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Assessment of the impact of 2,4-dichlorophenoxyacetic acid (2,4-D) on indigenous herbicide-degrading bacteria and microbial community function in an agricultural soil

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ABSTRACT

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) may influence soil microbial communities by altering the balance between resident populations. Our objective was to assess the effect of environmentally relevant levels (ERLs) of 2,4-D on microbial community function and on the population dynamics of 2,4-D degrading bacteria using a microcosm approach. The most probable number approach was used to enumerate 2,4-D-degrading soil bacteria. Carbon substrates utilization was tested with a microtiter-based oxygen sensor system to evaluate short-term functional shifts caused by herbicide treatment. Shifts in the community in response to potential toxicity of 2,4-D were assessed in the agricultural soil and a reference forest soil using the pollution-induced community-tolerance (PICT) approach. Results indicated that the agricultural soil had a stable 2,4-D degrading population able to use the herbicide as C and energy source, which increases immediately after an ERL dose of 2,4-D and remains high for about 1 month after exposure has ceased. An enhanced, dose-dependent response to 2,4-D as substrate was observed in the microtiter assay, while heterotrophic bacterial activity appeared mostly unchanged. The PICT assay showed higher tolerance to 2,4-D in the agricultural soil than in the unexposed forest soil. Our results suggest that agricultural use of 2,4-D at recommended level leads to selection for (1) a copiotrophic degrader population and (2) a persistently herbicide-tolerant, but functionally similar, microbial community.

1. Introduction

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is extensively applied in agricultural fields worldwide to control dicotyledonous weeds in cereal crops and pastures, and to improve weed control obtained with glyphosate in no-till systems (Roberts, 1998; Altieri and Pengue, January 2006). Although much is known about the physiological activity, mechanism of action, and environmental fate of 2,4-D, the effects on soil microbial communities are poorly understood, particularly for environmentally relevant levels (ERL) of 2,4-D (i.e. $\leq$5 mg kg^{-1}) in soil (Ka et al., 1995; Vieublé-Gonod et al., 2006; Merini et al., 2007; Zabaloy et al., 2008a; Bouseba et al., 2009).

Chlorophenoxyalcanoic acid herbicides (i.e. 2,4-D, 2,4-DB, MCPA and 2,4,5-T) have been shown to exert toxic effects on cultured bacteria (Arias and de Peretti, 1993; Castro et al., 1996; Loffhagen et al., 2003; Zabaloy and Gómez, 2005), aquatic hyphomycetes (Chandrashekar and Kaveriappa, 1989) and microbial communities in activated sludge (Meriç et al., 2003). 2,4-D toxicity to microbial cells is due to interference with DNA, RNA and protein synthesis, as well as with metabolism of polyamines and alteration of cell membranes (Arias and de Peretti, 1993; Castro et al., 1996; Smith and Beadle, 2008). Conversely, 2,4-D can be readily used as a carbon and energy source by environmental microorganisms (Tiedje et al., 1969; Loos, 1975; Han and New, 1994; Kamagata et al., 1997; Muller et al., 2001; Lerch et al., 2007). Numerous 2,4-D degrading bacteria have been isolated and characterized. Most of these strains are members of genera belonging to the β and γ subdivisions of the class *Proteobacteria* and were isolated from 2,4-D treated environments (Kamagata et al., 1997; Lee et al., 2005). These β and γ subgroups carry 2,4-D degrading genes homologous to the canonical *tfdABCDEF* genes found in *Cupriavidus necator* JMP134 (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Waustersia eutropha*) (Lerch et al., 2007).

2,4-D application to agricultural soils may trigger specific degradation pathways in existing degrading populations (Baelum et al., 2006, 2008), as well as structural and functional shifts in the overall microbial community by altering the balance between microbial populations in soil (Vieublé-Gonod et al., 2006; Chinalia and Killham, 2006; Macur et al., 2007; Lerch et al., 2009). In contrast,

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Bouseba et al. (2009) reported that dominant bacterial phylotypes did not differ between control and 2,4-D treatment in three sandy loam soils. Some studies have reported enhanced biodegradation of chlorophenoxyacetic acids in the rhizosphere of legumes (Shaw and Burns, 2004, 2005; Merini et al., 2007), probably as a result of one or more components of root exudates being structural analogs of 2,4-D (Shaw and Burns, 2004, 2005). Among the diversity of compounds produced in the rhizosphere, succinate is present in root and nodules exudates in leguminous plants and is also catabolically linked to 2,4-D (Velicer, 1999). Increased fitness on 2,4-D as a carbon source in lines of bacteria adapted to grow on succinate has been demonstrated (Velicer, 1999).

Functional or metabolic diversity of microbial communities, defined by the substrates used for energy metabolism, are crucial for the long-term stability of an ecosystem (Pankhurst et al., 1996). Functioning microbial communities are the basis of important ecosystem services (e.g. nutrient cycling, detoxification of pollutants in soil) which represent inherent economic value in accordance to the Millenium Ecosystem Assessment (2005). Methodological approaches that reflect *in situ* use of available substrates are preferred when assessing the ecologically relevant impacts of toxicants, such as pesticides, on microbial community function. The BD Oxygen Biosensor System (BDOBS, BD Biosciences, Bedford, MA) is a microplate platform with an O_2-sensitive fluorescent dye that enables rapid measurement of O_2 consumption in soil slurries for community-level physiological profiling (CLPP). Garland et al. (2003) found that the rate of fluorescent response of hydroponic rhizosphere samples was correlated with independent measures of *in situ* rates of substrate use, indicating that the BDOBS–CLPP approach produced functionally relevant profiles. BDOBS has been recently optimized to assess the use of low levels of C (<100 μg C g^{-1} soil) by soil microbial communities (Zabaloy et al., 2008b). Moreover, its flexibility to rapidly assess microbial community function in response to different amendments (e.g. toxic substances) makes it suitable to study aerobic degradation of compounds such as surfactants (Sharvelle et al., 2008) or other organic pollutants, and for ecotoxicological studies.

The pollution-induced community-tolerance (PICT) concept is based on the assumption that long-term exposure of a community to a given toxicant will lead to a higher tolerance for this pollutant (Blanck et al., 1988; Blanck, 2002). PICT is tested by collecting intact communities from polluted and reference sites and exposing these communities to contaminants under controlled conditions. Increased community tolerance resulting from the elimination of sensitive species and addition of tolerant species is considered strong evidence that changes were caused by the pollutant. A fundamental step in the PICT measurements is the selection of an ecologically relevant parameter as endpoint that reflects the toxic effects at the community level (Blanck, 2002). The PICT approach has been used to study effects of chemicals on microbial communities with various methods such as Biolog™ plates (Schmitt et al., 2004), respirometer (Gong et al., 2000) and methane oxidation assay (Seghers et al., 2003). The BDOBS tool can be readily adapted for use in PICT testing by evaluating the effect of increasing doses of toxicants on the respiration of ecologically relevant substrates. One such compound is coumaric acid, a phenylpropanoid derivative related to the degradation of lignin, a key process in C cycling in soil (Peng et al., 2003).

The goal of this work was to assess the impacts of ERL of 2,4-D on soil microbial community structure and function. Structural shifts were studied by enumerating culturable degrading and non-degrading bacteria in microcosms spiked with 2,4-D. The influence of succinate on degraders' populations was also assessed. Functional changes were assessed at the community level with BDOBS as a tool for CLPP and PICT assays.

2. Materials and methods

2.1. Soil sampling

The study area is located in Saldungaray (province of Buenos Aires) in the southern Pampas region, Argentina. The soil is a sandy loam classified as Typic Argiudoll, with the following characteristics: pH (water) 6.6; total organic carbon, 20.1 g kg^{-1}; total nitrogen, 1.51 g kg^{-1} soil. Samples were taken from an uncultivated plot that had received applications of 2,4-D until 2004, in April and September 2006. Ten soil cores were randomly collected from the upper layer (0–10 cm) and then pooled to make a composite sample. Fresh, field-moist soil was sieved (<5.6 mm) and kept at 4 °C until treatment within 2 weeks of sampling. A pristine forest soil with no history of herbicide exposure was sampled at the Harvard Forest Chronic Nitrogen Addition Study (Massachusetts, USA) in October 2006 to be used as a control for the PICT study. Samples (upper 10 cm of mineral soil) were collected from three replicate subplots within the control treatment plots. More information about this site can be found in Frey et al. (2004).

2.2. Experimental setup

The effects of 2,4-D on microbial abundances were conducted using soil sampled in April. Microcosms were prepared in 150 ml plastic flasks containing 40 g of soil, with caps loosely fitted to allow aeration. Before the experiment was initiated, soil microcosms were pre-incubated at their field moisture state for 7 days at 28 °C in the dark. One milliliter of a filter-sterilized aqueous stock solution of either 2,4-D or sodium succinate was spiked by dropping with pipette to give a final concentration of 5 mg kg^{-1} soil of each compound. Controls received only filter-sterilized distilled water (FSDW). The stock solutions were prepared by dissolution of 2,4-D (≥97% purity; Fluka, Germany) and sodium succinate (98% purity, Fluka, Germany) in FSDW. A total of 27 microcosms were set and allocated to an initial control (time = 0 day) and four treatments (2,4-D, succinate (S), 2,4-D+S and untreated control, with 6 flasks each). Microcosms were incubated in the dark in growth chamber (28 ± 1 °C) for up to 33 days. In the attempt to simulate the continuous flux of exudates from the rhizosphere, the S and 2,4-D+S treated microcosms were supplemented weekly with succinate solution at the same rate as the initial dose; 2,4-D and control received an equivalent volume of FSDW. Humidity content of the microcosms was 15% (w/w, 60% water-holding capacity [WHC]) at the beginning of the assay and was gradually increased to WHC (25%, w/w).

The effects of 2,4-D on the function of soil microbial communities was assessed using soil sampled in September. Microcosms were prepared with 15 g of soil in 50-ml centrifuge tubes with caps loosely fitted. Before the experiment was initiated, soil microcosms were pre-incubated at their field moisture state for 7 days at 28 °C in the dark. One milliliter of filter-sterilized aqueous stock solution of 2,4-D of appropriate concentration (see above) was added to give a final dose of either 5 mg kg^{-1} (ERL) or 50 mg kg^{-1} (high level, HL), or FSDW (used as control) increasing soil moisture to WHC. There were 12 replicate flasks for each of ERL and control treatments, and 6 microcosms were allocated to HL treatment. Microcosms were incubated in a growth chamber set at 28 °C and 80% relative humidity in the dark for up to 3 weeks.

2.3. Enumeration of culturable 2,4-D-degrading and aerobic heterotrophic bacteria

Three replicate flasks per treatment were destructively sampled 4 and 33 days after treatment, and soil sub-samples (10 g dry wt. basis) were suspended in 0.85% NaCl and used as the basis for a

10-fold dilution series to estimate the most probable numbers of 2,4-D-degrading bacteria ($MPN_{2,4\text{-}D}$). Aliquots (0.5 ml) of appropriate dilutions were transferred to 5 ml of Loos liquid culture medium (Loos, 1975) (4 replicates per dilution level) containing 100 mg l^{-1} 2,4-D. In order to estimate the culturable aerobic heterotrophic bacteria (AHB) population, aliquots (0.1 ml) were streaked onto 1/10 strength Nutrient Agar (Laboratorios Britania, Argentina) plates. The incubation times were 28 days for 2,4-D degraders and 7 days for AHB, and both media were incubated at 28 °C. The presence of 2,4-D degrading bacteria following acidification of the medium was determined by a color change and presumptive positive tubes were further confirmed by UV scanning (230–310 nm) in a UV-2100 Shimadzu spectrophotometer. Tubes were scored positive for 2,4-D degradation when partial (at least 30%) or complete disappearence of the 283-nm absorption peak was observed (Loos, 1975). $MPN_{2,4\text{-}D}$ were calculated using MPN tables (Woomer, 1994).

2.4. BDOBS-community-level physiological profiling

The 96-well BDOBS plates (BD Biosciences, Bedford, MA, USA) were purchased from the manufacturer. Substrates used were casein, sodium acetate, sucrose, D-mannose (Sigma, St. Louis, MO, USA), sodium succinate (Alfa Aesar, MA, USA), *p*-coumaric acid (MP Biomedicals, OH) and 2,4-dichlorophenoxyacetic acid (Acros Organics, NJ, USA). Cycloheximide was purchased from Sigma (St. Louis, MO, USA). All stock solutions and deionized water used were filter-sterilized (<0.22 μm) and stored at 4 °C before loading the plates.

Eighty μl of each substrate was dispensed from a stock solution (150 mg l^{-1}) to attain a final concentration of 50 mg l^{-1} in the plate. The un-amended wells were filled with an equivalent volume of FSDW. Half of the wells in each plate received either 80 μl of FSDW or a cycloheximide solution to achieve a final concentration of 11 mg ml^{-1}, to assess whether the herbicide causes shifts in the relative substrate utilization of the overall microbial community (fungi and bacteria) versus bacteria alone. Three replicate microcosms of each ERL treatment and control were destructively sampled after 3, 7, 14 and 20 days of incubation, and 5 g soil sub-samples were mixed with 12.5 ml of FSDW and 5 ml of sterile 2 mm-glass beads in 50-ml polypropylene tubes. Tubes were shaken for 1 min by hand and 80 μl per well were inoculated into pre-loaded microplates, increasing the final volume per well to 240 μl. Aerobic degradation of 2,4-D was further studied in a separate microplate assay, comparing the utilization of 2,4-D as a C source (50 mg l^{-1}) in triplicate ERL and HL microcosms sampled after 3 and 14 days of incubation.

2.5. BDOBS–pollution-induced community-tolerance

Chronic effects of 2,4-D exposure in the agricultural soil were assessed by the PICT method, evaluating the respiratory response to coumaric acid in control and ERL microcosms sampled at the end of the incubation (20 days) in comparison to a pristine forest soil. BDOBS plates were pre-loaded with 80 μl of coumaric acid stock solution (50 mg l^{-1} final concentration) and 80 μl of stock solutions of 2,4-D, so as to achieve increasing concentrations of herbicide (0, 25, 50, 125 and 250 mg l^{-1}) in the wells. Soil slurries were prepared and loaded as described earlier. Assuming homogeneous mixing of soil slurries, the lowest herbicide level represent a concentration of 187.5 μg 2,4-D g^{-1} soil in the plate.

2.6. Plate incubation conditions

The plates were incubated at 30 °C and kinetic fluorescence readings were obtained every 15 min for up to 48 h in a Synergy HT microplate reader (Bio-Tek Instruments, VT, USA), using a 485 nm wavelength excitation filter and a 590 nm wavelength emission filter.

2.7. Data analysis

Most probable numbers of degrading bacteria were reported as the median and 95% confidence interval of three replicates. Counts of AHB were log transformed and analyzed by two-way analysis of variance (ANOVA, $\alpha = 0.05$), with time and treatment as factors.

The kinetic fluorescent data obtained with BDOBS were reported as normalized relative fluorescence units (NRFUs) by dividing the readings at each time point by the response at 1 h. NRFU was plotted against incubation time for each substrate to obtain respiration curves, from which several parameters can be calculated. Both peak height and integrated area under curve (AUC) reflect the rate of O_2 consumption but AUC also integrates information on the lag in response (Garland et al., 2003). To assess the effect of herbicide treatment on substrates utilization we analyzed peak height, as no differences in lag were observed. To evaluate the effects of cycloheximide in the CLPP experiment, we selected AUC between 1 and 8 h (i.e. AUC versus $t = 8$ h), based on usual incubation time for the selective inhibition SIR to avoid the confounding effect of killed microbial biomass turnover (Velvis, 1997). A similar rationale was used for the PICT analysis in order to avoid: (1) turnover of microbes killed by the herbicide and (2) degradation of 2,4-D in the plate masking or decreasing the toxic effect on the existing biomass. The respiration curves with coumaric acid only (without 2,4-D) were visually inspected and the point of onset of exponential growth (i.e. synthesis of new biomass, Horwath and Paul, 1994) was determined by a visible increase in the oxygen consumption rate after a lag period of 12 h. A respiratory index (RI) was calculated for each soil as the average AUC within initial 10 h of incubation. RI of the control wells was set to 100% and that of all herbicide levels was expressed as a percentage of the control.

CLPP results were subjected to a two-way ANOVA ($\alpha = 0.05$), with time and treatment as factors, excluding cycloheximide from the analysis. The CLPP response with cycloheximide was analyzed with a two-way ANOVA ($\alpha = 0.05$) within each sampling day. When the interaction term was significant the differences between means were evaluated with Fisher's LSD test ($P < 0.05$). All ANOVA were performed using R version 2.8.1 (R Development Core Team, 2008).

3. Results

3.1. Population dynamics of 2,4-D-degrading bacteria

The density of AHB was constant over incubation time and was not affected by the addition of 2,4-D or S (Fig. 1a). The $MPN_{2,4\text{-}D}$ of untreated microcosms sampled at the start of the incubation was 4.6×10^2 bacteria g^{-1}, well above detection limit of 2.5 bacteria g^{-1} (Fig. 1b). There were significant differences in 2,4-D degrader abundances between the untreated and 2,4-D-treated microcosms 4 days after treatment. No significant differences were observed between S and control and between 2,4-D and 2,4-D+S, although succinate addition to 2,4-D-treated microcosms tended to increase degrader population. The same trend was observed in microcosms sampled 33 days after treatment. However, there was 100-fold increase in degrader abundances in both 2,4-D-treated microcosms.

3.2. CLPP–BDOBS assay

Only minor changes in substrate utilization profiles were induced in soil microcosms by treatment with 5 mg kg^{-1} 2,4-D

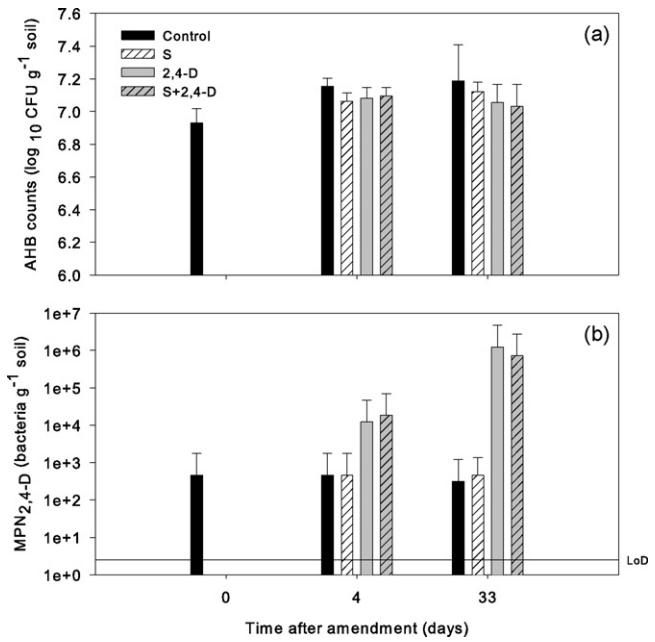

Fig. 1. Effect of combined amendments of 2,4-D and succinate on aerobic heterotrophic bacteria (AHB) counts (a) and most probable number of 2,4-D degraders ($MPN_{2,4\text{-}D}$) for soil microcosms sampled after 0, 4 and 33 days of incubation. AHB data are given as means ± S.E. ($n = 3$). $MPN_{2,4\text{-}D}$ data are represented as median of three replicates and 95% confidence intervals. LoD, limit of detection.

(Fig. 2). Casein consumption was higher in herbicide-treated microcosms over the 3-week incubation (ANOVA, $F_{1,16} = 7.71$, $P = 0.013$). Respiration with 2,4-D as a C source was stimulated in treated microcosms until day 14 (ANOVA T × H, $F_{3,15} = 11.37$, $P < 0.001$). Basal respiration (i.e. the response to background C) was slightly higher in treated than in control microcosms on day 7 (ANOVA T × H, $F_{3,16} = 18.05$, $P < 0.001$). By day 20, the use of all C sources

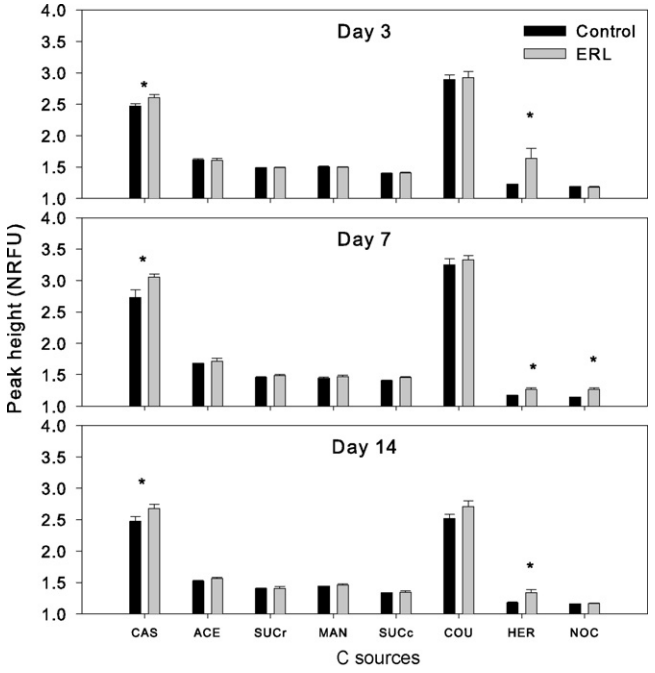

Fig. 2. Effect of 2,4-D (ERL = 5 mg kg^{-1}) on CLPP–BDOBS in soil microcosms sampled at different incubation times. CAS = casein, ACE = acetate, SUCr = sucrose, MAN = mannose, SUCc = succinate, COU = coumaric acid, HER = 2,4-D, NO C = unamended control. Values represent means of peak height ± S.E. ($n = 3$).

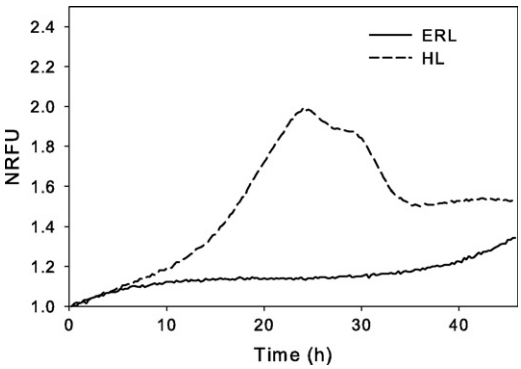

Fig. 3. Fluorescence curve (i.e. respiratory response) in BDODS plate with 2,4-D as sole source of C and energy, in 2,4-D-acclimated soil slurries after 14 days of incubation. Microcosms received 5 and 50 mg kg^{-1} 2,4-D (ERL and HL).

in herbicide-treated microcosms was the same as in the control soil (data not shown). Interactions between herbicide treatment and cycloheximide were not significant except for acetate on day 7 (ANOVA C × H, $F_{1,8} = 22.78$, $P = 0.001$), meaning that there was no differential effect of the herbicide on the fungal community in soil microcosms (data not shown). Use of 2,4-D as sole source of C and energy was retarded (>40 h) in both treatments at day 3, but after 14 days of incubation HL microcosms showed an earlier and larger respiratory response to 2,4-D in the plate in comparison to ERL microcosms (Fig. 3).

3.3. PICT–BDOBS assay

The PICT assay showed that the respiratory index with coumaric acid as substrate was slightly affected (<30%) by 2,4-D in a range of 25–125 mg l^{-1} in the agricultural soil, with no differences between treated microcosms and control. In contrast, coumaric acid respiration by the forest soil was reduced by >60% in the presence of herbicide doses of 50 mg l^{-1} and higher (Fig. 4).

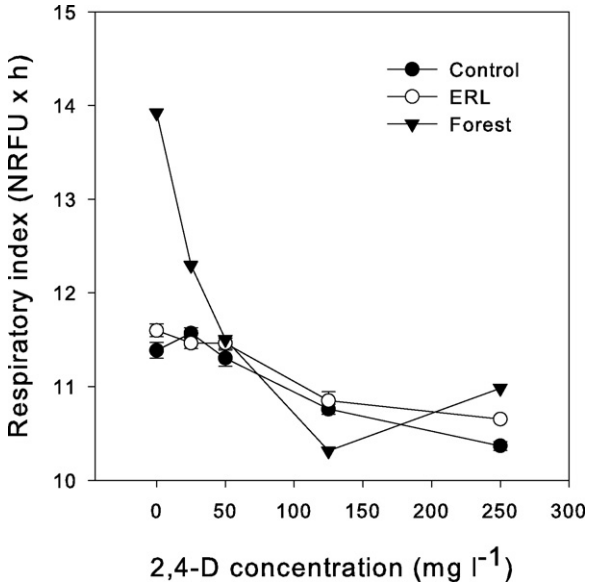

Fig. 4. Respiratory index with coumaric acid as C source, in agricultural soil treated with 5 mg kg^{-1} 2,4-D (ERL) or untreated (control) and forest soil, exposed to increasing doses of 2,4-D (25–250 mg l^{-1}) in BDOBS. Values represent means ± S.E. ($n = 3$); for forest soil is the average of two samples.

4. Discussion

4.1. Culturable heterotrophic and 2,4-D-degrading bacteria

In order to assess the microbial population response to the herbicide and the metabolically linked rhizospheric compound succinate, enumerations of aerobic heterotrophic and 2,4-D-degrading bacteria were performed in microcosms treated with either or both compounds. While aerobic heterotrophic bacteria were not affected by herbicide or succinate amendments, the 2,4-D-degrading population was significantly stimulated by the addition of herbicide to soil microcosms. The relatively high number of degraders at the start of the incubation suggests that this soil had a stable and measurable degrader population as a result of previous exposure to 2,4-D, which was rapidly enriched by freshly added herbicide. Similarly, Shaw and Burns (2005) found low numbers of 2,4-D degraders (<20 g^{-1}) in soils with no history of herbicide applications, while a garden soil previously treated with 2,4-D exhibited ten times higher densities. Overall, these results are in agreement with those reported by other authors in microcosms (Klappenbach et al., 2000; Merini et al., 2007) and in field (Ka et al., 1995) experiments for soils treated with 1–10 mg kg^{-1} of herbicide. The lack of effects of 2,4-D on heterotrophs should be interpreted with caution, given that less than 0.1–1% of soil microbes are cultivable (Torsvik et al., 1996). Several authors have investigated the effects of similar doses of 2,4-D on microbial community structure using molecular approaches, reporting either no effects (Bouseba et al., 2009) or transient enrichment of degraders, mainly proteobacteria (Klappenbach et al., 2000; Macur et al., 2007; Vieublé-Gonod et al., 2006).

Merini et al. (2007) found that the enrichment of 2,4-D degraders persisted after complete dissipation of 2,4-D from soil, and suggested that other 2,4-D-related compounds (metabolites or analogs) were sustaining these populations with time. If succinate was providing a C source for growth by 2,4-D degraders in our experiment, we would expected it to have an impact on the density of degraders even in the absence of herbicide, yet S microcosms showed similar MPN values than untreated soil. A 100-fold increase in $MPN_{2,4\text{-}D}$ in herbicide-treated microcosms was observed at the end of the incubation. We suggest that the degrading populations may have established stepwise, and had not reached the highest density of degraders 4 days after treatment. Similarly, Vieublé-Gonod et al. (2006) observed that 2,4-D mineralization (7.8 mg kg^{-1}) proceeded slowly during the first 2 days and reached a maximum after 15 days of incubation, as a result of growth of degrading populations initially present at low densities. Alternatively, the gradual increase in soil moisture may have led to herbicide residues desorption from the solid phase into the soil solution. Increasing soil water potential has been reported to influence the number of 2,4-D-degrading bacteria much more than the heterotrophic bacteria population (Han and New, 1994).

4.2. CLPP and 2,4-D degradation

The substrate utilization results indicate that ERL of 2,4-D induced only slight functional shifts (i.e. increase in the use of casein and 2,4-D) in the microbial community beyond a modest enrichment for 2,4-D degradation. Previous studies showed that 2,4-D caused minor temporary changes in functional diversity in this soil (Zabaloy et al., 2008a). Similarly, Ros et al. (2006) reported that a low dose of formulated atrazine (1 mg kg^{-1}) had negligible effects on potential functional diversity of soil; a moderate concentration (10 mg kg^{-1}) increased the diversity of carbohydrates utilization; while higher rates (100 and 1000 mg kg^{-1}) significantly reduced the use of carbohydrates, carboxylic acids and amino acids.

Maximal 2,4-D degradation potential of the microbial community was attained 2 weeks after treatment of soil microcosms. The slow mineralization of 2,4-D (50 mg l^{-1}) during the initial 3 days of incubation possibly corresponded to the lag phase of the specific degraders (Vieublé-Gonod et al., 2006; Lerch et al., 2009). During that period, 2,4-D degradation probably proceeded by co-metabolism in soil (Lerch et al., 2009), which explains the relatively low respiration rate when 2,4-D was supplied as the sole source of C and energy in the plate. Between the first 3 days and day 14 a shift in dominant degrader populations might have occurred in the HL microcosms, and the increase in use of 2,4-D in the BDOBS might reflect the activity of specific degraders (Lerch et al., 2009) that were favored by the high dose of herbicide. Macur et al. (2007) also reported that agriculturally relevant application rates of 2,4-D (10 mg kg^{-1}) provide a temporary selective advantage for microbes capable of using the herbicide as C and energy source. Overall, these results strengthen our hypothesis of a stepwise establishment of 2,4-D degrading populations after herbicide addition to soil.

4.3. PICT

The PICT concept states that the tolerance of a community to a toxicant is proportional to the past exposure of that community to the toxicant (Blanck, 2002). Our PICT study revealed that previous field exposure of the agricultural soil to 2,4-D, although additions had ceased 2 years before this study, was enough to develop resistant microbial populations. The presence of native 2,4-D-tolerant and degrading populations in the adapted agricultural soil was also suggested by our CLPP and MPN results. In contrast, increasing concentrations of 2,4-D in the plate had a more severe inhibitory effect on coumaric acid use in the forest soil that had never been directly exposed to 2,4-D. The differences in tolerance to the herbicide observed between the agricultural and the forest soils most probably reflect their contrasting exposure history. Even though pristine soils have been reported to harbor 2,4-D-tolerant or degrading bacterial species (Kamagata et al., 1997), the proportion or activity of such microbes was probably low. The high tolerance observed in the agricultural soil suggests that either previous exposure to 2,4-D led to a persistently tolerant community, or that the agricultural soils are more generally resistant to herbicide treatment. In a similar study, Seghers et al. (2003) reported that long-term use of atrazine and metolachlor selected towards a microbial community more tolerant to 2,4-D in an agricultural soil.

The absence of a clear difference in coumaric acid mineralization between the control soil and ERL treatment may mean that the dose of 5 mg 2,4-D kg^{-1} soil had not exerted a strong enough selection pressure to produce toxicant-induced succession in the ERL microcosms. As the coumaric acid response was evaluated within 10 h of incubation, 2,4-D degradation was not expected to occur to a significant extent, so we can exclude that the use of 2,4-D as a C source has confounded the ability to detect the toxic effects of the herbicide. Microbial activity (e.g. coumaric acid mineralization) may be affected by soil characteristics as well as other environmental factors other than contamination. However, increased tolerance to a specific contaminant is less sensitive to variation in physico-chemical variables, and more likely a direct results of contaminant exposure (Siciliano and Roy, 1999). In spite of the differences in physico-chemical properties between the forest and agricultural soils, the measurement of PICT on such microbial communities is still useful as a tool to distinguish an herbicide-exposed soil from an unpolluted one, as it has been reported that PICT response depends on the dose of toxicant and not on soil type (Gong et al., 2000).

4.4. Concluding remarks

The use of 2,4-D at recommended rates in agricultural soils may be considered sustainable in the long run, considering that it elicited only minor and transient effects on microbial community functional diversity. At ERL, 2,4-D produced an increase in degrading bacteria but not in heterotrophic bacteria counts, while the overall microbial community showed higher use of some substrates, including the herbicide itself. This may be seen as a beneficial capability of the microbial community that guarantees self-cleaning of herbicide-impacted agricultural soils. BDOBS proved to be useful for functional characterization and monitoring of 2,4-D degradation potential of microbial communities in agricultural soils intermittently exposed to 2,4-D. The PICT approach based on BDOBS successfully discriminated between the agricultural soil and a pristine forest soil. Future efforts should be directed towards validation of our PICT approach, with special emphasis on: (1) concurrent analysis of community structure with cultivation independent approaches; (2) assessing the robustness of the PICT response across various soils and toxicants.

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