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Nitrobacter in Mammoth Cave

by

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INTRODUCTION

Mammoth Cave, a large natural limestone cavern formed 20 to 30 million years ago in rocks laid down during the Mississippian Period, lies in west-central Kentucky and borders on the western coal basin and the Mississippian Plateau. Historically, over 1800 tons of nitrate sediments were mined from Mammoth Cave prior to and during the War of 1812, and were subsequently processed for gunpowder. The extensiveness of the operation is substantiated by the large number of mining archeological artifacts that remain in the cave (Faust, 1967).

Although the mechanism of saltpetre formation, $CaNO_3$, in cave ecosystems is unknown, various hypotheses have been suggested for saltpetre formation. Brown (1809) suggested that nitrates are leached into the cave sediments through drainage water since high concentrations of nitrates are sometimes found in cavernous sandstone rock. Priestley (1809) on the other hand suggested that weak nitrous acid produced in the atmosphere resulted in the deposition of saltpetre. Generally, it is thought that nitrate deposits in caves are formed by the degradation of bat guano (Clark, 1924); Hess (1900) reported, however, that deposits of nitrate extended over five miles into the cave, and such distances are not usually traversed by bats. Faust (1967) suggested that saltpetre formation was mediated by free-living (non-symbiotic) nitrogen fixing bacteria capable of fixing atmospheric nitrogen and using carbon dioxide as the sole energy source with the concomitant formation of $CaNO_3$. Yet, such an organism has never been reported nor isolated. Thus, the mode of formation of such large saltepetre deposits within Mammoth Cave and the role of bacteria in their formation remains unclear.

Cave ecosystems provide the microbial ecologist with a selective natural

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habitat in which to work. The environment for microbial growth is both extreme and constant in that the bacteria experience no light (in the non-tourist areas of the caves), constant temperatures, low nutrient levels, and a habitant whose pH is well buffered circumneutral. Such conditions provide selective pressures for the growth and proliferation of certain bacteria.

There are two basic approaches for studying bacteria in a natural ecosystem such as Mammoth Cave: the direct approach, which relies on viewing and recognizing bacteria in their natural habitat without enriching or culturing the bacterium on artificial media; secondly, the indirect approach, which removes the bacterium from its natural habitat and relies on the detection of the microbe or a microbial product in order to establish the presence of a given bacterium.

Isolated studies (Caumartin, 1963 and Gounot, 1967) have described indirect enrichment techniques for culturing organisms from cave sediments. Such techniques depend on observing the growth of the organisms after they are removed from their natural habitat and subjected to conditions dissimilar to those found *in situ*. Estimates of bacterial types and population densities by indirect procedures, i.e. plate counts, dilution plating, or most probable number analyses, may not reflect the bacteria present in the habitat (Wiebe, 1971). The indirect approach is limited by the fact that any single medium is not capable of supporting the growth of all bacterial types, thus certain bacteria will not be isolated, cultured and/or identified. On the other hand, the use of a wide variety of media and growth conditions is impractical and duplication of bacteria occurs. Additionally, separation of single bacterial colonies is often difficult, due to either the failure to separate single cells initially or the overgrowth of slower growing organisms. Since population estimates are based on visualization of colonies, the number of colonies on a given petri dish must be statistically numerous, yet not so large that crowding and overlapping occurs. Moreover, the development of colonies is a function in part of growth temperature, incubation time, and nutrient levels.

Direct procedures depend on the recognition of the bacterium of choice in its natural habitat without supplimental enrichment and growth. Such recognition is often very difficult, since most bacteria are not morphologically distinct. The development of the direct fluorescent antibody technique (Bohlool and Schmidt, 1968) and the implementation of the technique in natural ecosystems (Fliermans et al., 1974) has greatly expanded the field of microbial ecology and has made the direct approach to bacterial identification and quantification in various ecosystems possible. The fluorescent antibody technique has been described in detail elsewhere (Schmidt, 1973; Fliermans et al., 1974) and will only be outlined here.

The FA technique is derived from the high degree of specificity which occurs in an antigen-antibody reaction. A particular bacterium of interest (in this research, *Nitrobacter agilis* or *N. winogradskyi*) is isolated into pure cultures, cultivated, and used as the antigen for the preparation of specific antisera in rabbits. After a series of intravenous injections, specific antibodies against the injected antigen are produced. Antisera are then removed from the rabbit by

cardiac puncture and the globulins containing the active antibodies are separated then purified by ammonium sulfate precipitation. These antibodies are conjugated to a fluorochrome dye, usually fluorescein isothiocyanate (FITC), to form the fluorescent antibody (FA), is then used as a stain for samples taken from the natural environment. The bacterium of interest, if present in the sample, forms a specific antigen-fluorescent antibody complex which can be visualized by fluorescent microscopy. Such a technique is specific for the homologous system and highly sensitive, since as little as 10^{-15} g of FITC on a bacterium can be detected (Goldman and Carver, 1961).

We chose to use this direct fluorescent antibody technique to study the presence, distribution and population densities of the chemautotrophic nitrifiers, *Nitrobacter agilis* and *Nitrobacter winogradskyi*, in Mammoth Cave and other saltpetre caves in the southeastern United States. Recent studies (Fliermans et al., 1974) demonstrated that fluorescent antibodies for *Nitrobacter* were species specific and could be used to evaluate the presence of these organisms in saltpetre caves.

MATERIALS AND METHODS

Cultures. All cultures were maintained as described by Fliermans et al., 1974. New isolates of nitrifying bacteria were obtained from cave sediments through a series of selective enrichments and final isolates were picked from streak plates (Schmidt, 1973). Since *Nitrobacter* spp. are considered to be strict chemoautotrophs, unable to grow on organic compounds, all cultures were routinely checked using five different heterotrophic media for purity. The absence of *Nitrobacter* growth in these five media (Clark and Schmidt, 1967) and uniformity of organisms observed under light microscopy were confirmation of cultural purity.

Sampling. Cave sediment samples were aseptically taken with either an alcohol flamed spatula or soil corer, immediately placed in sterile Whirl Pak bags (NASCO), and returned to the laboratory for processing. All samples were processed within 24 hours of sampling.

Chimical Analysis. Each sediment sample was measured for pH, % moisture, nitrite and nitrate concentrations. Sediment moisture was determined gravimetrically by placing cave sediment samples into tared 35 mm metal screw-capped film cans directly in the field. In the laboratory the samples were weighed and dried to a constant weight at 110°C with the lids loose. The samples were then placed in a dessicator for temperature equilibration and reweighed. The amount of water lost was expressed as a percentage of the sediment sample. Sediment pH values were measured on a 1:1 w/v slurry with distilled water using an Orion portable pH meter with a combination electrode.

Qualitative spot tests for nitrate and/or nitrite were taken extensively throughout the cave ecosystem, using diphenylamine in concentrated sulfuric

acid (Pramer and Schmidt, 1964). Sediment samples were extracted with distilled water and filtered in the field using a filter holder (Swinnex-25, Millipore Corp.) and a 0.45μ membrane filter. Three drops of the filtrate were placed in white porcelain plates and an equal amount of reagent added. A complex between the diphenylamine and the nitrate or nitrite resulted in a deep blue color, indicating the presence of NO_3^- or NO_2^-.

Nitrites were measured quantitatively using the colorimetric procedure of Shinn (1941). Nitrates and nitrites from 50 g of cave sediment were extracted with 250 ml of 0.015M $CaSO_4$. The supernatant was filtered through a Whatman No. 42 filter and nitrite levels determined. Nitrate analyses were performed by passing the filtrate through a cadmium reduction column, measuring the nitrite concentration colorimetrically, and calculating the nitrate concentration by difference (Strickland and Parsons, 1968). The efficiency of nitrate reduction was 93-97%.

Leaching Studies. Composite samples each containing 300 g of Mammoth Cave sediments from thirty sites were placed in two chromatographic columns (40 x 600 mm) and leached free of detectable nitrates and nitrites with 400 ml filter sterilized distilled water. Leachate was collected aseptically in 50 ml aliquots and measured qualitatively for the removal of nitrates and nitrites. Total bacterial and *Nitrobacter* population densities in the soil column and in the leachate were measured by direct microscopy (Fliermans and Schmidt, 1975) and immunofluorescence (Fliermans et al., 1974), respectively.

RESULTS

Samples were taken from areas indicated by an "x" on the surveyed passages shown in Figure 1. Although the Mammoth Cave system contains more than 248 km of passageways, samples were taken from 55 km of passages, of which less than 10% were accessible to tourist. Samples were collected from areas within the passages where public influence was deemed negligible, i.e., ceilings, walls, crevasses, etc. Sampling was concentrated in the Rotunda and Booth's Amphitheater areas since archeological evidence indicates that extensive saltpetre mining took place in these areas. A more specific description of some of the sampling sites within Mammoth Cave along with chemical data of pH, NO_3^-, NO_2^- and percent moisture, are shown in Table I. Values for pH ranged from 5.95 to 8.99 with a mean of 7.94. This is as expected since the cave is formed in a limestone region where the buffering capacity of the parent material is high. Nitrite levels were generally less than 0.2 ppm NO_2^--N but did occur as high as 19.5 ppm. On the other hand nitrate levels were high, ranging from 1 to 660 ppm NO_3^--N with a mean of 223 ppm. Samples of water coming into the cave were always low in nitrates having less than 5 ppm, while soil samples above the cave were always less than 25 ppm NO_3-N. Moisture content of the sediment samples was low, except for samples taken where water was actively moving into the cave such as at Side Saddle Pit and Richardson's Spring. Sediment moisture levels ranged from 1.1 to 28.6% with a mean of 8.2%. The highest moisture levels occurred in the deepest part of the cave nearest the ground water, while lower moisture levels were generally observed in the upper passages.

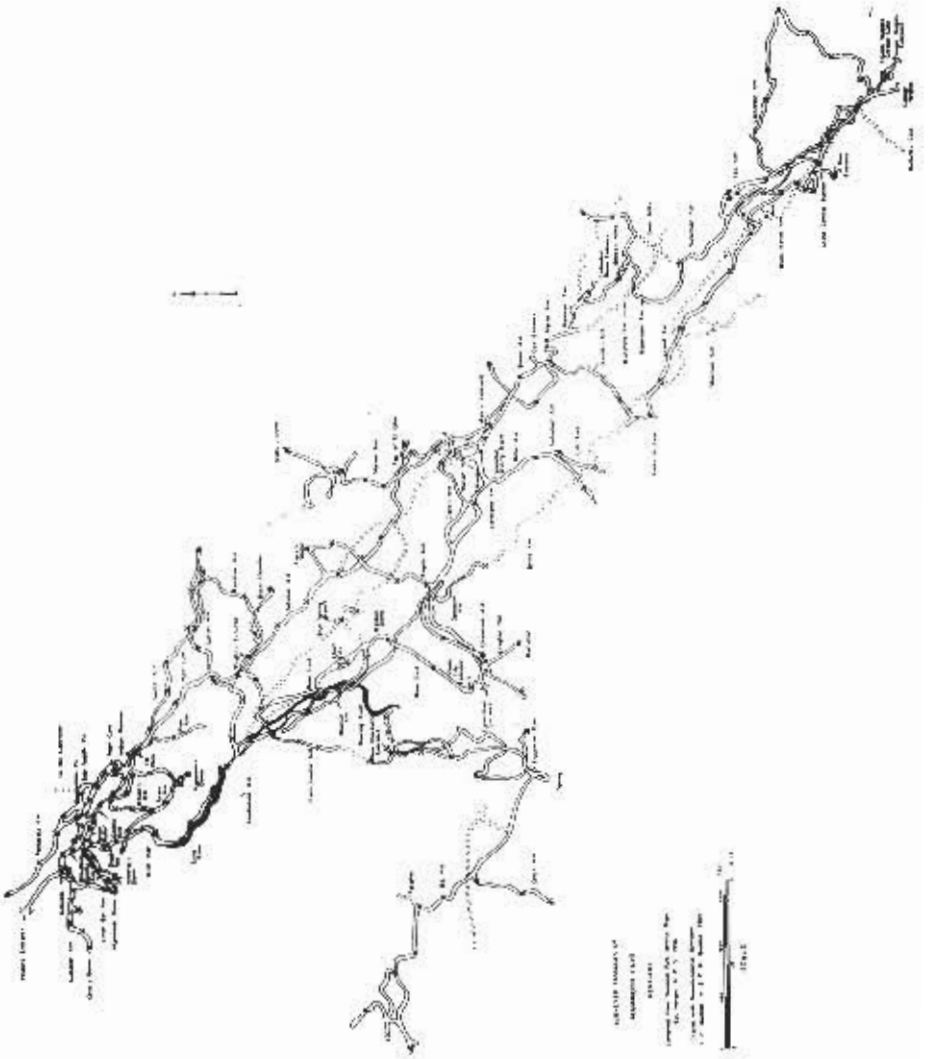

Fig. 1. Samples taken from areas designated by an "x" within passageways of Mammoth Cave. Only general sample locations are marked.

Table 1. Specific chemical and physical parameters of samples taken from a variety of habitats in Mammoth Cave.

Sample	Description	pH	% Moisture	NO_2^-	NO_3^-
114-1	Surface sample from 1st saltpetre vats in Rotunda	7.40	7.64	19.5	80
114-2	Subsurface sample (5 cm); 1st saltpetre vats in Rotunda	7.43	7.73	3.5	260
114-3	Subsurface sample (20 cm); 1st saltpetre bed gray powder material	7.89	5.51	<0.2	1
114-4	Adjacent 1st saltpetre vat in Rotunda; surface sample	7.08	N.D.	<0.2	190
114-5	Fine silt from 2nd saltpetre vat in Rotunda; from leaching trough	7.52	5.26	<0.2	390
115-1	Scrapings from collecting trough 2nd saltpetre bed; Rotunda	7.99	N.D.	17.5	115
115-2	Silt from final holding tank; Rotunda	7.60	N.D.	<0.2	275
115-3	Base of wall beyond Rotunda	7.14	N.D.	<0.2	275
115-4	Scrapings off wall 2M above 115-3	6.88	6.90	<0.2	420
115-5	East of Rotunda; surface sample 3M off trail near "Old Trail"	5.95	6.65	<0.2	410
115-6	East of Rotunda; base of wall near "Old Trail"	7.86	7.73	<0.2	470
115-7	Scrapings from wall above "Methodist Church"	7.42	9.05	<0.2	490
116-1	Silt from top of "Pulpit Rock"	7.86	N.D.	<0.2	440
116-2	Sample across from 2nd set of leaching vats	7.37	10.3	<0.2	455
116-3	Sample from reddish bank across from 2nd set of saltpetre vats	7.52	8.31	<0.2	660
116-4	Surface sample between 2nd set of leaching vats	7.42	N.D.	<0.2	140
116-5	Final leaching troughs; second set of leaching vats	7.63	N.D.	15.5	320
116-6	Scrapings from wall at "Boones Rock"	7.14	N.D.	<0.2	500
116-7	Sample below vats at "Booth's Amphitheatre"	7.46	5.31	4	245
117-1	Sample behind last leaching vat at "Booth's Amphitheatre"	6.54	N.D.	<0.2	455
117-2	Scrapings from side of ledge across from bleachers at "Gothic Avenue"	7.45	12.4	<0.2	510

117-3	Sample behind bleachers at "Gothic Avenue"	7.19	4.45	<0.2	140
117-4	Beneath ledge close to opening into main part of the cave at "Gothic Avenue"	7.28	N.D.	<0.2	142
117-5	Sample from floor of "Gothic Avenue"	7.52	7.61	<0.2	490
117-7	"Standing Rock," often used for excretory purposes	7.53	7.83	<0.2	455
117-8	Sample adjacent to old cart near "Standing Rock"	6.90	2.85	<0.2	120
117-9	Sample from under ledge at "Acute Angle"	6.79	7.65	<0.2	450
118-2	Sample from ledge inside "Acute Angle"	8.05	N.D.	<0.2	150
118-3	Sample near ceiling 1 m from gate at "Acute Angle"	8.15	N.D.	<0.2	290
118-4	Sample from floor at base of gate at "Acute Angle"	7.98	N.D.	<0.2	215
118-6	Sample from baseboard around the 1st "T. B. Hut"	7.67	7.81	<0.2	455
118-8	Sample from ledge at "Star Chamber"	8.12	11.6	<0.2	450
118-9	Sample between first two "T. B. Huts"	8.27	N.D.	2	425
119-2	Sample near "hoe marks" in "Cyclops Avenue"	7.27	4.18	<0.2	50
119-3	Sample from ledge in "Cyclops Avenue"	7.78	7.91	<0.2	160
119-4	Sample 1 m above 119-3	7.23	6.00	<0.2	200
119-5	Ceiling scrapings from "Backslider"	8.35	N.D.	<0.2	14
119-6	Sample from sediment wall in "Backslider" (0-2 mm)	7.85	15.9	<0.2	38
119-7	Same as 119-6 (2 mm-50 mm)	7.09	12.0	<0.2	14
120-1	Sample from floor of "Backslider" near 119-6	7.64	11.6	<0.2	20
120-2	Sample from ceiling at "Backslider"	8.99	7.29	<0.2	80
121-1	Sample from ledge in back passageway of "Wooden Bowl Room"	7.28	N.D.	<0.2	120
121-2	Same area as 121-1	7.51	4.04	<0.2	145
121-3	Sample from above plaque "Wooden Bowl Room"	7.91	6.52	<0.2	120
121-4	Sample on side wall of "Wooden Bowl Room"	7.38	6.34	<0.2	285
121-5	Sample near entrance to "Wooden Bowl Room"	7.40	11.2	<0.2	420
121-6	Sample 50 m beyond stairway below "Wooden Bowl Room"	8.54	4.26	<0.2	30
121-9	Water sample from "Richardson's Spring"	7.95	—	<0.2	1.5
121-10	Sample near "Richardson's Spring"	8.34	28.10	<0.2	26
122-1	Sample of reddish deposit near 2 fluorescent lights near "Blind Fish Aquarium"	7.78	10.4	<0.2	16

Sample	Description	pH	% Moisture	NO_2^-	NO_3^-
122-2	Sample on ledge near "Blind Fish Aquarium"	7.70	12.6	<0.2	100
122-4	Water sample from drippings at "Sidesaddle Pit"	7.50	—	<0.2	2.0
122-5	Sample across from "Sidesaddle Pit"	7.74	28.6	<0.2	4
122-6	Sample across dome cavity at "Sidesaddle Pit"	7.95	27.8	<0.2	5
122-8	Sample near floor at "College Heights Avenue"	7.05	18.6	<0.2	6
123-1	Same as 122-8; reddish clay	7.88	24.8	<0.2	70
123-2	Same area as 122-8; powdery sample	8.07	9.6	<0.2	10
123-3	Sample under ledge in "College Heights Avenue"	8.13	1.43	<0.2	10
123-4	Same as 123-3; much limestone	7.72	4.14	<0.2	13
123-5	Sample at "Flat Ceiling" behind fluorescent light	7.71	50.02	<0.2	220
123-6	Sample by rail at "Lover's Leap Canyon"	7.83	N.D.	<0.2	240
123-7	Sample below "Lover's Leap Canyon Trail"	8.20	N.D.	<0.2	90
123-8	Sample between "Flat Ceiling" and "Fairy Ceiling"	7.65	N.D.	<0.2	12.5
123-9	Sample from ledge 100 m beyond "Star Chamber"	7.08	10.8	<0.2	460
124-1	Sample 100 m from 123-9	8.07	4.76	<0.2	500
124-2	Sample 100 m from 124-1	7.80	N.D.	2	120
124-3	Same area as 124-2; gravel sample	8.81	N.D.	<0.2	93
124-4	Sample 100 m from 124-3	7.64	N.D.	<0.2	290
124-5	Sample 100 m from 124-4	8.13	5.37	<0.2	250
124-6	Same area as 124-5; other side of trail	8.16	N.D.	<0.2	10
124-7	Sample 100 m beyond 124-6	7.89	2.85	<0.2	310
124-8	Sample 100 m beyond 124-7	7.62	2.38	<0.2	405
125-1	Sample 100 m beyond 124-8	7.76	7.86	<0.2	430
125-2	Sample 100 m beyond 124-9	7.90	N.D.	<0.2	180
125-3	Sample near "Cataract Falls"	7.53	3.93	<0.2	330
125-4	Sample beyond waterfall at "Cataract Falls"	7.65	3.75	<0.2	410
125-5	Sample 100 m beyond 125-4	7.40	4.18	<0.2	300
125-6	Sample 100 m beyond 125-5	7.09	7.28	<0.2	356
125-7	Sample 100 m beyond 125-6	7.52	8.19	<0.2	450
125-8	Same area as 125-7	7.91	4.46	<0.2	410

126-1	Sample 30 m beyond 125-8	7.71	8.16	<0.2	440
126-2	Sample 150 m beyond 126-1; at "Chief City"	7.55	3.32	<0.2	460
126-3	Sample 100 m beyond 126-2	7.83	2.48	<0.2	410
126-5	Sample 100 m beyond 126-3	7.71	5.11	2	460
126-6	Sample above "Hains Dowe"	6.71	9.68	<0.2	290
126-7	Sample 100 m beyond 126-6	7.52	4.13	<0.2	440
126-8	Sample 100 m beyond 126-7	7.45	4.76	<0.2	150
126-9	Sample 100 m beyond 126-8; rocky sandy sample	7.70	1.60	<0.2	405
127-1	Sample 100 m beyond 126-9	8.05	5.00	<0.2	350
127-2	Sample 100 m beyond 127-1	7.13	9.82	<0.2	210
127-3	Sample 100 m beyond 127-2	7.81	7.0	<0.2	50
127-4	Sample 100 m beyond 127-3; base of wall	8.03	7.93	<0.2	10
127-5	Same area as 127-4	8.88	7.63	<0.2	115
128-1	Surface soil sample from "Backslider"	7.71	N.D.	<0.2	140
129-1	Surface soil sample from "Backslider"	7.09	N.D.	<0.2	27
130-1	Surface soil sample from "Backslider"	7.61	N.D.	<0.2	32
131-1	Surface soil sample from "Backslider"	7.90	N.D.	<0.2	260
132-1	Surface soil sample from "Backslider"	7.74	N.D.	<0.2	21
133-1	Sample from wall profile (0-5 cm) at "Backslider"; heavy clay	7.72	14.9	<0.2	40
133-2	Same as 133-1; 5-10 cm; heavy clay	7.61	16.7	<0.2	29
133-3	Same as 133-1; 10-15 cm; heavy clay	7.76	16.1	2	120
134-1	Same as 133-1; 20-25 cm; sandy	7.68	5.71	<0.2	19
135-1	Same as 133-1; 25-30 cm, base of profile; sandy	6.95	1.13	<0.2	28

(1) N.D. = Not Determined

Average NO_3^-: 222.8 ppm
range: 1 to 660 ppm
NO_2^-: range: <0.2 to 19.5 ppm
Average moisture: 8.21%
range: 1.13 to 28.6%
Average pH: 7.94
range: 5.95 to 8.99

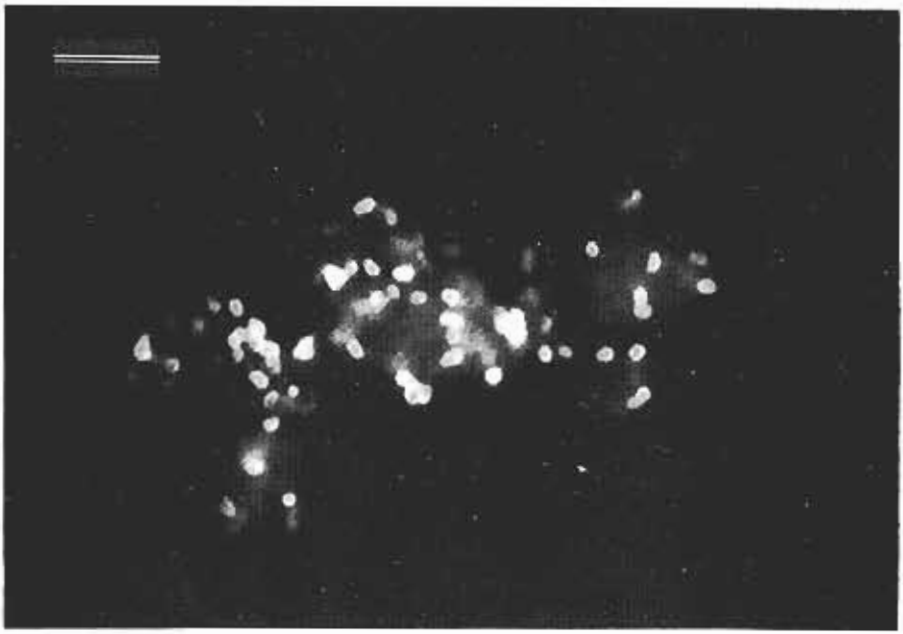

Fig. 2. *Nitrobacter* in Mammoth Cave sediments as stained by species specific fluorescent antibodies. Scale bar is 5 nm.

Since the fluorescent antibodies were species specific, the distribution of *N. agilis* and *N. winogradskyi* in the Mammoth Cave ecosystem were determined. The staining characteristics of *Nitrobacter* in Mammoth Cave sediments are shown in Figure 2. This black and white photomicrograph shows *Nitrobacter* as white cells, while in color photographs the cells would appear yellowish-green. The data in Table II indicate that 85% of the *Nitrobacter* population in Mammoth Cave was *N. agilis*. On the other hand, pure culture isolates obtained from a variety of agricultural soils were always *N. winogradskyi*, while only *N. agilis* was isolated from Mammoth Cave sediments (Table III).

The data summarizing nitrate concentrations and moisture content of the cave sediment samples are plotted with respect to *Nitrobacter* population densities in Figures 3 and 4, respectively. These data indicate that no strong correlation exists between the populations of *Nitrobacter* and either nitrate concentrations or sediment moisture. *Nitrobacter* densities in the cave sediments averaged 6.2×10^5 cells per gram of sediment, while soil samples taken above Mammoth Cave under a forest canopy had less than 10^3 *Nitrobacter* per gram of soil (Fliermans, unpublished data).

In order to determine if the presence of *Nitrobacter*, as observed in Mam-

Table 2. Population densities and species composition of chemoautotrophic nitrifiers in Mammoth Cave sediments.

Sample	Total *Nitrobacter* per gram sediment	#/Field *N. agilis*	*N. winogradskyi*
115-2	2.3×10^4	16	4
118-3	6.1×10^4	37	7
127-2	2.2×10^3	18	0
133-1	1.8×10^4	19	4
123-1	4.4×10^4	35	4
117-4	1.8×10^4	11	4
125-8	7.5×10^4	63	10
125-7	5.0×10^4	22	20
121-9	4.2×10^4	24	13
126-3	3.1×10^4	13	14
123-5	2.5×10^4	15	8
132-1	2.5×10^4	14	3
114-2	4.1×10^4	17	17
122-2	1.7×10^4	9	4
124-7	2.2×10^4	8	6
123-3	1.2×10^4	6	3
118-8	1.2×10^4	17	2
117-2	1.2×10^4	6	4
126-7	1.5×10^4	10	3
124-6	6.7×10^5	300	39
125-1	7.4×10^6	48	2
128-1	9.1×10^5	60	15
122-6	5.3×10^4	290	15
127-4	1.3×10^4	2	10
130-1	5.2×10^4	32	1
121-3	1.9×10^4	6	10
123-7	2.1×10^4	14	2
126-5	8.5×10^6	67	0.3
115-6	3.6×10^5	27	0.2
120-2	5.4×10^4	25	2
Average	6.2×10^5	41.0	7.55
	% of Total *Nitrobacter*	84.5	15.5

Table 3. Immunofluorescence specificity test with chemoautotrophic nitrifiers isolated from various habitats.

Culture	Source	*Immunofluorescence Reaction* *N. agilis*-FA	*N. winogradskyi*-FA
Nitrobacter			
Bearden 1	Minnesota Soil	Neg	3+
Bearden 2	Minnesota Soil	±	4+
Glencoe 1	Minnesota Soil	Neg	3+
Glencoe 2	Minnesota Soil	Neg	3+
Tara 1	Minnesota Soil	Neg	3+
Tara 2	Minnesota Soil	Neg	3+
F-A	Moroccan Soil	Neg	3+
F-B	Moroccan Soil	Neg	4+
Iceland 1	Iceland Soil	Neg	4+
133-2	Mammoth Cave, Ky.	3+	Neg
128-1	Mammoth Cave, Ky.	4+	Neg
125-8	Mammoth Cave, Ky.	4+	Neg
115-4	Mammoth Cave, Ky.	4+	Neg
123-1	Mammoth Cave, Ky.	3-4+	Neg
130-1	Mammoth Cave, Ky.	4+	Neg
122-6	Mammoth Cave, Ky.	3+	Neg

Absorbed *Nitrobacter* fluorescent antibodies were tested with pure cultures of autotrophic nitrifiers from diverse environments.

moth Cave was a widespread phenomenon in other saltpetre caves, samples were taken from 23 known saltpetre caves primarily in the southeastern United States. As shown in Table IV all but two of the caves had *Nitrobacter* present in sediment samples, as detected by immunofluorescence.

Leaching studies indicated that *Nitrobacter* populations in the cave sediments remained stable during the leaching process as compared to the change in the total bacterial population (Table V). Sediment samples from thirty different sites within Mammoth Cave were composited into a single sample and homogeneously mixed. Hydrometrical texture analyses of the pooled sample indicated that the mixture was 64% sand, 19.8% silt and 16.2% clay. The composite sample was then placed in a chromatographic column and continuously leached until free of nitrates and nitrites, using 400 ml of filter sterilized distilled water. The effluent was aseptically collected in 50 ml aliquots and the population densities of *Nitrobacter* and total bacteria were determined by direct microscopy (Fliermans and Schmidt, 1975). Before leaching, the total bacterial population, as measured directly with FITC staining, was 7.2×10^6 bacteria/g of sediment, and decreased by 57% to 4.1×10^6/g of sediment after 400 ml of filter sterilized distilled water had been passed through the sediment column. On the other hand, *Nitrobacter* populations, as measured by immuno-

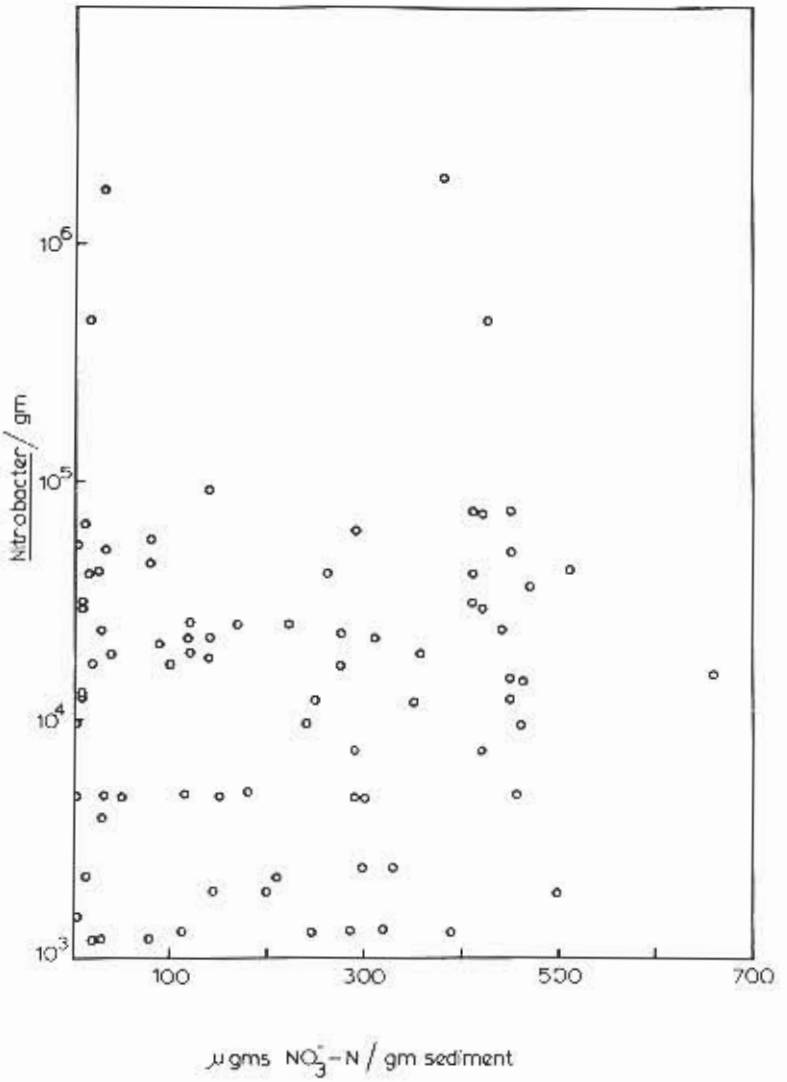

Fig. 3. Relationship between the number of *Nitrobacter* spp. per gram of cave sediments and the nitrate concentrations in the sediments.

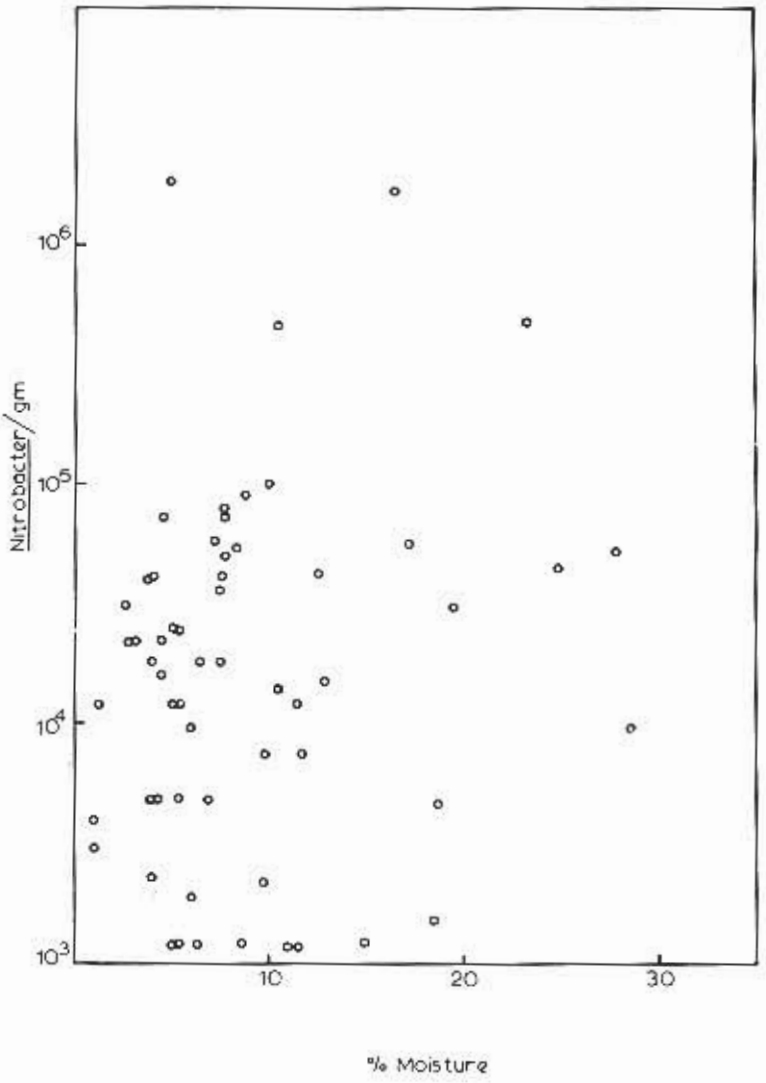

Fig. 4. Relationship between the number of *Nitrobacter* spp. per gram of cave sediments and the percent moisture in the sediments.

Table 4. Presence of *Nitrobacter* spp. in saltpetre caves as determined by immunofluorescence.

Cave	Location	*Nitrobacter*
Dan Boone Hut Cave	Bath Co., Ky.	—
Breathing Cave	Bath Co., Va.	+
Minor Saltpetre Cave	Lee Co., Va.	+
Perry Saltpetre Cave	Boutertate Co., Va.	+
Lawson Saltpetre Cave	Scott Co., Va.	+
Big Boone Cave	Van Buren Co., Tenn.	+
Petre Cave	Polaski Co., Ky	+
Crawford Cave	Randolph Co., W. Va.	+
Ellison's Cave	Walker Co., Ga.	+
Faust Saltpetre Cave	Wise Co., Va.	+
John Rogers Cave	Jackson Co., Ky.	+
Wind Cave	Wayne Co., Ky.	+
John Friends Saltpetre Cave	Garrett Co., Md.	+
Me Bane Saltpetre Cave	Pulaski Co., Va.	+
Saltpetre Cave	Buffalo River St. Park, Ark.	+
Greenville Saltpetre Cave	Logan Co., W. Va.	+
Madison Cave	Madison Co., Va.	—
Cave Mountain Cave	Grant Co., W. Va.	+
Henshaw's Cave	Warren Co., Tenn.	+
Carter Caves	Carter Co., Ky.	+
Dyers' Cave	Hardy Co., W. Va.	+
Saltpetre Cave	Mineral Co., W. Va.	+
Lobelia Saltpetre Cave	Greenbriar Co., W. Va.	+

fluorescence, were initially 4.8×10^4/g of sediment and showed no significant change to 5.2×10^4/g of sediment after leaching.

DISCUSSION

Although Mammoth Cave is a national park, it provides a unique speleological ecosystem for microbiological studies, since the touristic impact is restricted to about 10% of the known cave passages. Such an ecosystem is unique in that weathering occurs at a reduced rate since natural elements of rain, wind, sunlight, erosion, freezing and thawing are removed from the habitat. Air temperature in the deeper parts of the cave is relatively stable, fluctuating between 13.2 and 14.0°C with a mean of 13.6°C, while the relative himidity rarely drops below 80% and is generally between 95 and 100% (Barr and Kuehne, 1971). Light penetration into the cave is negligible and only where artificial lighting provides a source of energy do photosynthetic organisms occur. These organ-

Table 5. Effect of leaching on the removal of *Nitrobacter* spp. and other bacteria from Mammoth Cave sediments.

Volume Leached (ml)	Microorganisms/Microscope Field	
	Total Bacteria[a]	*Nitrobacter* spp.[b]
50	33.0	0.02
100	4.2	0.05
150	5.0	0.04
200	6.4	0.06
250	7.7	0.06
350	8.5	0.02
400	6.3	0.06
	Microorganisms/gm sediment	
Before Leaching	7.2×10^6	4.8×10^4
After Leaching	4.1×10^6	5.2×10^4

[a] Calculations based on 10 microscope fields.
[b] Calculations based on 50 microscope fields.

isms are primarily heterocystic filamentous bluegreen algae and diatoms (Fliermans, unpublished data). Moisture content of the cave sediments varied substantially from one site to the next within the cave. Mammoth Cave has five different passage levels with the lowest one being in contact with the underground Echo River. In general, moisture levels are highest in the lower region of the cave, although the majority to sediments contained less than 10% moisture. Isolated packets of high sediment moisture was apparent where seepage from natural springs arose.

The classical approach for the mining of saltpetre relied on the observation of a variety of physical phenomena within the cave. Some of these observations are consistent with the growth conditions required by the chemautotrophic nitrifier, *Nitrobacter*. Faust (1967) described the following ecological phenomena which were generally observed in saltpetre formations:

1. Caves must contain alkaline sediments with a stable year-round temperature of 11-14°C.
2. Free flowing air circulation must occur and running water or flood waters must not reach the saltpetre deposits.
3. Saltpetre sediments disturbed by running a sharp object through them became smooth in 2 to 5 days.
4. Sediments which were leached free of nitrates and subsequently returned to the cave ecosystem would regenerate comparable levels of nitrate in 3 to 5 years.
5. Saltpetre deposits are generally found in areas low in organic matter.

Nitrobacter spp. have a pH range of 6.5 to 8.5 with an optimum for growth

between 7.5 and 8.0 (Watson, 1975), thus the slightly alkaline conditions of Mammoth Cave sediments are close to the pH optimum required for *Nitrobacter* growth. Pure cultures of the nitrifiers are optimally adapted to a temperature near 25 to 30°C with a range from 5 to 40°C. Therefore, the mean cave temperature of 13.6°C for Mammoth Cave may not be optimal for *Nitrobacter* growth unless these bacteria are adapted to a different temperature optima *in situ*. Additionally, saltpetre deposits are found where air circulation occurs and water drainage is absent. Since the nitrifying bacteria are strict aerobes, they require oxygen as a terminal electron acceptor, and thus air circulation may help maintain the necessary aerobic conditions. The prevention of high water levels in the caves facilitates the formation of saltpetre deposits, since either seepage or flooding conditions promote leaching of the soluble nitrate ions from the cave sediments. In addition, saturated conditions produce anaerobic environments which prevent the growth of the chemoautotrophic nitrifiers.

The phenomenon of disturbing the sediments with a sharp object and having the sediments return to a smooth surface cannot be explained microbiologically. Since these sediments are at a low moisture content any disruption may result in a moisture equilibration with the high relative himidity of the cave and thus the saltpetre deposits swell due to water of hydration and cause a smoothing of the disturbed sediments.

The process of nitrate regeneration is interesting, since historically saltpetre sediments were often leached free of nitrates, returned to the cave ecosystem and a regeneration of saltpetre to initial nitrate concentrations occurred in 3 to 5 years. Laboratory leaching experiments with 300 g of Mammoth Cave sediments indicated that the nitrates were easily removed from the sediments but the nitrifying bacteria were not. Total bacterial populations before leaching were 7.2×10^6/g of sediment measured by direct FITC staining and decreased by 57% after 400 ml of distilled water had been leached through the sediments. On the other hand, *Nitrobacter* populations, as measured by immunofluorescence were 4.8×10^4/g of sediment and showed no significant change to 5.2×10^4/g. Thus, it appears that leaching of the sediments selectively maintains the *Nitrobacter* populations while removing some of the other bacteria. Likewise, leaching of the sediments appeared to promote the oxidation of nitrite to nitrate in that much higher levels of nitrite were oxidized after leaching than before (Fliermans, unpublished data). Such an increase in nitrite oxidation may result from the removal of nitrate which serves an end product inhibitor for *Nitrobacter* spp.

In order for nitrification to occur and deposits of saltpetre to form, a supply of inorganic nitrogen must be available. Since the nitrifiers in Mammoth Cave are chemoautotrophs, their metabolic activity is not affected directly by the concentration of organic matter. However, preliminary micro-kjeldahl studied indicated that these cave sediments were very low in organic matter (Fliermans, unpublished data), which is probably due to the lack of photosynthesis and thus the deposition of plant debris and humus material in cave ecosystems. Mammoth Cave is an old geological structure and the bacteriological events

observed in the cave are a result, in part, of this long period of time. The low levels of total organic matter (0.02 to 0.04%) may be the result of a continuous but very slow decomposition process. Many saltpetre caves have had at one time large populations of bats living in the cave which may have been a supply of organic material. It is possible that these guano deposits were eventually decomposed through deaminization and/or ammonification and NH_4^+ released, which in turn was used as substrate for the nitrifiers. The process of saltpetre formation may be near termination in that very little organic matter is now being deposited naturally in Mammoth Cave due to the absence of extensive bat populations.

Although the stochiometry of nitrogen transfer through the various components of the cave ecosystem remains unknown, the detection of a specific group of chemoautotrophic nitrifying bacteria, *Nitrobacter*, has been shown in saltpetre cave sediments. The population densities present in Mammoth Cave may be sufficient to account for the levels of saltpetre found in the sediments. Caverns such as Mammoth Cave, with stable parameters of temperature, pH, light, moisture and organic nutrients, may provide or at one time provided unique habitats for the chemoautotrophic nitrifiers.

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SUMMARY

Mammoth Cave, a large limestone cavern in Mammoth Cave National Park in the Central Kentucky karst, was first mined for saltpetre in 1808 and was a major source of nitrates used in the manufacture of gunpowder during the War of 1812. The mechanism of saltpetre formation is unknown, although hypotheses encompassing both biotic and abiotic functions have been suggested.

Present studies were conducted in various saltpetre caves using species specific fluorescent antibodies in order to determine if the chemoautotroph, *Nitrobacter*, were present. Population densities and species distribution of *Nitrobacter* were studied in relation to chemical and physical parameters for over 200 sediment samples from Mammoth Cave. Both the isolation and immunofluorescence data indicate that *Nitrobacter* are present in relatively high population densities in Mammoth Cave sediments, and that such bacteria are common among saltpetre caves in the southeastern United States. Immunofluorescence data further indicates that *N. agilis* dominates the *Nitrobacter* population in Mammoth Cave. The possibility that *Nitrobacter* is the etiological agent for saltpetre formation is suggested.

RÉSUMÉ

"Mammoth cave", une vaste caverne calcaire du parc national de Mammoth cave dans le karst

du Kentucky central, a d'abord été exploitée pour le salpêtre en 1808; elle a été la principale source de nitrate utilisé dans la fabrication de la poudre pendant la guerre de 1812. Le mécanisme de la formation du salpêtre est inconnu, quoique des hypothèses comportant à la fois des arguments biotiques et abiotiques aient été suggérées.

Les présentes recherches ont été conduites dans diverses grottes à salpêtre, en utilisant des anticorps fluorescents spécifiques, afin de déterminer si le chimioautotrophe *Nitrobacter* était présent. La densité de population et la distribution du genre *Nitrobacter* ont été étudiées, en rapport avec des paramètres physique et chimique, sur plus de 200 échantillons de sédiments de "Mammoth cave". Les données établies par isolement et fluorescence indiquent que Nitrobacter est représenté par une densité de population relativement élevée dans les sédiments de "Mammoth cave" et qu'une telle bactérie est commune dans le salpêtre des cavernes du Sud-Est des Etats-Unis. Les résultats de l'immunofluorescence indiquent de plus que *Nitrobacter agilis* domine parmi la population de *Nitrobacter* de "Mammoth cave". La possibilité que Nitrobacter soit l'agent étiologique de la formation du salpêtre est suggérée.

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