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Biofilms in the Potable Water Distribution Network

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Thesis submitted for the degree of Doctor of Philosophy

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May 1998

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Abbreviations

A	Attenuance
AMPS	Ammonium persulphate
AOC	Assimilable organic carbon
ATP	Adenosine triphosphate
BATH	Bacterial adhesion to hexadecane
BDOC	Biodegradable organic carbon
CCD	Charge coupled device
CFU	Colony forming units
cm	Centimetre
CSLM	Confocal scanning laser microscopy
CTC	5-cyano-2,3-tetrazolium chloride
CTP	Cytidine triphosphate
°C	Degrees celsius
D	Dilution rate
DAPI	4, 6-diamidino-2-phenyl indole
DGGE	Denaturing gradient gel electrophoresis
dl	Decilitre
DNA	deoxyribonucleic acid
DPD	Diethyl- <i>p</i> -phenylenediamine
DVC	Direct viable count
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethelyeneglycol-bis(β -aminoethyl ether) N,N,N',N'- tetraacetic acid
EPS	Exopolysaccharide
ESD	Equivalent spherical diameter
g	Gram
<i>g</i>	Gravitational force
GAC	Granular activated carbon
GTP	Guanosine triphosphate

HIC	Hydrophobic interaction chromatography
HPC	Heterotrophic plate count
hr	Hour
INT	2-(<i>p</i> -iodophenyl)-3-(<i>p</i> -nitrophenyl)-5-phenyl tetrazolium chloride
kbp	Kilobase pairs
kDa	KiloDalton
kHz	KiloHertz
l	Litre
LPS	Lipopolysaccharide
µg	Microgram
µl	Microlitre
µm	Micrometre
M	Molar
MATH	Microbial adhesion to hexadecane
MIC	Minimum inhibitory concentration
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
nm	Nanometre
OD	Optical density
<i>p</i>	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
%	Percent
pmole	Picomole
PVC	Polyvinyl chloride

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Scanning electron micrograph
SRB	Sulphate reducing bacteria
TC	Total count
TEM	Transmission electron micrograph
TEMED	N,N,N',N'-tetramethylethylenediamine
THM	Trihalomethane
TOC	Total organic carbon
TTP	Thymidine triphosphate
Tris	Tris-hydroxymethylaminomethane
TVC	Total viable count
V	Volt
VNC	Viable but non-culturable
v/v	volume by volume
w/v	weight by volume

Acknowledgements

I would like to thank all those that have helped me throughout this project. In particular, thanks to Crawford Dow for all his advice and encouragement and his help with the preparation of this manuscript. I am very grateful to those at Severn Trent Water who have supported this project and provided the data presented in the Appendix. Many thanks to Anne Morse for helpful discussions and for providing an insight into the workings of the local distribution network. Thanks also to everyone in the lab for making it such a lively place and for assisting me with unfamiliar protocols. I am especially grateful to Sharon and Ian for the crash courses in practical molecular biology.

Finally, thanks to my parents for backing me throughout my education and to Kate for her unfailing support and understanding.

Declaration

The work contained in this thesis is the result of original work by myself under the supervision of Dr C. S. Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

Nicholas S. Jakubovics

May 1998

Summary

The roles of vegetative dormancy and attachment to surfaces in the survival and growth of bacteria in potable water systems were investigated. Species present in the water were identified following isolation or direct observation of static batch enrichment cultures. Using the latter approach, many prosthecate and other stalked bacteria were found. Prosthecate bacteria undergo bi- or poly-phasic life cycles involving asymmetric division to produce reproductive cells and dormant swarmer cells and their presence in tap water supports the theory that vegetative dormancy is an important survival mechanism in this environment. A continuous flow model was established to analyse the metabolic activity of planktonic and attached bacteria in potable water. A physiological dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), was shown to stain active cells specifically in batch cultures of a *Sphingomonas* sp. and *Caulobacter crescentus*. Unsuccessful attempts were made to identify cellular proteins of *Sphingomonas* sp. cells that were specific to the attached or planktonic phenotype.

By comparing the total bacterial counts in potable water with the total viable counts it was shown that a large proportion of the microflora was not cultivable on heterotrophic media. However, a proportion of these cells became culturable following enrichment with peptone. After ceasing the exogenous nutrient addition cellular aggregation occurred, presumably reflecting physiological changes in response to nutrient depletion. No clear trend in the activity of attached cells during biofilm development was detected. However, firmly attached cells were buffered against changes in the chemistry of the water. Growth within biofilms and release into the water column elevated the concentration of bacteria in the water. Attached cells were resistant to 0.3 mg free chlorine l⁻¹ added for 3 hrs, although this did weaken the architecture of the biofilm. Long term biofilms (one year-old) were almost devoid of bacteria - an observation that could not be adequately explained.

CHAPTER 1

1 Introduction

1.1 Introduction to biofilms

Since the time of Pasteur conclusions on bacterial growth and physiology have been drawn from observations made on cells cultured in nutrient-rich laboratory monocultures. The importance of this classical approach to bacteriology cannot be overstated - the discovery of penicillin alone constitutes one of the major scientific breakthroughs of this century. However, it is becoming increasingly apparent that bacteria growing in natural environments are phenotypically distinct from those cultured in the laboratory and are usually more robust, showing an enhanced ability to survive a range of adverse conditions. The MIC of an antimicrobial agent is often much lower when determined against a laboratory-cultured population of cells than when applied to environmental bacteria. It is therefore essential to study the physiology of natural bacterial cells directly in order to predict their responses to physical or chemical treatments.

There are two fundamental differences between routine laboratory culture of bacteria and growth of cells in the environment which lead to the observed phenotypic differences: (i) the growth rate tends to be far lower in natural environments where nutrients are scarce and competition is abundant and (ii) the vast majority of bacteria in natural environments are associated with surfaces. Many aspects of the physiology and ecology of slow-growing bacteria have been elucidated by the use of laboratory chemostat culture techniques (reviewed by Koch, 1997). However, the numerous and complex effects of surface attachment on mixed microbial populations remain poorly understood despite a recent upsurge of interest in the field.

1.1.1 Definitions

There is currently no consensus of opinion on a general definition of a biofilm. Hamilton used the term biofilm to define “the discrete aggregation of organisms, generally microorganisms, and their metabolic products at an interface” (Hamilton,

1987). However, it has been argued that aggregates of bacteria dissociated from a solid substratum should be included in the definition as they are enclosed within a matrix, interact with other cells and enjoy a similar degree of protection from antimicrobial agents as do attached bacteria. More recently, Costerton *et al.* defined biofilms as “matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” (Costerton *et al.*, 1995). This does not include the eukaryotic organisms present at surfaces in most hydrated environments or tissue culture cells attached to the wall of a flask (Palmer & White, 1997). When considering the specific case of potable water systems, the definition proposed by Hamilton (above) is perhaps the most useful. This includes bacteria at the air-water interface but excludes free bacterial aggregates which do not benefit from the elevated concentration of nutrients at interfaces (Marshall, 1980) that provides one of the major advantages of attachment in systems where nutrients are scarce.

The term ‘glycocalyx’ was adopted for the polysaccharide components of bacterial cells lying distal to the outer membrane of Gram-negative cells or the peptidoglycan of Gram-positive bacteria (Costerton *et al.*, 1981). This definition included two major types of surface structure: S-layers and capsules, which could be further subdivided into rigid, flexible, integral and peripheral forms. Biofilm matrices contain macromolecules other than polysaccharides that are derived from bacteria and are important for several diverse functions. These include proteins, nucleic acids and lipids. Therefore in this thesis the term ‘glycocalyx’ will refer to the entire macromolecular matrix of bacterial origin, not just the polysaccharide component of it. The abbreviation ‘EPS’ will denote the exopolysaccharides associated with bacterial cells and biofilms.

1.1.2 A generalised biofilm

Biofilm structure is highly variable and is influenced by, for example, the types of microorganisms present, the availability of nutrients, the rate of flow of fluid over the surface and other factors (see Section 1.5 for a more detailed discussion). However, certain aspects of surface-associated growth are common to most or all biofilms

(Fig. 1.1). Biofilms are heterogeneous systems containing many different microenvironments. Cells are held within close proximity and interactions between cells occur. Biofilms are dynamic, continuously changing in response to environmental fluctuations. The advantages of sessile growth are considered in Section 1.2.

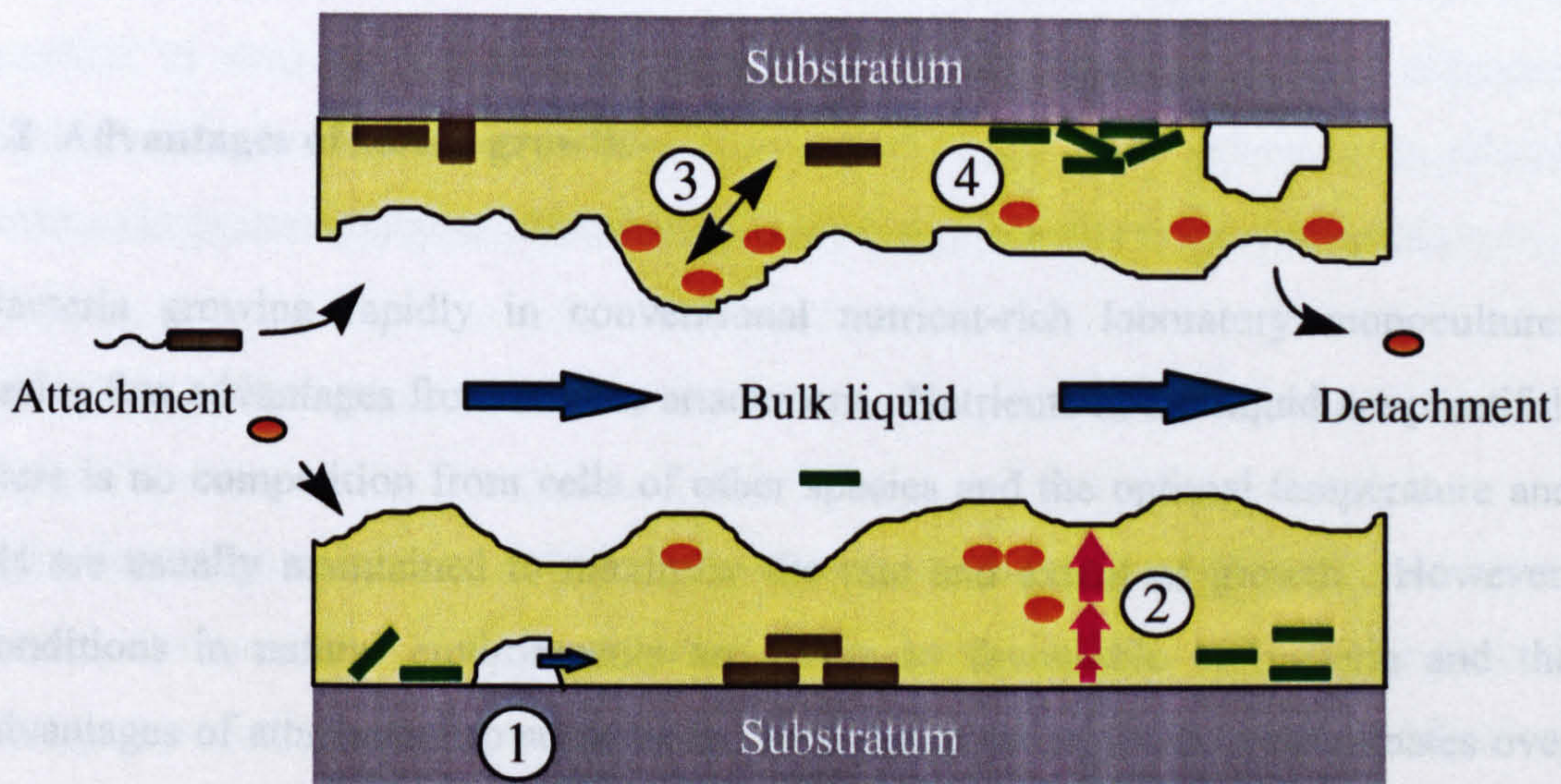


Figure 1.1 A generalised biofilm structure, e.g. a cross-section through a pipe. Attached cells are held within a matrix of extracellular polymeric substances (EPS). The biofilm is dynamic and attachment and detachment occur continuously. Some structural and physiological elements of the biofilm are shown: 1. Water channels flowing through the structure can aid the flow of metabolites to cells and the removal of waste products, 2. Gradients (e.g. nutrients, pH, O_2) occur through the matrix providing different microenvironments suitable for colonisation by different bacteria, 3. Interactions between cells facilitate genetic exchange or recycling of metabolites, 4. Cells divide to produce microcolonies.

1.1.3 Biofilms in potable water distribution systems

Biofilms were suggested to have a major influence on the microbiological quality of tap water as long ago as 1973 (Characklis, 1973). The first direct evidence for the presence of biofilms on the inner surfaces of potable water distribution pipes was provided by a survey using scanning electron microscopy (SEM) (Ridgway & Olson, 1981). It has since been shown that the vast majority of microbial growth in distribution systems occurs associated with solid surfaces (van der Wende *et al.*, 1989). The structure of pipeline biofilms is described in Section 1.5.3, the problems arising from them are discussed in Section 1.7 and potential remedies for these problems are considered in Section 1.8.

1.2 Advantages of sessile growth

Bacteria growing rapidly in conventional nutrient-rich laboratory monocultures derive few advantages from surface attachment. Nutrients in the liquid are plentiful, there is no competition from cells of other species and the optimal temperature and pH are usually maintained to maximise the rate and extent of growth. However, conditions in natural environments are never so favourable to bacteria and the advantages of attachment to surfaces are such that sessile growth predominates over planktonic growth in almost every low nutrient environment (Lappin-Scott & Costerton, 1989). The major advantages conferred on cells by attachment are:

- (a) cells are brought closer together, increasing the occurrence of genetic exchange and enabling recycling of metabolites, which maximises the total energy yield from nutrients in the system;
- (b) cells are protected from antimicrobial agents;
- (c) cells are brought into the area where the concentration of nutrients is greatest;
- (d) attachment enables cells to persist in an environment in the absence of growth.

This is particularly important since periods of inactivity are one of the key

bacterial survival mechanisms in nutrient depleted systems. Surface attachment is also a fundamental strategy to allow bacterial persistence in eukaryotic hosts.

1.2.1 Genetic exchange

The close interactions between biofilm cells results in a high frequency of conjugation relative to that of free-living cells (e.g. Angles *et al.*, 1993). It has been suggested that natural transformation is most likely to occur environmentally if DNA leaked from a lysed bacterial cell is held within a biofilm matrix (Baur *et al.*, 1996). Natural transformation has been demonstrated in at least one biofilm system, the river epilithon, and there is concern that it may increase the rate of spread of undesirable characteristics through natural ecosystems (Williams *et al.*, 1996). It is essential to evaluate the rate of transfer of genetic information to indigenous populations before releasing genetically modified bacteria into nature. Any role of biofilms in bacteriophage-mediated transduction also remains to be elucidated.

1.2.2 Metabolic interactions

Many important natural microbial processes cannot be performed by a pure culture, but can be carried out by a mixed microbial consortium. These are termed community-level processes and include biodegradation, tooth decay, denitrification, methanogenesis, biofouling, food spoilage, natural fermentations, microbially influenced corrosion and other processes (Caldwell & Costerton, 1996). Within biofilms cells of different species can attain the optimal spatial distribution to maximise the efficiency of metabolite exchange. For example, the spatial arrangement of members of a biofilm community capable of degrading the herbicide diclofop methyl was found to be tightly regulated (Wolfaardt *et al.*, 1994b). A looser regulation of spatial association occurred between two toluene-degrading bacteria in a seven-species microbial biofilm, presumably since either organism could degrade the substrate alone but a synergistic interaction increased the efficiency of the process (Møller *et al.*, 1998). Therefore, biofilms can increase the metabolic diversity of the

component cells and in turn the metabolic interactions can influence the structure of the biofilm.

The spatial distribution of cells can also be affected by chemical (e.g. O₂) gradients through the biofilm matrix (Ramsing *et al.*, 1993). However, such gradients will only form in thick biofilms and may be of little significance in the open architecture of potable water biofilms (see Section 1.5.3.2). Recent reviews have examined the range of naturally occurring microbial consortia (Paerl & Pinckney, 1996) or proposed novel approaches to the analysis of intact communities (Caldwell & Costerton, 1996; Molin & Molin, 1997).

1.2.3 Cell-cell communication

Intercellular communication is common amongst bacteria. Peptide signals regulate a wide range of cellular mechanisms in Gram-positive bacteria, including morphogenesis of *Myxococcus xanthus*, spore formation by *Bacillus subtilis*, and aggregation and conjugation of *Enterococcus faecalis* (Kaiser & Losick, 1993). Signalling between Gram-negative cells is also common, although in this case the process is usually mediated by *N*-acyl homoserine lactones (AHLs). Processes controlled by these signals include bioluminescence of *Photobacterium fischeri*, conjugation of *Agrobacterium tumefaciens* and antibiotic production by *Erwinia carotovora* (reviewed by Salmond *et al.*, 1995). In many cases the signals are secreted constitutively at low levels by cells and are effective only when they reach a threshold concentration. They therefore act to enable quorum sensing by bacteria (Fuqua *et al.*, 1994; Salmond *et al.*, 1995). The cell densities required to produce a response by cells are often higher than the planktonic cell concentrations found in natural environments. For example, 10¹⁰ *Nitrosomonas europaea* cells ml⁻¹ were required to produce sufficient AHL to stimulate a *lux E. coli* reporter system (Batchelor *et al.*, 1997). Cell densities as high as this occur only in biofilms or cell aggregates, which suggests that in general, quorum sensing may be more relevant to biofilm cells than to planktonic populations..

Surprisingly few studies of bacterial cell signalling have concentrated on biofilm bacteria and the range of biofilm responses known to be controlled by cell-cell communication is consequently limited. However, it has been shown that starved attached *Nitrosomonas europaea* cells recover more rapidly than starved planktonic cells after nutrient addition, probably due to accumulation of an AHL (Batchelor *et al.*, 1997). There is some preliminary evidence that the strength of attachment of a *Pseudomonas aeruginosa* strain is increased by the accumulation of an AHL within the biofilm matrix (Heys *et al.*, 1997). McLean *et al.* (1997) recently demonstrated AHL activity in natural aquatic biofilms, but the physiological role of cell-cell signalling was not examined. The strongest evidence that cellular communication is important during the formation of bacterial biofilms has come from analysis of signalling mutants of *Pseudomonas aeruginosa*. Biofilms produced by mutant cells, deficient in one of two known intercellular signalling pathways, were flat, tightly packed and more sensitive to removal by detergent than wild-type biofilms (Davies *et al.*, 1998). Further work is required to explore the range of communication processes that occur within biofilms and their specificity to individual species within mixed consortia.

1.2.4 Protection

1.2.4.1 General antimicrobial agents

Biofilm bacteria are relatively resistant to a range of antimicrobial agents including amoebae, white blood cells, bacteriophage, surfactants, biocides and antibiotics, compared to their planktonic counterparts (reviewed by Costerton *et al.*, 1987). Several explanations have been proposed to account for this and have been reviewed by Brown & Gilbert (1993). These authors concluded that growth rate, nutrient limitation, exclusion by EPS and the expression of adherence phenotypes each contribute to the observed recalcitrance. It should be noted that the obstacle to penetration of antimicrobial agents produced by bacterial exopolymers is not simply a diffusion barrier (Nichols *et al.*, 1988 and 1989). Instead the EPS can act as an ion-exchange or a reaction matrix to neutralise the agent. This is important since potable

water biofilms tend to be open structures that could not effectively retard mass transport (see Section 1.5.3).

The role of EPS in protection of bacteria in infection is complicated since cells must evade a wide range of host defence mechanisms. The array of functions of one exopolysaccharide, alginate, in protection of *Pseudomonas aeruginosa* in infection has been discussed in intricate detail by Govan & Deretic (1996).

Care must be taken when using plate counts as the sole method of measuring biocide efficacy since this technique is hyper-responsive to disinfection compared with other estimates of cellular activity (Stewart *et al.*, 1994; Yu & McFeters, 1994).

1.2.4.2 Free chlorine and monochloramine

Residual free chlorine or monochloramine concentrations in distribution pipelines are insufficient to prevent bacterial growth in the system (Ridgway & Olson, 1981; LeChevallier *et al.*, 1987 and 1988a; van der Wende *et al.*, 1989). Vess *et al.* (1993) demonstrated that recalcitrance of a number of bacterial strains commonly found in drinking water to a range of germicides increased when the cells attached to solid PVC surfaces. Biofilm bacteria are up to 150 to 3000 times more resistant to chlorine and monochloramine than planktonic cells (LeChevallier *et al.*, 1988a,b).

Monochloramine has been shown to be a slightly more effective residual disinfectant than free chlorine against biofilm bacteria (LeChevallier *et al.*, 1988b; Yu *et al.*, 1993). The efficacy of chlorine was reduced to a greater extent than that of monochloramine by the presence of an extensive EPS matrix (Samrakindi *et al.*, 1997). Even in the absence of cells, EPS can retard the progression of chlorine (Xu *et al.*, 1996). These observations suggest that being less reactive than chlorine, monochloramine can penetrate the biofilm more easily. However, an apparently contradictory study showed that (a) possession of an extracellular capsule *per se* did not increase the recalcitrance of *Klebsiella pneumoniae* cells to either free chlorine or monochloramine and (b) sparsely distributed attached cells were hundreds of times

more resistant to free chlorine than planktonic cells (LeChevallier *et al.*, 1988a). It seems likely that additional mechanisms act independently of EPS production to increase the resistance of attached cells to biocides. The authors suggested that the reduced exposure of cells to the aqueous medium resulting from firm attachment of one side of the cell to a surface may contribute to recalcitrance to biocides. Alternatively, phenotypic changes induced by attachment to a surface could produce cells that are relatively resistant to antimicrobial agents.

Enhanced resistance to antimicrobial agents is one of the major advantages that biofilm bacteria possess over monodispersed planktonic cells in potable water and must be considered when developing strategies for controlling bacterial growth in distribution pipelines.

1.2.5 Survival in low-nutrient systems

In most natural environments nutrients are scarce and interfaces play two important roles in the growth and survival of bacteria: (a) they provide areas of relatively high nutrient concentration (Marshall, 1980) and in certain cases biofilm matrices can facilitate nutrient storage (Wolfaardt *et al.*, 1995) and (b) they enable maintenance of slowly- or non-growing cells in systems with a limited retention time (for example, *Cryptosporidium parvum* oocysts were retained in a potable water biofilm for many weeks (Keevil *et al.*, 1995)). The relevance of the latter point is obviously dependant on the preponderance of dormant bacteria in nutrient-depleted systems. There is now substantial evidence supporting the theory that the vast majority of bacteria in natural environments exist in a dormant state but retain the capacity to grow and divide in response to favourable environmental changes.

1.2.5.1 Vegetative dormancy

Around the start of the last decade the concept was formed that two distinct types of bacteria exist: oligotrophs growing in low-nutrient environments and copiotrophs which require high concentrations of nutrients for growth (Poindexter, 1981a,b;

Kuznetsov *et al.*, 1979). However, the terms 'oligotroph' and 'copiotroph' were poorly defined and were not readily applicable to natural heterogeneous ecosystems (Morgan & Dow, 1985). Schut *et al.* (1997) recently reviewed numerous studies relating to oligotrophy in the marine environment and concluded that 'the oligotrophic way of life is a transient characteristic'. Rather than attempt to divide all natural ecosystems into two vaguely-defined groups and try to identify the differences between them, it seems more logical to look for similarities between the mechanisms exhibited by all bacteria to survive in a variety of hostile environments.

For many decades it has been recognised that cells of particular microbial genera such as *Bacillus* or *Clostridium* can differentiate to produce spores or cysts capable of withstanding extreme conditions (Slepecky, 1972). However, it has only recently become apparent that most or all vegetative bacteria also undergo major structural and physiological changes in response to stress (Table 1.1). Most studies have employed a gradual deprivation of nutrients to bring about the stress. Although the early stages of starvation are nutrient dependant, cells eventually conform to a 'general starvation response' (Siegele & Kolter, 1992). A similar response occurs when bacteria encounter other stresses, such as prolonged incubation in low temperature (Weichart & Kjelleberg, 1996). Therefore, vegetative dormancy appears to be a secondary effect following an initial specific response to the stress encountered. This explains why expression of a starvation-induced sigma factor, σ^S , results in cross-protection against a variety of other stresses, including heat shock, oxidative stress and osmotic shock (Loewen & Hengge-Aronis, 1994; Jenkins *et al.*, 1990; Jenkins *et al.*, 1988; Yildiz & Schoolnik, 1998; Rockabrand *et al.*, 1998).

Adaptation to stress	Selected references
Reduction in cell volume	Novitsky & Morita, 1976; Morita, 1985
Decreased DNA content	Novitsky & Morita (1977)
Nucleoid condensation	Baker <i>et al.</i> , 1983; Swoboda <i>et al.</i> , 1982; Robertson, 1996
Strengthening of cell wall	Tuomanen <i>et al.</i> , 1988; Nyström & Kjelleberg, 1989; Weichart & Kjelleberg, 1996
Reduced cell wall permeability	Robertson (1996)
Increased cell surface hydrophobicity	Kjelleberg <i>et al.</i> (1987)
Low rate of protein and RNA synthesis; no DNA synthesis	Dow <i>et al.</i> (1983)
Maintained or enhanced adenylate energy charge	Roth <i>et al.</i> , 1988; Emala & Weiner, 1983; Porter, 1984
Reduced proton motive force	Kaprelyants & Kell (1992)
Low respiratory activity	Novitsky & Morita (1977)
Reduced number of ribosomes	Wada <i>et al.</i> (1990)

Table 1.1 The general stress response. Limiting cells of a range of bacterial genera for essential nutrients or producing a stress, such as cold storage, brings about a universal set of adaptations, listed here. The implications of each characteristic of the starved or dormant cells listed are discussed in the text.

Cell volume reduces in response to starvation by means of a series of reductive cell divisions without an increase in biomass (Novitsky & Morita, 1978). It has been hypothesised that by increasing the number of cells in this way the probability of some of them encountering nutrients is increased (Morita, 1986). A reduction in cell volume will increase the surface area/volume ratio, facilitating nutrient scavenging

and may protect against grazing predators (Morita, 1985). Starvation also induces condensation of the nucleoid, presumably to stabilise the chromosome. In *E. coli* two catalases, HPI and HPII, are under control of σ^s and expression of these enzymes helps to prevent oxidative DNA damage (Loewen & Hengge-Aronis, 1994). The cell wall synthesised under amino acid starvation has a different structure from that synthesised during growth which appears to protect starved cells against autolysis (Tuomanen *et al.*, 1988; Nyström & Kjelleberg, 1989). Kjelleberg & Hermansson (1984) demonstrated a starvation-induced increase in the hydrophobic character of several strains examined. This may aid adhesion to surfaces (see Section 1.4.2.3), although the authors did not find a correlation between cell surface hydrophobicity and adhesion in that particular study.

The physiological responses to stress outlined in Table 1.1 function to maintain a low level of endogenous metabolism to enable cells to respond to favourable conditions (Siegele & Kolter, 1992). Clearly dormancy is a common secondary response to starvation and other stresses amongst vegetative bacteria, but the laboratory studies described above provide little information on the prevalence of vegetative bacterial dormancy in nature. Instead we must turn to a related area of research which has explored two different phenotypes of bacteria in natural environments: small cells (ultramicrobacteria) and the viable but non-culturable (VNC) state.

1.2.5.1.1 Ultramicrobacteria

Torrella & Morita (1981) used time-lapse phase contrast microscopy to monitor the growth of cells from sea water on nutrient-rich agar. They found two types of bacteria: zymogenic strains which enlarged and grew rapidly and small cells (<0.3 μ m diameter) which did not enlarge, but grew slowly on the agar. The authors termed the latter cells 'ultramicrobacteria' and speculated that they may constitute the autochthonous flora in marine environments. Very small cells have subsequently been found in a number of natural ecosystems (Roszak & Colwell, 1987).

From the point of view of assessing vegetative dormancy in nature it is necessary to determine whether these small cells represent dormant forms of normal bacteria, normal forms of small bacteria or both. Several accounts of resuscitation and enlargement of small cells from estuarine waters or soil have been published (MacDonell & Hood, 1982; Bakken & Olsen, 1987; Kjelleberg *et al.*, 1987; Kaprelyants *et al.*, 1993). However, it has proved far more difficult to find ultramicrobacteria that cannot enlarge under any circumstances, since failure to observe enlargement may simply reflect failure to provide the optimal conditions for growth (Morita, 1988). Recently *Sphingomonas* sp. strain RB2256 has been isolated from the marine environment and shown to maintain a small cell volume (0.03 to 0.07 μm^3) in media containing concentrations of dissolved organic carbon ranging from 0.8 to 800 mg l^{-1} (Eguchi *et al.*, 1996). Irrespective of whether obligate ultramicrobacteria exist, it is clear that many types of vegetative bacteria occur in natural environments as small, dormant cells.

1.2.5.1.2 Viable but non-culturable (VNC) cells

The term 'viable but non-culturable' was introduced by Colwell *et al.* (1985) to describe cells of certain Gram-negative species (*E. coli*, *Salmonella* and *Vibrio* spp.) that appeared after starvation and could not form colonies on solid media despite retaining the capacity to elongate in response to nutrients. There have since been numerous reports of the VNC state in Gram-positive and Gram-negative bacteria under environmental conditions (reviewed by Oliver, 1993). Unfortunately the terminology is confusing. There have been several examples of resuscitation of VNC cells (e.g. Oliver *et al.*, 1995; Oliver & Bockian, 1995; Magariños *et al.*, 1997; Jones *et al.*, 1991; Kaprelyants & Kell, 1992) so instead of 'non-culturable' it is more accurate to use the term 'not immediately culturable' (Barer, 1997). Viability has been inferred from a variety of different characteristics including the ability of cells to elongate (Rollins & Colwell, 1986; Xu *et al.*, 1982; Byrd *et al.*, 1991; Colwell *et al.*, 1985; Oliver *et al.*, 1995), retention of antigens (Turpin *et al.*, 1993), ability to uptake methionine (Rahman *et al.*, 1994), pathogenicity (Hussong *et al.*, 1987) or the presence of intact cells alone (Kaprelyants & Kell, 1992; Magariños *et al.*, 1997).

Kaprelyants *et al.* (1993) argued that dormancy must be a reversible state so the only correct definition of viability is the ability of a cell to divide.

In view of the obvious similarities between laboratory-induced dormancy and the VNC state (which can also be induced by starvation in low-nutrient laboratory media (Kaprelyants & Kell, 1992)) it seems logical that the observed loss of culturability is merely an extension of a general stress response. The failure of slowly- or non-growing bacteria to respond to high levels of nutrients is reminiscent of the phenomenon of substrate-activated death, described over thirty years ago (Postgate & Hunter, 1963). Recently two possible mechanisms have been proposed to account for the lack of immediate culturability of VNC cells on nutrient-rich media: cell death from osmotic shock (Koch, 1997) or death from oxidative damage invoked by a sudden increase in respiration prior to synthesis of all the necessary metabolic enzymes (Bloomfield *et al.*, 1998). However, it is difficult to believe that bacteria would not have evolved mechanisms to cope with these problems. It is possible that such high nutrient concentrations are simply not the correct conditions to activate dormant cells, but confirmation of this hypothesis awaits analysis of the metabolic activity of individual VNC cells following addition of nutrients.

1.2.5.2 Role of the cell division cycle

From the above review it is clear that dormancy is common amongst vegetative bacteria in nature. Dow *et al.* (1983) proposed a general mechanism by which entry into the dormant state may be regulated as a function of the bacterial cell division cycle. The basis of this mechanism was that under conditions of stress the bacterial cell cycle involves an asymmetric division producing a reproductive mother cell specialised for surface attachment and a dormant daughter cell programmed to survive in the fluid phase. The daughter remains inactive until it detects a stimulus, possibly an increase in the local nutrient concentration or the presence of a surface, whereupon it begins an obligate sequence of maturation steps causing it to become a reproductively competent mother cell. This life cycle was shown to occur in at least three unrelated prosthecate bacteria and was suggested to be common or universal

amongst the eubacteria (see below). The original hypothesis of Dow *et al.* (1983) has been adapted here (Fig. 1.2) to emphasise the relationship between dormancy and surface attachment. The mother cell is shown growing at an interface where the nutrient concentration is highest and the daughter cell is suggested to function to disperse the biofilms. Evidence for this life cycle is presented below.

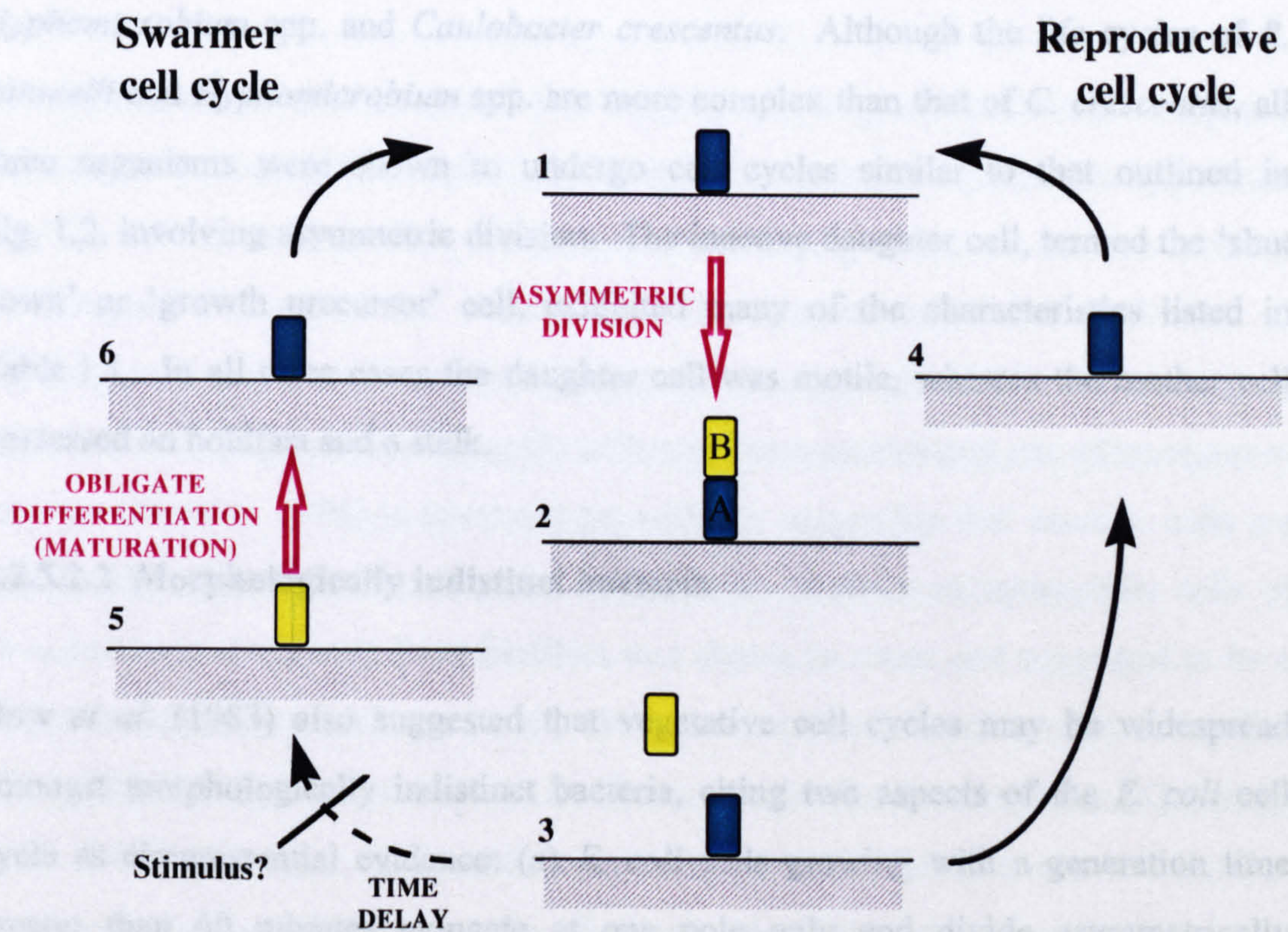


Figure 1.2 A proposed generalised life cycle for bacteria in low-nutrient environments. 1. As shown, cell type A is a *reproductive mother cell*, specialised for attachment to a surface by possession of a stalk and a holdfast (prosthecate bacteria), by being hydrophobic or by some other mechanism (other bacteria). 2. This cell divides asymmetrically, producing a *dormant daughter cell* (B), programmed to exist in the aqueous phase by possession of a flagellum (prosthecate bacteria) and/or by some active mechanism to cause release from a surface (other bacteria). 3. The daughter cell detaches, leaving the mother cell to immediately initiate another round of replication (4). 5. The daughter cell remains inactive and motile until it receives a stimulus. This may be attachment to a surface or another stimulus, e.g. increased nutrient concentration. It then embarks on an obligate sequence of maturation events culminating in gaining reproductive competence (6).

1.2.5.2.1 Prosthecate bacteria

Prosthecate bacteria provide excellent models to analyse the cell division cycle since they differentiate into distinct morphotypes which can be correlated with physiological activity (Whittenbury & Dow, 1977). Dow *et al.* (1983) reviewed the life cycles of three unrelated prosthecate bacteria: *Rhodocyclidium vanniellii*, *Hyphomicrobium* spp. and *Caulobacter crescentus*. Although the life cycles of *R. vanniellii* and *Hyphomicrobium* spp. are more complex than that of *C. crescentus*, all three organisms were shown to undergo cell cycles similar to that outlined in Fig. 1.2, involving asymmetric division. The inactive daughter cell, termed the 'shut down' or 'growth precursor' cell, exhibited many of the characteristics listed in Table 1.1. In all three cases the daughter cell was motile, whereas the mother cell possessed an holdfast and a stalk.

1.2.5.2.2 Morphologically indistinct bacteria

Dow *et al.* (1983) also suggested that vegetative cell cycles may be widespread amongst morphologically indistinct bacteria, citing two aspects of the *E. coli* cell cycle as circumstantial evidence: (a) *E. coli* cells growing with a generation time greater than 60 minutes elongate at one pole only and divide asymmetrically (Donachie & Begg, 1970; Begg & Donachie, 1977) and (b) when grown with a generation time in excess of 3 hours *E. coli* lysates contained two distinct types of nucleoids differing in their sedimentation coefficients. Since then many other traits, characteristic of dormant but not active *E. coli* cells, have been elucidated (Loewen & Hengge-Aronis, 1994; Robertson, 1996), further supporting the evidence for the involvement of a biphasic life cycle in growth of this organism in nutrient-limiting conditions.

However, the two cell types appear indistinct microscopically so if they differ in their potential to attach to surfaces the differences must be at the molecular level. Allison *et al.* (1990b) showed that *E. coli* cell surface hydrophobicity decreased with growth rate and that cells detaching from biofilms in a membrane elution system were

significantly more hydrophilic than those remaining. The relationship between cell surface hydrophobicity and surface attachment is reviewed in Section 1.4.3; in general, there is a positive correlation between hydrophobicity and surface attachment. The evidence of Allison *et al.* (1990b) therefore supports the involvement of the cell cycle model described above in the production of dormant *E. coli* cells.

The prevalence of dormancy and the ubiquity of the general starvation response amongst other vegetative morphologically indistinct bacteria supports the hypothesis that the cell cycle-dependant mechanism for bringing about an inactive state is homologous throughout the eubacteria. The demonstration that starvation of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* resulted in size reduction, an increase in the proportion of motile cells and hence an elevated rate of transport to surfaces (Mueller, 1996) is in agreement with the suggestion that inactive cells are specifically adapted for the dispersal of biofilms. Release of hydrophilic cells of *Pseudomonas aeruginosa* from biofilms was shown to occur and suggested to be a dispersal mechanism (Allison *et al.*, 1990a). A role of dormant cells in biofilm dispersal in the marine environment had been postulated a decade earlier (Kjelleberg *et al.*, 1987). However, these authors had not proposed a link between cell division and the production of inactive cells. Exploring the role of the proposed cell-cycle-dependant regulation of dormancy and surface attachment in potable water distribution systems is the major aim of the work presented here.

1.3 Methods for analysis of biofilms

Before discussing the formation and structure of biofilms it seems pertinent to review some of the recent technical advances that have had a significant impact on our understanding of biofilm systems. This will not be an exhaustive study of the relevant methodology, but will concentrate on the following four areas in which new techniques have recently been introduced: (i) modelling biofilms, (ii) bacterial identification, (iii) analysis of the physiology of attached cells and (iv) microscopic techniques for observation of biofilm structure.

1.3.1 Modelling biofilm development

The published literature contains numerous descriptions of *in vitro* systems designed to mimic biofilm growth. Each model of a biofilm system must be tailored to the individual needs of the experiment. Use of an inappropriate model produces data that is difficult to interpret or even unusable. Some of the advantages and drawbacks of models employed to assess the sensitivity of biofilms to antimicrobial agents were discussed by Brown & Gilbert (1993). To mimic conditions found in potable water distribution pipelines the following features should be incorporated into a laboratory system:

- (a) there should be a continuous throughput of water;
- (b) the inoculum should contain a mixed microbial population, preferably the complete natural potable water microflora;
- (c) the appropriate physical and chemical conditions should be attained;
- (d) a simple non-disruptive method for sampling biofilms should be included.

Of course, the significance of each of the above conditions is dependant upon the objective of the study. For example, a non-disruptive sampling method is crucial to the assessment of biofilm structure. Some of the model systems that have been employed, together with their advantages and disadvantages, are shown in Table 1.2.

Model	Advantages	Disadvantages	Reference
Flow cell	Can observe biofilms as they form	Cannot manipulate samples	de Beer <i>et al.</i> (1994b)
RotoTorque	Mimics hydrodynamics closely	Must disrupt biofilm to sample	van der Wende <i>et al.</i> (1989)
Modified Robbins device	Simple flow-through model Can examine different materials Easy to sample	Biofilms not uniform through device Impossible to get coupons flush (alters local hydrodynamics)	Nickel <i>et al.</i> (1985)
Coupons suspended in chemostat	Can control inoculum Easy to sample	Hydrodynamic conditions differ from those in pipes	Rogers <i>et al.</i> (1994)

Table 1.2 Models of potable water biofilms. See text for a brief description of each model.

Flow cells, such as that used by de Beer *et al.* (1994b), allow observation of the biofilm as it forms. Liquid is continuously flushed through a cell placed under the objective lens of a light or confocal scanning laser microscope. By using time-lapse photography it is possible to monitor biofilm formation. This technique is excellent for analysis of biofilm structure but is not useful for monitoring the physiology of individual cells. van der Wende *et al.* (1989) established a RotoTorque system to mimic the physical and chemical conditions in distribution pipelines, particularly hydrodynamic conditions and the changes in water quality along a distribution mains. Unfortunately sampling of attached cells required biofilm disruption so no data on the structure of biofilms or cellular physiology could be obtained.

One of the most widely-used biofilm systems has been the modified Robbins device (Nickel *et al.*, 1985). This consists of a pipe of rectangular section, into which retractable pistons are inserted. Circular discs are attached to the pistons so that they lie flush against the wall of the pipe. The pistons can be withdrawn to remove the discs. In theory, this model should allow similar hydrodynamic conditions to those present in pipelines *in situ* to be attained. However, it has proved impossible to make the biofilm coupons lie flat against the wall of the tube, causing localised perturbations to the flow of the water around the discs. When installing a Robbins device into a drinking water distribution system in Sweden, Manz *et al.* (1993) adjusted the orientation of the test samples, making no attempt to leave them flush against the pipe surface.

The model employed by Rogers *et al.* (1994) used a chemostat to seed a second vessel containing removable coupons. This enabled the substratum material to be changed easily and allowed tight control over the inoculum. However, the hydrodynamic conditions in distribution pipes were not reproduced and the experiments employed a defined population of organisms rather than the complete natural microflora of potable water.

It is essential to determine the key criteria for a model before setting up an experiment and to keep in mind the potential problems of that system when interpreting data.

1.3.2 Bacterial identification

Information on natural bacterial populations can be extracted without identification of the species or even the genera present. For example, a Gram stain index has recently been introduced to profile natural aquatic populations (Saida *et al.*, 1998). Although this and similar techniques are simple and applicable to many environments, the information they yield is limited. Identification of organisms cannot be avoided when studying the microbiology of a system in detail. Unfortunately, until the latter part of the last decade bacterial identification has relied

almost exclusively on isolation. Even with the use of specialised media, such as R2A (Reasoner & Geldreich, 1985) for isolating tap water microorganisms, only a small proportion of the total number of species present in natural environments can be cultivated in the laboratory (Amann *et al.*, 1995). Most culture techniques therefore lead to selection for certain organisms. Only by dilution of samples to extinction can this bias be eliminated (Schut *et al.*, 1993).

Rather than try to remove selectional bias, an alternative approach is to isolate bacteria then develop specific molecular probes to quantify the isolated organisms in the original sample. Antibodies generated for bacterial isolates have been used for their *in situ* detection in aquatic ecosystems (Faude & Höfle, 1997). Antibodies have proved particularly useful for following the fate of allochthonous organisms introduced into water or soil microcosms (e.g. Turpin *et al.*, 1993; Xu *et al.*, 1982; Buswell *et al.*, 1998).

However, polyclonal antibodies often do not give reproducible data and production of monoclonal antibodies is a costly and laborious process. Instead, nucleic acid analysis has created a new branch of molecular microbial ecology which has developed rapidly over the last ten years. The bacterial 16S rRNA subunit or the gene encoding it is becoming the standard target for *in situ* bacterial identification. Oligonucleotide probes for *in situ* hybridization can be designed on the basis of DNA or RNA sequences amplified directly from natural samples, obviating the requirement for isolation of bacteria. Alternatively a top-down approach can be employed initially using a universal and three domain-specific probes before closing in with probes of increasing specificity. *In situ* bacterial identification without cultivation has been the subject of several recent reviews (Amann *et al.*, 1995 and 1997; Head *et al.*, 1998).

A notable success in identification of the dominant species in potable water using 16S rRNA probes has recently been reported. A bacterial isolate from a drinking water population was phylogenetically characterised in the laboratory and shown to be the dominant microorganism *in situ* (Kalmbach *et al.*, 1997b). rRNA fluorescence

in situ hybridization can also be employed to estimate the physiological activity of individual cells (see below).

1.3.3 Metabolic activity of attached bacteria

Lazarova & Manem (1995) extensively reviewed techniques for estimation of total biomass and total bacterial activity in biofilms. However, biofilms are heterogeneous systems and cannot be accurately described using averaged data. Several protocols for examining the metabolic activity of individual attached cells that were not considered in the above article are discussed below.

1.3.3.1 Direct viable counts

The method of Kogure *et al.* (1978) has provided the basis for direct estimation of the number of viable cells without cultivation in many studies (e.g. Kogure *et al.*, 1979; Byrd *et al.*, 1991; Xu *et al.*, 1982). This procedure involves incubating samples in yeast extract and nalidixic acid. Viable cells elongate but cannot synthesise DNA in the presence of nalidixic acid and therefore cannot divide. Elongated cells can then be enumerated microscopically after staining with a fluorochrome. The DVC technique was adapted by Yu *et al.* (1993) for staining immobilised cells. However, it can be difficult to distinguish between elongated and non-elongated cells particularly in mixed microbial populations. Nalidixic acid or other antibiotics may have different effects against different species and this must be carefully considered before using the technique. When used in conjunction with other methods, the DVC procedure can provide useful information.

An interesting modification of the DVC technique is the ‘probe active count’ method for enumeration of active cells (Kalmbach *et al.*, 1997a). This involves incubation of samples in an appropriate carbon source and a division-inhibiting antibiotic, followed by hybridization with 16S rRNA-targeted fluorescent probes to detect cells containing ribosomes. The incubation step was found to increase the percentage of hybridized cells from 50% to 80%.

1.3.3.2 Respiratory chain activity

Zimmerman *et al.* (1978) first reported use of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to measure the electron transfer chain activity of bacteria. Reduction of INT produces an insoluble red formazan crystal which can be visualized by light microscopy. In complex biofilms the INT-formazan count can be artificially elevated by the inclusion of acellular material. Use of another tetrazolium compound, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to measure redox activity was reported by Severin *et al.* (1985). The insoluble formazan deposit produced by cellular reduction of CTC is fluorescent-orange and can be detected in the absence of background interference or on opaque surfaces using a fluorescence microscope equipped with the appropriate filter sets. CTC was first used for microbiological applications by Rodriguez *et al.* (1992) and has been employed for estimating the activity of attached and planktonic cells in numerous other studies (e.g. Yu & McFeters, 1994; Schaule *et al.*, 1993; Yamaguchi & Nasu, 1997). The sites of bacterial CTC reduction were found to be the primary dehydrogenases in *E. coli*, whereas INT may also be reduced by ubiquinone and possibly cytochromes *b*_{555,556} (Smith & McFeters, 1997). Addition of nutrients prior to staining with INT or CTC significantly increases the extent of formazan production (Blenkinsopp & Lock, 1990; Smith & McFeters, 1996). Comparisons of INT and CTC staining of prokaryotic cells suggest that CTC staining is more tightly linked to respiratory chain activity (Smith & McFeters, 1996), but that fluorescent CTC-formazan production is subject to inhibition by phosphate concentrations above 10 mmol l⁻¹, pH above 6.5 and possibly other factors (Smith & McFeters, 1996 and 1997; Walsh *et al.*, 1995; Pyle *et al.*, 1995). The inhibition of fluorescence may reflect production of weakly fluorescent or diffuse CTC-formazan rather than a decrease in CTC reduction *per se* (Smith & McFeters, 1996 and 1997). Production of extracellular CTC-formazan crystals has been observed in laboratory media in the presence or absence of cells (Bovill *et al.*, 1994).

Although the INT reduction method may be useful for single cells on filters or small aggregates, CTC is a more powerful tool for opaque or optically dense samples,

including complex biofilms (Posch *et al.*, 1997). Since the exact mechanisms of bacterial reduction of tetrazolium dyes and inhibition of staining are not yet clear, it is best to combine the use of INT or CTC with other methods of measuring microbial activity if possible.

1.3.3.3 Other measures of metabolic activity

A variety of methods of measuring *in situ* bacterial activity with fluorescent probes have been reviewed (McFeters *et al.*, 1995). A range of dyes have been reported to measure membrane potential, including rhodamine 123 (Kaprelyants & Kell, 1992), oxonols (López-Amorós *et al.*, 1995) and DiOC₆ (Ratinaud & Revidon, 1996; Monfort & Baleux, 1996). These may all be potentially useful for analysing the metabolic activity of attached cells, although they have not yet been evaluated for this purpose. The total RNA content of cells (Back & Kroll, 1991) or the specific amount of rRNA (Poulsen *et al.*, 1993) have also been used as *in situ* reporters of cellular activity. However, the total RNA or rRNA content may vary between species and this can cause problems when applying RNA determination to measure the activity of mixed populations. A novel approach, based on the ability of cells to undergo plasmolysis in response to a pulse application of 1.5 M NaCl, was applied to distinguish between viable and non-viable cells of *Salmonella enteritidis* and *Pseudomonas fluorescens* in biofilms (Korber *et al.*, 1996). Unfortunately this technique is limited to defined laboratory cultures since not all bacteria undergo plasmolysis. At present the best method for analysing elements of the physiological state of bacteria in biofilms is a combination of techniques to provide information on different aspects of cellular activity (Yu & McFeters, 1994).

1.3.4 Microscopy techniques

Microscopy is, of course, the most powerful tool a microbiologist has for studying biofilms. Many of the techniques for analysis of prokaryotic envelopes, reviewed by Beveridge (1993), are useful for biofilm studies. An important aspect of examination

of bacterial attachment to surfaces is the quantification of attached, usually stained, cells. Image analysis can greatly reduce the time required for enumeration of cells and can provide exact information on cell size and positioning. The introduction of confocal scanning laser microscopy (CSLM) into biofilm research (Lawrence *et al.*, 1991) has provided a new dimension for digital image analysts. Techniques for 2D and 3D digital imaging have been discussed by Caldwell *et al.* (1993).

A comprehensive review of the most important microscopic techniques for the examination of biofilms was published recently (Surman *et al.*, 1996).

1.4 Initial attachment to surfaces

Immersion of a solid substratum in a liquid results in the almost instantaneous deposition of a conditioning layer. This is formed by the adsorption of organic molecules to the surface. Bacterial attachment takes place in several phases (Fig. 1.3). Strong adhesive forces mediated by bacterial surface components have little effect until cells are within about 1.5 nm of a substratum. The initial phases of attachment are therefore approximately described by colloidal chemical theories such as the DLVO theory (van Loosdrecht *et al.*, 1990), with the exception that transport to the surface may be an active process if the cells are motile and may involve bacterial sensory mechanisms such as chemotaxis. Convective or diffusive forces can also bring cells close to surfaces. However, diffusion is slow and may be several orders of magnitude slower than active transport or convection (van Loosdrecht *et al.*, 1990).

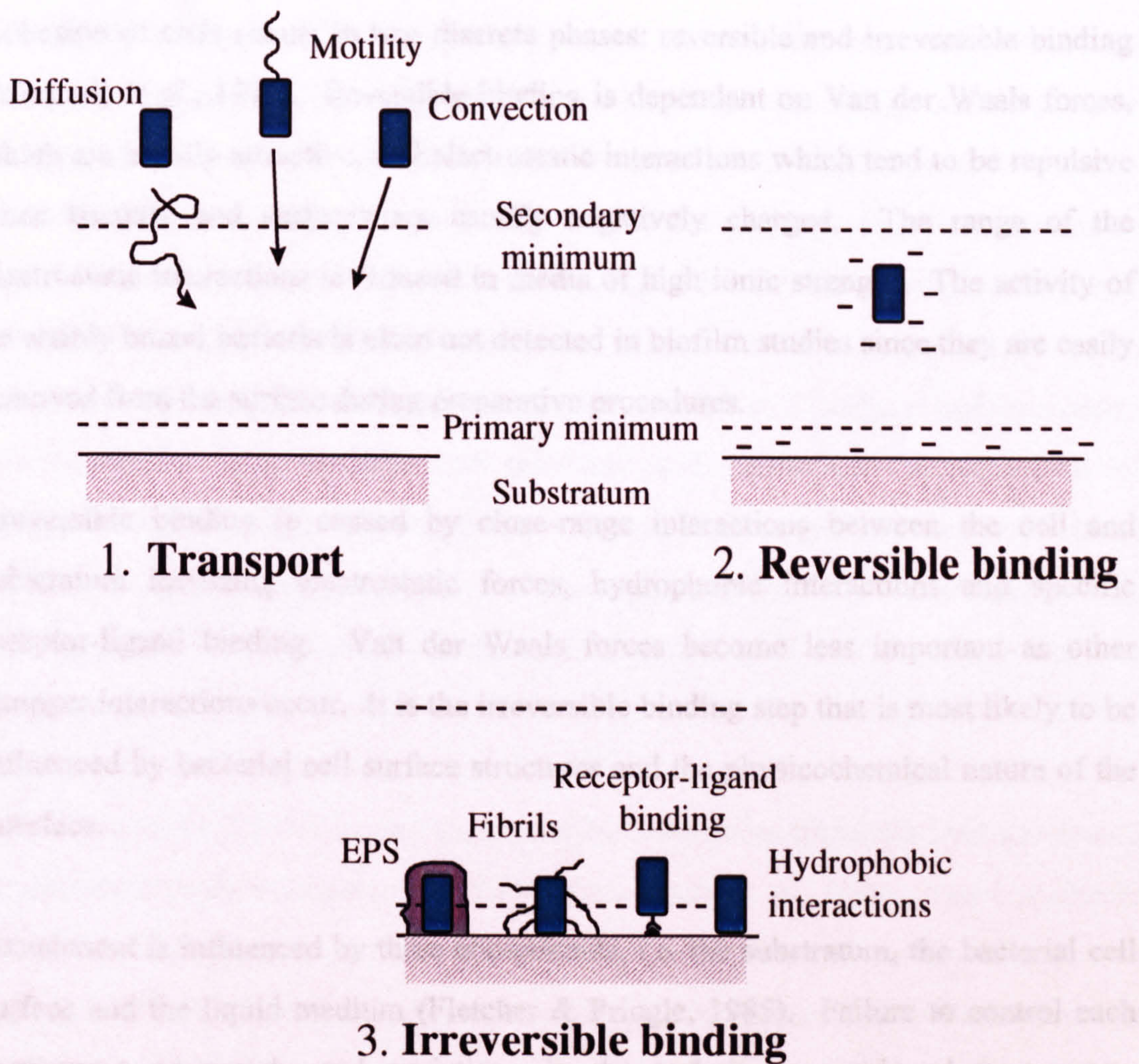


Figure 1.3 Schematic representation of the steps in the adhesion of bacteria to a solid surface. Cells actively move towards the surface or are passively transported by convective or diffusive forces. Reversible binding occurs when cells pass through a secondary energy minimum. The depth of this layer is dependant on the charge on the substratum and the cell surface and the ionic strength of the medium. Irreversible binding occurs when cells enter a primary energy minimum. The strength of attachment may be increased by cell surface components including EPS, specific adhesins and fibrils or by net changes in physicochemical properties of the cell surface such as charge or hydrophobicity.

Adhesion of cells occurs in two discrete phases: reversible and irreversible binding (Marshall *et al.*, 1971). Reversible binding is dependant on Van der Waals forces, which are usually attractive, and electrostatic interactions which tend to be repulsive since bacteria and surfaces are usually negatively charged. The range of the electrostatic interactions is reduced in media of high ionic strength. The activity of reversibly bound bacteria is often not detected in biofilm studies since they are easily removed from the surface during preparative procedures.

Irreversible binding is caused by close-range interactions between the cell and substratum including electrostatic forces, hydrophobic interactions and specific receptor-ligand binding. Van der Waals forces become less important as other stronger interactions occur. It is the irreversible binding step that is most likely to be influenced by bacterial cell surface structures and the physicochemical nature of the interface.

Attachment is influenced by three components, i.e. the substratum, the bacterial cell surface and the liquid medium (Fletcher & Pringle, 1985). Failure to control each component adequately and variations in the techniques employed to measure bacterial surface characteristics have led to apparently contradictory conclusions on the importance of individual physicochemical properties in microbial attachment (Gilbert *et al.*, 1991; Palmer & White, 1997). In natural environments the situation is further complicated by fluctuations in physical and chemical properties of the aqueous phase, including temperature, pH and electrolyte concentrations, which affect adhesive interactions. However, some interesting findings have emerged from numerous studies on the mechanisms of bacterial adhesion.

1.4.1 Electrostatic interactions

Fletcher & Loeb (1979) found an inverse correlation between the electronegativity of different substrata and the extent of attachment of a marine *Pseudomonas* sp.. Feldner *et al.* (1983) demonstrated that electrostatic interactions acted to inhibit binding of mycoplasmas to glass, but that this effect was overcome by changes in

cell shape of metabolically active cells which reduced the area over which the electrostatic repulsion was effective. No correlation between charge and irreversible binding was found for seven marine isolates when electrostatic interaction chromatography was used to measure cell surface charge (Kjelleberg & Hermansson, 1984). However, non-specific non-ionic binding in the chromatography column may have invalidated this technique. Comparison of the electrokinetic potential of cells, measured by electrophoresis, and measurements of cell surface hydrophobicity indicated that surface charge had relatively little influence on attachment of hydrophobic cells to polystyrene beads, but that electronegativity was much more important for controlling adhesion of hydrophilic cells (van Loosdrecht *et al.*, 1987a). This observation was corroborated by studies on *E. coli* and *Staphylococcus epidermidis* (Gilbert *et al.*, 1991).

According to the DLVO theory, increasing the ionic strength of the medium should reduce electrostatic repulsion effects (van Loosdrecht *et al.*, 1990). van Loosdrecht *et al.* (1989) observed increased irreversible adhesion of several species to polystyrene beads with increasing ionic strength. However, observations of single cells using a three-dimensional tracking microscope showed no dependence of the tendency of motile *E. coli* cells to approach a glass surface or the amount of time cells remained reversibly attached on the ionic strength of the medium (Vigeant & Ford, 1997). It will be interesting to see if this finding holds true for other bacterial species.

1.4.2 Hydrophobicity

Several studies have shown a greater tendency of bacteria to adhere to hydrophobic surfaces than to hydrophilic materials (e.g. Fletcher & Loeb, 1979; Pringle & Fletcher, 1983; Jones *et al.*, 1996). Marshall & Cruickshank (1971) observed that discrete areas of hydrophobicity on *Flexibacter* sp. and *Hyphomicrobium* sp. cell surfaces caused cells to orient perpendicularly to air-water, solid-water and water-oil interfaces.

Several simple protocols have been developed to assess the hydrophobicity of bacterial cell surfaces, based on partitioning between an aqueous and a hydrocarbon phase (Rosenberg *et al.*, 1980), hydrophobic interaction chromatography (Smyth *et al.*, 1978), measurement of the contact angle of water on bacterial lawns (Absolom *et al.*, 1983) and salt-induced aggregation (van Oss, 1978). The salt aggregation test is only semi-quantitative and does not always correlate with other measures of cell surface hydrophobicity (Jones *et al.*, 1991). A reasonable agreement was found between contact angle measurements and the behaviour of cells partitioning between an aqueous and a hydrocarbon phase (van Loosdrecht *et al.*, 1987b). However, Mozes & Rouxhet (1987) only obtained a good correlation between five hydrophobicity measurements for very hydrophobic or hydrophilic strains. A similar disparity was found between microbial adhesion to hexadecane (MATH) and hydrophobic interaction chromatography (HIC) (Flint *et al.*, 1997). It is therefore important to consider the validity of the technique employed when drawing conclusions on bacterial cell surface hydrophobicity.

A role of cell surface hydrophobicity has been implicated in the adhesion of many different microorganisms to solid surfaces, including *E. coli* (Smyth *et al.*, 1978), *Staphylococcus epidermidis* (Gilbert *et al.*, 1991), *Serratia marcescens* (Bar-Ness *et al.*, 1988), *Rhodococcus rhodochrous* (Sunairi *et al.*, 1997), benthic cyanobacteria (Fattom & Shilo, 1984) and others (van Loosdrecht *et al.*, 1987b). van Loosdrecht *et al.* (1987a) suggested that increased cell hydrophobicity has relatively little effect on bacterial adhesion for hydrophilic strains of bacteria. In agreement with this was the finding that adhesion of hydrophilic *Bacteroides fragilis* strains was independent of hydrophobicity (Oyston & Handley, 1990).

Cell surface hydrophobicity is dependant on the physiological activity of cells and increases progressively with growth rate in chemostat cultures (Allison *et al.*, 1990a; van Loosdrecht *et al.*, 1987a). In the specific case of the substrate being hydrophobic, incorporation of the substrate into the bacterial cell wall may directly modulate the hydrophobicity of the cell surface (Marchesi *et al.*, 1994). For other substrates the link between hydrophobicity and growth rate must be indirect. Any

molecule exposed on the outside of a cell may influence the physicochemical nature of the surface, but an attractive hypothesis is that the controlled release of microbial surface active compounds could direct rapid alterations in cell surface hydrophobicity (Neu, 1996). Alternatively, LPS production may control the hydrophobicity of Gram-negative cell surfaces since an inverse correlation between LPS production and growth rate has been noted (Brown & Gilbert, 1985), which would tend to confer hydrophilic characteristics on slowly growing cells. This is an interesting area for future research.

The importance of modulation of cell surface hydrophobicity for dispersal of cells from biofilms has already been discussed (Section 1.2.5.2.2). However, it should be emphasised that hydrophobic interactions constitute only a proportion of the total adhesive forces involved in development of biofilms (Kjelleberg & Hermansson, 1984).

1.4.3 Specific adhesins and pili

The surfaces of many bacteria contain adhesins which participate in specific receptor-mediated binding. These may be single protein molecules or filamentous structures termed fimbriae or pili (Saunders *et al.*, 1993). The role of specific adherence molecules in bacterial attachment to eukaryotic host cells has been extensively analysed. Over 50 adhesins or classes of adhesins have been identified in the Streptococci alone (Jenkinson & Lamont, 1997). The combination of specific adherence molecules expressed on the surface of oral bacteria determines their exact location within the human oral cavity (Jenkinson, 1994). Intra- or inter-generic coaggregation enables oral bacteria to attach to existing biofilms (Kolenbrander & London, 1993; Whittaker *et al.*, 1996). This phenomenon may also be important in aquatic environments. Buswell *et al.* (1997) have shown that lectin-like adhesins mediate coaggregation between bacteria isolated from a potable water biofilm model. The nature of the adhesins involved remains to be elucidated.

Specific adhesins may also contribute to attachment to inanimate surfaces. Removal of exposed proteins from the surface of *Pseudomonas fluorescens* cells reduced adhesion to tissue culture plates and petri dishes (Fletcher & Marshall, 1982). The application of molecular strategies to identify bacterial surface proteins involved in non-specific adherence to surfaces in natural environments will enhance our understanding of the molecular mechanisms of biofilm formation in these conditions (Baty *et al.*, 1996).

1.4.4 S-layers and capsules

S-layers are two dimensional crystalline arrays which cover the cell surface of members of every taxonomic group of walled bacteria and are almost universal amongst the Archaea (Sleytr, 1997). Since S-layers provide a rigid and uniform matrix covering the cell surface they may enable localization of adhesins or hydrophobic groups to specific areas of the cell envelope. They may also contribute to adhesion by masking the net negative charge of the peptidoglycan in bacterial cell walls.

Possession of capsules is also a widespread feature amongst both Gram-negative and Gram-positive bacteria. Capsules are composed of essentially the same extracellular polymers that form the glycocalyx of mature biofilms (Section 1.5.1.3). It would therefore be expected that capsules will increase bacterial adherence to surfaces. However, since capsular exopolysaccharides are generally negatively charged and hydrophilic, they tend to inhibit adhesion and aggregation (Wrangstadh *et al.*, 1986; Bayer & Bayer, 1994). This apparent paradox may be resolved by analysing the range over which the exopolysaccharides act. At long range their negative charge and hydrophilicity hinders cells approaching surfaces. When cells are already close to surfaces exopolysaccharides may bridge the repulsion barrier to anchor them (Geesey, 1982). Binding of cations stabilises the EPS matrix formed.

It appears that bacteria have mechanisms to regulate EPS production in response to surface attachment. Exopolymer synthesis was enhanced in a range of bacteria

isolated from the subsurface in response to surface attachment (Vandevivere & Kirchman, 1993). Using a reporter construct, the temporal regulation of one of the key genes in the polysaccharide production pathway of *Pseudomonas aeruginosa*, *algD*, was monitored following adhesion of cells to silicone rubber (Hoyle *et al.*, 1993). Production of EPS was transiently up-regulated immediately after attachment. Exopolysaccharide production by a different *Pseudomonas* sp. and a diatom, *Amphora coffaeiformis*, was enhanced by attachment to hydrophilic, but not hydrophobic surfaces (Becker, 1996). Attachment was strengthened on these surfaces by the polysaccharides. The strains studied adhered only weakly to the hydrophobic materials, suggesting that strong attachment was necessary to stimulate exopolysaccharide production.

Clearly exopolymers are important in the attachment of bacteria to surfaces. However, it is impossible to make general rules about the function of EPS in the initial stages of adhesion since the structures and chemical properties of different polymers are diverse. It is certain that in some situations EPS contributes to the recalcitrance of biofilms to removal processes.

1.5 Biofilm structure

Biofilms can be considered to be combinations of structural elements including single cells, microcolonies and a matrix composed primarily of EPS. Individual elements, responsible for many of the advantages that cells derive from sessile growth (Section 1.2), are discussed in Section 1.5.1. However, a comprehensive understanding of the benefits of biofilm growth requires detailed structural information on the complex biofilms assembled from these components. Models drawn in the 1980s to describe the structure of biofilms depicted a relatively uniform architecture, suggested to be common amongst bacterial biofilms from a wide diversity of environments (Hamilton, 1987; Costerton *et al.*, 1987; Lappin-Scott & Costerton, 1989). The increased use of non-destructive techniques in biofilm research over the last decade has led researchers to question the validity of this type of model, particularly for describing biofilms in low-nutrient environments.

Wimpenny & Colasanti (1997b) have compared the recent observations regarding biofilm structure and proposed three broad groups, based on nutrient availability, to summarise the range of biofilms found in nature: (i) thin biofilms (around 5 μm) with stacks projecting into the milieu (ii) thicker biofilms with mushroom-shaped structures, often fused at the top creating voids and channels running through the structure and (iii) densely packed biofilms with few pores or channels. These categories are not precisely defined but are useful to provide the basis for mathematical predictions of biofilm development.

1.5.1 Common structural elements

This section is intended to review the constituent elements of biofilms, the way they are influenced by surface attachment and the role each component plays in biofilm processes. The mechanisms by which complete biofilms are built from these components and the factors that influence the final biofilm structure are considered in Section 1.5.2.

1.5.1.1 Single cells

Individual cells obviously constitute the primary unit of biofilm structure. A number of alterations of bacterial phenotype have been reported to occur in response to adhesion which may contribute to their success in biofilm systems. The levels of 30% of the cellular proteins that could be resolved by 2D gel electrophoresis varied between biofilm and planktonic cells of the same species (Costerton *et al.*, 1995). Differences between the cell wall protein profiles obtained from *Enterococcus faecalis* cells grown in liquid media or attached to surfaces have been reported (Gilbert *et al.*, 1997). Dagostino *et al.* (1991) employed transposon mutagenesis to insert a promoterless *lacZ* construct into the genome of *Pseudomonas* sp. strain S9. β -galactosidase activity was demonstrated in a number of transformants growing on polystyrene microtitre plates, but not in liquid or on agar media. However, in the above examples, the function of the surface-induced phenotypic changes was unknown.

A reduction in the permeability of the Gram-negative cell wall to cephalosporins in slowly growing surface-attached cells indicates that modifications in the composition of the cell envelope may contribute to the enhanced resistance of biofilm bacteria to antimicrobial agents (Brown & Gilbert, 1985).

The system that has received the most attention, however, has been the biosynthesis of alginate in *Pseudomonas aeruginosa*. The expression of two genes encoding enzymes in this biosynthesis pathway, *algC* and *algD*, is up-regulated in response to attachment of cells to surfaces or surface-associated growth (Davies *et al.*, 1993; Hoyle *et al.*, 1993). This leads to increased production of the exopolysaccharide and enhanced resistance of cells to a range of stresses (Section 1.5.1.3). Expression of the *alg* gene locus is under the control of a sigma factor, AlgU, which is homologous to other sigma factors including the extreme heat shock sigma factor in *E. coli*, σ^E (DeVries & Ohman, 1994; Martin *et al.*, 1994; Deretic *et al.*, 1994; Govan & Deretic, 1996). This suggests the existence of fundamental mechanisms in bacteria for sensing surface attachment and coupling it to changes in levels of gene expression. However, much work is required to identify other elements of this signalling pathway and other responses controlled by AlgU.

1.5.1.2 Microcolonies

Division of cells within biofilms results in the formation of cell clusters, termed microcolonies. These confer a heterogeneous structure on biofilms, particularly those that are not densely populated by bacteria. Penetration of antimicrobial agents to the centre of microcolonies is limited by reaction with peripheral cells and diffusion inhibition by the densely packed clusters (Lappin-Scott *et al.*, 1992). Microcolonies may therefore have an important protective function for the central cells. However, diffusion of oxygen and nutrients into microcolonies is also limited. Using a microprobe to detect local oxygen concentrations, de Beer *et al.* (1994b) demonstrated the presence of anoxic regions in the centre of microcolonies in an

otherwise aerobic biofilm. The cells at the centre of the microcolony are therefore likely to be physiologically distinct from those at the edges.

1.5.1.3 The glycocalyx

In multicellular eukaryotes, cells are held in place by attachment to an extracellular matrix. Processes mediated by extracellular matrix components range from the migration of cells during tissue morphogenesis and repair to the filtration of molecules passing from blood to urine in the kidney glomerulus. In a similar manner, the matrix surrounding microbial cells is an integral part of biofilm systems. The basic structure of a variety of bacterial glycocalyces has been described by Costerton *et al.* (1992). Functions of glycocalyces include:

1. Adhesion of cells to surfaces or to preformed biofilms;
2. Protection;
3. Positioning of cells within biofilms;
4. Nutrient trapping.

The role of exopolymers in initial adhesion (Section 1.4.5) and protection (Section 1.2.4) has previously been discussed. Protection of cells results from the capacity of the glycocalyx to act as a reaction or ion-exchange matrix. However, probably the most important function of the glycocalyx is precisely the opposite: to enable the flow of nutrients and oxygen around entrapped cells. The hydrated matrix allows cells to integrate into the biofilm at a distance from other preattached cells. The glycocalyx retards diffusion to a lesser extent than cellular clusters.

The reduction of diffusion by exopolymers can lead to the establishment of gradients. For example, diffusion of oxygen to the base of biofilms around 200 μm thick was inhibited even through areas that were not densely populated (de Beer *et al.*, 1994b). Gradients of oxygen or nutrients may in turn lead to gradients of cellular activity, increasing from the base of the biofilm upwards. Kinniment & Wimpenny (1992) demonstrated a small increase in the adenylate energy charge through horizontal

sections of *Pseudomonas aeruginosa* biofilms from the base upwards. However, in this study the adenylate measurements were not correlated with the structure of the biofilm so it was impossible to determine the proportion of cells in each section that were exposed on the surface of the biofilm. CSLM in combination with fluorescent probes will provide a more detailed picture of the gradients of cellular activity that occur within biofilms. This technique has been employed to evaluate activity gradients established by fleroxacin treatment of *Pseudomonas fluorescens* biofilms (Korber *et al.*, 1994). A similar approach, using fluorescence microscopy to analyse biofilm sections, was employed to demonstrate gradients of alkaline phosphatase expression within biofilms (Huang *et al.*, 1998).

Glycocalyxes can trap nutrients (Wolfaardt *et al.*, 1994a) and metal cations (Lünsdorf *et al.*, 1997). Costerton *et al.* (1992) have suggested that the Gram-negative periplasmic space may trap protons and may be two full pH units lower than the surrounding milieu. In view of the negative charge on many exopolysaccharides, it is tempting to speculate that EPS may also act to retain protons extruded from cells by chemiosmotic pumping. In support of this is the demonstration that metal ion binding to exopolysaccharide capsules is reduced by conditions of low pH, suggesting that protons can compete with metal cations for binding to negatively charged groups in these polymers (Geddie & Sutherland, 1993).

1.5.1.4 Channels and voids

Mass transport may be enhanced in some biofilms by interstitial voids running through all depths of the matrix. These have been demonstrated using CSLM with or without particle image velocimetry using fluorescent latex spheres (de Beer *et al.*, 1994a,b; Stoodley *et al.*, 1994; Lawrence *et al.*, 1991). Flow of liquid through voids and channels may aid distribution of nutrients and removal of waste products.

1.5.2 Environmental influences on biofilm structure

1.5.2.1 Nutrients

The proposal of Wimpenny & Colasanti (1997b) (see above) that nutrient loading is the primary determinant of biofilm structure was supported by a compilation of structural data from a range of natural biofilm systems, by observations on the morphologies of colonies on solid media containing different nutrient concentrations and by predictions made using a cellular automaton model. These authors suggested that at very low substrate concentrations, the substrate at the base of the biofilm is rapidly used up and competition between cells results in the formation of stacked structures. At very high substrate concentrations it is space at the base of the biofilm which becomes the limiting factor and biofilms become very densely populated. This appears to be an excellent starting point for developing cellular automaton models and the resemblance of structures predicted even by very simple models to natural biofilms is remarkable.

It should be noted that substrate diffusion is not always the primary means of nutrient mass transfer in biofilms. Convection may be more important than diffusion in many biofilms, particularly at high flow velocities (de Beer & Stoodley, 1995) and substrate diffusion does not occur in photosynthetic biofilms. However, the mode of nutrient distribution will not greatly affect the predictions of cellular automata. There are situations in which dynamic changes in the rate of nutrient flow determine the positioning of cells. Thus a web-like structure was formed by cells in porous sandstone media following pulsed nutrient injections (Paulsen *et al.*, 1997). A more complex model would be required to predict the structure of this type of biofilm.

Many other environmental factors also play a role in the determination of biofilm structure. Interactions between cells of different species are clearly important since monospecies biofilms are invariably up to an order of magnitude thinner than those formed from binary or mixed populations (Murga *et al.*, 1995; Peyton, 1996). In natural biofilms numerous intercellular interactions occur (Section 1.2.2) which undoubtedly influence the overall structure to some extent. Some observations on

other factors that affect biofilm architecture are reviewed below. In most cases the data have been obtained from artificial laboratory systems so their possible relevance to newly-forming or mature potable water biofilms can only be inferred.

1.5.2.2 Flow conditions

van Loosdrecht *et al.* (1995 and 1997) have suggested that biofilm structure is mediated by a combination of substrate concentration gradients at interfaces and hydrodynamic shear. Using a biofilm airlift suspension reactor these authors showed that at a constant substrate concentration different biofilm architectures could be formed by varying the shear rate. It was postulated that protruding structures would be removed in high shear conditions. However, increasing shear also increases eddy diffusion rates and consequently raises the substrate concentration in the biofilm (Wimpenny & Colasanti, 1997a).

Flow characteristics can influence the ordering of cells on surfaces. Increasing laminar flow conditions caused *Pseudomonas* sp. cells to align in the direction of flow. When the flow became turbulent this general order was lost (Rao *et al.*, 1997). Stoodley *et al.* (1998) have observed streamer structures in turbulent flow conditions. These dissipate energy by oscillating and are therefore relatively resistant to shear stress. The influence of flow conditions on the structure potable water distribution system biofilms may be resolved by analysis of *in situ* biofilms at different points in the pipelines.

1.5.2.3 EPS

A direct correlation between EPS production and microcolony formation has been demonstrated using two mucoid strains isolated from freshwater and a non-mucoid derivative of one of them (Allison & Sutherland, 1987). Microcolonies encased in EPS were also formed in binary population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumophila* (Stewart *et al.*, 1995). Therefore EPS deposition tends to increase structural heterogeneity in biofilms.

1.5.2.4 Electric currents and pH

Electric fields can increase the efficacy of biofilm removal by antibiotics (Costerton *et al.*, 1994), although understanding of the mechanism for this action has remained elusive. It has recently been demonstrated that oscillating electric currents cause pre-existing biofilms to expand and contract. A biofilm developed on platinum wire electrodes expanded by approximately 4% when the wire was cathodic, but contracted to 74% of the original thickness when the wire became anodic (Stoodley *et al.*, 1997). This effect was reproduced by adjusting the pH of the medium. The biofilm thickness was unaffected by raising the pH from pH 7 to pH 10, but reduced to 69% of the original thickness at pH 3. A similar effect of pH was noted by van der Mei *et al.* (1994). The diffusion coefficients of cells with structural surface features, such as fibrils or fuzzy coats, were increased at pH 2 relative to pH 7 due to collapse of the surface features. The collapse of biofilm structures or surface features was presumably caused by loss of stabilising electrostatic interactions between charged acidic groups in the fibrils, EPS or cell envelopes.

1.5.2.5 Substratum hydrophobicity

Biofilms developed by an hydrophobic marine bacterium, SW5, showed markedly different morphologies on hydrophobic and hydrophilic substrata (Dalton *et al.*, 1994). Therefore substratum hydrophobicity may affect biofilm structure but it is difficult to determine the net effects in a complex microbial ecosystem containing species with a wide variety of cell surface characteristics.

Other factors including temperature, chlorine concentration, seasonal fluctuations and grazing may undoubtedly affect the architecture of biofilms. The recent advances in the development of non-destructive microscopic techniques (Section 1.3.4) will aid empirical assessments of the importance of each of these conditions in determining the final biofilm structure.

1.5.3 Potable water biofilms

1.5.3.1 Species present

Before considering how the above discussion relates to observations made on the structure of potable water biofilms it seems pertinent to examine the types of bacteria commonly found in drinking water distribution systems. Since isolation procedures extract only a small proportion of the species present in natural environments, direct observations on bacterial diversity in potable water are discussed. For a comprehensive analysis of freshwater bacteria the reader is referred to Holt *et al.* (1994).

1.5.3.1.1 Routinely isolated bacteria

Research into the species present in potable water has generally focused on detection of indicator organisms and pathogens (reviewed by Baker, 1994). However, it is becoming clear that a good understanding of all the bacteria present in potable water systems is essential in order to predict the outcome of water treatment procedures. Although molecular techniques for the identification of bacteria *in situ* have advanced rapidly in recent years (Section 1.3.2), most of the current knowledge of the species present in drinking water originates from studies involving isolation of bacteria. Twenty genera of bacteria that have been found in drinking water are listed alphabetically in Table 1.3. It is important to note that this table does not reflect the *in situ* abundance of each genus and is biased for three reasons:

- (i) isolation selects for certain organisms;
- (ii) at least two of the five reports cited were initiated to study problematic drinking water systems so the number of pathogens and coliforms is over-represented;
- (iii) identification is also selective unless every strain isolated is identified. From most of the reports it is not clear what proportion of the isolated strains were identified. However, Buswell *et al.* (1997) identified just four of twenty isolates.

Table 1.3 Bacterial genera isolated from potable water systems.

Bacterial genus	Reference ^a	Bacterial genus	Reference ^a
<i>Acinetobacter</i>	1,3,4	<i>Escherichia</i>	4
Actinomycete ^b	3	<i>Flavobacterium</i>	1,3,4
<i>Aeromonas</i>	3	<i>Klebsiella</i>	4
<i>Agrobacterium</i>	4	<i>Legionella</i>	3
<i>Alcaligenes</i>	1,4	<i>Methylobacterium</i>	1,5
<i>Arthrobacter</i>	(1) ^c ,4	<i>Micrococcus</i>	1,4,5
<i>Bacillus</i>	1,4	<i>Moraxella</i>	4
<i>Brevundimonas</i>	5	<i>Mycobacterium</i>	2
<i>Corynebacterium</i>	(1) ^c ,4	<i>Pseudomonas</i>	1,3,4,5
<i>Enterobacter</i>	4	<i>Sphingomonas</i>	1,3

Footnotes

- a
- 1 - Percival *et al.* (1998).
 - 2 - Schulze-Robbecke *et al.* (1992). This was a specific analysis of mycobacteria in biofilms.
 - 3 - Rogers *et al.* (1994). This study employed a natural population from the sludge at the base of a calorifier implicated in an outbreak of Legionnaires' disease to seed a potable water biofilm model.
 - 4 - LeChevallier *et al.* (1987). A distribution system that had been troubled by repeated coliform detection was examined.
 - 5 - Buswell *et al.* (1997).
- b
- Generic identification was not performed.
- c
- Identification did not distinguish between the corynebacteria or arthrobacter groups.

Strong evidence that our understanding of the total microflora of drinking water is still rudimentary is emerging from studies using 16S rRNA sequencing for bacterial identification. In a recent investigation, eight strains isolated from potable water on R2A medium were phylogenetically characterised by sequencing and analysing the genes encoding 16S rRNA. None of these isolates could be placed in established species on the basis of sequence homology (Kalmbach *et al.*, 1997b).

1.5.3.1.2 Stalked and appendaged bacteria

A few bacteria can be identified without isolation on the basis of cell morphology alone. Planctomycetes and prosthecate bacteria account for most of these recognisable cell types, although a few strains form characteristic chains or helical structures (e.g. *Gallionella* sp. or *Seliberia* sp.). The prosthecate bacteria are particularly useful models for cell cycle studies, since morphological cell type expression is tightly regulated in the cell cycle and correlates with the physiological state of the cell (Section 1.2.5.2.1).

Morphologically distinct bacteria, including planctomycetes and prosthecate bacteria, have often been observed in a variety of habitats including sea water, brackish water, fresh water rivers and lakes, soil and sewage (Staley *et al.*, 1992; Hirsch, 1974; Moore, 1981). However, observations of these organisms in chlorinated water supplies have been relatively rare, presumably since few investigators have searched for them. In the first documented study of potable water distribution network biofilms, (Ridgway & Olson, 1981) observed *Gallionella* sp. and *Prosthecomicrobium* sp. attached to the walls of distribution pipes. Another presumptive identification of *Gallionella* sp. in mains water biofilms was made recently (Percival *et al.*, 1998). After concentration of potable water by centrifugation, Manz *et al.* (1993) observed organisms resembling *Caulobacter*, *Hyphomicrobium* and *Ancalomicrobium* spp.. However, only rod-shaped bacteria and a few spirilla were observed in biofilms in this study. Sly *et al.* (1988) observed colonisation of sampling devices by budding hyphal bacteria.

The lack of information on appendaged bacteria in potable water is surprising in view of the fact that these organisms are commonly observed in the rivers and lakes that seed drinking water systems. It appears that the inclination to study microorganisms that can easily be isolated or are pathogenic has led to gaps in our understanding of the overall ecology of potable water.

1.5.3.2 Structure of potable water biofilms

At present the best model of the structure of potable water distribution system biofilms is that proposed by Keevil *et al.* (1995). This group applied a novel microscopic technique, episcopic differential interference microscopy (EDIC), to examine biofilms formed by a natural microbial population in a two-vessel chemostat model (Walker & Keevil, 1994). This non-destructive procedure allowed real-time observation of biofilms on glass surfaces in flow cells. Stacks protruding from the biofilms were clearly apparent as was grazing by protozoa. The structure of the biofilm observed is represented diagrammatically in Fig. 1.4. Although the microscopy technique produced excellent images, it should be stressed that the biofilms were developed in a model system. *In situ* analysis is required to confirm that this structure predominates in drinking water pipelines.

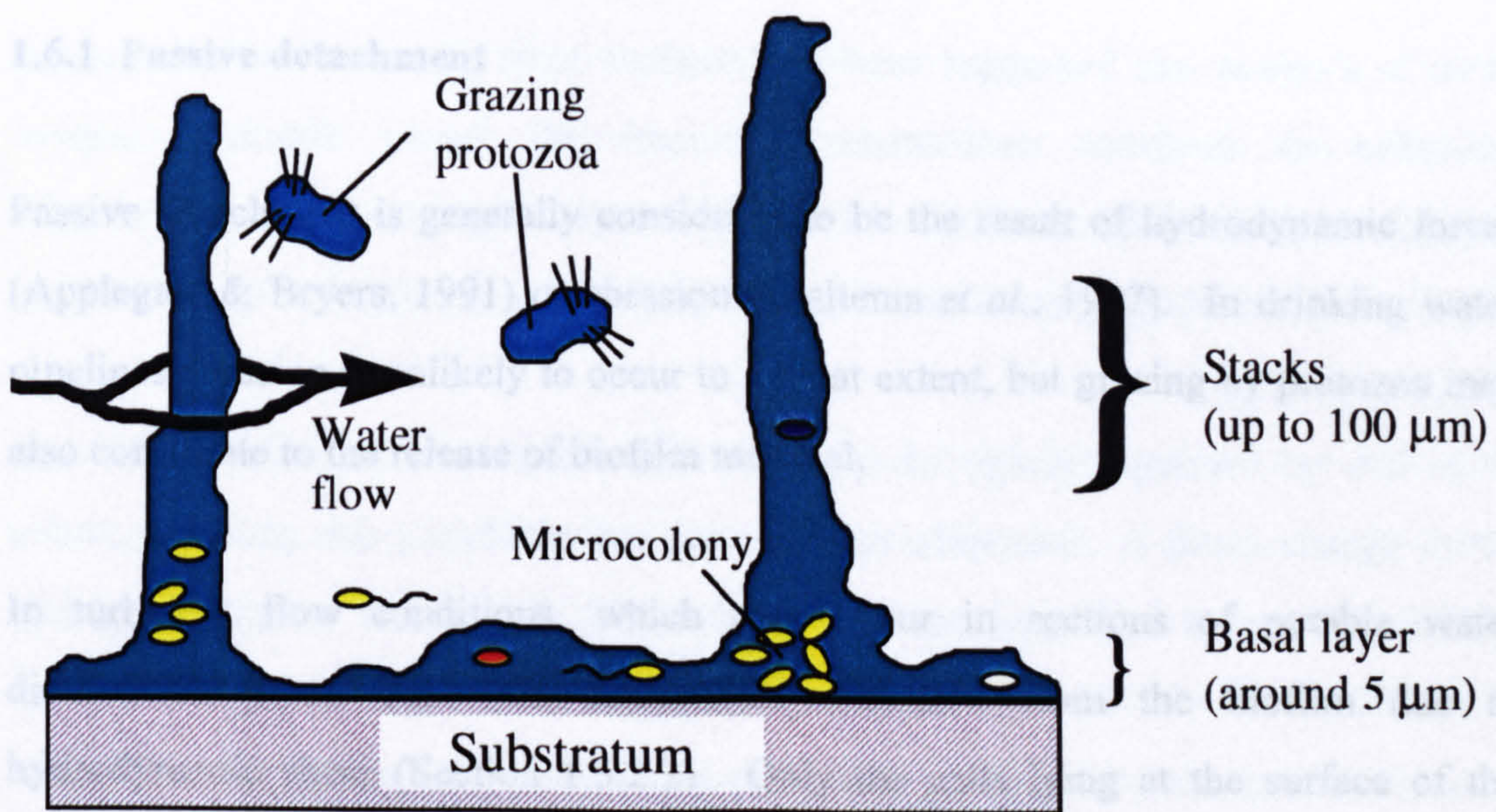


Figure 1.4 Schematic representation of a cross-section through a potable water biofilm (adapted from Keevil *et al.*, 1995). An open architecture exists with a thin basal layer and stacks projecting into the water. Protozoa move between these stacks and graze on the biofilm.

Most cells in this structure lie within about 5 μm from the biofilm-water interface and are therefore potentially prone to release from the biofilm by active or passive processes. These detachment mechanisms are discussed in the following section.

1.6 Detachment and dispersal

In order to colonise remote locations bacteria must be able to detach from surfaces (Gilbert *et al.*, 1993). Several proposed mechanisms for bringing about this detachment are reviewed in Section 1.6.2. The possibility that active detachment is linked to the cell cycle has been considered in Section 1.2.5.2 and is part of the central theory to be tested in this project. In addition to active release, in certain circumstances cells may be detached from surfaces by forces acting on the biofilm. In potable water systems this passive release may have a considerable impact on the quality of the water, particularly if the cohesive forces in the biofilm are loosened by the pulse addition of a disinfectant such as chlorine.

1.6.1 Passive detachment

Passive detachment is generally considered to be the result of hydrodynamic forces (Applegate & Bryers, 1991) or abrasion (Gjaltema *et al.*, 1997). In drinking water pipelines abrasion is unlikely to occur to a great extent, but grazing by protozoa may also contribute to the release of biofilm material.

In turbulent flow conditions, which may occur in sections of potable water distribution pipes, cells are continuously released from the biofilm due to hydrodynamic shear (Section 1.5.2.2). Only the cells lying at the surface of the biofilm are affected by this process. Random sloughing events may also occur which result in the removal of large chunks of the biofilm and can even dislodge the cells adjacent to the substratum. Sloughing may occur after the production of bubbles within the biofilm, for example by nitrogen formation by denitrifying bacteria or by production of gas at the surface of an electrode. Using carbon and oxygen limitation to modulate the amount of EPS in *Pseudomonas putida* biofilms, Applegate & Bryers (1991) found that increased amounts of EPS in the biofilm led to reduced shear but increased sloughing. Presumably the strong cohesive forces of the EPS caused increased frictional resistance leading to catastrophic sloughing events.

The role of protozoa in potable water systems is only beginning to come under scrutiny. Protozoa have been shown to swim between biofilm stacks (Keevil *et al.*, 1995) and direct evidence for protozoan grazing activity in model distribution system has recently been published (Sibille *et al.*, 1998). In theory grazing at the base of a stack could result in release of most of the structure into the water column, but the contribution of protozoa to passive detachment has not yet been determined.

1.6.2 Active release

The molecular basis of de-adhesion has been discussed by Neu (1996). Shedding of molecules or structures (i.e. fimbriae) involved in adhesion will cause release of cells from surfaces. Neu & Marshall (1991) have demonstrated that cells leave 'microbial

footprints' after detaching from surfaces and have suggested that analysis of these footprints should reveal the important interactions involved in adhesion. Alternatively, production of enzymes (Lee *et al.*, 1996) or emulsans (Rosenberg *et al.*, 1982) may free cells from EPS or hydrophobic interactions respectively.

Any of the above mechanisms could in theory be tightly regulated by cell cycle events, although this possibility has not yet been addressed. A direct change in the hydrophobicity of the *E. coli* cell surface has been suggested to account for the specific release of daughter cells from membranes (Allison *et al.*, 1990b; Gilbert *et al.*, 1993). In prosthecate bacteria, attachment is mediated by the production of a holdfast which is developmentally regulated. The genetic basis of this system is becoming clear (Kurtz & Smith, 1994) and it will be interesting to see if homologous regulatory systems exist in other bacteria.

1.7 Problems arising from potable water biofilms

The major concern arising from the presence of distribution pipeline biofilms is their potential to act as pathogen reservoirs. Biofilms can also contribute to corrosion and blockage of pipes, taste and odour complaints and possibly the presence of unacceptable levels of endotoxins in the water.

1.7.1 Biocorrosion and blockage

Microbial growth in biofilms can cause the biodegradation of a variety of materials including many of the metals routinely used in water mains and household installations. Corrosion occurs as a result of anaerobic conversion of protons to H₂ at a metal surface, often by sulphate reducing bacteria (SRB). The reduction of a nearby metal provides the electrons required for this reaction. Sulphides produced also react with the metal. Microbially influenced corrosion of metals is therefore a direct consequence of biofilm heterogeneity in two planes. Vertical O₂ gradients allow the maintenance of anaerobes at the metal/biofilm interface and horizontal heterogeneity results in the formation of corrosion cells which lead to localised pit

formation. Deposition of EPS alone is sufficient to generate a corrosion potential, but corrosion is far more extensive in the presence of an appropriate microbial consortium (Steele *et al.*, 1994). For a clear and concise description of the mechanism of biocorrosion see Lappin-Scott & Costerton (1989) or Geesey (1991).

Microbially influenced corrosion of pipes carrying potable water has often been observed. For example, the presence of a corroding consortium was detected in tubercles found on a cast iron distribution pipeline in Columbus, Ohio (Tuovinen & Hsu, 1982). A range of anaerobic bacteria were isolated from the tubercle, including SRB. Unidentified aerobic heterotrophs were also present, presumably providing the anaerobic conditions required for the corroding organisms. Copper pipes in domestic water installations are also subject to biofouling. Stainless steel has been suggested as a possible alternative material for domestic pipes. In a recent study minimal corrosion of stainless steel pipes was found after exposure to a continuous flow of drinking water for one year (Percival *et al.*, 1998). However, biofilms containing metal ions were present on all the test coupons analysed in this investigation. It was assumed that the metal ions originated from the substratum. Microbial activity has been implicated in the corrosion of stainless steel in deionized water systems (Jain, 1995).

Certain species of bacteria in potable water biofilms are capable of oxidising metals, resulting in insoluble deposits on the pipe walls which lead to constriction and reduction of the efficiency of water distribution. Thus iron deposition has been observed in the presence of *Gallionella* sp. (Ridgway & Olson, 1981) and manganese, iron and calcium was deposited onto surfaces by budding hyphal bacteria, presumably *Hyphomicrobium* or *Pedomicrobium* sp. (Sly *et al.*, 1988).

1.7.2 Chlorine assimilation

The chlorine residual of chlorinated potable water decreases as the water moves away from the treatment plant due to (i) the intrinsic instability of free chlorine and (ii) assimilation by biofilms. In a large scale study of a drinking water utility in New

Jersey, LeChevallier *et al.* (1987) found that the species diversity in the water and the coliform count increased as water flowed further down the line. Assimilable organic carbon (AOC) was utilised by bacteria in the mains pipelines. This investigation clearly demonstrated that the microbiological quality of water deteriorates as the water flows along distribution pipes. Chlorine can also react with metal oxides in biofilms so biocorrosion may aid the ability of the corroding consortium to evade the chlorine residual (LeChevallier, 1991).

1.7.3 Harbours of indicator organisms and pathogens

The study by LeChevallier *et al.* (1987) was instigated after a water utility had experienced problems from the repeated detection of coliforms in the network. Since the coliform concentration was one of the parameters routinely analysed to indicate the microbiological quality of the water, the chronic presence of these bacteria in the distribution system would mask a true breakdown of treatment processes and was therefore unacceptable. However, none of the control measures assessed in the study, including maintenance of a free chlorine residual of 1 mg l⁻¹, flushing or pigging the pipes, was effective in removing the coliforms.

Although the persistent presence of indicator organisms in mains water can be a very costly nuisance, it is the maintenance of pathogens in distribution network biofilms that is of most concern to the water industry. Many recent studies have demonstrated extended survival of bacterial pathogens in biofilms formed by drinking water bacteria. A syntrophic interaction enabled a pathogenic *E. coli* strain unable to degrade benzoate to grow within a biofilm of a benzoate-degrading water bacterium in a laboratory reactor fed only with benzoate (Szewzyk *et al.*, 1994). *Legionella pneumophila* cells were shown to survive within a drinking water biofilm in the absence of host cells (Rogers & Keevil, 1992). The persistence of *Campylobacter* spp. was considerably enhanced in the presence of the autochthonous drinking water microflora compared to sterile microcosms (Buswell *et al.*, 1998). The factors affecting the survival of allochthonous bacteria in aquatic systems have recently been reviewed (Barcina *et al.*, 1997).

The survival of non-bacterial pathogens in potable water biofilms is only beginning to be studied. Viruses are the etiological agent responsible for more than one-third of waterborne disease outbreaks in North America (Cubitt, 1991) or in Europe (Hunter, 1994), but very few investigators have searched for viruses in biofilms. One exception is a recent study where poliovirus-1 was shown to accumulate within biofilms in a pilot-scale distribution system (Quignon *et al.*, 1997a,b). This increased the viral residence time in the system by roughly three times but did not reduce susceptibility of the virus to chlorine. Protozoa can also cause waterborne diseases. Of particular concern are *Giardia* spp. and *Cryptosporidium parvum*. A single outbreak of cryptosporidiosis affected over 400,000 people in Milwaukee, USA in 1993 (MacKenzie *et al.*, 1994). It has been shown that *C. parvum* oocysts can survive in biofilms for many weeks in an infectious state, leading to the suggestion that sloughing of fragments of the biofilm may be the source of the many sporadic cases of unknown origin (Keevil *et al.*, 1995).

Improved molecular techniques for the *in situ* detection of pathogens will undoubtedly increase the number of findings of waterborne pathogens in distribution systems. It is a challenge for the water industry to find new methods for preventing unwanted organisms from becoming sequestered in biofilms.

1.7.4 Taste, odour and colour problems

Other unwanted organisms that may be harboured within potable water biofilms include those species that cause strong tastes or odours. For example, actinomycetes are commonly found in drinking water and have been linked to taste and odour complaints (Berman, 1973). However, the majority of taste, odour and colour problems in tap water probably arise from leaching of metal ions into the water as a result of the biodeterioration processes discussed above.

1.7.5 Endotoxin release

One other potential problem arising from the presence of bacteria in distribution pipelines is that of endotoxin release into the water. Endotoxins are heat stable LPS-protein complexes derived from the outer membrane of Gram-negative bacteria, including cyanobacteria. They have been implicated in a variety of human diseases including asthma, dermatitis and toxic shock syndrome. At present the most effective assay for the detection of endotoxins is the Chromogenic *Limulus* Amoebocyte Lysate Assay (Grabow *et al.*, 1991). Low levels of endotoxins are routinely detected in drinking water and are not removed by distillation or sterilization with steam or ethylene oxide. This may cause problems when distilled water is used for kidney dialysis machines and intravenously infused nutrients. In addition, several cases of gastrointestinal disease have been linked to cyanobacterial blooms in drinking water reservoirs (Hunter, 1995). However, the overall health implications of endotoxins in drinking water remain unclear.

The endotoxin concentration is reduced during water treatment, although some treatment processes, notably filtration through granular activated carbon, can actually increase the amount of endotoxins in the water (Burger *et al.*, 1989). As yet no studies have specifically addressed the issue of the contribution made by distribution pipeline biofilms to the endotoxin concentration in tap water and the subsequent impact on the health of the consumer.

1.8 Control and manipulation of biofilms.

It is not realistic to aim for complete and sustained exclusion of bacteria from water distribution systems. Removal of bacteria from pipes or installation of new pipes presents clean surfaces for colonisation by bacteria in the water column, which in turn leads to a defined succession of organisms before a mature biofilm becomes established. Therefore in order to minimise the diversity of bacteria released from biofilms into drinking water it is more appropriate to try to manipulate existing biofilms than to aim to dislodge mature biofilms and build a defined population on

the clean surface. Attached cell systems are commonly used in industry to carry out bioremediation of polluted soil or wastewater and to treat drinking water. Some of these systems are discussed in Section 1.8.2 with a view to targeting potential areas for the manipulation of existing distribution pipeline biofilms to carry out beneficial processes (Section 1.8.3). However, when unwanted organisms become integrated into biofilms removal of at least part of the attached population is essential and it is a review of some existing and potential methods for biofilm removal that begins this discussion of the control and manipulation of biofilms.

1.8.1 Removal of attached bacteria

1.8.1.1 Physical methods

There are currently two physical methods in use for the removal of attached cells from the surfaces of distribution pipes. Flushing involves increasing the rate of flow of water dramatically to dislodge bacteria by hydrodynamic shear. This method is cheap and can easily be applied to large sections of pipeline. The alternative is pigging, whereby swabs are sent through sections of the pipe to mechanically disrupt biofilms. This procedure removes up to 95% of the attached cells (LeChevallier *et al.*, 1987), but requires opening the mains pipes to insert and remove the swab. This can be a very costly and time-consuming process if biofilms are to be removed from long sections of pipe. Although flushing and pigging remove large numbers of attached cells, the biofilm regrows within around a week with little change to the bacterial population (LeChevallier *et al.*, 1987).

Of the new physical techniques that are becoming available, the use of ultrasound carries the most promise for control of distribution system biofilms. Ultrasound creates bubbles of high energy that cause biofilm disruption by cavitation. In a recent study short bursts (30 sec) of ultrasound at a frequency of 150 kHz proved effective for removing biofilms of *Proteus mirabilis* along 50 cm of the inside of a glass pipe, 18 mm in diameter (Mott *et al.*, 1997). Of course the dimensions over

which the effects of the ultrasound are transmitted must be increased dramatically for this technology to be of use in distribution systems.

Pulsed laser beams (Sadoudi *et al.*, 1997) and electric currents in combination with antibiotics (Costerton *et al.*, 1994; Jass & Lappin-Scott, 1996) have been shown to increase the efficiency of elimination of biofilm bacteria, but it is difficult to see how these technologies could be applied to distribution biofilms.

1.8.1.2 Chemical treatments

Chemical treatments for the inactivation of biofilm bacteria have been considered previously (Section 1.2.4). At least two mechanisms have been shown to enhance biofilm removal independently of bactericidal effects.

A combination of two polysaccharide-lysing enzymes, a mutanase and a dextranase, caused a 1-2 log drop in the number of adherent cells present in model plaque biofilms (Johansen *et al.*, 1997). The use of enzymes for the degradation of the EPS material binding cells to surfaces is a particularly attractive approach since enzymes have high specificity and could therefore release one unwanted strain without total disruption of the biofilm. In view of the open architecture of potable water biofilms penetration of highly specific enzymes may not be a major problem. However, enzymes are expensive to produce and tend to be less effective at the relatively low temperatures of drinking water than at 37°C.

Since EPS is co-ordinated by metal ions, ion chelation should lead to a loosening of the biofilm matrix. Turakhia *et al.* (1983) demonstrated immediate detachment of cells from a biofilm upon chelation of Ca²⁺ ions with EGTA. Removal was not complete, suggesting that additional mechanisms were acting to hold cells to the surface. The potential of metal ion chelators to act synergistically with other treatments to aid biofilm removal in drinking water systems has not been assessed. Depletion of metal ions as part of water treatment may help to prevent the formation

of stable EPS matrices, although it could lead to increased rates of metal ion leaching from the substrata.

1.8.1.3 Biological control

It has been suggested that protozoan grazing may be the main factor that determines the removal of allochthonous bacteria from aquatic systems (Barcina *et al.*, 1997). The rate of grazing is dependant on the strain and metabolic state of the prey. Gram-negative bacteria are more rapidly eliminated than Gram-positive cells because they are more easily digested (Iriberry *et al.*, 1994). Large, active cells are grazed preferentially to small cells (González *et al.*, 1993). A recent survey of protozoa in distribution systems found a very low grazing activity against biofilms and undetectable levels of grazing in the planktonic phase (Sibille *et al.*, 1998). However, artificially introduced *E. coli* were more rapidly lost from granular activated carbon (GAC)-treated water than from nanofiltered water, presumably because nanofiltration removed eukaryotic grazers. Understanding of the characteristics of grazing may enable the addition of protozoa to distribution systems to aid the specific removal of unwanted bacteria.

Infection by *Bdellovibrio* or bacteriophage may contribute to the removal of allochthonous bacteria from aquatic systems (Barcina *et al.*, 1997), suggesting another possible mechanism for the control of sessile bacteria. However, it should be noted that starvation increases the resistance of vegetative bacteria to bacteriophage infection (Kokjohn *et al.*, 1991).

1.8.2 Industrial use of biofilms

Biofilms are used for a variety of industrial processes, including vinegar production, microbial leaching and wastewater treatment (Bryers, 1993). There are several advantages of using fixed rather than free cells: (i) fixed cells often have a higher productivity, (ii) the products are easily separated from the cells and (iii) the reaction is localised and easily controlled. The use of immobilised cells in wastewater

treatment has been reviewed annually in *Water Environment Research* for several years and the latest of these reviews (Brower & Barford, 1997) provides an insight into the incredible variety of fixed-film bioreactors that have been developed. The aim of all these systems is to clean water, but this may be by removal of specific organic pollutants such as aromatic or halogenated compounds, nitrate removal or by reduction of the total concentration of organic material present.

Biofilms on sand or GAC are used to reduce the levels of organic carbon in potable water prior to distribution. It has been suggested that a sufficient reduction in the level of organic carbon could be an effective mechanism for preventing growth in distribution pipes. Concentrations of $<10 \mu\text{g l}^{-1}$ assimilable organic carbon (AOC) (van der Kooij, 1992) or $<150 \mu\text{g l}^{-1}$ biodegradable dissolved organic carbon (BDOC) (Servais *et al.*, 1995) have been proposed for the effective control of growth, although tests with an experimental distribution system demonstrated that a BDOC concentration of $60 \mu\text{g l}^{-1}$ could still support a heterotrophic biomass (Sibille *et al.*, 1997).

1.8.3 Manipulation of distribution system biofilms

Having considered some of the ways in which biofilms have been exploited in industry, I will now propose some potential aims for the manipulation of existing biofilms in distribution pipes to carry out reactions that would enhance the quality of tap water.

1. Biofilms in distribution pipes remove DOC from the water. If this removal could be enhanced by biofilms close to the distribution plant then growth downstream would be limited. Similarly, removal of nitrates and/or phosphates might help to control bacterial growth in the distribution system.
2. Chlorination of water results in the formation of trihalomethanes (THMs) which are carcinogenic. Fixed-film bioreactors can remove up to 98% of THMs from wastewater (Qaisi & Qasem, 1996). Manipulation of biofilms to enhance THM

removal from finished water might allow the use of higher concentrations of chlorine for water treatment. In theory, other harmful chemicals (e.g. phenols or toxic metals) could also be removed by *in situ* biofilms.

3. Exclusion of unwanted organisms from biofilms is essential to prevent colonisation of the distribution system. If biofilms could be engineered to exclude pathogens and other unwanted organisms this would clearly be of substantial benefit to the water industry and of course, to the consumer.

4. The engineering of consortia incapable of biocorrosion could increase the life span of metal pipes. Wolfaardt & Cloete (1992) have observed that removal of sulphate from water limits biocorrosion. Obviously this would need to be carried out at the treatment plant or by biofilms on non-metal pipes.

Before manipulation of distribution pipelines can be performed, it is essential to understand the mechanisms that bacteria use to survive and grow in distribution systems. This project is directed towards increasing our understanding of these mechanisms.

1.9 The local distribution network

Studies described here were performed on biofilms developed from tap water at Warwick University. This water is treated at Strensham treatment plant. The water undergoes flocculation, rapid gravity filtration, filtration through GAC and chlorination. Water leaving the plant is adjusted to pH 7.2-7.6, around 0.7 mg free chlorine l^{-1} and must contain 0 coliforms $100\ ml^{-1}$. The water is distributed through a series of bitumen-coated steel and prestressed concrete pipes before reaching the University (Fig. 1.5). There is no storage reservoir between the treatment plant and the University, but there is a chlorine booster about halfway along the mains.

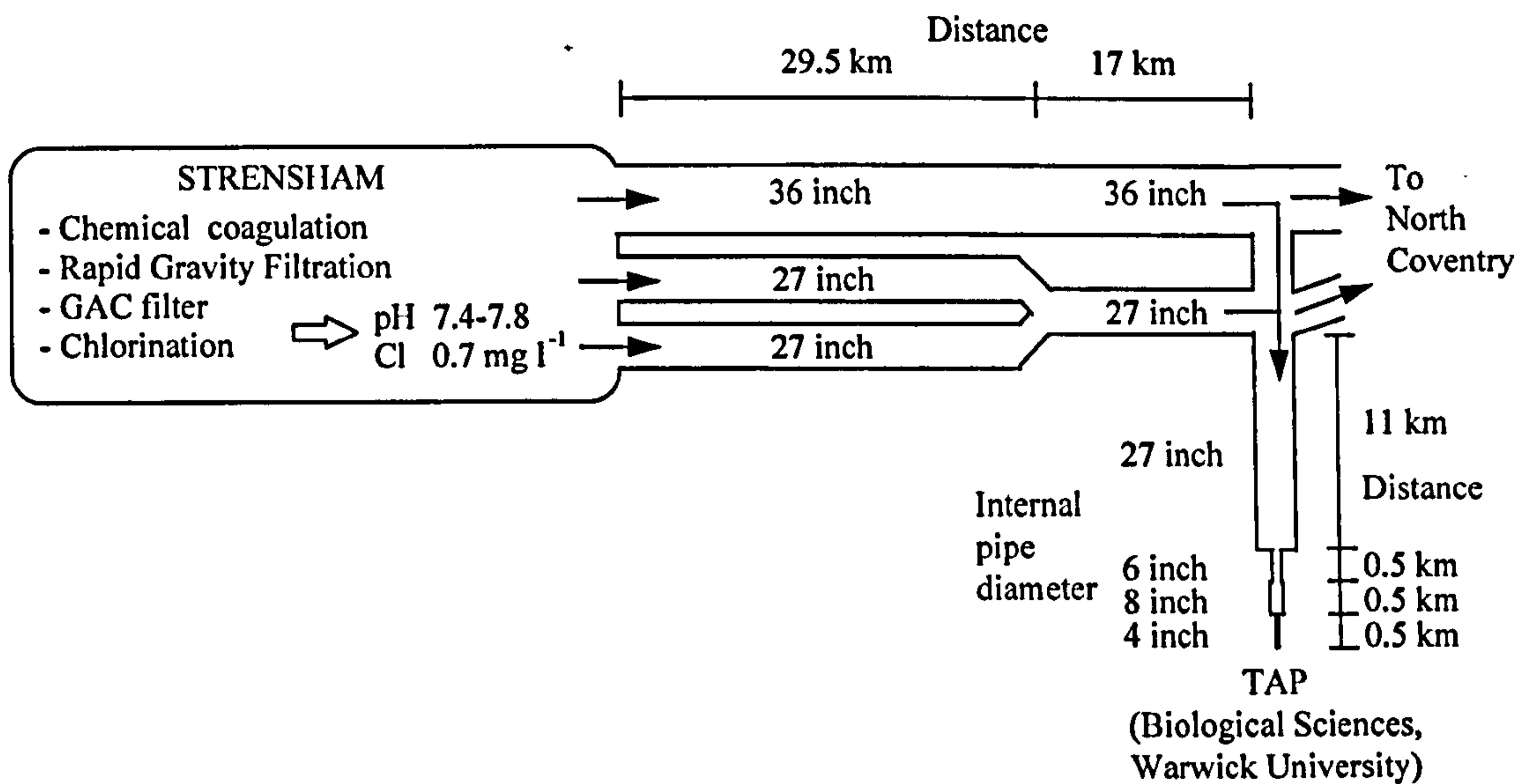


Figure 1.5 Schematic diagram of the local distribution system supplying Warwick University. Water is treated at Strensham Treatment Works and leaves the plant containing around 0.7 mg free chlorine l⁻¹ and 0 coliforms 100 ml⁻¹. It is then distributed along three mains pipelines, two of which merge 29.5 km downstream from Strensham. A single pipeline draws water from the remaining two pipes to supply southern Coventry including Warwick University. Other connections drawing water from the mains pipelines are not shown. Near the University a succession of smaller pipes distributes water to the Department of Biological Sciences where the work in this thesis was performed.

1.10 Aims

The overriding aim of this project was to assess the role of bi- or multi-phasic life cycles and vegetative dormancy in the survival of heterotrophic bacteria in potable water and in partitioning between biofilms and the aqueous phase. Specific targets towards this goal included:

- Identification of bacteria isolated from tap water at Warwick University to obtain an indication of some of the genera present.

- Analysis of cellular activity through the batch growth cycle of two tap water isolates to:
 - look for signs of a bi- or multi-phasic life cycle expressed by a morphologically indistinct bacterium by comparison with a prosthecate isolate;
 - develop a protocol for *in situ* analysis of physiological activity.
- Assessment of the correlation between expression of cell surface characteristics or specific proteins and attachment to identify mechanisms by which the metabolic state of cells may be linked to surface adhesion.
- Direct microscopic examination of morphologically distinctive cell types present in tap water to assess the diversity of known cell cycle-dependant adaptations for survival and growth in low nutrient systems.
- Development of a laboratory model for biofilm development from potable water to estimate:
 - the activity of planktonic cells and their ability to respond to an increase in the nutrient concentration in the water;
 - the succession of microorganisms on a solid substratum during biofilm accumulation;
 - the activity of cells in mature biofilms and their ability to respond to dissolved organic carbon;
 - the contribution of biofilms to the planktonic population.

CHAPTER 2

2 Materials and Methods

2.1 Bacterial strains

Caulobacter crescentus CB15 (NCIB) was cultured at 30°C in PYE medium with shaking. All other strains studied were isolated from tap water at Warwick University.

2.2 Bacterial identification

Isolated bacterial strains were identified initially on the basis of colonial and cellular morphology, motility, ability to grow anaerobically, Gram stain, oxidase and catalase tests with reference to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Where relevant, API20NE strips (Bio Mérieux, France) were employed in accordance with the manufacturer's instructions. The pattern of reactions was coded into a numerical profile and identification made with the computer-based API Profile Recognition System.

2.3 Media

2.3.1 Tryptone yeast extract glucose (TYG) medium

Sphingomonas sp. was routinely cultured in TYG containing:

Tryptone	5 g
Yeast extract	2.5 g
Glucose	1 g

made up to one litre with distilled water and sterilised by autoclaving at 121°C for 15 min.

2.3.2 Peptone yeast extract (PYE) medium

The following were added (per litre):

Bacto-peptone	2 g
Yeast extract	1 g
MgSO ₄ .7H ₂ O	0.2 g
Riboflavin	1.0 mg

The pH was adjusted to 7.0 and the medium was sterilised by autoclaving at 121°C for 15 min. After cooling, a final pH of 6.9 was obtained by addition of filter-sterile 0.1 M phosphate buffer (17.6 g l⁻¹ Na₂HPO₄.12H₂O, 8.0 g l⁻¹ NaH₂PO₄.2H₂O) to a final concentration of 5% (v/v). Filter-sterile D-glucose was added to give a final concentration of 0.2% (w/v).

2.3.3 R2A broth

The medium of Reasoner & Geldreich (1985) was employed for subculture of microorganisms isolated from tap water.

2.3.4 Minimal salts media

2.3.4.1 Carbon limited medium

The following ingredients were added (per litre):

Na ₂ HPO ₄	2.0 g
KH ₂ PO ₄	1.0 g
NH ₄ Cl	1.9 g
NH ₄ NO ₃	1.9 g
Na ₂ SO ₄	2.0 g
MgSO ₄ .7H ₂ O	0.2 g

The pH was adjusted to 7.0 and volumes less than two litres were sterilised by autoclaving at 121°C for 15 min. For larger volumes, filter sterilised phosphates were added after cooling to avoid precipitation. Filter-sterile glucose was added immediately before use to produce a concentration of 0.1% (w/v).

2.3.4.2 Nitrogen limited medium

The following were added (per litre):

Na ₂ HPO ₄	2.0 g
KH ₂ PO ₄	1.0 g
NH ₄ Cl	0.1 g
NH ₄ NO ₃	0.1 g
Na ₂ SO ₄	2.0 g
MgSO ₄ .7H ₂ O	0.2 g

This was adjusted to pH 7.0 and autoclaved at 121°C for 15 min. For large volumes (≥ 2 l), filter sterilised phosphates were added after cooling to avoid precipitation. Filter sterile glucose was added to a final concentration of 0.5% (w/v) immediately before use.

2.3.4.3 Hyphomicrobium basal (HB) medium

The medium developed by Attwood & Harder (1972) was used. The following ingredients were included (per litre):

KNO ₃	5.0 g
K ₂ HPO ₄	1.74 g
NaH ₂ PO ₄ .2H ₂ O	1.38 g
(NH ₄) ₂ SO ₄	0.5 g
MgSO ₄ .7H ₂ O	0.2 g
CaCl ₂ .2H ₂ O	0.025 mg
FeCl ₂ .4H ₂ O	3.5 mg

The pH was adjusted to 7.0 and the solution was sterilised by autoclaving at 121°C for 15 min. To avoid precipitation of calcium and iron salts, the calcium chloride and iron (II) chloride were autoclaved separately and added after cooling. 0.05% (v/v) Pfennig's trace elements (500 mg EDTA, 200 mg FeSO₄, 10 mg ZnSO₄, 3 mg MnCl₂.4H₂O, 30 mg H₃BO₃, 20 mg CaCl₂.6H₂O, 1 mg CuCl₂, 2 mg NiCl₂, 3 mg Na₂MoO₄.2H₂O per litre of distilled water) were added after cooling. 0.5% (v/v) filter-sterile methanol was added immediately before use.

2.3.5 Firm agar

Plate count agar (PCA) and R2A (both Oxoid) were used for isolation and routine culture and maintenance. Agar plates were produced from minimal salts media or PYE by inclusion of 1.5% (w/v) bacto-agar (Difco) before autoclaving.

2.3.6 Low nutrient sloppy agar

This medium was employed for the isolation of oligotrophic bacteria from potable water. A lower layer containing 0.005% (w/v) bacto-peptone and 1.5% (w/v) bacto-agar (Difco) in tap water was poured and allowed to set. The upper sloppy agar layer, containing 0.3% (w/v) bacto-agar in tap water, was autoclaved, cooled to 50°C, then inoculated with 200 µl of neat tap water or tap water diluted in sterile distilled water and poured immediately.

2.4 Maintenance of cultures

To avoid attenuation of isolates, cells from exponential phase cultures were harvested, washed and resuspended in sterile saline (0.85% (w/v) NaCl). An equal volume of glycerol was added and cultures were stored at -20°C. Cells were streaked onto PCA or R2A once a month to limit adaptation to the laboratory environment.

2.5 Batch culture

2.5.1 Aerobic

Liquid cultures were propagated overnight by inoculating 10 ml medium in a 25 ml universal bottle with a single colony picked from an agar plate. Batch culture experiments were started by adding 5 ml of an overnight culture to 95 ml medium in a 250 ml conical flask. The flask was shaken at 220 rpm in a Gallenkamp orbital shaker at 30°C.

2.5.2 Anaerobic

For anaerobic batch culture, flasks were fitted with subaseals and flushed with sterile oxygen-free nitrogen for 2 min before inoculation.

2.6 Static batch enrichment of tap water

400 ml of potable water were poured into a sterile 500 ml conical flask. Surfaces for colonisation, in the form of glass coverslips, were inserted along the length of several pieces of silicone tubing. These were placed vertically in the flask, which was then covered with foil and stored in the dark at room temperature for a total of 74 weeks.

2.7 Agar plate biofilms

Biofilms were developed on 45 mm diameter Millipore GS-membranes with pore size 0.22 μm by a modification of the method described by Bühler *et al.* (1998). To generate carbon-limited *Sphingomonas* sp. biofilms, cells were cultured to the exponential phase in carbon-limited minimal salts medium. Total cell counts were determined using *CellFacts* (Microbial Systems Ltd, Coventry) and cultures were diluted to 10^6 cells ml^{-1} in carbon-limited no glucose medium (carbon limited medium without glucose). The filter apparatus was rinsed with 10 ml of carbon-limited no glucose medium, 10 ml of the cell suspension were filtered and the membrane was rinsed again with the carbon-limited no glucose medium. The filter was placed in the centre of a prewarmed carbon-limited agar plate, which was incubated at 30°C for up to 60 hrs. To produce nitrogen-limited *Sphingomonas* sp. biofilms, the same protocol was applied except that nitrogen-limited minimal salts medium was used throughout in place of the carbon-limited medium.

Biofilm-grown cells were harvested at 12, 36 or 60 hrs by gently scraping the membrane with a sterile razor blade and resuspending in 4 ml of sterile saline (0.85% (w/v) NaCl). Examination of the filter by scanning electron microscopy before and after scraping confirmed that the vast majority of cells had been removed by this technique. The bacterial load on the filter was determined by $\text{OD}_{600\text{nm}}$, *CellFacts* analysis and protein assay (Section 2.30).

2.8 Chemostat culture

To obtain a continuous culture of *Sphingomonas* sp., cells were introduced into a chemostat in either carbon or nitrogen limited minimal salts medium. Use of a chemostat allowed control of the specific growth rate by regulation of the rate of flow of nutrients through the vessel. The layout of the chemostat is illustrated in Figs 2.1 and 2.2.

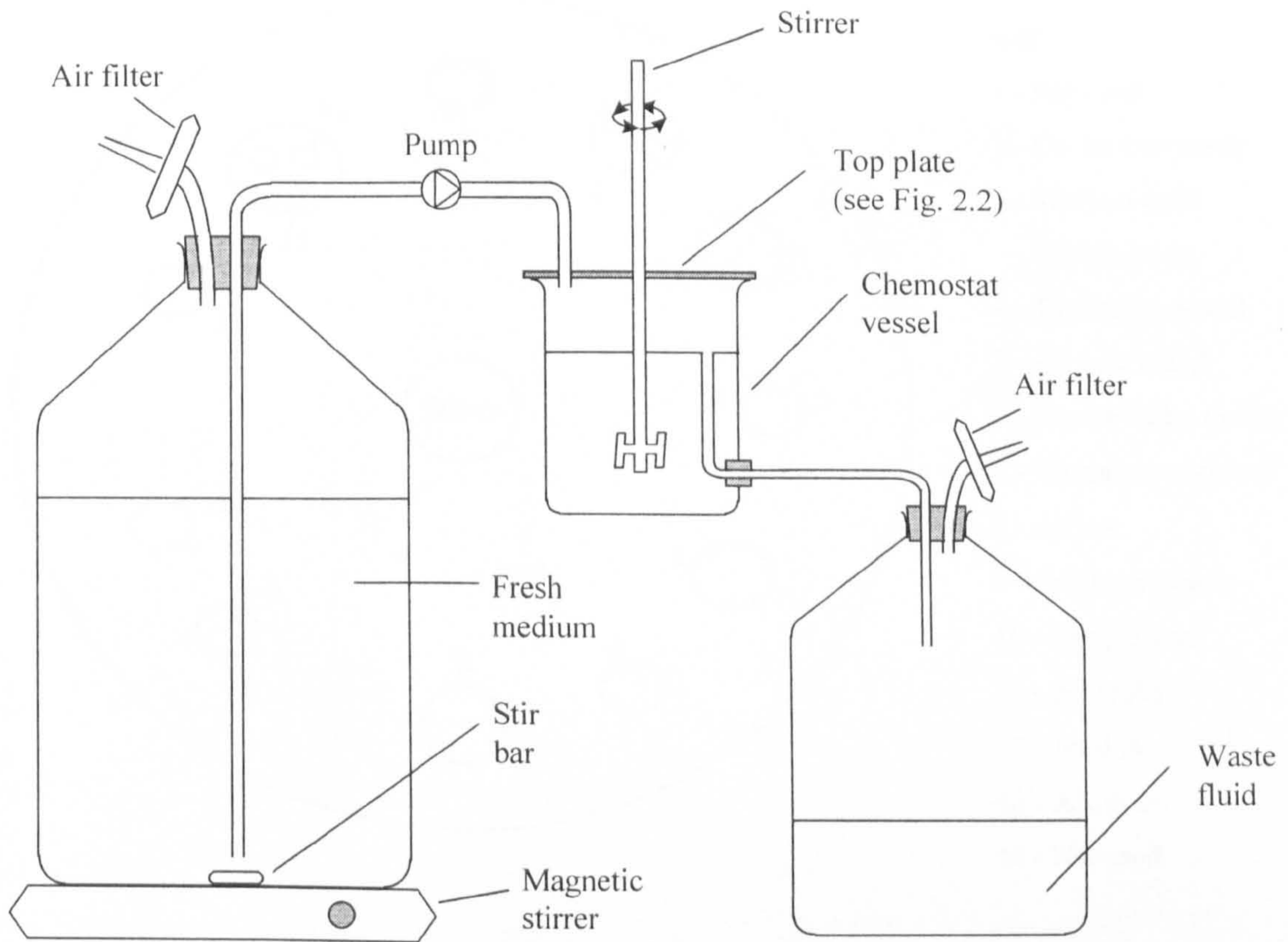


Figure 2.1 Chemostat set-up. *Sphingomonas* sp. cells were cultured in the chemostat vessel. The specific growth rate was controlled by maintaining a constant throughput of growth medium. For clarity, other controls, including pH, temperature and aeration (see text) are not shown.

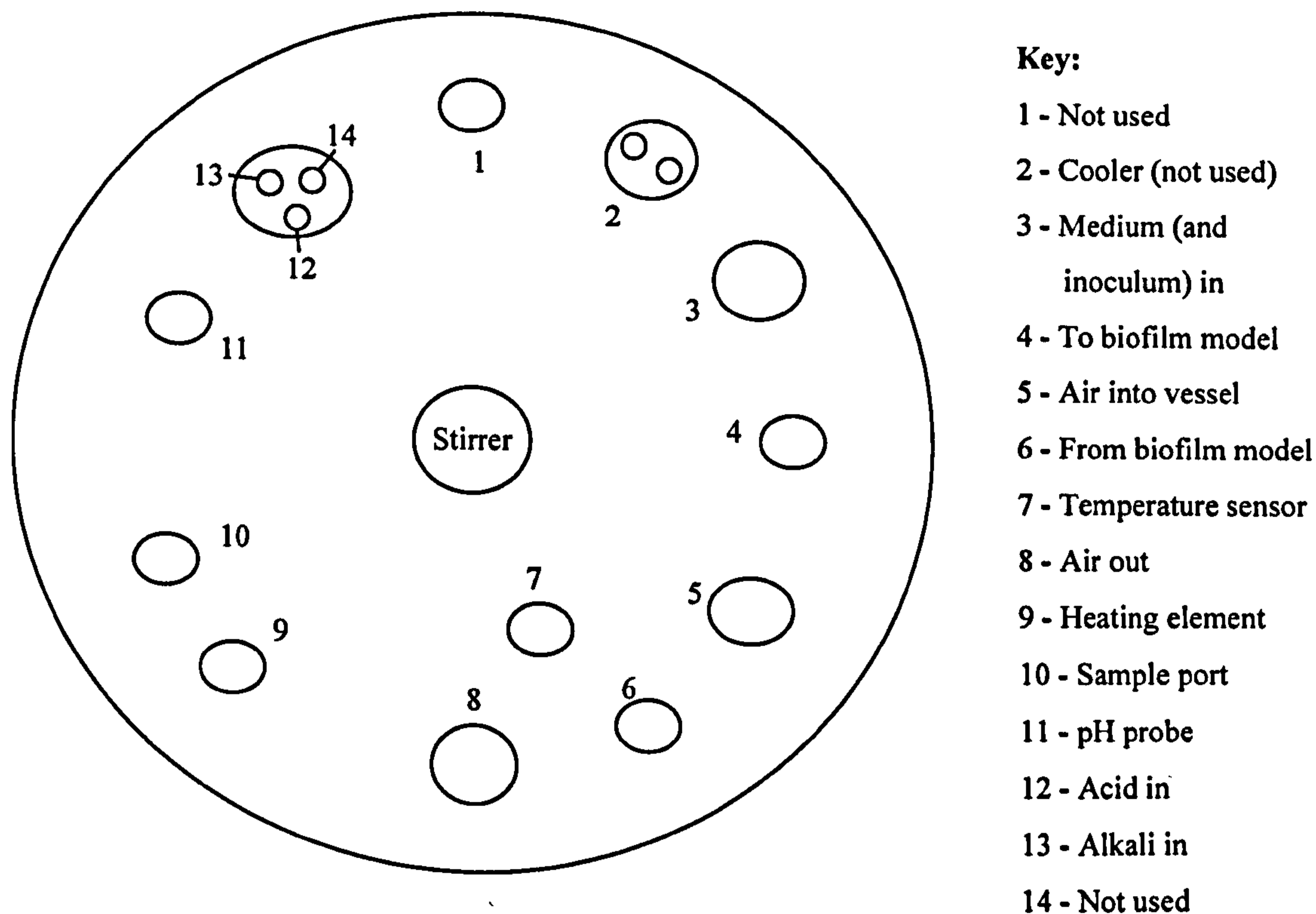


Figure 2.2 Layout of the top plate of the chemostat. The conditions used are described in the text.

Cells were cultured in a 2 l continuous culture vessel (LH fermenter). Fresh carbon- or nitrogen-limited medium was pumped into the chemostat at a constant rate. Cells were aerated at a rate of 250 ml min^{-1} and stirred at 500-750 rpm. The temperature was controlled at 30°C and pH was maintained at 6.8-7.2 by the automated addition of 1M sulphuric acid (H_2SO_4) or 1M potassium hydroxide (KOH).

2.9 Biofilm development

Biofilms were developed from chemostat cultures on glass coverslips held within a biofilm development vessel (Fig. 2.3). This was introduced to the chemostat on a recirculating loop after steady state growth kinetics had been reached. The glass coverslips were removed aseptically at intervals for analysis.

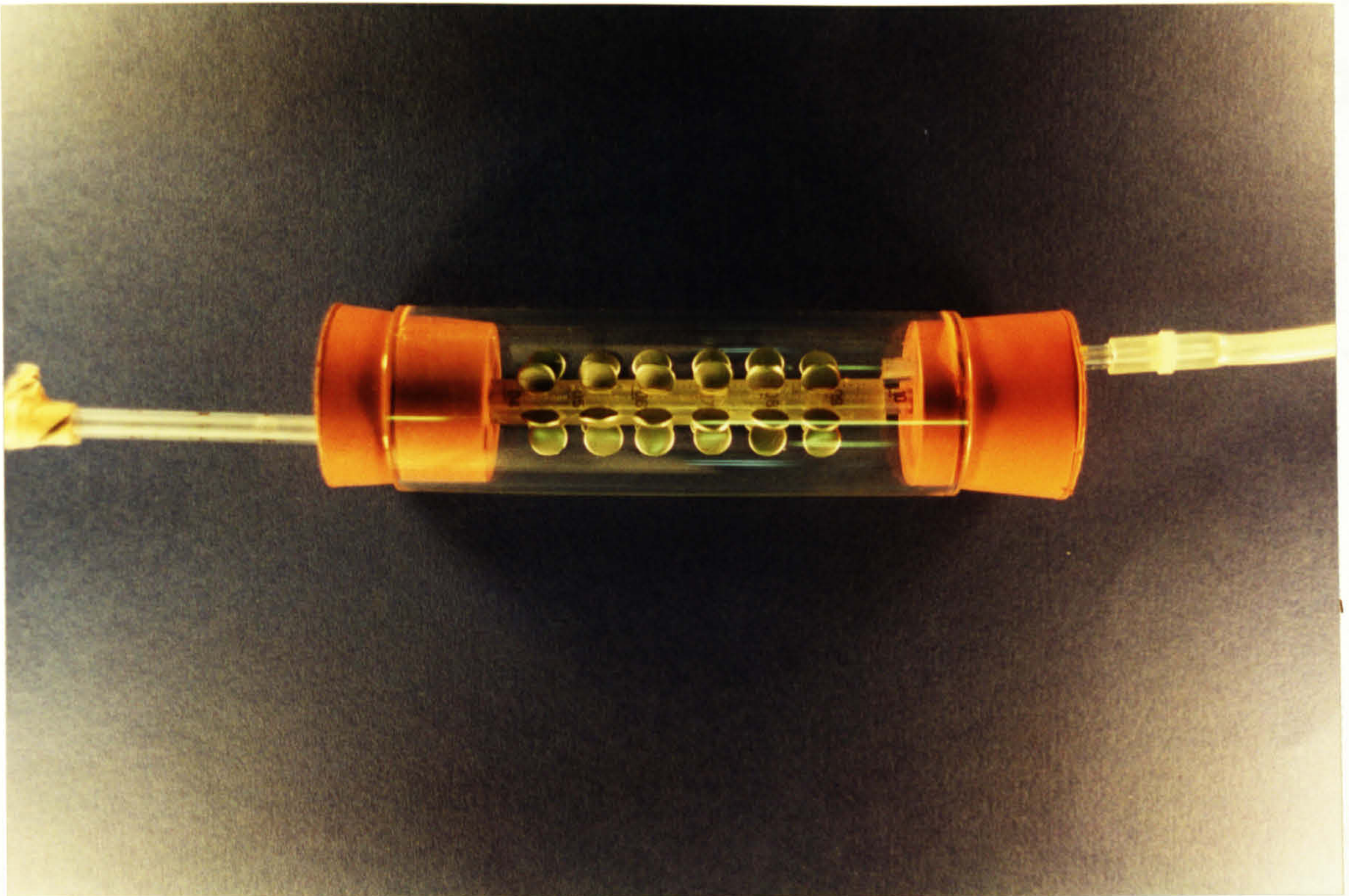


Figure 2.3 The biofilm development vessel. Liquid entered the vessel through tubing pushed through the rubber bungs at either end. The bungs were held in place using metal clamps. Surfaces for microbial attachment (shown here as steel discs, although glass coverslips were employed throughout this study) were inserted into slits cut in a length of silicone tubing surrounding a central glass rod, which was partially inserted into each of the rubber bungs.

2.10 Total particle counts and size analysis

Total particle counts and sizes were measured using *CellFacts* (Microbial Systems Ltd, Coventry). This instrument contains an orifice through which electrolyte flows. The flow of electrolyte carries an electric current between electrodes on either side of the orifice. Samples for analysis are mixed with the electrolyte before it is drawn through the orifice. Particles passing through the orifice impede the flow of electrical current and the instrument detects each impedance event as a voltage pulse, the size of which is directly proportional to the volume of the particle causing it.

Samples for analysis were removed from bacterial cultures or from the continuous flow model, maintained at room temperature and analysed within 10 min. The data collected were analysed using the *CellFacts Industrial* software (supplied). For each sample a profile was obtained which related the particle size, given as equivalent spherical diameter (ESD), to the number of particles of that size measured. The software also allowed calculation of the total number of particles detected within a given size range. The orifice used for these studies allowed detection of particles within the size range 0.75 to 9.5 μm ESD. The minimum particle concentration required for analysis was around 10^5 particles ml^{-1} .

Each sample was analysed in triplicate. Replicates were always tightly matched and standard deviations between the total counts or the counts within specified size ranges were usually too small to be clearly visible on graphs. Standard deviations were therefore not displayed unless they were large enough to be seen.

2.11 Total viable count determination

Total viable counts were determined by spreading 100 μl of an appropriate range of serial ten-fold dilutions onto the surface of prewarmed agar plates. Cells from monocultures were diluted in phosphate-buffered saline (PBS, pH 7.4) and each dilution was spread in triplicate. *Caulobacter crescentus* CB15 cells were spread onto PYE agar and PCA was used for counting *Sphingomonas* sp. cells. After incubation at 30°C for 48 hrs, colonies were enumerated and the original number of colony forming units (CFU) ml^{-1} was calculated.

Triplicate samples from potable water were diluted in sterile distilled water and each dilution was spread in duplicate onto prewarmed R2A plates. After incubation at 30°C for 5 days, colonies were counted and calculations of CFU ml^{-1} were made.

2.12 Specific viable counts

Viable counts of individual species from a mixed microbial population in tap water were determined by counting all colonies expressing an identical morphology. Serial dilutions were prepared and spread over R2A as described in Section 2.11. Plates containing 30-300 colonies in total were selected for manual enumeration of specific colony types. For a rough check that identical colony morphologies were not produced by different groups of bacteria, wet mounts of cells from several colonies of each type were prepared and examined by phase contrast microscopy using a x100 objective.

2.13 Spectrophotometric measurements

A Shimadzu UV-150-02 Double Beam spectrophotometer was used for determination of attenuation of light of a specific wavelength. 1 ml of the sample was placed in a clear plastic cuvette giving a 1 cm light path. The attenuation, in relative units, was determined by reference to a sample blank and a second blank was used to zero the instrument. For monitoring the kinetics of bacterial batch growth, triplicate samples were measured, but the standard deviations between them were not displayed on graphs as they were always too low to be visible.

2.14 CTC staining

Samples taken from batch culture were divided into 3 x 1 ml aliquots for staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Park Scientific Ltd). A negative control (growth medium alone for fluorimetry; no CTC for microscopy) was also included. 0.1 ml of 5 mM CTC was added to each sample and mixed, then samples were incubated in the dark for 30 min at 30°C. The concentration and time of staining was chosen on the basis of empirical measurements for each species (Section 4.3). Samples were then analysed fluorimetrically or microscopically, as described below.

Biofilms on glass coverslips were rinsed with sterile distilled water before staining with fluorescent dyes. The coverslips were then immersed in 1 ml of 0.5 mM CTC in 10% TYG and incubated in the dark for 30 min. A negative control (no CTC) and a positive control (actively growing cells from a laboratory culture) were included each time and three coverslips were stained for quantitative analysis.

2.14.1 Fluorimetry

Samples were transferred to 3 ml cuvettes and diluted in 2 ml of sterile distilled water prior to fluorimetric analysis. The fluorescence of liquid samples was determined using a Perkin Elmer LS-5 Luminescence Spectrometer. 3 ml of each sample were placed in a clear plastic cuvette, kept dark and analysed within five minutes of taking the sample. The instrument was set to zero using a blank (no fluorescent dye). For measurement of CTC-formazan fluorescence, the excitation wavelength was set to 450 nm \pm 10 nm and the emission wavelength to 630 nm \pm 2.5 nm.

2.14.2 Microscopic quantitation

After CTC staining, cells in suspension or on coverslips were washed twice with sterile saline and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma), as described below. Samples were analysed by epifluorescence microscopy (Section 2.19) and active and total cell counts were estimated using digital image analysis (Section 2.21).

2.15 DAPI staining

Samples were stained with 0.2 $\mu\text{g ml}^{-1}$ DAPI in the dark at room temperature for 15 min. Cells in suspension were then washed three times in sterile saline (0.85% (w/v) NaCl) and resuspended in 1 ml of saline. 10 μl were transferred to a well of 4 mm diameter on a quantitative microscope slide (ICN) and dried at 37°C in the dark. Slides were mounted in a Mowiol solution (Turpin *et al.*, 1993) to reduce

photofading. A glass coverslip was placed over the Mowiol and the sample was analysed by fluorescence microscopy.

Coverslips were rinsed six times with sterile saline, mounted in Mowiol solution and imaged under the fluorescence microscope.

2.16 Intracellular ATP

Intracellular ATP concentrations were determined using the ATP Bioluminescence Assay Kit HS II (Boehringer Mannheim, Germany) in accordance with the manufacturer's instructions. The reagents (dilution buffer and cell lysis reagent) were filter sterilised before each use to reduce the background luminescence. For high cell densities (i.e. *Sphingomonas* sp. monocultures), 25 µl of cell lysis reagent were added to an equal volume of cells, mixed and incubated for 5 min at room temperature. Samples were measured in triplicate and a negative control (growth medium without cells) was included. For low cell densities (i.e. potable water), cells were concentrated from 200 ml of solution by filtration onto a cellulose nitrate filter of pore size 0.2 µm (Sartorius, Germany). Sterile distilled water was also filtered each time to provide a negative control. ATP was extracted from the filter by adding 1 ml of cell lysis reagent and incubating at room temperature for 5 min. In order to minimise the relatively large deviations inherent in the measurement of such small quantities of ATP, samples were taken in triplicate and each of these was measured in triplicate. Means and standard deviations of the three independent samples were plotted graphically. For each set of samples a standard curve was produced by serial dilutions of the ATP standard included in the kit to allow compensation for slight variations in activity of the luciferase enzyme.

After incubation in cell lysis reagent, 50 µl of samples or standards were transferred to a black microtitre plate. This was inserted into a Luminoscan RS luminometer (Labsystems, UK). A protocol was set up to automatically inject 50 µl of luciferase into a well, mix for 1 sec then measure luminescence for 10 secs. The measurement

was integrated to produce a final reading in relative light units (rlu). This was converted into moles ATP ml⁻¹ by reference to the standard curve.

2.17 Cell surface hydrophobicity

Bacterial adhesion to hexadecane (BATH) was applied to estimate the hydrophobicity of the bacterial cell surface. This was an adaptation of the method of Rosenberg *et al.* (1980). Cells were harvested by centrifugation at 2,111g for 15 min in an MSE Mistral 1000 benchtop centrifuge, washed twice and resuspended in PUM buffer (22.2 g K₂HPO₄·3H₂O, 7.26 g KH₂PO₄, 1.8 g urea and 0.2 g MgSO₄·7H₂O dissolved in distilled water and made up to 1 litre), pH 7.1. This suspension was adjusted to OD_{400nm} = 1.5 ± 0.1 and 1.2 ml were transferred to a glass test tube. 100 µl of n-hexadecane (Sigma) were added and the mixture was allowed to stand at 30°C for 10 min. It was then vortexed vigorously for 2 min and left to stand at room temperature for 15 min to allow phase separation. The lower phase was carefully removed and placed in a plastic 1 ml cuvette. The OD_{400nm} was determined and the % adhesion to hexadecane calculated using the following equation:

$$\% \text{ Adhesion to hexadecane} = \frac{(\text{Original OD}_{400\text{nm}} - \text{OD}_{400\text{nm}}) \times 100}{\text{Original OD}_{400\text{nm}}}$$

Six replicates were performed for each analysis and mean values and standard deviations were calculated. Student's t-test was used to determine the level of significance of differences between the means of different samples.

2.18 Light microscopy

Light microscopy was performed using a Leitz Orthoplan microscope fitted with x40 and x100 objective lenses. Immersion oil was used in conjunction with the x100 objective lens. Photomicrographs were taken with a Leitz Orthomat camera and processed as described in Section 2.20.

2.18.1 Wet mounts

To assess cell morphology and motility, cells were picked from a colony on an agar plate using a sterile metal loop and suspended in a drop of water on a microscope slide. A glass coverslip was dropped onto the suspension and cells were examined by phase contrast under the x40 or x100 lens.

2.18.2 Gram stain

Preston and Morrell's modification of Gram's method (Cruikshank *et al.*, 1975) was employed with one modification. Instead of using an iodine-acetone mixture, iodine was rinsed from the slide with tap water. An equivolume mixture of acetone and ethanol was carefully pipetted over the slide until the colour ceased to run. The slide was immediately rinsed thoroughly with tap water prior to carbol-fuschin staining.

2.18.3 Acid fast stain

The Ziehl-Neelsen method for acid fast staining (Cruikshank *et al.*, 1975) was used. Slight modifications were required for direct acid fast staining of biofilms on coverslips. The coverslip was air dried, heat-fixed and placed on a microscope slide. This was flooded with carbol-fuschin stain and heated gently for 5 min. The slide was washed very gently to avoid displacing the coverslip. After the standard decolorizing and washing steps, the coverslip was attached to the slide with Sellotape, mounted in immersion oil and examined under the x100 objective lens.

2.18.4 Congo Red stain

Exopolysaccharides (EPS) were stained using the protocol of Allison & Sutherland (1984) without adaptation.

2.19 Epifluorescent microscopy

A Zeiss Axioskop microscope fitted with Plan-Neofluor x40 and x100 lenses was used for epifluorescent microscopy. An HBO 50 mercury short arc lamp provided the exciting light and the following filter sets were used: for DAPI stain - excitation G365, dichroic mirror FT395 and emission LP420; for CTC stain - excitation BP546/12, dichroic mirror FT560 and emission BP575-640. Fluorescence micrographs were taken using a Yashica 108 multi program camera and processed as described in Section 2.20.

2.20 Photography

Photographs were taken using a Pentax K1000 camera. Kodak Gold ISO 100 film was used for colour photographs, which were processed by Colab (Coventry). Black and white images were taken on TMax 400 Professional film. This was developed according to the manufacturer's instructions and printed onto Kodak Kodabrome II RC photographic paper.

2.21 Digital image analysis

Images were collected using an Hamamatsu C4742 CCD camera attached to the Zeiss Axioskop microscope. Image analysis was performed using the Biovision 2.2 software (Improvision, Coventry). Threshold levels were set subjectively and discrete particles were counted by the software. At least ten fields were counted for each coverslip or quantitative microscope slide. The area of a field was calibrated using a stage graticule. The extent of surface coverage by biofilm bacteria was expressed as cells cm^{-2} . Since the wells of the quantitative microscope slides were known to be 4 mm diameter, the number of fluorescent particles per ml of the original solution could be calculated.

2.22 Scanning electron microscopy

Cells in biofilms were fixed overnight in 2.5% glutaraldehyde at 4°C. They were then rinsed twice with distilled water and dehydrated through a series of ethanol concentrations by immersion for 5 min in 10%, 25%, 50%, 70%, 90% and finally twice in 100% ethanol. Samples were air dried or substituted with amyl acetate by immersion for 15 min in a range of amyl acetate:ethanol concentrations (1:3, 1:1, 3:1 then neat amyl acetate). After substitution, samples were dried at the critical point of carbon dioxide in a Polaron E3000 critical point drier (VG Microtech, Sussex). This procedure minimised sample distortion during drying.

The glass coverslips were mounted onto electron microscope stubs using electrodag 915 high conductivity paint (Agar Scientific Ltd). A fine layer of gold was deposited onto each sample for 120 secs using a BioRad-E5200 sputter-coater. Coated samples were observed under vacuum using a JEOL T330A scanning electron microscope and photomicrographs were taken on Polaroid 53 or 55 film.

2.23 Transmission electron microscopy

Samples were prepared prior to negative staining or heavy metal shadowing as follows: a drop of liquid containing bacterial cells was placed on a formvar-coated copper EM grid (300 mesh, Agar Scientific Ltd). After 2-15 min, excess fluid was removed with blotting paper. Samples were fixed by adding a drop of 2.5% (v/v) EM grade glutaraldehyde (Agar Scientific Ltd) and excess fluid was drawn off with blotting paper. Grids were washed twice with sterile distilled water and blotted carefully to remove the fluid.

Samples were negatively stained by placing a drop of 1% (w/v) phosphotungstic acid on a prepared EM grid and immediately removing excess fluid with blotting paper. Alternatively, samples were shadowed with heavy metal ions. Prepared grids were placed on a piece of blotting paper in an Edwards E306A vacuum coating unit. A piece of gold-palladium wire (Agar Scientific Ltd) about 3 cm in length was wound

around a V-shaped filament made of tungsten wire of diameter 1.5 mm (Agar Scientific Ltd). This was positioned in the vacuum coating unit to create an angle from the wire to the grids of around 45°. The unit was evacuated and grids were shadowed according to the manufacturer's instructions.

Stained or shadowed grids were analysed in a JEOL JEM-100S transmission electron microscope at 80 kV. Photomicrographs were taken on Kodak electron microscope 4489 film, developed and printed as described in Section 2.20.

2.24 Extraction of DNA from cells

Crude DNA preparations were made for amplification of the gene encoding the 16S subunit of rRNA by polymerase chain reaction (PCR). Cells were scraped from colonies on plates using a sterile wooden toothpick and suspended in 50 µl of TE buffer (50 mM Tris, 10 mM EDTA, pH 8). Alternatively, 1 ml of a liquid culture of cells was pelleted by centrifugation (MSE Micro Centaur, 11,600g, 10 min) and resuspended in 50 µl TE. Samples were sonicated on ice at an amplitude of 6 µm peak to peak for three pulses of 1 min, using a Jencons ultrasonicator fitted with a 4 mm probe. Cellular debris was removed by centrifugation at 4°C (MSE Micro Centaur, 11,600g, 5 min). The supernatant containing DNA was transferred to an Eppendorf tube and stored at 4°C.

2.25 PCR Techniques

The PCR reaction mix was made up to a final volume of 50 µl with sterile distilled water and consisted of the following: chromosomal DNA (10 ng or an approximation to this), primer 1 (100 ng), primer 2 (100 ng), dimethylsulphoxide (2.5 µl), 25 mM MgCl₂ (Perkin Elmer Cetus), 10x PCR buffer (Perkin Elmer Cetus, MgCl₂ free), 2.5 U Perkin Elmer Cetus Amplitaq and 10 mM each of dATP, dCTP, dGTP and dTTP. The chromosomal DNA was added last to prevent contamination of the stocks and the mixture was overlaid with DNase free mineral oil (Sigma). Amplifications were performed in a Hybaid thermal cycler. The cycle temperatures

and times are specified in the relevant section below. The sequences of the primers employed in this study are shown in Table 2.1.

Primer name	Primer sequence	Position in <i>E. coli</i> 16S rRNA gene	(°C) annealing
P _A	5'-AGA GTT TGA TCC TGG CTC AG-3'	8-28	60
P _H	5'-AAG GAG GTG ATC CAG CCG CA-3'	1542-1522	64
P ₁	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3'	341-357	58
P ₂	5'-ATT ACC GCG GCT GCT GG-3'	534-518	56

Table 2.1 Sequence of primers employed for PCR. P₁ contains a 40-bp GC-rich sequence at its 5' end which is not homologous to the template DNA, but prevents separation of the strands in denaturing gradient gel electrophoresis (DGGE) analysis. Annealing temperatures were calculated as $T_A = 2(A+T) + 4(G+C)$.

2.25.1 PCR between primers P_A and P_H

Primers P_A and P_H (Edwards *et al.*, 1989) are homologous to the DNA sequence of regions surrounding the gene encoding the 16S rRNA subunit. They are universal primers and can be used to amplify the same gene from almost all known bacteria. The concentration of template DNA was not determined. To ensure that one reaction was performed with roughly the correct amount of template DNA, two sets of reactions were carried out for each template, one using 1 µl of neat template DNA, the other using 1 µl of a 1:50 dilution of the template. Reaction mixtures were heated to 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 45 secs at 62°C and 45 secs at 72°C. The mixtures were maintained at 72°C for a further 5 min to maximise the final annealing of the DNA strands.

The success of the PCR was judged by analysing 2 µl of the product on a 1% (w/v) agarose gel. If a clear band of the correct size (about 1,600 bp) was observed, the DNA was purified for sequencing. The mineral oil used to cover the PCR reactions was removed by transferring the contents of an Eppendorf onto a piece of Parafilm. The resulting drop was rolled across the Parafilm until the oil was clearly separate from the aqueous solution. The solution containing the PCR product was transferred to a clean Eppendorf. The DNA was purified from proteins and small molecules using Microcon™ 3 filters (Amicon inc., USA). The recommendations of the manufacturer were followed. Before preparing DNA for sequencing, a 2 µl sample was run on a 1% (w/v) agarose gel. An estimation of the volume of solution required to give a suitable DNA concentration for sequencing was made based on the relative brightness of the band. For at least one sample from each isolate, the purity of the DNA was checked by denaturing gradient gel electrophoresis (DGGE) after PCR amplification between primers P₁ and P₂. The DNA extraction and PCR was repeated 2-3 times for each bacterial isolate to generate enough data to determine a reliable consensus sequence.

2.25.2 PCR between primers P₁ and P₂

The primary sequences of primers P₁ and P₂ (Muyzer *et al.*, 1993) are homologous to two regions within the gene encoding 16S rRNA that are highly conserved between different eubacterial species. P₁ contains a 40-nucleotide GC-rich sequence (a GC clamp), making it suitable for preparing DNA for DGGE. PCR was performed using the following thermal cycles: reaction mixtures were heated to 95°C for 10 min, followed by 35 cycles of 1 min at 94 °C, 45 secs at 60°C and 1 min at 72°C. Samples were incubated at 72°C for a further 10 min. The success of the PCR was assessed by running 2 µl of the product on a 1% (w/v) agarose gel and DNA producing a clear band was analysed by DGGE.

2.25.3 Labelling reactions for DNA sequencing

Microcon-purified PCR products were prepared for automated sequencing by primer extension using the Perkin Elmer Applied Biosystems ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit. The reaction mixture for each reaction contained 4-10 µl of template DNA, 8 µl of Terminator Ready Reaction Mix and 3.2 pmole of primer P_A. This was made up to 20 µl with sterile distilled water. For DNA extracted from Gram-positive organisms, 1 µl of dimethylsulphoxide was included to aid separation of the DNA strands. The mixture was overlaid with mineral oil and mini-Eppendorf tubes were transferred to a Perkin-Elmer DNA Thermal Cycler Model 480. Thirty cycles of 30 secs at 96°C, 15 secs at 50°C and 4 min at 60°C were performed, followed by storage of the samples at 4°C. The DNA was precipitated (see below) prior to automated sequencing.

2.26 Gel electrophoresis of DNA

2.26.1 Agarose gel electrophoresis

Agarose (1% (w/v) final concentration) was melted in TBE (10.8 g Tris base, 5.5 g boric acid, 0.93 g EDTA) containing ethidium bromide (0.1 µg ml⁻¹). The molten gel was poured into a Bio-Rad mini cell with a gel comb. When set, this was submerged in 1x TBE containing 0.1 µg ml⁻¹ ethidium bromide. DNA samples were mixed with 0.2 volumes loading buffer (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue), loaded and electrophoresed at 100 V for 1 hr. The size of DNA fragments was determined by comparison with 1 kb ladder commercial size markers (BRL). DNA was visualised on a UV transilluminator (UVP inc., USA) and photographed using Polaroid 665 film.

2.26.2 DGGE

A 40% (w/v) polyacrylamide gel containing a gradient from 0-100% denaturant (urea and formamide) was poured using the Model 475 Gradient Delivery System (Bio-

Rad). The gel was loaded into the tank and heated to 60°C in recirculating 0.5x TAE buffer (from dilution of a 50x TAE stock containing 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0). Sample DNA (2 µl) was mixed with an equal volume of agarose gel loading buffer and samples were loaded onto the gel. Samples were electrophoresed at 150 V for 6 hrs, then the gel was extracted from the gel rig and submerged in 0.5x TAE buffer (100 ml) containing 1 µg ml⁻¹ ethidium bromide for 5 minutes on a horizontal shaker (Luckham, UK). This solution was then removed, replaced with 100 ml distilled water and shaken for 15 minutes. DNA was visualised on a UV transilluminator (UVP inc., USA) and photographed using Polaroid 665 film.

2.27 DNA precipitation

Following the labelling reactions, DNA was precipitated before automated sequencing. Mineral oil was removed by rolling the sample over Parafilm and the aqueous product was transferred to a clean Eppendorf tube. 20 µl of 2 mM MgCl₂ and 55 µl of 95 % ethanol were added, the mixture was vortexed and left to stand at room temperature for 30 min. The DNA was pelleted by centrifugation (MSE Micro Centaur, 11,600g, 30 min) and the supernatant was discarded. To remove traces of supernatant the DNA pellet was incubated at 90°C for 1 min.

2.28 DNA sequencing

DNA precipitates were given to L. Ward (Warwick University) for automated DNA sequencing using an ABI PrismTM 377 cycle sequencer (Perkin Elmer Applied Biosystems).

2.29 DNA sequence analysis

2.29.1 Strains and accession numbers

Strains and GenBank accession numbers for the DNA sequences used for construction of a phylogenetic tree are listed in Table 2.2.

2.29.2 Generation of consensus sequences

Sequence data was analysed in the first instance using Lasergene software (DNASTAR, London). Sequences were edited in EditSeq and consensus sequences were generated using SeqMan. Alignments were made by the Clustal method in Megalign. The three closest matching sequences from the GenBank database (Benson *et al.*, 1998) were selected along with several unrelated sequences for construction of a phylogenetic tree.

2.29.3 Construction of a phylogenetic tree

A phylogenetic tree was drawn up using the Neighbour-joining method. Distances were calculated using the method of Jukes and Cantor (Jukes & Cantor, 1969). Calculations were performed using MEGA software (Kumar *et al.*, 1993) running on a PC.

Species	Strain	Accession number
<i>Azospirillum</i> sp.	BAL31	U63951
<i>Bacillus subtilis</i>	NCDO 1769	X60646
<i>Caulobacter subvibrioides</i>	CB81	M83797
<i>Helicobacter pylori</i>	85D08	U00679
<i>Hyphomicrobium vulgare</i>	MC-750	X53182
<i>Methylobacter capsulatis</i>	NCIMB 11128	L20843
<i>Mycobacterium chelonae</i>	ATCC 35752	X82236
<i>Mycobacterium farcinogenes</i>	NCTC 10955	Y11581
<i>Mycobacterium fortuitum</i>	ATCC 14472	X52921
<i>Mycobacterium nonchromogenicum</i>	ATCC 19530	X52928
<i>Mycobacterium senegalense</i>	NCTC 10956	Y11582
<i>Mycobacterium tuberculosis</i>	taxon:1773	X52917
<i>Mycobacterium wolinsky</i>	ATCC 700010	Y12873
<i>Neisseria meningitidis</i>	NCTC 10025 T	X74900
<i>Planctomyces limnophilus</i>	IFO 3507	X62911
<i>Pseudomonas fluorescens</i>	IFAM 1008	U63901
<i>Pseudomonas</i> sp.	-	Z79594
<i>Rhodococcus globerulus</i>	DSM43953	X80620
<i>Rhizomonas</i> sp.	BAL11	U63939
<i>Rhodomicrobium vanniellii</i>	EY33	M34127
<i>Staphylococcus aureus</i>	ATCC 29740	AF015929
<i>Streptomyces coelicolor</i>	M145	X60514
<i>Sphingomonas asaccharolytica</i>	IFO 15499 T	D28517
<i>Sphingomonas paucimobilis</i>	IFO 13935	D13725
<i>Sphingomonas pruni</i>	IFO 15498	D28568
<i>Sphingomonas yanoikuyae</i>	B1	U37524
<i>Streptococcus pyogenes</i>	ATCC 12344	AB002521
unidentified alpha proteobacterium	S23322	D84605
unidentified gamma proteobacterium	-	L25718
unidentified eubacterium	LX1	AJ001271

Table 2.2 Strains from which sequences of the gene encoding 16S rRNA were derived and GenBank accession numbers.

2.30 Isolation of bacterial outer membranes

Bacterial outer membranes were isolated by detergent solubilization. Cells were harvested, washed and resuspended in 10 mM Tris-HCl pH 8.0. The protein concentration was calculated (Section 2.30) and standardised to 200 $\mu\text{g ml}^{-1}$. The cells were ruptured by five pulses of sonication (15 secs each, at an amplitude of 6 μm peak to peak using a Jencons ultrasonicator fitted with a 4 mm probe) with intervals of 1 min to allow cooling. Unbroken cells were removed by centrifugation at 1,500g for 20 min at 4°C. The resulting supernatant was centrifuged at 48,400g in a JA-20 fixed-angle rotor in a Beckman centrifuge for 60 min at 4°C. The pellet was resuspended in 50 μl of distilled water and stored at -20°C. When required, samples were thawed and extracted with 400 μl of freshly-made membrane extraction detergent (1.67% (w/v) of N-laurylsarcosine and 11.1 mM Tris pH 7.6) at room temperature for 20 min. The insoluble outer membranes were pelleted at 48,400g for 90 min at 20°C. The pellet was resuspended in 50 μl of Laemmli sodium dodecyl sulphate (SDS) sample buffer containing:

Deionised water	3.8 ml
0.5M Tris-HCl pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% (w/v) bromophenol blue	0.4 ml.

2.31 Solubilization of proteins

To obtain whole cell proteins, bacterial cells cultured in the chemostat or in agar plate biofilms were harvested by centrifugation (8,000g for 20 min). Supernatant fluids were discarded and cells were washed and resuspended in saline (0.85% (w/v) NaCl). The protein concentration was calculated (Section 2.30) and standardised to 100 $\mu\text{g ml}^{-1}$. Cells were pelleted by centrifugation (MSE Micro Centaur, 11,600g, 10 min) and resuspended in 150 μl of SDS sample buffer for SDS-PAGE or 200 μl of

first dimension sample buffer (Section 2.31.2) for isoelectric focusing. Samples were prepared by boiling for 5 min prior to SDS-PAGE or by incubation in Laemmli sample buffer for 15 min at room temperature for isoelectric focusing.

2.32 Protein concentration determination

Protein concentration was determined by comparison with a bovine serum albumin standard using the 690-A Micro Protein Determination kit (Sigma Diagnostics) and following the recommendations of the manufacturer. This assay was sensitive above $150 \mu\text{g ml}^{-1}$ of protein. To maximise the accuracy of the assay a fresh standard curve was prepared each time, standards were assayed in duplicate and six replicates of each sample were performed.

2.33 Gel electrophoresis of proteins

2.33.1 Linear sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

A discontinuous system was employed for SDS-PAGE (Laemmli, 1970) in which the buffer ions in the gel and the electrolyte solution differed. A polyacrylamide stacking gel with low ionic strength and large pores was cast on top of the resolving gel to concentrate the protein sample and improve the final resolution of the protein separation. The ingredients of the resolving and stacking gels used are listed in Table 2.3. Linear polyacrylamide slab gels were produced and run using the Mini-PROTEAN II system (Bio-Rad).

Resolving Gel	12%	7.5%
Deionised water	3.35 ml	4.85 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl
Acrylamide-Bis	4 ml	2.5 ml
10% (w/v) AMPS	50 µl	50 µl
TEMED	5 µl	5 µl
Total monomer	10 ml	10 ml
Stacking Gel	4%	
Deionised water	6.1 ml	
1.5 M Tris-HCl pH 8.8	2.5 ml	
10% (w/v) SDS	100 µl	
Acrylamide-Bis	1.33 ml	
10% (w/v) AMPS	50 µl	
TEMED	10 µl	
Total monomer	10 ml	

Table 2.3 Ingredients for linear SDS-PAGE slab gels. Percentages refer to the final acrylamide concentration.

Acrylamide polymerisation was catalysed using an ammonium persulphate-N,N,N¹,N¹-tetramethylethylenediamine (TEMED) complex. TEMED was included to catalyse the formation of free radicals from ammonium persulphate (AMPS), which in turn initiated acrylamide polymerisation. AMPS and TEMED were added to the gel mixture last to avoid premature polymerisation. The gel chamber was filled with the resolving gel mixture using a Pasteur pipette, leaving sufficient space for the stacking gel and the formation of sample wells. The gel was overlaid gently with n-butanol both to exclude oxygen which inhibits gel polymerisation, and to

ensure a flat meniscus. Polymerisation was complete within 30 min, at which time the stacking gel solution was prepared. The overlay was removed and the stacking gel mixture was used to fill the remaining space in the gel chamber. Immediately, a 10-well PTFE comb was inserted into the stacking gel mixture and the polymerisation of the stacking gel allowed to continue. After polymerisation was complete, the comb was removed and the sample wells rinsed with distilled water. The gel assembly was transferred to an electrophoresis tank and Laemmli running buffer (3 g Tris, 14.4 g glycine and 10 ml 10% (w/v) SDS made up to one litre with distilled water) added to the lower reservoir. Any air bubbles settling under the gel were removed to ensure a uniform electrical contact between the gel and the running buffer. Running buffer was added to the upper reservoir.

Samples (20 μ l) prepared as described previously were added to each lane. Molecular weight markers (low molecular weight standards, Pharmacia Biotech) were loaded in both end wells. The assembled apparatus was connected to a power supply (Bio-Rad 3000/300) with the anode connected to the lower reservoir. Electrophoresis was performed at a constant voltage (160 V) until the dye front had migrated to about 1 cm from the bottom of the gel (*ca* 40 min). After electrophoresis, gels were carefully removed from the apparatus, fixed and stained (Section 2.34).

2.33.2 Two dimensional O'Farrell gels

In this gel system proteins were separated by their isoelectric points in the first dimension and according to molecular weight in the second dimension to obtain maximum resolution of proteins from complex mixtures (O'Farrell, 1975). Since ionic detergents alter the charge distribution on proteins, the first dimension was run in the absence of SDS.

Gels were produced using the Mini-PROTEAN II 2-D Cell (Bio-Rad). Ampholytes and acrylamide stock solutions were supplied by Bio-Rad.

The following solutions were prepared:

(i) 30% (w/v) acrylamide stock for isoelectric focusing:

Acrylamide	28.38 g
Bisacrylamide	1.62 g
Distilled water to	100 ml

(ii) 10% (w/v) Triton X-100 stock solution:

10g Triton X-100 was diluted to a final volume of 100 ml with distilled water. This solution was deionized overnight with 5 gm of AG 501-X8 (Bio-Rad) ion exchange resin.

(iii) First dimension sample buffer:

9.5 M urea	5.7 g
2.0% (w/v) Triton X-100	2.0 ml (10 % stock solution)
5% (v/v) 2-mercaptoethanol	0.5 ml
1.6% (v/v) Bio-Lyte 5/7 ampholyte	400 μ l
0.4% (v/v) Bio-lyte 3/10 ampholyte	100 μ l
Distilled water to	10 ml

This solution was warmed to 42°C to dissolve the urea, aliquoted into 0.5 ml volumes in Eppendorf tubes and stored at -70°C.

(iv) First dimension sample overlay buffer containing:

9 M urea	5.41 g
0.8% (v/v) Bio-Lyte 5/7 ampholyte	200 μ l
0.2% (v/v) Bio-Lyte 3/10 ampholyte	50 μ l
Bromophenol blue	500 μ l (of a 0.05% (w/v) Bromophenol blue stock solution)
Distilled water to	10 ml

This was warmed to 42°C to dissolve the urea, aliquoted into 0.5 ml volumes in Eppendorf tubes and stored at -70°C.

(v) First dimension gel monomer solution:

9.2 M urea	5.5 g
4% (w/v) acrylamide	1.33 ml (first dimension acrylamide stock (Bio-Rad))
2% Triton X-100	2 ml (10% (w/v) stock solution)
1.6% (v/v) Bio-Lyte 5/7 ampholyte	400 μ l
0.4% (v/v) Bio-Lyte 3/10 ampholyte	100 μ l
0.01% (w/v) AMPS	10 μ l (fresh 10% (w/v) stock)
0.1% TEMED	10 μ l
Distilled water to	10 ml

This produces sufficient volume for one set of tube gels. The solution was warmed to dissolve. AMPS and TEMED were added immediately before pouring.

Gel tubes were washed thoroughly with detergent, rinsed with distilled water then ethanol and dried before use. The gel tubes were inserted into a casting tube (supplied) and the bottom was covered with several layers of Parafilm. The first dimension gel monomer was poured into the tubes to about $\frac{3}{4}$ of the length of the tube. The gel was allowed to polymerise for 1-2 hrs. The Parafilm was carefully removed and excess acrylamide wiped from the tubes. Any tubes containing air bubbles within the gel were discarded since the presence of bubbles would break the electrical circuit and inhibit electrofocusing.

Tubes were transferred to the tube adapter and each sample reservoir was filled with 100 mM sodium hydroxide (NaOH). Air bubbles were expelled from the necks of the tubes using an Hamilton syringe. The gel tank was filled with 800 ml of 10 mM phosphoric acid (H_3PO_4) and the tube adapter was inserted. Air bubbles under the gel tubes were carefully removed with a Pasteur pipette with a bent tip. The upper chamber of the tube adapter was filled with 100 mM NaOH. 20 μ l of samples (see Section 2.29 for sample preparation) were loaded and overlaid with 20-40 μ l sample overlay buffer. The apparatus was connected to a power supply (Bio-Rad 3000/300) and the proteins focused by electrophoresis at 500 V for 10 min and 750 V for 3.5 hrs.

While the first dimension was running, 12% (w/v) acrylamide resolving slab gels were cast, allowed to set and overlaid with stacking gels (Section 2.33.1). A comb was not inserted into the stacking gel. Instead, a gap of about 1 cm was left at the top of the gel. The stacking gel was overlaid with n-butanol, which was removed immediately before use. Markers for the second dimension were produced by diluting 20 μ l of prepared LMW markers (Pharmacia Biotech) in 180 μ l 1% (w/v) agarose and casting in a gel tube. When set, the gel was extracted and cut to lengths of 0.6 cm to leave 10 μ g of marker proteins in each piece of agarose. After electrofocusing, gels were extruded from the tubes onto Parafilm using a water-filled syringe or frozen at -20°C for storage. Tube gels were loaded onto the second dimension slab gels. A piece of the marker in agarose was placed by the side of the tube gel and the space at the top of the gel was filled with Laemmli running buffer. The gel was run as described in section 2.33.1.

2.34 Staining of polyacrylamide gels

2.34.1 Coomassie blue stain

Following one dimensional SDS-PAGE, gels were fixed and stained with 0.1% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid for a minimum of 30 min. The gels were carefully removed from the glass supporting plates and placed in a plastic tray containing sufficient protein stain solution to immerse the gel. The gels were stained for 30 min at room temperature with gentle agitation. Unbound dye was subsequently removed by transferring the stained gel into destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid) and gently shaken to encourage decolourisation within 30 min. Gels were stored in a final fixative solution of 7% (v/v) acetic acid.

2.34.2 Silver stain

Silver staining is approximately 10-20 times more sensitive than Coomassie Blue staining, requiring a minimum of 5-10 μg per track. The method of Wray *et al.* (1981) was used to stain proteins that had been subjected to two dimensional gel electrophoresis. Gels were soaked in 50% (v/v) methanol for a minimum of 8 hrs with three changes. 1.6 g of silver nitrate were dissolved in 8 ml distilled water and slowly added, with shaking, to a solution containing 42 ml of 0.36% (w/v) NaOH and 2.5 ml of ammonia solution. The volume was made up to 200 ml with distilled water and used to stain the gel for 15 min. After two washes in distilled water for 5 min each, the gel was soaked in developer solution (2.5 ml 1% (w/v) citric acid, 0.4 ml formaldehyde made up to 500 ml with distilled water) until the bands appeared. The reaction was stopped by soaking the gel in a solution containing 10% (v/v) acetic acid and 45 % (v/v) methanol.

2.35 The continuous flow model

A simple model for biofilm development from the complex natural microflora of potable water was designed. The biofilms were developed on glass surfaces held within biofilm development vessels (Fig. 2.3). The layout of the model is shown schematically in Fig. 2.4. Glass coverslips were washed thoroughly in detergent, rinsed in water then ethanol, and dried prior to insertion into the biofilm vessel and the entire model was sterilised by autoclaving at 121°C for 15 min before use. Tap water was continuously introduced into the first reservoir and a fraction of this was pumped at a constant rate (117 ml min^{-1}) through the model. A second reservoir was included to allow sampling of the planktonic population and monitoring of the response of planktonic cells to exogenous addition of a carbon source. The model was kept dark during use. Three sample ports were included to allow sampling of the planktonic population (i) before the biofilm vessels, (ii) immediately after the series of vessels and (iii) in a separate reservoir after the series of biofilms in which the carbon concentration could be amended. A comparison of the planktonic population at port 1 with that at port 2 gives an indication of the effect of biofilms on the natural

planktonic microflora. Biofilms were analysed after the aseptic removal of glass coverslips from the development vessels.

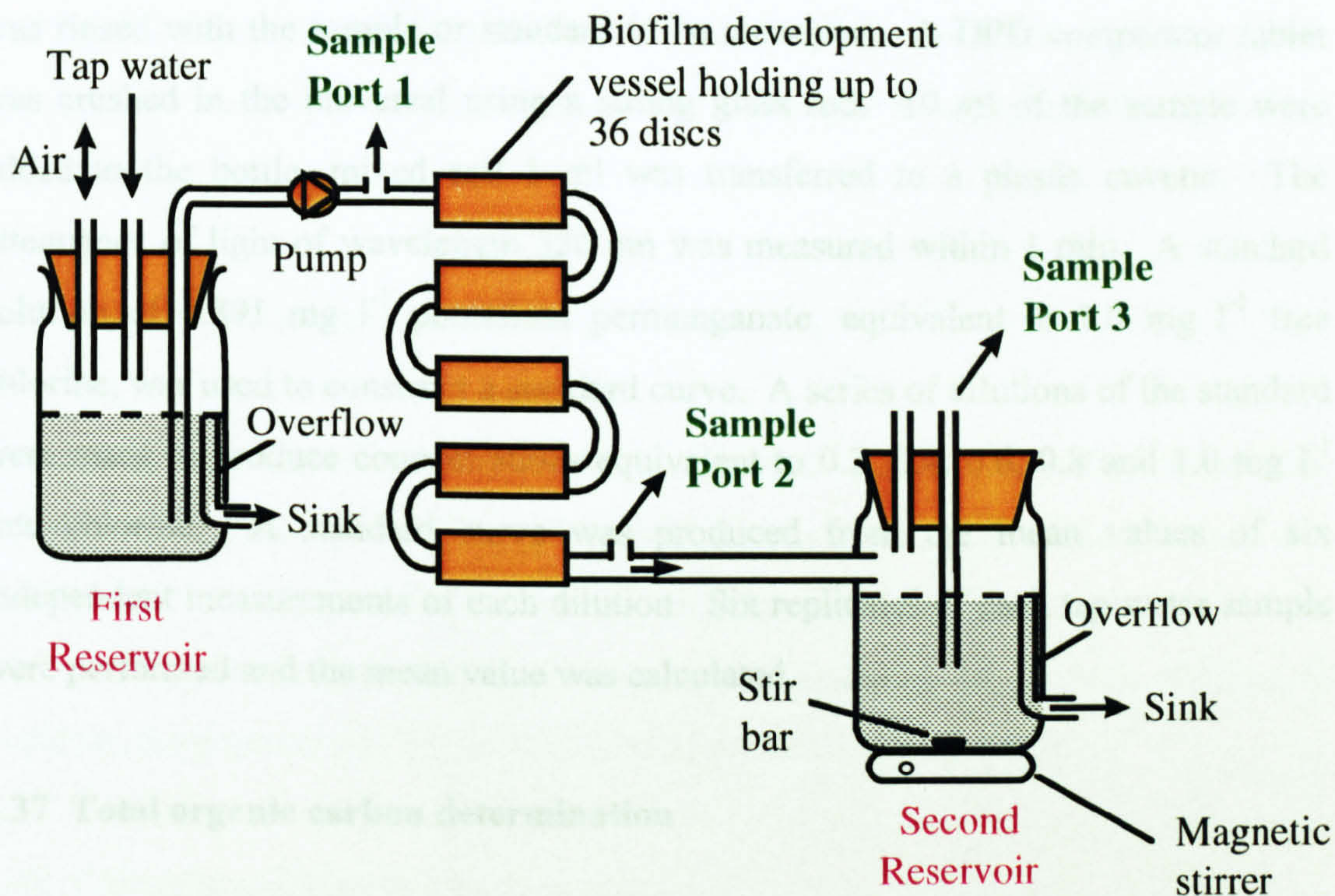


Figure 2.4 The continuous flow model. Tap water was continuously introduced to the first reservoir and a proportion of this was pumped at a defined rate over glass coverslips held within each of the five biofilm development vessels (Fig. 2.3). The second reservoir was included to monitor the response of planktonic cells to carbon addition. Biofilm samples were removed at intervals from the vessels. Water samples were taken before the series of biofilms (port 1), after the series of biofilms (port 2) or from the second reservoir (port 3).

During the period of biofilm development, the ATP, free chlorine and total organic carbon (TOC) concentrations, total particle count, total viable count, pH and temperature of the tap water were determined weekly. The parameters affected by bacterial cells (ATP concentration and particle and viable counts) were measured at sample ports 1 and 2. The extent of biofilm development and activity of attached cells was assessed by SEM and microscopic counts of CTC and DAPI stained cells.

2.36 Measurement of free chlorine

The free chlorine concentration of tap water was measured using diethyl-*p*-phenylenediamine (DPD) comparator tablets No. 1 (BDH). A glass universal bottle was rinsed with the sample or standard to be measured. A DPD comparator tablet was crushed in the universal using a strong glass rod. 10 ml of the sample were added to the bottle, mixed and 1 ml was transferred to a plastic cuvette. The attenuation of light of wavelength 550 nm was measured within 1 min. A standard solution of 0.891 mg l⁻¹ potassium permanganate, equivalent to 1.0 mg l⁻¹ free chlorine, was used to construct a standard curve. A series of dilutions of the standard were made to produce concentrations equivalent to 0.2, 0.4, 0.6, 0.8 and 1.0 mg l⁻¹ free chlorine. A standard curve was produced from the mean values of six independent measurements of each dilution. Six replicates of each tap water sample were performed and the mean value was calculated.

2.37 Total organic carbon determination

Total organic carbon (TOC) was measured in a Beckman 915-B TOC analyser. Inorganic carbon was removed by acidification of approximately 10 ml of sample with one drop of hydrochloric acid (HCl). Inorganic carbon precipitates were removed by bubbling nitrogen gas through the sample for 4 min. Standard curves were produced by dilution of a potassium hydrogen phthalate stock solution (2.125 g in one litre of distilled water; 1 g l⁻¹ TOC) to produce standards of 0, 10, 20, 40, 60, 80 and 100 mg l⁻¹ TOC. These were measured in triplicate to produce a fresh standard curve each time the analyser was used. Tap water samples were also measured in triplicate. From the standard curves, the sensitivity limit of the analyser was found to be around 10 mg TOC litre⁻¹.

2.38 Peptone enrichment of planktonic cells

To monitor the response of planktonic cells to exogenous nutrient addition, a final concentration of 0.001% (w/v) bacto-peptone was produced in the second reservoir.

The holding volume of this reservoir was 10.1 litres, so a concentration of 0.001% (w/v) peptone was produced by the addition 0.101 g of bacto-peptone, dissolved in 10 ml of tap water and sterilised by autoclaving at 121°C for 15 min. This concentration was maintained in reservoir 2 for 24 hrs by the introduction of sterile 0.1% (w/v) bacto-peptone at a rate of 1.17 ml min⁻¹ for 24 hrs using a peristaltic pump. The addition was then stopped and the peptone was gradually diluted to extinction.

2.39 Peptone enrichment of attached cells

To monitor the effect of exogenous peptone addition on the activity of attached cells, a final concentration of 0.001% (w/v) bacto-peptone flowing over the biofilms was achieved by pumping sterile 0.1% (w/v) peptone into port 1 at a rate of 1.17 ml min⁻¹. This was maintained for 24 hrs. After cessation of peptone addition to biofilms, the peptone was left to be diluted to extinction in tap water.

2.40 Chlorine addition to attached cells

Free chlorine was added to attached cells from a sodium hypochlorite solution (Fisher Scientific Ltd). The free chlorine concentration, determined empirically, was found to be 26.3 g l⁻¹. To produce a final concentration of 0.3 mg free chlorine litre⁻¹, a stock solution containing 30 mg ml⁻¹ was produced by dilution of the sodium hypochlorite and introduced into the continuous flow model through sample port 1 at a rate of 1.17 ml min⁻¹ for 3 hrs.

CHAPTER 3

3 Isolation and identification of bacteria found in drinking water

3.1 Introduction

Before undertaking a microbiological study of a natural system it is necessary to identify the major taxa present. In oligotrophic environments, including potable water, bacteria tend to be slow-growing and unable to adapt easily to rapid growth in standard laboratory media. Many of the common biochemical tests for identification of isolated strains rely on rapid growth and cannot be applied to bacteria that require several days to produce a turbid culture. These technical difficulties place practical constraints on the number of organisms that can be identified in a study of potable water and the level of identification attainable. In one recent study the authors were able to identify just four strains from a total of twenty isolated from tap water (Buswell *et al.*, 1997). Another limitation of taxonomic surveys of bacteria from potable water is caused by the presence of microorganisms that cannot be cultured by any known means. Total viable counts (TVCs) often underestimate the total number of bacteria quantified microscopically by one or two orders of magnitude (e.g. Roszak & Colwell, 1987). This discrepancy is undoubtedly caused in part by bacteria that do not produce colonies on agar.

In situ techniques, discussed in Section 1.3.2, will lead to a more accurate estimation of the diversity of microorganisms and frequency of each strain in natural ecosystems. However, at present these are limited by the range of molecular probes available.

Isolation is essential to obtain monocultures of strains from the autochthonous microflora of potable water for further laboratory studies. For rare or fastidious organisms, isolation often requires specialised media and/or an enrichment step. One of the major hypotheses underlying this project was that a distinctive bi- or multi-phasic life cycle occurs in most, if not all, eubacteria and acts as a mechanism by which attachment and release from surfaces can be related to the physiological activity of a cell. According to this theory, physiologically distinct cell types would

be responsible for performing different functions even in the absence of morphological differentiation. Dormant cells would act to disperse biofilms and reproductive cells would grow and divide at interfaces. If a marker could be found that would distinguish between active and dormant cells *in situ* then the distribution of cell types within drinking water pipes could be monitored. This would enable the question of whether multiphasic life cycles do play an important role mediating dormancy and biofilm dispersal in low-nutrient systems to be resolved. Since the correlations between cell type, activity and attachment to surfaces are most easily monitored for prosthecate bacteria, for which cell type can be determined microscopically, selective isolation of a prosthecate strain was desired. For comparison, a non-prosthecate isolate was also required.

3.2 Aims

There were two distinct aims to this section:

1. To carry out a taxonomic survey of the bacteria present in the tap water at Warwick University in as much detail as possible.
2. To isolate two organisms from the University drinking water for further laboratory studies.

For simplicity the taxonomic study was restricted to identification of those organisms that could be cultured on standard laboratory media. No *in situ* identification techniques were employed.

3.3 Morphology and biochemistry of isolated microorganisms

A range of techniques were employed to isolate bacteria from the potable water at Warwick University. These experimental approaches and the major species isolated are described in Table 3.1.

Table 3.1 Identification of microorganisms isolated from potable water. This list is not comprehensive but covers the major colony types isolated from tap water in the batch (Chapter 5) and continuous flow (Chapter 6) models of biofilm development.

Isolate	Isolation protocol ^a	Colonial morphology ^b	Cellular morphology	Motility	Gram	Oxidase	Catalase	API20NE performed	16S rDNA sequenced	Identification
B1	1	small, rd, translucent	pear-shaped, prosthecate	+	-	ND	ND	-	-	<i>Hyphomicrobium</i> sp. or <i>Pedomicrobium</i> sp.
B2	2	lemon, rd	cocci	-	+	+	+	-	-	<i>Micrococcus luteus</i>
B3	1,2	white, crumbly	branched rods	-	+	ND	ND	-	-	<i>Actinomyces</i> sp.
E1	3	brown, rd	rods	+	-	+	+	+	-	<i>Pseudomonas fluorescens</i>
E2	3	yellow, rd	rods	+	-	+	+	+	-	<i>Pseudomonas</i> sp.
E3	3	cream, rd	rods	+	-	+	+	+	-	<i>Comamonas acidovorans</i>
E4	3	white, smooth, rd	rods	+	-	-	+	+	-	<i>Xanthomonas maltophilia</i>
CF1	4	white, rd, sticky	rods	-	+	+	-	-	+	<i>Mycobacterium</i> sp.
CF2	1,2,4	orange, shiny, rd	rods	-	-	+	-	-	-	<i>Flavimonas</i> sp.
CF3	2,4	translucent, rd	rods	+	-	+	+	-	+	Alpha or gamma proteobacterium
CF4	4	white, rd, sticky	rods	-	+	+	-	-	+	As CF1
CF5	1,2,4	orange, shiny, rd	pleomorphic rods	-	-	-	+	-	+	Alpha proteobacterium
CF6	4	white, irreg.	large, coccoid	-	-	+	-	-	-	<i>Azotobacter</i> sp.
CF7	1,2,3,4,5	pink, rd	rods	+	-	+	+	-	+	<i>Methylobacterium</i> sp.
CF8	4	white, rd	rods	-	+	-	-	-	-	<i>Bacillus</i> sp.
CF9	4	brownish red, translucent, rd	rods	+	-	+	+	-	+	As CF3
CF10	1,2,4	dark orange, sticky	large flexible rods	-	-	-	-	-	-	<i>Flexibacter</i> sp.
CF11	4	orange, irreg., crumbly	rods	-	+	+	-	-	+	<i>Mycobacterium</i> sp.
CF12	6	white, swirling pattern	rods	-	+	ND	ND	-	-	<i>Bacillus cereus</i>
CF13	3,6	yellow, rd	rods	+	-	-	+	+	+	<i>Sphingomonas</i> sp.
CF14	6	white, irreg. crumbly	rods	-	+	+	-	-	+	<i>Mycobacterium</i> sp.

Footnotes

- (a) 1 - Isolated from the batch model on low nutrient sloppy agar (this was used specifically to isolate an appendaged microorganism - Section 3.5.2).
2 - Isolated from the batch model on PCA.
3 - Isolated from the continuous flow model on PCA after enrichment with 0.001% (w/v) bacto-peptone for 24 hrs.
4 - Isolated from the continuous flow model on R2A.
5 - Isolated from the batch model after enrichment with 0.5% (v/v) methanol.
6 - Isolated from the continuous flow model on R2A after enrichment of biofilms with 0.001% (w/v) bacto-peptone for 24 hrs.
- (b) rd = smooth-edged round colonies.
irreg. = irregular shaped colonies.

ND = not determined.

Only a few strains selectively enriched by the exposure of planktonic cells to 0.001% (w/v) bacto-peptone for 24 hrs grew sufficiently rapidly in the laboratory for the API20NE test to be applicable.

3.4 Identification of bacterial isolates by sequencing the gene encoding the 16S rRNA subunit

Strains for which no positive identification was obtained by any of the morphological and biochemical (including API20NE) tests, were grouped phylogenetically on the basis of partial 16S rDNA sequence data, as outlined below. The approach taken for DNA extraction and sequencing of part of the gene encoding the 16S rRNA subunit is shown schematically in Fig. 3.1.

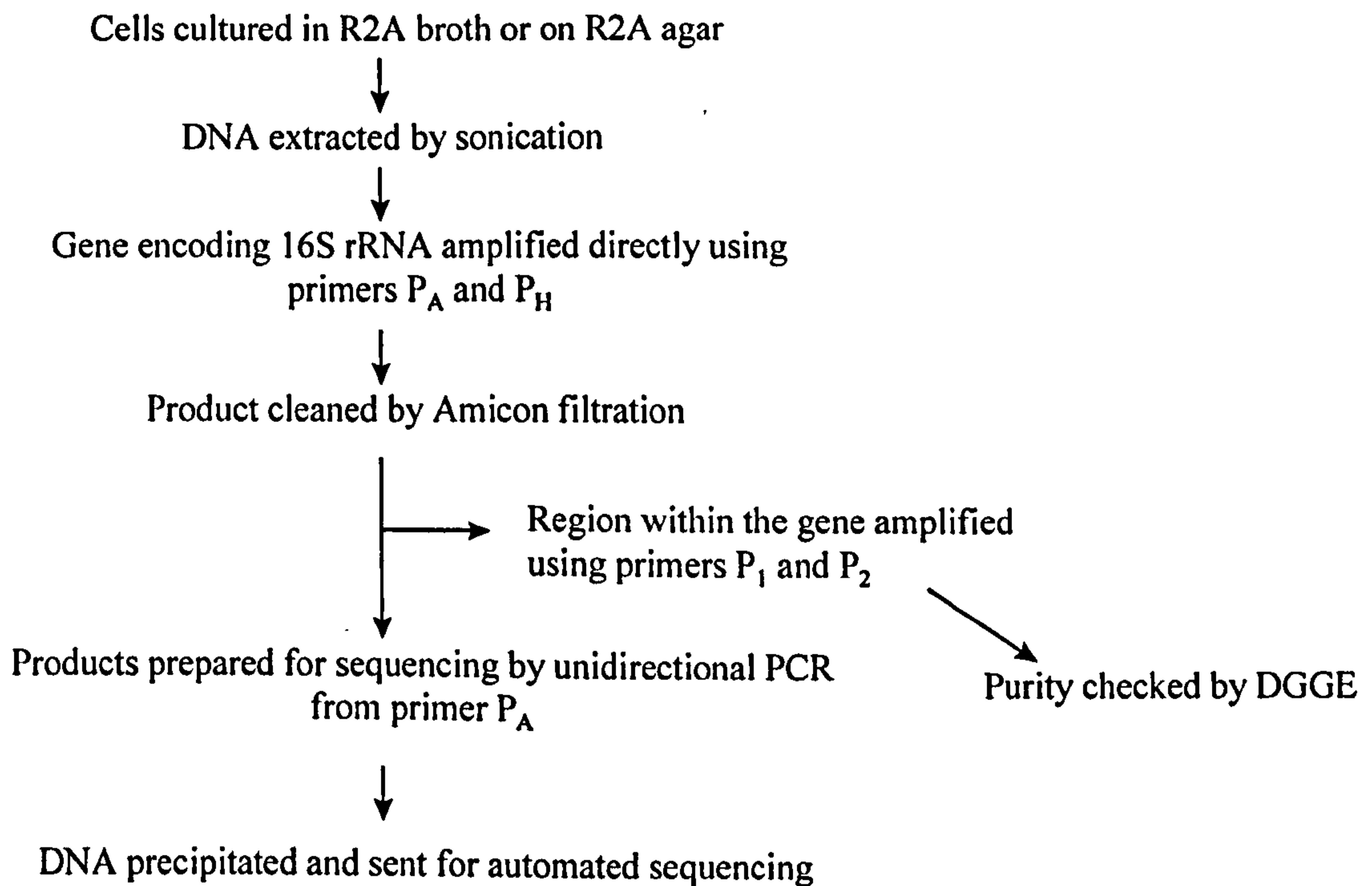


Figure 3.1 Strategy for sequencing the gene encoding 16S rRNA from a variety of potable water isolates. Each step is described in detail in the text. Primers P_A and P_H were homologous to regions at the 5' and 3' ends of the gene respectively (for details see Section 2.25). Primers P_1 and P_2 were homologous to regions within the gene, towards the 5' end. P_1 contained a GC clamp to produce a product suitable for denaturing gradient gel electrophoresis (DGGE).

Since primers P_A and P_H are homologous to regions around the 16S rRNA gene of almost all known eubacteria, the introduction of a contaminant before the first PCR step would lead to amplification of at least two gene products of similar size. These would not be separated by linear gel electrophoresis and could lead to inaccurate sequence data being obtained. To avoid this DNA was prepared from at least two different cultures of each organism. Each culture was checked for purity microscopically and by subculture. In addition, purified PCR products were analysed by DGGE. This technique separates DNA on the basis of GC content as well as size. In theory it is sensitive enough to detect single base pair differences between DNA fragments.

3.4.1 Amplification of the gene by PCR

Strains isolated from potable water were cultured in R2A broth or on R2A agar for 3-7 days. Crude total DNA preparations were produced and used as the template for PCR between primers P_A and P_H. A negative control was included in each PCR reaction to ensure that the primers were amplifying only the template DNA. To confirm the success of the PCR, DNA was separated by electrophoresis through a 1% (w/v) agarose gel. Samples containing the expected 1.6 kbp product were purified by Microcon centrifugation. Once again, DNA was checked by electrophoresis through a 1% (w/v) agarose gel. An example of one of these gels is shown in Fig. 3.2.

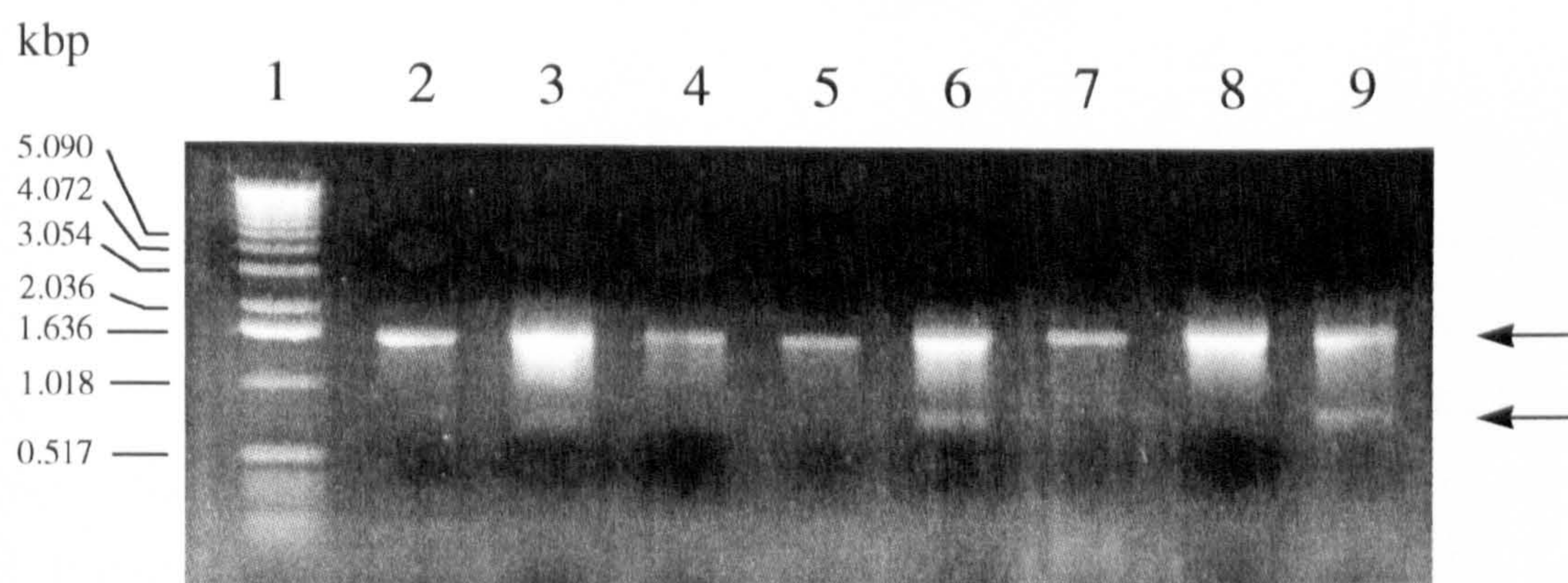


Figure 3.2 Analysis of the product of PCR reactions using primers P_A and P_H on a 1% (w/v) agarose gel. Total cellular DNA extracted from a range of bacterial isolates was used as a template for the PCR reactions. Products were purified by Microcon filtration before analysis. Arrows indicate clear bands and sizes of marker bands in kbp are shown. Lanes: 1 - markers; 2-9 - PCR products from: 2 - CF1, 3 - CF3, 4 - CF4, 5 - CF5, 6 - CF9, 7 - CF11, 8 - CF13 and 9 - CF14.

3.4.2 Analysis of the PCR product by DGGE

To ensure that no contaminating DNA fragments had been amplified by PCR, products were separated by denaturing gradient gel electrophoresis (DGGE). This

required a further PCR step to obtain a small (about 200 bp) product from within the gene itself. Primer P₁ contained a GC clamp, ensuring that the products were suitable for DGGE. A positive control, *Streptomyces griseus* DNA, was included to confirm that the system was working properly. An example of a DGGE gel is shown in Fig. 3.3. DNA containing a relatively high concentration of G+C migrates further through the gel. Bands from CF1, CF4, CF11, CF14 (all subsequently identified as members of the group of high GC Gram positive eubacteria) and the positive control migrated further than bands from the other isolates. In each lane only one dsDNA band was present, confirming the absence of contaminating PCR products. The faint shadowing bands, each at the same distance above the clear dsDNA band, were still present because the gel was not run for long enough to resolve cleanly.

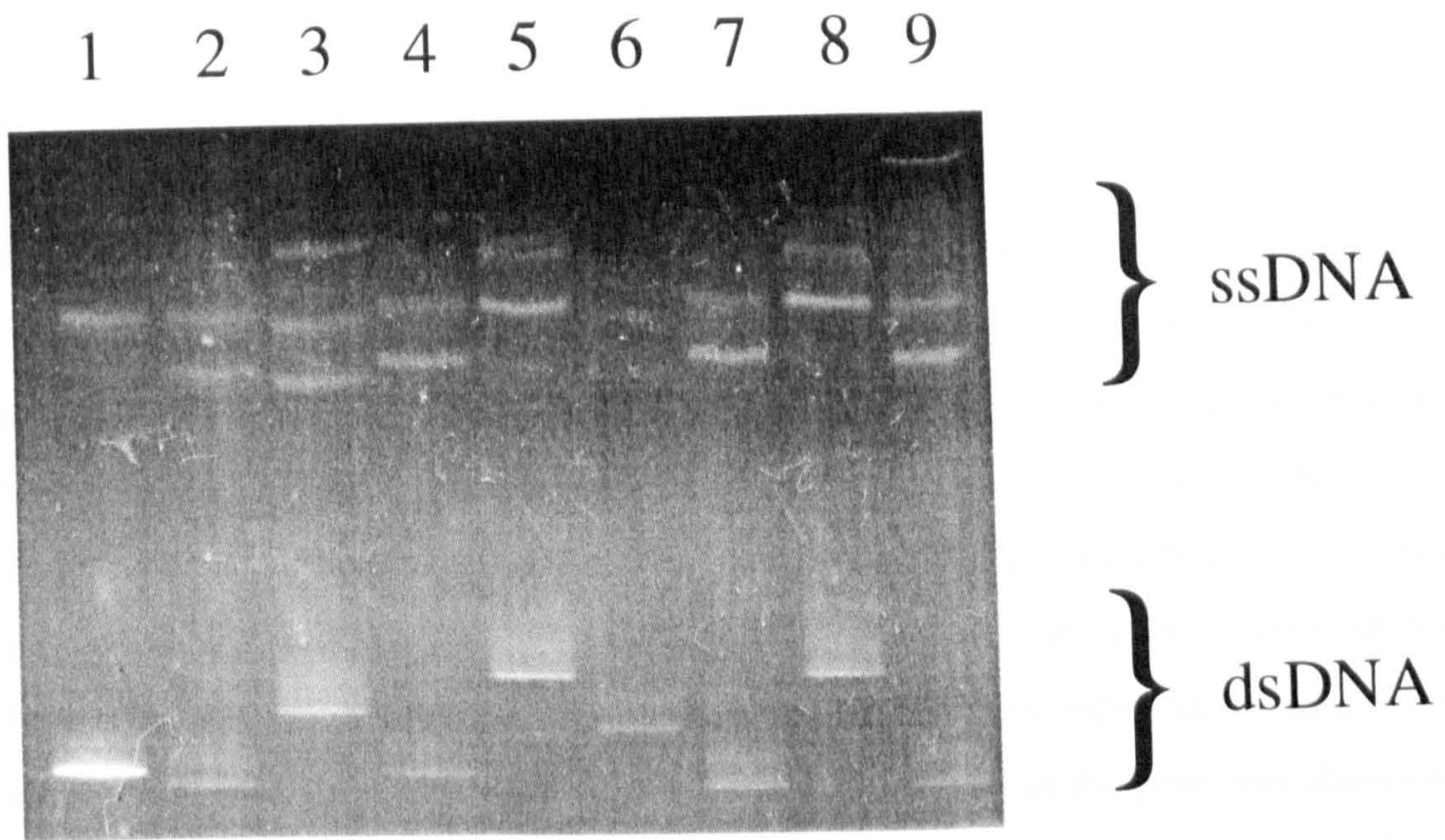


Figure 3.3 Analysis of the products of PCR reactions using primers P_A and P_H by DGGE. PCR products from a range of bacterial isolates were purified by Microcon filtration and subjected to a second PCR step, this time using primers P_1 and P_2 . The products were electrophoresed through a 40% (w/v) polyacrylamide gel containing a gradient from 0-100% of a denaturant. 'ss' indicates the area of the gel containing bands resulting from single stranded DNA. 'ds' denotes the area of the gel containing bands of double stranded DNA. Lanes: 1 - *Streptomyces griseus* (positive control), 2 - CF1, 3 - CF3, 4 - CF4, 5 - CF5, 6 - CF9, 7 - CF11, 8 - CF13 and 9 - CF14.

3.4.3 Partial gene sequence

The purified 1.6 kbp PCR products were used as templates for unidirectional PCR from primer P_A. The reaction mixture contained equimolar amounts of the four standard deoxynucleotide bases. Four deoxynucleotide bases, each labelled with a different fluorescent moiety, were also included to act as chain terminators, yielding a set of DNA fragments of different lengths, each containing one of four fluorescent labels. Total DNA was precipitated with ethanol and sent for automated sequencing. This process separated the DNA by polyacrylamide gel electrophoresis, then employed image analysis to match the DNA fragment size with a particular terminal base pair. Consensus sequences were determined from 2-4 sequences obtained for each strain. Sequence data starting from around the 5' end of the gene was obtained. Sequences were aligned with each other and with others in the GenBank database (Benson *et al.*, 1998) (Fig. 3.4).

3.4.4 Phylogenetic analysis based on the sequence data

Sequence data from the eight potable water isolates were compared to published data from a variety of strains in the GenBank database. An unrooted neighbour-joining tree was constructed using the Jukes-Cantor method for calculation of phylogenetic distances (Fig. 3.5).

214		293
<i>E. coli</i>	TGTGCCCAGA TGGGATTAGC TAGTAGGTGG GGTAACGGCT CACCTAGGCG ACGATCCCTA GCTGGTCTGA GAGGATGACC	
CF3/CF9	GGAACCTACG TCGGATTAGC TAGTTGGTGA GGTAATGGCT CACCAAGCGG ACGATCCGTA ACTGGTCTGA GAGGATGATC	
CF5	GTAGCCCGCG TATGATTACC TAGTTGGTGA GGTAAGGCT CACCAGGCGG ACGATCCTTA CCTGGTCTGA GAGGATGATC	
CF13	TGAGCCCGCG TAGGATTAGC TAGTTGGTGG GGTAAGGCC CACCAAGCGG ACGATCCTTA GCTGGTCTGA GAGGATGATC	
CF1/CF4	TGGGCCCGCG GCCTATCAGC TTGTTGGTGG GGTAATGGCC TACCAAGCGG ACGACGGGTA GCCGGCCTGA GAGGGTGACC	
CF11	TGGGCCCGCG GCCTATCAGC TGGTTGGTGG GGTAATGGCC TACCAAGCGG ACGACGGGTA GCCGGCCTGA GAGGGTGACC	
CF14	TGGGCCCGCG GCCTATCAGC TTGTTGGTGG GGTAATGGCC TACCAAGCGG ACCACGGGTA GCCGGCCTGA GAGGGTGACC	
<i>M. tub.</i>	TGAGCCCGCG GCCTATCAGC TTGTTGGTGG GGTAAGGCG ACGACGGGTA GCCGGCCTGA GAGGGTGACC	
		372
294		
<i>E. coli</i>	AGCCACACTG GAACTGAGAC ACGTCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAAATGGG CGCAAGC-TG	
CF3/CF9	AGTCACACCG GAACTGAGAC ACGTCCGGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GGACAAATGGG CGAAAGCCTG	
CF5	AGCCACACTG GAACTGAGAC ACGGCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GGACCAATGGG CGAAAGCCTG	
CF13	AGCCACACTG GAACTGAGAC ACGGCCATA CTCCTACGGG AGGCAGCAGT AGGGAATATT GGACAAATGGG CGAAAGCCTG	
CF1/CF4	GGCCACACTG GAACTGAGAT ACGGCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAAATGGG CGCAAGCCTG	
CF11	GGCCACACTG GAACTGAGAT ACGGCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAAATGGG CGCAAGCCTG	
CF14	GGCCACACTG GAACTGAGAT ACGGCCACA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAAATGG- CGCAAGCCTG	
<i>M. tub.</i>	GGCCACACTG GAACTGAGAT ACGGCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GCACAAATGGG CGCAAGCCTG	
		414
373		
<i>E. coli</i>	ATGCAGCCAT GCCCGTGTA TGAAGAAGGC -TTCGGGTTG TA	
CF3/CF9	ATCCAGCAAT ACCCGGTGTG TGAAGAAG-C CTTCCGGGTTG TA	
CF5	ATCCAGCAAT GCCCGGTGAG TGATGAAG-C CTTAGGGTTG TA	
CF13	ATCCACCAAT GCCCGGTGAG TGATGAAGGC CTTAGGGTTG TA	
CF1/CF4	ATGCAGCGAC TCCCGGTGAG GGATGACGGC CTTCCGGGTTG TA	
CF11	ATGCAGCGAC TCCCGGTGAG GGATGACGGC CTTCCGGGTTG TA	
CF14	ATGCACCGAC CCCCGGTGAG GGATGACCGC CTTCCGGGTTG TA	
<i>M. tub.</i>	ATGCAGCGAC GCCCGGTGGG GGATGACGGC CTTCCGGGTTG TA	

Figure 3.4 Sequence alignment of a 202 bp fragment of the gene encoding 16S rRNA. Alignments were calculated using the Clustal method. Numbering runs from base 214 to 414 in the *E. coli* sequence. *M. tub.* = *Mycobacterium tuberculosis*.

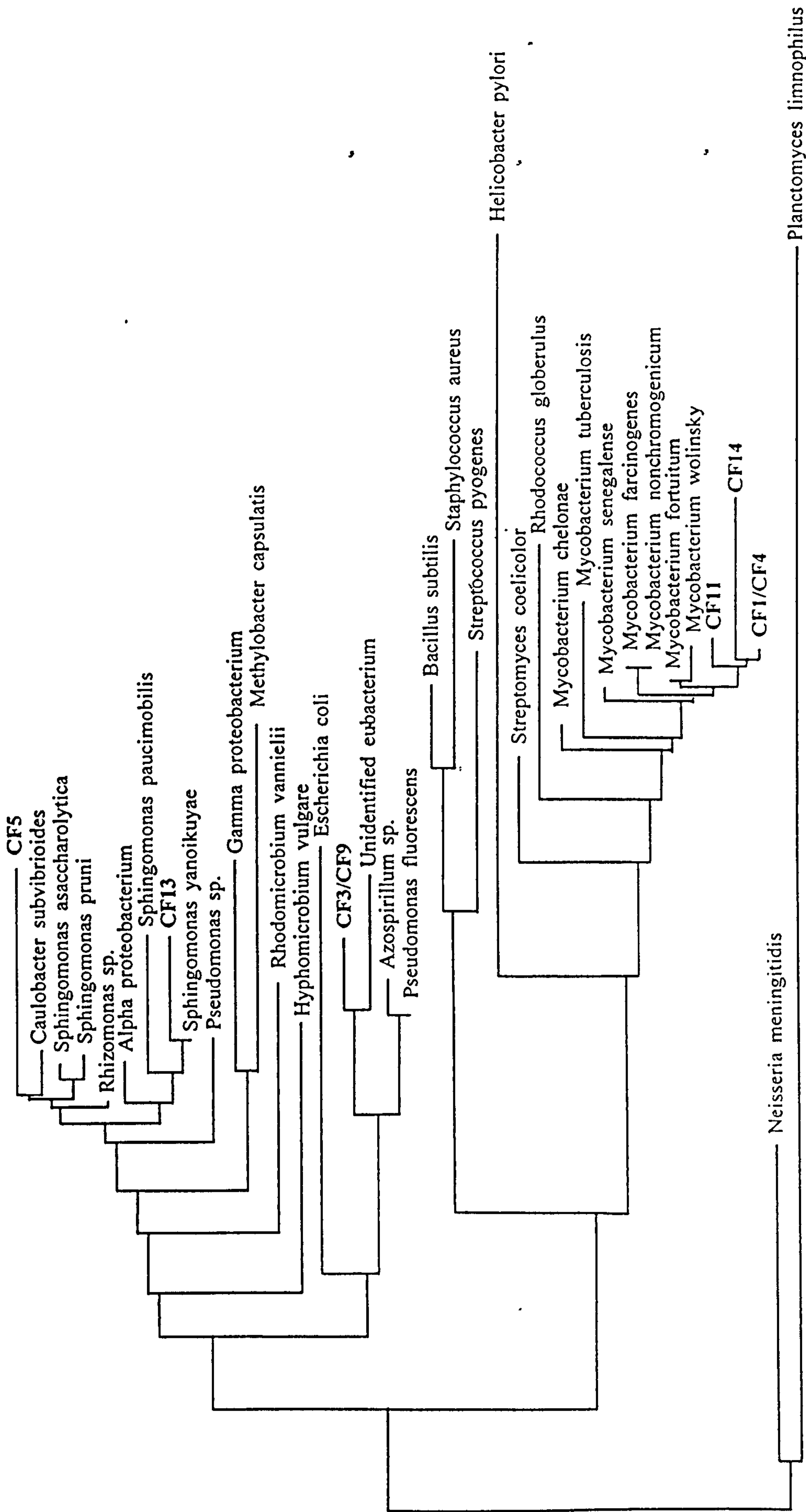


Figure 3.5 An unrooted neighbour-joining tree based on the DNA sequence shown in Fig. 3.4. Strains and culture collection numbers are listed in Table 2.2. Distances were calculated by the Jukes-Cantor method. Bar indicates number of substitutions per nucleotide position.

Despite the small number of bases analysed, the tree shows branches similar to those obtained from more extensive studies (Woese, 1987). The only major eubacterial groups that are mixed together are the α and γ subgroups of the *Proteobacteria*, although even here members of the α -4 subclass (Takeuchi *et al.*, 1994) containing the genera *Sphingomonas* and *Rhizomonas* group together.

Although no specific names can be ascribed to any of the isolates, it is possible to place five of them within individual genera: CF3 = *Sphingomonas* sp.; CF1, CF4, CF11 and CF14 = *Mycobacterium* sp. Additionally CF5 can be placed within the α subgroup of the *Proteobacteria* and CF3 and CF9 belong to a single species within either the α or γ subgroup of the *Proteobacteria*. None of these identifications contradicts morphological or biochemical data (Section 3.3).

It should be noted that several of the isolates group very closely to each other. It is not surprising that CF3 and CF9 belong to the same species as these strains differed only in production of a red-brown pigment by CF9. The tight grouping of the mycobacteria is more interesting. CF1 and CF4 almost certainly belong to the same species as they differed only slightly in the morphology of colonies produced on R2A medium. However, colonies produced by CF11 and CF14 were clearly distinct from each other as well as from CF1/CF4 (see Table 3.1). It is likely that the four isolates represent three distinct species. More biochemical and/or sequence data are required to ascertain whether the strains are truly more closely related to each other than to any other mycobacteria, as suggested by Fig. 3.5.

3.4.5 Mycobacteria in potable water

The common occurrence of mycobacteria in the Warwick University drinking water system is not unexpected, but the growing concern over the health risks associated with this group of organisms merits a brief discussion here. Mycobacteria are widespread in the environment, existing most commonly in water or watery habitats such as surface water, mud, soil and piped water supplies (Collins *et al.*, 1984; Grange, 1996). Only a few species are obligate pathogens but many are responsible for opportunistic infections. It is generally thought that infections caused by non-tuberculous mycobacteria originate from environmental sources, although little is known about the mode of transmission. A study of cooling and spray water from several dental units demonstrated that patients were exposed to large numbers of non-tuberculous mycobacteria during treatment. Swallowing, inhalation or inoculation into oral wounds could occur, potentially resulting in colonisation or infection (Schulze-Röbbecke *et al.*, 1995). The degree of mycobacterial colonisation of tap or bottled waters may act as an index of the hygienic quality for consumption by immunocompromised patients (Kubalek & Komenda, 1995; Kubalek & Mysak, 1996; Papapetropoulou *et al.*, 1997).

The hydrophobic nature of the mycobacterial cell wall and the fact that, unlike most aquatic bacteria, mycobacteria are non-flagellate, suggest that these organisms are specialised for adhesion to surfaces. Cells readily attach to surfaces (Schulze-Röbbecke *et al.*, 1992 and 1995) and cell division at surfaces can result in mycobacterial microcolonies (Schulze-Röbbecke *et al.*, 1992). Enrichment of mycobacteria by the presence of solid surfaces is investigated in Chapter 6.

3.5 Isolation of a prosthecate bacterial species

A prosthecate bacterial isolate from potable water was desired for laboratory studies (see Section 3.1).

3.5.1 Methanol enrichment

An *Hyphomicrobium* sp. or *Pedomicrobium* sp. was observed after short periods of static batch enrichment (Chapter 5). Since hyphomicrobia are virtually unique in their ability to utilise methanol and nitrate as the sole carbon and nitrogen sources respectively in the absence of oxygen (Moore, 1981), three enrichment flasks were set up containing HB medium with 0.5% (v/v) methanol added. Flasks were incubated anaerobically at 30°C with shaking for up to 8 weeks. At intervals, samples were removed and spread onto HB agar with 0.5% (v/v) methanol. Plates were examined after aerobic or anaerobic incubation at 30°C for 4 weeks. Two of the enrichment flasks became overgrown with *Methylobacterium* sp., there was no bacterial growth in the other.

3.5.2 Isolation on low-nutrient sloppy agar

Prosthecate bacteria are most commonly found in oligotrophic waters. Low-nutrient sloppy agar plates were used as a direct attempt to isolate a prosthecate species in order to limit substrate accelerated death, growth of heterotrophic organisms and exposure to stress from desiccation. As prosthecate organisms were enriched by batch storage of tap water (Chapter 5), water taken from the batch enrichment model after 20 weeks was added to the top sloppy agar layer before pouring and plates were incubated at 30°C or room temperature for 4 weeks. Discrete colonies were selected and subcultured in fresh sloppy agar. After another 4 week incubation at 30°C or room temperature, colonies were screened for the presence of prosthecate bacteria by light microscopy. One of the plates was found to contain a pure culture of a prosthecate bacterial species. The isolate was subcultured on low-nutrient sloppy agar and stocks were made for storage at -20°C.

A variety of culture conditions were tested to obtain the maximum growth rate and yield. Incubation on peptone agar, containing 0.001% (w/v) bacto-peptone, 1.5% (w/v) bacto-agar dissolved in tap water, or PCA at 30°C in high humidity yielded small colonies up to 1 mm in diameter after 14 days. Colonies were extremely tough

and it was difficult to scrape cells from them. Growth in liquid culture was very slow. Turbid cultures were obtained after aerobic or anaerobic incubation of cells in 10 ml of HB medium containing 0.5% (v/v) methanol or 0.1% (v/v) trimethylamine in a glass universal bottle for 8 weeks.

3.5.3 Characterisation of the prosthecate isolate

Cells appeared to be pear-shaped under phase contrast optics, usually bearing one or two polar prosthecae. Cells stained Gram negative and contained large granules of poly- β -hydroxybutyrate. Prosthecae were extensively branched and colonies on agar consisted of tangled cellular matrices. The majority of cells in liquid culture were attached to the glass sides of the vessel and for around 3-4 weeks after inoculation only a few single cells were observed, some of which were motile. After about 8 weeks most cells that were not attached to the sides of the vessel were present in aggregates. Division occurred by budding at the tips of the prosthecae and cells were classified as *Hyphomicrobium* sp. or *Pedomicrobium* sp. on the basis of cell morphology. Cells analysed by TEM after negative staining with phosphotungstic acid are shown in Fig. 3.6.

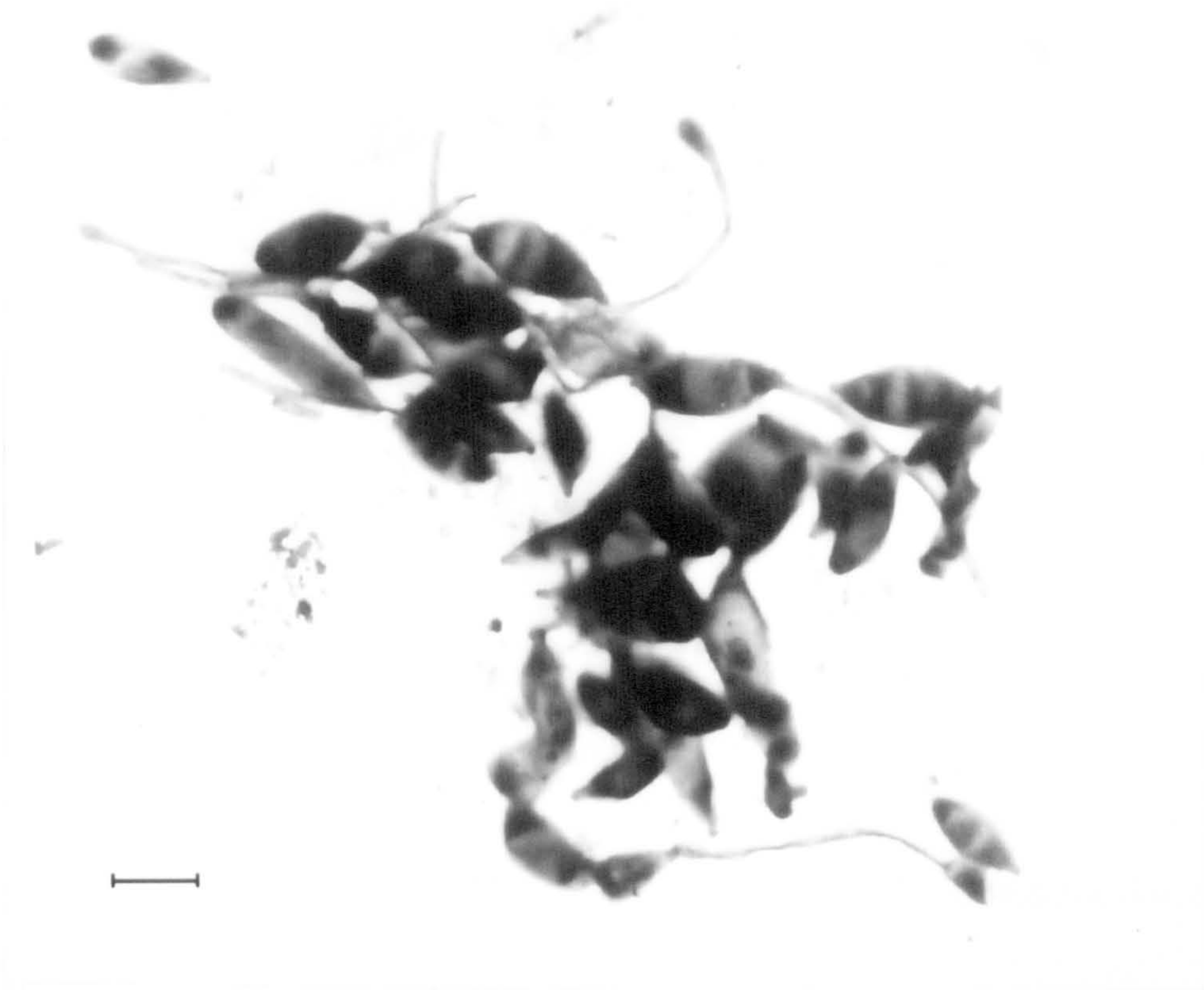


Figure 3.6 TEM of an *Hyphomicrobium* or *Pedomicrobium* sp. isolated from **potable water**. This organism was isolated on low-nutrient sloppy agar after enrichment for 20 weeks in the static batch biofilm model. Cells were negatively stained with phosphotungstic acid. Bar = 1 μm .

The slow growth of the *Hyphomicrobium* sp. or *Pedomicrobium* sp. and the difficulty of separating individual cells frustrated attempts to use it in laboratory studies. Since no other prosthecate bacteria were isolated, *Caulobacter crescentus* CB15 was obtained from the NCIB culture collection for laboratory work.

3.6 Conclusions

A wide range of bacteria, almost exclusively regular rods, were isolated from potable water. No coliforms or *Aeromonas* spp. were observed, although no specific attempts were made to search for these organisms. Most of the isolates grew slowly (greater than four days to form medium-sized colonies or turbid liquid cultures) and

could not be identified by API20NE tests. Bacteria that could not be identified by other means were placed into phylogenetic groups based on partial sequence of the gene encoding 16S rRNA. As the database of full and partial 16S rDNA sequences grows, this technique is becoming more powerful (Palys *et al.*, 1997).

Three distinct *Mycobacterium* spp. were identified, which grouped very closely to each other by DNA sequence analysis. The tight grouping may reflect a current limitation of the 16S rDNA database or it may show a close relationship between the three organisms. However, since the length of DNA sequenced was short and very few differences separated any of the mycobacteria analysed, it is impossible to draw clear conclusions about the inter-relationships between the species on this evidence alone. Further DNA sequencing or identification by other means (Jenkins *et al.*, 1992) is required to group these isolates into individual species, and without that knowledge it is impossible to discern whether any of the isolates are recognised opportunistic pathogens.

An appendaged microorganism was isolated but it could not be used for the planned laboratory studies, due to its slow growth rate and tendency to form tightly bound 'mycelia'. The fact that this organism was only isolated after the application of specialised techniques supports the theory that many bacteria are present in the system that are not isolated on rich laboratory media. This is further demonstrated by the wide range of cellular morphologies observed directly after static batch enrichment of tap water (Chapter 5) compared to the predominance of regular rod-shaped bacteria isolated on rich media.

CHAPTER 4

4 The physiology of isolated organisms

4.1 Introduction

Vegetative dormancy has been shown to occur in a variety of non-sporulating Gram-positive and Gram-negative bacteria under nutrient starvation or other stresses in the laboratory and in natural environments (Section 1.2.5.1). It has been suggested that production of dormant cell types that are physiologically and sometimes morphologically distinct from metabolically active cells of the same species is regulated through the bacterial cell division cycle. From studies on three unrelated prosthecate bacteria it was shown that in each case a life cycle involving an asymmetric division played a key role in bringing about two different cell types: an inactive motile daughter cell and a reproductive mother cell specialised for attachment to solid surfaces such as fixed surfaces, solid inert particles or other cells (Whittenbury & Dow, 1977). In a potable water distribution system this type of life cycle would allow growth and division of attached cells, while enabling dispersal by release of motile daughter cells into the water column. Since prosthecate bacteria are present in drinking water, it is clear that life cycles involving an asymmetric division step play a role in the production of vegetative dormant cell types and dispersal in this system.

From laboratory studies, there is strong evidence that asymmetric division also occurs in the life cycles of many non-prosthecate bacteria (Section 1.2.5.2.2). However, the role of bi- or multi-phasic life cycles in the survival, growth and dispersal of morphologically indistinct bacteria in natural environments has not been investigated. This is the major area to be explored in this thesis.

The first step is to find a marker to distinguish between dormant and active cell types *in situ*. Cell types can be distinguished on the basis of nucleoid sedimentation coefficients (Swoboda *et al.*, 1982; Robertson, 1996), but use of this technique is limited to planktonic laboratory monocultures. An attempt to identify a structural cell surface marker that could distinguish between active and dormant *E. coli* K-12

cells failed to produce cell type-specific antibodies (Robertson, 1996). An alternative approach is to apply physiological markers to differentiate between cell types *in situ*. Many different stains have the capacity to highlight exclusively the active cells in a population (Section 1.3.3). Of these, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has increasingly been employed to stain bacterial cells with an active respiratory chain in natural systems since it was first introduced to microbiology in 1992 (Rodriguez *et al.*, 1992). This tetrazolium salt is reduced by respiratory chain enzymes to an insoluble fluorescent formazan product, which can be observed microscopically. The mechanism of CTC reduction has not been fully elucidated (Section 1.3.3.2). Before applying a technique such as CTC staining to measure the metabolic activity of bacteria in complex systems, such as those predominating in the natural environment, the methodology must be refined in the laboratory.

4.2 Aims

The primary aims of this section were:-

- (a) to develop a technique to distinguish between distinct cell types of the same species *in situ*;
- (b) to analyse changes in cell type associated with surface attachment.

Three approaches were taken to achieve these objectives:-

1. The growth of two strains in batch culture was studied to determine the potential of a physiological stain (CTC) to distinguish between cell types. The strains selected for analysis were *Caulobacter crescentus* CB15 which undergoes a well-characterised dimorphic life cycle and *Sphingomonas* sp. (CF13) isolated from potable water at Warwick University.
2. A chemostat model of biofilm development was used to investigate the relationship between cell surface hydrophobicity and attachment proclivity.
3. Differences between cellular proteins of attached and planktonic cells were assessed with a view to finding markers that could be used to differentiate between cell types *in situ*.

4.3 Batch culture studies

Two organisms were selected for use in batch culture studies to establish the capacity of a physiological stain, CTC, to determine the cellular activity of populations of cells and individual members of them. *Caulobacter crescentus* CB15 was chosen because it undergoes a well-defined dimorphic life cycle. Although *C. crescentus* CB15 was not isolated from Warwick University potable water, *Caulobacter* species are ubiquitous freshwater organisms and at least one member of this group was observed directly in static batch tap water enrichments (data not shown).

The other strain chosen for laboratory studies was a *Sphingomonas* sp. isolated from tap water at Warwick University. *Sphingomonas* spp. are pathogenic to animals and are opportunistic human pathogens. They can degrade copper water pipes and have been found associated with a variety of biofilms (White *et al.*, 1996).

4.3.1 Cell physiology during culture of *Sphingomonas* sp.

4.3.1.1 Cell size

Bacterial cell size is a good indicator of physiological activity, larger cells being more active. This is true both in the laboratory where *E. coli* cells reach the minimum unit cell size only at relatively slow growth rates (Donachie, 1984), and in many natural environments where ultramicrobacteria abound (Morita, 1985). Cell size was monitored through batch culture of *Sphingomonas* sp. using the *CellFacts* particle analyser to assess changes in cellular activity through batch growth (Figs 4.1 and 4.2).

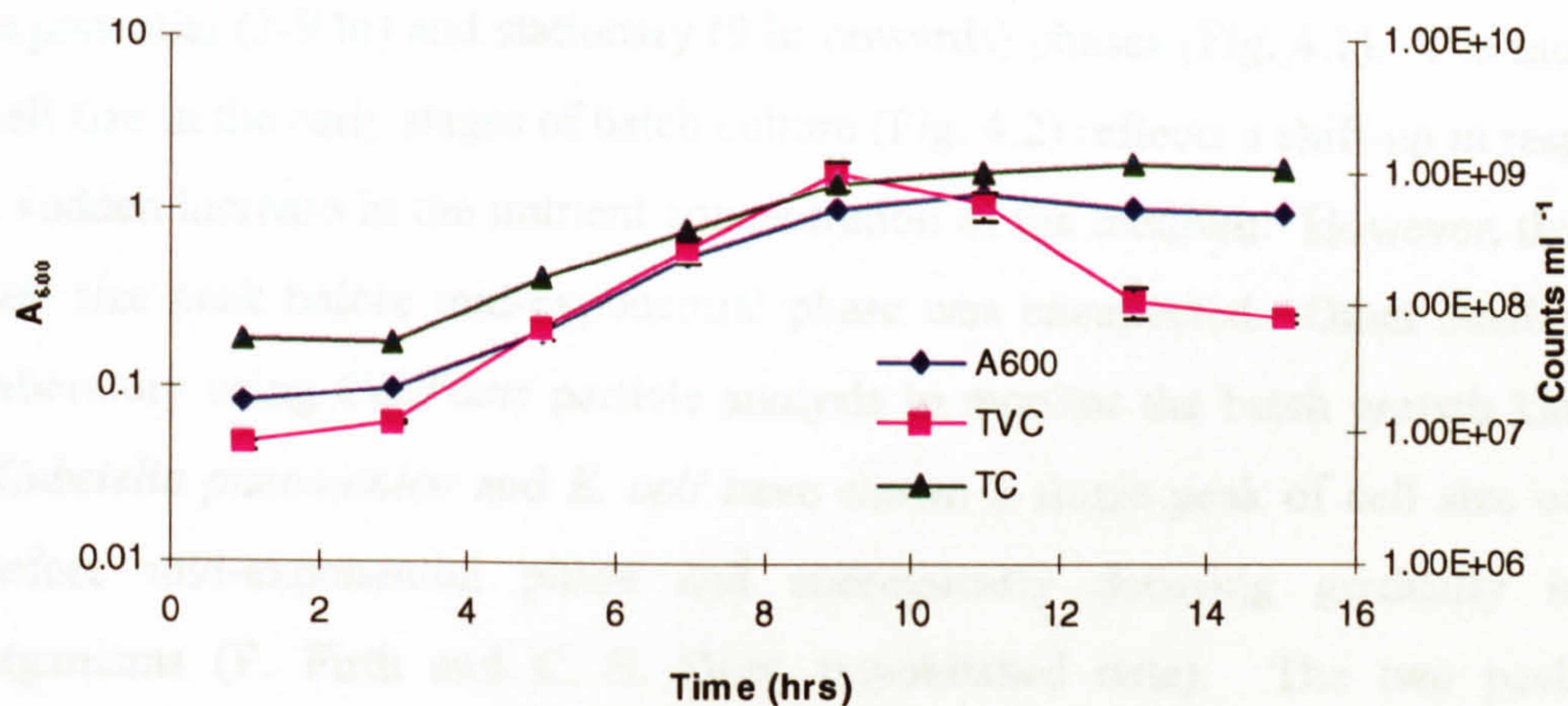


Figure 4.1 *Spingomonas* sp. batch culture growth curve. The growth of *Spingomonas* sp. in TYG broth was monitored. TVC = total viable counts ml⁻¹ on PCA; TC = total cell counts ml⁻¹ determined using *CellFacts*. Error bars shown for total viable counts represent standard deviations from triplicate samples. This graph shows one of a duplicate set of experiments, both of which gave similar profiles.

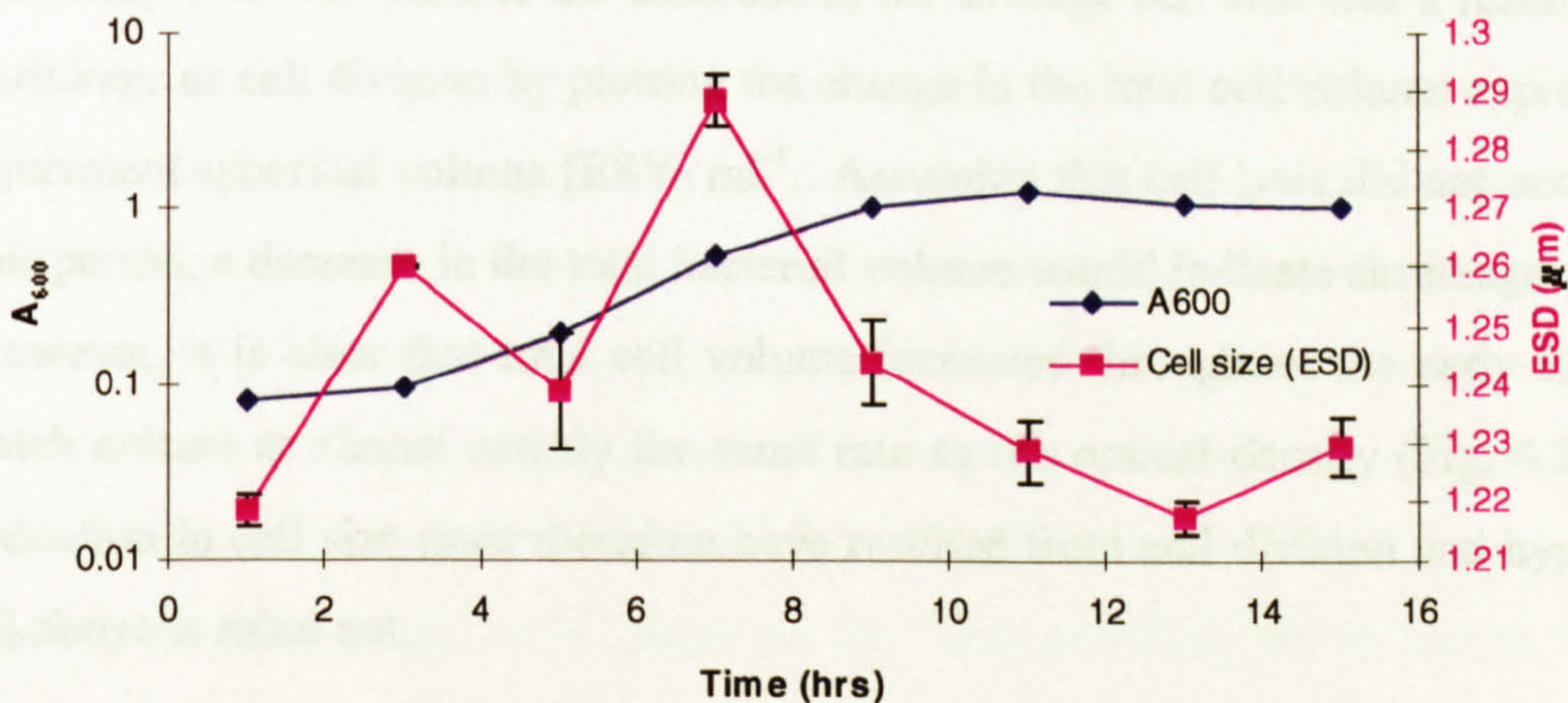


Figure 4.2 Cell size changes through batch culture of *Spingomonas* sp. in TYG broth. The mean cell size, given as ESD, was determined using *CellFacts*. Error bars are shown where large enough to be visible and indicate the standard deviation of triplicate samples. This graph shows one of a duplicate set of experiments, both of which gave similar data.

The batch growth curve showed the classic lag (0-3 hr as assessed by A_{600}), exponential (3-9 hr) and stationary (9 hr onwards) phases (Fig. 4.1). The increase in cell size in the early stages of batch culture (Fig. 4.2) reflects a shift-up in response to a sudden increase in the nutrient concentration of the medium. However, the double cell size peak before mid-exponential phase was unexpected. Other studies in this laboratory using *CellFacts* particle analysis to monitor the batch growth kinetics of *Klebsiella pneumoniae* and *E. coli* have shown a single peak of cell size occurring before mid-exponential phase and subsequently decaying gradually in these organisms (P. Firth and C. S. Dow, unpublished data). The two peaks were reproduced in a repeat of the experiment. There are two explanations that could account for the double peak:-

(a) The strain studied may have preferentially utilised one minor carbon source in the medium. If this was fully metabolised within 3 hrs of inoculation then the cells may have started to reduce in size before adapting to growth on a different substrate.

(b) Since the lag phase lasted until 3 hrs after inoculation (Fig. 4.1), the decrease in cell size between 3 and 5 hrs may have been caused by cell division. Continuation of the shift-up response until 7 hrs after inoculation would then create a second peak.

It is simple to test whether the decrease in the average cell size was a result of cell shrinkage or cell division by plotting the change in the total cell volume expressed as equivalent spherical volume (ESV) ml^{-1} . Assuming that cell lysis did not occur over this period, a decrease in the total bacterial volume would indicate shrinkage of cells. However, it is clear that total cell volume increased throughout the early stages of batch culture at almost exactly the same rate as the optical density (Fig. 4.3). The reduction in cell size must therefore have resulted from cell division and hypothesis (a) above is ruled out.

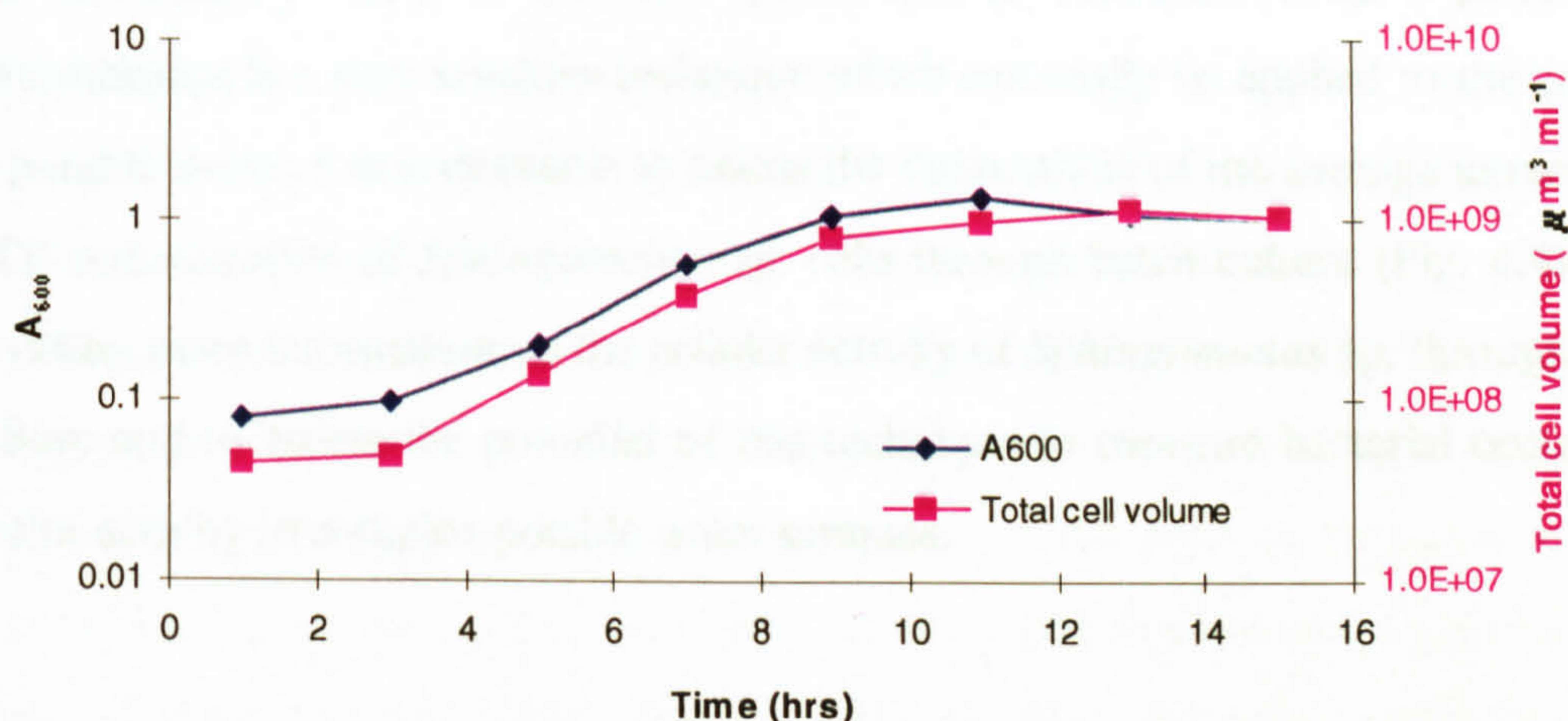


Figure 4.3 Changes in the total cell volume through batch culture of *Sphingomonas* sp. in TYG broth. The total cell volume was calculated as the product of the total cell number (Fig. 4.1) and the average cell volume.

There was another small increase in the average cell volume at the end of the batch growth cycle, between 13 and 15 hrs after inoculation (Fig. 4.2). This increase was statistically significant ($p < 0.05$) and was reproduced when the experiment was repeated. However, it should be noted that the statistical analysis was performed on just three samples at each time point and the confidence is therefore questionable. Since the cell size increase may have reflected the onset of dormancy in a proportion of cells in the population, the batch culture of *Sphingomonas* sp. was monitored for 24 hrs to analyse the peak more closely. However, the peak was not then observed and was not seen in a further repeat of the experiment (data not shown). It is therefore impossible to draw any conclusions on cell size changes in the early stationary phase of this strain based on the data obtained, but it would be an interesting area to investigate further (e.g. using different media, different strains and more frequent sampling).

4.3.1.2 Intracellular ATP concentration

The concentration of ATP within bacterial cells reflects their activity. ATP is retained in dormant cells (Porter, 1984; Emala & Weiner, 1983; Siegele & Kolter, 1992), but

lost immediately when a cell dies (Lazarova & Manem, 1995). Since ATP determination is a very sensitive technique which can easily be applied to the analysis of potable water, it was desirable to assess the fluctuations of the average intracellular ATP concentration of *Sphingomonas* sp. cells through batch culture (Fig. 4.4), both to obtain more information on the cellular activity of *Sphingomonas* sp. through batch culture and to assess the potential of this technique to measure bacterial occurrence and/or activity in complex potable water samples.

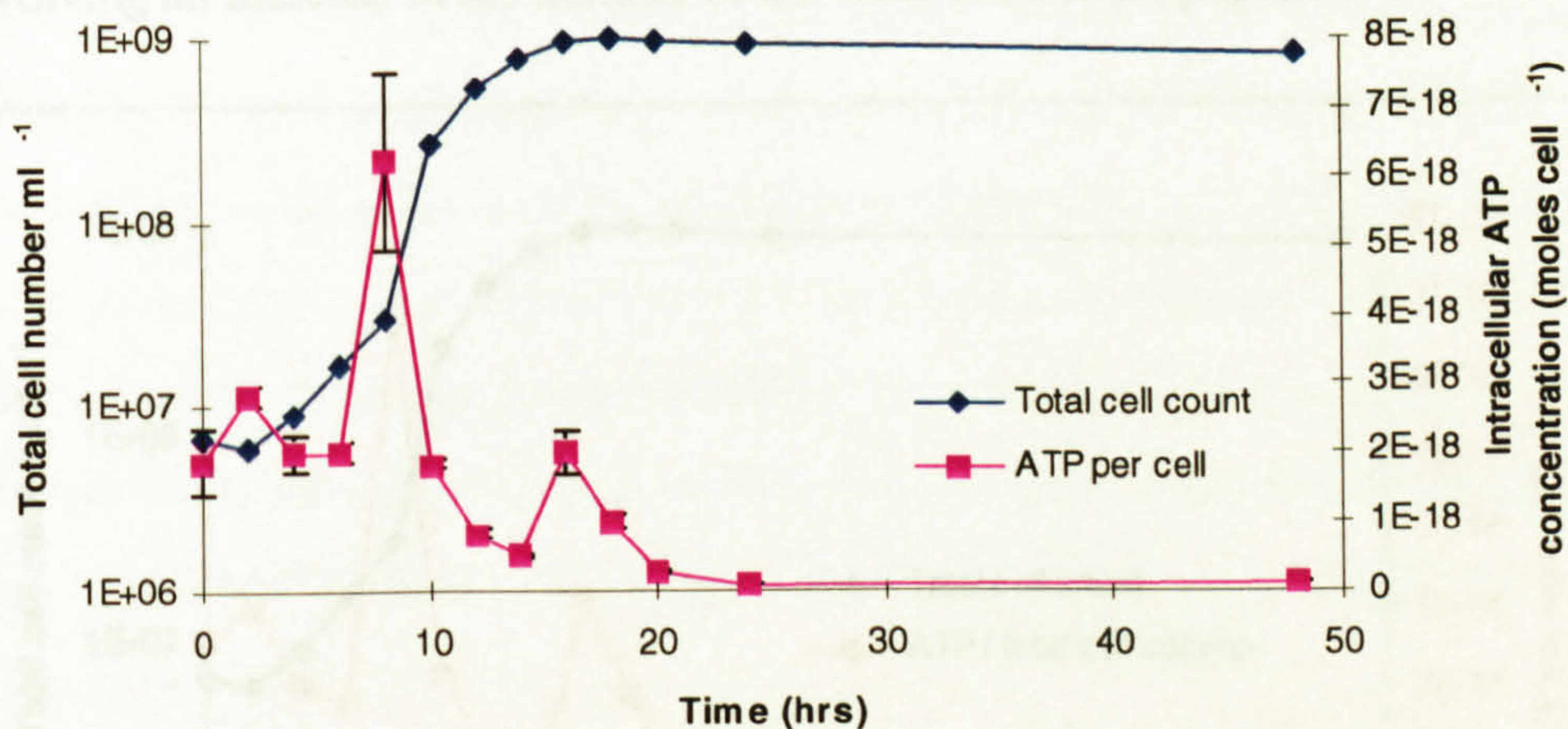


Figure 4.4 Intracellular ATP concentrations through *Sphingomonas* sp. batch culture. Intracellular ATP was measured using a luciferin-luciferase based assay, as described in Section 2.16. The ATP concentration of three samples was measured at each time point and error bars represent standard deviations from the mean. This graph shows one of a duplicate set of experiments, both of which gave similar data.

The peaks at 2 hrs and 8 hrs closely resemble the cell size maxima seen in the previous experiment (Fig. 4.2). The simplest explanation for this would be that the total intracellular ATP concentration was linked to the total volume of cellular material during the early stages of batch culture. However, an analysis of the relationship between these two parameters shows that this explanation is not valid (Fig 4.5). The first peak, two hours after inoculation, may have arisen from a shift-up in the average intracellular ATP concentration prior to utilisation of the ATP for cell division. The second sharp peak occurred in the middle of the exponential phase of growth, 8 hrs after inoculation of the culture, when virtually all the cells in the population would have been actively growing and dividing. A third peak of intracellular ATP was apparent at the onset of stationary phase, 16 hrs after inoculation. It is interesting to note that this was the point at which a transient increase in the cell size had been previously been observed (Fig. 4.2). It is likely that the changes in cell size and ATP concentration at this point reflected an active cellular response to nutrient depletion, involving an increase in the number of dormant cells in the population.

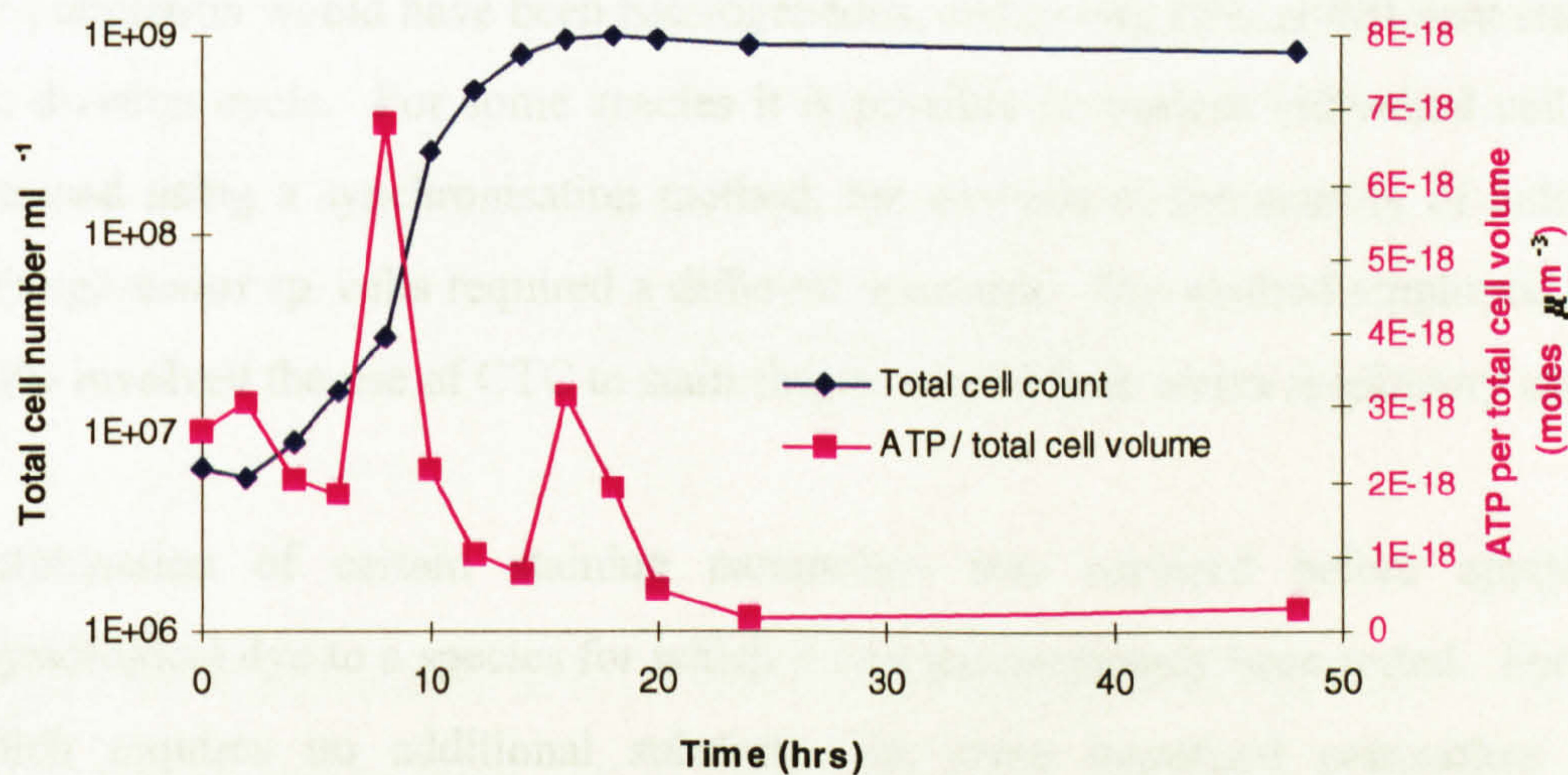


Figure 4.5 Relationship between the total intracellular ATP concentration and the total volume of cellular material through batch culture of *Sphingomonas* sp. in TYG broth. The total bacterial volume was calculated from *CellFacts* particle analysis data as the product of the mean cell volume and the total particle count between 0.75-2.0 μm.

The specific intracellular ATP concentration remained roughly constant between 24-48 hrs. Therefore in this *Sphingomonas* sp., as in other bacteria (Roth *et al.*, 1988; Emala & Weiner, 1983; Porter, 1984), ATP was maintained in non-growing cells. Assuming that most of the planktonic bacterial population in potable water is not actively growing and that intracellular ATP concentrations (i.e. amount of ATP per unit cell volume) are similar within dormant cells of all bacterial strains in tap water, then ATP should be a reasonable indicator of biomass. These assumptions appear to hold for most of the limited number of studies performed to date, but it will be necessary to analyse the intracellular ATP concentrations of many other tap water bacteria before a clear universal link between ATP and bacterial biomass in potable water can be confirmed.

4.3.1.3 Respiratory chain activity

Of course, the measurements performed above provided an average value of the intracellular ATP concentration of a non-synchronous population of cells. In fact, the population would have been heterogeneous, containing cells at different stages of the division cycle. For some species it is possible to analyse individual cell types obtained using a synchronisation method, but analysis of the activity of individual *Sphingomonas* sp. cells required a different approach. The method employed in this study involved the use of CTC to stain single cells with an active respiratory chain.

Optimisation of certain staining parameters was required before applying a physiological dye to a species for which it had not previously been tested. For CTC, which requires no additional substrate, the most important parameters to be considered were the concentration of dye and time of staining. Exponentially growing *Sphingomonas* sp. populations were always incubated in the dark at 30°C after CTC addition and the effects of different incubation times and CTC concentrations on staining were monitored by fluorimetry (Fig. 4.6).

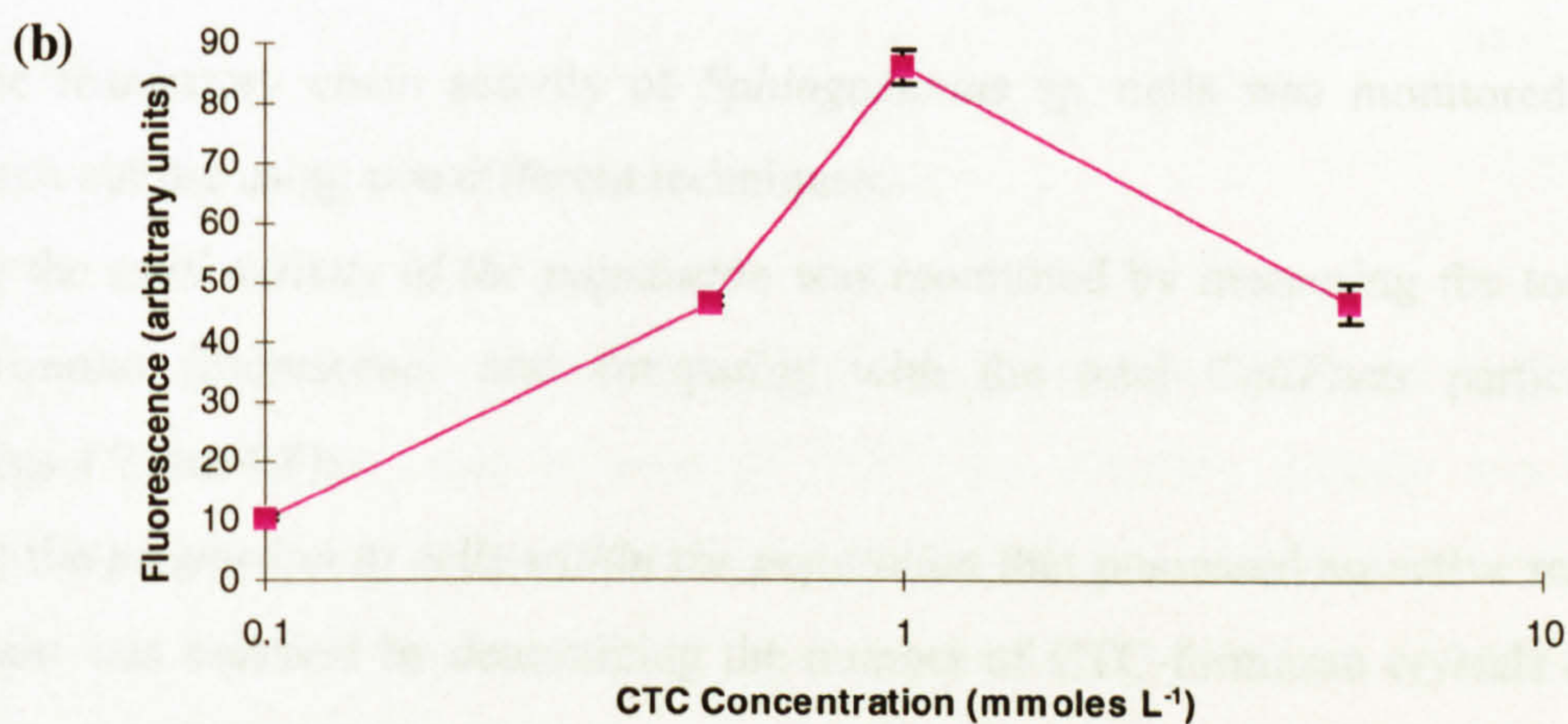
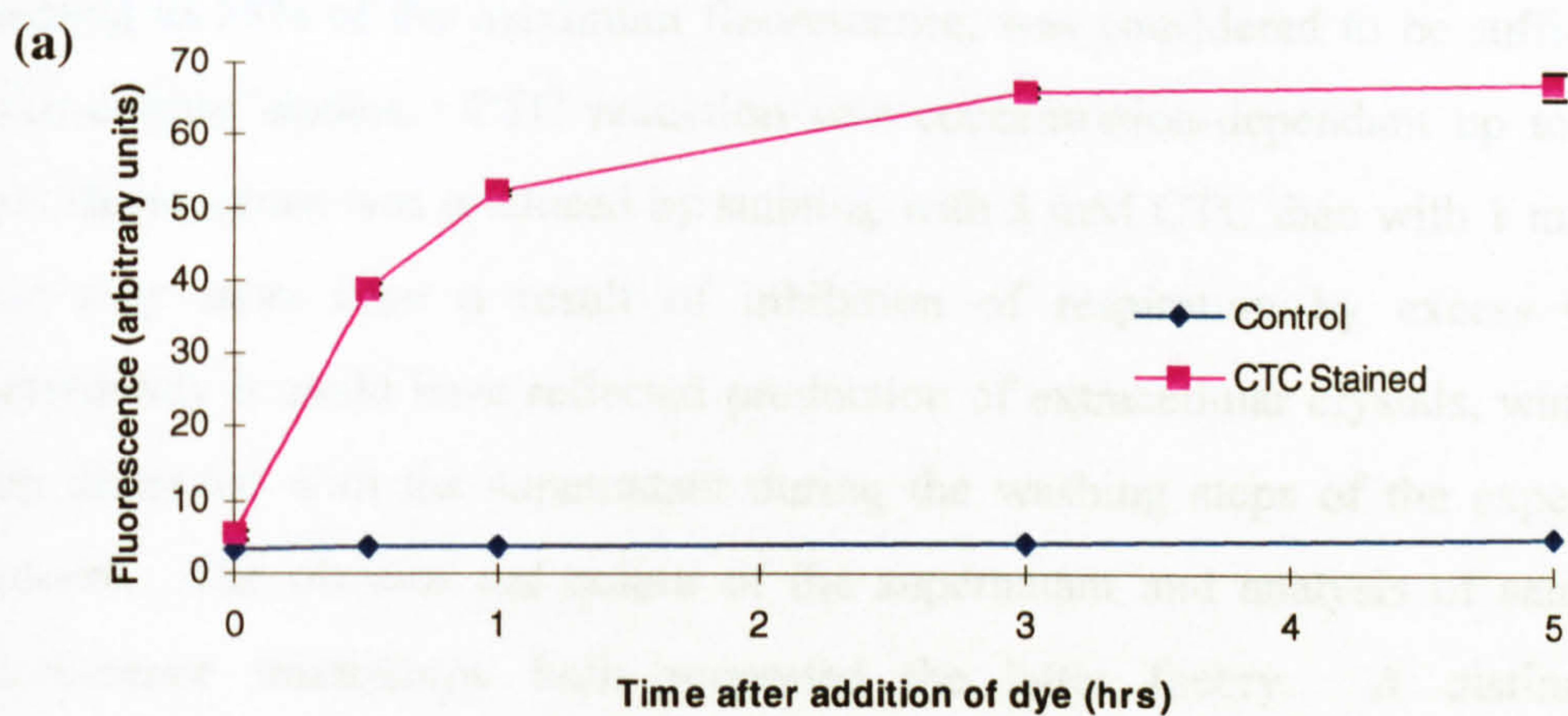


Figure 4.6 Optimisation of CTC staining of *Sphingomonas* sp. cells. The effects of (a) time of incubation of cells in 0.5 mM CTC and (b) CTC concentration using a 30 minute incubation time were assessed. Cells were incubated at 30°C in the dark and the amount of CTC staining was measured as the relative fluorescence emission at 630 nm after excitation at 450 nm. Control samples (no CTC) were included when assessing the effect of incubation time on CTC fluorescence. Error bars represent standard deviations from triplicate experiments.

Reduction of CTC followed first order kinetics. An incubation time of 30 min, resulting in 58% of the maximum fluorescence, was considered to be sufficient for batch culture studies. CTC reduction was concentration-dependant up to 1 mM. Less fluorescence was produced by staining with 5 mM CTC than with 1 mM CTC. This may have been a result of inhibition of respiration by excess CTC or alternatively it could have reflected production of extracellular crystals, which were then discarded with the supernatant during the washing steps of the experimental protocol. The obvious red colour of the supernatant and analysis of samples by fluorescence microscopy both supported the latter theory. A distinctly red supernatant was also observed when washing cells after staining with 1 mM CTC so a concentration of 0.5 mM was chosen for further work to minimise formation of extracellular crystals.

The respiratory chain activity of *Sphingomonas* sp. cells was monitored through batch culture using two different techniques:-

- (a) the *total activity of the population* was monitored by measuring the total CTC-formazan fluorescence and comparing with the total *CellFacts* particle count (Figs 4.7 and 4.8);
- (b) the *proportion of cells within the population* that possessed an active respiratory chain was assessed by determining the number of CTC-formazan crystals observed microscopically and comparing this with the total cell count obtained by counterstaining with DAPI (Fig. 4.8).

The total CTC-formazan fluorescence provided a bulk measurement of the activity of the population, comparable to measurements of cell size and ATP. The microscopic count produced an assessment of the heterogeneity of the population and was a technique that could be applied to quantify the active cells in potable water biofilms. The total cell count obtained by DAPI staining was consistently lower than the *CellFacts* count. This was a result of loss of cells during staining and preparation of slides. It seems fair to assume that there would be no bias towards the loss of either active or inactive cells so the CTC:DAPI ratio should not have been affected.

The total activity of the population, in terms of the ability to reduce CTC, followed similar kinetics to the optical density of the culture, except that no lag was observed before the initial increase in fluorescence. Fluorescence of the control (no CTC) remained relatively low.

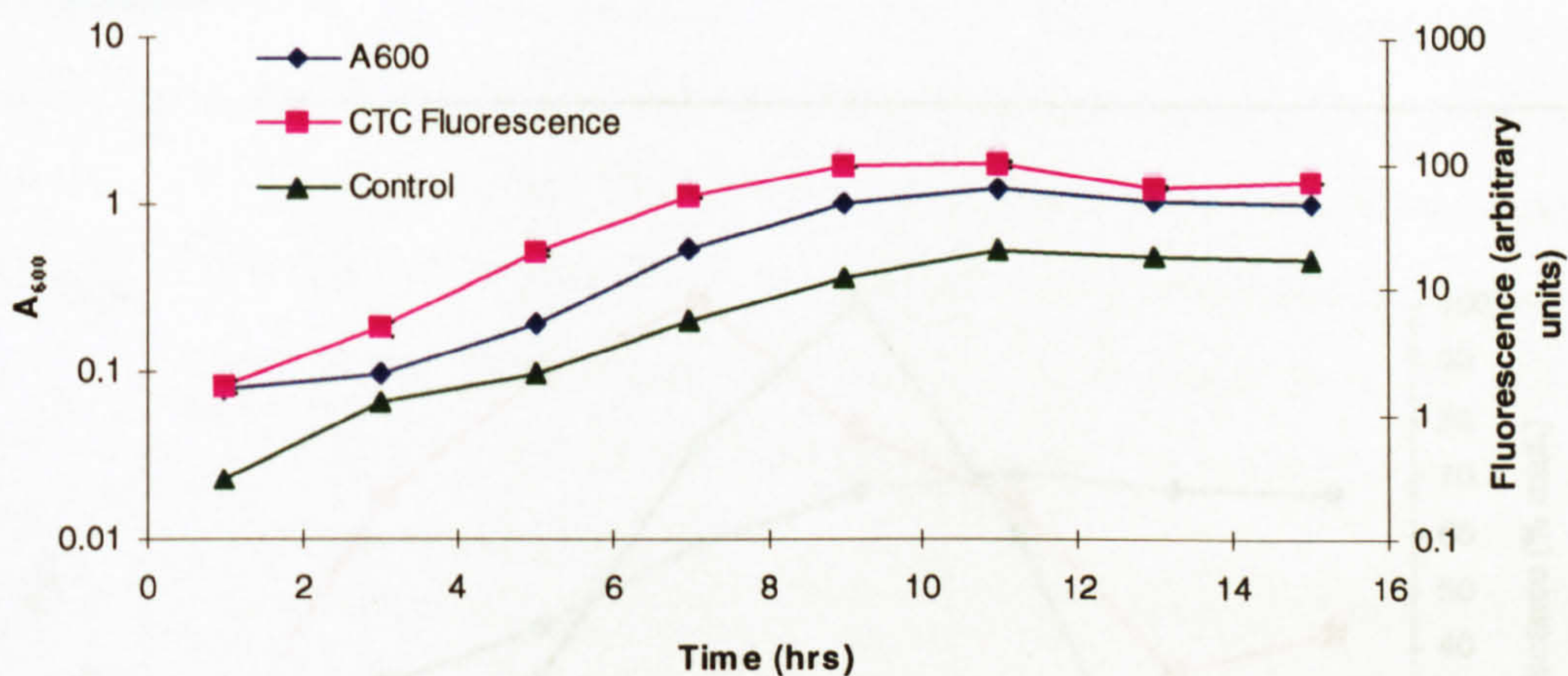


Figure 4.7 Total CTC fluorescence through *Sphingomonas* sp. batch culture. Samples were removed at intervals and CTC reducing activity was measured fluorimetrically. Error bars represent standard deviations of triplicate samples. A control (no CTC) was performed at each time point. One of a duplicate set of experiments is shown. Both sets gave similar data.

The average specific cellular activity, expressed as the total fluorescence per *CellFacts* particle count, increased in a hyperbolic manner until reaching a peak seven hours after inoculation of the culture (Fig. 4.8). This showed different kinetics from the exponential increase in total cell volume or the double peaks of average cell volume and intracellular ATP observed over the same period (Figs 4.2 - 4.5). The rapid increase in CTC reduction was produced by a small proportion of the population since the CTC:DAPI ratio was relatively low during the first seven hours of batch culture (Fig. 4.8). Between 13-15 hrs, there was a slight increase in the activity of the population and in the total number of CTC-formazan crystals produced per cell. This

corresponds to the point at which the cell size and the intracellular ATP concentration increased (Figs 4.2 - 4.5) and is interpreted as part of an active stationary phase response to produce dormant cells. The pattern of CTC staining towards the end of batch culture was complicated by the production of extracellular CTC-formazan crystals (Fig. 4.9). These may have artificially elevated the microscopic CTC counts, but would have decreased the fluorimetric yield through loss of crystals in the supernatant.

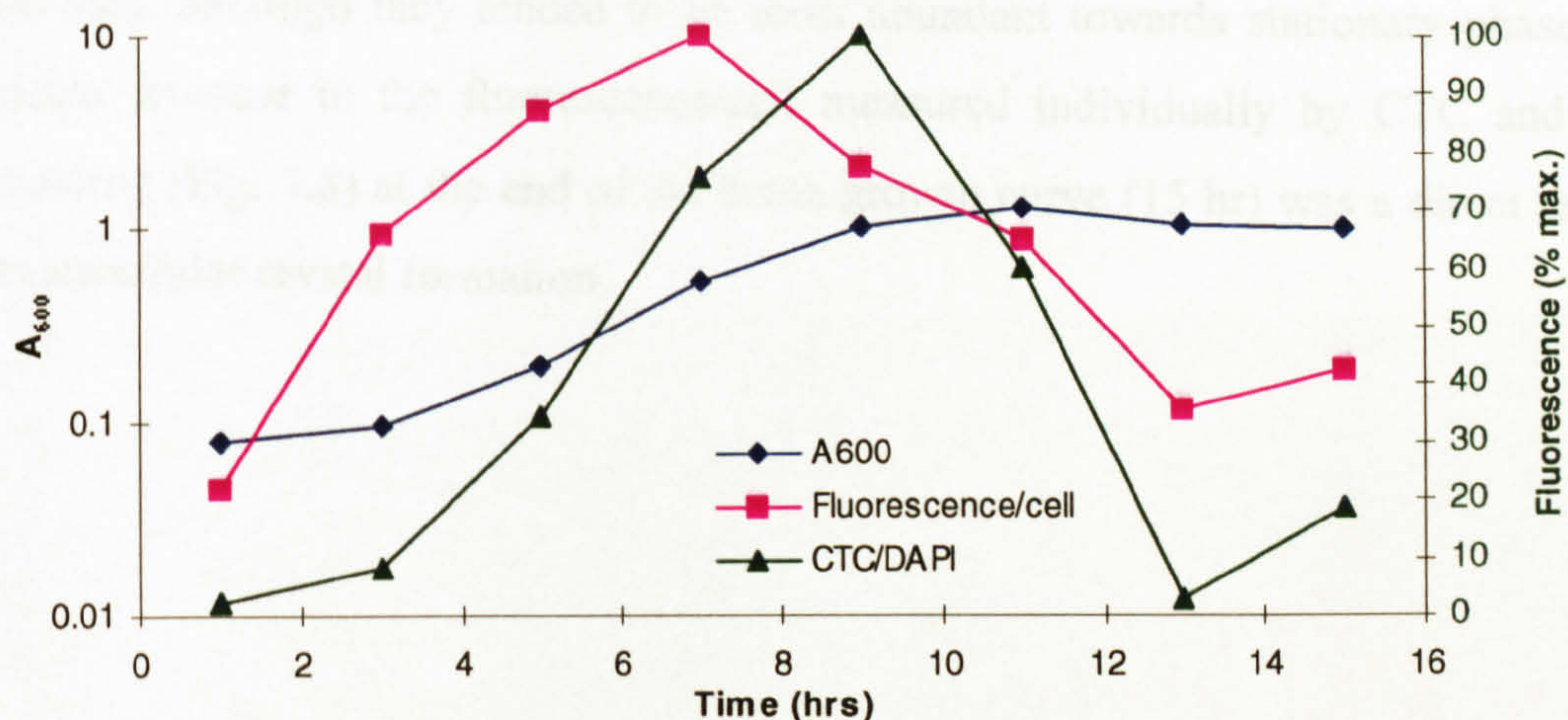
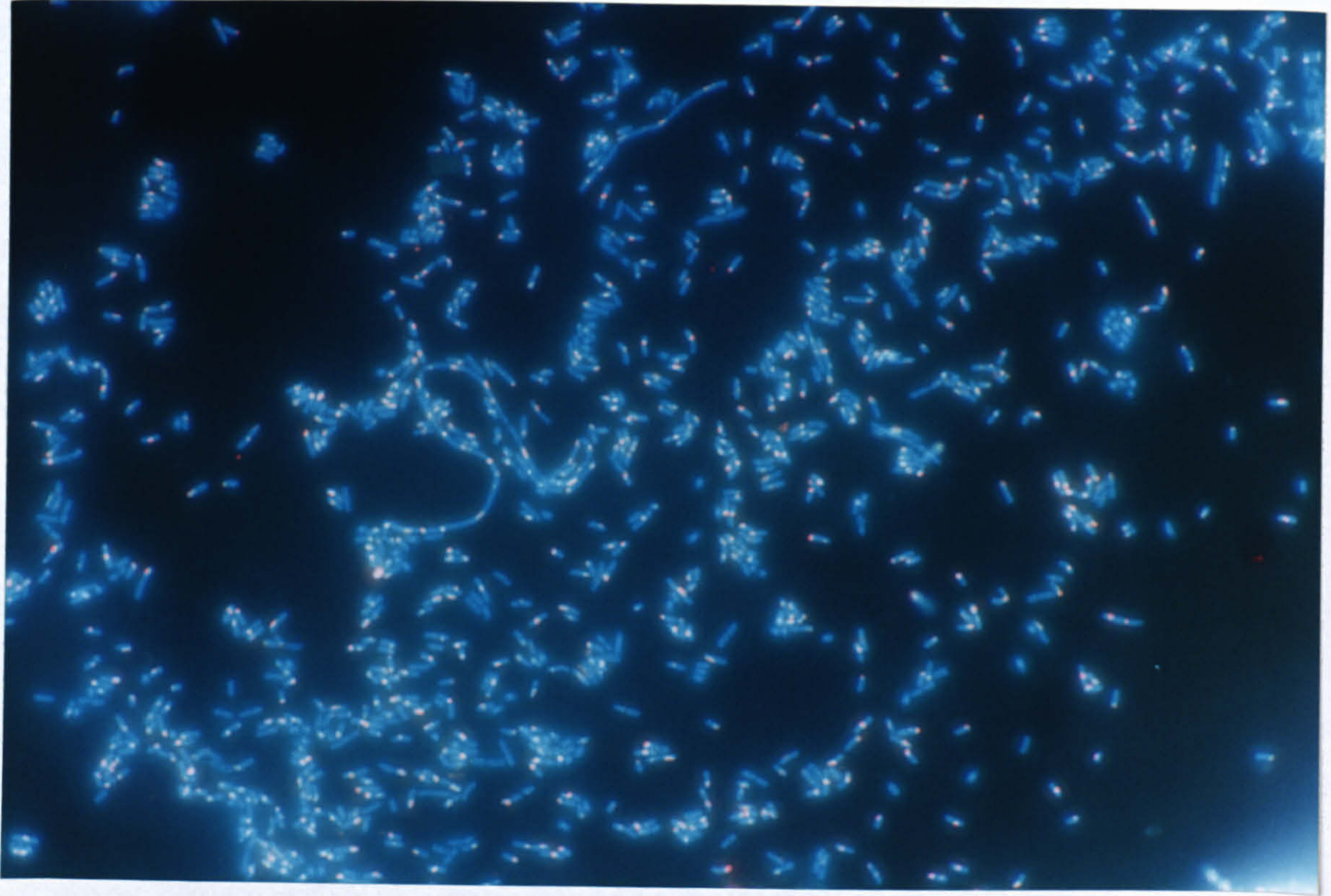


Figure 4.8 CTC fluorescence per cell through *Sphingomonas* sp. batch culture. Samples were removed at intervals and CTC reducing activity per cell was measured using two different techniques: (a) fluorescence/cell = fluorescence measured by fluorometry per total cell no. determined by *CellFacts* and (b) CTC/DAPI = fluorescence/cell calculated as microscopic CTC counts per microscopic DAPI counts, both obtained using fluorescence microscopy with the relevant filter set and image analysis. This graph shows one of a duplicate set of experiments, both of which gave similar data.

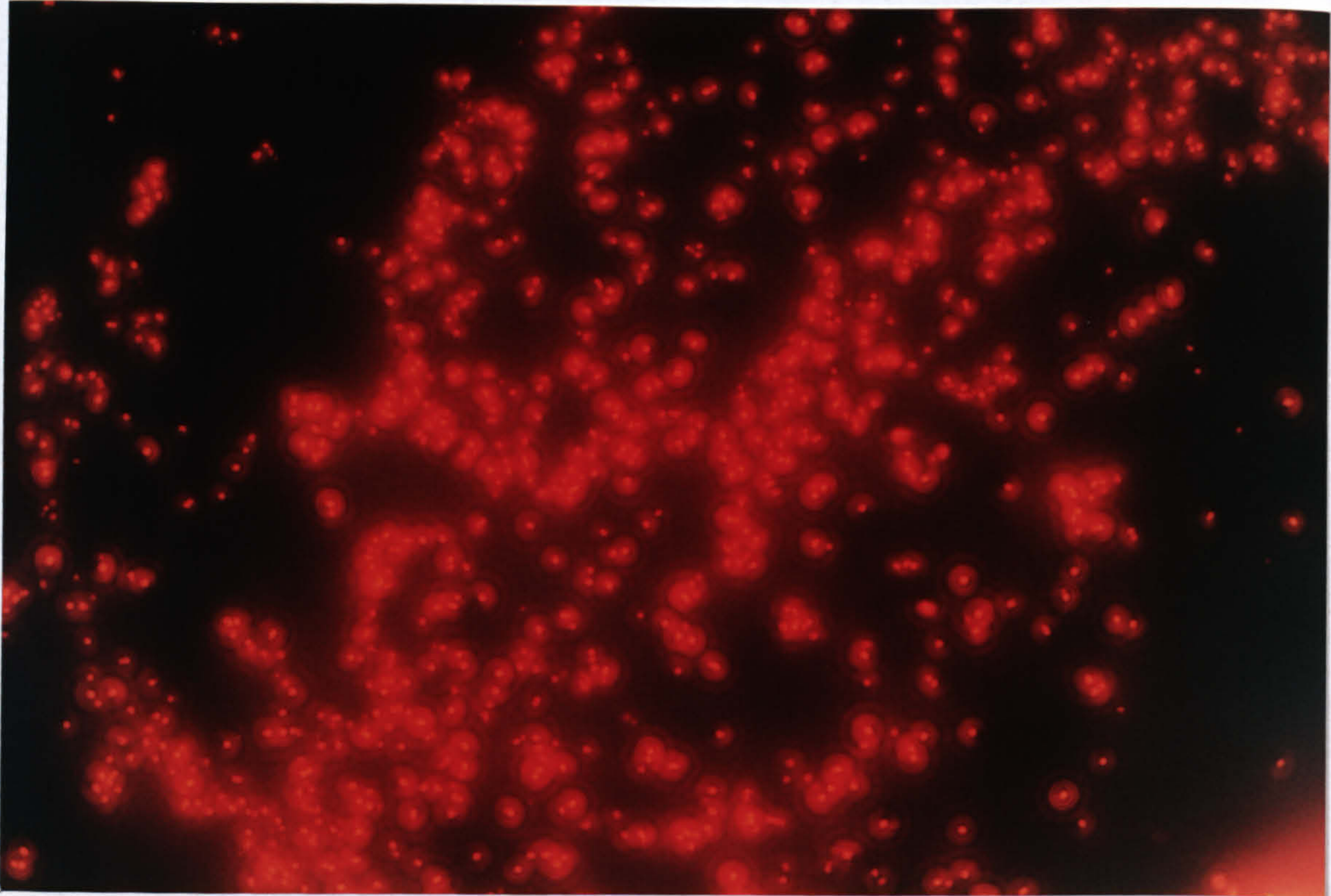
Despite the differences in the kinetics of total CTC reduction, the proportion of cells that reduced the dye and other measures of cellular activity (cell size and ATP) over

the early stages of batch culture, all measurements showed a peak during the mid-or late-exponential phase of growth and a second peak at around 15 hrs.

For most samples each cell contained a single formazan crystal, usually localised at one pole (Fig. 4.9(a and b)). Some abnormally long cells were seen, containing formazan crystals at regular intervals. Occasionally extracellular crystals were observed. However, some samples were strewn with relatively small extracellular crystals in addition to the larger intracellular formazan deposits (Fig. 4.9(c and d)). The conditions under which the extracellular crystals were formed were not well defined, although they tended to be most abundant towards stationary phase. The slight increase in the fluorescence/cell measured individually by CTC and DAPI staining (Fig. 4.8) at the end of the batch growth curve (15 hr) was a direct result of extracellular crystal formation.

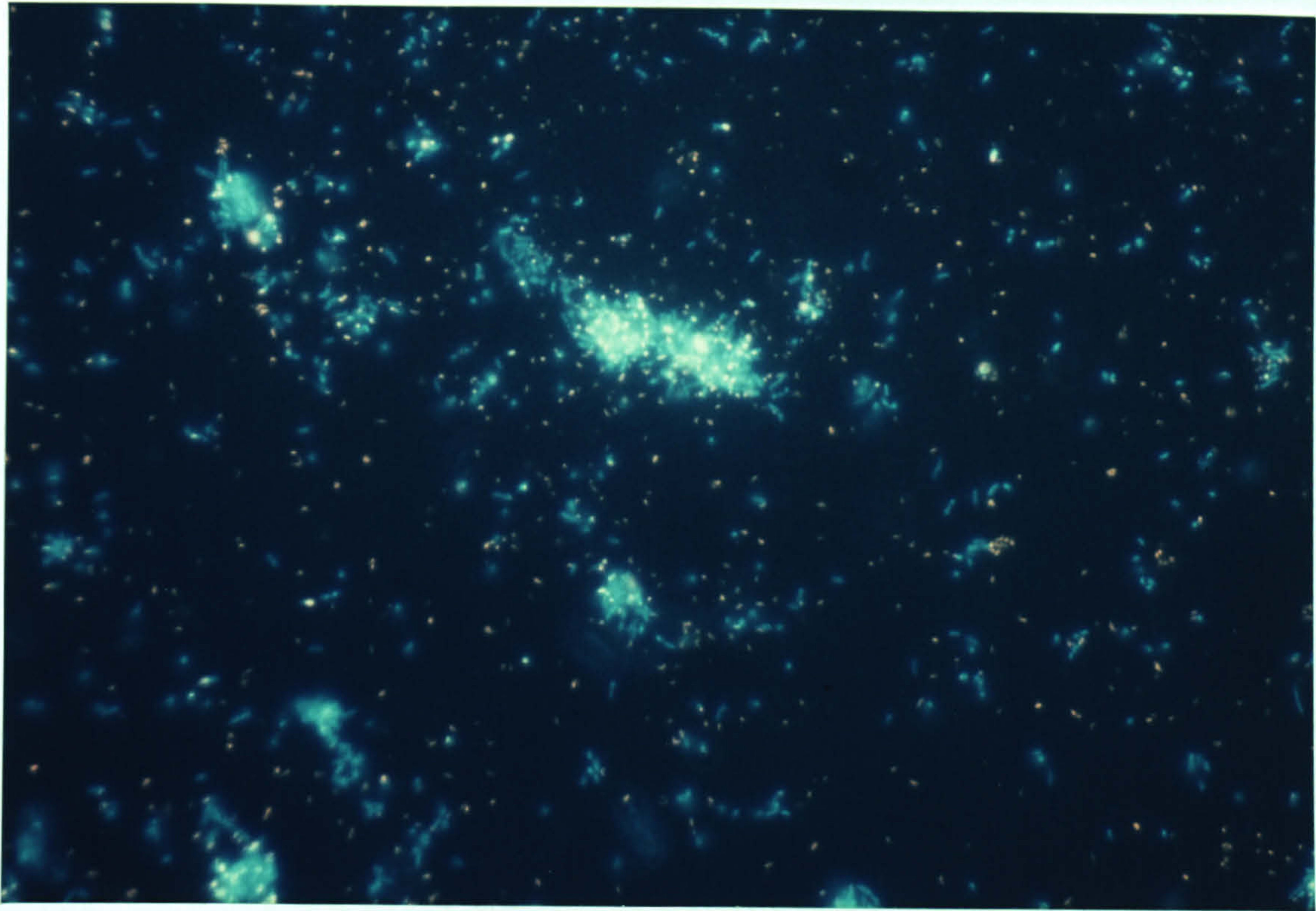


(a)

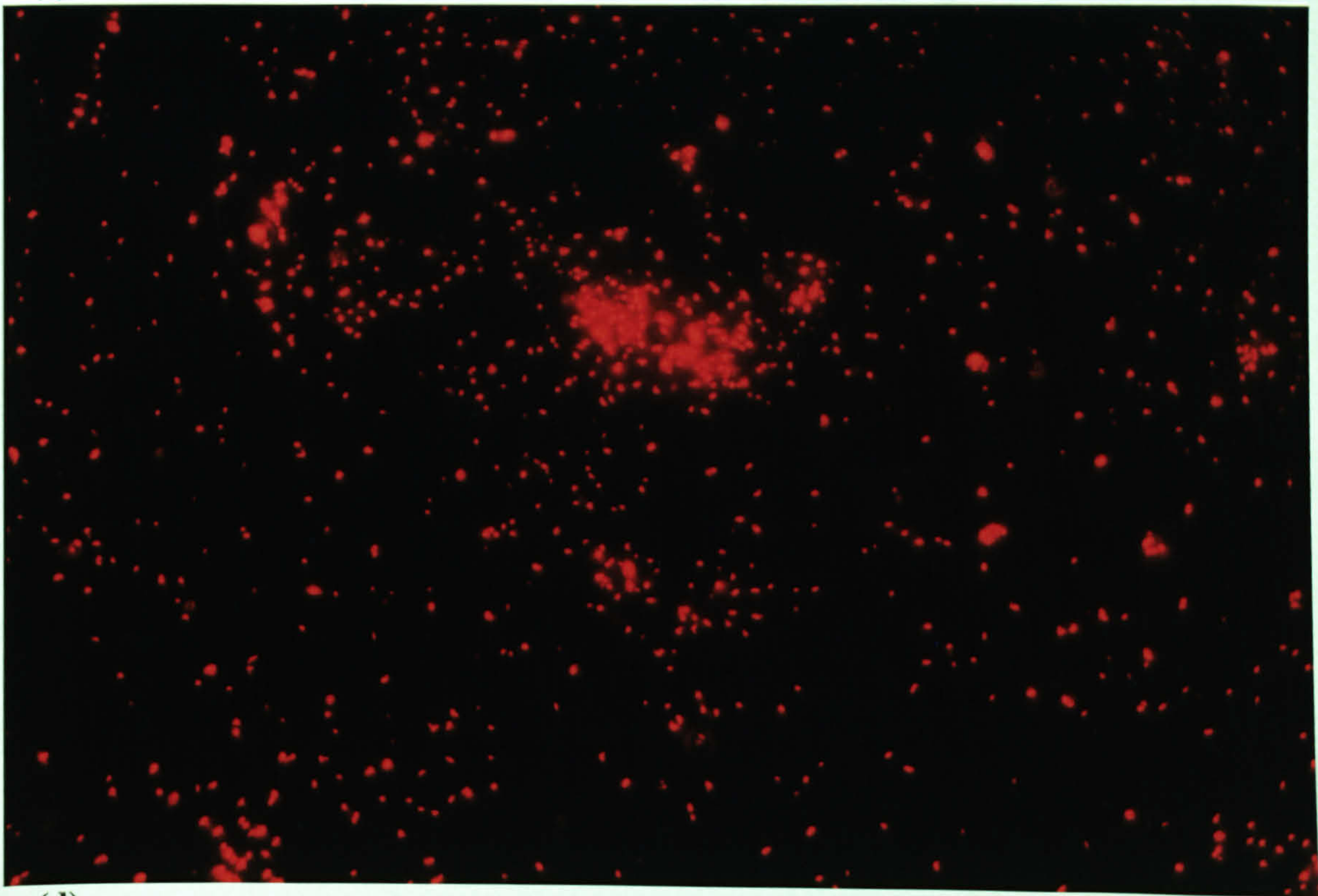


(b)

Figure 4.9 Fluorescence micrographs of *Sphingomonas* sp. cells stained with CTC and counterstained with DAPI. (a) Cells cultured for 8 hrs in TYG observed using the DAPI filter set, (b) the same field under the CTC filter set, (c) cells cultured



(c)



(d)

for 15 hrs in TYG observed under the DAPI filter set and (d) the same field under the CTC filter set. (a) and (b): most cells contain a single formazan crystal; (c) and (d): extensive extracellular formazan deposition is apparent. (a) and (b), bar = 10 μm ; (c) and (d), bar = 50 μm .

There are three possible explanations for the abundance of extracellular formazan deposits seen under certain conditions in this study:-

- (a) in certain circumstances the cells can detect the accumulation of a toxic formazan deposit and actively secrete it,
- (b) cells sometimes secrete enzymes capable of catalysing CTC reduction outside the cell, or
- (c) cells may have lysed (see below).

Although the extracellular crystals observed were generally smaller than those located inside cells, it seems unlikely that they could have escaped the cell without lysis of the membrane. Since extensive cell lysis was not seen in samples containing large numbers of extracellular crystals, it is presumed that extracellular CTC reduction occurred. In a previous investigation, CTC reduction occurred in nutrient-rich medium in the absence of cells (Bovill *et al.*, 1994). However, in this study no CTC was reduced in TYG broth without cells or in TYG broth containing fixed cells (cells and 1% (v/v) glutaraldehyde). Care must be taken to avoid extracellular staining when applying CTC *in situ* since it is difficult to relate the number of formazan crystals to the number of actively respiring cells if a single cell is capable of producing more than one fluorescent crystal.

4.3.2 Cell physiology during culture of *Caulobacter crescentus* CB15

4.3.2.1 Cell size and morphology

The life cycle of *C. crescentus* is well-documented (Cooper, 1991; Fig. 4.10). The morphology of the *C. crescentus* CB15 cell types is shown in Fig. 4.11.

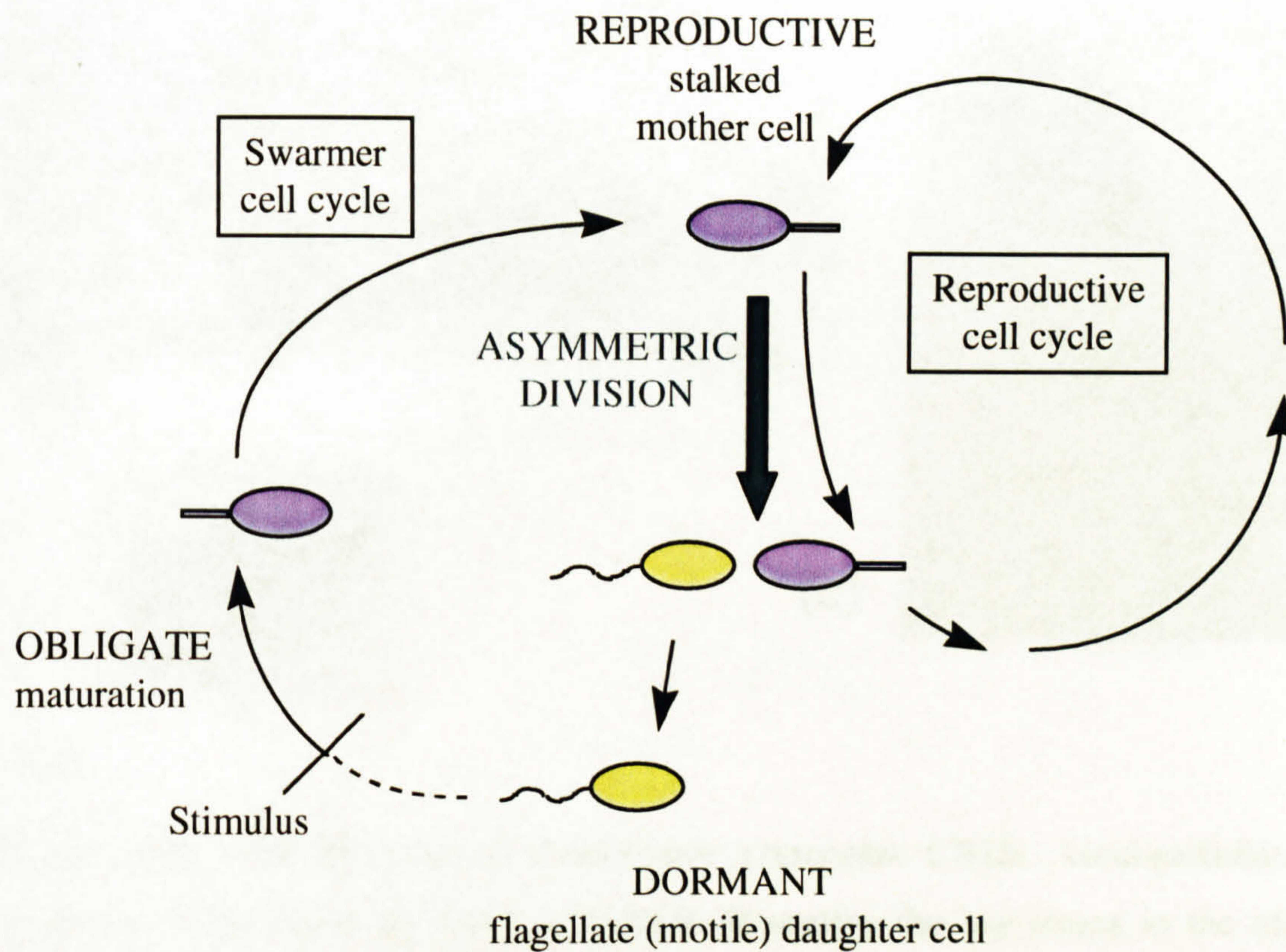


Figure 4.10 Schematic representation of the life cycle of *Caulobacter crescentus*. The life cycle of this organism is biphasic, involving an asymmetric division step. The stalked mother cell produced is capable of undergoing another round of cell division immediately. The flagellate daughter cell is dormant. Upon receiving a stimulus it embarks on an obligate sequence of maturation events, including shedding of the flagellum and production of a stalk. Once mature, this cell begins to divide.

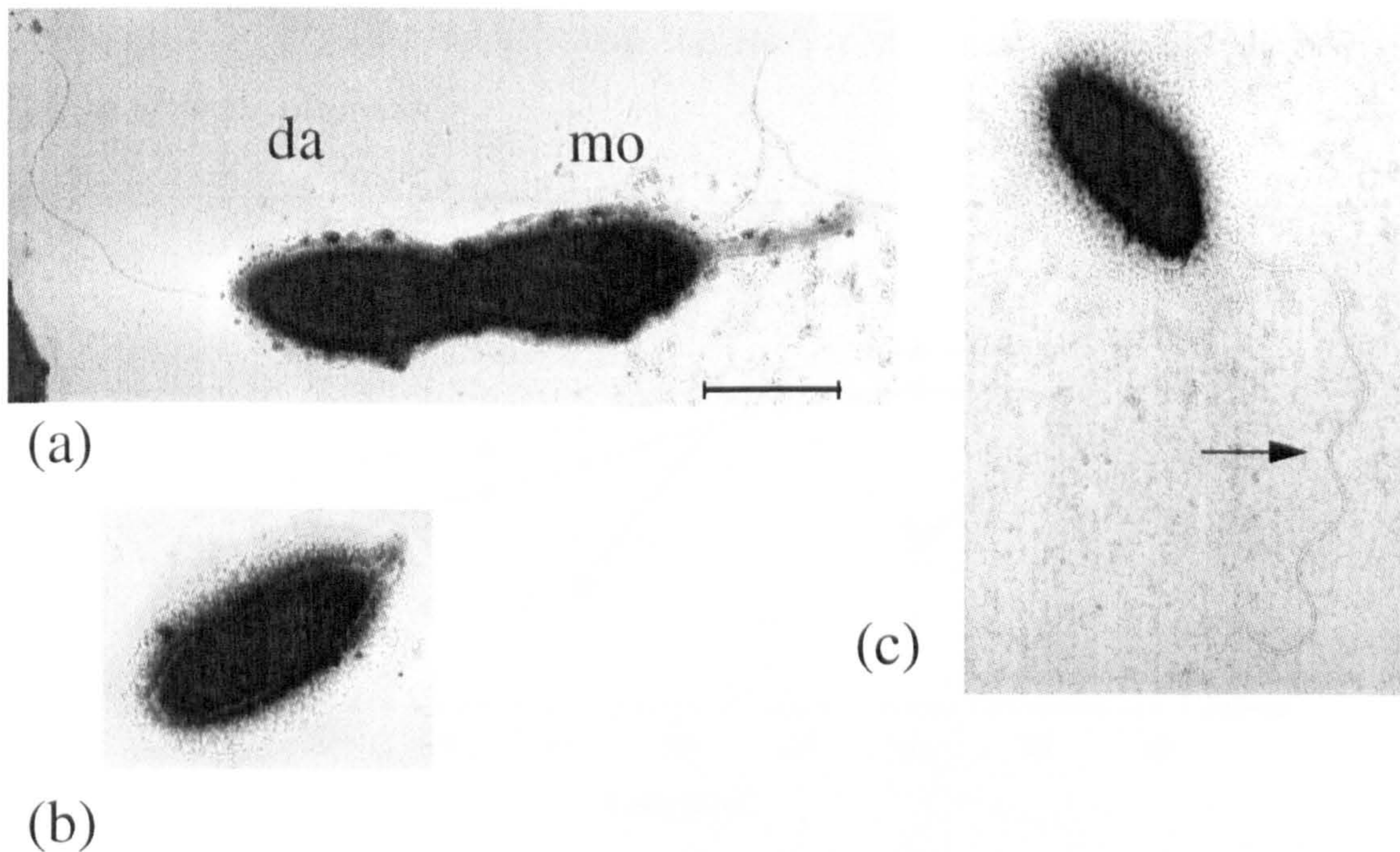


Figure 4.11 The life cycle of *Caulobacter crescentus* CB15. Gold-palladium shadowed transmission electron micrographs illustrating the key stages in the life cycle of *C. crescentus* CB15. (a) A cell dividing asymmetrically into a stalked mother cell (mo) and a flagellate daughter cell (da), (b) a single mother cell possessing a polar stalk and (c) a daughter cell with a polar flagellum (arrow). Bar = 1 μm .

The kinetics of *C. crescentus* CB15 batch culture and cell size fluctuations are shown in Figs 4.12 and 4.13. Stationary phase was not reached during the first 30 hrs of batch culture. By the time the second sample was taken, after 4 hrs, cell division had started (Fig. 4.12). Therefore the absence of a second cell size peak in the early phase of batch culture, as was produced by *Sphingomonas* sp. (Fig. 4.13; compare with Fig. 4.2), was expected. More frequent sampling would be needed to determine whether a second peak of cell size occurred, but was not detected, or whether there was only one peak at this stage of growth, as seen during the batch culture of *K. pneumoniae* or *E. coli* (P. Firth and C. S. Dow, unpublished). The discrepancy between the size of mother and daughter cells was insufficient to be detected by *CellFacts* analysis. However, an increase in cell size was detected upon entry into stationary phase, after 30 hrs. This is similar to the increase in the size of

Sphingomonas sp. when approaching stationary phase and may reflect an active stationary phase response.

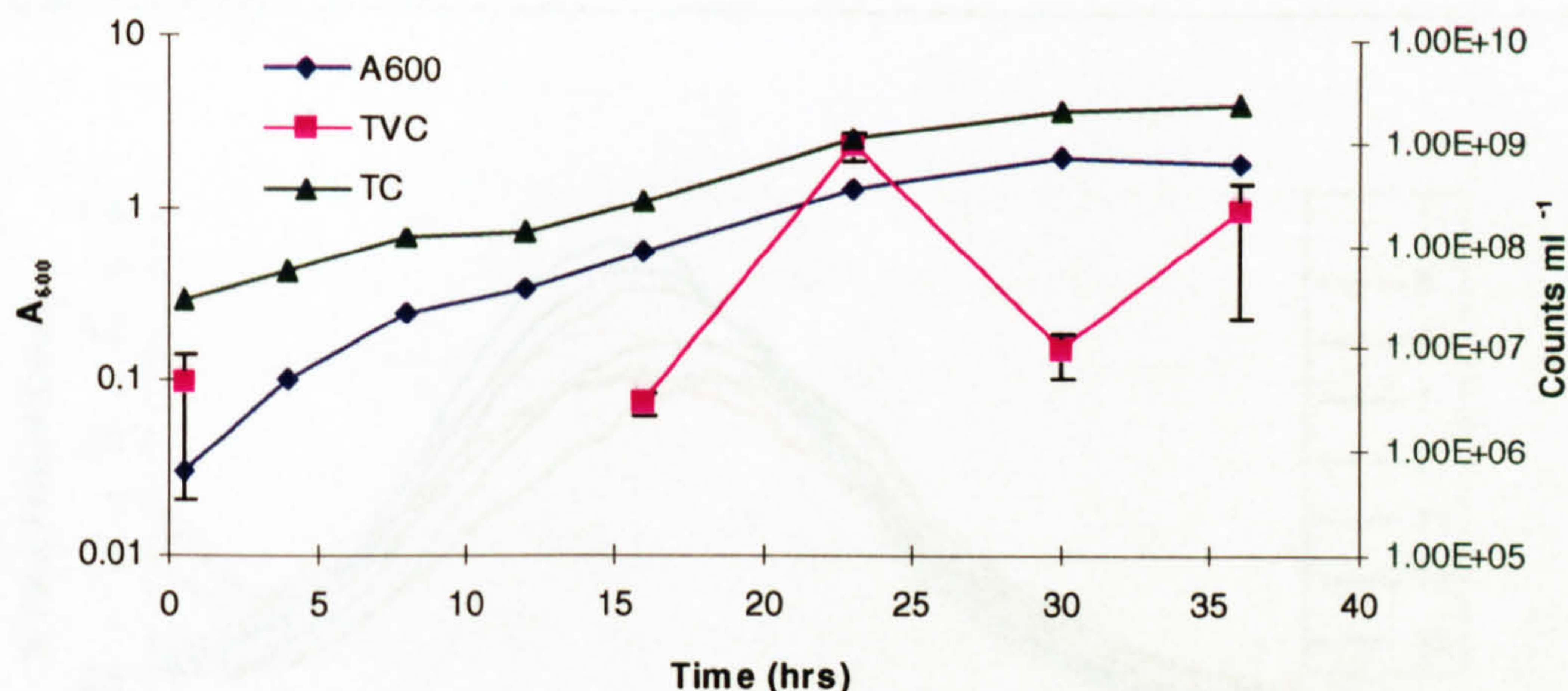


Figure 4.12 Growth curve of *C. crescentus* CB15 in PYE broth. Samples were removed at intervals and A_{600} , total viable counts (TVC) on PYE agar and total *CellFacts* counts (TC) were measured. Error bars shown for total viable counts represent standard deviations from triplicate samples. The graph shows one of a duplicate set of experiments, both of which gave similar data.

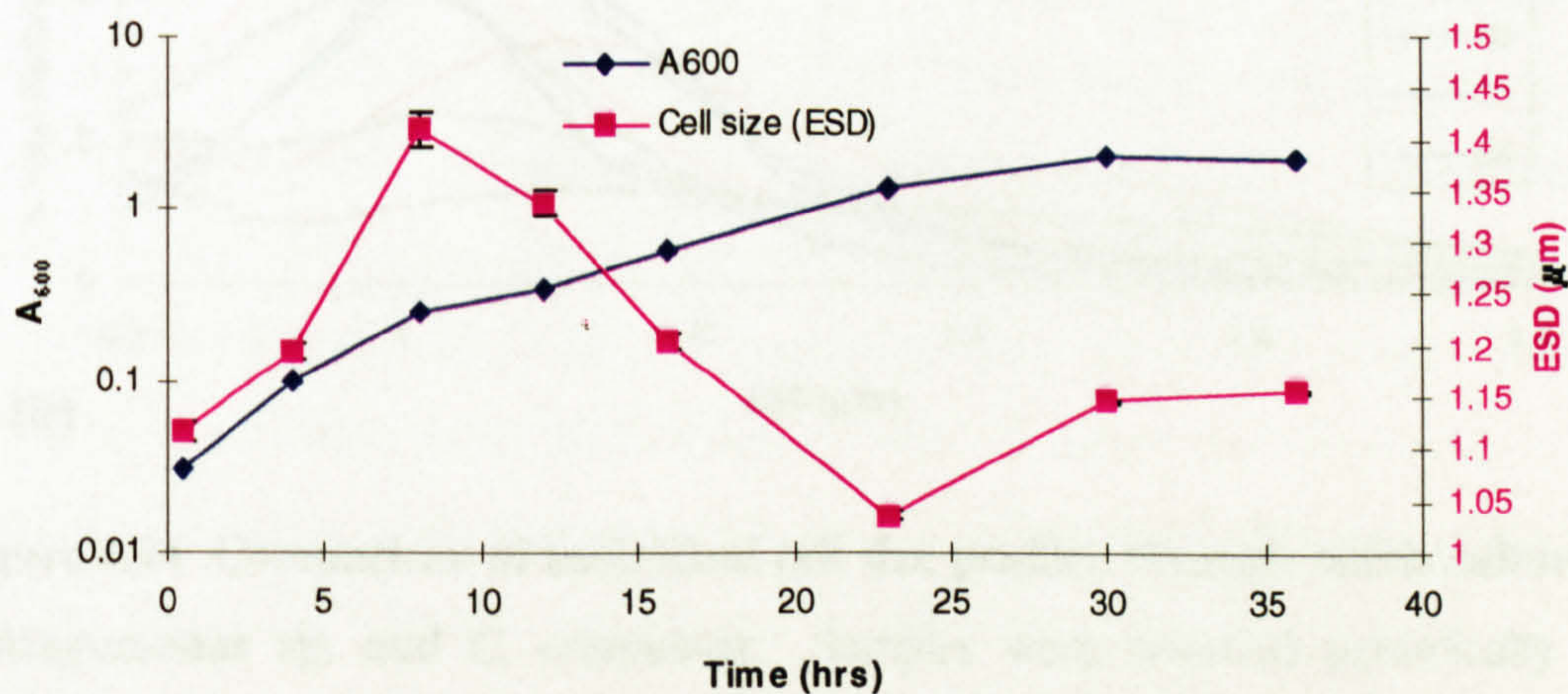


Figure 4.13 Cell size variation through *C. crescentus* CB15 batch culture. Cell size, expressed as ESD, was determined using *CellFacts*. Standard deviations of triplicate cell size analyses are shown, where large enough to be visible. The experiment was performed in duplicate, giving similar data each time. The graph uses data from one of the two replicates.

A closer analysis of the cell size profiles of *Sphingomonas* sp. and *C. crescentus* CB15 shows obvious differences between growth of cells of the two species in batch conditions (Fig. 4.14).

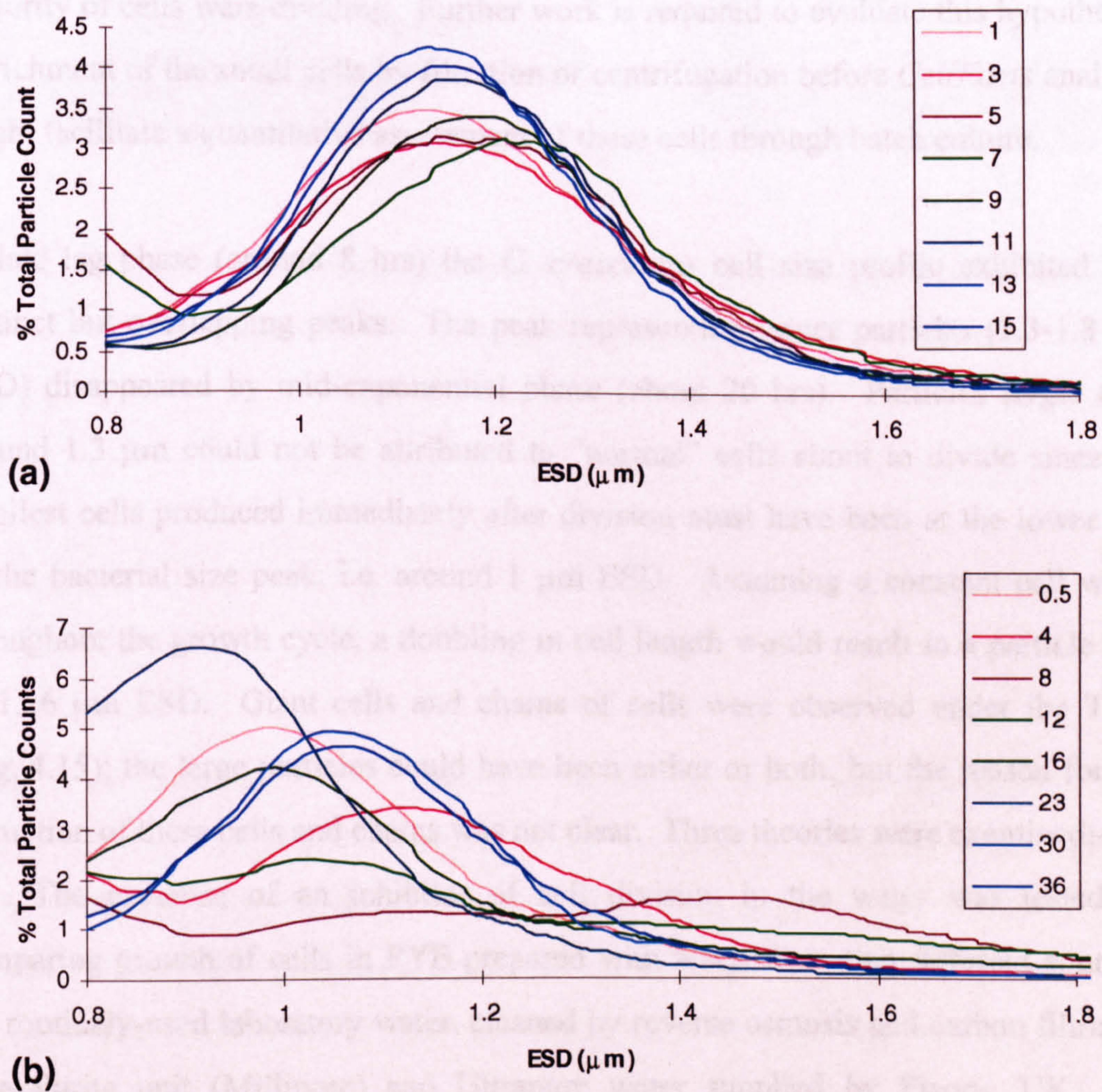


Figure 4.14 Comparison of individual cell size profiles through batch culture of *Sphingomonas* sp. and *C. crescentus*. Samples were removed periodically and analysed using *CellFacts*. At each time point three profiles were obtained. These were averaged and the resulting profiles were normalised to allow for variations in total particle counts. Profile labels indicate the number of hours after inoculation of batch cultures. (a) *Sphingomonas* sp. cultured in TYG and (b) *C. crescentus* CB15 cultured in PYE.

A shoulder was apparent on most of the *Sphingomonas* sp. cell size profiles at about 0.9 μm ESD, but was too small to analyse quantitatively. In chemostat studies (Section 4.4) when *Sphingomonas* sp. cells were cultured at low growth rates, the average cell size was around this value, suggesting that the shoulder represented a small proportion of the total cell population which remained inactive while the majority of cells were dividing. Further work is required to evaluate this hypothesis. Enrichment of the small cells by filtration or centrifugation before *CellFacts* analysis might facilitate a quantitative assessment of these cells through batch culture.

In late lag phase (around 8 hrs) the *C. crescentus* cell size profile exhibited two distinct but overlapping peaks. The peak representing larger particles (1.3-1.8 μm ESD) disappeared by mid-exponential phase (about 20 hrs). Particles larger than around 1.3 μm could not be attributed to "normal" cells about to divide since the smallest cells produced immediately after division must have been at the lower end of the bacterial size peak, i.e. around 1 μm ESD. Assuming a constant cell width throughout the growth cycle, a doubling in cell length would result in a particle size of 1.26 μm ESD. Giant cells and chains of cells were observed under the TEM (Fig. 4.15); the large particles could have been either or both, but the reason for the formation of these cells and chains was not clear. Three theories were examined:-

- (1) The presence of an inhibitor of cell division in the water was tested by comparing growth of cells in PYE prepared with water from two different sources: the routinely-used laboratory water, cleaned by reverse osmosis and carbon filtration in a Purite unit (Millipore) and Ultrapure water supplied by Fisons, UK. No difference in batch growth kinetics or cell size of *C. crescentus* CB15 was observed.
- (2) The effect of pH on cells was assayed by culturing cells in PYE adjusted to pH 5.5, 5.8, 6.1, 6.4, 6.7, 7.0, 7.3, 7.6, 7.9 or 8.2 before autoclaving. Large cells and chains of cells were observed microscopically in all samples where conspicuous cell growth occurred.
- (3) To ensure that laboratory attenuation of the *C. crescentus* strain studied had not taken place, two other strains were cultured. Chains of cells were formed by *C. crescentus* CB15N, a modified form of strain CB15, and by *C. crescentus* CB13, indicating that the phenomenon was not unique to *C. crescentus* CB15.

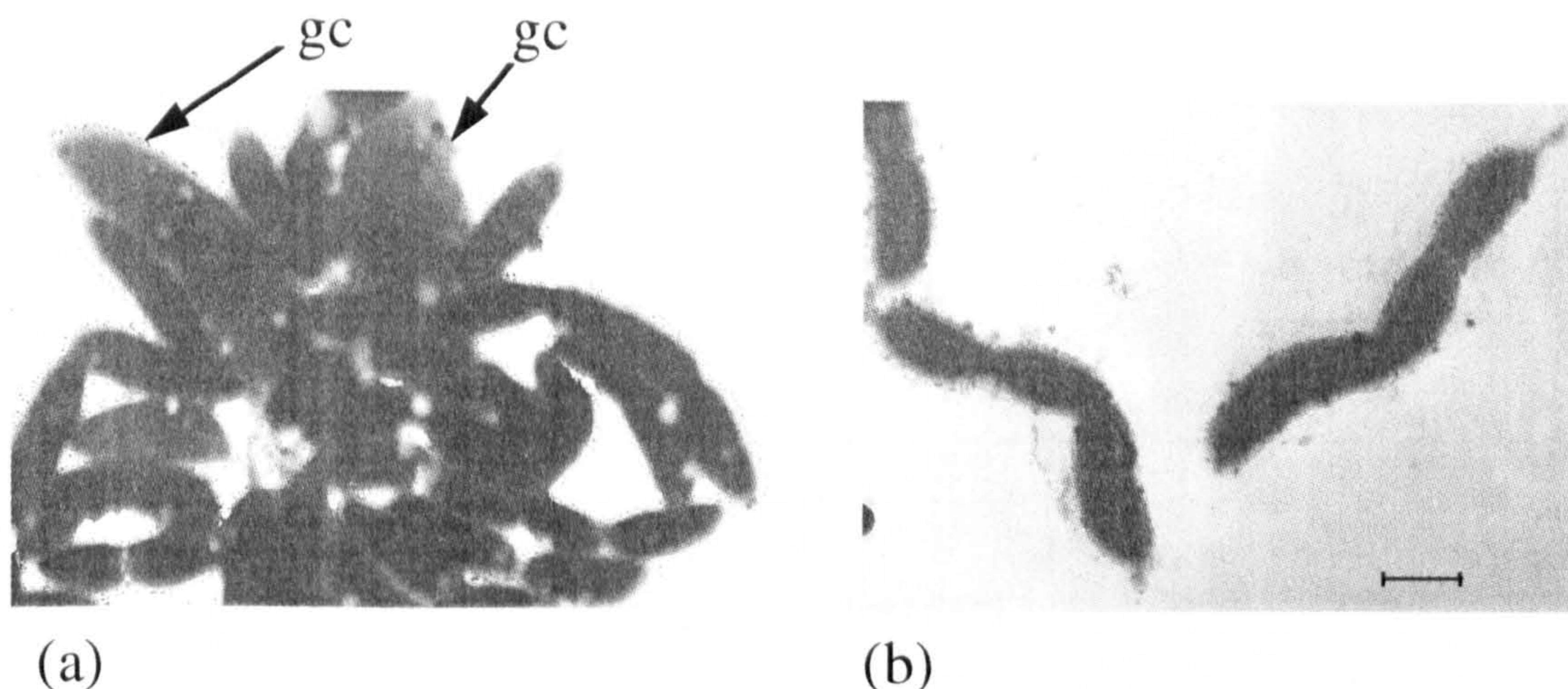
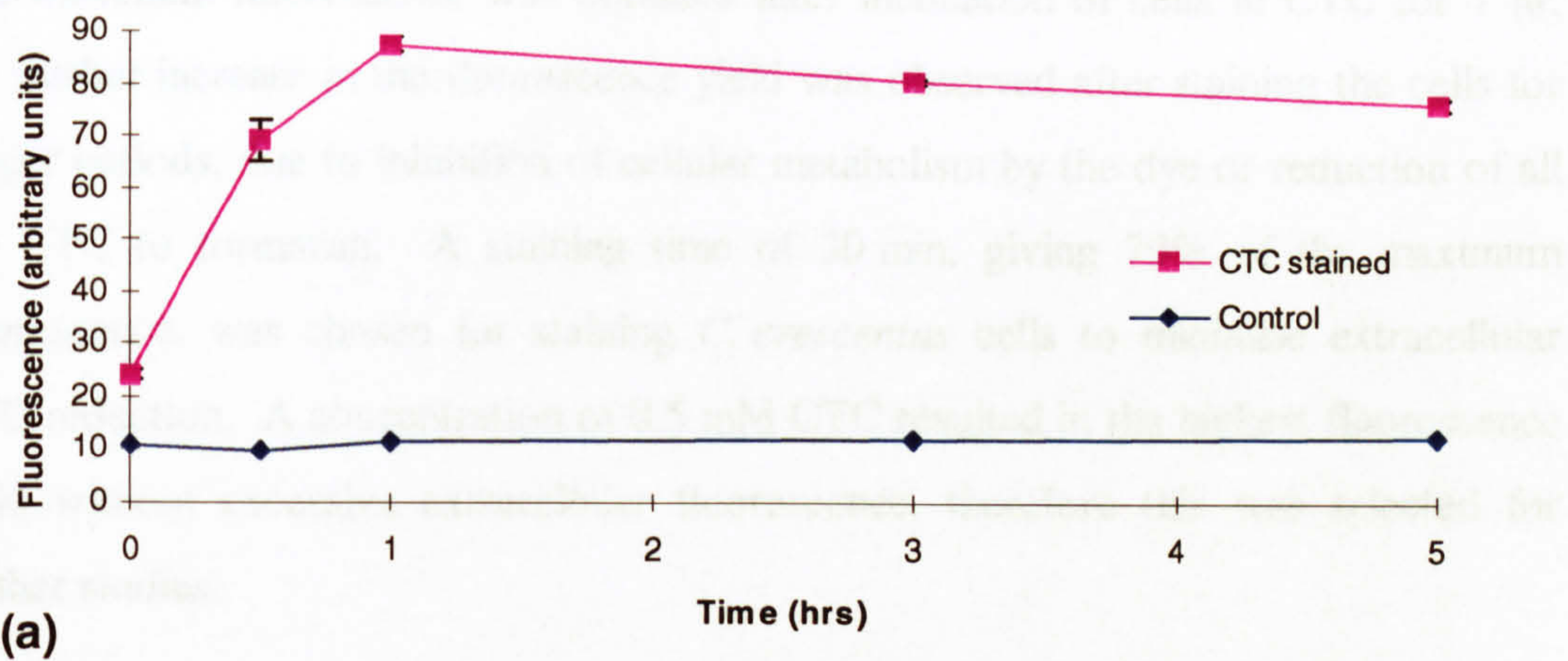


Figure 4.15 Giant cell and chain formation by *C. crescentus* CB15 cells. Gold-palladium shadowed TEMs showing (a) giant cells (gc) and (b) a chain of cells. Bar = 1 μm .

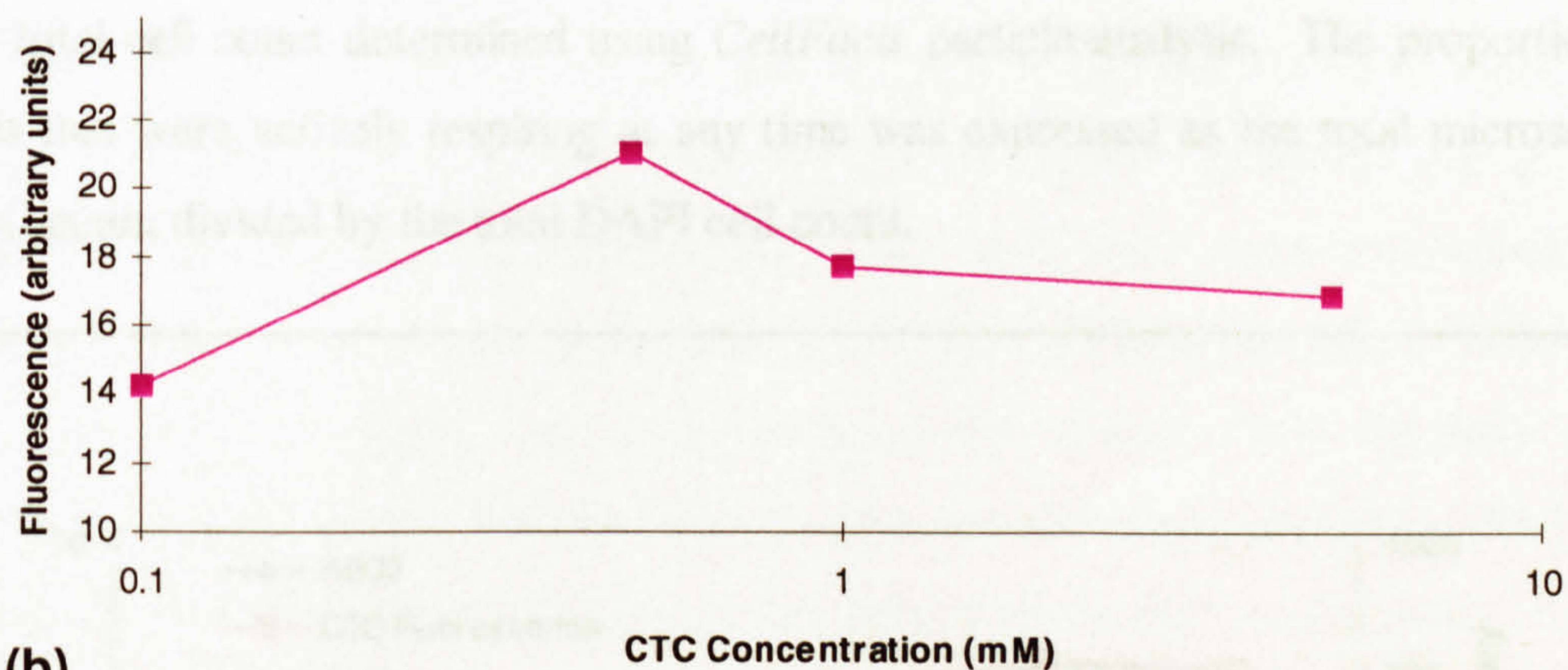
In a culture of bacterial cells it is not uncommon to see chain formation by a proportion of the population. Indeed, chains of *Sphingomonas* sp. cells were sometimes observed (e.g. Fig. 4.9). However, it was the large number of unusually large particles detected by *CellFacts* particle size analysis and the clear division of the particles into two distinct but overlapping peaks that was surprising and the cause remains unclear.

4.3.2.2 Respiratory chain activity

Staining of *C. crescentus* CB15 was optimised using the same techniques applied to *Sphingomonas* sp. (Section 4.3.1.3). The effects of time of incubation of cells in CTC and concentration of CTC were monitored (Fig. 4.16).



(a)



(b)

Figure 4.16 Optimisation of CTC staining of *C. crescentus* CB15 cells. The effects of (a) time of incubation of cells in 0.5 mM CTC and (b) CTC concentration using a 30 minute incubation time were assessed. Cells were incubated at 30°C in the dark and the amount of CTC staining was measured as the relative fluorescence emission at 630 nm after excitation at 450 nm. Control samples (no CTC) were included when assessing effect of incubation time on CTC fluorescence. Error bars represent standard deviations from triplicate experiments.

The maximum fluorescence was obtained after incubation of cells in CTC for 1 hr. No further increase in the fluorescence yield was observed after staining the cells for longer periods, due to inhibition of cellular metabolism by the dye or reduction of all the CTC to formazan. A staining time of 30 min, giving 79% of the maximum fluorescence, was chosen for staining *C. crescentus* cells to minimise extracellular CTC reduction. A concentration of 0.5 mM CTC resulted in the highest fluorescence yield without excessive extracellular fluorescence, therefore this was selected for further studies.

The activity of the *C. crescentus* CB15 respiratory chain was assessed through batch growth both fluorimetrically (Figs 4.17 and 4.18) and microscopically (Fig. 4.18). The average fluorescence per cell was calculated by dividing the fluorescence yield by the total cell count determined using *CellFacts* particle analysis. The proportion of cells that were actively respiring at any time was expressed as the total microscopic CTC count divided by the total DAPI cell count.

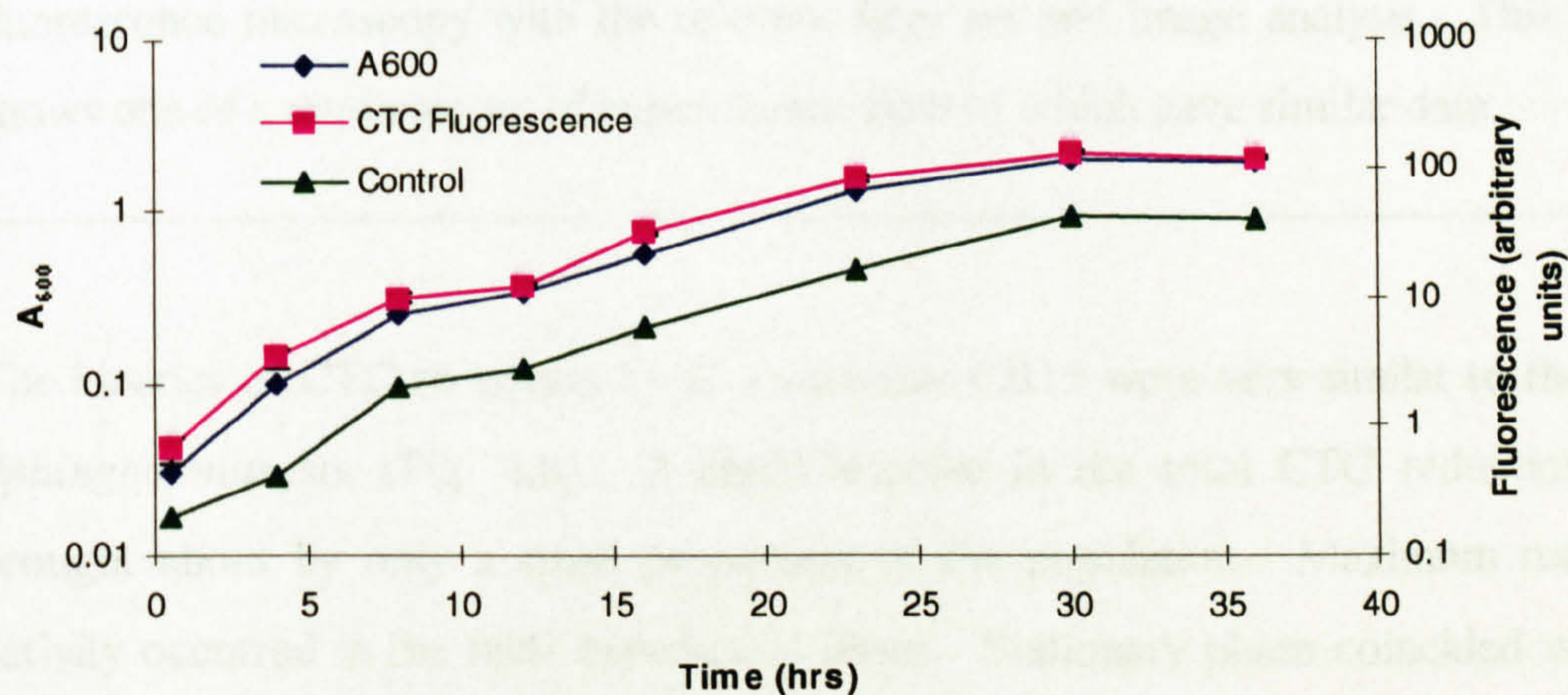


Figure 4.17 Total CTC fluorescence through *C. crescentus* CB15 batch culture. Samples were removed at intervals and CTC reducing activity was measured fluorometrically. Error bars represent standard deviations of triplicate samples. A control (no CTC) was performed at each time point. One of a duplicate set of experiments is shown. Both sets gave similar data.

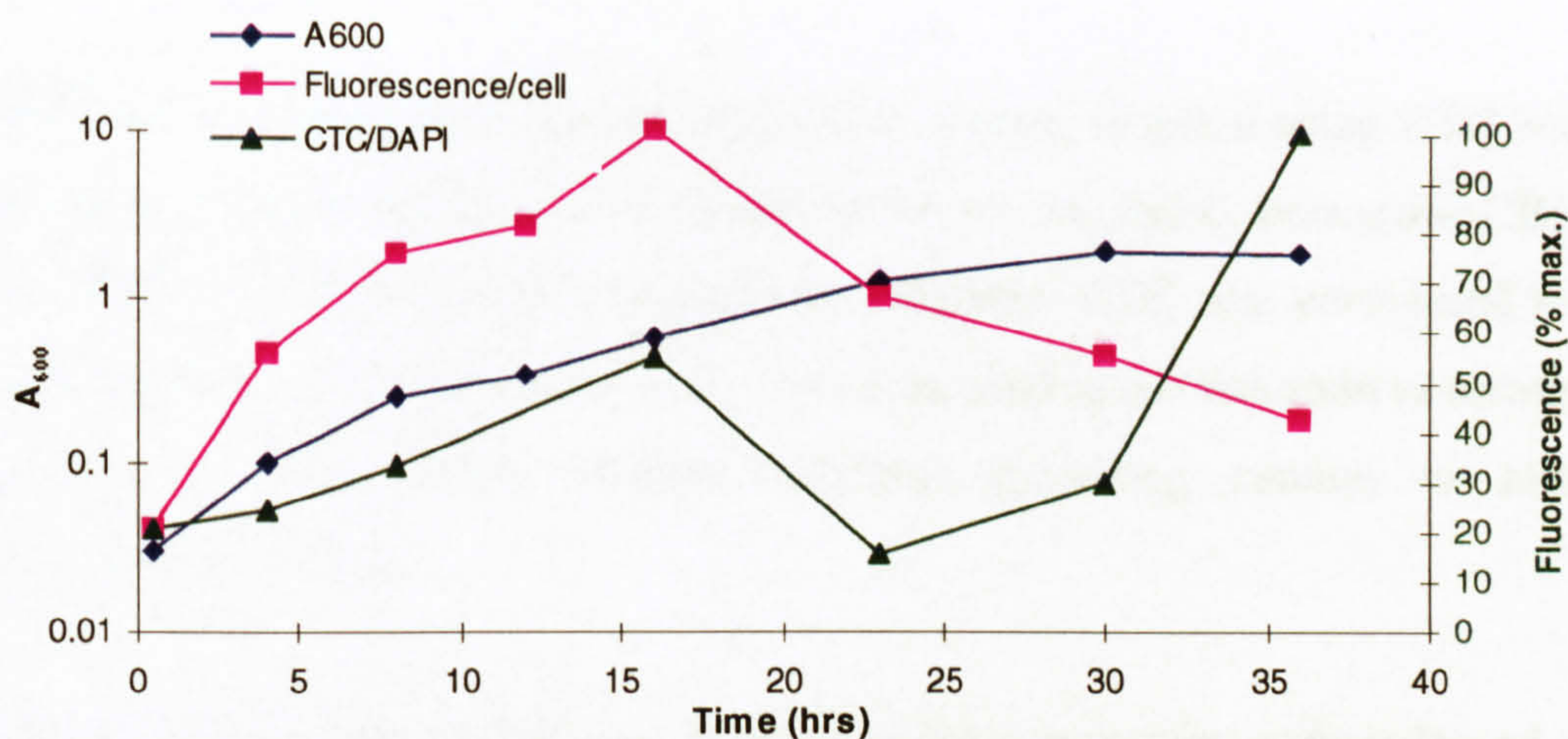


Figure 4.18 CTC Fluorescence per cell through batch culture of *C. crescentus* CB15. Samples were removed at intervals and CTC reducing activity per cell was measured using two different techniques: (a) fluorescence/cell = fluorescence measured by fluorimetry per total cell number determined by *CellFacts* and (b) CTC/DAPI = microscopic CTC counts per microscopic DAPI counts, both obtained using fluorescence microscopy with the relevant filter set and image analysis. This graph shows one of a duplicate set of experiments, both of which gave similar data.

The kinetics of CTC reduction by *C. crescentus* CB15 were very similar to those of *Sphingomonas* sp. (Fig. 4.8). A rapid increase in the total CTC reduction was brought about by only a small proportion of the population. Maximum reducing activity occurred in the mid- exponential phase. Stationary phase coincided with an increase in the proportion of cells that reduced CTC. However, total CTC fluorescence did not increase and the observed effect was a result of production of small extracellular formazan crystals in the same manner as those formed by *Sphingomonas* sp. cells (Figs 4.8 and 4.9). The extracellular staining by *C. crescentus* CB15 cells was more extreme than that produced by *Sphingomonas* sp. cells and the possibility that cell type plays a role cannot be excluded. Examination

of CTC staining by synchronous populations of *C. crescentus* CB15 is required to explore this effect in more detail.

Since the maximum cellular respiratory chain activity detected using CTC occurred during the exponential phase of both *Sphingomonas* sp. and *C. crescentus* CB15, i.e. when the maximum growth rate had been reached, CTC was considered to be a useful indicator of metabolic activity. It was decided to use this stain to measure the activity of cells within complex biofilms, exercising caution to minimise extracellular staining.

4.4 Analysis of planktonic and attached *Sphingomonas* sp. cells cultured under carbon- and nitrogen-limited conditions

4.4.1 Chemostat culture

By maintaining a continuous throughput of nutrients in the chemostat it is possible to regulate the rate of growth of a bacterial population. Since the hydrophobicity of bacterial cell surfaces alters with changes in growth rate, a chemostat model was employed to investigate the relationship between the nutrient limitation, cell surface hydrophobicity and the ability of the cells to attach.

It has been proposed that cell surface hydrophobicity may be modulated through the bacterial growth cycle and that production of cells with relatively hydrophilic surfaces may lead to detachment from surfaces (Allison *et al.*, 1990a,b). In most cases hydrophobic cells are more likely to attach to solid surfaces (Spencely, 1993; Fattom & Shilo, 1984; van Loosdrecht *et al.*, 1987a,b; Gilbert *et al.*, 1991), although exceptions to this general rule have been reported (Flint *et al.*, 1997). Apart from the direct influence of the hydrophobicity of the substratum and the aqueous phase, the type of organism and the prevailing environmental conditions (e.g. nutrient availability, temperature or the presence of antimicrobial agents) may also affect the relationship between bacterial cell surface hydrophobicity and attachment. To assess the role of cell surface hydrophobicity in modulating bacterial attachment to potable

water distribution pipes, an isolate from the system was cultured in the chemostat until steady-state growth was reached. The cell surface hydrophobicity was then measured before introducing a biofilm development vessel on a recirculating loop to determine the potential of the cells to attach to surfaces.

Sphingomonas sp. was chosen as the organism for analysis and glass surfaces were employed for attachment studies. Glass was selected as the surface for attachment as an inert surface amenable to microscopic analysis. The influence of substratum material on biofilm accumulation has been analysed in detail (Wolfaardt & Cloete, 1992; Rogers *et al.*, 1994a,b; Fletcher & Loeb, 1979; Kerr *et al.*, 1997; Hood & Winter, 1997) and was not the subject of investigation here. The layout of the chemostat model is described in Sections 2.8 and 2.9.

4.4.1.1 Steady-state carbon- and nitrogen-limited growth

Sphingomonas sp. cells were cultured under carbon-limited conditions at two different growth rates and under nitrogen limitation at a single growth rate. The population was monitored daily by *CellFacts* particle analysis. When no change in the profiles had been observed over six consecutive readings the system was taken as being in steady-state. The hydrophobicity was measured by bacterial adhesion to hexadecane (BATH) and a biofilm development vessel was introduced on a recirculating loop. Attachment to surfaces within this vessel was quantified at intervals by removing three coverslips, staining them with DAPI and enumerating cells by fluorescence microscopy and image analysis.

The effects of different growth conditions on steady-state particle size profiles and cell surface hydrophobicity are shown in Fig. 4.19 and Table 4.1. There was a small, but significant difference between the average particle size of cells cultured at the two different growth rates in carbon-limited medium and also between the average hydrophobicity of these two populations ($p < 0.001$ in both cases), but the effect was more notable when the limiting nutrient was changed to nitrogen. It is possible that the carbon-limited cells at the slower growth rate were the minimum unit cell size

and that increasing the growth rate five-fold resulted in increased biomass primarily through a greater number of cells rather than enlargement of individual cells. Studies of cells cultured in the same medium under batch conditions would be necessary to confirm this. The smallest cells, growing at a rate of 0.02 hr^{-1} were neither the most nor the least hydrophobic indicating that growth rate was not the sole determinant of cell surface structure.

Table 4.1: Steady state characteristics of planktonic *Sphingomonas* sp. cells of different steady states in the chemostat. Total cell no. and average cell size were determined by *CellFacts* analysis. Steady state growth conditions are expressed as N ltd (carbon-limited) or C ltd (nitrogen-limited), followed by the dilution rate. The hydrophobicity is expressed as % adherence to benzaldehyde. Mean values from at least six replicates are shown with standard deviations in brackets.

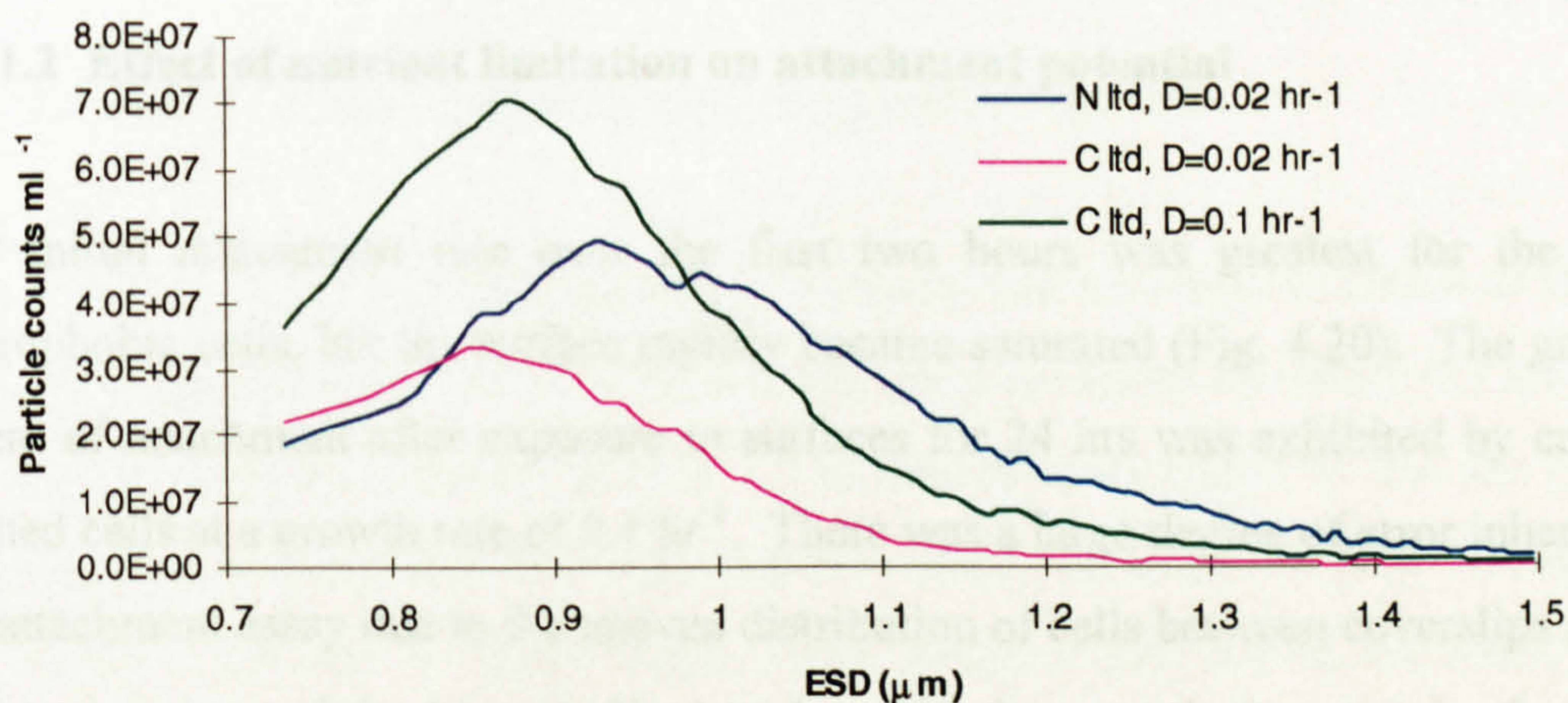


Figure 4.19 Cell size profiles of steady state *Sphingomonas* sp. chemostat cultures under various nutrient limitations. Cells were cultured to steady state in the chemostat under nitrogen limitation or carbon limitation at two different growth rates, as described in Section 2.8. Samples were removed and analysed by *CellFacts*. The cell size profiles shown are averages of at least six replicates.

	N ltd, D=0.02 hr ⁻¹	C ltd, D=0.02 hr ⁻¹	C ltd, D=0.1 hr ⁻¹
Total cell no. ml ⁻¹	1.19E+09 (1.3E+8)	4.74E+08 (8.24E+7)	1.14E+09 (1.55E+8)
Average ESD (μm)	1.04 (0.016)	0.92 (0.011)	0.96 (0.021)
% Adherence to hexadecane	18.7 (2.64)	8.6 (1.85)	3.4 (1.88)

Table 4.1 Some characteristics of planktonic *Sphingomonas* sp. cells at different steady states in the chemostat. Total cell no. and average cell size were determined by *CellFacts* analysis. Steady-state growth conditions are expressed as N ltd (nitrogen-limited) or C ltd (carbon-limited), followed by the dilution rate. The hydrophobicity is expressed as % adherence to hexadecane. Mean values from at least six replicates are shown with standard deviations in brackets.

4.4.1.2 Effect of nutrient limitation on attachment potential

The initial attachment rate over the first two hours was greatest for the most hydrophobic cells, but the surface rapidly became saturated (Fig. 4.20). The greatest extent of attachment after exposure to surfaces for 24 hrs was exhibited by carbon-limited cells at a growth rate of 0.1 hr⁻¹. There was a large degree of error inherent in the attachment assay due to the uneven distribution of cells between coverslips and in different regions of single coverslip samples. No clear conclusions can be drawn on the relationship between cell surface hydrophobicity and the attachment proclivity on the basis of this analysis.

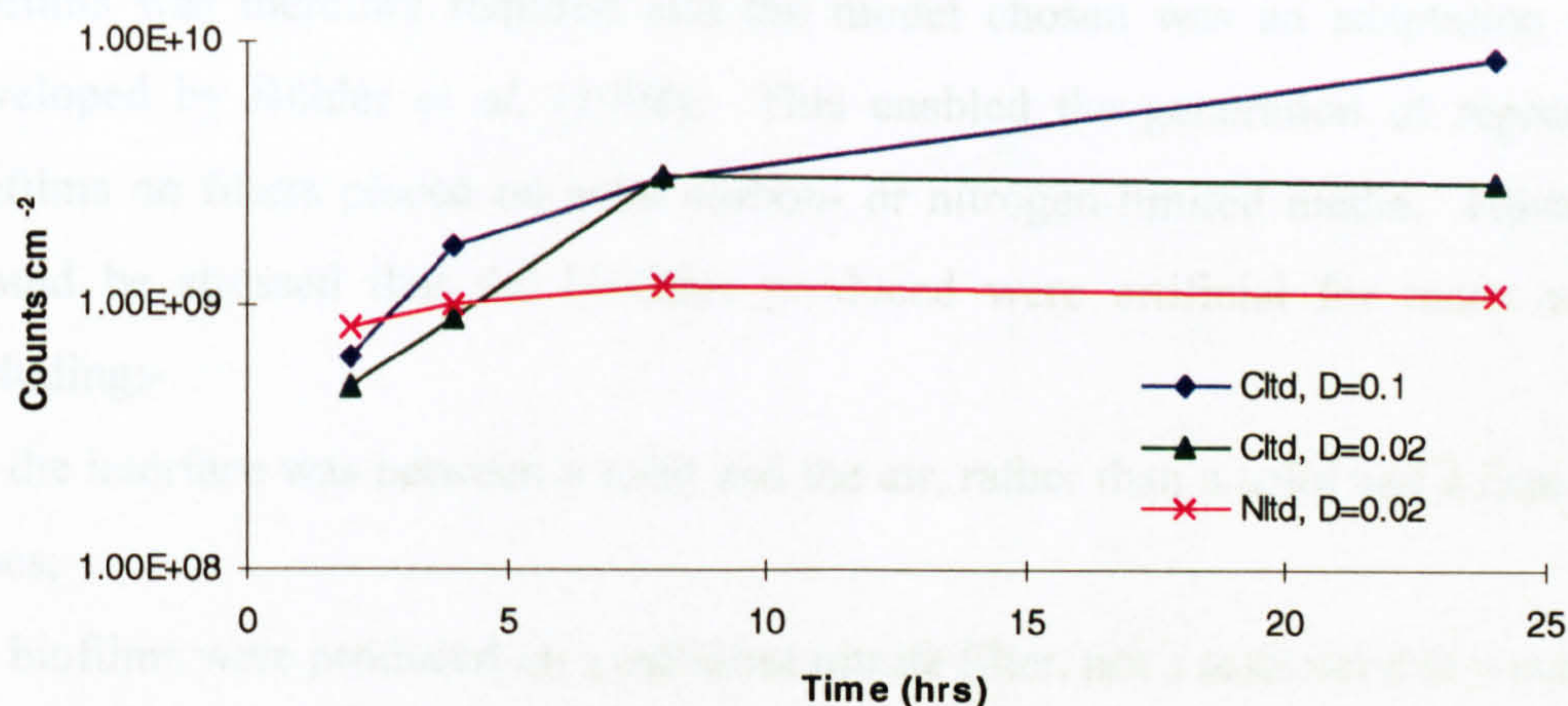


Figure 4.20 Rate of attachment of *Sphingomonas* sp. chemostat-cultured cells to glass surfaces. The extent of attachment of *Sphingomonas* sp. cells to glass surfaces was measured after introduction of a biofilm development vessel to the chemostat on a recirculating loop. Glass coverslips were removed at intervals and attachment was measured by DAPI staining and fluorescence microscopy in conjunction with image analysis.

4.4.2 Culture of surface-associated *Sphingomonas* sp. cells

There is strong evidence that vegetative dormancy and the ability to attach to surfaces are phenotypic characteristics of distinct cell types produced by dimorphic life cycles in a wide range of bacteria (Section 1.2.5), but the importance of this life cycle in natural systems is unclear. One approach is to search for a specific protein, expressed in dormant but not in active cells, or in attached but not in free-living cells. Production of specific antibodies to this protein would enable its use as a marker to determine whether the attached or planktonic phenotype correlated with a particular state of activity or dormancy *in situ*. Attempts to recover cells for protein analysis from the biofilms formed in the continuous culture model were unsuccessful: both sides of 36 glass coverslips were scraped, cells were suspended in 100 μ l of PBS and analysed by SDS-PAGE but no bands were seen on the gel, in contrast to the positive control (20 μ g of planktonic whole cell proteins). Another model for formation of

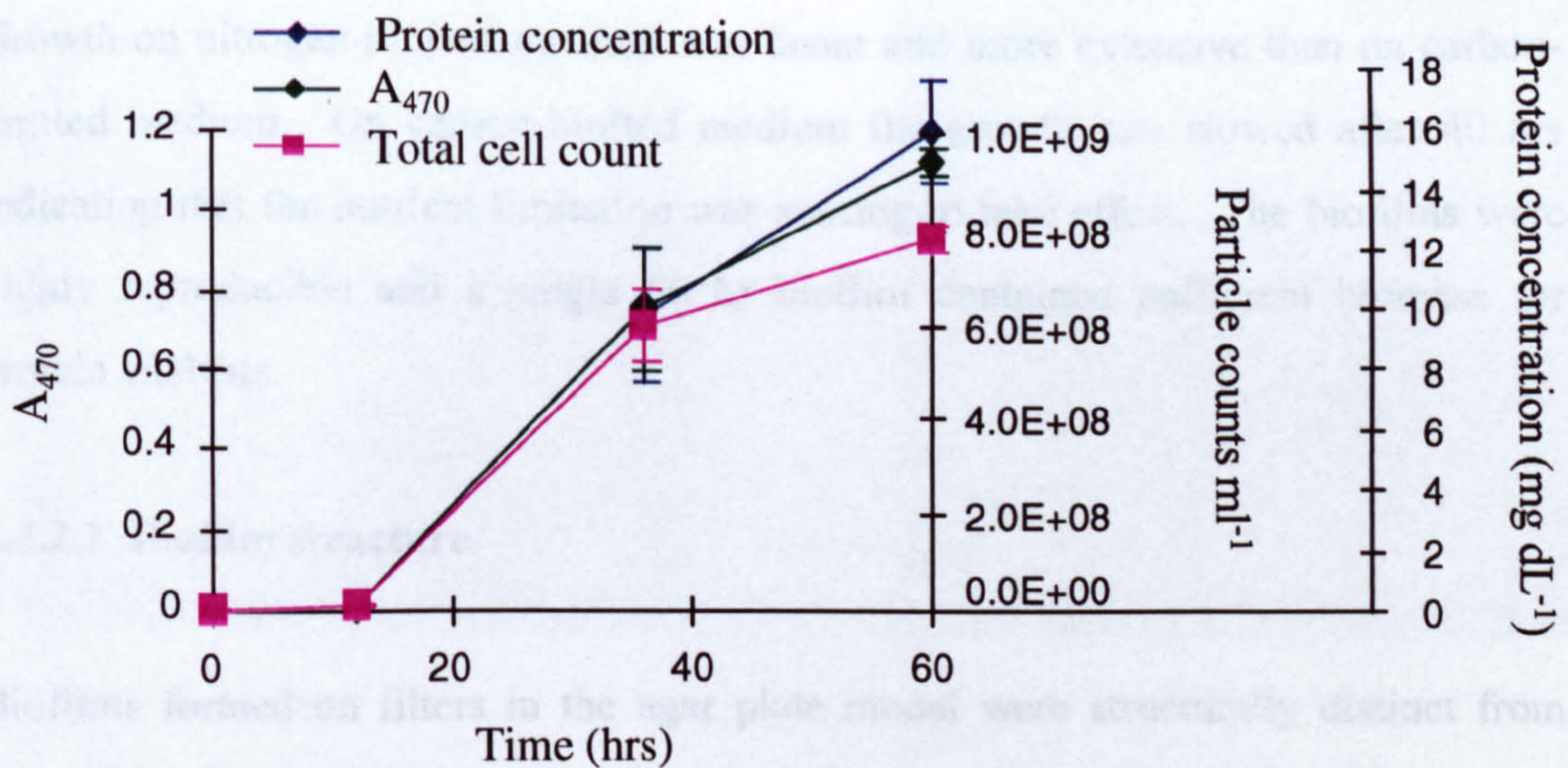
biofilms was therefore required and the model chosen was an adaptation of that developed by Bühler *et al.* (1998). This enabled the generation of reproducible biofilms on filters placed on solid carbon- or nitrogen-limited media. However, it should be stressed that the biofilms produced were artificial for many reasons, including:-

- (a) the interface was between a solid and the air, rather than a solid and a liquid as in pipes;
- (b) biofilms were produced on a cellulose nitrate filter, not a material that would have been present in a distribution system;
- (c) the nutrients were supplied from the underside of the biofilm while the oxygen entered from the top. Therefore different parts of the biofilm may have been limited for different substances at the same time;
- (d) biofilms were formed from a single species at 30°C; again not conditions that would be found in a distribution network;
- (e) the filter was inoculated once, not continuously, and cells were forced onto the filter rather than actively attaching.

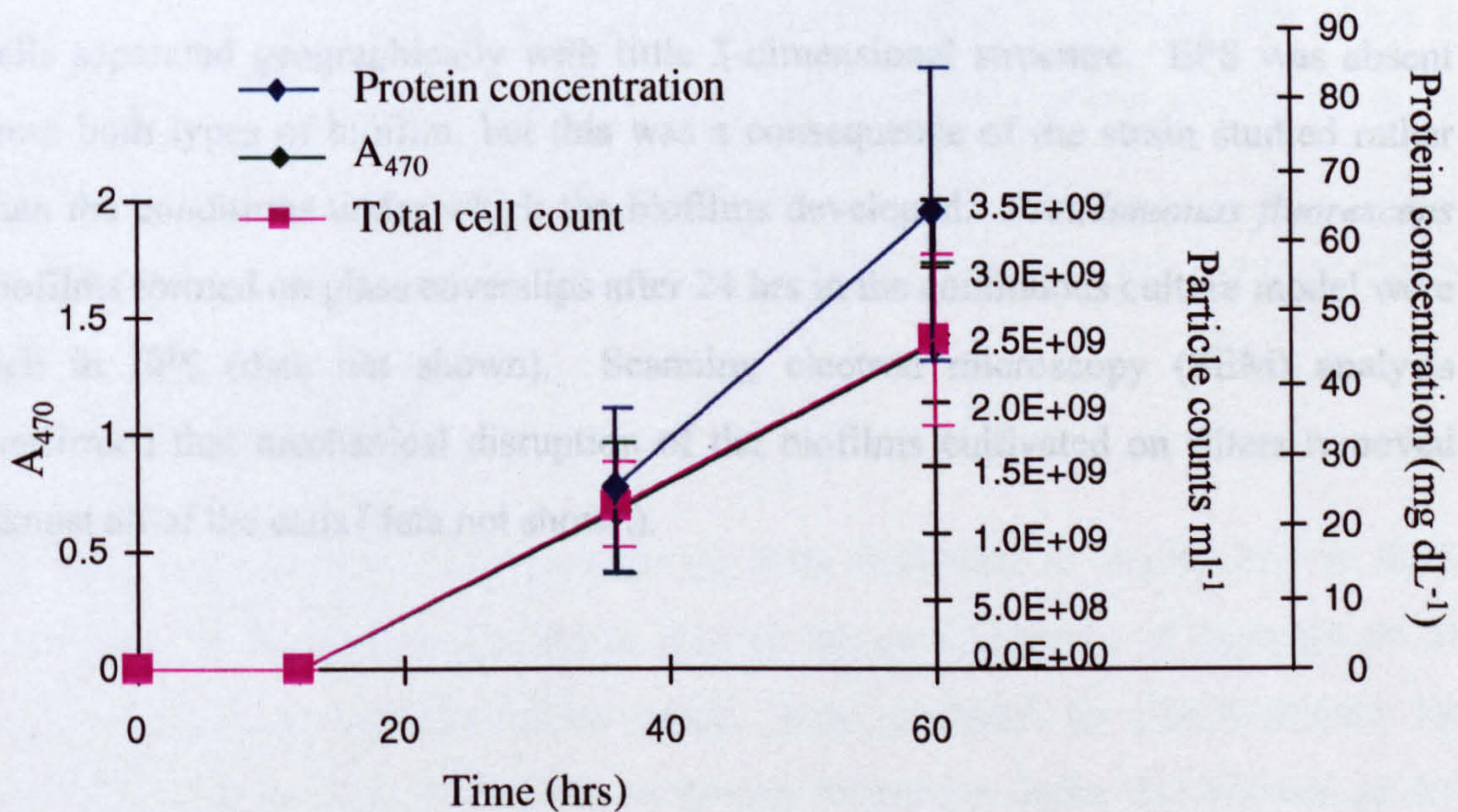
However, despite all these disadvantages, essentially all of the cells in the biofilm would have grown attached to a surface and would therefore have been expected to have produced any cell surface molecules specific to the attached phenotype.

4.4.2.1 Growth kinetics

The kinetics of bacterial growth in biofilms was measured by scraping the cells from the filters, resuspending in PBS and measuring the attenuation of light at 470 nm, the total count by *CellFacts* and the protein concentration (Fig. 4.21).



(a)



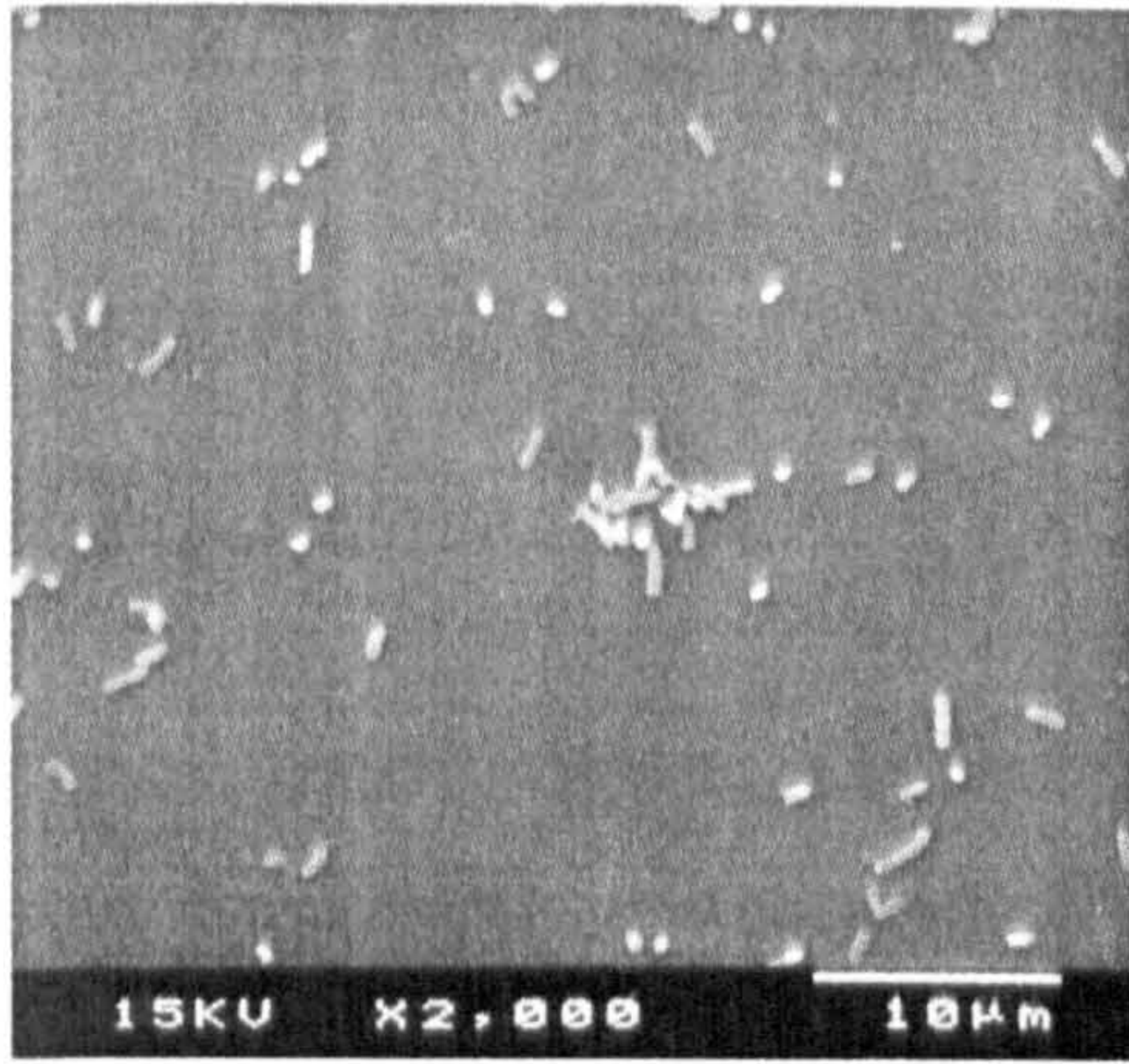
(b)

Figure 4.21 Kinetics of biofilm development by *Sphingomonas* sp. cells on solid media. Biofilms were developed on filters on (a) carbon- or (b) nitrogen-limited medium. Total cell counts were determined by *CellFacts*. Data points represent mean values from at least three different biofilms and error bars indicate standard deviations. Total counts and protein concentrations were determined in triplicate for each biofilm.

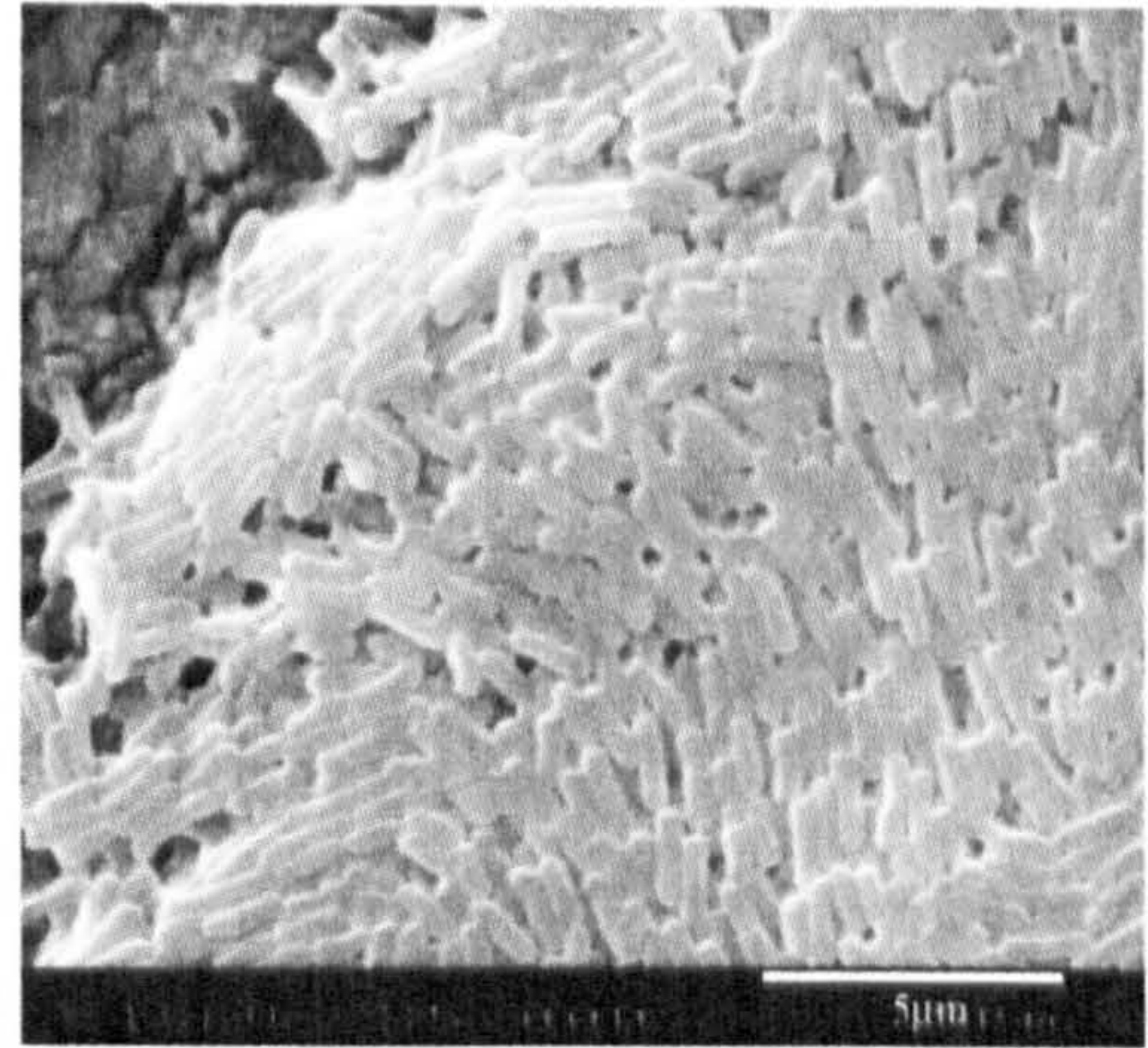
Growth on nitrogen-limited medium was faster and more extensive than on carbon-limited medium. On carbon-limited medium the growth rate slowed after 40 hrs indicating that the nutrient limitation was starting to take effect. The biofilms were highly reproducible and a single 60 hr biofilm contained sufficient biomass for protein analysis.

4.4.2.2 Biofilm structure

Biofilms formed on filters in the agar plate model were structurally distinct from those formed on glass in the continuous culture model (Fig. 4.22). The former consisted of confluent growth, several layers of cells thick, covering most of the surface. The biofilms developed on glass contained groups of cells or individual cells separated geographically with little 3-dimensional structure. EPS was absent from both types of biofilm, but this was a consequence of the strain studied rather than the conditions under which the biofilms developed. *Pseudomonas fluorescens* biofilms formed on glass coverslips after 24 hrs in the continuous culture model were rich in EPS (data not shown). Scanning electron microscopy (SEM) analysis confirmed that mechanical disruption of the biofilms cultivated on filters removed almost all of the cells (data not shown).



(a)



(b)

Figure 4.22 Structure of *Spingomonas* sp. biofilms. Scanning electron micrographs of *Spingomonas* sp. biofilms formed (a) on glass surfaces after 24 hrs in the chemostat-linked model and (b) on solid carbon-limited medium after 36 hrs in the agar plate model.

4.4.3 Cellular proteins

Cellular proteins from *Spingomonas* sp. cells cultivated to steady-state in liquid carbon- or nitrogen-limited media or after 60 hrs on the surface of filters placed on solid carbon- or nitrogen-limited media, were analysed by polyacrylamide gel electrophoresis to look for differential protein expression under the different growth conditions. If a protein could be found that was specifically expressed by attached cells then this would provide the basis for further studies on the function and *in situ* expression of this molecule.

4.4.3.1 One dimensional protein profiles

4.3.3.1.1 Total proteins

Cellular proteins were initially separated by one dimensional SDS-PAGE. To increase the resolution of the gels high and low molecular weight proteins were analysed separately (Figs 4.23 and 4.24). Differences between the density of the clearest bands in each lane were quantified by densitometry. Some differences were seen between high molecular weight proteins (Fig. 4.25), particularly an 83 kDa protein overexpressed in nitrogen-limited planktonic cells, a 55.3 kDa protein underexpressed in biofilm cells, a 44.8 kDa protein underexpressed in nitrogen-limited biofilm cells and a 37.2 kDa protein overexpressed in nitrogen-limited biofilm cells. Few obvious differences were seen in the low molecular weight proteins (data not shown), probably because they were too abundant to be resolved by this technique. The 43-44 kDa protein underexpressed in nitrogen-limited biofilm cells was observed and a 20-21 kDa protein was seen that was overexpressed in the same population.

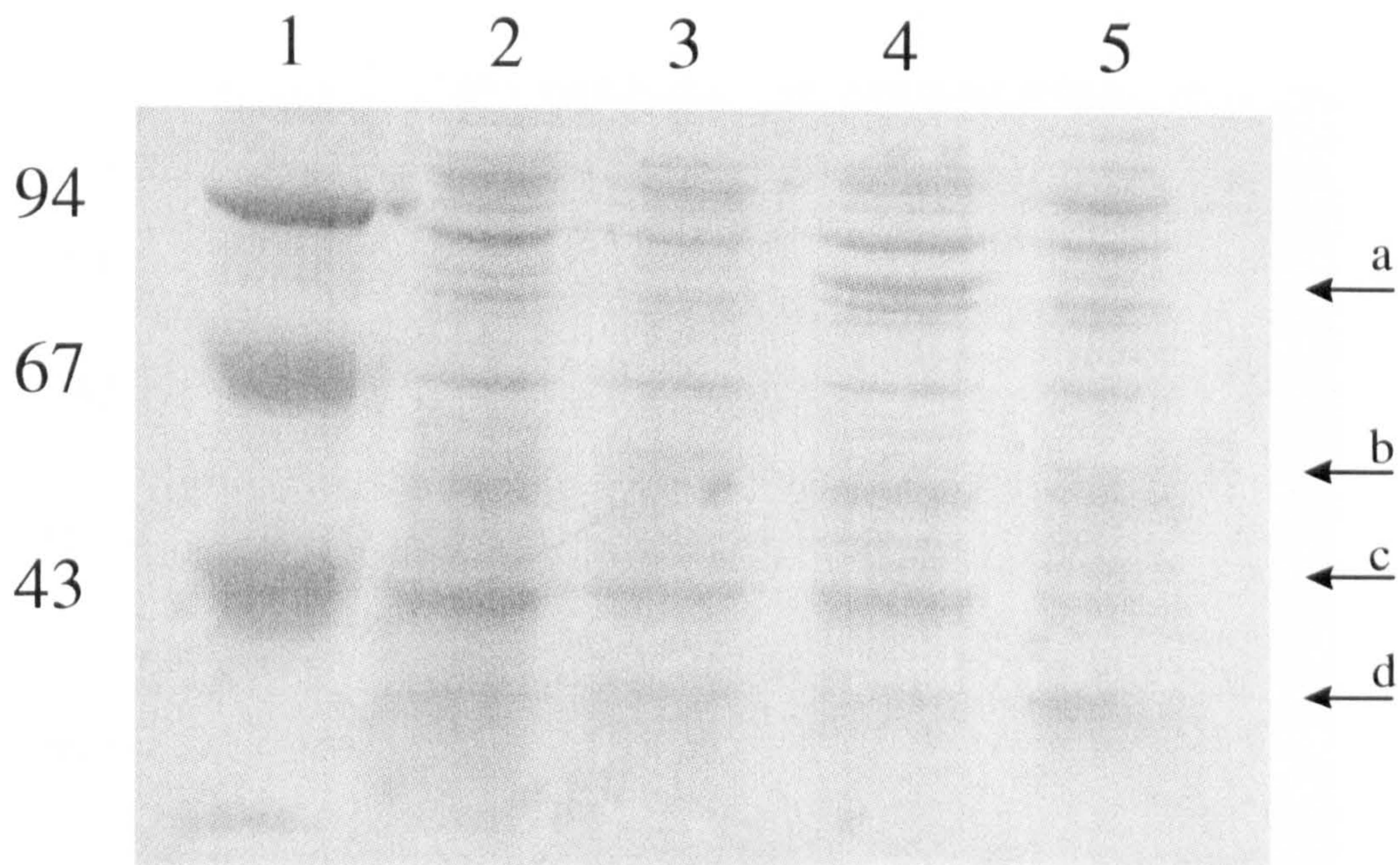


Figure 4.23 Analysis of high molecular weight proteins from *Sphingomonas* sp. cells cultured under a range of conditions. Cellular proteins were analysed by SDS-PAGE using a 15% (w/v) polyacrylamide gel. Lane 1 - molecular weight standards, lanes 2-5 - *Sphingomonas* sp. cellular proteins after culture as follows: lane 2 - in the chemostat under carbon limitation, lane 3 - on carbon-limited agar plates, lane 4 - in the chemostat under nitrogen limitation and lane 5 - on nitrogen-limited agar plates. Sizes of the molecular weight standards are indicated in kDa. The following bands are highlighted: (a) an 83 kDa protein overexpressed in nitrogen-limited planktonic cells, (b) a 55.3 kDa protein underexpressed in biofilm cells, (c) a 44.8 kDa protein underexpressed in nitrogen-limited biofilm cells and (d) a 37.2 kDa protein overexpressed in nitrogen-limited biofilm cells.

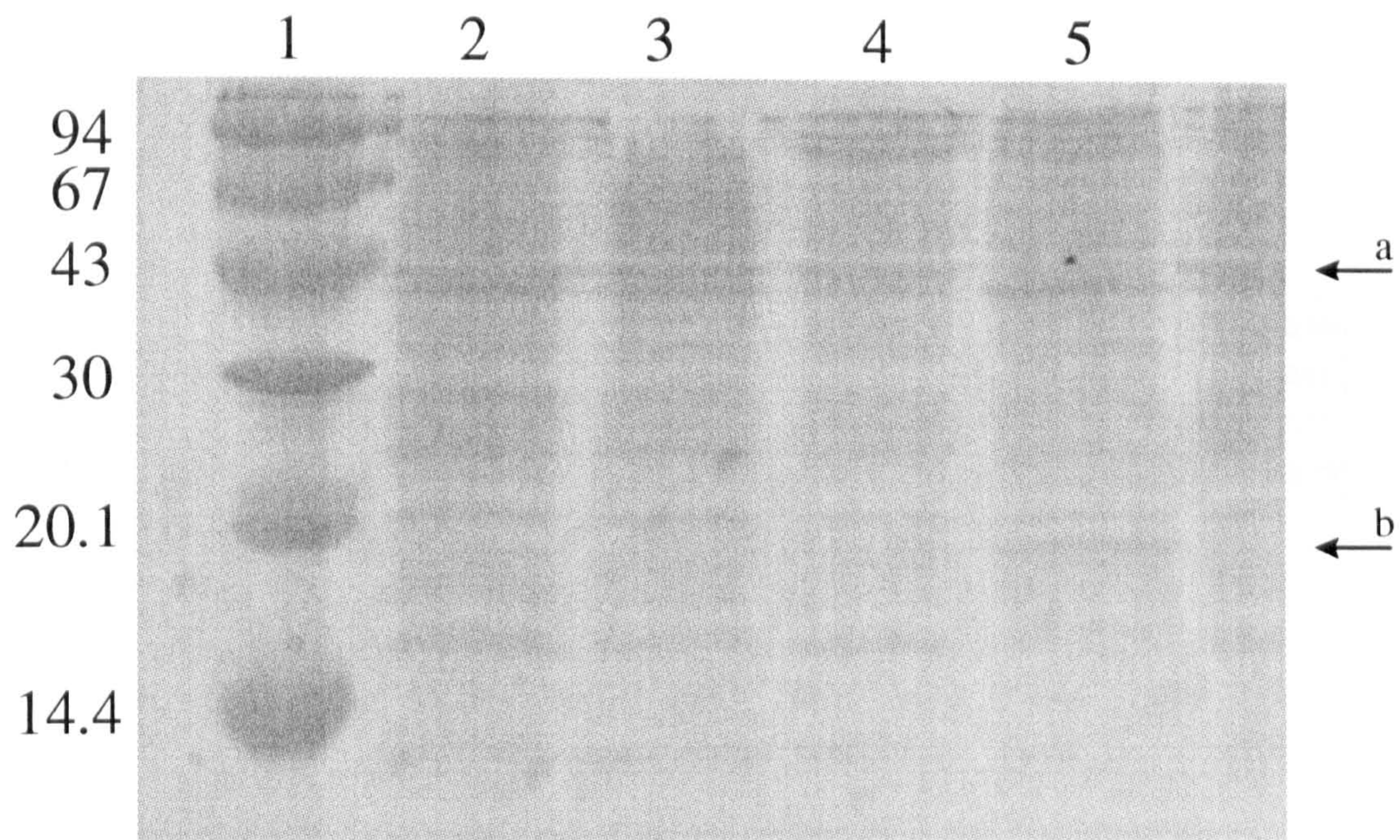


Figure 4.24 Analysis of low molecular weight proteins from *Spingomonas* sp. cells cultured under a range of conditions. Cellular proteins were analysed by SDS-PAGE using a 7.5% (w/v) polyacrylamide gel. Lane 1 - molecular weight standards, lanes 2-5 - *Spingomonas* sp. cellular proteins after culture as follows: lane 2 - in the chemostat under carbon limitation, lane 3 - on carbon-limited agar plates, lane 4 - in the chemostat under nitrogen limitation and lane 5 - on nitrogen-limited agar plates. Sizes of the molecular weight standards are indicated in kDa. The following bands are highlighted: (a) a 43-44 kDa protein underexpressed in nitrogen-limited biofilm cells and (b) a 20-21 kDa protein that was overexpressed in the same population.

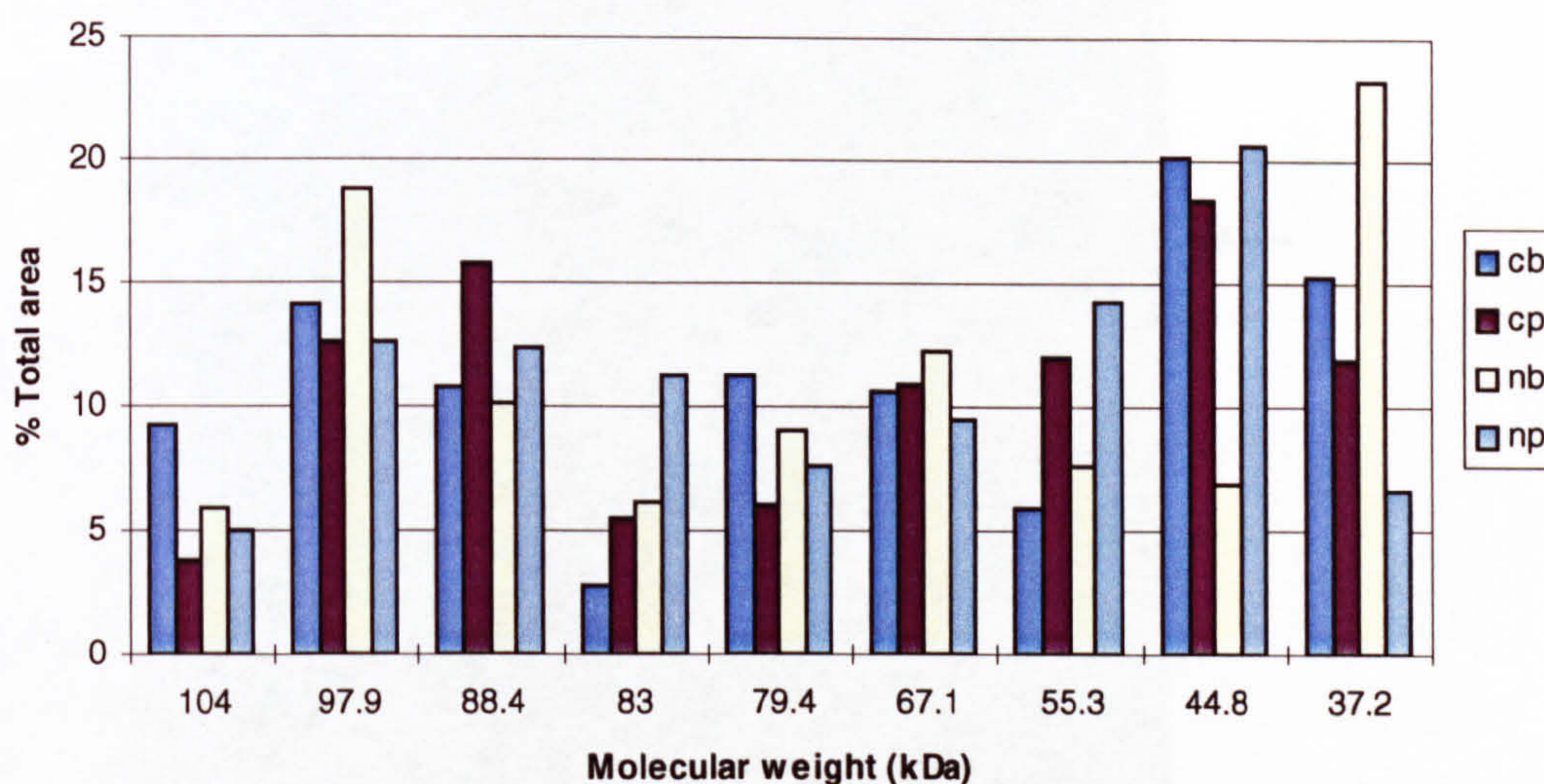


Figure 4.25 Comparison of protein bands from Fig. 4.23. Some of the bands in Fig. 4.23 were quantified by densitometry. The relative proportions of each of these bands are shown graphically. Legend (numbers in brackets refer to the lane on the polyacrylamide gel, Fig. 4.23): cb = carbon-limited biofilm cells (3), cp = carbon-limited planktonic cells (2), nb = nitrogen-limited biofilm cells (5), np = nitrogen limited planktonic cells (4).

4.4.3.1.2 Outer membrane proteins

The majority of proteins influencing attachment would be expected to occur in/on the outer membranes of cells. To identify outer membrane proteins involved in attachment, outer membranes from cells cultivated under the four different conditions (Section 4.4.3) were isolated by ultracentrifugation and proteins were analysed by SDS-PAGE (Fig. 4.26). Differences in the relative densities of the clearest bands were quantified by densitometry (Fig. 4.27).

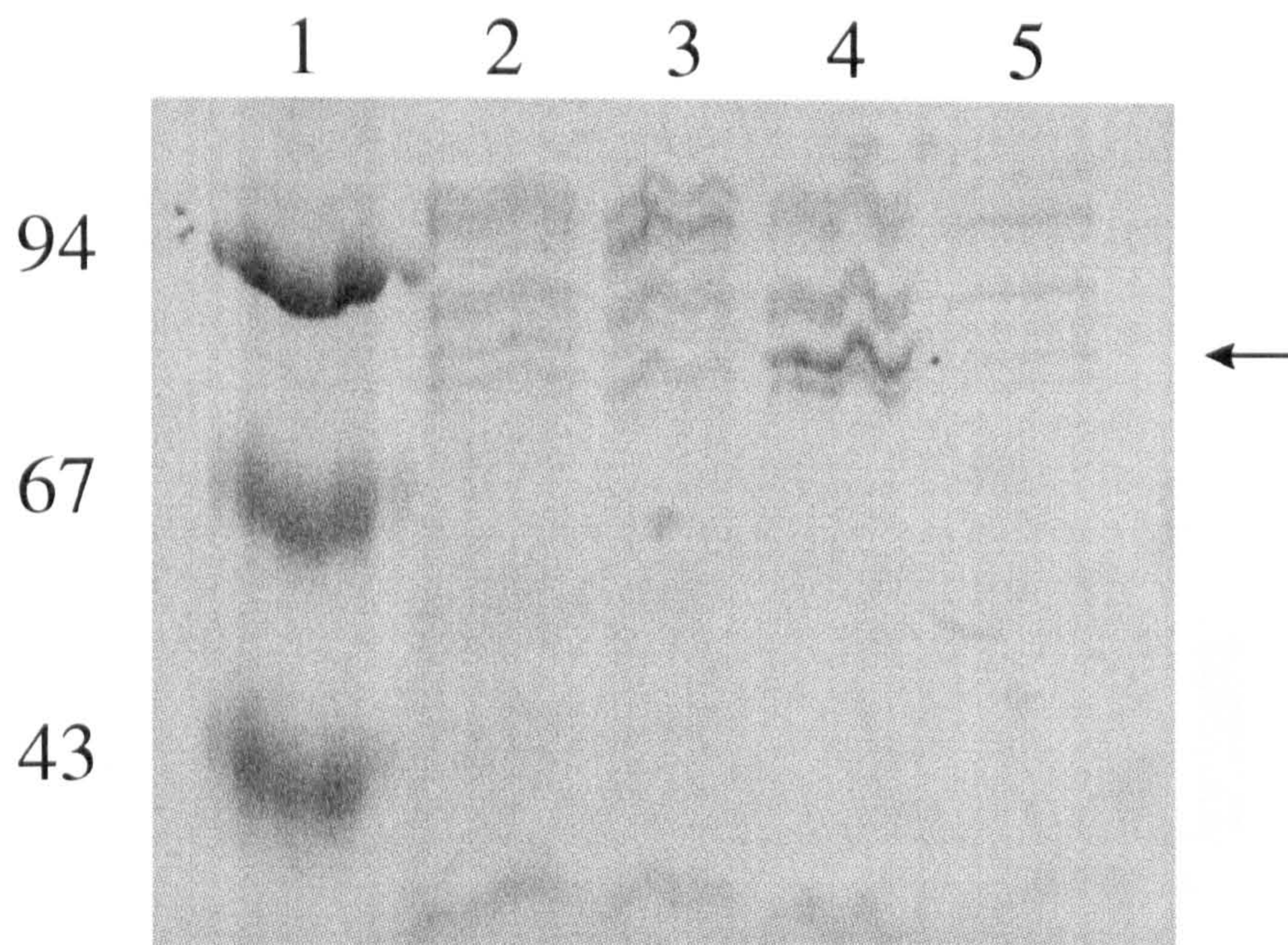


Figure 4.26 Analysis of high molecular weight outer membrane proteins from *Sphingomonas* sp. cells cultured under a range of conditions. Outer membrane proteins were purified and analysed by SDS PAGE using a 15% (w/v) polyacrylamide gel. Lane 1 - molecular weight standards, lanes 2-5 - *Sphingomonas* sp. cellular proteins after culture as follows: lane 2 - in the chemostat under carbon limitation, lane 3 - on carbon-limited agar plates, lane 4 - in the chemostat under nitrogen limitation and lane 5 - on nitrogen-limited agar plates. Sizes of the molecular weight standards are indicated in kDa. A single band at around 85 kDa, which was overexpressed in nitrogen-limited planktonic cells, is highlighted.

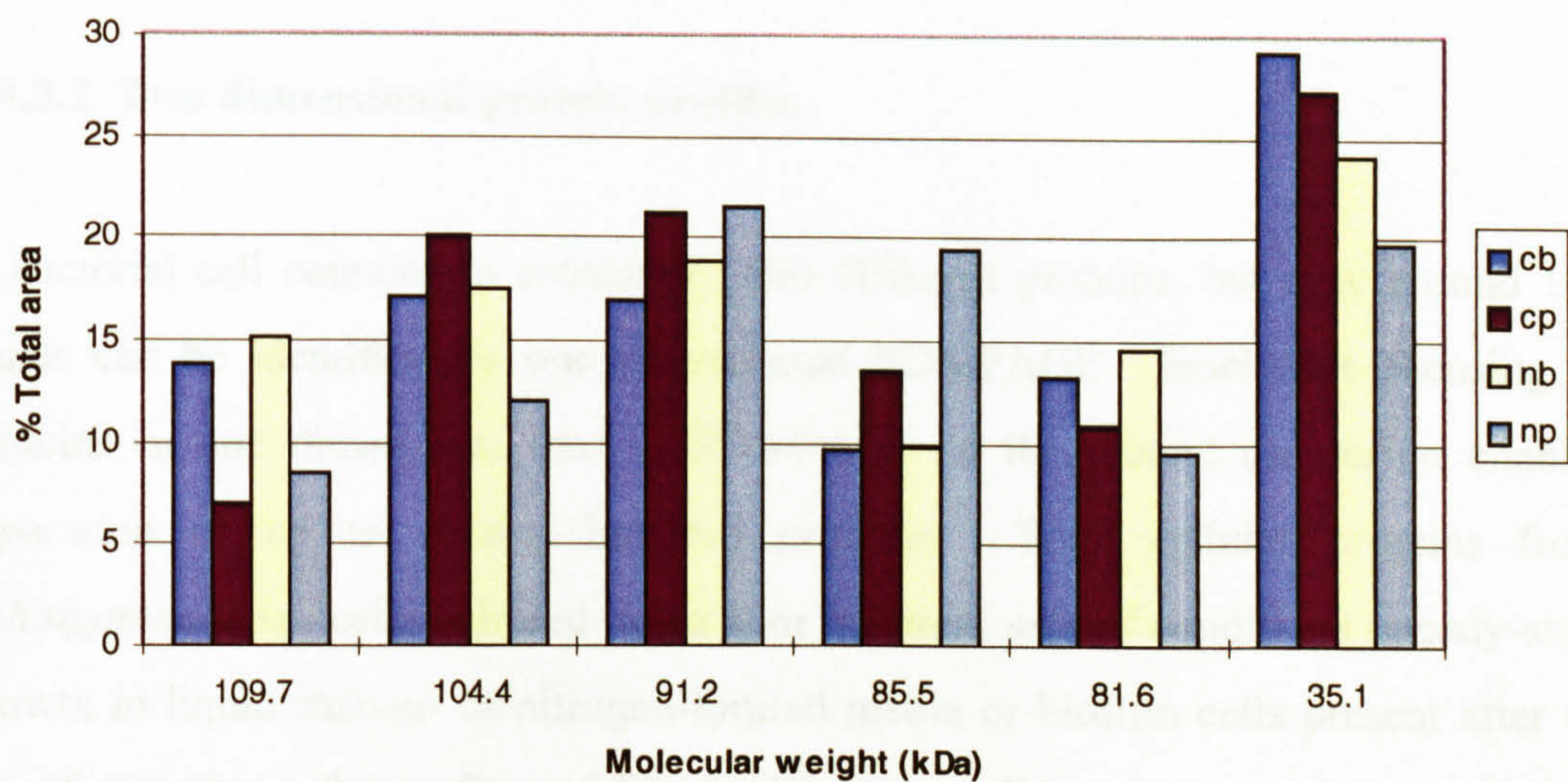


Figure 4.27 Comparison of protein bands from Fig. 4.26. Some of the bands in Fig. 4.26 were quantified by densitometry. The relative proportions of each of these bands are shown graphically. Legend (numbers in brackets refer to the lane on the polyacrylamide gel, Fig. 4.26): cb = carbon-limited biofilm cells (3), cp = carbon-limited planktonic cells (2), nb = nitrogen-limited biofilm cells (5), np = nitrogen limited planktonic cells (4).

The only obvious difference in protein expression was the roughly two-fold increase in expression of an 85.5 kDa protein in nitrogen-limited planktonic cells relative to cells from other culture conditions. This protein, identified as an 83 kDa molecule from whole cell extracts (Fig. 4.25), may have been expressed as part of the nitrogen starvation response in *Sphingomonas* sp. cells. Since the biofilm cell population was not homogeneous (cells on the upper surface of the biofilm being relatively starved of nutrients and those on the lower surface limited by oxygen) the growth rate would not have been uniform and starvation proteins would only have been expressed in a proportion of the cells. As there was no indication of nutrient limitation affecting the biofilm population after 60 hrs of growth (Fig. 4.21(b)), extensive expression of

starvation proteins would not have been expected. No bands specifically overexpressed in the outer membranes of biofilm cells were observed.

4.4.3.2 Two dimensional protein profiles

A bacterial cell contains in excess of 1000 different proteins, but only around 100 bands can be identified by one dimensional SDS-PAGE. Isoelectric focusing of proteins in one dimension prior to SDS-PAGE in the second dimension enables separation of up to several hundred proteins. Total cellular proteins from *Sphingomonas* sp. cells cultured under four different sets of conditions (steady-state growth in liquid carbon- or nitrogen-limited media or biofilm cells present after 60 hrs of growth on the surface of filters placed on solid carbon- or nitrogen-limited media) were extracted and analysed by two dimensional PAGE (Fig. 4.28).

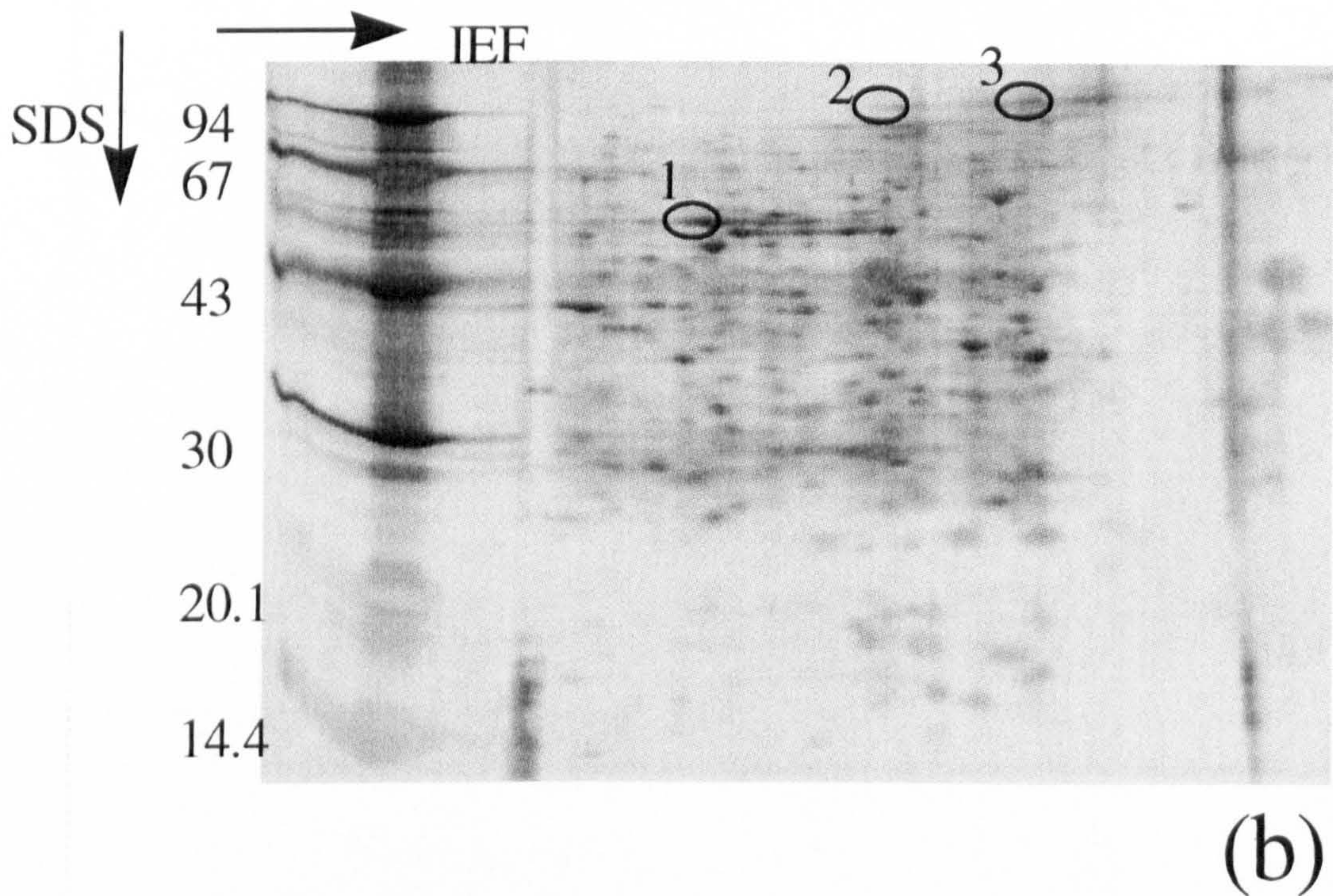
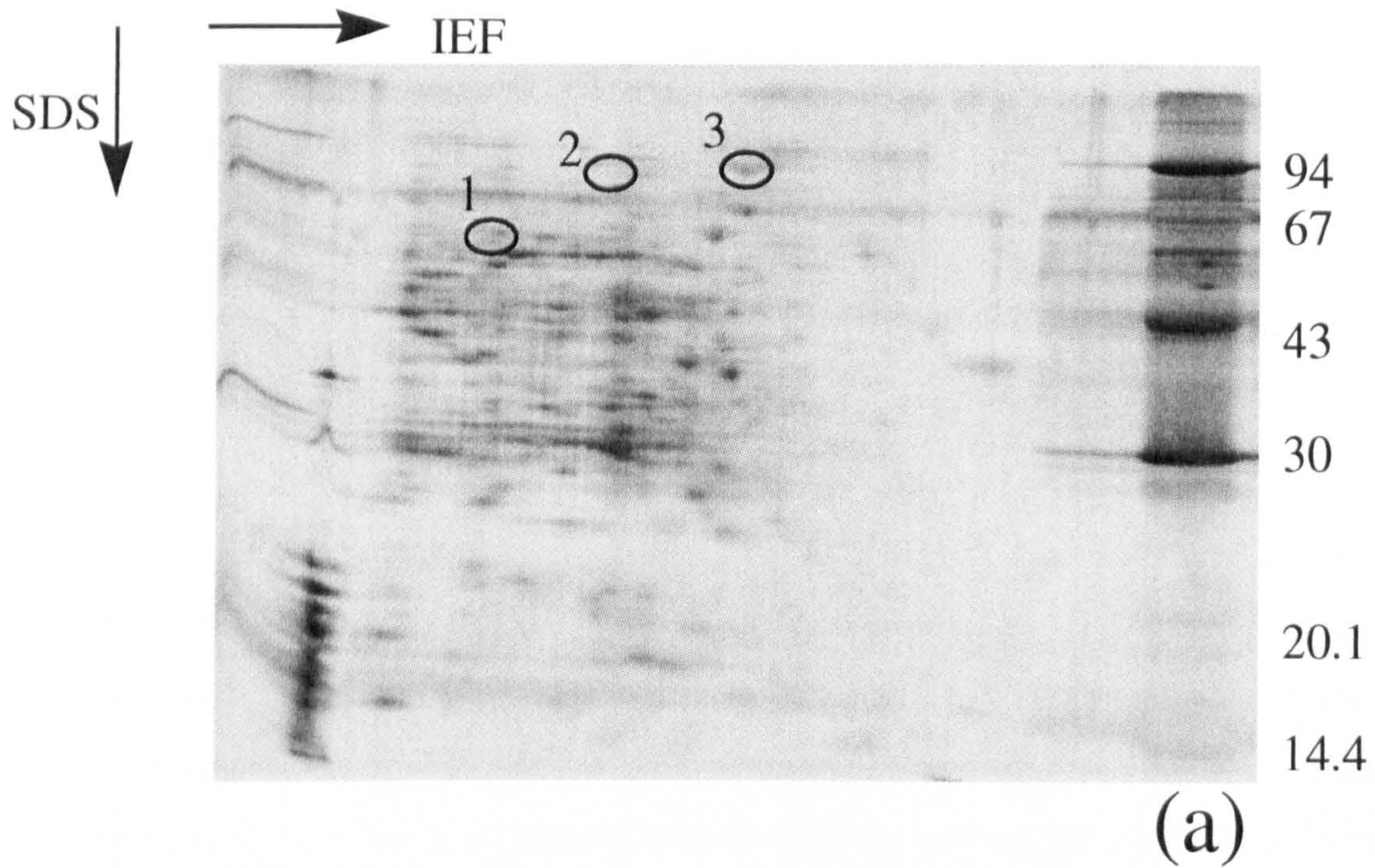
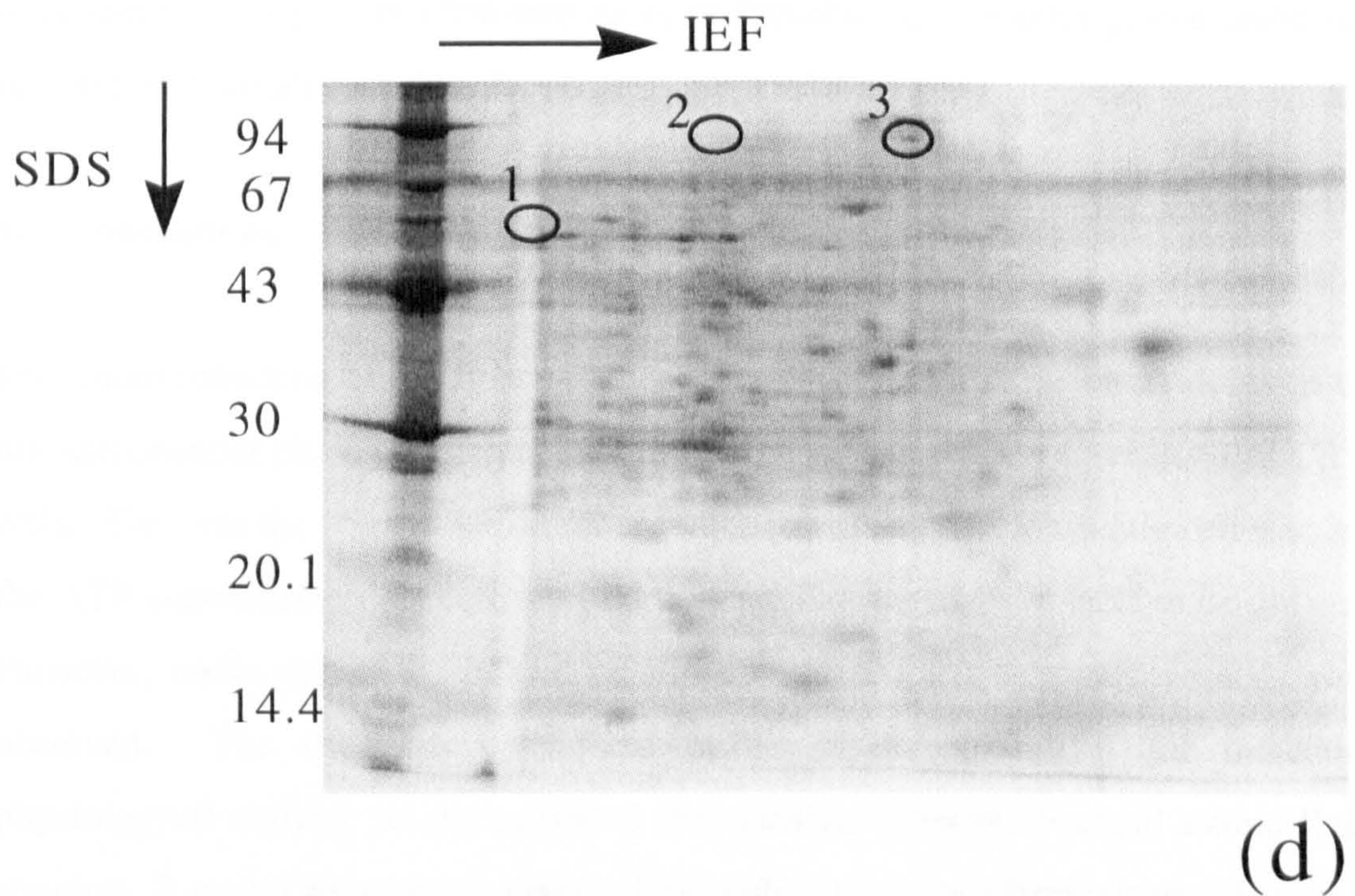
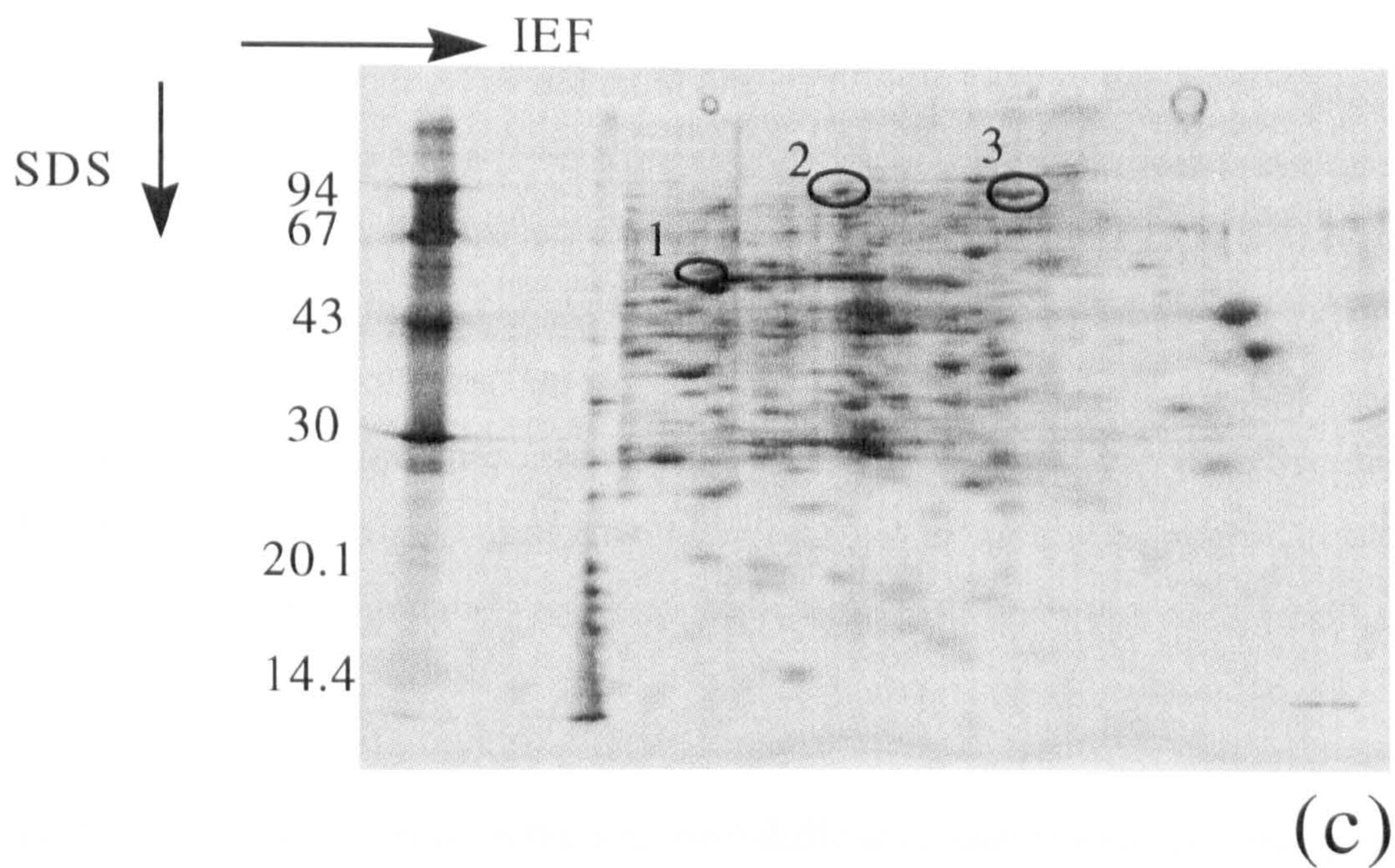


Figure 4.28 Analysis of proteins extracted from *Sphingomonas* sp. cells cultured under a range of conditions by 2D gel electrophoresis. Proteins were extracted from cells cultured under the following range of conditions: (a) at steady-state in the chemostat under carbon limitation, (b) after 60 hrs on carbon-limited solid medium (c) at steady-state in the chemostat under nitrogen limitation and (d) after 60 hrs on



nitrogen-limited solid medium. 10 μg of protein were loaded on each gel and spots were visualised by silver staining. Three proteins are indicated which were more strongly expressed by one cell type than the others: 1. most strongly expressed by carbon-limited biofilm cells, 2. and 3. most strongly expressed by nitrogen-limited planktonic cells. Molecular weight standards were run in the second dimension and molecular weights in kDa are indicated.

Three proteins that were clearly present at different densities on the four gels are indicated. Two were most abundant in nitrogen-limited planktonic cells. They both ran at around 85 kDa and are therefore candidates for the protein seen overexpressed in these cells by one dimensional SDS-PAGE. The third protein, around 55-60 kDa, was most abundant in the carbon-limited biofilm cells. It is not clear whether this protein was also expressed by the nitrogen-limited biofilm cells as the relevant portion of the gel is missing. However, since it was almost absent from the carbon-limited planktonic cells, it must have been regulated by surface-associated growth at least under carbon-limitation and may provide a useful indicator of a specific cell type.

Differences between spots on the gels were difficult to quantify and no other proteins were identified that were obviously more or less abundant in cells grown under one or two sets of conditions than the others.

4.5 Conclusions

Maximum reduction of the fluorescent redox probe CTC was shown to occur in the mid-exponential phase of batch culture of *Sphingomonas* sp. and *C. crescentus* CB15 cells. This was the point at which other indicators of activity, including cell size and the ATP concentration per cell, showed the metabolic activity of cells to be greatest. However, under certain ill-defined conditions extracellular formazan crystals were observed. The dye was considered to be potentially useful for indicating physiological activity *in situ* provided that caution is taken to avoid extracellular staining. It would be useful to analyse CTC reduction by synchronous populations of *C. crescentus* cells to determine whether staining is cell type specific. The dye could also be used in conjunction with flow cytometry to split slow-growing *E. coli* cells into populations based on cellular activity. Analysis of proteins from these populations may highlight cell type-specific protein expression.

By culturing *Sphingomonas* sp. cells in the chemostat it was shown that cell surface hydrophobicity and cell size were dependant on growth rate and the limiting nutrient.

However, it was not possible to correlate these changes with the ability of cells to attach to surfaces.

Analysis of proteins isolated from *Sphingomonas* sp. cells cultured under a range of conditions showed relatively few differences between the populations of cells. One 83-86 kDa protein, present in/on the outer membrane of nitrogen-limited planktonic cells at a concentration around twice that seen in other cell types, was thought to be linked to the nitrogen starvation response. Another protein, around 55-60 kDa, was shown by two dimensional PAGE to be expressed in carbon-limited biofilm cells with virtually no expression in their planktonic counterparts. The function and cellular localisation of this protein is unknown, but it may be involved in surface attachment. It would be useful to separate outer membrane proteins from the different cell populations by two dimensional PAGE to determine whether this protein is present in the outer membrane and whether any other outer membrane proteins are specific to any of the cell types.

CHAPTER 5

5 The tap water microflora at interfaces in a static batch enrichment system

5.1 Introduction

Reliance on direct culture of bacteria can give a misleading view of the range of cell types present in oligotrophic waters. For example, Lawrence (1978) demonstrated that the morphological diversity of bacteria in lake waters increases dramatically after storage of the water in the dark in static batch culture conditions in the absence of added nutrients for 3-6 months. The bacteria concentrated at interfaces, since these were the areas where the highest nutrient concentrations occurred (Marshall, 1980). The morphologically diverse species were part of the autochthonous microflora but were enriched by the period of static batch culture. Prosthecae bacteria are particularly well adapted for growth in low-nutrient environments (reviewed by Morgan & Dow, 1986) and the depletion of nutrients resulting from storage of the water would have enhanced the potential of these bacteria to compete effectively with other types present. In such enrichments the presence of trace amounts of volatile organic compounds in the atmosphere may provide an additional nutrient source for organisms that can utilise it (Morita, 1985). Since many *Hyphomicrobium* spp. exhibit a preference for growth on C₁ compounds such as methanol or formate, these may be enriched by trace amounts of volatile organic substances dissolved in the water.

5.2 Aims

The predominant morphology of bacteria identified by isolation from potable water was a regular or flexible rod. Some cocci were isolated but only one appendaged microorganism was recovered and then only after the employment of several specific isolation procedures (Chapter 3). In order to assess the role played by dimorphic life cycles in bacterial survival and growth in potable water it was important to analyse the range of morphological cell types present. Direct electron microscopic

observation of interfaces after static batch enrichment was considered to be the best approach.

A system was established with the aim of observing the microflora present at interfaces after:-

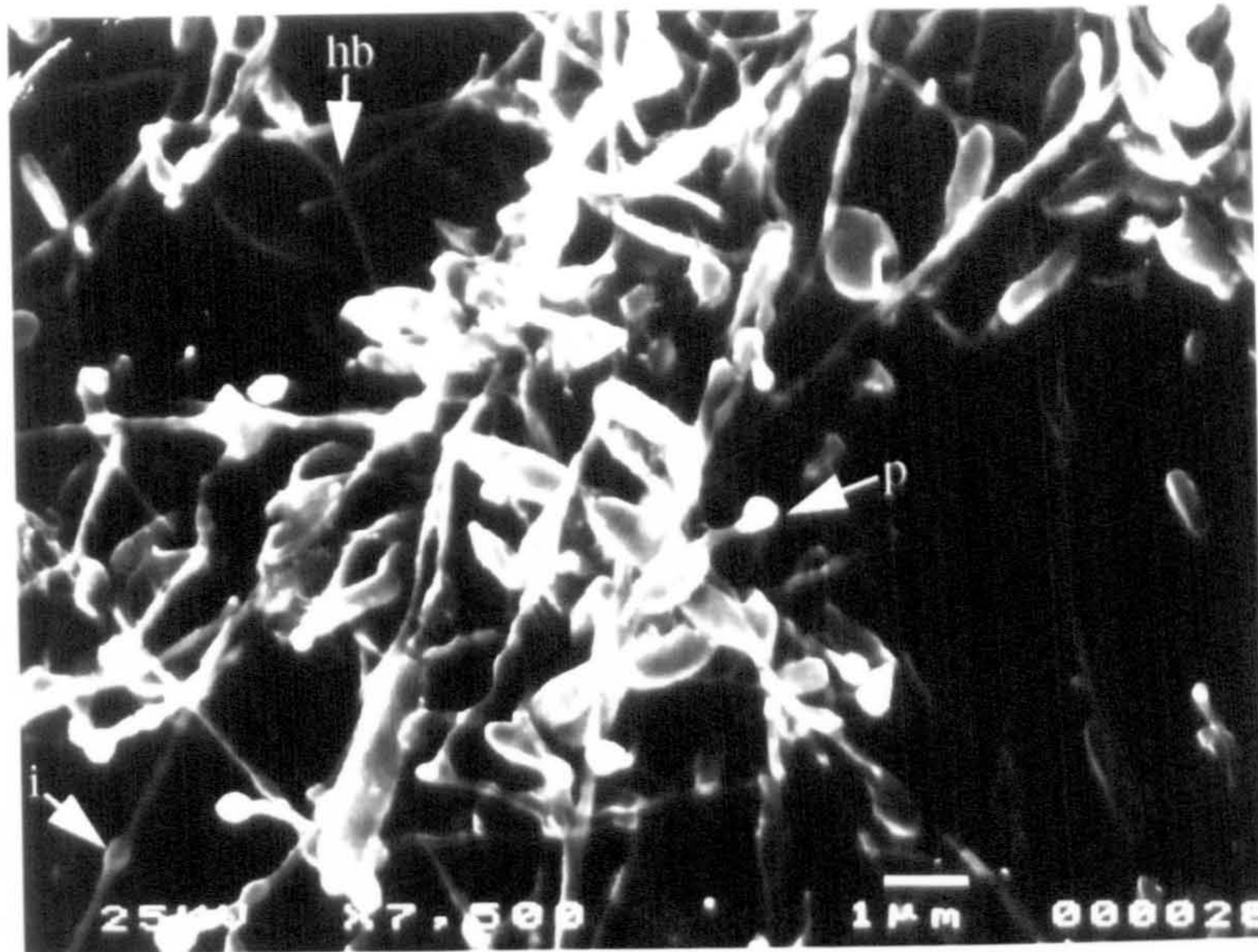
1. One month to detect the species responsible for initial surface colonisation.
2. Six months to find the maximum number of morphologically distinct cell types.
3. 18 months to discover whether conversion of dissolved compounds in the water had led to an increase in the total available carbon in the system and if so, whether this enabled other bacteria to grow and outcompete the appendaged microorganisms.

5.3 The static batch model

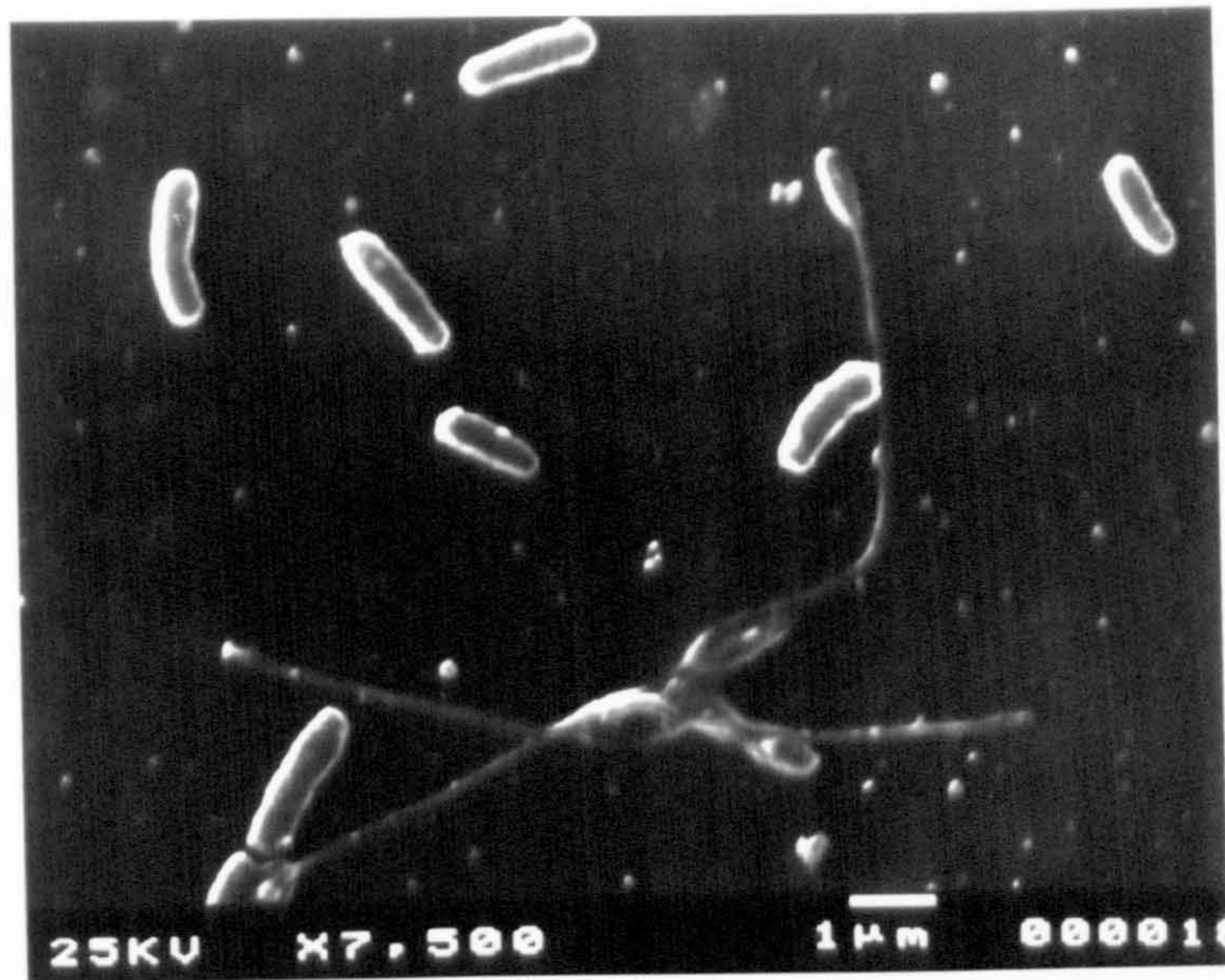
The static batch enrichment model employed contained glass coverslips, 13 mm in diameter, inserted into slits cut in silicone tubing and suspended at different levels in a 5 l Erlenmeyer flask. The flask was partially filled with 4 l potable water, the top was covered with aluminium foil and it was stored at room temperature in the dark for 18 months. Bacteria attached to the coverslips were observed by scanning electron microscopy (SEM). Samples of the air-water interface were taken and analysed by transmission electron microscopy (TEM).

5.4 Bacteria attached to surfaces after 3-5 weeks

A matrix of prosthecate bacteria, either *Hyphomicrobium* or *Pedomicrobium* sp. was present at the glass surface after 22 days of static batch enrichment (Fig. 5.1(a)) indicating that cells had attached and had already started to grow and divide. Budding occurred at the tips of prosthecae. Hyphae were not uniform in width and many contained integral cellular expansions. Other budding *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells were observed adjacent to regular rod-shaped cells (Fig. 5.1(b)).



(a)



(b)

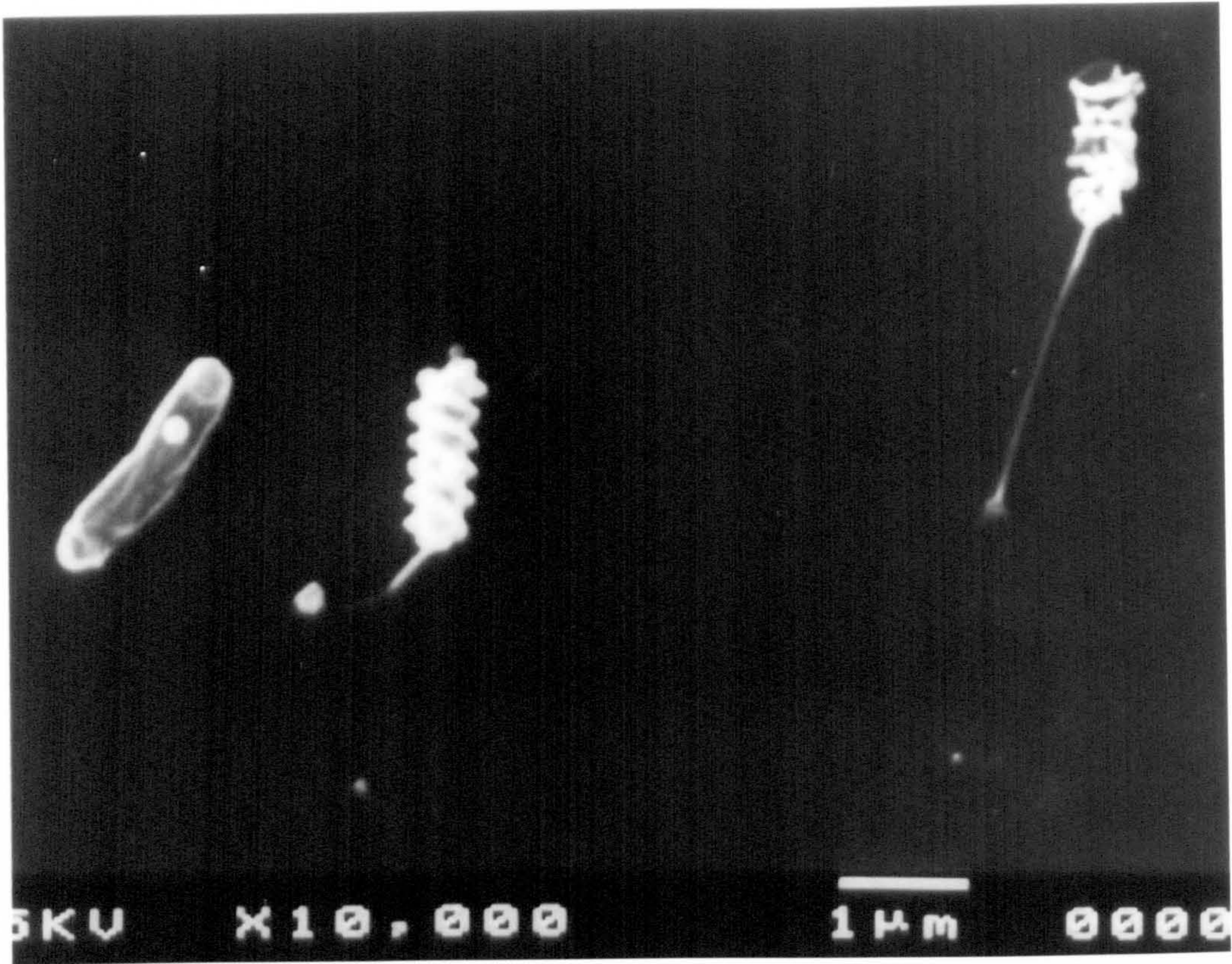
Figure 5.1 SEM analysis of bacteria attached to glass surfaces after 3-5 weeks in the static batch enrichment model. Coverslips were removed from the model after (a) 22 days and (b) 37 days. (a) A matrix primarily of *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells. Hyphal branching (hb) is indicated. Initiation of bud formation has occurred at poles of prosthecae (p). Integral expansions are apparent within hyphae (i). (b) Single rod-shaped cells and cells of *Hyphomicrobium* sp. or *Pedomicrobium* sp..

The presence of extensive networks of *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells within 22 days after starting the static batch enrichment suggests that a period of static batch culture was not required for selection of this strain, but that the glass interface itself was needed for matrix development. However, no other morphologically distinct cells were observed - all other cell types seen at this stage were regular rods.

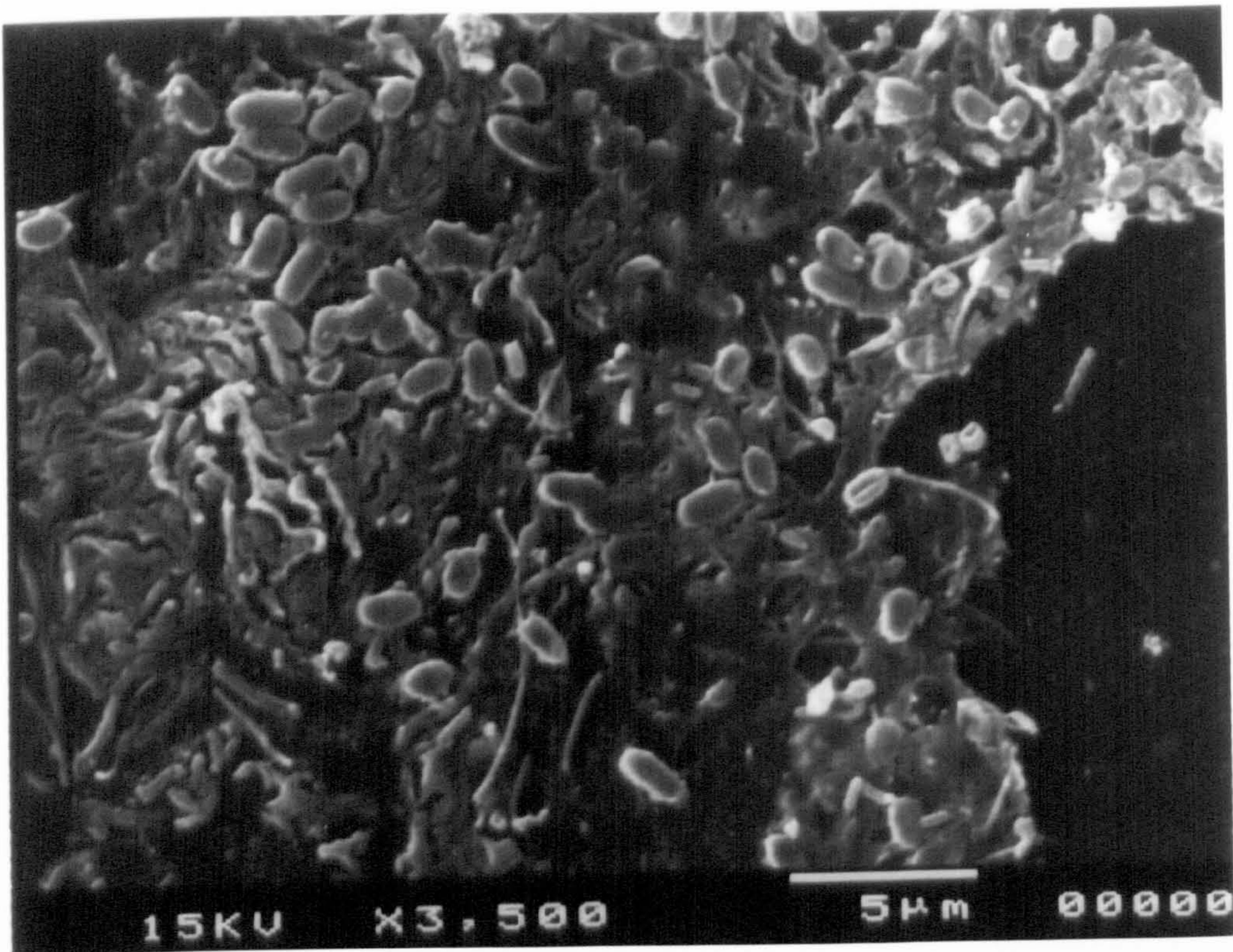
5.5 A survey of bacteria present after 20-25 weeks

5.5.1 Solid-liquid interfaces

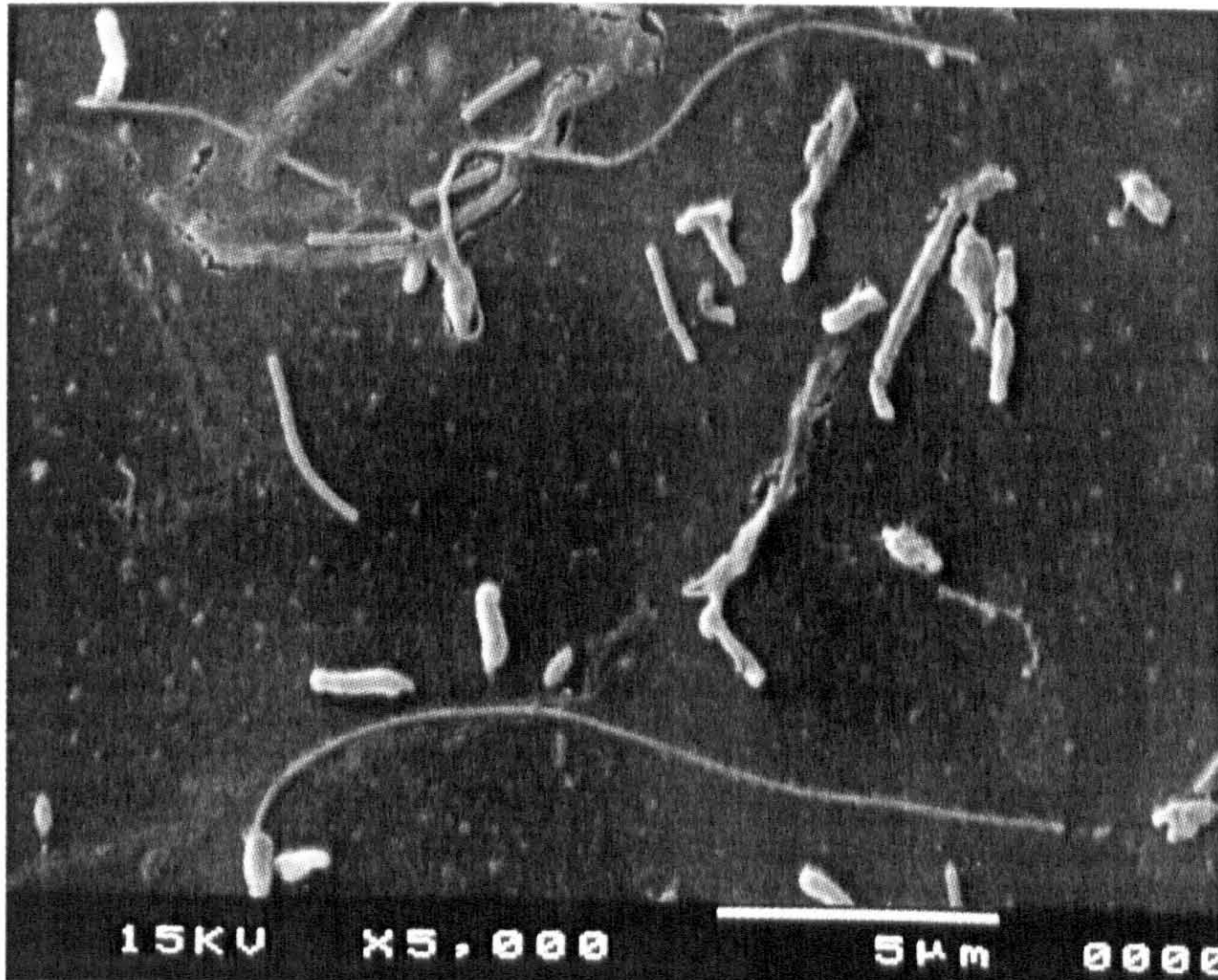
A wide diversity of cellular morphologies was observed at interfaces after 20-25 weeks. Apart from the *Hyphomicrobium* or *Pedomicrobium* sp., the most common distinctive morphology observed on the glass surfaces was a helical cell type possessing a single polar cellular appendage (Fig. 5.2(a)). This cell morphology, which was also seen by TEM in the air-water layer (Fig. 5.3(b)), has not previously been reported. The stalk was approximately 0.1 μm in diameter and could not therefore be resolved by light microscopy. Only individual cells of this type were ever observed so the mode of division could not be ascertained. Attempts to isolate the strain using procedures described in Chapter 3 were unsuccessful. It was only ever observed after static batch enrichment of potable water and was not seen in the continuous flow model (Chapter 6). Possible explanations for this are:- (i) the organism only attached to surfaces under static conditions; (ii) it was initially present in very low numbers and was selectively enriched by static batch culture or (iii) it was present in the continuous flow model but did not express the distinctive morphology there. When 0.5 l of water was held in the dark for 8 weeks in a similar static batch system the stalked helical cells were once again commonly observed at glass surfaces suggesting that an absolute minimum of 2 cells per litre of potable water were present. By diluting the potable water before introducing it to the static batch model it would be possible to obtain a quantitative assessment of the initial concentration of cells in the water. Other distinctive cell types at the glass surfaces included spirilla and cells of *Seliberia* sp. (not shown).



(a)



(b)

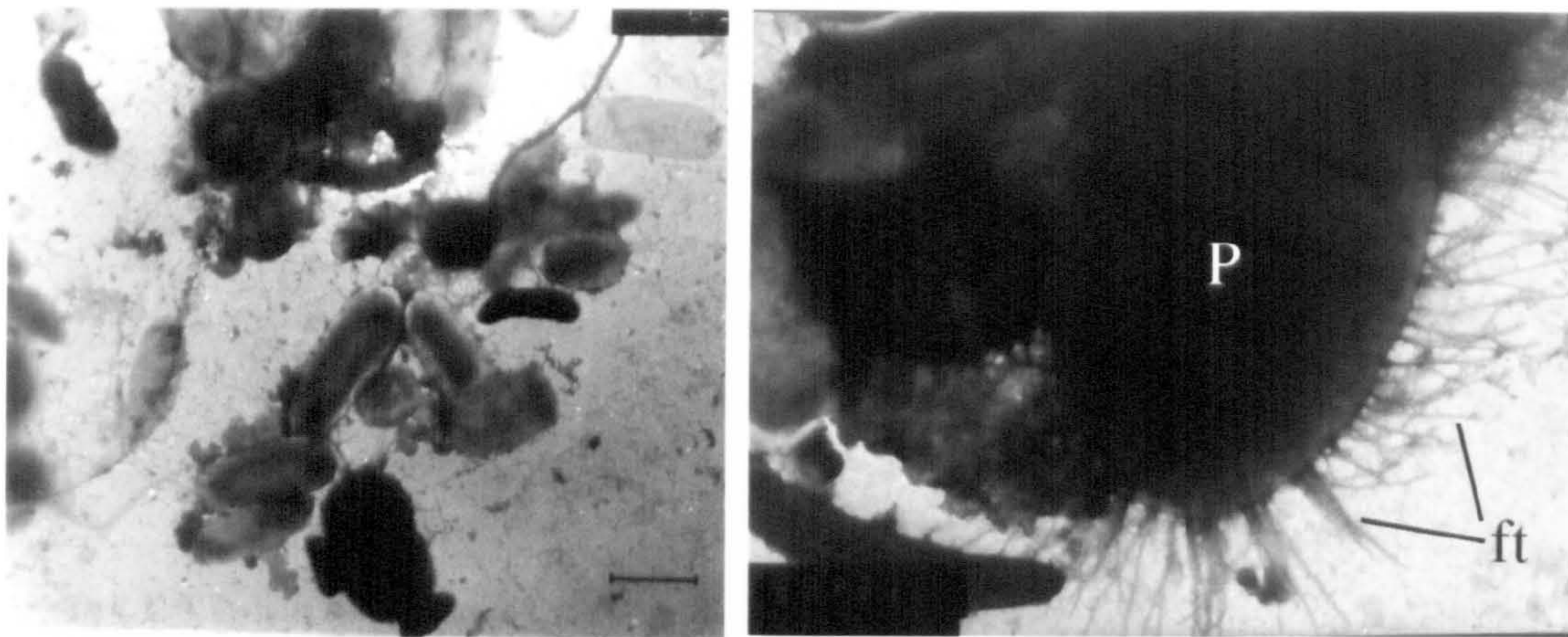


(c)

Figure 5.2 SEM observation of bacteria at solid / liquid interfaces after 20-25 weeks in the static batch enrichment system. Coverslips were removed from the model after (a) 140 days and (b,c) 200 days and prepared immediately for SEM examination. (a) A stalked helical cell type; (b and c) a mixture of cell types held within a matrix of EPS (b) or in the absence of obvious extracellular polymers (c).

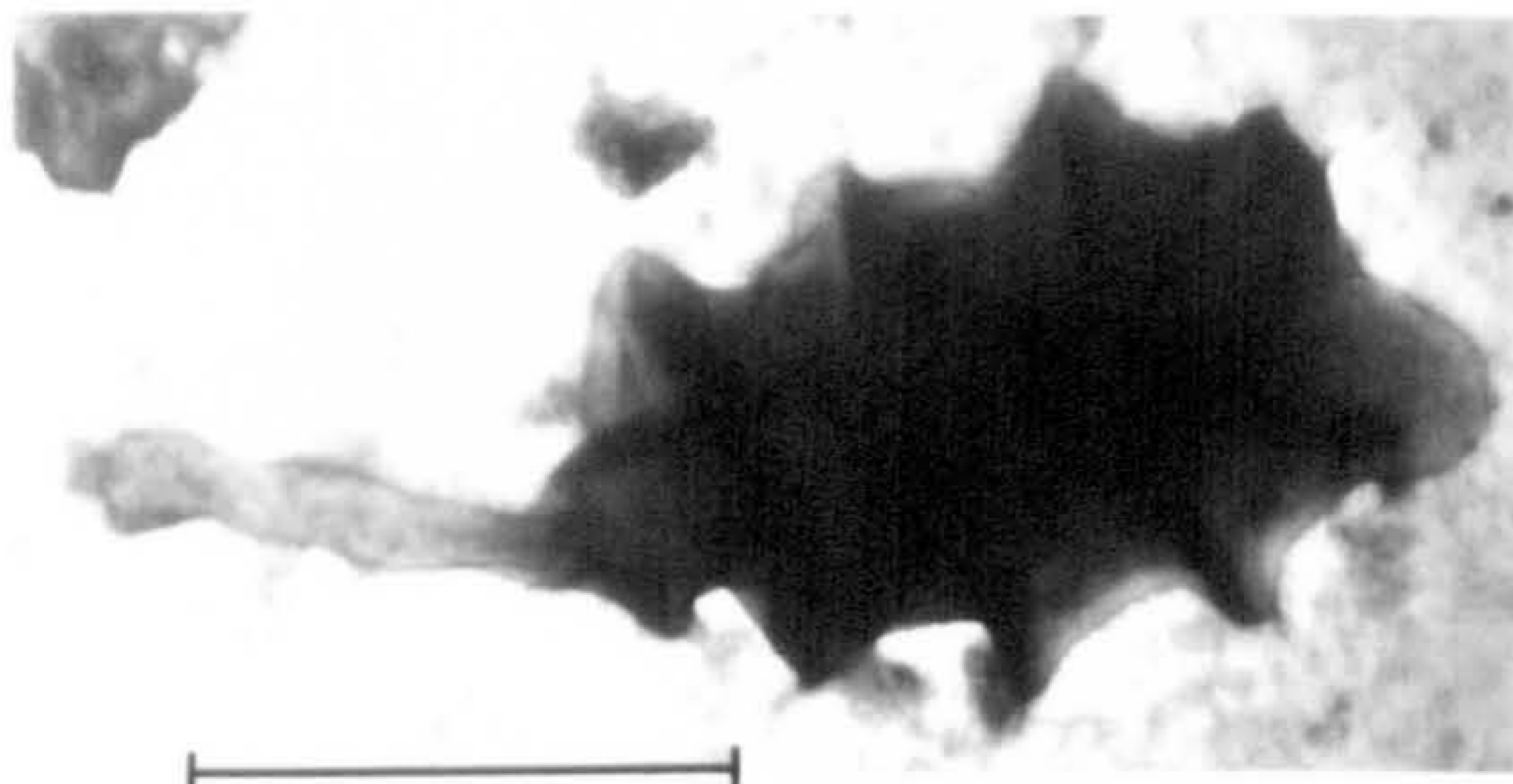
5.5.2 The air-water interface

A greater diversity of bacteria was recognised by TEM than by SEM, partly as a result of the increased resolving power of the transmission microscope. *Hyphomicrobium* sp. or *Pedomicrobium* sp., *Planctomyces* sp., and the stalked helical cell type were seen (Fig. 5.3). Rods encapsulated in EPS and cocci covered with pili were also present.

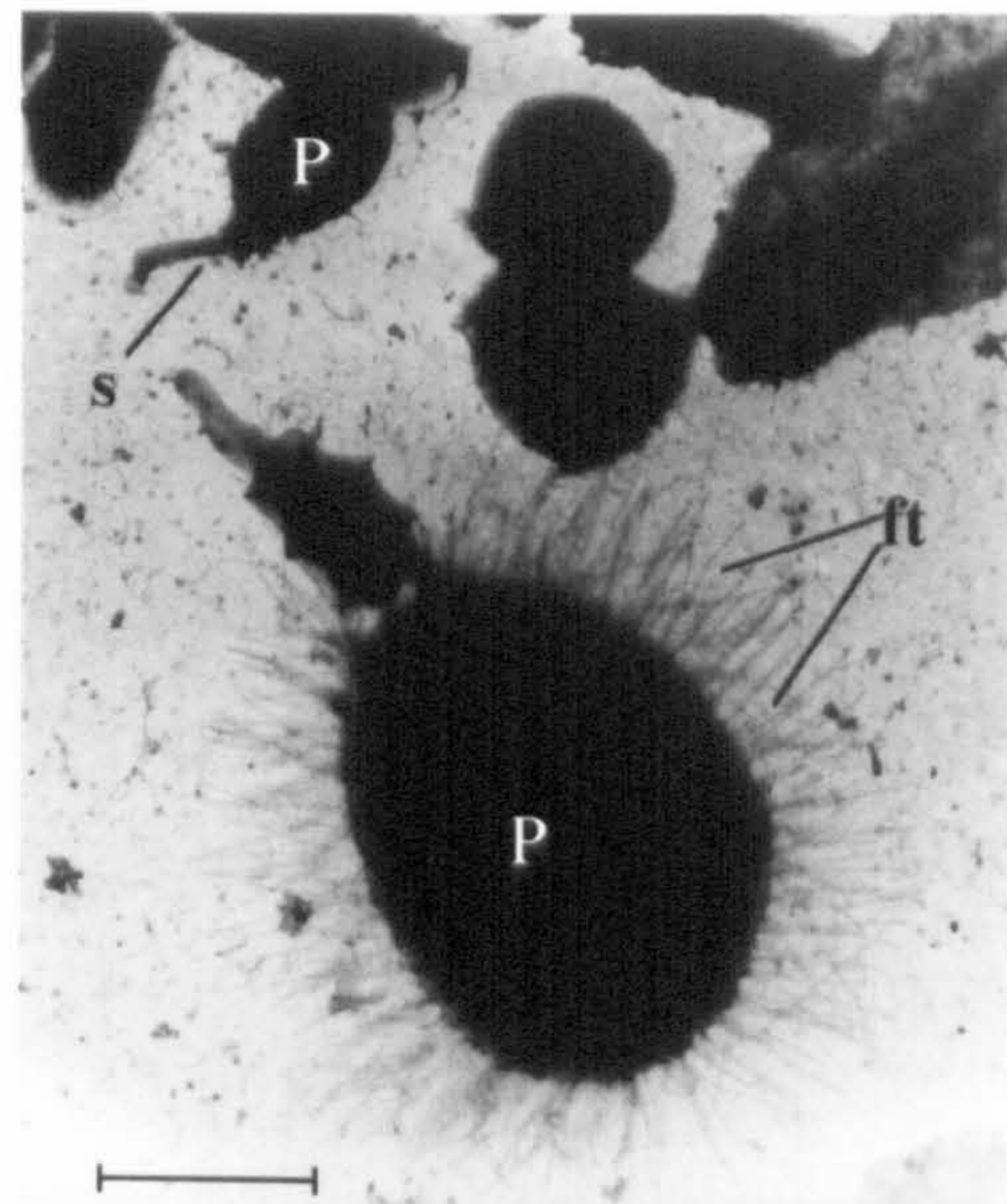


(a)

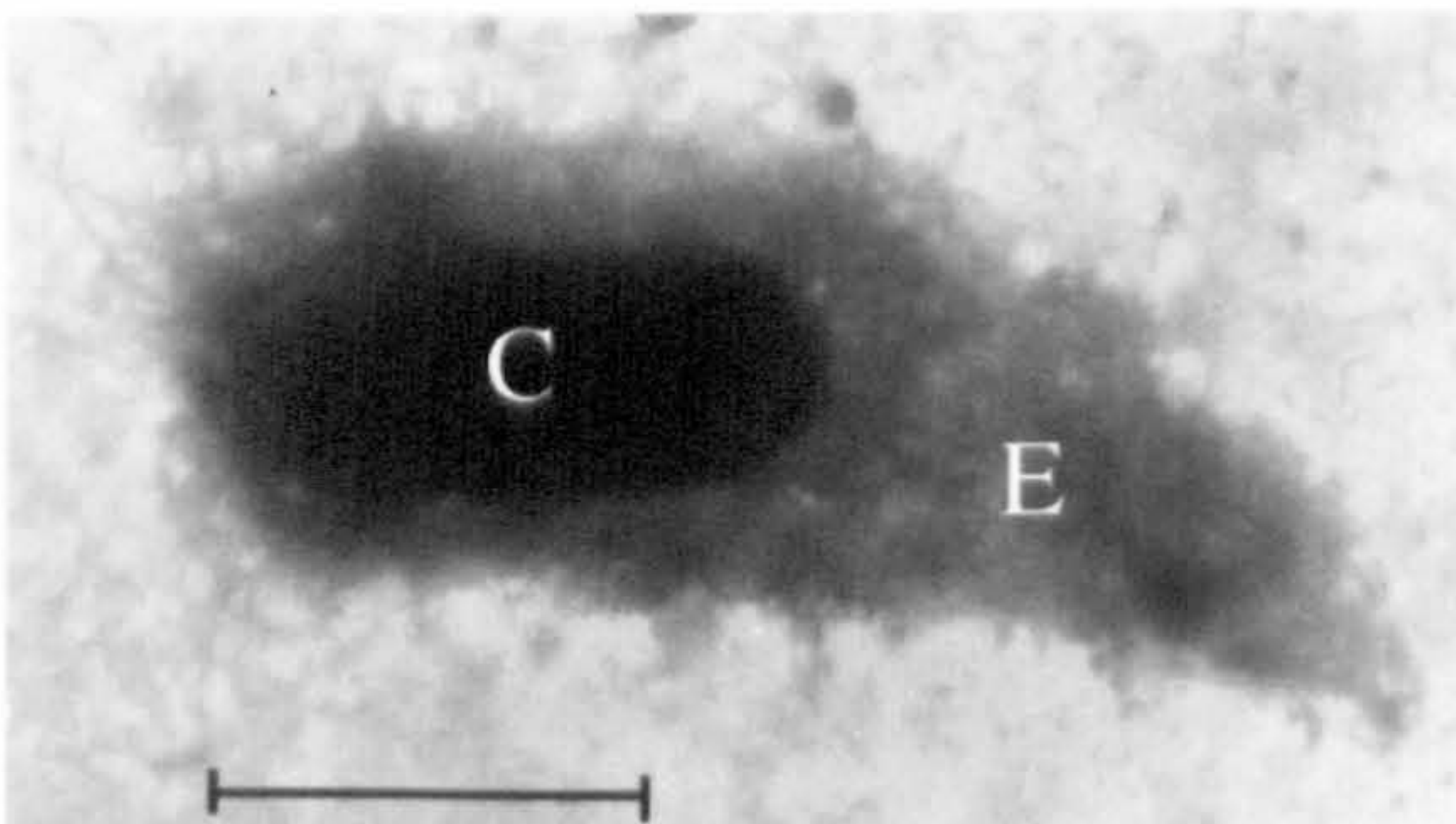
(d)



(b)

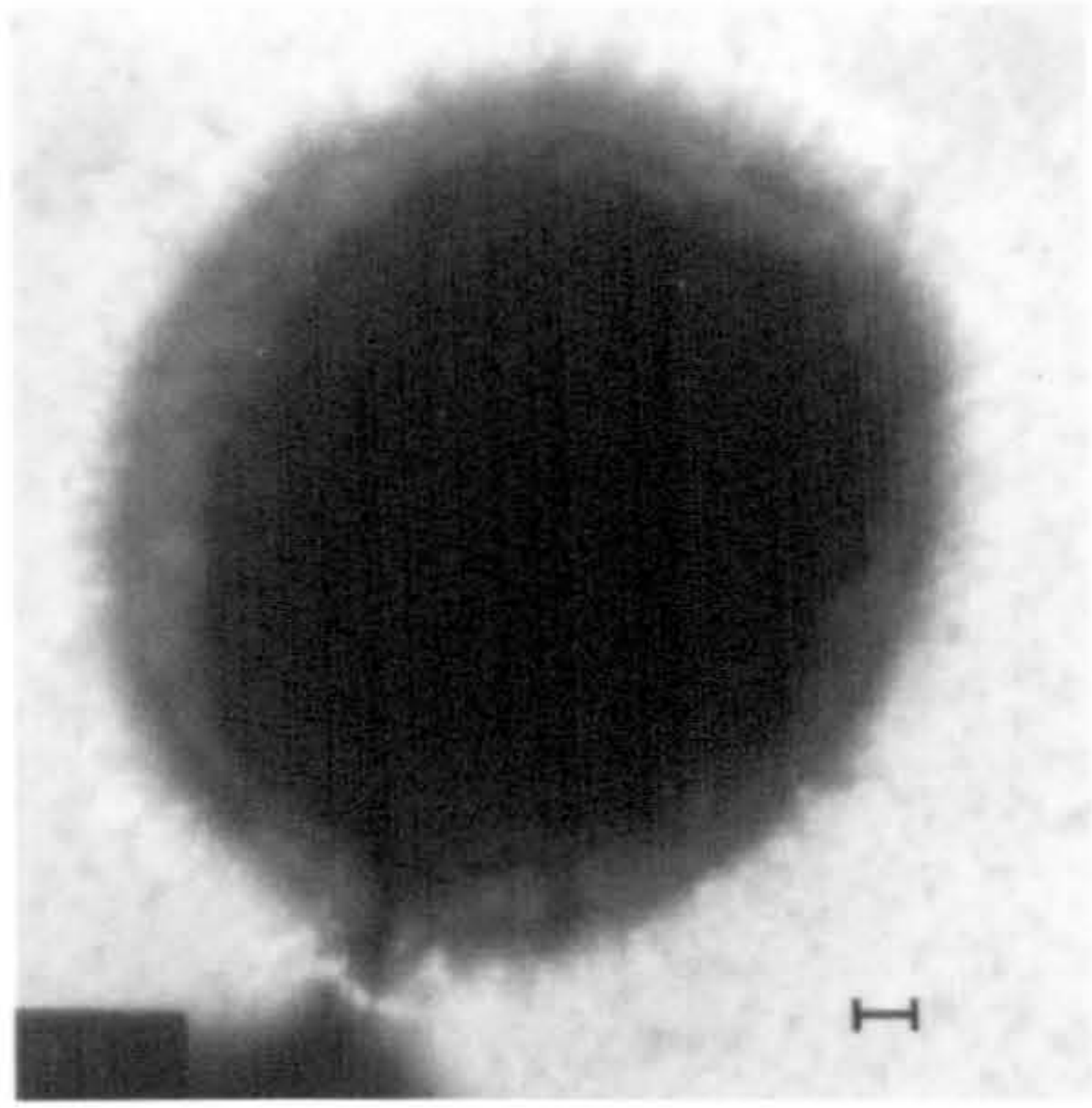


(e)

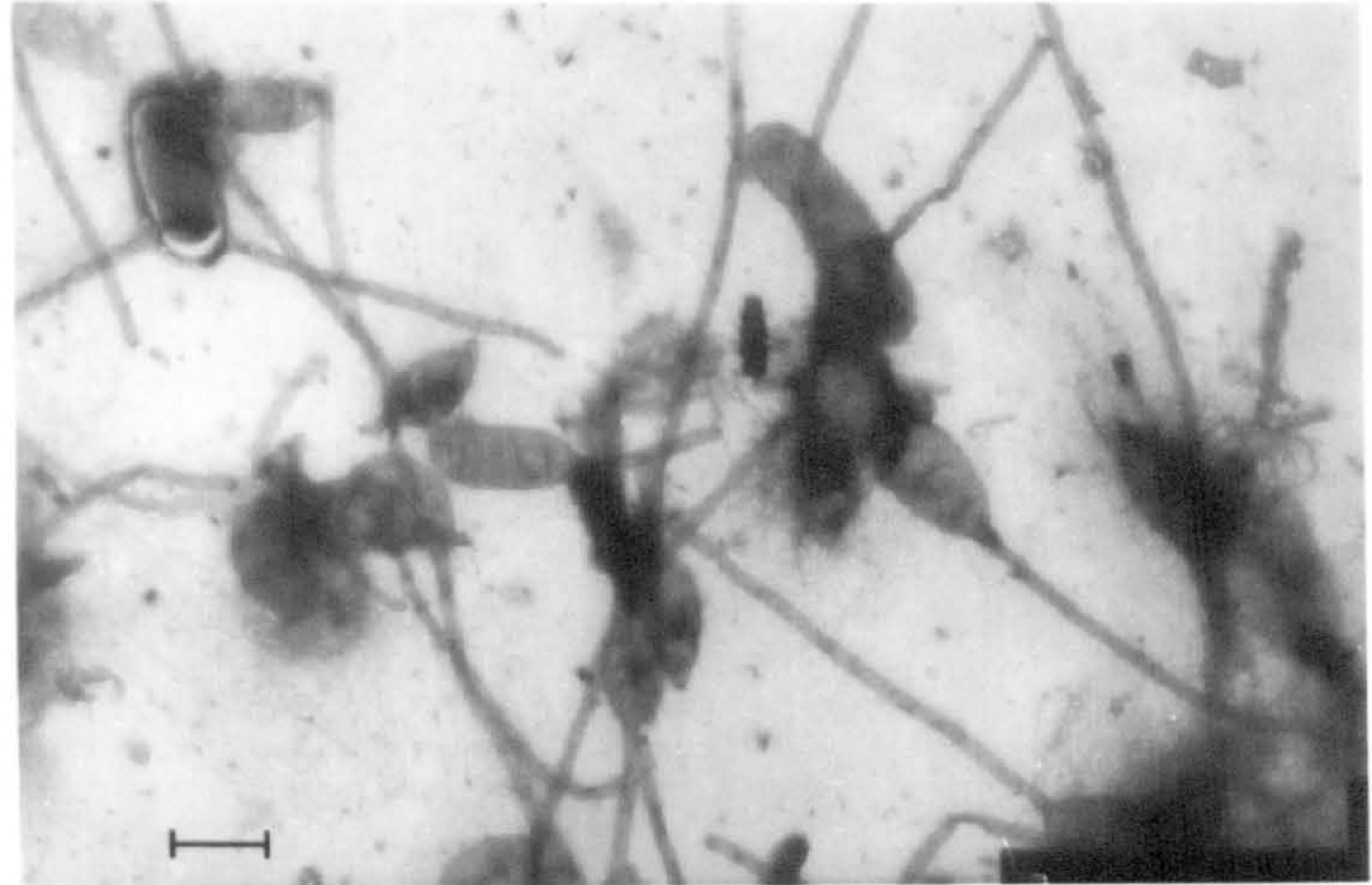


(c)

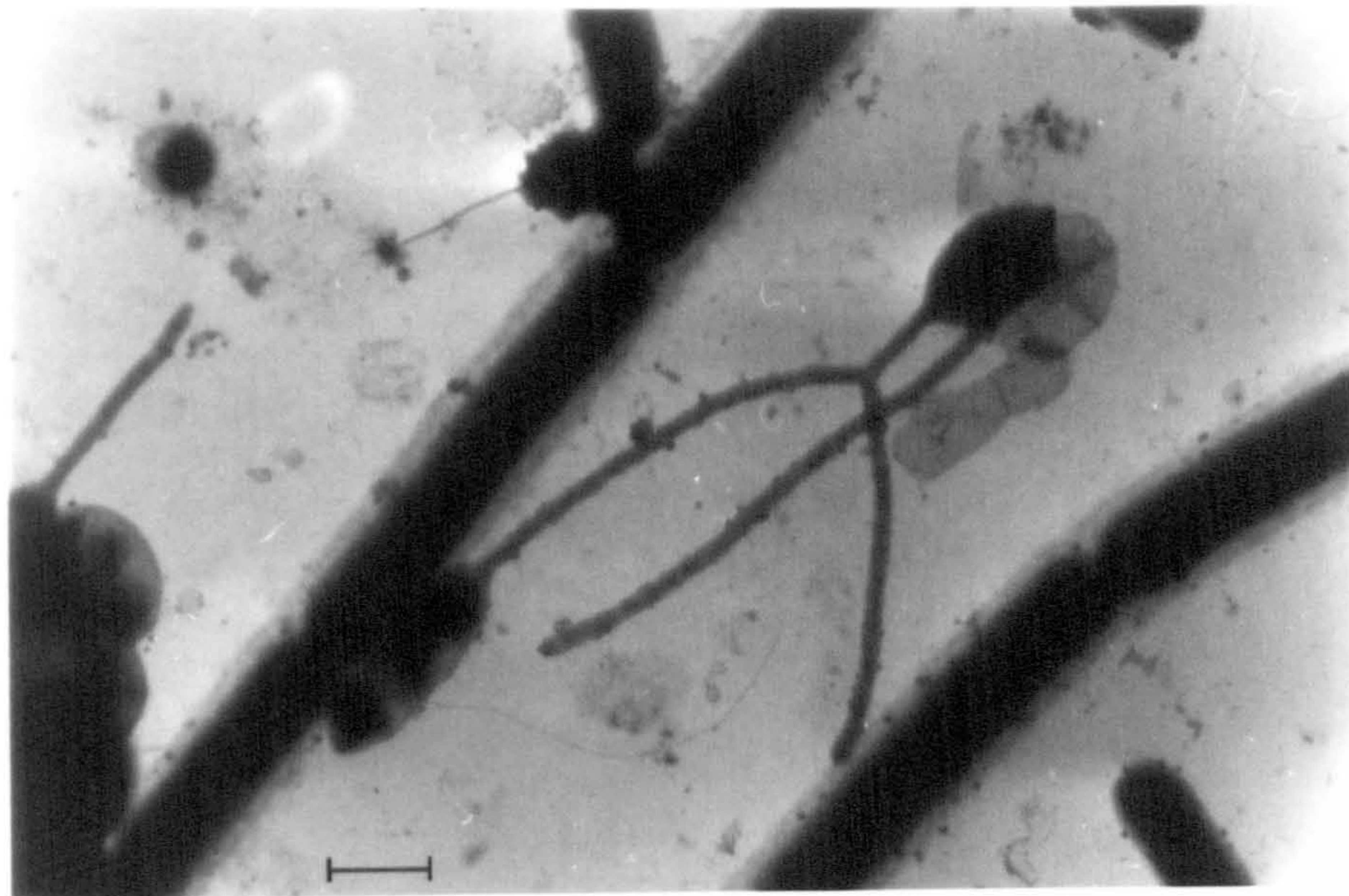
Figure 5.3 TEM analysis of bacteria present at the air / water interface after 24 weeks in the static batch enrichment model. A drop of water was removed from the surface of the liquid and placed on a copper grid for 30 mins. Excess liquid was then removed and the grid was prepared as detailed in Section 2.23. (a-g) Negative stained with phosphotungstic acid; (h) gold-palladium shadowed. (a) An overview of some of the different morphologies that were present; (b) an helical cell type



(f)



(g)



(h)

possessing a polar prosthecum; (c) a rod-shaped cell (C) encapsulated by EPS (E); (d) and (e) *Planctomyces* sp. cells (P), some with fibrillar tufts (ft) and others with a polar stalk (s); (f) a coccus with many pili covering the cell surface; (g) *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells with prosthecae emerging from one or both poles; (h) a variety of cell types including cells of *Hyphomicrobium* sp. or *Pedomicrobium* sp. and a stalked helical cell. (a)-(e) and (g)-(h), bar = 1 μm ; (f) bar = 0.1 μm .

Many cell types were seen that were only observed after static batch enrichment, yet all of these cells must have been present in the original potable water used to seed the model.

5.6 Structure of biofilms after 74 weeks

The model was maintained for 74 weeks to monitor the effect of prolonged incubation on the structure and cellular composition of biofilms present.

5.6.1 Isolation and identification of bacteria

In addition to strains routinely isolated directly from tap water, *Micrococcus luteus* was recovered from the static batch model after 74 weeks. Although most commonly isolated as a laboratory contaminant, *M. luteus* has previously been isolated from potable water (e.g. LeChevallier *et al.*, 1987; Buswell *et al.*, 1997). The static batch model was not maintained in a sterile environment so it is unclear whether *M. luteus* was initially present or whether airborne cells entered the system later. However, since cells of this species require relatively high levels of nutrients for growth, the detection of viable *M. luteus* cells is indicative of an increase in the nutrient concentration since the last sample had been taken. The TOC of the bulk water in the model was found to be $35.3 \pm 1.8 \text{ mg l}^{-1}$, confirming this conclusion.

5.6.2 Direct observation of the microorganisms present

Bacterial hyphae were still abundant when glass surfaces were viewed under phase contrast optics. Phase-bright rods and cocci were seen. The cocci were actively dividing and were probably *M. luteus*. Therefore, although the nature of the biofilm had changed with many more regular rods and cocci actively growing, the initial *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells were still present. Yeast cells were observed but other eukaryotes such as diatoms or amoebae were not.

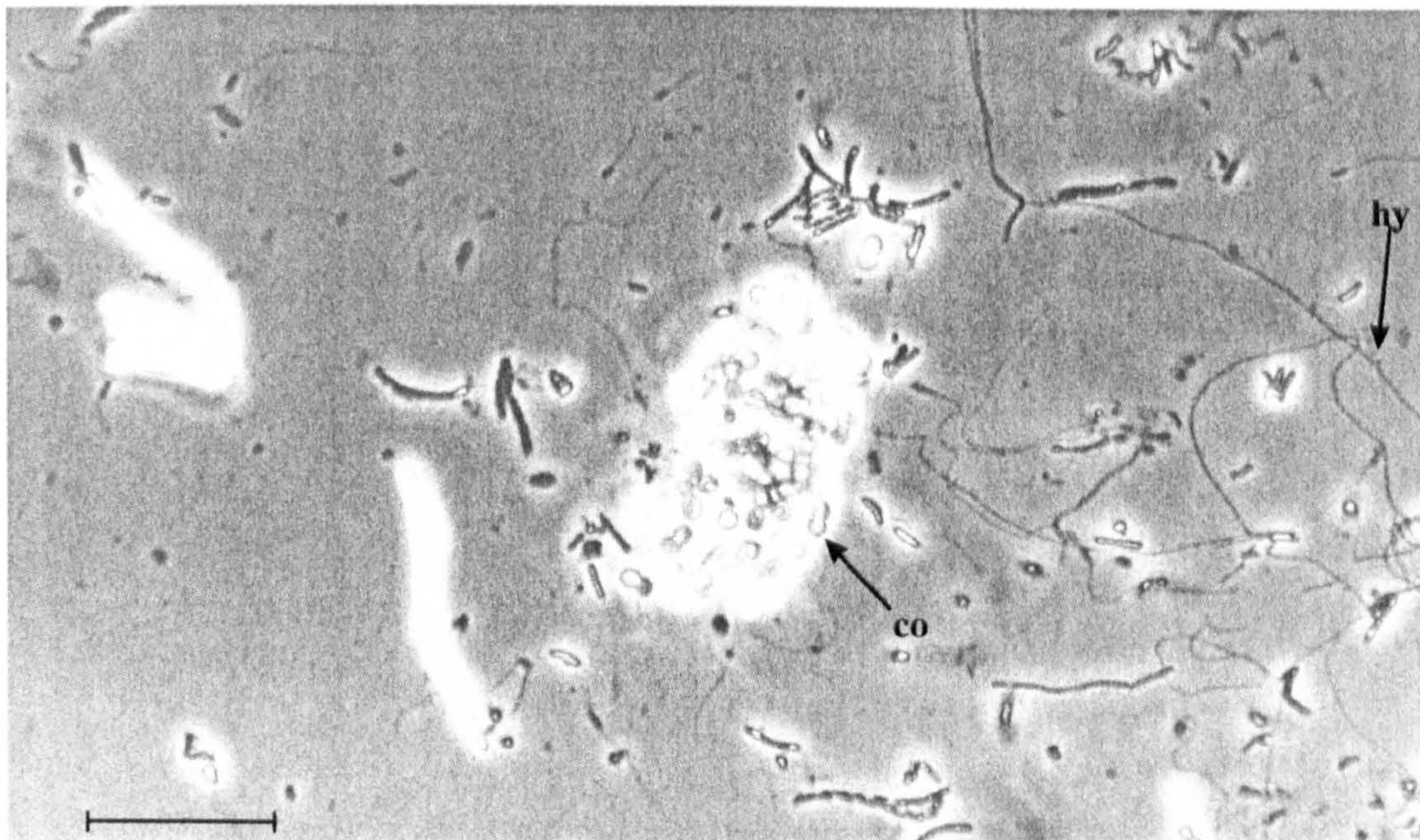


Figure 5.4 Phase contrast micrograph of a 74 week old biofilm produced after static batch enrichment. Hyphae produced by *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells are indicated (hy) as are phase-bright cocci of the same dimensions as isolated *Micrococcus luteus* (co). Several other cell types are also visible. Bar = 10 μ m.

5.6.3 EPS

Congo Red stain was employed to assess the extent of EPS production on the glass surfaces. Most of the biofilm stained red, highlighting the presence of extensive matrix material (Fig. 5.5). The EPS matrix was not continuous and even in this mature biofilm there were areas of the coverslip that were not covered by the matrix. Cells were usually, but not always, embedded within the EPS.

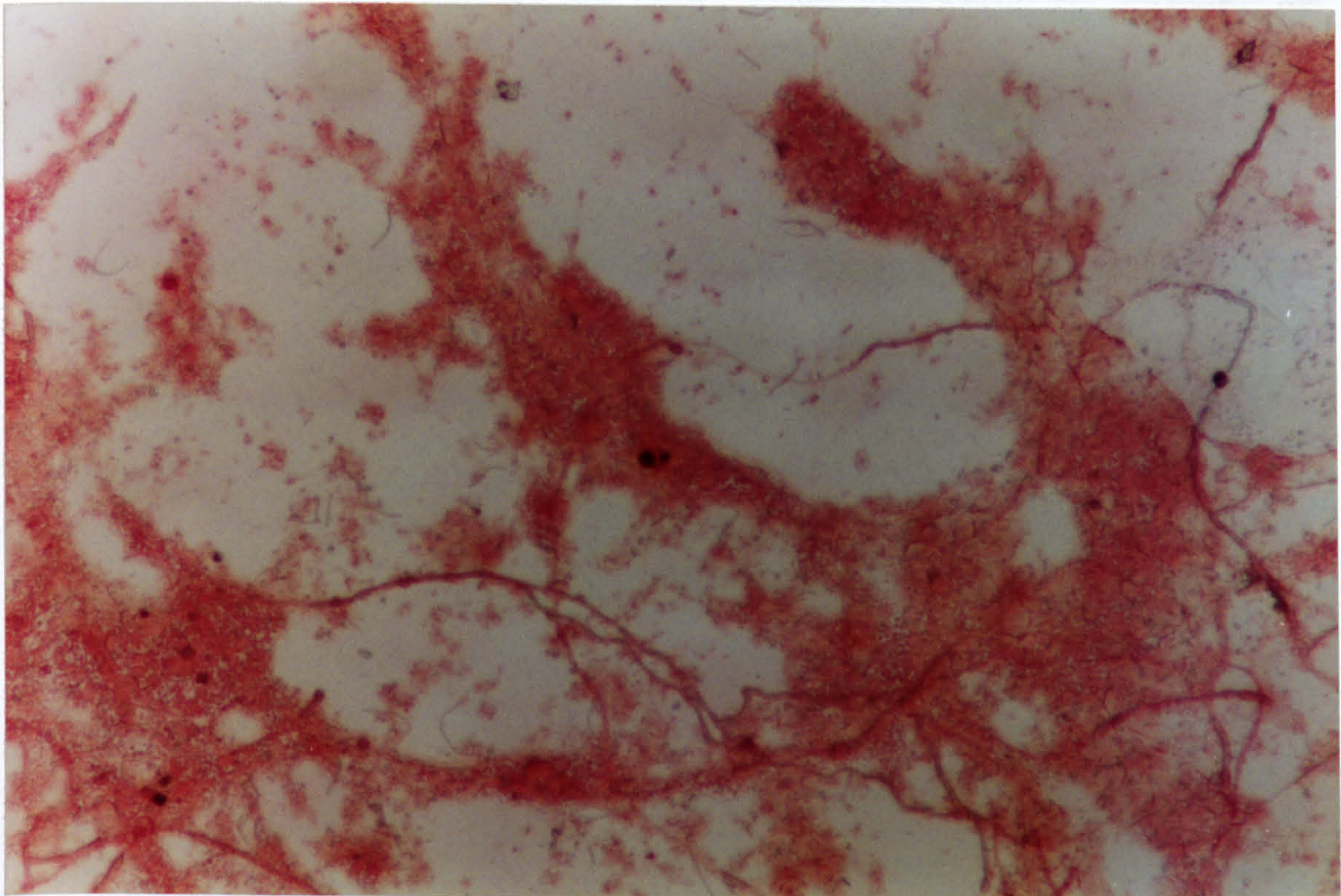


Figure 5.5 Light micrograph of a 74 week biofilm stained with Congo Red for EPS. Areas rich in EPS appear red. Actinomycete-like filaments are apparent, but are not always associated with the EPS. Bar = 50 μm .

5.7 Conclusions

Although not routinely isolated, cell types bearing distinctive morphologies are present in potable water and can be observed at interfaces after static batch enrichment for 6 months. The primary reason for the frequent failure to detect these organisms in water is undoubtedly the sampling technique employed. Most of the appendaged bacteria are adapted to growth in oligotrophic environments and cannot be isolated on nutrient-rich media. The growth cycle of prosthecate bacteria, which involves a motile cell stage lacking the prosthecum, may also contribute to their ability to escape detection. The flagellate daughter cells are not distinctive themselves and only the appendaged mother cells can easily be recognised

microscopically. However, specific rRNA probes will eventually overcome the difficulty of identifying the daughter cells *in situ*.

The role of appendaged bacteria in distribution pipe biofilms is unclear. *Hyphomicrobium* spp. can deposit iron or manganese which may lead to constriction or blockage of pipes (Sly *et al.*, 1988). The ability of *Hyphomicrobium* spp. to grow on C₁ compounds, particularly methanol and methylamine, may be of importance in potable water. By removing the methanol produced as a result of bacterial methane oxidation, hyphomicrobia can prevent the build-up of methanol to levels that would inhibit the methane-oxidising bacteria (Moore, 1981). The frequent isolation of *Methylobacter* sp. further supports the theory that sufficient methanol is present in treated potable water for bacterial utilisation.

The fact that so many types of di- or poly-morphic bacteria can be found in potable water demonstrates that life cycles involving an asymmetric division step are common in this environment and may be the standard mode of bacterial replication in potable water.

CHAPTER 6

6 Application of a laboratory model of potable water distribution network biofilms to study biofilm development and stability

6.1 Introduction

Drinking water and the related microbial planktonic and sessile bacterial populations constitute one of the most extensively studied oligotrophic systems. However, the difficulty of sampling and manipulating *in situ* biofilms in the drinking water distribution network necessitates the use of laboratory models. Various models for biofilm development have been constructed with a variety of different objectives (see Section 1.3.1). To study the role of dimorphic life cycles in potable water biofilms, the key requirements for any model are (i) that it must have a continuous throughput of potable water and (ii) that physical and chemical conditions must be as close as possible to those present in the distribution pipelines.

Previous investigations of potable water systems, reviewed in Section 1.7, have not assessed the preponderance of life cycles involving asymmetric division in this environment. In the absence of a specific universal probe for distinct cell types produced by asymmetric cellular division, only an analysis of the metabolic activity of cells can distinguish between cell types *in situ*. Many reports have shown that vegetative dormancy is common in potable water (e.g. Kalmbach *et al.*, 1997b; Morita, 1988; Byrd *et al.*, 1991; Kaprelyants *et al.*, 1993; Roszak & Colwell, 1987), although the ratio of active to inactive cells varies with the strain studied (Kalmbach *et al.*, 1997b). In order to determine whether dormant cells are produced by asymmetric division and whether they are specifically adapted for biofilm dispersal, it is necessary to assess the activity of sessile and planktonic cells and the relationship between them. If dispersal cells are actively released from biofilms as a function of the growth cycle it should be possible to detect a sustained increase in the number of planktonic cells as surfaces become colonised. Passive release from biofilms (e.g. by sloughing) would result in more random fluctuations in numbers of

planktonic bacteria and would elevate the number of particles larger than bacteria in the water.

The question of what triggers the reactivation of dormant cells produced by asymmetric division and the initiation of the sequence of events leading to maturation and reproduction remains unresolved (see Section 1.2.5.2). Undoubtedly the onset of maturation is delayed until the nutrient availability can support growth (Morgan & Dow, 1985), but attachment to a surface may also stimulate maturation either directly or indirectly by concentration of nutrients at the interface. In potable water distribution pipes the limiting nutrient is usually carbon (van der Kooij, 1992), but the carbon concentration is unstable. An investigation into the ability of sessile and planktonic cells to reactivate in response to a slight elevation in the assimilable organic carbon concentration of tap water will increase our understanding of bacterial dormancy and dimorphism in this environment.

Another factor limiting bacterial growth in treated water supplies is the chlorine residual. Chlorine affects the structure and composition of biofilms in the early stages of formation (van der Wende *et al.*, 1989), but the effect on mature complex biofilms has not been characterised.

6.2 Aims

The aim of this section of the project was to develop a simple continuous flow model to produce complex biofilms similar to those present on the inner surfaces of distribution pipes. This model would then be used to study the following aspects of the attached and free-living microflora:-

1. The responses of planktonic cells to a small increase in the concentration of dissolved organic carbon in the water.
2. The rate of biofilm accumulation and the effect of biofilm growth on the quantity and activity of planktonic cells.
3. The responses of mature biofilms to a small increase in the concentration of dissolved organic carbon in the water.
4. The responses of mature biofilms to chlorine.

6.3 Development of a continuous flow model

A model was established to enable regulation of the rate of flow of water over glass surfaces held within a series of biofilm development vessels (Fig. 2.3). Glass was selected to form the substratum, rather than a material used in the local distribution network, because glass is inert and easy to analyse microscopically. The substratum material affects the extent and composition of biofilms formed from potable water, but this area has been analysed previously (Rogers *et al.*, 1994a,b; Kerr *et al.*, 1997), and was beyond the scope of this thesis. The layout of the continuous flow model is shown schematically in Fig. 6.1. Three sample ports were included to allow sampling of the planktonic population (i) before the biofilm vessels, (ii) immediately after the series of vessels and (iii) in a separate reservoir after the series of biofilms in which the carbon concentration could be amended. A comparison of the planktonic population at port 1 with that at port 2 gives an indication of the effect of biofilms on the natural planktonic microflora. The model was kept dark during use.

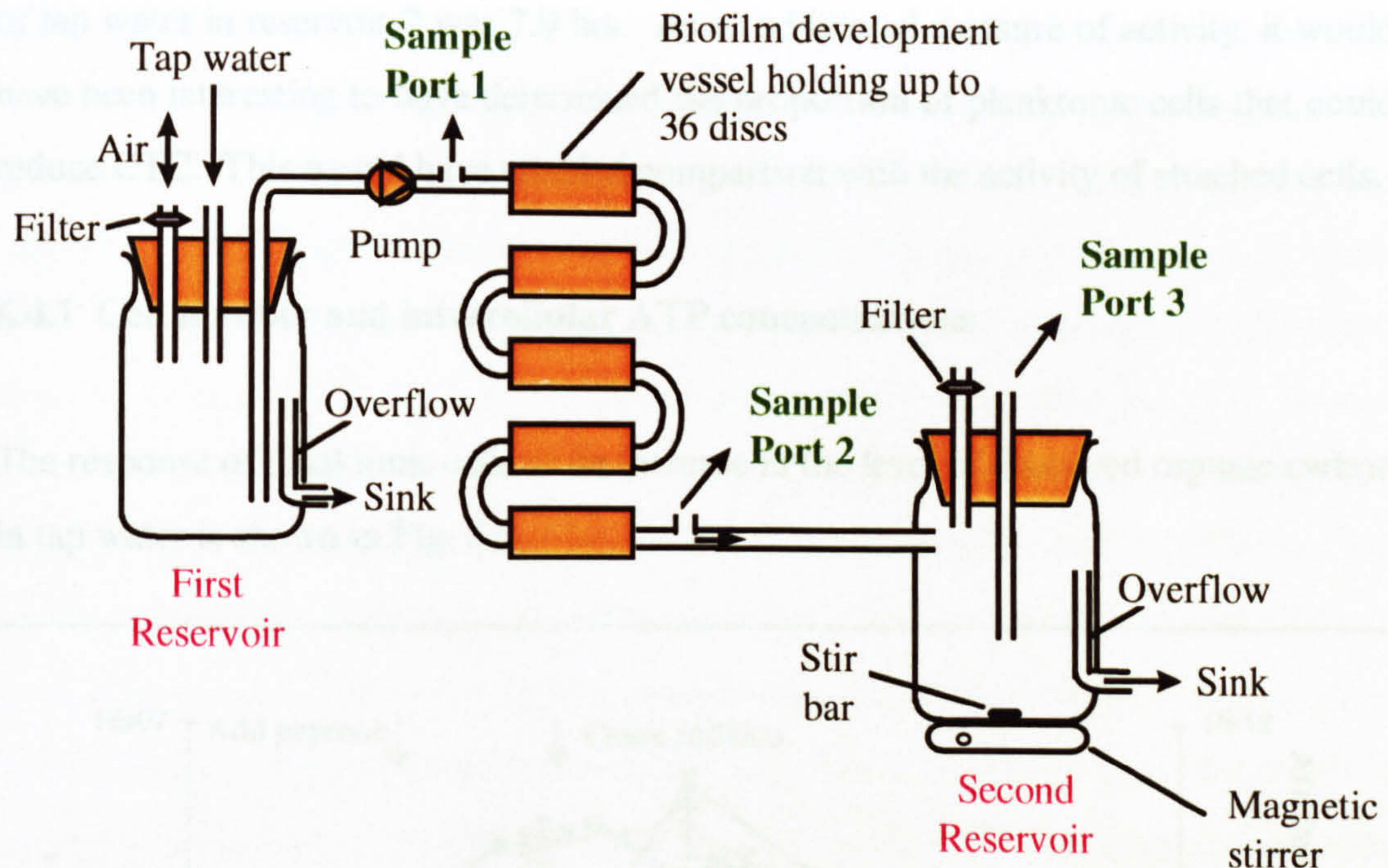


Figure 6.1 The continuous flow model. Tap water was continuously introduced into the first reservoir and a proportion of this was pumped at a defined rate over glass coverslips held within each of the five biofilm development vessels (Fig. 2.3). The second reservoir was included to monitor the response of planktonic cells to carbon addition. Biofilm samples were removed at intervals from the vessels. Water samples were taken before the series of biofilms (port 1), after the series of biofilms (port 2) or from the second reservoir (port 3).

6.4 Physiology of planktonic cells and their responses to an increase in the concentration of bioavailable carbon

The planktonic microflora of potable water was sampled from reservoir 2 in the continuous flow model (Fig. 6.1). Total counts and the particle size distribution were monitored on-line. The background intracellular ATP concentration and total viable count, determined on PCA, were measured and these parameters were subsequently monitored at intervals following addition of an exogenous carbon source (a final concentration of 0.001% (w/v) bacto-peptone) for 24 hrs. The carbon substrate was chosen to mimic the range of complex organic molecules that may periodically

become available to bacteria in distribution mains water. Note that the retention time of tap water in reservoir 2 was 7.9 hrs. As an additional measure of activity, it would have been interesting to have determined the proportion of planktonic cells that could reduce CTC. This would have enabled comparison with the activity of attached cells.

6.4.1 Cell number and intracellular ATP concentrations

The response of planktonic cells to an increase in the level of dissolved organic carbon in tap water is shown in Fig. 6.2.

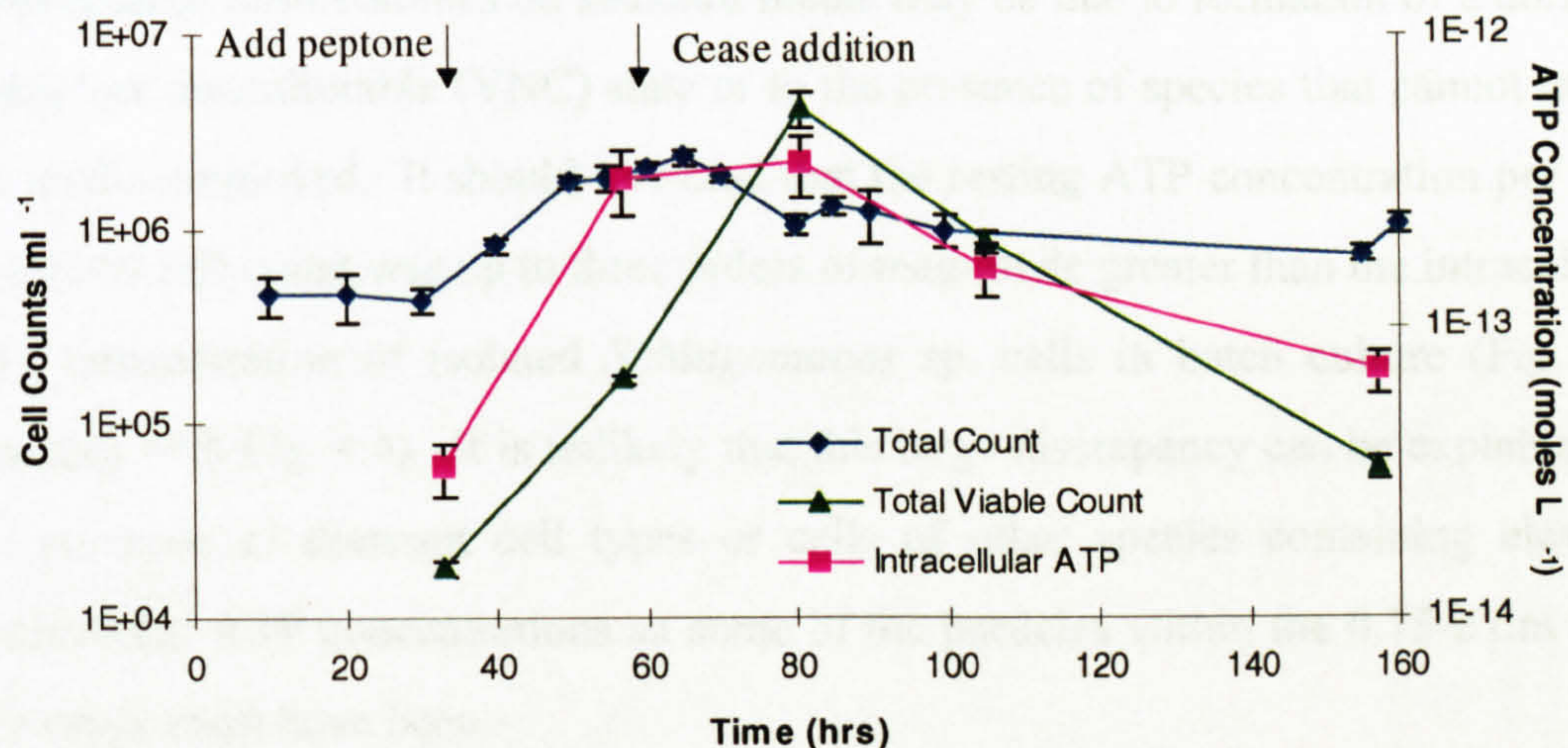


Figure 6.2 Responses of planktonic cells to extraneous peptone addition. A final concentration of 0.001% bacto-peptone was added to reservoir 2 of the continuous flow model at time = 37.3 hrs, as described in Section 2.38. This concentration was maintained for 24 hrs as indicated on the graph. The effect of this increase in dissolved organic carbon in the water on total viable counts on PCA, total counts measured on-line using the *CellFacts* particle analyser and the total intracellular ATP concentration is shown. Each total count data point represents a mean of five readings surrounding that point and standard deviations of these five measurements are shown. Three independent samples were taken for ATP and total viable count (TVC) determinations; data points indicate the means of these replicates and standard deviations are shown.

The background total particle count, within the bacterial size range of 0.75-2 μm equivalent spherical diameter (ESD), was more than ten-fold higher than the total viable count before the addition of nutrients. The *CellFacts* particle analyser was shown to have a sensitivity limit of around 1×10^5 counts ml^{-1} when applied to tap water filtered through a membrane of pore diameter 0.2 μm (data not shown). This limit was significantly below the background particle count of unfiltered tap water. The occurrence of bacteria in tap water that are not culturable on agar is well documented and has been termed 'the great plate count anomaly' by Staley and Konopka (1985). Some of these bacteria may be nonviable, but the majority are metabolically active (Byrd *et al.*, 1991; Kalmbach *et al.*, 1997b). The inability of these cells to form colonies on standard media may be due to formation of a dormant viable but nonculturable (VNC) state or to the presence of species that cannot utilise the media employed. It should be noted that the resting ATP concentration per total *CellFacts* cell count was up to three orders of magnitude greater than the intracellular ATP concentration of isolated *Sphingomonas* sp. cells in batch culture (Fig. 6.3; compare with Fig. 4.4). It is unlikely that this large discrepancy can be explained by the presence of dormant cell types or cells of other species containing elevated intracellular ATP concentrations so some of the particles within the 0.75-2 μm ESD size range must have been:-

(a) dead cells / inert particles,

(b) highly resistant cells not lysed during the ATP sampling protocol (the cell lysis reagents used in the ATP assay reduced the TVC in the sample by more than two orders of magnitude (data not shown), but highly resistant VNC cells may have avoided lysis), and/or

(c) small aggregates.

Addition of peptone resulted in almost a two-fold increase of the total cell count within 3 hrs. The total intracellular ATP concentration, the total cell count and the TVC increased by about one order of magnitude within 15 hrs. The total cell count and total intracellular ATP concentration then stabilised around 15 hrs after starting nutrient addition (Fig. 6.2), but the total viable count and the ATP per total cell continued to rise for a further 24 hrs (Fig. 6.3). It is not clear why the TVC

continued to increase when the total ATP concentration did not, but since a decrease in the total ATP concentration was observed in batch cultures of *Sphingomonas* sp. following nutrient depletion (Fig. 4.4), changes in cellular physiology may have counterbalanced the increase in viable cell number. 80 hrs after beginning the experiment the total viable count appeared to be higher than the total count. This was probably due to disruption of cellular aggregates by spread plating but not during direct counting using *CellFacts*. Note that around 15 hrs after starting peptone addition an increase in particles larger than single bacterial cells occurred (Section 6.4.2), presumably cellular aggregates. The sustained rise in the ATP concentration per total cell until 80 hrs after the start of the experiment can also be explained by cellular aggregation.

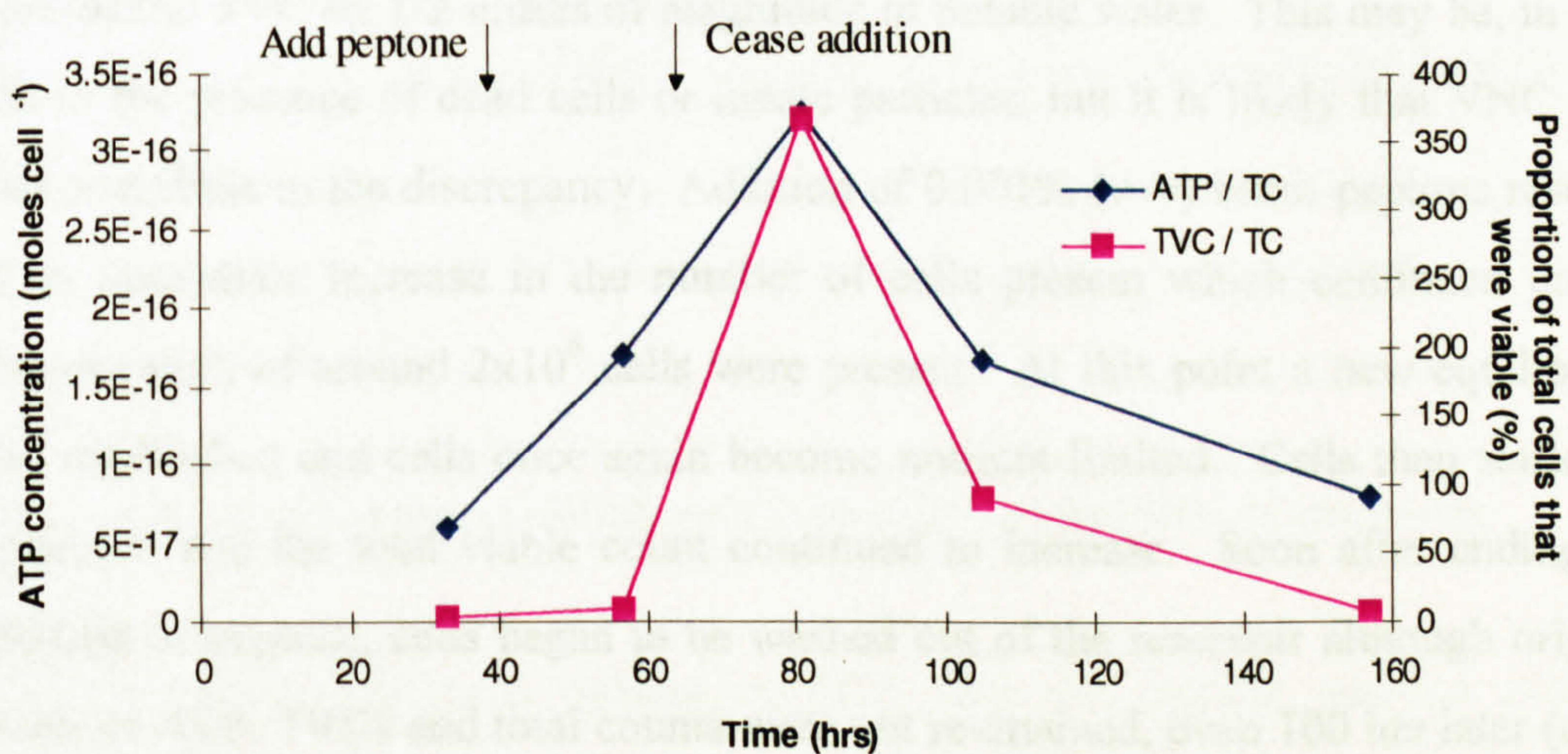


Figure 6.3 Responses of the total intracellular ATP concentration and proportion of planktonic cells that were viable following peptone addition to reservoir 2 of the continuous flow model. The data presented in Fig. 6.2 have been recalculated to show the average intracellular ATP concentration per particle of size 0.75-2 μm ESD (ATP/TC) and the proportion of these particles that gave rise to colonies on agar (TVC/TC). The TVC/TC value was greater than 100% at one point, which can be explained by the dispersal of aggregates by spread plating but not during *CellFacts* analysis (see text).

None of the three measures of bacterial occurrence/activity returned to its original level, even 100 hrs after ceasing peptone addition. The kinetics of washout of cells from the system would be affected by the retention time of water in the system and bacterial growth in the reservoir. Two factors could have prevented a return to the original cell count in the reservoir:-

(a) growth in the absence of exogenous nutrients at a rate greater than the retention time of water in the vessel (7.9 hrs), and/or

(b) growth on the glass walls of the vessel.

From the subsequent experiments on biofilm formation on glass, it seems certain that biofilms would have developed on the vessel walls and cell counts would not have been expected to have returned to initial values. However, the system was not monitored until the cell counts had stopped reducing, so this was not confirmed.

Therefore, it is clear that the total particle count within the bacterial size range exceeds the TVC by 1-2 orders of magnitude in potable water. This may be, in part, due to the presence of dead cells or innate particles, but it is likely that VNC cells also contribute to the discrepancy. Addition of 0.001% (w/v) bacto-peptone resulted in an immediate increase in the number of cells present which continued until a concentration of around 2×10^6 cells were present. At this point a new equilibrium was established and cells once again become nutrient-limited. Cells then started to aggregate and the total viable count continued to increase. Soon after ending the addition of peptone, cells began to be washed out of the reservoir although original levels of ATP, TVCs and total counts were not re-attained, even 100 hrs later (equal to 12.5 residence times in reservoir 2).

6.4.2 Particle size distribution

A closer analysis of the particle size distribution in tap water following peptone addition confirms that aggregates of cells started to appear around 15 hrs after the onset of peptone addition (Fig. 6.4). Most particles smaller than 2 μm ESD were single bacterial cells; larger particles were mainly aggregates of cells.

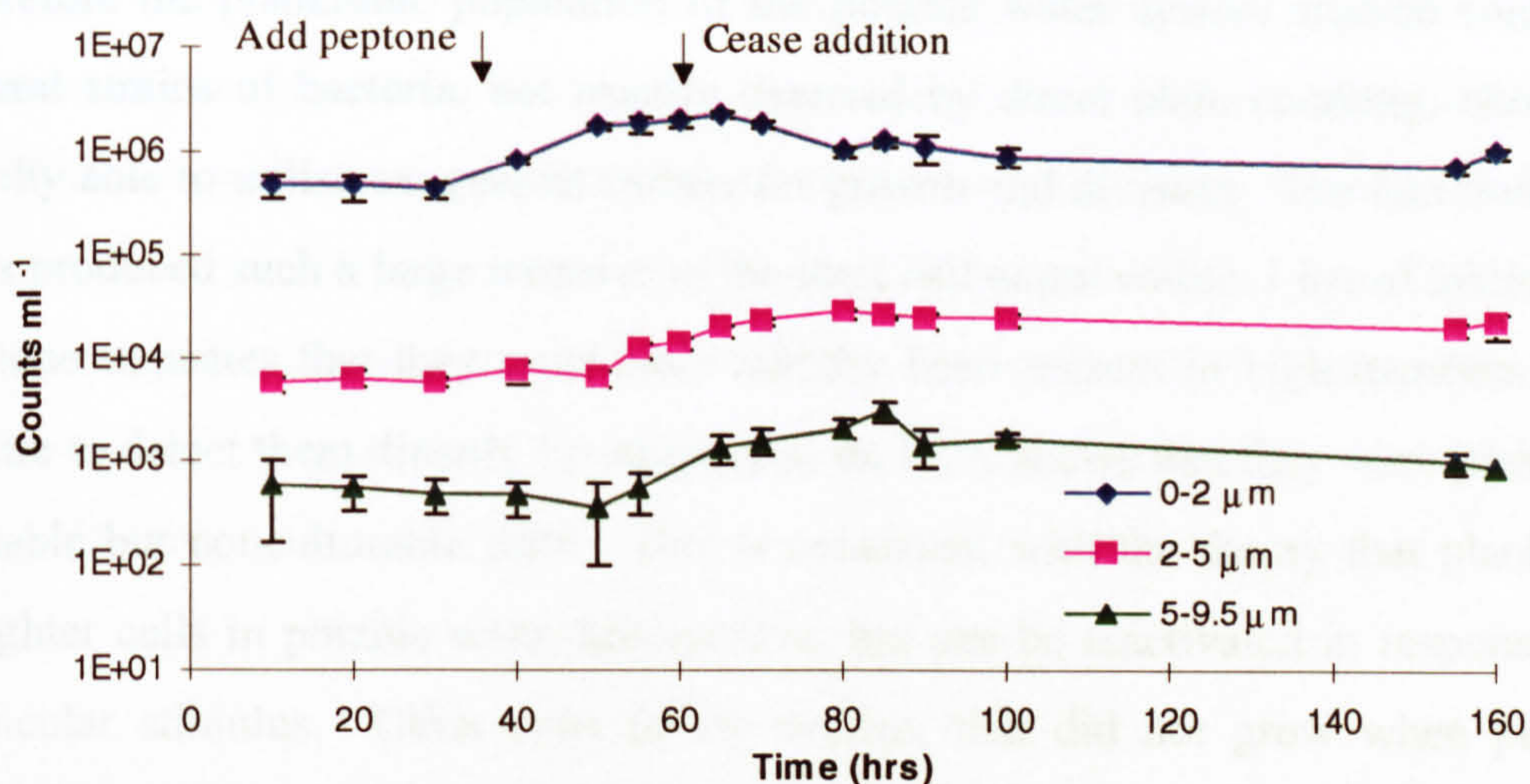


Figure 6.4 Effects of peptone addition to tap water on the particle size distribution. Particle count data shown in Fig. 6.1 were analysed in greater detail. Particles were grouped into three different size ranges, labelled according to the limits of ESDs encompassed and each data point shown represents the mean of the five surrounding points. Standard deviations are shown where they were sufficiently large to be visible.

6.4.3 Effects of carbon addition on the species composition of the water

Analysis of the cell types isolated on PCA after the nutrient addition indicated selection for rapidly-growing *Proteobacteria* had occurred (see Table 3.1). Previous investigations have shown that cultivation of cells from drinking water selectively enriches members of the alpha and gamma subclasses of *Proteobacteria*, often suppressing bacteria belonging to the beta subclass (e.g. Kalmbach *et al.*, 1997b). Selection for bacteria belonging to each of these three subclasses occurred (e.g. *Sphingomonas* sp. from the alpha subclass, *Comamonas acidovorans* from the beta subclass and *Pseudomonas fluorescens* from the gamma subclass) but the relative amounts of each were not quantified. The concentration of other bacteria in the water did not increase noticeably.

Therefore the planktonic population of the potable water system studied contained several strains of bacteria, not usually detected by direct plate counting, that were rapidly able to utilise exogenous carbon for growth and division. The fact that these cells produced such a large increase in the total cell count within 3 hrs of addition of peptone indicates that they must have initially been present in high numbers. The failure to detect them directly by cultivation on PCA shows that they were present in a viable but nonculturable state. This is consistent with the theory that planktonic daughter cells in potable water are inactive, but can be reactivated in response to a particular stimulus. Other cells in the system, that did not grow when peptone became available, presumably require a different stimulus to initiate maturation.

6.5 The accumulation of biofilm

The continuous flow model was employed to monitor the accumulation of biofilm and the influence of attached growth on planktonic cell numbers and activity. The model was sterilised before use. The quantity and metabolic activity of cells attached to glass were determined microscopically after fluorescent staining. Total planktonic cells were enumerated using the *CellFacts* particle analyser and the TVC by plating on solid R2A medium and counting colonies produced after incubation for 5 days at 30°C. Cellular activity was estimated by measuring the total intracellular ATP concentration in the aqueous phase. Staining planktonic cells with CTC would have been useful to allow a direct comparison between the activity of attached and planktonic cells. A simple protocol for the assessment of the CTC-staining capacity of planktonic cells, based on filtration of samples and staining of the filters has been developed (Pyle *et al.*, 1995), but this analysis was omitted here.

6.5.1 Tap water quality

The physical and chemical stability of the tap water supplying the continuous flow was monitored weekly over the course of the experiment (Table 6.1). Chlorine and TOC were consistently low throughout. The pH remained stable but the temperature increased slightly in the seventh week of the experiment and subsequently remained

relatively high. During the time that the continuous flow model was employed for the assessment of biofilm formation, the concentration of heterotrophic bacteria in the final water leaving Strensham distribution plant, which serves Warwick University, was stable and remained below 10 CFU ml⁻¹ (see Appendix, Fig a1). The concentration of unicellular eukaryotes in the same water increased sharply at the beginning of August 1997, around 8-9 weeks after beginning the laboratory (Appendix, Fig. a2).

Time (days)	0	7	14	21	28	35	42
pH	7.22	7.76	7.64	7.78	7.58	8.05	7.83
Temperature (°C)	16.5	17.5	17.5	16.7	16.0	16.8	19.1
TOC (mg l ⁻¹)	<5	<5	<5	<5	<5	<5	<5
Chlorine (mg l ⁻¹)	0.02	0.03	0.03	0.04	0.04	0.02	0.03

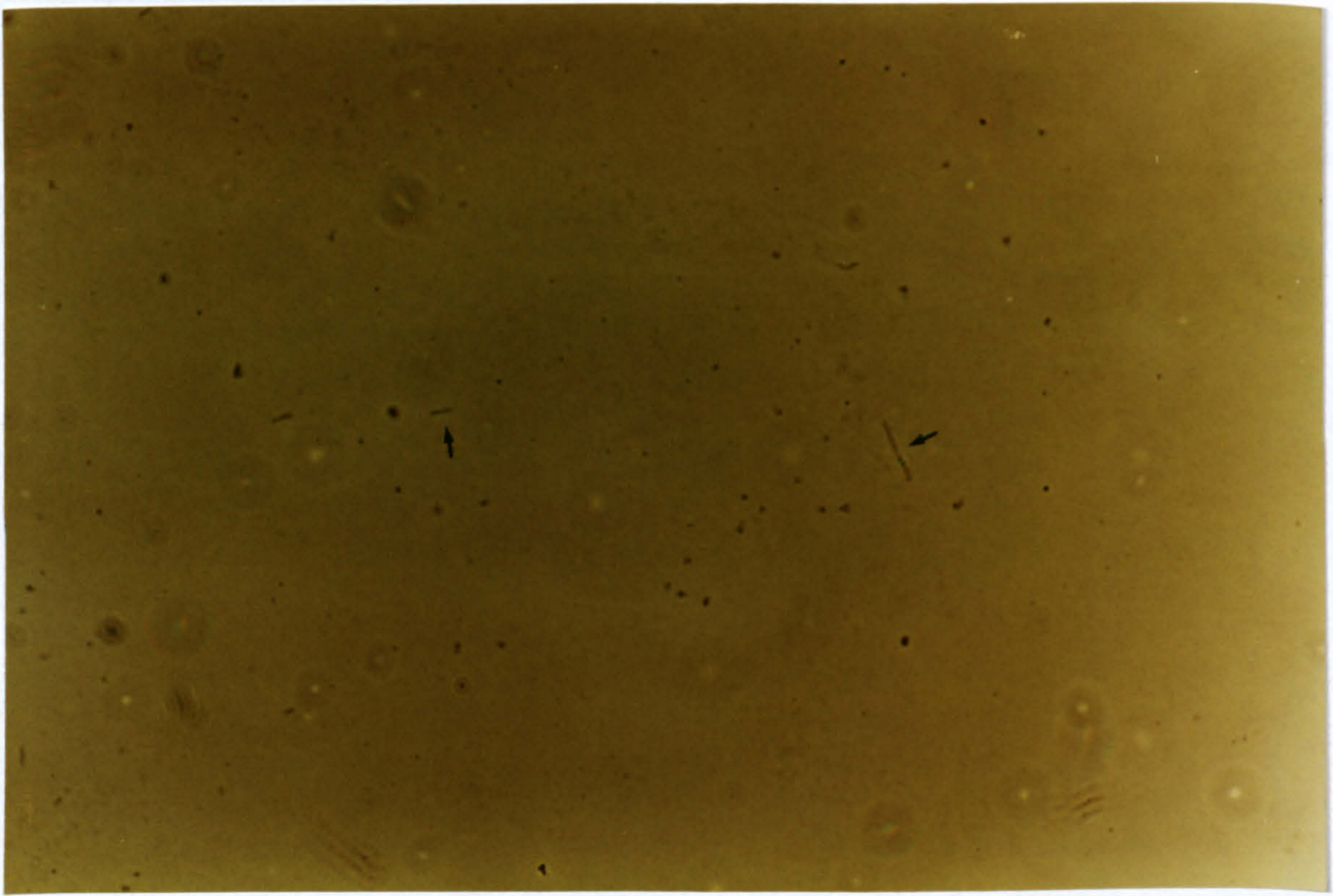
Time (days)	49	56	63	70	77	84
pH	7.71	7.54	7.65	7.61	7.73	7.68
Temperature (°C)	19.4	20.0	19.1	21.1	21.9	20.8
TOC (mg l ⁻¹)	<5	N/D	N/D	N/D	N/D	N/D
Chlorine (mg l ⁻¹)	0.01	0.04	0.02	0.02	0.03	0.02

Table 6.1 Water quality through biofilm development in the continuous flow model. Several physical and chemical properties of the tap water serving the continuous flow model were measured weekly on the same day that the microbiological tests were performed. N/D = not determined.

6.5.2 Total and active attached cell numbers at glass surfaces

Glass coverslips removed from the continuous flow model were stained with CTC to detect and quantify attached cells possessing an active respiratory chain. Counterstaining with DAPI enabled quantification of the total cell count. Three coverslips were prepared at each time point and these were analysed under a fluorescence microscope. A minimum of 10 fields were randomly selected for

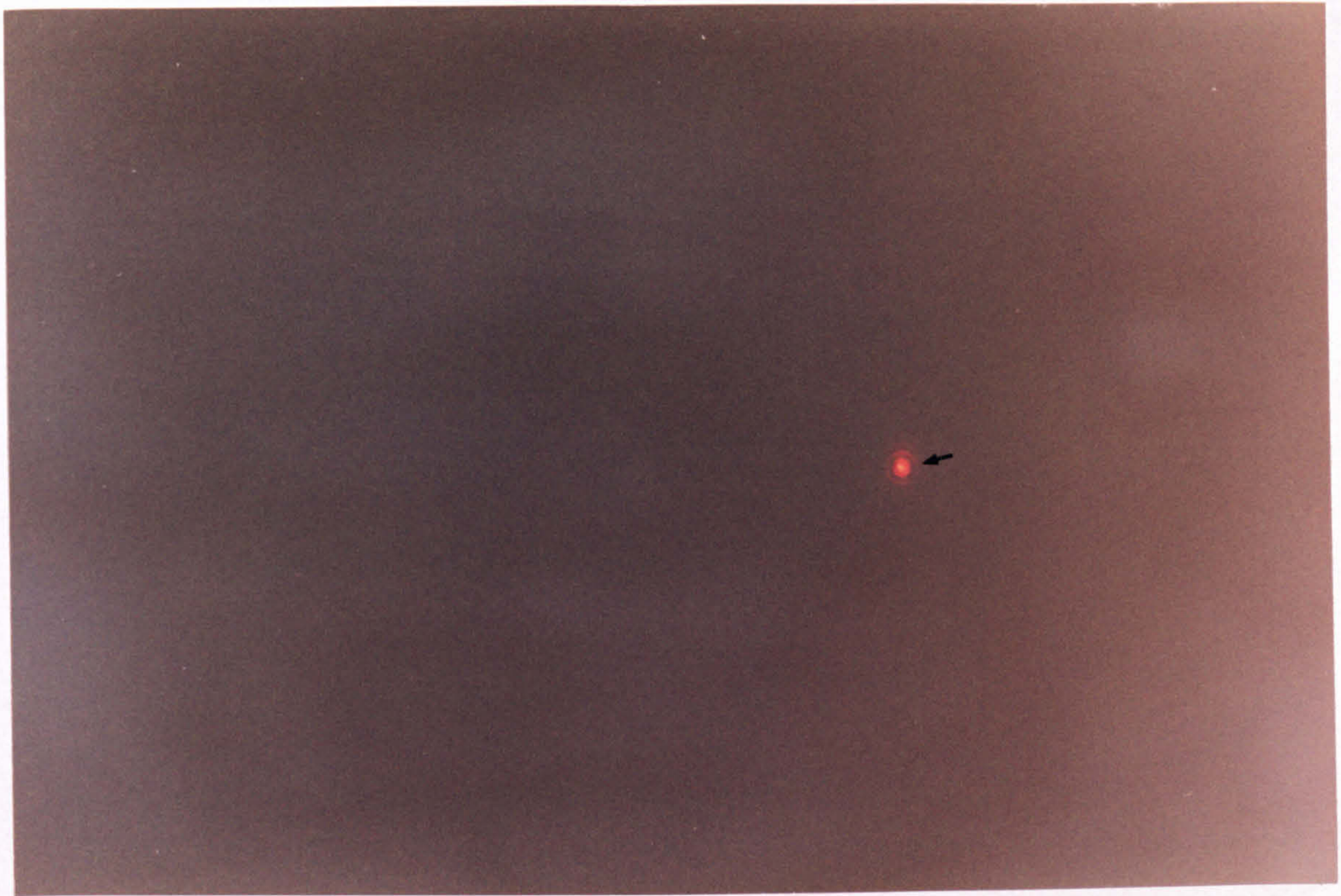
enumeration using both DAPI- and CTC-specific filter sets. If the total cell count in these 10 fields was below 200 cells then more fields were counted until the figure of 200 cells was reached. Images were captured using a CCD, relayed to an Apple Macintosh computer and quantified by image analysis. Examples of images obtained after staining are shown in Figs 6.5 and 6.6.



(a)



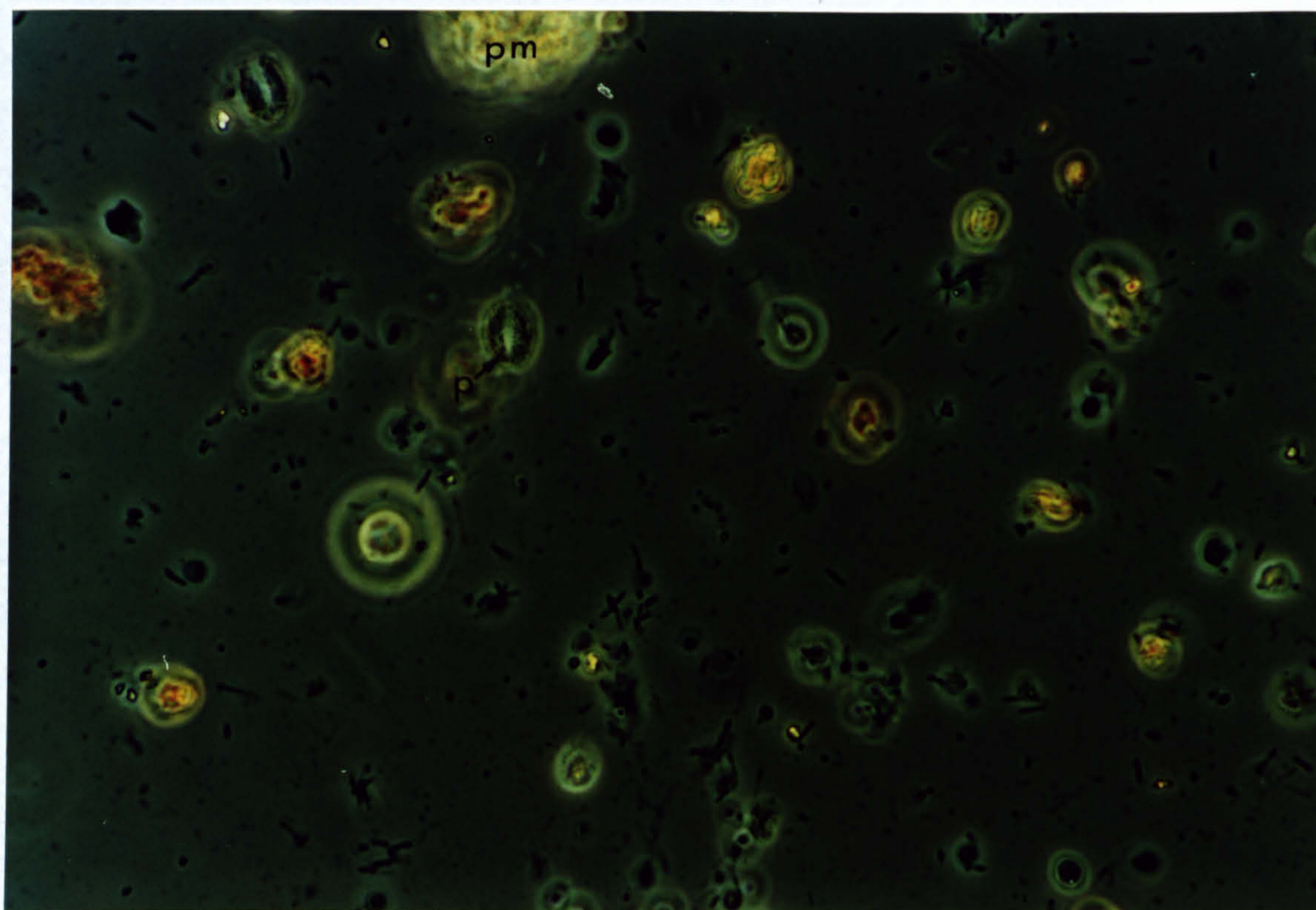
(b)



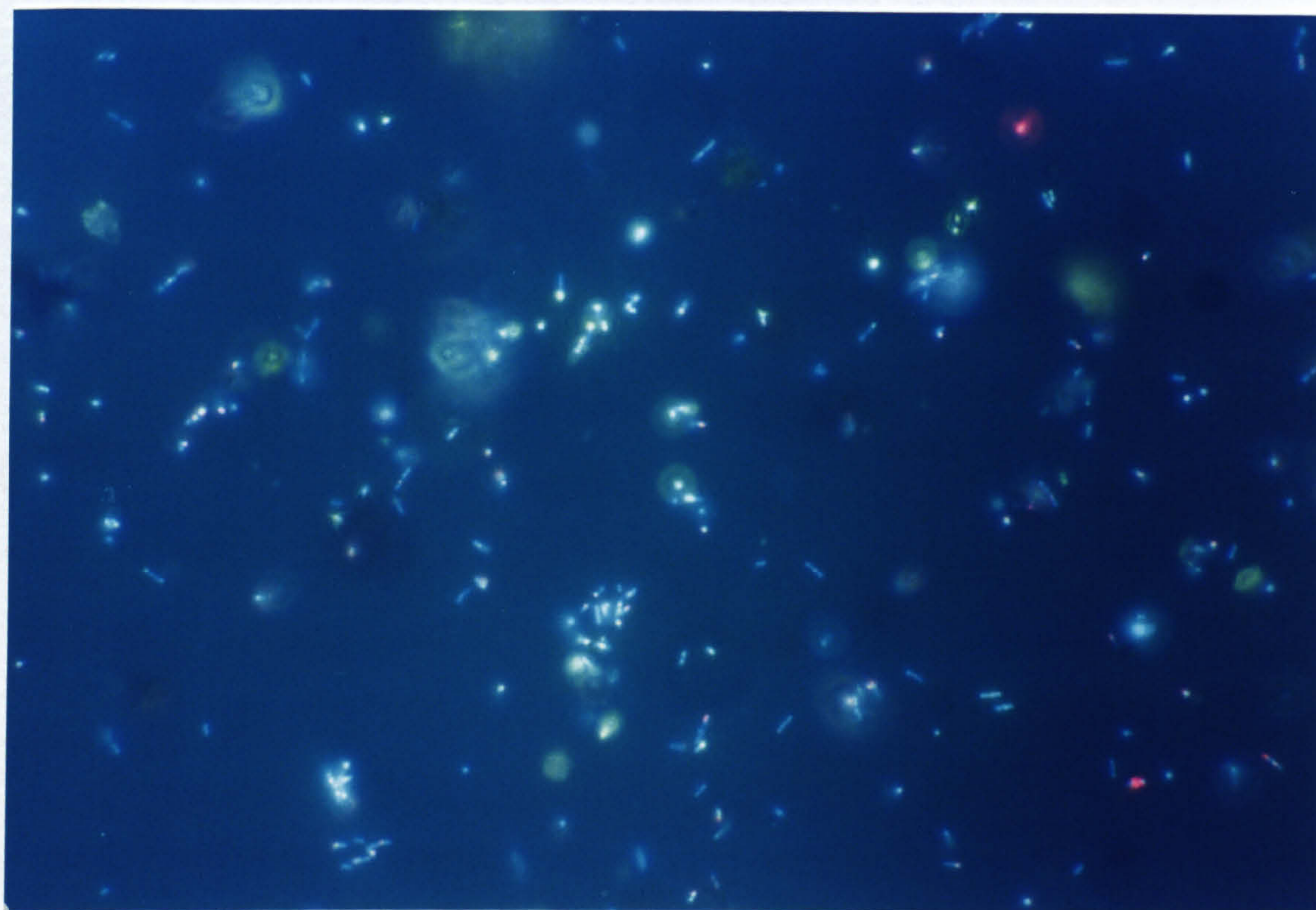
(c)



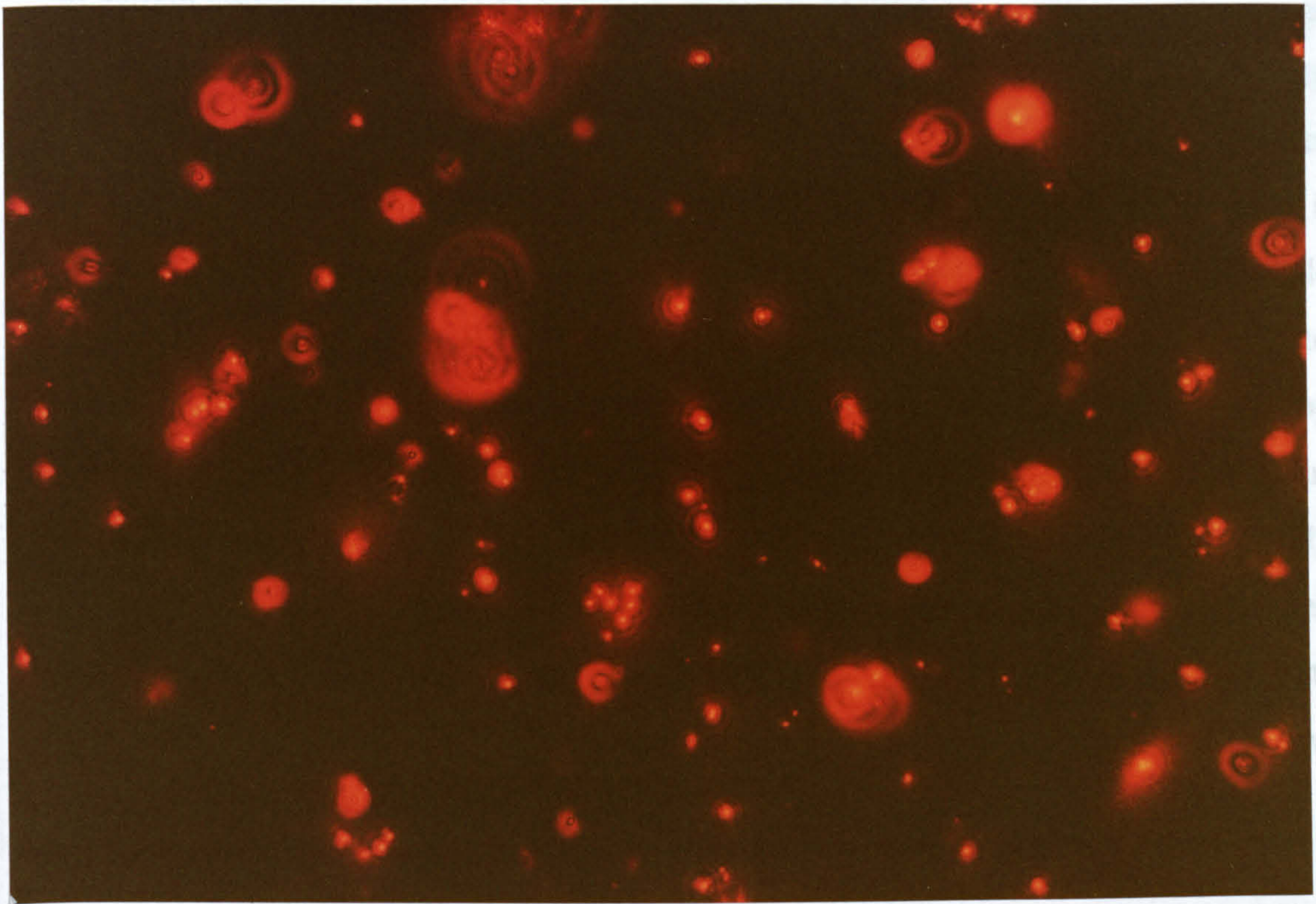
Figure 6.5 Phase contrast and fluorescence micrographs of glass surfaces after one week in the continuous flow model. A glass coverslip was removed from the model after one week, stained with CTC and DAPI, then examined microscopically. (a) Phase contrast image showing a relatively clean surface with only a few cells present. The arrows indicate some of the bacterial cells in the image. (b) The same field showing blue DAPI-stained cells (arrows). (c) The same field again, this time illuminated to highlight CTC-formazan crystals. A single crystal was present (arrow). Bar = 10 μm .



(a)



(b)

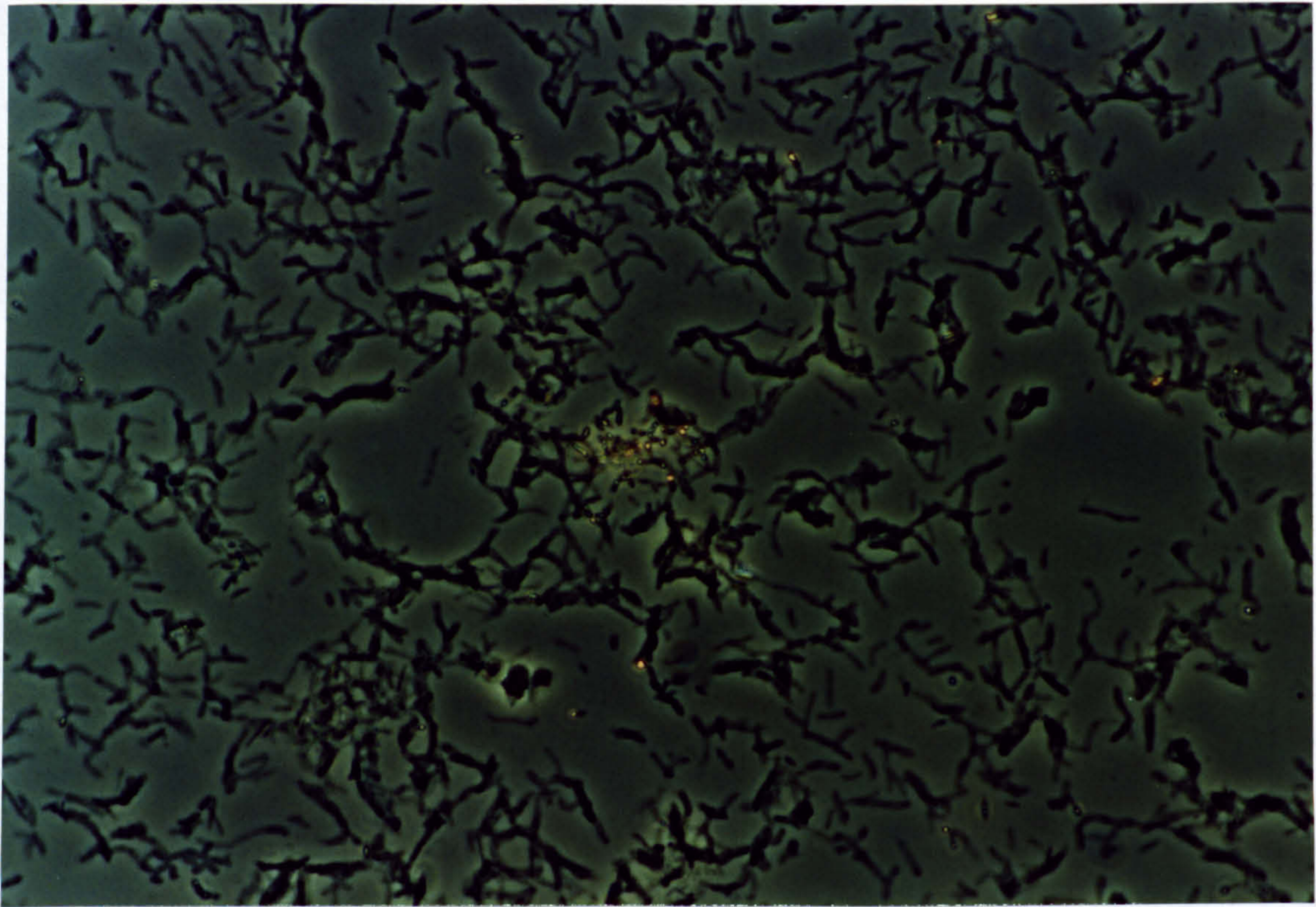


Problems were also encountered when examining CTC-stained sections. Sections stained with CTC and DAPI (Fig. 6.6c) showed the majority of formazan crystals associated with the bacteria. Negative controls, performed by incubating coverslips in CTC only for 15 min prior to CTC staining, were devoid of formazan crystals, indicating that CTC reduction only occurred in the presence of metabolically active bacteria. Therefore CTC was considered to be a good indicator of bacterial respiratory chain activity.

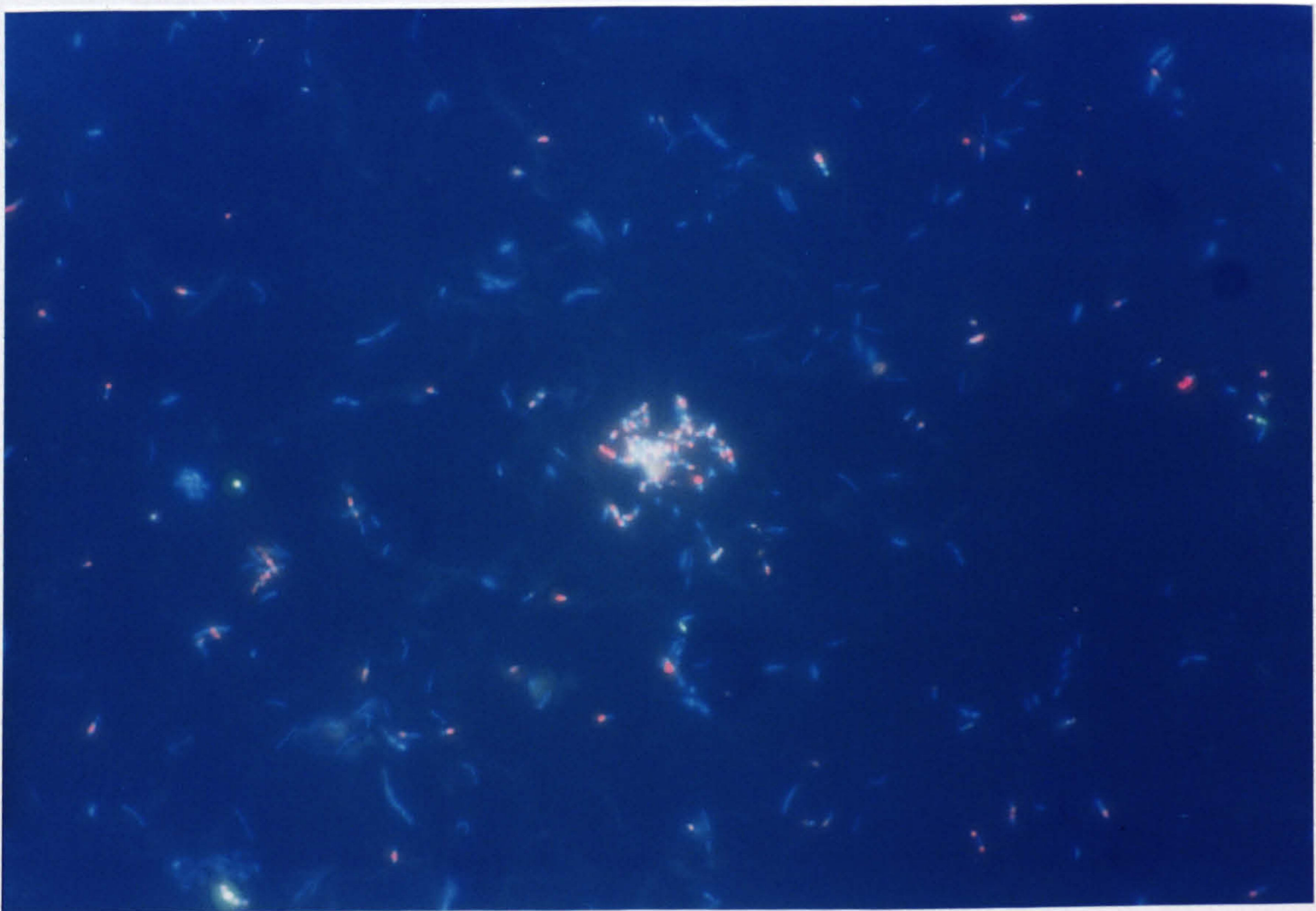
Figure 6.6 Phase contrast and fluorescence micrographs of glass surfaces after eight weeks in the continuous flow model. A glass coverslip was removed from the model after eight weeks, stained with CTC and DAPI, then examined microscopically. The same field is shown under phase contrast (a), DAPI (b) and CTC (c) illumination. In addition to bacteria, protozoa (p) and pigmented material (pm) were observed. Bar = 10 μ m.

In certain situations both DAPI and CTC appeared to give misleading results. Some areas that were covered with a network of actinomycetes did not stain extensively using DAPI (Fig. 6.7). Occasionally single cells observed by phase contrast did not fluoresce after DAPI staining. From the structures observed by phase contrast it seems unlikely that the cells had lysed and lost their DNA. A more probable explanation for the observed phenomenon was that some cells were impermeable to DAPI stain. To determine the extent of underestimation of the total cell count by DAPI, cells in ten microscopic fields of each coverslip removed from the continuous flow model over the first 70 days of biofilm accumulation were counted manually under phase contrast illumination. DAPI counts of identical fields were determined using image analysis. No significant difference was found between the two data sets (data not shown) indicating that although care should be taken when quantifying bacteria with DAPI, in this case a sufficiently accurate total cell count was obtained.

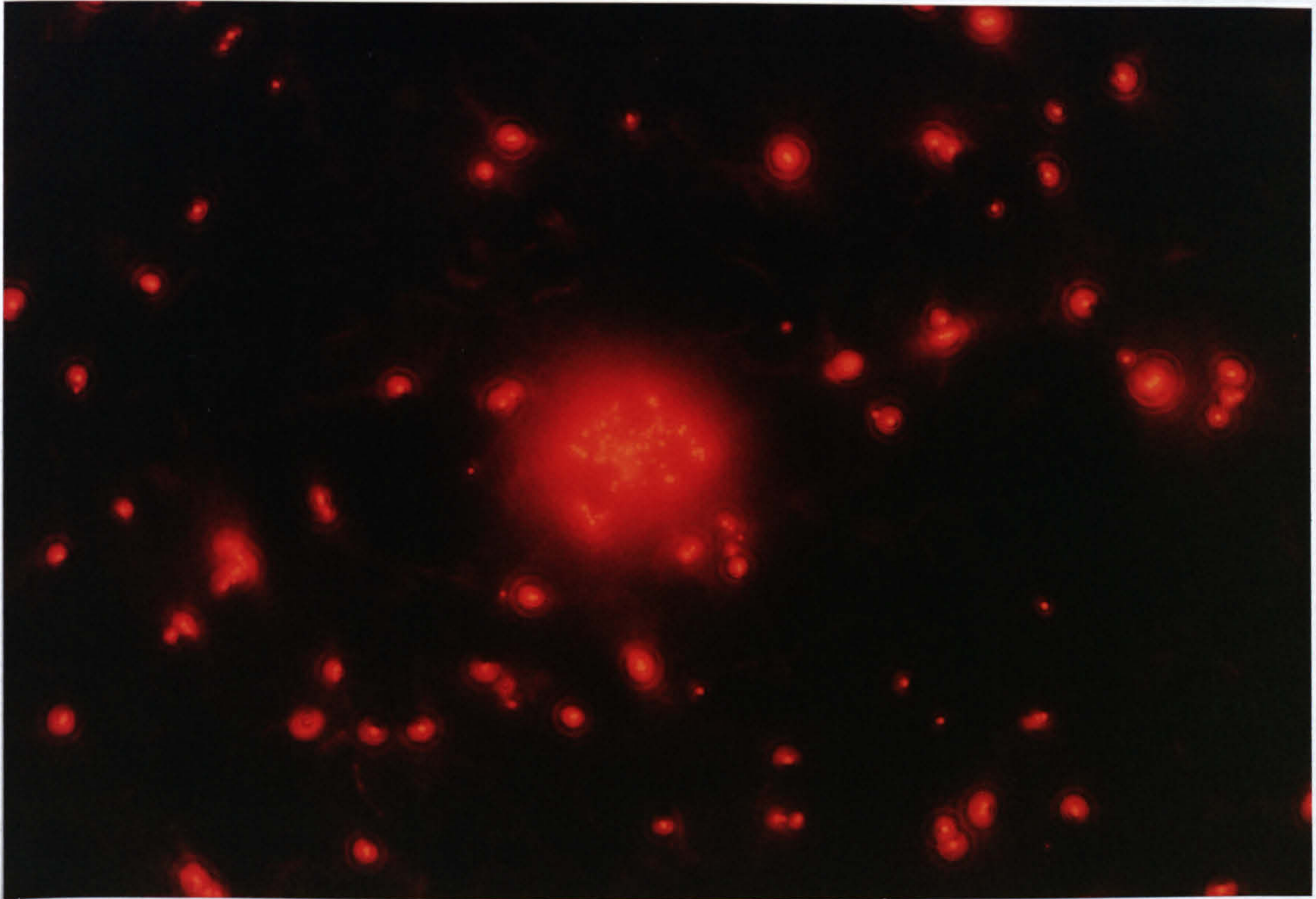
Problems were also encountered when employing CTC stain. Extracellular staining (see Sections 4.3.1.3 and 4.3.2.2) was sometimes observed (Fig. 6.8). However, the majority of formazan crystals observed were intracellular. Negative controls, performed by incubating coverslips in 1% (v/v) glutaraldehyde for 15 mins prior to CTC staining, were devoid of formazan crystals, indicating that CTC reduction only occurred in the presence of metabolically active bacteria. Therefore CTC was considered to be a good indicator of bacterial respiratory chain activity.



(a)



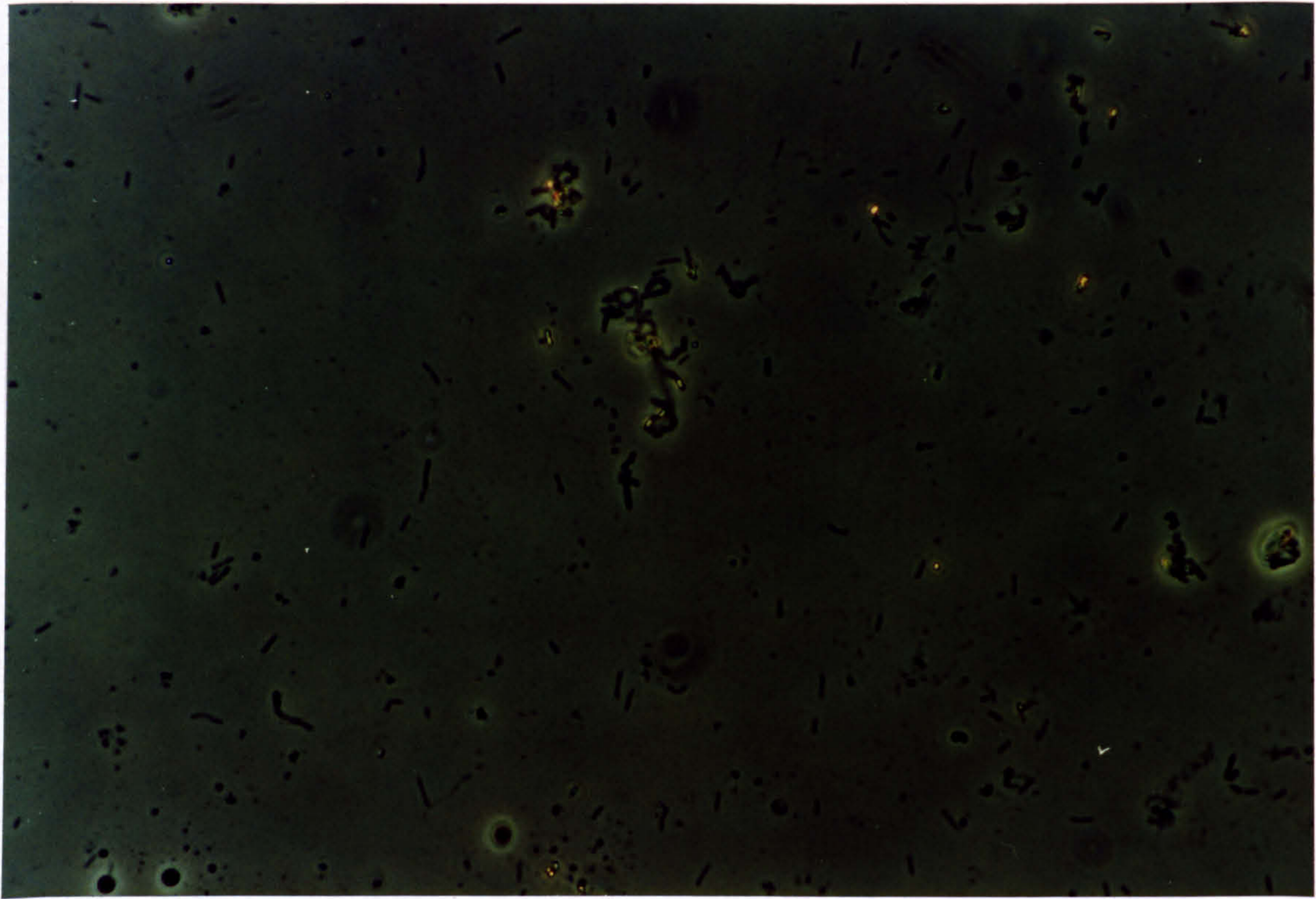
(b)



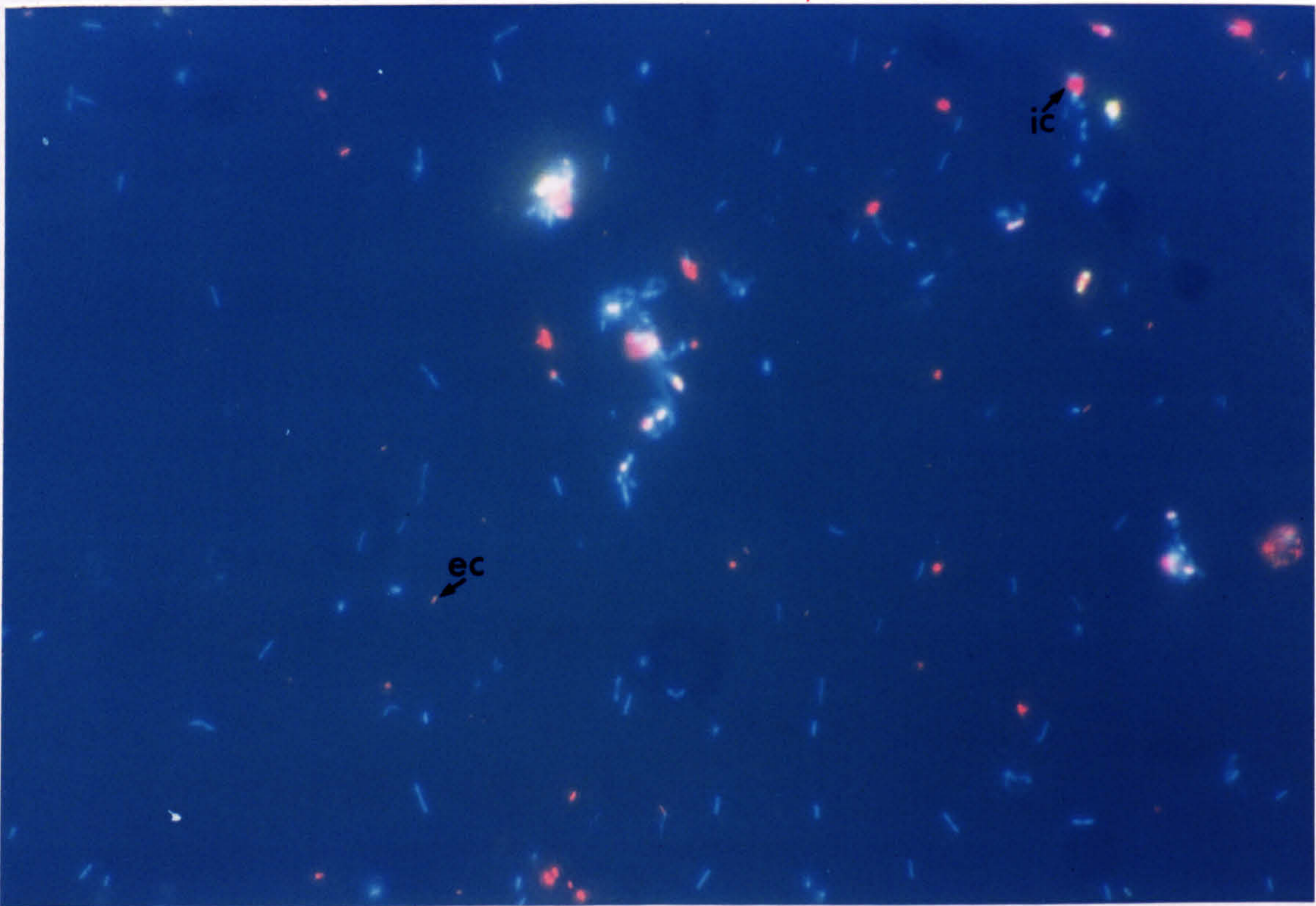
(c)



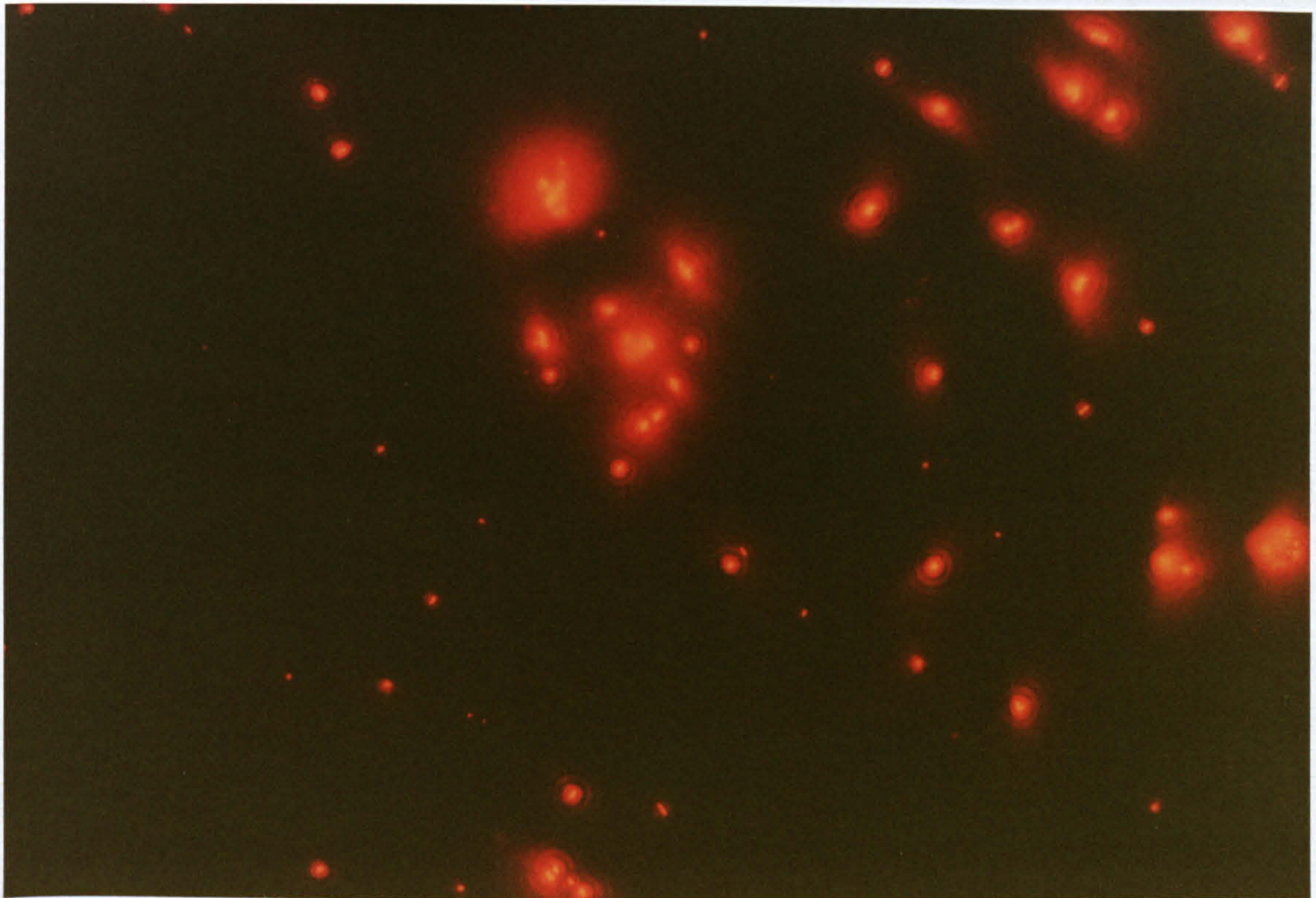
Figure 6.7 A potential drawback of DAPI stain: phase contrast and fluorescence micrographs of glass surfaces after eight weeks in the continuous flow model. A glass coverslip was removed from the model after eight weeks, stained with CTC and DAPI, then examined microscopically. (a) The primary matrix viewed by phase contrast consists of branched rod-shaped bacteria, probably belonging to the actinomycete group. (b) The same field viewed using the DAPI-specific filter set. The extensive network of rod-shaped cells cannot be seen. (c) CTC image of the same field. In addition to CTC-formazan crystals some non-crystalline red autofluorescence can be seen. Bar = 10 μm .



(a)



(b)



(c)



Figure 6.8 A potential drawback of CTC stain: phase contrast and fluorescence micrographs of glass surfaces after eight weeks in the continuous flow model. A glass coverslip was removed from the continuous flow model after eight weeks, stained with CTC, then counterstained with DAPI. One field is shown under (a) phase contrast, (b) DAPI-specific fluorescent and (c) CTC-specific fluorescent illumination. Several large intracellular CTC crystals are visible (ic) but smaller extracellular crystals can also be seen (ec). Bar = 10 μm .

Attached cell numbers and activities are shown in Fig. 6.9. The proportion of cells that were actively respiring was expressed as the ratio of CTC counts to DAPI counts.

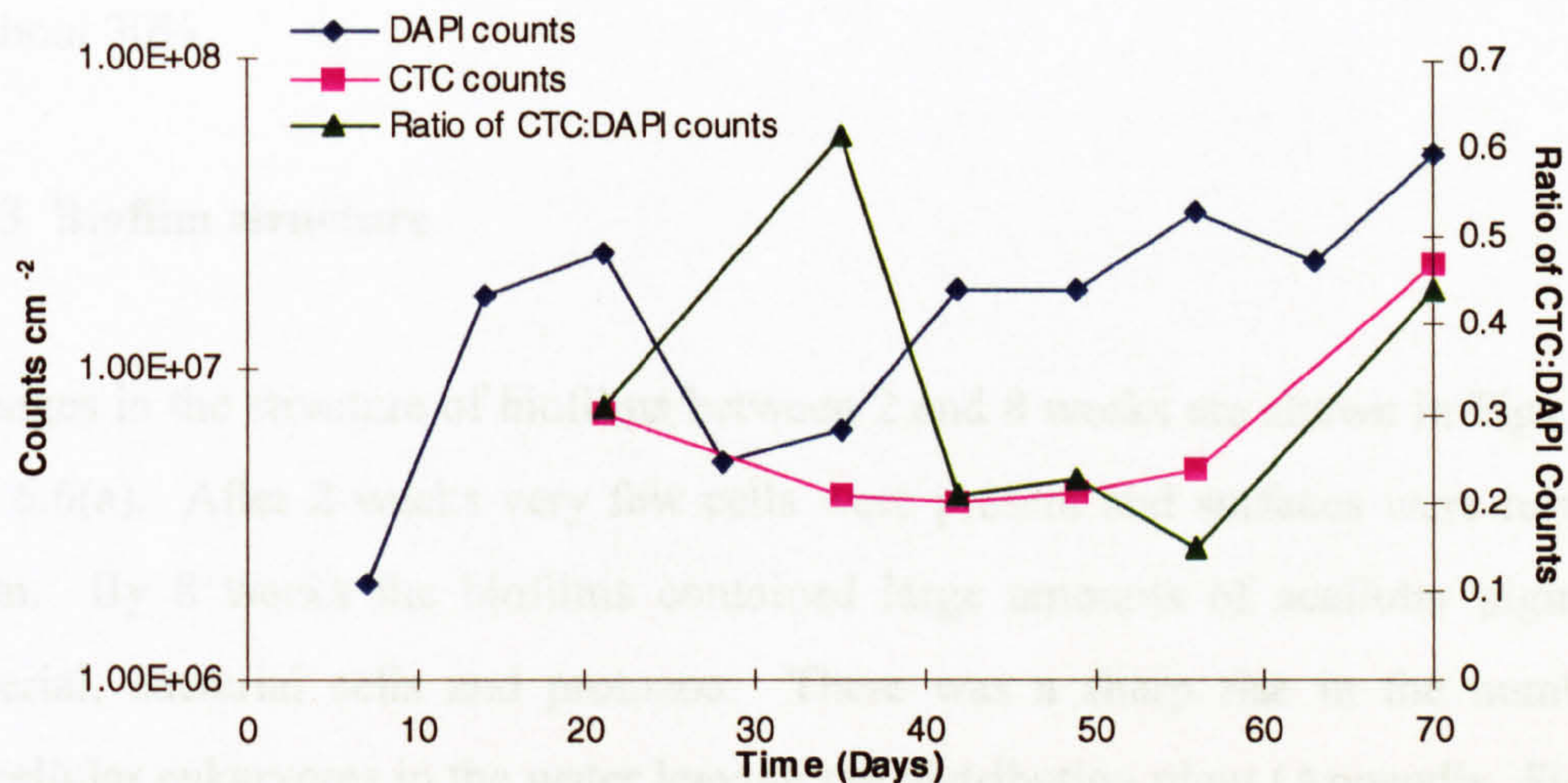


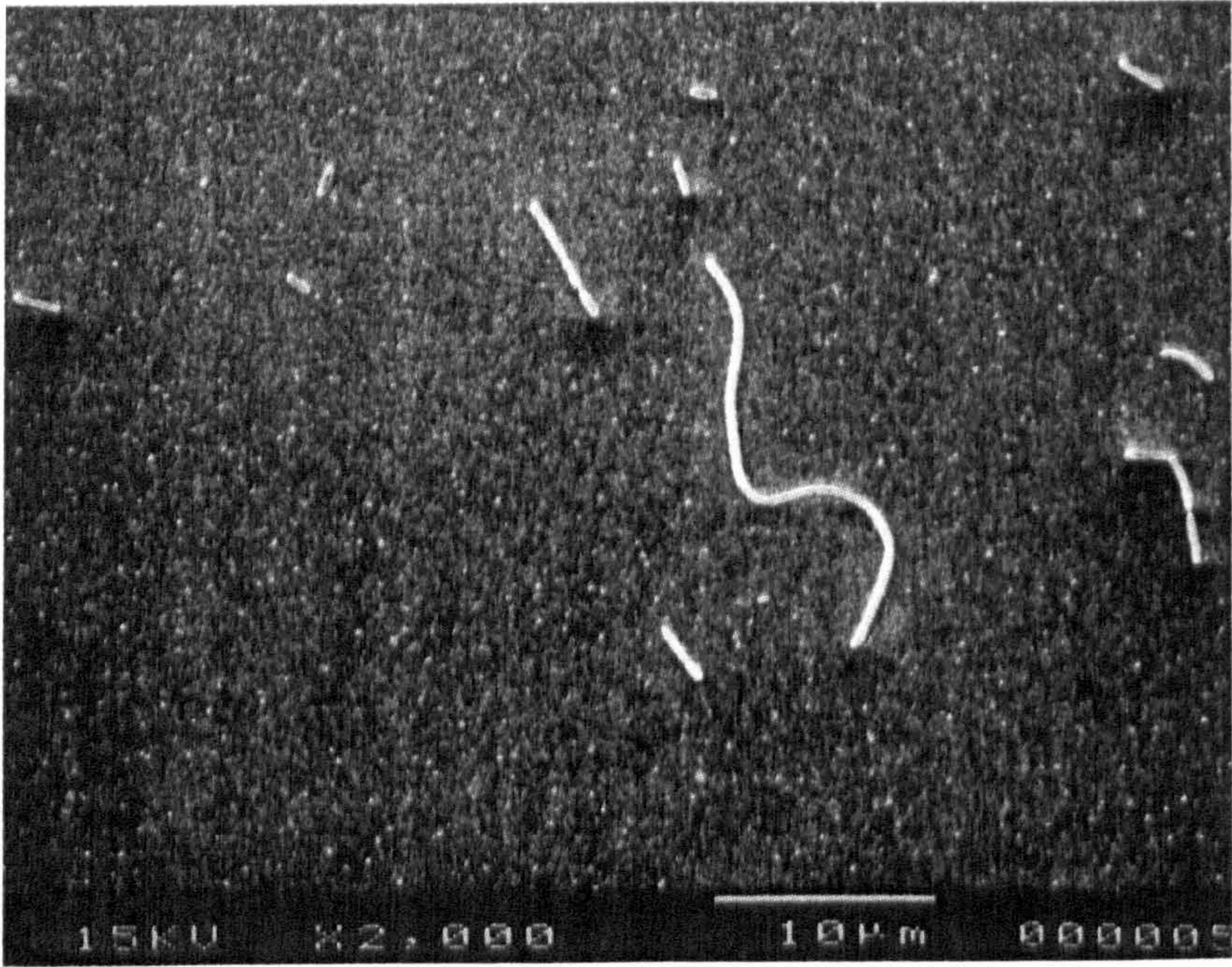
Figure 6.9 Total and active cell counts at glass surfaces during biofilm development in the continuous flow model. Counts were determined microscopically in conjunction with two fluorescent dyes: CTC which stains actively respiring cells red and DAPI which fluoresces blue on binding DNA and therefore stains all cells present. The proportion of cells that were actively respiring is indicated as the CTC:DAPI ratio. Note that no data were obtained from CTC staining until 21 days after the start of the experiment.

The total number of sessile cells increased in an approximately linear manner over the ten-week period of biofilm accumulation. No CTC staining was observed until 21 days after the start of the experiment. Until this time PBS was used to rinse coverslips prior to staining. Phosphates inhibit CTC staining (Smith & McFeters, 1996), so after 21 days saline (0.85% w/v NaCl) was used for all rinses. The data obtained from using PBS for rinses were discarded. After 21 days there was no clear trend in the proportion of cells that were active. A recent study in Berlin using a similar approach for developing and staining biofilms demonstrated that nearly 90%

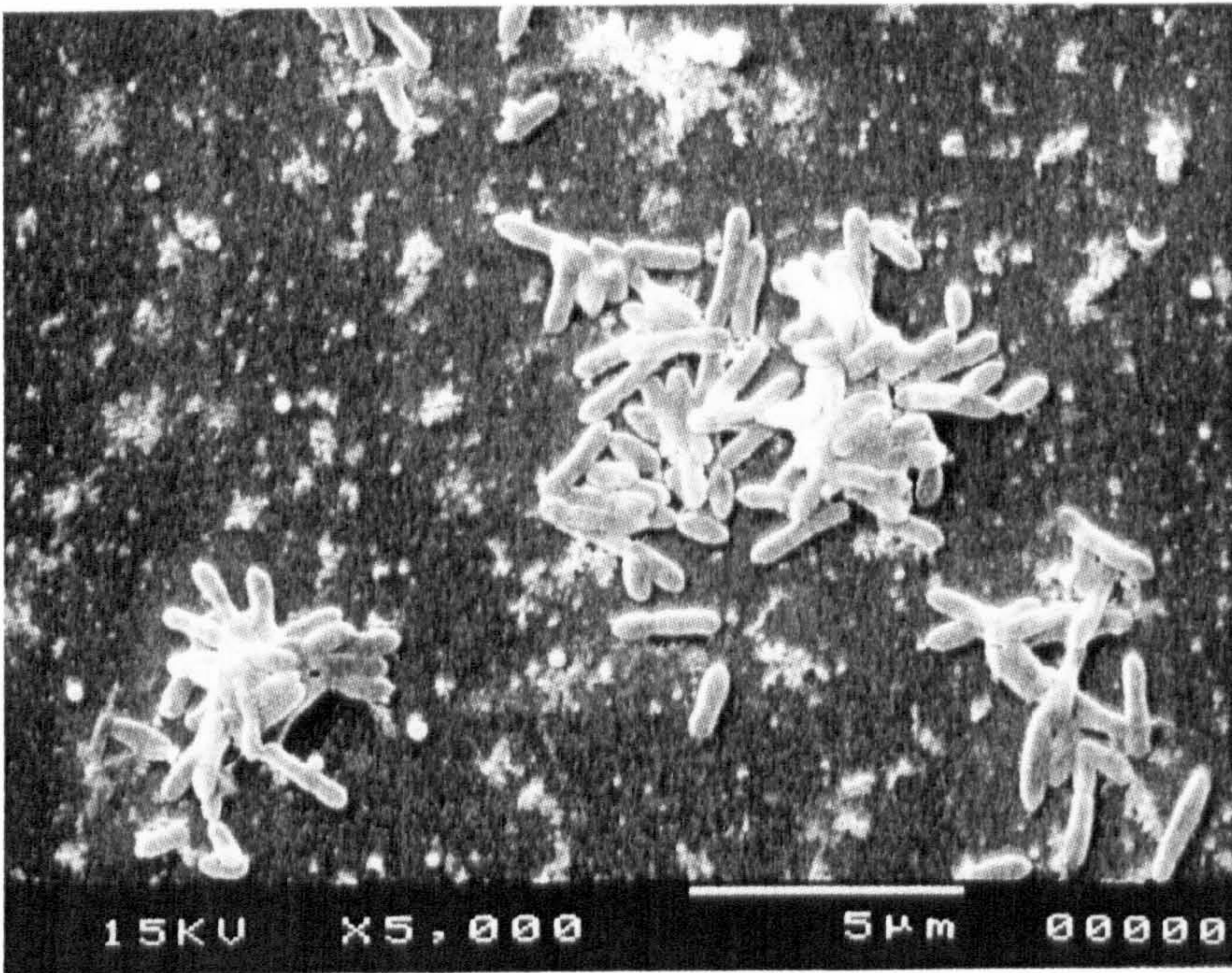
of the cells that attached within the first 2 days of biofilm accumulation were respirometrically active and this figure dropped to around 30% after 21 days and then remained fairly stable (Kalmbach *et al.*, 1997a). Extensive heterogeneity of biofilms produced in this study resulted in large errors of both DAPI and CTC counts. The observed ratio of CTC counts to DAPI counts is not inconsistent with a stable figure of about 30%.

6.5.3 Biofilm structure

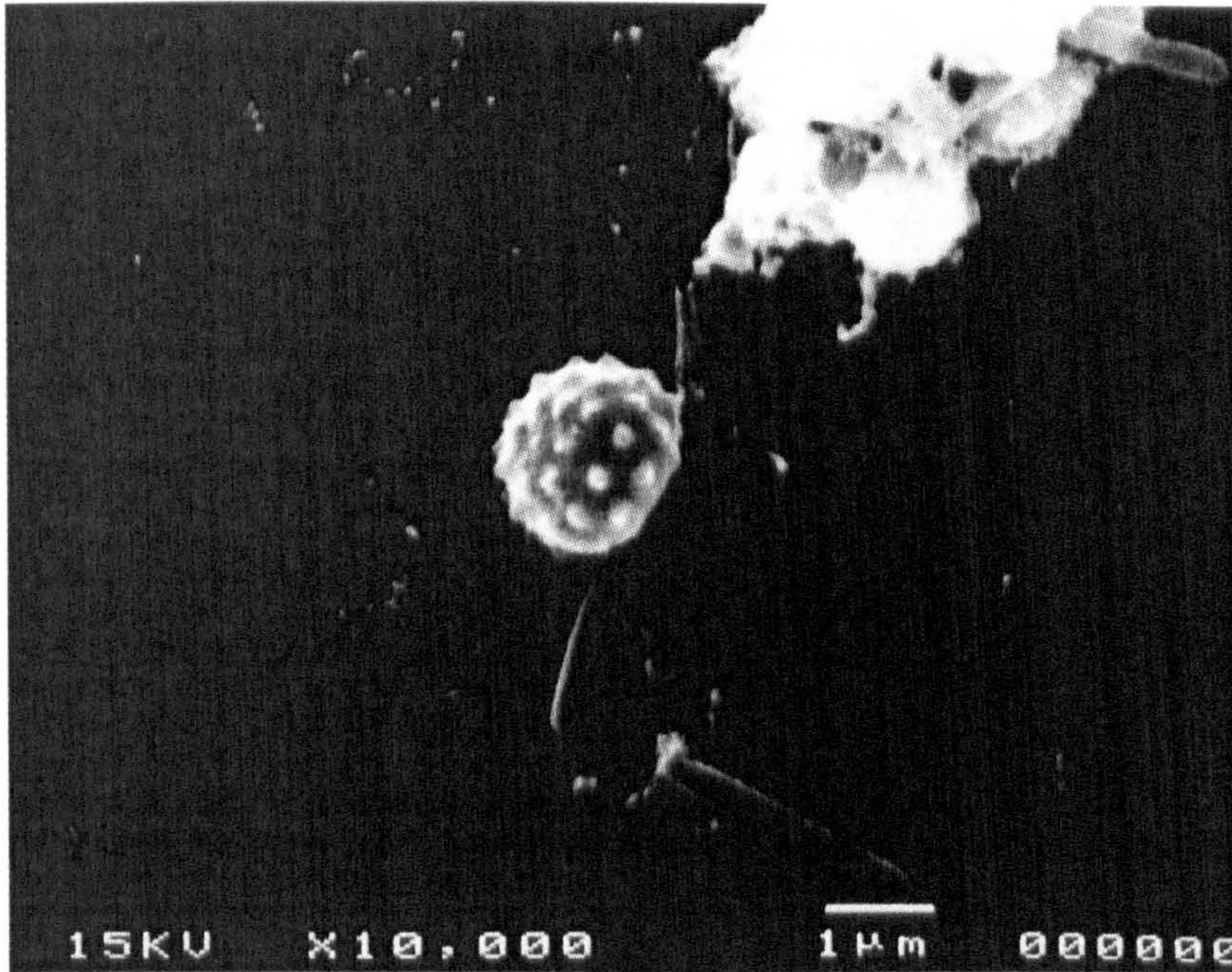
Changes in the structure of biofilms between 2 and 8 weeks are shown in Figs 6.5(a) and 6.6(a). After 2 weeks very few cells were present and surfaces were relatively clean. By 8 weeks the biofilms contained large amounts of acellular pigmented material, bacterial cells and protozoa. There was a sharp rise in the number of unicellular eukaryotes in the water leaving the distribution plant (Appendix, Fig. a2), but this did not occur until after the 8 week sample had been taken. Therefore, the background concentration of eukaryotes was sufficient to allow incorporation of material derived from these organisms into the biofilm. A higher power analysis of the surface by SEM showed that some bacterial division at the surface occurred after 2 weeks (Fig. 6.10(a)) and after 10 weeks microcolonies were present (Fig. 6.10(b)). Most of the cells observed by SEM were regular straight or flexible rods, although one unusual cell type was found (Fig. 6.10(c)). This was coccoid with multiple appendages and may have been a prosthecate bacterium or a spore state.



(a)



(b)



(c)

Figure 6.10 Scanning electron micrographs (SEMs) showing the structure of tap water biofilms developed in the continuous flow model. Glass coverslips were removed from the model after 14 days (a) and 70 days (b). Most bacteria observed were regular rods, often in the process of binary fission. After 70 days microcolonies and acellular biological material were abundant. One unusual coccoid cell type with multiple appendages was observed in the biofilm after 63 days (c), but was not identified.

6.5.4 Effects of attached growth on the planktonic cell population

Samples of the planktonic cell population were taken each week from ports 1 and 2 of the continuous flow model during the course of biofilm accumulation. Only the water taken from port 2 had flowed through the series of biofilm development vessels so comparison of the samples from each port enabled assessment of the effect of biofilm development on the planktonic microflora. Planktonic cells were enumerated by *CellFacts* particle counting and by TVC determination on R2A. Cellular activity was assessed by measurement of the total intracellular ATP concentration in the aqueous phase. The degree of cellular aggregation was monitored by analysis of particle size distribution data obtained using *CellFacts* and the quantities of each distinct colony type formed on R2A were noted to provide an analysis of the effect of biofilms on individual strains of bacteria.

6.5.4.1 Particle counts and size distribution

A small effect of biofilms on the particle size distribution in tap water was perceived (Fig. 6. 11), but the differences observed between particle counts at the two sample ports were not statistically significant in any of the size ranges analysed (data not shown). Particle counts were close to the limit of sensitivity of the analyser (about 1×10^5 total counts ml^{-1}) and the confidence ranges of the data sets were relatively large. To improve accuracy of the assay three independent samples were taken from each port at each time point and these were analysed in triplicate. Background noise was reduced by subtracting the mean particle count of six analyses of tap water sterilised by filtration through a membrane of pore diameter $0.2 \mu\text{m}$ from the particle count of each sample.

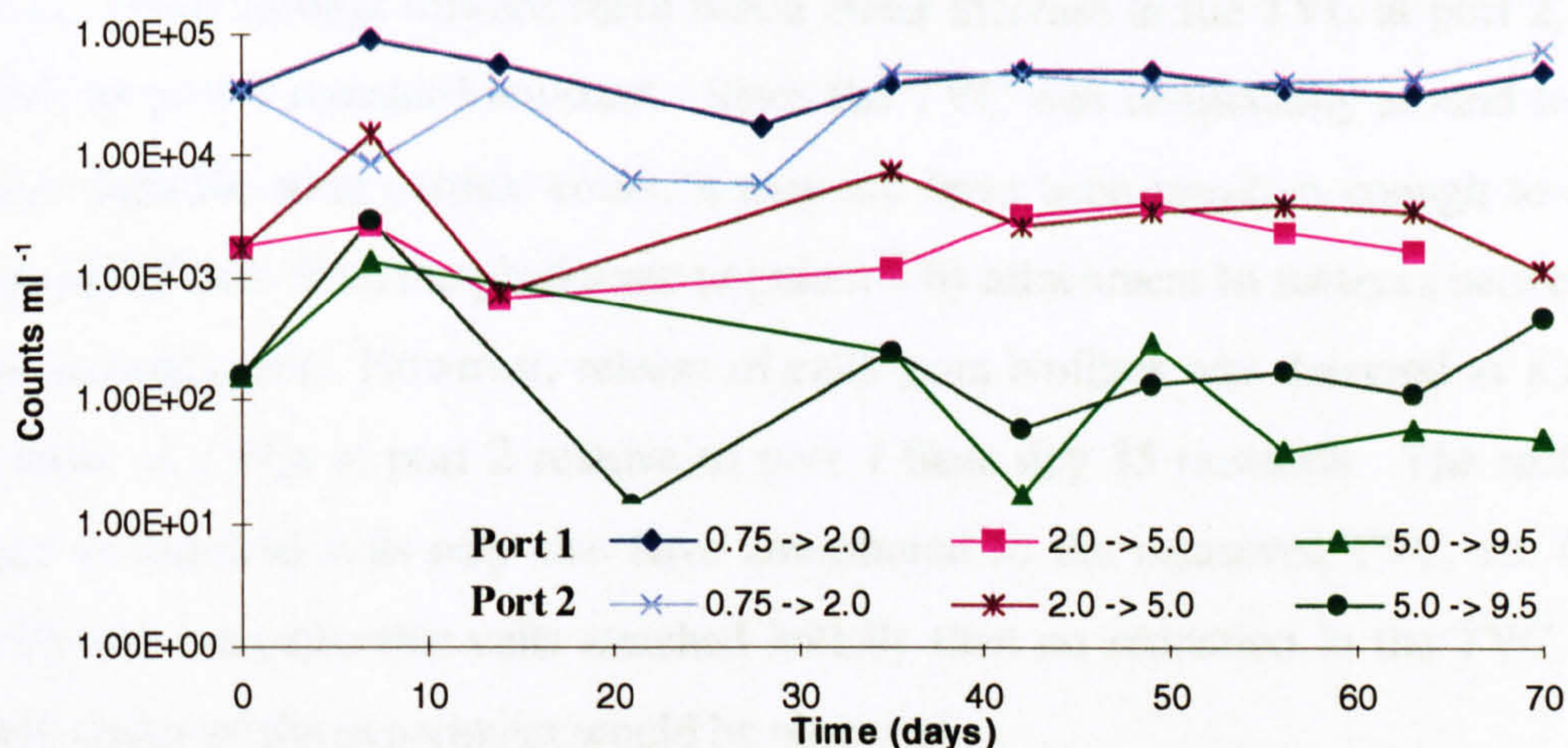


Figure 6.11 Effects of biofilms on the particle size distribution in the aqueous phase. Total particle counts and sizes at the two sample ports in the continuous flow model were measured weekly during the initial stages of biofilm formation, using *CellFacts*. Particles counts were divided into different size ranges. Each line shows fluctuations in counts in one size range (numbers refer to ESD in μm) at one sample port during the course of the experiment. The particle count obtained from filtered tap water was subtracted from each reading.

For the first 28 days of running the continuous flow system, particle counts in the bacterial size range (0.75-2.0 μm ESD) were slightly higher at port 1 than port 2. This situation gradually reversed and between 56-70 days counts in all size ranges were higher at port 2 than port 1. These observations can be explained by the initial removal of bacteria from the water by surface attachment gradually being counterbalanced by release of cells from biofilms into the water. In addition to release of individual cells, sloughing of cellular aggregates accounts for the increased numbers of large particles at port 2 between 56-70 days.

6.5.4.2 Total viable counts and intracellular ATP concentrations

The presence of surfaces did not appear to affect the total viable counts in tap water flowing through the continuous flow model in the first 35 days of the experiment (Fig.

6.12). From 35 days onward there was a linear increase in the TVC at port 2, while TVCs at port 1 remained constant. Since the TVC was consistently around ten-fold lower than the total particle count, it may not have been sensitive enough to detect removal of cells from the planktonic population by attachment to surfaces between the two sample ports. However, release of cells from biofilms was detected as a steady increase in TVCs at port 2 relative to port 1 from day 35 onwards. The metabolic state of bacterial cells may also have contributed to the measured TVC, i.e. if only viable but nonculturable cells attached initially then no reduction in the TVC in the early stages of the experiment would be observed.

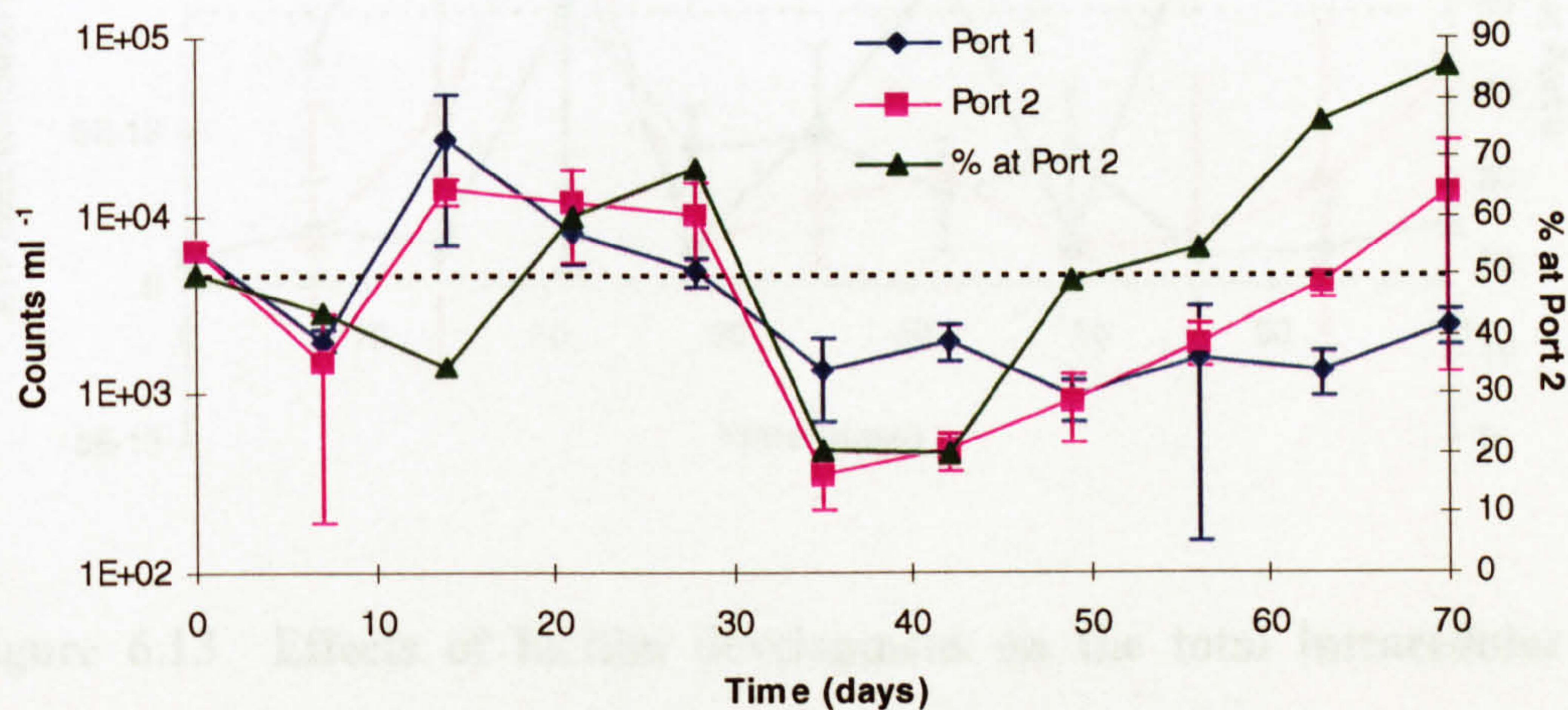


Figure 6.12 Effects of attached cells on the free-living total viable cell population. The total viable count on R2A was determined weekly after commencing the run of the continuous flow model. TVCs were measured at each of the two sample ports. The contribution of attached cells to the planktonic population is given as the percent of the overall TVC which occurred at port 2. The area at which the TVC at the two sample ports were equal is indicated by a dashed line. Data points represent averages of five or six experimental replicates and standard deviations are shown.

A similar trend was observed when the activity of planktonic bacterial cells was assessed by measuring the total intracellular ATP (Fig. 6.13). No trend in the ATP at port 2 relative to port 1 was observed until day 35. From then on it increased steadily (with one exception at day 49).

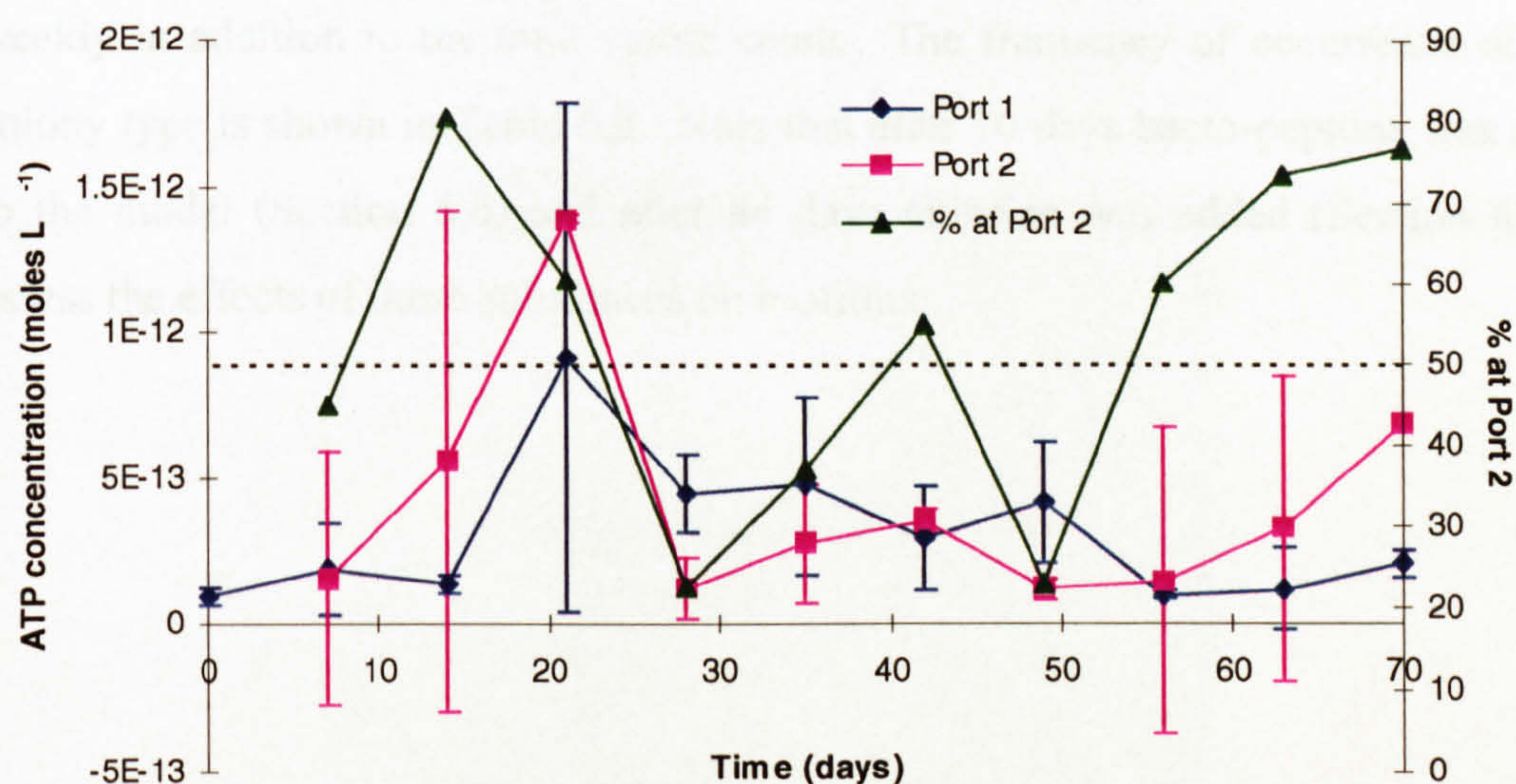


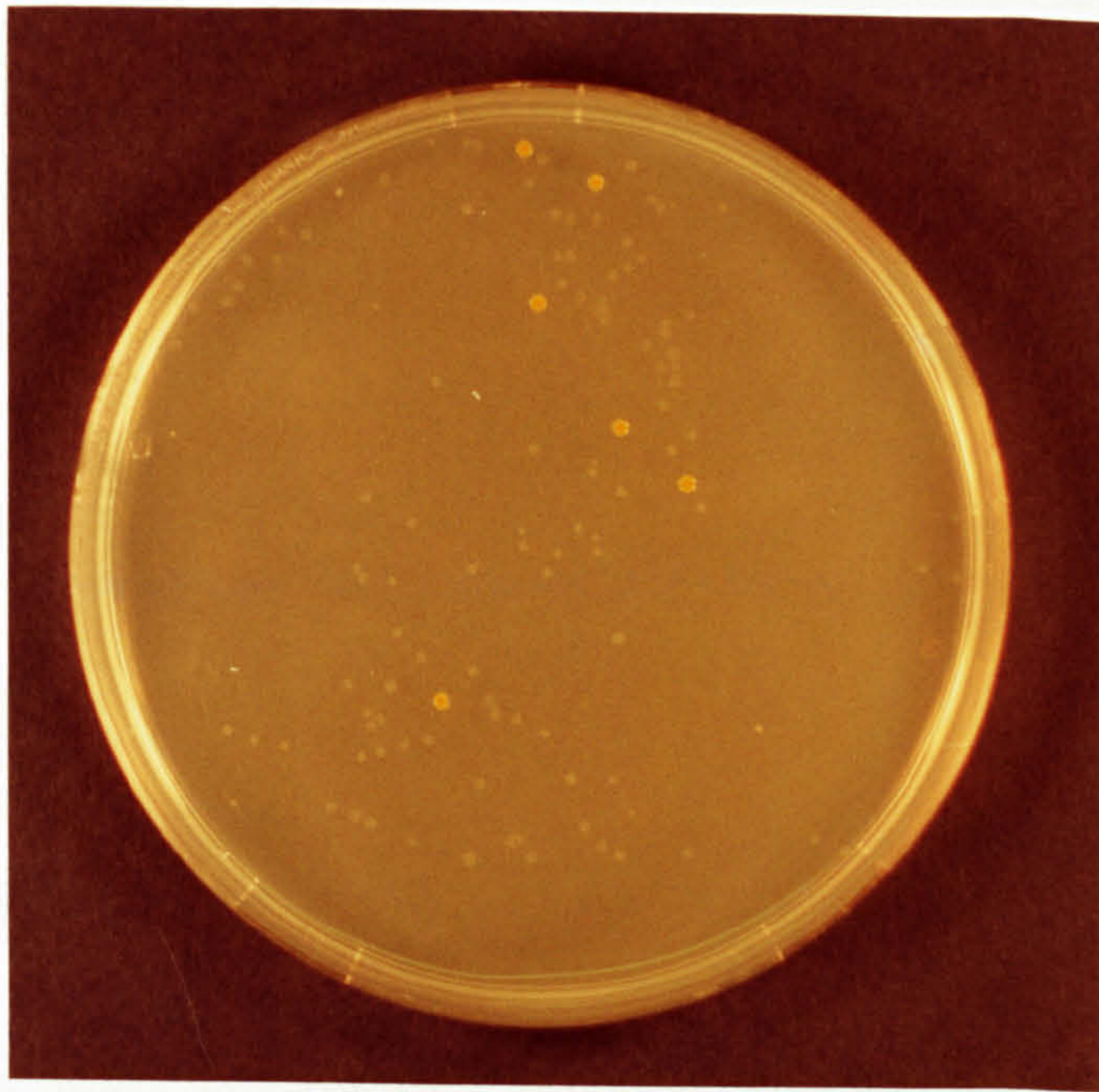
Figure 6.13 Effects of biofilm development on the total intracellular ATP concentration in the water column. Total intracellular ATP concentrations were measured at the two sample ports in the continuous flow model weekly after setting up the experiment. The contribution of biofilm cells to the planktonic population is expressed as the percent of total ATP present at port 2. The area at which the ATP was equal at the two sample ports is indicated by the dashed line. Data points represent averages from nine readings and standard deviations are shown. At each time point the ATP in sterile distilled water was measured and the reading obtained was subtracted from those of the samples.

6.5.4.3 Species composition

Examination of colonies formed on R2A from TVC samples 49 days after establishing the continuous flow model indicated an increased diversity of colony morphologies at port 2 relative to port 1 (Fig. 6.14). This provided further evidence that attachment to surfaces in the continuous flow model was affecting the planktonic population.

To quantify the affect of surfaces on bacterial diversity, colony types were described and identified (Table 3.1), and viable counts of each individual type were enumerated weekly in addition to the total viable count. The frequency of occurrence of each colony type is shown in Table 6.2. Note that after 70 days bacto-peptone was added to the model (Section 6.6) and after 84 days chlorine was added (Section 6.7) to assess the effects of these substances on biofilms.

(a)



(b)

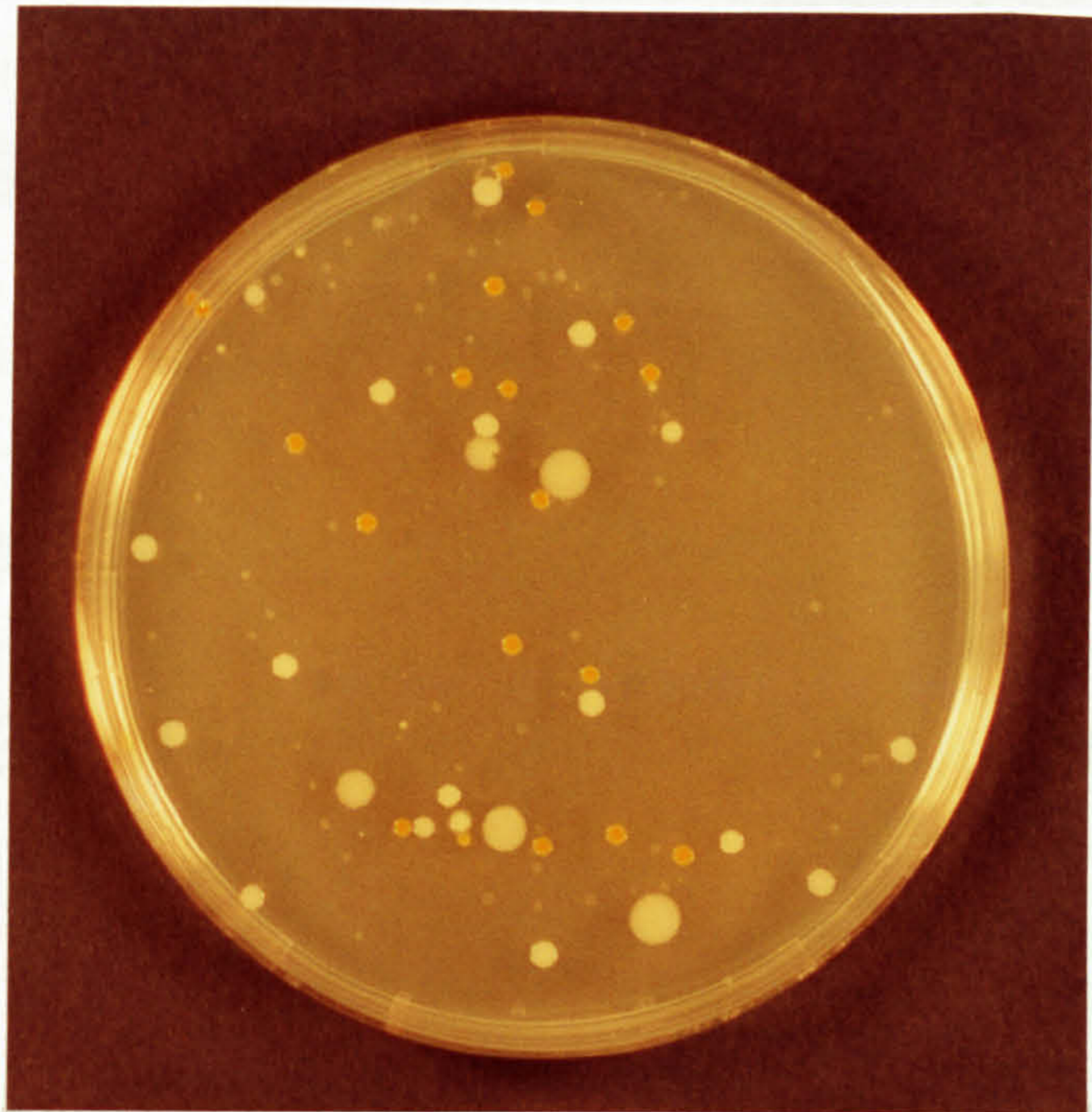


Figure 6.14 Species composition in the continuous flow model after 49 days. 100 μ l of tap water from port 1 (a) and port 2 (b) of the continuous flow model were spread onto R2A and incubated at 30°C for 5 days. An increase in colony diversity at port 2 relative to port 1 is clear. The most obvious colony types were isolated on R2A for identification. They are as follows: CF1 (port 2 only) - medium round cream colonies with entire edges; CF2 (both ports) - orange colonies; CF3 (both ports) - small translucent colonies; CF4 (port 2 only) - relatively large round cream colonies, slightly greyer than CF1.

Time (days)	Port	Colony type													
		CF1	CF2	CF3	CF4	CF5	CF6	CF7	CF8	CF9	CF10	CF11	CF12	CF13	CF14
49	1	3.3	16.7	935	0	0	0	0	0	0	0	0	0	0	0
	2	110	142	423	23.3	3.3	1.7	33.3	0	0	0	0	0	0	0
56	1	11.7	40	1350	1.67	1.7	0	81.7	0	23.3	3.33	0	0	0	0
	2	227	170	515	48.3	0	0	10	0	497	0	0	0	0	0
63	1	3.3	8.3	367	0	6.7	1.7	1.7	0	40	944	0	0	0	0
	2	700	593	210	45	0	0	13.3	0	710	0	0	0	0	0
70	1	11.7	23.3	597	0	0	0	0	0	31.7	1700	0	0	0	0
	2	925	790	1120	145	0	0	0	0	0	10	0	0	0	0
71	1	6	12	710	0	0	0	0	0	44	32	0	0	0	0
	2	917	9230	2650	0	0	0	150	0	2730	0	0	0	0	0
72	1	10	192	562	0	0	0	13.3	0	51.7	10	0	0	0	0
	2	1710	543	730	0	0	0	50	0	40	167	0	0	0	0
73	1	8	556	444	0	12	0	0	0	0	734	0	0	0	0
	2	1780	2290	353	20	0	0	0	0	0	0	0	0	0	0
77	1	25	155	255	0	0	0	0	0	0	10	0	0	0	0
	2	2380	1720	1230	50	16.7	0	16.7	0	317	16.7	0	0	0	1850
84	1	40	40	1100	0	0	0	0	0	0	0	0	0	0	0
	2	6100	1820	920	0	320	0	40	0	240	0	0	0	0	120
85	1	13.3	8.33	16.7	0	1.7	0	0	0	20	0	0	0	0	1480
	2	6200	667	133	0	783	0	66.7	0	583	0	0	0	0	1.7
86	1	35	55	115	0	1.7	0	0	0	13.3	0	0	0	0	33.3
	2	6870	1930	383	0	3650	0	0	0	900	0	0.5	0	0	0
87	1	13.3	5	102	0	0	0	0	1.7	18.3	0	0	0	0	0
	2	5280	733	100	0	1320	0	967	16.7	450	0	0	33.3	0	100
91	1	143	1020	335	0	283	0	0	0	72.5	0	0	10	0	50
	2	3480	940	240	0	340	0	0	0	200	0	0	80	0	570
															340

Table 6.2 Species composition in tap water during biofilm development in the continuous flow model. The major colony types appearing after incubation of tap water from the two sample ports of the continuous flow model on R2A were quantified. Numbers in the table represent CFU ml⁻¹. Until 49 days after commencing the experiment types CF2 and CF3 were observed almost exclusively, in roughly equal numbers at the two sample ports. Between 70-71 days peptone was added to a final concentration of 0.001% (w/v) and 0.3 mg free chlorine l⁻¹ was added for three hours immediately preceding the 85 day sample, as described in the text. All colony types were isolated on R2A and identification is outlined in Chapter 3.

The presence of surfaces selectively enriched certain species. This is clearly seen when the data obtained from colony types CF1/CF4 (isolated separately but later shown to be members of the same species) and CF3 are plotted graphically (Fig. 6.15).

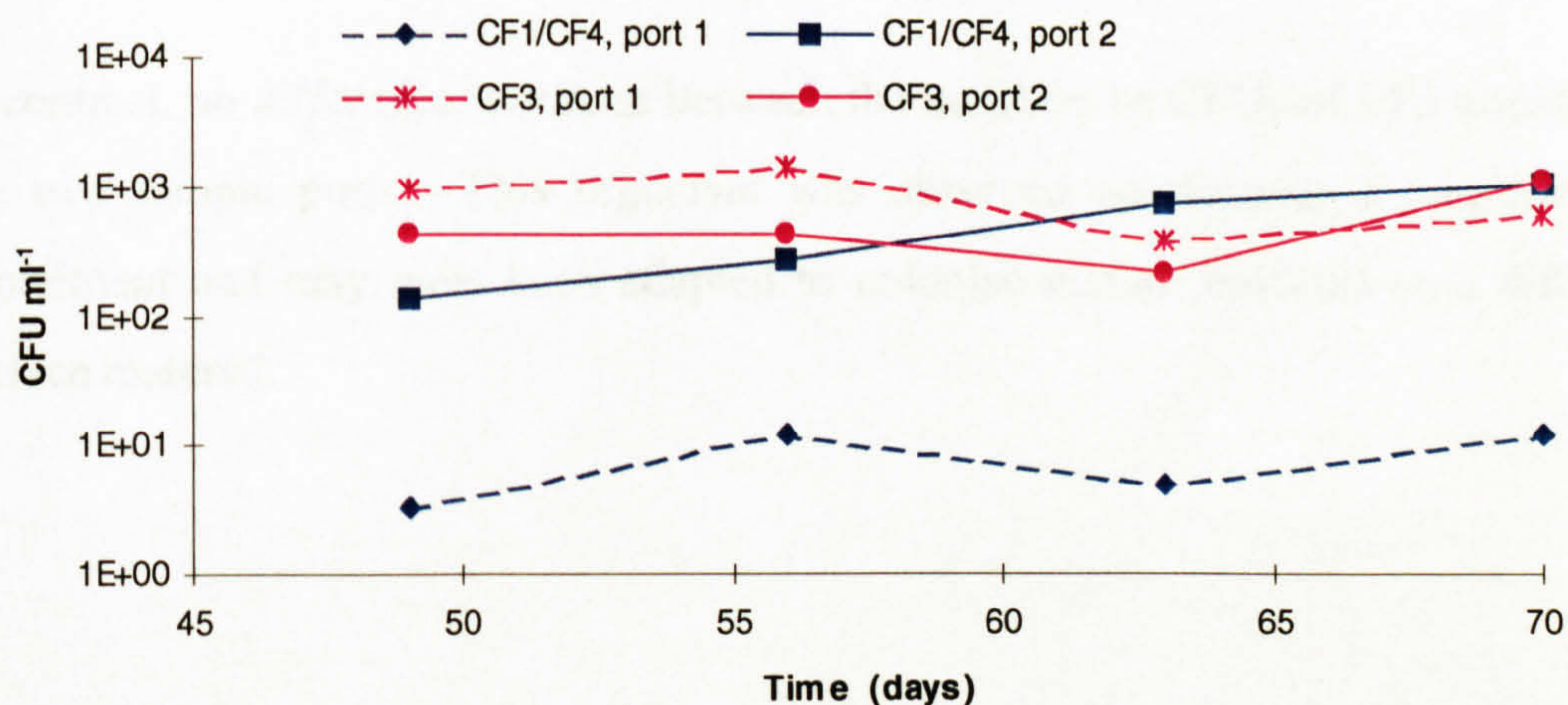
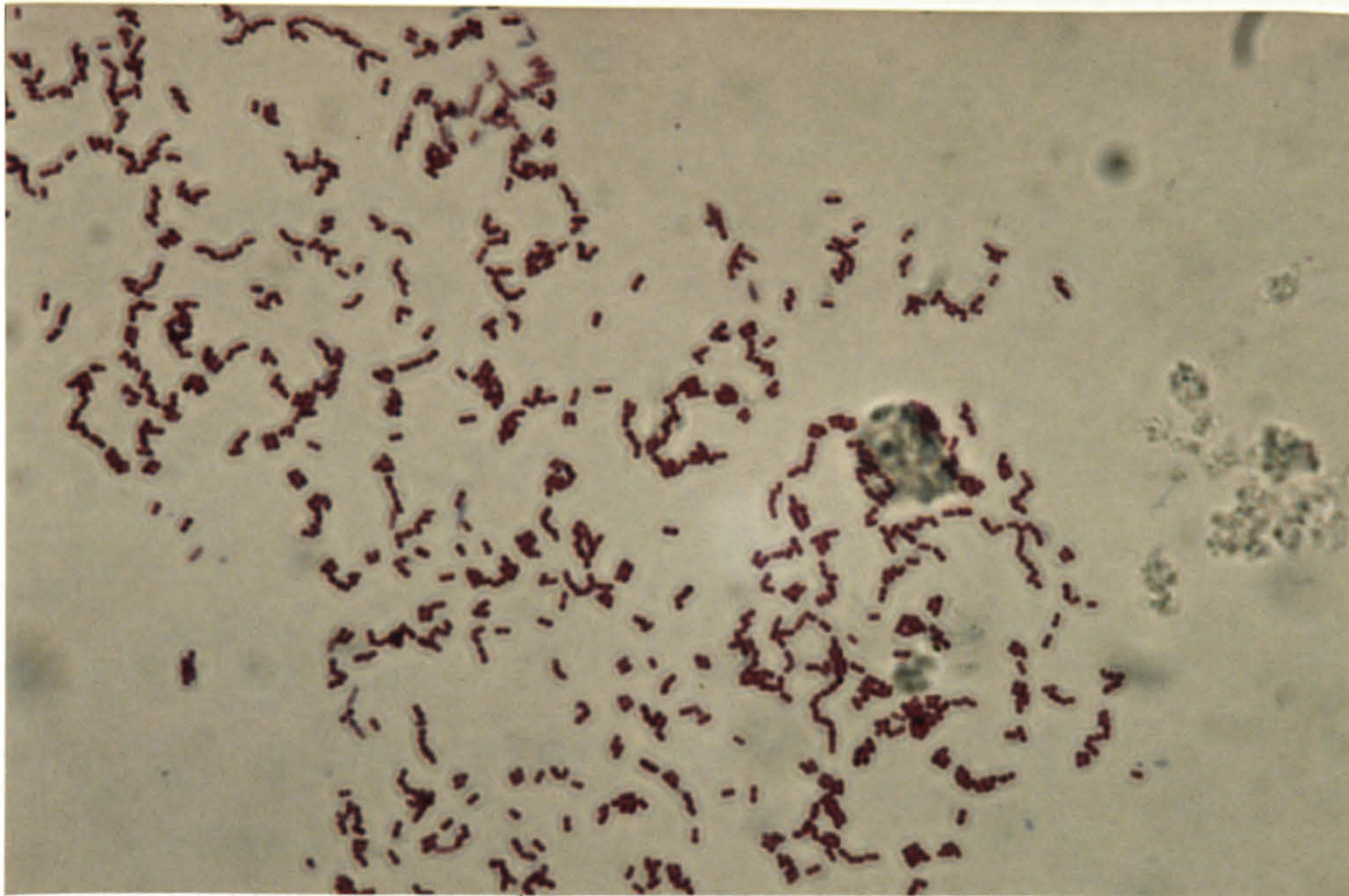


Figure 6.15 Comparison of the effect of surfaces on the total viable count in the water column of two different species. The number of colonies of CF1/CF4 (*Mycobacterium* sp.) and CF3 (unidentified α or γ proteobacterium) appearing after incubation on R2A for 5 days at 30°C, assessed at the two sample ports of the continuous flow model at weekly intervals (see Table 6.2). Port 2 was affected by the series of biofilm vessels; port 1 was not.

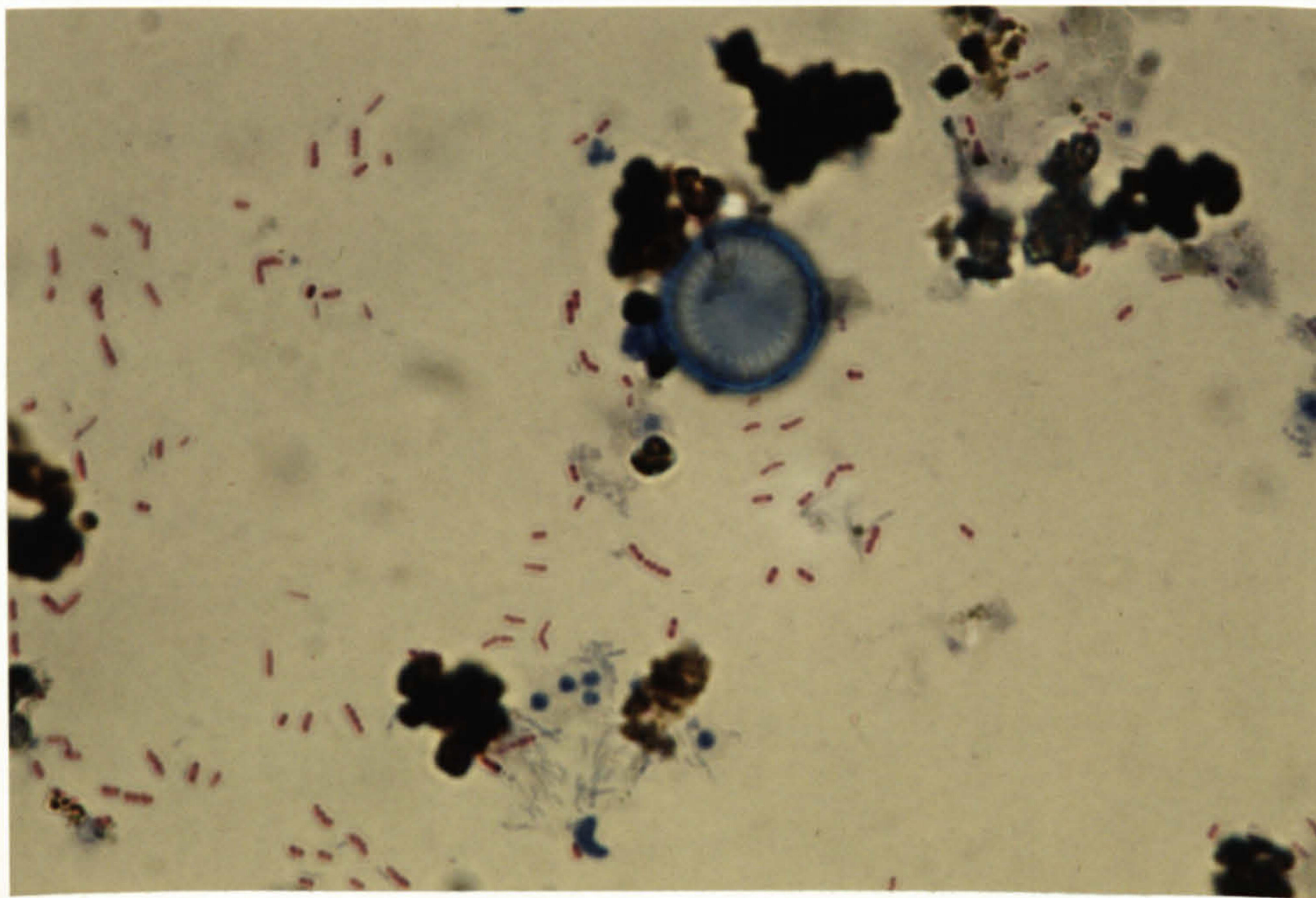
The R2A medium used did not completely prevent spreading of fungi derived from the potable water and several of the replicate plates could not be counted. This was only a problem on plates from undiluted tap water and did not affect the TVC. However, when counting low numbers of single colony types it was often impossible to obtain five or six replicates so standard deviations were not calculated. Even so, it is unlikely that the large sustained difference of two orders of magnitude between counts of CF1/CF4 at the two sample ports was due to experimental variation. Mycobacteria have previously been identified as primary colonisers of clean glass slides (Schulze-Röbbecke *et al.*, 1992) and attachment to glass surfaces, attached

growth and release of cells back into the water has undoubtedly occurred here, resulting in the elevated CF1/CF4 count at port 2 relative to port 1. The continued rise in numbers of CF1/CF4 CFUs at port 2 between 49-70 days indicates that surface growth was sustained over this period. Direct evidence that mycobacteria were actively growing and dividing within the biofilms was obtained by staining the surfaces with acid-fast stain (Fig. 6.16).

In contrast, no difference was seen between the numbers of CFUs of CF3 counted at the two sample ports. This organism was observed consistently throughout the experiment and may have been adapted to colonise mature biofilms or a different surface material.



(a)



(b)

Figure 6.16 Light micrographs of biofilms after 70 days in the continuous flow model stained by the acid-fast technique. The acid-fast staining procedure distinguishes mycobacteria (stained red) from other bacilli (stained blue). Regions of the biofilm (a) where mycobacteria were actively growing and (b) where mycobacteria were integrated into other biofilm structures, including non-acid-fast bacilli, diatoms and pigmented material were seen. All mycobacteria possessed capsules, seen here as rings of blue surrounding the cells. Bar = 10 μm .

6.6 Responses of attached cells to extraneous carbon addition

To further characterise the metabolic potential of attached cells, their response to a small increase in the carbon concentration of the water was monitored. 0.001% (w/v) Bacto-peptone was selected as the added nutrient to mimic pulses of DOC that may occur in potable water distribution pipes in response to environmental fluctuations, changes in the chlorination regime or other stimuli. The nutrient was added for 24 hrs via sample port 1 of the continuous flow model. Therefore only the attached cells within the biofilm vessels would have been exposed to the peptone and the resultant effects on these cells could be monitored directly by analysing the surfaces or indirectly as subsequent changes in the composition or activity of the planktonic cells sampled from port 2.

6.6.1 Total and active cell counts at the glass surfaces

Glass coverslips were removed at intervals following the addition of peptone to biofilms in the continuous flow model and stained with CTC and DAPI. Active cells that produced formazan crystals and total cells stained by DAPI were enumerated by digital image analysis and the proportion of cells that were actively respiring was calculated as the CTC:DAPI ratio (Fig. 6. 17).

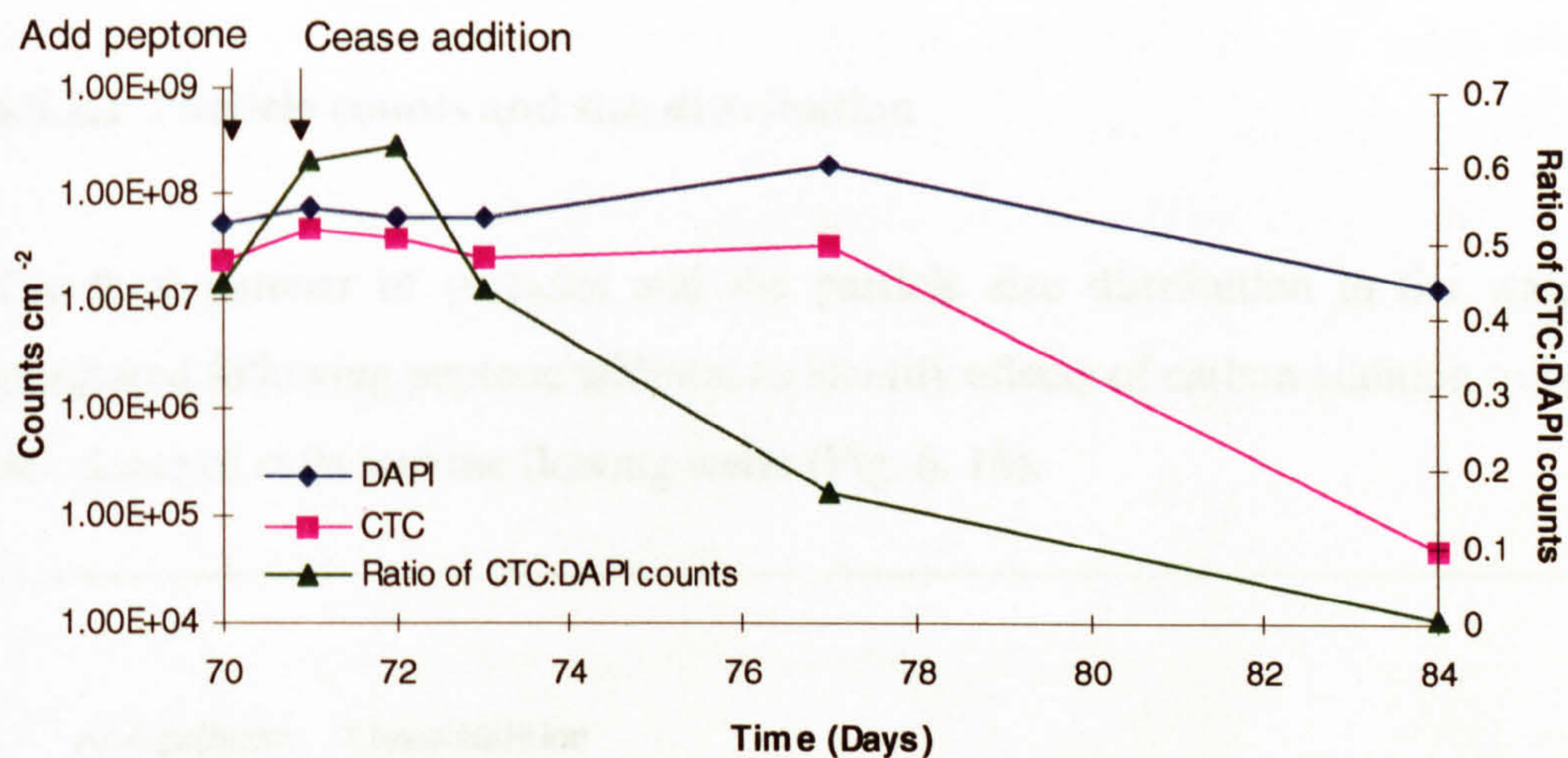


Figure 6.17 Responses of attached cells to extraneous peptone addition. 0.001% Bacto-peptone was added to 70-day biofilms in the continuous flow model for 24 hrs as indicated. The response of cells in terms of total attached (DAPI) counts and active (CTC) cell counts is shown. The proportion of total cells that were active is given as the CTC:DAPI ratio.

The addition of peptone resulted in a transient peak in the proportion of cells at the interface that were actively respiring, indicating that a fraction of the cells at the surface that were not responsive to CTC directly were still capable of metabolic activity after enrichment. After 73 days the proportion of cells possessing an active respiratory chain dropped to less than 0.01. This was unexpected, but may have been caused by bacteria re-entering a state of dormancy following a rapid drop in the nutrient concentration of the water. A longer-term study of the period following withdrawal of nutrients would have been required to confirm this. The total number of attached cells did not increase in response to carbon, suggesting either that carbon did not stimulate growth and division of attached cells or that growth was counteracted by release of cells from the surfaces.

6.6.2 Effects of biofilm cell responses on planktonic cells

6.6.2.1 Particle counts and size distribution

The total number of particles and the particle size distribution in the water was monitored following peptone addition to identify effects of carbon addition to biofilms on release of cells into the flowing water (Fig. 6. 18).

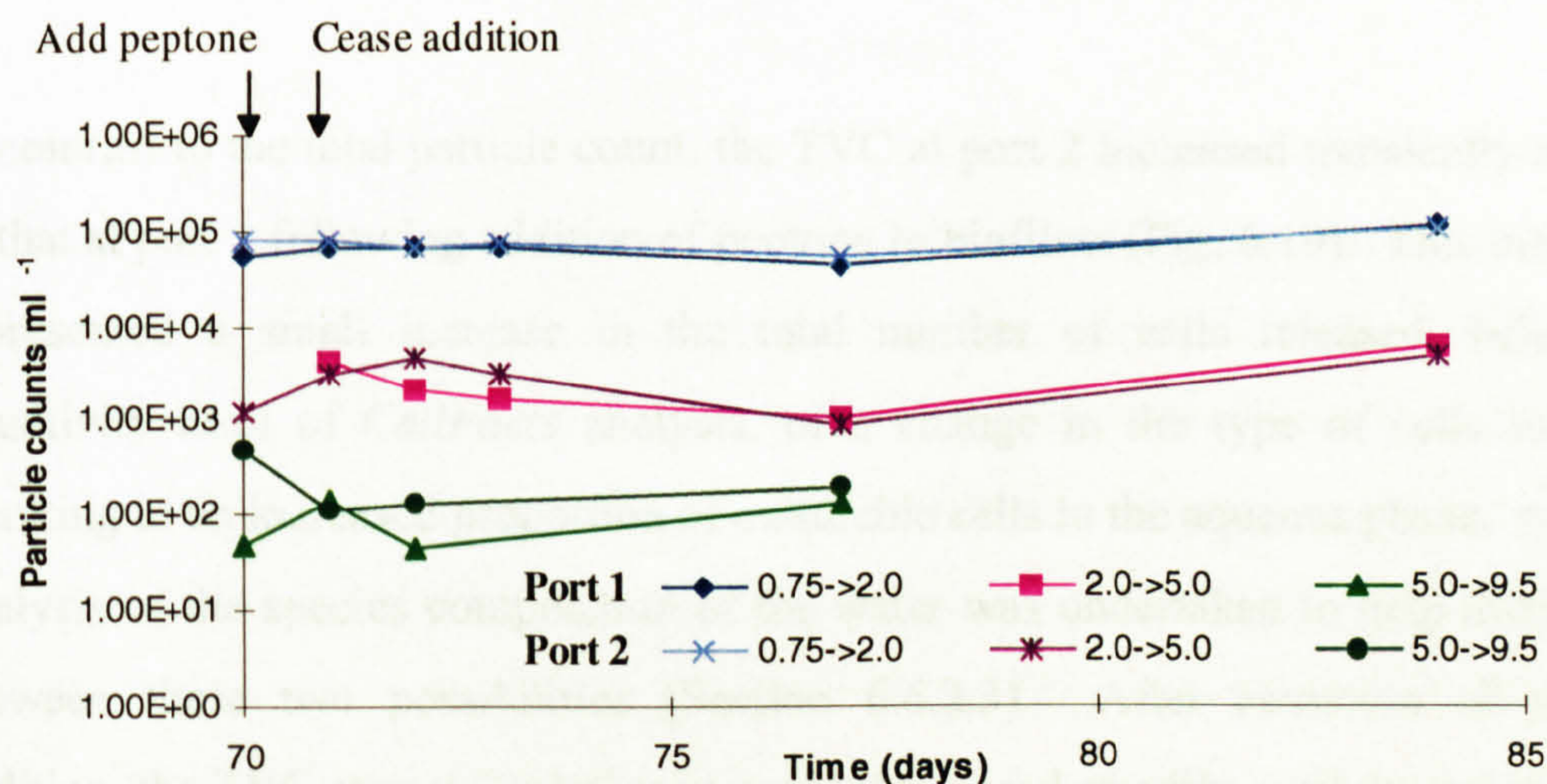


Figure 6.18 Effects of peptone addition to biofilms on the total particle count and size distribution in the aqueous phase. The response of cells in tap water after addition of 0.001% (w/v) bacto-peptone to biofilms for 24 hrs was measured using *CellFacts*. Particles were grouped into three different size ranges. The legend for each line indicates the range of ESDs in μm . Particle size ranges from each of the sample ports in the model are shown to highlight differences caused by release of biofilm cells.

Total particle counts and counts within each size range were nearly identical at the two sample ports of the continuous flow model following carbon addition to the biofilms, indicating that (i) exogenous nutrients did not stimulate growth of attached cells (since the total number of cells at interfaces did not increase following nutrient

addition - Section 6.5.4.1) and (ii) addition of carbon actually suppressed the release of cells from biofilms. This would suggest that biofilm cells were relatively unresponsive to fluctuations in the DOC concentration of the water. However, in this instance the *CellFacts* analyser was operated close to its limit of sensitivity and the data obtained from it should therefore be treated with caution. Further evidence of the state of planktonic cells released from biofilms was required to support these data.

6.6.2.2 Total viable counts and ATP concentrations

In contrast to the total particle count, the TVC at port 2 increased transiently relative to that at port 1 following addition of peptone to biofilms (Fig. 6.19). This may have represented a small increase in the total number of cells released, below the sensitivity limit of *CellFacts* analysis, or a change in the type of cells released, resulting in an increased proportion of culturable cells in the aqueous phase. A closer analysis of the species composition of the water was undertaken to help distinguish between these two possibilities (Section 6.6.2.3). After cessation of peptone addition, the TVC at port 2 relative to port 1 decreased steadily until the point, at day 77, where release of viable cells from the biofilm was undetectable. At this stage cellular activity at surfaces, measured by CTC staining, was also low demonstrating that as cells became dormant, release of cells diminished. This suggests that at least part of the total cellular detachment was an active process.

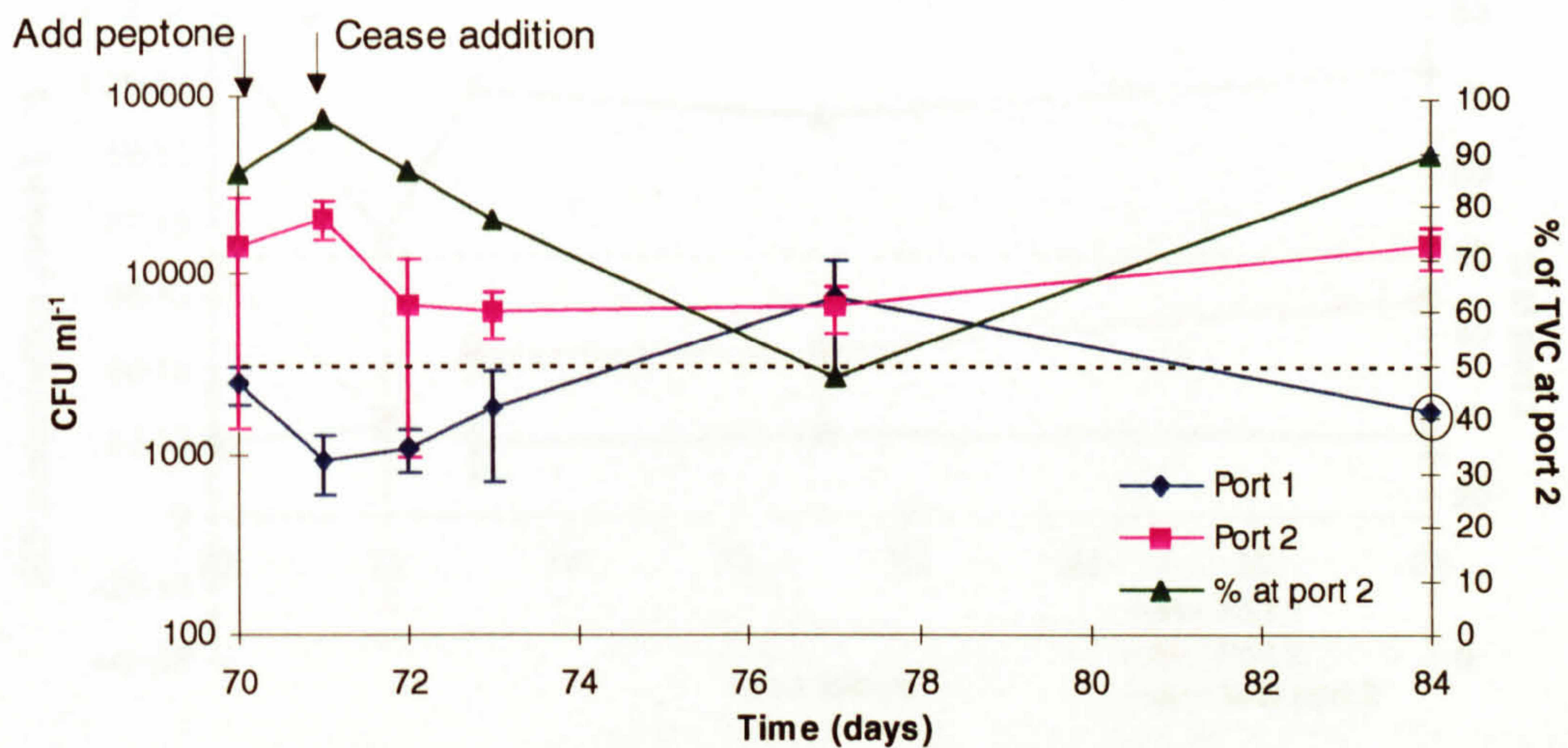


Figure 6.19 Responses of the total viable planktonic cell population to peptone addition to attached cells in the continuous flow model. 0.001% Bacto-peptone was added to sessile cells for 24 hrs. TVCs on R2A were then assessed for several days. The contribution of biofilms to the planktonic cell count is expressed as the percent of the overall TVC that was present at port 2. The point at which TVC at port 1 = TVC at port 2 is indicated by the dashed line. Data points represent means of five or six replicate samples, except the encircled point which was obtained from a single reading. Error bars show standard deviations from the mean.

To estimate the cellular activity of the planktonic population following addition of peptone to biofilm cells, the total intracellular ATP concentration was measured (Fig. 6.20).

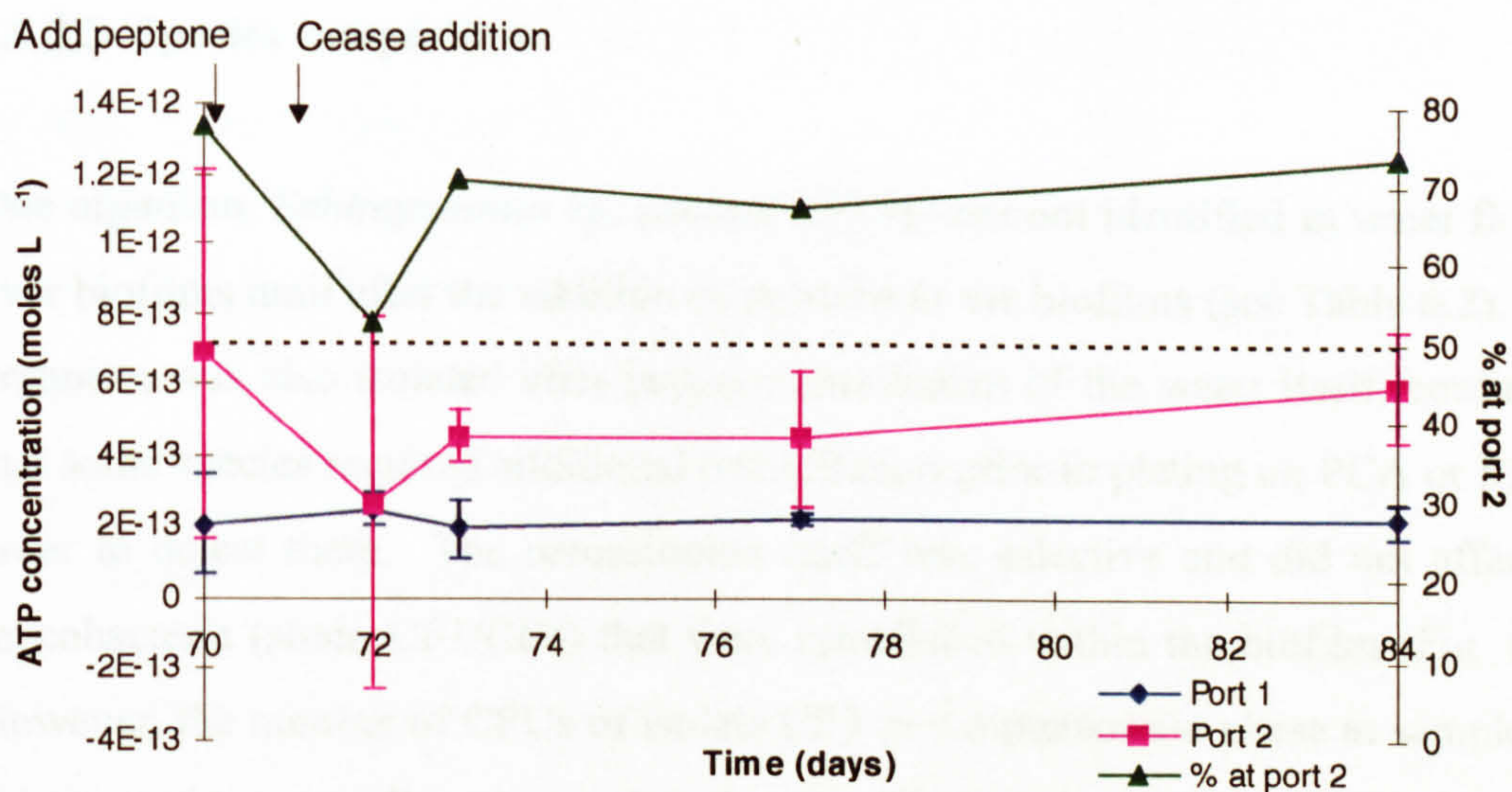


Figure 6.20 Total intracellular ATP concentration in the aqueous phase following peptone addition to attached cells. The total intracellular ATP concentration was monitored at the two sample ports in the continuous flow model after the extraneous addition of 0.001% bacto-peptone to biofilms for 24 hrs from day 70 to day 71. Data points represent the mean values from 9 readings and standard deviations are indicated. The points at which the ATP concentration is identical at the two sample ports are indicated by the dashed line.

Unfortunately a technical problem prevented determination of the immediate effect of nutrient addition to biofilms on the ATP concentration within planktonic cells. A transient reduction in the activity of planktonic cells at port 2 relative to port 1 was observed 24 hrs after cessation of the addition of peptone. At this point the total particle count and TVC at port 2 relative to port 1 were about equal to their values immediately before peptone addition so the reduction of the total intracellular ATP concentration reflected a drop in the activity of cells rather than a decrease in the number of cells *per se*. This is consistent with entry of cells into a dormant state following a decline in the nutrient concentration in the water. After 73 days the percentage of the total intracellular ATP that was detected at port 2 stabilised at around 70% indicating that biofilms continued to influence the planktonic population over this period.

6.6.2.3 Species composition

One organism, *Sphingomonas* sp. (isolate CF13), was not identified in water flowing over biofilms until after the addition of peptone to the biofilms (see Table 6.2). This organism was also isolated after peptone enrichment of the water itself, confirming that some species required additional resuscitation prior to plating on PCA or R2A in order to detect them. The resuscitation itself was selective and did not affect the mycobacteria (strain CF1/CF4) that were established within the biofilm (Fig. 6.21). However, the number of CFUs of isolate CF3 in the planktonic phase at sample port 2 increased temporarily. An explanation for this may be that strong attachment of bacteria to surfaces, in this case the mycobacteria, results in recalcitrance to agents beneficial to the cells in addition to the well-documented resistance to detrimental substances (Brown & Gilbert, 1993). Cells of strain CF3 loosely adhered to the substratum or on the outer layer of the biofilm retained the capacity to respond to an increased nutrient concentration in the water. Studies of the spatial distribution of cells within the biofilm would be required to test this hypothesis, although further information was obtained by measurement of the response of cells to added chlorine (Section 6.7).

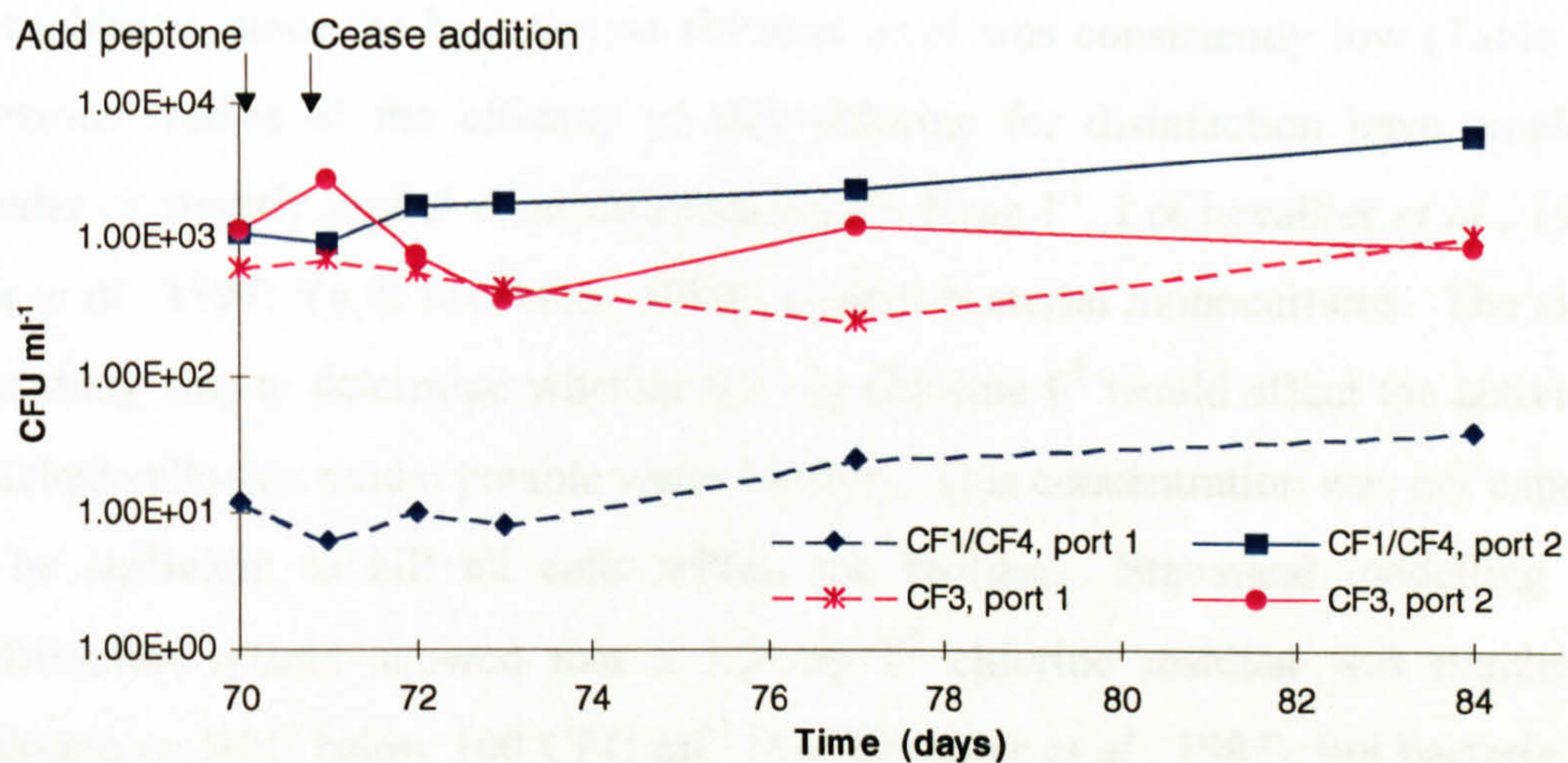


Figure 6.21 Effects of peptone addition to attached cells on the planktonic populations of two individual species. The specific viable counts were determined by enumeration of distinct colony types after incubation of serial dilutions of tap water on R2A for 5 days at 30°C. The water was taken from the two sample ports of the continuous flow model at intervals following the addition of 0.001% bacto-peptone to biofilms between the ports.

6.7 Responses of biofilm cells to extraneous chlorine addition

Chlorine is the disinfectant routinely used to treat water supplying Warwick University. The chlorine concentration leaving Strensham treatment plant is about 0.7 mg l⁻¹, but decreases as the water flows away from the treatment works. There is a booster about halfway along the network to add chlorine if necessary, so the chlorine residual after this point is subject to relatively large variations. However, the effects of fluctuations in the chlorine concentration on mature biofilms in the distribution system have not been thoroughly investigated.

To assess the effects of chlorine on mature complex biofilms in the continuous flow model extraneous chlorine was added via port 1. A fairly low concentration (0.3 mg l⁻¹) of free chlorine was selected for comparison with other studies.

Variations in the background chlorine concentration would not have interfered with this addition, since the background chlorine level was consistently low (Table 6.1). Previous studies of the efficacy of free chlorine for disinfection have employed similar or slightly higher concentrations (up to 1 mg l⁻¹, LeChevallier *et al.*, 1988a; Mir *et al.*, 1997; Yu & McFeters, 1994), against bacterial monocultures. The aim of this study was to determine whether 0.3 mg chlorine l⁻¹ would affect the activity of attached cells in a model potable water biofilm. This concentration was not expected to be sufficient to kill all cells within the biofilm. Statistical modelling of a distribution system showed that a 1.2 mg l⁻¹ chlorine residual was required to maintain an HPC below 100 CFU ml⁻¹ (LeChevallier *et al.*, 1987), but bacteria have been isolated from environments containing up to 2 mg l⁻¹ free chlorine (Mir *et al.*, 1997). The elevated chlorine concentration was maintained for 3 hrs.

6.7.1 Total and active counts at the glass surfaces

Addition of chlorine had no immediate effect on the total number of bacterial cells stained with DAPI at the glass surfaces, although the total attached cell count decreased slightly over the following 6 days (Fig. 6.22), presumably due to release of individual cells or aggregates. The chlorine may have loosened the extracellular matrix in addition to killing cells. However, the proportion of cells at interfaces that were metabolically active increased after chlorine addition. This is unlikely to have been a response to the chlorine and may have reflected recovery of cells after withdrawal of peptone at day 71.

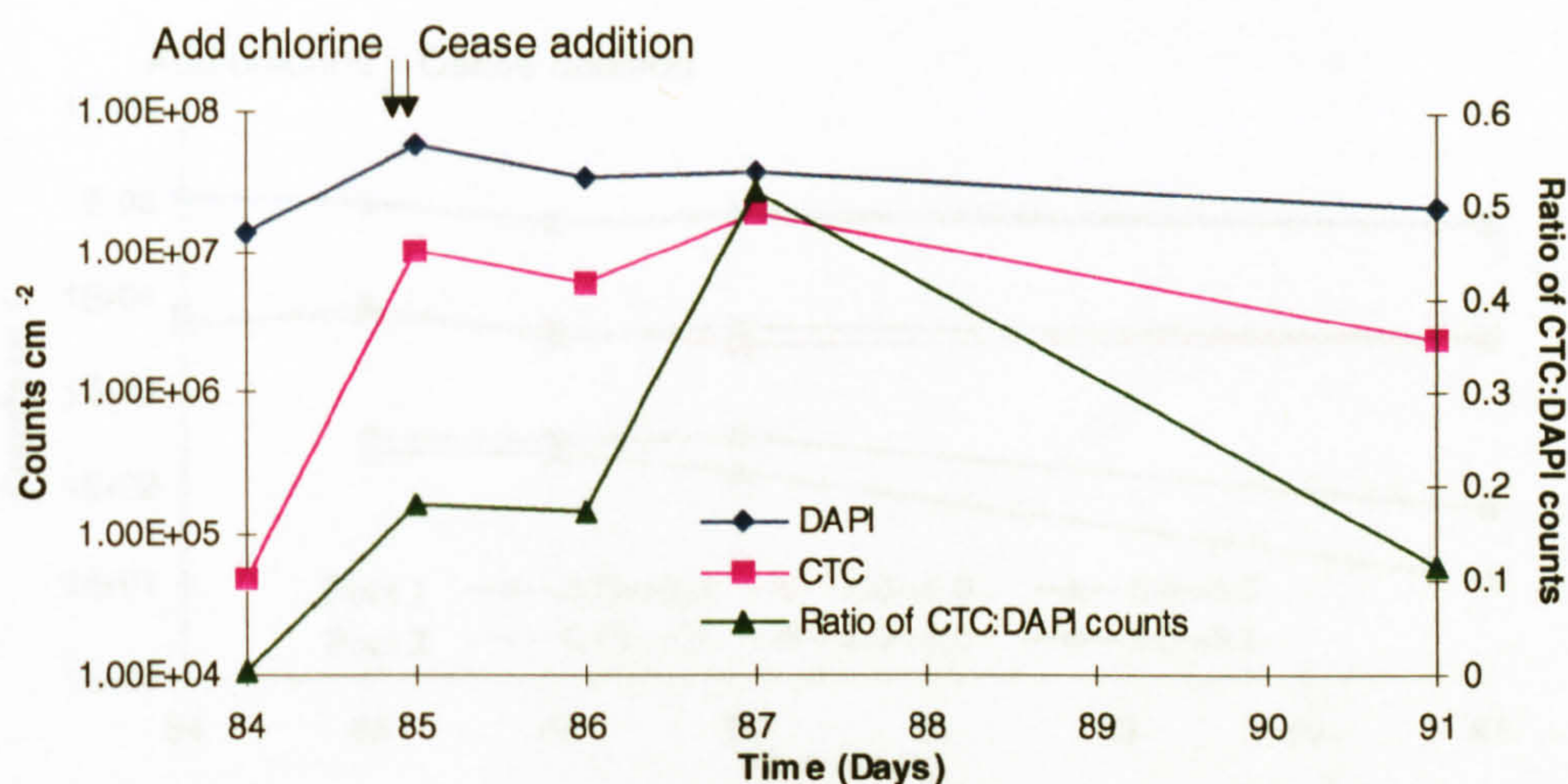


Figure 6.22 Responses of attached cells to the extraneous addition of 0.3 mg l⁻¹ free chlorine for 3 hrs. Total (DAPI) and active (CTC) cell counts at glass surfaces were determined microscopically. The proportion of total cells that were actively respiring is represented by the CTC:DAPI ratio.

6.7.2 Effects of biofilm cell responses on planktonic cells

6.7.2.1 Particle counts and size distribution

An increase in the number of large particles (5-9.5 μm ESD) at port 2 relative to port 1 was observed following addition of chlorine to the biofilms (Fig. 6.23), reaching almost a ten-fold difference between the two readings at 91 days. This was caused by release of cellular aggregates or acellular material from the biofilm after loosening by chlorine treatment.

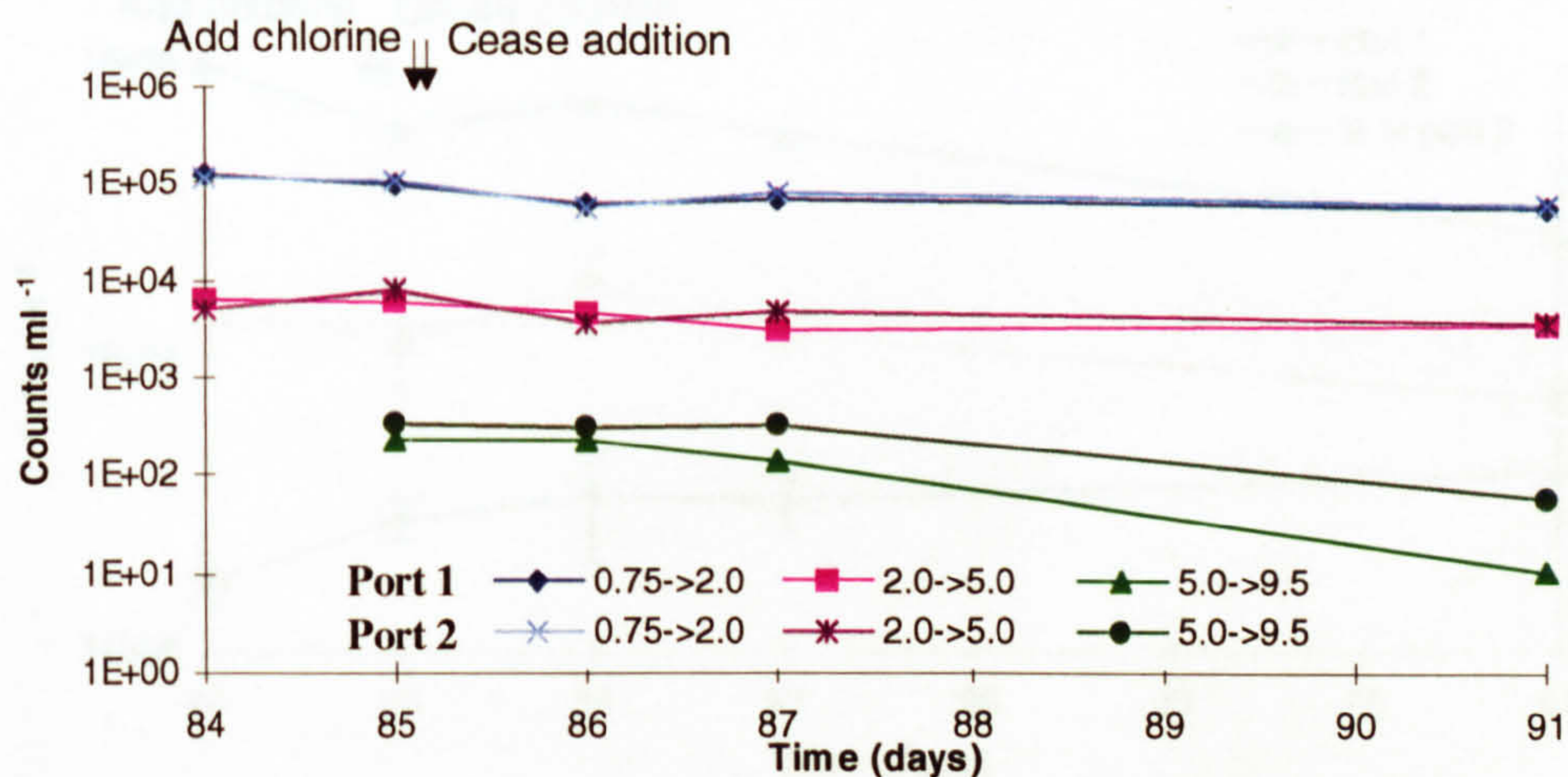


Figure 6.23 Effects of chlorine addition to attached cells on the particle size distribution in the water column of the continuous flow model. Total counts and particle sizes were measured at intervals following the addition of 0.3 mg l^{-1} free chlorine to biofilms for 3 hrs. Particles were divided into three size groups: $0.75\text{-}2.0 \mu\text{m}$, $2.0\text{-}5.0 \mu\text{m}$ and $5.0\text{-}9.5 \mu\text{m}$ ESD to distinguish between changes in numbers of individual bacterial cells and changes in counts of larger particles.

6.7.2.2 Total viable counts and intracellular ATP concentrations

The TVC and the total intracellular ATP concentration in the water both declined at port 2 relative to port 1 in the 6 days following addition of 0.3 mg l^{-1} chlorine to the biofilms (Figs 6.24 and 6.25), indicating that the influence of the biofilms on the planktonic population had diminished as a result of loss of cells from the biofilms. However, the chlorine did not completely remove the biofilms and they continued to affect the planktonic phase for the duration of the experiment.

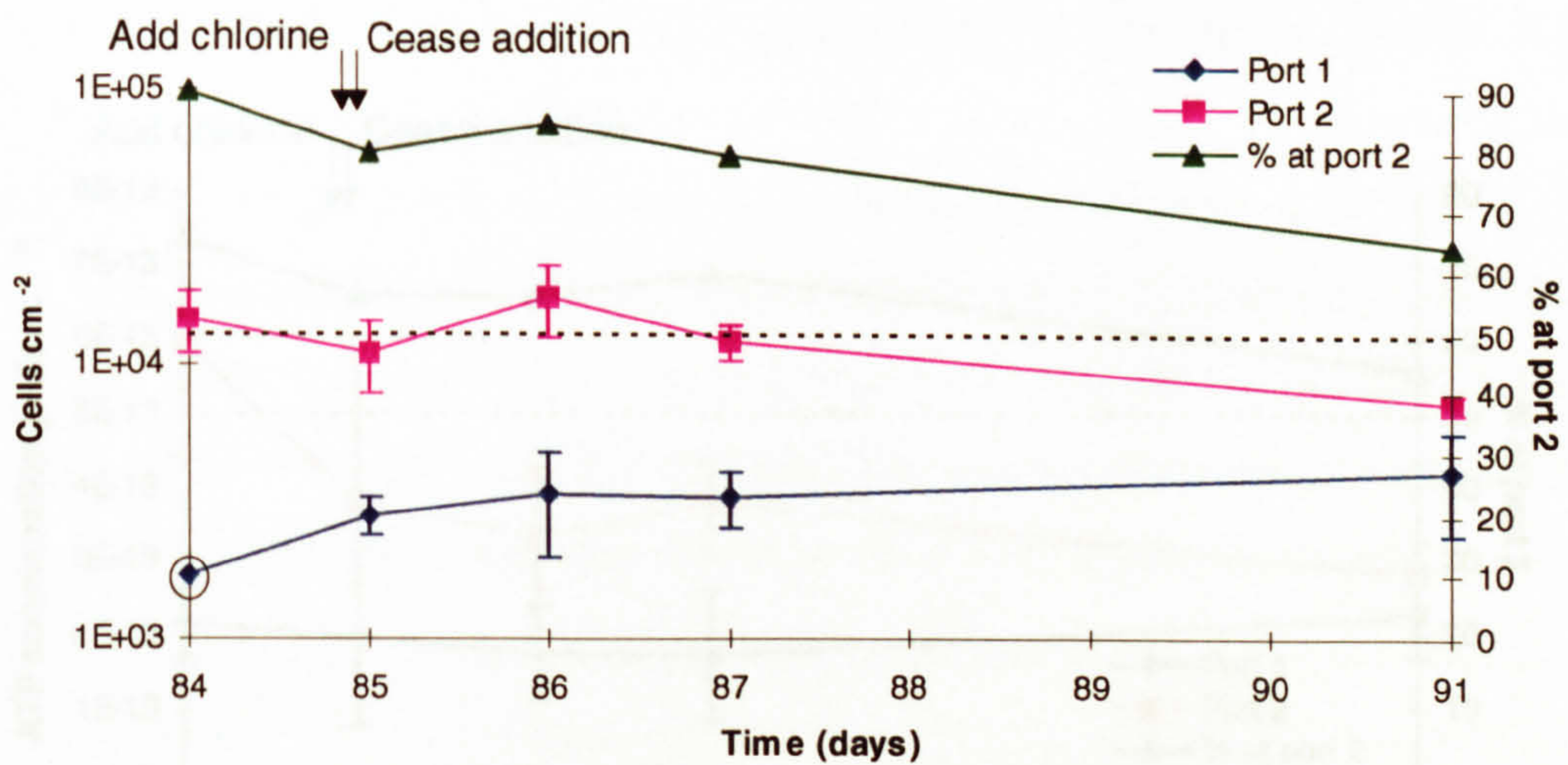


Figure 6.24 Effects of chlorine addition to sessile cells in the continuous flow model on the total viable planktonic cells. TVCs at the two sample ports were determined at various times after introducing 0.3 mg l⁻¹ free chlorine into water flowing over the glass coverslips in the model. The percentage of the overall TVC present at port 2 indicates the contribution of release of cells from biofilms. The dashed line highlights the set of points for which this figure is 50%. Data points represent the mean of 5 or 6 replicate samples, except the ringed point which was obtained from a single reading. Standard deviations are indicated by the error bars.

6.7.2.3 Species composition

The concentration of *Mycobacterium* sp. (F1074) at port 2 decreased relative to that at port 1 following addition of chlorine to the biofilms (Fig. 6.25). The decay kinetics for products based reduction over 6 days were similar to those observed for other parameters including DAPI staining of cells at surface, number of large (> 5.0 µm) ESCs present, TVC and total intracellular ATP concentration, providing further evidence that these cells were disrupted into the biofilms and were probably released into the medium after chlorination.

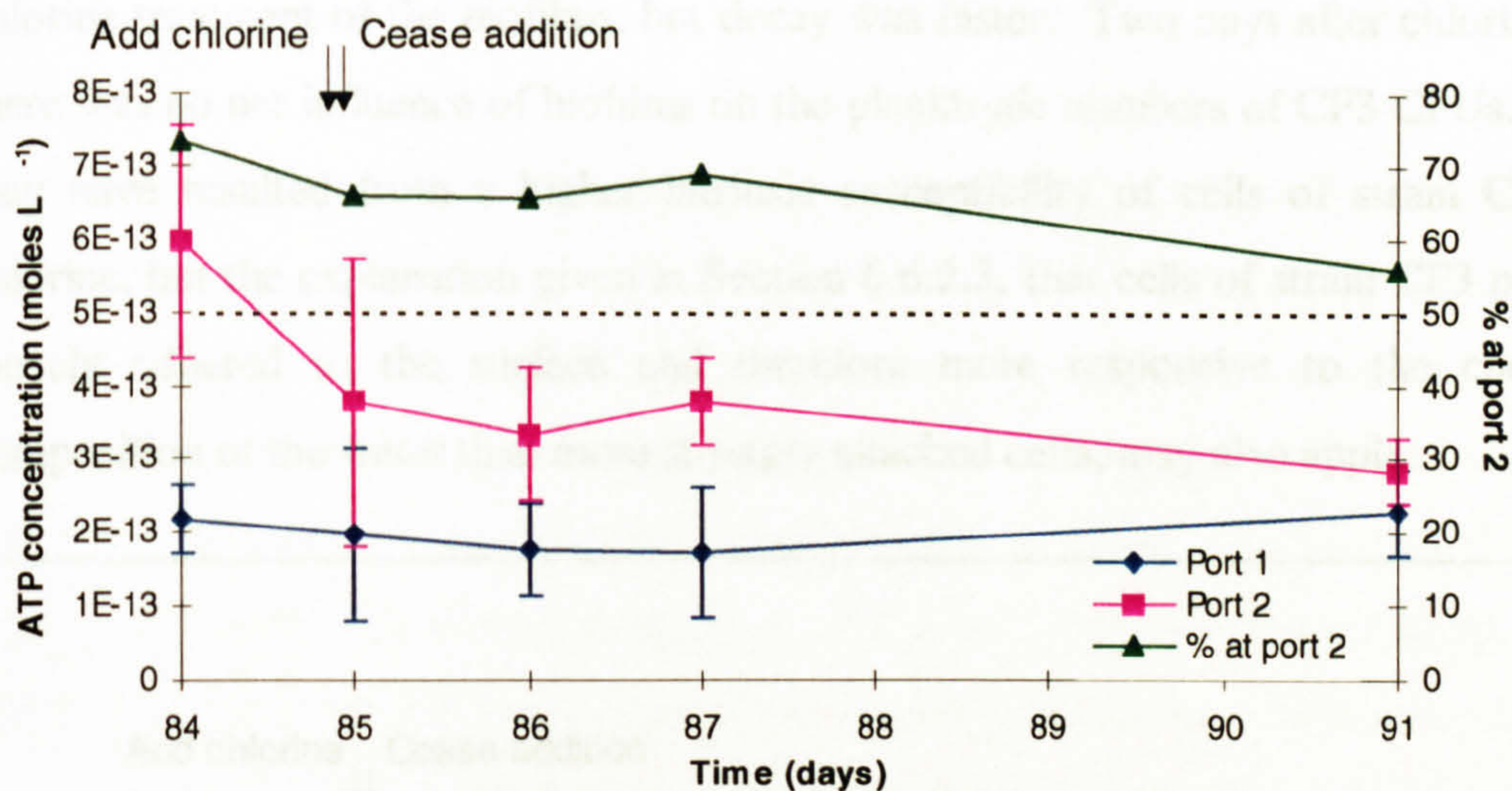


Figure 6.25 Effects of chlorine addition to attached cells in the continuous flow model on the total intracellular ATP concentration in the running water. Free chlorine (0.3 mg l^{-1}) was added to water flowing over the glass coverslips for 3 hrs. The total intracellular ATP in the water before (port 1) and after (port 2) the coverslips was then monitored. The influence of cells released from biofilms is inferred from the percentage of the total intracellular ATP detected at port 2. The set of points at which this release is essentially zero is marked by the dashed line.

6.7.2.3 Species composition

The concentration of *Mycobacterium* sp. CF1/CF4 at port 2 decreased relative to that at port 1 following addition of chlorine to the biofilms (Fig 6.25). The decay kinetics (a gradual linear reduction over 6 days) were similar to those observed for other parameters including DAPI staining of cells at surfaces, number of large ($0.5\text{-}0.95 \mu\text{m}$ ESD) particles, TVC and total intracellular ATP concentration, providing further evidence that these cells were integrated into the biofilm and were gradually released from the biofilm after chlorination.

The concentration of strain CF3 also decreased at port 2 relative to port 1 after chlorine treatment of the biofilms, but decay was faster. Two days after chlorination there was no net influence of biofilms on the planktonic numbers of CF3 CFUs. This may have resulted from a higher intrinsic susceptibility of cells of strain CF3 to chlorine, but the explanation given in Section 6.6.2.3, that cells of strain CF3 may be loosely adhered to the surface and therefore more responsive to the chemical composition of the water than more strongly attached cells, may also apply.

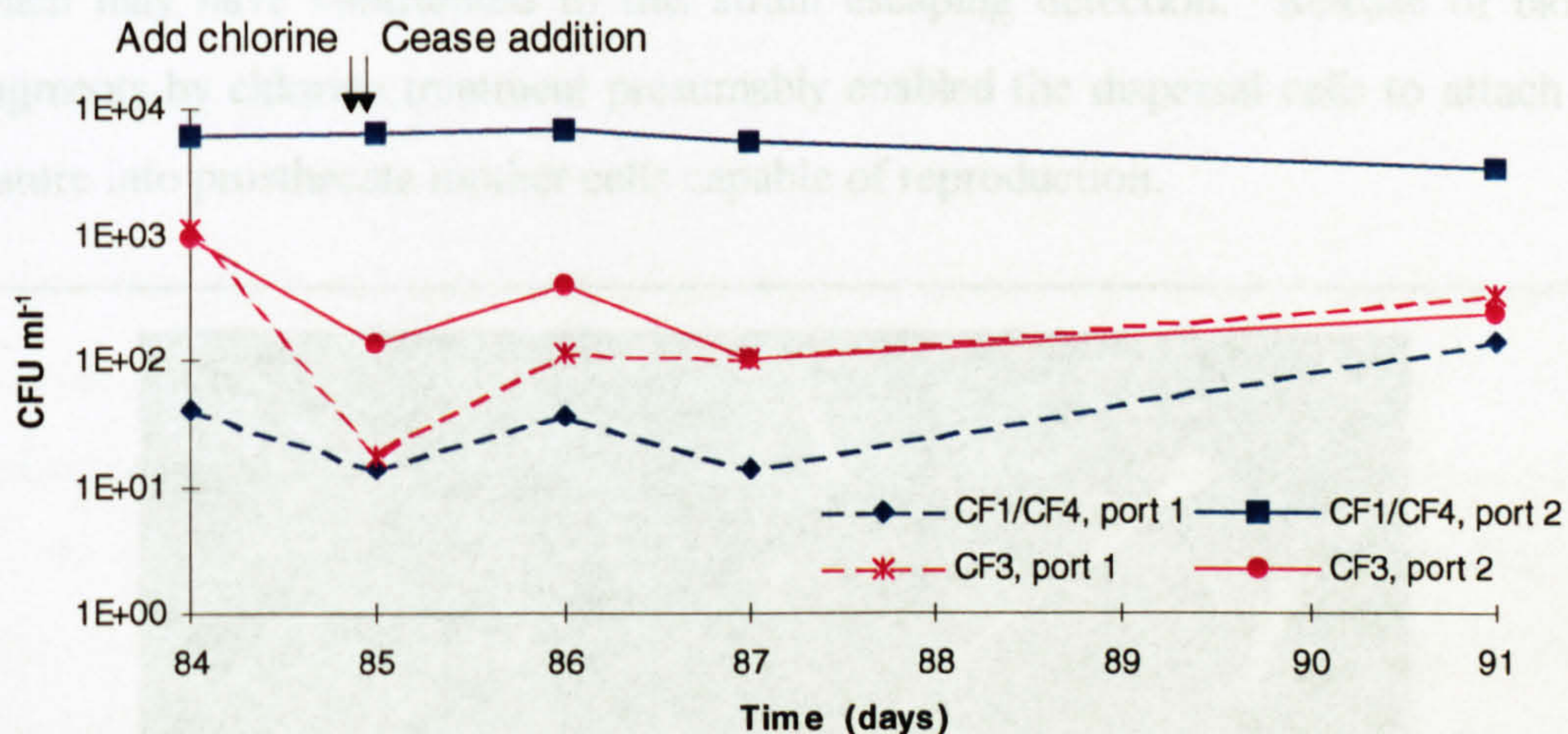


Figure 6.26 Effects of chlorine addition to sessile cells on the planktonic viable cell count of two individual species. The viable count of two different species, CF1/CF4 (*Mycobacterium* sp.) and CF3 (unidentified α or γ proteobacterium) was determined after incubation of tap water from the two sample ports of the continuous flow model on R2A for 5 days at 30°C. Release of cells from biofilms is indicated by elevated colony counts at port 2 relative to port 1.

An appendaged cell type was observed by SEM (Fig. 6.27) and by phase contrast microscopy after chlorination of the biofilm. This organism had not been observed prior to chlorine treatment despite weekly observation of the biofilms by light microscopy and SEM. It was morphologically similar to an *Hyphomicrobium* sp. or *Pedomicrobium* sp., with a prosthecum that was sometimes branched. Reproduction

occurred by budding at the pole of the stalk. However, the shape of the cell body differed from other *Hyphomicrobium* sp. or *Pedomicrobium* sp. cells observed in the static batch enrichment model (Figs 5.1 and 5.3(g)) and the cell bodies became charged in the SEM. The cells were metabolically active and staining with CTC resulted in large formazan crystals within the cell body (data not shown). The concentration of this organism in the tap water must have been quite high since large numbers of these distinctive cells were observed at interfaces after chlorine treatment, but it had not been seen previously and was not isolated. Prosthecate bacteria undergo dimorphic life cycles usually involving an indistinct rod-shaped morphology which may have contributed to this strain escaping detection. Release of biofilm fragments by chlorine treatment presumably enabled the dispersal cells to attach and mature into prosthecate mother cells capable of reproduction.

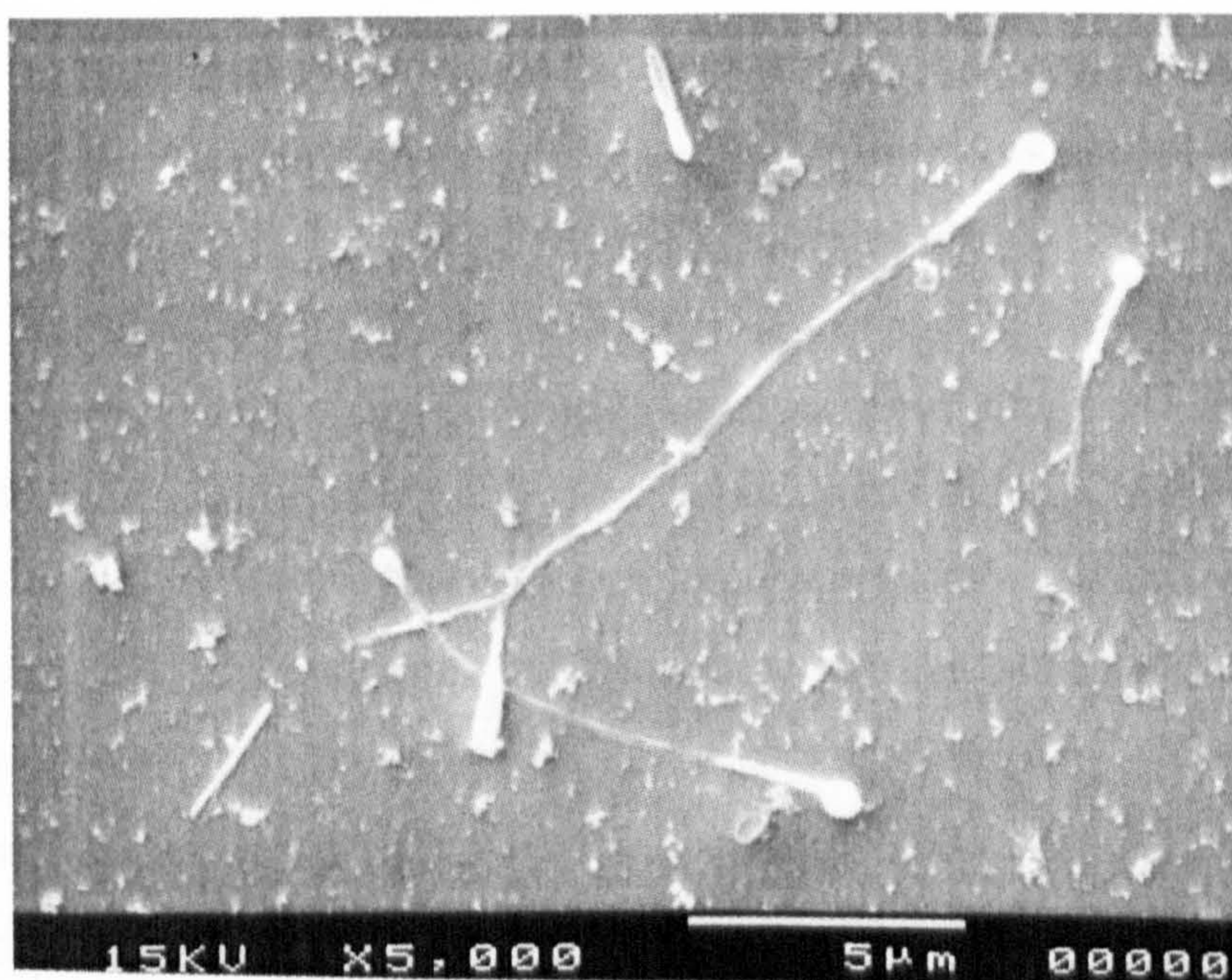


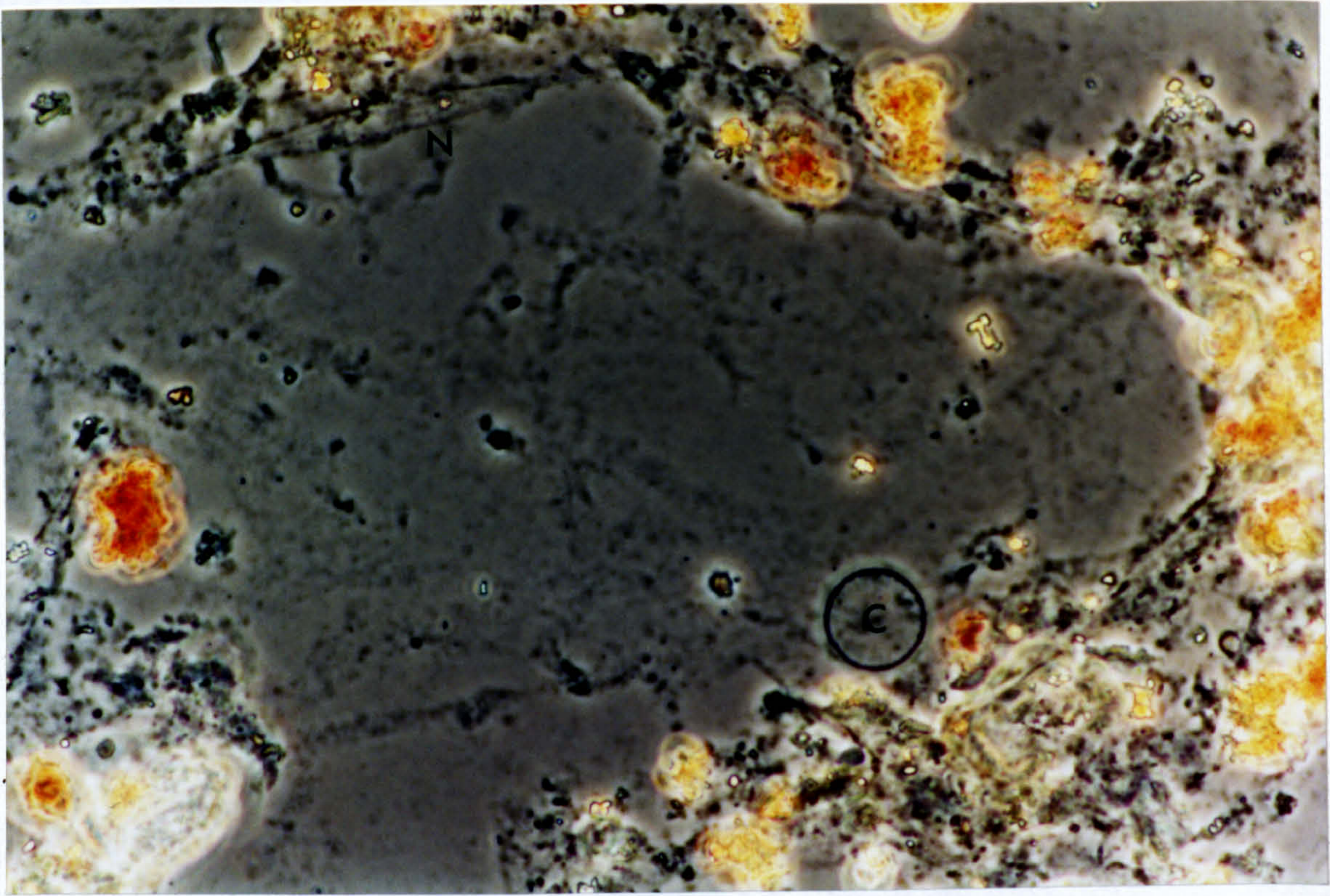
Figure 6.27 A prosthecate cell type observed by SEM following addition of chlorine to the biofilms. A coverslip removed from the continuous flow model 91 days after starting the run was dehydrated in ethanol, critical point dried and sputter-coated with gold before SEM analysis. Note the distinctive shape of the cell body, the thickening of the stalk close to the cell body and the charging of these areas under the microscope.

6.8 Analysis of biofilms formed on glass surfaces after 24 months in the continuous flow model

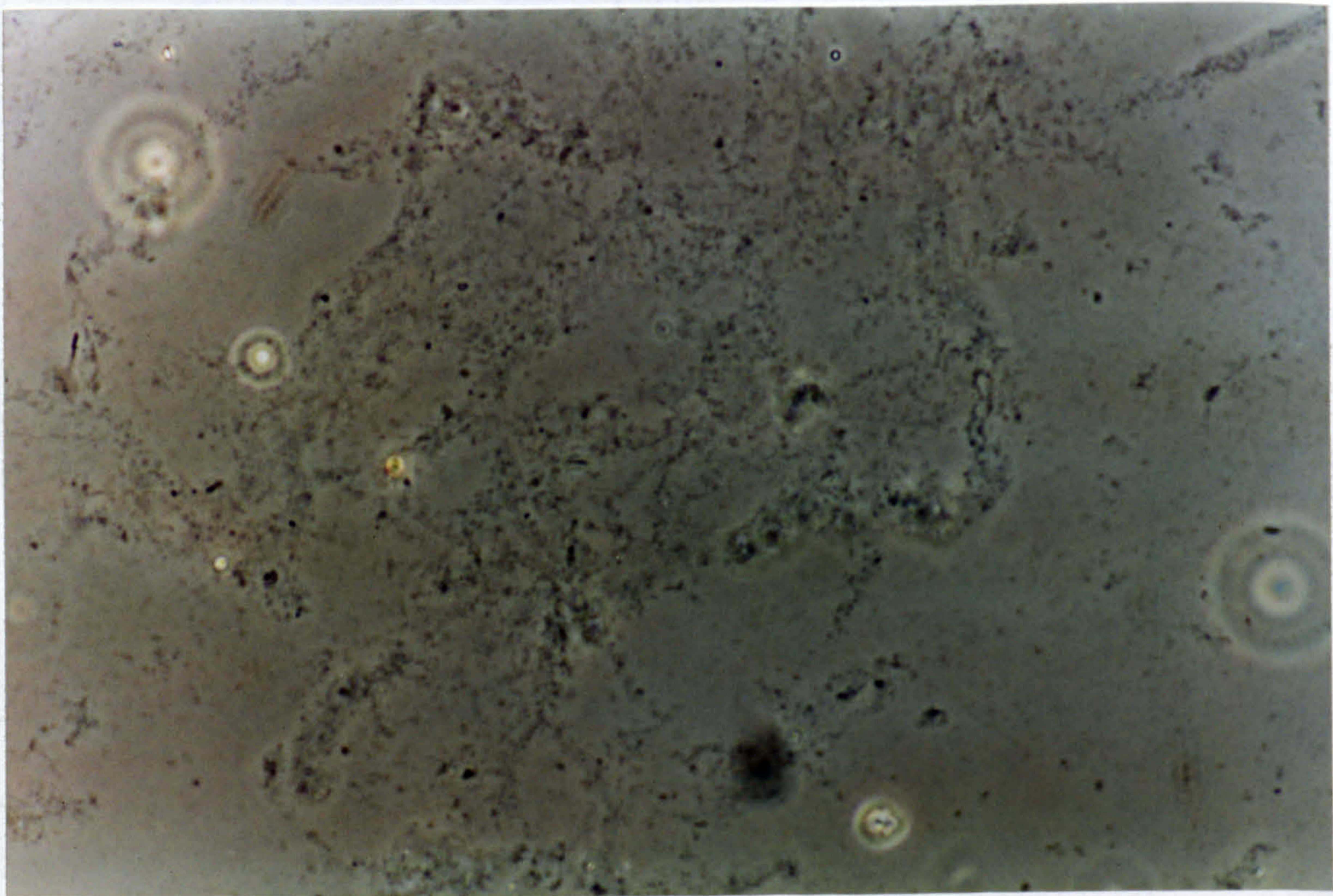
To determine the extent to which a ten-week biofilm changed over the following months, the continuous flow model was set up again and left to run for 24 months without interruption. After this time a range of techniques was employed to examine the structure of the biofilms. It was intended that the activity of cells within mature biofilms and their response to exogenous nutrients and chlorine would be assessed by staining coverslips with CTC and DAPI.

6.8.1 Biofilm structure

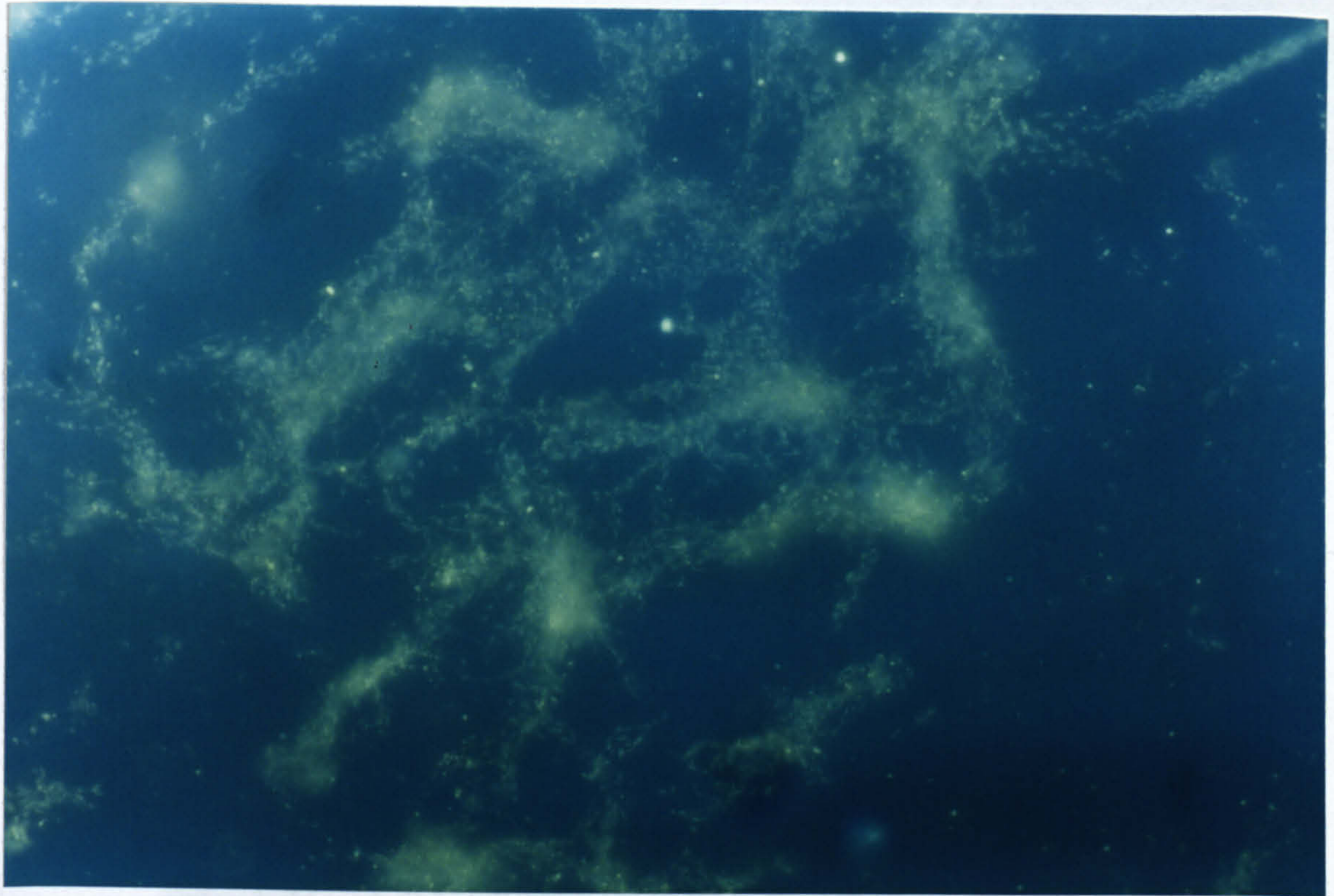
Direct observation of surfaces by phase contrast microscopy showed heterogeneity of structure with regions rich in biological material including diatom frustules, pigmented matter and other material (Fig. 6.28(a)), which were similar to areas of the biofilm observed after 10 weeks (Fig. 6.6(a)). However, the mature biofilm was almost completely devoid of bacterial cells. There were regions covered only with a layer of non-pigmented material which fluoresced blue after staining with DAPI (Fig. 6.28(b and c)), but the fluorescence was short-lived and therefore was not caused by staining of DNA. The only area where any bacteria were observed was the small region of the coverslip that was held within the silicone tubing in the biofilm development vessel. Here hyphomicrobia or pedomicrobia were seen that reduced CTC to produce formazan crystals. Other rod-shaped cells were also observed (data not shown).



(a)



(b)



(c)

Figure 6.29 SEM demonstrating the structure of a mature biofilm formed after 18 months in the continuous flow model. A field of view was selected from the nodal and prepared for SEM analysis. *Acetabularia* and a diatom (*Nitzschia* sp.) can be seen, but structures resembling those of *Cyprina* are not apparent.

Figure 6.28 Phase contrast and fluorescence micrographs showing the structure of mature biofilms formed after 18 months in the continuous flow model. (a) Phase contrast micrograph showing an area of the biofilm rich in pigmented material. Diatoms can be seen (e.g. *Cyclotella* sp. (C) and *Nitzschia* sp. (N)), but bacteria are not visible. (b) Phase contrast micrograph and (c) fluorescence micrograph of an identical field of view after staining with DAPI. Acellular material is visible which fluoresced emitting blue light. Unlike DAPI fluorescence after intercalation with DNA, the blue coloration was short-lived, lasting only a few seconds. Bar = 50 μm .

A more detailed examination of the surface by SEM confirmed the absence of bacteria. Again diatom frustules and acellular biological material were seen (Fig. 6.29).

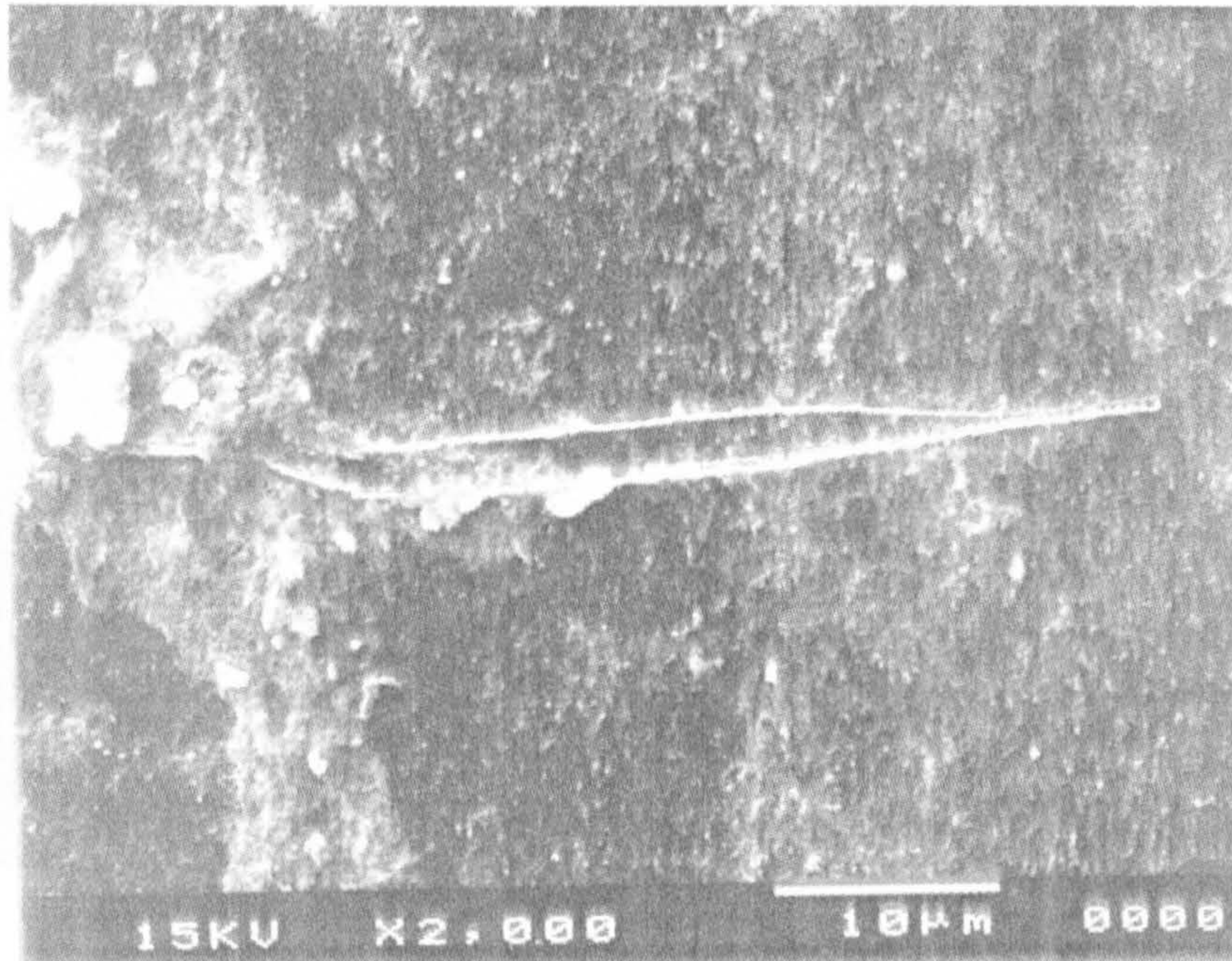


Figure 6.29 SEM demonstrating the structure of a mature biofilm formed after 18 months in the continuous flow model. A glass coverslip was removed from the model and prepared for SEM analysis. Acellular material and a diatom frustule (*Nitzschia* sp.) can be seen, but structures resembling bacterial cells are not apparent.

6.8.2 EPS

The background material stained only faintly with Congo Red (Fig. 6.30), suggesting that it was not made up of extensive bacterial slime material as seen in static batch biofilms (Fig. 5.5).

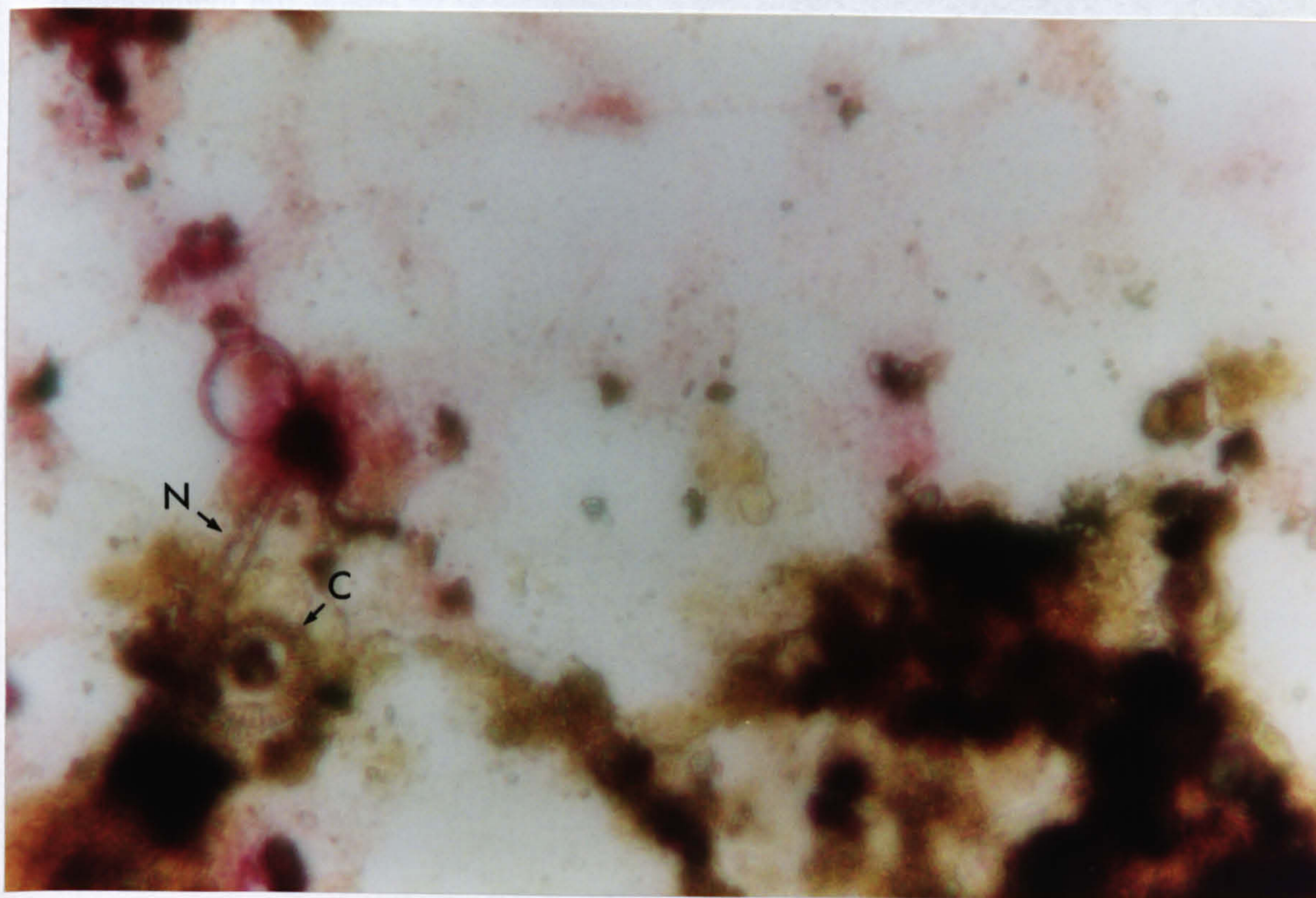


Figure 6.30 Congo Red stain of a mature biofilm developed after 18 months in the continuous flow model. A glass coverslip taken from the biofilm model was stained with Congo Red to highlight EPS. Pigmented matter now appears brown or orange. Diatoms are indicated (C = *Cyclotella* sp., N = *Nitzschia* sp.). Most of the acellular material appears only faintly pink, indicating an absence of extensive EPS. Bar = 50 μm .

6.8.3 Response to a challenge with *Sphingomonas* sp. cells

The absence of bacteria was unexpected and difficult to explain. Grazing of biofilms by protozoa often controls cell numbers but usually an equilibrium is established between bacterial growth and protozoan grazing (e.g. Kalmbach *et al.*, 1997a).

Natural senescence has been proposed as a mechanism of controlling biofilm development but again this is unlikely to result in complete removal of bacteria from interfaces (Palmer & White, 1997). The possibility that an inhibitory substance was present at the surface was considered. To test this the biofilm was challenged with a monoculture of *Sphingomonas* sp., originally isolated from potable water.

One biofilm development vessel was removed from the continuous flow model and attached to the continuous culture model (Section 4.4.1) on a recirculating loop. At this stage *Sphingomonas* sp. cells in the chemostat were at a steady state growth rate (μ) of 0.1 hr^{-1} . The rate of attachment was monitored by DAPI staining, fluorescence microscopy and image analysis. However, after just 4 hrs several layers of *Sphingomonas* sp. cells had attached and it was difficult to obtain a reliable estimation of the number of cells present. After 8 hrs attachment of *Sphingomonas* sp. cells was so extensive that the DAPI stain did not penetrate through to the lower layers. Cells attached non-specifically, covering diatoms and other biological material in the biofilms (Fig. 6.31).

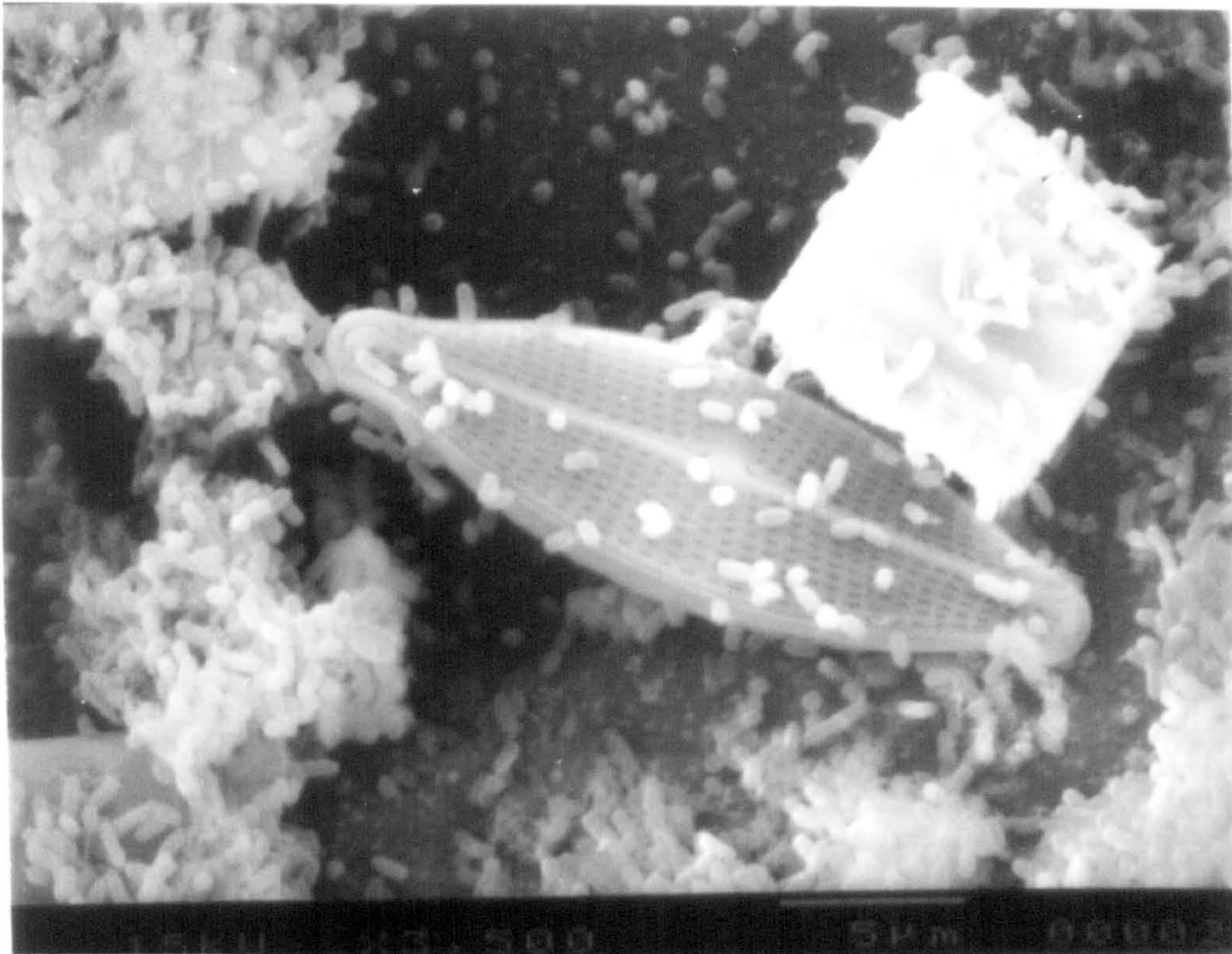


Figure 6.31 SEM of a mature biofilm formed after 18 months in the continuous flow model after challenge with a monoculture of *Sphingomonas* sp. cells. Extensive attachment of bacterial cells has occurred even covering the diatoms (*Navicula* sp., centre and *Tabellaria* sp., top right hand corner).

Clearly the mature biofilm did not inhibit attachment of a monoculture of bacterial cells. In fact attachment to the mature potable water biofilm was faster and more extensive than to a clean glass surface (Section 4.4.1.2). Therefore the biofilm was not inhibitory to bacterial attachment. An examination of *in situ* biofilms on the surfaces of pipes serving Warwick University is required to discover how closely biofilms in the continuous flow model resemble those in the distribution mains and to provide spatial information on the distribution of bacteria in pipeline biofilms. A study of pipe coupons of a range of sites in North America found bacteria associated with all surfaces sampled, although bacterial numbers were often quite low; in one case just one CFU cm⁻² was detected (LeChevallier *et al.*, 1987). In this study

coliform bacteria were only found at discrete sites (tubercles) in the mains. It is possible that most colonisation of the continuous flow model had moved from glass surfaces to more favourable sites. Bacteria were found on the small areas of glass inserted into silicone tubing, but the surfaces of the tubing or the rubber bungs in the biofilm development vessels were not analysed.

6.9 Conclusions

Most bacterial growth in low nutrient environments, including potable water, is associated with interfaces. In a study of a model distribution system, growth of planktonic cells in tap water was found to be negligible (van der Wende *et al.*, 1989). This is consistent with the theory that the role of most planktonic cells in such environments is to disperse the biofilms and locate new attachment sites, rather than to increase bacterial numbers in the water. The present study examined the ability of planktonic cells to respond to peptone, the rate of colonisation of surfaces, the activity of attached bacteria, their contribution to the planktonic phase and their response to exogenous carbon and chlorine. The following key observations and conclusions were made:-

- *Activity of the planktonic population*
 - The total particle count within the bacterial size range in potable water exceeded the TVC by at least one order of magnitude. Dead cells or inert particles may have accounted for some of this, particularly since the ATP concentration per particle was very high compared to the concentration per cell in batch cultures. However, cellular aggregates would have been underestimated by particle counting compared to TVCs.
 - Taken together with data on the response of planktonic cells to peptone and data from other studies showing a high count of total cells in tap water relative to TVCs (e.g. Kalmbach *et al.*, 1997a), the *CellFacts* data strongly support the theory that the majority of bacteria in potable water are not culturable on a single heterotrophic medium.

- *Response of planktonic cells to peptone*
 - A proportion of the microflora, primarily pseudomonads and other Gram-negative rods, was able to respond rapidly by growth and cell division despite not being culturable directly on agar before the enrichment.
 - After the nutrient addition was stopped cells aggregated, presumably to obtain protection from the harsh environment.
 - This evidence supports the hypothesis that, in general terms, the free-living cells in potable water are dormant but able to respond to stimuli and the actively growing cells must protect themselves by attaching to a surface.

- *Kinetics of biofilm accumulation*
 - The total number of bacteria at interfaces increased in a linear manner over 10 weeks. No trend was observed when the activity of attached cells was monitored over this period.
 - Almost all of the cells that attached were regular non-appendaged bacilli, in direct contrast to the wide diversity of cellular morphologies observed at glass surfaces in a static batch culture system (Section 5). It was only after treatment of 12 week-old biofilms with chlorine that appendaged cells were seen regularly at interfaces in the continuous flow model and then only one species was observed. Glass appears to be an inappropriate material to support the irreversible attachment of appendaged bacteria in a continuously flowing system. Many prosthecate bacteria deposit metals and would therefore be expected to adhere more readily to metal substrata.

- *Effects of biofilms on the planktonic population*
 - Growth of bacteria in biofilms clearly affected the planktonic cell population. This was particularly noticeable between 56-70 days when the TVC and the total intracellular ATP concentration in the water was elevated immediately after the series of biofilms.
 - Some species were affected more than others and cells of one *Mycobacterium* sp. in particular (CF1/CF4) were shown to have become established in early biofilms, grown within them and detached resulting in

an increase of almost two orders of magnitude in the free-living population.

- *Response of biofilms to peptone*

- A small increase in the proportion of the cells that were metabolically active and a small increase in the TVC of the water flowing out of the model was observed following peptone addition to biofilms.
- The response was not uniform and comparison of two species indicated that cells of the species that was most firmly associated with biofilms were least affected by the carbon addition. This was also true when biofilms were treated with chlorine.

- *Response of biofilms to chlorine*

- Boosting the free chlorine concentration to 0.3 mg l^{-1} for 3 hrs had little immediate effect on the biofilm, but weakened the overall structure. Relatively large particles (5-9.5 μm ESD) were released from the biofilm for at least 6 days after treatment.
- No decrease in the activity of the cells at the glass surfaces was detected.

- *Long term potable water biofilms*

- Biofilms developed in the continuous flow model after 10 weeks contained many structural elements that were present in mature 1 year-old biofilms developed in the same model, including diatoms and large quantities of acellular pigmented material. However, the mature biofilms were almost completely devoid of bacteria, although they were not inhibitory to bacterial attachment. This paradox was difficult to explain and further investigations are required to analyse the long-term stability of bacterial biofilms in potable water.

CHAPTER 7

7 Conclusions

In the past the autochthonous microflora of drinking water was generally considered to be harmless and microbiological analyses of drinking water therefore focused exclusively on allochthonous microorganisms, in particular pathogens and coliforms, that were directly detrimental to the quality and/or safety of the water. Disinfection was, and still is, intended to remove these bacteria specifically and their presence in finished water is indicative of a breakdown of the treatment process. It is now clear that pathogens, coliforms and other unwanted bacteria can survive and grow within biofilms on the inner surfaces of distribution pipes, causing deterioration of the microbiological quality of the water as it moves away from the treatment plant. Growth within biofilms depends upon complex interactions with many indigenous drinking water bacteria. Therefore, to understand the microbial ecology of the system, it is essential to analyse the total microbial population present. When the cellular mechanisms behind biofilm formation, function and dispersal are understood it will be possible to predict the effects of manipulating existing biofilms. This in turn will enable better control of microbial growth in distribution pipes and perhaps the use of biofilms to maintain clean water throughout distribution.

After isolation it was possible to identify around 15 bacterial genera present in the tap water at Warwick University. Isolation was selective even when using R2A, a medium designed for the cultivation of heterotrophic bacteria from drinking water. After peptone enrichment many pseudomonads and related organisms were isolated that were not routinely found by plating directly onto solid agar. Virtually all the isolates were regular rod-shaped bacteria or cocci. It was only by employing low-nutrient sloppy agar to minimise substrate shock and desiccation that an appendaged organism was isolated. Identification of bacteria was often difficult since strains grew slowly and rapid identification methods, such as API20NE could not be applied to fastidious microorganisms. However, the application of molecular techniques to microbial ecology promises to revolutionise bacterial identification. Nucleic acid sequencing is a simple and universal method for the identification of isolates and was employed here to confirm the generic identification of five strains and to narrow the

range of taxa that three other strains could have been assigned to. Since isolation is selective it must be avoided when estimating the frequency of strains *in situ*. Molecular methods based on PCR or oligonucleotide hybridization will soon enable the abundance of each strain in a complex natural microbial population to be determined.

Although studies on mixed populations are needed to define the inter-species interactions that occur within biofilms, investigations using single species are invaluable for assessing cellular mechanisms of adhesion and dispersal and for developing analytical techniques prior to applying them to complex populations. It has been proposed that in natural systems many bacteria, including those that express a single morphological form, undergo an asymmetric division resulting in two physiologically distinct cell types, a reproductive mother cell and a dormant daughter cell which is adapted for survival in the aqueous phase and therefore for biofilm dispersal (Dow *et al.*, 1983). In order to analyse vegetative dormancy in drinking water bacteria, the activity of a *Sphingomonas* sp. isolated from tap water was assessed through batch growth. Two peaks of activity, measured as cell size or as the specific intracellular ATP concentration, occurred before or during mid-exponential phase and a further transient increase in activity was observed shortly after the onset of stationary phase. It is hypothesised that the initial peak was caused by a shift-up of macromolecular and ATP synthesis which was offset by cell division at the end of the lag phase. The second peak then may have represented continuation of the shift-up before nutrients began to run out. The final peak of activity may have been caused by a proportion of the population entering a dormant state with other cells gradually dying and degrading ATP. However, a more detailed analysis of substrate utilisation kinetics and long-term survival of this strain in the absence of growth is required to confirm this hypothesis.

Since it is difficult to assess cellular activity in heterogeneous populations, similar studies were performed using *Caulobacter crescentus* CB15, an organism that undergoes a well-defined dimorphic life cycle. It is possible to synchronise this strain to measure the activity of defined populations, although this was not performed

in the present study. A single cell size peak was produced early in the exponential phase, presumably a result of nutrient shift-up. This was similar to the pattern of cell size increases observed through batch growth of *E. coli* and *Klebsiella pneumoniae* previously obtained in this laboratory. It will be interesting to see if this cell size peak correlates with the intracellular ATP concentration through batch growth of *C. crescentus* CB15 and to use synchronous populations to examine cell size and ATP concentration in mother and daughter cells. It was not possible to distinguish between mother and daughter cells in batch cultures on the basis of cell size alone.

To develop a technique for the analysis of dormancy in potable water biofilms, the ability of *Sphingomonas* sp. and *C. crescentus* CB15 cells at different stages in batch growth to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was assessed by two different procedures: (i) the total CTC reduction was measured fluorimetrically and (ii) the total number of cells that reduced CTC was determined microscopically. In both strains the two different procedures produced different kinetics. Total CTC reduction increased hyperbolically to a peak at mid-exponential phase, but the proportion of cells that reduced CTC increased exponentially to a peak around the middle of exponential growth. The observed differences may have highlighted the heterogeneity of the population, i.e. a few cells rapidly became active, but this activity was limited by competition for nutrients. Alternatively, the differences may have been an artefact produced by the limitations of each technique. To distinguish between these possibilities it will be necessary to refine the experimental protocols. Using a dye that produces a soluble tetrazolium salt, such as sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulphonic acid (XTT, Park Scientific Ltd) will improve the fluorimetric assay. The microscopic procedure should be carried out in strictly defined medium since phosphate, pH and nutrients can affect the staining.

The role of cell surface hydrophobicity in attachment and release from surfaces was assessed using *Sphingomonas* sp. cultured in a chemostat. Hydrophobicity was dependant on the growth rate of the population, with slowly-growing cells tending to be most hydrophobic. Hydrophobicity was increased by using nitrogen rather than

carbon as the limiting nutrient. However, no changes in the ability of cells to adhere to glass surfaces was observed, probably due to the lack of sensitivity of the assay employed. Perhaps it would be more useful to look for a correlation between cell surface hydrophobicity and the activity of cells, measured as their ability to reduce CTC or the intracellular ATP concentration. This would show if changes in cell surface hydrophobicity are part of a general mechanism for the detachment of dormant cells. If this does prove to be the case then understanding the molecular mechanisms that mediate hydrophobicity will enable analysis of the role of the cell cycle in vegetative dormancy and biofilm dispersal.

To assess whether distinct planktonic and biofilm phenotypes are regulated as part of the cell cycle of the *Sphingomonas* sp. isolate, two dimensional gel electrophoresis was employed to analyse the proteins produced by cells growing attached to a surface or those in liquid culture. Attempts were made to produce the same nutrient limitations in the two types of cell culture, although a very artificial biofilm system was required to obtain sufficient biomass for protein analysis. Only one or two proteins were identified that were differentially expressed by biofilm and planktonic cells. Separation may have been improved by use of a larger gel kit. Alternatively, restricting the analysis to the outer membrane proteins would have simplified the quantification of the levels of expression of each protein.

The information obtained from studies on *Sphingomonas* sp. is insufficient to prove or disprove the theory that this drinking water isolate employs asymmetric division as a mechanism to produce morphologically identical, but physiologically distinct cell types when nutrients are scarce. However, in combination with some of the work suggested above, data presented here should help to resolve this issue.

It is clear that many species in potable water undergo bi- or multi-phasic life cycles. This was deduced from a simple microscopic analysis of the wide diversity of cell types present, many of which are known to be produced from asymmetric division, following a period of static batch enrichment. Cell types resembling *Hyphomicrobium* or *Pedomicrobium*, *Planctomyces* and *Caulobacter* spp. were

observed, as well as a helical appendaged cell type that has not previously been reported. The frequency of appendaged bacteria in potable water is often underestimated since these organisms are generally difficult to cultivate and only exist for part of the life cycle in an easily recognisable appendaged form. However, prosthecate bacteria and planctomycetes are likely to play a significant contribution to the microbial ecology of potable water since they possess a wide range of metabolic capabilities, some of them unique amongst the eubacteria.

To assess the partitioning of these and other microorganisms between biofilms and the aqueous phase in a natural tap water system a continuous flow model was developed that allowed sampling of the water before and after a series of glass surfaces. The extent of vegetative dormancy in the water column was examined by the addition of nutrients (0.001% w/v bacto-peptone) to the water for 24 hrs. A rapid increase in the total and viable cell number was detected. Identification of the predominant colony types indicated that the increases counts were almost exclusively pseudomonads and similar organisms (e.g. *Comamonas*, *Xanthomonas* and *Sphingomonas* spp.) that were not routinely detected on R2A. The response was too rapid to have been caused by cryptic growth of a very small number of cells - some resuscitation of viable but nonculturable cells must have occurred. This clearly demonstrates that a proportion of the planktonic vegetative bacteria in the potable water were in a dormant state. Around ten hours after the onset of nutrient addition, aggregation of cells was detected by particle size distribution analysis. At this stage competition for nutrients may have been starting to take effect. An increase in the propensity of cells to attach following nutrient depletion is consistent with a model in which planktonic cells grow and divide when nutrients are plentiful, but become sticky once the nutrients begin to run out.

The attachment process was analysed in detail following the establishment of the continuous flow model with clean glass surfaces. No trend was observed in the activity of adhering cells, although this may have been a result of the failure to employ an effective CTC staining protocol until 21 days into the experiment. A similar study found that 90% of cells attached to glass after 2 days reduced CTC, but

that this figure decreased exponentially until around 21 days, when it stabilised at about 30% (Kalmbach *et al.*, 1997a). This is not inconsistent with the present study, given the high degree of intra- and inter-sample heterogeneity here. The activity of planktonic cells was not assessed by CTC staining in either investigation, but it is unlikely that 90% of planktonic cells would have reduced the dye. In a previous study Schaule *et al.* (1993) found that between 1-5% of planktonic cells in drinking water could reduce CTC. A similar discrepancy was found between viable counts and total counts in this study. Therefore it appears that adhesion is primarily an active mechanism, dependant upon cellular activity. A model where dormant planktonic cells require a stimulus to initiate maturation and regaining of cellular activity and the ability to adhere could explain this observation.

Kalmbach *et al.* (1997a) also found that colonisation of a clean surface followed a defined succession. This was confirmed here by analysis of the viable cells released from biofilms. Mycobacteria, which have previously been shown to be primary colonisers of domestic drinking water systems (Schulze-Röbbecke *et al.*, 1992), rapidly adhered to glass surfaces and divided. Attached growth caused an increase in the number of planktonic cells of this species of 1-2 orders of magnitude. The role of succession in biofilm formation should not be overlooked. Countless investigations have analysed biofilm formation by single species, but in nature many microorganisms may only attach to a developing or mature biofilm.

After ten weeks the response of attached cells to nutrients (0.001% bacto-peptone, 24 hrs) or chlorine (0.3 mg ml⁻¹, 3 hrs) was monitored. A slight increase in CTC staining was observed after the addition of nutrients, but there was no dramatic rise in the total number of attached cells. In particular it appeared that the species that were most firmly attached were relatively buffered and unresponsive to changes in the water chemistry. This may be explained by failure of nutrients or chlorine to penetrate the biofilm or by phenotypic changes in the cells. This was not investigated in this study since it has been the topic of many other experiments.

Perhaps the most surprising result from the continuous flow model was the observation that after one year biofilms were practically devoid of bacteria. It is difficult to propose a reasonable explanation for this. Surfaces were covered in biological material ('microbial footprints') and diatoms were abundant. The surfaces were not inhibitory to attachment of *Sphingomonas* sp. - in fact attachment was enhanced in the presence of the organic material. Bacteria were present in the areas of the glass that were inserted into silicone tubing, but it is not feasible that catastrophic sloughing could account for the complete and sustained removal of bacteria from the surfaces. It would be interesting to see if this effect is reproducible with other substratum materials. The best test would be to analyse the mature biofilms present *in situ* to determine the extent and activity of bacteria present.

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APPENDIX

Appendix - The microbiological quality of drinking water leaving Strensham treatment works

Some of the data obtained from routine microbiological analyses at Severn Trent Water's Strensham Treatment Plant are presented below, with permission. The concentration of *E. coli*, other coliforms and heterotrophic bacteria in the water leaving the plant via the south mains, which serves Warwick University, is determined daily. No coliforms or confirmed *E. coli* 100 ml⁻¹ were observed at any time during 1997. The concentration of heterotrophic bacteria, enumerated after incubation on R2A at 37°C for 48 hrs or at 20°C for 72 hrs, is shown graphically in Fig. a1. The 1997 data on the numbers of unicellular eukaryotes in the water leaving Strensham via the south mains, determined microscopically each week, are shown in Fig. a2.

The continuous flow model was started on 3rd June 1997 in order to monitor biofilm accumulation (Sections 6.5-6.7). The period over which the continuous flow model was running for this purpose is indicated in Figs a1 and a2. Peptone was added to the biofilms on 12th Aug 1997, chlorine was added on 26th Aug 1997 (Sections 6.6 and 6.7 respectively) and the experiment was terminated on 2nd Sep 1997.

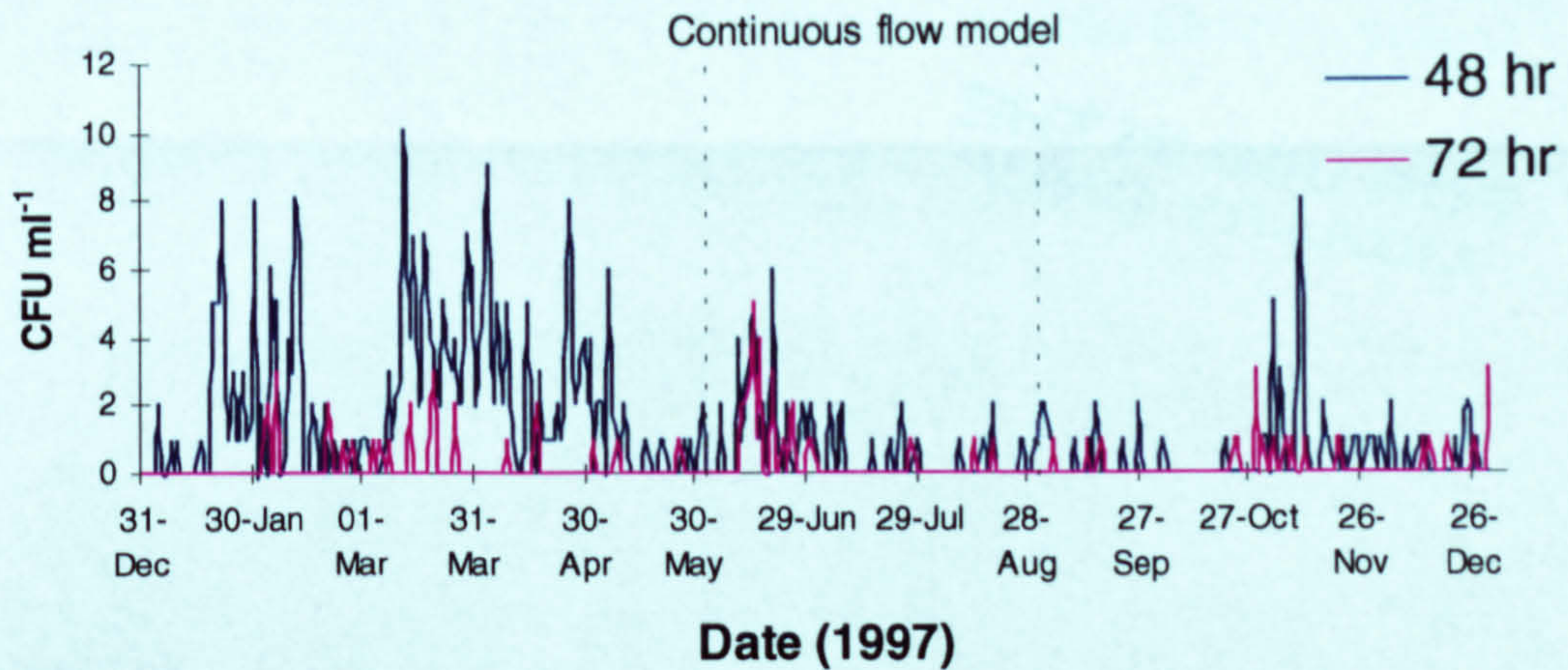


Figure a1 The concentration of heterotrophic bacteria in the final water distributed from Strensham treatment plant along the south mains during 1997. The data were provided by Severn Trent Water plc. Heterotrophic plate counts were determined by spreading samples onto R2A and incubating at 37°C for 48 hrs or at 20°C for 72 hrs. The period when the continuous flow model was run at Warwick University to monitor biofilm accumulation and responses to peptone and chlorine is indicated.

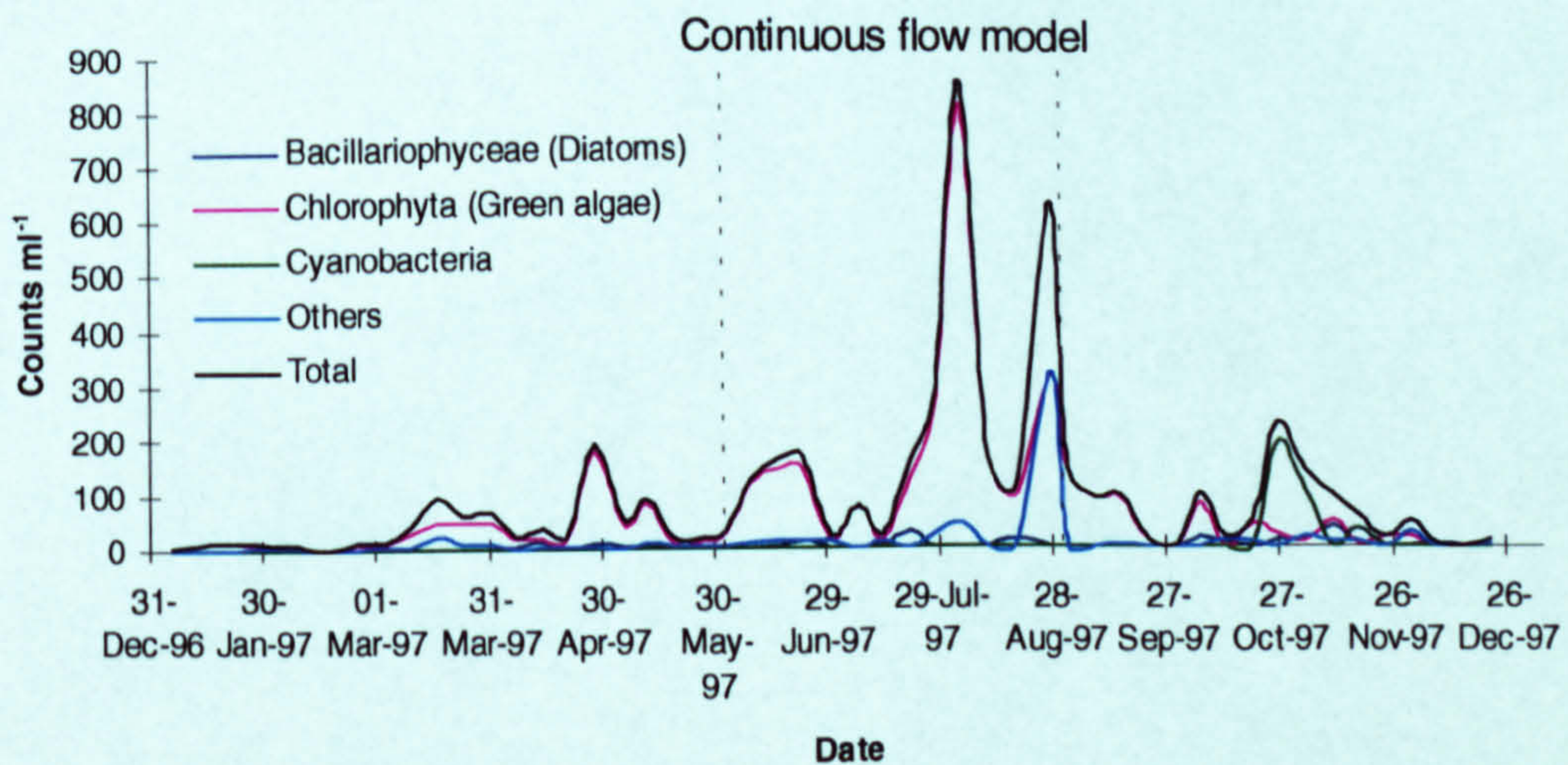


Figure a2 The concentration of unicellular eukaryotes in the finished water distributed from Strensham treatment plant along the south mains throughout 1997. The data were provided by Severn Trent Water plc. Unicellular eukaryotes were enumerated after microscopic analysis using a haemocytometer. The period when the continuous flow model was run at Warwick University to monitor biofilm accumulation and responses to peptone and chlorine is indicated.