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ORIGINAL ARTICLE

Linuron effects on microbiological characteristics of sandy soils as determined in a pot study

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Abstract The aim of this study was to find what dosages of linuron introduced into soil can be tolerated by microorganisms, and whether a dosage equal to 100-times higher than the predicted environmental concentration (PEC) is harmful. A pot study was performed to determine the effects of the herbicide at PEC (4 mg kg^{-1}), 5 times PEC (20 mg kg^{-1}), and 100 times PEC (400 mg kg^{-1}) on substrate-induced respiration (SIR), four enzyme activities (dehydrogenase, acid and alkaline phosphatases, and urease), ammonification and nitrification rates, plate counts of total bacteria, fungi, N_2-fixing bacteria, nitrifiers and denitrifiers, and distribution of r- and K-strategists in sandy soils of different texture throughout 28 days of incubation. Linuron increased SIR and ammonification, especially at 100 times PEC. In contrast, a decrease in nitrate concentrations was detected in both soils treated with the highest dosage of linuron. Although some changes in microbial numbers were ascertained, they were transient (i.e. total bacteria, N_2-fixing bacteria and nitrifiers) or not significant (i.e. total fungi and denitrifiers). Among the enzymes tested, dehydrogenase was the most sensitive to linuron, showing decreased activity for all treatments and two higher dosages in the loamy sand (LS) and sandy loam (SL) soils, respectively. The addition of 100 times PEC of linuron to LS resulted in the domination of slow-growing K-strategists on days 1 and 14. However, r-strategists dominated on both days 1 and 28 in SL. Our results indicate that linuron may disturb indigenous soil microorganisms, especially when released at high concentrations.

Keywords Enzyme activity · Linuron · Microbial numbers · Nitrogen transformation · r- and K-strategists · Substrate-induced respiration · Pot study

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Introduction

Modern agriculture applies million tons per year of different synthetically produced pesticides, such as insecticides, fungicides and herbicides, to control plant pests and improve food quality. As an outcome of this extensive environmental input, soils and waters have been contaminated with these compounds or intermediate metabolites formed during their biodegradation. Several laboratory and field studies have revealed that continuous application of these agrochemicals may lead to dangerous changes in the functioning of soil ecosystems. Pesticides affect microorganisms by changing their numbers, biochemical activity and functional biodiversity (Martinez-Toledo et al. 1998; Chen et al. 2001; Araújo et al. 2003; Cycoń et al. 2006; Lupwayi et al. 2009). Approaches based on application of molecular methods have indicated changes in structural diversity of microbial communities in soil exposed to different pesticides (el Fantroussi et al. 1999; Seghers et al. 2003; Valle et al. 2006; Lin et al. 2008).

The phenylurea herbicides are an important group of pesticides, and one of the most commonly used compounds is linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea]. Linuron has been used extensively in the conventional production of corn, cereals, vegetables and fruit to control annual and perennial broadleaf and grassy weeds on both crop and non-crop sites. The main mode of linuron action is the disruption of photosynthesis in target weed plants through inhibition of the enzymes involved in the photosynthetic electron transport chains (Snel et al. 1998). Moreover, this herbicide and some of its metabolites are suspected of exerting toxic effects on some aquatic and soil organisms (Caux et al. 1998). Linuron is moderately persistent and mobile in soils, with field half-life values ranging from 22 to 150 days in different soils and under various conditions (Caux et al. 1998). After its introduction into soil, many processes are involved in herbicide diminution, but the most important process responsible for linuron loss is microbial degradation through metabolic and co-metabolic pathways (Sørensen et al. 2003; Accinelli et al. 2004; Grenni et al. 2009). Dejonghe et al. (2003) isolated a *Variovorax* strain capable of degrading linuron; however, its activity was dependent on synergistic interactions with other bacteria. Results reported by Breugelmans et al. (2007) showed that degradation of linuron was performed by a group of metabolically interacting bacteria rather than by individual strains. Most linuron-degrading consortium members were identified as *Variovorax*, but a *Hydrogenophaga* and an *Achromobacter* strain capable of linuron degradation were also obtained. It would appear that interactions between consortium members are complex and can involve an exchange of both metabolites and other nutrients (Breugelmans et al. 2007).

Soil microorganisms are the first biota to undergo the direct and indirect impact of hazardous substances introduced into soil. They are recommended as more suitable indicators than other organisms or chemical parameters because they respond immediately to stressful environmental conditions (Nannipieri et al. 2002; Filip 2002). In this study, we examined microbial responses to different dosages of linuron added to two sandy soils of different texture using a range of microbiological tests, including substrate-induced respiration (SIR), soil enzyme activities, numbers of total culturable bacteria, fungi and specific groups of bacteria involved in soil nitrogen turnover. In addition, changes in the establishment of r- and K-strategists among culturable bacteria as well as changes in concentrations of ammonium and nitrate ions, indicating the herbicide effect on ammonification and nitrification in soils, were assessed.

Since herbicides are applied at different concentrations resulting in varying amounts being accumulated in soil, the response of indigenous microorganisms to these compounds is still far from being understood. The aim of this study was to find what dosages of linuron introduced into soil can be tolerated by microorganisms, and whether a dosage equal to 100-times higher than the predicted environmental concentration (PEC) in field conditions could be harmful. This latter point is of great interest, especially when one considers undesirable events such as herbicide spills in high amounts into soil, which can occur during damage of devices used to apply it, and due to uncontrolled disposal in soil of waste and water used to wash equipment as well as via transport or industrial accidents.

Materials and methods

Soils

Composite samples of two agricultural soils, prepared from ten different sub-samples taken from the areas of 25 m^2, were collected from the top layer (0–20 cm) at grass-covered fields located in two sites within the area of Pszczyna in Upper Silesia, southern Poland. The sampling places had not been used for agricultural purposes during the past 5 years; no application of pesticides, or organic and inorganic fertilisers had been used. The detailed physical and chemical characteristics of loamy sand (LS) and sandy loam (SL) soils are presented in Table 1. Particle size distribution was determined by the modified Bouyoucos areometric method (PN-ISO 11277:2005), while the pH values of the aqueous soil extracts (1:5, w/v) were measured in triplicate with a glass electrode by a Jenway 3510 pH-meter at 20°C (PN-ISO 10390:1997). The barium chloride method was used to determine soil cation exchange capacity (PN-ISO 11260:1999), and concentrations of analysed ions were estimated using a Perkin-Elmer 1100 atomic absorption spectrometer with a Mg hollow

Table 1 Selected physicochemical properties of soils: *LS* Loamy sand, *SL* sandy loam

Textural classification	LS	SL
Sand (2,000–50 μm) (%)	83	67
Silt (<50–2 μm) (%)	13	25
Clay (<2 μm) (%)	4	8
Density g cm^{-3}	1.3	1.6
pH_{H2O} (1:2.5)	6.4	6.7
Cation exchange capacity (cmol+ kg^{-1})	11.5	22.0
Water holding capacity (%)	33.60	45.80
C_{org} (%)	1.10	2.40
N_{tot} (%)	0.09	0.18
Microbial biomass (mg kg^{-1} dry weight)	648	1,075

cathode lamp (wavelength 285.2 nm, slit width 0.7 nm). The water holding capacity (WHC), organic carbon content (C_{org}) and total nitrogen content (N_{tot}) were determined by gravimetric method (PN-ISO 14239:2000), dichromate oxidation in the presence of concentrated sulphuric acid (PN-ISO 14235:2003) and the Kjeldahl method (PN-ISO 11261:2002), respectively. Soil microbial biomass was measured with the glucose-induced respiration method (PN-ISO 14240-1:2001). In the laboratory, the soil was air-dried at 18°C to about 10% moisture content and sieved (2 mm) prior to study.

Soil treatment

Phenylurea herbicide linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] (45% active ingredient), in soluble concentrate (SC) formulation, was used in this study. It was applied at three different dosages of 4, 20 and 400 mg kg^{-1} soil. The lowest dosage corresponded to the maximum predicted environmental concentration (PEC) of linuron in field conditions, assuming a homogeneous distribution of the herbicide to a depth of 5 cm and a soil density of 1.5 g cm^{-3}. Two higher dosages of linuron corresponded to 5 or 100 times of the PEC. The PEC and five-fold higher dosages are applied in routine ecotoxicological studies concerning the impact of pesticides on soil microbial activity, which are required to obtain the permission for selling and use of the plant protection compounds in practice. The highest dosage was used to evaluate the potential hazards of linuron on soil biota under accidental discharge of uncontrolled amounts of the compound to a soil.

The soils used were divided into four portions of equal weight (3,000 g) that were placed into plastic pots. Three portions of each soil were treated with corresponding concentrations of water suspension of the herbicide, and the fourth portion (control) received the same volume of water. There were three replicates of each treatment, giving a total of 24 pots in the experiment (i.e. two soils × four treatments × three replicates). The water content of the soils was adjusted to 50% of the maximum water holding capacity. To avoid linuron photodegradation and evaporation of water from soil, the pots were covered with perforated polypropylene sheets and incubated in darkness at 20±2°C for 28 days. Throughout the incubation period, water losses exceeding 10% of the initial values were compensated for by the addition of distilled water.

Determination of soil respiration

After 1, 7, 14 and 28 days, SIR short-term respiration was measured to characterise potentially active soil microbial biomass. Soil samples (100 g) were thoroughly mixed with glucose (2,000 mg kg^{-1} dry weight soil) and total quantities of oxygen consumption were determined within 12 h of glucose addition using the Sensomat Measurement System (LOVIBOND®, Dortmund, Germany). The principle of operation was based on the measurement of the pressure difference in the closed system. During respiration, CO_2 was bound to an absorber (45% KOH), and oxygen consumption resulted in the pressure drop that was proportional to the soil respiration. The quantity of oxygen consumed was estimated according to the calculations recommended by the manufacturer.

Determination of enzyme activities

After 1, 7, 14 and 28 days, activities of selected enzymes were determined in the soil samples. Dehydrogenase activity (DHA) was determined using 2,3,5-triphenyltetrazoliumchloride (TTC) as a substrate, incubating the soil samples (5 g) mixed with Tris buffer (pH 7.6) at 25°C for 20 h. The triphenyl formazan (TPF) produced was extracted from the reaction mixture with acetone and measured at 546 nm in a Jenway 6300 visible spectrophotometer (wavelength range 320–1,000 nm, spectral bandwidth 8 nm). The activity of dehydrogenase was expressed as µg TPF g^{-1} h^{-1} (Alef 1995).

Acid and alkaline phosphatase activities (PHOS-H and PHOS-OH) were determined using *p*-nitrophenyl phosphate as a substrate (0.05 M, 1 ml). Soil samples (1 g) were mixed with the modified universal buffer (MUB) of pH 6.5 and 11 for acid and alkaline phosphatase assays, respectively, and the substrate solution and incubated for 1 h at 37°C. After incubation, 0.5 M $CaCl_2$ and 0.5 M NaOH were added to stop the reaction and avoid the colouration caused by organic matter. The extracted *p*-nitrophenol (*p*-NP) was measured at 400 nm using the Jenway 6300 spectrophotometer. PHOS-H and PHOS-OH were expressed as µg *p*-NP g^{-1} h^{-1} (Tabatabai and Bremner 1969).

Urease activity (URE) was determined using urea (10%, w/v) as a substrate, which was added to wet soil (10 g) supplemented with citrate buffer (pH 6.7) followed by incubation at 37°C for 5 h. After incubation, NH_4^+ concentrations in the reaction mixtures were determined. The analysis was based on measurement of the intensity of the blue colour yielded during the reaction of ammonium with sodium chlorate (0.9% active Cl_2) and sodium phenolate (phenol 50%, NaOH 21.6%, 1:1) at 630 nm with the Jenway 6300 spectrophotometer. URE was expressed as mg NH_4^+ kg^{-1} h^{-1} (Gianfreda et al. 1994).

Ammonification and nitrification rates

In the treated and control soils, ammonium and nitrate concentrations were determined on days 1, 7, 14 and 28 to

characterise the overall activity of soil microorganisms mineralising organic nitrogen sources. Soil samples (10 g) were extracted with 100 ml 0.1% K_2SO_4 for 24 h followed by colorimetric determination of the ions in filtrates. For ammonium, the intensity of blue colour yielded during the reaction with sodium chlorate (1% of active Cl_2) and sodium phenolate (phenol 50%, NaOH 21.6%, 1:1) was measured at 625 nm using the Jenway 6300 spectrophotometer. For nitrate, determination of the intensity of the yellow colour resulting from the reaction with phenyldisulphonic acid (25% in concentrated H_2SO_4) was measured at 410 nm. The amounts of ammonium and nitrate ions were estimated by reference to calibration curves and the blank values obtained, and the results were calculated per kilogram dry weight soil.

Microbial enumeration

After 1, 14 and 28 days, soil samples (10 g) were placed in Erlenmeyer flasks containing 90 ml 0.1% sterile sodium pyrophosphate (pH 7.0) for shaking (130 rpm, 30 min) and preparing serial dilutions for plate counts. The total numbers of culturable bacteria were estimated on one-strength trypticase soy broth agar (0.1 TSBA: Difco TSB 3 g, agar 15 g per litre, pH 7.0). The inoculated plates were incubated at 28°C for 3 days, before the colonies were counted. Viable counts of fungi were performed on a Rose Bengal-Streptomycin Agar (glucose10 g, peptone 5 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, Rose Bengal 0.033 g, agar 15 g, streptomycin 30 mg ml^{-1} per litre, pH 5.6). The plates were incubated at 22°C and fungal colonies were counted after 7 days. The enumeration of culturable free nitrogen-fixing bacteria was conducted using Ashby's Mannitol Phosphate Agar (mannitol 10.0 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.2 g, $CaSO_4$ 0.1 g, $CaCO_3$ 5.0 g, agar 15 g per litre, pH 8.3). The plates were incubated in nitrogen atmosphere at 28°C for 14 days (Monkiedje et al. 2002). Culturable nitrifying bacteria were estimated on the agar medium (NaCl 0.30 g, $(NH_4)_2SO_4$ 0.68 g, KH_2PO_4 0.12 g, $MgSO_4 \cdot 7H_2O$ 0.23 g, $CaCO_3$ 1 g, agar 15 g per litre, pH 7.5) after incubation at 22°C for 10 days. Numbers of denitrifying bacteria were determined on the agar medium (KNO_3 5 g, meat extract 3 g, peptone 5 g, agar 15 g per litre, pH 7.0) after incubation at 22°C for 10 days under anaerobic conditions.

Bacterial growth strategies

To determine growth strategies among culturable bacteria, colonies appearing on the unselective medium (0.1-strength TSBA) were counted considering r (rapid colony formation within 24 h) and K (colony formation after 24–48 h) strategists (De Leij et al. 1993). For this purpose, plates were incubated at 27°C for 10 days and enumerated daily seven times (days 1–8) and, in addition, on day 10. Thus, eight classes of culturable bacteria (i.e. ecotypes) were defined per plate. The number of bacteria in each class was expressed as proportion (%) of the total number of culturable bacteria. The different distributions of the classes gave an insight into the distributions of r- and K-strategists in each soil.

Statistical analysis

Statistical analyses were performed on three replicates from each treatment on each time-point using ANOVA (Statistica 7.0, PL, http://www.statsoft.com/) and the least significant difference (LSD) test. Values were considered significantly different at the 95% confidence level. In this way, effects of the herbicide dosages on the microbial characteristics were assessed for each soil throughout the experiment.

Results and discussion

Soil respiration

The substrate-induced respiration method is commonly accepted for estimation of potential perturbations in microbe-mediated degradation of organic matter in soil treated with pesticides (Domsch et al. 1983). In this study, SIR results showed that microbial activity increased in linuron-treated soils, but this effect was transient (Fig. 1). In both soils, linuron stimulated SIR values, especially on day 1. In addition, the higher the concentration of herbicide added to the soils, the higher the increase in microbial activity. At the end of incubation, no difference was found between soil samples amended with linuron and respective controls. Dinelli et al. (1998) observed a similar transient increase of SIR in soils amended with other herbicides, e.g. triasulfuron, primisulfuron methyl and rimsulfuron. In general, several studies have indicated the variable effects of herbicides on potential microbial activity depending on the herbicide used and soil type (Ismail et al. 1998; Zabaloy et al. 2008a). Accinelli et al. (2002) studied the short-term effect of six agrochemicals on microbial activity and reported that sulfonylurea herbicides applied up to 20 μg g^{-1} soil stimulated soil respiration, whereas application of these herbicides at the agricultural rate did not exert any significant impact on soil microbial activity. Soil respiration was also stimulated by hexazinone over a 3-week period, when it was applied at a level up to 100 times higher than the recommended field rate (Vienneau et al. 2004). It would appear that some microorganisms are able to use applied herbicides as sources of energy and nutrients, resulting in an increase in their metabolic activity.

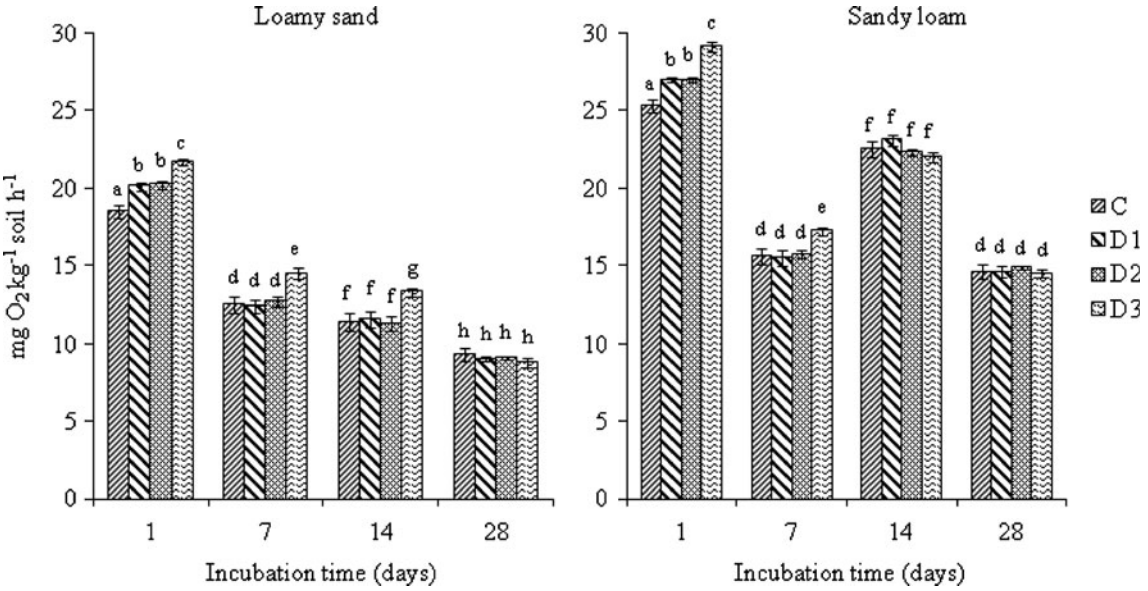

Fig. 1 Substrate-induced respiration in the loamy sand (LS) and sandy loam (SL) soils treated with linuron at different dosages (mg kg^{-1} soil): *C* 0 (control soil), *D1* 4, *D2* 20, *D3* 400. The values are the means ± standard deviations. For each soil, means with different letter(s) are significantly different at $P<0.05$. *LSD* Least significant difference ($n=3$)

Enzyme activities

Enzyme activities are often proposed as early and sensitive indicators of soil ecological stress in both natural and agricultural ecosystems (Badiane et al. 2001; Sannino and Gianfreda 2001). In this study, the results showed differences in enzyme activities in response to linuron dosages added to the soils (Table 2). In LS soil treated with linuron at a concentration of 400 mg kg^{-1} soil, DHA decreased compared to the control. However, this effect was not found on day 1. In addition, decreased DHA was also observed in soil treated with linuron at concentrations of 4 and 20 mg kg^{-1} soil on days 14 and 28. In contrast, the negative effect of linuron was observed only in SL treated with the highest dosage on days 14 and 28 (Table 2). The different impact of herbicides on DHA is associated with differences in soil organic matter content, type of herbicide, their dosage and farming history (Zabaloy et al. 2008a, b). The positive effect on DHA of glyphosate applied up to 200 mg kg^{-1} soil has been reported by several authors (Sannino and Gianfreda 2001; Accinelli et al. 2002; Araújo et al. 2003). Grenni et al. (2009) found no changes in DHA values between control and soils treated with linuron at the agricultural rate.

In contrast to DHA, both acid and alkaline phosphatase activities were generally stimulated by linuron in both soils (Table 2). In LS soil, the herbicide applied at the highest dosage caused a significant increase ($P<0.05$) in PHOS-H by 12–16% in comparison to the control throughout the incubation time. In contrast, this stimulatory effect of linuron in SL was observed only until day 14. For PHOS-OH, the highest dosage of linuron significantly reduced enzyme activity in LS on day 1. By the next sampling time, this dosage linuron had increased PHOS-OH significantly ($P<0.05$) by 6–20% as compared to the control. In SL soil, the highest linuron dosage significantly increased ($P<0.05$) PHOS-OH by 3–11% in comparison to the control at 14 days. This transient increase in PHOS-OH was also observed in soil treated with linuron at 20 mg kg^{-1} soil (Table 2). In contrast to our results, Peruci et al. (2000) observed detrimental effects of sulfonylurea and imidazoline herbicides applied at field and ten-fold field rates on both alkaline and acid phosphatase activities. A decrease in both phosphatase activities was also found in soil treated with trifluralin (Wyszkowska and Kucharski 2004), glyphosate and paraquat (Sannino and Gianfreda 2001) or alachlor (Pozo et al. 1994). However, for alachlor, PHOS-H and PHOS-OH decreased significantly shortly after the introduction to soil at concentrations of 5.0 to 10.0 kg ha^{-1} soil, but with time the enzyme activities reached levels similar to that found in the control (Pozo et al. 1994).

Generally, the impact of linuron on URE was similar in both soils (Table 2). On day 1, both soils treated with linuron at 400 mg kg^{-1} soil showed significantly lower ($P<0.05$) URE in comparison to the control. However, URE values were higher in all linuron treatments in SL soil on day 14. Results for URE activity reported by different authors showed high variability. Depending on the type of herbicide, dosage, application rate and soil type, a decrease (Ismail et al. 1998), no consistent changes (Jorge et al. 2007), or an increase (Yang et al. 2007) of URE have all been reported.

Table 2 Enzyme activities in sandy soils treated with different dosages of linuron. For each soil, means with different letter(s) are significantly different at $P<0.05$. *LSD* least significant difference ($n=3$). *DHA* Dehydrogenase activity, *PHOS-H* acid phosphatase activity, *PHOS-OH* alkaline phosphatase activity, *URE* urease activity, *TPF* triphenyl formazan, *p-NP* *p*-nitrophenol

Enzyme	Dosage (mg kg^{-1} soil)	LS (days after application)				SL (days after application)			
		1	7	14	28	1	7	14	28
DHA (μg TPF g^{-1} soil h^{-1})	0	27 a	27 a	26 a	28 a	56 a	48 b	52 a	38 c
	4	26 a	26 a	23 b	23 b	57 a	49 b	53 a	38 c
	20	26 a	28 a	22 b	22 b	57 a	46 b	53 a	40 c
LSD 2	400	28 a	22 b	17 c	13 d	55 a	48 b	38 c	32 d
PHOS-H (μg *p*-NP g^{-1} soil h^{-1})	0	101 a	123 c	118 c	144 f	136 a	155 c	127 e	153 c
	4	101 a	124 c	122 c	153 g	137 a	166 d	136 a	153 c
	20	104 a	121 c	130 e	161 h	137 a	172 d	145 b	153 c
LSD 3	400	115 b	141 d	137 d	161 h	146 b	167 d	151 b	152 bc
PHOS-OH (μg *p*-NP g^{-1} soil h^{-1})	0	83 a	61 c	84 a	90 e	96 a	89 c	93 a	101 d
	4	81 a	68 d	84 a	92 e	95 a	91 a	93 a	97 ad
	20	83 a	69 d	84 a	90 e	105 b	89 a	92 a	101 bd
LSD 2	400	76 b	74 b	89 e	99 f	107 b	95 a	96 a	97 ad
URE (mg NH_4^+ kg^{-1} soil h^{-1})	0	22 a	42 c	43 c	25 d	53 a	46 b	53 a	54 a
	4	19 ab	41 c	46 c	25 d	50 a	44 b	59 c	53 a
	20	19 ab	43 c	46 c	26 d	51 a	51 a	62 c	55 a
LSD 2	400	16 b	45 c	47 c	24 d	47 b	45 b	58 c	54 ac

Generally, the low activities of enzymes in soils treated with pesticides could result from the death of sensitive microorganisms; enzymes released from dead cells have only short-lived activity because they can be rapidly denatured, degraded or irreversibly inhibited (Marx et al. 2005). However, a certain proportion of free enzymes may undergo stabilization through adsorption to humic materials, which, despite affecting their catalytic potential may enable persistence of enzyme activity in soil (Badiane et al. 2001). This fact could explain the generally lower inhibition of enzyme activities by linuron in SL soil, which is characterised by a higher proportion of clay fraction and organic matter in comparison to LS.

Nitrification and ammonification rates

With regard to the great importance of nitrogen turnover for soil fertility and health, nitrification and ammonification processes are commonly used for assessment of microbial activity in soils amended with pesticides (Kara et al. 2004). Soil treatment with linuron at the highest dosage caused a significant decrease ($P<0.05$) in nitrate concentrations. This effect was observed throughout the incubation period and for only 7 days in LS and in SL, respectively. Lower dosages of linuron did not decrease the nitrate contents in both soils, except the dosage of 20 mg g^{-1} soil in LS on day 7 (Fig. 2). Marsh and Davies (1981) observed long-term strong inhibition of nitrification in dichlorprop- and mocoprop-treated soils. Moreover, nitrification was suppressed significantly by bensufuron-methyl applied at concentrations of 0.1 and 1.0 μg g^{-1} soil (El-Ghamry et al. 2002). It seems reasonable to assume that the chemical nature of herbicides is responsible for their effects on this process.

For ammonium, the addition of linuron at all dosages into LS increased the ion concentrations on day 1 (Fig. 2). In addition, the higher the concentration of linuron added to the soil, the higher the increase in ammonium content. At next sampling days, the stimulatory effect on the ammonification rate was observed in LS treated with linuron at 400 mg kg^{-1} soil, and ammonium concentrations were about six-fold higher as compared to the control in soil treated with this dosage on day 14 (Fig. 2). In contrast, an increase in ammonium concentrations was found in SL treated with linuron at 400 mg kg^{-1} soil during 14 days (Fig. 2). It reasonable to assume that some ingredients of the linuron formulation could stimulate ammonifying bacteria, resulting in enhanced production of ammonium. However, putative photodegradation of linuron might have resulted in production of increased ammonium concentrations. Nevertheless, a stimulating effect of linuron could not be excluded, as suggested by the data from treatment with the highest dosage. Kara et al. (2004) reported that the herbicide topogard significantly stimulated ammonification in neutral and alkaline soils, whereas acid soils showed significantly lower ammonium contents in comparison with

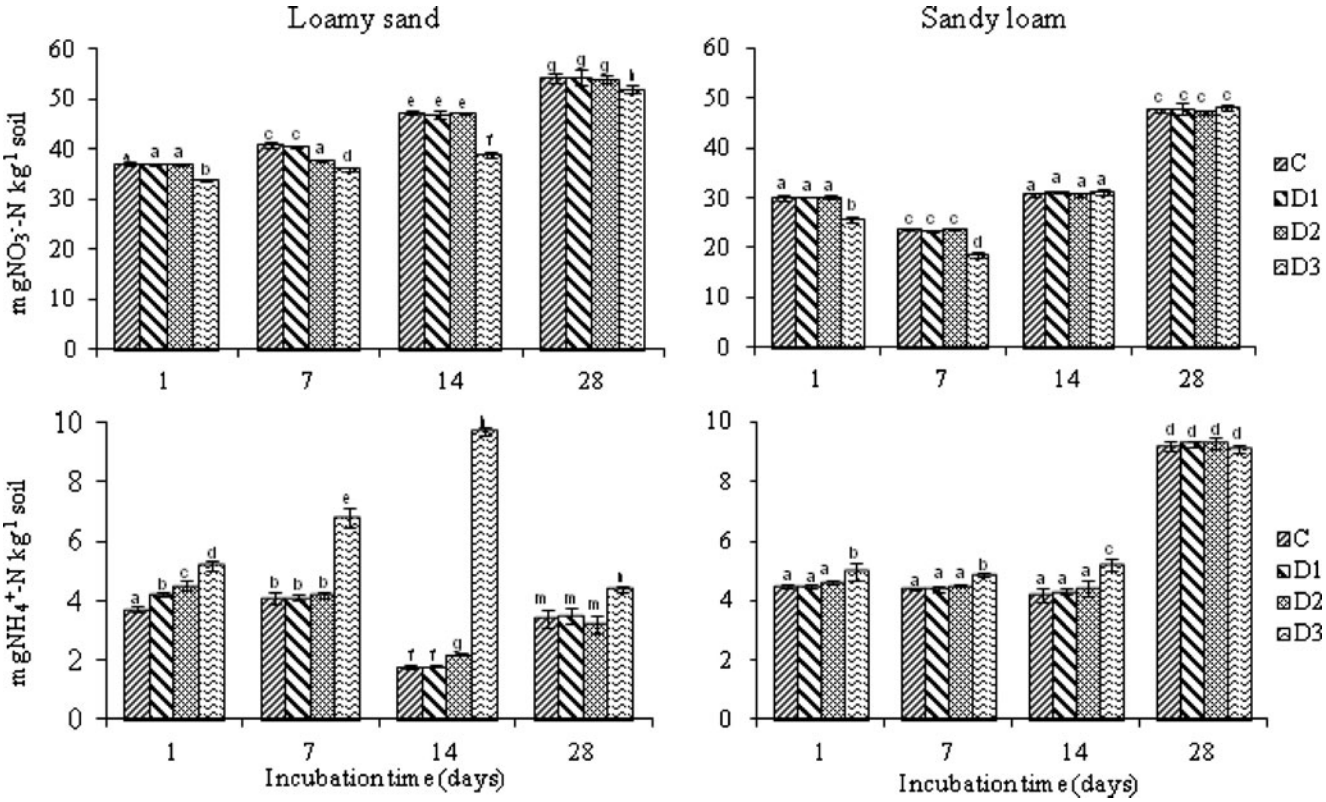

Fig. 2 Changes in nitrate and ammonium concentrations in LS and SL soils treated with linuron at different dosages (mg kg^{-1} soil): *C* 0 (control soil), *D1* 4, *D2* 20, *D3* 400. The values are the means ± standard deviations. For each soil, means with different letter(s) are significantly different at $P<0.05$. *LSD* Least significant difference ($n=3$)

controls. Ammonification was also initially inhibited by triazine herbicide hexazinone; however, ammonium contents were similar or greater in all treatments compared to controls after 4 weeks (Vienneau et al. 2004). Studies on successive application of different pesticides in field conditions over 2 years, carried out by Schuster and Schröder (1990), showed that herbicides had only slight and transient side-effects on the ammonification process.

Microbial group numbers

The plate-count results indicated that the two higher dosages of linuron increased the total numbers of culturable bacteria in LS. In turn, increased bacterial numbers were detected in SL treated with the highest dosage of the herbicide and soil portions treated with linuron at 20 mg kg^{-1} soil on all sampling days and on day 14, respectively (Table 3). Linuron is a phenylurea compound that can be degraded by different bacteria and might be used as a source of C and N, resulting in a significant increase in bacterial counts (Dejonghe et al. 2003). Recently, Grenni et al. (2009) indicated the lack of a linuron effect on the numbers of viable bacteria in a treated soil because of their adaptation to the presence of the herbicide. Zabaloy et al. (2008b) also observed increased numbers of culturable bacteria in different soils treated with metsulfuron-methyl and glyphosate. In addition, Araújo et al. (2003) reported increased counts of soil heterotrophic bacteria in response to applied herbicides. Ratcliff et al. (2006) did not find changes in culturable bacterial numbers between control and glyphosate-treated soil when the herbicide was used at a dosage of 50 mg kg^{-1} soil, whereas a 100-fold higher dosage increased both heterotrophic bacterial counts and the bacteria:fungi ratio. Admittedly, bacteria differ in their abilities to grow in the presence of different concentrations of herbicides. As a result, changes in bacterial community structure, associated with decreased or increased species (rybotypes) diversity due to different reactions of the unculturable fraction, have been reported in different soils (el Fantroussi et al. 1999; Valle et al. 2006; Lin et al. 2008). In contrast to bacterial counts, linuron treatments did not change ($P<0.05$) the fungal numbers significantly in either soil as compared to the corresponding controls (Table 3). It would appear that culturable fungi can tolerate linuron concentrations in both soils. Several soil fungi, including different species of *Cunninghamella*, *Mortierella*, *Talaromyces*, *Rhizopus*, *Rhizoctonia* and *Aspergillus* (Sørensen et al. 2003), are able to degrade linuron and other phenylurea herbicides.

Table 3 Numbers (log cfu g^{-1} dry soil) of culturable microorganisms in sandy soils treated with different dosages of linuron. For each soil, means with different letter(s) are significantly different at $P<0.05$. *LSD* Least significant difference ($n=3$). *TSBA* Trypticase soy broth agar

Microbial group	Dosage (mg kg^{-1})	Loamy sand (days after application)			Sandy loam(days after application)		
		1	14	28	1	14	28
Total bacteria on 0.1 TSBA	0	7.60 a	7.73 b	7.54 ad	8.50 a	8.68 b	8.71 b
	4	7.61 a	7.74 b	7.53 d	8.49 a	8.69 b	8.72 b
	20	7.75 b	7.83 c	7.70 b	8.48 a	8.77 c	8.70 b
LSD 0.03	400	7.54 a	7.88 c	7.74 b	8.59 b	8.92 d	8.87 d
Fungi	0	4.67 a	4.49 b	4.57 b	5.50 a	5.55 a	5.49 a
	4	4.68 a	4.50 b	4.58 b	5.50 a	5.56 a	5.50 a
	20	4.67 a	4.60 b	4.57 b	5.52 a	5.54 a	5.53 a
LSD 0.04	400	4.49 b	4.68 a	4.59 b	5.38 b	5.64 a	5.67 a
N_2-fixing bacteria	0	4.60 a	4.50 c	4.62 a	5.79 a	5.86 d	5.67 b
	4	4.55 a	4.52 ac	4.61 a	5.78 a	5.85 d	5.69 b
	20	4.52 a	4.40 d	4.64 a	5.64 b	5.87 d	5.67 b
LSD 0.04	400	4.14 b	4.34 e	4.56 a	5.42 c	5.76 a	5.68 b
Nitrifying bacteria	0	4.87 a	4.90 a	5.10 c	5.38 a	5.54 c	5.77 d
	4	4.83 a	4.91 a	5.09 c	5.39 a	5.53 c	5.78 d
	20	4.80 a	4.90 a	5.10 c	5.37 a	5.55 c	5.77 d
LSD 0.03	400	4.58 b	4.66 b	5.09 c	5.15 b	5.36 a	5.78 d
Denitrifying bacteria	0	5.03 a	4.95 a	5.00 a	5.86 a	5.71 b	5.91 a
	4	5.04 a	4.96 a	5.02 a	5.83 a	5.72 b	5.93 a
	20	5.10 a	5.01 a	5.05 a	5.87 a	5.73 b	5.91 a
LSD 0.03	400	4.96 a	4.95 a	5.06 a	5.93 a	6.02 c	5.93 a

Transient changes in the numbers of some bacteria involved in soil nitrogen transformations were also found in herbicide treated soils in comparison to untreated controls. On the 1st day after linuron application, an adverse effect on nitrogen-fixing bacteria was found for both soils treated with the highest dosage. This effect was also found in both soils on day 14 (Table 3). A decrease in numbers of nitrogen-fixing bacteria has often been found for different pesticides introduced into soil (Martinez-Toledo et al. 1998; Cycoń et al. 2006; Lin et al. 2008). For nitrifying bacteria, cfu counts decreased in both soils treated with linuron at 400 mg kg^{-1} soil; however, this effect was found only on days 1 and 14 (Table 3). Martinez-Toledo et al. (1996) also observed the negative effect of another herbicide (simazine) applied at 50 to 300 μg g^{-1} soil on nitrifying bacteria. However, this effect was particularly evident after the second application of the herbicide. In addition, the synergistic action of two herbicides (terbutryn and terbuthylazin) inhibited activity of nitrifying bacteria in acid and neutral soils (Kara et al. 2004). Conversely, nitrifying bacteria were not affected upon addition of the herbicide alachlor to an agricultural soil (Pozo et al. 1994). In contrast to nitrifying bacteria, the numbers of denitrifying bacteria were not affected by linuron in either soil (Table 3). Application of the herbicide glyphosate to grass resulted in a 20- to 30-fold increase in denitrification compared with untreated soil (Tenuta and Beauchamp 1996). Similar results have been obtained for pesticides, indicating that, as a group, denitrifiers may be regarded as being more tolerant of pesticides than nitrogen-fixing bacteria and nitrifiers (Martinez-Toledo et al. 1998; Cycoń et al. 2006).

Bacterial growth strategies

It is widely acknowledged that pesticides affect not only the numbers of microorganisms and their metabolic activities, but may also change the microbial community structure in soil (el Fantroussi et al. 1999; Saeki and Toyota 2004; Ratcliff et al. 2006). In our studies, we applied the method developed by De Leij et al. (1993), who used the r-/K-strategy concept for the characterisation of bacterial communities in rhizosphere and non-rhizosphere soil. For LS treated with linuron at 4 or 20 mg kg^{-1} soil, the distribution of bacterial classes (ecotypes) was similar to the control (Fig. 3). However, the domination of r-strategists was seen in these samples only on day 14. When the highest concentration of linuron was added to LS, the bacterial community was dominated by K-strategists on days 1 and 14. However, r-strategists were revealed as the dominant group in the soil on day 28. In

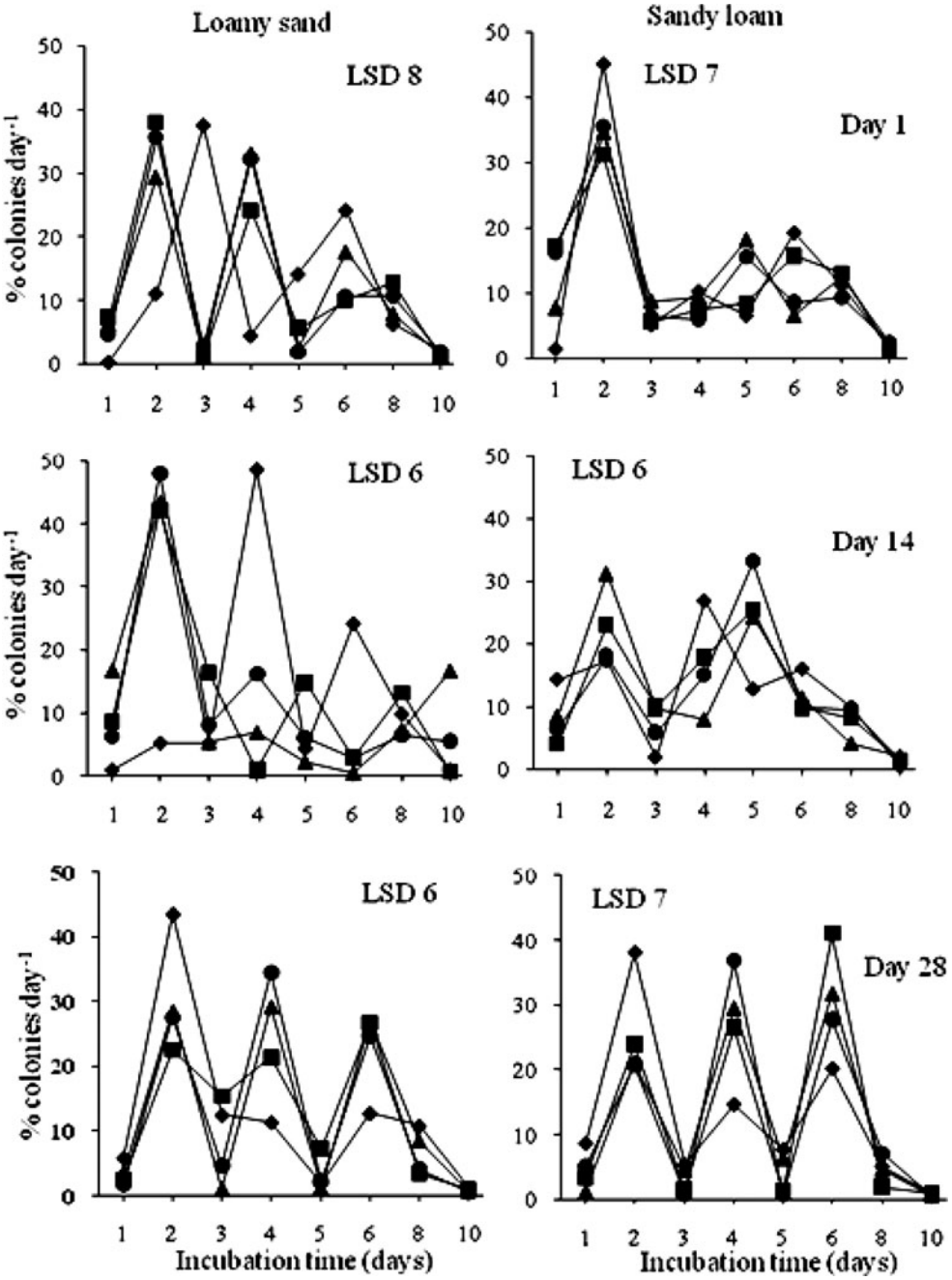

Fig. 3 Distribution of growth strategy pattern within culturable bacterial community in LS and SL soils treated with linuron at different dosages (mg kg^{-1} soil): ■ 0 control soil, ● 4, ▲ 20, ♦ 400. *LSD* Least significant difference (n=3). On 0.1-strength trypticase soy broth agar, fast growing bacteria (r-strategists) formed visible colonies within 48 h, and slow growing bacteria (K-strategists) formed colonies later

contrast, r-strategists dominated in SL soil treated with all dosages of the herbicide as well the untreated control on day 1, whereas an even distribution of bacterial ecotypes was found at subsequent sampling times, the exception being soil amended with linuron at 400 mg kg^{-1} soil. For the latter treatment, an increase in the numbers of r-strategists was observed on day 28 (Fig. 3). From these data, we can assume that only the highest dosage of linuron affects the distribution of r-/K-strategists within the culturable bacteria community in soil. In soil with lower contents of clay and organic matter, the higher availability of linuron may have caused a shift in the community structure towards domination of slow-growing bacteria. However, both the higher content of linuron and associated soil components may have been used as substrates supporting the growth of r-strategists in the treated soils on day 28. To our knowledge, this is the first study on the impact of herbicides on the culturable bacterial community in terms of differential selection of bacterial ecotypes in contaminated soil.

Although the bacterial community was dominated by one ecotype class for the highest linuron treatment, an increase in the numbers of heterotrophic bacteria was found after herbicide application. Taiwo and Oso (1997)

reported similar results using the plate count method. They also observed an increase in bacterial and fungal abundance, whereas the number of microbial species decreased simultaneously in soils treated with other pesticides (i.e. atrazine, metobromuron, metachlor and pyrethrin). It is reasonable to suppose that these findings result from the fact that several bacterial species sensitive to the herbicides are replaced by tolerant species belonging to the specific ecotype that dominates the bacterial community in treated soils. The ability to degrade herbicides, or adaptation of soil culturable bacteria to the compounds, may be different strategies revealed by r- and K-strategists, respectively, for survival in contaminated soil.

In conclusion, linuron appears to be well tolerated by soil microorganisms when applied at the recommended and even five-fold higher dosages to sandy soils. However, some significant disturbances in different microbial characteristics are apparent in soils treated with 100-fold PEC simulating undiluted chemical spill of the herbicide. In addition, comparison of plate counts and DHA data indicates that other soil microorganisms than those cultured on the medium used may have been affected by the higher contents of linuron with time. For example, el Fantroussi et al. (1999), using group-specific primers, showed that long-term application of this herbicide affected mostly bacterial species belonging to an uncultivated group of *Acidobacterium*. Hence, further direct microbial analyses should be undertaken to decipher the nature of the linuron effect in soil at a microbial scale.

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References

Accinelli C, Screpanti C, Dinelli G, Vicari A (2002) Short-time effects of pure and formulated herbicides on soil microbial activity and biomass. Int J Environ Anal Chem 82:519–527

Accinelli C, Dinelli G, Vicari A, Mallegnir R (2004) Metolachlor and linuron soil degradation after repeated applications under laboratory conditions. Agrochimica 48:132–140

Alef K (1995) Dehydrogenase activity. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic, London, pp 228–231

Araújo ASF, Monteiro RTR, Abarkeli RB (2003) Effect of glyphosate on the microbial activity of two Brazilian soils. Chemosphere 52:799–804

Badiane NNY, Chotte JL, Pate E, Masse D, Rouland C (2001) Use of soil enzyme activities to monitor soil quality in natural and improved fallows in semi-arid tropical regions. Appl Soil Ecol 18:229–238

Breugelmans P, D'Huys PJ, De Mot R, Springael D (2007) Characterization of novel linuron-mineralizing bacterial consortia enriched from long-term linuron-treated agricultural soils. FEMS Microbiol Ecol 62:374–385

Caux PY, Kent RA, Fan GT, Grande C (1998) Canadian water quality guidelines for linuron. Environ Toxicol Water Qual 13:1–41

Chen S-K, Edwards CA, Subler S (2001) A microcosm approach for evaluating the effects of the fungicides benomyl and captan on soil ecological processes and plant growth. Appl Soil Ecol 18:69–82

Cycoń M, Piotrowska-Seget Z, Kaczyńska A, Kozdrój J (2006) Microbiological characteristics of a loamy sand soil exposed to tebuconazole and λ-cyhalothrin under laboratory conditions. Ecotoxicology 15:639–646

De Leij FAAM, Whipps JM, Lynch JM (1993) The use of colony development for the characterisation of bacterial communities in soil and roots. FEMS Microbiol Ecol 27:81–97

Dejonghe W, Berteloot E, Goris J, Boon N, Crul K, Maertens S, Höfte M, De Vos P, Verstraete W, Top EM (2003) Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. Appl Environ Microbiol 69:1532–1541

Dinelli G, Vicari A, Accinelli C (1998) Degradation and side effects of three sulfonylurea herbicides in soil. J Environ Qual 27:1459–1464

Domsch KH, Jabnow G, Anderson TH (1983) An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. Res Rev 86:65–105

el Fantroussi S, Verschuere L, Verstraete W, Top EM (1999) Effects of phenylurea herbicides on soil microflora communities estimated by analysis of the 16S rRNA gene fingerprints and community level physiological profiles. Appl Environ Microbiol 65:982–988

El-Ghamry AM, Xu JM, Huang CY, Gan J (2002) Microbial response to bensulfuron-methyl treatment in soil. J Agric Food Chem 50:136–139

Filip Z (2002) International approach to assessing soil quality by ecologically-related biological parameters. Agr Ecosyst Environ 88:169–174

Gianfreda L, Sanninio F, Ortega N, Nannipieri P (1994) Activity of free and immobilzed urease in soil: effects of pesticides. Soil Biol Biochem 26:777–784

Grenni P, Caracciolo AB, Rodríguez-Cruz MS, Sánchez-Martín MJ (2009) Changes in the microbial activity in a soil amended with oak and pine residues and treated with linuron herbicide. Appl Soil Ecol 41:2–7

Ismail BS, Yapp KF, Omar O (1998) Effects of metsulfuron-methyl on amylase, urease, and protease activities in two soils. Aust J Soil Res 36:449–456

Jorge M, Martin A, Carballas T, Diaz-Ravina M (2007) Atrazine degradation and enzyme activities in an agricultural soil under two tillage systems. Sci Total Environ 378:187–194

Kara EE, Arli M, Uygur V (2004) Effects of the herbicide Topogard on soil respiration, nitrification, and denitrification in potato-cultivated soils differing in pH. Biol Fertil Soils 39:474–478

Lin X, Zhao Y, Fu Q, Umashankara ML, Feng Z (2008) Analysis of culturable and unculturable microbial community in bensulfuron-methyl contaminated paddy soils. J Environ Sci 20:1494–1500

Lupwayi NZ, Harker KN, Clayton GW, O'Donovan JD, Blackshaw RE (2009) Soil microbial response to herbicides applied to glyphosate-resistant canola. Agric Ecosyst Environ 129:171–176

Marsh JAP, Davies HA (1981) Effects of dichlorprop and mecoprop on respiration and transformation of nitrogen in two soils. Bull Environ Contam Toxicol 26:108–115

Martinez-Toledo MY, Salmerón Y, Rodelas B, Pozo C, Gonzalez-López J (1996) Studies of the effects of the herbicide simazine on microflora of four agricultural soils. Environ Toxicol Chem 15:1115–1118

Martinez-Toledo MY, Salmerón Y, Rodelas B, Pozo C, Gonzalez-López J (1998) Effects of the fungicide Captan on some functional groups of soil microflora. Appl Soil Ecol 7:245–255

Marx M-C, Kandeler E, Wood M, Wermbter N, Jarvis SC (2005) Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fraction. Soil Biol Biochem 37:35–48

Monkiedje A, Ilori MO, Spiteller M (2002) Soil quality changes resulting from the application of the fungicides mefenoxam and metalaxyl to a sandy loam soil. Soil Biol Biochem 34:1939–1948

Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns G, Dick RP (eds) Enzymes in the environment: activity, ecology and applications. Dekker, New York, pp 1–33

Peruci P, Dumontet S, Bufo SA, Mazzatura A, Casucci C (2000) Effects of organic amendment and herbicide treatment on soil microbial biomass. Biol Fertil Soils 32:17–23

Pozo C, Salmerón Y, Rodelas B, Martinez-Toledo MY, Gonzalez-López J (1994) Effects of the herbicide alachlor on soil microbial activities. Ecotoxicology 3:4–10

Ratcliff AW, Busse MD, Shestak CJ (2006) Changes in microbial community structure following herbicide (glyphosate) addition to forest soils. Appl Soil Ecol 34:114–124

Saeki M, Toyota K (2004) Effect of bensulfuron-methyl (a sulfonyl-urea herbicide) on the soil bacterial community of a paddy soil microcosm. Biol Fertil Soils 40:110–118

Sannino F, Gianfreda L (2001) Pesticide influence on soil enzymatic activities. Chemosphere 45:417–425

Schuster E, Schröder D (1990) Side-effects of sequentially-applied pesticides on non-target soil microorganisms: field experiments. Soil Biol Biochem 22:367–373

Seghers D, Verthé K, Reheul D, Bulcke R, Siciliano SD, Verstraete W, Top EM (2003) Effect of long-term herbicide applications on the bacterial community structure and function in an agricultural soil. FEMS Microbiol Ecol 46:139–146

Snel JFH, Vos JH, Gylstra R, Brock TCM (1998) Inhibition of photosystem II (PSII) electron transport as a convenient endpoint to assess stress of the herbicide linuron on freshwater plants. Aquat Ecol 32:113–123

Sørensen SR, Bending GD, Jacobsen CS, Walker A, Aamand J (2003) Microbial degradation of isoproturon and related herbicides in and below agricultural fields. FEMS Microbiol Ecol 45:1–11

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenylphosphate to assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Taiwo LB, Oso BA (1997) The influence of some pesticides on soil microbial flora in relation to changes in nutrient level, rock phosphate solubilization and P release under laboratory conditions. Agr Ecosyst Environ 65:59–68

Tenuta M, Beauchamp EG (1996) Denitrification following herbicide application to a grass sward. Can J Soil Sci 76:15–22

Valle A, Boschin G, Negri M, Abbruscato B, Sorlini C, D'Agostina A, Zanardini E (2006) The microbial degradation of azimsulfuron and its effect on the soil bacterial community. J Appl Microbiol 101:443–452

Vienneau DM, Sullivan CA, House SK, Stratton GW (2004) Effects of the herbicide hexazinone on nutrient cycling in a low-pH blueberry soil. Environ Toxicol 19:115–122

Wyszkowska J, Kucharski J (2004) Biochemical and physicochemical properties of soil contaminated with herbicide Triflurotox 250 EC. Pol J Environ Stud 13:223–232

Yang C, Sun T, He W, Zhou Q, Chen S (2007) Single and joint effects of pesticides and mercury on soil urease. J Environ Sci 19:210–216

Zabaloy MC, Garland JL, Gómez MA (2008a) Microbial respiration in soils of the Argentina pampas after metsulfuron-methyl, 2, 4-D and glyphosate treatments. Commun Soil Sci Plant Anal 39:370–385

Zabaloy MC, Garland JL, Gómez MA (2008b) An integrated approach to evaluate the impacts of the herbicides glyphosate, 2, 4-D and metsulfuron-methyl on soil microbial communities in the Pampas region, Argentina. Appl Soil Ecol 40:1–12