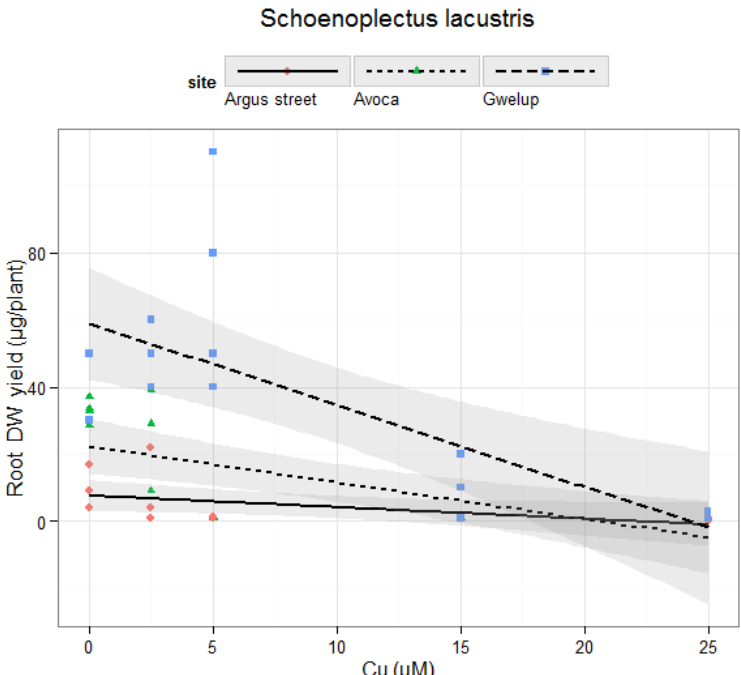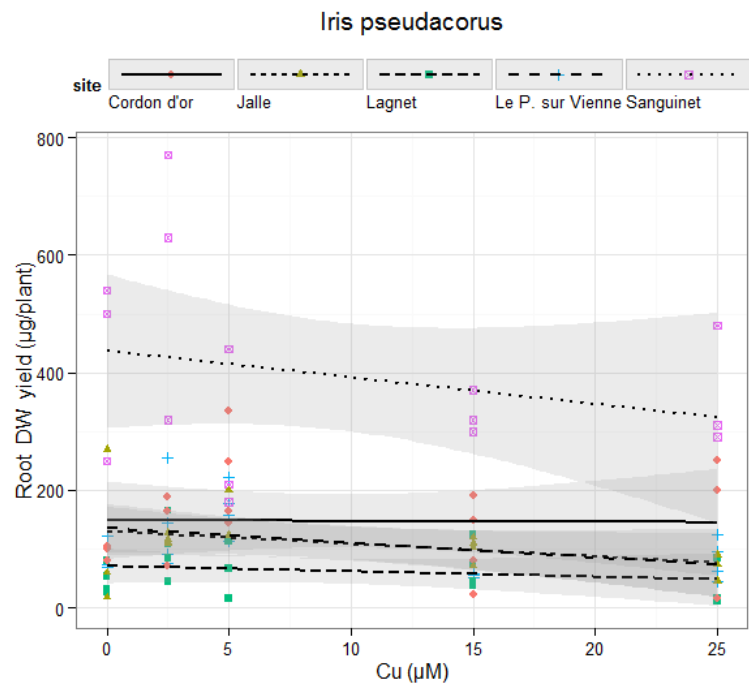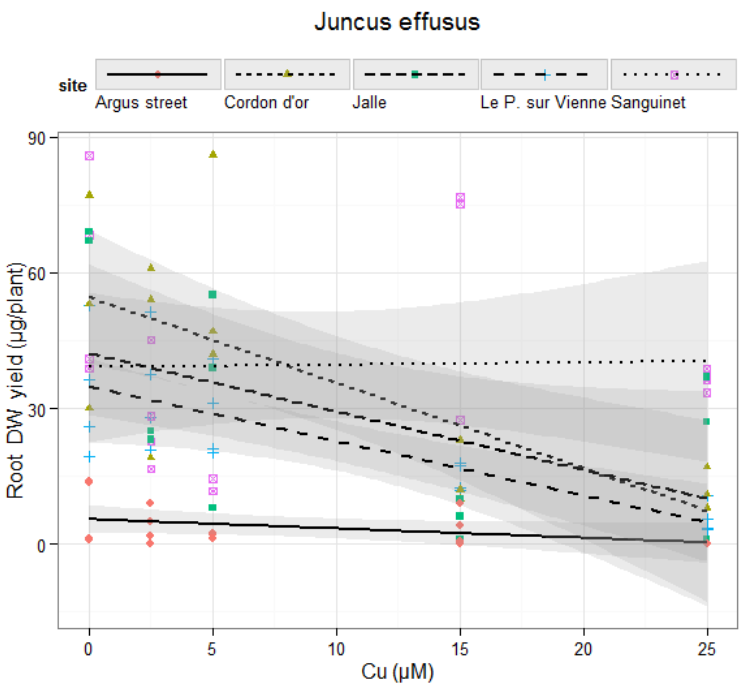
Figure 2 ANCOVA plot for root biomass production (µg DW plant^{-1}) of six macrophytes after a 3-week exposure in the 0.08-25 µM Cu range

Supplementary Material