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HIGHLIGHTS

- Degradation rate decreased with increasing pesticide concentration.
- The lowest dose of pesticides did not cause changes in soil microbial communities.
- Higher pesticide concentration did not consistently increase impact on microorganisms.
- Pesticides increased soil NO_3^-, suggesting beneficial effect on the bacteria involved.

Keywords:
Ammonium-oxidizing bacteria
Denitrification
Nitrification
Soil enzymes
Soil quality
Soil health

ABSTRACT

An experiment was performed to study non-target effects of difenoconazole (fungicide), deltamethrin (insecticide) and ethofumesate (herbicide) on microbial parameters in a clay-loam soil. Pesticides were applied as commercial formulations to soil samples at different concentrations (5, 50 and 500 mg kg^{-1} DW soil) and then incubated under laboratory conditions for 3 months. Throughout the incubation period, microbial parameters were determined at days 7, 30, 60 and 90. At 5 mg kg^{-1} DW soil, none of the three pesticides caused significant changes in soil microbial parameters. In contrast, at 500 mg kg^{-1} DW soil, pesticide application decreased overall soil microbial activity, negatively affecting the activity of soil enzymes. Similarly, at 500 mg kg^{-1} DW soil, difenoconazole and ethofumesate, but not deltamethrin, caused a pesticide-induced stress on soil microbial communities, as reflected by the respiratory quotient. Besides, deltamethrin and ethofumesate at 50 and 500 mg kg^{-1} DW soil resulted in lower values of denitrification potential. It was concluded that, although pesticide concentration had a somewhat inconsistent and erratic effect on soil microbial parameters, pesticide application at 500 mg kg^{-1} DW soil did have an impact on many of the microbial parameters studied here.

1. Introduction

As a result of the use and/or misuse of agricultural pesticides, pesticide contamination is nowadays an environmental problem of great concern. A considerable amount of the applied pesticides frequently ends up in the soil, where it can undergo biological and physicochemical transformations. Once in the soil, microbial degradation is the main route of pesticide removal (Bending et al., 2006). Pesticide application can result in harmful effects on non-target organisms, including soil microorganisms, with adverse consequences for soil quality (Johnsen et al., 2001; Niemi et al., 2009).

Microorganisms play a key role in many soil processes and the delivery of essential soil ecosystem services (Jeffery et al., 2010). Microbial parameters reflecting the biomass, activity and diversity of soil microbial communities are useful indicators of the impact of disturbances (including pesticide application) on soil quality (Epelde et al., 2009; Garbisu et al., 2011). In particular, microbial parameters that provide information on the soil nitrogen cycle have been reported to be very sensitive to pesticide application (Ahtiainen et al., 2003).

Abbreviations: DW, dry weight; Q_R, respiratory quotient; N_{min}, potentially mineralizable nitrogen; H', Shannon's diversity; T-SQI, treated-soil quality index.

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Depending on several factors (*e.g.*, pesticide composition, soil type, soil physicochemical and biological properties), pesticides frequently have slow rates of degradation in the soil environment. In consequence, repeated application of pesticides can ultimately lead to their accumulation at concentrations detrimental to soil microorganisms (Munier-Lamy and Borde, 2000; Rice et al., 2002).

In the present work, three scarcely studied pesticides were used:

(1) The fungicide difenoconazole (1-[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-ylmethyl]-1*H*-1,2,4-triazole): a systemic sterol demethylation inhibitor used against Ascomycetes, Basidiomycetes and Deuteromycetes. Its recommended field application rate is 75–125 g active ingredient (ai) ha^{-1} (300–500 mL ha^{-1} of a 25% formulation).
(2) The insecticide deltamethrin ((*S*)-cyano(3-phenoxyphenyl)methyl (1*R*,3*R*)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate): a disruptor of intracellular sodium channels used against Homoptera, Lepidoptera, Diptera, and Coleoptera. Its recommended field application rate is 7.5–17.5 g ai ha^{-1} (300–700 mL ha^{-1} of a 2.5% formulation).
(3) The herbicide ethofumesate (2-ethoxy-2,3-dihydro-3,3-dimethyl-5-benzofuranyl methanesulfonate): its mechanism of action is the inhibition of lipid biosynthesis. Ethofumesate is used against some grasses (Gramineae) and Dicotyledones. Its recommended field application rate is 750–1000 g ai ha^{-1} (1.5–2.0 L ha^{-1} of a 50% formulation).

In our study, commercial formulations of these pesticides were applied to soil samples, under laboratory conditions, at different concentrations (5, 50 and 500 mg ai kg^{-1} DW soil) in order to (i) determine their dissipation kinetics and (ii) assess their impact on soil microbial parameters. The recommended approach for assessing the effects of pesticides on soil microbial communities includes the simultaneous measurement of multiple ecological end-points (Zabaloy et al., 2008) and, thus, throughout the 3-month experiment, several microbial parameters with potential as bioindicators of soil quality were determined at regular intervals: basal respiration, substrate-induced respiration, potentially mineralizable nitrogen (N_{min}), nitrification rate, diversity of ammonium-oxidizing bacteria, denitrification potential and enzyme activities (dehydrogenase, β-glucosidase, urease, arylsulfatase, alkaline phosphatase). Finally, from the values of the enzyme activities, the treated-soil quality index (T-SQI) proposed by Mijangos et al. (2010) was calculated.

2. Materials and methods

2.1. Soil characterization

Soil (top 0–25 cm) was collected from an area belonging to the unsaturated riparian zone of the Salburua wetland, located in the Vulnerable Zone of the Quaternary Aquifer of Vitoria-Gasteiz (northern Spain). This area was not subjected to pesticide application for the last 15 years. In particular, soil analysis by GC–MS (see below) did not reveal any of the three pesticides studied here.

Immediately after collection, soil samples were taken to the laboratory in dark plastic bags, homogenized, air-dried at 25 °C during 48 h, sieved to <2 mm, and subjected to physicochemical characterization according to Sparks et al. (1996). The soil is of a Chernozem calcic character (FAO) with a clay-loam texture (sand–clay–silt: 29.8–38.7–31.5%), a pH of 8.3 (1:2.5 w/v in water), 17.0 g organic C kg^{-1} DW, 2.3 g total N kg^{-1} DW, a C/N ratio of 7.8 and an electrical conductivity of 0.18 dS m^{-1}.

2.2. Experimental design

The three pesticides studied here (difenoconazole, deltamethrin, ethofumesate) are intensively used in the abovementioned Vulnerable Zone, as they are included in the Code of Good Agricultural Practices for sugar beet cultivation in this agricultural area. Although these pesticides are normally applied on the plants themselves, soil applications were here conducted for research purposes only, not in accordance with agricultural practice.

Pesticides were applied as commercial formulations since adjuvants and surfactants present in such formulations may affect both pesticide degradation rates and their impact on soil microorganisms (Beigel et al., 1999). Thus, our soil was subjected to the following commercial formulations: Score® is an emulsifying concentrate containing 25% of difenoconazole, Audace® is an emulsifying concentrate containing 2.5% of deltamethrin; and Kemitran® is a concentrated emulsion with 50% of ethofumesate.

A three-month mesocosm study was carried out as previously described in Muñoz-Leoz et al. (2011). For each pesticide concentration (5, 50 and 500 mg ai kg^{-1} DW soil), a set of four replicated mesocosms was prepared by transferring samples of 4 kg DW soil (homogenized, air-dried, sieved soil from the 0–25 cm top layer; see above) to 10 L plastic trays, resulting in a soil layer of approximately 10 cm depth. Each soil sample was treated with the abovementioned commercial formulations diluted in deionized water, at a rate of 5–50–500 mg ai kg^{-1} DW soil and 60% water holding capacity (WHC).

Pesticide-treated soil samples were thoroughly mixed with a rotary mixer (Philips handmixer, HR1570) to assure uniform pesticide distribution. Trays were then covered with perforated polypropylene sheets and incubated in the dark at 22 ± 1 °C, in order to minimize evaporative losses of water from soil and avoid photodegradation of pesticides. Throughout the incubation period, water content was held constant by the weekly addition of deionized water.

From each mesocosm, samples of 250 g fresh weight (FW) soil were collected from the trays after 0, 7, 30, 60 and 90 days of incubation. Subsequently, soil samples were sieved (<2 mm) and stored at 4 °C until analysis.

2.3. Pesticide concentration

Soil samples for pesticide determination were randomly taken from different locations within the tray and then mixed to get a composite sample. The concentration of pesticide residues was quantified through two successive extractions with acetonitrile (firstly) and isopropanol (secondly), followed by GC–MS analysis as described in Muñoz-Leoz et al. (2012). Pesticide recovery from soil samples was >99%.

2.4. Soil microbial parameters

Throughout the incubation period, the impact of pesticide application on soil quality was assessed using a variety of soil microbial indicators. Soil microbial basal (R_B) and substrate-induced (SIR) respiration were determined following ISO 16072 Norm-2002 and ISO 17155 Norm-2002, respectively. The respiratory quotient Q_R, or the ratio of basal respiration to substrate-induced respiration ($Q_R = R_B/SIR$) was also calculated (Anderson and Domsch, 1985).

Urease activity was determined according to Kandeler and Gerber (1988). Arylsulfatase, β-glucosidase, alkaline phosphatase and dehydrogenase activities were determined according to Dick (1997) and Taylor et al. (2002), as described in Epelde et al. (2008) and Rodríguez-Loinaz et al. (2008).

Potentially mineralizable nitrogen (N_{min}) was assessed following Powers (1980). For nitrification rate, nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) concentrations in soil were determined following Sparks et al. (1996). Denitrification potential was determined according to a modified method of Šimek et al. (2002): 75 mg $N\text{-}NO_3^-$ kg^{-1} DW soil and 75 mg C-glucose kg^{-1} DW soil (in 10 mL of deionized water) were added to 10 g DW soil placed in 120 mL serum bottles with a helium atmosphere containing 10% v/v acetylene. After 24 h of incubation at

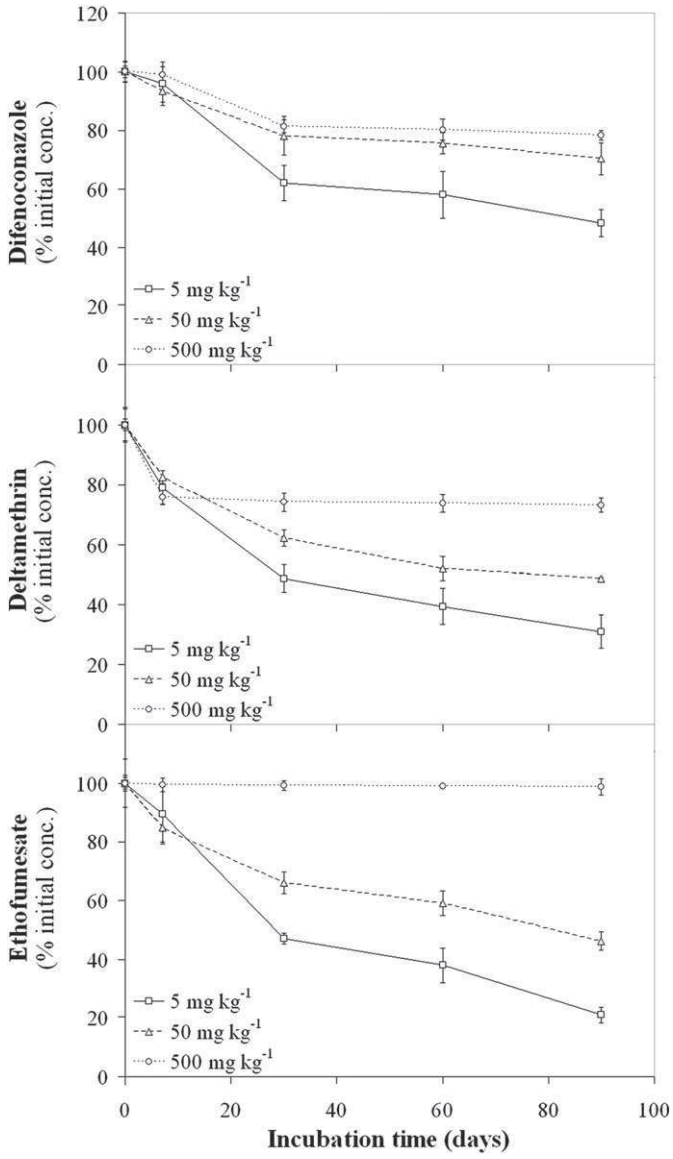

Fig. 1. Pesticide (difenoconazole, deltamethrin, ethofumesate) concentration in soil throughout the experiment. Pesticide concentration at a given incubation time is expressed as % of initial pesticide concentration. Mean values (n = 4) ± S.D.

25 °C in a rotary shaker, N-N_2O production was measured by gas chromatography (KNK 3000 HRGC) using a thermal conductivity detector and a Porapak Q 80/100 3 m × 1/8″ (Sugelabor) packed column. Operation conditions were as follows: column temperature, 25 °C; injection temperature, 25 °C; detector temperature, 150 °C; and helium as carrier gas at a flow rate of 16 mL min^{-1}.

Regarding microbial diversity parameters, in the soil, *amoA* gene copies of Crenarchaeota (Archaea) can be up to 3000-fold more abundant than bacterial *amoA* genes (Leininger et al., 2006). However, in our unsaturated N-rich clay-loam soil, ammonium oxidation is expected to be functionally dominated by bacteria rather than Archaea, as previously reported for N-rich grassland soils (Di et al., 2009; Jia and Conrad, 2009; Schleper and Nicol, 2010). Therefore, we estimated diversity of ammonium-oxidizing bacteria through PCR-DGGE analysis according to Avrahami et al. (2003). DNA was extracted from soil samples (0.25 g FW soil) using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, California, USA) following manufacturer's instructions. Diversity of ammonium-oxidizing bacteria was estimated using the Shannon's diversity index ($H' = -\sum p_i \log_2 p_i$), where p_i is the ratio between specific band intensity and total intensity of all bands in a lane sample after subtracting the background of each lane.

2.5. Data analyses

Statistical analyses were performed using SPSS Software (SPSS 17, SPSS Inc., 2010). Data on soil parameters were analyzed using a three-way analysis of variance (ANOVA), with pesticide concentration, type of pesticide and incubation time as factors. Differences between controls and samples treated with different concentration of pesticides, at a given incubation time, were compared using one-way ANOVA and Fisher's PLSD post-hoc test. Values were considered to be significantly different at $P<0.05$. Pearson's correlations and principal component analysis (PCA) were performed to establish relationships among soil parameters, and regression equations were assessed to evaluate relationships between microbial parameters and pesticide concentration at each incubation time. STATISTICA 6.0 Software (Statsoft Inc., 2004) was used to fit experimental data on pesticide degradation to a bi-exponential model.

From the values of the enzyme activities, the treated-soil quality index (T-SQI) proposed by Mijangos et al. (2010) was calculated at each incubation time:

$$\mathrm{T-SQI} = 10\log m + \frac{\sum_{i=1}^{n}(\log n_i - \log m) - \sum_{i=1}^{n}|\log n_i - \log \overline{n}|}{n}$$

where m is the reference (mean value of enzyme activity in the control untreated "reference" soil at each incubation time, set to 100%) and n is the measured values for each enzyme activity as percentages of the reference. This index takes into account (i) the magnitude of the increment of each enzyme activity, compared to the value for that specific enzyme activity shown by the reference soil (100%; first Σ of the numerator) and (ii) the maintenance of the evenness

Table 1
Kinetic parameters of pesticide dissipation in soil for difenoconazole, deltamethrin and ethofumesate.

Pesticide	Concentration (mg kg^{-1} DW)	*A*	*k*1 (d^{-1})	*B*	*k*2 (d^{-1})	$t_{1/2}$ (d)	r^2
Difenoconazole	5	2.6	0.0375	2.6	0.0008	84.9	0.983
	50	39.8	0.0013	10.5	0.0643	362	0.995
	500	263.1	−0.0035	245.2	0.0217	546	0.975
Deltamethrin	5	2.3	0.0783	2.7	0.0061	29.0	1.000
	50	31.9	0.0031	18.0	0.0804	78.0	0.999
	500	372.4	0.0002	127.6	0.4032	1381	1.000
Ethofumesate	5	1.9	0.0473	3.2	0.0116	28.5	0.994
	50	39.7	0.0057	10.4	0.1331	81.2	0.997
	500	304.8	0.0014	194.4	−0.0017[a]	5923	0.960

Pesticide dissipation in soil was described by a bi-exponential model [$PC(t) = A \cdot e^{(-k1 \cdot t)} + B \cdot e^{(-k2 \cdot t)}$, where $PC(t)$ = pesticide concentration at t time; A and B = constants; $k1$ and $k2$ = dissipation kinetic constants for the first and second component of the curve; t = time]. $t_{1/2}$ = half-life or time required for a 50% dissipation of initial pesticide concentration.
[a] Although mathematically speaking a negative value was obtained, it does not make sense from a kinetics point of view.

Table 2
Analysis of variance for soil microbial parameters as affected by type of pesticide (P), pesticide concentration (C), incubation time (T) and corresponding interactions. Non-significant values ($P > 0.05$) were excluded.

Factor	$N\text{-}NO_3^-$	$N\text{-}NH_4^+$	N_{min}	DEN	DEH	Q_R	T-SQI	H'-AOB
P	0.020	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
C	<0.001	<0.001	0.009	<0.001	<0.001	<0.001	<0.001	–
T	<0.001	<0.001	<0.001	0.009	<0.001	0.007	<0.001	–
P×C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–
P×T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.023	–
C×T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–
P×C×T	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.018	–

$N\text{-}NO_3^-$, nitrate concentration; $N\text{-}NH_4^+$, ammonium concentration; N_{min}, potentially mineralizable nitrogen; DEN, denitrification potential; DEH, dehydrogenase activity; Q_R, respiratory quotient; T-SQI, treated-soil quality index; H'-AOB, Shannon's index of ammonium-oxidizing bacteria diversity.

among the enzyme activities shown by the reference soil (second Σ of the numerator).

3. Results and discussion

3.1. Pesticide concentration

The evolution of pesticide concentration in soil fitted more accurately to a bi-exponential kinetic model [$PC(t) = A \cdot e^{(-k1 \cdot t)} + B \cdot e^{(-k2 \cdot t)}$, where $PC(t)$ = pesticide concentration at t time; A and B = constants; $k1$ and $k2$ = dissipation kinetic constants for the first and second component of the curve; and t = time] than to classical first-order models (Fig. 1). The biphasic pattern of degradation can be attributed to different adsorption sites or increased sorption over time: at the beginning of the experiment, microorganisms were able to use the pesticides as C source for growth as a fraction of the added pesticides was still in the dissolved phase and/or weakly adsorbed, to then become strongly adsorbed to clay and organic matter, thus resulting in lower bioavailability and slower degradation (Lee et al., 2004; Muñoz-Leoz et al., 2011). The additives present in commercial formulations that increase solubility or dispersion of the pesticides are also expected to modify their sorption. This might explain the lack of relationship between pesticide persistence in soil and their adsorption coefficients ($K_{oc} = 1.02 \times 10^7$, 600 and 97.8 mL g^{-1} for deltamethrin, difenoconazole and ethofumesate, respectively) (PPDB, 2012).

In soils treated with 5 mg pesticide kg^{-1} DW, by the end of the incubation, pesticide concentration was reduced by 52, 69 and 89% for difenoconazole, deltamethrin and ethofumesate, respectively, with corresponding half-life values of 84.9, 29.0 and 28.5 days (Table 1). On the other hand, higher values of half-life time were observed at increasing pesticide concentrations. Regarding ethofumesate, no significant dissipation was observed at the highest concentration tested. Our half-life values at 5 mg kg^{-1} DW are in accordance with those observed by Muñoz-Leoz et al. (2012) for the same commercial formulations and clay-loam soil. Our results also agree with those reported by other authors for difenoconazole (Guo et al., 2010) and ethofumesate (Siimes et al., 2006) when added as commercial formulations, and for deltamethrin (Roberts, 1998) when applied as active ingredient.

As abovementioned, microbial degradation has been reported as the main factor responsible for pesticide dissipation in soils (Bending et al., 2006). In this respect, the three pesticides studied here have a low vapor pressure and are insensitive to hydrolysis at the pH value of our experimental soil (PPDB, 2012). The capacity of soil microbial communities to degrade pesticides has been found to be reduced by increasing concentrations of fungicides (Chen and Edwards, 2001; Wang et al., 2009) and herbicides like alachlor (Felsot and Dzantor, 1995), but not deltamethrin (Muñoz-Leoz et al., 2009). The sudden lower deltamethrin degradation found at 500 mg kg^{-1} DW from day 7 could be associated with non-biological causes. A concentration of additives may have resulted in a drop of pesticide sorption into the

Table 3
Relationships between microbial parameters (P; for units, see figures) and pesticide concentration (C; in mg kg^{-1} DW soil) at each incubation time.

Parameter	Day	Difenoconazole		Deltamethrin		Ethofumesate	
		Regression	r^2	Regression	r^2	Regression	r^2
$N\text{-}NO_3^-$	7	$P = 1.89 \cdot C + 26.14$	0.652	$P = -8.66 \cdot C + 45.03$	0.933	$P = 3.60 \cdot C + 20.34$	0.941
	30	$P = 6.87 \cdot C + 7.65$	0.891	$P = 15.82 \cdot C - 20.95$	0.830	$P = 13.71 \cdot C - 16.58$	0.993
	60	$P = 12.77 \cdot C - 1.96$	0.907	$P = 29.97 \cdot C - 51.93$	0.833	$P = 19.33 \cdot C - 24.95$	0.999
	90	$P = 12.57 \cdot C - 5.19$	0.985	$P = 27.33 \cdot C - 44.83$	0.900	$P = 20.55 \cdot C - 27.92$	1.000
$N\text{-}NH_4^+$	7	$P = -0.28 \cdot C + 9.60$	0.379	$P = 8.32 \cdot C - 11.47$	0.930	$P = 3.34 \cdot C - 2.99$	0.773
	30	$P = -0.18 \cdot C + 5.20$	0.666	$P = -0.38 \cdot C + 5.27$	0.451	$P = -0.02 \cdot C + 4.35$	0.050
	60	$P = -0.06 \cdot C + 3.91$	0.239	$P = -1.15 \cdot C + 6.67$	0.743	$P = -0.039 \cdot C + 3.99$	0.078
	90	$P = 0.04 \cdot C + 3.32$	0.301	$P = -1.12 \cdot C + 6.15$	0.812	$P = -0.107 \cdot C + 3.82$	0.660
N_{min}	7	$P = -1.80 \cdot C + 37.38$	0.793	$P = 8.88 \cdot C + 20.47$	0.892	$P = -3.60 \cdot C + 44.49$	0.752
	30	$P = 0.44 \cdot C + 3.18$	0.744	$P = 2.48 \cdot C - 0.36$	0.682	$P = 0.23 \cdot C + 4.66$	0.072
	60	$P = 0.42 \cdot C + 3.03$	0.960	$P = 0.18 \cdot C + 4.43$	0.965	$P = 1.32 \cdot C + 1.18$	0.693
	90	$P = -1.64 \cdot C + 11.35$	0.913	$P = -1.60 \cdot C + 10.92$	0.947	$P = -1.14 \cdot C + 10.97$	0.970
DEN	7	$P = 11.26 \cdot C + 14.09$	0.774	$P = -6.26 \cdot C + 42.23$	0.947	$P = -3.19 \cdot C + 33.90$	0.513
	30	$P = 18.96 \cdot C - 15.16$	0.707	$P = -5.97 \cdot C + 41.33$	0.993	$P = -8.23 \cdot C + 48.98$	0.944
	60	$P = 11.32 \cdot C + 5.05$	0.808	$P = -6.65 \cdot C + 42.95$	0.912	$P = -6.45 \cdot C + 45.30$	0.913
	90	$P = 9.73 \cdot C + 10.16$	0.917	$P = -8.02 \cdot C + 52.02$	0.832	$P = -3.47 \cdot C + 39.43$	0.532
DEH	7	$P = -24.55 \cdot C + 162.63$	0.890	$P = -26.01 \cdot C + 191.96$	0.342	$P = -5.28 \cdot C + 143.51$	0.632
	30	$P = -6.71 \cdot C + 46.09$	0.962	$P = -10.93 \cdot C + 64.36$	0.532	$P = -0.15 \cdot C + 39.18$	0.001
	60	$P = -12.06 \cdot C + 77.18$	0.978	$P = -12.72 \cdot C + 77.41$	0.894	$P = -5.10 \cdot C + 56.01$	0.911
	90	$P = -8.13 \cdot C + 60.29$	0.658	$P = -6.15 \cdot C + 65.46$	0.186	$P = -18.46 \cdot C + 95.29$	0.984
Q_R	7	$P = 0.10 \cdot C + 0.03$	0.977	$P = 0.01 \cdot C + 0.21$	0.132	$P = 0.01 \cdot C + 0.24$	0.073
	30	$P = 0.12 \cdot C - 0.04$	0.981	$P = 0.01 \cdot C + 0.23$	0.001	$P = 0.063 \cdot C + 0.09$	1.000
	60	$P = 0.06 \cdot C + 0.14$	0.671	$P = 0.01 \cdot C + 0.15$	0.285	$P = 0.09 \cdot C - 0.01$	0.988
	90	$P = 0.03 \cdot C + 0.19$	0.871	$P = 0.02 \cdot C + 0.15$	0.551	$P = 0.10 \cdot C - 0.02$	0.997
T-SQI	7	$P = -12.81 \cdot C + 130.69$	0.822	$P = -16.61 \cdot C + 131.15$	0.982	$P = 0.26 \cdot C + 97.08$	0.015
	30	$P = -19.54 \cdot C + 143.17$	0.790	$P = -27.47 \cdot C + 155.84$	0.868	$P = -7.38 \cdot C + 112.67$	0.992
	60	$P = -20.88 \cdot C + 139.78$	0.907	$P = -25.03 \cdot C + 140.33$	0.864	$P = -11.04 \cdot C + 110.61$	0.732
	90	$P = -16.82 \cdot C + 137.24$	0.913	$P = -25.07 \cdot C + 149.08$	0.945	$P = -15.17 \cdot C + 128.51$	0.971
H'-AOB	7	$P = 0.16 \cdot C + 1.07$	0.246	$P = 0.32 \cdot C + 0.19$	0.655	$P = -0.03 \cdot C + 1.60$	0.047
	30	$P = -0.01 \cdot C + 1.25$	0.000	$P = 0.23 \cdot C + 0.37$	0.997	$P = -0.19 \cdot C + 2.44$	0.703
	60	$P = 0.14 \cdot C + 1.13$	0.172	$P = -0.32 \cdot C + 2.05$	0.831	$P = -0.36 \cdot C + 2.64$	0.996
	90	$P = 0.16 \cdot C + 0.72$	0.544	$P = 0.12 \cdot C + 0.59$	0.134	$P = -0.28 \cdot C + 2.38$	0.415

soil matrix, with a concomitant reduction in its bioavailability (Krogh et al., 2003; Crouzet et al., 2010).

Finally, during the quantification of pesticide concentration by GC–MS, degradation metabolites were not determined. In this respect, deltamethrin degradation products, dibromovinyl chrysanthemic acid and phenoxybenzoic acid, have been reported to be less toxic than the active ingredient (Grant et al., 2002); on the other hand, no information has been found regarding the toxicity of difenoconazole (triazolylalanine and triazolylacetic acid) (Lucini et al., 2009) and ethofumesate (2,3-dihydro-2-hydroxy-3,3-dimethyl-5-benzofuranyl methanesulfonate) (Kawahigashi et al., 2002) degradation products on soil microbial communities.

3.2. Pesticide impact on soil microbial parameters

The nature and concentration of pesticides are among the main factors affecting the existence and extent of non-target effects (Chen et al., 2001). In our study, the impact of pesticides on microbial parameters was dependent upon type of pesticide, pesticide concentration and incubation time (Table 2). Significant interactions between these three factors were found for dehydrogenase activity, Q_R, N_{min}, N-NH_4^+, N-NO_3^-, denitrification potential and T-SQI. However, diversity of ammonium-oxidizing bacteria only showed dependence upon type of pesticide.

Table 3 shows the relationships between microbial parameters and pesticide concentration at each incubation time. All pesticides showed a more adverse effect on dehydrogenase activity at higher concentrations (Table 3). Higher values of Q_R were obtained at higher difenoconazole and ethofumesate concentrations. By contrast, pesticide concentration had an erratic effect on N_{min}, N-NH_4^+ and H'-AOB. On the other hand, a direct relationship was observed between pesticide concentration and N-NO_3^- concentration. Finally, an inverse relationship was found between pesticide concentration and values of the T-SQI.

Dehydrogenase activity showed higher values at day 7 than at days 30, 60 and 90 (Fig. 2A). In soils treated with 5 and 50 mg kg^{-1} DW, pesticides had no clear effect on dehydrogenase activity. At 500 mg kg^{-1} DW, significantly lower values of dehydrogenase activity were found at all incubation times for both difenoconazole- and deltamethrin-treated soils (on average, 53.6 and 52.4% lower, respectively, compared to controls); similarly, 500 mg ethofumesate kg^{-1} DW soil resulted in significantly lower values of this enzyme activity at days 60 and 90. Both stimulation and inhibition of dehydrogenase, as a result of pesticide application, has been reported (Zabaloy et al., 2008; Crouzet et al., 2010; Muñoz-Leoz et al., 2011). Dehydrogenase activity is a good indicator of overall microbial activity in soil as it occurs only in viable cells but not in stabilized soil complexes (Nannipieri et al., 2002). Then, at 5 and 50 mg kg^{-1} DW, pesticides appear to have no clear effect on overall soil microbial activity. However, at 500 mg kg^{-1} DW, overall microbial activity in soil at days 60 and 90 was negatively affected by the three pesticides.

Regarding Q_R (Fig. 2B), at 5 mg pesticide kg^{-1} DW soil, no clear differences were observed between pesticide-treated and control soils. However, higher values of Q_R were generally found in samples treated with 50 and 500 mg kg^{-1} of difenoconazole and ethofumesate compared to untreated soils. In addition, in difenoconazole-treated soils, higher Q_R values were observed at 500 *versus* 50 mg kg^{-1} DW at days 7, 30 and 60; likewise, in ethofumesate-treated soils, higher Q_R values were observed at 500 *versus* 50 mg kg^{-1} DW at days 60 and 90. The respiratory quotient is an ecophysiological index which can

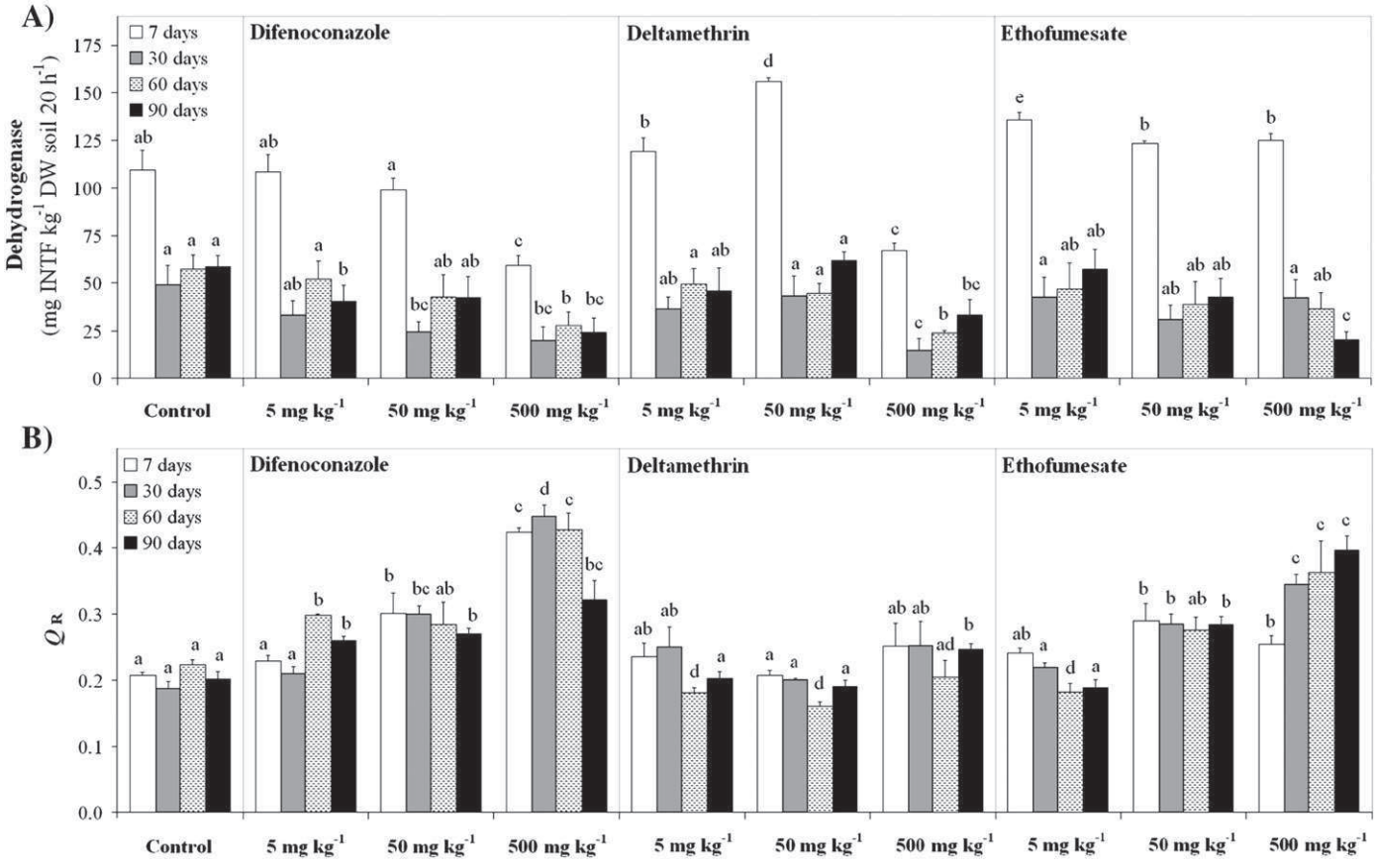

Fig. 2. Effect of pesticides (difenoconazole, deltamethrin, ethofumesate) at 5, 50 and 500 mg kg^{-1} DW soil on (A) dehydrogenase activity and (B) the respiratory quotient (Q_R: ratio of basal respiration to substrate-induced respiration). Mean values (n = 4) ± S.D. Different letters indicate statistically significant differences among treatments according to Fisher's PLSD test at each incubation time.

reflect environmental stress in microbial communities (Anderson and Domsch, 1985). Indeed, a higher respiratory activity related to the size of microbial biomass (SIR is an indicator of active microbial biomass) can reflect stressing conditions for microbial communities, forcing them to use a higher amount of their energetic resources for maintenance and survival, leading to a lower incorporation of organic C into microbial biomass (Anderson and Domsch, 1985).

N_{min} values (an indicator of biologically active soil N) were higher at day 7 than at days 30, 60 and 90 (Fig. 3A). This might be due to the fact that, during sample pre-treatment, soil homogenization and sieving induce the release of easily available nutrients from the breaking down of soil aggregates, resulting in a flush of N and C mineralization (Franzluebbers, 1999), as reflected here by N_{min} and dehydrogenase activity values. Pesticide application at 5 mg kg^{-1} DW had no effect on N_{min}. By contrast, at day 7, the addition of 50 and 500 mg kg^{-1} DW led to significantly lower values of N_{min} in difenoconazole- and ethofumesate-treated soils, and significantly higher values in deltamethrin-treated soils. It might be hypothesized that deltamethrin and/or adjuvants present in its commercial formulation might act as an available source of N- and C-compounds for N-mineralizing microorganisms (Devare et al., 2007; Mijangos et al., 2009). Nonetheless, these N-mineralizing microorganisms could, in some way, be damaged by the presence of difenoconazole and ethofumesate, especially at high concentrations (Černohlávková et al., 2009). At the highest concentration, significantly lower values of N_{min} were observed at day 90 for all three pesticides.

Nitrification and ammonification are closely related processes. Importantly, the simultaneous measurement of NH_4^+ and NO_3^- concentration can be used as an indicator of disruption in soil N transformations (Černohlávková et al., 2009). Except for ethofumesate at 5 mg kg^{-1} DW, all soils treated with pesticides showed significantly higher values of N-NH_4^+ concentration at day 7, compared to untreated controls (Fig. 3B). In deltamethrin-treated soils, at day 7, significantly higher values of N-NH_4^+ concentration were observed at 500 *versus* 50 mg kg^{-1} DW. In deltamethrin-treated samples, lower values of N-NH_4^+ concentration were observed at 500 *versus* 50 mg kg^{-1} DW at days 60 and 90. On the other hand, difenoconazole had a stimulatory effect on N-NO_3^- concentration (Fig. 4A). Similarly, at days 30, 60 and 90, values of N-NO_3^- concentration in soils treated with 50 and 500 mg kg^{-1} DW of deltamethrin and ethofumesate were higher than in controls. At these last three sampling times, values of N-NO_3^- concentration in pesticide-treated soils were higher at 500 *versus* 50 mg kg^{-1} DW. The higher values of N-NH_4^+ observed at day 7 in treated *versus* untreated soils could be interpreted as pesticide-induced inhibition of nitrification, as generally reported for pesticides (Černohlávková et al., 2009; Cycoń et al., 2010; Muñoz-Leoz et al., 2011). However, as reflected by N-NO_3^- values, nitrification appears stimulated by the presence of pesticides, particularly at the highest concentration. Alternatively, the higher N-NH_4^+ values might be due to pesticide-induced stimulation of ammonification, resulting from the mineralization of organic compounds present in pesticide formulations or in dead microbial biomass from microorganisms negatively affected by pesticide application (Monkiedje et al., 2007). In any case, other pyrethroid insecticides such as λ-cyhalothrin have been found to stimulate both N mineralization and nitrification (Cycoń et al., 2006; Devare et al., 2007).

Difenoconazole and ethofumesate increased the diversity of ammonium-oxidizing bacteria (*H′*-AOB) (Fig. 5). Nitrifying bacteria are very sensitive to pesticide application (Sáez et al., 2003). When comparing our results with those obtained with other pesticides, the herbicide atrazine at 10 mg kg^{-1} DW was found to increase AOB population abundance; by contrast, at 100 and 1000 mg kg^{-1} DW, atrazine induced a marked decrease of such abundance (Chang et al., 2001). Cycoń et al. (2006) observed that, at high concentrations, the insecticide

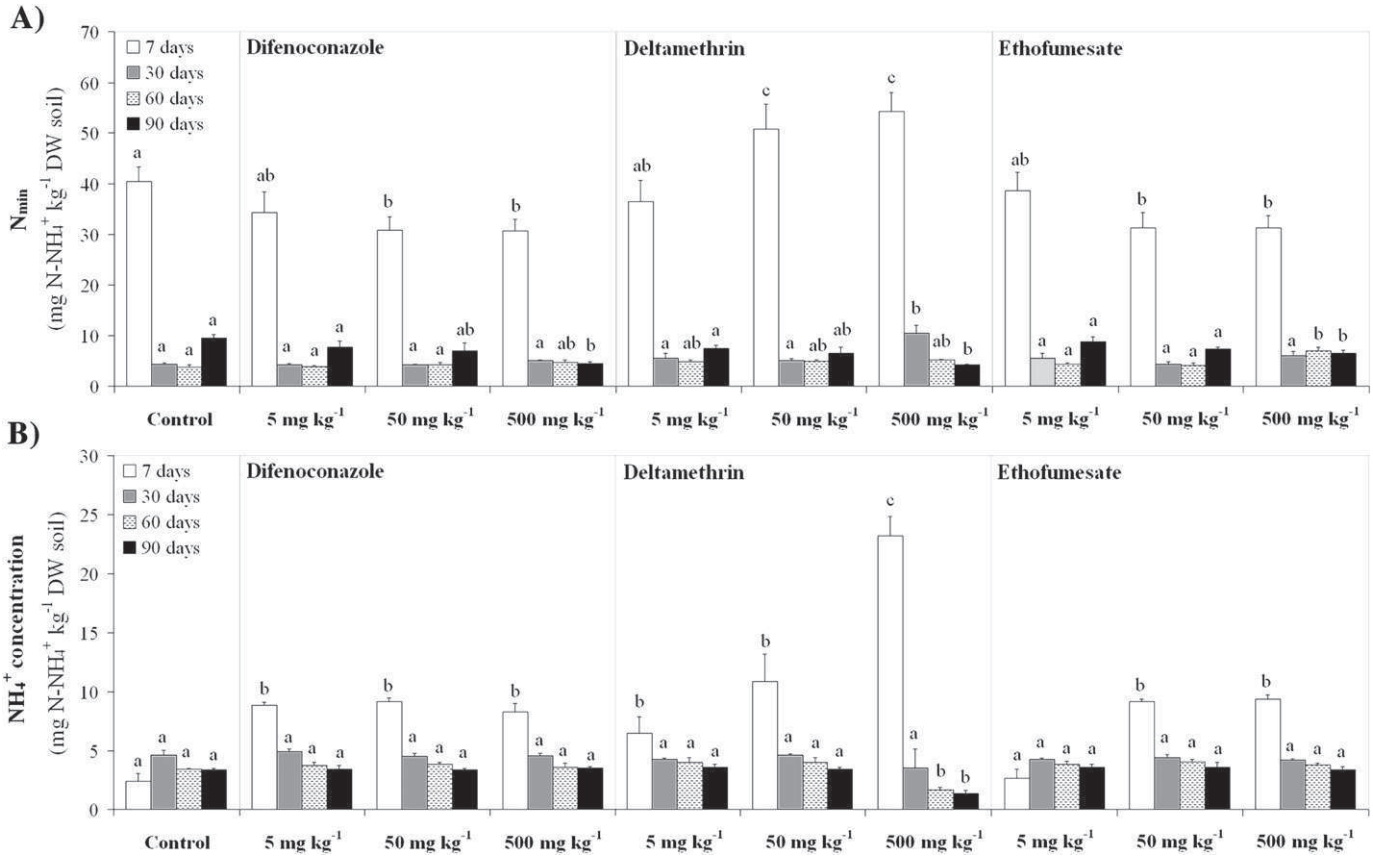

Fig. 3. Effect of pesticides (difenoconazole, deltamethrin, ethofumesate) at 5, 50 and 500 mg kg^{-1} DW soil on (A) potentially mineralizable nitrogen (N_{min}) and (B) N-NH_4^+ concentration. Mean values (n = 4) ± S.D. Different letters indicate statistically significant differences among treatments according to Fisher's PLSD test at each incubation time.

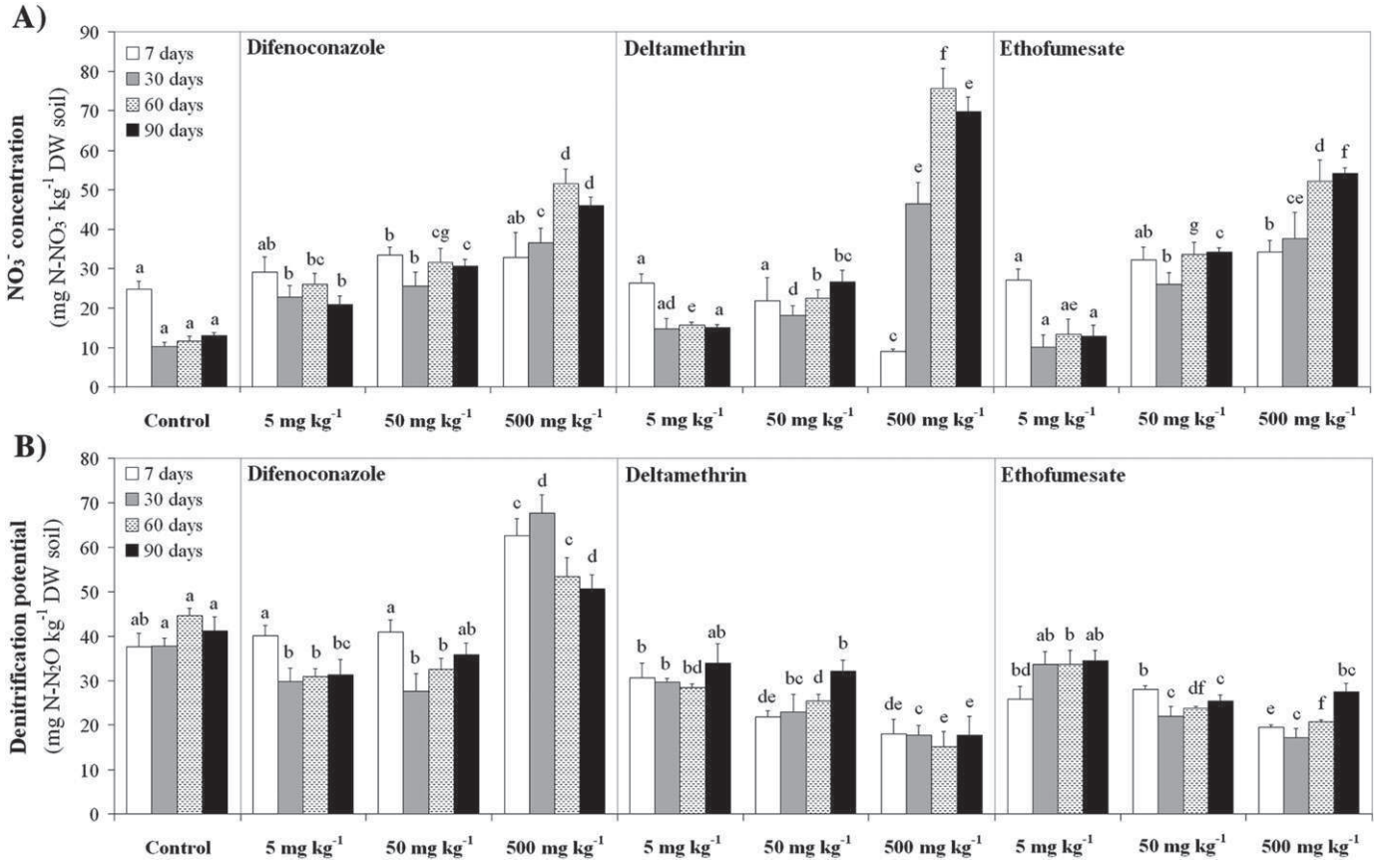

Fig. 4. Effect of pesticides (difenoconazole, deltamethrin, ethofumesate) at 5, 50 and 500 mg kg^{-1} DW soil on soil (A) $N\text{-}NO_3^-$ concentration and (B) denitrification potential. Mean values ($n=4$) ± S.D. Different letters indicate statistically significant differences among treatments according to Fisher's PLSD test at each incubation time.

λ-cyhalothrin had negative effects on nitrifying bacteria, while the application of the fungicide tebuconazole resulted in a stimulation of nitrifying bacteria.

Deltamethrin and ethofumesate at 50 and 500 mg kg^{-1} DW resulted in lower values of denitrification potential (Fig. 4B). On the contrary, difenoconazole at 500 mg kg^{-1} DW increased denitrification potential at all sampling times (however, at 5 and 50 mg kg^{-1} DW, lower values were observed at the three last sampling times). Pesticide impact on denitrification depends on many factors such as soil properties, incubation conditions, type of pesticide, specific adjuvants present in commercial formulations, *etc.* K_{oc} of pesticides conditions their fixation to soil organic matter and, hence, their non-target effects on microorganisms. However, here no correlation was found among K_{oc} of pesticides and their subsequent impact on denitrification potential. On the other hand, solvents and other compounds present in commercial formulations might act as alternative C sources for denitrifying microorganisms, which could explain the high values of denitrification potential observed in difenoconazole-treated soils at the highest concentration. In any case, most of published studies deal with pesticides applied as active ingredients, thus not taking into consideration the effects of the adjuvants and surfactants present in commercial formulations. For example, both deltamethrin-induced stimulation and inhibition of denitrification has been reported by Widenfalk et al. (2004) and Muñoz-Leoz et al. (2009), respectively. Yeomans and Bremner

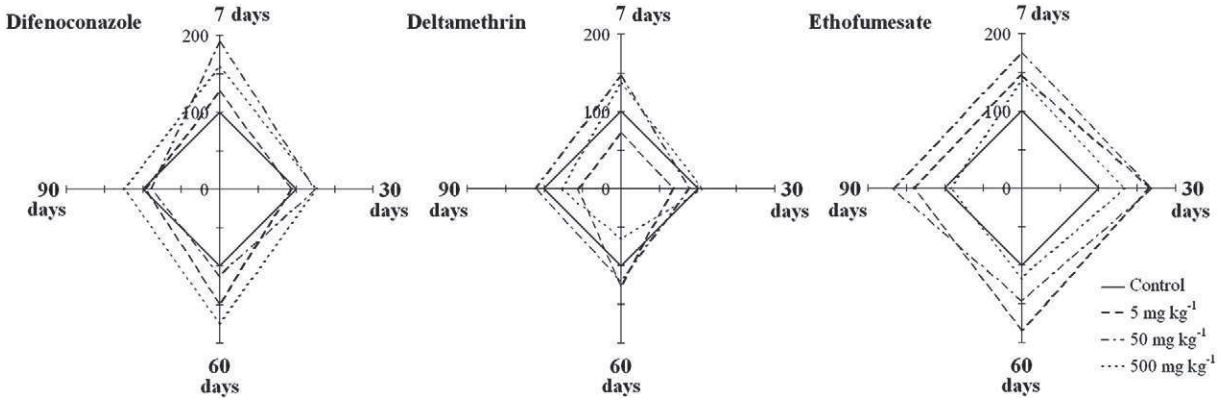

Fig. 5. Effect of pesticides (difenoconazole, deltamethrin, ethofumesate) at 5, 50 and 500 mg kg^{-1} DW soil on diversity of ammonium-oxidizing bacteria (as reflected by the values of the H' index from data of the *amoA*-DGGE analysis). Values of H' in control untreated soil at each incubation time were used as reference samples (set to 100%). H' values in these control soils were: 0.971 (day 7), 1.190 (day 30), 1.059 (day 60) and 1.156 (day 90).

Table 4
Effect of difenoconazole, deltamethrin and ethofumesate on the treated-soil quality index (T-SQI) at 7, 30, 60 and 90 days of incubation in the presence of 5, 50 and 500 mg pesticide kg^{-1} DW soil.

Time (days)	Pesticide	Pesticide concentration (mg kg^{-1})		
		5	50	500
7	Difenoconazole	101.6 ± 1.7^{a}	99.2 ± 1.7^{a}	76.0 ± 4.8^{b}
	Deltamethrin	96.6 ± 2.3^{a}	89.3 ± 5.8^{ab}	63.4 ± 5.0^{c}
	Ethofumesate	96.4 ± 7.0^{a}	100.3 ± 1.4^{a}	96.9 ± 1.2^{a}
30	Difenoconazole	98.3 ± 7.3^{ab}	96.2 ± 3.4^{a}	59.2 ± 6.5^{c}
	Deltamethrin	94.7 ± 7.1^{ab}	85.8 ± 5.0^{b}	39.8 ± 7.3^{d}
	Ethofumesate	97.5 ± 2.7^{a}	91.3 ± 2.7^{ab}	82.7 ± 4.5^{b}
60	Difenoconazole	94.2 ± 7.4^{ab}	84.9 ± 4.2^{b}	52.4 ± 2.7^{c}
	Deltamethrin	84.5 ± 6.5^{b}	76.7 ± 2.7^{b}	34.5 ± 7.5^{d}
	Ethofumesate	84.7 ± 8.9^{b}	85.2 ± 5.2^{b}	62.6 ± 5.5^{e}
90	Difenoconazole	100.6 ± 5.5^{a}	92.8 ± 6.1^{ab}	67.0 ± 0.3^{c}
	Deltamethrin	95.4 ± 3.1^{ab}	80.9 ± 3.9^{d}	45.3 ± 1.2^{e}
	Ethofumesate	96.6 ± 6.7^{ab}	86.0 ± 6.5^{bd}	66.3 ± 2.8^{c}

Mean values ($n = 4$) ± S.D. Different letters indicate statistically significant differences between treated and control "reference" samples (T-SQI = 100) at each incubation time, according to Fisher's PLSD test.

(1985a,b) observed that many pesticides did not negatively affect denitrification, but even stimulated it. By contrast, Cycoń et al. (2006) found denitrifying microorganisms to be sensitive to tebuconazole. Sáez et al. (2003) observed that some herbicides, as well as organochlorinated and organophosphorus insecticides, showed inhibitory effects on *Paracoccus denitrificans*.

The T-SQI can integrate information from different enzyme activities into one unique measure of soil functioning. At 5 mg kg^{-1} DW, pesticide application showed no clear significant effect on T-SQI values (apart from deltamethrin and ethofumesate-treated samples at day 60) (Table 4). By contrast, at 50 and 500 mg kg^{-1} DW, significantly lower values of this index were observed for many of the studied soil samples: as a general trend, it was found that the higher the pesticide concentration, the lower the T-SQI value.

On the other hand, N-NH_4^+, N_{min} and dehydrogenase activity were positively correlated among each other (Table 5). N-NO_3^- was negatively correlated with soil N-NH_4^+, N_{min} and dehydrogenase activity. The T-SQI was negatively correlated with Q_R and N-NO_3^- (these two parameters were positively correlated) and positively with dehydrogenase activity and N_{min}.

According to the PCA (Fig. 6), three principal components (PC) explained 82.6% of the total variance: PC1 (33.2% of total variance) was positively correlated with dehydrogenase activity, N_{min}, and N-NH_4^+, whereas PC2 (29.2% of total variance) was negatively correlated with N-NO_3^- and positively with T-SQI; PC3 (20.2% of total variance) was characterized by a high positive Eigen-value (>0.75) for denitrification potential and Q_R. For the PCA plot, PC1 was not considered as it did not show significant differences among treatments.

Table 5
Correlations among soil microbial parameters. Marked correlations are significant at 0.05 (*) and 0.01 (**) level of probability. Non-significant correlations ($P > 0.05$) were excluded.

	N-NO_3^-	N-NH_4^+	N_{min}	DEN	DEH	Q_R	T-SQI	H'-AOB
N-NO_3^-	1							
N-NH_4^+	−.274**	1						
N_{min}	–	.718**	1					
DEN	–	–	–	1				
DEH	−.230**	.426**	.787**	–	1			
Q_R	.482**	–	–	.265**	–	1		
T-SQI	−.683**	–	.158*	–	.447**	−.289**	1	
H'-AOB	–	–	–	–	–	–	–	1

N-NO_3^-, nitrate concentration; N-NH_4^+, ammonium concentration; N_{min}, potentially mineralizable nitrogen; DEN, denitrification potential; DEH, dehydrogenase activity; Q_R, respiratory quotient; T-SQI, treated-soil quality index; H'-AOB, Shannon's index of ammonium-oxidizing bacteria diversity.

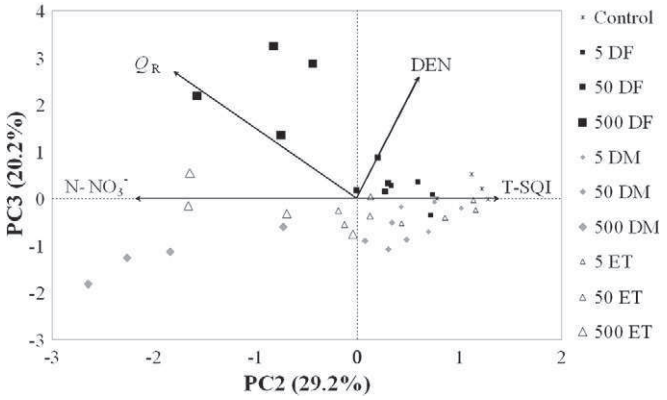

Fig. 6. Principal component analysis based on correlations between soil microbial properties and principal components (PC) 2 and 3, at the three pesticide concentrations studied here (5, 50 and 500 mg kg^{-1} DW soil). DEN, denitrification potential; DF: difenoconazole; DM: deltamethrin; ET: ethofumesate.

According to PC2 and PC3, soils treated with 5 mg kg^{-1} DW were located together with untreated controls, independently of the pesticide tested, towards the positive side of PC2; by contrast, soils treated with 50 and, specially, 500 mg kg^{-1} DW migrated to the negative side of PC2 (sample location was dependent upon pesticide type, with deltamethrin-treated soils being observed towards the positive side of PC3, while difenoconazole- and ethofumesate-treated soils were found towards the negative side of PC3).

The physiological similarity between target and non-target organisms can determine pesticide non-target effects. Not surprisingly, fungicides have been reported to induce more non-target harmful effects on soil microbial communities, compared to insecticides and herbicides (Chen et al., 2001; Cycoń et al., 2006; Muñoz-Leoz et al., 2011). However, deltamethrin and ethofumesate application also resulted in changes in the soil microbial parameters characterized in this study. We speculate that these changes could be related to non-specific effects of the active ingredients themselves as well as of the additives present in commercial formulations. Nonetheless, scarce information has been published about the impact of surfactants and adjuvants on soil microorganisms (Krogh et al., 2003; Katagi, 2008; Pereira et al., 2009). Although they can be used as C- and N-sources by soil microorganisms, some adjuvants such as alcohol ethoxylates and alkylamine ethoxylates can be toxic to microeukaryotes at low concentrations and to bacteria at high concentrations (Beigel et al., 1999; Krogh et al., 2003; Crouzet et al., 2010).

Finally, the low degradation rates found here for the three pesticides (especially, for difenoconazole) can ultimately lead to their progressive accumulation in soil as a result of repeated application. In our study, at 5 mg kg^{-1} DW, non-target effects of pesticides on soil microbial communities were limited and short-lived. As reported for other fungicides (Chen et al., 2001; Černohlávková et al., 2009), difenoconazole caused the most significant impact on soil microbial parameters (Q_R, N-NH_4^+ and N-NO_3^- concentration, denitrification potential), as its mechanism of action can also affect a wide range of soil microorganisms (including bacteria). However, at higher concentrations, non-target effects were more pronounced. This fact highlights the importance of considering increasing pesticide concentrations, due to low degradation rates and repeated applications, when assessing the environmental impact and potential non-target effects of pesticides on soil microbial communities and, hence, soil quality.

4. Conclusions

Pesticide degradation rates were dependent upon concentration: higher values of half-life time were observed at increasing pesticide concentrations. At 5 mg kg^{-1} DW none of the three pesticides caused

relevant changes in soil microbial communities. However, at higher pesticide concentrations, adverse impacts on soil microbial communities were detected. In particular, at 500 mg kg^{-1} DW soil, pesticide application decreased overall soil microbial activity, negatively affecting the activity of soil enzymes (it was found that the higher the pesticide concentration, the lower the T-SQI value). At high concentrations, difenoconazole and ethofumesate, but not deltamethrin, caused a pesticide-induced stress on soil microbial communities, as reflected by the respiratory quotient. At 500 mg kg^{-1} DW, lower values of N_{min} were observed at the end of the incubation. In turn, deltamethrin and ethofumesate at 50 and 500 mg kg^{-1} DW resulted in lower values of denitrification potential. It was concluded that, although pesticide concentration had a somewhat inconsistent and erratic effect on soil microbial parameters, pesticide application at 500 mg kg^{-1} DW soil did have an impact on many of the microbial parameters studied here.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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