UNIVERSITY OF CENTRAL LANCASHIRE

THE INTEGRATION OF AN AVIRULENT *LEGIONELLA PNEUMOPHILA* INTO AQUATIC BIOFILMS

A THESIS SUBMITTED TO THE UNIVERSITY OF CENTRAL LANCASHIRE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

SUSANNE BARBARA SURMAN BSc. (Hons.) C.BIOL. M.I.BIOL.

Collaborating Establishment: Research Division, Centre of Applied Microbiology and Research, Porton Down, Salisbury. SP4 0JG.

SEPTEMBER 1994

DECLARATION

I DECARE THAT WHILE REGISTERED AS A CANDIDATE FOR THE DEGREE WHICH SUBMISSION IS MADE I HAVE NOT BEEN REGISTERED AS A CANDIDATE FOR ANOTHER AWARD OF AN ACADEMIC ISTITUTION. NO MATERIAL CONTAINED IN THIS THESIS HAS BEEN USED IN ANY OTHER SUBMISSION FOR AN ACADEMIC AWARD.

Dedication

This thesis is dedicated to my husband Michael and our children Nicola and David, for without their constant support and encouragement this thesis would never have been completed.

Acknowledgments.

I wish to thank Professor Glyn Morton, Dr. R. England and Dr. F.Bolton for their continued support, friendship and encouragement throughout this research project.

I am especially indebted to the following staff from CAMR, Porton Down, particularly Dr Bill Keevil who has contributed both encouragement and practical support. I am also grateful to his staff Dr. J. Walker and Dr. J. Rogers for their help and friendship throughout this project and to Dr. R. Fitzgeorge and Mrs A. Williams for the provision of the *Legionella* strains. I am also very grateful to A. B. Dowsett and E. Elphick for their help with SEM micrographs presented in this study.

I also wish to thank Dr T. Rowbotham, Leeds PHLS, for the provision of the *Klebsiella aerogenes* and the axenic strain of *Acanthamoeba polyphaga* used in this project and also for his help in the identification of amoebae and for his continued friendly advice.

I am also indebted to Dr D. Goddard from the Company Research Laboratory, British Nuclear Fuels plc. Preston, for his help with the Atomic force microscopy which is presented as part of this study.

I am very grateful to A. Skinner, John Radcliffe Hospital, Oxford for his help in the preparation of the TEM micrographs which are also presented here.

I wish to thank Zeneca Specialities, Blakeley, for the provision of Vantocil IB and also for the use of their SEM. I would also like to thank Pro-lab Diagnostics, UK, for the provision of *Legionella* FITC and Latex agglutination kits.

I wish to thank the staff of the media department at the Public Health Laboratory, Preston, for their helpfulness during this project.

A special thanks goes to the following staff of the University of Central Lancashire for their friendship and help during this research project; the microbiology technicians, especially Mrs. C. Hughes; the Faculty of Science workshop for their prompt service with a smile, Bill Cowell in reprographics and Mike Peel in the library.

This study was made possible by the award of a grant by the Science and Engineering Research Council under the Instant Award Scheme.

ABSTRACT.

A continuous culture model system was set up in the laboratory and inoculated with a diverse range of microorganisms, including several bacteria and protozoa, obtained from the local mains water tap supply. This inoculum and was added to the system without any prior culture or other selection process. Biofilms readily developed on glass tiles suspended in the planktonic phase of the system. An avirulent *Legionella pneumophila* was inoculated into the system and was subsequently isolated from both biofilms and also from the aqueous (planktonic) phase of the chemostat. The attenuation of this strain, determined by its inability to cause disease and death in guinea pigs, remained unaltered despite the long term survival of this strain within the system.

Investigations to determine whether the avirulent *L. pneumophila* was able to infect and proliferate within protozoa were carried out. The results of the present study show that this avirulent *L. pneumophila* did not proliferate intracellularly and suggest that the association of *L. pneumophila* with protozoa although probably important in the long term survival of this bacterium especially during periods where adverse conditions prevail, is not essential but opportunistic. In chapter 3 the importance of the presence of these non-legionellae bacteria, which included *Flavobacterium* sp., *Acinetobacter* spp. and several species of *Pseudomonas*, was investigated. The results suggest that the presence of the non-legionellae are relevant to the survival of *Legionella* especially in environments which favour it's growth, for example water distribution systems.

In order that we may gain a further insight into the ecology of microorganisms in their natural environment, it is necessary to visualise them in conditions which allow them to interact in a way which mimics as closely as possible the natural environment. Biofilms were developed on surfaces which could be removed from the model system in their entirety. Direct visualisation techniques, including atomic force microscopy and Hoffman modulation microscopy could then be used which allowed the *in vivo* examination of biofilms *in situ* on the surface upon which they had developed. More traditional microscopy methods were also used. Atomic force microscope images of biofilms and individual biofilm bacteria including *Legionella* were obtained, which clearly showed the presence of exopolymeric substances (EPS). Hoffman modulation contrast microscopy and scanning electron microscopy showed the diverse nature of the biofilms being studied. The results of these investigations suggest that a more complete understanding of the complex nature of biofilms is achieved by the use of a combination of several microscopy techniques.

The response of a *L. pneumophila* serogroup 6 and the avirulent *L. pneumophila* serogroup 1 to a commercially available biocide, Vantocil IB, was investigated. Both the serogroup 6 and the avirulent serogroup 1 could not be detected following biocide teatment in either the planktonic phase or biofilms. These results suggest that this avirulent *L. pneumophila* is a suitable model substitute for the virulent *L. pneumophila*.

CONTENTS

CHAPTER 1.

INTRODUCTION 1

1.3. LEGIONELLOSIS 6

1.4.. TREATMENT ..... 1(

1.5. SOURCES OF INFECTION 1

1.6. LABORATORY DETECTION OF *LEGIONELLA* 1:

1.6.1. SUBTYPING. 1:

1. 7. PATHOGENESIS 1:

1.9. INTERDEPENDENCE BETWEEN *LEGIONELLA* AND OTHER ORGANISMS. 2:

1.10. BIOFILMS. 2:

1.11. BIOFILMS IN WATER DISTRIBUTION SYSTEMS. ..... 3(

1. 12. BIOFILMS AND *LEGIONELLA* 3:

1.13. PREVIOUS BIOFILM STUDIES 3:

1.14 BACKGROUND TO THIS STUDY 3:

1.15 AIMS AND OBJECTIVES ..... 3(

CHAPTER 2 ..... 4(

2. THE INTEGRATION OF AN AVIRULENT *L. PNEUMOPHILA* INTO A MODEL BIOFILM SYSTEM 41

2.1. INTRODUCTION. 41

2. 2.AN INVESTIGATION TO DETERMINE WHETHER AN AVIRULENT STRAIN OF L. PNEUMOPHILA SEROGROUP 1 IS ABLE TO INTEGRATE INTO A NATURALLY OCCURRING CONSORTIUM OF AQUATIC MICROORGANISMS. 4:

2. 2. 1. MATERIALS AND METHODS. 4:

2.2.1.1. THE CHEMOSTAT 4:

2. 2. 1.2 CONTINUOUS CULTURE MEDIA PREPARATION ..... 4(

2.2.1.3. INOCULUM PREPARATION ..... 4(

2.2.1.4. BIOFILM DEVELOPMENT 4:

2.2.1.5. THE *LEGIONELLA PNEUMOPHILA* SEROGROUP 1 PONTIAC (CORBY) STRAINS 4:

2.2.2. BACTERIAL ENUMERATION ..... 5(

2.2.2.1. PREPARATION OF INOCULA FOR ENUMERATION ON SPREAD PLATES. ..... 5(

2.2.2.2. GROWTH MEDIA ..... 5(

2.2.2.3. HEAT TREATMENT. 5

2. 2. 3. IDENTIFICATION METHODS 5

2.2.3.1. BACTERIAL IDENTIFICATION. 5

2.2.3.2. API 20NE 5

2.2.3.3. IDENTIFICATION BY BIOLOG. 5

2.2.3.4. CONFIRMATION OF IDENTITY OF *L. PNEUMOPHILA* ISOLATES. 5

2.2.4. FUNGAL IDENTIFICATION. 5

2.2.5. AMOEBAL IDENTIFICATION. 5

2. 2. 6. RESULTS. 5

2. 3. AN INVESTIGATION TO DETERMINE IF THE AVIRULENT STRAIN OF *L. PNEUMOPHILA* USED IN THIS STUDY CAN BE RECOVERED FOLLOWING INCUBATION AT 50 °C. 6

2. 3. 2. RESULTS. 6

2.4. AN INVESTIGATION TO DETERMINE WHETHER THE AVIRULENT *L. PNEUMOPHILA* STRAIN REVERTS TO VIRULENCE WHEN INTEGRATED INTO A MIXED CONSORTIUM OF AQUATIC MICROORGANISMS WITHIN A MODEL CONTINUOUS CULTURE BIOFILM SYSTEM 6

2.4.1. MATERIALS AND METHODS. 6

2.3.1.2. VIRULENCE TESTING. 6

2.4.2. RESULTS. 6

2.5. DISCUSSION 6

CHAPTER 3 7

AMOEBAL STUDIES 7

3.1. INTRODUCTION 7

3.1.4. VIRULENCE STUDIES USING AMOEBAE. 7

3.2. MATERIALS AND METHODS 7

3.2.1. CYCLOHEXIMIDE STUDIES. 7

3.2.1.2. CYCLOHEXIMIDE TUBE ASSAY. 7

3.2.1.3. CYCLOHEXIMIDE ADDITION TO THE CHEMOSTAT AND MEDIA SUPPLY. 7

3.2.1.5. ENUMERATION OF ISOLATES FROM THE CHEMOSTAT. 7

3.2.1. COMPARISON OF VIRULENCE USING THE STREAK PLATE METHOD 7

3.2.2. *ACANTHAMOEBA POLYPHAGA* STUDIES 8

3. 3. RESULTS 8

3.3.3. CYCLOHEXIMIDE TUBE ASSAY 8
3.3.2. CYCLOHEXIMIDE TUBE ASSAY 8
3.3.3. VIRULENCE STUDIES. 8
3.4. DISCUSSION 9
CHAPTER 4 ..... **9**
LEGIONELLA PNEUMOPHILA AND OTHER AQUATIC BACTERIA 9
4.2. MATERIALS AND METHODS 1
4.2.1. BIOFILM DEVELOPMENT AND BACTERIAL GROWTH AND ISOLATION. 1
4.2.2 GROWTH ON R2A. 1
4.2.2.1.WHOLE BACTERIAL CELLS. 1
4.2.2.2.HEAT TREATMENT 1
4.2.2.3.CELL FREE SUSPENSIONS 1
4.3. RESULTS. 1
4. 4. DISCUSSION. 1
CHAPTER 5 ..... **1**
5. MICROSCOPY 1
5.1.INTRODUCTION 1
5.2.1. BIOFILM PRODUCTION AND PREPARATION 1
5. 2. 2. PHASE CONTRAST MICROSCOPY. 1
5. 2. 3. MODULATION CONTRAST MICROSCOPY 1
5. 2. 4. DIFFERENTIAL INTERFERENCE CONTRAST (DIC) AND FLUORESCENCE MICROSCOPY 1
5.2.5.STAINING WITH PROPIDIUM IODIDE AND DAPI. 1
5.2.6. IMMUNOGOLD STAINING 1
5.2.7. FITC STAINING. 1
5.2.8.ATOMIC FORCE MICROSCOPY 1
5.2.9.SCANNING ELECTRON MICROSCOPY 1
5.3.RESULTS AND DISCUSSION 1
5.4.CONCLUSIONS 1
CHAPTER 6 ..... **1**

6. BIOCIDE STUDIES 1
6.1. INTRODUCTION. 1
6. 1. 2. VANTOCIL 1B. 1
6.1.3. MECHANISM OF ACTION. 1
6. 2. MATERIALS AND METHODS. 1
6. 2.1. MINIMUM INHIBITORY CONCENTRATION (MIC) STUDIES. 1
6.2.2. BIOFILM DEVELOPMENT 1
6.3. RESULTS 1
6. 3.1. IDENTIFICATION OF MICROORGANISMS. 1
6.3.2. MIC RESULTS. 1
6.3.3. THE EFFECT OF VANTOCIL 1B ON THE PLANKTONIC HETEROTROPHIC POPULATION. 1
6.3.4. EFFECTS OF VANTOCIL 1B ON THE HETEROTROPHIC BIOFILM POPULATION 1
6.3.5. EFFECTS OF VANTOCIL 1B ON *L. PNEUMOPHILA*. 1
6.4.DISCUSSION. 1
CHAPTER 7 1
DISCUSSION. 1
CONCLUSION. 1
REFERENCES 1
APPENDIX 1 2
R2A AND R3A (REASONER AND GELDREICH, 1985). 2
APPENDIX 3 2
LEGIONELLA MEDIA 2
2.1.BASAL MEDIUM BUFFERED CHARCOAL YEAST EXTRACT MEDIUM (BCYE) 2
2.1.4.BCYE PREPARATION 2
2.2.BUFFERED CHARCOAL YEAST EXTRACT MEDIUM WITHOUT L-CYSTEINE. 2
2.3.*LEGIONELLA* SELECTIVE MEDIUM. BCYE WITH SELECTIVE SUPPLEMENTS. 2
2.3.1.SUPPLEMENTS. 2
2.4.GVPC PREPARATION. 2
5. QUALITY CONTROL. 2

3.1.PAGES AMOEBAL SALINE.....21

3.2.AMOEBAL AGAR.....21

3.3. AMOEBAL LAWNS.....21

APPENDIX 4.....21

PEPTONE YEAST EXTRACT GLUCOSE BROTH.....21

APPENDIX 5.....21

5.1.PROPIDIUM IODIDE STOCK SOLUTIONS.....21

5.2. DAPI STOCK SOLUTION.....21

CHAPTER 1

INTRODUCTION

1. INTRODUCTION.

1.1. Background

Legionella pneumophila, was first recognised as the principal aetiological agent of Legionnaires' disease, following an outbreak of pneumonia among delegates attending an American Legion Convention in Philadelphia in 1976 (McDade *et al.*, 1977). *L. pneumophila* did not grow on conventional laboratory media available at the time, so the search for the causative organism of Legionnaires' disease took months of painstaking research before the bacterium was finally visualised on slides prepared from hens' egg yolk sacs which had been inoculated with samples taken from infected patients (Ager and Tickner, 1985). It has since been identified in retrospect, as being responsible for outbreaks of pneumonia from as long ago as 1947 (Mc Dade *et al.*, 1979).

To date there are at least 39 known species of *Legionella* (Dennis *et al.*, 1993a), with 53 serogroups. *L. pneumophila*, the species most often associated with Legionnaires' disease is separated into at least 14 different serogroups (Anon., 1991a) and 40 subtypes (Barbaree, 1993). Not all species of *Legionella* however, are known to cause disease in man. Five new species were recently isolated as the result of a survey to determine the factors governing colonisation of UK buildings by *Legionella* spp. (Dennis *et al.*, 1993a). It is likely that as more research is carried out into the environmental incidence of *Legionella,* further species will be isolated.

1.2.1. PHENOTYPIC CHARACTERISTICS OF *LEGIONELLAE.*

The classification of *Legionellaeceae* is based on DNA homology (Brenner *et al.*, 1984; Brenner, 1986). Enzyme activity analysis, slide agglutination, fatty acid composition and analysis of isoprenoid quinones are all used as confirmation of species (Dennis *et al.*, 1993a; Wilkinson *et al.*, 1990).

Legionellae are Gram negative, aerobic rods which divide by non-septate binary fission. The cytoplasm is rich in ribosomes and nuclear elements and vacuoles are often present (Rodgers, 1979). The cell wall has two triple unit membranes with a peptidoglycan layer containing *m*-diaminopimelic acid (Hoffman, 1984). Morphological variations occur depending on the source and age of the culture. Legionellae are 0.3-1.0 µm in width, but their length varies. Three different forms have been noted; coccobacillary forms 1-3 µm in length; larger filamentous forms 8-20 µm in length have been seen in both lung material and on artificial culture media (Rodgers, 1979) and longer filamentous forms up to 100 µm in length have also been reported in agar cultures (Chandler *et al.*, 1979).

L. pneumophila have either one or two polar flagella 8-10 µm in length and 14-25 nm in diameter (Surgot *et al.*, 1988; Rodgers *et al.*, 1980) and are usually poorly motile although non motile strains can occur (Harrison and Taylor, 1988). Rodgers *et al.* (1980) found that in some media 14 out of 21 strains of *Legionella* possessed pili, 5-8 nm in width and varying between 0.3-2.25 µm in length, distributed evenly around the bacterial surface. However, no flagella or pili have been reported to occur in isolates from clinical or environmental sources (Rodgers *et al.*, 1980; Surgot *et al.*, 1988). Gram's stain is only successful with an appropriate counterstain; saffranin or basic fuchsin give satisfactory results (Harrison and Taylor, 1988).

1.2.2. NUTRITIONAL / PHYSIOLOGICAL CHARACTERISTICS

Bergey's Manual Of Systematic Bacteriology (1994) defines *Legionella* as a chemo-organotroph utilising amino acids as carbon and energy sources, carbohydrates are not fermented or oxidised. Amino acids utilised include: arginine, cysteine, isoleucine, leucine, methionine, threonine, valine, phenylalanine and tyrosine. Some strains also have a requirement for proline, whilst serine and threonine may supply their total carbon and energy requirements (Rowbotham, 1980).

Legionella spp. may be differentiated from other aquatic bacteria in that they are nutritionally fastidious on laboratory media. *Legionella* spp. have a requirement for L-cysteine and metal ions for growth, particularly iron (Pine *et al.*, 1986). For optimal growth *Legionella* requires iron in excess of 20 μM of iron compared with the requirements of other pathogenic Gram-negative and facultative intracellular bacteria of between 0.3 and 1.6 μM (Mengaud and Horwitz, 1993). The mechanism of iron uptake has not yet been fully elucidated but is possibly achieved by means of a periplasmic iron reductase (Johnson *et al.*, 1991). The availability of intracellular iron has been shown to be a factor in the permissiveness of certain cells for the proliferation of *L. pneumophila* within them (Gebran *et al.*, 1994). Zinc, magnesium and calcium have also been shown to stimulate growth in defined media (Reeves *et al.*, 1981).

L. pneumophila is nitrate negative and although it does not produce catalase (Pine *et al.*, 1986) it reduces hydrogen peroxide by the action of peroxidase thus giving a positive catalase test result with 3% H_2O_2 (Harrison and Taylor, 1988). The oxidase reaction gives variable results and therefore it is not useful for identification purposes. Most strains of *Legionella* will produce gelatinase (exceptions are *L. micdadei* and *L. feeleii*). Most legionellae are able to hydrolyse hippurate to form benzoic acid and glycine, with the exceptions of two serogroup 4 strains, Los-Angeles-1 and San Francisco-6 (Harrison and Taylor, 1988). Some species of *Legionella,* but not *L. pneumophila,* exhibit autofluorescence when examined under UV light at a wavelength of approximately 365nm.

Legionella is acid tolerant, withstanding exposure to pH 2.0 for short periods. Although the optimum pH for laboratory media is between 6.6 to 6.9, *Legionella* has been isolated from environmental sources within the pH range of 5.5 to pH 8.3 (Anand *et al.*, 1983).

L. pneumophila has been shown to be thermotolerant, it is able to withstand temperatures of 50 °C for several hours and 60 °C for several minutes. However at

temperatures in excess of 70 °C they are killed almost instantly (Dennis *et al.*, 1984; Dennis, 1988; Anon, 1991b). *Legionella* has been isolated from thermal ponds and springs, and from aquatic sources in the vicinity of a volcano (Tison and Seidler, 1983; Verissimo *et al.*, 1991; Campbell *et al.*, 1984). Isolates are readily obtained from many different environmental aquatic sources between 30 and 70°C (Fliermans, 1984), with temperatures between 40 and 60°C giving the best yields (Dennis, 1988). Yee and Wadowsky (1982), showed that naturally occurring *L. pneumophila* survived and multiplied in water at temperatures between 25° and 45°C, the optimum temperature being within the range 32 to 42°C, with the greatest increase in viable counts occurring between 37 and 42°C. As the temperature falls below 37°C the multiplication rate decreases with no observable growth below 20°C.(Anon, 1991b).

It was a considerable time before a suitable *in vitro* medium which would sustain the *Legionellaeceae* was devised (Feeley *et al.*, 1978; 1979). Prior to this, isolation in guinea pigs or hens' eggs was necessary (Mc Dade *et al.*, 1977; Morris *et al.*, 1979). In the laboratory *L. pneumophila* is fairly slow growing and it can take up to 10 days for distinctive colonies to form (Dennis, 1988). When grown on buffered charcoal yeast extract, (BCYE) and BCYE with added glycine, vancomycin, polymixin and cycloheximide (GVPC), it forms colonies with a distinctive ground glass morphology and an entire smooth edge. The colonies are pigmented and vary from a bluish/green in younger colonies to a pinkish/purple in slightly older colonies. As the colonies get older this colouration becomes less distinctive to give a 'fried egg' appearance in aged colonies, though a pinkish tinge may still sometimes be seen on the edges of the colony (Harrison and Taylor, 1988).

1.3. LEGIONELLOSIS

1.3.1.Legionellosis

Legionellosis is the term used to describe all the clinical manifestations caused by organisms within the genus *Legionella* (Beaty, 1984). *L. pneumophila* can cause both severe pneumonias and systemic infections in susceptible patients and also a milder form of legionellosis termed Pontiac fever. Epidemiological evidence strongly implies that the mode of infection is via the inhalation of *L. pneumophila* infected aerosols (Baskerville *et al.*, 1981; Hambleton *et al.*, 1983; Ager and Tickner, 1985). Droplet size plays an important role in *L. pneumophila* infection. Droplets of 5 μm or less are able to penetrate deeply into the lung (Fitzgeorge *et al.*, 1983), these droplets may contain in excess of 1000 colony forming units (CFU) of *L. pneumophila* (Rowbotham, 1986). There are no known cases of man to man or man to animal transmission (Yu *et al.*, 1983). The dose of *Legionella* required to cause disease has not yet been established, since the individual's immune status and the presence of predisposing conditions are factors in the susceptibility of the host (Anon, 1991a; Joly, 1993). Previous studies have shown that many infections with relatively mild symptoms have occurred which have not been recognised at the time as being due to *Legionella* (Joly, 1993). A significant number of the general population have been shown to have antibodies to *L. pneumophila* (Schlick 1993; Paszlo-Kolva *et al.*, 1993). *L. pneumophila* serogroup 1 is the most pathogenic of the legionellae accounting for approximately 90% of the cases of legionellosis (Roig *et al.*, 1993). Sixteen other species of *Legionella* have also been implicated in disease (Anon. 1991a; Hoge and Breiman, 1991; Facklam and Breiman, 1991).

1.3.2. Legionnaires' disease

Legionnaires' disease is a severe pneumonia, which is difficult to distinguish clinically from other pneumonias (Edelstein, 1993). The clinical definition of a person with Legionnaires' disease as adopted by the World Health Organisation is " A person with acute respiratory disease confirmed by abnormal chest x-ray, positive culture and/or a four-fold rise in antibody titre to *L. pneumophila* serogroup 1. Presumptive cases include positive urinary antigen and direct fluorescent antibody tests" (Plouffe, 1993). There are approximately 100-275 cases reported to the Communicable Disease Surveillance Centre in England and Wales each year, accounting for approximately 2% of the community acquired pneumonias in the UK (Anon, 1991b). The incidence of nosocomial Legionnaires' disease varies from approximately 0-14% (Joly, 1993). This figure does not appear to be solely dependent upon whether *L. pneumophila* is present or not in the hospital water supply but also on various other factors which probably include the virulence of the *Legionella* spp. in question. The usual incubation period is 2-10 days (Beaty, 1984), a recent report, however, suggests that colonisation of the respiratory tract by *L. pneumophila* may occur several weeks before the onset of Legionnaires' disease (Marrie *et al.,* 1992a).

The symptoms of Legionnaires' disease include fever, with a body temperature exceeding 40°C in some cases, headache, myalgia and malaise followed by a dry non-productive cough. Frequently neurological symptoms are presented which may include hallucination, coma, stupor and grand-mal seizures (Schlick, 1993). In approximately 50% of cases the patients present with gastrointestinal symptoms, which may include nausea, vomiting, abdominal pain and diarrhoea (Schlick, 1993; Mayaud and Dournon, 1988). In severe cases, extrapulmonary multiple organ failure may occur (Fumarola and Pece, 1992) and 20% of patients with severe Legionnaires' disease suffer from bacteriaemia. The attack rate for Legionnaires' disease is thought to be approximately 1% (Anon, 1991a) with a fatality rate, usually due to respiratory failure, of

approximately 12% (Anon; 1991a; Anon, 1991b); however, in immunocompromised patients this may be as high as 90% (Roig *et al.*, 1993).

1. 3. 3. Pontiac Fever

L. pneumophila is also the agent responsible for Pontiac fever. The first incidence of this occurred in 1968 in the County Health Department, Pontiac, Michigan (Hedlund, 1981). Pontiac fever is a self-limiting 'flu like' illness lasting on average 2-3 days, with an incubation period ranging from 5 hours to 3 days (Anon, 1991a). Symptoms include malaise, headaches, muscle pains, dizziness, cough, nausea and mental confusion, with some patients also suffering from diarrhoea. The infection rate is much higher than for Legionnaires' disease and an attack rate of 95% was reported in the outbreak in Pontiac, USA.

It is as yet unclear as to why exposure to *Legionella* results in two such different disease states; several theories exist which include as factors, the inoculum size, virulence, host factors (Rowbotham, 1986), viability of the *Legionella* or an allergic response to protozoa (Miller *et al.*, 1993).

1. 3 4. Non-*L. pneumophila* infections.

Infection by *L. pneumophila* other than serogroup 1 usually only occurs in those patients who have been immunocompromised (Mayaud and Dournon, 1988). In these patients other species of *Legionella*, e.g. *L. micdadei*, *L. feelei* and *L. dumoffii* have also been shown to cause pneumonic illness (Tomkins and Louitt, 1993; Harrison and Taylor, 1988). *Legionella micdadei* has also been shown to be responsible for a form of Pontiac fever called Lochgoilhead fever, which is a non-pneumonic illness similar to Pontiac fever but with a longer incubation period and a recurrence of the symptoms in some

cases. This epidemic was unusual in that a high proportion of the children present at the site in question, 31 out of 35, became infected (Fallon *et al.*, 1993).

1.3.5. Extra-pulmonary infections.

There have been several reports of *Legionella* causing infections where the respiratory tract is not involved, these are primarily in immunocompromised patients. These infections may be as a result of *Legionella* contaminated water used for irrigation or to wash wounds. *Legionella* has also been shown to be responsible for incidences of colitis and bowel abscesses (Edelstein, 1993)

1. 3.6. Predisposing factors to Legionnaires' disease

The incidence of Legionnaires' disease in healthy children and young adults is very low unless there are predisposing factors, for example, heart disease (Pastoris *et al.*, 1984). There have been however, cases of Pontiac fever and Lochgoilhead fever reported in children (section 1.3.4).

In adults there are several factors which determine a person's susceptibility to Legionnaires' disease; particularly at risk are patients who have an immunocompromised status (Arata *et al.*, 1992), especially patients receiving high doses of glucocorticosteroids and those receiving anti-rejection therapy following transplantation (Edelstein, 1993). Nosocomial infections are a significant risk for patients undergoing treatment in hospitals which have infected water supplies (Joly and Alary, 1993). Males are three times more likely to be infected than females, and persons over 50 years old are more likely to contract the disease. Other high risk patients include those who have recently undergone surgery, or who suffer from existing chronic diseases e.g. congestive heart failure, cancer, renal insufficiency, liver cirrhosis or those who have an existing respiratory disease or diabetes. The risk is also increased for people who are heavy

smokers or who are alcoholics (Mayaud and Dournon, 1988; Anon 1991b; Ramsey and Roberts, 1992; Edelstein, 1993).

1.4.. TREATMENT

The antibiotic treatment of choice is erythromycin, followed by rifampicin, though in some cases a cocktail of two or more antibiotics may be required (Ramsey and Roberts, 1992; Mayaud and Dournon; 1988, Facklam and Breiman, 1991). Patients treated with erythromycin have a higher survival rate compared with those treated with aminoglycosides, ß-lactams or chloramphenicol (Roig *et al.*, 1993). However erythromycin is only inhibitory and not bactericidal. A relapse may occur and there can be serious side effects including severe phlebitis, gastrointestinal disturbances and loss of hearing. Other possible side effects include; elongation of the Q-T interval and torsades de pointes arrythmias (Edelstein, 1993). Initial treatment is usually given intravenously as erythromycin lactobionate followed by erythromycin ethylsuccinate orally once the patient is apyrexic (Roig *et al.*, 1993). Treatment is usually continued for 2-3 weeks depending upon the patients' response and whether there was any other underlying disease or immuno-incompetence.

Although the ß-lactam antibiotics are active against legionellae *in vitro*, these results do not correlate well with the treatment of clinical infections. This is due to the inability of these antibiotics to permeate into the infected cells (Ramirez *et al.*, 1993). The failure of community acquired pneumonias to respond to ß-lactam antibiotics is an indication that *Legionella* may be the causative microorganism. However, animal studies with combinations of β-lactams used together with β-lactamase inhibitors appear to be effective. Other antibiotics suggested as possibilities for future treatment of Legionnaires' disease include the newer macrolides such as clarithromycin (Roig *et al.*, 1993), a new azalide, azithromycin (Donowitz and Earnhardt, 1993) and the

fluoroquinolones which have been shown to be bactericidal for *Legionella* (Ramirez *et al.*, 1993; Smith *et al.*, 1993).

1.5. SOURCES OF INFECTION

Investigations into outbreaks of Legionnaires' disease have shown that sources of infection often include hot water systems, especially in large institutions such as hospitals (Wadowsky *et al.*, 1982; Hsu *et al.*, 1984; Stout *et al.*, 1985; 1992, Alary and Joly, 1991; Bezanson *et al.*, 1992). Other sources of infection include cooling towers (Tobin *et al.*, 1981; Barbaree *et al.*, 1986; 1987), fountains, machine cutting coolants, misting devices, spa baths (Anon 1991), whirlpools (Henke and Seidel, 1986) and nebulisers (Arnow *et al.*, 1982). In all of these cases the source of infection had the ability to produce an aerosol.

Factors which may predispose man-made environments to infection with *L. pneumophila* include: the working temperature of the system remaining below 60°C, stagnation, which can occur in the dead ends of distribution system pipework and in storage tanks , the presence of certain nutritional sources (which may include the material of the system itself), scale, sediments and non-legionellae microorganisms (Anand *et al.*, 1983; Stout *et al.*, 1985; Barbaree *et al.*, 1986; Vickers *et al.*, 1987; Anon, 1991b; Nahapetian *et al.*, 1991; Lück *et al.*, 1991; Verissimo *et al.*, 1991; Stout, *et al.*, 1992).

Epidemiological evidence implies that Legionnaires' disease is caused by the inhalation of *L. pneumophila* in an aerosol derived from such an infected source (Hambleton *et al.*, 1983; Ager and Tickner, 1985). Survival of the bacteria in an aerosol depends on various factors including relative humidity, wind speed, temperature and bacterial metabolic activity and viability (Hambleton *et al.*, 1983; Anon, 1991b). Outbreaks have been recorded where the infective source was over 1 mile from the

exposed patients. In this case the humidity was high and atmospheric conditions were such that widespread dissemination of infected aerosols could occur (Addiss *et al.*, 1989). Recent studies have shown that *L. longbeachae* is also able to survive in damp potting soils in a free living form for several months (Steele, 1993).

1.6. LABORATORY DETECTION OF *LEGIONELLA*

Advances in technology have increased the choice of alternative methods which are available for the detection of *Legionella* in the laboratory. Because of the difficulties encountered in the culture of *Legionella*, laboratory diagnosis of Legionnaires' disease and environmental sampling tends to be carried out in relatively few laboratories. Important factors in the selection of diagnostic tests are not only the sensitivity and specificity of the test in question but also the technical ease of its' performance. Of the more conventional diagnostic methods, culture is still regarded as the 'gold standard' (Tomkins and Louitt, 1993). Specificity is 100% with a sensitivity varying between laboratories and ranging from 50 to 80% (Finkelstein *et al.*, 1993). An important advantage of culture is that isolates can be kept and subsequently sub-typed for epidemiological purposes. The appropriate training of laboratory staff is an important factor in the development and sensitivity of culture methods. Edelstein (1993) reported that a survey carried out by the College of American Pathologists showed that 32% of otherwise competent laboratories were unable to culture a pure heavy growth of *L. pneumophila*. *Legionella* may take several days to appear on laboratory media, such a delay may mean inappropriate antibiotic therapy being prescribed. Further cases may occur due to a delay in the detection and treatment of an environmental source of the disease.

Legionella in environmental samples may be inhibited by the presence of other microorganisms (Gomez-Lus *et al.*, 1993) or by the selective procedures used in the routine isolation from water samples (Reinthaler *et al.*, 1993). These factors together

with the occurrence of viable non-culturable *L. pneumophila* (Colbourne *et al.*, 1988; Byrd *et al.*, 1991; Yamamoto *et al.*, 1993, Tomkins and Louitt, 1993), mean that other methods of detection are necessary. Detection of viable non-culturable forms may be especially important from within fixed tissues and also following antibiotic or biocide treatment (Harrison and Taylor, 1988).

Indirect methods for the detection of *L. pneumophila* include radio immunoassay (RIA) for urinary antigens (serogroup 1 only), monoclonal antibody detection and DNA probe tests. The sensitivity of clinical serologic diagnosis of *L. pneumophila* serogroup 1 is approximately 70 to 80% by either microagglutination or indirect immunofluorescence (IFA). Specificity is dependent upon the type of antigen used, heat-treated antigen is less reliable than using formalised yolk sac-grown antigen which is between 99 and 99.6% specific for *L. pneumophila* serogroup 1. Detection of other serogroups is less successful (Edelstein, 1993). Direct fluorescent antibody (DFA) testing has a high specificity (99-99.3%) for serogroup 1 but the sensitivity is variable (25-70%), a major factor being the experience of laboratory personnel in the interpretation of results. There are however, several reports of cross reactivity with both IFA and DFA when these tests are used for environmental sampling (Alary and Joly, 1992). Cross reactivity occurs not only between serogroups of *L. pneumophila* and between non-pneumophila species (Wilkinson *et al.*, 1990) but also between non-legionellae (Alary and Joly, 1992; Kfir and Genthe, 1993). This technique therefore, should be used in conjunction with other methods for environmental sampling.

Latex agglutination has been shown to be a useful rapid method for presumptive identification of *Legionella*, especially from environmental samples to screen out the non-legionellae isolates (Wilkinson *et al.*, 1990). However, as with DFA, problems with cross reactivity can occur.

The urinary antigen test has a high specificity (>99.5%) with a sensitivity of between 80 and 90% but this may be lower in cases where the Legionnaires' disease is community acquired, culture negative and sero-positive (Edelstein, 1993). This test is available only for *L. pneumophila* serogroup 1 infections although these account for 90% of Legionnaires' disease infections (Roig *et al.*, 1993).

DNA probe tests are less technically challenging than DFA and one advantage is that they are able to detect all species of *Legionella* (Edelstein, 1993). Commercial kits are now available with both high specificity [100%] and sensitivity [75%] (Finkelstein *et al.*, 1993).

New methods of detection include polymerase chain reaction (PCR) and ligase chain reaction (LCR), though their usefulness as diagnostic aids has not yet been fully established. PCR is a method by which very small amounts of specific sequences of nucleic acids are rapidly amplified to detectable levels allowing analysis of genetic material (Pickup, 1991; Raymond *et al.*, 1992). The specific oligonucleotide primers and probes used vary according to the research establishment (Tomkins and Louitt, 1993). Some workers use a clone of an 800 base pair fragment and also a metalloprotease gene (*pro*). Other workers use primers and probes as templates which contain the 5S rRNA to detect all *Legionella* species (Louitt and Tomkins, 1993) whilst others use a region of the macrophage infectivity potentiator protein (MIP) gene (Nowicki *et al.*, 1993). PCR can be used to fingerprint successfully legionellae from both environmental and clinical sources (Miller *et al.*, 1993, Kessler *et al.*, 1993; Maiwald *et al.*, 1994) and to show the presence of *Legionella* in amoebal trophozoites and cysts (Jauhac *et al.*, 1993). A major advantage of this method is that it can also detect non-culturable forms of *Legionella* and it is not inhibited by the presence of non-legionellae microorganisms which can produce bacteriocins which may inhibit *Legionella* growth on conventional culture media (Gomez-Luz *et al.*, 1993b). The sensitivity of PCR when compared with culture was 57%, but with a high specificity of

93% (Tomkins and Louitt, 1993).

The main disadvantages of PCR are:-

1. high cost (Alvarez *et al.*, 1993),
2. it cannot be used retrospectively, since formalin fixation decreases the sensitivity of PCR (Schlenk *et al.*, 1993),
3. cells which are damaged and devoid of nucleic acids would not be detected (Palmer *et al.*, 1993) and
4. the presence of rust in the samples has been shown to inhibit amplification (Maiwald *et al.*, 1994).

LCR is a recent development which has an advantage over PCR in that it is able to detect minor mutations, and therefore it may be possible in the future to select between specific strains of *Legionella*, differentiating between avirulent and virulent environmental isolates (Tomkins and Lovitt, 1993). Its application in the future as an epidemiological tool looks promising.

A recent advance in the detection of *L. pneumophila* in environmental samples is described by Walker *et al.* (1993). *L. pneumophila* species specific fatty acids were detected by gas chromatography-mass spectrometry from within water distribution system biofilms which had been developed in a model biofilm system. This method, however, has not yet been tried in the natural environment.

Because of the limitations of each of the individual methods discussed a combination of techniques would seem to be the most appropriate approach to detect *Legionella* in environmental samples.

1.6.1. Subtyping.

Because *Legionella* is so prevalent in the environment it is necessary to identify with some degree of certainty the particular strain responsible for causing an outbreak of

legionellosis. This is essential in order to identify links between cases of community acquired Legionnaires' disease and the site responsible for the dissemination of *Legionella,* so that disinfection and any engineering modifications can be carried out as quickly and cost effectively as possible. There are 14 recognised serogroups of *L. pneumophila* which can be subdivided further into subgroups using various phenotypic, immunologic and genotypic variations (Winn, 1993). The detection of differences in strains of the same species (subtyping) is therefore an important epidemiological tool.

L. pneumophila serogroup 1 was initially separated into three major subgroups: Pontiac, Bellingham and Olda (Watkins *et al.*, 1985) by an indirect immunofluorescent antibody technique using monoclonal antibodies (MAbs). As more strains were discovered it became necessary to differentiate further into minor subgroups using panels of several MAbs. The definition of a subgroup depends to a large extent on the monoclonal antibody panel used. In order to introduce a degree of standardisation, collaboration between laboratories in the USA, UK and Canada resulted in the setting up of an International panel of seven MAbs for the subtyping of *L. pneumophila* serogroup 1 into 10 subgroups or subtypes (Joly *et al.*, 1986). This has now been extended to 12 (Barbaree, 1993). MAb subtyping may be useful as a marker for virulence as a positive reaction with the panel for MAb2 may be indicative of increased virulence which would suggest that whole or part of the surface epitope recognised as MAb2 may be involved in the virulence of this microorganism. This marker would be a further useful tool in epidemiological investigations (Lever, 1993; Mamolen *et al.*, 1993).

There are now several methods of subtyping using serologic techniques with both polyvalent and monovalent antibodies using DFA, IFA, slide agglutination and dot enzyme linked immunoassays. Recently some authors have found that MAb typing was not sufficiently discriminatory to establish a link between clinical and environmental isolates (Streulens *et al.* 1993, Ehret *et al.*, 1993); further separation was required using genotypic subtyping by a combination of other methods.

Other methods of subtyping reported in the literature include plasmid profiling, electrophoretic allo-enzyme typing, ribotyping, DNA digest analysis and outer membrane protein analysis using polyacrylamide gel electrophoresis (Edelstein *et al.*, 1986, Barbaree, 1993, Bej *et al.*, 1993) and hydroxy-fatty acid profiling (Jantzen *et al.*, 1993). Barbaree (1993) in his State Of The Art Lecture, reviews the techniques used and concludes that the range of methods are complementary but he expresses reservations about plasmid analysis as many strains, especially from clinical isolates, have been reported to be free of plasmids. This may be partly due to the procedures in the preparation of strains which could give misleading results (Edelstein *et al.*, 1986).

Molecular fingerprinting methods have been used to link some clinical and environmental isolates (Montoya *et al.*, 1992) leading to the positive identification of the source of the disease. Using a modified PCR technique, subtyping of *Legionella* spp. is now theoretically possible from a single colony within a few hours (Gomez-Luz *et al.*, 1993). However, this technique has not yet been fully evaluated for its usefulness in epidemiological monitoring purposes and it should still be used in conjunction with more orthodox techniques.

1. 7. PATHOGENESIS

Pathogenicity is the capability of a microorganism to cause disease in a host cell. In order for the disease process to occur the pathogen must be capable of entering the host cell and to replicate inside it. It does this whilst resisting or avoiding the hosts' natural defence mechanisms whilst continuing to cause subsequent damage or death to the host (Smith, 1977). *L. pneumophila* is described as a facultative intracellular pathogen and has been shown to infect and multiply in several cell types including human monocytes and alveolar macrophages (Daisy *et al.*, 1981; Rechnitzer *et al.*, 1992; Yamamoto *et al.*, 1992a) and in certain protozoa including *Hartmanella vermiformis, Acanthamoeba* spp. *Naegleria* spp. and *Tetrahymena pyriformis* (Collins, 1986; Fields *et al.*, 1986;

Rowbotham, 1980; Yamamoto *et al.*, 1992). *L. pneumophila* is able to proliferate in intracellular phagosomes in macrophage cells producing proteases with cytotoxic activity which results in localised tissue destruction (Hoffman *et al.*, 1990; Rechnitzer *et al.*, 1992). This growth pattern is similar to that observed in certain species of protozoa (Rowbotham, 1980; Moffatt and Tomkin, 1992). *Legionella* has also been reported to infect macrophages of susceptible animals e.g. guinea pigs, rats, gerbils (Collins, 1986; Arata *et al.*, 1992) and a certain susceptible strain of mouse A/J (Arata *et al.*, 1992). Not all animals are susceptible however, *L. pneumophila* challenges of horse and some species of bird e.g. leghorn chickens, quails, pigeons and most strains of mice (Marra and Shuman, 1992)) have proved negative. It would therefore appear that infection is not purely a consequence of the virulence of *L. pneumophila* but can also depend on the susceptibility of the host (Collins, 1986).

The main mode of uptake of *Legionella* by macrophage cells is by coiling phagocytosis (Horwitz, 1993). In humans, monocyte complement receptors CR1 and CR3, C3 complement component fragments and the major outer membrane protein (MOMP) of *L. pneumophila* mediate phagocytosis (Horwitz, 1993). Once internalised the legionellae are then able to multiply in the cytoplasm where they appear to utilise the host cell mitochondria, smooth vesicles and ribosomes (Marra and Shuman, 1993) thus rupturing the cell and releasing increased numbers of *Legionella* to infect further numbers of macrophages (Ramirez *et al.*, 1993). *Legionella* has been shown to survive the antimicrobial activity of human polymorphonuclear leukocytes (PMN) which would normally act as the body's second line defence against bacterial invasion (Summersgill *et al.*, 1988). Other authors, however, have shown that *L. pneumophila* phagocytosed by PMNs are usually killed. This was substantiated by experiments in which there was found to be a subsequent increase in the numbers of infected macrophages when PMN activity was prevented (Fitzgeorge *et al.*, 1988).

This capacity to proliferate intracellularly, resisting phagosome -lysosome fusion (Horwitz, 1983; Ramirez *et al.*, 1993) and inhibiting phagosome acidification and the host cells anti-bacterial mechanisms (Rechnitzer *et al.*, 1992; 1993; Lochner *et al.*, 1985) is linked to the virulence of the *Legionella* sp.

1. 7.1. Virulence mechanisms

Several studies have been undertaken to elucidate the mechanisms involved in determining the virulence of *Legionella,* using the following models:-

1. human alveolar macrophages (Ott *et al.,* 1991; Facklam and Breiman, 1991; Rechnitzer *et al.*, 1992),
2. U937, HL60, human embryonic lung fibroblastic (MRC5 and MRC9) and HeLa cell lines (Rodgers and Gibson, 1993; Kwaik *et al.*, 1993; Hacker *et al.*, 1991; Hoffman *et al.*, 1990)
3. rat alveolar epithelial cells (Mody *et al.*, 1993),
4. macrophages from the A/J strain of mouse (Yamamoto *et al.*, 1992),
5. various animal models including guinea pigs (Baskerville *et al.*, 1986; Rechnitzer *et al.*, 1992),
6. and certain protozoa (Hacker *et al.*, 1993).

The pathogenicity of *L. pneumophila* is still not fully understood but from these studies it would appear that there are several pathogenic factors contributing to the virulence of the bacterial cell which are regulated by genetic mechanisms. One of the factors which has been shown to be important in the ability of *L. pneumophila* to infect human alveolar macrophages and certain protozoa, is the macrophage infectivity potentiator [*mip*-gene] (Cianciotto and Fields, 1992). Mutations of this gene have been linked to decreased virulence (Cianciotto *et al.*, 1990) resulting in an approximate 80-fold decrease in the infection of both cell lines and guinea pigs (Sadosky *et al.*, 1993). Another role proposed for this gene is that it may be involved in protein folding mechanisms but the effect of this property on the pathogenicity of the microorganism is

as yet undetermined (Hacker *et al.*, 1993). Belyi (1993) proposed that the mechanism by which *Legionella* inhibits the host cells' microbiocidal activity, allowing the proliferation of the bacteria within the cell, may be by interference with the hosts' intracellular signalling system. The *mip*-gene is thought also to have a role in this antibacterial resistance activity (Cianciotto and Fields, 1992) together with other factors which have not yet been fully determined but which, in some *Legionella* sp., could include the activity of enzymes *eg*. acid phosphatases and catalase in the so called 'respiratory burst system' (Tully *et al.*, 1992).

L. pneumophila cytotoxic protease is believed to be another important factor in the pathogenicity of this microorganism (Baskerville *et al.*, 1986; Rechnitzer *et al.*, 1992; Williams *et al.*, 1993). This enzyme is also described in the literature as a zinc metalloprotease (Moffat *et al.*, 1994), tissue destructive protease/factor or *Legionella* major secretory protein (Rechnitzer and Kharazmi, 1992). This extracellular protease which is in the 38-42kDalton (kDa) size range, is unusual among bacterial proteases in that it exhibits three phenotypic properties: proteolysis, haemolysis and cytotoxicity (Grandi and Galli, 1992). It has been shown to be responsible for tissue necrosis and cell lysis in the lungs of infected guinea pigs and it is also thought to play a role in inhibiting the development of the cell mediated immune response (CMI). This is achieved by either, the deactivation of cytokines or by degradation of T-cell surface proteins (Mintz *et al.*, 1993). However, the activity of this protease is not essential for the infective capability of this microorganism, as aerosols of protease deficient mutants of *L. pneumophila* have still proved to be pathogenic to guinea pigs.

It has been proposed by Hoffman *et al.* (1990) that expression of heat shock proteins and cytotoxic proteases (Kwaik *et al.*, 1993) may be a stress response of *L. pneumophila* to the unfavourable environment within the host cell. Kwaik *et al.* (1993) found protein expression was markedly modified when the *L. pneumophila* were internalised in

macrophage cells, exhibiting a response similar to that shown by the *L. pneumophila* when exposed to other stress conditions.

Other proteins which have been linked to the expression of virulence include:- the MOMP (High *et al.*, 1993), heat shock proteins [HSP] (Kwaik *et al.*, 1993), two haemolytic proteins, one which lyses human erythrocytes (legiolysin) and a second with both proteolytic and cytotoxic activity (Hacker *et al.*, 1991). There are also several membrane associated proteins which have been implicated as virulence factors although their precise role has still to be established (Belyi *et al.*, 1993; Hacker *et al.*, 1991; Marra and Shuman, 1992).

Although in some species of pathogenic bacteria *eg. Salmonella; Shigella*, and *Yersinia* the role of plasmid inclusion is associated with virulence (Mintz *et al.*, 1992), this does not appear to be the case for *L. pneumophila* (High *et al.*, 1993). It is thought that plasmid inclusion increases survival in the natural aquatic environment (Tully, 1993), but the transfer of certain plasmids into avirulent strains did not have any effect on their infectivity of eukaryotic cells (Mintz *et al.*, 1992, Marra and Shuman, 1993). It is suggested that the genes for virulence are located within the chromosomal DNA (Marra and Shuman, 1992).

Flagella have an important role in pathogenicity in some bacterial species, for example, in *Salmonella* and *Pseudomonas aeruginosa*. Ott *et al.* (1991) compared the flagella on both virulent and avirulent strains of *L. pneumophila* and concluded that the possession of flagella was temperature dependant with increased expression at lower temperatures suggestive of an environmental role rather than a virulence mechanism. There was no appreciable difference observed between the virulent and avirulent strains.

1.7. 2.. Avirulent *L. pneumophila*

Environmental isolates of *Legionella* differ in their virulence towards eukaryotic host cells and this is not solely related to species differences. Bollin *et al.* (1985) found a difference between the virulence of two environmental isolates belonging to the same serogroup, the more virulent strain was also detected in patient samples.

L. pneumophila has been shown to lose virulence by passage over laboratory culture media (McDade and Shepard, 1979; Hambleton *et al.,* 1983). Early work suggested that virulence could be regained by passage through guinea pigs, embryonated hens' eggs or human embryonic lung fibroblasts (Elliott and Johnson, 1982, Wong *et al.*, 1981). Catrenich and Johnson (1988), however, showed that the virulent to avirulent conversion of *L. pneumophila* passaged over laboratory media was stable and that this virulence was not regained by passage through Guinea pigs. More recently, Yamamoto *et al.* (1993) suggested that the technique used in the passage of strains is important in the selection of a stable population of the selected strain. There have, however, been no instances of strains which are avirulent in model systems causing infection *in vivo* (Engleberg, 1993).

Recent studies, using avirulent *L. pneumophila* have shown that avirulent strains are unable to detect and respond to the intracellular environment, showing marked differences relative to the isogenic virulent strain in protein expression initiated as a stress response to survival following phagocytosis (Hoffman *et al.*, 1993). Fields *et al.* (1993) found that an axenic culture of the amoeba *Hartmanella vermiformis* was unable to take up avirulent *L. pneumophila* although initial attachment occurred at the same rate as the virulent strain. They proposed that these avirulent strains lack an infectivity factor essential for internalisation of bacteria into the protozoa under study. These avirulent strains are therefore not able to replicate in monocytes, macrophages (Hacker *et al.*, 1993) or protozoa (Moffat and Tomkins, 1992; Fields *et al.,* 1986; Fields, 1993; Ott *et al.*, 1993). It has been suggested that this inability of *Legionella* spp. to replicate

within protozoa can be used to distinguish between avirulent and virulent strains of legionellae (Ott *et al.*, 1993; Nahapetian *et al.*, 1993).

1.8.. *LEGIONELLA* AND PROTOZOA

Drozanski (1963) described bacterial amoebal parasites that had been isolated from soil but which failed to grow on laboratory media. From his description it is quite possible that these bacterial parasites were *Legionella* sp. Since the development of a suitable medium for *Legionella,* as described earlier, many workers have investigated the relationship between protozoa and *L. pneumophila.* Rowbotham (1980) was the first to report the relationship between amoebae and *L. pneumophila* and these findings have subsequently been confirmed and extended by several workers including Fields *et al.* (1984; 1986), Tyndall and Domingue (1982), and Wadowsky *et al.* (1988; 1991). The ability of *Legionella* to survive and replicate both in protozoal and mammalian cells is thought to be unique although there are unconfirmed suggestions that *Listeria* and *Mycobacterium* spp.,which are known human pathogens, may also be able to survive in protozoa (Fields, 1993). The ingestion of *L. pneumophila* (Fields, 1993) and its subsequent multiplication in amoebae and certain ciliates has been described by several workers. It has been also shown that *L. pneumophila* can survive in certain encysted amoebal cells (Skinner *et al.,* 1983; Harf and Monteil, 1988). This appears to be a species related occurrence which has been observed in *Acanthamoeba* spp. but not *Hartmanella* spp. (Fields, 1993). It is postulated that this could be the mechanism by which *L. pneumophila* is able to survive adverse conditions such as low winter temperatures and biocide treatments (Anand *et al.,* 1984; Rowbotham, 1980; 1984; Barbaree *et al.,* 1986; King *et al.,* 1988). Intra-amoebal.grown *L. pneumophila* have been shown to exhibit variations in growth patterns compared with those grown *in vitro,* including modifications in the lipopolysaccharide and fatty acid content of the *L. pneumophila* cell envelope (Barker *et al.,* 1993). It has been postulated, (Barker *et al.,* 1993) that this may be one of the mechanisms by which intra-amoebally grown *L.*

pneumophila express increased resistance to biocides. However, Steele (1993), has shown that free living legionellae can survive in soil for several months although intra-amoebal amplification of *Legionella* in the potting soil mixes occurred .

The mechanisms which enable *L. pneumophila* to survive once it has been ingested by amoebae have still not been elucidated fully. There does appear to be a degree of host specificity involved as not all species of amoebae are acceptable hosts. If *L. pneumophila* is ingested by an unsuitable host amoeba then it is either egested or digested (Rowbotham, 1984). It has also been suggested that host acceptability may be a temperature dependent phenomenon. At 35°C the *L. pneumophila* are able to proliferate inside amoebae (Rowbotham, 1980), whilst at 22°C the amoebae are able to digest the *Legionella* (Nagington and Smith, 1980; Anand *et al.*; 1983; Panikov *et al.*, 1993). Multiplication of *L. pneumophila* has been observed in various amoebae including *Acanthamoeba, Naegleria* and *Hartmanella* spp. and in the ciliate *Tetrahymena pyriformis* (Fields *et al.*, 1984; Tyndall and Dominique, 1982; Rowbotham, 1980; 1986; Wadowsky *et al.*, 1991; Smith-Somerville *et al.*, 1991). Although the exact role that this ability plays in the transmission of *Legionella* to man is still undetermined, there are various anecdotal theories.

Fry *et al.* (1991) have used amoebae to recover a non-culturable *Legionella* from clinical specimens. Rowbotham (1993) describes several cases of *Legionella*-like amoebal pathogens (LLAP) causing respiratory disease but which are not culturable on current media for *Legionella*. This raises the possibility that there may still be *Legionella* spp. which exist in the environment but which remain undetected because there is not, as yet, a suitably defined laboratory medium for their culture. .

1.9. INTERDEPENDENCE BETWEEN *LEGIONELLA* AND OTHER ORGANISMS.

L. pneumophila is widespread within the environment. It is an opportunistic human pathogen found in high numbers in both natural and man-made aquatic environments (Grimes, 1991). Water alone is not sufficient to allow *L. pneumophila* to proliferate. Skaliy and McEachern (1979) and Fields *et al.*, (1984), showed that in sterile distilled water and sterile tap water *L. pneumophila* showed long term survival but no multiplication. Yee and Wadowsky (1982), however, showed that naturally occurring *L. pneumophila* did survive and multiply in non-sterile tap water. Growth of *L. pneumophila* must therefore have been sustained either by nutrients already available within the tap water, supplied directly or indirectly by non-*Legionella* bacteria, or other associated microorganisms (Yee and Wadowsky, 1982; Stout *et al.*, 1985).

L. pneumophila appears to be capable of thriving in association with many different micro-organisms. The association of *L. pneumophila* with different species isolated from aquatic sources is well documented and includes protozoa, cyanobacteria, algae and other bacteria (Rowbotham, 1980; Tison *et al.*, 1980; Tesh and Miller, 1981; Fliermans *et al.*, 1981; Bohach *et al.*, 1983; Wadowsky *et al.*, 1983; 1988; Grimes, 1991; Pope *et al.*, 1982 and Hume and Hann, 1984). However, our understanding of the interdependence of *L. pneumophila* with other aquatic microorganisms is still far from complete (Yee and Wadowsky, 1982; Stout *et al.*, 1985).

1.10. BIOFILMS.

The effect of the presence of a surface resulting in increased bacterial activity was first noted by Whipple (1901). Zobell and Anderson (1936) concluded that this effect occurred in low nutrient conditions, possibly because the nutrients are concentrated at the solid/liquid interface (Zobell, 1943). Since this initial work many studies have recognised the importance of surfaces as sites of increased microbial activity. Surface-

associated microbial activity and colonisation, or 'biofilm formation' is a phenomenon that occurs in both natural and man-made environments. Biofilms are formed as a survival mechanism to withstand adverse conditions, such as nutrient limitation or temperature extremes (Dawson *et al.*, 1981; Kjelleberg *et al.*, 1983; Keevil *et al.*, 1989).

Conditioning of a surface by the adsorption of organic molecules, followed by colonisation of the surface are the first steps leading to the subsequent formation of a biofilm on a material (Trulear and Characklis, 1979; Allison, 1993). The mechanisms involved in bacterial adhesion are dependent not only on the physiological status of the microorganism (Boyle *et al.*, 1991), but also on the nature of the substratum. Adhesion to non-biological surfaces is thought to be non-specific, although the electrochemical nature and relative hydrophobicity of the surface are important factors in this process (Fletcher and Loeb, 1979; Dahlbäck *et al.*, 1981; Fletcher and Pringle, 1983; Konhauser *et al.*, 1994). Binding to living surfaces may also involve receptor interactions and may therefore be of a more specific nature. Rougher surfaces are preferentially colonised, providing niches protected from the effects of shear stresses, turbulent flow and biocide activity (Lytle *et al.*, 1989; Konhauser *et al.*, 1994).

The processes by which microorganisms adhere to surfaces in natural and industrial environments are still unclear, although the role of extra-cellular polysaccharide substances (EPS), or the glycocalyx, secreted by the cells, is thought to be important. It may also play a role in secondary colonisation by different species (Costerton *et al.*, 1985). Current studies suggest that these high molecular weight EPS molecules do not act directly as an adhesin, but that other factors, possibly low molecular weight polysaccharides which have been shown to be produced in trace amounts, mediate the initial colonisation process followed by higher molecular weight EPS production as a response to later events (Allison, 1993; 1994). It has recently been proposed that glycocalyx formation may be a microbial co-operative response to cell density limitations initiated by bacterial pheromones (Williams and Stewart, 1994). The

glycocalyx is a complex hydrated polyanionic polysaccharide matrix produced by polymerases affixed to the lipopolysaccharide component of the cell envelope and may be composed of a mixture of several species specific polysaccharides (Costerton *et al.*, 1978; 1981; 1985; Geesey, 1982; Gaylarde and Beech, 1989). The composition of this glycocalyx is thought to be dynamic and subject to change as the biofilm develops (Trulear and Characklis, 1982). The glycocalyx acts as an ionic exchange matrix which is able to trap metal ions (Geesey *et al.*, 1988; Ferris *et al.*, 1987) and nutrients which may then be transported into the cell by highly efficient permeases (Costerton and Geesey, 1979). It also plays a role in the conservation and concentration of digestive enzymes released by the bacteria, thus increasing the metabolic efficiency of the cells (Costerton *et al.*, 1978). The substrate itself, or its corrosion products, may then be incorporated into the biofilm (Keevil *et al.*, 1989, Ellis, 1990, Walker *et al.*, 1991, Beech *et al.*, 1991).

Biofilms in nature are not homogeneous. They consist of a consortium of microorganisms which may exhibit differing physiological and metabolic properties from their planktonic counterparts in response to the pH, oxygen and nutrient gradients which occur within this exopolysaccharide matrix (Kepkay *et al.*, 1986; Gilbert and Brown, 1994). As a result, various niches occur which may permit the co-existence, within a biofilm, of microorganisms with conflicting growth requirements eg. both anaerobic and aerobic bacterial populations may be isolated from the same biofilm (Keevil, 1994). Metabolic interdependence may occur between species, which may be a factor in the increased resistance to physical and chemical stresses exhibited by biofilm members (Caldwell *et al.*, 1993).

Resistance to biocide treatments has been shown to be increased in bacteria which are attached to surfaces and particulate matter within a system (Ridgway and Olsen, 1982, Kutchta *et al.*, 1985, King *et al.*, 1988; Vess *et al.*, 1993). A major role of the glycocalyx is that it constitutes a barrier affording the various constituents of the biofilm

partial protection from antibacterial agents (Costerton *et al.*, 1981; Cloete *et al.,* 1989), and from the possible toxic effects of the substrate upon which a biofilm may form, for example, copper pipes within water distribution systems (Keevil *et al.*, 1989). Nichols (1994) reviews the mechanisms which may account for this protective property of the glycocalyx and suggests that the answer is not solely due to the physical impedence of the antimicrobial agent in question, but that there may be other factors such as absorption or catalytic destruction of the agent by microbes on the biofilm surface. It is unclear whether a phenotypic response of the microbial population to surface growth also plays a role in this increased resistance (Jass and Lapin-Scott, 1994). The answer may well prove to be a combination of several factors.

A biofilm is not a static entity as sloughing and erosion processes can result in the detachment of portions of a biofilm as a result of the hydrodynamic conditions or shearing forces occurring within a system (Characklis, 1981; Taylor and Bishop, 1985). The rate of this detachment may be related to the specific bacterial population, since some species have been shown to be more susceptible to these shear stresses (Oga *et al.*, 1991). The growth limiting factor in a system may also have an effect on the rate of shear and/or sloughing. Applegate and Bryers (1990), showed that appreciable differences in both shear and sloughing events could be observed between biofilms that were oxygen limited compared with those undergoing carbon limitation.. Sloughing or erosion may occur at any time during biofilm development resulting in the re-suspension of the microorganisms from the biofilm within the planktonic phase of the system (Trulear and Characklis, 1982). These may include potential human pathogens such as *L. pneumophila*, *Cryptosporidium* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Staphylococcus* spp, *Rotavirus*, and *Giardia*, enteroviruses, mycoplasmas and protozoa (Rowbotham 1980; van der Wende *et al.*, 1988; Reasoner, 1988; Keevil *et al.*, 1989; Alary and Joly, 1991; Boyle *et al.*, 1991; Emde *et al.,* 1992). Cells may also actively detach from the surface and subsequently relocate on the substratum, a process termed desorption (Escher and Characklis, 1988).

Biofilms then, are composed of areas consisting of microcolonies or stacks of dense microbial population and glycocalyx. These stacks are interspersed with sparsely populated areas created by the sloughing of the biofilm or grazing of the biofilm by higher organisms such as protozoa. These less dense areas act as channels which allow the flow of nutrients and extracellular products around biofilm stacks (Costerton *et al.*, 1994).

Biofilms are ubiquitous, they are found world-wide in a very diverse range of habitats, both natural and man-made. They may exist as beneficial epilithic communities in rivers and streams, in waste water treatment plants on trickling filter beds and in the alimentary canal of mammals (Hanaki *et al.*, 1982, Costerton *et al.*, 1986, Bryers and Characklis, 1982). Within biofilms pathogenic microorganisms, including those resident in humans and animals, may be able to evade the immune response of the host, gain increased protection against antimicrobial drugs and exhibit an increased level of virulence (Allison, 1994; Williams, 1994; Brown *et al.*, 1991; Evans *et al.*, 1994). They may occur for example, as dental plaque on teeth and dentures causing caries and gum disease (Keevil *et al.*, 1987, Marsh *et al.*, 1994). They may also form on medical prostheses, including pacemakers, replacement joints and indwelling catheters, where the colonising microorganisms cause chronic infections in the surrounding tissue which may develop into septicaemia or cause embolisms (Wilcox, 1994). In many cases the removal of the infected prosthetic is required to prevent recurrent life threatening infections (Finch, 1994; Wilcox, 1994). Biofilm formation has also been implicated in gallstone formation and in the plugging of urinary catheters and biliary stents (Stickler *et al.*, 1993; Stickler and Winters, 1994; Sung *et al.*, 1992).

Biofilms can be the cause of costly problems for many industries including the water industry. The costs are both direct and indirect.

Direct costs include:-

1. a decrease in product quality (Väisänen *et al.*, 1994),

2. spoilage of product eg. in the food and paint industry (Holah *et al.*, 1994; Eastwood, 1994),
3. physical and chemical biofilm removal ,
4. increased maintenance requirements and the replacement of corroded pipelines.

Indirect costs may be due to :-

1. biofouling of pipes which increases frictional resistance, (McCoy *et al.*, 1981),
2. reduced efficiency due to decreased pipeline capacity (Shariff and Hassan, 1985),
3. increases in energy requirements (Picologou *et al.*, 1980; Bott, 1994),
4. the cost of research programmes into the cause and successful elimination of biofilms which is also not inconsiderable.

Microbial biofilms then, by their very nature, are extremely complex microbial ecosystems that are difficult to study by conventional microbiological techniques. They are often heterogeneous and may consist of bacteria, algae, and grazing protozoa which may display morphological features not usually associated with the microorganisms when grown in pure culture (Cloete *et al.*, 1989).

1.11. BIOFILMS IN WATER DISTRIBUTION SYSTEMS.

Drinking water is not sterile. The main purpose of water treatment is to remove potential pathogenic microorganisms and to reduce turbidity (Mackerness *et al.*, 1991; Hutchinson and Ridgway, 1977). There is a diverse range of microorganisms which can exist naturally within potable water systems, these usually occur as mixed consortia and may include bacteria, fungi, viruses, yeasts ,protozoa, diatoms and algae (Keevil *et al.*, 1989; Haudidier *et al.*, 1988; Walker *et al.*, 1991; Le Chevallier *et al.*, 1987). The occurrence of biofilms within domestic and industrial aquatic systems is well documented (Costerton, 1984; Costerton *et al.*, 1987; Le Chevallier *et al.*, 1988; Colbourne *et al.*, 1988). Biofilms within such systems create not only a hazard to health

by the harbouring of potential pathogens including *L. pneumophila* which may exhibit increased resistance to biocide treatment (Colbourne and Dennis, 1987; Keevil *et al.*, 1989; Vess *et al.*, 1993), but can also be responsible for the deterioration of the quality of the water affecting its odour and taste (Le Chevallier and McFeter 1985; Haudidier *et al.*, 1988). Biofilm formation occurs not only on the surfaces of the water distribution systems themselves, but may also occur on particulate matter which may be suspended within the system. It may also form on sediment or on the surfaces of algae or micro-crustaceans within a system (Herson *et al.*, 1991). Metal oxidation and deposition and corrosion have also been associated with biofilms within distribution system pipework (Le Chevallier *et al.*, 1987; Lee *et al.*, 1980; Geesey *et al.*, 1988; 1994; Sly *et al.*, 1988, Gaylarde and Beech, 1989; Keevil *et al.*, 1989; Walker *et al.*, 1991).

The use of polymeric materials to replace traditional plumbing materials has been on the increase for many years. The use of PVC (polyvinyl chloride) pipes within water distribution systems is commonplace within industrial, hospital and domestic systems (Vess *et al.*, 1993). Organic compounds which leach from the surfaces of these components may provide a source of nutrients for those microorganisms which subsequently colonise them (Ashworth and Colbourne 1985; Pedersen, 1990; Rogers *et al.*, 1991). Biofilm formation on such surfaces may lead to a rapid decrease in water quality and subsequent failure to meet the required standards (van der Wende *et al.*, 1988, Anon, 1982; 1983). Although there is a British standard (BS6920) for these materials, it is concerned with growth of microorganisms in the planktonic phase and does not take into account biofilm formation. Materials which perform satisfactorily in the laboratory may, when used in certain circumstances, allow microbial attachment and support subsequent growth. This may result from their use in combination with less suitable materials or where local conditions do not meet the required standard, for example, in large buildings where unlagged hot and cold water system pipework run side by side (Ashworth and Colbourne, 1987).

Rogers *et al.* (1990) compared a range of different materials used in plumbing systems for their ability to support the growth of microorganisms. Copper was the most resistant to biofilm formation whilst ethylene-propylene and latex were the most susceptible. In a similar study Bezanson *et al.* (1991) found that PVC was significantly more susceptible to colonisation than copper or brass. Despite the natural resistance of copper to biofilm formation, corrosion of copper pipework has been shown to be due to microbially mediated biodeterioration and this is a particular problem in certain Scottish hospitals which are situated in areas which are fed by soft water (Keevil *et al.*, 1989; Walker *et al.*, 1991). Dosing regimes with chlorine based biocides have, in some cases, lead to the failure of plumbing systems requiring costly replacement (Grosserode *et al.*, 1992; Keevil *et al.*, 1989a).

Regrowth or aftergrowth, are the terms used to describe the continued proliferation of potential pathogens in a water distribution system downstream of a treatment plant (Keevil *et al.*, 1989b; Jaeggi and Schmidt-Lorenz, 1988; Power *et al.*, 1988; LeChevallier *et al.*, 1991). Regrowth, following microbial recovery from sub-lethal injury and biofilm regrowth or recontamination may be a consequence of the reduced effectiveness of biocide treatments (Wright *et al.*, 1991; Le Chevallier *et al.*, 1988; Yamamoto *et al.*, 1991; Power *et al.*, 1988). The effect of temperature is also an important factor in the regrowth of biofilms within water distribution systems, with periods of warm weather corresponding to increased incidences of bacterial growth (Le Chevallier *et al.*, 1991).

1. 12. Biofilms and *Legionella*

The growth of *L. pneumophila* in water systems is well documented (Wadowsky *et al.*, 1982; Wadowsky and Yee, 1985; States *et al.*, 1987; Le Chevallier *et al.*, 1987; Keevil *et al.*, 1989; Marrão *et al.*, 1993). Particularly at risk are cooling towers (Broadbent *et al.*, 1991; Grabow *et al.*, 1991), 54% of cooling towers examined in England and Wales

were found to be contaminated with *Legionella* (Colbourne and Trew, 1986). Other risk areas include the water distribution systems in large buildings such as hotels and office blocks and especially hot water supplies in hospitals , where nosocomial infection as a result of the presence of *Legionella* is not uncommon (Stout *et al.*, 1992). In public buildings such as hospitals, where energy conservation and fears of scalding patients have resulted in the temperature of hot water supplies being lowered, incidences of Legionnaires' disease have occurred (Plouffe *et al.*, 1983). Water tanks kept below 60 °C are significantly more likely to be associated with *L. pneumophila* isolation (Vickers *et al.*, 1987).

Legionella has been shown to occur in the sediments which settle at the bases of cooling towers. It has often been found in association with both free living and encysted protozoa and in biofilms colonising pipework, showerheads and washers within water distribution systems (Colbourne *et al.*, 1984; Schofield and Locci, 1985; Le Chevallier *et al.*, 1987). States *et al.* (1985) and Vickers *et al.* (1987) found that certain metals and their corrosion products found in plumbing systems, notably iron, zinc, potassium, magnesium and calcium, also enhanced the growth of *L. pneumophila.* The construction material of the system and the working temperature of the system are also other important factors in the continued survival of *L. pneumophila* in water systems (Walker *et al.*, 1993; Bentham, 1993). It has also been shown that *Legionella* associated with biofilms in water distribution systems has developed an increased resistance to biocides and can survive concentrations of chlorine which are lethal to coliform bacteria (Hsu *et al.*, 1984; Kuchta *et al.*, 1985).

1.13. PREVIOUS BIOFILM STUDIES

Previously the main areas of microbiological research in water distribution systems have concentrated on the problems associated with turbidity and coliform presence (Reasoner, 1988). The difficulties associated with studying a water distribution system *in situ*

suggest that a model of such a system in the laboratory would be the most practical approach. Some model biofilm systems used in previous studies, have not effectively mimicked the natural systems because recirculating or closed loop systems have been used or nutrients have been added to the system (Lake, 1988; Hanaki *et al.*, 1982). Addition of nutrient will result in the production of biofilms not representative of those in water distribution systems, since in the environment, biofilm formation often occurs in low nutrient conditions rather than in the relatively high nutrient concentrations often provided in the laboratory.

Other investigations with *L. pneumophila* have included batch culture studies (Pine *et al.*, 1979). Under these conditions *L. pneumophila* exhibited varying phenotypic characteristics. In batch culture the environment within the system is constantly altering as nutrients are utilised and the concentrations of metabolic products, which may be toxic to the microorganisms being studied, rise. Growth limiting factors may result in phenotypic variations which may not be representative of those found in the environment that the model system is meant to simulate.

Continuous culture of microorganisms allows steady state conditions to be established in which microorganisms can grow at a constant rate in a maintained environment. Parameters such as oxygen concentration, temperature, nutrient concentration and pH can each be altered as necessary to resemble as closely as possible those in the environment being modelled (Herbert *et al.*, 1956). Continuous culture studies of *L. pneumophila* have been carried out by Berg *et al.* (1984, 1985) using nutrient enhanced media. Although these studies have increased our understanding of the biochemistry and physiology of *L. pneumophila,* they have not added to our knowledge of the way *L. pneumophila* obtains nutrients in the natural environment. Strains which have been grown on agar, differ from freshly isolated environmental strains, Wadowsky *et al.* (1984), suggest that *L. pneumophila* grown in a natural environment under low nutrient conditions exhibited increased resistance to biocide treatments when compared with *L.*

pneumophila isolated from solid culture media. It is therefore essential that the growth conditions of the microorganism in the laboratory resemble as closely as possible the conditions found in the environment.

The model system chosen for use in this study was a continuous culture model biofilm system first developed by Keevil *et al.* (1987), to study dental plaque formation. This system had subsequently been used (Keevil *et al.*, 1988; 1990; Rogers *et al.*, 1991;1993) as a model water system to successfully develop aquatic biofilms including a virulent strain of *L. pneumophila.* Plans were kindly supplied by PHL, CAMR, for the in-house construction of the titanium head plate. The materials used, titanium and glass, were chosen for their chemical stability. The titanium head plate had ports which allowed easy insertion and removal of supports for biofilm formation.

1.14 BACKGROUND TO THIS STUDY

Although it is many years since *Legionella* was first isolated, it is still difficult to isolate and culture. It is now almost twenty years since the Philadelphia epidemic and *Legionella* still remains a cause of concern for public health authorities. Outbreaks of Legionnaires' disease still occur and cause deaths. Geographically isolated incidences of Legionnaires' disease may be the result of infection from the same source, the ease of travelling long distances in a short space of time means that a common source may not be quickly identified. The validity and cost effectiveness of routine sampling of potentially infective sources is questioned because:-

- *Legionella* spp. are ubiquitous within the natural environment,
- it is likely that there are still species as yet unidentified which may or may not be pathogenic,
- the numbers of *Legionella* needed to infect a water system before there is a disease threatening situation, is still unknown
- several species of *Legionella* may be isolated from sites within close proximity

to each other (Marrie *et al.*, 1992; Bezanson *et al.*, 1992), and

- their presence does not necessarily cause disease (Fliermans *et al.*, 1979).

Where nosocomial infection is a serious potential problem to high risk patients, routine testing for *Legionella* is a sensible precaution (Liu *et al.*, 1993). As stated earlier (Section 1.9.) it has been established that *L. pneumophila*, although widespread within aquatic systems, cannot grow in sterile water. Biofilms in man made aquatic environments such as those within distribution systems provide ecological niches ideally suited to the survival and growth of *L. pneumophila* (Colbourne and Trew, 1986). It is clear that biofilm formation within such systems is of economic and epidemiological importance. Kutchta *et al.* (1985), suggested that infection of a system may occur by the seeding of a small number of *L. pneumophila.* Therefore, small numbers of resistant *L. pneumophila* surviving biocide treatment, can have important implications in the subsequent proliferation of *L. pneumophila* within water distribution systems particularly in hospitals where nosocomial infection is a problem for a susceptible population. A better understanding of the inter-relationships between *L. pneumophila* and other aquatic microorganisms within such a system may lead to more efficient eradication methods and a subsequent decrease in the incidence of Legionnaires' disease. The ability, therefore, of *Legionella* spp. to exist within man made water systems and man's inability to eradicate it successfully by either physical or chemical methods together with the problems involved with undertaking routine sampling as discussed above, mean that there are still many areas where there is a need for further research.

A major problem for many laboratories wishing to handle *L. pneumophila* is that of containment. To investigate *L. pneumophila* within a water system means that there is a real risk for the researcher of being exposed to infected aerosols. Keevil *et al.* (1988; 1989; Keevil, 1991), Rogers *et al.* (1991; 1993), use class III cabinets and positive laboratory pressure to house their chemostats in order to reduce the risks of aerosol

exposure to acceptable limits. Class III containment facilities are very expensive and have to be checked and maintained at regular intervals.

1.15. AIMS AND OBJECTIVES

1. In this research, an investigation was carried out into the feasibility of using an avirulent strain of *L. pneumophila* serogroup 1 in a model water system.

2. Once it had been established that this avirulent strain was able to integrate into the biofilm and planktonic phases of the model system, further studies were carried out into the inter-relationships between *L. pneumophila* and other aquatic microorganisms.

3. Successful integration of this avirulent strain allowed investigations to determine if this avirulent strain was suitable for safer evaluation of biocides for use in aquatic systems susceptible to *L. pneumophila* infection, without the need for extensive containment facilities.

An initial study was undertaken therefore, to determine if an avirulent *L. pneumophila* could integrate into a model system containing a naturally occuring consortium of water distribution system microorganisms. Microorganisms within a continuous culture system must be able to multiply at least at the same speed as the dilution rate if they are not to be lost from the system, a process generally known as washout. Thus, in order for *L. pneumophila* to survive in a continuous culture system it must be able to replicate at the dilution rate. This is especially relevant in a model system containing a consortium of naturally occurring microorganisms, all competing for the low concentrations of nutrients that are available within the filter sterilised water used as the medium. This avirulent *L. pneumophila* had not previously been introduced into a 'natural' situation in which there is a mixed microbial community including bacteria and protozoa.

Chapter 2 presents an account of an experimental investigation into whether an avirulent strain would be able to survive when introduced into a model system containing a consortium of microorganisms isolated from the local water distribution system and whether this avirulent strain would integrate successfully into biofilms developed in this system.

Heat treatment is routinely used to increase the recovery of *L. pneumophila* from environmental samples. Since it was not known if this attenuated strain would exhibit the same degree of heat tolerance; an investigation into the relative heat tolerance of this strain was also undertaken.

The avirulent *L. pneumophila* serogroup 1 Pontiac (Corby Strain) used in this study was obtained by passage 32 times over BCYE (Jepras and Fitzgeorge, 1985). It was unable to survive in cultured alveolar macrophages, did not cause infection or death in guinea pigs exposed to the aerosolised avirulent *L. pneumophila* and had shown no reversion to virulence over a four year period (R. Fitzgeorge, personal communication). This attenuated strain had not however, been introduced into a mixed consortium of aquatic microorganisms where its continued survival would be dependent on virulence factors. A study was therefore carried out to determine whether this loss of virulence was stable in such a mixed consortium, this study is also described within chapter 2.

In order to gain further insight into the proliferation of this microorganism in the environment a greater understanding of the relationship between virulence and *L. pneumophila* survival in natural systems is essential. If the hypothesis that *L. pneumophila* survives adverse conditions by replication within protozoa is true, then it follows that virulence is an important factor in the long term survival of *L. pneumophila*. It is not known whether the survival of *Legionella* within such an ecosystem is dependent on this relationship with protozoa. If this avirulent form of *L. pneumophila* is to survive then it must either revert to a virulent form or it must possess other

mechanisms of proliferation and survival. One of the goals of this study was to determine whether the survival of this *L. pneumophila* serogroup 1 Pontiac (Corby strain) once integrated into a continuous culture model biofilm system was linked to virulence. Studies were undertaken to establish whether the avirulent *Legionella* used in this study was able to infect and multiply within an axenic strain of *Acanthamoeba polyphaga*. Chapter 3 provides an account of this work.

Legionella has also been shown to grow in association with other microorganisms, including cyanobacteria and bacterial species including *Flavobacterium* sp. which have been shown to support the growth of *L. pneumophila* on cysteine free media by producing extracellular-cysteine or a substitute. Bacterial members of the biofilm community were investigated to determine whether some of these were able to support *L. pneumophila* growth in batch culture and on cysteine free BCYE. An account of this work is presented in Chapter 4.

To assess biofilm development, direct visualisation techniques are required. Chapter 5 describes a comparative account of various methods which were carried out including the new techniques of atomic force microscopy and Hoffman modulation contrast microscopy. Methods for the direct detection of *L. pneumophila* within biofilms were also carried out.

Chapter 6 presents an account of experimental work undertaken to determine whether this avirulent strain was useful in terms of a model for the virulent strain. Because of the obvious restrictions in the use of virulent strains of *L. pneumophila* in industrial test methods, the use of an avirulent substitute is particularly appealing. The avirulent test organism must, however, show similar responses under test as the virulent *L. pneumophila*. The testing of biocides for use in water systems is an important area in industrial microbiology therefore, a comparative study was carried out on the effects of a biocide on *L. pneumophila* serogroup 6 and the avirulent strain.

CHAPTER 2

THE INTEGRATION OF AN AVIRULENT *L. PNEUMOPHILA* INTO A MODEL BIOFILM SYSTEM.

2. The integration of an avirulent *L. pneumophila* into a model biofilm system.

This chapter describes a series of investigations to determine:

1. whether an avirulent strain of *L. pneumophila* serogroup 1 is able to integrate into a naturally occurring consortium of aquatic microorganisms;

2. whether the avirulent strain of *L. pneumophila* used in this study can be recovered following incubation at 50 °C; and

3. if this avirulent strain is successful in integrating into the system, whether the loss of virulence of this strain is maintained in such a system.

2.1. Introduction.

It is nearly twenty years since the Philadelphia epidemic and *Legionella* still remains a cause of concern for public health authorities. Outbreaks of Legionnaires' disease still occur and cause deaths. Although there has been much research since *L. pneumophila* was first isolated and there have been four international conferences devoted solely to this research, *L. pneumophila* has still not been eradicated from man-made aquatic systems. There are still many fundamental questions which remain unanswered concerning the relationship between the causative organism *L. pneumophila* and Legionnaires' disease in man. Such questions include:

1. What is the the number of *Legionella* in a water system required to produce a disease threatening situation ? (Breiman and Barbaree, 1993)
2. Are there still species, as yet unidentified, which may be pathogenic ?

3. Why their presence does not necessarily cause disease (Fliermans *et al.*, 1979) and is this due solely to the susceptibility of the patient or are there other factors which increase the risk of infection ?
4. Are these ecological or virulence factors? (Joly, 1993)
5. What exactly is the role of amoebae in the proliferation and survival of *L. pneumophila* in the natural environment ? (Breiman and Barbaree, 1993).
6. Can *L. pneumophila* survive without the presence of amoebae?
7. How many less severe cases are occurring which are not identified? (Joly, 1993).
8. How can *L. pneumophila* be successfully eradicated from within man-made aquatic systems?

A better understanding of the inter-relationships between *L. pneumophila* and other microorganisms within an aquatic system may lead to the development of more efficient eradication methods and a subsequent decrease in the incidence of Legionnaires' disease. A major problem for many laboratories wishing to handle *L. pneumophila* is that of containment. To investigate *L. pneumophila* within a water system means that there is a real risk of the researcher being exposed to infected aerosols. Keevil *et al.* (1988; 1989; 1993), Rogers *et al.* (1991; 1993) and Mackerness *et al.* (1993), used class III cabinets and positive laboratory pressure in which to house their chemostats in order to reduce the risks of exposure to aerosols to acceptable limits. Class III containment facilities are very expensive and have to be checked and maintained at regular intervals.

The first part of this study was to determine whether an avirulent *L. pneumophila* could integrate into a model system containing a naturally occurring consortium of water distribution systems microorganisms. The avirulent *Legionella pneumophila* serogroup 1 Pontiac (Corby Strain) used in this study had lost virulence by passage 32 times over BCYE. This strain was unable to survive in cultured alveolar macrophages and did not

cause infection or death in Guinea pigs exposed to it (Tully *et al.*, 1992). This avirulent strain had shown no reversion to virulence over a four year period (R. Fitzgeorge, personal communication.). Virulence is the capacity of a microorganism to infect and cause disease and must therefore, be an important factor in the ability of *L. pneumophila* to proliferate in the natural environment. The survival of an avirulent *L. pneumophila* which has not previously been introduced into a 'natural' environment containing a mixed community of bacteria, protozoa, fungi and yeasts cannot be taken for granted, particularly in a situation where the avirulent *L. pneumophila* would be in competition for the low concentrations of nutrients in potable water. To be useful in terms of a model for the virulent strain, this avirulent strain must remain avirulent in such a situation. This second part of the study sets out to determine whether an avirulent *Legionella pneumophila* serogroup 1 Pontiac (Corby strain) can integrate into continuous culture model biofilm system and if it can remain stable or whether it would revert to its virulent form.

L. pneumophila has been shown to be more thermotolerant than many other aquatic isolates (Dennis, 1984; 1988; Anon, 1991b). Because *L. pneumophila* grow in association with other microorganisms (Stout *et al.*, 1985; 1992; Anon, 1991; Barbaree *et al.*, 1986) environmental samples are often heavily contaminated with a mixed bacterial flora which may outgrow and mask the more slow growing *L. pneumophila* (Dennis, 1988). Some bacterial isolates have been shown to inhibit the growth of *L. pneumophila* on solid laboratory media (Gomez-Lus *et al.*, 1993). This property of thermotolerance has been used to increase the recovery of *L. pneumophila* from environmental samples by pre-treatment with heat prior to culture (Dennis *et al.*, 1988). Because the continuous culture model used in this study was inoculated with a mixed consortium of microorganisms, the use of heat treatment would optimise the recovery of the *L. pneumophila* from the system. It was not known whether the avirulent strain would exhibit the same degree of heat tolerance and therefore, whether it could be recovered following heat treatment in the same manner as the

virulent strain. A study was carried out therefore, to determine whether there was any difference in the thermotolerance of this avirulent *L. pneumophila* and the corresponding virulent *L. pneumophila* serogroup 1 Pontiac (Corby) strain. This work forms the third part of this study.

2. 2. An investigation to determine whether an avirulent strain of *L. pneumophila* serogroup 1 is able to integrate into a naturally occurring consortium of aquatic microorganisms.

2. 2. 1. Materials and Methods.

Previous studies by Keevil *et al.* (1987, 1989), Walker *et al.* (1991), Rogers *et al.* (1990) used an existing consortium of aquatic microorganisms from the source of an outbreak of Legionnaires' disease, which included *L. pneumophila* serogroup 1. Unlike these previous studies the inoculum used in this study was obtained from a local water distribution system and did not initially contain *L. pneumophila*. The materials used in the system were chosen to avoid any alteration in the chemistry of the water.

2.2.1.1. The chemostat.

A continuous culture model biofilm system based on that described by Keevil *et al.*, (1987, 1988) was set up in the laboratory (Fig 2.1.). The materials used in the construction of the model system were chosen for their relative chemical stability, to minimise any changes to the water chemistry. It has been previously determined that attachment of bacteria and their subsequent activity can be affected by the chemical composition of the substratum (Fletcher, 1979). The head was constructed 'in house' from titanium which is increasingly used in orthopaedic and dental implants for its physiological properties and its corrosion resistance (Kohavi *et al.*, 1991). Titanium, unlike stainless steel does not leach metal ions eg. iron, manganese and chromium (Rogers and Keevil, 1992) which would alter the water chemistry and which therefore, may have an effect on the growth of the microorganisms within the system. All tubing used was of autoclavable food grade silicone rubber (Esco Rubber Ltd.). The top plate of the vessel had eight large ports (22mm), and four small ports

(10mm) and was clamped to the Pyrex glass base (Jencons Ltd, UK.) and sealed with a silicone rubber O-ring. The medium which was filter sterilised mains tap water was introduced into the vessel via an anti-growback device through one of the small ports at a dilution rate (D) of 0.05 h^{-1}. The aqueous phase was maintained at 1000 ml via a weir system attached to a peristaltic pump to a discard bottle (Fig. 2.2.). A temperature probe housed in a glass sheath (Brighton Systems, Ltd), pH and oxygen electrodes (Uniprobe), were fitted into the head via silicone rubber bungs. Temperature was monitored by the probe inserted into the aqueous phase of the vessel and maintained at 30 °C ± 1 by use of an external heater pad under the base of the vessel, via an Anglicon Biotech Solo Controller (Brighton Systems, UK.). The dissolved oxygen concentration was maintained at approximately 20% by adjusting the stirrer speed. The pH was monitored but not adjusted.

2. 2. 1.2 Continuous culture media preparation

The local mains tap water is soft lowland catchment. Ten litres of this mains tap water was sterilised by the method of Colbourne *et al.* (1988) to avoid any alteration in the chemical and nutrient status of the water. A peristaltic Watson and Marlow pump was used to aid the passage of the water through a 0.22 μm nylon membrane (Pall, Portsmouth) housed in a 90 mm Millipore stainless steel filter holder. The whole unit containing the filter was autoclaved prior to use. The sterile water was filtered directly into pre-sterilised 10 litre Nalgene containers via a modified titanium sampling port. The sterile water was then used as the continuous culture growth medium.

2.2.1.3. Inoculum Preparation

The initial inoculum contained a naturally occurring consortium of aquatic microorganisms which had not previously been cultured and was obtained from the local mains tap water by resuspending the deposit on the nylon filter membrane as described above. One litre of

local mains tap water was filtered as above, the nylon membrane was then rolled and cut into small pieces with sterile scissors. The deposit was resuspended by vigorously shaking the pieces in 50 ml of the filter sterilised tap water in a pre-sterilised wide top jar (Colbourne and Trew, 1986). From this concentrate 25 ml was added to the chemostat and the system was allowed to stabilise. The remaining 25 ml was stored at 4 °C for future use.

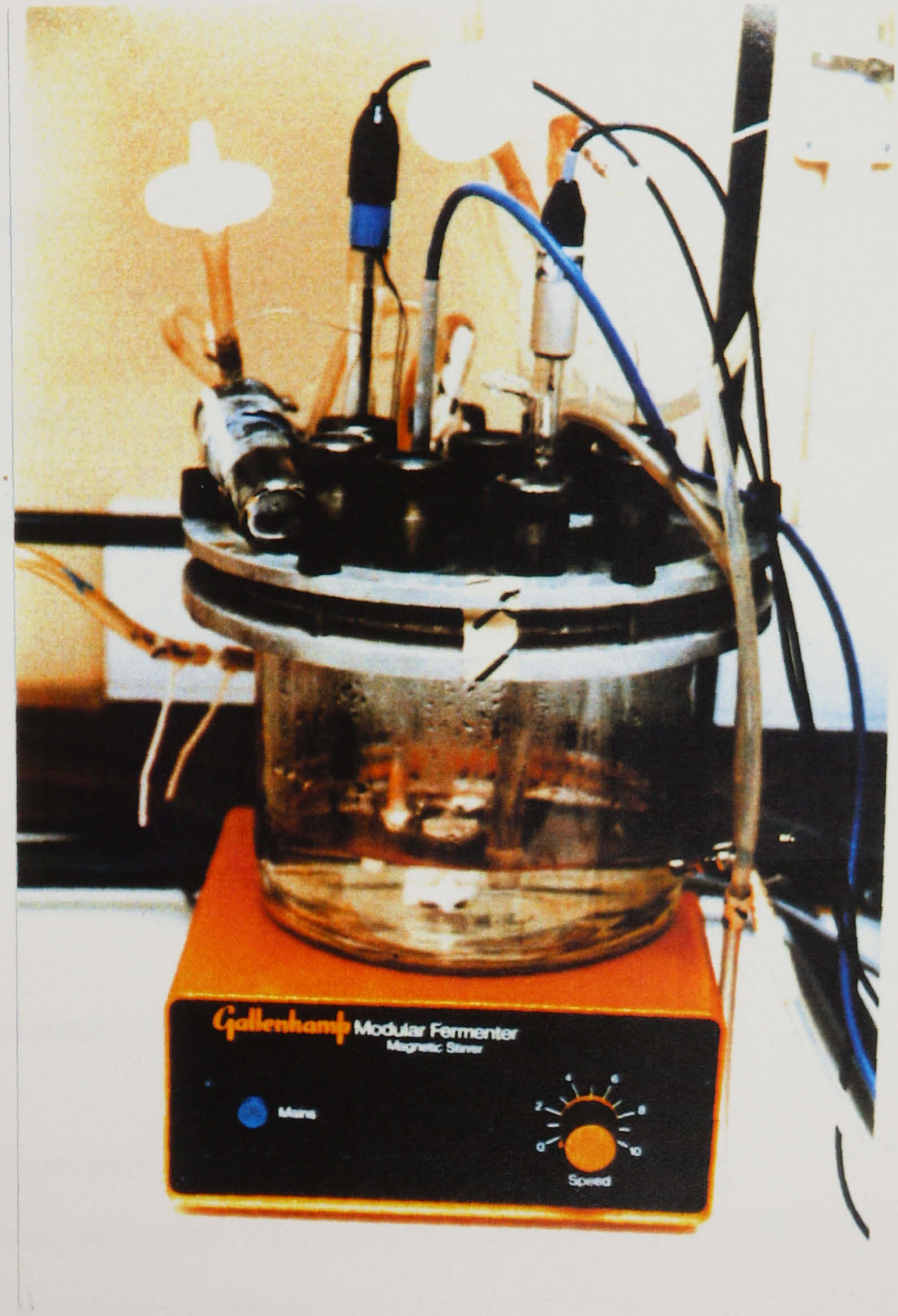

Fig 2.1. The model biofilm system as set up in the laboratory.

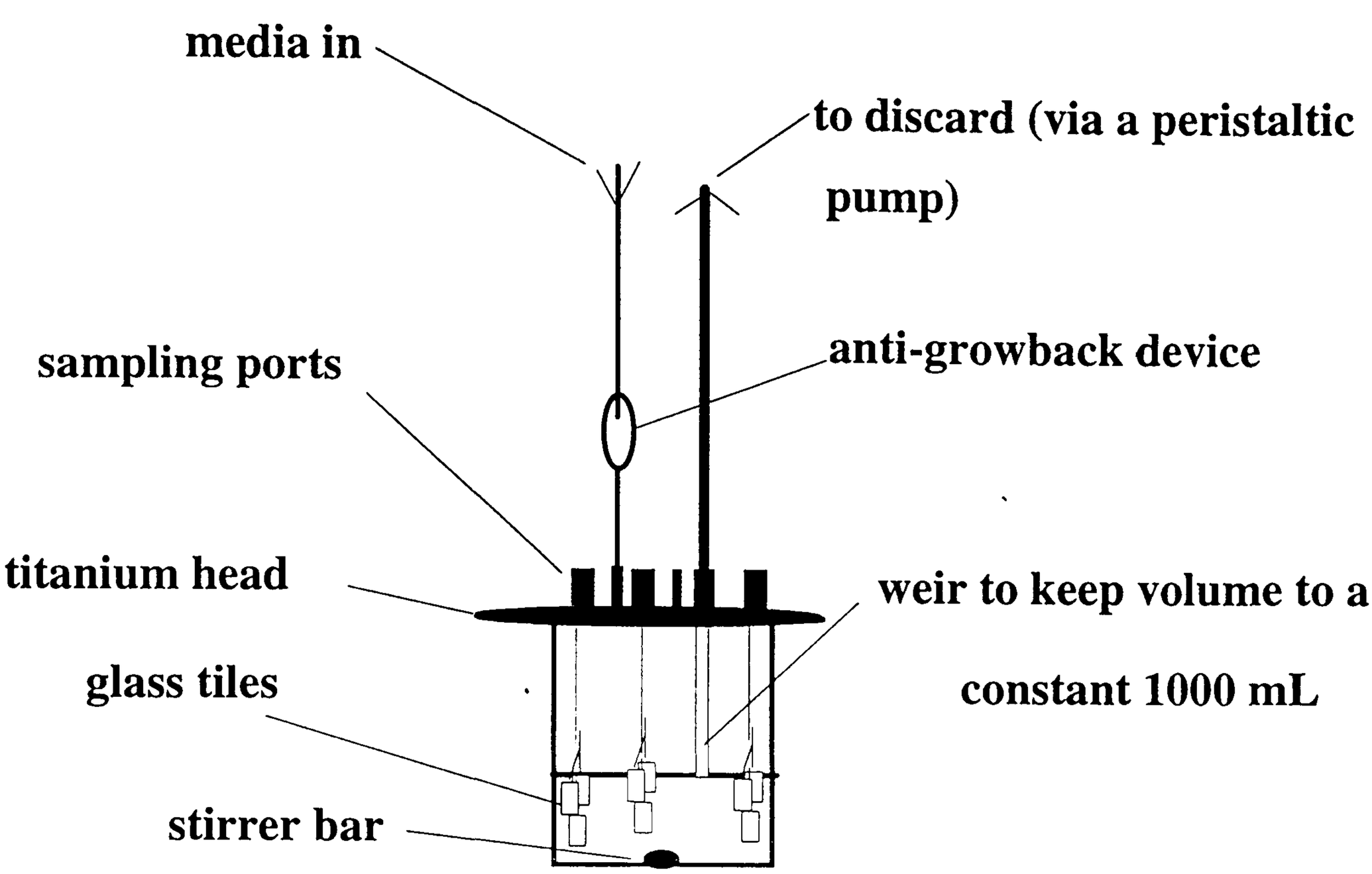

Fig. 2. 2. Diagrammatic view of the model biofilm system.

The weir system was used to maintain the volume at the required level. The stirrer speed was altered as necessary to maintain the oxygen concentration at approximately 20%. Temperature , oxygen and pH probes are not shown.

2.2.1.4. Biofilm Development

Photographic glass tiles ($1cm^2$) which had been pre-cleaned with acetone were suspended on titanium wire from silicone bungs (Fig. 2.3), inserted into glass bottles, and autoclaved. The tiles were then inserted aseptically into the aqueous phase of the model system, via the large ports, to provide a support for biofilm development.

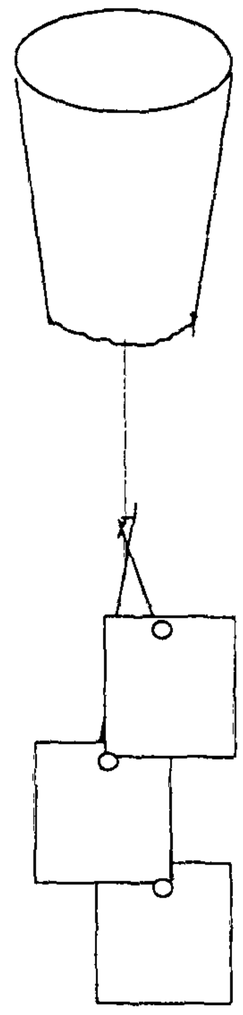

Fig 2. 3. The photographic glass tiles.

The tiles were inserted into the model system suspended from a silicone bung on titanium wire inserted into the model system.

2.2.1.5. The *Legionella pneumophila* serogroup 1 Pontiac (Corby) strains.

The *L. pneumophila* used in this study were *L. pneumophila* serogroup 1 Pontiac (Corby) strains (avirulent and virulent) kindly given by Dr. R. Fitzgeorge and Miss. A. Williams, PHLS, CAMR, Porton Down.. The avirulent strain had lost virulence by multiple passage (32 times) on BCYE (Jepras and Fitzgeorge, 1986) Both cultures were maintained at -70°C in vials containing cryopreservative (Pro-Lab Diagnostics Ltd, UK) and subcultured onto BCYE prior to use. An aliquot of approximately 10^{11} avirulent

L. pneumophila ml^{-1} was diluted with sterile water, before inoculation into the chemostat, to give a cell density in the system of 10^4 *L. pneumophila* ml^{-1}.

2.2.2. Bacterial Enumeration

2.2.2.1. Preparation of inocula for enumeration on spread plates.

Tiles were periodically removed aseptically from the system via the sampling ports, to assess biofilm growth. They were first rinsed in 10 ml of sterile water to remove any unattached microorganisms and the biofilms were then removed from both sides of the tile by scraping with a dental probe into 2 ml of sterile water. The suspensions were then vortexed and serial dilutions made in sterile microfuge tubes. Duplicate spread plates were made on non-selective media and, following heat treatment, onto media selective for *L. pneumophila* (see section 2.2.2.3). Scraping and vortexing was chosen in preference to sonication to remove the biofilm as Keevil *et al.* (1989b), have reported that sonication may disrupt the more fragile aquatic bacteria. The planktonic population was sampled by removing 10 ml from the aqueous phase of the chemostat and serially diluting as above.

2.2.2.2. Growth Media

The total heterotrophic population was assessed by growth on R2A medium [Appendix 1.] (Reasoner and Geldreich, 1985). R2A is a defined low nutrient medium and has been shown to reduce the effects of substrate shock which can occur when microorganisms are taken from a low nutrient environment and cultured in a high nutrient laboratory medium (Mackerness *et al.*, 1991).

BCYE [Appendix 2]was used as an enriched non-selective medium to grow the more fastidious bacterial isolates including *L. pneumophila* (Pasculle, 1980; Edelstein, 1981). After inoculation, plates were sealed in polythene bags to prevent drying. The R2A and BCYE were incubated aerobically at 30°C for seven days before enumeration.

2.2.2.3. Heat treatment.

Following the inoculation of the R2A and BCYE plates, the cultures in the microfuge tubes previously described (section 2.2.2.1.) were incubated in a dry heating block at 50 °C for 30 minutes to enhance the recovery of *L. pneumophila* (Dennis *et al.*, 1984). These heat-treated cultures were then used to inoculate BCYE plates with added glycine, vancomycin polymixin and cycloheximide (GVPC) which was used as the selective medium for the isolation of *L. pneumophila* (Dennis *et al.*, 1984). The GVPC plates were sealed in plastic bags as above, incubated at 37°C and examined after 5 days for the growth of *L. pneumophila*.

2. 2. 3. Identification methods

2.2.3.1. Bacterial identification.

Presumptive identification was determined by colony morphology and the use of the first stage diagnostic tables for Gram-negative bacteria (Cowan and Steel, 1993). Oxidase tests were carried out using the rapid identification test tablets (LabM, UK).

Further identification was then carried out using either the API 20NE or Biolog GN-Microplate system.

2.2.3.2. API 20NE

The API 20NE system (API- Biomerieux, Basingstoke) is for the identification of non-enteric Gram-negative rods. The method used was as described in the instruction leaflet (version G) supplied with the test strips, with the exception that some of the more slow growing bacterial isolates required incubation for a further 24 hours before a result could be obtained.

2.2.3.3. Identification by Biolog.

The Biolog GM Gram-negative identification system GN-Microplate, (Atlas Bioscan, Hayward, California, USA) is a colormetric microtitre plate identification system (see

Fig.2.4) using 95 carbon sources plus one control well (Biolog, 1989). A positive reaction is indicated by the formation of a purple coloration due to the reduction of a tetrazolium dye. This reduction occurs by actively respiring bacteria during the oxidation of the substrate. Bacterial isolates to be identified were spread over two R2A plates and incubated at 30°C for 24 to 48 hours depending on the rate of growth. A suspension of bacterial cells was then made in 0.85% saline (pH 5.5-7.0) to correspond to the optical density range of the standards supplied with the system, taking care not to carry over any agar from the culture plate. Aliquots (150 µl) of this suspension were immediately inoculated into each well of the Biolog microtitre plates and the plate was incubated at 30°C overnight (16-24 hours.). The plates are then read either by eye or using a microtitre plate reader at 590nm. An obvious increase in purple coloration over the negative control well is taken as a positive result if the results are read by eye. If a microtitre plate reader is used, a 40% increase in absorbance over the control well is accepted as a positive result.

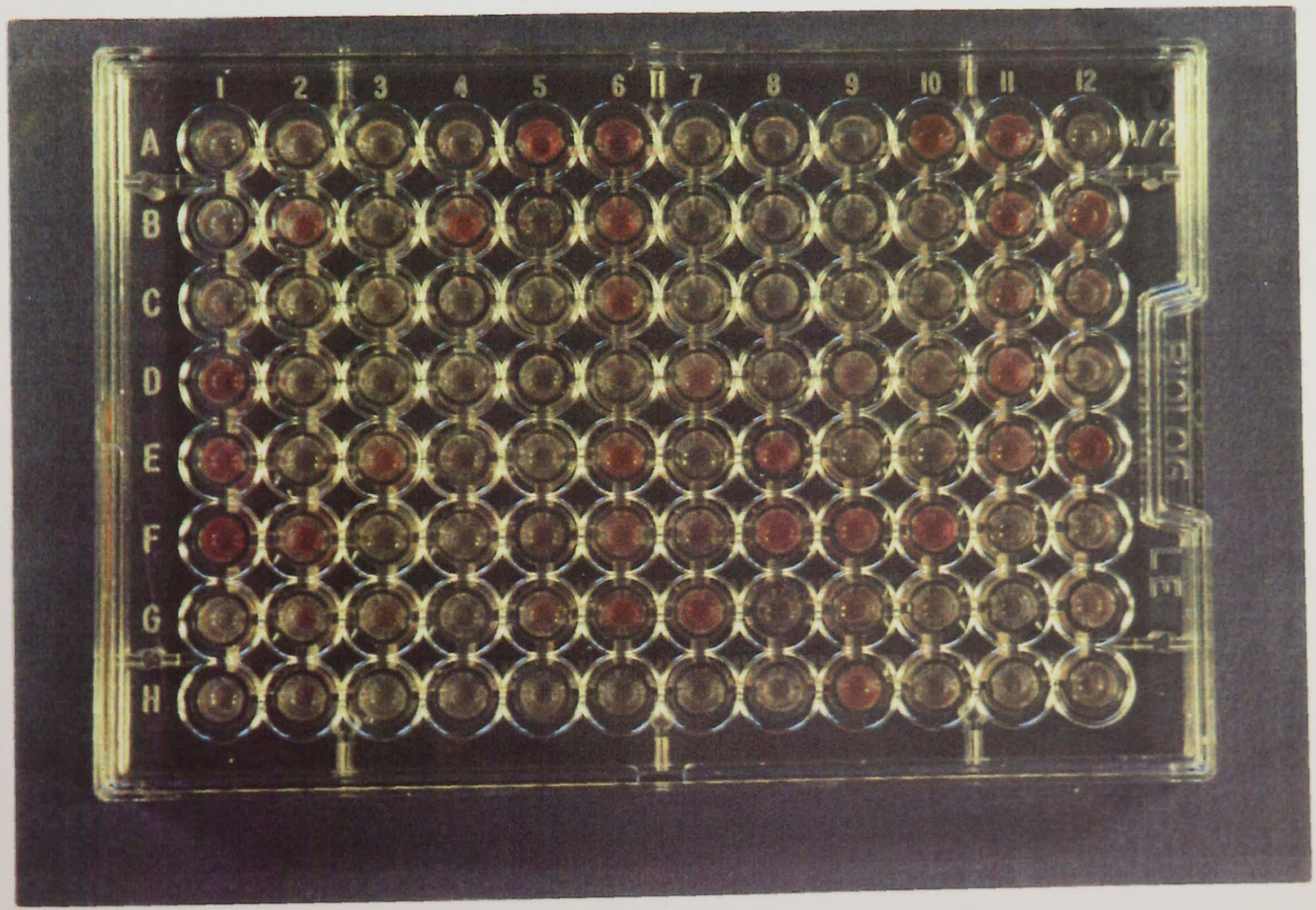

Fig. 2.4. A GN-microtitre plate. A positive well is a 40% increase in absorbance at 590 nm over the negative control well A1.

2.2.3.4. Confirmation of identity of *L. pneumophila* isolates.

Initial identification of *L. pneumophila* was by colony morphology, as *L. pneumophila* have a distinctive ground glass appearance on BCYE and GVPC. Isolates which grew on GVPC and failed to grow on BCYE without cysteine, were tentatively identified as *L. pneumophila*. This was confirmed by a latex agglutination test using the "Prolex" rapid latex kit (Pro-Lab Diagnostics, UK).

2.2.4. Fungal Identification.

Fungal isolates were tentatively identified by morphological characterisation and were confirmed by Professor L. H. G. Morton.

2.2.5. Amoebal Identification.

Amoebal identification utilised schemes described by Page (1976, 1988) and Rowbotham (1980 and personal communication).

Amoebae were removed by washing the plate with Page's amoebal saline (Page, 1976) which was gently pipetted over the surface and collected in a sterile universal bottle. For safety reasons this procedure must be carried out in a laminar flow cabinet. Hanging drops of this suspension are prepared by placing a drop on a coverslip which was kept in a dampened chamber for a minimum of 15 minutes to allow excystation and then inverted over the well of a microscope slide which was sealed with glycerol before being examined microscopically. The hanging drops were then examined using a long working distance (×25) lens on a microscope to observe trophozoite motility and also cyst shape.

Several drops of the amoebal suspension were streaked diagonally over amoebal agar plates covered with lawns of *Klebsiella aerogenes* NCTC 7427 [Appendix 3.]. These

plates were incubated at 30 °C and examined daily for plaques corresponding to grazed areas on the lawns. Using microscopic examination of these plates single clones were obtained by marking the area surrounding isolated cysts and removing them by cutting out the area of agar with a sterile scalpel. These agar plugs were carefully transferred to a fresh lawn of UV killed *Klebsiella aerogenes*. Suspensions of the single clones were made as described above and streaked across six *K. aerogenes* lawns which were then sealed in polythene bags and incubated at 45°C, 42°C, 37°C, 35°C, 30°C and room temperature (approx. 21°C) for up to three weeks. The plates were examined frequently for trophozoites and cysts and these were then tentatively identified using identification keys (Page, 1976; 1988).

2. 2. 6. Results.

2. 2. 6. 1.Bacterial enumeration.

Bacterial enumeration was carried out by culture onto R2A and BCYE. After seven days the planktonic phase had reached steady state (Fig 2.5.). Over a six month period the total numbers in the planktonic phase ranged from 4.36-6.3 log_{10} cfu ml^{-1} with a mean of 5.35 log_{10} cfu ml^{-1} [SD 0.53 (n=20)]. The avirulent *L. pneumophila* achieved steady state when inoculated into an existing consortium of microorganisms but not when inoculated as a pure culture (Fig. 2.6). Over the same time period the *L. pneumophila* counts ranged from 3.54-4.65 log_{10} cfu ml-^{1} [mean 3.54 log_{10} cfu ml^{-1}, SD 0.7 (n=14)].

Biofilms readily developed on the glass tiles within the system (Fig. 2.7) and contained a mixed population of bacteria including *L. pneumophila* (Table 2.1).

time (days)	mean total count log_{10} cfu cm^{-2}	SD	number of samples	mean *L. pneumophila* count log_{10} cfu cm^{-2}	SD	number of samples
7	5.45	0.43	11	3.2	1.2	8
14	6.057	0.17	8	4.3	0.56	8
21	6.34	0.09	6	4.4	0.33	6
28	6.45	0.36	5	4.57	0.9	5

Table 2.1. Mean counts of total bacterial population and *L. pneumophila* from biofilms removed from the chemostat.

Microbial growth in the planktonic phase following system inoculation

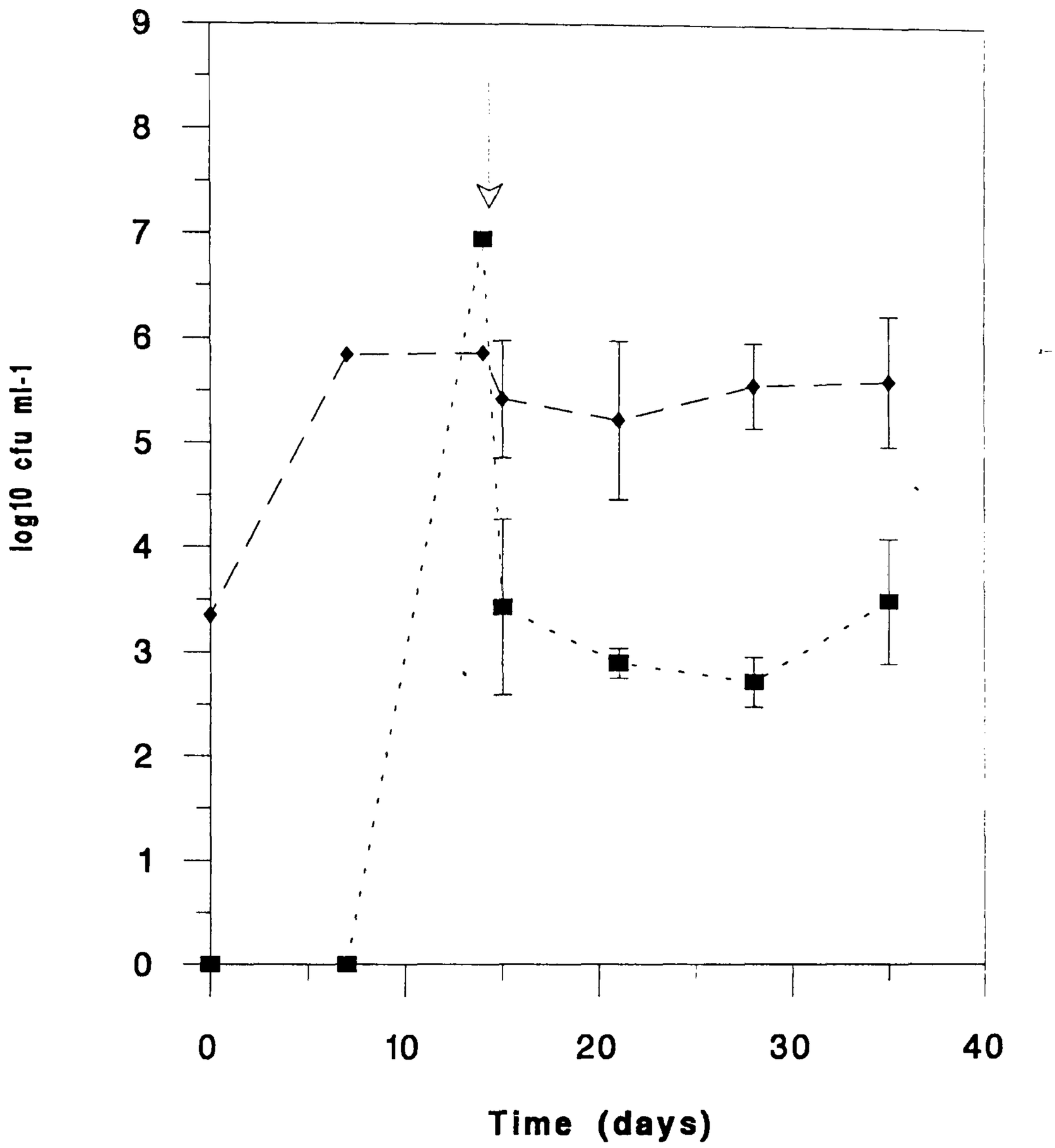

Fig 2. 5.Microbial growth in the planktonic phase following system inoculation. Total bacterial count (♦) **and** *L. pneumophila* (■). Arrow indicates *L. pneumophila* addition to the chemostat.

***L. pneumophila* in pure culture in the continuous culture biofilm system.**

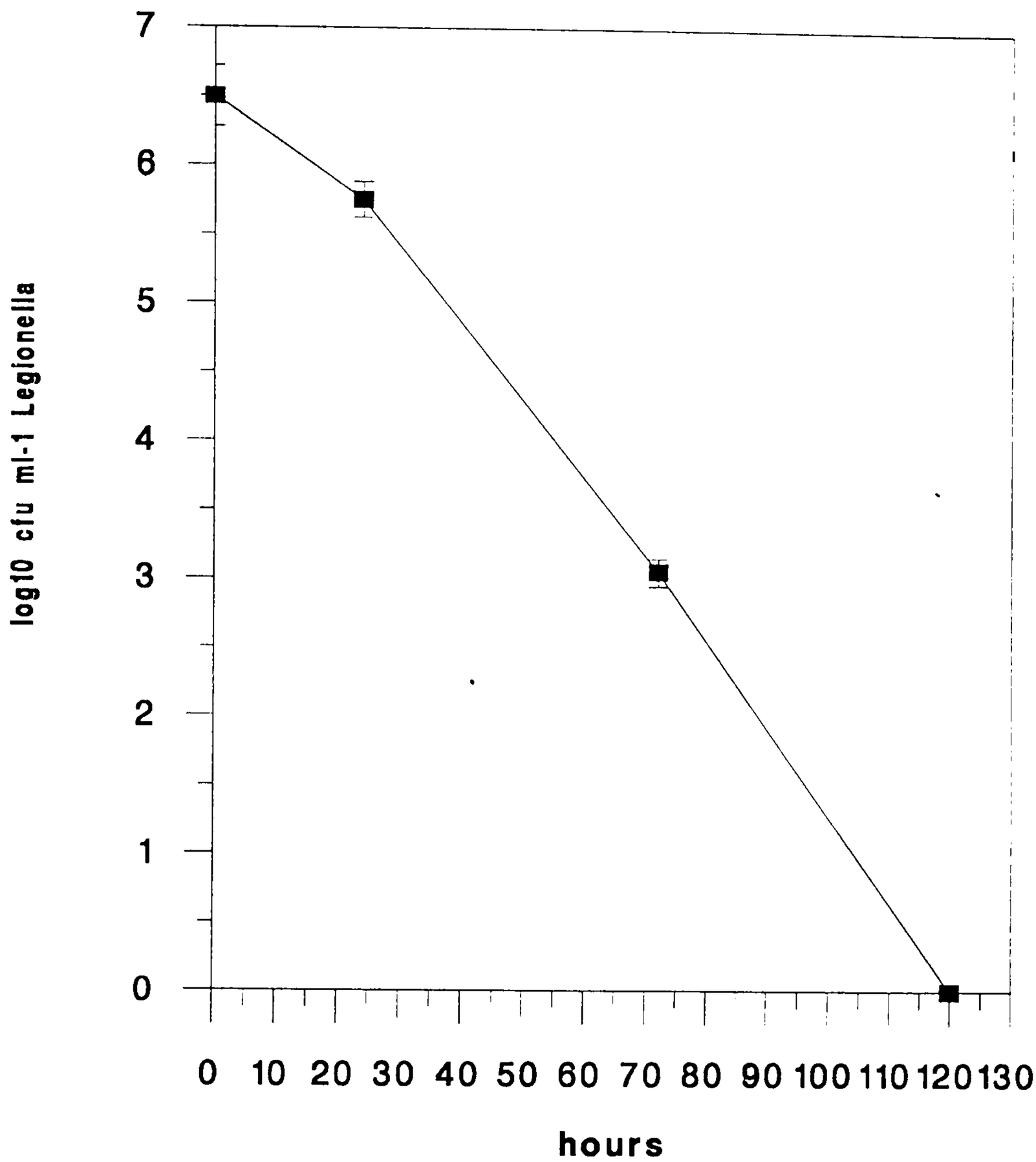

Fig. 2. 6. *L. pneumophila* when in pure culture in the continuous culture biofilm system.

After 5 days *L. pneumophila* could not be detected by culture onto BCYE. Results are the mean of two separate additions to the chemostat, subcultured onto three plates of BCYE.

Integration of *L. pneumophila* into 28 day biofilms

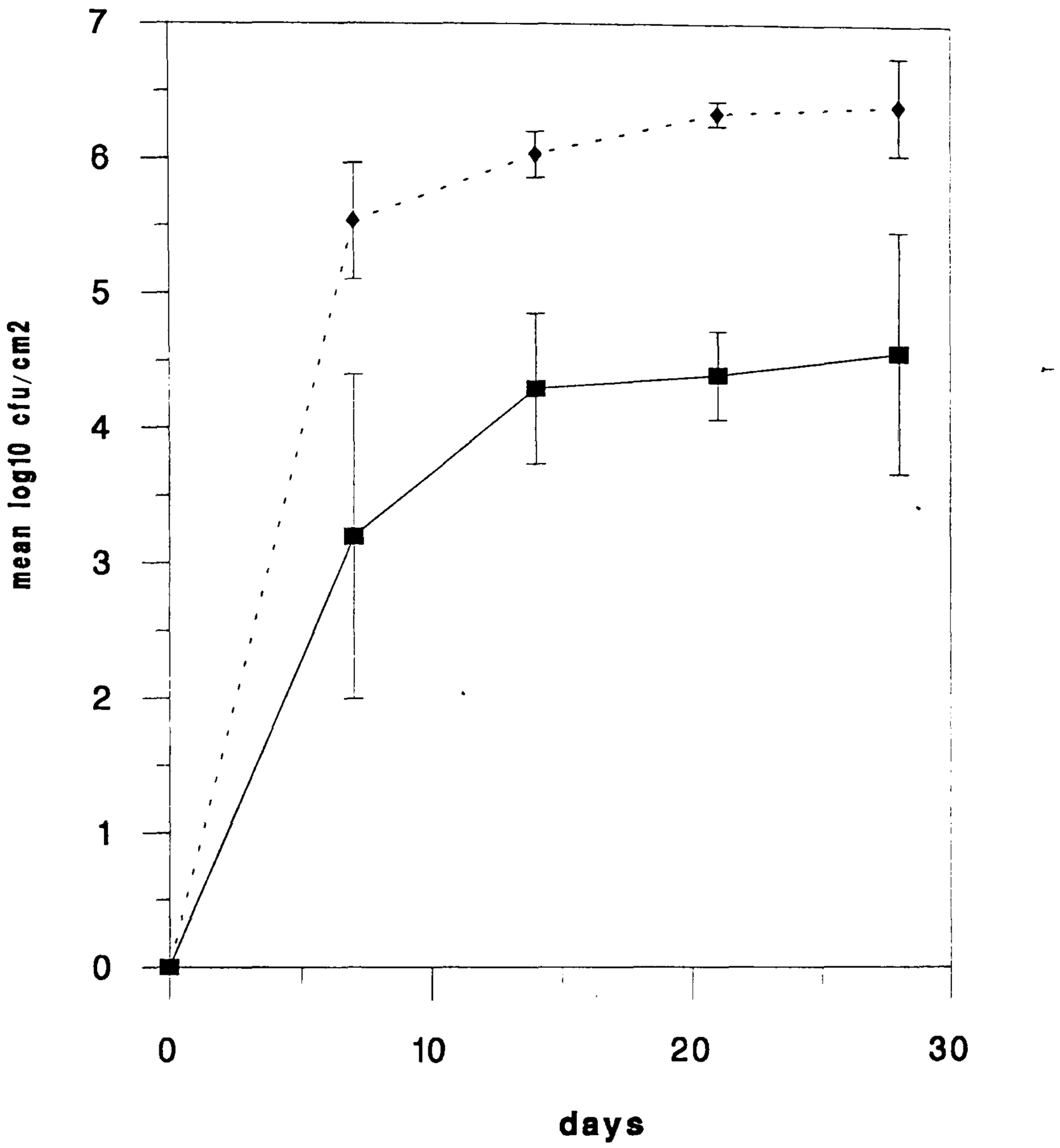

Fig. 2. 7. Mean biofilm development over 28 days. Total number of aquatic microorganisms ◆ and *L. pneumophila* ■ within the biofilm (Table 2.1).

The presence of protozoa which graze on biofilm microorganisms was detected by the formation of plaques on the R2A plates used for enumeration. These plaques were more readily observed in the undiluted planktonic and sessile phases (Fig 2. 8.).

Microscopic examination of these biofilms showed that they contained a diverse range of microorganisms including bacteria, fungi, protozoa and diatoms (Fig 2.9-2.10). Bacterial species isolated from the system included several yellow and pink pigmented colonies. Those which could be identified (Table 2.2.) were predominantly *Pseudomonas, Flavobacterium, Methylobacterium, Acinetobacter* and *Alcaligenes* spp. Fungi were tentatively identified as *Aspergillus flavus and Acremonium strictum.*

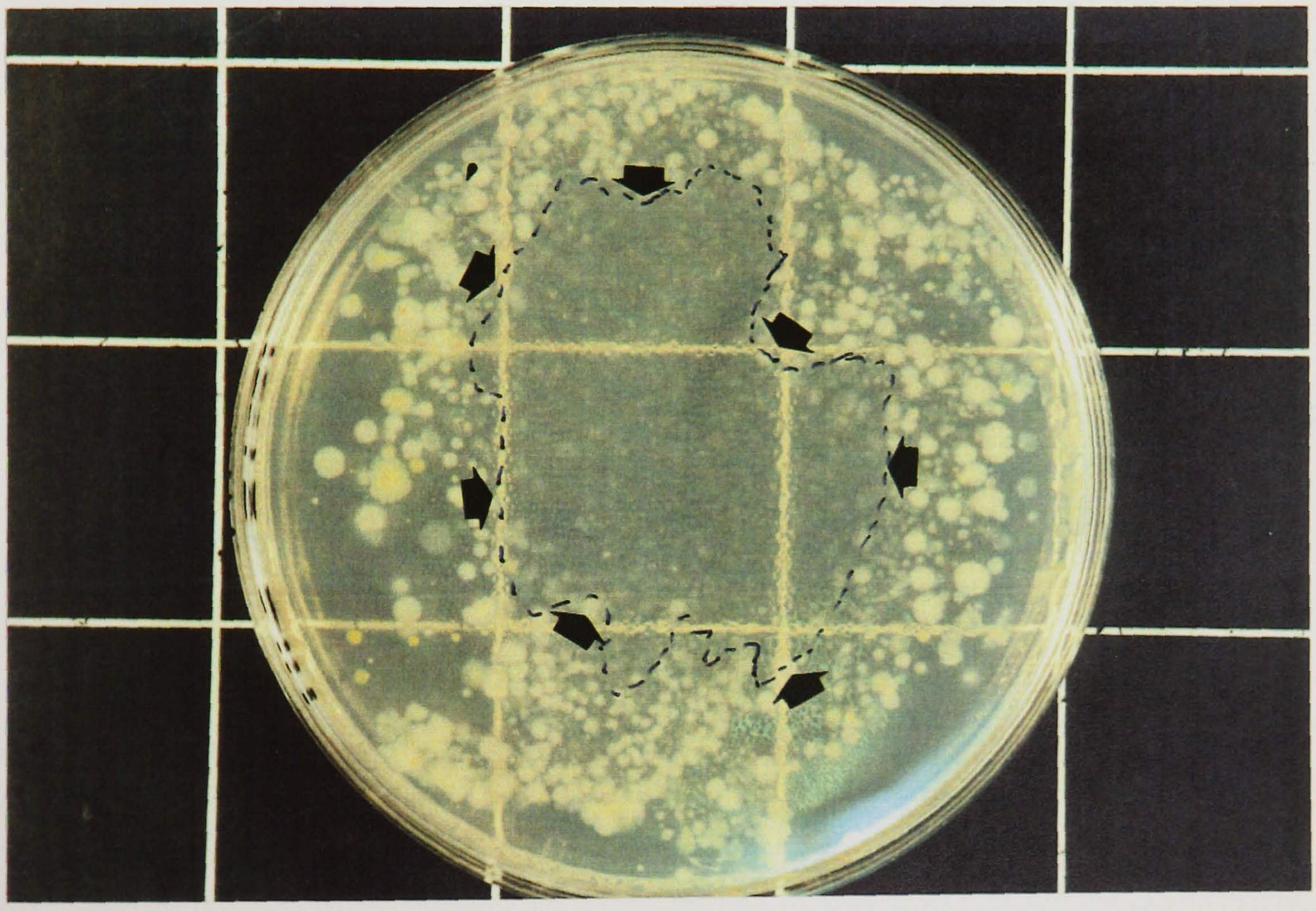

Fig 2.8 Plaque formation by grazing protozoa. An R2A plate inoculated with 100 µl of the undiluted planktonic phase of the continuous culture biofilm system. The clearer central zone delineated corresponds to the area grazed by protozoa (arrows).

Acinetobacter junii/johns	*Vibrio fischeri*
Sphingomonas paucimobilis	*Acinetobacter calcoaceticus*
Pseudomonas fluorescens	*Acidovorax p.v.Delafieldii*
Pseudomonas vesicularis	*Vibrio sp.*
Pseudomonas mesophilica	*Methylobacterium mesophilicum*
Pseudomonas putida	*Alcaligenes latus*
Moraxella lacunata	*Agrobacterium radiobacter*
Commamonas sp.	*Sphingomonas multivorum*
Ochrobacter anthropi	*Flavobacterium* sp.

Table 2.2 .Identifiable bacterial isolates from the continuous culture biofilm system.

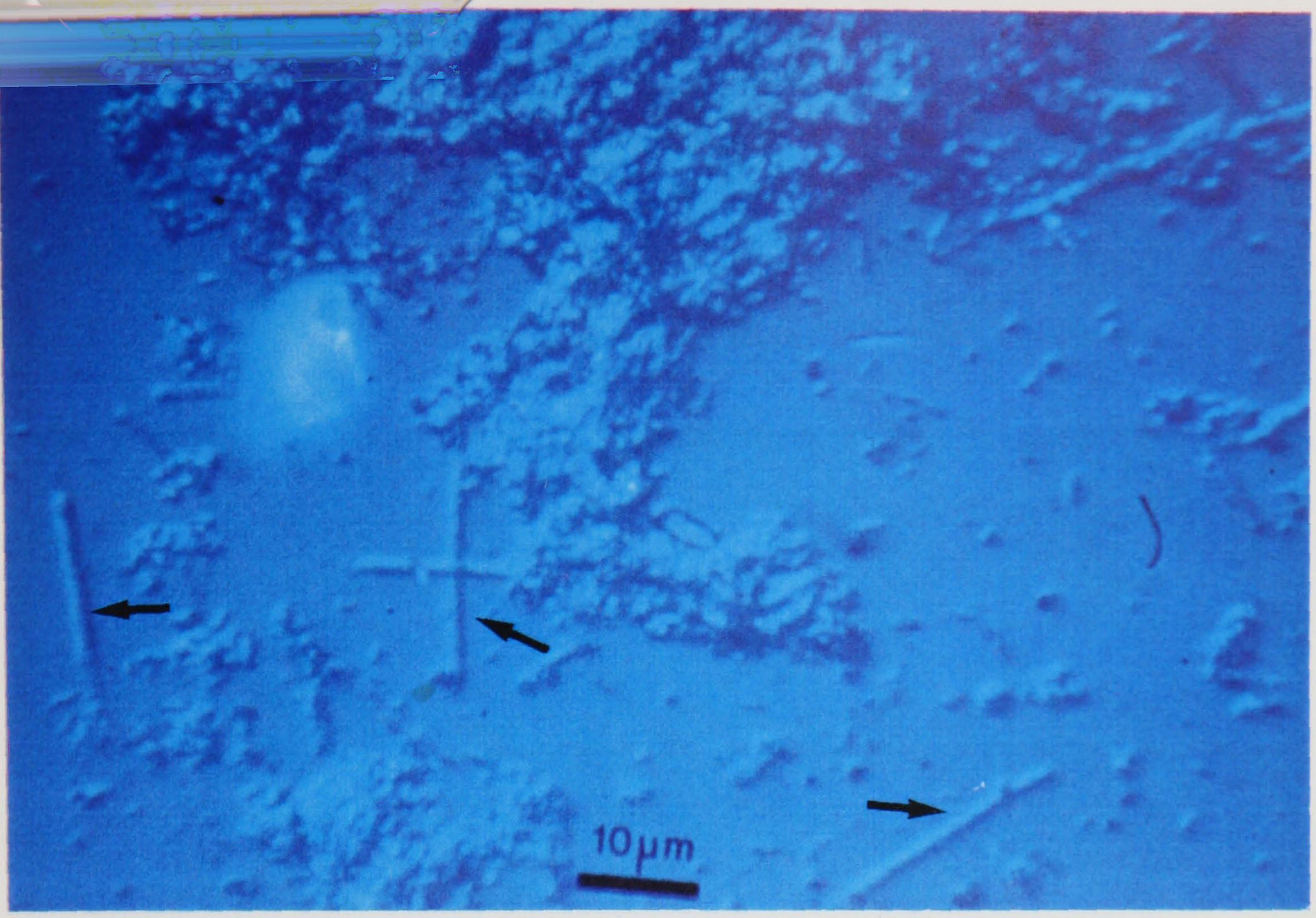

Fig. 2.9. An eight week biofilm imaged with differential interference contrast microscopy. A diverse consortium of microorganisms including diatoms can be seen clearly in the biofilm (arrows).

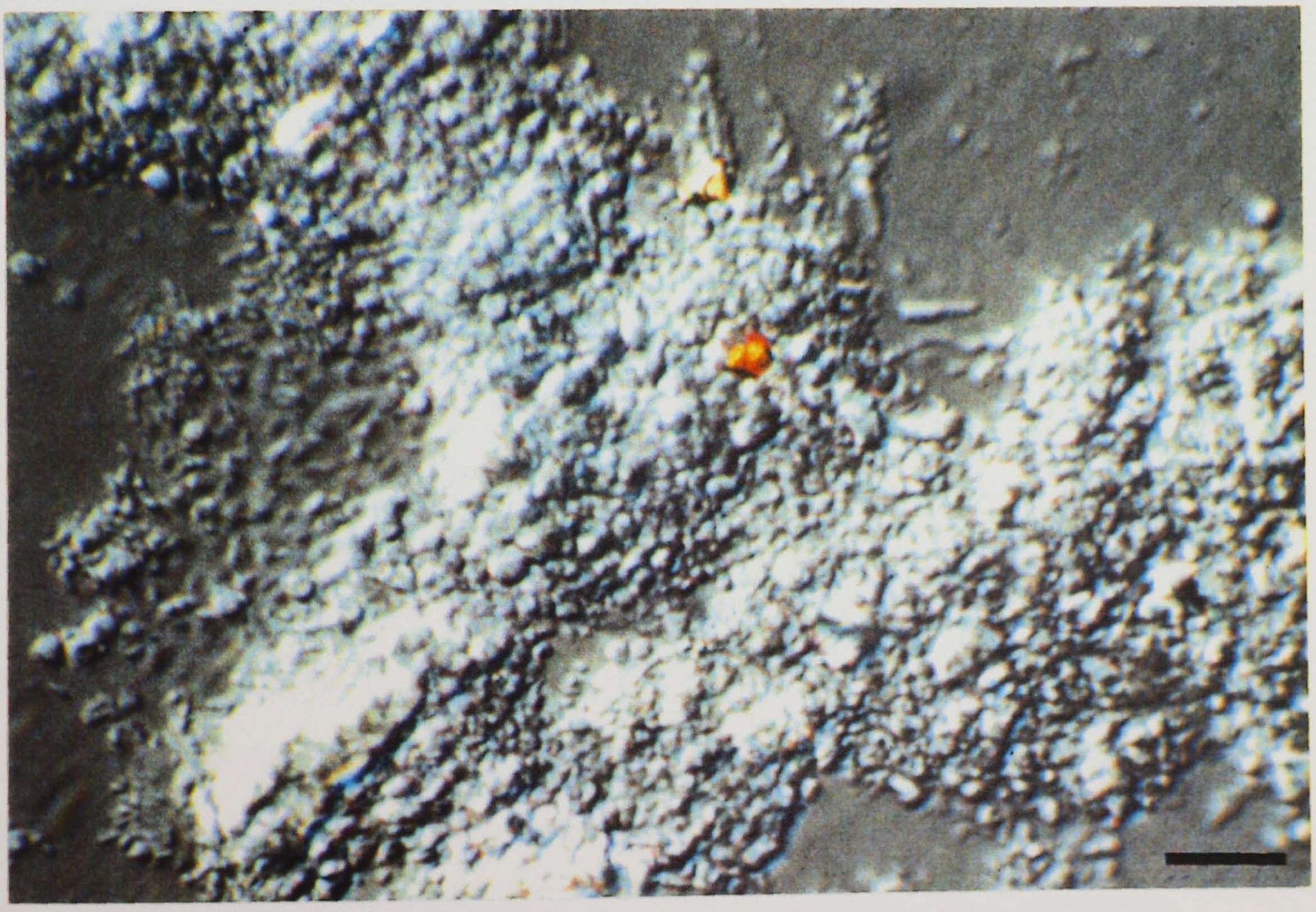

Fig 2.10. An two week biofilm imaged with Hoffman modulation contrast microscopy. The biofilm is a complex heterogeneous matrix of microorganisms. The biofilm is not uniformly dispersed over the tile surface. The denser areas measured between 80-100 µm measured on the microscope micrometer (bar = 10 µm).

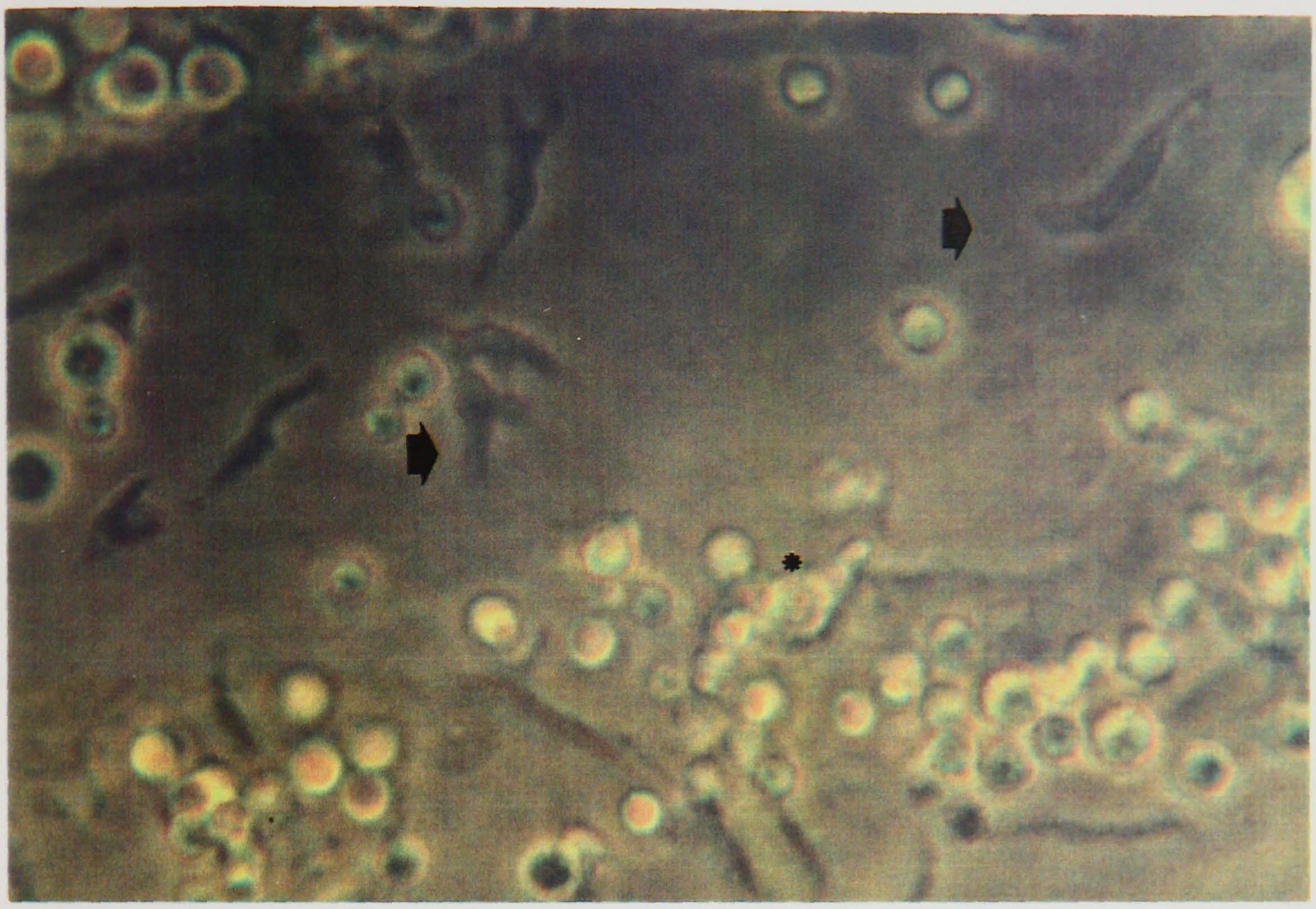

Fig 2.11. Amoebae tentatively identified as *Hartmanella vermiformis* (arrows) and cysts grazing on a lawn of *Klebsiella aerogenes*. ✸ denotes an excysting amoeba. Photographed under phase contrast. Initial microscope magnification × 400.

The protozoa in the system were identified with the help of Dr. Tim Rowbotham, from Leeds PHLS laboratory. Those tentatively identified as *Hartmanella vermiformis* were approximately 16 μm in length and with cysts of approximately 4.4 μm in diameter (Fig 2.11). This species is widespread in the environment and found in Europe, North America and Australia (Page, 1988). These amoebae formed plaques on lawns of *Klebsiella aerogenes* when incubated at all the temperatures ranging from room temperature to 45 °C as described earlier. Normally *Hartmanella vermiformis* would not be expected to grow above 37 °C. These isolates have been termed as "high temperature *Hartmanella vermiformis"* (T. Rowbotham, personal communication).

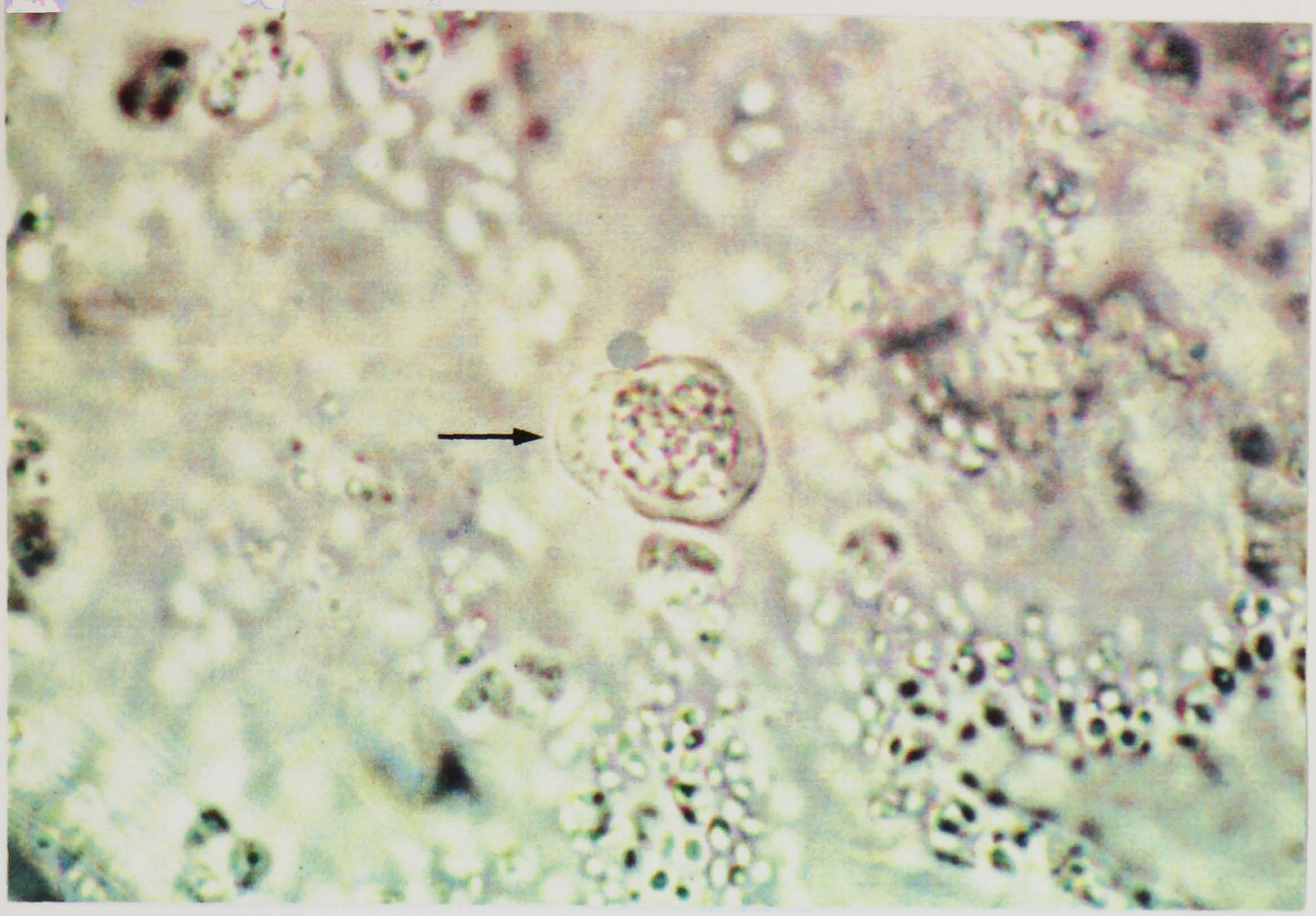

Fig 2.12. Amoeba tentatively identified as *Vannela* sp. Initial microscope magnification × 1000. This is a fairly common genus isolated in Europe, North and South America and parts of Asia depending upon species (Page, 1988)

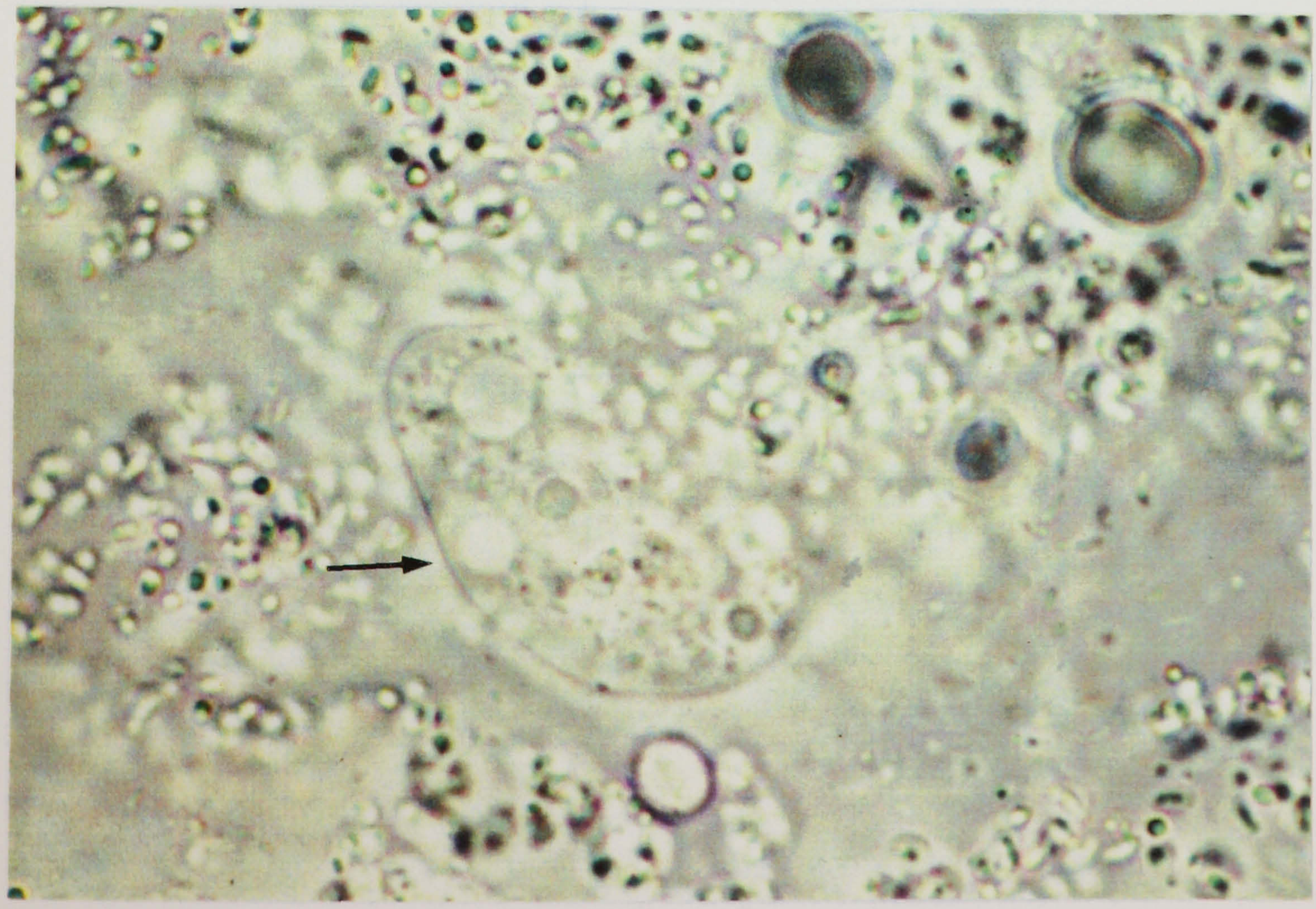

Fig 2.13. Unidentified amoeba. Initial microscope magnification × 1000.
This large amoeba could not be cultured on various food supplies and as yet remains unidentified (The above two photographs were taken by Dr. Tim Rowbotham, Leeds PHLS.)

Other amoebae were tentatively identified as *Acanthamoeba* sp. and *Vannella* sp.(Fig 2.12). A third large amoeba (Fig 2. 13) could not be cultured following initial isolation. This could be due to the inappropriate supply of a food source. Lawns of *Klebsiella aerogenes, E.coli* and algae were tried as a food source but no plaques could be observed. It is possible that this larger amoeba fed on trophozoites or cysts of the smaller protozoa.

2. 3. An investigation to determine if the avirulent strain of *L. pneumophila* used in this study can be recovered following incubation at 50 °C.

2.3.1. Materials and Methods.

A suspension of both the avirulent and virulent *L. pneumophila* were prepared by swabbing the surface of fresh cultures grown on BCYE into sterile water to give an OD at 590 nm. of 1.0. Triplicate serial dilutions (1 ml) were prepared in sterile microfuge tubes to give a dilution series of 10^5-10^1 cfu ml^{-1} for each strain of *L. pneumophila*. These diluted cultures were then incubated in a dry heating block at 50 °C and samples taken after 30 minutes and at regular time intervals to 24 hrs.

2. 3. 2. Results.

Table 2.3. shows the tolerance of the virulent and avirulent strains to 50 °C. Over the 30 minute incubation time which is used to increase *L. pneumophila* recovery from environmental samples (Dennis *et al.*, 1988), there was no reduction in viability of either the avirulent or the virulent strain. The numbers of bacteria in the sample seemed an important factor in the viability over an increased time interval, a higher bacterial count in the sample corresponded to a longer time over which viable *L. pneumophila* could be recovered (Table 2.3.).

	Time (hours)									
	0.5	1	2	4	6	8	10	12	18	24
Numbers of *L. pneumophila* (avirulent)										
10^1	+	+	±	-	-	-	-	-	-	-
10^2	+	+	+	+	±	-	-	-	-	-
10^3	+	+	+	+	+	±		-	-	-
10^4	+	+	+	+	+	+	±	±	-	-
10^5	+	+	+	+	+	+	+	+	-	-
(virulent)										
10^1	+	+	±	-	-	-	-	-	-	-
10^2	+	+	+	+	-	-	-	-	-	-
10^3	+	+	+	+	±	-	-	-	-	-
10^4	+	+	+	+	+	±	±	±	-	-
10^5	+	+	+	+	+	+	+	+	-	-

Table 2.3. Recovery of the avirulent and virulent *L. pneumophila* Corby strain after incubation at 50 °C.

+, confluent growth on subculture onto BCYE following incubation, ±, scanty growth only, - = no growth, following subculture onto BCYE.

2.4. An investigation to determine whether the avirulent *L. pneumophila* strain reverts to virulence when integrated into a mixed consortium of aquatic microorganisms within a model continuous culture biofilm system.

2.4.1. Materials and methods.

2.4.1.1. Isolation of *L. pneumophila*.

Legionella pneumophila was recovered from the continuous culture model biofilm system. Several colonies from both the planktonic and sessile phases were isolated and their identity confirmed as previously described (2.2.3.4.) and purified on BCYE. Care was taken to avoid more than three passages on solid media before virulence testing. This was to avoid the possibility of any attenuation of the *L. pneumophila* due to multiple passage on laboratory media (Jepras and Fitzgeorge, 1986; Catrenich and Johnson, 1989).

2.3.1.2. Virulence testing.

Virulence tests were performed at PHLS, CAMR, Porton Down by Dr. R. Fitzgeorge using the guinea pig aerosol model system (Fitzgeorge *et al.*, 1983). For each test, eight female Dunkin-Hartley guinea pigs weighing between 350 and 450g were used. When this method is used with *L. pneumophila* of unknown virulence a dilution series from 10^6 to 10^{10} is used. As previous studies had established that this *L. pneumophila* was likely to be avirulent the highest concentration was used initially in order to avoid the unnecessary use of guinea pigs. The guinea pigs were exposed for 5 minutes to an aerosol of 10^{10} bacteria ml^{-1} suspended in sterile water in a Collinson spray. Two guinea pigs were sacrificed immediately after dosing and the lung tissue cultured to

verify the dose received. The remainder were observed for signs of illness for a period of seven days after aerosol exposure.

2.4.2. Results.

In the guinea pig animal model system, the avirulent *L. pneumophila* showed no reversion to virulence when tested at intervals over a three year period. When a guinea pig is exposed to the virulent strain there is an observed increase in temperature within a short time following exposure and obvious signs of illness occur within a few days (Baskerville *et al.*, 1981). Following exposure to the avirulent *L. pneumophila* none of the guinea pigs showed an increase in temperature or any signs of illness even after exposure to 10 times the LD_{50} of the virulent strain (Fitzgeorge, personal communication) [LD_{50} *L. pneumophila* Corby strain Log_{10} 2.2. (Tully *et al.*, 1992)].

2. 5. Discussion.

For a system to be a valid model for the natural environment which it aims to imitate, the conditions within the system must be as close as possible to those found in the natural environment. This is especially true when modelling bacterial consortia normally found in water systems, where the environment may contain many different sub populations each existing within its own particular niche or microenvironment. Each population will be influenced by the available substratum and nutrients within their immediate vicinity.

In order to model as closely as possible a 'natural' water system, the initial inoculum was taken from the local mains water supply. To reduce the risk of bacterial selection by growth media and possible phenotypic alterations due to passage on laboratory media, the inoculum was obtained by filtering and introduced directly into the system. The materials used in the manufacture of the system were chosen for their relative chemical stability. The medium used was mains tap water from the same source as the original inoculum. This was filter sterilised by the method of Colbourne *et al.* (1988) to avoid any alteration in the chemistry of the water. The use of continuous culture allows the control of parameters including pH, nutrient concentration, temperature and oxygen concentration (Herbert, 1956). In order to maintain as natural a system as possible, pH and nutrient concentration were not controlled. By the definition of a 'natural' system as described by Brock (1971), it could be argued that because of this lack of complete control, this model was a itself a 'natural' system.

This model water system allowed the development of reproducible biofilms (Table 2.1). A diverse but fairly constant consortium of aquatic microorganisms including fungi, bacteria and protozoa can be maintained in this system. The relative numbers of planktonic to biofilm bacteria, in biofilms older than 7 days , were similar to those described by others. Studies by Costerton *et al.*, (1986) have found that biofilm bacteria

in natural ecosystems may be 10-100 times greater than those found in the planktonic phase. During the course of this investigation the mean of the planktonic counts was approximately 10 times less than the mean counts for biofilms of between 14-28 days old (Table 2.1). The bacterial species isolated from this system were similar to those isolated in other studies from geographically distant sources (Mackerness *et al.*, 1991, Walker *et al.*, 1994). This similarity in bacterial flora suggests that these species are ubiquitous within water systems. By microscopic examination of these biofilms it was noticed that those produced in this system contained many protozoa (Fig 2.7). *Hartmanella, Vannella* and *Acanthamoeba* spp are naturally occurring inhabitants of water systems and their continued presence suggests that the environmental parameters of this system remained acceptable. The biofilms produced were fairly fragile which could be a consequence of soft water. Rogers (unpublished data) have noted that biofilms produced from harder waters are thicker due to carbonate deposition which forms a support matrix for biofilm formation.

The importance of the presence of other bacteria is emphasised by the decline in *L. pneumophila* numbers when introduced into the model system in pure culture (Fig. 2.6). These results agree with those of other authors who found that *L. pneumophila* did not grow in experiments using sterile distilled water as the growth medium (Skaliy and McEachern, 1979; Fields *et al.*, 1984). However, *L. pneumophila* was successfully isolated from both the planktonic phases and the sessile phase of this model system, when integrated into an existing consortium of aquatic microorganisms and could be maintained in this system for future study.

GVPC was insufficiently selective to inhibit the growth of large numbers of non-legionellae bacteria from the system. Further selective measures in the form of heat treatment were necessary. Heat treatment was chosen in preference to acid treatment for two reasons; firstly as small volumes were removed from the chemostat, dilution during the course of the acid treatment would introduce an extra error factor. Secondly, with

heat treatment the same dilutions used to inoculate the non-selective media could be heated and subsequently used to inoculate the GVPC.

It had not previously been established whether the attenuation of this avirulent strain of *L. pneumophila* serogroup 1 affects the tolerance to heat shown by environmental isolates (Dennis *et al.*, 1984). The results of the heat tolerance study obtained during the course of this investigation may have important implications on the control of potential pathogens by heat. *L. pneumophila* has previously been detected in biofilms at 50 °C despite the general acceptance that *L. pneumophila* will not survive for long periods of time at this temperature (Rogers and Keevil, 1992; Rogers *et al.*, 1993). The results of the present investigation would suggest that the relatively high numbers present in the original inoculum were the important factor in the increased thermal resistance exhibited rather than the presence of the biofilm itself. However, it is possible that where there were lower numbers of cells present in the initial inoculum, these cells were actively growing. Thermal stress has previously been shown to be increased in cells which are in early exponential growth compared with slower growing cells (Koch, 1958). Where there was a higher number of cells in the initial inoculum, the available nutrients in the water would be more rapidly utilised and growth would then cease. Bacterial cells in biofilms grow at a reduced rate compared with their planktonic counterparts and have been shown to exhibit increased resistance to biocides (Gilbert and Brown, 1994; Kuchta *et al.*, 1985). It would appear from this study that the growth rate itself could be an important consideration in the resistance of bacteria to environmental factors. Further study is needed to elucidate fully the mechanisms involved in this heat resistance.

The avirulent *L. pneumophila* serogroup 1 Pontiac (Corby) strain used in this study is unable to grow in alveolar macrophages and does not cause infection and death in guinea pigs. The reason(s) for this lack of virulence are as yet undetermined but may be associated with the loss of peroxidase/catalase activity (Jepras and Fitzgeorge, 1986).

Tully *et al.* (1992) suggest that the loss of virulence in this strain is probably due to more than one mutation and subsequently to more than one metabolic alteration. If this is the case then it would seem unlikely that this attenuation could be easily reversed. This avirulent strain of *L. pneumophila* isolated from the model system remained avirulent when tested in the animal model system.

In conclusion, this model system can be used to grow reproducible biofilms from a naturally occurring consortium of aquatic microorganisms. An avirulent strain of *L. pneumophila* can be maintained in both the planktonic and sessile phases of the system. This strain can be successfully recovered using heat treatment following the method described for the virulent strain (Dennis, 1988). Furthermore the stability of the attenuated strain means that this *L. pneumophila* may be safely used by competent personnel without the need of costly class III containment facilities.

The relationship between amoebae and *L. pneumophila* is believed to be important in the survival of *L. pneumophila* in the natural environment (Rowbotham, 1980). Virulence is important in the capacity of *L. pneumophila* to infect and multiply within amoebae. The importance of the role of the protozoa in the survival of *L. pneumophila* within this model system will be discussed in the next chapter.

CHAPTER 3.

AMOEBAL STUDIES

Chapter 3. Amoebal studies.

3.1. Introduction.

Although it is fourteen years since Rowbotham, (1980) suggested that the relationship between amoebae and *L. pneumophila* may be an important factor in the amplification of *Legionella* in the environment, the relevance of this association to the survival of *L. pneumophila* in aquatic sources and the incidence of Legionnaires' disease is not yet fully understood. Since then many studies have shown that amoebae are important growth factors for *L. pneumophila* in aquatic systems (Barbaree *et al.,* 1986; Fields *et al.,* 1984; 1986; Tyndall and Domingue, 1982; King *et al.,* 1991; and Wadowsky *et al.* 1988; 1991).

3.1.2. Virulence.

Fields, (1993) states that "protozoa are the main sources of *Legionella* growth in the environment and that to date, there are no documented instances of *L. pneumophila* growth in the natural environment in the absence of these hosts". Virulence has been shown to be an important factor in the ability of *L. pneumophila* to infect and subsequently multiply within amoebae (Fields *et al.*, 1986; Moffat and Tomkins, 1992), however, the avirulent strain used in the present study was both able to survive and to proliferate in the microenvironment within the model sytem (Chapter 2. 3). This avirulent *L. pneumophila* does not replicate within human alveolar macrophages or cause infection and death in guinea pigs (Tully *et al.*, 1992; this volume, 2.3.2.) However, it is not known whether this strain is also avirulent for protozoa. Tully *et al.* (1992) have shown that some strains which exhibit reduced virulence are still able to multiply within certain host cells. A greater understanding of the relationship between the avirulent *Legionella pneumophila* used in this study and the protozoa which are resident within the model system is necessary to determine whether *Legionella* are able

to proliferate other than via parasitization of a protozoan species. Such a study may enable us to gain further insight into the proliferation of this microorganism in natural systems.

3.1.3. Cycloheximide.

Cycloheximide inhibits protein synthesis in eukaryotic cells, inhibiting all three stages of initiation, elongation and termination (Oleinick, 1977). Emetine, which is structurally similar to cycloheximide has been shown to affect ribosomal aggregation in certain amoebae (Flicklinger, 1972). Cycloheximide has previously been used in ecological studies to inhibit protozoal bacteriovores without any detectable detrimental effect on the aerobic heterotrophic population (Tremaine and Mills, 1987). The same species of amoebae as those isolated from this system, which were tentatively identified as *Hartmanella vermiformis and Acanthamoeba polyphaga*, have previously been shown to be suitable hosts for environmental isolates of *L. pneumophila* serogroup 1 (Rowbotham, 1980; 1983; Wadowsky *et al.*, 1988; Fields, 1993). If intracellular multiplication is essential for the proliferation of *L. pneumophila*, inhibition of the trophozoites within the model system would prevent any further growth of *L. pneumophila.* This would result in a decrease in *Legionella* numbers due to dilution by the continuous culture medium. Cycloheximide therefore, was added both to the continuous culture vessel itself and to the filter sterilised water used as the growth medium. This was to maintain a constant concentration of cycloheximide in the system to inhibit the protozoa, whilst maintaining the integrity of the aerobic heterotrophic population including the avirulent *L. pneumophila*.

3.1.4. Virulence studies using amoebae.

Rowbotham, (1980), devised a method to demonstrate the virulence of an environmental isolate of *L. pneumophila.* This method has been adapted in the present study to

compare the effect of the avirulent and corresponding virulent strains of *L. pneumophila* on the amoebae isolated from the chemostat. Further qualitative studies were carried out to determine whether these strains of *L. pneumophila* were able to infect and to subsequently multiply within an axenic strain of *Acanthamoeba polyphaga*. This was achieved by co-culturing with the appropriate *L. pneumophila* and then visualising with transmission electron microscopy and Hoffman modulation contrast microscopy.

3.2. Materials and Methods.

3.2.1. Cycloheximide studies.

Initial studies were carried out to determine the sensitivity of the protozoal population isolated from the model system, to cycloheximide by a tube assay method.

3.2.1.1. Cycloheximide preparation

A ImM stock solution of cycloheximide (molecular weight = 241.4) was prepared by dissolving 0.2814 g in a small amount of ethanol and then adding sterile water to one litre. The solution was then stored at 4 °C and diluted with sterile water as necessary to the required concentration.

3.2.1.2. Cycloheximide tube assay.

The chemostat, inoculum and media were all as previously described (Chapter 2.2.1). Two R2A plates were inoculated with 100 µl of the planktonic phase of the chemostat, spread-plated, sealed in plastic bags and then incubated at 30 °C. After seven days these plates were washed with PAS (Appendix 3) into sterile universal bottles as previously described (Chapter 2. 2.). The amoebal suspensions, obtained from the plates, were then centrifuged at 1000 x g for 10 minutes, the supernatant was then removed and discarded. The amoebae were prepared for enumeration by taking 900 µl of the amoebal suspension and adding 100 µl of 1% formalin to immobilise the trophozoites and then counted in an improved Nebauer counting chamber. The remaining trophozoite suspension, which consisted of a mixed suspension of amoebae tentatively identified as *Hartmanella vermiformis* and *Acanthamoeba polyphaga*, was then standardised by diluting with PAS to give a suspension containing $\cong 10^5$ trophozoites ml^{-1}. A dilution series was prepared in PAS from the cycloheximide stock solution and equal volumes of the amoebal suspension added to give final concentrations in duplicate sterile tubes

ranging from 0-50 μM solutions. The tubes were incubated at 30 °C, which was the temperature of the chemostat, 50 μl from each tube was then inoculated onto the centre of three *K. aerogenes* lawn plates after time intervals of 30, 60 and 120 minutes. Viability was assessed by the formation of plaques on the *Klebsiella* lawn plates and by microscopic examination for the presence of motile amoebal trophozoites.

3.2.1.3. Cycloheximide addition to the chemostat and media supply.

Cycloheximide was added to the chemostat to give an final concentration of 100 μM. To avoid dilution by the filter sterilised tap water used as the growth medium, cycloheximide was also added to the medium before filtration to maintain this concentration. Higher concentrations of cycloheximide were prepared in a similar manner.

3.2.1.4. Cycloheximide plate assay.

An assay system was developed to monitor the concentration of cycloheximide in the system. The sensitivity of several species of yeast, including *Candida albicans, Candida pseudotropicalis, Sporobolomyces salmonicolor, Rhodotorula rubrum* and *Saccharomyces cerevisiae* to cycloheximide, was examined by swabbing a dilute suspension of the appropriate organism over duplicate plates of malt agar. Wells measuring 9 mm in diameter were cut into the plates with a sterile cork borer and a standard solution of 100 μl of 100 μM cycloheximide was added to duplicate wells. A control well containing 100 μl of distilled water was used. The diameters of the zones, formed by the inhibition of the yeast by cycloheximide were measured and compared with the standard.

When used to assay cycloheximide concentrations in the model system and in the media, *Saccharomyces cerevisiae* on malt agar plates proved to give the most consistent and

accurate results. The concentration of cycloheximide in the system was then monitored at regular intervals.

3.2.1.5. Enumeration of isolates from the chemostat.

Both the total heterotrophic population and the avirulent *L. pneumophila* were removed from the system and prepared and enumerated as described earlier (Chapter 2.2.2.2).

3.2.1.6. Amoebal viability studies following cycloheximide addition.

To assess the viability of protozoa in the chemostat 10 ml samples of the planktonic phase were removed from the chemostat, centrifuged at 1000 x g for 10 minutes. and the deposit inoculated onto the centre of two replicate *K. aerogenes* lawn plates. For assessment of the viability of protozoa in the biofilm, two glass tiles were aseptically removed from the chemostat, rinsed in sterile water and each placed on the centre of a *Klebsiella* lawn plate. A control plate was inoculated from an amoebal suspension prepared with an axenic strain of *Acanthamoeba polyphaga*. The plates were then sealed in plastic bags, incubated at 30 °C and examined at frequent intervals for up to 21 days. Microscopic examination of the undiluted planktonic phase and the glass tiles was also carried out.

3.2. 2. Virulence studies.

3.2.1. Comparison of virulence using the streak plate method

The method described by Rowbotham (1980) was adapted to compare the effect of both the avirulent and virulent *L. pneumophila* serogroup 1 Pontiac (Corby) strain on a mixed culture of amoebae isolated from the chemostat. UV killed *K. aerogenes* lawns were prepared as described [Appendix 3]. A small square was cut from a plaque corresponding to a grazed area on an R2A plate which had been previously inoculated with a sample of undiluted planktonic phase from the chemostat. This was then inverted and placed in the centre of a *K. aerogenes* lawn plate. A heavy suspension of fresh *L. pneumophila* was prepared in sterile distilled water, from the cultures which had been

maintained at -70 °C. A line of the appropriate *L. pneumophila* was made by streaking a plastic loop inoculated with the suspension across the plate at a distance of approximately 1cm away from the edge (Fig. 3.1.). The plates were then sealed in plastic bags containing dampened tissue to avoid any dehydration of the plates, and incubated at 30 °C. The plates were examined daily for plaques the presence of which indicated grazing by the amoebal inoculum.

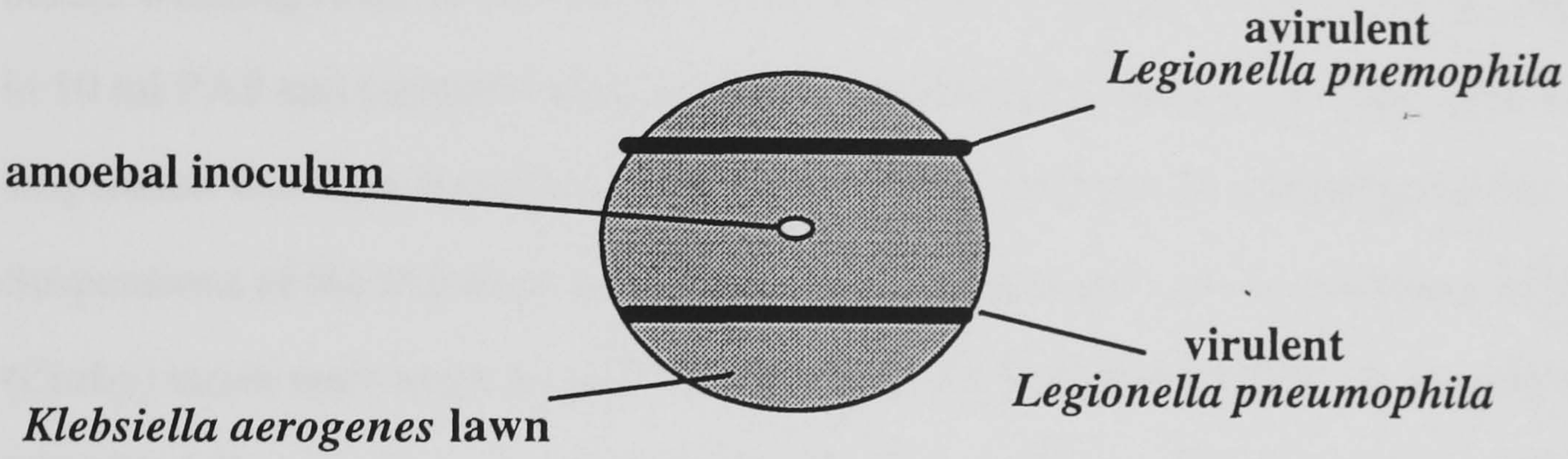

Fig. 3.1. A schematic diagram of the inoculated *K. aerogenes* lawn plates. The avirulent and virulent *L. pneumophila* were streaked across a *Klebsiella aerogenes* lawn to compare their effect on a mixed amoebal culture which had been isolated from the chemostat.

3.2.2.*Acanthamoeba polyphaga* studies

3.2.2.1. The *Acanthamoeba polyphaga*.

An axenic strain of *Acanthamoeba polyphaga* was kindly supplied by Dr. T. Rowbotham from Leeds PHLS. This *Acanthamoeba polyphaga* had previously been isolated from the source of the outbreak of Legionnaires' disease which occurred at the Lincoln Police Headquarters and had been shown to be a suitable host for *L. pneumophila* serogroup 1 (Rowbotham, personal communication). A further advantage of using this particular *Acanthamoeba polyphaga* is that it is a non-pathogenic strain as it was able to multiply at 35 °C but not at 37 °C.

3.2.2.2. The effect of virulence on intracellular replication of *L. pneumophila*

3.2.2.2.1. Method.

Three universals of 20 ml peptone yeast extract glucose broth (PYG) for the culture of amoebae [Appendix 4] were inoculated with 0.5 ml of the axenic culture and incubated at 35 °C. After 48 hours the amoebal suspensions were centrifuged at 1000 x g for 10 minutes to pellet the amoebae. The pellets were then resuspended in PAS and pooled before washing twice in fresh PAS. After the final washing the pellet was re-suspended in 10 ml PAS and counted using an improved Nebauer counting chamber. The amoebal suspension was then dispensed in 900 µl amounts into 9 sterile microfuge tubes. Suspensions of the avirulent and virulent *Legionella pneumophila* serogroup 1 Pontiac (Corby) strain were made to give an approx 0.5 McFarland standard. A suspension of UV killed *K. aerogenes* was prepared in the same way as a control organism. Aliquots (100 µl) of the virulent and avirulent *Legionella* suspension were added to three tubes each and 100 µl of *K. aerogenes* was added to the remaining three tubes. These were then incubated overnight (16-24 hours) in a 35°C waterbath. The suspensions were then viewed with a Nikon Labophot microscope fitted with Hoffman modulation contrast optics.

3.2.2.3.Transmission Electron Microscopy (TEM).

Amoebal suspensions were grown as above in PYG and then washed in amoebal saline and centrifuged at 1000 x g for ten minutes to pellet. The supernatant was removed and 1ml of 4% v/v glutaraldehyde was added to fix the pelleted amoebae and *L. pneumophila*. Further preparation and microscopy was carried out by Andrew Skinner at John Radcliffe Hospital, Oxford as follows:

At the start of the preparation and between each stage below, a pellet of cells was obtained by centrifuging the sample at 3000 x g for 1-2 minutes and then removing the supernatant.

The pellet was then suspended overnight (16-24 hours) in phosphate buffered saline PBS pH 7.2, followed by the addition of 4% $^{v}/_{v}$ OsO_4 for 45 minutes. The stained pellet was then put through the following dehydration series of alcohol/water ($^{v}/_{v}$) 35%, 70%, 95% for 30 minutes each, followed by industrial methylated spirits and finally absolute alcohol.

The pellet was then suspended in propylene oxide for 45minutes, followed by a 50:50 mixture of propylene oxide and spurr resin for 1 hour. Neat spurr resin was then added for 1 hour, followed by fresh spurr resin, taking care not to disturb the pellet at this stage. This was then placed at 70 °C overnight (16-24 hours) to polymerise before sectioning and viewing by Phillips 301 Transmission Electron Microscope.

3. 3. Results.

3.3.1. Cycloheximide tube assay.

The results of the tube assay showed that 50 μM of cycloheximide successfully inhibited plaque formation, corresponding to trophozoite grazing. This occurred after a contact time greater than 30 mins. (Table 3.1)

cycloheximide concentration (μM)	0 mins	30 mins	60 mins	120 mins
50	+	-	-	-
40	+	+	+	+
30	+	+	+	-
20	+	+	+	+
0	+	+	+	+

Table 3.1. Effect of cycloheximide on amoebal trophozoites. Tube assay results showing the susceptibility of the amoebal trophozoites from the chemostat to cycloheximide. + denotes plaque formation on the *Klebsiella* lawn plates, – denotes no plaque formation or motile trophozoites after 21 days incubation.

3.3.2. Cycloheximide plate assay.

Saccharomyces cerevisiae (strain X2180-1A) proved to be the most suitable species for this assay. It gave the most consistent results when the zones of inhibition formed by the action of cycloheximide at known concentrations were measured. No loss of activity was detected either in the medium supply or in the chemostat itself, using the assay.

Both the medium and the planktonic phase sample gave consistently the same zone size as the 100 μM standard (Table 3.2).

zone size with 100 μM cycloheximide standard (mm)	**zone size with cycloheximide in filtered tap water (mm).**	**zone size with cycloheximide in chemostat (mm)**
27.7 (SE= 0.33)	28	28 (SE= 0.33)

Table 3.2. A comparison of cycloheximide activity in the filter sterilised tap water used as the continuous culture medium and in the chemostat with a standard solution of cycloheximide. Zones produced by the inhibition of *Saccharomyces cerevisiae* (strain X2180-1A) growth by the action of cycloheximide (n=3).

The initial concentration of cycloheximide (100 μM) resulted in the proliferation of a black slime in the chemostat which covered the walls of the vessel, trailed from the suspended tiles and clogged up the tube to the sampling port. Microscopic examination of the film showed that this slime was of fungal origin. A slow growing fungus tentatively identified as *Phialophora* sp. was isolated on malt agar. This would not grow on further attempts at subculture onto solid media presumably due to the toxic effects of the cycloheximide.

The concentration of cycloheximide was then progressively increased in the system, until complete inhibition of grazing, and no visible trophozoites were observed in samples taken from both the planktonic and sessile phases. The effect of the cycloheximide is apparent when the undiluted planktonic phase R2A plate was compared with the one obtained prior to cycloheximide addition (Fig. 3.2).

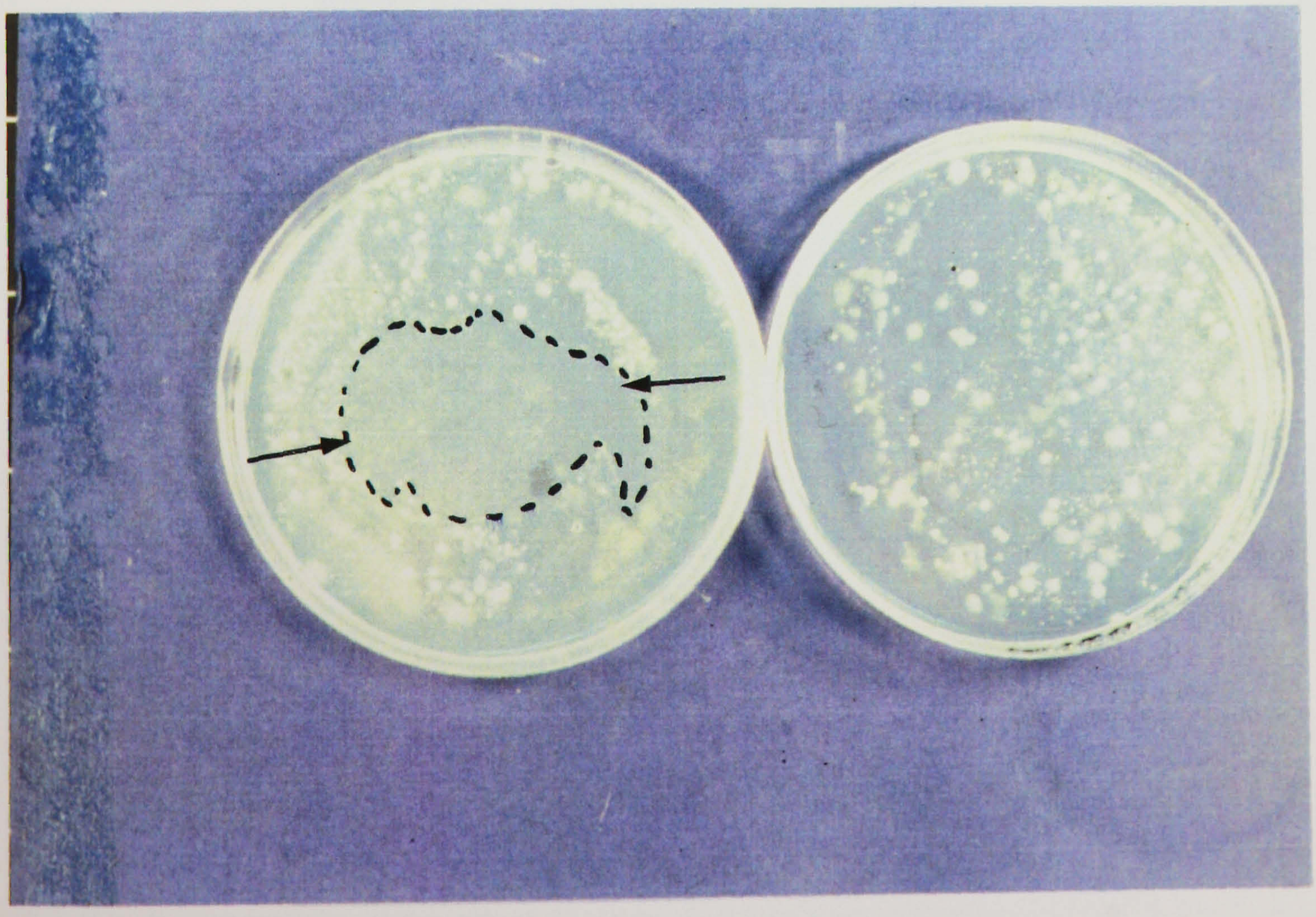

Fig. 3.2. The effect of cycloheximide on the planktonic heterotrophic population.
Undiluted planktonic phase samples cultured on R2A, pre-cycloheximide addition (LHS) and post cycloheximide (RHS). Plaques (arrows) due to grazing of the heterotrophic bacteria can clearly be seen on the plate from the untreated sample.

The counts of the heterotrophic bacterial population increased in both the planktonic and sessile phases of the system following cycloheximide addition. (Figs. 3.4-3.5). The numbers of *L. pneumophila* also increased from 2.48 $\log_{10}$ cfu ml^{-1} to 4.92 $\log_{10}$ cfu ml^{-1} (Fig. 3.4) in the planktonic phase and from 3.52 $\log_{10}$ cfu cm^{-2} cfu cm^{-2} to 5.35 $\log_{10}$ cfu cm^{-2} (Fig. 3.5) in the biofilm.

3.3.3. Virulence studies.

The virulence study using the streak plate method showed that the advancing amoebae were not inhibited or infected by the avirulent strain of *L. pneumophila*. The amoebae progressed and grazed through the line of avirulent *L. pneumophila*. Conversely the virulent *L. pneumophila* inhibited the progress of the amoebae (Fig. 3.3) and microscopic examination of this area showed numerous cysts and atypical forms of amoebae.

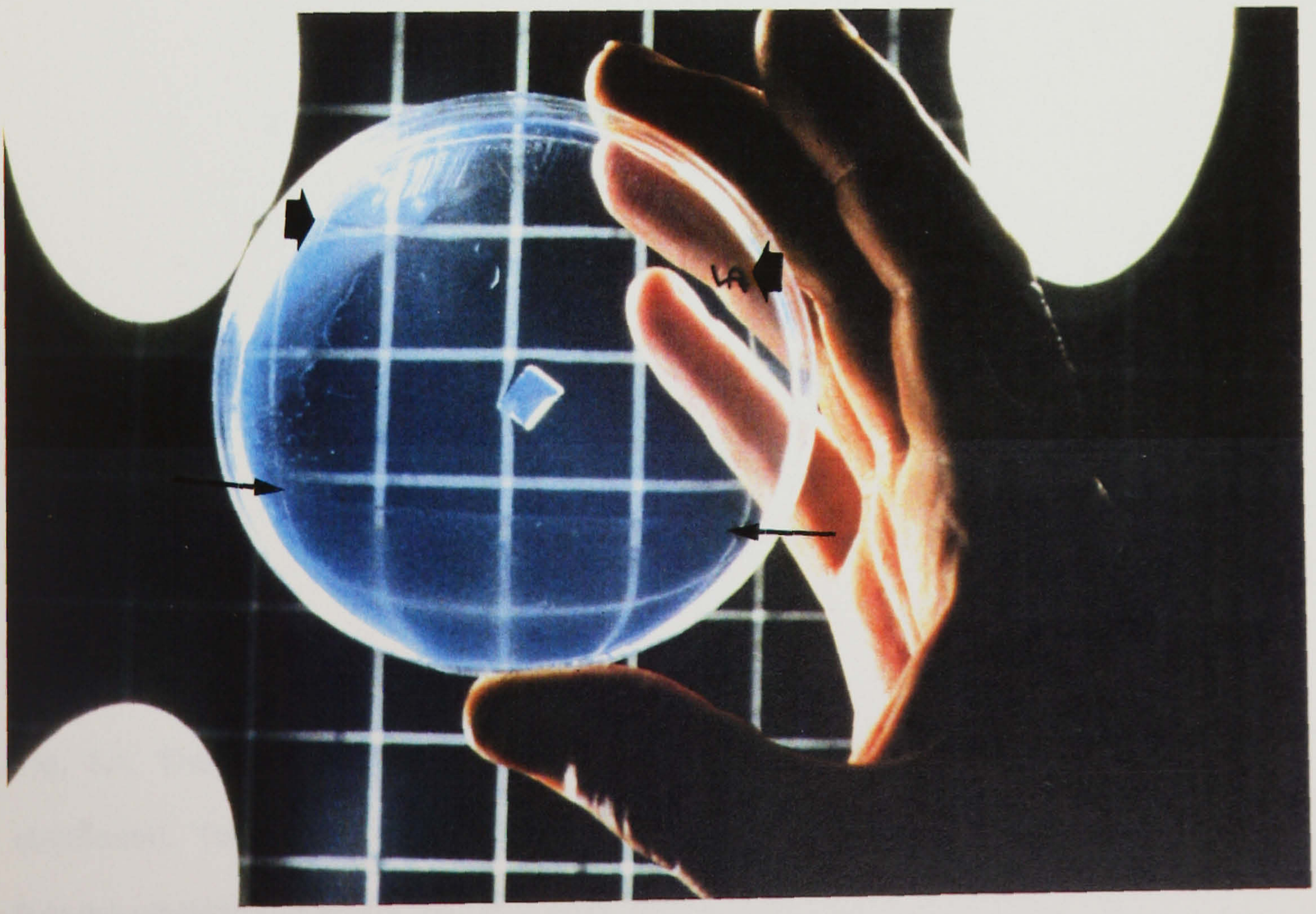

Fig. 3.3. A comparison of the effect of virulent and avirulent *L. pneumophila* on amoebal grazing. The mixed amoebal culture obtained from the chemostat was inhibited by the line of virulent *L. pneumophila* (long arrows), but not by the line of avirulent *L. pneumophila* (short arrows).

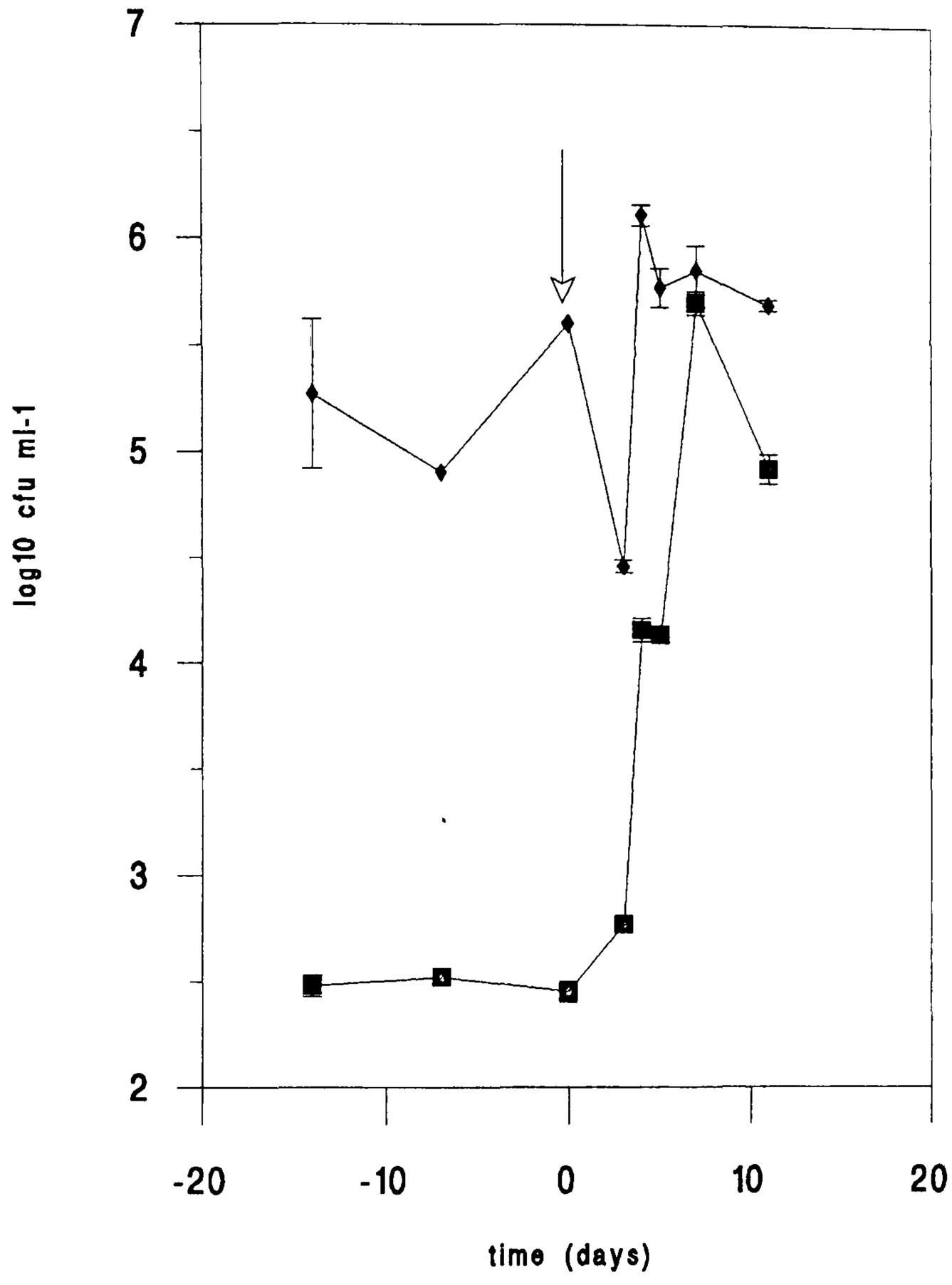

Fig. 3.4. The effect of cycloheximide on the planktonic bacterial population of the chemostat. Total counts of the heterotrophic population ♦, and the *L. pneumophila* population ■ in the planktonic phase of the continuous culture model system, before and following addition of cycloheximide (arrow) [n=3]

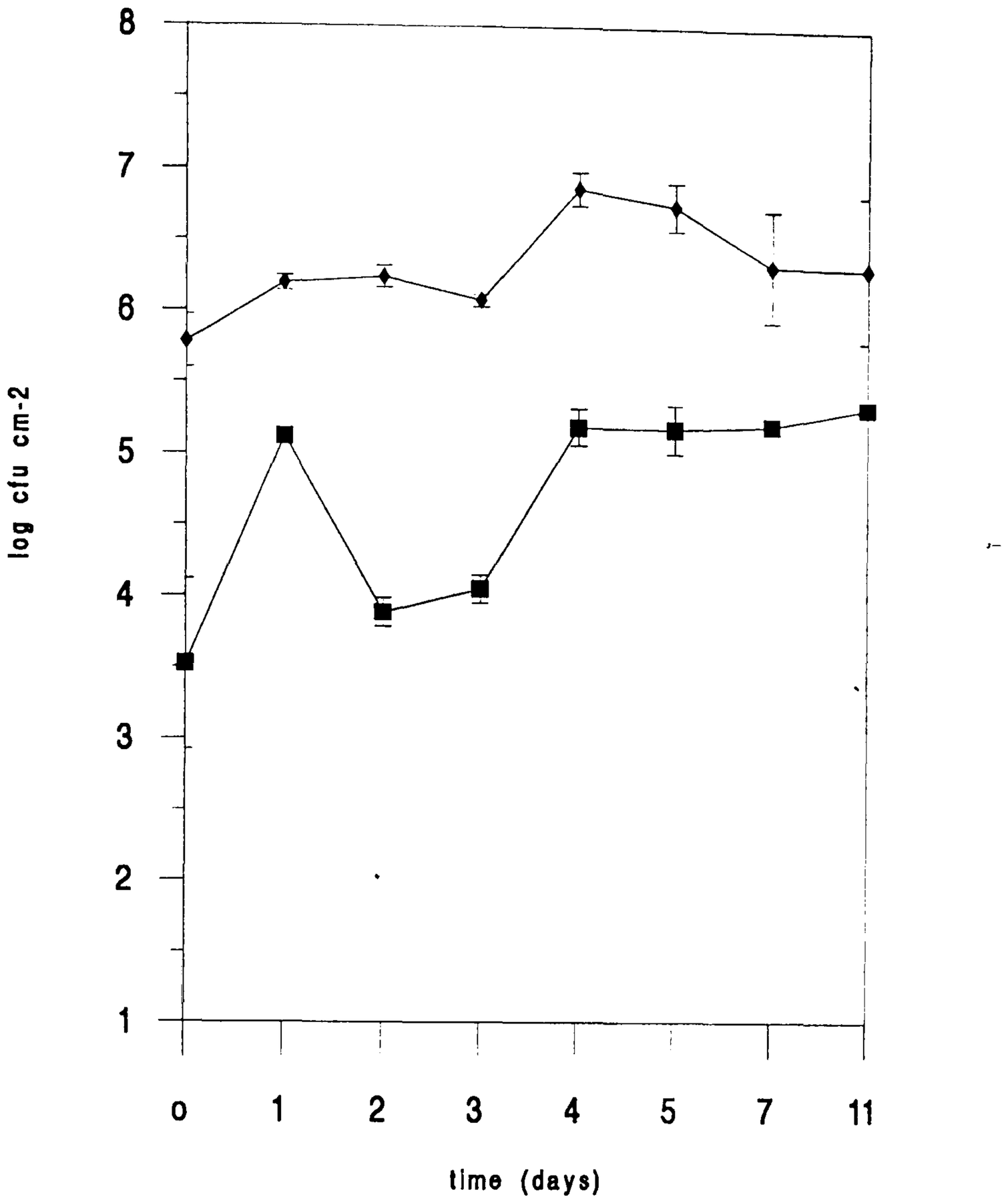

Fig 3. 5. Effect of cycloheximide addition on the biofilm bacterial population. Total biofilm counts of heterotrophic bacteria ♦and of *L. pneumophila* ■, in seven day biofilms following cycloheximide addition.

The results of the virulence studies using the axenic strain of *L. pneumophila* show that the virulent *L. pneumophila* infects and multiplies within the amoebae. Fig. 3.5 shows *Acanthamoeba polyphaga* after overnight incubation with the virulent strain viewed by Hoffman modulation contrast microscopy. There were many amoebae packed with *L. pneumophila*, some in the process of bursting , together with much debris. The *Acanthamoeba polyphaga* which had been incubated with the avirulent strain however, was still motile, with no signs of infection and no burst amoebae (Fig. 3.6). The *Acanthamoeba polyphaga* which had been incubated with the avirulent strain when viewed by TEM showed that some of them contained vacuoles with *L. pneumophila* inside (Fig. 3.7). Some of these vacuoles contained degenerate material including what appeared to be the remains of the *Legionella* (Fig. 3.8). These amoebae, apart from the presence of *Legionella* in the vacuoles, did not differ from the control (Fig. 3.9). The *Acanthamoeba polyphaga* which had been incubated with the virulent strain when viewed by TEM showed several intracellular *L. pneumophila* and damage caused to the amoebae by the cytotoxic activity of these bacteria (Fig. 3.10).

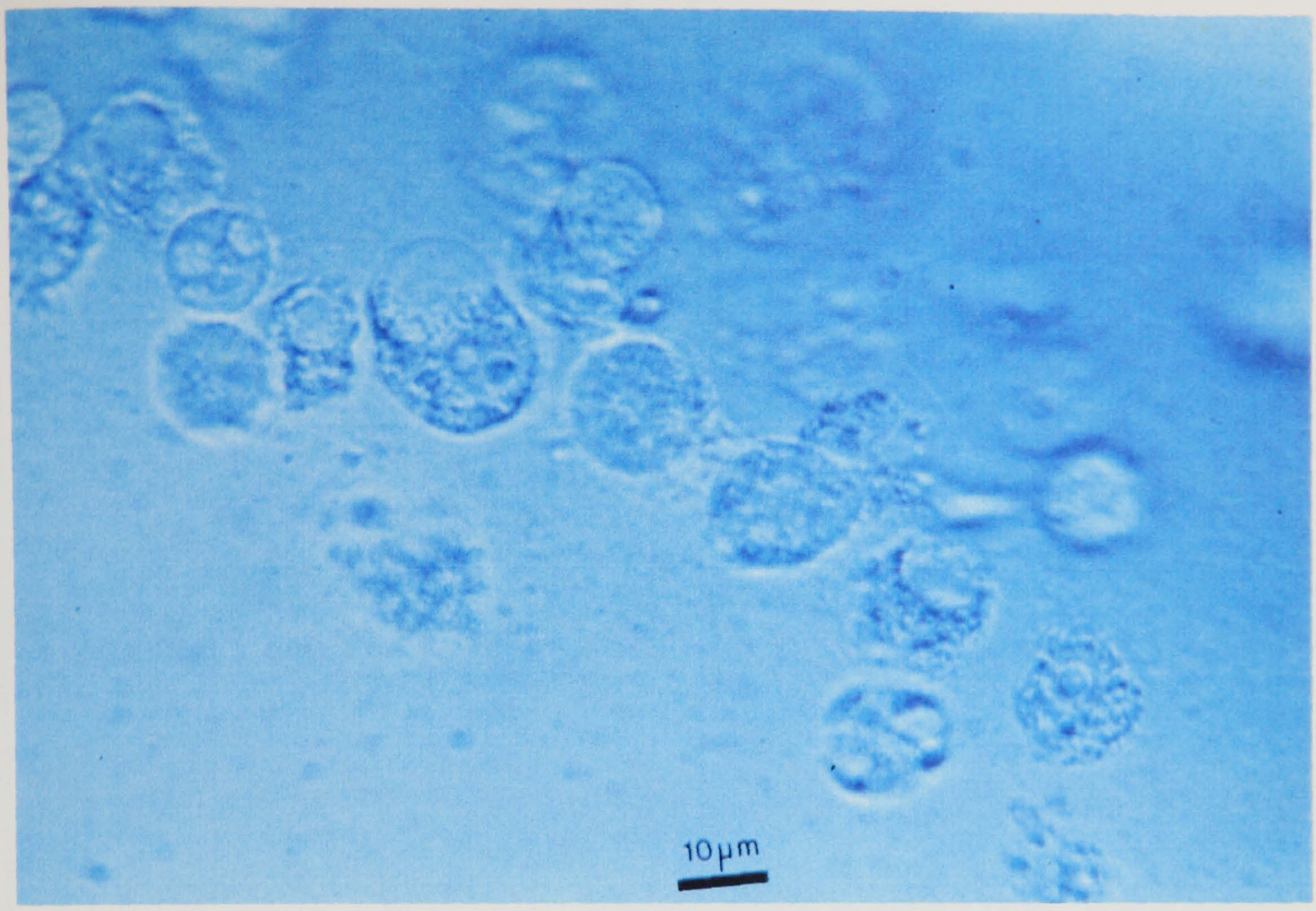

Fig.3.5. *Acanthamoeba polyphaga* overnight incubation with virulent *L. pneumophila*.

These amoebae were non-motile and packed with *L. pneumophila*

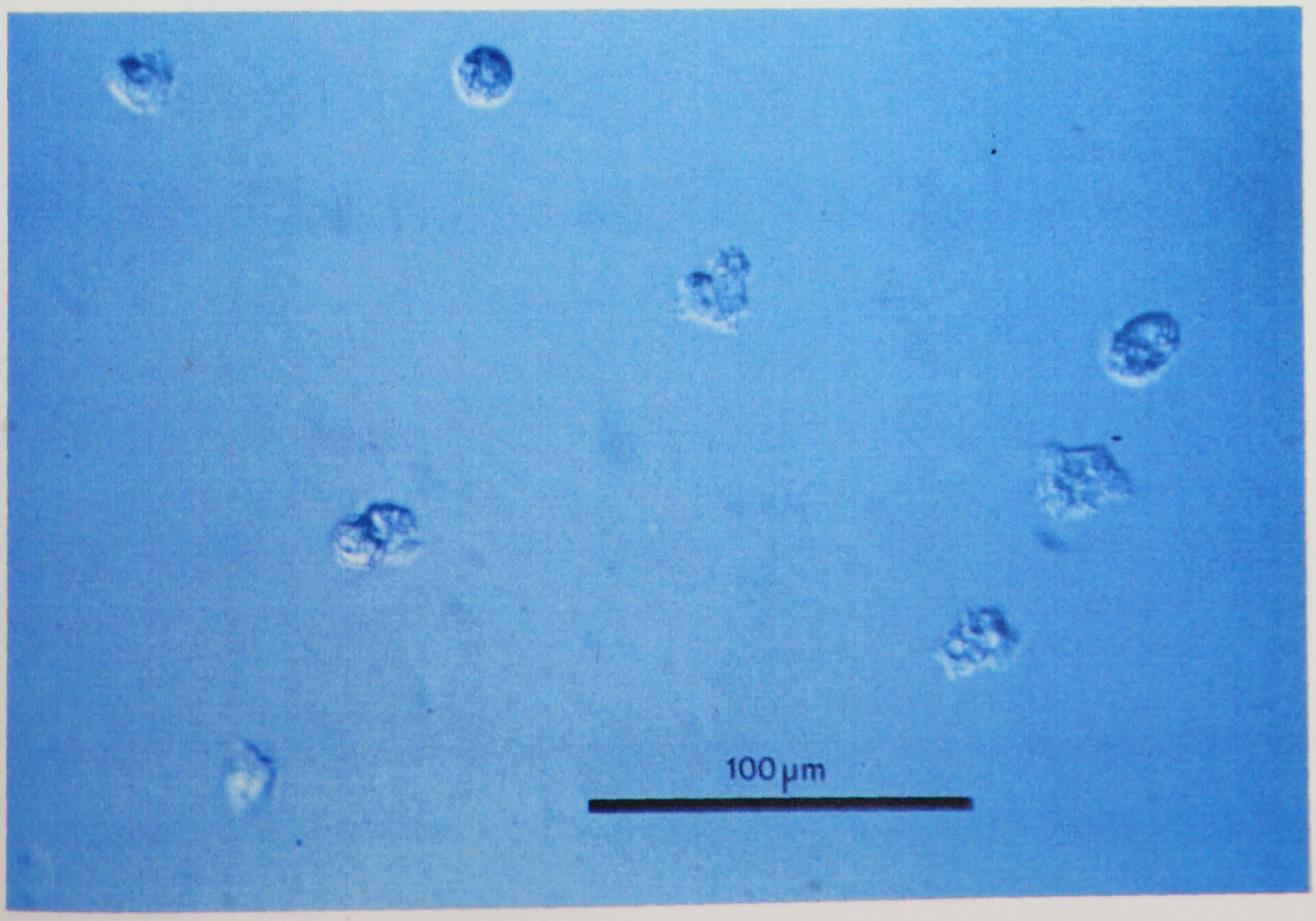

Fig. 3.6. *Acanthamoeba polyphaga* after overnight incubation with the avirulent *L. pneumophila*. These amoebae were still motile and there were no signs of infection or debris.

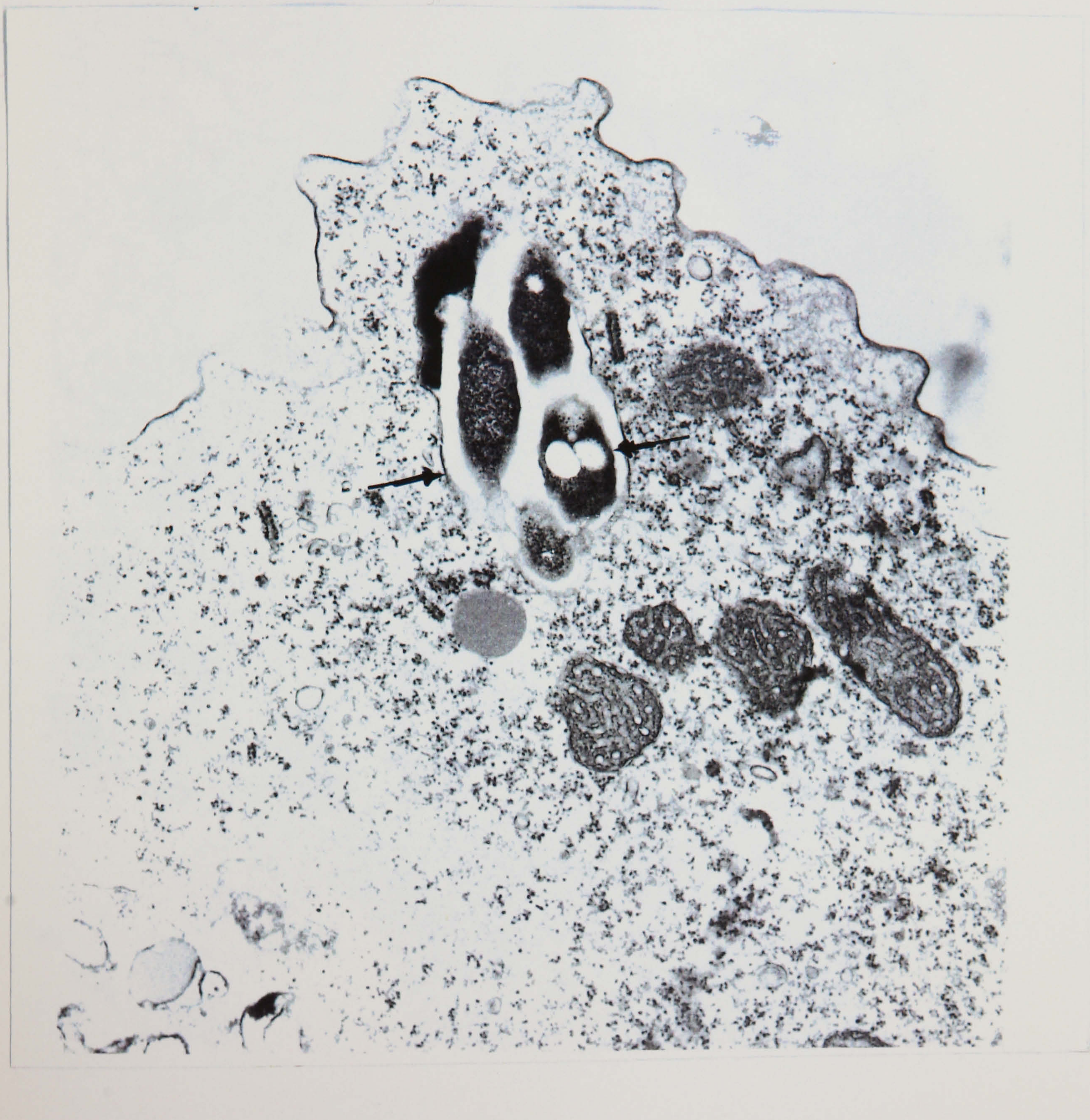

Fig. 3.7. TEM micrograph of an *Acanthamoeba polyphaga* following incubation with the avirulent *L. pneumophila*. A vacuole is visible containing *Legionella* (arrows). (magnification x 33,000)

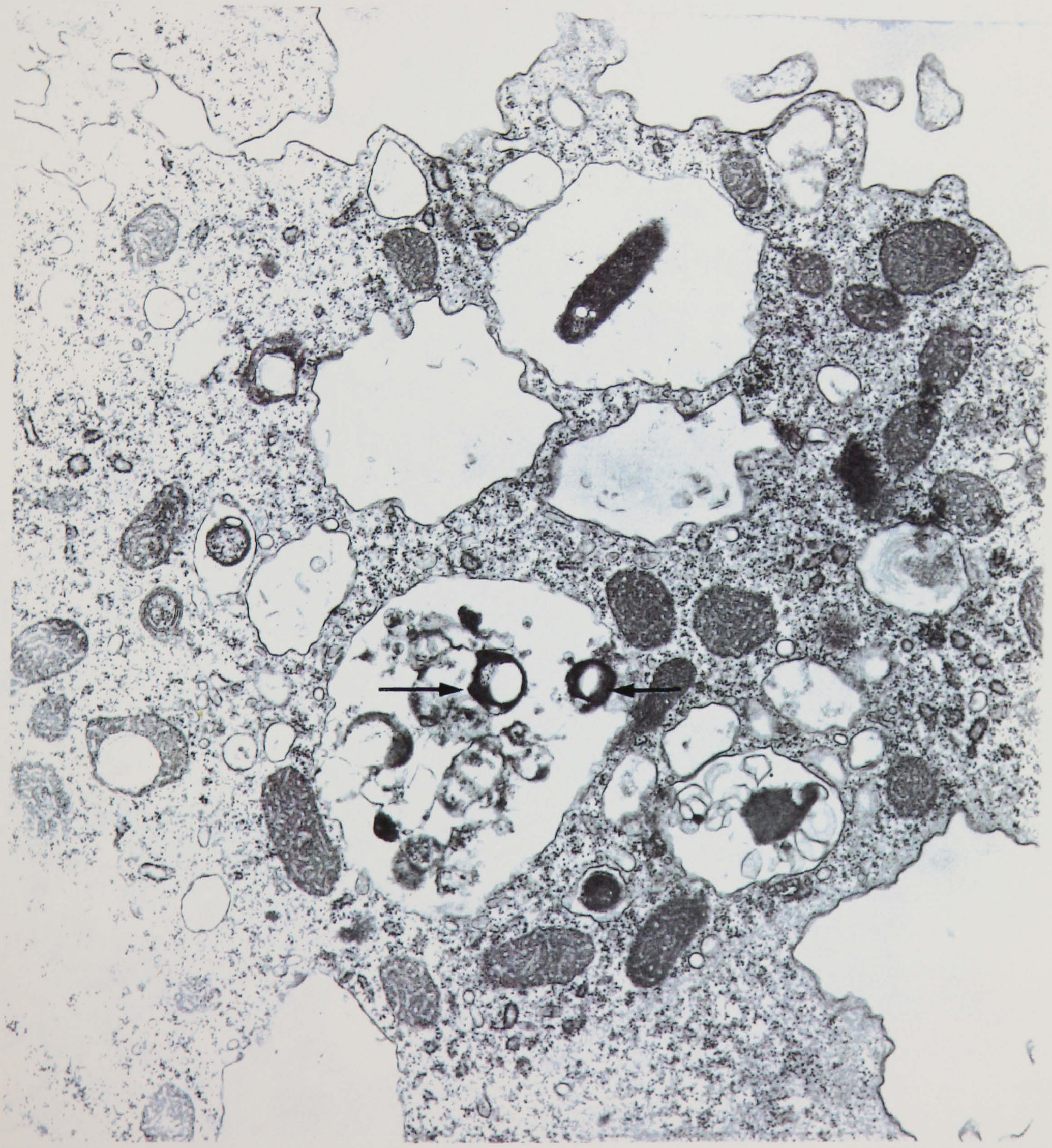

Fig. 3.8. *Acanthamoeba polyphaga* following incubation with avirulent containing *L. pneumophila.* One vacuole with an avirulent *L. pneumophila* and a vacuole containing degenerate material which appears to be *L. pneumophila* (arrows) are visible. (Magnification 19,800).

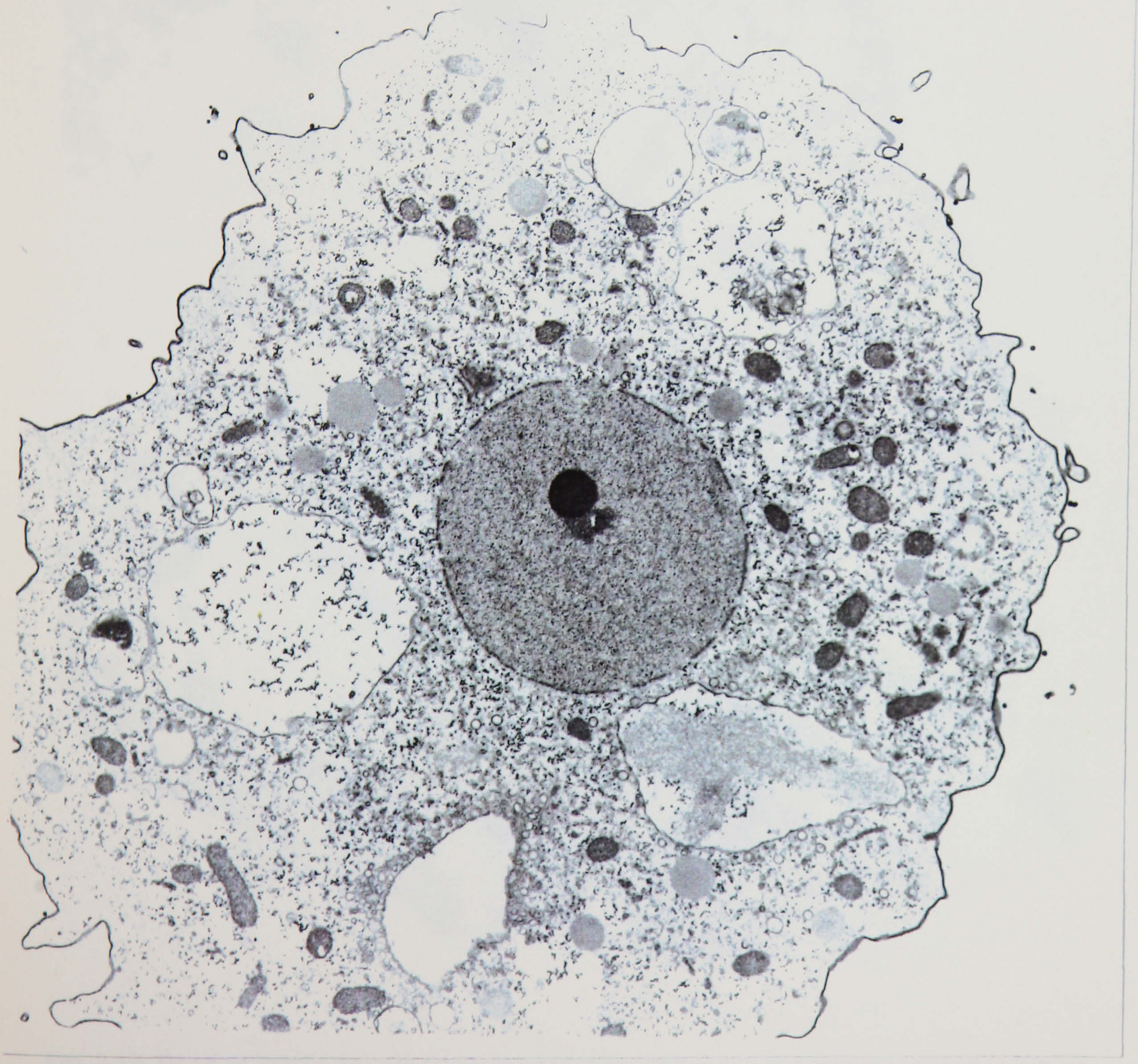

Fig.3.9. Control *Acanthamoeba polyphaga* following overnight incubation (16-24 hours).

(Magnification 9,600)

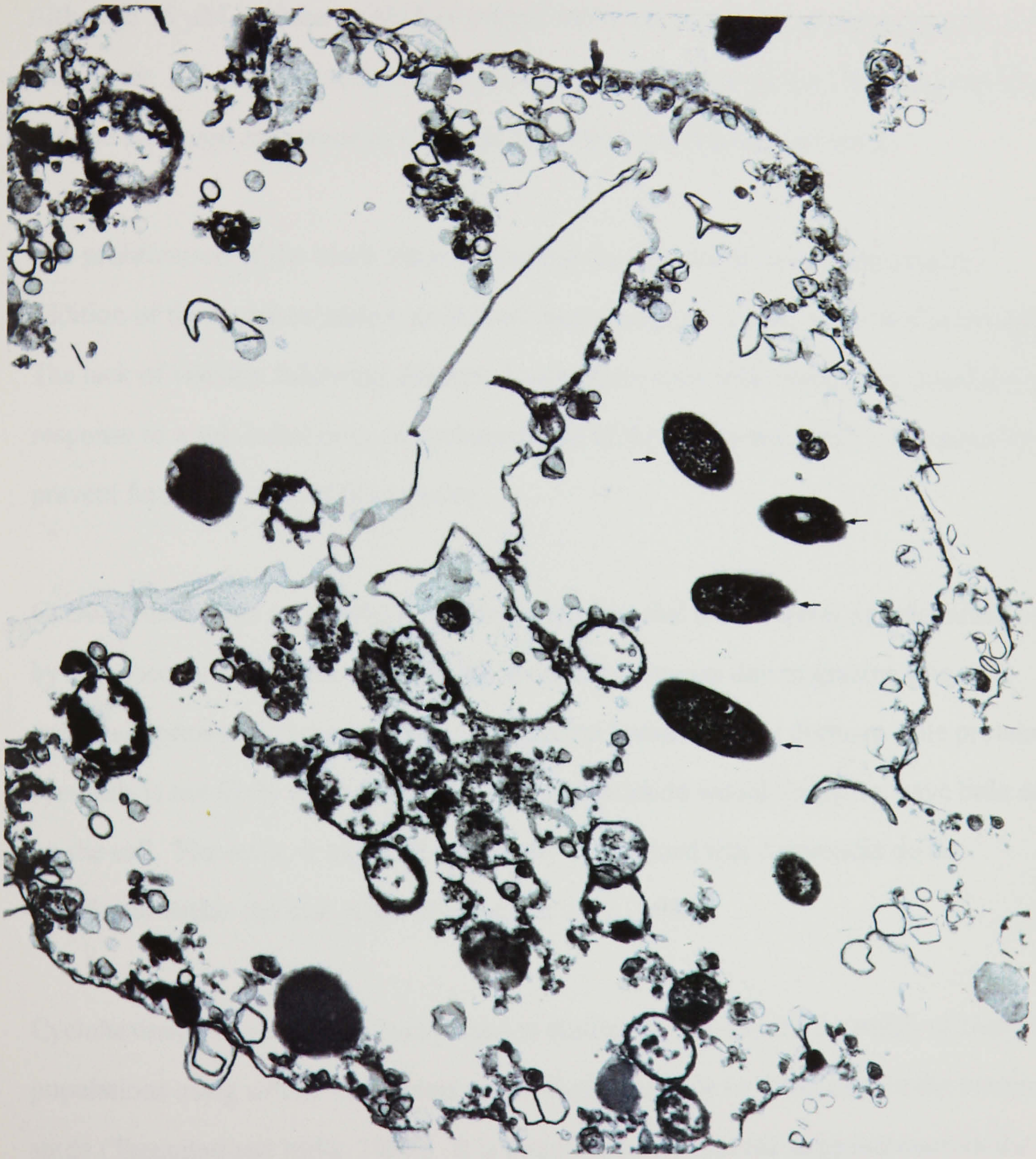

Fig. 3.10. Dead or dying *Acanthamoeba polyphaga* following overnight incubation (16-24 hours) with the virulent strain of *L. pneumophila*. Several intracellular *Legionella* (arrows) can be seen with severe internal damage to the amoebae, presumably due the cytolytic activity of the virulent *L. pneumophila*.

(Magnification x 25,200)

3.4. Discussion.

Although 50 μM cycloheximide had proved sufficient to inhibit protozoal grazing in the tube assay, 100 μM was added to the model system because of the presence of biofilms and the increased resistance to chemical treatments associated with them.

The proliferation of the black slime containing the *Phialophora* sp. following the addition of the cycloheximide was in itself interesting and worthy of further investigation. The lack of viability following attempted subculture onto solid media was possibly as a response to a sub-lethal dose of cycloheximide in the chemostat,which was sufficient to prevent further culture of this species.

Cycloheximide was successful in inhibiting the amoebal trophozoites as determined both by microscopic examination and by the absence of plaques due to grazing (Fig. 3.2). The continued presence of cysts was not unexpected because in this dormant state protein synthesis is not likely to be occuring and cycloheximide would therefore, have little effect on the cell. However, it has been previously determined that *Legionella* do not proliferate within the cyst stage (Bercouvier *et al.*, 1986).

Cycloheximide has been previously used in studies to quantify the mortality of bacterial populations using similar concentrations of cycloheximide to those used in the present study (Tremaine and Mills, 1987). It is therefore likely that the large increases in the bacterial counts observed for samples taken from both the planktonic and sessile phases of the chemostat (Figs. 3.5-3.6) were due to the successful inhibition of the trophozoite population.

The results of the virulence studies show that the avirulent *L. pneumophila* is grazed by the protozoal population of the model system (Fig.3.3). The amoebae were not infected or inhibited by the line of avirulent *Legionella* and they continued to advance to the edge

of the plate. The microscopic studies using both Hoffman modulation contrast microscopy and TEM confirm that the virulent strain (Fig. 3.10) not only infected and proliferated within these amoebae but also resulted in the death of the infected protozoa. The avirulent *L. pneumophila* however, did not infect and proliferate within these protozoa (Figs. 3.5-3.8) and like the control, remained motile (Fig. 3.9.). It is unlikely therefore, that the increase in *L. pneumophila* counts obtained for both the planktonic phase and the biofilms following the addition of cycloheximide (Figs. 3.4-3.5) was due to the release of *Legionella* from dead or dying protozoa.

The results observed for the virulent strain on the streak plate tests are in agreement with those previously described by Rowbotham (1980). He also observed that a *L. pneumophila* serogroup 1 Pontiac strain caused cyst formation and inhibited the progression of amoebae past that virulent strain.

The results of this study are important in two respects; firstly the effect that trophozoite grazing has on the bacterial population is quite substantial as seen by the rise in bacterial counts. This has not been taken into account in previous studies (Keevil *et al.*, 1988; Walker *et al.*, 1991). Grazing will affect the development of the biofilm consortia, by providing new niches for recolonisation which may not necessarily be by the same bacterial species. Furthermore, the release of excretory and other products by the protozoa would increase the available nutrients to the remaining bacterial population. Secondly, and more importantly to the present study, inhibition of the protozoal population did not result in the loss of *L. pneumophila* from the chemostat vessel. This finding suggests that *L. pneumophila* is not an obligate parasite of protozoa.

Intracellular proliferation is therefore probably opportunistic, allowing this bacterial species to survive in otherwise hostile conditions, for example, in periods of drought. In systems where conditions are conducive to the survival and proliferation of *L. pneumophila*, such as man-made aquatic systems, the presence of biofilms rather than protozoa may be the important factor in the continued presence of *L. pneumophila*. The

importance of the non-protozoal biofilm consortia upon the survival of *L. pneumophila* will be considered in the next chapter.

CHAPTER 4.

LEGIONELLA PNEUMOPHILA AND OTHER AQUATIC BACTERIA

4.1 Introduction

Drinking water is not sterile. There is a diverse range of microorganisms which can occur naturally within water distribution systems, usually occurring as mixed consortia which may include protozoa, fungi, yeasts, bacteria and viruses, (Keevil *et al.*, 1989; Mackerness *et al.,* 1991; Rogers *et al.,* 1991). As previously discussed (Chapter 1), the presence of non-legionellae microorganisms, including protozoa, has been shown to be an important factor in the proliferation and survival of *L. pneumophila* in the natural environment (States *et al.,* 1985, Fields *et al.,* 1989; Nahapetian *et al.*, 1991; Wadowsky and Yee, 1985; Wadowsky *et al.*, 1991).

Water alone is not sufficient to allow *L. pneumophila* to proliferate. Skaliy and McEachern (1979), Hsu *et al.*, (1984) and Fields *et al.*, (1984), demonstrated that *L. pneumophila* showed long term survival in sterile distilled water and sterile tap water but did not multiply. Yee and Wadowsky (1982), however showed that naturally occurring *L. pneumophila* did survive and multiply in non-sterile tap water. The association of *L. pneumophila* with different species isolated from aquatic sources is well documented. The relationship between *L. pneumophila* and protozoa has been discussed in the previous chapter, but other aquatic microorganisms have also been implicated in maintaining the growth and survival of *L. pneumophila* in the environment (Wadowsky and Yee, 1983; Wadowsky *et al.,* 1988; Grimes, 1991).

It is known that *L. pneumophila* has a nutritional requirement for amino acids (Rowbotham, 1980; Pine *et al.*, 1986) and this is supported by the finding of Wadowsky and Yee, (1983), who observed that *Flavobacterium breve* was able provide enough cysteine or a suitable alternative, to support the growth of *L. pneumophila* on a solid laboratory medium (BCYE) prepared without cysteine. Conversely other authors have found that certain bacterial species inhibited the growth of *L. pneumophila* both on laboratory media (Gomez-Lus *et al.*, 1993b) and also in a model aquatic system (Toze *et al.*, 1993).

An investigation was carried out therefore, to determine whether the non-legionellae microorganisms, isolated from the system, influenced the survival and growth of *L. pneumophila*. The model system was as previously described (2.2.1.1). The planktonic phase and the biofilms which developed, contained the microorganisms of a water distribution system which had not previously been cultured and therefore not selected or altered phenotypically by passage over laboratory media. The *L. pneumophila* was then introduced into this consortium within the model system. The presence of these non-legionellae microorganisms, which co-existed in the system with *L. pneumophila,* had been shown to be essential for the continued survival of *L. pneumophila* in this environment. In the absence of other microorganisms *L. pneumophila* was quickly lost from the system (Chapter 2. 3.1).

R2A medium (Reasoner and Geldreich, 1985), is a low nutrient culture medium devised to improve the isolation of heterotrophic bacteria from potable water. *Legionella* spp. require complex media containing various supplements including iron and cysteine and will not therefore normally grow on R2A. However, it was found that *L. pneumophila* could be recovered from this medium when co-cultured with the non-legionellae bacteria isolated from the biofilm. To determine whether living microorganisms were necessary for the increased survival of the *L. pneumophila*, the effect of certain viable and non-viable non-legionellae biofilm microorganisms, on the survival of *L. pneumophila* on R2A was investigated. Investigations were also undertaken to determine whether isolates from the model system used in the present study supported the growth of *L. pneumophila* on cysteine free media or caused inhibition of *L. pneumophila* when inoculated onto BCYE.

4.2. Materials and Methods

4.2.1. Biofilm development and bacterial growth and isolation.

In this study microorganisms derived from a local mains tap water were inoculated into a continuous culture model biofilm system as previously described (Chapter 2.2.1.1). The system was inoculated with a *L. pneumophila* serogroup 1 Pontiac (Corby strain). Filter sterilised tap water from the same source as the initial inoculum was used as the growth medium (Chapter 2.2.1.2). Biofilms containing both the tap water microorganisms and the *L. pneumophila* formed on the glass tiles suspended within the planktonic phase of the system.

Initial isolation of the bacteria from the glass tiles following aseptic removal and suspension of the biofilms was achieved using R2A medium (Reasoner and Geldreich, 1985). R3A medium [a modified R2A, (Reasoner and Geldreich, 1985)], was used to maintain the bacterial isolates in pure culture. Tentative identification was by the API 20 NE (API Biomerieux, Basingstoke, UK.) and /or Biolog systems (Biolog Inc., Hayward, California).

The *L. pneumophila* was grown on BCYE plates for four days and suspended in sterile filtered water to form a dense inoculum which was swabbed uniformly over R2A plates (LP/R2A).

4.2.2 Growth on R2A.

Bacteria isolated from the biofilms using the method described above were subcultured onto R2A plates and then treated as follows:

4.2.2.1.Whole bacterial cells.

The isolates were swabbed off the R2A plates and streaked across the LP/R2A plates (Fig. 4.1.). A cross streak with saline only was used as a control. An aqueous suspension of each isolate (100 µl) was inoculated into each of two wells cut from a plate containing BCYE prepared without cysteine. Sterile water was used as a control. The plates were incubated at 35°C for seven days and then sealed in plastic bags and left at room temperature for up to six weeks. A sample from each of the cross-streaks was subcultured at seven day intervals onto BCYE and BCYE supplemented with glycine, vancomycin, polymixin and cycloheximide (GVPC). The presence of *L. pneumophila* was confirmed by morphological characterisation, by its failure to grow on BCYE medium minus cysteine and by latex agglutination (Pro-Lab Diagnostics Ltd.).

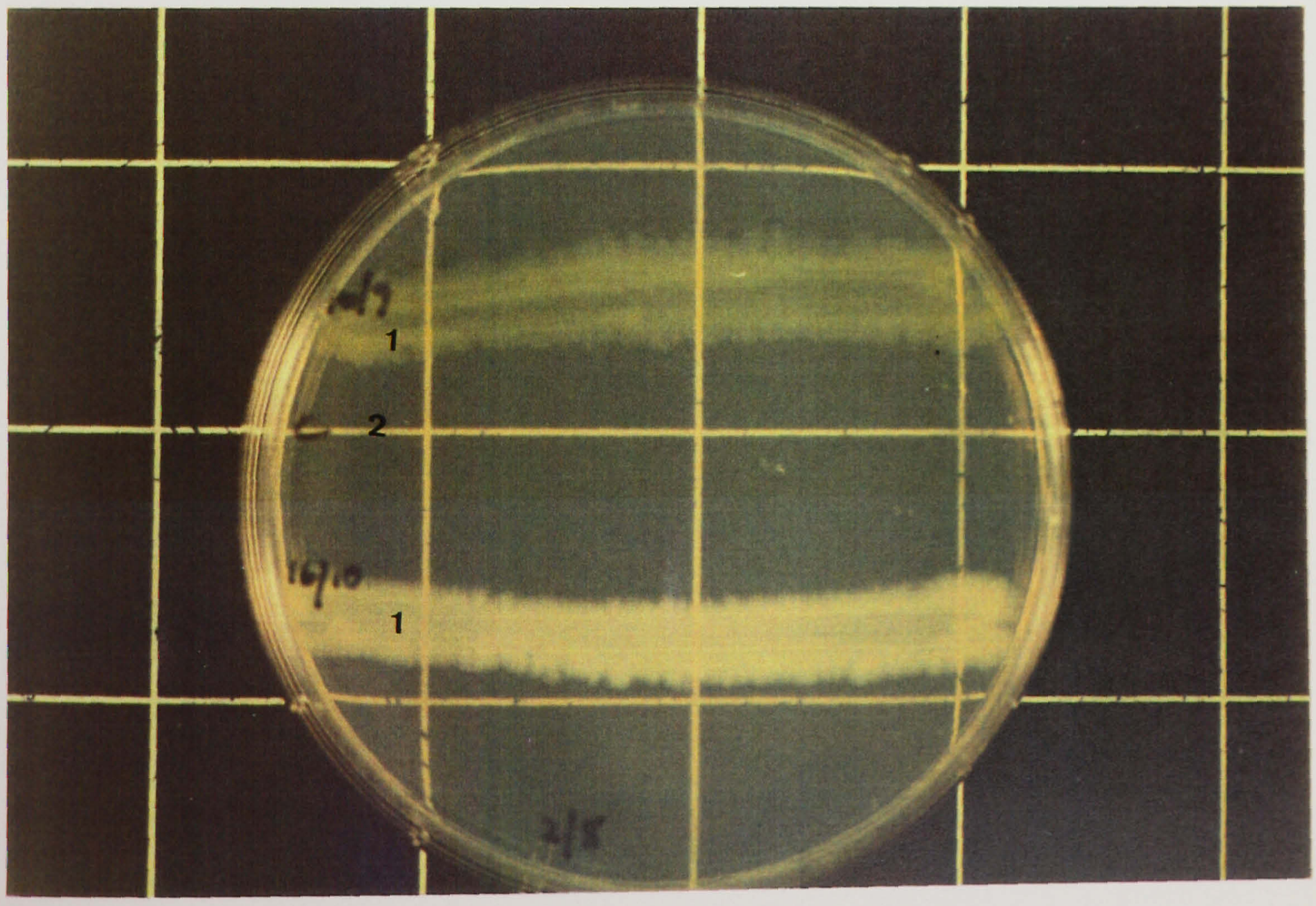

Fig. 4.1. LP/R2A plates. A suspension of *Legionellae* was spread over the entire surface of the plate with a sterile swab. The isolates from the model system were streaked across the plate in parallel lines (1) together with the control (2).

4.2.2.2.Heat treatment

The same bacterial isolates (see section 4.2.2.) were grown up overnight in 100 ml R2A broths. A 20 ml aliquot of each of these overnight cultures was heat treated by immersing in a 60°C water bath for ten minutes. This suspension was then cross streaked onto the LP/R2A plates and incubated at 35°C for seven days. These cross-streaks were then subsequently subcultured onto GVPC and BCYE plates. An aliquot of each suspension (100 µl) was inoculated into two wells in BCYE minus cysteine agar. Sterile R2A broth heated as above was used as a control.

4.2.2.3.Cell free suspensions

A 20 ml aliquot of the overnight culture was centrifuged at 10,000 rpm for ten minutes at 4°C. The resulting pellet was re-suspended in 2 ml of sterile water and sonicated using an MSE Soniprep 150 on full amplitude for a 10 second burst followed by a 20 second rest for a total of 3 minutes. It was confirmed that the cells were no longer viable. The resulting suspension was filtered through a 0.2 µm Millipore filter and the filtrate streaked across the surface of duplicate R2A plates, incubated at 35°C for seven days and subcultured as above. A 100 µl aliquot of the sonicated filtrate was added to two wells cut into BCYE minus cysteine medium. Sonicated R2A broth was used as a control.

4.3. Results.

L. pneumophila could not be retrieved from R2A medium when inoculated onto the plates as a pure culture. Table 4. 1 Shows that the *L. pneumophila* used in this study was still viable after seven days co-culture with intact bacterial cells isolated from a water distribution system. Viability could still be demonstrated with some of these isolates after six weeks. However when these same bacterial cells had been subjected to heat treatment or prepared as cell free extracts and were no longer viable, as determined by lack of growth on R2A, *L. pneumophila* could not be recovered from the R2A plates by subculture onto either BCYE or GVPC.

Only viable non-legionellae biofilm isolates supported the satellite growth of *L. pneumophila* (Fig 4. 2.) on BCYE minus cysteine. Satellite growth occurred consistently with both *Pseudomonas vesicularis* and the *Flavobacterium* sp. Satellite growth could not be detected with either the heat treated or sonicated non-legionellae bacterial extracts. Two species isolated from the system, *Acinetobacter junii and Acinetobacter calcoaceticus,* were found to inhibit the growth of *L. pneumophila* on BCYE.

Fig.4.2. Satellite colony formation of *L. pneumophila*. BCYE without cysteine showing satellite colonies (arrows) of *L. pneumophila* around a well cut into the agar and inoculated with viable *Pseudomonas vesicularis*.

	Whole cells	Heat-treated cells	Cell free extracts
Pseudomonas vesicularis	+	-	-
Pseudomonas sp.	+	nd	-
Pseudomonas paucimobilis	+	-	-
Flavobacterium sp	+	-	-
Methylobacterium sp.	+	-	-
unidentified	+	+*	-
unidentified	+	-	-

Table 4.1. Recovery of *Legionella pneumophila* on BCYE after seven days co-culture with aquatic bacterial isolates from the biofilms developed within the model system. += growth of *Legionella pneumophila*. – =no growth of *Legionella pneumophila*. nd= test not carried out. +* this bacterium survived heat treatment. The isolates labelled as 'unidentified' were oxidase positive Gram negative bacilli, which did not give an acceptable profile with either the API or BIOLOG systems.

4. 4. Discussion.

Several workers have suggested that in the aquatic environment *L. pneumophila* is nutritionally dependent on other micro-organisms (Rowbotham 1980; Stout *et al* 1985; Tesh and Miller, 1981; Wadowsky *et al* 1985;1988) including: cyanobacteria (Tison *et al.,* 1980 Bohach and Snyder, 1983; Fliermans *et al.*, 1981), algae (Pope, *et al.,* 1982) and other bacterial species (Wadowsky and Yee, 1983; Wadowsky *et al.,* 1988; Grimes, 1991,). The nature of these associations is still the subject of some speculation, for example, Tison *et al.*, (1980); have demonstrated that the active photosynthesis of cyanobacteria can support the growth of *L. pneumophila* but as *L. pneumophila* is found in conditions where photosynthesis does not occur this association is likely to be opportunistic and perhaps demonstrates the exploitive nature of *Legionella* species. Bacteria isolated from the biofilms developed in the continuous culture system were shown to prolong the viability of *Legionella pneumophila* when inoculated onto R2A medium. Heat killed and cell free extracts of the same bacteria were not able to support the survival of *L. pneumophila* on R2A.

From Table 4.1 it can be seen that *L. pneumophila* remained viable and culturable only when incubated in the presence of other viable bacteria, as determined by its growth on both the BCYE and GVPC plates. Although *L. pneumophila* could be recovered from the R2A plates visible colonies could not be detected, even when these plates were viewed with a dissecting microscope. The bacterium which was still viable following heat treatment also supported the survival of the *Legionella* which suggests that the intact bacterial cells contribute in some way to the continued survival of the *L. pneumophila.* These cells may be producing a substance which is required either in much larger quantities than found in the cell free extract preparation, or is rapidly utilised by the *L. pneumophila* and therefore must be continually produced by these micro-organisms. Tesh and Miller (1981) demonstrated that the carbon and energy requirments for *L. pneumophila* growth can be obtained solely from amino acids and

Wadowsky and Yee (1985) suggest that the *L. pneumophila* are able to obtain this amino acid requirement from the exogenous products of other aquatic bacteria. They found however, that the the survival of *L. pneumophila* in tap water cultures was enhanced only if large numbers of non-legionellae bacteria were present. The growth of *L. pneumophila* as satellite colonies around two of the isolates, *Flavobacterium* sp. and *P. vesicularis*, suggests that this work concurs with that of Wadowsky and Yee (1985), in that *L. pneumophila* is able to utilise amino acids produced by other aquatic microorganisms. Not all of the microorganisms used in this study were able to support satellite growth and two isolates were shown to inhibit *L. pneumophila* growth on solid media. However, the continued presence of *L. pneumophila* in the model system suggests that this was either a localised effect, possibly due to bacteriocin production, or as a consequence of growth on laboratory media.

Further explanation of the mechanism which enhanced the survival of *L. pneumophila* is needed. The non-legionellae may have been producing an acceptable l-cysteine alternative. A further possibility is that the non-legionellae were removing substances which are inhibitory to *L. pneumophila* survival, a view consistent with the use of charcoal in laboratory media for the culture of *L. pneumophila*. A further possibility is that, as *L. pneumophila* were not visible on the R2A plates and could only be recovered from within the streak of viable bacteria, the non-legionellae may be protecting the *L. pneumophila* from environmental factors such as dehydration which would be an important determinant in the survival of *L. pneumophila* in the environment.

The results of this study suggest therefore, that the non-legionellae play a role as yet undefined in the survival of *L. pneumophila*. Further work is required to establish these processes, which may be important determinants in the continued proliferation of this opportunistic pathogen in the natural environment. Ecological studies are required which will determine whether certain co-inhabitants of biofilms within a system are more important in the survival of *L. pneumophila* than others.

Microscopic techniques play an important role in ecological studies. Chapter 5 discusses the application of various microscopic techniques which allow *in vivo* visualisation of entire biofilms *in situ* on the surface upon which they have developed.

CHAPTER 5

MICROSCOPY

5. MICROSCOPY

5.1.Introduction

In order that we may gain a greater insight into the ecology of the microorganisms that exist in biofilms, it is necessary not only to be able to isolate them individually, but also to have some understanding of the way in which the individual microorganisms interact. Microscopic examination enables us to gain some insight into the spatial organisation that occurs within biofilms on the surface supporting their development. Other information which may be gained from various microscopic techniques include measurement of the depth of biofilm (Bakke and Olsson, 1986), fluorescent labelling to determine cell viability (Walker *et al.*, 1994) and the identification of a particular species, eg. *Legionella pneumophila* within the mixed biofilm consortium (Rogers and Keevil, 1992). The ability to visualise the biofilm and the supporting surface simultaneously can demonstrate the heterogeneity which exists within biofilms. For the *in vivo* visualisation of intact unstained biofilms, modification of the light microscope is required to produce sufficient contrast between the specimen and the background to produce a clear image.

Conclusions.

A number of alternative techniques were used to examine intact biofilms, these included some recent advances which required no prior preparation and which therefore, minimised loss, shrinkage and disturbance of the biofilm and associated microorganisms. Non-disruptive light microscopy techniques included, bright field transmitted light microscopy with phase contrast optics, Hoffman modulation contrast microscopy and Nomarski episcopic differential interference contrast (DIC) microscopy. For higher magnification Atomic Force Microscopy was used.

Other techniques for visualisation of the biofilms which required prior fixation and staining were scanning electron microscopy, fluorescent staining with propidium iodide (PI) and 4' 6-diamidino-2-phenylindole (DAPI). Techniques for the labelling of *L. pneumophila* in the biofilm included immunogold and fluorescein isothiocyanate (FITC). This study compared the results from the use of each of the above methods. Intact biofilms were viewed at various stages of their development *in vitro* on their glass support surface.

5.2. Materials and Methods

5.2.1. Biofilm Production and Preparation

Biofilms were developed on glass coupons within a continuous culture model biofilm system as previously described (Chapter 2). The coupons were removed aseptically from the system and gently rinsed in 10 ml sterile water to remove any unattached cells before either direct visualisation, or fixation and staining, prior to microscopy by one of the methods described below.

5. 2. 2. Phase Contrast Microscopy.

Phase contrast microscopy enables visualisation of transparent objects and gives clear detail and good contrast. This technique can be used to detect small differences in thickness and/or density within the object which change the phase of light without staining or other processing. Light passing through an object is scattered *i.e.* diffracted and retarded compared with the light passing through the surrounding area. If two parallel rays of light in phase pass through different density substances, e.g. air and a biofilm, the ray passing through the denser substance will be delayed or retarded. The amount of retardation depends on the density and thickness of the biofilm. In the eyepiece these two sets of light waves combine and interfere with each other producing brightness, shade or darkness. The microscopist uses this effect of retarding the light

waves to enhance the contrast of transparent and low density materials by inserting an annulus consisting of material of two different densities.(Fig. 5. 1).

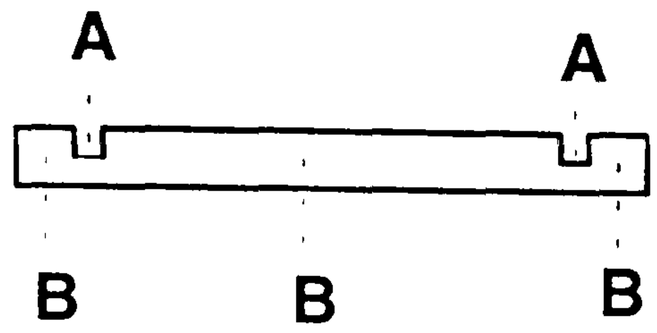

Fig.5. 1. A cross section of a typical phase plate. Light passing through the groove AA has less glass to penetrate than light passing through BBB. (After Cassartelli,1965)

The image of AA, the light source annulus, and the rays produced by this image are direct rays that are retarded by ¼ of a wave length by the phase annulus. The object produces diffracted rays that usually produce a retardation of ¼ of a wavelength. When these two are added together the effect is to produce a retardation of ½ a wavelength which is the optimum for good contrast (Cassartelli, 1965). Without the addition of phase optics and on microscopes that only have an incident light source, it is not possible to obtain true phase contrast.

5. 2. 3. Modulation contrast microscopy

The Hoffman modulation contrast microscope is an adaptation of a brightfield microscope. This adaptation allows non-invasive imaging, with no need for prior staining or preparation of the biofilm. Biofilms can be imaged with high contrast resolution resulting in an image with a three dimensional appearance. The 3-D effect is obtained by conversion of opposite phase gradients to opposite intensities in the image. One side of the object appears bright, whilst the other appears dark against a grey background (Hoffman, 1988). The image should have good contrast and be without any artefacts such as the halos which are often observed in phase contrast microscopy. A Nikon Optiphot-2 was modified by the addition of an objective incorporating a special amplitude filter (a modulator) (Fig. 5. 2). A slit is located at the front focal plane of the

condenser, which is partially covered by a contrast control polariser. The objectives, [Hoffman modulation contrast 40 times long working distance with a 0.5 numerical aperture (NA) and a 100 times oil immersion (1.25 NA)], were manufactured by Modulation Optics Inc. USA.

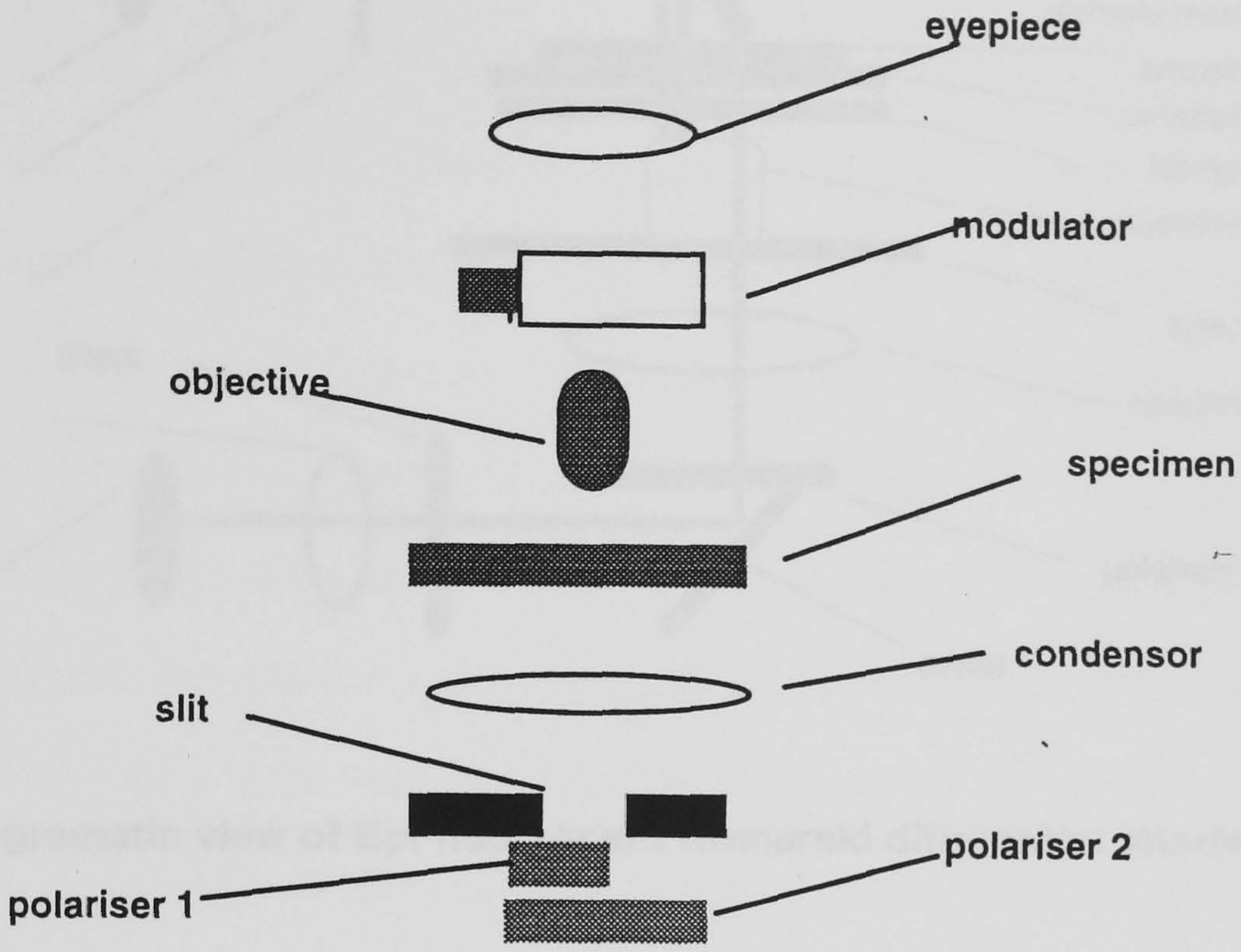

Fig. 5. 2. Schematic representation of the Hoffman Modulation Microscope. (After Hoffman, 1988).

5. 2. 4. Differential Interference contrast (DIC) and Fluorescence Microscopy.

Episcopic light Normaski differential interference contrast microscopy used in combination with a non-coverslip corrected long working distance lens, allows visualisation of the intact surface contours of a biofilm. This technique is most successful when the object is viewed directly without a coverslip. The air/object interface reflects the episcopic light that interferes with light returning to the specimen (Fig 5. 3). This method allows rapid clear visualisation of the surface topography of an object without the need for staining or other disruptive procedures.

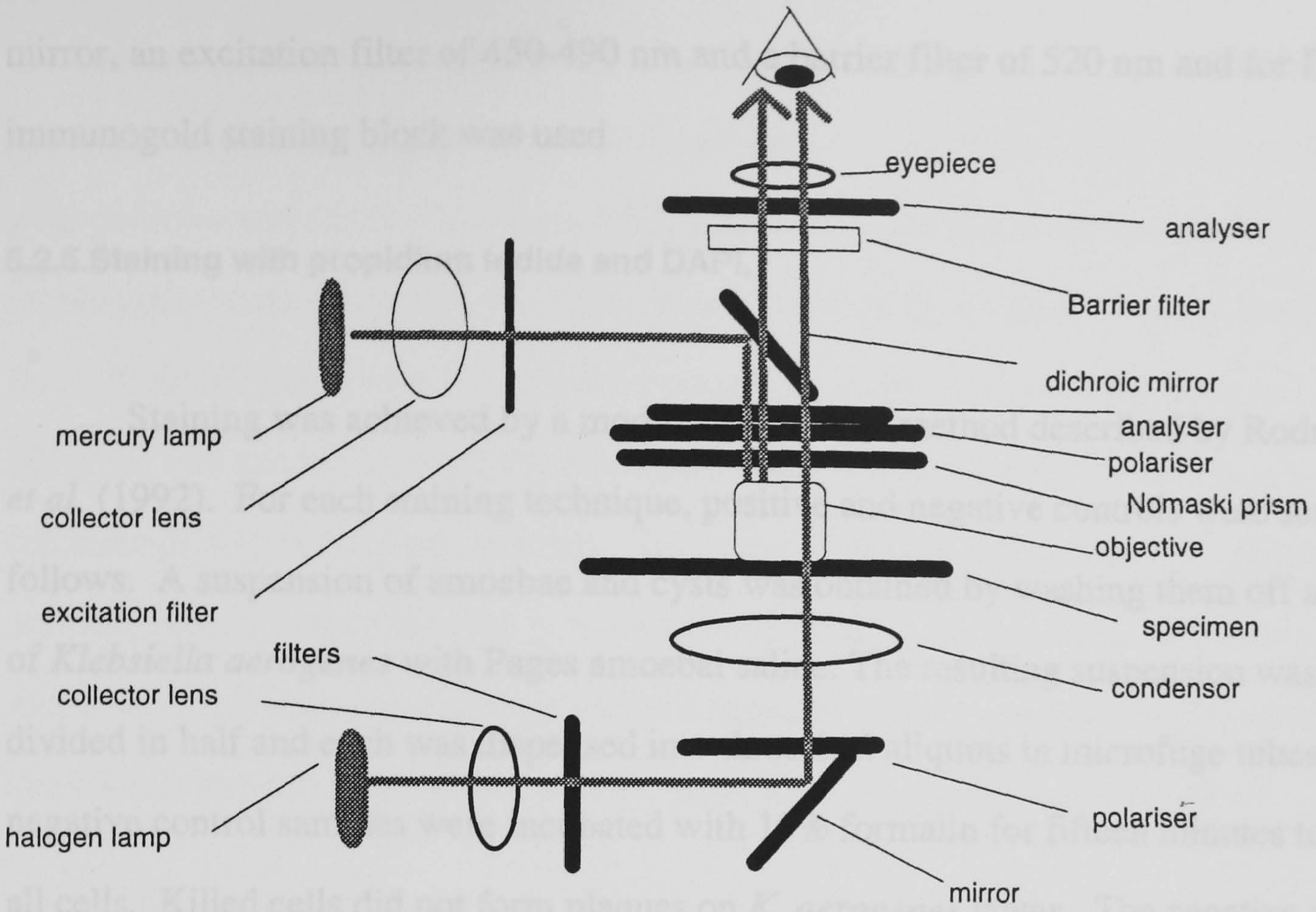

Fig. 5. 3. A diagramatic view of Epi-fluorescent Nomarski differential interference contrast microscope.

The differential interference microscope (Nikon Labophot-2) has been described previously (Keevil and Walker, 1992; Rogers and Keevil, 1992) and consists of a conventional light microscope, a Nikon Labophot (Nikon, UK), fitted with transmitted light with UV fluorescence. The light source was a 100 W halogen lamp. The episcopic UV light source was a mercury lamp with neutral density filters to control the light as necessary. This microscope has a large housing to accommodate interchangable excitation filter blocks for easy change of the filters. Other adaptations include the siting of the polariser above the specimen, which allows opaque specimens to be viewed and the presence of mirror plates in the mercury lamp housing to increase the light intensity. The objective lenses were long working distance and non-contact which are usually used in metallurgical studies. They included an M Plan Apo 40 x (0.5 NA), 100 x (0.8 NA) and 150 x (0.95 NA). A zoom was fitted to allow the magnification to be increased. As the objectives used were non-coverslip corrected, a coverslip was not necessary. For epi-fluorescence, a B-2A filter block was used, which has a 510 dichroic

mirror, an excitation filter of 450-490 nm and a barrier filter of 520 nm and for DIC, an immunogold staining block was used

5.2.5.Staining with propidium iodide and DAPI.

Staining was achieved by a modification of the method described by Rodriquez *et al.* (1992). For each staining technique, positive and negative controls were set up as follows. A suspension of amoebae and cysts was obtained by washing them off a lawn of *Klebsiella aerogenes* with Pages amoebal saline. The resulting suspension was divided in half and each was dispensed into three 1ml aliquots in microfuge tubes. The negative control samples were incubated with 10% formalin for fifteen minutes to kill all cells. Killed cells did not form plaques on *K. aerogenes* lawns. The negative and positive control samples were then incubated at 28 °C with 2 ml of the appropriate stain for 30 minutes . Glass tiles which had been prepared as described above (5. 2. 1.) were then flooded with the appropriate working solution of the stain (Appendix 5) and incubated in a moist chamber for 30 minutes as above. The microscope used was the Nikon Labophot fitted with episcopic UV illumination as described above. The filter block contained a 545 nm excitation filter.

5.2.6. Immunogold Staining

The method used was based on that described by Rogers and Keevil (1992). Glass tiles supporting the development of the biofilms were removed aseptically from the system and rinsed as described above. The tiles were then flooded with 1% formalin and allowed to air dry with gentle heat. The biofilms were then fixed by covering with 10 % formalin for ten minutes, rinsed with distilled water and flooded with acetone for 15 minutes to dehydrate. The tiles were then covered with 100 µl of lipopolysaccharide specific monoclonal antibody for *Legionella pneumophila* (Sethi/LP 45; Cogent Ltd., Edinburgh) which had been diluted 1/40 in phosphate buffered saline (PBS) prior to use. The tiles were then incubated at 37°C for 30 minutes in a moist chamber. Removal

of unbound monoclonal antibody was achieved by washing three times in PBS with gentle agitation. Goat anti-mouse immunoglobulin G conjugated (100 µl), with 5nm gold particles and diluted 1/40 with PBS, was then added to the tile surfaces and incubated at room temperature for four hours followed by washing in approximately three 20 ml aliquots of PBS to remove excess conjugate. A silver enhancing kit (Biocell) was used to increase the resolution to the desired amount by checking microscopically at frequent intervals. This stage took only a few minutes. The reaction was stopped by rinsing the tiles in tap water. A positive control consisted of the avirulent *Legionella pneumophila* whilst a negative control consisted of *Pseudomonas vesicularis* isolated from the chemostat. Both were fixed and stained as above.

5.2.7. FITC staining.

A direct fluorescent antibody kit (Pro-Lab Diagnostics Limited), was used to stain duplicate glass tiles which had been removed from the chemostat and rinsed as described earlier. Each tile was mounted onto a glass slide with clear nail varnish. A positive control suspension of the avirulent *L. pneumophila* in 1% formalin (to approx. McFarland 1.0 standard) was spotted on to two multi-well slides. The slides were then air dried with gentle heat and fixed by flooding with 10% formalin for ten minutes. Following fixation the tiles and slides were rinsed with distilled water and air dried. *L. pneumophila* DFA reagent FITC-antibody specific for serogroup 1 was added to one of the tiles and one of the multiwell slides and the negative control conjugate supplied with the kit was added to the second tile and multiwell slide. The slides were then incubated in a petri-dish lined with damp tissue for 45 minutes at 37 °C, which had been previously determined as being the optimum incubation time (Smith, 1994, personal communication). The test samples were then thoroughly rinsed with PBS pH 7.5-7.7 followed by a rinse with distilled water and allowed to air dry. Three to four drops of the mounting medium supplied were then applied to the surface of each tile and a coverslip placed on top. Examination of the slides was by UV fluorescence using a

Zeiss × 40 objective followed by a 100 × oil immersion objective for confirmation. It is preferable to view immediately but if necessary the specimens may be stored overnight in a dark container at 4 °C. Suspensions of pure cultures of the chemostat bacterial flora were spotted onto multi-well slides and stained as the positive control.

5.2.8.Atomic Force Microscopy.

For imaging biofilms *in vivo* at a higher magnification atomic force microscopy was used. The Atomic Force MIcroscope (AFM) was developed in 1986 (Fig 5.4) and is a form of scanning probe microscopy which enables the visualisation of a surface at atomic resolution (Yamada *et al.*, 1992; Hansma *et al.*, 1988). Initially materials imaged by this technique were of a non-biological nature and included metals, superconductors, semiconductors and catalysts (Haggerty and Lenhoff, 1993). More recently the advantages of using this technique to visualise biological molecules, including DNA, various proteins and intact cells, have been explored by biologists (Haggerty and Lenhoff, 1993, Kasas *et al.*, 1993; Azumi *et al.*, 1991).

To build up the image of the surface using contact mode, the sample is scanned in a raster pattern by a silicon-nitride tip. The tip or probe is mounted on a delicately sprung cantilever. Attractive forces cause the cantilever to deflect as the tip approaches the surface until at the surface the cantilever is deflected in the opposite direction by repulsive forces which is due to overlapping of electron clouds. These repulsive forces are very small and are usually less than 1 nanometre (Edstrom *et al.*, 1990). The cantilever is deflected by these forces until a maximum load on the cantilever, pre-determined by the microscopist, is reached. As the tip leaves the surface, the cantilever is subjected to adhesive forces until a point of maximun adhesive force is reached and the cantilever returns to a resting position (Burnham *et al.*, 1991). The image is obtained by measurement of the position of the laser beam which correspond to the cantilever deflection as the tip scans the contours of the sample surface (Goddard *et al.*,

1993). The laser beam falls onto a split photodetector, which produces a feedback signal to the piezoelectric scanner. The piezo scanner undergoes changes in physical dimensions in response to voltage changes. This results in the fine adjustment of the sample height as the surface is scanned so that the cantilever deflection remains the same. The signal is then converted by a microcomputer image processing programme to produce a 3-D computer image. Each pixel in the image corresponds to a single change in voltage applied to the piezoscanner. The resulting image corresponds to the variation in the sample height detected during the scan of the surface of the sample.

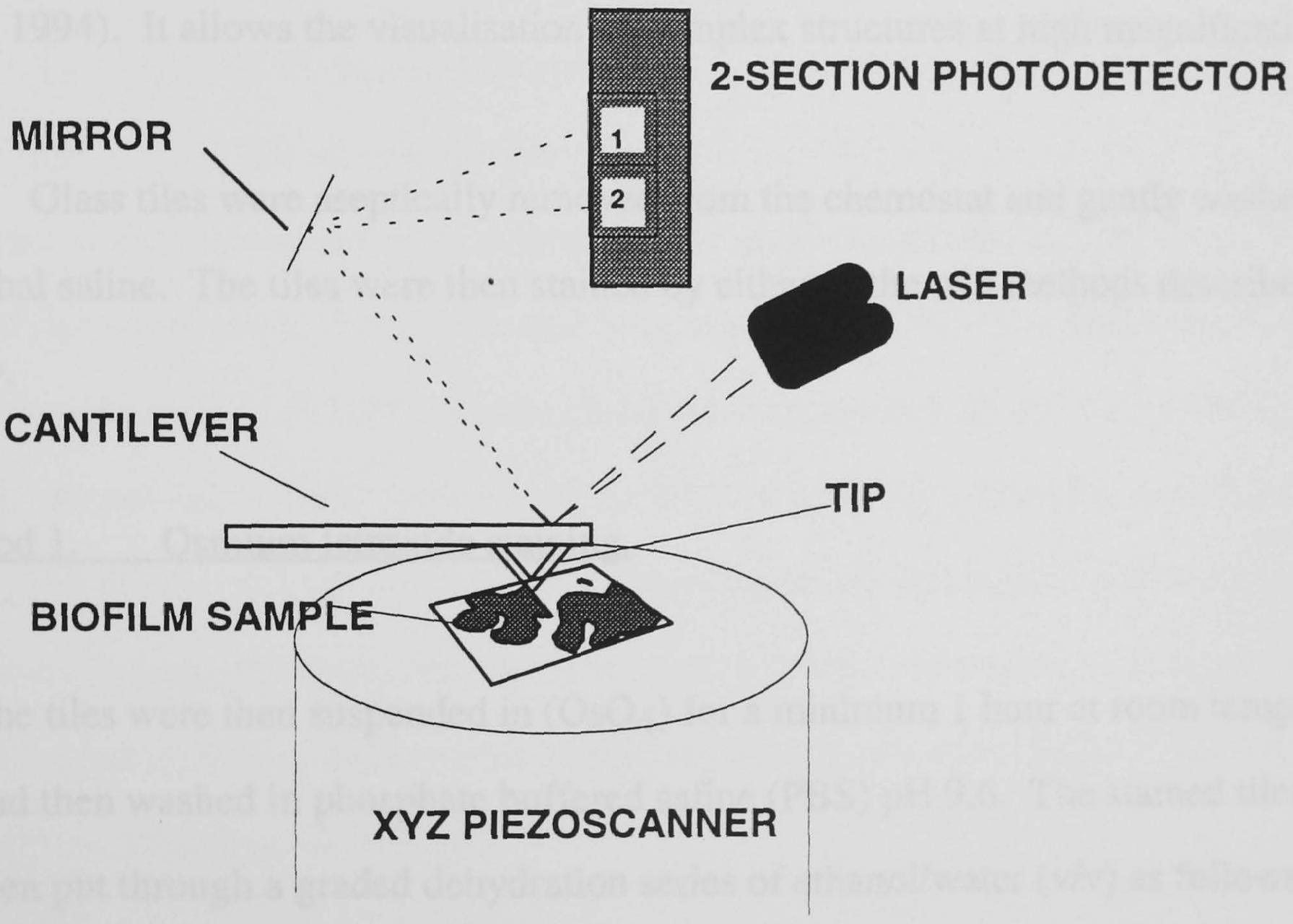

Fig. 5. 4. Diagrammatic representation of the atomic force microscope. The movement of the cantilever is detected by the laser. The photodetector produces a feedback signal to the piezoelectric scanner which continuously adjusts the sample height to maintain a constant deflection of the cantilever.

In this study intact biofilms on the glass tile supporting their development were placed on the AFM sample stage and viewed directly using the Nanoscope III (Digital Instruments Inc. USA.). Biofilms were scanned as soon as possible after removal from the aqueous phase of the system to keep dehydration to a minimum. Images of biofilms were also obtained by perfusing a flow cell containing the glass tile with filter sterilised tap water.

5.2.9. Scanning electron microscopy.

Scanning electron microscopy and its application to examining the surfaces of bacterial biofilms has been previously described (Wheatley, 1981; Costerton *et al.,* 1986; Sutton *et al.*, 1994). It allows the visualisation of complex structures at high magnification.

Glass tiles were aseptically removed from the chemostat and gently washed in amoebal saline. The tiles were then stained by either of the two methods described below.

Method 1. Osmium tetroxide staining.

The tiles were then suspended in (OsO_4) for a minimum 1 hour at room temperature and then washed in phosphate buffered saline (PBS) pH 9.6. The stained tiles were then put through a graded dehydration series of ethanol/water (v/v) as follows:-

i).	30 %	15 minutes
ii).	50 %	15 minutes
iii).	70 %	30 minutes
iv).	90 %	60 minutes.

The stained tiles were then kept dry in a dessicator before gold sputter coating and viewing as secondary electron images (8kV) in a Cambridge Stereoscan, S2a SEM by Barry Dowsett at CAMR, Porton Down

Method 2. Ruthenium red staining

The method used was based on that of Luft (1965). After removal and rinsing as above, the tiles were washed in cacodylate buffer pH 6.8. The tiles were then immersed, for 1-2 hours at 4 °C, in a ruthenium red/ glutaraldehyde solution comprising of equal volumes of:-

i). ruthenium red (1500 ppm in distilled water)

ii). 0.2 % v/v cacodylate buffer

ii). 3 % v/v glutaraldehyde

The tiles were then washed in cacodylate buffer and resuspended in 2 ml 0.5% v/v glutaraldehyde in cacodylate buffer and left overnight at 4 °C. The following morning the tiles were washed in buffer and suspended overnight in 5 ml of 1% v/v OsO_4 plus 1 ml of the ruthenium red / glutaraldehyde solution as above. The tiles were then put through an alcohol dehydration series as described above and kept in a dessicator. Following staining the specimen was fixed onto a metal stud and then made conductive by gold splutter coating until examined microscopically by a Jeol SEM at Zeneca Specialities, Blakeley, Manchester.

5.3. Results and Discussion.

Fig. 5.5. shows a four week biofilm imaged by phase contrast microscopy clearly demonstrates the heterogeneity which exists within these water distribution biofilms including amoebal cysts and trophozoites, denoted by the arrows. Fig.5.6 shows a thicker region of the biofilm. Phase contrast is not very sucessful for viewing dense biofilms or biofilms developed on opaque surfaces.

Hoffman modulation transmission microscopy can be used successfully to examine more abundantly populated areas of the biofilm. The biofilm viewed using this microscope revealed a clear image of a dense area of the biofilm and shows the heterogeneous matrix consisting of a very diverse and active consortium of microorganisms (Fig 5.7). Amoebal trophozoites, depicted by arrows, and other protozoa could be seen to be motile in the biofilm. Fig. 5.8 shows a sparsely populated area of the same biofilm. A large number of amoebal cysts are visible and grazing trophozoites (denoted by arrows) were motile at the time of examination. As a coverslip is required for viewing the biofilms, some compression of the biofilm is inevitable, however the advantages are that the biofilm remained intact and hydrated for a considerable time.

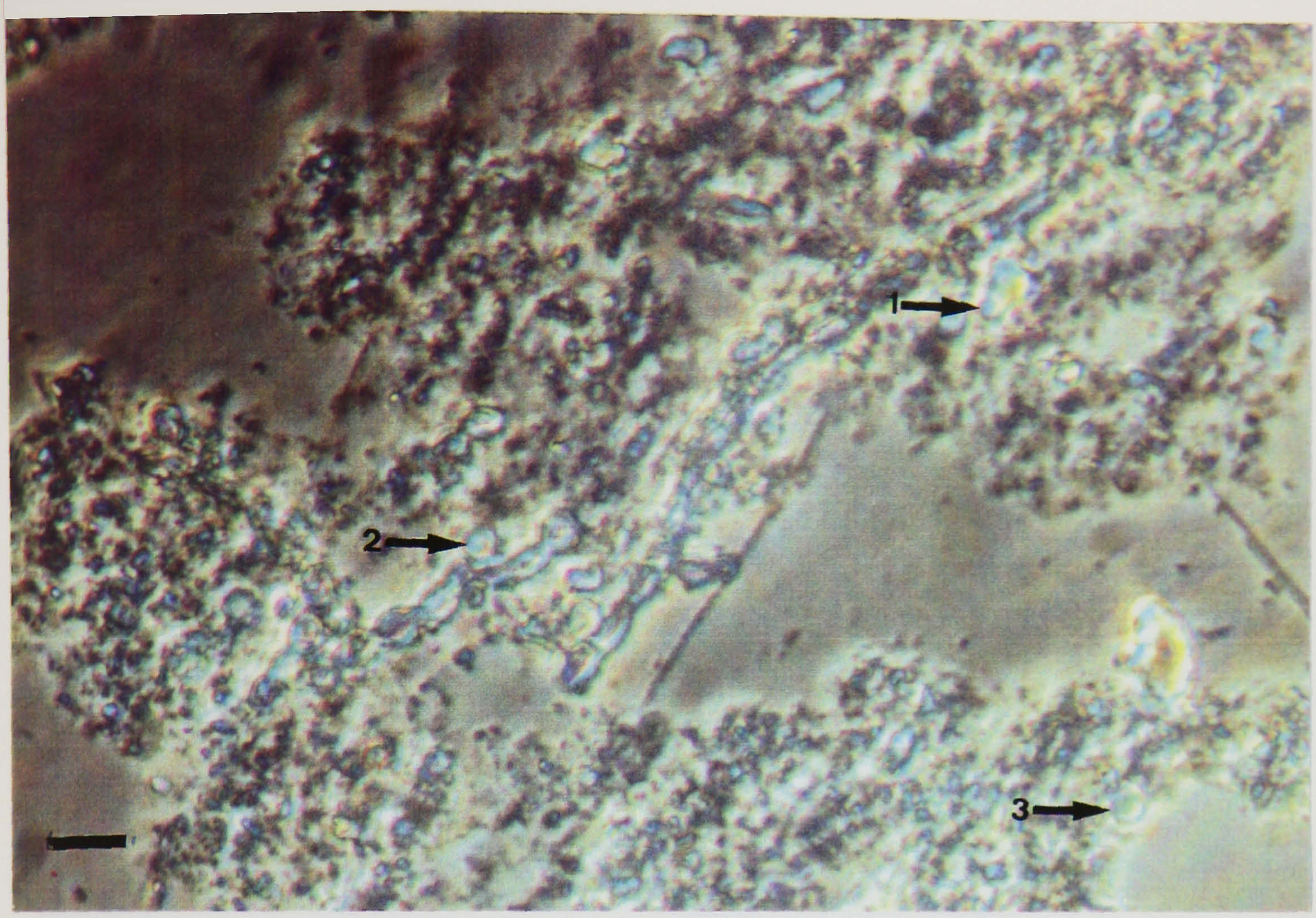

Fig. 5. 5. A 4 week biofilm as viewed by phase contrast under oil immersion. The arrows denote a grazing amoeba (1), and amoebal cysts (2) and (3). (bar = 10μm)

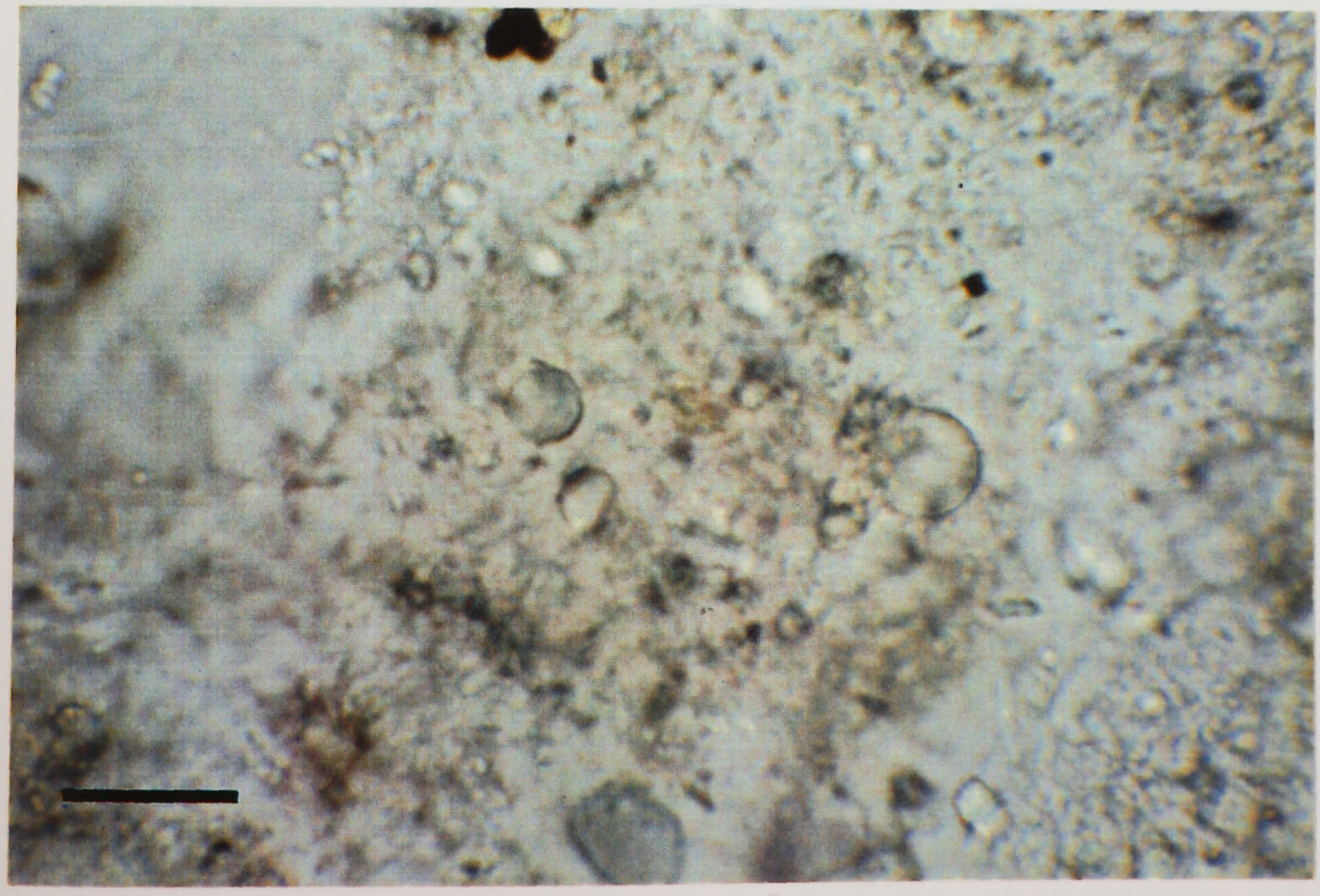

Fig. 5. 6. **A denser region of the same 4 week biofilm as above.** (bar = 10μm)

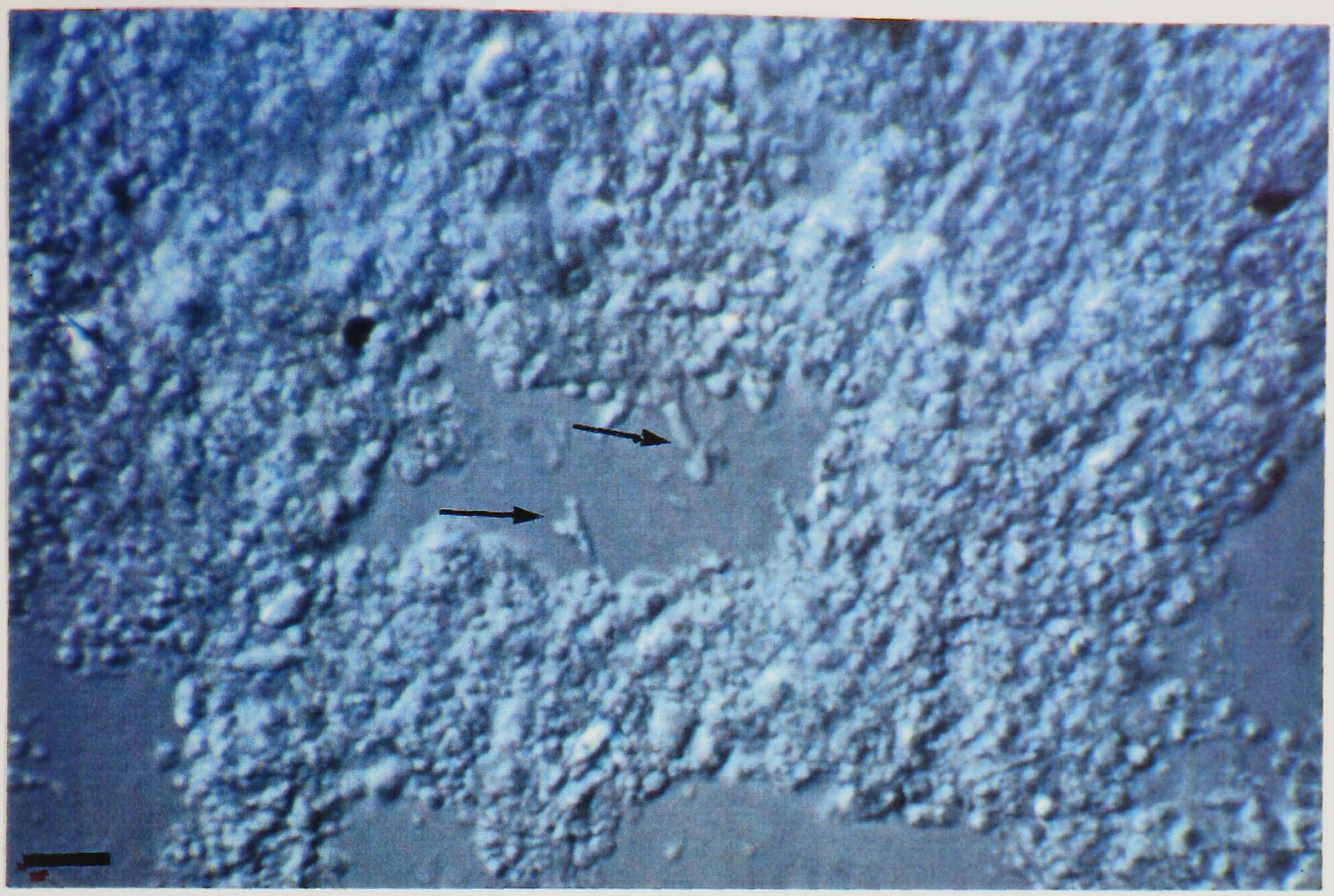

Fig. 5.7. An 8 week old biofilm as viewed by Hoffman modulation contrast microscopy. The arrows denote grazing amoebae (bar = 10μm)

Fig. 5.8. A less dense area of the same biofilm as above. Many amoebal cysts and grazing trophozoites (arrows) are visible (bar = 10μm).

Episcopic DIC microscopy can also be used to visualise dense areas of biofilms and the use of a non-coverslip corrected lens eliminates the problem of compression of the biofilm and allows for measurement of the depth of the biofilm when used in conjunction with the microscope micrometer. The more dense areas of the biofilm measured 80-100 μm in thickness using this method. An advantage of DIC microscopy over conventional phase contrast is that the 3-dimensional effect enables the clear visualisation of the surface of dense areas of the biofilm. Fig. 5. 9 shows the surface topography of an intact eight week biofilm and gives a clear image which includes several ameobal cysts visible on the biofilm surface. Fig. 5. 10 shows a dense area of biofilm with volcano-like structures, similar to those structures previously described in SEM photomicrographs of much denser biofilms from anaerobic digestors (Robinson *et al.*, 1984). These structures are thought to be associated with transport of nutrients and waste products within the biofilm matrix.

Episcopic DIC can also be used in conjunction with epi-fluorescence microscopy to reveal microorganisms on opaque substrata and to visualise biofilms on irregular and on curved surfaces e.g. plumbing tube materials which require optics with a large depth of field (Rogers and Keevil, 1992). This form of visualising biofilms can also be used in conjunction with fluorescent and vital stains to distinguish between viable and non-viable domains within the biofilm and to provide information on the bacterial composition of the biofilm (Keevil and Walker, 1992).

DAPI and PI are both fluorogenic vital dyes which have been used to differentiate viable and non-viable oocysts of *Cryptosporidium parvum*. PI specifically binds to double stranded DNA (Smith and Smith, 1989) and is not able to enter the intact cell wall of viable oocysts and therefore only stains disrupted / damaged cells which therefore, are no longer viable (Horan and Kappler, 1977). DAPI is DNA-AT-selective and is able to pass through the cell wall of viable oocysts and which results in an increase in fluorescence when bound to the DNA in viable stained cells (Campbell *et al.*, 1992).

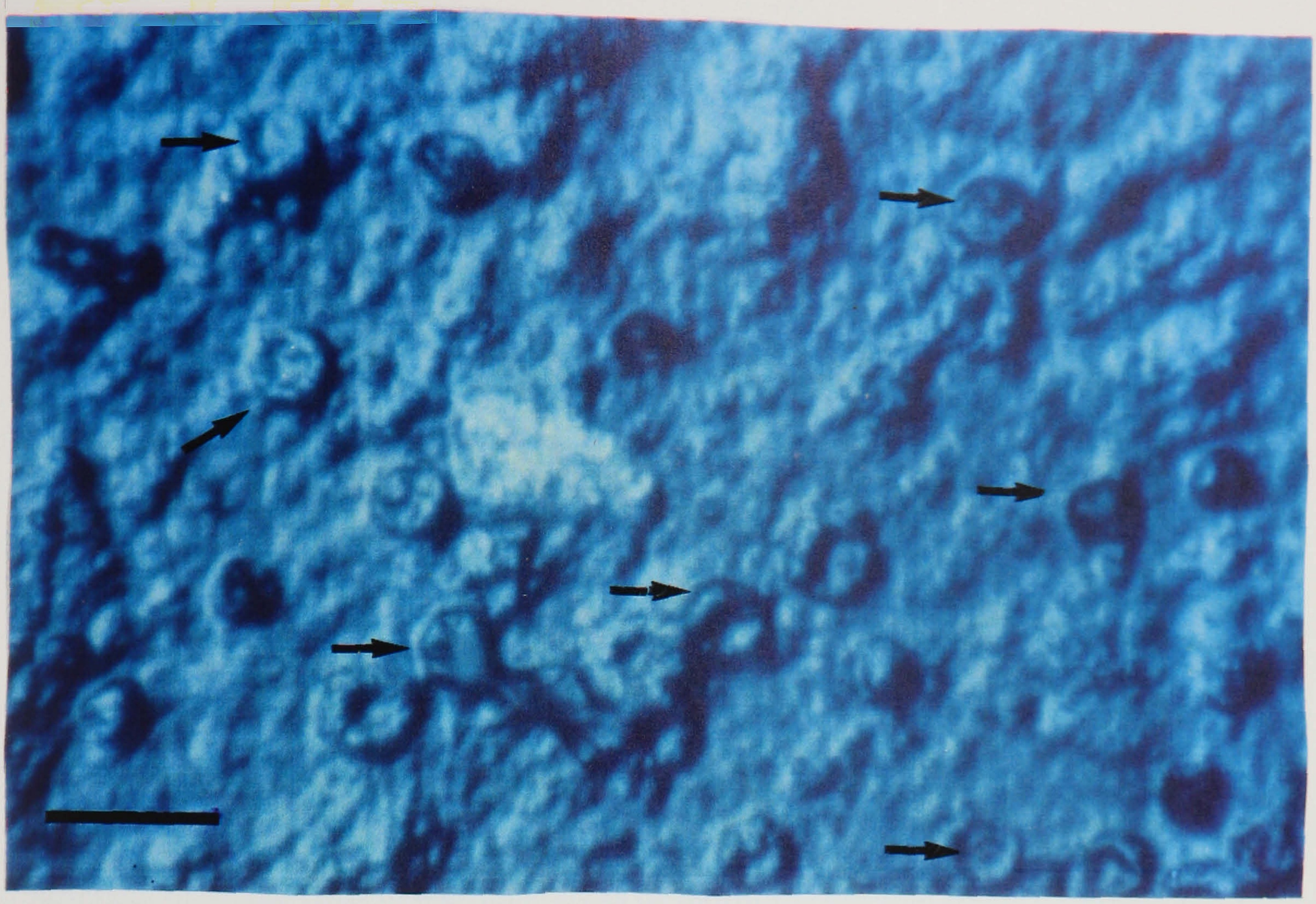

Fig. 5.9. An eight week biofilm as viewed with DIC microscopy. Numerous amoebal cysts (arrows) can be seen on the surface of the biofilm (bar = 10µm).

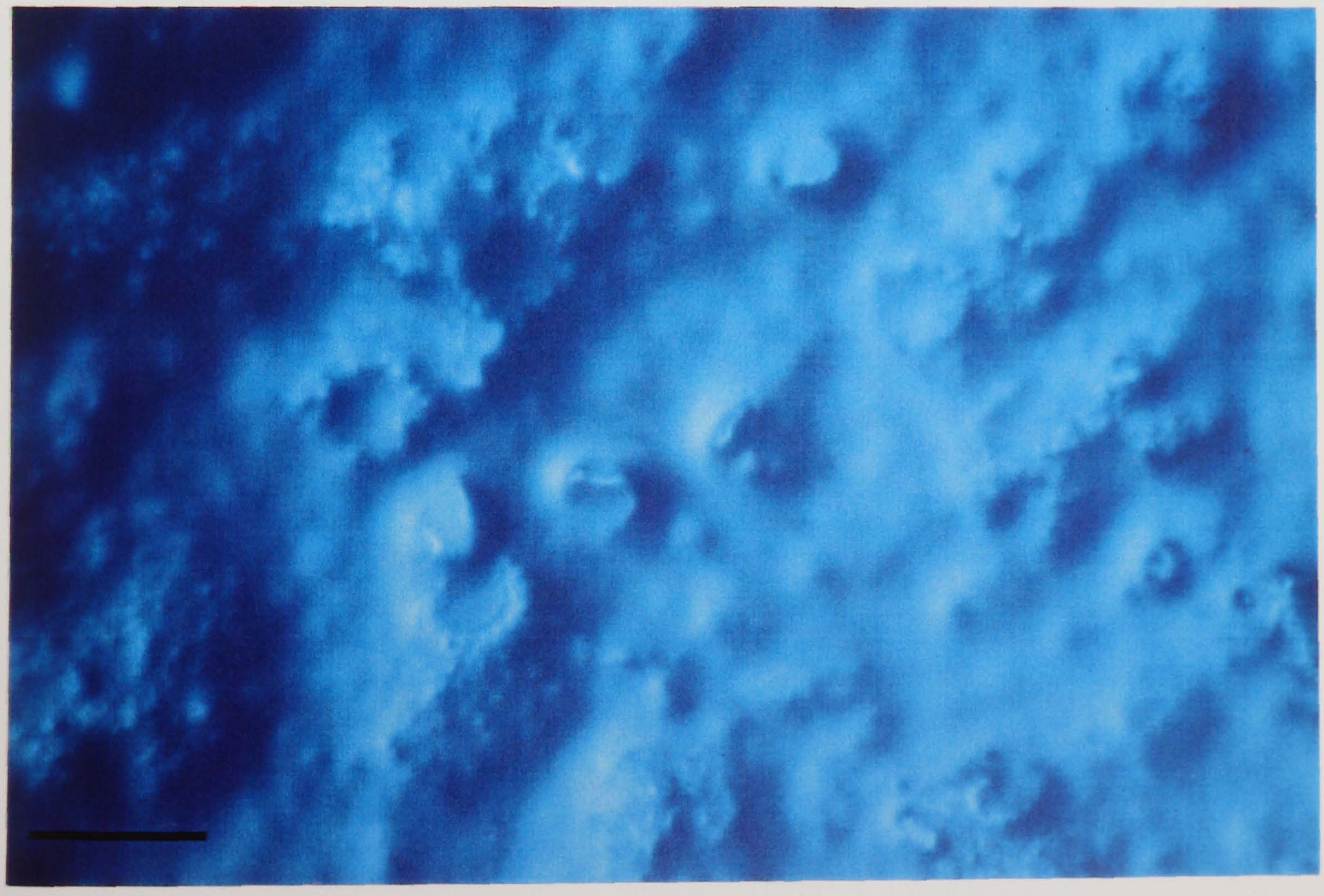

Fig. 5.10. A dense area of an eight week biofilm viewed by DIC microscopy. The volcano-like structures are thought to be associated with the transport of nutrients. (bar = 10µm).

Initial studies showed that both DAPI and PI stained both viable and formalin killed amoebae and amoebal cysts isolated from the system, and PI therefore was not useful for distinguishing between intact viable cells and those with disrupted cell membranes. The differentiation observed with these stains for viable and non-viable *Cryptosporidium parvum* oocysts does not occur with either the amoebal trophozoites or cysts. This difference in staining ability is probably associated with *Cryptosporidium parvum* oocysts having relatively impermeable thick cell walls [4-7 μm] (Smith *et al.*, 1989). However, it was noted that these cells were more easily visualised within the biofilm following PI staining. Staining the biofilm with PI highlights the high density of amoebal cysts present in this biofilm (Fig 5.11) and gives a good indication of depth within the biofilm, the higher intensity of the surface staining may relate to increased numbers of viable cells in the outer areas of biofilm.

Fig 5.12. shows a biofilm of the same age as in Fig. 5.11, stained with DAPI. A grazing amoeba can be seen (large arrow) and some amoebal cysts (small arrows) though far fewer are visible than in the previous figure. It is possible that this differentiation in staining is due to differences in viability of the cysts, those stained with PI being non-viable and that those few cysts stained with DAPI being those which are viable. However initial studies suggest that this is not likely as both viable and non-viable controls stained by both methods. It is well known that bacteria isolated from within biofilms are more resistant to biocides and that this resistance may be associated with altered cell physiology among other factors (Hamilton *et al.*, 1994). It is possible therefore, that there may be differences between the amoebal trophozoites and cysts which were isolated from the biofilm and those used as controls. Further work is necessary to determine if this is the case.

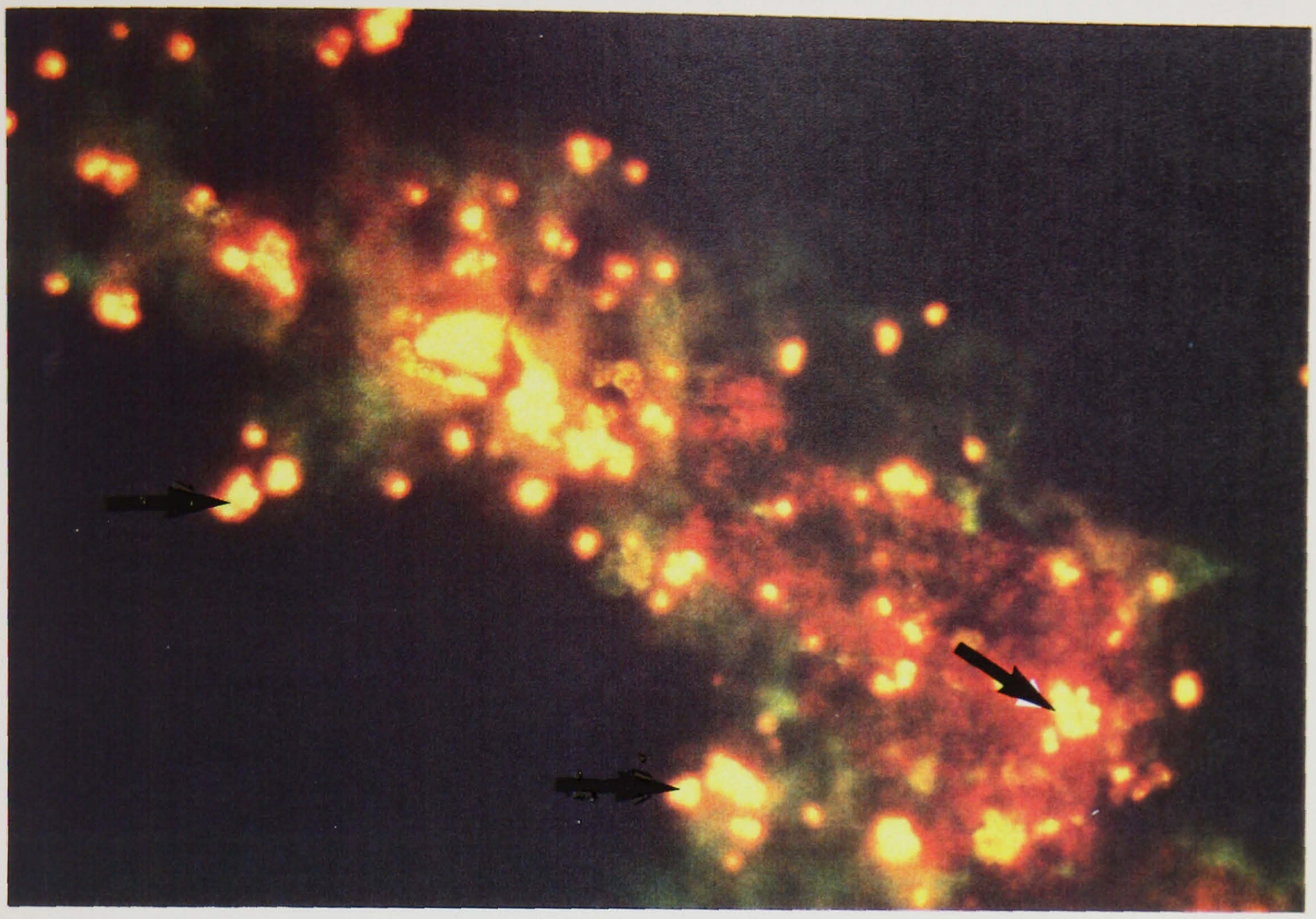

Fig. 5.11. An eight week biofilm stained with the vital stain propidium iodide. Numerous amoebal cysts (arrows) can be seen in the biofilm, (microscope magnification x 400).

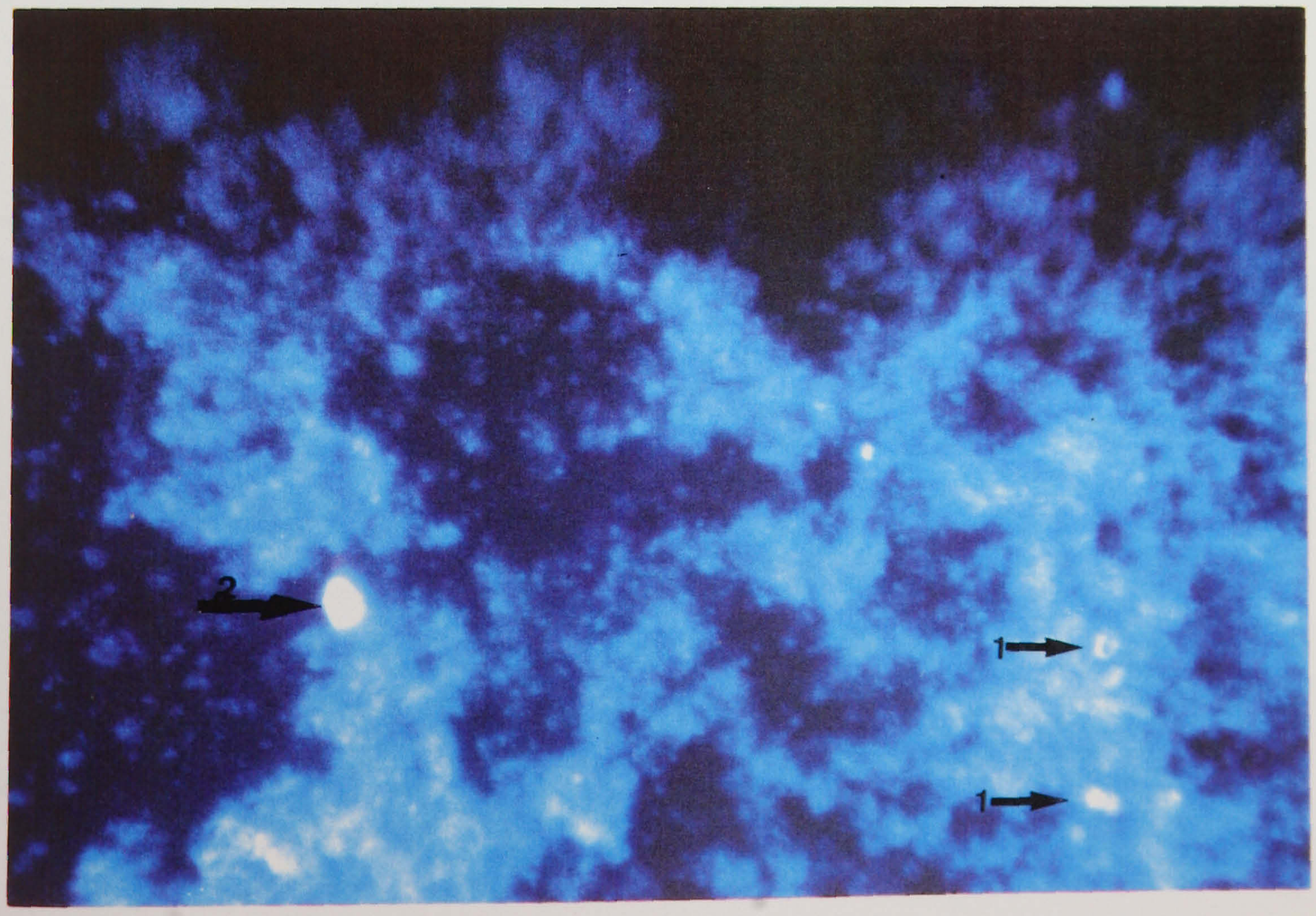

Fig. 5.12. A dense area of an eight week biofilm stained with DAPI. Several ameobal cysts (1) and a grazing amoebal trophozoite (2) can be seen, (microscope magnification × 400).

FITC staining of the avirulent *L. pneumophila* was positive showing that the surface epitope is conserved in the avirulent strain. There was no detectable difference in fluorescence intensity between the virulent and avirulent control strains. FITC staining of biofilms from the chemostat (Fig. 5.13) showed that *L. pneumophila* is able to exist within microcolonies dispersed throughout the biofilm which confirmed the results obtained with the immunogold staining. There was no staining observed with the non-legionellae bacterial population from the chemostat. Non-specific staining within the biofilm is easily discernible from stained *Legionella* (Fig. 5.14). This FITC kit is therefore, useful for monitoring the population within a model system. Although the specificity of DFA is reported to be between 99-99.3% (Edelstein, 1993), cross reactions have been noted by some authors with some DFA reagents (Alary and Joly, 1992). Further work is necessary to determine the specificity of this antibody when used to establish the presence of *L. pneumophila* within the natural environment.

Staining with immunogold confirmed the results obtained with the FITC kit. No staining occurred with the negative control and any non-specific staining was easily distinguished from the bacterial population. A shorter incubation time was used in the method described above, 4 hrs compared with 8 hrs as used by Rogers and Keevil, (1992). The results however, were as described by these authors in that positive staining of *L. pneumophila* was visible, dispersed in microcolonies throughout 24hr and 14 day biofilms (Fig. 5.15 and 5. 16). Insufficient numbers of biofilms were processed by this method to determine if this shorter incubation time would be suitable for all ages of biofilm. Immunogold staining, even with the reduced incubation time, is more time consuming than FITC staining. A big advantage is that both the biofilm and the *Legionella* can be seen simultaneously.

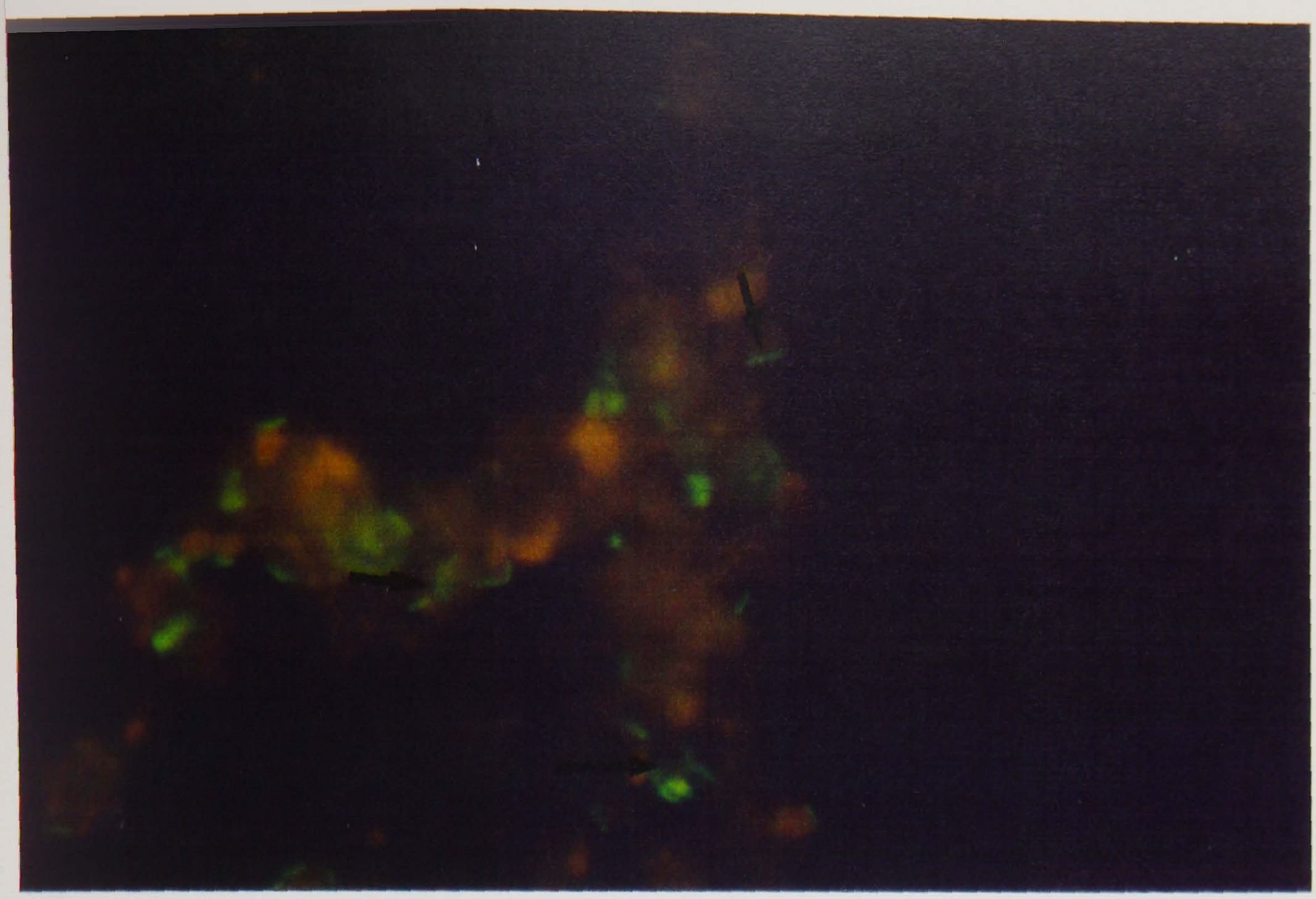

Fig. 5.13.FITC staining of the biofilm. *Legionella pneumophila* can be seen dispersed in microcolonies within a seven day biofilm (microscope magnification × 1000).

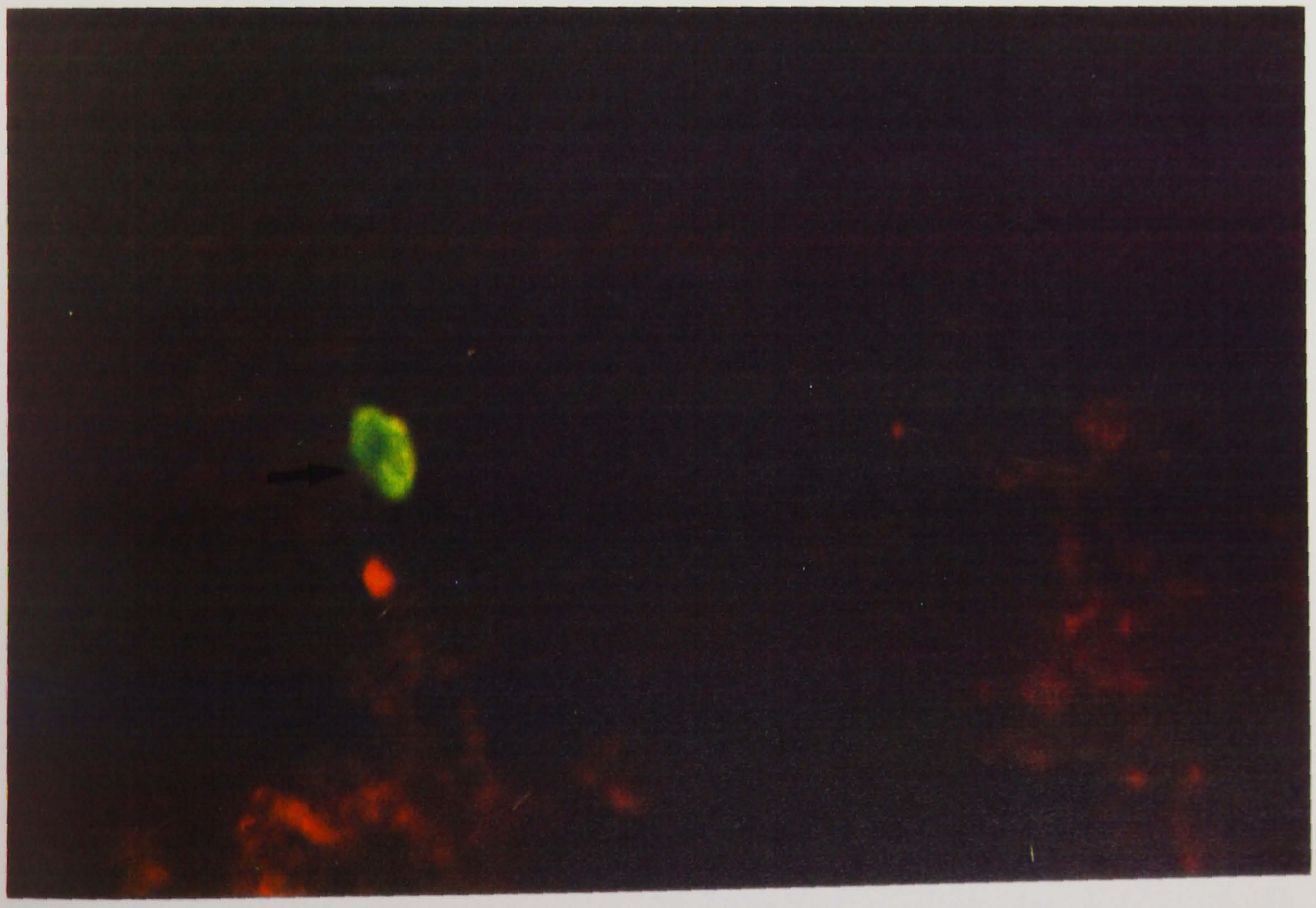

Fig. 5.14. Non-specific fluorescent staining. Non-specific fluorescent staining (arrow) was easily distinguishable from the *L. pneumophila*.(Microscope magnification × 1000).

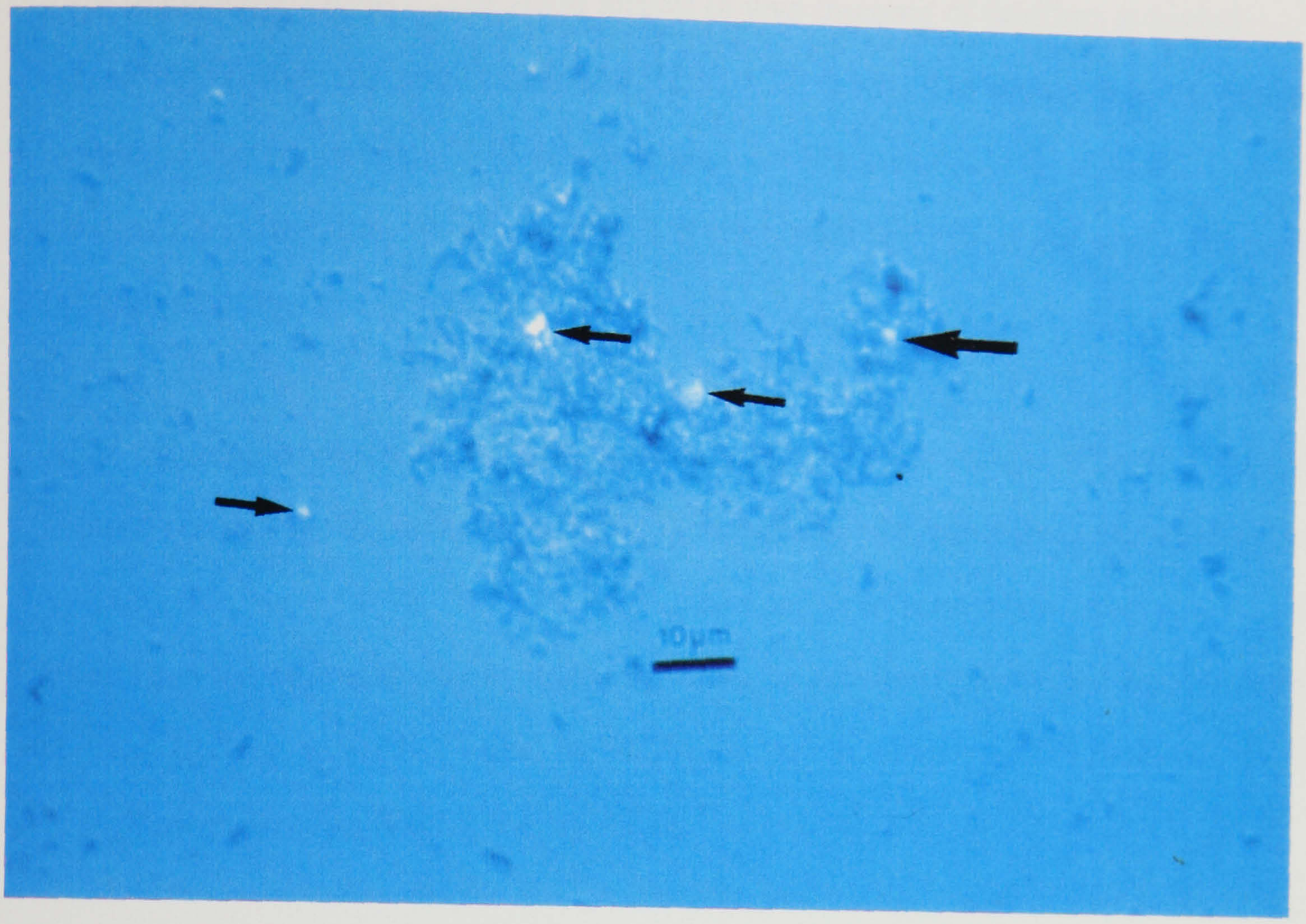

Fig. 5.15. Immunogold staining of *L. pneumophila.* A 24 hour biofilm with positive immunogold staining (arrows).

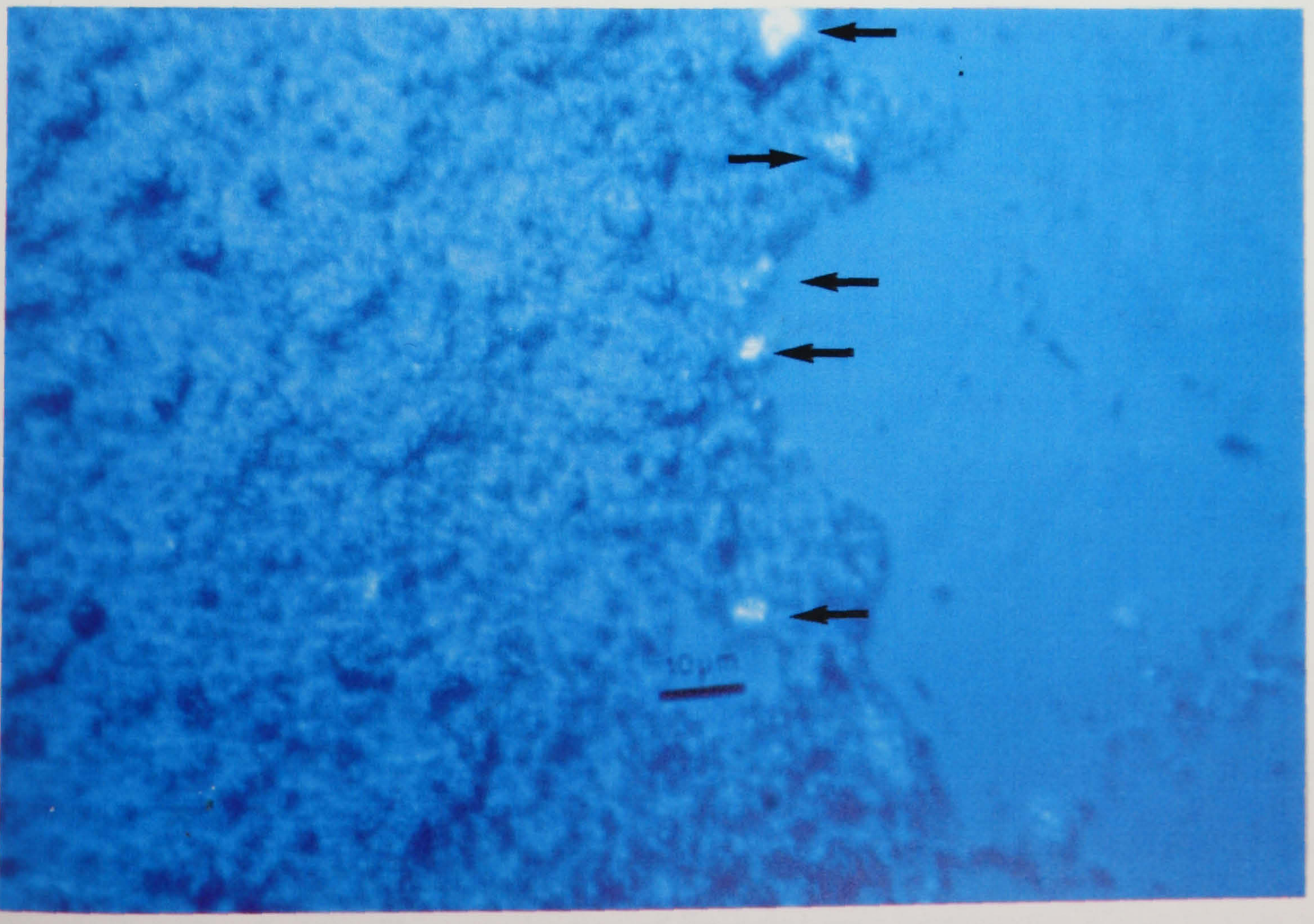

Fig. 5.16. A 14 day biofilm positively stained for *Legionella pneumophila* (arrows).

A higher magnification technique which does not require pre-treatment is the atomic force microscope (AFM). The AFM allows *in vivo* imaging of biofilms at higher magnification and with good resolution (Figs. 5.17, 5.18). The ability to zoom in to an individual bacterium (Fig. 5.19) and to manipulate the image to gain accurate information on the dimensions of individual bacteria (Fig 5. 20) is not easily achievable by other techniques.

AFM scanning of pure cultures of *L. pneumophila* produced images (Fig. 5. 21) which showed similarities in surface structure to those obtained previously of the Pontiac strain by SEM (Rodgers, 1979). The avirulent *L. pneumophila* showed the same non-parallel sides, rounded ends and visible outer membrane as previously described for the virulent form by Rodgers (1979). Unlike the SEM micrograph, however, EPS is clearly visible at high magnification (Fig. 5. 22) by AFM.

The technique of examining biological samples using AFM in a fully hydrated state, is still in its early stages. Difficulties in imaging may be a consequence of the presence of capillary forces between the surface of the sample and the tip (Weisenhorn *et al.*, 1989). Although capillary forces are not significant when imaging biofilms under liquid, imaging proved to be more difficult. An image was obtained but the resolution was not as good as the previous images (Fig. 5. 23). One of the factors in the disappointing results obtained, maybe as a result of the natural compliance present in living biological specimens. This compliance tends to be greater when a specimen is immersed in a fluid (Blackford *et al.*, 1991). Further difficulties may be have been due to movement of the living sample itself.

Specimen damage which may occur with the atomic force microscope is usually due to the effect of tip forces on the sample. Problems occur with larger biological structures (Southam *et al.*, 1993) due to the tip geometry, especially when imaging steeply sided samples (Fig. 5. 24). The introduction of finer tips may increase the ability to scan into

finer features upon the sample surface but large structures would still be prone to tip-induced damage. Sharper tips may increase the problems of tip-surface interactions. However, the combination of sharper tips and the introduction of modulation modes of scanning, e.g. tapping mode, may lead to clearer images.

Tapping mode is a form of high amplitude resonance, where the tip oscillates at approximately 300kHz with an amplitude of between 10 and 100 nm. Tip-surface interactions are lower in this mode because of the short time that the tip is in contact with the sample surface.

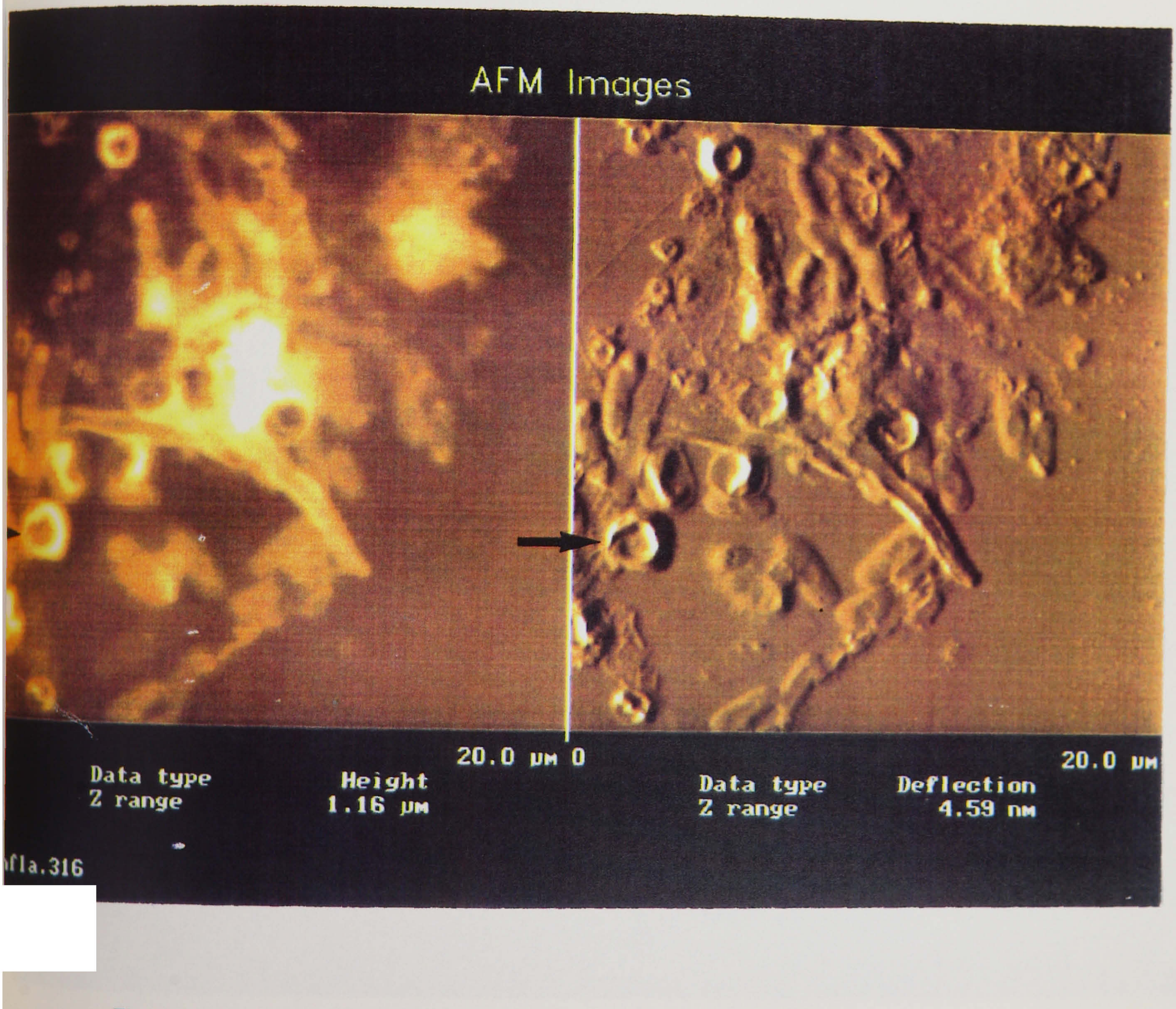

Fig. 5.17. A seven day biofilm as imaged by atomic force microscopy. Ameobal cysts (arrows) and a microcolony of bacteria can be seen . The image on the LHS is processed to show the relative height of the sample. The image on the RHS corresponds to the deflection of the tip.

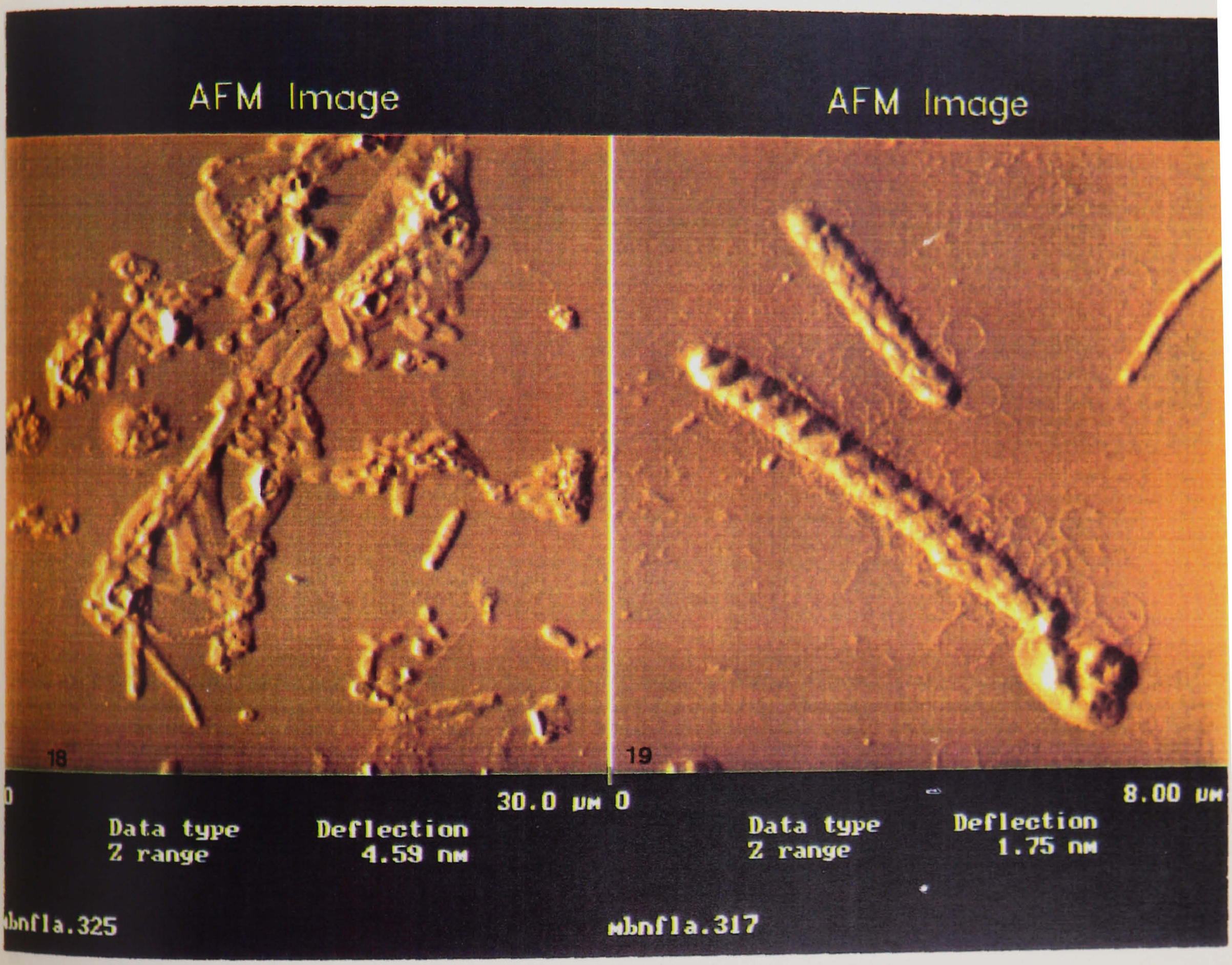

Fig. 5.18. An AFM image of a seven day biofilm. This figure shows the diversity that exists within the biofIm. **Fig. 5.19 The image of two biofilm bacteria.** This image was achieved by zooming in to 15 times the magnification of the biofilm shown in previous image (Fig. 5.18).

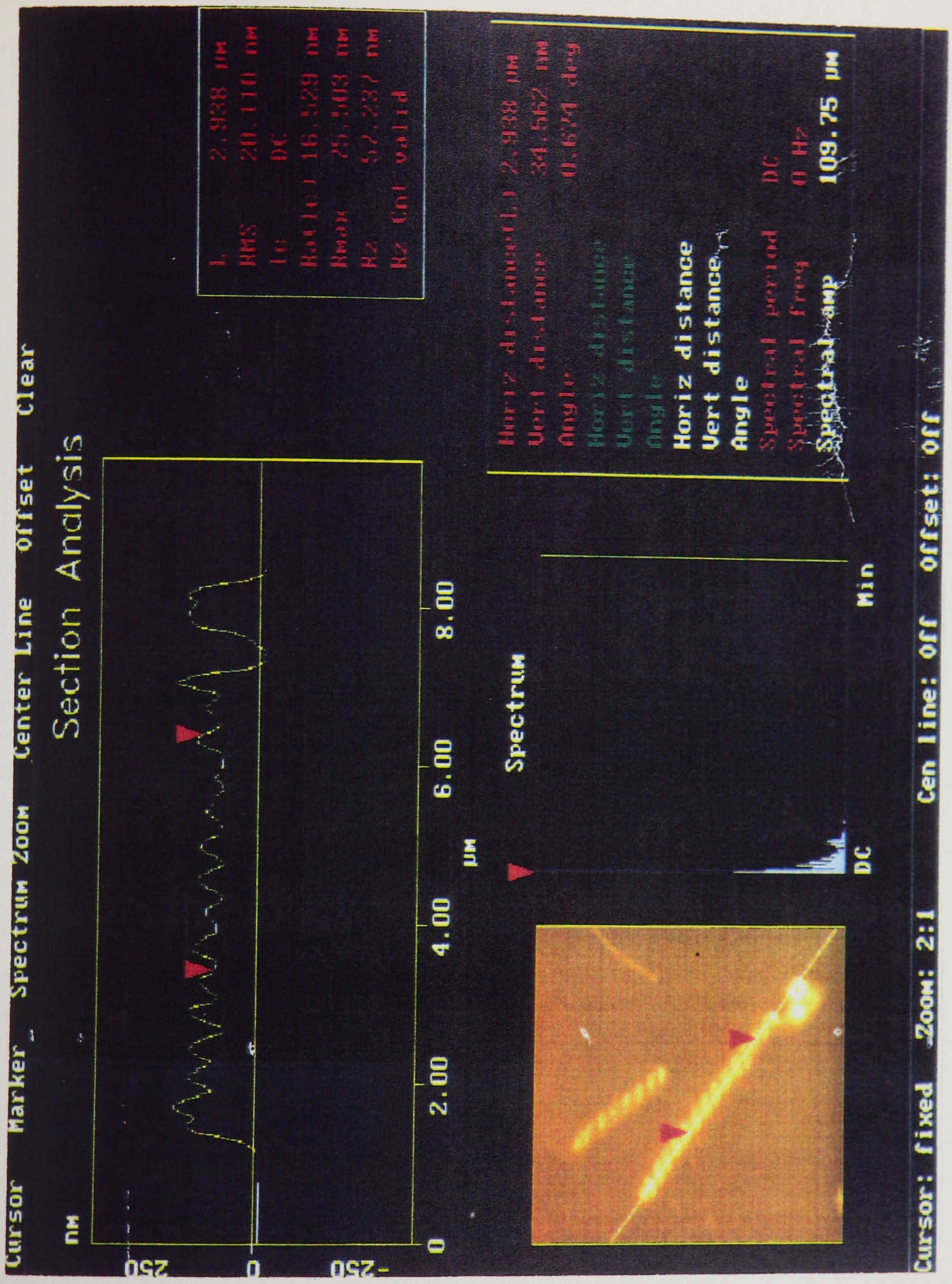

Fig. 5. 20. Section analysis of the biofilm bacteria. These are the bacteria previously shown in Flg. 5.19. The red arrows visible in the image are markers which can be moved to any position on the image. The image may then be manipulated to allow the various parameters to be calculated which will give accurate imformation of the height, width and cross sectional parameters of individual bacteria.

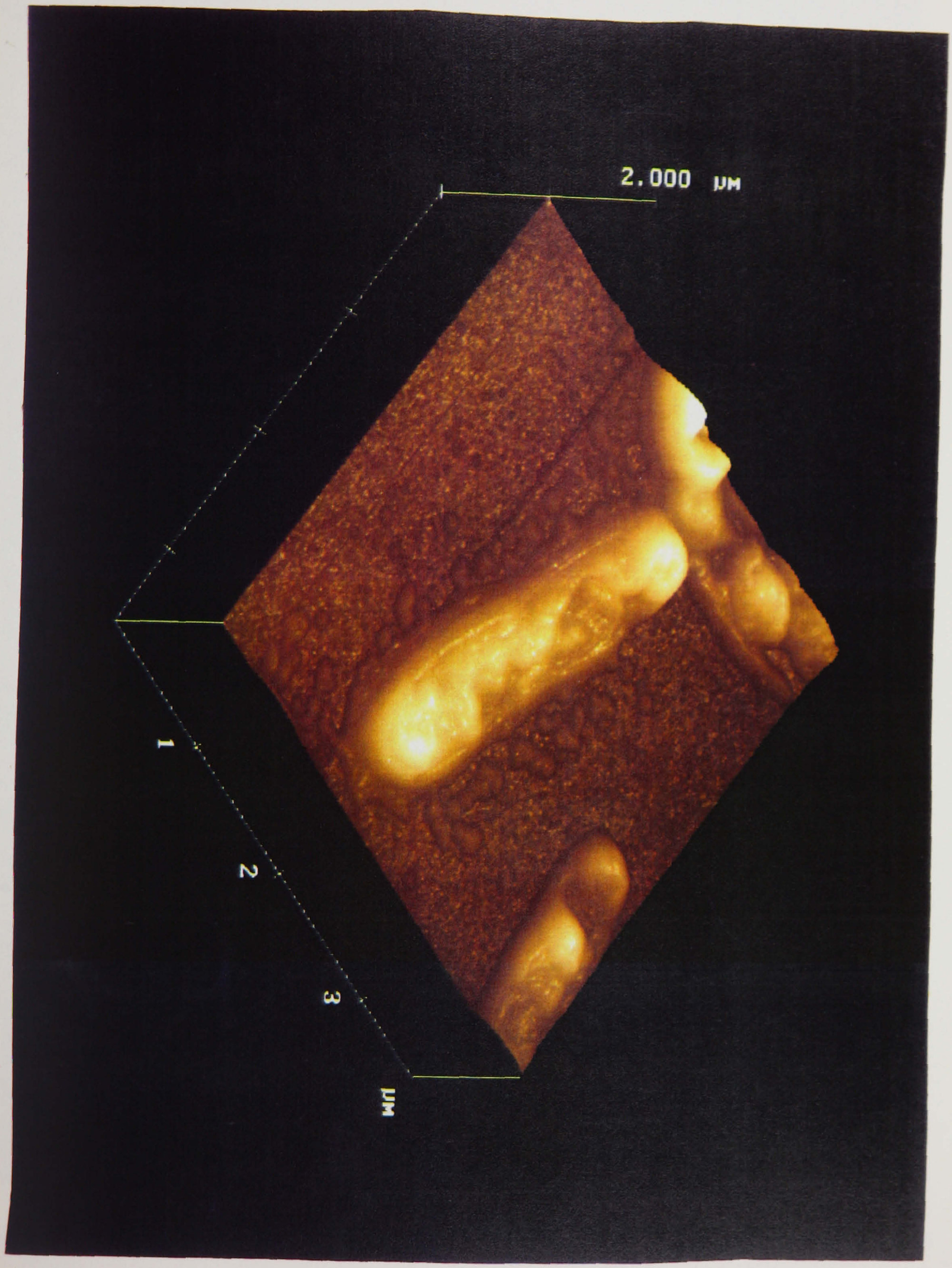

Fig. 5.21. An AFM image of *L. pneumophila*. An overnight (16-24hour) biofilm developed from a pure culture of the avirulent *L. pneumophila*. The image of the *L. pneumophila* showed the non-parallel sides, rounded ends and visible outer membrane.

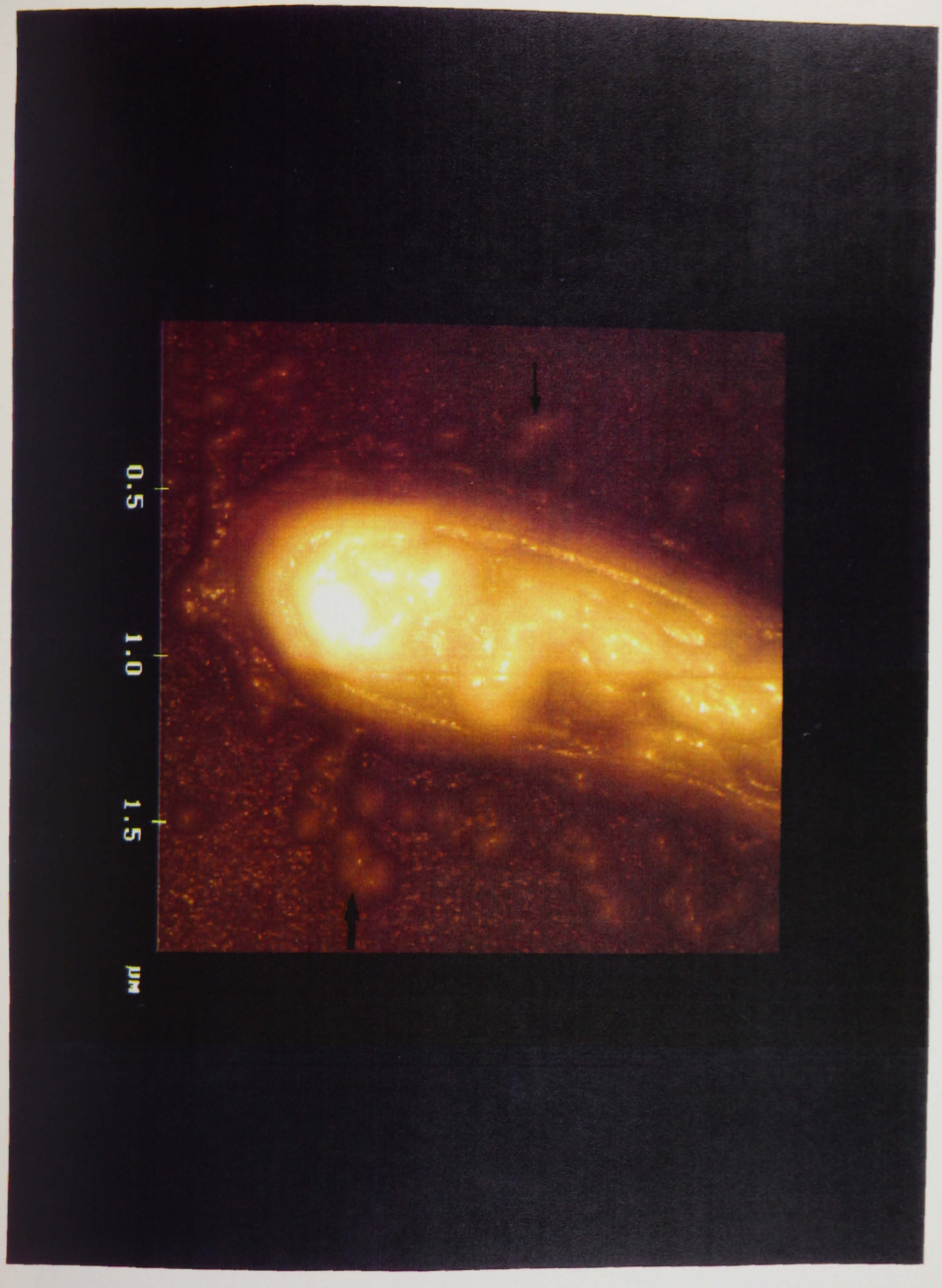

Fig. 5. 22. An AFM image of *L. pneumophila.* This image is at a higher magnification than Fig. 5.21 and demonstrates that EPS can be seen clearly around the bacterium (arrows).

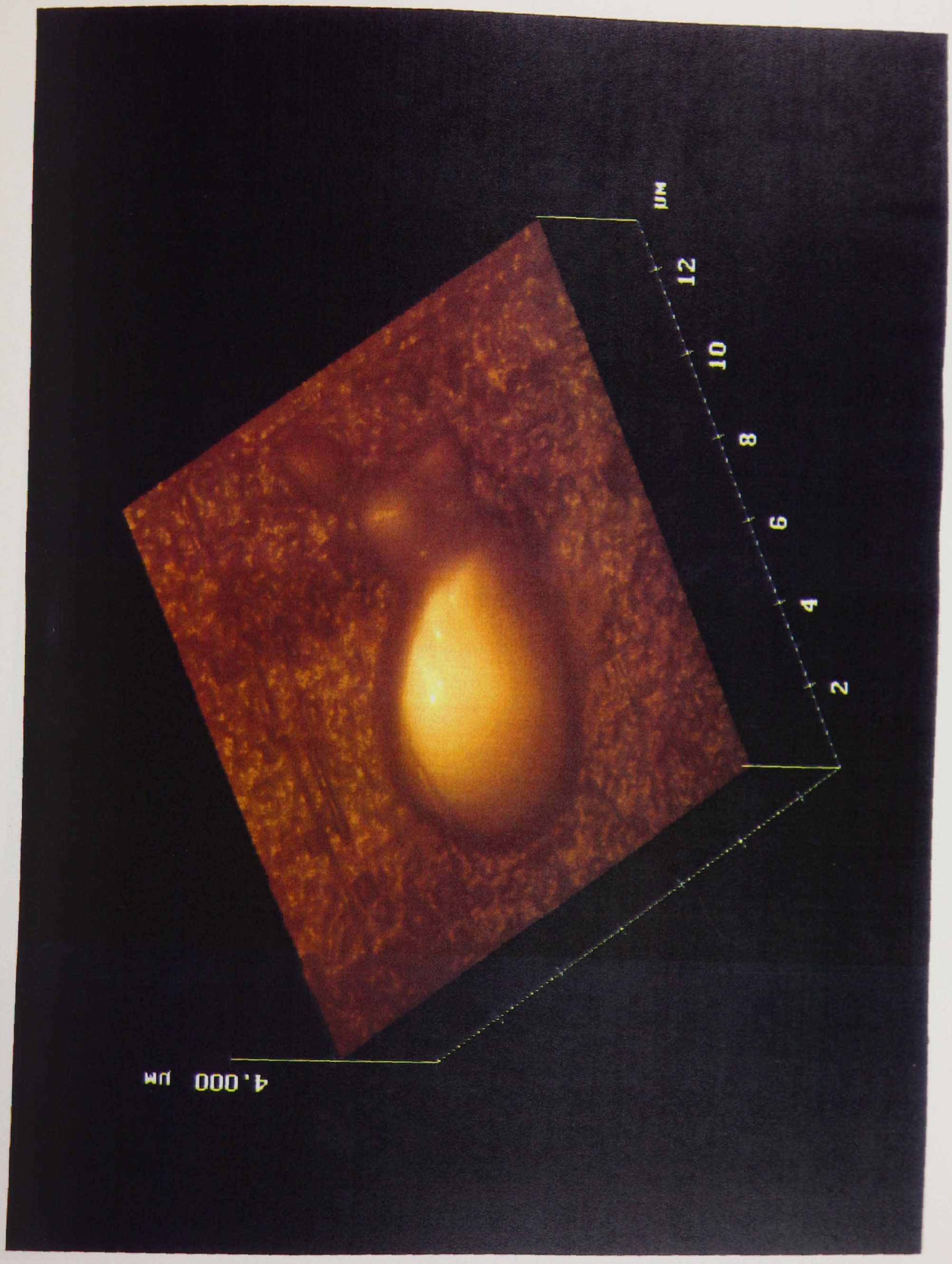

Fig. 5.23. An image of a fully hydrated biofilm bacterium. This image was achieved whilst the biofilm, on the glass tile support, was fully hydrated within a flow cell.

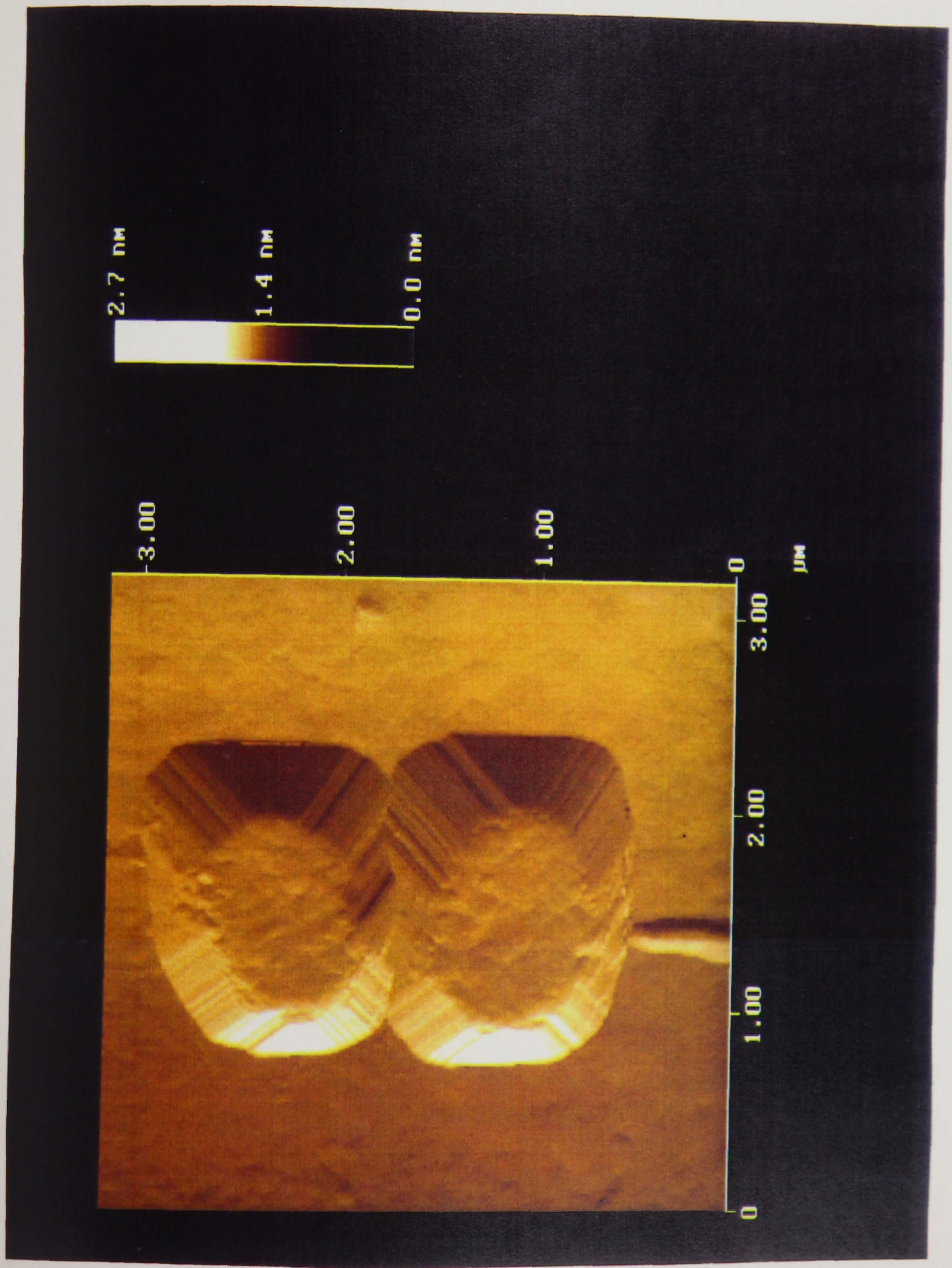

Fig. 5. 24. An AFM image of two bacteria. This image shows the distortion of the image which is due to tip surface interactions.

At higher magnification using SEM, sample preparation in the form of fixation, dehydration and staining is required. SEM offers good resolution with the capacity to image complex shapes. Osmium Tetroxide (OsO_4) fixes the cytoplasm bound water (Wheatley, 1981). Fig. 5. 25 a-d show OsO_4-stained sparse and dense areas of the same eight week biofilm. EPS (1) can be seen to be covering the more dense areas (a-c) together with grazing protozoa (2) and numerous cysts (3) denoted by arrows. Fig. 5. 25d shows a clump of grazing protozoa which are probably *Hartmanella vermiformis.*

Ruthenium red and (OsO_4) together are used to show the presence of EPS (Luft, 1965; Jones *et al.*, 1989). This staining method forms a complex with acidic polysaccharides, due to the reduction of OsO_4 to lower insoluble oxides to form an electron dense structure (Luft, 1965; Pate and Ordal, 1967). Ruthenium red/OsO_4 staining of the biofilm clearly shows the electron dense EPS on the surface of the biofilm (Fig 5. 26), the chemical nature of the interaction of the stain with this structure has been previously described by Luft (Pate and Ordal, 1969).

As a biological material bacterial biofilms may be sensitive to the harsh treatments required for visualisation by traditional electron microscopes. Because biofilms are highly hydrated entities dehydration processes have a shrinking effect on biofilms, which leads to to the production of artefacts (Sutton *et al.*, 1994) and may also lead to the loss of fine surface structures (van Doorn *et al.*, 1990). Care must be taken therefore, in the interpretation of these photomicrographs to *in vivo* biofilm structures. Comparative visualisation by other microscopy techniques and/or confirmatory biochemical tests maybe necessary to aid in the discrimination between artefacts and genuine structures (Costerton and Geesey, 1979).

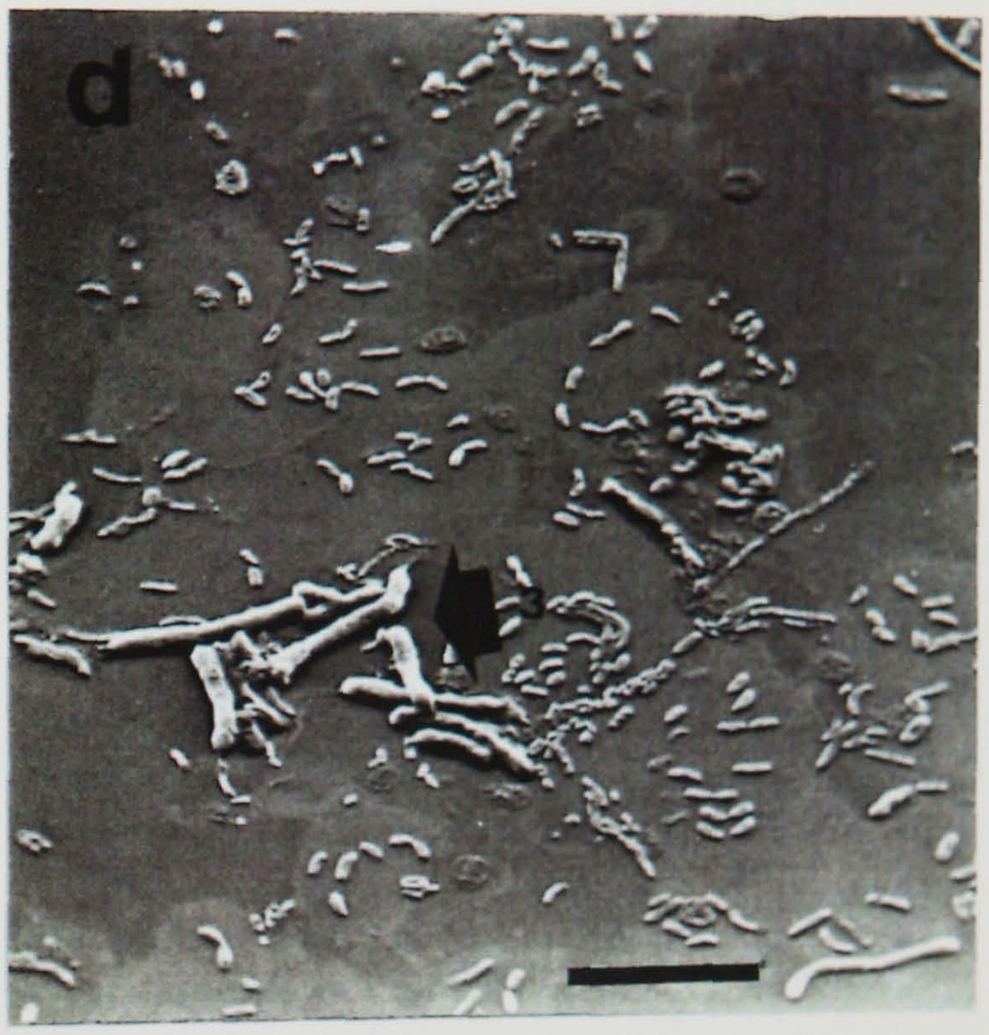

Fig. 5.25 a-d. An eight week OsO4 stained biofilm examined by SEM. (Magnification x 3000, (bar = 10μm). EPS can be seen over the more dense regions of the biofilm (1), many amoebal cysts are present (2) and several grazing amoebal trophozoites (3). Fig. 5.25d. A clump of grazing protozoa which are probably *Hartmanella vermiformis* can be seen in a less dense area.

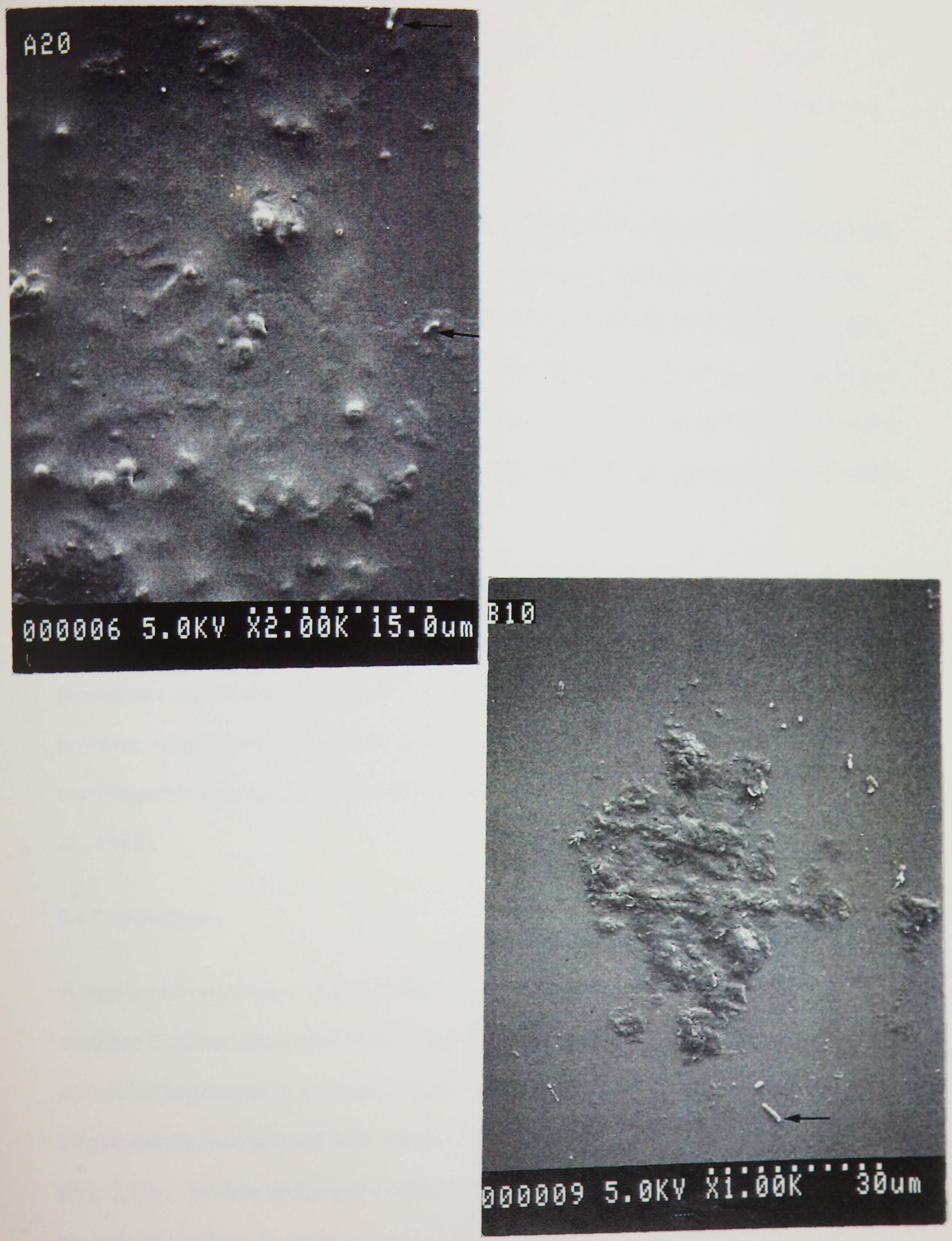

Fig. 5.26 a-b. Biofilms stained with Ruthenium red/OsO_4. Staining clearly shows the EPS layer covering the dense areas of biofilm. Bacteria can be seen embedded in the biofilm (arrows).

Two other notable microscopic methods which are available are the scanning laser confocal and the environmental scanning electron microscope (ESEM). These microscopes were not available for use in this study but have been used previously to examine hydrated biofilms (Sutton *et al.*, 1994).

Scanning laser confocal microscopy uses a combination of fluorochrome staining and image analysis technology to build up a 3-dimensional image of thin sections of the object (Schormann and Jovin, 1992). An advantage of this method is that the surface topography of an opaque substratum beneath the biofilm may be imaged (Cummins *et al.*, 1992; Walker *et al.*, 1994). A disadvantage is the loss of contrast and detail which may occur in the construction of the 3-D image during the processing required to stack the sections (Schormann and Jovin, 1992).

With the ESEM a pressure chamber is necessary to maintain the specimen in a hydrated state. Artefacts as a result of prior preparation in the form of dehydration and staining procedures, do not therefore occur. As with other forms of electron microscopy however, imaging of untreated biological samples with the ESEM may result in damage occuring after a relatively short time (minutes) as a result of the electron beam (Little *et al.*, 1991).

5.4.Conclusions.

A number of established and recently developed microscopic techniques were used to visualise biofilms developed from water distribution system microorganisms. Biofilms are not homogeneous in composition but are complex matrices composed of microcolonies interspersed with channels allowing the movement of fluid and nutrients (Fig. 5.10). No one technique can be said to be better than another in the structural analysis of biofilms in order to build a more complete picture showing the complexity of biofilm structure. Despite the lower magnification possible with optical microscopy compared with the more complex higher magnification techniques, there are several advantages of using the optical techniques described for *in vivo* examination of biofilms.

The initial cost of the microscope is likely to be a lot less and preparation and staining techniques are generally less time consuming and costly. In addition the light beam is non-destructive, with only a very small dose of radiation compared with the electron microscope beam. The biofilm can also be relatively easily maintained in suitable environmental conditions ie. maintenance of hydrated samples for *in vivo* visualisation. With the addition of a thermostatically controlled heated stage the temperature of the sample can also be maintained. Hoffman modulation contrast microscopy has an advantage over phase contrast microscopy in that dense areas of biofilm can be easily visualised.

DIC is useful for giving excellent topographical information because there is no compression of the specimen. When used in conjunction with vital and species specific stains DIC together with fluorescence can give valuable information on the viability and ecology of the biofilm (Figs.5.11.- 5.14). Imaging with AFM is still in its early days. The potential to image under liquid at high magnification needs further work to overcome the difficulties discussed earlier. The possibility of performing time course studies on specific antibody labelling within a mixed consortium would be a useful tool in the future.

The ability to manipulate the AFM image to take measurements of individual bacteria is another area in which this microscope has much to offer to future microbiological research, particularly in mixed consortium studies (Fig. 5.20). The prospect of using the capability of the AFM to provide accurate measurements of the morphological detail of individual bacteria and to build up a database of the results is worth investigating for future ecological studies. The use of the AFM tip itself to manipulate bacterial surface structures is another intriguing possibility for future study.

Each of the techniques used added a different dimension to the understanding of the spatial composition of biofilms. It follows therefore, that a combination of as many

niques as available is required to overcome the problems of recognising artefacts to give the most accurate picture of the true biofilm structure and organisation.

PAGE

MISSING

IN

ORIGINAL

CHAPTER 6.

BIOCIDE STUDIES

6. Biocide studies

6.1. Introduction.

The occurrence of biofilms within domestic, hospital and industrial aquatic systems is well documented (Geesey *et al.,* 1978; Costerton, 1984; Costerton *et al.,* 1987; Le Chevallier *et al.*, 1988; Colbourne *et al.,* 1988b). Biofilms within such systems, are of economic (Le Chevallier, *et al.,* 1987; Geesey *et al.*, 1988; Sly *et al.,* 1988; Gaylarde and Beech, 1989; Keevil *et al.*, 1989), aesthetic (Le Chevallier and McFeter 1985) and health related importance (Cloete *et al.*, 1989b; Keevil *et al.*, 1989). Biofilms in man made aquatic environments such as those within distribution systems provide ecological niches ideally suited to the survival and growth of *L. pneumophila* (Colbourne and Trew, 1986).

Resistance to biocide treatments has been shown to be increased in bacteria which are attached to surfaces and to particulate matter within a system (Ridgway and Olsen, 1982; Kutchta *et al.,* 1985; King *et al.,* 1988). The mechanisms involved in conferring resistance to biocide treatment upon biofilm microorganisms are still not fully understood. The presence of the glycocalyx on the biofilm surface is thought to be important in that it constitutes a barrier, inhibiting the action of antimicrobial agents on the biofilm microflora (Costerton *et al.*, 1981; Cloete *et al.,* 1989b). The biofilm consortium itself is thought to play a role in the inactivation of biocides by absorption or catalytic destruction of the agent (Nichols, 1994). It is unclear whether a phenotypic response of the microbial population to surface growth also plays a role in this increased resistance (Jass and Lapin-Scott, 1994).

Regrowth, the continued proliferation of potential pathogens following treatment (Keevil *et al.,* 1989) is a consequence of this reduced effectiveness of biocide treatment (Keevil *et al.,* 1989, Le Chevallier *et al.,* 1988a-c, Yamamoto *et al.,* 1991). The potential for regrowth of *L. pneumophila* is of particular importance within water systems where there is the potential for aerosol production.

In aquatic systems where growth conditions are often nutrient limited, bacteria may exhibit increased biocide resistance patterns when compared with their broth grown counterparts. *L. pneumophila* often exhibits higher resistance to chlorine than some aquatic microorganisms which are commonly used as indicators of water quality, especially where chlorine is regularly added to the system (Kuchta *et al.*, 1985). As infection of a system may occur by the seeding with a small number of *L. pneumophila*, a few resistant *L. pneumophila* surviving biocide treatment could have important implications in the subsequent proliferation of this microorganism (Kuchta *et al.*, 1985).

6. 1. 2. Vantocil 1B.

Vantocil is a broad spectrum industrial biocide. It is polyhexamethylene biguanide hydrochloride (PHMB). The active ingredient is a polydisperse mixture (PHMB) which is a mixture of hexamethylene biguanides with the general formula :-

$$\left\{ (CH_2)_3\text{—NH—C(=NH)—NH—C(=NH.HCL)—NH—}(CH_2)_3 \right\}^{HCl}_{n}$$

were n =12. (ICI Biocides, 1991)

Biguanides have been recognised for several years as effective antimicrobial agents (Curd and Rose, 1946; Rose and Swain, 1956) with the advantage that they exhibit low toxicity to mammals (Broxton *et al.*, 1983). Vantocil 1B is used in many industrial processes such as pre-process pasteurisation, wash waters from latex and paint manufacturing processes and in the hydrotesting of oil pipelines. These are processes where there is the potential for both biofilm formation and aerosol production and where a biocide which is effective in controlling biofilm microorganisms including *Legionella pneumophila* is required.

6.1.3. Mechanism of action.

Vantocil 1B is active against both Gram positive and Gram negative bacteria. The mechanism of action of the biguanides is by the reversible binding of the biocide to the bacterial membrane phospholipids, phosphatidylglycerol and diphosphatidylglycerol. This binding causes a change in membrane conformation, which rapidly alters cell permeability to potassium and allows the subsequent loss of cellular components including inorganic phosphates (Broxton *et al.*, 1983; 1984). Barker *et al.* (1992) found that Vantocil was effective against *Legionella pneumophila* when grown in broth and amoebal culture. The effect of this biocide against *L. pneumophila* in a mixed consortium of aquatic microorganisms, either in a planktonic or sessile population has not previously been assessed.

6.1.4.This study.

Experimental work was undertaken to determine whether the avirulent *L. pneumophila* serogroup1 Corby strain was useful as a model substitute for the virulent strain in biocide studies, alleviating the need for costly class three containment facilities for future biocide research.

A comparative study was carried out on the effects of a biocide, Vantocil 1B, on an environmental isolate of *L. pneumophila* serogroup 6 and the avirulent *L. pneumophila* serogroup 1 strain. Vantocil IB was added to the model systems used in this investigation to assess its effect on the *Legionella* and also on the heterotrophic planktonic and biofilm populations. Seven day biofilms were used in this study because they have been shown to exhibit higher resistance to biocides than less developed biofilms (Le Chevallier *et al.*, 1988).

6. 2.Materials and Methods.

6. 2.1. Minimum inhibitory concentration (MIC) studies.

In order to determine the dose of biocide necessary to eradicate the *Legionella* from the chemostat minimum inhibitory concentrations (MIC) were carried out. In conventional MIC tests a nutrient medium inoculated with the test organism is incubated with serial dilutions of the antimicrobial agent. The MIC is the lowest concentration at which no growth occurs following incubation.

Microorganisms present in water are usually in a low nutrient environment. The use of a high nutrient broth medium to determine the dose of biocide is therefore inappropriate. The susceptibility of the planktonic population in the chemostat, containing *L. pneumophila* serogroup 6, to the biocide was assessed by removing samples of water containing planktonic phase microorganisms from the chemostat and adding aliquots of this to replicate serial dilutions of the biocide. These were sampled at intervals and inoculated onto R2A and buffered charcoal yeast extract (BCYE) with the addition of glycine, vancomycin, polymixin and cycloheximide (GVPC) plates to assess growth of both the heterotrophic population and *L.pneumophila.* The R2A plates were incubated at 30°C and examined after 48 hours, 72 hours and 5 days. The GVPC plates were incubated at 37 °C and examined after 5 days.

6.2.2. Biofilm development

A model water system based on that of Keevil *et al.* (1987, 1989), Walker *et al.* (1991), and Rogers *et al.* (1990), was set up as previously described (Chapter 2. 2. 2). The initial inoculum was derived from the local mains tap water supply and was the same for both chemostats. Mains tap water from the same source as the initial inoculum was filter sterilised, (Chapter 2. 2.3), using a 2 µm nylon filter [Pall, UK] (Colbourne *et al.*, 1988). The sterile water was subsequently used as the continuous culture growth

medium in the vessel to give a dilution rate (D) of 0.05 h^{-1}. The depth of the aqueous phase was maintained at 1000 ml via a weir system. Temperature was monitored by a glass temperature probe inserted into the aqueous phase of the vessel and maintained at 30°C (±1) by use of an external heater pad under the base of the vessel linked to a microprocessor control unit (Brighton Systems, UK)

Biofilms were allowed to develop for seven days on glass tiles suspended on titanium wire within the aqueous phase of the system. Tiles were removed aseptically from the system via the sampling ports, in order to assess biofilm growth. The tiles were first rinsed in 10 ml of sterile distilled water to remove any unattached microorganisms. Adherent biofilms were subsequently removed by scraping them into 1 ml of sterile water with a dental probe. This suspension was then vortexed to break up any clumps of microorganisms. Serial dilutions were then prepared for spreading onto two replicate plates of selective and non-selective media. Scraping and vortexing was chosen in preference to sonication to remove the biofilm as Keevil *et al.* (1989), have reported that sonication may disrupt the more fragile aquatic bacteria. The planktonic population was sampled by aseptically removing 10 ml from the aqueous phase of the chemostat and serially diluting as above. After 7 and 12 days the chemostat was shock dosed with 200 ppm (40 ppm active ingredient), of Vantocil IB added via one of the small ports.

The numbers of *L. pneumophila* present were assessed by spread-plating onto GVPC following heat treatment (Chapter 2.).The total heterotrophic population was assessed by growth on R2A medium (Reasoner and Geldreich, 1985). After inoculation the plates were sealed in polythene bags to prevent dehydration. The R2A plates were incubated aerobically at 30 °C for up to 5 days before enumeration. The GVPC plates were incubated at 37 °C and examined after 5 days. Identification of the heterotrophic bacteria and protozoa was as previously described (Chapter 2. 2. 3). The presence of *L. pneumophila* was confirmed by colony morphology, absence of growth on BCYE without cysteine and latex agglutination (Pro-Lab Diagnostics, UK).

6.3. Results

6. 3.1. Identification of microorganisms.

The continuous culture model system contained a diverse population of microorganisms. This included two species of protozoa tentatively identified as *Acanthamoeba* sp., and two strains of *Hartmanella vermiformis*, one of which grew at 42 °C. The heterotrophic population included *Pseudomonas* spp., *Flavobacterium* sp. and *Methylobacterium* sp. (Table 6 1.)

Agrobacterium radiobacter	*Pseudomonas fluorescens*
Commamonas sp.	*Pseudomonas mesophilica*
Flavobacterium sp.	*Pseudomonas paucimobilis*
Methylobacterium mesophilicum	*Pseudomonas vesicularis*
Moraxella lacunata	*Sphingomonas multivorum*
Acinetobacter calcoaceticus	

Table 6.1. Heterotrophic microorganisms identified from the chemostat population. Initial identification was by use of the first stage diagnostic table for Gram-negative bacteria (Cowan and Steele, 1966, 1993). Further identification was by API 20NE (API- Biomerieux, Basingstoke) or the Biolog GM Gram-negative identification system (Atlas Bioscan, Hayward, California, USA). Identification was only accepted if rated "good" or better by the scheme utilised.

6.3.2. MIC results.

The effect of Vantocil 1B on the *L. pneumophila* serogroup 6 from the planktonic phase (Table 6.2) shows that 200 ppm was bactericidal after 4 hours contact time. When the contact time was increased to 24 hours, a concentration of 20 ppm or above inhibited growth (Table 6.3). The same degree of inhibition was also seen with the total planktonic population,

		ppm Vantocil							
hours	**control**	**1000**	**500**	**250**	**200**	**100**	**50**	**25**	**20**
1	+	+	+	+	+	+	+	+	+
2	+	nd	+	+	+	+	+	+	+
4	+	nd	nd	nd	nd	+	+	+	+
8	+	nd	nd .	nd	nd	+	+	+	+
12	+	nd	nd	nd	nd	+	+	+	+
24	+	nd	nd	nd	nd	nd	nd	nd	nd

Table 6. 2. The effect of biocide on *L. pneumophila* serogroup 6. *L. pneumophila* had previously been isolated from the chemostat on GVPC. += growth; nd= growth not detected.

When the R2A plates were incubated for 48 hours (Table 6. 3), 100 ppm of the biocide appeared to be effective after only four hours contact time. However when these same plates were incubated for an additional 24 hours, further growth occurred and it was found that 250 ppm of biocide and a contact time of 12 hours was required to achieve the same result. After 5 days incubation, greater numbers of slower growing pink pigmented bacteria were present and bacterial survival persisted in the presence of

higher concentrations of biocide. A longer contact time was required to have a bactericidal effect.

Incubation time			**ppm Vantocil IB**							
	hours	**control**	**1000**	**500**	**250**	**200**	**100**	**50**	**25**	**20**
48	**1**	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+
hours	**4**	+	nd	nd	nd	nd	nd	+	+	+
	8	+	nd	nd	nd	nd	nd	nd	nd	nd
	12	+	nd	nd	nd	nd	nd	nd	nd	nd
	24	+	nd	nd	nd	nd	nd	nd	nd	nd
	hours	**control**	**1000**	**500**	**250**	**200**	**100**	**50**	**25**	**20**
	1	+	+	+	+	+	+	+	+	+
72	**2**	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+
hours	**8**	+	+	+	+	+	+	+	+	+
	12	+	nd	nd	nd	+	+	+	+	+
	24	+	nd	nd	nd	nd	nd	nd	nd	nd
	hours	**control**	**1000**	**500**	**250**	**200**	**100**	**50**	**25**	**20**
120	**1**	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+
hours	**4**	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+
	12	+	+	+	+	+	+	+	+	+
	24	+	nd	nd	nd	nd	nd	nd	nd	nd

Table 6. 3.The effect of biocide on the growth of the heterotrophic planktonic population following incubation with biocide. Growth was determined after 48 hour, 72 hours and 5 days incubation on R2A medium. += growth; nd= growth not detected.

6.3.3. The effect of Vantocil 1B on the planktonic heterotrophic population.

At the beginning of the experiment the mean of the planktonic heterotrophic population counts in the model systems were 5.96 and 5.89 $\log_{10}$ cfu ml^{-1} (Table 6.4) for the system containing the *L. pneumophila* serogroup 6 (SG6) and the system containing the avirulent *L. pneumophila* serogroup 1(AV) respectively.

Following the first addition of biocide (200 ppm) the planktonic population was reduced by 97 % (Fig. 6. 1.) in both systems after 24 hours. A second dose after 5 days, reduced the recovering population further, to 99 % overall reduction for the SG6 strain and to below detectable limits in the system containing the avirulent strain.

6.3.4. Effects of Vantocil 1B on the heterotrophic biofilm population

Prior to the addition of biocide the mean heterotrophic biofilm counts were 6.2 $\log_{10}$ cfu cm^{-2} for the SG6 strain and 6:64 $\log_{10}$ cfu cm^{-2} for the AV strain (Table 6.5) which were reduced after 8hours by 99% (SG6) and 98% (AV) following the initial dose of biocide (Fig.6.2.). Twenty four hours after biocide addition the biofilm bacterial count had increased in both vessels. The second dose of Vantocil1B reduced the original heterotrophic biofilm population by 92.5 % (SG6) and 98.7 % (AV) after 24hours.

The difference in the biofilm, following biocide addition can be clearly seen in the atomic force microscope images (Fig.6.3a and 6.3b). Following biocide addition there is very little EPS (6.3b) and the glass tile support is sparsely populated compared with a biofilm of the same age with no biocide added.

• hours	Log_{10} planktonic mean (SG6) ml^{-1}	Log_{10} SE planktonic mean (SG6)	Log_{10} planktonic mean (AV) ml^{-1}	Log_{10}SE planktonic mean (AV)
0	5.96	2.27	5.89	1
1	4.88	0.78	4.72	1.02
4	4.68	1.2	4.64	0
8	4.49	0.57	4.4	0.27
24	4.37	0.9	4.37	0.663
120	5.34	1.8	6.26	1.6
121	4.13	0.84	5.4	1.26
124	4.01	0.76	4.78	0.65
144	4.02	0.32	nd	nd

Table 6.4. Log_{10} heterotrophic planktonic counts. Duplicate samples were taken from each system at the above time intervals following biocide addotion. Ten fold dilutions were made and each dilution was spread plated onto duplicate R2A plates prior to counting. nd = growth not detected. (SE = standard error of the mean)

hours	Log_{10} biofilm mean (SG6 ml^{-1}	Log_{10} SE biofilm mean (SG6)	Log_{10} biofilm mean (AV) ml^{-1}	Log_{10}SE biofilm mean (AV)
0	6.2	1.35	6.64	0.07
1	5.7	0.84	5.69	0.01
4	4.73	0.63	5.16	0.06
8	4.25	0.45	5.00	0.09
24	5.19	1.58	5.64	0.03
120	5.43	0.95	7.1	0.01
121	4.64	1.23	5.52	0.12
124	4.68	1.62	5.944	0.07
144	5.08	0.75	4.77	0.4

Table 6.5. Log_{10} heterotrophic biofilm counts. Duplicate samples were taken from each system at the above time intervals. Following scraping and vortexing, 10 fold dilutions were made and each dilution was spread-plated onto duplicate R2A plates prior to counting.

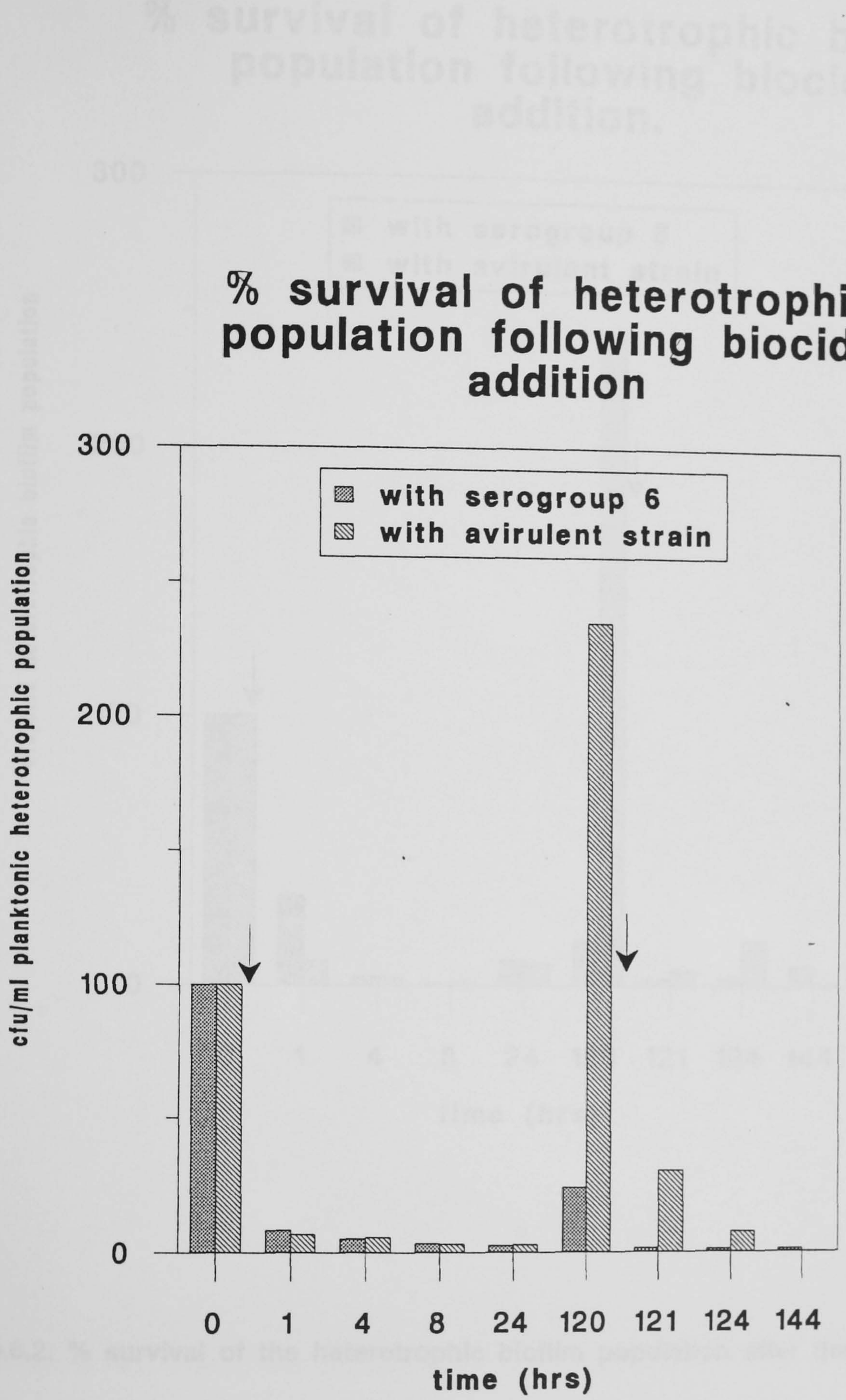

Fig.6.1. % survival of the heterotrophic planktonic population after dosing with 200 ppm. Vantocil 1B at t=0 hours and t=120 hours (arrows). Results are expressed as a percentage of the heterotrophic population recovered on R2A medium at the start of the experiment.

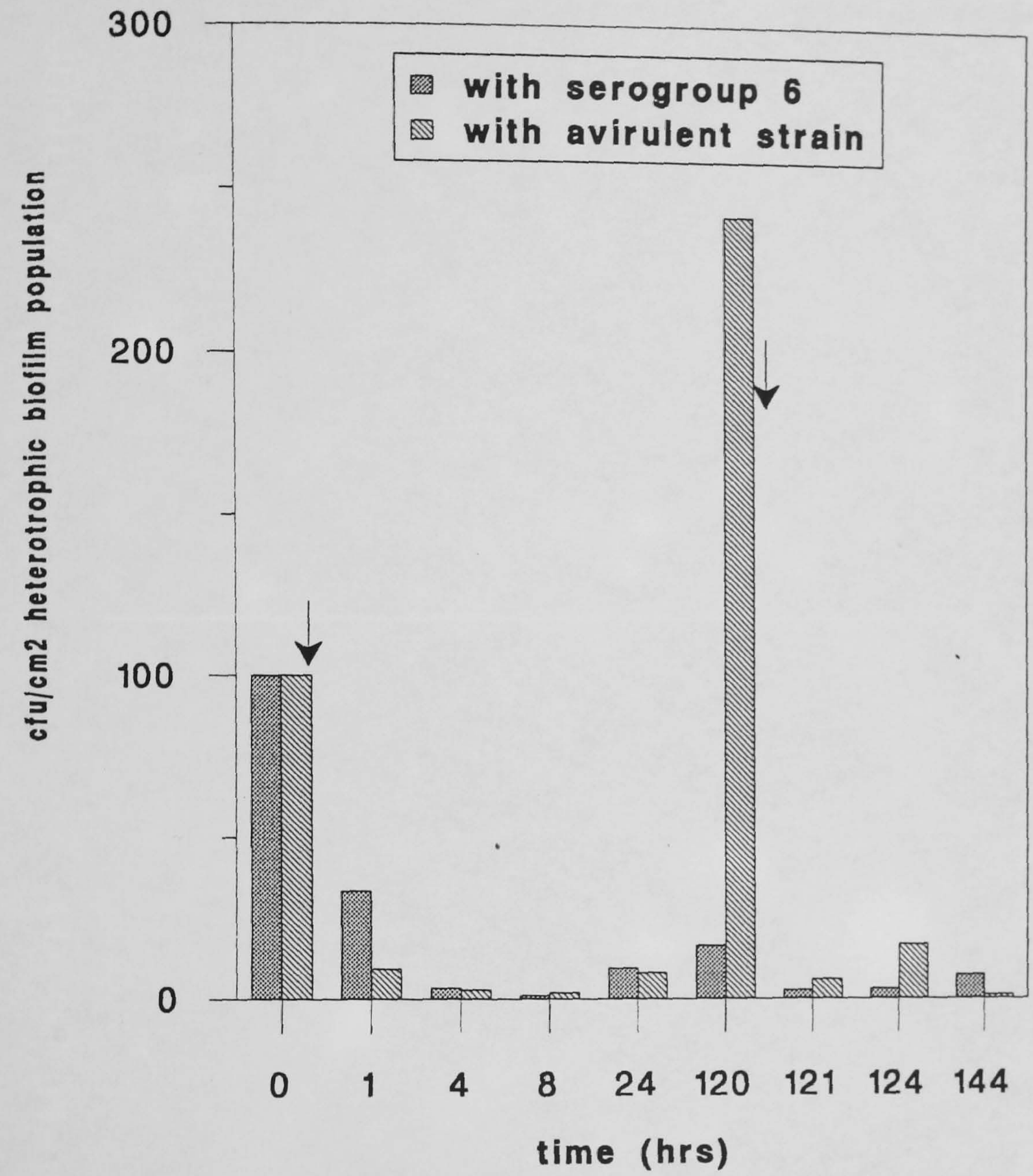

Fig.6.2. % survival of the heterotrophic biofilm population after dosing with 200 ppm Vantocil 1B at t= 0 hours and t=120 hours (arrows). Results are expressed as a percentage of the heterotrophic biofilm population recovered on R2A medium at the start of the experiment.

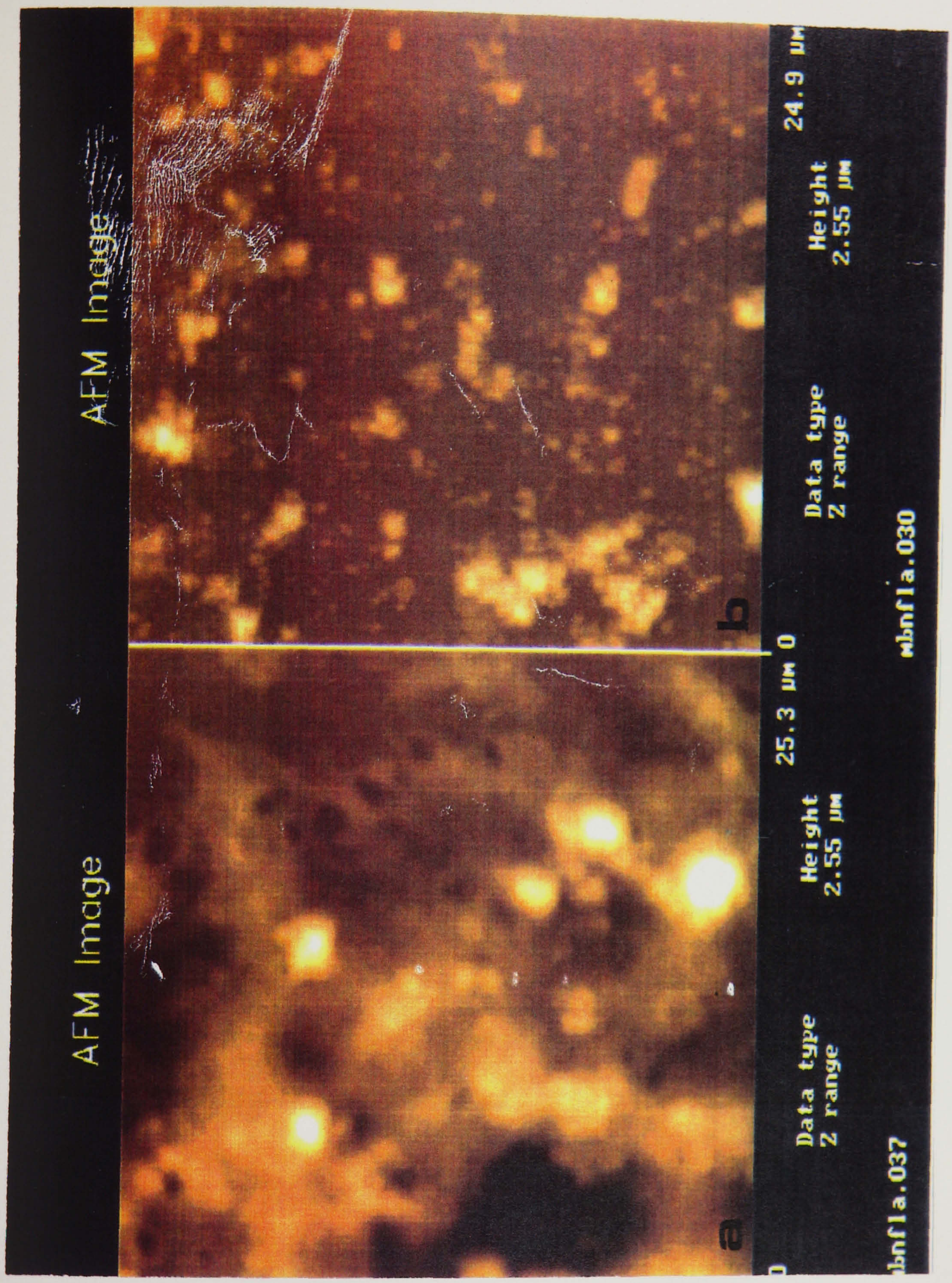

Fig.6.3. Atomic force microscope surface images of biofilms without biocide (a) and (b) after biocide addition. Prior to biocide addition (a), a reduction in the biofilm population can be clearly seen after the addition of biocide (b).

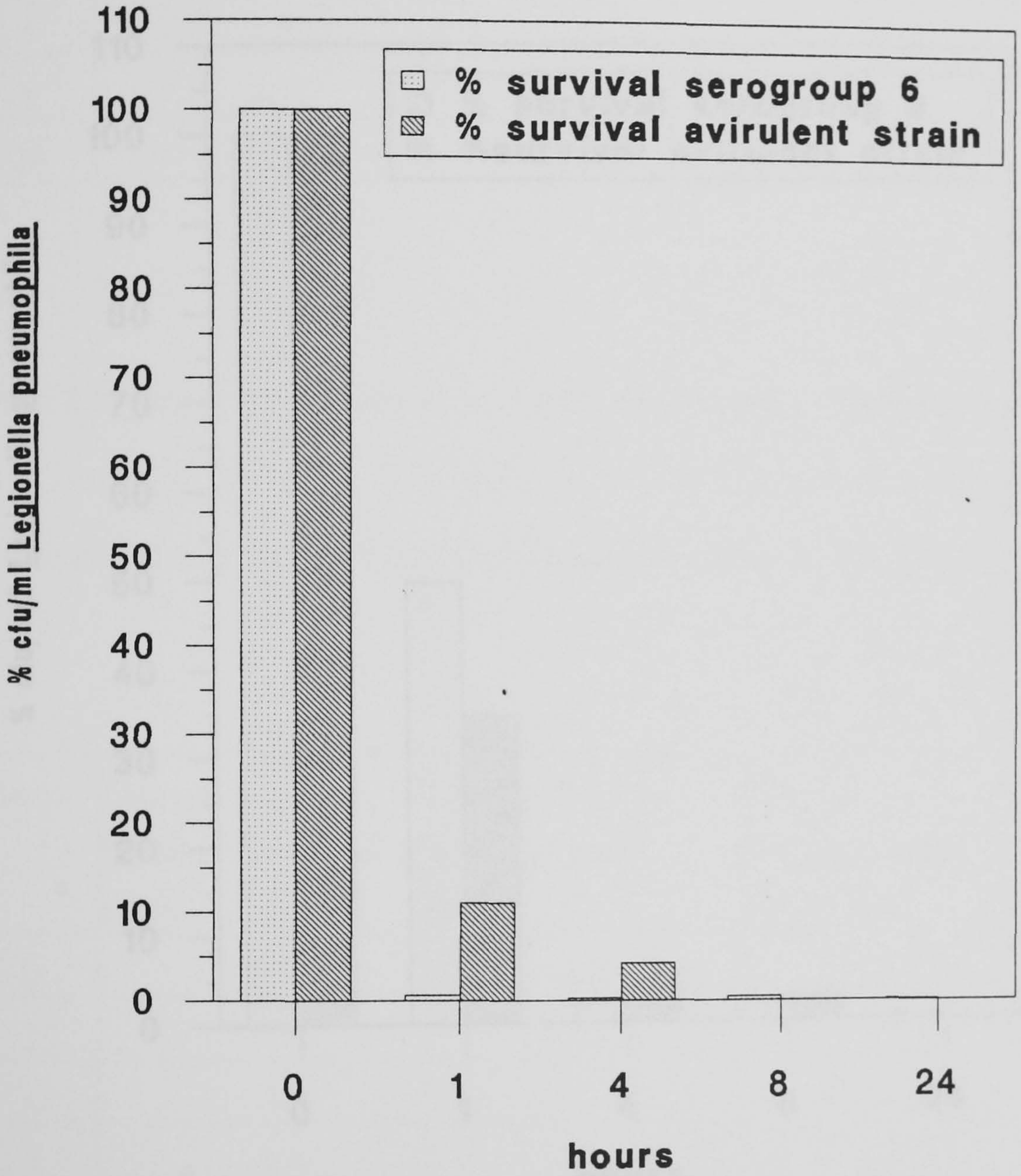

Fig. 6.4. % survival of planktonic *L. pneumophila* after addition of 200 ppm Vantocil 1B. Results are expressed as the pertentage of the original biofilm *Legionella* population recovered on GVPC plates before the addition of biocide.

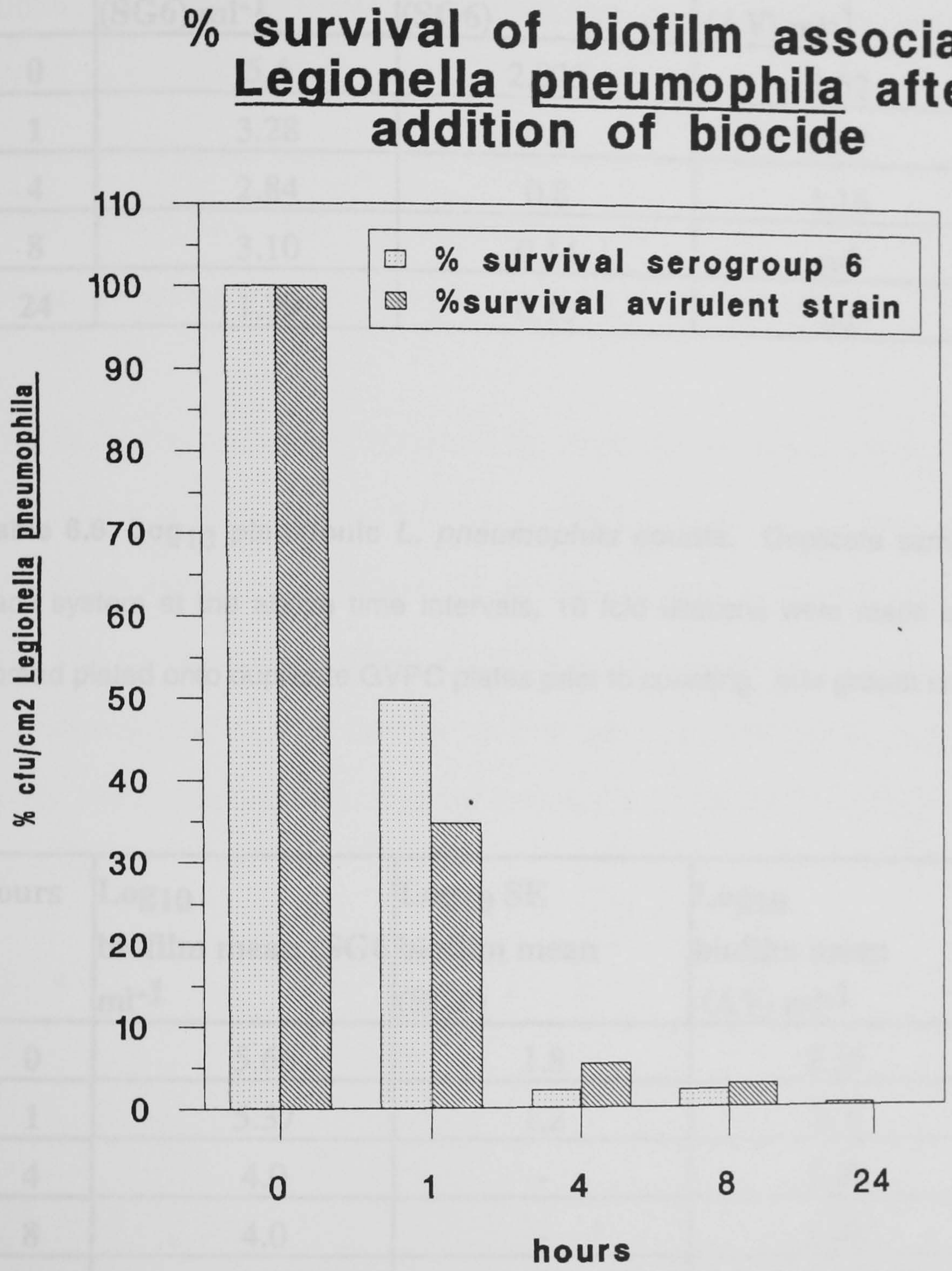

Fig. 6. 5. % survival of biofilm associated *L. pneumophila* after addition of 200 ppm. Vantocil 1B. Results are expressed as a percentage of the original population in the planktonic phase before the addition of biocide.

• hours	Log_{10} planktonic mean (SG6) ml^{-1}	Log_{10} SE planktonic mean (SG6)	Log_{10} planktonic mean (AV) ml^{-1}	Log_{10}SE planktonic mean (AV)
0	5.4	2.025	3.52	0.14
1	3.28	0.7	2.57	0.22
4	2.84	0.8	1.16	0.16
8	3.10	0.14	nd	nd
24	1.39	0.13	nd	nd

Table 6.6. Log_{10} planktonic *L. pneumophila* counts. Duplicate samples were taken from each system at the above time intervals, 10 fold dilutions were made and each dilution was spread plated onto duplicate GVPC plates prior to counting. nd= growth not detected. (n=3)

hours	Log_{10} biofilm mean (SG6 ml^{-1}	Log_{10} SE biofilm mean (SG6)	Log_{10} biofilm mean (AV) ml^{-1}	Log_{10}SE biofilm mean (AV)
0	5.66	1.8	2.56	0.2
1	5.37	1.2	2.1	1.7
4	4.0	-	1.29	0.51
8	4.0	-	1.01	0.01
24	3.2	.015	nd	-

Table 6.7. Log_{10} biofilm associated *L. pneumophila* counts. Duplicate samples were taken from each system at the above time intervals. Following scraping and vortexing, 10 fold dilutions were made and each dilution was spread-plated onto duplicate GVPC plates prior to counting. (n=3)

6.3.5. Effects of Vantocil 1B on *L. pneumophila.*

Figure 6.4 shows that in the presence of 40 ppm active ingredient of Vantocil IB the numbers of *L. pneumophila* in the planktonic phase (Table 6.6) were reduced by 99.99% (SG6) and to below detectable levels for the avirulent strain after 24 hours. Figure 6.5 shows that after the addition of Vantocil at time=0 hours, after 24 hours the viable count of the biofilm associated *L. pneumophila* (Table 6.7.) was reduced by 96.6% and 97.2% for the serogroup 6 and avirulent *L. pneumophila* respectively.

Five days after addition of Vantocil IB, when the biocide concentration in the system would have been diluted to well below effective levels, no *L. pneumophila* could be detected in either the biofilm or the planktonic phases and could still not be detected after 144 hours. After 4 weeks a culture of the system which had contained the avirulent *L. pneumophila,* showed that the heterotrophic population had returned to pre-biocide levels, but *L. pneumophila* was not detected in either the planktonic or sessile phases of the system.

6.4.Discussion.

The MIC work was carried out using only planktonic microorganisms but the results obtained were in accord with those obtained in the chemostat. A most interesting aspect of this MIC work was the effect that the incubation time had on the results obtained. It was found that assessments made using plates incubated for relatively short periods of time could lead to an over-estimation of the efficacy of the biocide if it was assumed that lack of visible growth was due to a bactericidal effect. It has been shown that longer incubation times allowed the development of the slower growing pigmented bacteria which had a greater resistance to the biocide (Fig.6.2). The results demonstrate the distinction between bactericidal and bacteriostatic effects.

The results of the chemostat studies confirm the increased biocide resistance of sessile microorganisms noted by other workers (Walker *et al.*, 1994). The extent of kill recorded for planktonic microorganisms after the first hour was significantly greater than that recorded with the sessile microorganisms (Figs. 6.1-6.2). When the chemostats were dosed with a second addition of biocide it was found that regrowth of the microorganisms occurred significantly faster in the sessile phase than in the planktonic phase. Nevertheless, Vantocil IB was found to be effective in reducing the viable counts of both the sessile and planktonic microorganisms used in this study. More importantly, is the fact that the use of 200 ppm (40 ppm PHMB) of the biocide reduced the viable count of both the *Legionella pneumophila* serogroup 6 and the avirulent serogroup 1 to below detectable limits over the time period of the experiment.

In a previous study *L. pneumophila* has been shown to reappear within the system following initial suppression following biocide addition (Bornstein *et al.*, 1986). This suggests that either sub-lethal doses were being used or that the biocide used had a bacteriostatic rather than a bacteriocidal effect. To establish whether Vantocil IB was bacteriocidal in action a further sample was taken four weeks later. Although the

heterotrophic populations had recovered the avirulent *L. pneumophila* could not be detected in either the planktonic or the sessile phases of the system. These results are similar to those by Walker *et al.* (1994) working with a virulent serogroup 1 strain and using a bromine containing biocide. As in the present study, the heterotrophic population recovered following dilution of the biocide from the system. *L. pneumophila* remained below detectable levels in both the planktonic and sessile phases of the system.

The atomic force microscope images (Fig.6.3) at high resolution show a marked difference in the biofilm after the addition of the biocide . As there is no preparation needed prior to imaging using this method, it would be a useful tool to monitor not only the effect of the biocide on a biofilm population but to monitor also the effect on the surface once the biofilm had been removed. This would be particularly relevant on metal substrata where pitting corrosion is suspected.

In conclusion, the increased resistance of the sessile population confirms the importance of monitoring the biofilm population in biocide studies. In the natural environment the continued proliferation of a resistant heterotrophic population would be a cause for concern. Biofilms have been shown to both support the growth of *L. pneumophila* in the natural environment and to have a protective effect from the action of biocides (Colbourne and Dennis, 1988; Keevil *et al.*, 1989; Vess *et al.*, 1993). However, the results of this study show that Vantocil 1B is effective against both planktonic and biofilm associated *L. pneumophila*. This is in accord with the results of Barker *et al.* (1992) who found that Vantocil 1B was effective against both broth grown and amoebal grown *L. pneumophila*. Amoebae grown *Legionella*, like those in biofilms have been shown to exhibit increased resistance to antimicrobial agents (Barker *et al.*, 1992). These results suggest that Vantocil 1B would be effective in reducing the risk of Legionnaires' disease in industrial processes where there is a risk of aerosol production.

The similarity in the response of the avirulent strain shows that there is potential in the future use of this avirulent *L. pneumophila* in the evaluation of biocides in a model water system.

CHAPTER 7.

DISCUSSION.

Chapter 7. Discussion.

It is many years since *L. pneumophila* was first isolated. Since then a great deal of time, money and effort has been put into research aimed at preventing Legionnaires' disease and eradicating the causative organism from potentially infective sources.

As our understanding of the nature of the Legionnaires' disease bacterium has increased many of the earlier problems associated with the identification, culture and isolation of legionellae have been overcome (Barbaree, 1993; Dennis *et al.*, 1993; Harrison and Taylor, 1988; Feeley *et al.*, 1978; 1979). We are now aware that there are many more species of *Legionella*, other than *L. pneumophila,* some of which may also cause disease (Dennis *et al.*, 1993; Anon, 1991). However, as our knowledge increases we become aware of other factors which may have profound affects on the way we tackle the problems caused by the presence of this bacterium. The diverse range of habitats found to be colonised by these bacteria (Tison and Seidler, 1983; Verissimo *et al.*, 1991; Yee and Wadowsky, 1982; Anon, 1991), the association of *Legionella* with other non-related species and their resistance to biocide regimes are just some of these factors which make this a fascinating and and ongoing research area (Rowbotham, 1980; Anand *et al.*, 1983; Stout *et al.*, 1985; 1992b; Barbaree *et al.*, 1986; Kuchta *et al.*, 1985; 1993).

In Chapter 2 some of the questions, which remain unanswered about the growth and survival of *L. pneumophila*, are highlighted. Because of the practical difficulties involved in studying a water distribution system in its entirety there is a need for a model system which will mimic as closely as possible the 'natural' environment. Although model systems have their limitations in that they can never reproduce all the parameters that exist in the natural environment they are an important stage in the development of our understanding of the macroenvironment. Any ecological study, involving a diverse consortium of microorganisms including different species of bacteria, is limited by the ability of that population to grow on the available media. The

bacterial population isolated may not necessarily be totally representative of the actual population which co-existed either in the model system or in the natural environment. There are several reports of different bacterial species existing in a viable but non-culurable state (Xu *et al.*, 1982; Rollins and Colwell, 1986; Berry *et al.*, 1991; Weichart *et al.*, 1992). The system used in the present study was inoculated with a consortium of microorganisms which had not previously been cultured and therefore not selected or subjected to stresses which may cause phenotypic alterations, for example by passage over laboratory media. The system should therefore, contain those species which although non-culturable may play an important role in the survival of *Legionella* in the natural environment.

The microorganisms isolated from the model system used in this study closely resembled those isolated in the course of other similar studies involving aquatic systems (Mackerness *et al.*, 1991; Walker *et al.*, 1994). The continued survival of the bacterial population together with the continued presence of several protozoa suggest that the environment within the system remained within acceptable parameters.

The successful integration and survival of the avirulent *L. pneumophila* in both the biofilm and the planktonic phase of this model system posed some interesting questions regarding the association of the *L. pneumophila* with the other microorganisms within the system. This strain of *L. pneumophila* used in the present study was shown to remain avirulent in the authentic animal model following removal from the model system. The importance of virulence to the survival of *Legionella* in an aquatic system is linked with its ability to infect and subsequently proliferate with protozoa (Moffat *et al.*, 1992).

The investigations described within Chapter 3 were undertaken in order to establish whether the association with protozoa was essential for the survival of the *L. pneumophila* within the model system used in the present study. This particular

avirulent strain was not able to infect and grow within the amoebae from the chemostat or within an axenic strain of *Acanthamoeba polyphaga* which had previously been shown to be an acceptable host for the corresponding virulent strain of *L. pneumophila* (Rowbotham, 1980). The subsequent increase in counts of the non-legionellae and the avirulent *L. pneumophila* following addition of cycloheximide (Figs.3.4-3.5) suggested that this treatment had been successful in inhibiting the protozoal population. This difference in counts before and after cycloheximide treatment, can therefore, be attributed to bacterial mortality due to grazing by bacteriovorous protozoal species. This is a very important factor which does not appear to have been taken into account in other studies. Grazing by protozoa will alter the nature of a particular niche or microenvironment, opening up areas for recolonisation by bacterial species which may not necessarily be the same as the previous occupants. Furthermore, the recolonising population may be survivors following biocide treatment and may therefore exhibit increased resistance to further treatments. Both *L. pneumophila* and some cysts of protozoa have been shown to exhibit increased resistance when isolated from systems which have been subjected to chemical treatment (Kuchta *et al.*, 1985; 1993; Barker *et al.*, 1992). Protozoa may well preferentially select certain bacterial species upon which to graze, this would alter the ecology of the biofilm and have either a detrimental, or beneficial effect upon the neighbouring populations. This is obviously an area which needs further investigation.

Of more relevance to this study, *L. pneumophila* was shown not to be an obligate parasite for protozoal species, so that it is likely that its capability to infect and proliferate intracellularly, whether in human macrophages or protozoa, is opportunistic. It is generally accepted that *L. pneumophila* cannot proliferate in sterile water but is able to grow successfully in non-sterile aquatic systems (Skaliy and McEachern, 1979; Hsu *et al.*, 1984; States *et al.*, 1985; Yee and Wadowsky, 1982). Its ability to grow and survive in the absence of protozoa suggests therefore, that the other non-protozoal

population must play an important role in *L. pneumophila* persistance in aquatic systems.

Chapter 4 investigates the role of the non-legionellae population in *L. pneumophila* survival and examines the role of viability on the ability of these other bacteria to support the growth of *L. pneumophila.* These investigations suggest that the viability of the other microorganisms is important (Table 4.1.). Non-viable cells unlike their viable counterparts, did not support the survival of *L. pneumophila* on R2A medium. Other authors have found that cysteine production by *Flavobacterium breve* supported the growth of *L. pneumophila* on a cysteine deficient medium (Wadowsky and Yee, 1985). The present study also finds that not only a *Flavobacterium* sp. but also *Pseudomonas vesicularis* supported the growth of *L. pneumophila* on a similar cysteine deficient medium. However the ability to produce sufficient extracellular cysteine is not the complete answer to *L. pneumophila* survival on R2A medium. *L. pneumophila* did not form satellite colonies, on the medium minus cysteine, around other bacterial isolates from the biofilms, including other Pseudomonads which had supported the survival of *L. pneumophila* on R2A. The results of the heat recovery study described in Chapter 2, suggest that the bacterial loading of a system may be an important factor in *L. pneumophila* survival. This factor may have played a role in the survival of *L. pneumophila* on the R2A plates, the cross streaks representing a biofilm population. However, the importance of the viability of these bacteria on *L. pneumophila* survival suggests that the role of the non-legionellae may be multifactorial and may possibly include the 'mopping up' of substances which would otherwise inhibit *L. pneumophila* survival, a role played by the addition of charcoal to laboratory media for the successful culture of *L. pneumophila.* The precise role of the biofilm is therefore still unclear, whether the primary role of the biofilm is to provide nutrients or to protect the legionellae from adverse environmental factors. This therefore, is is an area which needs further investigation.

Visual examination of the subject in any ecological study is essential to understand the complex nature of the system being investigated. In chapter 5 a number of established techniques together with some more recently developed microscopic methods were used to visualise biofilms *in situ* on the surface which had supported their development. The biofilms visualised were not homogeneous in composition, comprising of stacks of biofilm microorganisms together with grazing protozoa. The stacks were interspersed with channels, some with a volcanic appearance (Fig. 5.10), possibly to allow the movement of nutrients, excretory and other products, to and from the biofilm consortia. By use of a combination of methods the misinterpretation of artefacts produced by some of the techniques was avoided. However, each of the methods used had its own particular advantage which contributed to an increased understanding of the complexity of the biofilm matrix and associated microorganisms. The capability of the atomic force microscope to image bacteria in a hydrated state, and the ability to manipulate an image allowing accurate measurement of bacteria in three dimensions, suggested that there are intriguing possibilities for the use of this microscope as a tool in further research.

In chapter 6 investigations were carried out to assess the suitability of the avirulent *L. pneumophila* used in the present study as a model substitute for virulent *L. pneumophila*. The results of this study suggest that the avirulent strain used in this study is suitable for use, in place of the virulent strain, in industrial test models. The response of *L. pneumophila* to biocides may be assessed by suitably trained personnel without the need for class III containment facilities.

In conclusion.

The avirulent strain of *L. pneumophila* used in this study successfully integrated into the model biofilm system. This avirulent strain did not revert to virulence, did not require protozoa for survival and growth within the system but did require the presence of other bacterial species. The presence of a biofilm together with *L. pneumophila* is therefore

particularly relevant in aquatic sources where there is the potential for aerosol formation. The response of this strain to a commercially available biocide suggests that this avirulent *L. pneumophila* is a suitable substitute for the virulent strain, and would reduce the risk of Legionnaires' disease during industrial testing procedures.

REFERENCES

Addiss, D. G., J. P. Davis, M. LaVenture, P. J. Wand, M. A. Hutchinson, and R .M. McKinney, (1989). Community-aquired Legionnaires' disease associated with a cooling tower: evidence for longer-distance transport of *Legionella pneumophila.* American Journal of Epidemiology, 130, 557-568.

Ager, B. P., and J.A. Tickner (Eds.), (1985). The control of microorganisms responsible for Legionnaires' disease and humidifier fever. Science Reviews Ltd. Occupational Hygiene Monograph no.14. pp 9-14

Alary, M., and J. R. Joly, (1991). Risk Factors for Contamination of Domestic Hot Water Systems By *Legionella.* Applied Environmental Microbiology. 57, 2360-2367.

Alary, M. and J. R. Joly, (1992), Comparison of culture methods and immunofluorescence assay for the detection of *L. pneumophila* in domestic hot water devices. Current Microbiology, 25, 19-23.

Allison, D. G, (1993), Biofilm-Associated exopolysaccharides, Microbiology Europe, Nov/Dec. 16-19

Allison, D. G., (1994), Exopolysaccharide synthesis in bacterial biofilms. In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 35-30.

Alvarez, A. J., E. A. Hernández-Delgado and G. A. Toranzos, (1993), Advantages and disadvantages of traditional and molecular techniques applied to the detection of pathogens in water, Water Science Technology. 27, 3/4, 253-256.

Anand, C.M., A. R. Skinner, A. Malic, (1983), Interaction of *Legionella pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*). Journal of Hygiene Cambridge, 91,167-178.

Anon, (1991a), The control of legionellosis including Legionnaires' disease. Health and Safety Series booklet HS(G)70, HMSO.

Anon, (1991b), Minimising the risk of Legionnaires' disease. CIBSE Technical Memoranda 13.

Anon (i), 1982. EC Directive relating to the quality of Water intended for human consumption (80/778/EEC). Department of the Environment Circular 20/82. London HMSO.

Anon (ii), (1982). Specification of requirements for the suitability of materials for use in contact with water for human consumption with regard to their effect on the quality of the water. Draft for Development 82. London: British Standards Institution.

Anon (iii), 1983. The Bacteriological Examination of Drinking Water Supplies. 1982. Reports on Public Health and Medical Subjects No. 71. London HMSO.

Applegate, B. and J. D. Bryers, (1991), Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processess, Biotechnology and Bioengineering, 37,17-25.

Arata, S., C. Newton, T. Klein and H. Friedman, (1992). Enhanced growth of *Legionella pneumophila* in tetrahydrocannabinol- treated macrophages. Proceedings of the Society for Experimental Biology and Medicine, 199, 1, 65-67.

Arnow, P. M., T. Chou, D. Weil, E. N. Shapiro and C. Kretzschmar, (1982). Nosocomial Legionnaires' disease caused by aerosolised tap water from respiratory devices. Journal of Infectious Diseases, 146, 460-467.

Ashworth, J. and J. S. Colbourne, (1987). The testing of non-metallic materials for use in contact with potable water, and the inter-relationships with in service use. Industrial Microbiological Testing, Technical series 23. Hopton, J. W., and E. C. Hill.(Eds). Blackwell Scientific Publications. Oxford, London, Edinburgh.

Ashworth, J. and J. S. Colbourne (1987). Microbial alterations of drinking water by building services materials-field observations and the United Kingdom water fittings testing scheme. Biodeterioration of Constructional Materials. Proc. Biodeterioration Soc. Summer Meeting. Delft. The Netherlands.

Azumi, R., M. Matsumoto, Y. Kawabata, T. Ichimura, T. Mizuno and H. Miyamoto, (1991). Atomic force microscopic study of vesicles of synthetic surfactant, vesicles of thylakoid membrane and whole cells of bacteria. Chemistry Letters, 1925-1928.

Bakke, R. and P. Q. Olsson, (1986), Biofilm thickness measurements by light microscopy. Journal of Microbiological Methods, 5, 93-98.

Barbaree, J. M., B. S. Fields, J. C. Feeley, G. W. Gorman and W. T. Martin, (1986). Isolation of protozoa from water associated with a Legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila.* Applied and Environmental Microbiology, 51, 2, 422-424.

Barbaree, J. M.,G. W. Gorman, W. T. Martin, B. S. Fields and W. E. Morrill, (1987). Protocol for sampling environmental sites for Legionellae. Applied and Environmental Microbiology, 53,7,1454-1458.

Barbaree, J. M., (1993), Selecting a subtyping technique for use in investigations of Legionellosis epidemics. *in Legionella*, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 169-172.

Barker, J., M. R. W. Brown, P. J. Collier and P. Gilbert, (1992). Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: Physiological status and susceptibility to chemical inactivation. Applied and Environmental Microbiology, 58, 8, 2420-2425.

Baskerville, A., M. Broster, R. B. Fitzgeorge, P. Hambleton and P. J. Dennis, (1981). Experimental transmission of Legionnaires' disease by exposure to aerosols of *Legionella pneumophila*. Lancet, ii, 1389-1390.

Baskerville, A., J. W. Conlan, L. A. E. Ashworth and A. B. Dowsett, (1986). Pulmonary damage by a protease from *Legionella pneumophila*. British J. Experimental Pathology, 67, 527-536.

Beaty, H. N., (1984), Clinical features of Legionellosis. *In*: Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C.

Beech, I. B., C. C. Gaylarde, J. J. Smith and G. G. Geesey, (1991), Extracellular polysaccharides from *Desufovibrio desufuricans* and *Pseudomonas fluorescens* in the presence of mild and stainless steel. Applied Microbiology Biotechnolology. 35, 65-71.

Beech, I. B. and C. C. Gaylarde, (1991), Microbial Polysaccharides and Corrosion, In International Biodeterioration, 27, 2, 95-107.

Bej, A. K., M. H. Mahbubani and R. M. Atlas, (1993), Detection and molecular serogrouping of *Legionella pneumophila* by poymerase chain reaction amplification and restriction enzyme analysis. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992pp. 173-174.

Belyi, Y. F., (1993), Intracellular parasitism of *Legionella* and signaling in eukaryotic cells. FASEB Journal, 7, 1010-1015.

Bentham, R. H., (1993) Environmental factors affecting the colonisation of cooling towers by *Legionella* spp. in South Australia, International Biodeterioration Biodegradation, 31, 55-63.

Bercouvier, H., B. Fattal and H. Shuval, (1986), Seasonal distribution of *Legionellae* isolated from various types of water in Israel. Israel Journal of Medical Sciences, 22, 9, 644-646.

Berg, J. D., J. C. Hoff, P. V. Roberts, and A. Matin, (1984), Growth of *Legionella pneumophila* in continuous culture and its sensitivity to inactivation by Chlorine dioxide, In Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C.

Berg, J. D., J. C. Hoff, P. V. Roberts, and A. Matin, (1985), Growth of *Legionella pneumophila* in continuous culture, Applied and Environmental Microbiology. 42. 5. 768-772.

Bergey's Manual of Determinative Microbiology, 9th Edition, (1994) J. Holt, (Ed.) ISBN, 0-683-00603-7

Berry, C., B. J. Lloyd and J. S. Colbourne, (1991), Effect of heat shock on recovery of *Escherichia coli* from drinking water. Water Science Technology, 24, 2, 85-88.

Bezanson, G. S., Burbridge, D. Haldane, and T. Marrie. (1992). In situ colonisation of polyvinyl chloride, brass, and copper by *Legionella pneumophila*. Canadian Journal Microbiology, 38, 328-330.

Binnig, G. and C. F. Quate, (1986), Atomic force microscope. Physical Review Letters, 56, 9, 930-933.

Biolog Reference Manual, (1989). Metabolic reactions of Gram-negative species, T. A. Marello aand B. R. Bochner (Eds.) Biolog, Inc. USA.

Blackford, B. L., M. H. Jericho and P. J. Mulhern. (1991). A review of scanning tunneling microscope and atomic force microscope imaging of large biological structures: problems and prospects. Scanning Microscopy, 5, 5, 1991.

Bohach, G. A., and I. S. Snyder. (1983). Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. Applied and Environmental Microbiology,46, 2, 528-531

Bollin, G. E., J. F. Plouffe, M. F. Para and R. B. Prior. (1985). Difference in virulence of environmental isolates of *Legionella pneumophila*. Journal of Clinical Microbiology, 21, 5, 674-677.

Bornstein, N., C. Vieilly, M. Nowicki and J. C. Paucod, (1986), Epidemiological evidence of legionellosis transmission through domestic hot water supply systems and possibilities of control. Israel Journal of Medical Sciences, 22, 655-661.

Bott, T. R. (1994), The control of biofilms in industrial cooling water systems, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 173-180.

Boyle, M., T. Ford, J. S. Maki and R. Mitchell, (1991). Biofilms and the survival of opportunistic pathogens in recycled water, Waste management and Research, 9, 465-470.

Breiman, R. F. and J. M. Barbaree, (1993). Ten years progress in *Legionella* research: summary of the fourth international symposium on *Legionella* . *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992. (301-302).

Brenner, D. J., J. C. Feeley and R. E. Weaver. (1984). Family VII *Legionellaceae*. In : Kreig, N. R. and Holt, J. G. (Eds), Bergey's manual of systematic bacteriology, vol. 1. Baltimore: Williams and Wilkins, pp. 279-288.

Brenner, D. J., (1986), Classification of *Legionellaceae,* current status and remaining questions. Israel Journal of Medical Sciences, 22, 620-632.

Broadbent, C. R., L. N. Marwood, R. H. Bentham.(1991). *Legionella* in cooling towers: Report of a field study in South Australia. p55-60

Brock, T. D. (1971).Microbial growth rates in nature. Bacteriological Reviews, 35, 39-58.

Brown, M. R. W., J. W. Costerton and P. Gilbert, (1991), Extrapolating to bacterial life outside the test-tube, Journal of Antimicrobial Chemotherapy, 27, 565-567.

Broxton, P., P. M. Woodcock and P. Gilbert, (1983). A study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. Journal Applied Bacteriology, 54, 345-353.

Broxton, P., P. M. Woodcock, F. Heatley and P. Gilbert. (1984), Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Esherichia coli.* Journal Applied Bacteriology, 57, 115-124.

Bryers, J. D. and W. G. Characklis.(1982). Processes governing primary biofilm formation. Biotechnology and Bioengineering. 24, 2451-2476

BSI (1988). Suitability of non metal products for use in contact with water intended for human consumption with regards to their effect on the quality of the water. British Standards Institute, BS6920 section 2.4 growth of aquatic microorganisms.

Burnham, N. A., R. J. Colton and H. M. Pollock, (1991). Interpretation issues in force microscopy. Journal of Vacuum Science and Technology, A9, 4, 2548-2553.

Butt, H. J., (1991a). Electrostatic interaction in atomic force microscopy. Biophysical Journal, 60, 777-785.

Butt, H. J., (1991b). Measuring electrostatic, van der Waals and hydration forces in electrolyte solutions with an Atomic Force Microscope, Biophysical Journal. 60, 1438-1444.

Byrd, J. J., H. Xu, and R. R. Colwell, (1991). Viable but non-culturable bacteria in drinking water. Applied and Environmental Microbiology, 56, 796-802.

Caldwell, D. E., D. R. Korber and J. R. Lawrence, (1993), Analysis of biofilm formation using 2D and 3D imaging, Microbial cell envelopes:interactions and biofilms. Soc. for Applied Bacteriology Symposium series no. 22, Quesnel, B., P. Gilbert And P. S. Handley. (Eds.) pp. 52S-66S.

Campbell, J., W. F. Bibb, M. A. Lambert, S. Eng, A. G. Steigerwalt, J. Allard, C. W. Moss and D. J. Brenner. (1984). *Legionella sainthelensi*: a new species of *Legionella* isolated from water near Mt. St. Helens. Applied and Environmental Microbiology, 47, 369-373.

Campbell, A. T., L. J. Robertson and H. V. Smith. (1992). Viability of *Cryptosporidium parvum* oocysts: correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. Applied and Environmental Microbiology, 58, 3488-3493.

Cassartelli, J. D. (1965), Microscopy for Students. McGraw-Hill, London.

Catrenich, C. E. and W. Johnson. (1988). Virulence conversion of *Legionella pneumophila*: a one-way conversion. Infection and Immunity, 56, 3121-3125.

Chandler, F. W., R. M. Cole, M. D. Hicklin, J. A. Blackmon and C. S. Callaway. (1979). Ultrastructure of the Legionnaires' disease bacterium. A study using transmission electron microscopy. Annals of Internal Medicine. 90, 642-647.

Characklis, W. G. (1981), Bioengineering report, Fouling biofilm development: A process analysis, Biotechnology and Bioengineering. 23, 1923-1960..

Cianciotto, N. P., B. I. Eisenstein, C. H. Mody and N. C. Engleberg, (1990). A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence.

Cianciotto, N. P. and B. S. Fields, (1992), *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. Proceedings National Academy of Science, USA. 89, 5188-5191.

Cloete, T. E., V. S. Brozel and J. Pressly. (1989).A bacterial population structure study of water cooling sysytems in South Africa. Water SA. 15, 37-42.

Cloete, T. E., F. Smith and P. L. Steyn. (1989b). The use of planktonic bacterial populations in open and closed recirculating water cooling systems for the evaluation of biocides. International Biodeterioration. 25, 115-122.

Colbourne, J. S., D. J. Pratt, M. G. Smith, S. P. Fischer-Hoch and D. Harper, (1984), Water fittings as sources of *Legionella pneumophila* in a hospital plumbing system. Lancet i, 210-213.

Colbourne, J.S. and Trew. R.M, (1986). Presence of *Legionella* in London's water supplies. Israel Journal of Medical Sciences. 22, 633-639.

Colbourne, J.S., P. J. Dennis, R. M. Trew, C. Berry and G. Vesey. (1988b). *Legionella* and public water supplies. Water Science Technology. 20, 11/12:5-10.

Colbourne, J.S., R.M Trew and P.J. Dennis, (1988). Treatment of water for aquatic bacterial growth studies. Journal of Applied Bacteriology, 65, 299-324.

Colbourne, J. S., and P. J. Dennis, (1988), *Legionella* : a biofilm organism in engineered water systems. International Biodeterioration 7, 36-42.

Collins, M.T., (1986). *Legionella* infections in animals. Israel Journal of Medical Sciences. 22, 662-673.

Costerton, J.W., G. G. Geesey and K. J. Cheng, (1978). How Bacteria Stick. Scientific American, 238, 86-95

Costerton, J.W. and G. G. Geesey, (1979). Which populations of aquatic bacteria should we enumerate. Native Aquatic Bacteria. Enumeration, Activity and Ecology,ASTM STP 695, J. W. Costerton and R. R. Colwell (Eds). American Society for testing and Materials. pp. 7-18.

Costerton, J.W., R. T. Irvin, K. J. Cheng. (1981). The bacterial glycocalyx in nature and disease. Annual Review of Microbiology. 35, 299-324

Costerton, J. W., (1984). The formation of biocide-resistant biofilms in industrial, natural and medical systems. In: Developments in Industrial Microbiology, Symposium: Biocide applications and functions. 25, 363-372

Costerton, J.W., T. J. Marrie and K. J. Cheng, (1985). Phenomena of bacterial adhesion. In Bacterial Adhesion,.D. C. Savage and M. Fletcher Eds. Plenum London pp.3-40

Costerton, J.W., J.G. Nickel and T.I. Ladd, (1986). Suitable methods for the comparative study of free living and surface associated bacterial populations. In Poindexter, J.S. and E.R. Leadbetter. (Eds) Bacteria in Nature Vol. 2. Plenum Press. New York.

Costerton, J.W., K. J. Cheng, G. G. Geesey, T.I. Ladd, J.G. Nickel, M. Dasgupta and T. J. Marrie (1987). Bacterial biofilms in nature and disease. Annual Review of Microbiology. 41, 435-464.

Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber and G. James. (1994). Biofilms, the customised microniche. Journal of Applied Bacteriology, 176, 2137-2142.

Barrow, G.I and R. K. A. Feltham, (Eds). (1993) Cowan and Steel's Manual for the Identification Of Medical Bacteria. Third Edition Cambridge University Press, London.

Cummins, D., M. C. Moss, C. L. Jones, C. V. Howard and P. G. Cummins, (1992). Confocal microscopy of dental plaque development. Binary, 4, 86-91.

Curd, F. H. S. and F. L. Rose, (1946). Synthetic antimalarials. Part X. Some aryldiguanide-("biguanide") derivatives. Journal of the Chemical Society, 2, 729-737.

Dahlbäck, B., M. Hermannsson, S. Kjelleberg and B. Norkrans. (1981). The hydrophobicity of bacteria- an important factor in their initial adhesion at the air-water interface. Arch. Microbiol. 128, 267-270.

Daisy, J. A., C. E. Benson, J. McKitrick and H. M. Friedman. (1981). Intracellular replication of *Legionella pneumophila*. Journal of Infectious Diseases, 143, 460-464

Dawson, M. P., B. A. Humphrey and K. C. Marshall, (1981), Adhesion: A tactic in the survival strategy of a marine vibrio during starvation, Current Microbiology, 6, 195-199.

Dennis, P. J., D. Green and B. P. C. Jones, (1984), A note on the temperature tolerance of *Legionella* , Journal Applied Bacteriology, 56, 349-350

Dennis, P.J. (1988). Isolation of *Legionellae* from environmental specimens, *In* T.G. Harrison and A.G. Taylor (ed.) A Laboratory Manual for *Legionella*. John Wiley & Sons Ltd. Chichester New York Brisbane Toronto and Singapore, p31-44.

Dennis, P. J., D. J. Brenner, W. L Thacker, R. Wait, G. Vesey, A. G. Steigerwalt and R. F. Benson. (1993a), Five new *Legionella* species isolated from water. International Journal of Systematic Bacteriology, 43, 2, 329-337.

Dennis, P. J. (1993), Potable Water Systems: Insights into control, *In*: Legionella: current status and emerging perspectives. Barbaree, J. M., Breiman, R. F. and Dufour A. P. (Eds). Proc. The 4th International Symposium on Legionella American Society for Microbiology, Washington, D.C.

Donowitz, G. R. and K. I.Earnhardt, (1993), Azithromycin inhibition of intracellular *Legionella micdadei*, Antimicrobial Agents Chemotherapy, 37, 11, 2261-2264.

Drozanski, W. (1963). Studies of Intracellular parasites of free-living amoebae. Acta Microbiologica Polonica. 12, 3-8

Eastwood, I. M. (1994), Problems associated with biocides and biofilms, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 169-172.

Edelstein, P. H. (1993), Laboratory diagnosis of Legionnaires' disease an update from 1984. *In*: Legionella: current status and emerging perspectives. Barbaree, J. M., Breiman, R. F. and Dufour A. P. (Eds). Proc. The 4th International Symposium on Legionella American Society for Microbiology, Washington, D.C.

Edelstein, P. H., C. Nakahama, J. O. Tobin, K. Calarco, K. B. Beer, J. R. Joly and R. K. Selander, (1986), Paleoepidemilogic investigation of Legionnaires' disease at Wadsworth veterans administration hospital by using three typing methods for comparison of Legionellae from clinical and environmental sources, Journal of Clinical Microbiology, 23, 6, 1121-1126.

Edelstein, P. H., (1981). Improved semi-selective media for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. Journal of Clinical Microbiology, 14, 298-303.

Edstrom, R. D., X. Yang, G. Lee and D. Fennel Evans. (1990). Viewing molecules with scanning tunneling microscopy and atomic force microscopy. FASEB Journal, 4, 3144-3151.

Ehret, W., G. Anding, I. Tartakovskii and G. Ruckdeschel, (1993), Molecular epidemiology of outbreak-associated serogroup 1 isolates of *Legionella pneumophila*, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp.186-189.

Elliott, J. A. and W. Johnson. (1982). Virulence conversion of *Legionella pneumophila* serogroup 1 by passage through guinea pigs and embryonated eggs. Infection and Immunity. 35, 943-946.

Ellis, D. M., (1990), Microorganisms: their control in cooling systems. The Plant Engineer, Jan/Feb. pp.35-36

Emde, K. M. E., H. Mao, and G. R. Finch, (1992), Detection and Occurrence of waterborne bacterial and viral pathogens. Water Environment Research, 64, 4, 641-647.

Engleberg, N. C. (1993). Genetic studies of *Legionella* pathogenesis. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, p63-68.

Escher, A. R. and W. G. Characklis. (1988). Microbial colonisation of a smooth substratum: A kinetic analysis using image analysis. Water Science Technology, 20, 277-283.

Evans, E., M. R. W. Brown and P. Gilbert, (1994), Iron chelator, exopolysaccharide and protease production in *Staphylococcus epidermidis*: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. Microbiology, 140, 153-157.

Facklam, R. R. and R. F. Breiman. (1991), Current trends in bacterial respiratory pathogens. American Journal of Medicine, 91, 6A, 3-11S q2

Fallon, R. J., Goldberg, D.J, Wrench, J. G., Green, S. T., and Emslie, J. A. N. (1993), Pontiac fever in children. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992. 50-51.

Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel and H. W. Smith. (1978). Primary isolation media for Legionnaires' disease bacterium. Journal of Clinical Microbiology, 8, 3, 320-325.

Feeley, J.C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine. (1979). Charcoal yeast extract agar: primary isolation medium for *Legionella pneumophila*. Journal of Clinical Microbiology, 10, 437-441.

Ferris, F. G., S. Schultze, T. C. Witten, W. S. Fyfe and T. J. Beveridge. (1987). Metal interactions with microbial biofilms in acidic and neutral pH environments. Applied and Environmental Microbiology, 55, 5, 1249-1257.

Fields, B. S., E. B. Shotts Jnr, J. C. Feeley, G. W. Gorman, and W. T. Martin. (1984). Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. Applied and Environmental Microbiology, 47, 3, 467-471.

Fields, B. S., E. B. Shotts Jnr, J. C. Feeley, G. W. Gorman, and W. T. Martin, (1984b). Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*.In Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C. p327-328

Fields, B. S., J. M. Barbaree, E. B. Shotts Jnr, J. C. Feeley, W. E. Morrill, G. N. Sanden and M. J. Dykstra. (1986). Comparison of Guinea pig and protozoan models for determining virulence of *Legionella* species. Infection and Immunity, 53, 3, 553-559.

Fields, B. S., G. N. Sanden, J. M. Barbaree,W. E. Morrill, R. M. Wadowsky, E. H. White and J. C. Feeley, (1989).Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. Current Microbiology. 18,131-137.

Fields, B. S. (1993). *Legionella* and protozoa: interaction of a pathogen and its natural host. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992,129-136.

Fields. B. S., S. R. Utley Fields, J. N. Chin Loy, E. H. White, W. L. Steffens and E. B. Shotts. (1993b). Attachment and entry of *Legionella pneumophila* in *Hartmanella vermiformis*. Journal of Infectious Diseases, 167, 1146-1150.

Finch, R. G., (1994), Medical problems associated with biofilms, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 88-92.

Finkelstein, R., P. Brown, W. A. Palutke, B. B. Wentworth, J. G. Geiger, G. D. Bostic and J. D. Sobel. (1993). Diagnostic efficiency of a DNA probe in pneumonia caused by *Legionella* species, Journal of Clinical Microbiology, 38, 183-186.

Fitzgeorge, R. B., A. S. R. Featherstone and A. Baskerville. (1988). Effects of polymorphonuclear leukocyte depletion on the pathogenesis of experimental Legionnaires' disease. British Journal of Experimental Pathology, 69, 105-112.

Fitzgeorge, R.B., A.Baskerville, M.G. Broster, P. Hambleton and P. J. Dennis, (1983). Aerosol Infection of animals with strains of *Legionella pneumophila* of different virulence: comparison with intraperitoneal and intranasal route of infection. Journal of Hygiene. Cambridge, 90, 81-90.

Fletcher, M. and G. I. Loeb, (1979), Influence of substratum characteristics on the attachment of a marine Pseudomonad to solid surfaces, Applied and Environmental Microbiology, 37, 1, 67-72.

Fletcher, M. and J. H. Pringle, (1983). The effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces, Journal of Colloid and Interface Science, 104, 1, 5-14.

Flicklinger, C. H., (1972), Ribosomal aggregates in amoebae exposed to the protein synthesis inhibito emetine. Experimental Cell Research, 74, 541-546.

Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker. (1979). Isolation of *Legionella pneumophila* from nonepidemic-related aquatic habitats. Applied and Environmental Microbiology, 37, 6, 1239-1242.

Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison and D. H. Pope. (1981). Ecological distribution of *Legionella pneumophila*. Applied and Environmental Microbiology, 41, 1, 9-16.

Fliermans, C. B., (1984), In Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C. pp.285-289

Fry. N. K., T. J. Rowbotham, N. A. Saunders and T. M. Embley. (1991). Direct amplification and sequencing of the 16S ribosomal DNA of an intracellular *Legionella* species recovered by amoebal enrichment from the sputum of a patient with pneumonia. FEMS Microbiology Letters. 83, 165-168.

Fumarola. D. and S. Pece. (1992). Multiple organ involvement by *Legionella pneumophila*: pathogenic mechanisms. European Journal of Epidemiology, 8, 1, 142-143.

Gaylarde, C. C. and Beech, I. B. (1989), Bacterial polysaccharides and corrosion. In Biocorrosion, Proceedings of joint meeting between the Biodeterioration Society and the French Microbial Corrosion Group, C. C. Gaylarde and L. H. G. Morton (Eds).p 85-98.

Gebran, S. J., C. Newton, Y. Yamamoto, R. Widen, T. W. Klein and H. Freidman. (1994). Macrophage permissiveness for *Legionella pneumophila* growth modulated by iron. Infection and Immunity. 62, 2, 564-568.

Geesey, G. G., R. Mutch, J. W. Costerton and R. B. Green. (1978). Sessile bacteria: An important component of the microbial population in small mountain streams.Limnology Oceanography 23, 6, 1214-1223.

Geesey, G. G., (1982), Microbial exopolymers: Ecological and economic considerations, ASM News, 48, 1, 9-14.

Geesey, G. G., L. Jang, J. G. Jolley, M. R. Hankins, T. Iwaoka, and P. R. Griffiths, (1988), Binding of metal ions by extracellular polymers of biofilm bacteria, Water Science Technology, 20, 11/12, 161-165..

Geesey, G. G., P. J. Bremer, W. R. Fischer, D. Wagner, C. W. Keevil, J. Walker, A. H. Chamberlain and P. Angell, (1994). Unususal types of pitting corrosion of copper tubes in potable water systems. In: Biofouling and corrosion in industrial water systems. Geesey, Lewandowski and Fleming Eds. Lewis Publishers, London. pp.243-263

Gilbert, P. and M. R. W. Brown, (1994), Experimental approaches to the physiological properties of biofilm cells. In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 49-52.

Goddard, D. t., W. Weaver, A. Steele and H. Harron, (1993). Imaging soft and delicate materials. Materials world, November, 616-617.

Gomez-Lus, P., B. S. Fields, R. f. Benson, W. T. Martin, S. P. O'Connor and C. M. Black, (1993), Comparison of arbitarily primed polymerase chain reaction, ribotyping, and monoclonal antibody analysis for subtyping *Legionella pneumophila* serogroup 1, Journal of Clinical Microbiology, 31, 7, 1940-1942.

Gomez-Lus, R., E. Lomba, M. S. Abarca, S. Gomez-Lus, A, Martinez, E, Duran and M. C. Rubio, (1993b), In vitro antagonistic activity of *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Aeromonas* spp. against *Legionella* spp. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp.265-267.

Grabow, N. A., R. Kfir and J. L. Slabbert. (1991). Microbiological methods for safety testing of drinking water directly reclaimed from wastewater. Water Science Technology. 24, 2, 1-4.

Grandi, G. and G. Galli, (1992), Metaloproteases and their role in biotechnology, Metal Ions In Biological Systems. 28, 415-453.

Grimes, D. J. (1991). Ecology of estuarine bacteria capable of causing human disease. Estuaries 14, 4,334-360

Grosserode, M., C. Helms, M. Pfaller, N. Moyer, N. Hall, and R. Wenzel, (1992), Continuous hyperchlorination for control of nosocomial Legionellosis: A ten year follow up to the effiency, environmental effects and costs. In Abstracts, American Society for Microbiology, 1992 International Symposium on Legionella, Orlando, Florida, p.10.

Hacker, J., M. Ott, E. Wintermeyer, B. Ludwig and G. Fischer. (1993). Analysis of virulence factors of *Legionella pneumophila*. Zbl. Bakt. Hyg, 278, 348-358.

Hacker, J., M. Ott, B. Ludwig and U. Rdest, (1991), Intracellular survival and expression of virulence determinants of *Legionella pneumophila*. Infection 19, Suppl. 4, S198-S201

Haggerty, L. and A. M. Lenhoff, (1993). STM and AFM in Biotechnology. Biotechnology Progress, 9, 1-11.

Hambleton, P., M. G. Broster, P. J. Dennis, R. Henstridge, R. Fitzgeorge and J. W. Conlan. (1983). Survival of *Legionella pneumophila* in aerosols. Journal of Hygiene, Cambridge, 90, 451-460.

Hamilton, W. A. and D. J. Stickler. (1994), In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. 41-45.

Hanaki, K., S. Kawasaki and J. Matsumoto, (1982), Characteristics of the degradation of organic matter by attached biofilms in rivers. Technology Reports, 47, 2, 225-235.

Hanaki. K. S., S. Kawasaki and J. Matsumoto, (1982). Characteristics of the degradation of organic matter by attached biofilm in rivers. Technology Reports, Tohoku Univ. 47, 2, 225-235.

Hansma, P. K., V. B. Elings, O. Marti and C. E. Bracker. (1988), Scanning tunneling microscopy and atomic force microscopy: Application to Biology And Technology. Science, 242, 209-216.

Harf, C. and H. Monteil. (1988). Interactions between free-living amoebae and *Legionella* in the environment. Water Science Technology, 20, 235-239

Harrison, T. G., G. Taylor, (1988), *In:* A laboratory Manual for *Legionella*, T. G. Harrison and A. G. Taylor (Eds). John Wiley and Sons Ltd.

Haudidier, K., J. L. Paquin, T. Francais, P. Hartemann, G. Grapin, F. Colin, M. J. Jourdain, J. C. Block, J. Cheron, O. Pascal, Y. Levi and J. Miazga. (1988). Biofilm growth in a preliminary water network: a preliminary industrial pilot plant experiment. Water Science Technology, 20, 109-115.

Hedlund, K. W., (1981). *Legionella* Toxin. Pharmacolology Therapeutics 15, 123-130.

Henke, M. and K. M. Seidel. (1986). Association between *Legionella pneumophila* and amoebae in water. Israel Journal of Medical Sciences. 22, 690-695.

Herbert, D., R. Elsworth and R. C. Telling, (1956). The continuous culture of bacteria; a theoretical and experimental study. Journal of General Microbiology, 14, 601-622

Herson, D. S., D. R. Marshall K. H. Baker and H. T. Victoreen. (1991). Association of microorganisms with surfaces in distribution systems. Research and Technology. J. AWWA, 103-106.

High, A. S., S. D. Torosian and F. G. Rodgers, (1993), Cloning, nucleotide sequence and expression in *Escherichia coli* of a gene (omp M) encoding a 25kDa major outer-membrane protein (MOMP) of *Legionella pneumophila.* Journal of General Microbiology, 139, 1715-1721

Hoffman, P., (1984), State of the Art Lecture, Bacterial physiology, *In:* Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C. p 61-67.

Hoffman, R., (1988). Applications of the Modulation contrast microscope. International Laboratory, 32-39.

Hoffman, P. S., L. Houston and C. A. Butler, (1990). *Legionella pneumophila htpAB* heat shock operon: nucleotide sequence and expression of the 60-kilodalton antigen in *L. pneumophila* -infected HeLa cells. Infection and Immunity. 58, 3380-3387..

Hoffman, P. S., M. Ripley, R. Weeratna, G. Fernandez, G. Faulkner, D. Hoskin, T. J. Marrie, S. M. Logan and M. A. Trevors. (1993). *Legionella pneumophila* surface proteins: gene regulation and cellular immunity. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 74-78

Hoge, C. W. and R. F. Breiman. (1991). Advances in the epidemiology and control of *Legionella* infections. Epidemiologic Reviews. 13, 329-340

Holah, J. T., S. F. Bloomfield, A. J. Walker and H. Spenceley, (1994), Control of biofilms in the food industry, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 163-168.

Horan, S. J. and J. W. Kappler, (1977). Automated fluorescence analysis for cytotoxicity assays, Journal of Immunology Methods. 18, 309-316.

Horwitz, M. A., (1983). The Legionnaires' disease bacterium (*Legionella pneumophila)* inhibits phagosome-lysosome fusion in human monocytes. J. Experimental Medicine, 158, 2108-2126.

Horwitz, M. A., (1993). Toward an understanding of host and bacterial molecules mediating *Legionella pneumophila* pathogenesis. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 55-62.

Hsu, S.C., Martin,R. and Wentworth,B.B.9, (1984). Isolation of *Legionella* species from drinking water. Applied and Environmental Microbiology, 48, 830-832.

Hume, R. D., and W. D. Hann, (1984), Growth relationships of *Legionella pneumophila* with green algae (Chlorophyta), In *Legionella, Proceedings of the Second International Symposium, American Society for Microbiology*, Eds. C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, Washington D. C. p 323-324.

Hutchinson, M. and Ridgway. (1977), Microbiological aspects of drinking water supplies. In Microbiology of Drinking water, 179-218.

ICI Biocides, 1991. Vantocil 1B, 37-8E, Morpeth, F. (ed.).

Jaeggi, N. E. and W. Schmidt-Lorenz.(1988). Bacterial regrowth in drinking water. Zbl. Bakt. Hyg. 186, 494-503.

Jantzen, E., A. Sonesson, T. Tangen and J. Eng, (1993), Hydroxy-fatty acid profiles of *Legionella* species: diagnostic usefulness assessed by principal component analysis. Journal of Clinical Microbiology, 31, 6, 1413-1419.

Jass, J. and H. Lapin-Scott, (1994), Sensitivity testing for antimicrobial agents against biofilms. In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 73-76.

Jauhac. B., C. Hare, M. Nowicki and H. Monteil. (1993). Detection of *Legionella* spp. in environmental water samples and free-living amoebae by using DNA amplification. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 151-153.

Jepras, R. I. , R. B. Fitzgeorge and A. Baskerville. (1985). A comparison of virulence of two strains of *L. pneumophila* based on experimental aerosol infection of guinea pigs. Journal of Hygiene, Cambridge, 95, 29-38.

Jepras, R. I. and R. B. Fitzgeorge, (1986). The effect of oxygen dependent antimicrobial systems on strains of *L. pneumophila* of different virulence. Journal of Hygiene, Cambridge, 97, 61-69.

Johnson, W., L. Varner, M. Poch, (1991), Aquisition of iron by *Legionella pneumophila*: Role of iron reductase. Infection and Immunity, 59, 2376-2381.

Joly, J. R., R. M. McKinney, J. O. Tobin, W. F. Bibb, I. D. Watkins and D. Ramsay, (1986), Development of a standardised subgrouping scheme for *Legionella pneumophila* serogroup 1 using monoclonal antibodies, Journal of Clinical Microbiology, 23, 768-771.

Joly, J. R., (1993), State of the Art Lecture, Monitoring for the presence of *Legionella*: where, when, and how?, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp. 211-222.

Joly, J. R. and M. Alary, (1993), Occurence of Nosocomial Legionnaires' disease in hospitals with contaminated potable water supply, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, p.39

Jones, H.C., I. L. Roth and W. M. Sanders III, (1989). Electron microscopic study of a slime layer. Journal of Bacteriology, 99, 316-325.

Kasas, S., V. Gotzos and M. R. Celio, (1993). Observation of living cells using the atomic force microscope. Biophysical Journal, 64, 539-544.

Keevil, C. W., D. J. Bradshaw, A. B. Dowsett, and T. W. Feary. (1987), Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture, Journal Applied Bacteriology, 62, 129-138.

Keevil, C. W., D. A. Glenister, K. E. Salamon, P. J. Dennis and A. A. West. (1988), A continuous culture biofilm model for the study of medical and industrial corrosion, In Proceedings of the Biodetioration Society, 4: Biofilms, 48-62

Keevil, C. W., J.T. Walker, J. McEvoy and J. S. Colbourne. (1989a). Detection of biofilms associated with pitting corrosion of copper pipework in Scottish hospitals. Proceedings of the Biodetioration Society, Corrosion. 5: pp. 99-117

Keevil, C. W., A. A. West, J.T. Walker, J. V. Lee, P. J. L. Dennis and J. S. Colbourne, (1989b), Biofilms: Detection, Implications and Solutions, In Watershed 89, Proc. IAWPRC. Conference, Guildford, UK. D. Wheeler, M. L. Richardson, and J. Bridges, (Eds), Pergamon Press, Oxford. pp 367-374.

Keevil, C. W., C. W. Mackerness, and J. S. Colbourne, (1990), Biocide treatment of biofilms, International Biodeterioration, 26, 169-179.

Keevil, C. W., (1991), *Legionella* biofilms. Proc. 6th Meeting European Working Group Legionella Infections.

Keevil, C. W. and J. Walker, (1992). Nomarski DIC Microscopy and image analysis of biofilms. Binary, 4, 93-95.

Keevil, C. W. (1994), Methods for assessing the activity of biofilm microorganismsin situ. In Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp.45-48.

Keevil, C. W., A. B. Dowsett and J. Rogers, (1994), *Legionella* Biofilms and their control. In. Microbial Biofilms: Formation and Control. Soc. Applied Bacteriology.

Kepkay, P. E., P. Schwinghamer, T. Willar and A. J. Bowen. (1986). Metabolism and metal binding by surface colonising bacteria: results of microgradient measurements. Applied and Environmental Microbiology, 51,1,163-170.

Kessler, H. H., F. F. Reinthaler, A. Pschaid, K. Pierer, B. Kleinhappl, E. Eber and E. Marth, (1993) rapid detection of *Legionella* species in bronchoalveolar lavage fliuds with the enviroamp *Legionella* PCR amplification and detection kit. Journal of Clinical Microbiology, 31, 3325-3328.

Kfir, R. and B. Genthe, (1993), Advantages and disadvantages of the use of immunodetection techniques for the enumeration of microorganisms and toxins in water, Water Science Technology, 27, 243-252.

King, C. H., E. B. Shotts, R. E. Wooley and K. G. Porter, (1988). Survival of coliforms and bacterial pathogens within protozoa during chlorination. Applied and Environmental Microbiology 54, 3023-3033.

King, C. H., B. S. Fields, E. B. Shotts, and E. H. White, (1991). Effects of cytochalasin D and Methylamine on intracellular growth of *Legionella pneumophila* in amoebae and human monocyte like cells. Infection and Immunity. 59, 758-763..

Kjelleberg, S., B. A. Humphrey and K. C. Marshall, (1983), Initial phases of starvation and activity of bacteria at surfaces, Applied and Environmental Microbiology, 46, 978-984.

Koch, A. L. (1958). Death of bacteria in growing cultures. Journal of Bacteriology, 77, 623-629.

Kohavi, D., Z. Schwartz, D. Amir, C. Muller Mai, U. Gross and J. Sela. (1992). Effect of titanium implants on primary mineralization following 6 and 14 days of rat tibial healing. Biomaterials. 13, 4, 255-260

Konhauser, K. O., S. Schulze-Lam, F. G. Ferris, W. S. Fyfe,F. J. Longstaffe and T, J, Beveridge, (1994), Mineral precipitation by epilithic biofilms in the Speed River Ontario, Canada. Applied and Environmental Microbiology, 60,2, 549-553.

Kuchta, J. M., S. J.States, J. E. McGlaughlin, J. H. Overmeyer, R. M. Wadowsky, A. M. McNamara, R. S. Wolford and R. B. Yee. (1985),.Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium passaged strains. Applied and Environmental Microbiology, 50:1:21-26.

Kuchta, J. M., J. S. Navratil, M. E. Sheperd, R. M. Wadowsky, J. N. Dowling, S. J. States and R. B. Yee, (1993), Impact of chlorine and heat on the survival of *Hartmanella vermiformis* and subsequent growth of *Legionella pneumophila*, Applied and Environmental Microbiology, 59, 12, 4096-4100.

Kwaik, Y. A., B. I. Eisenstein and N. C. Engleberg. (1993). Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infection and Immunity, 61, 4, 1320-1329.

Lake, A. (1988), A reactor for the continuous culture of fouling biofilms. In Biofilms, Proceedings of the Spring Meeting of the Biodeterioration Society. L. H. G. Morton (Ed.) 133-138.

LeChevallier, M.W. and McFeters, G. A. (1985), Interactions between heterotrophic plate count bacteria and coliform organisms. Applied and Environmental Microbiology, 49, 1338-1341.

Le Chevallier, M.W., T. M. Babcock and R. G. Lee. (1987). Examination and characterisation of distribution system biofilms.Applied and Environmental Microbiology, 53, 12, 2714-2724.

Le Chevallier, M.W., C.D.Cawthon, and R.G. Lee, (1988a). Inactivation of biofilm bacteria. Applied and Environmental Microbiology,54, 10, 2492-2499.

Le Chevallier, M.W., C.D.Cawthon, and Lee, R.G. (1988b). Mechanisms of bacterial survival in chlorinated drinking water.Wat. Sci. Tech. 20, 11/12, 145-151

Le Chevallier, M.W., C.D.Cawthon, and Lee, R.G. (1988c). Factors Promoting Survival of Bacteria in Chlorinated Water Supplies. Applied.and Environmental.Microbiology. 54 , 2492-2499.

Le Chevallier, M.W., W. Schulz, and R.G. Lee. (1991). Bacterial nutrients in drinking water. Applied and Environmental Microbiology, 57, 857-862.

Lee, S., J. T. O'Connor and S. Banerji, (1980), Biologically mediated corrosion and its effects on water quality and distribution, Research and Technology, J. AWWA, 636-645.

Lever, M. S. (1993). Production and characterisation of *Legionella pneumophila* specific monoclonal antibody. FEMS Microbiology Letters, 107, 5-10.

Little, B., Wagner P., Ray R., Pope R. and Scheetz R. (1991) Biofilms: An ESEM evaluation of artefacts introduced during SEM preparation. J. Industrial Microbiology, 8, 213-222.

Liu, W. K., D. E. Healing, J. T. Yeomans and T. S. J. Elliott, (1993), Monitoring of hot water supplies, Journal of Hospital Infection, 24, 1-9.

Lochner, J. E., R. H. Bigley and B. H. Iglewski. (1985). Defective triggering of polymorphonuclear leukocyte oxidative metabolism by *Legionella pneumophila* toxin. Journal of Infectious Diseases, 151, 42-46

Louitt, J. S. and L. S. Tomkins, (1993), Evaluation of a DNA aplification procedure for detection of *Legionella pneumophila* and *Legionella dumoffii* in water. *In*: Legionella: current status and emerging perspectives. Barbaree, J. M., Breiman, R. F. and Dufour A. P. (Eds). Proc. The 4th International Symposium on Legionella American Society for Microbiology, Washington, D.C. pp.176-178

Lück, P. C., L. Bender, M. Ott, J. H. Helbig and J. Hacker. (1991). Analysis of *Legionella pneumophila* serogroup 6 strains isolated from a hospital warm water supply over a three-year period by using genomic long-range mapping techniques and monoclonal antibodies. Applied and Environmental Microbiology, 57, 3226-3231.

Luft, J. H., (1965), Ruthenium red and violet. 1. Chemistry, purification, methods of use for electron microscopy and mechanism of action. The Anatomical Record, 171, 347-368.

Lytle, M. S., J. C. Adams, D. G. Dickman and W. R. Bressler, (1989). Use of nutrient response techniques to assess the effectiveness of chlorination of rapid sand filter gravel. Applied and Environmental Microbiology, 55, 29-32.

Mackerness, C. W. J. S. Colborne and C. W. Keevil. (1991). Growth of Aeromonas hydrophila and Escherichia coli in a distribution system biofilm model. Proc. of the UK. Symposium on Health-Related Microbiology. University of Strathclyde. 3-5th September. In IAWPRC Edited by Morris, R., L. M. Alexander, P. Wyn-Jones and J. Sellwood.

Maiwald, M., K. Kissel, S. Srimuang, M. Vob Knebel Doeberlitz and H. G. Sonntag, (1994) Comparison of polymerase chain reaction and conventional culture for the detection of *Legionella* in hospital water samples. Journal Applied Bacteriology, 76, 216-225.

Mamolen, M., R. F. Breiman, J. M. Barbaree, R. A. Gunn, K. M. Stone, J. S. Spika, D. T. Dennis, S. H. Mao and R. L. Vogt. (1993). Use of multiple molecular subtyping techniques to investigate a Legionnaires' disease outbreak due to identical strains at two tourist lodges. Journal of Clinical Microbiology, 31, 10, 2584-2588.

Marra, A. and H. A. Shuman. (1992). Genetics of *Legionella pneumophila* virulence. Annu. Rev. Genet. 26, 51-69.

Marrão, G., A. Veríssimo, R. G. bowker and M. S. da Costa, (1993), Biofilms as major sources of *Legionella* spp. in hydrothermal areas and their dispersion into stream water, FEMS Microbiology Ecology, 12, 25-33.

Marrie, T. J., G. Bezanson, D. J. M. Haldane and S. Burbridge, (1992a), Colonisation of the respiratory tract with *Legionella pneumophila* for 63 days before the onset of pneumonia, Journal of Infection, 24, 81-86.

Marrie, T. J., D. J. M. Haldane, G. Bezanson, and R. Peppard, (1992), Each water outlet is a unique ecological niche for *L. pneumophila*, Epidemiol. Infect. 108, 261-270.

Marsh, P. D., D. J. Bradshaw, G. K. Watson and D. Cummins, (1994). Factors affecting the development and composition of defined mixed culture biofilms of oral bacteria, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 13-18.

Mayaud, C. and E. Dournon, (1988), In A laboratory Manual for Legionella, T. G. Harrison and A. G. Taylor (Eds).John Wiley and Sons Ltd. p5-12

McCoy, W. F., J. D. Bryers, J. Robbins and J. W. Costerton, (1981), Observations of fouling biofilm formation, Canadian J. Microbiology. 27, 910-917.

McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle and the Laboratory Investigation Team, (1977). Legionnaires' disease: isolation of a bacterium and demonstration of its role in respiratory disease. New England Journal of Medicine, 297, 1197-1203.

McDade, J.E., C.C. Shepard, (1979). Virulent to avirulent conversion of Legionnaires' disease bacterium (*Legionella pneumophila*)-its effect on isolation techniques. J. Inf. Dis. 139, 707-711.

McDade, J. E., D. J. Brenner and F. M. Bozeman. (1979), Legionnaires' disease bacterium isolated in 1947. Annals of Internal Medicine, 90, 659-661.

Mengaud, J. M. and M. A. Horwitz, (1993), The major iron-containing protein of *L. pneumophila* is an aconitase homologous with the human iron-containing responsive element-binding protein, Journal Applied Bacteriology, 175, 17, 5666-5676.

Miller, L. A., J. L. Beebe, J. C. Butler, W. Martin, R. Benson, R. E. Hoffman, B. S. Fields, (1993) Use of a polymerase chain reaction in an epidemiologic investigation of Pontiac Fever. Journal of Infectious Diseases, 168, 769-772.

Mintz, C. S., B. S. Fields and C. Zou. (1992). Isolation and characterisation of a conjugative plasmid from *Legionella pneumophila*. J. Gen. Microbiol. 138, 1379-1386.

Mintz, C.S., R. D. Miller, N. S. Gutgsell and T. Malek. (1993). *Legionella pneumophila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. Infection and Immunity. 61, 8, 3416-3421.

Mody, C. H., R. Paine, M. S. Shahrabadi, R. H. Simon, E. Pearlman, B. I. Eisenstein and G. B. Toews. (1993). *Legionella pneumophila* replicates within rat alveolar epithelial cells. Journal of Infectious Diseases, 167, 1138-1145.

Moffat, J. F. and L. S. Tomkins. (1992). A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoebae castellanii.* Infection and Immunity. 60, 296-301.

Moffat, J. F., W. J. Black and L. S. Tompkins. (1994). Further characterisation of the cloned *Legionella pneumophila* zinc metalloprotease. Infection and Immunity. 62, 2, 751-753.

Montoya, M. L., M. S. Leal, L. Espinosa, M. J. C. Mundo, I. C. M. y M. Foz Sala, (1992), Utilidad de los marcadores epidemiologicos moleculares en el estudio de un brote epidmico de enfermedad del legionario de origen nosocomial, Med. Clin. (Barc), 99, 761-765.

Morris, G. K., C. M. Patton, J. C. Feeley, S. E. Johnson, G. Gorman, W. T. Martin, P. Skaliy, G. F. Mallison, B. D. Politi and D. C. Mackel. (1979). Isolation of the Legionnaires' disease bacterium from environmental samples. Annals of Internal Medicine, 90, 664-666.

Nagington, J., and D. J. Smith, (1980), Pontiac fever and amoebae, Lancet, ii, 1241.

Nahapetian, K., O. Challemel, D. Beurtin, S. Dubrou, P. Gounon and F. Squinazi. (1991).The intracellular multiplication of *Legionella pneumophila* in protozoa from hospital plumbing systems. Institut Pasteur. Res. Microbiol. 142, 677-685

Nahapetian, K., O. Challemel, S. Dubrou, C. Tram and F. Squinazi. (1993). Relationships between different serogroups of Legionellae within the ciliated protozoan *Tetrahymena pyriformis*. in Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 156-159.

Nichols, W., (1994), Biofilm permeability to antibacterial agents, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 141-150.

Nowicki, M., N, Bornstien, B, Jaulhac, Y, Piemont, H, Monteil, and J. Fleurette, (1993), Rapid detection of *Legionella* in clinical and environmental samples by polymerase chain reaction, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp. 178-181.

Oga, T., S. Suthersan and J. J. Ganczarczyk, (1991), Some properties of aerobic biofilms, Environmental Technology, 12, 431-440.

Oleinick, N. L., (1977), Initiation and elongation of protein synthesis in growing cells: differential inhibition by cycloheximide and emetine, Arch. Bioch. Biophys. 182, 171-180.

Ott, M., P. Messner, J. Heesemann, R. Marre and J. Hacker. (1991). Temperature-dependent expression of flagella in *Legionella*. Journal of General Microbiology, 137, 1955-1961.

Ott, M., M. Steinert, L. Bender, B. Ludwig, P. C. Lück and J. Hacker. (1993). temperature dependent replication of virulent and avirulent isolates of *Acanthamoebae castellanii*. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 149-151.

Page, F.C.(1976), An illustrated key to freshwater and soil amoebae, Ambleside. Freshwater Biological Association,

Page, F.C. (1988).A new key to Freshwater and Soil Gymnamoebae, Culure Collection of Algae and Protozoa, Ambleside, Freshwater Biological Association, ISBN 1 871105 02 1

Palmer, C. J., Y. Tsai, C. Paszko-Kolva, C. Mayer, and L. R. Sangermano, (1993), Detection of *Legionella* species in sewage and ocean-water by polymerase chain reaction, direct fluorescent antibody and plate culture methods, Applied and Environmental Microbiology 59, 11, 3618-3624.

Panikov, N. S., A. E. Merkurov and I S. Tartakovski. (1993). Kinetic studies of *Legionella* interactions with protozoa. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992,153-156.

Pasculle, A., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling, (1980). Pittsburgh pneumonia agent: direct isolation from human lung tissue. Journal of Infectious Diseases, 141,727-732

Pastoris, M. C., G. Nigro, M. F. Mazzotti, and M. Midulla, (1984), In Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C. p14-15.

Paszko-Kolva, C., M. Shahamat and R. R. Colwell, (1992), Long term survival of *L. pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiology Ecology, 102, 45-55.

Paszko-Kolva, C., M. Shahamat, J. Keiser And R. R. Colwell, (1993), Prevalence of Antibodies against *Legionella* species in healthy and patient populations. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp. 24-26.

Pate, J. L. and E. J. Ordal, (1967). The fine structure of *Chondrococcus columnaris*. Journal of Cell Biology, 35, 37-51.

Pedersen, K. (1990). Biofilm development on stainless steel and PVC surfaces in drinking water. Wat. Res. 24, 2, 239-243.

Pickup, R. W., (1991) Development of molecular methods for the detection of specific bacteria in the environment, J. Gen. Microbiol. 137, 1009-1019.

Picologou, B. F., N. Zelver and W. G. Characklis, (1980), Biofilm growth and hydraulic performance, J. Of The Hydraulics Division, 106, 733-746.

Pine, L., J. R. George, M. W. Reeves and W. K. Harrell, (1979), Development of a chemically defined medium for growth of *Legionella pneumophila*. Journal of Clinical Microbiology, 9, 615-626.

Pine, L., P. S. Hoffman, G. B. Malcolm, R. F. Benson and M. J. Franzus. (1986). Role of keto-acids and reduced-oxygen-scavenging enzymes in the growth of *Legionella* species. Journal of Clinical Microbiology, 32, 1, 33-42.

Pine, L., P. S. Hoffman, G. B. Malcolm, R. F. Benson and M. J. Franzus, (1993), Role of Keto-acids and reduced oxygen scavenging enzymes in the growth of *Legionella* species, Journal of Clinical Microbiology, 23, 1, 33-42.

Plouffe, J. F., L. R. Webster and B. Hackman, (1983), Relationship between colonisation of hospital buildings with *Legionella pneumophila* and hot water temperatures. Applied and Environmental Microbiology, 56, 3, 769-770.

Plouffe, J. E., (1993), Evolution of chemotherapy and diagnostic tests, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992.

Pope, D. H., R. J. Sorracco, H. K. Gill and C. B. Fliermans, (1982), Growth of *Legionella pneumophila* in two membered cultures with green algae and cyanobacteria, Curr. Microbiol., 7, 319-322

Power, K., B. Cooper, H. Watts and L. A. Nagy. (1988). Aftergrowths in drinking water systems: an Australian perspective. Wat. Sci. Tech. 20, 11/12, 437-439.

Ramirez, J. A., J. T. Summersgill, R. D. Miller, T. L. Meyers and M. J. Raff, (1993), Comparitive study of the bactericidal activity of ampicillin/sublactam,and erythromycin against intracellular *L. pneumophila*, Journal of Antimicrobial Chemotherapy, 32, 93-99.

Ramsey, M. K. and G. H. Roberts, (1992), *Legionella pneumophila*: the organism and its implications, Laboratory Medicine, 23,4,244-247.

Raymond, J., T. Picone and M. A. Zoccoli, (1992). the polymerase chain reaction: the use of chemistry for the analysis of *L. pneumophila* in water samples. Abstracts of papers of the american Chemical Soc. 203, 71.

Reasoner, D. J., and E. E. Geldreich, (1985). A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology, 49,1,1-7.

Reasoner, D. J. (1988). Drinking water microbiology research in the United States: an overview of the past decade. Water Science Technology, 20, 101-107.

Rechnitzer, C., A. Williams, J. B. Wright, A. B. Dowsett, N. Milman and R. B. Fitzgeorge. (1992). Demonstration of the intracellular production of tissue destructive protease by *Legionella pneumophila* multiplying within guinea pig and human alveolar macxrophages. Journal of General Microbiology, 138, 1671-1677.

Rechnitzer, C. and A. Kharazmi, (1992). Effect of *Legionella pneumophila* cytotoxic protease on human neutrophil and monocyte function. Microbial Pathogenesis, 12, 115-125.

Rechnitzer, C., J. M. Bangsborg and G. H. Shand, (1993), Effect of *Legionella pneumophila* sonicate on killing of *Listeria monocytogenes* by polymorphonuclear neutrophils and monocytes, APMIS, 101, 249-256.

Reeves, M. W., L. Pine, S. H. Hutner, J. R. George and W. K. Harrel, (1981), Metal requirements of *Legionella pneumophila,* Journal of Clinical Microbiology, 13, 4, 688-695.

Reinthaler, F. F., J. Sattler, K. Schaffler-Dullnig, B. Weinmayr and E. Marth, (1993), Comparitive study of procedures for isolation and cultivation of *Legionella pneumophila* from tap water in hospitals, Journal of Clinical Microbiology, 31, 5, 1213-1216.

Ridgway, H. F. and B. H. Olson, (1982). Scanning electron microscope evidence for bacterial colonisation of a drinking-water distribution system. Applied and Environmental Microbiology, 41, 274-287.

Robinson, R. W., D. E. Akin, R. A. Nordstedt, M. V. Thomas and H. C. Aldrich. (1984). Light and electron microscopic examinations of methane-producing biofilms from anaerobic fixed-bed reactors. Applied and Environmental Microbiology, 48, 1, 127-136.

Rodgers, F. G. (1979), Ultrastructure of *Legionella pneumophila*, Journal of Clinical Pathology, 32,1195-1202.

Rodgers, F. G., P. W. Greaves, A. D. Macrae and M. J. Lewis, (1980), Electron microscopic evidence of flagella and pili on *Legionella pneumophila*, Journal of Clinical Pathology, 33, 1184-1188.

Rodgers, F. G. and F. C. Gibson. (1993). Opsonin-independent adherence and intracellular development of *Legionella pneumophila* within U-937 cells. Canadian J. Microbiology, 39, 718-722.

Rodriguez, G. G., D. Phipps, K. ishiguro and H. f. Ridgway. (1992). Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. Applied and Environmental Microbiology, 58, 6, 1801-1808.

Rogers, J., A. B. Dowsett, J. V. Lee and C. W. Keevil. (1990) Chemostat studies of biofilm development on plumbing materials and the incorporation of *Legionella pneumophila*. Biodeterioration and Biodegradation 8: proceedings of the 8th International Biodeterioration and Biodegradation Symposium, 1990 :Windsor Ontario / edited by H.W. Rossmore.

Rogers, J., J. V. Lee, P. J. Dennis and C. W. Keevil, (1991), Continuous culture biofilm model for the survival and growth of *Legionella pneumophila* and associated protozoa in potable water systems. Proc. UK. Symposium on health related microbiology. IAWPRC. Morriss, R., L. M. Alexander, P. Wyn-Jones and J. Sellwood. (Eds). University of Strathclyde, Glasgow, 3-5th Sept. 1991.

Rogers, J. and C. W. Keevil, (1992). Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Applied and Environmental Microbiology, 58,7,.

Rogers, J., P. J. Dennis, J. V. Lee and C. W. Keevil, (1993). Effects of water chemistry and temperature on the survival and growth of *L. pneumophila* in potable water systems. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992. 248-250.

Roig, J., A. Carreres and C. Domingo, (1993), Treatment of Legionnaires' disease, Drugs 46 (1), 63-79.

Rollins, D. M. and R. R. Colwell, (1986), Viable but nonculturable stage of *Campylobacter jejuni* and its role in the natural aquatic environment, Applied and Environmental Microbiology, 52, 3, 531-538.

Rose, F. L. and G. Swain, (1956). Bisdiguanides having antibacterial activity. Journal of the Chemical Society, 4, 4422-4425.

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. 33, 1179-1183

Rowbotham, T., (1983) Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interactions of those and other isolates with amoebae. Journal of Clinical Pathology, 36, 978-986.

Rowbotham, T. J. (1984). Legionellae and Amoebae, In Legionella, Proceedings of the Second International Symposium, C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski, (Eds). American Society for Microbiology, Washington D. C. p 325-327

Rowbotham, T. J. (1986). Current views on the relationships between amoebae, Legionellae and man. Israel Journal of Medical Sciences. 22, 678-689.

Rowbotham, T. J. (1993), *Legionella* -like amoebal pathogens, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp.137-140.

Sadosky, A. B., L. A. Wiater and H. A. Shuman. (1993). Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infection and Immunity. 61, 12, 5361-53738.

Schlenk, R., A Wildfeuer and O. Haferkamp, (1993), Identification of *Legionella pneumophila* in variuos specimens by the polymerase chain reaction, Arzneimittel Forschung/Drug research, 43, 11, 1249-1252.

Schlick, W. (1993), The problems of treating atypical pneumonias, Journal of Antimicrobial Chemotherapy, 31, suppl. C, 111-120.

Schofield, G. M. and R. Locci, (1985), Colonisation of components of a model hot water system by *L. pneumophila*. Journal Applied Bacteriology, 58, 151-162.

Schormann, T. and T. M. Jovin, (1992). Contrast enhancement and depth perception in three- dimensional representations of differential interference contrast and confocal scanning laser microscope images. Journal of Microscopy, 166, 2, 155-168.

Shariff., N. and R.S. Hassan. (1985). Engineering and nutritional parameters affecting biofilm development. Effluent and Water Treatment Journal. 25, 12, 423-425.

Skaliy P., and H. V. McEachern. (1979). Survival of Legionnaires' disease bacterium in water. Annals of Internal Medicine. 90, 662-663.

Skinner, A. R., C. M. Anand, A. Malic, and J. B. Kurtz, (1983), *Acanthamoebae* and environmental spread of *Legionella pneumophila*, Lancet ii, 289-290.

Sly. L. I., M. C. Hodgkinson and V. Arunpairojana. (1988). Effect of water velocity on the early development of manganese-depositing biofilm in a drinking water distribution system. FEMS. Microb. Ecol. 53, 175-186.

Smith, A. L. and H. V. Smith. (1989). A comparison of fluorescein diacetate and propidium iodide staining and in vitro excystation for determining *Giardia intestinalis* cyst viability. Parasitology, 99, 329-331.

Smith, H. (1977), Microbial Surfaces In Relation To Pathogenicity, Bacteriological Reviews, 41, 2, 475-500.

Smith, G. M., K. H. Abbott, M. J. Wilkinson and R. Sutherland, (1993), A rat model of *Legionella pneumophila* pneumonia for efficacy studies with β-lactam antibiotics, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 19-22.

Smith-Somerville, H. E., V. B. Huryn, C. Walker, and A. L. Winters. (1991). Survival of *Legionella pneumophila* in the cold water ciliate *Tetrahymena vorax*. .Appl. Environ. Microbiol.57, 9, 2742-2749

Southam, G., M. Firtel, B. L. Blackford, M. H. Jericho, W. Xu, P. J. Mulhern and T. J. Beveridge. (1993). Transmission electron microscopy, scanning tunneling microscopy and atomic force microscopy of the cell envelope layers of the archaebacterium *Methanospirillum hungatei* GPI.Journal of Bacteriology, 175, 7, 1946-1955.

States, S. J., L. F. Conley, M. Ceraso, T. E. Stephenson, R. S. Wolford, R. M. Wadowsky, A. M. McNamara and R. B. Yee. (1985). Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. Applied and Environmental Microbiology, 42, 5, 1149-1154.

States, S. J., L. F. Conley, S. G. Towner, R. S. Wolford, T. E. Stephenson, A. M. McNamara, R. M. Wadowsky, and R. B. Yee. (1987). An alkaline approach to treating cooling towers for control of *Legionella pneumophila*. Applied and Environmental Microbiology, 53, 8, 1775-1779.

Steele, T. W. (1993). Interactions between soil amoebae and soil Legionellae. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 140-142

Stickler, D. J., J. King, J. Nettleton and C. Winters, (1993), The structure of urinary catheter encrusting bacterial biofilms, Cells and Materials, 3, 3, 315-320.

Stickler, D. and C. Winters, (1994), Biofilms and urethral catheters, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 97-104.

Stout, J. E., V. L. Yu and M. G. Best, (1985). Ecology of *Legionella pneumophila* within water distribution systems. Applied and Environmental Microbiology, 56, 3, 796-802.

Stout, J. E., V. L. Yu, P. Muraca, J. Joly, N. Troup, L. Tompkins, (1992), Potable water as a cause of sporadic cases of community aquired Legionnaires' disease, New England Journal of Medicine. 326, 151-155.

Stout, J. E., V. L. Yu, Y. C. Yee, S. Vaccarello, W. Diven and T. C. Lee, (1992). *Legionella pneumophila* in residential water supplies: environmental surveillance with clinical assessmenrt for Legionnaires' disease. Epidemiol. Inf. 109:49-57.

Streulens, M. J., N. Maes, A. Deplano, N. Bornstein and F. Grimont, (1993), Comparison of methods for subtyping *Legionella pneumophila*, *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, pp. 183-186.

Summersgill, J. T., M. J. Raff and R. D. Miller, (1988). Interactions of virulent and avirulent *Legionella pneumophila* with human polymorphonuclear leukocytes. Microbial Pathogenesis, 5, 41-47.

Surgot, M., C. M. Barioz, M. Nowicki, N. Bornstein and J. Fleurette, (1988), an electron microscopy study of *Legionella pneumophila* after in vitro and in vivo culture, Zbl. Bakt. Hyg. A 269, 26-33.

Sutton, N. A., N. Hughes and P. S. Handley. (1994). A comparison of conventional SEM techniques, low temperature SEM and the electroscn wet scanning electron microscope to study the structure of a biofilm of *Streptococcus crista* CR3. Journal Applied Bacteriology, 76, 5, 448-455.

Taylor Eighmy, T.. and P. L. Bishop. (1985). Effect of reactor turbulence on the binding protein-mediated aspartate transport system in thin wastewater biofilms. Applied and Environmental Microbiology, 50, 1, 120-124.

Tesh, M. J. and R. D. Miller, (1981), Amino acid requirements for *Legionella pneumophila* growth. *J. Clin. Microbiol.*, 13, 5, 865-869.

Tison, D. L. and R. J. Seidler. (1983). *Legionella* incidence and density in potable drinking water supplies. Applied and Environmental Microbiology, 45, 1, 337-339.

Tobin, J. O. H., R. A. Swann and C. L. R. Bartlett. (1981). Isolation of *Legionella pneumophila* from water systems: methods and preliminary results. British Medical Journal, 282, 515-517.

Tomkins, L. S. and J. S. Louitt, (1993), Detection of *Legionella* by molecular methods. *In*: Legionella: current status and emerging perspectives. Barbaree, J. M., Breiman, R. F. and Dufour A. P. (Eds). Proc. The 4th International Symposium on Legionella American Society for Microbiology, Washington, D.C. pp163-168.

Toze, S., L. Sly, C. Hayward and J. Fuerst, (1993), Bactericidal effect of inhibitory non-*Legionella* bacteria on *Legionella pneumophila*. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 269-273.

Tremaine, S. C. and A. L. Mills, (1987), Inadequacy of the eukaryote inhibitor cycloheximide in studies of protozoan grazing on bacteria at the freshwater-sediment interface. Applied and Environmental Microbiology, 53, 8, 1969-1972.

Trulear, M.G. and W.G. Characklis. (1982). Dynamics of biofilm processes. J. Water Pollut. Control Fed. 54, 1288-1301

Tully, M., A. Williams And R. B. Fitzgeorge, (1992). Transposon mutagenesis in *Legionella pneumophila* 11.-Mutants exhibiting impaired intracellular growth within cultured macrophages and reduced virulence *in vivo*. Research in Microbiology, 5,143,481-489.

Tully, M., (1993). A *Legionella* plasmid mediates resistance to UV light but not to solar radiation. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 120-123.

Tyndall, R. L. and E. L. Domingue. (1982). Cocultivation of *Legionella pneumophila* and free-living amoebae..Appl. Environ. Microbiol. 44, 4, 954-949

Väisänen, G. M., E. Nurmiaho-Lassila, S. A. Marmo and M. S. Salkinoja-Salonen, (1994), Structure and composition of biological slimes on paper and board machines. Applied and Environmental Microbiology, 60, 2, 641-653.

van Belkum, A., M. Streulens and W. Quint. (1993), Typing of *Legionella pneumophila* strains by polymersae chain reaction-mediated DNA fingerprinting. Journal of Clinical Microbiology, 31, 8, 2198-2200.

van der Wende. E., W. G. Characklis and J. Grochowski. (1988). Bacterial growth in water distribution systems. Wat. Sci. Tech. 20, 11/12, 521-524

van Doorn, W. G., F. Theil and A. Boekstein. (1990). Cryoscanning electron microscopy of a layer of extracellular polysaccharides produced by bacterial colonies. Scanning. 12, 297-299.

Veríssimo, A., G. Marrão, F. Gomes Da Silva, and M. S. Da Costa. (1991). Distribution of *Legionella* spp. in hydrothermal areas in continental Portugal and the Island of São Miguel, Azores. Applied and Environmental Microbiology, 57, 2921-2927.

Vess, R. W., R. L. Anderson, J. H. Carr, W. W. Bond and M. S. Favero, (1993), the colonisation of solid PVC surfaces and the aquisition of resistance to germicides by water microorganisms. Journal Applied Bacteriology, 74, 215-221.

Vickers, R. M., V. L. YU, S. S. Hanna, P. Muraca, W. Diven, N. Carmen, and F. B. Taylor, (1987), Determinants of *Legionella pneumophila* contamination of water distribution systems: 15-hospital prospective study, *Infection and Immunity,* 8, 9, 357-363.

Wadowsky, R. M., R. B. Yee, L. Mezmar, E. J. Wing and J. N. Dowling. (1982). Hot water systems as sources of *Legionella pneumophila* in hospital and non-hospital plumbing supplies. Applied and Environmental Microbiology, 56, 3, 796-802.

Wadowsky, R. M., and R. B. Yee. (1983). Satellite growth of *Legionella pneumophila* with an environmental isolate of *Flavobacterium breve*. Applied and Environmental Microbiology, 46, 1447-1449

Wadowsky, R. M., R. Wolford, A. M. McNamara and R. B. Yee., (1984), Enhanced chlorine resistance of tap water adapted *Legionella pneumophila* as compared with agar passed strains, Applied and Environmental Microbiology, 50, 21-26.

Wadowsky, R. M., and R. B. Yee, (1985), Effect of Non-*Legionellaceae* bacteria on the multiplication of *Legionella pneumophila* in potable water. Applied and Environmental Microbiology, 49,5,1206-1210.

Wadowsky, R. M., L. J. Butler, M. K. Cook, S. M. Verma, M. A. Paul, B. S. Fields, G. Keleti, J. L. Sykora and R. B. Yee. (1988). Growth supporting activity for *Legionella pneumophila* in tap water cultures and implication of Hartmannellid amoebae as growth factors. Applied and Environmental Microbiology, 54,11,2677-2682.

Wadowsky, R. M., T. M. Wilson, N. J. Kapp, A. J. West, J. M. Kutcha, S. J. States, J. N. Dowling and R. B, Yee. (1991). Multiplication of *Legionella* spp. in tap water containing *Hartmanella vermiformis.* Applied and Environmental Microbiology, 57,7,1950-1955.

Walker. J.T., A.B. Dowsett, P. J. L. Dennis and C. W. Keevil, (1991), Continuous culture studies of biofilm associated with copper corrosion. International Biodeterioration, 27, 121-134

Walker, J., A. Sonesson, C. W. Keevil and D, C. White, (1993). Detection of *L. pneumophila* in biofilms containing a complex microbial consortium by gas chromatography-mass spectrometry analysis of genus-specific hydroxy fatty acids. FEMS Microbiology Letters, 113, 139-144.

Walker, J. T., J. Rogers and C. W. Keevil, (1994), An investigation of the efficacy of a bromine containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila.* Biofouling, 8, 47-54.

Walker, J., D. Wagner, W. Fischer and C. W. Keevil, (1994a). Rapid detection of biofilm on corroded copper pipes. Biofouling, 8, 55-63.

Walker, J., D. E. Caldwell, K. Hanson and C. W. Keevil. (1994) , Use of scanning confocal laser microscopy to study biofilm corrosion. In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp.69-72.

Watkins, I. D., J. O'H Tobin, P. J. Dennis, W. Brown, R. Newnham, and J. B. Kurtz, (1985). *Legionella pneumophila* serogroup 1 subgrouping by monoclonal antibodies-an epidemiological tool, Journal of Hygiene Cambridge, 95, 211-216.

Weichart, D., J. D. Oliver and S. Kjelleberg, (1992), Low temperature induced non-culturability and killing of *Vibrio vulnificus*. FEMS Microbiology Letters, 100, 205-210.

Weisenhorn, A. L., P. K. Hansma, T. R. Albrecht and C. F. Quate, (1989), Forces in Atomic Force Microscopy in air and water. Applied Phys. Letters, 9343-9347.

Wheatley, A. D., (1981). Investigations into the ecology of biofilms in waste treatment using scanning electron microscopy. Environmental Technology Letters, 2, 419-424.

Whipple, G. C., (1901), Changes that take place in the bacterial contents of waters during transportation. Tech. Quart., 14, 21-29.

Wilcox, M. H., (1994), Treating medical device associated infections, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 155-158.

Wilkinson, I. J., N, Sangster, R. M. Ratcliff, P. A. Mugg, D. E. Davos, and J. A. Lanser, (1990), problems associated with identification of *Legionella* species from the environment and isolation of six possible new species, Applied and Environmental Microbiology 56, 3, 796-802.

Williams, P. (1994), Host immune defences and bacterial biofilms, In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp. 93-96.

Williams, P. and G. S. A. B. Stewart, (1994), Cell density dependent control of gene expression in bacteria- implications for biofilm development and control. In: Bacterial biofilms and their control in medicine and industry, J. Wimpenny, W. Nichols, D. Stickler and H. Lapin-Scott, (Eds.) Bioline, Cardiff. pp.9-12.

Williams, A., C. Rechnitzer, M. S. Lever and R. B. Fitzgeorge. (1993). intracellular production of *Legionella pneumophila* tissue destructive protease in alveolar macrophages. *in* Legionella, Current status and emerging perspectives. J. M. Barbaree, R. F. Breiman, and A. Dufour, (Eds.) American Society for Microbiology. The 4th International Symposium on Legionella, Florida, 1992, 88-90.

Winn, C. Jnr. (1993), *Legionella* and the clinical microbiologist, Laboratory Diagnosis of Infectious Diseases, 7, 2, 377-392.

Wong, M. C., W. L. Peacock Jr., R. M. McKinney and K. H. Wong. (1981). *Legionella pneumophila*: avirulent to virulent conversion through passage in cultured human embryonic lung fibroblasts. Curr. Microbiol. 5, 31-34.

Wright, J. B., I. Ruseka and J. W. Costerton, (1991), Decreased biocide susceptibility of adherent *Legionella pneumophila,* Journal Applied Bacteriology, 71, 531-538.

Xu, H., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes and R. R. Colwell, (1982), Survival and Viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbiol Ecology, 8, 313-323.

Yamada, H., S, Akamine and C. F. Quate, (1992). Imaging of organic molecular films with the atomic force microscope. Ultramicroscopy, 42-44, 1044-1048.

Yamamoto, H., T. Ezaki, M. Ikedo, and E. Yabuuchi, (1991), Effects of biocidal treatments to inhibit the growth of *Legionella*e and other microorganisms in cooling towers, Microbiol. Immunol. 35, 9, 795-802.

Yamamoto, Y., T. W. Klein, C. Newton and H. Friedman. (1992a). Differing macrophage and lymphocyte roles in the resistance to *Legionella pneumophila* infection. Journal of Immunology, 148, 584-589.

Yamamoto, Y., T. W. Klein and H. Freidman. (1992). Genetic control of macrophage susceptibility to infection by *Legionella pneumophila*. FEMS Microbiology Immunology, 89, 137-146.

Yamamoto, Y., T. W. Klein and H. Friedman. (1993). *Legionella pneumophila* virulence conserved after multiple single-colony passage on agar. Current Microbiology, 27, 241-245.

Yamamoto, H., Y. Hashimoto And T. Ezaki, (1993b), Comparison of detection methods for *Legionella* species in environmental water by colony isolation, fluorescent antibody staining, and polymerase chain reaction, Microbiology Immunology, 37, 8, 617-622.

Yee, R. B. and R. M. Wadowsky. (1982). Multiplication of *Legionella pneumophila* in unsterilised tap water. Appl. Environ. Microbiol. 43, 6, 1330-1334.

Yu, V. L., J. J. Zurayleff, L. Gavlik and M. H. Magnussen, (1983). Lack of evidence for person to person transmission of Legionnaires' disease. J. Infect. Dis., 147, 362.*(Tully 1992)

Zobell, C. E. (1943). The effect of solid surfaces upon bacterial activity. J. Bacteriology, 46, 39-56.

Zobell, C. E. and D. Q. Anderson. (1936). Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. Biol. Bull.,71, 324-342.

Appendix 1

R2A and R3A (Reasoner and Geldreich, 1985).

	R2A (g/litre)	R3A (g/litre)
Yeast Extract (Oxoid)	0.5	1.0
Difco proteose peptone No 3	0.5	1.0
Casamino acids	0.5	1.0
Glucose	0.5	1.0
Soluble starch	0.5	1.0
sodium pyruvate	0.5	1.0
K_2HPO_4	0.3	0.5
$MgSO_4.7H_20$	0.05	0.6
Agar (Oxoid)	15	15

Make up to volume with distilled H_2O, autoclave at 15 lbs for 15 minutes.

Appendix 2.

Legionella media

(BCYE , GVPC and supplements as described below, were all prepared to the formulations as used in the PHLS laboratory in Preston.)

2.1.Basal medium Buffered charcoal yeast extract medium (BCYE)

Yeast extract	10.0 g
New Zealand Agar	12.0 g
Activated charcoal	1.5 g
α-ketoglutarate,monopotassium salt	1.0 g
ACES buffer	
(N-2-acetoamido-2-amino ethane sulphanilic acid)	10.0 g
KOH (pellets)	2.8 g
L-cysteine HCl (0.4g / l presterilised solution)	10.0 ml
Ferric pyrophosphate ($Fe_4(P_2O_7)3$)	
(0.28g/l presterilised solution)	10.0 ml
Distilled water	to 1000 ml

2.1.2.Preparation of L-cysteine HCl solution

L-cysteine HCl	0.4 g
distilled H_2O	10 ml

Dissolve the L-cysteine in water and filter sterilise through a 0.22 µm cellulose ester filter.

Store at -20 °C for not more than 3 months. Thaw at room temperature before use.

2.1.3.Preparation of ferric pyrophosphate solution.

$Fe_4(P_2O_7)_3$	0.28 g
distilled H_2O	1000 ml

prepare and use as above.

2.1.4.BCYE preparation

Add ACES buffer to 500ml of distilled water and dissolve by standing the mixture in a water bath at 45-50 °C. Add 480 ml distilled water and all the KOH pellets and mix thoroughly until dissolved.

<u>* It Is Important That The Above Sequence Is Followed To Avoid Denaturation Of The Yeast Extract By The Aces Buffer.*</u>

Add charcoal, yeast extract, α-ketoglutarate and agar, mix well and autoclave at 121± 1° C for 15 minutes. Add the L-cysteine and ferric pyrophosphate solutions aseptically. Adjust the pH to 6.9 ± 0.2 with sterile 0.1 mol / l KOH or sterile 0.1M H_2SO_4. Dispense in 20 ml portions into petri dishes of 90-100 mm diameter. dry plates and store in the dark at 4 ± 1 °C for a maximum of four weeks.

2.2. Buffered charcoal yeast extract medium without L-cysteine.

Prepare as above, omitting the L-cysteine.

2.3. *Legionella* selective medium. BCYE with selective supplements.

Prepare the BCYE as above adding the supplements as described below:

2.3.1.Supplements.

i. Ammonium free glycine	3 g
ii. Polymixin B sulphate (2 g/l)	5.5 ml*
iii. Vancomycin hydrochloride (0.01 g/l)	1 ml
iv. Cycloheximide (0.2 g/l)	4 ml

*to give a final concentration of 79,200 IU

Supplement preparation

i. Add the ammonium free glycine after the rehydration of the ACES buffer and addition of α-ketogluarate, but before autoclaving.

ii. Polymixin. Add 200 mg of polymixin B sulphate to 100 ml of distilled water (600 units/100 ml), mix and filter sterilise as previously described. Dispense in 5.5ml volumes into sterile containers and store at -20 °C. Thaw at room temperature prior to use.

iii. 20 mg of vancomycin hydrochloride to 20 ml distilled water, mix and filter sterilise as previously described, Dispense in 1 ml volumes into sterile containers and store and use as above.

iv. 2 g cycloheximide** to 100 ml distilled water, filter sterilise as previously described. Dispense in 4ml volumes and srore and use as above.

** Cycloheximide is a hepatotoxin, Use gloves and mask when handling.

2.4.GVPC preparation.

Prepare as BCYE with the addition of the ammonium free glycine as described above. Add one specified volume of the three antibiotic supplements to a one litre volume of the final medium immediately prior to pH adjustment. Mix well.

5. Quality control.

BCYE and its supplements are heat sensitive, prolonged heating or too high temperatures during the sterilisation process can alter the nutritional properties of the medium. Batch to batch variations in ingredients (particularly α- ketoglutarate can result in poor preformance. Each new batch should be tested with a fresh environmental isolate of *Legionella pneumophila.* Growth should occur within three days of inoculation.

Appendix 3

3.1.Pages Amoebal Saline.

10 ml solution A.

10 ml solutionB.

980 ml distilled water.

Mix solutions A and B with the water, adjust pH to 6.9 if necessary, and autoclave at 15 lbs/ 15 minutes.

3.1.1.Solution A.

NaCl	6 g
MgSo4	0.2 g
Na_2HPO_4	7.8 g
KH_2PO_4	6.1 g
H_2O	500 ml

3.1.2.Solution B

$CaCl_2.2H_2O$	0.2 g
H_2O	500 ml
0r	
anhydrous $CaCl_2$	0.2 g
H_2O	662 ml

Autoclave each stock solution 15lbs / 15minutes.

3.2.Amoebal Agar

To make 1litre

H_2O	980 ml.
Amoebal saline solution A	10 ml
Amoebal saline solution B	10 ml
Malt extract	0.1 g.
yeast extract	0.1 g.
Agar	15 g.

3.3. Amoebal lawns.

Amoebal agar plates were flooded with a suspension of *Klebsiella aerogenes* NCTC 7427 in amoebal saline to give an approximate MacFarlane 5 standard. The excess suspension was removed by pasteur pipette and the plates incubated at 30 °C overnight. These lawns were killed if required by exposing them to UV radiation. (It is necessary to predetermine the length of time for each particular UV source by exposing plates of *K. aerogenes* lawns to the UV source for increasing time intervals (approx. 1-10 minutes) and culturing to assess viability.

Appendix 4.

Peptone Yeast Extract Glucose Broth.

For axenic growth of *Acanthamoeba polyphaga*. Make up in 2 l amounts.

Glucose	20 g
Proteose peptone	30 g
Yeast Extract powder	10 g
Ferrous sulphate $FeSO_4$ $7H_2O$	11 mg.

Dissolve in 2 l of amoebal saline.

Adjust pH to 6.8 with HCl or KOH

Filter (NB.) even if the medium looks clear.

Dispense into 20 ml amounts into universals

Autoclave at 10 lbs for 15mins.

The medium will fall to the desired pH 6.6 if the above autaclaving instructions are closely adhered to. If they are not this medium turns a dark brown it is useless.

Appendix 5.

5.1.Propidium iodide stock solutions

Stock solution 10 mg Propidium iodide is dissolved in 1 ml of Dulbecco PBS

100 µl is made to up to 50 ml to make a working solution

5.2. DAPI Stock solution

10 mg DAPI in 1 ml.

5 µl is made up to 50 ml to give a working solution.

Each stock solution was stored in the dark at 4 °C until required.