Denitrifier Community Dynamics in Soil Aggregates under Permanent Grassland and Arable Cropping Systems

M. N. Miller
Agriculture and Agri-Food Canada
Potato Research Centre
Fredericton, NB, Canada E3B 4Z7
and
Univ. of Guelph
Dep. of Environmental Biology
Guelph, ON, Canada N1G 2W1
currently at
AgriTECH Park
90 Research Dr.
Bible Hill, NS, Canada B6L 2R2

B. J. Zebarth*
Agriculture and Agri-Food Canada
Potato Research Centre
Fredericton, NB, Canada E3B 4Z7

C. E. Dandie
Agriculture and Agri-Food Canada
Potato Research Centre
Fredericton, NB, Canada E3B 4Z7
currently at
CSIRO Land and Water
Waite Rd
Glen Osmond, SA 5064, Australia

D. L. Burton
Nova Scotia Agricultural College
Dep. of Environmental Sciences
Truro, NS, Canada B2N 5E3

C. Goyer
Agriculture and Agri-Food Canada
Potato Research Centre
Fredericton, NB, Canada E3B 4Z7

J. T. Trevors
Univ. of Guelph
Dep. of Environmental Biology
Guelph, ON, Canada N1G 2W

A better understanding of the spatial distribution of denitrifiers and their activity may lead to an improved understanding of the denitrification process in soil. This study determined the spatial distribution of the total bacterial community (16S rRNA), components of the denitrifier community (*cnorB*$_P$; *Pseudomonas mandelii* and related species; *nosZ*), and denitrification activity across a range of soil aggregate size fractions (4–8, 1–2, and 0.25–0.5 mm) under permanent grassland (PG) and arable cropping (AC) systems. Aggregate size fraction had no significant effect on the abundance of the *nosZ* or *cnorB*$_P$ gene-bearing bacteria in soil from the AC system. The highest abundance of denitrifier bacteria was measured in the smallest size fraction in the PG system. Respiration did not differ among aggregate size fractions within the PG system; however, respiration was higher for the PG system than the AC system for all aggregate size fractions. For the AC system, higher respiration was measured in the 0.25- to 0.5-mm aggregate fraction than the 4- to 8-mm aggregate fraction. Denitrifying enzyme activity (DEA) was higher in the largest size fraction of the PG system than the AC system; however, DEA did not differ among aggregate size fractions within each management system. Cumulative denitrification during a 72-h incubation was significantly higher in the largest aggregate size fractions under both management systems. The results indicate that the differences among the aggregate size fractions were small in magnitude and that the spatial location of the denitrification activity and the abundance of the denitrifier bacteria were uncoupled across aggregate size fractions in the contrasting management systems.

Abbreviations: AC, arable cropping; DEA, denitrifying enzyme activity; EOC, extractable organic carbon; PCR, polymerase chain reaction; PG, permanent grassland.

Denitrification has received attention because it is one of the primary soil microbial processes leading to the production of N_2O, a greenhouse gas and natural catalyst of stratospheric ozone degradation (Intergovernmental Panel on Climate Change, 2001). Bacteria capable of denitrification and N_2O production are widely distributed in the environment and exhibit a high taxonomic diversity (Philippot et al., 2007). In soil, the heterogeneity of their habitat affects the distribution of these bacteria. This heterogeneity arises from the combined biotic and abiotic factors present within the soil, such as competition, predation, temperature, pH, O_2 concentration, water content, and available substrates (Ladd et al., 1996; Franklin and Mills, 2003). Most microbial research related to denitrification has been performed on a macroscale basis (i.e., bulk soil); however, the bacteria responsible for this process live and interact with their environment at a microscale level.

One approach used to examine soil microbial habitats is in the context of soil aggregates. Soil aggregates vary in stability from weak, large macroaggregates (>0.25 mm) to tightly bound microaggregates (<0.25 mm) (Tisdall and Oades, 1982). Microaggregates generally form by microbially mediated processes and are relatively stable. They form as a result of the binding between organic and mineral colloids, in part by multivalent cations and by polysaccharides of microbial and plant origin. Macroaggregates are less stable and form by loose associations of microaggregates, minerals, and particulate organic matter, largely held together by fungal hyphae and plant roots (Tisdall and Oades, 1982).

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*Corresponding author (bernie.zebarth@agr.gc.ca).
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Soil aggregation can undergo permanent or temporal alterations due to various soil management practices including tillage and the amount and quality of the organic residues incorporated into the soil (Plante and McGill, 2002). Tillage, for example, can affect the organic matter content of the soil, which is involved in the formation and stabilization of aggregates (Tisdall and Oades, 1982). Macroaggregate stability is thought to respond rapidly to changes in soil management (Tisdall and Oades, 1982). Greater aggregate stability and soil organic matter contents of macroaggregates have been reported in native grassland or under reduced tillage in comparison with conventionally tilled systems (Beare et al., 1994; Six et al., 1998).

Microorganisms and their activities are heterogeneously distributed among soil aggregates and their distribution may change in response to management practices that affect aggregate formation. Fractionation studies have shown that the microbial community differs within and among aggregate size fractions (Gupta and Germida, 1988; Lensi et al., 1995; Ranjard and Richaume, 2001; Sessitsch et al., 2001). Previous research examining soil aggregation and denitrifying activity have reported conflicting results, with higher amounts of denitrification reported in both the smallest (Seech and Beauchamp, 1988; Sey et al., 2008) and largest (Drury et al., 2004) aggregate fractions studied. Little is known about the abundance of denitrifiers and their function among soil aggregate size fractions. Lensi et al. (1995), using the most probable number method, found that the number of denitrifiers increased as aggregate size decreased in both permanent pasture and continuous cultivation management systems.

Molecular-based tools have improved our ability to examine soil denitrifier communities. One such method, quantitative polymerase chain reaction (PCR), targets functional genes involved in the denitrification process including nitrate reductase (*narG*, *napA*), nitrite reductase (*nirS*, *nirK*), nitric oxide reductase (c*norB*, *qnorB*), and nitrous oxide reductase (*nosZ*). Several studies have used quantitative PCR to quantify denitrifiers in bulk soil samples (Henry et al., 2006; Dandie et al., 2007); however, this approach has not been used to investigate soil denitrifiers in soil aggregate fractions. Examining small-scale soil heterogeneity (i.e., soil aggregates) by using quantitative PCR may provide insight into the spatial distribution of denitrifiers and their activity. Denitrification has been reported to be unevenly distributed in soil, leading to the concept of "hot spots" that are linked to local, transient, available C for microbial growth and activity (Parkin, 1987). This type of microscale approach should be utilized to strengthen our understanding of denitrification (Philippot et al., 2007) since the soil environments experienced by organisms are not always reflected by measurements of these conditions performed on bulk soil samples (Parkin, 1987).

The objective of this study was to determine the spatial distribution of denitrification, DEA, the bacterial community (quantified using the 16S rRNA gene), and components of the denitrifier community (*nosZ* and *cnorB* gene-bearing bacteria) across a range of soil aggregate size fractions under both PG and AC systems. These systems were chosen to reflect contrasting managements that may influence the aggregate size distribution and properties. Quantification of the denitrifier community was done using a broad-range primer set used for analysis of the *nosZ* gene (Henry et al., 2006) and a narrow-range primer set based on the *cnorB* gene targeting *Pseudomonas mandelii* and closely related species ($cnorB_P$) guild (Dandie et al., 2007) developed because it is one of the dominant culturable denitrifiers found in our experimental potato (*Solanum tuberosum* L.) cropping system. This denitrifier guild was previously found to be responsive to C addition and to vary with both time and space under laboratory and field conditions (Miller et al., 2008; Dandie et al., 2008).

MATERIALS AND METHODS

Soil

Soil was collected from two fields located about 500 m apart in Fredericton, NB, Canada (45°52'N, 66°31' W) in October 2006. They belong to the Research Station soil association (coarse loamy morainal ablational till over coarse loamy morainal lodgement till), and are classified in the Canadian soil classification system as Orthic Humo-Ferric Podzols (Rees and Fahmy, 1984), equivalent to Typic Haplorthods in the U.S. Soil Taxonomy. Irrigation was not applied to either field.

In the field under AC, soil (0–15 cm) was collected from the potato phase of a potato–spring wheat (*Triticum aestivum* L.) rotation to represent an arable cropping system under continuous cultivation. Potato is the dominant arable crop in Atlantic Canada. The plot was managed according to standard recommended practices (Dandie et al., 2008). Fertilizer was banded at planting at 200 kg N ha^{-1} as NH_4NO_3, 150 kg P_2O_5 ha^{-1} as triple superphosphate (0–46–0), and 150 kg K_2O ha^{-1} as KCl (0–0–60). Soil was collected from the potato hill after vine desiccation when vines and tubers were still present.

In the second field, soil (0–15 cm) was collected from a PG management system that had not been tilled for at least 10 yr. The dominant grass species was timothy (*Phleum pratense* L.) and very little legume was present in the stand. Fertilizer was surface broadcast in May 2006 at 75 kg N ha^{-1} as NH_4NO_3. The field is generally fertilized every 2 yr with a similar rate of fertilizer application. Hay is removed annually in a one-cut system.

Due to constraints in sampling area locations, soil was collected from one representative location in each field. At each location, several soil samples were obtained and combined to form a single composite sample from which aggregates were obtained. Soil was collected by carefully loosening the upper 15-cm layer of soil from below with a flat spade to preserve the natural aggregates and minimize aggregate disturbance. The soil was placed in crush-resistant containers and stored at 3°C. The soils were laid out between sheets of brown paper and allowed to dry for 24 h to a gravimetric water content of 0.15 kg kg^{-1} before fractionation. This process was conducted at 3°C to minimize the impact of air drying on microbial communities and activities. Using a dry-sieving procedure, three air-dry aggregate fractions, 4 to 8, 1 to 2, and 0.25 to 0.5 mm, were separated by placing 200-g subsamples of soil on the top of a stack of six sieves (8, 4, 2, 1, 0.5, and 0.25 mm) and agitating for 60 s with a sieve shaker at amplitude 2 (Octagon 2000, Endecotts Ltd., London). Aggregate size fractions <0.25 mm represented a small (<2%) proportion of the total soil in both management systems and consequently were not used in the study. The aggregates were sieved and stored in polyethylene bags at 3°C in the dark before experimentation. Soil texture was determined by the pipette method following organic matter removal. Soil concentrations of organic C and total N were determined by combustion (LECO CNS 1000, LECO Corp., St. Joseph, MI). Soil pH was determined using a 1:1 soil/water suspension.

Experimental Design

Our objective was to determine the spatial distribution of the total bacteria community and components of the soil denitrifier bacteria and their activity (denitrification and DEA) in the soil aggregate fractions from contrasting AC and PG systems. The experiment was a completely randomized experimental design with six treatments replicated five times. Treatments included aggregate size fractions of 4 to 8, 1 to 2, and 0.25 to 0.5 mm collected from each soil management system. These aggregate fractions were chosen based on the results from preliminary experimentation conducted in November 2006 (2 wk after the initial soil collection) in the AC system, which showed that cumulative denitrification during a 72-h incubation period was highest for the 4- to 8- and 2- to 4-mm aggregate fractions, intermediate for the 2- to 4-mm fraction and bulk soil (<8 mm), and lowest for the 0.5- to 1- and 0.25- to 0.5-mm aggregate fractions (data not shown). The three aggregate fractions used in the current study were representative of the range of cumulative denitrification observed.

Soil treatments were assembled at 3°C in January 2007, 10 wk following the initial soil collection. Soil aggregate fractions (50 g) were gently placed on a weigh boat and then transferred to the bottom of a 1-L canning jar. Each jar received 50 mg N kg^{-1} as KNO_3, by slowly pipetting sufficient solution on the soil to achieve a water content of 0.43 kg kg^{-1}. This water content was chosen to represent the water content of a soil at 70% water-filled pore space, assuming a bulk density of 1 Mg m^{-3} and a particle density of 2.65 Mg m^{-3}. The jars were sealed with lids fitted with a septum to allow gas sampling. Each jar was then evacuated to 0.27 kPa and flushed with He. Each jar then received a 10% volume of C_2H_2, generated from CaC_2 and water, to block N_2O reduction to N_2. The jars were immediately incubated at 25°C for 72 h. Time zero was the time at which the jars were placed in the incubation chamber. Jars were destructively sampled at 0, 24, 48, and 72 h to allow DNA extraction, real-time PCR assays, and DEA and biochemical measurements.

Extraction of DNA and Quantitative Polymerase Chain Reaction

Soil samples in 15-mL tubes were freeze-dried overnight until completely dry and stored at −80°C before nucleic acid extraction. Soil DNA was extracted from 0.25 g of freeze-dried soil as previously reported by Griffiths et al. (2000) and modified by Dandie et al. (2007). The DNA was quantified using the Picogreen assay (Invitrogen, Burlington, ON, Canada) and fluorescence was measured on a Fluoroskan Ascent fluorometer (Thermo Scientific, Waltham, MA). Amplification of quantitative PCR products was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Real-time PCR amplification of the 16S rRNA gene (as a measure of total community numbers), *$cnorB_P$* gene, and *nosZ* gene was conducted as previously reported by Dandie et al. (2007) and Miller et al. (2008). The T4 gene 32 protein (250 ng per reaction; New England Biolabs, Pickering, ON, Canada) was added to reactions to reduce inhibition by soil-derived components.

Standard curves relating the threshold cycle (C_T) value and 16S rRNA, *cnorB*, or *nosZ* gene copy numbers were generated with three replicate 10-fold serial dilutions of a known quantity of plasmid DNA containing a fragment of the gene (Dandie et al., 2007; Miller et al., 2008). Standard curves were used for each 96-well real-time PCR plate. Due to the large sample numbers, multiple plates were used to analyze samples from the experiment and replicate calibrator samples were used on each plate.

Sampling and Analysis

Moisture content was determined gravimetrically at each sampling time. The NO_3^-, NH_4^+, and extractable organic C (EOC) were extracted by shaking 25 g of soil for 30 min with 50 mL of 0.5 mol L^{-1} K_2SO_4. Extracts were filtered and stored at −20°C pending analysis. Concentrations of NO_3^-, NH_4^+, and EOC in the filtered aqueous extract were measured using a Technicon Auto Analyzer II (Bran+Luebbe, Norderstedt, Germany) system (Miller et al., 2008).

Headspace gas samples were taken at 0, 24, 48, and 72 h and analyzed for N_2O and CO_2 by gas chromatography. Gas analysis was performed using a Varian Star 3800 gas chromatograph (Varian, Walnut Creek, CA) as previously described (Miller et al., 2008). The N_2O and CO_2 concentrations were corrected for temperature and pressure changes in the jars, and gas dissolved in the water was estimated (Moraghan and Buresh, 1977) in all calculations. Denitrification was measured as the accumulation of N_2O emissions in the presence of C_2H_2. Cumulative CO_2 emissions were used as a measure of respiration in the soil treatments.

Denitrification enzyme activity was measured at each sampling period, as previously described in Miller et al. (2008). Soil (25 g) was amended with 25 mL of a solution containing 10 mmol L^{-1} glucose, 10 mmol L^{-1} KNO_3^-, 10 mmol L^{-1} K_2HPO_4, and 0.100 g L^{-1} chloramphenicol. Samples were made anoxic by flushing with He; C_2H_2 was added and the samples were incubated for 1 h. The N_2O concentration was measured as described above.

Statistical Analysis

All statistical analyses were conducted using the general linear model of SAS (Version 8, SAS Institute, Cary, NC). All non-normal data was logarithmically transformed. Treatment means were calculated using uniform minimum variance unbiased estimators (Parkin and Robinson, 1994). Results were similar to the arithmetic means, therefore treatment means and standard errors presented in tables and figures were calculated from untransformed data. Means comparisons were performed using the LSmeans test, although if the interaction was not significant, treatment means were compared using a protected LSD test. The relationships between denitrifier abundance and other measured parameters were explored using linear regression analyses and Pearson correlation coefficients. Significance was accepted at a level of probability of $P < 0.05$.

RESULTS

Aggregate Properties

The aggregate size distribution differed between the PG and AC management systems (Table 1). Soil from the PG system had a greater proportion of soil in the 1- to 2-mm aggregate fraction and a lower proportion of soil in the 0.25- to 0.5-mm aggregate fraction compared with soil from the AC system. The proportion of soil in the 4- to 8-mm aggregate fraction did not differ between soils from the AC and PG systems.

Organic C was significantly lower in the 0.25- to 0.5-mm aggregate fraction than the 4- to 8-mm aggregate fraction in the AC system (Table 1). In the PG system, organic C was significantly higher in the 1- to 2-mm aggregate fraction than the two other size fractions. When comparing between management systems, there was significantly higher organic C in the 4- to 8- mm aggregate fraction of the soil for the AC system than the PG system, whereas organic C concentration did not differ between the two management systems for the 1- to 2- and 0.25- to 0.5-mm

Table 1. Initial soil contents of organic C, total N, sand, silt, and clay of three soil aggregate fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems.

Aggregate fraction	Aggregate distribution	Organic C	Total N	Sand	Silt	Clay
mm	%	g kg^{-1}				
AC 4–8	24.3 (1.9)†	36 (3.8) A‡	2.1 (0.10) A	408 (4.8) C	467 (3.9) A	123 (0.8) B
AC 1–2	22.0 (0.6)	31 (2.6) AB	1.8 (0.14) A	416 (6.6) BC	460 (6.7) A	123 (0.3) B
AC 0.25–0.5	9.7 (1.0)	27 (0.3) B	1.7 (0.02) A	457 (8.8) A	425 (6.5) C	116 (2.3) C
PG 4–8	23.7 (1.2)	28 (0.4) B	1.9 (0.03) A	425 (0.8) BC	437 (1.2) B	137 (0.9) A
PG 1–2	28.4 (2.0)	32 (1.7) A	2.2 (0.21) A	429 (3.3) B	435 (4.0) B	135 (2.8) A
PG 0.25–0.5	2.3 (0.7)	26 (0.3) B	1.8 (0.02) A	437 (11.0) B	427 (12.0) C	135 (1.3) A

† Values in parentheses are standard errors ($n = 5$).

‡ Means within a column followed by the same letter are not significantly different at $P < 0.05$.

aggregate fractions (Table 1). The soil total N concentration was not significantly different among the aggregate size fractions within or between the soils from the AC and PG systems (Table 1). The sand and silt contents differed significantly among the aggregate size fractions in both management systems, whereas the clay content only differed significantly among the AC aggregate fractions (Table 1). The sand content generally increased with decreasing aggregate size fraction in both the AC and PG systems. The lowest contents of silt and clay were measured in the 0.25- to 0.5-mm aggregate fraction in the AC management system.

The initial soil NO_3^- concentrations were significantly higher in the soil from the AC system than from the PG system, with average values of 2.1 and 0.1 mg NO_3^-–N kg^{-1} soil, respectively (Table 2). The initial soil NO_3^- concentrations did not differ significantly among the aggregate size fractions within each management system.

The initial soil NH_4^+ concentrations varied among aggregate size fractions in each management system; however, values were very low, <1 mg NH_4^+–N kg^{-1} soil, in all cases (Table 2). Soil NH_4^+ concentrations were highest for the 1- to 2-mm aggregate fraction in the AC system and for the 0.25- to 0.5-mm aggregate fraction in the PG system.

The initial soil EOC concentrations did not differ significantly among the aggregate size fractions within the PG management system, with an average value of 44 mg EOC kg^{-1} soil (Table 2). In soil from the AC system, the EOC concentration was lowest in the 4- to 8-mm aggregate fraction.

Nitrate, Ammonium, and Extractable Organic Carbon

Following the addition of 50 mg NO_3^-–N kg^{-1} to each treatment, no significant decrease in soil NO_3^- was measured with time in any treatment (data not presented). Soil NO_3^- concentration varied significantly among the aggregate size fractions from both systems (Fig. 1). For both the AC and PG systems, the soil NO_3^- concentration was significantly lower in the 4- to 8-mm aggregate fraction than the 0.25- to 0.5-mm aggregate fraction. For the same aggregate size fractions, the soil from the AC system had significantly lower NO_3^- concentrations than the soil from the PG system.

Table 2. Initial soil NO_3^-, NH_4^+ and extractable organic C (EOC) concentrations of three soil aggregate size fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems.

Aggregate fraction	NO_3^-	NH_4^+	EOC
mm	mg N kg^{-1} soil		mg EOC kg^{-1} soil
AC 4–8	2.05 (0.66) A†	0.18 (0.03) DE	46.1 (2.6) BC
AC 1–2	2.35 (0.75) A	0.49 (0.04) BC	51.9 (0.5) AB
AC 0.25–0.5	2.09 (0.57) A	0.15 (0.01) E	52.7 (0.7) A
PG 4–8	0.15 (0.02) B	0.33 (0.11) CD	45.7 (1.5) BC
PG 1–2	0.13 (0.01) B	0.50 (0.01) B	43.5 (1.1) C
PG 0.25–0.5	0.13 (0.01) B	0.80 (0.03) A	44.5 (1.0) C

† Values in parentheses are standard errors ($n = 5$). Means within a column followed by the same letter are not significantly different at $P < 0.05$.

The soil EOC concentrations were significantly lower in the 4- to 8-mm aggregate fraction than in the 0.25- to 0.5-mm aggregate fraction for both the AC and PG systems (Fig. 1). Aggregate size fractions from the PG system had significantly higher EOC concentrations than the same size fractions from the AC system, except in the 4- to 8-mm aggregate fraction.

Soil NH_4^+ concentrations did not differ significantly among the aggregate size fractions from the AC system (Fig. 1). For the PG system, the soil NH_4^+ concentration was significantly higher in the 0.25- to 0.5-mm aggregate fraction, 4.4 mg NH_4^+–N kg^{-1} soil, in comparison with the 1- to 2- and 4- to 8-mm aggregate fractions, an average of 3.4 mg NH_4^+–N kg^{-1} soil (Fig. 1). Aggregate size fractions from the AC system had significantly lower NH_4^+ concentrations than the same aggregate size fractions from the PG system.

Denitrifying Enzyme Activity, Respiration, and Denitrification

Within each management system, DEA was not significantly different among the aggregate size fractions (Table 3). The management system did not have a significant effect on DEA except for the 4- to 8-mm aggregate fraction, which had a higher value of DEA for the PG system, 0.30 mg N kg^{-1} h^{-1}, compared with the AC system, which had 0.12 mg N kg^{-1} h^{-1}.

Cumulative respiration during the 72-h incubation varied with aggregate size fraction and management system. Cumulative respiration at the end of the incubation for the PG system averaged 62 mg CO_2–C kg^{-1} soil and did not differ among aggregate size fractions (Table 4). For the AC system, greater cumulative respiration at the end of the incubation was measured in the 0.25- to 0.5-mm fraction, 56 mg CO_2–C kg^{-1} soil, compared with the 4- to 8-mm aggregate fraction, 47 mg CO_2–C kg^{-1} soil. Cumulative respiration at the end of the incubation was higher for the PG system than the AC system for all aggregate size fractions.

Cumulative denitrification during the 72-h incubation was <0.18 mg N kg^{-1} soil in all treatments (Table 4). There was no significant treatment × time interaction on cumulative denitrification. When averaged across aggregate size fraction, management system had no effect on cumulative denitrification; however, there was a significant effect of aggregate size fraction on

cumulative denitrification. Within the AC system, cumulative denitrification was highest in the 4- to 8-mm aggregate fraction, intermediate in the 1- to 2-mm aggregate fraction, and lowest in the 0.25- to 0.5-mm aggregate fraction, whereas in the PG system, cumulative denitrification was higher in the 4- to 8-mm aggregate fraction than in the 1- to 2- and 0.25- to 0.5-mm aggregate fractions. In addition, there was also a significant effect of time on cumulative denitrification, with a significant increase in cumulative denitrification between time zero and 72 h.

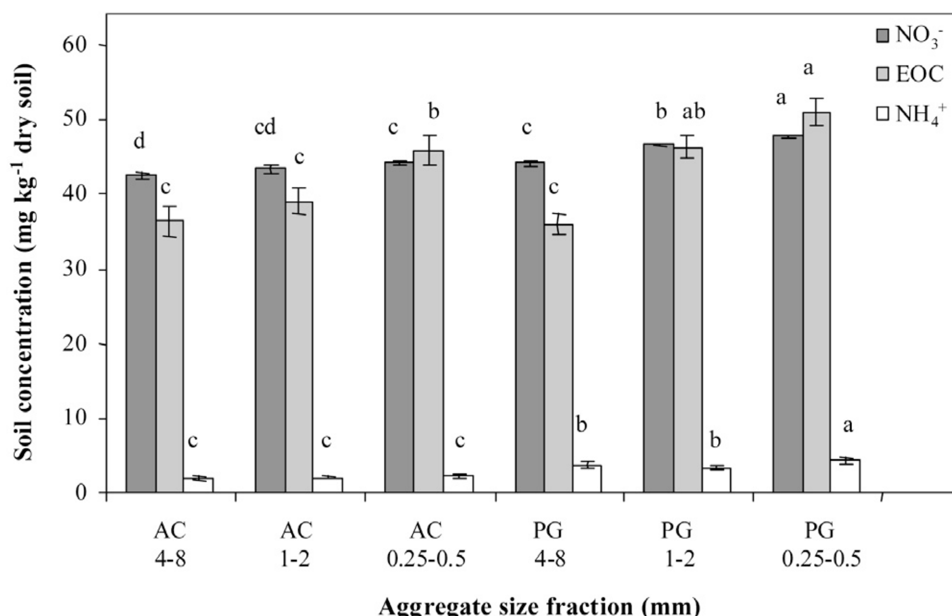

Fig. 1. Concentrations of NO_3^-, NH_4^+, and extractable organic C (EOC) of soil aggregate fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems averaged over time. Treatment means with the same letter are not significantly different (*P* < 0.05) across both soil types based on a protected LSD test. Values represent means (*n* = 20) ± standard error.

Total Bacteria and Denitrifier Abundance

There was no significant effect of aggregate size fraction from the PG system on the total bacterial community measured as 16S rRNA gene copy numbers (Fig. 2). In contrast, the smallest aggregate size fraction from the AC system had a significantly higher total bacterial community, an average of 1.0×10^{11} 16S rRNA gene copies kg^{-1} dry soil, compared with the largest aggregate size fraction, average of 6.1×10^{10} 16S rRNA gene copies kg^{-1} dry soil. The 16S rRNA gene copy numbers for the 1- to 2- and 0.25- to 0.5-mm aggregate fractions were significantly higher for the AC system than the PG system, whereas there was no effect of management system on total bacterial community in the 4- to 8-mm aggregate fraction.

There was no significant effect of aggregate size fraction on the abundance of *nosZ* gene-bearing bacteria for the AC system (Fig. 2). In contrast, the smallest aggregate size fraction for the PG system had significantly more *nosZ* gene-bearing bacteria, an average of 4.0×10^{10} *nosZ* gene copies kg^{-1} dry soil, compared with the 1- to 2-mm aggregate fraction, which had an average of 2.5×10^{10} *nosZ* gene copies kg^{-1} dry soil. The management system had a significant effect on the abundance of *nosZ* gene copy numbers, with both the 4- to 8- and 1- to 2-mm aggregate fractions having significantly more *nosZ* gene-bearing bacteria in the AC system than in the PG system. The abundance of *nosZ* gene copy numbers for the 0.25- to 0.5-mm aggregate fraction averaged 4.1×10^{10} *nosZ* gene copies kg^{-1} dry soil and did not differ significantly between management systems.

There was also no significant effect of aggregate size on the abundance of $cnorB_P$ gene-bearing bacteria for the AC system. In contrast, their abundance was significantly higher, an average of 3.1×10^9 $cnorB_P$ gene copies kg^{-1} dry soil, in the 0.25- to 0.5-mm aggregate fraction in the PG system than the 1- to 2-mm aggregate fraction, which had an average of 1.8×10^9 $cnorB_P$ gene copies kg^{-1} dry soil (Fig. 2). The management system did not have a significant effect on the abundance of $cnorB_P$ gene copy numbers.

DISCUSSION

The aggregates were carefully handled to preserve them and minimize disturbance, and the method of aggregate fractionation used a low energy input, therefore the aggregate size fractions obtained should reflect those present under field conditions. Various techniques have been used to obtain soil aggregate fractions, with many studies using either a dry or wet sieving

Table 3. Average value of denitrifying enzyme activity (DEA) measured at four sampling times during a 72-h incubation in three soil aggregate size fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems.

Aggregate fraction	DEA
mm	mg N kg^{-1} soil h^{-1}
AC 4–8	0.12 (0.03) A†
AC 1–2	0.15 (0.04) A
AC 0.25–0.5	0.10 (0.03) A
PG 4–8	0.30 (0.04) B
PG 1–2	0.27 (0.07) AB
PG 0.25–0.5	0.19 (0.05) AB

† Values in parentheses are standard errors (*n* = 20). Means within a column followed by the same letter are not significantly different at *P* < 0.05.

Table 4. Cumulative respiration and denitrification (cumulative N_2O emissions in the presence of C_2H_2) at the end of a 72-h incubation in three soil aggregate size fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems.

Aggregate fractions	Respiration	Denitrification
mm	mg CO_2–C kg^{-1} soil	mg N_2O-N kg^{-1} soil
AC 4–8	47 (2.1) C†	0.18 (0.04) A
AC 1–2	52 (1.4) BC	0.07 (0.03) A
AC 0.25–0.5	56 (0.8) B	0.009 (0.003) B
PG 4–8	63 (3.5) A	0.10 (0.08) A
PG 1–2	63 (2.5) A	0.01 (0.003) B
PG 0.25–0.5	60 (3.0) A	0.01 (0.001) B

† Values in parentheses are standard errors (*n* = 5). Means within a column followed by the same letter are not significantly different at *P* < 0.05.

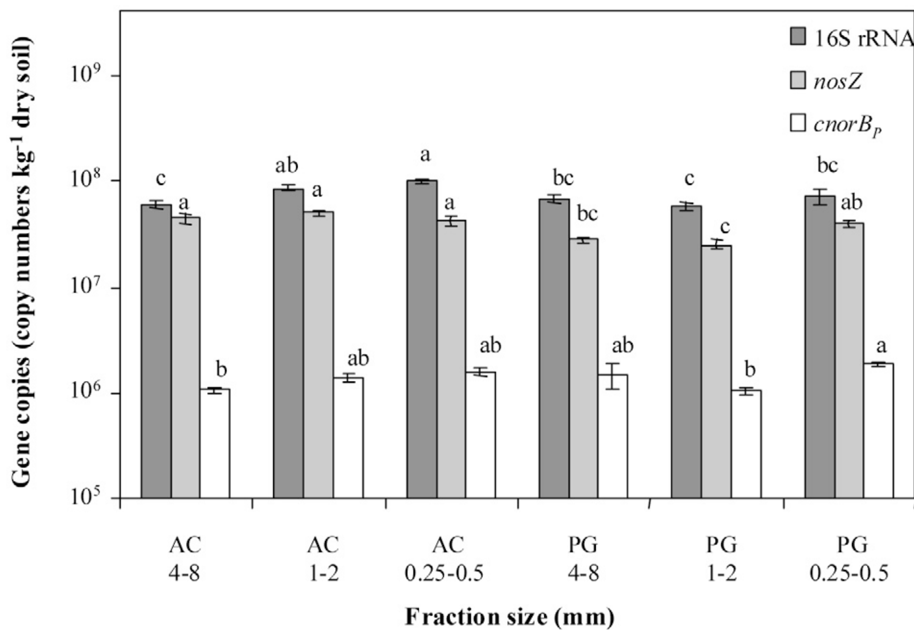

Fig. 2. Real-time polymerase chain reaction quantification of 16S rRNA, *nosZ*, and *cnorB*$_P$ gene copy numbers in soil aggregate fractions from a potato field in arable cropping (AC) and permanent timothy grassland (PG) management systems averaged over time during a 72-h incubation. Standard curve descriptors and detection levels: 16S rRNA gene: R^2 = 0.997, *y* intercept = 37.8, *E* (amplification efficiency) = 103%, NTC (no template control) = undetected; *nosZ* gene: R^2 = 0.998, *y* intercept = 37.3, *E* = 88.2%, NTC = undetected; *cnorB*$_P$: R^2 = 0.999, *y* intercept = 36.2, *E* = 98.0%, NTC = undetected. Treatment means with the same letter are not significantly different ($P < 0.05$) across both soil types based on a protected LSD test. Values represent means (n = 20) ± standard error.

procedure (Darbyshire et al., 1993). Aggregate size distributions are sensitive to sampling conditions, sieving technique, and the duration of sieving (Beare and Bruce, 1993). Wet sieving was not utilized because this method can rupture weak aggregates, cause increased disruption of the microbial physical habitat, and underestimate the C and N pools in aggregates because of the loss of water-soluble C and N (Sainju, 2006). In the current study, soil aggregate samples were fragmented by dry sieving at a uniform oscillation speed to limit the amount of disruption to the soil aggregates and the soil microbial properties (Schutter and Dick, 2002). The soil moisture content before fractionation can affect the final aggregate size distribution (Dickson et al., 1991). A preliminary study determined, however, that the aggregate size class distribution of the soil did not change significantly when the soil was dried to water contents of 0.05, 0.10, and 0.15 kg kg^{-1} dry soil before fractionation (data not shown). This sieving method, in combination with the sandy loam texture, resulted in microaggregates representing <2% of the soil because microaggregation in soils is generally a feature of heavy clay soils, whereas sandy soils contain relatively few microaggregates (Jocteur-Monrozier et al., 1991).

Responses to Aggregate Size

Cumulative denitrification was greatest, while cumulative respiration was least, in the largest aggregate size fraction of the AC management system. Likewise, within the PG management system, cumulative denitrification was greatest in the largest aggregate size fraction; however, aggregate size had no effect on respiration. Denitrifying enzyme activity was not significantly different among the aggregate size fractions in either management system. In comparison with this study, Drury et al. (2004), using a clay loam soil and a dry sieving method, found that denitrification was greater in the largest aggregate size fraction (4–8 mm), whereas respiration decreased with increasing aggregate size. These results are in contrast to those reported by Seech and Beauchamp (1988), who used a silt loam soil and a dry sieving method but found that denitrification was greater in smaller aggregate fractions (<0.25 and 0.25–0.5 mm) and generally decreased as aggregate size increased. They also reported that respiration increased with decreasing aggregate size and suggested that C availability may partially explain denitrification activity in the soil aggregate fractions. No significant relationship was found between respiration and denitrification in the current study. It is unknown what is controlling the differences in the amount of denitrification among the aggregate fractions in this study. The atmosphere in the headspace was replaced with He, therefore O_2 diffusion and consumption should not be a factor in this experiment. Likewise, soil pH was similar among the aggregate fractions, averaging 6.31 ± 0.01 (mean ± SE). No correlation was found between denitrification and the initial soil NO_3^- concentration. The larger size soil aggregate fractions may have more active and efficient soil denitrifiers, leading to a higher amount of denitrification in the larger aggregate fractions.

Soils from both systems were developed from the same parent material and were subjected to the same climatic conditions but under contrasting management systems. Therefore, this provided an opportunity to study the distribution of 16S rRNA, *nosZ*, and *cnorB*$_P$ gene-bearing bacteria among the soil aggregates from contrasting systems. Several studies have examined the diversity of microbial communities among soil aggregate fractions and found that the composition of the microbial community can vary across microenvironments (Lupwayi et al., 2001; Chotte et al., 2002; Izquierdo and Nüsslein, 2006). Information on the distribution (i.e., abundance) of soil denitrifiers among aggregate size fractions, however, is sparse and to date no study has quantified the denitrifiers using quantitative PCR in soil aggregates within or between management systems. Denitrifier bacteria gene copies (*nosZ* and *cnorB*$_P$) did not significantly change with time. Previous research measured increases in the *cnorB*$_P$ gene-bearing bacteria only after addition of a significant C input (≥250 mg C kg^{-1} soil) (Miller et al., 2008, 2009).

In the current study, aggregate size had no significant effect on the abundance of the *nosZ* and *cnorB*$_P$ gene-bearing bacteria in the soil from the AC system. Within the AC management system, there is frequent mixing of the soil during cultivation, which breaks up the soil structure and alters the physical and chemical environment (Kennedy and Smith, 1995). This fragmentation can result in a large degree of similarity among aggregate size fractions, which may result in the microbial communities being more evenly disturbed among soil aggregates (Petersen et al.,

1997; Burns et al., 1999). While microaggregates are considered to remain stable for long periods of time, macroaggregates, such as those used in this study, are considered to be more transient and sensitive to soil management (Tisdall and Oades, 1982).

Aggregate size did have a significant effect on the denitrifiers within the soil from the PG system, with the greatest *nosZ* and $cnorB_P$ gene-bearing bacteria measured in the smallest aggregate size fraction (0.25–0.5 mm). Increases in bacterial cell numbers in smaller aggregate size fractions have been attributed to adsorption of bacterial cells to clay (Kandeler et al., 2000; Selesi et al., 2007). This is unlikely in our study because the clay content did not significantly differ among the aggregate size fractions in the PG system. Pore structure, including connectivity between pores, can also influence microhabitat characteristics and thus microbial distribution (Young and Crawford, 2004; Mummey et al., 2006). Smaller aggregate size fractions typically contain smaller sized pores, therefore the denitrifiers located in the pores within smaller sized aggregates may have been better protected from predators (Hattori, 1988, Jocteur-Monrozier et al., 1991). Although quantitative methods were not used, Sessitsch et al. (2001) did observe a greater diversity of microbes in smaller than in larger aggregate size fractions, and suggested that the bacterial diversity in the various aggregate size fractions was more closely related to the characteristics of these aggregates, such as pore size, than to environmental constraints.

Responses to Management Systems

Soil from the PG system was expected to have a greater organic C content than soil from the AC system. The field in the PG system had not been plowed for at least 10 yr, whereas intensive tillage practices are practiced annually in the field in the AC system. Studies have shown that the organic C content of macroaggregates was greater in native grassland or under reduced tillage than conventionally tilled systems (Beare et al., 1994; Six et al., 1998). Soil organic C content measured previously at 126 grid points in the 5-ha field under the AC system averaged 21 g kg^{-1} soil, with a range of 13 to 56 g kg^{-1} soil (unpublished data, 2001). Consequently in the present study, organic C levels may have been similar or greater in the AC aggregate fractions than in the corresponding aggregates of the PG management system primarily because of spatial variation in soil properties. Despite the limited variation in soil organic C between management systems, respiration was greater in the aggregate fractions from the PG soil than the AC soil. Even though the quantity of organic C may not have differed, the quality of the C and its accessibility to microbes may have led to greater respiration in the PG management system.

Cumulative denitrification was low in each management system compared with unamended bulk soil core treatments used in previous experimentation (Miller et al., 2008, 2009). Similarly, denitrification in the current study was lower then previously quantified in aggregated soil (Seech and Beauchamp, 1988: Drury et al., 2004). Experimental conditions, including the aerobicity of the soil, soil type, and time of soil sampling, all varied among theses studies and thus may have influenced the amount of denitrification. For example, soil was collected in the fall for the current study, while Drury et al. (2004) and Seech and Beauchamp (1988) collected their soil in the spring. The availability of C in the soil may have been lower in the fall given that there were no C additions other than root exudates during the growing season. Alternatively, many soil bacteria are inactive at a given time due to low nutrient availability (Coleman, 2001). Sexstone et al. (1985) reported that denitrifying activity was not detected in all of the soil aggregates even if the center of the aggregate was anaerobic. It has been suggested that the spatial discontinuity of the available C, NO_3^-, and denitrifying bacteria were the major factors limiting denitrification in aggregated soil (Sexstone et al., 1988). In the current study, although C substrate was available as determined by respiration, C availability may have limited denitrification. Production of CO_2 can be undertaken by all heterotrophic microorganisms, not just soil denitrifiers. Other microorganisms may have been responsible for the measurable CO_2 production. For example, fungi can play a large role in both the formation and stabilization of soil aggregates (Lynch and Bragg, 1985). Studies have found that fungal populations are more abundant in macroaggregates than in microaggregates (Gupta and Germida, 1988; Guggenberger et al., 1999; Schutter and Dick, 2002). Fungal growth may have also been encouraged during storage of the soil at 3°C. Pietikäinen et al. (2005) reported that fungi were more adapted to low-temperature conditions than bacteria.

Few differences in the abundance of the denitrifiers of the same aggregate size fractions were measured between the two management systems. The only difference was measured within the *nosZ* gene-bearing bacteria, which had significantly greater abundance in the 4- to 8- and 1- to 2-mm aggregate fractions of soil from the AC system than the PG system. The abundance of the $cnorB_P$ gene-bearing bacteria was similar between the same aggregate size fractions of both management systems. Previous research using bulk soil samples found increases in the $cnorB_P$ gene-bearing bacteria, but only following addition of a significant C input (≥250 mg C kg^{-1} soil) (Dandie et al., 2007).

Differences may have been measured in the *nosZ* copy numbers in the AC and not the PG soil aggregates because potato cropping systems commonly receive high fertilizer N inputs (Zebarth et al., 2003), which may have resulted in the selection or adaptation of denitrifiers in this soil. The presence of lower concentrations of NO_3^- in the PG system may have removed the competitive advantage of bacteria having *nosZ*, resulting in a decrease in gene copy numbers. Alternatively, differences may reflect the crop species grown in the field, since crop type has been shown to directly affect the soil microbial community (Garbeva et al., 2004; Patra et al., 2006). Philippot et al. (2002) reported that the structure of the NO_3–reducing community (*narG*) differed between soils planted with and without maize (*Zea mays* L.), while Wakelin et al. (2007), using quantitative PCR, found that *nifH* (N_2 fixation) and *napA* (denitrification) gene abundance was affected by management of maize stubble. An increase in gene abundance was measured in soil where the maize stubble was retained and plowed under at the end of the growing season in comparison with soil in which the stubble was burned. It is unclear in the current study if plant species affected the abundance and distribution of the denitrifier bacteria and further investigation is required.

Bulk soil is a very heterogeneous environment, and it was hypothesized that study of aggregate size fractions would be a useful approach to address soil heterogeneity in understanding the spatial distribution of denitrification activity and denitrifier abundance in soil. In this soil, however, aggregate size fractions were of

limited effectiveness in explaining the variation in total bacterial abundance, denitrifier abundance, DEA, and denitrification, as indicated by few differences, and differences of small magnitude, in these parameters among aggregate size fractions. Limited effects of aggregate size fractions might have been expected in a potato cropping system due to intensive tillage, but were not expected in the perennial grassland. Greater differences may have been measured among soil aggregate fractions and between the two management systems if microaggregates were analyzed. Few soil microaggregates were collected in this sandy loam soil with the dry sieving method. Wet sieving may have been more effective in the collection of soil microaggregates; however, it would also have been a more disruptive sampling method and would be expected to have a greater effect on the soil chemical, physical, and microbial properties (Sainju, 2006). Previous studies have shown that that there are more bacteria in soil microaggregates than macroaggregates (Lensi et al., 1995; Ranjard and Richaume, 2001).

CONCLUSIONS

This study examined the distribution of denitrifiers and their activity across a range of soil macroaggregates from contrasting management systems. Measurement of DEA, denitrification, and denitrifiers confirmed that denitrification characteristics are not homogenously distributed among the soil aggregate size fractions. Denitrification was significantly higher in the largest aggregate size fraction (4–8 mm) under both management regimes. The location of the denitrification activity and the abundance of the denitrifier bacteria (*nosZ* and $cnorB_P$), however, were uncoupled across aggregate size fractions in the contrasting management systems.

From an aggregation point of view, the AC and PG soils differed in the amount of aggregates contained in each fraction, but there was little variation in the measured biochemical nature of these aggregates, with relatively few differences found between the denitrifier bacteria dynamics of the same aggregate fraction of both management systems. The aggregate size had no significant effect on the abundance of the *nosZ* and $cnorB_P$ gene-bearing bacteria in the soil from the AC system but did have a significant effect on the denitrifier bacteria within the soil from the PG system, with the highest gene copy numbers observed in the smallest aggregate size fraction (0.25–0.5 mm). These smaller sized aggregates may have provided better protection to the denitrifiers from predators since they generally have smaller sized pores.

In this soil, aggregate size did have an effect on denitrification but only a small effect on the abundance of the denitrifiers. The distribution of bacteria within the soil is a key factor affecting their survival, activity, and ecological function. Future work should focus on aggregates, including microaggregates, in a variety of soil types and cropping systems to obtain a more comprehensive view of denitrifier bacteria dynamics within the soil matrix.

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