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BIOSTIMULATION OF AGRICULTURAL BIOBEDS WITH NPK FERTILIZER ON CHLORPYRIFOS DEGRADATION TO AVOID SOIL AND WATER CONTAMINATION

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ABSTRACT

Degradation of the insecticide chlorpyrifos (160 a.i mg kg^{-1}) using a biomix of a biobed system biostimulated with inorganic fertilizer (NPK) was investigated. Three concentrations of the fertilizer (0.1%, 0.5% and 1.0% ww^{-1}) were evaluated on chlorpyrifos degradation, TCP (3, 5, 6-trichloro-2-pyrinidol) accumulation and biological activity of the biomix. The chlorpyrifos was dissipated efficiently (>75%) after 40 days of incubation and no additional dissipation was obtained with increasing concentration of NPK after 20 days of incubation. TCP accumulation occurred in all evaluated NPK concentrations and its concentration increased with the increment of NPK addition raising the probability of leaching of this compound. Biological activity (FDA and ligninolytic enzyme activity) in the biomix increased by the NPK presence in all evaluated concentrations. The DGGE analyses showed that combined treatments with lower amounts of NPK (0% and 0.1%) and chlorpyrifos showed no significant modifications in the microbial community in the biomix. However, combined overdoses of NPK (0.5 and 1.0%) and chlorpyrifos caused significant modifications in the bacterial communities that could be associated with TCP degradation reduction in the biomix. In conclusion, the obtained results demonstrated that the biomix prepared with Andisol and biostimulated with NPK nutrient can be recommended in biobeds as a viable alternative of chlorpyrifos dissipation avoiding soil and water contamination probability.

Keywords: Chlorpyrifos, biostimulation, NPK fertilizer, biobed, Andisol

INTRODUCTION

Pesticides play an important role in the success of modern farming and food production. However, surface and groundwater can be contaminated due to their inadequate management. The release of pesticides into the environment generally occurs by diffuse (nonpoint) or localized (point) sources. Point sources like spillages, tank filling, or cleaning the spraying equipment have been identified as the major risks of contamination of soil and water in agricultural system.

Biobed is a biological technology developed in Sweden and widely used in Europe to minimize point source contamination by pesticides in agricultural system (Torstensson and Castillo, 1997). Typical Swedish biobeds

are built with simple and cheap materials and a biomix mainly composed of a volumetric proportion of straw (50%), peat (25%) and soil (25%) (Torstensson and Castillo, 1997). Straw is the main component for ligninolytic fungi growth, soil provides sorption capacity and favors microbial activity, and peat contributes to sorption capacity and moisture control in the biomix. Several studies have demonstrated that these biological systems can effectively retain and degrade pesticides including chlorpyrifos (Castillo *et al*., 2008, Coppola *et al*., 2007; Vischetti *et al*., 2008).

Chlorpyrifos [*O,O*-diethyl *O*.(3,5,6-trichloro-2-pyridyl) phosphorothiate] is a broad-spectrum organophosphorus insecticide widely used throughout the world including in Chilean agriculture. It has a low water solubility (1.39 mg L^{-1}) and a high affinity for soil organic carbon (K_{oc}= 8498 mL g^{-1}) (Racke, 1996). When chlorpyrifos is introduced into the soil it can cause a reduction of soil microbial populations (Chu *et al.,* 2008). In addition, chlorpyrifos bound to soil constituents may be introduced into rivers by surface runoff from agricultural lands (Wu and Laird, 2004). Phillips *et al.* (2003) reported that this compound bound to suspended colloid is toxic to aquatic organisms.

Pesticide degradation in soil or biobeds can be limited by many factors, for example: soil properties, moisture, temperature, pH and initial concentration or repeated applications of pesticides (Castillo and Torstensson, 2007; Fogg *et al.,* 2003; Vischetti *et al.,* 2008). Moreover, biostimulation of the indigenous microorganisms by addition of nutrients is an important aspect to be taken into account because the enrichment of the indigenous microbial populations is the most widely tool used in bioremediation procedure. Several works have demonstrated the positive effects of biostimulation, mainly with NPK fertilizers, on contaminant biodegradation in soil (McGhee and Burns, 1995; Margesin and Schinner, 2001; García-Blanco *et al.,* 2007). McGhee and Burns (1995) reported that the addition of a combined NPK fertilizer increased significantly degradation of the herbicides 2,4-D and MCPA. In another work, García-Blanco *et al.* (2007) determined that fertilization with N and P improved oil disappearance in a contaminated site. Similar results were found by Margesin and Schinner (2001), where biostimulation with NPK showed a significant reduction of hydrocarbons in a soil contaminated with diesel, and all biological parameters were also significantly enhanced. Although it has been demonstrated that biostimulation is an effective technique for the treatment of contaminated sites, no information is reported in the literature about the use of biostimulation in a biobed system. Therefore, the objective of our study was to evaluate the effect of the biostimulation with NPK of the biomix of a biobed on the degradation of chlorpyrifos and on the biological parameters of the biomix.

MATERIALS AND METHODS

Chemicals

Analytic-grade (99% purity), chlorpyrifos (O, O-diethyl-O-trichloro-2-pyridylphosphorothioate), and TCP (3,5,6-trichloro-2-pyridinol) were purchased from Sigma-Aldrich. Formulated chlorpyrifos (Clorpirifos S480) of 48% w/v was purchased from ASP Chile. MBTH (3-methyl-2-benzothiazolinone hydrazone), DMAB (3-(dimethylamino) benzoic acid) were purchased from Aldrich. All other

chemicals and solvents were of analytical reagent grade and were purchased from Equilab and Merck Chile. Commercial NPK fertilizer (20-5-5%) was purchased from Anasac.

Preparation of biomix and incubation conditions

The biomix was prepared by mixing an allophanic top soil (Andisol) without chlorpyrifos application history (37.1% sand, 34.2% silt, 28.7% clay), commercial peat-free compost (organic carbon 39.67%) and winter wheat straw (organic carbon 43%) in the volumetric proportion of 1:1:2, respectively. The biomix (organic carbon 21.16%, pH 6.05) was macerated using a food processor and composted for 15 days at 20 ± 1°C in a polypropylene bag before being used in the experiments. All the materials used for the biomix preparation were passed through a 3 mm sieve and stored at 4ºC until use. The chemical analysis of used soil (Andisol) is presented in the Table 1.

Table 1. Characteristics of the Andisol used in this study.

Parameters	Value
Organic carbon (%)	8.8 ± 0.96
Nitrogen (mg kg^{-1})	29 ± 1.52
Phosphorous $_{Olsen}$ (mg kg^{-1})	25 ± 0.99
Potassium (mg kg^{-1})	485 ± 2.45
pH (in water)	6.13 ± 0.04

The values are the average of three replicates (n=3).

Available mineral nitrogen ($N\text{-}NH_4^+$ + $N\text{-}NO_3^-$) was determined by extraction with KCl 2 M and quantification by titration with HCl and specific electrode, respectively. Available P was determined by extraction with sodium bicarbonate (0.5 M, pH 8.5) and quantified colorimetrically with the molybdate-ascorbic acid method.Oración muy larga la cortaría en respectively. K was quantified by atomic absorption spectrophotometry (Shimadzu GBC SensAA) after extraction with ammonium acetate 1 M, pH 7.0

Effect of NPK on chlorpyrifos degradation

The biomix samples (25 g) at 60% of water holding capacity (WHC) were placed in glass flasks (500 mL) and amended with NPK fertilizer as nutrient source (0.1, 0.5 and 1% w w^{-1}) Formulated chlorpyrifos (160 mg a.i. kg^{-1} of biomix) was added to each flask. The chlorpyrifos concentration used in the experiments corresponded to approximately 100 times of field dose. After mixing, the samples were incubated in the dark for 40 days at 20 ± 1ºC. The biomix moisture level was maintained by the periodic application of distilled water. Biomix samples (from each treatment) were removed at 0, 5, 10, 15, 20, 30 and 40 days and stored at -20ºC until their analysis. In each sample, residual chloroyrifos, TCP formation, ligninolytic enzymes activity, total microbial activity and microbial community were evaluated. All experiments were made in triplicate.

Chlorpyrifos degradation in biomix was described with the first-order kinetic equation as $C= C_0\ e^{-kt}$, and from the equation, we obtained (Eq. [1]):

$$t_{½} = Ln\ (2)/k \qquad [1]$$

Leaching potential of chlorpyrifos was also determined by the GUS index (Groundwater Ubiquity Score) (Eq. [2]) (Gustavson, 1989). This is an important

index, because it is used for evaluating the potential for groundwater pollution and for leaching of pesticides into surface water via the drainage systems. This index is based on adsorption coefficient (K_{oc}) and half-life ($t_{1/2}$):

$$GUS = log_{10}(t_{1/2}) \times [4 - log_{10}(K_{oc})] \quad [2]$$

Biomix analyses

Ligninolytic enzyme activity. The analysis was performed using the MBTH/DMAB assay according to the methodology described by Castillo *et al.* (1994). Briefly, samples (10 g) were weighed into Erlenmeyer flasks and 50 mL of a 100 mM succinate-lactate buffer (pH 4.5) were added to each sample. The flasks were shaken at 100 rpm for 2 h, and 10 mL of the supernatant were collected and centrifuged at 4,000 rpm for 20 min and filtered through 0.45 µm membrane (National Scientifics filter unit). The reaction mixture contained 300 µL of 6.6 mM DMAB, 100 µL of 1.4 mM MBTH, 30 µL of 20 mM $MnSO_4$, 10 µL of 10 mM H_2O_2, and 1560 µL of the sample in a 100 mM succinic/lactic acid buffer pH 4.5. The reaction was followed at 590 nm (ε= 0.053 $\mu M^{-1} cm^{-1}$) in a Spectronic Genesis™ 2PC. As no correction was made for the possible presence of lignin peroxidase (LiP) and laccase (Lac) activity, this measurement may represent the sum of manganese peroxidase, LiP and laccase (Castillo and Torstensson, 2007).

Total microbial activity. The total microbial activity was measured by monitoring fluorescein diacetate hydrolysis (FDA) according to the methodology described by Schnurer and Rosswall (1982) with slight modifications. Briefly, 1g of biomix from glass flasks was incubated in a 30 mL conical flask with 9.9 mL of sterile 60 mM sodium phosphate buffer,at pH 7.8. The reaction was started by adding 0.1 mL of an FDA solution (2.0 mg mL^{-1}). After 1 h incubation at 25 ± 1 ºC, 10 mL of acetone were added to stop the reaction. A_{490} was determined after removal of the biomix by centrifugation and filtration. The concentration of the released fluorescein was calculated by a calibration curve with standard quantities of FDA and the results were expressed as µg FDA $g^{-1} h^{-1}$.

Microbial community analyses

The microbial community composition in biomix was evaluated at 15 days of incubation after the addition of NPK by DGGE by using specific primer sets for bacteria (Table 2). Briefly, biomix DNA extraction was carried out by using Power Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA). For bacterial community analysis, fragments of 16S rRNA gene were amplified by touchdown polymerase chain reaction (PCR) with the primer set EUBf933-GC/EUBr1387 (454 bp of variable regions V6–V8) (Cea *et al.*, 2010). All PCR amplifications were carried out with reagents supplied with GoTaq® DNA Polymerase (Promega, Co. Madison,WI, USA). DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). Twenty microliters of PCR product were loaded onto a 9% (w/v) polyacrylamide gel with 20% and 70% gradient (urea and formamide). Electrophoresis was run for 16 h at 100V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30min and photographed on an UV transilluminator. Modifications in the microbial community composition of each sample were identified through the ImageJ 1.43u program (Wayne Rasband National Institutes of Health, USA). Clustering

representation was performed on the data set obtained from DGGE analysis with the software ClustalX.

Pesticide extraction and analysis

Residual concentration of chlorpyrifos in biomix was determined by HPLC after extraction with 6 ml of acidified acetone (acetone + water + concentrated phosphoric acid, 98 + 1 + 1 by volume) per gram of substrate (Racke *et al.,* 1996). 5 g (in duplicate) of biomix were mixed with 30 mL of acidified acetone and incubated under shaking (350 rpm) for 2 h at 25ºC. Later, the samples were sonicated for 30 min, centrifuged at 10,000 rpm and filtered with PTFS membrane (0.2 µm pore size; Millipore) and then injected in a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20 µL loop and a Merck Hitachi L-7455 diode array detector. The detector was set at 290 nm and the column was a C18 column (Superspher RP-C18, 5 µm 4.6 x 150 mm). The mobile phase consisted of 60% CH_3CN and 40% water/acetic acid (95.3/4.3 v/v) with a flow rate of 1 mL min^{-1} at 25ºC. The chlorpyrifos and TCP recovery was chlorpyrifos and TCP recovery was > 85%. Retention times were 3.7 min for TCP and 23 min for chlorpyrifos, detection limits were 0.02 and 0.01 mg L^{-1} in the aqueous phase for TCP and chlorpyrifos, respectively.

RESULTS AND DISCUSSION

Effect of NPK on chlorpyrifos degradation

Chlorpyrifos (160 mg a.i kg^{-1}) degradation in biomix with different NPK doses (0, 0.1, 0.5 and 1% w/w) is shown in Figure 1. A high degradation was found in all treatments with NPK. More than 70% of initial applied chlorpyrifos was dissipated after 40 days of incubation in all treatments. Moreover, an initial rapid dissipation of chlorpyrifos was found during the first 10 days of incubation mainly when 0.1 and 0.5% of NPK were added to the biomix (Table 2). However, there were no statistically significant differences in the residual chlorpyrifos concentrations in respect to the control after 40 days.

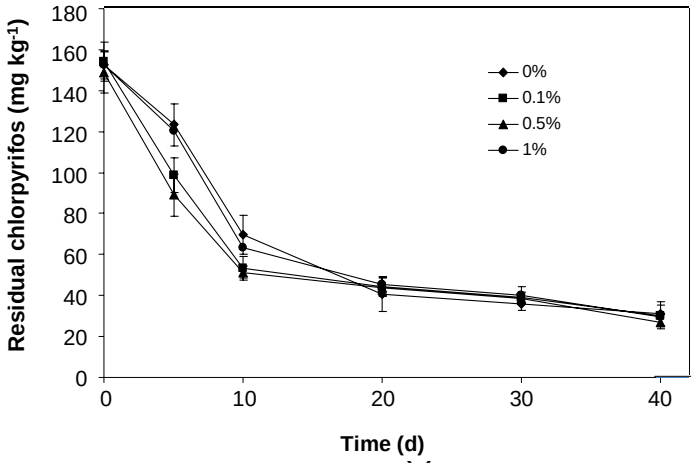

Figure 1. Chlorpyrifos (160 mg kg^{-1}) degradation in biomix of a biobed with different concentrations of NPK fertilizer.

Biostimulation of the biomix with low NPK concentration (0.1% and 0.5%) on the chlorpyrifos degradation demonstrated that this one has a significant effect during the first 10 days of incubation. We think that a short and fast biostimulation of some microbial population present in the biomix occurred due to the presence of nutrients added. After this time nutrient necessity of the biomix microorganisms could be supported by the available native concentration of nutrients in the biomix. Besides, the opportunity of co-metabolism was enhanced due to the component of the biomix (straw and peat). After 20 days of incubation, nutrients addition did not improve the degradation of chlorpyrifos, probably due to limitation of available fertilizer or by the use of carbon and energy more usable compounds in the biomix (Williams *et al*., 1993). Biostimulation can improve the degradation of several pesticides in soil (Silva *et al.,* 2004; de Lipthay *et al.,* 2007). Therefore, assays with other nutrient (macro and micronutrients) resources could be evaluated in the biomix to try to improve the degradation of chlorpyrifos in the biomix during the whole incubation period.

Table 2. Degradation of chlorpyrifos (%) in the biomix with different concentrations of NPK fertilizer.

	NPK (%)			
Time (d)	**0**	**0.1**	**0.5**	**1.0**
5	23	46	37	24
10	58	68	67	60
20	72	73	72	72
40	75	75	75	74

Half-life values for chlorpyrifos in the different studied NPK concentrations are shown in Table 3. As it can be observed, chlorpyrifos degradation in the biomix biostimulated with NPK fertilizer was not modified significantly ($p \leq 0.05$) showing half-life values between 19 and 24 days. These values are very low compared with values reported in the literature. A half-life value for chlorpyrifos between 43 and 59 days has been reported in studies with biomix of biobeds in Europe (Vischetti *et al.,* 2008; Fogg *et al.,* 2003). The differences in the results may be due to the different characteristics of the soil used in our biomix, which has high organic carbon content (8.8%) and acidic pH (5.9). On the other hand, chlorpyrifos has a half-life between 1 and 200 days, depending on the soil characteristics as soil type, pH, and moisture among others (Racke *et al*., 1990, Racke *et al*., 1993, Singh *et al.,* 2003). On the other hand, the Andisol used in this study has a great capacity to adsorb chemical compounds as chlorophenols and chlorpyrifos (Diez *et al.,* 1999; Navia *et al.,* 2003; Cea *et al.,* 2007; Tortella *et al.,* 2010) that decrease the half-life value of this contaminant.

Table 3. Half-life values (t½) and Groundwater Ubiquity Score (GUS) index of chlorpyrifos obtained in biomix with different concentrations of NPK.

NPK (% w w^{-1})	**K (d^{-1})**	**$t_{1/2}$**	**r^2**	**GUS**
0	0.04	18.9 **a**	0.89	0.03 **a**
0.1	0.03	24.4 **a**	0.91	0.04 **a**
0.5	0.03	23.9 **a**	0.95	0.04 **a**
1.0	0.03	19.9 **a**	0.92	0.03 **a**

*Different letters refer to significant differences between mean values (n=3) with Duncan Test ($p \leq 0.05$).

The GUS index obtained (Table 3) for chlorpyrifos was similar between treatments and no significant differences ($p \leq 0.05$) were found. The values were lower than 2.8, which is a limit value between a leachable and non- leachable compound (Gustavson, 1989). This indicates that this compound has a high probability of being retained in the biomix, thereby reducing its lixiviation potential.

Figure 2 shows TCP accumulation in the biomix amended with NPK fertilizer. A clear trend in the TCP accumulation was observed when fertilizer concentration increased in the biomix. Indeed, the highest TCP level was found in the biomix containing 1.0% of NPK and the lowest TCP level was found in the biomix without fertilizer. TCP is a main metabolite produced during chlorpyrifos degradation and it is an undesirable compound due to its chemical properties. TCP has higher water solubility, lower adsorption coefficient (K_{oc}), higher GUS index and higher half-life value than chlorpyrifos. We assume that the fertilizer added to the biomix can have a stimulatory effect on microbial populations different from TCP-degrading microorganism. Moreover, NPK addition could result in an imbalance of elements, and competition for nutrients within the microbial communities may limit the overall microbial growth and slow contaminant degradation (Biddlestone *et al.,* 1987). The results indicate that the addition of fertilizer as an easily usable nutrient resource in the biomix may affect the adaptation of microorganism to use and to degrade TCP. Similar results were reported by Coppola *et al*. (2007), where a significant TCP accumulation was observed in the mixtures of biobeds with low content of straw and amended with urban and garden compost. In addition, Castillo *et al.* (2008) reported that high nitrogen or no lignin content in the biomix may not support an adequate microbial activity for the degradation of pesticides or their metabolites.

Biological activities in the biomix

The most widely adopted method to estimate microbial activity in soil amended with organic residues and to estimate the effect of stress by the presence of xenobiotics is the hydrolysis of fluorescein diacetate (FDA) (Gaspar *et al.,* 2001; Sanchéz-Monedero *et al.,* 2008)

During the incubation time, it was observed that FDA hydrolysis increased by the addition of different doses of NPK fertilizer up to 0.5% (Figure 3). The highest FDA values were found in the biomix containing 0.1% (11 $\mu g\ g^{-1}\ h^{-1}$) at 20 days of incubation and 0.5% (9 $\mu g\ g^{-1}\ h^{-1}$) at 30 days of incubation. The lowest FDA values were found in the biomix containing 1% of NPK being lower than values obtained in the control biomix (0% NPK). In a previous work, we found that chlorpyrifos in the same concentration used in this study caused an initial decreasing in FDA activity (Tortella *et al.,* 2010). According to our results, the global microbial activity, quantified through FDA activity, was stimulated by the presence of NPK, except when 1% of NPK was added.

The NPK addition to the biomix in all used concentrations increased the ligninolytic activity in the biomix compared with the biomix without fertilizer (Figure 4). The highest values of ligninolytic activity were obtained when 0.1% of NPK fertilizer was added. Ligninolytic enzyme activity (LiP, MnP and laccase) is used to estimate the activity of lignin-degrading fungi (peroxidases and phenoloxidases) from straw solid substrate (Castillo *et al.,* 1997). In some works, ligninolytic activity has been correlated to the

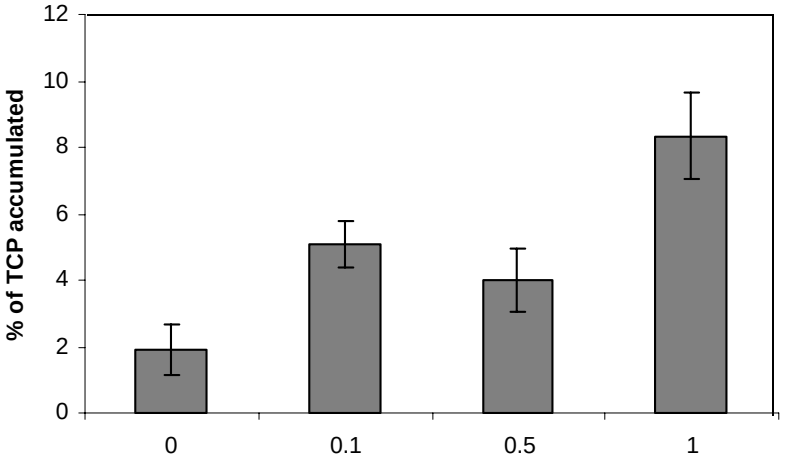

Figure 2. Percentage of TCP (3,5,6-trichloro-2-pyridinol) accumulation with respect to initial concentration of chlorpyrifos (160 mg kg^{-1}) in biomix of a biobed biostimulated with different concentrations of NPK fertilizer after 40 days of incubation.

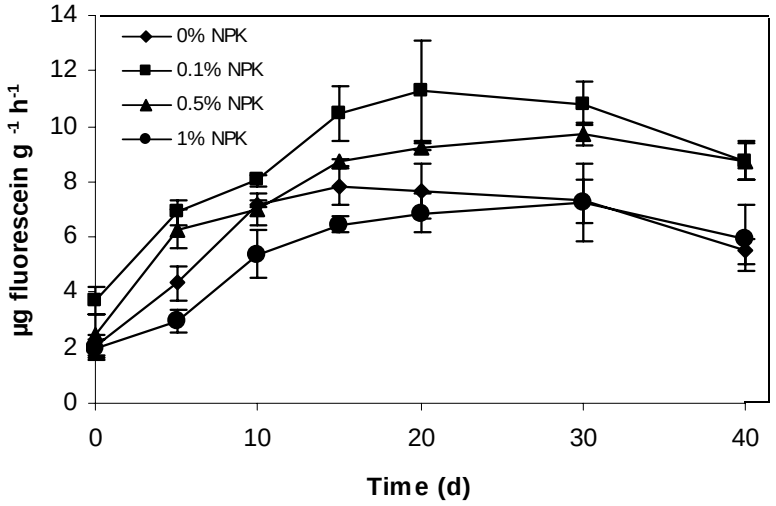

Figure 3. FDA activity in biomix of biobed biostimulated with different concentrations of NPK fertilizer.

degradation of several pesticides, such as isoproturon (Castillo and Torstensson, 2007; Von Wirén-Lehr *et al.,* 2001), chlorpyrifos and TCP (Coppola *et al.,* 2007; Pizzul *et al.,* 2009).

The increment in chlorpyrifos degradation and in biological activities with the addition of NPK fertilizer until 20 days of incubation is associated with half-life values obtained for this pesticide (aprox.

21 days). The major activity in the biomix due to NPK nutrients probably favored degradation of other lignocellulosic materials in the biomix and not only chlorpyrifos and TCP. No information is provided by the literature in relation to the effects of nutrients in the pesticide degradation in biobed system. It has been reported that ligninolytic activity, although being an important biological process in biomix (Castillo *et al*., 2008) may not be the only biological activity that governs the processes of pesticide dissipation. Monitoring the FDA and ligninolytic activity in the biomix of biobeds is only one/an approach to the effects of pesticides on biological activity, since biodegradation of pesticides in the biomix or soil is a series of physical, chemical and biological interactions. Moreover, it has been demonstrated that several enzymes can act in pesticide degradation in soil, such as phosphatases (Hasan, 1999), hydrolases and carboxylesterases (Lan *et al.,* 2005).

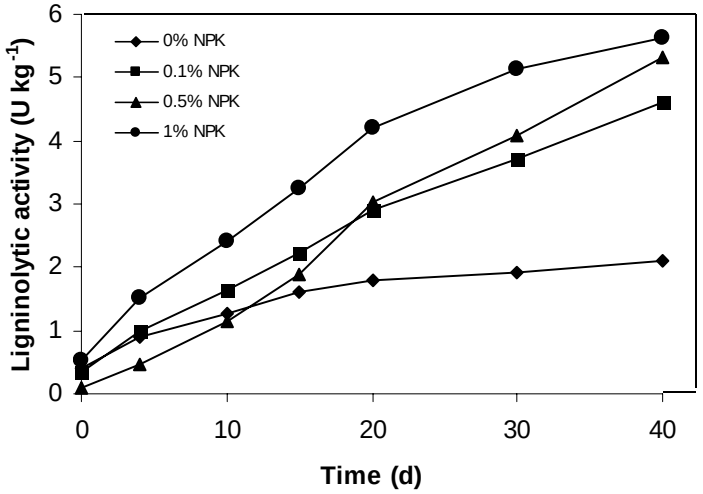

Figure 4. Accumulated ligninolytic activity in biomix of biobed biostimulated with different concentration of NPK fertilizer.

Microbial community analyses

The effect of NPK application after 15 days of incubation on microbial community modifications were assessed with DGGE and classified by hierarchichal cluster analysis (HCA) (Figure 5). DGGE profiles demonstrated that increments in NPK fertilizing stimulated some bacterial strains (Nº 3, 10, 11, 12, 15, 18, 19, 20, 21, 22 and 24). The sample treated with 1.0% NPK included a higher number of stimulated bacterial strains that were not present in control and sample treated with 0.1% fertilizer. On the other hand,

combined treatments with lower amounts of NPK (0% and 0.1%) and chlorpyrifos showed no significant modifications in their microbial community profiles, as compared with the same NPK treatments without chlorpyrifos. However, samples with highest NPK doses plus chlorpyrifos displayed a reduction of bacterial strains Nº 11 and Nº 12. HCA shows that biostimulation with different NPK fertilizing conditions modified the structure of bacterial communities. Furthermore, HCA allowed the classification of different treatments in three clusters into the dendrogram. The first group included the controls (0%) and treatments with 0.1% of NPK fertilization that were also enclosed in two subgroups of samples treated with and without chlorpyrifos. A second group consisted of samples treated only with 0.5% and 1.0% NPK supply. The third group included samples that were treated with combined conditions of high NPK doses (0.5% and 1.0%) and chlorpyrifos that displayed a? significant structural difference in their populations as compared with the samples described before.

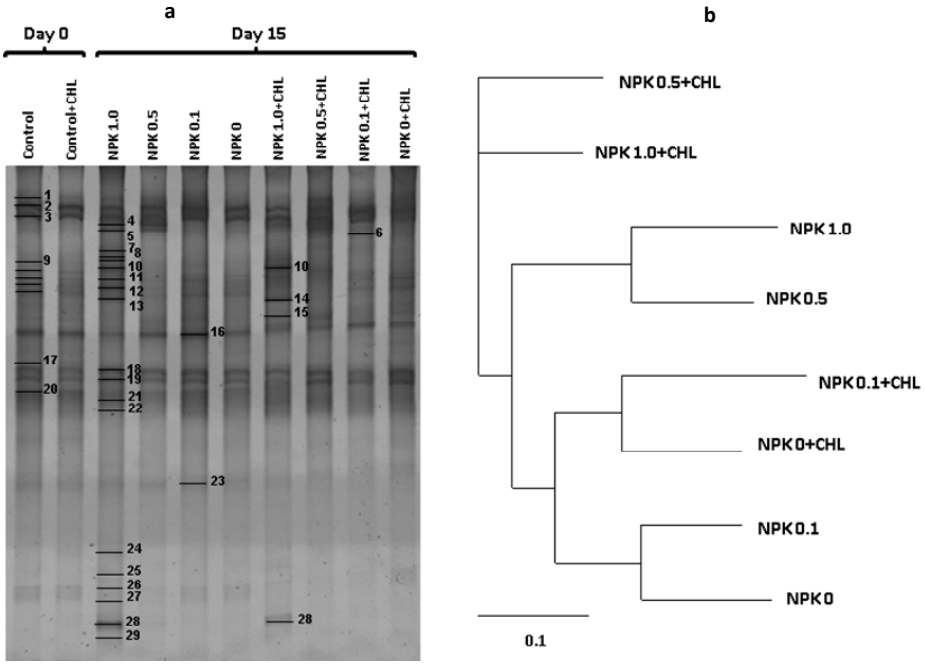

Figure 5. The DGGE banding pattern (a) and clustering representation (b) of the bacterial 16S rRNA genes amplified with the primers sets EUBf933- GC EUBr1387.

CONCLUSIONS

The results of this study indicate that the addition of nutrients (NPK) up to 0.5%, increased chlorpyrifos degradation during the first days of incubation and concomitant with an increase of the biological activity in the biomix. On the other hand, high NPK doses (0.5 and 1.0%) and chlorpyrifos caused significant

modifications in the bacterial communities that could be associated with the observed TCP (3, 5, 6-trichloro-2-pyrinidol) increment. Moreover, combined treatments with lower amounts of NPK (0 and 0.1%) and chlorpyrifos did not show any significant modifications in the microbial community in the biomix. Finally, it is necessary to study more details about the effect of nutrients addition in the increase of TCP accumulation in the biomix in long term assays.

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