

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

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ABSTRACT

Investigations were carried out to evaluate and quantify colonization of laboratory-scale drinking water biofilms by a chromosomally green fluorescent protein (*gfp*)-tagged strain of *Salmonella* Typhimurium. *Gfp* encodes the green fluorescent protein and thus allows *in situ* detection of undisturbed cells and is 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 recolonization elsewhere, indicates the potential for a persistent health risk to consumers once a network becomes contaminated with this bacterium.

Key words | biofilm, drinking water, GFP, *Salmonella* Typhimurium

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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 *Escherichia 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), *Salmonella* Typhimurium has been associated with the consumption of contaminated groundwater 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.

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Biofilms in 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 study of 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 *S. 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 1,000 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). To establish 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*, *P. cineris*, *Paenibacillus* sp., *Bacillus* sp. and *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 63×/1.4 Zeiss FS10 Neofluor objective. The images

were captured using a charge-coupled device 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 tubes and incubated at 37 °C for 24 h and 10 µl was transferred to 9.9 ml of Rappaport-Vassiliadis broth (Oxoid) and incubated at 42 °C for 24 h. A loopful from each dilution was plated onto xylose lysine deoxycholate (XLD) agar (Oxoid), 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 agar.

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

A flow cell with channels of 2×2×15 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 double-distilled water. Thereafter, sterile tap water supplemented with 1,000 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 10⁶ colony-forming units (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 10² or 10⁶ 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 living, 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 vigorously for 20 s. Colonies were recovered by incubation for 24 h at 37 °C on Luria–Bertani agar with 100 µg ml⁻¹ kanamycin (Roche). Thereafter colonies were tested for the presence of GFP and their identity confirmed on XLD agar.

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) × 0.79 (wall size) × 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 10² and 10⁶ CFU ml⁻¹, and after allowing 2 h for attachment at 25 °C 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 double-distilled water. One ml of these suspensions was 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 1,000 µg l⁻¹ of acetate was used to supplement sterile tap water at a temperature of 37 °C to enhance the visibility of the *gfp*-tagged *Salmonella* cells. A concentration of 100 µg 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 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 assimilable organic carbon 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 1(a) and (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 five-member inoculum formed a substantial biofilm in drinking water by 72 h, with no autofluorescence observed. Individual *Salmonella* cells could be visualized 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 1(c)–(f)) rather than being evenly distributed. After 144 h, the established biofilm had formed very thick intermixed

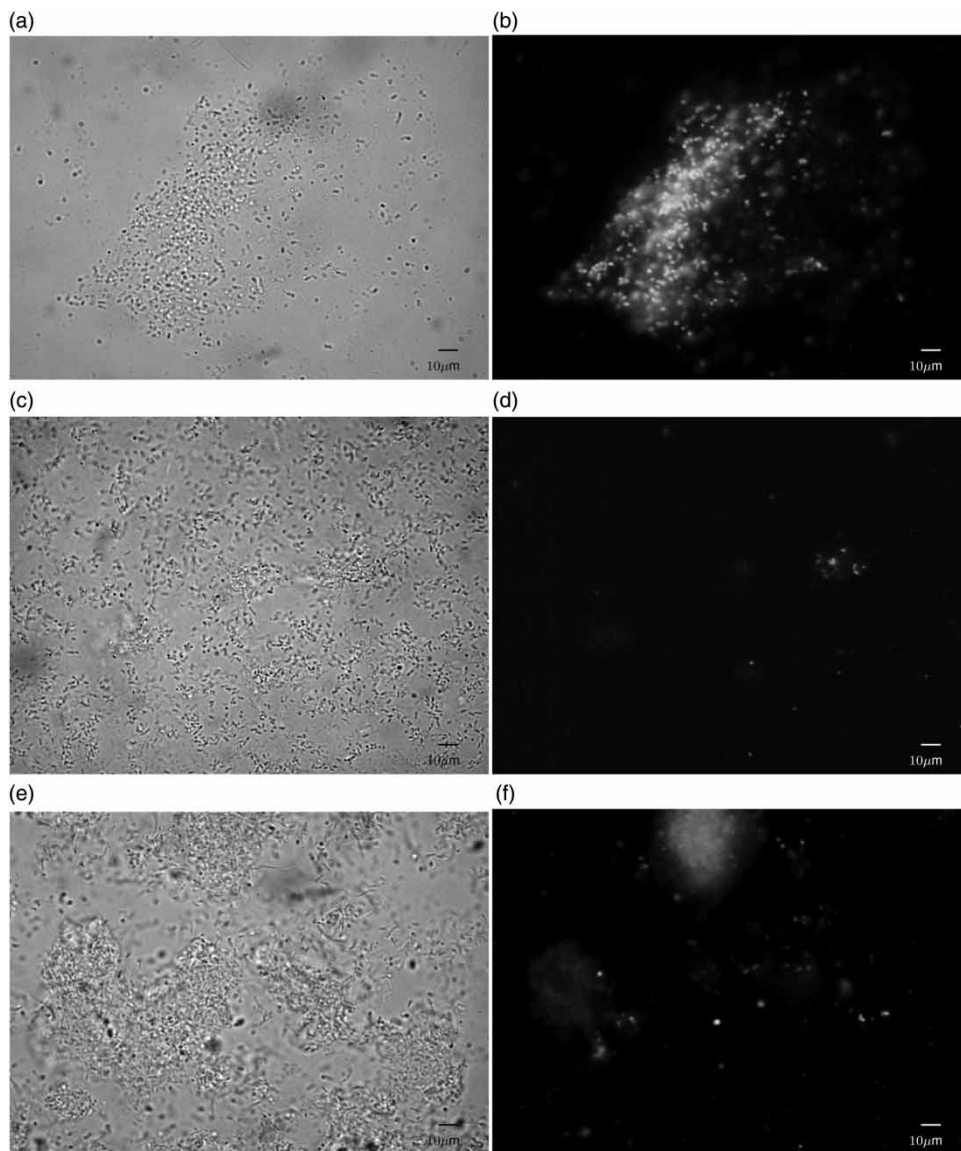


Figure 1 | Photomicrographs of GFP-tagged *Salmonella* growing as biofilm for 48 h on glass in a flow cell fed tap with water with $1,000 \mu\text{g l}^{-1}$ acetate at 0.4 mm s^{-1} . *S. Typhimurium* was spiked into a sterile system ((a), (b)) or into a 3-day-old, five-strain biofilm comprised of non-fluorescing isolates from drinking water distribution systems at 10^2 CFU mL⁻¹ ((c), (d)) or 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)).

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* (Jones & Bradshaw 1997). 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. 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.

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 1(c) and (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 $>10^5$ 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 h 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 with tap water containing 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 10¹ MPN cm⁻², while that of the high inoculum was $>10^3$ MPN cm⁻². Within 24 h *Salmonella* slowly began to accumulate. Both inocula steadily increased until 72 h, the high inoculum increasing by approximately 2 log and the low inoculum by approximately 1 log (Figure 2). Thereafter, the low inoculum biofilm

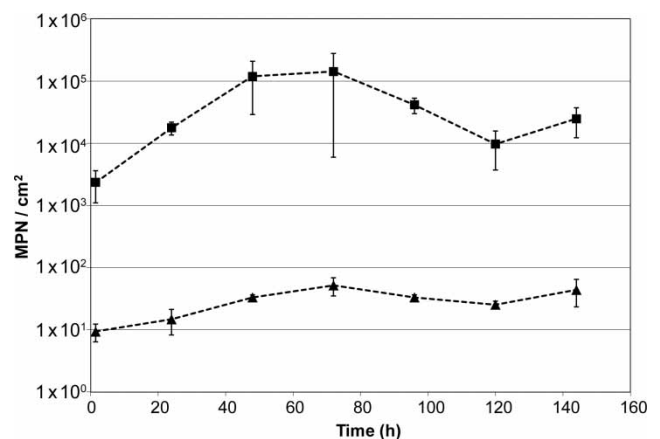


Figure 2 | Average *Salmonella* MPN cm⁻² from *S. Typhimurium* biofilm on silicone tubing fed sterile tap water supplemented with 100 µg l⁻¹ acetate. *S. Typhimurium* was inoculated at either 10² CFU ml⁻¹ (low inoculum) (▲) or 10⁶ CFU ml⁻¹ (high inoculum) (■). The data represent the average of three independent experiments and error bars represent one standard error of the mean.

reached a steady state and remained fairly constant. The high inoculum biofilm dropped by one order of magnitude at 120 h, but increased by 0.5 log at 144 h (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.

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 10^5 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 h, *Salmonella* increased by approximately 1 log for the high inoculum and about 0.5 log for the low inoculum; 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 h of growth, reaching 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 h (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 h, *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*

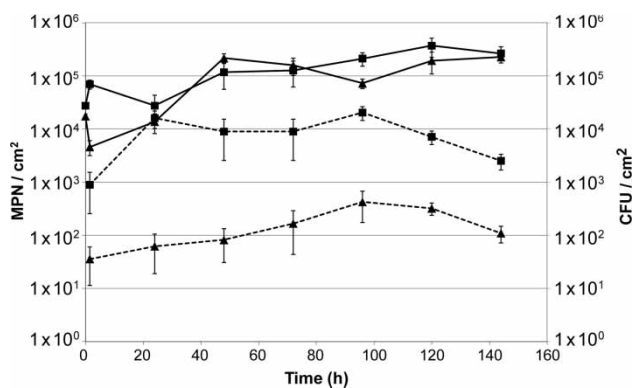


Figure 3 | Average *Salmonella* MPN cm $^{-2}$ (dashed line) and CFU cm $^{-2}$ (solid line) from a multi-strain spiked model biofilm on silicone tubing fed sterile tap water supplemented with $100 \mu\text{g l}^{-1}$ acetate. *S. Typhimurium* was inoculated at either 10^2 CFU ml $^{-1}$ (low inoculum)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

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 $^{-1}$.

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 at 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 $^{-2}$ (Figure 3), indicating that stable biofilm levels were reached at 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*-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 recolonization elsewhere and possible subsequent infection of consumers.

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