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Research Article

PHYTOREMEDIAL EFFECT OF *MEDICAGO SATIVA* ON SOIL IRRIGATED WITH TREATED WASTEWATER AND FERTILIZED WITH SEWAGE SLUDGE

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ABSTRACT

The present study aims to evaluate the growth and the phytoemediation effect of Medicago sativa, a forage and industrial plant, on soil irrigated with treated urban wastewater and/or amended with urban sewage sludge. Tap water was used for control. The seeds were brought from the oasis of Gabes in the southeast of Tunisia. The sewage sludge was tested at 5 and 25 t/h doses. Results showed that the best production was obtained when cultivating M. sativa on soil amended with 25 t/ha of sewage sludge and irrigated by tap water. Metal trace elements abatements were recorded in soils cultivated by alfalfa and irrigated by tap water; alfalfa thus exerts a phytoremediator power on the soil, it absorbs the metal trace elements and accumulates them in its aerial parts. However, this effect has not been demonstrated when alfalfa grown on soil amended with sewage sludge and irrigated with treated wastewater.

INTRODUCTION

The water deficit is a major abiotic constraint on agricultural production in Tunisian semi-arid and arid regions. The water mobilizable resources in this country are evaluated during the year 2017 to 4665 million m3 with an availability of 460 m^3/inhabitant/year, knowing that globally, a country is considered in water shortage when the estimated water availability is less than 500 m^3/inhabitant/year (DGRE, 2017). Faced with this situation, long-term strategies have been put in place. These strategies have taken into account the major water-consuming sectors and the effects of climate change by determining the volume of supply and demand and exploring ways to ensure water and food security in a sustainable way and provide solutions to reduce the risks of water scarcity. The reuse of treated wastewater is one of the solutions that Tunisia has adopted since the early 1980s in order to increase its available water resources. Currently, wastewater is considered an undervalued and sustainable resource of water, energy, nutrients and other recoverable byproducts, rather than something to eliminate (WWAP, 2017). Demographic, economic and urban increases are responsible for various sources of water contamination, which, if raw and without prior treatment, can act as a vector of potentially dangerous agents that can harm human health and environment. Thus, wastewater sanitation has become an imperative for modern societies by the establishment of wastewater treatment plants with the objective of improving water quality before their reintroduction into the natural environment (Benoudjit, 2016). However, an efficient operation of the water purification systems inevitably results in the production of secondary residues called "sewage sludge" whose quantities have increased over time posing an additional problem.

The agricultural application of sewage sludge has grown considerably in the last decade. The agronomic interest of these materials lies mainly in their nitrogen and phosphorus content (Afgane, 2016) and may contain also high concentrations of metal elements usually present in trace amounts in agricultural soils (Cd, Pb, Cu, Ni, Zn...). The plants response to the heavy metals brought by sewage sludge depends on the soil conditions, the cultivation techniques adopted and especially the nature of the sewage sludge, which the properties vary according to the origin of wastewater and the treatment system

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adopted in the wastewater treatment plants (Morel *et al*, 1988). However, a significant plants absorption of these elements can be responsible for physiological disturbances at each link of the food chains. Excess metal trace elements in soil and the environment is a critical factor that can affect plant productivity, endanger animal health, and disrupt human communities at all levels (Ali-Zade *et al*, 2010). At the same time, soils treated with sewage sludge retain moisture longer and vegetation elaborates on such soils a more developed root system compared to untreated soils (Garcia *et al*, 2017).

The remediation of soils amended by sewage sludge is possible by mechanical or physicochemical remediation technologies, such as stabilization / immobilization and thermal desorption. These techniques are generally expensive and disrupt the soil biological mechanisms (Atma, 2016). However other techniques, such as phytoremediation, which consists of using plants and the microorganisms associated with them to reduce the level of pollution present in soil by removing or degrading organic and inorganic contaminants from soil and water (Suthersan, 2001; Ali *et al*, 2013). The plant species that is subject of this study is *Medicago sativa* L. (alfalfa), it has a soil phytoremediator power (Licinio, 2017). Alfalfa is one of the most common forage legumes in all continents and the most nutritious because of its high protein, mineral and vitamin content (Mouafek, 2010). This species can adapt to very different climatic conditions and can withstand severe drought. At the root level, it has nodules filled with symbiotic bacteria fixing atmospheric nitrogen in the form of organic molecules, which makes it very little dependent on the supply of nitrogen fertilizers (Tidjani and Tounsi, 2005) and also allows the enrichment of poor soils with a high carbon / nitrogen ratio (Leys *et al*, 2005). Alfalfa is used in phytoremediation around the world because of its effectiveness in the treatment of hydrocarbons contaminations (Beauchamp, 2017) and also contaminations based on metal trace metals (Licinio, 2017). The aim of this study is to evaluate the bioremediation effect of alfalfa grown on irrigated soil by treated urban wastewater and/ or amended with two massive doses of urban sewage sludge.

MATERIALS AND METHODS

Set-up of the Experiment

The *M. sativa* seeds used in this study were collected in 2017 year from a local cultivar of the oasis of Chenini in Gabes southeast Tunisia and stored in hermetically sealed containers under ambient laboratory condition with temperature varying from 15 °C to 35 °C respectively in winter and summer seasons. Healthy alfalfa seeds were sown manually in April 2018 at a rate of $4g/m^2$ and at a depth of no more than 1cm in pots containing 2.5 kg of sandy loam soil with low clay contents. The experimental setup adopted includes 6 treatments as shown in Table 1; each treatment has been repeated 3 times. The pots were sheltered at ambient temperature and irrigated at a rate of 13 m^3 per hectare. Physicochemical characteristics of irrigation waters (Tap water and urban treated wastewater) and urban sewage sludge used as fertilizer are shown in Table 2. The first cut of the plants aerial parts was carried out at the beginning of August and was used for the alfalfa chemical analysis. The study was conducted at the experimental field of INRGREF located in Tunis, North of Tunisia. Climatic conditions prevailing during the period of experimentation (April-August) in the year 2018 were given in Fig 1 (Source: National Institute of Metrology, Tunisia).

Morphological Characterization of Medicago sativa

Plant development was studied from sowing (April) to blooming (August). Several morpohological parameters relating to vegetative and reproductive developments were used to analyze the phenotypic variability according to the different treatments applied (Table 3).

Medicago Sativa Chemical Characterization

The alfalfa aerial parts (leaves and stems) were dried at 70◦C for 48 h and dry weights determined. A quantity of 1g of plant powder was wet-digested with $HNO_3/HClO_4$ (1/1, V/V) and the concentration of P in aerial parts was determined on the digests with colorimetric standard method (Model AE-11, Erma Optical Works Ltd., Japan; Pauwels *et al*, 1992). The concentrations of Na and K were determined with flame spectrophotometry (Jenway PFP7, U.K.; Pauwels *et al*, 1992) and the concentrations of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn in aerial parts were dosed using flame atomic absorption spectrophotometry (PerkinElmer A Analyst 400, U.S.A.) according to Jedrzejczak and Szteke (1990) method. All samples were analyzed in triplicate with reagent blanks.

Soil Sampling and Analysis

Soil sampling was performed initially before the irrigation and at the end of the experiment after mixing the pots contents. The samples were dried, sieved at 2 mm and analyzed. The pH was measured by the electrometric method in distilled water (1:2.5). Soil electrical conductivity (EC) and major ions (Ca^{2+}, Mg^{2+}, K^+, Na^+, Cl^-, HCO_3^- and SO_4^{2-}) were determined in an extract of saturated paste according to the standard methods suggested by the American Public Health Association (APHA, 1995). Metal trace elements (MTE) content was extracted by means of acid digestion using a mixture of concentrated nitric, hydrochloric and hydrofluoric acids (1:3:3 v/v/v) in Teflon bombs placed in a hot plate with 1 g of each soil sample. After digestion, the samples were filtered and adjusted to 100 ml with distilled water (ISO 14869-1, 2001). MTE were dosed in the filtrates using flame atomic absorption spectrophotometer (PerkinElmer A Analyst 400, U.S.A.). The accuracy of the chemical analyses was carefully studied using a repeated analysis of soil samples and by calculating the percent charge balance error (% CBE). The results of the analysis were judged to be acceptable when the CBE does not exceed ±5%.

Statistical Analysis

The statistical model used incorporates two dependent factors (irrigation water quality and sewage sludge dose) explaining the variations of the independent quantitative variables (all chemical parameters used for plant and soil characterization). Data normality of quantitative variables was assessed by means of the Shapiro-Wilks test. A two-way ANOVA was performed using SPSS software (IBM SPSS statistics, version 20) to study variability and interactions between the variables and was complemented by a Duncan's multiple range test.

Table 1 The different treatments performed during the experiment

Treatment	Description
T1 (control)	Unfertilized soil and irrigated with tap water (TW)
T2	Soil amended by sewage sludge at a rate of 5 t / ha and irrigated by TW
T3	Soil amended by sewage sludge at 25 t / ha and irrigated by TW
T4	Unfertilized soil and irrigated with treated wastewater (TWW)
T5	Soil amended by sewage sludge at a rate of 5 t / ha and irrigated by TWW
T6	Soil amended by sewage sludge at 25 t / ha and irrigated TWW.

Table 2 Physicochemical characteristics of tap water, treated wastewater and sewage sludge

Parameters	Tap water	Treated wastewater	Sewage sludge
pH	7.63	7.67	7.63
CE	1.94 µS/cm	2.29 µS/cm	8.02 µS/cm
DCO	-	105.6 mgO_2/l	-
DBO_5	-	15 mgO_2/l	-
N %	1.3 %	5.4 %	21.4 %
Cl	4 mg/l	15.51 mg/l	1949.4 mg/l
SO_4	4.28 mg/l	7.53 mg/l	1945.2 mg/l
HCO_3	1.83 mg/l	5 mg/l	457.6 mg/l
Na	3.95 mg/l	11.14 mg/l	919.2 mg/l
K	0.654 mg/l	0.78 mg/l	78.18 mg/l
Mg	0.52 mg/l	5.5 mg/l	668.25 mg/l
Ca	0.7 mg/l	7.5 mg/l	501.25 mg/l
Cd	0.004 mg/l	0.034 mg/l	36 mg/kg
Co	0.01 mg/l	0.054 mg/l	18.34 mg/kg
Cr	0.018 mg/l	0.027 mg/l	8.45 mg/kg
Cu	0.008 mg/l	0.046 mg/l	151 mg/kg
Fe	0.126 mg/l	0.261 mg/l	1581 mg/kg
Mn	0.02 mg/l	0.032 mg/l	22.6 mg/kg
Ni	0.013 mg/l	0.031 mg/l	19.9 mg/kg
Pb	0.016 mg/l	0.045 mg/l	66.4 mg/kg
Zn	0.186 mg/l	0.205 mg/l	255 mg/kg

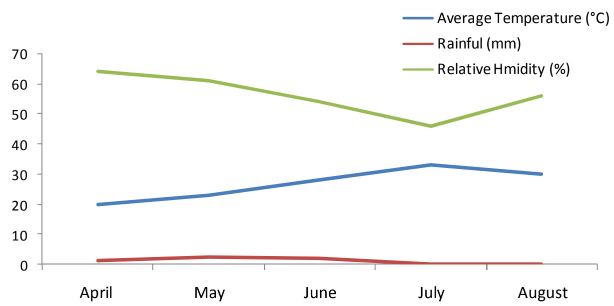

Figure 1 Rainfall, relative Humidity and average temperature of the experimental site from April to August during 2018.

Table 3 Morphological parameters used (Chaabena *et al*, 2004)

Parameters informing about forage production capacity	Parameters informing on plant architecture
Leaves number per plant (LNP) Leaf area [a] (cm²) (LA) Fresh material weight (g) of the aerial part (FW) Dry weight (g) of the aerial part (DW) Production index [b]	Branches number (NB) Stem height (SH) (cm) Stem diameter (SD) (mm)

[a] is determined using a "Delta-T Devices LTd" type planimeter, [b] is a production index which is equal to the dry weight / leaf area (Noitsakis, 1986).

RESULTS AND DISCUSSION

Impact of Treated Wastewater Irrigation and Sewage Sludge input on the Morphological Behavior of Medicago sativa

Alfalfa growth was studied by varying the irrigation water quality (tap water and treated wastewater) and the sewage sludge dose applied to the soil (0 t/ha, 5 t/ha and 25 t/ha) through parameters presented in Table 3. Results showed that the highest values for all parameters tested were recorded with alfalfa irrigated by TW and fertilized with the sewage sludge dose of 25 t / ha; while the lowest values were noted with plants irrigated by TWW and fertilized with the 5 t / ha dose (Fig 2). The statistical analysis showed that the soil quality factor had a significant effect on the variations of the parameters SD, LA, FW and DW ($P < 0.05$). While the irrigation water quality affected significantly only the stem height of alfalfa ($P < 0.05$). The interaction between the two factors irrigation water quality and soil quality did not significantly influence the variations of the parameters studied ($P > 0.05$). In irrigating alfalfa with TW, treatments with 5 and 25 t / ha of sewage sludge were among the increases in dry matter production of *M. sativa* by 26.9 % and 93.96 % respectively, relative to the control. While with the TWW, this production is increased by 27.27% and 61.26%, respectively (Fig 2). The addition of sewage sludge improved thus the dry matter production of alfalfa aerial part (stem and leaf). This result is consistent with the works of Mazen *et al* (2010) on wheat and jews mallow plants. They noted a marked improvement in the development and yield of plants on amended soils compared to non amended soils due to the presence of essential nutrients for the growth of plants in sewage sludge. According to Cottenie *et al* (1983), the tolerance index (Ti) is defined as the ratio between the production in enriched medium and the control production (Ti = enriched soil growth / control growth). When this expression equals 1, this indicates that there is no influence on growth. Ti is greater than 1 when there is a favorable action and it is less than 1 when growth is affected by environmental conditions. Table 4 presents the calculated tolerance indices for alfalfa stem and leaves for each of the applied treatments. It turns out that by fertilizing the soil with sewage sludge, the tolerance indices of alfalfa stem and leaves are higher when irrigating with TW compared to irrigation with TWW (Table 4). The beneficial effect of fertilization is less pronounced in irrigation with TWW, probably because of the competitive effect of MTE in sewage sludge and TWW and some macroelements (Lassoued Aouini, 2016). Thus, the use of fertilizers under irrigation conditions with treated wastewater does not improve the production, on the contrary it risks disturbing the crop and causing contamination of the water table (Xanthoulis *et al*, 2002).

Impact of Treated Wastewater Irrigation and Sewage Sludge input on the Chemical Composition of Medicago sativa

Plant growth requires a supply of nutrients such as nitrogen, potassium, phosphorus, sodium and trace elements. These elements are essential for plant life and are found, in appreciable quantities but in very variable proportions relative to the needs of vegetation, in TWW as shown in Table 2. Results showed that the highest concentrations for all chemical elements tested were recorded with aerial parts of alfalfa irrigated by TWW and fertilized with the sewage sludge dose of 25 t/ha. While the lowest contents was measured in plants irrigated with TW and grown on soil not amended by sewage sludge (Fig 3). Overall, nutrient levels (especially Na) and Fe increased in aerial parts of alfalfa irrigated by TWW and fertilized with sewage sludge. Ni, Pb and Cd contaminations was also observed in the aerial parts of plants irrigated by TWW and fertilized with sewage sludge (especially at 25 t/ha dose) compared to the witnesses. So irrigation with TWW results in significant enrichment of nutrient and micronutrient plant tissues (Charfi, 1995, Yadav *et al*, 2002, Fars *et al*, 2003). However, in case of irrigation with such quality of water, it is necessary to avoid the addition of fertilizer such as sewage sludge. Because in fact, in case of accumulation in vegetative tissues, nutrients and metal cations compete and interfere in essential functions such as photosynthesis, which is at the origin of the metals phytotoxicity and may is noticed by some visual symptoms as the stop of growth and the destruction by necrosis of plants aerial parts (Atma, 2016).

Soil Phytoremediation

After 4 months of growing alfalfa on soils amended by two doses of sewage sludge (5 and 25 t / ha) and irrigated with tap water (for controls) and treated wastewater, results in Table 5 showed a slight increase in pH values but these variations were not significant according to irrigation water and soil qualities ($P > 0.05$). The increase in pH can be due to the fact that soil solution becomes concentrated after irrigation and sewage sludge input whereby calcite, fluorite and Mg-silicate precipitate, Ca^{2+} and Mg^{2+} molalities decrease, while pH increases (Marlet *et al*, 1998). Increases in electrical conductivity (EC) values were noted ($P < 0.05$) particularly in the treatment receiving 25 t/ha of sewage sludge (Table 5). At the same time, enrichments in salt-forming ions (Cl^-, K^+, Na^+, HCO_3^-, SO_4^{2-}, Ca^{2+}, Mg^{2+}) have been noted, which implies an increase in the salinity of irrigated, initially non-saline soils (Table 5). Increased EC and soil salinization may negatively affect plant metabolism and growth due to osmotic effects of salts causing food imbalance (Machado and Serralheiro, 2017). The highest concentrations of all chemical elements studied were recorded in soils irrigated with TWW and amended with sewage sludge ($P < 0.05$). The levels of most of these chemical elements were significantly lower in the soils cultivated and irrigated by tap water compared to initial soils ($P < 0.05$) which proves that they have been absorbed by *M. sativa* (Table 5) and reminds all the studies that have reported the accumulation of metallic elements in the aerial parts of plant species (Cataldo and Wildung, 1978; Greger, 2004; Vamerali *et al*, 2010). Fertilized and irrigated soil with TWW could be contaminated and become toxic to plants but Flogeac *et al* (2007) reported that antagonistic effects between MTE in contaminated soils may reduce their uptake by plants. Agnello (2014) reported that Alfalfa can tolerate a heavy metal soil, which is an essential characteristic of any plant species used in phytoremediation but it could not be considered as an active heavy metal removal species as it was not able to phytoextract significant amounts of heavy metals in amended soil. These conclusions do not agree with our results since we noted that the Fe, Cd, Ni and Pb contents showed significant variations according to the soil quality factor ($P < 0.05$). For nitrogen, soil concentrations did not decrease on the contrary they increased (Table 5) due to the fertilization by sewage sludge and the irrigation with TWW on the one hand, and on the other hand, through the cultivation of alfalfa characterized by its nitrogen fixation capacity (Mathieu, 2003). However, the combination of sewage sludge spreading and treated wastewater irrigation will have a negative effect on plant growth (Bourioug, 2013).

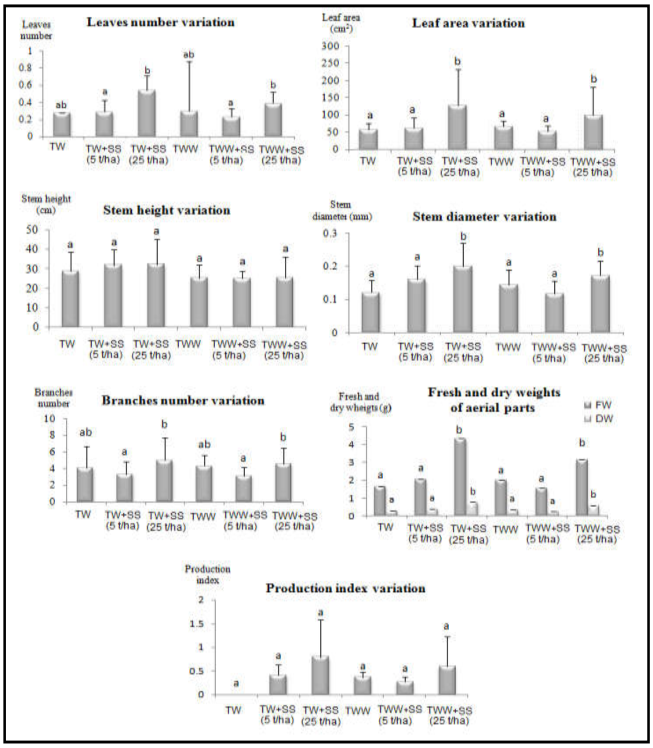

Figure 2 Variation of morphological parameters depending on irrigation water quality and use of sewage sludge. TW: tap water irrigation, TWW: treated wastewater irrigation, SS: fertilization with sewage sludge, 5 t/ha and 25 t/ha were the doses at which sewage sludge is applied. Different letters indicate significance between parameters values at α= 0.05 according to Duncan test.

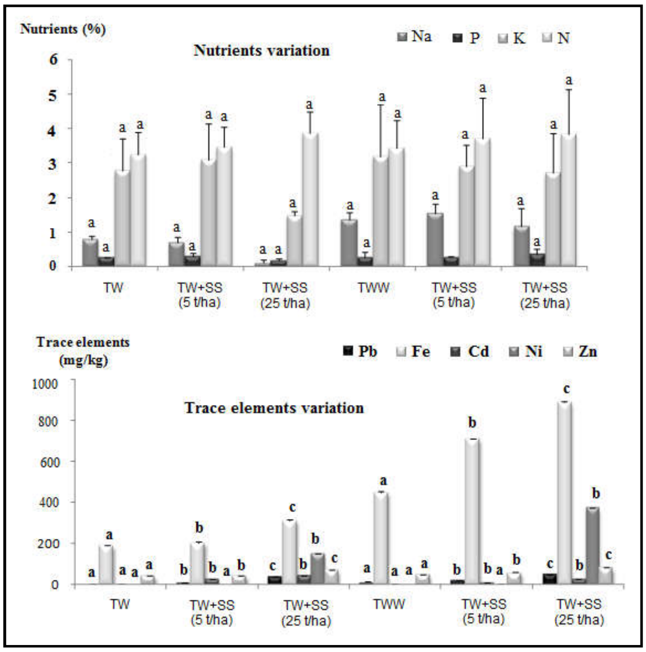

Figure 3 Variation of aerial parts chemical composition depending on irrigation water quality and use of sewage sludge. TW: tap water irrigation, TWW: treated wastewater irrigation, SS: fertilization with sewage sludge, 5 t/ha and 25 t/ha were the doses at which sewage sludge is applied. Different letters indicate significance between parameters values at α= 0.05 according to Duncan test.

Table 5 Comparison of soil physicochemical characteristics before and after amendment with sewage sludge, alfalfa cultivation and irrigation

Water quality	Sewage sludge	Initial soil			Soil cultivated by alfalfa and irrigated					
					Tap water			Treated wastewater		
Soil quality		0 t/ha	5 t/ha	25 t/ha	0 t/ha	5 t/ha	25 t/ha	0 t/ha	5 t/ha	25 t/ha
pH	7.63±0.3^{b}	7.03±0.04^{a}	6.86±0.07^{a}	7.24±0.04^{a}	7.17±0.02^{a}	7.11±0.1^{a}	7.3±0.44^{a}	7.04±0.03^{a}	7.11±0.05^{a}	7.14±0.03^{a}
EC (dS/cm)	8.02±0.3^{c}	3.29±0.3^{a}	4.17±0.3^{b}	6. 45±0.8^{a}	4.77±0.37^{b}	5.07±0.47^{a}	6.53±0.74^{c}	9.68±0.5^{b}	12.06±2.46^{a}	12.96±0.29^{c}
Cl (mg/l)	1949.4±26^{c}	625.4±16^{a}	849.7±38^{b}	949.7±43^{b}	399.7±12^{a}	646.2±6.85^{a}	941.7±17.8^{b}	620.4±28^{a}	2449.2±19^{a}	2699.2±28 ^{b}
HCO_3 (mg/l)	457.6±18.7^{a}	610.1±10^{b}	496.6±7.8^{a}	549±26.3^{a}	488.1±16.7^{a}	386.6±7.7^{a}	518.6±5.36^{a}	457.6±18.8^{a}	447.6±15^{a}	396.6±7.80^{a}
SO_4 (mg/l)	1945.2±20^{c}	2135.4±53^{a}	2317.9±24^{d}	2618.6±13^{b}	2001.7±4.8^{b}	2134.4±26^{a}	2775.8±58^{c}	2896.3±43^{b}	3318.8±3^{a}	4268.9±23.5^{c}
Ca (mg/l)	501.25±4.4^{b}	420.7±7.3^{a}	498.85±15^{b}	601.5±4.7^{a}	341.75±4.3^{b}	461.4±27^{a}	601.5±4.86^{c}	802.25±4^{b}	941.6±6.7^{a}	1203±3.51^{c}
Mg (mg/l)	668.25±116^{b}	303.7±34^{a}	379.45±16^{b}	729±13^{a}	267.3±22.5^{b}	121.5±11.3^{a}	607.5±3.26^{c}	324.25±1.9^{b}	407.5±1.8^{a}	874.8±28.17^{c}
K (mg/l)	78.18±15^{a}	95.2±10^{a}	122.1±3.3^{a}	162.6±10^{a}	51.6±2.5^{a}	50.1±1.10^{a}	67.2±5.29^{a}	93.4±2.52^{a}	111±1.53^{a}	130.6±9.95^{a}
Na (mg/l)	919.2±13^{c}	442.3±60^{a}	481.8±16^{a}	566±6.8^{b}	418.5±19.9^{a}	565.8±15^{a}	596.8±10.6^{a}	848.2±9.5^{a}	953±11^{a}	1296±10.5^{a}
N (mg/l)	364±30.08^{a}	7.6±1^{a}	8.4±1.17^{a}	11.2±0.5^{a}	8.06±0.36^{a}	10.08±0.88^{a}	12.88±0.55^{a}	8.4±0.68^{a}	8.96±0.5^{a}	13.08±0.86^{a}
Ni (mg/kg)	19.9±2.78^{c}	2.47±0.5^{a}	4.9±0.8^{a}	6.8±1.91^{b}	1.92±0.76^{a}	3.66±0.25^{b}	6.1±0.47^{c}	2.32±0.32^{a}	5.6±0.6^{b}	8.1±0.3^{c}
Zn (mg/kg)	255±10.65^{b}	54.8±5^{a}	57±15.65^{a}	61±9.50^{a}	51.5±5.69^{a}	51.7±2.97^{a}	54.1±4.52^{a}	54.5±4.03^{a}	59.9±4.51^{a}	76.4±3.52^{a}
Cd (mg/kg)	36±2.08^{c}	2.8±1^{a}	14.2±4.41^{b}	22±2.40^{b}	2.68±0.13^{a}	10.57±1.36^{b}	21.9±4.14^{c}	2.21±0.97^{a}	16.2±1.02^{b}	29±3.51^{c}
Fe (mg/kg)	1581±26.46^{a}	1552±104^{a}	1557±207^{a}	1873±260^{a}	1525±56.2^{ab}	1470±134^{a}	1558±89.95^{b}	1511±103^{ab}	1565±77^{a}	1962±50.95^{b}
Pb (mg/kg)	66.4±4.58^{b}	8.02±0.55^{a}	91.6±4.85^{c}	157.1±4.7^{d}	6.98±004^{a}	91.3±4^{b}	132.8±28^{c}	4.5±0.85^{a}	95.2±13^{b}	166.9±3.93^{c}

Initial soil: soil before sowing alfalfa seeds and irrigation; 5 t/ha and 25 t/ha: the doses of sewage sludge brought to soil; EC: electrical conductivity. Values are means (± SD), n = 3; Letters in the same column indicate differences at $p < 0.05$ (Duncan's test).

CONCLUSION

The cultivation of *Medicago sativa* (alfalfa) on a soil irrigated by tap water and fertilized with sewage sludge showed that this species absorbs significant quantities of metal trace elements and accumulates them in its aerial parts allowing thus soil phytoremediation. However, this effect has not been demonstrated when alfalfa grown on soil amended with sewage sludge and irrigated with treated wastewater. The combined application of treated wastewater and sewage sludge does not improve the production and constitute, in the short term, a polluting load.

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