Environmental Pollution 170 (2012) 32–38

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Protozoan predation in soil slurries compromises determination of contaminant mineralization potential

Nora Badawi [a,b,*], Anders R. Johnsen [a], Kristian K. Brandt [b], Jan Sørensen [b], Jens Aamand [a]

[a] Department of Geochemistry, Geological Survey of Denmark and Greenland, Øster Voldgade 10, DK-1350 Copenhagen K, Denmark
[b] Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 1 February 2012
Received in revised form
4 June 2012
Accepted 7 June 2012

Keywords:
Cycloheximide
Protozoan inhibition
Small volume samples
MCPA
MPN

ABSTRACT

Soil suspensions (slurries) are commonly used to estimate the potential of soil microbial communities to mineralize organic contaminants. The preparation of soil slurries disrupts soil structure, however, potentially affecting both the bacterial populations and their protozoan predators. We studied the importance of this "slurry effect" on mineralization of the herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA, ^{14}C-labelled), focussing on the effects of protozoan predation. Mineralization of MCPA was studied in "intact" soil and soil slurries differing in soil:water ratio, both in the presence and absence of the protozoan activity inhibitor cycloheximide. Protozoan predation inhibited mineralization in dense slurry of subsoil (soil:water ratio 1:3), but only in the most dilute slurry of topsoil (soil:water ratio 1:100). Our results demonstrate that protozoan predation in soil slurries may compromise quantification of contaminant mineralization potential, especially when the initial density of degrader bacteria is low and their growth is controlled by predation during the incubation period.

1. Introduction

Microbial degradation of organic contaminants in soil has often been studied using soil slurries, e.g. polycyclic aromatic hydrocarbons (Doick and Semple, 2003), hexachlorocyclohexanes (Bachmann et al., 1988), atrazine (Jablonowski et al., 2010), and chlorobenzoates (Brunsbach and Reineke, 1993). These studies focus on the impact of such parameters as pH, agitation and soil:-water ratio on mineralization rate and the mineralization potential of bacterial degraders (Doick and Semple, 2003; Woo et al., 2004; Stroud et al., 2009; Fenlon et al., 2011). Slurry studies have also been used to optimize the environmental conditions for introduced contaminant-degrading bacteria in soil bioremediation studies (Bachmann et al., 1988; Brunsbach and Reineke, 1993; Arshad et al., 2008). These and other studies based on soil slurries are often justified on the grounds that the use of slurries eliminates heterogeneity and hence major data variability. Provided the incubation period is short, soil slurry studies may sometimes reliably reflect pre-existing population activity levels. They do not necessarily mimic *in situ* soil conditions, however, as preparation of the slurry disrupts soil structure, dissolves and transports nutrients and microorganisms, and facilitates protozoan predation on the soil bacteria (Kuikman et al., 1989; Stroud et al., 2009).

Protozoan and metazoan predation plays a crucial role in microbial population dynamics in soil (Habte and Alexander, 1977; Lee and Welander, 1994; Rønn et al., 2002), and these organisms contribute importantly to organic matter degradation and nutrient release. Protozoa are most abundant in the topsoil, where organic matter and bacterial prey abound, but may also be found in subsoil (Sinclair and Ghiorse, 1987). Within the heterogeneous soil matrix active soil protozoa typically require the presence of a 30-μm thick water film; small amoeba may thrive in water films as thin as 5 μm, however (Hausmann et al., 2003). In a previous study, protozoan predation on bacterial cells was found to increase when the water content of the soil samples was raised, with the introduced bacteria being almost eliminated at 80% water holding capacity (Vargas and Hattori, 1986). In addition to water distribution, protozoan predation is also affected by soil porosity as pores with a diameter of less than approx. 3 μm are inaccessible to protozoa and the bacteria inhabiting them may therefore escape predation (Darbyshire et al., 1985; Vargas and Hattori, 1986).

In contrast to the situation in intact soil, protozoan motility (and hence predation) in slurries is not restricted to water films and slurries lack the heterogeneous soil matrix and microcavities that would otherwise protect the bacteria from protozoan predation. Depending on initial substrate conditions, population densities and duration of incubation, the use of soil slurries may therefore lead to growth of both bacterial and protozoan populations during mineralization assays. Despite the frequent use of soil slurries in

* Corresponding author.
E-mail address: nba@geus.dk (N. Badawi).

0269-7491/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.envpol.2012.06.013

studies of contaminant mineralization, however, no studies appear to exist that validate the slurry approach for quantification of soil mineralization potential. We hypothesized that specific populations of degrader bacteria may be subject to intensive protozoan predation in soil slurries, thereby compromising the validity of soil mineralization potentials determined in this manner. To investigate this hypothesis we prepared slurries from two soil layers differing in initial bacterial and protozoan population density, pesticide exposure and degradation potential. The mineralization of the pesticide 2-methyl-4-chlorophenoxyacetic acid (MCPA) in slurries of different soil:water ratio was compared to that in the intact soil using the eukaryotic inhibitor cycloheximide to reveal the impact of protozoan predation.

2. Materials and methods

2.1. Soil and slurry samples

The study was conducted using soil from the plough layer (median sampling depth was 8 cm, denoted topsoil) and subsoil (48 cm depth) from an agricultural field site at Højbakkegård, Taastrup, Denmark. The soil is a sandy loam. The composition of the topsoil is 48% silt + clay, 51% sand and 1.2% gravel (>2 mm), C_{org} 0.99% and pH 6.6, while that of the subsoil is 42% silt + clay, 51% sand and 7.7% gravel (>2 mm), C_{org} 0.27% and pH 6.3. The field is cultivated in the conventional manner with the use of pesticides, but a phenoxy acid herbicide has only been applied once (dichlorprop, Basagran M75, 0.75 L ha^{-1} in 2007). The soil samples were stored at 5 °C overnight and sieved (2 mm) prior to use.

The slurries were prepared by transferring 50-g (wet wt, water content: 14–16% of dry wt) samples of either topsoil or subsoil to 250 mL Bluecap bottles and adding sterile (autoclaved) Millipore water to yield soil:water ratios of 1:3, 1:10 and 1:100 (w/v) for topsoil and 1:3 (w/v) for subsoil. The slurries were placed on a horizontal shaker (140 rpm) for 30 min prior to use. Sieved samples of intact topsoil and subsoil were included for comparison in all the mineralization experiments in order to determine the impact of soil slurry preparation on mineralization.

2.2. Microbial populations

Culturable heterotrophic soil bacteria were enumerated from 100-μL aliquots of 10-fold dilution series (3 subsamples of each) of a 1:10 (w/v) slurry prepared from 50 g soil as described above and plated on quarter strength Tryptone Soy Agar (TSA, Bacto, France) containing 200 mg L^{-1} Delvocid (inhibits fungal growth, DSM food specialities, The Netherlands). The plates were incubated for 6 days at 15 °C and colony-forming units (CFU) then counted as described in Niemelä (1983).

Culturable MCPA-degrading bacteria were determined using a most probable number (MPN) ^{14}C-radio-respirometric microplate assay based on incubation of 3-fold dilution series of the 1:10 (w/v) slurry used for enumeration of the culturable heterotrophs ($n = 5$; 200 μL per well) (Johnsen et al., 2009). All wells were spiked with 20 μL of ^{14}C-ring-labelled MCPA solution (2500 dpm $well^{-1}$), corresponding to an initial MCPA concentration of 10 mg L^{-1}. Wells were scored as positive when the accumulated amount of $^{14}CO_2$ activity exceeded 10% of the initially added ^{14}C-labelled MCPA. The ^{14}C-radio-respirometric microplate assay is described in further detail below.

A total of all culturable protozoa (flagellates, amoeba and ciliates) were enumerated by a most probable number (MPN) assay in 96-well flat-bottomed microplates (Sarstedt Inc., Newton, USA) using a 3-fold dilution series ($n = 6$; 200 μL per well) of topsoil and subsoil in Neffs' amoeba saline (Page, 1988; Rønn et al., 1995) containing 0.3 g L^{-1} Tryptic Soy Broth (TSB, Bacto, France). The microplates were incubated at 15 °C and examined twice under low magnification (200×) over a 3-week incubation period using an inverted microscope (Olympus CK40). Microplate wells with visibly active protozoa were scored as growth-positive and used for calculation of MPN according to Hurley and Roscoe (1983).

2.3. Mineralization of MCPA

Mineralization of MCPA in the intact soil and slurry was determined using the ^{14}C-radio-respirometric microplate method of Johnsen et al. (2009) for measuring mineralization of ^{14}C-labelled compounds in small sample volumes. A radiolabelled working solution of ^{14}C-ring-labelled MCPA was freshly prepared for each experiment by dissolving analytical grade MCPA (purity 99%, Dr. Ehrenstorfer GmbH, Ausburg, Germany) and [ring-U-^{14}C]-MCPA (159.7 μCi mg^{-1}, purity > 95%, IZOTOP, Budapest, Hungary) in Millipore water. Deep-well microplates (96-well MegaBlock, 1.2 mL, Sarstedt, Switzerland) were used with 200 mg intact soil or 200 μL soil slurry in each well. Each well received 20 μL of ^{14}C-MCPA working solution to yield an initial MCPA concentration of 10 mg L^{-1} and 2500 dpm $well^{-1}$. The $^{14}CO_2$ produced from the mineralization of MCPA in each well was captured with $Ca(OH)_2$-amended filter paper discs (Whatmann 542 filter, 6 mm dia.). Each filter was loaded with 10 μL $Ca(OH)_2$ (0.5 M) suspended in a 1:15 dilution of wet-room wallpaper adhesive solution (Bostik Våtrumslim 78, Silvan, Denmark) and placed on microplate sealing tape (Seal Plate-PCR-SP, Axygen Inc., Union City, CA) in a pattern corresponding to the microplate wells. The sealing tape was changed weekly and the trapped $^{14}CO_2$ from each well quantified from a standard series of $NaH^{14}CO_3$ using digital autoradiography and subsequent digital image analysis (Johnsen et al., 2009).

2.4. Effect of protozoan predation on MCPA mineralization in slurry samples

As the effect of protozoan predation on contaminant mineralization was to be determined by using cycloheximide to inhibit protozoan activity, we first performed experiments to verify the duration of its inhibitory effect. Cycloheximide has previously been shown to inhibit both protozoan and metazoan activity (Lee and Welander, 1994). Cycloheximide (97.7% purity, Sigma Aldrich) dissolved in Millipore water was added to 100-mL Bluecap bottles ($n = 4$) each containing 10 mL of topsoil or subsoil slurry (soil:water ratio 1:3) to yield a final cycloheximide concentration of 400 mg L^{-1}. Parallel assays without cycloheximide were included for comparison ($n = 4$). All the bottles were incubated on a rotary shaker in the dark at 15 °C. After 0, 1, 2, 3, 6, 9 and 15 days, 100-μl aliquots were sampled from each flask, diluted in 3-fold series and used for MPN enumeration of protozoa as described above. Since protozoan growth during the MPN assay was inhibited by carry-over of cycloheximide from the lower dilutions, the detection limit in the MPN assay was defined as the lowest dilution at which at least one well contained active protozoa. Protozoan growth took place in the MPN wells when the cycloheximide concentration was reduced below approx. 1 mg L^{-1}, corresponding to an approx. detection limit of 500 protozoan cells mL^{-1} slurry.

The effect of protozoan activity on MCPA mineralization in topsoil slurries of differing dilution (soil:water ratio 1:3, 1:10 or 1:100, made as serial dilutions) was studied by adding cycloheximide (final concentration 400 mg L^{-1}) to the deep-well microplate wells as described above and comparing the results to mineralization in intact soil and slurries devoid of cycloheximide ($n = 21$). The subsoil studies were only performed using dense slurry (soil:water ratio 1:3). Independent control experiments (without cycloheximide) in which bacterial activity was completely inhibited by the respiratory inhibitor azide (25 μL 1% w/v sodium azide per well) showed that no abiotic mineralization of MCPA took place in the slurries (data not shown). The plates were sealed and incubated at 15 °C in the dark.

2.5. Statistics

The effect of cycloheximide on protozoan number in the slurry samples on each sampling day was tested by two-way analysis of variance. A modified student's *t*-test was used to test the pair-wise differences in culturable populations (protozoa, heterotrophic bacteria and MCPA degraders) in the topsoil and subsoil (Niemelä, 1983). All MPN estimates were log_{10}-transformed prior to statistical analysis.

To elucidate the statistical significance of the protozoan predation and slurry dilution on MCPA mineralization, comparison of the lag phases for the mineralization of MCPA in soil and slurry at different dilutions with and without cycloheximide was done – the lag phase was defined as the time period needed to achieve 10% MCPA mineralization. Mineralization curves not reaching 10% mineralization within the incubation period were given the value 60 days as the maximum time of incubation. The lag phase was selected as the most appropriate parameter for comparing the mineralization of MCPA, as at 10% mineralization of MCPA the mineralization curves were in the linear or exponential phase, thereby allowing comparison of the time lag. Furthermore, at a 10% cut-off the observed accumulated mineralization was not affected by mineralization of radiolabelled impurities in the MCPA stock solution and accumulation of background noise from the cyclone scanner. Survival analysis of the time lags was done and the groups: soil, slurry with and without cycloheximide, and dilution level were pair-wise compared using log–rank test.

The analyses were made using Excel 2003 or R version 2.10.1 (www.r-project.org) with a 5% significance level.

3. Results

3.1. Culturable populations of heterotrophic bacteria, MCPA degraders and protozoa

Culturable populations of heterotrophic bacteria, MCPA-degrading bacteria and protozoa were present in both the topsoil and the subsoil from the agricultural field site, but population densities were significantly lower in the subsoil ($p < 0.0001$). Thus the number of culturable heterotrophs decreased from $7.2 \pm 0.6 \times 10^6$ cells g^{-1} in topsoil to $4.6 \pm 0.4 \times 10^6$ cells g^{-1} in subsoil, while the estimated number of MCPA degraders decreased from approx. 610 cells g^{-1} in topsoil to approx. 15 cells g^{-1} in subsoil, and the estimated protozoan abundance decreased from 6.7×10^3 cells g^{-1} in topsoil to 1.1×10^3 cells g^{-1} in subsoil.