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Interactions between accumulation of trace elements and major nutrients in *Salix caprea* after inoculation with rhizosphere microorganisms

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Abstract

Although the beneficial effects on growth and trace element accumulation in *Salix* inoculated with microbes are well known, little information is available on the interactions among trace elements and major nutrients. The main purpose of this study was to assess the effect of inoculation with rhizobacteria *Agromyces* sp. AR33, *Streptomyces* sp. AR17, and the combination of each of them with the fungus *Cadophora finlandica* PRF15 on biomass production and the accumulation of selected trace elements and major nutrients (Cd, Zn, Fe, Ca, K and Mg) in *Salix caprea* grown on a moderately polluted soil. Dry matter production was significantly enhanced only upon inoculation with *Agromyces* AR33. Microbial treatments differently affected the accumulation of Zn and Cd in plants. Both the inoculation with *Streptomyces* AR17 and the co-inoculation of *C. finlandica* with *Agromyces* AR33 were most efficient in enhancing the accumulation of Zn and Cd in leaves. These two treatments showed also a higher translocation factor from roots to the leaves for both Cd and Zn. Concentrations of major nutrients in shoots were generally increased in the treatments with the fungus compared to those without, except for K in plants inoculated with bacterial strain *Streptomyces* AR17. Co-inoculation of *C. finlandica* plus *Agromyces* AR33 resulted in a better accumulation of both Zn and Cd and Ca, K and Mg in shoots. This study suggests that the phytoextraction of Zn and Cd can be improved by inoculation with selected microbial strains.

Keywords

Salix caprea; phytoextraction; rhizosphere microorganisms; trace elements; major nutrients

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1. Introduction

Contamination of soils with heavy metals is one of the most serious environmental problems and several technologies have been developed to remove contaminants from the soil or render them harmless. Plants with the natural capability to accumulate heavy metals and metalloids in their aboveground biomass were selected and tested for the development of phytoextraction technologies (e.g. Baker and Brooks, 1989; Raskin et al., 1997; Wenzel and Jockwer, 1999; Pulford and Watson, 2002; Prasad and Freitas, 2003; Gamalero et al., 2009). Most trace elements do not have any known nutritional function (e.g. Cd, Hg, Pb), but others are important micronutrients (e.g. Fe, Cu, Zn), however, all are toxic when available at high levels in the soil. Plants have developed several mechanisms to control the accumulation of nutrients. Such mechanisms ensure that the plant obtains adequate levels of essential nutrients, while prevent their excessive accumulation (Grusak et. al., 1999). The uptake of toxic metals by plants is depending on several mechanisms that are both specie and metal specific. Heavy metals are taken up in root tissue via transport proteins of cells in the root cortex (Marschner et al., 1995). It has been reported that toxic metal, such as Cd, compete for the same transmembrane carriers with other essential nutrients (e.g. Ca, Mg, K, Fe, Zn) due to relative lack of selectivity of these transport systems (Clarkson and Lüttge 1989; Ghosh and Singh, 2005). After heavy metals have passed the membranes they are either stored in the root or translocated to the shoots. Inside the cell heavy metals are rendered less toxic through several mechanisms including chelation with phytochelatins, vacuolar compartmentalization and sequestration, induction of mechanisms to compensate the effects of reactive oxygen species such as the biosynthesis of antioxidant molecules and stress proteins, and upregulation of peroxidase synthesis (Salt et al., 1995; Sanita` di Toppi and Gabbrielli, 1999; Bricker et al., 2001). The cell wall and vacuole are the main sites of heavy metal sequestration.

The genus *Salix* is considered highly tolerant to excess heavy metal concentrations and is potentially suitable for Cd and Zn phytoextraction (van der Heijden, 2001; Pulford and Watson, 2002; Kuzovkina and Quigley, 2004; Dos Santos and Wenzel, 2007; Unterbrunner, 2007). Phytoextraction efficiency depends on metal tolerance and accumulation capacity, and can be improved by beneficial interaction between plant roots, soil and microorganisms (Jing et al., 2007; Wenzel, 2009). Several studies reported an increase of heavy metal concentrations in the tissues of terrestrial plants by application of rhizosphere microorganisms that can influence the bioavailability of contaminants and nutrients, the composition of root exudates and plant root development through the release of chelating substances, the acidification of the microenvironment, and the induction of changes in redox potential (Lebeau, et al., 2008; Wenzel, 2009; Glick, 2010). Moreover, microbial inoculation can improve nutrient supply and enhance plant tolerance to excess amounts of toxic trace metals when grown on contaminated soil (Leyval et al., 2002; Krupa et al., 2007; Gamalero et al., 2009). It is well known that some bacteria can synthesize siderophores which solubilize and sequester Fe from the soil solid phase, thus increasing its phytoavailability (Crowley et al., 1991; Wang et al., 1993). It has been demonstrated that some bacteria are able to synthesize several plant growth regulators including indole-3-acetic acid, auxins and cytokinins (Glick et al., 1998), which can increase the root surface absorption area resulting

in a better uptake of water and nutrients (Lasat, 2002; Wu et al., 2005). Chen and Zao (2009) reported improved nutrient uptake in *Astragalus sinicus* grown on contaminated soil, when inoculated with arbuscular mycorrhiza. It has been also reported that soil bacteria may affect gene expression in plants by providing additional nutrients from the soil (Gamalero et al., 2009); positive effect on nutrient uptake were reported also in *Trifolium pratense* when inoculated with both mycorrhizal and bacterial strain (Vivas et. al., 2006).

Although some recent studies have focused on the effects of microbial inoculation on growth and heavy metal accumulation in *Salix* sp. (Baum et al., 2006; Dos Santos Utmazian et al., 2007; Kuffner et al., 2008; 2010), the interaction of microorganisms on biomass production, nutrient and heavy metal accumulation are still poorly understood and results are often contrasting.

The main objective of this study was to assess the efficiency of three rhizosphere microorganisms (two bacterial and one fungal strain) on the phytoextraction capacity of *Salix caprea*. All inocula were already tested before on a different heavily polluted soil. However, since phytoextraction will be mainly feasible on moderately contaminated soil, we have chosen such a soil to assess the effects of these inocula on Zn and Cd phytoextraction on a less polluted substrate. In addition, the effect of rhizosphere microorganisms on the accumulation of selected nutrients (Fe, Ca, K and Mg) was assessed and compared with the Zn and Cd concentrations.

2. Material and methods

2.1 Bacterial and fungal strains

The bacterial strains tested were isolated from rhizosphere soil and were affiliated by sequencing of the 16S rRNA gene and analysis of 16S rDNA to the genera *Streptomyces* and *Agromyces* in a previous study conducted by Kuffner et al. (2008). The arbuscular mycorrhizal (AM) fungal strain *Cadophora finlandica* was isolated and characterized by PCR-amplification and sequencing of the ITS-region in a previous research of Dos Santos Utmazian et al. (2007).

2.2 Inoculation of *Salix caprea* plantlets

Cuttings of *Salix caprea*, clone Boku 04 CZ-024 (derived from Kutna Hora, Czech Republic, as described in Kuffner et al., 2010) were pre-grown for one year in a sand-soil mixture under controlled environmental conditions (14/20°C day/night temperature; 80% air moisture, 16 h light day⁻¹). The heavy metal contaminated soil used in this experiment was collected in Celje (Slovenia), air dried, sieved (2 mm) and gamma-ray irradiated with 25 kGray for 24h by Mediscan GmbH (Seibersdorf, Austria). The main properties of the experimental soil are given in Table 1. Each pot was filled with 800 g of the soil. The bacterial inocula were prepared as described in Kuffner et al. (2010). The fungal inoculum was prepared as described in Dos Santos Utmazian et al. (2007).

The contaminated soil was inoculated with microorganisms to test six treatments: autoclaved inoculum of *Streptomyces* and *Agromyces* as control (C); *Streptomyces* sp. (AR17);

Agromyces sp. (AR33); *C. finlandica* PRF15 plus autoclaved inoculum of AR17 + AR33 (F); *C. finlandica* PRF15 + AR17 (F_AR17); *C. finlandica* PRF15 + AR33 (F_RA33).

For all treatments with *C. finlandica* PRF15, the fungal inoculum was mixed into the soil before planting the willows. For all treatments inoculated with bacterial strains, 10 ml of bacterial suspension were applied to the soil surface surrounding the plantlet. The pots were positioned in a greenhouse in a randomized design, and each treatment was replicated four times. After a growth period of 12 weeks, plants were harvested, separated into roots, shoots and leaves and washed using tap water. To remove metals from the apparent free space of the root tissues, roots were sonicated for 10 min in 0.05 M CaCl₂ and rinsed with deionized water. All samples were dried at 80 °C for 24 hours before determining the dry matter (DM). For analysis of trace element and selected nutrient concentration in the tissues, each sample was finely ground and a subsample of 0.2 g was digested using a mixture of HNO₃/HClO₄ (4:1) in an open digestion system. Cd and Zn concentrations were determined by using an ICP-MS (Elan 9000 DRCE, PerkinElmer), whereas concentrations of Ca, Mg, K and Fe were determined with an ICP-OES (Zeiss, Plasmaquant 100). The translocation factor (TF) was calculated as element concentration in the leaves divided by the concentration in roots.

2.3 Statistical Analysis

Statistical analysis was performed by R software (version 2.10.1). All variables were tested with one way Analysis of Variance (ANOVA) followed by Duncan's test. A Student's *t* test was performed to determine statistical differences between Cd and Zn translocation factor within the same treatment. Values of elements concentrations in shoots were analyzed with two-way factorial ANOVA to evaluate the interaction between microbial treatments and selected elements (Cd, Zn, K, Ca and Mg); all data, then were plotted by using the normalized element concentration values calculated through a *z* score standardization. The *z* score value is given by $z=(x-\mu)/\sigma$, where *x* is the element concentration of each sample; μ and σ are respectively the mean and the standard deviation of each element.

3. Results

3.1 Biomass production

Except for root biomass, the microbial treatments had no significant effect on the biomass of leaves and branches of *Salix caprea*; however, some non-significant tendency for increased leaf and branch biomass was found after inoculation with *Agromyces* AR33 (Fig. 1 A). With the same bacterial inoculum, root biomass was significantly higher than the control, whereas no significant differences were observed in the other microbial treatments (Fig. 1 B), including *Agromyces* AR33 that was ineffective when co-inoculated with the AM fungus *C. finlandica* (Fig. 1 B).

3.2 Cadmium and zinc concentrations in leaves and roots

The concentrations of Zn and Cd in roots and leaves of *S. caprea* were significantly enhanced by microbial treatments (Fig. 2). For both elements, foliar concentrations always exceeded those in the roots. In particular, Zn concentrations in leaves varied from 187 mg kg⁻¹ in the control to 320 mg kg⁻¹ in the F_AR33 treatment, and in roots between 104 mg

kg⁻¹ of the control and 161 mg kg⁻¹ in the F treatment. Foliar Zn concentrations were significantly increased in the treatments F_AR33 and AR17 compared with the others treatments which were not significantly different from each other. Instead, for all microbial treatments root Zn concentrations were statistically different compared to the control. As for Zn, the highest Cd foliar concentrations were observed in the treatments F_AR 33 and AR 17 (7.4 and 7.1 mg kg⁻¹, respectively), whereas the lowest was found in the other combined treatment F_AR17 (3.6 mg kg⁻¹) and in the control (5.1 mg kg⁻¹), that were not significantly different compared to the other treatments. In the roots, the highest Cd concentrations were found in F and in F_AR33 (3.1 mg kg⁻¹) and the lowest in the control (1.94 mg kg⁻¹), whereas no differences were found among the others treatments (2.3 mg kg⁻¹). Considering all treatments, a close relationship was found between Zn and Cd concentrations in plant tissues ($r^2=0.80$) (Fig. 3).

Table 2 shows the translocation factors (TF) from roots to leaves for Cd and Zn. A one-way ANOVA showed that the investigated parameters were significantly affected by treatments ($P<0.05$). Zinc TF was significantly higher in the treatment with co-inoculation of the bacterial strain AR33 and *C. finlandica* (F_AR33), and significantly lower in the treatments AR33 and F; however no significant differences were observed between individual microbial treatments and the control. The TF for Cd showed that the co-inoculation of fungus *C. finlandica* generally decreased translocation from roots to leaves with respect to the corresponding treatments without fungus strain, except for F_AR33 which was not different from the bacterial strain AR33. Independent from the treatments, TF for Cd were generally higher than for Zn; comparing Cd and Zn TF in each microbial treatment, Student's *t* test showed significant differences ($P \leq 0.05$) only for the control and for both bacterial treatments AR17 and AR33 (Table 2).

3.3 Trace element and selected nutrient concentrations in shoots

The concentration of trace elements and selected nutrients in shoots are shown in Table 3. Except for Fe, all elements were significantly affected by the microbial treatments. Ca and Mg concentrations were generally higher in the co-inoculated treatments compared to those without the fungus *C. finlandica* (Table 3). In particular, the co-inoculated treatment F_AR33 showed a significant increase of 33 % of Ca and 27 % of Mg compared to AR33. For K, no differences were observed for all treatments except for AR17 which was significantly higher compared to the corresponding treatment with *C. finlandica* (F_AR17). As for leaves, both Cd and Zn concentrations in shoots of *Salix* showed similar trend with the highest values in F_AR33 and the lowest in F_AR17; in this latter, Zn and Cd concentrations were reduced by 30% and 46% respectively, with respect to the corresponding treatments without fungus (AR17) (Table 3).

Table 4 reports the statistical parameters of the two way ANOVA performed on the concentration of all elements (except Fe). The single factors (microbial treatments and selected elements) and the interaction between them were found to be significant ($p < 0.05$). This results indicate that treatments affected the accumulation of trace elements and major nutrients in shoots of *Salix* (Table 4). Indeed, as shown in Figure 4 where the *z* scores values of selected elements (Cd, Zn, Ca, Mg and K) are plotted, the data are grouped distinctly as a

function of treatments. Indeed, comparing the bacterial strain AR17 and the co-inoculated F_AR17 treatment, standardized values are arranged in two clearly distinct groups, i.e. Ca-Mg and Cd-Zn-K. In particular, high levels of Cd-Zn-K scores in AR17 treatment match high Ca-Mg values in F_AR17 and *vice versa*. To the contrary, in both AR33 and the corresponding combined treatment F_AR33, z scores values are grouped all together with the highest values in the combined treatment. Instead, in C and in F treatments z scores values are similar and generally coupled together.

4. Discussion and conclusions

Interaction between plants and soil microorganisms can affect plant growth, accumulation and tolerance to elevated trace element concentrations, as well as nutrient acquisition (Chen and Zao, 2009; Wenzel, 2009; Weyens et al., 2009). In the experiment reported here, no significant differences were observed on leaves and branches dry matter after inoculation with specific microorganisms, except for *Agromyces* AR33 where a significant increase of root dry matter and a slight trend of enhanced aboveground dry matter was found (Fig. 1). Although several studies showed that root growth is associated with enhanced nutrient and trace element accumulation (Marschner et al., 1995; Vessey, 2003), our results showed that the increase of root biomass observed with *Agromyces* AR33 inoculation did not lead to increase the concentration of both trace elements and selected nutrients, except for Zn in roots. On the other hand, *Streptomyces* AR17 and *C. finlandica* plus *Agromyces* (F_AR33) showed an increase of all selected elements in shoots, independently of biomass production. These results suggest that a mechanism other than root growth promotion was responsible for the increase of selected elements observed after inoculation with *Streptomyces* AR17 and with *C. finlandica* plus *Agromyces* (F_AR33).

It has been reported that rhizosphere microorganisms can differently alter bioavailability of contaminants and nutrients through the release of chelating substances, acidification of the microenvironment and by changing the redox potential, modifying soil conditions that influence metal accumulation in plants (Whiting et al., 2001; Lasat, 2002; Gadd, 2004; Jing et al. 2006; Kidd et al. 2009). About trace elements in relation to the microorganisms tested, our results (Figure 2 and Table 3) showed that in each microbial treatment Cd and Zn concentrations in tissues of *Salix* were affected similarly. Indeed, considering all treatments together, Cd and Zn concentrations in tissues were well correlated ($r^2=0.80$). Similar results were reported by Kuffner et al. (2008) who, by testing several bacterial strains, suggested that released bacterial metabolites may affect Zn and Cd concentration similarly. Instead, considering specific treatments, it is interesting to note that bacterial strain *Streptomyces* AR17 enhanced both Cd and Zn concentration in leaves while it decreased these elements in the corresponding treatment co-inoculated with *C. finlandica*. Conversely, the bacterial strain *Agromyces* AR33 increased both trace elements and major nutrients when associated to the fungus *C. finlandica*. Similar differences were observed for both Cd and Zn TF, i.e. Cd was translocated to a larger extent from roots to leaves compared to Zn, particularly in treatments with the bacterial strain only (Table 2). These results confirm the findings of Gamalero et al., (2009), who reported that metal uptake by plants treated with arbuscular mycorrhiza fungi can vary significantly as a function of the interaction with a specific bacterial strain. In addition it was also reported that mycorrhizal associations may reduce

trace elements translocation from roots to shoots by binding large amounts on cell wall components such as chitin, extrahyphal slime or through intracellular immobilization with metallothioneins and polyphosphates or compartmentalization within vacuoles (Leyval et al., 1997), suggesting a beneficial symbiosis of fungi and bacteria association.

In our study, the inoculation with microorganisms affected also the concentration of selected nutrients in shoots, except for Fe which was the only nutrient not statistically significant, probably because the two investigated bacterial strains did not produce siderophores (Kuffner et al., 2008). It is well known that some bacteria can synthesize siderophores which solubilize and sequester Fe from the soil solid phase, thus increasing its phytoavailability (Crowley et al., 1991; Wang et al., 1993). Except for Fe, among microbial treatments the uptake of nutrients generally increased in the co-inoculated *C. finlandica* plus *Agromyces* (F_AR33) treatment. Leyval and Berthelin (1991) suggested a positive interaction between ectomycorrhizal fungi and soil bacterial strains, hypothesizing that bacteria modify availability of soil nutrients, whereas the fungal partner may increase the absorptive area around roots. This is not the case for the co-inoculation of *C. finlandica* with *Streptomyces* (F_AR17) where a lower Cd, Zn and K concentration in shoots and a higher Ca and Mg concentrations was found compared to the *Streptomyces* (AR17) treatment. Although it is known that bacteria affect bioavailability of elements in the rhizosphere modifying their accumulation, our results showed that the co-inoculation of a fungus and bacterial strains modifies the rhizosphere in different ways with respect to the individual microorganisms.

Considering all data together, the interaction between microbial treatments and all selected elements in shoots was found to be significant (Table 4 and Fig.4), indicating that the tested microorganisms had different effects on shoot concentrations of both trace elements and major nutrients; thus, it is difficult to identify univocal behavior in the accumulation of all elements and to infer relationships between toxic and essential metals. Interesting results were found for K in relation to Zn and Cd; indeed, standardized values of K in all microbial treatments showed always values near to those of Cd and Zn, indicating that increased Cd concentration in shoots were associated with a concomitant increase of K concentrations. This trend can indicate a possible synergistic effect between Cd and K as previously found in tomato (Moral et al., 1994) and in Indian mustard (Jiang et al., 2004). Wenzel and Jockwer (1999) hypothesized that K may be related to compartmentation of toxic metals in several hyperaccumulator plants. Gussarsson et al. (1994) suggested that trace elements including Cd may interfere with nutrient uptake, including K, by altering the plasma membrane permeability and by affecting element transport processes across the membrane.

As for Zn, Cd and K, standardized values of Ca and Mg concentrations in shoots were similar, suggesting an analogous mechanism of accumulation for Ca and Mg in *S. caprea*, in which their concentrations increase or decrease depending on the microbial treatment tested. According to von Fircks et al. (2001) the uptake of Ca and Mg is mainly passive and depends to a great extent on the availability of these elements in soil solution. Moreover, Ca and Mg accumulation was high in all fungal treatments similar to bacterial strains. Following Rousseau et al. (1994), the high uptake of Ca and Mg found in *S. caprea* inoculated with *C. finlandica*, could be ascribed to an increased root absorbing surface area associated with the extensive mycelium system. Furthermore, the fungal hyphae penetrate

the soil around plant roots and consequently the plant has access to a larger volume of soil (Chen and Zao, 2009).

Finally, among the microorganisms tested the bacterial strain *Streptomyces* AR17 was most efficient to increase the accumulation of Zn and Cd, thus this particular strain is a good candidate to increase the phytoextraction efficiency of *Salix caprea* on both highly (Kuffner et al., 2008) and moderately contaminated soil. Instead, the combination of *Cadophora finlandica* plus *Agromyces* AR33 resulted in an enhanced accumulation of both major nutrients and trace elements. Considering the complex role of rhizosphere in phytoremediation technologies, further study are necessary to fully understand the role of bacteria, mycorrhiza and their combinations to exploit their synergistic mechanisms for phytoextraction of contaminated soils.

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Research Highlights

- > In this study we assess the effect of different rhizosphere microbes on the phytoextraction efficiency of *Salix caprea*.
- > The different inocula have significantly influenced the root biomass and the accumulation of Zn and Cd as well as the accumulation of some major nutrients.
- > Higher concentrations of Zn and Cd in leaves were associated with increased accumulation of K.
- > Rhizosphere microbes may enhance the phytoextraction capacity also on moderately polluted soils.

Fig. 1. Effect of microbial inoculation on: 1A) shoots and 1B) root dry matter. Data are means $\pm$ s.e. (n= 4); bars with same letter code are not significantly different for $p < 0.05$ based on Duncan's test.

Fig. 2.

Zinc (2A) and cadmium (2B) concentration in leaves and roots of *Salix caprea* inoculated with several microorganisms. Data show means (n= 4), bars with same letter code are not significantly different for $p < 0.01$ based on Duncan's test.

Fig. 3.

Relationship between Zn and Cd concentrations in roots (white symbols) and leaves (black symbols). Each data point represents measurements from an individual plant.

Fig. 4. Mean values of z-scores ($\pm$ standard error) performed on concentrations of Cd, Zn, Ca, Mg and K in shoots of *Salix caprea* inoculated with different microorganisms.

Table 1

Experimental soil properties.

Characteristic	Value
Sand (g kg ⁻¹)	450
Silt (g kg ⁻¹)	340
Clay (g kg ⁻¹)	210
pH(H ₂ O)	7.54
Carbonate content (g kg ⁻¹)	7.30
Cation exchange capacity (mmol kg ⁻¹)	273
Organic Carbon (g kg ⁻¹)	38.5
Total element concentration (<i>aqua regia</i>)	
Zn (mg kg ⁻¹)	608
Cd (mg kg ⁻¹)	4.93
Fe (mg kg ⁻¹)	2.30
Ca(g kg ⁻¹)	24.6
K (g kg ⁻¹)	3.79
Mg (g kg ⁻¹)	15.8
Extractable element concentration (1 M NH ₄ NO ₃)	
Zn (mg kg ⁻¹)	0.27
Cd (mg kg ⁻¹)	0.01

Table 2

Root-to-leaves translocation factors (TF) for Zn and Cd.

Treatment	Translocation Factor		<i>t test p-value</i>
	Zn	Cd	
C	1.83 ± 0.17 abc	2.80 ± 0.38 a	0.05*
AR17	2.17 ± 0.30 ab	3.05 ± 0.20 a	0.05*
AR33	1.42 ± 0.26 c	2.42 ± 0.47 ab	0.02*
F	1.48 ± 0.10 c	1.78 ± 0.17 bc	0.25
F_AR17	1.57 ± 0.30 bc	1.48 ± 0.22 c	0.86
F_AR33	2.28 ± 0.13 a	2.44 ± 0.19 ab	0.51
<i>F probability</i>	0.04**	0.008*	

Values are means ± s.e. (n=4).

Means with the same letter within a column are not significantly different according to Duncan's test. The statistical analysis was a one-way ANOVA.

* Significant differences between means within a line (P < 0.05 of *t*-Student test) are marked with asterisks.

Table 3Concentrations of selected elements in shoots of *Salix caprea*

Treatments	Ca	Mg	K	Fe	Cd	Zn
	(g kg ⁻¹ of DM)			(mg kg ⁻¹ of DM)		
C	20.6 ± 2.1 b	5.4 ± 0.2 bc	18.8 ± 1.6 b	57.7 ± 4.5	5.56 ± 0.1 abc	155.9 ± 6.7 c
AR 17	23.3 ± 1.2 ab	5.5 ± 0.2 bc	23.6 ± 0.9 a	62.6 ± 2.9	6.56 ± 0.4 ab	238.3 ± 24.1 ab
AR 33	20.0 ± 0.5 b	4.9 ± 0.1 c	20.1 ± 0.7 ab	58.8 ± 2.8	4.77 ± 0.5 cd	168.8 ± 20.8 c
F	24.5 ± 1.7 ab	5.5 ± 0.3 bc	20.1 ± 0.7 ab	71.1 ± 4.9	5.21 ± 0.3 bc	201.9 ± 14.7 bc
F AR 17	26.7 ± 2.7 a	6.0 ± 0.2 ab	18.0 ± 1.9 b	60.1 ± 3.3	3.53 ± 0.5 d	166.8 ± 13.9 c
F_AR 33	26.6 ± 1.1 a	6.2 ± 0.1 a	21.2 ± 0.9 ab	59.7 ± 3.3	6.63 ± 0.8 a	258.6 ± 18.5 a
<i>F probability</i>	<i>P</i> < 0.05	<i>P</i> < 0.01	<i>P</i> = 0.05	<i>P</i> = 0.15	<i>P</i> < 0.001	<i>P</i> < 0.01

Values are means ± s.e. (n=4). Means with the same letter within a column are not significantly different according to Duncan's test. The statistical analysis was a one-way ANOVA.

Table 4

Statistical parameters of two-way ANOVA performed on selected elements concentration (Cd, Zn, Ca, Mg and K) for all microbial treatments.

Source of variation	df	MS	F
Treatments (T)	5	11 620 295	3.43 **
Selected elements (E)	4	3 043 665 220	898.66 ***
T × E	20	9 323 321	2.8 ***
Residuals	90	3 386 875	

df, degree of freedom; MS mean of square; F variance ratio

**
P < 0.01

P < 0.001;