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Nitrogen Mineralization by *Acanthamoeba polyphaga* in Grazed *Pseudomonas paucimobilis* Populations

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Nitrogen mineralization was studied in a simple grazing system in which the protozoan *Acanthamoeba polyphaga* was grown with the bacterium *Pseudomonas paucimobilis* (two soil organisms isolated from the shortgrass prairie in northern Colorado). In different experiments, either carbon or nitrogen was adjusted to be in limiting amounts. When carbon was limiting, grazers were almost entirely responsible for nitrogen mineralization, with bacteria themselves contributing little. When nitrogen was limiting, nitrogen mineralization by grazers permitted continued growth by the grazed bacteria and a greater bacterial biomass production. The increased growth of the grazed bacteria did not result in an increased total amount of carbon used, but the grazed bacteria used carbon more efficiently than the ungrazed bacteria.

Many studies on decomposition which have included protozoa in addition to the primary decomposer organisms show that protozoa are beneficial to decomposition (11). Most evidence indicates that the primary decomposers directly decompose organic detritus, but are not very efficient in quickly releasing minerals from their own biomass (5). Secondary decomposers, such as protozoa, consume primary decomposers and release mineral nutrients as waste products that are tied up in the primary decomposer biomass. In this way, grazers are able to make nutrients available that would otherwise remain inaccessible much longer. Most studies of the effects of grazers on decomposition have attributed their beneficial actions to mineral regeneration, which relieves nutrient limitations for primary decomposers (10). Under conditions of available carbon but a mineral limitation, the regeneration of minerals permits continuing growth by primary decomposers. With no available carbon source, the mineralized nutrients build up in the environment, being available to other organisms such as the primary producers (2).

These experiments were designed to quantify the rate of nitrogen mineralization of a common soil grazer, *Acanthamoeba polyphaga*, to compare it with that of ungrazed bacteria, and to observe the effects of nitrogen mineralization on the growth of nitrogen-limited bacteria.

MATERIALS AND METHODS

Pseudomonas paucimobilis and *A. polyphaga* were isolated from grassland soil (rhizosphere of *Bouteloua gracilis*, blue grama) of the Central Plains Experimental Range (18 km northeast of Nunn, Colo.) where both commonly occur. Stock cultures of *A. polyphaga* were maintained in 1% proteose peptone-1% glucose medium and grazed cultures were inoculated with 50,000 washed amoebae per ml. In both the grazed (*A. polyphaga* and *P. paucimobilis*) and ungrazed (*P. paucimobilis* only) treatments, there were three replicate cultures, each consisting of 200 ml of medium in a 500-ml Erlenmeyer flask. The medium was a carbonate-buffered saline with salts designed to match the soil solution (8). Cultures were aerated by bubbling CO_2-free air through the medium and incubated at 26°C.

For the nitrogen-limited experiment, there was a period in which all nutrients except nitrogen were available, and for the carbon-limited experiment, there was a period in which all nutrients except a carbon source were available. In the nitrogen-limited experiment, 2,000 μg of glucose per ml and 15 μg of $(NH_4)_2SO_4$-N per ml were used. The nutrient levels for the carbon-limited experiments were 1,000 μg of glucose per ml and 50 μg of asparagine-N per ml in experiment 1 and 500 μg of glucose per ml and 20 μg of asparagine-N per ml in experiment 2. In the nitrogen-limited experiment, of the 15 μg of $(NH_4)_2SO_4$-N per ml supplied to the cultures, 5 μg/ml was supplied upon inoculation of the organisms, and growth was permitted for 48 h. During this time, the bacterial population grew to an appreciable size and used nutrients released by the amoebae from their postinoculation die-off. After this time, 10 μg of $(NH_4)_2SO_4$-N per ml was added, and we assumed that the subsequent nutrient status and growth conditions were equal in the grazed and ungrazed treatments. In the carbon-limited experiments, all nutrients were added when the organisms were inoculated, since the amoebae would not grow any other way.

The bacterial numbers were determined with a Helber counting chamber. Bacterial size was determined by measuring the length and width of 20 bacteria from each replicate with a filar micrometer and by calculating the volume with the assumption that the bacteria were cylinders. Amoebae were counted by placing 0.02 ml of culture on a slide and covering it with a 22-mm cover glass. The amoebae were counted in five transects across the cover glass, and the number per milliliter was calculated from the average number per transect. Amoebal size was determined with a Coulter Counter. Bacterial and amoebal carbon contents were determined from assays of pure cultures of these organisms with a furnace (Leco Corp., St. Joseph, Mich.). Ammonium was determined with Nesslerization or the alkaline phenol method (4). Glucose was determined with the Glucostat method (Worthington Diagnostics, Freehold, N.J.). CO_2 was assayed by trapping the CO_2 exiting the flasks in 1 N NaOH in a 20-cm test tube, precipitating the carbonate with $BaCl_2$, and titrating the excess alkali with 1.00 N HCl in a Gilmont microburette.

RESULTS

Nitrogen from the bacterial biomass began to be mineralized when the glucose was exhausted in the carbon-limited experiment (Table 1). This occurred at 47 h in the grazed treatment and 71 h in the ungrazed treatment. There was a greater ammonium release in the grazed treatment; however, since too much nitrogen was added in the nutrients, not all of the mineral nitrogen came from the bacterial biomass via grazers. The amount of ammonium-N released by the amoebae was calculated by subtracting the amount of ammonium in the grazed treatment from that in the ungrazed treatment at 168 h or 8.1 μg of ammonium-N per ml.

The bacterial C (Table 1) increased initially in the grazed treatment after the glucose was exhausted (47 h grazed versus 71 h ungrazed) and dropped in the grazed treatment after 96 h. Amoebal growth continued through the experiment, but showed a trend toward encystation near the end of the experiment.

CO_2 release (Table 1) by the grazed and ungrazed treatments slowed after glucose was exhausted, but continued in amounts larger than would be expected from the amount of bacterial biomass present.

In a follow-up ungrazed experiment in which less nitrogen was added to the nutrients and the experiment lasted longer, bacteria had a chance to use up any residual carbon sources. The CO_2 release in this experiment (Table 2) showed that bacteria seemed to have a residual carbon source, but that between 306 and 378 h this was mostly depleted, because little CO_2 was produced during this time. Also, at this time, the ungrazed bacteria did not mineralize any nitrogen (Fig. 1).

Ammonium was rapidly taken up in the nitrogen-limited experiment, and the net amount present through most of the experiment in both the grazed and ungrazed treatments was very low (Fig. 2).

The nitrogen limitation resulted in a cessation of growth of the ungrazed bacteria after 9.5 h (Table 3). The amount of biomass C of the ungrazed bacteria was depressed to less than one-sixth of that of the ungrazed population by

TABLE 1. *Carbon data for carbon-limited experiment 1*

Form of carbon	Amt of carbon used (μg/ml) at following time (h)[a]:						
	0	24	47	71	96	120	168
Grazed bacterial C	10.2	67.7	77.8	90.4	108.4	61.7	46.0
Amoebal C	21.9	24.2	30.4	34.5	43.3	32.5	41.2
Ungrazed bacterial C	5.5	11.4	89.9	122.5	98.6	103.5	99.3
Glucose-C in grazed treatment	388.8	33.1	0.0	0.0	0.0	0.0	0.0
Glucose-C in ungrazed treatment	400	369	103	0.0	0.0	0.0	0.0
Cumulative CO_2-C from grazed treatment		177	219	241	269	301	343
Cumulative CO_2-C from ungrazed treatment		34	153	268	309	336	379

[a] Amount of carbon added initially was 442 μg of glucose and asparagine carbon per ml.

TABLE 2. *Carbon data carbon-limited experiment 2*

Form of carbon	Amt of carbon used (μg/ml) at following time (h):									
	0	24.5	42	65	114	141	162	235.5	306	378
Bacterial C	33.2	65.8	83.1	161.2	136.8		138.5	134.9	84.6	108.7
Glucose-C	159.0	92.0	57.0	0	0		0	0	0	0
Cumulative CO_2-C		32.0	84.0	125.0		157.0	170.0	190.0	209.0	225.0

grazing during the nitrogen limitation. Amoebal growth was greatest between 54 and 139 h.

Both glucose use and CO_2 production were less in the grazed treatment than in the ungrazed treatment during nitrogen limitation.

DISCUSSION

The importance of grazers to nitrogen mineralization was established in carbon-limited experiment 1 by the accelerated rate at which ammonium appeared in the grazed treatment (Fig. 3). The amount of ammonium previously calculated to have been mineralized by the amoebae was 8.1 μg of ammonium-N per ml. If this ammonium build-up occurred between 47 and 168 h when glucose was exhausted and bacteria could not grow, it would be equivalent to 0.067 μg of ammonium-N per ml mineralized per h. If the experimentally determined values for amoebae of 10% dry weight and amoebal N as 10% of dry weight are used, the value would be 0.014 μg of N per μg of amoebal N per h. This could be lower than the actual ammonium release, since the amount of CO_2 released after glucose was exhausted suggested that there was a carbon source that the bacteria were slowly using, possibly waste or metabolite C. In the ungrazed treatment from 71 h, when the glucose was exhausted, to 168 h, there was between 122.5 and 99.3 μg of bacterial C per ml, but 111 μg of CO_2-C per ml was respired during this same time. This would mean that, since the experiment was not entirely carbon limited, the grazed bacteria grew to some extent and used mineral-

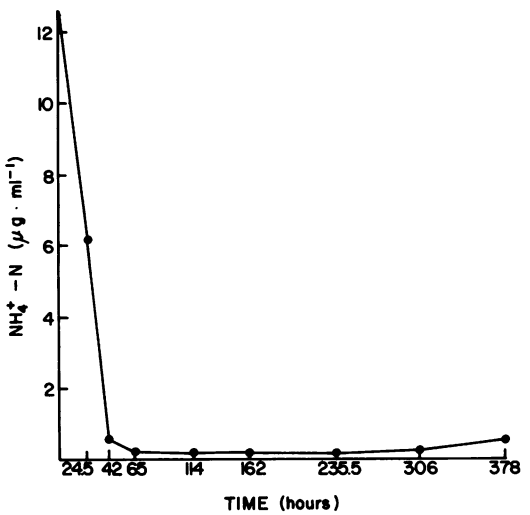

FIG. 1. *Ammonium-N for carbon-limited experiment 2.*

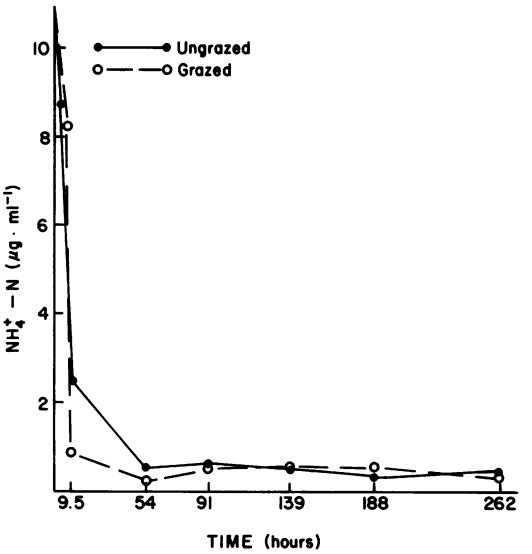

FIG. 2. *Ammonium-N for the nitrogen-limited experiment. Ammonium is quickly used up so that only negligible amounts remain.*

TABLE 3. *Carbon data for the nitrogen-limited experiment*

Form of carbon	Amt of carbon used (μg/ml) at following time (h)[a]:						
	0	9.5	54	91	139	188	262
Grazed bacterial C	18.8	38.5	30.2	8.0	8.0	8.6	10.5
Amoebal C	17.0	18.0	26.4	49.9	73.0	67.5	60.3
Ungrazed bacterial C	14.7	41.3	49.0	51.3	32.6	48.5	47.8
Glucose-C in grazed treatment	718	578	453	318		306	269
Glucose-C in ungrazed treatment	727	631	489	271		209	114
Cumulative CO_2-C in grazed treatment	40.1	73.1	112.0	192.0	230.3	239.5	282.4
Cumulative CO_2-C in ungrazed treatment	59.3	97.3	146.0	234.5	263.9	278.1	323.1

[a] Amount of carbon added initially was 800 μg of glucose carbon per ml.

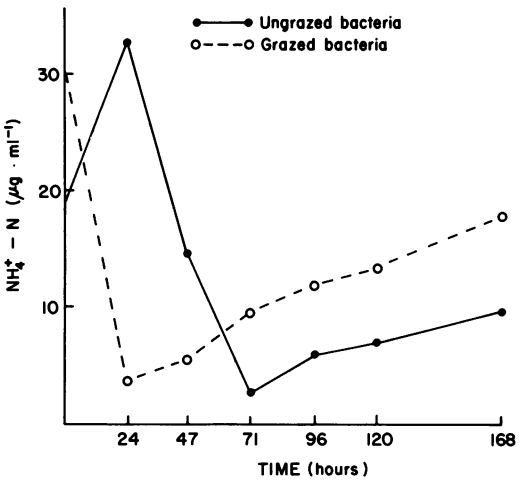

FIG. 3. *Ammonium-N for carbon-limited experiment 1.*

ized nitrogen for biomass production, thus reducing the apparent nitrogen mineralization of the amoebae.

Carbon-limited experiment 2 demonstrated that bacteria by themselves released nitrogen from the biomass sparingly, even during a fairly complete carbon limitation. This was seen from 306 and 378 h, at which times a bacterial biomass between 85 and 109 μg of C per ml respired only 16 μg of CO_2-C per ml. This implied that very little carbon was available to the bacteria; however, there was no ammonium released by the bacteria.

In the nitrogen-limited experiment, nitrogen mineralized by the amoebae was not directly detectable, since bacteria removed it from the medium as rapidly as it was released to form a continuous internal cycle (6, 9). Nitrogen mineralization was deduced from bacterial consumption by amoebae. The amoebae assimilated a portion of the bacteria up to 139 h that was equivalent to 12.9 of the total 15 μg of $(NH_4)_2SO_4$-N per ml that was supplied to the bacteria. We calculated this value by comparing the bacterial C at 139 h, or 8 μg of bacterial C per ml with that which grew from the initial 5 μg of $(NH_4)_2SO_4$-N per ml or 18 μg of bacterial C per ml. Therefore, 8/18 × 5 μg of N per ml or 2.1 μg of N per ml remained, meaning that 5 − 2.1 or 2.9 μg of N per ml was assimilated by the amoebae. In addition to 2.9 μg of N per ml, all of the bacterial biomass that grew from the 10 μg of pulse of nitrogen per ml was assimilated, meaning that 2.9 + 10 or 12.9 μg of bacterial N per ml was assimilated by the amoebae. If the C/N ratios of the bacteria and amoebae were equal and if a growth efficiency of 0.37 (7) for *Acanthamoeba* is used (supported by a growth efficiency of 0.4 of [1]), then 63% of the ingested nitrogen would be in excess and would be excreted. Therefore, if the amoebae used 37% of the ingested nitrogen for growth, then 12.9/0.37 or 34.8 μg of N per ml was ingested by the amoebae. If 34.8 μg of N per ml was ingested by the amoebae, then 34.8 × 0.63 or 22.0 μg of N per ml was excreted. This would be equal to 0.1579 μg of N per h or 0.0199 μg of N per μg of amoebal N per h. The difference in nitrogen excretion rates between the carbon- and nitrogen-limited experiments (0.014 μg of N per μg of amoebal N per h in the carbon-limited experiment versus 0.0199 μg of N per μg of amoebal N per h in the nitrogen-limited experiment) can probably be accounted for by the large fraction of the amoebal population that was beginning to encyst, and thus in a less active state, in the carbon-limited experiment, plus the presence of a residual carbon source in the carbon-limited experiment.

Despite the availability of nitrogen from mineralization by grazers, the grazed treatment did not use more glucose than the ungrazed treatment during the period of nitrogen limitation of 54 to 262 h (Table 3) of the nitrogen-limited experiment. During this time, 184 μg of glucose-C per ml was used in the grazed treatment versus 375 μg of glucose-C per ml in the ungrazed treatment. Nonetheless, the grazed bacteria were more productive than the ungrazed bacteria. This can be seen by considering how much bacterial biomass was produced to support the observed amoebal growth. From 54 to 262 h, when nitrogen was limiting, there was a gain of 46.6 μg of amoebal C per ml. With a growth efficiency of 0.37 (7), the amount of bacterial C consumed by the amoebae would be 46.6/0.37 or 126 μg of bacterial C per ml. Also, during 54 to 262 h, the bacterial population dropped by 19.8 μg of C per ml as a result of grazing. Thus, 126 − 19.8 or 106.2 μg of bacterial C per ml would have been produced during this period. If the maintenance requirements of the consumed bacteria are considered, then 42.8 μg of bacterial C per ml would have been used had the bacteria remained alive from 54 to 262 h. The maintenance requirement is based on the amount of CO_2 released by the bacteria near the end of carbon-limited experiment 2 (0.002 μg of CO_2-C per μg of bacterial C per h). Therefore, 106.2 − 42.8 or 63.4 μg of bacterial C per ml would be the bacterial C production during the period of nitrogen limitation. With 184 μg of glucose-C per ml used from 54 to 262 h, the growth efficiency would be 63.4/184 or 34.5%. In the ungrazed treatment, there was no biomass formed from 54 to 262 h, but 375 μg of glucose-C per ml was used for an apparent growth efficiency of 0%. Al-

though the grazed bacterial growth had to be extrapolated from amoebal growth, there was probably no corresponding cryptic growth in the ungrazed treatment. If the ungrazed bacteria had a large die-off and regrowth, it would have shown in the CO_2 production of the ungrazed treatment of the carbon-limited and follow-up carbon-limited experiments. Also, in carbon-limited experiment 2, cryptic growth would have led to a release of ammonium from excess nitrogen of dead bacteria when nutrients of dead bacteria were used by newly growing bacteria.

Thus, increased growth of the grazed bacteria in response to mineralized nitrogen from amoebae was accomplished by more efficient use of carbon rather than increased carbon use. Hence, during nitrogen limitation, 34.5% of the carbon used became bacterial biomass in the grazed treatment versus 0% in the ungrazed treatment. Cooney et al. (3) reported that under nitrogen limitation *Enterobacter aerogenes* used a sizeable fraction of the carbon source for metabolite production. The carbon budget of the nitrogen-limited experiment tended to support this finding. In the grazed treatment from 54 to 262 h (during the nitrogen limitation), 170.4 μg of CO_2-C per ml was produced and 184 μg of glucose-C per ml was used, meaning that after metabolism by both bacteria and amoebae, 170.4/184 or 93% of the glucose-C used became CO_2-C. In the ungrazed treatment, during the same time period, 177.1 μg of CO_2-C per ml was produced with 375 μg of glucose-C per ml used, meaning that 177.1/375 or 47% of the glucose-C used became CO_2-C. Since no biomass was formed, all of the remainder of this carbon must have gone into metabolite production. Therefore, it would appear that grazing under nitrogen limitation results in a more efficient use of carbon by grazed bacteria. Tempest and Dicks (12) and others have shown that faster growing bacteria grow more efficiently than slower growing bacteria. Since the grazed bacteria had a nitrogen source and were able to grow, compared with the ungrazed bacteria, perhaps they also used carbon more efficiently for this reason.

These results demonstrated the role of grazers in rapidly releasing mineral nitrogen from bacterial biomass under carbon limitation and its effect on bacterial growth under nitrogen limitation. The carbon and nitrogen transformations described in these experiments provide specific information for gaining a better understanding of the results of soil microcosm studies (1) and assist in understanding microbe dynamics and nutrient fluxes in grassland soils.

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