Fate of *Salmonella* Typhimurium in laboratory scale drinking water biofilms

L.M. Schaefer[1,3], V.S. Brözel[2,3], S.N. Venter[3]

[1]Division of Natural Resources and the Environment, CSIR, Pretoria, 0001, South Africa

[2]Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA[2]

[3]Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa[3]

Corresponding author: Lisa Schaefer

Tel: +2712 841 4279

LSchaefer@csir.co.za

ABSTRACT

Investigations were carried out to evaluate and quantify colonization of laboratory scale drinking water biofilms by a chromosomally *gfp*-tagged strain of *Salmonella* Typhimurium. A *Salmonella* strain genetically labelled with GFP, encoding the green fluorescent protein allowed *in situ* detection of undisturbed cells and was ideally suited for monitoring *Salmonella* in biofilms. The fate and persistence of non-typhoidal *Salmonella* in simulated drinking water biofilms was investigated. The ability of *Salmonella* to form biofilms in monoculture and the fate and persistence of *Salmonella* in a mixed aquatic biofilm was examined. In monoculture *S.* Typhimurium formed loosely

structured biofilms. *Salmonella* colonized established multi-species drinking water biofilms within 24 hours, forming micro-colonies within the biofilm. *S*. Typhimurium was also released at high levels from the drinking water-associated biofilm into the water passing through the system. This indicated that *Salmonella* could enter into, survive and grow within, and be released from a drinking water biofilm. The ability of *Salmonella* to survive and persist in a drinking water biofilm, and be released at high levels into the flow for re-colonization elsewhere, indicates the potential for a persistent health risk to consumers once a network becomes contaminated with this bacterium.

Keywords

Salmonella Typhimurium, drinking water, biofilm, GFP

INTRODUCTION

Salmonella is frequently isolated from water sources (Baudart *et al*. 2000; Ho & Tam 2000; Gannon *et al*. 2004), which serve as potential reservoirs for infection. Compared to *E. coli*, *Salmonella* appears to withstand a wider variety of environmental fluctuations and may persist in water environments for extended periods (Winfield & Groisman, 2003). Although food has been implicated as the major source of non-typhoidal *Salmonella* infections (Guillot & Loret, 2010), *S.* Typhimurium has been associated with the consumption of contaminated ground water and surface water supplies (WHO, 2011) and the role of non-typhoidal *Salmonella* in the transmission of disease in developing countries is therefore of concern. 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 the organism is not well understood.

Biofilms of potable water distribution systems have the potential to harbour pathogenic bacteria (Diergaardt *et al.* 2004; September *et al*. 2007), posing a risk of release into the water stream (Szewzyk *et al,* 2000). The attached populations could also serve as a reservoir for subsequent spread through the system following detachment (Camper *et al.* 1999). Bacteria in biofilms are less sensitive to disinfection procedures, and therefore resistant to residual disinfectant (Costerton *et al.* 1995). There is very little understanding of how pathogenic organisms in a drinking water biofilm contribute to enteric disease. Although water-treatment plants disinfect water, it is still possible for pathogenic bacteria to enter a drinking water distribution system. Long retention times in extensive networks lead to dissipation of disinfectant, and bacteria released from biofilms are therefore less challenged, constituting a health risk to consumers (Lee & Kim 2003).

Tracking specific bacteria in a biofilm poses distinct challenges. Not all bacteria present in a biofilm may be culturable, and organisms can generally not be identified based on their morphology (Camper *et al.* 1999). Tagged strains distinguishable *in vivo* from the rest of the bacterial community facilitate studying the fate and persistence of specific pathogens. Tagging a bacterial strain with the gene encoding the green fluorescent protein (GFP) allows for non-destructive visualization using fluorescent microscopy (Möller *et al*. 1998). Chromosomal tagging results in maintenance of the gene and little, if any effect on *in situ* fitness (Burke *et al*. 2008). In this way, a more realistic indication of population dynamics in a biofilm can be obtained.

The aim of this study was to evaluate and quantify surface colonization of a *gfp*-tagged strain of *Salmonella* Typhimurium in both a sterile system and in a mixed culture drinking water biofilm. This was evaluated in a flow cell at 37°C in tap water supplemented with

1000 µg l^{-1} acetate and in silicone pipes at 25°C in tap water supplemented with 100 µg l^{-1} acetate.

MATERIALS AND METHODS

Bacterial strains

Salmonella enterica subsp. *enterica* ser. Typhimurium strain with the mini Tn5-Km-*rrnB* P1-RBSII-*gfp* mut3b*-To-T1 cassette inserted randomly into the chromosome was chosen as a stable GFP-tagged strain distinguishable *in vivo* from the rest of the bacterial community (Burke *et al*. 2008). For establishing a mixed culture biofilm, bacteria were isolated from tap water on R2A agar (Difco). Five isolates that did not display auto-fluorescence were analysed by 16S rRNA sequencing as described previously (September *et al*. 2004) and were identified as *Paenibacillus favisporus*, *Bacillus* sp, *Paenibacillus cineris*, *Paenibacillus* sp., *Enterococcus mundtii*.

Fluorescence microscopy

The bacterial cells were viewed by phase contrast and epifluorescence microscopy using an inverted Zeiss Axiovert 200 fluorescent microscope (Excitation: BP 450-490 nm; Emission: BP 515-565 nm and beam splitter FT510), with a 63x/1.4 Zeiss FS10 Neofluor objective. The images were captured using a charge-coupled device (CCD) camera (Nikon). A variety of microscopic fields were examined for each flow cell lane.

Most Probable Number analysis

Ten-fold dilutions of *Salmonella* samples were made in triplicate in buffered peptone water (BPW) tubes. The BPW was incubated at 37°C for 24 h and 10 µl was transferred to 9.9 ml of Rappaport-Vassiliadis (RV) broth (Oxoid) and incubated at 42°C for 24 h. A

loop-full from each dilution was plated onto XLD agar and incubated for 24 h at 37°C. Black growth on XLD was scored positive and the most probable number (MPN) was estimated from an MPN table (Beliaeff & Mary 1993). Colonies were selected to determine if they were still fluorescing green after transfer to Luria Bertani (LB) agar.

Operation of the flow-cell with *Salmonella* in monoculture biofilm and in an established mixed culture biofilm

A flow cell with channels of 2 x 2 x 15 mm was used to simulate biofilm formation in a water distribution system, with flow through delivered at 0.4 mm s^{-1} using a Watson Marlow 205S peristaltic pump. A glass cover-slip was fixed over the channels of the flow cell. Initially 3.5% (w v^{-1}) sodium hypochlorite was pumped through the flow cell for 30 min, followed by rinsing for 30 min with sterile ddH_2O. Thereafter, sterile tap water supplemented with 1000 µg l^{-1} acetate was pumped through the flow cell. The system was maintained at 37°C. A sterile syringe with a 0.45 mm gauge needle was used to inoculate the flow cell immediately upstream with 1 ml of culture. The pump was left off for 1 h after inoculation to allow for attachment, before the pump was switched on and set at a flow rate of 0.4 mm s^{-1} through the flow cell. For evaluation of monoculture *Salmonella* biofilm formation, 1 ml volumes of an overnight culture of the tagged *Salmonella,* grown in $^1/_{10}$ strength R2A medium (Reasoner & Geldreich 1985), and pre-diluted to 10^6 CFU ml^{-1}, were inoculated into separate channels of the flow cell and fed with acetate supplemented sterile tap water. For evaluation of *Salmonella* in an established mixed culture biofilm, five bacterial water isolates were inoculated separately into 10 ml of $^1/_{10}$ strength R2A broth and incubated at 37°C overnight. These cultures were combined and 1 ml was inoculated into the flow cell. The flow was left off for 1 h and after 72 h the system was spiked with 1 ml of *Salmonella* at a density of either 10^2 or

10^6 CFU ml^{-1}. A further channel of established biofilm remained unspiked to serve as a control. Flow cells were run for 144 h once the flow was resumed and viewed daily by fluorescent microscopy. To determine release of *Salmonella* from the biofilm, effluent from the flow cell was collected at 72 h of operation and *Salmonella* was quantified by the MPN technique. For verification that *Salmonella* in the mixed culture biofilm were live, sections of cover-slip were removed at 144 h. The biofilm on the cover-slip was suspended in ¼ strength Ringer's solution (Merck) and vortexed aggressively for 20 s. Colonies were recovered by incubation for 24 h at 37°C on LB agar with 100 µg ml^{-1} kanamycin (Roche). Thereafter colonies were tested for the presence of GFP and their identity confirmed on Xylose Lysine Deoxycholate (XLD) agar (Oxoid).

Quantitative evaluation of *Salmonella* biofilm population density in monoculture biofilm and in an established mixed culture biofilm

In order to simulate a drinking water distribution system, silicone pipes of 1.59 (outer diameter) x 0.79 (wall size) x 1.41 (internal diameter) mm (Sigma-Aldrich) were connected to a Watson Marlow peristaltic pump. Pipes were cleaned with sodium hypochlorite as described above. Thereafter, sterile tap water supplemented with 100 µg l^{-1} acetate was pumped through the pipe at 0.4 mm s^{-1}, and maintained at 25°C. To study formation of monoculture biofilms, pipes were inoculated with *Salmonella* at 10^2 and 10^6 CFU ml^{-1}, and after allowing 2 h for attachment at 25 rather than 37 °C, flow was resumed. Each experiment was replicated three times and a separate pipe remained unspiked as a control for contamination. For quantification of *Salmonella* in a mixed culture biofilm, a biofilm was established in silicone tubing by inoculating with 1 ml of mixed culture as described above. The tubes were fed for six days and then *Salmonella* was inoculated into the tubes, and 2 h later flow was resumed. Counts on R2A agar (for

mixed culture biofilm) and MPN (*Salmonella)* counts were determined for the three separate tubes in triplicate 1.5 h after resumption of flow, and thereafter daily until day six, using three separate pieces of pipe for each of the experiments. Five cm sections from each pipe were removed and manually manipulated to loosen the biofilm. The biofilm was rinsed from the pipe with 5 ml of sterile ddH_2O. One ml of these suspensions were transferred to 9 ml of ¼ strength Ringer's solution and serial dilutions were plated onto R2A agar and incubated at 37°C for 24 h. Density of *Salmonella* was determined by the MPN technique. Release of *Salmonella* from the biofilm was quantified by collection of the pipe effluent at 144 h of operation and analysis by R2A counts (for mixed culture biofilm) and the MPN technique.

RESULTS AND DISCUSSION

Biofilm formation conditions

A laboratory scale simulated drinking water distribution system was set up to investigate the ability of *Salmonella* to form a monoculture biofilm, or colonize an established mixed culture biofilm. For the initial flow cell experiments a concentration of 1000 ug l^{-1} of acetate was used to supplement the sterile tap water at a temperature of 37°C to enhance the visibility of the *gfp*-tagged *Salmonella* cells. A concentration of 100 ug l^{-1} 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. Huck *et al.* (1991) reported that the assimilable organic carbon (AOC) levels of raw water varied seasonally with average values just above 100 µg acetate carbon equivalents per litre (C eq/l) in summer and more than 200 µg l^{-1} in spring. Grundlingh *et al*. (1999) found that the average AOC levels for distribution end point water was 60 µg acetate C eq/l. A room

temperature of 25˚C was also chosen for the quantitative evaluation to be representative of optimal growth conditions in an actual water distribution system. Armon *et al.* (1997) showed that when *S.* Typhimurium was introduced into a non-sterile simulated biofilm flow system it survived well at both 24 and 36°C for at least 30 d. In this study, the *Salmonella* strain formed single culture biofilm at both 25 and 37°C.

Visual evaluation of *Salmonella* in flow cell biofilm

In monoculture, *Salmonella* formed sparse biofilms in drinking water with acetate. Microcolonies were formed by 24 h and grew to form, thin layered biofilms by 48 h. (Figure 1a & b). The less compact, less structured growth in monoculture biofilm that *Salmonella* displayed indicated that *Salmonella* does not form biofilm well under these nutrient limited conditions. Although *Salmonella* appeared to form single species biofilms poorly, they could attach and survive in a mixed culture biofilm. The 5 member inoculum formed a substantial biofilm in drinking water by 72 h, with no autofluorescence observed. Individual *Salmonella* cells could be visualised within 24 h of spiking with both 10^6 CFU ml^{-1} and 10^2 CFU ml^{-1}. *Salmonella* persisted in the established biofilm, growing to form larger colonies (Figure 1c, d, e & f) rather than be evenly distributed. After 144 h the established biofilm had formed very thick intermixed micro-colonies and *Salmonella* could still be detected as multicellular conglomerates (data not shown). *Salmonella* attached and maintained stable populations in a mixed culture biofilm, with establishment within 24 h of spiking at both high and low inoculum concentrations and persistence in the established biofilm (Figure 1). The enhanced biofilm development by *Salmonella* in an established drinking water biofilm points to association and 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). A laboratory reactor that simulated

biofilm formation in water pipes was used to study the interactions of biofilm formation between a nitrogen-fixing strain of *Klebsiella pneumoniae* and *S.* Enteritidis. The level of attachment of *S.* Enteritidis was higher in the binary than the single species biofilm. 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 (Jones & Bradshaw 1997). Habimana *et al*. (2010) showed that dual-species biofilms promoted the growth of *Salmonella* compared to *Salmonella* in mono-species biofilms in a drip flow biofilm reactor. The ability of *Salmonella* to form less structured and less compact monoculture 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. Pure cultures of either *K. pneumoniae* or *Pseudomonas aeruginosa* biofilms in a laboratory reactor were thinner, whereas a biofilm containing both species was thicker. This could be because one species enhanced the stability of the other.

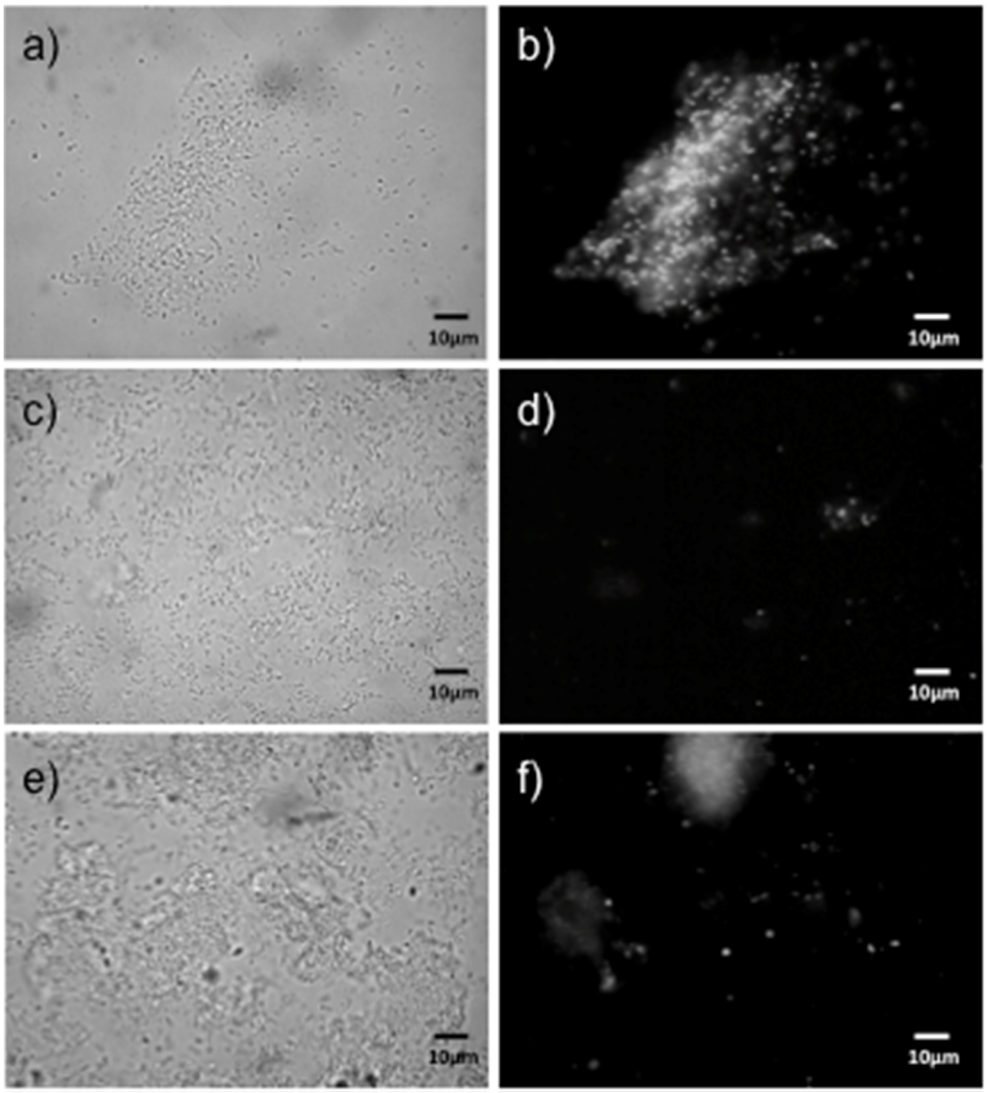

Figure 1 Photomicrographs of GFP-tagged *Salmonella* growing as biofilm for 48 h on glass in a flow cell fed tap water with 1000 µg l^{-1} acetate at 0.4 mm s^{-1}. *S.* Typhimurium was spiked into a sterile system (a,b), or into a three-day old, 5-strain biofilm comprised of non-fluorescing isolates from drinking water distribution systems at 10^2 CFU mL^{-1} (c,d) or at 10^6 CFU mL^{-1} (e,f). Images on the left were taken under phase contrast (a,c,e), and images on the right were taken under green fluorescence (b,d,f).

Recovery and release of the tagged *Salmonella* from flow cell biofilm

Salmonella were removed from 144 h old biofilm, and formed black colonies on XLD agar, produced growth on kanamycin agar and fluoresced green. Although fluorescent cells were viewed in the low inoculum biofilm (Figure 1c & d), *Salmonella* could not be recovered from the biofilm. This may be due to suboptimal performance of the recovery protocol. As the influent medium was sterile, the presence of *Salmonella* in the effluent proves release from the biofilm. *Salmonella* was released from the flow cell at $>10^4$ MPN ml^{-1} for the high inoculum and $>10^3$ MPN ml^{-1} for the low inoculum biofilm. 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). Twenty-five colonies selected at random all fluoresced green, indicating that the *gfp* gene was maintained in all *Salmonella* cells during the 144-hour period in a biofilm environment.

Quantitative evaluation of *Salmonella* in silicone tube biofilms

The quantitative evaluation of *Salmonella* in monoculture biofilm indicated that *Salmonella* established a population in the pipes fed tap water with 100 µg l^{-1} acetate. Surface coverage levels reached > 10^5 MPN cm^{-2} for the high inoculum and < 10^2 MPN cm^{-2} for the low inoculum (Figure 2). At 1.5 h after turning on the flow, the quantitative evaluation indicated that 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}. Within 24 hours *Salmonella* slowly began to accumulate. Both inoculums steadily increased until 72 hours, the high inoculum increasing by approximately 2 log and the low inoculum by approximately 1 log (Figure 2). Thereafter, the low inoculum biofilm reached a steady state and 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 (Figure 2). The effluent from the pipes taken at 144 h of operation indicated that *Salmonella* was released from the high inoculum system at a concentration of > 10^3 MPN ml^{-1} and > 10^2 MPN ml^{-1} from the low inoculum system.

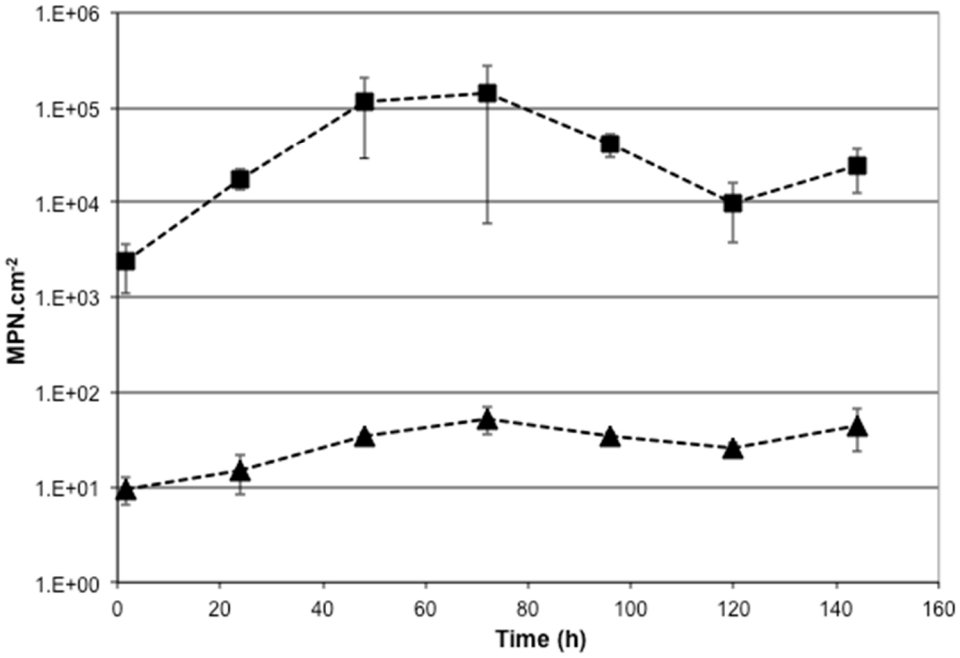

Figure 2 Average *Salmonella* MPN $cm^{2\ -1}$ from *S.* Typhimurium biofilm on silicone tubing fed sterile tap water supplemented with 100 µg l^{-1} acetate. *S.* Typhimurium was inoculated at either 10^2 CFU ml^{-1} (low inoculum) (▲), or 10^6 CFU ml^{-1} (high inoculum) (■). The data represents the average of three independent experiments and error bars represent one standard error of the mean.

The ability of *Salmonella* to attach to the mixed culture biofilm was quantitatively confirmed. At 1.5 h after spiking, the high inoculum spiked biofilm harboured

approximately 10^3 MPN cm^{-2} and for the low inoculum < 10^2 MPN cm^{-2} (Figure 3). *Salmonella* population densities increased in both low and high inoculum biofilm. At 24 hours, *Salmonella* increased by approximately 1 log for the high inoculum and about 0.5 log for the low inoculum (Figure 3). The number of cells remained relatively constant between 48 and 72 h for the high inoculum, while the low inoculum increased slightly (Figure 3). *Salmonella* showed rapid and increased levels of integration and at 96 hours of growth, reached concentrations of > 10^4 MPN cm^{-2} for the high inoculum and < 10^3 MPN cm^{-2} for the low inoculum (Figure 3). This measurement of population increase is only slightly less when compared with the independent biofilm formation of *Salmonella* at 96 hours (Figure 2), 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. At 144 hours, *Salmonella* declined slowly to > 10^3 MPN cm^{-2} for the high inoculum and to approximately 10^2 MPN cm^{-2} for the low inoculum (Figure 3). *Salmonella* appeared to replicate without hindrance in the mixed culture biofilm. The effluent released from the pipes at 144 h yielded > 10^3 MPN ml^{-1} and > 10^2 MPN ml^{-1} *Salmonella* from the high and low inoculum respectively. The R2A counts for the both the high and the low inoculum followed the same trend (Figure 3). 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}. The effluent taken from the pipes at 144 h yielded > 10^4 CFU ml^{-1}.

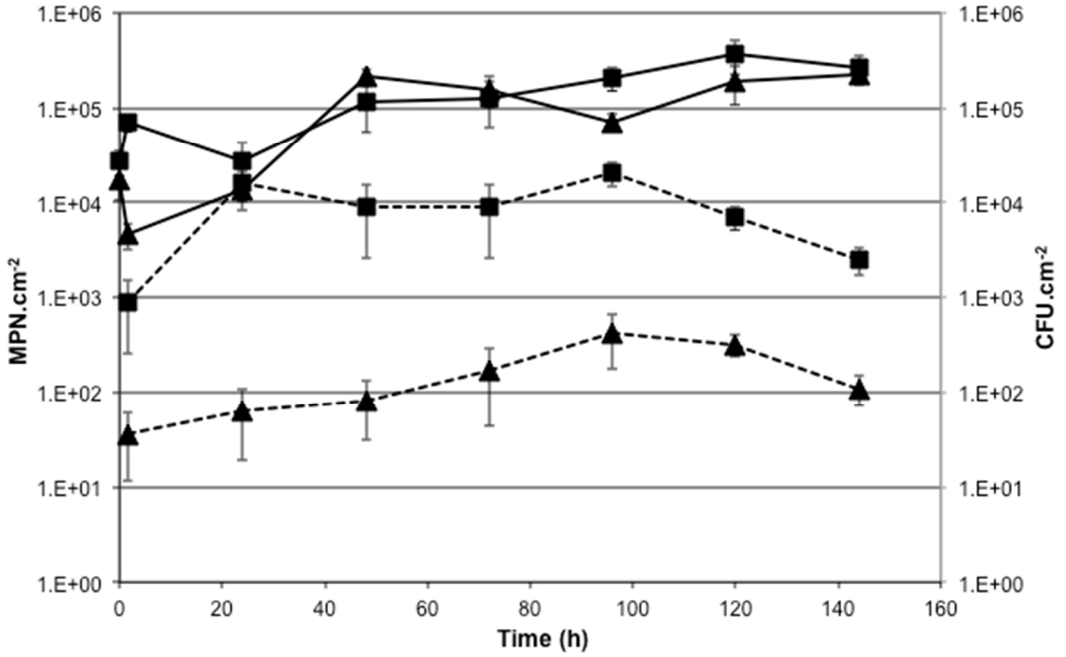

Figure 3 Average *Salmonella* MPN $cm^{2\ -1}$ (dashed line) and CFU $cm^{2\ -1}$ (solid line) from a multi-strain spiked model biofilm on silicone tubing fed sterile tap water supplemented with 100 µg l^{-1} acetate. *S.* Typhimurium was inoculated at either 10^2 CFU ml^{-1} (low inoculum) (▲), or 10^6 CFU ml^{-1} (high inoculum) (■). The data represents the average of three independent experiments and error bars represent one standard error of the mean.

Salmonella produced high surface accumulation in the simulated drinking water distribution pipe studies for both monoculture and mixed culture biofilm formation in low nutrient tap water. The pipes containing the mixed culture biofilm represent an open niche system where only limited spaces are available for colonization. This indicates that if contamination of *Salmonella* occurs in a water distribution system, it can quickly become established within the biofilm. The lack of a significant lag time in both the high and low spiking concentrations (Figure 3) suggests that initial colonization is

independent of the concentration of the inoculum. The colonization of *Salmonella* would, therefore, be dependent on the colonization spaces available and the surface type. The R2A counts for the mixed culture biofilm reached levels of $>10^5$ CFU/$cm^{2\ -1}$ (Figure 3), indicating that stable biofilm levels were reached as this level. This was similar to the average optimal internal density of cells observed in South African urban drinking water distribution systems (September *et al*. 2007).

CONCLUSIONS

A stable *gfp* genetically labelled *Salmonella* strain allowed non-destructive *in situ* detection and was suited for monitoring *Salmonella* in monoculture or a mixed species biofilm. *S. enterica* subsp. *enterica* ser. Typhimurium formed less structured and less compact independent biofilms, but showed enhanced biofilm development when colonizing an established biofilm. In drinking water distribution system biofilms, planktonic *Salmonella* may form a new biofilm or attach to an existing biofilm. Absorption, growth and subsequent detachment pose a public health risk 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 confirmed that *Salmonella* can enter into, survive and grow within an existing mixed culture biofilm and be released at high levels into the flow for re-colonization elsewhere and possible subsequent infection of consumers.

ACKNOWLEDGEMENTS

We thank Raynard MacDonald for valuable assistance with setting up flow cells, and the Laboratory of Microscopy and Microanalysis at the University of Pretoria for assistance with fluorescent microscopy. This research was supported by a grant from the Water Research Commission of South Africa (WRC K5/1276) to VSB and SNV, and National

Research Foundation (NRF) grant 2046811 to VSB. LMS was supported by a scholarship from the NRF of South Africa.

REFERENCES

Armon, R., Starosvetzky, J., Arbel, T. & Green, M. 1997 Survival of *Legionella pneumophila* and *Salmonella typhimurium* in biofilm systems. *Water. Sci. Technol.* **35**, 293-300.

Baudart, J., Lemarchand, K., Brisabois, A. & Lebaron, P. 2000 Diversity of *Salmonella* strains isolated from aquatic environment as determined by serotyoing and amplification of the ribosomal DNA spacer regions. *App. Environ. Microbiol.* **66**, 1544-1552.

Beliaeff, B. & Mary, J-Y. 1993 The “most probable number” estimate and its confidence limits. *Water Res.* **27,** 799-805.

Burke, L.M., Brözel, V.S. & Venter, S.N. 2008 Construction and evaluation of a gfp-tagged *Salmonella* Typhimurium strain for environmental applications. *Water SA* **34**, 19-24.

Camper, A., Burr, M., Ellis, B., Butterfield, P. & Abernathy, C. 1999 Development and structure of drinking water biofilms and techniques for their study. *J. Appl. Microbiol. Symp*. **85**, 1S-12S.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. & Lappin-Scott, H.M. 1995 Microbial Biofilms. *Annu. Rev. Microbiol.* **49,** 711-745.

Diergaardt, S.M., Venter, S.N., Spreeth, A., Theron, J. & Brözel, V.S. 2004 The occurrence of Campylobacters in water sources in South Africa. *Water Res.* **38**, 2589-2595.

Donlan, R.M. (2002). Biofilms: Microbial life on surfaces. *Emerg. Infect. Dis.* **8,** 1-19.

Gannon, V.P.J., Graham, T.A., Read, S., Ziebell, K., Muckle, A., Mori, J., Thomas, J., Selinger, B., Townsend, I., & Byrne, J. 2004 Bacterial pathogens in rural water supplies in southern Alverta, Canada. *J. Toxicol. Environ. Health A*, **67**, 1643-1653.

Grundlingh, J.A., Nel, C., Kotze, E., & de Wet, C.M.E. 1999 Biodegradable compounds and microbial regrowth in water. Report to the Water Research Commission, South Africa, no. 541/1/99.

Guillot, E. & Loret, J-F. 2010 *Waterborne pathogens: Review for the drinking water industry.* Global Water Research Coalition Report Series. IWA Publishing, London

Habimana, O., Møretrø, T., Langsrud, S., Vestby, L.K., Nesse, L.L., & Heir, E. 2010 Micro ecosystems from feed industry surfaces and biofilm study of *Salmonella* versus resident flora strains. *BMC Vet. Res.* **6**, 1-10.

Ho, B.S.W. & Tam, T.-Y. 2000 Rapid enumeration of *Salmonella* in environmental waters and wastewater. *Water Res.* **34,** 2297-2399.

Huck, P.M., Fedorak, P.M. & Anderson, W.B. 1991 Formation and removal of assimilable organic carbon during biological treatment. *J. Amer. Water Works Assoc.* **83**, 69-80.

James, G.A., Beaudette, L., & Costerton, J.W. 1995 Interspecies bacterial interactions in biofilms. *J. Ind. Microbiol.* **15**, 257-262.

Jones, K. & Bradshaw, S.B. 1997 Synergism in biofilm formation between *Salmonella enteritidis* and a nitrogen-fixing strain of *Klebsiella pneumoniae*. *J. Appl. Microbiol.* **82,** 663-668.

Lee, D.G. & Kim, S.J. 2003 Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. *J. App. Microbiol.* **95**, 317-324.

Möller, S., Sternberg, C., Andersen, J.B., Christensen, B.B., Ramos, J.L. Givskov, M. & Molin, S. 1998 In situ gene expression in mixed culture biofilms evidence of metabolic interaction between community members. *Appl. Environ. Microbiol.* **64,** 721-732.

Reasoner, D.J. & Geldreich, E.E. 1985 A new medium for enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* **49**, 1-7.

September, S.M., Brözel, V.S. & Venter, S.N. 2004 Diversity of nontuberculoid *Mycobacterium* species in biofilms of urban and semiurban drinking water distribution systems. *Appl. Environ. Microbiol.* **70**, 7571-7573.

September, S.M., Els, F.A., Venter, S.N. & Brözel, V.S. 2007 Prevalence of bacterial pathogens in biofilms of drinking water distributiom systems. *J. Water Health* **5**, 219-227

Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer, K-H. (2000) Microbiological safety of drinking water. *Annu. Rev. Microbiol.* **54,** 81-127.

Winfield, M.D. & Groisman, E.A. 2003 Minireview. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol.* **69**, 3687-3694.

World Health Organisation (WHO). 2011 Guidelines for drinking-water quality. Forth Edition. Malta: Gutenberg.