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EFFECT OF HEXACHLOROCYCLOHEXANE ISOMERS ON SOME SOIL MICROBIOLOGICAL PROPERTIES

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RESUMEN: Se estudió el efecto de la contaminación con altas cantidades de un residuo compuesto de isómeros de 1,2,3,4,5,6-hexaclorociclohexano (HCHs) (10, 50 y 100 g HCHs kg^{-1} suelo) sobre la densidad en el suelo de microorganismos heterótrofos, algas y cianobacterias fijadoras de N_2, sobre algunos microorganismos implicados en los ciclos de C y N (amonificantes, amilolíticos, y celulolíticos) así como sobre la capacidad de las comunidades microbianas edáficas para degradar varios sustratos carbonados (perfiles fisiológicos de las comunidades microbianas, CCLP). Estas propiedades microbianas se analizaron a las 2 semanas y los 2 y 4 meses de la aplicación del contaminante. También se estudió el efecto de la contaminación con HCHs aplicado a distintas concentraciones y en forma de partículas de distinto tamaño, sobre la respiración del suelo.

A corto plazo tras la adición del contaminante al suelo, la densidad de los microorganismos heterótrofos aumentó, la abundancia de algas descendió y la población de cianobacterias no vario significativamente. A los 4 meses de la contaminación el efecto de las concentraciones de contaminante más altas (50 y 100 g HCHs kg^{-1} suelo) sobre los grupos microbianos mencionados fue negativo. El residuo de HCHs tuvo un efecto sobre la población amonificante similar a la observada en los microorganismos heterótrofos. Las densidades de amilolíticos y celulolíticos no se vieron afectadas o aumentaron, a corto plazo después de la adición de contaminante, aunque, a largo plazo (4 meses tras la contaminación) experimentaron una reducción con las concentraciones más altas de HCHs. A los dos meses de la contaminación, la capacidad de las comunidades microbianas del suelo para degradar sustratos carbonados (CCLP) se vio negativamente afectada por el residuo de HCHs. Cabe resaltar el descenso en la degradación de aminoácidos, que fue especialmente importante en los suelos con 50 y 100 g HCHs kg^{-1} suelo.

El residuo de HCHs también afectó a la respiración del suelo, aunque el efecto dependió de la cantidad y tamaño de partícula del contaminante y de la presencia o ausencia de microorganismos procedentes de un emplazamiento contaminado con HCHs por largo tiempo (adaptados a la contaminación con HCHs). Cuando estuvieron presentes microorganismos adaptados a HCHs y el contaminante se aplicó en forma de partículas 4-10 mm, la respiración del suelo se vio estimulada con concentraciones entre 20 y 100 g HCHs kg^{-1} suelo e inhibida con concentraciones mayores de 200 g HCHs kg^{-1} suelo. Al aplicar el residuo de HCHs en forma de partículas < 1mm, el contaminante no estimuló la respiración y la redujo a concentraciones de 100 g HCHs kg^{-1} suelo o superiores. La adición de HCHs en forma de partículas de 4-10 mm en concentraciones de 100 g HCHs kg^{-1} suelo o superiores a un suelo con microorganismos no adaptados al contaminante redujo la respiración del suelo y con concentraciones menores no se modificó la cantidad de CO2 desprendida.

Palabras clave: Isómeros de HCH, lindano, contaminación del suelo, respiración del suelo, microorganismos del suelo, bacterias del suelo

SUMMARY: The effect of heavy contamination with a residue composed of 1,2,3,4,5,6-hexachlorocyclohexane isomers (HCHs) (10, 50 and 100 g HCHs kg^{-1} soil) on the densities of cultivable soil heterotrophs, algae and N_2-fixing cyanobacteria, on soil microorganisms involved in the N and C cycles (ammonifiers, amylolytics and cellulolytics) as well as on the ability of soil microbial communities to degrade several C substrates (community level physiological profiles, CCLP) was studied. These microbial properties were analysed 2 weeks, and 2 and 4 months, after the soil was artificially contaminated. The effect of HCHs residue applied at different concentrations, and in the form of particles of different sizes on soil respiration was studied. The influence of the presence or absence of microorganisms from a long term polluted site on the effect of HCHs on soil respiration was also analysed.

In the short term after the addition of the contaminant to the soil, the density of heterotrophs increased, the abundance of algae decreased, while the population of cyanobacteria did not change significantly. Four months after contamination the effect of the highest concentrations of HCHs (50 and 100 g HCHs kg^{-1} soil) on these microbial groups was negative. The HCH residue had a similar effect on the ammonifying population to that on heterotrophs. The densities of amylolytics and cellulolytics were either not modified, or were stimulated by the contaminant in the short term but, in the longer term (4 months after the contamination), were inhibited by HCHs applied at the highest concentrations. Two months after contamination, the ability of soil microbial communities to degrade C substrates (CCLP) was negatively affected by HCHs. It was noteworthy the decrease in the ability for degrading aminoacids, which was particularly important in the soils with 50 and 100 g HCHs kg^{-1} soil.

HCHs also affected soil respiration but the effect depended on the HCH concentration, particle size of the contaminant, and on the presence or absence of microorganisms from a long term HCH-contaminated site (adapted to HCH contamination). When microorganisms adapted to HCH contamination were present and the contaminant was applied in form of particles of 4-10 mm, the soil respiration was stimulated by HCH at concentrations between 20 and 100 g HCH kg^{-1} and inhibited with concentrations higher than 200 g HCH kg^{-1} soil. If the HCHs were applied as particles < 1 mm, soil respiration was not stimulated by the contaminant and decreased with concentrations of 100 g HCH kg^{-1} or higher. The addition of HCHs in form of particles of 4-10 mm at concentration of 100 g HCH kg^{-1} or higher to a soil with microorganisms non-adapted to the contaminant reduced soil respiration, while lower concentrations of contaminant did not affect the amount of CO_2 evolved.

KEYWORDS: HCH isomers, lindane, soil contamination, soil respiration, soil microorganisms, soil bacteria

INTRODUCTION

The organochlorine 1,2,3,4,5,6 hexachlorocyclohexane (HCH) is a broad-spectrum insecticide that was used on a large-scale worldwide since the 1940s. There are eight isomers of HCH, which are differentiated by the axial or equatorial positions of the chlorine atoms around the cyclohexane ring and among them only the gamma isomer (lindane) has insecticidal properties. In spite of this, a formulation known as technical-grade HCH, composed of a mixture of different isomers, mainly α- (60–70%), β- (5–12%), γ- (10–15%), and δ-HCH (6–10%)) was used for many years as insecticide. Moreover, after the use of technical-grade HCH was banned, a residue of several HCH isomers was generated in great amounts by the chemical industry producing lindane. Nowadays the use of lindane is also restricted or completely banned in most countries, but, due to their persistence and high toxicity, the contamination with these compounds continues to pose serious environmental and health concerns, particularly on highly contaminated former production or dumping sites (Lal *et al.* 2006; Schwitzguébel *et al.* 2006).

Soil microorganisms play an essential role in degrading HCH isomers in the environment, both in oxic and anoxic conditions. Regarding aerobic biodegradation, several bacterial strains, most of them members of the genus *Sphingomonas*, able to mineralize different HCH isomers have been isolated and the degradation pathway has been elucidated and studied in detail (recent reviews Lal *et al*. 2006, 2010, Nagata *et al*. 2007, Phillips *et al*. 2005, Singh *et al*. 2000; Schwitzguébel *et al.* 2006). Biodegradation of lindane and other HCHs in soil has also been analysed by several authors (Calvelo-Pereira *et al.* 2006; Kidd *et al.* 2008; Kumar *et al.* 2006; Phillips *et al*. 2004, 2005, 2006). Nevertheless, the effect of these compounds on soil microbial community composition and function has received less attention (Neufeld *et al.* 2005) and some of the studies have focus on soils containing low amounts of HCHs (Das and Mukherjee 2000; Rodriguez and Toranzos 2003; Singh and Singh 2005a,b,c;) or were addressed to analyse specific microbial groups or processes (Hussain et al 2009; Johnsen et al 2001; Mertens *et al.* 2005; Singh and Prasad 2001).

The present study aims to obtain information about the effect of heavy contamination with HCHs isomers on several soil microbial groups and on the soil respiratory activity. The microbial groups analysed include cultivable heterotrophs, algae and N2-fixing cyanobacteria as well as microorganisms involved in the C and N cycles (amylolytics, cellulolytics and ammonifiers). The effect of HCHs on soil respiration is analysed in soil samples with microorganisms previously exposed to HCH contamination (microorganisms from a site contaminated with HCHs in the 1950s) and in soil samples with microorganisms non-adapted to HCHs.

MATERIAL AND METHODS

HCH-residue, soils and contamination protocol

The 1,2,3,4,5,6-hexachlorocyclohexane (HCH) residue, used for artificial contamination of soil, was obtained from a former lindane factory. The mean composition of the residue was: 77% α-HCH, 16% β-HCH, 5% δ-HCH, and 2% γ-HCH (Calvelo Pereira *et al.* 2006). The residue was ground and sieved to obtain the different particle fractions used in this study.

The soil used for artificial contamination was a garden soil located in Santiago de Compostela, NW Spain, developed over granite with a C content of 3.7 % and pH of 5.5. The contaminated soil used as source of microorganisms adapted to long term contamination and able to degrade HCHs was collected in a HCH-contaminated site in Porriño (Pontevedra), NW Spain, where residues from lindane fabrication were disposed of during the 1950s and 1960s. Soils were sieved and the < 2 mm fraction was stored at 4 ºC until use.

For the analysis of the long term effect of HCHs on soil microbial properties, the non contaminated garden soil was artificially contaminated with appropriate amounts of ground HCH residue (particles < 4 mm) to reach levels of contamination of 0, 10, 50 and 100 g HCHs kg^{-1} soil. In addition 5% of long term contaminated soil from Porriño were mixed with to the artificially contaminated soil to ensure the presence of HCH degrading microorganisms. Previous studies showed that degradation of HCH isomers was not detectable in the absence of microorganisms from the contaminated site (unpublished data). The contaminated soil mixtures were transferred to pots (55 kg), fertilized (300 kg N ha^{-1} + 150 kg P2O5 ha^{-1} + 250 kg K ha^{-}

[1]) and the water content adjusted to 75% of the field capacity. The pots were placed in a greenhouse and periodically watered during the experiment. Two weeks (end of July) and 2 and 4 months (September and November respectively) after the artificial contamination, three aliquots of 5 g were taken from each pot for microbiological analyses.

The effect of different levels of HCH contamination on soil respiration was analysed in 2 different experiments: a) HCH particles 4-10 mm b) HCH particles < 1mm. For these experiments, non contaminated garden soil was thoroughly mixed with appropriate amounts of ground and sieved HCH residue: a) 0, 20, 40, 60, 80, 100, 200, 300, 400 and 500 g HCH residue kg^{-1} soil; b) 0, 20, 50, 100, 200, 300, 400, 500, 600, 700, g HCH residue kg^{-1} soil. These artificially contaminated soils were inoculated with microorganisms from the long term HCH contaminated site. The inocula were prepared by shaking (for 10 min) 10 g soil from the long term contaminated site with 90 ml of deionized water and filtrating the suspension through 100 µm pore size (1 ml of the filtrate was used to inoculate 50 g of artificially contaminated soil). Four 50 g replicates of each treatment were prepared. In the case of experiments a) replicates of artificially contaminated soil without inoculum were also prepared.

Microbial and chemical analysis and measurement of soil respiration

Cultivable heterotrophic bacteria, algae, N_2-fixing cyanobacteria, ammonifying, amylolytic and cellulolytic populations were determined by the most probable number (MPN) technique, as follows. Five grams of soil were shacked with 45 ml sodium hexametaphosphate solution (1%) for 30 min in an end-over-end shaker. These soil suspensions were diluted in 10-fold series and 50 µl aliquots were used to inoculate microtiter plates containing selective liquid media (150 µl/well). Four wells were inoculated per suspension-dilution. Heterotrophic population was estimated in yeast-extract medium (1.0 g yeast extract, 1.0 g glucose, 0.5 g KNO_3, 0.2 g $MgSO_4.7H_2O$, 0.5 g K_2HPO_4, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.01 g $FeCl_3$, in 1 l deionised water) plus oligoelements (1.5 mg $FeSO_4 \cdot 7H_2O$, 0.3 mg H_3BO_3, 0.19 mg $CoCl_2$, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4 \cdot 5H_2O$, 0.036 mg $NaMoO_4$, 0.024 mg $NiCl_2 \cdot 6H_2O$). Amylolytic microorganisms were cultured in Winogradsky's saline medium (containing 0.25 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_4NO_3, 0.1 g NaCl, 2 mg $Fe_2(SO_4)_3 \cdot 7H_2O$, 2 mg $MnSO_4$ and 1.5 g soluble starch in 1 l deionised water) plus oligoelements (as above). Ammonifiers were evaluated in Winogradsky`s saline solution with L-asparagine (0.2 g l−1) as the only N and C source. The redox dye, tetrazolium violet (2,5-Diphenyl-3-(α- naphthyl) tetrazolium chloride (TV), 15 mM) was used to indicate growth, and was added to media using the ratio 1:100 (v/v, TV:media). Tetrazolium violet serves as an indicator of dehydrogenase activity, forming a deep purple precipitate upon reduction by electrons flowing through the electron transport system and by superoxide radicals (Kennedy 1994). Cyanobacteria, algae and cellulolytics were cultivated in tubes containing 9 ml of the appropriate medium which were inoculated with 1 ml of the ten fold dilution prepared as described above (4 tubes per level of dilution). Medium for culturing N2-fixing cyanobacteria contained in 1 l of deionised water 0.08 g K_2HPO_4, 0.02 g KH_2PO_4, 0.08 g $MgSO_4$ $7H_2O$, 0.02 g $CaCl_2$, 0.02 g Na_2CO_3 plus oligoelements (as above). Medium for the determination of algae was composed of 0.33 g $Ca(NO_3)_2$, 0.08 g KCl, 0.08g $MgSO_4$ $7H_2O$, 0.08 g KH_2PO_4, 0.1 g $FeCl_3$ $6H_2O$, plus oligoelements (as above) in 1 l of

deionised water. Cellulolytics were grown in Winogradsky's saline medium with a strip of cellulose paper as C source. Positive wells and tubes were recorders after 3 weeks of incubation at 28 ºC in the darkness, except for algae and cyanobacteria which were incubated with a photoperiod of 16 hours light and 8 hours darkness and counted after 6 weeks of incubation at 25 ºC. MPN was determined from the appropriate table, corrected for the initial dilution and inoculated volume, and expressed as log10MPN g^{-1} dry soil.

The community level physiological profiles were determined (CLPP) analysis). Carbon substrate microtiter (MT) plates were prepared with 24 carbon sources (Table 1) from those included in EcoplateTM from Biolog Inc. or recommended by Kennedy (1994). Individual carbon source stock solutions were prepared at a concentration of 10%, passed through a sterile filter and added to sterile saline medium (1.75 g K_2H_2PO4, 0.5 g $KHPO_4$, 0,582 g NH_4Cl and 0.25 g $MgSO_4 \cdot 7H_2O$ in 1 l deionised water, adjusted to pH 7.0) (1:100 v/v, C source:saline medium). The indicator TV was added as above. Each well of microtiter plates was filled by dispensing 100 µl of C substrate:saline medium-TV, and each set of carbon sources was replicated three times in a single 96 well MT plate. Wells containing saline medium-TV without C substrates were also prepared as controls. A tenfold dilution of the first soil suspension (10^{-2}) was used to inoculate the MT plates. The soil suspension was allowed to sediment for 1 h and 100 µl of the supernatant were inoculated into each well of the MT plate. Substrate utilisation was indicated by colour development of the tetrazolium violet redox dye, and recorded after 7 days incubation at 28 °C.

The soil pH was measured in soil/water suspensions (1:2.5 w/v) with a glass electrode. After pH measurement the soil suspension was filtered (100 µm pore size) and chlorine was measured in the filtrate. Chorine determination was carried out by the mercury(II) thiocyanate method (Adriano and Doner 1982) based on measuring the colour development originated after the addition of $Hg(SCN)_2$) and $Fe(NO_3)_3$ to the sample and the consequent formation $HgCl_2$ and coloured compound Fe(SCN). A standard curve was prepared with NaCl.

The soil respiration (50 g soil) was measured in a Respicond II apparatus (Nordgren 1988). This equipment measures continuously the conductivity of a 0.2 N KOH which acts as trap of CO2 evolved from the soil.

Data presented are means of triplicate or quadruplicate determinations of each analysis

Table 1. List of carbon sources used in metabolic profiling of microbial communities

Chemical group	Carbon substrate
Carbohydrates	D-(+) cellobiose
	α-lactose
	β-methyl D-glucoside
	D-(+) xylose
	i-erythritol
	Maltose
	N-acetyl-D-glucosamine
Carboxylic acids	D-galactonic acid δ-lactone
	Galacturonic acid
	o-hydroxybenzoic acid
	p-hydroxybenzoic acid
	Malonic acid
	α-keto butyric acid
	Malic acid
Amino acids	L-arginine
	L-asparagine
	L-phenylalanine
	L-serine
	L-glutamic acid
Polymers	Tween 40
	Tween 60
	α-cyclodextrin
	Glycogen
Miscellaneous	α- D-glucose-1-phosphate

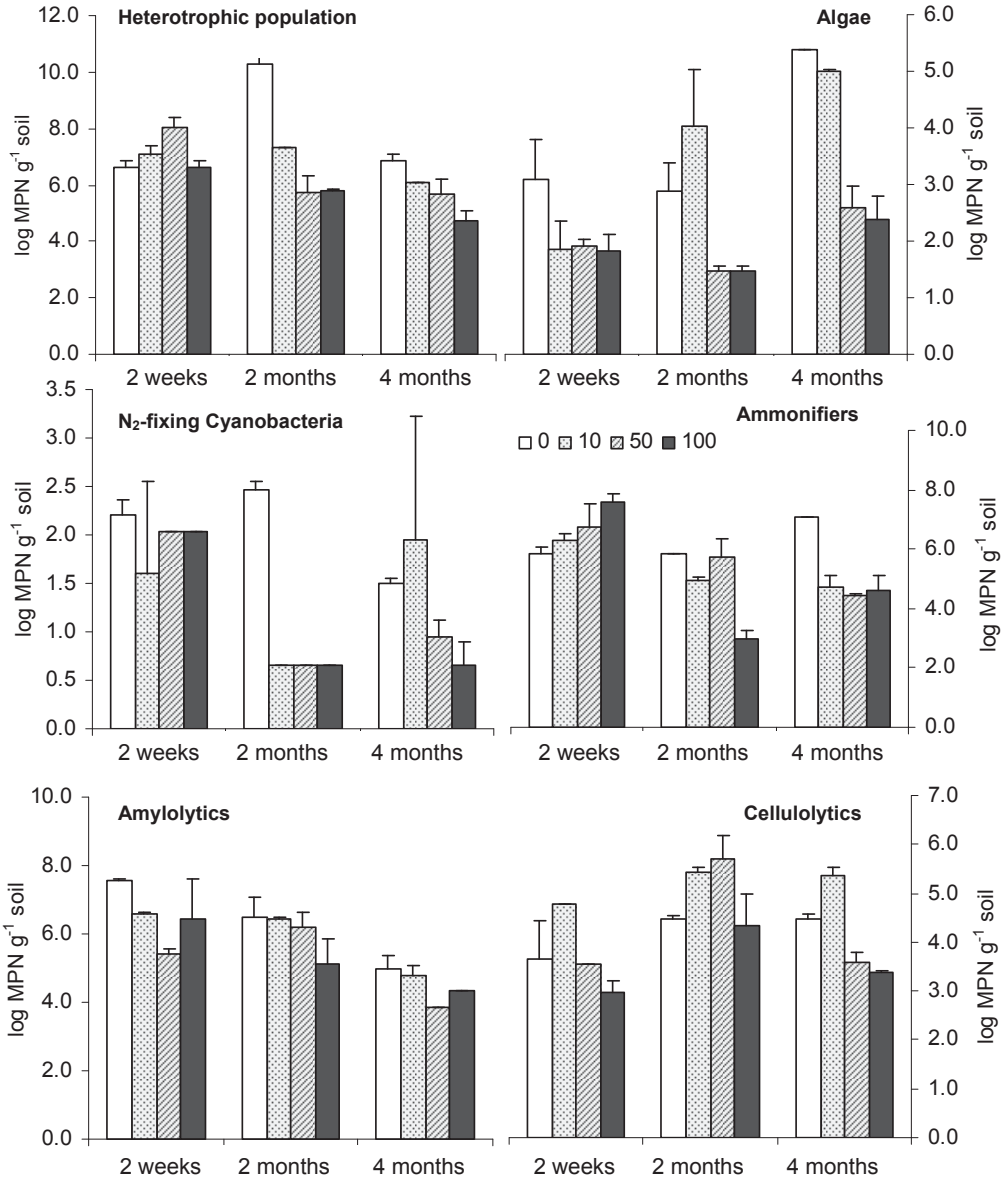

Fig. 1. Microbial densities in non contaminated and artificially contaminated soil inoculated with microorganism from a long term HCH-contaminated site.

MPN: most probable number; 0, 10, 50 and 100: g HCH residue kg^{-1} soil; 2 weeks, 2 months, 4 months: time after the application of the contaminant

RESULTS

In the short term, 2 weeks after contamination, the presence of HCHs at concentrations of 10 or 50 g HCHs kg^{-1}, slightly increased the density of cultivable heterotrophs, negatively affected the algae and did not significantly affect N2-fixing cyanobacteria (Fig. 1). The highest concentration of HCHs (100 g kg-1) also reduced the abundance of algae. Two months after the application, the population of both the cultivable heterotrophs and autotrophs was lower in the contaminated than in the non-contaminated soils, except in the case of the abundance of algae in soil with the lowest concentration of HCHs which was similar to that in the pristine soil. The negative effect of HCHs was particularly intense at the higher concentrations (50 and 100 g HCHs kg^{-1}) and on the N_2-fixing cyanobacteria. In the soil with 50 or 100 g HCHs kg^{-1}, the reduction of microbial densities was still detectable 4 months after the contamination, especially in the case of autotrophs.

Two weeks after the contamination, the population of ammonifiers was stimulated by HCHs; thereafter, the effect of the contaminant was reversed and 4 months later, the contaminated soil showed a lower density of ammonifiers than the non contaminated soil (Fig. 1). The abundance of amylolytics decreased two weeks after the contamination. In the longer term, the negative effect of HCH on this microbial group tended to disappear, although, 4 months after contamination amylolytics were less abundant in the contaminated than in the pristine soil. Regarding the cellulolytics, the concentration of 10 g HCHs kg^{-1} stimulated the population of these microorganisms and the positive effect was prolonged in time. The dose of 50 g HCHs kg^{-1} soil had also a positive effect on cellulase producers which turned negative four months after the contamination. The highest concentration of HCHs tested, initially did not significantly modify the density of cellulolytics but, 4 months after the contamination, had a negative effect.

Two weeks after the contamination the community level physiological profiles (CCLP) analysed were not affected by HCHs and the microbial communities of the non contaminated and contaminated soils samples studied were able to degrade all the substrates tested. Nevertheless, two months after the contamination, the microbial communities in the contaminated soil were able to degrade less C substrates than those of the pristine soil. Thus, soils with HCHs failed to degrade α- D-glucose-1-phosphate and i-erythritol; moreover, some amino acids were not utilized by the microorganisms in the soils with the higher contaminant concentration (L-asparagine L-phenylalanine L-serine L-arginine in the case of the soil with 100 g HCHs kg^{-1} and the first two amino acids in the case of in the case of the soil with 50 g HCHs kg^{-1}). Surprisingly, α-ketobutyric acid was not degraded by the communities in the soil with 10 or 50 g HCHs kg^{-1}, but was degraded by those of the soil with more contaminant, while malic acid was not decomposed by the microorganisms in the pristine soil. Four months after the contamination, the microbial communities in the soil with 10 g HCHs kg^{-1}, recovered the ability to degrade α- D-glucose-1-phosphate and i-erythritol but failed to degrade the aminoacid L-phenylalanine, while, in the case of the highest concentrations (50 or 100 g HCHs kg^{-1}), the negative effect of the contaminant on CCLP already described was maintained until the end of the experiment.

After the contamination, soil pH decreased and chlorine amount increased (Fig. 2). The main changes in pH and chlorine were observed 2 months after the addition of HCHs and were similar in the soils with 50 or 100 g HCHs kg^{-1} and more pronounced than in the

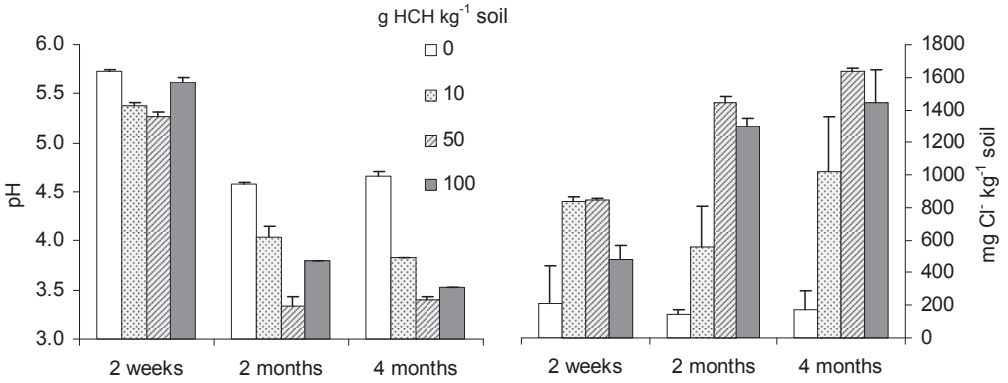

Fig. 2. Soil pH and chlorine concentration in non contaminated and artificially contaminated soil inoculated with microorganism from a long term HCH-contaminated site.

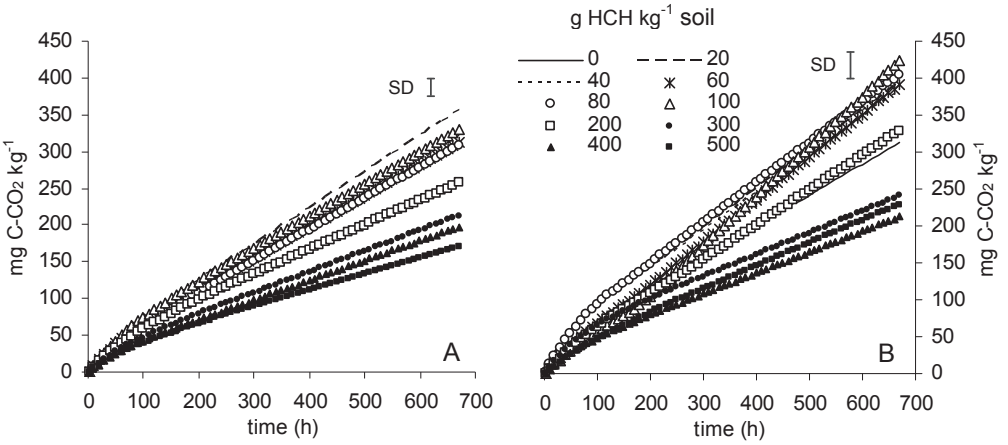

Fig. 3. Respiration of soil non-contaminated and artificially contaminated with HCHs (particles 4-10 mm), non-inoculated (A) or inoculated (B) with microorganisms from a long term HCH-contaminated site. SD: average standard deviation

soil with 10 g HCHs kg^{-1}. After 4 months, pH tended to decrease and chlorine increased, though the variation was less pronounced than that observed in the first two months.

The inoculation of non contaminated soil with microorganisms from the long term polluted site did not affect soil respiration (Fig. 3). When microorganisms from the long term polluted site were not present, the contamination with HCH (particles 4-10 mm) at concentrations 20-100 g HCHs kg^{-1}, did not affect or even slightly stimulated (dose of 20 g HCHs kg^{-1}) soil respiration. At higher concentrations of HCHs soil respiration decreased progressively with the amount of contaminant (Fig. 3). The respiration of the soil inoculated with microorganisms from the long term polluted site was increased by HCHs (particles 4-10 mm) at concentrations of up to 100 g HCHs kg^{-1}, was not modified when the contaminant was applied at a concentration of 200 g HCHs kg^{-1}, and

decreased at higher concentrations of HCHs (300-500 g HCHs kg^{-1}) (Fig. 3). In general the stimulation observed at concentrations higher than 60 g HCHs kg^{-1} was not observed immediately after the application of the contaminant, but after about 200 hours of incubation. When the contaminant was applied in form of fine particles (< 1 mm) to the soil inoculated with microorganisms from the long term polluted site, the respiration did not change at concentrations of 20 or 50 g HCHs kg^{-1} but at higher concentrations soil respiration decreased progressively with the amount of contaminant at (Fig.4). At concentrations higher than 500 g HCHs kg^{-1}, soil respiration was below the detection limit of the equipment used (data not shown)

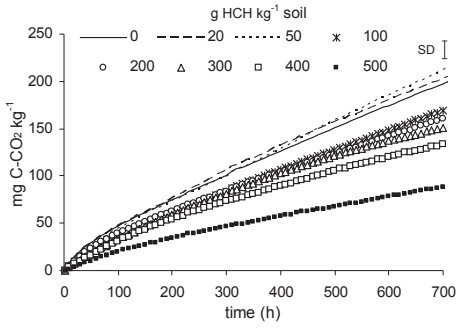

Fig. 4. Respiration of soil non-contaminated and artificially contaminated with HCHs (particles, < 1mm) inoculated with microorganisms from a long term HCH-contaminated site.

SD: average standard deviation

DISCUSSION

In general, soil microorganisms showed a high resistance to the contamination with HCHs, although four months after the contamination with 50 g or 100 g HCH kg^{-1} soil the density of the microbial groups analysed was lower than in non-contaminated soils. Autotrophs (algae and N2-fixing cyanobacteria) were more affected than heterotrophs, but even for these microorganisms the toxic effect of HCHs was mainly observed with the highest concentration of contaminant (50 and 100 g HCH kg^{-1} soil), while at the lower concentration (10 g HCH kg^{-1} soil) the populations of autotrophs were able to recover.

HCHs had a transitory positive effect on the density of heterotrophs, which agrees with the stimulation of respiration observed in some of the experiments carried out in soils with microorganisms adapted to HCH. Other authors, analysing soils with a much lower amount of contaminant than in the present study, have found increases, little variation or even decreases in the bacterial density (Das and Mukherjee 2000; Kidd *et al.* 2008; Rodriguez and Toranzos 2003; Singh and Singh 2005c). The initial increase in the density of heterotrophs and ammonifiers after contamination may be related to the proliferation of microorganisms able to degrade these compounds, which are present in the soil used for inoculation (unpublished results) and have been frequently found in other long term contaminated soils (Phillips *et al.* 2005, 2006; Kumar *et al.* 2006). The decrease in pH and the increase in chlorine concentration indicate that degradation of HCH took place in the soils analysed. On the other hand the proliferation of heterotrophs could also be related to a deleterious effect of the contaminant on some soil microorganisms, which would leave soil niches free for colonization and whose biomass may have served as labile C substrate for those adapted and able to survive. Ammonification is a common metabolic ability among soil heterotrophs, and densities of soil ammonifiers are usually of the same order magnitude than heterotrophs (Acea *et al.* 2001, 2003). The negative effect of HCHs on algae is in agreement with studies demonstrating the high toxicity of lindane to

these microorganisms (KrishnaswamyChang 1997; Mostafa and Helling 2002). Nevertheless algae densities recovered in the long term in the less polluted soil, suggesting adaptation of some of these organisms to the presence of the contaminant. Regarding cyanobacteria, some studies indicate that, lindane concentrations much lower than those used in this study, have a toxic effect on this bacterial group (Babu *et al.* 2001; Bueno *et al*. 2004).

The contamination with HCH altered the structure of the microbial communities (CCLP). Martínez and Toranzo (2003) also detected some temporary changes in the Biolog patterns in a soil contaminated with 100 mg lindane kg^{-1}. One of the main changes in soil CCLP after the contamination was the decrease in the ability to degrade amino acids observed in the soils with higher concentration of contaminant, which agrees with the reduction of ammonifiers in the long term after the contamination. Other authors have observed increases or decreases in soil organic N mineralization caused by lindane (Singh and Prasad 1991; Singh and Singh 2005b). The reduction in the capacity of the microbial community for degrading amino acids may lead to a reduction in the availability of N to microorganisms and plants, which could hamper the biodegradation or phytoremediation of HCHs in soil and suggests that N fertilization of HCH contaminated soils may be necessary to avoid this limitation. The amounts of HCH applied in this study are higher than those usually found in soils candidates for bio- or phytoremediation, nevertheless, a deficiency in N availability could occur locally, around HCH particles, even in soils with much lower HCH amount.

It is noteworthy that in contaminated soils an important decrease in pH and increase in chlorine concentration was observed. These variations likely reflect the degradation of HCHs, which, according to the known degradation pathways, would generate six molecules of HCl from each molecule of HCH (Lal *et al*. 2006, 2010, Nagata *et al*. 2007, Phillips *et al*. 2005, Singh *et al*. 2000). The changes in pH and chlorine concentration may have had a side toxic effect on microbial population and affect community structure, thus, further studies are needed to analyse this potential toxicity. Nevertheless, variation in the abundance of some microorganisms between the second and fourth month after the application of the contaminant appeared not to be related to changes in pH or chlorine, which showed little variation during this period.

Hexachlorocyclohexane affected soil respiration and the effect depended no only on the amount of contaminant but also on the size of particles added to the soil. HCH applied in the form of small particles caused decreases in soil respiration at a lower concentration than when added as larger sized particles. This observation suggests that the increase in respiration detected in some experiments, after addition of HCH to the soil, is not reflecting mineralization of the contaminant. Das and Mukherjee (2000) observed an increase in C mineralization in soils treated with low amounts of lindane, which attributed to the stimulation of the microbial mineralization of endogenous soil organic matter. The dead biomass of microorganisms affected by HCH toxicity may have served as C substrate for those surviving to the presence of the contaminant.

The solubility, bioavailability and toxicity of the contaminant is expected to be higher when it is present in the form of small particles than in the case of bigger particles; consequently, the negative effect on soil respiration could be observed at lower concentrations of HCHs in the first case, as it was detected in the present study. On the other hand, when the contaminant is present

in larger size particles, some soil niches, not directly in contact with the surface of HCH particles, may be colonized by surviving microorganisms, while in the case of high HCH concentration and/or presence of fine particles HCH-free niches may be less abundant. In the soil with microbiota not exposed previously to HCHs the soil respiration was inhibited by a lower amount of contaminant than in soil with microorganisms from a long term polluted site. This observation indicates that soil microbial communities adapt to the presence of HCHs and become less sensitive to the contaminant, as it was observed for other pesticides (Hussain et al 2009)

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