Fate of *Salmonella* Typhimurium in biofilms of drinking water distribution systems

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted for a degree at any other university.

Date _____

Signature _____

CONFERENCE CONTRIBUTIONS

The following aspects of this work have been presented as posters at international conferences:

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SUMMARY

Fate of *Salmonella* Typhimurium in biofilms of drinking water distribution systems

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The propensity of *Salmonella* to persist in water environments under unfavourable conditions is of concern as these water environments serve as contamination reservoirs. The role of contaminated water in the transmission of *Salmonella* in developing countries is largely unknown. The fate and persistence of non-typhoidal *Salmonella* in water environments and the specific influence of the indigenous microbiota on the survival and growth of *Salmonella* is poorly understood. A tagged *Salmonella* strain distinguishable *in vivo* from a mixed bacterial community would greatly facilitate the study of *Salmonella* in water environments.

The clinically relevant *S. enterica* subsp. *enterica* ser. Typhimurium isolate was chromosomally tagged using the pUT mini–Tn5 Km transposon with the green fluorescent protein gene *gfp*mut3b*. Southern Blot hybridisation confirmed that the *gfp* gene had integrated into the chromosome. The *gfp* gene was stably maintained and the *gfp*-labelled recombinants were not growth rate impaired under low nutrient

conditions. No significant changes were observed between the wild-type and the tagged strain. The survival fitness studies indicated the incorporation of the *gfp* gene did not have any noted detrimental effects on the survival and behaviour of the tagged strains. These tagged strains could therefore be used to study the fate and survival of *Salmonella* in biofilms of drinking water distribution systems.

Genetic tagging of the target organism with the *gfp* gene, encoding the green fluorescent protein, allows *in situ* detection of undisturbed cells and is ideally suited for monitoring *Salmonella* as a monospecies or in a complex mixed community. The fate and persistence of non-typhoidal *Salmonella* in drinking water biofilms was investigated. The ability of *Salmonella* to form biofilms independently and the fate and persistence of *Salmonella* in an aquatic biofilm was examined.

In monoculture *S.* Typhimurium formed loosely structured biofilms. *Salmonella* colonized established multi-species drinking water biofilms within 24 hours, growing to form micro-colonies within the biofilm. *S*. Typhimurium was also released at high levels from the drinking water-associated biofilm into the flow, and was seen to re-colonize elsewhere. Results showed that *Salmonella* can enter into, survive and grow within, and be released from a drinking water biofilm. Once *Salmonella* has entered into a distribution system, it will be able to colonize an existing biofilm, grow in it and be released into the flow for re-colonization elsewhere, and possible subsequent infection of consumers.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µl	microlitre
Abs	absorbancy
Amp	ampicillin
Amp^R	ampicillin resistance
ARC	Agricultural Research Council
ATP	adenosine triphosphate
ave	average
BG	brilliant green
bp	base pair
BPW	buffered peptone water
CCD	charged coupled device
CDC	Centres for Disease Control and Prevention
CFU	colony forming unit
CSLM	confocal scanning laser microscopy
CTC	5 cyan-2, 3-ditolyl tetrazolium chloride
DAPI	4,6 diamino-2-phenylindole
ddH_2O	double distilled water
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
EDTA	ethylenediamine-tetra-acetic acid
eg.	for example
Fig.	figure
FISH	fluorescent *in situ* hybridisation
$FMNH_2$	reduced flavin mononucleotide
GEM's	genetically engineered microorganisms
GFP	green fluorescent protein
h	hour
H_2S	hydrogen sulphide

HCl	hydrochloric acid
HIV	human immunodeficiency virus
Km	kanamycin
Km^R	kanamycin resistance
l	litre
LB-medium	Luria-Bertani medium
Log	Logarithm
M	Mol
m/v	mass per volume
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mM	millimol
MPN	most probable number
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
OD	optical density
PCR	polymerase chain reaction
PI	propidium iodide
PM	phenotypic microarray
rdar	red, dry and rough
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RV	Rappaport-Vassiliadis
Sp.	species
U	unit
US	United States
USA	United States of America
UV	ultraviolet
VBNC	viable but non-culturable
vs	versus

w/v	weight per volume
XLD	xylose-lysine-deoxycholate

CHAPTER ONE
LITERATURE REVIEW

1.1 INTRODUCTION

The provision of safe drinking water is one of the most important challenges faced by countries due to an increasing population and industrialisation, which leads to poor living standards and inadequate sanitation. It is imperative that water supplies are protected so that the water that reaches the consumer is safe for consumption and free from any substances that are harmful including pathogenic bacteria. *E.coli, Salmonella* and *Vibrio* are the most reported water-borne bacterial pathogens (Szewzyk *et al.,* 2000).

Salmonella is an endemic public health concern worldwide. *Salmonella* is a primary agent in gastroenteritis in both humans and animals and many large outbreaks have been documented. Each year an estimated 1, 4 million cases of salmonellosis occur among humans in the United States (Mead *et al.,* 1999). The agent is usually transmitted by food (D'Aoust *et al.,* 1997) or water (Harrey *et al.,* 1967). *Salmonella* is a commonly isolated agent of bacteraemic HIV-positive patients (Gordon *et al.,* 2000). Although eradication of salmonellae is not possible, provision of safe potable water, sanitary food and waste treatment may reduce the incidence of salmonellosis (National Academy of Sciences, 1969).

The genus *Salmonella* contains facultatively anaerobic, gram-negative rods, and belongs to the family Enterobacteriaceae. According to the latest proposed classification system, the genus *Salmonella* contains two species, each of which has multiple serotypes. The two species are *Salmonella enterica*, the type species, and *Salmonella bongori* (Brenner *et al.,* 2000). There are currently 2,463 serotypes (serovars) of *Salmonella* (Popoff *et al.,* 2000).

Salmonella is continually released into the environment through infected hosts such as humans, farm animals, pets and wildlife (Baudart *et al.,* 2000). *Salmonella* has been isolated from a large number of animal species (Scherer and Miller, 2001). This

suggests that *Salmonella* lack special host adaptations and are capable of colonising a wide variety of organisms such as mammals, birds and reptiles (CDC, 2005). Many potential routes are used for the transmission of these excreted enteric pathogens (Baudart *et al.,* 2000). The ubiquitous nature of *Salmonella* may assist a cyclic lifestyle consisting of the passage through a host into the environment and back into a new host (Thomason *et al.,* 1977). The ability of *Salmonella* to be transmitted by any of these routes depends largely on its resistance to environmental factors, which control its survival and its capacity to be carried by water as it moves through the environment (Baudart *et al.,* 2000). Long-term survival of *Salmonella* in the secondary habitat ensures its passage to the next host (Winfield and Groisman, 2003). The enhanced survival of *Salmonella* in soil and water and on a variety of surfaces provides the bacterium with an increased probability of infecting a new host. This tendency of *Salmonella* to persist in hostile environments further heightens public health concerns.

The fate and persistence of non-typhoidal *Salmonella* in water environments has not been studied in detail due to the lack of a tagged strain distinguishable *in vivo* from the rest of the bacterial community. In order to protect and manage the quality of water sources, a clear understanding of the fate, survival and transport of this pathogenic bacterium is necessary.

1.2 THE IDENTIFICATION AND DETECTION OF BACTERIA

1.2.1 Conventional culturing and detection

Viable plate counts or most probable number (MPN) techniques are frequently used for quantification of active cells in environmental samples. It is estimated that only a small fraction, possibly below 10%, of micro-organisms have been grown in pure culture and characterised (Staley and Konopka, 1985; Torsvik *et al*., 1990). Plate count measures are time consuming due to the lengthy incubations that are required. Plate counts do not provide useful information regarding microbial activity and viability in the absence of exogenous nutrient supplementation. Bacteria are also physically removed from the native sample and are therefore no longer subject to possible inhibitory substances and conditions that may limit their activity in the native

sample (Rodriguez *et al*., 1992). Plate count procedures can also not be used to directly observe active cells at the single cell level. It has been shown that plate count techniques used to monitor the survival of enteric pathogenic bacteria in river water can greatly underestimate the viability and survival potential of *Salmonella* species (Santo Domingo *et al.,* 2000).

In MPN determinations of *Salmonella* it is normal practice to inoculate sample volumes into enrichment medium. The removal of *Salmonella* from environmental samples to enrichment media that supports its growth merely allows for an estimate of the initial number of *Salmonella.* Enrichment and pre-enrichment broths can dilute inhibitory compounds produced by competing bacteria in the sample as well as aid in the recovery of injured, stressed and lag-phase *Salmonella* cells (Clark and Ordal, 1969). Resuscitation techniques for the detection of injured *Salmonella* cells involve a pre-enrichment broth such as buffered peptone water. Buffered peptone water allows for the resuscitation of some of the sub lethally injured cells (Vanderzant and Splittstoesser, 1992). This step is usually followed by selective enrichment and cultivation. Rappaport Vassiliadis enrichment broth is usually recommended as selective enrichment medium when isolating *Salmonella* from environmental samples (Van Schothorst and Renaud, 1983). Xylose-lysine-deoxycholate (XLD) and brilliant green (BG) -phenol red-lactose-sucrose agars have been found to be the most selective media for the culturing of all *Salmonella* serotypes (Moriñigo *et al.,* 1989). XLD selects those strains that are *Salmonella*-like, because they contain indicators of H_2S production and pH changes.

It has also been shown that microbial inoculants grown in the laboratory may loose their ability to be cultured on agar medium after long-term incubation (Oliver, 2000). Gram-negative bacteria, like *Salmonella* enter this viable but non-culturable state (VBNC) in which they no longer grow on conventional media, but remain intact and retain their viability (Chowdbury, 2000). The VBNC status has previously been described for *Salmonella* Enteritidis (Morinigo *et al.,* 1990), *Salmonella* Typhimurium (Jimenez *et al.,* 1989) and *Salmonella* Salamae (Monfort and Baleux, 1994) under conditions of starvation. It is reported that serotypes such as *S.* Typhimurium, which have entered the VBNC state, retain their capacity to cause disease and are therefore still active (McDougald *et al.,* 1998). This information is

crucial as pathogenic bacteria in aquatic habitats that are not immediately culturable may still serve as a risk for human exposure.

1.2.2 Direct counts

Direct counts allow enumeration of total bacteria in a given environment and although it is not species specific, it provides additional information regarding the size and morphology of cells. This method cannot be used for samples containing spiked populations, as there is no way to distinguish a specific organism from the rest of the bacterial community. This technique is therefore only applicable for the detection of micro-organisms when working with pure cultures under sterile conditions. It is frequently reported that direct microscopic counts exceed culturable cell counts by several orders of magnitude for oligotrophic to mesophyllic habitats. This is also true for sediments and soils where it is known that the majority of microscopically visualised cells are viable, but do not form visible colonies on plates (Amann *et al.,* 1995).

Direct counts are often combined with staining methods to determine the number of viable cells. Dyes such as acridine orange or 4,6 diamino-2-phenylindole (DAPI) have traditionally been used to estimate bacterial numbers in diverse environmental samples. These dyes function by staining any DNA-containing cell. They eliminate unspecific staining of dead cells that do not contain significant amounts of DNA, but include inactive bacteria in the count. Some staining methods are based on the retention of cell membrane integrity as a determining characteristic of the bacterial viability status. The fluorescent nucleic acid stain propidium iodide (PI) only enters cells with a compromised plasma membrane. Advantages of theses methods are ease of application and the potential for *in situ* use such as biofilm studies. It was found that PI-based counts of *E. coli* in ground water and effluent were considerably higher than plate counts for most samples taken, indicating the presence of damaged or dying cells (Banning *et al.,* 2002).

A more direct indicator of viability is the detection of respiratory activity. The redox dye 5 cyano-2, 3-ditolyl tetrazolium chloride (CTC) has been employed for direct epifluorescent microscopic enumeration of actively respiring bacteria in

environmental samples (Rodriguez *et al.,* 1992). Oxidised CTC is almost colourless and is non-fluorescent, however the compound is reduced by electron transport activity to fluorescent insoluble CTC-formazan, which gathers intracellularly in organisms such as *Salmonella* Typhimurium (Rodriguez *et al.,* 1992). CTC is however, only reduced in low redox environments (e.g. in the presence of sodium dithionite). Carbon supplementation of most native samples is required to maximise the number of bacteria that can actively reduce CTC in the selected time frame (Rodriguez *et al.,* 1992). This method is therefore not effective for the investigation of starving cells or cells exposed to changing environments (Sternberg *et al*., 1999). The addition of nutrients alters the native environment and does not truly reflect the metabolic capabilities of the bacterial community.

1.2.3 Polymerase chain reaction for identification

Techniques based on the polymerase chain reaction (PCR) provide rapid and sensitive detection of bacteria, even when cells cannot be cultured. This technique does however provide limited information on the number of microbes and their spatial distribution within a particular environmental sample. When detecting pathogens in environmental samples, problems arise in removing colloidal debris or humic substances which can inhibit the PCR reaction (Pillai *et al*., 1991). PCR often requires enrichment of environmental samples to increase the *Salmonella* numbers and to dilute potential inhibitors that may interfere with the PCR reaction. One of the concerns regarding the use of PCR for water monitoring is the recovery of PCR-compatible DNA (Purohit and Kapley, 2002). Another disadvantage is that there is no discrimination between active or inactive organisms. Masters *et al.,* (1994) showed that the precise correlation of cell viability with detection of DNA was poor as DNA persisted in actively killed cells for significant periods of time. *Salmonella* has been identified in river water samples by PCR targeting the *invA* locus. Multiplex PCR analysis has been used to allow for the detection of *Salmonella* species in environmental samples from soil and water using *phoP*, *Hin* and *H-li* primer sets (Kapley, 2000; Way *et al*., 1993).

1.3 *In situ* DETECTION IN MIXED CULTURE COMMUNITIES

In most natural environments, bacteria do not exist as monospecies, but as complex communities consisting of more than one microbial species. It is often necessary to obtain knowledge regarding complex multi-species microbial communities, as they would occur in the environment. Populations are usually arranged in a specific way, such as biofilms, and these communities display activities that cannot be achieved by individual organisms (Lawrence *et al.,* 1995). Techniques are required to observe the activity and distribution of microbes directly in minimally disturbed native samples, to determine how many cells of a certain species are in a defined spatial element in a given time and to determine the *in situ* activity of an individual microbial cell in a native environment. Recent developments enhancing the ability to observe the structure and function of microbial communities *in situ* have been developed.

1.3.1 Fluorescent antibodies for identification

Immunofluorescent microscopy is a well-established technique (Bohlool and Schmidt, 1980) that makes use of fluorescent antibodies that target specific antigens. One disadvantage of this technique is non-specific staining of bacteria due to antibody cross-reactivity. Since the immunofluorescence approach requires prior isolation of pure cultures for raising specific antibodies, it is not cultivation independent (Amann *et al.,* 1995). Some bacterial antigens are only expressed under specific conditions. When fluorescent antibodies were used for the detection and identification of sulphate-reducing bacteria in nature, it was found that culture conditions influence the expression of surface antigens. For this reason, fluorescent antibodies produced against culture collection strains have limited applications in detecting, enumerating and identifying these micro-organisms in nature (Cloete and de Bruyn, 2001). A microcolony fluorescent antibody procedure was used to detect *Salmonella* in environmental, food, and feed samples and sufficiently detected all specimens found positive by culture (Thomason, 1971). Specific analyses of spatial distributions in biofilm systems can be obtained by using immunofluorescence (Bohlool and Schmidt, 1980). Once labelled, however, the sample is usually no longer biologically active, making this approach inappropriate for real-time studies (Camper *et al.,* 1999). Fluorescent antibodies have also been used to detect *Legionella pneumophila* in thin

mixed population biofilms (Rogers and Keevil, 1992). Stewart *et al.*, (1997) used the fluorescent antibody labelling method to identify specific organisms in intact and dispersed mixed population biofilms. However, the methods were developed using defined mixed population biofilms. Desmonts *et al.,* (1990) used a fluorescent antibody technique for detecting viable but non-culturable *Salmonella* species in chlorinated wastewater.

1.3.2 Fluorescent *in situ* hybridisation (FISH)

Fluorescent *in situ* hybridisation with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments (Amann *et al.,* 2001). FISH is as sensitive as the well-established immunofluorescent techniques (Bohlool and Schmidt, 1980), but instead of targeting antigens, it is based on the recognition of specific nucleic acid sequences. The rRNA's are the main target molecules for FISH as they are found in all living organisms, they are relatively stable, occur in high numbers in actively growing cells (usually several 1000 per cell), and they also include both variable and highly conserved domains (Amann *et al.,* 1990; Amann *et al.,* 1995). Signature sequences unique to chosen groups of organisms from whole phyla to individual species can therefore be identified (Amann *et al.,* 2001). The oligonucleotide probes used for FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5'-end to a single fluorescent dye molecule. This method is said to provide species identification, quantification and it provides information about the growth state of cells due to the correlation between ribosome content and cellular growth rate. Microscopic analysis has not yet been automated sufficiently to allow high sample throughput, which would be desirable in many ecological investigations (Glöckner *et al.,* 1999).

The disadvantages of this technique are that there may be permeability concerns and that stability of ribosomes under a variety of conditions may differ in different bacteria and therefore determinations of cellular ribosome concentrations are not directly comparable (Sternberg *et al.,* 1999). It is only in cells growing under steady state conditions that the content of ribosomes truly reflects the growth state of the cells (Möller *et al.,* 1995). The correlation between growth rate and ribosome content

may only be relevant for bacteria able to grow under optimal conditions, which carry several copies of the rRNA operon (Sternberg *et al.,* 1999). Monitoring of growth activity using this method, may therefore only give indications of actively growing cells when there is significant nutrient supply. This method is not completely accurate, as ribosomes remain intact for a considerable time after the *de nova* synthesis of rRNA has ceased (Sternberg *et al.,* 1999). This approach reveals the history of the cells rather than the current physiological growth state of the organisms.

FISH has played an important role in determining the abundance and spatial organisation of complex communities. The use of these fluorescently labelled ribosomal probes allows the identification of specific organisms within a biofilm and can be used to determine the spatial distribution within the biofilm (Donlan and Costerton, 2002). Fang *et al.,* (2003) have used FISH with 23S rRNA probes for the detection and identification of *Salmonella* species in naturally contaminated food samples. FISH has also been used as an *in situ* identification method for bacteria in drinking water and adjoining biofilms (Manz *et al*., 1993). Poulsen *et al*., (1993) used rRNA *in situ* hybridisation to measure the activity of single cells in young and established biofilms. FISH has been used to identify strains of bacteria that are the main constituents in drinking water biofilms and indicates an approach to describing bacterial diversity and community structure and function (Kalmbach *et al.,* 1997).

1.3.3 Biomarkers

An alternative molecular tool for the identification of cells *in situ* is the use of biomarkers as specific tags. A biomarker or marker gene is defined as a DNA sequence introduced into an organism, which confers a distinct genotype or phenotype to enable monitoring in a given environment (Jansson and de Bruijn, 1999). Marker gene tagging is a specific method to identify and quantify populations of micro-organisms in a certain locations. The choice of a biomarker depends on the bacterial strain as well as the sensitivity and specificity of the detection that is required (Jansson *et al.,* 2000). Examples of biomarkers are luciferase and green fluorescent protein.

1.3.3.1 Luciferase biomarker

The luciferase biomarker system allows micro-organisms to be tagged with the firefly luciferase gene (*luc*), or the bacterial luciferase genes (*luxAB*). The bacteria can be easily detected and counted as luminescent colonies on agar plates. Plate-counting detection is however also possible with other markers and is subject to the limitations of culture-based methods. The main advantage of luminescent markers is the ability to directly monitor light output in a luminometer, without the necessity for cultivation of the cells (Rattray *et al.,* 1990) or a scintillation counter in chemiluminescence mode (Belas *et al.,* 1986). Luciferase activity can also be directly monitored by visual or microscopic observations (Flemming *et al.,* 1994). Although bioluminescence represents a powerful tool for *in situ* analysis, it may be problematic to use, since the reaction requires the addition of an exogenous substrate for the light reaction to occur. When using *lux*AB genes as a reporter, an aldehyde substance, usually *n*-decanal, must be supplied to the cells during the assay procedure. Although this penetrates the cells readily, it can be toxic in low doses. Alternatively, *luxCDABE*, which contains all five genes of the *lux* operon, can be used. This is a completely independent light-generating system that does not require an additional substrate (Applegate *et al*., 1998). It has, however, been found that cells containing the entire *lux* operon exhibit reduced viability compared to cells containing only *luxAB* (Amin-Hanjani *et al*., 1993). Luciferase enzymes are dependent on cellular activity reserves or reducing equivalents for bioluminescence. ATP is required for firefly luciferase and $FMNH_2$ for bacterial luciferase. Therefore, the light output is indicative of a metabolically active population of cells. If the cells are growing, the light output is proportional to the number of cells in the sample (Jansson *et al.,* 2000).

After long-term incubation in soil environments or other harsh environments, microbial cells become starved or stressed. The light production from luciferase enzymes declines in response to energy status (Duncan *et al.,* 1994). When *Pseudomonas fluorescens* cells tagged with *lux*AB genes were monitored in sterile soil microcosms, the light output decreased over time, but the number of viable cells remained relatively constant (Meikle *et al.,* 1994). Therefore, *in situ* bioluminescence is not a reliable indicator of microbial biomass under starvation conditions (Jansson *et al.,* 2000).

An environmental isolate of *Salmonella* Typhi was chromosomally tagged with *lux*AB genes encoding luciferase. This *lux*-tagging method was compared to a *gfp*-tagging method for verifying a VBNC state of bacteria in the environment. The survival of *Salmonella* Typhi introduced into groundwater microcosms was examined by luminescence-based plate counts and the measurement of bioluminescence. A VBNC state of *S.* Typhi could not be verified by a *lux*-based system, however, in comparison, the *gfp*-based direct viable counts and plate counts could verify the VBNC state of *S.* Typhi (Cho and Kim, 1999a).

1.3.3.2 Green Fluorescent Protein (GFP) biomarker

The GFP gene encoding green fluorescent protein is obtained from the jellyfish *Aequorea victoria*. Depending on the nature of the study, GFP fluorescence can be monitored by exposing the cells to UV light (Tresse *et al.,* 1998), by fluorometric detection (Burlage *et al.,* 1996), epifluorescence microscopy (Chalfie *et al.,* 1994), confocal laser scanning microscopy (Eberl *et al.,* 1997) or flow cytometry (Tombolini *et al.,* 1997). No other energy source or substrate addition is required other than the presence of oxygen during the initial formation of the chromophore. The chromosomal tagging of bacterial strains using GFP allows for the possibility of performing survival studies under mixed culture conditions. In this way, a more realistic indication of the growth potential of *Salmonella* is obtained due to the interaction with competing indigenous micro-organisms. GFP has recently been optimised as a marker for bacteria in environmental samples (Unge *et al.,* 1997; Tombolini and Jansson, 1998). GFP is ideally suited to the quantification of tagged organisms in environmental samples as no substrate addition is required and the detection of fluorescence is independent of energy reserves. GFP can be detected *in situ* in single cells and GFP is a very stable protein (Andersen *et al.,* 1998). GFP is a good choice for non-disruptive studies of bacterial systems that require live cells to be studied at the single cell level (Andersen *et al.,* 1998). The wild-type GFP from the jellyfish *Aequorea victoria* must undergo a series of self-modifications in order to become fluorescent (Tsien, 1998). This requires oxygen and results in the appearance of fluorescence lagging behind the synthesis of the actual protein (Albano *et al*., 1996). Wild-type GFP is usually resistant to proteolysis, (Tsien, 1998) which means that once made, GFP will persist in a cell after the promoter that drives its expression is shut down (Leveau and Lindow, 2001). GFP variants, which have significantly

reduced maturation times such as enhanced GFP (EGFP) (Patterson *et al*., 1997) and GFPmut3 (Cormack *et al*., 1996) have been created to circumvent these properties of wild-type GFP. Unstable variants of GFP with reduced half-lives such as GFP [ASV], GFP [AAV], and GFP [LVA] have been engineered to be susceptible to degradation by indigenous housekeeping proteases (Andersen *et al*., 1998). The stable GFPmut3 is about 20 times more fluorescent than wild-type GFP when excited at 488nm (Cormack *et al*., 1996). It has been found that GFP fluorescence is maintained in viable but non-culturable cells, but is lost from dead cells (Lowder *et al.,* 2000). When GFP is fused with a growth regulated promoter, a system like this will show cell growth activity, which is very useful for monitoring of bacterial development in environmental samples. In biofilms, this system also allows the growth activity to be tracked to the level of individual cells, cell clusters and microcolonies (Sternberg *et al.,* 1999). Recently, spectral variants of GFP with red- and blue-shifted fluorescence emissions have been characterised, which allows for the possibility of double labelling with two different coloured GFP fusion proteins (Ellenberg *et al.*, 1999). The use of the cyan (*cfp*) and yellow (*yfp*) variants of *gfp* has also allowed studies of multiple cellular processes within a single cell (Feucht and Lewis**,** 2001).

Bacteria tagged with *gfp* have been used to study the distribution and dynamics of bacterial populations in soils (Errampalli *et al.,* 1998; Tresse *et al.,* 1998), aqueous systems (Stretton *et al.,* 1998), activated sludge (Eberl *et al.,* 1997; Olofsson *et al.,* 1998), rhizospheres (Gage *et al.,* 1996; Tombolini *et al.,* 1997), and biofilms (Möller *et al.,* 1998; Skillman *et al.,* 1998). Eberl *et al.,* (1997) found that by using epifluorescent microscopy individual cells of the tagged *Pseudomonas putida* strain could be visualised in activated sludge due to their bright fluorescence. This allowed for online monitoring of this strain after introduction into a complex sludge community. Tombolini *et al.,* (1997) demonstrated the use of *gfp*-tagged *Pseudomonas fluorescens* for *in situ* study in rhizospheres. Although some autofluorescence from soil particles was observed, the *gfp*-tagged cells were clearly distinguishable. The green fluorescent phenotype was detected in all growth phases even under nutrient-limited conditions. GFP has been used to tag an environmental isolate of *Salmonella* Typhi. A comparison was made between GFP-based direct viable counts and plate counts. It was shown that *S.* Typhi enters a VBNC state in groundwater and pond water. *S.* Typhi was shown to survive longer in ground water

than in pond water as both a culturable and VBNC state (Cho and Kim, 1999b.). A chromosomally labelled GFP strain of *Pseudomonas putida* was used to assess the single cell activity of this strain in biofilms (Sternberg *et al.,* 1999). Möller *et al.,* (1998) has demonstrated that that the GFP system is ideal for studying multiple-species bacterial communities. It was found that *gfp*-tagged *Pseudomonas putida* tended to colonise the outermost layer of a mixed species biofilm while an *Acinetobacter* strain primarily attached to the substratum supporting the biofilm (Möller *et al*., 1998).

1.4 METHODS FOR TAGGING CELLS WITH GFP

1.4.1 Plasmids for GFP-tagging

In the past, plasmids have been the favoured tools for gene cloning and expression, but they do present many difficulties for maintaining a recombinant phenotype under working environmental conditions. Recombinant DNA inserted into the chromosome is usually very stably maintained, whereas plasmid recombinant DNA may be lost during cell division. Another disadvantage of using plasmids is that plasmid copy number, which is often hard to predict, may affect gene expression and may even cause physiological changes in the cell (Jensen *et al.,* 1993). The possibility of gene transfer from one species to another is also a risk with plasmids. For strains that are destined for use in an environmental situation, it is imperative that the desired traits are encoded and maintained on the chromosome, ensuring stability and predictability of the engineered phenotype (Eberl *et al.,* 1997).

Leff and Leff (1996) used a plasmid-borne green fluorescent protein gene as a marker for monitoring the survival of genetically engineered microrganisms (GEM's) in aquatic environments. However, this may not precisely reveal the true survival of GEM's, as during starvation, plasmid loss has been reported (Caldwell *et al.,* 1989). Frana and Carlson (2001) developed and used a plasmid encoding *gfp* in *Salmonella* Typhimurium phagetype DT104. It was found that fluorescence was maintained for up to 50 propagations under non-selective conditions.

1.4.2 Allelic exchange and temperature sensitive plasmids for GFP-tagging

The *gfp* gene can be integrated into the *ara* locus in the chromosome of *Salmonella* by means of allelic exchange between a chromosomal gene and a temperature sensitive plasmid (Gandhi *et al.,* 2001). The *ara* gene encodes 2-arabinose isomerase, an enzyme that mediates the bioconversion of arabinose to ribulose. This gene is not essential when alternative carbon sources are available. The temperature sensitive plasmid fails to replicate under non-permissive growth temperature (41-43°C). This results in selection for chromosomal integration at regions of DNA homology in the presence of an antibiotic. The temperature sensitive plasmid, pSY101 (Gandhi *et al.,* 2001), contains the constitutive promoter of the β-lactamase gene, *gfp,* a segment of the *ara*A gene and an antibiotic resistance gene (kanamycin). Integration of the plasmid into the chromosome was accomplished by plating on media containing kanamycin and incubation at 43°C, the non-permissive temperature.

1.4.3 Minitransposons for GFP-tagging

Minitransposons provide a straightforward tool to clone and insert foreign genes stably into the chromosomes of various gram-negative bacteria (Eberl *et al.,* 1997). Chromosomal tagging eliminates problems associated with plasmid instability. Minitransposons are often derived from functional segments of DNA from naturally occurring transposons. These have been artificially arranged to create shorter mobile elements (de Lorenzo *et al.,* 1998). Natural transposons are mobile DNA elements found in the genomes of prokaryotic organisms that are capable of moving from one site in DNA to another by a process of transposition. A protein called transposase catalyses this action. Natural transposons are large and cumbersome for genetic manipulations. Their termini are composed of repeated sequences, which tend to cause DNA rearrangements. Their transposases, which mediate transposition, also mediate transposition immunity, which prevents multiple cloning in the same strain (de Lorenzo *et al.*, 1998). Minitransposons have the advantages of natural transposons, but lack their disadvantages (de Lorenzo, 1992). Mini-Tn10 vectors have generally been used for designing vaccine strains (Tzchaschel *et al.,* 1996), but their use as general genetic tools has been rare (de Lorenzo *et al.,* 1998).

1.4.3.1 Mini-Tn7 Transposons

The bacterial transposon Tn7 inserts at high frequency into a specific intergenic site *att*Tn7 on the chromosome on a number of gram-negative bacteria (Craig, 1989). Tn7-based systems allow stable insertion of marker genes to enable specific detection of tagged bacteria in natural environments (Højberg *et al.,* 1999). The main advantage of the Tn7 transposon is that it has a specific site of insertion, whereas Tn5-based vectors are inserted randomly into the chromosome.

Koch *et al* (2001) have developed *gfp* delivery plasmids (pBKmini-Tn7-*gfp*), which contain a Tn7 element with the intensely fluorescent mutant *gfp* gene, *gfp*mut3* (Cormack *et al.,* 1996; Andersen *et al.,* 1998). The *gfp* gene is controlled by a modified *lac* promoter $P_{A1/04/03}$ (Andersen *et al.,* 1998) and is constitutively expressed. Expression from the lac promoter has been observed in several other gram-negative bacteria. The *gfp* gene is flanked by two strong transcriptional terminators, T_0 (derived from phage λ) and T_1 (derived from the rrnBP1 operon of *E.coli*) (Andersen *et al.,* 1998) and by various antibiotic resistance genes. In many gram-negative bacteria, including *Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Desulfovibrio desulfuricans* and *Sphingomonas yanoikuyae,* Tn7 transposes into a specific target site on the chromosome located downstream from the *glmS* gene (Craig, 1989; Lichtenstein and Brenner, 1982; Wall *et al.,* 1996; Wang and Lau, 1996). However, the sequence of the insertion site (*att*Tn7) is not conserved between different bacteria (Craig, 1989). Tagging with the pBKmini-Tn7-*gfp* vector resulted in random insertion of two *Pseudomonas putida* strains (Staley *et al.,* 1997).

1.4.3.2 Mini-Tn5 Transposons

Mini-Tn5 vectors are often combined with novel markers and reporter genes such as GFP, which can be monitored through optical means such as light emission or fluorescence. A general problem of the pUT mini-Tn5 Km transposon delivery plasmid (de Lorenzo *et al.,* 1990) is that its insertion involves the disruption of a chromosomal sequence. Environmentally important functions can thus be affected (de Lorenzo *et al.,* 1998). Therefore, all *gfp* strains generated from the transposon insertion must be screened against growth defects. The transposase gene is adjacent to, but outside of the mobile DNA segment. Due to the loss of the transposase gene after insertion, the mini-transposon is stably inherited (Herrero *et al.,* 1990 and de

Lorenzo *et al.,* 1990). The mini Tn5 transposon's mobility is determined by two insertion sequences flanking the DNA region encoding the kanamycin resistance gene. The pUT mini–Tn5 Km element has an *sfi*I cassette containing the kanamycin resistance gene and a single NotI site outside the cassette (de Lorenzo *et al.,* 1998). Sternberg *et al.,* (1999) have developed a transposon delivery plasmid by inserting a fragment of the pSM1690 plasmid (*rrnB*P1-RBSII-*gfp*mut3b*-T_0T_2) (Andersen *et al.,* 1998), that is flanked by NotI sites into the NotI site of the pUT-mini Tn5 Km. The delivery plasmid contains *gfp*mut3b* (Andersen *et al.,* 1998), a very bright and stable variant of the green fluorescent protein. The delivery plasmid has a growth-regulated 72bp promoter (*rrnB* P1) from *E.coli* (Bartlett and Gourse, 1994) that is inserted in front of the *gfp* gene to drive the expression of the marker gene. There is a synthetic ribosome binding site, stop codons in all three reading frames and two strong transcriptional terminators, T_0 (derived from phage λ) and T_1 (derived from the *rrnB* P1 operon of *E.coli*) (Andersen *et al.,* 1998).

When a mini-Tn5 transposon was used to label *Pseudomonas putida*, a strongly fluorescent mutant was obtained. The labelled strain was found to be completely indistinguishable from the wild-type with respect to growth and starvation survival and the green fluorescent cells were determined to be perfectly viable (Eberl *et al*., 1997). Cho and Kim (1999b) confirmed that as the Tn5-based system does not rely upon plasmids, it is a good system to monitor pathogenic bacteria and genetically engineered micro-organisms introduced into natural environments. The capability of gene transfer does not exist due to the chromosomally tagged gene.

1.5 SURVIVAL OF *Salmonella* IN THE ENVIRONMENT

1.5.1 *Salmonella* survival in composted biosolids

Sludge stabilization, by means of composting, attempts to convert biosolids into a marketable product. Composting can effectively stabilize wastewater biosolids and reduce pathogens to low levels (Millner *et al.,* 1987). There is, however, a potential risk associated with the use of biosolids as organic fertilizer as it may facilitate the transmission of human diseases. *Salmonella* is frequently found in treated wastewater sludge and has been found in compost and is therefore a pathogen of major concern in

the utilization of biosolids (Gibbs *et al.,* 1997). Rainfall can also result in pathogen spread by run-off from places where manure or biosolids have been applied by leaching through the soil profile. It is known that bacterial and viral ground water contamination increases during heavy rainfall (Satamaría and Toranzos, 2003). As *Salmonella* cells are not inactivated by freezing and are relatively resistant to drying (Hay, 1996; Plym-Forshell and Ekesbo, 1993), *Salmonella* can survive the composting process in low numbers (Russ and Yanko, 1981) and then regrow in soil amendments and stored biosolids under certain conditions (Skanavis and Yanko, 1994; Gibbs *et al.,* 1997). The specific growth rates of *S*. Typhimurium isolates in sterilized compost determined by the MPN method was significantly higher than other bacterial strains (Sidhu *et al.,* 1999). Bacterial indicators such as faecal streptococci and faecal coliforms were found to be more susceptible to sludge treatment than *Salmonella* (Gibbs and Ho, 1993).

1.5.2 *Salmonella* survival in soil

The adhesion of *Salmonella* to soil particles is linked with cell surface hydrophobicity (Stenstrom, 1989). The outer membrane of the bacterial cell is modified in response to changes in the environment. *Salmonella* can be widely disseminated in soil, even in the absence of active fertilization with biosolids, as a result of water currents, underground springs and rainwater run-off carrying contaminated material (Abdel-Monem and Dowidar, 1990; Chao *et al.,* 1987). Percolation of wastewater through the soil filters out bacteria that become trapped in this environment (Chao *et al.,* 1987). *Salmonella* has been detected frequently in environmental soil samples collected from agricultural and recreational areas by cultivation on selective media without enrichment (Abdel-Monem and Dowidar, 1990) or cultivation and fluorescent antibody tests (Thomason *et al.,* 1975; Thomason *et al.,* 1977).

1.5.3 *Salmonella* survival in water sources

Salmonella is frequently isolated from water sources (Cherry *et al.,* 1972), which serve as reservoirs and may result in transmission between hosts (Foltz, 1969). *Salmonella* species are often detected in sewage, fresh water, marine and coastal water and ground water (Baudart *et al.,* 2000). The potential of wastewater to transmit pathogenic organisms is well known and is a primary reason for sewage treatment. Faecally contaminated water is a likely vehicle for the spread of salmonellae. Flood events may mobilize *Salmonella* reservoirs located within the watershed and contribute to the dissemination of a wide variety of species, which are transmitted to surface waters (O'Shea and Field, 1992). Sewage and storm run-off and seepage from septic tanks as well as from sewage injection well fields, facilitates bacterial passage into surface waters (Paul *et al.,* 1995). A recent study in Marrakech (Morocco) indicated that 80% of wastewater samples used as irrigation sources tested positive for *Salmonella*, which serves as a risk for the contamination of vegetables (Melloul *et al.,* 2001).

Salmonella appears to withstand a variety of stressors associated with environmental fluctuations and it may persist in water environments for some time (Winfield and Groisman, 2003). Compared to other bacteria, *Salmonella,* when monitored using differential media has a high survival rate in aquatic environments (Chao *et al.,* 1987). *Salmonella* outlives *Staphylococcus aureus* and the water-borne *Vibrio cholera* in ground water and heavily eutrophied river water (DiRita, 2001). *Salmonella enterica* serovar Typhimurium mr-DT-104 was found to survive for several months in aquatic environments, with enhanced survival in sediments in comparison to the overlying water (Moore *et al.,* 2003) as determined by plate counts. Water samples remained culture positive for *Salmonella* for up to 54 days (Moore *et al.,* 2003). The presence of *Salmonella* in marine environments determined by enrichment and plate counts does not vary seasonally and does not depend on the water temperature, which varied in this study from 26°C to 9°C (Alonso *et al.,* 1992). When *Salmonella* abundance was evaluated by indirect enumeration of colony forming units on selective media, it was found that *Salmonella* had a high survival rate in sewage effluent mixed with brackish water, which causes a dramatic increase in salinity (Mezrioui *et al.,* 1995). This is in

contrast to *E. coli*, where osmotic stress appears to be the reason for apparent death in *E. coli* in seawater (Bogosian *et al.,* 1996).

1.5.4 *Salmonella* survival in sediment

Sediment particles are believed to function as micro-ecological niches in which bacteria can survive and perhaps replicate (Brettar and Hofle, 1992). Bacteria absorbed to sediment particles may be protected from the influence of factors such as UV radiation (Bitton *et al.,* 1972), high salinity (Ghoul *et al.,* 1986), heavy metal toxicity (Jones, 1964) and attack by bacteriophage (Roper and Marshall, 1979) or grazing protozoans (Fish and Pettibone, 1995). Sediments may protect enteric bacteria from certain stressors associated with aquatic environments and they are known to act as nutrient sources that may support bacterial growth (Lim and Flint, 1989).

The accumulation of indicator bacteria and viruses in sediments has been well documented (Pommepuy *et al.,* 1992). This is due to the absorption of the micro-organisms to particles suspended in the water, which then sediment out (Davies *et al.,* 1995). Sediments may contain 100-1000 times as many faecal indicator bacteria as the overlying water (Ashbolt *et al.,* 1993; Van Donsel and Geldreich, 1971).

Fish and Pettibone (1995), measured the density of *Salmonella* in sediments and water using direct counts, direct viable counts and standard plate counts. *Salmonella* survived in microcosms containing autoclaved water and sediment for at least 28 days. *Salmonella enterica* serovar Typhimurium mr-DT-104 remained culture positive for up to 119 days in sterile sediment samples (Moore *et al.,* 2003). While Thomason *et al.,* (1977) found using culturing and fluorescent antibodies that *Salmonella* could survive and multiply in a soil ecosystem for at least one year. Nearly 100% of the *Salmonella* samples taken downstream of a sewage treatment plant over a one-year period were samples obtained from bottom sediment, rather than samples obtained from surface waters (Hendricks, 1971). Surface water bodies are thought to be more vulnerable to faecal contamination than ground water reservoirs due to the lack of natural soil protection and filtration (Kistemann *et al.,* 2002). Grimes (1980), has shown that coliforms detected in the water column may be a result of the resuspension of sediment-bound cells rather than faecal contamination.

1.5.5 *Salmonella* survival in aquatic biofilms

1.5.5.1 Biofilm formation

A biofilm is defined as an assemblage of microbial cells that are irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). Biofilms can be composed of single species populations or mixed communities of bacteria depending on the environmental parameters (Davey and O'Toole, 2000). Biofilms have been shown to exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002).

The formation of a well-developed biofilm is thought to occur in a sequential process consisting of initial attachment, formation of microcolonies followed by the formation of well-developed biofilms (Van Loosdrecht *et al*., 1990). Biofilm attachment of cells is affected by surface properties, nutrient availability, hydrodynamics and the cell surface properties (Donlan, 2002). A material surface exposed in an aqueous medium will become conditioned or coated with polymers from that medium resulting in chemical modification that will affect the rate and degree of microbial attachment (Donlan, 2002). Bacteria are transported to the attachment surface by Brownian motion, convective transport due to liquid flow, or active bacterial movement (Fig. 1.1) (Van Loosdrecht *et al*., 1990). This is followed by initial adhesion which can be reversible or irreversible. After the bacterium has been deposited on the solid surface, attachment occurs and the surface structure polymers may form strong links between the cell and the solid surface. The interaction of cells with the surface is hastened by force-generating organelles such as type IV pili and flagella. Once temporary contact with the surface is made, bacteria use either flagella or pili to move along the surface in two dimensions until other bacteria are encountered and microcolonies are formed or enlarged (O'Toole and Kolter, 1998). Lastly, surface colonization occurs when the attached cells start growing and newly formed cells remain attached to each other (Van Loosdrecht *et al*., 1990). Microcolonies develop which are characterized by mushroom or pillar-like structures surrounded by extrapolsaccharides and interspersed with fluid-filled channels (Kuchma and O'Toole, 2000). These bacterially produced extrapolysaccharides, play a role in resisting shear forces (Donlan and Costerton, 2002) and are required to stabilize the biofilm (Watnick and Kolter, 1999). There are

also motile cells that keep their association with the fixed biofilm cells for long periods of time, swimming between pillars of biofilm associated bacteria (Watnick and Kolter, 2000). Cells which are loosely attached may detach and these cells together with offspring of other biofilm cells may recolonize previously uncolonized surfaces, (Korber *et al*., 1989) which extends the biofilm.

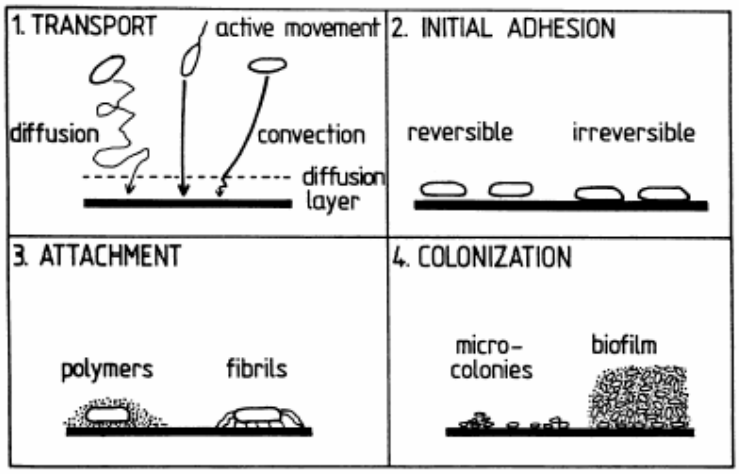

Figure 1.1 Schematic representation of the sequence of steps in biofilm formation (Modified from Van Loosdrecht *et al*., 1990).

1.5.5.2 Biofilm formation in drinking water distribution systems

Biofilms appear to be a common occurrence in water distribution systems (Szewzyk *et al.,* 2000) and the growth and persistence of bacteria in distribution systems is well-documented (Camper *et al.,* 1999). Drinking water biofilms would appear to be a hostile environment for microbial growth due to the low organic carbon concentrations and the presence of disinfectant (Camper *et al*., 1999). Although water treatment plants disinfect water, it is a fallacy that sterile conditions exist in the distribution system and that few, if any micro-organisms should be able to survive in disinfection procedures employed by water authorities (Szewzyk *et al*., 2000). The recovery and growth of bacteria after water treatment is of concern for public health reasons (Kerr *et al*., 1999). Biofilms are found on all surfaces of drinking water distribution systems, from the treatment plants through to installations in the house of the consumer (Szewzyk *et al.,* 2000). Cells attach and form biofims in water distribution systems and on surfaces of piping material, irrespective of the type of material used (Szewzyk *et al.,* 2000). Attachment to the surfaces of pipes gives the

cell a nutritional advantage in this low nutrient environment (Camper *et al.,* 1999). Biofilms of potable water distribution systems have the potential to harbour enteric pathogens such as *Salmonella*. This is problematic, as these pathogens could be released back into the flow (Szewyk *et al.,* 2000). The attached cells could also serve as a reservoir for subsequent spread through the system following detachment (Camper *et al.,* 1999). Biofilms are known to provide a safe haven for many bacteria as these bacteria are more resistant to disinfection procedures (Costerton *et al.,* 1995). Bacteria growing in biofilms tend to become up to one thousand fold more resistant to treatment or disinfection than plantonic cells (Costerton *et al.,* 1995). Other problems associated with biofilms in drinking water distribution systems include taste and odour problems as a result of actinomycetes or fungi, corrosion of pipe material and the possible regrowth of micro-organisms (Camper *et al.,* 1999). Survival of pathogenic bacteria such as *Flavobacterium* and *L. pneumophila* was enhanced in a non-sterile biofilm containing other heterotrophic micro-organisms (Wadowsky and Yee, 1983, 1985).

1.5.5.3 Methods for culturing biofilms and analysing biofilm formation

Biofilms can be analysed by conventional culturing or by direct microscopy (Camper *et al.,* 1999). Both these approaches have limitations, as not all organisms present in biofilm may be culturable and they cannot be identified based on morphology alone (Camper *et al.,* 1999). Electron microscopy was originally the method of choice to analyse biofilm formation under high resolution. A disadvantage of electron microscopy is that it requires dehydrated samples (Davey and O'Toole, 2000). Confocal scanning laser microscopy (CSLM) is the method of choice for studying the three-dimensional structure of thick hydrated biofilms in a non-destructive manner. CSLM, however, uses a laser as a light source and the equipment is very expensive (Lawrence *et al*., 1991).

Bacterial biofilms can be grown by batch culture or by continuous culture. The batch culture method is convenient as it involves suspending a sterile surface, such as a glass coverslip, in inoculated growth medium. After incubation, the cover-slip is examined by microscopy on a microscope slide. Disadvantages of batch culture are the accumulation of metabolites in the culture vessel which may alter or inhibit biofilm formation and potential nutrient starvation if cultures are incubated for

extended time periods (O'Toole *et al*., 2001). Flow cells are often used to culture biofilms using the continuous culturing method. Flow cells are small continuous-flow systems through which fresh medium is pumped continuously and waste leaves the system. The medium is not recycled through the flow cell. The flow cell has a viewing port which allows direct observation of the biofilm without disrupting the culture system (Davey and O'Toole, 2000). This system is also thought to closely mimic the conditions bacteria are exposed to in the natural environment. Fairly constant nutrient conditions are maintained and the developing biofilm may be observed under the microscope continuously without disturbing the culture medium over a long period of time (O'Toole *et al*., 2001).

Biofilms in drinking water distribution systems can be studied by setting up models of the system that have been fitted with reactors such as Robbin's and Pederson's devices (Manz *et al.,* 1993; Pederson, 1982). These devices can be placed in the actual drinking water distribution system where slides that serve as a surface for biofilm growth can be removed for analysis. The biofilm can then be analysed by conventional culturing or by direct microscopy (Camper *et al.,* 1999).

1.5.5.4 Biofilm formation of *Salmonella*

Salmonella enterica serotype Typhimurium expresses multicellular and aggregative behaviour and harbours the genetic information for the expression of the red, dry and rough (rdar) morphotype (Römling *et al*., 1998a; Römling *et al*., 1998b). This multicellular behaviour is characterized by the expression of cellulose and curlifimbriae (thin aggregative fimbriae) which form part of the extracellular matrix produced by biofilm-forming *S.* Typhimurium (Römling *et al*., 1998b; Solano *et al*., 2002). The matrix protects against disinfectants (Solano *et al*., 2002) and plays a role in survival outside the host (Gerstel and Römling, 2001). This suggests that biofilm formation may be an important factor in the survival of *Salmonella* on surface environments. The rdar morphotype is regulated by the *agf*D operon (Römling *et al*., 1998b). Cells expressing the rdar morphotype exhibit many different modes of multicellular behaviour such as forming large cell clusters in liquid culture, adhering to hydrophyllic and hydrophobic abiotic surfaces and spreading on agar plates in the stationary phase of growth (Römling and Rohde, 1999).

Salmonella species are capable of adhering and forming biofilms on metal, glass, rubber and plastic surfaces (Hood and Zottola, 1997; Sinde and Carballo, 2000; Stepannović, 2004) Flagella, pili and fimbriae all play a role in the adherence of *Salmonella enterica*. The resistance of *Salmonella* to treatment with sanitizers varies depending on the surface. Biofilm cells on stainless steel were found to be much more sensitive than those formed on plastic surfaces (Joseph *et al*., 2001). The contents of the *Salmonella* biofilm also vary depending on the surface. It was found that cellulose was the primary polysaccharide in *Salmonella* biofilms formed on glass (Solano *et al*., 2002) however, cellulose was not a major component in the biofilm production on gallstone (Prouty and Gunn, 2003).

1.5.5.5 *Salmonella* survival in drinking water biofilms

Survival and growth of pathogenic micro-organisms within biofilms may be enhanced by the association and metabolic interactions with indigenous organisms (Donlan, 2002). Camper *et al*., (1998) used fluorescent antibodies to monitor *S.* Typhimurium in a model distribution system. *Salmonella* cells were found to attach and persist in the model distribution system containing undefined heterotrophic bacteria and chlorine from an unfiltered osmosis system for more than 50 days, which suggests that the normal biofilm flora of this water system provided niche conditions capable of supporting the growth of this organism. The cells in the biofilm became impossible to culture on selective media over time, but could be resuscitated if the biofilm was incubated in non-selective medium. The presence of fimbriae, flagella and surface associated polysaccharides or proteins may provide a competitive advantage for one organism where a mixed community is involved (Donlan, 2002). Armon *et al.,* (1997) showed that when *S.* Typhimurium was introduced into a non-sterile simulated biofilm flow system it had a good survival determined by plate counts at both temperatures of 24°C and 36°C for at least 20 to 30 days. A laboratory reactor, which simulates biofilm formation in water pipes, was used to study the interactions of biofilm formation between a nitrogen-fixing strain of *Klebsiella pneumoniae* and *Salmonella* Enteritidis. The level of attachment of *S.* Enteritidis determined by heterotrophic plate counts was higher in the binary biofilm than in the single species biofilm. In the initial colonization phase the binary biofilm contained a much higher proportion of metabolically active cells of *S.* Enteritidis than in the single species

biofilms particularly during the initial colonization period. This was determined by using a redox dye as an indicator of electron transport activity (Jones and Bradshaw, 1997).

1.6 CONCLUDING REMARKS

The propensity of *Salmonella* to persist in water environments under hostile conditions is of concern as it serves as a contamination reservoir. Many ongoing efforts are being implemented to protect and manage the quality of our water sources. These approaches will however only be successful if they are based on a clear understanding of the origin, fate, survival and transport of pathogens such as *Salmonella*. The role of contaminated water in the transmission of *Salmonella* in developing countries is largely unknown. The fate and persistence of non-typhoidal *Salmonella* in water environments and the specific influence of the indigenous microbiota on the survival and growth of *Salmonella* is not understood. A tagged *Salmonella* strain distinguishable *in vivo* from a mixed bacterial community would greatly facilitate the study of *Salmonella* in water environments. A detection method was sought that does not require culturing. A recently developed approach allows for the prior genetic labelling of the target organism with the *gfp* gene, encoding the green fluorescent protein (Eberl *et al.,* 1997). As the system allows *in situ* detection of undisturbed cells and does not require any external substrates, it is ideally suited for monitoring *Salmonella* as a monospecies or in a complex mixed community.

The objective of this study was therefore,

(i) to chromosomally tag and evaluate a selected clinically relevant *Salmonella* strain,

(ii) to investigate the fate and persistence of non-typhoidal *Salmonella* in drinking water biofilms in monoculture and in mixed culture, and

(iii) to determine the potential build-up of *Salmonella* in drinking water biofilms and the possible effect thereof on the microbial quality of the water phase.

CHAPTER TWO
CONSTRUCTION AND EVALUATION OF GFP-TAGGED *Salmonella* ISOLATES

2.1 INTRODUCTION

The fate and survival of bacterial pathogens like *Salmonella* in water environments is not well understood due to the difficulty of monitoring a specific species in complex mixed populations. Culture-based enumeration techniques may significantly underestimate viable numbers of cells due to the presence of starved or injured cells or potential 'viable but non-culturable' cells (Smith *et al*., 1994; Cho and Kim., 1999b). The use of specifically targeted fluorescent antibodies may provide an alternate detection technique, but this method can result in non-specific staining of bacteria due to antibody cross-reactivity or to differential expression of the target antigen (Cloete and de Bruyn, 2001). There is often the need to observe the activity and distribution of bacteria in minimally disturbed native samples. Fluorescent *in situ* hybridisation (FISH) has allowed the presence and interaction of bacteria in various environments to be examined by targeting the rRNA molecules. It is, however, only in cells growing under steady state conditions that the content of ribosomes truly reflects the growth state of the cells (Möller *et al*., 1995). FISH can have limited success when applied to starved cells as their low rRNA content can be problematic due to high background fluorescence present in some environmental samples (Banning *et al*., 2002). Biomarkers such as luciferase have been used to detect individual cells *in situ*, however, the detection of *lux*-encoded luciferase fluorescence activity is dependent on energy reserves within cells. The energy reserves of bacteria in the environment may be too low to allow *in situ* detection of these cells (Meikle *et al*., 1994).

The marker gene GFP encoding green fluorescent protein, obtained from the jellyfish *Aequorea victoria* (Chalfie *et al*., 1994) overcomes the above shortcomings. Unlike other detection methods, GFP-labelling does not require staining or fluorescent probe addition to visualise the bacteria and cells may be viewed alive allowing temporal observation. Samples can be analysed directly without substrate addition. Detection of GFP only requires irradiation with near-UV or blue light and is an excellent marker system for

studies of complex ecosystems (Chalfie *et al.,* 1994; Valdivia *et al*., 1996). The detection of GFP is independent of energy reserves and green fluorescence phenotypes are detectable in all growth phases even under starved conditions (Tombolini *et al*., 1997). GFP is also a very stable protein in bacteria (Andersen *et al*., 1998). GFP was subsequently chosen as a marker with which to tag *Salmonella* in order to monitor its interaction with other micro-organisms in a drinking water biofilm environment. In order to eliminate the risks associated with plasmids, the pUT mini-Tn5 Km transposon was used to transpose the GFP onto the chromosome of the target strain. It is essential that strains destined for use in an environmental situation have the desired traits encoded on the chromosome, to ensure stability and predictability of the engineered phenotype (Eberl *et al.,* 1997). The pUT mini-Tn5 Km transposon delivery plasmid contains *gfp*mut3b* (Andersen *et al.,* 1998), a very bright and stable variant of the green fluorescent protein and a growth-regulated promoter (*rrnB* P1) inserted in front of the *gfp* gene to drive the expression of the marker gene. This allows the *gfp* gene to be expresed when the cell is alive or growing, while a constitutive promoter would allow expression of the gene irrespective of environmental or developmental factors. A general problem of the pUT mini-Tn5 Km transposon delivery plasmid is that its random insertion involves the disruption of a chromosomal sequence. Environmentally important functions can therefore be affected (de Lorenzo *et al*., 1998). For this reason all GFP strains generated from transposon insertion must be screened for the occurrence of growth defects.

In order to study the fate and survival of *Salmonella* in drinking water biofilms, *Salmonella* strains were selected for tagging with GFP. The aims of this part of the investigation were, therefore, (i) to isolate and identify clinically relevant *Salmonella* strains from fresh water environments, (ii) to chromosomally tag selected *Salmonella* strains with the marker gene *gfp*mut3b* under control of the *rrnB* P1 promotor, (iii) to verify that the *gfp* gene is present on the chromosome of the tagged strains, (iv) to compare the growth activity of the tagged strains to that of the wild-type strains under nutrient limiting conditions, and to ascertain whether the gene was stably maintained during prolonged periods of growth.

2.2 MATERIALS AND METHODS

2.2.1 Isolation and identification of *Salmonella* strains

Sediment samples were collected in sterile bottles from rivers in the Venda and Free State regions of South Africa. Ten grams of each of the sediments were pre-enriched in 50 ml of buffered peptone water (BPW) (Oxoid) and incubated for 24 hours at 37°C. Thereafter, 1ml of the pre-enrichment sediment was inoculated into 9 ml of Rappaport-Vassiliadis (RV) broth (Oxoid) for selective enrichment and incubated for 24 hours at 42°C. Following incubation of the RV enrichment broth, a loop-full of broth from each positive tube was plated on to XLD (Xylose Lysine Deoxycholate) agar (Oxoid) and incubated for 24 hours at 37°C. Once pure black colonies were obtained, API 20E (bioMérieux) biochemical test strips were inoculated with presumptive *Salmonella* isolates and incubated for 24 hours at 37°C. After the addition of the prescribed reagents to the cupules, the results were recorded and identifications were made on the basis of the profile index. Presumptive *Salmonella* isolates were chosen to undergo serological testing at the ARC-Onderstepoort Veterinary Institute, Pretoria, South Africa.

2.2.2 Chromosomal tagging

S. ser. Typhimurium isolates were cultured on Luria-Bertani (LB) agar (1% tryptone; 0.5% yeast extract; 1% NaCl; 1.2% bacteriological agar) containing 100 µg/ml of ampicillin (Amp100) (Roche) and LB agar containing 100 µg/ml of kanamycin (Km100) (Roche) to confirm susceptibility to these two selective agents. The pUT mini–Tn5 Km transposon (de Lorenzo *et al*, 1990) was used to insert GFP into the chromosome of the chosen *Salmonella* strains. The delivery plasmid contains *gfp*mut3b* (Andersen *et al.,* 1998), a very bright and stable variant of the green fluorescent protein. A growth-regulated 72bp promoter (*rrnB* P1) from *E.coli* (Bartlett and Gourse, 1994) is inserted in front of the *gfp* gene to drive the expression of the marker gene. There is a synthetic ribosome binding site, stop codons in all three reading frames and two strong transcriptional terminators, T_0 (derived from phage λ) and T_1 (derived from the *rrnB* P1 operon of *E.coli*) (Andersen *et al.,* 1998).

Salmonella was transformed by tri-parental mating using the *E.coli* strains HB101 (pRK2013) as a helper strain (Figurski and Helinski, 1979), CC118λpir (pSM1695) as the donor strain (Sternberg *et al*, 1999) and the *Salmonella* isolate as the recipient (Table 2.1). The donor and helper strain were kindly supplied by J.B Andersen (Department of Microbiology, Technical University of Denmark). A loop-full of each of the donor, helper and recipient strains were mixed together on LB agar plates and incubated for 24 hours at 37°C. After 24 hours the growth obtained was cultured on XLD media containing 100 µg/ml of kanamycin and incubated at 37°C for 24 hours. Black colonies were selected and confirmed as tri-transconjugants by their ability to fluoresce green when exposed to blue light. Confirmed tri-transconjugant colonies were inoculated into 10 ml of LB broth (1% tryptone; 0.5% yeast extract; 1% NaCl) and incubated at 37°C. After 24 hours, serial dilutions (10^{-1} – 10^{-6}) were made by transferring 100 µl of LB broth to 900 µl of ¼ strength Ringer's solution (Merck). One hundred µl of the dilutions were plated out on LB Km100 plates and incubated at 37°C for 24 hours. A dilution plate was selected containing suitably spaced colonies to undergo replica plating. Sterile velvet strips were pressed lightly onto colonies on the LB Km100 plates and transferred to LB Amp100 plates by pressing the fabric on to the replica plates. The LB Amp100 plates were incubated at 37°C for 24 hours. A comparison of the colonies on the replica plates was made. Colonies that did not grow on the LB Amp100 plates were selected from the LB Km100 plates. For each colony, 10 µl of nuclease-free water (Promega) was mixed with the single colony on a glass slide. A cover slip was mounted onto the glass slide and sealed with transparent nail varnish to prevent the samples from drying out. These colonies were examined for fluorescence with an inverted Zeiss Axiovert 200 fluorescent microscope (Excitation - 490 nm and Emission - 510 nm), fitted with a 100x/1.4 Zeiss Neofluor objective, and fluorescent isolates were selected. Different microscopic fields were examined for each isolate. The images were captured using a Nikon charge-coupled device (CCD) camera.

Table 2.1 Strains and plasmids used for tri-parental mating

Strains and Plasmids	Relevant Characteristics	Reference or Source
***E.coli* K-12 Strains**		
HB101	*E.coli* K-12/B hybrid	J.B. Andersen
CC118λpir	λpir lysogen	J.B. Andersen
***Salmonella* Strains**		
S. Typhimurium	Recipient strain; Environmental isolate	This study
Tagged *S.* Typhimurium	S. Typhimurium X HB101 (pRK2013) X CC118λpir (pSM1695); S. Typhimurium with mini Tn5-Km-*rrnB* P1-RBSII-*gfp* mut3b*-To-T1 casette randomly inserted into chromosome; Km^R	This study
Plasmids		
pRK2013	RK2 transfer function; Helper plasmid in tri-parental matings	Figurski and Helinski, 1979
pSM1695	Amp^R Km^R; Delivery plasmid for mini Tn5-Km-*rrnB* P1-RBSII-*gfp* mut3b*-To-T1	Sternberg *et al* ., 1999

2.2.3 Verifying chromosomal tagging

The location of the *gfp* gene on the genome was confirmed by PCR and Southern Blot hybridisation.

2.2.3.1 PCR

Chromosomal DNA was extracted from tagged strains, wild-type strains and the donor strain using the Dneasy tissue kit (Qiagen). The isolated DNA was used directly for PCR amplification. The *gfp* gene of 720 bp was amplified in a PCR reaction using the primers $P_{gfp(up)}$ (5' -ATATAGCATGCGTAAAGGAGAAGAACTTTTCA- 3') and $P_{gfp(down)}$ (5' -CTCTCAAGCTTATTTGTATAGTTCATCCATGC- 3') (Anderson *et al*, 1998). The 50 µl PCR reaction mixture contained 50 pmol/µl of each primer, 2.5 mM deoxynucleotide phosphate (dNTP), 5µl of 10 x PCR buffer (containing 15 mM $MgCl_2$), 1U of Taq Supertherm Gold DNA polymerase (Southern Cross) and 50-100 ng of genomic template DNA. For control purposes, a reaction mixture containing nuclease-free water and all other reagents but no template DNA was included. The reaction mixture was subjected to thirty amplification cycles of 92°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute on a GeneAmp® 2700 PCR System (Applied

Biosystems). An initial start of 92°C for 5 minutes and a final elongation step at 72°C for 5 minutes was also included.

Aliquots of each PCR reaction mixture were analysed by agarose gel electrophoresis in the presence of a 100 bp DNA molecular weight marker (Marker XIV, Roche). A 1% (w/v) agarose gel was cast and samples electrophoresed at 100 V in 1 x TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA). The agarose gel was stained with ethidium bromide at a final concentration of 1 mg/ml. Prior to electrophoresis, the PCR products were resuspended in 2 µl of loading buffer (0.25% bromophenol blue; 40% (w/v) sucrose in dH_2O). The DNA was visualised by UV-induced fluorescence on a transilluminator.

2.2.3.2 Southern Blot hybridisation

Prior to Southern Blot hybridisation the *gfp* gene was amplified from the plasmid DNA of the donor strain (pSM1695) in a PCR reaction as previously described. The 720 bp PCR product was excised from an agarose gel and purified using the QIAquick® PCR purification kit protocol (Qiagen). The purified PCR product of the *gfp* gene was estimated to be a concentration of 200 ng/µl. In order to make a probe for Southern Blot hybridisation, random primed labelling of the DNA was carried out using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche).

For Southern Blot hybridisation, chromosomal DNA was extracted from tagged strains, wild-type strain and the donor strain using the Dneasy tissue kit (Qiagen). Plasmid DNA was extracted from tagged strains, wild-type strain and the donor strain using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). The plasmid and genomic DNA of the donor strain, wild-type and tagged strains was loaded on a 1% (w/v) agarose gel without ethidium bromide. The *gfp* gene, which was previously amplified, was included as a positive control. The gel was run in the presence of a 100 bp DNA molecular weight marker (Marker XIV, Roche) for an hour at 100V as described in section 2.2.3.1 to allow the DNA be well separated. The gel was subsequently stained briefly in 0.50 µg/ml ethidium bromide to assess the quality of the target DNA.

The DNA was fragmented by partial depurination by submerging in 250 mM HCl, with shaking at room temperature for 10 minutes. The gel was rinsed with ddH_2O and then submerged for 2 x 15 minutes at room temperature in denaturation solution (0.5 M NaOH; 1.5 M NaCl) with gentle shaking. Following denaturation, the gel was rinsed with ddH_2O and submerged in neutralisation solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 x 15 minutes at room temperature. The gel was equilibrated for 10 minutes in 10 x SSC (1.5 M NaCl; 150 mM sodium citrate, pH 7.0).

The DNA was transferred from the gel to a Hybond^{TM}-N+ nylon membrane (Amersham) by vacuum blotting using a Model 785 Vacuum Blotter (Bio-Rad) according to the standard procedure. The gel was transferred in 10 x SSC for 180 minutes at 5 inches Hg. After transfer, the membrane was placed on Whatman 3MM paper soaked in 2 x SSC (0.3 M NaCl; 30 mM sodium citrate, pH 7.0) and the wet membrane was exposed to UV light on a UV transilluminator for 5 minutes. The membrane was rinsed in ddH_2O and then hybridised using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche). A hybridisation temperature of 64°C was used with 20 ng of the random primed labelled probe. Chromogenic detection was carried out with NBT/BCIP according to the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche).

2.2.4 Survival Studies

2.2.4.1 Stability and maintenance of the *gfp* gene

The tagged strains and wild-type strain were inoculated separately into 10 ml of medium and incubated at 37°C with agitation at 180 rpm. After 24 hours, 10 µl of culture was transferred to another 10 ml of fresh medium and incubated at 37°C with agitation. This process was repeated every 24 hours for thirty days. The presence and stability of the label was verified by fluorescent microscopy initially, and after constant growth for thirty-days in both nutrient rich (LB broth) and low nutrient medium ($^1/_{10}$ strength R2A) medium, facilitated by daily transfer into fresh medium.

2.2.4.2 Survival Fitness

The wild-type and tagged strains were inoculated separately into 30 ml of LB broth and incubated overnight at 37°C with agitation at 180 rpm. One ml of the overnight culture

was inoculated into 250 ml side-arm flasks containing 100 ml LB broth. The OD readings of the culture were measured with a spectrophotometer set at 600 nm from time 0 hours. Single readings were taken every 30 minutes. After each reading, the side-arm flasks were incubated at 37°C with agitation at 180 rpm. This was repeated in duplicate in side-arm flasks containing $^{1}/_{10}$ strength R2A medium. For the growth curves in $^{1}/_{10}$ strength R2A medium, culturable counts were prepared by making dilutions in 9 ml of ¼ strength Ringer's solution (-1 to -10) and plating out 100 µl of the dilutions on R2A agar (Oxoid). Plate counts were taken on an hourly basis.

Phenotype Microarrays (Biolog) were used to compare the phenotypic profiles of the wild-type and tagged (A) strain. The Phenotypic Microarrays (PM) were performed in 96-well microplates containing different nutrients or inhibitors in which cell respiration is measured with a redox indicator. Carbon, nitrogen, phosphorous and sulphur sources were examined. One set of microarrays was performed for the wild-type and the tagged (A) strain. Each microplate has a control to determine whether the strain tested has a positive or negative ability to utilize that source. Results were analysed with an EL_X800 Universal Microplate Reader at 590 nm (Bio-tek instruments inc.).

2.3 RESULTS

2.3.1 Isolation and Identification of *Salmonella* Strains

Several black colonies, indicative of *Salmonella* were isolated from the sediment samples. Colonies on the XLD that appeared black due to H_2S production were selected for further characterisation. The API 20E system identified three of the isolates as *Salmonella* species. These biochemical tests were confirmed by serotyping in order to identify presumptive *Salmonella* isolates. Three isolates were typed as follows:

Isolate 1: *S. enterica* subsp. *enterica* ser. Typhimurium
Isolate 2: *S. enterica* subsp. *enterica* ser. Muenchen
Isolate 3: *S. enterica* subsp. *salamae*

2.3.2 Chromosomal tagging

The pUT mini–Tn5 Km transposon (de Lorenzo *et al*, 1990) was used to insert the *gfp* gene into the chromosome of the *S.* ser. Typhimurium strain. Prior to tagging, *S.* ser. Typhimurium was sensitive to both ampicillin and kanamycin. Following tri-parental mating, two tagged *S.* ser. Typhimurium strains were obtained. Both strains were sensitive to ampicillin, but resistant to kanamycin. Both tri-transcongugants fluoresced green when exposed to blue light (Fig. 2.1).

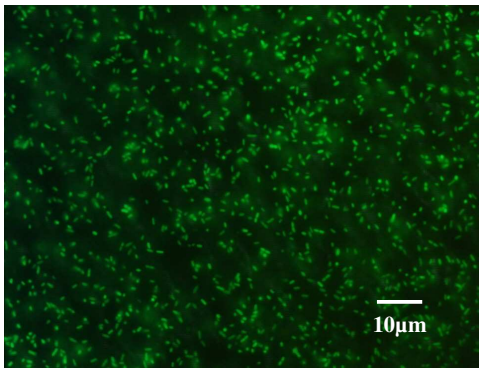

Figure 2.1 Green fluorescence emitted by the recipient tri-transconjugant *Salmonella* cells visualised by fluorescence microscopy.

2.3.3 Verifying chromosomal tagging

2.3.3.1 PCR

In order to verify whether transposition had taken place, a PCR detecting the *gfp* gene was performed on chromosomal DNA of the tagged strains. Genomic DNA was successfully extracted and positive PCR reactions indicated that the *gfp* gene was present on the genome (Figure 2.2).

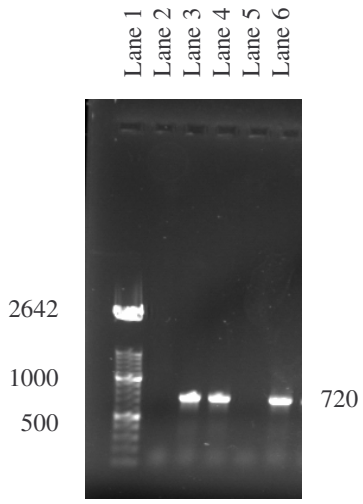

Figure 2.2 Amplification of the 720 bp *gfp* gene.

Lane 1: Marker XIV (Roche). Lane 2: Negative control. Lane 3: Tagged (A) isolate. Lane 4: Tagged (B) isolate. Lane 5: Wild-type strain. Lane 6: Positive control plasmid pSM1695. The sizes of the molecular weight marker in bp are indicated to the left of the figure and the size of the PCR amplicon in bp is indicated to the right of the figure.

2.3.3.2 Southern Blot hybridisation

In order to confirm that the *gfp* gene integrated into the chromosome of the tagged strains, Southern Blot hybridisation was carried out. The gel to be transferred to a nylon membrane contained the *gfp* gene and both the plasmid and genomic DNA of the tagged, wild-type and donor strains. The wild-type strain served as a negative control, while the *gfp* gene and the donor strain DNA served as positive controls (Fig. 2.3). The *gfp* probe hybridised with the genomic DNA of both the tagged strains and the control indicating the *gfp* gene is present on the genome of the tagged strains (Fig. 2.4). The probe did not hybridise with the plasmid DNA, which confirms that the *gfp* gene is not maintained on a plasmid, but has integrated into the chromosome of the tagged strains.

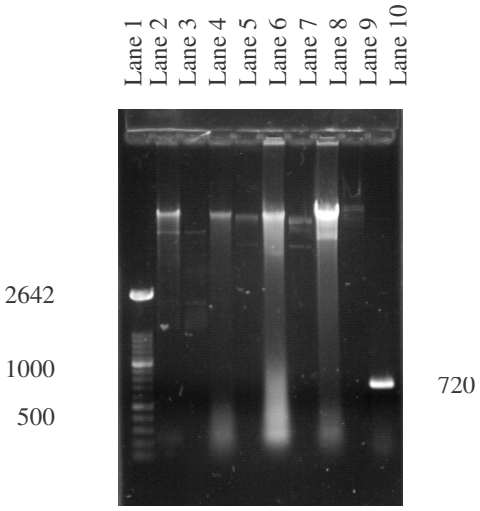

Figure 2.3 Agarose gel electrophoresis separating DNA fragments for Southern Blot analysis.

Lane 1: Marker XIV (Roche). Lane 2: Wild-type genomic DNA. Lane 3: Wild-type plasmid DNA. Lane 4: Tagged (A) isolate genomic DNA. Lane 5: Tagged (A) isolate plasmid DNA. Lane 6: Tagged (B) isolate genomic DNA. Lane 7: Tagged (B) isolate plasmid DNA. Lane 8: Donor strain genomic DNA. Lane 9: Donor strain plasmid DNA. Lane 10: The 720 bp *gfp* gene. The sizes of the molecular weight marker in bp are indicated to the left of the figure and the sizes of the PCR amplicons in bp are indicated to the right of the figure.

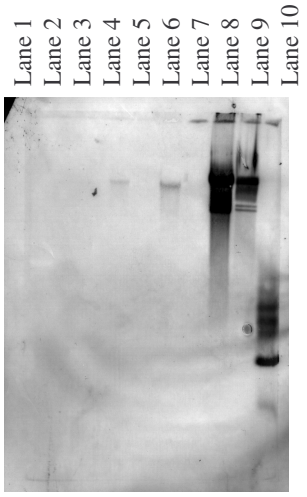

Figure 2.4 Southern Blot analysis of DNA on a nylon membrane.

Lane 1: Marker XIV (Roche). Lane 2: Wild-type genomic DNA. Lane 3: Wild-type plasmid DNA. Lane 4: Tagged (A) isolate genomic DNA. Lane 5: Tagged (A) isolate plasmid DNA. Lane 6: Tagged (B) isolate genomic DNA. Lane 7: Tagged (B) isolate plasmid DNA. Lane 8: Donor strain genomic DNA. Lane 9: Donor strain plasmid DNA. Lane 10: The 720 bp *gfp* gene.

2.3.4 Stability and maintenance of the *gfp* gene

The stability of the GFP labelling was tested by exposing the isolates to a thirty-day growth period under non-selective conditions. The presence of the label was verified by fluorescence microscopy. After 30 days, the GFP was visible in all tagged cells, indicating stable maintenance (Fig. 2.5). The GFP fluorescence was also visible in cells under low nutrient conditions (Fig. 2.6) indicating that even when cells are subjected to such stress, the *gfp*mut3b* gene is still maintained.

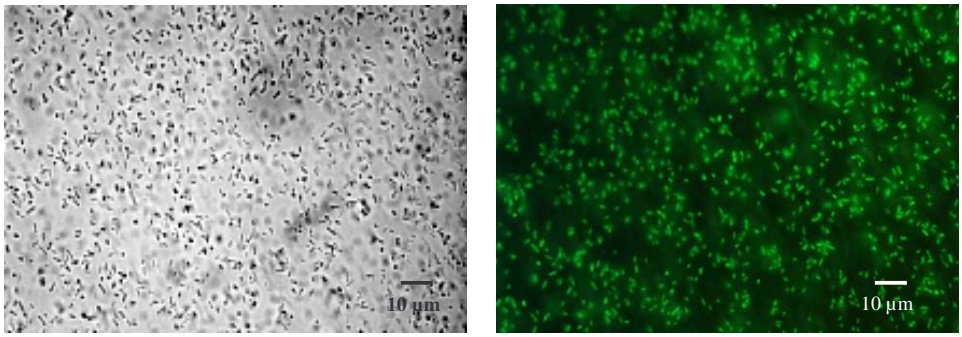

Figure 2.5 Tagged isolate cultured in LB broth viewed with phase contrast (left panel) and fluorescence microscopy (right panel).

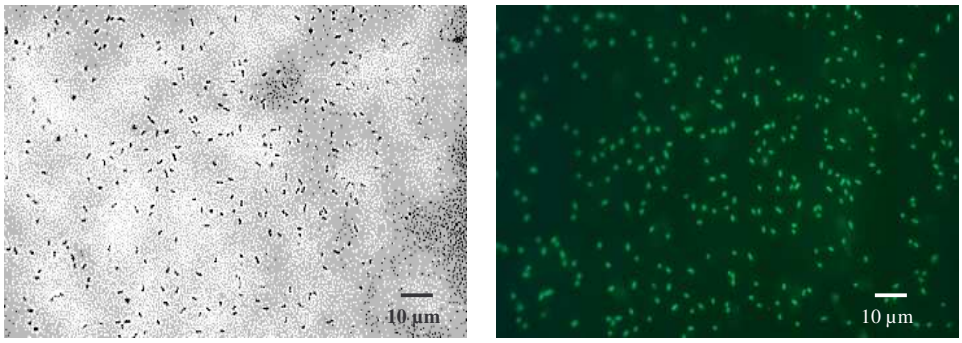

Figure 2.6 Tagged isolate cultured in $^{1}/_{10}$ strength R2A broth viewed with phase contrast (left panel) and fluorescence microscopy (right panel).

2.3.5 Survival Fitness

2.3.5.1 Fitness under nutrient rich and low nutrient conditions

The labelled strains did not display growth rate deficiencies when compared to the wild-type strains, both under nutrient rich (LB broth) (Fig. 2.7) and low nutrient ($^{1}/_{10}$ strength R2A medium) (Fig. 2.8) conditions. GFP continued to fluoresce in cells cultured in low nutrient medium. Plate counts in the low nutrient medium indicated that the cells remained culturable (Fig. 2.9). This shows that the incorporation of the *gfp* gene did not have any detrimental effects on the behaviour or survival of the isolates.

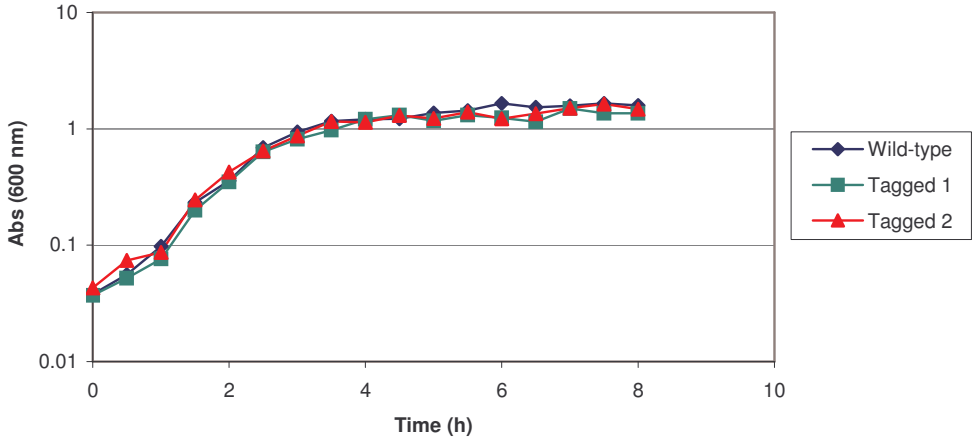

Figure 2.7 Growth of *S.* ser Typhimurium wild-type vs. tagged strains in LB broth at 37°C.

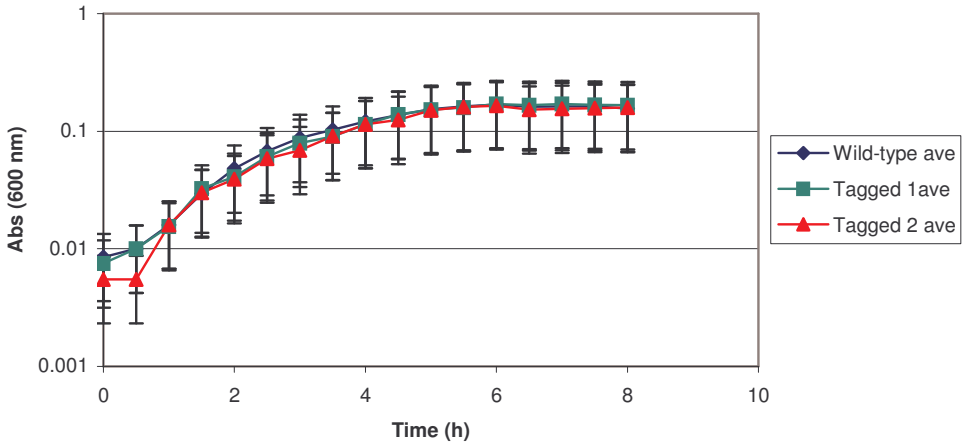

Figure 2.8 Average growth of *S.* ser Typhimurium wild-type vs. tagged strains in $^{1}/_{10}$ strength R2A medium at 37°C. The error bars denote the standard deviation from the mean.

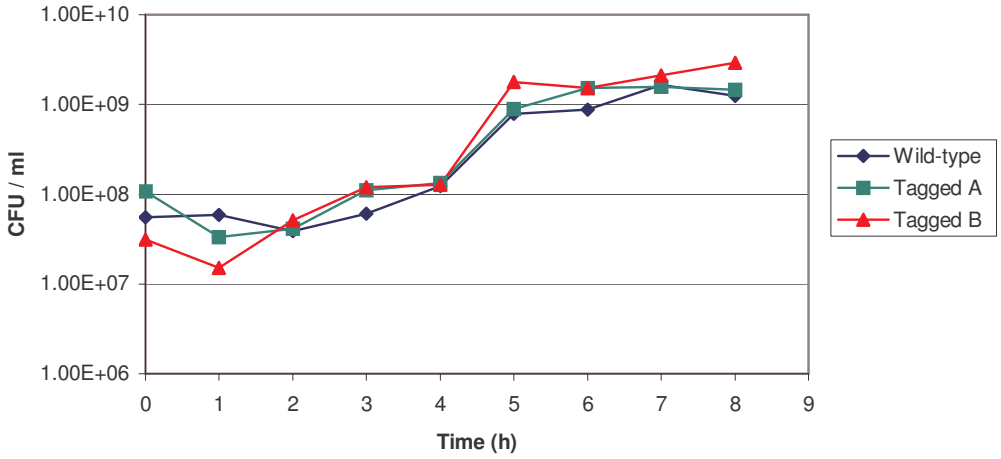

Figure 2.9 Culturable counts of *S.* ser Typhimurium wild-type and tagged strains in $^{1}/_{10}$ strength R2A medium measured in CFU/ml at 37°C.

2.3.5.2 Phenotypic Comparison

The Biolog Phenotype Microarrays indicated 21 out of a total of 379 phenotypic changes in the tagged (A) strain in comparison to the wild-type strain. Seven changes were observed in the ability of the tagged strain to utilize carbon sources, eight changes were observed in the utilisation of nitrogen sources, and six changes in the utilization of the phosphorous and sulphur sources. An assumption was made that a ratio of colour change of the tetrazolium dye of 0.5 or above constituted a difference in respiration. A positive sign indicates that the tagged strain showed greater rates of respiration than the wild-type. Negative signs indicate that the wild-type strain showed greater rates of respiration than the tagged strain (Table 2.2). These changes between the wild-type and tagged strains are not central or important for general growth and energy utilization processes. This indicates that the incorporation of the *gfp* gene is not of detriment to the fitness of the tagged strains or their ability to utilize certain substrates.

Table 2.2 Phenotypic comparisons between the *S*. Typhimurium wild-type strain and the *gfp*-tagged (A) strain

Carbon sources (PM 1)	**Wild-type strain colour change ratios of tetrazolium dye**	**Tagged strain colour change ratios of tetrazolium dye**	
D-Aspartic Acid	0.767	0.267	- [(a)]
M-Inositol	-0.11	1.089	+
Bromo Succinic Acid	0.025	0.845	+
Tricarballylic Acid	-0.131	1.003	+
Carbon Sources (PM 2)			
Dihydroxy Fumaric Acid	0.073	1.716	+
2-Hydroxy Benzoic Acid	0.148	1.841	+
D-Tartaric Acid	0.383	1.027	+
Nitrogen sources (PM 3)			
D,L –α-Amino-Caprylic Acid	0.062	0.92	+
L-Arginine	0.181	0.721	+
L- Asparagine	-0.023	0.797	+
D-Asparagine	0.735	0.077	-
D-Aspartic Acid	0.671	0.094	-
N-Acetyl-D-Mannosamine	1.241	0.019	-
Xanthine	0.368	0.923	+
Ala-Glu	0.092	1.156	+
Phosphorous and Sulphur sources (PM 4)			
D-Mannose-1-Phosphate	-1.189	-0.195	+
D-Mannose-6-Phosphate	-0.965	-0.168	+
Cysteamine-5-Phosphate	-1.251	-0.85	+
O-Phospho-D-Serine	-0.84	-0.267	+
Inositol Hexaphosphate	-0.532	-1.211	-
D-Methionine	-1.209	-0.273	+

(a) Positive signs indicate that the tagged strain showed greater rates of respiration than the wild-type. Negative signs indicate that the wild-type strain showed greater rates of respiration than the tagged strain.

2.4 DISCUSSION

Salmonella strains were isolated and identified from fresh water sediments and a clinically relevant *Salmonella enterica* subsp. *enterica* ser. Typhimurium strain was selected for tagging with GFP. Several *Salmonella* colonies could be isolated from the sediment samples. The high prevalence of *Salmonella* isolated from the sediments suggests that *Salmonella* is widely abundant in fresh water sediment of rivers in the Venda and Free State region of South Africa, where it serves as a possible contamination reservoir of drinking water sources. The pre-enrichment in the buffered peptone water allows resuscitation of the injured or stressed bacteria (Vanderzant and Splittstoesser, 1992). Rappaport Vassiliadis enrichment broth is usually recommended as selective enrichment medium when isolating *Salmonella* from environmental samples (Van Schothorst and Renaud, 1983). Xylose-lysine-deoxycholate (XLD) agar has been found to be the most selective media for the culturing of all *Salmonella* serotypes (Moriñigo *et al.,* 1989). Metabolic and biochemical tests are the basis of presumptive identification of microorganisms. Due to the genetic variability arising from mutations and the exchange of plasmids encoding biochemical traits, the API tests were confirmed by serotyping in order to identify presumptive *Salmonella* isolates. Isolate 3 (*S. enterica* subsp. *salamae*) belongs to group II of the *Salmonella* species. Serotypes belonging to this group are usually isolated from cold-blooded animals and the environment, but rarely from humans (Farmer *et al.,* 1984). Isolate 1 (*S. enterica* subsp. *enterica* ser. Typhimurium) and isolate 2 (*S. enterica* subsp. *enterica* ser. Muenchen) both belong to the Enterica subspecies of *Salmonella* (group I). This is the only subspecies of *Salmonella* that are responsible for infections in warm-blooded animals and humans (Popoff and Le Minor, 1997). *S.* Muenchen is one of approximately 2400 *Salmonella* serotypes that cause illness in humans. It is, however, an infrequently isolated serotype, which accounts for 1.6% of human *Salmonella* isolates reported in 1997 to the U.S. Public Health Laboratory Information System (Martin *et al.,* 1995 and CDC, 1998). *S*. Typhimurium was the most common isolate in the United States in 1999, causing 25% of culture confirmed infections according to the CDC *Salmonella* Surveillance 1999 Annual Summary (CDC, 2003). *Salmonella* ser. Typhimurium was selected for GFP tagging, as this is a clinically relevant environmental *Salmonella* isolate.

The pUT mini–Tn5 Km transposon (de Lorenzo *et al.,* 1990) was used to introduce GFP into the chromosome of the *S.* ser. *Typhimurium* strains. The pUT mini–Tn5 Km element has an *sfi*I cassette containing the kanamycin resistance gene and a single NotI site outside the cassette (de Lorenzo *et al.,* 1998). The fragment of the pSM1690 plasmid carrying rrnBP1-RBSII-*gfp*mut3b*-T_0T_2 is flanked by NotI sites and is inserted into the NotI site of the pUT-mini Tn5 Km (Sternberg *et al.,* 1999). The delivery plasmid contains *gfp*mut3b* (Cormack *et al*., 1996; Andersen *et al*., 1998) a very bright and stable variant of the green fluorescent protein. A growth-regulated 72 bp promoter (*rrnB*P1) from *E.coli* (Bartlett and Gourse, 1994) is inserted in front of the *gfp* gene to drive the expression of the marker gene. The delivery plasmid has a synthetic ribosome binding site, stop codons in all three reading frames and two strong transcriptional terminators (Sternberg *et al.,* 1999).

Tri-parental mating was used to transform the *Salmonella* recipient strains with the *gfp*-carrying transposon. Tri-parental mating involves donor, helper and recipient strains. The helper strain contains an RK2 origin of transfer function. The donor strain contains an R6K-based suicide delivery plasmid (Kolter *et al.,* 1978). Plasmids having the R6K origin of replication require R6K-specified replication protein π. The plasmid is only maintained in pir protein producing bacteria. The plasmid also has an origin of transfer (RP4 OriT) function, which enables the donor plasmid to be mobilized through the aid of the helper strain into target cells through RP4 conjugal transfer functions (de Lorenzo *et al.,* 1998). Prior to tagging, *S.* ser. Typhimurium was sensitive to both ampicillin and kanamycin. The media used to select transconjugants (XLD Km100) counter-selects the helper and donor strains, but selects recipient strains carrying the transposon marker. Once the transposon has integrated onto the chromosome, the recipient should only have resistance to kanamycin. If the suicide delivery plasmid replicates in the recipient and is not lost, the recipient will display resistance to kanamycin and ampicillin. Following tri-parental mating, two *S.* ser. Typhimurium isolates were obtained. Both strains were sensitive to ampicillin, but resistant to kanamycin. Both strains fluoresced green when exposed to blue light at 488 nm.

It is necessary to verify that the suicide delivery plasmid from the donor strain, carrying the *gfp* gene, does not replicate in the recipient strain, but is lost. This will allow the random integration of the *gfp* gene onto the chromosome to occur by homologous

recombination. A PCR reaction, detecting the *gfp* gene was carried out using the chromosomal DNA of the tagged strains to determine if transposition had taken place. Positive PCR reactions indicated that the gene was present on the chromosome. This was confirmed by Southern Blot hybridisation. Since PCR is a very sensitive technique it was necessary to eliminate the possibility of genomic DNA containing traces of the GFP-carrying plasmid, which could result in a false positive reaction. Southern Blot hybridisation confirmed that the *gfp* gene is not maintained as a plasmid, but has integrated into the chromosome of the tagged strains.

It is suggested that testing of growth under different nutrient conditions is required in order to compare the fitness of wild-type and genetically modified strains under environmentally relevant conditions. The stability of the GFP labelling was tested by exposing the isolates to a thirty-day growth period under non-selective conditions. The presence of the label was verified by fluorescence microscopy. After 30 days, the GFP was visible in all tagged cells, indicating stable maintenance (Fig. 2.5). Low nutrient conditions reflect the nutrient stress induced on micro-organisms that could occur in aquatic environments. The GFP fluorescence was also visible in cells under low nutrient conditions (Fig. 2.6) indicating that even when cells are subjected to nutritional stress, the new gene is still maintained. This indicates that the inserted fragment remains stably inherited and does not serve as a burden to the carrier strain.

A general problem of the mini-Tn5 transposon is that its insertion is a random process and involves the disruption of a chromosomal sequence. Environmentally important functions can thus be affected (de Lorenzo *et al.,* 1998). Therefore, all *gfp* strains generated from the transposon insertion must be screened for growth defective lesions. Reduced competitive abilities of *gfp*-tagged strains could also result from the continuous production and presence of GFP in the cell, which may slightly disturb the intracellular metabolism and nutrient transport mechanisms of the cell (Tombolini *et al*., 1997). The expression of the GFP protein itself may account for increased metabolic burden and reduced competitiveness (Füchslin *et al*., 2003). The labelled strains did not display growth rate deficiencies when compared to the wild-type strains, both in nutrient-rich (LB broth) and nutrient-poor ($^{1}/_{10}$ strength R2A) medium. GFP continued to fluoresce in cells that were cultured in low nutrient medium. Plate counts in the low nutrient medium indicated that the cells also remained culturable. This shows that the incorporation of the

gfp gene did not have any noted detrimental effects on the survival and fitness of the isolates.

Phenotypic Microarrays were used to determine the phenotypic changes in the tagged strain in comparison the the wild-type strain. Phenotypic Microarrays (PM) (Bochner *et al.,* 2001) permit examination of cellular phenotypes in a high throughput format. The Biolog Phenotypic Microarrays indicated 21 phenotypic changes in the tagged strain when compared to the wild-type strain out of a total of 379 phenotypes tested (Table 2.2). These changes between the tagged and the wild-type strains do not appear to have an influence on metabolites associated with general growth and energy. In addition, the compounds do not appear to be major components of aqueous environments. The Phenotype Microarrays are advantageous as a large number of phenotypes can be tested simultaneously. PM technology also provides a simple way to determine mutational effects on a genome-wide scale (Zhou *et al*., 2003). Phenotyping is not, however, all-inclusive. It may miss phenotypes involving surface structures and functions such as flagella attachment, biofilm formation, motility and chemotaxis as well as functions turned on only under anaerobic conditions (Zhou *et al.,* 2003).

Since the *gfp*-tagged *Salmonella* cells were indistinguishable from the wild-type with respect to growth, fitness and starvation survival, the use of chromosomally *gfp*-tagged *Salmonella* cells indicates a superior technique to monitor cells in a water environment. GFP is a very stable protein in bacteria, and allows rapid detection of *Salmonella* in a complex environment. As the *Salmonella* strain is chromosomally tagged, the possibility of gene transfer is minimised. No additional co-factors are required for the *Salmonella* cells to fluoresce. As the *gfp* gene is under the control of the *rrnB*P1 growth-regulated promoter the cells fluoresce under a variety of conditions, including starvation, but is lost from dead cells. This allows the *gfp* gene to be expresed when the cell is alive or growing, while a constitutive promoter would allow expression of the gene irrespective of environmental or developmental factors. Chromosomal tagging with GFP will allow long-term monitoring of *Salmonella* survival in nutrient limited conditions and mixed microbial populations, improving knowledge on the structure and function of *Salmonella* within a multi-species community such as a drinking water distribution system. GFP-tagging aids studies of real time gene expression *in situ*.

These studies indicate that the incorporation of the *gfp* gene did not have any noted detrimental effects on the survival and fitness of the tagged strains. These tagged strains can therefore be used *in vivo* to study the fate and survival of *Salmonella* in water environments.

2.5 CONCLUSIONS

Salmonella was successfully isolated from fresh water sediments. The clinically relevant *S.* ser. Typhimurium isolate was selected for GFP-tagging. The pUT mini-Tn5 Km transposon (de Lorenzo *et al*., 1990) was used to introduce GFP onto the chromosome of the *S.* Typhimurium strain by tri-parental mating. The *S.* Typhimurium strain was successfully tagged with GFP and displayed a bright green fluorescence when exposed to blue light. Southern Blot hybridisation confirmed that the *gfp* gene had integrated into the chromosome. The *gfp* gene was stably maintained during continuous growth for 30 days in nutrient-rich (LB broth) or nutrient–poor ($^{1}/_{10}$ strength R2A broth) medium. The *gfp*-labelled recombinants were not growth rate impaired, in nutrient-rich or nutrient–poor medium. The Phenotype Microarray changes between the wild-type and tagged strain did not appear to have an influence on metabolites associated with general growth and energy. The survival fitness studies indicate the incorporation of the *gfp* gene did not have any noted detrimental effects on the survival and behaviour of the tagged strains. These tagged strains can therefore be used in vivo to study the fate and survival of *Salmonella* in water environments.

CHAPTER THREE
FATE OF *Salmonella* IN A DRINKING WATER BIOFILM

3.1 INTRODUCTION

Salmonella is frequently isolated from water sources (Cherry *et al.*, 1972) where it serves as a reservoir for infection. *Salmonella* has a high survival rate in aquatic environments (Chao *et al.*, 1987). Compared to *E. coli*, *Salmonella* appears to withstand a wider variety of environmental fluctuations and may persist in water environments for some time (Winfield and Groisman, 2003). Although food has been implicated as the major source of non-typhoidal *Salmonella* infections in the USA (Mead *et al.,* 1999), the role of contaminated water in the transmission of the disease in developing countries is unknown.

Biofilms appear to be a common occurrence in water distribution systems (Szewzyk *et al.*, 2000). A biofilm is defined as an assemblage of microbial cells that are irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). Attachment to the surfaces of pipes gives the cell a nutritional advantage in this low nutrient environment (Camper *et al.*, 1999). Biofilms of potable water distribution systems have the potential to harbour enteric pathogens. This is problematic, as these pathogens could be released back into the flow (Szewyk *et al.,* 2000). The attached cells could also serve as a reservoir for subsequent spread through the system following detachment (Camper *et al.,* 1999). Biofilms are known to provide a safe haven for many bacteria and these bacteria can also become resistant to disinfection procedures (Costerton *et al.,* 1995).

Biofilms in drinking water distribution systems can be studied by setting up models of the system that have been fitted with reactors such as Robbin's devices and Pederson's devices (Manz *et al.,* 1993 and Pederson, 1982). These devices can be placed in the actual drinking water distribution system where stubs or slides that serve as a surface for biofilm growth can be removed for analysis. The biofilm can then be analysed by conventional culturing or by direct microscopy (Camper *et al.,* 1999). Both these approaches have limitations. Not all organisms present in biofilm may be culturable and

organisms can generally not be identified based on their morphology alone (Camper *et al.,* 1999).

There is very little understanding of how the interaction and growth of pathogenic organisms in a biofilm result in an infectious disease process. Although water-treatment plants disinfect water, it is still possible for pathogenic bacteria to enter a drinking water distribution system (Lee and Kim, 2003). At the end of a distribution system, there is a high probability of microbiological problems due to low chlorine concentrations, increased retention time and the accumulation of detached bacteria from the upstream distribution system (Lee and Kim., 2003). The period of pathogen survival in drinking water distribution systems is of concern as it may have serious health consequences. Camper *et al*., (1998) used fluorescent antibodies to monitor *S.* Typhimurium in a model distribution system. *Salmonella* cells were found to attach and persist in the model distribution system containing undefined heterotrophic bacteria and chlorine from an unfiltered osmosis system for more than 50 days, which suggests that the normal biofilm flora of this water system provided niche conditions capable of supporting the growth of this organism. The cells in the biofilm became impossible to culture on selective media over time, but could be resuscitated if the biofilm was incubated in non-selective medium. Armon *et al.,* (1997) showed that when *S.* Typhimurium was introduced into a non-sterile simulated biofilm flow system it had a good survival determined by plate counts at both temperatures of 24°C and 36°C for at least 20 to 30 days. A laboratory reactor, which simulates biofilm formation in water pipes, was used to study the interactions of biofilm formation between a nitrogen-fixing strain of *Klebsiella pneumoniae* and *Salmonella* Enteritidis. The level of attachment of *S.* Enteritidis determined by heterotrophic plate counts was higher in the binary biofilm than in the single species biofilm. In the initial colonization phase the binary biofilm contained a much higher proportion of metabolically active cells of *S.* Enteritidis than in the single species biofilms, particularly during the initial colonization period. This was determined by using a redox dye as an indicator of electron transport activity (Jones and Bradshaw, 1997).

A tagged strain distinguishable *in vivo* from the rest of the bacterial community would greatly aid the study of the fate and persistence of non-typhoidal *Salmonella* in drinking water biofilms. Techniques used to aid the microscopic visualisation of specific

organisms have been developed. The use of fluorescently labelled ribosomal probes allows the identification of specific organisms within a biofilm and can be used to determine the spatial distribution within the biofilm (Donlan and Costerton, 2002). A more recent approach to study the behaviour of a specific organism in a biofilm is by tagging the bacterial cell with the gene encoding the green fluorescent protein or GFP (Möller *et al.*, 1998). The chromosomal tagging of bacterial strains using GFP allows for the possibility of performing survival studies under mixed culture conditions. In this way, a more realistic indication of the growth potential of *Salmonella* in biofilm can be obtained due to the interaction with competing indigenous microorganisms.

The aims of this part of the investigation were therefore, (i) to visually evaluate *Salmonella* biofilm formation in drinking water biofilm supplemented with acetate, (ii) to visually evaluate the fate and survival of *Salmonella* in an established mixed culture drinking water biofilm, (iii) to quantify *Salmonella* in an independently formed biofilm, (iv) to quantify *Salmonella* in an established mixed culture drinking water biofilm and (v) to establish whether *Salmonella* is released from a drinking water biofilm.

3.2 MATERIALS AND METHODS

3.2.1 Visual evaluation of *Salmonella* biofilm formation

3.2.1.1 Visual evaluation of *Salmonella* in independent biofilms

Flow cells are small continuous-flow systems through which fresh medium is pumped continuously and waste leaves the system. The medium is not recycled through the flow cell. The flow cell has a viewing port which allows direct observation of the biofilm without disrupting the culture system (Davey and O'Toole, 2000). A flow cell was used to simulate biofilm formation in a water distribution system. A Watson Marlow 205S peristaltic pump was connected to the flow cell to allow the media to move through the flow cell channels, each having an individual dimension of 2 x 2 x 15 mm. A glass cover-slip was fixed over the channels of the flow cell with silicone sealant (Bostik®). Initially 3.5 % (m/v) sodium hypochlorite was pumped through the flow cell to sterilize it, followed by extensive rinsing with sterile ddH_2O. Thereafter, sterile tap water supplemented with 1000 µg/l acetate was pumped through the flow cell. Acetate was chosen to supplement the water as it is an easily assimilable organic substrate and aids in

the visualisation of fluorescence. The acetate-supplemented medium and the flow cell were incubated at 37°C. A sterile syringe with a 0.45 mm x 13 mm gauge needle was used to inoculate the flow cell slowly with 1 ml of culture at the point of inflow. The pump was left off for 1 hour after inoculating to allow bacteria to attach to the inner surface of the glass slide. The influent line is clamped to prevent back-growth during the inoculation. After an hour, the pump was switched on and set at a flow rate facilitating a flow of 0.4 mm/s through the flow cell. The biofilms that developed on the slides were viewed by phase contrast microscopy and epifluorescence microscopy using an inverted Zeiss Axiovert 200 fluorescent microscope (Excitation - 490 nm and Emission - 510 nm), fitted with a 100x/1.4 Zeiss Neofluor objective. Different microscopic fields were examined for each flow cell lane. The images were captured using a Nikon charge-coupled device (CCD) camera.

In order to characterise biofilm development of *Salmonella* in monoculture, one ml volumes of an overnight culture of the tagged (A) *Salmonella* and the wild-type strain, grown in a $^{1}/_{10}$ strength R2A medium, and pre-diluted to 10^{6} CFU/ml, were inoculated into separate channels of the flow cell. It was determined that *Salmonella* grown in $^{1}/_{10}$ strength R2A reached counts of 10^{7} CFU/ml at an OD of 0.007 (Section 2.3.5.1, Fig. 2.8 and 2.9), which enabled the *Salmonella* culture to be pre-diluted to the correct concentration of 10^{6} CFU/ml prior to inoculating. The flow cell was fed with acetate supplemented sterile water and was run for 72 hours.

3.2.1.2 Visual evaluation of *Salmonella* in an established mixed culture biofilm

The flow cell was set up as described in section 3.2.1.1.

3.2.1.2.1 Isolation of cultures from tap water

In order to observe the entry, fate, growth and release of *Salmonella* in a biofilm under mixed culture conditions, drinking water biofilms were cultured. In order to obtain a representative mixed culture established biofilm, bacteria were isolated from tap water. Serial dilutions were made from water taken from the laboratory tap, streaked out on R2A agar and incubated at 28°C for 48 hours. Colonies with different morphologies were selected at random and screened for auto-fluorescence by fluorescence microscopy. Five

isolates that did not display auto-fluorescence were selected and their phylogenetic position ascertained by determining the 16S rDNA sequence.

3.2.1.2.2 Identification of water isolates

Chromosomal DNA was extracted from the five water isolates using the Dneasy tissue kit (Qiagen). The isolated DNA was used directly for PCR amplification. A 1300 bp region of the 16S rRNA gene was amplified in a PCR reaction using the primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998). The 50 µl PCR reaction contained 50 pmol/µl of each primer, 2.5 mM deoxynucleotide phosphate (dNTP), 5 µl of 10 x PCR buffer, 25 mM $MgCl_2$, 1 U of taq DNA polymerase (Southern Cross) and 50-100 ng of genomic template DNA. The reaction mix was subjected to 30 amplification cycles of 94˚C for 1 minute, 55˚C for 1 minute, and 72˚C for 1 minute on a GeneAmp® 2700 PCR system (Applied Biosystems). An initial start of 94˚C for 5 minutes and an elongation step at 72˚C was also included. For control purposes, a reaction mixture containing nuclease-free water and all other reagents but no template DNA was included.

Aliquots of each PCR reaction mixture were analysed by agarose gel electrophoresis in the presence of Marker XIV (Roche) 100 bp DNA molecular weight marker. A 1% (w/v) agarose gel was cast and electrophoresed at 100 V in 1 x TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA). The agarose gel was stained with ethidium bromide at a final concentration of 1mg/ml. Prior to electrophoresis, the PCR products were resuspended in 2 µl of loading buffer (0.25% bromophenol blue; 40% (w/v) sucrose in dH_2O). The DNA was visualised by UV-induced fluorescence on a transilluminator. The 1300 bp region of the 16S rDNA gene from the five water isolates were excised from the agarose gel and purified using the QIAquick® PCR purification kit protocol (Qiagen).

The purified 16S PCR product was sequenced using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin Elmer). The forward primer 63f was used in the sequencing reactions. Each reaction mixture contained 150 ng of template DNA, 3.2 pmol/µl sequencing primer, 2 µl Terminator Ready Reaction Mix, 1 µl 5 x buffer and nuclease-free water in a final reaction volume of 10 µl. Cycle sequencing was performed using the following program

for 25 cycles: denaturation at 96ºC for 10 seconds; annealing at 50ºC for 5 seconds and extension at 60ºC for 4 minutes. An initial temperature of 96ºC for 5 seconds was also included. Following brief centrifugation, the extension products were precipitated by the addition of 16 µl of 100% ethanol and 2 µl 3M sodium acetate (pH 4.8). The tubes were incubated on ice for 20 min, centrifuged at 15000 rpm for 30 minutes and the supernatant was carefully aspirated. The pellets were rinsed twice with 50 µl of 70% ethanol, vacuum-dried for 10 minutes and then stored at -20ºC.

Prior to electrophoresis, the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer, denatured for 2 min at 90 ºC and loaded onto a model 377 automated DNA sequencer (Perkin Elmer). The obtained nucleotide sequences were edited using BioEdit Sequence alignment Editor Version 5.09. The BLAST alignment program (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for nucleotide sequences similar to the obtained sequences.

3.2.1.2.3 Establishing a mixed culture biofilm

The water isolates were inoculated separately into 10 ml of R2A media and incubated at 37°C overnight. These cultures were combined and 1 ml of the combined water isolates was inoculated into the flow cell. These water isolate biofilms were allowed to grow for 72 hours in order to obtain a thick mixed culture biofilm. After 72 hours, the established biofilm was spiked with *Salmonella*.

3.2.1.2.4 Spiking the established biofilm with *Salmonella*

One ml volumes of an overnight culture of tagged *Salmonella* grown in a $^{1}/_{10}$ strength R2A medium and pre-diluted to about 10^{6} CFU/ml and 10^{2} CFU/ml, were inoculated into separate channels containing the established biofilm and fed with acetate supplemented medium. A further channel of established biofilm remained unspiked to serve as a control. The flow cell was run for 144 hours.

3.2.1.2.5 Recovery of the tagged *Salmonella* from an established biofilm

In order to verify that *Salmonella* were growing in the mixed culture biofilm, a section of the cover-slip was removed at the end of the flow cell operation. The biofilm on the cover-slip was suspended in 9 ml of ¼ strength Ringer's solution (Merck) and vortexed.

Serial dilutions were made and 100 µl of the dilutions were plated out onto Luria-Bertani (LB) agar (1% tryptone; 0.5% yeast extract; 1% NaCl; 1.2% bacteriological agar) supplemented with 100 µg/ml kanamycin (Roche) and incubated at 37°C for 24 hours. The colonies were subsequently transferred to XLD (Xylose Lysine Deoxycholate) agar (Oxoid) and incubated at 37˚C for 24 hours. A section of the cover-slip was also suspended directly in 9 ml of buffered peptone water (BPW) (Oxoid) and vortexed. One ml of the same dilutions in Ringer's was transferred to 9 ml of BPW. The BPW was incubated at 37°C for 24 hours. One hundred µl of the BPW was transferred to 9.9 ml of Rappaport-Vassiliadis (RV) broth (Oxoid) and incubated at 42°C for 24 hours. Following incubation of the RV enrichment broth, a loop-full from each dilution of the broth was plated onto XLD (Xylose Lysine Deoxycholate) agar (Oxoid) and incubated for 24 hours at 37°C. Colonies were selected and screened for fluorescence.

3.2.1.2.6 Release of *Salmonella* from the drinking water biofilm

In order to determine whether *Salmonella* was released from the biofilm, effluent from the flow cell was collected at 72 hours post inoculation, and serial dilutions were made directly in 9 ml of BPW in triplicate and incubated at 37°C for 24 hours. After 24 hours, 100 µl of the BPW dilutions were transferred to 9.9 ml of RV broth and incubated at 42°C for 24 hours. Following incubation of the RV enrichment broth, a loop-full from each dilution of the broth was plated onto XLD agar and incubated for 24 hours at 37°C. The black colonies were selected and screened for fluorescence.

3.2.2 Quantitative evaluation of biofilm formation

3.2.2.1 Quantitative evaluation of *Salmonella* from an independent biofilm

In order to simulate a drinking water distribution system, silicone pipes of 1.59 x 0.79 x 1.41 mm (Sigma-Aldrich) were connected to a Watson Marlow peristaltic pump. Initially 3.5 % (m/v) sodium hypochlorite was pumped through the pipes, followed by extensive rinsing with sterile ddH_2O. Thereafter, sterile tap water supplemented with 100 µg/l acetate was pumped through the pipe. The acetate-supplemented medium and the pipes were incubated at a room temperature of 25°C. A sterile syringe with a 0.45 mm x 13 mm gauge needle was used to inoculate the pipes slowly with culture. The pump was left off for two hours after inoculating to allow bacteria to attach to the inner surface of the pipes.

After two hours, the pump was switched on and set at a flow rate facilitating a flow of 0.4 mm/s through the pipes. Quantitative counts were taken 1.5 hours after switching on the pump and on a daily basis thereafter for the duration of 144 hours. The presence of *Salmonella* was determined in triplicate using three separate pieces of pipes for each of the treatments. A separate pipe remained unspiked to serve as a control for contamination. Plate counts on R2A agar were taken and selective enrichment was carried out for MPN determinations initially at 1.5 hours and every 24 hours thereafter for the duration of 144 hours. Five cm sections from each pipe were removed and the biofilm was rinsed from the pipe with 5 ml of sterile ddH_2O. One ml of these suspended biofilm cells was transferred to 9 ml of ¼ strength Ringer's solution and serial dilutions were made. One hundred µl of each dilution was plated out onto R2A agar (Oxoid) and incubated at 37°C for 24 hours. After incubation the colonies were counted. One ml of the suspended biofilm cells and one ml of the same dilutions in the Ringer's solution was also transferred, in triplicate to 9 ml tubes of BPW. The BPW was incubated at 37°C for 24 hours. After 24 hours the 100 µl of the BPW was transferred to 9.9 ml of RV broth and incubated at 42°C for 24 hours. A loop-full from each tube of RV broth was plated out onto XLD agar and incubated at 37°C for 24 hours. After determining the number of positives for each dilution in triplicate, the most probable number (MPN) estimate was determined from the MPN table (Beliaeff and Mary, 1993). In order to quantify the release of *Salmonella* from the biofilm, the effluent of the pipes was collected at 144 hours of operation and dilutions were made in ¼ strength Ringer's solution. Each dilution was plated out onto R2A and dilutions were also transferred to BPW, followed by RV broth and plating on XLD as described previously for MPN determinations. Colonies were selected at random from the R2A plates and screened for fluorescence.

In order to quantify biofilm development of *Salmonella* in monoculture, the silicone pipes were inoculated with an overnight culture of the tagged (A) *Salmonella* strain in $^1/_{10}$ strength R2A medium. The cultures were pre-diluted to 10^6 CFU/ml and 10^2 CFU/ml and each concentration was inoculated into three separate pipes and fed with the acetate supplemented sterile tap water.

3.2.2.2 Quantitative evaluation of *Salmonella* in a mixed culture biofilm

In order to quantify *Salmonella* survival in an established mixed culture biofilm, the silicone pipes were set up as previously described in section 3.2.2.1. Overnight cultures of the water isolates used previously for flow cell study, grown in R2A medium, were combined and inoculated into the pipes. The mixed culture biofilm was allowed to establish for 144 hours. After 144 hours, an overnight culture of the tagged (A) strain of *Salmonella*, grown in a $^{1}/_{10}$ strength R2A medium and pre-diluted to 10^{2} CFU/ml and 10^{6} CFU/ml, were each inoculated into three separate pipes and fed with acetate supplemented medium. A further pipe of established biofilm remained unspiked to serve as a control. The pipes were run for 144 hours. Plate counts on R2A agar were taken and selective enrichment was carried out as described previously for MPN determinations initially at 1.5 hours and every 24 hours thereafter for the duration of 144 hours. In order to quantify the release of *Salmonella* from a mixed culture biofilm, the effluent of the pipes was collected at 144 hours of operation and dilutions were made in ¼ strength Ringer's solution. Each dilution was plated out onto R2A and dilutions were also transferred to BPW in triplicate, followed by RV broth and plating on XLD as described previously for MPN determinations. Colonies were selected at random from the R2A plates and screened for fluorescence.

3.2.2.3 Evaluation of *Salmonella* growth on R2A versus the MPN technique

To evaluate the growth of *Salmonella* in monoculture on R2A and using the MPN method, serial dilutions were made from an overnight culture of *Salmonella* grown in a $^{1}/_{10}$ strength R2A medium. These dilutions were plated directly on R2A agar and the same dilutions were also transferred to BPW in triplicate, followed by RV broth and subsequent plating on XLD agar, as described previously in section 3.2.2.

3.3 RESULTS

3.3.1 Visual evaluation of *Salmonella* in independent biofilms

Salmonella did attach and form biofilms in drinking water supplemented with 1000 µg/l acetate at 37°C. *Salmonella* did not appear to form compact biofilms under these low nutrient conditions, but accumulated on the surface with sparsely aggregated clumps. At 24 hours, a thin monolayer of cells was formed (Fig. 3.1 and Fig. 3.4). Within 48 hours

small micro-colonies began to develop (Fig. 3.2 and Fig. 3.5). However, the colonies did not amalgamate and cells did not appear to migrate from one micro-colony to another. After 72 hours larger micro-colony clusters were visible which were not dispersed across the surface but aggregated in clumps (Fig. 3.3 and Fig. 3.6). Both the wild-type *Salmonella* and the tagged strain showed similar biofilm formation.

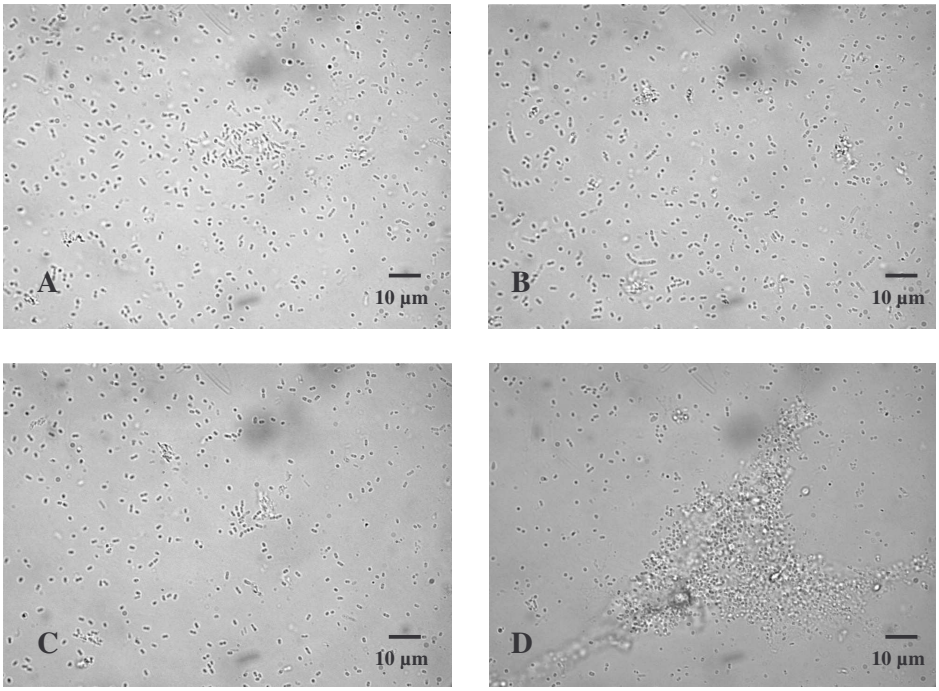

Figure 3.1 Wild-type strain (A, B, C, D) after 24 hours viewed by phase contrast microscopy.

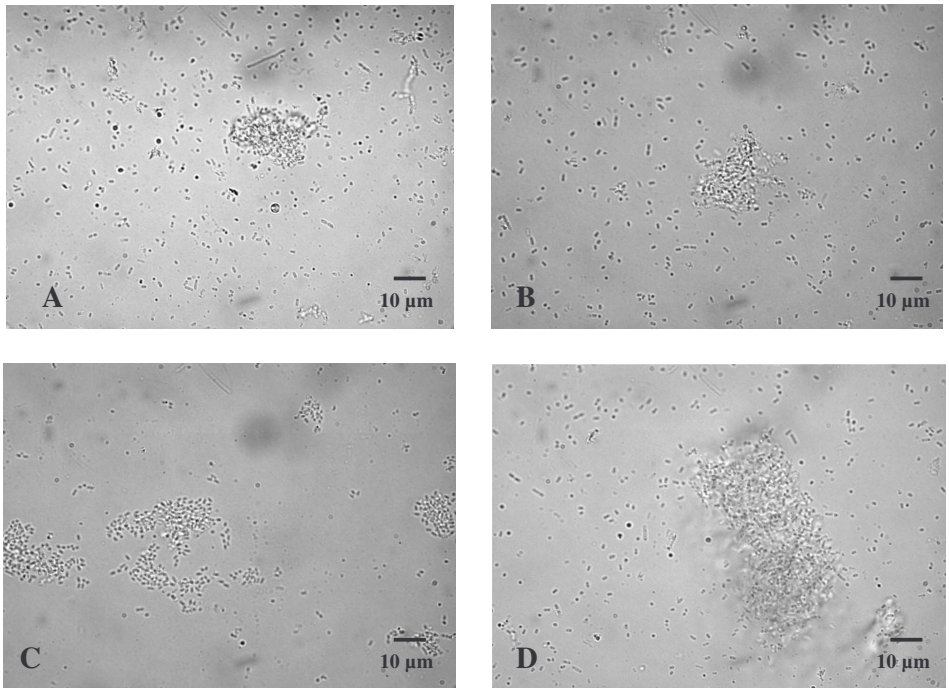

Figure 3.2 Wild-type strain (A, B, C, D) after 48 hours viewed by phase contrast microscopy.

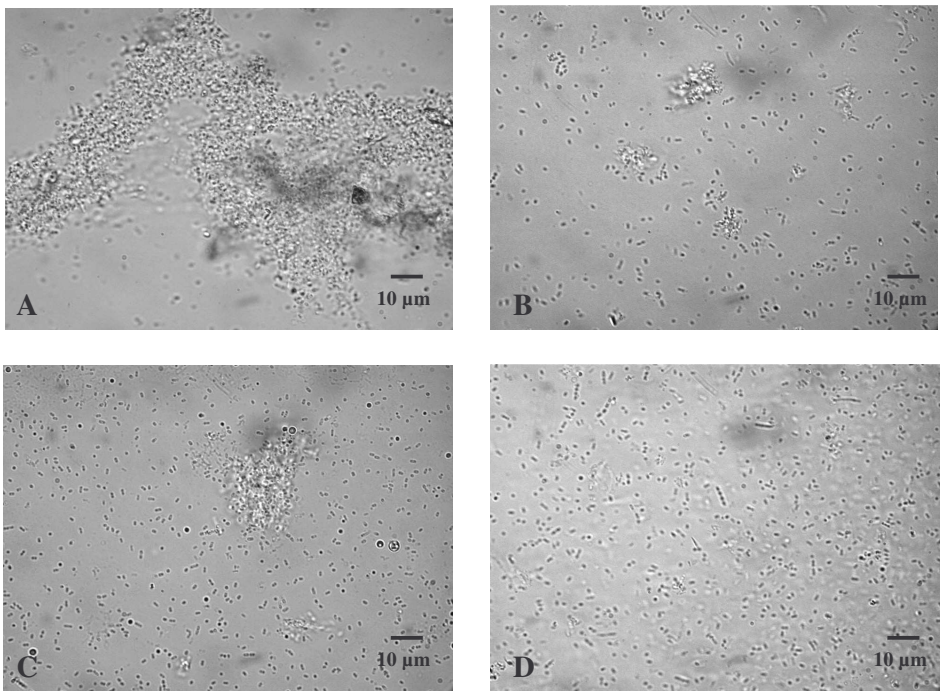

Figure 3.3 Wild-type strain (A, B, C, D) after 72 hours viewed by phase contrast microscopy.

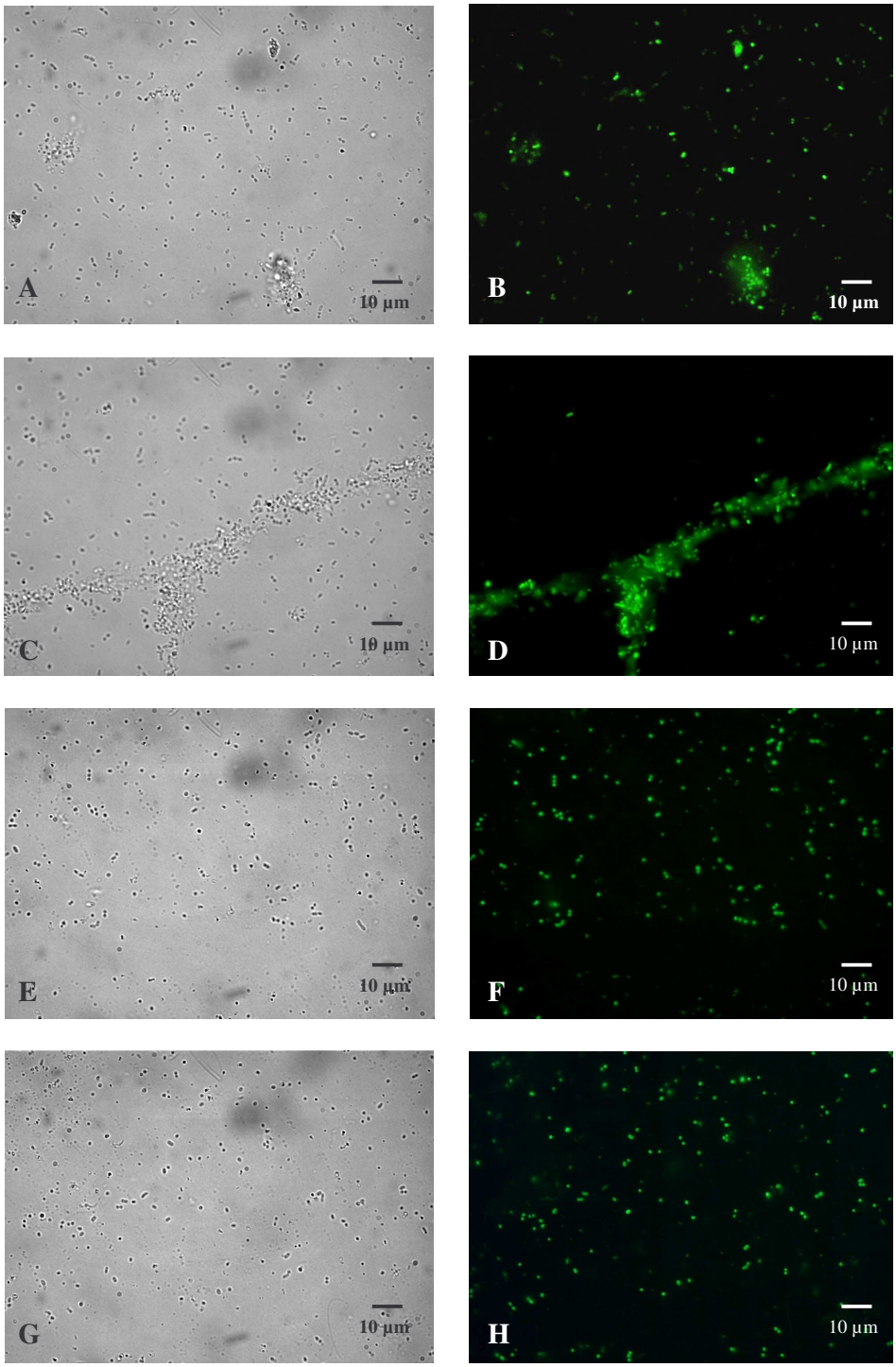

Figure 3.4 Tagged strain after 24 hours viewed by phase contrast (A, C, E, G) and fluorescence microscopy (B, D, F, H).

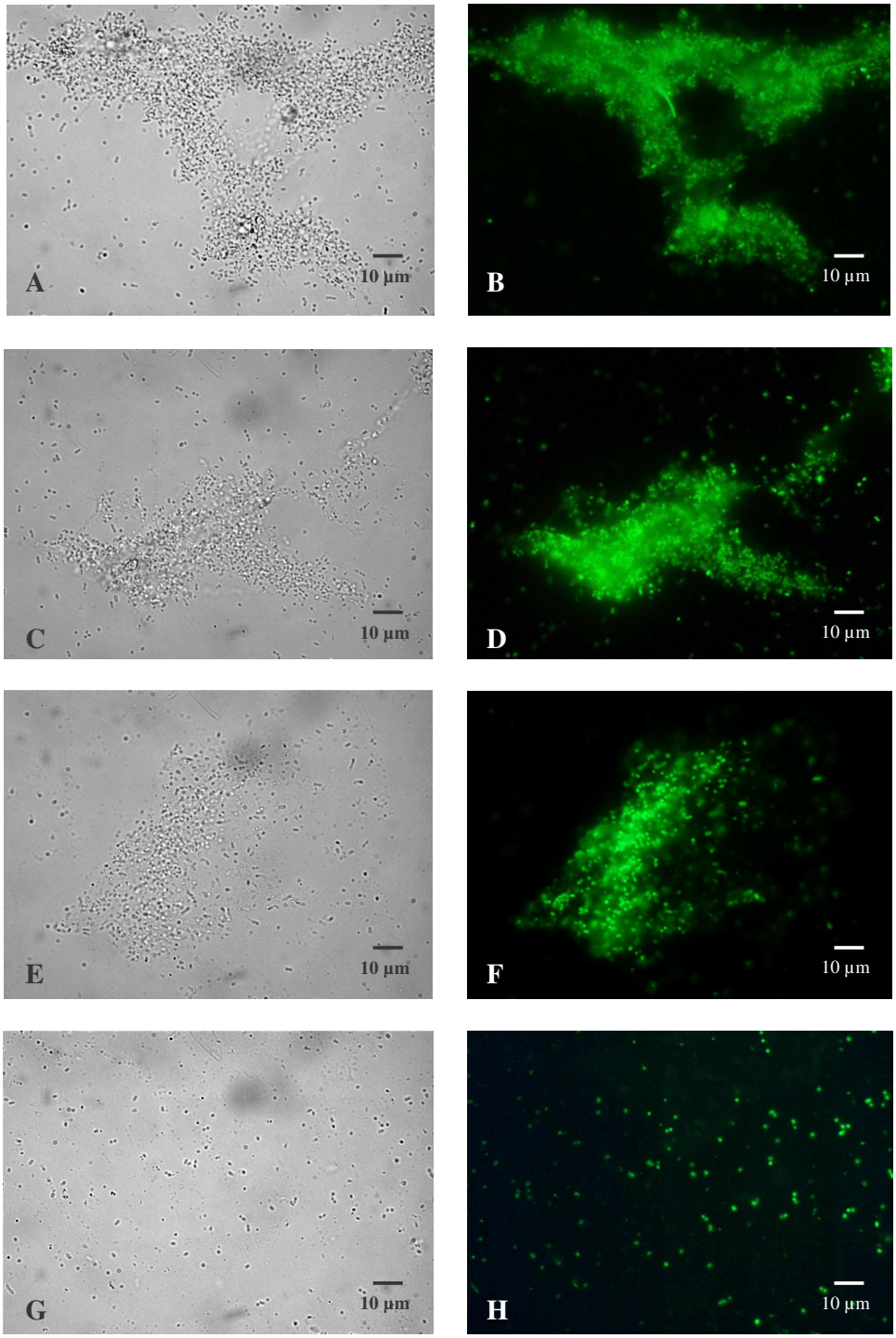

Figure 3.5 Tagged strain after 48 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

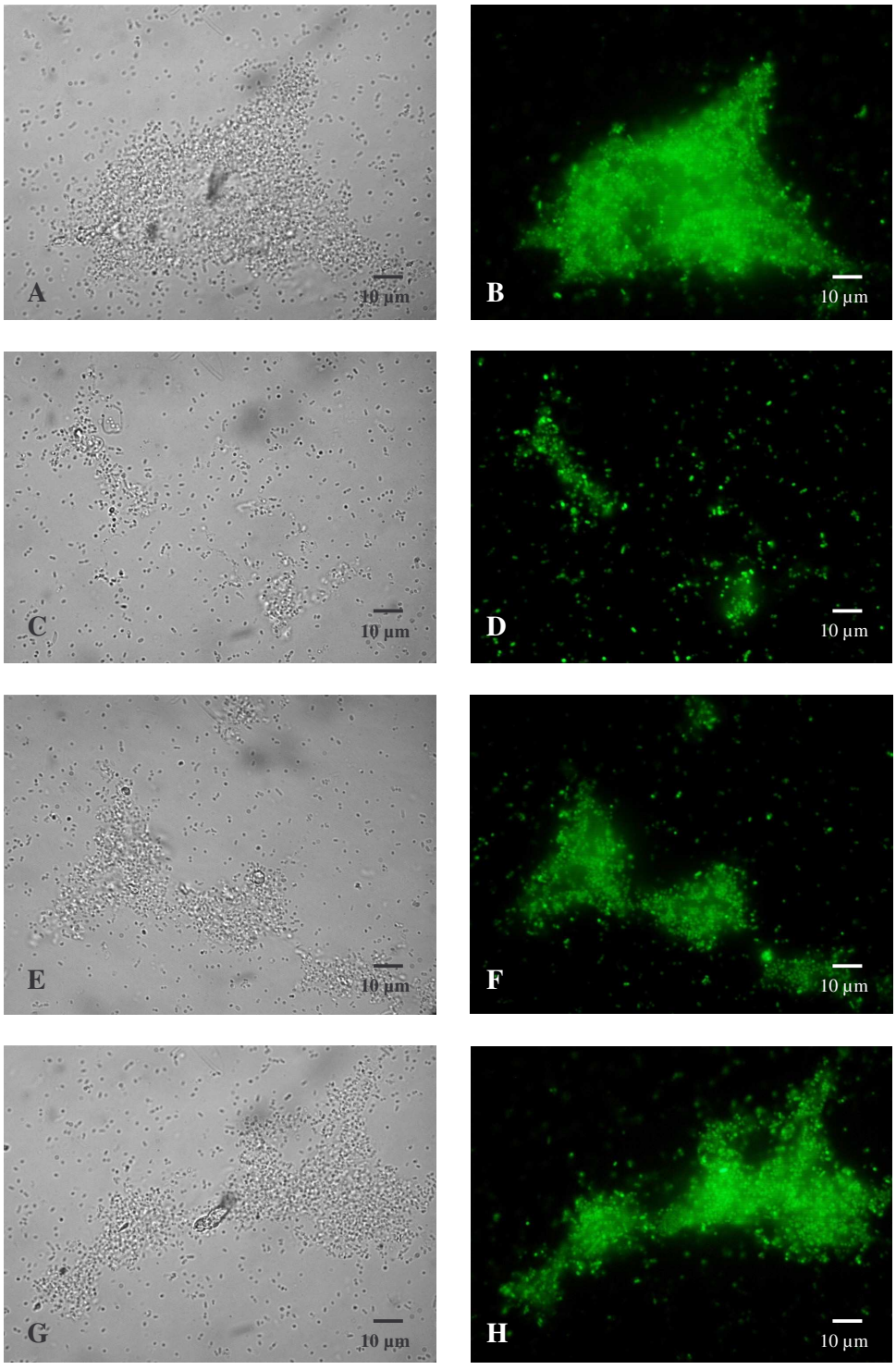

Figure 3.6 Tagged strain after 72 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

3.3.2 Quantitative evaluation of *Salmonella* from an independent biofilm

A significant difference in surface accumulation of *Salmonella* was observed between the spiked high inoculum (10^6 CFU/ml) and the low inoculum (10^2 CFU/ml) of drinking water biofilms supplemented with 100 µg/l acetate and grown at 25°C. At 1.5 hours after spiking, the attachment levels of the low inoculum were approximately 10^1 MPN/cm^2, while that of the high inoculum was > 10^3 MPN/cm^2. Both inoculums steadily increased until 72 hours, the high inoculum increasing by approximately 2 log and the low inoculum by approximately 1 log. Thereafter, the low inoculum biofilm culturable number remained fairly constant. The high inoculum biofilm dropped by one order of magnitude at 120 hours, but increased by 0.5 log at 144 hours (Fig. 3.7). The effluent from the pipes taken at 144 hours of operation indicated that *Salmonella* was released from the high inoculum biofilm at a concentration of > 10^3 MPN/ml and > 10^2 MPN/ml from the low inoculum biofilm (Fig. 3.7).

In comparison, the R2A counts for both the high and the low inoculum were higher than the MPN counts. At 72 hours, the high inoculum counts were approximately 0.5 log higher than the MPN counts, while the low inoculum counts were 1 log higher than the MPN counts (Fig. 3.8). The effluent from the pipes taken at 144 hours of operation indicated that *Salmonella* was released from the high inoculum at a concentration of > 10^3 CFU/ml and > 10^2 CFU/ml from the low inoculum (Fig. 3.8).

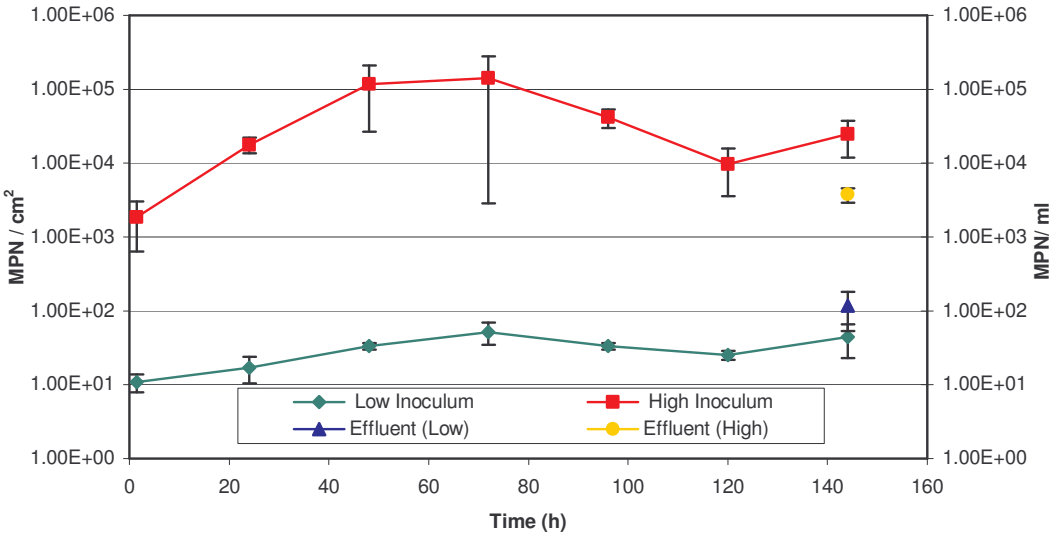

Figure 3.7 Average independent *Salmonella* biofilm MPN counts for high and low inoculum spiking concentrations and effluent counts following detachment from the biofilm system. The error bars denote the standard deviation from the mean.

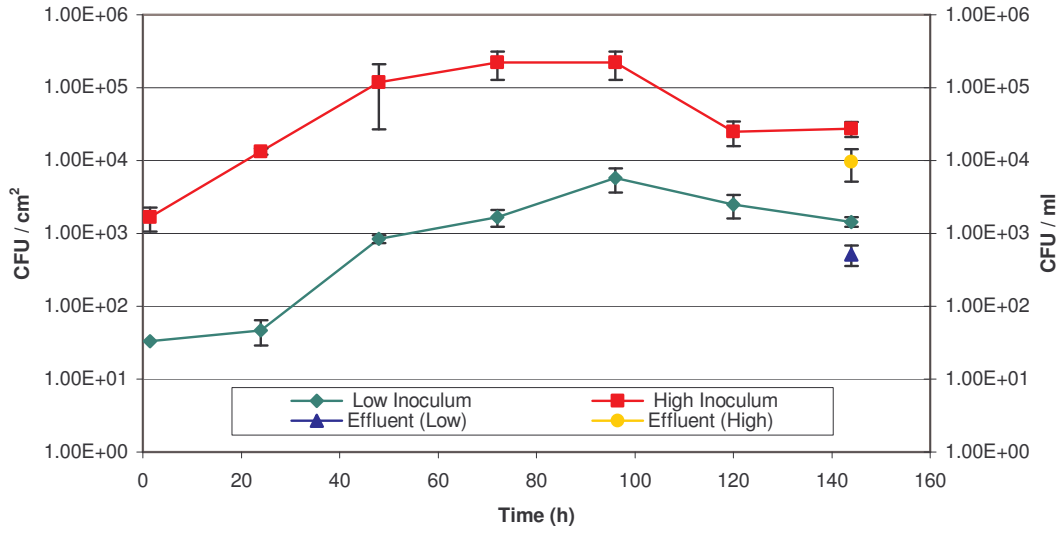

Figure 3.8 Average independent *Salmonella* biofilm R2A counts for high and low inoculum spiking concentrations and effluent counts following detachment from the biofilm system. The error bars denote the standard deviation from the mean.

3.3.3 Visual evaluation of *Salmonella* in an established mixed culture biofilm

3.3.3.1 Isolation of cultures from tap water

Several colonies were obtained on the R2A agar and the five isolates that did not display autofluorescence were selected for sequencing.

3.3.3.2 Sequencing of water isolates

A BLAST search against all nucleotide sequences contained in the GenBank database revealed a significant sequence homology of 96-100% between the water isolate sequences and the database sequences. The probable identity of these water isolates is summarized in Table 3.1.

Table 3.1 16S rRNA closest homologue of water isolates

Water Isolate	Closest Homologue
1	*Paenibacillus favisporus*
2	*Bacillus* sp.
3	*Paenibacillus cineris*
4	*Paenibacillus* sp.
5	*Enterococcus mundtii*

3.3.3.3 Establishing a mixed culture biofilm

After 72 hours a mixed culture biofilm was formed containing thick layers of intermixed micro-colonies, which did not autofluoresce. The tagged strain was inoculated into this biofilm and its fate and survival was investigated.

3.3.3.4 Spiking the established biofilm with *Salmonella*

Salmonella can attach and survive in a mixed culture biofilm in drinking water supplemented with 1000 µg/l acetate at 37°C. After 24 hours individual *Salmonella* cells could be visualised among the mixed culture biofilm at spiking concentrations of both 10^6 CFU/ml and 10^2 CFU/ml (Fig. 3.9 and Fig 3.15). *Salmonella* persisted in the established biofilm, forming small microcolonies after 48 hours (Fig. 3.10 and Fig. 3.15). *Salmonella* continued to persevere in the established biofilm forming small masses at 72

(Fig. 3.11 and Fig. 3.16) and 96 hours (Fig 3.12 and Fig. 3.17). Bubbles appear from time to time in the medium feed due to the permeability of the silicon tubing to atmospheric gases. This may lead to a bubble moving through the flow cell, exerting a shear force on the attaching cells, resulting in detachment of these cells. It was shown that once a layer of established biofilm had been removed by a bubble, *Salmonella* had the ability to recolonise a cleared zone. This indicates that *Salmonella* growing as part of a drinking water biofilm may be disseminated and re-colonize another part of the biofilm. After 144 hours the established biofilm had formed very thick intermixed micro-colonies and *Salmonella* could still be detected as multicellular conglomerates (Fig. 3.13 and Fig. 3.18). This indicates that *Salmonella* can grow and survive in a mixed culture biofilm.

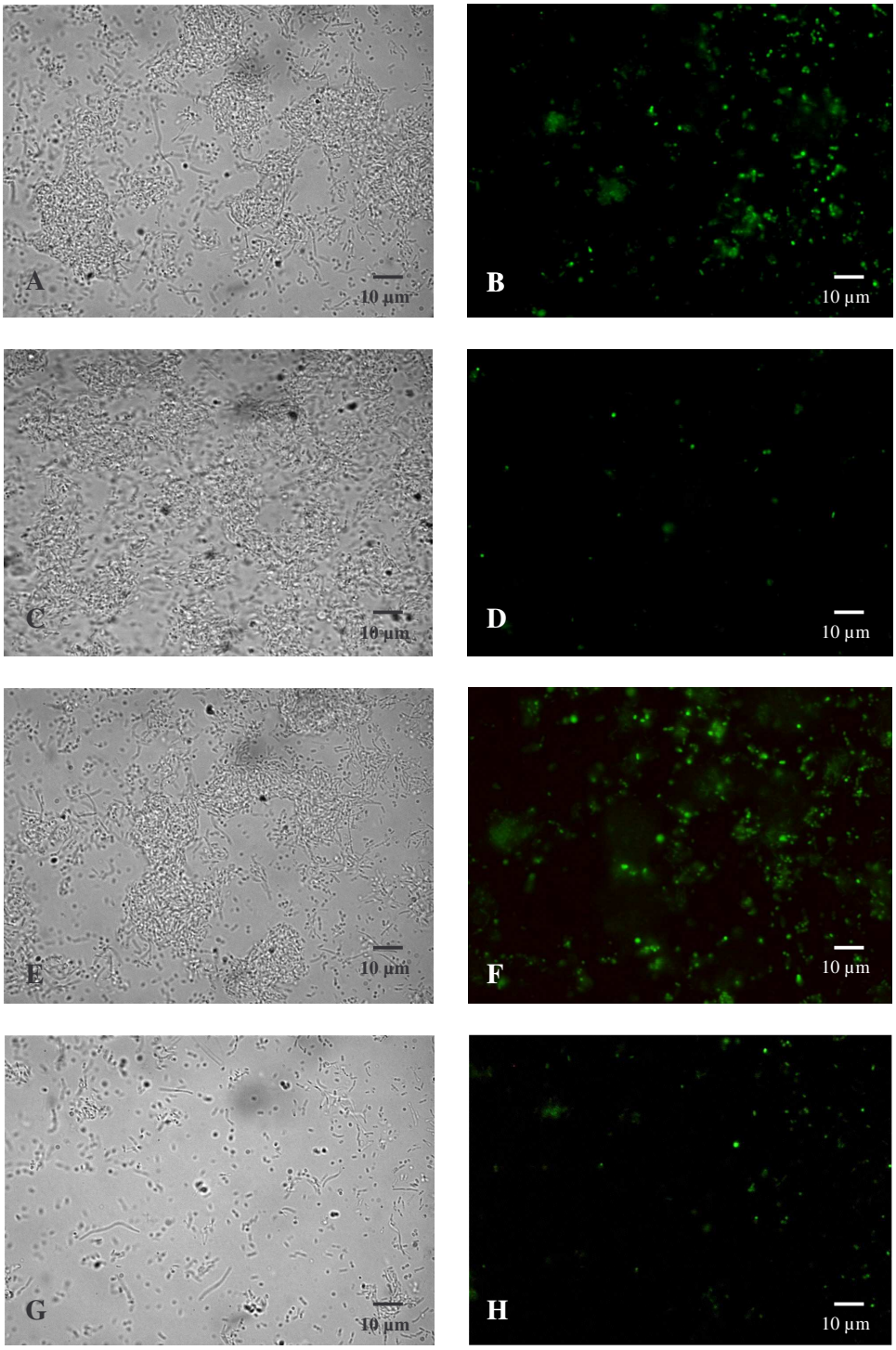

Figure 3.9 Mixed culture biofilm spiked with *Salmonella* of 10^6 CFU/ml after 24 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

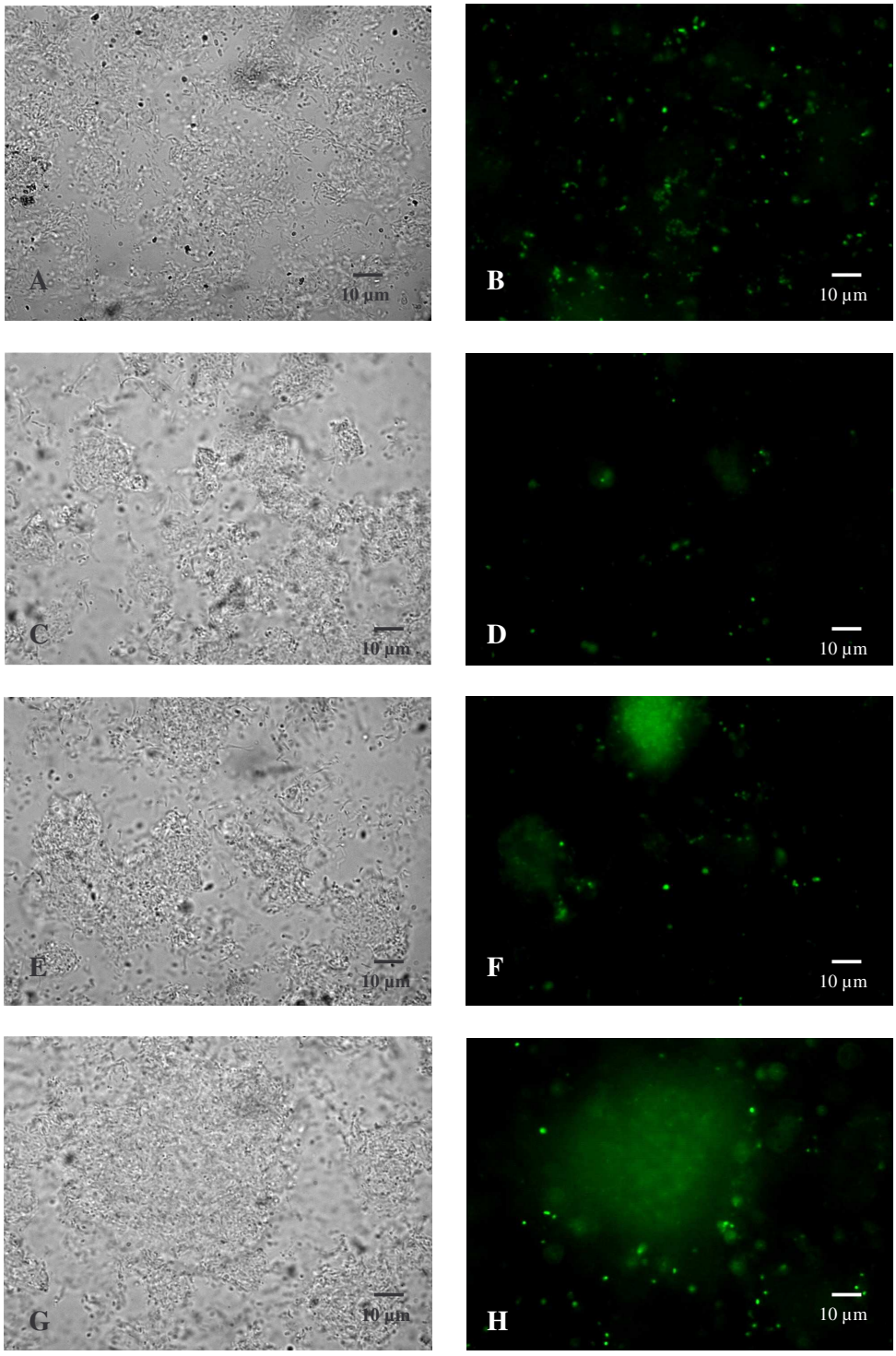

Figure 3.10 Mixed culture biofilm spiked with *Salmonella* of 10^6 CFU/ml after 48 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

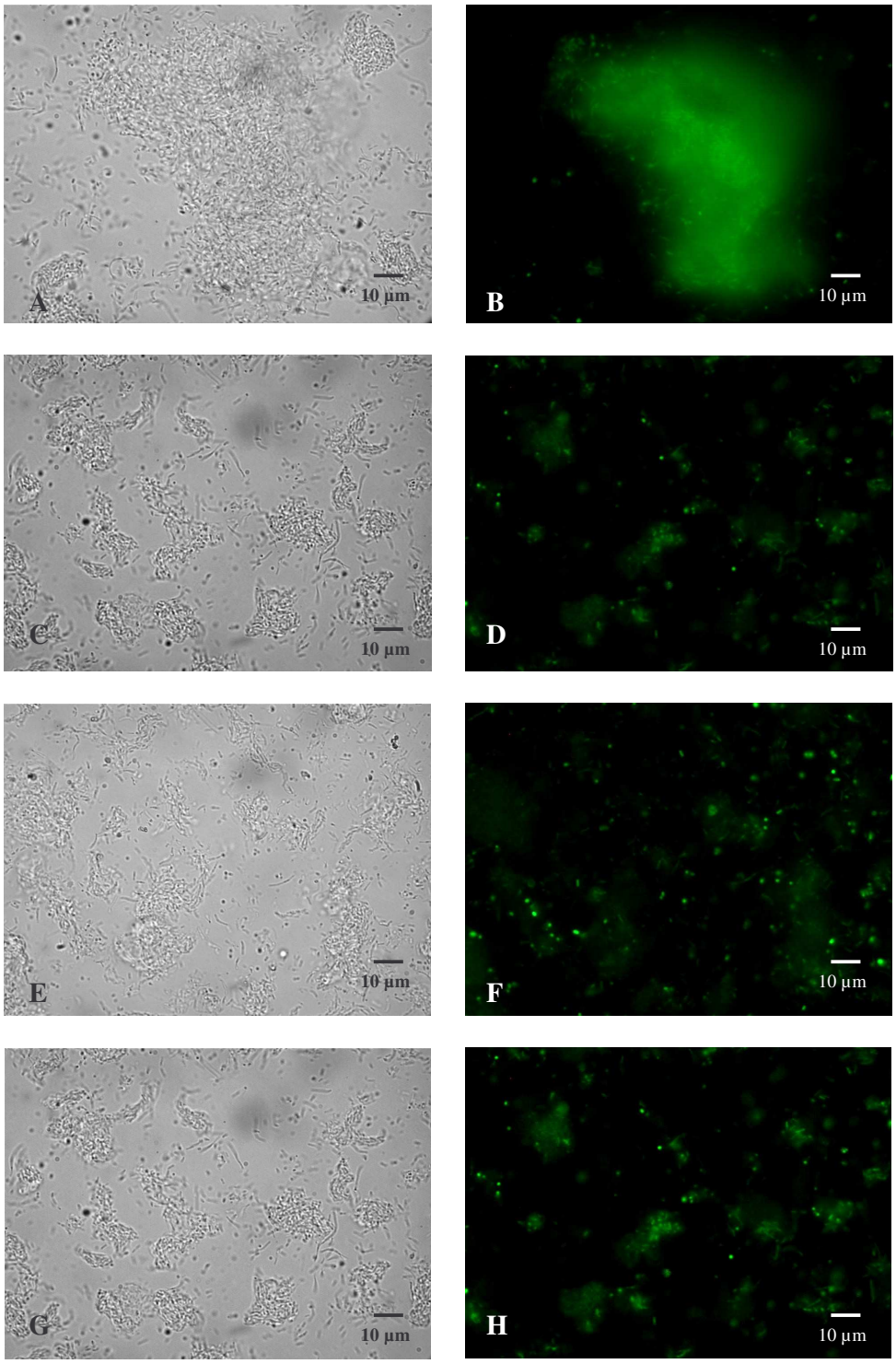

Figure 3.11 Mixed culture biofilm spiked with *Salmonella* of 10^6 CFU/ml after 72 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

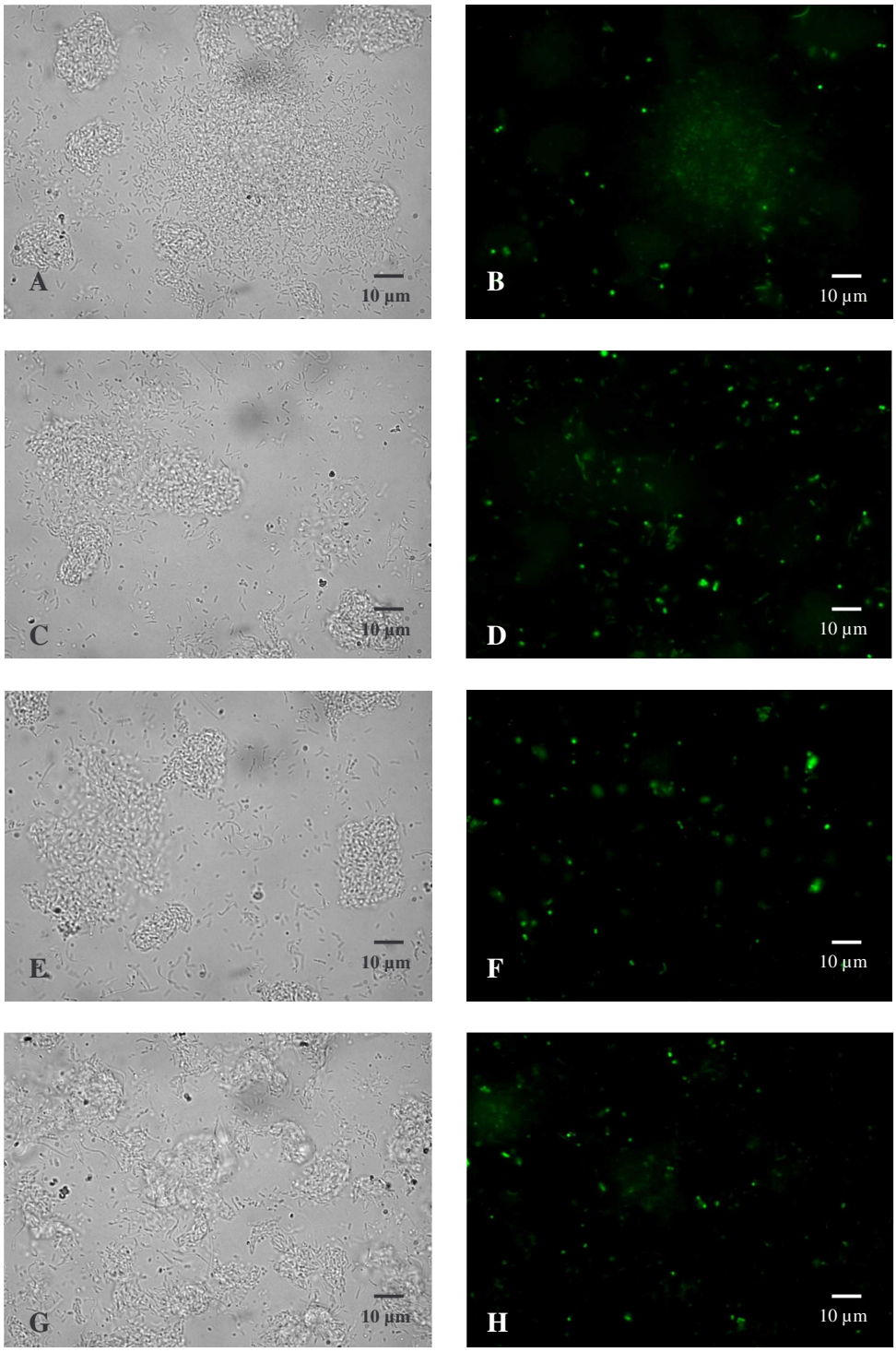

Figure 3.12 Mixed culture biofilm spiked with *Salmonella* of 10^6 CFU/ml after 96 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

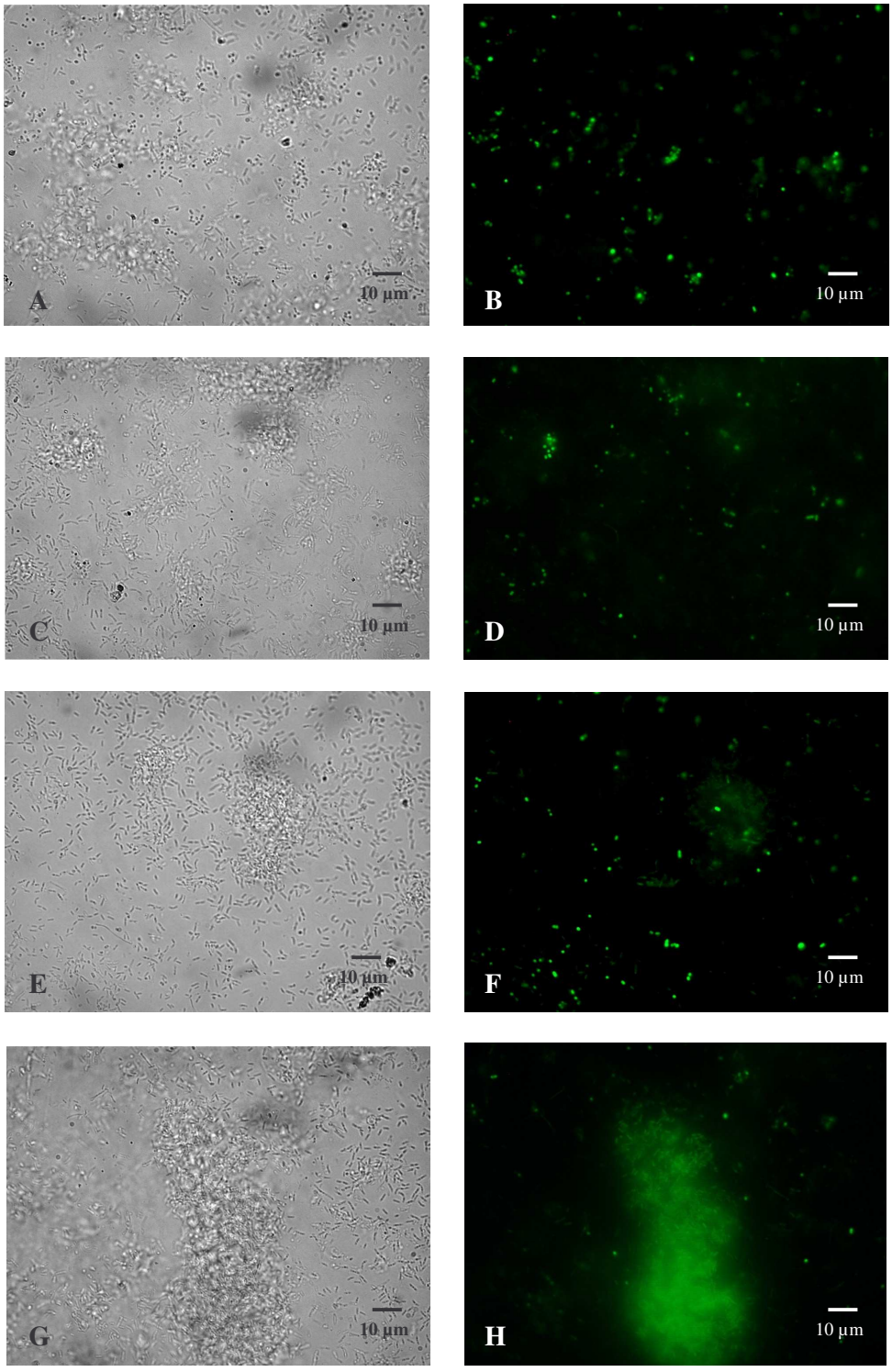

Figure 3.13 Mixed culture biofilm spiked with *Salmonella* of 10^6 CFU/ml after 144 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

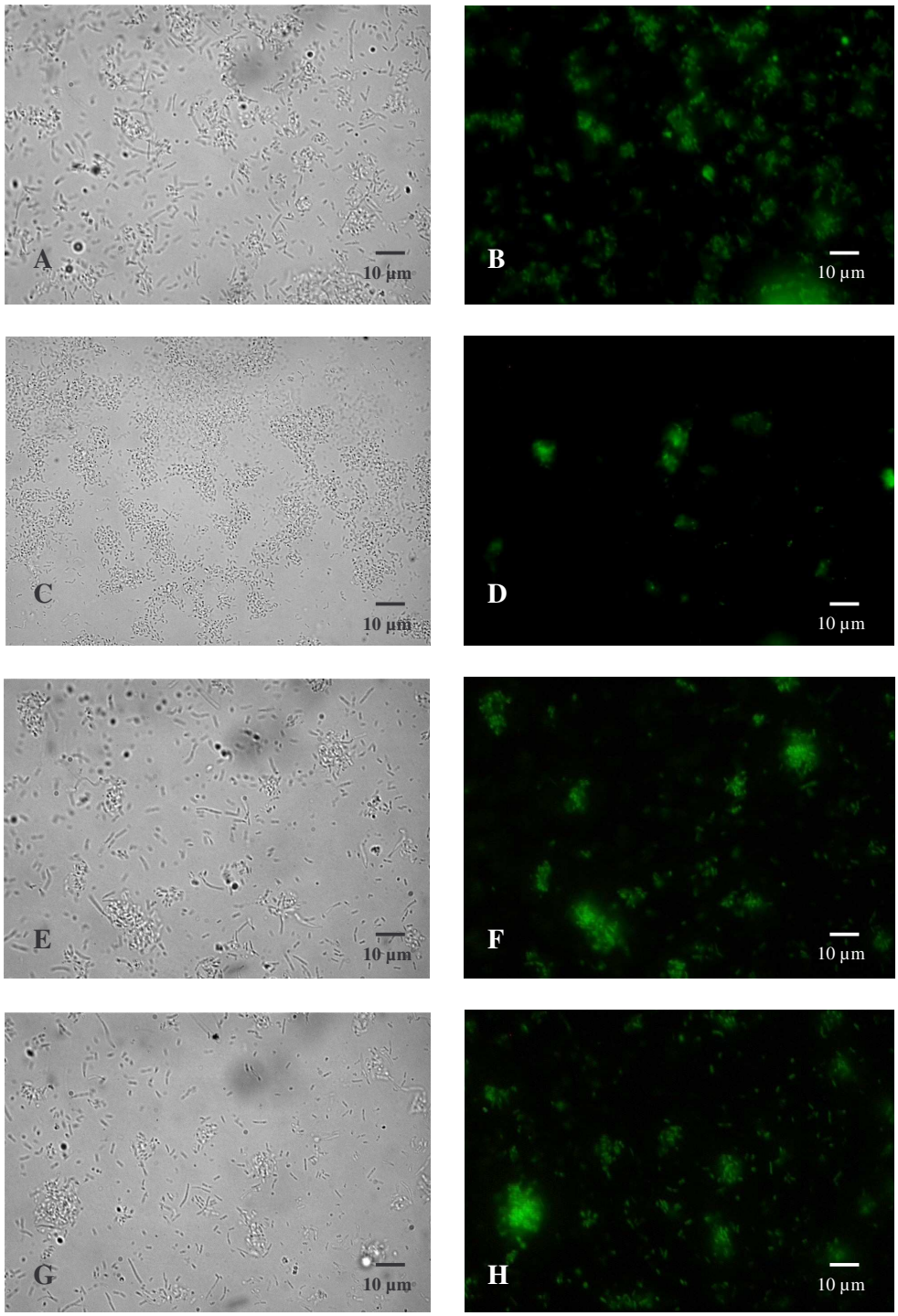

Figure 3.14 Mixed culture biofilm spiked with *Salmonella* of 10^2 CFU/ml after 24 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

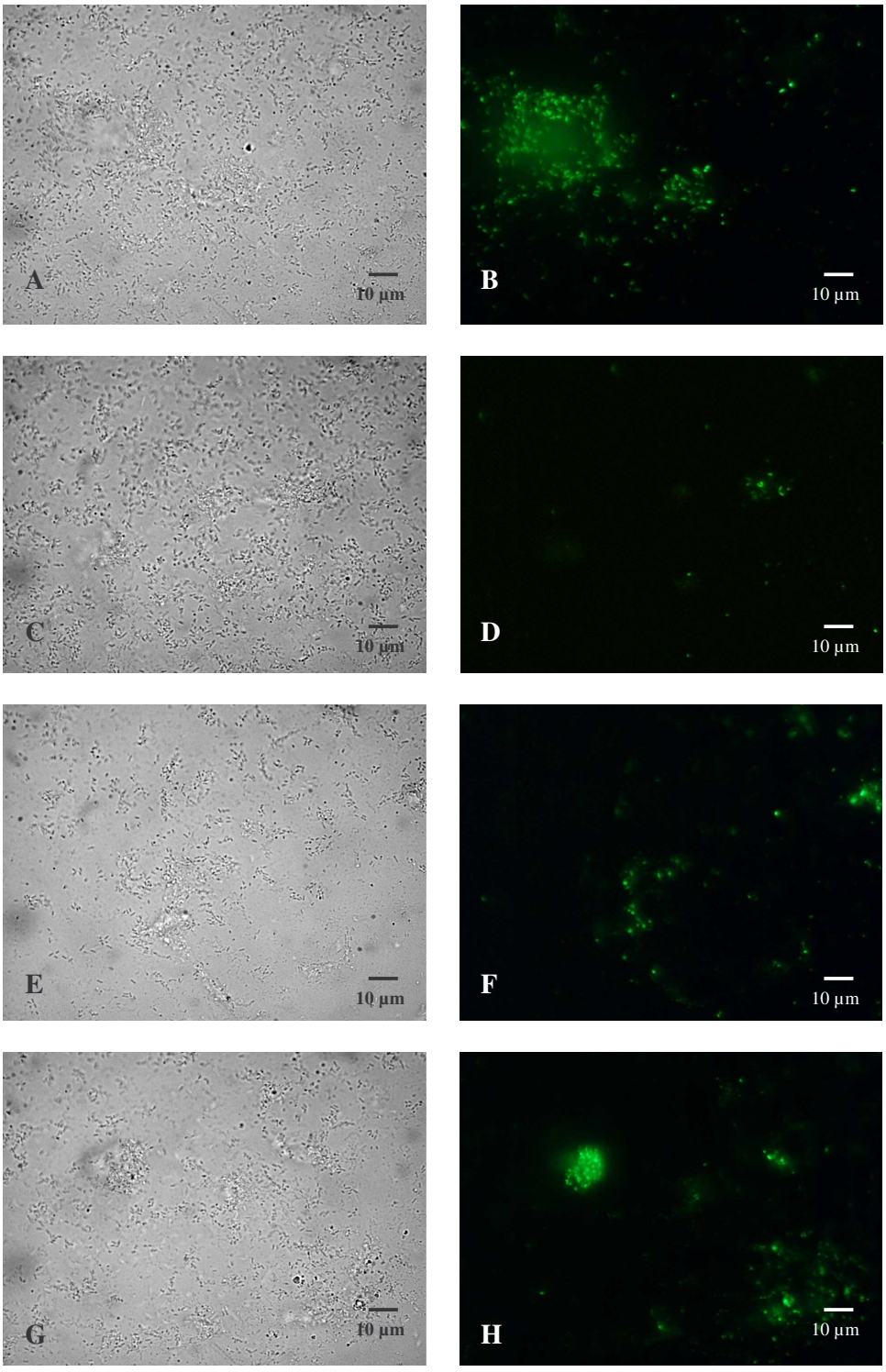

Figure 3.15 Mixed culture biofilm spiked with *Salmonella* of 10^2 CFU/ml after 48 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

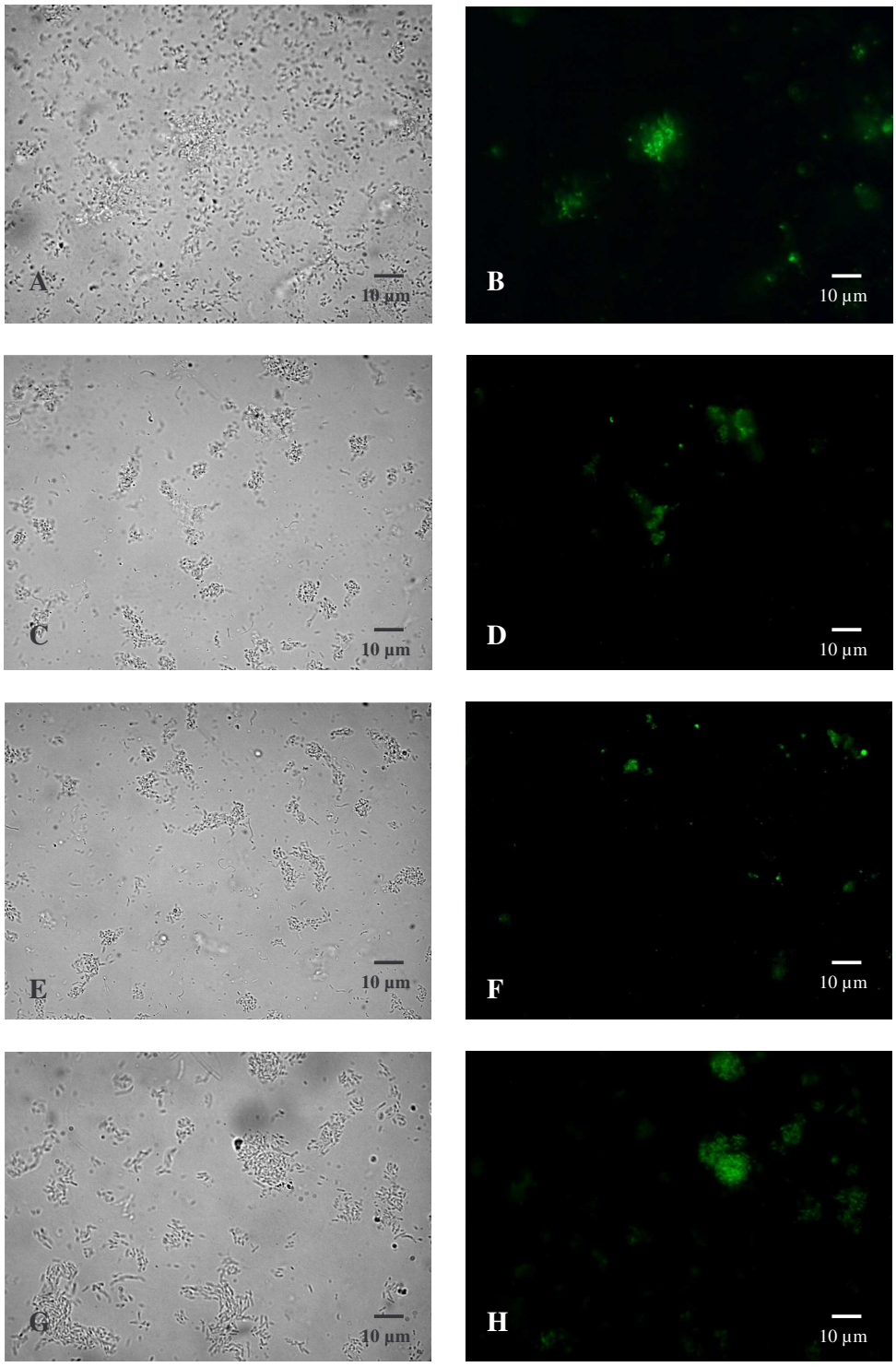

Figure 3.16 Mixed culture biofilm spiked with *Salmonella* of 10^2 CFU/ml after 72 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

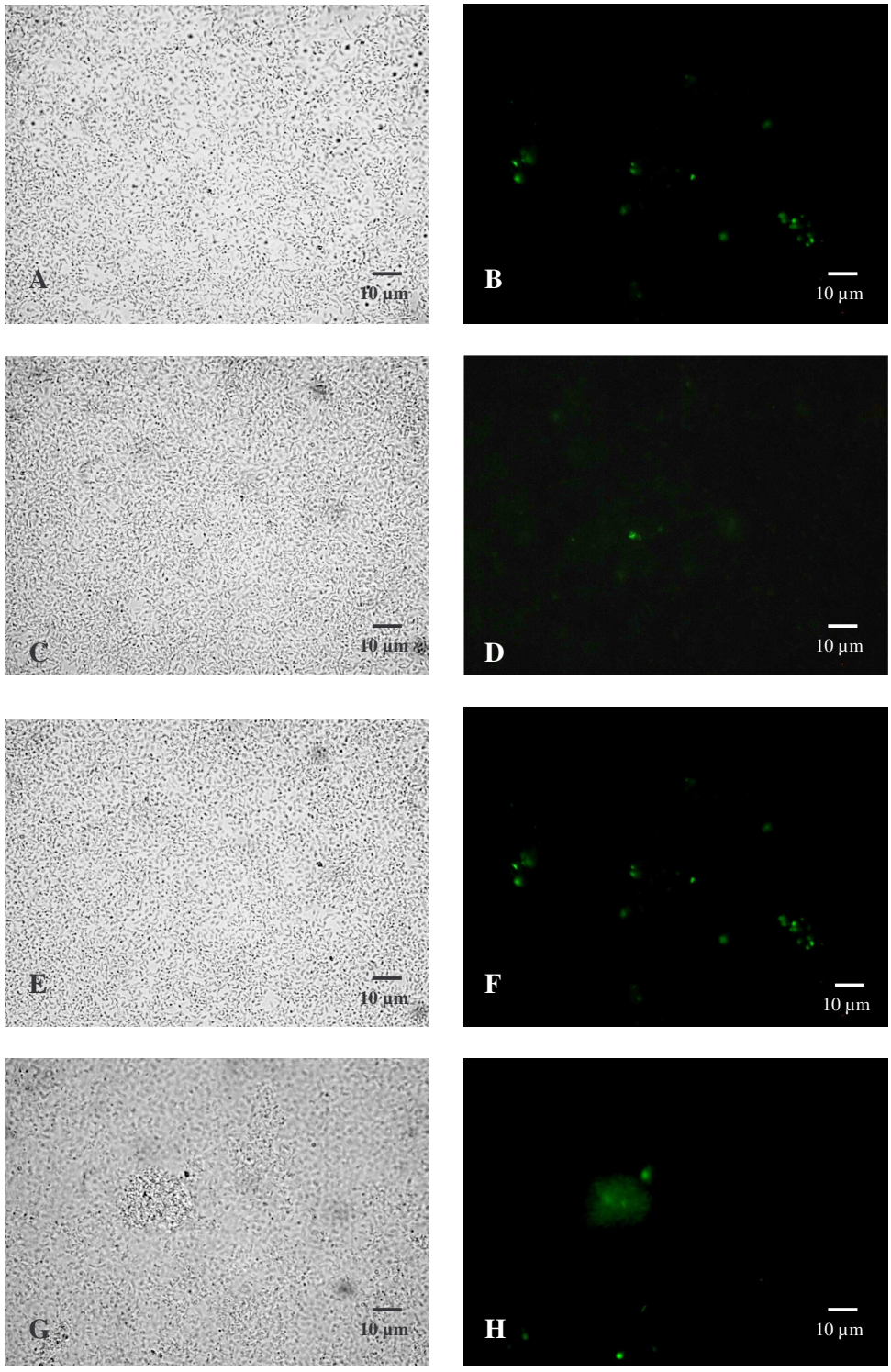

Figure 3.17 Mixed culture biofilm spiked with *Salmonella* of 10^2 CFU/ml after 96 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

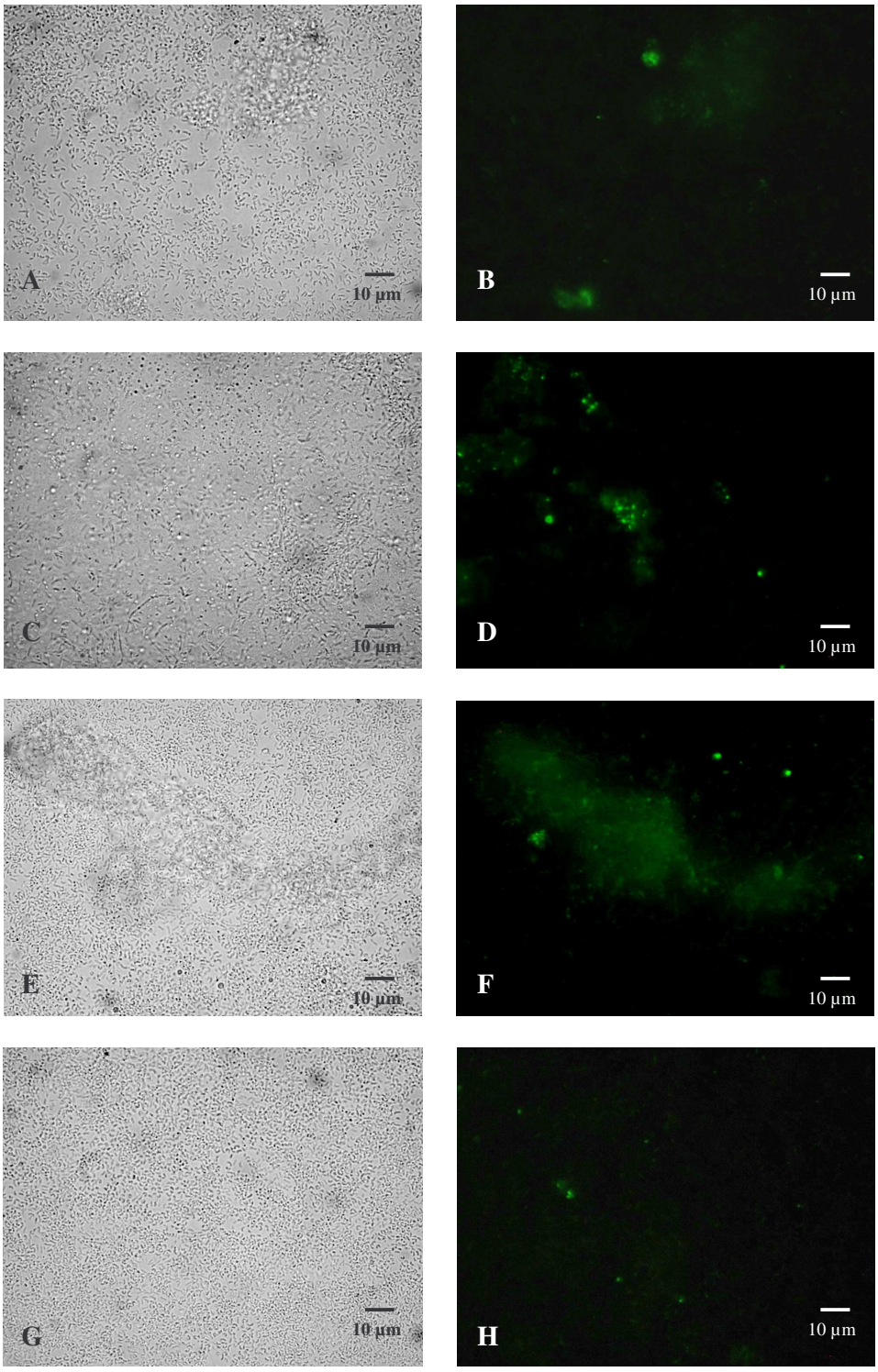

Figure 3.18 Mixed culture biofilm spiked with *Salmonella* of 10^2 CFU/ml after 144 hours & viewed by phase contrast (A, C, E, F) and fluorescence microscopy (B, E, G, H).

3.3.3.5 Recovery of the tagged *Salmonella* from an established biofilm

Dilutions of the 144 hour-old biofilm from the initial high inoculum (10^6 CFU/ml) made directly in BPW all subsequently yielded black colonies on XLD, confirming the presence of *Salmonella* in the biofilm. Plating of suspensions onto kanamycin-containing agar yielded 44 colonies on the highest dilution showing growth. All 44 colonies grew on XLD agar, producing black growth and showing that all kanamycin-resistant colonies were *Salmonella* and that all fluorescing cells were *Salmonella. Salmonella* could not be recovered directly from the biofilm on the coverslip with a low initial inoculum (10^2 CFU/ml).

3.3.3.6 Release of *Salmonella* from the drinking water biofilm

In order to determine whether *Salmonella* was released from the biofilm, effluent from the flow cell was subjected MPN determination. All colonies from the RV broth appeared black on XLD and $>10^4$ CFU/ml of *Salmonella* was obtained for the high inoculum of 10^6 CFU/ml and $>10^3$ CFU/ml for the low inoculum of 10^2 CFU/ml was obtained, showing that *Salmonella* was released from the drinking water biofilm. Twenty-five colonies were selected from both the high and low inoculum effluent plates and screened for fluorescence. All colonies fluoresced green, confirming that the *gfp* gene was maintained in all *Salmonella* cells during the 144-hour period in a biofilm environment.

3.3.4 Quantitative evaluation of *Salmonella* in an established mixed culture biofilm

Salmonella survived well in the mixed culture biofilm in drinking water supplemented with 100 µg/l acetate grown at 25°C. At 1.5 hours after spiking with *Salmonella*, the attachment levels of the high inoculum were approximately 10^3 MPN/cm^2 and for the low inoculum were $< 10^2$ MPN/cm^2. After 1.5 hours, *Salmonella* increased by approximately 1 log for the high inoculum and about 0.5 log for the low inoculum. After 96 hours of growth, *Salmonella* reached concentrations of $> 10^4$ MPN/cm^2 for the high inoculum and $< 10^3$ MPN/cm^2 for the low inoculum. Thereafter, *Salmonella* declined slowly to $> 10^3$ MPN/cm^2 at 144 hours for the high inoculum and to approximately 10^2 MPN/cm^2 for the low inoculum (Fig. 3.19). The effluent from the pipes taken at 144 hours of operation indicated that *Salmonella* was released from the high inoculum at a concentration of $> 10^3$ MPN/ml and $> 10^2$ MPN/ml from the low inoculum (Fig 3.19).

The R2A counts for the both the high and the low inoculum followed the same trend (Fig. 3.20). The time 0 count displayed on the graph indicates the levels of mixed culture biofilm before spiking with *Salmonella* which was > 10^4 CFU/cm^2. The R2A counts reached levels of > 10^5 CFU/cm^2. This level is close to the average bacterial density of cells observed in South African drinking water distribution systems (S.M September, personal communication, 2005). The effluent from the pipes taken at 144 hours of operation indicated that the total cells were released from both the high inoculum and the low inoculum biofilm at > 10^4 CFU/ml (Fig. 3.20).

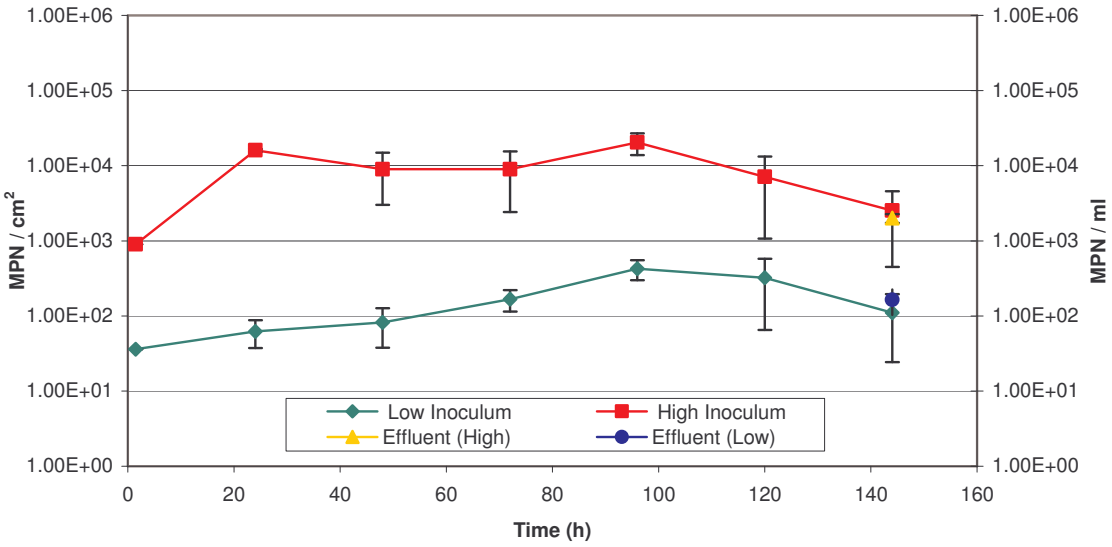

Figure 3.19 Average mixed culture biofilm MPN counts for high and low inoculum spiking concentrations and effluent counts following detachment from the biofilm system. The error bars denote the standard deviation from the mean.

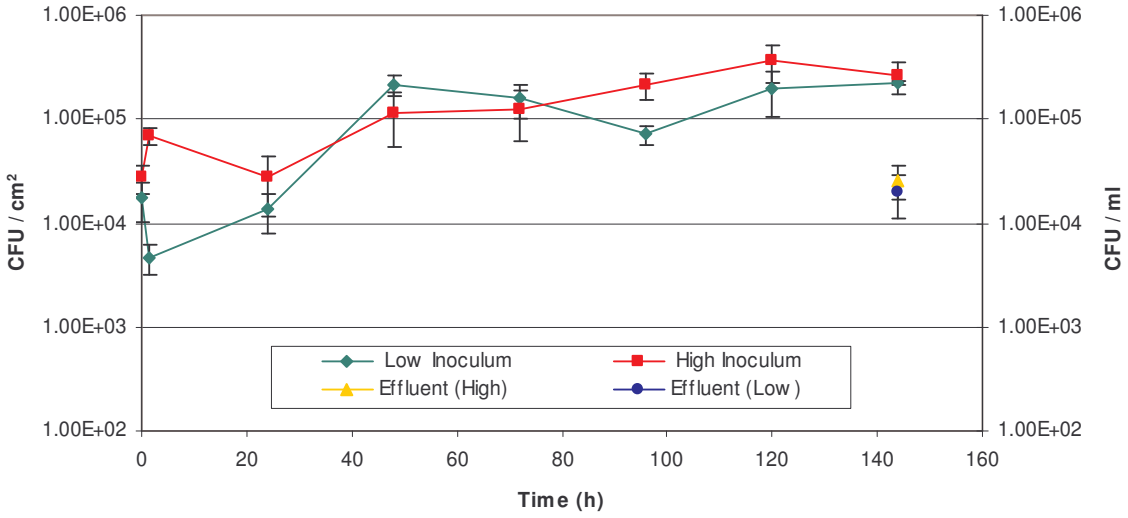

Figure 3.20 Average mixed culture biofilm R2A counts for high and low inoculum spiking concentrations. The time 0 count indicates the levels of mixed culture biofilm before spiking with *Salmonella*. The error bars denote the standard deviation from the mean.

3.3.5 Evaluation of *Salmonella* growth on R2A versus the MPN technique

Counts obtained for *Salmonella* were approximately 1 log higher than the counts obtained using the MPN method when the same sample was analysed. The highly selective RV broth could be responsible for fewer cells obtained in the MPN estimate.

3.4 DISCUSSION

A laboratory biofilm system was set up to evaluate bioflm formation in a drinking water distribution system and to investigate the capability of *Salmonella* to survive either independently in biofilm or in an established mixed culture biofilm. Only the high inoculum concentration (10^6 CFU/ml) of cells was used for spiking during the visual evaluation of *Salmonella* biofilm formation in monoculture. To determine if the initial concentration of cells plays a role in biofilm formation, a low inoculum concentration (10^2 CFU/ml) of *Salmonella* was included for spiking for the visual evaluation of *Salmonella* in a mixed culture biofilm and the quantitative evaluation of *Salmonella* in monoculture and in a mixed culture biofilm. These concentrations of *Salmonella* were chosen to account for a worst-case scenario of the concentration of pathogens present in tap water and the probability of this occurrence is very low. Initially a lower concentration of acetate (500 ug/l) was used to supplement the sterile tap water used as influent media in the flow cell for the visual evaluation of biofilm formation. Although enough biomass of *Salmonella* was obtained with the lower acetate concentration, the visibility of the GFP was diminished. A concentration of 1000 ug/l of acetate was therefore used to supplement the sterile tap water to enhance the visibility of the *gfp*-tagged *Salmonella* cells. A concentration of 100 ug/l of acetate was chosen to supplement the tap water for the quantitative evaluation to be more representative of the actual carbon sources present in tap water. A room temperature of 25˚C was also chosen for the quantitative evaluation to be truly representative of an actual water distribution system.

Salmonella did attach and form biofilms independently in drinking water supplemented with 1000 µg/l acetate at 37°C. *Salmonella* did not appear to form compact biofilms

under these low nutrient conditions, but accumulated on the surface with sparsely aggregated clumps. Since the *Salmonella* cells are tagged with the *gfp*mut3b* gene, driven by a growth-regulated promoter, expression of the green fluorescent phenotype indicates that the cells are alive and growing. Both the wild-type *Salmonella* and the tagged strain showed similar biofilm formation. The biofilms were patchy and detached readily under conditions of high flow. The composition of the medium affects *Salmonella* biofilm formation (Gerstel and Römling, 2001). It has been shown that the *agf*D promoter is involved in *Salmonella* species biofilm formation. Maximal expression of the *agf*D promoter under aerobic conditions was in nutrient-limited medium rather than nutrient-concentrated medium (Gerstel and Römling, 2001). It was found that the most effective medium in promoting *Salmonella* biofilm formation on plastic was diluted tryptic soy broth (Stepanović *et al*., 2004). Römling and Rohde, (1999) demonstrated that *Salmonella* Typhimurium cells form a pellicle with a tight bacterial network at the air-liquid interface when incubated in rich medium in standing culture at room temperature. In drinking water supplemented with 1000 µg/l acetate *Salmonella* formed sparsely aggregated clumps of biofilm.

The quantitative evaluation of *Salmonella* in independent biofilm indicated that *Salmonella* survived well in the pipes, reaching surface coverage levels of $> 10^5$ MPN/cm^2 for the high inoculum and $< 10^2$ MPN/cm^2 for the low inoculum (Fig. 3.7). This shows a significant difference in surface accumulation between the high inoculum (10^6 CFU/ml) and the low inoculum (10^2 CFU/ml) of spiked *Salmonella*. Within 24 hours *Salmonella* slowly began to accumulate (Fig. 3.7). This corresponds with the visual evaluation where a thin monolayer of cells was formed (Fig. 3.1 and Fig. 3.4). Both inoculums steadily increased until 72 hours, the high inoculum increasing by approximately 2 log and the low inoculum by approximately 1 log (Fig. 3.7). This correlates with the visual evaluation where *Salmonella* increased in number to aggregate in clumps (Fig 3.3 and Fig. 3.6). In comparison, the R2A counts for both the high and the low inoculum were higher than the MPN counts (Fig. 3.8). The highly selective RV broth could be responsible for fewer cells obtained in the MPN estimate.

In nature most bacteria do not exist as pure cultures, and significant proportions of all microorganisms are associated with surfaces in complex multi-species communities called biofilms (Costerton *et al.,* 1987). *Salmonella* can attach, colonize, grow and

survive in a mixed culture biofilm. The water isolates obtained from tap water, used to attain a mixed culture biofilm, were all Gram-positive bacteria. The high proportion of Gram-positive bacteria isolated from tap water could be due to the increased resistance of Gram-positives to chlorination or superior growth in the fluid phase (Murgel *et al*., 1991). After 24 hours individual *Salmonella* cells could be visualised among the mixed culture biofilm at spiking concentrations of both 10^6 CFU/ml and 10^2 CFU/ml (Fig. 3.9 and Fig. 3.14). This shows the ability of *Salmonella* to incorporate into the existing biofilm. In a laboratory reactor, which simulates biofilm formation in water pipes, the level of attachment of *S.* Enteritidis was higher in binary biofilm with *Klebsiella pneumoniae* than in a single species biofilm. The initial colonization phase contained a much higher proportion of metabolically active cells of *S.* Enteritidis in the binary biofilm than in the single species biofilms (Jones and Bradshaw, 1997).

Salmonella persisted in the established biofilm, forming small microcolonies after 48 and 72 hours (Fig. 3.10; Fig. 3.15 and Fig 3.11; Fig 3.16). The enhanced biofilm development by *Salmonella* in association with an established drinking water biofilm points to association and metabolic interactions with indigenous organisms. The presence of fimbriae, flagella and surface associated polysaccharides or proteins may provide a competitive advantage for one organism where a mixed community is involved (Donlan., 2002). The ability of *Salmonella* to form less structured and less compact independent biofilms, but enhanced biofilm development when colonizing an established biofilm is also in agreement with James *et al.,* (1995), who showed that biofilm thickness could be affected by the number of component organisms, indicating that one species could enhance the stability of the other. After 144 hours the established biofilm had formed very thick intermixed micro-colonies and *Salmonella* could still be detected as multicellular conglomerates (Fig. 3.13 and Fig. 3.18). This indicates that *Salmonella* can grow and survive in a mixed culture biofilm and is supported by other studies (Camper *et al*., 1998; Armon *et al.,* 1997).

This ability of *Salmonella* to attach and survive in the mixed culture biofilm was confirmed by the quantitative evaluation in the established biofilm. At 1.5 hours after spiking with *Salmonella*, the attachment levels of the high inoculum were approximately 10^3 MPN/cm^2 and for the low inoculum were $< 10^2$ MPN/cm^2 (Fig. 3.19). The pipes containing the mixed culture biofilm represent an open niche system where only limited

spaces are available for colonization. Fewer bacteria were able to attach at 1.5 hours than the initial inoculum spiked. No significant lag time was observed. This indicates that if contamination of *Salmonella* occurs in a water distribution system, it can quickly become established within the biofilm. Inoculum size and lag time are usually inversely related, therefore, the lack of a significant lag time in both the high and low spiking concentrations indicates that initial colonization is independent of the concentration of the inoculum. The colonization of *Salmonella* would thus be dependent on the colonization spaces available and the surface type. At 24 hours, *Salmonella* increased by approximately 1 log for the high inoculum and about 0.5 log for the low inoculum (Fig. 3.19). This correlates with the visual evaluation as after 24 hours *Salmonella* cells were seen amongst the mixed culture biofilm at both spiking concentrations (Fig. 3.9 and Fig. 3.14). In the visual evaluation, *Salmonella* formed small micro-colonies in the established biofilm at 48 and 72 hours (Fig. 3.10; Fig. 3.15 and Fig. 3.11; Fig. 3.16). In the quantitative evaluation, the number of cells remained relatively constant for this time period for the high inoculum, while the low inoculum increased slightly (Fig. 3.19). *Salmonella* showed rapid and increased levels of integration at 96 hours of growth, reaching concentrations of $> 10^4$ MPN/cm^2 for the high inoculum and $< 10^3$ MPN/cm^2 for the low inoculum (Fig. 3.19). This measurement of population increase is only slightly less when compared with the independent biofilm formation of *Salmonella* at 96 hours (Fig. 3.17) which further portrays the enhanced growth of *Salmonella* in the mixed culture biofilm and its ability to compete for a limited number of binding sites. The concentration of the inoculum in mixed culture does affect the amount of cells in the biofilm, but this relationship is not linear. At 144 hours, *Salmonella* could still be detected visually as multi-cellular conglomerates (Fig. 3.13 and Fig. 3.18) confirmed by the quantitative data by declining slowly to $> 10^3$ MPN/cm^2 at 144 hours for the high inoculum and to approximately 10^2 MPN/cm^2 for the low inoculum (Fig. 3.19). *Salmonella* appeared to replicate without hindrance in the mixed culture biofilm. The R2A counts for the both the high and the low inoculum followed the same trend reaching levels of $> 10^5$ CFU/cm^2. This level is close to the average density of bacterial cells observed in South African drinking water distribution systems (S.M. September, personal communication, 2005).

It was determined that *Salmonella* could be recovered from the mixed culture biofilm in the flow cell in the visual evaluation from the high inoculum biofilm. When growth from

the biofilm on kanamycin-containing agar was transferred to XLD, it was confirmed that all kanamycin-resistant colonies were *Salmonella*. As the *gfp* gene is located directly alongside the kanamycin resistance gene, the synonymity of kanamycin resistance with *Salmonella* showed that the *gfp* gene had not been transferred to other bacterial species in the biofilm, and that all fluorescing cells were *Salmonella. Salmonella* could not be recovered from the biofilm formed on the small cover-slip area when spiked with a low initial inoculum (10^2 CFU/ml). This is in contrast to the quantitative evaluation where *Salmonella* was recovered from the effluent at 10^2 MPN/ml from the low inoculum.

Mature biofilms are known to shed cells, releasing them into the flow. Biofilm cells may be dispersed either by shedding of daughter cells from active growing cells, detachment as a result of nutrient levels or quorum sensing, or due to the shearing of biofilm aggregates because of flow effects (Donlan, 2002). In order to verify the notion that *Salmonella* would be released from a drinking water biofilm, isolations were performed on the effluent from the flow cell in the visual evaluation. As the influent medium was sterile, the presence of *Salmonella* in the effluent proves release from the biofilm. All colonies from the RV broth appeared black on XLD and $>10^4$ CFU/ml of *Salmonella* was obtained for the high inoculum and $>10^3$ CFU/ml for the low inoculum was obtained, showing that *Salmonella* was released from the drinking water biofilm. When colonies were screened for fluorescence, all fluoresced green, showing that the *gfp* gene was maintained in all *Salmonella* cells during the 144-hour period in the biofilm environment. These numbers are higher than those obtained from the effluent of the established biofilm for the quantitative evaluation which was $> 10^3$ MPN/ml for the high inoculum and $> 10^2$ MPN/ml for the low inoculum (Fig. 3.19). This is to be expected as the influent medium for the flow cell in the visual evaluation was sterile tap water with 1000 µg/l acetate at a temperature of 37˚C, whereas the influent medium in the pipes for the quantitative evaluation was sterile tap water with 100 µg/l acetate at room temperature. Lee and Kim, (2003) showed that samples of effluent and biofilm from the end region of a water distribution system showed more Enterobacteriaceae and higher colony formation frequency on selective media than that of the influent. This variation results from changes and detachment of bacteria in the biofilm, which can be potentially harmful to health.

In the quantitative evaluation of *Salmonella* in an independent biofilm, the effluent from the pipes taken at 144 hours of operation indicated that *Salmonella* was released from the

high inoculum biofilm at a concentration of > 10^3 MPN/ml and > 10^2 MPN/ml from the low inoculum biofilm (Fig. 3.7). The R2A effluent counts correspond well with the MPN effluent counts (Fig. 3.8). It was found that in a pure culture biofilm generating system, clumps of aggregated *Salmonella* Enteritidis biofilms adhering to Teflon and stainless steel were also readily sloughed off (Austin *et al*., 1998).

The release of *Salmonella* from the drinking water distribution system biofilm has huge implications from a public health point of view. Although relatively high numbers of *Salmonella,* which are unlikely to reflect the true situation in a drinking water distribution system were chosen for inoculation, the possibility that *Salmonella* may detach from their biofilm support under favourable conditions and be released into the supply system cannot be ruled out. *Salmonella* had the ability to form biofilms, producing high surface accumulation in the quantitative evaluation of the pipe studies for both independent and mixed culture biofilm formation in low nutrient tap water. The MPN determinations of biofilm cells could also be possibly lower and an underestimate of the actual numbers due to the high selectivity of the RV broth.

In drinking water distribution system biofilms, large numbers of free bacteria may form a new biofilm or absorb to the already existing biofilm. This absorption, growth and detachment in biofilms can be potentially lethal where human pathogenic bacteria, such as *Salmonella* enter a drinking water distribution system and become part of these already existing biofilms. Both the visual evaluation and the quantitative evaluation confirm that *Salmonella* can enter into, survive and grow within a mixed culture biofilm and be released at high levels into the flow for re-colonization elsewhere. It has been shown that when *Salmonella enteritidis* is attracted to a biofilm, it is protected from biocides which are bactericidal in the water column (Dhir and Dodd, 1995). This protection offered by biofilms also has serious implications for the water industry.

3.5 CONCLUSIONS

S. enterica subsp. *enterica* ser. Typhimurium colonized established multi-species drinking water biofilms within 24 hours, growing to form micro-colonies within the biofilm. *S*. Typhimurium was also released at high levels from the drinking water-associated biofilm into the flow, and was seen to re-colonize elsewhere. This showed that

Salmonella can enter into, survive and grow within, and be released from a drinking water biofilm. The results indicate that once *Salmonella* has entered into a distribution system, it will be able to colonize an existing biofilm, grow in it and be released into the flow for re-colonization elsewhere, and possible subsequent infection of consumers.

CHAPTER FOUR
CONCLUDING REMARKS

In most natural environments, bacteria do not exist as monospecies, but as complex mixed communities consisting of more than one microbial species. The ultimate goal of research into bacterial pathogens in water environments such as biofilms is to refine management approaches to supply safer drinking water. This can be achieved by monitoring the activities of a specific species within a complex mixed population, as it would occur in its natural environment. The objectives of this study were to chromosomally tag a clinically relevant *Salmonella* isolate with GFP and to investigate its fate and survival in a drinking water biofilm.

In the first part of the study a *Salmonella* strain was isolated from fresh water sediment and identified as an isolate of the clinically relevant *S. enterica* subsp. *enterica* ser. Typhimurium. This isolate was successfully chromosomally tagged with the marker gene GFP and displayed a bright green fluorescence when exposed to blue light at 488 nm. It was determined by Southern Blot hybridization that the *gfp* gene had integrated into the genome of the *Salmonella* recipients. The *gfp* gene was stably maintained during continuous growth for 30 days without selective pressure. The *gfp*-labelled recombinant strains were not growth-rate impaired in nutrient-rich (LB broth) or low nutrient ($^{1}/_{10}$ strength R2A) broth. Phenotypic microarrays indicated no noteworthy changes between the wild-type and tagged strain. The survival fitness studies showed that the incorporation of the *gfp* gene did not have any detrimental effects on the survival or behaviour of the tagged *Salmonella* strains. These tagged strains could therefore be used effectively *in vivo* to study the fate and survival of *Salmonella* in a drinking water biofilm.

In the second part of the study *Salmonella* was evaluated both visually and quantitatively on its ability to form independent biofilm and its ability to grow and survive in a mixed culture biofilm. In monoculture *S. enterica* subsp. *enterica* ser. Typhimurium formed loosely structured biofilms in drinking water supplemented with 1000 µg/l acetate. The biofilms were patchy and detached readily under conditions of high flow. When drinking water was supplemented with 100 µg/l of acetate, *Salmonella* was released from the high inoculum independent biofilm at a

concentration of > 10^3 MPN/ml and from a low inoculum biofilm at a concentration of > 10^2 MPN/ml.

S. enterica subsp. *enterica* ser. Typhimurium colonized established multi-species drinking water biofilms supplemented with 1000 µg/l acetate within 24 hours, growing to form small micro-colonies within the biofilm. *S*. Typhimurium was also released at high levels from the drinking water-associated biofilm into the flow and was seen to recolonize elsewhere. When drinking water was supplemented with 100 µg/l acetate, *Salmonella* was released from the high inoculum mixed culture biofilm at a concentration of > 10^3 MPN/ml and from the low inoculum mixed culture biofilm at a concentration of >10^2 MPN/ml. This showed that *Salmonella* can enter into, grow within and be released from a drinking water-associated biofilm. The results indicate that once *Salmonella* has entered into a drinking water system it will be capable of colonizing an existing biofilm, grow in it and be released into the flow for re-colonization elsewhere, and possible subsequent infection of consumers.

Since the chromosomally *gfp*-tagged *Salmonella* isolate proved to be stable and it was demonstrated that the tagged strain displayed few differences in strain-specific characteristics in comparison to the wild-type strain, it would be feasible to continue research using this recombinant strain. Suggestions regarding future research would involve further studies of the fate, transport, survival and behaviour of the tagged *Salmonella* isolate in a variety of different water environments.

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