

1 **Bioaccumulation of heavy metals from wastewater through a *Typha***
2 ***latifolia* and *Thelypteris palustris* phytoremediation system**

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22 *Abbreviations:* DM, dry matter; f.w., fresh weight, ICP-MS, inductively coupled plasma mass
23 spectrometry.

24 **Declaration of interest**

25 The authors declare no competing financial interests.

26 **Highlights:**

27 The increasing biomass showed that *T. latifolia* and *T. palustris* grew normally.

28 The increase of Zn and Cu in plants was related by a decrease of metals in water.

29 Both plants are able to phytoremediate Zn and Cu from contaminated wastewater.

30

31 **Abstract**

32 Animal production is a source of heavy metals in livestock wastewater and also a key link
33 in the food chain, with negative impacts on human and animal health. In intensive animal
34 production systems, the most critical elements are zinc and copper. In order to development of
35 innovative non-invasive strategies to reduce the environmental impact of livestock, this study
36 assessed the ability of two plants, *Typha latifolia* and *Thelypteris palustris*, to bioaccumulate
37 the heavy metals used in animal nutrition, from wastewater. Four mesocosms (width 2.0 m,
38 length 2.0 m, 695 L of water, 210 kg of soil) were assembled outdoors at the Botanical Garden.
39 Two of them were planted with *T. latifolia* (TL treated, n=30; TL control, n=30) and two with
40 *T. palustris* (TP treated, n=60; TP control, n=60). In T0 a solution of a mineral additive premix
41 (Zn 44.02 mg/L; Cu 8.63 mg/L) was dissolved in the treated mesocosms. At T0, d 15 (T1) and
42 d 45 (T2) samples of roots, leaves, stems, soil and water were collected, dried, mineralized and
43 analyzed using ICP-MS in order to obtain HMs content. We found that *T. latifolia* and *T.*
44 *palustris* accumulate and translocate Zn, Cu from contaminated wastewater into plant tissues
45 in a way that is directly related to the exposure time (T2 for Zn: 271.64±17.70, 409.26±17.70
46 for Cu: 47.54±3.56, 105.58±3.56 mg/kg of DM, respectively). No visual toxicity signs were
47 observed during the experimental period. This phytoremediation approach could be used as an
48 eco-sustainable approach to counteract the output of heavy metals.

49

50

51 **Keywords:** phytoremediation, heavy metals, *Typha latifolia*, *Thelypteris palustris*, swine
52 livestock, environmental impact.

53 **Short title:** Bioaccumulation of heavy metals from wastewater through phytoremediation
54 system.

55 **1. Introduction**

56 The contamination of wastewater with heavy metals and metalloids (HMs) has become a
57 worldwide concern in areas of intensive agriculture (Bhargava *et al.* 2012). The long-term
58 consequences of the accumulation of HMs can reduce the quality of cultivation and increase
59 the pollution of agricultural lands (Gul *et al.* 2015; Jakubus *et al.* 2013; Liu *et al.* 2018; Lopez-
60 Alonso *et al.* 2012; Rossi *et al.* 2013, 2014 a,b). The major routes of HMs into the soil include
61 atmospheric deposition, agrochemicals, inorganic fertilizers and also animal manure, the latter
62 reflecting the content of HMs from animal feed (Nicholson *et al.* 2003).

63 Animal production is thus a possible source of HMs which can contaminate the food chain
64 with a negative impact on human and animal health (Dumont *et al.* 2012; Jarup 2003;
65 Lyubenova *et al.* 2013; Ma *et al.* 2016; Hejna *et al.* 2019). HMs can enter the animals' diet both
66 as contaminants or undesirable substances and as essential nutrients (Fink-Gremmels 2012;
67 Hejna *et al.* 2018). Elements such as cobalt (Co), copper (Cu), iron (Fe), iodine (I), manganese
68 (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) are some of the numerous enzymes that
69 coordinate biological processes, and consequently should be integrated into the animal diet as
70 mineral additives by respecting the maximum admitted level (EC N° 1831/2003; Lopez-Alonso
71 *et al.* 2012).

72 The previous study showed that the content of HMs in manure reflected their
73 concentration in the diet (Hejna *et al.* 2019), and that Zn and Cu, widely used in high doses to
74 control enteric bacterial infections as well as to enhance the integrity of the immune system
75 (Liu *et al.* 2018) represent the most critical output from swine livestock.

76 The scenario of livestock have changed significantly in the last decade. In fact, after the
77 antibiotics ban in 2006 in Europe (EC, Reg. 1831/2003), there was an increased use of high
78 dosages of zinc salts, possible after veterinary prescription as an alternative to in-feed
79 antibiotics to control the enteric disease in the growing phases. Despite the antibacterial activity

80 of zinc salts, the use of zinc in feed might have contributed to the emergence of methicillin-
81 resistant *Staphylococcus aureus* (MRSA). There is worldwide concern that MRSA has become
82 a zoonotic pathogen in animal production. For these reasons together with the environmental
83 issues, the EU has banned the inclusion of pharmacological levels of ZnO after 2022
84 (EMA/394961/2017).

85 Since the bioavailability of mineral sources is limited and they are partially absorbed by
86 organisms, the excess is eliminated by excretion. In swine farms, wastewater-derived
87 conventional techniques of civil and livestock waste, could be valuable for agricultural
88 irrigation; however, HM contamination (Chardon *et al.* 2012; Hejna *et al.* 2018; Moral *et al.*
89 2005; Nicholson *et al.* 2003) drastically reduces their potential use in irrigation.

90 Since the use of contaminated irrigation water would be responsible for the distribution
91 of large numbers of metallic ions in the environment, the removal of HMs from manure
92 wastewaters is essential in order to improve the soil quality (Gul *et al.* 2015; Jakubus *et al.*
93 2013; Liu *et al.* 2018; Lopez-Alonso *et al.* 2012).

94 Thus, the aim of this study was to assess the ability of two aquatic species, *Typha latifolia*
95 (Broadleaf cattail) and *Thelypteris palustris* (Marsh fern), to remove Zn and Cu from
96 contaminated livestock wastewaters, given that these species have already been used to
97 decontaminate water and soils from metals (Chandra and Yadav, 2010; Hazra *et al.* 2015;
98 Manios *et al.* 2003a, b; Salem *et al.* 2017). Cattail is a wetland specie that can be grown under
99 different climatic conditions such as brackish and polluted water and because of their rapid
100 growth and easily harvesting they can be used in phytoremediation (Milam *et al.* 2004; Ahmad
101 *et al.* 2017; Rodriguez-Hernandez *et al.* 2017). Marsh fern also could be ideal aquatic plant for
102 phytoremediation due to its wide range of habitat and easy of cultivation in many environments
103 including agricultural sites, endangered coastal wetlands and urban brownfield sites (Anderson
104 *et al.* 2007). A phytoremediation pilot mesocosms system was developed, which could be easily

105 managed in animal production systems. In addition, to enable plants to work in the system for
106 a long time and to reduce the amount of exhausted plants that need disposing of a mineral
107 additive premix was dissolved in the wetland water to obtain a concentration of zinc fourteen
108 times higher than the regulation limit.

109

110 **2. Material and methods**

111 **2.1. Plant culture**

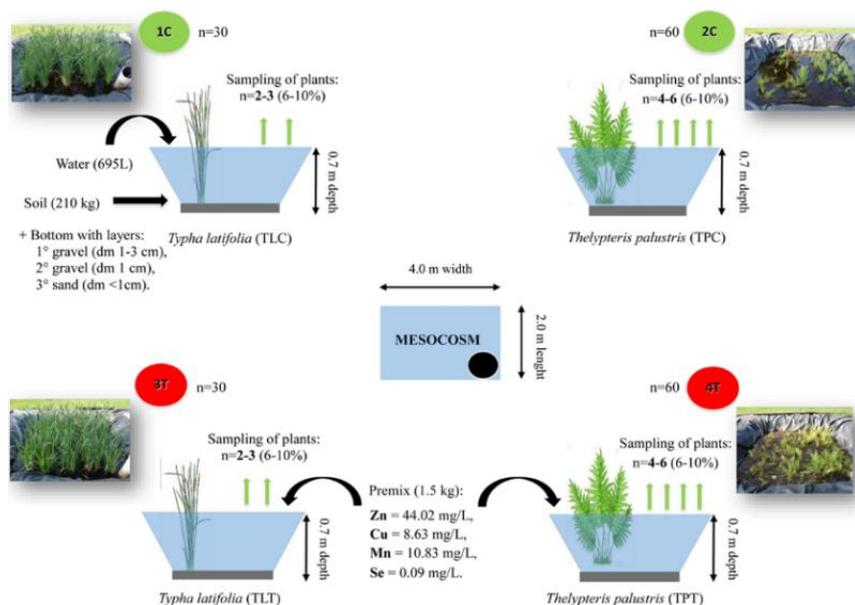
112 A pilot wetland system containing four mesocosms (width: 2.0 m; length 2.0 m; depth
113 1.2 m) was assembled outdoors at the Botanical Garden of the University of Milan (Italy). The
114 mesocosms were aligned in one row parallel to the sun's pathway to receive the same intensity
115 of light radiation. Each mesocosm had a constant flow-through capacity by a horizontal
116 submerged flow system, which was combined with the open input-output pipe.

117 Mesocosms were filled by waterproof cloths, two layers of stone chippings (1st gravel
118 with diameter 1-3 cm; 2nd gravel with diameter 1 cm) and sand (diameter <1cm) was poured
119 into the basis. In order to create positive drainage, gravel was placed, and compacted on the
120 bottom. This substratum was then induced to create a sediment upon water addition, and finally
121 210 kg of loam for plant culture composed of acid peat, pumice, clay and manure NPK (0.3 s/m
122 of electric conductibility, 300 kg/m³ dry density and 90% v/v total porosity; mature commercial
123 compost Flox Containerpflanzen, Blumenerde VitaFlor) was layered on the substratum.

124 The commercial compost used in the experimental trial contained 45.45% of ashes as
125 fresh weight (f.w.) with 8.57% humidity (Supplementary Table 1). Fresh water (650 L) was
126 added to each mesocosm. Then young healthy plants (purchased from Centro Flora) were
127 planted and were left in the substrate for one week for the adaptation. Two mesocosms were
128 used for *T. latifolia* (TL control: control, n=30; TL treated: treatment, n=30) and two

129 mesocosms were used to test *T. palustris* (TP control: control, n=60; TP treated: treatment,
 130 n=60).

131 After the adaptation (T0), 1.5 kg of a mineral commercial additive premix (feed Maxi
 132 CRC 0.5%, Alpha, Zn 20.400 mg/kg, Cu: 4.000 mg/kg, Mn: 5.020 mg/kg, Se 41 mg/kg,
 133 Vitamin K: 150 mg/kg; Vitamin B2: 440 (mg/kg); Vitamin A: 1.100.000 UI/kg; Vitamin D3:
 134 220.000 UI/kg) was dissolved, more than 14 times higher concentration for Zn referring to the
 135 maximum admitted level established by Italian regulation (for Zn: 3 mg/L according D. 337
 136 152/2006 and for Cu: 2 mg/L according 98/83/EC.) in the treatment mesocosms planted with
 137 *T. latifolia* (TL treated) and *T. palustris* (TP treated), respectively. The mineral commercial
 138 additive premix contain all essential trace elements and macronutrients for animal diet and it is
 139 normally added to the feed. The theoretical final concentrations were calculated: 44.02 mg/L
 140 of Zn; 8.63 mg/L of Cu (Figure S1). The mineral premix was added carefully to the surface of
 141 the water taking care not to spill outside the mesocosm.



142
 143 1C: *T. latifolia* control mesocosm; 2C: *T. palustris* control mesocosm; 3T: *T. latifolia* treated mesocosm; 4T: *T.*
 144 *palustris* treated mesocosm.

145 **Figure 1.** The outdoor mesocosms with the amount of mineral additive premix dissolved on the
 146 first d of the experiment (T0).

147 **2.2. Plants, soil and water sampling**

148 The experiment took place over a period of 10 weeks. Before the sampling procedure,
149 each mesocosm was separated into three homogenous areas and plants were then collected from
150 these three areas. At T0 and 15 d later (T1), and 45 d later (T2), samples of plants (aerial –
151 leaves/stem and subaerial – rhizomes/roots organs), samples of water (5 mL) and soil (300 g)
152 were collected. A total of 70% of each soil sample were collected near to the plants' roots, and
153 the remaining 30% were collected from the different mesocosm parts. The water samples were
154 derived from the horizontal submerged flow system, and were then combined with a special
155 pipe in order to proceed with the sampling process. The plants were collected from three
156 different mesocosm regions (n=2-3 of *T. latifolia* and n=4-6 of *T. palustris*; around 5-10% of
157 total amount) at T0, T1 and T2. Each plant collected was rinsed twice with the distilled water
158 in order to wash off any soil particles.

159 **2.3. Chemical composition of plant samples**

160 The dry matter (DM) of plants (subaerial organs and aerial organs separately, TL control
161 n=14; TL treated n=28; TP control n=14; TP treated n=28) was obtained by inserting the
162 samples in preweighed aluminum bags which were dried in a forced-air oven at 80°C for 72 h
163 (AOAC 2005 method; proc. 930.15; CR No. 152/2009). All dried plants were ground with a
164 laboratory mill to 0.5 mm (Cyclone Sample Mill, Model 3010-019, pbi International, Milan,
165 Italy) and were evaluated from two time experimental points (T0 and T2). Crude protein (CP)
166 was measured following the Kjeldahl method (AOAC 2005 method, proc. 2001.11). Crude
167 fiber (CF) was determined by the Filter Bag technique (AOCS 2005 method, proc. Ba 6a-05).
168 Lipid content (EE) was measured by the Soxhlet method, with prior hydrolysis (European
169 Commission Regulation No. 152/2009). Ashes were measured using a muffle furnace at 550°C
170 (AOAC 2005 method; proc. 942.05). The amylose ratio in starch, on a dry weight basis (DW)
171 was calculated (Megazyme total starch kit) by spectrophotometric evaluation at 510 nm.

172 **2.4. Evaluations of HMs in plants, soil and water samples by inductively coupled plasma**
173 **mass spectrometry (ICP-MS)**

174 A total of 0.3g of each dried plant (subaerial organs and aerial organs separately, TL
175 control n=14; TL treated n=28; TP control n=14; TP treated n=28) and 0.3 g of dried soil (0.3
176 g/DM of each; TL control n=6; TL treated n=6; TP control n=6; TP treated n=6) were
177 mineralized by an ultrawave single reaction chamber microwave digestion system (Anton Paar
178 MULTIWAVE 3000) in Teflon tubes filled with 10 ml of HNO₃ (65% concentrated) by
179 applying a one-step temperature ramp (at 120°C in 10' and maintained for 10). The mineralized
180 samples were cooled for 20 min and the homogenous samples solutions were transferred into
181 polypropylene test tubes. Plant samples (250 µl) were then diluted 1:40 with a standard solution
182 containing an internal standard (100 µl) and H₂O (9.75 ml). The soil samples (100 µl) were
183 diluted 1:100 with a standard solution containing an internal standard (100 µl) and HNO₃ (0.3
184 M, 10 ml). Water samples were analyzed without dilution (5,0 mL; TL control n=4; TL treated
185 n=4; TP control n=4; TP treated n=4).

186 An aliquot of 2 mgL⁻¹ of an internal standard solution (⁷²Ge, ⁸⁹Y, ¹⁵⁹Tb) was added to the
187 samples and calibration curve to obtain a final concentration of 20 µgL⁻¹. All samples were
188 analysed in triplicate by inductively coupled plasma mass spectrometry (ICP-MS; Bruker
189 Aurora M90 ICP-MS, Bremen, Germany) in order to detect the following elements: Na, Mg,
190 K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb (Supplementary Tables 2 and 3).
191 The accuracy of the results obtained using ICP-MS was evaluated using internal reference
192 materials supplied by LGC Standards Company: sewage sludge (LGC 61812); poultry feed
193 (LGC7173); and waste water (SPS-WW2 1). The typical polyatomical analysis interferences
194 were removed using the collision-reaction interface (CRI) with an H₂ flow of 75mL/min⁻¹
195 through a skimmer cone.

196 **2.5. Statistical analysis**

197 In order to evaluate any statistically significant differences among mean values, all data
198 were analyzed using Glimmix of SAS software (9.4., SAS Inst. Inc., Cary, NC). The analysis
199 accounted for the fixed effects of treatment, time, plant type and part, and associated two-way
200 interactions and the random effect of plant (treatment). Repeated measures were used as the
201 time (treatment). Means were considered different when $P < 0.05$ and tended to differ if $0.05 <$
202 $P \leq 0.10$. Tukey-Kramer studentized adjustments were used to separate treatment means within
203 the two-way interactions. Within significant two-way interactions, the slice option was used to
204 separate means within a specific time and plant type. The results are reported as least squares
205 means and standard errors of the means.

206

207 **3. Results**

208 ***3.1. Biomass and chemical composition of plants***

209 In order to detect the effect of Zn and Cu exposure on plant growth, the amount of dry
210 matter (DM)/plant was measured as an indicator of the biomass. *T. latifolia* and *T. palustris*
211 plants grew normally in the control and metal-treated mesocosms from T0 to T2 as showed
212 increasing trend of the plant biomass (Table 1). In fact, with respect to T0, the growth rate for
213 TL treated and TP treated was higher (504.17% and 183.33%, respectively) comparing to TL
214 control and TP control (329.63% and 131.58%, respectively). Interestingly, for both species,
215 the biomass mostly increased in T2 treated with respect to control mesocosms.

216 The principal chemical components of the control and treated plants (aerial organs and
217 subaerial organs) are presented in Table 1 for *T. latifolia* and *T. palustris*. For both plants, fiber
218 and ash content increased from T0 to T2 in all the organs in parallel to the decrease in water
219 content. However, slight differences were observed in plants grown in the control with respect
220 to the treated mesocosms. On the other hand, there was a decrease in total lipids in *T. latifolia*
221 and *T. palustris* after 45 d of metal exposure compared to the control (see T2 with respect to

222 T0; Table 1). In the control leaves of both plants, lipids slightly increased from T0 to T2, while
 223 in treated samples there was a decrease of about 10% and 25% for *T. latifolia* and *T. palustris*,
 224 respectively. In rhizome, a decrease of total lipids was observed both in the control and treated
 225 plants particularly in marsh ferns (Table 1; compare T0 and T2). However, in T2 there was a
 226 greater decrease of lipids in the treated plants than in the control plants (Table 1).

227 Proteins showed different trends in *T. latifolia* and *T. palustris*, although they increased
 228 in both plants from T0 to T2. This increase was more pronounced in marsh ferns than in
 229 Monocot plants.

230 The quantification of starch showed that the amount of this polymer was different
 231 depending on the organ or the plant. In *T. latifolia*, no differences were observed for aerial
 232 organs in the treated and control samples (Table 1; see T2 with respect to T0). An opposite
 233 trend of starch content was observed in the aerial and subaerial organs of *T. palustris* after metal
 234 exposure with respect to the control. In fact, in the control, starch decreased in leaves and
 235 increased in rhizomes during plant growth (compare T0 and T2). On the other hand, in T2
 236 treated plants, an increase of starch in leaves was accompanied by a decrease of starch in the
 237 subaerial organs with respect to T0 (Table 1).

Chemical composition

Part of plants	Treatment	Time points	Humidity (%)	Crude protein (g/100g)	Crude fiber (g/100g)	Lipids (g/100g)	Ash (g/100g)	Starch (g/100g)
<i>T. latifolia</i>								
Aerial organs	Control	T0	21.04	5.34	17.42	2.19	9.41	19.43
		T2	10.05	8.56	32.71	2.52	9.75	10.81
	Treated	T0	13.86	10.78	17.92	2.93	9.36	19.90
		T2	8.83	11.54	29.04	2.60	10.70	10.12

Subaerial organs	Control	T0	12.53	2.97	26.43	0.94	7.27	-
		T2	8.33	4.96	26.01	0.91	9.82	-
	Treated	T0	9.53	4.49	26.31	1.05	7.73	-
		T2	6.55	5.62	26.78	0.78	10.54	-

Biomass of *T. latifolia* (kg DM/Plant)

Control	T0	0.027±0.010						
	T2	0.089±0.054						
Treated	T0	0.024±0.010						
	T2	0.121±0.075						

T. palustris

Aerial organs	Control	T0	24.85	7.69	24.51	1.68	6.75	8.90
		T2	16.03	7.92	27.08	1.84	8.87	8.09
Subaerial organs	Treated	T0	23.88	7.26	24.25	1.76	7.23	6.02
		T2	22.20	10.20	29.26	1.27	9.01	12.53
Aerial organs	Control	T0	18.72	5.48	20.93	1.23	6.68	18.07
		T2	14.92	6.81	23.26	0.55	9.06	20.67
Subaerial organs	Treated	T0	15.31	4.46	21.54	3.66	8.71	22.32
		T2	13.34	11.46	27.01	0.72	10.61	16.30

Biomass of *T. palustris* (kg DM/Plant)

Control	T0	0.019±0.011						
	T2	0.025±0.018						
Treated	T0	0.018±0.011						
	T2	0.033±0.014						

238 T0: first d of the experiment, T2: 45 d later.

239 **Table 1.** The chemical composition (on DM basis) and the biomass of *T. latifolia* and *T.*
 240 *palustris* plants (for aerial organs and subaerial organs) in time points (T0 and T2) for control
 241 (TL control) and treatment (TL treated) mesocosms.

242

243 **3.2. Content of Cu²⁺ and Zn²⁺ in plants, soil and water from *T. latifolia* plants by ICP-MS.**

244 To evaluate the ability of *T. latifolia* to accumulate Zn and Cu and thus phytoremediate
 245 contaminated water, the concentrations of metals in plants, water and soil were measured in T0,
 246 T1 and T2.

247 In control mesocosms, whole *T. latifolia* plants showed the same concentration of Zn²⁺
 248 and Cu²⁺ in samples collected in T0, T1 and T2 (Table 2). The same behavior was observed
 249 when subaerial and aerial organs were considered separately. However, there was an increase
 250 of Cu²⁺ in subaerial organs of T2 controls plants, although not significant (p < 0.05), showing
 251 that root and rhizomes can accumulate Cu²⁺, which was naturally present in the soil and water.
 252 In treated samples, the plants began to accumulate Zn²⁺ and Cu²⁺ after 15 d of metal exposure,
 253 since there was only a significant increase in metal concentration in T2 (Zn: p < 0.001; TL
 254 treated = 271.64±17.71 vs. TL control = 55.79±17.71 mg/kg; Cu: p < 0.001; TL treated
 255 =47.54±3.56 vs. TL control =15.20±3.56 mg/kg; Table 2).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
TL control	T0	56.35±17.70 ^{aA}	12.64±3.56 ^{aA}
	T1	57.61±17.70 ^{aA}	10.47±3.56 ^{aA}
	T2	55.79±17.70 ^{aA}	15.20±3.56 ^{aA}
TL treated	T0	81.14±17.70 ^{aA}	13.81±3.56 ^{aA}
	T1	105.80±17.70 ^{aA}	25.92±3.56 ^{aA}
	T2	271.64±17.70 ^{bB}	47.54±3.56 ^{bB}

256 TL control: *T. latifolia* control mesocosm; TL treated: *T. latifolia* treated mesocosm; T0: first d of the experiment,
257 T1: 15 d later, T2: 45 d later.

258 a-b: the obtained values are expressed as means \pm SE; means with different superscriptions (ab) are significantly
259 different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with
260 different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control
261 and TL treated ($p < 0.001$).

262 **Table 2.** The average Zn and Cu concentration in *T. latifolia* (TL) plants in the control and
263 treatment mesocosms (TL control; TL treated) at the three time points (T0, T1, T2).

264 However, even if no significantly different was observed for the Zn and Cu concentration
265 in aerial and subaerial organs, Zn was mostly accumulated in TL treated subaerial organs, with
266 the maximum concentration at T2 (177.28 ± 30.66 mg/kg). At the same time, TL control showed
267 a concentration of zinc of about 77.16 ± 30.66 mg/kg. Similarly, the Zn concentration of aerial
268 organs was higher in T2-TL treated than T2-TL control (59.29 ± 30.66 vs. 31.26 ± 30.66 mg/kg,
269 respectively).

270 Higher Cu concentrations were also observed in aerial and subaerial organs of metal
271 treated plants with respect to the control. In addition, rhizomes/roots showed a higher Cu
272 content compared with aerial organs (33.29 ± 6.16 vs. 14.73 ± 6.16 mg/kg, respectively).

273 The increase of Zn^{2+} and Cu^{2+} concentrations in plant organs was related by a decrease
274 of these metals in water (Table 3). Zn^{2+} and Cu^{2+} were higher in the water of T0 treated
275 mesocosms with respect to the controls due to the addition of the commercial mineral additive
276 premix containing metals used in the experimental trial. The metals in the water had already
277 decreased after two weeks (T1, Table 3) remaining constant for Zn, and slightly decreasing for
278 Cu in T2 water. The decrease of metals in water was in parallel with the increase of metals in
279 soil, particularly in T2 samples (Table 3; $p < 0,001$).

Experimental groups	Time points	Concentration of heavy metals (mg/kg)
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		Zn	Cu
	T0	59.19±30.66 ^{aA}	8.88±6.16 ^{aA}
TL control soil	T1	46.01±30.66 ^{aA}	6.10±6.16 ^{aA}
	T2	58.94±30.66 ^{aA}	6.71±6.16 ^{aA}
	T0	87.18±30.66 ^{aA}	12.14±6.16 ^{aA}
TL treated soil	T1	179.72±30.66 ^{aA}	29.97±6.16 ^{aA}
	T2	578.36±30.66 ^{bB}	94.59±6.16 ^{bB}
Concentration of heavy metals (mg/L)			
	T0	0.001	0.009
TL control H₂O	T1	0.001	0.004
	T2	0.005	0.007
	T0	0.187	0.204
TL treated H₂O	T1	0.023	0.033
	T2	0.022	0.024
pH of H₂O			
	T0	7.36±0.07 ^a	
TL control	T1	7.14±0.07 ^a	
	T2	7.58±0.07 ^a	
	T0	7.00±0.07 ^a	
TL treated	T1	7.07±0.07 ^a	
	T2	7.25±0.07 ^a	

280 TL control: *T. latifolia* control mesocosm; TL treated: *T. latifolia* treated mesocosm; T0: first d of the experiment,

281 T1: 15 d later, T2: 45 d later.

282 a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly

283 different within the same time points (T0, T1, T2) between TL control and TL treated (p <0.001); means with

284 different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control
285 and TL treated ($p < 0.001$); for pH: $p < 0.05$.

286 **Table 3.** The average Zn and Cu concentration in soil and water and pH of water of *T. latifolia*
287 (TL) mesocosms in the control and treatment mesocosms (TL control; TL treated) in the three
288 time points (T0, T1, T2).

289 Since the bioavailability of metals depends on the pH in the environment, the pH values
290 has been measured. During the experiment, the pH of water varied from neutral to slightly
291 alkaline. However, even if no significantly different, in TL control the pH values remained
292 higher with respect to TL treated mesocosm (Table 3). Moreover, the mineral additive premix
293 inclusion led to a reduction of pH at the beginning of the experiment (T0 7.36 vs 7.00).

294 **3.3. Content of Cu^{2+} and Zn^{2+} in plants, soil and water from *T. palustris* plant by ICP-MS.**

295 As observed in *T. latifolia*, whole plants of *T. palustris* were also able to accumulate Zn^{2+}
296 and Cu^{2+} in their organs. In fact, higher concentrations of metals were detected in TP treated
297 than in the control already 15 d (T1) after metal addition (Table 4; $p < 0.001$) and there was a
298 slight decrease in T2 plants.

299 There was a similar trend in the aerial and subaerial organs separately, in which high
300 concentrations of Zn^{2+} and Cu^{2+} were reached in T1-TP treated samples (Table 5). Zn was
301 mostly accumulated in TP treated subaerial organs, with the maximum concentration at T2
302 (Table 5). At T2, the Zn concentration of aerial organs was higher in TP treated than TP control
303 (Table 5).

304 Cu concentration also significantly increased in T1 and T2-TP treated subaerial organs
305 compared with TP control (Table 5; $p < 0,001$), and likewise for T1-TP treated aerial organs.
306 Surprisingly, 45 d after metal addition, Cu decreased significantly in leaves of *T. palustris*
307 (Table 5, T2). Translocation of metals from subaerial organs to leaves was higher with respect

308 to *T. latifolia*, however, *T. palustris* accumulated Zn²⁺ and Cu²⁺ preferentially in subaerial
 309 organs (Table 5).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
TP control	T0	113.33±17.70 ^{aA}	11.30±3.56 ^{aA}
	T1	85.62±17.70 ^{aA}	18.25±3.56 ^{aA}
	T2	88.36±17.70 ^{aA}	16.50±3.56 ^{aA}
TP treated	T0	89.11±17.70 ^{aA}	12.46±3.56 ^{aA}
	T1	414.67±17.70 ^{bB}	136.12±3.56 ^{bB}
	T2	409.26±17.70 ^{bB}	105.58±3.56 ^{bB}

310 TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment,

311 T1: 15 d later, T2: 45 d later.

312 a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly
 313 different within the same time points (T0, T1, T2) between TL control and TL treated (p <0.001); means with
 314 different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control
 315 and TL treated (p <0.001).

316 **Table 4.** The average Zn and Cu concentration in *T. palustris* (TP) plants in the control and the
 317 treatment mesocosms (TP control; TP treated) in the three time points (T0, T1, T2).

Experimental groups	Time point	Concentrations of heavy metals (mg/kg DM)	
		Zn	Cu
<i>T. palustris</i> aerial organs			
TP control	T0	35.49±30.66 ^{aA}	7.08±6.16 ^{aA}
	T1	43.95±30.66 ^{aA}	8.94±6.16 ^{aA}
	T2	22.04±30.66 ^{aA}	8.98±6.16 ^{aA}
TP treated	T0	22.32±30.66 ^{bA}	6.59±6.16 ^{aA}

	T1	235.08±30.66 ^{bB}	119.48±6.16 ^{bB}
	T2	201.63±30.66 ^{bB}	33.03±6.16 ^{aA}
<i>T. palustris</i> subaerial organs			
	T0	191.96±30.66 ^{aA}	15,21±6.16 ^{aA}
TP control	T1	93.94±30.66 ^{aA}	31.79±6.16 ^{aA}
	T2	134.51±30.66 ^{aA}	24.19±6.16 ^{aA}
	T0	175.79±30.66 ^{aA}	18.12±6.16 ^{aA}
TP treated	T1	527.37±30.66 ^{bAB}	204.70±6.16 ^{bB}
	T2	786.49±30.66 ^{bB}	235.10±6.16 ^{bB}

318 TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment,

319 T1: 15 d later, T2: 45 d later.

320 a-b: the obtained values are expressed as means ± SE; means with different superscriptions (ab) are significantly
321 different within the same time points (T0, T1, T2) between TL control and TL treated (p <0.001); means with
322 different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control
323 and TL treated (p <0.001).

324 **Table 5.** The average Zn and Cu concentration in *T. palustris* (TP) subaerial organs and the
325 average Zn and Cu concentration in *T. palustris* aerial organs in the control and in the treatment
326 mesocosms (TP control; TP treated) in the three time points (T0, T1, T2).

327 The increase of metals in plants was related by a decrease of Zn²⁺ and Cu²⁺ in water
328 (Table 6). As observed in *T. latifolia* mesocoms, Zn²⁺ and Cu²⁺ were higher in T0 treated water
329 than in the controls; during the experimental proceed the metal concentration decreased both in
330 T1 and T2 samples (Table 6). Unlike the *T. latifolia* mesocoms, both Zn²⁺ and Cu²⁺ were present
331 at significantly higher concentrations in soil two weeks after the metals had been added (Table
332 6; p < 0.001). There was then a significant decrease in the Zn²⁺ and Cu²⁺ concentration in T2
333 soil samples (Table 6; p < 0.001), confirming the idea that the uptake of metals by plants occurs
334 preferentially by soil.

Experimental groups	Time points	Concentration of heavy metals (mg/kg)	
		Zn	Cu
TP control soil	T0	112.53±30.66 ^{aA}	11.60±6.16 ^{aA}
	T1	118.97±30.66 ^{aA}	14.02±6.16 ^{aA}
	T2	108.55±30.66 ^{aA}	16.34±6.16 ^{aA}
TP treated soil	T0	69.24±30.66 ^{aA}	12.66±6.16 ^{aA}
	T1	481.55±30.66 ^{bB}	84.17±6.16 ^{bB}
	T2	239.65±30.66 ^{aA}	48.60±6.16 ^{aA}
		Concentration of heavy metals (mg/L)	
TP control H ₂ O	T0	0.001	0.007
	T1	0.001	0.003
	T2	0.002	0.005
TP treated H ₂ O	T0	0.381	0.240
	T1	0.053	0.025
	T2	0.036	0.013
		pH of H ₂ O	
TP control	T0	7.18±0.03 ^a	
	T1	7.12±0.03 ^a	
	T2	7.29±0.03 ^a	
TP treated	T0	6.99±0.03 ^a	
	T1	7.27±0.03 ^b	
	T2	7.41±0.03 ^a	

335 TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment,

336 T1: 15 d later, T2: 45 d later.

337 a-b: the obtained values are expressed as means \pm SE; means with different superscriptions (ab) are significantly
338 different within the same time points (T0, T1, T2) between TL control and TL treated ($p < 0.001$); means with
339 different superscriptions (AB) are significantly different among different time points (T0, T1, T2) in TL control
340 and TL treated ($p < 0.001$); for pH: $p < 0.05$.

341 **Table 6.** The average Zn and Cu concentration in soil and water and pH of water of *T. palustris*
342 (TP) mesocosms in the control and treatment mesocosms (TL control; TL treated) in the three
343 time points (T0, T1, T2).

344 During the experiment, water pH varied from neutral to slightly alkaline both in the
345 control and in treated mesocosms. After the premix had been added in T0 the pH decreased in
346 TP treated compared with TP control. However, even if later pH mostly increased in T1-TP
347 treated and T2-TP treated with respect to T1-TL treated T2-TL treated mesocosms (Table 6, p
348 < 0.05 in T1).

349

350 **4. Discussion**

351 The intensive animal production system is a source of HM input into environment and
352 also a key link in the food chain. This has led to the development of approaches to increase the
353 sustainability of intensive livestock farming. Animal manure reflects the composition of their
354 diet and is frequently used as an organic fertilizer given that it contains a broad range of
355 nutrients such as nitrogen, phosphorus, potassium, as well as micronutrients and HMs.
356 Although the maximum permitted levels are well defined by EU regulations (EC N°
357 1831/2003), they are often above the physiological requirements.

358 In line with the major topics of agroecology, multidisciplinary strategies are required that
359 take into account the needs of animals (health, welfare and nutrition productivity) and farmers
360 (profitability and productivity) together with the environment. Phytoremediation system is used
361 to refine pre-treated wastewaters before they are used for irrigation (Peterson, 1998).

362 The tolerance threshold for HM accumulation in the tissues in each plant differs from
363 species to species and is determined by genetical, environmental and physiological features (Ali
364 *et al.* 2013; Lone *et al.* 2008; Mukhopadhyay *et al.* 2010; Thangavel *et al.* 2004). However, our
365 approach showed that both *T. palustris* and *T. latifolia* removed Zn and Cu from pilot wetland
366 systems contaminated by a mineral additive premix normally used in animal diets.

367 ***4.1. T. latifolia and T. palustris could work in series to refine wastewater by Cu and Zn***
368 ***phytoremediation.***

369 The ability of *T. latifolia* to accumulate metals is well known (Fediuc and Erdei 2002;
370 Hemmati *et al.* 2012; Klink *et al.* 2013; Klink *et al.* 2016; Klink 2017; Kumari and Tripathi,
371 2015; Lyubenova and Shroder, 2011; Manios *et al.* 2002, 2003 a,b; Maric *et al.* 2013; Peralta
372 *et al.* 2001; Rafati *et al.* 2011; Rai *et al.* 1995; Ye *et al.* 1997). On the other hand, the potential
373 of *T. palustris* in phytoremediation systems has only been tested for arsenic (Anderson *et al.*
374 2011).

375 In order to mimic the condition of wastewater refining systems in the livestock, an
376 outdoor pilot wetland system was used. In this system, *T. latifolia* and *T. palustris* showed
377 different capability to accumulated Cu and Zn contained after the mineral additive premix has
378 been added to the water in the TL treated and TP treated mesocosms. The decreasing trend for
379 Zn and Cu in the water and soil was accompanied by an increase of metal concentration in the
380 TL treated and TP treated plants. Our phytoremediation pilot system decontaminated the
381 wastewater from the toxic elements in line with Petroselli *et al.* 2015.

382 Analyses of HM concentrations in plants (in the whole plants or in the aerial and subaerial
383 organs) suggested that *T. palustris* was more effective than *T. latifolia* in accumulating metals
384 in subaerial organs and in translocating them to leaves in a short time. The low capacity of *T.*
385 *latifolia* to translocate metals is already reported and is considered a metal tolerance strategy
386 (Feriuc end Erdei, 2002; Klink *et al.* 2013, 2017).

387 Already after 15 d of exposure to metals, *T. palustris* was able to efficiently uptake Zn
388 and Cu, while *T. latifolia* started the accumulation process later. This difference could be due
389 to the high metal concentration in the soil. When we added metals to the water in the treatment
390 mesocoms, Zn and Cu concentrations were higher in the water than in the soil. The
391 concentrations of the metals then decreased in water and increased in soil. It has been reported
392 that in wetlands, the binding of metals to substrate is the major process for water to remove
393 metals (Almeida *et al.* 2017; Yadav *et al.* 2012). Our data suggest that metal uptake occurs
394 preferentially by the soil rather than by the water. In addition, the concentrations of Zn and Cu
395 increased earlier in the soil of *T. palustris* compared to *T. latifolia* mesocoms.

396 It is possible to hypothesize that marsh ferns modify the chemical features of soil by
397 increasing the adsorption capacity of the matrix. In fact, several molecules were released by the
398 roots into the rhizosphere and could thus modify the availability of nutrients and the matrix
399 composition (Dakora and Phillips, 2002; Lyubenova *et al.* 2013). The significant decrease of
400 metal concentrations in the soil in T2 samples suggested that *T. palustris* was more efficient in
401 short-term phytoremediation processes. The co-presence of two species which work in series
402 could increase the efficiency of the phytoremediation wetland systems.

403 In wetland systems, the degree of metal translocation by soil to plants depends on several
404 environmental conditions (Yang and Ye, 2009). The pH influences the bioavailability of metal
405 ions, and low pH promotes metal accumulation in rooted wetland plants (Emamverdian *et al.*
406 2015; Yang and Ye, 2009). The optimal condition for the uptake of several nutrients in *T.*
407 *latifolia* is a pH value of 6.5 (Brix *et al.* 2002; Dyhr-Jensen and Brix 1996). The addition of
408 mineral additive led to a decrease in water pH, which during the experiment subsequently
409 increased to slightly alkaline values. This trend has been observed in other phytoremediation
410 systems (Barakat 2011; Han *et al.* 2015; Kumari *et al.* 2015) and could be due to the ability of

411 plants to modify the pH condition in the rhizosphere (Brix *et al.* 2002; Dyhr-Jensen and Brix
412 1996). The increase in water pH to slightly alkaline values did not seem to affect plant uptake.

413 **4.2. *T. latifolia* and *T. palustris* differently respond to metal exposure in a pilot wetland** 414 **system.**

415 Our results showed that the metal concentrations used in the pilot system were not toxic
416 for the two plants, in fact the biomass increased over time. Biomass is a relevant factor for metal
417 exchange and an important aspect of the health status of plants. In fact, according to Maric *et*
418 *al.* (2013) the ideal plant for removing HMs should have a very large biomass and a rapid
419 growth. Although *T. latifolia* showed a lower capacity to absorb metals in a short period of
420 time, it may be better than hyperaccumulator plants because it produces more biomass and has
421 a higher growth rate (Ali *et al.* 2013). Interestingly, in our treated plants the biomass increase
422 was higher with respect to the control suggesting that although the metal concentrations used
423 were fourteen times higher than that permitted by Italian regulations, they stimulate plant
424 growth.

425 Zn^{2+} and Cu^{2+} are essential trace metals involved in many physiological processes in
426 plants (Arif *et al.* 2016; Emamverdian *et al.* 2015; Manios *et al.* 2002). It is possible to
427 hypothesize that these concentrations provide an amount of heavy metals which accelerates the
428 growth of *T. palustris* and *T. latifolia*. Alternatively, the increase in biomass could be a
429 tolerance mechanism of plants which grow in order to increase the number of tissues where
430 metals could be accumulated or diluted.

431 Despite the increase of biomass, some chemical variations were recorded by ICP-
432 analyses. Most relevant alterations in treated with respect to T2 control plants were detected for
433 proteins, lipids and starch. The different behaviors of protein content observed in T2-TL treated
434 and T2-TP treated with respect to the control suggested that the early uptake of metals by *T.*
435 *palustris*, could activate stress and tolerance mechanisms that enabled plants to grow in the

436 contaminated mesocosms. It is known that HMs trigger the expression of those genes that codify
437 for proteins involved in stress responses (Hasan *et al.* 2017), such as phytochelatins and
438 metallothioneins or enzymes with antioxidant activities to scavenge active oxygen species
439 (REF). These tolerance mechanisms could also be activated in *T. palustris* during metal
440 exposure.

441 After metal treatment, in T2 samples, the amount of lipid decreased with respect to the
442 control in both plants. This difference was similar in subaerial organs and in leaves, but
443 appeared more pronounced in marsh ferns compared to *T. latifolia*. This effect could be due to
444 a lower *T. palustris* metal tolerance or to a rapid accumulation of metals in this plant
445 (accompanying paper Stroppa *et al.* 2019). The ability of metals to induce a decrease in lipids
446 and changes in lipid composition has been reported in other plants (Elloumi *et al.* 2014; Oves
447 *et al.* 2016). The reduction of lipids that we detected in T2 metal exposed plants, particularly in
448 *T. palustris*, could also be due to an alteration in the carbohydrate metabolisms. In fact, in *T.*
449 *palustris*, the increase of starch in aerial organs suggests an evolution of chloroplasts into
450 amyloplasts, as also observed in microscopical analyses (accompanying paper Stroppa *et al.*
451 2019). Plastids transformation could trigger a reduction in thylakoid and thus a reduction of
452 lipid content. The decrease of starch in roots and rhizomes of both plants was different from
453 what has been reported elsewhere for other plants in phytoremediation systems since in this
454 case the starch content in roots and rhizomes increased (Frossard *et al.* 1989; Higuchi *et al.*
455 2015; Todeschini *et al.* 2011). The modification of carbohydrate metabolisms was considered
456 a response of plants to metal accumulation. In *L. perenne*, the increase in Zn induced a fructan
457 accumulation, while the increase in Cu induced an increase of starch (Frossard *et al.* 1989). In
458 our study, the presence of high amounts of starch in the leaves of *T. palustris*, suggests that it
459 has greater sensitivity to metal exposure than *T. latifolia*. (12.53 vs 10.12 g/100g in T2 treated
460 mesocosms, respectively).

461 Since these modifications occurred in the absence of visible symptoms of phytotoxicity,
462 it appears that in *T. latifolia* and *T. palustris*, some mechanisms of metal tolerance have been
463 present. However, it is not possible to exclude that some effects to metal exposure could also
464 be due to the toxicity of metals. Further analyses could better clarify this point.

465 Moreover, tested plants after the bioaccumulation process can be used as eco-material for
466 building constructions (Melià et. al, 2014). Contemporary building materials (cement concrete,
467 steel) require high energy for their production and are responsible for the emission of
468 greenhouse gases (Morel *et al.* 2001; Venkatarama Reddy and Prasanna Kumar, 2010). The use
469 of natural materials is encouraged by its availability, large quantities, affordable cost and less
470 energy needed during the production process (Melià *et al.* 2014); Thus, once at the end of life
471 the natural material is recyclable with no impact on the environment (Delgado and Guerrero,
472 2006).

473

474 **5. Conclusions**

475 The mesocosms treated with *T. latifolia* and *T. palustris* in our experiment were highly
476 contaminated with a heavy metal mineral additive premix widely used in swine nutrition. *T.*
477 *latifolia* and *T. palustris* exhibited relatively high Zn and Cu accumulation and translocation
478 abilities. In addition, *T. latifolia* and *T. palustris* tolerated high levels of Zn and Cu, with no
479 visual toxicity signs and no significant visual effect on their development throughout the
480 experimental period. To conclude, both *T. latifolia* and *T. palustris* can accumulate and
481 translocate the Zn and Cu form contaminated wastewater. However, in order to decrease critical
482 amounts of Zn and Cu in swine livestock output, when its level is critical, *T. palustris* can be
483 used to reduce the Zn and Cu content in a short period of time. On the other hand, the wastewater
484 phytoremediation for a long time could be achieved by *T. latifolia* working in series with respect
485 *T. palustris*.

486 The results suggest that the ability of the two plants to survive different concentrations of
487 Zn and Cu indicates that they could be used in a phytoremediation strategy to counteract the
488 output of zinc and copper, and possibly other HMs from the livestock industry.

489

490 **Declaration of interest**

491 The authors declare no competing financial interests.

492

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497

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