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Modification of enzymatic activity in soils of contrasting pH contaminated with 2,4-dichlorophenol and 2,4,5-trichlorophenol.

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Abstract

According to previous studies, acidic soils may receive larger quantities of 2,4-dichlorophenol (2,4-DCP) and of 2,4,5-trichlorophenol (2,4,5-TCP) than the concentrations indicated in the prevailing legislation for defining a soil as contaminated, without any important changes in their biochemical properties. In this study, we investigated whether neutral or slightly alkaline soils behave in the same way as acidic soils in response to contamination by these compounds. For this purpose, a large number of acidic soils (pH between 4.2 and 5.9) and calcareous soils (pH between 6.5 and 8.0) were contaminated in the laboratory with different doses of 2,4-DCP (up to 10000 times the GRL) and of 2,4,5-TCP (up to 500 times the GRL). After an incubation period of three days, the activities of several enzymes (dehydrogenase, catalase, ß-glucosidase and phosphomonoesterase) were measured in the soils. The effects of 2,4,5-TCP were much greater than those of 2,4-DCP in both the acidic and calcareous soils, regardless of the dose applied. Phosphomonoesterase and ß-glucosidase activities were scarcely affected by either of the contaminants in any of the soils, whereas the catalase activity decreased slightly. The dehydrogenase and urease activities were strongly affected in all soils and in some cases even disappeared, particularly after the application of 2,4,5-TCP. Multiple regression analysis of the percentage reductions in dehydrogenase and urease activities in relation to contaminant dose and different soil properties indicated that the reduction in enzyme activity depended, in decreasing order, on the dose of contaminant applied, total carbon content and soil pH. We suggest that the processes that regulate the toxicity of these compounds in soils are their adsorption by soil organic matter and the dissociation of the non-adsorbed compound into phenolate ions (which are toxic to microorganisms). In fact, the chlorophenols scarcely affected the biochemical properties of the soils under study because of their high organic matter contents (A horizons with total carbon contents of up to 11%). Moreover, both chlorophenols had slightly stronger effects on the calcareous soils than on the acidic soils, probably because the dissociation process was favoured at higher pH. On the other hand, the 2,4,5-TCP had stronger effects on soil biochemical properties than 2,4-DCP, which may be explained by the lower p*Ka* value of 2,4,5-

TCP (6.9) than that of 2,4-DCP (7.9). The results show that the GRL values established by the legislation are not appropriate for either of these chlorophenol compounds.

Key words: Soil enzyme activities; Soil contamination; 2,4-dichlorophenol; 2,4,5-trichlorophenol; Temperate-humid soils

1. Introduction

Chlorinated compounds are organic compounds widely used as bacteriological agents (Hutzinger et al., 1985). The use of some of these compounds, such as certain isomers of hexachlorocyclohexane (lindane), has been prohibited because they are toxic to various species, including humans (Willet et al., 1998). However, other compounds such as 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) are widely used as antifungal agents in silviculture to slow down the decomposition of cut timber in forests, and as precursors of different herbicides in agriculture (Hajslová et al., 1988). The presence of 2,4-DCP and 2,4,5-TCF in soils is generally due to their use as antifungal agents or to the degradation of certain herbicides and pesticides (Hutzinger et al., 1985; Czaplicka, 2004). These compounds may also be present in soil as a result of the accidental spillage of waste water generated in the paper industry, as they may be formed during the process of whitening paper (Kookana and Rogers, 1995; Cea et al., 2007). Both compounds are considered toxic to the environment (USEPA, 1979), and their presence in soils should therefore not surpass a certain concentration. Unfortunately, the frequent use of excessive amounts of these compounds, and of many other xenobiotic compounds, leads to contamination of soils and of the environment in general (Doran, 2002).

Although the legislation regarding soil contamination is less well established than that concerning contamination of water or the atmosphere (Oldeman et al., 1991), national governments and supranational organizations have recently made efforts to restrict the use of various compounds with the aim of protecting soils from external aggressions, including aggressions caused by the improper use of organic compounds (European Directive on Contaminated Soils, 2006). Thus, a soil can be declared contaminated, and therefore not suitable for certain uses, if the amount of any contaminant in soil exceeds 100 times the value of the concentration established by legislation. This value is denominated the generic reference level (GRL) and is defined for each compound and each soil use. For 2,4-DCP and 2,4,5-TCP, the Spanish legislation (Real Decreto 9/2005), similarly to the European legislation (European Directive on Contaminated Soils, 2006), establishes GRLs of 0.1 mg kg^{-1} for 2,4-DCP and 10 mg kg^{-1} for 2,4,5-TCP for soils, irrespective of the type of soil use. In addition, a soil is considered as contaminated if the amount of a toxic product is greater than 100 times the GRL, i.e. 10 mg kg^{-1} for 2,4-DCP, and 1000 mg kg^{-1} for 2,4,5-TCP.

Various studies carried out by our research group have shown that Galician soils, which generally have acid surface horizons (pH between 4.2 and 5.9) and high organic matter contents (values of total carbon between 5% and 12%), are capable of receiving much higher concentrations of 2,4-DCP and 2,4,5-TCP than the levels indicated in the prevailing legislation

for diagnosing a soil as contaminated, without any apparent effects on the biochemical, biological or physiological properties and, therefore, without any loss of soil quality (Moscoso et al., 2007; Bello et al., 2008; Bello et al., 2011). Similar results have recently been obtained by Martí et al. (2011) for soils in the Mediterranean area, although some properties of these soils, such as the total C content, are very different from those of Galician soils. In other words, it is difficult to demonstrate the loss of quality in soils that receive such high quantities of chlorophenols, as the indicators listed in the legislation for determining deterioration of soil quality do not always change or respond in a similar way to the presence of these compounds (Bello et al., 2008). Nevertheless, although the responses of biochemical properties such as respiration and nitrogen mineralization are rather inconsistent and increase or decrease randomly, the changes in enzyme activities are generally more consistent and usually decrease in response to increasing doses of chlorophenols (Moscoso et al., 2007; Bello et al., 2008).

Moreover, as observed by other researchers working on diverse chlorophenols (He et al., 2006; Cea et al., 2007), contamination by 2,4-DCP and 2,4,5-TCP generally has less effect on the biochemical properties of acidic soils with high contents of organic matter than on the biochemical properties of other types of soils (Moscoso et al., 2007; Bello et al., 2008). These observations were established in studies involving large numbers of soils, always of pH (in water) lower than 6. Despite the relatively narrow range of pH in these soils (between 4.2 and 5.9), it was observed that as the pH increased, the effect produced by the chlorophenols generally increased. This suggests that soil pH may be an important factor in determining soil deterioration in response to contamination, although the effect may be masked by other edaphic properties (Bello et al., 2008).

Taking the latter into account, and as only acidic to strongly acidic soils have been considered in previous studies, the objectives of the present study were: a) to investigate the behaviour of different oxidoreductases and hydrolytic enzymes in neutral to slightly alkaline soils in response to contamination with 2,4-DCP and 2,4,5-TCP; b) to compare the degree to which the properties are affected in these soils and in acidic soils with similar characteristics; c) to further our knowledge of the mechanisms of action of these chlorophenols in soil, and d) to shed some light on the reason why the GRLs indicated in the prevailing legislation are in many instances not applicable to soils.

2. Materials and methods

2.1. Soils

Thirteen neutral and slightly alkaline soils developed over limestone (calcareous soils), and 17 moderate and strongly acidic soils developed over different siliceous parent materials (acidic soils) were sampled at sites distributed throughout Galicia (NW Spain) and under different types of use (forest, pasture, crop). The calcareous soils are mainly Leptosols, Phaseozems and Luvisols, whereas the acidic are Umbrisols and Regosols (ISSS Working Group R.B., 1998). At each site, 10-15 samples of the A horizon (0-10 cm) were collected at random and pooled in the field to produce a composite sample. The samples were transported in isothermal bags to the laboratory where they were sieved (< 4 mm). A sub-sample from each site was air-dried to determine general soil properties, and the remainder was stored at 4 ºC until analysis of different

enzymatic activities and for the experiment involving contamination of soils with 2,4-DCP and 2,4,5-TCP. The enzymatic analysis of the soil samples and preparation of the soil contamination experiment were carried out within one week of obtaining the soil samples.

2.2 Soil contamination

Triplicate aliquots of the fresh soils were artificially contaminated with four different doses of 2,4-DCP (0, 100, 500 and 1000 mg kg^{-1}, i.e. 0, 1000, 5000 and 10000 times the GRL established by the Spanish legislation) or with 5 doses of 2,4,5-TCP (0, 100, 500, 1000 and 5000 mg kg^{-1}, equivalent to 0, 10, 50, 100 and 500 times the GRL for this compound). As both chlorophenols are sparingly soluble in water (Czaplicka, 2004), they were first mixed with quartz sand (in the proportions required to generate the doses indicated), and the mixtures were then shaken for 48 h in a rotary shaker to achieve homogeneity (Moscoso et al., 2007). The soils were contaminated by addition of the sand/contaminant mixture to the moist soil in a proportion of 10% (10 g of the mixture was added to an amount of moist soil equivalent to 100 g of oven-dried soil) to obtain the above-indicated concentrations of contaminant. Distilled water was added to maintain the system at field capacity (Gardner, 1986), i.e. the amount of water retained by the soil layers at a -33 kPa (1/3 bar) matrix potential, which corresponds to the water potential traditionally used to afford optimal moisture conditions in mineralization experiments (Leirós et al., 1999). After addition of the water, the mixtures were homogenized carefully and maintained at 20 °C for 72 h. This contact time was selected on the basis of the results of prior experiments that indicated that the major modifications to soil properties occur 72 h after contamination (Bello et al., 2008). The control soils were mixed with sand only (the same amount added for spiking soil samples). At the end of incubation period (72 h), the soils were analyzed to determine the enzymatic activities.

2.3. Analysis of soil physical and chemical properties

The following properties were determined by the methods described by Guitián and Carballas (1976): pH in water (1:2.5, soil:water ratio), pH in 1 M KCl (1:2.5, soil:solution ratio), total carbon (dichromate oxidation in acid medium), nitrogen content (Kjeldahl procedure), particle size distribution (Robinson pipette, with Calgon® as dispersant) and amorphous Al_2O_3 and Fe_2O_3 (oxalic-oxalate solution extractable). The mean values and standard deviations of these properties in both groups of soils (acidic and calcareous) are shown in Table 1.

2.4. Determination of enzyme activities

Dehydrogenase activity was determined with iodonitrotetrazolium violet (INT) as substrate, after incubation of the soil samples with 1 M TRIS-HCl buffer (pH 7.5) at 40 ºC for 1 h. The iodonitrotetrazolium formazan (INTF) produced was extracted with a 1:1 (v:v) mixture of ethanol and dimethylformamide, and measured spectrophotometrically at 490 nm (Camiña et al., 1998). The activity was quantified by reference to a calibration curve obtained using INTF standards incubated with soil under the same conditions as described above, and is expressed as µmol INTF g^{-1} h^{-1}.

Catalase activity was determined according to the method of Johnson and Temple (1964), including the modifications reported by Trasar-Cepeda et al. (1999). The soil samples were

incubated with 8.8 mM H_2O_2 for 10 min, and the residual H_2O_2 was subjected to peroxidase-catalyzed decomposition, yielding O_2, which through oxidative coupling with 4-aminoantipyrine and phenol, forms a coloured product that absorbs at 505 nm (Trasar-Cepeda et al., 1999). The activity is expressed as mmol H_2O_2 consumed g^{-1} h^{-1}.

The activity of urease was determined as described by Nannipieri et al. (1980). Briefly, urease activity was determined with urea as substrate, after incubation of the soil samples with 0.2 M phosphate buffer (pH 8.0) and 37 ºC for 1.5 h and measurement of the NH_4^+ released with an ammonia-selective electrode. The enzymatic activity is expressed in µmol NH_3 g^{-1} h^{-1}.

Acid phosphomonoesterase activity was determined with *p*-nitrophenyl phosphate as substrate, after incubation at pH 5.0 (maintained with Modified Universal Buffer following the procedure described by Trasar-Cepeda et al., 1985) and 37 ºC. After 30 min, 2 M $CaCl_2$ was added (to avoid the coloration caused by organic matter), and the *p*-nitrophenol released was extracted with 0.2 M NaOH and measured spectrophotometrically at 400 nm (Tabatabai and Bremner, 1969; Saá et al., 1993). ß-glucosidase activity was determined as described for phosphomonoesterase activity, except that the substrate was *p*-nitrophenyl-ß-D-glucopyranoside, the incubation time was 1 h and the *p*-nitrophenol released was extracted with 0.1 M THAM-NaOH (pH 12) (Eivazi and Tabatabai, 1988). The phosphomonoesterase and β-glucosidase activities were both quantified by reference to calibration curves constructed for *p*-nitrophenol (PNP) standards incubated with soil under the same conditions as described above, and are expressed in µmol PNP g^{-1} h^{-1}.

For each soil, the results for each enzyme, dose and type of contaminant were expressed as percentages of the values in the corresponding control soil (dose 0).

2.5. Statistical analysis

All determinations were performed in triplicate, and for each soil sample the mean value of the three determinations in the replicate samples were calculated. Calculation of means, deviations, multiple regression analysis, etc. were performed with Statistica 6.0 (StafSoft®) for Windows (StatSoft Inc., 2001).

The significance of the differences between means (dose effect and soil type effect) were tested by the analysis of variance (ANOVA) and by Student's t test.

3. Results

3.1. General soil properties and soil enzyme activities

The mean pH of the calcareous soils (both in water and in KCl) was obviously higher than that of the acidic soils. For both types of pH measurements the differences between the mean values for the two groups of soils were slightly more than 2 units, and were significant at $P<0.05$ (Table 1). In contrast, the concentrations of total C, and of amorphous Fe_2O_3 and Al_2O_3 extracted from the acidic soils were higher than those extracted from the calcareous soils, with a difference of almost 1% in the mean concentration of Al_2O_3 in acidic and calcareous soils (Table 1).

As regards the texture, silt fraction was significantly more predominant in the calcareous soils than in the acidic soils. In the acidic soils, the texture was mainly sandy loam, whereas in the soils developed on limestone the texture was silt loam (Table 1).

Except for the catalase activity, which was generally higher in the calcareous soils, the other enzyme activities were generally higher in the acidic soils. In all cases, and independently of the pH of the soils, the deviations within each group were very high, and only the differences in the mean values of the phosphomonoesterase activity were significant (P<0.05) (Table 1).

3.2. Effect of 2,4-DCP on enzyme activities

The effect of 2,4-DCP on enzymatic activities was generally slight, even at the highest dose used (1000 mg kg^{-1}). For both the acidic and calcareous soils, the dehydrogenase and urease activities were the most strongly affected and followed similar patterns in both groups of soils, with a significant (P<0.05) decrease in activity as the dose of contaminant increased (Table 2). For both enzymes, the reduction in the enzyme activity with the dose of contaminant was greater in the calcareous soils than in the acidic soils, although for each dose the difference between acidic and calcareous soils was not significant (P < 0.05) for either of these enzymes.

Independently of the dose of contaminant, the ß-glucosidase and phosphomonoesterase activities were scarcely affected in either group of soil, as the largest mean decrease (ß-glucosidase in the acidic soils) was equivalent to only 6% of the activity with respect to that of the control soils (Table 2). Furthermore, the catalase behaviour was anomalous, as it was affected to a greater extent in the acidic soils than in the calcareous soils (non significant differences). Thus, although a mean value of 89% of the control activity was reached in the calcareous soils at the maximum dose added, in the acidic soils, the mean value was only 69%. However, the values of the catalase activity in the acidic soils were the most variable (largest deviations), so that the decreases were not significant either in relation to the dose (only in the acidic soils, the mean values for the higher dose was significantly different from that obtained for the intermediate dose) or with respect to the group of soils considered in relation to pH (Table 2).

3.3. Effect of 2,4,5-TCP on enzyme activities

In general, the effect of 2,4,5-TCP on the soil enzyme activities was higher than that of 2,4-DCP. Similarly to the latter compound, the activities most affected were dehydrogenase and urease, and the least affected were ß-glucosidase and phosphomonoesterase (Table 2).

In both groups of soils, the dehydrogenase and urease activities were clearly and significantly affected in relation to the dose (P<0.05; Table 2), so that the highest dose (5000 mg kg^{-1}) caused the almost complete disappearance of both enzyme activities in many of the soils (particularly in the calcareous soils). For both enzymes, the acidic soils were less affected than the calcareous soils, although the differences were only significant (P<0.05) for dehydrogenase (Table 2). Thus at the highest dose of 2,4,5-TCP used, on average only 27% of the dehydrogenase activity remained, and 22% of the urease activity remained in acidic soils, whereas in the calcareous soils, the levels of activity were 5 and 9% for dehydrogenase and urease respectively (Table 2).

The decreases in the ß-glucosidase and phosphomonoesterase activities with increasing dose of 2,4,5-TCP were much lower than those undergone by the dehydrogenase and urease activities (Table 2). In the calcareous soils, the ß-glucosidase and phosphomonoesterase activities were 73-74% of those measured in the controls for the dose of 5000 mg kg^{-1}, being

these differences significant at $P<0.05$ (Table 2), whereas in the acidic soils the phosphomonoesterase activity was not affected and the mean ß-glucosidase activity was, for this dose, 83% of that in the control soils (Table 2).

The changes in the catalase activities in relation to the dose were scarce and very similar in the acidic and calcareous soils, and at the highest dose of the compound, the activity was approximately 70% of the control values in acidic soils and 67% of the control values in the calcareous soils (Table 2). However, despite the scarce difference in this activity in response to contamination, the effect of the dose was significant in both groups of soils, due to the low deviations obtained (Table 2).

4. Discussion

The results clearly show that the activities of the various enzymes considered and, in some cases both groups of soils, responded differently to 2,4-DCP and 2,4,5-TCP contamination. To facilitate the interpretation, we will discuss the results on the basis of the following three points: *i)* sensitivity of the enzymatic activities to chlorophenols, *ii)* influence of the type of chlorophenol on enzymatic activities, and *iii)* influence of soil properties on the toxicity of chlorophenols.

4.1. Sensitivity of soil enzymatic activities to chlorophenols

For both groups of soils and for both contaminants, catalase, ß-glucosidase and phosphomonoesterase activities were generally not affected, or were only slightly affected, independently of the dose of contaminant added to the soils. The response of these activities was very different to that shown by the dehydrogenase and urease activities, which, for both cholorophenols and in both groups of soils, decreased with increasing dose of contaminant added to the soil. This parallel response was highlighted by the fact that for both chlorophenols, the correlations between the reduction in the urease and in dehydrogenase activities ($r= 0.76$ and $r=0.84$, $P<0.001$, for 2,4 DCP 2,4,5-TCP, respectively) were higher than the correlations between the reduction in any other of the enzyme activities measured (r values lower than 0.57). It is important to emphasize that the behaviour of catalase activity was surprisingly different from that of dehydrogenase activity. Both are intracellular enzymes, which are considered to reflect microbial activity (Skujins, 1976; Nannipieri, 1994), so that if the toxic products affect the edaphic microbiota, the activities of both enzymes (dehydrogenase and catalase) might be expected to undergo similar reductions. However, the decreases in dehydrogenase activity were not accompanied by similar reductions in catalase activity, because for the same dose of contaminant, the decrease in catalase activity was always less than the decrease in dehydrogenase activity (Table 2). The lack of any change in catalase activity in response to contaminants has been interpreted as being the result of an abiotic-type reaction capable of decomposing the hydrogen peroxide used as a substrate for catalase and thus masking the behaviour of the enzyme (Bello et al., 2008). The possibility of abiotic catalysis of H_2O_2 has been considered by other researchers (Skujins, 1976) and creates the problem (also widely reported) that catalase activity cannot be considered a clear indicator of soil contamination (Gil-Sotres et al., 2005).

Among the hydrolytic enzymes assayed, only urease responded clearly to the presence and dose of contaminants. One possible explanation for this behaviour is the different source and location of these enzymes in the soils. The hydrolytic activity in soils is associated both with the production of enzymes by active organisms in the presence of substrate, and with the activity of enzymes stabilized by soil colloids (Ladd, 1978; Burns, 1982). It is therefore possible that the urease in the soils under study is basically derived from live organisms, whereas the other two hydrolytic enzymes (ß-glucosidase and phosphomonoesterase) are mainly found in the stabilized fraction. Thus, as the edaphic microorganisms die as a result of the toxic effects of the contaminant, the urease activity would disappear, whereas the stabilized activities would scarcely be affected by the contamination. Nevertheless, the urease activity has been described by several authors as being largely stabilized on the colloidal soil components (Burns et al., 1972; Nannipieri et al., 1978), and there is no reason to consider that the same does not occur in the present case. It must be considered that although the changes in urease and dehydrogenase activities appeared to be very similar, the reduction in urease activity as a result of the presence of chlorophenols was generally lower than that of dehydrogenase, as reflected by the slope of the regression line obtained for urease compared with that obtained for dehydrogenase (0.94 for 2,4,5-TCP and 0.85 for 2,4-DCP) (Fig 1 a and b). Furthermore, for soils contaminated with 2,4-DCP and those contaminated with 2,4,5-TCP, the regressions yielded y_0 (ordinate at $x = 0$) values of 13 and 15%, respectively, which represent the mean proportion of urease that remains active when the dehydrogenase activity has totally disappeared (Fig 1 a and b). As various authors have reported (West et al., 1988; Nannipieri et al., 1996), such values may reflect the presence of a stabilized fraction of urease that is highly resistant to contaminants. However, at the highest doses of 2,4,5-TCP added to the soils, the urease activity in many of the samples (independently of the pH of the soil) reached values very close to 0% of the activity of the corresponding control (Fig 1 b), which suggests that the fraction of the enzyme that does not depend directly on microbial activity (i.e. the stabilized fraction) has been damaged by the presence of the contaminant. This, in turn, suggests that the active centre of the urease may be inactivated by the contaminants, thus impeding the hydrolytic action of the enzyme. If such inactivation of the active centre does actually occur, the effect of the chlorophenols on soil functioning should not only be considered in relation to their toxicity, but also in relation to the damage suffered by the enzymes stabilized by the soil colloids. Inactivation of extracellular ureases stabilized by soil colloids has been reported by other authors in studies of contamination of soil by heavy metals (Moreno et al., 2001).

4.2. Influence of the type of chlorophenol on enzymatic activities

In those cases in which there was a large reduction in enzymatic activity (dehydrogenase and urease), 2,4,5-TCP had the greatest effect (Table 2). Although this may be a consequence of the larger quantities of 2,4,5-TCP than of 2,4-DCP used to contaminate the soils (maximum doses of 5000 mg kg^{-1} for 2,4,5-TCP, and of 1000 mg kg^{-1} for 2,4-DCP), it has also been suggested that this may also be related to the number of Cl atoms in the benzene ring (Bello et al., 2008; Bello et al., 2011), as in chlorinated derivatives the toxicity generally increases with the number of Cl atoms in the molecule (Liu, 1981). The legislation (Real Decreto 9/2005; European Directive on Contaminated Soils, 2006) does not appear to have taken this

characteristic into account, and the GRLs are much lower for 2,4-DCP than for 2,4,5-TCP (0.1 mg kg^{-1} and 10 mg kg^{-1}, respectively). As the legislation uses the concept of GRL to define a soil as contaminated, the doses used in relation to the GRL must be indicated in order to enable comparison of the effects of the different compounds on the soil. The maximum amounts of 2,4-DCP and 2,4,5-TCP used represent 10000 and 500 times the GRL respectively. Although the amount of 2,4,5-TCP added was much lower in terms of the GRL, this compound clearly had a much stronger effect on the soils than 2,4-DCP. The GRLs of these compounds may have been considered in the legislation, not because of any real effect on the soil, but because of some physicochemical parameter of the compound, e.g. its solubility in water. Such oversimplification may lead to errors in the definition of soils as contaminated. This is illustrated by the results of the present study, as doses of 2,4-DCP as high as 1000 times the GRL (100 mg kg^{-1} of 2,4-DCP, the lowest dose used here) scarcely affected the biochemical properties of the soils.

4.3. Influence of soil properties on the toxicity of chlorophenols

Both 2,4-DCP and 2,4,5-TCP affected the calcareous soils to a greater extent than the acidic soils (Table 2). These results are consistent with the previously mentioned idea that the effects of the compounds increase with soil pH (Díez et al., 1999). The effect of pH on the action of chlorophenols is explained by considering that it is the phenolate anion (derived from the dissociation of chlorophenol) that is actually toxic to the microbiota. Because of its charge and relatively small size, the phenolate anion can cross cell membranes, and once inside the cell cytoplasm, its presence will cause the death of microorganisms (Packham et al., 1982; Bello et al., 2008). As the pK_a values for the dissociation of 2,4-DCP and 2,4,5-TCP are respectively 7.9 and 6.9 (Westall et al., 1985; Severtson and Banerjee, 1996), the proportion of dissociated forms will be higher in the soils developed over limestone (pH in water between 6.5 and 8.0) than in the acidic soils (pH in water between 4.2 and 5.9), for both of the chlorophenols, which explains the greater decrease in the enzyme activity in calcareous than in acidic soils. This interpretation may also explain the different toxicity of the chlorophenols because in all of the soils under study, there should always be a higher concentration of the phenolate anion in the soils contaminated with 2,4,5-TCP than in the soils contaminated with 2,4-DCP, as the pK_a of 2,4,5-TCP is lower than that of 2,4-DCP.

However, acidic soils are not only differentiated from calcareous soils by pH, but also by total carbon content (Table 1). The total carbon contents in soils derived from acidic parent material were between 1.98 and 11.19% (mean value 5.24%), whereas in soils derived from limestone, the total carbon contents ranged between 1.07 and 6.06% (mean value 2.97%). In many acidic soils, the organic matter content is one of the main factors that determines the resistance of such soils to degradation by chlorophenols, as the soil organic matter may be capable of adsorbing part of the chlorophenol added, thus impeding its presence in the soil solution and making dissociation difficult (or even totally impeding it). The same hypothesis has also been proposed in other studies (Díez et al., 1999; Cea et al., 2007, Bello et al, 2008). Furthermore, since the structure of the soil organic matter may be different under acid conditions than under conditions in which the calcium carbonate is present (Duchaufour, 1976; Stevenson, 1982), the greater effect observed in the calcareous soil may be the result of the combined effect of soil pH, organic matter content and the characteristics of the organic matter.

Stepwise multiple correlation analysis of the enzyme activities that clearly changed after the addition of the chlorophenols (i.e. dehydrogenase and urease), the dose of chlorophenol added and the general soil properties, revealed highly significant multiple correlation coefficients (Table 3), and also that the order in which the different variables intervened was generally the same, independently of the contaminant applied. The variable that best explained the percentage variation was always the dose, followed by the total carbon content of the soil, and finally the pH, although for the dehydrogenase activity in the soils contaminated with 2,4,5-TCP, the effect of pH was slightly higher than that of the carbon content. Higher correlation coefficients were obtained when all soils were considered together than when calcareous and acidic soils were considered separately (data not shown). This indicates that the type of organic matter does not have any appreciable effect on the soil response, but that the most important factor determining the response of soils to chlorophenols is the total carbon content. Therefore, the soils most resistant to contamination with these products are always the soils under forest vegetation, in which carbon contents of up to 11% can be reached in the surface horizon (Trasar-Cepeda et al., 2008). It was also seen that other properties related to soil components capable of participating in adsorption processes (clay, extractable oxides) did not participate in the multiple correlation, or when they did, they always accounted for very low, non significant percentages of variation. Contrary to our expectations, pH had much less effect than the organic matter, and for some activities and contaminants (2,4-DCP), the effect of pH in regard to reducing enzyme activity was only significant at $P<0.05$.

Although it is difficult to determine the mechanisms responsible for the toxicity of chlorophenols in soils (Peuravuori et al., 2002), the results obtained in the present study suggest that the main process that regulates the toxicity of these compounds is their adsorption by organic matter, as this would limit the concentration of the contaminant in the soil solution. In fact, experiments carried out in our laboratory (unpublished data) with the A horizon of a strongly acidic soil (pH in water 3.9, i.e. a pH at which chlorphenols are scarcely dissociated) and with a high content of organic matter (13% total C) contaminated with either 1000 mg kg^{-1} of 2,4-DCP or with the same dose of 2,4,5-TCP revealed an adsorption capacity of 98.5% for 2,4-DCP and of 81.5% for 2,4,5-TCP. Adsorption on organic matter would limit the presence of large quantities of phenolate in the soil solution, which at least partly explains the low sensitivity of soils under study to the toxic action of the chlorophenols. The fact that the undissociated compound was strongly adsorbed by the organic matter is not surprising, as both contaminants are sparingly soluble in water and the values of the octanol/water partition coefficient ($\log K_{ow}$ = 3.06 for 2,4-DCP and 4.10 for 2,4,5-TCP) reflect their hydrophobicity and their tendency to bind to non polar surfaces such as soil organic matter, so that the distribution between the aqueous phase and the adsorbed phase would be approximately 1:1000 (McBride, 1994).

We are currently investigating the adsorption capacity of soils containing different amounts and types of organic matter, for these chlorophenol compounds, by determining adsorption isotherms that will enable estimation of the adsorption parameters. We are also carrying out other studies to elucidate how these compounds affect soil biochemical properties.

5. Conclusions

The various enzyme activities investigated showed different sensitivities to the presence of 2,4-DCP and 2,4,5-TCP. The enzymes least affected were ß-glucosidase and phosphomonoesterase, and the most strongly affected were dehydrogenase and urease.

The lack of response of the ß-glucosidase and phosphomonoesterase activities suggests that the chlorophenols used in the study only act on edaphic microorganisms and not on the enzymes stabilized on soil colloids. However, the large reduction in urease activity (enzyme considered to be largely stabilized on soil components) suggests that chlorophenols may inhibit the activity of urease by inactivating their active centre.

Independently of the soil properties, 2,4-DCP is much less toxic and affects the biochemical soil properties to a much lesser extent than 2,4,5-TCP. This shows that the GRLs outlined in the legislation regarding these compounds are not appropriate, as the GRLs indicated for 2,4-DCP are much lower than those indicated for 2,4,5-TCP.

The effects of the chlorophenols on the biochemical properties that are sensitive to these compounds are less intense at lower soil pH and in soils with a high organic matter content. The main process that regulates the effect of chlorophenols on the biochemical properties of soil is the absorption of the compounds on soil organic matter, and dissociation of the chlorophenol that remains in soil solution is less important.

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Table 1. Mean values and standard deviations for the general soil properties and enzyme activities in the acidic soils (n = 17) and in the calcareous soils (n = 13). For each property, mean values followed by the same lower case letter are not significantly different (P<0.05).

	Acidic soils	**Calcareous soils**
pH in water	5.27±0.51*a*	7.38±0.52*b*
pH in KCl	4.22±0.43*a*	6.59±0.61*b*
Total C (%)	5.24±2.64*a*	2.97±1.70*b*
Total N (%)	0.33±0.18*a*	0.30±0.15*a*
Amorphous Fe_2O_3 (%)	0.79±0.30*a*	0.44±0.26*b*
Amorphous Al_2O_3 (%)	1.28±1.01*a*	0.28±0.28*b*
Sand (%)	64±14*a*	34±10*b*
Silt (%)	19±11*a*	53±9*b*
Clay (%)	17±4*a*	13±4*b*
Dominant texture	sandy loam	silt loam
Dehydrogenase (µmol INTF g^{-1} h^{-1})	0.42±0.24*a*	0.35±0.20*a*
Catalase (µmol H_2O_2 consumed g^{-1} h^{-1})	1.25±0.75*a*	3.07±1.64*a*
Urease (µmol NH_3 g^{-1} h^{-1})	11.34±13.02*a*	6.6±5.50*a*
ß-glucosidase (µmol PNP g^{-1} h^{-1})	0.96±0.47*a*	0.75±0.33*a*
Phosphomonoesterase (µmol PNP g^{-1} h^{-1})	4.08±2.89*a*	1.29±1.14*b*

Table 2. Mean percentage variations (relative to control) in the enzyme activities in acidic and calcareous soils after the addition of 2,4-DCP and 2,4,5-TCP. The same capital letters indicate that for each enzyme and dose, the behaviour of both groups of soils was not significantly different (P<0.05). The same lower case letters indicate that there were no significant differences (P<0.05) in the responses to the different doses of the compounds.

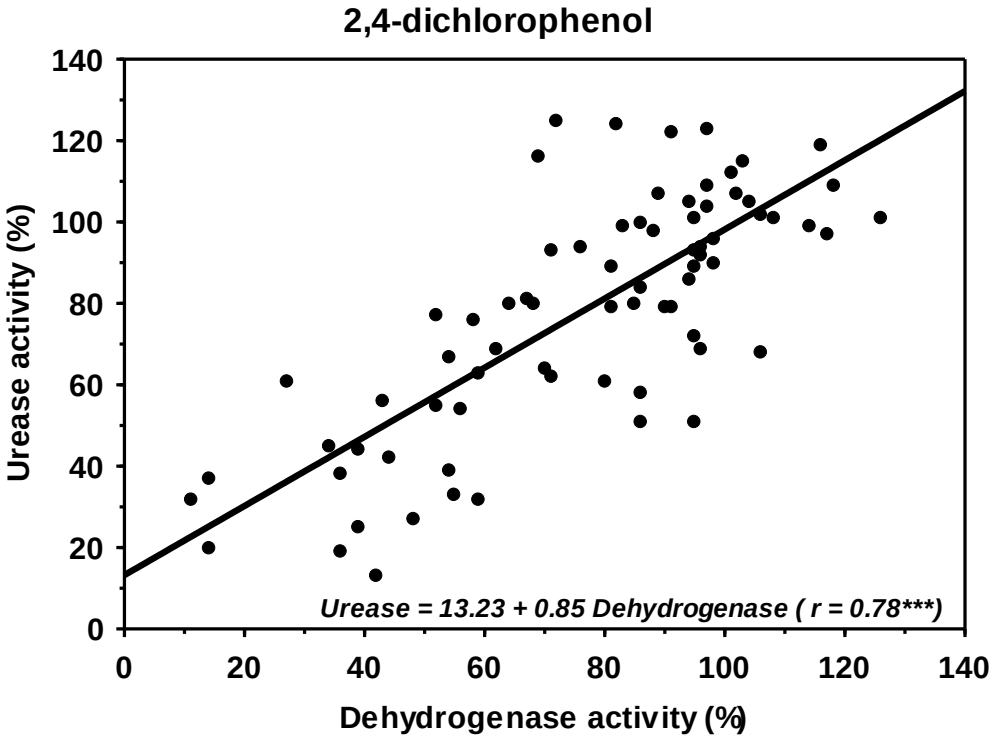
Dose	**Dehydrogenase**		**Catalase**		**Urease**		**ß-glucosidase**		**Phosphomonoesterase**	
	Acidic	***Calcareous***	***Acidic***	***Calcareous***	***Acidic***	***Calcareous***	***Acidic***	***Calcareous***	***Acidic***	***Calcareos***
2,4-DCP										
100	99±11*aA*	94±16*aA*	108±29*aA*	96± 6*aA*	104± 9*aA*	95±15*aA*	97± 6*aA*	103±10*aA*	98± 8*aA*	99±18*aA*
500	84±15*bA*	84±19*aA*	94±28*aA*	96± 9*aA*	83±26*bA*	90±23*aA*	95± 8*aA*	105±13*aB*	100± 8*aA*	97±13*aA*
1000	56±17*cA*	44±26*bA*	69±40*bA*	89±14*aA*	55±28*cA*	43±21*bA*	94±10*aA*	96±19*aA*	100± 8*aA*	97±12*aA*
2,4,5-TCP										
100	97±12*aA*	84±17*aB*	106±*24aA*	97±5*aA*	104±12*aA*	95±10*aA*	100± 9*aA*	96± 8*aA*	*96*±2*aA*	94± 9*aA*
500	70±16*bA*	46±13*bB*	89±26*abA*	98±5*aA*	85±22*bA*	80±19*aA*	92±10*bA*	90±11*abA*	99±12aA	90±11*aA*
1000	56±19*cA*	21±16*cB*	83±30*bA*	88±11*bA*	68±28*bA*	47±21*bA*	92±12*bA*	87± 9*bA*	102± 9aA	90±17*aA*
5000	28±20*dA*	5± 5*dB*	70±28*bA*	67±12*cA*	22±20*cA*	9± 6*cA*	83±19*bA*	73±15*cA*	96±13aA	74±17*bB*

Table 3. Multiple linear regression (forward stepwise) between percentages of enzyme activities (dependent variable) and dose and soil variables (independent variables). The independent variables are indicated in order of importance, and the sign of the regression coefficient is indicated in brackets. R^2, multiple regression coefficient.

Activity	**Variables in order of importance**					**R^2**
	1st	**2nd**	**3rd**	**4th**	**5th**	
2,4-DCP (n = 90)						
Dehydrogenase	Dose (-)	Total C (+)				0.58***
Urease	Dose (-)	Total C (+)				0.54***
2,4,5-TCP (n = 120)						
Dehydrogenase	Dose (-)	pH in water (-)	Total C (+)	Al_2O_3 (+)		0.68***
Urease	Dose (-)	Total C (+)	pH in water (-)	Al_2O_3 (+)	Fe_2O_3 (+)	0.76***

* $P<0.05$; **, $P< 0.01$; ***, $P<0.001$

a)



b)

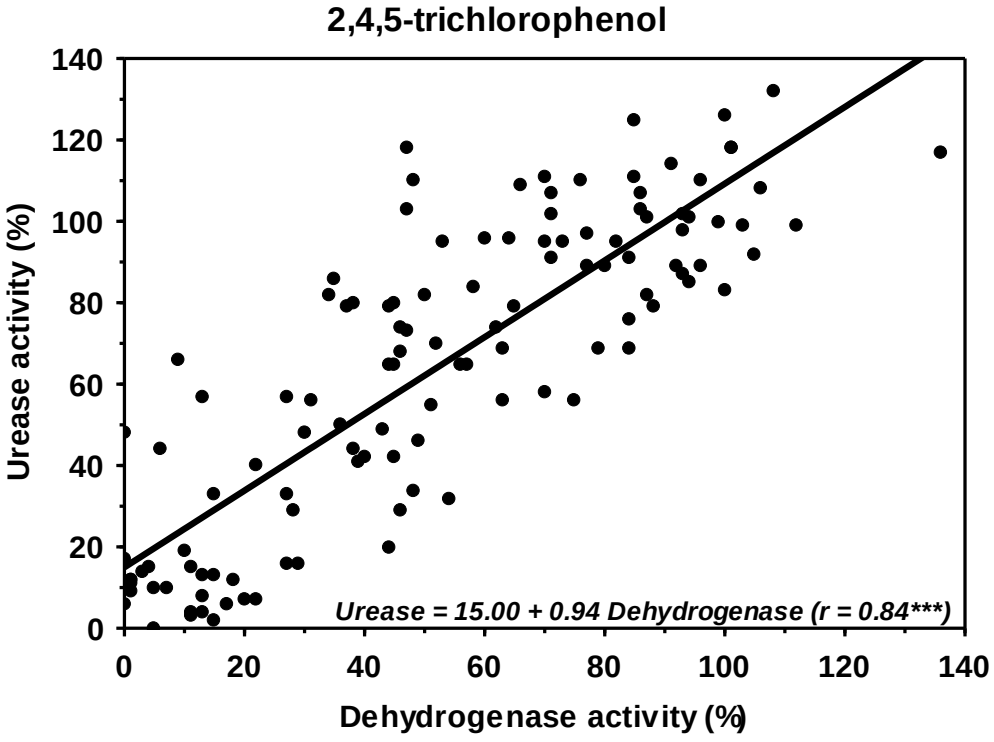


Fig. 1. Relationship between urease and dehydrogenase activities (expressed as percentages relative to controls) for soil samples contaminated with a) 2,4-DCP and b) 2,4,5-TCP (all soils and soil doses, except controls).