APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1989, p. 1074–1077
0099-2240/89/051074-04$02.00/0
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Vol. 55, No. 5

Free-Living Pathogenic and Nonpathogenic Amoebae in Maryland Soils

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Received 6 September 1988/Accepted 21 December 1988

Tests for potentially pathogenic amoebae were carried out on soil samples from the following sites: (i) farmlands fertilized with municipal sewage wastes, (ii) a stream receiving sewage effluent from a sludge lagoon, (iii) a ravine receiving storm runoff from a cattle farm, (iv) farmlands not fertilized with sewage wastes, and (v) a vegetated shoreline of a waterfront estate not used for farming or livestock production. Study sites were located on the eastern shore of Maryland, bordered to the north by Delaware and to the south by Virginia. Twenty-four species of soil amoebae, including five potentially pathogenic *Acanthamoeba* species (members of the family Acanthamoebidae), were identified. All of the sites yielded two or more of the potential pathogens.

Small, free-living amoebae belonging to the genus *Acanthamoeba* or *Naegleria* are now recognized as potential pathogens of humans and animals (11). Singh and Das (20) found that pathogenic species of both genera were recoverable from municipal sewage wastes, and DeJonckheere (4) found that they were common in thermally polluted waters. *Naegleria fowleri* Carter 1970 and *Acanthamoeba culbertsoni* Singh and Das 1972 have been recovered from human patients as well as experimentally infected laboratory animals. *N. fowleri* causes primary amoebic meningitis and is recognized as a primary cause of death in humans, and *A. culbertsoni* causes granulomatous amoebic encephalomyelitis and is considered to be an opportunistic pathogen, usually fatal to immunocompromised patients (11). More recently, several other *Acanthamoeba* species have been recovered from the eyes of human patients suffering from amoebic keratitis, especially among those wearing contaminated contact lenses (2). Although most species of free-living amoebae isolated from soil and water and tested in laboratory mice fail to cause death, temperature-tolerant species are often pathogenic. Today, it is widely accepted that foul or polluted environments are the principal sources of potentially pathogenic species of free-living amoebae.

The present study was designed to compare five different ecosystems with respect to their potential as sources of pathogenic free-living amoebae: (i) farm soils fertilized with municipal sewage wastes, (ii) farm soils not receiving sewage wastes, (iii) a freshwater stream receiving discharged effluent from sewage lagoons, (iv) a deep ravine receiving surface water runoff from a cattle farm, and (v) shoreline soil from a large waterfront estate not subject to runoff from sewage or animal sources. All study locations were in Talbot County on the Maryland eastern shore. The objective of the study was to determine whether sewage sludge used as farm fertilizer could serve as a source of human pathogens introduced into groundwater or tributaries of the Chesapeake Bay.

MATERIALS AND METHODS

Soil samples from five study sites (see below) were collected with sterile tongue depressors, placed in sterile plastic snap-cap culture dishes (Becton Dickinson Labware, Oxnard, Calif.), and stored on ice until they were returned to the laboratory. The samples were then stored in a refrigerator until used for culture preparation the next day. Cultures were prepared and amoebae were identified by using procedures previously described for soil or sediments (17, 19). The study sites, which were sampled in July and August 1986, were as follows: (i) open fields on two farms fertilized with sewage sludge (stations 1 and 2); (ii) shoreline areas on two farms not fertilized with sewage (stations 3 and 4); (iii) shoreline areas of a stream receiving effluent from sewage lagoons (station 5); (iv) a deep, rocky ravine subject to surface runoff from a cattle farm (station 6); and (v) shoreline areas of a large waterfront estate not subject to contamination by sewage or animal wastes (station 7). Shoreline waters were tested for salinity, pH, and temperature, and soils were tested for pH and temperature. Small colonies of amoebae or cysts appearing on primary cultures were removed on small agar blocks and subcultured for temperature tolerance at incubator temperatures of 37 to 39°C. Identifications were made by using existing keys (15) and references cited therein. Species lists were prepared to compare results among the seven study stations.

RESULTS

Station characteristics and amoeba diversity. (i) Station 1. Station 1 was located on a large farm that produced wheat and soybeans and that was fertilized with sewage sludge in winter 1984 and spring 1985. The soil sample was sandy, light brown, and extremely dense and compact as a result of prolonged heat and dry weather; the soil temperature was 28°C, and the pH was 5.5 to 5.9. Seven species were recovered, with five growing at 37 to 39°C, and two of these, *A. hatchetti* and *A. rhysodes*, were known to be pathogenic to laboratory mice (Tables 1 and 2). The station was not in proximity to water.

(ii) Station 2. Station 2 was located on a large wheat farm that was fertilized with sewage sludge in spring 1986. The soil sample was slightly brown to gray and hard as a result of prolonged hot dry weather; the soil temperature was 28 to 30°C, and the pH was 6.7 to 6.9. Twelve species of amoebae were recovered, with eight growing at 37 to 39°C, and four of these, *A. hatchetti*, *A. rhysodes*, *A. lenticulata*, and *Acanthamoeba* sp., were known to be pathogenic (Tables 1 and 2). The station was not in proximity to water.

(iii) Station 3. Station 3 was located on a large farm that produced corn and that had never been fertilized with

TABLE 1. Amoebae recoveries by station

Species	Recovery from station:							Frequency
	1 (dry)	2 (dry)	3 (wet)	4 (wet)	5 (wet)	6 (damp)	7 (wet)	
Acanthamoeba spp.								
A. hatchetti	+	+	+	+	+	+	+	7/7
A. polyphaga	+	+	+	+	+	+	+	7/7
A. lenticulata	−	+	+	−	+	+	−	4/7
A. rhysodes	−	+	−	−	−	+	+	3/7
Acanthamoeba sp.	+	+	+	−	−	−	+	4/7
Naegleria								
N. gruberi	−	+	+	−	+	+	+	5/7
Naegleria sp.	−	−	−	−	+	+	−	2/7
Vahlkampfia								
V. avara	−	−	−	+	−	−	−	1/7
V. hartmannia	−	+	+	−	+	+	−	4/7
V. ustiana	+	−	−	−	+	−	−	2/7
V. aberdonica	−	+	−	−	−	−	−	1/7
V. lobospinosa	−	−	−	−	−	+	−	1/7
Vahlkampha sp.	−	−	−	−	−	−	+	1/7
Paratetramitus jugosus	−	−	−	+	−	−	−	1/7
Vannella sp.	−	−	−	+	−	−	−	1/7
Hartmannella sp.	+	+	+	+	−	+	+	6/7
Thecamoeba similis	+	+	+	−	−	+	+	5/7
Mayorella sp.	−	−	+	−	−	−	−	1/7
Platyamoeba								
P. schaefferi	+	+	+	−	+	+	−	5/7
P. stenopodia	−	−	−	−	−	+	−	1/7
Schizopyrenus sp.	−	−	−	−	+	−	−	1/7
Gephyramoeba sp.	−	+	−	−	−	−	−	1/7
Leptomyxa sp.	−	−	−	−	−	−	+	1/7
Cochliopodium sp.	−	−	−	−	+	−	−	1/7
Other taxa								
Testaceans	−	−	−	−	+	+	−	2/7
Ciliates	−	−	+	−	−	+	+	3/7
Nematodes	−	−	−	+	+	+	+	4/7

sewage sludge. The sample was made up of wet, highly organic soil from a vegetated shoreline near the headwaters of a brackish tributary that supports large populations of waterfowl during winter. The soil temperature was 24 to 27°C, the pH was 5.6 to 5.9, and the salinity was 9‰. Eleven species of amoebae were recovered, with six growing at 37 to 39°C, and three of these, *A. hatchetti*, *A. lenticulata*, and *Aconthamoeba* sp., were known to be pathogenic (Tables 1 and 2).

(iv) Station 4. Station 4 was located on a large farm that produced corn and that had never been fertilized with sewage sludge. The sample was well-drained, fine to medium wet sand from an open unvegetated beach. The soil temperature was 27 to 29°C, the pH was 5.5 to 6.3, and the salinity was 12‰. Six species of amoebae were recovered, with two growing at 37 to 39°C, and one of these, *A. hatchetti*, was known to be pathogenic (Tables 1 and 2).

(v) Station 5. Station 5 was located at a narrow, freshwater stream that received sewage effluent from Talbot County sewage lagoons. The soil sample was a thin (2-mm) brown mud overlying black, highly organic, foul-smelling muck. Red-brown deposits (presumably iron) were present as irregular streaks or patches in the shoreline muck. The soil temperature was 27 to 28°C, and the pH was 5.5 to 6.3. Ten species of amoeba were recovered, with six growing at 37 to 39°C, and two of these, *A. hatchetti* and *A. lenticulata*, were pathogenic, (Tables 1 and 2).

(vi) Station 6. Station 6 was located in a deep, rocky, shaded ravine with muddy soil but no running water except during rains and runoff from a cattle farm during periods of wet weather. The sample was dark brown to mahogany-colored damp mud; the soil temperature was 23°C, and the pH was 5.6 to 6.3. Twelve species of amoebae were recovered, with six growing at 37 to 39°C, and two of these, *A. lenticulata* and *A. rhysodes*, were pathogenic (Tables 1 and 2).

(vii) Station 7. Station 7 was located on a gently sloping shoreline at a private waterfront estate not in proximity to sewage or animal wastes. The site was bordered by aquatic grasses, with ducks, geese, and swans seasonally present. The sample was made up of fine to medium dark brown sand; the soil temperature was 30 to 35°C, the pH was 5.7 to 6.5, and the salinity was 12‰. Nine species of amoebae were recovered, with six growing at 37 to 39°C, and two of these, *A. lenticulata* and *A. rhysodes*, were pathogenic (Tables 1 and 2). The tidal cove bordering this station has periodically been closed to oyster harvesting because of seasonally high sewage bacteria contamination.

A total of 24 species of amoebae (Table 1) were recovered from Talbot County soils, with none of the test stations yielding more than 12 species. Twelve species grew at 37 to 39°C (temperature tolerant), with four known to kill experimentally infected mice. Farms fertilized with sewage sludge (stations 1 and 2) yielded 13 of 24 species, with 8 of 12 growing at high temperatures and 4 of 8 known to be pathogenic. Stations contaminated with human or farm animal wastes (stations 5 and 6) yielded 15 of 24 species, with 8 of 12 growing at high temperatures and 3 of 8 known to be pathogenic. The temperature-tolerant strain designated *Naegleria* sp. was not tested for pathogenicity and was recovered only at stations 5 and 6, both freshwater stations. The three stations not contaminated with known point source sewage or animal wastes (stations 3, 4, and 7) yielded 13 of 24 species, with 8 growing at high temperatures and 3 of 8 known to be pathogenic. Thus, with the exception of the two stations yielding *Naegleria* sp., there was little or no difference between the test and control stations.

TABLE 2. Heat-tolerant[a] amoebae by station

Species	Recovery from station:							Frequency
	1	2	3	4	5	6	7	
Acanthamoeba								
A. hatchetti[b]	+	+	+	+	+	−	+	6/7
A. lenticulata[b]	−	+	+	−	+	+	+	5/7
A. rhysodes	−	+	−	−	−	+	−	2/7
Acanthamoeba sp.	+	+	+	−	−	−	+	4/7
Naegleria sp.[b]	−	−	−	−	+	+	−	2/7
Vahlkampfia								
V. hartmannia	+	+	+	−	+	+	−	5/7
Vahlkampfia sp.	−	−	−	−	−	−	+	1/7
Hartmannella sp.	+	+	+	+	−	+	+	6/7
Schizopyrenus horticola	−	−	−	−	+	−	−	1/7
Platyamoeba schaefferi[b]	+	+	+	−	+	+	−	5/7
Gephyramoeba sp.	−	+	−	−	−	−	−	1/7
Leptomyxa sp.	−	−	−	−	−	−	+	1/7

[a] Growth at 37 to 39°C.

[b] Previously recognized as growing at 37°C or higher.

Of the 13 genera of amoebae identified, 6 had species (9 of 24 species) that were present at four or more of the seven stations: *Acanthamoeba, Naegleria, Hartmannella, Thecamoeba, Vahlkampfia,* and *Platyamoeba* (Table 1). Summary data showed that except for the limited distribution of *Naegleria* sp., there were few or no differences in the occurrence of temperature-tolerant or potentially pathogenic amoebae between control and test stations. The presence of one or more known pathogens suggested that all of the stations had been contaminated with sewage wastes at some time in the past. It is known that all of the brackish-water creeks at or near the shoreline stations have been perodically closed to shellfish harvesting in recent times (see Discussion). Summary data by station showed that station 2, with the greatest amoeba diversity (12 species) and the largest number of potential pathogens (4 species), had received sewage sludge several months prior to the study. In contrast, control station 4 had only six species, with one potential pathogen.

DISCUSSION

Land disposal of solid sewage wastes is considered to be an effective means of decreasing the organic loads that otherwise would be discharged into rivers, bays, and oceans. Burge and Marsh (1) listed several advantages of using solid wastes as a fertilizer for farmland soils: (i) depleted nutrient reserves are replaced; (ii) the physical structure of the soil is improved, and (iii) wastewater may be stored rather than discharged into the water bodies. The same authors also listed several disadvantages: (i) the potential toxicity of chemicals to plants and animals, and (ii) the accumulation of heavy metals, allergens, and pathogens in soil. Reddy and Dorn (16) reported that the presence of cadmium in sewage wastes was of concern because of possible inhalation of contaminated dust by persons working in sludge-treated fields; they also found that cattle raised on sludge-treated farmlands had three times more cadmium in their tissues than did control cattle. In further studies, Dorn et al. (5) investigated 47 farms that were treated with sludge and 46 that were not and found no significant differences in health-related problems in farm residents. They did caution, however, that care should be taken in using their data because of differences in levels of disease agents in sludge and differences in rates of land application. Dubinski et al. (6) studied freshwater tidal wetlands and found that they may retain heavy metals in excess of ambient concentrations during growing seasons because vegetation reduces soil erosion, whereas loss of these metals into the environment may increase during winter because of ice shear, storm runoff, and tidal action. Studies cited above illustrate the complexity of the pros and cons of land disposal of sewage sludge as an alternative to ocean dumping, e.g., physical characteristics of farmlands, potential for environmental contamination, and potential health-related problems in humans and animals.

Stations selected for the present study included sludge farms where storm runoff and soil erosion effects were minimal and control farmlands where runoff and tidal waters would have an influence on the diversity of amoebae present in soils. The cattle ravine and sewage lagoon streams were selected as point sources of fecal pollution. Diverse environmental and ecological characteristics of the eastern shore of Maryland include opportunities for fecal pollution from rural drain fields, city sewage disposal systems, and fecal droppings from wildlife that feed on sludge farms, etc. Most of the rivers and creeks in Talbot county have been closed to shellfish harvesting at one time or another because of high levels of sewage-associated bacteria in the water column.

I chose small, free-living amoebae for my study because several members of the genus *Acanthamoeba* present in soil and water are capable of causing fatal brain disease, blindness, or impaired vision in humans. Strains of the amoebae capable of killing experimentally infected mice are routinely recovered from municipal sewage wastes (20) and thermally polluted waters (4). Pathogenic species have also been recovered from Baltimore Harbor, Md. (19), ocean sludge disposal sites (14, 18), and waters closed to shellfish harvesting because of sewage pollution (12). Griffin (8) found that pathogenic *N. fowleri* grew at 42°C, *A. culbertsoni* grew at 42°C, and *A. rhysodes* grew at 37°C; and Sawyer et al. (19) found that pathogenic *A. hatchetti* grew at 40°C. Therefore, it was originally believed that disease caused by certain free-living amoebae was associated only with strains that grew at animal body temperatures. Griffin (8) also reported that nonpathogenic strains of *A. polyphaga, A. castellanii,* and *A. rhysodes* did not grow above 36 to 37°C. Since that time, however, these three species and *A. hatchetti* have been cultured from samples obtained from the eyes of human patients. All *Acanthamoeba* species must now be considered potential pathogens.

Attempts have not been made to estimate the numbers of amoebae or their cysts in polluted sediments. However, the numbers of *A. polyphaga* amoebae in sediments from a freshwater lake in Nebraska did not exceed 600/100 g from January to June but increased to 1,950/100 g in August (13). Numbers of nonpathogenic *N. gruberi* amoebae and cysts in Michigan soils (21) also were higher in summer months (16,280/100 g) than in cooler months (159/100 g). Therefore, there appears to be a seasonal influence on the numbers of amoebae in soil: a natural characteristic that probably is disrupted by the intentional loading of soils with sewage sludge. For example, the largest number of pathogenic and nonpathogenic amoeba species recovered in my study was from station 1, which received sludge only several months prior to sampling. Station 4, with the smallest number of species, was a shoreline station with little or no vegetation and subject to wind and tidal action. Results from my study suggest that land disposal of sewage wastes negates the natural checks and balances that minimize the health hazard posed by potentially pathogenic free-living amoebae.

The health hazard posed by contaminated airborne dust has been documented by Lawande (9), who briefly exposed opened culture plates during the hartmattan or dusty season in Nigeria and recovered five genera of soil amoebae, including two *Acanthamoeba* species that killed experimental mice. Lawande et al. (10) also cultured nasal swabs from 50 Nigerian children following a fatal case of *Naegleria* infection in a child. Of the 50 children, 12 (24%) had soil amoebae in their nasal passages; 2 had pathogenic *N. fowleri*. Wang and Feldman (22) cultured throat swabs from 2,289 patients and recovered amoebae from 33 of them, including *Hartmannella* and *Acanthamoeba* species. Airborne dust, contaminated water, infected wounds, contaminated contact lenses, etc., are now recognized as sources of infection by certain free-living amoebae.

The potential public health hazards associated with the land disposal of sewage wastes have not been fully recognized by public health officials, sanitarians, or the medical profession. The lack of concern is due to the relatively small number of human cases reported worldwide. This failure is due, in part, to a widespread lack of familiarity with the clinical symptoms of amoebic infection; the failure to recognize small, single-celled amoebae in histologic specimens; and probable natural immunity in healthy individuals. It has been suggested that humans are exposed to soil- and waterborne amoebae from early childhood, affording them the opportunity for long-term acquired natural immunity, and that fatal infections occur primarily in patients with deficient immune systems. Serum from healthy individuals has been shown to lyse both pathogenic *A. culbertsoni* and *A. rhysodes* (7). Much earlier, Culbertson et al. (3) published several significant findings from animal experiments: (i) mice infected with small numbers of *Acanthamoeba* species did not always die or become paralyzed; (ii) in nonfatal infections, granulomas were found for as long as 7 months postinfection; and (iii) amoebae were embedded in the nasal mucosa of infected monkeys but not in cerebral brain lesions. Experimental studies clearly indicate that subacute asymptomatic amoeba infections may be more widespread than currently appreciated. For example, fatal cases of meningitislike disease in which a causative agent is not isolated from patient tissues are not reportable to public health officials in Maryland. Second, some cases of human amoebic eye disease that did not respond to treatment were first thought to be due to herpesvirus infection. Increased awareness of free-living amoebae as agents of fatal or nonfatal human disease has resulted in an increase in the number of human infections reported in recent years.

In conclusion, my studies were designed to document the presence and persistence of potentially pathogenic, cyst-forming amoebae on farms fertilized with solid sewage wastes. Control stations not subject to truck-delivered sewage wastes and stations naturally contaminated with sewage lagoon effluent and animal wastes were also tested for pathogens. All stations were positive for pathogenic species, as expected in a location (Talbot County) inundated with rivers and creeks contaminated by discharges from sewage treatment facilities and land runoff. The study area also suffers from severe shoreline erosion and shorelines subject to sewage contamination by tidal waters. My studies clearly show that open farmlands, often bordered by tributaries of the Chesapeake Bay and contaminated with sewage and animal waste, are unreasonably degraded by further additions of municipal sewage wastes. The complex land and water use interaction in Talbot County, including the recent finding that the county sewage lagoon system was rated as the third worst in the state, would seem to provide a logical basis for rejecting applications or revising existing regulations for out-of-state sludge disposal in county farmlands, especially those bordered by tidal rivers and creeks. Finally, the public health risks associated with sewage wastes are difficult to assess, since subacute long-term effects associated with heavy metals, pesticides, and pathogenic organisms are not always immediately apparent in otherwise healthy individuals.

ACKNOWLEDGMENTS

I gratefully acknowledge the capable technical assistance of Tracy Mueller, Hood College, Frederick, Md., who held a summer student position at Foundation for Advanced Research in the Medical Sciences, Inc., during the course of this study. Special appreciation is expressed to the various landowners who granted permission to take samples from their properties.

LITERATURE CITED

1. **Burge, W. D., and P. B. Marsh.** 1978. Infectious disease hazards of landspreading sewage wastes. J. Environ. Qual. **7**:1–9.
2. **Centers for Disease Control.** 1986. *Acanthamoeba* keratitis associated with contact lenses—United States. Morbid. Mortal. Weekly Rep. **35**:405–408.
3. **Culbertson, C. G., P. W. Ensminger, and W. M. Overton.** 1966. Hartmannella (Acanthamoeba). Experimental chronic granulomatous brain infections produced by new isolates of low virulence. Am. J. Clin. Pathol. **46**:305–314.
4. **DeJonckheere, J. F.** 1981. Pathogenic and nonpathogenic *Acanthamoeba* spp. in thermally polluted discharges and surface waters. J. Protozool. **28**:56–59.
5. **Dorn, R. C., C. S. Reddy, D. N. Lamphere, J. V. Gaeuman, and R. Lanese.** 1985. Municipal sewage sludge application on Ohio farms: health effects. Environ. Res. **38**:332–359.
6. **Dubinski, B. J., R. L. Simpson, and R. E. Good.** 1986. The retention of heavy metals in sewage sludge applied to a freshwater tidal wetland. Estuaries **9**:102–111.
7. **Ferrante, A., and B. Rowan-Kelly.** 1983. Activation of the alternative pathway of complement by *Acanthamoeba culbertsoni*. Clin. Exp. Immunol. **54**:477–485.
8. **Griffin, J. L.** 1972. Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. Science **178**:869–870.
9. **Lawande, R. V.** 1983. Recovery of soil amoebae from the air during the hartmattan in Zaria, Nigeria. Ann. Trop. Med. Parasitol. **77**:45–49.
10. **Lawande, R. V., S. N. Abraham, I. John, and L. J. Egler.** 1979. Recovery of soil amebas from nasal passages of children during the dusty hartmattan period in Zaria. Am. J. Clin. Pathol. **71**:201–203.
11. **Martinez, A. J.** 1985. Free-living amebas: natural history, prevention, diagnosis, pathology, and treatment of disease. CRC Press, Inc., Boca Raton, Fla.
12. **Munson, D. A., and T. K. Sawyer.** 1987. Distribution of *Acanthamoeba hatchetti* (Amoebida: Acanthamoebidae) in the Chester River, Maryland. Trans. Am. Microsc. Soc. **106**:95–96.
13. **O'Dell, W. D.** 1979. Isolation, enumeration and identification of amoebae from a Nebraska lake. J. Protozool. **26**:265–269.
14. **O'Malley, M. L., D. W. Lear, W. N. Adams, J. Gaines, T. K. Sawyer, and E. J. Lewis.** 1982. Microbial contamination of continental shelf sediments by wastewater. J. Water Pollut. Control Fed. **54**:1311–1317.
15. **Page, F. C.** 1976. An illustrated key to freshwater and soil amoebae. Scientific Publication no. 34. Freshwater Biological Association, Ambleside, England.
16. **Reddy, C. S., and C. R. Dorn.** 1985. Municipal sewage sludge application on Ohio farms: estimation of cadmium intake. Environ. Res. **38**:377–388.
17. **Sawyer, T. K., and S. M. Bodammer.** 1983. Marine amoebae (Protozoa: Sarcodina) as indicators of healthy or impacted sediments in the New York Bight Apex., p. 337–352. *In* I. W. Duedall, B. H. Ketchum, P. K. Park, and D. R. Kester (ed.), Wastes in the ocean, vol. 1. Industrial and sewage wastes in the ocean. John Wiley & Sons, Inc., New York.
18. **Sawyer, T. K., E. J. Lewis, M. Galassa, D. W. Lear, M. L. O'Malley, W. N. Adams, and J. Gaines.** 1982. Pathogenic amoebae in ocean sediments near wastewater sludge disposal sites. J. Water Pollut. Control Fed. **54**:1318–1323.
19. **Sawyer, T. K., G. S. Visvesvara, and B. A. Harke.** 1977. Pathogenic amoebas from brackish and ocean sediments, with a description of *Acanthamoeba hatchetti*, n. sp. Science **196**:1324–1325.
20. **Singh, B. N., and S. R. Das.** 1972. Occurrence of pathogenic *Naegleria aerobia*, *Hartmannella culbertsoni*, and *H. rhysodes* in sewage sludge samples of Lucknow. Curr. Sci. **41**:277–281.
21. **Umeche, N.** 1983. The numbers of *Naegleria* spp. in Michigan soils and litters. Arch. Protistenkd. **127**:127–130.
22. **Wang, S. S., and H. A. Feldman.** 1967. Isolation of *Hartmannella* species from human throats. N. Engl. J. Med. **277**:1174–1179.