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AN INVESTIGATION OF AMOEBAE FROM AN ORGANICALLY-CONTAMINATED AQUIFER AT CAPE COD, MASS., USA

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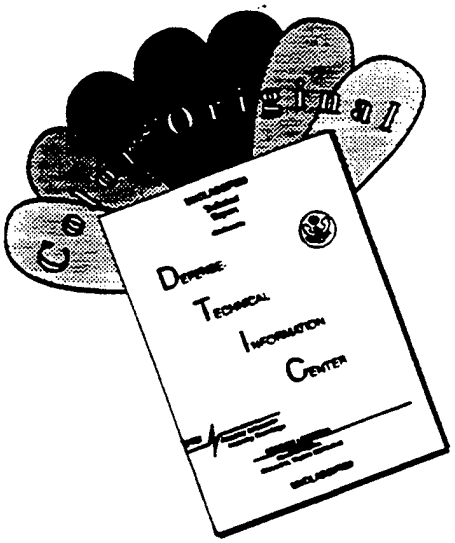

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Eukaryotic microbes (protists) are now thought to be widespread in subsurface environments and evidence is accumulating that they play an important role in the biodegradation of organic contaminants in polluted aquifers. The most commonly isolated protists are flagellates and amoebae. In previous investigations, populations of subsurface protists have been analyzed using culture and enumeration techniques designed for a broad range of organisms. No studies have been made using techniques designed specifically for amoebae. Since 1990, the Natural History Museum (in collaboration with the University of New Hampshire and the US Geological Survey) has studied the role of protists in an aquifer contaminated by treated sewage from the Otis Air Base at Cape Cod, Mass. Investigations to date have centered mainly on flagellates.

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EXECUTIVE SUMMARY

1. Eukaryotic microbes (protists) are now thought to be widespread in subsurface environments and evidence is accumulating that they play an important role in the biodegradation of organic contaminants in polluted aquifers. The most commonly isolated protists are flagellates and amoebae.

2. In previous investigations, populations of subsurface protists have been analyzed using culture and enumeration techniques designed for a broad range of organisms. No studies have been made using techniques designed specifically for amoebae.

3 Since 1990, The Natural History Museum (in collaboration with the University of New Hampshire and the US Geological Survey) has studied the role of protists in an aquifer contaminated by treated sewage from the Otis Air Base at Cape Cod, Mass. Investigations to date have centred mainly on flagellates.

4. The main objective of the present study was to assess the density of amoebae at two sites within the Cape Cod aquifer, one inside the contaminant plume the other at a pristine site outside the plume. A second objective, given the availability of time, was to compare the diversity of amoebae at the two sites.

5. The results of this study confirm that amoebae are present in the aquifer, but in low numbers (10^{-1} - 10^{0} per gram dry weight of sediment) relative to the flagellates. Most of the amoebae observed were in an active rather than encysted state.

6. Seven species of amoebae were isolated of which five were identified to genus, based on trophozoite and cyst morphology.

7. Both the density and diversity of amoebae were higher at the contaminated site than the pristine site. This probably reflects the greater abundance and variety of food available within the contaminant plume.

8. It is concluded that amoebae are likely to play to play only a minor role, relative to the flagellates, as consumers of bacteria and in the process of biodegradation of organic contaminants, within the Cape Cod aquifer.

INTRODUCTION

Until relatively recently eukaryotic microbes (protists) were not thought to form a significant part of the microbial populations in subsurface environments. Indeed, previous investigations involving sandy aquifer sediments suggested that they may be absent altogether (Ghiorse and Balkwill, 1983; Wilson *et al.*, 1983; Harvey *et al.*, 1984). Evidence is now accumulating that protists are normal inhabitants of both shallow and deep aquifer (Hirsch and Rades-Rohkohl, 1983; Sinclair and Ghiorse, 1987, 1989; Beloin *et al.*, 1988; Sinclair *et al.*, 1990; Madsen *et al.*, 1991; Sinclair, 1991; Sinclair *et al.*, 1993).

Heterotrophic protists (protozoa) have a very important role in most aquatic ecosystems. Many are effective predators of bacteria and they are often the primary consumers of these organisms. They may also feed on viruses, fungi, other protists or on dissolved and particulate organic matter. It is likely that protists play a similar role in groundwaters. Subsurface protists, however, remain largely unstudied due to the inaccessibility of their habitat and the difficulty in obtaining uncontaminated, representative samples.

Sinclair (1991) noted that despite their common occurrence in the subsurface, protists are present in very low numbers at pristine sites, with typically <1 to 100 cells per gram found when present. By contrast, elevated numbers of protists (up to 10^5 per gram) have been recorded in at least three organically-contaminated sites (Kinner *et al.*, 1991; Madsen *et al.*, 1991; Sinclair *et al.*, 1993) which suggests that protists may play an important role in natural biodegradation processes.

The most commonly isolated subsurface protists are flagellates and amoebae. Numerically, flagellates are generally dominant, although Sinclair *et al.* (1993) reported that the abundance of amoebae was roughly equal to that of the flagellates in an aquifer contaminated by aviation fuel. Since most studies of

subsurface protists rely on enumeration by most probable number (MPN) methods, the apparent dominance of the flagellates may simply reflect the preference of these organisms for the culture conditions used. Direct enumeration techniques have also been employed for enumerating subsurface protists (Kinner *et al.*, 1991). However, because amoebae are usually small, translucent and attached to surfaces, they are difficult to observe. Direct counting techniques therefore tend to greatly underestimate amoebae populations.

With the exception of the taxonomic study of flagellates by Novarino *et al.* (1994), studies of subsurface protists have centred almost exclusively on their numerical abundance and distribution. There is scant morphological documentation of subsurface protists, particularly the amoebae, and hardly any information is available on their taxonomic identity. This kind of information is essential if we are to gain a better understanding of the ecology of subsurface environments.

Since 1990, the NHM, UNH and USGS have been carrying out investigations of the protistan populations from a sewage-contaminated aquifer on Cape Cod, Mass. (Kinner *et al.*, 1991; Harvey *et al.*, 1992, in press; Novarino *et al.*, 1994). A survey of the aquifer (Kinner *et al.*, 1991) suggested that most (>99%) of the protists present are flagellates, although small numbers of amoebae are also present. Direct epifluorescence and MPN techniques were employed during the survey. Both techniques, however, would have tended to select for the flagellates which are generally faster growing and associate more loosely with grain surfaces than amoebae.

The purpose of the present research was to investigate the populations of amoebae at two sites, one inside the contaminant plume and one at a pristine site outside the plume, using techniques specifically designed for the enumeration and identification of amoebae.

MATERIALS AND METHODS

1. Study area

The area studied during the present investigation is the U.S. Geological Survey Cape Cod Toxic-Substances Hydrology Research Site located in Falmouth, Massachusetts, U.S.A. (Fig. 1). Since 1936, more than 1,700 m^3 per day of secondary sewage effluent from Otis Air Base has been disposed onto rapid infiltration sandbeds (Figs 2, 3), resulting in a plume of organic contamination within the underlying aquifer, which is 5 km long, 1 km wide and 23 m thick (Fig. 1). The plume is characterized by elevated temperature (up to 18^0C), specific conductance (up to 450 μS/cm) and dissolved organic carbon (4 mg/l) relative to pristine zones of the aquifer (10^0C, <80 μS/cm and <1 mg/l respectively). The plume also contains nitrate (60 mg/l), alkylbenzene sulfonate (ABS) detergents (2-4 mg/l) and concentrations of organics such as trichloroethylene, dichlorobenzene and nonylphenol isomers (<1 mg/l) (Harvey *et al.*, 1992). The aquifer sediments are composed largely of quartz and feldspar, with very little (<1%) clay. Hydraulic conductivity, average porosity and mean grain size in the area are ~0.1 cm sec^{-1}, 0.35 and ~0.59 mm respectively (LeBlanc, 1984).

2. Sampling

Cores of aquifer material were obtained aseptically using a wireline piston core barrel (Zapico *et al.*, 1987) in conjunction with a hollow stem auger drill. To prevent contamination from the non-sterile core sleeve, sediment was removed from the central portion of the core. Previous research at the Massachusetts site (Bunn, 1992) indicated that there was no significant difference between epifluorescence direct counts of protists in sediments collected from non-sterile and sterile core sleeves (confidence = 95%).

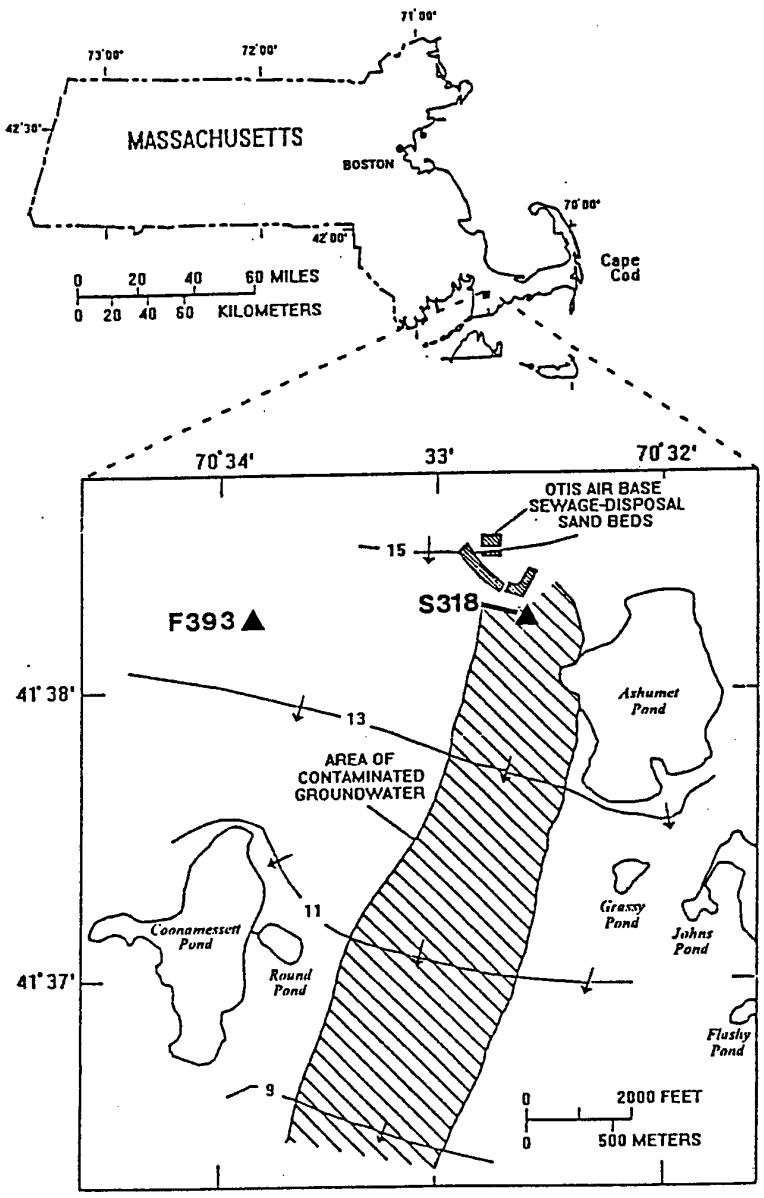

Figure 1. Location of study area and sampling sites. Numbers on contours show altitude of water table above sea level (in meters). Arrows show direction of groundwater flow.

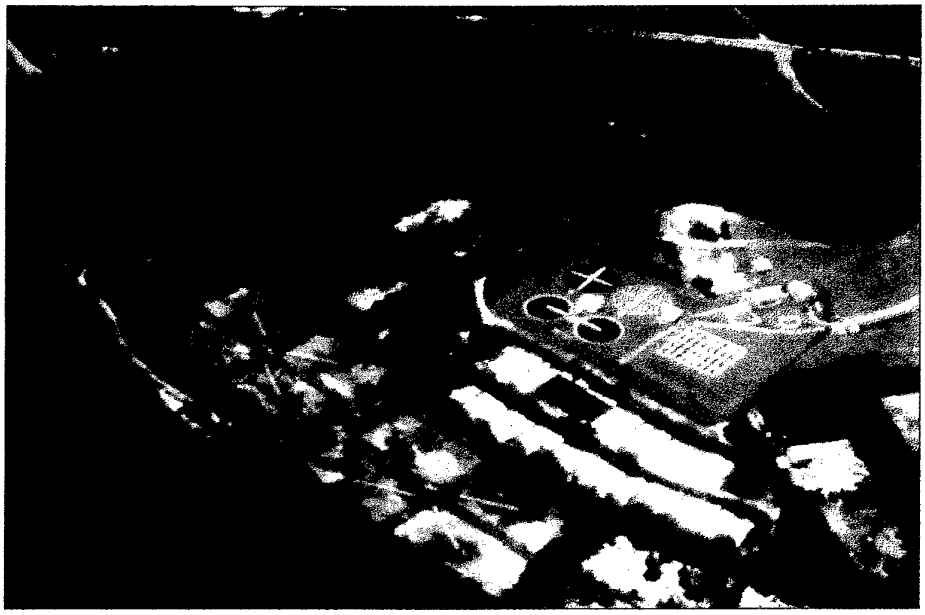

Figure 2. Aerial view of sewage treatment works at Otis Air Base, Cape Cod, MASS.

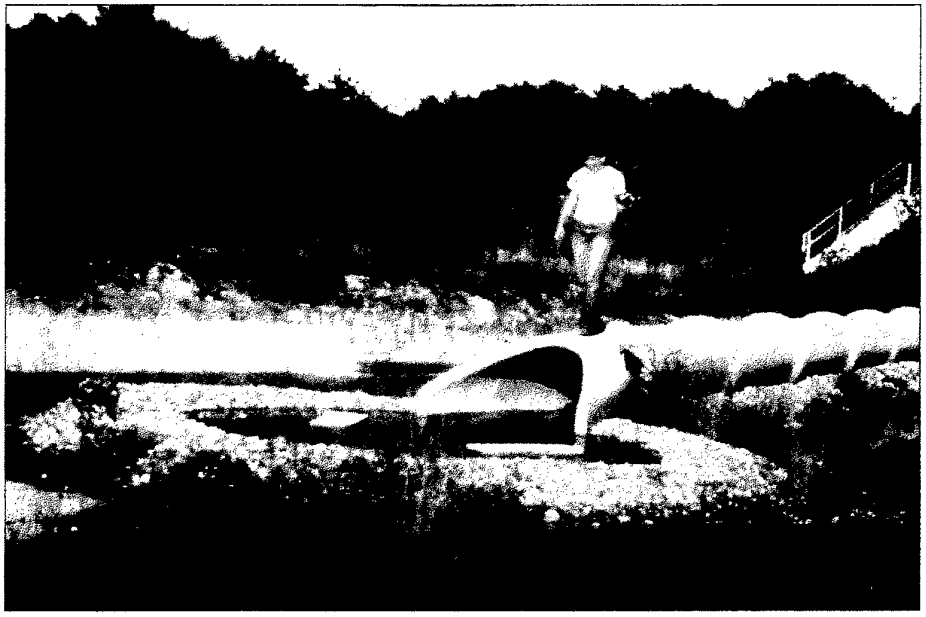

Figure 3. Disposal of treated sewage effluent onto rapid infiltration sandbeds.

The core barrel was removed from the ground and the ends were immediately sealed with plastic caps wiped with 95% ethanol. In order to minimize the risk of contamination, both ends of (about 0.15 m) of each 1.5 m core were cut away and discarded immediately after collection. The barrel was wiped with 95% ethanol in the area to be cut, and a pipe cutter wiped with 95% ethanol was used to make all cuts. Plastic caps were wiped with 95% ethanol were immediately placed on the ends of the core after cutting was complete. Caps were secured with several wraps of electrical insulating tape. Cores were stored upright at 4^0C for transport to the laboratory.

For the present investigation cores were collected at two sites between 13-15th April 1994. One core was obtained at a depth of 34.9 - 35.7 meters below surface (mbs) from site S318, which is located adjacent to the rapid infiltration sandbeds (Fig. 1); in the text this will be referred to as the 'contaminated site'. The other core was obtained at a depth of 34.4 - 35.1 mbs from site F393, which is located outside the plume of contamination (Fig. 1); this will be referred to as the 'pristine site'. These cores were shipped by international courier to the UK, where they arrived 24 hours after collection.

3. Culture, enumeration and identification of amoebae

Cores were processed on the day of arrival at the laboratory. Standard aseptic techniques were used throughout. The top 2 cm of each core was discarded. Subsamples of aquifer material was taken from the central part of the cores and analyzed for amoebae; care was taken to avoid the outermost 2 cm in order to minimize the risk of contamination. Other subsamples were weighed before and after drying at 100^0C to determine the water content of the material.

Amoebae were enumerated by a modified version of the enrichment

culture method described by Rogerson and Laybourn-Parry (1992), replicated using three different culture media; Cerophyl-Prescott's infusion (CP), soil extract with added salts (SE) and modified Neff's amoeba saline (AS) with added fragments of sterile rice grain to stimulate growth of bacteria (see Appendix). For each medium, a ten-fold dilution series was made of the aquifer sediment. Care was taken to resuspend the sediment grains before each dilution step. Aliquots of diluted sediment were inoculated into the wells of tissue culture plates containing culture medium. For each medium and each dilution, three replicate 24-well plates were set-up. Pipettes with cut-off ends (i.e. with wide bore) were used to prevent obstruction by sediment grains.

Replicate plates were set-up using aquifer sediment which had been treated overnight with 2% HCl and neutralized with 2% NaOH (Anderson *et al.*, 1978). This procedure kills trophic amoebae and allows the enumeration of encysted amoebae. All plates were wrapped in cling-film to prevent evaporation of the culture medium and incubated at 18^0C in the dark. They were scanned after one and two weeks to check for growth of fast-growing amoebae.

Amoebae were enumerated after between three and four weeks incubation, using an inverted phase contrast microscope at 400x magnification. This incubation time had been found to give optimal counts of amoebae from marine sediments. For each amoeba present in a well, it was assumed that at least one amoeba cell of this species had been present in the initial aliquot of inoculum. Counts were multiplied up to give numbers of amoebae g^{-1} undiluted aquifer sediment and expressed as numbers g^{-1} dry weight (gdw) of sediment. Amoebae were tentatively identified, using the key by Page (1988).

RESULTS AND DISCUSSION

Counts of amoebae in both cores were low relative to the number of flagellated protozoa observed in the cultures and were in the order of 10^{-1} to 10^{0} amoebae gdw of sediment (Table 1). These results are consistent with those reported previously for the same two sampling sites (Kinner *et al.*, 1991). This is despite the fact that the extraction and enumeration techniques used in the 1991 study were designed for protists such as flagellates, which are generally more loosely associated with the grain surfaces than the amoebae. In the only other study of a contaminated aquifer in which amoebae were enumerated, Sinclair *et al.* (1993) also reported the presence of elevated numbers in areas of contamination and where biotreatment was occurring. In contrast to the present study, however, Sinclair *et al.* (1993) reported that numbers of amoebae were roughly equal to numbers of flagellates in both contaminated and uncontaminated parts of the aquifer.

The reason that numbers of flagellates are so much higher than those of amoebae at Cape Cod may be that both are introduced as part of the sewage effluent and, since the flagellates are more mobile, can become dispersed through the aquifer more easily than the slower-moving amoebae. The transport behaviour of some common flagellates through the aquifer has been the subject of recent investigations (Harvey *et al.*, 1992, in press). Similar studies have yet to be carried out on amoebae. It is also possible that competition from flagellates keeps the number of amoebae low. In marine sediments for example, it has been found that amoebae are more abundant in finer-grained sediments which exclude most other protists. The aquifer sediment at Cape Cod has a relatively large grain size so a similar effect is not observed.

Higher numbers of amoebae were found in the sediment from the contaminated site than from the pristine site. This is consistent with previous findings for flagellate populations at

	Culture medium		
	CP	SE	AS
Contaminated sediment	0.81 (0.48)	2.28 (0.88)	0.10 (0.10)
Pristine sediment	0.41 (0.27)	0.31 (0.31)	0.20 (0.10)

Table 1. Mean numbers of amoebae g^{-1} dry weight aquifer sediment, with standard error of mean in parenthesis. (Means are from the results of 3 replicate 24-well plates at each dilution).

Cape Cod which were 10^1-10^2 gdw higher inside than outside the contaminant plume (Kinner *et al.*, 1993). Numbers of both free-living and surface-associated bacteria are also higher inside (~10^6 ml^{-1} and ~10^7 gdw, respectively) than outside (~10^4 ml^{-1} and 10^5 gdw) the contaminant plume (Harvey *et al.*, 1984). The greater abundance of amoebae inside the plume may be due to the higher numbers of bacteria which are able to support an increased population of bacterivorous protists. Nevertheless, this hypothesis would have to be confirmed experimentally since factor analysis has shown a far weaker relationship between the free-living bacteria and protists in the aquifer than might be expected for typical predator-prey relationships (Kinner *et al.*, 1993).

Very low numbers of amoebae were present in the acid-treated samples (data not shown) suggesting that, although the numbers of amoebae are low, the population is in an active rather than an encysted state. This is consistent with previous investigations which showed that 3-40% of the flagellate population in the Cape Cod aquifer was encysted, and at the majority of sites >70% of organisms were in an active, trophic state (Kinner *et al.*, 1993). It is possible, however, that acid treatment may inactivate cysts resulting in an underestimate of the number of encysted forms (Foissner, 1987).

This is the first study of subsurface protists in which a method specifically designed for the enumeration of amoebae has been employed. Nevertheless, the numbers of amoebae recorded are probably a conservative estimate of their true abundance in the sediment. This is because;

(1) not all amoebae will grow in culture
(2) it is assumed that all the amoebae observed in a well are derived from a single inoculum cell
(3) some amoebae may not have been seen in the cultures due to obstruction by the sediment grains
(4) cysts were frequently seen in cultures, some of which may have belonged to amoebae which had reproduced and encysted

before being scored.

It is unlikley that the true number of amoebae present in the aquifer will be known until more accurate methods of enumeration are developed, such as those based on oligonucleotide probes, monoclonal antibody probes or the measurement of biochemical markers.

The high standard errors of the means (Table 1) indicate that there was some microspatial variability in the numbers of amoebae in the aquifer sediments, perhaps caused by gradients of biogeochemical parameters. The variation in the number of amoebae which grew-up in different culture media illustrates the selective nature of the different culture media. The highest number of amoebae was obtained using soil extract medium. Cerophyl-Precott's infusion also supported good growth of amoebae, but amoeba saline with rice grains yielded low numbers. Dixon (1937) also noted that liquid soil extract medium gave good results for the enumeration of amoebae.

Seven species of naked amoebae were observed in cultures from the aquifer sediment (Table 2). Not all species, however, were observed in every culture medium, again showing the selectivity of different media. *Hartmannella* sp., *Vahlkampfia* spp. and unidentified amoeba sp. 1 were the most commonly observed taxa. Examples of these and of other amoebae isolated from the aquifer are shown in Figure 4. Only one subsurface amoeba has ever previously been figured; this was an unidentified filose amoeba, thought to represent a new family of the order Aconthulinida (Sinclair and Ghiorse, 1987). Therefore, those shown in Figure 4 represent the most comprehensive documentation of groundwater amoebae yet to be assembled.

All five of the genera identified in the present study are common inhabitants of soil and freshwater (Page, 1988) and may be endemic in the aquifer. However, four of these (*Acanthamoeba, Hartmannella, Vahlkampfia* and *Vannella*) have also been reported in sewage treatment processes (Ramirez *et al.*, 1993) and may have

Species	Size (µm)	Site	Media	Comments
Acanthamoeba sp.	20-35	P	CP AS	The cysts formed were irregularly-shaped, with the two-layered wall characteristic of this genus, approximately 10µm in diameter.
Hartmannella sp.	10-14	P,C	CP SE AS	Limax amoebae which did not move in an eruptive fashion. An anterior hyaline cap was present at all times, which was deeper antero-posteriorly than its breadth. Some uroidal filaments present on posterior of cells. No cysts observed.
Vahlkampfia sp.1	15-26	C	CP SE	Limax amoebae which moved rapidly with eruptive locomotion, changing direction very frequently. Uroidal filaments present on posterior of cells. Central nucleolus. Cysts round with no gelatinous coating, 7.5-10.5 µm in diameter.
Vahlkampfia sp.2	20-30	P	CP SE	Similar to *Vahlkampfia* sp.1, but changed direction less often and cysts had an outer coating. Possibly *V. avara*.
Vannella or *Platyamoeba* sp.[2]	5-8	C	SE	Semi-circular or oval-shaped amoebae. The anterior hyaline zone occupied approximately the anterior half of the cell. The posterior margin was straight or slightly convex. Cysts were round and about 4.5 µm in diameter. This isolate was smaller than any named freshwater species of either genus.
Unidentified amoeba sp. 1	7-20	C	CP SE AS	Rounded or fan-shaped amoebae, in which the granuloplasm was entirely surrounded by a very flattened hyaline margin. Contained cytoplasmic inclusions or crystals. Uroidal filaments present on posterior of cells. Round cysts, about 7.5 µm in diameter, were formed. May belong to the family Cochliopodiidae, which have a cuticle or microscales on the cell surface.
Unidentified amoeba[1] sp. 2	c.40	C	CP	Flattened amoebae with long, thin, branching pseudopodia.

[1] This amoeba only grew-up from sediment which had been acid treated, and was the only morphotype found in the acid-treated samples. [2] Separation of amoebae belonging to these two morphologically similar genera requires further study and possibly examination by TEM.

Table 2. Species of amoebae found in aquifer sediments with approximate size (maximum dimension), site from which isolated (C = contaminated; P = pristine) and culture media used.

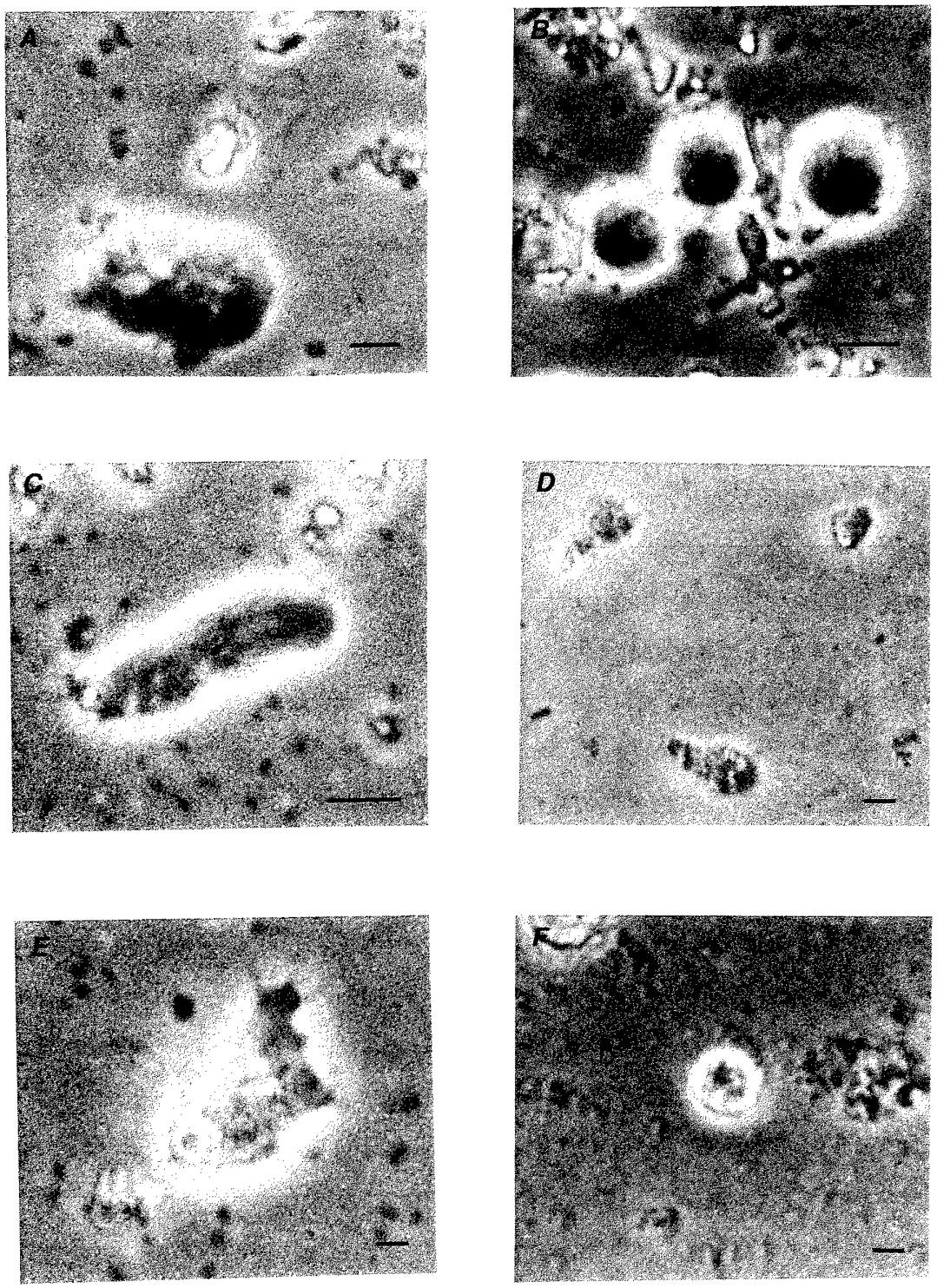

Figure 4. Photomicrographs of amoebae from Cape Cod aquifer using phase contrast microscopy.
A - *Vahlkampfia* sp.1, trophozoite; B - cyst;
C - *Vahlkampfia* sp.2, trophozoite; D - unidentified sp.1, trophozoites; E - *Acanthamoeba* sp., trophozoite;
F - cyst. Scale bars = 5 µm.

been introduced into the aquifer in the sewage effluent. A survey of amoebae present in the sewage effluent will help to determine if this is indeed happening.

The diversity of species was greater in cultures from the contaminated site than from the pristine site. This is consistent with previous findings which have shown that the diversity of flagellates from the same sampling sites was significantly higher in the contaminated than the uncontaminated part of the aquifer (Novarino *et al.*, 1994). The relationships between microbial diversity and organic contamination are not fully understood (Chapelle, 1993). In the Cape Cod aquifer, it may be hypothesized that the greater abundance and variety of food sources in the contaminant plume (e.g. bacteria, colloidal and dissolved organic matter) is capable of supporting a larger number of protistan species than the pristine site. All of the amoebae isolated are known to be bacterivorous. Little is known, however, about their feeding strategies *in situ* or whether they are capable of taking up dissolved or particulate organic matter within the contaminant plume.

CONCLUSIONS

1. Numbers of amoebae in the aquifer sediments were low relative to the flagellates. This may be a result either of competition from flagellates for food keeping numbers of amoebae low or the more mobile flagellates becoming dispersed through the aquifer sediment more efficiently than the slower-moving amoebae .

2. The density and diversity of amoebae were both higher in the contaminated site than the pristine site. This is probably a result of the greater abundance and variety of food available within the contaminant plume.

3. Most of the amoebae observed were in an active rather than encysted state. This suggests that conditions in the aquifer are favourable for the growth and survival of amoebae.

4. It is likely that amoebae play a minor role, relative to the flagellates, as consumers of bacteria and in the process of biodegradation of organic contaminants within the aquifer.

FUTURE WORK

Future work at the Otis Aquifer on Cape Cod will be centred on the flagellates which are numerically the dominant group. This will include further investigations of their taxonomy and of the feeding strategies they employ *in situ*.

Work on amoebae will be carried out on other organically-contaminated aquifers such as those in the León Valley and the Mezquital Valley in Mexico. At both these locations, prolonged use of untreated wastewaters for irrigation purposes has led to chronic pollution of the subsurface.

All of these studies will help to elucidate the role of protists in natural degradation processes in organically-contaminated aquifers.

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APPENDIX

Media used for culturing amoebae.

1. Cerophyl-Prescott Liquid (PC)

This is an infusion of "Cerophyl"* in Prescott's and James' (PJ) liquid. The infusion strength used for groundwater amoebae was 0.1% (weight/volume):

Cerophyl	1.0 g
PJ	1.0 litre

Boil for 5 min, filter out particulate matter and restore to original volume with distilled water.

** A commercially prepared dried grass powder with vitamin additives, available from International Marketing Corporation, 36 Lenexa Centre, 9900 Pflumma Road, Lenexa, Kansas 662115, USA.*

Prescott's and James' Solution

Stock solutions, each in 100 ml glass distilled water:

1.	$CaCl_2.2H_2O$	0.433 g
	KCl	0.162 g
2.	K_2HPO_4	0.512 g
3.	$MgSO_4.7H_2O$	0.280 g

Final solution:
Combine 1 ml of each sock solution and make up to 1 litre with glass distilled water.

2. Soil Extract Medium with Added Salts (SE)

Stock solutions:

		per litre
1.	K_2HPO_4	1.0 g
2.	$MgSO_4.7H_2O$	1.0 g
3.	KNO_3	10.0 g

Final solution:

#Soil extract solution	100.0 ml
Stock solutions 1-3	20.0 ml each

Make up to 1 litre with glass distilled water.

#Soil extract stock:
Mix 1 part air-dried, sieved soil with 2 parts distilled water. Adjust to pH 8 with NaOH or HCl and autoclave for one hour at 15 lb/in^2 pressure. Decant or filter the supernatant.

3. Amoeba Saline Solution (AS)

Stock solutions:

		per 500 ml
1.	NaCl	12.0 g
	$MgSO_4.7H_2O$	0.4 g
	$CaCl_2.6H_2O$	0.6 g
		per 500 ml
2.	Na_2HPO_4	14.2 g
	KH_2PO_4	13.6 g

Final solution:

Stock solutions 1 and 2	5.0 ml each

Make up to 1 litre with glass distilled water.