Partial stratification *in vitro* of microbial subpopulations from the Salt Marsh Microbial Mat, Sippewissett MA.

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Microbial Diversity Course

Summer 1995

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Introduction

Microbial mats are stratified communities of bacteria and few eukaryotic organisms, all closely related at the metabolic level. The community is based on very well defined gradients that support different living forms at different depths. There are several types of microbial mats: hot springs microbial mats, where purple sulfur bacteria and gliding bacteria live at temperatures ranging from 40 up to 70°C, approximately. In non-thermal environments, masses of photosynthetic bacteria (cyanobacteria, purple sulfur and non sulfur bacteria, green sulfur bacteria and sulfate reducers) bloom at intertidal zones in salt marshes. This second type of microbial mat has been extensively studied on the past years due to its ecological, taxonomical, biochemical (photosynthetic mechanisms, mainly) and evolutionary relevance.

Although great efforts have been made to isolate the organisms present in microbial mats, only the most represented or easy-to-cultivate ones have been identified. It has been estimated that they could represent about 5% of the whole community. New efforts and probably radically different methods of isolation have to be considered for future studies.

The development of microbial model systems under controlled laboratory conditions has been studied for relatively simple communities such as co-cultures or single species systems. There are several types of laboratory model systems: open model systems (multistage chemostats, gradostats, gradostats with cell separation), and closed model systems (batch cultures, gel stabilized systems) and biofilms.

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Gel stabilized systems are characterized by well defined boundaries: carbon source, proton and gas concentrations, etc. An interface develops when countergradients meet and a microenvironment is developed. Organisms capable of tolerate such conditions overgrow in this zone. After a while, a community becomes spatially ordered if solutes deep diffusing.

Model systems have been heavily criticized because they could oversimplify the natural environment, but their development and study should not be dismissed taking into account that direct field studies, in some cases, are close to impossible. We should also keep in mind that model systems for mat communities could have left aside key organisms unable to adjust to the new semi-artificial environments, as well as non-bacterial members such as bacteriophages and lower eukaryotes which could be playing very important roles in genetic information exchange and population control. Nevertheless, model systems for microbial communities can expand our knowledge of the complex relationships among their residents.

The present report includes experiments done in order to observe how could a microbial community spatially organize itself under laboratory conditions. The whole microbial community of the salt marsh in Sippewissett MA, was used as inoculum in semisolid sea water agar and poured on top of an agar slant containing different carbon sources. Different light conditions were also tested. All physical conditions and chemical composition of culture medium were kept as simple as possible.

Materials and Methods

A mixed sample (~30 ml) of the salt marsh microbial mat from Sippewisset MA was resuspended to a final volume of 45 ml with sterile and filtrated sea water. The sandy sample was shaken vigorously for 10 min and let it stand at room temperature for 15 min

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until a particle-free supernatant was obvious. The supernatant containing a mixed bacterial population among some other organisms (ciliates, nematodes, etc.) was diluted 1:10, 1:50 and 1:100 with sterile and filtrated sea water. Equal volumes of diluted samples were mixed with filtrated sterile sea water-agar 1.5% (final concentration 0.75% agar, in a final volume of 5 ml). The semisolid agar-sample mixture was poured into two different sets of carbon source-agar tubes: a) yeast extract 0.25%, filtrated sea water and agar 1.5%; or b) sterile liquid phase of boiled mud obtained from the salt marsh mixed with agar 1.5% in filtrated sea water. At least five tubes for each set of conditions were poured. The carbon source was poured in two different ways: as a plug at the bottom of the test tube, or as a gradient in tilted test tubes (slants). Once the semisolid agar was poured, the tubes were cooled down immediately on ice to prevent diffusion or local accumulation of the inoculum. The agar tubes were overlayed with sterile sand and exposed to two different light conditions: a) direct sunlight; and b) buried up to the sand overlay in a wet sand bucket or beakers covered with clear plastic film or glass Petri dishes, according to the number of tubes prepared. It is important to keep the sand wet, not flooded, in order to maintain a moisturized environment and prevent the agar tubes from drying out. The buried tubes were covered with hydrated dialysis membrane (cut off 12 000-14 000 Da) to let the sunlight go into the test tubes. The tubes exposed to direct sunlight were covered with plastic closures only.

Both sets of tubes were exposed to sunlight, maintaining a day-night cycle according to the local weather oscillations. Temperatures during incubations range from 30 up to 45°C during day, and 18 to 20°C at night.

All micrographs were taken under 40x inmersion oil objective.

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Results and Discussion

The salt marsh microbial mat in Sippewissett MA contain a great variety of microorganisms stratified within the first 10 cm (top to bottom): gold layer (diatoms and cyanobacteria); upper green layer (cyanobacteria); pink layer (purple sulfur bacteia); peach layer (purple sulfur bacteria, mainly *Thiocapsa pfennigi*); lower green (green sulfur bacteria); and, black layer (sulfate reducing bacteria). The thickness and color intensity varies within short distances (10-20 cm), suggesting different stratification stages and/or local chemical composition. In the present study, few benthic invertebrates, such as nematodes, and some heterotrophic protists were observed. The initial sample was collected from a well developed site (all the layers present, see Figs. 1 and 2).

Six days after setting up the semisolid agar gradient tubes, different types of colonies appeared at different depths in the tubes at 1:100 dilution exposed to direct sunlight. Colonies were more abundant on the tubes prepared with 0.25% yeast extract than in the tubes prepared with boiled sterile mud. Three different types of colonies are clearly visible (see Fig. 3a): white and tiny colonies at the bottom of the tube, deep red colonies at the middle portion of the tube, and small light brown colonies close to the top sand overlay. The same type of spatial distribution of the colonies was observed on the tubes prepared with boiled sterile mud but they developed after 8-9 days (Fig. 3b).

The tubes buried in the wet sand bucket developed only two types of colonies after 6-7 days: white small colonies all over the tube, and black colonies close to and within the sand overlay (Fig. 4a, b), although some tubes presented black colonies all over the semisolid agar. Some white colonies turned blackish after awhile, and some tubes only developed black colonies. Tubes that were not completely buried in the sand developed red and brown colonies close to the top and in the sand plug (results not shown).

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Samples of each colony type are shown in figure 5 . There is no obvious cell shape difference within the white/black colonies developed in the buried tubes: motile rod-shape cells (Fig. 5a, b, c). The fact that the black colonies developed in buried tubes and at the bottom of these tubes, suggests that they could be sulfate reducing bacteria, although no gas analysis of the headspace was done. The brown upper layer developed in the tubes exposed to sunlight contain non-refractile round cells and few spirilla, all motile (Fig. 5d). The deep red middle colonies contain ribbon-shaped cells actively swimming (Fig. 5e, g). These two colony pigmentations suggest the presence of purple non sulfur bacteria. The white colonies developed at the bottom of the tubes contain rods, round and drumstick-shaped cells, not very motile (Fig. 5f, h). It should be noted that no refractile sulfur granules inside the bacteira were obvious thirteen days after innoculation, suggesting that sulfur bacteria were not enriched at this point in time, although they were present in the initial sample (see Fig. 2).

Colony stratification was not observed in tubes inoculated with higher inoculum concentrations (dilutions 1:10 and 1:50) and exposed to sunlight. Anoxygenic photosynthetic bacteria (red and brown colonies) spread all over the tube. However, seven days after inoculation, light green filamentous colonies developed close to the top (within the first 1-1.5 cm, see Fig. 6a). These green colonies are rich in diatoms and non-photosynthetic flagellates, as well as the enriched photosynthetic bacteria (see Fig 6b, c, d). At lower concentrations of inoculum, only bacteria were enriched at the same time point, suggesting that if any flagellate or other organisms with more or less the same nutritional requirements were present, they are not able to persist until the bacterial population reaches an optimal concentration for its survival.

Due to the experimental design, direct measurments of oxygen concentrations cannot be assessed. The sand overlay is a mechanical barrier that could damage the

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oxygen microsensors. However, a more qualitative approach was taken and tubes containing 25 mg/ml of rezazurin (oxygen colorimetric indicator) in the semisolid-inoculum (1:100 dilution) mixture were poured. Rezazurin tubes with and without inoculum were pink during the first 24 hours after pouring them; a day later, all the tubes turned clear, suggesting that an anoxigenic or at least microaerophilic environment was developed. It is very likely that a pH gradient is formed during the experiment, but the same constrains as with oxygen measurements apply in this case. A solution for pH measurements could be the presence of a pH universal indicator (wide pH range) in the semisolid agar, although toxicity and color interference with the bacterial population should be kept in mind. Another alternative is retrieveing the intact semisolid agar/carbon source gradient, slice it and resuspend it distilled water for pH electrode measurements.

It should be stressed out that the main objective of the present work was to test the possibility of stratified enrichment of a microbial mat community. No further isolations of organisms of each layer was pursued, but it would be worth trying them, in order to have a more precise description of the type of orgamisms enriched in this study. Also it should be considered that the development of the layers was followed only for three weeks. More organisms could appeared beyond this time span. This observation suggest that an artificial species succession is taking place in the experimental model tested in this study, so it could be possible to isolate organisms not very abundant in the inital inoculum at the time the sample was taken.

Conclusions and Perspectives

A gel stabilized system with an artifical carbon source can be used to obtain a partial stratification of some members of a salt marsh microbial mat community. This could be considered as an initial step of enrichment for several of these organisms. The present

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experimental design avoided chemical defined culture media; instead, rich media containing yeast extract or salt marsh mud were suitable to support the enrichment of some organisms. Many other residents could have been left out of this experimental set up, but further improvements can be made. Some suggestions follow: changing carbon source composition, include some inorganic chemicals, increase the surface area by including glass beads or thoroughly washed sand grains. It would be worth trying to test the possibility of studying succession under the experimental conditions tested in this work.

Acknowledgments.

I want to thank Dr. Thomas Pitta for the micrographs, for taking me to Sippewissett to collect the samples, and for encouraging me to continue this work; and, to The Department of Navy, Office of Naval Research for giving me financial support.

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Figure legends

Fig. 1 Sliced microbial mat from the Salt Marsh, Sippewissett MA.
The most prominent layers are (from top to bottom): upper green (cyanobacteria), pink layer (purple sulfur bacteria), peach layer (purple sulfur bacteria, mainly *Thiocapsa*), and black layer (sulfate reducers). From the top layer to the beginning of the black layer there are 1.5-2.0 cm.
This picture was taken on July 22, 1995.

Fig. 2 Some residents of the Salt Marsh microbial mat.
Phase contrast micrographs from the resuspended mat used as inoculum. Cyanobacteria (a, c, d, h, where c is an epifluorescent micrograph of the bacteria shown in d). Sulfur bacteria (b, e, f), note a small spirochete in upper right corner in Fig. e, as well as the refractile sulfur granules inside the cells. A diatom (g).

Fig. 3 Partial stratification of some members of the microbial mat community in gel stabilized tubes exposed to direct sunlight.
Fig. a, tubes grown in 0.25% yeast extract; b, tubes grown in filtrated and boiled salt marsh mud. Note the interphase separation between the carbon source/agar at the bottom, and the white colonies developed in this zone. Also note the brown colonies close to the top sand overlay.

Fig. 4 Enrichment of sulfate reducer-like bacteria from the microbial mat in buried gel stabilized tubes.

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Note the development of a thick black zone within the sand overlay and tiny white colonies at the interphase of the semisolid agar/carbon source (a, yeast extract; b, boiled and filtrated salt marsh mud).

Fig. 5 Phase contrast of the stratified colonies developed under direct sunlight or in buried tubes.

Black layer observed in buried tubes and black colonies, a, b, c. Brown upper layer developed in the tubes exposed to sunlight, d. Deep red middle colonies, e, g. White colonies developed at the bottom of the tubes, f, h.

Fig. 6 High inoculum concentration (1:10) gel stabilized enrichments in tubes exposed to sunlight.

Gradient tube containing 0.25% yeast extract poured as slant, a. The tube was incubated for 7 days. Flagellated protists, b. Diatoms under phase contrast (c) or epifluorescence microscopy (d).

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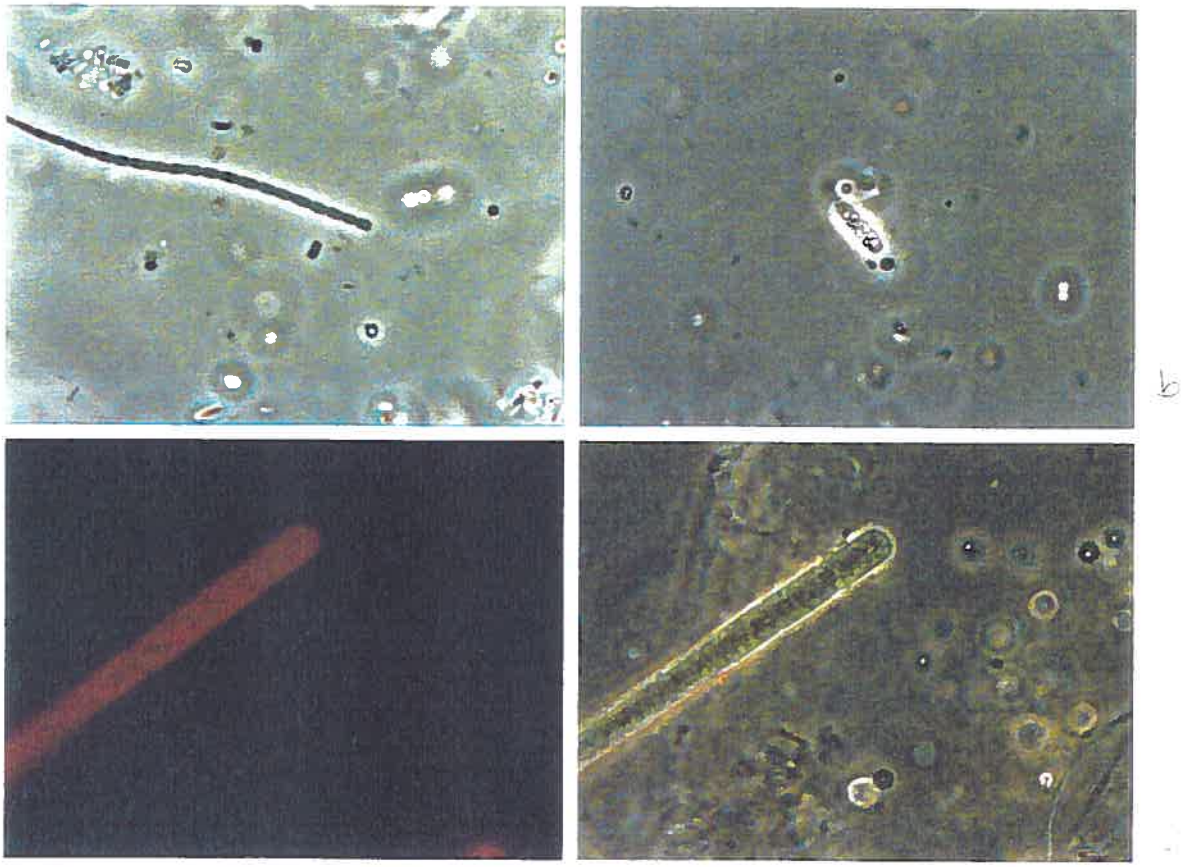
Fig 1



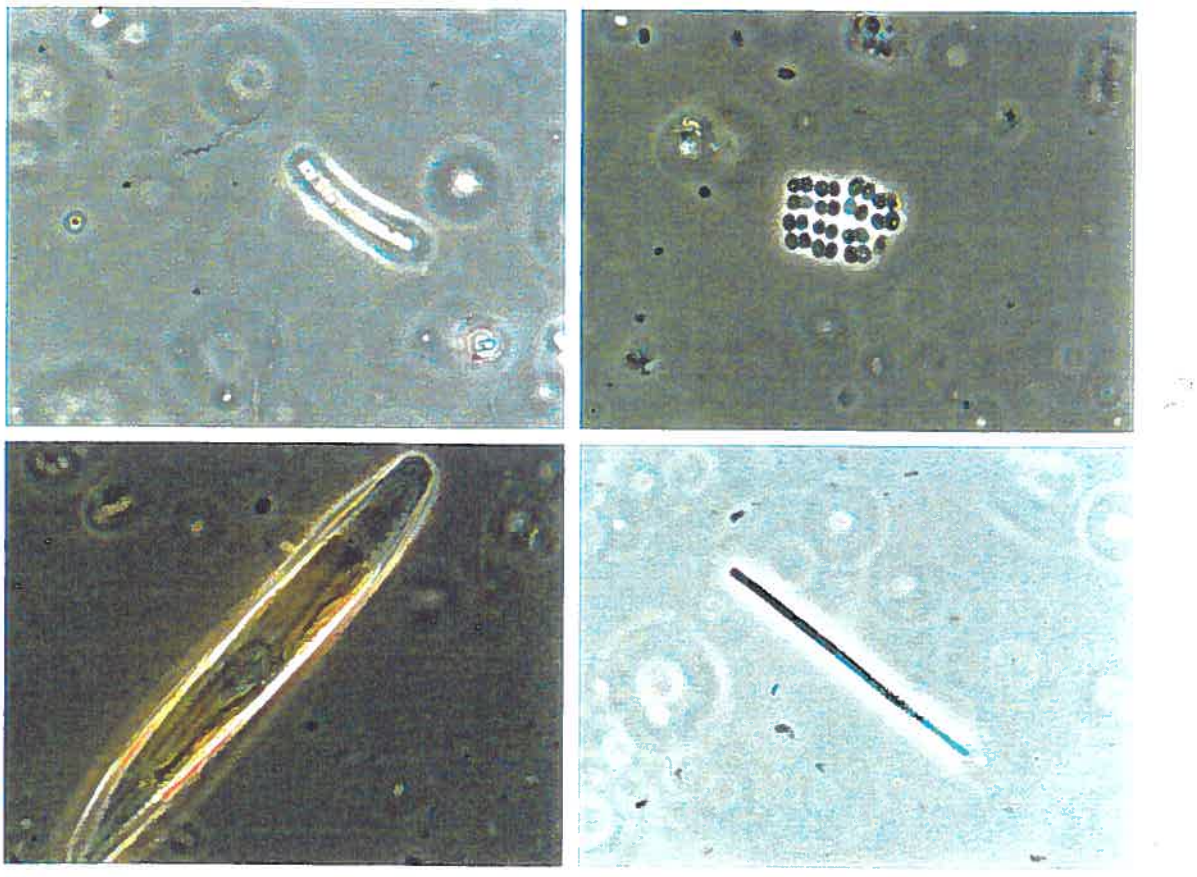


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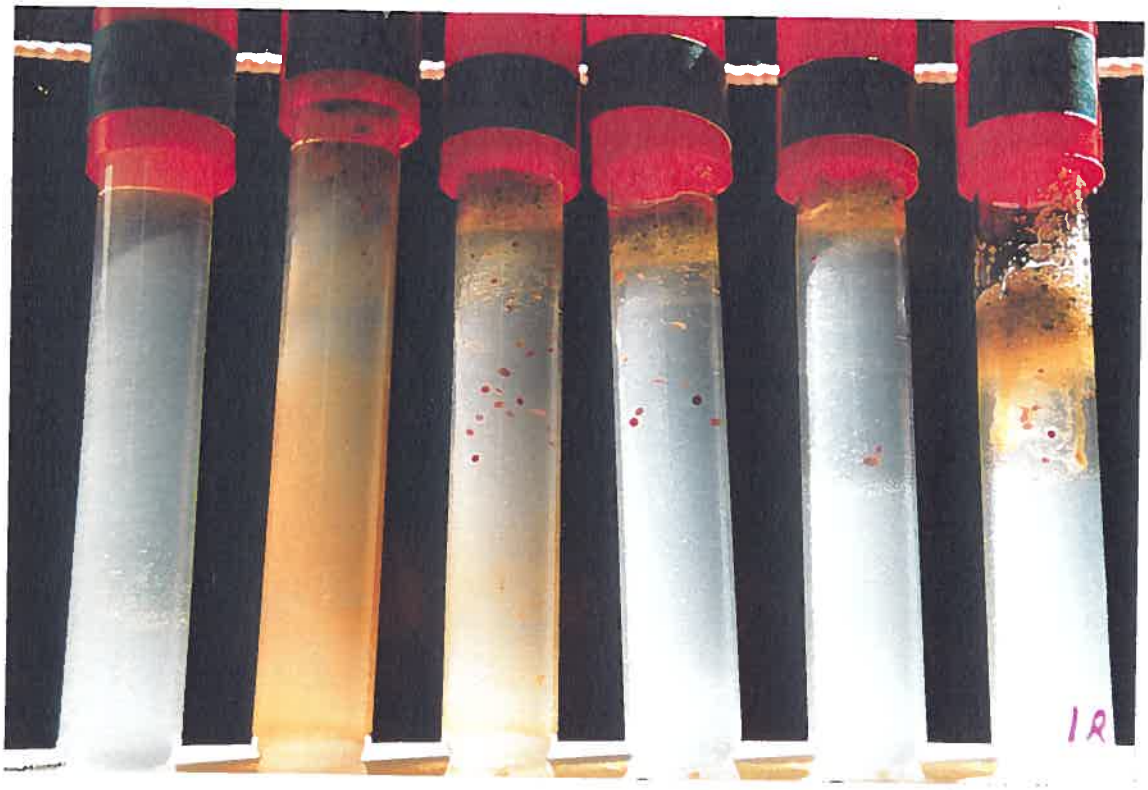
Fig 2



a

b

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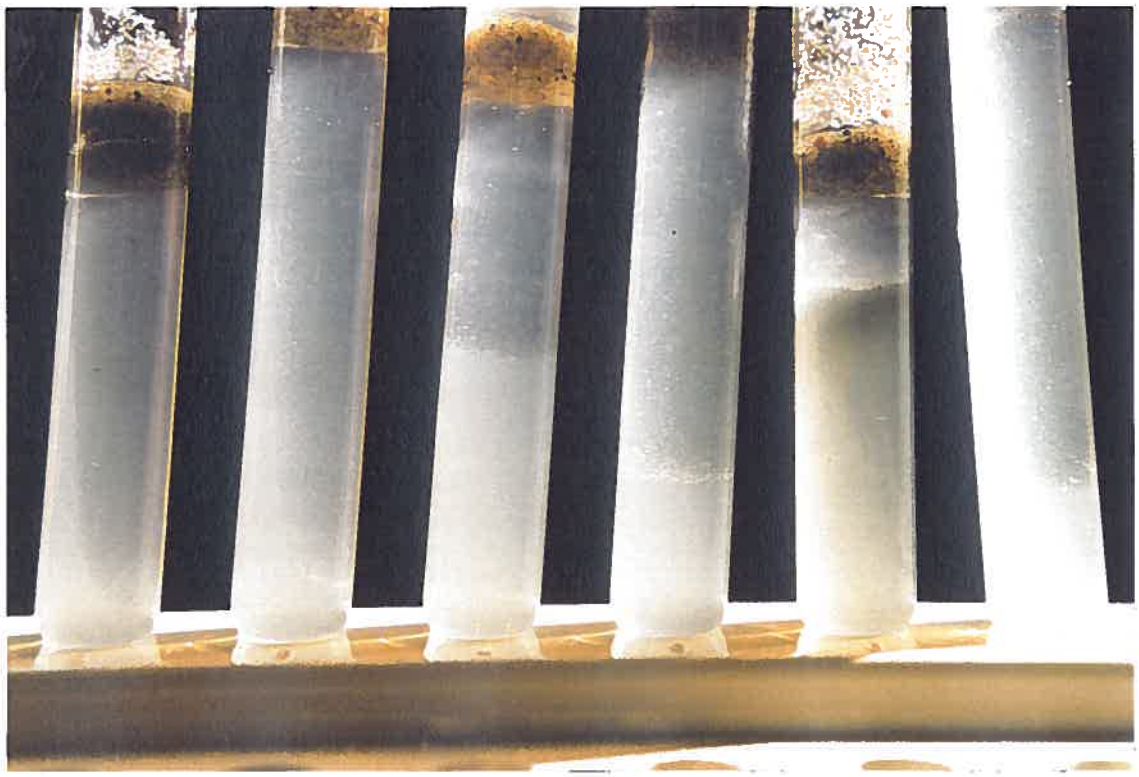
Fig 3



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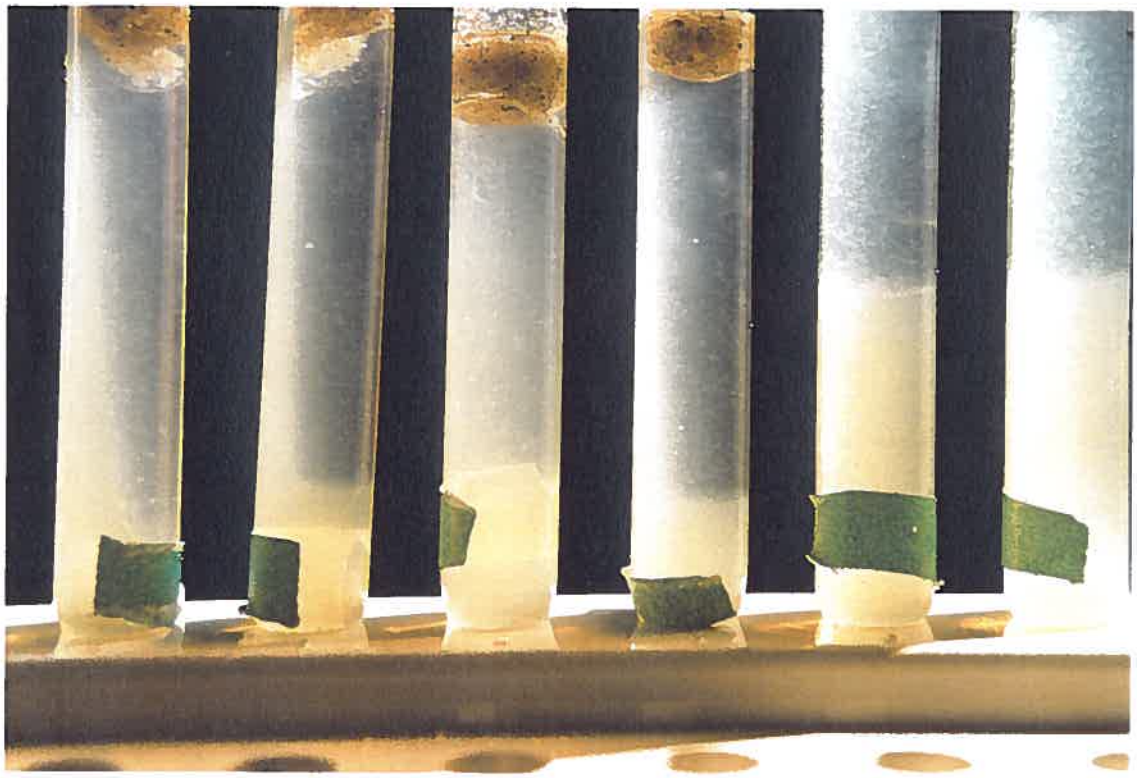

b

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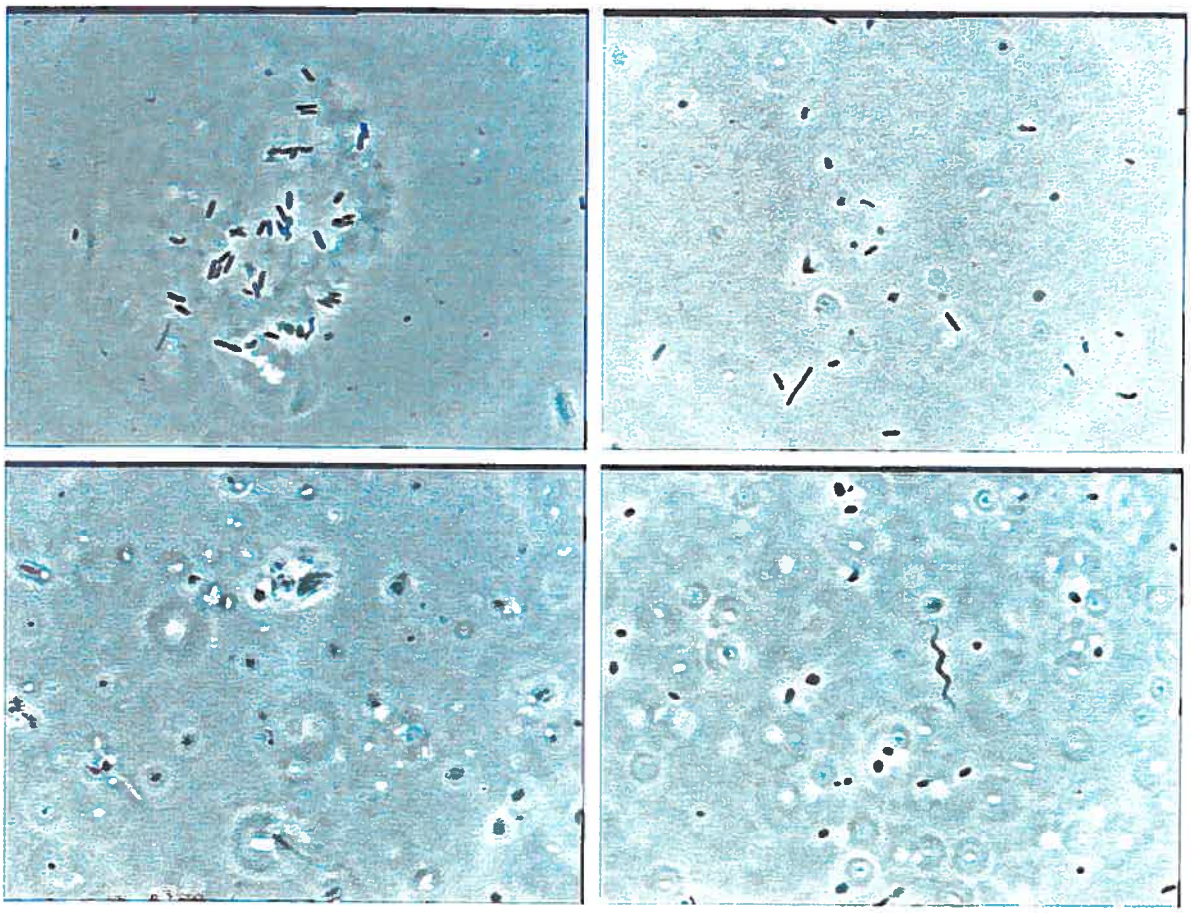
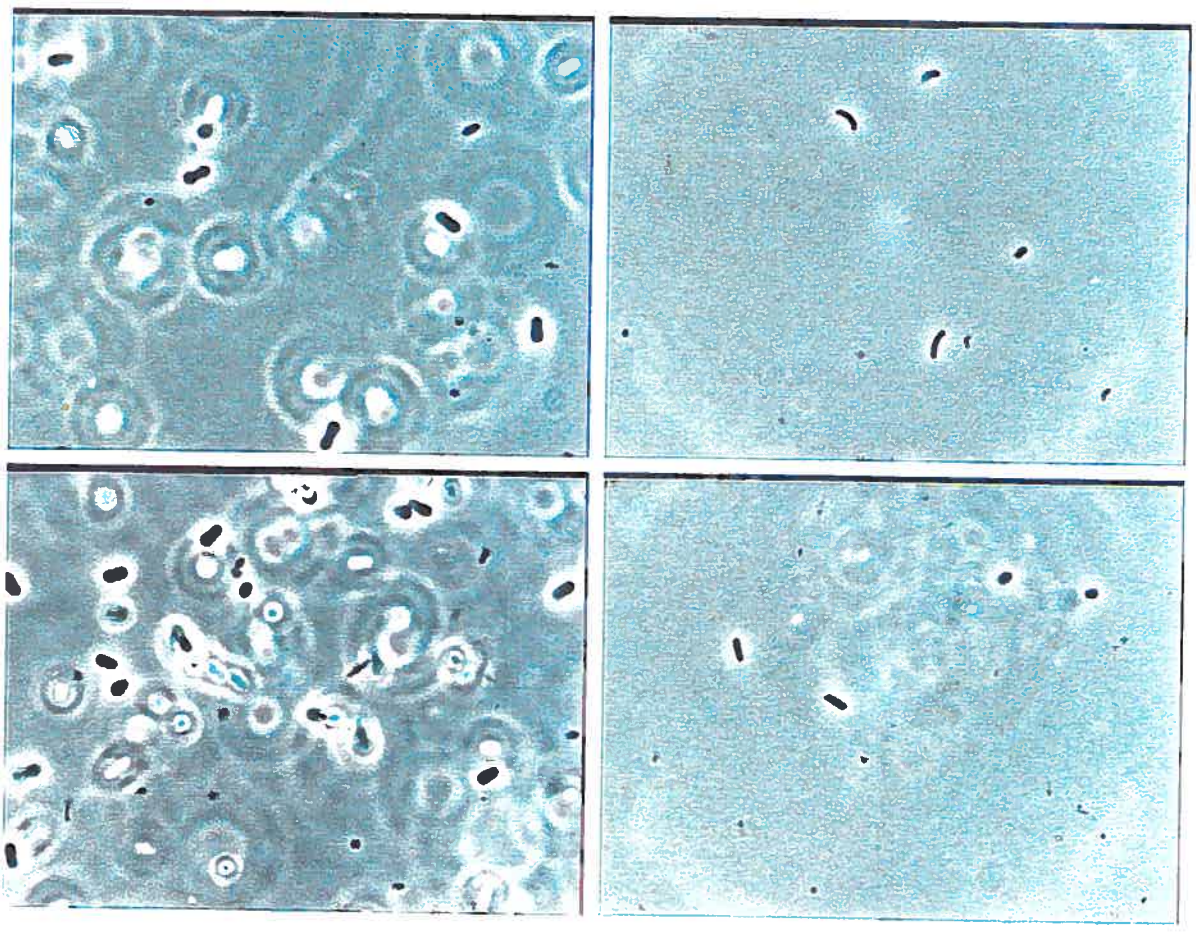
Fig 4





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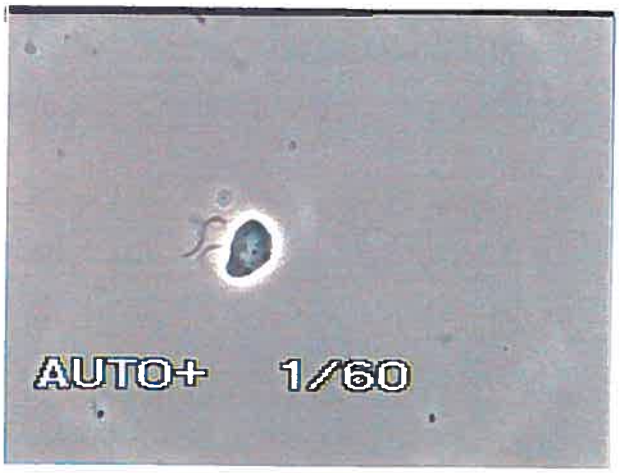
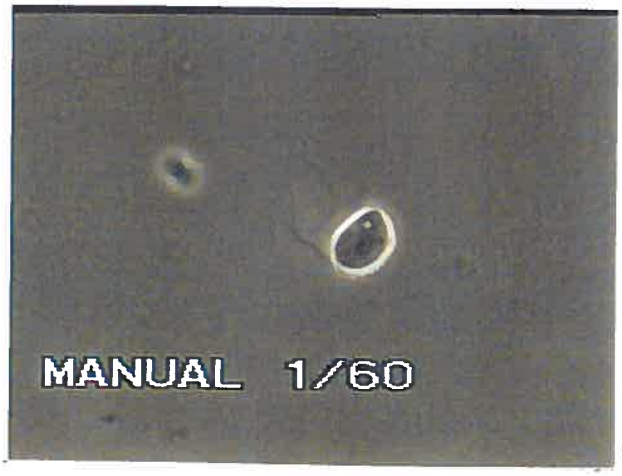
Fig'

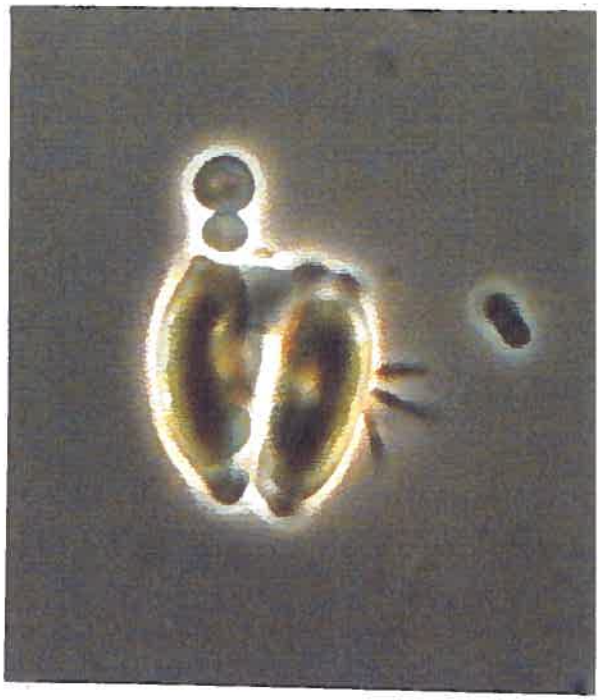
a





b

Fig 6



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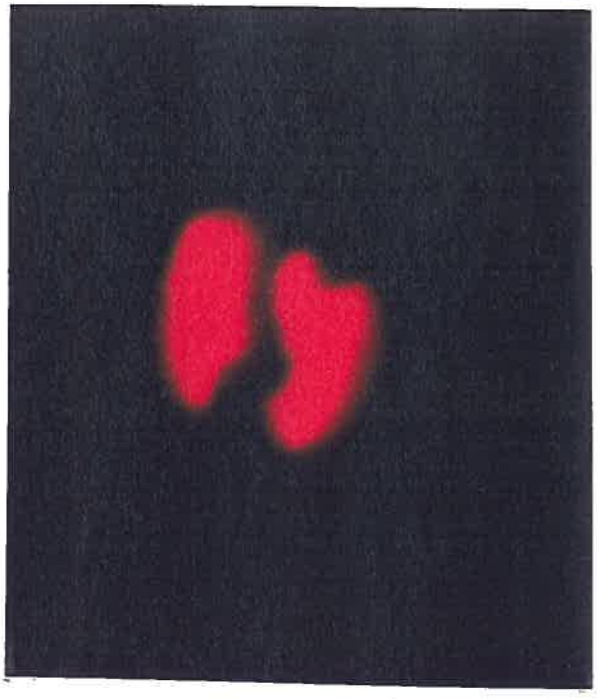

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