PROTOZOOLOGY OF LANDFILL

(Final Report)

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EXECUTIVE SUMMARY

1. The Department of Energy is interested in optimising the energy potential of landfill gas, and in obtaining a clear understanding of the identity and growth requirements of the methanogens and other microorganisms involved in gas production.

2. In the last few years it has been recognised that some methanogens also live symbiotically inside free-living anaerobic protozoa. Since the latter are extremely adaptable and demonstrably capable of colonising anaerobic environments, we set out to discover if anaerobic protozoa were also present in landfill.

3. This study had the objectives of establishing the presence or absence of protozoa in landfill, of verifying whether any species present contained methanogen symbionts, and of describing and quantifying the role of protozoan consortia in the generation of methane within landfill.

4. We have established that anaerobic protozoa do live in landfill; they were discovered in four of the five landfill sites investigated. At least nine species were found and there are undoubtedly others waiting to be discovered. They probably spend much of their time encysted, especially in the drier (<40% water) sites.

5. Many of the protozoa living in landfill contain symbiotic methanogenic bacteria which use the hydrogen gas released from the hydrogenosomes (essentially, anaerobic mitochondria) of the host. In the best described case (the ciliate *Metopus palaeformis*) the methanogens are probably *Methanobacterium formicicum*. In response to a scarcity of water, the protozoon encysts by secreting a thick protective coat. Viable methanogen symbionts are retained within the protozoan cyst. When sufficient water becomes available, the protozoon emerges from its cyst, bringing with it the methanogen symbionts, which resume growth and cell division within the ciliate.

6. We have isolated and cultured several species of protozoa from landfill. The best described species is the ciliate *Metopus palaeformis*, which is apparently ubiquitous in landfill sites. The ciliate is easily harvested and concentrated by gentle centrifugation. It can be manipulated on the open bench without the precaution of anaerobic handling. The ciliate can be grown in crude culture with the mixed landfill microbial community as food; its generation time is 30h (20°C) and densities of at least 3000 per ml can be achieved.

7. Unlike free-living methanogens, the *M. palaeformis*-methanogen consortium is not particularly sensitive to oxygen; the symbiotic methanogens remain viable following exposure of the consortium to atmospheric oxygen for several days. The protective mechanism probably involves the oxygen-scavenging capacity of the ciliates' hydrogenosomes. The wind-blown dispersal of methanogen-bearing protozoan cysts is feasible, possibly contributing to the widespread occurrence of *Methanobacterium formicicum* in landfill sites. The possibility that free-living and symbiotic methanogens are identical to each other could be easily tackled using gene probes.

8. It is unlikely that methanogen-bearing protozoa are responsible for a significant part of the methane generation in dry (i.e. <40% water) landfill, where the shortage of water will keep them encysted. When water is added to dry landfill, we have tentative evidence from laboratory experiments that protozoan activity stimulates methane generation by the complete landfill microbial community, that methane generation is initiated earlier, and that the sustained rate of methane generation is greater. This work should be followed up, in conjunction with a study directed at very wet landfill sites.

9. The point cannot be made too strongly that protozoa will live in landfill if it is wet enough. If water is not limiting, methane production by protozoan consortia can be estimated using data for protozoan densities and growth rates and the known

abundance and behaviour of the symbiotic methanogens in crude culture. Using two independent methods, the estimated methane emission from the *M.palaeformis*-methanogen consortium alone is in the range 0.29 to 0.90 m^3 CH_4/tonne/year. This is at the low end of the range of laboratory measurements of methane production by complete landfill and similar to laboratory measurements of methane production by landfill from two of the sites we visited. It seems clear that protozoa can contribute significantly to methane generation by landfill if water is not limiting.

INTRODUCTION

Methane is among the principal final products of organic matter degradation in the absence of oxygen. The production of methane in landfill sites is well-documented, and landfill gas, which is typically 40-60% methane, is an economically viable energy source.

The Department of Energy is interested in developing the energy potential of landfill gas and aware that the optimum methane production is not being realised. We believe that the attempted maximisation of methane production from landfill will benefit from a clear understanding of the identity and growth requirements of the organisms concerned.

It is usually assumed that the bacteria which produce methane are all free-living, but in the last few years it has become known that a variety of anaerobic protozoa harbour large numbers of symbiotic methanogenic bacteria (see Zwart et al. 1988 and refs therein).

Protozoa are the smallest and simplest of all animals. They are unicellular, they occur naturally wherever there is sufficient free water, and they feed on bacteria and other microbes. They have relatively high growth rates and they are usually abundant where organic decomposition is significant (e.g. sewage treatment plants, lake sediments) Almost all protozoa fall within the size range 0.002 - 2 mm.

Most protozoa are aerobic but a diversity also live in such anaerobic habitats as the anoxic layers of marine and freshwater sediments, bogs and septic tanks. None of these protozoa have 'typical' mitochondria but many have energy-yielding microbodies - known as hydrogenosomes because of their capacity to release reducing equivalents as hydrogen gas in the absence of oxygen (Finlay & Fenchel 1989b).

The ciliated protozoa are the best studied group of anaerobic protozoa. Most anaerobic ciliates contain symbiotic methanogens.

There is tentative evidence that interspecies hydrogen transfer operates between hydrogenosomes and methanogens - the latter benefiting from the waste hydrogen produced by the ciliate. The symbiotic methanogens will help maintain a low internal hydrogen pressure within the ciliate but there is no evidence that this biological consumption is quantitatively more important than diffusion of hydrogen to the external environment.

The work reported here is being carried out in the absence of any published information concerning the presence of protozoa in anaerobic landfill. If anaerobic protozoa do live in landfill, and if they do contain significant numbers of methanogens, we will have a more complete, if slightly more complex understanding of the microbiological origin of methane in landfill sites.

This work is primarily concerned with:

- establishing whether or not protozoa live in anaerobic landfill
- verifying the existence of methanogens in any protozoa that are discovered
- quantifying the relative contribution of methanogen -protozoon consortia to methane generation in landfill sites.

COLLECTION OF LANDFILL MATERIAL

Samples were obtained from five sites:

1. Lower Burgh, near Chorley in Lancashire
2. Slackhead, near Beetham in Cumbria
3. Brogborough in Bedfordshire
4. Stairfoot Quarry (Yorkshire Brick Co.), Area A, near Barnsley, South Yorkshire
5. Backford Burrow Pit, near Chester, Cheshire

The samples from Lower Burgh were obtained while the shell and auger was in operation. They were delivered directly into thick plastic bags which were immediately sealed and placed in an anaerobic jar filled with nitrogen (a cylinder of oxygen-free nitrogen was taken onto the landfill site).

The Slackhead samples were obtained by the operatives on site, who packed several kg from each depth into plastic bags. Within about one hour of sampling, subsamples were excavated, bagged and placed in an anaerobic jar for transfer to the laboratory at Windermere.

The Brogborough samples were posted to Windermere by Barry Croft at Harwell.

The Stairfoot material was freshly collected from a trench excavated with a JCB. The material was immediately enclosed in double polythene bags and returned to the lab.

The Backford Burrow material was obtained while the rotary drilling rig was in operation. The material was immediately enclosed in double polythene bags and returned to the lab. A sample of fresh leachate was also taken from the edge of the site and returned in a stoppered bottle to the lab.

At the Windermere Laboratory, all landfill material apart from that sent from Brogborough, was stored at 10°C. All subsequent

laboratory procedures were carried out within 24h of sample collection. The Brogborough samples were received between 5 and 12 days from the date of collection. They were dealt with directly upon receipt at Windermere.

The landfill from the different sites was collected using various types of drilling and excavating equipment. The material from only two of the sites (Lower Burgh and Slackhead) was sealed under nitrogen during transport to the lab. Nevertheless we were able to isolate methanogen-bearing protozoa from the four sites that we visited and material from all sites produced methane in the laboratory. We were always careful to sample from the centre of the bulk landfill material immediately after it was brought to the surface and to pack the material tightly, in glass anaerobic jars, or in thick plastic bags, before transporting it. It appears that if these simple precautions are carried out, the material will remain capable of generating methane and the anaerobic protozoan consortia will remain viable.

LABORATORY INVESTIGATIONS

The initial laboratory procedures were designed to verify:

- that the untreated landfill material would still produce methane in the laboratory
- the presence of free-living methanogens
- the presence or absence of protozoa. If protozoa were not immediately obvious, could their absence be attributed to something toxic in the landfill material, to insufficient free water, or to the lack of a suitable microbial food source for the protozoa?

LABORATORY METHODS

The dry weight of landfill samples was calculated from measurements of water lost by evaporation (80°C, 24h) from approximately 30g of sample.

Microbiological analyses were performed following dilution of the landfill material with anoxic water. We used the natural mineral water 'Volvic' as diluent; this has some buffering capacity and it is known to be a suitable mineral medium for the maintenance of protozoa. It was outgassed with N_2 and brought to pH 7 with CO_2.

Free-living methanogens were detected and enumerated using epifluorescence microscopy. Landfill (2 ml) was diluted with 15 ml anoxic 'Volvic' in a measuring cylinder. 2.5 ml was removed and chemically fixed with 2.5 ml of a 1:1 mixture of 1% formaldehyde solution and 1% glutaraldehyde. A 20 µl subsample was removed and added to 1 ml membrane-filtered (0.2 µm) 'Volvic' in a plastic tube, then dispersed using a vortex mixer for a few seconds. The sample was filtered (25 mm black Nuclepore, 0.2 µm), excited sequentially with ultraviolet and violet light (Leitz Ploemopaks, peak excitation at 350 and 420 nm respectively; [Doddema & Vogels, 1978]) and observed using a Zeiss Neofluar 100x (final magnification 1250x) in oil. Autofluoresing particles resembling bacteria were counted.

Methane production by landfill samples was measured by gas liquid chromatography (Simon & Jones, 1983). Samples of about 5g were handled in an anaerobic cabinet and enclosed in gas-tight teflon vials (30 ml). The samples were incubated at 25°C and the headspace periodically sampled (0.5 ml) with a syringe.

Direct observation of protozoa in fresh material is difficult because the landfill material is fairly dry and very heterogeneous, and because it is easy for any protozoa that are present to remain hidden. We mixed the landfill as best we could while it was still in the original bags, then submerged measured quantities in anaerobic 'Volvic'. This diluted landfill was then pipetted into Sedgewick-Rafter Cells and examined by light microscopy at a final magnification of 125x. We examined a volume of diluted landfill sufficient to achieve a limit of detection equivalent to 10 protozoa per ml in the original landfill (e.g. one protozoon recorded in 1 ml of 10x diluted original sample).

Protozoa were identified using standard reference texts (principally Curds 1982, Curds et al. 1983, Page 1988, Jankowski 1964, Kahl 1930-35 and Vickerman 1976)

We used two methods to culture any protozoa that might remain hidden and/or dormant in the landfill. The first method was aimed at encouraging the growth of ciliates. Approximately one gram of landfill was inoculated, under a stream of nitrogen, into a 160 ml serum vial containing 40 ml of either anoxic 'Volvic' or anoxic CP medium (see Appendix), both at an initial pH of 7. The vials were incubated with an headspace of N_2, at 20°C. Material from Stairfoot Quarry and Backford Burrow was incubated only in SES medium (+ 50 mg powdered cerophyll; see Appendix). The change to this medium was made after it demonstrated itself to support the growth of a wide range of anaerobic free-living protozoa.

One ciliate species frequently found growing in these crude liquid cultures was subsequently isolated with micropipettes and inoculated into SES medium (see Appendix) with additional powdered

cereal leaves (Sigma). The cereal leaves acted as the carbon source for the growing mixed microbial flora on which the ciliate fed.

The abundance and growth rate of ciliates in the serum vials was determined by periodically removing subsamples with a syringe, fixing the cells in a 4% formaldehyde solution, counting them in a Sedgewick-Rafter Cell, and replacing the headspace of the culture with N_2.

The second method was aimed at cultivating amoebae and other small protozoa that grow best when immersed in only a thin film of water. Landfill from each sampling depth was diluted 10x with anaerobic 'Volvic'. One ml was then pipetted onto an agar plate previously streaked with *E. coli*. The diluted samples from Lower Burgh were pipetted onto three types of agar plates : Glucose-Salts (GS) agar, Non-Nutrient (NN) agar and CPA (see Appendix).

Since we subsequently found amoebae growing only on the non-nutrient agar plates, these alone were used in inoculating material from further sites. Agar plates were incubated in an anaerobic jar filled with N_2, at 20°.

Symbiotic bacteria in protozoa

Methanogen symbionts in protozoa were initially detected as autofluorescing particles inside living protozoa. This fluorescence fades within a few seconds, in contrast to that produced by chemically-fixed cells. The original fixative we used was a 1:1 mixture of 1% formaldehyde solution and 1% glutaraldehyde. We subsequently discovered that the glutaraldehyde serves no useful purpose and that its omission from the fixative also cuts out some of the non-specific fluorescence in protozoan cytoplasm.

Fixed cells were filtered onto black Nuclepore (0.22 μm) membranes. The filters were blotted dry to remove excess water,

then impregnated with immersion oil and flattened between a glass cover slide and cover slip. Autofluorescence following excitation with UV (peak excitation 350 nm) or violet light (420 nm, Leitz Ploem filter block D mounted on a Leitz Dialux microscope [Doddema & Vogels, 1978]) was photographed using Professional Ektachrome 800 ASA colour transparency film.

We used a cytochemical method for the localisation of hydrogenase activity in protozoa (Zwart et al. 1988), employing nitroblue tetrazolium as electron acceptor, H_2 as substrate and N_2 as control (see also Appendix).

Electron microscopy

Protozoa were prepared for both scanning (SEM) and transmission electron microscopy (TEM) by gentle centrifugation of cells into a loose pellet followed by chemical fixation (see Appendix). Samples for TEM were embedded in Spurr resin and sectioned with an ultramicrotome. Samples fixed for SEM were frozen in slushed nitrogen (-210^{o}C), freeze-dried, mounted on stubs and sputter coated with gold. All material prepared for electron microscopy was examined using a Jeol 100CX TEMSCAN microscope.

RESULTS AND DISCUSSION

1. Water content

The sites we examined were all very dry (range 11-46% water content; mean, 26%), so far as the maintenance of most species of protozoa is concerned. Living, motile protozoa (i.e trophs, in contrast to cysts) were scarce in the freshly-collected and untreated landfill; they could not be counted with any accuracy and there were probably no more than several tens per ml.

The heterogeneity of landfill material extends also to water content (Fig. 1). The surface layer tends to be drier than the middle depths (approx. 4 - 10m) and the greatest depths, perhaps because of greater compression, are again slightly drier. The two wettest samples we obtained were from 5.5m at Stairfoot, and from 6m at Lower Burgh, (46% and 41% water respectively). It is noteworthy that in these two samples alone did we find a few protozoa, upon direct observation of the fresh material. In each case the protozoa were anaerobic flagellates, one of which was almost certainly *Chilomastix*, which shows signs of endosymbiotic methanogen fluorescence.

The extremely low abundance of trophic protozoa in the landfills we investigated is consistent with what is known of protozoan abundance and diversity in other natural environments. Landfill is similar to soils which periodically become depleted of water, encouraging the protozoa to encyst - indeed the capacity for encystment is an almost universal characteristic of soil protozoa (Fenchel, 1986; Finlay, 1990). Encystment by protozoa is considerably less common in lake sediments, where the water content is rarely less than 50% and is usually closer to 90% (Finlay, 1980).

2. Methane production.

With the exception of material collected from the aerobic surface layer, samples from Slackhead produced methane vigorously,

especially when supplemented with water. This methane production preceded the main thrust of population growth of symbiont-bearing ciliates so we must assume that most of this initial methane production was by free-living methanogens.

Untreated samples from Stairfoot and Backford Burrow also produced methane almost immediately (Figs 2, 3; Tables 4a, 5a). Since these samples were not supplemented with water, and since the encysted protozoa they contained could not, therefore start growing, we must assume that this early methane production was due to free-living methanogens.

The estimates of methanogen abundance in Lower Burgh (Table 1) should be commented upon. Our initial confidence in counting bacteria-sized fluorescing particles slowly faded as we realised that the bacteria were sometimes masked by non-fluorescing particles, that many of them were embedded in organic aggregates, and that some non-bacterial particles have fluorescence characteristics resembling those of methanogens. The overall trend of increasing abundance at 4m followed by decreasing abundance with increasing depth is probably real but the problems of enumeration mentioned above should be borne in mind when considering the accuracy of the actual figures.

Untreated subsurface material from Slackhead (2 - 3m) did not produce methane; we have no explanation for this. The same failure of material from 14m in Backford Burrow, may be due to contamination of the sample with oxygenated water during sampling. The best estimates of methane production by untreated landfill material in the lab. are 72 and 42 nmol CH_4/g /day, from Stairfoot and Backford Burrow respectively. The figure from Slackhead will be significantly higher if the data available from 1 - 2m (1369 nmol CH_4/g/day) are representative of the whole site. There is no obvious correlation between water content of landfill samples and the rate of methane generation. The most probable explanation for this is that although there was insufficient water to support active protozoa, none of the samples were so dry that the activity of free-living methanogens was inhibited.

3. Protozoa

The observation of protozoa in only the wettest of the freshly collected samples indicates that the water content of landfill may be critical for the maintenance of protozoa in the trophic state. This view is supported by the results of our attempts to cultivate protozoa from landfill; protozoa grew up in incubated material from all sites if water or an aqueous culture medium was added. We know that untreated landfill supports vigorous bacterial growth (evidence of microscopy, and measurements of methane generation), which could serve as food for protozoa - all we need do to stimulate the growth of protozoa is to add enough water to make it possible for them to excyst, transport themselves, and to feed.

We cannot account for the extremely poor growth of protozoa and for the absence of ciliates in incubated samples from Brogborough (Table 3). This was the only landfill material we examined that was not dealt with by us immediately after collection and which was sent to us through the post (where it spent several days).

The failure of most incubated material from Backford Burrow to produce protozoa is also a mystery. Protozoa were found only in a cultured sample of leachate. The leachate was collected at 14m but it appeared to have seeped down from 4.5m, the depth where the rotary drill cut the water table. The contamination of this (aerated) water at 14m is, we suspect, also the cause of inhibition of methane generation by free-living methanogens at this depth. The absence of protozoa in material from the greater depths at this site is, we believe due to the high temperatures at these depths. This was the only site we visited in which deep landfill material was delivered hot and steaming at the surface.

All of the protozoa that developed in the laboratory were bacteria-feeding representatives of the three principal taxonomic groups; ciliates, flagellates and amoebae. The full list of protozoa found in cultured landfill material from all sites appears in Table 6.

Relatively little is known about the ecology and systematics of anaerobic free-living protozoa but we can be fairly sure that the species we found are not unique to landfill. It is likely that they are all capable of encysting (as are most soil protozoa) and that they colonise landfill from anaerobic habitats in the surrounding soil (drainage ditches, septic tanks etc.). This is almost certainly the case for the ubiquitous landfill ciliate *Metopus palaeformis* which we and others (e.g. Kahl 1930-35) found in anaerobic sediments, soils and a septic tank.

The polymorphic species *M. palaeformis* is particularly interesting. Kahl's (1930-35) description fits the landfill ciliate in all respects, with the exception of a claimed reniform nucleus which is not present in our isolate (Figs 7a, 8a). He described several varieties of the species, and he conveyed the assumption that the varieties may subsequently be shown to be distinct species. Having observed all the variation in form he described as deriving from one isolate in culture, we can confidently say that *Metopus palaeformis* is a polymorphic ciliate, extremely plastic in its morphology, and capable of encystment. Encystment is a protective device, preventing both dehydration and, possibly, the inwards diffusion of oxygen. The cyst wall of *M. palaeformis* is particularly thick (Fig. 11).

We have obtained the ciliate in crude anaerobic culture, feeding on a variety of anaerobic bacteria. It is most easily grown in an infusion of soil in mineral salts (SES, see Appendix), with Cerophyll or cellobiose as carbon source for the food bacteria. It is not difficult to achieve a generation time of about 30h at 20^{o}C and to produce culture densities of more than 3000 ciliates per ml. This is a crude method of cultivation but it is reliable and it resembles landfill conditions more closely than refined methods using pure cultures. The conditions for the growth of the protozoa must be close to optimal since an anaerobic ciliate the size of *M. palaeformis* is unlikely to grow with a generation time of less than 30h (see Fenchel & Finlay, 1990).

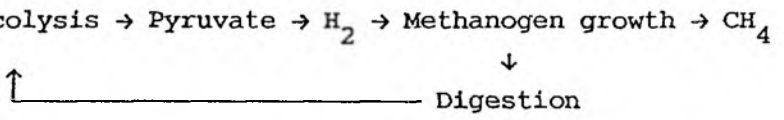
We have also kept the ciliate in a reduced mineral medium (CMV - see Appendix) and growing, albeit slowly, on an exogenous supply of baker's yeast. The ciliate is not particularly fragile and we are confident of refining and simplifying the culture conditions.

Cells vary greatly in size and shape, from short (60 μm) and fat, especially at the beginning of culture growth, to long (170 μm) and thin, when cells begin to starve at the end of batch growth (Figs 1a, 3a). Starvation leads to encystment of at least some cells in the population (Fig. 5a). The factors inducing excystment are unknown; cells grew out (presumably from cysts) from landfill when it was supplemented with water alone, but we have also induced excystment in a starving culture by renewing the carbon source (cellobiose) for the food bacteria. We do not know if the presence of food, or rehydration, or both, are responsible for inducing excystment.

Throughout the cycle of population growth and encystment, *Metopus palaeformis* contains particles which show the autofluorescence characteristic of methanogens (Figs 2a, 4a, 6a). Transmission electron microscopy confirms that these particles are indeed bacteria (Figs 12a- 15a) and that they resemble methanogens (shape, internal membranes [Fig. 17a]), especially endosymbiotic *Methanobacterium formicicum* (van Bruggen et al. 1984, 1988; Goosen et al. 1988). The methanogens vary in size (length ~1 to >5 μm) and in their abundance per ciliate. The mean number of fluorescing rods per ciliate is probably in the region of 500 per cell although some cells have over 1000 and the smallest ciliates have only a few hundred.

The bacteria grow and divide within the ciliate and their number stays relatively constant throughout the course of population growth of the ciliate. There is some evidence that the bacterial growth rate may exceed that of the ciliate since digestion of the methanogens has been observed in TEM sections (Figs 16a, 18a). This is also our understanding of the growth and fate of methanogen symbionts in ciliates from other anaerobic habitats

(Finlay & Fenchel 1989a,b), with digestion of the methanogens completing the recycling of waste within the ciliate:



We have demonstrated that the microbodies in *Metopus palaeformis* have an hydrogenase (Figs 9a, 11a) and that they are, therefore, probably similar to the microbodies in other anaerobic ciliates which release reducing equivalents as hydrogen gas. There is every reason to believe that an 'interspecies' hydrogen transfer mechanism operates between the ciliate hydrogenosomes and the symbiotic methanogens.

4. Further definition of the character of the landfill ciliate Metopus palaeformis.

All experiments were carried out at 20°C.

a) *The ciliate is an anaerobe*

Most ciliated protozoa make clear behavioural responses to the oxygen tension of the water. In particular, in an experimental situation with a tight oxic-anoxic boundary, microaerobic ciliates will aggregate in a zone of low oxygen tension. The landfill ciliate *Metopus palaeformis* is obviously not microaerobic (Figs 4,5). Several thousand cells from an anaerobic culture were introduced into a Sedgewick-Rafter cell left open at one end, and a dense band of microaerobic bacteria developed at some distance inwards from the meniscus. The ciliates totally avoided this zone, showing a sharp cut off at, and total avoidance of, oxygenated water. As yet we have no explanation for the tendency of only the long thin variants to remain in the main bulk of anaerobic water (Fig. 5).

b) *The endosymbiotic bacteria are methanogens*

We used bromoethane sulfonic acid (BES), a specific inhibitor of methanogens (Oremland & Capone, 1988), to confirm that the endosymbionmts of *Metopus palaeformis* are methanogens. BES was added (10mM) to an anaerobic culture of the ciliate in a hypovial. The number of methanogens remaining over several days was monitored by epifluorescence. The number of methanogens decreased in the presence of BES, the number falling by a factor of about two for each ciliate division, indicating that the methanogens had stopped growing and that their number was simply being diluted by continued cell division of the ciliate. The fluorescence of the remaining methanogens was also much less than that of the control. Figures 6 and 7 show a control ciliate and a ciliate after 5 days treatment with 10mM BES.

It appears that *M. palaeformis* receives little or no energetic advantage from its methanogen symbionts. Three anaerobic hypovial cultures were established at time 0h. Each contained 55ml SES medium and 50mg ground, dried cereal leaves (SIGMA). The medium in each was outgassed with N_2/CO_2 (95:5) and brought to pH 7 with sodium bicarbonate. At 112h, one of the cultures was opened and half the contents were gently poured into another hypovial containing BES (final conc. 10mM). Each of these two hypovials now contained about 25ml, each was plugged with cotton wool and, because each had a vigorous bacterial growth by this time, the contents of each can be considered 'microaerobic'. The depth of liquid in each hypovial was now about 12 mm.

At the same time (112h), one of the other two anaerobic cultures received BES to 10mM, so from this time in the course of population growth there were four treatments: Microaerobic, Microaerobic + BES; Anaerobic, and Anaerobic + BES. BES was added to one of the microaerobic treatments as a control - to test for possible toxicity of BES, and to verify that any methanogens remaining in microaerobic ciliates were still functional (if dead, the number in the latter would decrease at the same rate as in the BES treatment). All four cultures were incubated without stirring or shaking at a constant 20°.

The results (Fig. 8; Table 7) are conclusive and several points are clear:

- Ciliate population growth rate in the presence of BES is indistinguishable from the control; this applies if the ciliates grow anaerobically or microaerobically.

- BES inhibited the methanogens, reducing their number to about 10% of those in the controls. The remaining 10% may not have been functional - they showed very weak fluorescence. There seems to be no evidence that this ciliate species gets an energetic advantage from its (untransformed) methanogens.

- The final yield of ciliates is about twice as high in the microaerobic cultures as it is in those produced anaerobically. Final ciliate sizes in the cultures did not differ greatly and the yields in terms of biovolume are still about twice as high in the microaerobic cultures.

- Ciliate population growth rate is higher, and probably significantly so, when the cells grow microaerobically (microaerobic and anaerobic generation times are 28h and 35h respectively).

Lest we draw the apparently obvious conclusion that the ciliate-methanogen consortium grows better and faster because of some direct intervention of oxygen, we should consider the alternative, and probably more likely explanation that oxygen plays an indirect role. We made DAPI counts of total bacteria in the culture medium at 180h and at 235h:

Time (h)	Total bacteria in culture medium ($x10^8$/ml)	
	Anaerobic	Microaerobic
180	1.7	3.4
235	1.3	3.2

The abundance of bacterial 'food' is about twice as high in the microaerobic culture. This is presumably in line with what we would expect - that the bacteria grow more efficiently and more quickly in the presence of oxygen, creating a larger food resource for the ciliates. With such an abundance of bacteria, and with a consideration of the heterogeneity in the culture bottles (pieces of dried grass etc.), we think it most likely that the ciliates could have remained effectively anaerobic (note that they keep their methanogens), possibly with occasional access to low levels of (energetically irrelevant) oxygen, but that many of the bacteria in the microaerobic flask grew aerobically. The latter, more efficient process may, indirectly, be the source of the greater production of both ciliates and symbiotic methanogens in the microaerobic flasks. (Unfortunately we still cannot be sure if it is the greater number, or the new physiological types of bacteria (nutritionally enhanced with steroids etc.) that is most important).

c) *Polymorphic life cycle*

The main elements of the normal, polymorphic population growth cycle are shown in Fig. 9. The initial ciliate culture was anaerobic, with a volume of 80 ml in a 160ml hypovial with an head space of N_2/CO_2 (95:5). 50 mg ground cereal leaves was the carbon source for the food bacteria. The growth of these bacteria, and their subsequent consumption by the ciliates, fuelled growth and cell division of the latter. The ciliates reached their maximum size at the end of stationary phase; thereafter, their average size decreased through log phase. The majority of cells ended up as long thin forms while a minority (about 5%) transformed into cysts. This partition of the final starved stage may be viewed as a sensible strategy for optimising survival of this ciliate species in an unsuitable but unpredictable environment.

The cause of population decline was probably starvation. When the culture was supplemented with 57mg cellobiose (arrow A), there was a short lag (the time taken for reproduction of the food bacteria in the culture medium), before a period of rapid population growth

and exploitation of the new food source. Thereafter, the pattern of population development was as before, with the eventual production of long, thin starved forms, and of cysts.

At point B we removed the air-tight seal of the culture bottle and replaced it with cotton wool. After several days, there was another increase in ciliate number. This was probably in response to the increase in abundance of microaerobic food bacteria - as witnessed in the preceding experiment.

d) *Oxygen inhibition*

It is perhaps surprising that an anaerobic organism which contains large numbers of oxygen-sensitive symbionts is capable of surviving for several days in the presence of oxygen at atmospheric pressure. The data in Fig. 10 support this claim. Two normal anaerobic cultures were established in hypovials. At 118h, one of them was poured into a Petri dish, to a depth of 2-3 mm. Cysts and trophs remaining after 6 days exposure to oxygen in this dish are shown in Figs 11 and 12. So far as we can tell, the cysts contain normal, viable, methanogens and the symbiotic consortium returns to normal trophic growth following its return to anaerobic culture.

e) *Methane production +/- ciliates*

The protozoa living in landfill are part of a complex microbial community. Some of the bacteria in that community, including the free-living methanogens, serve as food for the protozoa. The protozoa will also change the nature of the microbial community, not least through their ability to secrete various high molecular weight compounds and to flocculate suspended bacteria and other particles (this process is of proven importance in the clarification of effluent from activated sludge plants, which have an abundant protozoan fauna). Bearing these points in mind, it becomes clear that the least ambiguous way of deciphering the role of protozoa in methane generation by landfill is to monitor methane production by the complete microbial community: when protozoa are present, and when they are absent.

In a preliminary experiment, we established four anaerobic cultures (SES + cereal leaves as carbon source) of the complete microbial community from Slackhead (1-2m). These cultures were left for 7 days before a loose pellet of living *Metopus palaeformis* from another culture was added to one of the hypovials (to B in Fig. 14). Methane production in all vials was monitored, as well as ciliate growth in vial B. The results (Fig. 14) provide evidence that the rate of methane production is slightly higher in the presence of ciliates but the large variation in the rate of methane production, especially between the 'bacteria only' cultures means that these results should be regarded as tentative.

The evidence is clearer that methane production is initiated earlier when ciliates are present. It is not possible to say yet if this is due to the additional symbiotic methanogens provided in the ciliate 'seed', or if the grazing activities of the protozoa help to maintain the 'vigour' of the mixed microbial community and hence the rate of methane production by the free-living methanogens. The same phenomenon of protozoan enhancement of the activity of predated bacteria is well-documented in the literature, being analogous in some ways to a stimulation of the growth of grass by the grazing (and excretion) of sheep. There is no doubt that grazing protozoa make a significant impact on the microbial community, removing most of the suspended bacteria and producing a clear liquor. The results presented are tentative, but they are promising.

The potential contribution by symbiotic methanogens to methane generation in landfill

We can estimate the maximum contribution that endosymbiotic methanogens could make to the generation of methane from wet landfill. This will give an indication of the potential economic significance of protozoa.

Assume that *Metopus palaeformis* is the only anaerobic protozoon in landfill, that each ciliate contains 500 methanogens, that the availability of water does not limit its growth, and that the landfill can sustain a population density of 3000 ciliates per gram. (All of these conditions can be produced in crude laboratory cultures). Assume also that the symbiotic methanogens have dimensions which are the average of the extreme sizes shown in Figs 13a and 15a and that the symbionts divide twice per ciliate generation. (This is an estimate - the methanogens must divide at least as often as the ciliates if they are to maintain a constant population density and if they are to simultaneously fuel continuous digestion by the host) and that the ciliate grows with a generation time of 30h.

Number of symbiotic methanogens	=	1.5×10^{6}/g wet landfill
Yield of symbiotic methanogens	=	3.0×10^{6}/g/30h
Average biovolume of one methanogen	=	0.3 μm^3
Average dry weight (DW) of one methanogen (assuming DW = 20% WW and s.g. = 1.1)	=	7×10^{-14}g
Yield (DW) of symbiotic methanogens	=	2.1×10^{-7}g/g/30h

Methanobacterium formicicum grows with a yield of 4.8g dry weight per mol CH_4 produced (Schauer & Ferry, 1980). With the assumption that the symbiotic methanogen grows with the same yield per mol and that all CH_4 produced leaves the ciliate:

Methane generation by symbiotic methanogens	=	$(2.1 \times 10^{-7})/4.8$
	=	0.044 µmol CH_4/g/30h
	=	0.99 µl CH_4/g/30h
	=	0.29 m^3/tonne/year

Probable methane generation can also be estimated from measured rates of emission from bacteria. Van Bruggen (1986) measured methane production by the *Methanobacterium formicicum* isolated from another *Metopus* species:

= 0.07 pmol/bacterium/day (at 20°C)
= 35 pmol/ciliate/day
= 0.11 μmol/g landfill/day
= 40 μmol/g landfill/year
= 896 μl /g landfill/year
= 0.90 m^3 /tonne/year

These figures can be put into context:

	m^3 CH_4/tonne/year
Estimated maximum production by one ciliate species	0.29 - 0.90
Backford Burrow (this report)	0.34
Stairfoot (this report)	0.58
Slackhead untreated (this report)	11
Slackhead + water (this report)	18
Various lysimeter studies, laboratory pot experiments and *in situ* measurements[#]	0.1 - 200

[#] *A Basic Study of Landfill Microbiology and Biochemistry*, ETSU B 1159, 1988. (p.47; assuming biogas containing 50% methane)

It is therefore theoretically possible for the protozoa in wet landfill to produce as much methane as is sometimes recorded from complete, untreated landfill. The figure we have calculated is similar to the rates of methane production measured from two of the sites we sampled although it should be

pointed out that these latter rates are at the low end of the complete range of measurements available from landfill. The estimate for production by protozoan symbionts may be criticised because it assumes sustained ideal conditions for the growth of the protozoa. On the other hand, we have calculated methane production by only one of the species of anaerobic protozoa that are known to live in landfill - the species for which we have the best information.

Recommendations for future work

The presence of anaerobic protozoa in landfill has been demonstrated and the most common species have been shown to contain symbiotic methanogens. If sufficient water is present, methane generation by protozoan consortia is theoretically significant. The grazing activity of protozoa may also stimulate overall metabolic activity within the landfill microbial community. Definitive conclusions concerning the role of protozoa in landfill will be reached only after some fundamental studies of the general biology of these protozoa is completed. These studies should be directed at the 'ubiquitous' landfill ciliate *Metopus palaeformis*.

1. Tolerance Experiments

The persistence of viable, active protozoa in landfill is probably determined by a) water content, and b) high temperatures.

We should:

a) precisely deterine the minimum water content required to support the growth of *Metopus palaeformis* and other protozoa in landfill.

b) determine the temperatures which are lethal to landfill protozoa and their cysts.

2. Protozoan stimulation of the microbial community

Protozoa are known to stimulate the rate of decomposition by microorganisms in aerobic environments. This is brought about by a combination of continuous cropping of the microbial production and by nutrient excretion from the protozoa. Both processes together maintain the grazed microbial populations in a state of 'vigour'.

We have tentative evidence that anaerobic protozoa stimulate the flux of

methane from mixed anaerobic microbial communities. We should identify and quantify the role played by these protozoa.

The simplest first approach would be to introduce crude landfill microbial communities, with and without protozoa, to sterile, anaerobic landfill; then monitor the rate of methane production and the nature and quantities of VFA's produced.

At the IFE, we are putting together a system (based on electromigration and GC-mass spec.) to isolate, identify and quantify the excretion products of anaerobic landfill protozoa.

3. Wet Landfill

Anaerobic protozoa with symbiotic methanogens are predicted to be active and relatively abundant in very wet landfill. This should be tested by examining the wettest landfill available.

4. Protozoan cysts as vectors of landfill methanogens

Wind-blown dispersal of protozoan cysts which contain methanogens is proposed as a mechanism by which an oxygen-sensitive microorganism (the methanogen) could become widely-distributed in landfill sites. The symbiotic methanogen is probably *Methanobacterium formicicum*. The same species of methanogen (although not necessarily the same strain) is widely reported from landfill. We have isolates of the symbiont-bearing protozoon (*Metopus palaeformis*) from landfill sites in the UK, from anaerobic lake sediment in Southern Germany, and from a domestic septic tank. If the symbiotic methanogens are shown to be identical to the free-living landfill strain, we would be encouraged in the belief that methanogens can be exchanged between ciliates and the surrounding landfill. If they are shown not to be identical or even similar, we would conclude that no such exchange takes place.

The simplest approach would be to sequence the 16s rRNA in both the symbiotic methanogens of *Metopus palaeformis* and in the free-living methanogens from

landfill, and to establish degrees of similarity between isolates. No one has yet attempted to sequence any of the rRNA's of symbiotic methanogens, mainly because of the lack of sufficient numbers of uncontaminated protozoan consortia, and because of inadequate technology for gene amplification. Both problems have recently been solved and the first step should now be to isolate very clean ciliates. This is relatively easy to do using techniques of electromigration (in an electric field the ciliates swim in a straight line towards the cathode; if the cathode is located at the end of a long tube filled with clean medium, the ciliates effectively clean themselves of external contaminating bacteria). We need isolate only a few protozoa by this method to obtain the material required for gene amplification by the polymerase chain reaction.

Some results should be guaranteed, e.g. a) confirmation that the symbiont is a methanogen, b) the best description yet of the identity of a symbiotic methanogen, and c) confirmation of the number of species of symbiotic methanogens in the protozoon (there is much debate about this in the literature).

5. Inoculation with protozoa

It is relatively easy to produce large scale cultures of the methanogen-bearing ciliate *Metopus palaeformis*. Both trophic cells and cysts can be produced. If cultures are exposed to the air there is only a gradual loss in the viability of both ciliates and their methanogen symbionts. Inoculation of 'sick' landfill with methanogen-bearing protozoa may be a more appropriate method of introducing methanogens which, in their free-living state, are extremely sensitive to oxygen. This procedure would have the greatest chance of success in wet landfill.

A useful first step would be to attempt inoculation of a solid waste digestor, where the higher water content should present a more equable environment for the establishment and growth of anaerobic methanogen-containing protozoa.

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TABLES

Table 1: LOWER BURGH 20 JUNE 1989

Mainly domestic waste, 8 - 10m deep. Filling stopped 1983 - now capped and grassed over. Boreholes sunk by shell and auger

	Depth (m)			
	2	4	6	8
Dry Weight (%)	74	84	59	61
Methanogens x 10^{-7} per ml	9.0	13.5	4.2	3.9
(two counts from each depth)	9.3	18.0	6.1	2.9

Protozoa: Initial direct observation

Ciliates	-	-	-	-
Flagellates	-	-	+[a]	-
Amoebae	-	-	-	-

Protozoa: Cultures

	Days				
Liquid	10	Volvic	-	-	-	-
		Cero	-	+[b]	-	-
	14	Volvic	-	+[b]	-	-
		Cero	-	+[b]	-	-
	19	Volvic	-	-	-	-
		Cero	-	+[b]	+[b]	-
	27	Volvic	-	-	-	-
		Cero	-	+[b]	+[b]	-
Agar	17	GS	-	-	-	-
		NN	+[c]	-	-	-
		CPA	-	-	-	-
	34	GS	-	-	-	-
		NN	+[d]	-	-	-
		CPA	-	-	-	-

a Two spp. bodonid flagellates observed
b *Metopus palaeformis*
c *Phreatamoeba* sp.
d *Mastigamoeba* sp.

Table 2: SLACKHEAD 25 JULY 1989

Mainly domestic waste. Filled site, uncapped. Trench dug 1 - 2m and boreholes (shell and auger) sunk in the trench

	Depth (m)				
	0-1	1-2	2-3	4.2	7.0
Dry weight (%)	76	79	86	69[c]	74

Protozoa Initial direct observation

Ciliates Flagellates Amoebae	No protozoa observed at any depth*

* We also attempted (without success) to isolate protozoa by electromigration (Wagener et al. 1986)

Protozoa Cultures

	Days					
Liquid	15	Volvic	-	+[a]	+[a]	-	-
		Cero	-	+[a]	-	-	+[a]
	29	Volvic	+[a]	+[a]	+[a]	-	+[a]
		Cero	-	+[a]	-	-	-
Agar	15		-	-	+[b]	-	-
	31		-	-	+[b]	-	-

a *Metopus palaeformis*
b *Acanthamoeba* cysts (no internal fluorescence)
c Drilling equipment lubricated with water at this depth

Table 2a: **SLACKHEAD**

Cumulative CH_4 produced (nmol/g wet wt)

Depth (m)	Untreated	+ 10 ml Volvic	+ 10 ml CP medium
After 3 days			
0-1	0	0	106
1-2	682	294	389
2-3	0	152	385
4.2	n/a	155	405
After 8 days			
0-1	0	0	0
1-2	6542	7097	8259
2-3	0	4521	6415
4.2	n/a	3193	11739
After 14 days			
0-1	0	0	0
1-2	19165	30485[a]	14855
2-3	0	21097	20212[a]
4.2	n/a	31076	93880

Formalin-killed controls were all zero

[a] *Metopus palaeformis* grew up in these samples

Table 3: BROGBOROUGH 15 - 20 SEPT. 1989

Data supplied by B. Croft (Harwell)	Sample A	Sample B	Sample C
Collection date	15-9-89	18-9-89	20-9-89
Received at Windermere	27-9-89	25-9-89	25-9-89
Origin	Cell #1 (normal density refuse)	Cell #2 (low density refuse)	Cell #3 (normal domestic refuse)
Depth into waste (m)	11	5	10
Approximate age (months)	24	14	23
Temp. (^{o}C)	48	37	37
Data obtained at Windermere			
Dry Weight (%)	73	67	71

Protozoa: Initial direct observation

Ciliates Flagellates Amoebae	No protozoa observed

Protozoa: Cultures

Days		
15 'Volvic'	A	some flagellates (*Heteromita*?)
	B	zero
	C	zero
30 'Volvic'	A	*Heteromita* (no fluorescence)
	B	zero
	C	zero

Table 4: YORKSHIRE BRICK (STAIRFOOT) 7 FEB 1990

Filled with domestic waste; tipping completed 1977-8. Material collected at the time of excavation by JCB

	Depth (m)				
	1	3	4.5	5.5	6.5
Dry weight (%)	82	80	74	54	74

Protozoa: Initial direct observation

Ciliates Flagellates Amoebae	No protozoa observed at any depth* apart from 5.5m (a few unidentified flagellates, probably *Chilomastix*)

*Neither did we find protozoa in samples of leachate obtained at the same time. We did find *Metopus palaeformis* and the odontostomatid *Discomorphella* in a sample collected from surface run-off.

Protozoa Cultures

After 22 days incubation, protozoa were found in cultures from all landfill depths. The same protozoan species were present, and all were abundant, in all cultures. The protozoa were: *Metopus palaeformis*, two species of the amoeboflagellate *Mastigamoeba*, the flagellate *Chilomastix* sp. and possibly other flagellate species (see Appendix). Of the flagellates, only the *Chilomastix* showed clear signs of methanogen autofluorescence. There was tentative evidence of the same in the larger *Mastigamoeba* sp. We are attempting to maintain all of these protozoa in reproducible cultures.

TABLE 4a

YORKSHIRE BRICK (STAIRFOOT)

(Methane production by samples collected 7 Feb 1990)

Cumulative CH_4 produced (nmol/g wet wt)

DAY	0	1	4	6	8	11	15	%moisture
DEPTH (m)								
1	0	0	0	0	0	0	0	16
3	0	13	169	299	465	888	1464	19
4.5	0	138	455	585	661	892	1073	28
5.5	0	50	311	417	554	918	1244	28
6.5	0	80	254	320	364	494	550	26

All data are means of four replicates.

Variation in methane production between subsamples of material collected at one depth (5.5m).

DAY	0	1	4	6	8	11	15	%moisture
DEPTH (m)								
5.5M	0	0	0	0	0	0	51	30
5.5M	0	81	413	558	668	968	1316	29
5.5M	0	117	553	759	1080	1972	2499	29
5.5M	0	0	280	349	466	732	1109	23

Table 5 **BACKFORD BURROW PIT 21 FEB 1990**

	Depth (m)			
	5	14	16	17.5
Dry weight (%)	89	68	81	73

Protozoa: Initial direct observation

Ciliates Flagellates Amoebae	No protozoa observed at any depth

Protozoa cultures

We examined incubated material at 1, 17 and 26 days. Only at 26 days did we find protozoa - large numbers of anaerobic flagellates (an unidentified retortamonad, probably *Chilomastix*) at 5m and the ciliate *Metopus palaeformis* in the incubated sample of leachate. No protozoa grew up in incubated landfill material colected deeper than 5m.

TABLE 5a

BACKFORD BURROW PIT

(Methane production by samples collected 21 Feb 1990)

Cumulative CH_4 produced (nmol/g wet wt)

DAY	0	1	4	6	10	13	21	28	31	38	%moisture
DEPTH(m)											
5	0	0	0	0	0	0	17	28	39	87	17
14	0	98	111	111	110	98	107	99	88	98	34
16	0	0	0	49	66	103	218	426	538	870	20
17.5	0	0	125	190	221	284	515	677	738	1066	28

All data are means of two replicates.

Table 6 Protozoa discovered in the five landfill sites

Ciliates	Source
Metopus palaeformis Kahl	All landfill sites apart from Brogborough
Discomorphella sp.	Stairfoot
Flagellates	
Heteromita sp.	Brogborough
Mastigamoeba (2 spp.)	Stairfoot; Lower Burgh
Chilomastix sp.	Stairfoot; possibly at other sites
unidentified bodonids	Stairfoot; Lower Burgh
Amoebae	
Acanthamoeba sp. (cysts)	Slackhead
Phreatamoeba sp.	Lower Burgh

We have the anaerobic flagellates and *Metopus palaeformis* in crude culture. The taxonomy of the flagellates is currently something of a mess and it is unlikely that anyone can give a precise identification until some TEM is carried out.

Table 7. Yield of the ciliate *Metopus palaeformis*, with various treatments, at 235h into the growth experiment (see also Fig. 8)

Treatment	Ciliates per ml	(mm^3/ml)	Methanogens per ciliate	Symbiotic methanogens ($x10^6$/ml)
Microaerobic	3732	0.071	423	1.6
Microaerobic + BES	3575	0.079	54	0.19
Anaerobic	1856	0.032	516	0.96
Anaerobic + BES	1980	0.038	61	0.12

TABLE 8. Methane production in the presence and absence of ciliates. Data are means (as shown in Fig. 14), followed by values for duplicate cultures (*italics*).

Time (days)	Methane (μmol) in headspace: Bacteria only (A)	Bacteria + ciliates (B)	Ciliate no. ($x10^{-2}$)/mL (B)
0	0 *(0, 0)*	0 *(0, 0)*	0 *(0, 0)*
4	0 *(0, 0)*	0 *(0, 0)*	0 *(0, 0)*
6	0 *(0, 0)*	0 *(0, 0)*	0 *(0, 0)*
7	0 *(0, 0)*	0 *(0, 0)*	0 *(0, 0)*
10	0 *(0, 0)*	0.9 *(0.1, 1.7)*	6.5 *(5.1, 7.8)*
12	0 *(0, 0)*	3.8 *(2.4, 5.3)*	15 *(11, 18)*
14	1.2 *(0, 2.4)*	7.8 *(5.9, 9.8)*	27 *(19, 35)*
17	3.1 *(0, 6.2)*	14 *(11, 18)*	31 *(29, 33)*
19	5.9 *(3.3, 8.6)*	17 *(13, 20)*	33 *(28, 38)*
21	9.7 *(6, 14)*	19 *(15, 24)*	30 *(25, 35)*
24	13 *(9, 17)*	24 *(18, 29)*	28 *(25, 33)*
26	14 *(9, 20)*	28 *(22, 34)*	28 *(25, 30)*
28	19 *(11, 28)*	34 *(30, 38)*	24 *(21, 26)*
31	24 *(13, 34)*	44 *(38, 50)*	22 *(21, 23)*

FIGURES

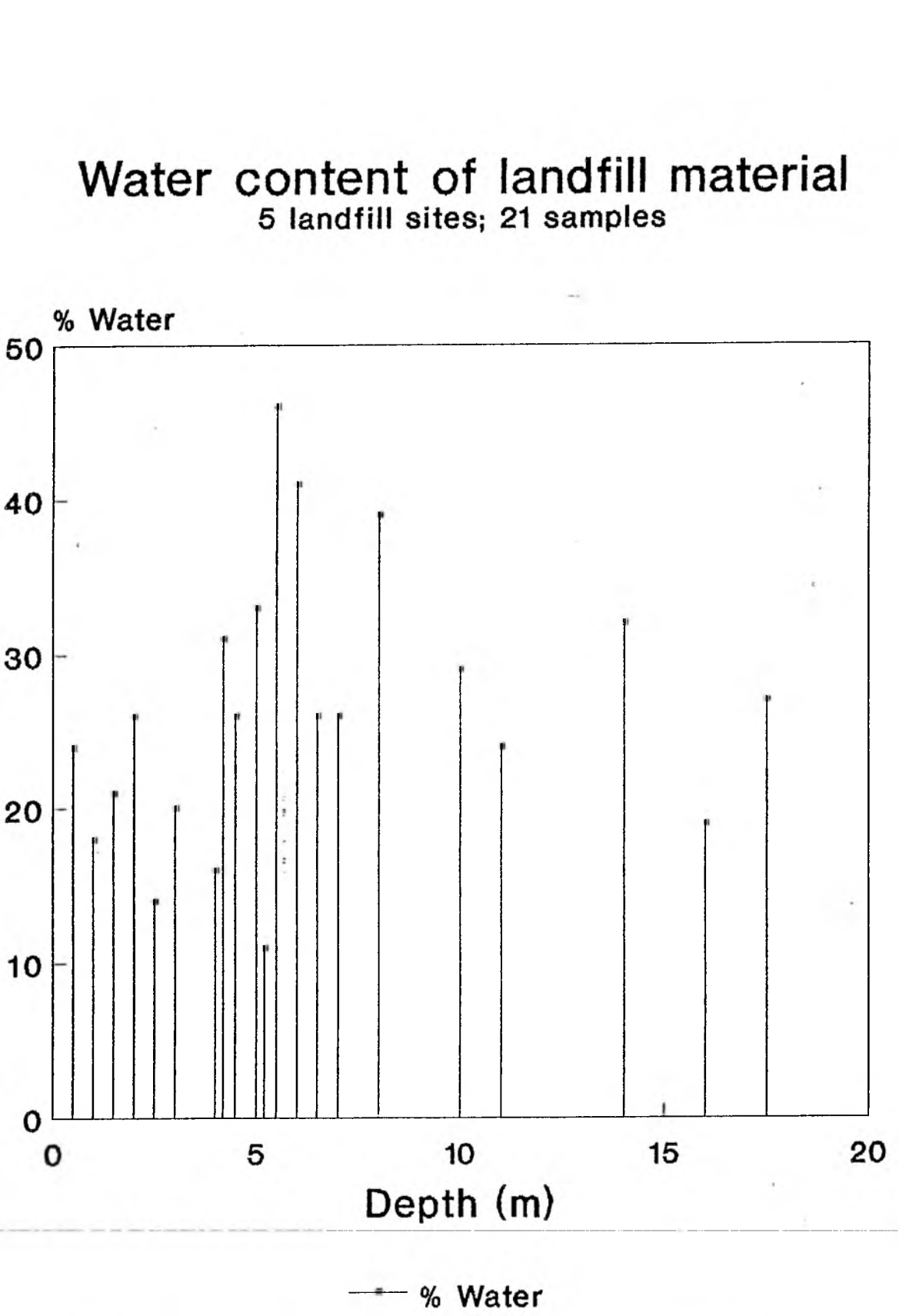


Fig. 1 Evaporative water loss from landfill material

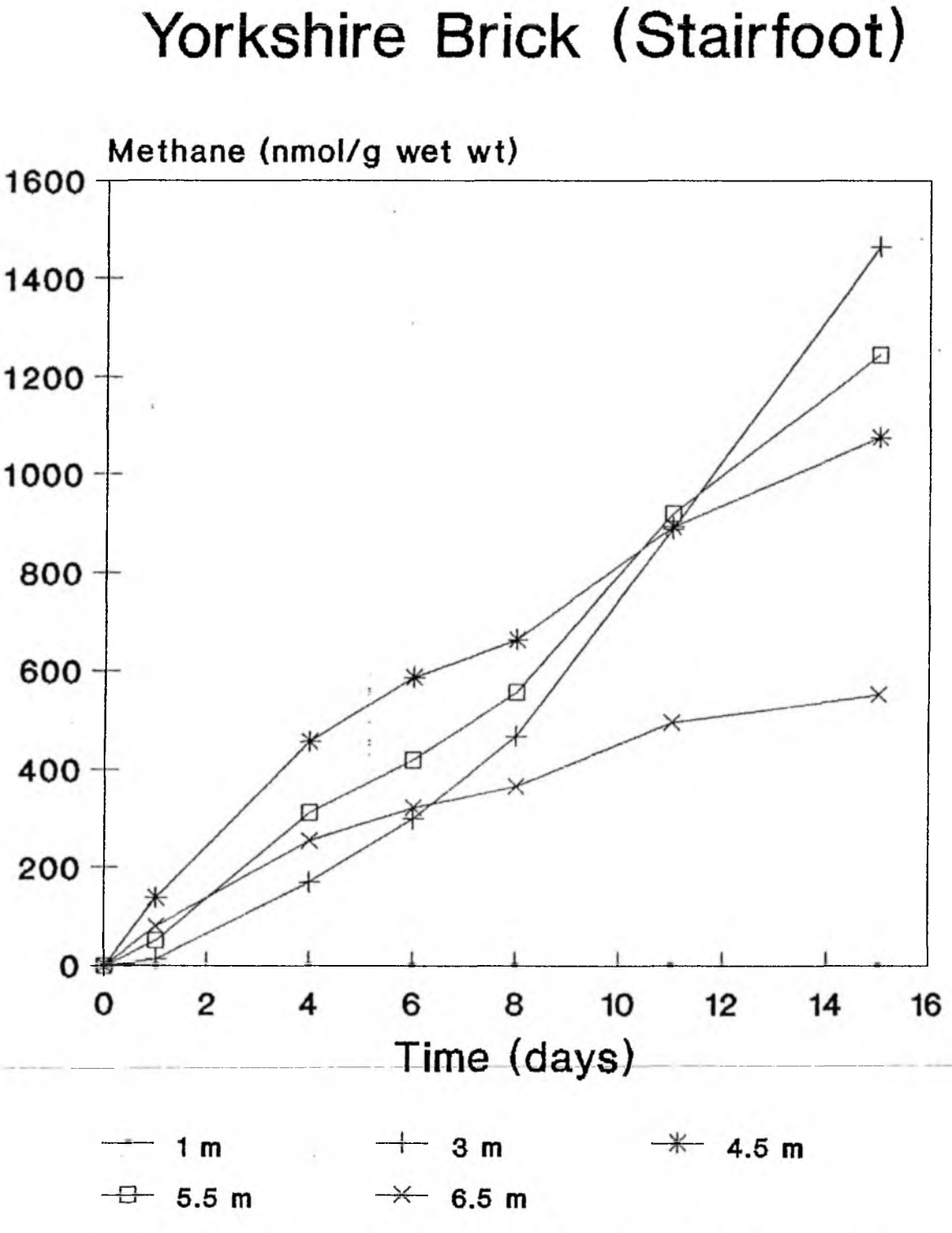


Fig. 2 Cumulative methane production by landfill from five depths in Stairfoot Quarry (no additions)

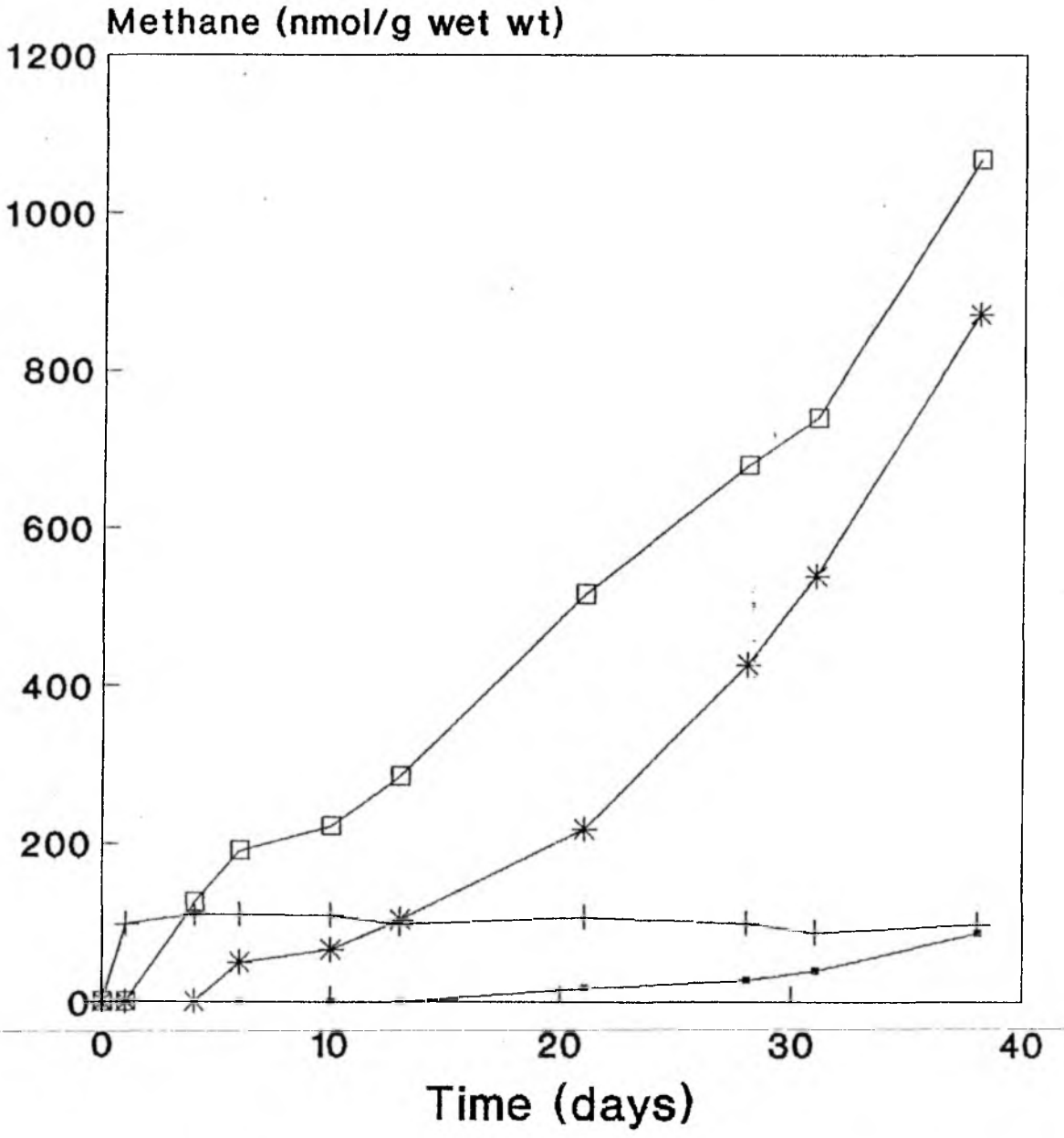


Fig.3 Cumulative methane production by landfill from four depths in Backford Burrow Pit (no additions)

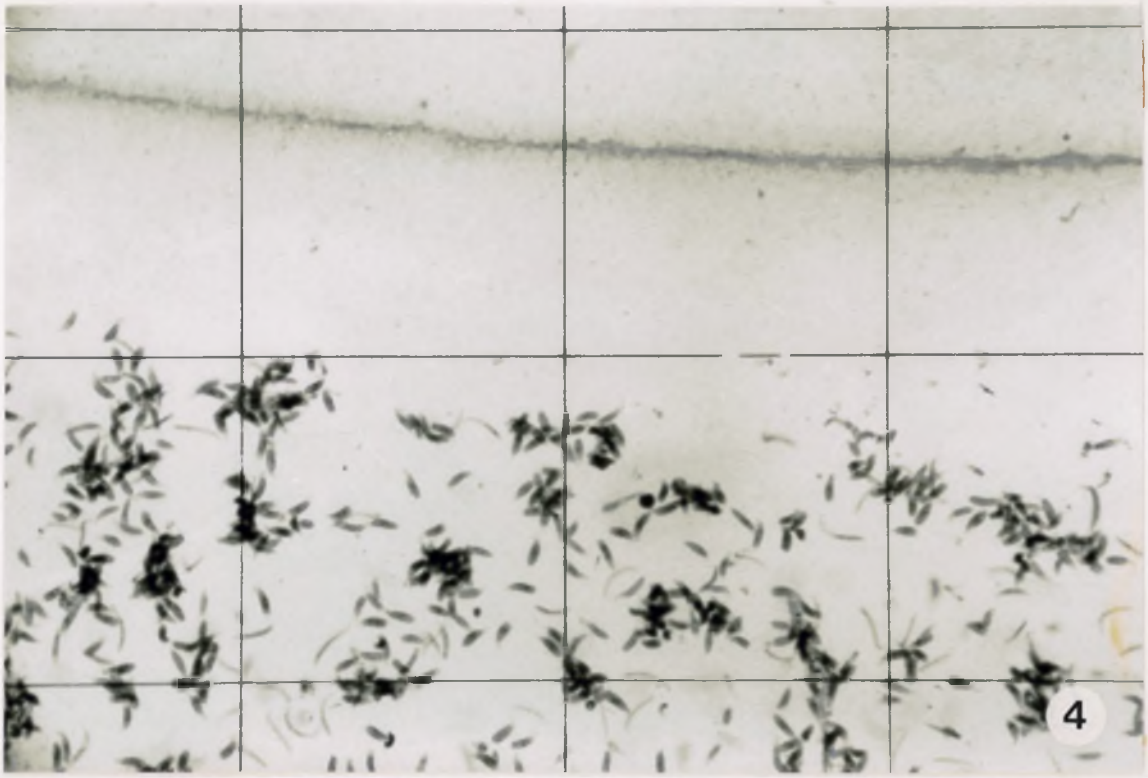


Fig. 4. Culture of *Metopus palaeformis* in a Sedgewick-Rafter Chamber, exposed to air at the top (off the photograph). A band of microaerobic bacteria has developed at some point in the oxygen gradient and, further into the chamber, the ciliates are confined to anaerobic water, totally avoiding residual oxygen lying beneath the bacterial band. The squares have a side of 1mm.

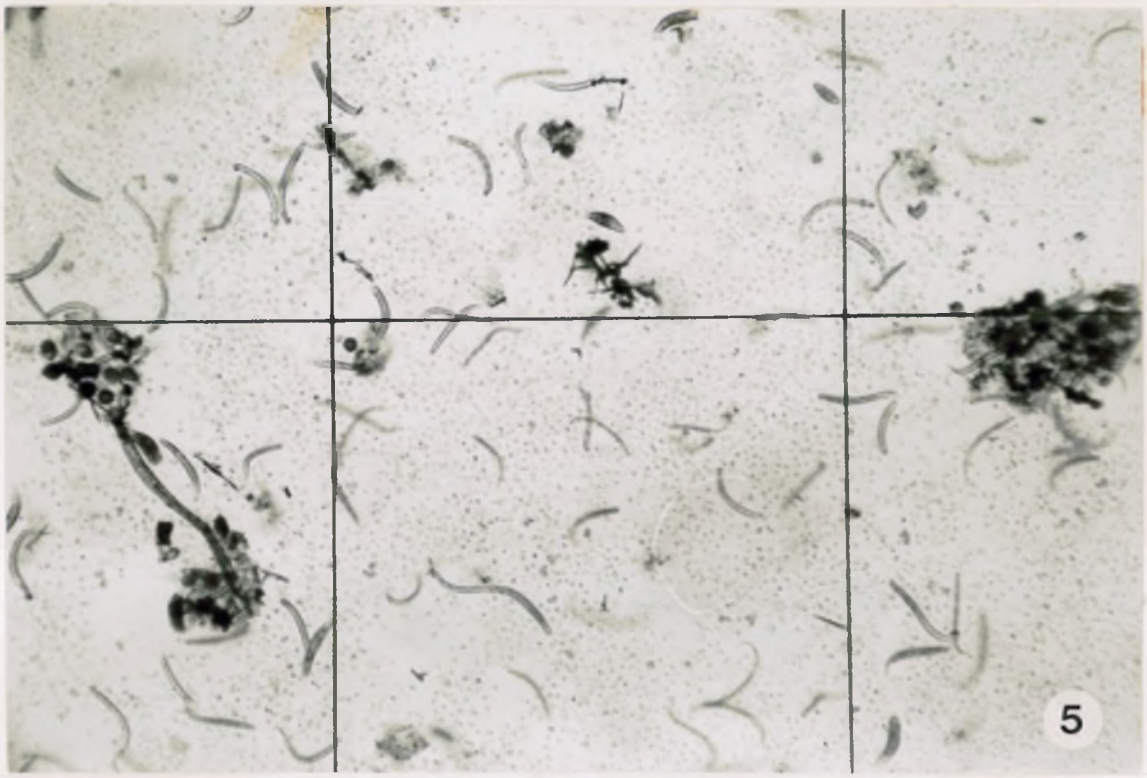


Fig. 5. *Metopus palaeformis* in the middle of the anaerobic zone in a Sedgewick-Rafter Chamber. Many cysts are obvious (eg clump towards the left). Most of the trophs are long and thin and apparently starved; we have no explanation for the scarcity of well-fed cells in this zone.

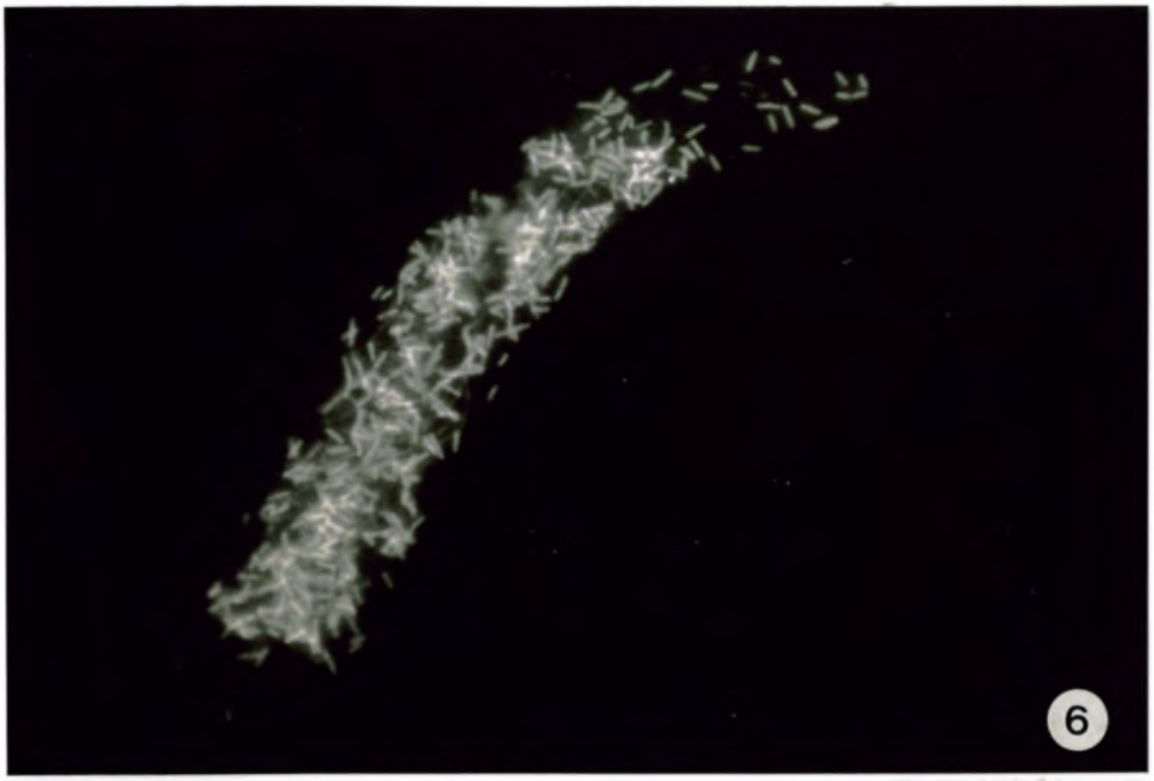


Fig. 6. Normal autofluorescence (UV excitation) of methanogens in anaerobic *Metopus palaeformis* (x750).

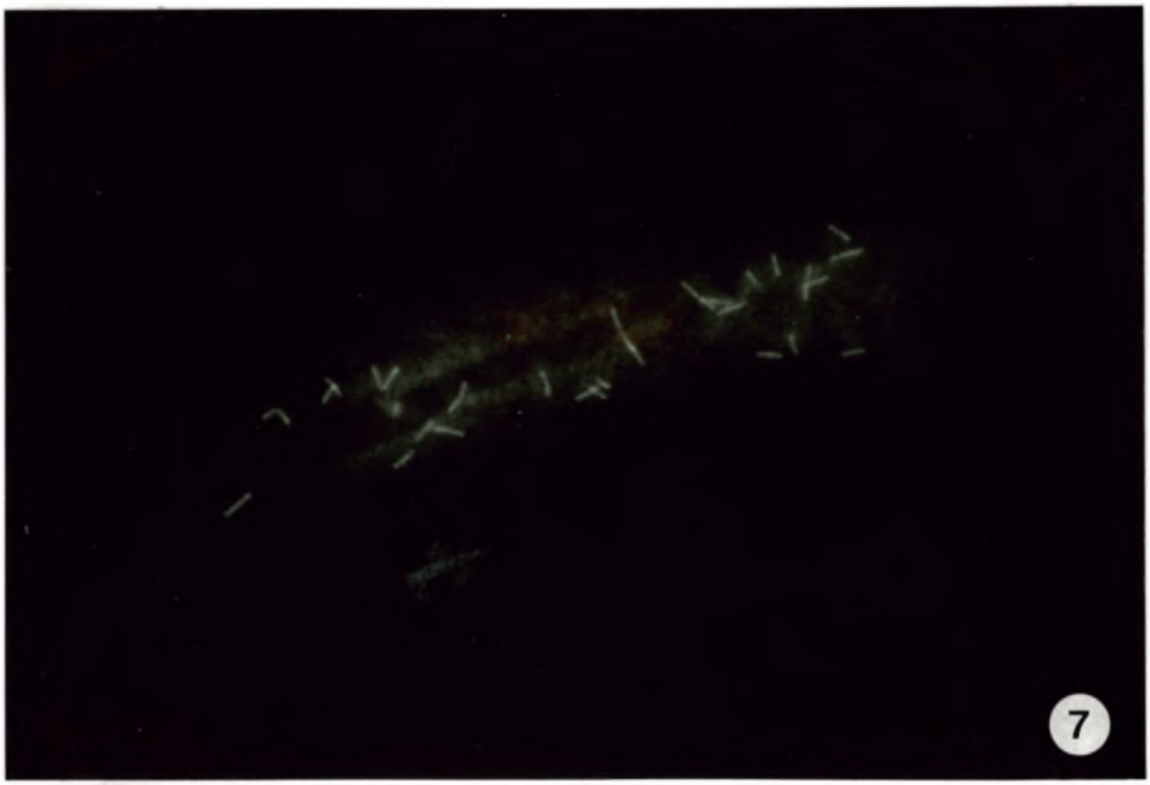


Fig. 7. Autofluorescence of *M. palaeformis* methanogens after 5 days exposure to 10mM BES (x750).

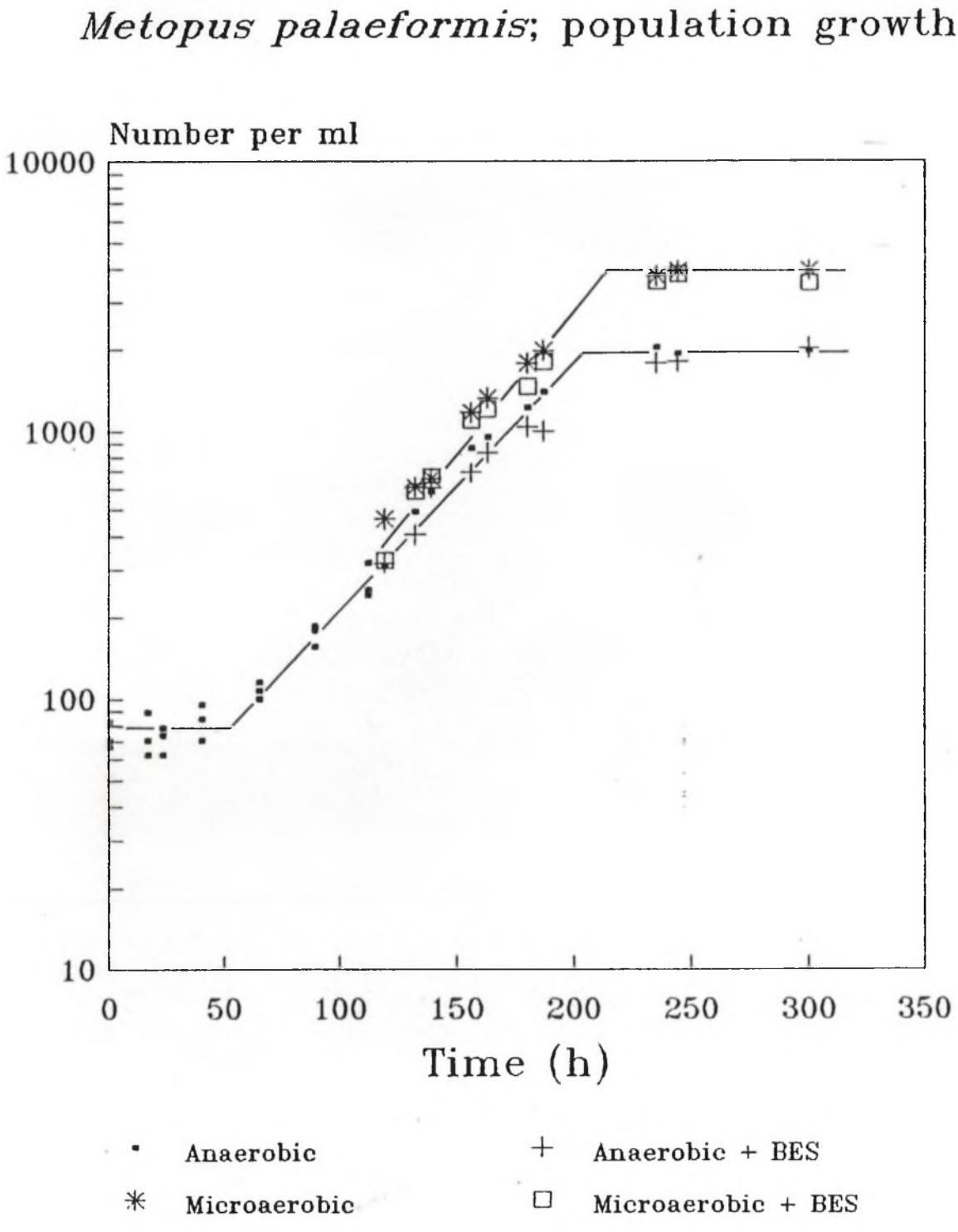
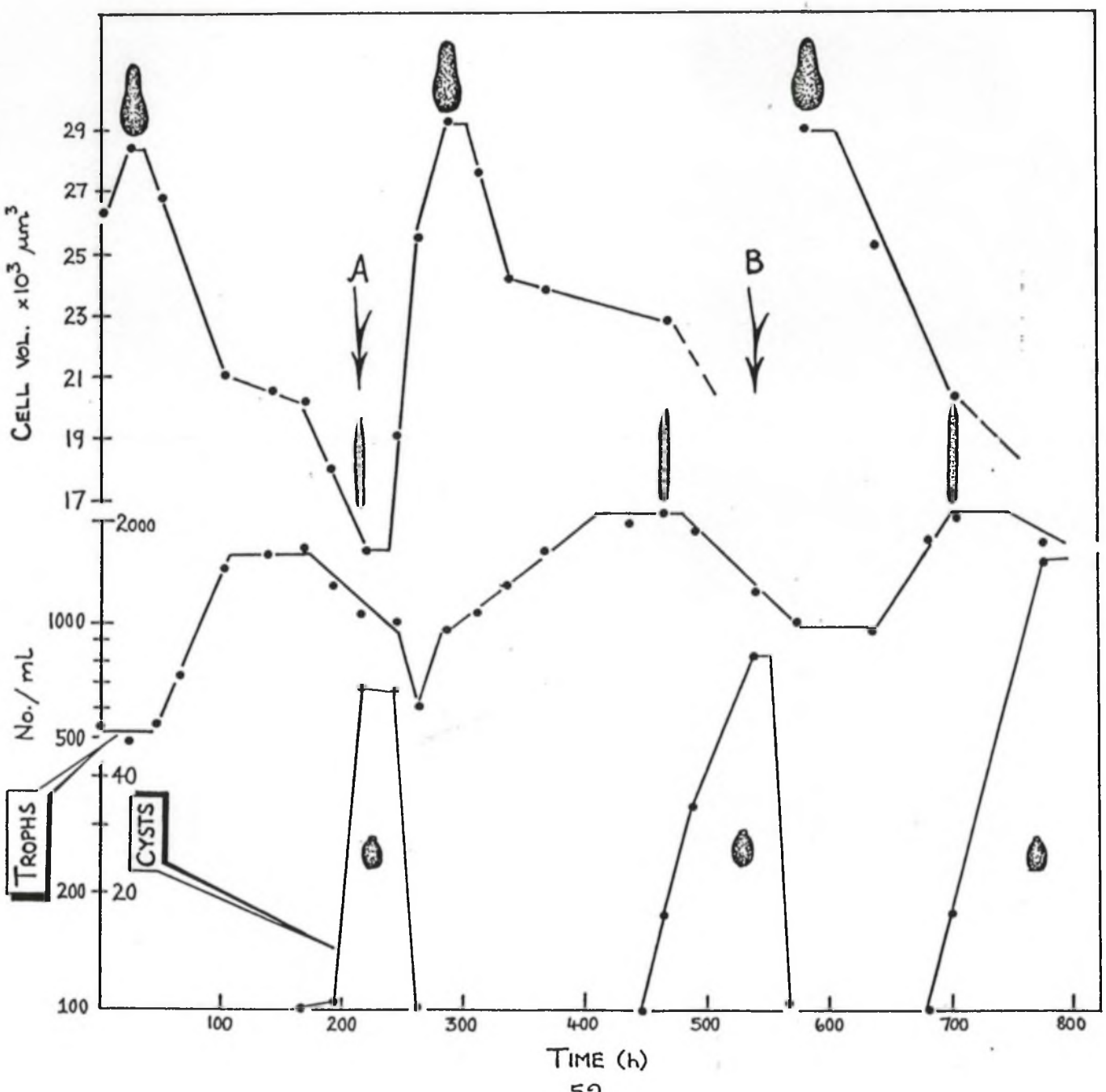


Fig. 8. Population growth of the landfill ciliate *Metopus palaeformis*, with and without access to oxygen, and in the presence and absence of the methanogen inhibitor BES.

FIGURE 9

FIGURE 9: Population growth and polymorphic life cycle of an anaerobic culture (80 ml) of *Metopus palaeformis*.

During the course of population growth, trophs feed and increase in size. As they continue to divide, they become smaller, and eventually long and thin, or they encyst. Introduction of a new bacterial food supply (A & B) stimulates a repetition of the cycle. See text for more details.



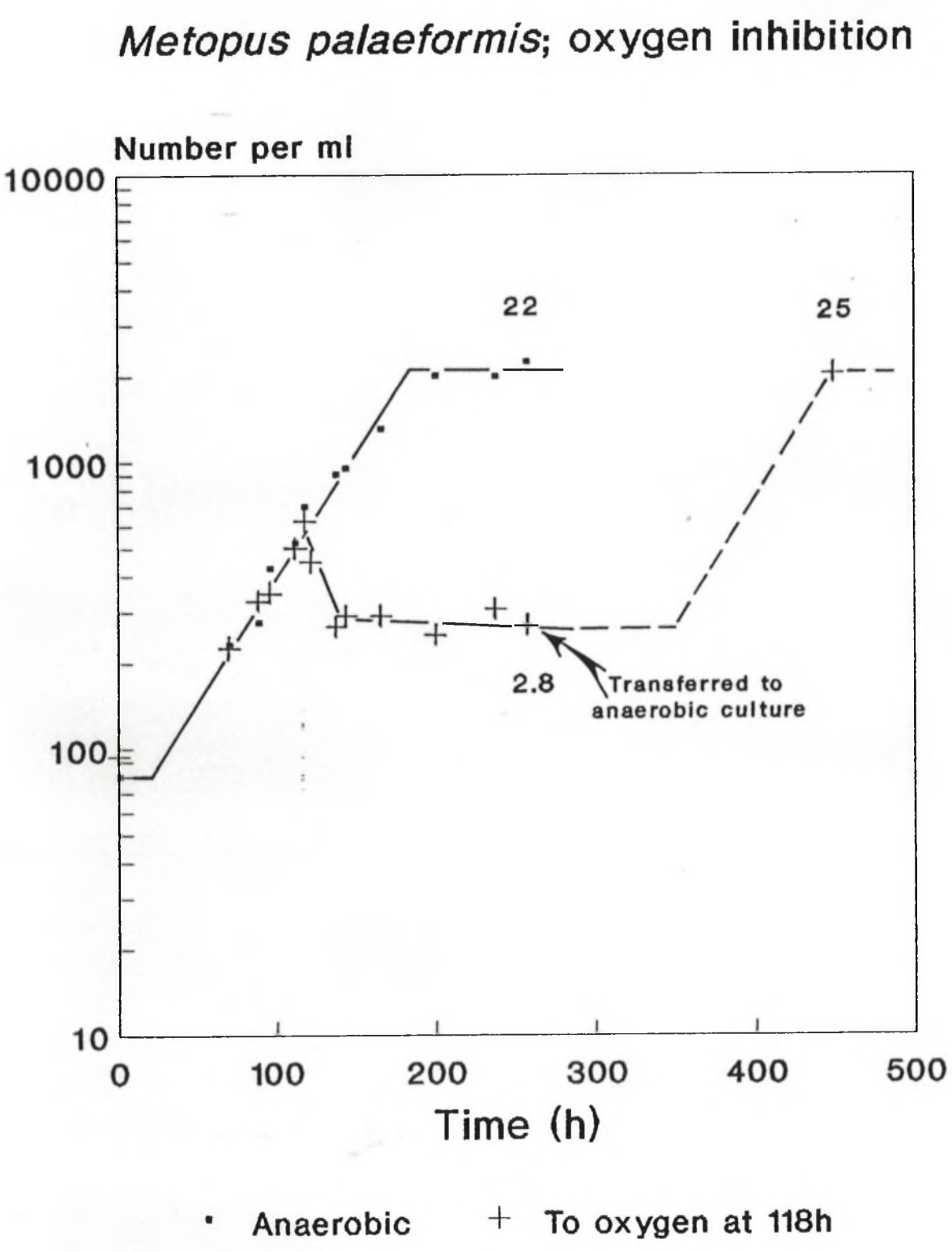
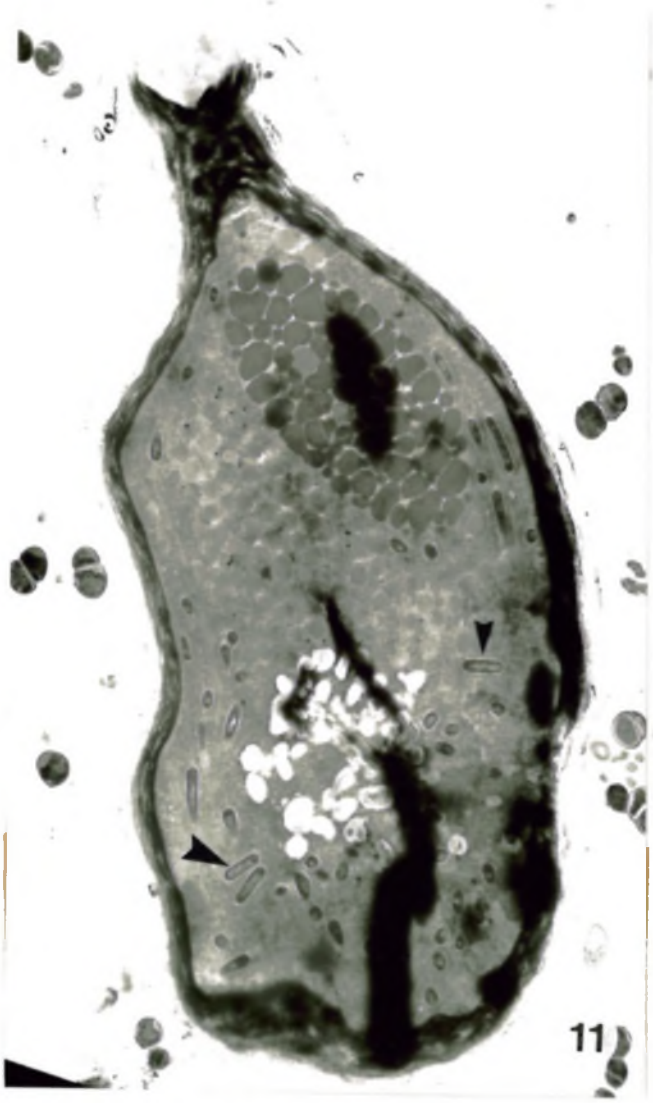


Fig. 10. Oxygen inhibition of population growth in *Metopus palaeformis*. Two anaerobic cultures were established. One was transferred to a Petri dish at 118h (exposure to atmospheric oxygen) then returned to anaerobic culture at 260h. Average numbers of symbiotic methanogens per cell are shown.



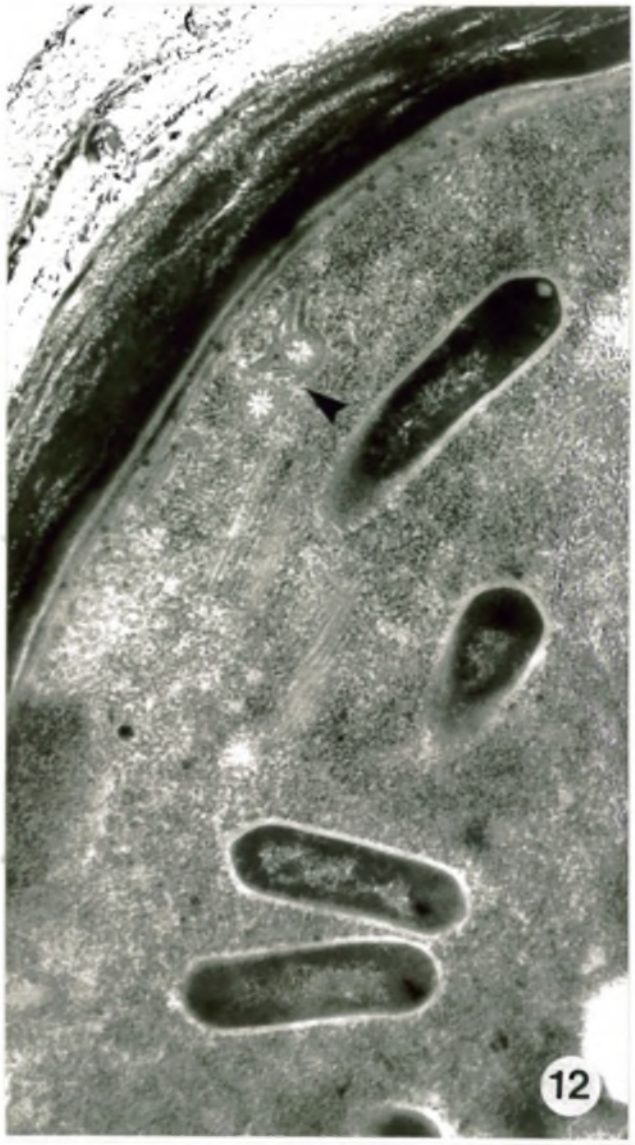
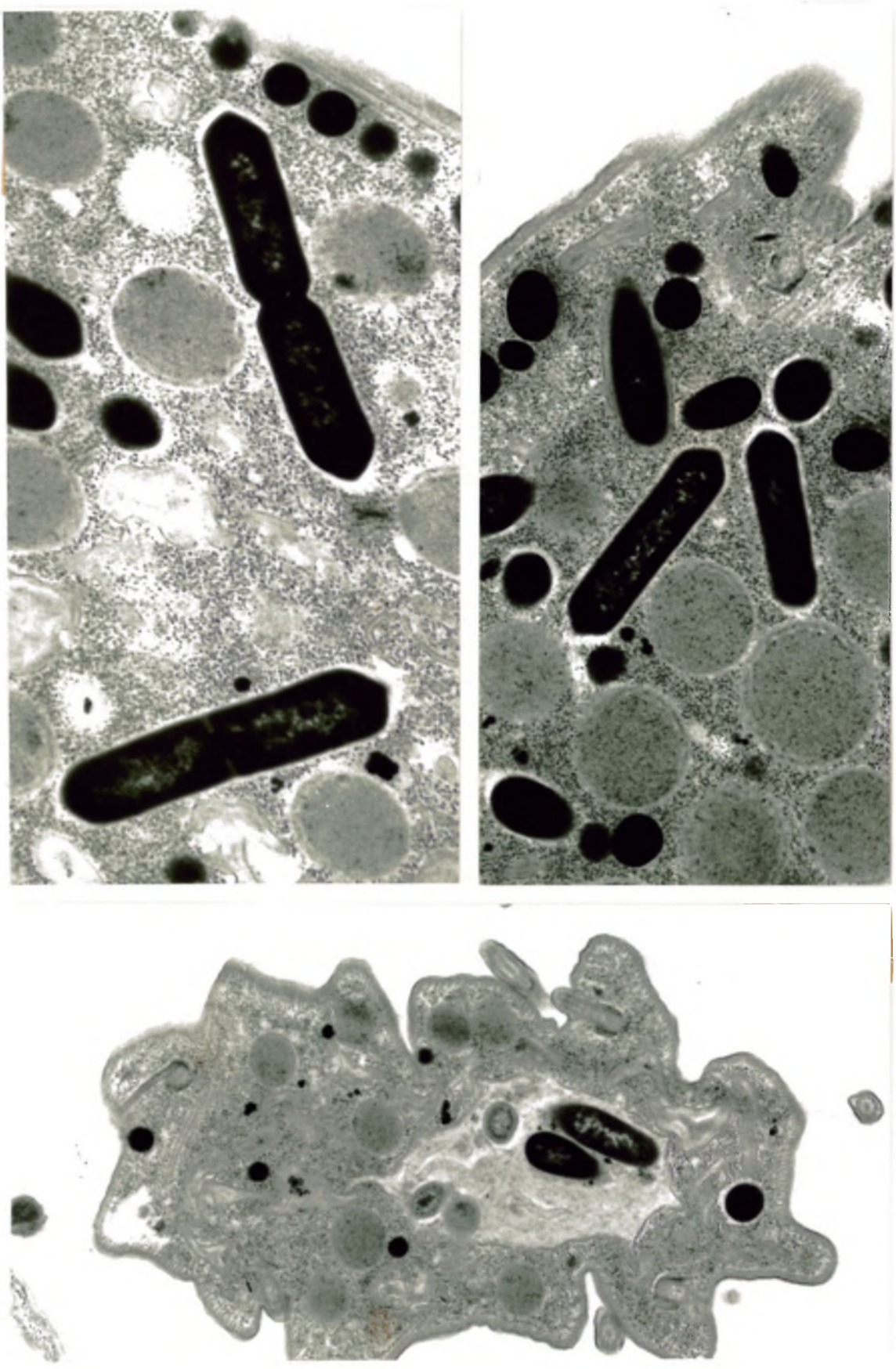


Fig. 11. The cyst of *Metopus palaeformis* after 6 days exposure to atmospheric oxygen. The symbiotic methanogens are clearly visible (arrows). x4,550.

Fig. 12. Part of Fig. 11 magnified to x26,000, showing laminated cyst wall, apparently normal methanogens, and remains of ciliary basal bodies (arrow).

Fig. 13. Sections of *Metopus palaeformis* after 6 days exposure to atmospheric oxygen, showing apparently normal, dividing methanogens (top left; x32,500), normal hydrogenosomes (top right; x26,000), and digestion of methanogens (bottom; x16600).



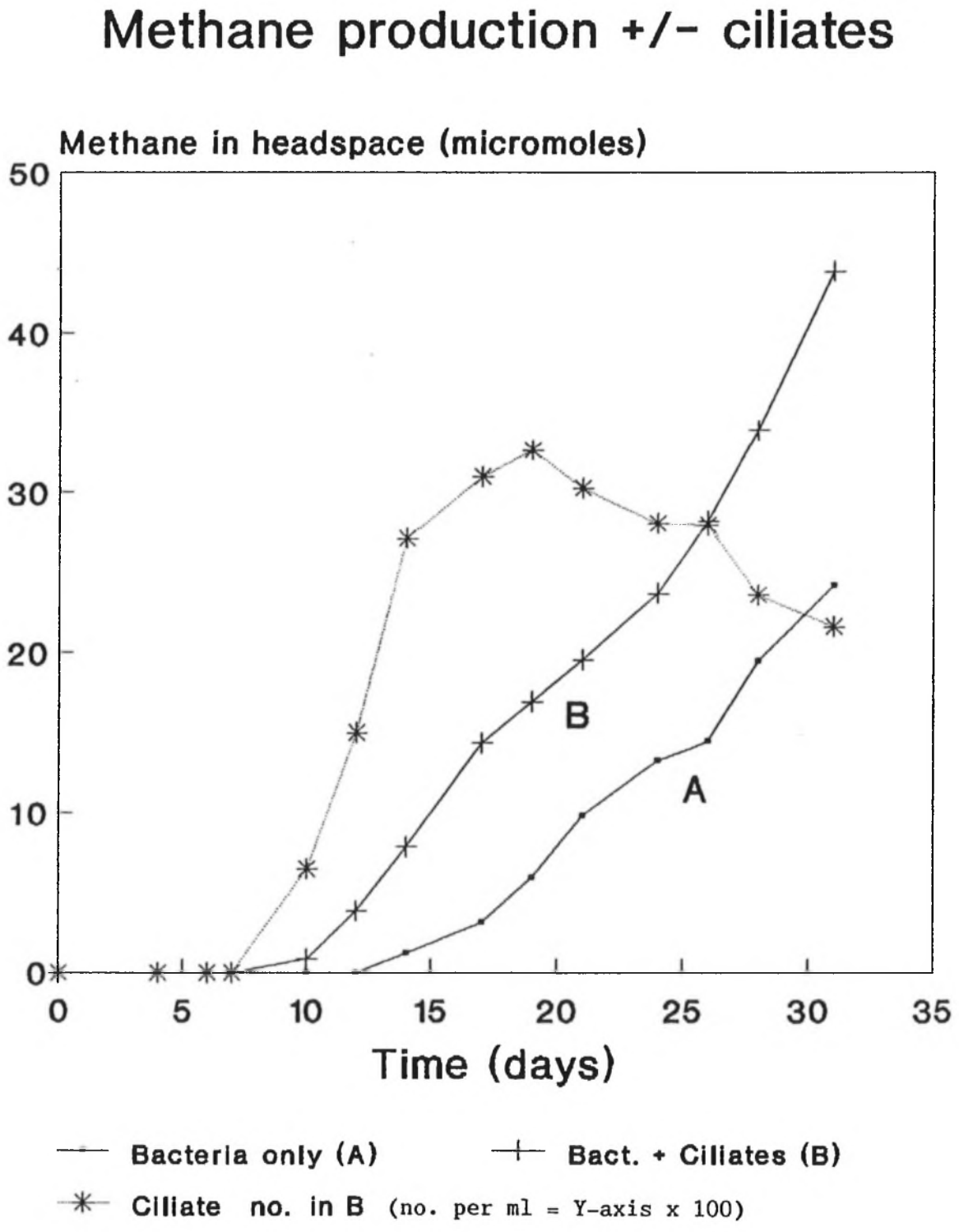


FIG. 14. Methane production in the presence and absence of *Metopus palaeformis* (means of duplicate cultures; see Table 8). Ciliates were separated from the 'Bacteria only' cultures by gentle centrifugation (200 x *g*, 20 sec).

FIGURE LEGENDS. All Figures are of *Metopus palaeformis* (Slackhead)

FIGURE 1a. Normal specimen, living; Nomarski. x500.

FIGURE 2a Autofluorescing methanogens in normal specimen. UV excitation; x500.

FIGURE 3a. Starving cell, living; Nomarski. x500.

FIGURE 4a. Autofluorescing methanogens in starving cell. UV excitation; x500.

FIGURE 5a. Living cysts of the ciliate; Nomarski. x500.

FIGURE 6a. Autofluorescing methanogens within cysts of the ciliate. UV excitation; x500.

FIGURES 7a and 8a. Variation in the ciliate nuclei. DAPI-stained; UV excitation; x1000.

FIGURE 9a. Hydrogenase activity in hydrogenosomes, visualised with reduced NBT.

FIGURE 10a. Whole cells; mouth and feeding organelles in central specimen. SEM x2750.

FIGURE 11a. Section close to anterior end, in region of mouth, showing hydrogenosomes (H) and methanogens (M). TEM x13400.

FIGURE 12a. Transverse section, showing mixed microbial flora in food vacuoles (FV). TEM x5550.

Figure 13a. Symbiotic methanogen (M) and hydrogenosomes (H). TEM x62300.

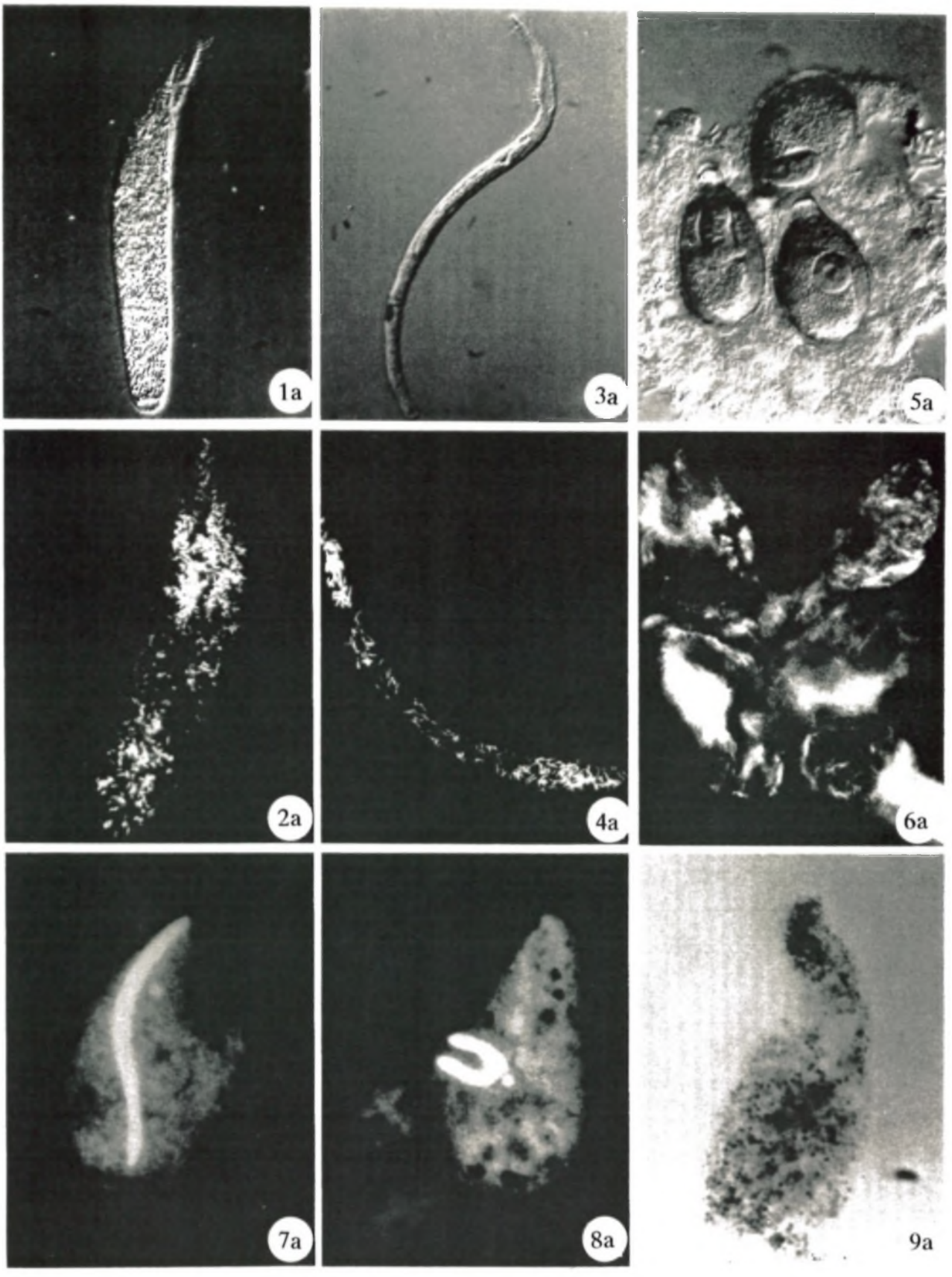
Figure 14a. Food vacuole (FV) and an elongate example of the symbiotic methanogen (M), close to binary fission. TEM x12750.

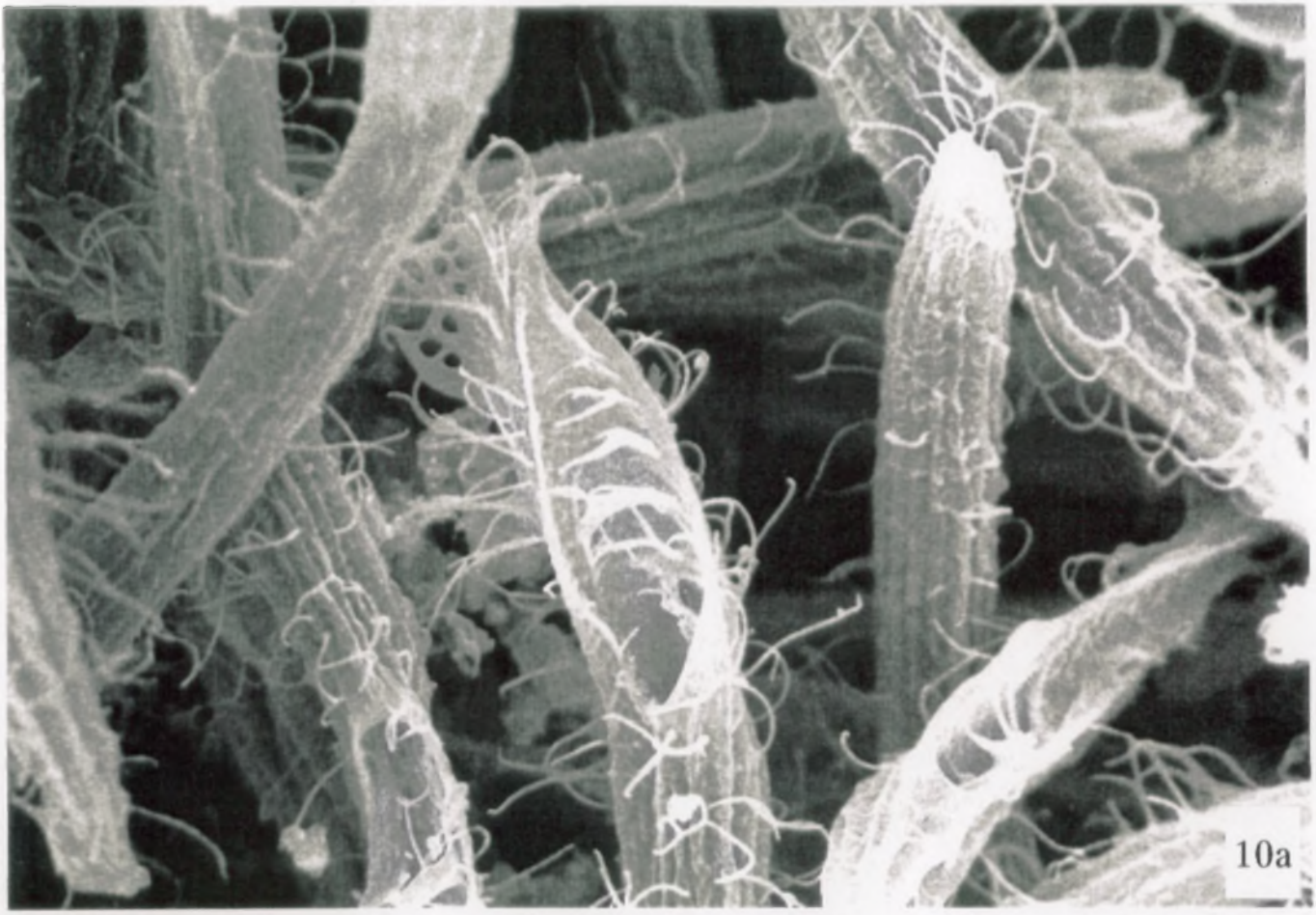
Figure 15a. The methanogen (M) from Fig. 14 and associated hydrogenosomes (H). TEM x18700.

Figure 16a. Symbiotic methanogen in a digestive vacuole. TEM x41500.

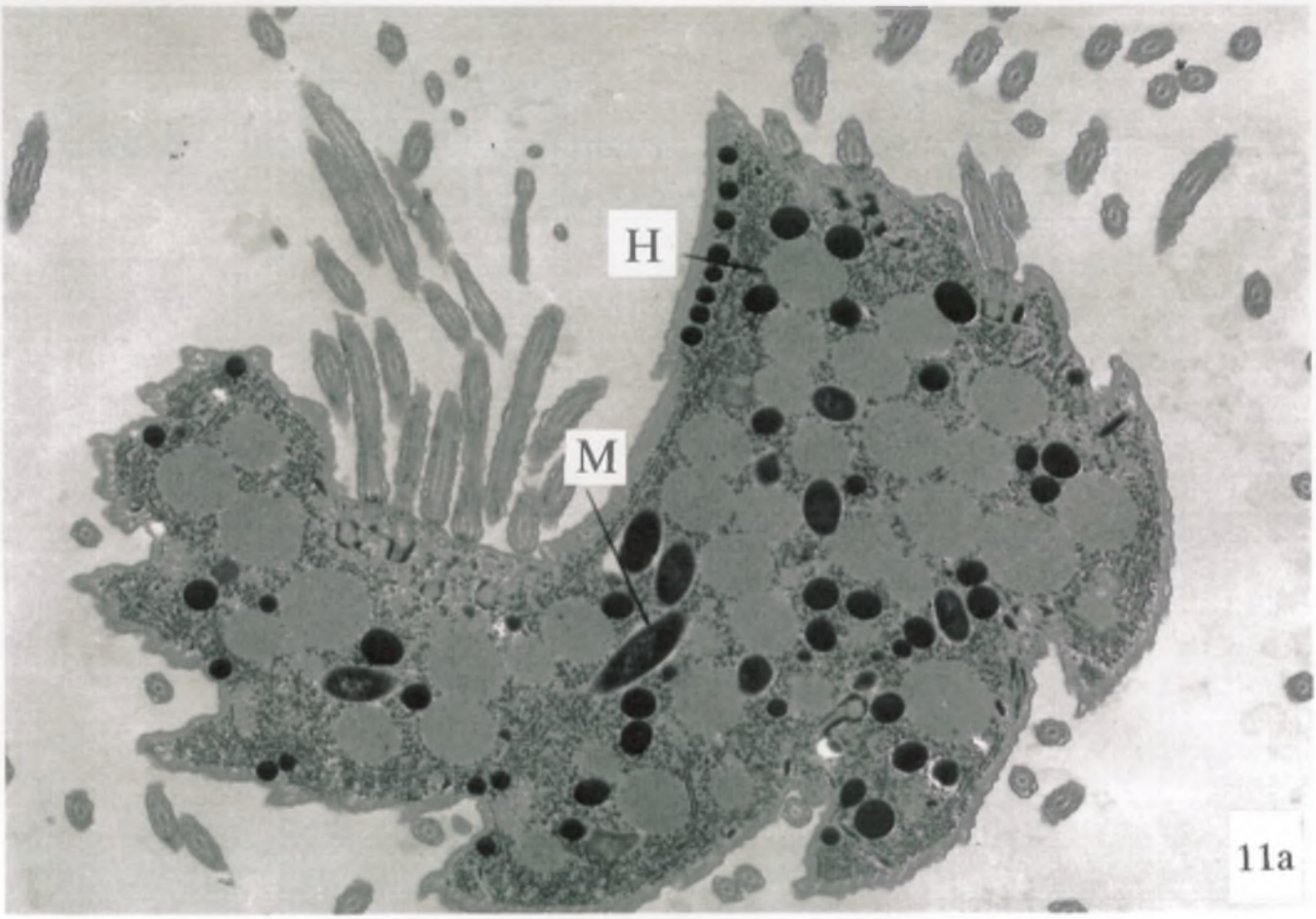
Figure 17a. Permanganate fixation showing internal membranes in symbiotic methanogens (M). TEM x31550.

Figure 18a. Symbiotic methanogens (M) - some in the process of digestion within a food vacuole (FV). Ma, macronucleus; Mi, micronucleus. TEM x15500.

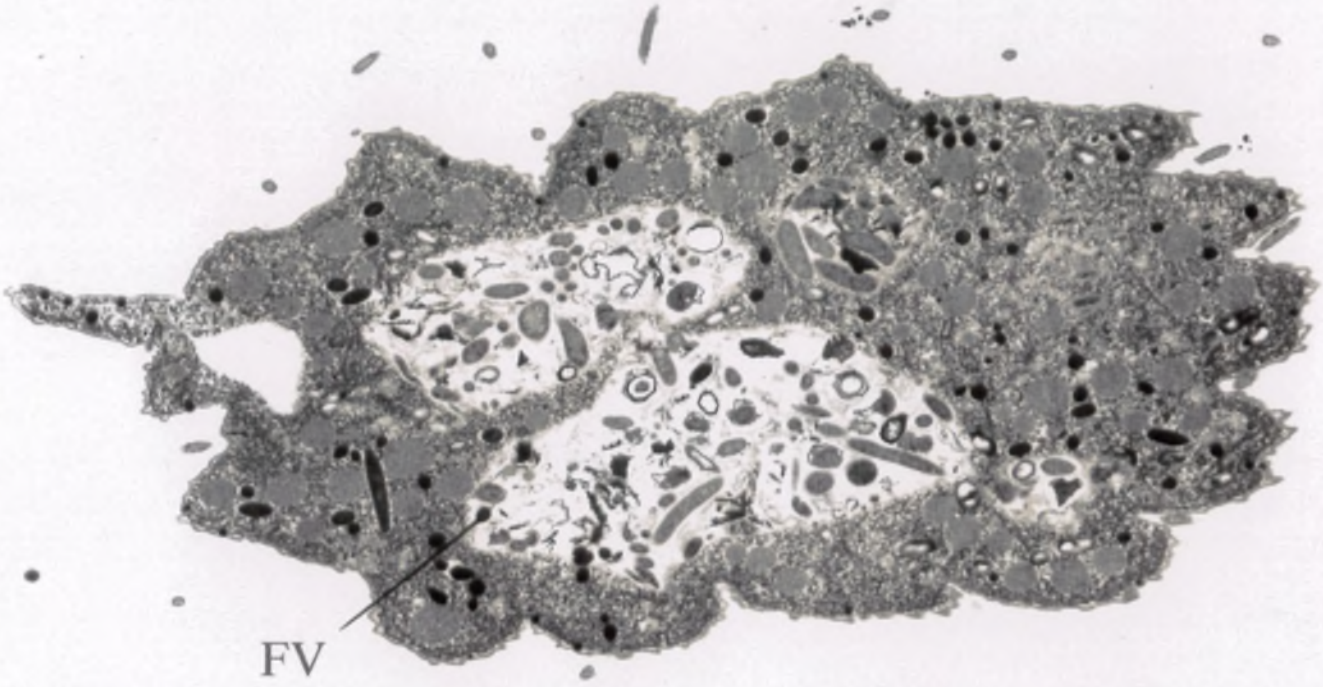

1a
3a
5a
2a
4a
6a
7a
8a
9a



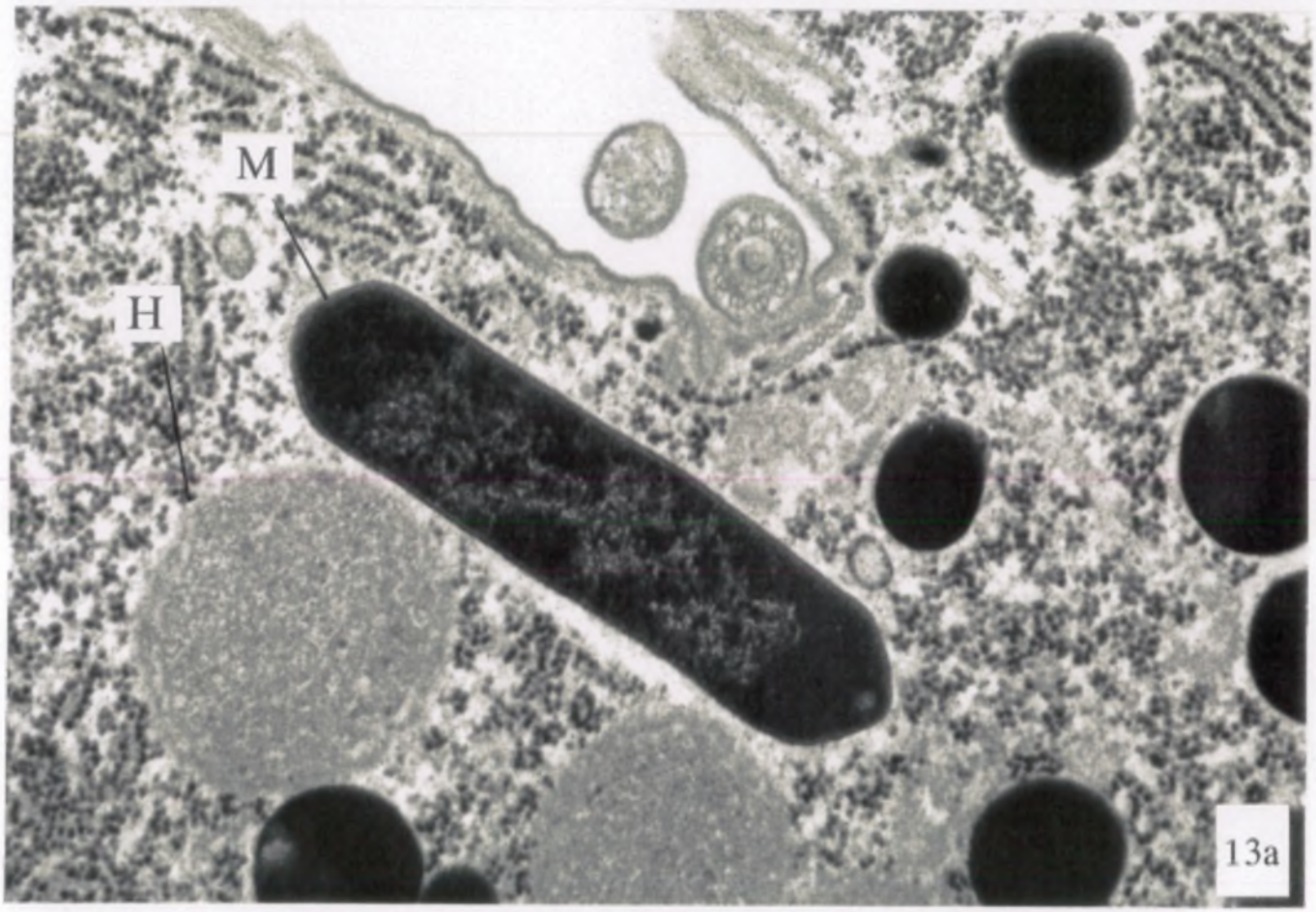
10a

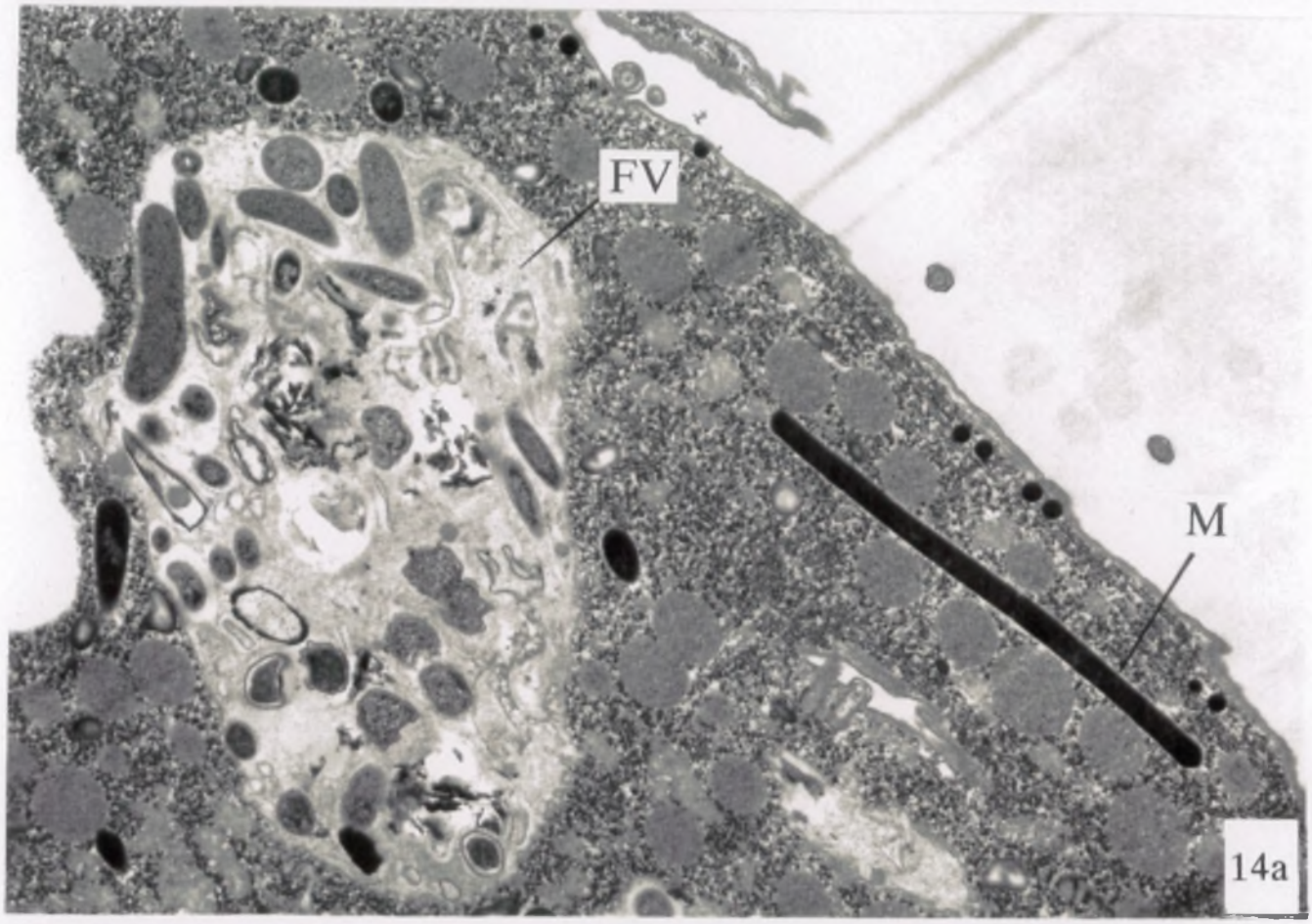


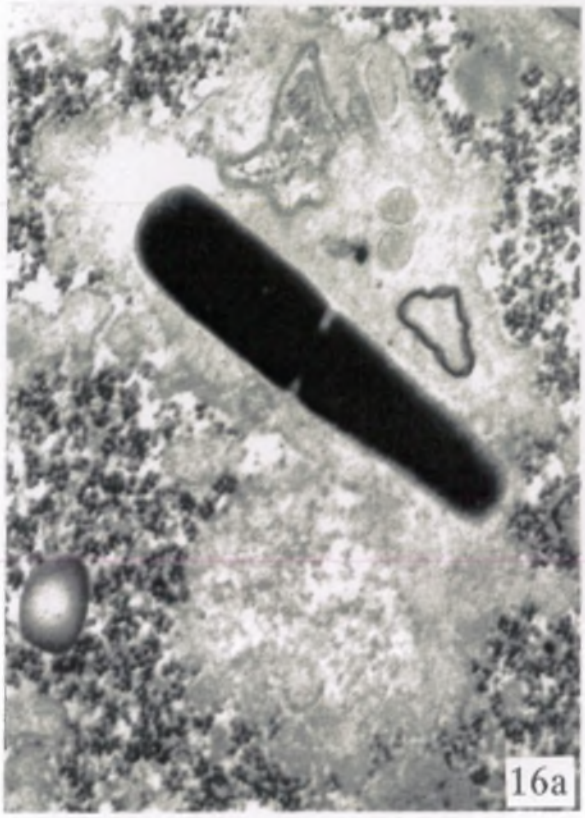
11a

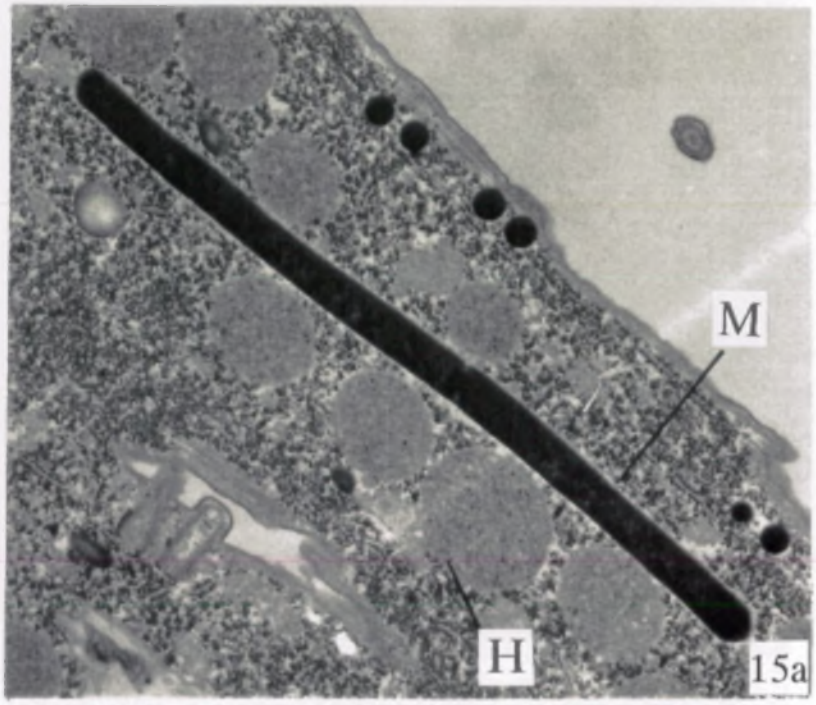


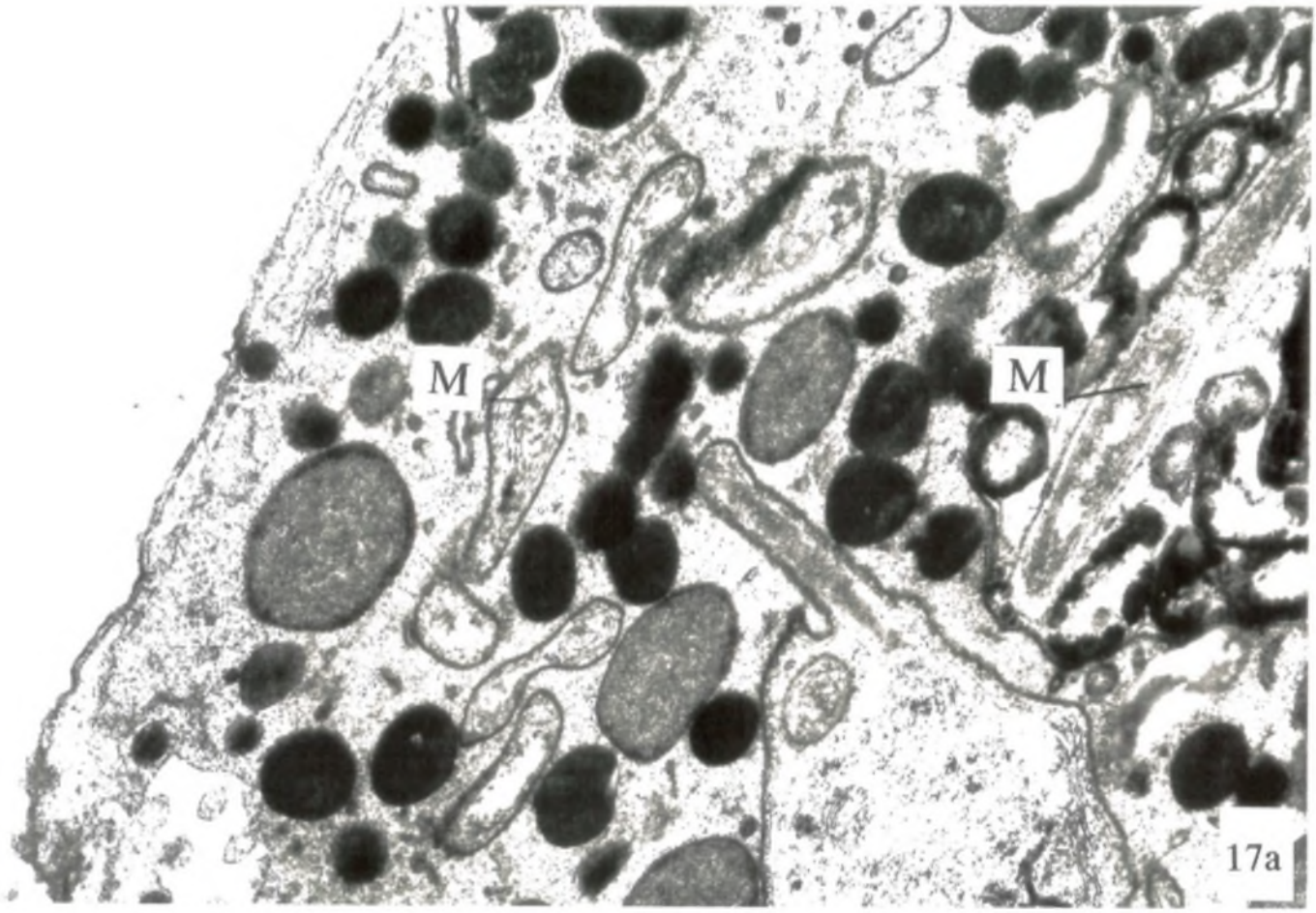
12a

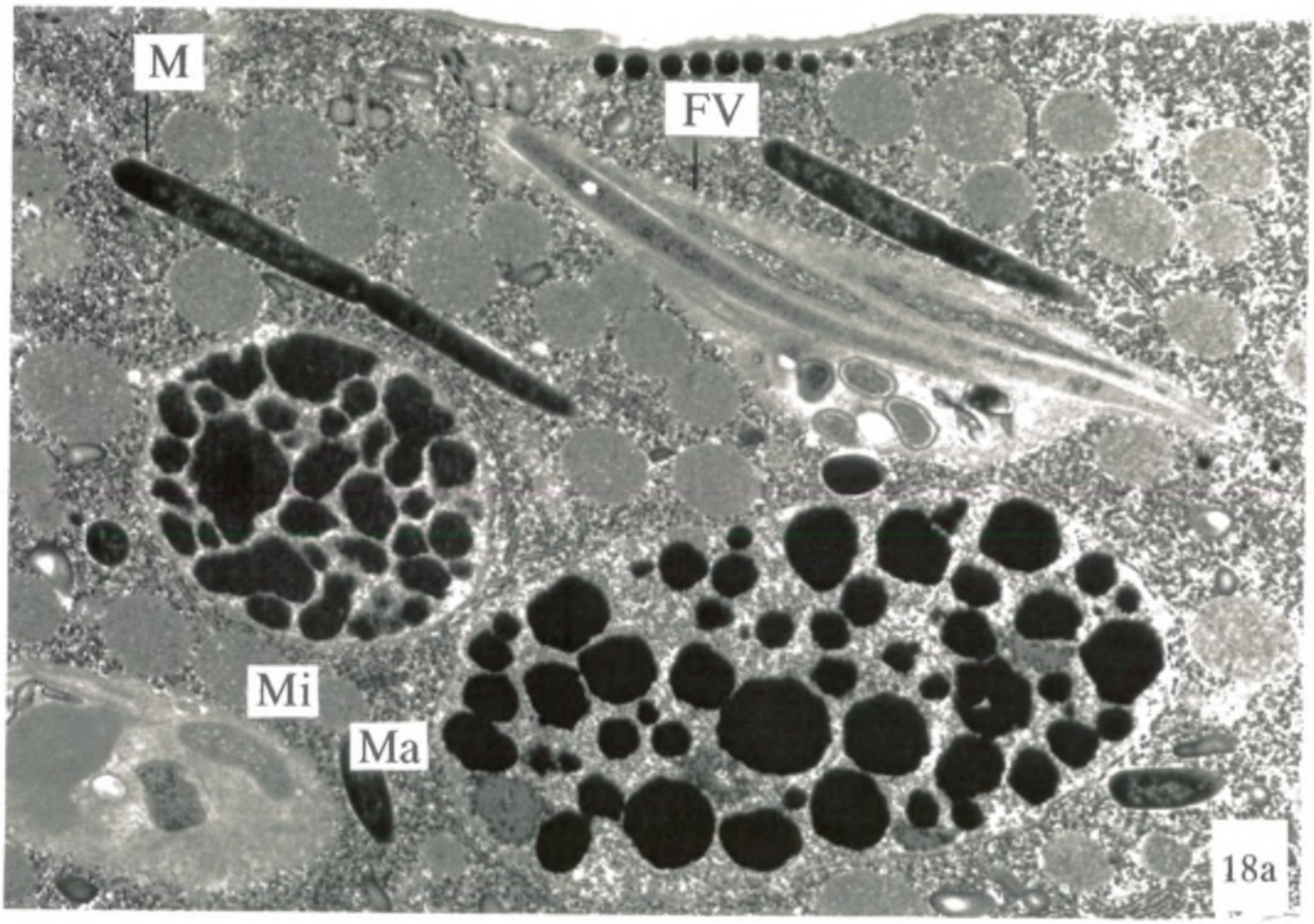


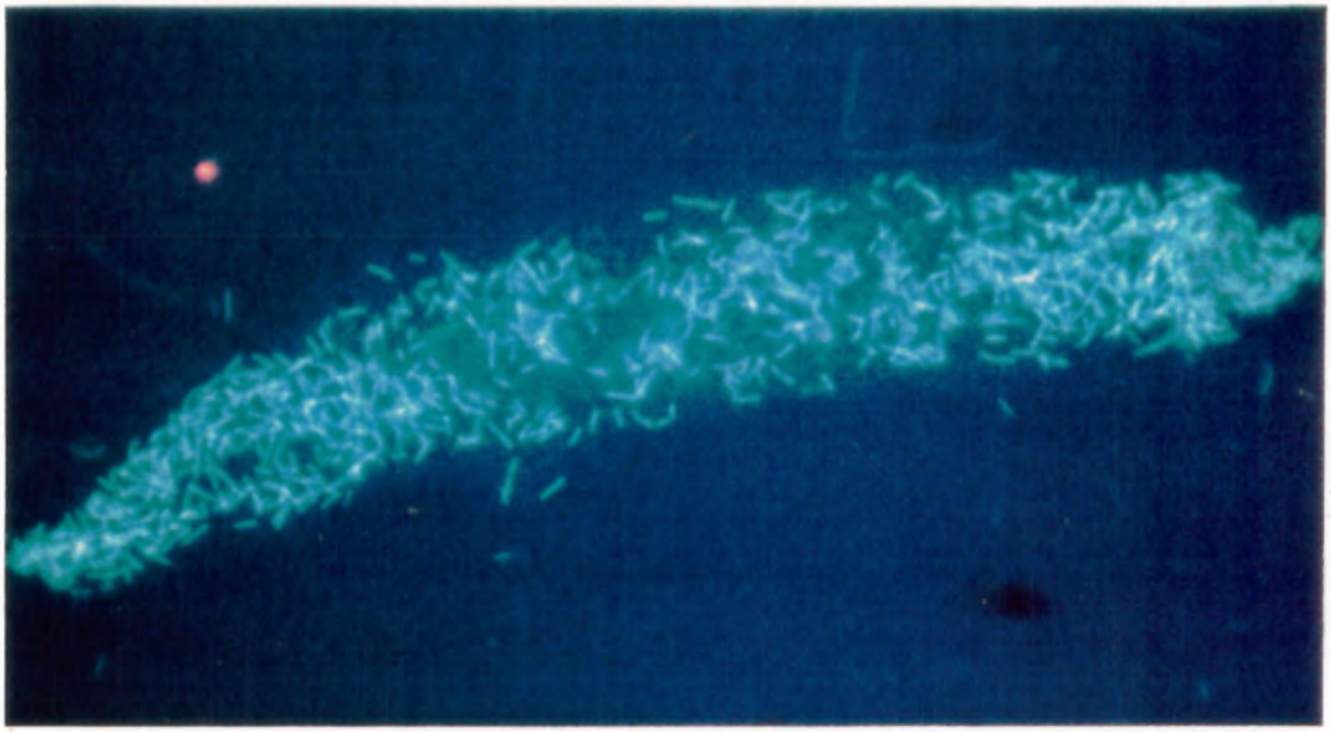

FV
M
14a


16a

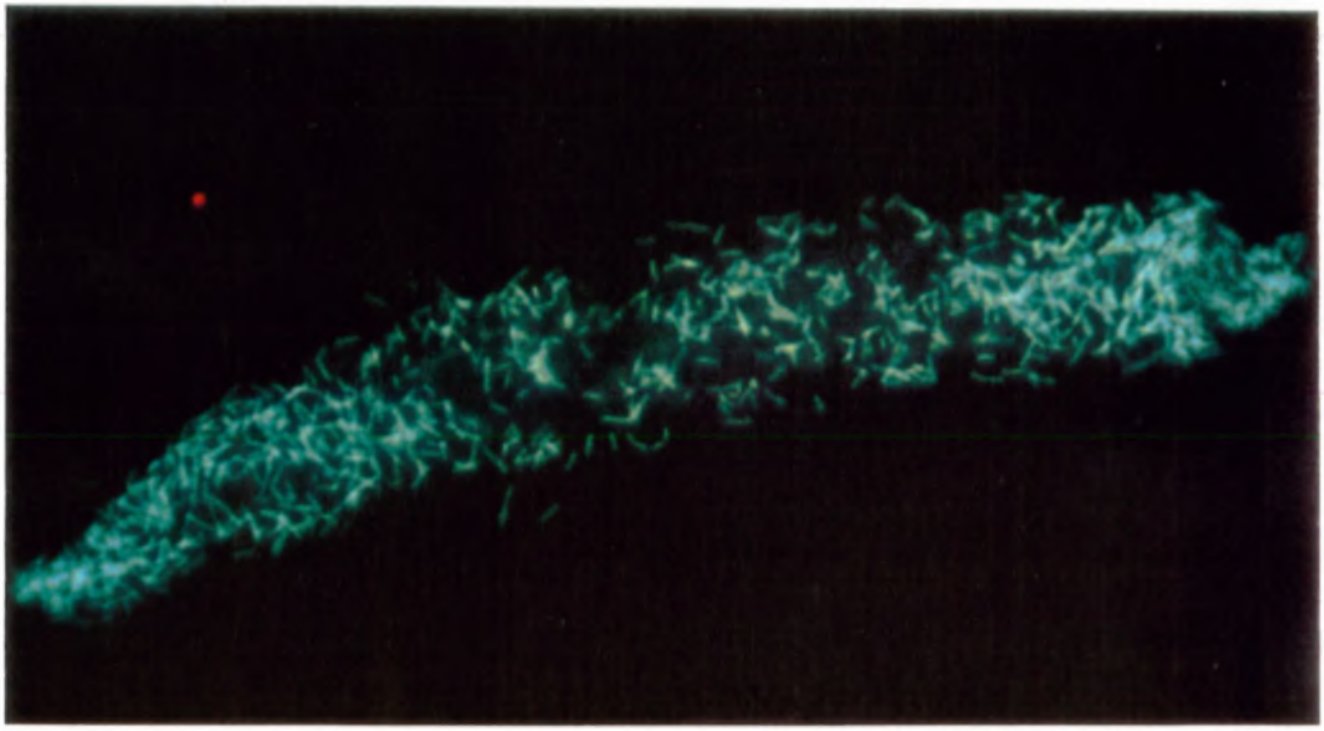

M
H
15a


M
M
17a

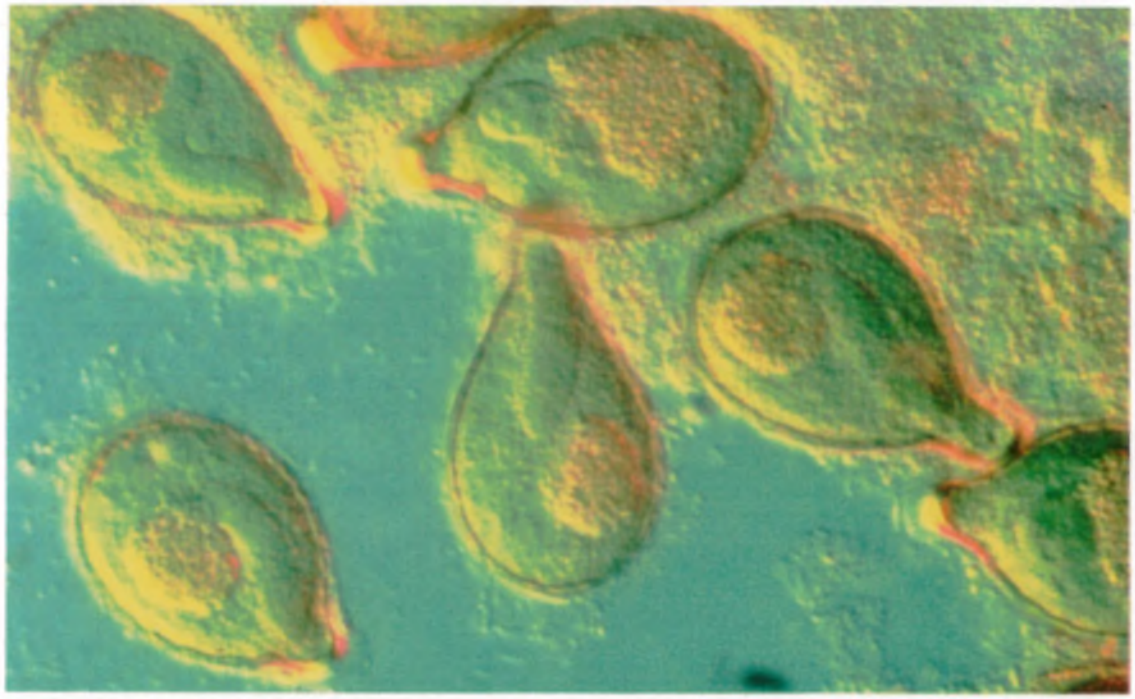

M
FV
Mi
Ma
18a



Metopus palaeformis (Slackhead) Violet excitation x1200

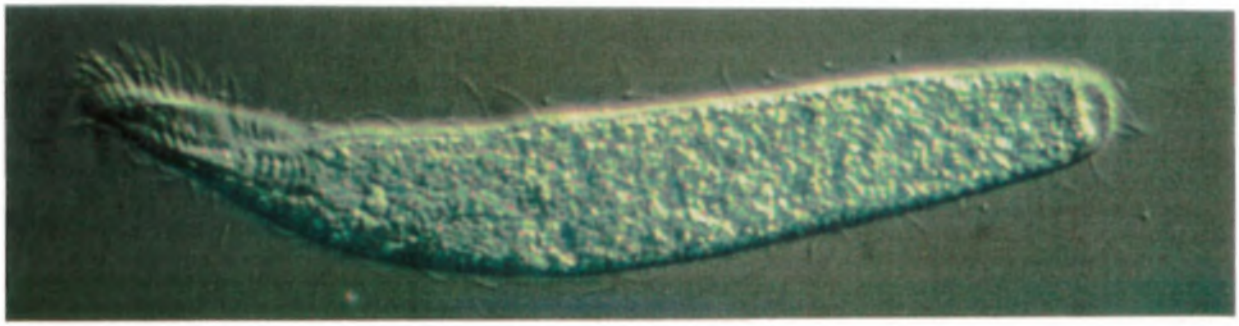


Metopus palaeformis (Slackhead) Ultraviolet excitation x1200

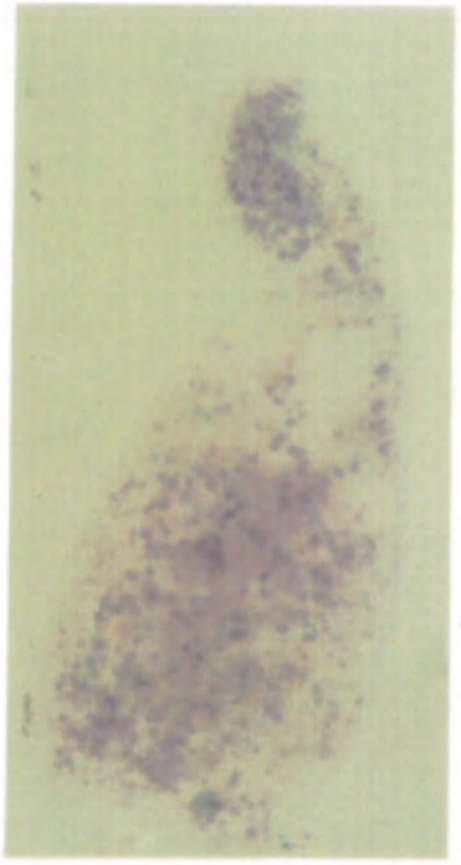


Metopus palaeformis (Slackhead) Cysts x900

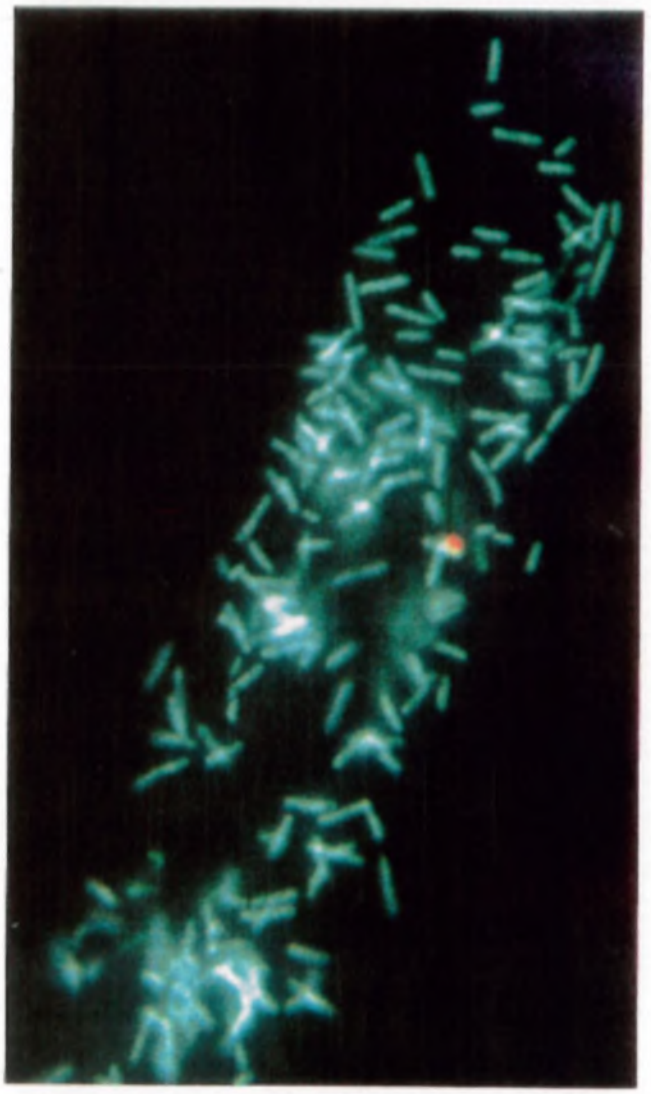
Metopus palaeformis (Slackhead) Starving cell x900



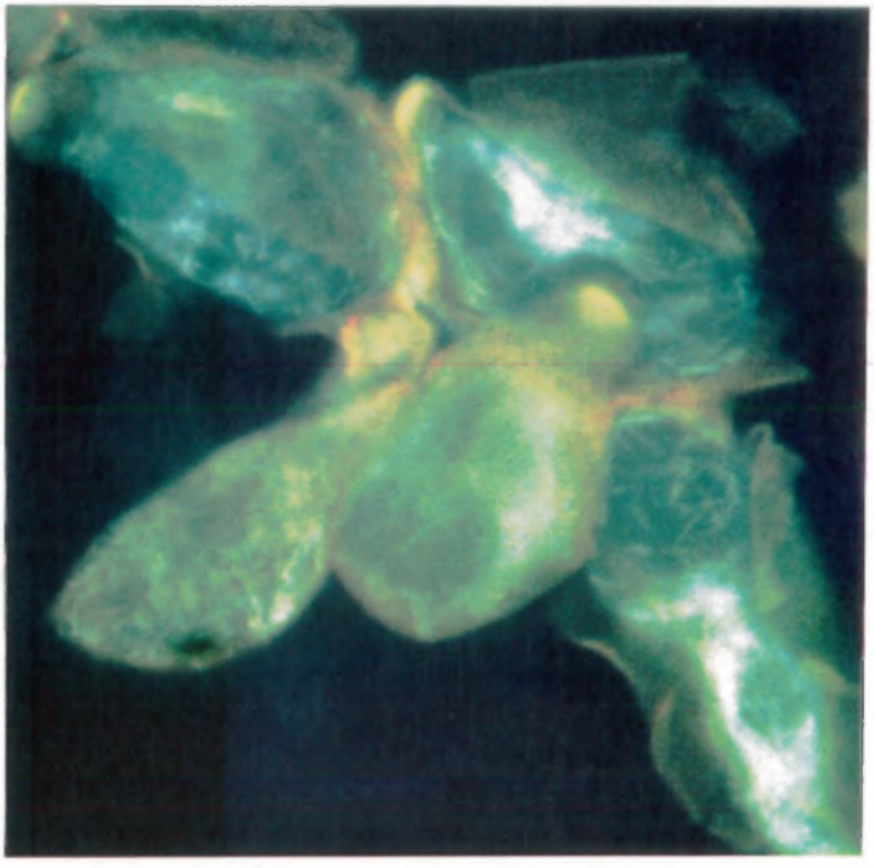
Metopus palaeformis (Slackhead) Normal cell x900



Metopus palaeformis (Slackhead)
Cytochemical staining for
x1200 hydrogenase



Metopus palaeformis (Slackhead) x2250
Fluorescing endosymbionts Ultraviolet excitation



Metopus palaeformis (Slackhead)
Cysts Violet excitation
x1200

APPENDICES

Appendices

TEM fixation procedure for landfill *Metopus*

1. Simultaneous fix with cold 3% glutaraldehyde and cold 1% osmium tetroxide.

2. The glutaraldehyde is prepared from 70% supplied in a sealed vial.

   The glutaraldehyde and the OsO_4 are diluted using a phosphate buffer with supplements:

   a) Phosphate buffer 100mM, pH 7
   b) Sucrose 1mM .....68 mg in 200 ml buffer
   c) Magnesium sulphate 0.01mM .....trace

3. Open vial of 70% Glutaraldehyde

   Add 1 ml to 15.5 ml supplemented buffer (= 4.5% Glut.)

4. Dissolve 0.2 mg OsO_4 in 10 ml supplemented buffer (= 2%)

5. Spin down living cells to 0.5 ml.

6. Add 1 ml cold 4.5% Glutaraldehyde, immediately followed by 1.5 ml cold 2% OsO_4. This gives final fixative concentrations of 3% Glutaraldehyde and 1% OsO_4.

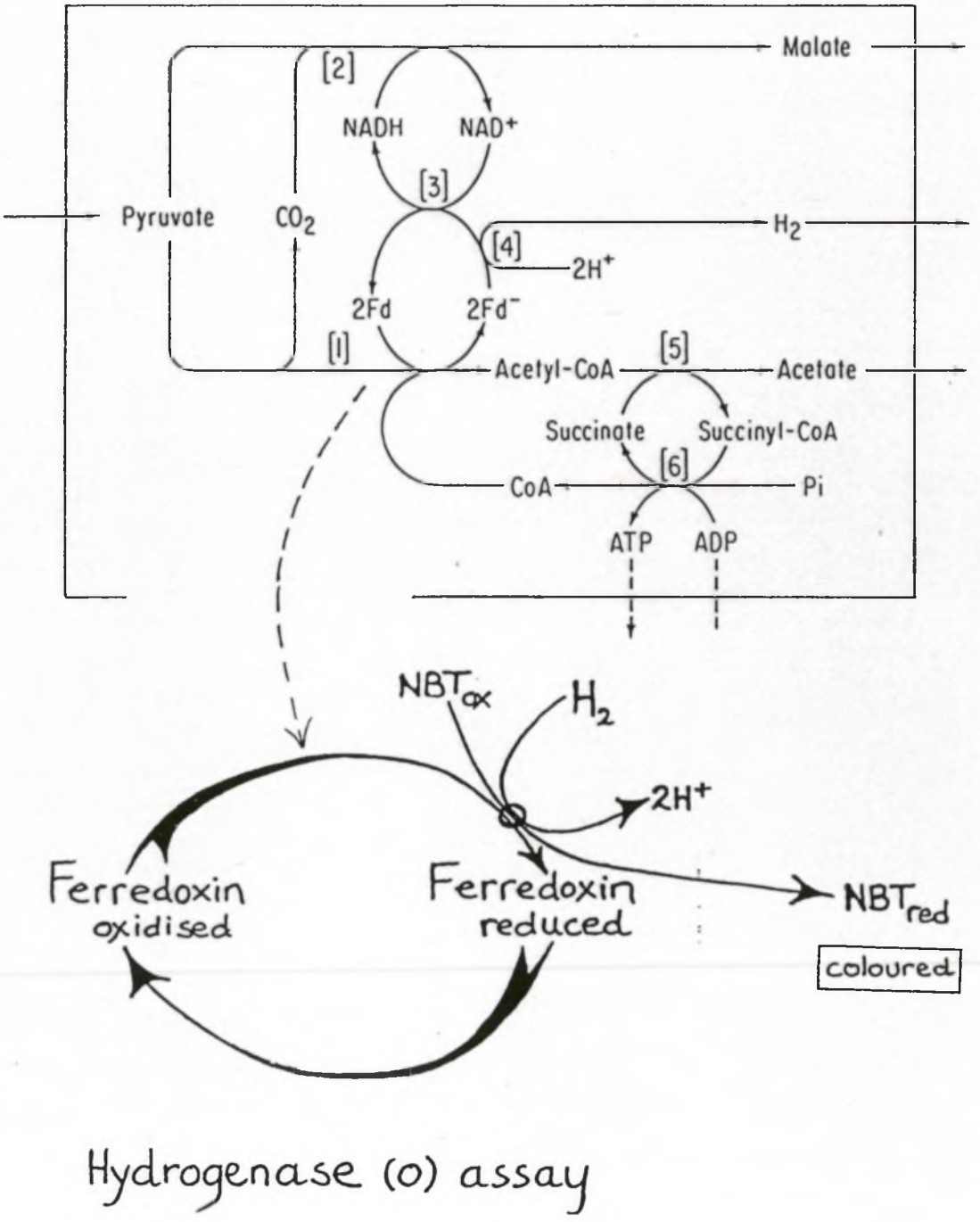
7. Leave at $4^{o}C$ for 20 min.

8. Wash twice with supplemented buffer then twice with distilled water.

9. Add alcoholic uranyl acetate (UA) in the dark. This is prepared in a brown bottle by adding UA to 70% ethanol to the point of saturation (an obvious precipitate forms). The UA is filtered through a 0.2 um membrane on the end of a syringe and added directly to the fixed cells. This procedure is carried out in the darkroom with a red safety light.

10. Cells remain in UA overnight at room temperature.

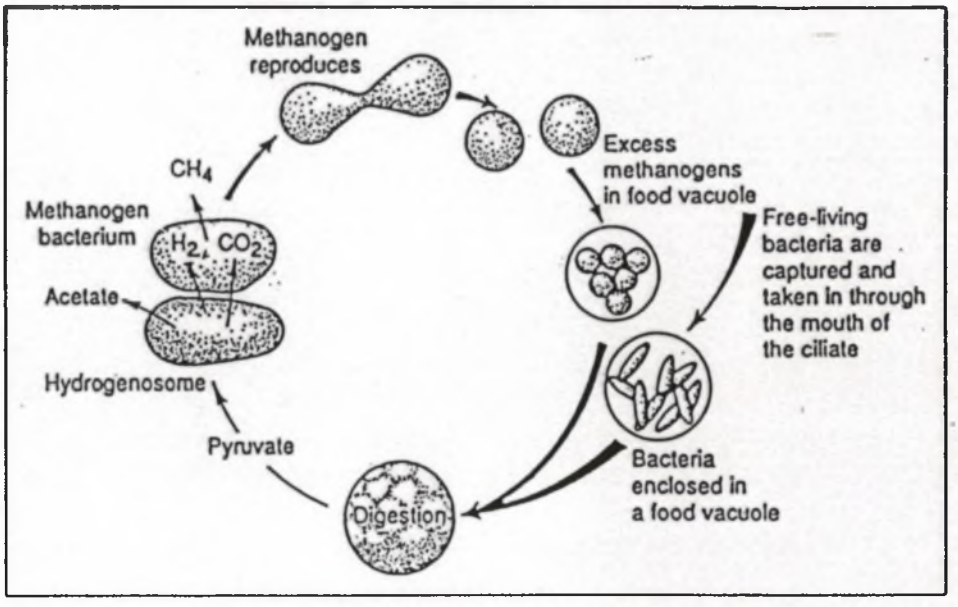
11. Wash several times in 70% ethanol in dim light, then dehydrate completely.



Hydrogenase (o) assay

APPENDIX

Cytochemical assay for hydrogenase in the hydrogenosomes of anaerobic protozoa. The method depends on hydrogenase reducing the tetrazolium salt nitroblue tetrazolium when supplied with exogenous hydrogen. Based on the metabolic map for trichomonads in Muller (1988), and methods described in Zwart et al. (1988) and Finlay & Fenchel (1989b).



APPENDIX

Proposed scheme for waste re-cycling in anaerobic ciliates, for which there is evidence in the electron micrographs of *Metopus palaeformis* from landfill (adapted from Finlay & Fenchel 1989b).

Permanganate fixation procedure for TEM

1. Gently centrifuge culture into a loose pellet.
2. Remove supernatant with a long syringe needle.
3. Push supernatant (10 ml) through a 0.2 μm membrane into a test tube containing 0.2g potassium permanganate.
4. Shake to dissolve.
5. Pour onto loose pellet (about 1 ml) of cells.
6. Leave for 1 h at room temperature.
7. Wash thoroughly with distilled water.

CMV medium (pers. comm. Dr S. Wagener, Konstanz) for *Metopus*

	g/l
KH_2PO_4	0.15
NH_4Cl	0.035
NaCl	0.4
$MgCl_2.6H_2O$	0.2
KCl	0.15
$CaCl_2.2H_2O$	0.25

Autoclave, outgas with N_2, add $NaHCO_3$ (12 mmol (100mg/100ml)), and reduce with a few crystals sodium dithionite

CP (Cerophyl-Prescott Liquid) Protozoa

This is an infusion of 'Cerophyl'* in Prescott's and James' liquid (see recipe for PJ). The infusion strength used depends on the type of protozoan culture; for most purposes 0.1% (weight/volume) is suitable:

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 Business Centre, 9900 Pflumma Road, Lenexa, Kansas 662115, USA. 'Cereal Leaves' from Sigma can be used instead of Cerophyll.

CPA (Cerophyl-Prescott Agar) Protozoa

1 litre of Cerophyl-Prescott Liquid (CP) and 15 g of non-nutrient agar.

NN (Non-nutrient (Amoeba Saline) Agar) Protozoa

Non-nutrient agar	15.0 g
*Amoeba saline solution	1.0 litre

* Amoeba saline solution:
Stock solutions:

		per 500 ml
1.	NaCl	12.0 g
	$MgSO_4.7H_2O$	0.4 g
	$CaCl_26H_2O$	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.

Glucose-Salts (GS) medium

19 mM NH_4Cl

1.1mM $MgSO_4.7H_2O$

22 mM KH_2PO_4

42 mM Na_2HPO_4

22 mM Glucose

1.5% (w/v) Difco Bacto-Agar made up in glass distilled water. Glucose added to the medium prior to sterilization. Adjust to pH 8.0 with 1N NaOH. Sterilize at 121°C for 15 min.

PJ (Prescott's & James's Solution) Protozoa

Stock solutions, each in 100 ml of 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 stock solution and make up to 1 litre with glass distilled water.

SES (Soil Extract Medium with Added Salts) Protozoa, freshwater and marine algae

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 stock	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/in² pressure. Decant or filter the supernatant.