

## Abundances of protozoa in soil of laboratory-grown wheat plants cultivated under low and high atmospheric CO<sub>2</sub> concentrations

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### Summary

With increasing evidence of elevated carbon dioxide in the atmosphere, there is considerable interest in the effects of this atmospheric change on aquatic and terrestrial biota. There is, however, little information on the effects of elevated atmospheric CO<sub>2</sub> on terrestrial protozoa even though other research studies have shown an increase in density of soil-dwelling metazoa due to enhanced plant productivity and increased root exudates. We examined densities of protozoa in rhizosphere soil of wheat plants grown under experimentally controlled CO<sub>2</sub> treatments. The mean density of protozoa (number g<sup>-1</sup> soil) in rhizosphere samples of wheat plants grown in environmental controlled chambers was c. 17,000 g<sup>-1</sup> for elevated CO<sub>2</sub> concentrations (750 μmol l<sup>-1</sup>) and 10,900 g<sup>-1</sup> for plants grown at near ambient CO<sub>2</sub> concentrations (365 μmol l<sup>-1</sup>). The statistically significant (p < 0.01) increased density of total protozoa in the rhizosphere of plants grown in elevated CO<sub>2</sub> atmospheres provides additional evidence of enhanced soil microbial activity in response to elevated atmospheric CO<sub>2</sub> concentrations. The increased mineralizing activity of the proliferated protists may enhance plant nutrient recovery and help support the increased growth observed in elevated CO<sub>2</sub> environments.

**Key words:** atmospheric CO<sub>2</sub> enrichment, ciliates, flagellates, gymnamoebae, protists, rhizosphere communities, soil fertility

### Introduction

There has been substantial research on the effects of elevated carbon dioxide on individual plant species in controlled environmental conditions, especially the role of increased CO<sub>2</sub> partial pressures on plant growth and productivity (e.g., Enoch and Kimball, 1986; Delgado et al., 1994; Bernston et al., 1997). Particular attention has been given to ecosystem level responses, since increased loading of CO<sub>2</sub> in the atmosphere could have dramatic effects on climate and the stability of plant and animal communities (Cure and Acock 1986; Strain, 1987; Bazzaz et al., 1990; Bazzaz and Fajer,

1992; Field et al., 1992; Bowes, 1993; Idso and Kimball, 1993; Gifford, 1994; Griffin and Seemann, 1996; Drake et al., 1997; Ceulemans, et al., 1999; Petersen et al., 1999). In addition to more direct responses of plants, there also has been increasing interest in possible secondary effects of elevated atmospheric CO<sub>2</sub> on plant-animal interactions, especially decomposition of litter and below-ground effects on terrestrial communities including interactions between microbiota and metazoans (e.g., Griffiths et al., 1991; Canadell et al., 2000). However, less attention has been given to the effects on soil microbial communities (Hu et al., 1999).

Although there has been some variability in the reported effects of increased atmospheric CO<sub>2</sub> on plant growth and productivity (e.g., Enoch and Kimball, 1986; Sage et al., 1989), elevated atmospheric CO<sub>2</sub> tends to stimulate plant photosynthesis and increase carbon release (including root exudates) into ecosystems (Bernston and Bazzaz, 1996; Paterson et al., 1997; Couëteaux and Bolger, 2000). There is some evidence for selectively increased microbial activity in the rhizosphere of plants when grown under elevated atmospheric CO<sub>2</sub> concentrations (Schortemeyer et al., 1996; Zak et al., 1996; Elhottova et al., 1997; Kandeler, et al., 1998), but not all studies report increased protozoan abundances (e.g., Treonis and Lussenhop, 1997; Hungate et al., 2000). Moreover, there may be little effect on bacterial community structure (Griffiths, et al., 1998). Many of these studies have concentrated on bacterial and fungal responses (Hu et al., 1999) or on metazoa such as nematodes (e.g., Griffiths et al., 1991; Yeates, et al., 1997). An increased bacterial biomass in the rhizosphere of plants grown under elevated CO<sub>2</sub> concentrations also may enhance the abundance of eukaryotic microbiota that feed upon them, especially bacterivorous protozoa. Elliott et al. (1979) using soil microcosms inoculated with bacteria (*Pseudomonas* sp.) and amoebae (*Acanthamoeba* sp.) found that microcosms with amoebae mineralized significantly more carbon, nitrogen and phosphorus than controls without amoebae. Therefore, an increase in microbial activity associated with increased organic nutrients in the rhizosphere may enhance remineralization of essential nutrients required by plants to sustain their growth in CO<sub>2</sub>-enriched atmospheres. In this study we investigated the hypothesis that there will be a greater abundance of protozoa (flagellates, gymnamoebae and ciliates) in the rhizosphere of wheat plants grown under elevated CO<sub>2</sub> compared to plants grown at near ambient CO<sub>2</sub> concentrations using laboratory environmental growth chambers.

## Material and Methods

### *Treatment conditions*

Since the objective of this study was to examine the hypothesis that elevated atmospheric CO<sub>2</sub> will increase the number of protozoa within the rhizosphere of wheat plants when grown under controlled laboratory conditions, we have employed a standard controlled environment experimental design used in elevated CO<sub>2</sub> studies with plants. Four controlled environment chambers were used. Two were at ambient atmospheric CO<sub>2</sub> concentrations (365 μmol l<sup>-1</sup> CO<sub>2</sub>) and served as the control preparations (labeled as Ambient CO<sub>2</sub>; Expt. 1 and Expt. 2 in Table 2). The other two were

maintained at (730 μmol l<sup>-1</sup> CO<sub>2</sub>) and were the experimental treatments (labeled as High CO<sub>2</sub>; Expt. 1 and Expt. 2 in Table 2). Thus, all variables are held constant, except for the variation in CO<sub>2</sub> concentration. We did not examine the effects of elevated CO<sub>2</sub> on non-rhizosphere soil in pots without plants, since there is considerable published evidence that protozoan populations in the rhizosphere soil are enhanced compared to non-rhizosphere soil, and our focus was on testing the main hypothesis of the effects of elevated CO<sub>2</sub> versus ambient CO<sub>2</sub> on rhizosphere-dwelling protozoa. One pot was placed in each of the chambers and sown with several wheat seeds. After sprouting, the wheat plants were thinned to three plants per pot. Thus, there were two replicate samples for each CO<sub>2</sub> regime with four environmental growth chambers in total. Wheat seeds were planted in plastic 2 l containers in a sandy loam potting soil with 16% (w/w) organic content determined by combustive analysis at 375° C. The soil mixture was prepared by mixing equal volumes of a commercially prepared potting soil (Earthgro™, Glastonbury, CT) and a sandy soil mix (ASB Greenworld™, Valdosta, GA). This is somewhat higher in organic content than typical agricultural soils, but we believed it would provide greater water retentive capacity during pot-growth of the plants in the controlled environmental chambers. The size-particulate composition of the soil mixture based on sieved samples was as follows: Particle sizes greater than 2000 μm (15%), 400 μm – 2000 μm (42%), 200 – 400 μm (28%), and less than 200 μm (15%). The soil mixture was prepared immediately before use from sealed bags as distributed by the vendors. The soil mixture was not sterilized before sowing the seed and was initially moistened with pond water to introduce the same source of potential protozoa in all of the pots. Thereafter, the soil was evenly moistened and maintained at field capacity (47% w/w) using tap water (pH = 7.2, Accumet™ Model 15 pH meter) supplied by the local water authority from natural wells (United Water, Rockland County, NY). Alkalinity (as CaCO<sub>3</sub>) was 161 mg/l and salinity (sodium ions) was 27 mg/l. No fertilizer or nutrient amendments were added to the soil. The containers were placed in Conviron™ controlled environment growth chambers (Model E-15) with a day/night temperature of 28° C/ 20° C respectively and a 16 hour day. The light level at the top of the plants was 450 μmol and the relative humidity was a constant 50%. Seeds sprouted within one week and the wheat plants were grown for five additional weeks before soil samples were taken.

### *Sampling and enumeration of rhizosphere protozoa*

After five weeks growth, the root and soil mass was released by gently tapping the inverted pot, and a 2 cm

**Table 1.** Soil pH and plant biomass

Soil pH	Mean plant biomass at harvest Dry weight
Initial 6.8 Final 7.4 - 7.5	Ambient CO <sub>2</sub> control pots 2.8 g/plant Elevated CO <sub>2</sub> treatment pots 4.4 g/plant

long core of rhizosphere soil was removed from the base where the root mass was concentrated, using a cylindrical (1 cm diam.) brass corer. We sampled the same location in the base of each experimental pot to ensure that we were getting a soil sample enriched in root mass and thus largely rhizosphere soil. The number of protozoa per unit weight of soil was determined as follows (e.g., Anderson, 2000). Each plug of soil was immediately weighed, thoroughly dispersed in micropore (0.45 µm) filtered pond water, and diluted to 1/60. A 10 µl aliquot of this suspension was added to each well of a 24-well, sterile, plastic culture dish containing 2 ml of micropore filtered pond water. Each well also contained a small cube of malt/yeast agar (Page, 1991) to support growth of food bacteria for the protozoa. Two dishes (48 wells in total) were prepared for each soil sample. The objective of the method is to determine how many protozoa were in each of the 10 µl samples added to each well of the culture dishes. Since it is not possible to count protozoa directly in the 10 µl sample due to many protozoa, especially gymnamoebae, being obscured by soil particles, we incubate the inoculated water in the dishes for two weeks. At that point, any protozoa that have proliferated can be readily identified, characterized for size and shape, and each morphotype tallied as an instance of one species introduced in the original 10 µl inoculum. After 14 days of incubation at 25° C, each well in the culture dishes was inspected for protozoan growth using a Nikon Diaphot inverted microscope with phase optics. The growth of a protozoan species or morphotype in the well that received 10 µl of inoculum indicated that at least one individual of that protozoan type was included in the 10 µl aliquot introduced into the well from the diluted soil suspension. It is necessary to incubate the inoculated dishes for at least 14 days to amplify the number of each kind of protozoa introduced in the inoculum. Therefore, it is not possible to determine how many of the protozoa were encysted at the time of inoculation. Consequently, the numbers obtained by this method include all viable forms (trophonts and cysts). The total number of protozoa (flagellates, gymnamoebae and ciliates) ml<sup>-1</sup> in the original solution was determined by computing the number of individuals in the original soil extract volume in proportion to the number observed in the 480 µl of soil suspension that had been

added to the 48 wells. Since the number of protozoa may be underestimated when a large proportion of the wells contain growth, due to possible multiple occurrences of the same species in the 10 µl introduced into a well, a Poisson distribution adjustment of the counts was made. This was based on procedures originally published for the most probable number technique (MPN) with bacteria (e.g., Halvorson and Ziegler, 1932). The transformation of the data used the following formula:  $M = N \cdot [-\ln(1 - k/N)]$ , where  $M$  = the estimated mean count of a protozoan morphotype in the plate wells,  $N = 48$  (the number of wells in total), and  $k$  = the number of positive wells containing growth of the protozoan morphotype. When the proportion of wells with growth is relatively low,  $M$  is almost equivalent to the original raw count. When, however, the proportion of wells with growth is high,  $M$  is larger reflecting the likelihood of multiple occurrences of a given morphotype in the initial inoculum that would not be detected by inspection of the culture dish well, and possibly undercounted. The abundances of protozoa obtained by this method were expressed as density ( $D$ ), number g<sup>-1</sup> of soil, based on the weight of the plug of soil dispersed in the original suspension as explained below. To estimate the standard deviation, we employed the formula used by Halvorson and Ziegler (1933) based on the estimation of the variance as  $V = N \cdot p \cdot (1-p)$  where  $V$  = variance and  $p = k/N$  (the proportion of wells with growth). The standard error was calculated as  $SD/(N)^{0.5}$  and is an estimate within the lower limit of the 97% confidence interval.

Using  $M$  as an estimate of the mean number of protozoa in the 48-well, culture dishes, the density of protozoa is given by the following formula:  $D = [(M \cdot V_m) / (I \cdot W)] / S$ , where  $D$  = density of a protozoan morphotype or species (number g<sup>-1</sup> soil),  $M$  = Poisson mean estimate of the numbers of the protozoan of interest,  $V_m$  = volume of soil suspension used to prepare the inoculum expressed as µl,  $I$  = volume of inoculum added to each well (here it was 10 µl),  $W$  = total number of wells used, and  $S$  = quantity of soil (g dry weight) used to prepare the soil suspension ( $V$ ). This is in accordance with our standard published techniques (Anderson and Rogerson, 1995; Darbyshire et al., 1996; Anderson and Bohlen, 1998; Anderson, 2000). It is important to note that the Poisson transformation of the data, while improving the estimate of protozoan numbers, does not bias the data statistically. The significant t-test difference between the grand means based on the transformed data (Table 2) is comparable to the difference obtained when the raw counts, based on direct observation, are used to analyze the data. A two-tailed t-test was used to analyze differences in mean densities of protozoa (e.g., Table 2).

**Table 2.** Abundances of protozoa (number g<sup>-1</sup>) in rhizosphere soil from pot-grown wheat plants cultivated under ambient and elevated carbon dioxide concentrations

	Ambient CO <sub>2</sub> (365 ppm) Control Preparation			High CO <sub>2</sub> (730 ppm) Experimental Treatment		
	Expt. 1	Expt. 2	Means Grand	Expt. 1	Expt. 2	Means Grand
Flagellates	1,714 (65)	6,559 (88)	4,137 (77)	11,245 (75)	5,296 (89)	8,271 (82)
Gymnamoebae	3,055 (73)	3,084 (74)	3,070 (74)	2,845 (73)	9,121 (53)	5,983 (63)
Ciliates	6,269 (92)	1,242 (56)	3,755 (74)	3,439 (84)	2,076 (64)	2,757 (74)
Totals	11,038 (230)	10,885 (218)	10,962 (224)*	17,529 (232)	16,493 (206)	17,011 (219)*

**Note:** There were two replicate experiments for each CO<sub>2</sub> treatment labeled here as Expt. 1 and Expt. 2. The mean values are the Poisson mean densities of the protozoa in the samples for each CO<sub>2</sub> concentration. Values in parentheses are the Poisson standard errors of the mean. Asterisks indicate statistically significant differences ( $t = 11.5$ ,  $p < 0.01$ ,  $df = 2$ ).

To ensure that the experimental treatments were as close to natural conditions as possible, we grew the wheat plants in soil rather than sand. Since roots cling to, and penetrate into soil particles, it is not possible to effectively separate them from the soil to obtain a root biomass as is possible with sand-cultured plants. We chose not to grow the plants in sand since this would be quite artificial compared to any natural or cultivated terrestrial location. Since it was not practical to assess productivity based on root mass, we measured the dry mass of the wheat leaves as an indicator of plant productivity.

## Results

The pH of the soil before and after plant growth, and the biomass (mean dry weight) of the leaves of the wheat plants at time of harvest for each CO<sub>2</sub> treatment are presented in Table 1. Since it has already been shown that there is greater root production, release of exudates in plants grown in elevated CO<sub>2</sub> environments and enhanced bacterial growth (e.g., Bernston and Bazzaz, 1996; Schortemeyer et al., 1996; Zak et al., 1996; Paterson et al., 1997; Kandeler, et al. 1998), we did not evaluate these parameters. The pH of the soil became slightly more alkaline (7.4 – 7.5) after plant growth. The plant dry weight biomass was larger in the elevated CO<sub>2</sub> treatment than in the ambient CO<sub>2</sub> control environment and this is consistent with prior published results of the positive effect of elevated atmospheric CO<sub>2</sub>

on plant growth (e.g., DeLucia et al., 1999). Early in the growth of the wheat plants, it was clear that the plants in elevated CO<sub>2</sub> were more robust than the controls at ambient CO<sub>2</sub>.

The densities of protozoa (number g<sup>-1</sup> soil) in the rhizosphere of plants in the high and low CO<sub>2</sub> treatments are shown in Table 2 for each of the replicates (experiment 1 and experiment 2). While there is some variability within a given group of protozoa (flagellates, gymnamoebae, and ciliates), the mean densities for total protozoa in the high CO<sub>2</sub> treatment are larger than in the ambient, control CO<sub>2</sub> environment (17,011 g<sup>-1</sup> and 10,962 g<sup>-1</sup>, respectively). Although there were only two experimental samples per treatment, the mean differences are highly statistically significant ( $t = 11.5$ ,  $p < 0.01$ ,  $df = 2$ ). Also the mean densities of flagellates and gymnamoebae tended to be larger in the high CO<sub>2</sub> treatment than in the low CO<sub>2</sub> treatment. The densities of flagellates and gymnamoebae were respectively 2.0 and 1.9 times greater in the high CO<sub>2</sub> compared to the low CO<sub>2</sub> treatment. There was no appreciable difference in the mean densities of ciliates within the error of the method. The diversity of protozoa based on a Shannon-Wiener formula (H) was similar among the four treatments (H = 3.0 – 3.2).

Most of the protozoa in the rhizosphere soil from high and low CO<sub>2</sub>-treated plants appeared to be of similar morphotypes at the generic level, however they differed in size as reported below and may represent different species. Small flagellates (< 10 µm) were commonly observed, but few amoeboflagellates.

Approximately 90% of all flagellates were less than 10  $\mu\text{m}$ . Most of the gymnamoebae morphotypes were greater than 10  $\mu\text{m}$ , but 56% of the gymnamoebae morphotypes were less than 20  $\mu\text{m}$ . Among the gymnamoebae, the major genera were *Acanthamoeba*, *Mayorella* or *Dactylamoeba* (*Korotnevella* group, Smirnov, 1999), *Saccamoeba*, *Vannella* and *Platyamoeba*. *Colpoda* sp. (c. 50 - 60  $\mu\text{m}$ ), a commonly occurring soil ciliate, and occasionally euplotid ciliates (c. 60 - 70  $\mu\text{m}$ ), were observed in most of the treatment samples. Other ciliates were smaller (15 to 20  $\mu\text{m}$ ) and occurred with variable frequency across the treatment conditions.

The mean sizes of gymnamoeba and flagellate morphotypes were larger in the high  $\text{CO}_2$  compared to the ambient  $\text{CO}_2$  treatments. Since morphology and size characterize each morphotype, we report the sizes as indicative of possible species-level taxonomic differences in the two treatment conditions. Although there were similar genera among the treatment conditions, differences in size of the protozoan morphotypes may indicate species differences that could not be detected solely by light microscopic, morphological examination. The mean length of gymnamoeba morphotypes in the high  $\text{CO}_2$  treatment was  $24.5 \pm 2 \mu\text{m}$  and for those in the low  $\text{CO}_2$  treatment  $18.8 \pm 1 \mu\text{m}$  ( $t = 2.3$ ,  $p = 0.02$ ,  $df = 114$ ). The mean length of flagellate morphotypes in the high  $\text{CO}_2$  treatment was  $7.5 \pm 0.2 \mu\text{m}$  and in the low  $\text{CO}_2$  treatment was  $6.5 \pm 0.4 \mu\text{m}$  ( $t = 2.1$ ,  $p = 0.04$ ,  $df = 125$ ). The mean size of ciliate morphotypes was larger in the low  $\text{CO}_2$  treatment ( $50 \pm 1 \mu\text{m}$ ) compared to the high ( $40 \pm 3 \mu\text{m}$ ) mainly due to a heavy bloom of large colpodids that occurred in one of the containers in the low treatment ( $t = 2.9$ ,  $p < 0.01$ ,  $df = 87$ ). We are not suggesting that the sizes reflect differences in nutritional states of the protozoa, but rather probably reflect the differences in species composition of the protozoan populations in the two treatment conditions. The high  $\text{CO}_2$  treatment appears to favor the growth of larger morphotype protozoa. Since the enumeration method requires enrichment culturing of the soil sample aliquots for 2 weeks before enumeration, we cannot suggest that the differences in size represent in-situ differences in nutritional status of the protozoa at the time of sampling.

## Discussion

These data add additional evidence that elevated atmospheric  $\text{CO}_2$  levels affect abundances of terrestrial microbiota in the rhizosphere and extend the observations beyond bacteria and fungi to include protozoa, one of the next higher level taxa in the food chain. This finding in conjunction with prior reports of increased

numbers of nematodes (e.g., Yeates, et al., 1997) indicates a taxonomically broad-based proliferation of protists and invertebrates within the rhizosphere of plants exposed to elevated  $\text{CO}_2$ . Previous research on the effects of elevated  $\text{CO}_2$  on terrestrial bacteria and protozoa has shown varied results. For example, Treonis and Lussenhop (1997) found no statistically significant differences in bacteria or protozoa in elevated  $\text{CO}_2$  treatments compared to ambient  $\text{CO}_2$ . They analyzed bulk soil samples from pots containing *Brassica nigra* plants when grown in open-top chambers in the natural environment. It is important to note that they did not find enhanced growth of shoot or root biomass of the *B. nigra* in elevated  $\text{CO}_2$  which is in contrast to some prior research studies. Hence, it may not be surprising that they found no effect on bacterial or protozoan growth. Moreover, they did not sample the dense rhizosphere as we have done, but assumed that most of the soil in the pots was occupied by root growth. They also used a multiple dilution technique, rather than the culture-enrichment technique we used, and assessed presence or absence of protozoa by making a wet-mount slide of the culture fluid. In our procedure, each well of the culture plate is examined under phase contrast illumination using an inverted microscope. We, thus, assess the entire contents of each well to determine size and shape of the morphotypes in addition to counting them. The plants in our experiment, moreover, showed increased dry-weight biomass in the high  $\text{CO}_2$  treatment (4.4 g/plant) compared to the ambient  $\text{CO}_2$  treatment (2.8 g/plant). Thus, it is possible more root exudates were released from plants in the high  $\text{CO}_2$  treatment compared to the controls at ambient  $\text{CO}_2$  concentrations. This could account for the increased number of protozoa observed in the high  $\text{CO}_2$  treatments in our experiments.

It is important also to consider the possible effect of different plant species in protozoan growth within the rhizosphere. The exudates of some plants may be less productive for protozoan growth compared to other species and more research is needed to determine possible differential effects of root exudates of different plants on protozoan proliferation and bacterial abundances. For example, although Treonis and Lussenhop (1997) found no statistical increase in bacterial abundances in their open-top chamber studies with *B. nigra*, Marilley et al. (1999) working with *Lolium perenne* and *Trifolium repens* grown in open-air,  $\text{CO}_2$  fumigating, ring systems (FACE), found enhanced growth of heterotrophic bacteria in soil fractions in elevated  $\text{CO}_2$  compared to ambient  $\text{CO}_2$  treatments. Until more controlled experiments are done with the same soil, container size, and illumination, it is difficult to assess how much of this variability is due to plant species versus environmental variables.

Our study used the same commercially prepared soil in all of the growth treatments, hence we believe we have carefully controlled this variable in our experiments. However, this soil has a higher organic content than most agricultural soils, so the results may not be representative of the effects of elevated CO<sub>2</sub> in agricultural settings. We also sampled the densest part of the root-filled soil at the base of the pots to ensure that we had a consistent, representative sample of rhizosphere soil from each treatment pot. Further research is needed to determine to what extent non-rhizosphere soil at some distance from the root mass is affected by elevated CO<sub>2</sub> treatment of plants with varying kinds of soil and different plant species. Samples were taken only at the end of the experiment (5 weeks of growth) and it would be interesting to also determine if there are changes in the protozoan population in a time series study at shorter intervals. A prior time series study of microbiota (0.3 – 2.7 µm size range) in the rhizosphere of winter wheat plants grown hydroponically in sand cultures showed that microbial biomass C increased in the elevated CO<sub>2</sub> treatment between 25 and 34 days, but not earlier (Elhottova et al., 1997). This is similar to the period of time used in our study. However, sand-based hydroponic plant cultivation may produce a different microbial response than soil-based cultivation, and much more research is needed to clarify how the structure and composition of the substratum affects responses of rhizosphere microbiota to elevated CO<sub>2</sub>.

In the current experimental study, both flagellates and gymnamoebae occurred in increased numbers in the elevated CO<sub>2</sub> treatments and are known to be effective grazers on bacteria, especially the smaller species. However, some small gymnamoebae are also effective mycophagous predators (e.g., Old and Chakraborty, 1986), and further research is needed to clarify the food web dynamics of protozoa in the rhizosphere of plants grown in ambient and elevated CO<sub>2</sub> concentrations. It is interesting to note that in our research, the relative numbers of flagellates, gymnamoebae, and ciliates varied among the four treatment conditions (Table 2). For example, in the control pot at ambient CO<sub>2</sub> (experiment 1, Table 2) a bloom of ciliates, mainly relatively large colpodids, was much higher in density (6,269 g<sup>-1</sup>) than in any of the other treatments. However, the density of flagellates in this control sample (1,714 g<sup>-1</sup>) was among the lowest observed in our experiment. This may be due to mutual exclusion between these two groups. The ciliates may have been better competitors for food, or may have been predators on the smaller flagellates, thus reducing their relative densities in this treatment. However, it should be noted that the overall densities of protozoa in both experiments at ambient CO<sub>2</sub> concentrations are similar, since there are larger numbers of flagellates in

experiment 2 (Ambient CO<sub>2</sub>, Table 2) where the larger colpodid ciliates were not so dominant. This adds further credence to the hypothesis that the larger ciliates may have excluded flagellates in experiment 1 at ambient CO<sub>2</sub> concentrations. A larger number of gymnamoebae (9,121 g<sup>-1</sup>) occurred in the soil of experiment 2 (high CO<sub>2</sub>) than in any of the remaining treatment conditions. It is not immediately clear why this occurred, although the flagellates are once again much lower in experiment 2 than in experiment 1 in the elevated CO<sub>2</sub> treatments. The abundance of gymnamoeba in conjunction with a lower number of flagellates may be due to predation by the gymnamoebae on the flagellates. It is known that some gymnamoebae prey on flagellates including species of *Mayorella* (Bovee, 1985) and *Vexillifera* (e.g., Anderson, 1994). Although there are variations among the different kinds of protozoa in the high CO<sub>2</sub> treatment, listed in Table 2, the overall means in the two experiments are rather similar (17,529 g<sup>-1</sup> and 16,493 g<sup>-1</sup>). No information is available in this study on the relative number of encysted versus trophic stages of protozoa. The enumeration technique assesses total viable protozoa (cysts and trophonts). However, in a prior study (Anderson, 2000) a rapid drying method was used to assess the number of desiccation-resistant cysts of gymnamoebae that grew up in the culture enrichment method compared to total amoebae in non-dried equivalent samples of soil that were enumerated by the culture enrichment procedure. A strong linear regression was obtained relating proportion of active forms to soil hydrature expressed as percent (w/w). Based on this equation, at a moisture content of 47% as occurred in the soil in the current study, most of the gymnamoebae would be active. However, this equation is based on soil sampled from a grassy knoll, and very likely represents a different set of relations than would be expected for the more organic-rich soil used here. More recently, however, an unpublished estimate of proportion of encysted to total gymnamoebae was determined using the rapid drying technique for more organically rich soil (c. 13% w/w) in laboratory culture similar to the soil composition used in this study. At a moisture content of 45% (w/w), 85% of the gymnamoebae were active and 15% were encysted. Further research is needed to determine if variations in atmospheric CO<sub>2</sub> concentrations affect the proportion of active protozoa in the rhizosphere.

Since c. 90% of all flagellate morphotypes in the current study were less than 10 µm, and c. 50% of the gymnamoebae morphotypes were less than 20 µm, these smaller protozoa may be among the more important members of the rhizosphere microbial community. Furthermore, since protozoa are known to increase the productivity of microbial communities (e.g., Stout, 1980; Clarholm, 1981; Griffiths, 1994;

Zwart, et al., 1994) and enhance available soil nutrients through mineralization of particle-bound carbon and inorganic nutrients during predation on bacteria and smaller eukaryotic microbiota, it is very likely that elevated CO<sub>2</sub> levels can increase soil fertility. The greater dry-weight biomass of the wheat leaves in the high CO<sub>2</sub> treatments (c. 1.6 x) compared to the low CO<sub>2</sub> treatments confirms previously published reports and gives further evidence of the dynamic complementarity between plant enhanced growth and microbial increased activity during CO<sub>2</sub> atmospheric enrichment. There is evidence that plants grown at elevated CO<sub>2</sub> concentrations require more nutrients than those grown at lower CO<sub>2</sub> concentrations (e.g., Gorissen, 1996; Hu, et al. 1999). This need may be partially balanced by the nutrient mineralizing activity of more robust soil microbial communities in response to increased root exudates of plants exposed to elevated atmospheric CO<sub>2</sub> and help to sustain their enhanced growth induced by the elevated atmospheric CO<sub>2</sub> concentrations. However, additional research is needed to determine the dynamics of nutrient exchange between plant roots and the surrounding soil microbial communities. Research with respect to nitrogen (e.g., Griffiths and Robinson, 1992) using mathematical models suggests that enhanced mineralization due to increased root exudates and elevated microbial activity may allow for efficient recycling of nitrogen, rather than supply extra nitrogen from the soil organic matter. However, under elevated demand for nutrient resources during enhanced growth of plants, even this saving of nutrient resources may help to sustain plant productivity. Other researchers (e.g., Thornley and Cannell, 2000) point out that even under conditions of enhanced nitrogen availability, ecosystems may take many decades or longer to come to a new equilibrium level of productivity in response to elevated atmospheric concentrations of CO<sub>2</sub>, though a variety of responses may occur in the range of 1 – 10 years. The generalizability of the data from these mathematical models needs to be evaluated in further experimental research. Additional evidence is needed for the role of rhizosphere microbiota in mineralization of major soil nutrients under a variety of environmental conditions, including elevated partial pressures of atmospheric CO<sub>2</sub>.

## Acknowledgements

This research was supported by a Packard Foundation Grant DLP 998306 to KLG & ORA. This is Lamont-Doherty Earth Observatory Contribution Number 0000.

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*The manuscript is presented by M. Farmer*