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1 ***Miscanthus x giganteus* culture on soils highly contaminated by**  
2 **metals: Modelling leaf decomposition impact on metal mobility and**  
3 **bioavailability in the soil–plant system**

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13 **Abstract:** *Miscanthus x giganteus* is suggested as a good candidate for phytostabilization of  
14 metal-polluted soils. Its late harvest in winter generates large amounts of leaf litter on the soil  
15 surface. However, little is known about the mobility and the bioavailability of metals following  
16 leaf decomposition and the consequences on the succeeding culture. *Ex situ* artificial aging for  
17 1, 3, and 6 months was conducted with miscanthus leaf fragments incorporated into three  
18 agricultural soils displaying a gradient concentration in Cd (0.6, 3.1 and 7.9 mg.kg<sup>-1</sup>), Pb (32.0,  
19 194.6 and 468.6 mg.kg<sup>-1</sup>), and Zn (48.4, 276.3 and 490.2 mg.kg<sup>-1</sup>) to simulate the leaf litter input  
20 over 20 years of miscanthus culture. We investigated the impacts on physicochemical and  
21 biological soil parameters, CaCl<sub>2</sub>-extractable metal, and their subsequent ryegrass shoot  
22 concentrations, and hence on ryegrass health. The results showed that the amended soils  
23 possessed higher pH along with greater available phosphorous and soil organic carbon values.  
24 The respiratory activity and microbial biomass carbon in the amended soils increased mainly  
25 after 1 month of aging, and decreased afterwards. Despite the higher Pb- and Zn-CaCl<sub>2</sub>  
26 extractability in the amended soils, the phytoavailability slightly increased only in the most  
27 contaminated soils. Moreover, leaf incorporation did not affect the ryegrass biomass,  
28 photosynthetic pigment contents, nor the antioxidative enzyme activities. Conclusively, leaf  
29 incorporation induced slight variations in soil physicochemical and biological parameters, as  
30 well as metal extractability, but not to an extent that might cause a considerable threat to the  
31 subsequent culture. Nevertheless, these results are preliminary data that require confirmation  
32 by long-term *in-situ* experimentations as they reflect the modelization of long-term impact of  
33 leaf decomposition on soil-plant system.

34

35 Keywords: *Miscanthus*, *ex-situ* experiment, organic matter restitution, metal cycle, metal  
36 behavior, ryegrass, stress biomarkers,

37

## 38 1. Introduction

39  
40 Soil metal pollution is a worldwide environmental issue, caused by detrimental human activities  
41 such as smelting and mining operations, industrial production, and intensive agriculture  
42 (Tchounwou et al., 2012). Consequently, human health and the ecosystem are threatened with  
43 alteration (Kim et al., 2015).

44 Elevated costs of traditional remediation techniques have urged the development of innovative  
45 cost-effective biological methods of soil treatments (Hernandez-Allica et al., 2007). These  
46 include phytoremediation comprising several techniques that depend on plants and associated  
47 microorganisms, which aims to remove pollutants from soil and/or reduce their mobility,  
48 thereby rendering them harmless by decreasing potential health and environmental risks (Ali et  
49 al., 2013; Tangahu et al., 2011). One of these various methods is phytostabilization, a process  
50 whereby plants are established and function primarily to accumulate metals in root tissue  
51 and/or aid in their precipitation in the rhizosphere. This process is mainly adopted to  
52 revegetate degraded soils and decrease metal bioavailability (Epelde et al., 2009).

53 A promising plant for phytostabilization is the C4 perennial, rhizomatous lignocellulosic grass  
54 *Miscanthus x giganteus* (hereafter termed miscanthus), well known for its high capacity to  
55 withstand a wide spectrum of metals and mainly accumulate them in its underground parts  
56 (Nsanganwimana et al., 2015; Nsanganwimana et al., 2016). *Miscanthus* species are also well  
57 known for their ability to sequester inorganic contaminants and promote bacterial  
58 degradation of persistent organic pollutants (Técher et al., 2012). The highly metal-polluted  
59 agricultural area (mainly Cd, Pb and Zn) surrounding the former lead smelter Metaleurop Nord  
60 (northern France) is a clear evidence of the efficiency of this plant in phytomanagement.  
61 Nsanganwimana et al. (2016) demonstrated that the miscanthus mainly accumulated metals in  
62 their underground parts, and Pelfrêne et al. (2015) shed light on its ability to reduce human  
63 risks by decreasing metal oral bioaccessibility. Moreover, Al Souki et al. (2017) demonstrated  
64 miscanthus positive effects on improving the contaminated soils' physicochemical and  
65 biological parameters. However, the Cd, Pb, and specifically Zn concentrations in the aerial  
66 parts were not negligible and accumulated in leaves more than in stems (Nsanganwimana et al.,  
67 2016). This raises the question of metal cycles after several years of leaf fall.

68 After the establishment phase of 3–5 years, miscanthus biomass remains stable for at least two  
69 decades (Dufossé et al., 2014; Lewandowski et al., 2000). Early harvest of miscanthus could be  
70 organized at the end of the growing period (September to October), to obtain the highest yield

71 by collecting both stems and leaves. On the other hand, late harvest (December to February)  
72 reduces the harvesting yield, but with drier biomass, which eventually lowers the costs by  
73 limiting the need of a drying storage place and special equipment (Nsanganwimana et al., 2014;  
74 Roncucci et al., 2015). Leaf fall reduces soil nutrient losses and prevents weed growth due to  
75 the cover formed by the fallen leaves. In addition, late harvest allows a complete nutrient  
76 translocation from the miscanthus above-ground organs to the rhizomes (Nsanganwimana et  
77 al., 2014; Roncucci et al., 2015). In the case of miscanthus plants established on contaminated  
78 areas, late harvest should be examined cautiously considering the potential risk posed by  
79 contaminated leaf incorporation into soils.

80 Some researchers claimed that after a 15- to 20-year period of establishment, the crop yield  
81 tends to decrease (Arundale et al., 2014; Clifton-Brown et al., 2007; Dufossé et al., 2014). Thus,  
82 economic reasons might lead to establishing another crop after the removal of the miscanthus  
83 stand. This crop change needs to be planned, especially with crop productions in contaminated  
84 areas. Indeed, the biodegradation of plant residues is considered a crucial step in the cycling of  
85 the potential polluting elements in soil (Boucher et al., 2005; Cui et al., 2011). This process  
86 would modify the cycle of elements such as carbon, nitrogen, phosphorus, and metals.  
87 Consequently, metal speciation and extractability, in addition to soil organism abundance and  
88 activities, are affected (Cui et al., 2011; Zhou et al., 2014). Concerning the miscanthus culture,  
89 the effects of litter incorporation on metal extractability as well as on the crops that succeed  
90 leaf litter incorporation are totally unknown and therefore require investigation.

91 In the present work, *ex situ* experiments in controlled conditions (in the laboratory) were  
92 launched simulating the input of 20 years of miscanthus leaf litter to three different soils. For  
93 this reason, we artificially aged soils mixed with an estimated input of 20 years of leaf litter and  
94 i) monitored the changes in certain soil physicochemical parameters, microbial biomass carbon,  
95 and basal respiration, ii) measured the pseudo-total and CaCl<sub>2</sub>-extractable metal  
96 concentrations, and iii) evaluated the phytoavailable metals in the succeeding culture (*Lolium*  
97 *perenne*) and their effects on plant health using biomarkers (photosynthetic pigments and  
98 antioxidative enzymatic activities).

99

## 100 2. Materials and Methods

### 101 2.1. Soil and leaf litter origin and preparations

102

103 Soils (plowed horizon, 0–25 cm) used throughout the experiment were sampled (February  
104 2015) from contaminated agricultural fields cultivated with miscanthus plants since 2007,  
105 located around the former Metaleurop Nord Pb smelter (northern France) and designated as  
106 M200 and M500 according to their approximate Pb topsoil concentrations (in mg kg<sup>-1</sup>). The

107 M200 plot (50°24'52"N, 3°01'51"E, Courcelles-les-Lens) is located 1.8 km from the former  
108 smelter and characterized by its sandy clay loam soil. The M500 plot (50°25'49"N, 3°02'13"E,  
109 Evin-Malmaison) is approximately 1.4 km from Metaleurop Nord and has a clay loam soil. A  
110 third uncontaminated plot, MC (50°20'46"N 2°12'15"E), was taken as the control and is located  
111 75 km from the smelter; a silt portion dominates. Soil samples were homogenized, oven-dried  
112 at 40°C and sieved through a 2-mm mesh before launching the experiment.

113 From each field, samples of the miscanthus leaf litter were collected from several 1-m<sup>2</sup> spots,  
114 weighed and then oven dried at 40°C in the laboratory, sieved, and ground to 1–2 mm.

## 115 2.2. Determination of metal concentration in miscanthus leaves 116

117 Metals in the miscanthus leaves were determined according to the method described by  
118 Waterlot et al. (2013), in which 300 mg of each sample were acid-digested with nitric acid  
119 (HNO<sub>3</sub>, 70%) and heated at 95°C for 75 min. Following the addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>,  
120 30%), the solution was heated for further 180 min before adding osmosed water. Cd, Pb, and Zn  
121 concentrations in the extracts were determined by atomic absorption spectrophotometry (AA  
122 6800, Shimadzu, Kyoto, Japan). Quality control of the chemical extraction and digestion was  
123 performed by including blanks, internal, and certified (Polish Virginia tobacco leaves, INCTPVTL-  
124 6, Poland) reference materials.

## 125 2.3. Soil aging experiment 126

127 To each soil (500 g, 2 mm), 1 g of sieved leaf samples (1–2 mm) was introduced. This leaf  
128 quantity was evaluated by measuring leaves deposition on several 1m<sup>2</sup> spots on each of the  
129 studied miscanthus fields for three years. Upon evaluation, the estimation of 20 years of leaves  
130 accumulation was calculated. Later on, the soils and leaves were mixed attentively with a  
131 powder mixer. The mixture was transferred to small plastic pots (9 × 9 × 9 cm; *n* = 6).  
132 Simultaneously, other plastic pots (*n* = 6) were filled with MC, M200, and M500 soils without  
133 leaf powder.

134 The aging experiments were conducted at different time intervals (1, 3, and 6 months, named  
135 I1, I3, and I6, respectively) at 25°C according to the experiments conducted by Cui et al. (2011)  
136 and Du et al. (2008). Deionized water was added to the pots to maintain soil moisture at 60% of  
137 their water-holding capacity (WHC). At the end of incubation, three pots of each treatment  
138 were sacrificed for analysis, whereas the other three were cultivated with ryegrass to simulate  
139 a post-miscanthus culture.

## 140 2.4. Soil analysis 141

142 Soil samples underwent several investigations, before and after the three aging periods. A  
143 portion was kept fresh and sieved to 2 mm for the measurement of biological activities. The  
144 remaining portion was oven-dried at 40 °C, sieved, and ground to pass a 2-mm and 250- $\mu$ m  
145 mesh for the physicochemical analysis, pseudo-total and CaCl<sub>2</sub>-extractable metal  
146 concentrations.

#### 147 2.4.1. Pseudo-total metal concentrations and physicochemical parameters of soils 148

149 Soil pseudo-total Cd, Pb, and Zn concentrations was determined through acid digestion utilizing  
150 a digestion plate (HotBlock™ Environmental Express, USA). Aqua regia solution (HCl + HNO<sub>3</sub>,  
151 3:1, 6 mL) was added, and the aliquot was heated at 120°C for 90 min. Quality control of the  
152 extraction and analysis was provided by the introduction of two internal reference samples and  
153 a certified soil reference (CRM 141, IRMM, Belgium). Cd, Pb, and Zn concentrations were  
154 determined by atomic absorption spectrophotometry (AA 6800, Shimadzu) (Waterlot et al.,  
155 2012).

156 Soil pH (H<sub>2</sub>O) was measured after agitating a mixture of soil and deionized water (1:5, v/v)  
157 according to the ISO 10390 standard. The available phosphorus (P<sub>2</sub>O<sub>5</sub>) concentration was  
158 measured according to Joret and Heber (1955) via extraction in ammonium oxalate solution  
159 ((NH<sub>4</sub>)<sub>2</sub> C<sub>2</sub>O<sub>4</sub>, 0.1 M, pH = 7) and afterwards quantified spectrophotometrically upon the  
160 addition of a coloring reagent. Soil organic carbon (SOC) content was measured through a  
161 colorimetric dosage following extraction by potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, 80 g L<sup>-1</sup>) according  
162 to the ISO 14235 standard.  
163

#### 164 2.4.2. Soil CaCl<sub>2</sub>-extractable metal concentrations 165

166 CaCl<sub>2</sub>-extractable Cd, Pb, and Zn concentrations in the different soil modalities were  
167 investigated as well. The soils were mixed with a 0.01 M calcium chloride (CaCl<sub>2</sub>) solution at a  
168 ratio of 1:10, m/v (Waterlot and Douay, 2012). The Cd, Pb, and Zn concentrations in extracts  
169 were determined by atomic absorption spectrophotometry (AA 6800, Shimadzu). The quality of  
170 analytical data was verified by including the certified reference material CRM BCR®-701.  
171

#### 172 2.4.3. Soil biological parameters 173

174 Soil basal microbiological respiration (BR) and microbial biomass carbon (MBC) were  
175 determined based on the quantification of the CO<sub>2</sub> liberated in the soil and via the chloroform  
176 fumigation–extraction method, respectively, according to Al Souki et al. (2017).

### 185 2.5. Ryegrass cultivation in the aged soil and analysis 186

187 The remaining three aged soils were cultivated with the 1 g of ryegrass seeds (*Lolium perenne*).  
188 The cultivation period lasted 2 months in a controlled environment (20 °C, 16 h photoperiod).  
189 Deionized water was added to soil samples to maintain moisture at 60% water-holding capacity  
190 (WHC). At the end of the cultivation period, the ryegrass shoots were harvested by cutting 1 cm  
191 above the soil with scissors. Part of the shoots were immediately flash frozen in liquid nitrogen  
192 directly after sampling and stored at –80 °C until biomarker analysis. The remaining part was  
193 then washed three times with osmosed water to remove any dust particles. The samples were  
194 oven-dried at 40°C for 48 h and weighed to determine their dry biomass. Finally, samples were  
195 ground into fine powder using a knife mill (GM 200, Retsch) to determine the metal  
196 concentration.

197

#### 198 2.5.1. Metal concentrations in the ryegrass shoots

199 The Cd, Pb, and Zn concentrations in the ryegrass shoots were determined by atomic  
200 absorption spectrophotometry (AA 6800, Shimadzu) following the same protocol described in  
201 section 2.2.

202

#### 203 2.5.2. Quantification of ryegrass photosynthetic pigment

204

205 Photosynthetic pigments were evaluated spectrophotometrically using a plate reader (Thermo  
206 Scientific Multiskan™ GO). Briefly, two foliar discs (0.5 cm in diameter) per plant sample were  
207 collected from frozen leaves using a manual punch and weighed. They were then transferred  
208 into in 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead per well, and ground twice  
209 for 1.5 min at 30 Hz under frozen conditions using a Mixer Mill MM 400 (Retsch). After the  
210 addition of 1.5 mL of ice cold 95% methanol in each well, samples were homogenized for 2 min  
211 at 15 Hz with the MM400 grinder. Plates were left in the dark for 24 hours of incubation.

212 After incubation, leaf extracts were homogenized for 2 min at 15 Hz with the Mixer Mill MM  
213 400. A total of 100 µL was collected for photosynthetic pigment analysis. The absorbance was  
214 measured at 470, 652, and 666 nm. Concentrations of total carotenoids (Car), chlorophyll a (Chl  
215 a) and b (Chl b), respectively, were calculated according to the extinction coefficients and  
216 equations reported by Lichtenthaler (1987). Finally, data were averaged and the mean  
217 concentration obtained was expressed as mg g<sup>-1</sup> FW of leaf.

218

#### 219 2.5.3. Quantification of ryegrass antioxidative enzymatic activity

220

221

222 Antioxidative enzymatic activity assays were evaluated spectrophotometrically using a plate  
223 reader (Thermo Scientific Multiskan™ GO) as well. Briefly, five foliar discs (0.5 cm in diameter)  
224 per plant sample were collected from frozen leaves using a manual punch. They were then

225 transferred into a 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead per well, and  
226 ground twice for 1.5 min at 30 Hz under frozen conditions using a Mixer Mill MM 400 (Retsch).  
227 After addition of 1 mL of ice cold Tris extraction buffer (pH 7.0), containing 0.01 M of  
228 ethylenediaminetetraacetic acid (EDTA), 0.4 M of polyvinyl pyrrolidone (PVP), 0.05 ascorbate,  
229 11.44 mM  $\beta$ -mercaptoethanol and protease cocktail inhibitor, samples were homogenized for 2  
230 min at 15 Hz with the MM400 grinder. Plates were then centrifuged at 5000 *g* for 15 min at 4°C.  
231 Supernatants were collected and protein content was determined according to Bradford  
232 (1976), using bovine serum albumin (BSA, Sigma) as standard.

233 Total superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the  
234 photochemical reduction of nitro blue tetrazolium (NBT) according to the method reported in  
235 Giannopolitis and Ries (1977). The reaction mixture contained 0.47 mM NBT (Sigma), 3.85  $\mu$ M  
236 riboflavin (Sigma), 19.23 mM methionine (Sigma), 36.54 mM phosphate buffer (pH 7.8), and 20  
237  $\mu$ L of enzyme extract. The test tubes containing the mixture were placed 30 cm below a light  
238 source (30 W fluorescent lamps). The reaction started by switching on the light and was  
239 allowed to run for 10 min. The reaction was stopped by switching off the light with the  
240 absorbance at 560 nm. An unirradiated reaction mixture that did not develop color served as  
241 the control, and its absorbance was subtracted from the test tube's absorbance. One unit of  
242 SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT  
243 reduction.

244 Ascorbate peroxidase (APX) activity was evaluated by the decrease of absorbance at 290 nm  
245 due to ascorbate oxidation (Nakano and Asada, 1981). The reaction mixture contained 434 mM  
246 phosphate buffer (pH 7.0), 3.77 mM H<sub>2</sub>O<sub>2</sub>, 0.56 mM ascorbic acid, and 10  $\mu$ L enzyme extract.  
247 One enzyme unit was defined as 1  $\mu$ mol of ascorbic acid oxidized per minute at 290 nm using an  
248 ascorbate standard curve. The enzyme activity was expressed in  $\mu$ moles of ascorbate oxidized  
249 min<sup>-1</sup> mg<sup>-1</sup> protein.

## 250 2.6. Statistical analysis

251 Analysis of variance was calculated to compare modalities. Fisher test was considered for  
252 significance ( $p \leq 0.05$ ). If statistically significant differences were found, Tukey HSD test was  
253 used for pair-wise comparisons. All statistical analyses were performed using XLSTAT software.

254

## 255 3. Results

### 256 3.1. Metal concentrations of the miscanthus leaves

257 Cd, Pb, and Zn concentrations in the miscanthus leaves collected on each miscanthus plot are  
258 presented in Table 1.



259 Leaves sampled from the uncontaminated MC plot displayed the lowest metal concentrations  
260 (< LD: 0.4, 7.7, and 32.8 mg kg<sup>-1</sup> corresponding to the Cd, Pb, and Zn concentrations,  
261 respectively). Metal concentrations increased in the leaves as soil contamination increased:  
262 leaves sampled from the M500 plot displayed, respectively, 3.7, 2.5, and 3.2 times greater Cd,  
263 Pb, and Zn concentrations than those in the MC soil.

### 264 3.2. Pseudo-total metal concentrations and physicochemical parameters of soils

265 Table 2 presents the pseudo-total metal concentrations, pH, available P<sub>2</sub>O<sub>5</sub>, and soil organic  
266 carbon (SOC) of the control, un-amended, and amended soils throughout the different aging  
267 periods (1, 3, and 6 months).

268 To begin with, the control MC soil displayed the lowest metal concentrations (0.6, 32.0, and  
269 48.4 mg kg<sup>-1</sup> corresponding to the concentrations of Cd, Pb, and Zn, respectively), followed by  
270 the M200 and M500 soils. The incubation did not influence the pseudo-total metal  
271 concentrations in un-amended soils (I1 NL, I3 NL, and I6 NL). Interestingly, the addition of the  
272 miscanthus leaves did not modify the metal concentrations in the three amended soils (no  
273 significant differences were detected between the different soil modalities).

274 In general, the un-amended soils did not undergo any significant variation in comparison with  
275 their corresponding controls throughout the entire incubation process regarding the  
276 physicochemical parameters investigated (pH, available P<sub>2</sub>O<sub>5</sub>, and SOC). However, certain  
277 variations were detected in the amended soils. The pH of the MC soil was slightly acidic (pH =  
278 6.1) in comparison to the M200 and M500 soils, which tended to be slightly alkaline (pH = 7.3  
279 and 7.4, respectively). The leaf incorporation increased the pH of MC soil solely. After 1 month  
280 of incubation, the pH augmentation in the amended MC soil reached 12.9 % in comparison to  
281 the control pots. No significant differences were observed in the M200 and M500 soils.

282 The available P<sub>2</sub>O<sub>5</sub> content in the M200 control soil was higher than the MC and M500 control  
283 soils (0.4, 0.2, and 0.2 mg kg<sup>-1</sup> in the M200, MC, and M500 control soils, respectively).  
284 Concentrations increased progressively in the MC and M200 amended soils (highest levels  
285 detected at I6 with 23.2 and 27.8% elevation with respect to their controls). The M500  
286 amended soil did not undergo significant variations in comparison with its control.

287 The highest SOC concentrations were detected in the M500 control soil, followed by the MC  
288 and M200 controls (20.1, 13.2, and 27.8 g kg<sup>-1</sup> corresponding to the MC, M200, and M500  
289 control soils respectively; significant differences existed between the three soils).  
290 Concentrations increased progressively in the amended MC and M500 soils throughout the  
291 incubation period up to 14.2 and 15% respectively at I6. The M200 amended soils did not  
292 undergo significant variations in comparison with their corresponding control.

### 293 3.3. Soil CaCl<sub>2</sub>-extractable metal concentrations 294

295 The 0.01 M CaCl<sub>2</sub>-extractable Cd, Pb, and Zn concentrations of the control, un-amended, and  
296 amended MC, M200, and M500 soils after 1, 3, and 6 months of incubation are presented in  
297 Table 3.

298 Globally, CaCl<sub>2</sub>-extractable concentrations of the three metals increased as soil metal  
299 contamination increased (MC < M200 < M500). The CaCl<sub>2</sub>-extractable Cd, Pb, and Zn  
300 concentrations in the un-amended and control soils were quite similar throughout the entire  
301 incubation period (no significant differences were detected).

302 The leaf amendment did not impact Cd CaCl<sub>2</sub>-extractable concentrations in the three soils, in  
303 which no significant differences were detected between the different soil modalities with the  
304 same metal concentration. On the other hand, leaf incorporation raised the Pb CaCl<sub>2</sub>-  
305 extractable concentrations throughout the entire incubation period in the M200 soil only after  
306 the 1st month of incubation in the MC and M500 soils. In 1 l pots, in comparison with the  
307 control ones, the Pb-CaCl<sub>2</sub> concentrations increased by 26.2, 27.8, and 23.0% in the MC, M200,  
308 and M500 amended soils, respectively.

309 Incorporating leaves increased the Zn CaCl<sub>2</sub>-extractable concentrations in the three amended  
310 soils, with values at I1 displaying the highest augmentations (36.2, 52.8, and 49.9%,  
311 corresponding to Zn-CaCl<sub>2</sub> concentrations of the amended MC, M200, and M500 soils,  
312 respectively). However, the values tended to decrease with time throughout the incubation  
313 phase, remaining higher than their controls and un-amended soils (significant differences were  
314 still detected).

### 315 3.4. Soil microbial carbon 316

317 The influence of soil aging and leaf incorporation was studied on soil microbial carbon (MBC)  
318 (Figure 1).

319  
320 The highest MBC was measured in the MC control soil, then M500 and M200 controls  
321 respectively (significant differences were noted among the soils). No significant variations were  
322 recorded between the un-amended soils and their controls.

323 On the other hand, leaf incorporation boosted the biomass in the MC soil mainly at I1 with a  
324 20.2% increase with respect to its control. Values tended to decrease with time yet remained  
325 significantly higher than their un-amended and control soils.

326 The MBC in the amended M200 and M500 soils were significantly higher than their controls and  
327 un-amended soils only after the 1st and 3rd month of incubation, with the largest biomass  
328 detected at I1, representing a rise of 16.7 and 15.0%, respectively, relative to their controls.

### 329 3.5. Soil basal respiration

330

331 The respiratory activities decreased as the contamination increased (Figure 2). MC soil control  
332 displayed the greatest activity, followed by M200 and M500 soils respectively (significant  
333 differences were determined among the soils). No significant variations were detected between  
334 the un-amended soils and their controls.

335 However, the leaf incorporation led to a significant activity increase in the MC soil during the  
336 entire incubation period, up to 31.4% at I1 in comparison with the activity in the control soil. On  
337 the other hand, the leaf incorporation improved the respiratory activity only after the 1st  
338 month of incubation in the M200 and M500 soils (22.7 and 24.9% rise respectively, compared  
339 with their controls). Afterwards, no significant differences were detected in the amended M200  
340 and M500 soils with their controls and un-amended soils.

341

### 342 3.6. Ryegrass shoot dry biomass

343

344 The ryegrass shoot dry biomass did not vary between those cultivated in the amended and un-  
345 amended soils (no significant differences were detected) whatever the metal contamination  
346 (Figure 3).

347 The ryegrass samples planted in the contaminated soils displayed a decline in their biomass  
348 (significant differences were detected between the shoots planted in the MC soil and those  
349 cultivated in the M200 and M500 soils) (Figure 3). The average shoot dry biomass of the  
350 ryegrass cultivated in the MC un-amended soil was 2.4 g pot<sup>-1</sup>, and decreased by 37.1 and 35.2  
351 % in the shoots cultivated in the M200 and M500 soils, respectively. However, no significant  
352 variations were recorded between the results obtained in the M200 and M500 soils.

### 353 3.7. Metal concentrations in ryegrass shoots

354

355 The Cd and Zn concentrations in the ryegrass shoots cultivated in both the amended and un-  
356 amended soils after incubation are presented in Table 4. Pb concentrations are not shown  
357 because they were below the detection limit (0.052 mg L<sup>-1</sup>; i.e., 4.3 mg kg<sup>-1</sup>) in all the samples  
358 studied.

359 Cd concentration in ryegrass shoots increased progressively with the soil metal concentration.  
360 It was below the detection limit ( $0.4 \text{ mg kg}^{-1}$ ) in the shoots of the MC soil. The Cd concentration  
361 fluctuated between  $0.4$  and  $0.6 \text{ mg kg}^{-1}$  in the ryegrass shoots cultivated in the M200 soil with  
362 no significant differences detected between plants cultivated in the different soil modalities.  
363 The ryegrass in the M500 soil displayed the highest Cd concentration, yet no significant  
364 variations were detected in the shoots except for those cultivated in the I1 L soils ( $1.9 \text{ mg kg}^{-1}$ ).

365 Zn concentrations in the ryegrass shoots increased progressively with the increase in soil  
366 contamination. No significant differences were detected in the shoot metal concentrations of  
367 the plants cultivated in the amended and un-amended MC and M200 soils. The ryegrass  
368 cultivated in the M500 soil exhibited the highest Zn concentration in their shoots, with a slight  
369 but significant increase recorded in the shoots cultivated in the leaf-amended soils, in which the  
370 Zn increased by an average of 13.7% in comparison with the plants in the un-amended soils.  
371

### 372 3.8. Ryegrass photosynthetic pigments

373  
374 The influence of soil modalities on photosynthetic pigments (Chl a, Chl b, and Car) contents are  
375 shown in Figures 4, 5, and 6, (refer to supplementary material) respectively.

376  
377 For these three biomarkers, no significant differences were observed between ryegrass plants  
378 cultivated in the amended or un-amended soils. The Chl a mean concentration in the ryegrass  
379 shoots cultivated in the MC soil was  $16.6 \text{ mg g}^{-1}$  FW (Figure 4, supplementary material). It  
380 decreased progressively in the ryegrass shoots cultivated in the M200 and M500 soils by 48.9  
381 and 67.4%, respectively.

382 Similar trends were observed for Chl b (Figure 5, supplementary material). The average Chl b  
383 concentration in the ryegrass shoots cultivated in the MC soil was  $26.0 \text{ mg g}^{-1}$  FW, and  
384 decreased by 47.1 and 68.7% in those cultivated in the M200 and M500 soils, respectively.

385 The Car content was also strongly affected by soil contamination (Figure 6, supplementary  
386 material). The average Car level in ryegrass plants grown in the MC soil was  $19.4 \text{ mg g}^{-1}$  FW,  
387 and decreased by 41.7 and 54.8% in the ryegrass shoots cultivated in the M200 and M500 soils,  
388 respectively. However, unlike Chl a and b, no significant differences were observed between  
389 plants cultivated in the M200 and M500 soils.

### 390 3.9. Ryegrass antioxidative enzymatic activity assay

391  
392 The antioxidative activities of SOD and APX are displayed in Figures 7 and 8 (refer to  
393 supplementary material) respectively.

394 SOD activities in the ryegrass samples significantly increased with the soil contamination (Figure  
395 7, supplementary material). The plants cultivated in the M200 and M500 soils displayed  
396 activities 3.3 and 5.1 times higher than those recorded in the plants grown in the MC soil (basal  
397 average value, 57.3 U mg<sup>-1</sup> FW).

398  
399 Similar trends were observed for APX (Figure 8, supplementary material). APX increased  
400 progressively in the plants cultivated in the M200 and M500 soils, respectively, recording values  
401 3.6 and 7.4 times higher than the basal values obtained on the MC soil (0.1 U mg<sup>-1</sup> FW). No  
402 significant variations were recorded between the SOD and APX activities in the ryegrass shoots  
403 cultivated in the amended and un-amended soils of the same metal concentrations.

404

## 405 4. Discussion

### 406 4.1. Influence of leaf incorporation on soil parameters

407 Miscanthus has been proved to be a suitable candidate for phytomanaging metal-polluted soils  
408 due to its capacity to accumulate high metal concentrations in its underground parts, and to a  
409 lesser extent in the aerial organs, as well as producing high biomass when cultivated in  
410 contaminated areas (Nsanganwimana et al., 2015; Nsanganwimana et al., 2016). Moreover, it  
411 displayed a positive influence on improving the physicochemical and biological parameters of  
412 the contaminated soils along with decreasing human health risks (Al Souki et al., 2017; Pelfrêne  
413 et al., 2015). However, the present study focuses on the impacts of leaf residue decomposition  
414 throughout the period of miscanthus culture on different soil parameters as well as on any  
415 potential crop that could replace the miscanthus plants after their removal. For this reason, a  
416 controlled artificial soil aging process incorporating powder of the miscanthus leaves (estimated  
417 quantity, 20 years of leaf fall) into different uncontaminated and contaminated soils was  
418 launched to determine the consequences.

419 To begin with, leaf incorporation did not impact the soil's pseudo-total metal concentrations  
420 (Table 2). Indeed, the quantity of leaves incorporated represented 2/1000 of the soil mass.  
421 Moreover, the leaf metal concentrations (in milligrams per kilogram) account for 18.9–19.3%  
422 for Cd, 4.1–24% for Pb, and 21.7–67.6% for Zn of the total soil metal concentrations in the MC,  
423 M200, and M500 plots from which they were collected. Thus, soil contamination was even  
424 slightly diluted. However, the main concern regarding leaf incorporation was not its influence  
425 on the pseudo-total metal concentration but rather on their available fractions.

426 This study demonstrates the influence of miscanthus leaf incorporation on CaCl<sub>2</sub> metal  
427 extractability in the amended soils studied (Table 3). It increased the Pb and Zn CaCl<sub>2</sub>-  
428 extractable metal concentrations in the amended soils mainly at I1 in comparison with the  
429 control and un-amended soils. However, no impacts were detected on the Cd CaCl<sub>2</sub>-extractable

430 concentrations throughout the incubation process. This increase in metal CaCl<sub>2</sub> extractability  
431 cannot be explained by the decrease of pH or organic carbon content, which are considered as  
432 two of the main parameters influencing metal extractability. The reason behind the increase in  
433 Pb and Zn CaCl<sub>2</sub> extractability might be the increase in the amount of the metals in the  
434 exchangeable fractions of soil due to their influx from the incorporated leaves (Du et al., 2008),  
435 as well as their mobility, which will be discussed in the following paragraph (section 4.2). As for  
436 pH, in spite the relatively high values during the experiment, the miscanthus leaves amendment  
437 caused a slight pH increase in the soil, mainly the MC amended soil (Table 2). Boucher et al.  
438 (2005) and Cui et al. (2011) demonstrated that *Arabidopsis halleri* and *Brassica juncea* leaf  
439 incorporation sharply increased soil pH, and the maximum level was obtained mainly after the  
440 1st few days of incubation. Afterwards, a trend showing a slight decline followed by consistency  
441 prevailed. Wang et al. (2015) recorded the same outcome by incorporating Cd-contaminated  
442 rice straw into soils. The fast rise in soil pH may be due to the adsorption of H<sup>+</sup> ions by organic  
443 materials or the rapid release of excess cations from the residues reacting with H<sup>+</sup> in the soil  
444 (Tang et al., 1999). Another factor affecting the soil pH might be the plant material being  
445 incorporated: cations may be released from plant residues during microbial degradation, thus  
446 increasing the corresponding soil pH (Cui et al., 2011; Vazquez et al., 2008; Xu et al., 2016).

447 Leaf incorporation increased the available P<sub>2</sub>O<sub>5</sub> and soil organic carbon (SOC) concentrations,  
448 compared to the control and un-amended soils. These results are in agreement with Xu et al.  
449 (2016), who demonstrated the augmentation of SOC concentrations in the soils amended with  
450 rice straw, and Wei et al. (2015) who stated that wheat straw incorporation resulted in higher  
451 available phosphorous and SOC concentrations in the amended soil. Many studies (Dufossé et  
452 al., 2014; Medina et al., 2015; Zhang et al., 2016) showed the significant effects of crop residue  
453 addition in improving the soil's organic matter dynamics and nutrient cycling, and thus  
454 replenishing the soil's organic matter content and supplying essential nutrients after  
455 mineralization (N, P, etc.). Moreover, factors such as suitable moisture levels and temperature  
456 might have promoted the subsequent decomposition of the plant residues incorporated and  
457 thus the increase of organic carbon and essential nutrient concentrations such as P in the  
458 amended soils (Surekha et al., 2003; Wei et al., 2015).

459 The present study also demonstrated an influence of leaf incorporation on soil microbial  
460 biomass carbon (Figure 1) and basal respiration (Figure 2). Globally, these biological parameters  
461 exhibited higher values in the uncontaminated MC soil and decreased with the soil's metal  
462 concentration. According to Al Souki et al. (2017), metal contamination negatively impacts soil  
463 functionality and affects the growth, morphology, and metabolism of soil microorganisms  
464 through functional disturbance and alteration of protein and cell membrane integrity. The BR  
465 and MBC varied as well between the amended and the un-amended soils. After 1 month of  
466 incubation (I1), both the respiratory activities and MBC increased to reach their maximum

467 values in all amended soils (significant differences were detected between the amended soils  
468 and their controls). On the other hand, the second phase (I3 and I6) represented a declining  
469 phase, where both parameters followed a descending pattern to reach their minimum levels by  
470 the end of the incubation period. The increase in BR and MBC values at the first stage could  
471 stem from the utilization of easily available organic compounds, most of which are water-  
472 extractable from the incorporated plant leaves (Shi and Marschner, 2014). Thus, the  
473 subsequent decline in both parameters to mimic the control levels might be due to the  
474 depletion of the carbon substrates and easily available compounds, in which the utilization of  
475 the more recalcitrant compounds required energy for enzyme synthesis and therefore left less  
476 energy for growth (Pan et al., 2016; Quinn et al., 2011; Shi and Marschner, 2014).

#### 477 4.2- Impacts of leaf incorporation on the ryegrass crop 478

479 Ryegrass is an important, widespread perennial cool-season turf grass. It was selected because  
480 it is a valuable forage crop that can be used for plant cover as well as for phytoremediation  
481 purposes due to its capacity to accumulate toxic substances in its biomass (Bai et al., 2015a;  
482 Bidar et al., 2009; Houben et al., 2013). The results reported herein show that ryegrass  
483 accumulated more Cd and Zn in its aboveground parts depending on the contamination  
484 gradient (Table 3). However, only the ones cultivated in the amended M500 soils exhibited  
485 higher Zn concentrations in their shoots, in comparison with those planted in the un-amended  
486 soils. These results are quite consistent with our data obtained on CaCl<sub>2</sub> metal extractability  
487 (except for Pb), even if the effect of leaf incorporation on metal accumulation in shoots is only  
488 notable on the M500 soil. However, the low accumulation of Pb in ryegrass shoots is due to its  
489 very low mobility into plants and its poor translocation from roots to shoots (Pourrut et al.,  
490 2013; Pourrut et al., 2011).

491 Our results on Cd and Zn are in agreement with those obtained by Perronnet et al. (2000) and  
492 Wang et al. (2015), who conducted similar experiments, in which soils were amended with Cd-  
493 and Zn-contaminated *Thalpi caerulea* leaves and Cd-contaminated rice straws,  
494 respectively. These authors demonstrated that the incorporated metals exhibited high mobility  
495 and were transferred in large amounts to the subsequent crop, proving the idea that the effects  
496 of plant leaf addition on metal speciation and phytoavailability in soils depend not only on soil  
497 pH and organic carbon, but also on the plant material and the amounts of metal within.

498 Du et al. (2008) also investigated the impact of amending soils with arsenic (As)-rich Chinese  
499 brake fern (*Pteris vittata* L.). After 1, 3, and 6 months of soil aging, germinated wheat was  
500 planted in the soils, and the results showed that the As concentration in the shoots significantly  
501 increased with the increasing amount of As in the leaves. In addition, the shoots planted in the  
502 soils incubated for 1 month exhibited the highest concentrations. The relative increase in the  
503 shoot metal concentration in the subsequent ryegrass might be attributed to the high mobility

504 of the metals contained in the incorporated miscanthus leaves, which were mainly transferred  
505 to the subsequent crop (Perronnet et al., 2000; Wang et al., 2015).

506 Even if the miscanthus leaf incorporation into soils slightly increased metal accumulation in  
507 ryegrass shoots on the most contaminated soils (Table 3), it did not affect the plant's health  
508 (Figures 3–8, supplementary material). Yet, there were significant variations between the  
509 ryegrass samples cultivated in the uncontaminated MC soils and those cultivated in the  
510 contaminated soils. The biomass results (Figure 3) showed a significant 37.1 and 35.2%  
511 decrease in the plants cultivated in M200 and M500 soils, respectively, relative to those in the  
512 MC. The impact of the soil gradient contamination was more evident in the photosynthetic  
513 pigment concentrations (Figures 4–6, supplementary material), in which Chl a decreased by  
514 48.9 and 67.4% in the shoots cultivated in the M200 and M500 soils, respectively. Similar trends  
515 were demonstrated for Chl b and Car levels. Finally, The SOD and APX activities in the plants  
516 sampled from the M200 and M500 soils were three to seven times higher than those recorded  
517 in the MC soil plants. Overall, the results obtained in the present study indicate that the  
518 ryegrass species suffered from stressful conditions in the contaminated amended and un-  
519 amended soils.

520 These results are consonant with the study reported by Wang et al. (2013), who revealed the  
521 significant negative effects on the Chl a, Chl b, and Car contents as well as the biomass of  
522 ryegrass seedlings upon exposure to Cd. Bai et al. (2015b) also demonstrated the negative  
523 impact Pb had on the ryegrass biomass, the net photosynthetic rate, in addition to the Chl and  
524 Car contents of their corresponding leaves. The decrease in the ryegrass biomass could be  
525 attributed to the negative influence exerted by the metals over the main metabolic processes  
526 including photosynthesis, respiration, as well as nutrient and water uptake (Chibuike and  
527 Obiora, 2014; Pourrut et al., 2013; Pourrut et al., 2011). Moreover, the metal concentration-  
528 dependent increase of SOD and APX activities in the present study agrees with several other  
529 experiments (Bonnet et al., 2000; Cao et al., 2016; Lou et al., 2015; Luo et al., 2011). In addition,  
530 the gene expression of SOD and APX were boosted in the ryegrass that was subjected to Pb  
531 stress in the experiment of Li et al. (2012).

532 Interestingly, in the current work, we demonstrated that ryegrass plants underwent substantial  
533 oxidative stress, while photosynthetic pigments were impacted to a lesser extent. The impact of  
534 metal contamination was less notable on plant biomass and no significant differences were  
535 observed between plants grown on the M200 and M500 soils. This different response could  
536 stem from the short cultivation period (2 months). Significant variations might appear in the  
537 shoot biomass in the contaminated soils had the duration been prolonged. The results obtained  
538 were in accordance with Li et al. (2012), who showed that the Chl contents of ryegrass were not  
539 affected by the increasing Cd concentrations in a 1-week experiment, in contrary to the



540 activities of the antioxidative enzymes, which responded immediately to the increasing metal  
541 stress. Luo et al. (2011) made similar observations while studying the impact of increasing levels  
542 of Cd on ryegrass, in which the Chl a and b levels in the plants treated with 0.2 and 0.5 mM Cd  
543 were the same. Overall, the results obtained indicate that the antioxidative enzymes  
544 responsible for the early stage of defense against metal-induced oxidative stress are the most  
545 severely affected.

546

## 547 5. Conclusions and perspectives

549 The main objective of the present study was to assess the impacts of incorporating leaf litter  
550 into the soil on several soil parameters, metal availability, as well as on the health of the crop  
551 succeeding the miscanthus culture (ryegrass). Nevertheless, miscanthus leaf incorporation  
552 induced slight variations in the soil physicochemical parameters studied, mainly after the 1st  
553 month of aging. Litter incorporation did not influence the pseudo-total metal concentrations in  
554 soils but significantly increased Pb and Zn CaCl<sub>2</sub>-extractable concentrations. Leaf incorporation  
555 increased the available P<sub>2</sub>O<sub>5</sub> and soil organic carbon concentrations compared to control and  
556 un-amended soils. Meanwhile, the soil respiratory activities and microbial biomass carbon rose  
557 mainly after the 1st month of incubation as a result of leaf amendment. However, no significant  
558 effects of leaf incorporation were observed on the subsequent ryegrass plant health. In  
559 conclusion, incorporating miscanthus leaves did not have significant and persistent impacts on  
560 the soil–plant system studied, which might be considered as a very positive point when  
561 contaminated areas are harvested late. However, the results obtained might be used with  
562 caution given that they were obtained *ex situ*, in controlled conditions, using artificial aging.  
563 They must be considered as preliminary data that need to be confirmed by long-term *in situ*  
564 experiments, which are ongoing on the Metaleurop Nord experimental sites.

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Fig 1: Microbial biomass carbon (MBC) of the control, non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 2: Basal respiration (BR) of the control, non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 3: Shoot dry biomass of the ryegrass cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 4: Chl a contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 5: Chl b contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 6: Car contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Fig 7: SOD activities in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

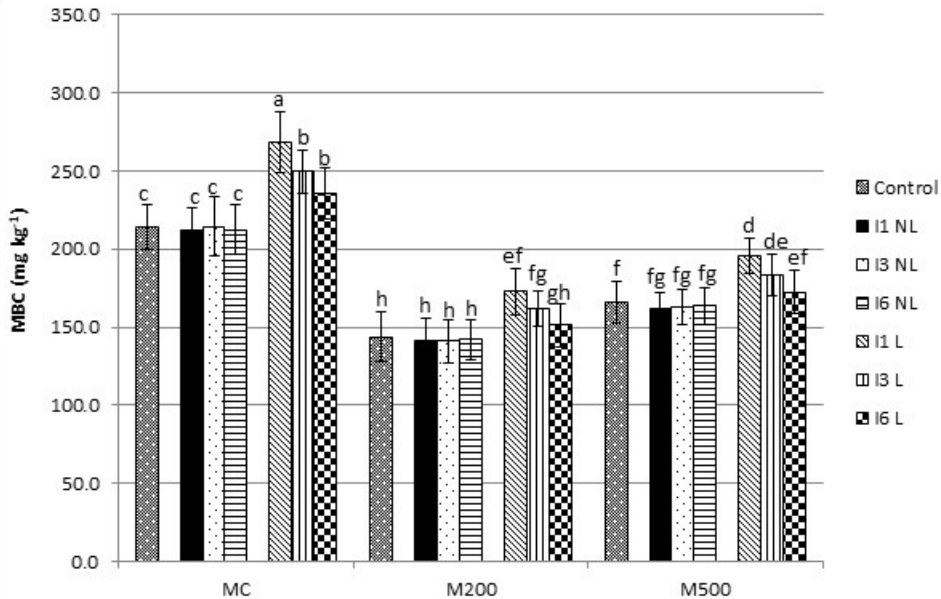
Fig 8: APX activities in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means  $\pm$  standard deviations. Different letters refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

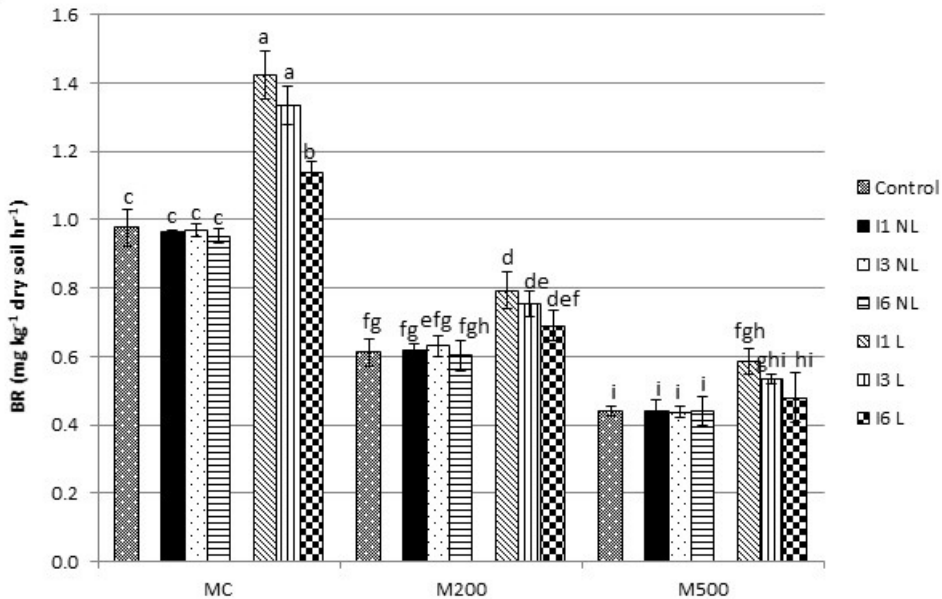
Table 1: Cd, Pb, and Zn concentration in the miscanthus leaves collected on MC, M200 and M500 soils. Values represent means  $\pm$  standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ ). LD: Limit of detection (Cd:  $0.0049 \text{ mg L}^{-1}$ ; i.e.  $0.4 \text{ mg kg}^{-1}$ )

Table 2: Evolution of pseudo-total metal concentrations, pH,  $\text{P}_2\text{O}_5$  content and Soil organic carbon (SOC) of the control, non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means  $\pm$  standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

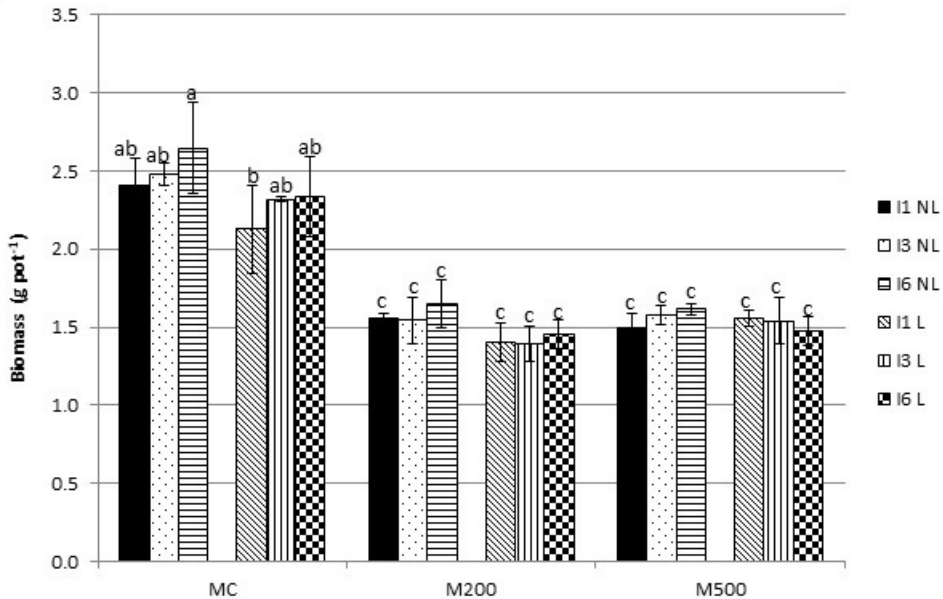
Table 3: Cd, Pb, and Zn  $\text{CaCl}_2$  extractable fractions of the control, non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means  $\pm$  standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ )

Table 4: Cd and Zn concentrations in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means  $\pm$  standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test,  $n = 3$ ,  $p \leq 0.05$ ).  
LD: Limit of detection (Cd:  $0.4 \text{ mg kg}^{-1}$ )

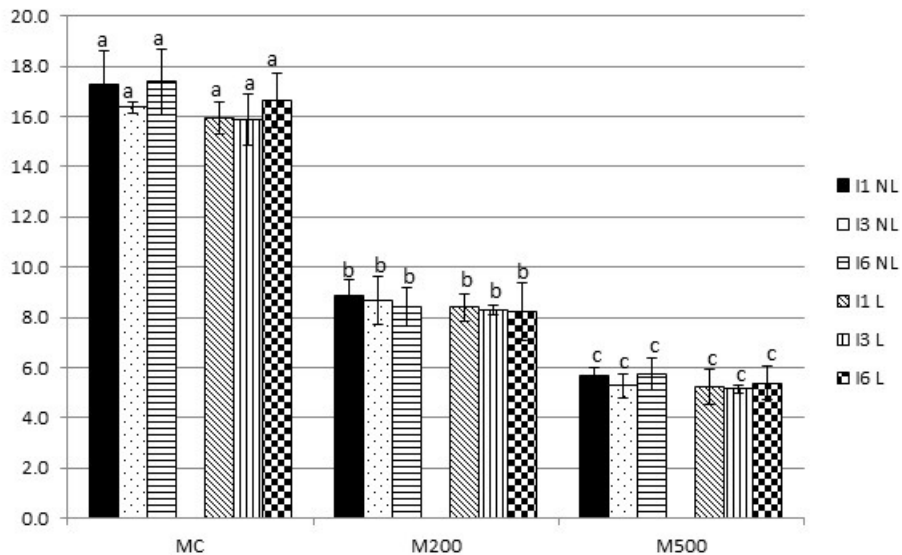


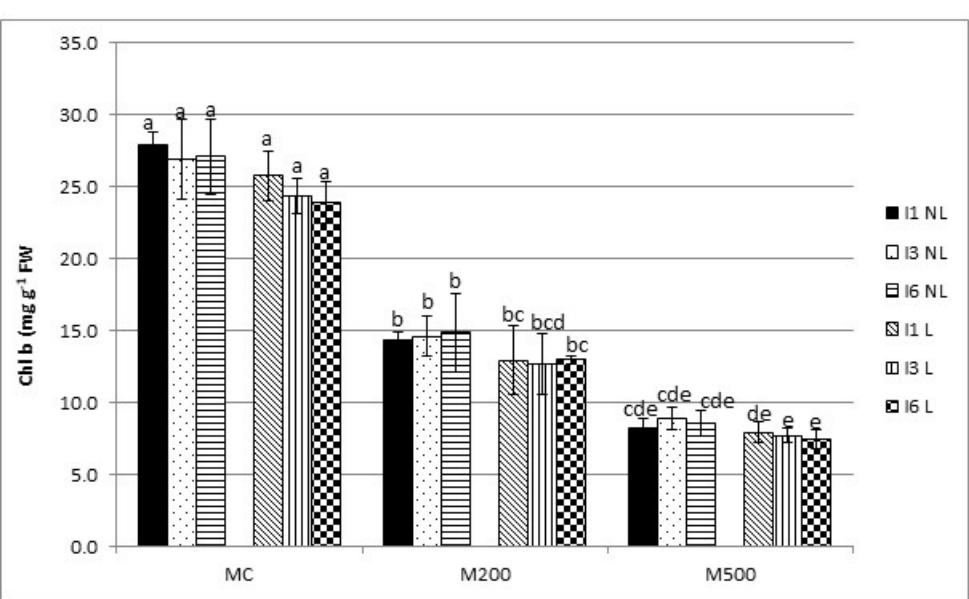


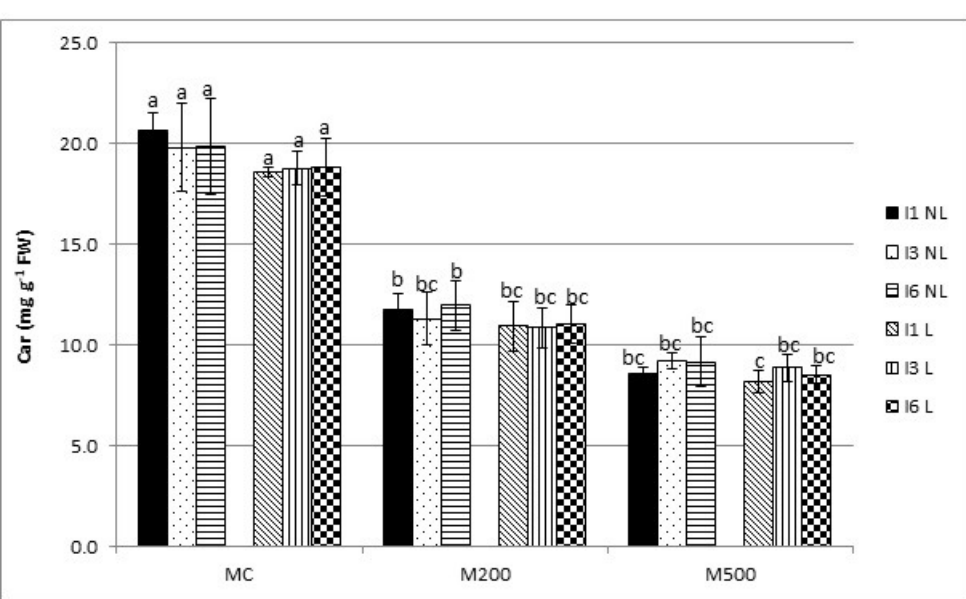




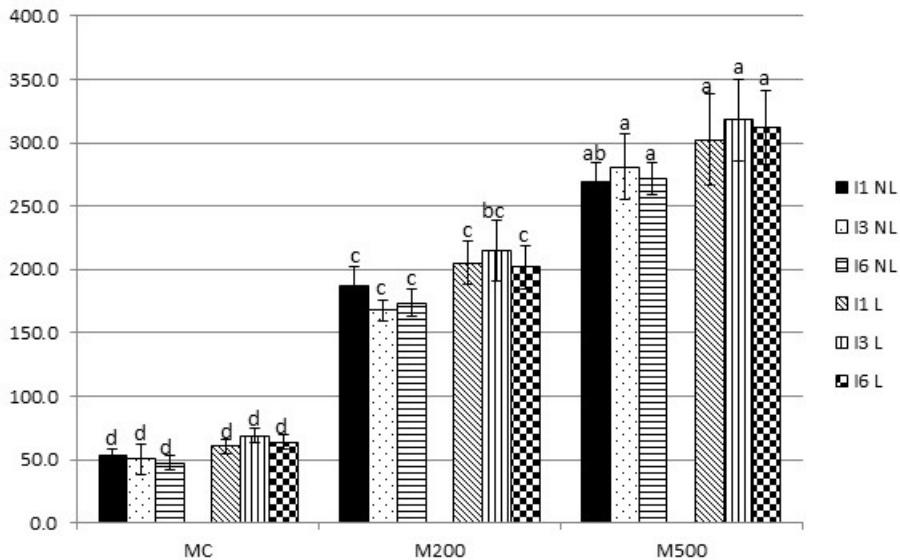
Chl a (mg g<sup>-1</sup> FW)

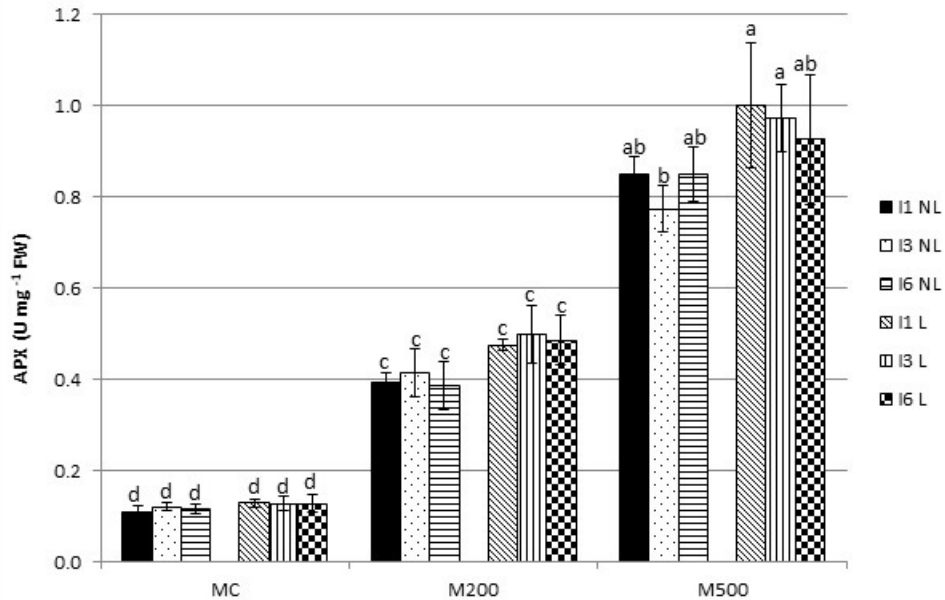






SOD (U mg<sup>-1</sup> FW)





	MC	M200	M500
Cd (mg kg <sup>-1</sup> )	< LD	0.6 ± 0.0 b	1.5 ± 0.1 a
Pb (mg kg <sup>-1</sup> )	7.7 ± 0.8 b	10.8 ± 1.7 b	19.5 ± 1.8 a
Zn (mg kg <sup>-1</sup> )	32.8 ± 3.0 c	73.1 ± 4.6 b	106.3 ± 8.1 a

Table 1.

		Control	Non-amended soil			Amended soil		
			I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd (mg kg <sup>-1</sup> )	MC	0.6 ± 0.1 c	0.5 ± 0.0 c	0.5 ± 0.1 c	0.6 ± 0.1 c	0.6 ± 0.0 c	0.6 ± 0.0 C	0.6 ± 0.1 c
	M200	3.1 ± 0.2 b	3.0 ± 0.2 b	3.1 ± 0.3 b	2.9 ± 0.3 b	3.5 ± 0.2 b	3.5 ± 0.3 b	3.5 ± 0.1 b
	M500	7.9 ± 0.2 a	8.0 ± 0.8 a	7.9 ± 0.5 a	7.9 ± 0.6 a	8.3 ± 0.6 a	8.3 ± 0.2 a	8.4 ± 0.2 a
Pb (mg kg <sup>-1</sup> )	MC	32.0 ± 6.7 c	29.0 ± 1.8 c	28.9 ± 4.0 c	29.1 ± 5.2 c	36.8 ± 3.5 c	33.9 ± 3.0 c	38.0 ± 5.1 c
	M200	194.6 ± 21.1 b	181.7 ± 13.1 b	185.9 ± 8.0 b	181.3 ± 3.3 b	201.3 ± 6.5 b	202.9 ± 10.9 b	198.9 ± 10.0 b
	M500	468.6 ± 6.8 a	458.4 ± 30.6 a	461.0 ± 12.0 a	475.0 ± 10.8 a	478.7 ± 11.3 a	477.9 ± 15.7 a	475.0 ± 10.8 a
Zn (mg kg <sup>-1</sup> )	MC	48.4 ± 6.4 d	50.3 ± 1.3 d	51.7 ± 1.6 d	50.8 ± 1.6 d	54.5 ± 3.0 d	54.9 ± 3.5 d	53.5 ± 5.2 d
	M200	276.3 ± 7.1 c	276.9 ± 9.6 c	274.8 ± 12.6 c	275.0 ± 7.8 c	287.6 ± 6.7 c	287.5 ± 6.0 c	288.0 ± 7.9 c
	M500	490.2 ± 4.1 ab	490.0 ± 10.3 ab	479.5 ± 18.6 b	488.6 ± 15.0 ab	510.4 ± 11.3 a	500.6 ± 9.7 ab	505.8 ± 6.9 ab
pH	MC	6.1 ± 0.1 i	6.1 ± 0.0 i	6.0 ± 0.3 i	6.0 ± 0.0 i	7.0 ± 0.1 fgh	6.9 ± 0.1 gh	6.8 ± 0.0 h
	M200	7.3 ± 0.1 bcde	7.3 ± 0.0 def	7.2 ± 0.0 ef	7.2 ± 0.1 efg	7.6 ± 0.1 ab	7.5 ± 0.0 abcd	7.4 ± 0.1 abcde
	M500	7.4 ± 0.1 abcde	7.4 ± 0.1 abcde	7.4 ± 0.0 bcde	7.3 ± 0.0 cde	7.7 ± 0.1 a	7.6 ± 0.1 a	7.6 ± 0.1 abc
P <sub>2</sub> O <sub>5</sub> (mg kg <sup>-1</sup> )	MC	0.2 ± 0.0 e	0.2 ± 0.0 e	0.2 ± 0.0 e	0.2 ± 0.0 e	0.3 ± 0.0 de	0.3 ± 0.0 de	0.3 ± 0.0 d
	M200	0.4 ± 0.0 c	0.4 ± 0.0 c	0.5 ± 0.0 c	0.4 ± 0.0 c	0.5 ± 0.0 b	0.6 ± 0.0 ab	0.6 ± 0.0 a
	M500	0.2 ± 0.0 e	0.2 ± 0.0 e	0.2 ± 0.0 e	0.2 ± 0.0 e	0.3 ± 0.0 de	0.3 ± 0.0 de	0.3 ± 0.0 de
SOC (g kg <sup>-1</sup> )	MC	20.1 ± 1.5 de	19.5 ± 1.4 e	19.8 ± 1.2 de	19.1 ± 1.7 ef	21.7 ± 1.9 cde	22.9 ± 1.3 cd	23.4 ± 1.8 c
	M200	13.2 ± 1.4 g	13.2 ± 1.7 g	13.0 ± 1.4 g	13.1 ± 1.2 g	15.2 ± 1.9 g	15.5 ± 1.2 g	16.0 ± 1.3 fg
	M500	27.8 ± 1.9 b	27.7 ± 1.6 b	27.3 ± 2.3 b	27.3 ± 1.6 b	31.7 ± 1.4 a	31.8 ± 1.9 a	31.8 ± 1.8 a

Table 2.

		Control	Non-amended soil			Amended soil		
			I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd-CaCl <sub>2</sub> (mg kg <sup>-1</sup> )	MC	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 ef	0.01 + 0.00 f	0.01 + 0.00 f
	M200	0.02 + 0.0 cd	0.02 + 0.00 cde	0.02 + 0.00 cd	0.02 + 0.00 de	0.03 + 0.00 c	0.02 + 0.00 cd	0.02 + 0.00 cd
	M500	0.04 + 0.0 ab	0.04 + 0.00 ab	0.04 + 0.00 ab	0.04 + 0.00 b	0.05 + 0.00 a	0.04 + 0.00 a	0.04 + 0.00 ab
Pb-CaCl <sub>2</sub> (mg kg <sup>-1</sup> )	MC	0.21 + 0.03 gh	0.21 + 0.02 gh	0.19 + 0.01 h	0.19 + 0.02 h	0.28 + 0.03 cdef	0.25 + 0.02 fgh	0.25 + 0.02 efgh
	M200	0.26 + 0.02 efgh	0.27 + 0.01 defg	0.27 + 0.02 defg	0.26 + 0.02 efgh	0.36 + 0.01 ab	0.35 + 0.02 b	0.35 + 0.02 bc
	M500	0.33 + 0.01 bcd	0.33 + 0.03 bcde	0.32 + 0.03 bcde	0.32 + 0.04 bcde	0.43 + 0.03 a	0.38 + 0.02 ab	0.37 + 0.02 ab
Zn-CaCl <sub>2</sub> (mg kg <sup>-1</sup> )	MC	0.08 + 0.01 ijk	0.06 + 0.00 jk	0.07 + 0.01 jk	0.06 + 0.01 k	0.12 + 0.01 fg	0.11 + 0.01 gh	0.10 + 0.00 h
	M200	0.09 + 0.01 hij	0.07 + 0.01 ijk	0.08 + 0.01 ijk	0.08 + 0.01 ijk	0.18 + 0.01 d	0.15 + 0.01 e	0.14 + 0.01 ef
	M500	0.14 + 0.00 ef	0.14 + 0.00 ef	0.14 + 0.01 ef	0.14 + 0.01 ef	0.28 + 0.01 a	0.25 + 0.01 b	0.23 + 0.00 c

Table 3.

		Non-amended soil			Amended soil		
		I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd (mg kg <sup>-1</sup> )	MC	< LD	< LD	< LD	< LD	< LD	< LD
	M200	0.4 + 0.0 d	0.4 + 0.0 d	0.5 + 0.0 d	0.6 + 0.0 d	0.5 + 0.0 d	0.5 + 0.0 d
	M500	1.3 + 0.1 c	1.4 + 0.2 bc	1.4 + 0.2 bc	1.9 + 0.1 a	1.7 + 0.3 ab	1.5 + 0.2 bc
Zn (mg kg <sup>-1</sup> )	MC	15.6 + 1.2 d	15.9 + 2.0 d	14.1 + 1.1 d	21.0 + 3.0 d	17.3 + 2.1 d	17.4 + 1.0 d
	M200	40.3 + 3.7 c	41.7 + 3.8 c	41.7 + 3.9 c	42.8 + 2.9 c	42.3 + 3.2 c	41.9 + 2.2 c
	M500	67.8 + 4.2 b	64.4 + 2.9 b	68.3 + 5.2 b	77.9 + 3.9 a	77.8 + 3.0 a	76.6 + 4.3 a

Table 4.