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Influence of Protozoa and Nutrient Availability on Nitrification Rates in Subsurface Sediments

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ABSTRACT

Protozoan abundance, nitrification potential, and related factors in saturated subsurface sediments and the overlying soil were compared at a nonfertilized grassland and an agricultural cropland site. In a 6-week laboratory experiment, DOC, ammonium, and protozoan abundance were manipulated in flasks containing groundwater-sediment slurries. Microbial abundance (protozoa, actively respiring bacteria, and total bacteria) and nutrient concentrations (extractable ammonium and nitrate) were measured.

Results from the soil profile analysis showed that protozoan abundance declined with depth at both sites, but significant numbers (392 cells g^{-1}dw) were found in groundwater sediments at the cropland site. Nitrification potential declined with depth at the grassland site and increased with depth at the cropland site. In the laboratory experiment, treatment responses generally were observed within 3 weeks, but had diminished by 6 weeks. Protozoa reduced bacterial populations through the first 3 weeks, but this effect was not significant by week 6. In the cropland sediments, increased net nitrate production occurred in the two reduced protozoa treatments that received ammonium, suggesting that nitrification was occurring and was limited by ammonium. High protozoan abundance in the cropland sediments increased the nitrate flux response, unless DOC was added; in this case, no response occurred. No such responses were recorded in the grassland sediments.

Apparently, appreciable nitrification can occur in some groundwater sediments, if sufficient ammonium is present and DOC availability is low. Furthermore, nitrification can be enhanced when protozoan abundance is elevated. Finally, our results suggest that surface land use practices can alter subsurface nitrification rates and microbial community structure.

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Introduction

Nitrate (NO_3^-) is a ubiquitous and increasing chemical contaminant in the world's aquifers [43]. High concentrations can restrict groundwater usability and have been linked to several deleterious medical conditions [2, 43]. Anthropogenic inputs of nitrogen on or near the soil surface and subsequent nitrification and nitrate leaching are believed to be the main sources of nitrate contamination. However, nitrification could also potentially occur within the groundwater environment, provided nitrifying bacteria, sufficient ammonium, and O_2 exist. Nitrifying bacteria probably exist in saturated subsurface sediments, as do most of the other major functional groups of microorganisms, but their metabolic rates are poorly documented [17, 19, 29]. Oxic groundwater is common in pristine aquifers, and detectable ammonium concentrations also occur, most likely as a result of in situ mineralization of organic nitrogen. Therefore, a significant portion of nitrate in groundwater may be a direct result of subsurface nitrification.

In addition to sufficient ammonium availability, the presence of nitrifying bacteria and O_2, dissolved organic carbon (DOC) availability may also influence subsurface nitrification. Specifically, under conditions where DOC availability is high, nitrification may be reduced. Using continuous culture experiments in chemostats, Verhagen and Laanbroek [47] showed that nitrate production can be reduced and even eliminated by increasing DOC concentrations. They presumed that, as DOC availability increased, the heterotrophic bacteria switched from being carbon limited to being nitrogen limited. Available ammonium and subsequent nitrification were reduced because the heterotrophic bacteria outcompeted nitrifying bacteria for ammonium.

Trophic interactions within the subsurface environment may also affect nitrification rates. Berninger et al. [4] suggested that, in freshwater environments, bacterial abundance is regulated by both substrate supply and protozoan grazing. Protozoa rely on bacteria as food in both aquatic and terrestrial habitats [16]. It has been estimated that flagellates can ingest (and digest) a total volume of particles per hour that equals their own cell volume [15]. Slow-growing bacteria, such as nitrifying bacteria, may be especially susceptible to grazing protozoa [48]. In fact, relatively slow-growing bacteria can be eliminated from the bacterial community in sewage water by protozoan grazing [32, 40]. Although fewer protozoa are found in groundwater than in other systems, their importance is not known.

Generally, intensive protozoan predation on bacteria lowers bacterial abundance [6, 27] (but see [39]). Hunt et al. [23] proposed a model, which has been well supported with empirical data (see [45]) suggesting that lowering bacterial biomass through predation will increase concentrations of a limiting nutrient. This, in turn, will increase the growth rate of bacteria (bacterial activity) and the uptake of nonlimiting nutrients. In agreement with this model, several studies in soil, surface water, and cultures have shown that intense protozoan grazing increases rates of nitrogen mineralization [7, 11, 18, 36, 38] and nitrification [20, 50]. However, the effects of protozoa on nitrification in the subsurface environment have not been determined.

In this study, protozoan populations and the nitrification process within groundwater sediments were examined at a nonfertilized grassland site and an agricultural cropland site in northeastern Kansas. This research had two primary objectives: first, to quantify the protozoan population and understand the potential for nitrification in the groundwater sediments compared to the overlying soil at both sites; and second, to determine the factors controlling nitrification in the subsurface environment below these sites. The factors considered included ammonium availability, DOC availability, and microbial interactions (protozoan grazing).

Materials and Methods

Study Sites

Both the cropland site and the grassland site, in this study, are located at the Konza Prairie Research Natural Area, a 3,487-ha tract of native prairie approximately 15 km south of Manhattan, Kansas, USA. Konza Prairie lies within the Flint Hills of northeast Kansas, and the underlying geology consists of alternating layers of limestone and shale. The soil at both sites consists of approximately 10 m of unconsolidated sediments overlaying the bedrock and has been mapped as a Reading silt loam (fine, mixed, mesic Typic Argiudolls). Soil textural analysis shows similar soils at both sites with no clear trends with depth. Previous research established that the groundwater in this area is oxic [13].

Despite the similar geology and soil, the surface land use at the two sites is very different. The grassland site has been in pasture since 1939 and was used for livestock grazing as recently as 1980, but has not been fertilized or grazed since then. At some point before 1976, the area was replanted with brome grass (*Bromus* sp.), but is slowly reverting back to tallgrass prairie. Currently, approximately 50% of the grass is reverting to native grasses found on Konza Prairie. The cropland site is about 1 km downstream (Kings Creek) from the grassland site and is currently under cultivation for wheat with regular additions of fertilizer. This site has been under continuous agriculture since sometime between 1939 and 1950, as evidenced by aerial photography.

Core Drilling and Sample Analysis

The grassland site cores were collected about 100 m up-slope from the Kings Creek stream bed. Cores from duplicate boreholes (7 m apart) were taken in 60-cm lengths to limestone bedrock (8 m) on 19 April 1993. At the time of drilling, the capillary fringe occurred at a depth of 4 m, and the top of the water table was approximately 4.3 m. Wells at the cropland site also were drilled about 100 m from the Kings Creek stream bed. Duplicate cores were collected (9 m apart) on 25 April 1993 down to bedrock (10.3 m), with the capillary fringe starting at 5.1 m and the water table at 5.3 m.

Wells were drilled and cores collected with a hollow stem auger with a split barrel. The center of the barrel was lined with 5.1-cm-diameter polycarbonate sleeves. No drilling fluids or muds were used, and all equipment was pressure washed with water before drilling at each site. The split core and sleeves were sanitized with 90:10 ethanol:H_2O and air-dried immediately before use. The cores in their polycarbonate sleeves were stored at 4°C until they were partitioned on 29 April 1993. Sanitized tools (dipped in 90:10 ethanol:H_2O, and flamed) were used to remove material from each core sleeve into sterile whirl-pak bags, where they were stored 4°C until analysis.

Protozoan populations were estimated using a modification of most probable number (MPN) techniques described by Singh [42] and Sinclair and Ghiorse [41]. From each soil or sediment sample, 10 g (wet weight) was transferred aseptically to a sterile blender containing 90 ml of sterile phosphate buffer (2.2 mM KH_2PO_4, 4.02 mM K_2HPO_4, pH 7) and blended for 30 s. This mixture was used to make seven additional tenfold serial dilutions (10^{-2} to 10^{-8}). Aliquots (200 μl) from each serial dilution were placed in six wells of a sterile Corning 96-well tissue culture plate to which 50 μl of sterile 1.5% agar had been added previously. Also added to each well was 10 μl of a nongrowing (stationary phase) *Enterobacter aerogenes*/phosphate buffer slurry (spectrophotometer absorbance of 0.680 at 750 nm) to provide a bacterial food source for the protozoa. Plates were incubated for 30 days, in the dark, at 25°C. Filter-sterilized reverse osmosis water was added periodically to each well throughout the incubation to maintain a moist environment. After incubation, a sample from each well was examined microscopically at 400× for the presence or absence of protozoa. A computer program [24] was used to calculate protozoan MPN values.

Nitrification potential of the core samples was estimated using a modification of the chlorate block technique described by Belser and Mays [3]. A single slurry was made from each core sample using 160 ml of site-specific groundwater and 15 g of soil or sediment. The groundwater used to make the slurries was collected the day of slurry preparation, and filter (0.2 μm) sterilized before use. Each sample was analyzed in triplicate by placing 50 ml of slurry into three separate 60-ml Wheaton borosilicate glass serum bottles. To prevent nitrite oxidation, 800 μl of 0.5-M $NaClO_3$ (10 mM $NaClO_3$ final concentration) was added to each bottle. Cotton was placed in the opening of each bottle to minimize microbial contamination, while allowing oxygen to permeate. The bottles were shaken (120 rpm) in the dark for 9 days at ambient groundwater temperature (12°C). Twelve-milliliter aliquots were removed from each bottle before and after the 9-day incubation, filtered (Whatman GF/F), and colorimetrically analyzed for nitrite [1] using a Hitachi U-2000 spectrophotometer. Nitrite accumulation during the incubation period was used to estimate nitrification potential.

Laboratory Experiment Design and Setup

A laboratory experiment to examine the factors controlling nitrification in the groundwater sediments from both sites was performed. Three different factors were examined: protozoan abundance, DOC availability, and ammonium availability. Each factor was included at two levels. For the protozoan factor, abundance was either above the ambient level (P), or below the ambient level (p). Additional DOC was either added weekly (C), or not added (c). Ammonium also was either added weekly (N), or not added (n). Levels of additions are described later. The treatments consisted of every possible combination of the factor levels, resulting in eight treatments for each site: PCN, PCn, PcN, Pcn, pCN, pCn, pcN, and pcn. Each treatment was triplicated.

Groundwater sediments were collected from the bottom of a single, open-bottom well at each site, using a PVC pipe sediment collector [44]. The terminal end of the wells at the grassland and the cropland sites were approximately 8 m and 10.5 m deep, respectively. Sediments were stored in buckets (rinsed in 95:5 ethanol:H_2O and air dried) on ice until arrival at the laboratory (approximately 1 h). A slurry (1.7 liter) was made immediately for each site and autoclaved for 1.5 h. To ensure complete sterilization, the slurries were autoclaved again 24 h later for 1.5 h [51].

Reinoculation of the sterile slurries was required to achieve the desired level of protozoan manipulation. Each 1.7 liter of slurry was divided into two 850-ml portions. For the ambient protozoan slurries, 5 ml of a site-specific protozoan culture was added. For the low protozoan slurries, 5 ml of 3-μm-filtered, site-specific protozoan culture was added.

Each of the four 850-ml slurries was subdivided into 12, 50-ml portions and placed into 150-ml Erlenmeyer flasks for additional treatment. All flasks received 50 ml of 3-μm-filtered, site-specific, fresh sediment slurry to inoculate the sediments with representative subsurface bacteria. The sediment densities of the final cropland and grassland slurries were 29.2 and 98.1 mg dw ml^{-1}, respectively.

Protozoan growth was discouraged in the low protozoa treatments by adding 400 μl of 0.936 mM fumagillin (SIGMA Chemical Co., St. Louis, Mo.) in dimethyl sulfoxide (DMSO) to the appropriate flasks. Flasks containing ambient protozoa treatments received 400 μl of DMSO, alone. (Fumagillin is a crystalline antibiotic that is derived from the fungus *Aspergillis fumigatus* H-3 [12] and is used regularly as a protozoan-specific biocide in apiculture [46, 52] and aquaculture [25, 28]. Recently, fumagillin also has been shown to be a successful biocide of soil protozoa [8].)

The DOC addition flasks initially received 100 μl of a 0.01 M dextrose solution (10 μM final concentration). A sufficient supply of DOC was maintained with weekly dextrose additions (10 μM final concentration). This rate of DOC application has been shown to maintain aerobic conditions in groundwater sediments from a nearby site (unpublished data). The flasks containing the ammonium addition initially received 200 μl of 1000 mg l^{-1} NH_4Cl-N (2

mg l^{-1} NH_4^+-N final concentration). Ammonium also was added weekly at 2 mg l^{-1} NH_4^+-N (final concentration) to maintain a supply of substrate for nitrification. This ammonium concentration stimulates nitrification in sediments from a nearby site [44]. Sterile cotton was placed in the opening of each flask, and they were incubated, in the dark, for 6 weeks at ambient groundwater temperature (11°C). The contents were swirled daily to prevent anaerobic conditions from developing in the sediments.

Laboratory Experiment Analysis Methods

Aliquots were removed from the flasks at various times throughout the experiment for enumeration of protozoa, actively respiring bacteria, and total bacteria. Additional aliquots were removed weekly to determine the solids content per slurry volume and to analyze for ammonium and nitrate.

Protozoa were counted from freshly collected sediments and from each flask at the beginning and end of the experiment. The MPN procedure used was identical to the one described earlier. Actively respiring and total bacteria were counted from freshly collected sediments and at the initial, 3-week, and 6-week time points of the experiment. A modification of a counterstaining procedure using 4′,6-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was employed [37, 53]. A 500-µl aliquot was removed and received 500 µl of filter-sterilized (0.2 µm), pH 7.0, 0.01% sodium pyrophosphate solution and 20 µl of 0.25 M CTC (5 mM final concentration). The vials were kept in the dark and shaken at room temperature, for 4 h, at 160 rpm. To counterstain with DAPI, 20 µl of each CTC-stained solution was placed into a separate dark vial along with 5 ml bacteria-free RO water and 50 µl of 100 mg l^{-1} DAPI (1 µg ml^{-1} final concentration). This solution was incubated at room temperature, in the dark, for 10 min.

A 1-ml subsample was added to a Teflon filter tower and filtered through a 0.2-µm black Poretics polycarbonate membrane filter. The filter then was mounted on a microscope slide with a drop of immersion oil (Cargille type DF). Another drop of oil was placed on top of the filter before the coverslip was applied. At least 50 fields were examined for CTC-stained bacteria, and at least ten fields were examined for DAPI-stained bacteria. Because CTC absorbs the fluorescent energy emitted by DAPI, total counts were obtained by adding the CTC and DAPI counts together [53].

Extractable ammonium and nitrate concentrations were determined from the fresh sediment and from each flask at weekly intervals. A 5-ml aliquot of each slurry and 5 ml of 4 *N* KCl (2 *N* KCl, final concentration) were placed in a 20-ml borosilicate glass scintillation vial and agitated for 30 min. Aliquots were taken from the samples that received weekly ammonium additions before the ammonium was added. The mixture was centrifuged at 1,064 *g* for 5 min. The liquid was then filtered through a 0.45-µm Millipore membrane filter and analyzed on an ALPKEM Flow Solution autoanalyzer for ammonium and nitrate, using modifications of the phenol hypochlorite method and the cadmium reduction method, respectively [35].

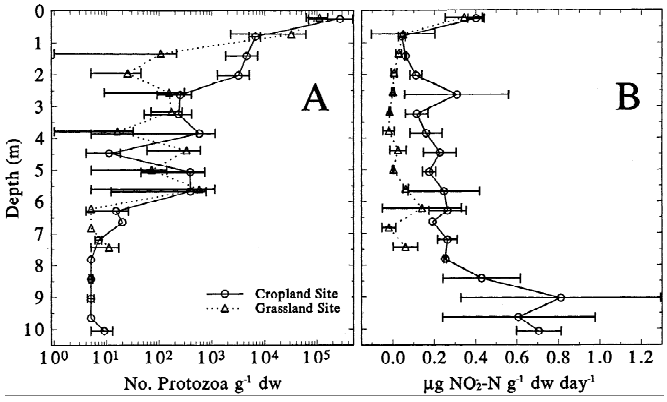

Fig. 1. Protozoan abundance (**A**) and nitrification potential (**B**) in soil profiles from a grassland site and a cropland site. *Error bars* show minimum and maximum observation and ±1 SD for protozoan abundance and nitrification potential, respectively.

Laboratory Experiment Statistical Methods

Experimental results were analyzed using analysis of variance of a balanced, completely randomized, multifactorial design. The factors considered in the analysis were protozoan manipulation, ammonium addition, organic carbon addition, and the interactions. Statistical analysis of the treatment effects in the cropland and grassland sediments usually were conducted separately, because within-site comparisons were thought to be more important than between-site comparisons. When between-site comparisons were made, the site effects were analyzed using site as a block effect. Log transformations were done before analysis of the microbial counts to aid in meeting the analysis of variance assumption of equal variances.

Results

Protozoa in the enrichment cultures made from the core samples were observed primarily in the encysted stage, but active flagellates and amoebae were also common. Ciliates were seen in only the surface sample of the grassland site. Protozoan populations were highest in the surface soils and decreased with depth at both sites (Fig. 1A). Between-site comparisons showed no significant differences at any depth. Nitrification potential was high in the surface soils at both sites and decreased within the first meter of soil (Fig. 1B). Potentials remained low in the grassland profile, but the rates in the cropland profile gradually increased with depth and, by 9 m, exceeded those of the surface.

Spearman correlation coefficients were calculated on nitrification potential values and log-transformed protozoan numbers for each site separately, and for the sites combined, to identify possible relationships between protozoa and ni-

Table 1. Characteristics of freshly collected sediments compared to sediment characteristics of the initial treatments (time = 0)[a]

Characteristic	Site	Fresh sediments	Ambient protozoa	Low protozoa
Total bacteria (No. cells g^{-1} dw)	Cropland	6.15×10^5 (2.86×10^5)	1.96×10^6 (5.17×10^5)	8.29×10^5 (1.42×10^5)
	Grassland	5.35×10^6 (5.45×10^5)	3.64×10^6 (8.81×10^5)	5.46×10^6 (2.71×10^6)
Actively respiring bacteria (No. cells g^{-1} dw)	Cropland	5.60×10^4 (3.67×10^4)	BLD[b]	7.47×10^3 (1.29×10^4)
	Grassland	BLD	7.15×10^4 (6.19×10^4)	BLD
Protozoa (No. protozoa g^{-1} dw)	Cropland	1.21×10^5 (6.91×10^4)	2.25×10^4 (1.49×10^4)	4.82×10^3 (4.27×10^3)
	Grassland	2.72×10^4 (8.09×10^3)	4.70×10^3 (2.99×10^3)	2.36×10^2 (1.00×10^2)
Nitrate ($\mu g\ NO_3^-$-N g^{-1} dw)	Cropland	13.47 (0.19)	15.50 (0.35)	0.14 (0.20)
	Grassland	BLD	0.09 (0.13)	1.12 (1.59)
Extractable ammonium ($\mu g\ NH_4^+$-N g^{-1} dw)	Cropland	BLD	5.13 (0.88)	7.43 (2.42)
	Grassland	1.00 (1.22)	2.36 (0.09)	2.27 (0.13)

[a] Numbers in parentheses represent one standard deviation of the mean of three observations
[b] BLD, Below limit of detection

trification. The only significant correlation observed was a negative relationship between protozoan abundance and nitrification potential within the cropland cores ($r = -0.55$, $P = 0.0007$).

In the laboratory experiment, sediments were reinoculated after autoclaving to reestablish a representative microbial assemblage. Total bacterial abundance was not significantly different between the freshly collected sediments and the autoclaved and inoculated treatments (Table 1). This suggests that inoculation brought bacterial populations back to their original levels. However, numbers of active bacteria in the low protozoa treatments remained similar to those in the fresh sediment; ambient protozoa treatments in the cropland sediments had fewer actively respiring bacteria than the fresh sediments, and the ambient protozoa treatments in the grassland sediments had more actively respiring bacteria than the freshly collected sediments (Table 1). Initial protozoan density was lower in the ambient protozoa treatments than the fresh sediments (Table 1). But the lowest protozoan densities were found in the low protozoa treatments ($P = 0.0001$, ANOVA), based on analysis of log-transformed protozoan numbers.

By the end of the 6-week incubation, the strong treatment differences in protozoan counts observed at the beginning of the experiment had diminished (Fig. 2). Analysis of variance on log-transformed protozoan numbers, however, still revealed significantly higher protozoan populations in ambient protozoa treatments ($P = 0.0415$). No significant relationships were discovered within the sites.

The 3-week CTC counts of actively respiring bacteria in the cropland sediments indicated very low numbers (Fig. 3A). Of the four treatments that did have detectable numbers (PCN, Pcn, pCN, and pCn), three were treatments to which DOC had been added. The one treatment with added DOC that did not show detectable numbers of actively respiring bacteria was an ambient protozoa treatment (PCn). By 6 weeks (Fig. 3B), actively respiring bacteria were observed in all treatments, but no pattern related to treatment could be discerned. In contrast, actively respiring bacteria were observed in all treatments by 3 weeks (Fig. 3C), and again at 6 weeks, in the grassland sediments (Fig. 3D). Unlike the cropland sediments, no significant relationships with respect to actively respiring bacteria were detected between the treatments at any time.

At 3 weeks, total bacterial abundance for the cropland sediments (Fig. 4A) revealed a significant interaction effect between the protozoan and ammonium addition factors ($P = 0.0166$, ANOVA). Bacterial densities were also found to be significantly greater in the low protozoa treatments ($P = 0.0281$, ANOVA), but because of the significant interaction,

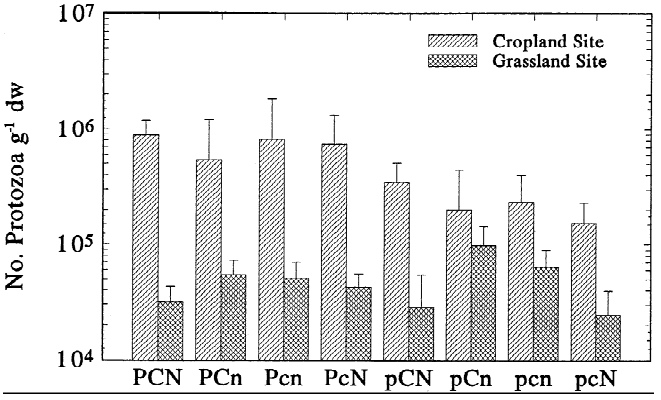

Fig. 2. Protozoan abundance after 6-week laboratory incubation. *P,* elevated protozoan abundance; *p,* reduced protozoan abundance; *C,* organic carbon added weekly; *c,* organic carbon not added; *N,* ammonium added weekly; *n,* ammonium not added. *Error bars* = ±1 SD.

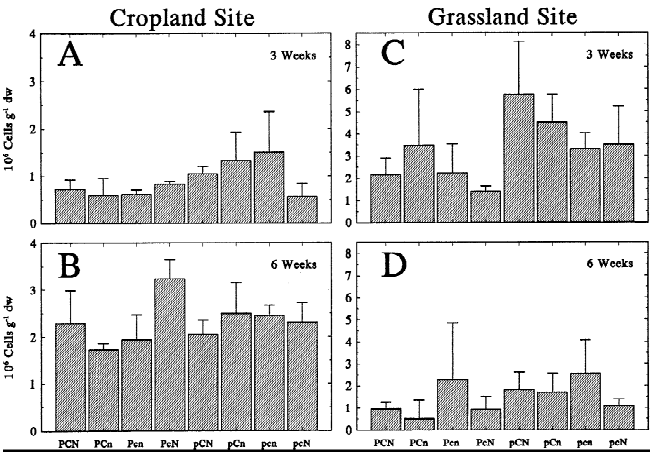

Fig. 4. Total bacterial abundance in the cropland (**A** and **B**) and grassland (**C** and **D**) sediments after 3 and 6 weeks of laboratory incubation. Treatment as in Fig. 2. *Error bars* = ±1 SD.

this relationship should be interpreted with caution [33]. However, when the data from both sites were combined, bacterial densities were found to be significantly greater in the low protozoa treatments (P = 0.0001, ANOVA). By 6 weeks, no significant differences between treatments were observed (Fig. 4B). In the grassland sediments, at 3 weeks (Fig. 4C), the total number of bacteria was significantly greater in the low protozoa treatments (P = 0.0001, ANOVA). But by 6 weeks, total bacterial abundance was not significantly different between treatments (Fig. 4D).

Fluctuations in ammonium concentration were more pronounced in the cropland sediments than the grassland sediments (Fig. 5). Ammonium concentrations in all treatments from both sites increased and decreased in unison throughout the experiment, resulting in no significant differences among treatments. However, the higher ammonium levels exhibited in the cropland samples indicates either greater mineralization or lower microbial uptake was occurring in the sediment from that site. Nitrate concentrations (Fig. 6) were also more dynamic in the cropland sediments, but, unlike ammonium, there were distinct treatment effects that occurred.

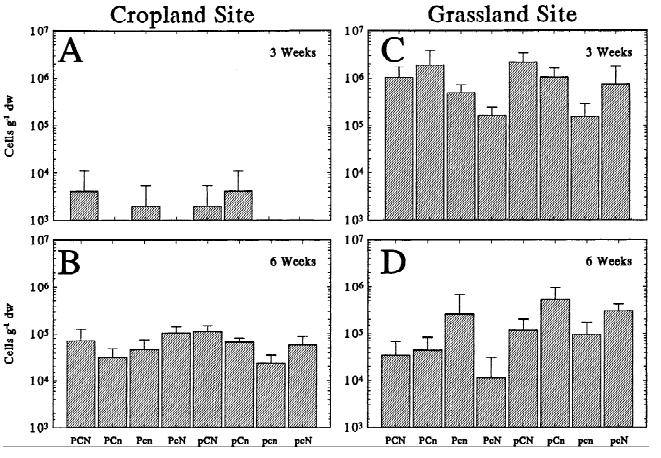

Fig. 3. Actively respiring bacterial abundance in cropland (**A** and **B**) and grassland (**C** and **D**) sediments after 3 and 6 weeks of laboratory incubation. Treatment as in Fig. 2. *Error bars* = ±1 SD.

After 1 week, in the cropland sediments, the treatments that had received ammonium, but not DOC, had elevated nitrate concentrations. After 2 weeks, all treatments that received ammonium additions showed high levels of nitrate, except for the ambient protozoa treatment with added ammonium and DOC. After 3 weeks, the only treatment with high nitrate was the ambient protozoan treatment with ammonium additions, but not DOC. The nitrate concentration in this treatment decreased substantially by week 4, but remained higher than the other treatments for the remainder of the incubation period.

Weekly change (flux) in nitrate concentration (Fig. 7) was used to indicate the balance between nitrate production (nitrification) and nitrate consumption (denitrification and/or nitrate assimilation). Nitrate production and consumption can be inferred by positive and negative values of change in nitrate concentration, respectively. Again, only the cropland sediments demonstrated significant fluctuations. In the cropland sediments, during the first week, nitrate production was greatest in the two low protozoa treatments with ammonium additions (P = 0.0001, one-way ANOVA). Of

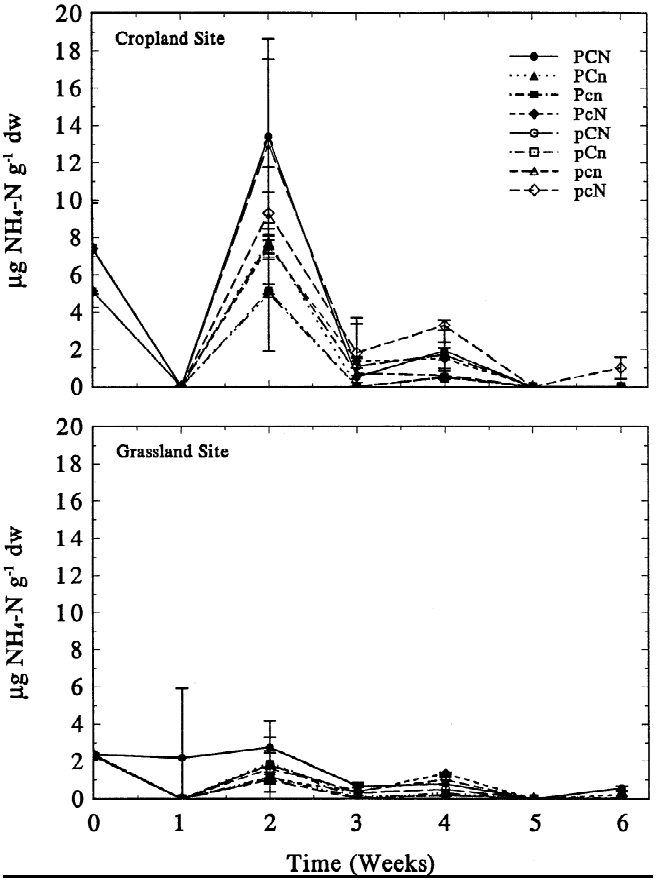

Fig. 5. Extractable ammonium by week for all treatments of the laboratory experiment. Treatment as in Fig. 2. *Error bars* = ±1 SD.

the two, pcN showed a greater response, but this was limited to the first week. In subsequent weeks, no nitrate flux was observed. During week 2, PcN and pCN had the highest nitrate production ($P = 0.0001$, one-way ANOVA) but were not significantly different from each other. During week 3, flux was reduced in the pCN treatment and never recovered. Also during week 3, PcN maintained the highest value of nitrate production ($P = 0.0001$, one-way ANOVA). By week 4, nitrate consumption had become the dominant transformation in the PcN treatment, and no net nitrate production occurred in any treatment. During weeks 5 and 6, nitrate concentrations had stabilized in all treatments, showing no net nitrate production or consumption.

Discussion

Protozoan populations in soils can range from 0 to 1.6×10^7 cells g^{-1}, and can be especially high in cropland soils or in "hot spots" influenced by living roots or dead organic matter [14]. Our results show that protozoan abundance was near the upper end of this range in the surface soil at the cropland (2.77×10^5 cells g^{-1}) and grassland (1.11×10^5 cells g^{-1}) sites. Protozoan populations observed in the saturated sediments at both the cropland site (5–392 cells g^{-1}) and the grassland site (5–573 cells g^{-1}) also were consistent (within an order of magnitude) with other values reported for subsurface communities. Madsen and Ghiorse [30] stated that 1–100 cyst-forming protozoa g^{-1} may be present in shallow, pristine aquifer sediments. Higher populations of protozoa may occur in sediments that have enriched levels of organic carbon. Madsen et al. [31] found more protozoa in saturated sediments associated with a polluted site containing buried coal tar than in sediments from an adjacent, pristine site. Similarly, Kinner et al. [26] discovered elevated numbers of protozoa in the groundwater at a site contaminated with sewage. Given the numbers of protozoa we observed, it is likely that row crop agriculture on the surface does not impact the groundwater protozoan community as greatly as episodes of organic contamination. In such organically en-

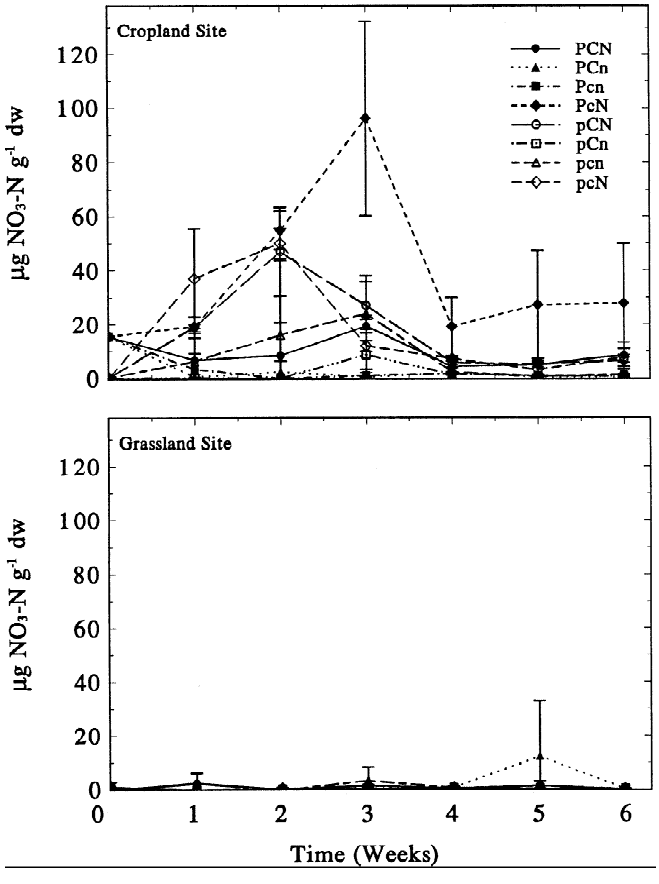

Fig. 6. Nitrate by week for all treatments of the laboratory experiment. Treatment as in Fig. 2. *Error bars* = ±1 SD.

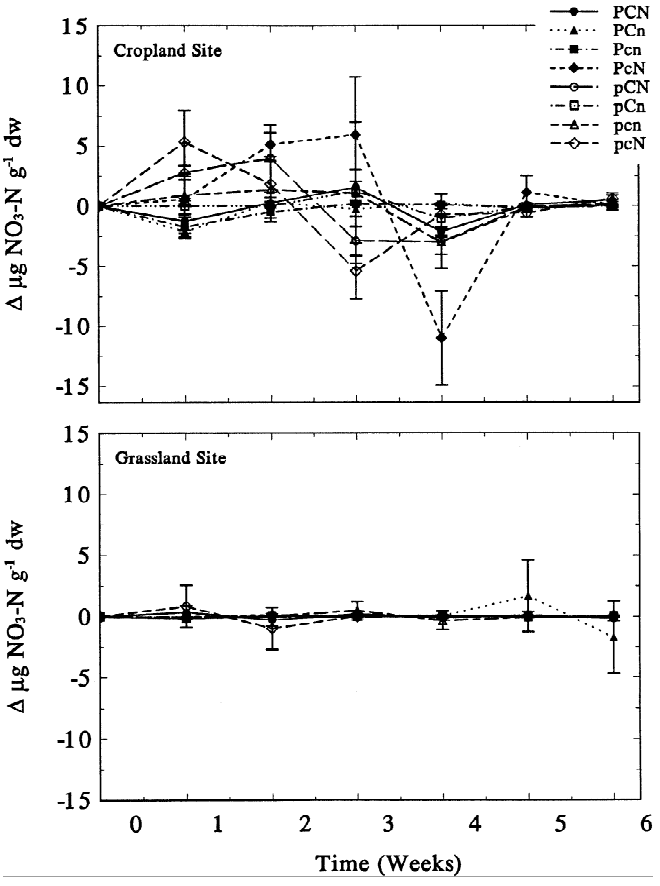

Fig. 7. Change in nitrate by week for all treatments of the laboratory experiment. Treatment as in Fig. 2. *Error bars* = ±1 SD.

riched areas, protozoan density may be high enough to exert a level of grazing pressure on the local bacterial community that could substantially affect nutrient cycling dynamics.

Nitrification rates were higher at the cropland site (surface and subsurface) than at the grassland site, in our study. This is consistent with other studies because soil and sediment from this grassland site have higher levels of soluble organic carbon [9], and low nitrification measurements have often been reported from systems high in organic matter. Thus, nitrification in cropland systems may have a greater impact on groundwater nitrate than nitrification in grassland systems. Potential nitrification rates measured in microcosms containing surface soil and subsurface sediments from our study were somewhat lower than rates reported for other systems (Table 2). Rates of nitrification observed in this study (at both sites and at all depths) were comparable in magnitude to the lower range values reported elsewhere.

The negative correlation between nitrification potential and protozoan abundance observed in the samples from the cropland site suggests that protozoan grazing may lower nitrification rates. However, spatial autocorrelation may have been a problem in this statistical analysis, and strong conclusions should not be developed regarding the interaction between protozoa and nitrification based on these results alone.

In the laboratory experiment, the initial protozoan manipulations were successful in establishing the desired differences in protozoan abundance between the ambient and the low protozoan abundance treatments. But, raising protozoan abundance above ambient levels may have created an environment more characteristic of sediments containing higher numbers of protozoa, such as those that are naturally or anthropogenically enriched with organic matter. Pristine sediments typically have fewer protozoa. Thus, the results of this experiment may be a better indicator of interactions that could occur in organically enriched sediments.

Unfortunately, the significant differences between the protozoan treatments were not sustained to the end of the experiment. We anticipated that the fumagillin treatment

Table 2. Comparison of nitrification rates in soils or sediments from different systems. All rates were determined from laboratory incubated microcosms

System	Depth	Nitrification rate (µg N g^{-1} dw day^{-1})	Reference
Mediterranean shrubland soil	0–5 cm	0.67–1.42	[5]
Norway spruce forest soil	Litter Layer	0–12.7	[31]
	Humus Layer	0–5.7	
	0–10 cm	0–2.6	
	10–20 cm	0.1–3.8	
	20–30 cm	0.3–2.9	
	30–40 cm	0.3–3.0	
	40–50 cm	0.2–3.2	
Cropland soil	0–1 m	0.04–0.40	This study
Grassland soil	0–1 m	0.03–0.34	This study
Cropland subsurface sediments	4–10 m	0.16–0.81	This study
Grassland subsurface sediments	4–7.5 m	0–0.13	This study
Oligotrophic lake sediment	0–1 cm	0.85–2.35	[21]
Mesotrophic lake sediment	0–1 cm	1.6–5.6	[21]
Eutrophic lake sediment	0–1 cm	1.15–17.35	[21]
Oligotrophic spring sediment	0–15 cm	0.84–49.05	[10]

would prevent protozoan proliferation in the low protozoa treatments, as it has in other studies [8], but this did not occur. Either the fumagillin concentration was too low to be effective, or the fumagillin, itself, was not effective at controlling protozoa. Because protozoan abundance stabilized by the end of the experiment, the effects of the protozoan treatments probably were strongest early, and diminished as the experiment progressed.

Actively respiring and total bacterial populations had stabilized by 6 weeks. Significantly fewer total bacteria were present in the ambient protozoa treatments at 3 weeks. This suggests that predation by protozoa influenced bacterial populations early in the experiment. By 6 weeks, the population density of bacteria in the sediments from both sites had stabilized, coinciding with the stabilization of protozoa. It is unclear whether the simultaneous stabilization of bacterial and protozoan populations was related. However, the effects observed on the bacterial populations could have been due to protozoa [21]. Protozoan grazing pressure could have stabilized along with protozoan population density, resulting in similar bacterial densities.

The early responses in the microbial counts to the protozoan treatments appeared to have no effect on extractable ammonium concentrations. In fact, no differences in ammonium concentration were observed within the samples for the two sites at any time in response to any treatment combination. A possible explanation for this could be that microbial ammonium uptake was high enough in all treatments to prevent treatment differences or ammonium accumulation.

The differences observed in nitrate production early in the experiment among the four cropland sediment treatments that received ammonium additions indicate two factors that may be controlling nitrification: ammonium availability and protozoan abundance. The observation of increased net nitrate production in the two low protozoa treatments that received ammonium additions suggests that nitrification was occurring and was limited by ammonium.

Protozoa were able to modify the nitrate production response even though it appeared that nitrification was limited by ammonium. Nitrate flux in the ambient protozoa treatment with only ammonium additions followed the same rise and decline pattern as that in the low protozoa treatments, but was delayed by 1 week. Even with the delay, this treatment achieved the highest cumulative amount of nitrate produced (highest total net nitrification for the whole experiment) of any treatment. Thus, when protozoan populations are high, nitrification may be enhanced in certain groundwater sediments. Protozoan-enhanced nitrification also has been shown in liquid culture and in an amended clay-loam soil [20].

In the ambient protozoa treatment where both ammonium and DOC were added, no nitrate flux response was observed. Because DOC was added, the heterotrophic bacteria may have been able to out-compete the nitrifying bacteria for ammonium [47, 49], limiting nitrification activity. Thus, elevated protozoan abundance increased nitrate production in response to ammonium additions, unless DOC also was added. In this situation, no response occurred.

The results from this laboratory study suggest that surface land use could temporarily impact the response to nutrient perturbations in the subsurface environment. Initially, the manipulations affected the microbial composition of the sediments from both sites. But the treatment effects soon stabilized, resulting in no significant differences by the end of the experiment. In general, microbial abundance in both sites quickly stabilized after alterations in the microbial community. With respect to the nutrient concentrations, only in the cropland sediments did these perturbations have significant effects, but these were short lived. By the end of the experiment, nutrient concentrations were at preperturbation levels. The grassland sediments were more resistant to these alterations, not showing any response to the nutrient additions.

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