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

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

Declaration of interest

The authors declare no competing financial interests.

Highlights:

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

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

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

Abstract

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

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

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

1. Introduction

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

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

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

The scenario of livestock have changed significantly in the last decade. In fact, after the antibiotics ban in 2006 in Europe (EC, Reg. 1831/2003), there was an increased use of high dosages of zinc salts, possible after veterinary prescription as an alternative to in-feed antibiotics to control the enteric disease in the growing phases. Despite the antibacterial activity of zinc salts, the use of zinc in feed might have contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). There is worldwide concern that MRSA has become a zoonotic pathogen in animal production. For these reasons together with the environmental issues, the EU has banned the inclusion of pharmacological levels of ZnO after 2022 (EMA/394961/2017).

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

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

Thus, the aim of this study was to assess the ability of two aquatic species, *Typha latifolia* (Broadleaf cattail) and *Thelypteris palustris* (Marsh fern), to remove Zn and Cu from contaminated livestock wastewaters, given that these species have already been used to decontaminate water and soils from metals (Chandra and Yadav, 2010; Hazra *et al*. 2015; Manios *et al*. 2003a, b; Salem *et al*. 2017). Cattail is a wetland specie that can be grown under different climatic conditions such as brackish and polluted water and because of their rapid growth and easily harvesting they can be used in phytoremediation (Milam *et al*. 2004; Ahmad *et al.* 2017; Rodriguez-Hernandez *et al.* 2017). Marsh fern also could be ideal aquatic plant for phytoremediation due to its wide range of habitat and easy of cultivation in many environments including agricultural sites, endangered coastal wetlands and urban brownfield sites (Anderson *et al.* 2007). A phytoremediation pilot mesocosms system was developed, which could be easily managed in animal production systems. In addition, to enable plants to work in the system for a long time and to reduce the amount of exhausted plants that need disposing of a mineral additive premix was dissolved in the wetland water to obtain a concentration of zinc fourteen times higher than the regulation limit.

2. Material and methods

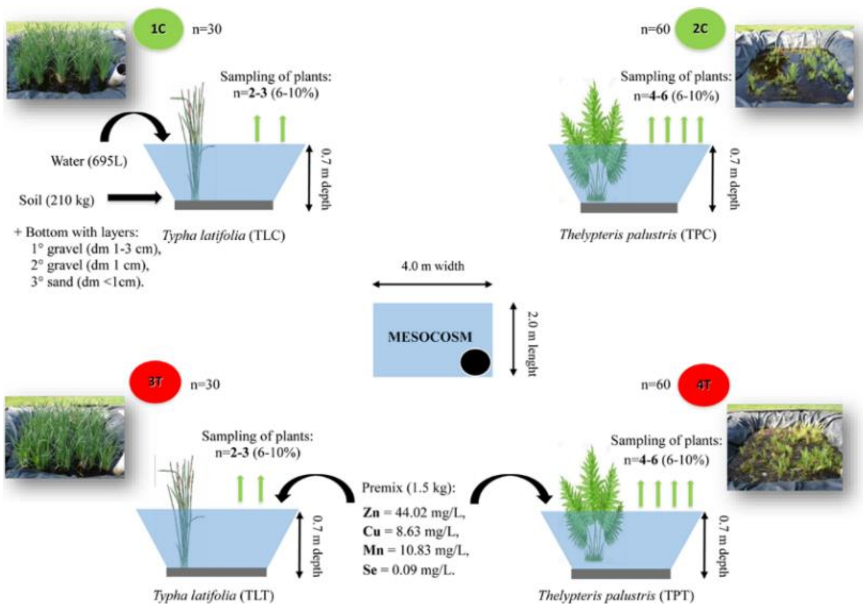
2.1. Plant culture

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

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

The commercial compost used in the experimental trial contained 45.45% of ashes as fresh weight (f.w.) with 8.57% humidity (Supplementary Table 1). Fresh water (650 L) was added to each mesocosm. Then young healthy plants (purchased from Centro Flora) were planted and were left in the substrate for one week for the adaptation. Two mesocosms were used for *T. latifolia* (TL control: control, n=30; TL treated: treatment, n=30) and two mesocosms were used to test *T. palustris* (TP control: control, n=60; TP treated: treatment, n=60).

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



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

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

2.2. Plants, soil and water sampling

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

2.3. Chemical composition of plant samples

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

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

A total of 0.3g of each dried plant (subaerial organs and aerial organs separately, TL control n=14; TL treated n=28; TP control n=14; TP treated n=28) and 0.3 g of dried soil (0.3 g/DM of each; TL control n=6; TL treated n=6; TP control n=6; TP treated n=6) were mineralized by an ultrawave single reaction chamber microwave digestion system (Anton Paar MULTIWAVE 3000) in Teflon tubes filled with 10 ml of HNO_3 (65% concentrated) by applying a one-step temperature ramp (at 120°C in 10' and maintained for 10). The mineralized samples were cooled for 20 min and the homogenous samples solutions were transferred into polypropylene test tubes. Plant samples (250 µl) were then diluted 1:40 with a standard solution containing an internal standard (100 µl) and H_2O (9.75 ml). The soil samples (100 µl) were diluted 1:100 with a standard solution containing an internal standard (100 µl) and HNO_3 (0.3 M, 10 ml). Water samples were analyzed without dilution (5,0 mL; TL control n=4; TL treated n=4; TP control n=4; TP treated n=4).

An aliquot of 2 mgL^{-1} of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added to the samples and calibration curve to obtain a final concentration of 20 μgL^{-1}. All samples were analysed in triplicate by inductively coupled plasma mass spectrometry (ICP-MS; Bruker Aurora M90 ICP-MS, Bremen, Germany) in order to detect the following elements: Na, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, and Pb (Supplementary Tables 2 and 3). The accuracy of the results obtained using ICP-MS was evaluated using internal reference materials supplied by LGC Standards Company: sewage sludge (LGC 61812); poultry feed (LGC7173); and waste water (SPS-WW2 1). The typical polyatomical analysis interferences were removed using the collision-reaction interface (CRI) with an H_2 flow of 75mL/min^{-1} through a skimmer cone.

2.5. Statistical analysis

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

3. Results

3.1. Biomass and chemical composition of plants

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

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

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

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

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

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

3.2. Content of Cu^{2+} and Zn^{2+} in plants, soil and water from T. latifolia plants by ICP-MS.

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

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

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

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

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

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

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

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

Experimental groups	Time points	Concentration of heavy metals (mg/kg)

		Zn	Cu
TL control soil	T0	59.19 ± 30.66^{aA}	8.88 ± 6.16^{aA}
	T1	46.01 ± 30.66^{aA}	6.10 ± 6.16^{aA}
	T2	58.94 ± 30.66^{aA}	6.71 ± 6.16^{aA}
TL treated soil	T0	87.18 ± 30.66^{aA}	12.14 ± 6.16^{aA}
	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)
TL control H_2O	T0	0.001	0.009
	T1	0.001	0.004
	T2	0.005	0.007
TL treated H_2O	T0	0.187	0.204
	T1	0.023	0.033
	T2	0.022	0.024
			pH of H_2O
TL control	T0	7.36 ± 0.07^{a}	
	T1	7.14 ± 0.07^{a}	
	T2	7.58 ± 0.07^{a}	
TL treated	T0	7.00 ± 0.07^{a}	
	T1	7.07 ± 0.07^{a}	
	T2	7.25 ± 0.07^{a}	

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

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

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

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

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

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

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

Cu concentration also significantly increased in T1 and T2-TP treated subaerial organs compared with TP control (Table 5; $p < 0,001$), and likewise for T1-TP treated aerial organs. Surprisingly, 45 d after metal addition, Cu decreased significantly in leaves of *T. palustris* (Table 5, T2). Translocation of metals from subaerial organs to leaves was higher with respect to *T. latifolia,* however, *T. palustris* accumulated Zn^{2+} and Cu^{2+} preferentially in subaerial 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}

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

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

Table 4. The average Zn and Cu concentration in *T. palustris* (TP) plants in the control and the 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**
	T. palustris 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	$22.32\pm30.66b^{aA}$	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}
	T. palustris subaerial organs		
TP control	T0	191.96±30.66^{aA}	15,21±6.16^{aA}
	T1	93.94±30.66^{aA}	31.79±6.16^{aA}
	T2	134.51±30.66^{aA}	24.19±6.16^{aA}
TP treated	T0	175.79±30.66^{aA}	18.12±6.16^{aA}
	T1	527.37±30.66^{bAB}	204.70±6.16^{bB}
	T2	786.49±30.66^{bB}	235.10±6.16^{bB}

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

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

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

The increase of metals in plants was related by a decrease of Zn^{2+} and Cu^{2+} in water (Table 6). As observed in *T. latifolia* mesocoms, Zn^{2+} and Cu^{2+} were higher in T0 treated water than in the controls; during the experimental proceed the metal concentration decreased both in T1 and T2 samples (Table 6). Unlike the *T. latifolia* mesocoms, both Zn^{2+} and Cu^{2+} were present at significantly higher concentrations in soil two weeks after the metals had been added (Table 6; $p < 0.001$). There was then a significant decrease in the Zn^{2+} and Cu^{2+} concentration in T2 soil samples (Table 6; $p < 0.001$), confirming the idea that the uptake of metals by plants occurs 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_2O	T0	0.001	0.007
	T1	0.001	0.003
	T2	0.002	0.005
TP treated H_2O	T0	0.381	0.240
	T1	0.053	0.025
	T2	0.036	0.013
		pH of H_2O	
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}	

TP control: *T. palustris* control mesocosm; TP treated: *T. palustris* treated mesocosm; T0: first d of the experiment, T1: 15 d later, T2: 45 d later.

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

In wetland systems, the degree of metal translocation by soil to plants depends on several environmental conditions (Yang and Ye, 2009). The pH influences the bioavailability of metal ions, and low pH promotes metal accumulation in rooted wetland plants (Emamverdian *et al*. 2015; Yang and Ye, 2009). The optimal condition for the uptake of several nutrients in *T. latifolia* is a pH value of 6.5 (Brix *et al.* 2002; Dyhr-Jensen and Brix 1996). The addition of mineral additive led to a decrease in water pH, which during the experiment subsequently increased to slightly alkaline values. This trend has been observed in other phytoremediation systems (Barakat 2011; Han *et al*. 2015; Kumari *et al*. 2015) and could be due to the ability of plants to modify the pH condition in the rhizosphere (Brix *et al*. 2002; Dyhr-Jensen and Brix 1996). The increase in water pH to slightly alkaline values did not seem to affect plant uptake.

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

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

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

Despite the increase of biomass, some chemical variations were recorded by ICP-analyses. Most relevant alterations in treated with respect to T2 control plants were detected for proteins, lipids and starch. The different behaviors of protein content observed in T2-TL treated and T2-TP treated with respect to the control suggested that the early uptake of metals by *T. palustris*, could activate stress and tolerance mechanisms that enabled plants to grow in the contaminated mesocoms. It is known that HMs trigger the expression of those genes that codify for proteins involved in stress responses (Hasan *et al*. 2017), such as phytochelatins and metallothioneins or enzymes with antioxidant activities to scavenge active oxygen species (REF). These tolerance mechanisms could also be activated in *T. palustris* during metal exposure.

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

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

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

5. Conclusions

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

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

Declaration of interest

The authors declare no competing financial interests.

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