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Distribution of Protozoa in Subsurface Sediments of a Pristine Groundwater Study Site in Oklahoma

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Sediment core samples were obtained at a groundwater study site in Oklahoma in January and June 1985. Most-probable-number estimates showed that protozoan numbers declined steeply with depth in subsoil. Flagellates and amoebae dominated the protozoan population, which declined to a most probable number of 28 · g (dry weight)$^{-1}$ in a clay loam layer at the bottom of the unsaturated zone. Samples from a texturally variable interface zone between 3 and 4 m down also were variable in their content of protozoa. Four contiguous clay loam samples in a single core from this zone contained variable numbers of amoebae ranging from 0.2 to 44 · g (dry weight)$^{-1}$. However, a sandy clay loam layer at the bottom of the core contained a mixture of flagellates and amoebae with a combined population density of 67 · g (dry weight)$^{-1}$. A slow-growing filose amoeba was isolated from interface zone samples and was tentatively classified in a new family in the order Aconchulinida. Protozoa were not detected in the saturated zone except in a very permeable gravelly, loamy sand layer at a depth of approximately 7.5 m. Low numbers (4 to 6 · g [dry weight]$^{-1}$) of surface-type flagellates and amoebae, as well as the filose amoeba seen in the interface zone, were observed in this layer. Acid-treated and untreated samples contained equivalent numbers of protozoa, showing that the majority of protozoa in the layer at 7.5 m and the interface zone samples were encysted. Increased numbers of bacteria also were found in the layer at 7.5 m, indicating that it was biologically more active than other saturated-zone layers. Cyanobacteria grew in illuminated samples from this layer, suggesting that it may be connected hydrologically to a nearby river.

Previous microbiological studies of oligotrophic sediments from unsaturated and saturated subsurface zones at sites in Oklahoma, Louisiana, and Texas (2, 10, 11, 27, 29, 30) have largely failed to demonstrate the presence of eucaryotic microorganisms or their characteristic long-chain polyenoic fatty acids (28). These negative results indicate that eucaryotic microorganisms are not dominant members of the subsurface microbiota; because the methods used may not have been sensitive enough to detect very low population densities, however, the results do not mean that eucaryotic microorganisms are absent from subsurface environments. Indeed, eucaryotic forms have been observed microscopically in some subsurface samples (4, 10), and recent work on the microbial community structure of subsurface soils (8) shows that eucaryotic fatty acids are present in most, if not all, subsoil horizons.

Most-probable-number (MPN) procedures based on enrichment culture principles are more sensitive than other methods for detection and enumeration of microorganisms. Such methods have been particularly effective for estimating numbers of protozoa in soil (1, 7, 22), because a nearly universal and complete food source, the bacterial cell, is readily provided. However, MPN procedures for protozoa are subject to problems of underestimation and airborne contamination, which may significantly affect counts in samples containing few or no protozoa. Protozoan cysts, like fungal and bacterial spores, are ubiquitous airborne contaminants. On the average, air contains two protozoan cysts m^{-3} (23).

Despite these problems, MPN and enrichment culture methods have been used successfully to demonstrate the presence of protozoa in subsurface samples. For example, groundwater (13), wastewater treatment wells (15), the waters and muds of caves (12, 16), unsaturated subsoils (19, 26), and subsurface clay sediment samples from as deep as 6.1 m (14) all have been found to contain viable protozoa. However, while these results reflect the ubiquity of protozoa, they do not reveal protozoan distribution in subsurface zones, nor do they indicate possible variations of protozoan population density in subsurface sedimentary layers.

In the present work, we applied MPN counting methods to determine the distribution of protozoa in a depth profile at a groundwater microbiology study site near Lula, Okla. (2, 29, 30). Aseptic procedures were used to ensure minimal airborne contamination of samples. Numbers of protozoa declined with depth, but amoebae or flagellates were present in all samples down to an interface zone just below the water table. Deeper in the profile, only samples from one layer of the saturated zone contained protozoa. The results emphasize the biological variability of deeper subsurface layers.

(Reports on this work have been presented previously [J. L. Sinclair and W. C. Ghiorse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q4, p. 258; R. Beloin, J. L. Sinclair, D. L. Balkwill, and W. C. Ghiorse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, N82, p. 255].)

MATERIALS AND METHODS

Description of study site and sampling procedures. Core samples were obtained aseptically from a pristine study site in Lula, Okla. (2, 29, 30) as described previously (29). The study site is located on the margin of the flood plain of a small river. The water table occurs at approximately 3 m. Clay loam layers of variable thickness form a relatively impermeable confining layer just below the water table (30). When the confining layer is penetrated, water rises approx-

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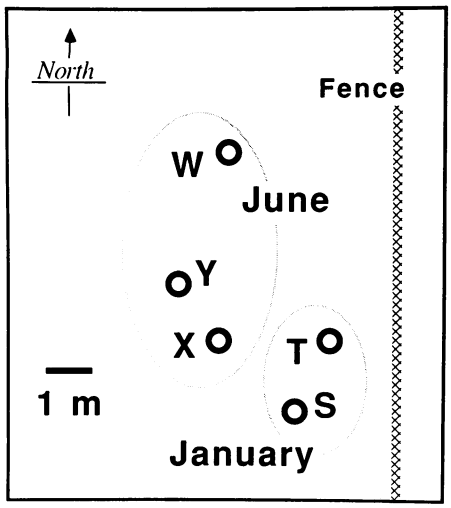

FIG. 1. Diagram of Lula, Okla. sample site showing location of boreholes (W, X, Y, T, and S) for the January and June 1985 profile sampling. The site is on the margin of the flood plain of a small river. The slope of the terrain is generally from northeast to southwest and can be used as a rough guide to the direction of water flow in the underlying aquifer (see references 2, 29, and 30 for more information on this site).

imately 0.5 m in the borehole, indicating that water in the saturated zone is under artesian pressure.

Four preliminary samples were obtained from two boreholes in November 1982 and September 1983. Profile samples were obtained in January and June 1985. The profile drilling area was located 21 m upslope from the nearest previous borehole. Cores were obtained from five boreholes drilled within 6 m of each other (Fig. 1). Subsurface samples consisted of approximately 250 to 500 g of sediment packed in sterile 0.9-liter canning jars. Soil samples from the root zone also were collected in sterile jars. The saturation status and appearance of the subsurface samples were recorded at the time of sampling. For this study, a simplified vertical profile diagram (Fig. 2) was constructed from field observations and texture analyses.

Shipping and handling of samples. All samples were chilled on ice and shipped to our laboratory in Ithaca, N.Y., by an overnight delivery service. Upon arrival, they were stored at 4°C. Sample jars were opened only in a laminar flow hood to minimize airborne contamination. For most samples, MPN counts were performed within 1 week after sampling. A few samples were reexamined after storage for up to 4 months. Moisture content and total bacterial counts (acridine orange direct counts [AODC]) were determined routinely as described previously (2, 10, 29).

Enumeration and cultivation of protozoa. To estimate the number of protozoa in a sample, 10 g of soil or sediment was weighed out aseptically from each sample jar and diluted in 90 ml of phosphate buffer (2.2 mM KH_2PO_4, 4.02 mM K_2HPO_4 [pH 7]). A modified Singh MPN method (21) was used in which five sterile, 2-cm-diameter, 1-cm-high glass rings were arranged symmetrically in a 9-cm-diameter petri dish. Thirty milliliters of sterile molten 1.5% agar in the phosphate buffer was added to the dish to surround the rings with agar. After the agar solidified, 1 ml of diluted sample or 1 g of undiluted sample dispersed in 1.0 ml of sterile phosphate buffer was added to each of the five replicate rings. One petri dish was used for each dilution level. At least three dilution levels were used for each sample.

Initially two different bacterial food sources were used in parallel dilution plates. One was a commonly used source, nongrowing *Enterobacter aerogenes* harvested from 1-day-old, half-strength Trypticase (BBL Microbiology Systems) soy agar plates. Because it was possible that subsurface protozoa might be adapted to oligotrophic conditions and thus be sensitive to high bacterial numbers as soil protozoa sometimes are (20), we also used a medium containing 3% (vol/vol) autoclaved subsurface sediment extract with the phosphate-buffered agar mixture. Indigenous subsurface bacteria that grew on nutrients in the sediment extract provided another food source for protozoa. It was found that both food sources gave equivalent MPN values; therefore, because *E. aerogenes* was the more reproducible source, use of the sediment-extract medium was discontinued in later work.

All cultures were incubated at room temperature (20 to 23°C).

To determine the presence or absence of protozoa and distinguish the types present, samples were removed aseptically from the rings and examined in a Zeiss standard phase-contrast microscope under a 16 or 40× objective lens. In most cases, up to 14 days of incubation was necessary before protozoa could be observed. If no protozoa were seen in samples from a dilution ring after 1 month of incubation, that ring was counted as negative. An MPN program for an Apple MacIntosh personal computer (18) and appropriate dilution factors were used to calculate the MPN · grams [dry weight]$^{-1}$ from the number of positive endpoint dilution rings. The lower limit of detection of the method was an

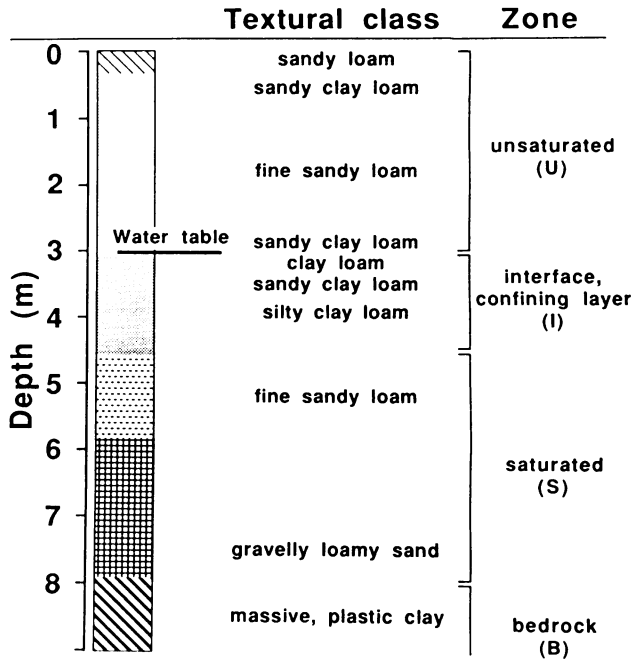

FIG. 2. Simplified diagram of the vertical profile at the Lula site, giving the textural class of the sediments in each zone. The interface zone is shown as a region of variable textured sediments below the water table, including relatively impermeable sediments that formed a confining layer. This diagram contains less detail than those published previously (2, 30).

MPN of 0.2 protozoa · g (dry weight)$^{-1}$. The standard error of the MPN counts was estimated to be 50%.

Amoebae were isolated in uniprotozoan cultures by a repeated endpoint dilution method under the same cultural conditions used for the MPN counts.

Determination of encysted protozoa. To estimate the number of encysted protozoa in a sample, 10 g of the sample was diluted in 90 ml of buffer in the usual manner, and 10 ml of 0.55 N HCl was added to kill the vegetative protozoa. After 15 min of incubation, the acid was neutralized by adding enough 1 N NaOH to bring the pH to 7. The neutralized sample was then subjected to the MPN procedure described above.

Sediment textural analysis. Sediment texture and particle size distribution were estimated by the field method (9, 24). For most samples, the error did not exceed 5% clay or 12% sand (Ray B. Bryant, Department of Agronomy, Cornell University, personal communication).

Microscopy and photomicrography. A Zeiss standard 18 microscope with phase-contrast optics and a Zeiss MC63 microscope with differential interference-contrast optics were used. Both microscopes were equipped with Zeiss MC-35 camera systems. Photomicrographs were recorded on Kodak Ektachrome 160T or Kodak Plus X film.

RESULTS

In 1982 and 1983, several samples from three different subsurface study sites were screened for protozoa (Sinclair and Ghiorse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). One species of a cyst-forming filose amoeba (Fig. 3) was detected at a density of approximately 110 · g (dry weight)$^{-1}$ in samples from the Lula, Okla. site. This amoeba was found in two samples from approximately 3 m down in an undefined region of the interface zone. No protozoa were detected in other samples from the saturated zone between 4.5 and 6.5 m at the Lula site or in samples from the saturated zones of the other two sites (Conroe, Tex. or Fort Polk, La.). These preliminary results prompted us to return to the Lula site in 1985 to confirm the presence of amoebae in the interface zone and to determine the distribution of protozoa in other zones of the subsurface profile.

The MPN of protozoa in surface soil, a continuous rhizosphere under luxuriant grassy vegetation, ranged from 1.9×10^5 · g (dry weight)$^{-1}$ in January (Table 1) to 6.4×10^6 · g (dry weight)$^{-1}$ in June (Table 2). The density of protozoa in these samples was high compared with that of typical agricultural soils, which usually contain between 10^3 and 10^5 protozoa · g (dry weight)$^{-1}$ (25). Protozoan MPN counts of surface soil samples were repeated, and the high numbers of protozoa were confirmed. The density of bacteria in the soil also was high. The range of AODC values was between 1×10^9 and 2×10^9 · g (dry weight)$^{-1}$ for both dates. The most abundant protozoa in the soil were small- and medium-sized flagellates and limax and filose amoebae (Fig. 4a). The morphology of some of the filose amoebae in the surface soil resembled that of the filose amoeba found in interface zone samples (compare Fig. 3 and 4a); however, sufficient differences were noted in the size of cysts, time for excystation, and growth rate of the two amoebae to indicate that they were different. The interface zone amoeba produced smaller but thicker-walled cysts (compare Fig. 3b and 4a), took longer to excyst, and grew more slowly than did the filose amoebae of surface soil.

Ciliates also were present in surface soil and subsoil, but they made up only 2% of the protozoan population (9U and 9S3, Table 1; 9X9 and 9X1, Table 2). No ciliates were detected in any samples from below 0.5 m in the profile.

Numbers of protozoa declined drastically with depth in the unsaturated zone, reaching equivalent MPN values of 50 and 40 · g (dry weight)$^{-1}$ at depths of 1.3 and 1.8 m, respectively (9S1, Table 1; 9X2, Table 2) and of 28 · g (dry weight)$^{-1}$ at 2.95 m (9W4, Table 2). The bacterial AODC also declined to 3×10^8 · g (dry weight)$^{-1}$ at 1.3 m and 10^7 · g (dry weight)$^{-1}$ at the lower depths. These samples contained flagellates and amoebae whose morphological types were similar to those in surface soil, suggesting that they had originated in the soil above. In the unsaturated zone, a general trend toward a higher proportion of flagellates as sampling depth increased was noted. In fact, some deeper samples from the unsaturated zone (e.g., 9X2 and 9W4, Table 2) contained only flagellates.

The region just below the water table between 3.0 and 4.0 m (Fig. 2) was designated as the interface zone. It was characterized by its textural variability and by the presence of relatively impermeable sediments that formed a clay loam confining layer. A core obtained from this zone in June was divided into contiguous 10-cm samples (9W4-9W1, Table 2). These samples were variable in their content of protozoa.

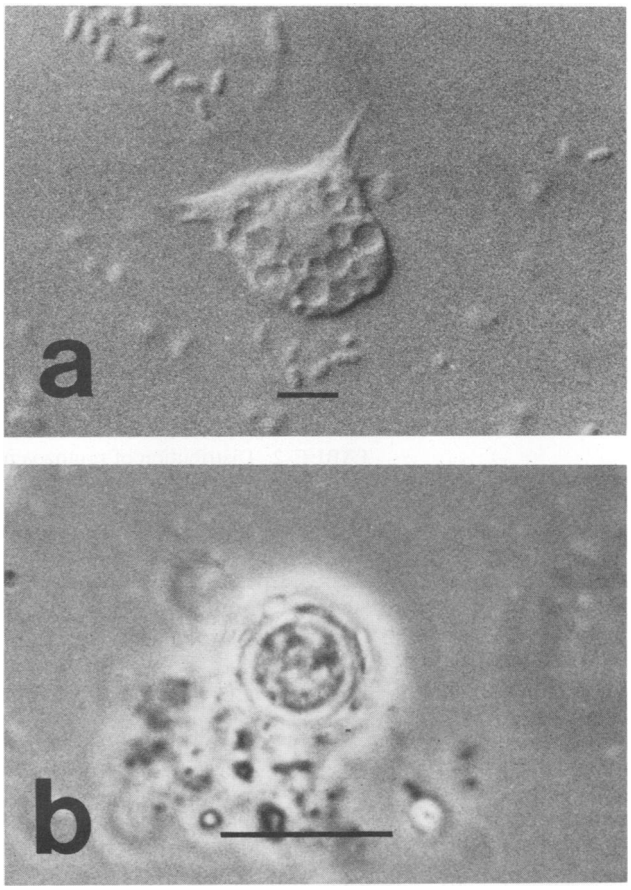

FIG. 3. (a) Filose amoeba isolated from sediments from the interface zone (see Fig. 2). This amoeba represents a new family of the order Aconchulinida. Food bacteria can be seen in the background. Photographed with differential interference-contrast optics. (b) Cyst of the Aconchulinida amoeba shown in Fig. 3a. The cyst is approximately 7.5 μm in diameter. Photographed with phase-contrast optics. Bar, 10 μm.

TABLE 1. Distribution of protozoa in a subsurface profile at Lula, Okla., January 1985

Sample[a]	Approx. depth from surface (m)	Textural class[b]	Estimated particle size distribution (%)			Zone[b]	Protozoa · g (dry wt)$^{-1}$		Proportion (%)[c]		
			Sand	Silt	Clay		MPN	95% Confidence interval	A	F	C
9U	0.10	Sandy loam	70	25	5	U	1.9×10^5	1.3×10^5–3.9×10^5	37	63	1
9S3	0.50	Fine sandy loam	57	28	15	U	4.3×10^2	2.8×10^2–8.3×10^2	12	86	2
9S1	1.30	ND[d]	ND	ND	ND	U	50	41–140	10	90	0
9T2	3.60	Clay loam	23	41	36	I	0[e]	—[e]	—	—	—
9S6	4.20	Fine sandy loam	67	22	11	S	0[e]	—	—	—	—
9S9	7.50	Gravelly, loamy sand	88	10	2	S	4	2.1–5.4	16	84	0
9T4	8.00	Silty clay[f]	5	49	46	S	0[e]	—	—	—	—

[a] Sample code: 9 indicates site location (Lula, Okla.), S and T are different boreholes (Fig. 1), the third number indicates the core segment, and 9U is a surface sample.
[b] For profile and abbreviations, see Fig. 2.
[c] A, Amoebae; F, flagellates; C, ciliates; —, not determined.
[d] ND, Not determined.
[e] Protozoa not detected, <0.2 · g (dry weight)$^{-1}$.
[f] Bedrock clay layer (see Fig. 2).

The uppermost sample (9W4) contained 28 flagellates · g (dry weight)$^{-1}$; the next lower one (9W3) contained only 2 flagellates g (dry weight)$^{-1}$, and the third (9W2) contained no flagellates. Instead, it contained amoebae at a density of 44 · g (dry weight)$^{-1}$ (Table 2). Significantly, the amoebae in the 9W2 sample were identical to the filose amoeba found during our initial screening work (Fig. 3). This amoeba was isolated in uniprotozoan cultures by repeated endpoint dilution methods. Studies of its ultrastructure and motility suggest that it can be classified in a new family of the order Aconchulinida (J. L. Sinclair, F. C. Page, and W. C. Ghiorse, manuscript in preparation).

To determine whether the amoebae in the 9W2 sample were present as vegetative cells or cysts, parallel dilutions were made on HCl-treated and untreated samples. The acid-treated samples yielded an MPN of 30 compared with 44 · g (dry weight)$^{-1}$ in the untreated sample (9W2 and 9W2 acid, Table 2). Because the 95% confidence intervals of the two MPN estimates overlapped, the numbers are not statistically different. These results suggest that most or all of the amoebae in the 9W2 sample were present as acid-resistant cysts.

A final contiguous 10-cm sample from the interface zone core contained two distinct textural layers. Its upper clay loam layer (9W1a, Table 2) contained only one amoeba in one dilution well. In contrast, its lower sandy clay loam layer (9W1b, Table 2) contained a mixture of amoebae and flagellates at a combined density of 67 · g (dry weight)$^{-1}$. At least four types of flagellates and one type of amoeba were present in this layer. These results show the marked biological variability that can occur between subsurface sedimentary layers of different texture, even over very short vertical distances (in this case less than 10 cm).

No protozoa were detected in samples taken from 3.6, 3.8, 4.2, or 5.2 m in the profile (Tables 1 and 2). Bacterial AODC values in these samples remained relatively constant—around 10^7 · g (dry weight)$^{-1}$. These results confirmed our previous negative findings in samples from the saturated zone between 4.5 and 6.5 m at this site (see above). In addition, no protozoa were detected in a sample (9T4, Table

TABLE 2. Distribution of protozoa in a subsurface profile at Lula, Okla., June 1985

Sample[a]	Approx. depth from surface (m)	Textural class[b]	Estimated particle size distribution (%)			Zone[b]	Protozoa · g (dry wt)$^{-1}$		Proportion (%)[c]		
			Sand	Silt	Clay		MPN	95% Confidence interval	A	F	C
9X9	0.10	Sandy loam	58	31	11	U	6.4×10^6	4.1×10^6–14×10^6	61	39	0.1
9X1	0.25	Sandy clay loam	60	19	21	U	2.9×10^3	2.0×10^3–5.3×10^3	67	31	2
9X2	1.80	Fine sandy loam	65	19	16	U	40	27–80	0	100	0
9W4	2.95	Sandy clay loam	53	25	22	U	28	19–51	0	100	0
9W3	3.05	Clay loam	30	38	32	I	2	1.0–2.8	0	100	0
9W2	3.15	Clay loam	27	43	30	I	44	30–80	100	0	0
9W2 acid	3.15	Clay loam	27	43	30	I	30	11–79	—	—	—
9W1a[d]	3.25	Clay loam	44	22	34	I	0.2[e]	0.1–1.0	100	0	0
9W1b[d]	3.35	Sandy clay loam	57	20	23	I	67	43–150	33	67	0
9X10	3.80	Silty clay loam	18	47	35	I	0[f]	—[e]	—	—	—
9X12	5.20	Fine sandy loam	70	18	12	S	0[f]	—[e]	—	—	—
9Y1	7.40	Gravelly, loamy sand	82	15	3	S	6	3.9–12	5	95	0
9Y1 acid	7.40	Gravelly, loamy sand	82	15	3	S	10	2.6–24	—	—	—

[a] Sample code: 9 indicates site location (Lula, Okla.), W, X, and Y are boreholes (Fig. 1), and the third number indicates the core segment. The acid designations indicate treatment with HCl to kill vegetative protozoa.
[b] For profile and abbreviations, see Fig. 2.
[c] A, Amoebae; F, flagellates; C, ciliates; —, not determined.
[d] These samples were removed from the top and bottom of the same 10-cm core segment.
[e] One amoeba was detected in one MPN well.
[f] Protozoa not detected, <0.2 · g (dry weight)$^{-1}$.

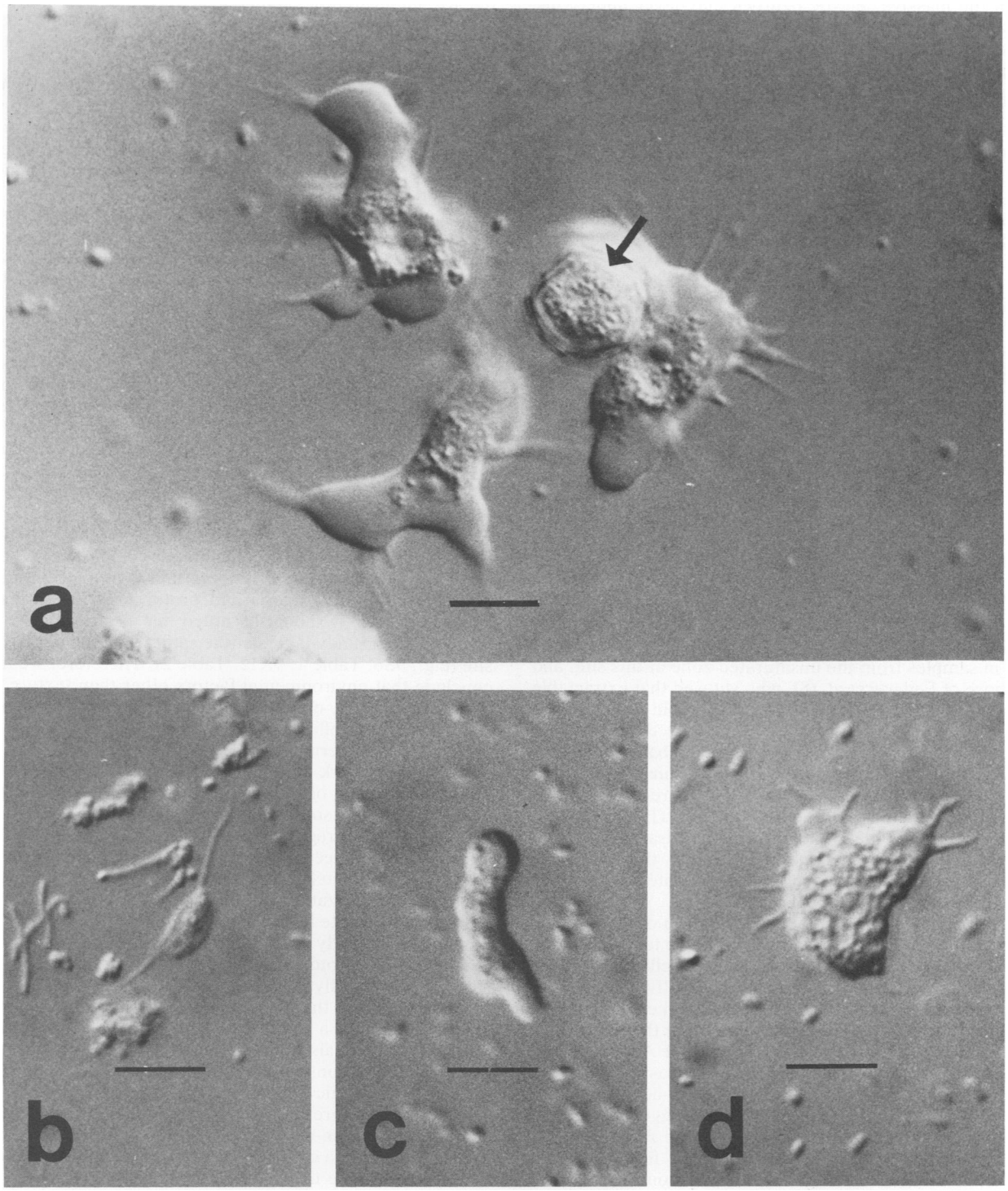

FIG. 4. (a) Amoeba detected in surface soil that closely resembled the Aconchulinida amoeba (Fig. 3a). The diameter of the cyst (arrow), however, is approximately 15 μm, twice that of the Aconchulinida cyst (Fig. 3b). (b) Small flagellate, possibly a *Cercomonas* sp. similar to those seen in the surface soil samples. (c) Limax amoeba detected in a sample (9Y1, Table 2) from the very permeable gravelly, loamy sand layer at 7.4 to 7.5 m (Fig. 2). (d) Amoeba detected in a different sample (9S9, Table 1) from the same permeable layer. This amoeba was morphologically indistinguishable from the Aconchulinida-type amoeba shown in Fig. 3a. All photomicrographs were produced by differential interference contrast. Bar, 10 μm.

1) from the massive, plastic bedrock clay layer underlying the saturated zone at this site.

Contrary to the general trend indicating that protozoa were absent from deeper layers in the profile, protozoa were found in two different samples from a very permeable gravelly loamy sand layer at 7.4 to 7.5 m in the saturated zone. This layer contained MPN values of 4 and 6 · g (dry weight)$^{-1}$ in January and June, respectively (9S9, Table 1; 9Y1, Table 2). The bacterial AODC in this layer increased from 1×10^7 in January to 6×10^7 · g (dry weight)$^{-1}$ in June. On both sampling dates, the protozoa found in this layer were small flagellates (Fig. 4b) and limax-type amoebae (Fig. 4c) similar to flagellates and amoebae found in surface and unsaturated-zone samples. They included forms tentatively identified as members of the genera *Bodo* and *Cercomonas* (17, 19). This layer also contained filose amoebae (Fig. 4d) that were morphologically indistinguishable from those observed in the interface zone samples (Fig. 3). Acid-treated samples from this layer gave approximately the same MPN values as those of untreated samples (9Y1 and 9Y1 acid, Table 2), indicating that the protozoa in this layer too were mostly encysted forms.

DISCUSSION

The vertical decline of protozoan population densities in the Lula subsurface profile agrees with previous findings of Sandon (19) and other soil microbiologists (14, 26), who reported that protozoan numbers declined drastically in subsoil. It also agrees with the recent findings of Bone and Balkwill (4), who observed a low number of eucaryotic forms in samples from the unsaturated zone at this site, and with those of Federle et al. (8), who showed that extractable phospholipids, including polyenoic fatty acid indicators of eucaryotic microorganisms, declined with depth in four contrasting types of subsurface soils in Alabama. This disappearance of ciliates below 0.5 m and dominance of flagellates and amoebae in the Lula profile also are in agreement with previous studies on subsoil protozoa (19, 26). These results support the idea that subsoil and unsaturated zone-sediments down to the confining layer at this site were colonized by surface protozoa which migrated down from more productive soil layers above.

The confining layer was located at the top of an interface zone which was characterized by its variable texture (Fig. 2) and its variable content of amoebae and flagellates (Tables 1 and 2). Because of the presence of the relatively impermeable confining layer in this zone, it was unlikely that downward migration of protozoa could account for the protozoa in this or deeper zones of the profile. In fact, the Aconchulinida amoeba isolated from the interface zone was not found in surface soil, but it was found in the 7.5-m-deep, gravelly, loamy sand layer in the saturated zone, suggesting that its origin may have been the deeper subsurface zone.

On the other hand, other amoebae and flagellates that were found in the layer at 7.5 m were common surface forms, indicating that they may have originated on the surface. Indeed, one possible source of the protozoa in the 7.5-m-deep layer is the nearby river, which could be connected hydrologically to it. This idea is supported by our recent finding of filamentous cyanobacteria growing in illuminated samples from this layer but not in other subsurface samples from the Lula site (J. L. Sinclair, unpublished observations). Further support is provided by evidence for generally increased biological activity in the layer at 7.5 m (R. M. Beloin, J. L. Sinclair, and W. C. Ghiorse, submitted for publication). Counts of viable bacteria and ATP content, as well as AODC and protozoa counts, were higher in the 7.5-m-deep layer compared with data from other layers of the saturated zone.

Protozoa require a certain minimum pore space in which to live (3, 6). Thus, the absence of protozoa in a given subsurface stratum may be related to a finer texture (i.e., higher clay content) of the sediments in that stratum. Indeed, some layers with moderate-to-high clay content in the interface zone (e.g., 9T2, Table 1; 9X10, Table 2) and the impermeable bedrock clay layer contained no protozoa detectable by our MPN method. In contrast, the very permeable layer at 7.5 m was the only layer below 3.35 m that did contain protozoa, suggesting that the coarse texture and resultant high permeability of this layer provided enough space and nutrients for protozoa to live there. Thus, a positive correlation appears to exist between sandy or gravelly sediment texture and the presence of protozoa in subsurface strata. These textural correlations were corroborated in samples from a deeper subsurface coring site in Kansas in which 2 to 20 cyst-forming protozoa · g (dry weight)$^{-1}$ were found in a gravelly layer at a depth of approximately 85 m, but no protozoa were found in high-clay layers immediately above and below the gravelly layer (J. L. Sinclair, unpublished observations).

Our observations imply that the coarse texture and high permeability of the protozoa-containing layers at the Lula site and the Kansas site were important factors affecting the presence of viable bacteria and protozoa in the saturated zone. However, it should be noted that protozoa were not detected in all sandy layers of the saturated zone at the Lula site (e.g., 9S6, Table 1; 9X12, Table 2). Therefore, we must conclude that environmental factors other than texture and permeability (e.g., organic or inorganic nutrient concentrations) also may influence the distribution of protozoa and bacteria in very permeable subsurface layers.

Finally, our work raises questions concerning the ecological role, if any, of protozoa in subsurface sedimentary environments. In soil environments where protozoa graze actively, they can regulate maximum bacterial density (1, 3), affect bacterial species composition (J. L. Sinclair and M. Alexander, submitted for publication), or increase the rate of bacterial mineralization processes (5). Because protozoan numbers were quite low in the Lula sediments and appeared to be present mostly as dormant cysts, we can conclude that the influence of protozoa on bacterial populations in the Lula subsurface normally is minimal. However, in keeping with the argument that the presence of protozoa in a subsurface layer may indicate a higher potential for biological activity in that layer, we can also argue that a potential for regulation of bacterial populations exists in that layer. Indeed, if during seasonal hydrologic fluxes or a pollution episode, excess organic matter were to enter a permeable subsurface layer such as the 7.5-m-deep layer of the Lula aquifer, then indigenous bacteria could proliferate, and protozoa would be present to regulate their population density and activity.

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