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Removal of pentachlorophenol in a rhizotron system with ryegrass *(Lolium multiflorum)*

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Abstract

Degradation of pentachlorophenol (PCP) in the rhizosphere of *Lolium multiflorum* (ryegrass) was studied in an Andisol of southern Chile. A rhizotron system was designed to evaluate the effect of different PCP concentrations (50, 100 and 250 mg kg^{-1}) on plant biomass, organic acid exudation, dehydrogenase and the β-glucosidase activity of *L. multiflorum*. Moreover, the microbial biomass was estimated using a molecular technique in different sections of rhizotrons. More than 96% of PCP was removed in the rhizotron system by adsorption in the soil and degradation by the rhizosphere of *L. multiflorum*. The plant biomass decreased when the PCP concentration was increased in the soil, the biomass for the treatment with 250 mg kg^{-1} being significantly lower. The estimation of the microbial biomass and β-glucosidase activity in the soil was not affected when the PCP concentration was increased, indicating a low toxicity of the contaminant due to its lesser availability. However, the dehydrogenase activity decreased when increasing the PCP concentration, showing negative effects on microorganisms. The organic acid exudates varied during the incubation time, influenced by the stress of the different treatments and the distribution of roots. Therefore, *Lolium multiflorum* is a promising candidate for designing field scale phytoremediation processes.

Keywords: Pentachlorophenol, phytoremediation, *Lolium multiflorum*, rhizosphere

1. Introduction

Pentachlorophenol (PCP) is a pollutant with a significant environmental impact not only on the ecosystem, but also on human health. The most commonly used methods for removing PCP from polluted soils are disposal in landfills, soil washing and chemical extraction. Many of these strategies are expensive and are not proven to be effective in eliminating contaminants from the environment. Bioremediation, an alternative biological method, that includes phytoremediation, has demonstrated its effectiveness, and this biotechnology will be our focus.

In phytoremediation the rhizosphere is very important place were pollutant degradation occurs, due to interaction between plants roots and soil microorganisms and takes place (Dams *et al*,2007). In the rhizosphere, pollutant degradation is stimulated by the release of root exudates, plant enzymes and soil microorganisms. The composition and concentration of them can vary depending on plant species (Zheng and Zhang 2000; He *et al*. 2005). Plant roots also improve the adsorption of pollutants in the soil, increasing their bioavailability, thereby generating several changes in the soil (Dams *et al*. 2007). These changes can be detected by measuring microbial-origin enzymes as they provide an indication of quality changes in contaminated soils. Similarly, *β*-glucosidase activity varies with soil management and has been proposed as an indicator of soil quality because it provides an early sign of changes in organic matter status and its turnover (Ndiaye *et al*. 2000; Madejón *et al*. 2001). In addition, dehydrogenase activity has been considered a measure of the total oxidative activity of soil microflora, and therefore of the metabolic activity of soil (Nannipieri *et al*, 2002).

He *et al*. (2005) and Nakamura *et al*. (2004) showed that the rhizosphere effect of *Lolium perenne* L. and *Allium tuberosum Rottler* effectively influences PCP degradation in soil, suggesting that the rhizosphere associated with microbial biomass increases with the ability to eliminate this compound. He *et al*. (2009) suggested that root exudates induced modifications to microbial communities in the PCP contaminated rhizosphere. To improve our understanding of this phenomenon, further research is necessary to provide clarity of the plant–soil–microbe interactions developed within this study.

Therefore, the aim of this study was to evaluate the degradation of PCP in the rhizosphere of Lolium multiflorum (ryegrass). In this assay, Lolium multiflorum was planted in the soil contaminated with different PCP concentrations and disposed in a rhizotron system composed of micro suctions tubes to investigate the chemical changes of the rhizosphere by the localized extraction of soil solution near the root. Furthermore, soil enzyme activities and bacterial biomass were determined via real time PCR.

2. Materials and Methods

2.1. Preparation of soil

An Andisol collected from the Temuco Series located in southern Chile (38º 42’ S, 73º 35’ W) was used for the experiments. This soil was collected at a depth of 0-20 cm and the pre-treatment involved sieving to select a particle size under 2 mm and air-drying. The major physic-chemical properties of the soil are; pH (in water) = 5.70±0.10; N (%) = 0.72±0.02; C (%) = 8.06±0.95; P (mg kg^{-1}) = 15.0±0.95; MO (%) 14.0±1.68; C/N = 11.0±1.25, values of mean (n=3) ± standard deviation.

The soil was spiked with a stock solution of PCP (Aldrich, 98% purity) diluted in acetone to reach a concentration of 50, 100 or 250 mg PCP kg^{-1} soil, homogenized by vigorous shaking and kept under a fume hood for 12 h to evaporate the solvent.

2.2. Seed preparation

Seeds of *Lolium multiflorum* were used to evaluate PCP degradation. The seeds were sterilized in 2% v v-1 sodium hypochlorite solution for 20 min, and then washed for 30 min with sterilized water. The seeds were incubated at 25 ºC in germination trays of 18 x 12 x 6 cm, containing moist autoclaved Whatman no. 44 filter paper (Rattray *et al*. 1995). After 2 weeks, the germinated seeds were placed on 4 liter plastic pots containing Taylor and Foy nutritive solution (Taylor and Foy 1985). The solution was changed after the first week and maintained under periodic aeration in a controlled temperature chamber at 20 ± 2 ºC for 2 weeks.

2.3. Rhizotron assay

The assays were performed in triplicate using the rhizotron system of 33 x 11.6 x 2 cm. The rhizotrons were filled with 620 g of soil contaminated with different PCP concentrations (0, 50, 100 or 250 mg kg^{-1}). Six ryegrass plants previously grown in Taylor and Foy nutritive solution with a root length of approximately 15 cm, were placed in the rhizotron system. Five micro suction tubes 3 mm in diameter were installed around the plant root to obtain root exudates (malic acid, succinic acid and citric acid). Further, control treatments with unplanted soil, with different PCP concentrations, and planted soil without PCP concentration were developed for comparative effect. Each experiment was conducted in triplicate.

All rhizotrons were incubated at 20 ± 2 ºC and their water holding capacity (WHC) controlled at 60% for 10 days. The root exudates were periodically measured at 2, 4, 6, 8 and 10 days. At the end of the experiment, the rhizotrons were divided into three sections of 11 cm depth each: top zone (section 1), central zone (section 2) and bottom of rhizotron (section 3) in order to determine the effect of root on the PCP degradation. In each section, the residual PCP concentration, enzymatic assays (dehydrogenase and β-glucosidase) and microbial estimates were determined. In addition, the plants were collected to determine the root length and aerial and root biomass quantification.

2.4. Plant biomass

Plant growth was evaluated by biomass (aerial and roots) evaluation on the basis of dry weight at 80 º C for 24–48 h. The results were expressed by the following equations:

% relative mass= treated plant dry weight/ control plant dry weight x100 (*Ec. 1*)

2.5. Extraction and analysis of PCP

The PCP extraction from soil was performed as described by Soto-Córdoba *et al*. (2001). The procedure was performed as follows: i) 4 g of soil were extracted with hexane for about 16 hours in a soxhlet extractor system, ii) the samples were evaporated at 65º C using a rotavapor under reduced pressure, iii) the sample was re-suspended in 5 mL of methanol HPLC grade and filtered through a 0.45 µm membrane and iv) an aliquot of the organic phase was used for analysis.

Residual PCP was determined by high performance liquid chromatography (HPLC) with an instrument equipped with a Merck-Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20 µL loop, a Merck-Hitachi L-7455 diode array detector operating at 215 nm, and a Hitachi D-7000 data processor. A Lichrosphere 60 RP select B 250 mm x 4 mm column of 5 µm particle size with a LichroCART 4-4 guard column (Merck) was used. The mobile phase consisted of acetonitrile and phosphoric acid (1% aqueous solution) 1:1 (v v^{-1}) with a flow rate of 1 ml min^{-1} (PCP retention time was 12 min). Instrument calibrations and quantifications were performed against the pure reference standard (0.05–5 mg L^{-1}). The procedure described was checked for recovery (which ranged from 86–100%). The detection limit was 0.03 mg l^{-1}, considering a noise-to-signal ratio greater than 2.

2.6. Collection and quantification of root exudates

Exudates were collected every two days until day 10, and frozen immediately after extraction. The concentration of organic acid (malic acid, succinic acid and citric acid) exudates was analyzed by HPLC. The samples were filtered through a 0.45 μm membrane before injection (20 μL) in the HPLC equipment. HPLC analyses were performed with a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with 20 μL loop diode array detector. The detection was set up at 210 nm and the column was a reverse phase (Merck, LiChrospher 100 RP-18. 5 μM). The mobile phase consisted of phosphoric acid 200 mM, pH 2.1 worked at 25 °C delivered at a flow rate of 2 mL min^{-1}, at room temperature (about 22 ºC). Standard solutions were prepared from a mixture of organic acids, to identify organic acids present in the exudates, which was done by comparing the retention times with standards.

2.7. Enzymatic activities

Dehydrogenase activity was determined using Mersi and Schinner (1991) method modified by Garcia *et al*. (1997). One gram of soil was incubated for 20 h at 25 ºC with 0.2 mL of 0.4% 2-p-iodophenyl-3-p-nitrophenyl-5 tetrazolium chloride (INT) as a substrate. Iodonitrotetrazolium formazan (INTF) produced in the reduction of INT was extracted with a mixture of acetone:tetrachloroethene (1.5:1), and measured in a spectrophotometer at 490 nm. Assays without soil and without INT were performed simultaneously as controls. The enzymatic activity is expressed in ug INTF g h^{-1}.

For the determination of β-glucosidase, 0.5 mL of 0.05 M 4-nitrophenyl-β-D-glucanopyranoside (PNG) was used as substrate (Tabatabai 1982). Soil samples (0.5 g) were incubated at 37 ºC for 2 h with 2 mL of maleate buffer at pH 6.5. The samples were kept at 2 ºC for 15 min to stop the reaction, and the p-nitrophenol (PNP) produced was extracted and determined at 398 nm (Nannipieri *et al*. 1982). The enzymatic activity is expressed in ug PNP g h^{-1}.

2.8. Soil–DNA extraction and real-time PCR assay

In this study, the total DNA was extracted from 0.5 g soil following the manufacturer's instructions for the MoBio UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA). To remove trace concentrations of PCR inhibitors, the second step (Inhibitor Removal Solution) was repeated. The DNA samples were checked for concentration and quality using the NanoDrop1 ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA).

Real-time PCR was performed to quantify the number of 16S rRNA copy in triplicate soil–DNA extracts. Universal specific primers for V3 hypervariable regions of 16S rRNA of eubacteria P1 and P2 (Muyzer, Dewaal, & Uitterlinden 1993) were used. Each 21 μL PCR reaction contained from 2 to 5 ng of the DNA, 10.5 mL 2× iQ SYBR Green Supermix (Bio-Rad, Munich, Germany), and 400 nM each primer. For each extracted DNA, real-time PCR experiments were conducted three times with the threshold cycle (Ct) determined in triplicate. The real-time PCR program consisted of 1.5 min at 50 ºC for carryover prevention, 15 min at 95 °C for enzyme activation, followed by 35 cycles of 15 s at 95 ºC, 30 s at 55 ºC and 30 s at 72 ºC, when the fluorescence signal was measured. PCR amplification procedure was checked with a heat dissociation protocol (from 70 ºC to 100 ºC) after the final PCR cycle. The DNA copy number was quantified on an iQ5 thermocycler using iQ5-Cycler software (Bio-Rad, Munich, Germany). The standard curve was generated by using a plasmid recombinant containing a copy of 16S rRNA fragment. The curve was drawn by plotting the Ct value as a function of the log of the copy number of 10-fold dilution serial of plasmid DNA. As Ct values may vary slightly between experiments, parallel sets of the three dilution series of pure standard DNA were run in all experiments. The relationship between Ct and the target gene copy number, and the copy numbers of the real-time standard were calculated as described by Qian *et al.* (2007).

2.9. Data analyses

Experiments were conducted using three independent replicates. PCP residual measurements in the different treatments were subjected to an analysis of variance (one-way ANOVA), and the averages were compared by Tuckey`s test at $p \leq 0.05$, to identify statistical differences among section of rhizotron at the same treatment. Relative mass were compared by Tuckey`s test at $p \leq 0.05$, to identify statistical differences among treatments with the control at the end of assay.

Microbial activities measurements in the different treatments at the end of assay were subjected to a one-way ANOVA and the averages were compared by Tuckey`s test at $p \leq 0.05$, to identify significant effects of sampling treatments in the same treatments in different sections of rhizotron and among treatments at the same section of rhizotron.

3. Results and Discussion

3.1. PCP removal

High PCP removal (> 96%) was observed in the different sections of rhizotrons with 50, 100 and 250 mg kg^{-1} of initial PCP concentration (Figure 1).). In the different sections of the rhizotrons, significant differences were observed only in the treatment with 250 mg kg^{-1} of PCP between section 3 and the other sections.

These results can be attributed to the absence of roots in section 3 of the rhizotron. In this respect, only the root growth in the rhizotron with 250 mg kg^{-1} did not reach section 3, with the root growth being inhibited by PCP concentration; consequently, there was less removal of PCP (Table 1). These results are consistent with the study performed by Dams *et al*. (2007), where it was demonstrated that root length of *Triticum aestivum* was affected by 100 mg kg^{-1} of PCP compared to the root length for plants in the absence of PCP. Therefore, our study suggests that PCP concentrations greater than 100 mg kg^{-1} inhibit root growth of *Lolium multiflorum* generated a reduced removal of PCP due to decreased root growth.

These results agree with several studies (He *et al*. 2005; Dams *et al*. 2007; He *et al*. 2009), which have demonstrated that PCP degradation in soil planted with ryegrass depended on the proximity of the roots to the contaminated soil. He *et al*. (2005) attributed this effect to interactions of PCP with root exudates and the soil microbial community.

The PCP removal in unplanted soil is presented in Figure 2. In soil with 50 mg kg^{-1} of removed PCP concentration, there were 17.35 mg kg^{-1} of PCP, corresponding to 34.5%, while in an unplanted soil with 100 mg kg^{-1} and 250 mg kg^{-1} they were 36% and 38%, respectively. PCP removal in unplanted soil is attributed mainly to PCP degradation by indigenous microorganisms and the adsorption process.

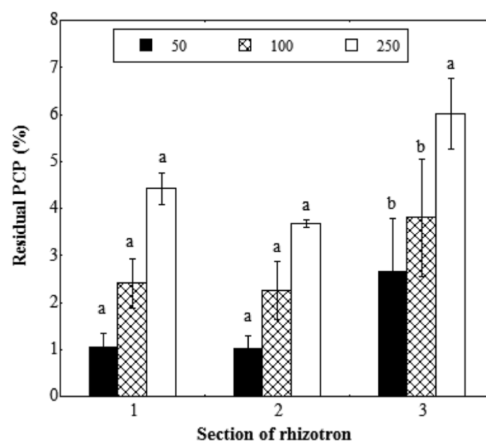

Figure 1. Residual PCP (%) in the soil contaminated with 50, 100 and 250 mg kg^{-1} of PCP in the different sections of rhizotron, during the degradation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Bars indicated by different letters show significant differences among section of rhizotron at the same PCP concentration.

In this context, in most studies on PCP removal in Andisols, the principal mechanism is adsorption (Navia *et al*. 2003; Diez *et al*. 2005; Cea *et al*. 2005; Rubilar *et al*. 2011) Rubilar *et al.* (2011) demonstrated that in an Andisol contaminated with 250 mg kg^{-1} of PCP, 55.7 ± 6.3% was removed, where the 41.2 ± 2.4% (measured in sterile soil) due to the adsorption process, with the highest adsorption rate during the first 7 days of incubation Cea *et al.* (2005) demonstrated that Chilean Andisols are particularly efficient sorbents for chlorophenols, mainly for allophane–ferrihydrite associations found in organic matter accumulated through the formation of stable complexes with FeOH and AlOH, so that these will allow hydrogen bond formation between hydroxyl groups and pentachlorophenol.

Table 1. Root long of *Lolium multiflorum* planted in soil contaminated with different PCP concentrations.

Soil contaminated with initial PCP concentration (mg kg^{-1})	Root long (cm)[a]
0	28.0 ± 1.2
50	26.0 ± 0.9
100	24.0 ± 1.1
250	15.0 ± 0.4

[a] Value of mean (n = 3) ± standard deviation

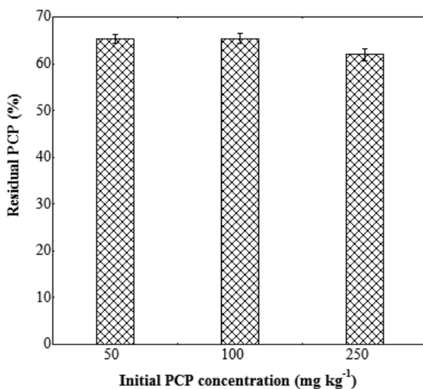

Figure 2. Residual PCP (%) in the unplanted soil contaminated with 50, 100 and 250 mg kg^{-1}. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

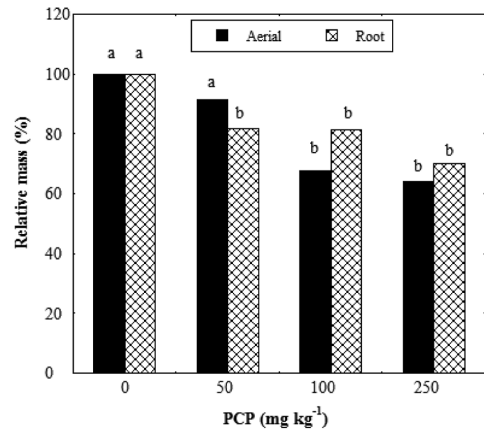

Figure 3. Plant biomass (aerial and root) in a rhizotron with a total of 6 *Lolium multiflorum* (ryegrass) expressed in relative mass (%) to different PCP initial concentrations. Bars indicated by different letters show significant differences among treatments with the control at the end of assay.

3.2. *Effect of PCP concentration on plant biomass of Lolium multiflorum*

Aerial and root plant biomass were measured after 10 days planted in PCP-contaminated soil (Figure 3). No significant differences between control treatment and the 50 mg kg^{-1} of PCP concentration were observed for the aerial biomass. However, significant differences were obtained between the control sample and treatments with 100 and 250 mg L^{-1}. The same effect was found in studies performed by Dams *et al*. (2007) with *Triticum aestivum*, demonstrating that a PCP concentration of 100 mg kg^{-1} decreased plant growth.

A similar effect was observed in the root biomass of *Lolium multiflorum* mainly in the thickness of the roots, with the dry weight of root being lowest in the rhizotron system with the highest PCP concentration. Significant differences were found between the control treatment and the other treatment. However, no significant differences among different PCP concentrations (50, 100 and 250 mg kg^{-1}) were observed.

3.3. *Effect of PCP concentration on enzymatic activities of soil*

The dehydrogenase enzyme is very often considered a reliable indicator of the active microbial biomass in soil, the activity of which depends on the survival of the living microbial cell (Trasar-Cepeda *et al*) 2000). Therefore, it is very sensitive to PCP contamination (Diez *et al*. 2006). The dehydrogenase activity PCP-contaminated soil decreased as the PCP concentration increased, especially in the rhizotron with an initial PCP concentration of 250 mg kg^{-1} (Figure 4). This suggested that although the PCP concentration was lower at the end of the assay, the soil microbes had not fully recovered from initial toxic responses of PCP. These results agree with Diez *et al.* (2006), who showed that dehydrogenase activity is affected further by PCP concentrations. In addition, McGrath and Singleton (2000) reported that, dehydrogenase activity decreased considerably in soils contaminated with PCP, although the PCP residual concentration was less than 2 mg kg^{-1} in soil after 6 weeks of incubation. This effect can be attributed to the toxicity of PCP degradation product. In this context, Rubilar *et al*. (2007) demonstrated that the main metabolite of pentachlorophenol degradation in soil is the pentachloroanisole. However, this compound is subsequently degraded by soil microorganisms. Besides, the dehydrogenase activity was not affected with respect to the different depths of rhizotrons in treatments of PCP at 0 and 50 mg kg^{-1}. This effect could be attributed to that the root length was detected in the three sections of rhizotron. Different behavior was demonstrated in the treatment with 100 and 250 mg kg^{-1} where the highest dehydrogenase activity was detected in the third section of rhizotron. In this case the high activity in section 3 of rhizotron could be attributed to a high accumulation of radical exudates (malic acid).

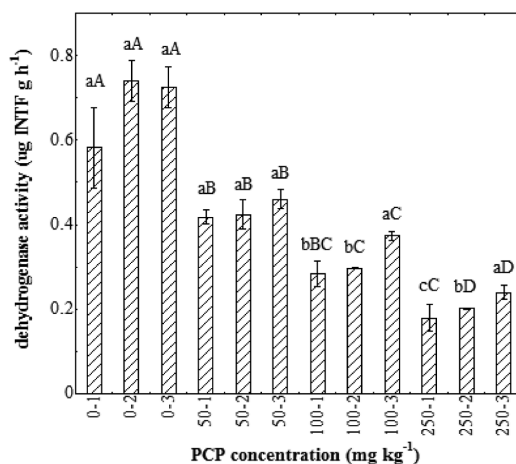

Figure 4. Dehydrogenase activity in the soil contaminated with 50, 100 and 250 mg kg^{-1} of PVP in the different sections of rhizotron, during the degradation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Bars indicated by different capital or lower case letters show significant effects of sampling treatments in the same treatment in different sections of rhizotron and among treatments at the same section of rhizotron.

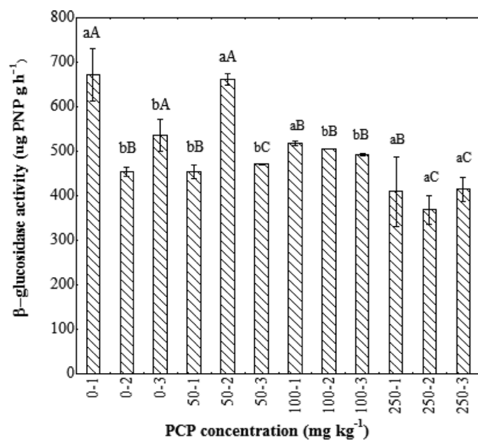

Figure 5. β-Glucosidase activity in the soil contaminated with 50, 100 and 250 mg kg^{-1} of PCP in the different sections of rhizotron, during the degradation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Bars indicated by different capital or lower case letters show significant effects of sampling treatments in the same treatment in different sections of rhizotron and among treatments at the same section of rhizotron.

The other enzyme evaluated was β-glucosidase as it has been proposed as a soil quality indicator, since it provides indications of changes in organic matter in the soil (Debosz *et al.* 1999). β-glucosidase activity is shown in Figure 5. In general, lower activities were observed in treatment contaminated with 250 mg kg^{-1} of PCP compared to the control in the three sections of rhizotron ($p \leq 0.05$). In addition, β-glucosidase activity in control treatment without PCP the values were significantly higher in section (1 and 3) than rhizotrons contaminated with 50 and 100 mg kg^{-1} of PCP at the end of assay. This result is consistent with studies of Moreno *et al*. (2011), who found a negative effect of herbicides on β-glucosidase activity.

3.4. Production of organic acids exuded by roots of Lolium multiflorum

Malic acid, succinic acid and citric acid exuded by roots of L. *multiflorum* are shown in Figure 6.

Malic acid was detected throughout the entire incubation time for rhizotron with 100 and 250 mg kg^{-1} of PCP, increasing as time went on and being highest in the rhizotron with 250 mg kg^{-1} of PCP (Figure 6a); by contrast, the malic acid production was detected from day 6 in the control rhizotron and malic acid was detected only on days 8 and 10 in the rhizotron with 50 mg kg^{-1}.

Succinic acid production was detected in all the rhizotrons with and without PCP. However, the results showed that the exudation of succinic acid was changeable in response to different PCP concentration stresses (Figure 6b).

Citric acid was detected in all treatments with and without PCP from the first day; it decreased for each treatment until it was not detected in any treatment on day 10 (Figure 6c).

Exudation of organic acid depends on diverse soil processes, such as plant metabolism, pollutant detoxification, nutrient available, soil composition, etc. It is difficult to determine the role of organic acid in phytoremediation due to the influence of many factors during their measurement, such as solid phase sorption/desorption reactions, complexation reactions, leaching and microbial degradation (Morita *et al*. 2004). The results showed that the exudation of organic acid was changeable in response to PCP concentration stress. Among the three organic acids detected in the experiment, malic acid showed the highest concentration in the soil, being exuded mainly in the rhizotron with the highest PCP concentration. Therefore, this organic acid could be important in controlling phytotoxity induced by PCP concentration. Walton *et al*. (1994) demonstrated that plants may respond to chemical stress by increasing or changing

the exudation radical affecting the microfloral composition or activity of the rhizosphere, being capable of degrading toxicants. In the same way, Amellal *et al*. (2002) and Ouvrard *et al.* (2006) demonstrated that plants subjected to chemical stress can change soil properties, varying the bioavailability of contaminants in the soil. However, we must consider that there might be other factors that affect the exudation of organic acids. With these results, the variation of exudates among the three treatments with the addition of PCP and control can be achieved, reflecting a change in the responses of ryegrass to the different environmental conditions to which they have been subjected, which can in turn influence soil decontamination processes.

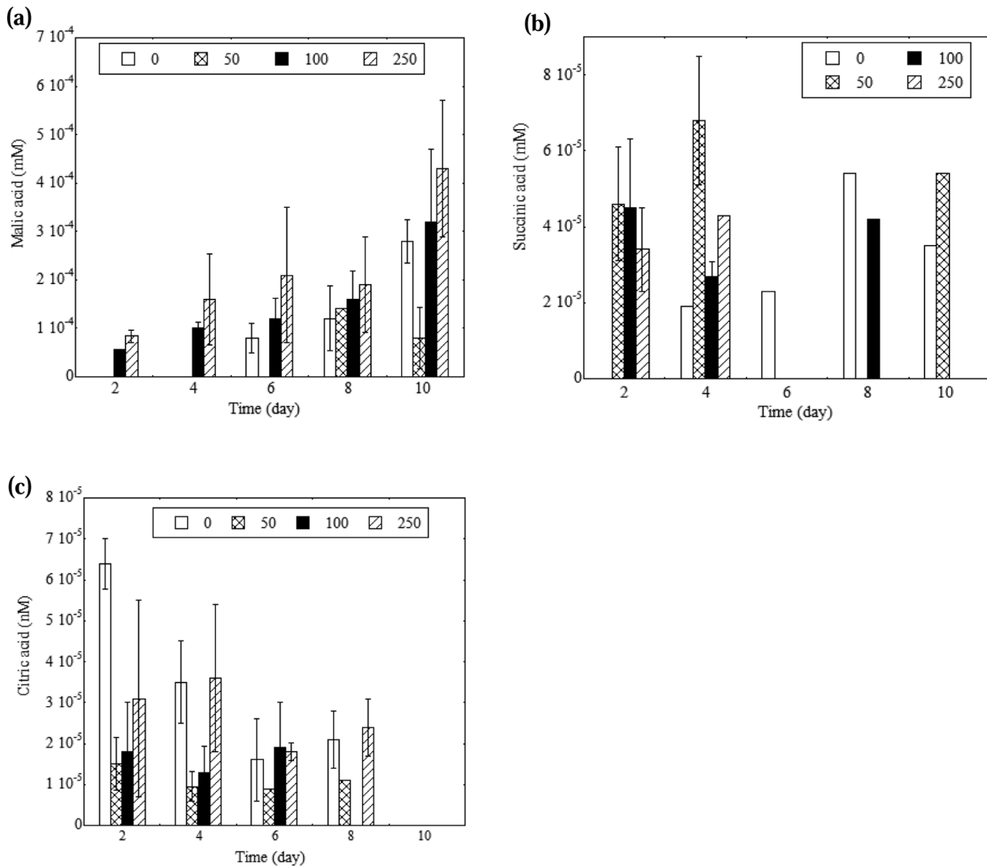

Figure 6. Exudation radical of malic acid (a), succinic acid (b) and citric acid (c) in the soil contaminated with 50, 100 and 250 mg kg^{-1}of PCP in the different sections of rhizotron, during the degradation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

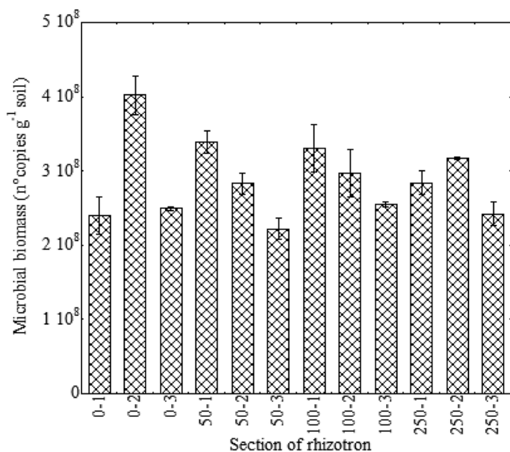

Figure 7. Estimation of the microbial biomass in the soil of PCP with 50, 100 and 250 mg kg^{-1} concentration in the different sections of rhizotron, during the degradation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

3.5. Real-time quantitative PCR

The estimation of the bacterial biomass was not affected by increasing the PCP concentration (Figure 7). Instead, the lowest quantity of copies detected in section 3 of the rhizotron are more likely associated with the lowest amounts of roots in this section than with the effect of PCP concentration (Figure 7). Studies conducted by He *et al*. (2005) demonstrated that microbial biomass was the largest in root proximity; as a result, the most effective PCP degradation, presumably due to the complementary effects between roots and associated microbial activity.

With respects to the different depths of rhizotron, the trend was not always the same for each treatment, which can be attributed to the sensitivity of microorganisms to different biotic and abiotic factors.

4. Conclusions

Some promising general, conclusions may be derived for the results obtained here:

1. PCP removal in contaminated soil was greater than 96% in the three concentrations studied, which could be caused either by adsorption in the soil matrix, or degradation by the rhizosphere, which showed higher PCP removal in planted treatments, than unplanted soil.

2. Aerial and radical biomass decreased when the PCP concentration was increased, resulting in significant differences, especially for the 250 mg kg^{-1} treatment of PCP with the control. The dehydrogenase activity decreased when the PCP concentration was increased, showing certain negative effects on some types of microorganisms.

3. The estimation of the microbial biomass was not affected by increasing the PCP concentration. With respect to the β-glucosidase activity, there was no significant difference between diverse PCP concentrations applied to the soil, indicating a low toxicity of the contaminant due to low PCP availability in the soil matrix at the end of the assay.

4. The organic acid exudates varied during the incubation time, influenced by the stress due to the different treatments and the root distribution. Therefore, *Lolium multiflorum* is a promising candidate for designing field scale phytoremediation processes.

Acknowledgements

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