THE PERSISTENCE OF VIRUSES IN BIOFILMS FROM WATER DISTRIBUTION SYSTEMS

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by

Sarah Anne Strugnell BSc (Hons)
Department of Microbiology
University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

STATEMENT

The work in this thesis was carried out by the author, unless otherwise stated in the text, during the period October 1996 to September 2000, under the supervision of Professors W.G. Grant and S. Myint in the Department of Microbiology, University of Leicester. This thesis is submitted for the degree of Doctor of Philosophy at Leicester University, and has not been submitted in full or part for any other degree.

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Sarah Anne Strugnell

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SYNOPSIS

Biofilms form in all water distribution systems providing a protective haven for waterborne pathogens such as *Legionella pneumophila*. Capable of entrapping substances from the bulk fluid phase, such dynamic systems also contribute to the dispersal of these pathogens back into the drinking water causing a potential health risk to the community concerned. In addition to bacteria, enteric viruses have also been isolated from treated drinking water. Their high resistance to bactericidal agents such as chlorine and their ability to persist in aqueous environments allows them to evade water treatment processes. This also has major health implications and raises the question of whether viruses are able to persist in water distribution systems and, if so, what mechanisms do they adopt?

This research aimed to investigate whether water distribution system biofilms could act as a potential reservoir for viruses. Model water distribution systems were set up in the laboratory and biofilm formation was monitored. Two model viruses, bacteriophage lambda and poliovirus, were introduced into the system and their interactions with the two phases of the biofilm model (planktonic and sessile) were followed over a period of time using molecular detection methods and traditional culture procedures. In the planktonic phase both viruses decreased in number eventually disappearing, while in the sessile phase some viral adsorption was initially apparent but this too disappeared with time. In some cases the virus persisted in the sessile phase for longer than it did in the planktonic phase but this was not reproduced consistently. Enumeration of virus showed no correlation between viral numbers in the two phases indicating that much of the virus was being destroyed or inactivated by the biofilm. The similar patterns observed for both viruses showed that this was a non-specific reaction by the biofilm and observations of virus in biofilm leachate alone suggested the biofilm matrix or its continual production of leachate was responsible. Molecular methods, as distinct from culture methods, revealed that poliovirus remained present in the model for the entire duration of every experiment. This suggested that the detrimental actions of the biofilm either caused virus inactivation or promoted its physical destruction to levels lower than the detection limits of the traditional culture methods.

Sarah Anne Strugnell

For Angela & Robert

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CHAPTER 1

INTRODUCTION

1.1The Biofilm

1.1.1 Background

Many microbiological investigations have traditionally centred on the analysis and growth of pure cultures of bacteria (Korber *et al*, 1995). Solid media techniques have allowed for the isolation of individual species from natural environments and their intensive study has served well in providing an accurate understanding of microbial genetics and metabolism. It has also facilitated the isolation and identification of pathogens in a variety of diseases and much data in this area has been compiled (Costerton *et al*, 1987). However it has now been clearly established that in nature, medicine and industry, the majority of bacteria exist in the sessile mode of growth within communities termed biofilms (Korber *et al*, 1995).

Biofilms and the advantages of surfaces in relation to bacterial growth were first discovered in 1943 by Claude Zobell. He noticed that in water samples taken from various environmental sources the rate of bacterial multiplication was related to the receptacle in which the sample was placed. The smaller the receptacle the greater the bacterial growth. From this he proposed that since smaller receptacles present a larger surface area per unit volume of water they must be beneficial to bacterial growth. His experiments confirmed this showing that many bacteria grow attached to solid surfaces. He deduced that their attachment to surfaces was facilitated by their production of a mucilaginous substance which also allowed for the concentration of nutrients in the vicinity of these sessile organisms thereby providing greater advantages to microbes. After this only a few experiments concerning biofilms were performed until 1978 when Geesey *et al* adapted a whole series of quantitative recovery methods for the enumeration of sessile bacteria in a mountain stream. These workers compared the numbers and activity of the sessile organisms with their planktonic counterparts and discovered that biofilm bacteria predominate in number and activity (Costerton *et al*, 1987). This finding launched a new interest in biofilm research in many different disciplines such as microbiology and engineering.

The full importance of biofilm research is now becoming clear with evidence of petrified biofilms in Precambrian Stromatolithic rocks dating back 3.5 billion years confirming that this mode of growth constitutes the oldest form of life on the planet (Flemming, 1993). In addition it has been estimated that approximately 99% of all bacterial life on earth exists within biofilm communities (Coghlan, 1996) and that there is almost no surface which is not or cannot be colonised by bacteria. Biofilms represent a unique form of life playing key roles in the production and degradation of organic matter and in the cycling of phosphorous, nitrogen,

sulphur and other inorganic compounds. A very successful form of organised microbial life, biofilms are capable of adapting to extreme environmental conditions such as that of temperature and pH (Flemming, 1993). Many types of hostile environments have been found to support biofilm growth including the leaves of parsley plants, prosthetic devices in the human body and even the aluminium basins used for the storage of spent nuclear fuel (Stickler, 1999).

1.1.2 The Structure and Function of Biofilms

A biofilm is defined as a complex consortium of microbes attached to a substratum and embedded in a matrix composed of extracellular polymeric substances (EPS) (Characklis & Marshall, 1990; Allison & Gilbert, 1992; Brown & Gilbert, 1993). Ecologists originally perceived biofilms to have homogeneous bacterial cell distributions with an essentially uniform extracellular polysaccharide matrix (Costerton *et al*, 1987). However the introduction of sophisticated digital imaging techniques such as confocal scanning laser (CSL) microscopy have shattered these theories. Such techniques have allowed for the study of live and fully hydrated biofilms in real time and have revealed a much more complex structure consisting of microbial cell clusters or microcolonies separated by interstitial voids (de Beer *et al,* 1994; Yang *et al*, 1995). The variable distribution of cells, microcolonies, extracellular polymers and void spaces has been termed "biofilm architecture" by Lawrence *et al* (1991). This biofilm architecture is not only a feature of mixed-culture biofilms but can also be observed in pure-culture communities suggesting that there is a fundamental relationship between biofilm structure and in situ function (Costerton *et al*, 1987). This and other evidence (Costerton *et al*, 1995; van Loosdrecht *et al*, 1995; Wimpenny, 1995; Zhang & Bishop, 1994) has lead to a general picture of biofilm structure to which the majority of biofilms adhere (see Figure 1.1).

The general consensus is that a biofilm system consists of various compartments. These are defined as the substratum, on which the biofilm accumulates, the biofilm itself and the bulk fluid which overlays it. The biofilm can then be subdivided into a further 2 compartments termed the base and surface films (Wilderer & Characklis, 1989).

The substratum plays a vital role in defining the subsequent biofilm formation. The characteristics of the substratum are decisive, especially during the initial stages of biofilm development, and may influence the rate of cell accumulation and the initial population distribution (Wilderer & Characklis, 1989). In general microbial communities found on inert sustrata are much greater and more diverse than those on biologically viable ones. However direct observations concerning the recovery of microbes from biological substrata, such as plant and animal tissue, have revealed very successful attached bacterial populations (Allison

Figure 1.1 - The Diagrammatic Representation of the Microcolonies and Water Channels of a Biofilm

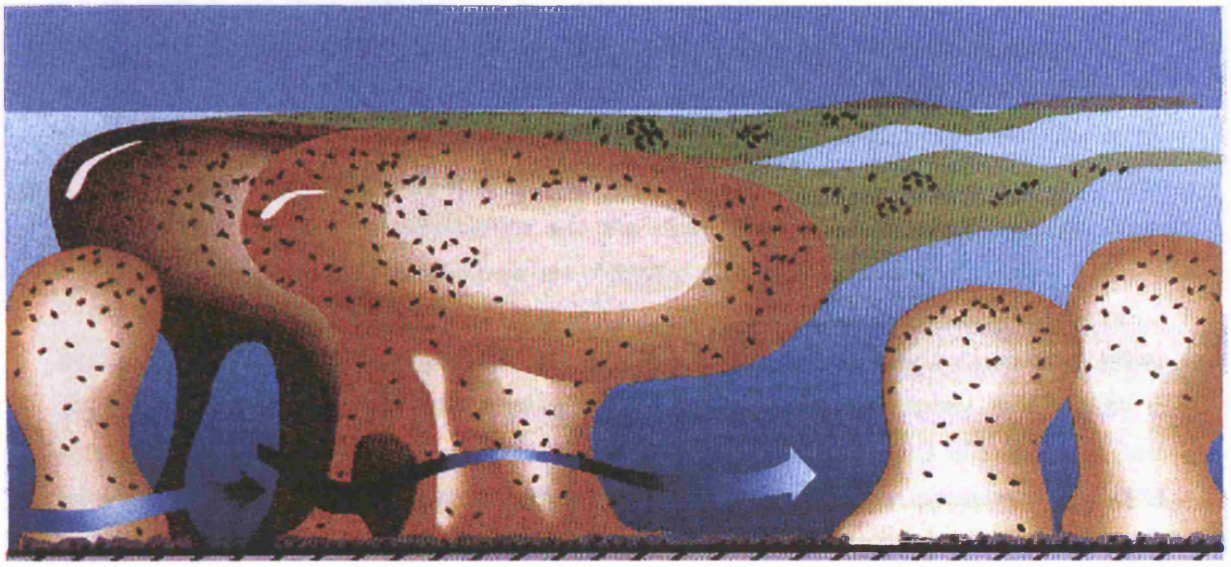


Figure 1.1 - The conceptual model of the architecture of a biofilm based on data collected by CSLM of living biofilms. This shows the matrix-enclosed microcolonies which are often deformed by high shear forces producing streamers that project into the bulk fluid. Convective fluid flow is seen in the water channels between and even below these microcolonies (Costerton *et al*, 1987). This picture was taken from Gilbert & Lappin-Scott (2000) Microbiology Today, vol 27.

& Gilbert, 1992). If the substratum is made of an inert impermeable, non-porous solid material then a well-defined interface will exist between biofilm and substratum. Additional surface properties such as roughness enhance biofilm development by providing the attached organisms with some protection from detachment (van Loosdrecht *et al*, 1995). However if the substratum is of a porous nature it may contain an additional gas or liquid phase providing more nutrients to the attached cells. If the pores are large enough microbes may settle within it thus causing the biofilm-substratum interface to become morphologically undefined. Semi-permeable membranes allow the feeding of the biofilm organisms with substrates from the substratum (Wilderer & Characklis, 1989). Similarly on biological substrata, for example on lung tissue, invading microbes may derive nutrients and moisture from the solid surface on which they colonise. Thus since microbes are metabolising entities the nature of their interaction with a potential substratum and the location of biofilm development will have profound effects on its expressed properties (Allison & Gilbert, 1992).

The lower levels of the biofilm are relatively firm and structured (Wilderer & Characklis, 1989) consisting of a dense layer of microbes and other matter bound tightly together by a polysaccharide matrix (Wimpenny, 1995). This high density of organisms is a result of their initial accumulation at the interface (Zhang & Bishop, 1994). They produce much EPS keeping them densely packed and function to anchor the biofilm to the surface. Above this lies the surface film which is much looser in structure with of irregular topography. It provides a transition between the bulk fluid compartment and the base film (Wilderer & Characklis, 1989). The basic structural and functional unit of this layer is the microcolony which is interspersed with less-dense regions or voids within the matrix (Costerton *et al,* 1994). Each microcolony consists of the progeny of cells whose stimulated growth established it (Costerton, 1995). It acts as a nucleus for attracting other cells of the same or different bacterial strains and thus it may be composed of either a single or multiple species of microbes (Khardori & Yassien, 1995). All organisms within it are clearly delineated by their exopolysaccharide matrix which holds them in stable juxtaposition and regulates their effective contact with the fluid phase. Multispecies microcolonies usually have symbiotic associations enabling them to utilise less readily biodegradable substances and to develop specific ecological niches promoting their improved survival. For example anaerobic zones may form within biofilms in aerobic environments and nutrient-rich compartments may develop in essentially oligotrophic systems (Flemming, 1993).

The microcolony appears to have great complexity. Simple proliferation of cells would produce a mound-like colony on a surface however direct CSL microscopy has revealed microcolonies to be mushroom-shaped (Costerton, 1995). This presupposes a measure of growth control by cell-cell communication and has been recently coined quorum sensing (Pennisi, 1995).

The interstitial spaces or voids that lie between these microcolonies have been found to be of great importance in the transport of substances to and from the biofilm. The polysaccharide matrix which is concentrated in dense proportions around the microcolonies is much less densely distributed in the interstitial spaces. This more sparse distribution produces ill defined but functional water channels (Costerton *et al*, 1994). Many workers have shown that in these voids liquid flow is possible while in the microcolonies liquid remains stagnant. Consequently, in the voids or water channels mass transport takes place by both convection or molecular diffusion, whereas, in the microcolonies, only diffusion can occur (de Beer *et al,* 1994; Yang *et al,* 1995). In essence the individual bacteria of the biofilm enjoy some of the advantages of multicellular life, having a primitive "circulatory system" delivering nutrients from the bulk fluid to the microcolonial niche and removing metabolic products by the same process (Costerton *et al,* 1994).

The bulk liquid compartment influences the biofilm structure. One of the major factors that determine the rate of biofilm formation is the nutritional status of the surrounding aqueous phase. In extremely oligotrophic environments, such as in water, organic nutrients tend to associate with available surfaces thus triggering biofilm formation (Costerton *et al*, 1995). The presence of flow rates has an effect on the types of organisms colonising the surface. For example, under turbulent conditions bacteria capable of rapid adhesion have a distinct advantage over slower colonisers. Furthermore organisms that secrete exopolymers with a high resistance to shear forces would also prosper under turbulent flow conditions (Allison & Gilbert, 1992).

One of the most important components of the biofilm is the extracellular polysaccharide matrix (EPS) produced by the bacteria themselves. The EPS consists of a network of both inorganic and organic compounds (Christensen & Characklis, 1990; Flemming, 1995) and performs many functions within the biofilm. It plays an important role in biofilm structure (Jahn & Nielsen, 1995), acts to various degrees as a diffusion barrier and molecular sieve (Lawrence *et al*, 1994) and provides the forces responsible for the cohesion of the microbes and adhesion to the substratum (Flemming, 1995). In addition the EPS has been described as a sorption site (Flemming, 1995) capable of entrapping nutrients and other substances (Robinson *et al*, 1995). This property has been exploited in many biotechnological applications, for example, it is used for sorbing copper from aqueous media (Flemming, 1995).

The biofilm mode of life offers significant advantages to microorganisms. In oligotrophic conditions characterised by low energy substrates and nutrients, most microbes are able to adjust by forming such microbial communities (Marshall, 1987). The few molecules already present and those originating as excretions or lytic products from other organisms tend to concentrate at interfaces thus providing these organisms with increased growth prospects. In

addition mixed species biofilms allow for the co-operative mobilisation of nutrients (Gilbert *et al,* 1993) and in flowing systems the EPS entraps nutrients encouraging the rapid growth and reproduction of colonising bacteria (Marshall, 1987, Flemming, 1993). A higher level of protection from hostile conditions such as fluctuations of pH, biocide concentration shocks and dehydration, are also afforded to biofilm bacteria (Flemming, 1993) and their close proximity allows for genetic exchange (Allison & Gilbert, 1992).

1.1.3 Biofilm Formation and Dynamics

The complex process of biofilm formation can generally be divided into 6 sequential steps. Firstly certain events bring the organisms into close proximity with the surface. This is followed by steps 2 and 3 which involve the adhesion of the organisms to the surface beginning with a reversible stage followed by an irreversible one. The organisms then adapt their phenotypes allowing for their colonisation and subsequent microcolony formation (step 5) and finally a dispersive phase (step 6) allows for their recolonisation of new surfaces and thus continues the cycle of events (Gilbert *et al,* 1993).

The initial interaction of bacteria with a surface is facilitated by many factors. These include environmental conditions such as electrolyte concentration, pH and temperature, the fluid dynamics of the liquid phase, the species of bacteria and their nutritional status and the physicochemical characteristics of the substratum. In flowing systems the fluid dynamic forces cause organic molecules, ions and some bacteria to concentrate at the viscous boundary layer forming a conditioning film (Allison & Gilbert, 1992). The physiochemical characteristics of this film and those produced by the substratum further attract bacteria. Surface roughness plays a vital role in presenting niches within which microbes may be protected from shear forces and thus influences the initial adhesion process (Gilbert *et al*, 1993). In static or quiescent systems the deposition of bacteria is governed by certain transport mechanisms. These include brownian motion, bacterial motility or settling via gravity (Lawrence *et al,* 1995). Recently Pratt and Kolter (1998) analysed the role of bacterial motility in the initiation of biofilm formation and discovered that mutant strains of *E. coli*, which lacked or had paralysed flagellae, were severely defective in their ability to form a biofilm. Additionally other workers (O'Toole & Kolter, 1998) studying surface attachment deficient (*sad*) mutants of *Pseudomonas fluorescens* confirmed that the presence of flagellae as well as some other factors played a role in this process. They also presented evidence that protein synthesis was required for the initiation of biofilm formation and that certain environmental signals were also involved.

Bacterial attachment to the surface follows by both a reversible and an irreversible stage. This is commonly explained in most review articles using the Derjarquin, Landau, Verwey and Overbeek (DLVO) theory (Lawrence *et al*, 1995; Gilbert *et al*, 1993). This theory dictates that the sum of the attractive (van der Waals) and repulsive (electrostatic) forces determine firm cell adhesion at the bacterial-substratum interface (Lawrence *et al*, 1995). Particle attraction to a substratum occurs over both short (≤1 nm) and long (5-10 nm) distances and these are termed the primary and secondary minima respectively. Between these 2 minima there is a zone of maximum electrostatic repulsion which decreases with solution electrolyte strength. Thus as the ionic strength is increased so the repulsive forces are decreased. In natural ecosystems intermediate ionic strengths predominate and in this case cells will accumulate firstly at the secondary (reversible attachment) and subsequently at the primary minimum (irreversible attachment). As discussed above the possession of appendages such as flagellae and fimbrae and the production of exopolysaccharide glycocalyx polymers serve to bridge the gap between primary and secondary minima and to anchor the cells in place. Alternatively the energy to propel the cells from the secondary to the primary minima may be provided by brownian motion (Gilbert *et al*, 1993).

Once attached, cell division produces sister cells bound within the matrix thus initiating the development of adherent microcolonies (Costerton *et al,* 1987). Other planktonic cells from the bulk liquid phase are also sequestered and a mature multispeces biofilm begins to develop. At this stage there is much evidence to suggest that these sessile organisms become phenotypically distinct from their planktonic counterparts (Costerton, 1995; Bradshaw, 1995; Brozel, 1994; Brown & Gilbert, 1993) having more active reproduction and general metabolic activity (Costerton *et al,* 1987). One such difference is that these sessile cells begin to secrete additional polymers (EPS) leading to the formation of an extensive glycocalyx which eventually envelopes the entire population. This functions to anchor the biofilm to the surface and stabilise it against fluctuations in the surrounding macro-environment (Allison & Gilbert, 1992; Gilbert *et al*, 1993). Consequently, the biofilm finally consists of both single cells and microcolonies embedded in a highly hydrated, predominately anionic matrix of bacterial exopolymers and trapped extraneous macromolecules (Costerton *et al*, 1987). The continued development of the biofilm involves microbial cell-cell communication or quorum sensing between primary and secondary colonisers possessing different nutritional requirements and the whole system eventually reaches a plateau with a certain thickness and metabolic capacity (Allison & Gilbert, 1992).

Bacterial growth in biofilms also brings with it the need to disperse. This is a survival strategy ensuring that the micro-organisms are able to recolonise new surfaces (Allison & Gilbert, 1992). As stated previously these organisms are phenotypically different from those in the sessile mode of growth. As their function is concerned with recolonising new surfaces they are more committed to motility than reproduction (Costerton *et al,* 1987). Dispersal is an

essential part of biofilm life as it allows the microorganisms in the biofilm to extend their area, promotes the survival of the microbes concerned and so continues the cycle of biofilm development (Gilbert et al, 1993).

1.1.4 Interactions and Succession in Biofilms

It is generally accepted that larger populations of organisms are more successful that their individual counterparts. This is evident when observing bacterial susceptibility to antimicrobial agents. A population of high density will fair better than a more dilute one. Additionally a larger population of pathogenic organisms will produce infection in a host while the individual strains or a smaller population will be unable to overcome the host's immune mechanisms. These examples suggest the ability of micro-organisms to interact with each other in a positive manner. Thus it is more favourable for the micro-organism to be a member of a community than to live autonomously. The major reason for biofilm formation is therefore to promote the success of the community (Atlas & Bartha, 1998).

Within these communities many interactions take place. Positive interactions are deemed co-operation and can be further classified as neutralism, commensalism, synergism and mutualism. Negative interactions also occur and in the long run can be beneficial to the community as a whole usually serving as feed back systems. These are divided into competition, parasitism and predation. Neutralism is a lack of interaction between two populations and generally occurs when they are spatially distant from each other. A commensal relationship is where one population benefits while the other remains unaffected. Micro-organisms interact in this way for a variety of reasons. Some organisms modify their surrounding habitat allowing other less able organisms to prosper, some produce needed growth factors while others transform insoluble substrates into soluble ones. Bradshaw *et al* (1995) showed that in the presence of a facultative anaerobe, *Fusobacterium nucleatum*, obligate anaerobes were able to survive and grow in oxidised environments. This type of interaction is especially important in biofilms where the creation of different oxygen gradients has been noted (Costerton *et al,* 1994). Other workers (Bell *et al,* 1974) found that *Flavobacterium brevis* excretes cysteine a required nutrient for the survival of *Legionella pneumophila.* These associations can also occur between bacterial and protozoan species. For example Barbaree *et al* (1986) repeatedly showed that an amoeba and a ciliate, *Tetrahymena sp*, were able to support intracellular multiplication of *L. pneumophila* in water cooling towers affording the latter organism protection.

1.1.5 The Distribution of Biofilms

Bacterial biofilms can develop on almost any surface where viable microorganisms are present (Allison & Gilbert, 1992). Their formation on surfaces is a universal strategy for the promotion of survival and optimum positioning with regard to available nutrients (Costerton *et al,* 1987). This much protected mode of growth allows for the development of consortia with co-operative metabolic responses and thus provides spatially defined niches for microorganisms with differing growth requirements (Costerton,1995; Brown & Gilbert, 1993; Gilbert & Allison, 1999).

Naturally formed biofilms can have both beneficial and detrimental roles depending on whether their development is controlled or unintentional (Bryers, 1993). Biofilms of benefit to the human population are actively encouraged to grow from a variety of reasons. In the sewage treatment process biofilms are part of the purification technology forming on trickling filters, rotating discs, membrane reactor sand aerobic and anaerobic fixed beds. The purification effect of sand filters in drinking water treatment is due to biofilms forming on the sand grains and biofilm reactor technology has been successfully applied to air purification processes (Flemming, 1993). The immobilisation of simpler microbial populations is also employed in industrial plants (Allison & Gilbert, 1992). For acetic acid production *Acetobactor spp.* biofilms are formed on large columns of packed beechwood chips and alcoholic solutions are trickled over them. In addition approximately 10-20% of the copper mined in the US is extracted by microbial leaching and this technology has been extended to other metals such as uranium, silver, cobalt, molybdenum, nickel and gold (Bryers, 1993). During biofilm formation the production of extracellular polysaccharides is critical and these are also exploited for commercial purposes. For example *Flavobacterium uligosum* MP-55 biofilms produce heteroglycans used in the treatment of cancer and *Erwinia tahitica* biofilms produce polysaccharides used for paint thickening (Bryers, 1993). Biofilms also form on biological substrata. Most sections of the gastrointestinal tract are colonised by autochthonous bacterial biofilms which provide a high degree of protection from other pathogenic organisms (Allison & Gilbert 1992).

A large number of biofilms forming in nature have detrimental effects and this is compounded by our inability to control or restrict their development (Allison & Gilbert, 1992). In industrial environments biofilms contribute a range of costly problems (McFeters *et al,* 1995). They cause reduced heat transfer efficiency in heat exchangers, lead to higher energy consumption and shorter lifetime of plant components and increase the frictional resistance in pipelines and even on ship hulls. In municipal drinking water biofilms cause contamination and on metal and rock surfaces they induce biofouling and weathering respectively (Flemming, 1993).

Of medical importance, biofilms of *Pseudomonas aeruginosa* forming on lung tissue produce persistent infections in cystic fibrosis patients, *E. coli* causes bladder infections and *Staphylococcus aureus* is associated with osteomyelitis (Allison & Gilbert, 1992). Classically associated with inanimate surfaces of biomaterials, they form on hip replacements and intravenous catheters. The most common implantable device is the central venous catheter. Biofilm formation on this produces severe infections of bacteraemia, fever, shock and endocarditis lasting for several months. While almost any microbe can be responsible, most are due to staphylococci, gram negative bacteria such as *Klebsiella spp.* and *Candida albicans* (Bayston, 1999). In the mouth biofilms form at stagnant sites between the teeth, in pits and fissures on the surfaces of molars and premolars and around the gums (gingival crevice). Here these biofilms are termed plaque and are the cause of 2 of the most prevalent diseases in the developed world namely dental caries and periodontal diseases (Marsh & Bradshaw, 1995).

The ubiquitous nature of the biofilm mode of growth and its importance in microbial ecology is no longer in dispute (Brown & Gilbert, 1993). Biofilms are a nuisance in industrial processes (de Beer *et al*, 1994), may be life threatening *in vivo* and, in contrast, may be exploited in biotechnological applications. In all these areas the control of biofilms is still being greatly researched and the mechanisms of biofilm resistance to antimicrobial agents is under continued discussion. Prevention of biofilm formation is still causing difficulties. Suggested methods include producing surface coatings containing antibiotics, blocking the adhesion process mediated by pili and flagella or interfering with cell-cell communication involved in biofilm development (Stickler, 1999). To date all attempts to control or prevent biofilm formation have been unsuccessful and further research in this area continues.

1.2 Water Distribution Systems

1.2.1 The Provision of Clean Drinking Water

Water occupies the majority of the earth's surface and is vital for life. For the human population surface and subsurface water plays a vital role as a source of drinking water and for waste disposal. For microorganisms water is a unique habitat within which they have developed many strategies for their survival. The contamination of this ground water by domestic and industrial wastes leads to many environmental concerns for the human population. In these situations, microorganisms can be either the route of the problem or its solution. Thus the provision of clean drinking water for the human population involves the removal of wastes and pathogenic microorganisms and this may be achieved, in part, with the help of certain heterotrophic microorganisms involved in the decomposition of organic matter (Prescott *et al*, 1990).

Liquid wastes (domestic sewage), produced daily by human activity, must first be treated to reduce the biological oxygen demand (BOD) before being discharged into surface waters such as rivers, lakes and oceans. BOD is a measure of the oxygen consumption required by the microbial oxidation of readily degradable organics and ammonia found in sewage. The reduction of BOD during the sewage process consists primarily of 3 stages termed primary, secondary and tertiary treatments. Following the removal of larger objects such as plastics, grits and floating scum, the primary treatment employs physical methods to remove smaller suspended solids. This is achieved in settling tanks or basins where solids are sedimented and drawn off at the bottom. This reduces the BOD by approximately 30-40%. The secondary treatment relies on microbial biodegradation to mineralise organic matter through the use of various devices such as aerobic and anaerobic tanks, trickling filters and sludge digesters, further reducing the BOD to about 80-90%. The tertiary treatment then involves physical, chemical and/or biological methods to complete the process. The resultant effluent is then discharged into surface waters which in turn is used as a source for drinking water, recreational activity and land irrigation (Atlas & Bartha, 1998).

Initially taken from surface waters, municipal drinking water must again undergo several purification steps before its distribution to the general public. This is a critical process for the promotion of public health allowing for the removal of chemical and microbial contaminants which may cause health problems. As for sewage treatment this process usually requires several steps most commonly these are aeration, sedimentation, coagulation, filtration and disinfection.

Raw water may contain large concentrations of ions which must be removed in the early stages of the purification process. Iron and manganese ions are common and can be removed by aeration of the water. Upon exposure to air these ions precipitate out and they can then be removed along with other suspended particles at the sedimentation stage. As for the primary treatment of sewage large sedimentation basins are employed to allow these materials to settle out. The partially clarified water is then coagulated using a mixture of alum and lime to allow for further precipitation of substances which are once again allowed to settle out of solution. This procedure adequately removes microorganisms, organic matter, toxic contaminants and suspended fine particles. The water is then passed through rapid sand filters which depend on the physical trapping of fine particles and flocs and further removes 99% of the remaining microbes present. Following this the water is disinfected usually by chlorination but the use of ozone at this stage is becoming increasingly common (Prescott *et al*, 1990). The chlorination of municipal drinking water further removes pathogenic microorganisms of public health concern. This was introduced in 1937 after the water supply was implicated in a large outbreak of typhoid fever in Croydon, Surrey (Walker *et al*, 1995). At present the dose used must be large enough to leave the residual free chlorine at a concentration of $0.2 - 2.0\ mgL^{-1}$.

In recent years it has been recognised that certain pathogens namely *Giardia*, *Cryptosporidium* and enteric viruses are able to evade these treatment processes. In 1990 *Giardia* was recognised as the most commonly isolated waterborne pathogen in the United States and it's increased incidence lead to the introduction of slow sand filters prior to the disinfection process. This treatment involves the slow passage of water through a sand bed in which a microbial film covers the surface of each grain. Any *Giardia* cysts and other pathogens will be removed by their adhesion to this surface biofilm. In addition, although coagulation and filtration processes reduce the viral load to between 90-99%, some viruses remain. Further viral inactivation is achieved through the use of chemical oxidants, elevated pH and photooxidation. None of these processes, however, is considered to provide the sufficient removal of all known pathogens (Prescott *et al*, 1990).

The importance of bacterial quality as a measure of water quality has been recognised for many years (Walker *et al*, 1995). To determine water quality workers have generally measured the levels of indicator organisms. The main group of such indicator organisms monitored being the coliforms with the rationale that the absence of these organisms is evidence of the absence of waterborne pathogens (McMath & Holt, 1999). The use of coliform detection in the regulation of water quality has been apparent since 1905 when workers recognised that *Bacillus coli* (later renamed *Escherichia coli*) was a good indicator of faecal contamination. Since no specific methodology existed, at that time, to adequately isolate this organism the total coliform levels were monitored instead and this technique is still employed today (McMath & Holt, 1999).

1.2.2 Biofilms in Water Distribution Systems

Water supplied for drinking and domestic purposes is treated to EEC and World Health Organisation (WHO) standards to prevent the spread of pathogens (Keevil *et al*, 1995). However this water is not sterile and consequently contains a diverse range of heterotrophic organisms (Robinson *et al*, 1995). The range of organisms recovered from potable drinking water include genera of *Alcaligenes, Pseudomonas, Flavobacterium, Achromobacter, Klebsiella, Spirillum, Arthrobacter, Gallionella* and *Lepothrix* species (Walker *et al*, 1995). Since direct estimates in bacterial drinking water suggest that there are around 10^6 cellsml^{-1} (Jakubovics & Dow, 1997) it is not surprising that biofilms form in all water distribution systems below 60°C (Keevil *et al,* 1995).

The proliferation of biofilms on the internal surfaces of pipes in water distribution systems has been frequently reported (McMath *et al*, 1997). However the thorough examination of biofilm accumulation and composition has proved difficult in these systems for a variety of reasons. Some workers (Le Chavallier *et al*, 1987; Herb *et al*, 1995) obtained samples directly from water distribution systems during flushing, pigging and cleaning procedures. Observations from these samples are accurate but the interpretation of data is limited because the extent of biofilm removal from the pipe surface and the relation between sample volume and pipe surface is unknown (van der Wende & Characklis, 1990). These problems have been combated by other workers who have either installed model devices, such as the Modified Robbins Device (MRD), directly into water distribution systems (Manz *et al*, 1993; van der Kooiji *et al*, 1995) or produced representative model biofilm systems in the laboratory (Rogers *et al*, 1994; Lisle & Rose, 1995; Frais *et al*, 1994; Szewzk *et al,* 1994; Robinson *et al*, 1995). The former method is the most accurate but has some limitations concerned with sample removal, handling and transportation. The latter method, on the other hand, is preferred by most workers because it is easier to perform and all parameters can be accurately monitored. In addition to this a variety of methods have evolved for the analysis of these samples. Most are cultured and enumerated using agar and microscopic methods (Le Chavallier, 1987; Frais *et al*, 1994), electron microscopy has been employed to examine morphology (Rogers *et al*, 1994) as well as fluorescent oligonucleotide probes (Manz *et al,* 1993) to determine composition. More recently Confocal Scanning Laser Microscopy (CSLM) (Lawrence *et al*, 1991; Lawrence *et al,* 1994; Costerton *et al*, 1994; Labarbe *et al*, 1997; Surman *et al*, 1996) and genetically engineered strains (Pratt & Kolter, 1998; O'Toole & Kolter, 1998) have been used to study fully hydrated biofilms in real time and biofilm formation methods respectively. The latter 2 methods being performed in model systems within the laboratory.

The extensive study of water distribution system biofilms is of increasing significance for many reasons. Such biofilms play a key role in the contribution of the microbial degradation of

both the pipe materials and the quality of the drinking water being distributed. They produce a variety of taste and odour complaints, cause the extensive corrosion of pipe materials and allow the regrowth of certain undesirable pathogenic microorganisms (Kerr *et al*, 1997). In addition owing to the large surface area provided by these pipe interiors, such biofilms represent a substantial reservoir of microorganisms and these sessile organisms are afforded approximately 600 times more resistance to disinfection regimes than their planktonic counterparts (Keevil *et al*, 1995). Recently workers have noted that potable water biofilms can be established with cell densities great enough to satisfy the primary conditions required for genetic exchange. Lisle and Rose (1995) demonstrated that natural transformation can occur in such biofilms at frequencies similar to those found in natural waters. This raises more health implications as the exchange of nucleic acids, such as antibiotic resistance genes on plasmids, in this particular environment will lead to the increased exposure of these more resistant organisms to the general population.

Another concern attributed to biofilms in water distribution systems is the production of taste and odour complaints in the distributed water. *Arthrobacter spp.* have been found to predominate in cast iron and galvanised systems and were reported to be responsible for rapid discolouration of drinking water (Le Chavallier *et al*, 1987). Certain species of *Actinomyces* and fungi have also been identified as the cause of reduced water quality and higher organisms, such as *Asellus* (water shrimp), which are able to proliferate in biofilms, have been detected at customers taps. As stated previously the use of chloramine for water disinfection has also produced complaints allowing for the growth of ammonia-oxidising bacteria such as *Nitrosomonas* which causes taste problems and failures of regulatory standards (O'Niell *et al*, 1997).

The corrosion or biofouling of pipe materials in water distribution systems is an added expense incurred by the water companies and has been attributed to the presence of biofilms. Corrosion is an interfacial process which depends on local heterogenities, redox potential, pH and the concentration of oxygen and electrolytes. All of these factors are influenced by biofilms. In addition certain microorganisms have been implicated in the biofouling process. Certain species of heterotrophic bacteria produce organic acids which concentrate in the biofilm-substratum interface and cause pitting. For example a community of aerobic pseudomonads have been shown to cause pitting of copper (Angell *et al*, 1993). Slime forming organisms contribute local heterogenities causing the formation of oxygen gradients and thus provide a habitat for anaerobic organisms such as sulphate reducing bacteria. These anaerobes further contribute to the acid corrosion of the pipes (Flemming, 1993) and have been shown to cause pitting in stainless steel (Angell & White, 1995). Where chloramine is used for water disinfection the low levels of ammonia eventually produced by its breakdown provide a substrate for nitrifying bacteria which again cause acid corrosion. These organisms also contaminate the water with nitrites (O'Niell *et al*, 1997). Iron and manganese oxidising

bacteria, such as *Gallionella* and *Lepothrix spp.*, also aid in the corrosion of drinking water lines forming tubercles which may provide a haven for bacterial pathogens (Flemming, 1993).

During the monitoring of drinking water it has been assumed that the presence of coliforms such as *E. coli* are the result of faecal contamination (Robinson *et al,* 1995). However a growing body of evidence has now become apparent to suggest that in certain cases their presence is due to regrowth and survival in biofilms (McMath & Holt, 1999). Le Chavallier *et al* (1987) detected a large increase in coliform densities in a 1.1km pipeline and proposed that, given the detention time between sampling points, bacteria in the water column would have had to grow at a faster rate than one division every 30 minutes. Since this is not possible under the low nutrient conditions, low temperature and high chlorine residuals present they deduced that such coliforms must have come from the biofilm in the pipeline. These findings complicate regulatory monitoring systems and pose the question that if these organisms can regrow in such oligotrophic environments can other more pathogenic organisms?

1.2.3 Biofilms Associated with Waterborne Infection

Despite extensive water treatment processes incidences of waterborne infection still occur in the developed world and potable drinking water has been implicated in many cases. Between 1977 and 1986 a total of 15 outbreaks associated with waterborne disease were identified in the UK with *Campylobacter enteritis* and viral gastroenteritis accounting for 66% (Walker *et al*, 1995). More recently cryptosporidiosis accounted for 64% of 11 outbreaks in the UK between 1987 and 1990 (Microbial biofilms) while in Milwaukee, Wisconsin (USA) a large outbreak of cryptosporidiosis occurred with 400,000 cases and more than 100 fatalities (Young, 1996). In fact over the last 10 years the waterborne transmission of *Cryptosporidium* has affected an estimated 418,000 individuals in the UK and United States and some of these incidences have been associated with water that has received adequate treatment and monitoring (Fricker & Smith, 1997). Moreover *Cryptosporidium*, *Giardia* and certain enteric viruses have been detected in water free of coliforms which was deemed fit for human consumption (McMath & Holt, 1999) and, of more concern, these organisms are more resistant to the chlorination procedures used in the water treatment process.

The apparent increase in the number of outbreaks is probably due to the more active surveillance of water systems and better techniques for detecting infection. However it is likely that there is still an underestimation of cases (McFeters, 1984). The reasons for this is due to the criteria for declaring outbreaks. Two cases must be recorded for an outbreak to be registered, the source of the outbreak may not always be apparent and the aetiological agent responsible may not be identified. Since approximately 45% of cases have an identifiable

cuase the majority of cases are excluded. Specific culture techniques available for certain microorganisms have previously implicated organisms such as *Salmonella, Shigella* and *Giardia* as the cause of the majority of waterborne infections (Walker *et al*, 1995). However this is probably not entirely correct.

Recently there has been a growing body of evidence to suggest that those organisms that can evade the water treatment process can become incorporated into the biofilms in water distribution systems. Such biofilms provide a haven for these potential pathogens against environmental stresses, grazing predators and disinfection (Keevil *et al*, 1995). Certain species of *Flavobacterium* and *Pseudomonas* have been isolated from water distribution system biofilms and are known opportunistic pathogens. *Peudomonas aeruginosa* being of major importance as it is a common problem in hospitals causing nosocomial infection (Walker *et al*, 1995). Other emerging pathogens such as *Aeromonas hydrophila,* which is ubiquitous in freshwater environments, have also been shown to evade the water treatment process and become incorporated in water system biofilms (Keevil *et al*, 1995). There is evidence to suggest that this organism causes diarrhoea associated infection and its presence in a water system in Australia was linked to an outbreak of this kind (Mackerness *et al*, 1993). *Cryptosporidium* oocysts have also been isolated from water distribution system biofilms where they were able to survive in an infectious state for many weeks (Keevil *et al*, 1995). Another alarming finding was recently reported by workers at the University of Aberdeen who successfully managed to grow *Helicobacter pylori* in a model water distribution system biofilm. This organism, which has been implicated in a wide variety of gastrointestinal infections such as stomach ulcers and gastric cancers, was able to survive in the biofilm even after unchlorinated water was flushed through for 192 hours (Copley, 1999).

Co-operative metabolic processes occurring in the biofilm further enhance the survival of potential pathogens. These processes allow more fastidious microorganisms to proliferate in oligotrophic environments such as water where they would otherwise be incapable. For example, it has previously been stated that certain microorganisms produce oxygen gradients in the biofilm. Thus microaerophillic habitats arise allowing organisms such as *Campylobacter spp.* to survive and grow (Keevil *et al,* 1995). Additionally, the colonisation of plumbing systems by *Legionella pneumophilla*, the agent responsible for Legionnaires' disease and Pontiac fever, is well documented (Rogers *et al*, 1994). Incapable of growth in sterile water, this organism obtains much required nutrients from other microorganisms such as *Flavobacterium* spp., cyanobactera and amoebae. Its association with *Acanthamoeba* is also well documented. Trophozoites of this organism have been shown to support the intracellular replication of *L. pneumophila* (Kilvington & White, 1994) and once encysted provides increased protection from chlorine residuals in water (Atlas & Bartha, 1998). Thus *L. pneumophilla* must associate with biofilms for its own survival (Keevil *et al*, 1993).

This area of research is relatively new but the evidence so far suggests that pathogenic organisms are able to persist and proliferate within biofilms in water distribution systems. Owing to the dynamics of such biofilms these organisms will be periodically dispersed back into the bulk liquid phase by both active and passive sloughing where their distribution to the general public may cause health problems. Additionally the increased resistance of these sessile organisms is also of major public concern as their eradication from the water systems will prove difficult.

1.3 The Viruses

1.3.1 Viruses in the Environment

Over the past 50 years researchers have attempted to determine whether (a) viruses of public health concern are present in the environment and (b) whether such viruses pose a potential health risk with regard to the transmission of infectious disease in humans (Metcalf *et al*, 1995). As a result of this a great abundance of literature has emerged demonstrating the presence of viral agents responsible for human infection in almost every section of the environment. A large amount of the evidence gathered has centred around sewage, soil, fomites and various aquatic environments including lakes, rivers, oceans and treated drinking water (Payment *et al*, 1988; Egglestone *et al*, 1999; Payment *et al*, 1983; Tani *et al,* 1995; Genthe *et al,* 1995; Chapron *et al*, 2000; Hurst, 1991; Tambini *et al*, 1993). The reasons for this stem from the routes by which viruses are eliminated from the human body and their passage from the environment back into the human population (Nester, 1995).

Over 100 different viruses are excreted from the human body through the gastrointestinal tract, the respiratory system, the genito-urinary system and in other bodily fluids (Cook & Myint, 1995). However those viruses most able to persist in the environment are those of enteric origin including adenoviruses, caliciviruses, enteroviruses and hepatoviruses. Their ability to persist is due to their extreme resistance to unfavourable conditions (Guyader *et al*, 1994). Large numbers of enteroviruses may be found in sewage. Research has shown that the rate of such viruses shed in raw sewage ranges from 100 to 1000 plaque forming units (PFU) per 100 ml (Wallis *et al*, 1972; Wallis *et al*, 1979). These sections of the environment thus act as reservoirs for viral infection. Problems of pollution or simply the increased resistance of some sewage treatment processes allows viral dissemination to occur. Upon treatment of the sewage a large proportion of the viral load will be greatly reduced but some viruses will remain.

Following treatment the surface water will run into lakes, rivers, estuaries and oceans while sewage sludge will be disposed of in landfill sites and some used for the fertilisation of crops. Lakes, rivers and oceans are used for recreational purposes allowing the spread of infection to swimmers and other users. Some studies have shown that varying concentrations of viruses were detected in lakes and seawater used for this purpose (Rao, 1982).

Water taken from lakes and rivers are commonly used for the irrigation of crops further spreading any virus pollution onto the surrounding soil and onto the crops themselves. The

crops in turn are consumed allowing infection to spread into the population. Oceans support the growth of many organisms consumed by the human population and one of major concern is the consumption of filter feeders such as shellfish. Although these organisms do not become infected by the viruses they accumulate within them and humans become infected by consuming them when poorly cooked. Outbreaks of shellfish transmitted viral disease occur periodically, causing public health problems and economic loss to the seafood industry (Guyader *et al,* 1994). The largest epidemic traced to shellfish occurred in Shanghai in 1988. Hepatitis A was implicated and it involved 300,000 cases (Metcalf *et al,* 1995).

1.3.2 Viruses Associated with Waterborne Infection

Viruses are an abundant and ubiquitous component of aquatic ecosystems (Suttle, 1982). Of major public health concern, the enteric viruses including enteroviruses, hepatitis A virus, norwalk virus, reovirus, rotavirus, adenovirus and astrovirus, can occur in ground and surface waters used as a source for drinking water (Eaton *et al*, 1995). Such viruses are a common cause of a wide spectrum of infections including gastroenteritis, myocarditis and meningitis (Fuchs *et al*, 1993). More alarmingly, these viruses are able to survive for extended periods in the environment and can resist wastewater treatment processes and disinfection by chlorine, chloramine and ozone. They achieve this by their adsorption and occlusion in particulate matter (Hejkal *et al*, 1979; Lipson & Stotzky, 1984). The waterborne transmission of these viruses has now been well documented (Gerba & Rose, 1990). Surveillance studies suggest that 10 to 15 million illnesses due to non-polio enteroviruses occur each year. In addition it has been estimated that 4 in a thousand school age children in developing countries are affected by paralysis due to poliovirus infection. Control of these infections is now a priority in order to reach the eradication objectives of poliomyelitis established by the WHO (Fuchs *et al,* 1993).

Although enteric viruses are now used to indicate viral contamination of waters, testing is not mandatory and there is a lack of reliability and consistency associated with these testing procedures (Cook & Myint, 1995). Difficulties encountered with the isolation of enteric viruses from water samples has caused major problems and probably accounts for the limited number so far identified as the cause of waterborne outbreaks (Gerba & Rose, 1990). Enteric viruses have now been recovered from finished drinking water (Atlas & Bartha, 1998). Keswick *et al* (1984) isolated both enteroviruses and rotaviruses from 4 out of 9 finished drinking water samples which met acceptable limits of turbidity (<1.0 NTU), residual chlorine (>0.2 mgL^{-1}) and total coliform bacteria (<1 $CFU100ml^{-1}$) (Keswick *et al*, 1984). Wild type astroviruses were detected in water where a concurrent gastroenteritis outbreak was reported

(Pinto *et al*, 1996). Payment (1981) isolated viruses from all 60 samples of both raw and finished drinking water taken between September 1978 and August 1979 from the Pont-Viau water treatment plant and Sekla *et al* (1980) isolated enteric viruses from 6.7% of drinking water samples collected from a waterway system in Manitoba. In addition to this in the US between 1946 and 1980 acute gastroenteritis of unknown aetiology accounted for over 50% of documented waterborne outbreaks and viruses were attributed to 12% of these (Gerba & Rose, 1990).

The direct association of gastroenteritis outbreaks with the presence of viruses in the water supply is rare. This is because only a small percentage of viral illnesses are recognised and reported, asymptomatic infection is common among enteroviruses, there is still a lack of methods and facilities and the viruses may be present in numbers below the detection limits of the assays used (Gerba & Rose, 1990). Recent improvements in viral concentration and isolation methods are now beginning to show the full impact of viruses in water systems. The development of field concentration methods and laboratory innovations in the propagation of such viruses has revealed greater numbers and wider varieties of them to be present in environmental samples. In the US the annual number of documented waterborne outbreaks has increased 4 fold since the 1960's and this is expected to continue (Gerba & Goyal, 1982). There is probably significantly more illness in the population due to the viral contamination of drinking water than is currently recognised (Gerba & Rose, 1990).

1.3.3 Viruses and Biofilms

Evidence for the existence of viruses in biofilms is lacking, although it has been noted that, in marine and freshwaters, the abundance of viruses is positively correlated with high bacterial concentrations (Suttle, 1994; Fuhrman, 1999; Middleboe, 1996). Other studies indicate that specific morphotypes appear to be associated with the collapse of bacterial communities in natural systems (Suttle, 1987). In addition, Doolittle *et al* (1995) demonstrated that an *E. coli* biofilm growing on a submerged surface in a Modified Robbins Device (MRD) was susceptible to infection by somatic coliphage. These workers later reproduced this result when they monitored the infection of two phages (T4D-RH phage and E79-RH phage) in biofilms containing *E.coli* and *Pseudomonas aeruginosa.* However, it must be noted that these interactions were observed to be host specific only (Doolittle *et al*, 1996).

In the past decade there has been an increased interest in the interaction of viruses with biofilms. Hennes *et al* (1995) fluorescently stained marine viruses and successfully used them as probes to label, identify and enumerate specific strains of bacteria and cyanobacteria in marine biofilms. Other workers have recognised the potential for viruses as a means of biofilm

control. Owing to the increased need for their removal in certain environments such as the food industry, Roy *et al* (1993) compared the use of quarternary ammonium compounds with that of listeriophage to reduce the levels of *Listeria monocytogenes* on stainless steel and polypropylene surfaces. They observed that phage concentrations of up to 3.8×10^8 $PFUml^{-1}$ were at least as efficient as a 20 ppm solution of this disinfectant (QUATAL). Their findings also suggested that such bacteriophage could be used in conjunction with the disinfectant as it had no adverse effect on their biological activity. More recently Quignon *et al* (1997) investigated the behaviour of poliovirus in a water distribution system. During their study they recovered a greater amount of viruses from the biofilm than from the water phase and suggested that such viruses do have a tendency to accumulate in these biofilms.

Under many conditions, viruses are able to adsorb to organic and inorganic substances and retain their infectivity. Hejkal *et al* (1979) suggested that viruses adsorbed to clay and other inorganic solids are afforded protection from inactivation by chlorine and other workers provide evidence to support this (Sekla *et al*, 1980). Thus since viruses evade water treatment processes by their ability to adsorb to particulate matter (Hejkal *et al,* 1979) and biofilms consist of a large fraction of adsorbed materials, it is not unreasonable to assume that viruses may also become entrapped within such microbial systems.

Biofilms are heterogeneous (van der Wende & Characklis, 1990) and, in nature are often joined by other microbes such as fungi, algae and protozoa (Allison & Gilbert, 1992; Gilbert *et al*, 1993). The presence of amoebae is of importance as they are predators which engulf organic particles in microbial systems. The ingestion and subsequent multiplication of the pathogen *Legionella pneumophila* has been reported in amoebae. This offers a possible mechanism by which these bacteria persist through adverse conditions (Barbaree *et al*, 1986). This poses the question can other pathogens, such as enteric viruses, become ingested by such protists and survive to persist within them?

1.4 Aims of this Research

The aim of the research described in this thesis was to investigate the persistence of enteric viruses within naturally formed biofilms from water distribution systems and to monitor any interactions which may occur within such microbial systems.

The first objective is to development an adequate representation of a water distribution system biofilm in the laboratory. Once established and shown to be reproducible the next objective is to determine the heterogenity of such a biofilm and identify the presence of amoebae and other protists within the system. A partial characterisation of the principle organisms present in the biofilm model will be essential both for comparison with other models and to monitor the continued development of the biofilm identifying any changes that may occur. In addition to observe morphological changes during biofilm development the use of Scanning Electron Microscopy (SEM) will be employed. Although this method collapses the otherwise hydrated biofilm it will still provide a wealth of information concerning biofilm morphology if this slight drawback is taken into account.

Once these objectives have been achieved the system can be inoculated with virus. For this 2 model viruses were chosen namely bacteriophage lambda and poliovirus sabin type 1. The first virus, although not an enteric organism, was chosen for it's ease of growth, isolation and detection. Prior to the use of this virus it was essential to confirm that no host for this organism was present within the biofilm model. In the absence of a host this virus was assumed to act in a similar manner to enteric viruses in this situation. The latter virus used, poliovirus, was chosen as a model enteric virus. This organism is prevalent in the environment due to extensive vaccination programs and is commonly used as a model virus. Quite a hardy virus, it has been shown to survive in the environment for prolonged periods and much of its structure and biochemistry is well documented. In addition this virus grows well in tissue culture and methods for its detection are well established.

CHAPTER 2

MATERIALS AND METHODS

2.1 Setting up the Biofilm Models

2.1.1 The Use of Vortex Flow Filtration to Produce the Initial Inoculum

To produce the initial inoculum for all complex biofilm experiments, the vortex flow filtration (VFF) unit (Membrex) was used (see Figure 2.1). Originally designed for use as a bioreactor, this machine allows the concentration of small insoluble materials, such as bacterial cells, from large volumes of fluid. It does this by vortex flow perfusion. A cylindrical filter, placed in a slightly larger chamber, rotates as the water to be concentrated is passed into the system. The toroidal vortices produced transport material (e.g. cells, cell debris, macromolecules etc) away from the membrane surface allowing the fluid water to pass through it thus separating the two phases. The resultant permeate was then discarded while the remaining concentrate in the chamber was recovered. This sophisticated fluid flow profile allows for the quick and effective concentration of materials while maintaining high cell viability and reducing the contamination risks of conventional filter designs.

For the desired initial inoculum, 50 litres of boosted tap water (i.e. water held in a storage tank) was collected in 5 litre portions from the cold water supply of lab 125, Medical Science Building, University of Leicester. Sodium thiosulphate was added to a final concentration of 0.01% (Le Chavallier *et al*, 1987) to remove any residual chlorine and each 5 litre portion was recirculated through the VFF until only 100 mls of fluid remained in the chamber. The water was pumped through the system using a hydraulic pump (Masterflex) at 200 mlsminute^{-1} and the membrane was rotated at 1500 rpm 20 psi pressure. These parameters were taken from Tsai *et al* (1993) who used them for the concentration of enteroviruses from seawater samples. The resultant concentrate was then collected in a sterile 1 litre duran and the process was repeated until the full 50 litres had been concentrated to 1 litre. The concentrate was then immediately inoculated into the apparatus for biofilm growth.

Following concentration, the system was washed according to the manufacturers instructions. Briefly 1 litre 1M NaOH was circulated through the system at 1000 rpm 20 psi pressure for 10 minutes. Sterile deioinised water was then passed through at 3000 rpm 20 psi pressure for 10 minutes and then at 1000 rpm 0 psi pressure until the pH had returned to neutral (pH 7). The membrane was then removed and stored in 70% ethanol at room temperature. This procedure was also performed prior to water concentration to ensure the system was always clean.

Figure 2.1 - The Vortex Flow Filtration (VFF) Unit used to Produce the Initial Inoculum

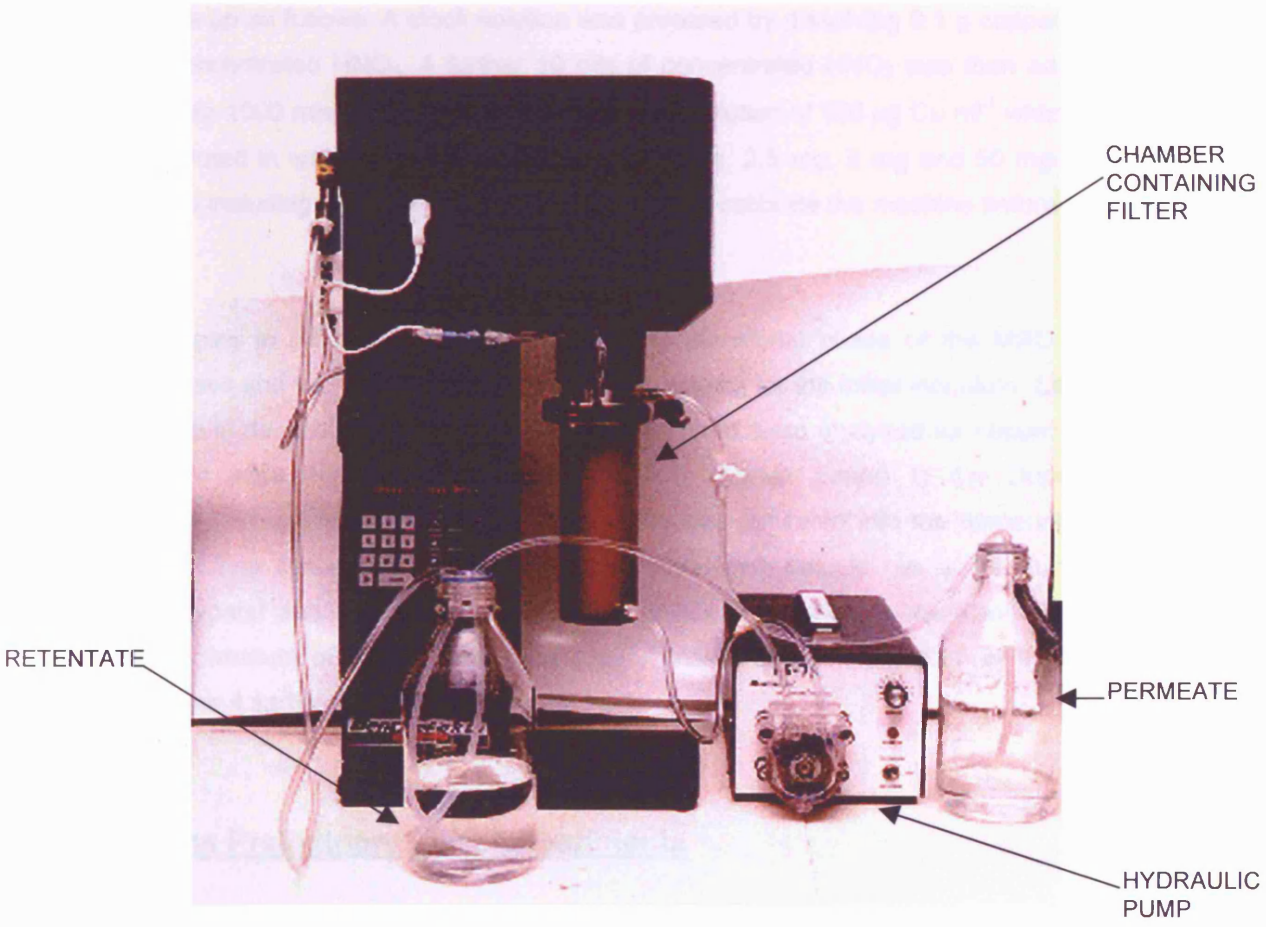


Figure 2.1 - A photograph of the Vortex Flow Filtration (VFF) unit (Membrex) showing the retentate bottle which contained the tap water being concentrated, the chamber containing the filter through which the water passed and the permeate bottle into which the water (free of solutes) was collected. The water was circulated through the machine until there was no more left in the retentate bottle, the 100mls remaining in the chamber was then used as part of the initial inoculum. The permeate was discarded.

2.1.2 Measuring Copper Ions in Water

To determine the amount of copper present in the planktonic samples of the MRD flame adsorption spectrometry was used. For this method a set of copper standards were required and made up as follows. A stock solution was prepared by dissolving 0.1 g copper metal in 2 mls of concentrated HNO_3. A further 10 mls of concentrated HNO_3 was then added before diluting into 1000 mls water. This produced a stock solution of 100 μg Cu ml^{-1} which was then further diluted in water to produce standards of 1 mg, 2.5 mg, 5 mg and 50 $mgml^{-1}$. These standards including a copper blank were then used to calibrate the machine before testing the samples.

The samples to be tested were taken from the planktonic phase of the MRD containing copper discs and from the tap water used as the source for the initial inoculum. Each sample was taken in duplicate as 10 ml portions. The samples were analysed for copper ions using an atomic adsorption spectrophotometer 1100B (Perkin Elmer) in the Department of Chemistry (University of Leicester). Each sample was aspirated into the flame where it was atomised. The amount of energy produced was then passed, as a beam, through a monochromater and detected at a wavelength of 324.7 nm which is characteristic of copper. Since the amount of energy emitted is proportional to the concentration of the copper its levels in the 4 samples could be deduced.

2.1.3 The Preliminary Tube Experiments

For these experiments 15 ml polypropylene tubes were used because viruses do not readily adsorb to this material (Ward & Winston, 1985). For each experiment virus was inoculated in 30 mls of filter sterilised water and this was then split into 3 lots of 10 mls in three separate tubes. The tubes were labelled 1-3. Eight sterile plastic discs were added to tube 2 and eight discs with preformed biofilm were added to tube 3. The biofilm was preformed for a period of 14 days in an MRD before being aseptically transferred to tube 3 using flame sterilised forceps. The three tubes were then placed on a rotor mixer (see Figure 2.2) in an incubator maintained at 22°C and samples were taken periodically after 1 hour, 1, 4, 7 and 14 days. At these time points assays for virus were performed by the aseptic removal of one disc and 100 μl of the planktonic phase. See sections 2.4.2, 2.5.3, 2.5.5 and 2.5.7 for the virus assays performed.

Figure 2.2 - The Apparatus used for the Preliminary Tube Experiments

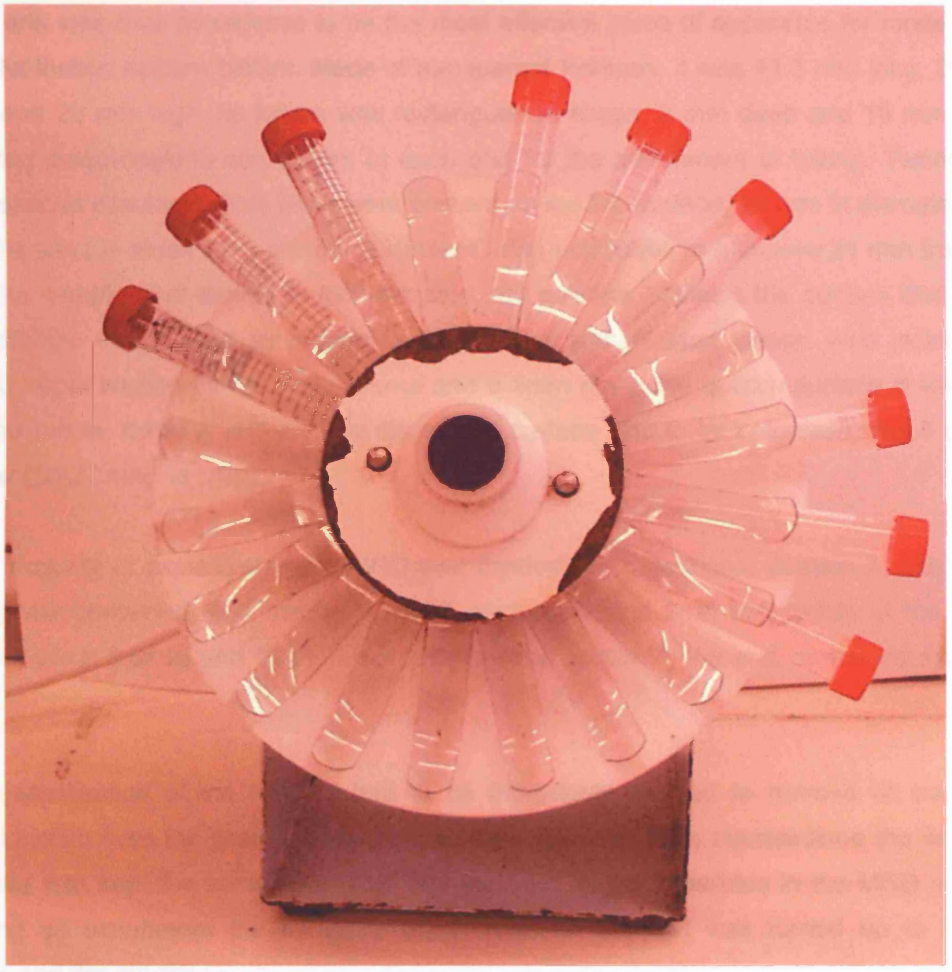


Figure 2.2 - A photograph of the apparatus used for all of the preliminary tube experiments using both bacteriophage and polioviruses. The picture shows the polypropylene tubes on a rotor mixer which was left rotating for the entire duration of the experiment. The whole system was placed in an incubator set at approximately 22°C.

2.1.4 The Modified Robbins Device (MRD)

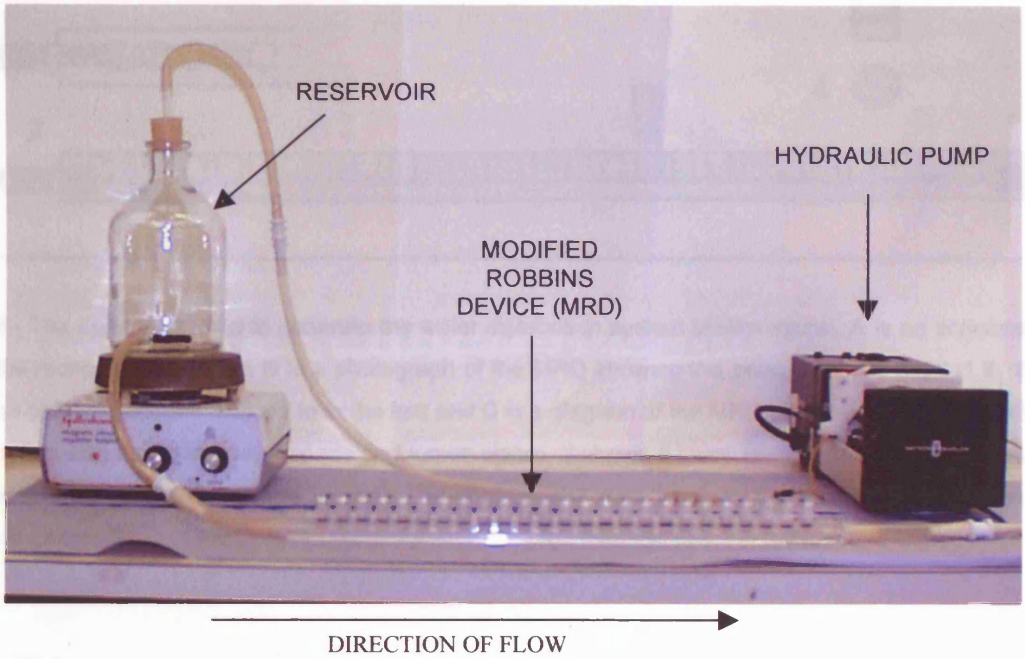
The Modified Robbins Device (MRD, manufactured at Exeter University; see Figure 2.3), used for the majority of experiments, was designed to mimic the conditions within a flowing system and was thus considered to be the most effective piece of apparatus for modelling a water distribution system biofilm. Made of transparent Perspex, it was 41.5 mm long, 26 mm across and 20 mm high. Its lumen was rectangular in shape, 2 mm deep and 10 mm wide, and it had polypropylene connectors at each end for the attachment of tubing. Twenty-five evenly spaced circular sample ports were present on the top surface, 11 mm in diameter, into which the sample studs were placed. A rubber O-ring positioned in a groove 21 mm from the top of the sample stud served to lock the stud into position, allowing the surface disc to be flush with the lumen, and to prevent leakage. The sample stud design also included a bevelled upper section for ease of removal and a 1mm rim in the bottom surface in which to place the rubber backing and surface discs. The surface disc to be colonised was 8 mm in diameter (50.27 mm^2 in area).

For the majority of experiments the MRD was divided into 4 sections. Section 1, nearest to the reservoir contained stud numbers 1 to 7, sections 2 and 3, in the middle of the MRD, contained studs 8 to 15 and 16 to 20 respectively and section 4, the end, contained studs 21 to 25.

Prior to sterilisation of the MRD it had to be thoroughly washed to remove all traces of residual biofilm from the previous experiment. To keep everything standardised the washing procedure was kept the same each time and used for all the apparatus in the MRD system. Following an experiment the hydraulic pump (Watson Marlow) was turned up to it's full capacity and the planktonic phase was discarded into a duran and autoclaved. Turning up the hydraulic pump allowed for the production of high shear forces dislodging some organisms from the biofilm. The planktonic phase was then replaced with a 2% solution of hycolin (William Pearson Chemicals, Coventry,UK) and circulated for approximately 1 hour at the normal speed of 500 $\mu l min^{-1}$. This served to disinfect the system and destroy the bacteria and FLA's present in the biofilm. The hycolin was then removed and the MRD was briefly rinsed through. The whole closed apparatus was then placed into a large bucket containing a solution of 10% virkon (Antec International, Suffolk,UK) and taken apart in this. Virkon is highly virucidal and thus was used to destroy any viruses left in the system. This also helped to kill any remaining bacteria present. The apparatus was left submerged in this solution for approximately 30 – 60 minutes and then rinsed with tap water. After the disinfection procedure the apparatus was transferred to another bucket containing pyroneg (Diversity Ltd,Northampton,UK) and left for 30 minutes. This is a detergent which is commonly used for the removal of oily substances from glass and thus it removed any residual biofilm

Figure 2.3 - The Apparatus used to Generate a Biofilm - The Modified Robbins Device (MRD)

A



B

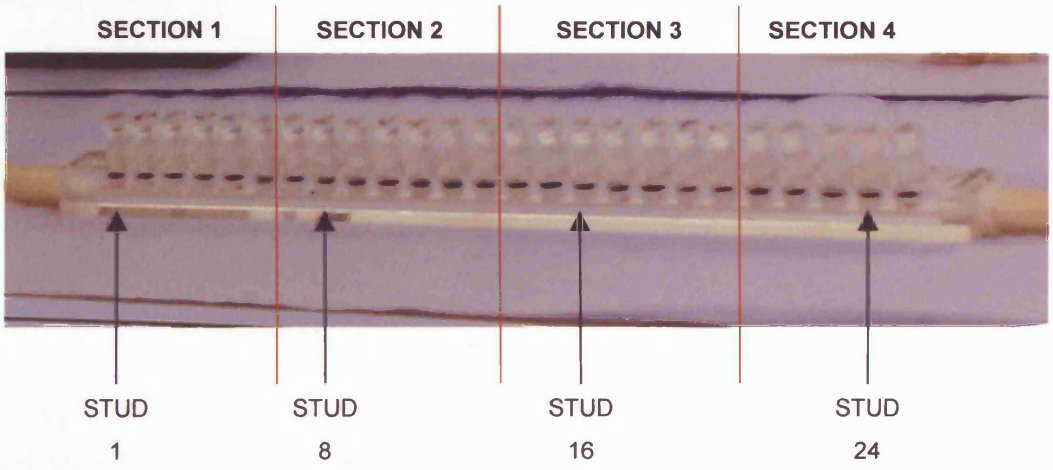


Figure 2.3 Cont. - The Apparatus used to Generate a Biofilm - The Modified Robbins Device (MRD)

C

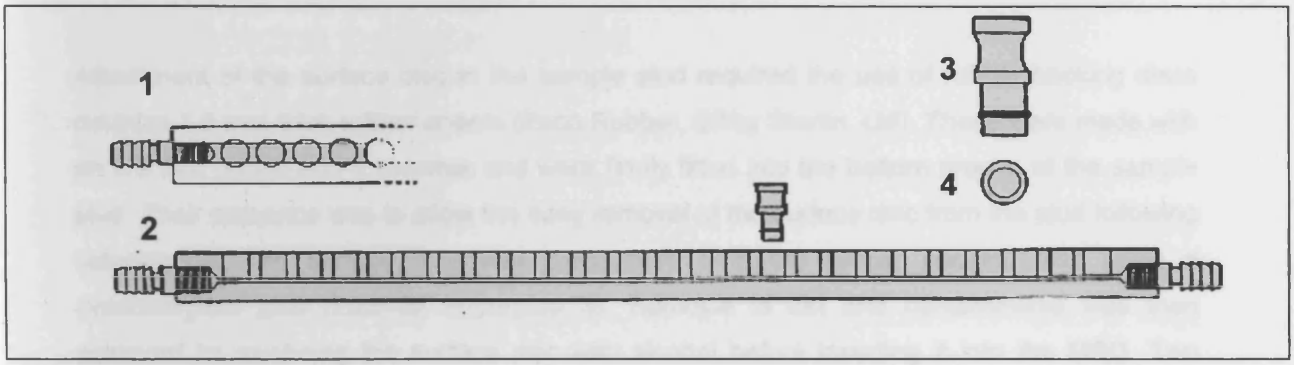


Figure 2.3 - The apparatus used to generate the water distribution system biofilm model. **A** is an annotated photograph showing the recirculating system, **B** is a photograph of the MRD showing the principle studs used (1,8,16 and 24) and the location of the 4 sections referred to in the text and **C** is a diagram of the MRD (1 shows a view from above, 2 shows a cross-section with the stud, sample ports and lumen visible, 3 shows a cross section of a stud and 4 shows a view of the stud bottom where the discs were placed). The photographs were taken by the author and the diagram was taken from Jass & Lappin-Scott (1992).

components still left on the inside of the apparatus. After rinsing the apparatus was then submerged in decon 90 (Decon Laboratories Ltd, Sussex, UK). This is another detergent which continues the washing process and is commonly used to degrease surfaces. This was followed by 3 rinses in tap water and 3 subsequent rinses in deionised water. The apparatus components were then dried and prepared for sterilisation.

Attachment of the surface disc to the sample stud required the use of rubber backing discs cut from 1.6 mm thick rubber sheets (Esco Rubber, Bibby Sterlin, Ltd). These were made with an 8.5 mm punch and a hammer and were firmly fitted into the bottom groove of the sample stud. Their presence was to allow the easy removal of the surface disc from the stud following colonisation. The surface disc was then glued onto the rubber backing disc using a cyanoacrylate glue (Locktite Superglue 3). Removal of dirt and contaminants was then achieved by swabbing the surface disc with alcohol before inserting it into the MRD. Two sample studs were allocated for each sample port, one for insertion into the MRD and one replacement stud. These studs were then numbered according to their sample ports so that in every experiment the same studs were used for their respective ports. This ensured the standardisation of experiments. Replacement studs were placed in a lidded plastic container and these and the MRD were then ready for sterilisation.

Sterilisation was achieved by autoclaving all the components of the MRD system except the MRD itself. The MRD was made of perspex which is a heat sensitive material. At the high temperatures required for autoclaving this material would warp and the O-rings on the studs, which allow for effective sealing and prevent leakage, would be damaged. Other methods of sterilisation such as the use of formalin, alcohol or UV proved insufficient. The formalin would have fixed any dirt to the MRD and alcohol and UV, like autoclaving, damage the perspex and the O-rings. Thus ethylene oxide was employed for MRD sterilisation. This highly toxic gas is also used for the sterilisation of surgical equipment and so the MRD and studs were sent to Glenfield hospital for this procedure. Once sterilised, the MRD apparatus is placed together inside the laminar flow cabinet under aseptic conditions.

2.1.5 The Alternative Jar Models

The alternative jar models were washed in a similar fashion to the MRD in order to keep everything standardised (see Section 2.1.4). Thin glass capillary tubes were threaded onto cotton strands of approximately 15cm in length and secured at one end using a small bead. Onto these were glued (Locktite superglue 3) three plastic discs and three copper discs (only plastic in some later experiments) and the thread was then dangled into the jar around its edge ensuring all discs were at the same level. A magnetic flea was then added to the jar and

the whole thing was sterilised in an autoclave at 121°C, 1 atmosphere pressure for 15 minutes. Once sterile the jar was placed on a magnetic stirrer (Gallenkamp) and was ready for inoculation.

2.1.6 Inoculating the Model Systems

Prior to inoculation of the concentrated water into the MRD it had to be primed and checked for leaks. Sterile deionised water was passed through the MRD at a rate of 200 mls minute^{-1} and the whole system was placed on blue tissue paper. This paper is ideal when attempting to spot any leaks that may be present in the system. Once checked the deionised water was removed and the concentrated water added to the reservoir. The hydraulic pump at this stage was left on full (200 mls minute^{-1}) so that the concentrated water could fully circulate the system. This took approximately 2 hours. Once fully circulated the pump was changed to 0.5ml/minute and the experiment begun.

2.1.7 Sampling the Biofilm

The method used for sampling the biofilm was adapted from that used by Jass *et al* (1995). This procedure had to be done both aseptically and quickly to lower the risk of contaminating the system. Thus all tubes and vials were prepared and labelled prior to sampling. When sampling from the MRD the pump was momentarily stopped and the tubes either side of the MRD clamped to prevent leakage when changing the studs. The desired studs were then carefully removed and placed upright into a sterile bijou for transport into another laboratory. Sterile studs were then placed into the MRD, clamps were removed and the pump switched back on. Discs taken from the other systems (tube experiments and alternative jar model) were not replaced in the same way as in the MRD. These were aseptically removed near a Bunsen burner using flame sterilised forceps.

Once removed from the systems the discs were washed three times using 3 mls filter sterilised water to remove any planktonic cells that may have been present. The surface of the discs was then scraped into 1 ml filter sterilised tap water using a sterile hypodermic needle . Both the needle and the disc were then dropped into the 1ml sample and subjected to 1 minute sonication in a sonic water bath (Kerry). After this, the sample was subjected to 1 minute vortexing treatment for maximum biofilm removal and to allow for an even suspension of cells. The resultant suspension was then ready for testing. When testing for poliovirus the protocol was changed slightly. The biofilm sample was scraped into 1 ml serum-free tissue

culture medium (MEM; Gibco) for ease of $TCID_{50}$. However this later caused unforeseen problems with PCR and DIG hybridisation experiments.

Figure 2.4 - The Apparatus used for the Alternative Jar Model

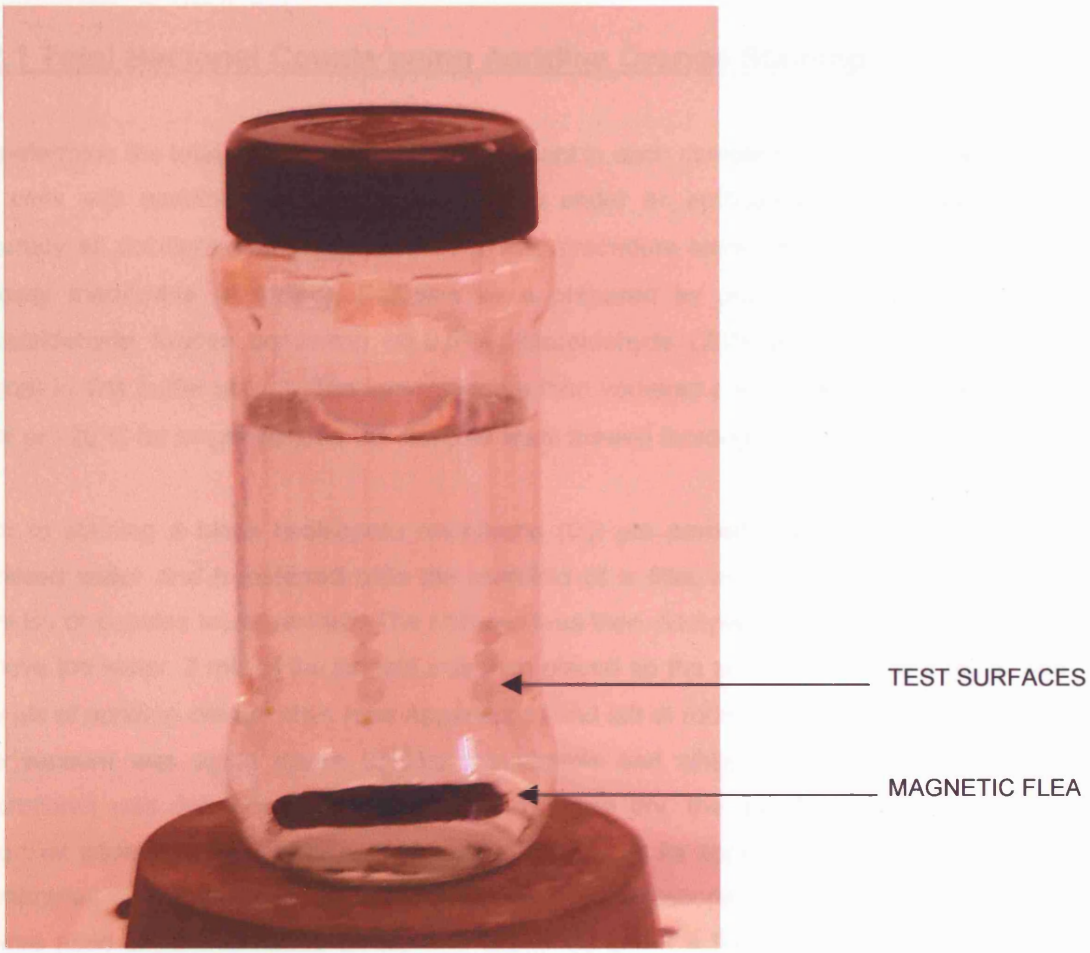


Figure 2.4 - A photograph of the Alternative jar Model showing the jar placed on a magnetic stirrer and the test surfaces hanging in place on cotton thread.

2.2 Evaluating the Biofilm Model Systems

2.2.1 Total Bacterial Counts using Acridine Orange Staining

To determine the total number of organisms present in each sample it was necessary to stain the cells with acridine orange and count them under an epifluorescent microscope. For accuracy all solutions and reagents used in this procedure were filtered through a 0.2 μm porosity membrane (Acrodisc). Samples were prepared by placing 500 μls in 4.5 mls glutaraldehyde fixative consisting of 0.5% glutaraldehyde (25% aqueous stock solution; Sigma) in Tris buffer pH 7.4. The samples were then vortexed and stored at either 4°C for 7 days or –20°C for longer periods. All samples were thawed thoroughly before staining.

Prior to staining a black nucleopore membrane (0.2 μm porosity) was pre-wetted in filter sterilised water and transferred onto the manifold of a filter apparatus, ensuring that any wrinkles or bubbles were avoided. The chimney was then clamped and the vacuum drawn to remove the water. 3 mls of the sample was then placed on the surface of the filter along with 100 μls of acridine orange stain (see Appendix 1) and left at room temperature for 5 minutes. The vacuum was again drawn to filter the sample and while maintaining it 1.5 mls of isopropanol was carefully added for destaining. Once dry, the membrane was transferred onto filter paper in a dark place and allowed to dry further for approximately 20 minutes. The membranes were then cut in half and placed on glass microscope slides. A drop of immersion oil was used to fix a coverslip on top and examined under a fluorescent microscope (Leitz Wetzer, Germany) with a mercury vapour light source. The excitation wavelength for acridine orange is 450-490 nm and this is within the range of the mercury light source. A grid was inserted into the eyepiece to allow ease of counting and 3 – 30 bacterial cells per grid area were counted. 10 fields were randomly evaluated using 100x magnification and an oil immersion lens.

Acridine orange stains the nucleic acids of the bacterial cells where DNA stains green and RNA stains orange. However this cannot always be distinguished and so any fluorescent images seen were counted and the total counts were calculated using the formula in Appendix 2.

2.2.2 Total Viable Counts

For the total viable bacterial counts, 10 fold serial dilutions of the sample were made in filter sterilised tap water. 100 µls of each dilution were plated out, using a flame sterilised glass plate spreader, onto the surface of both R_2A and MacConkey agars (Oxoid). R_2A was used for the enumeration of viable heterotrophs and was incubated for a period of 7 days at 30°C. Plates were placed in a plastic bag in the incubator to avoid drying out. This medium was chosen because it allows the effective isolation of heterotrophs from water minimising the nutrient shock of the cells (Le Chavallier *et al*, 1987). The other medium, MacConkey agar, was used for the enumeration of presumptive coliforms and plates were incubated for 48 hours at 37°C. This medium is commonly used for the isolation of coliforms and strains of pseudomonads and thus for these experiments these organisms were collectively termed presumptive coliforms.

Once grown, between 30 - 300 individual colonies were counted using a touch sensitive plate counter and from this the number of total viable organisms were calculated using the formula in Appendix 2.

2.2.3 Isolation and Enumeration of Free-Living Amoebae (FLA)

The isolation and enumeration of FLA's was achieved using non-nutrient agar (see Appendix 1) seeded with *E. coli* LE392. The plates were prepared as follows; 2 mls of molten NNA were dispensed into each well of 24 well plates and allowed to set. Once set a heavy suspension (approximately Macfarland 4) of *E.coli* LE392 in ¼ strength Ringer's solution was seeded dropwise onto the surface of each well using a sterile plastic pasteur pipette. Plates were allowed to dry in a 37°C incubator for approximately 2 hours before being wrapped in cling film and stored at 4°C until further use.

Samples were serially diluted 10 fold in filter sterilised tap water and 100µls of each dilution (usually neat sample to 10^{-3} dilution inclusive) were added to the surface of each well in the 24 well plate. Each dilution was tested in triplicate. Plates were then placed in a plastic bag to avoid drying out and incubated at 30°C for a period of 7 days.

Plates were examined on a daily basis using an inverted light microscope (Lieca DMIL), at a magnification of 50x, for the presence of FLA's. The enumeration of these organisms was achieved by examining the number of positive wells gained at each dilution and using the Reed and Muench formula (Appendix 2) to calculate the most probable number of FLA's in the original sample.

2.2.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was performed on the discs removed from the MRD to examine the morphology of the complex biofilm produced. Studs were removed from the MRD and washed to dislodge any planktonic cells (see section 2.1.7). The biofilm was then prepared for SEM as follows.

Firstly the biofilm was fixed onto the disc by placing it directly in 5mls 4% formaldehyde solution (25% aqueous stock solution; Sigma) for 30 - 60 minutes. The discs were then rinsed in double distilled water for 5 minutes 3 times before beginning the dehydration process. Dehydration of the biofilm was achieved by placing the discs in a series of acetone solutions for 15 minutes at a time. These solutions began at 30% through 50%, 70%, 90% and twice in 100%. Following this the discs were dried in a critical point dryer (Balzers CPD 030) before being coated with gold in a sputter coater (built in house, based on a polaron code sputter SEM machine). The discs were then glued onto mounts and stored under vacuum until examination by SEM could be performed. Specimens were viewed on a Cambridge S100 SEM at 25kV at magnifications between 500x and 5000x.

2.3 The Partial Characterisation of the Biofilm

2.3.1 Bacterial Morphology & Biochemical Tests

Following the enumeration of viable organisms on the R_2A and MacConkey agar plates the colonial morphology of some individual colonies was examined. Since the diversity of organisms was large only representative strains of organism groups were isolated for biochemical tests and further identification. Three groups from each agar type were distinguished and followed through the experiment due mainly colonial morphology and colour.

The isolation and purification of each respective organism was achieved by sub culturing them onto R_2A and incubating them at 30°C for those taken from the R_2A originally and both 30°C and 37°C for those taken from the MacConkey agar. Once pure, three simple biochemical tests were performed on each strain. These tests were the Gram stain and the determination of both oxidase and catalase activity.

The first test performed was the Gram stain. This is a procedure commonly used as the first step in the identification process of bacterial isolates. For this test a bacterial colony, taken from a pure culture, was suspended in a drop of sterile distilled water on a glass microscope slide. The resultant smear was allowed to dry for approximately 15 minutes at room temperature and then heat fixed in a bunsen flame. Crystal violet stain was then added to the surface of the slide and incubated for 1 minute to allow the primary staining of the cells. After rinsing the slide an iodine solution was added. This functioned as a mordant increasing the interactions of the stain and the cells in the smear. The slide was once again rinsed before being decolourised with acetone for approximately 30 seconds. This step generates the differentiation process between gram positive and negative cells. Gram positive cells will retain the crystal violet stain while the negative cells will be rendered colourless. Following this a solution of safronin, the counterstain, was added staining the now colourless Gram negative cells their characteristic pink colour. Once stained the smears were examined using an oil immersion lens at a magnification of x100 using a light microscope (Lietz Welzer, Germany).

To determine the oxidase activity of the isolates the method of Kovacs (Cowan & Steel, 1974) was used. For this 2 to 3 drops of a 1% solution of tetramethyl-p-phenylenediamine dihydrochloride was added to a piece of 3 mm whatman paper in a petri dish. Before the drops could dry a bacterial colony was smeared onto the surface of the impregnated paper.

The appearance of a purple colouration on the paper after approximately 10 seconds indicated a positive result.

To assess the catalase activity of the biofilm isolates a simple method utilising H_2O_2 was used. Approximately 200 µls 30% H_2O_2 was placed onto a glass microscope slide. A colony taken from a freshly grown pure culture was then emulsified into this solution using a sterile toothpick. The appearance of gas bubbles forming immediately after the addition of the organism indicated a positive result and was due to a reaction of the enzyme and H_2O_2 forming gaseous oxygen as a by-product. It is important to note that a toothpick was used instead of a wire loop as some of the materials used in wire loops cross react with the H_2O_2, producing false positive results. To be certain all the results were correct both negative and positive controls were employed and they were strains of *E. coli* and *Pseudomonas sp.* respectively.

2.3.2 SDS – PAGE

SDS polyacrylamide gel electrophoresis (PAGE) was used to determine similarities or duplicates present among isolates from the biofilm model. The method chosen was the discontinuous gel method of Laemmli (1970) in which an upper stacking gel concentrates the samples before the proteins are separated in the resolving gel. The gels and buffers used (detailed in Appendix 1) contained the anionic detergent sodium dodecyl sulphate (SDS). This protein denaturant binds to proteins producing linear complexes with a constant negative charge per unit mass. Thus the electrophoretic mobility of the protein is related to molecular weight only and in one-dimensional electrophoresis these proteins are separated as bands forming a protein profile or fingerprint.

In this experiment small gels were used and assembled for vertical gel electrophoresis using the Bio-Rad Mini PROTEAN II slab cell kit with 1 mm spacers between the glass plates. A 12% resolving gel was prepared and poured to a depth approximately 1 cm below the base of the wells. This was then covered with a layer of water to produce a level horizontal surface and allowed to set at room temperature. Once set the water was removed and the stacking gel was poured on top. A 10-toothed comb was used for the wells.

Biofilm isolates were purified on R_2A before being grown in LB broth at 30°C. The cells were pelleted by centrifuging (MSE Microcentaur microfuge) 1 ml broth at 13K rpm for 15 minutes and resuspended in 200 µls PAGE sample buffer. Samples were then thoroughly mixed by vortexing and placed on a preheated heating block set at 100°C for 15 minutes. This allows the breaking open of cells, destroys proteolytic activity and extracts the denatured whole-cell

proteins by solubilisation. After cooling on ice the extracts were once again centrifuged at 13K rpm for 15 minutes and the supernatant containing the extracted proteins was transferred to fresh clean eppendorfs. These extracts were stored at -70°C.

The protein extracts were once again heated at 100°C for 5 minutes, cooled and centrifuged at 13K rpm for a further 5 minutes to remove any debris prior to loading onto the gel. For each gel 1 well was loaded with a molecular weight marker (BDH 'Electran', molecular weight range 12,300 – 78,000) and the protein extracts were then loaded into the remaining 9 wells using a P20 gilson pipette. Proteins were electrophoresed using a Pharmacia Electrophoresis Constant Power Supply ECPS 3000/150 power pack, at a constant current of 50 mAmps per gel (i.e. 100 mAmps for 2 gels). The running time for electrophoresis was approximately $1^1/_2$ hours for 2 gels.

After electrophoresis the gels were transferred to a shallow plastic container filled with Coomassie Blue Stain. This was rocked gently on a DENLEY A600 Rocker for approximately 2-3 hours to simultaneously stain and fix the proteins. The stain was then poured off, the gel was briefly rinsed with distilled water and then a destain solution was added. The destain was periodically replaced with fresh solution until the gel background became clear showing only protein bands. This process generally took 2-3 hours.

The gels were then photographed on an Electrophoresis documentation and Analysis System 120 (Kodak Digital Science) before being dried using the NOVEX system (R & D Systems Europe Ltd.). The gel was briefly rinsed in distilled water, soaked in drying solution overnight and then dried between two pieces of cellophane clamped within an upright plastic frame at room temperature. Gels were dry in approximately 48 hours and kept flat between the pages of a thick book.

2.3.3 DNA Extraction, PCR and Sequencing of Bacterial Isolates

The extraction of the bacterial genomic DNA was achieved using the method of Chen *et al* (1993) as all isolates to be sequenced were previously found to be Gram negative. The DNA was extracted from a pure culture of each isolate grown in LB broth at 30°C. 1.5 ml broth culture was centrifuged at 13K rpm for 5 minutes to pellet the cells. For cell lysis the pellet was resuspended in 200 µls Lysis buffer and vigorously pipetted. To remove cell debris and protein, 66 µls 5M NaCl was added, mixed thoroughly before centrifuging at 13K for 10 minutes at 4°C. The supernatant was then transferred into a fresh eppendorf containing an equal volume of chloroform. This was then inverted 50 times until a milky solution was apparent. Following a further centrifugation step at 13K rpm for 5 minutes, the extracted

supernatant was placed in another clean eppendorf and the DNA was precipitated with 100% ethanol at -20°C. This process took approximately 30 – 60 minutes but could be left overnight if appropriate. Following precipitation the DNA was washed twice with 70% ethanol, dried at room temperature and resuspended in 50 µls nanopure water. 5 µls of this DNA was then electrophoresed on a 1% agarose gel supplemented with 0.1µl ml^{-1} ethidium bromide (Sigma) to determine an estimated yield of DNA. Once clean the DNA was diluted to produce a final concentration of approximately 1ng μl^{-1} ready for subsequent amplification.

The 16S rDNA gene was enzymatically amplified using PCR (Rolleke *et al*, 1996; *Herrick et al*, 1993; Borneman *et al*, 1996). The reaction mix consisted of 3 mM $MgCl_2$; 0.8 mM each of dGTP, dATP, dCTP and dTTP (Promega, Madison, WI, USA); 20 pmol each of primers FD1 (5' – AGAGTTTGATCCTGGCTCAG – 3') and RP1 (5' – ACGG(TCA)TACCTTGTTACGACTT – 3') for eubacteria and 1.5 units T*aq* polymerase in a buffer consisting of 750 mM Tris HCl (pH 8.8), 20 mM $(NH4)_2SO_4$ and 0.1% Tween 20 (Advanced Biotechnologies, Leatherhead, UK). The total volume of the reaction mix was 50 µls. This was transferred to a 0.5 ml eppendorf with approximately 1 ng template DNA and overlayed with 1 to 2 drops of mineral oil (Sigma Chemical Co., Ltd). PCR was performed using a Trio-Thermoblock (Biometra) with the following cycling procedure. The DNA was first denatured at 96°C for 2minutes. This was followed by 30 cycles of 95°C for 30seconds, 55°C for 40 seconds and 72°C for 2 minutes, with a final 10-minute extension at 72°C. The PCR products were then purified using a QIAquick PCR purification kit (QIAGEN, Crawley, UK) and their purity was confirmed by agarose gel (1% w/v) electrophoresis stained with 1µg ml^{-1} ethidium bromide (Sigma Chemical Co., Ltd) (Sambrook *et al*, 1989). The purified PCR product was then diluted to produce a final concentration of approximately 0.2 µg μl^{-1} and sent to PNACL (based at the University of Leicester) along with 1 pmol μl^{-1} FD1 primer for automated sequencing.

The sequencing reactions were performed using a BigDye terminator DNA sequencing kit. The 20 µl reaction volumes contained 8 µl reaction mix, 4 µls FD1 primer (1pmol μl^{-1}) and 8µls DNA template (0.2 µg μl^{-1}). Samples were then heated to 95°C for 5 minutes before 45 cycles of PCR were run at 96°C for 18 seconds, 55°C for 12 seconds and 60°C for 4 minutes using a PE Biosystems 9700 thermal cycler. Excess dye terminators were removed from the reaction mixture with Centri-Sep spin columns (Princeton Separations) and the reactions were analysed using a 377 automated sequencer (Mathilda). The sequences were then compared with those stored on the BLAST database (website: www.ncbi.nlm.nih.gov/BLAST/). It must be noted that the sequencing was only done in one direction. Bidirectional sequencing would have been more accurate but since the matches obtained were very high (between 98 – 99%) this was deemed unnecessary.

2.4 Bacteriophage Work

2.4.1 Growing up Bacteriophage Stock Solutions

To produce fresh phage stock for subsequent experiments the old stock was titrated using the method detailed below (see section 2.4.2). Following overnight incubation of plates single plaques were picked up using a sterile toothpick and transferred into 2 mls LB broth supplemented with 1% $MgSO_4$ and 100 µls overnight culture of *E.coli* LE392. The tubes were then incubated at 37°C on an orbital shaker for 6 hours or until the culture had become clear indicating that cell lysis had occurred. Cell debris was removed by centrifugation (Centaur 2 Centrifuge, MSE) at 2500 rpm (1120 xg) for 30 minutes and the supernatant was transferred into a fresh screw capped polypropylene tube. This phage stock solution was then stored for further use at 4°C.

2.4.2 The Titration of Bacteriophage

To assay the phage solutions 10 fold serial dilutions of phage were made in phage buffer (to about 10^{-8} dilution) in 1.5 ml Eppendorf tubes. A suspension of *E. coli* LE392 was then prepared by centrifuging the cells at 2500 rpm (1120 xg) for 10 minutes and resuspending the pellet in a solution containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$ to produce a final optical density (at a wavelength of 600 nm) of approximately 0.5. 100 µls of this *E. coli* suspension was then added to each phage dilution and incubated at room temperature for 20 minutes to allow phage adsorption to the *E. coli* strain.

3 mls molten soft top agar were dispensed into 3" glass test tubes and placed in a water bath set at 45°C. Once the tubes were equilibrated to this temperature, one tube was removed, .the excess water wiped away and 100 µls phage/cell dilution was added. The agar was then gently mixed to avoid bubbles and carefully poured onto the surface of a LB agar (LA) plate. This procedure was repeated for all dilutions and a negative control containing the *E. coli* strain diluted in phage buffer only. Once set the plates were inverted and incubated at 37°C overnight. The resultant plaques formed were enumerated using a touch sensitive cell counter and the concentration of phage calculated using the formula in Appendix 2.

2.4.3 Evaluation of Biofilm Removal Methods on Bacteriophage

As sonication and vortexing procedures were to be used for the removal of the biofilm from the disc, it was important to determine the effect of these two treatments on the bacteriophage alone. This was to ensure that any removal method used was not detrimental to the phage itself. For this experiment 100 µls of phage stock solution was added to 1.9 mls filter sterilised water. This was then divided into two 1 ml portions and placed into 3" glass test tubes. 100 µls was then removed from each tube and placed at 4°C for short term storage. One tube was then left in the test tube rack for 1 minute while the other was subjected to 1 minute sonication treatment in a sonic water bath (Kerry). 100 µls was, once again, taken from each test tube and all four samples were assayed using the agar overlay method detailed above (see section 2.4.2). To determine the effect of the vortexing treatment the above experiment was repeated substituting the sonication step with vortexing. These experiments were performed in triplicate.

2.4.4 Methods of Bacteriophage Isolation from the Biofilm

Bacteriophage lambda was detected and enumerated from the two phases of the biofilm model using several different methods. The predominant method used was the plaque assay method, the procedure for which is detailed above (see section 2.4.2). The other three methods evaluated were all related and required the enrichment of the bacteriophage. The first method, the simple enrichment, was a modification of the above method for growing up the phage stock (see section 2.4.1). For this serial 10 fold dilutions of biofilm sample (both planktonic and sessile) were made. 100 µls of each dilution was then filtered through a 0.2 µm porosity membrane (Acrodisc) before being placed in 2 mls of an enrichment broth (LB broth supplemented with 1% $MgSO_4$) containing the *E. coli* LE392 host. This broth was then incubated at 37°C for 6 hours and then checked for clarity. Any clear broths produced were recorded as positive and in theory could be used to determine the number of phage present in the original sample using the most probable number method. However, when applying this method to a known concentration of phage stock solution the results were found to be inaccurate. This is probably due to the nature of phage infection, it's release from the host cell or the growth of the host. Following phage infection the host will lyse releasing thousands of progeny phage into solution but a host cell can release from 10 to 1000 progeny phage particles at one time. If the progeny phage is low the broth may not reach clarity and will be recorded as negative while if the phage release is high the opposite will be true. This produces huge margins for error and so results could not be accurately read. When testing the phage stock with this method the results were usually much lower and so to this procedure was added another step whereby any turbid broths were assayed using the plaque

assay method (section 2.4.2) following the enrichment step. However, due to the previously described inaccuracies the plaques were not enumerated. The results from these experiments were simply used to verify the presence of the phage in the original sample and so biofilm samples no longer required serial dilution for this procedure. In addition to enriching and assaying the biofilm samples, the disc from the model was also subjected to this procedure after biofilm samples had been taken. This was to determine if the phage had been missed when sampling.

Since the enrichment method, by definition, was more sensitive than the plaque assay another enrichment method was evaluated to try and enumerate the low levels of phage found. This method was a modification of the $TCID_{50}$ procedure used for the enumeration of poliovirus (see section 2.5.3). For this 1 ml overnight culture of *E. coli* LE392 was added to 9mls enrichment broth. Using an electronic multichannel pipette (BIOHIT) 100 µl portions of this broth culture were dispensed into the wells of a 96 well flat bottomed microtitre plate. 10 fold serial dilutions of the biofilm sample were then made and filtered through a 0.2 µm porosity membrane (Acrodisc) before 100 µls of each dilution were inoculated onto 10 wells each of the microtitre plate. The plates were then incubated at 37°C for 6 hours following which time 100 µls of molten soft top agar was placed over the top of each well. The plates were then returned to 37°C and reincubated overnight. After incubation a clear well was recorded as a positive and a turbid well a negative result. The calculation for $TCID_{50}$ was then used for the phage enumeration. Although this method worked well the reproducibility was questionable and so this procedure was not continued.

2.5 Poliovirus Work

2.5.1 The Maintenance and Storage of the Cell Lines used

For the experiments concerning poliovirus two types of cell line were used, HT-29 and BGMK cells. The HT-29 or human colon adenocarcinoma grade II cells were originally taken from a primary tumour in a 44 year old caucasion female. These cells were used initially for the propagation of poliovirus as previous workers had found it to grow quite well in them (Patel *et al*, 1985). However later researchers (Kok *et al*, 1998) suggested that the use of a different cell line, BGMK cells, for the $TCID_{50}$ procedure would be more accurate. BGMK cells are an African Green Monkey Kidney cell line commonly used for the isolation of waterborne viruses (Grabow, 1999; Dahling & Wright, 1986; Chonmaitree *et al*, 1988).

Both cell lines were maintained in 75 cm^2 plastic tissue culture flasks and incubated at 37°C with 5% CO_2. The culture medium used for the HT-29 cells consisted of M199 tissue culture medium (Gibco) supplemented with 10% heat inactivated foetal calf serum, 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 1% fungizone (0.25 $\mu g\ ml^{-1}$ amphotericin; Gibco). A similar culture medium was used for the maintenance of the BGMK cells with MEM tissue culture medium (Gibco) instead of M199 and an additional supplement of 1% none essential amino acids (Gibco). Once confluent, cultures were split using a 0.25% trypsin in versene solution. For the HT-29 cells usually three flasks were seeded with approximately 5×10^5 cells while for the BGMK cells six flasks were seeded at a concentration of 5×10^4 cells before reincubation. Cultures were usually split every three days and media was changed periodically when the indicator in the spent medium began to change colour. For each new batch of medium opened 5 mls was placed in the incubator in a plastic universal tube. This was examined for turbidity on a daily basis and used to ensure that the medium was not contaminated with bacteria.HT-29 cells were used for poliovirus infection (see section 2.5.2) while BGMK cells were used for $TCID_{50}$ experiments (see section 2.5.3).

The cells were counted using a Neubauer counting chamber and Trypan Blue stain. For this 80 µls of trypsinised cell suspension was added to 20 µls Trypan Blue and incubated at room temperature for approximately 1 minute. The coverslip was firmly fixed onto the neubauer slide and the suspension carefully dispensed into the chamber using a plastic pasteur pipette. This was then placed onto an inverted microscope (Leica DMIL) for the enumeration of cells within the grid of the chamber. Once counted the total cell concentration was calculated using the formula in Appendix 2. The Trypan Blue stain was used to determine the viability of the cells. Viable cells are able to take up the stain while dead cells are not. Thus when enumerating the cells only those with a blue coloration were counted.

2.5.2 The Cultivation, Concentration and Storage of Poliovirus

Poliovirus was cultivated in confluent HT-29 cells and incubated at 37°C with 5% CO_2. The confluent cells were first washed with 10 mls PBS before a 1 ml solution of stock poliovirus at a final concentration of approximately 1 x $10^5 TCID_{50}u\ ml^{-1}$ was added to the cell surface. This was then incubated for 1 hour at 37°C to allow for virus adsorption to the cells. Following this the monolayer was once again washed and fresh maintenance medium (12 mls) was added. The maintenance medium used was similar to that used for cell growth with the exception that it was supplemented with only 2% foetal calf serum. The reason for this is that many viruses are inhibited by the presence of high foetal calf serum concentrations (Lee & Kurtz, 1981; Willcocks *et al*, 1992; Matsui & Greenberg, 1996). The flasks were then reincubated for approximately 48 hours or until cytopathic effect (CPE) was observed. An inverted light microscope (Leica DMIL) was used to examine the cells on a daily basis to determine the presence of CPE. CPE was apparent when the cells became rounded and came away from the surface of the flask into solution. For each infection process one flask of cells was left uninfected to ensure that the medium and conditions used were not the cause of the CPE.

Following the appearance of CPE the poliovirus was harvested ready for concentration. Since the cells readily came away from the flask surface into solution the scraping of cells was not required. The solution within all of the infected flasks was poured into a glass duran and frozen at -70°C. For concentration, the pooled poliovirus was freeze-thawed twice to break open the cells and expel any intracellular poliovirus. This was then centrifuged in 50ml polypropylene tubes at 3500 rpm (2195 xg) in a benchtop centrifuge (Centaur 2, MSE) to remove cell debris. The supernatant was then ultracentrifuged at 27,000 rpm (135,536 xg) for 3 hours in a Sorvall Ultraspeed centrifuge OTD – 50B (rotor = AH-629) at 4°C in 17 ml polyallomer tubes to pellet the virus. The tubes were then drained and allowed to dry before the viral pellet was resuspended in PBS. For each 17 ml portion of supernatant centrifuged 500 µls PBS was used for resuspension. The stock poliovirus was then alliquoted into 100 µl portions in 0.5 ml Eppendorfs and stored at -70°C for subsequent experiments.

2.5.3 The Enumeration of Poliovirus using $TCID_{50}$

$TCID_{50}$, the 50% infectious dose in tissue culture, is a commonly used method for the enumeration of poliovirus (Melnick & Rennick, 1980). Less tricky than the plaque assay it has been performed by previous workers using BGMK cells (Kok *et al*, 1998). Firstly, the plates must be made. This is done by first trypsinising confluent monlayers of BGMK cells and enumerating them using a neubauer counting chamber as detailed above (section 2.5.1). The cells were then centrifuged at 1000 rpm (179 xg) in a benchtop centrifuge (Centaur 2, MSE)

before being resuspend in tissue culture medium, supplemented with 10% foetal calf serum, to produce a final concentration of 5 x 10^5 cells/ml. 200 µls of this cell suspension was then dispensed into flat bottomed 96 well microtitre plates using an electronic multichannel pipette (BIOHIT). These plates were placed in a 37°C incubator with 5% CO_2 for a few hours to allow the cells to settle.

Poliovirus stock solution was serially diluted 10 fold in tissue culture medium. For each dilution 10 times 100 µls were then inoculated into 10 wells of the microtitre plate seeded with BGMK cells. The outer wells of the plates were not used as they tend to dry out when incubated. Once inoculated the plates were incubated at 37°C with 5% CO_2 for approximately 48hours. Plates were examined for CPE using an inverted light microscope (Lieca DMIL) on a daily basis to check for the appearance of CPE. Following their infection the plates were stained with crystal violet. The staining procedure began with formalising the plates. The tissue culture medium was carefully poured into a waste vessel containing a solution of 10% virkon (Antec International, Suffolk, UK). Into each well of the microtitre plates was then placed a solution of formal saline (see Appendix 1) and this was incubated at room temperature for approximately 1 hour to allow for the fixing of uninfected cells. The plates were then briefly rinsed with tap water and a solution of 0.25% crystal violet was added to each well using a plastic pasteur pipette and allowed to stain for 15 to 30 minutes. Following staining the plates were once again rinsed with tap water and allowed to dry at room temperature on blue tissue paper.

This staining procedure allows the final results to be read without the need for a microscope. Those cells, which became infected with the poliovirus, became detached from the plastic surface of the microtitre plate while those uninfected cells remained firmly attached. This means that at the formalising stage only those cells remaining will be fixed to the plate and thus only those uninfected cells will be subsequently stained by the crystal violet. Therefore a clear well depicts infection while a purple stained well does not. The number of clear wells for each dilution were then enumerated and the $TCID_{50}$ of the stock solution could be calculated using the formula in Appendix 2. This technique utilised cell suspensions rather than allowing the formation of a monolayer prior to sample inoculation. This was because previous workers had found that using suspended cells achieved greater sensitivity than using a preformed monolayer (Anderson *et al*, 1996).

2.5.4 Evaluating Physical Biofilm Removal Methods on Poliovirus

To determine whether the procedure to be used to remove the biofilm was detrimental to the poliovirus it was deemed necessary to test the two techniques of sonication and vortexing on

the poliovirus itself. This experiment was very similar to the one involving bacteriophage in that 100 µls poliovirus stock solution was placed in 1.9 mls filter sterilised tap water. This solution was then divided into two 1 ml portions and placed into two 3" glass test tubes. 100 µls was then removed from each of the two tubes and placed in the fridge for short term storage. One tube was then placed in a test tube rack while the other was subjected to 1 minute sonication treatment in a sonic water bath (Kerry). Following treatment, 100 µls was again removed from each of the two tubes and all four samples were enumerated for poliovirus using the $TCID_{50}$ procedure detailed above (section 2.5.3). This experiment was then repeated, substituting a vortexing step for the sonication treatment. Both experiments were performed in triplicate.

2.5.5 RT-PCR Protocols

PCR was another method used to detect the presence of poliovirus in the biofilm samples. This method was not only more sensitive than the $TCID_{50}$ assay but it also allowed for the detection of none viable viral particles. A single tube method was used for this taken from a procedure by Cook and Kurdziel (1998). This method eliminated the need for separate RNA extraction or reverse transcriptase steps thus reducing problems of contamination and loss of sample. For increased sensitivity a nested procedure was used. In the first step the rTth enzyme (Perkin Elmer) was used, instead of *Taq* polymerase, which can perform both the reverse transcriptase and the amplification step. The reaction mix for this consisted of 2 mM $Mn(OAc)_2$; 0.2 mM each of dGTP, dATP, dCTP and dTTP (Promega, Madison, WI, USA); 25 µM each of primers Po1 (5' – CAGTTCAAGAGCAAACACC – 3') and Po2 (5' – TCGTCCATA ATCACCACTCC – 3') for poliovirus (Egger et al 1995) and 2.5 units rT*th* polymerase in the EZ buffer (250 mM Bicine, 575 mM Potassium acetate, 40% (w/v) Glycerol, pH 8.2) taken from the EZ rT*th* RNA PCR Kit (Perkin Elmer, Warrington, UK). 25 µls of this reaction mix was then transferred to a 0.5 ml thin walled Eppendorf containing 25 µls of sample and overlayed with 1 to 2 drops of mineral oil (Sigma Chemical Co., Ltd). The PCR was performed in a Trio-Thermoblock (Biometra) using the following cycling procedure. The RNA was first extracted from the whole virus by heating for 5 minutes at 94°C before the reverse transcriptase step of 60°C for 30 minutes. This was followed by a denaturation step of 94°C for 1 minute and continued with 50 cycles of 94°C for 45 seconds and 62.5°C for 45 seconds with a final 7 minute extension at 72°C.

Following this first step the nested step was performed again in the Trio-Thermoblock (Biometra). This time 2 µls of the first round product was added to 48 µls of the following reaction mixture; 2 mM $MgCl_2$; 0.3 mM each of dGTP, dATP, dCTP and dTTP (Promega, Madison, WI, USA); 75 µM each of primers Po1N (5' – CATTCAGGGGCCGGAGGA – 3') and

Po2N (5' – AAGCACTTCTGTTTCC – 3'; Hyypia et al 1989) and 1 unit *Taq* polymerase in a buffer consisting of 750 mM Tris HCl (pH 8.8), 20 mM $(NH_4)2SO_4$ and 0.1% Tween 20 (Advanced Biotechnologies, Leatherhead, UK). This mixture was once again overlayed with mineral oil (Sigma Chemical Co., Ltd) and cycled as follows. The denaturation step was 94°C for 2 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 74°C for 1 minute, with a final 5 minute extension of 74°C. This procedure yielded a product of 139 bp which was detected by agarose gel electrophoresis with a 2% gel containing 1ug ml^{-1} Ethidium bromide (Sigma Chemical Co., Ltd).

Prior to performing PCR on the biofilm samples the sensitivity of the procedure was tested. This was done by serially diluting the stock poliovirus 10 fold in nanopure water. Each dilution was then amplified by PCR and enumerated by $TCID_{50}$. The sensitivity of the PCR reaction was then compared to the $TCID_{50}$ assay. The results obtained indicated a good level of sensitivity but a distinction between the lowest and highest dilutions could not be distinguished on a gel. Thus this method could not be effectively used for the quantitation of the virus present in the initial sample and so an alternative quantification procedure, that of DIG hybridisation, was used.

Some problems were encountered when attempting to PCR certain biofilm samples and this was attributed to the combination of serum-free media, in which the samples were placed, and inhibitors present in the biofilm sample itself. To overcome this problem the RNA was extracted from these samples prior to the above RT-PCR procedure.

2.5.6 RNA Extraction

Prior to RNA extraction all Eppendorfs and Gilson pipette tips were treated with 1µl ml^{-1} DEPC to remove any RNases present. All steps were done at 4°C to maintain the stability of the RNA.

For the extraction procedure, 200 µls of sample was placed in a 1.5 ml Eppendorf containing 200 µls chloroform and 200 µls RNA isolation reagent (Ultraspec). The mixture was vortexed for 15 seconds and stored at 4°C for 5 minutes before being centrifuged (MSE Microcentaur Microfuge) at 13K rpm for 15 minutes. The upper aqueous layer was then carefully removed and placed in a fresh Eppendorf containing an equal volume (approximately 400 µls) of isopropanol. This was then mixed by gently inverting the tube and the RNA was precipitated at -20°C for at least 30 minutes. Following a further centrifugation step at 13K rpm for 15 minutes, the resultant RNA pellet was washed with 70% ethanol before being air dried at

room temperature. The RNA was then resuspended in 30 µls RNase-free nanopure water and stored at –70°C for subsequent experiments.

2.5.7 DIG Hybridisation Methods

To quantify the total virus load present in the biofilm samples filter hybridisation was performed on them. An alternative to the use of radioactively labelled probes (with ^{32}P or ^{35}S), this simple method utilises a digoxygenin-11-dUTP (DIG) labelled probe. Following its hybridisation with the sample nucleic acid an anti-DIG antibody alkaline phosphatase conjugate was allowed to bind and the subsequent signal is detected, directly on the membrane, using a colourimetric alkaline phosphatase substrate. For quantitation, the intensity of the colour produced on the membrane could then be compared to standards and the amount of nucleic acid hybridised calculated.

This experiment involved several steps, the first of which was the generation of a labelled probe. This was done by incorporating the DIG-11-dUTP label during PCR using the PCR DIG probe synthesis kit (Boehringer Mannheim GmbH, Biochemica.). For this 2 µls of first round PCR product generated from the positive control in the previous PCR experiments (see section 2.5.5) was added to a second round PCR labelling reaction mix. The mix consisted of 2 mM MgCl2; 0.2 mM each of dATP, dATP and dGTP; 0.13 mM dTTP; 0.07 mM alkali-labile DIG-11-DUTP; 0.75 mM each of Po1N (5' – CATTCAGGGGCCGGAGGA – 3') and Po2N (5' – AAGCACTTCTGTTTCC – 3'; Hyypia et al 1989) with 1 unit of *Taq* polymerase in a buffer consisting of 750 mM Tris HCl (pH 8.8), 20 mM $(NH_4)2SO_4$ and 0.1% Tween 20 (Advanced Biotechnologies, Leatherhead, UK). This mix plus the target DNA was transferred to a 0.5 ml Eppendorf, overlayed with a drop of mineral oil (Sigma Chemical Co., Ltd), and cycled on a Trio-Thermoblock (Biometra). The cycling was begun with an initial denaturation step of 94°C for 2 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 74°C for 1 minute, with a final 5 minute extension at 74°C. The resultant PCR product was then cleaned using a QIAquick PCR purification kit (QIAGEN, Crawley, UK) according to the manufacturers instructions and 5 µls was electrophoresed on a 2% agarose gel containing 1µg ml^{-1} ethidium bromide (Sigma Chemical Co., Ltd) to confirm the success of the experiment.

The next step in this experiment was the quantification of the generated probe. This was required to allow for the optimisation of later filter hybridisation experiments. The chosen method for this was the spot test using a DIG-labelled control. This labelled DNA control was made using unlabelled control DNA and the DIG High Prime DNA labelling kit (Boehringer Mannheim GmbH, Biochemica) according to the manufacturers instructions. After prediluting, to produce a final concentration of 1ng μl^{-1}, the control DNA was further diluted in DNA

dilution buffer to produce standards of 100 pgμl^{-1}, 10 pgμl^{-1}, 1 pgμ^{-1} l, 0.1 pgμl^{-1} and 0.01 pgμl^{-1}. This procedure was then repeated for the labelled probe, using the intensity of the band produced by it on the agarose gel to estimate it's approximate concentration and thus predilute to produce about 1 ngμl^{-1} probe. 1 μl of each dilution, for both control and probe, was then spotted onto a nylon membrane and fixed by cross-linking with UV-light for 1 minute. After briefly rinsing the membrane it was placed in blocking solution for 30 minutes at room temperature before being submerged in antibody solution for a further 30 minutes at room temperature. After washing twice in washing buffer (15 minutes per wash), the membrane was ready for the detection procedure.

For detection of the hybridised probe, the membrane was placed in detection buffer for 2 minutes before being transferred to 5 mls colour substrate solution. The colour precipitate was then allowed to form in the dark. Once the colour had developed (usually after 4 hours), the reaction was stopped by washing the membrane in sterile TE buffer for 5 minutes at room temperature. The intensities of the experimental spots were then compared to those observed on the control strip and the probe concentration was calculated.

Prior to DIG hybridisation experiments a mock hybridisation was performed on the blank nylon membrane using differing amounts of probe. This was done to optimise the probe concentration required and to reduce any background. For this small pieces of the membrane were cut (approximately 1 cm^2), placed in sealed bags containing 10 mls hybridisation solution and prehybridised at 65 – 68°C for 2 hours. The probe was denatured, by boiling it at 100°C for 10 minutes, and diluted in hybridisation solution to produce final concentrations of 5 ngml^{-1}, 25 ngml^{-1} and 50 ngml^{-1}.

After prehybridisation the hybridisation solution was discarded, replaced with each of the above probe concentrations and hybridised overnight in a waterbath set at 65 - 68°C. A negative control, which did not contain probe, was also included. Following hybridisation the probe was poured into polypropylene tubes and stored at –20°C for reuse. The membranes were then washed twice in 2x wash solution at room temperature (15 minutes per wash). This process was repeated using 0.5x wash solution at 65 – 68°C and followed with an equilibration step in washing buffer for 1 minute at room temperature. After this the detection procedure outlined above was performed. Since no samples were placed on the membrane this method was simply done to determine how much background would be produced when using the different concentrations of probe. The results indicated little background and 25 ngml^{-1} probe was used as the stock probe concentration for all hybridisation experiments performed after this.

For initial hybridisation experiments the biofilm samples were placed directly onto the membranes in 10 µl portions and cross linked by baking at 120°C for 30 minutes. The theory was that this heat would rupture the viruses and expel the RNA. However, problems occurred at the detection stage because the samples were in tissue culture medium. This medium stained the membrane so the colourimetric assay could not be visualised. To combat this problem the RNA was extracted from the samples (see Section 2.5.6) prior to inoculation onto the membrane and it was then cross linked by subjecting it to 1 minute UV treatment. To maximise the sample placed on the membrane a slot blot method was used. For this, the slot blot manifold was cleaned and 4 sheets of heavy, adsorbant paper were prewetted in 20x SSC and placed on it. The membrane was then briefly prewetted in water, carefully placed on top ensuring that no bubbles formed underneath and the manifold was clamped together. The apparatus was then connected to a vacuum pump and all the slots were washed through with 10x SSC twice. Meanwhile the RNA was placed in a denaturing solution containing 20 µl 100% formamide, 7 µl 37% formaldehyde and 2 µl 20x SSC for every 10 µls RNA. This was incubated for 15 minutes at 68°C before being cooled on ice. 50 µls of the RNA preparation was then loaded into the slots and the vacuum was applied to draw it through. The membrane was then ready for cross linking which was achieved using the 1 minute UV treatment mentioned above.

For hybridisation the membrane was prehybridised at 65 – 68°C for 2 hours and the hybridised with 25 $ngml^{-1}$ probe in hybridisation solution overnight. The probe was boiled for 10 minutes at 100°C and plunged on ice prior to this. The membrane was then washed and the detection procedure begun. These methods are the same as those described above for the quantification of probe.

CHAPTER 3

RESULTS AND DISCUSSION I
THE BIOFILM MODEL

3.1 Generating A Biofilm In A Model System

3.1.1 The Initial Inoculum

In order to recreate the conditions within a water distribution system tap water was used as the initial inoculum. Boosted tap water (water held in a storage tank) was chosen, as high bacterial numbers had previously been isolated from it (Prof. S Myint, personal communication) and sodium thiosulphate was added to a final concentration of 0.01% to remove any residual chlorine present (Le Chavallier *et al.*, 1987). To accelerate biofilm formation and reduce microbial variation between experiments large quantities (50 litres) of this water were concentrated prior to inoculation into the Modified Robbins Device (MRD).

This method for generating a natural biofilm model was adapted from the method chosen by Rogers *et al.* (1994) who originally inoculated their system with a natural biofilm and used filter sterilised tap water as the nutrient source for it's culture. In this particular case not enough material could be obtained as an inoculum from the pipes in a water distribution system to produce an adequate biofilm model. Therefore, since most planktonic organisms in tap water have originated from a biofilm, tap water was concentrated and used in a recycling system. As the source for the initial inoculum it had the added advantage of being readily available and in great abundance. Therefore it was decided that this would produce the most accurate representation of a water distribution system biofilm.

Other methods for the production of a biofilm model were also considered but were found to have many disadvantages. Robinson *et al.* (1995) isolated the organisms from a natural biofilm and then inoculated them into their system. This method creates problems such as bias, for example the laboratory selection of strains and the exclusion of non-culturable isolates and other organisms such as free living amoebae (FLA). Other workers used normal drinking water as the sole source for the formation of their biofilm. Manz *et al.* (1993) installed an MRD directly into a water distribution system, Frias *et al.* (1994) circulated finished drinking water through their apparatus and Kerr *et al.* (1997) fed a continuous supply of potable water through an MRD. All research groups had to wait a long time, 3 to 8 weeks, 2 months and 11 days respectively, for biofilm development to occur. In contrast preliminary results from my investigations demonstrated steady state biofilm formation after 24 hours (as shown in Figure 3.1). These results reinforce the view that concentrated tap water accelerates biofilm formation.

Figure 3.1 shows the total viable heterotrophs recovered from the initial inocula and from the sessile phase of 4 separate experiments. This graph is displayed in log format because

Figure 3.1 - A Comparison of the Initial Inoculum with the Biofilm formed after 1 and 14 days in four Distinct MRD's

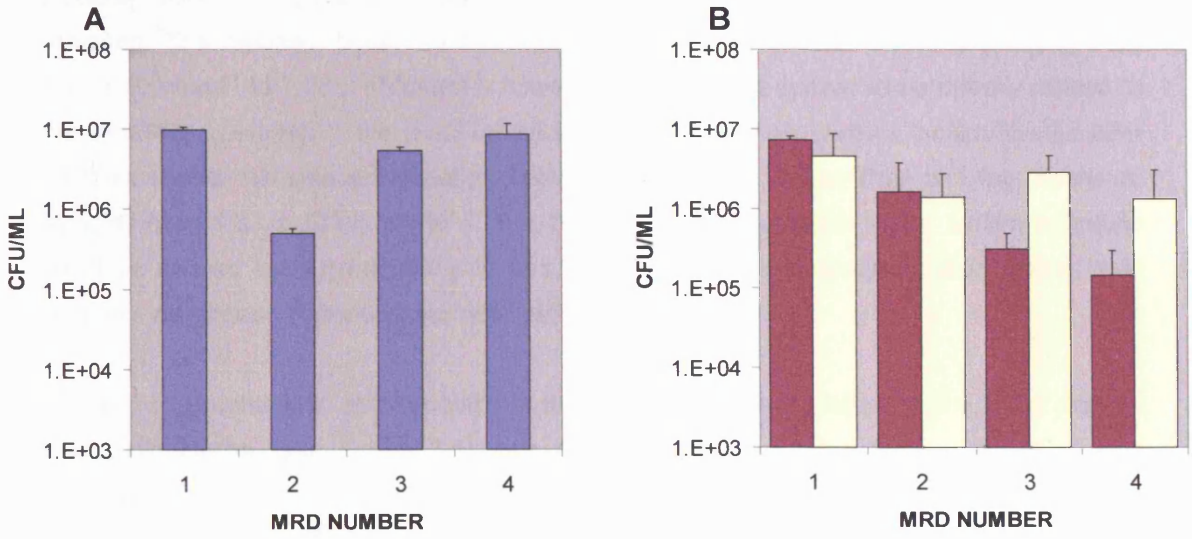


Figure 3.1 - The total viable heterotrophs isolated from **A** the initial innoculum ( ) and **B** the sessile phase after 1 ( ) and 14 days ( ) . The initial innoculum is expressed as the mean of four samples taken from four separate experiments. The sessile phase is also expressed as the mean of four samples but these are taken from two MRD's within each experiment.

natural variation occurs in microbial populations and only log changes were considered to be of significance to these experiments. The results show that the initial inoculum was variable between MRD's and ranged over approximately $1^1/_2$ logs (i.e. from $4.98 \times 10^5 cfuml^{-1}$ and $9.76 \times 10^6 cfuml^{-1}$). Since these experiments were done at separate times this variation was probably due to daytime temperature fluctuations and differences in tap usage when sampling. The resultant biofilm formed after 1 day exhibits similar variability (ranging from $1.46 \times 10^5 cfuml^{-1}$ to $7.35 \times 10^6 cfuml^{-1}$) however this does not appear to be directly related to the variability observed in the initial inoculum. The slightly more mature biofilm formed after 14 days shows the smallest variation between MRD's and is less than a 1 log difference (ranging from $1.33 \times 10^6 cfuml^{-1}$ to $4.50 \times 10^6 cfuml^{-1}$). This variation in the biofilm formation could be caused by a great many factors such as daytime temperature fluctuations, stud variation, roughness of the disc surfaces etc.

It should be emphasised at this point that there is little difference between the 1-day and the 14-day-old biofilm (see Figure 3.1) thus demonstrating that after 1 day the biofilm has reached steady state (as mentioned previously). However, since the variation between experiments is smaller for the more mature biofilm it was decided that the model would be left for this period of time. In addition since much of the variability in the initial innocula and the biofilm was attributed to daytime temperature fluctuations the systems were placed in a special air-conditioned room in a laminar flow cabinet. Temperature fluctuations were minimised and a chemostat was not required.

3.1.2 The Modified Robbins Device (MRD)

For the majority of experiments the biofilm was generated in a Modified Robbins Device (MRD). This apparatus was chosen because it resembles a pipe in shape and when attached to a peristaltic pump it allows a flowing system to be established. The peristaltic pump also permits the flow rate to be constant and for all experiments it was set at 500µls/minute. Shear forces are also present in a system where a flow has been established further mimicking the conditions within a pipe. The provision of ports in the top permitted the ease of sampling without disassembling the apparatus and allowed different materials to be examined.

The MRD was attached to the reservoir and the pump via silicon rubber tubing. This material is commonly used in such systems (Gilbert & Allison, 1993) because it is inert and thus has little or no effect on either the planktonic or the sessile phase. For comparative purposes the pipes were all the same length and breadth (except in some unavoidable cases where connectors were slightly different). In addition for reasons of reproducibility each stud was numbered so that after sampling similar studs were replaced in their respective ports. This

however did not allow for any differences in roughness of the discs attached to the studs and some unavoidable differences in biofilm formation may have been caused by this.

The system used was closed i.e. the culture was recirculated through the model. The advantages of this were mainly that the initial inoculum was not disposed of and that slow growing organisms were not washed away. In addition it allowed the rapid, simple and reproducible production of a model water distribution system biofilm (Gilbert & Allison, 1993). The disadvantages of this system were that of contamination when sampling and the fact that it is uncontrolled with respect to the growth rate (Brown *et al.*; 1988). The former problem was greatly reduced by placing the MRD in a sterile laminar flow cabinet. This was also beneficial in controlling the water temperature within the system. Although the tap water temperature used as the initial inoculum source fluctuated proportionally with changes in room temperature, the planktonic phase in the MRD remained constant at approximately 22°C throughout the course of the experiments eliminating the need for a temperature control.

The other factor taken into consideration while setting up the model biofilm was that of pH. The pH of this model was monitored during the course of one experiment and over a period of 14 days it remained constant at 7.2. Additionally the tap water used as the source of the initial innoculum was also measured and results indicated, once again, a constant pH of 7.2.

The pipes in a water distribution system are commonly made of copper throughout the household and medium density polyethylene (MDPE) going into the household. Thus biofilm formation was preliminarily examined on these two materials. Figure 3.2 shows the total viable heterotrophs isolated from biofilms formed on both copper and MDPE. The results indicate that a much thicker biofilm formed on the MDPE. When testing the biofilm on copper the planktonic phase was examined for copper ions to determine whether high levels were present due to leaching.

Table 3.1 – The Levels Of Copper Ions Present In Tap Water And The Planktonic Phase Of The MRD After 14 Days

SAMPLE	COPPER IONS (mgL^{-1})
Tap Water 1	0.49
Tap Water 2	0.48
MRD 1	0.34
MRD 2	0.37

Table 3.1 – The copper levels were measured by the atomic adsorption spectrometric method. Samples were compared to standards containing 1 to 5 mgL^{-1} copper ions and were duplicated.

Figure 3.2 - A Comparison of the Biofilms Formed on Copper and Plastic in the Small MRD's

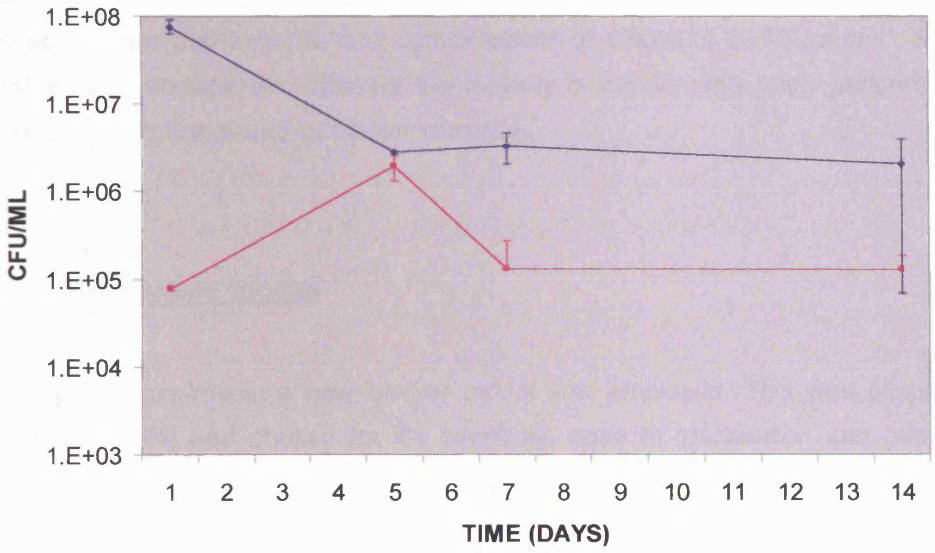


Figure 3.2 - The total viable heterotrophs isolated from MDPE (—◆—) and copper (—■—) from studs in two small MRD's. Data are expressed as the mean of triplicate studs with population standard deviations as error bars.

Table 3.1 shows the levels of copper ions in the tap water and in the planktonic phase of the MRD. Interestingly the results show that there were higher copper levels in the tap water than in the MRD suggesting that the biofilm may have absorbed the missing copper ions. This assumption is reinforced by the work of Rogers *et al.* (1994) who reported that copper had leached from suspended tiles into their control biofilm at a level of 23.75μgs cm^{-2}. Since the two biofilms were considerably different the majority of experiments were performed using MDPE as the principle material for biofilm formation.

3.1.3 An Alternative Model

For a few later experiments a new simpler model was employed. This was adapted from Rogers *et al.* (1994) and chosen for it's simplicity, ease of sterilisation and because an unlimited number of surfaces could be colonised. For comparative purposes this model was also a closed system and the shape of the jar was once again tubular mimicking a pipe but not as closely as the MRD. The surfaces were hung on cotton around the periphery of the jar and the movement of water was achieved using a magnetic stirrer. The shear forces produced in this model are assumed to be different to those produced by a flowing system but this was taken into account and some preliminary tests were performed using it.

3.2 Sampling the Biofilm

3.2.1 The Evaluation of Biofilm Removal Methods

Before beginning an experiment to monitor the biofilm formation in a model system it is essential to determine whether the removal methods to be used are adequate and that the organisms being removed remain unharmed. To effectively remove the biofilm from the discs physical methods were adopted. The reason for this is that biofilms are notoriously adherent to surfaces and thus are very difficult to remove (Eginton *et al.*, 1995). The chosen method was adapted from that used by Jass *et al.* (1995) and the techniques under investigation were sonicating and vortexing for both 1 and 5 minutes.

Figure 3.3 shows the number of heterotrophs released into the water phase following these two removal methods. The graph clearly shows that both methods for biofilm removal work relatively well causing the release of increased heterotroph numbers after 1 minute of treatment. Vortexing the sample appears to cause higher release of heterotrophs into the water phase than sonicating alone. This technique exhibited similar numbers of organisms after both 1 and 5 minutes indicating that sufficient organisms have been removed after the 1-minute treatment and that any further vortexing would be a waste of valuable time. In comparison the sonication treatment produced the highest number of heterotrophs after the 1-minute treatment but then declined after the 5-minute treatment. This suggests that this method is detrimental to the heterotrophs after prolonged periods and this result is further reinforced by results observed in Figure 3.4. Since both methods were adequate at removing heterotrophs from the biofilm after 1-minute treatments they were combined to ensure that the maximum number of organisms could be removed from the sessile phase.

3.2.2 The Effect of Removal Methods on Sessile Organisms

To be certain that these methods adequately removed the organisms without causing them harm the treatments were also tested on the same organisms in the planktonic phase. Figure 3.4 shows the results of this experiment. Once again the sonication treatment produced an increase in heterotroph counts after 1 minute. Since the organisms are already suspended in the water phase this result implies that the sonication treatment is breaking up bacterial aggregates. When this treatment was continued for a further 5 minutes it proved detrimental

Figure 3.3 - The Effect of the Physical Removal Methods on Viable Heterotrophs in the Sessile Phase

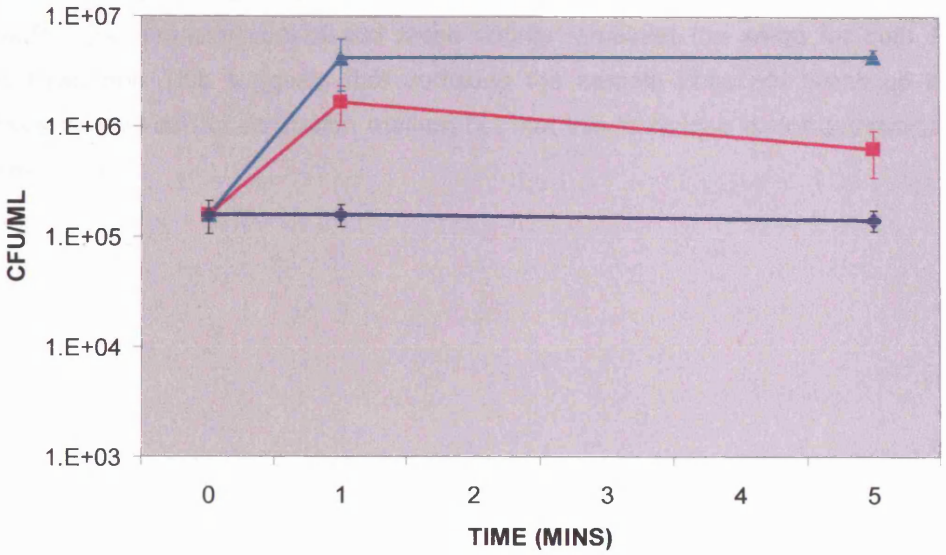
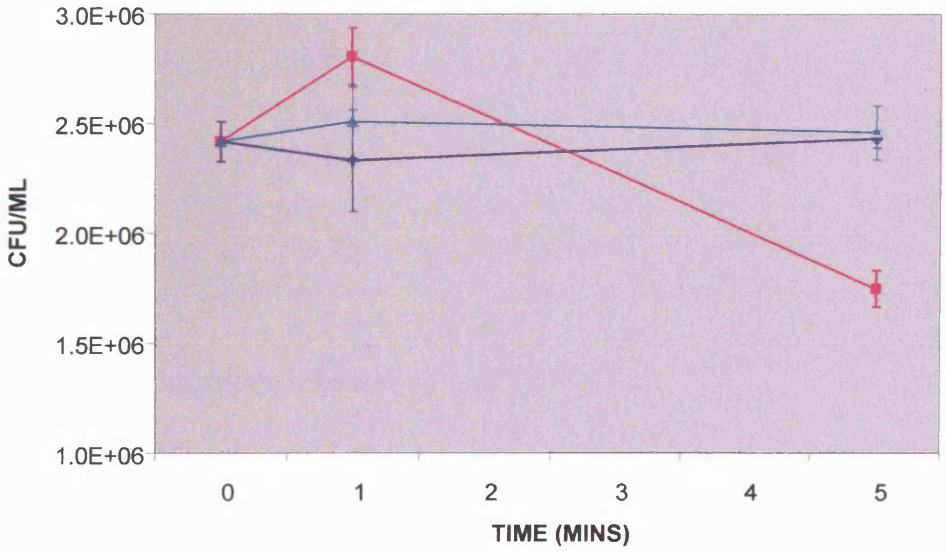


Figure 3.4 - The Effect of the Physical Removal Methods on Viable Heterotrophs in Water to Determine if they are Harmful



Figures 3.3 & 3.4 - The treatments include (◆) negative control, (■) sonication and (▲) vortexing. For Figure 3.3 neighbouring studs were removed from a small MRD for use. The data are expressed as the mean of triplicate experiments with population standard deviations as error bars.

to the organisms producing lower heterotroph counts than those observed at the onset. The inference made from this result is that the sonication treatment is harmful to the heterotrophs if employed for periods longer than 1 minute. This is strengthened by the results shown in Figure 3.3. When the sample was vortexed it too produced slightly higher heterotroph counts compared to the negative control and these counts remained the same for both 1 and 5 minutes treatment. This suggests that vortexing the sample does not break up bacterial aggregates as well as the sonication method but that this technique is not detrimental to the organisms tested.

3.3 The Evaluation of the Biofilm Model Systems

3.3.1 Biofilm Variation in the Model Systems

3.3.1.1 The MRD

Although a steady state biofilm was formed after 24 hours in the MRD, preliminary experiments suggested that a more reproducible biofilm would be formed if the system were left circulating for 14 days (see Figure 3.1). After this period of time the variation along the length of the MRD was evaluated. Figure 3.5 shows the total bacterial counts and free-living amoebae (FLA) present on 2 studs from each section of the MRD.

The total number of bacteria isolated from each of the studs remained constant along the entire length of the MRD. Little difference was observed between either distant or neighbouring studs. In every case, with the exception of stud 1, the total bacterial counts were between 0.5 and 1 log higher than those obtained for the total viable heterotrophs. This difference accounts for the number of dead or unculturable organisms present in the biofilm.

The total viable heterotroph counts displayed a similar pattern exhibiting a constant biofilm of approximately 1×10^7 cfu $disc^{-1}$ on the majority of the studs tested. The only stud exhibiting a significant difference in heterotroph numbers was stud 1, which was located closest to the reservoir. The number of heterotrophs present on this stud was shown to be 1 log lower compared to the others. This was originally attributed to higher shear forces producing an increase in sloughing of cells. However, this idea was abandoned when the total bacterial counts did not reinforce it. Since these are just as high on stud 1 as those on the other studs it must be inferred that this area in the biofilm contains a higher proportion of dead or unculturable organisms than the rest of the biofilm. This variation is probably due to stud location. As the stud is situated at the beginning of the MRD nearest both the reservoir and the connector a slightly harsher environment may be produced.

The presumptive coliforms were so named because they were enumerated on MacConkey agar. This medium is commonly used to isolate coliforms and was originally chosen for that reason. However all organisms detected on this agar were non-lactose fermentors suggesting that they were not coliforms but either strains of other members of the *Enterobacteriacae* or possibly *Pseudomonas spp (*or closely related*)*. The results show that most of the biofilm studs contain approximately 5×10^4 cfu $disc^{-1}$ of presumptive coliforms with the exception of stud 1 and stud 9 which showed both lower and higher numbers respectively.

Figure 3.5 - The Variation in Biofilm Formation Along the Length of the Large MRD

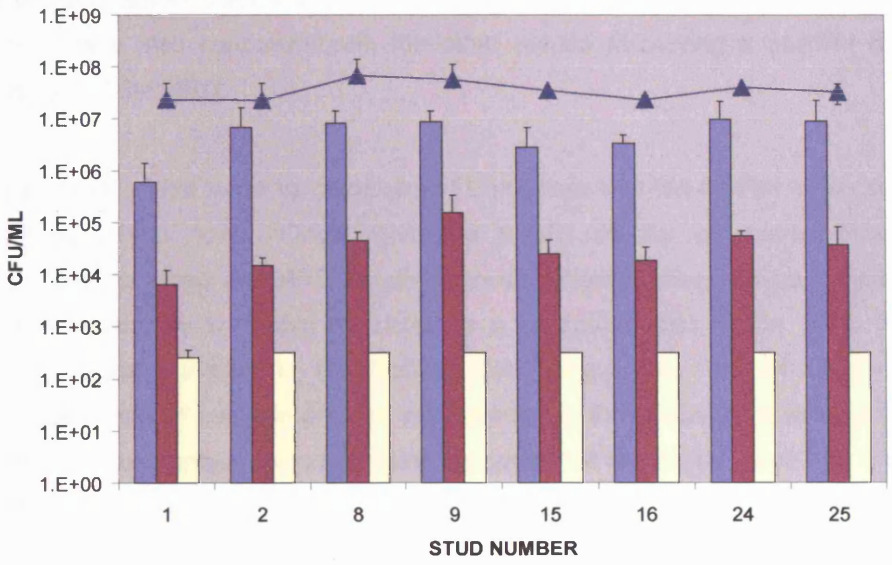


Figure 3.5 - The total bacterial counts (—▲—) and total viable heterotrophs (■), presumptive coliforms (■) and Free-Living Amoebae (□) isolated from the sessile phase of the large MRD at T0. Total counts were done using epifluorescence microscopy while the viable organisms were isolated on R_2A, MacConkey and none nutrient agar (NNA) respectively. NNA was seeded with *E.coli* and FLA counts were estimated using the most probable number method. The model system was left to stabilise for 14 days prior to sampling. Data are expressed as the means of duplicate experiments with the population standard deviations as error bars.

Since the heterotroph numbers are also lower on stud 1 and higher on stud 9 this is not an unexpected result. The reason for this is unclear but may be due to the studs themselves. They may sit at slightly different depths in the MRD causing some differences in biofilm thickness or the associated discs may have variations in roughness at the surface providing differing surface areas thus allowing for differences in the colonisation of the organisms. The FLA numbers were also consistent with the other results displaying a uniform distribution along the length of the MRD.

Figure 3.6 shows the total bacterial counts and FLA's present in the 4 different sections of the MRD at different time points. Once again the results display a uniform distribution of organisms occurring along the MRD length at these different times. Slight variations were observed but since they were not restricted to a particular area in the MRD they were attributed to natural variation in the biofilm. Interestingly after the 14 days of biofilm observation the levels of bacteria present were similar to those observed at time 0 with the exception of the presumptive coliforms. This suggests that the biofilm itself has a finite size and does not continue to grow exponentially. Other workers (Boyle *et al.*, 1999) have noted that flowcell studies, observations of trickling filters and other extended surface systems show biofilm reaching a constant limited thickness. Some reasons for this have been proposed. In addition it has been predicted that, in permeable beds, a constant flow rate and an increase in biofilm thickness would lead to an increase in local velocities and thus an increase in shear forces. Boyle *et al.* (1999) also suggested that, in homogeneous biofilms on surfaces, the transport of nutrients and oxygen (for aerobic films) becomes limiting as the biofilm thickens and that the theoretical maximum active population of cells per unit surface area is dependent on the biofilm morphology (Boyle *et al.*, 1999).

Although the presumptive coliforms remained uniformly distributed along the MRD length, they gradually increased with time to levels much higher than those previously observed at time 0. The reason for this is probably a result of the removal of studs during the experiment. The provision of fresh clean surfaces stimulates the organisms in the system to recolonise and the presumptive coliforms maybe more resistant to external pressures and faster growers than the other heterotrophs. This phenomenon is known as succession and has been noted previously in biofilm communities (Atlas and Bartha, 1998). Wahl (1989) showed that following the immersion of clean surfaces in natural waters a sequence of events occurs resulting in the succession of surface colonising species. Lawrence *et al.* (1995) also noted the occurrence of successional development in complex microbial communities beginning with colonisation of a surface.

In addition, the FLA's have also increased, following a similar successional pattern as the presumptive coliforms. Other workers have observed similar patterns during long term

Figure 3.6 - The Variation in Biofilm Formation in the 4 Sections of the MRD at Different Time Points

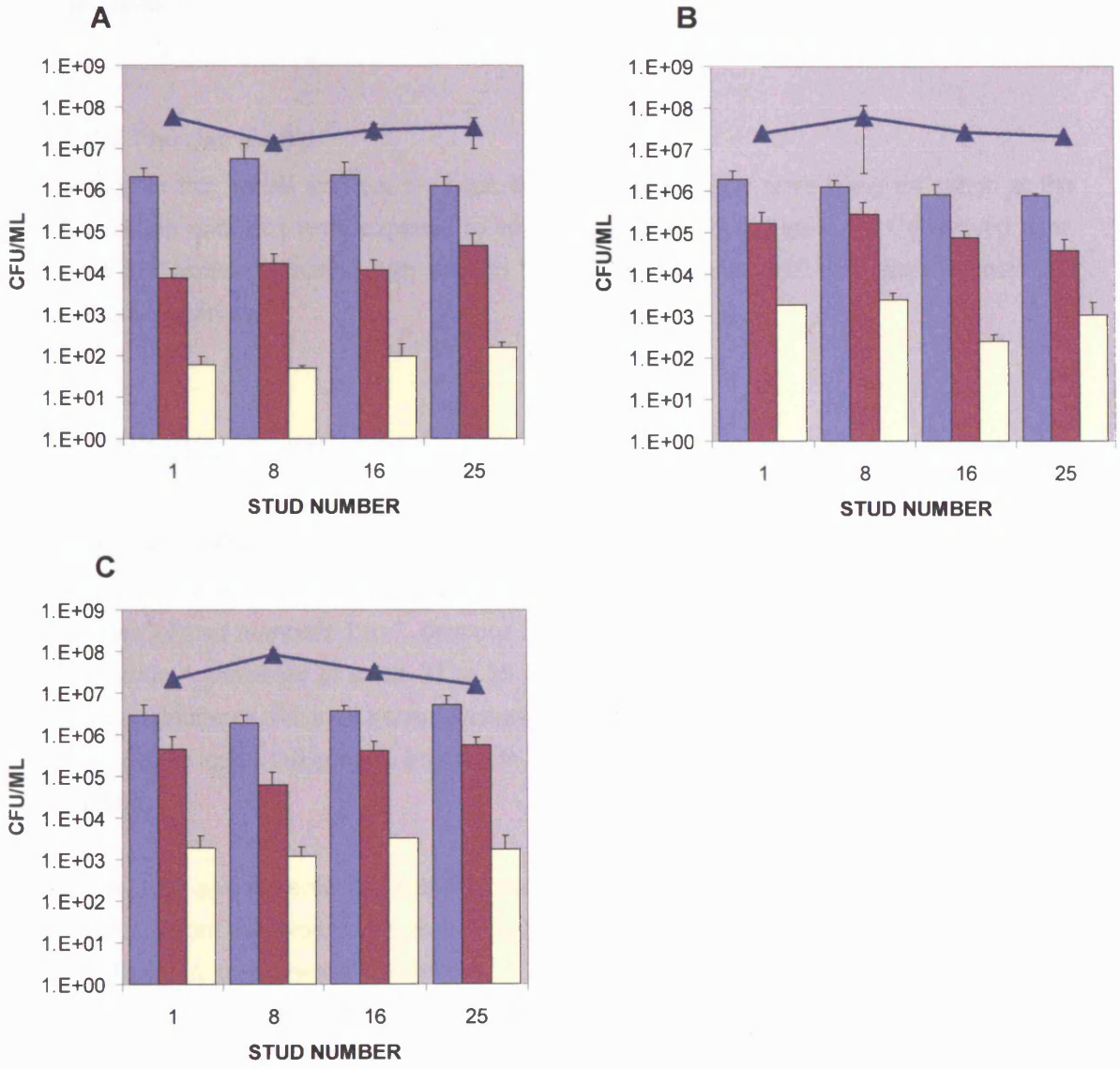


Figure 3.6 - The total bacterial counts (—▲—) ,total viable heterotrophs (■), presumptive coliforms (■) and Free-Living Amoebae (□) isolated from the sessile phase of the large MRD . The variation is shown after **A** 1 day; **B** 7 days and **C** 14 days. Total counts were done using epifluorescence microscopy while the viable organisms were isolated on R_2A, MacConkey and none nutrient agar (NNA) respectively. NNA was seeded with *E.coli* and FLA counts were estimated using the most probable number method. The model system was left to stabilise for 14 days prior to sampling. Data are expressed as the means of duplicate experiments with the population standard deviations as error bars.

observations of immersed surfaces in flowing or static systems. They showed that the primary colonisers are often rod shaped bacteria followed by more fastidious organisms with the eventual attraction of predators, such as amoebae, that feed on the biofilm (Wahl, 1989; Rittle *et al.,* 1990).

3.3.1.2 The Jar Model

Variation in this model was not an issue because all the discs were hung on cotton at the same length and thus were exposed to very similar conditions. Figure 3.11 displayed later, shows the biofilm formation with time in the jar experiments and error bars indicate the variations observed.

3.3.2 Biofilm Formation with Time

3.3.2.1 The MRD

Figure 3.7 shows the biofilm formation in the 4 different sections of the MRD. Section 1 consisted of stud numbers 1 to 7, sections 2 and 3 of studs 8 to 15 and 16 to 20 respectively and section 4 consisted of studs 21 to 25. Each graph displays the counts of the individual biofilm components, the total bacterial counts, viable heterotrophs, presumptive coliforms and FLA's. In this case the graphs are not in log format so that even minor changes can be observed.

Figures 3.7A and B show that a biofilm formed after 24 hours and remained at a constant level throughout the experiment. Some fluctuations were observed, especially in section 2 of the MRD, but these were considered to be insignificant and were attributed to natural variation within the biofilm itself. The presumptive coliforms and the FLA's, however, did not grow in the same way. They increased during the course of the experiment suggesting the occurrence of succession within the biofilm (previously discussed in section 3.3.1) This was probably a result of the sampling process whereby studs were periodically replaced with fresh clean surfaces.

Figure 3.7C shows that in the front end of the MRD (stud numbers 1 to 7) the presumptive coliforms increased exponentially with time. Although this was also the case in section 2, after day 7 a decrease of 2.2×10^5 cfudisc^{-1} was observed. In contrast the latter two sections exhibited a slow rate of growth until day 7 after which their growth rate accelerated by an average of 4.4×10^5 cfudisc^{-1}. The decrease observed in section 2 following the day 7 time point could account for the change of growth rate in the latter two sections. Alternatively the

higher levels of FLA's present in the end section of the MRD could be responsible for their slow rate of growth at the start of the experiment.

Figure 3.7D shows that after one day there are slightly higher FLA levels present in the end section of the MRD than in the other three sections. This suggests that the slightly lower turbulence present in the last section allowed them to colonise preferentially. Following this time point the FLA levels began to rise in the first three sections, if a little slowly in section 3, while remaining constant at the end until day 14 when they appear to have levelled off.

For comparative purposes it was necessary to monitor the levels of bacteria and FLA's in the planktonic phase of the MRD. Figure 3.8 shows these results. Once again the total bacterial counts and the total viable heterotrophs exhibit similar traits. After the initial 14 days a slight decrease in both total and heterotroph counts was observed. This is as expected since the majority of organisms from the initial inoculum will preferentially grow in the sessile phase. Active and passive sloughing in this dynamic system will return similar numbers of organisms back into the planktonic phase which explains why the decrease is only slight.

In contrast, the presumptive coliform and FLA levels have increased slightly suggesting that they may be growing within the system. Once again this is an expected result. The presumptive coliforms maybe faster growers than the majority of the heterotrophs and therefore compete better for space in the biofilm. The FLA's have little competition and since they feed on bacteria the MRD is ideal for their growth.

After 1 day (day 15 in Figure 3.8, owing to the initial 14 day circulation period) there was a dramatic increase in the levels of all the organisms tested. Prior to this a large number of studs were removed from the MRD and replaced with new clean studs. This momentarily upset the dynamics of the system. The presence of the clean studs may have exposed the remaining biofilm to different shear forces thus causing increased sloughing of cells and resulting in an increased number of organisms in the planktonic phase. Additionally it may be that the presence of new surfaces has stimulated the organisms to recolonise and therefore grow faster and compete with each other. Since the presumptive coliforms are quick growers and the FLA's have little competition these organisms will increase more rapidly than the others. This is apparent when examining the results obtained for these organisms.

At day 7 and day 14 (days 21 and 28 on Figure 3.8) the levels of organisms present in the planktonic phase decreased to similar numbers found at the start of the experiment. This indicates that the system is returning to a steady state. Willcock *et al.* (1997) stated that at steady state the numbers of cells detaching from the film, and being dispersed to the fluid phase, are balanced numerically by the growth and division of the attached organisms.

Figure 3.7 - The Biofilm Formation in the MRD with Time

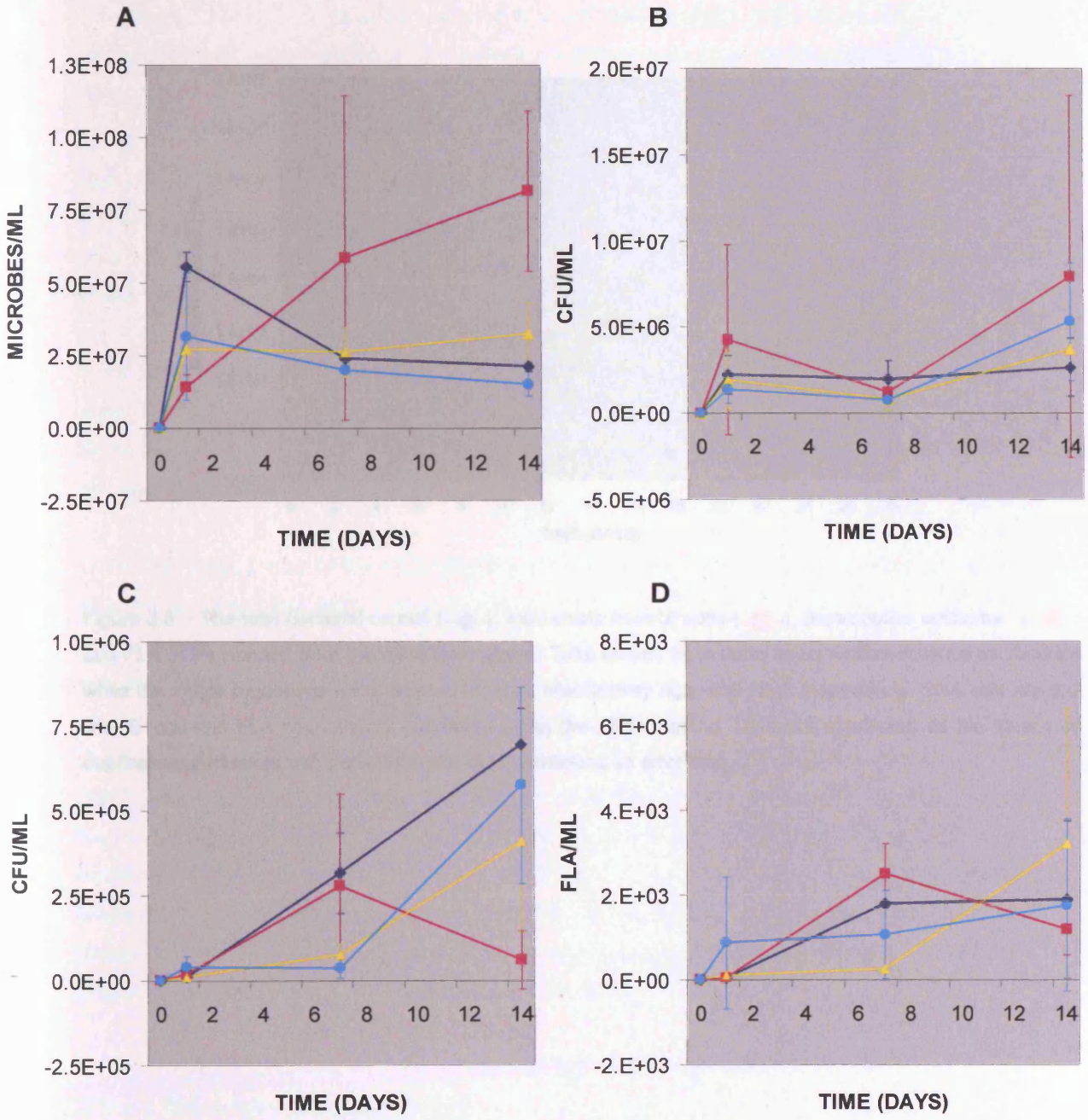


Figure 3.7 - The formation of the biofilm in the 4 sections of the MRD: section 1 (◆); section 2 (■); section 3 (▲) and section 4 (●). The parameters measured were - **A** total bacterial counts, **B** viable heterotrophs, **C** presumptive coliforms and **D** free-living amoebae (FLA). For total counts, epifluorescence microscopy was used while viable organisms were isolated on R_2A, MacConkey and NN agar respectively. Data are expressed as the means of duplicate experiments with population standard deviations as error bars.

Figure 3.8 - The Levels of Planktonic Organisms in the MRD with Time

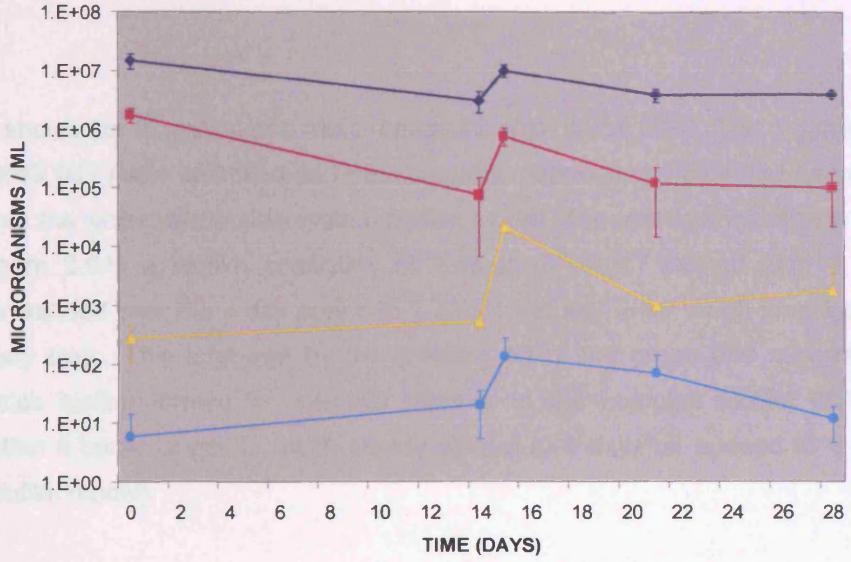


Figure 3.8 - The total bacterial counts (—◆—), total viable heterotrophs (—■—), presumptive coliforms (—▲—) and FLA (—●—) isolated from the planktonic phase. Total counts were done using epifluorescence microscopy while the viable organisms were isolated on R_2A, MacConkey agar and NNA respectively. NNA was seeded with *E. coli* and FLA counts were estimated using the MPN method. Data are expressed as the means of duplicate experiments with population standard deviations as error bars.

Although the levels of organisms in the planktonic and sessile phases are difficult to directly compare they do both exhibit a levelling off after the 1 day time point which is consistent with this theory. However it must be noted that a complete steady state could not be totally returned to the system owing to the selective pressures incurred by the sampling process itself.

Figure 3.9 shows the formation of a mono-species biofilm in the MRD. The organism used for this was ORG 001 (later identified as *Pseudomonas mendocina*) which had been previously isolated from the water distribution system biofilm model. The results show that in the sessile phase (Figure 3.9A) a biofilm consisting of 1.44 x 10^5cfuml^{-1} formed after 1 hour. This gradually increased over the 4 day period to 1.72 x 10^6cfuml^{-1} after which time it appeared to reach steady state. This is shown by the levelling off of the graph and suggests that this mono-species biofilm formed in a similar manner to the complex biofilm with the slight exception that it takes longer to reach steady state (i.e. 4 days as aposed to 1 day for the complex biofilm model).

In the planktonic phase of the MRD (see Figure 3.9B) low levels of viable bacterial cells (2.9 x 10^5cfuml^{-1}) were noted after 1 hour increasing rapidly to 1.65 x 10^6cfuml^{-1} after 1 day. This increase has been previously noted in the complex biofilm model and was attributed to the selective pressures incurred by the sampling process. The replacement of studs with fresh, clean surfaces at sampling would stimulate increased growth in the sessile phase, which in turn, would cause a high release of cells into the planktonic phase at this time. After 1 day the graph levelled off suggesting that steady state biofilm formation had occurred i.e. at this stage the planktonic cells will enter and leave the biofilm at a similar rate. However it did not occur in the sessile phase until day 4. The reason for this is proposed to be due to the increased growth rate stimulated by the presence of the new test surfaces. Although the planktonic phase has already reached the steady state the sessile phase has not and so the cells in this phase will still be at their exponential phase of growth.

3.3.2.2 The Alternative Jar Model

Figure 3.10 shows the biofilm formation on both copper and plastic surfaces in the 3 jar models over time. Jar A contained only one organism (ORG 001) previously isolated from the complex biofilm while jar B contained this and a FLA isolated from the source of the initial inoculum. The results indicate that in each of these jar models a biofilm formed on both surfaces after 1 hour. This biofilm remained constant throughout the time course of the experiment at approximately 3 x 10^7 bacteria disc^{-1}. It must be noted that when comparing total counts for the sessile phase on both copper and plastic in the 2 models, the bacterial numbers were, in general, slightly higher on the copper than on the plastic. This was an

Figure 3.9 - The Formation of a Mono-Species Biofilm in the MRD with Time

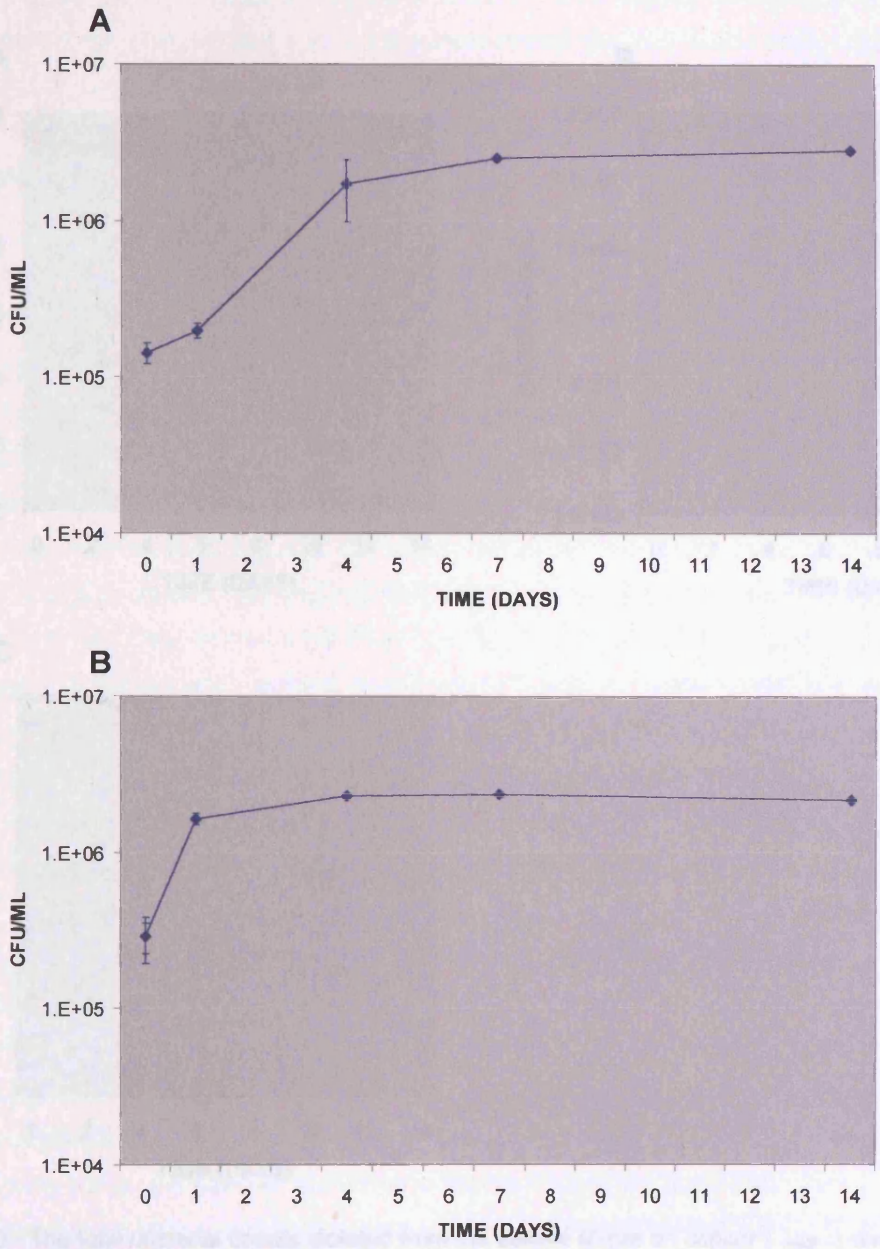


Figure 3.9 - The formation of a mono-species biofilm in the MRD with time. The graphs show the levels of viable bacterial cells in **A** the sessile phase and **B** the planktonic phase of the MRD. The mono-species biofilm was formed from a culture of *Pseudomonas mendocina* (otherwise called ORG 001) in filtered tap water previously isolated from the complex water distribution system biofilm model. Bacterial counts were done using PCA. Data are expressed as the means of duplicate experiments with population standard deviations as error bars.

Figure 3.10 - The Biofilm Formation in the Alternative Jar Models with Time

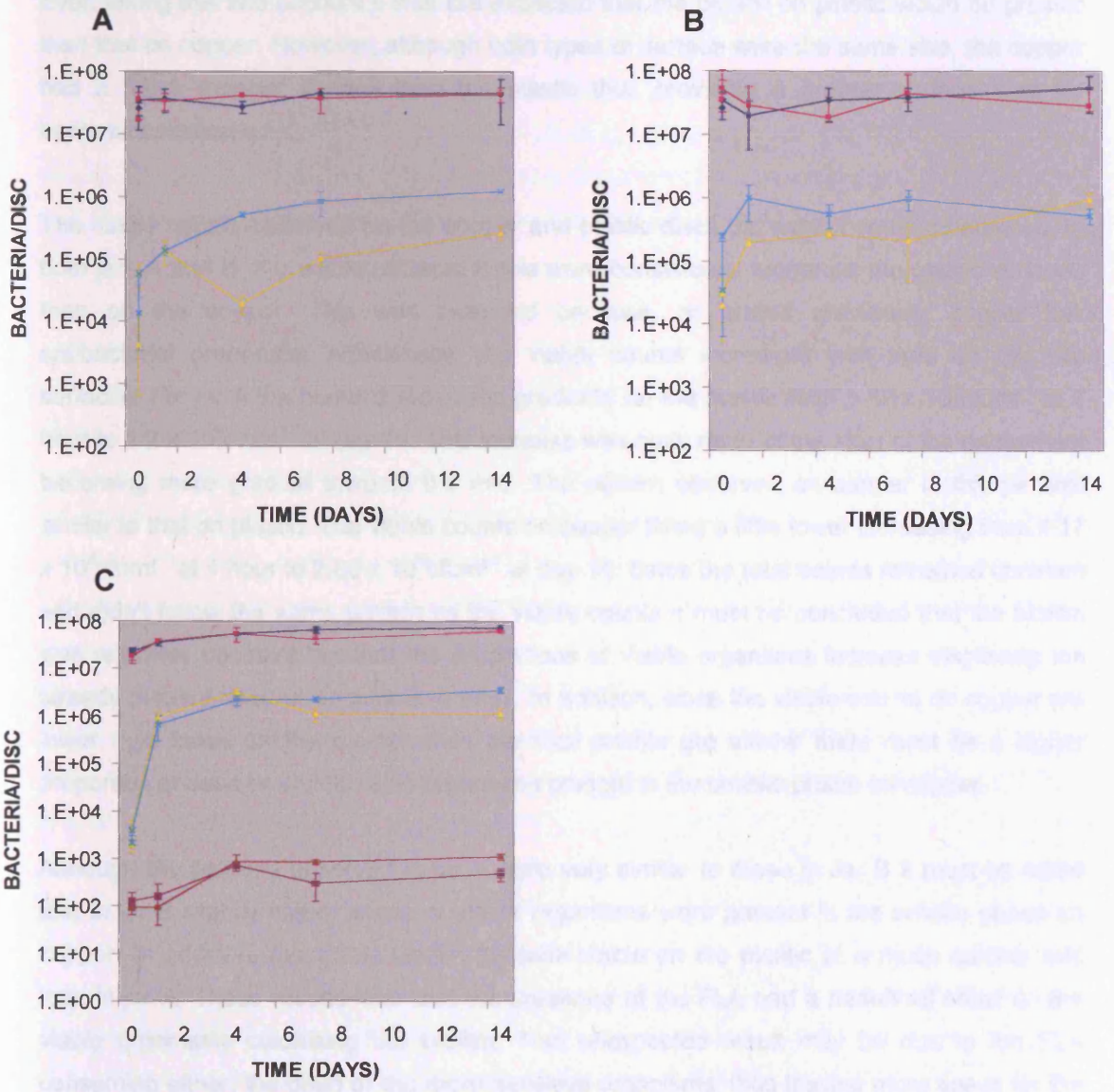


Figure 3.10 - The total bacterial counts isolated from the sessile phase on copper (—◆—) and plastic (—■—), total viable heterotrophs from the sessile phase on copper (—▲—) and plastic (—■—)and the presumptive coliforms from the sessile phase on copper (—■—) and plastic (—▲—) of the three jar models; **A** Mono- species biofilm (containing bacterium 001), **B** binary biofilm (containing bacterium 001 and a FLA) and **C** complex biofilm (containing concentrated water). Total counts were performed using epifluorescence microscopy while the viable organisms were isolated on R_2A and MacConkey agar. Data are expressed as the means of triplicate samples with population standard deviations as error bars.

unexpected result since copper has antibacterial properties (Hermansson *et al.*, 1987) and plastic is inert. However since the copper and plastic surfaces were in the same model it is likely that the biofilm on plastic was also affected by the copper ions present in the system. Even taking this into account it was still expected that the biofilm on plastic would be greater than that on copper. However, although both types of surface were the same size, the copper had a much rougher surface than the plastic thus providing a greater surface area for bacterial colonisation.

The viable counts observed on the copper and plastic discs did exhibit some differences. In both jars A and B, the viable bacterial levels were consistently higher on the plastic surfaces than on the copper. This was expected because, as stated previously, copper has antibacterial properties. Additionally, the viable counts increased with time on the two surfaces. For jar A the bacteria increased gradually on the plastic from $1.32 \times 10^4 cfuml^{-1}$ at 1 hour to $1.2 \times 10^6 cfuml^{-1}$ at day 14. This increase was quite rapid at the start of the experiment becoming more gradual towards the end. The pattern observed on copper in this jar was similar to that on plastic. The viable counts on copper being a little lower increasing from $4.17 \times 10^3 cfuml^{-1}$ at 1 hour to $2.63 \times 10^5 cfuml^{-1}$ at day 14. Since the total counts remained constant and didn't follow the same pattern as the viable counts it must be concluded that the biofilm was relatively constant but that the proportions of viable organisms increase displacing the already present dead or unculturable ones. In addition, since the viable counts on copper are lower than those on the plastic while the total counts are similar there must be a higher proportion of dead or unculturable organisms present in the sessile phase on copper.

Although the patterns observed in jar A were very similar to those in Jar B it must be noted that in jar B slightly higher levels of viable organisms were present in the sessile phase on copper. In addition, the viable biofilm became stable on the plastic at a much quicker rate than in jar A. These results infer that the presence of the FLA had a beneficial effect on the viable organisms colonising the biofilm. This unexpected result may be due to the FLA consuming either, the dead or the more sensitive organisms, thus leaving more space for the viable organisms and selecting for a much more resilient population in the system.

Figure 3.10C shows the biofilm formation occurring in a more complex community of organisms. In this case the initial inoculum was similar to that used for experiments in the MRD. The results obtained for the total counts show that the biofilm formed almost immediately on both the copper and the plastic and remained stable throughout the experiment. The viable counts, however, display a dramatic increase over the initial 1 day period before once again levelling off. These results are comparable to those obtained in the MRD showing stable biofilm formation after 1 day. Interestingly in this model there was little, if any, difference between the viable communities forming on either the copper or the plastic

surfaces. The reason for this is proposed to be that, because the initial inoculum was taken from concentrated tap water, which was transported through copper pipes, the organisms in this water must have some copper resistance. In addition, as stated previously, because the copper and plastic surfaces are in the same jar the copper ions will still affect the organisms in the biofilm forming on the plastic.

Table 3.2 shows the levels of FLA's isolated from the sessile phases of jars B and C. These results confirm that FLA's have become incorporated into the biofilms forming on both copper and plastic in jar B. In contrast, the FLA's were only isolated in the sessile phase of jar C at a much later stage of biofilm development.

Figure 3.11 shows the bacterial levels present in the planktonic phases of the 3 jar models with time. The results indicate stable levels of total and viable organisms in both jars A and B. The total counts fluctuate around a mean of $4 \times 10^7 cfuml^{-1}$ while the viable counts remain 1.5 logs lower at approximately $4 \times 10^6 cfuml^{-1}$. It must be stated that in this phase little growth occurs, the organisms present have mainly originated from the biofilm. Thus the levels are constant due to the biofilm dynamics. The similar counts displayed in both jars suggest that the presence of the FLA's in jar B had little effect on the planktonic organisms. This is further reinforced by the results in Table 3.2 showing low, if any, FLA's isolated from this phase of the model system. The lower viable counts at the start of the experiment reflect the lower counts also observed in the sessile phase. As stated previously these results are 1.5 logs lower than the total counts suggesting that the proportion of dead or unculturable organisms is similar in both phases of the model system.

Figure 3.11C shows the planktonic organisms present in the more complex biofilm model. This shows similar trends in the total and viable counts obtained. Additionally it shows an increase in organisms after 1 day which almost immediately returned to a normal level. This has also been shown to occur in the MRD and may be attributed to the dramatic increase in organisms colonising the sessile phase of the system. The presence of fresh surfaces allows bacterial colonisation and stimulates growth and reproduction. This in turn causes the increased release of organisms into the planktonic phase and is shown by the increase on the graph. The large numbers of planktonic organisms then enter the sessile phase and after the initial day the model stabilised forming a dynamic system. As in jar B, the levels of FLA's in the planktonic phase of this model were very low further suggesting that these organisms prefer the sessile mode of growth. They probably, as in jar B, contribute to the stabilisation and dynamics of the biofilm model.

Figure 3.12 shows the formation of a mono-species biofilm in the alternative jar model using plastic test surfaces only. For this the same organism (ORG 001, later identified as *Ps.*

mendocina) as was used for the above jar experiments. This model was considered more appropriate for later virus investigations than the previous jar models containing both copper and plastic test surfaces, because it would be more comparable to the other models (tube experiments and MRD's) which also contained plastic test surfaces only.

The results displayed in Figure 3.12 are similar to those observed for the mono-species biofilm formed in the MRD (see Figure 3.9). In the sessile phase4 (see Figure 3.12A) a gradual increase in viable cells was observed over the initial 3 day period which levelled off afterwards indicating the formation of a steady state biofilm over this time period. In the planktonic phase (see Figure 3.12B) a similar increase was observed which was rapid over the initial 1 day period and gradual until day 3 when it too levelled off confirming that the model had reached steady state. Although the patterns observed in this model were similar to those previously seen for the same biofilm in the MRD, the levels of bacterial cells in both phases of the alternative jar model were approximately 1 log lower. The reason for this may be attributed to the larger size and hence the larger surface area present in the MRD. Alternatively it may be the fact that the MRD has a flowing system incorporated into the model. This would allow for more nutrients to be transported to the biofilm and waste products to be removed by the same method. Both theories may be correct but the latter idea is thought to be the main reason as other workers have shown that higher flow rates do allow for the formation of thicker biofilms.

3.3.3 The Interactions within the Biofilm Model System

3.3.3.1 The Sessile Phase of the MRD

In the 3 separate MRD's 4 groups of heterotrophs and 3 groups of presumptive coliforms were monitored. These organisms were distinguished by their colonial morphology on their respective agar plates and followed during the course of the experiment to determine whether any relationships emerged. The initial inoculum for MRD's 2 and 3 was from the same sample of concentrated water, while the initial inoculum for MRD 1, although from the same source, was a different concentrated water sample. While slightly different populations were observed in the 3 MRD's the total bacterial counts and total viable organism numbers remained similar to each other and to those displayed in the previous figures (see Figures 3.5 – 3.8). The reasons for the differing populations are proposed to be due to selective pressures incurred within each MRD. These selective pressures are detailed below.

Table 3.2 – The levels of Free- Living Amoebae (FLA) in the Jar Model

A

TIME (DAYS)	JAR B (FLA ML^{-1})	JAR C (FLA ML^{-1})
0	0	0
0.04 *	0	0
0.25 *	0	0
1	1800	0
4	6	0
7	0	1800
14	0	0

B

TIME (DAYS)	JAR B (FLA ML^{-1})	JAR C (FLA ML^{-1})
0	-	-
0.04 *	180	0
0.25 *	3160	0
1	18	0
4	6	0
7	18	5600
14	6	18

C

TIME (DAYS)	JAR B (FLA ML^{-1})	JAR C (FLA ML^{-1})
0	-	-
0.04 *	1800	0
0.25 *	560	0
1	0	0
4	6	0
7	56	316
14	18	18

Table 3.2 – The levels of FLA's isolated from **A** the planktonic phase, **B** the biofilm formed on plastic test surfaces and **C** the biofilm formed on copper test surfaces in the two jar models. Jar B contained organism 001 and a FLA. Both were previous isolates from the water used for the initial inoculum. Jar C contained concentrated water (i.e. 25 litres concentrated to 500 mls). Viable FLA's were isolated using none nutrient agar seeded with *E.coli* and enumerated using the most probable number method. * 0.04 = 1 hour and 0.25 = 6 hours.

Figure 3.11 - The Levels of Planktonic Organisms in the Alternative Jar Model with Time

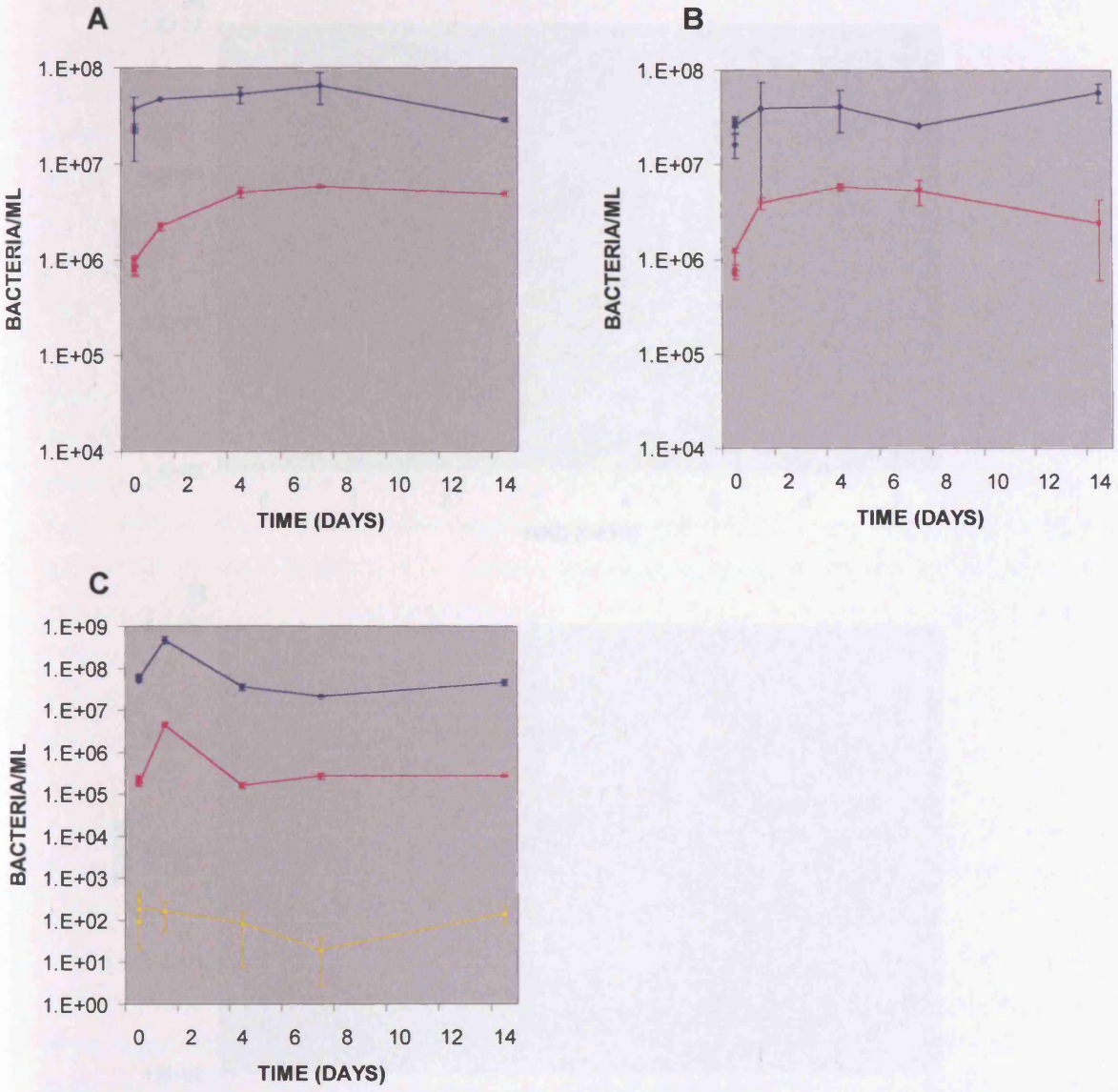


Figure 3.11 - The total bacterial counts (—◆—), total viable heterotrophs (—■—) and presumptive coliforms (—▲—) isolated from the planktonic phase of the three jar models; **A** Mono-species biofilm (containing bacterium 001), **B** Binary biofilm (containing bacterium 001 and a FLA) and **C** Complex biofilm (containing concentrated water). Total counts were performed using epifluorescence microscopy while the viable organisms were isolated on R_2A and MacConkey agar. Data are expressed as the means of triplicate samples with population standard deviations as error bars.

Figure 3.12 - The Formation of a Mono-Species Biofilm in the Alternative Jar Model with Time - using Plastic Test Surfaces Only

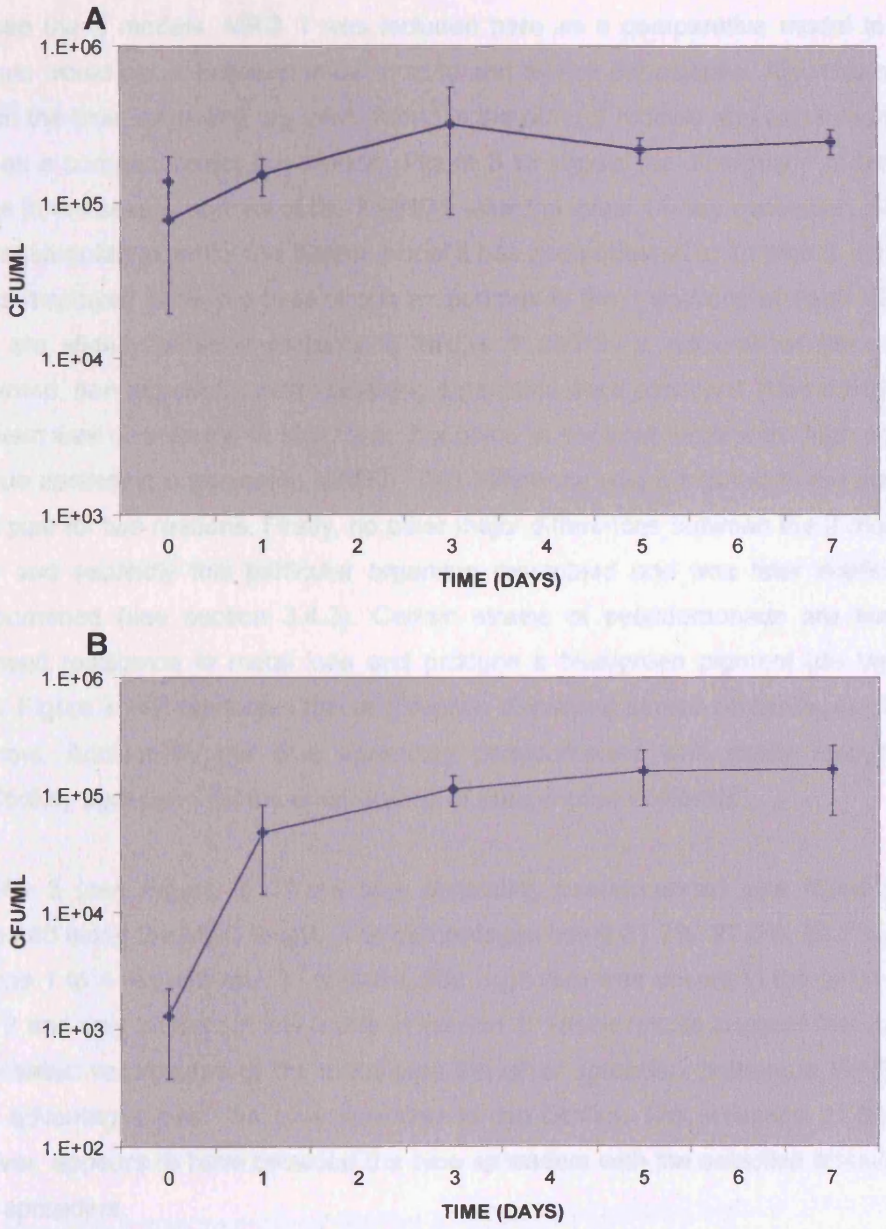


Figure 3.12 - The formation of a mono-species biofilm in the Alternative Jar Model with time using plastic test surfaces only. The graphs show the levels of viable bacterial cells in **A** the sessile phase and **B** the planktonic phase of the MRD. The mono-species biofilm was formed from a culture of *Pseudomonas mendocina* (otherwise called ORG 001) in filtered tap water previously isolated from the complex water distribution system biofilm model. Bacterial counts were done using PCA. Data are expressed as the means of duplicate experiments with population standard deviations as error bars.

As stated previously MRD's 2 and 3 contained the same initial inoculum. However, the apparatus of MRD 3 also contained a small metal pipe connector previously overlooked. It was this metal pipe that was believed to be the cause of the population differences observed between the 2 models. MRD 1 was included here as a comparative model to show if any changes would occur between initial inocula and biofilm populations. Also this model did not contain the blue spreading organism found in the other 2 models and can to some extend be used as a comparison for this reason. Figure 3.13 shows the distribution of the heterotroph groups in the sessile phases of the 3 MRD's after the initial 14-day circulation. Since this was the first sampling point for the biofilm model it has been referred to as time 0. Additionally, the graphs displayed show the heterotroph proportions in the 4 sections of each MRD. Although there are slightly different patterns in MRD's 1 and 2, in general, similar proportions of pigmented, non-pigmented and spreading organisms were observed. Also the blue spreading organism was undetected in MRD 2 at this point. In contrast there were high percentages of the blue spreading organism in MRD 3. This difference was attributed to the presence of the metal pipe for two reasons. Firstly, no other major differences between the 2 models could be found and secondly this particular organism resembled and was later confirmed to be a pseudomonad (see section 3.4.3). Certain strains of pseudomonads are known for their increased resistance to metal ions and produce a blue/green pigment (de Vincente *et al.*, 1990). Figure 3.14B reinforces this assumption displaying similar percentages of presumptive coliforms. Additionally the blue spreading pseudomonad was easily recognised on the MacConkey agar used for the enumeration of presumptive coliforms.

In MRD 3 (see Figure 3.13) the blue spreading pseudomonad was found to be evenly distributed along the MRD length. The percentages being 31.7%, 27.5%, 25.7% and 33.5% in sections 1 to 4 respectively. In contrast, this organism was absent in the latter 3 sections of MRD 2 and only present in low levels at section 1. These results suggest that in the absence of the selective pressure of the metal pipe the other spreaders present in MRD 2 may have more advantages over the blue spreader in the biofilm. The presence of the metal pipe, however, appears to have provided the blue spreaders with the selective advantage over the other spreaders.

The proportions of the pigmented and non-pigmented strains in MRD 3 are also similar to those in the other 2 MRD's and are probably only slightly lower due to the presence of the blue spreading organism. The pattern differences in the different sections of the MRD's can probably be attributed to natural variation in the biofilm and unavoidable differences in the MRD's themselves i.e. stud variation, disc roughness and shear forces.

Figure 3.14 displays the percentages of viable presumptive coliforms in relation to the heterotroph levels in the biofilms forming in the MRD's at time 0. Similar proportions of

Figure 3.13 - The Proportions of Distinguishable Viable Heterotrophs Present in the Sessile Phase of 3 distinct MRD's at Time 0

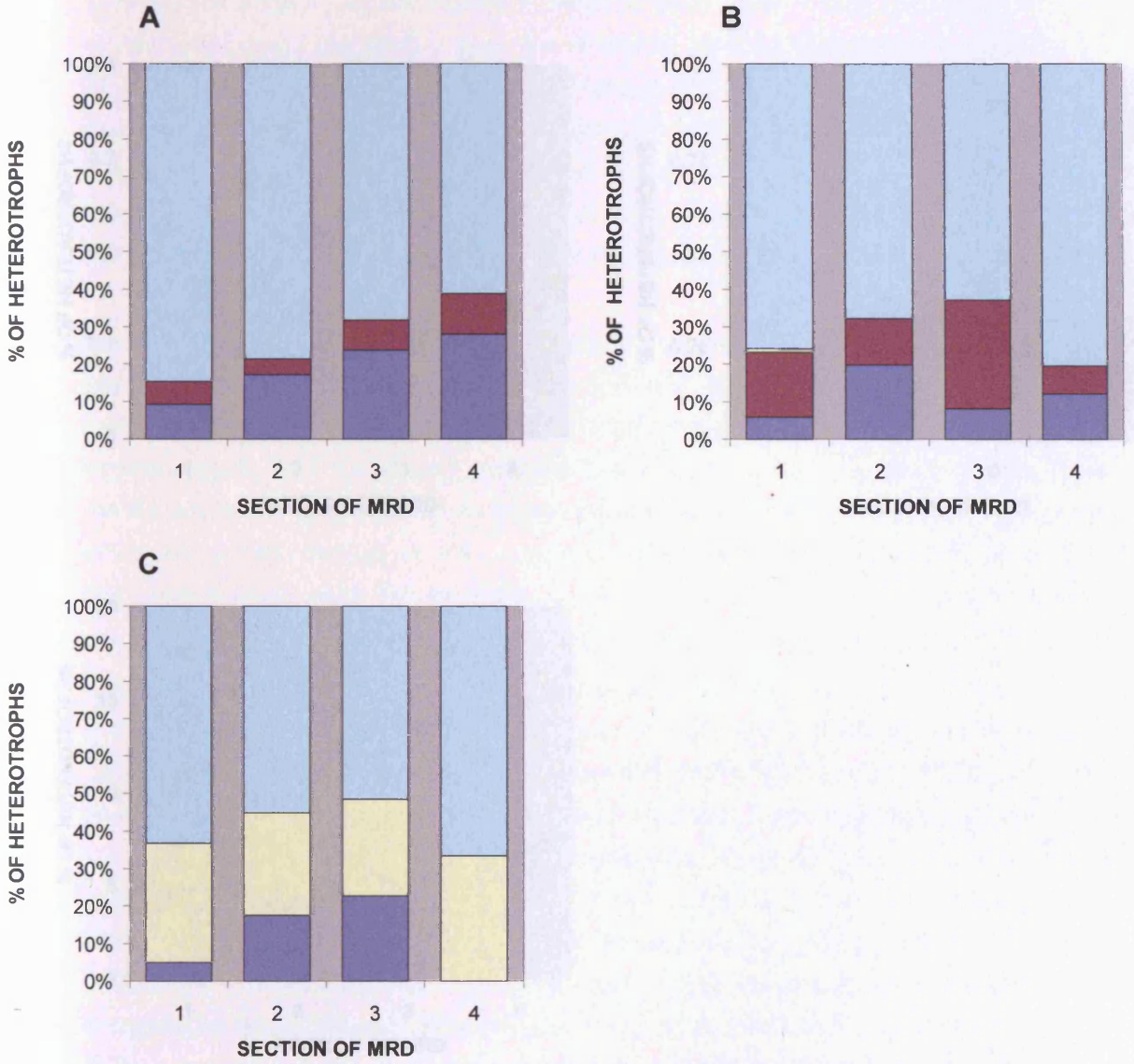


Figure 3.13 - The percentages of pigmented ( ), spreading ( ), blue spreading ( ) and miscellaneous ( ) heterotrophs present in the sessile phase of **A** MRD 1, **B** MRD 2 and **C** MRD 3. All heterotrophs were isolated on R_2A agar and the percentages were relative to the total viable counts also enumerated on R_2A agar.The model systems were left to stabilise for 14 days prior to sampling at T= 0.

Figure 3.14 - The Percentages of Viable Presumptive Coliforms Present in the Sessile Phase of 3 distinct MRD's at Time 0

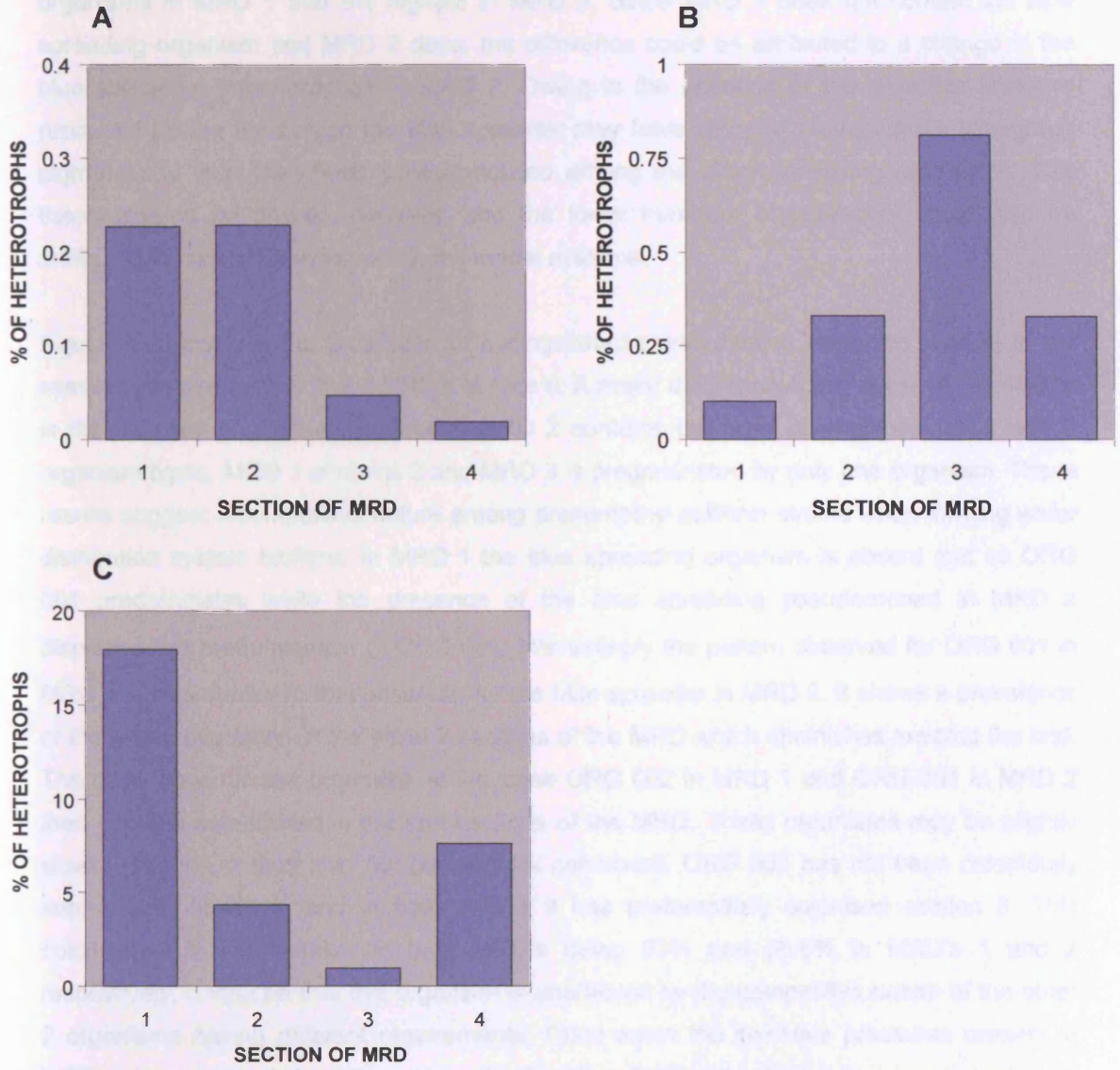


Figure 3.14 - The percentages of presumptive coliforms (■) present in the sessile phase of **A** MRD 1, **B** MRD 2 and **C** MRD 3. Presumptive coliforms were isolated on MacConkey agar and the percentages were relative to the total viable heterotroph counts enumerated on R_2A agar.The model systems were left to stabilise for 14 days prior to sampling at T= 0.

presumptive coliforms but slightly different patterns were observed in MRD's 1 and 2 while much higher presumptive coliform numbers appear in MRD 3. This reinforces the results obtained from the previous figure, which also shows the lowest proportion of spreading organisms in MRD 1 and the highest in MRD 3. Since MRD 1 does not contain the blue spreading organism and MRD 2 does, the difference could be attributed to a change in the blue spreading pseudomonad in MRD 2. Owing to the absence of the selective pressure produced by the metal pipe the blue spreader may have stopped producing it's blue/green pigment and thus may have gone unnoticed among the other spreading organisms. This theory cannot be proved, however, and the lower numbers of spreaders could also be attributed to natural variation within the model systems.

Figure 3.15 displays the proportion of distinguishable presumptive coliforms present in the sessile phase of each of the 3 MRD's at time 0. A major difference noted between the MRD's is the diversity of organisms present. MRD 2 contains the most diverse population with 3 organism types, MRD 1 contains 2 and MRD 3 is predominated by only one organism. These results suggest a competitive nature among presumptive coliform strains when forming water distribution system biofilms. In MRD 1 the blue spreading organism is absent and so ORG 001 predominates while the presence of the blue spreading pseudomonad in MRD 2 displaces this predomination of ORG 001. Interestingly the pattern observed for ORG 001 in MRD 1 is very similar to that observed for the blue spreader in MRD 2. It shows a prevalence of the respective strain in the initial 2 sections of the MRD which diminishes towards the end. The more opportunistic organism, in this case ORG 002 in MRD 1 and ORG 001 in MRD 2 then become established in the end sections of the MRD. These organisms may be slightly slower growers or they may not be as quick colonisers. ORG 002 has not been completely suppressed, however, and in both MRD's it has preferentially colonised section 3. This colonisation is very similar in both MRD's being 53% and 58.8% in MRD's 1 and 2 respectively. It may be that this organism is unaffected by the competitive nature of the other 2 organisms having different requirements. Once again the selective pressures present in MRD 3 has reduced the competition significantly allowing the blue spreading organism to completely dominate the biofilm.

Figure 3.16 shows the levels of heterotrophs present in the sessile phase of the 3 MRD's with time. Although some differing relationships exist, MRD's 1 and 2 exhibit very stable levels of organisms in the biofilm over the time period monitored. In contrast MRD 3 displays an increase in 3 groups of heterotrophs with a corresponding decrease in the other. The proposed reason for this concerns a new selective pressure incurred in all three MRD's. This is the affect of sampling the model. In MRD's 1 and 2 there are few selective pressures and so when fresh clean surfaces are introduced the organisms recolonise at a similar rate as they initially did. Thus organism levels will appear to be constant over time. However in MRD

Figure 3.15 - The Proportions of Distinguishable Presumptive Coliforms Present in the Sessile Phase of 3 distinct MRD's at Time 0

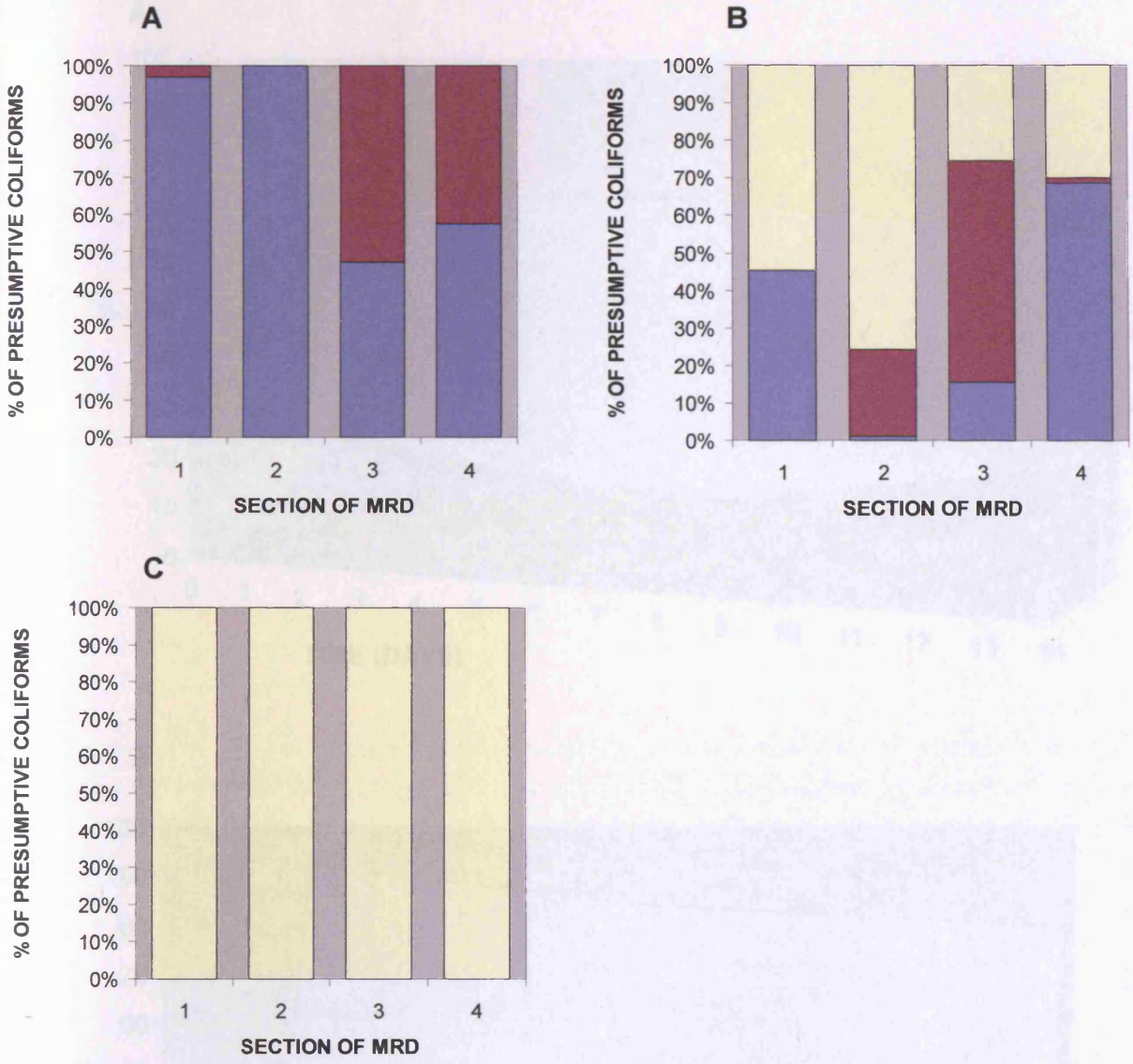
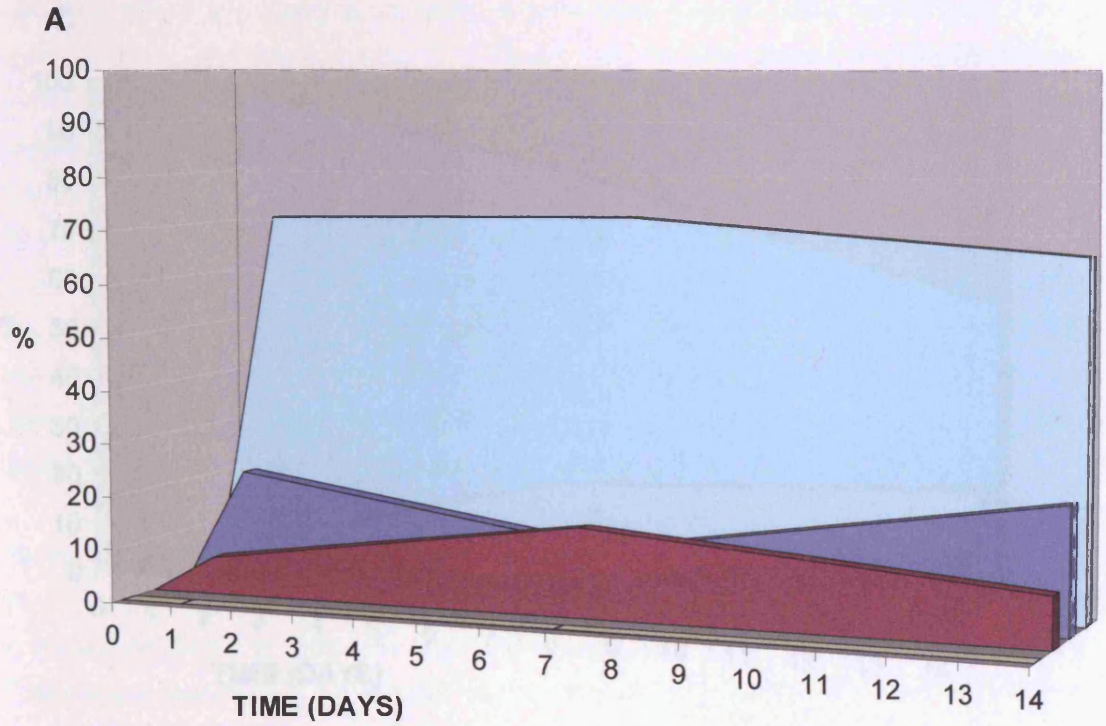


Figure 3.15 - The percentages of ORG 001 ( ), ORG 002 ( ) and blue spreading ( ) presumptive coliforms present in the sessile phase of **A** MRD 1, **B** MRD 2 and **C** MRD 3. Presumptive coliforms were isolated on MacConkey agar and the percentages were relative to the total viable coliform counts also enumerated on MacConkey agar.The model systems were left to stabilise for 14 days prior to sampling at T= 0.

Figure 3.16 - The Proportions of Distinguishable Heterotrophs in the Sessile Phase of 3 distinct MRD's with time



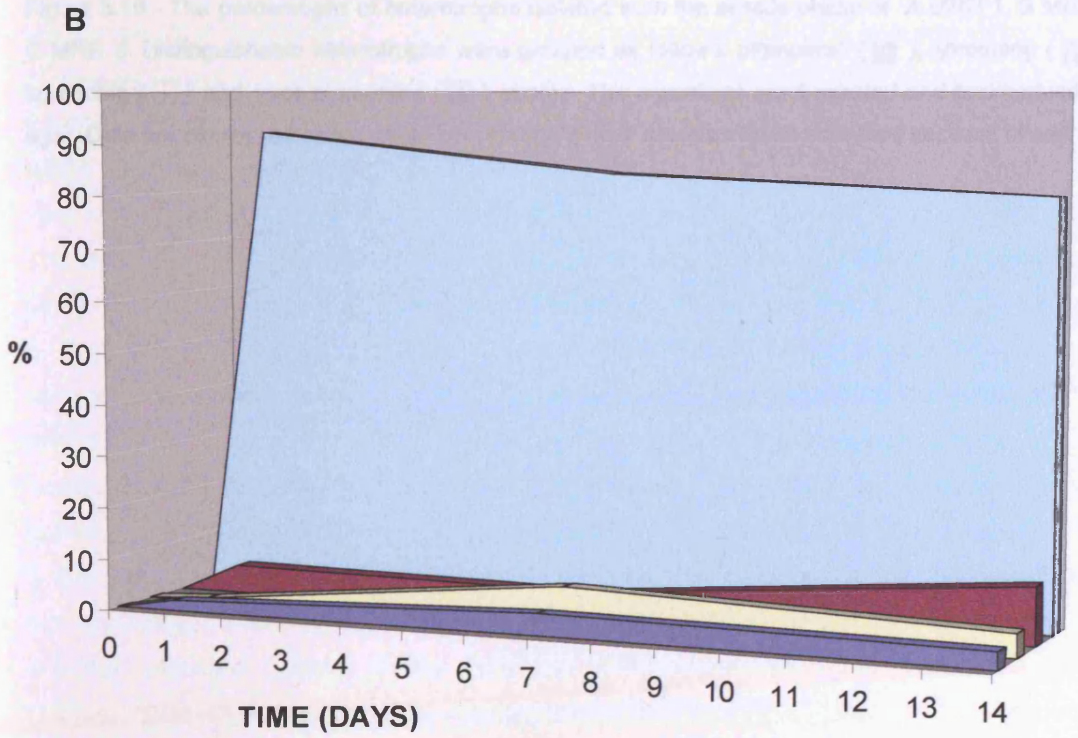


Figure 3.16 Continued - The Proportions of Distinguishable Heterotrophs Isolated From the Sessile Phase of 3 MRD's

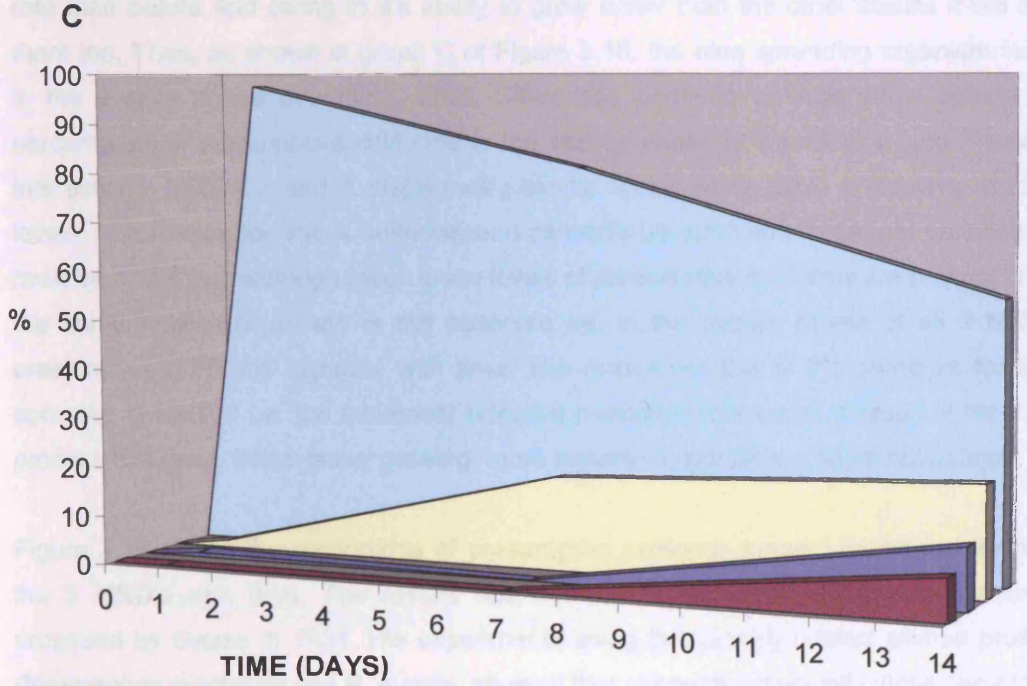


Figure 3.16 - The percentages of heterotrophs isolated from the sessile phase of **A** MRD 1, **B** MRD 2 and **C** MRD 3. Distinguishable heterotrophs were grouped as follows: pigmented (■), spreading (■), blue spreading (□) and none pigmented (□) strains. The organisms were isolated and enumerated on R_2A agar. Data are expressed as the mean percentage of four samples taken from the4 sections of each MRD.

3 the presence of increased metal ions has selected for a fast growing, relatively resistant organism and allowed it to flourish. In this case when fresh clean studs are made available this organism (the blue spreader), now at much higher levels will recolonise at a much faster rate than before and owing to it's ability to grow faster than the other strains it will outgrow them too. Thus, as shown in graph C of Figure 3.16, the blue spreading organism increases in the sessile phase over time. Stark differences begin to emerge when comparing the percentages of presumptive coliforms in the sessile phase of the MRD's (see Figure 3.17). Interestingly MRD's 2 and 3 display very similar levels while MRD 1 displays much lower levels. The reason for this is unknown and can only be attributed to natural variation. It also must be noted that although much lower levels of presumptive coliforms are present in MRD 1 the same pattern of growth is still observed i.e. in the sessile phase of all 3 MRD's the presumptive coliforms increase with time. The reason for this is the same as for the blue spreader in MRD 3 i.e. the additional selective pressure incurred as a result of the sampling process has given these faster growing, more resistant organisms a slight advantage.

Figure 3.18 shows the proportions of presumptive coliforms present in the sessile phase of the 3 MRD's with time. The results obtained mimic the theory of competitive exclusion proposed by Gause in 1934. His experiments using two closely related ciliated protozoans, *Paramecium caudatum* and *P. aurelia*, showed that although individually these two organisms grew well over a 16 day period, in mixed culture only the *P. aurelia* survived for this length of time. Since neither attacked the other or secreted toxic substances he concluded that *P. aurelia* simply outcompeted *P. caudatum* for available nutrients. In a contrasting experiment *P. caudatum* and *P. bursaria* were able to survive and reach a stable equilibrium when grown together. Although both still competed for the same food they occupied different locations within the flask thus minimising the competition and preventing either of their extinction. This may also be true for ORG's 001 and 002 in the sessile phase of MRD 1 (see Figure 3.18A). The two organisms compete, with ORG 001 increasing to a stable state to the detriment of ORG 002. However ORG 002 is not completely diminished and this is probably due to its location in the MRD. Figure 3.15A shows that ORG 001 completely dominates the first two sections of the MRD while ORG 002 occupies most of the latter two. It may be that this preference in location has allowed ORG 002 to remain in the population at relatively low levels. Similarly in MRD 2 ORG 002 is outcompeted by the other two organisms but still remains present at very low levels. Once again this is reinforced by the results in Figure 3.15B showing that the other two organisms preferentially colonise the former two sections of the MRD while ORG 002 is located mainly in section 3. In MRD 3, however, the additional selective pressure incurred by the presence of the metal pipe allows the blue spreading organism a greater competitive advantage thus totally excluding ORG 002 from the sessile phase. These results are similar to the initial experiment performed by Gause (1934).

Figure 3.17 - The Percentage of Viable Presumptive Coliforms Present in the Sessile Phase of 3 Distinct MRD's With Time

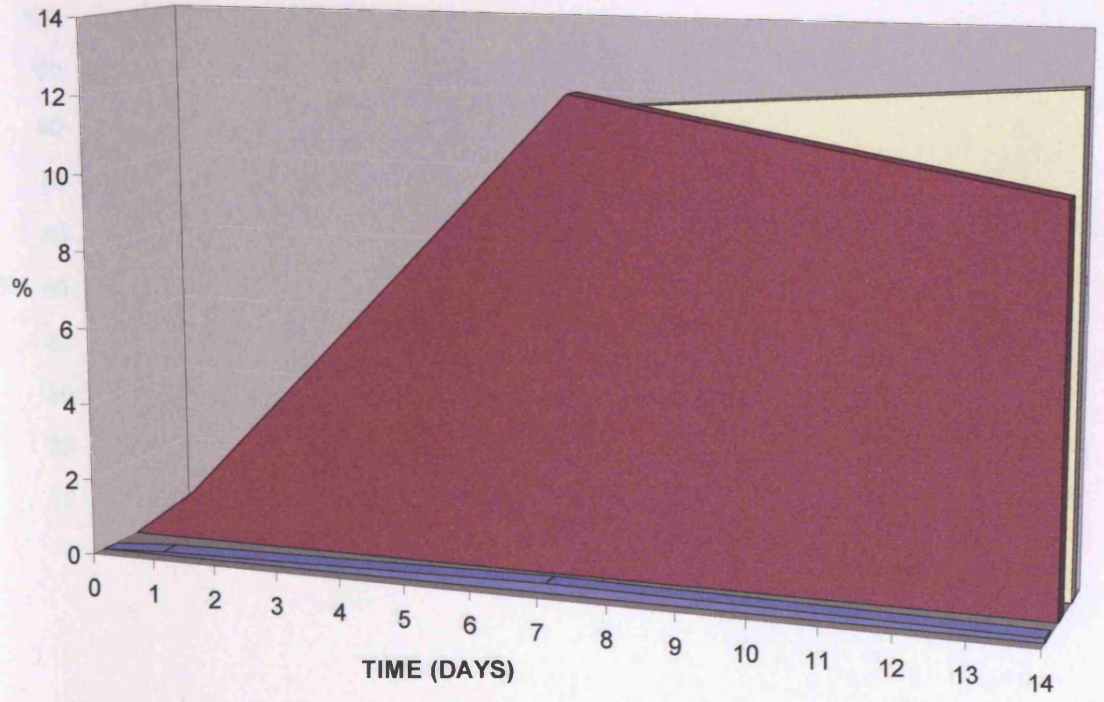


Figure 3.17 - The levels of presumptive coliforms present in the sessile phases of MRD's 1 ( ), 2 ( ) and 3 ( ). The data are expressed as the mean (of duplicate samples) percentages relative to the total heterotrophs isolated at different time points.

Figure 3.18 - The Proportions of Distinguishable Presumptive Coliforms Present in the Sessile Phase of 3 Distinct MRD's With Time

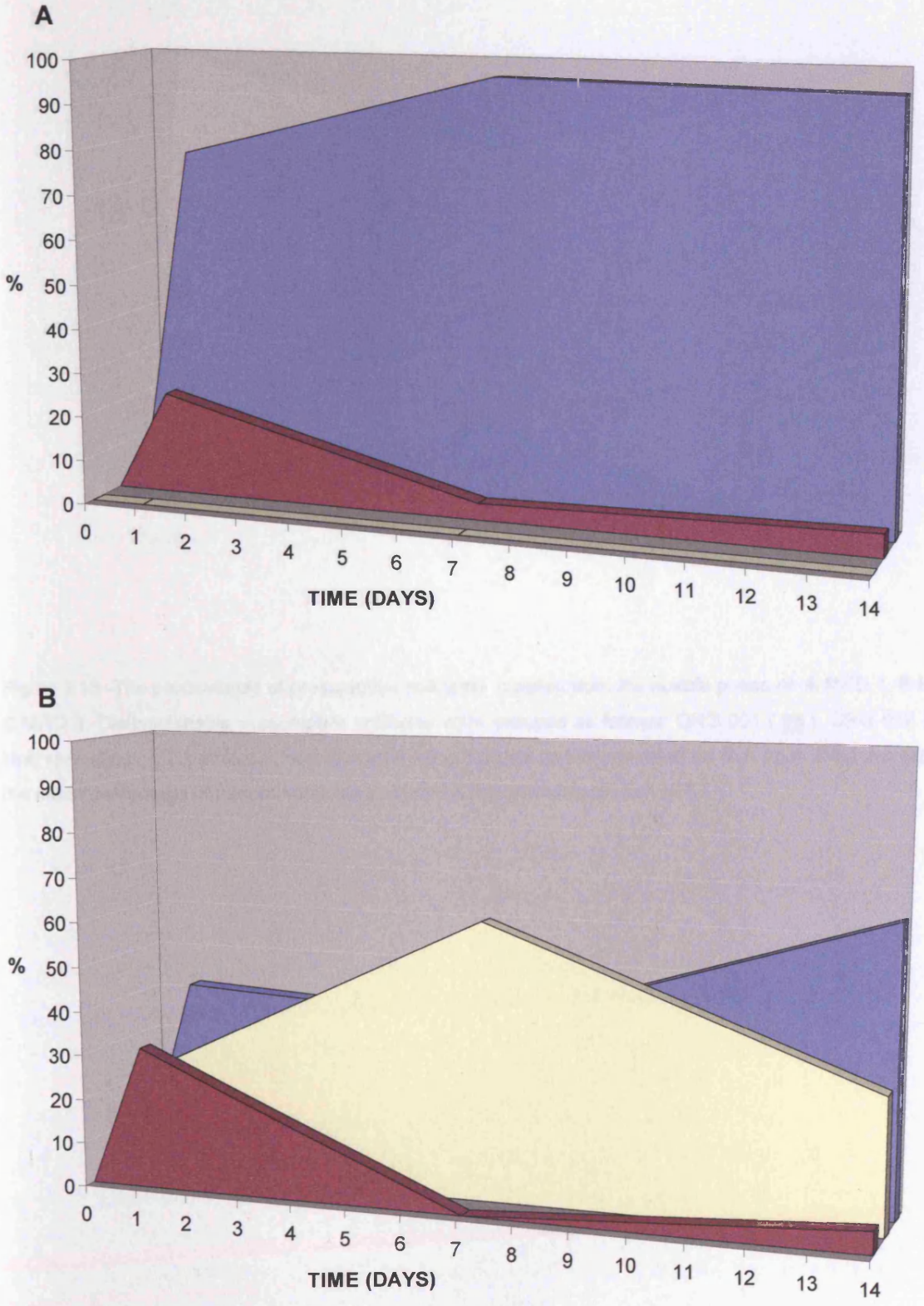


Figure 3.18 Continued - The Proportions of Distinguishable Presumptive Coliforms Present in the Sessile Phase of 3 Distinct MRD's With Time

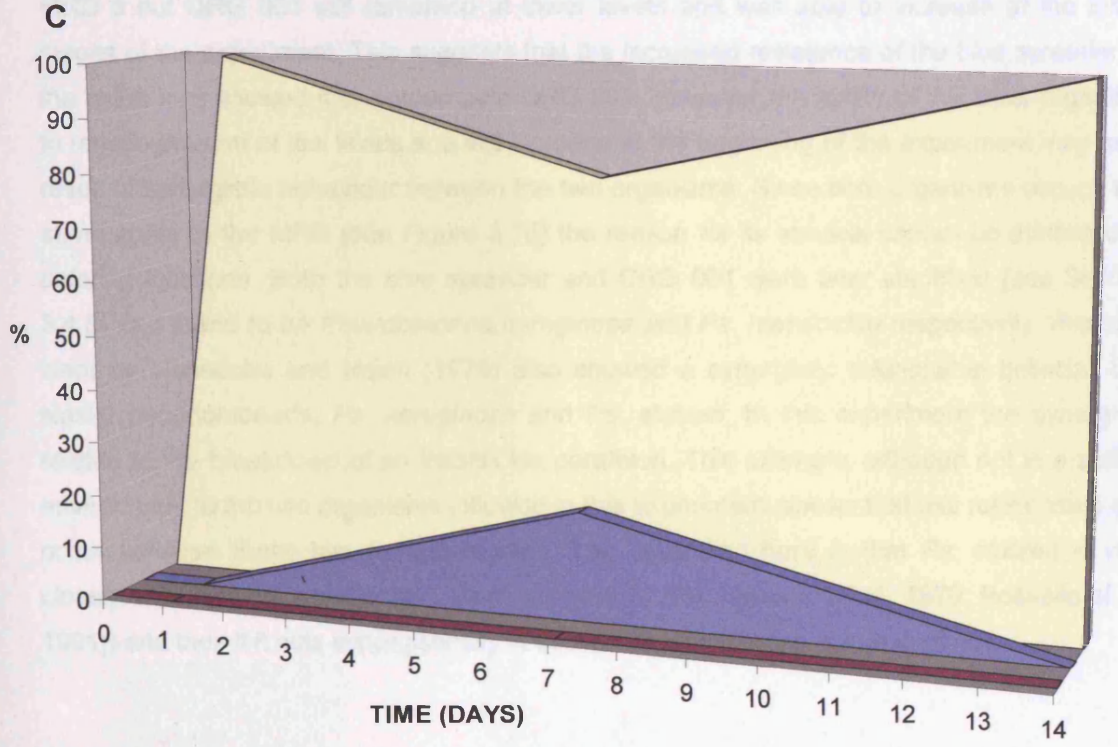


Figure 3.18 -The percentages of presumptive coliforms isolated from the sessile phase of **A** MRD 1, **B** MRD 2 and **C** MRD 3. Distinguishable presumptive coliforms were grouped as follows: ORG 001 (■), ORG 002 (■) and blue spreading (□) strains. The organisms were isolated and enumerated on R_2A agar. Data are expressed as the mean percentage of four samples taken from the four sections of each MRD.

ORG 001 and the blue spreader, however, appear to exhibit a more synergistic relationship (Atlas and Bartha, 1998). In MRD 2 the two organisms were able to grow and reach steady state neither one being harmed by the other. In contrast the spreading organism flourished in MRD 3 but ORG 001 still remained at lower levels and was able to increase at the initial stages of the experiment. This suggests that the increased resistance of the blue spreader to the metal ions allowed it to outcompete ORG 001. However the ability of the latter organism to remain present at low levels and still increase at the beginning of the experiment may be a result of synergistic behaviour between the two organisms. Since both organisms occupy the same areas in the MRD (see Figure 3.15) the reason for its survival cannot be attributed to differing locations. Both the blue spreader and ORG 001 were later identified (see Section 3.4.3) and found to be *Pseudomonas aeruginosa and Ps. mendocina* respectively. Previous work by Munnecke and Hsieh (1976) also showed a synergistic relationship between two similar pseudomonads, *Ps. aeruginosa* and *Ps. stutzeri*. In this experiment the synergism related to the breakdown of an insecticide parathion. This example, although not in a similar environment to the two organisms followed in this experiment, shows that this relationship can occur between these two pseudomonads. The reasoning here is that *Ps. stutzeri* is very closely related to *Ps. mendocina* (Skerman *et al.*, 1980; Palleroni *et al.*, 1970; Rossello *et al.*, 1991)) and thus if it acts synergistically in this environment it may do so in others.

3.3.3.2 The Planktonic Phase of the MRD

Unfortunately the planktonic phase of MRD 1 was not monitored and so cannot be compared but the results for the planktonic organisms isolated from MRD's 2 and 3 were and are displayed in Figure 3.19.

Figure 3.19A shows the proportions of the 4 groups of heterotrophs isolated from the 2 MRD's after an initial circulation of 14 days. Similar proportions of both pigmented and non-pigmented strains were observed in each MRD. However, although in low numbers, slightly higher proportions of the blue spreading organism were noted in MRD 3. The percentages of these organisms were found to be 0.43% and 1.64% for MRD's 2 and 3 respectively. This difference was, once again, attributed to the presence of the metal pipe in MRD 3.

Figure 3.19C shows the proportions of presumptive coliforms present in the planktonic phase of the 2 MRD's. These results display a diversity of organisms in MRD 2 and a predominance of one organism in MRD 3. Once again the blue spreading pseudomonad predominated in MRD 3. Interestingly in MRD 2 the proportions of the blue spreading pseudomonad are similar to those observed for ORG 001 and ORG 002. It must be noted that the percentages

Figure 3.19 - The Proportions of Distinguishable Bacteria in the Planktonic Phase of the MRD's at Time 0

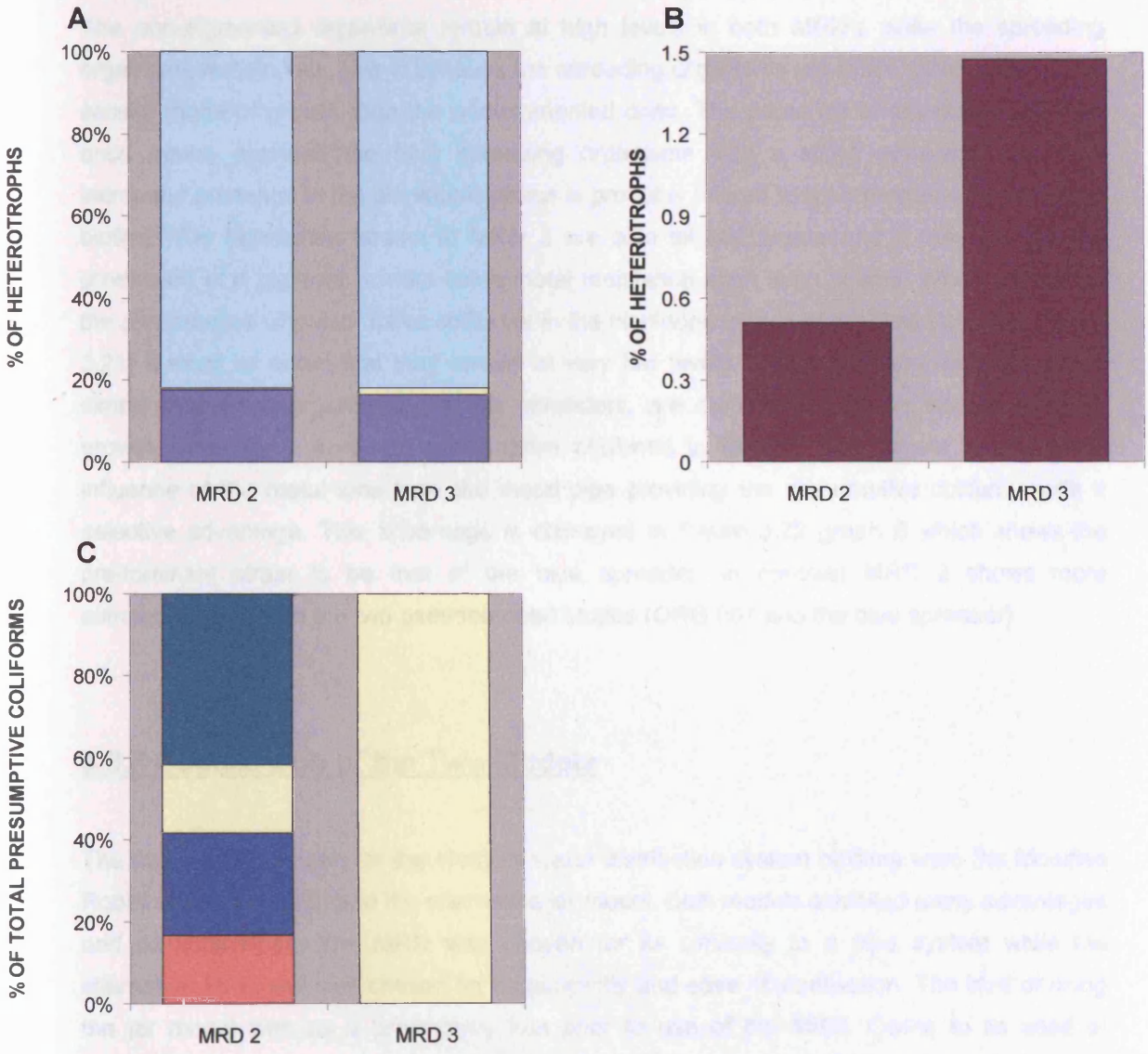


Figure 3.19 - The distribution of viable bacteria present in the sessile phase of MRD's 2 and 3 at time 0. Graph **A** shows the proportions of distinguishable heterotrophs: pigmented ( ), spreading ( ), blue spreading ( ) and non pigmented ( ). Graph **B** shows the percentage of presumptive coliforms present ( ) and Graph **C** shows the proportions of distinguishable presumptive coliforms: ORG 001 ( ), ORG 002 ( ), blue spreading ( ) and miscellaneous ( ). Note - the systems had been left to circulate for 14 days prior to sampling.

for the blue spreading pseudomonad were slightly lower when detected on the MacConkey agar. This is probably due to the selectivity of this medium causing additional selection. Figure 3.20 displays the heterotroph proportions in the planktonic phase of MRD's 2 and 3 over time. The patterns observed for the heterotroph groups monitored exhibit similar traits. The non-pigmented organisms remain at high levels in both MRD's while the spreading organisms remain low. This is because the spreading organisms are much more suited to the sessile mode of growth than the non-pigmented ones. The presence of the metal pipe has, once again, provided the blue spreading organisms with a slight advantage but their increased presence in the planktonic phase is probably related to their greater success in the biofilm. The pigmented strains in MRD 3 are also slightly higher and it may be that the production of a pigment confers some metal resistance upon such strains. When comparing the percentages of presumptive coliforms in the planktonic phase of the 2 models (see Figure 3.21) it must be noted that they remain at very low levels. The reason proposed for this is simply that these organisms, like the spreaders, are more suited to the sessile mode of growth. The higher levels of presumptive coliforms in MRD 3 once again reinforce the influence of the metal ions from the metal pipe providing the presumptive coliforms with a selective advantage. This advantage is displayed in Figure 3.22 graph B which shows the predominant strain to be that of the blue spreader. In contrast MRD 2 shows more competition between the two pseudomonad strains (ORG 001 and the blue spreader).

3.3.4 Comparison of the Two Models

The two models chosen for the study of water distribution system biofilms were the Modified Robbins device (MRD) and the alternative jar model. Both models exhibited many advantages and disadvantages. The MRD was chosen for its similarity to a pipe system while the alternative jar model was chosen for its simplicity and ease of sterilisation. The idea of using the jar model was as a preliminary test prior to use of the MRD. Owing to its ease of sterilisation and the abundance of jars in the laboratory many could be set up at one time containing different initial inocula and different conditions. Before proceeding the biofilm produced in both models had to be compared to determine its usefulness.

In the sessile phase the total counts observed after 1 day are similar for both models being on average $3.75 \times 10^7 cfuml^{-1}$ and $3.2 \times 10^7 cfuml^{-1}$ for the jar and the MRD respectively. In contrast the viable counts display lower levels in the jar model than in the MRD on average being $6.4 \times 10^5 cfuml^{-1}$ and $2.45 \times 10^6 cfuml^{-1}$ respectively. This may be due to design of the model systems. The MRD is a flowing system so the biofilm produces waste products into the planktonic phase which are then immediately carried away by the flow and diluted in the

Figure 3.20 - The Proportions of Distinguishable Viable Heterotrophs in the Planktonic Phase of the MRD's With Time

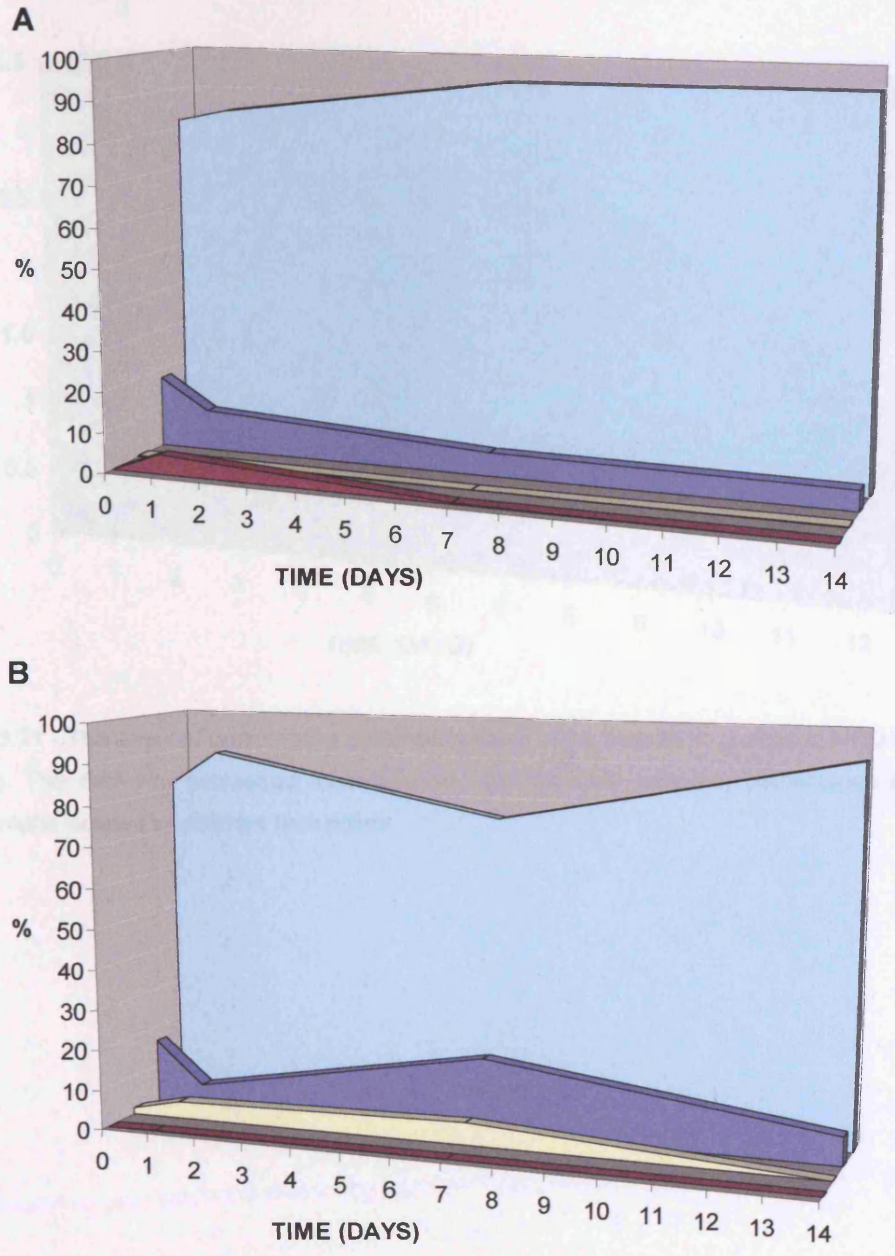


Figure 3.20 - The levels of four distinguishable heterotrophs isolated from the planktonic phase of **A** MRD 2 and **B** MRD 3. Heterotroph groups are as follows: spreading ( ), blue spreading ( ), pigmented ( ) and non pigmented ( ) strains. Data are expressed as the mean percentage of duplicate samples.

Figure 3.21 - The Percentage of Viable Presumptive Coliforms Present in the Planktonic Phase of Two MRD's

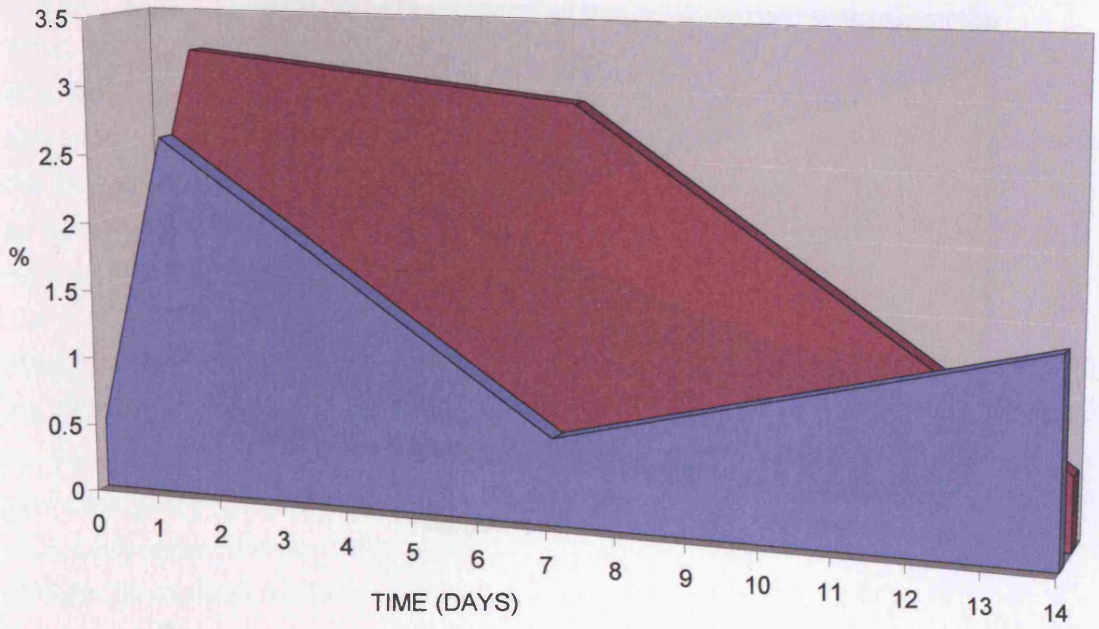


Figure 3.21 - The levels of presumptive coliforms present in the planktonic phases of MRD 2 (■) and MRD 3 (■). The data are expressed as the mean (of duplicate samples) percentages relative to the total heterotrophs isolated at different time points.

Figure 3.22 - The Proportions of Distinguishable Presumptive Coliforms Present in the Planktonic Phase of Two MRD's

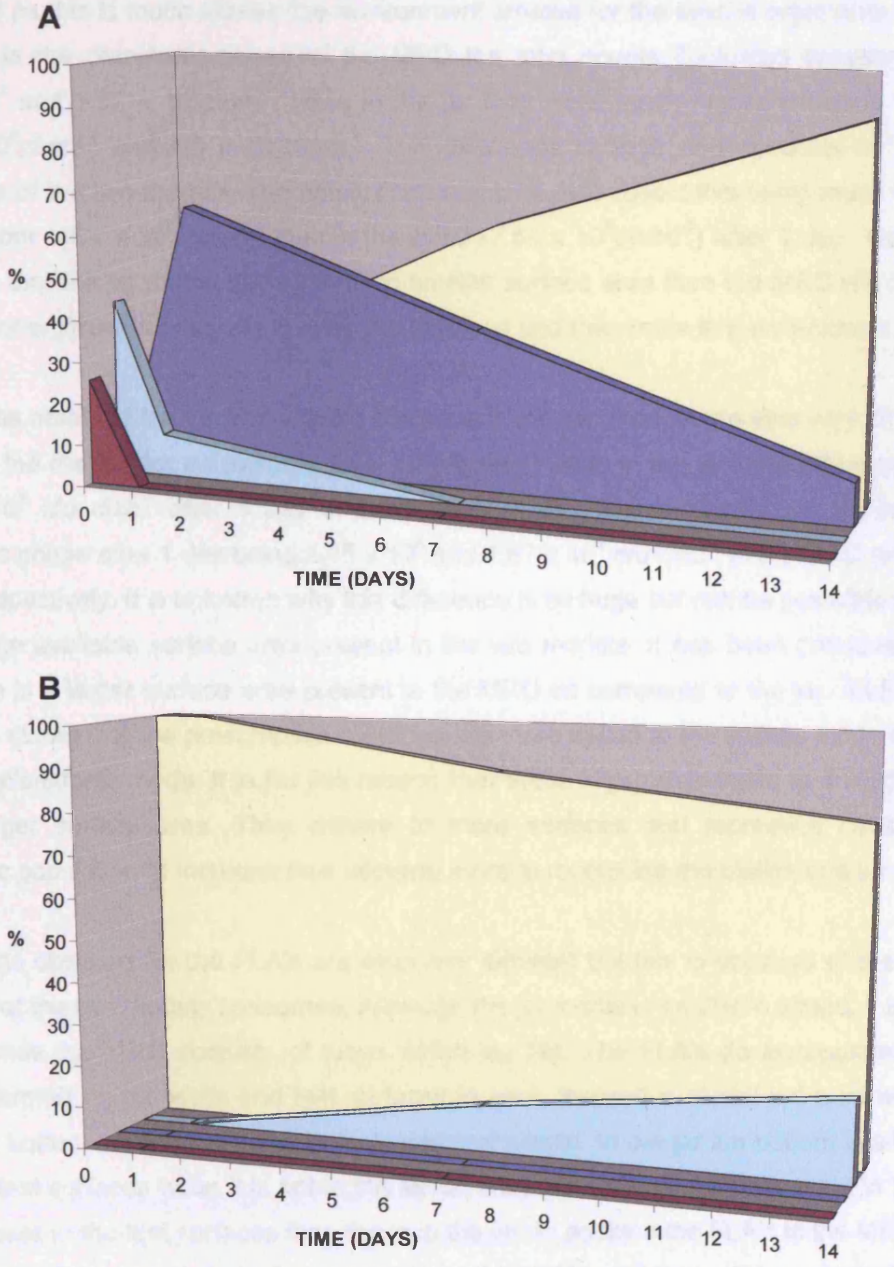


Figure 3.22 - The levels of four distinguishable presumptive coliforms isolated from the planktonic phase of **A** MRD 2 and **B** MRD 3. Presumptive coliform groups are as follows: ORG 001 (■), ORG 002 (■), blue spreading (□) and miscellaneous (□) strains. Data are expressed as the mean percentage of duplicate samples.

reservoir. The biofilm in the jar model, however, is effectively growing in the reservoir area. This means that although both models contain the same amount of fluid, the jar model does not have an efficient way of immediately removing waste products. Instead they just diffuse away and as this is much slower, the environment created for the sessile organisms is a little harsher. In the planktonic phase of the MRD the total counts fluctuated between 9.85 x 10^6cfuml^{-1} and 3.92 x 10^6cfuml^{-1} while in the jar they were much higher wavering between 4.75 x 10^8cfuml^{-1} and 4.5 x 10^7cfuml^{-1}. This difference is large and accounts for the size difference of the two models. The heterotroph numbers also reflect this being much higher in the jar model (4.4 x 10^6cfuml^{-1}) than in the MRD (7.54 x 10^5cfuml^{-1}) after 1 day. The reason for this is that the jar model having a much smaller surface area than the MRD will obviously have fewer organisms adhering to available surfaces and thus more in the planktonic phase.

The results obtained for the presumptive coliforms in the two models are also very different. In the MRD the results are on average 1.9 x 10^4cfu disc^{-1} while in the jar model they are as low as 1 x 10^2 cfu disc^{-1} after 1 day in the sessile phase. These results are similar in the planktonic phase after 1 day being 2.15 x 10^4 and 1.67 x 10^2 cfu disc^{-1} in the MRD and the jar model respectively. It is unknown why this difference is so huge but can be postulated to be to do with the available surface area present in the two models. It has been previously stated that there is a larger surface area present in the MRD as compared to the jar. Additionally it has been stated that the presumptive coliforms are more suited to the sessile mode of growth than the planktonic mode. It is for this reason that these organisms thrive in a model with a much larger surface area. They adhere to more surfaces and reproduce causing their planktonic population to increase thus allowing more to recolonise the biofilm and so on.

The results obtained for the FLA's are also very different but this is because of the differing structure of the two models concerned. Although the jar model is tubular in shape, it is tall and narrow while the MRD consists of tubes which lay flat. The FLA's do incorporate into the biofilms formed on the walls and test surfaces in each respective model but a lot will simply fall to the bottom of the model due to their size and weight. In the jar the bottom is a long way from the test surfaces while it is not in the MRD. Thus the residual FLA's present in the MRD will be closer to the test surfaces than those in the jar. In addition the FLA's in the MRD will be more successful owing to their close proximity to the biofilm and thus will be detected in much higher levels in the MRD. The results obtained for the presence of FLA's in the two models support this theory. In the sessile phase after 1 day the FLA's were not isolated from the jar model while they were from the MRD at an average of 265.8 disc^{-1}.

When monitoring the biofilm formation in the jar model the experiments performed were on copper and plastic test surfaces together. Since copper surfaces were not present in the MRD the two models could not be properly compared as copper ions from the test discs will affect

the biofilm formed on the plastic. This assumption was proved when comparing two mono-species biofilms forming in the jar model. The organism used for the mono-species biofilm was ORG 001 which had been previously isolated from a complex biofilm forming on plastic in the MRD. For comparative purposes and owing to the dynamics of the biofilm model the two phases were taken into account the sessile phase being expressed as a percentage of the planktonic phase at each time point. Figure 3.23 shows the results of this investigation, conclusively proving that the biofilm formed in the presence of copper was less than that observed in the presence of plastic only. This reinforces previous results and is expected as copper is renowned for having inhibitory properties.

In conclusion the alternative jar model has it's limitations but is an ideal model for the preliminary testing of a variety of biofilms formed under different conditions. Due to limitations of time this model was not fully utilised. Since the copper test surfaces affected the biofilm this model could not be used to compare with the MRD. Instead it was adapted to just contain plastic test surfaces but another problem was encountered when testing the effect of poliovirus on the mono-species biofilm formed. This problem concerned the virus itself adhering to the glass walls of the jar and the results obtained were backed up by the literature (see Section 5).

3.3.5 The Morphology of the Biofilm Model in the MRD

To evaluate the biofilm morphology in the 4 sections of the MRD Scanning Electron Microscopy (SEM) was used. This technique has the limitation that, during sample preparation, a dehydration process must be incorporated which causes the collapse of the formerly hydrated biofilm with the result that the original open structure cannot be observed. However, this taken into account, SEM does provide valuable information as to how the biofilm formed at the different sections of the MRD.

The results displayed in Figures 3.24 and 3.25 show the biofilm formation on studs 1, 8, 16 and 25 of the MRD following biofilm formation after 1 and 14 days respectively. The reason for evaluating the biofilm morphology at these 2 different time points was to determine whether similarities in biofilm formation occurred in these 4 sections of the MRD. The 2 time points were chosen because previous results had shown (see Figure 3.1) that although biofilm formation occurred after 1 day, similar levels of bacteria were still present at day 14. In addition, the 14 day old biofilm was found to be more reproducible between MRD's than the 1 day old biofilm.

Figure 3.23 - A Comparison of the Mono-Species Biofilm Formation in the Alternative Jar Model in the Presence and Absence of Copper

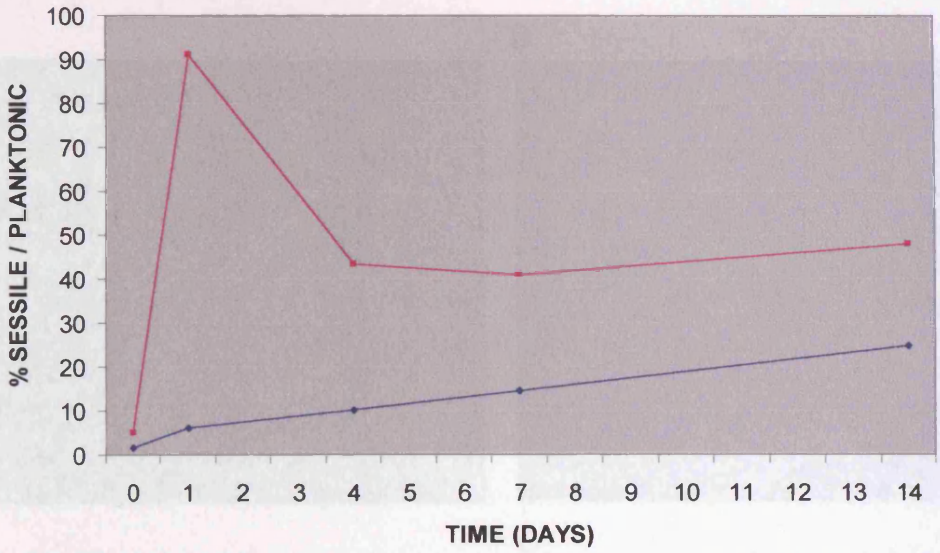


Figure 3.23 - The total viable heterotrophs isolated from a mono-species biofilm forming on plastic in the presence of copper (—◆—) and in the absence of copper (—■—) in two jar models. For comparative purposes data are expressed as the mean percentage of the planktonic phase in each of the respective jars.

Figure 3.24 - The Biofilm Morphology in the four Sections of the MRD After 1 day of Biofilm Formation

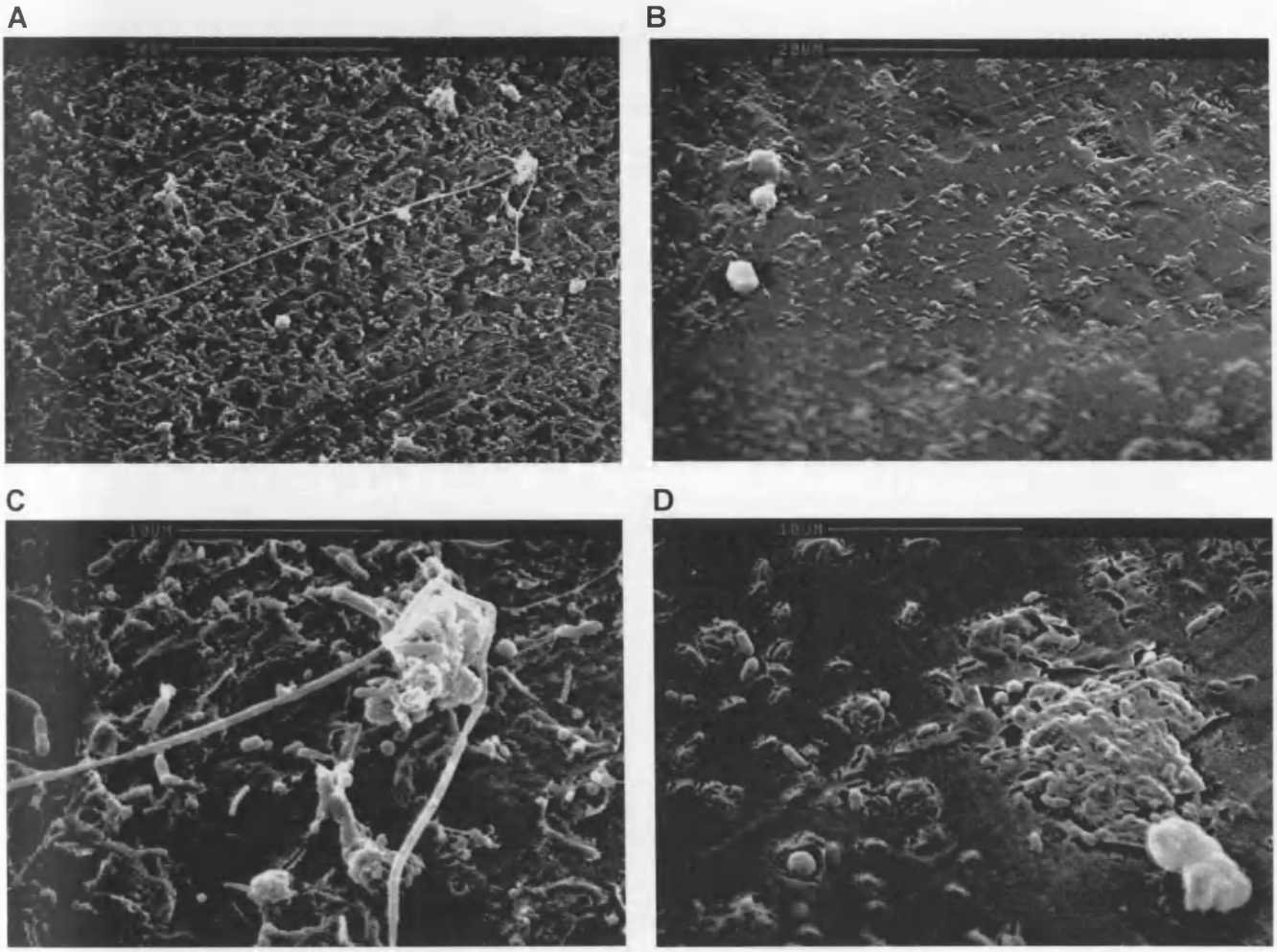


Figure 3.24 - The Scanning Electron Micrographs of the two beginning sections of the MRD after a 1 day circulation period. **A** and **C** show the micrographs of the biofilm on stud 1 at magnifications of 775X and 3.71KX respectively. **B** and **D** show the micrographs of the biofilm on stud 8 at magnifications of 1.36KX and 3.46KX respectively.

Figure 3.24 Continued - The Biofilm Morphology in the four Sections of the MRD After 1 day of Biofilm Formation

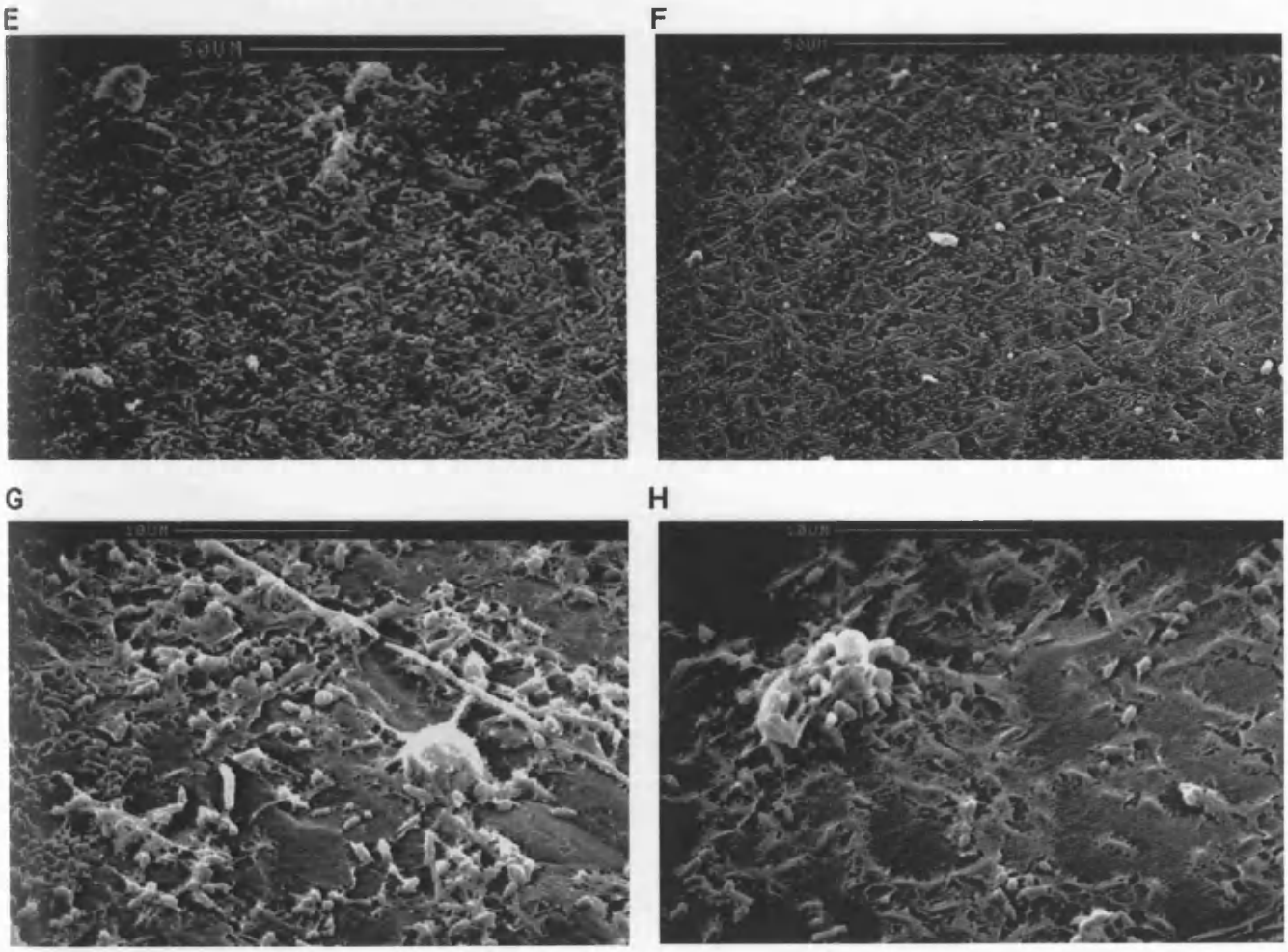


Figure 3.24 Continued - The Scanning Electron Micrographs of the two end sections of the MRD after a 1 day circulation period. **E** and **G** show the micrographs of the biofilm on stud 16 at magnifications of 643X and 3.14KX respectively. **F** and **H** show the micrographs of the biofilm on stud 24 at magnifications of 621X and 3.53KX respectively.

Figure 3.25 - The Biofilm Morphology in the four Sections of the MRD After 14 days of Biofilm Formation

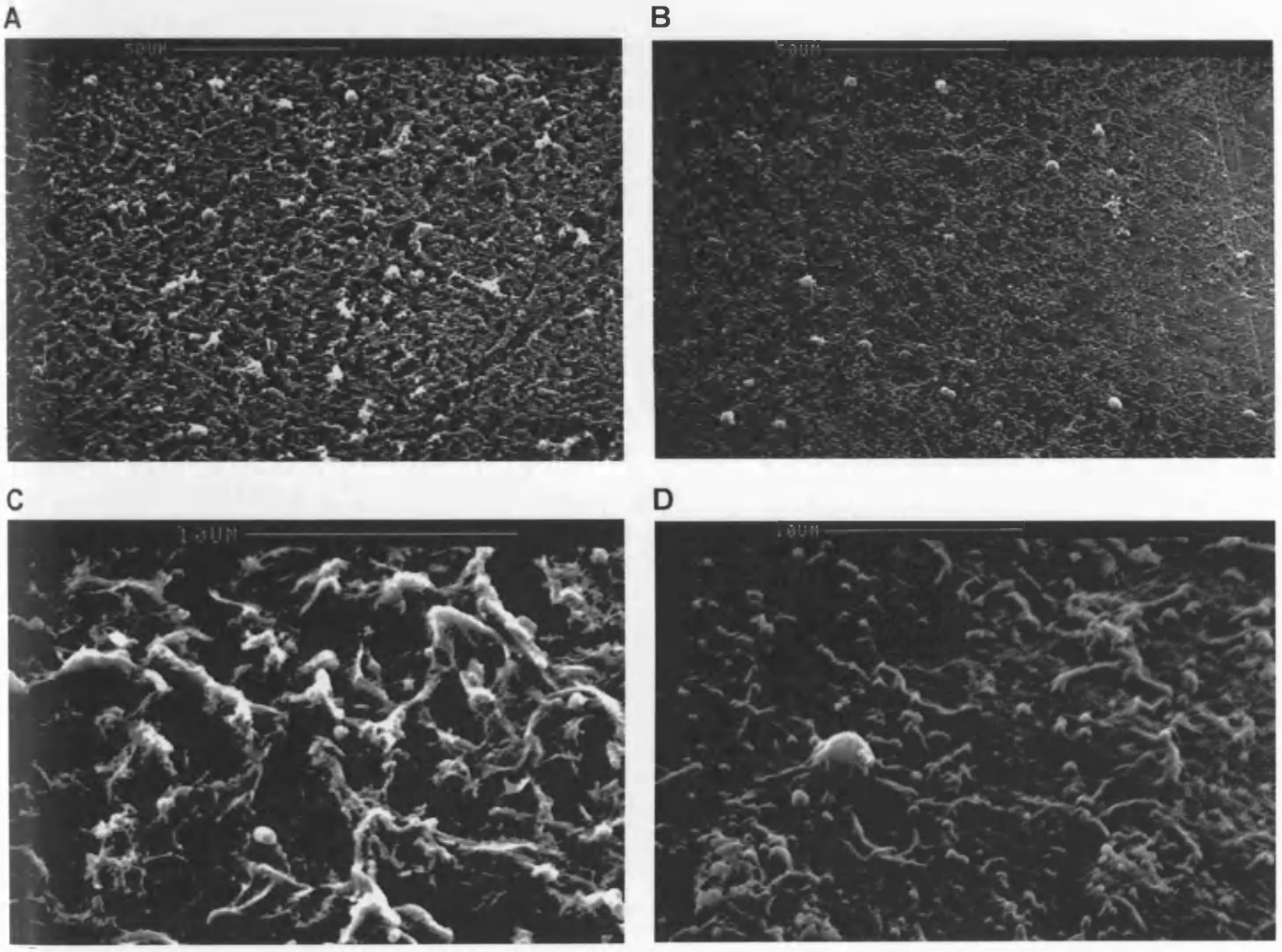


Figure 3.25 - The Scanning Electron Micrographs of the two beginning sections of the MRD after the initial 14 day circulation period. **A** and **C** show the micrographs of the biofilm on stud 1 at magnifications of 603X and 3.45KX respectively. **B** and **D** show the micrographs of the biofilm on stud 8 at magnifications of 652X and 3.53KX respectively.

Figure 3.25 Continued - The Biofilm Morphology in the four Sections of the MRD After 14 days of Biofilm Formation

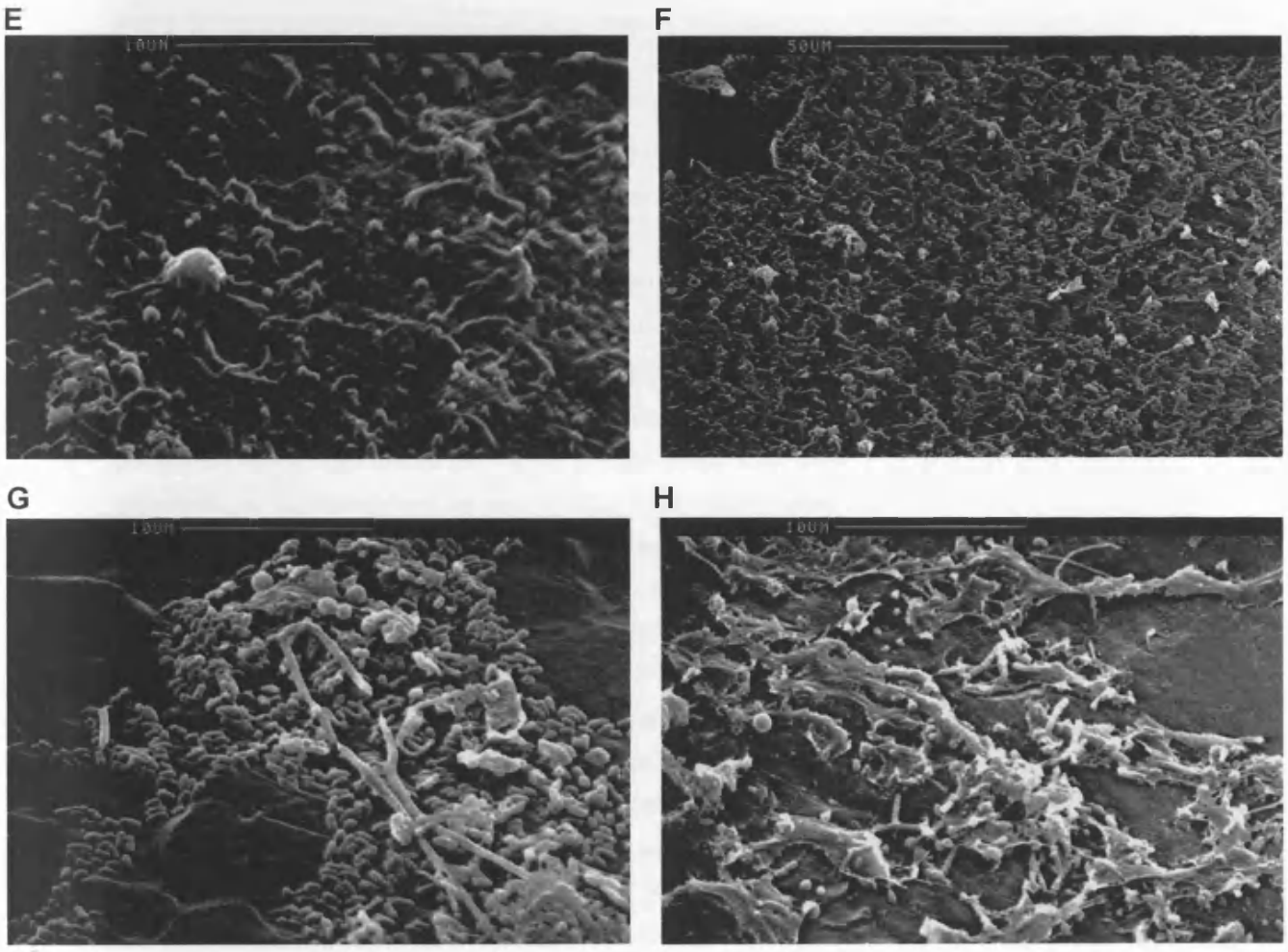


Figure 3.25 Continued - The Scanning Electron Micrographs of the two end sections of the MRD after the initial 14 day circulation period. **E** and **G** show the micrographs of the biofilm on stud 16 at magnifications of 609X and 3.48KX respectively. **F** and **H** show the micrographs of the biofilm on stud 24 at magnifications of 616X and 3.45KX respectively.

Although previous results (see Figures 3.5 and 3.6) indicated similar microbial levels at all four sections, these results show that the biofilm formed was not of uniform thickness displaying morphological differences along the MRD length. These differences, however, were similar at the two time points tested showing that this effect was reproducible. In addition, this finding has been previously reported by other workers who noted that biofilms in water distribution systems consist of both densely and sparsely populated regions (Surman *et al.*, 1996).

At both times, the biofilm formed on stud 1 exhibited a patchy appearance with parts of the test surface still visible. The micro-organisms appeared to be randomly stacked on top of each other and were covered by thick layers of EPS which was also patchy. At day 1 (see Figures 3.5A and C) a large microcolony was pictured with a long thin structure partially surrounding it and extending across the surface at a length of approximately 124 μm. It is unclear what this structure is but it may be either a water channel, the result of high turbulence in this region of the MRD or a pleomorphic bacterium. The latter idea has been previously noted by Wainwright *et al.* (1999) when they grew *E. coli* under conditions of starvation on silicon wafers. At stud 8 after 1 day (see Figure 3.5B) the biofilm morphology displayed marked differences to those observed on stud 1. Here the biofilm did not appear to be as thick showing individual organisms lying on the test surface. However, closer examination (see Figure 3.5D) revealed that the entire surface was covered by a thick layer of EPS and the micro-organisms were embedded within it. A large clump of cells proposed to be a microcolony was also observed. This microcolony differed to the one noted on stud 1 being much wider and flatter. These results suggest slight differences in the biofilm population at these two sections. Jass *et al.* (1995) evaluated the biofilm formation of two different strains of pseudomonads and found that, although they were similar, they formed very different biofilms. One strain produced adherent microcolonies while the other formed a more confluent biofilm. These findings may explain the differences observed here. The high amount of EPS produced at these two beginning sections of the MRD may be the result of slightly higher flow rates. Brading *et al.* (1995) deduced that at higher flow rates there was a greater production of EPS by sessile organisms. This has also been previously implied by the viable heterotroph counts (see Figure 3.5). At stud 1 the lower viable heterotroph numbers were attributed to the slightly harsher conditions produced in the front section of the MRD.

In the third section of the MRD (stud 16) at day 1 (see Figure 3.24E and G) the biofilm once again took on a patchy appearance similar to that observed on stud 1. However, in this case, the biofilm covered a larger area having very small gaps between organisms. Once again, the EPS was quite thick but had a slightly more fibrous appearance. More microcolonies were apparent and on closer examination (see Figure 3.24G) they appeared to be dome shaped. At section 4 (stud 24) of the MRD the biofilm (see Figure 3.24F and H) appeared similar to

that observed at stud 8 (see Figure 3.24B and D). However this stud had a greater area of covering with only a few patches where the test surface was visible. Once again, the EPS was thick and the biofilm took on a flatter more confluent appearance than that observed at studs 1 and 16. In addition many individual micro-organisms could be distinguished and a few microcolonies were observed.

These results suggest that in the latter two sections of the MRD the biofilm formed was very similar to that observed for the former two sections but this time the covering was slightly greater. The presence of thick EPS in the latter two sections rules out the above theory that it is caused by a higher flow rate in the beginning section. This is because thick EPS was observed throughout the MRD. The fact that the front section of the MRD has slightly harsher conditions may still be true, as more microbes appeared to favour the latter two sections when forming a biofilm. Since previous results (see Figure 3.5 and 3.6) suggest the presence of similar levels of micro-organisms at all four sections of the MRD these morphological differences are probably due to differing species at the different locations and not to large differences in organism numbers.

The biofilm formation in the same MRD after the 14 day period (see Figures 3.25A to H) was essentially similar to that observed after 1 day. This confirmed that the observed differences were due to location in the MRD and not to other variables present. This further demonstrates the reproducibility of the model used.

In the first 2 sections of the MRD (studs 1 and 8; see Figure 3.25A to D) the biofilm appeared to be more densely populated than previously noted. In contrast the latter two sections (studs 16 and 24; see Figures 3.25E to H) exhibited a more sparse biofilm than the front section or previously at day 1. Since the morphology of the biofilm was still similar and other results still exhibited similar micro-organism levels at all biofilm sections at this time point these results suggest some differences exist between young and mature biofilms. The micro-organisms have adapted to their surroundings and produced more extreme forms of the biofilm at their differing locations.

At stud 16 (see Figure 3.25E and G) the biofilm exhibited the greatest difference taking on a much rougher appearance than previously observed at day 1. In this case, it did not appear to have produced any EPS, the cells randomly sitting next to, or on top of, each other. Very large patches were also observed in which the test surface was visible. Upon closer examination (see Figure 3.26) it was noted that the cells were attached to each other by EPS exhibiting a much more fibrous appearance. This fibrous EPS was previously observed at the same location of the MRD after day 1 but was not as pronounced.

The reasons for the observed differences in biofilm morphology at the four different sections of the MRD are due mainly to location. Such differing locations probably promote the colonisation of different microbial species, which in turn, utilise different colonisation strategies producing different type of EPS leading to the heterogenities observed. The fact that these heterogenities were similar at both time points suggests reproducibility in the system.

Figure 3.26 - A Closer Look at the Biofilm on Stud 16 After 14 Days of Biofilm Formation

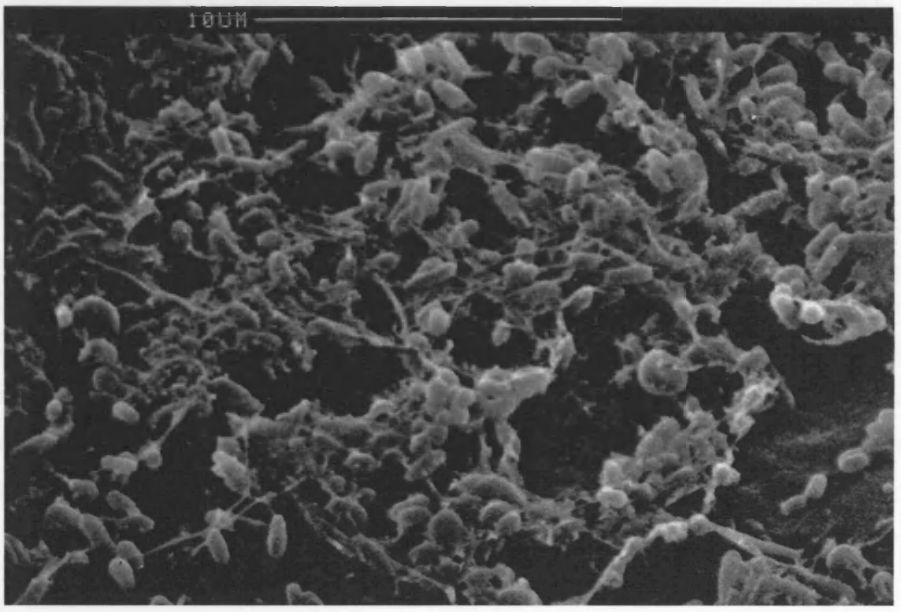


Figure 3.27 - The Scanning Electron Micrograph of the biofilm formed on stud 16 using a higher magnification of 4.6KX. This shows the presence of low levels of fibrous exopolymeric substance (EPS) among the individual bacterial cells in the biofilm.

3.4 Partial Characterisation of the Biofilm

Due to the extreme diversity of the bacteria present in the biofilm it would have been a huge task to characterise it completely. For this reason the organisms that could be easily distinguished from each other were monitored. When looking at the viable heterotrophs it was easy to distinguish the pigmented organisms from those without pigment. Likewise those organisms which spread across the plate were easy to distinguish from the others. Problems arose when looking between plates and at different time points, it was easy to distinguish the different types of pigmented organisms at one time point but later in the experiment and on different batches of R_2A it was not so easy to tell. For this reason a much simpler view of the biofilm was adopted. Those organisms that could be grouped and distinguished on every occasion were followed. Thus all organisms producing a pigment were grouped and representative species identified. In this case pigmented organisms consisted of yellow, pink and orange strains. Many different colonial morphologies for each of these strains were observed.

3.4.1 Bacterial Morphology and Biochemical Tests

The isolated bacteria were placed into 2 initial groups, heterotrophs and presumptive coliforms, according to the agar on which they were isolated. These were then subdivided into pigmented, non-pigmented, spreaders and blue spreaders for the heterotrophs and ORG 001, 002 and blue spreaders for the presumptive coliforms. The pigmented group of organisms were the easiest to distinguish and were further classified into yellow, orange and pink strains. Since several different types of each of these strains became apparent at different time points only representative strains were characterised. Also since these strains were present only in relatively low numbers and sometimes not at all it was considered ideal to group them together. This way any general trends could be observed and maybe linked to the characteristic of pigment production. The organisms grouped into the category termed spreaders were also easily distinguished from the others at all time points tested. These organisms all had the ability to spread across the agar plate. In contrast to the pigmented organisms these were difficult to distinguish from each other and so were grouped together for this reason. The blue spreaders were easy to distinguish and very clear on both R_2A and MacConkey agars. A sub-group of the spreaders they produced a very characteristic blue-green pigmentation which penetrated the medium on which they were grown. They were grouped separately from the spreaders because of their abundance when present. The non-

Table 3.3 – The Groups of organisms present in the Biofilm Model

A. Heterotroph Groups

Heterotroph Group	Colony Colour (on R_2A)	Colonial Morphology [1]				Biochemical tests [2]		
		SV	TV	A	T	Gr.	Ox	Cat
PIGMENTED	YELLOW	4	2	S	M	–B	+,–	$+^S$
	ORANGE	2,4	2	S	M	–B	+	+
	PINK	3,4	1	S	D	–B,Y	+	+
SPREADER	GREY WHITE	1	2,3	S	D	–B	+	+
BLUE SPREADER	BLUE/GREEN	1	3	S	D	–B	+	+
NON PIGMENTED[3]	GREY CREAM WHITE	V	V	V	V	V	V	V

B. Presumptive Coliform Groups

Presumptive Coliform Group	Colony Colour (on MacConkey)	Colonial Morphology [1]				Biochemical tests [2]		
		SV	TV	A	T	Gr.	Ox	Cat
ORG 001	PINK/ORANGE	3	1	S	M	–B	+	+
ORG 002	ORANGE	5	3	S	D	–B	+	$+^S$
BLUE SPREADER	PURPLE	1	3	S	D	–B	+	+

Table 3.3 – The colonial morphology and simple biochemical test results for the most common organisms in each representative group monitored during the course of the biofilm model experiments.

[1] For the colonial morphology SV = side view (1 flat, 2 Raised, 3 Low convex, 4 Domed or 5 umbonate); TV = top view (1 entire, 2 undulate or 3 crenated); A = appearance (S smooth or R wrinkled) and T = texture (M mucoid or D dull).

[2] For the biochemical tests Gr. = gram reaction (B bacilli, C cocci or Y yeast-like); Ox = oxidase test (S strong or W weak reaction) and Cat = catalase test (as for oxidase).

[3] V = variable results obtained.

pigmented group was all the indistinguishable organisms of grey, white and cream colouration on the R_2A medium. They were so grouped because they were difficult to distinguish. Owing to the selectivity of the MacConkey agar only a few strains could be isolated on it. These were easily distinguished from each other and were classified as ORG 001 and 002. In a few experiments another organism which resembled the blue spreader on R_2A was also isolated on the MacConkey. This organism was also enumerated on the MacConkey agar because it was in abundance when present. Table 3.3 summarises these organism groups and displays the colonial morphology as well as some simple biochemical test results obtained for the most abundant representative strains.

3.4.2 Protein Profiles

In order to confirm that the organisms monitored were the same at each time point and in each sample a simple protein profiling system was used. For this organisms from each time point and sample were denatured and their profiles on an SDS-PAGE gel were compared. Figure 3.27 shows the profiles of the blue spreading organism isolated from both phases of each of two MRD's. This figure also shows the profiles of these organisms isolated on both R_2A and MacConkey agars. The results conclusively prove that in all but one case (Lane 20) the organisms grouped as the blue spreaders were all identical. Although the organism in lane 20 is slightly different it's profile is very similar to the others suggesting that it is very similar and probably very closely related to the other organisms.

Figure 3.28 shows the profiles of the other spreading organisms. Results suggest the presence of 4 distinct organisms within this group but as stated previously they were very difficult to distinguish by morphological traits alone and were thus grouped accordingly. The profiles are once again quite similar indicating that these strains are probably related.

Figure 3.29 shows the results for the protein profiles of the pigmented strains. It should be noted here that the yellow strain profiled was consistently the most abundant organism but others, which were not profiled, were also present. The gel (A) shows that this particular organism was the same throughout the experiment and it was this one that was used as the representative strain. Likewise for the pink strains there were several other types observed on the plate but those profiled were the most common. Unlike the yellow strains the results show some differences. Unfortunately they all proved very difficult to isolate and purify. In contrast only one type of orange strain was noted among the heterotrophs and the results indicate these were the same organism.

Figure 3.27 - The Protein Profiles of the Blue Spreading Heterotrophs Isolated from the MRD's

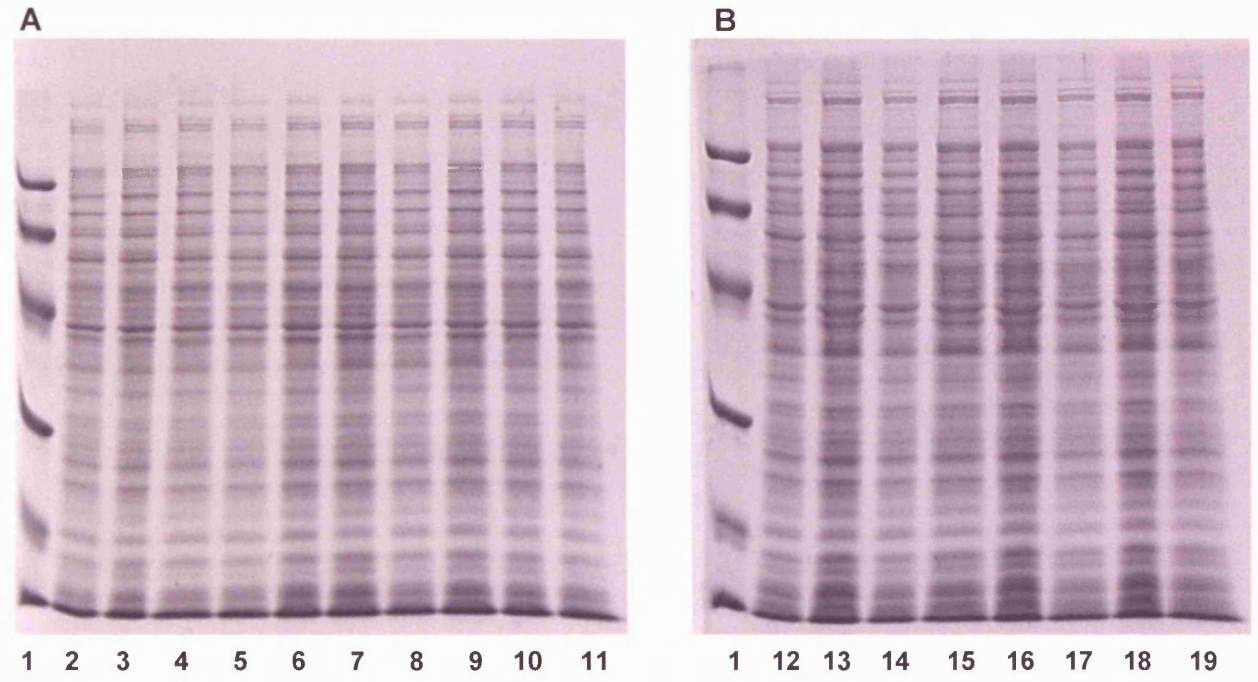


Figure 3.27 - SDS-PAGE of the blue spreading organisms isolated from **A** the biofilm formed in MRD 2, **B** the biofilm formed in MRD 3 and **C** the planktonic phase of the 2 MRD's. The lanes are as follows: 1 molecular weight marker; 2 & 3 were isolated at T=0; 4, 5 & 6 were isolated from the 1 day old biofilm ; 7, 8 & 9 were isolated from the 7 day old biofilm; 10 & 11 were isolated from the 14 day old biofilm. From the sessile phase of MRD 3: 12 & 13 were isolated at T=0; 14 & 15 were isolated from the 1 day old biofilm; 16 & 17 were isolated from the 7 day old biofilm and 18 & 19 were isolated from the 14 day old biofilm. From the planktonic phase of MRD 2: 20 & 21 were isolated at T=0; 22 was isolated at T=1and 23 & 24 were isolated at T=14. From the planktonic phase of MRD 3: 25 & 26 were isolated at T=0; 27 & 28 were isolated at T=1; 29 was isolated at T=7 and 30 & 31 were isolated at T=14. Heterotrophs in lanes 2, 4, 7, 10, 12, 14, 16, 18, 20, 23, 25, 27 and 30 were all isolated on R_2A agar while the others were isolated on MacConkey agar.

Figure 3.28 - The Protein Profiles of the Other Spreading Heterotrophs Isolated From the MRD's

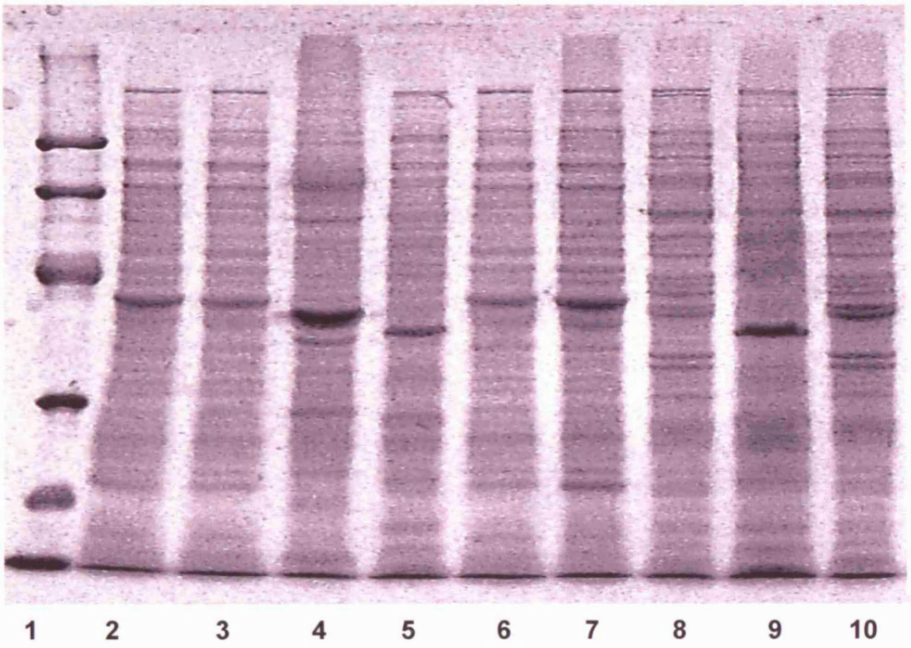


Figure 3.28 - SDS-PAGE of the other spreading heterotrophs isolated from the MRD's. Lanes are as follows: 1 molecular weight marker; 2 & 3 were isolated from the plankonic phase of MRD 1 at T=0 and T=7 respectively; 4, 5 & 6 were isolated from the sessile phase of MRD 1 at T=1, T=7 and T=14 respectively; 7 was isolated from the planktonic phase of MRD 2 at T=0; 8,9 & 10 were isolated from the sessile phase of MRD 2 at T=1, T=7 and T=14 respectively. All other spreaders were isolated on R_2A agar.

Figure 3.29 - The Protein Profiles of the Pigmented Heterotrophs Isolated from the MRD's

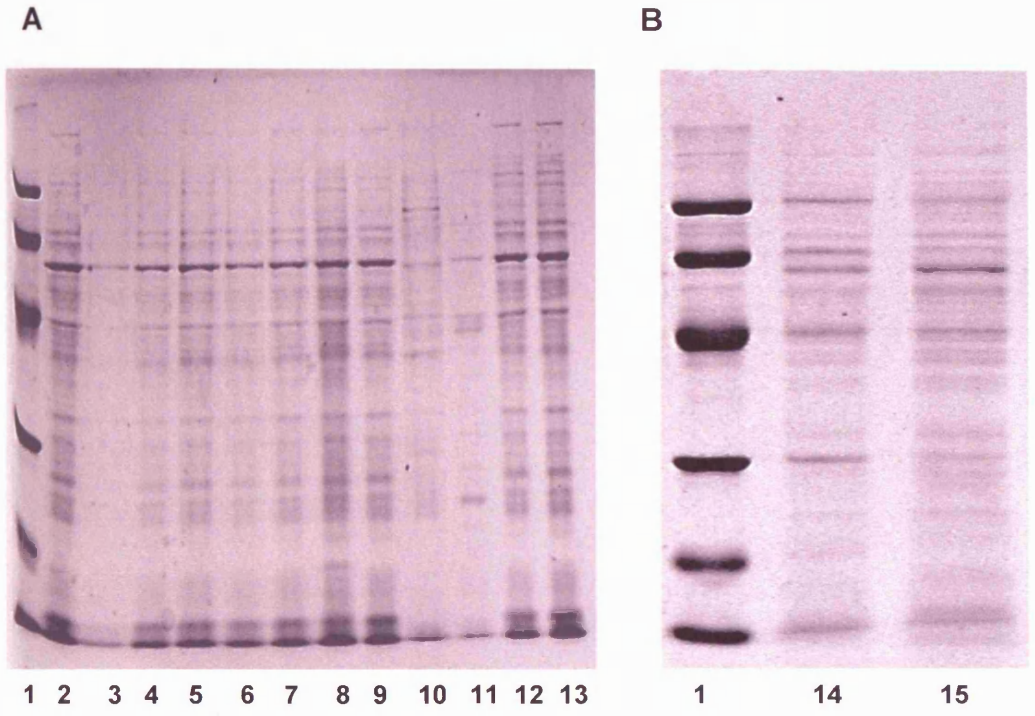


Figure 3.29 - SDS-PAGE of pigmented heterotrophs isolated from the 3 MRD's. **A** shows the profiles of some large yellow strains, **B** shows those for orange strains and **C** shows those for pink strains. Lane 1 = molecular weight marker, lanes 2 - 7 = yellow strains from MRD2, lanes 8 - 13 = yellow strains from MRD3, lane 14 = orange strain from MRD2, lane 15 = orange strain from MRD3, lanes 16 - 19 = pink strains from MRD2 and lanes 20 - 22 = pink strains from MRD3.

Figure 3.30 - The Protein Profiles of the two Presumptive Coliforms Isolated from the MRD's

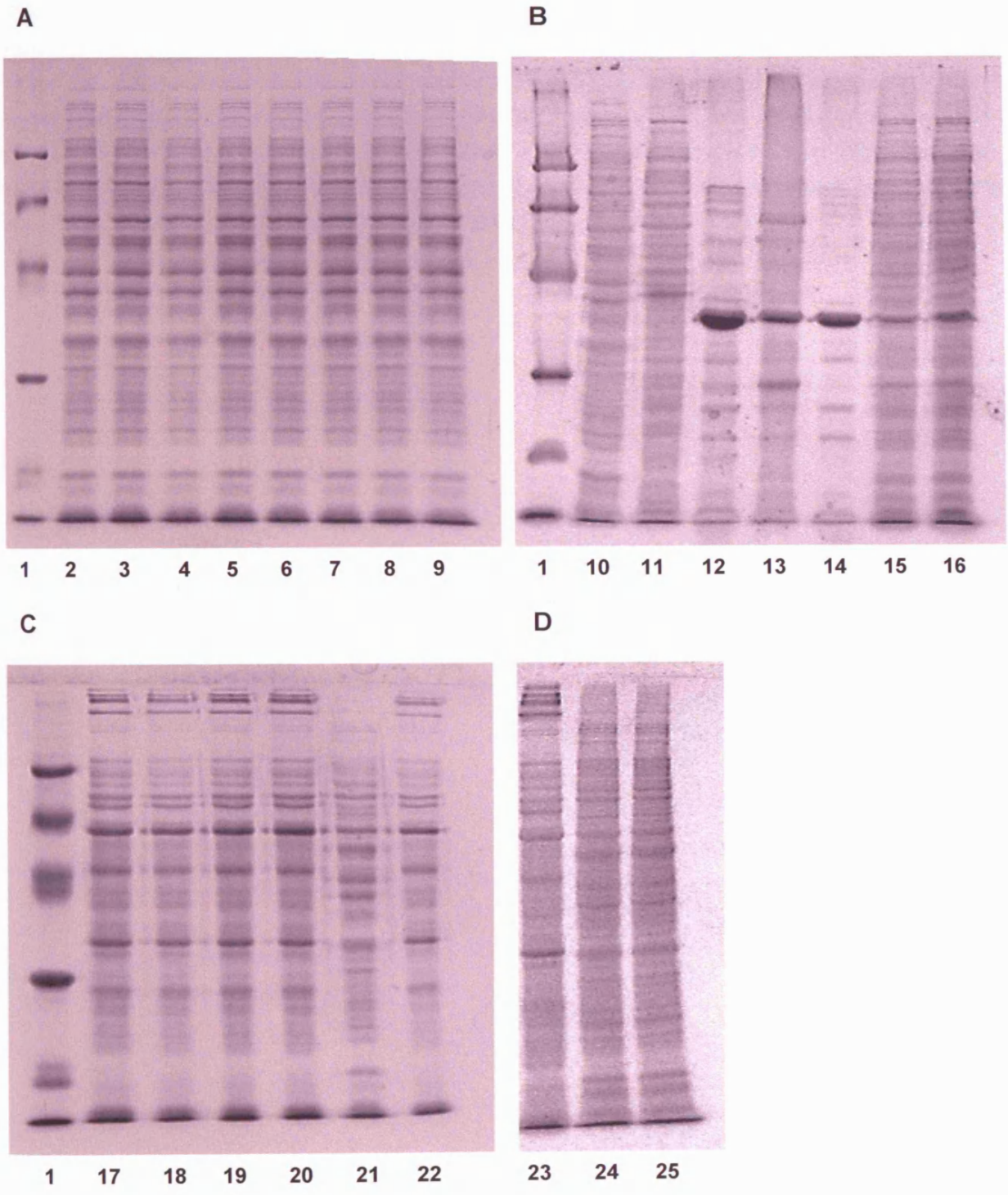


Figure 3.30 - SDS-PAGE of the two distinguishable presumptive coliforms isolated from the 3 MRD's. **A** & **B** show the profiles of ORG001 isolated from the sessile phase of MRD 1 and MRD 2 & 3 respectively. **C** & **D** show the profiles of ORG002 isolated from MRD 1 and MRD's 2 & 3 respectively. Lanes are as follows: 1 molecular weight marker; 2 & 3 were isolated at T=0; 4 & 5 at T=1; 6 & 7 at T=7 and 8 & 9 at T=14. Lanes 10, 11, 12, 13 & 14 were isolated from MRD 2 at T=0 from the planktonic phase and at T=0, 1, 7 and 14 from the sessile phase respectively. Lanes 15 & 16 were isolated from the planktonic phase of MRD 3 at T=0. Gel **C** shows isolates from the sessile phase of MRD 1. Lanes 17 & 18 were isolated at T=0; 19 & 20 at T=1; 21 at T=7 and 22 at T=14. Gel **D** shows isolates from MRD 2 from the sessile phase at T=1, T=7 and T=14 respectively. All strains were isolated on MacConkey agar except those in lanes 3, 5, 7, 9, 18 and 20 which were isolated on lauryl sulphate agar (LSA).

Figure 3.30 shows the results obtained for the presumptive coliforms, ORG 001 and ORG 002. Gel A shows the isolates from MRD 1 while gel B shows those from MRD's 2 and 3. Throughout the time course of the experiment in MRD 1 the organisms were identical, however from the other 2 MRD's the profiles were not so clear. They all appear to be related but not identical to either each other or to ORG 001 from MRD 1. The organisms in lanes 10 and 11 do display similarities to those in gel A suggesting a possible relatedness while the latter 5 lanes are all very similar to each other. Gels C and D display the profiles of those organisms classified as ORG 002. Once again the results suggest a close relationship between all the organisms but also exhibit some minor differences especially between isolates from MRD 1 and those from MRD'S 2 and 3. The organisms from the non-pigmented group were not profiled as they were so different. They also weren't sequenced for the same reasons.

3.4.3 Identification

The organisms further identified using partial 16S RNA gene sequencing were representative strains from each of the groups followed during the previous experiments. Table 3.4 shows the results of this method of identification. The strains identified were the blue spreading strain, two members of the pigmented group of heterotrophs and organisms 001 and 002 from the 3 MRD's monitored. The blue spreading organism was chosen to represent both it's own group and the group of spreading heterotrophs. This was because at least four strains were present in this group but none could be distinguished as being the most abundant and hence representative of the group. The blue spreading organism, however, was the most prominent and easily isolated strain. Originally placed in the group of spreading heterotrophs this organism was placed in a separate category because of its abundance. Two strains from the pigmented group were chosen owing to their ample presence in the biofilm and easy isolation. These isolates were not the only yellow and orange organisms but rather the most common. The pink strain was not included here owing to difficulties with the isolation of this organism and due to constraints of time. Additionally the non-pigmented strains were not included for the obvious reason that no representative strains could be distinguished from this group.

The results from Table 3.4 indicate that the blue spreading organism is a *Pseudomonas aeruginosa*. This organism is commonly isolated from aquatic environments and is of major importance owing to its pathogenic properties. This was an expected result as the organisms morphology indicated as such especially the production of the blue/green pigment known as pyocyanin which is commonly associated with this organism (Prescott *et al.*, 1990). The two pigmented strains were identified as *Sphingomonas yanoikuyae* and *Blastomonas natatoria*

for the yellow and orange strains respectively. These organisms are closely related and in fact *Blastomonas natatoria* used to be classified as a *Sphingomonas spp* (Yabuuchi *et al.*, 1999). Another interesting point is that the *Blastomonas natatoria* on the BLAST database which matched the strain isolated from this model was initially isolated from an aquatic biofilm. Other workers (Jess *et al.*, 1997) also isolated several *Sphingomonas* strains from a water distribution system biofilm. Organism 001 was confirmed to be the same in all three MRD'S while organism 002 was not. In MRD 1 organism 002 was identified as a *Delftia spp* while in MRD's 2 and 3 it was found to be a *Ralstonia spp*. These two organisms are however quite closely related to each other and both used to be classified as *Pseudomonas spp* (Wen *et al.*, 1999; Yacuuchi *et al.*, 1995).

Table 3.4 – The Identification of Representative strains using Partial 16s RNA Gene Sequencing

Organism Group	Other Information	Strain ID (Lab No)	Identification	% (No. of Matches)
BLUE SPREADER		R312	*Pseudomonas aeruginosa*	99% (505/509)
PIGMENTED	YELLOW	R302	*Sphingomonas yanoikuyae*	99% (554/566)
	ORANGE	R305	*Blastomonas natatoria*[1]	99% (551/553)
ORG 001	From MRD 1	M001	*Pseudomonas mendocina*	98% (389/393)
ORG 001	From MRD 2	M312	*Pseudomonas mendocina*	99% (406/410)
ORG 002	From MRD 1	M002	*Delftia sp.*[2]	98% (582/590)
ORG 002	From MRD 2	M305	*Ralstonia sp.*[3]	98% (330/335)

Table 3.4 – The identification of the representative strains isolated from the laboratory produced water distribution system biofilm models. The identification was done using partial 16S RNA gene sequencing and matches were made on the BLAST server.

[1] *Blastomonas natatoria* – was formerly known as *Sphingomonas natatoria* (the strain it was compared to was isolated from an established aquatic biofilm).

[2] *Delftia sp.* – certain strains of this species are related to *Pseudomonas sp.*

[3] *Ralstonia sp.* – certain strains of this species are related to *Pseudomonas sp.*

3.5 Chapter Summary

The results obtained for the biofilm model in the MRD suggest that, although some variation did occur, in general the model used was reproducible in size and structure. The resultant biofilm formed after a period of 24 hours and little variation occurred along the MRD length after this time. Some morphological differences, noted using SEM, did occur but these were found to be consistent at two different time points.

Many different organisms were isolated from the biofilm including heterotrophs, pseudomonad and related strains and FLA's. These organisms have been previously found to occur in water distribution system biofilms by other workers (Le Chavallier *et al.*, 1987). The interactions occuring between the organisms present were as expected and have all been previously described (Atlas & Bartha, 1998) and the bacterial isolates identified, mainly *Pseudomonas spp.* and *Sphingomonas spp,* have all been found to be common isolates of mature aquatic biofilms (Jess *et al.*, 1997). All of these findings suggest that the model used was both reproducible and a very close match to a normal water distribution system biofilm. In addition, the model was found to be sensitive to changes in the system. For example, the presence of a small metal pipe connector was found to completely alter the population of the biofilm.

The alternative jar model was also quite a reproducible system and its ease of sterilisation and allowance for many test surfaces served as a distinct advantage. It was however quite different to the MRD and was thus deemed an inappropriate model for the representation of a water distribution system biofilm model. However it may be a useful model for use in preliminary experiments. For example, it was easier to produce a mono-species biofilm in this model. The formation of such a biofilm proved to be similar to that observed in the MRD with slight differences observed for bacterial numbers.

In conclusion the two biofilm models were reproducible and thus could be used for subsequent experiments concerning the interaction of viruses with such biofilms.

CHAPTER 4

RESULTS AND DISCUSSION II
THE INTERACTIONS OF BACTERIOPHAGE WITH THE BIOFILM MODEL

4.1 The Evaluation of Methods Used

4.1.1 The Sensitivity and Reproducibility of the Methods

Before embarking on experimentation using bacteriophage it was necessary to evaluate the chosen methods for phage detection and enumeration. The most common method used for this is the plaque assay method (Campbell, 1996). In order to determine the sensitivity and reproducibility of this and some adapted methods these were all performed in triplicate on the same batch of stock phage suspension.

The results of these experiments are displayed below in Table 4.1. From this table it can be concluded that the amount of phage present in the stock solution was between 1.78×10^7 and 3.12×10^{12} $pfuml^{-1}$. This is a huge difference in calculation and shows that all methods are not precise. The enrichment broth method was low because it relies on the clearing of the broth for a positive result. The enrichment and assay procedure shows that a positive result does not always produce a clear broth and thus the enrichment method allowed for an underestimation of the amount of phage present. The enrichment and assay method thus produced a more precise result. However it must be noted that, upon cell lysis of the host, an unpredictable number of phage may be released further diminishing the accuracy and precision of this method for quantitative purposes.

The plaque assay, however, provided a direct count of the phage present while the other 3 methods were estimates. Additionally the last 3 methods were variations on the same theme culminating with the CID_{50} method. This latter method must therefore be a more precise estimation than the other two simply because it utilises at least 5 wells at each dilution per experiment. When comparing the two methods, the plaque assay and the CID_{50}, many advantages and disadvantages were apparent. The plaque assay is, as stated previously, a direct count of the phage present. The standard deviation observed with this method was low indicating good reproducibility and the method itself was both simple to perform and inexpensive. The CID_{50} method, however, did appear to be the more sensitive choice but it's high standard deviation indicated a lack of reproducibility. In addition it was much more expensive and time consuming.

In conclusion the plaque assay was chosen as the main procedure because it is a direct method and it is highly reproducible. In some of the experiments, however, it was necessary to confirm the presence or absence of the phage if the plaque assay produced a negative result and thus the enrichment and assay procedure was utilised. This was only used for isolation of low phage numbers as apposed to their quantitation. It must be noted that

although this method produced a lower phage count than the plaque assay it did still exhibit growth of the phage at dilutions as low as 10^{-9} and 10^{-12}. At these dilutions plaques were not visible in the direct method. Thus the enrichment and assay method exhibited a higher degree of sensitivity. The CID_{50} method was not used as the disadvantages outlined above outweighed the advantages.

4.1.2 The Effect of Biofilm Removal Methods on Bacteriophage λ

This was done in order to determine whether the techniques for biofilm removal would have any detrimental effects on the bacteriophage itself. To evaluate this the bacteriophage was subjected to 1 minute treatments of both sonication and vortexing. The results in Figure 4.1 show that neither treatment had an adverse effect but rather served to increase the phage levels in the tubes tested. This increase was not due to growth but may be attributed to the breaking up of viral aggregates (Campbell, 1996).

In all tubes prior to the treatment there was a mean of 1.3×10^9 $pfuml^{-1}$. In the control tubes after 1 minute this value had reduced slightly to 8.6×10^8 $pfuml^{-1}$. This reduction suggests that the bacteriophage were somehow being removed from the fluid. Two reasons are proposed for this; the phage may have become aggregated or it may have adhered to the surface of the glass tube. Both incidences have previously been referred to in the literature (Yates *et al*, 1997).

After the 1 minute sonication the phage levels increased. Since no host was present this cannot be attributed to rapid growth but rather to the breaking up of viral aggregates in the solution. Additionally the results after the 1 minute vortexing treatment were similar to that observed for the sonication but were not as high. This may mean that the latter treatment was not as efficient at breaking up viral aggregates as the former method.

Table 4.1 – The Sensitivity and Reproducibility of the Phage Isolation and Enumeration Methods

METHOD	RESULTS	INFERENCE
Plaque Assay [1]	255; 326; 212 pfu/100µl at 10^{-7}	$3.16 \times 10^{10} \pm 1.13 \times 10^{9}$ pfuml^{-1}
Enrichment broth [2]	3/3 +ve* at 10^{-6}; 2/3 at 10^{-7}	1.78×10^{7} pfuml^{-1} Ψ
Enrichment & Assay [2]	3/3 +ve* at 10^{-9}; 1/3 at 10^{-10} & 10^{-12}	1.00×10^{10} pfuml^{-1} Ψ
CID_{50} [3]	$10^{10.5}$; $10^{11.5}$; $10^{11.77}$ /100µl	$3.12 \times 10^{12} \pm 2.28 \times 10^{12}$ iu ml^{-1}

Table 4.1 – The results obtained using four methods for the isolation and quantitation of phage. For all methods *E. coli* LE392 was used as the host bacterium and the phage used came from the same stock solution. Experiments were done in triplicate and the means and standard deviations (where possible) are shown.

[1] Plaque Assay – utilised the agar overlay method.

[2] Enrichment broth & Assay – 100 µls serial dilutions were incubated overnight with host and then checked for clarity or assayed using the plaque assay. Both tests allowed the estimation of the initial phage using the Reed and Muench (1938) calculation of 50% end points.

* +ve refers to clear broth or plate in relation to the enrichment broth method and the enrichment & assay procedure respectively.

Ψ These results were the same for all three experiments.

[3] CID_{50} – 50% infectious dose in culture. An adaptation of the enrichment and assay procedure and of the $TCID_{50}$ (50% infectious dose in tissue culture) method used later for poliovirus.

Figure 4.1 - The Effect of the Biofilm Removal Methods on Bacteriophage Lambda to Determine Detrimental Effects

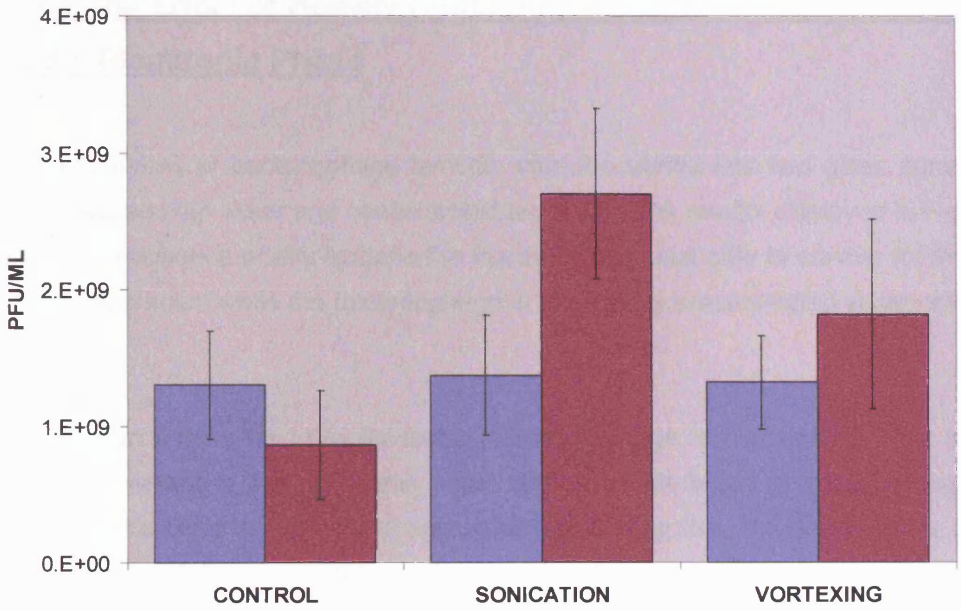


Figure 4.1 - The levels of bacteriophage lambda in tubes before ( ) and after ( ) 1 minute treatments of sonication and vortexing. Data are expressed as the means of triplicate experiments with population standard deviations as error bars.

4.2 Bacteriophage Levels in Preliminary Experiments

4.2.1 The Effect of Heavily Contaminated Water on Bacteriophage Levels in the Planktonic Phase

Equal volumes of bacteriophage lambda were inoculated into two glass durans containing filter sterilised tap water and concentrated tap water. The results displayed in Figure 4.2 show that in the absence of any bacteria the bacteriophage was able to survive for the full 15 days of the experiment while the bacteriophage in the heavily contaminated water only survived for 7 days.

In the clean water after 1 day the levels of bacteriophage increased by 0.1 log. Since the host was not present in the water this effect cannot be attributed to bacteriophage growth but rather to the breaking up of viral aggregates. Following this, the phage levels decreased by approximately 0.5 logs and remained at this level for 7 days. The reason for the decrease was unclear but proposed to be due to either the reforming of viral aggregates or to the adhesion of virus particles to the glass surface in the duran. Destruction of the virus was ruled out because a further increase in virus was later observed and it may be that a cycling of aggregation or adhesion to the bottle surface or both was occurring. In contrast, the contaminated water showed a decline in phage levels from the beginning of the experiment. This decline appeared to be inversely proportional with time and, although it was unclear at this point what was happening to the phage, it suggested that the presence of microbes in water had a negative effect on the persistence of bacteriophage. The proposed reasons for the negative interactions are as follows. Although not shown in this experiment, the microbes will have formed a biofilm on the bottle surface and this may act as an increased adsorbent for the phage thus removing it exponentially from the planktonic phase. Another reason may be that the organisms are producing enzymes which either promote increased viral aggregation, inactivate or destroy the bacteriophage.

4.2.2 The Effects of the Biofilm on Bacteriophage Lambda with Time A Simple Tube Experiment

Figure 4.3 shows the effect of a complex water distribution system biofilm on the levels of bacteriophage lambda in both the planktonic and sessile phases of the preliminary tube model. In the planktonic phase the bacteriophage survived for the full 14 day period of the experiment in both test (tube 3) and control tubes. However, in the two control tubes the

Figure 4.2 - The Effect of High Bacterial Numbers on Bacteriophage Lambda in Water

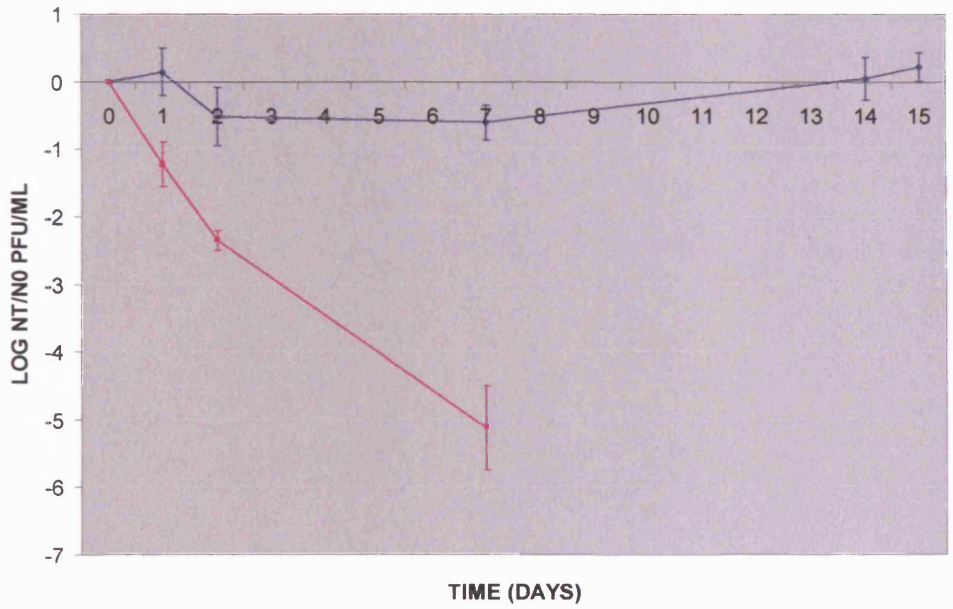


Figure 4.2 - The levels of Bacteriophage Lambda in sterile filtered water (—◆—) and concentrated water (—■—) over time. Phage levels are expressed as the log NT/N0 total PFU; where NT = total PFU at time point and N0 = initial PFU at time 0. The data show the means of duplicate sampling with population standard deviations as error bars.

Figure 4.3 - The Effect of a Biofilm on Bacteriophage Lambda With Time

A Simple Tube Experiment

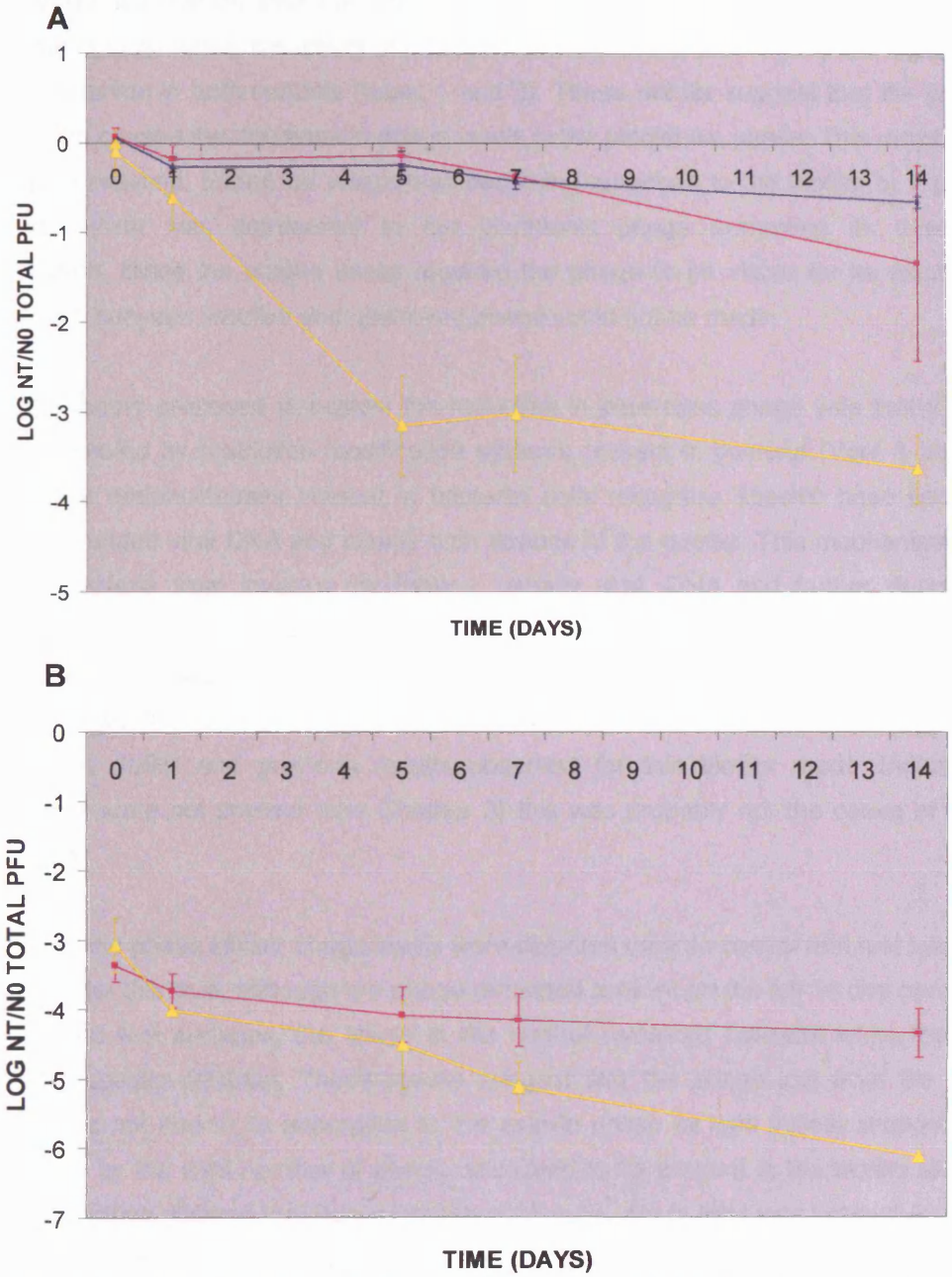


Figure 4.3 - Bacteriophage lambda in **A** the planktonic phase and **B** the sessile phase in the presence of a biofilm. The levels were measured in tube 1 (—◆—) containing filter sterilised water; in tube 2 (—■—) containing filter sterilised water and sterile plastic discs and in tube 3 (—▲—) containing filter sterilised water and 14 day old biofilm on plastic discs. The plastic discs with the biofilm were taken from an MRD. Phage levels were enumerated using the agar overlay method and are expressed as the log NT/N0 total PFU; where NT = total PFU at time point and N0 = initial PFU at time 0. Experiments were done in triplicate tubes and the means and population standard deviations are shown.

bacteriophage levels remained constant and followed a similar pattern, while in the test tube, containing the preformed biofilm on plastic discs, the phage levels declined considerably. This decline was exponential over the first 5 days, showing a decrease of approximately 0.6 logs day^{-1} which levelled off after this period. Although the phage levels were relatively constant after day 5 in all tubes, the levels of phage in tube 3 were approximately 2.5 logs lower than those observed in both controls (tubes 1 and 2). These results suggest that the presence of the biofilm caused the decrease in phage levels in the planktonic phase. This may be due to a variety of reasons. Either the phage was becoming adsorbed to the biofilm or it produced a product which was detrimental to the planktonic phage promoting its destruction or inactivation. Since the plaque assay required the phage to be viable for its enumeration, a distinction between inactive and destroyed phage could not be made.

Another theory proposed to explain the reduction in planktonic phage was that it may have been promoted by restriction modification systems present in bacteria (Voet & Voet, 1990). Restriction endonucleases present in bacterial cells recognise specific base sequences in double stranded viral DNA and cleave both strands of the duplex. This mechanism serves to protect bacteria from invasion by foreign, usually viral, DNA and further degradation by bacterial exonucleases would thus serve to destroy the virus (Brown, 1991). However this mechanism requires the infection of a host cell by the virus. Since bacteriophage lambda's natural hosts are *E. coli* and some other members of the Enterobacteriaceae family (Campbell, 1996) and previous results observed for this biofilm model indicated these organisms were not present (see Chapter 3) this was probably not the cause of the phage reduction.

In the sessile phase similar phage levels were detected on both control and test surfaces after 1 hour. After this time, although the phage remained present for the full 14 day period on both control and test surfaces, the levels in the control remained constant while those on the biofilm gradually declined. These results suggest that the phage lost from the planktonic phase was not due to its adsorption to the sessile phase as was initially proposed. This is supported by the total number of phage calculated to be present in the biofilm after 1 hour. This calculation showed that approximately 1.25×10^7 pfu in total was present in the sessile phase which only accounted for 0.46% of the total phage lost from the planktonic phase at the same time point. These results reinforce the previous assumption that the biofilm produced a product which promoted either the destruction or inactivation of the phage. This is further supported by the decline in phage levels noted in the sessile phase over time. The observed decline in phage levels appeared to be more pronounced in the planktonic phase compared to the sessile phase in the test model. This suggests that either the products produced by the biofilm diffused into the planktonic phase or that the virus was afforded more protection from them when adsorbed to it. The findings of previous workers have shown that viruses are able to adsorb to substances where they are afforded protection (Campbell,1996; Hejkal *et al*,

1997) thus supporting the latter idea. That the phage levels became constant in the planktonic phase after day 5. It also suggests that a small proportion of the planktonic phage population may have had an increased resistance to the detrimental effects of the biofilm products.

4.3 Bacteriophage Levels in the MRD

4.3.1 The Effect of a Biofilm on Copper

Figure 4.4 shows the results obtained for the persistence of phage in the planktonic phase in the presence of biofilms forming on both copper and plastic. It appears to show that overall the phage survives for a much longer period of time in the presence of the biofilm on copper than in the presence of a biofilm on plastic. However after the day 5 time point the numbers of phage recovered from the planktonic phase in the presence of copper are negligible being between 2 and 20 pfuml-1. In the presence of the plastic the phage was not isolated after this time point but it may be that low levels were still present but were missed in the detection procedure (see Section 4.1.1 for sensitivity of the plaque assay).

After 1 day the levels of phage in the presence of a biofilm formed on plastic increased very slightly by approximately 0.2 logs or 7.7% of the initial innoculum. This was probably due to the breaking up of viral aggregates and has been previously shown to be an effect of dilution in water (see Section 4.2.1). This result also shows that any detrimental effects on the phage in this model system occur after an initial 24-hour period. The results in table 4.2 show the levels of phage isolated from the sessile phase on triplicate studs. They show that after 1 day phage was isolated from the biofilm at approximately –2.27 logs or 0.5% of the initial inoculum. After this time point no phage could be isolated from any of the studs tested. This result confirms the previous finding that it takes at least 24 hours for the detrimental effects produced by the biofilm to either destroy or inactivate the phage in both phases of the MRD.

In contrast the phage levels in the presence of a biofilm on copper in the planktonic phase have dramatically decreased by more than 2 logs at day 1. This accounts for approximately 99.5% of the initial innoculum. This result greatly differs from that observed in the MRD containing plastic and the reasons for this could be either that the different biofilm forming on copper was more detrimental than that on plastic or that the copper itself had an effect. The latter theory is thought to be the most likely as previous results (chapter 3) indicate that, while a biofilm on copper did form at the same rate as one on plastic, it did not contain as many viable organisms. Additionally copper ions are known to leach into the fluid phase and such copper ions have been found by other workers to have detrimental effects on phage (Sagripanti *et al*, 1993). The results shown in Table 4.2 confirm that this idea may be valid exhibiting negative results for phage isolation from the sessile phase of a biofilm on copper at all the time points tested.

After day 5 the phage levels in the planktonic phase, although very low, remain at a constant level in the MRD containing copper while their presence is absent in the MRD containing plastic. The reason for this are unclear but may be due to the differences in biofilm populations present in the two MRD's. Previous results and other workers (Rogers *et al*, 1994) have shown that copper ions accumulate in the biofilm. The gradual removal of the copper from the planktonic phase may have saved the few phage left in the system. This, however, does not explain why the biofilm products have not destroyed or inactivated the phage and it may be that since the phage numbers are negligible similar levels were present in the MRD containing plastic but unfortunately were undetected. It must be noted that in order to determine whether the copper itself is the detrimental factor in the first MRD the same experiment should have been done without a biofilm. This was originally done but it became contaminated and since no other experiments utilising copper as the surface for biofilm formation were performed this experiment was left as it is.

Once again in the presence of the biofilm on plastic, more than 24 hours passed before destruction or inactivation of the phage begins. The previous results gained in chapter 3 show that the biofilm has fully formed at this time point and it may be that the biofilm itself as opposed to the individual members is the detrimental agent with regard to the phage.

Following the above experiment, at day 28, a total of 14 studs and two planktonic samples were removed from the MRD with the copper test surfaces. The discs taken from these studs and the planktonic samples were then tested for the presence of phage using the enrichment and assay procedure. This was done in order to determine whether any phage was present at levels lower than the sensitivity of the plaque assay method. Table 4.3 shows the results obtained. These results surprisingly show that some phage was present in the sessile phase of the biofilm model and that the distribution of the phage was at either end of the MRD. This was in contrast the planktonic samples, which displayed a lack of phage confirming the previous plaque assay results.

These results suggest that the phage was present in the biofilm at levels lower than the detection limits of the plaque assay method. The lack of phage in the planktonic phase suggests that the phage did leave the planktonic phase and become adsorbed into the biofilm where it may have been afforded some protection. Low levels of phage were previously demonstrated in the biofilm in the tube experiment and it may be that a minority of the phage population possess increased resistance to the detrimental effects produced. Another reason could be that those phage more strongly adsorbed to the disc surface are afforded more protection than those in the planktonic phase.

Figure 4.4 - The Effect of Biofilms Forming on Copper and Plastic on Bacteriophage Lambda Levels in the Planktonic Phase of the MRD

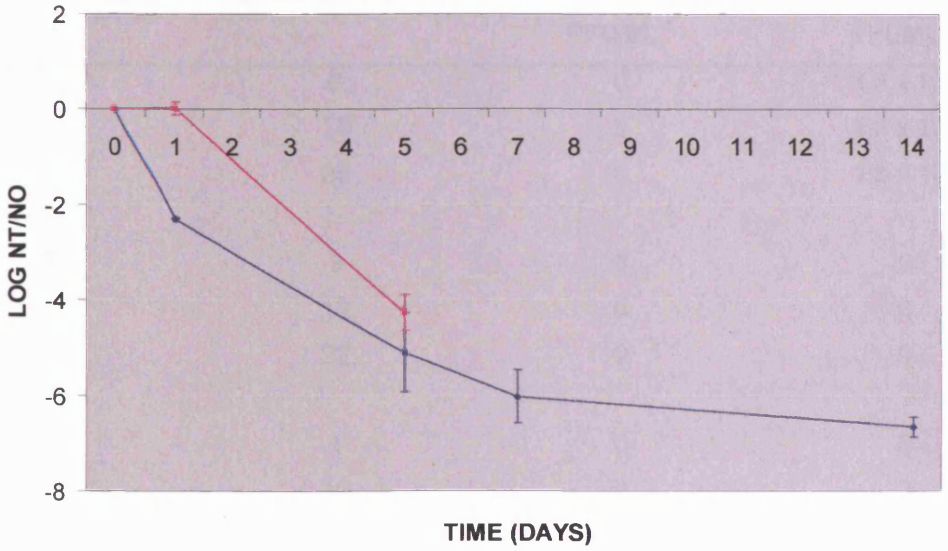


Figure 4.4 - The bacteriophage levels in the the planktonic phase of the MRD in the presence of biofilms forming on copper and plastic. The levels were measured in MRD 1 (—◆—) containing biofilms forming on copper and in MRD 2 (—■—) containing biofilms forming on plastic. Phage levels were enumerated using the agar overlay method and are expressed as the log NT/N0 total PFU; where NT = total PFU at time point and N0 = initial PFU at time 0. Duplicate samples were taken from each MRD and the means and population standard deviations are shown.

Table 4.2 – The Levels Of Bacteriophage Lambda Isolated From Developing Biofilms Forming On Copper And Plastic

TIME (DAYS)	STUD NUMBER	COPPER PFUML^{-1}	PLASTIC (MRD 5) PFUML^{-1}
1	6	0	3.4×10^3
	16	0	4.2×10^2
	25	0	1.8×10^2
5	3	0	0
	12	0	0
	22	0	0
7	1	0	0
	10	0	0
	20	0	0
14	2	0	0
	11	0	0
	19	0	0

Table 4.2 – The isolation of bacteriophage lambda from the sessile phase of two MRD's. One containing copper discs and the other plastic. At each sampling point 3 discs were removed at random from each MRD and the plaque assay method was used to enumerate the phage.

Table 4.3 – The Isolation of Phage from the Biofilm formed on copper using the Enrichment and Assay Procedure

STUD NUMBER	PHAGE PRESENCE[1]
1	+
3	+
4	+
6	-
10	-
11	-
12	-
13	-
19	-
20	-
21	-
22	-
23	-
24	+
PL [2]	-,-

Table 4.3 – The results obtained for the isolation of phage using the enrichment and assay technique. Studs were removed from the MRD containing copper surfaces at day 28 of the experiment. 14 days after the initial experiment had ended. The discs were placed directly into enrichment broth containing the host *E. coli,* incubated overnight and assayed at a dilution of 10^{-1} the following day. Results obtained are semi-quantitative showing the levels of phage isolated in relation to each other only.

[1] + is a positive result while – indicates no plaques observed.

[2] PL denotes the planktonic sample which was tested in duplicate. Both results are displayed.

4.3.2 The Effect of Biofilm Development on Plastic

The results displayed in Figure 4.5 show the levels of phage present in the planktonic phase of the MRD containing a biofilm forming on plastic. For this experiment the phage was inoculated at the same time as the biofilm inoculum. The results show that in the absence of a biofilm the phage survived for a period of 7 days in the MRD while in the presence of a biofilm it only survived in the planktonic phase for 5 days.

After 1 day the results in the three MRD's containing developing biofilms are variable. In two of the MRD's the phage levels are reduced to lower levels than in the control while in the other a slight increase was observed. These results were difficult to interpret because, although two of the test MRD's displayed similar results to the control, one showed a significant difference (MRD 4). If the results are taken as a whole however, taking biological variation into consideration, then it must be noted that two out of the three test MRD's displayed a decrease greater than that found in the control. This would thus infer that over the initial 1 day period the biofilm had a minor effect on the planktonic phage levels. The results obtained for the levels of phage in the sessile phase (displayed in Tables 4.4 and 4.5) show that some of the missing phage from the planktonic phase has adsorbed into the sessile phase. At this time point similar levels of phage were isolated from the three test MRD's but significantly higher levels were isolated from the control. This suggests that although the phage was adsorbing to the biofilm it was either being destroyed or inactivated by it.

At the 5 day time point all 4 MRD's exhibit a significant decrease in phage levels. In the control MRD this decrease was less pronounced showing an approximate 1.5 log reduction. This probably accounts for the number of phage particles adsorbing to the inside of the MRD. In contrast the MRD's containing the developing biofilms displayed much larger reductions in phage levels. These were 4.3, 4.5 and 2.9 logs lower than at the day 1 time point for MRD's 2, 3 and 4 respectively. Although the reduction observed in MRD 4 was not as large as in the other two MRD's this was because the initial reduction at day 1 was larger. It must be noted that at day 5 the levels of phage were all very similar in the three test MRD's. These results, once again, suggest that the biofilm was affecting the phage levels in the planktonic phase. The phage was either becoming adsorbed to the biofilm or being destroyed or inactivated by it. This reconfirms previous results obtained from tube experiments showing that it was the biofilms presence that had the effect rather than any component materials in the apparatus used. At this time point phage could only be isolated from one of the 3 test MRD's and from the control in the sessile phase. The levels of phage, however, were still lower than those observed in the control MRD and it may be that the biofilm developing in MRD 2 was doing so at a slower rate or had a slightly different biofilm population than those in MRD's 3 and 4.

Figure 4.5 - The Effect of a Developing Biofilm Forming on Plastic Surfaces on Bacteriophage Lambda in the Planktonic Phase of the MRD

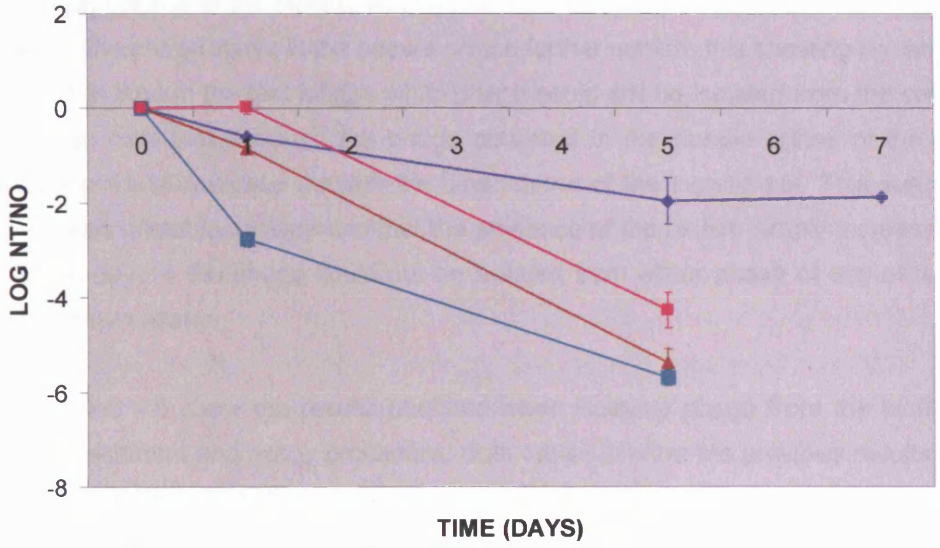


Figure 4.5 - The bacteriophage lambda levels in the planktonic phase of the MRD in the presence of a developing biofilm forming on plastic. Bacteriophage was inoculated into the MRD at time 0 of biofilm formation and the phage levels were measured in MRD 1 (—◆—) containing filter sterilised tap water and in MRD's 2 (—■—), 3 (—▲—) and 4 (—■—) containing water distribution system biofilms forming on plastic. Phage levels were enumerated using the agar overlay method and are expressed as the log NT/N0 total PFU; where NT = total PFU at time point and N0 = initial PFU at time 0. Duplicate samples were taken from each MRD and the means and population standard deviations are shown.

At day 7 the control MRD still contained relatively high levels of phage in the planktonic phase. In the MRD's containing the developing biofilm, however, the phage was undetected. This result was in contrast to the previous tube experiments which exhibited a low level of phage presence for up to 14 days. The reason for these contrasting results may be due to the differences in volume of the planktonic phase in the two types of apparatus used. The results obtained for the phage levels in the sessile phase further confirm this showing no more phage present at this time in the test MRD's while phage could still be isolated from the control. It is interesting to note that although the phage persisted in the sessile phase of the control it exhibited a gradual decrease through the time course of the experiment. This suggests that the phage was unstable anyway and that the presence of the biofilm simply increased its rate of decay. At day 14 the phage could not be isolated from either phase of any of the MRD's using the plaque assay.

Tables 4.5 and 4.6 show the results obtained when isolating phage from the biofilm model using the enrichment and assay procedure. Both tables confirm the previous results obtained using the plaque assay method showing that even the more sensitive method did not pick up any residual phage missed by the plaque assay in the planktonic phase. It must be noted that there were some exceptions in the sessile phase in both test and control MRD's.

In the test MRD at day 7, phage was enriched from the sessile phase on the disc but not from the neat sample (undiluted sample taken from the disc). Since the disc was directly enriched after the sampling process had been done this result suggests that low levels of phage were present in the biofilm at this location but that they were not removed from the disc during sampling and thus were missed during the plaque assay procedure. The level of phage recovered after the enrichment was low and may indicate that a minority of the phage were able to strongly adsorb to the test surface resisting the removal techniques applied. This is confirmed by the results obtained for the control MRD. In the sessile phase including at day 14, when phage was not detected using the plaque assay, phage could be enriched. Additionally the enrichment of the phage occurred in both the neat and disc samples tested. This confirmed that some of the phage remained in the system for at least 14 days at very low levels but also that a small level of phage adsorbed to the surface of the disc so strongly that their removal was almost impossible.

4.3.3 The Effect of a Mature Biofilm formed on Plastic

Figure 4.6 shows the results obtained for planktonic phage levels in the presence of a mature biofilm formed on plastic. For this experiment the biofilm was allowed to develop over a 14 day period prior to phage inoculation. The results display similar traits to those previously

observed in the presence of a developing biofilm (see Figure 4.5). In all 4 MRD's the planktonic phage levels decreased with time. Once again the phage remained present in the control for a period of 7 days while it only survived for 5 days in the three MRD's containing the biofilm. Slight differences were, however, observed and these are detailed below.

After 1 day there was a decrease in the planktonic phage levels in all 4 MRD's. This decrease was slight demonstrating a log reduction of between 0.1 and 0.6 in all the MRD's including the control. In fact the reduction in the control was greater than that observed in all of the test models. However since this reduction in the control was not significantly different from that observed in the three test models it must be inferred that the biofilm had little, if any, effect on the phage at this time point. This result was similar to that previously noted for planktonic phage levels in the presence of a developing biofilm (see Figure 4.5). When evaluating the planktonic phage it was previously deduced that the biofilm needed to form before its detrimental actions on the phage could occur. However, since the biofilm was already formed in this experiment these results show that this was not the case. This biofilm exhibited a similar 1 day time lapse prior to having a detrimental effect on the phage thus showing that this action happened regardless of the developmental stage or age of the biofilm. This prompts the assumption that this action was non-specific.

In the sessile phase after 1 day lower levels of phage were detected in the biofilm samples compared to the control (see Table 4.7). These results show that similar levels of phage were isolated from the sessile phase of both the mature and developing biofilms. Slightly higher levels were isolated from the mature biofilm in one of the test MRD's (MRD 5) but this was probably insignificant, caused by variations in the biofilm, because the other two test MRD's did not exhibit similar results. Once again, they display similar results to those obtained in the previous experiment.

After the 5 day time point the results for planktonic phage were variable between MRD's. Two of the test models displayed the expected decrease in phage levels while one test model (MRD 5) displayed higher levels than those observed in the control. The reason for this difference in results was unclear but it may be to do with the initial inoculum of phage in the respective MRD's. Even in this MRD, however no phage could be detected after this time point and these results were the same for all the test models. The control, once again, exhibited planktonic phage survival for the 7 day period. Interestingly the test model (MRD 5) displaying higher results in the planktonic phase also exhibited higher phage levels in the sessile phase further supporting the above assumption that the initial phage inoculum may have been slightly higher in this MRD.

Table 4.4 – The Levels Of Bacteriophage Lambda Isolated From Developing Biofilms Forming On Plastic

TIME (DAYS)	STUD NUMBER	MRD 1 (CONTROL) PFUML^{-1}	MRD 2 PFUML^{-1}	MRD 3 PFUML^{-1}	MRD 4 PFUML^{-1}
1	1	9.70×10^4	1.55×10^2	3.32×10^2	6.00×10^1
	2	1.50×10^4	1.54×10^2	2.80×10^2	1.04×10^2
5	3	1.00×10^3	4.87×10^2	0	0
	4	5.90×10^3	8.49×10^2	0	0
7	5	7.1×10^2	0	0	0
	6	4.4×10^2	0	0	0
14	7	0	0	0	0
	8	0	0	0	0

Table 4.4 – The isolation of bacteriophage lambda from the sessile phase of four MRD's. One containing filter sterilised tap water (MRD 1) and the other three containing concentrated tap water. Both phage and biofilm inoculum were inoculated at time 0. At each sampling point two discs were removed from each MRD. Data are expressed as the mean of the results. The plaque assay method was used to enumerate phage.

Table 4.5 – The Isolation of Phage from the Developing Biofilm Using the Enrichment and Assay Procedure

TIME (DAYS)	STUD NUMBER	MRD 1 (CONTROL)[1]		MRD 2 (TEST)[1]	
		NEAT[2]	DISC[3]	NEAT[2]	DISC[3]
1	1	+	+	+	+
	2	+	+	+	+
5	3	+	+	+	+
	4	+	+	+	+
7	5	+	+	-	+
	6	+	+	-	-
14	7	+	+	-	-
	8	+	+	-	-

Table 4.5– The isolation of bacteriophage lambda from the sessile phase using the enrichment method. The control MRD contained filter sterilised tap water (MRD 1) while the test MRD (MRD 2) contained concentrated tap water. Both phage and biofilm inoculum were inoculated at time 0. At each sampling point 2 discs were removed from the MRD's. They were taken from the MRD moving away from the reservoir. These MRD's are the same as those in Table 4.4.

[1] + is a positive result while – indicates no plaques observed.

[2] NEAT indicates that the sample tested was following the sampling of the disc. This sample was also tested using the plaque assay procedure and the results are displayed in table 4.4.

[3] DISC indicates that the disc was directly enriched following the sampling procedure.

Table 4.6 – The Isolation of Phage from the Planktonic Phase of a developing biofilm using the Enrichment and Assay Procedure

TIME (DAYS)	MRD 1 (CONTROL)[1]	MRD 2 (TEST)[1]
0	+	+
	+	+
1	+	+
	+	+
5	+	+
	+	+
7	+	-
	+	-
14	-	-
	-	-

Table 4.6 - The isolation of bacteriophage from the planktonic phase using the enrichment method. The control MRD contained filter sterilised tap water (MRD 1) while the test MRD (MRD 2) contained concentrated tap water. Both phage and biofilm inoculum were inoculated at time 0. At each sampling point 2 1ml samples were removed from the reservoir of the MRD's. These MRD's are the same as those in Tables 4.4 and 4.5.

[1] + is a positive result while – indicates no plaques observed.

Figure 4.6 - The Effect of a Mature Biofilm Formed on Plastic Surfaces on Bacteriophage Lambda in the Planktonic Phase of the MRD

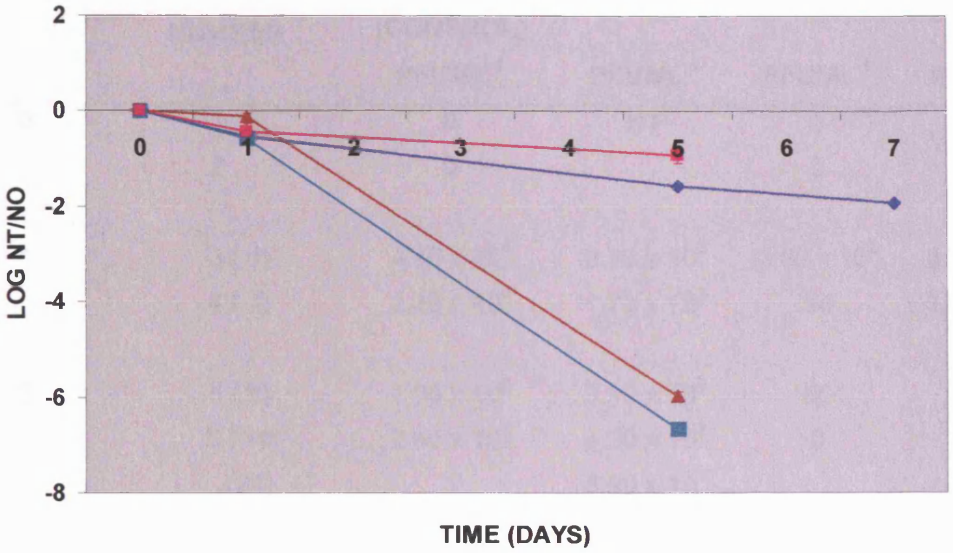


Figure 4.6 - The bacteriophage lambda levels in the planktonic phase of the MRD in the presence of a mature biofilm formed on plastic. Bacteriophage was inoculated into the MRD at time 14 of biofilm formation and the phage levels were measured in MRD 1 (◆) containing filter sterilised tap water and in MRD's 2 (■), 3 (▲) and 4 (■) containing water distribution system biofilms formed on plastic. Phage levels were enumerated using the agar overlay method and are expressed as the log NT/N0 total PFU; where NT = total PFU at time point and N0 = initial PFU at time 0. Duplicate samples were taken from each MRD and the means and population standard deviations are shown.

Table 4.7 – The Levels Of Bacteriophage Lambda Isolated From Mature Biofilms Forming On Plastic

TIME (DAYS)	STUD NUMBER [1]	MRD 1 (CONTROL) PFUML^{-1}	MRD 5 PFUML^{-1}	MRD 3 PFUML^{-1}	MRD 4 PFUML^{-1}
0	1	0	NT	0	0
	2	0		0	0
1	3 (4)	4.70×10^4	3.70×10^3	3.00×10^2	3.00×10^2
	4 (7)	2.70×10^4	1.70×10^3	54	3.00×10^2
5	5 (8)	1.00×10^3	3.10×10^2	80	62
	6 (14)	2.40×10^3	4.20×10^2	0	0
	(24)		8.90×10^2		
7	7 (9)	5.20×10^2	0	0	0
	8 (17)	3.40×10^2	0	0	0
	(23)		0		
14	9 (13)	0	1.60×10^2	0	0
	10 (15)	0	0	0	0
	(21)		0		

Table 4.7 – The isolation of bacteriophage lambda from the sessile phase of four MRD's. One containing filter sterilised tap water (MRD 1) and the other three containing concentrated tap water. Phage was inoculated into the system (at time 0) after an initial 14 day circulation allowing the development of the biofilm. At each sampling point two to three discs were removed from the MRD's. They were taken along it's length moving away from the reservoir except in MRD 5 where they were randomly selected. The plaque assay method was used to enumerate the phage.

NT = not tested; [1] The stud numbers in brackets are those from MRD 5.

The sessile phase results (see Table 4.7) in the presence of the mature biofilm did, however, exhibit similar patterns to each other and to previous results obtained from the experiment concerning the developing biofilm. The enrichment results for the sessile phage (see Table 4.8) showed differing results to the previous experiment. They confirm the above inference that the rate of decay of the phage is slower in the presence of the mature biofilm than in the presence of the developing one. This is because phage was isolated at day 7 on all the test surfaces and on one at day 14. It must be noted, however, that once again the rate of decay of the phage in the control was still slower.

The enrichment results for the planktonic phage (see Table 4.9) were once again similar to the results observed using the plaque assay method. One slight difference noted was the small amount isolated from the day 14 time point. Since no phage was isolated at the previous time point it may be inferred that this low level phage has come from the biofilm. However the amount of phage isolated even after enrichment was still incredibly low indicating that it may be a negligible result.

Table 4.8 – The Isolation of Phage from the Mature biofilm using the Enrichment and Assay Procedure

TIME (DAYS)	STUD NUMBER[1]	MRD 1 (CONTROL)[2]		MRD 5 (TEST)[2]	
		NEAT[3]	DISC[4]	NEAT[3]	DISC[4]
0	1	+	+	NT	NT
	2	+	+		
1	3 (4)	+	+	+	+
	4 (7)	+	+	+	+
5	5 (8)	+	+	+	+
	6 (14)	+	+	+	+
	(24)			+	+
7	7 (9)	+	+	+	-
	8 (17)	+	+	+	-
	(23)			+	+
14	9 (13)	+	+	-	-
	10 (15)	+	+	-	-
	(21)			+	+

Table 4.8– The isolation of bacteriophage lambda from the sessile phase using the enrichment method. The control MRD contained filter sterilised tap water (MRD 1) while the test MRD (MRD 5) contained a mature biofilm. Both MRD's were allowed to circulate for a period of 14 days prior to phage inoculation. At each sampling point 2 to 3 discs were removed from the MRD's. They were taken along it's length moving away from the reservoir except in MRD 5 where they were randomly selected. These MRD's were the same as those in Table 4.6.

NT = not tested

[1] The stud numbers in brackets are those from MRD 5.

[2] + is a positive result while – indicates no plaques observed.

[3] NEAT indicates that the sample tested was following the sampling of the disc. This sample was also tested using the plaque assay procedure and the results are displayed in table 4.4.

[4] DISC indicates that the disc was directly enriched following the sampling procedure.

Table 4.9 – The Isolation of Phage from the Planktonic Phase of a Mature biofilm using the Enrichment and Assay Procedure

TIME (DAYS)	MRD 1 (CONTROL)[1]	MRD 5 (TEST)[1]
0	+	NT
	+	
1	+	+
	+	+
5	+	+
	+	+
7	+	-
	+	-
14	+	+
	-	-

Table 4.9 - The isolation of bacteriophage from the planktonic phase using the enrichment method. The control MRD contained filter sterilised tap water (MRD 1) while the test MRD (MRD 5) contained a mature biofilm. Both MRD's were allowed to circulate for 14 days prior to phage inoculation. At each sampling point 2 1ml samples were removed from the reservoir of the MRD's. These MRD's are the same as those in Tables 4.6 and 4.7.

NT = not tested

[1] + is a positive result while – indicates no plaques observed.

4.4 The Activity of Bacteriophage λ on the Biofilm

4.3.1 Biofilm Development in the Presence of Bacteriophage λ

Figures 4.7 and 4.8 show the levels of viable bacteria isolated from both the planktonic and sessile phases respectively of the MRD, in the presence of phage. For this experiment the phage was inoculated at the same time as the initial inoculum used for biofilm formation. Thus it shows the biofilm development in the presence of the phage.

In the planktonic phase the levels of both heterotrophs and presumptive coliforms were similar at all time points tested. This is also true for the sessile phase and these results are comparable to those obtained previously for the biofilm model (see Chapter 3). This demonstrated that steady state biofilm formation occurred after the first 24 hours and remained throughout the continuation of the experiment. The observed levels of presumptive coliforms, however, were much higher than previously noted in the biofilm model but since they are high in both the test model and in the control it cannot be attributed to the presence of the phage. The reason for the high presumptive coliforms was attributed to the addition of luria broth to the system. Since the phage preparation used was a filtrate in luria broth the same amount of sterile broth was added to the control. Thus the increase in nutrients from this broth produced a slightly different biofilm than previously described (see Chapter 3).

The levels of bacteria in both the test and control models also showed no significant difference further demonstrating that the presence of the phage had little effect on the biofilm itself. Interestingly, however, it was noted that in the majority of cases the levels of bacteria, although very similar, were always higher in the presence of the phage. The reason for this was not thought to be attributed to the phage itself and later results displayed in section 4.3.2 confirm this. It was thought that although the phage preparation was filtered prior to addition to the system and although the negative control contained the same amount of luria broth the phage preparation contained additional nutrients resulting from the lysis of the *E. coli* it was originally grown in. Thus, although not significant, the biofilm model containing the phage had a slightly higher amount of nutrients than the control and thus produced a positive interaction in the biofilm.

Figure 4.7 - The Effect of Bacteriophage Lambda on the Viable Bacteria Present in the Planktonic Phase of the MRD

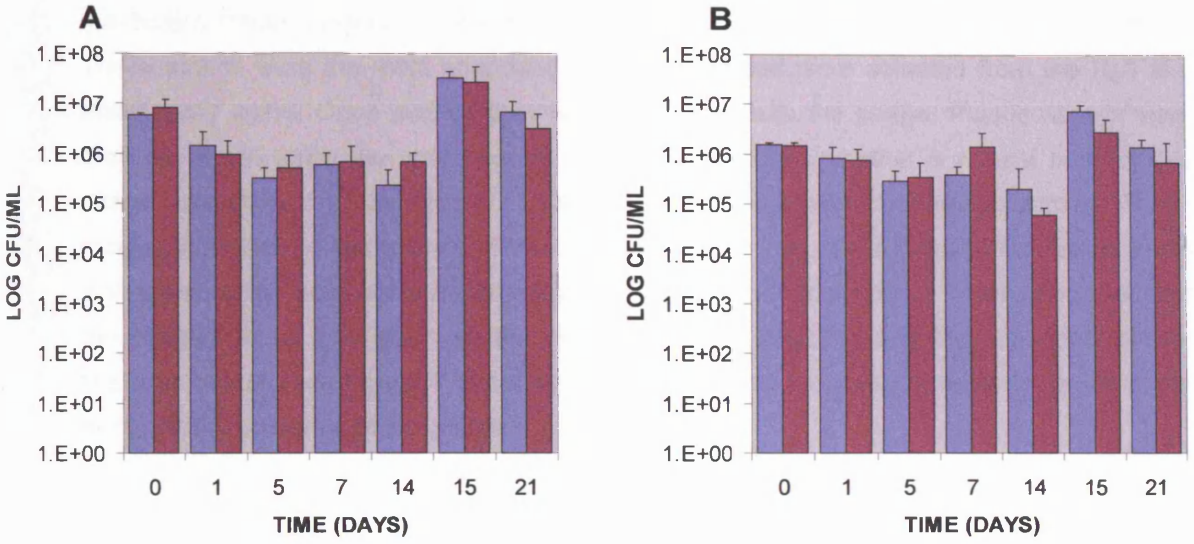
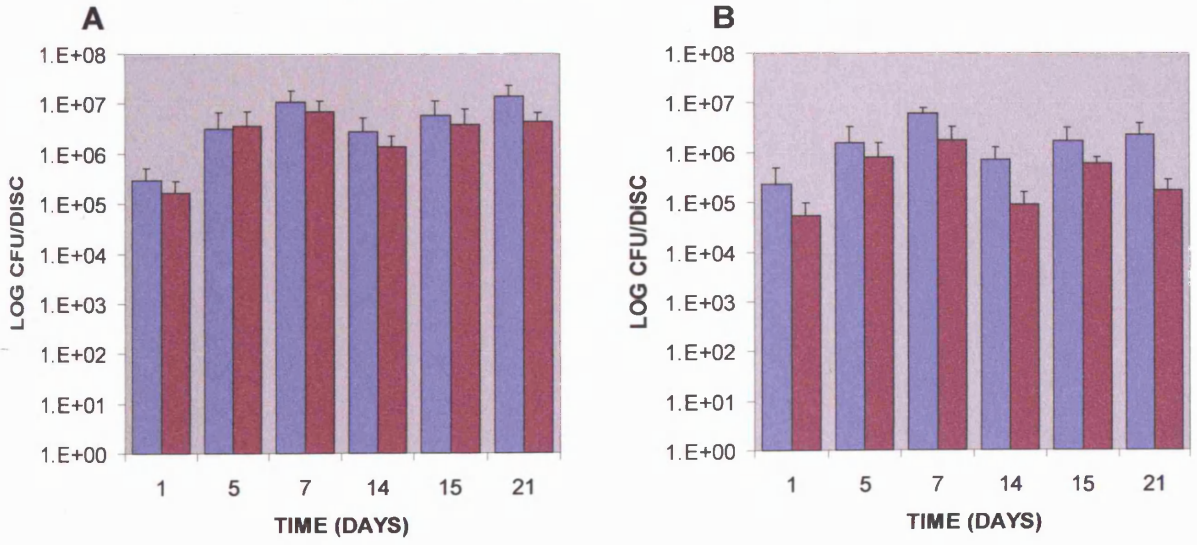


Figure 4.8 - The Effect of Bacteriophage Lambda on the Viable Bacteria Present in the Sessile Phase of the MRD



Figures 4.7 & 4.8 - The levels of **A** viable heterotrophs and **B** presumptive coliforms in the two phases of the biofilm model in the presence (■) and absence (■) of bacteriophage lambda.The heterotrophs and presumptive coliforms were isolated and enumerated using R_2A and MacConkey agar respectively. Data are expressed as the means of duplicate experiments with population standard deviations as error bars.

4.3.2 Bacteriophage Activity on Biofilm Isolates

Approximately 20 bacterial strains were isolated from both the sessile and planktonic phase of the biofilm model system to determine whether the phage had any activity against them. These strains were the most abundant on the plates and were selected from the R_2A and MacConkey agars. Once purified they were incubated with the phage. Plaque assays were then performed using the agar overlay method to ascertain whether a natural host for this phage was present in the system. Table 4.10 shows the results of phage activity. These results show that no lytic activity could be found with any of the isolates tested. Since these isolates were the most abundant organisms in the biofilm model system it was concluded that the phage had no lytic effect on the biofilm or on the organisms in the planktonic phase. Previous results (see Figure 4.8) confirm this showing no reduction in bacterial levels in the biofilm in the presence of the phage.

Table 4.10 – The Effect of Bacteriophage lambda on Bacterial Isolates from the Biofilm Model

STRAIN NUMBER [1]	ISOLATED FROM [2]	PLAQUES PRESENT [3]
R001	PL	-
R002	PL	-
R003	PL	-
R004	PL	-
R005	PL	-
R006	PL	-
R007	S	-
R008	S	-
R009	S	-
R010	S	-
R011	S	-
R012	S	-
R013	S	-
R014	S	-
R015	S	-
M001	S	-
M002	S	-
M003	S	-
M004	S	-

Table 4.10 – Bacteria isolated from the two phases of the MRD and tested for phage activity. The most abundant strains were tested and most were taken from the sessile phase as the most diversity was apparent in this phase. All organisms were distinct from each other and experiments were done in triplicate.

[1] R = strain isolated from R_2A; M = strain isolated from MacConkey agar.

[2] PL = strain isolated from the planktonic phase; S = strain isolated from the sessile phase

[3] - = no plaques observed; + = plaques observed

4.4 Chapter Summary

The results obtained when evaluating the interactions of bacteriophage lambda with the complex water distribution system biofilm model show that in both phases the biofilm had a detrimental effect. Since the plaque assay only allowed the detection and enumeration of viable virus while the enrichment method only detected the presence of viable virus it could not be ascertained whether the detrimental effect produced promoted destruction or the inactivation of the bacteriophage.

In the planktonic phase all the models indicated an acceleration of phage decay which was more dramatic than that observed in the sessile phase. Although in the tube experiment the phage in the planktonic phase remained present for the full 14 days, its levels were much reduced compared to the control. In the MRD the phage did not survive for this long having a survival time of 5 and 7 days for test and control respectively. This difference between models was probably due to the high initial inoculum of phage in the tube experiment and the low water volume in the tube compared with the MRD (10 mls as opposed to 500 mls).

The effect noted in this phase was not thought to be due to the developmental stage of the biofilm since the patterns of decline in the presence of both developing and mature biofilms were similar. Neither did the pattern of phage decline differ greatly when evaluating the planktonic phage in the presence of a different biofilm i.e. one forming on copper. These results suggested that the detrimental actions of the biofilm were probably non-specific.

In the sessile phase the phage did adsorb to the biofilm but always at lower levels than observed in the control. This confirms the previous finding that the biofilm produced a detrimental effect on the bacteriophage. In addition phage could usually be detected in the biofilm at day 1 and occasionally at day 5. This supports the theory that the detrimental effects of the biofilm took time (approximately 24 hours) to act on the bacteriophage. The lower phage levels in the mature biofilm support the idea that the detrimental product accumulated in the sessile phase and this may explain why the filtered planktonic phase had little effect on the phage levels in a previous tube experiment. In the biofilm forming on copper, bacteriophage was only detected using the enrichment procedure indicating that it was present but only at levels lower than the detection limits of the plaque assay. The inhibitory effects produced by copper probably caused this.

Upon evaluating the biofilm itself, similar levels of bacteria were present in both the control (without phage) and test MRD's indicating that the presence of the phage did not effect the biofilm. Screening bacterial isolates confirmed this indicating that there was an absence of the

host bacterium in the model. This also supported a previous inference made which suggested that the action of restriction endonucleases may have been the cause for the phage decline in the presence of the biofilm. However, for this to be true a host for the phage must be present in the system. Some results suggested an increased presence of the presumptive coliforms in the system but this was due to the fact that the phage suspension was not purified but simply a filtrate of luria broth. To confirm it was the effect of the increased nutrients the broth was also added to the negative control. The majority of the presumptive coliforms were non-lactose fermentors and those which were not were screened to determine if phage infection within them was possible.

CHAPTER 5

RESULTS AND DISCUSSION III
THE INTERACTIONS OF POLIOVIRUS WITH THE BIOFILM

5.1 The Evaluation of Poliovirus Isolation & Enumeration Methods

5.1.1 The Sensitivity & Reproducibility of the Methods used

Poliovirus detection was achieved through the use of 3 separate methods namely 50% Infective Dose in Tissue Culture ($TCID_{50}$), Reverse Transciptase – Polymerase Chain Reaction (RT-PCR) and Digoxigenin (DIG) Hybridisation.

5.1.1.1 $TCID_{50}$

$TCID_{50}$ was used both to establish the presence of poliovirus in the sample and for its quantitation. This method was chosen over the plaque assay method because it was inexpensive and easy to perform. Since a large number of samples were required to be screened this was deemed important. Although slightly less accurate than the plaque assay, many workers have successfully used the $TCID_{50}$ method for the enumeration of a variety of enteroviruses (Kok *et al*, 1998; Melnick & Rennick, 1980; Montagnon *et al*, 1998).

For purposes of reproducibility it was essential to use one cell line for all assays performed. Kok *et al* (1998) evaluated 5 cell lines using $TCID_{50}$ and noted that certain cells were more or less sensitive to enteroviral infection. Dahling and Wright (1986) gathered data from 58 laboratories and concluded that the BGM cell line was superior for virus isolation compared to other cell types and many other workers have noted the growth of poliovirus in these cells (Pfirrmann & Bossche, 1994; Chapnon *et al*, 2000; Grabow *et al*, 1999). In addition, this cell line was easy to maintain and cultivate and its rapid growth enabled a large number of assays to be performed at one time. Thus the BGM cell line was chosen for this procedure. Figure 5.1 shows a $TCID_{50}$ assay result for poliovirus.

Prior to the use of this assay its reproducibility and sensitivity was assessed. For reproducibility the $TCID_{50}$ assay was performed on the poliovirus stock solution in triplicate and the mean and population standard deviation was determined. Calculations are shown in Table 5.1 below. The results show that some variation was apparent with this method but the population standard deviation indicates that this variation is relatively low. In order to monitor this slight variation, replicate samples were always taken and each of these was tested in triplicate. This thus allowed for any large variations to be detected during experimentation.

The determination of the sensitivity of the $TCID_{50}$ assay was difficult, as this was the only method of quantitation of viable virus used. In this assay, the lowest detectable number of virus particles would produce cytopathic effect (CPE) in 1 out of the possible 5 wells in a neat sample and this equates to a $TCID_{50}$ of log 0.5 which is equivalent to 3.16 infectious units

Figure 5.1 - A $TCID_{50}$ Plate

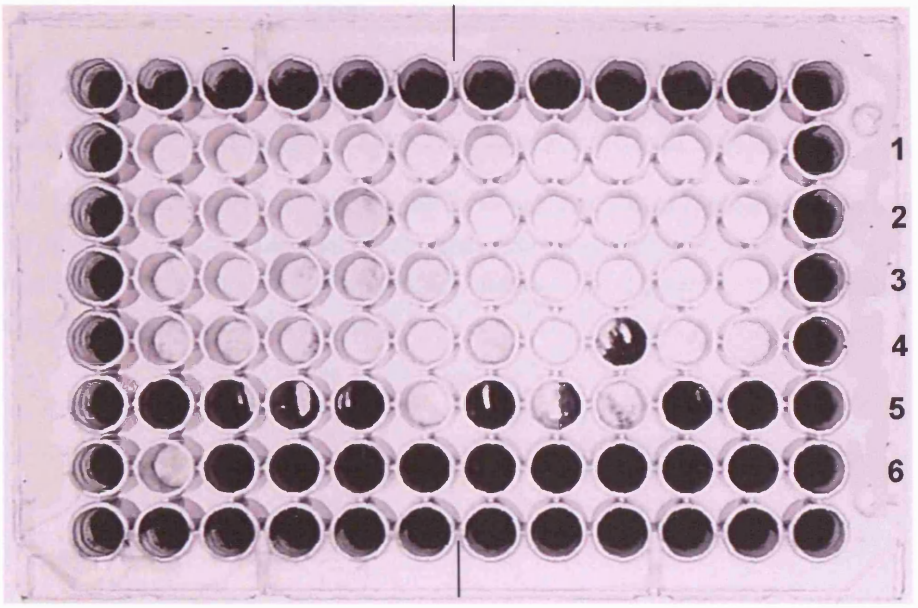


Figure 5.1 - An example of the results obtained for poliovirus stock solution using the $TCID_{50}$ method in duplicate. The clear wells indicate CPE while the stained wells indicate no CPE. The outside wells were not used as they dry out quickly. The plate was usually split in to two portions as shown by the lines above and five wells were used for each dilution; 1 = 10^{-3}, 2 = 10^{-4}, 3 = 10^{-5}, 4 = 10^{-6}, 5 = 10^{-7} and 6 = 10^{-8}. The calculation used for $TCID_{50}$ is shown in Appendix 2.

Table 5.1 – The Reproducibility of the $TCID_{50}$ Assay

SAMPLE	$TCID_{50}$ $100\mu ls^{-1}$		MEAN ± STANDARD DEVIATION
PV 1	$10^{8.50}$	3.16×10^8	
PV 2	$10^{8.17}$	1.48×10^8	$3.8 \times 10^8 \pm$
PV 3	$10^{8.83}$	6.76×10^8	0.22×10^8

Table 5.1 – The $TCID_{50}$ assay was performed on three separate samples taken from the stock poliovirus solution to determine the reproducibility of the assay. The stock poliovirus was grown up in HT-29 cells and concentrated according to the procedures outlined in chapter 2. $TCID_{50}$ calculations were determined using the formulae in appendix 2.

($TCID_{50}$ units) in the inoculated sample. Since 100 µls of sample dilution was inoculated onto the cells the lowest viral levels to be detected with this procedure were calculated to be 31.6 $TCID_{50}$ units ml^{-1} assuming that the sample contained fully distributed and dispersed virus particles.

Problems of viral aggregation were also considered. Following the concentration procedures outlined in chapter 2 (Materials and Methods; see Section 2.5.2), the poliovirus was always resuspended in phosphate buffered saline (PBS). Previous workers have shown that when poliovirus is suspended in PBS it is usually in its dispersed state (Floyd & Sharp, 1977) and thus it was assumed that the stock solution was fully dispersed. The biofilm samples, however, were resuspended in tissue culture solution (MEM) supplemented with 10% foetal bovine serum (FBS) to maximise the efficiency of the $TCID_{50}$ assay. Since this solution is buffered in a similar manner to the PBS any virus present was also assumed to be in its dispersed form. It must be noted that in some cases the presence of 10% FBS has detrimental or inhibitory effects upon virus infection in cell culture. This is because, in many cases, trypsin is used to aid the viral infection of cells. For example a 10 $\mu g ml^{-1}$ trypsin solution is commonly used to promote the infection of CaCO2 cells with astrovirus particles (Pinto *et al*, 1996). The presence of high levels of protein, such as FBS, would thus block the effects of the trypsin and subsequently inhibit infection. However, trypsin is not required for the promotion of poliovirus infection in either of the cell lines (HT-29 & BGM) used and so its presence was not expected to inhibit or lower the rate of infection. This was checked by simply inoculating poliovirus onto a monolayer of cells in the presence and in the absence of FBS. The results show that, in both HT-29 and BGM cell lines, similar virus levels were produced at similar rates whether FBS was present or not.

The FBS was left in the tissue culture medium during $TCID_{50}$ for the following reason: Previous workers had found that the sensitivity of this method was improved if the virus samples were inoculated into microwells of suspended cells rather than onto the surface of preformed monolayers. They deduced that when in suspension the virus is exposed to a greater cell surface area than when it has formed a monolayer. Uninfected cells will then form a monolayer while those that are infected will not. Therefore the same result is obtained but, for this to work, the cells required FBS. Initially the full 10% FBS levels were used in the assay, however subsequent RT-PCR experiments (detailed below) indicated some problems with its use and so the FBS levels, once again, had to be re-evaluated.

Problems arose when it was discovered that FBS levels were required for the $TCID_{50}$ assay but were inhibitory to the RT-PCR method. Since 200 µls of cell suspension was inoculated into each well in the microtitre plates and 100 µls of biofilm sample was then inoculated into this, the maximum amount of FBS could be 6.67%. This level of FBS was then used for the subsequent cultivation of BGM cells and it was found to be adequate for their growth and monolayer formation. Thus all biofilm samples taken could be placed in serum-free tissue culture medium without hindering the $TCID_{50}$ method.

Although the $TCID_{50}$ assay was used as the principle method of choice for poliovirus detection and quantification it did have some limitations concerning its low sensitivity and the fact that it relied on the viruses ability to infect the cells. Thus an additional two methods which were considered to be more sensitive and did not rely on virus viability were also used in conjunction.

5.1.1.2 RT – PCR

A technique commonly employed for the detection of enteroviruses in environmental samples (Schwabbb *et al*, 1995; Kopecka *et al*, 1993; Egger *et al*, 1995; Tsai *et al*, 1993; Guyader *et al*, 1994; Abbaszadegan *et al*, 1993), RT-PCR was chosen to supplement the information obtained from the $TCID_{50}$ results. This technique is used to detect viral nucleic acids in a sample by enzymatic amplification. Since poliovirus is a positive sense single stranded RNA virus (Cann, 1997) an RNA extraction procedure and a reverse transcription step had to be incorporated prior to DNA amplification. In most cases the RNA is extracted from the virus particle by chemical means, transferred to another tube containing the reverse transcriptase reaction mix to convert it to cDNA before being amplified by PCR using the *Taq* polymerase enzyme. However this method is labour intensive and each time the sample is transferred between tubes there is a possibility of loss thus reducing the sensitivity of the procedure. This was overcome by the use of a single tube technique which utilised a simple heat extraction method to isolate the RNA an enzyme (rTth, Perkin Elmer) which can perform both RT and PCR steps depending on the temperature of the reaction mix. Therefore three steps could be performed in a single tube which significantly reduced the possibility of loss retaining a high level of sensitivity (Cook & Kurdziel, 1998). One tube transfer was, however, performed because the PCR required 2 rounds of amplification. The use of nested PCR increased the sensitivity of the method further and this finding was supported by other workers who also concluded that two rounds of amplification detected more enteroviruses in stool samples than one (Abebe *et al*, 1992).

Prior to its use the poliovirus stock solution was amplified by PCR to check the stock and the method and the resultant 129 bp product is shown in Figure 5.2A. This experiment was performed in triplicate and shown to be highly reproducible.

To determine the sensitivity of the PCR serial dilutions of the poliovirus stock were amplified and the results in Figure 5.2B show that a dilution as low as 10^{-10} still produced a PCR product following nested PCR, while a 10^{-12} dilution did not. When comparing this with the $TCID_{50}$ results of the same poliovirus stock it was calculated that a 10^{-10} dilution corresponded to 0.38 $TCID_{50}$units ml^{-1} and approximately 0.01 $TCID_{50}$units $25\mu ls^{-1}$. At the 10^{-12} dilution the levels were calculated to contain 0.0001 $TCID_{50}$units $25\mu ls^{-1}$. This amount was considered negligible and since the 10^{-10} dilution exhibited a high sensitivity to the poliovirus, further dilutions were not considered appropriate. It should be noted at this stage that all dilutions tested produced

Figure 5.2 - The PCR Products After Amplification of Poliovirus Controls under Different Conditions

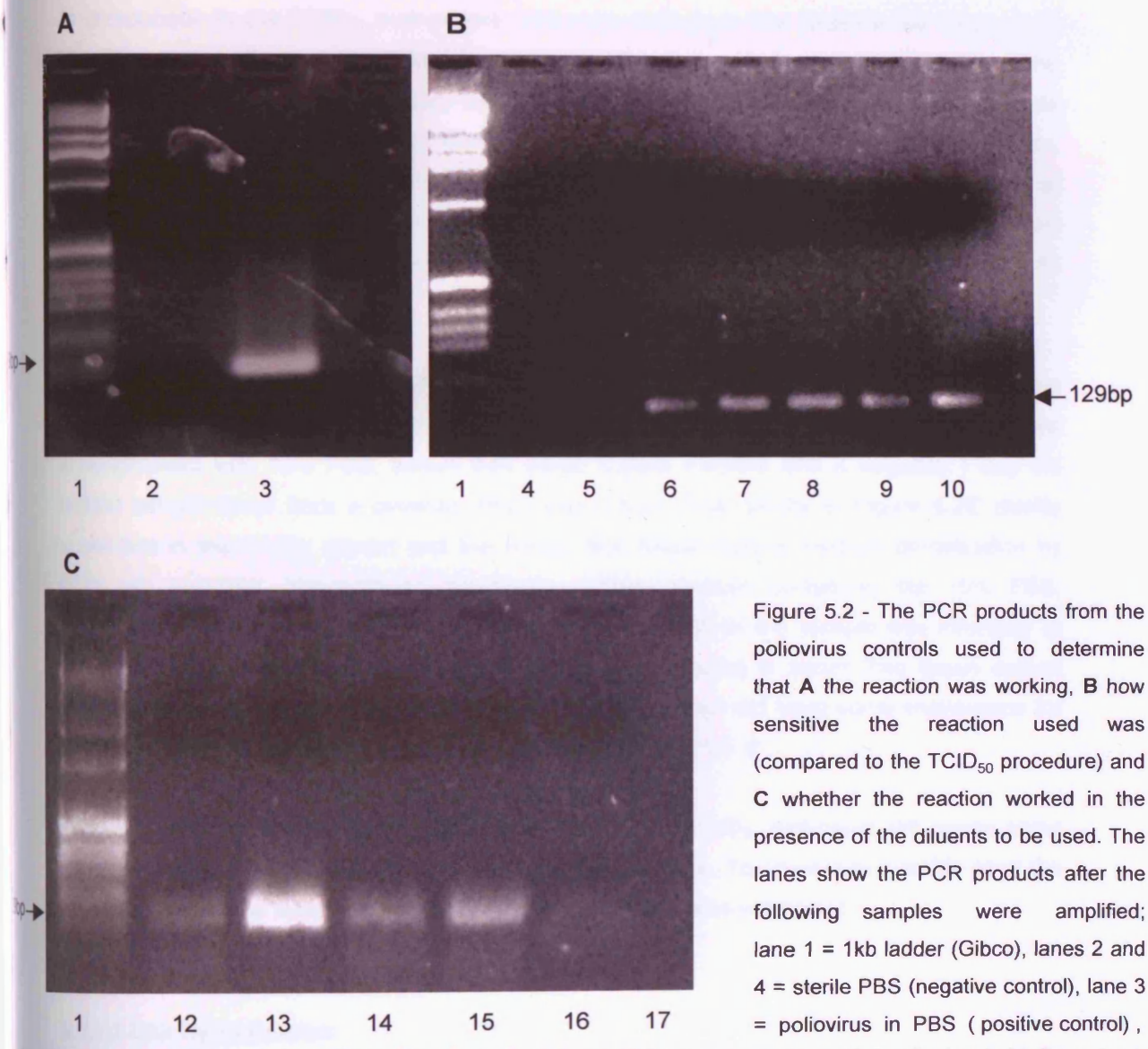


Figure 5.2 - The PCR products from the poliovirus controls used to determine that **A** the reaction was working, **B** how sensitive the reaction used was (compared to the $TCID_{50}$ procedure) and **C** whether the reaction worked in the presence of the diluents to be used. The lanes show the PCR products after the following samples were amplified; lane 1 = 1kb ladder (Gibco), lanes 2 and 4 = sterile PBS (negative control), lane 3 = poliovirus in PBS (positive control) , lanes 5 - 10 = poliovirus dilutions 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} and 10^{-2} respectively, lane 12 = poliovirus in biofilm extract and serum free tissue culture medium, lane 13 = poliovirus in PBS (positive control), lane 14 = poliovirus in biofilm extract, lane 15 = poliovirus in serum free tissue culture medium, lane 16 = poliovirus in tissue culture medium supplemented with 10% FBS and lane 17 = sterile tissue culture medium (negative control). In **A** the positive control was 10^{-2} dilution of poliovirus stock while in **C** all controls were 10^{-8} dilutions.

bands on the agarose gel of similar intensity (Figure 5.2B). The reason for this is unclear but is probably to be due to the large number of cycles during the nested amplification process. This result indicates that the quantitation of PCR products could not be done using this technique.

In comparison to the $TCID_{50}$ method this molecular technique was found to be much more sensitive and was thus used for this reason. In addition, it does not rely on the viability of the virus and will produce supplementary data as to the presence of the virus whether it is viable or not. However, since this method relies on the detection of viral nucleic acids in the sample, it cannot distinguish between whole virus or its nucleic acid. Since the nucleic acid of poliovirus is single stranded RNA which is a very unstable molecule in the environment (Jones *et al*, 1994) we can assume that a positive result is from whole virus as opposed to its nucleic acid.

Following the evaluation of sensitivity, further controls were amplified by PCR to check that inhibitory agents were not present. For this, poliovirus was seeded into tissue culture medium supplemented with 10% FBS, serum free tissue culture medium and a negative 1 day old biofilm extract taken from a previous MRD experiment. The results in Figure 5.2C clearly show that in the biofilm extract and the serum free tissue culture medium amplification by PCR still occurred. However, in the tissue culture medium containing the 10% FBS, amplification was poor suggesting that the presence of FBS in the sample was inhibitory to the PCR reaction. The method of placing the biofilm samples in serum free tissue culture medium was thus adopted. This slight change in methodology did have some implications for the $TCID_{50}$ method but these were corrected (see Section 5.1.1.1).

The much increased sensitivity of the RT-PCR over the $TCID_{50}$ technique did cause some problems when trying to quantify viable and inactivated virus. To attempt to quantify all of the virus present, a new molecular method, DIG Hybridisation, was employed.

5.1.1.3 DIG Hybridisation

The method of DIG Hybridisation was proposed to provide supplementary data to the two previous methods outlined above. Although not considered to be as sensitive as the PCR, the quantification of all viral nucleic acid in the sample was considered important for comparison with the $TCID_{50}$. The theory was that the amount of viral nucleic acid could be compared to the amount of infective virus and the difference would account for the inactivated virus in the sample. For this, the PCR product obtained during the previous method was labelled with DIG and used as the probe (see Figure 5.3A). This was then quantified (see Figure 5.3B) before being hybridised with the sample which was blotted onto a nylon membrane. The amount of

Figure 5.3 - The Results Obtained From the DIG Hybridisation Experiments

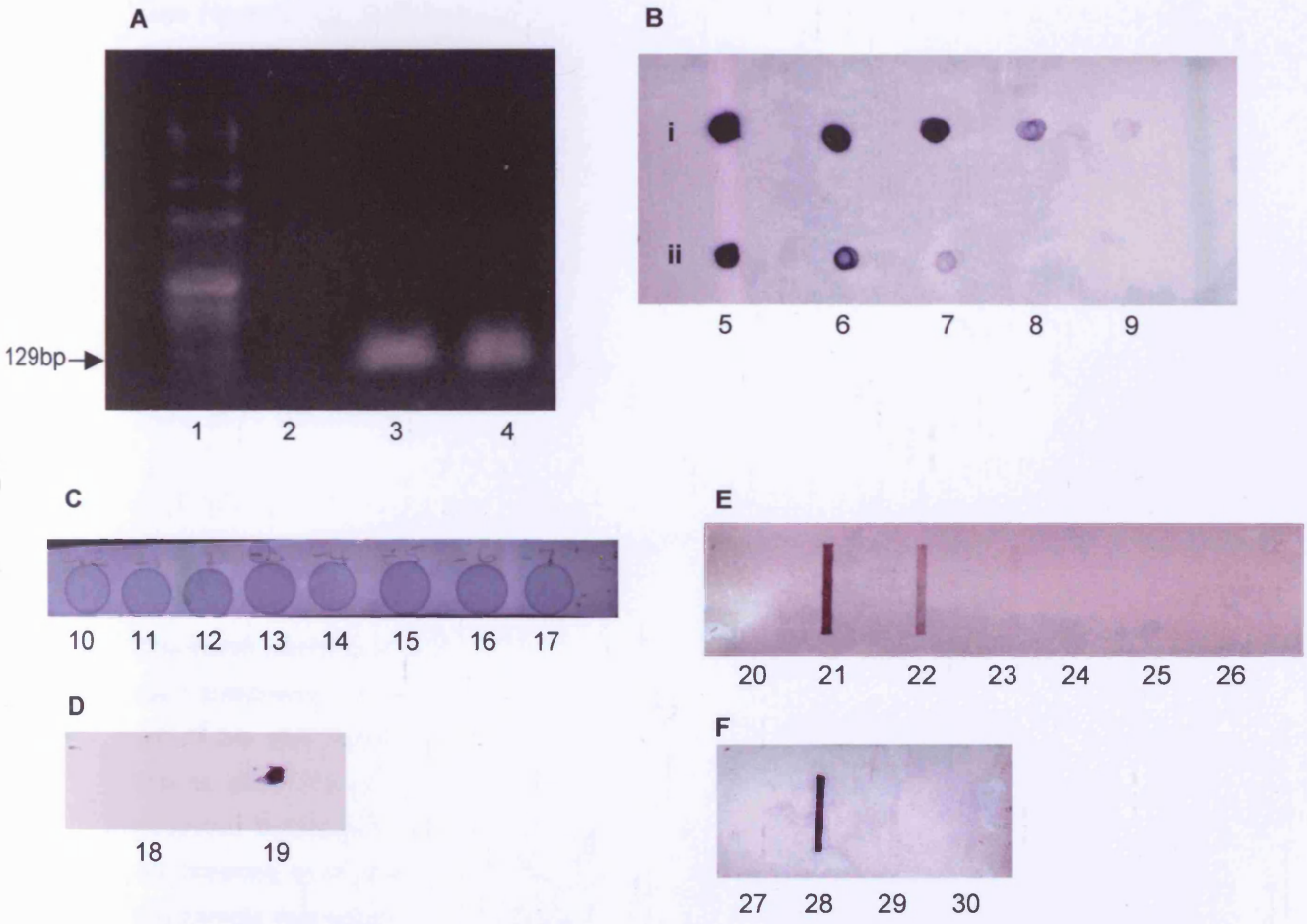


Figure 5.3 - The results obtained following preliminary experiments using DIG hybridisation: **A** the DIG probe after amplification and DIG labelling by PCR (lane 1 = molecular weight marker, lane 2 = -ve control, lane 3 = +ve control and lane 4 = DIG probe), **B** Quantification of the DIG probe (i = control DNA, ii = DIG probe; lanes 5, 6, 7, 8 & 9 = approximate concentrations of DNA = 100 $pg\mu l^{-1}$, 10 $pg\mu l^{-1}$, 1 $pg\mu l^{-1}$, 0.1 $pg\mu l^{-1}$, 0.01 $pg\mu l^{-1}$ respectively), **C** hybridisation of the probe to dilutions of poliovirus after baking onto the membrane at 120°C for 30 minutes (lane 10 = -ve and lanes 11 - 17 = poliovirus dilutions 10^{-1} - 10^{-7} respectively), **D** hybridisation of the probe to poliovirus after RNA extraction and 1 minute UV cross linking (lane18 = -ve, lane 19 = +ve), **E** hybridisation of the probe to slot blotted RNA dilutions (lane 20 = -ve, lane 21 - 26 = 10^{-1} - 10^{-6} dilutions) and **F** hybridisation of the probe to planktonic samples after RNA extraction and slot blotting (lane 1 = -ve, lane 2 = +ve, lanes 3 & 4 = planktonic samples from MRD 2 at time 0).

probe hybridised could then be detected by a colourimetric assay and the intensity of the colour produced would then allow for quantitation. Initial problems encountered were during the RNA extraction and cross linking procedure on the membrane. Baking the sample at 120°C for 30 minutes was thought to cause the virus particles to release their RNA and allow cross linking of such RNA to the membrane. However, because the sample was in tissue culture medium, this also baked onto the membrane and inhibited the hybridisation reaction (see Figure 5.3C). This was rectified by chemically extracting the RNA and cross linking it to the membrane using UV light. This method worked well as shown in Figure 5.3D but unfortunately the sensitivity of this procedure proved to be much lower than originally thought. Only the 10^{-2} dilution of the stock poliovirus solution (equivalent to approx. 9.5 x 10^5 $TCID_{50}$units per 25 µl sample) produced a colour reaction (see Figure 5.3E). This experiment was subsequently tried on four planktonic samples which had previously been found to be positive both with $TCID_{50}$ and RT-PCR. The results were negative using this method (see Figure 5.3F). Owing to constraints of time this method could not be efficiently optimised for use during this investigation and so it was abandoned. If time had allowed a similar method using more sensitive radioactive labels, such as ^{32}P, would have been considered.

5.1.2 The Effect of Biofilm Removal Methods on Viable Poliovirus

The harsh methods used to remove the biofilm were tested on the poliovirus to ensure that such treatments had no detrimental effects upon it. Figure 5.4 shows the results obtained which are very similar to those obtained for the bacteriophage lambda. Once again, the results show that both removal treatments did not have any detrimental effect on the poliovirus but rather served to increase their levels. This observed increase was attributed to the breaking up of viral aggregates in the two test samples and was more pronounced when the sample was subjected to the 1 minute sonication treatment. In contrast, the control tubes showed a slight decline in virus levels after 1 minute. This suggests that the virus had either continued to aggregate or that it had become adsorbed to the glass walls of the test tube. These results reinforce those previously observed with the bacteriophage lambda and suggest that aggregation and adsorption may be a common phenomenon among a variety of viral species.

5.1.3 Poliovirus in Tap Water

Figure 5.5 shows the survival of poliovirus in tap water following different initial inocula. In the first experiment 1 x 10^5 $iuml^{-1}$ poliovirus was inoculated while in the second a higher level of 1 x 10^7 $iuml^{-1}$ was used. Interestingly, the results appear to show that the lower viral inoculum allowed a greater survival in the water than the higher one. However, when evaluating these results additional factors must be taken into consideration. One such factor is the dilution rate of the stock virus into the water. In this experiment the dilution rate differed by a factor of 10.

In the first experiment the lower inoculum was achieved by diluting the stock virus into the water 100 fold while in the second a 10 fold dilution was used. This in turn would have changed the aggregating properties of the virus itself in the two resultant solutions. Floyd and Sharp (1977) examined the aggregation properties of poliovirus in water and found that when diluted 10 fold in water a previously dispersed preparation began to form aggregates but when the same preparation was diluted further (e.g. 1:100 or 1:1000) the virus remained dispersed. Thus the 1:100 virus dilution would have been dispersed in the water, while the 1:10 virus dilution would promote the aggregated form. In a more aggregated virus suspension it is much more likely that sampling would miss the virus. Since only one time point followed the day 7 point this may have been why virus was isolated from the water containing the more dispersed form than from the experiment containing the aggregated form. This theory is further supported by the result observed after 1 hour in the experiment with the lower viral load. It shows a slight increase in viral levels, indicating that any viral aggregates that may have initially been present have been fully dispersed.

After the 1-day time point, the virus levels decreased by approximately 1 log in both experiments showing that initially little difference between the two experiments was apparent and that the virus behaves in a similar fashion. However, following this time point, the second experiment containing the higher viral load shows a marked decline in virus which continues exponentially throughout the rest of the experiment. This is in contrast to the first experiment which displays a much slower decline in virus levels over the time period investigated. Particle concentration and collision frequency are proposed to be the prime factors causing this observation. A higher particle concentration has been previously shown to produce a greater collision frequency in a suspension (Floyd & Sharp 1977). The collision rate is therefore proportional to the frequency of aggregation. Thus in a suspension containing higher levels of poliovirus greater collisions between virions will occur prompting an increase in viral aggregation. Since the $TCID_{50}$ method does not distinguish between dispersed and aggregated virus the more aggregated a suspension becomes the greater its decline in solution will appear. Therefore, these two experiments appear to demonstrate the aggregating properties of poliovirus when diluted in water. The lack of poliovirus observed at day 14 in the second experiment was probably due to the sensitivity of the $TCID_{50}$ assay i.e. the virus was so aggregated as to have been missed.

Figure 5.4 - The Effect of Biofilm Removal Methods on Poliovirus

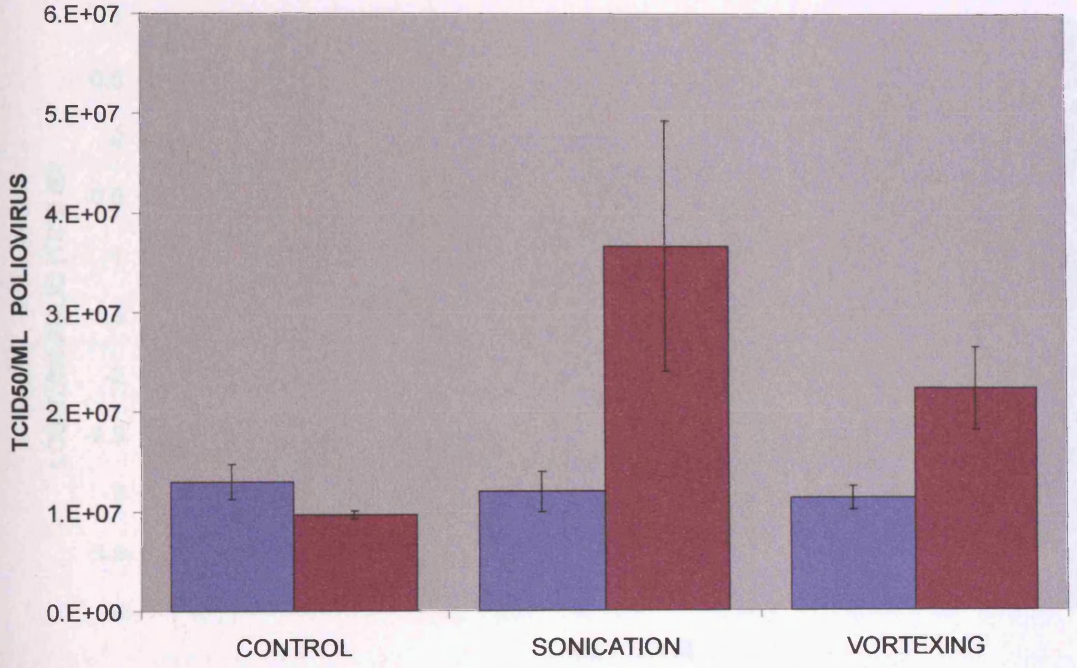


Figure 5.4 - The levels of poliovirus in tubes before (■) and after (■) 1 minute treatments of sonication and vortexing. Data are expressed as the means of triplicate experiments with population standard deviations as error bars.

Figure 5.5 - The Decay of Poliovirus in Sterile Tap Water

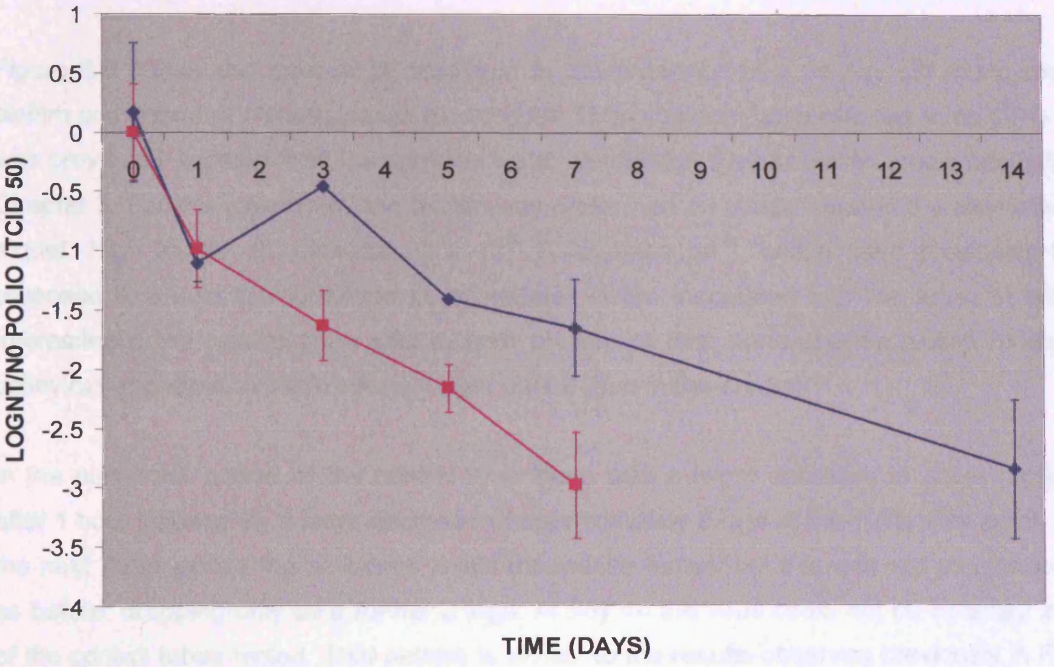


Figure 5.5 - The levels of poliovirus in filter sterilised tap water following different initial inocula. The first experiment (—◆—) was inoculated with 1 x 10^5 iuml^{-1} while the second (—■—) was inoculated with 1 x 10^7 iuml^{-1}. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

5.2 The Interactions of Poliovirus with a Mono-species Biofilm Evaluated using $TCID_{50}$

5.2.1 The Levels of Poliovirus in Preliminary Tube Experiments

Figure 5.6 shows the survival of poliovirus in the presence of a 14 day old mono-species biofilm composed of *Pseudomonas mendocina*. This organism, also referred to as ORG 001, was previously isolated from the complex water distribution system biofilm model evaluated in Chapter 3. For this experiment the biofilm was preformed on plastic discs in the alternative jar model. High levels of poliovirus (1×10^7 $TCID_{50}$units ml^{-1}), which have previously been proposed to cause the formation of aggregates, were inoculated into the tubes at time 0. Interestingly, the results show that in both phases of this mono-species biofilm model the poliovirus appeared to survive for a longer period than in the control.

In the planktonic phase of the control tube there was a slight decrease in poliovirus levels after 1 hour followed by a large decrease of approximately 2 logs at the 1 day time point. Over the next 7 day period the poliovirus levels decreased further but this was not as pronounced as before, dropping only by a further 2 logs. At day 14 the virus could not be detected in any of the control tubes tested. This pattern is similar to the results observed previously in Figure 5.5 for higher levels of poliovirus in water and thus reconfirms the reproducibility of this model. As stated previously (see Section 5.1.3), the observed pattern of virus decay in the planktonic phase of the control tube may be attributed to the aggregation properties of the virus. Since dispersed virus particles appear to survive for longer periods (as shown in Figure 5.5) and since aggregated viruses are afforded greater protection to environmental factors (Floyd & Sharp, 1978; Sharp *et al*, 1980; Sharp & Leong, 1980) it is likely that, although not detected at day 14, the virus is still present in the control tube.

In contrast, the poliovirus levels in the planktonic phase of the mono-species biofilm model appear to remain at higher levels throughout the experiment. After 1 hour, a slight increase in viral numbers suggests the dispersal of aggregated virus has occurred. Since this did not occur in the control it must be assumed that the mono-species biofilm caused this effect. This finding has been shown to occur in water when the initial viral load was lower and the dilution factor was higher (see Figure 5.5). Floyd & Sharp (1977) deduced that a higher dilution factor promoted a decrease in the ionic strength of the water and that this allowed for virus dispersal in an environment where virus aggregation was usually observed. Thus, in this case, it may be that the mono-species biofilm changes the composition of the water enabling virus dispersal to occur. In addition to this, the virus level continued to decrease at a similar rate as that observed for dispersed virus in water and survived for the same period. This phenomenon was attributed to the low viral numbers initially inoculated into the water in Figure 5.5 and hence to the lower collision frequency. In this experiment this is not the case.

Figure 5.6 - The Effect of a Mono-species Biofilm on the Levels of Poliovirus in the Preliminary Tube Experiments

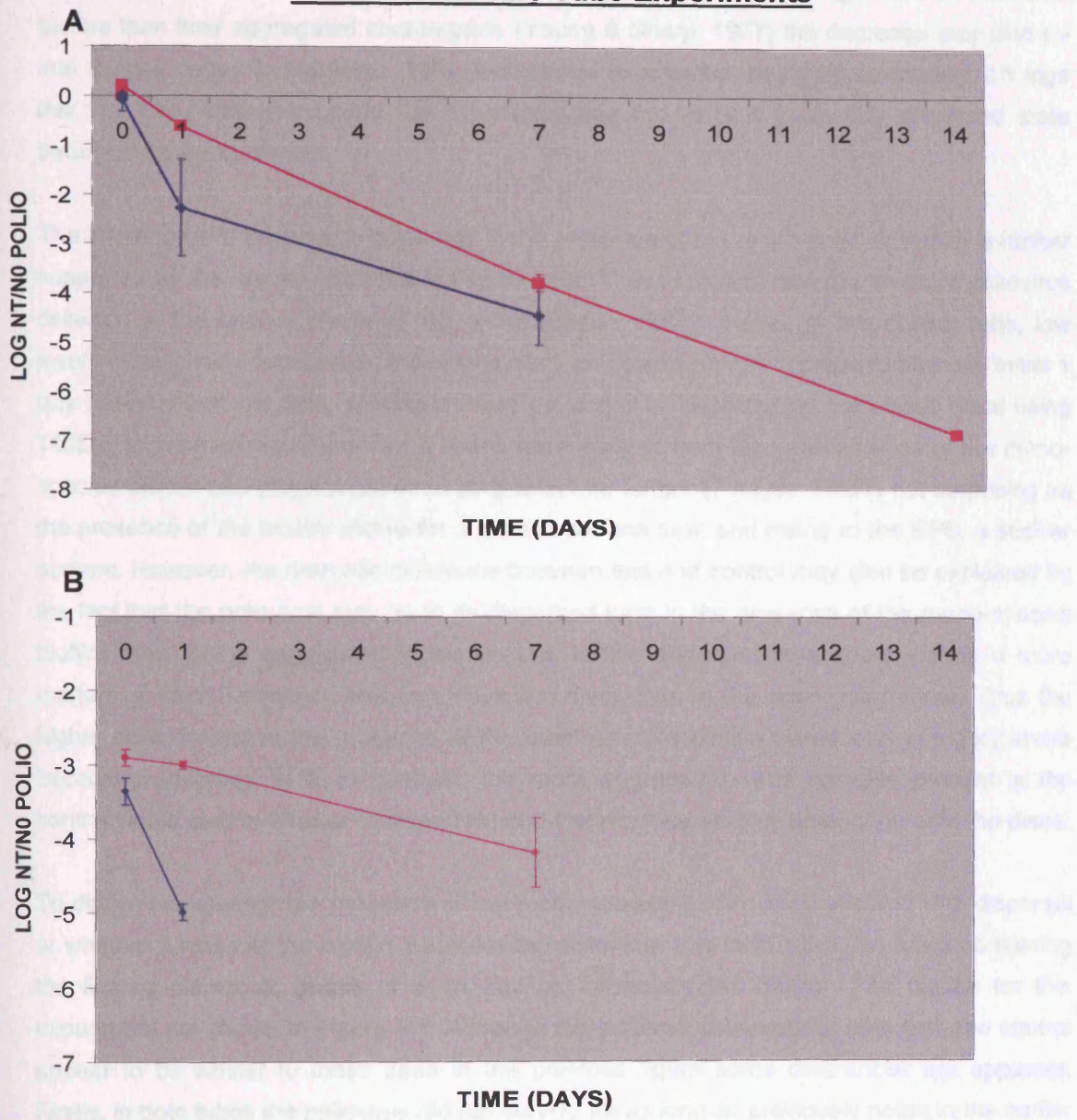


Figure 5.6 - The isolation of poliovirus from **A** the planktonic phase and **B** the sessile phase of tube experiments with time. Tube 1 (—◆—) contained sterile plastic discs and tube 2 (—■—) contained preformed mono-species biofilm on plastic discs. The mono-species biofilm was preformed in a jar model (see chapter 3) over a period of 14 days. It consisted of the ORG 001 *(Pseudomonas mendocina)* previously isolated from the biofilm model evaluated in chapter 3. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

The high virus levels added, at the high dilution factor (1:100), would usually promote virus aggregation. Therefore the mono-species biofilm must in some way promote the dispersal of the virus either by maintaining a lower ionic strength or by producing substances which have a direct action. Since dispersed virions are afforded less protection against environmental factors than their aggregated counterparts (Young & Sharp, 1977) the decrease may also be due to their decay in the water. This decrease is exponential being approximately 0.5 logs day^{-1} and this further supports the hypothesis that the virus is in its fully dispersed state throughout the experiment.

The inference that the virus is dispersed in the presence of the mono-species biofilm is further supported by the results observed in Figure 5.6B. These results show the levels of poliovirus detected in the sessile phase of the mono-species biofilm model. In the control tube, low levels were initially detected on the plastic discs and these rapidly decreased after the initial 1 day period. After this time, poliovirus could no longer be detected on the plastic discs using $TCID_{50}$. In contrast higher poliovirus levels were isolated from the sessile phase of the mono-species biofilm and they continued to be present for longer (7 days). This is not surprising as the presence of the biofilm allows for a greater surface area and owing to the EPS, a stickier surface. However, the dramatic difference between test and control may also be explained by the fact that the poliovirus may be in its dispersed form in the presence of the mono-species biofilm while being aggregated in the control. In the dispersed state there will be a more marked collision frequency between virus and discs than in the aggregated state. Thus the higher collision rate in the presence of the mono-species biofilm would lead to higher levels becoming adsorbed to it. In contrast, the more aggregated virus particles present in the control would lead to a lower collision rate and therefore lower virus absorption onto the discs.

To determine whether the presence of the mono-species biofilm itself affected viral dispersal or whether it was just the biofilm leachate, the poliovirus was inoculated into tubes containing the filtered planktonic phase of a 14 day old mono-species biofilm. The results for this experiment are shown in Figure 5.7. Although the patterns observed for both test and control appear to be similar to those seen in the previous figure some differences are apparent. Firstly, in both tubes the poliovirus did not survive for as long as previously noted in the earlier experiment (i.e. it only survived for 7 and 5 days in the test and control respectively). The reasons for this are unclear and may be due to biological variation. The second difference occurred after 1 day, once again, a decrease in virus levels was observed in the control indicating probable viral aggregation while an increase occurred in the test prompting the assumption of dispersal. In the control, the pattern was the same as before (see Figure 5.6) while in the test the increase in viral load was much greater and appears to take a slightly longer time i.e. 1 day as aposed to 1 hour (as seen in the previous Figure – Figure 5.6). Another difference noted was that the further viral decline, observed in the period following the initial increase, was not as pronounced as it was when the actual mono-species biofilm was present being only 0.28 logs day^{-1}. These differences infer that the living cells within the

Figure 5.7 - The Effect o f Mono-species Biofilm By Products on the Levels of Poliovirus in the Planktonic Phase

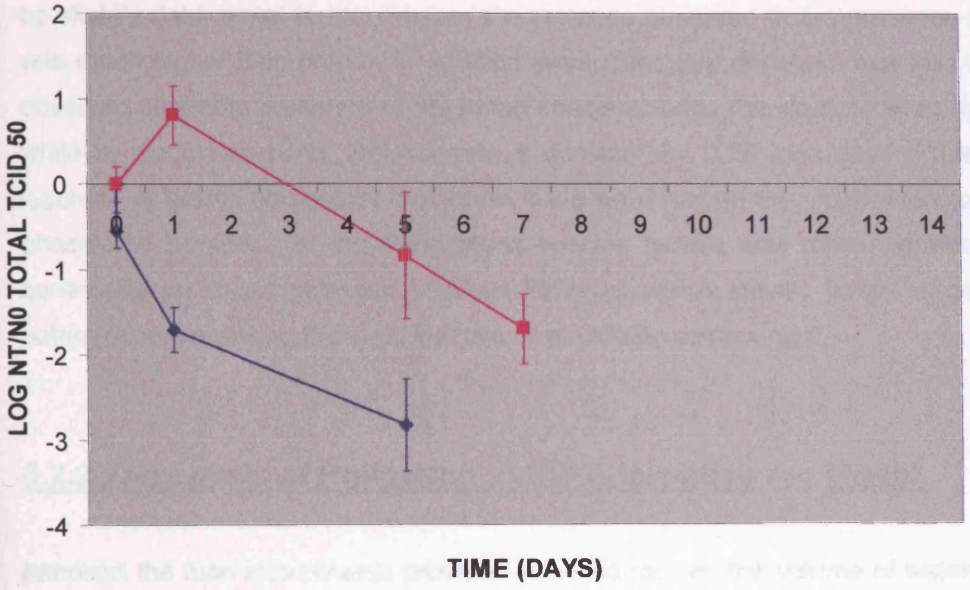


Figure 5.7 - The levels of poliovirus in the presence of waste products from a mono-species biofilm. Tube 1 (—◆—) contained filter sterilised tap water and tube 2 (—■—) contained filtered water taken from the jar model containing a mono-species biofilm of *Pseudomonas mendocina* strain. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

biofilm must, not only promote virus dispersal but they must actively destroy or inactivate it too. In addition, it appears to take approximately 24 hours for the leachate of the mono-species biofilm to cause virus dispersal while it took only 1 hour when the live cells were present. Thus the presence of the living biofilm may have a direct affect upon the virus probably by it's continued production of by-products. The living cells in the biofilm must also be slightly detrimental to the virus as the increase observed in the presence of the leachate was much higher than before. In addition the subsequent decrease was less than previously observed i.e. in the presence of the mono-species biofilm the virus declined by 0.5 logs day^{-1} while in the presence of the leachate it declined by 0.28 logs day^{-1}. Thus, although the leachate of the mono-species biofilm did have an effect on the virus levels in the planktonic phase, the presence of the living mono-species biofilm was more significant. It probably continually produced products of short life-span which initially broke up viral aggregates before either rendering the virus inactive or physically destroying it.

5.2.2 The Levels of Poliovirus in the Alternative Jar Model

Although the tube experiments provided valuable results, the volume of water used was very low being 10 mls. This small volume enabled high virus levels and low dilution factors to be evaluated. However, since larger amounts of water were to be used in the MRD and since water distribution systems have large quantities of water passing through them, it was deemed necessary to evaluate the virus levels in a model containing a much larger volume of water. The alternative jar model was considered to be similar to the tube experiment in that it was a contained system, but a scale up version containing the amount of water to be used in the MRD (500 mls). Owing to its larger size some slight differences must be noted. Firstly, movement of the water was achieved through the use of a magnetic stirrer as aposed to physically moving the model and the test surfaces on which the mono-species biofilm formed were held static on a cotton line. Secondly, the jar model was made of glass whereas the tube model was made of polypropylene. This major difference did cause some later problems due to viral adsorption to the glass surface. This problem was not encountered with the tube model as previous workers had noted that viruses do not adsorb to polypropylene (Ward & Winston, 1985).

The initial inoculum used for this experiment consisted of 1ml of the poliovirus stock solution. This was calculated to produce an initial level of 3.8×10^5 $TCID_{50}$units ml^{-1} and was confirmed to be 3.5×10^5 $TCID_{50}$units ml^{-1} at time 0. Since the dilution factor was 1:500, the virus was assumed to be in the dispersed state according to the findings of Floyd & Sharp (1977). The large volume of water into which the virus was added further contributed to virus dispersal as the lower viral load would cause a decline in the virus collision frequency (Floyd & Sharp, 1978; Totsuka *et al*, 1978). All this taken into consideration, the virus levels in the control jar should, theoretically, exhibit a similar pattern to previous results observed for dispersed virions in tap water (see Figure 5.5). However this was not the case.

Figure 5.8 shows the levels of poliovirus detected in the planktonic phase of the alternative jar model in the presence of a mono–species biofilm. After 1 hour post virus inoculation, the levels of poliovirus decreased by approximately 0.5 logs. This is in contrast to the dispersed tube experiment with a similar inoculum (see Figure 5.5) which displayed a slight increase in viral numbers. Since a similar decline occurred in both jars, at this time, this effect was assumed to be due to the model and was proposed to be caused by the viral adsorption to the glass surface of the jar. This theory was further supported by the findings of Ward & Winston (1985) who revealed that high percentages of virus particles, sometimes >99%, were lost through their adherence to containers. They noted that Pyrex glass caused the most rapid adherence and that the movement of the water served to increase this phenomenon. Thus the further 2 log decrease apparent at day 1 in the control jar was probably also due to virus adsorption. In the test jar, however, a much greater decline, of approximately 3.5 logs, was observed at day 1. Although the virus in this model would also adhere to the glass surface its removal from the planktonic phase was more marked than that observed in the control. This result must, therefore, be due to the presence of the mono-species biofilm in the test model. If it is assumed that the virus adheres to the glass at the same rate in both test and control models then the mono-species biofilm must produce an effect on the missing 1.5 logs of the virus. Since the virus was likely to be in its dispersed state during this experiment and the previous tube experiments indicated that the mono-species biofilm promotes further dispersal of the virus, this effect cannot be due to increased viral aggregation but rather to its destruction or inactivation.

At this point it must be noted that the glass surface of the jar will also promote bacterial adsorption and hence biofilm formation. The presence of a biofilm on the inside surface of the jar would produce a slightly larger surface area, as it would no longer be smooth, and would be of stickier texture due to EPS production. This could also promote the increased loss of virus from the planktonic phase. Table 5.2 shows the results of virus detection on the test surfaces in the sessile phase of the jar model. After 1 hour two out of three of the test surfaces in the control exhibited virus while all three showed the presence of virus in the test model. In addition to this, the levels of poliovirus were much higher in the sessile phase of the test than in the control supporting the above theory to some extent. After 1 hour virus was not detected, by $TCID_{50}$, on the plastic test surfaces of the control, while one surface in the test displayed low levels of virus. This too supports the above theory that more viruses will adsorb to the biofilm than to the plastic surfaces alone. Since plastic is a slightly more hydrophobic surface than glass the virus will preferentially adsorb to the glass than to the test surface, further suggesting that this model is inadequate for the purposes of this investigation.

Figure 5.8 - The levels of Poliovirus in the Planktonic Phase in the Presence of a Mono-Species Biofilm formed in the Jar Model

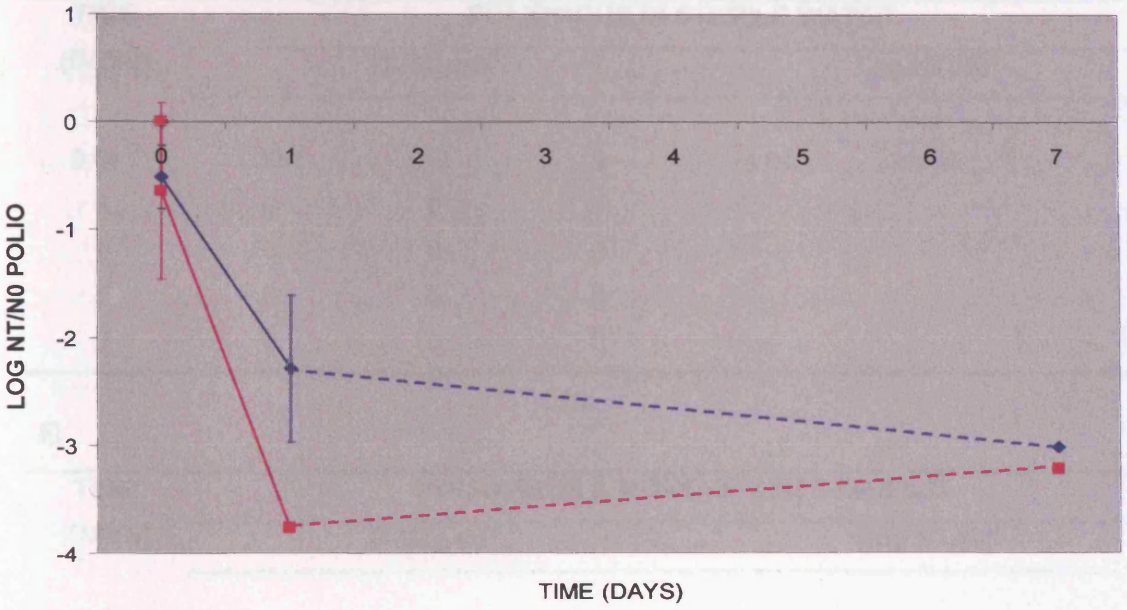


Figure 5.8 - The poliovirus levels detected in the planktonic phase of the jar model.The negative control jar (—◆—) contained filter sterilised tap water while the test jar (—■—) contained a mono-species biofilm. This mono-species biofilm was produced using *Pseudomonas mendocina* (ORG 001) which was previously isolated from the complex water distribution system biofilm. This organism was allowed to circulate for 14 days prior to poliovirus inoculation to allow biofilm development to occur. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars

Table 5.2 - The Levels of Poliovirus in the Sessile Phase in the Presence of a Mono-species Biofilm formed in the Jar Model

A

TIME (DAYS)	POLIOVIRUS IN STERILE WATER					
	$TCID_{50}$ ml^{-1}			Log $NT/N0^2$		
0.04 [1]	32	32	0	-4.04	-4.04	-
1	0	0	0	-	-	-
3	0	0	0	-	-	-
5	0	0	0	-	-	-
7	0	0	0	-	-	-

B

TIME (DAYS)	POLIOVIRUS & MONO-SPECIES BIOFILM					
	$TCID_{50}$ ml^{-1}			Log $NT/N0^2$		
0.04 [1]	692	100	32	-2.71	-3.5	-4.04
1	0	0	32	-	-	-4.04
3	0	0	0	-	-	-
5	0	182	0	-	-3.29	-
7	0	32	0	-	-4.04	-

Table 5.2 – The levels of poliovirus detected in the sessile phase of the mono-species biofilm formed in **A** the control jar model containing filter sterilised tap water and **B** the test jar model containing a monobiofilm. The monobiofilm was formed using a *Ps mendocina* strain (ORG 001) which was previously isolated from the complex biofilm. This model was left for 14 days, to allow biofilm formation to occur, prior to poliovirus inoculation.

[1] 0.04 days is equivalent to 1 hour.

[2] Log NT/N0; where NT = $TCID_{50}$units/ml at time point and N0 = initial $TCID_{50}$units/ml at time 0. In this case N0 = 3.53 x 105 $TCID_{50}$units/ml.

At the subsequent time points of days 3 and 5, no virus was detected in the planktonic phase of either jar model. However at day 7 poliovirus was detected at similar levels in the planktonic phase of both jar models. Similarly, although virus was not detected in the sessile phase of the control, some of the surfaces in the test jar did display the presence of poliovirus at days 5 and 7. In addition, the levels of virus on the test surface at day 5 in the presence of the mono-species biofilm, were higher than those observed at day 1. These results suggest that in both models the virus remained present throughout the experiment and that the mono-species biofilm may not have been inactivating or destroying it. Removal of the virus from the planktonic phase was simply due to the high proportion of glass promoting viral adsorption and the slow movement of the water probably facilitated this. The lack of virus on the test surfaces may be attributed to the higher hydrophobicity of the plastic and thus the virus preferentially adsorbed to the glass. In addition the detection of the virus at day 7 in both jars suggests a dynamic nature in which the virus adsorbs and desorbs from the glass at certain instances.

5.2.3 The Levels of Poliovirus in the MRD

The experiment monitoring the interaction of poliovirus in the MRD showed conclusively that this mono-species biofilm had little or no detrimental effect on the poliovirus. Once again, the initial inoculum consisted of a 1:500 dilution producing approximately 3.5×10^5 $TCID_{50}$units ml^{-1} at time 0, allowing virus dispersal to occur. Figure 5.9 shows the results of these experiments. After 1 hour post virus inoculation, the results show the expected virus increase observed previously in the tube experiments (see Figure 5.5). This confirms that in this system the dispersal of aggregated virus has occurred. Since this increase occurred at a similar level in both test and control it must be attributed to the high dilution factor upon addition of the virus in the tap water. The results also display similar virus decay in both test and control models. In addition the graph observed is similar to that seen in the previous experiment showing the decay of dispersed poliovirus in tap water (see Figure 5.5). This confirms the reproducibility between the two models used in this investigation (discounting the jar model due to its uncontrollable extra parameters). It also suggests that the presence of the mono-species biofilm in a flowing system had little or no effect on the levels of poliovirus in the planktonic phase.

In the sessile phase, the poliovirus was detected at slightly higher levels in the mono-species biofilm than on the control discs and for a longer time period. This confirms previous findings that the mono-species biofilm did not have a detrimental effect upon the virus in the sessile phase and that due to an increase in surface area and the sticky texture produced by the biofilm, increased viral adsorption occurred. As stated previously the hydrophobicity of the plastic surface is probably the reason why the poliovirus went undetected in the sessile phase of the control MRD after day 4 of the experiment.

Figure 5.9 - The Effect of a Mono-Species Biofilm on the Levels of Poliovirus in the MRD

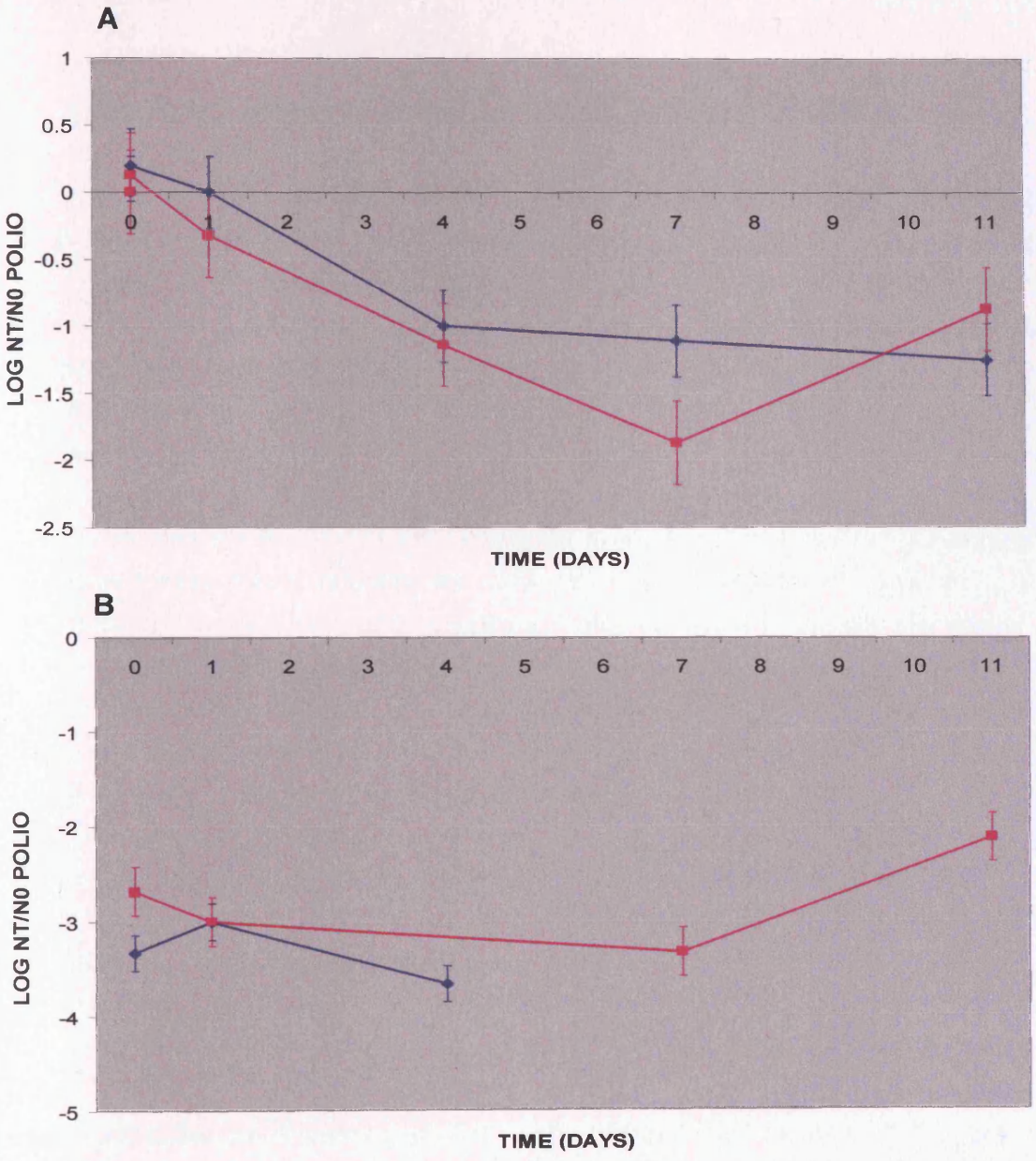


Figure 5.9 - The levels of poliovirus detected in A the planktonic phase and B the sessile phase of an MRD containing a mono-species biofilm. The negative control MRD (—◆—) contained filter sterilised tap water while the test MRD (—■—) contained a monoculture composed of *Pseudomonas mendocina* (ORG 001). This organism was previously isolated from the complex water distribution system biofilm evaluated in chapter 3. The monoculture was inoculated into filter sterilised water and allowed to circulate in the MRD for 14 days prior to poliovirus inoculation. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of duplicate samples from each MRD with population standard deviations as error bars.

Although these results prove that poliovirus does adsorb to the biofilm, higher levels were still apparent in the planktonic phase. However since the surface area of the biofilm is large and due to the dynamics of the virus adsorption shown in the jar experiment, it may be that the accumulated virus in the biofilm acts as a significant reservoir for the planktonic phase.

5.3 Poliovirus Levels in the Presence of a Mono-species Biofilm Evaluated by RT-PCR

5.3.1 The Levels of Poliovirus in the Alternative Jar Model

Figure 5.10 shows the PCR products gained following amplification of the planktonic samples in the alternative jar model. This model contained a mono-species biofilm composed of *Ps mendocina* formed in filter sterilised tap water. The results shown were positive for all time points tested in both control and test models indicating that the poliovirus was present in the planktonic phase throughout the 7 day experiment. Although the intensity of the bands observed were similar, this does not indicate that similar virus levels were present. Previous results (see Figure 5.2) show that this assay could not be used for quantitation, as low virus levels produced bands of similar intensity to high virus levels. The corresponding $TCID_{50}$ results obtained for the planktonic samples taken from the same model (see Figure 5.8) confirm these results indicating the presence of poliovirus at low levels at day 7. However, using $TCID_{50}$, the virus went undetected at days 3 and 5 in the test and control models respectively. The absence of poliovirus at these time points was attributed to its adsorption to the glass surface of the jar. The similar results occurring in the control jar suggested that the presence of the mono-species biofilm was not the cause. Virus removal by adsorption to the glass was still thought to be the case but the results suggest that not all of the virus was removed in this way. Instead, the virus either dropped to levels below the detection limits of the $TCID_{50}$ procedure or was present in a non-infective form. It must be noted that the RT-PCR used could be detecting the viral nucleic acid as aposed to the whole virus particles. However, since poliovirus contains positive sense RNA it was assumed that this was not the case. RNA is a labile molecule susceptible to breakdown by environmental RNases (Jones *et al,* 1994) and therefore it would not persist for 7 days even in filter sterilised tap water.

Of the above two theories proposed for the discrepancies between the two assays it was considered that the former idea was more likely. This theory proposed that the virus dropped to levels below the $TCID_{50}$ detection limits probably because it had become adsorbed to the glass surface of the jar. That the virus was detected at day 7 by the $TCID_{50}$ indicates that it had not been inactivated in either the test or control jar and that it's levels may have been increased by its desorption from the glass surface by a mechanism such as sloughing.

Figure 5.11 shows the PCR products obtained after amplification of the sessile samples in the alternative jar model. These results show that poliovirus adsorbed to the test surfaces in the control and to the mono-species biofilm. Although this assay was not quantitative, a semi-quantitative result may be inferred by assessing the number of surfaces yielding a positive

Figure 5.10 - The PCR Products After Amplification of the Planktonic Phase of the Mono-Species Biofilm in the Alternative Jar Model

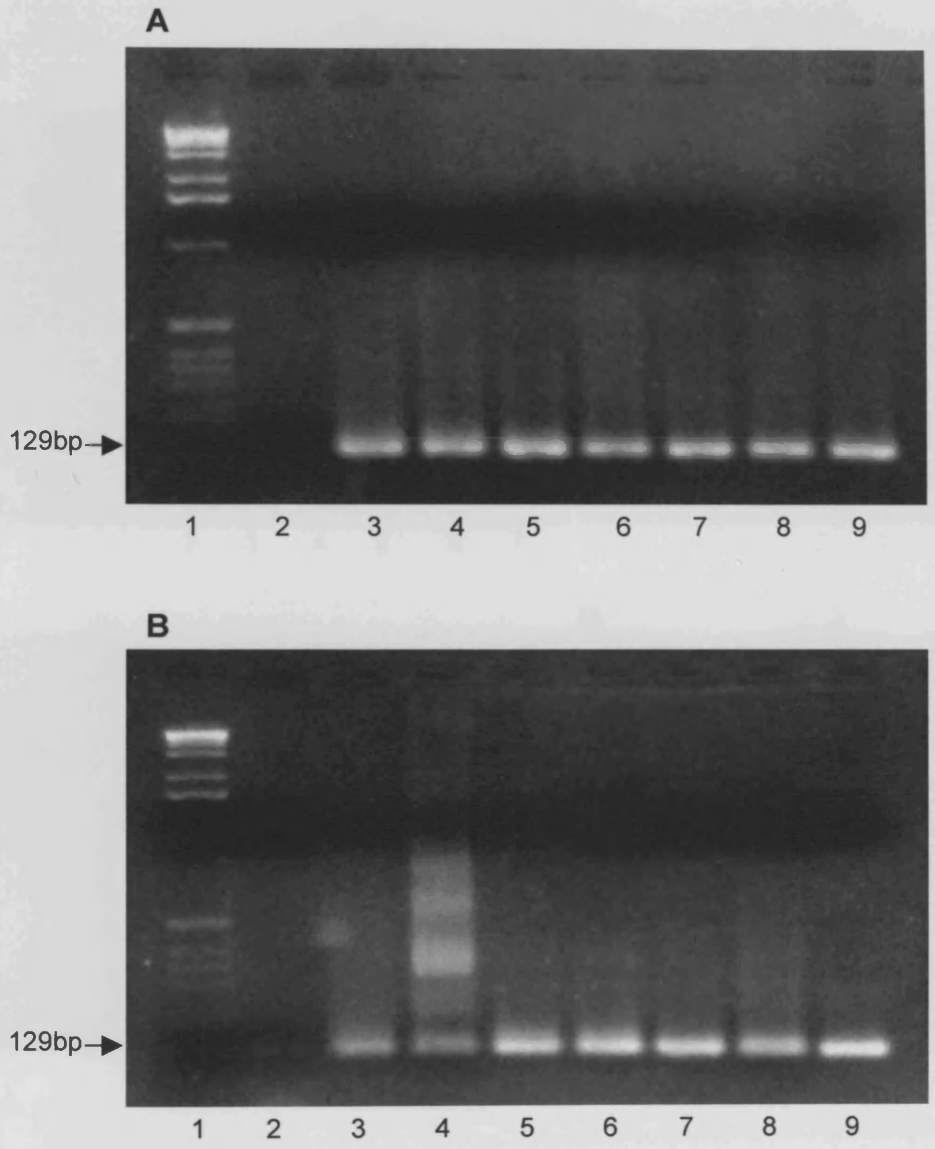


Figure 5.10 - The PCR products after the amplification of samples taken from the planktonic phase of **A** the control Jar containing sterile tap water and **B** the test Jar containing a mono-species biofilm. The biofilm was allowed to circulate for 14 days prior to poliovirus inoculation. This was produced by inoculating a monoculture composed of *Pseudomonas mendocina* (ORG 001) into filter sterilised water in a Jar. This organism was previously isolated from the complex water distribution system biofilm evaluated in chapter 3. The biofilm was allowed to circulate for 14 days prior to poliovirus inoculation. Lane 1 =- 1kb molecular weight ladder, lane 2 = negative control, lane 3 = positive control and lanes 4 - 9 = PCR products after amplification of the planktonic samples at time 0 (4), 1 hour (5), 1 day (6), 3 days (7), 5 days (8) and 7 days (9). Samples were PCRed in duplicate but only the first experiment is shown.

Figure 5.11 - The PCR Products After Amplification of the Sessile Phase of the Mono-Species Biofilm in the Alternative Jar Model

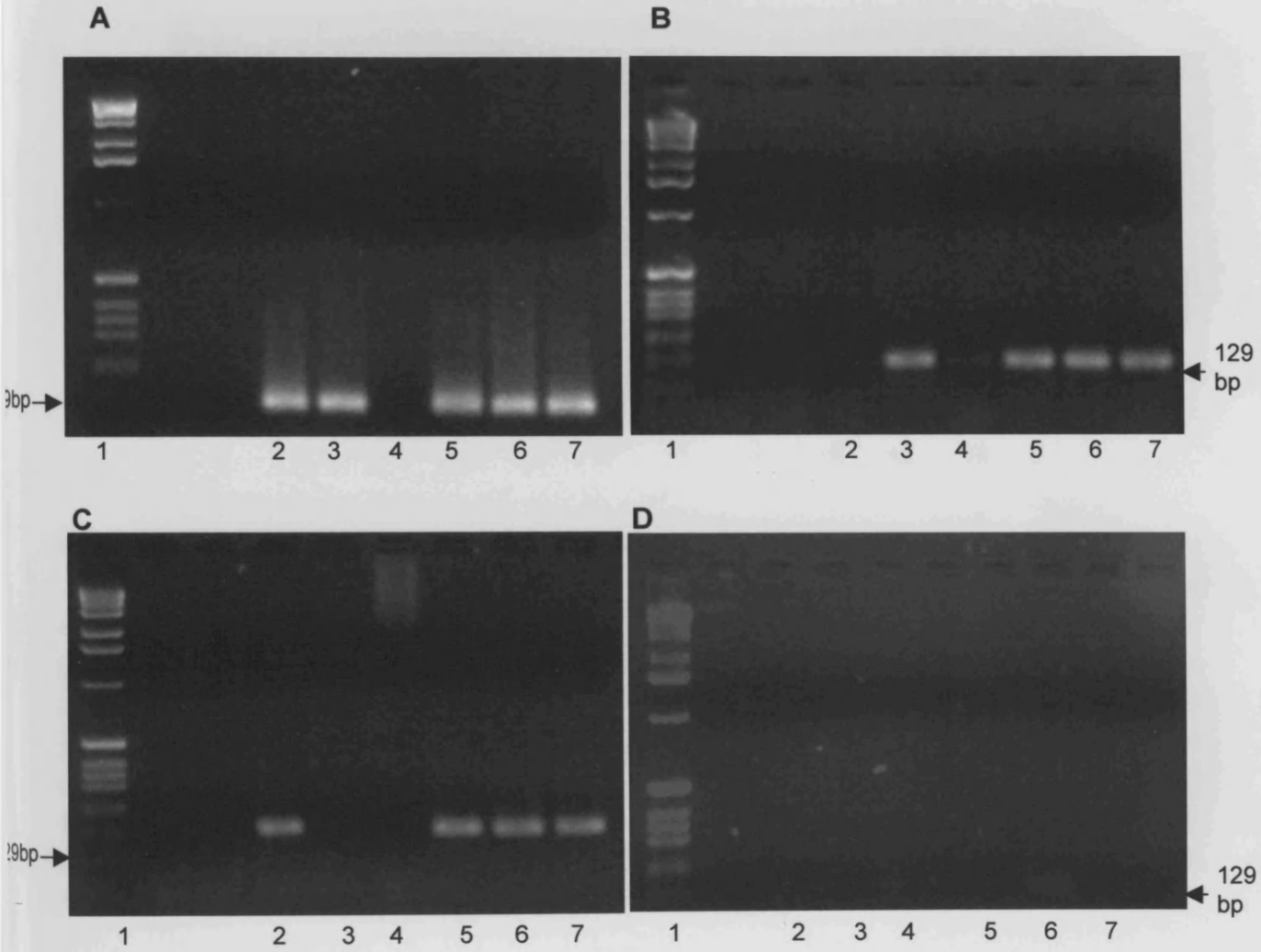


Figure 5.11 Continued - The PCR Products After Amplification of the Sessile Phase of the Mono-Species Biofilm in the Alternative Jar Model

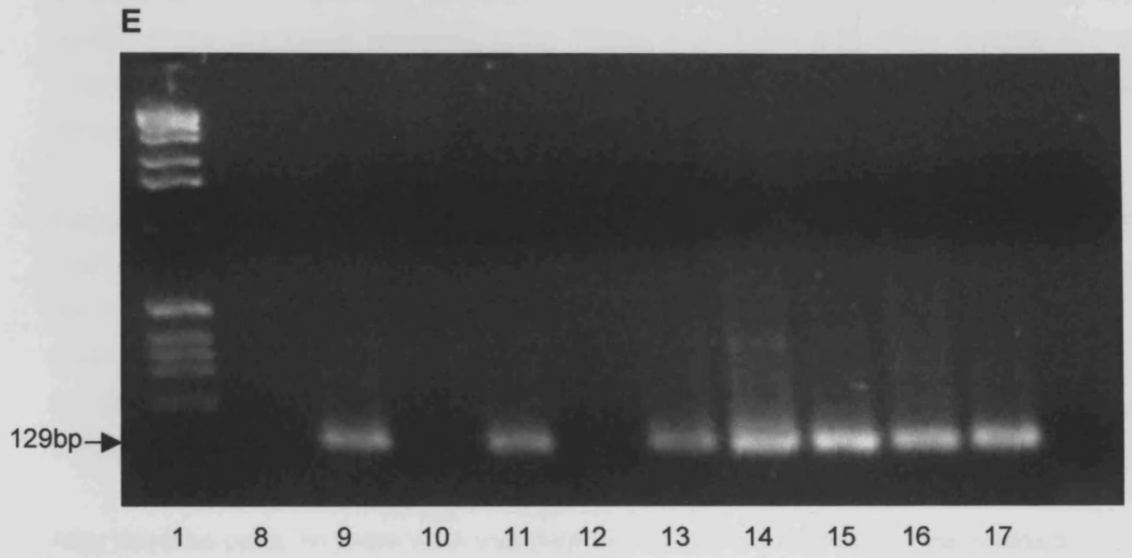


Figure 5.11 - The PCR products after the amplification of samples taken from the sessile phase of the control Jar (lanes 2, 3 and 4) containing sterile tap water and the test Jar (lanes 5, 6 and 7) containing a mono-species biofilm. Samples were taken at **A** day 1, **B** day 3, **C** day 5, **D** day 7 and **E** the PCR products after the amplification of controls placed in the biofilm extracts. Lane 1 = 1kb molecular weight ladder and lanes 8 & 10 = negative controls (sterile water), lanes 9 & 11 = positive controls (poliovirus in sterile water), lane 12 = negative control (biofilm extract) and lanes 13, 14, 15, 16 & 17 = poliovirus in 1hour, 1 day, 3 day, 5 day and 7 day old filtered biofilm extract. The biofilm was allowed to circulate for 14 days prior to poliovirus inoculation. This was produced by inoculating a monoculture composed of *Pseudomonas mendocina* (ORG 001) into filter sterilised water in a Jar. This organism was previously isolated from the complex water distribution system biofilm evaluated in chapter 3. Samples were PCRed in duplicate but only the first experiment is shown.

result at the different time points. Using this method, the results indicate that higher levels of poliovirus adsorbed to the mono-species biofilm samples than to the control surfaces. In addition, over the initial 5 days, the virus levels in the sessile phase of the control model showed a decline while they remained constant in the mono-species biofilm. At day 7, the poliovirus was no longer detected on the control surfaces and also displayed a rapid decline on the biofilm showing a very faint positive result on only 1 of the 3 surfaces. These results confirm those previously observed using $TCID_{50}$ (see Table 5.2). They indicate that more virus adsorbed to the mono-species biofilm than to the test surface alone and that poliovirus persisted in the biofilm for a longer time period.

Table 5.3 summarises the semi-quantitative data obtained for both $TCID_{50}$ and RT-PCR methods and, once again, shows the RT-PCR procedure detected more positive results than the $TCID_{50}$. After one hour, both $TCID_{50}$ and RT-PCR results were the same, showing that poliovirus was detected on all three of the surfaces present in the test model while only on two of the three surfaces in the control. This confirms the initial quantitative result obtained for $TCID_{50}$ (see Table 5.2) showing greater numbers of virus adsorbed to the biofilm.

After this time point, no more virus was detected on the test surfaces of the control by $TCID_{50}$. In contrast, poliovirus was still detected using RT-PCR at day 5. This result suggests that on the surfaces in the control model the virus levels present were lower than the $TCID_{50}$ limits of detection. In the test model, both assays indicated the presence of higher poliovirus levels. By $TCID_{50}$ the virus was detected at all time points, except at day 3, but not on all test surfaces. Similarly RT-PCR detected virus at all time points but also on all surfaces except those tested at day 7. These results indicate that although poliovirus did adsorb to the biofilm at higher levels than the control it also declined in the biofilm albeit at a slower rate than that observed for the control. Since the biofilm consists of EPS which acts as a sorption site (Flemming, 1995) and the presence of a biofilm would produce an increase in the surface area of the disc this result is not surprising. In addition, the presence of the biofilm probably reduced the hydrophobicity of the plastic surface thus making this easier for the virus to adsorb to. This would also explain why lower virus levels were present on the surfaces in the control model. The greater number of positive results obtained using PCR may reflect the higher sensitivity of this assay, indicating either, the presence of virus at levels below the detection limits of the $TCID_{50}$ method or the presence of inactivated virus.

5.3.2 The Levels of Poliovirus in the MRD

Figure 5.12 shows the results obtained after PCR amplification of both planktonic and sessile samples taken from the MRD containing a mono-species biofilm. In this experiment the results were positive for all samples tested. This was expected confirming the previous $TCID_{50}$ results (see Figure 5.9) which also showed that the poliovirus was present in all samples of the test MRD. A slight exception must be noted for the sessile phase of the control

MRD which generated negative results by $TCID_{50}$ after day 4. Since the plastic surfaces have been noted to be hydrophobic in nature, this conflicting result is probably due to the higher sensitivity of the RT-PCR method. The virus was probably present in these samples at levels below the detection limits of the $TCID_{50}$ procedure and thus missed.

Table 5.3 – Poliovirus Persistence in the Sessile Phase of a Mono-species Biofilm in the Alternative Jar Model – Comparison of $TCID_{50}$ and RT-PCR Results

TIME (DAYS)	CONTROL JAR		TEST JAR	
	$TCID_{50}$	RT-PCR	$TCID_{50}$	RT-PCR
0.04[1]	2/3	2/3	3/3	3/3
1	0/3	2/3	1/3	3/3
3	0/3	1/3	0/3	3/3
5	0/3	1/3	1/3	3/3
7	0/3	0/3	1/3	1/3

Table 5.3 – The levels of poliovirus detected in the sessile phase of the alternative jar model using the $TCID_{50}$ and RT-PCR methods. The control jar contained filter-sterilised tap water while the test jar contained a mono-species biofilm composed of *Ps mendocina* (ORG 001). This bacterium was isolated from the water distribution system biofilm model previously evaluated in chapter 3. The jar was left for 14 days prior to poliovirus inoculation.

[1] 0.04 days is equivalent to 1 hour

Figure 5.12 - The PCR Products After Amplification of the Mono-species Biofilm in the MRD

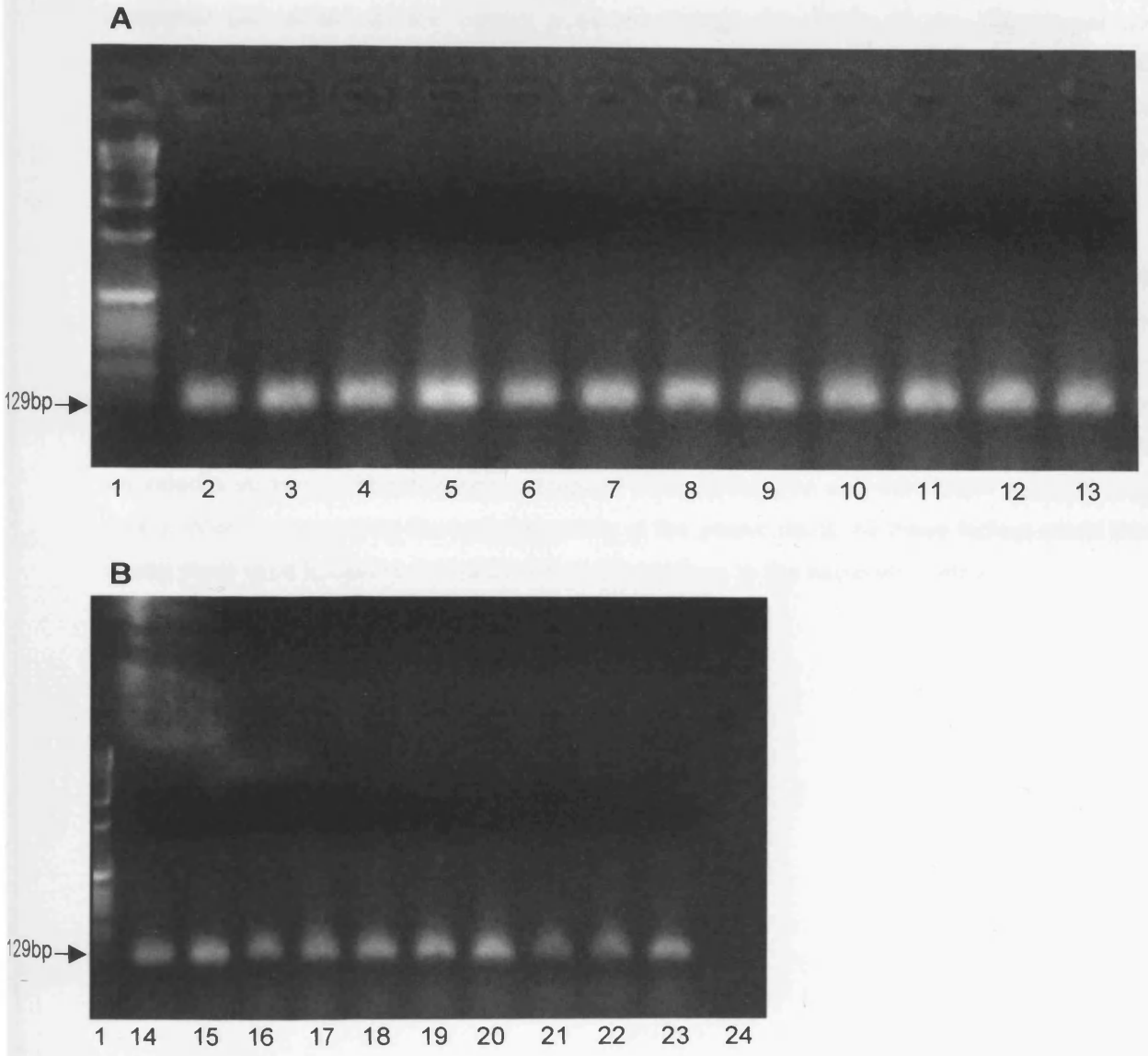


Figure 5.12 - The PCR products after the amplification of samples taken from **A** the planktonic phase and **B** the sessile phase of the MRD containing the Mono-species biofilm. This was produced by inoculating a monoculture composed of *Pseudomonas mendocina* (ORG 001) into filter sterilised water in an MRD. This organism was previously isolated from the complex water distribution system biofilm evaluated in chapter 3. The biofilm was allowed to circulate for 14 days prior to poliovirus inoculation. The negative control MRD for this experiment is as shown in Figure 5.20. Lane 1 = 1kb molecular weight marker, lanes 2 - 13 are the PCR products after amplifying the planktonic samples at time 0 (2 & 3), 1 hour (4 & 5), 1 day (6 & 7), 4 days (8 & 9), 7 days (10 & 11) and 11 days (12 & 13). Lanes 14 - 23 are the PCR products after amplifying the sessile phase at 1 hour (14 & 15), 1 day (16 & 17), 4 days (18 & 19), 7 days (20 & 21) and 11 days (22 & 23). Lane 24 = negative control.

Additionally, the results obtained for the control MRD were also positive for all the samples at all time points tested (see Figure 5.22A which shows the PCR results obtained for a negative control MRD). These also confirmed the previous results using $TCID_{50}$ for the planktonic phase but were in contrast to those obtained for the sessile phase. In the latter phase using $TCID_{50}$, poliovirus was only shown to persist for 4 days. This suggests that very low levels of poliovirus did remain on the control surfaces through the entire 11 day experiment but probably at levels lower than the detection limits of the $TCID_{50}$. These results suggest that the presence of the mono-species biofilm did not have any detrimental effects upon the poliovirus in either of the two phases. This is supported by previous results obtained from other models containing the mono-species biofilm.

In this model, high levels of poliovirus were detected in the mono-species biofilm and this was shown by the continually positive $TCID_{50}$ results and confirmed by the RT-PCR results. Since this biofilm was not detrimental the virus adsorption was continual and its increased presence in comparison to the control was due to the increase in surface area of the test surfaces produced by the biofilms presence. In addition, the EPS produced by the biofilm would have provided a stickier surface allowing increased virus adsorption and the biofilm would also be responsible for decreasing the hydrophobicity of the plastic discs. All these factors would thus cause more virus to adsorb to the biofilm in comparison to the negative control.

5.4 The Interactions of Poliovirus with a Complex Water Distribution System Biofilm Model – Evaluated using $TCID_{50}$

5.4.1 The Levels of Poliovirus in Preliminary Tube Experiments

Figure 5.13 shows the results observed when the two different levels of inocula were placed in the presence of a mature biofilm. This Figure shows the effect of the biofilm on both dilute (possibly dispersed) and more concentrated (possibly aggregated) virus (see Figures 5.13A & B). The results generate two very different graphs but one very similar result is apparent and that is that the poliovirus does not survive for as long in the presence of a biofilm in either case. This suggests that either the virus is becoming incorporated into the biofilm itself or that the biofilm is destroying or rendering the poliovirus inactive in some way. In either case the virus appears to leave the planktonic phase.

The results displayed in Figure 5.13A show that in all tubes after 1 hour the viral load has increased. This supports the previous theory that when poliovirus is introduced into water as a low dilution any aggregates will be dispersed. The fact that all three tubes exhibit the same result suggests that the biofilm had little or no effect at this point. Following this at the 1-day time point, the viral load decreases quite dramatically by approximately 2 logs. Once again this result is similar in all three tubes demonstrating that the biofilm had little or no effect on the virus particles at this time. The reason for this decrease is unclear but since it occurs in the controls as well it can be considered to be a normal reaction of the virus to its dilution in tap water. It may be due to some aggregation of the virus as this water probably contains traces of calcium ions which are known to promote viral aggregation (Floyd & Sharp 1977). After the 1-day time point, poliovirus could no longer be detected in the tube containing the biofilm while it continued to be present in the two controls. This suggests that the poliovirus is becoming incorporated into the sessile phase or that the biofilm had a detrimental effect on the virus in the planktonic phase. In contrast to this, the results displayed in Figure 5.13B show that the more concentrated virus survived in the planktonic phase for slightly longer being detected at day 5 post viral inoculation. This result indicates that the biofilm had a detrimental effect but shows that the concentrated (possibly aggregated) form of the virus is more resistant to it. This finding is consistent with previous observations indicating that aggregated virus is substantially more resistant than suspensions of single particles. The theory behind this is that these aggregates exert a protective effect on the interior particles (Floyd & Sharp 1977; Young & Sharp 1977).

Figure 5.13 - The Effect of a Mature Biofilm on the Levels of Poliovirus in the Planktonic Phase with Time

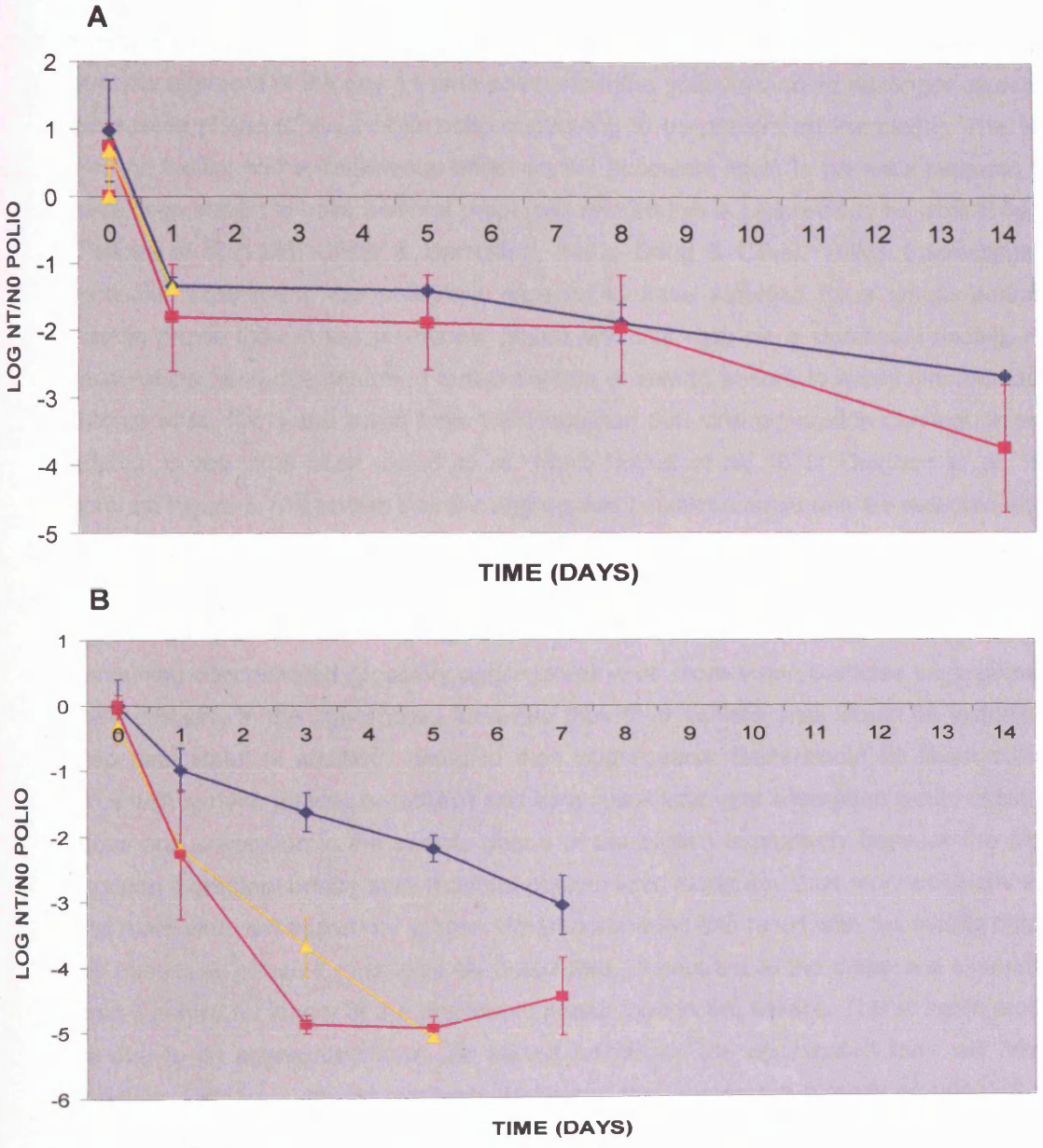


Figure 5.13 - The enumeration of poliovirus from the planktonic phase of three tube experiments with time after an inoculum of **A** 1 x 10^5 iuml^{-1}and **B** 1 x 10^7 iuml^{-1}. Tube 1 (—◆—) contained filter sterilised tap water, tube 2 (—■—) contained filter sterilised tap water and sterile plastic discs and tube 3 (—▲—) contained filter sterilised tap water with preformed biofilm on plastic discs. The biofilm was preformed in an MRD for a period of 14 days before being aseptically placed in tube 3. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

The results of poliovirus detection in the sessile phase of these experiments also demonstrate marked differences between the dilute and the more concentrated virus suspensions. In Figure 5.14A the results show that high levels of poliovirus have adsorbed to both the sterile plastic discs and to the preformed biofilm at a similar rate. This suggests that the presence of the biofilm had little effect on the adsorption properties of the virus and therefore the interactions observed are probably non-specific. Differences in the poliovirus adsorption become apparent at the day 14 time point when the poliovirus could no longer be detected in the sessile phase of the biofilm while continuing to be present on the plastic. This indicates that the biofilm had a detrimental effect on the poliovirus itself. In previous research bacteria have been shown to have antiviral properties and so this is proposed to be what is happening (Fujioka *et al*, 1980; Cliver & Herrmann, 1972; Deng & Cliver, 1995). Interestingly in this particular experiment, the poliovirus appears to have survived for a longer period in the sessile phase than in the planktonic phase and this may be a significant finding. Previous researchers have demonstrated that poliovirus is able to absorb to many different substrates (Moore *et al*, 1981) and some have demonstrated that viral adsorption can induce protective effects on the virus itself (Abad *et al*, 1994; Hejkal *et al,* 1979; Quignon *et al*, 1997). In contrast Figure 5.13B shows that the aggregated poliovirus could only be detected after day 1 on the sterile plastic discs and after day 3 on the preformed biofilm. This result suggests that the aggregated form of virus adsorbs and remains adsorbed to the biofilm for longer periods than to the plastic. This may again be a factor of the virus state. Although in the tube containing concentrated (possibly aggregated) virus more virion particles were present, they were probably in the aggregated form and thus their surface area would be less than in the dispersed state. In addition, owing to their aggregation, there would be fewer collisions of virus with surface (plastic or biofilm) and as a result less viral adsorption would occur. Slightly more viral adsorption in the sessile phase of the biofilm is probably because the biofilm will produce a greater surface area than the plastic discs alone and thus more collisions will occur and more virus will adsorb for longer. When comparing this result with the results obtained for the planktonic phase it must also be noted that, in contrast to the dispersed experiment, the virus survived for longer in the planktonic phase than in the sessile. This is again proposed to be due to its aggregated form. As stated previously the aggregated form will have fewer collisions with the surfaces available and since the viruses are already aggregated they are afforded more protection in the planktonic phase and so will be able to remain there for longer.

To determine whether the detrimental effect was produced by the biofilm or its by-products a further tube experiment was performed and the results are displayed in Figure 5.15. For this experiment the virus was inoculated at the lower level of 1 x 10^5 $TCID_{50}$ units ml^{-1} thus promoting virus dispersal. The planktonic phase was taken from an MRD after 14 days biofilm formation and filtered to remove any live cells. The results show that, although the filtered leachate did have a slight detrimental effect on the virus levels, it was still able to survive for the full 14 day period of the experiment. This is in stark contrast to the previous experiment (see Figure 5.13A) the results of which show that virus could not be detected after the 1 day

Figure 5.14 - The Effect of a Mature Biofilm on the Levels of Poliovirus in the Sessile Phase with Time

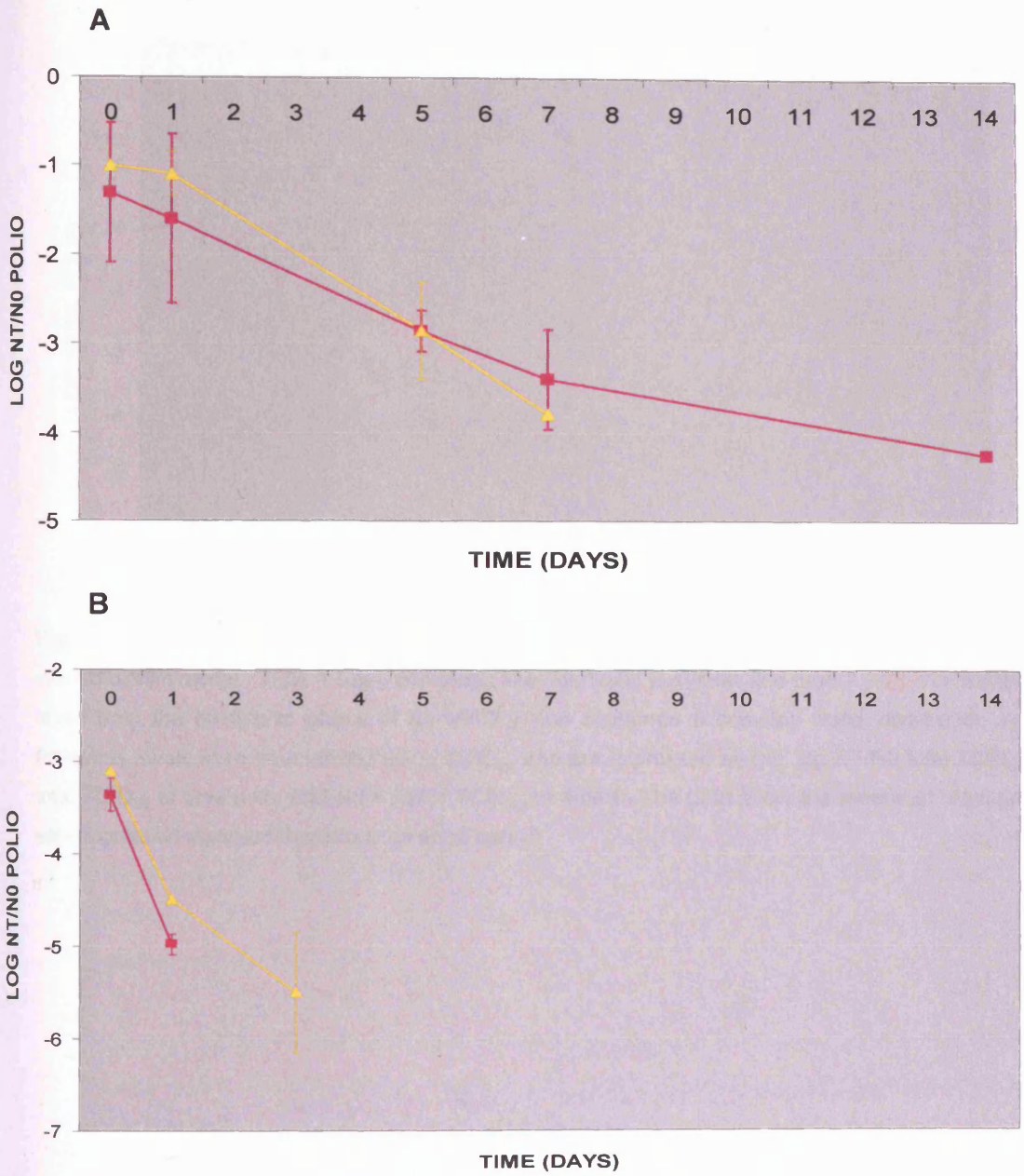


Figure 5.14 -The enumeration of poliovirus from the sessile phase of two tube experiments with time after an inoculum of **A** 1 x 10^5 iuml^{-1}and **B** 1 x 10^7 iuml^{-1}. Tube 2 (—■—) contained sterile plastic discs and tube 3(—▲—) contained preformed biofilm on plastic discs. The biofilm was preformed in an MRD for a period of 14 days before being aseptically placed in tube 3. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

Figure 5.15 - The Effect of Biofilm Waste Products on the Levels of Poliovirus in the Planktonic Phase

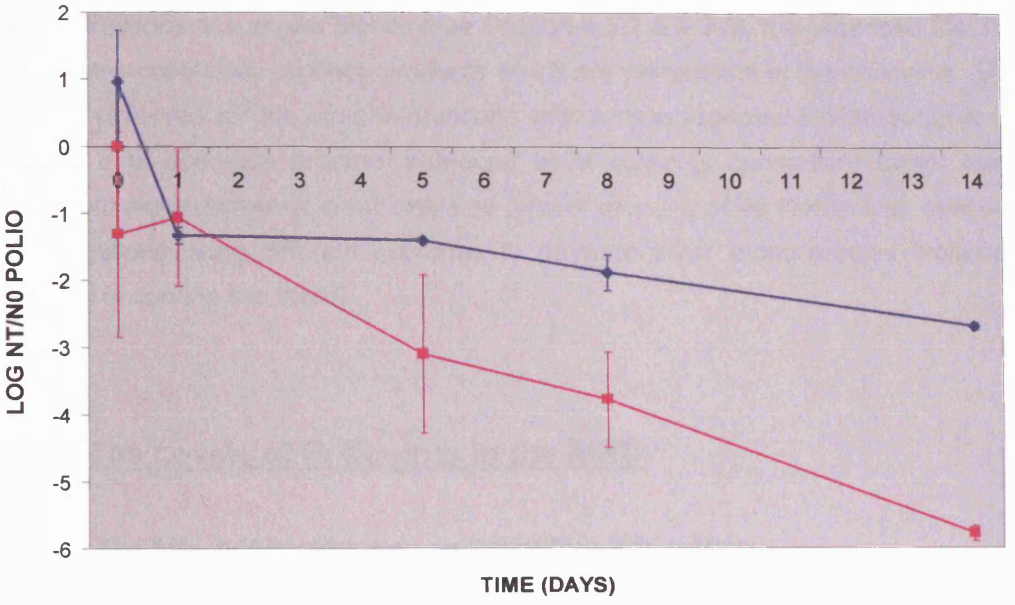


Figure 5.15 - The levels of poliovirus in the presence of waste products from a complex water distribution system biofilm model . Tube 1 (◆) contained filter sterilised tap water and tube 2 (■) contained filtered water taken from the planktonic phase of an MRD which contained a complex water distribution system biofilm . Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of triplicate experiments with population standard deviations as error bars.

time point. These results suggest that, although the leachate did significantly reduce the virus levels in the planktonic phase, the presence of the biofilm had a more detrimental effect. This result is further supported by the previous findings of other workers (Deng & Cliver, 1995; Fujioka *et al*, 1980) who noted the detrimental actions of micro-organisms in water on certain enteric viruses. It is also supported by the previous results obtained for bacteriophage lambda in the presence of a similar biofilm (see Section 4.3.2 & 4.3.3). It is proposed that such sessile organisms continually produce products which are detrimental to the poliovirus. The previous results observed for the virus interactions with a mono-species biofilm suggest that maybe this is a co-operative process produced by a complex consortium rather than a single organism alone. However since only one type of mono-species biofilm was evaluated, further investigations using different microbes to produce other mono-species biofilms would be needed to confirm this theory.

5.4.2 The Levels of Poliovirus in the MRD

5.4.2.1 The Effect of Biofilm Age on Poliovirus Adsorption

Figure 5.16 shows the results obtained when investigating the adsorption properties of biofilms of differing age. They show that with increasing biofilm age, increased levels of poliovirus adsorb. However, in both cases little difference was observed between the adsorption properties of the older biofilms. In fact the greatest difference in poliovirus adsorption occurred between the negative control and the 1-hour-old biofilm. These results suggest that the presence of a biofilm, regardless of its age, allows for the greater adsorption of virus. This is probably because the presence of a biofilm on a surface serves to increase the surface area of the substrate on which it grows thus allowing for more viruses to adsorb. Additionally the EPS produced by the members of the biofilm is both sticky, serving to increase viral adsorption.

Interestingly these results also reinforce previous results concerning biofilm formation. The 1 hour old biofilm allowed greater adsorption than the negative control and in turn the 1 day old biofilm allowed greater adsorption than the 1 hour old one. However the much older biofilms, of 7 and 14 days, allowed similar numbers of virus to adsorb as the 1 day old biofilm. This result suggests that the absorptive surface area of the biofilm is fully formed after 1 day and remains at that absorptive capacity there after.

The results also indicate a relatively uniform distribution of poliovirus along the length of the MRD at all ages of the biofilm suggesting that there is not any particular part of the MRD for poliovirus adsorption.

Figure 5.16 - The Adsorption of Poliovirus to Biofilms of Differing Ages

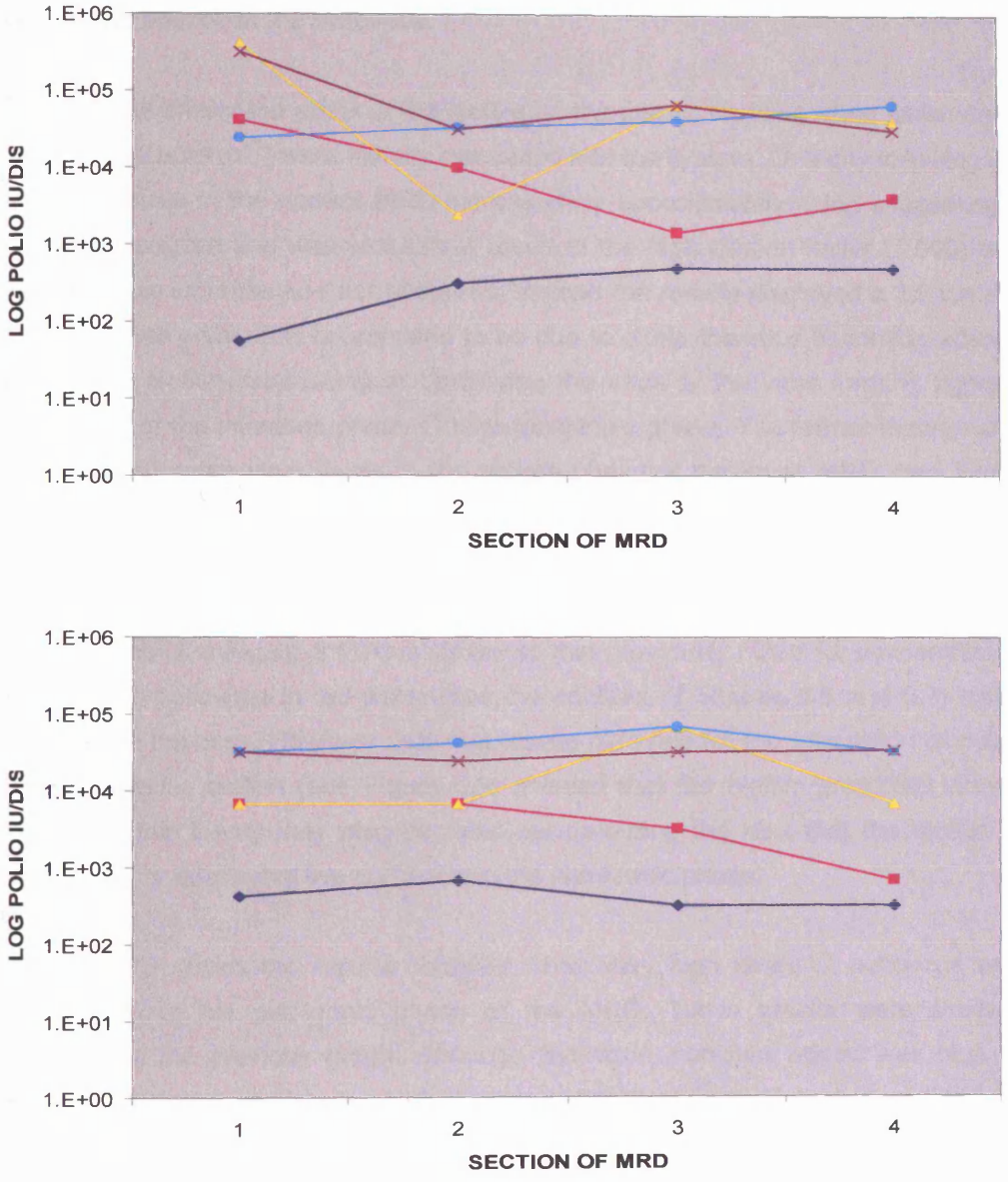


Figure 5.16 - The isolation of poliovirus from different sections of two MRD's 1 hour after poliovirus inoculation. Biofilms of varying age were formed in the MRD by changing the studs at varying time points prior to the inoculation of the poliovirus. The biofilm ages were as follows; negative control (◆) containing filter sterilised water only, a 1 hour old biofilm (■), a 1 day old biofilm (▲) , a 7 day old biofilm (●) and a 14 day old biofilm (✕).

5.4.2.1 The Poliovirus Levels in the MRD with Time

The results obtained for the poliovirus levels in the two phases of the biofilm model in the MRD with time are shown in Figures 5.17 and 5.18. For the three experiments performed, three different initial inocula of poliovirus were evaluated and, although these results are not clear cut, they all show that the presence of a water distribution system biofilm had detrimental effects on the poliovirus.

Figure 5.17A shows the effect of the biofilm on the planktonic virus when lower virus levels (1 x 10^5 $TCID_{50}$ units ml^{-1}) were initially inoculated into the system. One day following inoculation, the virus levels in the control MRD increased by approximately 1 log suggesting that virus dispersal occurred and was probably a result of the high dilution factor (1:500) used. In the test MRD this increase was not observed, instead the results displayed a 2.5 log reduction at the 1 day time point. This is proposed to be due to either the virus becoming adsorbed to the biofilm, the biofilm inactivating or destroying the virus or the virus forming aggregates with each other or the microbes present in the planktonic phase. The former theory was ruled out after evaluating the virus levels in the sessile phase of the same MRD (see Figure 5.18A). This showed that, although poliovirus was detected in the biofilm, its levels were initially similar and then considerably lower than those observed in the control. The latter theory of virus-virus and virus-microbe aggregation is a possibility. The fact that the pattern observed on the graph (see Figure 5.17A) is similar to that previously noted for concentrated (possibly aggregated) poliovirus in tap water (see the controls of Figures 5.6 and 5.7) also suggests this may be the case. However previous results obtained for the interaction of poliovirus with a mono-species biofilm (see Figure 5.9) showed that the biofilm promoted virus dispersal. Therefore, this theory may also be ruled out favouring the idea that the biofilm was either inactivating or destroying the poliovirus in the planktonic phase.

Figure 5.17B shows the results obtained when very high levels of poliovirus were initially inoculated into the planktonic phase of the MRD. These results were similar to those observed in the previous graph. Although the virus inoculum added was high enough to maintain virus aggregation the high dilution factor (1:100) used was considered to promote virus dispersal according to the theories of Floyd and Sharp (1977) previously mentioned. Following virus inoculation into the control MRD, the expected virus increase was observed but this appeared to take longer than before (see Figure 5.17A) reaching its full 1 log increase after 4 days. The reason for this is proposed to be due to conflicting conditions promoting both aggregation and dispersal. Following this, a decrease in the viral load was observed which was similar to that previously noted for the control in Figure 5.17A. This was thus considered to be the normal virus decay in the absence of a biofilm.

In the test MRD, the viral load decreased by approximately 2 logs and remained at this level for 1 day following poliovirus inoculation. This result was also similar to the previous observation in graph A, allowing for similar conclusions to be drawn. Once again, the loss of the virus in the planktonic phase could not be attributed to increased virus adsorption to the

biofilm as lower levels of poliovirus were detected in the sessile phase compared to the control (see Figure 5.18B).

After day 1 the virus levels in the planktonic phase of the test MRD continued to decrease and the graph observed had a similar slope to that seen in graph A. However, in this experiment poliovirus was detected in both the test and control MRD's until day 7. This was not observed previously. The reason for this was attributed to the high levels of poliovirus initially inoculated. Although the virus decay was similar to that seen before, this higher initial inoculum allowed for the process of virus decay to take longer. In addition, the higher particle levels present may have caused an increase in the collision frequency of the virus causing some aggregation to occur and in this state the virus would be afforded some protection (Young & Sharp, 1977). Although the virus appeared to survive in both the test and control MRD's for the same length of time its levels in the test were much lower, once again, suggesting that the presence of the biofilm had detrimental effects on the planktonic virus. In addition to this, there were no more sampling points between days 7 and 14 so it is unclear whether the virus did survive for the same period of time in both MRD's. The next experiment indicated that the virus did not survive for as long in the test MRD.

Figure 5.17C shows the results obtained after a lower level of virus (1×10^6 $TCID_{50}$units ml^{-1}) was inoculated into the planktonic phase of the MRD. For this experiment, an extra sampling time was incorporated between days 7 and 14 to determine the survival length of poliovirus in this phase. In this case the virus in the planktonic phase of the test MRD survived for the initial 7 day period while in the control it survived for 11 days. These results display marked differences to those previously observed in graphs A and B but the conclusions that may be drawn were essentially the same. In this experiment the low virus levels and high dilution factor (1:500) probably did promote virus dispersal and this was clearly shown by the increase in virus levels after 1 hour in both MRD's. In contrast to previous results this increase was observed in the test MRD at slightly higher levels than in the control and for a longer period (1 day) than the control. These results confirm a previous finding that the presence of a biofilm did promote virus dispersal in the planktonic phase. In the control MRD the virus levels declined slightly producing the expected graph for dispersed virus in sterile tap water. In contrast the virus levels in the test MRD decreased exponentially by approximately 0.6 logs day^{-1} following the initial increase at day 1. At day 7, the virus levels were approximately 2 logs lower in the test MRD than those observed in the control. At day 11 poliovirus was no longer detected in the test MRD while continuing to be present in the control. The conclusions drawn from this are, once again, similar to those previously stated for Figures 5.17 and B. The initial increase at the start of the experiment demonstrated that the virus was probably in it's dispersed state, and the fact that similar virus levels were observed in the sessile phase of the same MRD ruled out the theory that the virus removal was due to its adsorption to the biofilm. Thus all three graphs show that the presence of a complex water distribution system biofilm had detrimental effects on the poliovirus levels in the planktonic phase causing either its destruction or inactivation. These results are reinforced by those previously observed for

bacteriophage lambda levels in the presence of a similar biofilm and other workers have noted the antiviral activities of microbes in water (Fujioka et al, 1980; Cliver & Herrmann, 1972).

In the sessile phase (see Figure 5.18), poliovirus could be detected in both test and control MRD's for at least 4 days. When 1 x 10^5 $TCID_{50}$units ml^{-1} was initially inoculated (see Figure 5.18A) the virus appeared to survive in the sessile phase for the same length of time (7 days) in both MRD's but much higher levels of virus were detected in the control than in the biofilm. In the next experiment using the higher initial inoculum of 1 x 10^8 $TCID_{50}$units ml^{-1} (see Figure 5.18B), similar results were observed. However, in this case poliovirus could be detected at day 14 in the test MRD while it was absent in the control. These results suggest that poliovirus did adsorb to the biofilm but the lower levels observed may have been due to the detrimental actions of the biofilm previously noted in the planktonic phase (see Figure 5.17). It must be noted that in both cases the poliovirus appeared to survive for a longer period in the sessile phase of the test MRD than it did in the planktonic phase while the virus survival in the two phases of the control were the same. This infers that, although the biofilm does produce a detrimental effect on the virus it does preferentially adsorb to the biofilm where it may be afforded a limited amount of protection. This phenomenon may be a result of its aggregation but this is unclear.

The results displayed in Figure 5.18C however, contradict this theory displaying similar virus adsorption to surfaces in both the control and test MRD's initially with a slight decrease in virus numbers observed at day 4 in the presence of the biofilm. In addition, the virus could not be detected in the sessile phase of either MRD after the day 4 time point. Thus in this experiment the virus appeared to survive for longer in the planktonic phase than in the sessile phase of both MRD's. The reasons for this are unclear.

Figure 5.17 - The Effect of a Complex Biofilm on the Levels of Poliovirus in the Planktonic Phase of the MRD

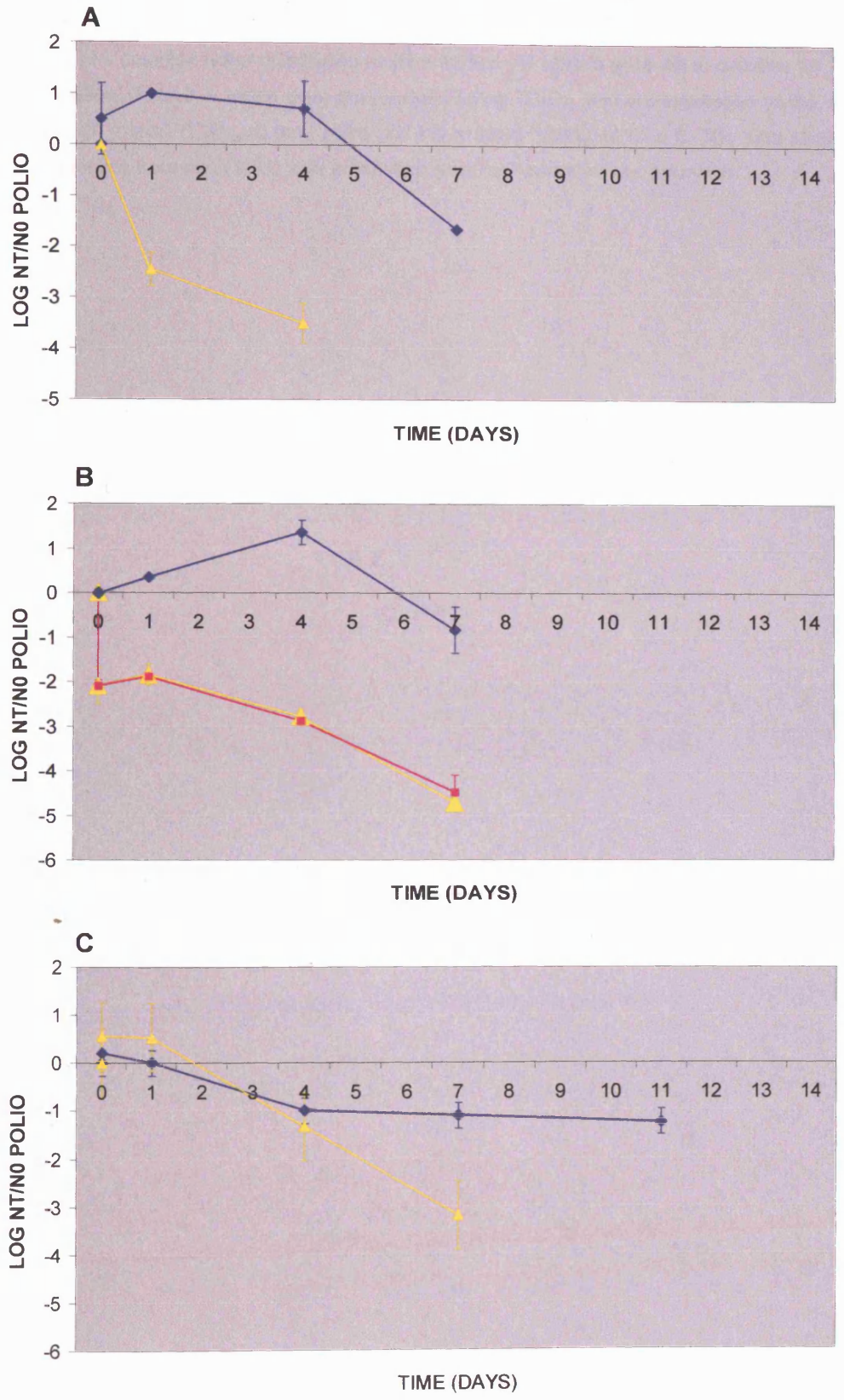


Figure 5.17 - The levels of poliovirus detected in the planktonic phase of the MRD in the presence of a complex water distribution system biofilm. The three graphs display results after different levels of poliovirus were initially inoculated into the systems. Graph **A** was inoculated with 1 x 10^5 iuml^{-1}, Graph **B** with 1 x 10^8 iuml^{-1} and Graph **C** with 1 x 10^6 iuml^{-1}. Negative control MRD's (—◆—) contained filter sterilised tap water while MRD'S 1 (—■—) and 2 (—▲—) contained a complex water distribution system biofilm. All MRD's were left to circulate for 14 days prior to poliovirus inoculation. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of quadruplicate samples from each MRD with population standard deviations as error bars.

Figure 5.18 - The Effect of a Complex Biofilm on the Levels of Poliovirus in the Sessile Phase of the MRD

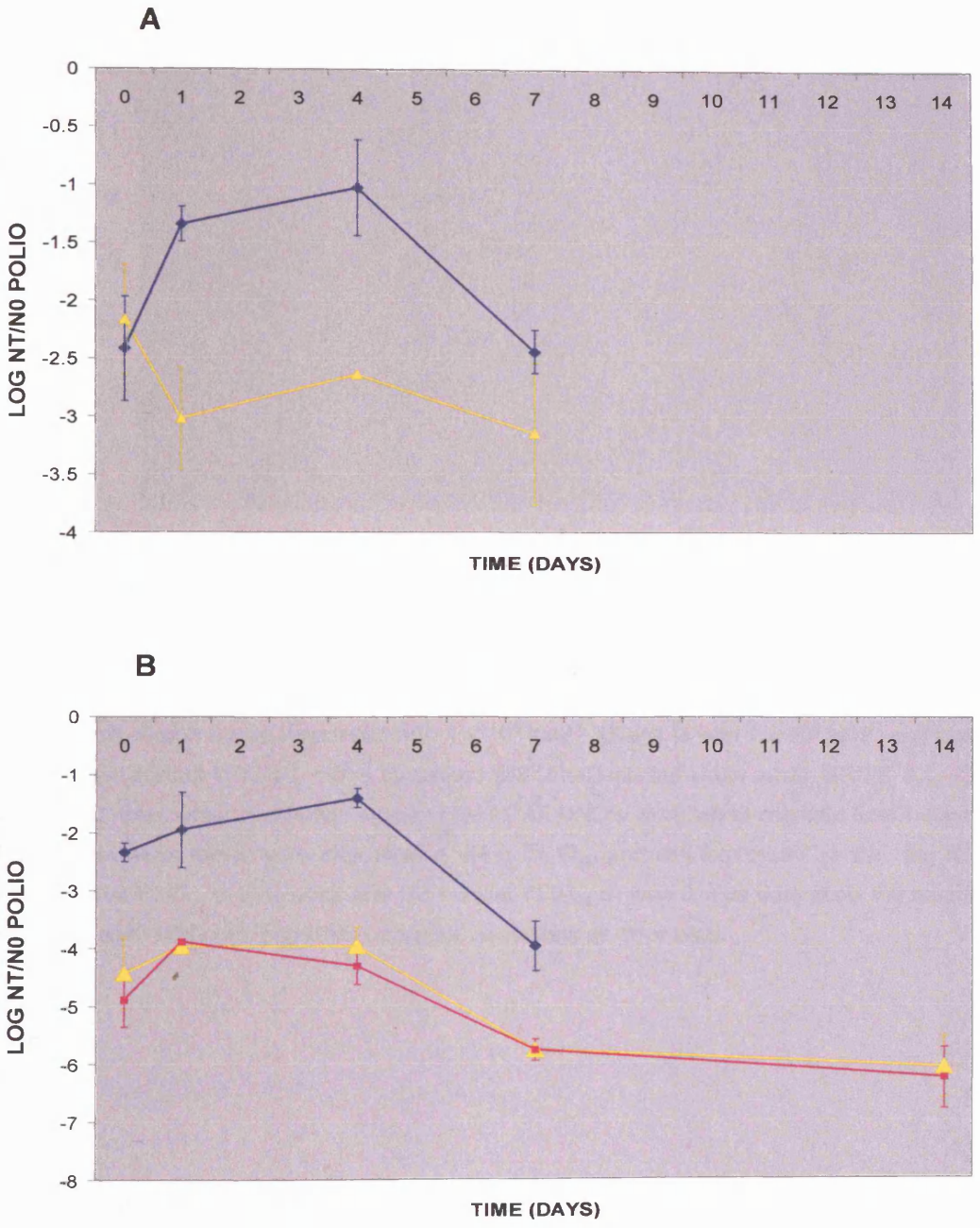


Figure 5.18 Continued - The Effect of a Complex Biofilm on the Levels of Poliovirus in the Sessile Phase of the MRD Continued

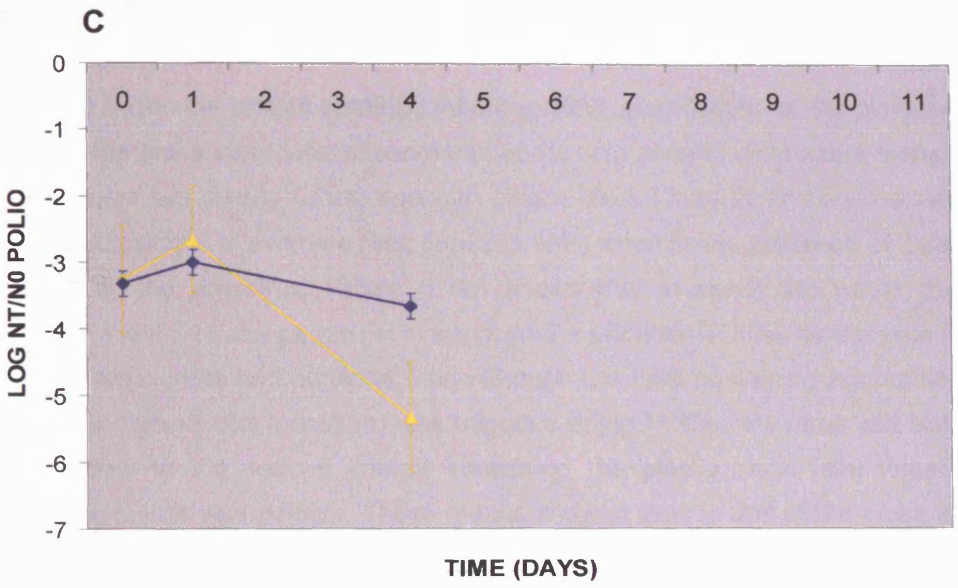


Figure 5.18 - The levels of poliovirus detected in the sessile phase of the MRD in the presence of a complex water distribution system biofilm. The three graphs display results after different levels of poliovirus were initially inoculated into the systems. Graph **A** was inoculated with 1 x 10^5 iuml^{-1}, Graph **B** with 1 x 10^8 iuml^{-1} and Graph **C** with 1 x 10^6 iuml^{-1}. Negative control MRD's (◆) contained filter sterilised tap water while MRD'S 1 (■) and 2 (▲) contained a complex water distribution system biofilm. All MRD's were left to circulate for 14 days prior to poliovirus inoculation. Poliovirus levels were enumerated using $TCID_{50}$ and are expressed as the log NT/N0 total $TCID_{50}$; where NT = total $TCID_{50}$ at time point and N0 = initial $TCID_{50}$ at time 0. The data show the means of quadruplicate samples from each MRD with population standard deviations as error bars.

5.5 Poliovirus Levels in the Presence of a Water Distribution System Biofilm Model - Evaluated by RT-PCR

5.5.1 The Levels of Poliovirus in Preliminary Tube Experiments

Figure 5.19 shows the results obtained following PCR amplification of the planktonic samples taken from the preliminary tube experiments containing a water distribution system biofilm. In this experiment two control tubes, one with plastic discs (Tube 2) and another without (Tube 3), were incorporated to evaluate (see Figure 5.19B) whether the presence of these discs had any effect on the poliovirus. Figure 5.19A shows that in sterile tap water, the poliovirus survived for the full 14 day period. This supports the previous $TCID_{50}$ results (see Figure 5.13) for the dispersed virus and confirms that, although the tube containing aggregated poliovirus (i.e. with the higher initial inoculum) was negative using $TCID_{50}$, the virus was still present as was expected. In the second control containing the plastic discs (see Figure 5.19B), a difference in results was evident. These results showed that, in one of the tubes at day 5 and in both tubes at day 14, the virus was not detected, suggesting that the plastic discs did influence the poliovirus in the planktonic phase. However, at day 7 the virus was detected by PCR in both tubes and the $TCID_{50}$ results also indicated the presence of the virus at days 5 and 7 in all control tubes tested. This suggests that at the day 5 time point the PCR may have been inhibited by an unknown factor and this may also have occurred at day 14. In the control tubes containing the plastic discs and the dilute virus (see Figure 5.13A), the poliovirus was detected for the full 14 days using $TCID_{50}$ while it was only detected at day 7 in the corresponding control containing the concentrated virus. Therefore the reason for the negative result at day 14 is unclear but it may be due to an inhibitory factor producing a false negative result.

In the tube containing the preformed biofilm on plastic discs (see Figure 5.19C) the virus was consistently detected by PCR until day 3. At day 5, one tube produced a negative result while the other produced a faint positive and at day 7 both tubes produced positive results. Again at day 14 only one tube exhibited the presence of the virus. These results suggest that the virus was present for the full 14 days of the experiment in at least one of the tubes and is in contrast to the previous results obtained by $TCID_{50}$. These indicated virus survival times of 1 and 5 days for dilute and concentrated poliovirus respectively. The inference from this is that the virus was present either at levels below the detection limits of the $TCID_{50}$ or it was in a non-infective form. The negative results produced may be a result of PCR inhibition caused by a build up of products in the planktonic phase. Since this model was of low volume (10 mls) any inhibitory substances produced by the biofilm may have built up in this phase.

Figure 5.19 - The PCR Products From the Planktonic Phase of the 3 Tube Experiments

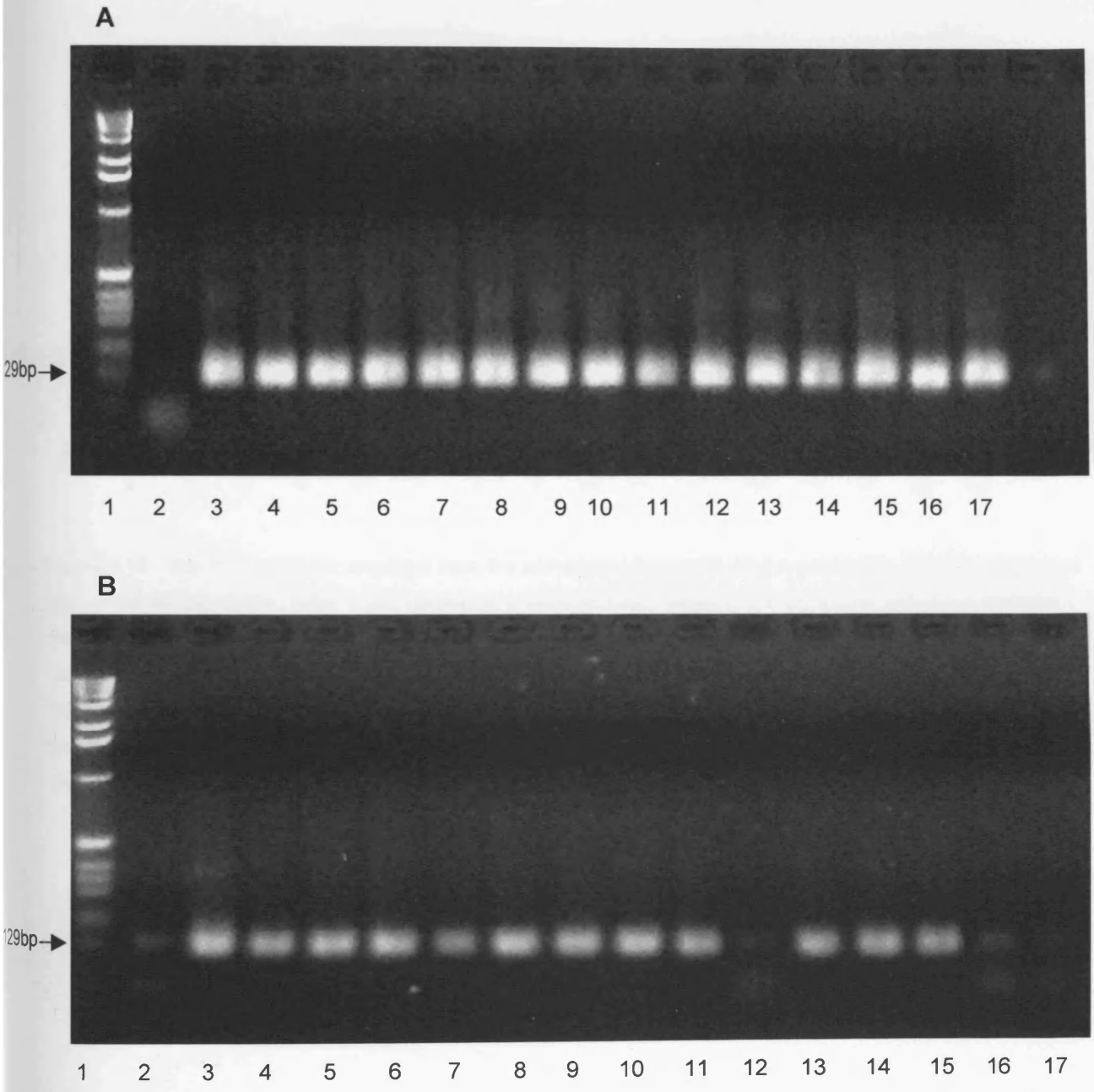


Figure 5.19 Continued - The PCR Products From the Planktonic Phase of the 3 Tube Experiments Continued

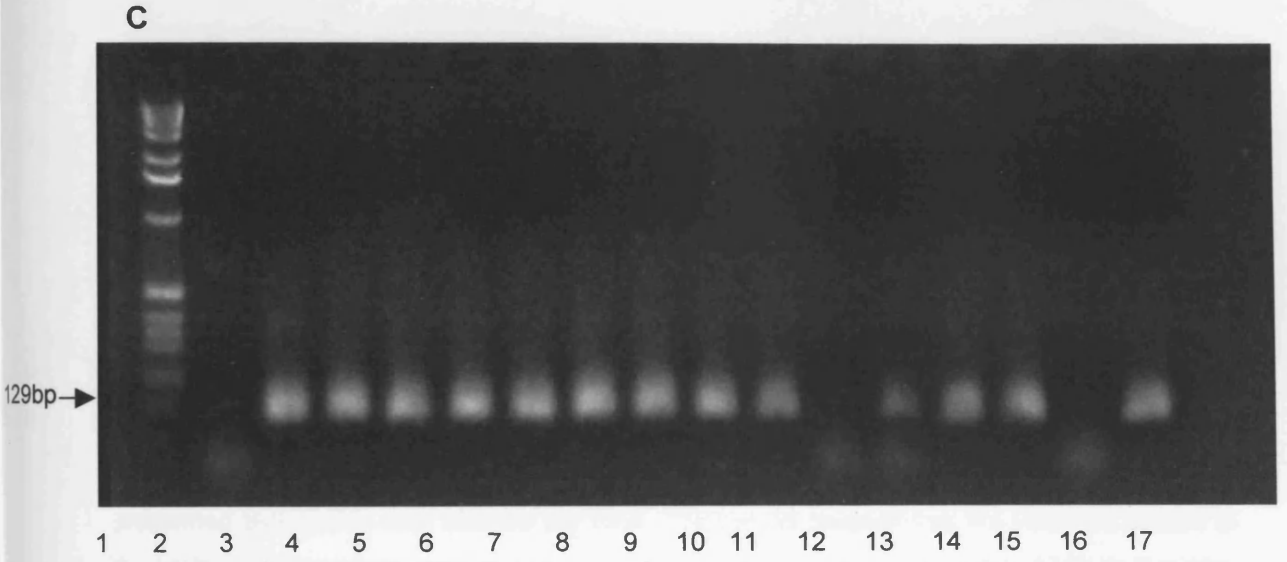


Figure 5.19 - The PCR products amplified from the planktonic phase of 3 tube experiments. All tubes contained filter sterilised tap water. Tube 1 (A) contained poliovirus only, Tube 2 (B) contained poliovirus and sterile plastic discs and Tube 3 (C) contained poliovirus and preformed biofilm on plastic discs. The preformed biofilm was generated using a Modified Robbins Device (MRD) and was allowed to circulate for 14 days. Lanes 1 = 1kb molecular weight marker, 2 = negative control, 3 = positive control and lanes 4 - 17 = samples taken at time 0 (4 & 5), 1hour (6 & 7), 1 day (8 & 9), 3 days (10 & 11), 5 days (12 & 13), 7 days (14 & 15) and 14 days (16 & 17).

Figure 5.20 shows the results obtained following PCR amplification of the sessile phase of the tube experiments. Interestingly, the standard PCR procedure, which utilised high temperature RNA extraction repeatedly did not work, confirming a previous inference that the biofilm produced a substance which was inhibitory to the PCR. To overcome this problem the RNA was chemically extracted from all of the sessile samples prior to amplification by the same PCR procedure. The chemical RNA extraction method would not only provide the RNA for the PCR but would also remove any inhibitors present in the sample.

Following amplification of the viral RNA it was revealed that virus was present in all of the sessile samples tested except in one tube of the control (Tube 2) at day 7. Since the other tube at day 7 was positive it might be that the RNA was lost during the extraction procedure for this sample. In the sessile samples of the control tube the corresponding $TCID_{50}$ results (see Figure 5.14) confirmed this for the dilute virus. However the concentrated virus control tube did not. Since the RT-PCR was unaffected by the state of the virus, i.e. whether it was dispersed or aggregated, the positive RT-PCR result supports the previous theory (see Section 5.4.1). This theory suggested that in its aggregated form, owing to the lack of a distinction between these two forms using $TCID_{50}$, the virus levels appeared to decrease at a faster rate to levels below the detection limit of the $TCID_{50}$. In the test tube containing the preformed biofilm this may also be the case. This would suggest that the virus did adsorb to the biofilm and remained present either at levels below the $TCID_{50}$ detection limits or in a non-infective form throughout the 14 day experimental period.

5.5.2 The Levels of Poliovirus in the MRD

Figure 5.21 shows the results obtained following amplification of the planktonic samples taken from the MRD containing a water distribution system biofilm. These results confirm those observed previously in the tube experiments, showing that poliovirus was present in the planktonic phase of this model throughout the 14 day experimental period. For these samples, the extraction of the RNA by chemical means was not required as no PCR inhibition was observed. The reason for this may be that such inhibitory substances initially accumulated in the biofilm but upon dispersal into the planktonic phase they became diluted in the large volume of water (500mls) present in this model. The fact that the results were similar in both the control and test MRD's suggests little difference occurred between the two models and thus the biofilm had little effect upon the virus in the planktonic phase. However, the corresponding $TCID_{50}$ results (see Figure 5.17) suggest the contrary. They consistently showed lower virus levels were present in the test MRD compared to the control. Since the $TCID_{50}$ is quantitative while the RT-PCR shows the virus presence at low levels regardless of infectivity, there are two possibilities to explain these results. The first, is that the biofilm produced a substance which actively destroys the virus eventually reducing its levels to lower than the detectable limits by $TCID_{50}$. The second, suggests that the biofilm does not destroy the virus but inactivates it rendering it non-infective and thus lowering its detection by $TCID_{50}$.

Figure 5.20 - The PCR Products From the Sessile Phase of 3 Tube Experiments

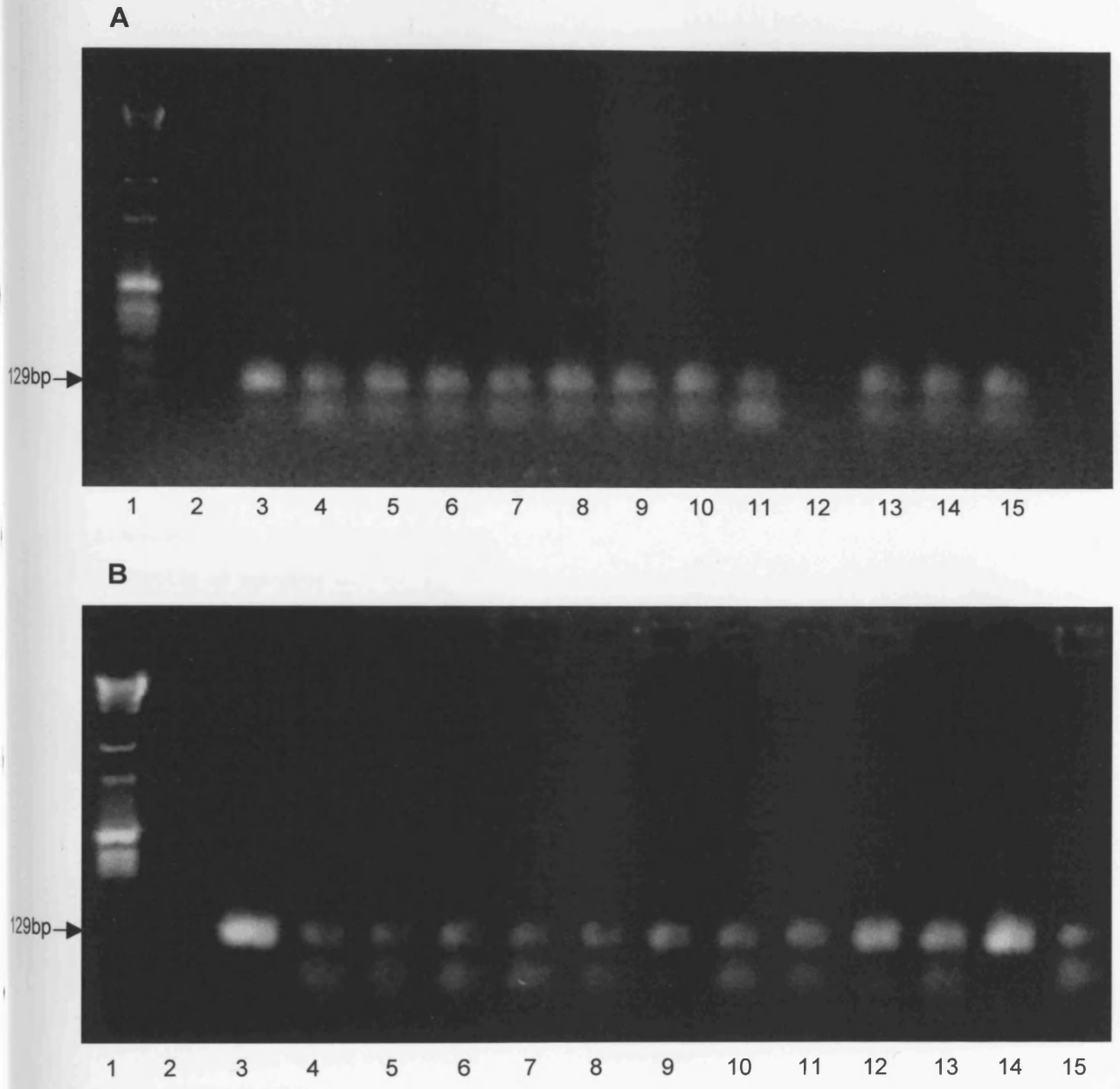


Figure 5.20 - The PCR products amplified from the sessile phase of 3 tube experiments. All tubes contained filter sterilised tap water. Tube 2 **(A)** contained poliovirus and sterile plastic discs and Tube 3 **(B)** contained poliovirus and preformed biofilm on plastic discs. The preformed biofilm was generated using a Modified Robbins Device (MRD) and was allowed to circulate for 14 days. Lanes 1 = 1kb molecular weight marker, 2 = negative control, 3 = positive control and lanes 4 - 15 = samples taken at time 1 hour (4 & 5), 1 day (6 & 7), 3 days (8 & 9), 5 days (10 & 11), 7 days (12 & 13) and 14 days (14 & 15).

For a clearer picture, a quantitative method for the detection of all whole virus regardless of infectivity was required for comparison with the $TCID_{50}$ results and the DIG hybridisation method would have provided this. However this method needed to be optimised and this could not be done due to constraints of time.

Upon PCR amplification of the sessile samples from the test and control MRD's (see Figure 5.22) the results were similar to those previously noted in the tube experiments. Once again, the conventional PCR, done on the biofilm samples, was inhibited and the RNA had to be extracted by chemical means prior to amplification. Since no problems occurred when amplifying the planktonic samples, this inferred that the inhibitory substance was produced and accumulated in the biofilm. Previous experiments concerning poliovirus interactions with a mono-species biofilm exhibited slight inhibition of PCR with very low virus levels but the samples tested did not require chemical RNA extraction. This infers that either the water distribution system biofilm produced larger quantities of this inhibitory substance than did the mono-species biofilm or that the combined activities of the microbial consortium produced a different inhibitory effect.

Following RNA extraction and PCR amplification, the results showed that the virus was present in all samples of both test and control MRD's at all time points. However, differences in the intensity of the PCR products were noted and this was found to be marked between test and control MRD's. In the control MRD the PCR products produced were of low intensity and a lot of primer dimers were apparent. In contrast, in the test MRD, the PCR products gave more intense bands on the agarose gels in the majority of the samples tested. Although this PCR could not be used for quantitative purposes, it may be that higher levels of virus were present in the biofilm samples as opposed to the control. This is consistent with previous results suggesting that the increased surface area, greater stickiness of the EPS and the decreased hydrophobicity of the surface produced by the biofilm would all cause increased virus adsorption. However, this is not consistent with the corresponding $TCID_{50}$ results (see Figure 5.18) which showed greater virus levels were present in the sessile phase of the control MRD than the test. These results may suggest that the presence of the biofilm had a detrimental effect on the virus levels in both phases of the biofilm model but that instead of totally destroying it they have rendered it non-infective. Further work employing a quantitative molecular technique is needed here to conclusively prove the above theory. The optimisation of the DIG hybridisation assay may have done this if time had allowed.

Figure 5.21 - The PCR Products After Amplification of the Planktonic Phase of the Complex Biofilm in the MRD

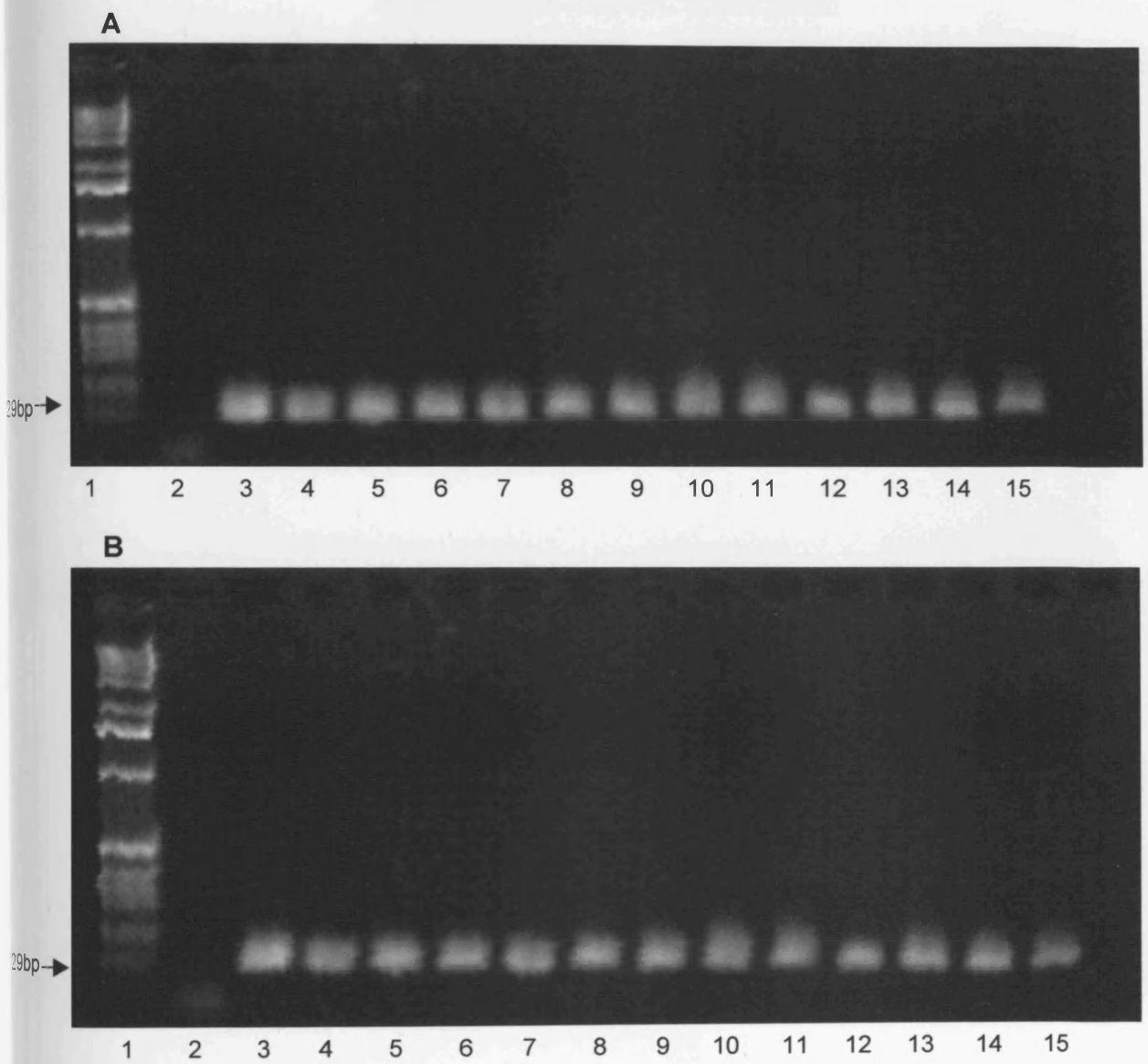
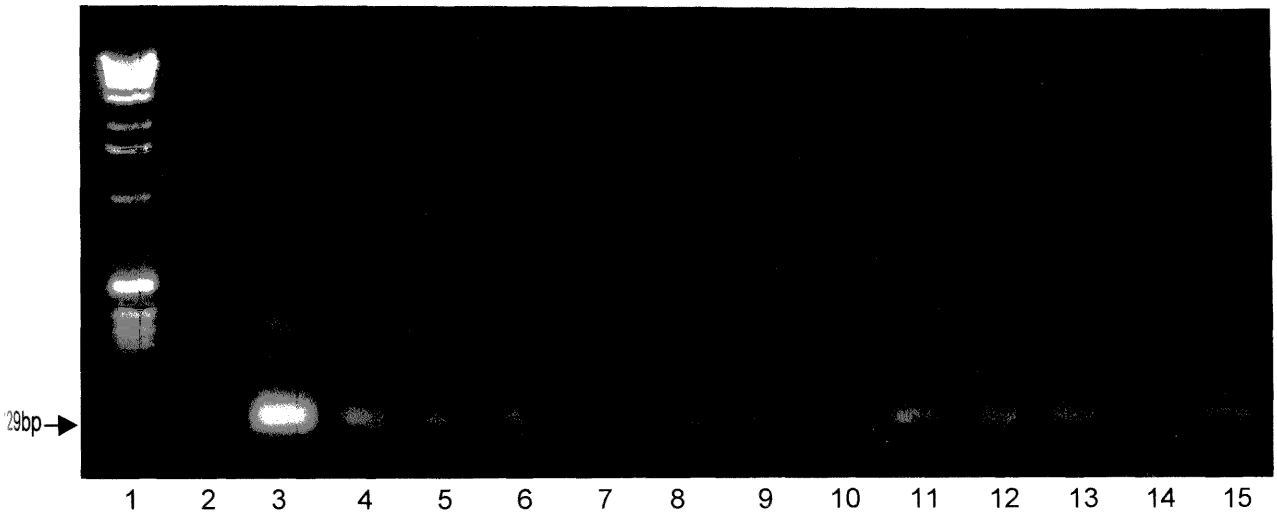


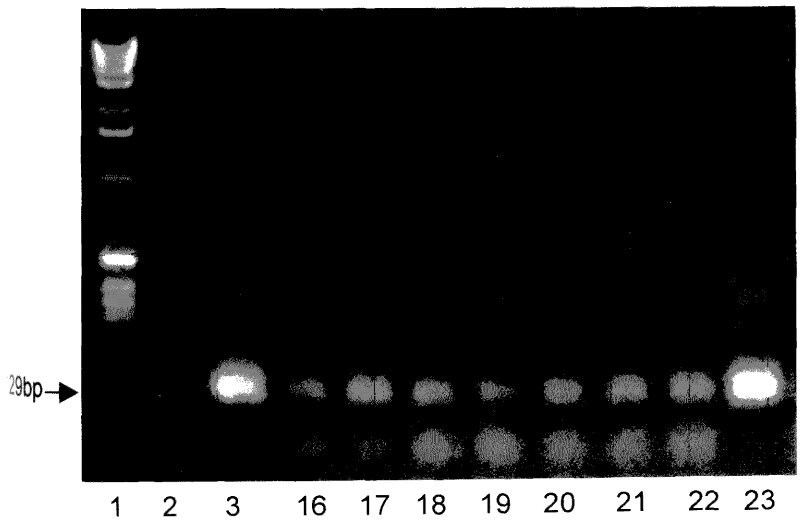
Figure 5.21 - The PCR products after the amplification of samples taken from the planktonic phase of **A** the control MRD containing sterile tap water and **B** the test MRD containing the complex water distribution system biofilm model. The biofilm was allowed to circulate for 14 days prior to poliovirus inoculation. Lanes 1 = 1kb molecular weight marker, 2 = negative control, 3 = positive control and lanes 4 - 15 = samples taken at time 0 (4 & 5), 1 hour (6 & 7), 1 day (8 & 9), 4 days (10 & 11), 7 days (12 & 13) and 14 days (14 & 15). The results displayed are duplicate samples taken from MRD 2. These results were the same for all other experiments on other MRD's.

Figure 5.22 - The PCR Products After Amplification of the Sessile Phase of the Complex Biofilm in the MRD

A



A Continued



5.6 Chapter Summary

In filter sterilised tap water, poliovirus may be present in two states, dispersed or aggregated, depending on the initial virus inoculum and the dilution factor used. The use of a high initial inoculum and a low dilution factor promotes virus aggregation while a low initial inoculum with a corresponding high dilution factor promotes virus dispersal (Floyd & Sharp, 1977). In its aggregated form, previous workers have shown that the virus is afforded greater protection to environmental fluctuations and some of the results displayed in this chapter have confirmed this finding (Young & Sharp, 1977).

In the presence of a mono-species biofilm composed of *Ps. mendocina*, a common isolates from water distribution system biofilms (Le Chavalleir *et al*, 1987; Jess *et al*, 1997), virus dispersal appeared to be promoted in the planktonic phase even under conditions which would normally favour aggregation. This suggests that the biofilm either produced a substance, which actively broke up the viral aggregates or it actively altered the physicochemistry of the water by its presence. Inoculating low virus levels into filtered planktonic phase appeared to promote greater initial virus dispersal followed by a slower virus decay compared to the planktonic phase in the presence of this biofilm. This suggested that the substance promoting virus dispersal was a leachate of the biofilm that had accumulated in the planktonic phase. The fact that the virus decay was less pronounced in the filtered leachate, may indicate that it had a short life-span and was continually produced by the biofilm.

In the sessile phase, higher levels of virus observed in the mono-species biofilm compared to the non-biofilm control further supported the above inference of virus dispersal and showed that this biofilm had few, if any, detrimental effects on the virus. The presence of the biofilm probably served to increase the surface area of the test discs, reduce the hydrophobicity of the plastic and provide a stickier surface. All these factors and the higher collision frequency promoted by virus dispersal would contribute to increased virus adsorption to the mono-species biofilm. RT-PCR confirmed the virus presence in both phases of the test and control models. The negative result produced by $TCID_{50}$ for the sessile samples, taken from the non-biofilm control in the latter stages of the experiment, confirm that the biofilm was a better sorption site than the plastic alone.

In the presence of a complex water distribution system biofilm, the $TCID_{50}$ results obtained from both models suggested that the biofilm caused the accelerated virus decay in the planktonic phase. In the sessile phase, virus adsorption was apparent but in all cases, except for the concentrated (possibly aggregated) virus in the tube experiment, the virus was at lower levels than the control. This suggests that the biofilm also had a detrimental effect on the poliovirus adsorbed to the sessile phase but that it's adsorption possibly afforded it limited

protection. The reason for the exception, may also be attributed to the aggregated virus being afforded greater protection in this form. This result contrasted with the previous findings concerning the interaction of poliovirus with the mono-species biofilm and suggests that such detrimental effects were the result of a combination of products produced by different species in the multi-species biofilm.

Although neither concentrated nor dilute virus survived for as long as it did in the control models, the tube experiments revealed that the concentrated (possibly aggregated) form appeared to survive for longer in the planktonic phase while the dilute (possibly dispersed) form favoured the sessile phase. This was proposed to be due to a higher collision frequency in the dispersed tube promoting increased adsorption. Additionally, an aggregated virus would be afforded more protection from harsh environmental factors compared to the dispersed form and so would survive for longer in the planktonic phase. Filtration of the accumulated leachate of the biofilm and subsequent inoculation of the dispersed virus appeared to remove some of the detrimental effects produced by the active biofilm. Although the virus decay was still greater than in tap water alone, the virus managed to survive for the same length of time as the control. This suggested that the presence of the living biofilm was more effective in removing the virus from the planktonic phase than the biofilm leachate alone. This suggests that the detrimental products of the biofilm had a short life-span and were continually being produced by the live biofilm.

In the MRD, virus adsorption was found to be related to biofilm surface area as opposed to age. The similar levels observed in biofilms of one, seven and fourteen days old confirmed the previous findings (see Chapter 3) that the biofilm formed after day 1 and, in so far as the virus adsorption was concerned, remained constant after that period. The uniform distribution of virus along the MRD length indicated that there was no a preferential adsorptive areas in the MRD. $TCID_{50}$ of the planktonic phase of the MRD revealed similar results to the tube experiments for the planktonic phase i.e. greater virus decay in the presence of the biofilm. In the sessile phase, the virus appeared to survive for as long as the control but at much lower levels. In the case where a higher initial inoculum was used, the virus appeared to survive for longer in the sessile phase compared to the control. This was attributed to the higher collision frequency in this MRD promoting increased virus adsorption.

All RT-PCR results indicate that the poliovirus was present in most of the samples at all of the time points tested. This was again in contrast to $TCID_{50}$ results prompting two conclusions to be drawn. Either the virus levels were reduced to below the $TCID_{50}$ detection limits or the biofilm rendered the virus non-infective. At this stage it was unclear which was true and further work, using a quantitative molecular technique, such as DIG hybridisation, would have allowed the distinction to be made. Unfortunately the full optimisation of this technique was not completed due to constraints of time.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

Biofilms are ubiquitous in nature and their existence in water distribution systems is well documented (Frais *et al*, 1994; Keevil *et al*, 1995; Schwartz *et al*, 1998; Momba *et al*, 2000). The EPS, a major component of the biofilm, consists of a network of microbially produced compounds (Christensen & Characklis, 1990; Flemming, 1995) which perform many functions. Such functions include the ability to act as a diffusion barrier (Lawrence *et al*, 1994) and as a sorption site (Flemming, 1995) capable of entrapping nutrients and other substances (Robinson *et al*, 1995). This mode of growth confers many additional advantages upon the sessile organisms (Brozel, 1994) affording them increased protection from biocidal agents, such as antibiotics and disinfectants (Brown & Gilbert, 1993), and allowing them to influence their own microenvironment (Nivens *et al*, 1995).

The co-operative metabolic processes which occur in such biofilms thus allows them to form in oligotrophic conditions such as those, found in water distribution systems. In this environment they not only contribute to the microbial degradation of both pipe materials and water quality (Kerr et al, 1997), but can also act as a haven for waterborne pathogens (Keevil *et al*, 1995). Pathogens found to be associated with biofilms in water systems include *Legionella pneumophilla* (Rogers *et al*, 1994), *Aeromonas hydrophila, Cryptosporidium parvum* (Keevil *et al*, 1995) and, more recently, *Helicobacter pylori* (Copley, 1999, Mackay *et al*, 1998). Since biofilms are dynamic systems which not only involve bacterial attachment and colonisation but also active cell detachment, they must therefore contribute to the dispersal of such pathogens and other sorbed substances back into the water phase (Flemming, 1995; Gilbert *et al*, 1993).

In addition to bacteria, enteric viruses have also been isolated from treated drinking water (Sekla *et al*, 1980; Payment, 1981; Keswick *et al*, 1984) suggesting that conventional water treatment processes do not always adequately remove them from water designated for human consumption. The presence of enteric viruses in potable drinking water has major public health implications (Gerba & Rose, 1990) which is compounded by their extended survival, coupled with their ability to produce infection even when present in low numbers (Abbazadegan *et al*, 1993).

One hundred and ten types of enteric viruses have already been identified and new ones are discovered almost every year (Gerba & Rose, 1990; Melnick, 1996). The majority of such viruses cause a variety of infections ranging from gastro-enteritis to aseptic meningitis (Abbazadegan *et al*, 1993). The enteroviruses, cause paralysis, meningitis, respiratory problems and diarrhoea, while the rotaviruses, adenoviruses 40/41 and SRSV's cause gastro-enteritis. Hepatitis A and E viruses cause liver degeneration characterised by jaundice. The Norwalk viruses have been shown to cause diarrhoea and vomiting and are a major cause of

waterborne disease in the US while outbreaks of astrovirus infection have been associated with oysters and drinking water (Gerba & Rose, 1990). Additionally, each year, in the UK, more than 20,000 cases of rotavirus and 2,500 cases of SRSV are reported to the PHLS Communicable Disease Surveillance Centre, while hepatitis A has become endemic (Selwood, 1997).

Although the water transmission of enteric viruses is well recognised, the direct association of these viruses in water distribution systems is rarely shown and there is probably significantly more illness due to the viral contamination of drinking water than is currently recognised. This may be due to the lack of methods and facilities available for virus investigations, the viruses may be present in numbers below the detection limits of existing methods and the occurrence of asymptomatic infections within the population which go unnoticed is an unknown factor (Gerba & Rose, 1990). In recent years, the viral outbreaks and the contamination of drinking water have been increasingly reported and thus their significance in water distribution systems is now becoming clearer.

Since enteric viruses are an abundant component of aquatic ecosystems (Suttle, 1987) capable of evading water treatment processes, their entry into the water distribution system has raised many questions. Can they persist in such water distribution systems to transmit subsequent infections in the population? And, if so, what mechanisms do they adopt? Many previous researchers have demonstrated the persistence of enteric viruses in environmental waters (Gerba *et al*, 1996; Kukkula *et al*, 1997a, Schvoerer *et al*, 1999; Hafliger *et al*,2000) but few have studied the interactions of such viruses in biofilms from water distribution systems. Sakoda *et al* (1997) showed that viruses were able to adsorb to a variety of materials in the environment and Hejkal et al (1979) inferred that such adsorbed viruses were afforded greater protection from inactivation by substances such as chlorine than their unadsorbed counterparts. In addition, Quignon *et al* (1997) demonstrated the short term accumulation of poliovirus in a biofilm from a water distribution system.

Since biofilms act as huge sorption sites (Flemming, 1995) and viruses are able to adsorb to surfaces where they are afforded much protection (Quignon *et al*, 1997b) it may be reasonable to assume that viruses could persist in water distribution systems by adsorbing to the biofilm. In addition to this, the large surface area provided by the pipe interiors would represent a substantial reservoir (Keevil *et al*, 1995) for such adsorbed viruses and their accumulation.

This research aimed to determine whether such enteric viruses could adsorb and persist within biofilms of water distribution systems. For this, a suitable model of a water distribution system was required and the persistence of virus within it was monitored in both the planktonic and sessile phases. Two viruses were monitored in the system, bacteriophage

lambda and poliovirus sabin 1. Although bacteriophage lambda is not an enteric virus of concern to public health, it was used as the first model virus for its ease of cultivation and enumeration. Additionally, this virus has been extensively studied, is commonly used as a model for the understanding of other viruses (Eaton et al, 1995) and the lack of any host for it in the system meant that the results obtained should mimic any non-specific interactions typical of any virus. A common environmental isolate, this virus is relatively hardy and many workers have suggested the use of bacteriophages as indicator organisms in a similar manner to the use of coliforms (Abad *et al*, 1994; Ueda & Horan, 2000). Poliovirus was used as a model enteric virus and, similarly to the bacteriophage lambda, it was easily detected in samples. Additionally much research has focused on this virus (Metcalf et al, 1995) and thus it was considered to be an accurate model virus representative of the enteric virus family.

6.2 Discussion of Results and General Remarks

The principle apparatus chosen for the development of a model water distribution system biofilm was a Modified Robbins Device (MRD) attached to a recirculating system. This apparatus was chosen because it produces a flow of water similar to that observed in water distribution systems and its shape mimics that of a pipe. Previous workers have used the MRD for this purpose (Jass *et al*, 1995; Linten *et al*, 1999) and the ease of sample removal must be noted. Although the recirculation of water does not closely mimic the conditions within a normal water distribution system, this was considered easier to use for a long time period and it allowed for a reduced number of variables in the model. For example, once added, the number of viruses could be monitored at each time point and their levels compared to the initial inoculum. The system was such that all viruses inoculated should be accounted for at all times.

For the source of the biofilm, a natural inoculum was used which was considered to form the closest match to the water distribution system biofilm. Previous researchers also used natural inocula (Rogers *et al*, 1994) and noted that this was superior to the use of laboratory grown isolates as this would cause the involuntary selection of strains and therefore biases in the system. Boosted tap water (water from a storage tank) was used as it contained much higher bacterial levels than normal tap water. Since large quantities of water pass through a distribution system a large water volume of 25 L was used and the organisms concentrated to a 500 ml volume so that it could be placed in the recirculating apparatus.

The results observed when evaluating this model water distribution system biofilm were found to be highly reproducible. A biofilm took approximately 24 hours to reach a dynamic state where the bacterial levels became constant in both phases and once formed remained uniform in size and structure throughout the experiments performed. The levels of organisms isolated were consistent with previous results obtained by other researchers examining similar biofilms and the organisms isolated have been previously noted to be common isolates from water distribution system biofilms (Jass *et al*, 1997). However, this system was found to be very sensitive to selective pressures incurred on it. This was noted by the accidental inclusion of a small metal pipe connector which produced a marked change in the observed biofilm population allowing an opportunistic pathogen, *Ps aeruginosa,* to flourish.

The presence of this organism, *Ps aeruginosa*, in two of the systems tested was different to previous experiments while the bacterial levels remained similar. This change was noted and the connector was removed prior to performing subsequent experiments. This infers that some other variations may have occurred within the biofilm populations of other experiments but since all of the organisms could not be properly isolated and identified in all of the model systems, this could not be verified but was duly noted.

In addition to the MRD model, an alternative jar model was also evaluated to determine if it could be used for later experiments. This model contained the same volume of water as the MRD system but was much easier to set up and sterilise. Additionally, an unlimited number of test surfaces could be used. Although the resultant biofilm formed did appear to be similar to the biofilm in the MRD, some marked differences, particularly in organism levels, were noted and therefore this model was not considered to be an adequate representation of a water distribution system. A later preliminary experiment using this model also demonstrated other inadequacies with its use.

Upon the addition of bacteriophage lambda to the biofilm model systems, a decline was consistently noted in the planktonic phase, which was always greater than that observed in the control models. This lead to the assumption that either the virus was being removed from this phase by its adsorption to the biofilm or that the biofilm was destroying or rendering the virus inactive. Since the plaque assay method used relied on the viability of this virus a distinction between inactivated and totally destroyed virus could not be made. Similarly, when monitoring the poliovirus levels in the planktonic phase of the biofilm model, a decline in virus levels was consistently observed with time. This led to similar conclusions to those noted for the bacteriophage and since it was similar for both viruses it was assumed that it was a non-specific effect of the biofilm and not related to the particular virus.

When monitoring the poliovirus levels in sterile tap water over a time period, differences in survival times were noted when differing initial virus inocula were used. Interestingly, lower virus inocula appeared to survive for much longer periods of time than higher inocula and the higher inocula also appeared to decay at a faster rate. However, in the presence of a biofilm, the reverse was observed i.e. the virus survival time appeared to be longer in the planktonic phase when a higher initial inoculum was used. This phenomenon was attributed to the aggregation properties of the poliovirus. Previous researchers have noted that a higher virus inoculum achieved through the use of a lower dilution factor would cause the virus to aggregate. In contrast, a lower virus inoculum with a corresponding higher dilution factor would cause virus dispersal. In addition to this, they noted that a higher level of virus particles would, in turn, produce a higher collision frequency among viruses and accelerate the aggregation process further (Floyd & Sharp, 1977). Since the $TCID_{50}$ method did not distinguish between such dispersed and aggregated viral forms, the occurrence of virus aggregation in sterile tap water would be accompanied by what would appear to be a rapid decline in virus when using this method. In contrast, a dispersed virus would appear to be decaying at a much slower rate compared to the aggregated form and would be more evenly distributed thus allowing for its increased detection at the time points and making it appear to survive for longer. The same researchers also noted that in its aggregated form, the poliovirus was afforded much more protection from harsh environmental conditions, than its dispersed

counterparts. The theory behind this being that, when aggregated, the external viruses protect those on the inside (Young & Sharp, 1978; Floyd & Sharp, 1977).

In the MRD model, the poliovirus was assumed to be in its dispersed form in all experiments because of the dilution factor imposed by the inoculum. This assumption was confirmed by the viral increase observed in all the controls following virus inoculation. Once again, the results consistently showed a decline in viral load in the planktonic phase which was greater in the presence of the biofilm than in the corresponding controls. The virus survival times were also less in the presence of the water distribution system biofilm than in the control and in some experiments (i.e. when the lower inoculum was used, 1×10^5 $TCID_{50}$units ml^{-1}) the virus survival time was also shorter than both the controls and the other experiments utilising higher virus levels at the start. These results further support the theory that the biofilm had a detrimental effect on the virus in the planktonic phase.

In the sessile phase, bacteriophage lambda did adsorb and persist in the biofilm. In some cases this persistence was longer in the sessile phase than in the corresponding planktonic phase. However, in the sessile phase, the phage numbers also exhibited a decline over the time period evaluated. In addition to this, the phage numbers on the test surfaces of the non-biofilm control were either similar or higher than those in the biofilm itself. This result ruled out the theory that phage decline in the planktonic phase was simply due to its adsorption to the biofilm and reconfirmed the idea that the biofilm was detrimental to its presence.

In the MRD model, when the sessile phase was monitored by $TCID_{50}$ for poliovirus the levels present were variable in comparison to the controls. In some cases higher levels were observed on the control surfaces compared to the biofilm while in other cases the reverse was true. In all cases, however, the poliovirus levels declined in the presence of the biofilm. Interestingly when a very high initial virus inoculum was used (1×10^8 $TCID_{50}$units ml^{-1}) the poliovirus appeared to persist in the sessile phase of the MRD model for a longer period than it did in the corresponding planktonic phase. In addition to this, although at much lower levels, it appeared to persist in the biofilm for longer compared to the control. This suggests that in higher numbers the virus would fair better in the presence of the biofilm. In this case it is likely that the high viral load was accompanied by an increased collision frequency. In the control model this would result in an increase in virus aggregation and by $TCID_{50}$ this would appear as a viral decrease probably reducing the numbers to below the $TCID_{50}$ detection limits. Additionally, the high levels of viral aggregates would then reduce the collision frequency of such aggregates to surfaces thus resulting in fewer adsorbed virus in the sessile phase of the control. In contrast, the virus in the test MRD, containing the complex biofilm, would be acted upon by the detrimental products of the biofilm reducing its levels. This would produce a decreased collision frequency, in comparison to the control and so fewer aggregates would form. Also previous results evaluating the interactions of this virus with a mono-species

biofilm suggested that some sessile bacteria promote virus dispersal even under conditions where aggregation should occur. Therefore in this model the virus is more dispersed and so its collision frequency with the surfaces would be greater and hence its detection in this phase would be more frequent than in the corresponding control.

These results confirm those previously observed for the bacteriophage lambda, showing that the biofilm had a detrimental effect on the viable virus. Since both viruses exhibited similar patterns this effect was probably non-specific resulting from the harmful enzymes bacteria may produce. This is supported by previous workers who have reported the antiviral effects of bacteria (Cliver & Herrmenn, 1972) and that viruses survive for shorter periods when placed in bacterially contaminated water (Fujioka *et al*, 1980; Ward & Winston, 1985).

When monitoring the levels of bacteriophage lambda in the presence of a biofilm, two developmental stages, developing and mature, were evaluated. This was done to determine whether the detrimental actions of the biofilm occurred during biofilm development or whether the products causing the effect accumulated in the mature biofilm. The results were similar to both types of biofilm indicating that the developmental stage was not relevant. To determine whether the biofilm by-products had a similar effect to the biofilm itself, poliovirus was inoculated into filter sterilised planktonic phase which had been circulating in a biofilm model system for 14 days. This revealed that dispersed virus was able to survive for approximately the same length of time as it did in the control.

When performing experiments with poliovirus an additional detection method, RT-PCR was used. This more sensitive method allowed for the detection of both inactivated and viable virus but could not be used for virus quantitation. It should be noted that this method relied on the detection of viral nucleic acid and so may have only detected this instead of whole virus. However, poliovirus contains RNA and since this is very unstable in the environment (Jones *et al*, 1994), a positive result was assumed to be from whole virus. Surprisingly, this technique was positive for all test and control samples in both phases of the biofilm model and at all time points. This suggests that poliovirus was able to persist in both phases of the biofilm model but since it was similar to the control this cannot be attributed to the presence of the biofilm. Since this method was not quantitative, differences in viral levels between test and control could not be ascertained. The virus was probably present at levels below the detection limits of the $TCID_{50}$ method or it may have been present at higher levels but in an inactivated form. The fact that the virus could be detected still has major health implications for water distribution systems but since all controls were similar it cannot be directly attributed to the presence of the biofilm at this stage.

When monitoring the levels of poliovirus in the presence of a mono-species biofilm, very different results were observed. In this case, the virus was able to survive for long time

periods in both phases of the biofilm models and at similar levels to the corresponding controls. Indeed this biofilm did not appear to have a significant detrimental effect on the virus. One thing noticed was that even under conditions where virus aggregation was likely to be promoted, the virus in the presence of the mono-species biofilm decreased slowly indicating that it may have been dispersed. Thus this biofilm may have had the effect of promoting virus dispersal and the conclusion drawn from this was that the sessile organisms produced substances which promoted this effect. Since this biofilm was not particularly detrimental, the virus was able to adsorb to it and remain viable for the duration of the experiments. Additionally, the large surface area, stickiness of the EPS and the reduced hydrophobicity produced by the biofilms presence allowed the virus to adsorb to it in higher numbers than in the control. This result was considered to be important to establish what the interactions between biofilms and viruses but since this type of biofilm is unlikely to be present in a water distribution system it was not considered to be of importance to public health at this stage.

In general these results show that viruses can adsorb to biofilms from water distribution systems for short periods where they may be afforded limited protection. Such biofilms appear to produce substances which have a detrimental effect on the viruses present particularly on those residing in the planktonic phase. This effect produces viral decline in both phases which is non-specific promoting either destruction or inactivation of the virus. However, the use of RT-PCR revealed some conflicting results to the $TCID_{50}$ methods, showing that virus remained in both phases for the entire duration of the experiments performed. This suggests that that the viral numbers are either too low for detection by $TCID_{50}$ or that the virus is in a non-infective state.

6.2.1 Summary of Results

- Steady state biofilm formation was achieved after 24 hours in this model system and remained constant throughout the experiments and in all sections of the MRD.

- Better reproducibility between model biofilm systems was observed in biofilms after a 14 day circulation period.

- Some bacterial succession was observed particularly with the presumptive coliforms. These were later identified as either pseudomonad or pseudomonad related species.

- Using traditional culture techniques both bacteriophage lambda and poliovirus declined in the planktonic phase with time in numbers greater than those observed in the sessile phase. This suggested that the biofilm had detrimental effects on the planktonic viruses promoting either virus destruction or inactivation. This effect was non-specific.

- In the presence of a mono-species biofilm poliovirus remained present in both phases of the experiment and for it's duration. This suggested that the observed effect was due to the multiple species present in the more complex biofilm.

- In the sessile phase some viral adsorption occurred but this also declined over time. The decline was not as great in this phase compared to that observed in the planktonic phase.

- Viral adsorption to the biofilm was uniform along the length of the MRD and found to be unrelated to biofilm age.

- PCR results revealed poliovirus was present in both phases of the experiments at all time points tested. This contrasted with the $TCID_{50}$ results suggesting the virus was present in numbers below the detection limits of this method or in a non-infective form.

6.3 Future Work

At present the results show that the complex water distribution system biofilm had a detrimental effect on both types of virus tested. However, this effect was only noted on viable virus. The use of molecular methods revealed that the poliovirus remained present even when viable virus was completely undetectable. Since the PCR method used was much more sensitive than the $TCID_{50}$ method it could not be deduced whether the biofilm caused virus inactivation or whether the virus remained present in numbers below the detection limits of the latter method. In order to determine whether the biofilm caused virus destruction or rendered it inactive the total amount of virus must be detected and quantified. This could be done by using a variety of methods. DIG hybridisation was tried but constraints of time meant it could not be adequately optimised. If more difficulties arose then a quantitative PCR method would be an appropriate alternative. These results could then be compared to those obtained for viable virus and the quantity of inactivated virus could then be calculated.

The use of a mono-species biofilm showed that not all biofilms produced detrimental effects upon the virus and, at this stage, the conclusion drawn was that a complex biofilm would be required for virus destruction or inactivation. However, since only one mono-species biofilm was evaluated for its interactions with poliovirus, more mono-species biofilms composed of different bacterial species would need to be investigated to conclusively prove that a complex multi-species biofilm caused the effect. Since most biofilms in nature are multi-species biofilms this result, showing some virus persistence in the biofilm, is probably not significant.

More work could also be done to determine how the biofilm produces this non-specific detrimental effect on the virus. The microbial components of the biofilm would need to be studied in great depth to find out which enzymes and other products they produced. These products could then be incubated with the virus in combinations to determine if any of them were responsible. This information could then be extrapolated to pin point any key organisms in the biofilm that produced this effect. Further confirmation could be made through the use of mutant strains to form the initial biofilm. Problems at this stage could be encountered particularly when trying to produce a natural biofilm composed of mutant strains caused by laboratory selection and bias. Since the biofilm appears to have a negative effect on the virus in water distribution systems and the above procedures would be labour intensive, time consuming and costly these methods may not be appropriate for this field of research.

REFERENCES

1. Abad, F.X., Pinto, R.M. & Bosch, A. (1994) Survival of enteric viruses on environmental fomites. *Applied and Environmental Microbiology*, **60**, 3740-3710.

2. Abbaszadegan, M., Huber, M.S., Gerba, C.P. & Pepper, I.L. (1993) Detection of enteroviruses in groundwater with the polymerase chain reaction. *Applied and Environmental Microbiology*, **59**, 1318-1324.

3. Allison, D.G. & Gilbert, P. (1992) Bacterial biofilms. *Science Progress*, **36**, 305-321.

4. Anderson, N., Seyfried, P.L. & Doane, F.W. (1996) A co-cultivation method developed for recovery of animal and bacterial viruses from biomedical waste solids. *Journal of Microbiological Methods*, **26**, 287-290.

5. Angell, P., Arrage, A.A., Mittleman, M.W. & White, D.C. (1993) On line, non-destructive biomass determination of bacterial biofilms by fluorometry. *Journal of Microbiological Methods*, **18**, 317-327.

6. Angell, P. & White, D.C. (1995) Is metabolic activity by biofilms with sulfate-reducing bacterial consortia essential for long-term propogation of pitting corrosion of stainless steel? *Journal of Industrial Microbiology*, **15**, 329-332.

7. Atlas, R.M. & Bartha, R. (1998) Interactions among microbial populations. In: *Microbial Ecology: Fundamentals and Applications.*, pp. 60-98. Ed. Atlas, R.M. & Bartha, R. Benjamin/Cummings Science Publishing, California.

8. Atlas, R.M. & Bartha, R. (1998) Ecological aspects of biodeterioration control and soil, waste and water management. In: *Microbial Ecology: Fundamentals and Applications.*, pp. 462-510. Ed. Atlas, R.M. & Bartha, R. Benjamin/Cummings Science Publishing, California.

9. Atlas, R.M. & Bartha, R. (1998) Development of microbial communities. In: *Microbial Ecology: Fundamentals and Applications.*, pp. 174-217. Ed. Atlas, R.M. & Bartha, R. Benjamin/Cummings Science Publishing, California.

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

11. Bayston, R. (1999) Biofilms in medicine and disease: An overview. In: *Biofilms: The Good, the Bad and the Ugly.*, pp. 1-6. Ed. Wimpenny, J., Gilbert, P., Walker, J.T., Brading, M.G. & Bayston, R. Bioline, Cardiff.

12. Bell, W.H., Lang, J.M. & Mitchell, R. (1974) Selective stimulation of marine bacteria by algal extracellular products. *Limnology and Oceanography*, **19**, 833-839.

13. Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., Nienhuis, J. & Triplett, E.W. (1996) Molecular microbial diversity of an

agricultural soil in Wisconsin. *Applied and Environmental Microbiology*, **62**, 1935-1943.

14. Boyle, J., Dodds, I., Lappin-Scott, H.M. & Stoodley, P. (1999) Limits to growth and what keeps a biofilm finite. In: *Biofilms: The Good, the Bad and the Ugly*, pp. 303-315. Ed. Wimpenny, J., Gilbert, P., Walker, J.T., Brading, M.G. & Bayston, R. Bioline, Cardiff.

15. Brading, M.G., Boyle, J. & Lappin-Scott, H.M. (1995) Biofilm formation in laminar flow using *Pseudomonas fluorescens* EX101. *Journal of Industrial Microbiology*, **15**, 297-304.

16. Bradshaw, D.J. (1995) Metabolic responses in biofilms. *Microbial Ecology in Health and Disease*, **8**, 313-316.

17. Brown, M.R.W., Allison, D.G. & Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: A growth rate related effect. *Journal of Antimicrobial Chemotherapy*, **22**, 777-789.

18. Brown, M.R.W. & Gilbert, P. (1993) Sensitivity of biofilms to antimicrobial agents. *Journal of Applied Bacteriology*, **74**, 87S-97S.

19. Brown, T.A. (1991) *Gene cloning - An introduction.* Chapman & Hall, Manchester.

20. Brozel, V.S. (1994) Biofilms: a neglected bacterial habitat. *South African Journal of Science*, **90**, 200

21. Bryers, J.D. (1993) The biotechnology of interfaces. *Journal of Applied Bacteriology*, **74**, 98S-109S.

22. Campbell, A.M. (1996) Bacteriophages. In: *Virology*, pp. 587-607. Ed. Fields, B.S., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Roizman, B. & Monath, T.P. Lippincott - Raven Publishers, Philedelphia.

23. Cann, A.J. (1997) *Principles of molecular virology.* Acedemic Press, San Diego.

24. Chapron, C.D., Ballester, N.A., Fontaine, J.H., Frades, C.N. & Margolin, A.B. (2000) Detection of astroviruses, enteroviruses and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an intergrated cell culture-nested PCR procedure. *Applied and Environmental Microbiology*, **66**, 2520-2525.

25. Chen, W.P. & Kuo, T.T. (1993) A simple and rapid method for the preparation of gram negative bacterial genomic DNA. *Nucleic Acids Research*, **21**, 60

26. Chonmaitree, T., Ford, C., Sanders, C. & Lucia, H.L. (1988) Comparison of cell cultures for rapid isolation of enteroviruses. *Journal of Clinical Microbiology*, **26**, 2576-2580.

27. Christensen, W.G. & Characklis, W.G. (1990) Physical and chemical properties of biofilms. In: *Biofilms*, pp. 93-130. Ed. Characklis, W.G. & Marshall, K.C. John Wiley and Sons, Inc., New York.

28. Cliver, D.O. & Herrmann, J.E. (1972) Proteolytic and microbial inactivation of enteroviruses. *Water Research*, **6**, 797-805.

29. Coghlan, A. (1996) Slime city. *New Scientist*, 32-36.

30. Cook, N. & Myint, S. (1995) Modern methods for the detection of viruses in water and shellfish. *Reviews in Medical Microbiology*, **6**, 207-216.

31. Copley, J. (1999) Dangerous bugs may be lurking in your pipes. *New Scientist*, 14

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

33. Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D.E., Korber, D.R. & James, G. (1994) Biofilms, the customised microniche. *Journal of Bacteriology*, **176**, 2137-2142.

34. Costerton, J.W. (1995) Overview of microbial biofilms. *Journal of Industrial Microbiology*, **15**, 137-140.

35. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. & Lappin-Scott, H.M. (1995) Microbial biofilms. *Annual Reviews in Microbiology*, **49**, 711-745.

36. Cowan, S.T. & Steel (1974) *Manual for the identification of medical bacteria.* Cambridge University Press, London.

37. Dahling, D.R. & Wright, B.A. (1986) Optimization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. *Applied and Environmental Microbiology*, **51**, 790-812.

38. De Vincente, A., Aviles, M., Codina, J.C., Borrego, J.J. & Romero, p. (1990) Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. *Journal of Applied Bacteriology*, **68**, 625-632.

39. DeBeer, D., Stoodley, P. & Lewandowski, Z. (1994) Liquid flow in heterogeneous biofilms. *Biotechnology and Bioengineering*, **44**, 636-641.

40. Deng, M.Y. & Cliver, D.O. (1995) Persistence of inoculated Hepatitis A virus in mixed human and animal wastes. *Applied and Environmental Microbiology*, **61**, 87-91.

41. Doolittle, M.M., Cooney, J.J. & Caldwell, D.E. (1995) Lytic infection of *Escherichia coli* biofilms by Bacteriophage-T4. *Canadian Journal of Microbiology*, **41**, 12-18.

42. Doolittle, M.M., Cooney, J.J. & Caldwell, D.E. (1996) Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. *Journal of Industrial Microbiology*, **16**, 331-341.

43. Eaton, A.D., Clesceri, L.S. & Greenberg, A.E. (1995) *Standard methods for the examination of water and wastewater.* American Public Health Association & Water Environment Federation, New York.

44. Egger, D., Pasamontes, L., Ostermayer, M. & Bienz, K. (1995) Reverse transcription multiplex PCR for differentiation between polio and enteroviruses from clinical and environmental samples. *Journal of Clinical Microbiology*, **33**, 1442-1447.

45. Egglestone, S.I., Caul, E.O., Vipond, I.B. & Darville, J.M. (1999) Absence of human astrovirus RNA in sewage and environmental samples. *Journal of Applied Microbiology*, **86**, 709-714.

46. Eginton, P.J., Gibson, H., Holah, J., Handley, P.S. & Gilbert, P. (1995) Quantification of the ease of removal of bacteria from surfaces. *Journal of Industrial Microbiology*, **15**, 305-310.

47. Flemming, H.C. (1993) Biofilms and environmental protection. *Water Science and Technology*, **27**, 1-10.

48. Flemming, H.C. (1995) Sorption sites in biofilms. *Water Science and Technology*, **32**, 27-33.

49. Floyd, R. & Sharp, D.G. (1977) Aggregation of poliovirus and reovirus by dilution in water. *Applied and Environmental Microbiology*, **33**, 159-167.

50. Floyd, R. & Sharp, D.G. (1978) Viral aggregation: quantitation and kinetics of the aggregation of poliovirus and reovirus. *Applied and Environmental Microbiology*, **35**, 1079-1083.

51. Floyd, R. & Sharp, D.G. (1978) Viral aggregation: effects of salts on the aggregation of poliovirus and reovirus at low pH. *Applied and Environmental Microbiology*, **35**, 1084-1094.

52. Floyd, R. & Sharp, D.G. (1979) Viral aggregation: buffer effects in the aggregation of poliovirus and reovirus at low and high pH. *Applied and Environmental Microbiology*, **38**, 395-401.

53. Frais, J., Ribas, F. & Lucena, F. (1994) Substrate affinity from bacterial strains and distribution water biofilms. *Journal of Applied Bacteriology*, **76**, 182-189.

54. Fricker, C. & Smith, H. (1997) *Cryptosporidium* and Cryptosporidiosis. *SGM Quarterly*, **24**, 52-53.

55. Fuchs, F., Leparc, I., Kopeca, H., Garin, D. & Aymard, M. (1993) Use of cRNA digoxygenin-labelled probes for detection of enteroviruses in humans and in the environment. *Journal of Virological Methods*, **42**, 217-226.

56. Fuhrman, J.A. (1999) Marine viruses and their biogeochemical and ecological effects. *Nature*, **399**, 541-548.

57. Fujioka, R.S., Loh, P.C. & Lau, L.S. (1980) Survival of human enteroviruses in the hawaiian ocean environment: evidence for virus-inactivating microorganisms. *Applied and Environmental Microbiology*, **39**, 1105-1110.

58. Gause, G.F. (1934) *The struggle for existence*. Williams and Wilkins, Baltimore.

59. Geesey, G.G., Mutch, R., Costerton, J.W. & Green, R.B. (1978) Sessile bacteria: an important component of the microbial population in small mountain streams. *Limnol.Oceanography*, **23**, 1214-1223.

60. Genthe, B., Gericke, M., Bateman, B., Mjoli, N. & Kfir, R. (1995) Detection of enteric adenoviruses in South-African waters using gene probes. *Water Science and Technology*, **31**, 345-350.

61. Gerba, C.P. & Rose, J.B. (1990) Viruses in source and drinking water. In: *Drinking Water Microbiology*, pp. 380-396. Ed. McFeters, G.A. Springer verlag, New York.

62. Gerba, C.P., Rose, J.B., Haas, C.N. & Crabtree, K.D. (1996) Waterborne rotavirus: A risk assessment. *Water Research*, **30**, 2929-2940.

63. Gilbert, P. & Allison, D.G. (1993) Laboratory methods for biofilm production. In: *Microbial Biofilms: Formation and Control*, pp. 29-49. Ed. Denyer, S.P., Gorman, S.P. & Sussman, M. Blackwell Scientific Publications, Oxford.

64. Gilbert, P., Evans, D.J. & Brown, M.R.W. (1993) Formation and dispersal of bacterial biofilms *in vivo* and *in situ*. *Journal of Applied Bacteriology*, **74**, 67S-78S.

65. Gilbert, P. & Allison, D.G. (1999) Dynamics in microbial communities: A lamarkian perspective. In: *Biofilms: The Good, the Bad and the Ugly.*, pp. 263-268. Ed. Wimpenny, J., Gilbert, P., Walker, J.T., Brading, M.G. & Bayston, R. Bioline, Cardiff.

66. Grabow, W.O.K., Botma, K.L., DeVilliers, J.C., Clay, C.G. & Erasmus, B. (1999) Assessment of cell culture and polymerase chain reaction procedures for the detection of poliovirus in wastewater. *Bulletin of the World Health Organisation*, **77**, 973-980.

67. Guyader, F.L., Dubois, E., Menard, D. & Pommepuy, M. (1994) Detection of Hepatitis A virus, Rotavirus and Enterovirus in naturally contaminated shellfish and sediment by reverse transcription-seminested PCR. *Applied and Environmental Microbiology*, **60**, 3665-3671.

68. Hejkal, T.M., Wellings, F.M., LaRock, P.A. & Lewis, A.L. (1979) Survival of Poliovirus within organic solids during chlorination. *Applied and Environmental Microbiology*, **38**, 114-118.

69. Hennes, K.P., Suttle, C.A. & Chan, A.M. (1995) Fluorescently labelled virus probes show that natural virus populations can control the structure of marine microbial communities. *Applied and Environmental Microbiology*, **61**, 3623-3627.

70. Herb, S., Stair, J.O., Ringelberg, D.B., White, D.C. & Flemming, H.C. (1995) Characterisation of biofilms on corroded concrete surfaces in drinking water reservoirs. *Water Science and Technology*, **32**, 141-147.

71. Hermansson, M., Jones, G.W. & Kjelleberg, S. (1987) Frequency of antibiotic and heavy metal resistance, pigmentation and plasmids in bacteria of the marine air - water interface. *Applied and Environmental Microbiology*, **53**, 2338-2342.

72. Herrick, J.B., Madsen, E.L., Batt, C.A. & Ghiorse, W.C. (1993) Polymerase chain reaction amplification of naphthalene-catabolic and 16S rRNA gene sequences from indigenous sediment bacteria. *Applied and Environmental Microbiology*, **59**, 687-694.

73. Hfaliger, D., Hubner, P. & Luthy, J. (2000) Outbreak of gastroenteritis due to sewage contaminated drinking water. *International Journal of Food Microbiology*, **54**, 123-126.

74. Homma, W.C. & Melnick, J.L. (1972) A portable virus concentrator for testing water in the field. *Water Research*, **6**, 1249-1256.

75. Hurst, C.J. (1991) Presence of enteric viruses in freshwater and their removal by the conventional drinking water treatment process. *Bulletin of the World Health Organisation*, **69**, 113-119.

76. Hyypia, T., Auvinen, P. & Maaronen, M. (1989) Polymerase chain reaction for human picornaviruses. *Journal of General Virology*, **70**, 3261-3268.

77. Jahn, A. & Nielson, P.H. (1995) Extraction of extracellular polymeric substances (EPS) from biofilms using a cation exchange resin. *Water Science and Technology*, **32**, 157-164.

78. Jakubovics, N.S. & Dow, C.S. (1997) The succession of microorganisms on interfaces immersed in potable water. In: *Biofilms: Community Interactions and Control*, pp. 203-214. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

79. Jass, J., Costerton, J.W. & Lappin-Scott, H.M. (1995) Assessment of a chemostat-coupled modified Robbins device to study biofilms. *Journal of Industrial Microbiology*, **15**, 289

80. Jess, J., High, N.J., Epton, H.A.S., Sigee, D.C., O'Niell, J.G., Meier, H. & Handley, P.S. (1997) Comparison of the API 20NE biochemical identification system and partial 16S rRNA gene sequencing for the characterisation of culturable heterotrophs from a water distribution system biofilm. In: *Biofilms: Community Interactions and Control*, pp. 149-155. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

81. Jones, P., Qiu, J. & Rickwood, D. (1994) *RNA - Isolation and analysis.* BIOS Scientific Publishers Limited, Oxford.

82. Keevil, C.W., Dowsett, A.B. & Rogers, J. (1993) Legionella biofilms and their control. In: *Microbial Biofilms: Formation and Control.*, pp. 201-216. Ed. Denyer, S.P., Gorman, G.W. & Sussman, M. Blackwell Scientific Publications, Oxford.

83. Keevil, C.W., Rogers, J. & Walker, J.T. (1995) Potable-water biofilms. *Microbiology Europe*, **3**, 10-14.

84. Kerr, C.J., Osborn, K.S., Robson, G.D. & Handley, P.S. (1997) The effect of substratum on biofilm formation in drinking water systems. In: *Biofilms: Community Interactions and Control*, pp. 167-174. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

85. Keswick, B.H., Gerba, C.P., DuPont, H.L. & Rose.J.B. (1984) Detection of enteric viruses in treated drinking water. *Applied and Environmental Microbiology*, **47**, 1290-1294.

86. Khardori, N. & Yassien, M. (1995) Biofilms in device-related infections. *Journal of Industrial Microbiology*, **15**, 141-147.

87. Kilvington, S. & White, D.G. (1994) *Acanthamoeba*: biology, ecology and human disease. *Reviews in Medical Microbiology*, **5**, 12-20.

88. Kok, T.W., Pryor, T. & Payne, L. (1998) Comparison of rhabdomyosarcoma, buffalo green monkey epithelial, A549 (human lung epithelial) cells and human embryonic lung fibroblasts for isolation of enteroviruses from clinical samples. *Journal of Virological Methods*, **11**, 61-65.

89. Kopecka, H., Dubrou, S., Prevot, J., Marechal, J. & Lopez-Pila, J.M. (1993) Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction and hybridization. *Applied and Environmental Microbiology*, **59**, 1213-1219.

90. Korber, D.R., Lawrence, J.R., Lappin-Scott, H.M. & Costerton, J.W. (1995) Growth of microorganisms at sufaces. In: *Microbial Biofilms*, pp. 15-45. Ed. H.M.Lappin-Scott & J.W.Costerton Cambridge University Press, Cambridge.

91. Kukkula, M., Arstila, P., Klossner, M.L., Maunula, L. & Bonsdorf, C.H.V. (1997) Waterborne outbreak of viral gastroenteritis. *Scandanavian Journal of Infectious Diseases*, **29**, 415-418.

92. Labarbe, R., Treloar, R., Watson, K. & Allison, C. (1997) Structural analysis of oral biofilms using optical microscopy methods. In: *Biofilms: Community Interactions and Control.*, pp. 11-14. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

93. Lawrence, J.R., Korber, D.R., Costerton, J.W. & Caldwell, D.E. (1991) Optical sectioning of microbial biofilms. *Journal of Bacteriology*, **173**, 6558-6567.

94. Lawrence, J.R., Wolfaart, G.M. & Korber, D.R. (1994) Determination of diffusion coefficients in biofilms by confocal laser microscopy. *Applied and Environmental Microbiology*, **60**, 1166-1173.

95. Lawrence, J.R., Korber, D.R., Wolfaart, G.M. & Caldwell, D.E. (1995) *Behavioural strategies of surface-colonising bacteria.* Plenum Press, New York.

96. LeChavallier, M.W., Babcock, T.M. & Lee, R.G. (1987) Examination and characterization of distribution system biofilms. *Applied and Environmental Microbiology*, **53**, 2714-2724.

97. Lee, T.W. & Kurtz, J. (1981) Serial propogation of Astrovirus in tissue culture with the aid of trypsin. *Journal of General Virology*, **57**, 421-424.

98. Lipson, S.M. & Stotzky, G. (1984) Effect of proteins on Reovirus adsorption to clay minerals. *Applied and Environmental Microbiology*, **48**, 525-530.

99. Lisle, J.T. & Rose, J.B. (1995) Gene exchange in drinking water and biofilms by natural transformation. *Water Science and Technology*, **31**, 41-46.

100. Mackay, W.G., Gribbon, L.T., Barer, M.R. & Reid, D.C. (1998) Biofilms in drinking water systems - A possible reservoir for *Helicobacter pylori. Water Science and Technology*, **38**, 181-185.

101. Mackerness, C.M., Colbourne, J.S., Dennis, P.J., Rachwal, T. & Keevil, C.W. (1993) Formation and control of coliform biofilms in drinking water distribution systems. In: *Microbial Biofilms: Formation and Control.*, pp. 217-226. Ed. Denyer, S.P., Gorman, G.W. & Sussman, M. Blackwell Scientific Publications, Oxford.

102. Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K.H. & Stenstrom, T.A. (1993) *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. *Applied and Environmental Microbiology*, **59**, 2293-2298.

103. Marsh, P.D. & Bradshaw, D.J. (1995) Dental plaque as a biofilm. *Journal of Industrial Microbiology*, **15**, 169-175.

104. Marshall, K.C. (1987) Colonisation, adhesion and biofilms. In: *Manual of Environmental Microbiology*, pp. 358-365. Ed. Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. & Walter, M.V. ASM Press, Washington DC.

105. Matsui, S.M. & Greenberg, H.B. (1996) Astroviruses. In: *Fields Virology*, pp. 811-824. Ed. Fields, D.M., Knipe, D.M. & Howley, P.M. Lippincott-Raven Publishers, Philadelphia.

106. McFeters, G.A., Yu, F.P., Pyle, B.H. & Stewart, P.S. (1995) Physiological methods to study biofilm disinfection. *Journal of Industrial Microbiology*, **15**, 333-338.

107. McMath, S.M., Chamberlain, A.H.L., Lloyd, B.J. & Holt, D.M. (1997) The natural history of biofilms in a model water distribution system. In: *Biofilms: Community Interactions and Control*, pp. 193-202. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

108. McMath, S.M. & Holt, D.M. (1999) Are biofilms the source of low-level coliforms in water distribution systems? In: *Biofilms: The Good, the Bad and the Ugly.*, pp. 147-153. Ed. Wimpenny, J., Gilbert, P., Walker, J.T., Brading, M.G. & Bayston, R. Bioline, Cardiff.

109. Melnick, J.L. & Rennick, V. (1980) Infectivity titers of enterovirus as found in human stools. *Journal of Medical Virology*, **5**, 205-220.

110. Melnick, J.L. (1996) Enteroviruses: Polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: *Virology*, pp. 655-711. Ed. Fields, B.S., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Roizman, B. & Monath, T.P. Lippincott - Raven Publishers, Philedelphia.

111. Metcalf, T.G., Melnick, J.L. & Estes, M.K. (1995) Environmental virology: From detection of virus in sewage and water by isolation to identification by molecular biology - A trip of over 50 years. *Annual Reviews in Microbiology*, **49**, 461-487.

112. Middelboe, M., Jorgensen, N.O.G. & Kroer, N. (1996) Effects of viruses on nutrient turnover and growth efficiency of noninfected marine bacterioplankton. *Applied and Environmental Microbiology*, **62**, 1991-1997.

113. Momba, M.N.B., Kfir, R., Venter, S.N. & Cloete, T.E. (2000) An overview of biofilm formationin distribution systems and its impact on the deterioration of water quality. *Water, SA*, **26**, 59-66.

114. Munnecke, D.M. & Hsieh, D.P.H. (1976) Pathway of microbial metabolism of parthion. *Applied and Environmental Microbiology*, **31**, 63-69.

115. Nestor, I. (1995) Human viruses possibly present in the environment. *Central Europian Journal of Public Health*, **3**, 84-91.

116. Nivens, D.E., Palmer, R.J. & White, D.C. (1995) Continuous nondestructive monitoring of microbial biofilms: a review of analytical techniques. *Journal of Industrial Microbiology*, **15**, 263-276.

117. O'Niell, J.G., Banks, J. & Jess, J. (1997) Biofilms in water mains - Now under control. In: *Biofilms: Community Interactions and Control*, pp. 259-268. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

118. O'Toole, G.A. & Kolter, R. (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS356 proceeds via multiple convergent signalling pathways: a genetic analysis. *Molecular Microbiology*, **28**, 449-461.

119. Patel, J.R., Daniel, J. & Mathan, V.I. (1985) A comparison of the susceptibility of three human gut tumour-derived differentiated epithelial cell lines, primary monkey kidney cells and human rhabdomyosarcoma cell line to 66-prototype strains of human enteroviruses. *Journal of Virological Methods*, **12**, 209-216.

120. Payment, P. (1981) Isolation of viruses from drinking water at the Pont-Viau water treatment plant. *Canadian Journal of Microbiology*, **27**, 417-420.

121. Payment, P., Ayache, R. & Trudel, M. (1983) A survey of enteric viruses in sewage. *Canadian Journal of Microbiology*, **29**, 111-119.

122. Payment, P., Gamache, F. & Paquette, G. (1988) Microbiological and virological analysis of water from two water filtration plants and their distribution systems. *Canadian Journal of Microbiology*, **34**, 1304-1309.

123. Pennisi, E. (1995) The secret language of bacteria. *New Scientist*, 30-33.

124. Pinto, R.M., Abad, F.X., Gajardo, R. & Bosch, A. (1996) Detection of infectious astrovirus in water. *Applied and Environmental Microbiology*, **62**, 1811-1813.

125. Pratt, L.A. & Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type 1 pili. *Molecular Microbiology*, **30**, 285-293.

126. Prescott, L.M., Harley, J.P. & Klien, D.A. (1993) The aquatic environment. In: *Microbiology*, pp. 818-837. AnonymousWm.C.Brown Publishers., Dubuque.

127. Quignon, F., Sardin, M., Kiene, L. & Schwartzbrod, L. (1997) Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Applied and Environmental Microbiology*, **63**, 978-982.

128. Quignon, F., Kleine, L., Levi, Y., Sardin, M. & Schwartzbrod, L. (1997) Virus behaviour within a water distribution system. *Water Science and Technology*, **35**, 311-318.

129. Rao, V.C. (1982) Introduction to environmental virology. In: *Methods in Environmental Virology*, pp. 1-13. Ed. Gerba, C.P. & Goyal, S.M. Dekker, New York.

130. Reed, L.J. & Muench, H. (1938) A simple method for estimating fifty percent endpoints. *American Journal of Hygiene*, **27**, 493-497.

131. Rittle, K.H., Helmstetter, C.E., Meyer, A.E. & Baier, R.E. (1990) *Escherichia coli* retention on solid surfaces as a function of substratum surface energy and cell growth phase. *Biofouling*, **2**, 121-130.

132. Robinson, P.J., Walker, J.T., Keevil, C.W. & Cole, J. (1995) Reporter genes and fluorescent probes for studying the colonisation of biofilms in a drinking water supply line by enteric bacteria. *FEMS Microbiology Letters*, **129**, 183-188.

133. Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V. & Keevil, C.W. (1994) Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Applied and Environmental Microbiology*, **60**, 1585-1592.

134. Rollecke, S., Muyzer, G., Wawer, C., Wanner, G. & Lubitz, W. (1996) Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, **62**, 2059-2065.

135. Roy, B., Ackermann, H.W., Pandian, S., Picard, G. & Green, R.B. (1993) Biological inactivation of adhering *Listeria monocytogenes* by listeriaphages and quaternary ammonium compounds. *Applied and Environmental Microbiology*, **59**, 2914-2917.

136. Rueckert, R.R. (1996) Picornaviridae: The viruses and their replication. In: *Virology*, pp. 609-653. Ed. Fields, B.S., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Roizman, B. & Monath, T.P. Lippincott - Raven Publishers, Philedelphia.

137. Sagripanti, J.L., Rouston, L.B. & Lytle, C.D. (1993) Virus inactivation by copper or iron ions alone and in the presence of peroxide. *Applied and Environmental Microbiology*, **59**, 4374-4376.

138. Sakoda, A., Sakai, Y., Hayakawa, K. & Suzuki, M. (1997) Adsorption of viruses in water environment onto solid surfaces. *Water Science and Technology*, **35**, 107-114.

139. Sambrook, K.J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning: A laboratory manual.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

140. Schvoerer, E., Bonnet, F., Dubois, V., Rogues, A.M., Gachie, J.P., Lafron, M.E. & Fleury, H.A. (1999) A hospital outbreak of gastroenteritis possibly related to the contamination of tap water by a small round structured virus. *Journal of Hospital Infection*, **43**, 149-154.

141. Schwab, K.J., De Leon, R. & Sobsey, M.D. (1995) Concentration and purification of beef extract mock eluates from water samples for detection of enteroviruses, Hepatitis A virus and Norwalk virus by reverse transcription-PCR. *Applied and Environmental Microbiology*, **61**, 531-537.

142. Schwartz, T., Hoffman, S. & Obst, U. (1998) Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. *Water Research*, **32**, 2787-2797.

143. Sekla, L., Stackiw, W., Kay, C. & VanBuckenhout, L. (1980) Enteric viruses in renovated water in Monitoba. *Canadian Journal of Microbiology*, **26**, 518-523.

144. Sharp, D.G., Young, D.C., Floyd, R. & Johnston, J.D. (1980) Effect of ionic environment on the inactivation of poliovirus in water by chlorine. *Applied and Environmental Microbiology*, **39**, 530-534.

145. Sharp, D.G. & Leong, J. (1980) Inactivation of poliovirus I (Brunhilde) single particles by chlorine in water. *Applied and Environmental Microbiology*, **40**, 381-385.

146. Stickler, D. (1999) Biofilms. *Current Opinion in Microbiology*, **2**, 270-275.

147. Surman, S.B., Walker, J.T., Goddard, D.T., Morton, L.H.G., Keevil, C.W., Weaver, W., Skinner, A., Hanson, K., Caldwell, D.E. & Kurtz, J. (1996) Comparison of microscopic techniques for the examination of biofilms. *Journal of Microbiological Methods*, **25**, 57-70.

148. Suttle, C.A. (1987) Community structure: Viruses. In: *Manual of Environmental Microbiology*, pp. 272-277. Ed. Hurst, C.J., Knudsen, G.R., McInerney, M.J. & Stetzenbach, L.D. ASM Press, Washington DC.

149. Suttle, C.A. (1994) The significance of viruses to mortality in aquatic microbial communities. *Microbial Ecology*, **28**, 237-243.

150. Szewzyk, U., Manz, W., Amann, R., Schleifer, K.H. & Stenstrom, T.A. (1994) Growth and *in situ* detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water bacterium by use of 16S and 23S-rRNA directed fluorescent oligonucleotide probes. *FEMS Microbiology Ecology*, **13**, 169-176.

151. Tall, B.D., Williams, H.N., George, K.S., Gray, R.T. & Walch, M. (1995) Bacterial succession within a biofilm in water supply lines of dental air-water syringes. *Canadian Journal of Microbiology*, **41**, 647-654.

152. Tambini, G., Andrus, J.K., Marques, E., Boshell, J., Pallansch, M. & de Quadros, C.A.K.O. (1993) Direct detection of wild Poliovirus circulation by stool surveys of healthy children and analysis of community wastewater. *Journal of Infectious Disease*, **168**, 1510-1514.

153. Tani, N., Dohi, Y., Kurumatani, N. & Yonemasu, K. (1995) Seasonal distribution of adenoviruses, enteroviruses and reoviruses in urban river water. *Microbial Immunology*, **39**, 577-580.

154. Totsuka, A., Ohtaki, K. & Tagaya, I. (1978) Aggregation of enterovirus small plaque variants and polioviruses under low ionic strength conditions. *Applied and Environmental Microbiology*, **38**, 519-533.

155. Tsai, Y.L., Sobsey, M.D., Sangermano, L.R. & Palmer, C.J. (1993) Simple method of concentrating enteroviruses and Hepatitis A virus from sewage and ocean water for rapid detection by reverse transcription-polymerase chain reaction. *Applied and Environmental Microbiology*, **59**, 3488-3491.

156. Ueda, T. & Horan, N.J. (2000) Fate of indigenous bacteriophage in a membrane bioreactor. *Water Research*, **34**, 2151-2159.

157. van der Kooij, D., Vrouwenvelder, H.S. & Veenendaal, H.R. (1995) Kinetic aspects of biofilm formation on surfaces exposed to drinking water. *Water Science and Technology*, **32**, 61-65.

158. van der Wende, E. & Characklis, W.G. (1990) Biofilms in potable water distribution systems. In: *Drinking Water Microbiology*, pp. 249-268. Ed. McFeters, G.A. Springer-Verlag, New York.

159. van Loosdrecht, M.C.C., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L. & Heijnen, J.J. (1995) Biofilm structures. *Water Science and Technology*, **32**, 35-43.

160. Voet, D. & Voet, J.G. (1990) *Biochemistry*. John Wiley & Sons, New York.

161. W.G.Characklis & K.C.Marshall (1990) Biofilms: A basis for an interdisciplinary approach. In: *Biofilms*, pp. 3-15. Ed. W.G.Characklis & K.C.Marshall John Wiley & Sons, Inc., New York.

162. Wahl, M. (1989) Marine epibiosis I. Fouling and antifouling: Some basic aspects. *Marine Ecological Progress Series*, **58**, 175-189.

163. Wainwright, M., Canham, L.T., al-Wajeeh, K. & Reeves, C.L. (1999) Morphological changes (including filamentation) in *Escherichia coli* grown under starvation

conditions on silicon wafers and other surfaces. *Letters in Applied Microbiology*, **29**, 224-227.

164. Walker, J.T., Mackerness, C.M., Rogers, J. & Keevil, C.W. (1995) Heterogeneous mosaic biofilm - A haven for waterborne pathogens. In: *Microbial Biofilms*, pp. 196-203. Ed. H.M.Lappin- Scott & J.W.Costerton Cambridge University Press, Cambridge.

165. Wallis, C., Melnick, J.L. & Gerba, C.P. (1979) Concentration of viruses from water by membrane chromatography. *Annual Reviews in Microbiology*, **33**, 413-437.

166. Ward, R.L. & Winston, P.E. (1985) Development of methods to measure virus inactivation in fresh waters. *Applied and Environmental Microbiology*, **50**, 1144-1148.

167. Wilderer, P.A. & Characklis, W.G. (1989) Structure and function of biofilms. In: *Structure and Function of Biofilms*, pp. 5-17. Ed. Characklis, W.G. & Wilderer, P.A. John Wiley & Sons Ltd, Hamberg.

168. Willcock, L., Holah, J., Allison, D.G. & Gilbert, P. (1997) Population dynamics in steady - state biofilms: Effects of growth environment upon dispersal. In: *Biofilms: Community Interactions and Control*, pp. 23-32. Ed. Wimpenny, J., Handley, P.S., Gilbert, P., Lappin-Scott, H.M. & Jones, M. Bioline, Chippenham.

169. Willcocks, M.M., Carter, M.J. & Madeley, C.R. (1992) Astroviruses. *Reviews in Medical Virology*, **2**, 97-106.

170. Wimpenny, J. (1995) Biofilms: Structure and organisation. *Microbial Ecology in Health and Disease*, **8**, 305-308.

171. Yang, S.N. & Lewandowski, Z. (1995) Measurement of local mass-transfer coefficient in biofilms. *Biotechnology and Bioengineering*, **48**, 737-744.

172. Yates, J.Y., Thompson, S.S. & Jury, W.A. (1997) Sorption of viruses during flow through saturated sand columns. *Environmental Science and Technology*, **31**, 548-555.

173. Young, D.C. & Sharp, D.G. (1977) Poliovirus aggregates and their survival in water. *Applied and Environmental Microbiology*, **33**, 168-177.

174. Young, P. (1996) Safe drinking water: A call for global action. *ASM News*, **62**, 349-352.

175. Zhang, T.C. & Bishop, P.L. (1994) Structure, activity and composition of biofilms. *Water Science and Technology*, **29**, 335-344.

176. Zobell, C. (1943) The effect of solid surfaces on bacterial activity. *Journal of Bacteriology*, **46**, 39-56.

APPENDICES

APPENDIX 1 – Formulations of Solutions and Buffers

Solutions for Evaluating the Biofilm

1. Acridine Orange stain

50 mg acridine orange stain (BDH, microscopical stains), 10 mls Potassium phosphate buffer pH 7.4

Sterilisation was achieved by filtering through a 0.45 μm membrane (Acrodisc). This stain could only be used for a few days if stored at 4°C in the dark.

2. Stock Potassium Phosphate buffer

42 mls Potassium phosphate dibasic solution, 8 mls Potassium phosphate monobasic solution.

3. Stock Potassium Phosphate Solutions – Monobasic

3.40 g KH_2PO_4 (BDH), 500 mls distilled H_2O

Dibasic

4.35 g K_2HPO_4 (BDH), 500 mls distilled H_2O

The pH was adjusted to 7.5 in the monobasic solution and sterilisation was achieved by autoclaving.

4. Non-Nutrient Agar (NNA)

7.5 g Agar (Lab M), 1 tablet ¼ strength Ringers (Sigma), 500 mls distilled H_2O

Sterilisation was achieved by autoclaving.

SDS - PAGE Mini Gels

GEL COMPOSITION	12% RESOLVING GEL	STACKING GEL
H_2O	3.3 mls	3.4 mls
30% Acrylamide solution	4.0 mls	830 μls
1.5M Tris Buffer (pH 8.8)	2.5 mls	-
1.0M Tris Buffer (pH 6.8)	-	630 μls
10% Ammonium Sulphate[1]	100 μls	50 μls
10% SDS	100 μls	50 μls
TEMED	5 μls	5 μls

[1] Ammonium sulphate was made up fresh every time as it goes off

SDS - PAGE Solutions

1. SDS PAGE Running Buffer	30.3 g Tris Powder, 144 g Glycine, 10 g SDS dissolved in 5 L H_2O
2 SDS PAGE Sample Buffer	50 mM Tris pH 6.8, 100 Mm DDT, 2% SDS, 0.1% Bromophenol Blue, 10% Glycerol.
3. Coomassie Blue Solution	0.1% Coomassie Blue powder, 10% acetic acid (v/v), 25% Isopropanol (v/v), 65% H_2O
4. Destain Solution	10% acetic acid (v/v), 25% Isopropanol (v/v), 65% H_2O (v/v)

Solutions For DNA Extraction, PCR And Sequencing

1. Lysis Buffer	40 mM Tris-acetate pH 7.8, 20 mM sodium - acetate,1 mM EDTA, 1% SDS.

Solutions For Phage Work

1.Phage Buffer	10 mM Tris pH 7.5, 10 mM $MgSO_4.7H_2O$, 0.01% Gelatin solution. Sterilisation was achieved by autoclaving the former 2 chemicals in solution and adding the filter sterilised gelatin aseptically afterwards.
2. Soft Top Agar	Tryptone 10 gL^{-1}, Yeast extract 5 gL^{-1}, NaCl 5 gL^{-1}, Agar 7 gL^{-1}. Sterilisation was achieved by autoclaving.

Solutions For Poliovirus Work

1. Versene Solution	0.1 g EDTA, I tablet PBS (Oxoid), 100 mls distilled H_2O Sterilisation was achieved by autoclaving.
2. Formal Saline	5 g NaCl, 15 g Na_2SO_4, 900 mls distilled H_2O, 100 mls 40% Formalin*

Sterilisation was achieved by autoclaving prior to the addition of Formalin*.

3. Crystal Violet stain — 2.5 g crystal violet stain (BDH), 1 L distilled H_2O

Solutions for DIG Hybridisation

1. Blocking Solution — 10 g Blocking reagent (vial 6)[1],100 mls Maleic acid buffer

2. Hybridisation Solution — 5x SSC, 0.1% (w/v) N-lauryl-sarcosine[2], 0.02% (w/v) SDS[2], 1% Blocking Reagent

3. Washing Buffer — Maleic acid buffer, 0.3% (w/v) Tween 20[2]

4. 2x Wash Solution — 2x SSC, 0.1% SDS

5. 0.5x Wash Solution — 0.5x SSC, 0.1% SDS

6. Antibody Solution — 1 µl Anti-Dig alkaline phosphatase (vial 4)[1], 5 mls blocking solution

7. Detection Buffer — 100 mM Tris[3] pH 9.5, 100 mM NaCl

8. Colour Substrate Solution — 200 µls NBT/BCIP (vial 5)[1], 10 mls detection buffer

9. Maleic Acid Buffer — 0.1 M Maleic acid, 0.15 M NaCl pH 7.5[4]

10. 20x SSC — 3 M NaCl, 300 mM sodium citrate pH 7.0

All solutions were treated with DEPC to remove RNases and sterilised by autoclaving unless otherwise stated below.

[1] All reagents were taken from the DIG High Prime Labelling and Detection Starter Kit I (for colour detection with NBT/BCIP; Boehringer Mannheim, GmbH, Biochemica).

[2] Sterilisation of these solutions was achieved by filtration through a 0.2-0.45 µm membrane.

[3] Tris was treated with DEPC as it has an adverse effect.

[4] For this solution the pH was adjusted using concentrated NaOH

APPENDIX 2 – Formulae used For Calculations

Calculating Total Bacterial Counts following Acridine Orange staining

Area of Filter = 490.9 mm^2

Area of Grid = 1×10^{-3}

∴ There are 4.91×10^5 grid areas per filter

$$\frac{B\ (4.91 \times 10^5)}{Vf} \times DF = \text{No. Bacteria } ml^{-1}$$

Where B = mean bacteria from 10 fields

Vf = volume filtered (usually 3 mls)

DF = dilution factor (usually 10)

Calculating the Amoebae Counts

This method utilised the Reed and Muench (1938) Calculations

E.G.

Dilution	Reading	Ratio	+ve	-ve	Total +ve	Total -ve	Ratio	%
10^{-1}	3	3/3	3	0	8	0	8/8	100
10^{-2}	3	3/3	3	0	5	0	5/5	100
10^{-3}	2	2/3	2	1	2	1	2/3	66.6
10^{-4}	0	0/3	0	3	0	4	0/4	0

$$\frac{\%\text{ Next Above 50\% - 50\%}}{\%\text{ Next Above 50\% - \% Next Below 50\%}}$$

66.6 – 50% / 66.6 – 0 = 16.6 / 66.6 = 0.25

Add this value to the dilution next above 50% (3) = 3.25

Anti-log of 3.25 = 1778.3 = **1.78×10^3 amoebae ml^{-1}**

Calculating Cell Numbers using a Neubauer Counting Chamber

$$A \times 25 \times 10^4 = \text{No. of cells } ml^{-1}$$

Where A = mean number of cells per square

$TCID_{50}$ Calculation

E.G. See Figure 2.1 for the $TCID_{50}$ result for a stock poliovirus suspension.

Virus Dilution	CPE Ratio	No CPE (NC)	CPE (C)	Σ NC	Σ C	Total Ratio	% C/C+ NC
10^{-1}	10/10	0	10	0	67	67/67	100
10^{-2}	10/10	0	10	0	57	57/57	100
10^{-3}	10/10	0	10	0	47	47/47	100
10^{-4}	10/10	0	10	0	37	37/37	100
10^{-5}	10/10	0	10	0	27	27/27	100
10^{-6}	9/10	1	9	1	17	17/18	94.4
10^{-7}	7/10	3	7	4	8	8/12	66.67
10^{-8}	1/10	9	1	13	1	1/14	7.14

Here the 50% CPE point is between 10^{-7} and 10^{-6}. It is located at the proportionate distance (PD) from 10^{-7}

$$PD = \frac{\text{CPE Above 50\% - 50\%}}{\text{CPE Above 50\% - CPE below 50\%}}$$

$$PD = 66.67\text{-}50 \,/\, 66.67 - 7.14 = 16.67 \,/\, 59.53 = 0.28$$

The titre of the virus suspension which produced CPE in 50% of the wells was:

$$10^{7+0.28} = 1.905 \times 10^{7}\ TCID_{50}\text{units}/100\mu\text{ls}$$

$$\therefore = \mathbf{1.91 \times 10^{8}\ TCID_{50}units\ ml^{-1}}$$

NB This calculation is a variation on the Reed and Muench (1938) Calculation used for the enumeration of amoebae.

APPENDIX 3 – The Identification of Bacterial Isolates

The following bacterial matches were made using the BLAST database at the website address: www.ncbi.nlm.nih.gov/BLAST/

1. The Blue Spreader – R312

```
Gb|AF216704.1|AF216704 Pseudomonas aeruginosa 1001 16S ribosomal RNA gene,
partial sequence
Length = 1491

Score = 985 bits (495), Expect = 0.0
Identities = 505/509 (99%)
Strand = Plus / Plus

Query: 1   agcggcggacgggtgagtaatgcctaggaatctgcctggtagtgggggataacgtccgga  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 68  agcggcggacgggtgagtaatgcctaggaatctgcctggtagtgggggataacgtccgga 127

Query: 61  aacgggcgctaataccgcatacgtcctgagggagaaagtgggggatcttcggacctcacg 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:128  aacgggcgctaataccgcatacgtcctgagggagaaagtgggggatcttcggacctcacg 187

Query:121  ctatcagatgagcctaggtcggattagctagttggtggggtaaaggcctaccaaggcgac 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:188  ctatcagatgagcctaggtcggattagctagttggtggggtaaaggcctaccaaggcgac 247

Query:181  gatccgtaactggtctgagaghatgatcagtcacactggaactgagacacggtccagact 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:248  gatccgtaactggtctgagaghatgatcagtcacactggaactgagacacggtccagact 307

Query:241  cctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgc 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:308  cctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgc 367

Query:301  cgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagta 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:368  cgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagta 427

Query:361  agttaataccttgctgttttgacgttaccaacagaataagcaccggctaacttcgtgcca 420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:428  agttaataccttgctgttttgacgttaccaacagaataagcaccggctaacttcgtgcca 487

Query:421  gcagccgcggtaatacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgc 480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:488  gcagccgcggtaatacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgc 547

Query:481  gtargtgggtcaacaagttggatttgaaa 509
           ||| |||| ||| ||||||||| |||||
Sbjct:548  gtaggtggttcagcaagttggatgtgaaa 576
```

2. The Yellow Pigmented strain – R302

ab|U37525.1|SYU37525 Sphingomonas yanoikuyae Q1 16S rRNA
Length 1406

Score = 1090 bits (550), Expect = 0.0
Identities = 564/566 (99%), Gaps = 2/566 (0%)
Strand = Plus / Plus

```
Query:  1   cgagatcttcggatctagtggcgcacgggtgcgtaacgcgtgggaatctgcccttgggtt  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36   cgagatcttcggatctagtggcgcacgggtgcgtaacgcgtgggaatctgcccttgggtt  95

Query: 61  cggaataacttctggaaacggaagctaataccggatgatgacgtaagtccaaagatttat 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 96   cggaataacttctggaaacggaagctaataccggatgatgacgtaagtccaaagatttat 155

Query:121   cgcccaaggatgagcccgcgtaggattagctagttggtgaggtaaaggctcaccaaggcg 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:156   cgcccaaggatgagcccgcgtaggattagctagttggtgaggtaaaggctcaccaaggcg 215

Query:181   acgatccttagctggtctgagaggatgatcagccacactgggactgagacacggcccaga 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:216   acgatccttagctggtctgagaggatgatcagccacactgggactgagacacggcccaga 275

Query:241   ctcctacgggaggcagcagtagggaatattggacaatgggcgaaagcctgatccagcaat 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:276   ctcctacgggaggcagcagtagggaatattggacaatgggcgaaagcctgatccagcaat 335

Query:301   gccgcgtgagtgatgaaggccttagggttgtaaagctcttttacccgggatgataatgac 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:336   gccgcgtgagtgatgaaggccttagggttgtaaagctcttttacccgggatgataatgac 395

Query:361   agtaccgggagaataagctccggctaactccgtgccagcagccgcggtaatacggaggga 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:396   agtaccgggagaataagctccggctaactccgtgccagcagccgcggtaatacggaggga 455

Query:421   gctagcgttgttcggaattactgggcgtaaagcgcacgtaggcggctattcaagtcagag 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:456   gctagcgttgttcggaattactgggcgtaaagcgcacgtaggcggctattcaagtcagag 515

Query:481   gtgaaagccc-gggctcaaccccggaactg-ctttgaaactagatagcttgaatccagga 538
            |||||||||| |||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct:516   gtgaaagcccggggctcaaccccggaactgcctttgaaactagatagcttgaatccagga 575

Query:539   gaggtgagtggaattccgagtgtaga 564
            |||||||||||||||||||||||||
Sbjct:576   gaggtgagtggaattccgagtgtaga 601
```

3.Orange Pigmented strain- R305

ab|U20772.2|SSU20772 Blastobacter sp. SMCC B0477 16S small subunit RNA gene, partial sequence
Length = 1307

```
Score = 1079 bits (543), Expect = 0.0
Identities = 551/553 (99%), Gaps = 1/553 (0%)
Strand = Plus / Plus

Query:  1   tagtggcgcacgggtgcgtaacgcgtgggaatctgcccttgggttcggcataacagtgag  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 69   tagtggcgcacgggtgcgtaacgcgtgggaatctgcccttgggttcggcataacagtgag 128

Query: 61   aaattactgctaataccggatgatgacttcggtccaaagatttatcgcccaaggacgagc 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:129   aaattactgctaataccggatgatgacttcggtccaaagatttatcgcccaaggacgagc 188

Query:121   ccgcgtaagattagctagttggtgaggtaaaggctcaccaaggcgacgatctttagctgg 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:189   ccgcgtaagattagctagttggtgaggtaaaggctcaccaaggcgacgatctttagctgg 248

Query:181   tctgagaggatgatcagccacactgggactgagacacggcccagactcctacgggaggca 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:249   tctgagaggatgatcagccacactgggactgagacacggcccagactcctacgggaggca 308

Query:241   gcagtggggaatattggacaatgggcgaaagcctgatccagcaatgccgcgtgagggttg 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:309   gcagtggggaatattggacaatgggcgaaagcctgatccagcaatgccgcgtgagggttg 368

Query:301   aaggccttagggttgtaaagctcttttaccagggatgataatgacagtacctggagaata 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:369   aaggccttagggttgtaaagctcttttaccagggatgataatgacagtacctggagaata 428

Query:361   agctccggctaactccgtgccagcagccgcggtaatacggagggagctagcgttgatcgg 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:429   agctccggctaactccgtgccagcagccgcggtaatacggagggagctagcgttgatcgg 488

Query:421   aattactgggcgtaaagcgcacgtaggcggccattcaagtcagaggtgaaagcccggggc 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:489   aattactgggcgtaaagcgcacgtaggcggccattcaagtcagaggtgaaagcccggggc 548

Query:481   tcaaccccggaactgcctttgaaactagatggcttgaatcttgga-aggcgagtggaatt 539
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct:549   tcaaccccggaactgcctttgaaactagatggcttgaatcttggagaggcgagtggaatt 608

Query:540   ccgagtgtaragg 552
            |||||||||| |||
Sbjct:609   ccgagtgtagagg 621
```

4. ORG 001 From MRD 1 – M001

ab|AF232713.1|AF232713 Pseudomonas mendocina 16S ribosomal RNA gene, partial sequence
Length = 1439

```
Score = 743 bits (373), Expect = 0.0
Identities = 389/393 (98%), Gaps = 2/393 (0%)
Strand = Plus / Plus
```

```
Query:  1  ccggaaacgggcgctaataccgcatacgtyctacgggagaaagcaggggaccttcgggcc  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:113  ccggaaacgggcgctaataccgcatacgtcctacgggagaaagcaggggaccttcgggcc 172

Query: 61  ttgcgctatcagatgagcctaggtcggattagctagttggtgaggtaatggctcaccaag 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:173  ttgcgctatcagatgagcctaggtcggattagctagttggtgaggtaatggctcaccaag 232

Query:121  gcgacgatccgtaactggtctgagaggatgatcagtcacactggaactgagacacggtcc 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:233  gcgacgatccgtaactggtctgagaggatgatcagtcacactggaactgagacacggtcc 292

Query:181  agactcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatccagc 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:293  agactcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatccagc 352

Query:241  catgccgcgtgtgtgaagaargtcttcggattgtaaagcactttaagttggga-gaaggg 299
           |||||||||||||||||| ||||||||||||||||||||||||||||||| ||||||
Sbjct:353  catgccgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaaggg 412

Query:300  cattaacctaatacgttagtgttttgacgttaccgacagaataagcaccggctaacttcg 359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:413  cattaacctaatacgttagtgttttgacgttaccgacagaataagcaccggctaacttcg 472

Query:360  tgccagcagsccgcggtaatacgaagggtgcaa 392
           ||||||||| ||||||||||||||||||||||
Sbjct:473  tgccagcag-ccgcggtaatacgaagggtgcaa 504
```

5. ORG 001 From MRD's 2 and 3 – M312

ab|AF232713.1|AF232713 Pseudomonas mendocina 16S ribosomal RNA gene, partial sequence
Length = 1439

Score = 800 bits (373), Expect = 0.0
Identities = 406/410 (99%)
Strand = Plus / Plus

```
Query:101  gaaacgggcgctaataccgcatacgtcctacgggagaaagcaggggaccttcgggccttg 160
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:116  gaaacgggcgctaataccgcatacgtcctacgggagaaagcaggggaccttcgggccttg 175

Query:161  cgctatcagatgagcctaggtcggattagctagttggtgaggtaatggctcaccaaggcg 220
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:176  cgctatcagatgagcctaggtcggattagctagttggtgaggtaatggctcaccaaggcg 235

Query:221  acgatccgtaactggtctgagaggatgatcagtcacactggaactgagacacggtccaga 280
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:236  acgatccgtaactggtctgagaggatgatcagtcacactggaactgagacacggtccaga 295

Query:281  ctcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatycagccat 340
           |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct:296  ctcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctgatccagccat 355
```

```
Query:341  cccgcgtgtgtgaagaargtcttcggattgtaaagcactttaagttgggaggaagggcat 400
           ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct:356  cccgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcat 415

Query:401  taacctaatacgttagtgttttgacgttaccgacagaataagcaccggctaacttcgtgs 460
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:416  taacctaatacgttagtgttttgacgttaccgacagaataagcaccggctaacttcgtgs 475

Query:461  cagcagccgcggtaatacgaagggtgcaagcgttaatcggaattactggg 510
           |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:476  cagcagccgcggtaatacgaagggtgcaagcgttaatcggaattactggg 525
```

5. ORG 002 From MRD 1 – M002

ab|AF274316.1|AF274316 Delftia sp. JK-2 16S ribosomal RNA gene, partial sequence
Length = 1495

Score = 800 bits (325), Expect = 0.0
Identities = 582/590 (98%)
Strand = Plus / Plus

```
Query:  1  gctgacgagtggcgaacgggtgagtaatacatcggaacgtgcccagtcgtgggggataac  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:119  gctgacgagtggcgaacgggtgagtaatacatcggaacgtgcccagtcgtgggggataac 178

Query: 61  tactcgaaagagtagctaataccgcatacgatctgaggatgaaagcgggggaccttcggg 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:179  tactcgaaagagtagctaataccgcatacgatctgaggatgaaagcgggggaccttcggg 238

Query:121  cctcgcgcgattggagcggccgatggcagattaggtagttggtgggataaaagcttacca 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:239  cctcgcgcgattggagcggccgatggcagattaggtagttggtgggataaaagcttacca 298

Query:181  agccgacaatctgtagctggtctgagaggacgaccagccacactgggactgagacgcggc 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:299  agccgacaatctgtagctggtctgagaggacgaccagccacactgggactgagacgcggc 358

Query:241  ccagactcctacgggaggcagcagtggggaattttggacaatgggcgaaagcctgatcct 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:359  ccagactcctacgggaggcagcagtggggaattttggacaatgggcgaaagcctgatcct 418

Query:301  agccaacaatctgtagcggtctgagaggacgaccagccacactgggactgagacgcggc 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:419  agccaacaatctgtagctggcctgagaggacgaccagccacactgggactgagacgcggc 478

Query:361  atccgacaatctgtagctggtctgagagsacgaccagccacactgggactgagacgcggc 420
           |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct:479  atccgacaatctgtagctggtctgagaggacgaccagccacactgggactgagacgcggc 538

Query:421  aaccgacaatctgta-ctggtctgagaggacgaccagccacactgggactgagacgcggc 480
           |||||||||||||||| |||||||||||||||||||||| ||||||||||||||||||||
Sbjct:539  aaccgacaatctgtagctggtctgagaggacgaccagccatactgggactgagacgcggc 598
```

```
Query:481  agccgacaatctgtagctggtctgagaggacgaccagccacactgggactgagacgcggc 540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:599  agccgacaatctgtagctggtctgagaggacgaccagccacactgggactgagacgcggc 658

Query:541  tcaatgccgcgtccaggatgaaggccttcgtgttgtaaa-tgcttttgta 590
           |||||||||||||| |||||||||||||||||| |||||||| ||||||||||
Sbjct:659  tcaatgccgcgtgcaggatgaaggccttcgggttgtaaactgcttttgta 709
```

6. ORG 002 From MRD's 2 and 3 – M305

ab|AF098288.1|AF098288 Ralstonia sp. FRA01 16S ribosomal RNA gene, partial sequence
Length = 1495

Score = 631 bits (313), Expect = e-179
Identities = 330/335 (98%), Gaps = 2/335
Strand = Plus / Plus

```
Query: 97  taataccgcatacgacctgagggtgaaagcgggggaccgaaaggcctcgckcagcaggag 156
           |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct:146  taataccgcatacgacctgagggtgaaagcgggggaccgaaaggcctcgcgcagcaggag 205

Query:157  cggccgatgtctgattagctagttggtggggtaaaggcccaccaaggcgacgatcagtag 216
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:206  cggccgatgtctgattagctagttggtggggtaaaggcccaccaaggcgacgatcagtag 265

Query:217  ctggtctgagaggacgatcagccacactgggactgagacacggcccagact-ctacggga 275
           |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct:266  ctggtctgagaggacgatcagccacactgggactgagacacggcccagactcctacggga 325

Query:276  ggcagcagtggggaattttggacaatgggggcaaccctgatycagcaatgccgcgtgtgt 335
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct:326  ggcagcagtggggaattttggacaatgggggcaaccctgatccagcaatgccgcgtgtgt 385

Query:336  gaagaaggccttcgggttgtaaagcacttttgtccggaaagaaattgctctggctaatac 395
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:386  gaagaaggccttcgggttgtaaagcacttttgtccggaaagaaattgctctggctaatac 445

Query:396  ctggggtagatggac-gthccggaagaataagcacc 429
           |||||||||||||| || ||||||||||||||||
Sbjct:446  ctggggtagatggac-gthccggaagaataagcacc 480
```