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Microbial indicators for assessing the adverse impact of technical-grade hexachlorocyclohexane on soil quality

Indicadores microbianos para evaluar el impacto adverso de hexaclorociclohexano de grado técnico sobre la calidad del suelo

Indicadores microbianos para avaliar o impacto adverso de grau técnico do hexaclorociclohexano na qualidade do solo

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ABSTRACT

Technical-grade hexachlorocyclohexane (HCH) has been widely used for human health and agricultural purposes. Consequently, HCH residues have entered the soil ecosystem with concomitant deleterious effects on soil quality. The aim of this study was to assess the impact of HCH on soil microbial properties as biological indicators of soil quality. To this end, non-polluted soil was spiked with different amounts of a heavily HCH-polluted soil in order to obtain a concentration gradient between 0 and 1,500 mg HCH kg^{-1} dry matter soil. The mixtures were incubated under laboratory conditions for 2 months. Dehydrogenase activity, fluorescein diacetate hydrolysis activity (FDA), basal respiration, substrate-induced respiration (SIR), microbial biomass carbon, metabolic potential, and the soil quality index were negatively affected by increasing HCH concentrations in soil, in many cases following an exponential pattern. FDA and SIR appear a priori suitable indicators for the impact of HCH on soil microbial properties and, hence, soil quality.

RESUMEN

El hexaclorociclohexano (HCH) ha sido ampliamente utilizado para fines agrícolas y relacionados con la salud humana. En consecuencia, residuos de HCH han entrado en el ecosistema edáfico con efectos perjudiciales concomitantes sobre la calidad del suelo. El objetivo de este estudio fue evaluar el impacto del HCH sobre las propiedades microbianas edáficas como indicadores biológicos de la calidad del suelo. Con este fin, se añadieron diferentes cantidades de un suelo muy contaminado por HCH a un suelo no contaminado, con el fin de obtener un gradiente de concentración entre 0 y 1.500 mg HCH kg^{-1} de materia seca de suelo. Las mezclas se incubaron en condiciones de laboratorio durante 2 meses. La actividad deshidrogenasa, la actividad de hidrólisis del diacetato de fluoresceína (FDA), la respiración basal, la respiración inducida por substrato (SIR), el carbono de la biomasa microbiana, el potencial metabólico, y el índice de calidad del suelo se vieron afectados negativamente por el aumento en las concentraciones de HCH en el suelo, en muchos casos siguiendo un patrón exponencial. FDA y SIR parecen a priori indicadores adecuados para evaluar el impacto del HCH sobre las propiedades microbianas edáficas y, por lo tanto, en la calidad del suelo.

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RESUMO

O Hexaclorociclohexano (HCH) tem sido amplamente utilizado para fins de saúde humana e agrícolas. Como consequência, os resíduos de HCH entram no ecossistema dos solos com os consequentes efeitos nocivos sobre a qualidade do solo. O objetivo deste estudo foi avaliar o impacto de HCH nas propriedades microbianas do solo, considerando-as indicadores biológicos de qualidade do solo. Para este fim, o solo não contaminado foi misturado com diferentes quantidades de um solo fortemente poluído com HCH, a fim de obter um gradiente de concentração entre 0 e 1.500 mg HCH kg^{-1} de matéria seca do solo. As misturas foram incubadas em condições de laboratoriais durante 2 meses. Observou-se que a atividade da desidrogenase, a atividade hidrolítica do diacetato de fluoresceína (FDA), a respiração basal, a respiração induzida pelo substrato (SIR), o carbono da biomassa microbiana, o potencial metabólico, e o índice de qualidade do solo foram negativamente afectados pelo aumento das concentrações de HCH no solo, em muitos dos casos, seguindo um padrão exponencial. A FDA e SIR parecem a priori indicadores adequados para avaliar o impacto de HCH nas propriedades microbianas do solo e, consequentemente, na qualidade do solo.

KEY WORDS
Contamination, lindane, microbial parameters, pollution, soil health

PALABRAS CLAVE
Contaminación, lindano, parámetros microbianos, polución, salud del suelo

PALAVRAS-CHAVE
Contaminação, lindano, parâmetros microbianos, poluição, a saúde do solo

1. Introduction

Hexachlorocyclohexane is manufactured by the photochemical chlorination of benzene and mainly comprises five variously stable isomers: α, β, γ, δ and ε (Lal et al. 2010). The mixture of these isomers is also known as technical-grade hexachlorocyclohexane (HCH). The γ-isomer (lindane) possesses insecticidal properties and for decades has been purified from HCH for human health and agricultural purposes. Nonetheless, because of its suspected carcinogenic, bioaccumulative and endocrine disrupting properties, the use of lindane has been banned in at least 52 countries (Vijgen et al. 2011). However, an assessment of the distribution of γ-hexachlorocyclohexane in European soil and water highlighted that lindane emissions, despite the marked decreasing trend, persist beyond the provisioning of existing legislation (Vizcaíno and Pistocchi 2010).

The historic use of HCH and lindane has left a legacy of HCH-polluted sites that continues to impact natural resources and human health on a global scale (Weber et al. 2008). During the production of lindane, a substantial fraction of other HCH isomers lacking insecticidal properties was produced as a by-product: for each tonne of lindane produced, 8-12 tonnes of other HCH isomers were generated as waste, which was largely deposited in an uncontrolled manner (Vijgen et al. 2011).

Pesticides have been reported to have a negative impact on soil microbial communities (Hussain et al. 2009; Imfeld and Vuilleumier 2012). This is of concern since the microbial communities play a key role in many soil processes and the delivery of essential ecosystem services (Garbisu et al. 2011; Guimarães et al. 2010). Indeed, soil microorganisms (mainly bacteria and fungi) are to a great extent responsible for soil fertility owing to their involvement in nutrient cycling and organic matter (OM) decomposition. Soil microbial parameters are being increasingly used as indicators of the impact of anthropogenic activity on soil quality due to their rapid response, sensitivity, ecological relevance and capacity to provide information that integrates many environmental factors (Doran and Zeiss 2000; Mijangos et al. 2006). In particular, microbial parameters which provide information on the biomass (*e.g.*, microbial biomass C, substrate-induced respiration), activity (*e.g.*, basal respiration,

enzyme activities) and diversity (*e.g.*, DGGE profiles) of soil microbial communities have shown to be valuable monitoring tools of the impact of pollutants on soil quality (Epelde et al. 2008, 2009).

The aim of the current work was to assess the impact of HCH on soil microbial properties as biological indicators of soil quality. To this aim, we determined a variety of soil microbial parameters that provide information on the biomass, activity and diversity of soil microbial communities in soils polluted with different concentrations of HCH. Likewise, we aimed at finding the most suitable microbial indicators for the impact of HCH on soil microbial communities.

2. Materials and methods

2.1. Sampling and experimental design

The HCH-polluted soil was collected (upper 0-20 cm) from an uncontrolled dumpsite located near Bilbao (northern Spain). Immediately after collection, it was taken to the laboratory, dried at room temperature for 48 h, sieved to < 2 mm and characterized according to standard methods (MAPA 1994). The polluted soil had a pH of 2.9 and an OM content of 34.4%. The concentration of the five main HCH isomers was determined by gas chromatography with an electron-capture detector. The soil showed a very high concentration of HCH, ca. 20,000 mg HCH kg^{-1} dry matter (DM) soil. The proportion (%) of the five main isomers was: α (17), β (21), γ (1), δ (48) and ε (13).

A non-polluted soil was collected from a natural, polyphita grassland located in Derio (northern Spain). The soil was a clay loam, with a pH of 6.2 and an OM content of 6.3%. This non-polluted soil was spiked, by weight, with different proportions of the heavily HCH-polluted soil collected from the uncontrolled dumpsite, in order to obtain a concentration gradient between 0 and 1,500 mg HCH kg^{-1} DM soil. The proportion of heavily HCH-polluted soil from the dumpsite was equal to only 0.25 and 4% in the mixture with the lowest and highest HCH concentration respectively. Mixtures were prepared in 10 cm-diameter pots containing a total of 100 g DM soil (three replicates per mixture). Soil mixtures were incubated in the dark at 25 ºC for 2 months at 80% water holding capacity. At the end of this incubation, soils were thoroughly mixed manually, randomly sampled, and stored at 4 ºC for less than one month prior to analysis.

2.2. Determination of soil properties

2.2.1. Physico-chemical parameters

Dry matter content (%) was calculated from weight loss following oven drying at 105 ºC for 24 h. Total organic carbon was determined according to Nelson and Sommers (1996). Water soluble organic carbon (C_{ws}) was determined following Wei et al. (2008): 1 g of soil was suspended in 5 mL of deionized water and the suspension was horizontally shaken at 175 rpm for 1 h. After centrifugation at 2400 × g, organic carbon was determined according to Wu et al. (1990). Finally, soil pH (in water, 1:2.5 w/v) and particle size distribution (Mastersizer 2000 laser diffractometer) were also determined in the soil samples.

2.2.2. Microbial indicators

β-glucosidase (GLU) and β-glucosaminidase (GLM) enzyme activities were determined according to a modified Taylor et al. (2002) and Parham and Deng (2000), respectively. Briefly, 1.6 mL of modified universal buffer (20 mM pH 6.0 for GLU and 100 mM pH 5.5 for GLM) were added to 1 g of soil and heated to 37 ºC. Then, 0.4 mL of temperate substrates were added (50 mM 4-nitrophenyl β-D-glucopyranoside for GLU and 10 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide for GLM) and the reaction mixtures were left at 37 ºC for 45 min. The reactions were stopped by adding 0.4 mL of 0.5 M calcium chloride and 1.6 mL of 0.1 M Tris(hydroxymethyl)aminomethane (pH 12.0)

for GLU or 1.6 mL of 0.5 M sodium hydroxide for GLM. Finally, samples were centrifuged at 2400 × g for 5 min, and absorbances read at 410 nm.

Dehydrogenase activity (DEH) was determined following Taylor et al. (2002): 1 g of soil was mixed with 0.4 mL of 100 mM Tris(hydroxymethyl) aminomethane buffer (pH 7.0) and 0.4 mL of substrate [iodonitrotetrazolium chloride (0.5% w/v)]. The mixture was incubated at 25 ºC for 3 h and the reaction stopped with 8 mL of methanol. After centrifugation (2400 × g, 3 min), the absorbance value of the samples was read at 490 nm.

Fluorescein diacetate hydrolysis activity (FDA) was determined as described in Schnürer and Rosswall (1982): 50 µL of 0.2% fluorescein diacetate was added to 0.5 g of soil in 4 mL universal modified buffer (100 mM, pH 7.6) at 25 ºC. The reaction was stopped after 15 min with 4 mL of acetone, and the samples centrifuged at 17,000 × g for 2 min. The absorbance value of the samples was read at 490 nm.

Microbial biomass carbon (C_{mic}) was measured by the fumigation-extraction method (Vance et al. 1987): 5 g of soil was fumigated for 24 h with amylene-stabilized $CHCl_3$ and extracted with 20 mL of 0.5 M K_2SO_4. Then, 3.5 mL of chromium reagent [chromium (VI) oxide (0.06% w/v); sulfuric acid (65% v/v)] was added to 2 mL of extract and incubated at 150 ºC for 60 min. Organic carbon concentration was determined colorimetrically at 445 nm. C_{mic} was calculated as the difference between organic carbon concentration of the fumigated and unfumigated extracts (Wu et al. 1990).

Soil basal respiration (R_B) was determined by measuring CO_2 evolution in hermetic flasks incubated at 28 ºC for 72 h according to ISO norm 16072 (2002). Subsequently, 80 g of glucose, 13 g of di-ammonium sulphate and 2 g of potassium di-hydrogenous phosphate were added (1 g of this substrate mixture per 100 g DM soil) for the determination of substrate-induced respiration (SIR) according to ISO norm 17155 (2002). The amount of CO_2 evolved from the soil was quantified after 6 h of incubation at 28 ºC.

The respiratory quotient (*q*R) was calculated as the ratio of basal respiration to substrate-induced respiration (*q*R = R_B/SIR). The metabolic potential (MP = dehydrogenase activity/C_{ws}) was also calculated.

DNA was extracted from soil (0.25 g) using the DNA PowerSoil™ Isolation Kit (MO Bio Laboratories, CA) according to the manufacturer's instructions. Prior to DNA extraction, soil samples were washed twice in 120 mM K_2HPO_4 (pH 8.0) to wash away extracellular DNA (Kowalchuk et al. 1997). Extracted DNA concentrations were determined spectrophotometrically (Nanodrop, ND-1000).

*q*PCR analyses for the determination of bacterial and fungal abundances (N) were carried out according to Dhanasekaran et al. (2010). Primers used to assess gene copy number for total bacteria (16S rRNA) were Ba519f and Ba907r (Lueders et al. 2004). Primers used to assess gene copy number for total fungi (18S rDNA) were FF390r NIOO and Fung5f NIOO (Vainio and Hantula 2000). Each reaction mixture contained, in a final volume of 25 µL, 2.5 µL DNA, 12.5 µL SYBER® PremixExTaq™ (Takara Bio Inc.), 0.25 µL of each primer (at a 30 µM concentration for total bacteria and 20 µM concentration for total fungi), 1.25 µL bovine serum albumin (40 mg mL^{-1}), 0.5 µL ROX™ dye and 7.5 µL of sterile deionized water. PCR thermal cycling conditions for total bacteria were as follows: a single step of 15 min at 95 ºC, 40 cycles of 30 s at 94 ºC, 30 s at 52 ºC and 60 s at 72 ºC. Immediately after the PCR assay, melting curve analyses were performed by heating samples for 15 s to 95 °C, followed by cooling for 60 s to 60 °C, and then heating again to 95 °C for 30 s with continuous fluorescence recording and a final extension for 15 s at 60 ºC. For total fungi, PCR conditions were: 15 min at 95 ºC, 40 cycles of 30 s at 94 ºC, 30 s at 50 ºC and 60 s at 72 ºC. The melting curve analyses were performed as described previously for total bacteria. Known template standards were made from whole genomes extracted from pure bacterial and fungal isolates as described in Dhanasekaran et al. (2010).

For the estimation of bacterial richness (S) through PCR-DGGE, the 16S rDNA was

amplified by using the primer pair F968-GC/R1378. PCRs were carried out in 25 µL volumes containing 1 µL of template, 12.5 µL of 2X PremixExtaq™ (Takara Bio Inc.), 1.5 µL of each primer (10 µM) and 8.5 µL of sterile deionized water. The reaction mixture was preheated at 94º C for 2 min, followed by 35 thermal cycles of 30 s at 92 ºC, 60 s at 55 ºC, 45 s at 68 ºC (+1 s cycle^{-1}) and a final extension of 5 min at 68 ºC. For the estimation of fungal richness, the 18S rDNA was amplified by using the primer pair FR1GC/FF390. PCRs were carried out in 25 µL volumes containing 1 µL of template, 12.5 µL of 2X PremixExtaq™, 0.5 µL of each primer (30 µM) and 10.5 µL of sterile deionized water. Again, the reaction mixture was preheated at 94 ºC for 5 min, followed by 30 thermal cycles of 30 s at 92 ºC, 60 s at 50 ºC, 60 s at 68 ºC and a final extension of 10 min at 68 ºC. Amplifications were carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA). For DGGE analysis, a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA) was used. The denaturating gradient was from 35 to 60% of denaturant with 8% acrylamide for bacteria (16S rDNA) and from 40 to 55% of denaturant with 7.5% acrylamide for fungi (18S rDNA). DGGE was performed using 20 µL of the PCR product in 1X TAE buffer (40 mM tri-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) at 60 ºC. Gradient gels were topped with 5 mL of acrylamide containing no denaturant. Electrophoresis was performed at 100 V for 10 min followed by 50 V for 16 h. The gels were stained with SYBER® SafeDNA Gel Stain (Invitrogen, USA) following the manufacturer´s instructions, and the bands visualized under UV light in a G:BOX (Syngene). Banding patterns were analysed using the Gene Tools (Syngene) program.

2.2.3. Data analysis

In order to provide a visual illustration of overall soil microbial functionality in response to the presence of different concentrations of HCH in soil, an amoeba diagram was plotted (**Figure 5**). Likewise, in order to obtain an integrative measurement of the impact of HCH on microbial parameters, the Soil Quality Index (SQI) was determined according to Bloem et al. (2006):

$$SQI = 10^{\log m - \frac{\sum_{i=1}^{n} |\log m - \log n_i|}{n}}$$

where *m* is the reference (mean value of control non-polluted soil, set to 100%) and *n* are the measured values as percentages of the reference.

Relationships between microbial parameters and HCH concentration were examined by means of principal component analysis (PCA) applied to the correlation matrix of these variables, running Canono 5 (Ter Braak and Šmilauer 2012). Variation partitioning analyses were also performed to test the unique and combined effects of HCH concentration and pH on soil microbial parameters. The response of soil microbial and physicochemical parameters to increasing HCH concentrations was adjusted to single two-parameter exponential decay equations using SigmaPlot software. *q*R data were adjusted to a single three-parameter exponential rise to maximum equation. The F-test was used to check the significance of the observed regressions.

3. Results

In spite of the heterogeneity of pollutant distribution typical of uncontrolled dumpsites and the well-known spatial heterogeneity of the soil matrix itself, when spiking the non-polluted soil with the heavily HCH-polluted soil, we did obtain a good ($r = 0.93$, $p < 0.0001$) concentration gradient from 0 to 1,500 mg HCH kg^{-1} DM soil in the experimental pots. As expected, the properties of the non-polluted soil were not greatly modified due to spiking with heavily HCH-polluted soil, but some physicochemical parameters were indeed affected: soil pH decreased at increasing HCH concentrations ($r \geq 0.92$, $p < 0.001$) from 6.4 ± 0.00 (mean value ± standard deviation) at 0 mg HCH kg^{-1} DM soil to 4.6 ± 0.1 at 1,500 mg HCH kg^{-1} DM soil. Concerning the OM content of the non-polluted soil, no effect was observed as a result of the addition of heavily HCH-polluted soil. By contrast, C_{ws} showed a positive correlation ($r \geq 0.93$, $p < 0.001$) with HCH concentration (data not shown). Finally, regarding soil

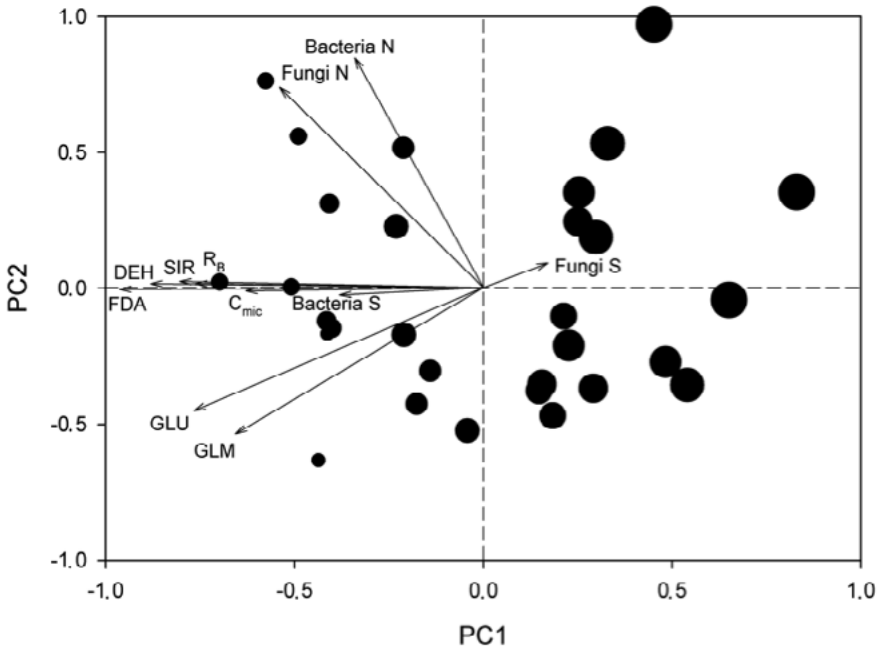

Figure 1. Principal component analysis of soil microbial parameters in soils subjected to different concentrations of HCH (the bigger the circle size the higher the HCH concentration). DEH, dehydrogenase activity; FDA, fluorescein diacetate hydrolysis activity; R_B, basal respiration; SIR, substrate-induced respiration; C_{mic}, microbial biomass carbon; GLU, β-glucosidase; GLM, β-glucosaminidase; Bacteria N, bacterial abundance; Fungi N, fungal abundance; Bacteria S, bacterial richness; Fungi S, fungal richness.

texture, no differences were found among the experimental pots.

According to the variation partitioning analysis performed to test the effects of HCH concentration and pH on soil microbial parameters, 82% of the explained effect was combined, while 15 and 3% were due to solely HCH concentration and pH, respectively. In the PCA (**Figure 1**), a HCH concentration gradient was observed along PC1 (PC1 explained 44% of the total variance; PC2 explained 16% of the total variance). Apart from fungal richness, all microbial parameters were negatively correlated with soil HCH concentration. FDA, DEH, SIR, R_B and C_{mic} showed the strongest negative correlations with soil HCH concentration (**Figure 1**). Values

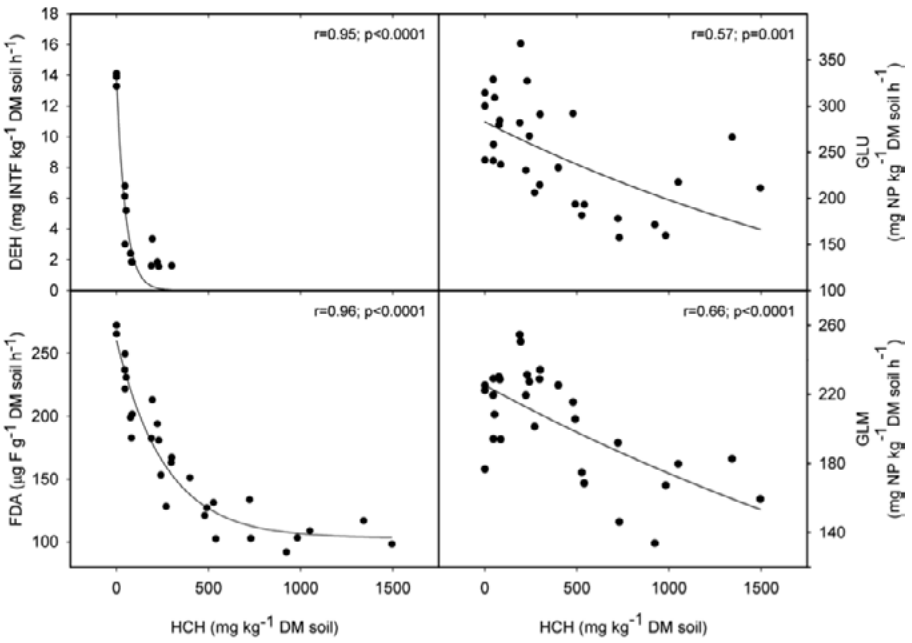

Figure 2. Effect of HCH concentration on soil dehydrogenase (DEH), fluorescein diacetate hydrolysis (FDA), β-glucosidase (GLU) and β-glucosaminidase (GLM) activity. INTF, iodonitrotetrazolium violet-formazan; F, fluorescein sodium salt; NP, nitrophenol.

of DEH, FDA (**Figure 2**) and SIR (**Figure 3**) decreased at increasing HCH concentrations following an exponential pattern ($r \geq 0.90$, $p < 0.0001$). The HCH concentration required to observe a 50% reduction, as compared to values found in non-polluted soil, was 36, 420 and 260 mg kg^{-1} DM soil for DEH, FDA and SIR, respectively. DEH decreased sharply at the lower HCH concentrations: at approximately 50 and 100 mg HCH kg^{-1} DM soil, DEH was reduced by 56 and 80%, respectively. Values of R_B and C_{mic} (**Figure 3**) also appeared to decrease at increasing HCH concentrations but, in this case, the values did not follow a clear pattern ($r = 0.58$ and 0.56 for R_B and C_{mic}, respectively). At the highest HCH concentration (1,500 mg kg^{-1} DM soil), C_{mic} values were reduced by 84%. Similarly, the other microbial parameters determined here (GLU, GLM, bacterial and fungal abundance, bacterial and fungal richness) did not follow a clear pattern in response to increasing HCH concentrations in soil (**Figures 2 and 4**).

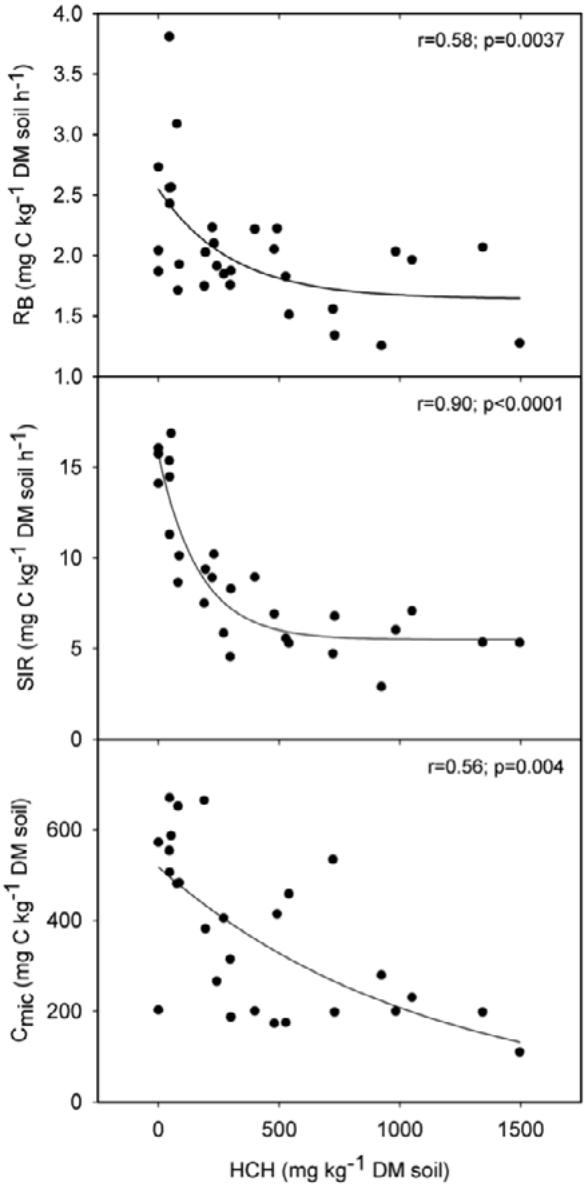

Figure 3. Effect of HCH concentration on soil basal respiration (R_B), substrate-induced respiration (SIR) and microbial biomass carbon (C_{mic}).

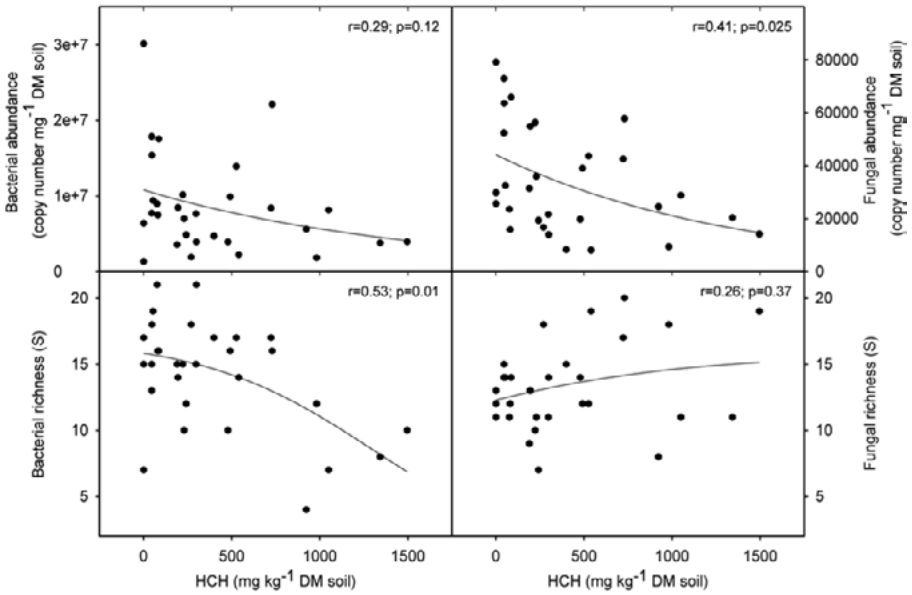

Figure 4. Effect of HCH concentration on soil bacterial and fungal abundances determined by qPCR, and bacterial and fungal richness determined by PCR-DGE.

An amoeba diagram was plotted to provide a visual illustration of overall soil microbial response to the presence of HCH in soil (**Figure 5**). For this analysis, data were grouped into four HCH-pollution levels: non-pollution, low pollution (0-200 mg kg^{-1} DM soil), medium pollution (200-500 mg kg^{-1} DM soil) and high pollution (500-1,500 mg kg^{-1} DM soil). In agreement with our PCA results (**Figure 1**), when grouping the response of microbial parameters in these four categories, values of DEH, FDA and SIR also decreased at increasing HCH-pollution levels (**Figure 5**). DEH was most sensitive to the presence of HCH, being highly inhibited even at the low pollution level. GLU activity was inhibited at medium (12% lower compared to non-polluted) and high (32% lower compared to non-polluted) pollution levels. In turn, GLM activity was lower (20% lower compared to non-polluted) only at the highest HCH pollution level.

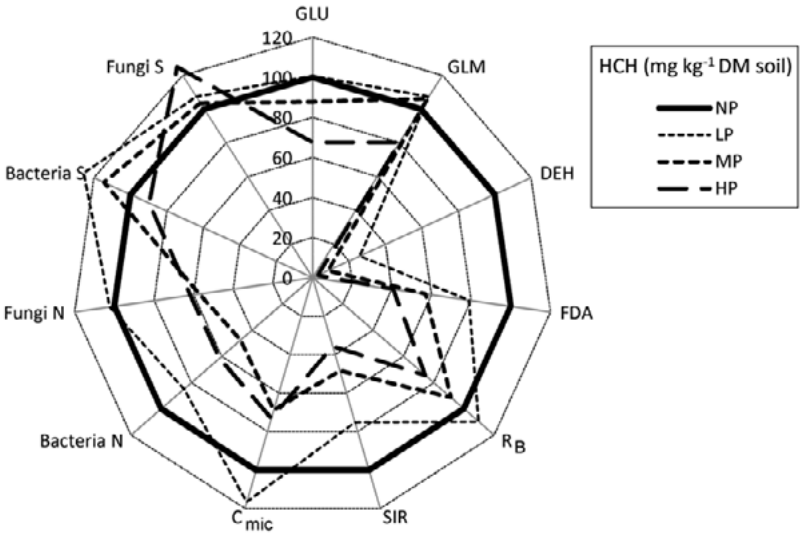

Figure 5. Overall soil microbial response to the presence of HCH in soil. Data were grouped into four HCH-pollution levels: non-pollution, low pollution (0-200 mg kg^{-1} DM soil), medium pollution (200-500 mg kg^{-1} DM soil) and high pollution (500-1500 mg kg^{-1} DM soil). Abbreviations as in **Figure 1**.

Finally, values of C_{mic} and R_B, as well as bacterial and fungal abundance, were inhibited at medium and high pollution levels.

From all the microbial parameters studied here, the SQI was calculated (**Figure 6**), finding out a decreasing pattern (r = 0.82, p < 0.0001) at increasing HCH concentrations. By contrast, the respiratory quotient followed an increasing exponential pattern (r = 0.75, p < 0.0001) when exposed to increasing HCH concentrations. The metabolic potential also decreased, following an exponential pattern (r = 0.96, p < 0.0001), at increasing HCH concentrations; in this respect, DEH was completely inhibited at HCH concentrations higher than ca. 250 mg kg^{-1} DM soil (**Figure 2**).

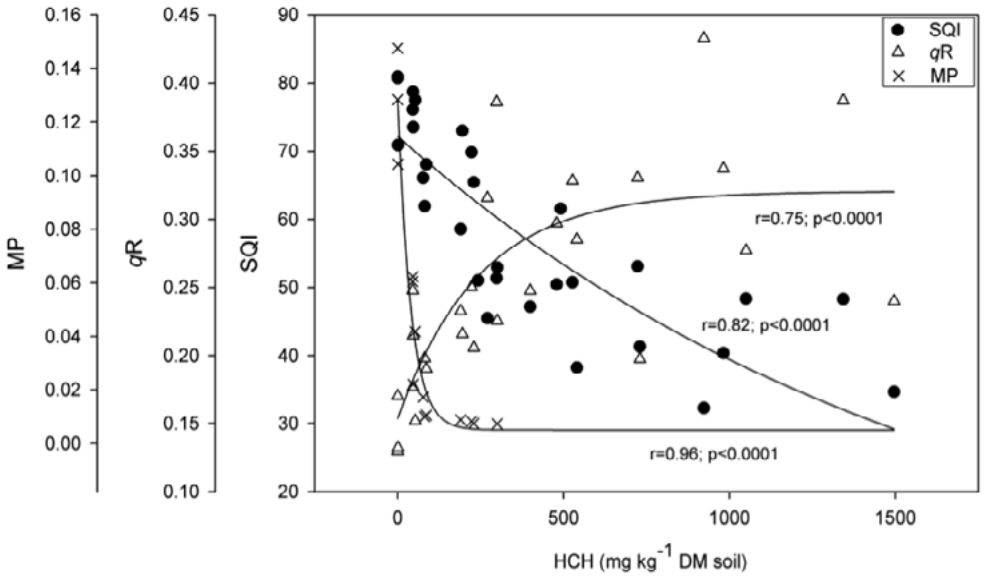

Figure 6. Effect of HCH concentration on the soil quality index (SQI), the respiratory quotient (*q*R = R_B/SIR) and the metabolic potential (MP = DEH activity/water soluble organic carbon).

4. Discussion

Hexachlorocyclohexane and lindane can impact soil quality directly by application to crops or indirectly by pollution from stockpilings accumulated near production areas and uncontrolled dumpsites (Weber et al. 2008). The impact of pesticides on soil microorganisms depends on a variety of factors such as pesticide concentration, application rate, exposure time, soil type and so on (Eisenhauer et al. 2009; Hussain et al. 2009; Imfeld and Vuilleumier 2012; Mijangos et al. 2009). In our study, a considerable reduction in soil pH was observed due to spiking with heavily HCH-polluted soil. Other authors (Zargar and Johri 1995) reported the effects of γ-HCH on soil amylolytic microorganisms and amylase activity. Baldwinder et al. (2005) found an initial decrease in soil microbial counts, which showed subsequent recovery, as a result of lindane application. Similarly, a 50% reduction in bacterial cell concentration (this reduction was correlated with a reduction in the rate of substrate utilization as observed by Biolog™) was found by Rodríguez and Toranzos (2003) in lindane-amended soil; on the contrary, these authors did not observe lindane-induced effects on genetic diversity determined by DGGE. In a study on the effects of lindane on agricultural soil, lindane inhibited nitrifying bacteria at concentrations of 3.5 to 15 kg ha^{-1} (Martínez-Toledo et al. 1993); however, the total number

of heterotrophic bacteria, fungal populations, denitrifying bacteria and aerobic dinitrogen fixing bacteria were not affected by lindane application. Methane oxidation has also been reported (Mertens et al. 2005) to be negatively affected by long-term HCH pollution; in particular, the type I methanotrophic community was less evenly distributed in historically HCH-polluted soils compared to less polluted reference soils. Finally, HCH application resulted in increased rates of organic C and N mineralization (Das and Mukherjee 2000).

Dehydrogenase activity, an intracellular process that occurs in every viable microbial cell, is used as an indicator of overall soil microbial activity (Nannipieri et al. 2002). DEH proved to be extremely sensitive to the presence of HCH, even at the lowest concentrations tested here. In agreement with our results, in a groundnut field treated with lindane, soil DEH activity suffered a 30-35% reduction (Singh and Singh 2005). DEH activity has previously been reported to decrease as a result of the application of insecticides such as, for instance, chlorpyrifos, quinalphos (Pandey and Singh 2006) and methamidophos (Yu et al. 2011). On the other hand, DEH activity has been shown to be very sensitive to a fungicidal preparation consisting of mancozeb supplemented with dimethomorph (Cycon et al. 2010).

Furthermore, FDA and SIR appear a priori (our results were obtained with only one soil type; responses might be different in other soil types) suitable indicators for the impact of HCH on soil microbial communities. FDA activity, an indicator of soil hydrolytic activity, is a measurement of the contribution of different enzymes (non-specific esterases, proteases and lipases, involved in the decomposition of OM in soil) and reflects overall soil microbial activity (Dick 1997). Nonetheless, as indicated by Nannipieri et al. (2003), one must be very cautious when interpreting data on FDA because the measured activities depend on the contribution of both extracellular (associated with soil colloids) and intracellular enzyme activities, and, strictly speaking, only intracellular enzyme activities can truly reflect microbial activity, because the contribution of free extracellular enzymes released by active microbial cells is negligible. On the other hand, FDA has been reported to be strongly correlated with microbial biomass (Aseri and Tarafdar 2006). SIR is an indicator of potentially active microbial biomass (Hassink 1995) and then it is not surprising to observe lower values when soil microorganisms are exposed to increasing HCH levels.

Soil enzyme activities are valid indicators of the functional status of the soil ecosystem (Naseby and Lynch 2002) and have widely been used to study the impact of pesticides on soil quality (Floch et al. 2011; Muñoz-Leoz et al. 2011, 2012). Here, β-glucosidase and β-glucosaminidase activities were inhibited by the presence of HCH and, thus, contributed to the lower SQI values observed at increasing HCH concentrations. These two enzyme activities play a key role in the cycling of nutrients (Ekenler and Tabatabai 2002; Turner et al. 2002), which highlights the impact of HCH on soil fertility and hence soil quality. Muñoz-Leoz et al. (2011) found an inhibition of β-glucosidase activity in soils treated with tebuconazole (fungicide) at all incubation times. In any event, under our experimental conditions and at the range of HCH concentrations tested here, these two enzyme activities were not suitable indicators of the impact of HCH on soil functioning, because they only responded to medium-high HCH concentrations and without following an identifiable pattern.

An inhibition of C_{mic} was also observed at increasing HCH concentrations (**Figure 3**). Organic pollutants can be a source of carbon for heterotrophic microorganisms. Then, it is not surprising that other authors (Kumar et al. 2012) found that the application of insecticides (chlorpyrifos and cartap hydrochloride) and an herbicide (pretilachlor) resulted in higher C_{mic} values for 15-30 days after pesticide application. Nevertheless, our HCH-polluted soil was taken from an uncontrolled dumpsite where HCH residues were illegally dumped at least 40 years ago. Thus, the HCH currently present in this dumpsite is presumably highly recalcitrant and of low bioavailability. On the other hand, soil sieving can affect both the availability of organic pollutants (Ter Laak et al. 2007) and the

activity of soil microorganisms (Černohlávková et al. 2009; Thomson et al. 2010). In fact, it can increase both pollutant bioavailability and microbial activity through aggregate disruption.

The respiratory quotient (*q*R) followed an increasing exponential pattern when exposed to increasing HCH concentrations (**Figure 6**). Ecophysiological indices, such as qCO_2 (R_B/C_{mic}) and *q*R (R_B/SIR), can reflect stress in soil microbial populations (Anderson and Domsch 1985; Pal et al. 2008; Wardle and Ghani 1995), leading to a diversion of energy from growth and reproduction to cell maintenance (Eisenhauer et al. 2009; Gómez et al. 2009; Moreno et al. 2007). Indeed, a higher respiratory activity (R_B) for the same microbial biomass (SIR is an indicator of potentially active microbial biomass) is indicative of higher levels of maintenance energy and may be evidencing a lower metabolic efficiency due to a pesticide-induced stressing effect (Gómez et al. 2009). The metabolic potential (DEH/C_{ws}) was also sensitive to the presence of increasing HCH concentrations, once more highlighting the adverse impact of HCH on soil microbial metabolism. It has been suggested that DEH, especially when referred to the energetic and immediately available carbon substrate (C_{ws} represents the most labile fraction of soil OM because it is susceptible to microbial attack), gives an idea of the metabolic potentiality of soil rehabilitation (Masciandaro et al. 2000).

Finally, soil quality, as reflected by the values of the SQI (Bloem et al. 2006), decreased at increasing HCH concentrations (**Figure 6**). The SQI, calculated from the values of all the microbial parameters determined here (DEH, FDA, GLU, GLM, R_B, SIR, C_{mic}, bacterial and fungal N, bacterial and fungal S), is an integrative measurement of the combined effect of HCH on the biomass, activity and diversity of soil microbial communities, thereby providing valuable information on the overall microbial functionality of the HCH-impacted soil. Similarly, the area and shape of the amoeba plot (**Figure 5**) provide an integrated fingerprinting for assessing the adverse impact of increasing concentrations of HCH on overall soil microbial functionality. In any case, although the SQI is indeed very useful to integrate different results, it must be interpreted with caution as it is only a simplified reflection of the extremely complex soil ecosystem and, inevitably, entails a loss of useful information about the response of each specific parameter considered during the calculation of the SQI.

5. Conclusions

The spiking of non-polluted soil with heavily HCH-polluted soil has shown to decrease soil pH and negatively impact soil microbial biomass and activity, and hence soil quality. In particular, values of DEH, FDA, basal respiration, SIR, microbial biomass C, metabolic potential and the soil quality index were negatively affected by increasing HCH concentrations in soil, in many cases following an exponential pattern. By contrast, the respiratory quotient increased exponentially at increasing HCH concentrations, suggesting an HCH-induced stressing effect on soil microorganisms. At the range of concentrations studied here, FDA and SIR appear a priori suitable indicators for the impact of HCH on soil microbial communities, as they followed a clear exponential pattern with values of $r \geq 0.90$. Although values of DEH and metabolic potential also decreased at increasing HCH concentrations in soil following an exponential pattern with values of $r \geq 0.95$, they were too sensitive to the presence of HCH to be considered suitable indicators for the impact of HCH at the range of concentrations tested here. Soil microbial properties have proved to be very useful biomonitoring tools for the assessment of the impact of HCH on soil quality.

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