The Effects of Tannery Wastewater on the Development of Different Plant Species and Chromium Accumulation in *Phragmites australis*

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Abstract Toxicity tests were performed to assess the effect of tannery wastewater with different treatment levels on two wetland plants, *Phragmites australis* and *Typha latifolia*, which are frequently used in constructed wetlands (CWs) for water treatment, and thus deepen the knowledge on their capacity to withstand the application of industrial wastewater. *Trifolium pratense*, a plant generally used as an indicator in toxicity tests, was included as a control. End points measured were germination percentage, shoot length, root elongation, and biomass growth of the plants. When tannery effluent, with a low treatment level, was supplied to the wetland plants germination occurred even at effluent concentrations of 100%, whereas germination of *T. pratense* was completely inhibited, almost invariably, at effluent concentration of 50%. Higher germination levels were achieved when the plants were exposed to effluent originating from the outlet of constructed wetland pilot units, allowing germination of all tested plants, indicating a significant decrease in its toxicity level. Experiments conducted with the same plants using different growing substrata as the germination matrix, namely expanded clay aggregates (Filtralite® MR 3-8 and Filtralite® NR 3-8) and two types of sand (fine gravel and standard sand) have shown that higher germination levels were achieved in standard sand and that *P. australis* was the plant species showing higher germination in all cases, reinforcing the robustness of this plant to environmental stress. The phytoextraction potential of *P. australis*, was evaluated by subjecting the plant to tannery wastewater supplemented with 50 and 150 mg Cr/L. After 6 weeks of exposure, levels up to 4825, 883, and 627 mg Cr/kg were found in the rhizome, shoot, and leaves, respectively, although phytotoxic signs in the plant were evident. This plant might not be considered a chromium hyperaccumulator, but the potential to extract and accumulate this metal on its rhizomes is high.

Keywords Toxicity · Tannery wastewater · Chromium · Constructed wetlands · *Phragmites australis*

Introduction

The tanning industry is one of the oldest and most traditional industries in Portugal (INETI 2000). The discharge of effluents from this industrial sector is a matter of concern due to its high complexity and the serious pollution problems that can cause (Calheiros et al. 2007; INETI 2000; Karunyal et al. 1994; Mant et al. 2006; Sinha et al. 2002; Tišler et al. 2004). Chromium (Cr), when used in the productive cycle, is one of the most problematic pollutants discharged by the tanning industry (INETI 2000; Mant et al. 2006; Sinha et al. 2002; Zayed and Terry 2003). Its effects and mechanisms causing plant stress have been the subject of many studies (Shanker et al. 2005, Sharma et al. 2003; Sinha et al. 2002; Zayed and Terry 2003). Cr is a non-essential element to plants and it is considered to cause toxicity at multiple levels (Shanker et al. 2005). The search of plants suitable for phytoextraction of this metal is gaining a great interest.

Toxicity tests are an important tool in monitoring programs for controlling the quality of effluent discharges mainly when the composition of the wastewater might vary (Tišler et al. 2004; Wang 1990). Chemical and physical tests alone are not sufficient when assessing the potential effects of wastewaters in aquatic and terrestrial biota. The effluents might contain a wide variety of substances, not sufficiently characterized in terms of their chemical profile to

acknowledge their environmental hazard or effects, and for that, the use of the "whole effluent" as a toxicity approach might be a useful tool to point out effluents of concern (OSPAR 2005). A range of tests encompassing bacteria, algae, plants, and fishes can be used (APHA 1998; OSPAR 2005; Tišler et al. 2004). The use of plants to evaluate toxicity has several advantages (Rosa et al. 1999; Wang 1990) and can be conducted to gain knowledge on the requirements of a plant to develop under adverse conditions. The effects of water contaminants on seed germination and seedling growth of emergent plants can be a measure of a toxic response (Wang 1990). Considering that those parameters represent the first phase of plant development, a significant inhibition of this phase will affect the ability of plants to compete and survive in their environment (APHA 1998). Red clover (*Trifolium pratense*) is a plant historically used as an indicator in toxicity tests (OECD 2006); examples are the use of this plant to evaluate the impacts from limed sewage and landfill wastewater application on soil (Vasseur et al. 1998) and to assess the toxicity of composted oily waste (Juvonen et al. 2000).

Emergent plants are important components of aquatic and wetland ecosystems (APHA 1998). The use of aquatic macrophytes in water quality studies has been considered appropriate because they are commonly exposed to water pollution (Aksoy et al. 2005; Wang 1990). *Typha latifolia* and *Phragmites australis* have been used in several phytoremediation applications (Aksoy et al. 2005; Calheiros et al. 2007; Dunbabin and Bowmer 1992; Mant et al. 2006; Weis and Weis 2004). Toxicity tests constitute a valuable help in assessing the capacity of these plants to withstand the inflow of a certain wastewater, which is crucial for their successful application in a phytoremediation strategy based on constructed wetlands (CWs).

The objectives of the present study were to investigate (1) the effect of tannery wastewater originating from different stages of a wastewater treatment plant and from the outlet of constructed wetland pilot units (CWUs) on seed germination and seedling growth of a toxicity test indicator species, *T. pratense*, (2) the effect of tannery wastewater coming from the inlet and outlet of CWUs on the germination of wetland species (*P. australis* and *T. latifolia*) (3) the effect of substrata commonly used in CWs on the germination of *T. pratense*, *P. australis*, and *T. latifolia*, and (4) the capacity of *P. australis* to phytoextract Cr from tannery wastewater.

Materials and Methods

Toxicity Tests

Seed germination and seedling growth tests were conducted according to Standard Methods for the Examination of Water and Wastewater (APHA 1998) and the Organisation for Economic Co-operation and Development (OECD 2006), in a plant-growth room [photoperiod of 16/8 h, 450 μm/m^2/s photosynthetically active radiation (PAR)] for 20 days. The temperature and relative humidity were kept within 18–26 °C and 69–98%, respectively. Different treatments were applied: different types of wastewater, growth substratum, and plant species were tested. In each experiment, 15 seeds were placed in Petri dishes (85 × 15 mm) filled with the appropriate substratum and 30 ml of the test effluent solution were added at the beginning of the experiment. For each treatment, four replicates were used. Deionzed water (DW) (electrical conductivity < 0.1 μS/cm) was used as a control solution in all of the experiments and as dilution water for the different concentrations of the wastewater applied. A seed was considered to be germinated when the radicle presented a length of at least 5 mm.

Substratum Material

The substratum material used in the toxicity tests included standard sand (SS) with particle size ranging from 0.5 to 1.0 mm (AGS 0.5–1.0, from Areipor - Areias Portuguesas, Lda, Portugal), fine gravel (FG) with particle size ranging from 4 to 8 mm (AGH 4–8, from Areipor - Areias Portuguesas, Lda – Portugal), Filtralite® MR 3–8 (FMR) and Filtralite® NR 3–8 (FNR) with particle size ranging from 3 to 8 mm (from maxit - Argilas Expandidas, SA, Portugal). According to the supplier description, Filtralite® NR 3-8 has a higher hydraulic conductivity and a lower particle density than MR 3-8. All of the materials were rinsed with DW and dried in an oven at 40 °C for 4 days prior to its use. The substrata were analyzed for pH and conductivity (Houba et al. 1995).

Plant Material

Seeds of *T. pratense* were acquired from a local specialized shop. Seeds of *P. australis* and *T. latifolia* were collected from an industrial polluted site in Estarreja, Portugal and were cold-shocked at 4°C for 3 days before germination (Oliveira et al. 2001).

Tannery Wastewater

Wastewater samples corresponding to different stages of a tannery wastewater treatment plant (TWTP) were collected from a leather company, for which details on the production process are given in Calheiros et al. (2007). As detailed in Figure 1, sample A was collected after an equalization stage of the effluent, sample B was collected after a sedimentation

tank (which also corresponded to the inlet of all the CWUs), and sample C was collected after a series of filter beds (composed of gravel) placed after the sedimentation tank. Samples U1 to U6 corresponded to the outlet of six horizontal subsurface flow (HSF) CWUs placed in parallel, operating with substratum FMR and planted with different plant species: U1, *Canna indica*; U2, *T. latifolia*; U3, *P. australis*; U4, *Stenotaphrum secundatum*; U5, *Iris pseudacorus*; U6, unvegetated unit. Samples UP1 and UP2 corresponded to the outlet of two HSF CWUs operating in series and composed of substratum FMR and the plant *P. australis*, and samples UT1 and UT2 corresponded to the outlet of two HSF CWUs operating in series and composed of substratum FMR and the plant *T. latifolia.*

All of the samples were analyzed before the toxicity tests. The following parameters were determined, based on Standard Methods (APHA 1998): pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total suspended solids (TSS), Kjeldahl nitrogen (TKN), nitrate nitrogen (NO_3^--N), ammonia nitrogen (NH_3-N), total phosphorus (total P), total chromium (total Cr), and hexavalent chromium [Cr(VI]. The sulfates determination (SO_4^{2-}) was done based on Association of Official Analytical Chemists (AOAC 1995). Conductivity was registered with a WTW hand-held multi-parameter instrument 340i.

Experimental Design

In Experiment I, samples A, B, and C at different concentrations (2%, 5%, 10%, 25%, 50%, and 100%) were added to Petri dishes filled with SS and seeded with *T. pratense*. Germination percentage, root elongation, shoot length, and inhibition of growth based on biomass were assessed; the biomass of the plants was determined after drying samples at 70 °C for 48 h in an oven (Wallinga et al. 1989).

In Experiment II, sample B (inlet of CWUs) and samples U1 to U6 (outlet of CWUs) at different concentrations (3%, 10%, 25%, 50%, 70%, and 100%) were added to Petri dishes filled with SS and seeded with *T. pratense*. The seed germination was assessed.

In Experiment III, sample B (inlet of the CWUs) and samples UP1, UP2, UT1, and UT2 (outlet of CWUs) at different concentrations (25%, 50%, and 100%) were added to Petri dishes filled with SS and seeded with either *P. australis*, *T. latifolia*, or *T. pratense*. The seed germination of each plant was assessed.

In Experiment IV, the wetland plants *T. latifolia*, *P. australis*, and *T. pratense* were exposed to sample B (inlet of the CWUs) and sample U2 (outlet of CWU). Three different substrata (FMR, FNR, and FG) and SS media were used. The seed germination of each plant in each of the substrata was assessed.

Inhibition Parameters

Inhibition parameters were determined as effective concentration causing 50% of inhibition (EC_{50}) of seed germination and growth inhibition (biomass based) expressed as [(biomass of control) − (biomass of sample)]/(biomass of control).

Cr Accumulation by P. australis

Nine pots (3.5 L) were established with FMR and with three rhizome cuttings of *P. australis* each. All plants were

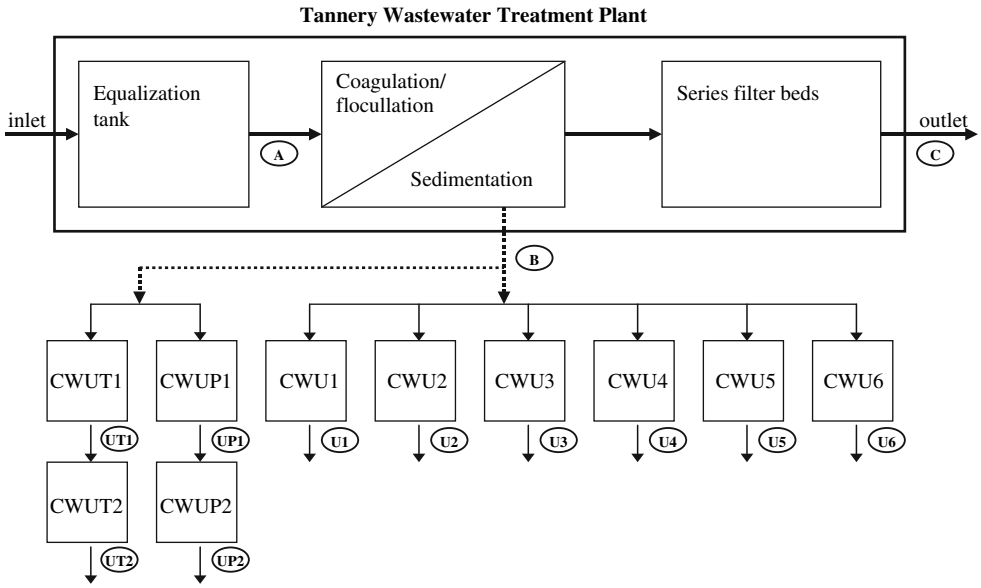

Fig. 1 Schematic representation of the sampling points in the tannery wastewater treatment plant and from the inlet and outlet of the constructed wetland units (CWUs)

collected from pots in which rhizome cuttings have been developed in FMR for 3 months and have been regularly fed with a solution of tannery wastewater at 50%. Although no monitoring of the Cr content in the wastewater was undertaken during this time, that content was expected to be low since its use in the production process is not continuous. The pots were filled with solutions to the level just below the substratum. The test solutions used in this experiment were as follows: T1, tannery wastewater at 50%; T2, tannery wastewater at 50% plus 50 mg Cr/L; T3, tannery wastewater at 50% plus 150 mg Cr/L. These concentrations were chosen because according to INETI (2000) the concentration of Cr (III) found in non-treated tannery wastewater, when it is applied in the production process, is typically around 143 mg L^{-1}. The tannery wastewater was collected after the primary sedimentation tank of the TWTP (corresponding to sample B). Cr(III) was applied in the form of basic chromium sulfate (Cromitan® B; BASF, Germany), the salt employed in the tanning company in the retanning stage and, in general, in the tanning industry in Portugal (INETI 2000).

Approximately 200 ml of fresh solution was added every week (for 6 weeks) to maintain the same level of the liquid in the pots. The experiment was carried out in a plant-growth room (photoperiod of 16/8 h, 450 µm/m^2/s PAR). The temperature and relative humidity registered during the 6 weeks of experiment were respectively 23.6 ± 1.4 °C and 42 ± 5%.

Plant Tissue Analysis

The biomass of the plant material was determined according to Wallinga et al. (1989) as described previously. For the determination of Cr content, dried rhizomes, shoots and leaves were ground and sieved to < 1 mm. The resulting samples were then digested following the determination of "total" trace elements and heavy metals by means of aqua regia. The Cr content was determined by flame atomic absorption spectrometry (Houba et al. 1995).

Root-to-Shoot Ratios

The translocation factor (TF) was determined using the following ratio (Marques et al. 2006): {[(Cr concentration in the shoot) × (shoot biomass) + (Cr concentration in the leaves) × (leaves biomass)]}/[(Cr concentration in the rhizome) × (rhizome biomass)]. The biomass ratio relating rhizome and aboveground parts was also determined (Chiu et al. 2006; Rotkittikhun et al. 2007).

Statistical Analysis

All of the data were analyzed using two-way analysis of variance (ANOVA) relating the germination percentage with different sample concentrations applied, different plant species, and different substratum used, depending on the experimental design; in Experiment IV, for *T. pratense*, a one-way ANOVA was performed. For the experiment of Cr accumulation in *P. australis*, one-way ANOVA was used to relate each ratio with the different test solution applied. When a significant *F*-value was obtained ($p < 0.05$), observation means were compared using Duncan's multiple range test. All statistical analyses were performed using the software SPSS, ver. 12.0 (SPSS Inc., Chicago, IL, USA). All of the values mentioned in this study after the symboll ± correspond to standard error.

Results and Discussion

The wastewater samples tested in this study originated from a TWTP and from the outlet of different CWUs. The characterization of each sample is shown in Table 1. The tannery wastewater used in this study showed, in general, some variability in its composition, typical of tannery wastewaters as several authors have also reported (Calheiros et al. 2007; Karunyal et al. 1994; Tišler et al 2004). The physicochemical analyses were not exhaustive due to the complex mixtures of chemicals used in the productive cycle. The tannery wastewater presented high concentrations of COD, BOD_5, and TSS, and, in general, high concentrations of TKN, NH_3, NO_3^-, and SO_4^{2-}, with lower levels when samples originated from the outlet of CWUs. Cr is not continuously used in the productive cycle and it was detected at low levels.

Toxicity of Wastewater Collected at Different Stages of a TWTP

Wastewater collected from the three different stages of the TWTP varied in composition (Table 1).

Significant differences in toxicity were found between sample A and samples B and C concerning seed germination and root elongation, although none of the wastewater samples allowed seed germination at concentrations higher than 25% (Table 2). The higher germination and development of root and shoot occurred with sample B at 2% and at 5% and sample C at 2%, when compared to the control, which could be due to

Table 1 Characteristics of the samples collected for each toxicity experiment

Experiments		Parameters											
	Samples	pH	COD (mg/L)	BOD_5 (mg/L)	TSS (mg/L)	TKN (mg/L)	NH_3 (mg/L)	NO_3^- (mg/L)	T. P (mg/L)	T. Cr (mg/L)	Cr(VI) (mg/L)	SO_4^{2-} (mg/L)	Conductivity (mg/L)
I	A	4.24	3740	1100	320	183	105	65	3.1	0.17	0.002	1120	12.43
	B	6.72	2218	940	93	150	87	50	0.41	0.04	<0.001	720	9.67
	C	6.95	1825	810	89	146	71	49	0.52	0.03	<0.001	615	9.71
II	B	7.08	2124	810	79	90	65	20	0.30	0.30	0.002	742	7.71
	U1	8.06	680	420	25	74	48	18	0.20	0.03	<0.001	507	8.13
	U2	8.14	632	430	24	63	43	15	0.21	0.01	<0.001	510	8.26
	U3	8.03	615	410	18	70	52	17	0.18	0.02	<0.001	529	8.14
	U4	8.33	743	460	23	65	58	19	0.20	0.02	<0.001	556	8.21
	U5	8.42	693	480	22	72	56	18	0.18	0.03	<0.001	521	8.10
	U6	8.01	786	490	17	68	45	16	0.22	0.02	<0.001	549	8.47
III	B	6.39	1940	770	50	120	72	43	0.71	0.21	0.010	430	5.83
	UT1	8.13	480	300	15	71	41	26	0.61	0.10	<0.001	284	5.50
	UT2	8.11	265	180	9	61	28	20	0.57	0.06	<0.001	197	5.52
	UP1	8.15	500	290	14	80	41	30	0.58	0.16	<0.001	227	5.83
	UP2	8.24	250	160	8	63	27	24	0.53	0.04	<0.001	176	4.87
IV	B	5.53	1722	680	73	109	78	48	1.2	0.10	0.078	220	7.98
	U2	8.14	695	380	21	83	47	32	0.40	0.02	<0.001	107	5.93

nutrients present in the tannery effluent. Rosa et al. (1999) have reported, for raw textile effluent, greater biomass of exposed plants when compared to control plants, which could be explained by nutrients present in that effluent.

Table 2 shows that, in general, as the concentration of the wastewater increased, inhibition of germination and growth also increased, with shoot length and root elongation decreasing. At a concentration of 100% and 50%, no germination occurred. Karunyal et al. (1994) reported that the germination of *Oryza sativa*, *Acaia holosericca*, and *Leucaena leucocephala* is restrained by tannery wastewater applied at 25% and 50% and is totally inhibited when the concentration rises to 75% and 100%. On the other hand, when that effluent is diluted to 25% and used for irrigation, it could improve the growth of *Grossypium hirsutum*, *Vigna mungo*, *Vigna unquiculata*, and *Lycopersicon esculentum*. However, plants respond differently to different effluent samples and a comparison between reports is complicated due to different test conditions (Rosa et al. 1999; Wang 1990).

For *T. pratense*, the EC_{50} was much lower for sample A when compared to samples B and C (Table 2). Wastewater originating from the equalization tank of the TWTP does not have the same level of treatment as the other two, so it can cause an inhibitory effect even when diluted to a higher extent.

Toxicity of Wastewater Collected at the Inlet and Outlet of CWUs

Wastewater Samples Derived from CWUs Established with Different Plants

In Experiment II, the toxicity of samples collected from the inlet (B) and outlet of CWUs (U1 to U6), which have been operating with different plant species for approximately 1 year, was assessed. Concerning the organic content, a reduction in COD between 63% and 71%, in BOD_5 between 40% and 49% and TSS between 68% and 78% occurred within the different CWUs. For the other parameters, a reduction occurred to lower extents (Table 1).

The type of sample and its concentration had a significant influence in seed germination (Table 3). Significant differences in germination were found between plants exposed to tannery wastewater collected from the inlet and outlet of the CWUs. No germination occurred for concentrations higher than 25% when sample B (inlet of the CWUs) was applied. However, germination of the standard plant, *T. pratense*, occurred even when samples collected from the outlet of the CWUs were applied at 100% concentration. The EC_{50} values was much higher for the outlet of the pilot units. The wastewater went through a depurative process along the CWUs and, thus, its toxicity at the

Table 2 Seed germination, shoot length and root elongation of *T. pratense* after exposure to wastewater originating from different stages of a TWTP (Experiment I)

Samples	Concentration	Germination (%)	Shoot length (mm)	Root elongation (mm)	Inhibition of growth (%)
A	2%	30 ± 10 abc	55 ± 5 de	44 ± 8 de	−3
	5%	27 ± 6 abc	39 ± 4 c	34 ± 6 cd	35
	10%	18 ± 3 ab	15 ± 3 ab	16 ± 3 ab	67
	25%	10 ± 4 a	7 ± 2 a	11 ± 4 a	78
	50%	0	0	0	100
	100%	0	0	0	100
	EC_{50} (%)	6			
B	2%	62 ± 9 d	53 ± 5 cde	63 ± 6 f	−8
	5%	52 ± 6 d	43 ± 6 cd	54 ± 5 ef	21
	10%	32 ± 6 bc	23 ± 5 b	26 ± 4 abc	48
	25%	23 ± 4 ab	11 ± 3 ab	17 ± 4 ab	61
	50%	0	0	0	100
	100%	0	0	0	100
	EC_{50} (%)	22			
C	2%	53 ± 9 d	58 ± 5 e	61 ± 5 f	−10
	5%	45 ± 6 cd	48 ± 6 cde	41 ± 7 cde	26
	10%	30 ± 7 abc	20 ± 2 ab	29 ± 4 bcd	46
	25%	22 ± 5 ab	8 ± 1 a	12 ± 2 a	57
	50%	0	0	0	100
	100%	0	0	0	100
	EC_{50} (%)	19			
	Control	50 ± 9	41 ± 7	45 ± 7	
Sample(Samp)		***	NS	**	
Concentration(Conc)		***	***	***	
Samp × Conc		NS	NS	NS	

Note: Means of four observations (±SE) followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $p < 0.05$. For two-way ANOVA: **significant effect at the level of $p < 0.01$, ***significant effect at the level of $p < 0.001$, NS = nonsignificant effect. The data were analyzed without including the respective control and concentration 100% and 50%

outlet of the CWUs was lower than at the outlet of the TWTP. In fact, the CWUs allowed a higher reduction in organic load than the TWTP. Furthermore, the fact that different plants were present in each CWU did not affect the toxicity of the effluent at the outlet of each CWU. The similar germination levels observed for the wastewater collected from the planted units and the unvegetated unit might be due to the fact that the former units had not reached maturity in terms of plant growth. This is supported by studies reported by Calheiros et al. (2007), in which a similar performance of the CWUs was observed after 17 months of operation, independently of the existence of plants in the units. CWs comprise numerous mechanisms to improve water quality and, thus, are used for wastewater treatment. The plants used in these systems contribute to the treatment of wastewater in a number of ways, although the time that takes for the system to achieve maturity might vary depending on several factors such as type of plant, environmental conditions, and type of wastewater (USEPA 1995).

Wastewater Samples Derived from CWUs Established in Series with Two Plants

In Experiment III, samples collected from the outlet of the second CWUs of the series UT (*T. latifolia*) and UP (*P. australis*) units, presented a reduction of respectively 86% and 87% for COD, 77% and 79% for BOD_5, 82% and 84% for TSS, 48% and 49% for TKN, 61% and 63% for NH_3, 53% and 44% for NO_3^-, 20% and 25% for total P, and 54% and 59% for SO_4^{2-}, comparing to the inlet tannery effluent (sample B). Table 4 shows that as the concentration of the wastewater increased, the inhibition of germination increased, in general, for all the plant species under investigation. Regardless the differently planted CWUs, the germination percentages were always higher for the samples at the outlet of the second unit, indicating a further reduction in toxicity. *T. pratense* did not germinate at a concentration of 100% of sample B, and low germination occurred at a level of 50%. For all of the samples, the plant species had a significant effect on the germination level. *P.*

Table 3 Seed germination of *T. pratense* after exposure to wastewater collected at the inlet and outlet of constructed wetland units with different plants (Experiment II)

Concentration	Germination (%)						
	Samples						
	B	U1	U2	U3	U4	U5	U6
3%	42 ± 11bcdefghijk	67 ± 9 klm	70 ± 9lm	72 ± 6m	73 ± 10m	62 ± 10ijklm	65 ± 11jklm
10%	28 ± 7abcdef	53 ± 7fghijklm	55 ± 12ghijklm	57 ± 10hijklm	45 ± 8defghijkl	50 ± 10efghijklm	44 ± 9cdefghijk
25%	17 ± 4ab	40 ± 7abcdefghij	37 ± 8abcdefghi	32 ± 6abcdefgh	30 ± 7abcdefg	34 ± 6abcdefgh	28 ± 6abcdef
50%	0	18 ± 6abc	20 ± 5abcd	25 ± 6abcde	15 ± 4a	23 ± 4abcd	22 ± 3abcd
70%	0	12 ± 3	17 ± 8	20 ± 7	10 ± 4	13 ± 3	12 ± 6
100%	0	5 ± 3	10 ± 4	9 ± 4	7 ± 3	4 ± 2	5 ± 2
Control	55 ± 6						
EC_{50} (%)	11	41	38	39	28	38	26
Sample (Samp)**							
Concentration (Conc)***							
Samp × Conc NS							

Note: Means of four observations (±SE) followed by the same letters are not significantly different according to Duncan's multiple range test at the level of $p < 0.05$. The data were analyzed without including the respective control, concentration 100% and 70%, and 50% from sample B

For two-way ANOVA: **significant effect at the level of $p < 0.01$; ***significant effect at the level of $p < 0.001$; NS = nonsignificant effect

australis always presented a higher percentage of germination. The concentration factor had a significant influence for samples coming from the first units (UT1 and UP1) but not for the samples coming from the second units (UT2 and UP2), reinforcing the reduced toxicity of the wastewater at that stage.

A higher level of treatment, encompassing two units in series, decreased the toxicity of the wastewater allowing for a higher germination percentage. The different responses in germination obtained for each plant—*P. australis* being the most tolerant species—might be related to their sensitivity to toxicants and environmental conditions. Factors such as genetics, physiology, and toxicological pathways should be involved (Rosa et al. 1999). For untreated textile effluents Rosa et al. (1999) reported that, depending on the type of plant used, the responses ranged from nontoxic (*Avena sativa*) to highly toxic (*Mucuna aterrima*, *Triticum aestivum*, *Glycine max* and *Phaseolus mungo*), or even highly stimulatory (*Vicia benghalensis* and *Oryza sativa* cvs 108 and 109).

T. latifolia and *P. australis* are often used in CWs due to their characteristics and tolerance to wastewaters with relative high and often variable concentrations of pollutants. They are considered to promote high levels of pollutant removal and *P. australis* is referred as persistent and highly invasive (USEPA 1995). Studies with CWs applied to tannery wastewater (Calheiros et al. 2007) have shown that *P. australis* and *T. latifolia* were the only species to establish successfully when compared to other plants, namely *I. pseudacorus*, *C. indica*, and *S. secundatum*.

Plant Germination in Different Substrata with Wastewater Derived from CWUs

In Experiment IV, four substrata with different characteristics were tested for their suitability for germination of two wetland species frequently used in CWs, plus the indicator plant. The wastewater samples were collected from the inlet (sample B) and from the outlet of a CWU (U2) and applied without previous dilution (100%). Germination results for *P. australis*, *T. pratense*, and *T. latifolia* are presented in Figure 2. No germination occurred for *T. pratense* in any of the four substrata when sample B was applied. For the two wetland species, the type of sample and type of substratum had a significant influence on the germination percentage. Germination was higher in SS, which might be due to its characteristic size. SS presents a different platform for the seeds to germinate when compared to the FG and to the expanded clay aggregates, a material that has been lately used in CWs. The testing of different substrata is very important because

Table 4 Seed germination of *P. australis*, *T. latifolia*, and *T. pratense* after exposure to wastewater collected from the inlet and outlet of constructed wetland units operating in series (Experiment III)

Plant species	Concentration		Germination (%)				
			Samples				
			B	UT1	UT2	UP1	UP2
P. australis	25%		45 ± 11 cde	58 ± 13 cde	73 ± 5 c	63 ± 9 d	70 ± 11 b
	50%		33 ± 7 bcd	60 ± 9 de	69 ± 4 c	61 ± 11 d	68 ± 9 b
	100%		23 ± 8 abc	55 ± 10 cde	64 ± 7 c	59 ± 9 d	65 ± 10 b
	Control	69 ± 8	e	E	c	d	b
T. latifolia	25%		31 ± 7 bcd	48 ± 9 abcde	59 ± 10 bc	50 ± 9 cd	64 ± 9 b
	50%		25 ± 5 abc	44 ± 11 abcde	53 ± 9 abc	48 ± 5 bcd	56 ± 7 ab
	100%		18 ± 3 ab	32 ± 11 abcd	46 ± 11 abc	40 ± 8 abcd	54 ± 12 ab
	Control	58 ± 14	e	cde	abc	d	ab
T. pratense	25%		11 ± 2 ab	26 ± 9 abc	34 ± 8 ab	29 ± 7 abc	33 ± 8 a
	50%		4 ± 2 b	23 ± 6 ab	31 ± 6 ab	21 ± 7 ab	30 ± 7 a
	100%		0	16 ± 4 a	30 ± 6 a	19 ± 4 a	28 ± 6 a
	Control	53 ± 10	de	bcde	abc	cd	ab
Plant species (PSp)			**	***	***	***	***
Concentration (Conc)			***	*	NS	*	NS
PSp × Conc			NS	NS	NS	NS	NS

Note: Means of four observations (± SE) followed by the same letters within each column and plant species experiment are not significantly different according to Duncan's multiple range test at the level of $p < 0.05$. For two-way ANOVA: *significant effect at the level of $p < 0.05$; **significant effect at the level of $p < 0.01$; ***significant effect at the level of $p < 0.001$; NS-non-significant effect

The data were analyzed without including the sample B at concentration 100% for *T. pratense*

they act as the support material for plants to develop in CWs. If plants face more adequate conditions, a greater success will be expected in their establishment. In terms of plant germination, in general, the higher percentage was achieved for *P. australis*. Normal plant growth and establishment might be affected by pH and tolerance to salt content in the root medium. Values of pH below 3 and above 9 might cause adverse effects on plants (Shu et al. 2001). In the case of *T. latifolia* and *P. australis*, the pH range to support growth varies between 3.0 and 8.5 and between 3.7 and 8.0, respectively (USEPA 1995). In this study, the pH was 9.75 for substratum FMR (Calheiros et al. 2007), 8.90 for FNR, and 6.97 for FG, and the pH of sample B was 5.53 and that of sample U2 was 8.14. Concerning the conductivity, in general, all species survive in the range 0–2 mS/cm and sensitive species are affected by a conductivity of 4–8 mS/cm, whereas only tolerant species can achieve satisfactory growth when conductivity is greater than 8 mS/cm (Shu et al. 2001); the conductivity for the substrata FMR, FNR, and FG were respectively 0.177, 0.132, and 0.035 mS/cm, and in the samples, the conductivity was 5.93 (U2) and 7.98 (B) mS/cm. Taking into account these variations in pH and conductivity, it is possible that they might have influenced plant development in some way. At the end of the experiment, no significant differences were registered among the germination occurring in FMR, FNR, and FG for the three plants, although further studies considering other stages of the plant development would be interesting.

Cr Accumulation by P. australis

The accumulation of the metal in the plant was found in the decreasing order rhizome > shoot > leaf (Table 5). The concentration of Cr in the leaf, shoot and rhizome increased with the concentration applied, effect also noted by other authors (Sharma et al. 2003; Sinha et al. 2002). *P. australis* grows in wet wastelands (Aksoy et al. 2005) and is used in CWs for the treatment of wastewater containing metals (Bragato et al. 2006; Weis and Weis 2004).

According to Weis and Weis (2004), the degree of upward translocation is dependent on the plant species, the metal, and several environmental conditions. Sinha et al. (2002) demonstrated that aquatic macrophytes vary greatly in the ability to accumulate Cr in the tissues and this accumulation influences the physiological status of the plants. The mechanism of partitioning is a common strategy used by plants that manage to concentrate toxic ions in the roots preventing any effect on leaves, the site of photosynthesis, and other metabolic activities (Sinha et al. 2002). In this study, the Cr level found in *P. australis*

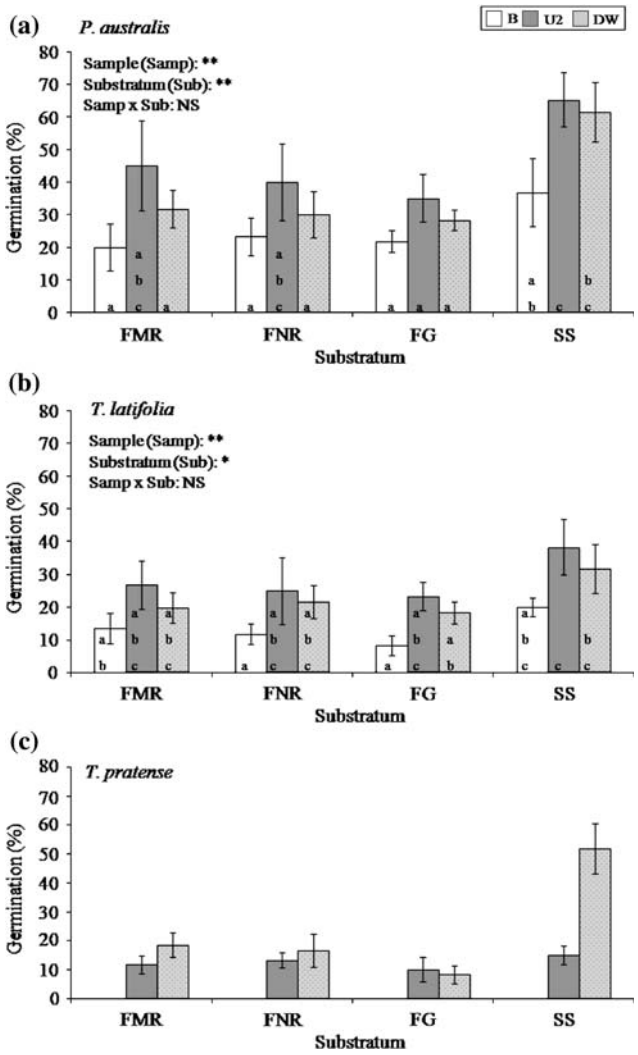

Fig. 2 Germination percentage of **(a)** *P. australis*, **(b)** *T. latifolia*, and **(c)** *T. pratense* in different substrata when subject to the inlet (sample B) and outlet of constructed wetland unit (U2) and to deionized water, for Experiment IV. Values are means of four observations ± SE. Columns marked with different letters differed significantly according to Duncan's multiple range test at of $p < 0.05$. For (a) and (b), two-way ANOVA was performed: *significant effect at the level of $p < 0.05$; **significant effect at the level of $p < 0.01$; ***significant effect at the level of $p < 0.001$; NS = nonsignificant effect. For (c), one-way ANOVA without including the data from sample B was performed

varied between 1647 and 4825 mg/kg in the rhizomes, 109 and 627 mg/kg in the leaves, and 369 and 883 mg/kg in the shoots. Other authors have also reported that *P. australis* retains a greater metal fraction in the roots than in leaves (Aksoy et al. 2005). Similar Cr levels of root accumulation (about 5000–6000 mg/kg) have been reported for *Eichhornia crassipes* after supplying 10 mg/L of Cr(VI) as potassium dichromate in nutrient solutions for a period of 14 days (Lytle et al. 1998). Another plant, *Zea mays* L. cv Ganga 5, has been shown to accumulate up to 2538 mg Cr/kg in roots and up to 611 mg Cr/kg in young leaves when subject to a solution with Cr supplied as sodium dichromate superimposed on a basal nutrient solution after 16 days of exposure (Sharma et al. 2003). Also, *Alternanthera sessilis* has been shown to accumulate 1017 ± 55 mg Cr/kg in the roots and 201 ± 35 mg Cr/kg in the leaves when subject to a Cr solution (8 mg Cr/L) for 9 days (Sinha et al. 2002). Metals in tannery wastewater occur in complex form and vary in their availability to the plants (Gupta and Sinha 2007), but in the present study, in order to account for such complexity, real tannery wastewater was used, without no addition of nutrients, as it could interfere or interact with the tested material.

In order for a phytoextraction process to be effective, substantial amounts of the Cr removed from the root medium must be translocated to the harvestable plant parts (Zayed and Terry 2003). Also, only those plants that concentrate more than 1000 mg Cr/kg are considered as an hyperaccumulators plant; this happens for species capable of accumulating metals at levels 100-fold greater than those typically measured in shoots of common nonaccumulator plants (Lasat 2002). In this study, *P. australis* accumulated low concentrations of Cr in aboveground parts. Additionally, there were also visible toxicity symptoms, such as discolored leaves and necrosis, with more incidences in the plants exposed to higher Cr concentrations (T3). Another indication for a plant to be a good candidate for phytoextraction is to have a high translocation factor (Marques et al. 2006). In this study, the TF determined for each treatment was low (Table 5), indicating that Cr accumulation is occurring mainly in the belowground parts.

The root-to-shoot ratio (biomass based) was determined, as it is considered an indicator of environmental stress by plants (Chiu *et al.* 2006; Rotkittikhun *et al.* 2007). The highest ratio obtained in this study (3.37) was exhibited for plants exposed to higher Cr concentrations. Sinha et al. (2002) analyzed the root-to-shoot ratios in the plants *A. sessilis* and *P. flavidum* subject to a tannery discharge point with Cr, and that varied between 0.87 and 16. Although *P. australis* showed phytotoxic symptoms, its use should not be neglected because its capacity to extract and concentrate high amounts of Cr in the plant belowground tissues.

Conclusion

The responses, in terms of root elongation, shoot length and germination for *T. pratense*, and in terms of germination for *T. latifolia* and *P. australis*, were assessed concerning a tannery wastewater collected from a TWTP and from the inlet and outlet of different CWUs was assessed. The samples were heavily loaded with organic and inorganic compounds. In general, as the proportion of

Table 5 Chromium content in substratum and plant tissues of *P. australis* exposed to tannery wastewater with different metal levels

Chromium level		T1	T2 (50 mgCr/L)	T3 (150 mgCr/L)	T1	T2 (50 mgCr/L)	T3 (150 mgCr/L)
		Total Cr (mg/kg dry weight)			Dry weight (g/pot)		
Plant part[a]	Leaf	109 ± 32a	568 ± 26ab	627 ± 41ab	0.47 ± 0.07	0.33 ± 0.03	0.53 ± 0.12
	Shoot	369 ± 28ab	827 ± 57b	883 ± 51b	0.60 ± 0.17	0.57 ± 0.07	0.73 ± 0.09
	Rhizome	1647 ± 27c	4585 ± 246d	4825 ± 525d	0.73 ± 0.15	2.30 ± 0.21	4.20 ± 0.30
Chromium level (CrL)		***					
Plant part (PP)		***					
CrL × PP		***					
Ratio[b]	R/SL	0.74 ± 0.08a	2.67 ± 0.08b	3.37 ± 0.34b			
	TF	0.22 ± 0.05b	0.06 ± 0.01a	0.05 ± 0.01a			

[a] Means of three observations (±SE) followed by the same letters are not significantly different according to Duncan's multiple range test at the level of $p < 0.05$. For two-way ANOVA: ***significant effect at the level of $p < 0.001$

[b] Means of three observations (±SE) followed by the same letters within each row by the same letters are not significantly different according to Duncan's multiple range test at the level of $p < 0.05$. For one-way ANOVA: significant effect at the level of $p < 0.05$

the wastewater increased, the inhibition of germination also increased. The concentration 100, 70 and 50%, for wastewater with low level of treatment, completely inhibited the germination of *T. pratense*. However, its toxicity was decreased after the wastewater had passed through a treatment stage, especially after passing through different CWUs. Higher seed germination occurred always for *P. australis*, with similar profiles in different substrata, further indicating that *P. australis* is a robust plant tolerant to different wastewaters and growth conditions.

Chromium accumulation occurred mainly in the rhizomes of *P. australis*, and the low Cr translocation factor indicates that this plant might not be the most adequate for phytoextraction because the highest Cr accumulation is not occurring in the harvestable parts of the plants and phytotoxic symptoms also occurred. However, this plant might be used to accumulate this metal in its rhizomes.

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