Effects of Metal Contamination on Physical-Chemical Properties and Microbial Activities in *Phragmites Australis* Cav. Rizosphere along the Sarno River (Italy)

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Abstract

The Sarno River is one of the most polluted rivers in Italy (Campania region) marked by the presence of tanneries and skin processing factories located along its course and the large-scale dumping of untreated agricultural and industrial waste, in addition to domestic effluents. The aim of this work is to provide a preliminary analysis of the rizosphere of *Phragmites australis* Cav., interstitial and above waters. The metal contents (Cd, Cr, Cu, Ni, Pb, V, Zn and Fe, Mn) were evaluated together with their possible relationships with the physical-chemical properties of the rizosphere samples from along the river and its tributaries, which are now almost entirely fed by urban and industrial waste. With the aim to assess the possible relationships with microbial communities and their possible responses to perturbations, some enzyme activities in the rhizosphere sediments were assayed (acid and alkaline phosphatase, protease, β-glucosidase and peroxidase). The data obtained were compared to Interim Sediment Quality Guidelines (ISQGs), and a "Pollution Load Index" (PLI) was also calculated for each site. Assay analysis, in particular the activity of peroxidase, which was positively correlated with metals, showed a high level of stress. The PLI evaluated showed a progressive level of contamination from the source of the Sarno River to the delta.

Keywords: Rizosphere; *Phragmites australis* Cav; Metal; Microbial Activity

Introduction

Sediments of the riparian zone are a matrix that is complex and variable over time [1-3], in which the presence of chemical and physical gradients, biological activities and bioturbation affect the bioavailability of the substances that may be present. The surface layer (i.e. 5cm) is the active portion, while the deeper layers represent a finger-pint of the historical events or legacy of contamination that have occurred in the ecosystem [2]. For this reason, even allowing for the high biological component in terms of biodiversity and vulnerability, due to the natural and anthropogenic pressure on the system load, there is a need for continuous monitoring in order to manage and preserve the quality of these delicate ecosystems [4,5]. The riparian zone, periodically flooded, presents different plant communities with a clear dominance of *Phragmites australis* Cav., which have a large extension of the rhizosphere that makes quantitatively significant processes that occur in it [6,3]. In addition, it is precisely at the level of the rhizosphere that all chemical and biological interactions occur between the root and the surrounding sediment, which, because of

the radical exudates, is particularly rich in microorganisms. In fact, in the rhizosphere their number may be two or three times the level found in the part of the sediment not influenced by the roots [7]. In this way, a real symbiotic relationship between plants and microorganisms is created in the sediment: the plant gives to the microorganism the nutrients necessary for their development, releasing, through the roots, exudates containing organic carbon (i.e. sugars, alcohols, amino acids, organic acids and enzymes). The nutrients released stimulate and intensify the biological and degradative activity.

Microorganisms in turn provide the plant with a less toxic environment in which to grow, providing many nutrients that are released through the decomposition of organic matter. In turn, the rhizosphere can affect different parameters of the sediment, such as the nutrients transport, texture, temperature and pH, thus improving conditions for the proliferation of microorganisms [8-10].

The relationship between the sedimentary particles surrounding the rhizosphere, which in turn is closely interlinked with plant-organism relationships and the potential availability of metal sand metalloids for the biotic component of the sediment, is not completely known, and this requires greater investigation. Due to its high biological activity, the rizosphere is often a "hot spot" of biogeochemical transformations and related element fluxes [8,11,9,10].

The Sarno River is one of the most polluted rivers in Italy (Campania region) marked by the presence of tanneries and skin processing factories located along its course and the large-scale dumping of untreated agricultural and industrial waste, in addition to domestic effluents [12-15]. The Sarno is 24 km long and its basin covers 439 sq. km. That area is densely populated, containing 1,193,000 in habitants. Large-scale dumping of untreated agricultural and industrial waste into the river further aggravates the situation in addition to domestic effluents. Additionally, the area is also affected by frequent flooding and mudslides; in the last 20 years, the embankments have ruptured two or three times a year, causing a spillover of polluted waters into the adjacent countryside with all the associated risks to public health. Therefore, the aim of this work, which is part of a wider work that includes a study of "phytoremediation", is to provide a set of results and its interpretation that help in answering to these questions. In particular we analyzed the interstitial and column waters and sediments near the *Phragmites australis* Cav. roots and we evaluated metal contents (i.e. Cadmium, Cd; Cromium, Cr; Copper, Cu; Nichel, Ni; Lead, Pb; Vanadium, V; Zinc, Zn; Iron, Fe; Manganese, Mn) and their possible relationships with the physical-chemical properties of the rizosphere samples from along the Sarno River and its tributaries (Solofrana and Cavaiola), which are now almost entirely fed by urban and industrial waste water. Moreover, to evaluate the possible relationships with microbial communities and their possible responses to perturbations,

some enzyme activities (acid and alkaline phosphatase, protease, β-glucosidase and peroxidase) in the rhizosphere sediments were assayed. The data obtained were analyzed with reference to Interim Sediment Quality Guidelines (ISQGs), and a "Pollution Load Index" (PLI) was also calculated for each site to evaluate the possible contamination degree.

Material and Methods

Study area and sample collection

Five sites (Fig. 1) were chosen along the River Sarno at the following locations: site S1, about 1 km from the source of the Sarno River in the town of Foce (40 ° 49'54 "N; 14 ° 35'33" E); site S2 is located in the town of Sarno (40 ° 48'52 "N; 14 ° 37 '04" E).
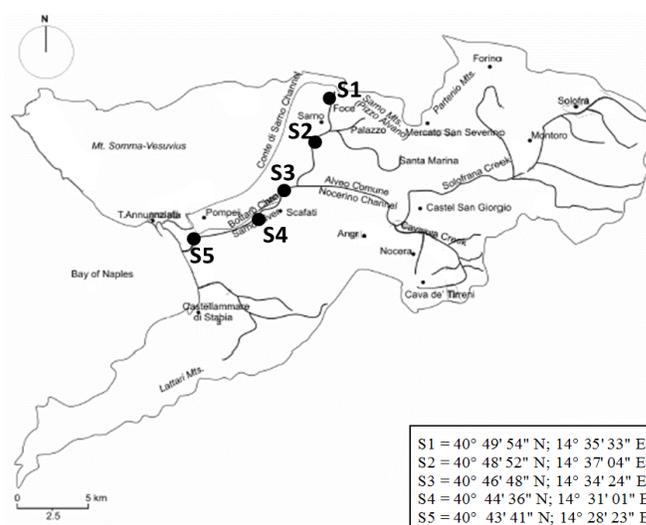

Fig. 1 Map illustrating the sampling sites along Sarno River (Italy).

In site S2, the waters of the river become more turbid and riparian vegetation is poor; site S3 is located in the countryside of San Marzano, downstream of the Cavaiola confluence and of the town, (40 ° 46'48 "N; 14 ° 34'24" E); the waters in this site are turbid and muddy, the embankments are high with dense riparian vegetation, and deposits of solid and special waste are visible; site S4 is located in the vicinity of Scafati (40 ° 44'36 "N; 14 ° 31'01" E), downstream of the town. Its waters are turbid and on its banks can be found discharges of different kinds of wastes (municipal and industrial wastewater) and downstream of the sampling site appear partially cementified; the site S5 (40 ° 43'41 "N; 14 ° 28'23" E) is situated about 400m from the delta in the zone of the transitional water. The river at this point is wide and the waters are turbid; there are numerous industrial discharges in the area. In the study area, the greatest contamination is caused by both agricultural (e.g., tobacco crops, sunflower, wheat, corn, grapes, olives and vegetables) and industrial (food, textile, paper, rubber, tanneries

and plastics, chemical, pharmaceutical, metallurgical and mineral products) activities as well as by urban-municipal waste water and landfill of all the basin municipalities. Agriculture is carried out using the river waters; in addition, agricultural wastewater, containing high levels of nitrates, phosphates and pesticides, promotes eutrophication [16,17]. The textile, tanning, metallurgical, pharmaceutical and paper industries, agricultural and domestic effluents introduce into the environment large quantities of organic compounds (current use pesticides (CUPs), PCBs, PAHs, pharmaceuticals and personal care products (PPCPs) and detergents/surfactants) that contain metals, as well as Cu, Cr, Cd, Ni, Pb and Zn.

Sample collection and analytical Methods

Rhizosphere sediment samples were collected in September and October 2011 (i.e. at the end of the summer dry season) with the purpose of comparing the differences in the accumulations along the river. The rationale to select this sampling period was due to the fact that, as is known, during floods, sediment and pollutants their associated are re-mobilized and transported further downstream, or to the final receptor or, in the event of floods, deposited on floodplain. In this regard, it is important to note that pollutant concentrations contained in the river sediments after a flood event are generally lower than those measured in the low water periods. Consequently, the most representative sampling will be made at the end of a lean period in which there was a more or less continuous material deposition [8].

As is well known, the large extent of the *Phragmites australis* rhizosphere [3] sediment samples were collected in the rhizosphere simultaneously with sampling *Phragmites australis* (data object of study for a subsequent paper).

At each sampling site, five sediment cores were collected by a snapper (Ø 5cm) from a layer between 0-15cm deep. After decantation, the above water was recovered and used for an immediate determination of pH, using an electronic pH meter (HI 8424, HANNA Instruments, Sarmeola di Rubano PD, Italy). All samples were transferred to special containers and transported in the darkness at 4°C to the laboratory for subsequent analysis [18].

In the laboratory, interstitial water pH was determined on each sediment samples, after removing roots; for enzyme activity measurements, samples were stored at -80°C until further analysis. The interstitial water was assayed by cold ultracentrifugation (4°C) [19], and, subsequently, pH was measured using an electronic pH meter, as aforementioned.

The pH of the rhizosphere sediment was measured by shaking an aliquot of sediment in distilled water (10 g of dry sediment in 25 ml of water) for 10 min. The suspension was left to stand for 10 min. The pH of the supernatant was measured with an

electronic pH meter (HI 8424, HANNA Instruments, Sarmeola di Rubano PD, Italy).

A representative portion (500 g) of each sample was used for the determination of the coarse sand, fine sand, silt and clay composition in accordance with the USPRA (U.S. Public Roads Administration) classification [20]. A second portion of each sample, oven-dried at 75°C until constant weight, was sieved (pore diameter 2 mm and nylon sieves) and ground to a fine powder using a Fritsch Germany pulverisette 6 with an agate pocket, to prevent trace element contamination.

An NCS Analyzer (Carlo Erba NA 1500) conducted total carbon (TC), inorganic carbon (IC) and nitrogen analyses in triplicate on a powdered sediment aliquot. Soil organic carbon was determined as the difference between the total carbon and the inorganic carbon evaluated treating the micro samples at 550°C for 2 h before the combustion into Elemental Analyser.

Metal (V, Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb) analyses were carried out in triplicate by atomic absorption spectrometry (SpectrAA 20 Varian) complete with graphite furnace and flame and quantified using standard solutions (STD Analyticals, Carlo Erba). Aliquots of the powdered sediment samples (250 mg) were mineralized in a Milestone Microwave Laboratory Systems Ethos 900 lab-station, endowed with temperature control, using a combination of hydrofluoric and nitric acid (HF 50% : HNO_3 65% = 1:2). After digestion the solutions were diluted with deionized water to a final volume of 50 ml. Nickel, Cr, Pb, Cu, V and Cd concentrations were measured using a graphite furnace AAS and the Fe, Zn and Mn concentrations with a flame AAS [21].

Accuracy was checked by the concurrent analysis of standard reference materials (Tab. 1) by the Resource Technology Corporation, Laramie, WY; the recovery ranged from 91 to 102%.

Metal	Certified value	Confidence interval	% recovery
Cd	0.82		101
Cr	48.1	43.4 – 52.8	95
Cu	36.4	34.8 – 37.9	102
Ni	26	23.8 – 28.1	96
Pb	95.3	90.0 - 101	96
V	44.4	39.8 – 49.0	98
Fe	33.042	31.29 – 34.80	102
Mn	260.8	249 - 272	94
Zn	133.5	126 - 141	91

Table 1. Accuracy analysis and % of recovery for each metal assayed compared to standard reference materials (Resource Technology Corporation, Laramie, WY).

In the laboratory, rhizosphere sediments collected from each

site were thawed, mixed, and analyzed for enzyme activities. In particular, acid and alkaline phosphatase activities were assayed in accordance with Tabatabai and Brenner [22] and Eivazi and Tabatabai [23], protease activity in accordance with Ladd and Butler [24], β-glucosidase activity in accordance with Tabatabai [25] and Eivazi and Tabatabai (1988) and peroxidase activity in accordance with Leatham and Stahmann [26].

Statistics

All data were inspected for outliers according to the Q-Test. Data were checked for normality and heteroscedasticity. Differences between the samples were tested with one-Way ANOVA explaining the variance followed by Tukey test (MINITAB INC 13). Pearson's simple correlation coefficient was used to determine relationships between chemical and biological data.

Means and standard deviations, reported in tables and figures, were calculated from three sampling replicates for each study site.

Results and discussion

Table 2 shows the chemical-physical properties of the sediments sampled along the Sarno River. The sediments showed an alkaline pH exhibiting values ranging from 7.71 to 7.92 and was consistent within that reported by Chen and Lin [27], with lack of release of metals other than Mn and Ni ($2.3 < pH > 7.44$ and $2.3 < pH > 7.02$ respectively). In fact, sediment pH is dependent on the buffering ability of the sediment itself and when the pH reaches a certain value (i.e. $2.3 < pH > 5.37$), the metals are released [27]. Generally, the metals solubility decreases as pH increases due to the formation of oxides and hydroxides resulting in a decreasing bioavailability. The lowest pH (7.71) in sediments was found in the site near the delta (S5) and the highest (7.92) in the site located in the town of Sarno (S2).

ment, but may be mobilized by chemical and biological agents both in sediments and in the water column [28, 29]. For this reason, pH measurements were made of the water column above the rhizosphere sediment and the interstitial water (Tab. 2). The correlation between the sediment pH and that of the above water showed significantly difference for site S2 ($p < 0.001$), where the river receives waters from other sources. As is well known, a strong influence on pH variation is exerted by the root system, strongly altering the characteristics of the rhizosphere and the metals bioavailability. As a result, the roots create favourable conditions for the microorganisms involved to modify the metals bioavailability and nutrients [30]. However, the microorganisms may also interact with the same roots in order to increase the potential for metal uptake. The potential availability of heavy metal ions and metalloids for the biotic component of the sediment is also dependent on the particles size around the rhizosphere.

Another factor that influences the pollutants distribution in the sediment is its texture and composition [31]. In this study, there was a predominance of coarse and fine sand in all sites, with a range of 54-95.5%. Silt and clay fractions, instead, showed values in the range of 0.08 to 3.14% (Tab.2). The greatest sedimentary dynamics detected in the S5 rhizosphere sediments, only for particles larger granulometry, is mainly due to the block and retention of sediment transport by *Phragmites australis* (excessive transport) favored by the embankments upstream cementified (S5 site is located near the estuary and bordered to upstream and downstream embankments cementified). Likewise, the organic matter (OM) content is very important because it is strongly related to metals. OM acts on the balance of the solution metals by means of complexation reactions, for example influencing the metals' solubility, altering their distribution between oxidized and reduced forms, altering their bioavailability and consequently their toxicity, influencing the processes of metals adsorption on

Sites	pH$_{sedriz}$	pH$_{H2Oabove}$	pH $_{H2Oint}$	Gravel %	Coarse sand %	Fine sand %	Silt and clay %	OC mg/g
S1	7.75 (0.01)	7.88 (0.01)	7.77 (0.01)	45.3	51.6	3.03	0.08	58.35 (2.91)
S2	7.92 (0.01)	8.03 (0.01)	8.08 (0.01)	43.48	52.56	3.8	0.145	50.64 (3.62)
S3	7.73 (0.01)	7.91 (0.01)	7.68 (0.01)	23.26	37.75	35.84	3.139	31.55 (1.60)
S4	7.87 (0.01)	7.99 (0.01)	7.54 (0.01)	4.38	57.85	37.65	0.102	42.25 (2.22)
S5	7.71 (0.01)	7.60 (0.01)	7.37 (0.01)	44.5	51.47	3.80	0.219	19.60 (0.99)

Table 2. Chemical - physical properties of rizosphere sediment sampled along the Sarno River. The rizosphere sediments pH (pH$_{sedriz}$), the water interstitial pH (pH $_{H2Oint}$) and above pH (pH$_{H2Oabove}$) were also reported. Standard deviation is in parenthesis.

ANOVA tests showed that the only statistically significant differences occurred between sites S2-S3 ($p < 0.05$) and S2-S5 ($p < 0.01$).

As is known, metals are not indissolubly linked to the sedi-

suspended material, and influencing the stability of metal-containing compounds [32]. The organic carbon (OC) concentration varied between 19.60 mg/g dw and 58.35 mg/g dw. (Tab. 2). In site S5 (19.6 mg/g dw), where we found the lowest pH$_{H2O}$int value (7.37), OC content was significantly lower than

in the others (p<0.001). Probably, this might be due to: 1) a high OM decomposition rate in the Scafati - delta stretch; or 2) to industrial wastes; or 3) to the formation of H_2CO_3 during OM oxidation [33]. Instead, the increase of OC in S4, S1 and S2, and the highest pH values were probably due to the release of untreated wastewaters. This was confirmed by the strongly significant correlations found between OC and the sediment pH, the above water column and the interstitial water (p<0.001).

herbicides [34,35] and also from industry. All metals assayed appeared strongly and negatively correlated to above and interstitial water pH and OC. (Tab. 5). However, no correlation were found between metals and the rizosphere sediment pH; instead, no correlation was found for the others. The metal concentrations were significantly lower (p<0.01) than those of the samples collected in areas nearby to the sampling sites but far from the rhizosphere (data not shown).

Table 3. Metal concentrations, mean ± SD (µg/L), found in the above and interstitial water in the different sites of the Sarno River. The limits set for surficial waters by Italian Legislative Decree 152/2006 are shown in parentheses.

Sites	V_{above}	V_{int}	Cd_{above} (2.3 µg/L)	Cd_{int} (2.3 µg/L)	Cu_{above} (40 µgL)	Cu_{int} (40 µg/L)	Ni_{above}	Ni_{int}	Pb_{above} (10 µg/L)	Pb_{int} (10 µg/L)	Cr_{above} (20 µg/L)	Cr_{int} (20 µg/L)
S1	12.91±0.6	11.54±0.8	3.30±0.1	3.14±0.15	3.32±0.2	3.25±0.15	12.38±0.6	11.51±0.5	3.41±0.1	3.56±0.11	2.99±0.12	2.73±0.11
S2	8.84±0.4	8.7±0.4	5.51±0.2	5.45±0.23	16.51±0.7	17.41±0.8	10.61±0.5	10.28±0.5	5.04±0.2	4.77±0.22	3.55±0.11	2.99±0.13
S3	8.17±0.5	8.12±0.4	6.53±0.3	6.38±0.26	18.15±0.8	18.60±0.8	12.46±0.6	12.31±0.6	8.19±0.3	9.06±0.43	4.83±0.20	4.44±0.20
S4	2.58±0.1	2.51±0.1	14.32±0.8	14.10±0.7	20.11±1.0	20.09±0.9	27.62±0.9	28.78±1.3	23.48±1.1	22.05±1.1	8.96±0.40	8.22±0.40
S5	2.01±0.1	1.95±0.1	22.01±1	22.21±1	202.05±9.0	198.54±8.0	36.81±1.2	36.38±1.7	35.21±1.5	35.07±1.5	47.06±2.2	47.42±2.20

Since it is known that sediments release contaminants into the water column and into interstitial water by means of a diffusion mechanism, the metals content of the water column and interstitial water were assayed (Tab. 3). When comparing the metal contents in the different waters, there were no significant differences among sites (data not shown).

These data were compared with metal concentrations reported by other authors on some of the major river of the world (Tab. 6). In particular, it was evidenced that the concentrations occurring in this study were: (a) lower than the Lambro river except for Pb in sites S4 and S5 [31] , (b) higher than the Olona river, except for sites S1, S2 and S3 for Pb [36], (c) higher than the Astura river, except for Cu in sites S1, S2, S3 and S4, and for Ni in site S1[37], (d) lower than the Calore river

Sites	V	Cd	Cu	Ni	Pb	Cr	Mn	Fe	Zn
S1	143±12.14	0.532±0.069	27.94±2.84	29.08±2.6	13.34±1.2	96.86±9.3	724± 5	14272±150	194±1.3
S2	150±14.64	0.654±0.061	33.35±3.53	36.06±3.3	45.34±4.4	97.55±9.34	730± 5	25558±159	236±2.1
S3	226±12.79	0.928±0.74	34.79±3.27	37.07±4.2	47.02±4.5	156±9.5	906± 6	29630±161	238±2.1
S4	248±13.06	1.071±0.1	63.12±6.41	52.52±7.2	74.19±6.3	232±21.3	1442±8	43457±269	297±2.2
S5	326±36	1.227±0.14	117±11.7	54.30±4.7	109±11.6	279±13.6	1341±8	47867±289	363±2.9

Table 4. Trace metal contents, mean ± SD (µg/g d.w), in the rizosphere sediment from different sites sampling along Sarno River.

Metal concentrations in the above and interstitial water were compared with the water quality guideline limits that determine the suitability of water quality for fish life (Italian Legislative Decree 152/2006). The comparison showed that the Cd limit (i.e. 2.3µg/L) was always exceeded, while the Cu, Cr and Pb limits (40µg/L, 20µg/L and 10 µg/L, respectively) were exceeded only in the sites close to the delta.

Table 4 shows metal concentrations in the rizosphere sediments sampled along the Sarno River. These concentrations increased progressively from the S1 site, at the Sarno River headwaters, to the S5 site. In particular, these increments were significantly higher (418% and 817%) for Cu and Pb. These metals may be originated from leaching of surrounding farmland, because they are used in agriculture as a soil improver and are contained in a number of pesticides, fertilizers and

except for Cd and Pb in S4 and S5 sites and for Zn in all sites [21], generally (e) higher than the Guadaia and Yamuna rivers, except for Cd [38,39], (f) and higher than the Ganga, Genesse e Gomti rivers except for Pb in S1 site. The data obtained in this study, therefore, were analyzed by reference to ISQGs [40-42]. Effects range-low (ERL) and effects range-median (ERM) guidelines [43] were re-named ISQG-Low and ISQG-High guidelines, respectively [40]. These values correspond to the lower 10th percentile (ERL) and 50th percentile (ERM) of chemical concentrations associated with adverse biological effects in field studies and laboratory bioassays from a large database compiled from studies across all three coastlines of North America [43]. These guidelines identify three ranges of concentrations of sediment-associated contaminants; the first, rarely associated with adverse effects (<ERL), the second, occasionally (<ERLs and <ERMs), and the third, frequently (>ERMs). Within this framework, it was observed that: (a)

Rizosphere sediment trace metals	pH$_{sed}$	pH$_{H2O}$ above	pH $_{H2O int}$	OC
V	NS	r= -0.71; p<0.001	r= -0.87; p<0.001	r= -0.93; p<0.001
Cd	NS	r= -0.58; p<0.01	r= -0.82; p<0.001	r= -0.89; p<0.001
Cu	NS	r= -0.71; p<0.001	r= -0.78; p<0.001	r= -0.79; p<0.001
Ni	NS	r= -0.54; p<0.01	r= -0.74; p<0.001	r= -0.71; p<0.001
Pb	NS	r= -0.52; p<0.01	r= -0.70; p<0.001	r= -0.84; p<0.001
Cr	NS	r= -0.66; p<0.001	r= -0.88; p<0.001	r= -0.82; p<0.001
Mn	NS	r= -0.58; p<0.01	r= -0.82; p<0.001	r= -0.63; p<0.001
Fe	NS	r= -0.53; p<0.01	r= -0.71; p<0.001	r= -0.75; p<0.001
Zn	NS	r= -0.57; p<0.01	r= -0.74; p<0.001	r= -0.82; p<0.001

NS=not significant

Table 5. Correlation between trace metals and pH$_{sed}$. pH$_{H2O}$ above. pH $_{H2Oint}$ and OC.

Rizosphere sediment trace metals	pH$_{sed}$	pH$_{H2O}$ above	pH $_{H2O int}$	OC
V	NS	r= -0.71; p<0.001	r= -0.87; p<0.001	r= -0.93; p<0.001
Cd	NS	r= -0.58; p<0.01	r= -0.82; p<0.001	r= -0.89; p<0.001
Cu	NS	r= -0.71; p<0.001	r= -0.78; p<0.001	r= -0.79; p<0.001
Ni	NS	r= -0.54; p<0.01	r= -0.74; p<0.001	r= -0.71; p<0.001
Pb	NS	r= -0.52; p<0.01	r= -0.70; p<0.001	r= -0.84; p<0.001
Cr	NS	r= -0.66; p<0.001	r= -0.88; p<0.001	r= -0.82; p<0.001
Mn	NS	r= -0.58; p<0.01	r= -0.82; p<0.001	r= -0.63; p<0.001
Fe	NS	r= -0.53; p<0.01	r= -0.71; p<0.001	r= -0.75; p<0.001
Zn	NS	r= -0.57; p<0.01	r= -0.74; p<0.001	r= -0.82; p<0.001

Table 6. Global compassions of heavy metal concentrations (mg/kg) detected in sediments of different rivers of the world.

Cd occurred below the ISQG-Low limit value (1.5 mg/kg) in all sites; (b) Cr, and Ni were above the ISQG-Low limit in all-sites (80 mg/kg and 21 mg/kg) and Zn in almost all sites (200 mg/kg); (c) Ni was above the ISQG-High limit (52 mg/g) only in the S4 and S5 sites; (d) Cu was above the ISQG-Low limit (65 mg/kg) only in site S5; (e) Pb was above the ISQG-Low limit (50 mg/kg) only in sites S4 and S5; (f) and Zn A further assessment of the degree of metal pollution was performed using the Pollution Load Index (PLI) [44].This index is based on the values of concentration factors (CF) calculated, for our study, by dividing the concentration of each element of the sample (sample C) by the ISQG "Low" limit (C reference sites). The PLI, for each site, was calculated as the nth root of the product of the individual values of CF. This index provides a simple and comparative way to assess the level of pollution. PLI values close to 1 indicate that the loads are close to the background level, and values greater than 1 indicate the de

PLI values close to 1 indicate that the loads are close to thebackground level, and values greater than 1 indicate the degree of Containmation.

The PLI evaluated showed a progressive increase from site S1 (town of Foce) to site S5 near the delta. In particular, sites S3, S4 and S5 show all values of PLI > 1 and site S5 even shows values of PLI > 2 (Fig. 2). The PLI calculated for each metal, (Fig.2) showed values higher than 1 for the majority of metals considered except Cd, Cu and Pb. The high PLI values found for Cr, Ni, V, Mn, Fe and Zn may be due to discharges from a variety of industries: chemical, leather, food and/or discharges affecting the urban and rural areas, in addition to domestic effluents.

In most aquatic ecosystems, a significant portion of energy linked to the nutrient cycle, is derived from microorganisms.

	Peroxidase	β-Glucosidase	Phosphatase acid	Phosphatase basic	Protease	V	Cd	Cu	Ni	Pb	Cr	Mn	Fe
Peroxidase	1												
β-Glucosidase	NS	1											
Phosphatase acid	$p<0.05$	$p<0.001$	1										
Phosphatase basic	$p<0.05$	$p<0.001$	$p<0.001$	1									
Protease	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$	1								
V	$p<0.001$	NS	NS	NS	NS	1							
Cd	$p<0.001$	NS	NS	$p<0.05$	$p<0.05$	$p<0.001$	1						
Cu	$p<0.001$	NS	$p<0.05$ (-)	NS	NS	$p<0.001$	$p<0.001$	1					
Ni	$p<0.001$	NS	NS	$p<0.01$	$p<0.01$	$p<0.001$	$p<0.001$	$p<0.001$	1				
Pb	$p<0.001$	NS	NS	NS	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	1			
Cr	$p<0.001$	NS	NS	$p<0.05$	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	1		
Mn	$p<0.001$	$p<0.01$	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	1	
Fe	$p<0.001$	NS	NS	$p<0.01$	$p<0.01$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	1
Zn	$p<0.001$	NS	NS	NS	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$

NS= not significant

Table 7. Correlation between trace metals and enzyme activities of sediments sampled in the different sites along the Sarno river.

Extracellular enzymes produced by microbial communities, working in combination, promote the degradation of organic matter and the release of nutrients that appear to be limiting [45]. In addition, enzyme activity can be used to measure the flow of microbial communities and their responses to perturbations [46,47]. In this study, we assayed different extracellular enzymatic activities, such as alkaline phosphatase and acid β-glucosidase, protease and peroxidase in different sampling sites along the Sarno River (Fig. 3). A similar trend was observed in the acid and alkaline phosphatase, β-glucosidase and protease, which returned the highest values in site S4 and the lowest in sites S2 and S5. Peroxidase, instead, shows a gradual increase from site S1 to site S5. The metals, originating from anthropogenic sources influence microbial communities, also altering enzyme activity. Many studies have shown an increase in peroxidase activity in the presence of stress [48-51].The correlations between the various enzymatic activities and the content of metals were tested in the different sampling sites along the Sarno River (Tab. 7). In particular, correlation analysis may suggest a common origin in the case of positive relationships and different emission sources in the case of negative results.

Peroxidase activity was positively correlated with all metals, suggesting that these metals have induced a state of stress in the organisms (Tab. 7). Only Cd, Ni and Cu showed correlations with protease activity and alkaline phosphatase activity. It is known that metals can cause oxidative stress in organisms [52].On the other hand, other enzymatic activities showed no correlations with the metals tested in the sediments. Besides, all the trace metals tested showed a high degree of correlation, suggesting a common origin.
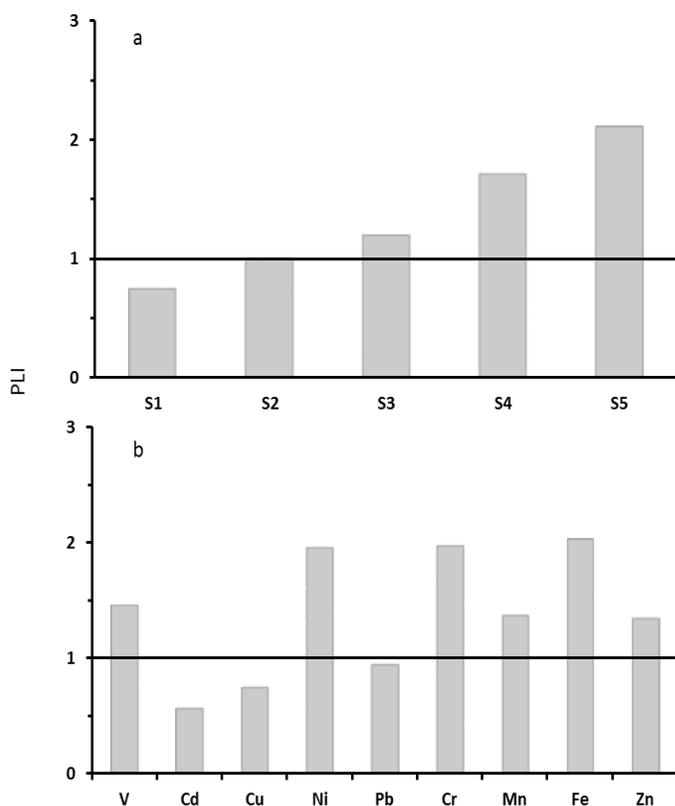

Figure 2. PLI calculated for each site (a) and metal (b) calculated using I.S.Q.G. limits (1). PLI for each site (a) increased from SI to S5, with sites S3, S4 and S5 exceeding the ISQG limit (solid lines). PLI for each metal (b) showed values higher than one for the majority of metals considered.
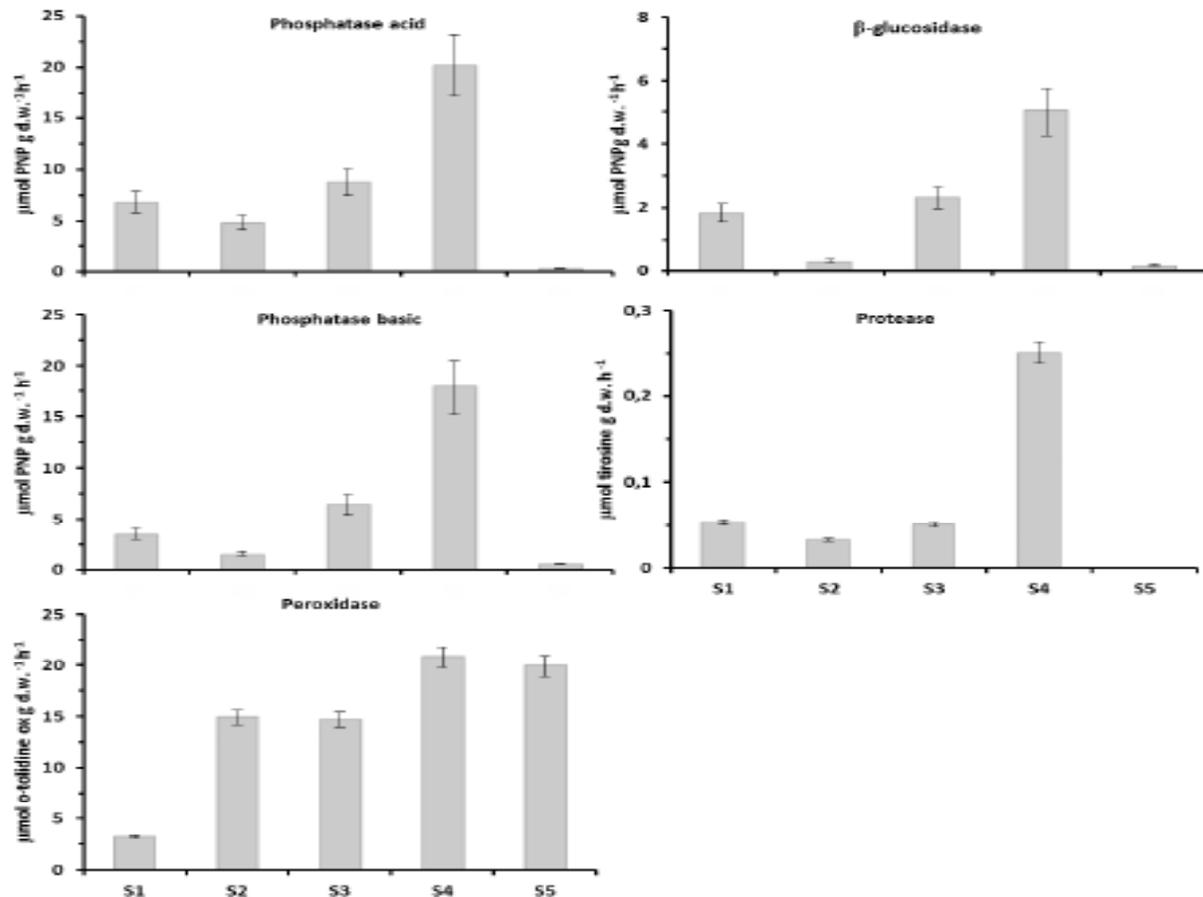
Figure 3. Extracellular enzymatic activities, such as alkaline phosphatase and acid β-glucosidase, protease and peroxidase assayed in the rizosphere sediments from different sampling sites along the Sarno River. Data are mean ± SD. In general, site S4 showed the highest extracellular enzymatic activities.

Conclusion

In our study, the metal concentrations showed a progressive accumulation towards the delta and the enzymatic analyses assayed, in particular the activity of peroxidase and protease, which were positively correlated with metals, showed a high level of stress. These findings suggest a breaking of the physiological balance between the production and the elimination, by antioxidant defence systems, of chemical species oxidants, related to the presence of metals. Exceeding of the limits proposed by Italian Legislative Decree 152/2006 and those of ISQG, the PLI data, and also the enzyme activity values, in particular peroxidase, indicate a gradual increase in pollution, with an alteration of the rhizosphere, which would result in a change in the roots of *Phragmites australis* Cav.

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