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Chemical and ecotoxicological effects of the use of drinking-water treatment residuals for the remediation of soils degraded by mining activities

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ABSTRACT

The aim of this study was to evaluate the use of drinking-water treatment residuals (DWTR) in the amendment of a soil affected by mining activities (Aljustrel mine, Portuguese sector of the Iberian Pyrite Belt), considering the effects on its chemical, biochemical and ecotoxicological characteristics. The DWTR had neutral characteristics (pH 6.7) and an organic matter (OM) content of 575 g kg^{-1} dry matter (DM), which makes them a potential amendment for the remediation of mine degraded soils, as they may correct soil acidity and reduce the extractable metal fraction. An incubation assay, with soil and DWTR, with or without lime, was carried out to test the doses to be used in the assisted-phytostabilization experiment. Based on the results obtained, the doses of DWTR used were the equivalent to 48, 96, and 144 t DM ha^{-1}, with and without lime application ($CaCO_3$ 11 t DM ha^{-1}). *Agrostis tenuis* Sibth was used as the test plant. Some amendments doses were able to improve soil characteristics (pH and OM content), to decrease metal extractability by 0.01 M $CaCl_2$ (especially for Cu and Zn), and to allow plant growth, that did not occur in the non-amended soil. Copper, Pb and Zn concentrations in the plant material were lower than the maximum tolerable level for cattle feed, used as an indicator of risk of entry of those metals into the human food chain. The simultaneous application of DWTR (96 and 144 t ha^{-1}), with lime, allowed a reduction in the mine soil ecotoxicity, as evaluated by some lethal and sub-lethal bioassays, including luminescence inhibition of *Vibrio fischeri*, *Daphnia magna* acute immobilization test, mortality of *Thamnocephalus platyurus*, and 72-h growth inhibition of the green microalgae *Pseudokirchneriella subcapitata*. However, DWTR were unable to increase soil microbial activity, evaluated by dehydrogenase activity, an important soil-health indicator. Also, OM content and $N_{Kjeldahl}$, concentrations increased slightly but remained low or very low (P and K extractable concentrations were not affected). In general, the bioassays highlighted a decrease in soil ecotoxicity with the presence of lime and DWTR (144 t DM ha^{-1}). In conclusion, DWTR are recommended to amend acidic soils, with high concentrations of trace elements, but an additional application of organic or mineral fertilizers should be considered.

I Introduction

In Portugal, mining exploration is ancient, at least since the Roman occupation of the peninsula (Alvarenga et al., 2004). Generally, the ore extraction and processing led to the production of large volumes of waste rocks and tailings, which were deposited in the surroundings of the mine (Matos and Martins, 2006). Nowadays, many of these mines are abandoned, but the erosion of these soils, by wind and water, contributes to the progressive enlargement of the contaminated area (Alvarenga et al., 2004; Matos and Martins, 2006). In Portugal, thousands of hectares of abandoned mine lands, affected by low pH, poor nutritional conditions, and high concentrations of potentially toxic trace elements, need reclamation (Alvarenga et al., 2012; 2103; Matos and Martins, 2006; Abreu et al., 2010; Silva et al., 2009).

The most conventional option for the rehabilitation of areas affected by mining activities consists in digging out the polluted soil and its confinement, without treatment, in a controlled landfill, followed by surface capping using unpolluted material, excavated from elsewhere. This option is often expensive and can have a huge environmental impact in both sites, the one to be recovered and the one where the soil was deposited (Sarkar et al., 2007; Volchko et al., 2014; Soderqvist et al., 2015). Therefore, it is very important to develop strategies that can treat and stabilize contaminants *in situ*, in an efficient and cost-effective manner (Sarkar et al., 2007; Volchko et al., 2014; Soderqvist et al., 2015; Pena et al., 2015). *In situ* chemical immobilization, where land applied amendments are used to retain contaminants *via* adsorption and/or precipitation reactions, is an effective and cost-effective option (Adriano et al., 2004). Even more promising, is to combine the *in-situ* immobilization of metals with phytoremediation, in a strategy that can be called “aided phytostabilization”. The latter could be a realistic, environmentally sound, and cost-effective alternative, especially for vast industrial sites, like abandoned mine areas, because not only the potentially toxic trace elements are immobilized, but also because the soil is protected from erosion by the establishment of a plant cover (Vangronsveld et al., 1995; Tordoff et al., 2000; Mench et al., 2003; Wong, 2003; Adriano et al., 2004; Perez-de-Mora et al., 2006; Alvarenga et al., 2009a, 2009b; Pardo et al., 2011; Galende et al., 2014; Pena et al., 2015). In a phytostabilization strategy, it is very important to improve the soil capacity to sustain a plant cover, which can be achieved by properly amending the soil, for instance with organic and inorganic amendments, like those obtained in the valorisation of wastes (Alvarenga et al., 2008a, 2009a, 2014; Pardo et al., 2011; Galende et al., 2014; Pena et al., 2015). The soil amendment can promote the re-establishment of a vegetative cover on these soils by: adding essential nutrients for plant growth, increasing the organic matter content, raising the pH, increasing the water-holding capacity, and by rendering the metals less mobile/bioavailable by shifting them from “plant- available” forms, extractable with water or solutions of neutral salts, to fractions associated with organic matter, carbonates or metal oxides (Alvarenga et al., 2008a, 2009a, 2014; Pena et al., 2015). Numerous inorganic (*e.g.* clays, red mud, Al/Fe/Mn oxides and hydroxides; Lombi et al., 2004; Sarkar et al., 2007; Rodriguez-Jorda et al., 2010a; Rodriguez-Jorda et al., 2010b; Castaldi et al., 2014; Garau et al., 2014) and/or organic amendments (*e.g.* sewage sludge, municipal solid waste compost, green waste compost, biochar; Beesley et al., 2010; Perez-de- Mora et al., 2011; Pardo et al., 2011; Galende et al., 2014) have been land-applied with success, reducing trace elements mobility.

Polyelectrolytes, like Fe and Al salts (*e.g.* $FeCl_3$, $Fe_2(SO_4)_3$, Al_2O_3), are often used to remove particulate and dissolved constituents from water supplies, acting as coagulant agents, in the production of potable drinking water (Ippolito et al., 2011). Besides the desired treated water, a waste sludge is produced, called drinking-water treatment residuals (DWTR), which are rich in Fe/Al (hydr)oxides. Elliott and Dempsey (1991), reported one of the first studies of the agronomic effects of land application of these sludges, emphasizing the need for their useful use, as opposed to the large costs for their landfill disposal. However, they alerted for the fact that DWTR should be mostly used as a soil conditioner, for their liming and water-holding capacities, and not because of their organic matter or nutrient content (Elliott and Dempsey, 1991). That liming capacity, associated with the potential ability of Fe/Al (hydr)oxides to bind As in soils (Lombi et al., 2004; Sarkar et al., 2007; Castaldi et al., 2014; Garau et al., 2014), may turn these residual materials into interesting conditioners in trace elements contaminated soils. Several authors have evaluated the capacity of DWTR to immobilize As and other metalloids in contaminated soils, because it is known that the adsorption of As in soils is primarily controlled by Fe/Al (hydr)oxides (Sarkar et al., 2007; Garau et al., 2014; Castaldi et al., 2014). In fact, Fe/Al (hydr)oxides can be very effective in decreasing metalloids bioavailability in soils due to their high specific surface area and reactive surficial functional groups (Sarkar et al., 2007; Garau et al., 2014). Other authors have studied the capacity of DWTR, in combination with diammonium phosphate, composted biosolids, or lime-stabilized biosolids, to act as metal adsorbents in tailings contaminated with Pb, Zn and Cd, and found promising results with one of the combinations, which allowed the revegetation of the tailing (Ippolito et al., 2011; Brown et al., 2007).

The improvement in soil quality, because of its remediation, can be assessed by general physicochemical properties, which are related to soil fertility status, and using chemical extraction procedures, as surrogate measures of trace elements immobilization (Rao et al., 2008; Alvarenga et al., 2009a, 2013; Davidson, 2013). However, chemical data should be complemented with results from biochemical and ecotoxicological tests, which allow an integrated evaluation of the toxic effects of pollutants on organisms and the interactions between contaminants, matrix and biota (ISO 17402, 2008; Leitgib et al., 2007; Alvarenga et al., 2009b; Epelde et al., 2009; Epelde et al., 2014). Ecotoxicological tests using aqueous soil extracts can be used to assess soil toxicity, not only because chemical compounds present in the soil aqueous phase affect soil organisms, but also because they evaluate the impact of soil composition on ground water and runoff to surrounding receiving waters (*i.e.* soil retention function) (van Gestel et al., 2001; Loureiro et al., 2005; Leitgib et al., 2007; Antunes et al., 2008; Alvarenga et al., 2008b, 2009b, 2016).

Another important indicator of soil fertility and ecological status are soil enzymatic activities. Dehydrogenase is an intracellular oxidor- eductase, related to the oxidative phosphorylation process, which, because of that, can be used as an overall indicator of microbial activity in a soil (Tabatabai, 1994; Gil-Sotres et al., 2005; Izquierdo et al., 2005; Tejada et al., 2006). That is why the measurement of its activity has been used by several authors as an early and sensitive indicator of soil health recovery in remediation processes (Perez-de-Mora et al., 2005, 2006; Tejada et al., 2006; Hinojosa et al., 2008).

Taking all these facts into account, the aim of this study was to evaluate the effectiveness of DWTR, from the Roxo water treatment plant (Alentejo - Portugal), as the amendment of a soil affected by mining activities (Aljustrel mine, Iberian Pyrite Belt), in order to assess: (i) the effects of the amendments in soil chemical properties, (ii) the capacity of the soil to establish a plant cover using *Agrostis tenuis* Sibth., (iii) the capacity of DWTR to immobilize metals in the soil, avoiding their transference to the plant, and (iv) the effects of the amendments in the soil ecotoxicological and biochemical properties.

2. Materials and methods

2.1. Characterization of the drinking water treatment residuals

Drinking water treatment residuals were obtained from a water treatment plant (WTP) which is located at the Roxo dam in Alentejo (Portugal). The water is captured in the dam and submitted to a sequence of processes and operations to produce a safe drinking water. First, the water passes through a screen to remove coarse material, and then to a pre-chlorination, by the injection of ClO_2, for algae control and arresting biological growth. The addition of a polyelectrolyte, Al_2O_3, promotes the coagulation and flocculation steps, which neutralizes the negative charge of the suspended colloids, promoting coagulation and the formation of thicker flocs, which settle during the sedimentation. The clear water obtained on top, after the settlement, will pass through sand and activated carbon filters, to remove dissolved particles, and, afterwards it is submitted to a final disinfection, again with ClO_2, which has a strong oxidizing potential and will not only kill any remaining microorganism, but also allow the elimination of iron and manganese.

Drinking water treatment residuals are produced during the sedimentation and filtration stages and are submitted to thickening to increase their solid content. First, an organic flocculant is added (Superfloc C-496®), which will promote the liquid-solid separation, and then they are mechanical dewatered in a belt-filter press. At the time of this experiment, all DWTR produced at Roxo WTP were sent to a municipal landfill.

The characterization of the DWTR was performed in accordance with the Decree-Law No. 276/2009, which regulates the use of sewage sludge, and similar sludges, in agricultural practices (Table 1), with techniques described elsewhere (Alvarenga et al., 2015). The DWTR have neutral characteristics (pH 6.7), which may contribute to the

Table 1
Characterization of the drinking-water treatment residuals (DWTR) (mean ± standard-deviation, n = 3), and the Portuguese legal limits for different classes of contaminants in sewage sludges intended to be land applied.

		DWTR	Legal limits[a]
Agronomic parameters			
	Dry matter (%) pH	19.0 ± 0.2 6.7 ±	-
	EC (mS cm^{-1})	0.0 0.92 ± 0.02	
	OM (g kg^{-1} DM)	575 ± 1	
	N(gkg^{-1} DM)	6.3 ± 0.8	
	P(gP_2O_5 kg^{-1} DM)	<6	
	K(g K_2O kg^{-1} DM)	490 ± 16	
	Ca (g kg^{-1} DM)	295.0 ± 5.5	-
	Mg (g kg^{-1} DM)	6.2 ± 0.1	-
Metals	Cd (mg kg^{-1} DM)	1.8 ± 0.1	20
	Cr (mg kg^{-1} DM)	< 6.67	1000
	Cu (mg kg^{-1} DM)	20.4 ± 0.4	1000
	Hg (mg kg^{-1} DM)	0.05 ± 0.00	16
	Ni (mg kg^{-1} DM)	18.2 ± 0.5	300
	Pb (mg kg^{-1} DM)	2.7 ± 1.2	750
	Zn (mg kg^{-1} DM)	28.9 ± 0.8	2500
Organic contaminants	LAS (mg kg^{-1} DM)	26	5000
	NPE (mg kg^{-1} DM)	< 0.05	450
	PCB (mg kg^{-1} DM)	< 7.8	0,8
	PAH (mg kg^{-1} DM)	18	6
	PCDD/F (ng TE kg^{-1} DM)	8.7 ± 3.0	100
Pathogenic microorganisms	*Escherichia coli* (CFUg^{-1})	< 10	< 1000
	Salmonella spp (Present/Absent 50g^{-1})	Absent	Absent 50g^{-1}

[a] Decree-Law No. 276/2009; DWTR: drinking-water treatment residuals; EC: electrical conductivity; OM: organic matter; DM: dry matter; LAS: linear alkylbenzene sulphonate; NPE: Nonylphenol ethoxylates; PCB: Polychlorinated biphenyls (sum of the concentrations of seven compounds); PAH: Polycyclic aromatic hydrocarbons (sum of the concentrations of 16 compounds); PCDD/F: Polychlorinated dibenzodioxins and polychlorinated dibenzofurans; TE: toxicity equivalents; DM: dry matter; CFU: colony forming units.

increase in soil pH, important to reduce the bioavailable fraction of trace elements, and high OM content (575 g kg^{-1} DM), which makes them promising to improve soil nutritional characteristics. Trace elements and pathogenic indicator microorganisms content were below the limit values established by law (Decree-Law No. 276/2009), but the polycyclic aromatic hydrocarbon (PAH) content was above legal limits, which restricts its use in generalized agricultural practices. However, they can be used in the remediation of mine degraded soils, as suggested in this study.

2.2. Soil characterization

The soil used in the study was collected in the Aljustrel mine (Alentejo), in the Portuguese sector of the Iberian Pyrite Belt. The weather in this area is characterized by a Mediterranean mesothermic humid climate, with hot and dry summers, and with low pluviosity (annual average rainfall of 500-650 mm; Reis and Gonçalves, 1987). The mineralization is characterized by the dominance of pyrite (FeS_2), associated with other sulphides, like chalcopyrite ($CuFeS_2$), sphalerite (ZnS) and galena (PbS) (Alvarenga et al., 2004). Pyrite was extracted from 1850 to 1980, when its production was discontinued, and was the main responsible for the pollution observed (in soils, tailings, superficial water, and sediments) (Matos and Martins, 2006). The mine exploration was re-established by ALMINA - Minas do Alentejo, S.A., after 2009, with a complete different approach towards environmental issues, but the area formerly affected by the mining activities, still needs to be recovered. The mine site rehabilitation is being done by EDM (http://edm.pt/), a state company, which has mostly used constructive techniques to dig and contain the contaminated tailings and other waste materials which were deposited in the area, but the soil that was left uncovered still needs to be ameliorated with the best available techniques.

The soil was collected from the 20-cm topsoil, air-dried and sieved through a 2-mm non-metallic sieve. Soil physicochemical analysis were performed using well described methodologies (Alvarenga et al., 2008a): particle-size distribution was determined by the pipet method (Gee and Bauder, 1986); soil pH (H_2O) was determined in a soil to deionised water suspension of 1:2.5 (w/v); electrical conductivity (EC) was determined in a soil to deionized water suspension of 1:5 (w/v); total nitrogen was analysed by the Kjeldahl method ($N_{Kjeldahl}$); total oxidizable organic carbon (C_{org}) was determined according to Walkley and Black (1934), and converted to organic matter content (OM) by multiplying by a factor of 1.72; extractable P and K were determined using the Egner-Riehm method (Riehm, 1958); and pseudo-total metal concentrations (Cd, Cr, Cu, Ni, Pb and Zn) were determined by flame atomic absorption spectrometry after digestion of the samples with aqua regia according to ISO 11466 (1995), using a Varian apparatus (SpectrAA 220FS, 220Z, and 110Z). Three independent replicates were performed for each sample and blanks were measured in parallel.

The soil used in the study can be classified as a loamy-sand soil (76% sand, 18% silt and 6% clay), very acidic (pH 3.05), with high electrical conductivity (EC 3.65 mS cm^{-1}), low in OM content (5.3 g kg^{-1} DM), in essential nutrients (0.6 g kg^{-1} DM $N_{Kjeldahl}$, 32.2 mg P_2O_5 kg^{-1} DM, and 12.4 mg K_2O kg^{-1} DM), and with high levels of some trace elements (296.9 mg Cu kg^{-1}, 1269.4 mg Pb kg^{-1} and 801.0 mg Zn kg^{-1}, all in a DM basis).

2.3. Incubation assay

An incubation assay was conducted to evaluate the ability of the DWTR to correct soil acidity and to decrease trace elements ex- tractability. The results from this incubation assay will allow the selection of the conditions to be used in the phytostabilization assay (Section 2.4). Different doses of DWTR: 0, 4, 8, 16, 32 and 64 g DM kg^{-1} (equivalent, approximately, to 0, 6, 12, 24, 48 and 96 t DM ha^{-1}, considering 20 cm depth, 1.5 t m^{-3} bulk density, and 50% soil fraction < 2-mm), were tested, with and without lime application (13.3 g DM kg^{-1} $CaCO_3$, equivalent approximately to 11 t ha^{-1}), using 200 g of soil, three replicates per treatment. Mixtures were maintained at 60% water-holding capacity, in aerated boxes, at 20 ± 2 °C, for one month.

The effects of the amendments on the soil physicochemical characteristics were assessed by measuring different soil properties: pH (1:2.5 w/v, in deionised water), EC (1:5 w/v, in deionised water), and OM content, using the methods referred in 2.2 sub-chapter. Copper, Pb and Zn in a readily available fraction were assessed by extraction using a 0.01 M $CaCl_2$ solution (Alvarenga et al., 2009c). Extractions were performed for 2 h, in a horizontal reciprocate shaking, on a 1:10 (w/v) soil to solution ratio, at room temperature. The extract was separated from the solid residue by filtration through a Whatman® 40 filter, acidified to pH < 2 with HNO_3, and kept at 4 °C until analysis (Alvarenga et al., 2009c). Copper, Pb and Zn were analysed using a Varian apparatus (SpectrAA 220FS). Three independent replicates were performed for each sample and blanks were measured in parallel.

The highest dose of sludge (96 t DM ha^{-1}), caused a two-fold increase in the soil OM content and a decrease in its salinity (Table 2). The increase in the soil pH was more pronounced with the simultaneous application of $CaCO_3$. A significant decrease in the Cu and Zn $CaCl_2$ 0.01 M extractable concentrations was observed: extractable Cu decreased from 111.4 to 0.2 mg kg^{-1} DM, and Zn from 712.0 to 8.1 mg kg^{-1} DM, respectively, with the simultaneous application of 96 t DM ha^{-1} DWTR and $CaCO_3$ (Table 2). Lead extractable content was very low, even for the non-amended soil, lower that the detection limit of the technique (< 1.67 mg kg^{-1}, data not shown).

Table 2
Results obtained in the incubation assay (mean ± standard-deviation, n = 3). Values in a column marked with the same letter are not signi ficantly different (Tukey test, $p > 0.05$).

Treatments		Soil properties									
DWTR (t ha^{-1})	$CaCO_3$ (t ha^{-1})	pH		EC (mS cm^{-1})		Organic matter (g kg^{-1} DM)		Extractable Cu (mg kg^{-1} DM)		Extractable Zn (mg kg^{-1} DM)	
0	0	3.05	e	3.65	f	5.3	a	111.4	h	712.0	ab
0	11	3.89	c	2.99	a	5.4	a	63.5	f	707.8	ab
6	0	3.46	f	3.34	e	5.7	a	90.8	g	742.6	a
6	11	4.24	ab	2.94	ab	6.7	ab	39.7	e	734.4	a
12	0	3.83	c	3.11	ae	5.9	a	70.8	f	772.1	a
12	11	4.33	a	2.90	ab	6.8	ab	26.0	cd	685.4	ab
24	0	4.14	b	3.06	a	9.0	abc	30.0	de	635.9	abd
24	11	4.30	ab	3.00	a	7.3	abc	9.3	ab	481.3	bcd
48	0	4.33	a	2.87	abd	8.6	abc	14.7	bc	489.6	bcd
48	11	4.67	d	2.72	bcd	9.9	bc	3.8	ab	407.6	cd
96	0	4.64	d	2.54	c	10.9	c	3.6	ab	271.3	c
96	11	5.34	g	2.64	cd	10.8	c	0.2	a	8.1	e

DWTR: drinking-water treatment residuals; EC: electrical conductivity; DM: dry matter.

2.4. Assisted-phytostabilization experiment

Considering the results from the previous incubation assay, a pot experiment was assembled to evaluate the effects of the DWTR on soil properties in a less controlled scenario. As the acidity correction and OM content improvement were not very effective following the application of 12 and 24 t DWTR ha^{-1}, they were not used in the pot experiment, and a higher application dose was tested: 144 t DWTR ha^{-1}. In this experiment, the pots were filled with 3 kg of soil, amended with different doses of DWTR: 0, 32, 64, and 96 g DM kg^{-1} (equivalent, approximately, to: 0, 48, 96, and 144 t DM ha^{-1}), with and without lime application (13.3 g DM kg^{-1} $CaCO_3$, equivalent approximately to 11 t ha^{-1}), four replicates per treatment. Pots were watered *ad libitum* and maintained outdoors. *Agrostis tenuis* Sibth (equivalent to 250 kg seeds ha^{-1}) was seeded one week after amendments application, and the pots were left outdoors for three months. *A. tenuis* has been used because it is considered suitable for the reclamation of metalliferous sites, due to its tolerance to acid lead/zinc wastes, and copper wastes (Alvarenga et al., 2014; Williamson and Johnson, 1981). Its metal tolerance may result from a metal exclusion strategy, comprising avoidance of metal uptake and restriction of metal transport to the shoots; this plant is therefore used to revegetate bare soil areas, including abandoned mines (*e.g.* in phytostabilisation technology) (Alvarenga et al., 2014).

Three months later, plant aboveground material was collected, washed thoroughly with tap water to remove any attached particles, and then rinsed three times with deionised water. The samples were dried at 70 °C for 48 h, weighed, and ground in an electric mill. Shoot dry biomass was registered.

Approximately 1 g of dried plant material was ashed in a muffle furnace at 500 °C for 6 h, dissolved with 10 mL of 3 M HCl and evaporated to near dryness twice, dissolved again with the same acid solution, filtered (Whatman® 40), and adjusted to a volume of 100 mL with ultra-pure water. The digested samples were analysed for total Cu, Pb and Zn using the procedures described previously by Alvarenga et al. (2008a).

The effects of the amendments on the soil chemistry were assessed by measuring the properties already referred: pH(H_2O), EC, OM, $N_{Kjeldahl}$, extractable P and K, total trace elements in soils (Cu, Pb and Zn; aqua-regia digestion), and extractable trace elements in soils (Cu, Pb and Zn; extracted by 0.01 M $CaCl_2$).

2.5. Soil biochemical and ecotoxicological characterization

Biochemical status of the soil was assessed by measuring dehydrogenase activity, according to Tabatabai (1994), with modifications.

Soils sub-samples, at their “field moisture content” were passed through a 2-mm sieve and incubated for 16 h, at 25 °C, with 0.1% (w/v) triphenyltetrazolium choride (TTC) in a Tris-buffer (0.1 M, pH 7.8 for acid soils, pH 7.6 for neutral soils), which allows the reduction of TTC to triphenylformazan (TPF), which is measured spectrophotometrically at 546 nm. Dehydrogenase activity was expressed in ^g TPF g^{-1} h^{-1}, on an oven-dried soil weight basis (105 °C, 48 h).

The effect of the DWTR application on the soil retention function was evaluated using the soil water-extract, obtained according to the DIN 38414-S4 (1984) methodology. Briefly, leaching was carried out using a batch test, with a single leaching cycle, at room temperature, during 24 h, under constant agitation, using deionised water in a solid- to-liquid ratio of 1:10 (w/v). The leachate was separated by centrifugation (3000*g*, 30 min), and filtered through a membrane filter Whatman® 40 of pore size 0.45 ^m. The soil water-extracts obtained were analysed for pH and EC and used in the different bioassays with their intrinsic pH values.

Four different bioassays were carried out with organisms representative of different trophic levels: (i) luminescence inhibition of *Vibrio fischeri* (ISO, 11348-2, 2007); (ii) *Daphnia magna* acute immobilization test (ISO, 6341, 2012); (iii) 24-h mortality test with *Thamnocephalus platyurus* (Persoone, 1999), and (iv) 72-h growth inhibition of the green microalgae *Pseudokirchneriella subcapitata* (OECD 201, 1984).

2.5.1. Luminescence inhibition of *V. fischeri*

Inhibitory effects of soil water-extracts on the light emission of *V. fischeri* (NRRL B-11177) were determined according to ISO (1134)8-2 (2007). Soil water-extracts, and their dilutions with a non-toxic control (2% w/v NaCl solution), 3.1%, 6.2%, 12.5%, 25.0% and 50.0% (v/v), were tested and compared with the control. All measurements were carried out in duplicate. The decrease in luminescence was measured after 30 min contact using a LUMIStox 300 equipment and EC_{50} (%; the concentration of each sample that reduced 50% of the bacterial luminescence) was determined. The sensitivity of *V. fischeri* organisms was tested with the reference substance ($K_2Cr_2O_7$) from Merck®.

2.5.2. *D. magna* immobilization test

The *D. magna* acute immobilization test was performed according to the standardised method ISO 6341 (2012). Soil water-extracts (100% v/ v), and their dilutions, 50%, 25%, 12.5% and 6.3% (v/v), were tested. Holding and dilution water was prepared according to ISO 6341 (2012), and this solution was also used as the negative control. Five juveniles, aged less than 24 h at the start of the test, were exposed to 20 mL of the test solution, at different concentrations, for a period of 48 h. Tests were conducted in environmental chambers at 20 ± 2 °C, with a 16 h light/

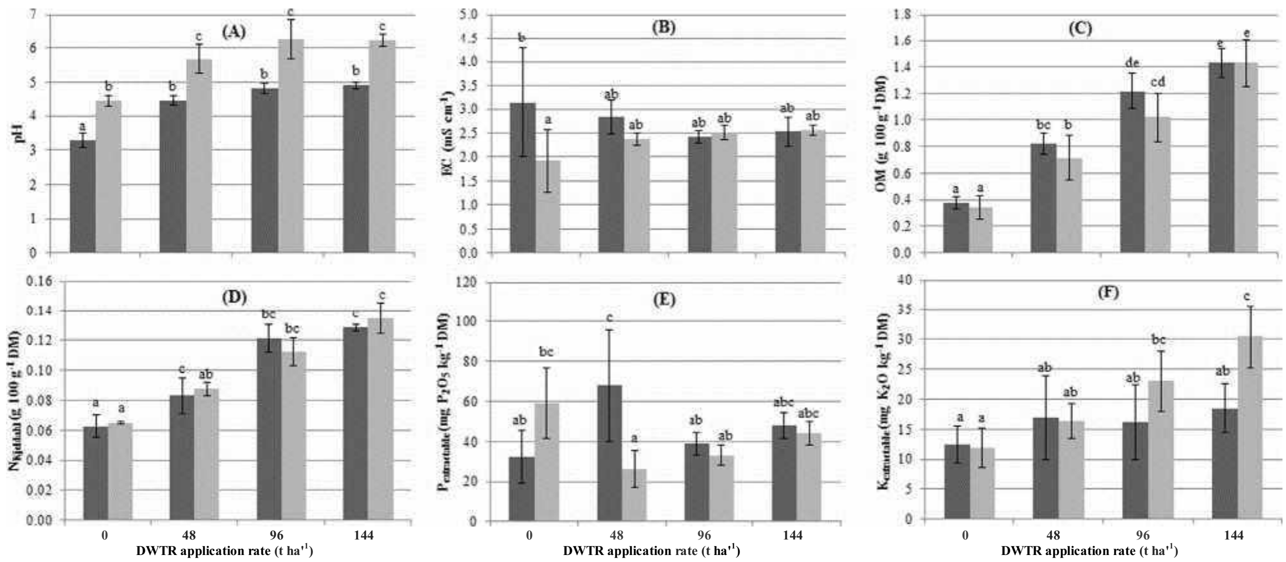

Fig. 1. Effects of the treatments with diff erent doses of DWTR, with and without lime, on: soil pH (A), electrical conductivity (EC) (B), organic matter (OM) (C), $N_{Kjeldahl}$ (D), extractable P (E), and extractable K (F) (mean ± standard deviation, n = 4). Columns marked with the same letter within the same graph are not significantly different (Tukey test, p > 0.05).

8h dark cycle, using three replicates per treatment. Immobilization was recorded, by visual observation, after 48 h exposure and compared with the control, and the EC_{50} (% v/v) was determined. A reference test with potassium dichromate ($K_2Cr_2O_7$) was conducted as a positive control.

2.5.3. *T. platyurus* bioassay

The effect of the soil water-extracts on the mortality of *T. platyurus* was evaluated in accordance with the standard operational procedure provided in the THAMNOTOXKIT FTM kit (Persoone, 1999). The concentrations initially tested were 100.0%, 50.0%, 25.0%, 12.5% and 6.3% (v/v), but, in the cases of higher toxic responses, dilutions were increased in a geometric sequence with a common ratio of % (3.1%, 1.6%, 0.8%, 0.4% and 0.2% v/v). Dilutions were made with synthetic freshwater (included in the test kit, and also used as the nontoxic control). Larvae of shrimp *T. platyurus* (< 24 h), obtained by the hatching of cysts, were incubated in 24-well plates, with 1.0mL of test solution and ten crustaceans per well, using three replicates per treatment, at 25 °C for 24 h in the dark. Animals were not fed during the test. A reference test with potassium dichromate ($K_2Cr_2O_7$) was performed as a positive control. The sensitivity of *T. platyurus* was in accordance with the protocol included in THAMNOTOXKIT FTM Standard Operational Procedure (Persoone, 1999), the 24-h EC_{50} value ranged between 0.087 and 0.116 mg L^{-1}. The number of dead shrimps after 24-h contact with each test solution was used as the selected endpoint, and EC_{50} (% v/v) values were calculated.

2.5.4. Growth inhibition of the green microalgae *P. subcapitata*

The potential inhibitory effect of the soil water-extracts on the growth of the unicellular algae *P. subcapitata* was assessed based on the protocol of OECD 201 (1984). The microalgae (100 ^L of an inoculum with 3-5 $x10^4$ cells mL^{-1}) were exposed to the soil water-extract (900 ^L; 100% v/v), or its dilutions with MBL growth medium (6.3%, 12.5%, 25%, and 50% v/v), which was also used as the negative control, in a 24-well microplate (eight replicates for the control and six replicates for each test sample). Test vials were randomly incubated in an orbital shaker for 72 h at 21 ± 2 °C and with a constant luminous intensity (60-120 pEm^{-2} s^{-1}, equivalent to 6.000-10.000 lx). The initial concentration of algae and the concentrations after 72 h exposure were calculated using a Neubauer chamber.

The average specific growth rate, for a specific period, was calculated as the logarithmic increase in biomass after 72 h, from the equation:

$$^{\wedge}{i\text{-}j} = (\ln B_j - \ln B_t)/ij\text{-}t_i \quad (1)$$

Where: $p_{i\text{-}j}$ is the average specific growth rate from time i to j; t_i is the time for the start of the exposure period; t_j is the time for the end of the exposure period, B_i is the biomass concentration at time i, and B_j is the biomass concentration at time j.

The inhibition of algal growth was estimated as percentage of reduction of growth rate with respect to the control:

$$\% I = [(p_{\cdot c}\text{-}^{\wedge}t)/^{\wedge}c] \text{ X } 100 \quad (2)$$

Where: % I is the mean percentage of inhibition for specific growth rate; p_c is the mean value for the growth rate in the control, and p_t is the mean value for the growth rate in the water samples.

2.6. Statistical treatment of data

All physicochemical data were subjected to one-way ANOVA to evaluate statistical differences between tested treatments. Whenever significant differences were found ($p < 0.05$), a post hoc Tukey HSD test was used to further elucidate differences among means ($p < 0.05$).

The EC_{50} values corresponding to *T. platyurus* mortality and to *D. magna* immobilization tests were calculated using the Probit Method (Finney, 1971). For the *V. fischeri* bioluminescence inhibition test EC_{50} values were determined using LUMISsoft 4 Software™. The effects on growth inhibition of algae were checked for normality by the Kolmogorov-Smirnov test and variance homogeneity (Levene's tests). As the ANOVA assumptions were not met, data were analysed non-parametrically using Kruskal-Wallis ANOVA by ranks test. When significant differences were found ($P < 0.05$), a post-hoc Dunnet's test was used to compare sampling stations with the control with a p-value of 0.05 as the minimum significant level (Zar, 1996). All statistical analyses were performed with the STATISTIC 6.0 Software™ (StatSoft, Inc., 2001).

3. Results and discussion

3.1. Effects on physicochemical soil properties

The amendments led to a significant increase in soil pH (Fig. 1-A), especially with the simultaneous application of lime. The application of DWTR alone, caused an increment in soil pH from 3.3 to 4.9, for the highest application dose, but the application of 11 t $CaCO_3$ h^{-1} allowed an increase in soil pH of about 1.2-1.5 units, in addition to the pH increment brought by the DWTR application, for each treatment. This seems an important contribution, since, considering the results, the increment of the DWTR application rate by itself, from 48 to 96, and from 96 to 144, was not able to promote a proportional significant increase in the soil pH, which remained acid.

Electrical conductivity values were not affected by the DWTR application, that is, the treatments did not increase soil salinity (Fig. 1-B). In fact, DWTR salinity (EC 0.92 mS cm^{-1}) is lower than the soil salinity itself (EC 3.65 mS cm^{-1}). This is an interesting characteristic, since most of the soil conditioners, both organic and inorganic, can promote and undesired increase in soil secondary salinity and, because of that, their application doses need to be restricted (Alvarenga et al., 2015). This is an extra concern when the salinity of the soil to be remediated is already high, which is the case.

Soil OM content increased significantly: the highest dose of sludge (144 t DM/ha), caused a three-fold increase in the soil OM content (Fig. 1-C), while $N_{Kjeldahl}$ content (Fig. 1-D) doubled with the same application rate of sludge. However, despite this high increase, the classification of the OM content only turned from a 'very low' to 'low' OM content classification, in accordance with LQARS (2000), for the highest dose of sludge (144 t DM/ha). Elliott and Dempsey (1991), have already alerted for that, soil conditioning effects, resulting from increased organic matter content through DWTR application, are quite small. The same is true for N in DWTR, although its agronomic benefits depend more on its availability than on the total amount present, which is governed by the mineralization rate (the rate at which waste-borne nutrients are converted into forms available to plants; Elliott and Dempsey, 1991). Several studies proved that the addition of DWTR to soil did not increase N mineralization rate, and that a low percentage of N in DWTR is available to crops in the first growing season (Elliott and Dempsey, 1991).

As for the $P_{extractable}$ content (Fig. 1-E), it did not increase after DWTR application, while $K_{extractable}$ content increased significantly only for the application of 96 and 144 t DM/ha of DWTR with lime (Fig. 1-F). Moreover, both P and K extractable concentrations are still considered as 'very low' or 'low' (LQARS, 2000) in the amended soils, indicating that additional mineral fertilization should be considered when DWTR are applied, to establish a wealthy plant cover. The results for the low content of P in the amended soils could be expected, because there are studies showing that DWTR have a strong capacity to adsorb P, and that the adsorption process is fast and nearly irreversible (Elliott and Dempsey, 1991; Ippolito et al., 2011). In fact, Elliott and Dempsey (1991) stated that the reduced availability of P to crops is a main disadvantage of DWTR application to agronomic soils. Therefore, since DWTR application can induce P deficiency in crops, further studies are needed to ascertain application doses and the need for additional fertilization with phosphate salts when DWTR are used in mine soil remediation.

3.2. Effects on trace elements extractable fractions

As expected, total metal concentrations, extracted by *aqua regia*, did not change because of amendments application, maintaining values that were not statistically different for each treatment: 269 ± 89 mg kg^{-1} DM for Cu; 1479 ± 443 mg kg^{-1} DM for Pb; and 576 ± 99 mg kg^{-1} DM for Zn, mean values ± standard deviation, n = 32. Similar results have been obtained by Garau et al. (2014) when applying 3% w/w Fe-DWTR to a circumneutral contaminated soil.

In contrast, a significant decrease in the Cu and Zn $CaCl_2$-extractable content was observed (Figs. 2-A and 2-B, respectively), emphasizing the ability of the DWTR to immobilize trace elements by adsorption/occlusion. That ability was higher for Cu, which $CaCl_2$-extractable content decreased from 75.2 to 2.0 mg kg^{-1} DM, with the application of 144 t DM/ha DWTR. The decrease in Zn $CaCl_2$-extractable content was more pronounced with the simultaneous application of lime, allowing a further decrease in its extractability to less than half of the value that was accomplished by the single application of DWTR. In fact, as previously discussed, the increase in soil pH was more efficient by the simultaneous application of a liming agent, and not by the sole application of DWTR, which has a pH of 6.7. In fact, the significant increase in soil pH, relatively to the mine soil, by the application of the DWTR, was only possible because the soil was very acidic. Consequently, DWTR oxides played an important role on metal adsorption/occlusion, but that effect would be more pronounced if higher soil pH values were obtained, that is, if higher $CaCO_3$ application rates were applied.

Lead $CaCl_2$-extractable content was determined in the sequence of the amendments. The values were very low (< LD =1.67 mg kg^{-1}), even before the amendments application, and despite the high total Pb concentration in the non-amended soil. The authors have also noticed that fact in previous studies in mines from the Iberian Pyrite Belt, where Pb was pointed as the trace element with the lower bioavailable values (Alvarenga et al., 2012).

3.3. Effects on plant parameters

Plants germinated but died in the non-amended pots and in the pots amended with 48 t ha^{-1} DWTR, without lime (Fig. 2-C). The other combinations of amendments allowed the establishment of a plant cover, however, with high differences between replicates.

Copper, Pb and Zn concentrations in the aboveground plant material were measured and the concentrations found with the application of 144 t ha^{-1} DWTR were used to evaluate the possibility of using the plant in a phytostabilization strategy (Table 3). Considering the concentrations in contaminated plants from Kabata-Pendias and Pendias (2001), Pb concentrations found in the plant material were lower than the typical values for contaminated plants, which could be expected, taking in consideration the low Pb $CaCl_2$-extractable concentrations found in the soils. The same was not true for Cu and Zn, especially for Zn in the higher application rate (Table 3), with concentrations which are typical of contaminated plants. However, Cu, Pb and Zn concentrations in the plant material were still lower than the maximum tolerable level for cattle feed, according to the National Research Council (2005). Those limit values can be used as an indicator of risk of entry of those metals into the human food chain (Table 3), which suggests that there is a low risk of their transference if this phytotechnology were to be applied.

3.4. Effects on soil biochemical and ecotoxicological properties

There are several microbial endpoints (*e.g.* microbial biomass-C, dehydrogenase activity, N-mineralization, number of culturable heterotrophic bacteria, soil basal and substrate-induced respiration, soil exoenzymes activities) which can be used to assess the ability of different amendments to improve soil health in remediation studies (Lombi et al., 2004; Pardo et al., 2011; Perez-de-Mora et al., 2011; Alvarenga et al., 2014; Garau et al., 2014; Galende et al., 2014). It is commonly acknowledged that, since dehydrogenase is an oxidor- eductase, which is only present in viable cells, the results from dehydrogenase activity represent a good indicator of the active microbial population of a soil (Tabatabai, 1994; Izquierdo et al., 2005), and, consequently, that microbial endpoint was selected to be used in this study. In this case, the treatments did not affect dehydrogenase activity, which remained with very low activities, lower than the quantification limits, QL, of the technique (< QL = 1 ^g TPF g^{-1} DM h^{-1}). Because of that, it is possible to conclude that DWTR were not able to improve the soil overall microbial activity, which is partly a consequence of their own low microbial activity. Garau et al. (2014) also found that the level of soil microbial biomass-C remained the same after the application of 3% Fe-DWTR to a circumneutral trace element contaminated soil, which indicates a deficient ability of DWTR to improve soil microbial

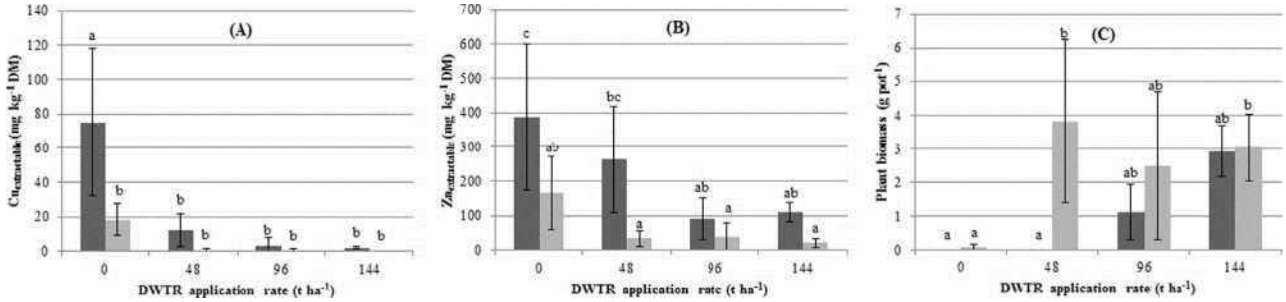

Fig. 2. Effects of the treatments with different doses of DWTR, with and without lime, on: Cu (A) and Zn (B) extractable factions, and on plant biomass yield (C) (mean ± standard deviation, n = 4). Columns marked with the same letter within the same graph are not significantly different (Tukey test, $p > 0.05$).

Table 3
Trace elements concentration in the aboveground plant material with the application of 144 t ha^{-1} of DWTR (mean ± standard deviation, n = 8), typical concentrations found in the literature for contaminated plants, and limit values for trace elements in feedstuff.

	Concentration in the plants (mg kg^{-1} DM)	Concentrations in contaminated plants[a] (mg kg^{-1} DM)	Maximum tolerable level for cattle[b] (mg kg^{-1} DM)
Cu	34 ± 5	20 - 100	40
Pb	24 ± 12	30 - 300	100
Zn	477 ± 259	100 - 400	500

[a] Kabata-Pendias and Pendias (2001). [b] National Research Council (2005); DWTR: drinking-water treatment residuals; DM: dry matter.

activity. However, the same authors found a significant increase in the number of culturable heterotrophic bacteria after the application of Fe-DWTR, suggesting that the number of culturable bacteria can be a more sensitive indicator of the microbial status of a polluted soil than microbial biomass-C (Garau et al., 2014).

Considering the results from the ecotoxicological tests, some amendment doses of DWTR, especially with the simultaneous application of $CaCO_3$, led to a reduction in the mine soil water-extract ecotoxicity (Table 4 and Fig. 3).

Soil water-extract toxicity towards *V. fisheri* decreased in the higher DWTR application rates, 96 and 144 t ha^{-1}, with or without lime (Table 4). For *D. magna*, the toxicity decreased with the application of the higher application rates of DWTR, 96 and 144 t ha^{-1}, and the toxic response disappeared with the simultaneous application of DWTR and lime (Table 4). The toxicity towards *T. platyurus* also decreased as a response to DWTR application, especially with the simultaneous application of lime. The bioassay with *T. platyurus* was the most sensitive, when we evaluate the acute toxicity (EC_{50} values), induced by the soil water-extractable trace elements fraction, with and without amendments. The sensitivity of this crustacean already has been reported by the authors, in other liquid matrices, such as domestic wastewaters and irrigation waters (Palma et al., 2016). The growth inhibition of the microalgae *P. subcapitata* was only positively reverted with the simultaneous application of DWTR and lime, with values of 27% reduction in the growth inhibition, comparing with the control, for the higher application rate.

Considering these results, it is possible to conclude that the application of the DWTR, especially for the higher application rates, 96 and 144 t ha^{-1}, positively affected mine soil water-extract composition, by correcting its acidity and trace elements extractability - the soil was effectively chemically stabilized.

4. Conclusion

The application of DWTR, especially at the higher application rates, 96 and 144 t ha^{-1}, with the simultaneous application of lime (11 t ha^{-1} $CaCO_3$), corrected soil acidity and decreased metal extractability (especially for Cu and Zn), relatively to the non-amended soil, which allowed plant growth. This fact had also a positive effect on soil mine water-extract composition and, consequently, the toxicity towards aquatic organisms decreased, showing that DWTR application can contribute to an improvement in soil retention function, due to the capacity of their oxides to adsorb/occlude potentially toxic trace elements, and, to a lesser extent, to increase soil pH.

However, the soil nutritional status was not so positively affected; DWTR by their own were not sufficient to increment soil OM, N, P, and K to optimum levels, which are essential to maintain a healthy plant cover and to increase soil microbial activity. It is important to bear in mind that this residual material, by itself, is insufficient to act as a fertilizer, requiring addition of nutrients, especially P, because its extractable concentration in the soil remained low after the amendment with DWTR. In fact, DWTR could be contributing to P deficiency to the plant. So, further research is needed, to evaluate different application rates of DWTR and $CaCO_3$, to achieve higher pH values, and additional correction with N, P and K fertilizers.

Table 4
Soil water-extract characteristics: pH, electrical conductivity (EC; mS cm^{-1}) (mean ± standard-deviation, n = 3), and ecotoxicological responses calculated as EC_{50} values (mean values % v/v; 95% con fidence interval, CI; n = 2 for *V. fisheri*, and n = 3 for *T. platyurus* and *D. magna*).

DWTR (t ha^{-1})	$CaCO_3$ (t ha^{-1})	Soil water-extract pH	Soil water-extract EC	*V. fisheri* 30 min-EC_{50}	*T. platyurus* 24h-EC_{50}	*D. magna* 48 h-EC_{50}
0	0	2.8 ± 0.0	2.14 ± 0.16	43.2 (42.4-44.0)	0.5 (0.4-0.6)	5.1 (3.0-7.0)
0	11	4.9 ± 0.5	1.35 ± 0.06	nt	2.2 (1.8-2.8)	4.0 (1.8-5.7)
48	0	4.2 ± 0.0	1.90 ± 0.17	27.2 (27.0-27.5)	0.4 (0.3-0.5)	4.7 (2.6-6.6)
48	11	5.9 ± 0.4	1.40 ± 0.03	53.6 (53.4-53.8)	21.6 (9.9-31.8)	nt
96	0	4.8 ± 0.3	1.35 ± 0.06	nt	12 (7.7-16.2)	22.0 (17.0-19.5)
96	11	6.1 ± 0.4	1.57 ± 0.11	nt	19.7 (15.4-24.5)	nt
144	0	4.7 ± 02	1.59 ± 0.07	95.6 (94.4-96.8)	4.4 (2.6-6.1)	31.2 (25.7-39.4)
144	11	6.1 ± 0.2	1.71 ± 0.01	nt	51.0 (36.1-57.0)	nt

DWTR: drinking-water treatment residuals; EC_{50}: soil water-extract concentration, % v/v, at which a toxic effect on 50% of the exposed organisms can be observed; nt: non-toxic.

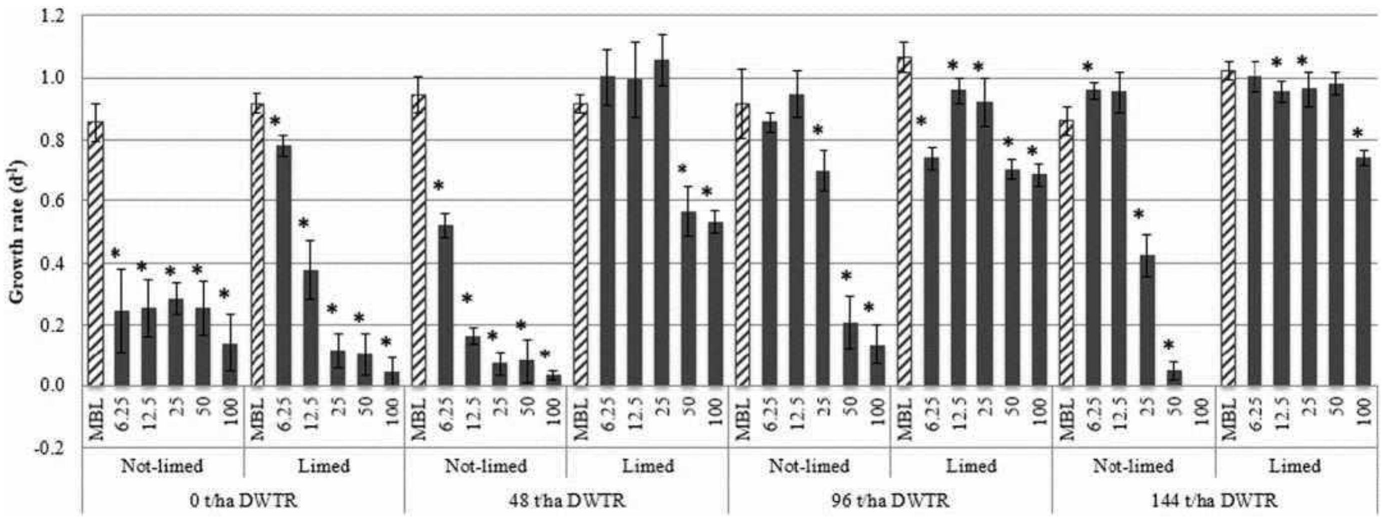

Fig. 3. Growth rate (d^{-1}) from the microalgae *P. subcapitata* after 3 days exposed to the treatments with different doses of DWTR, with and without lime (mean ± SD; n = 6; * $p < 0.05$, Dunnett's test with a control (MBL).

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