

Recovery of *Salmonella* from biofilms in a headwater spring ecosystem

James P. Gaertner, Joseph A. Mendoza, Michael R. J. Forstner and Dittmar Hahn

ABSTRACT

Salmonellae are pathogenic bacteria often detected in waters impacted by human or animal wastes. In order to assess the fate of salmonellae in supposedly pristine environments, water and natural biofilm samples along with snails (*Tarebia granifera*) and crayfish (*Procambarus clarkia*) were collected before and up to 7 days following four precipitation events from sites within the headwater springs of Spring Lake, San Marcos, TX. The samples were analyzed for the presence of salmonellae by polymerase chain reaction (PCR) after semi-selective enrichment. Salmonellae were detected in one water sample directly after precipitation only, while detection in ten biofilm and two crayfish samples was not related to precipitation. Salmonellae were not detected in snails. Characterization of isolates by rep-PCR revealed shared profiles in water and biofilm samples, biofilm and crayfish samples, and biofilm samples collected 23 days apart. These results suggest that salmonellae are infrequently washed into this aquatic ecosystem during precipitation runoff and can potentially take up residency in biofilms which can help facilitate subsequent long-term persistence and eventual transfer through the food chain.

Key words | contamination, *invA* gene, PCR, runoff, salmonellae

James P. Gaertner
Joseph A. Mendoza
Michael R. J. Forstner
Dittmar Hahn (corresponding author)
Department of Biology,
Texas State University,
601 University Drive,
San Marcos, TX 78666,
USA
E-mail: dh49@txstate.edu

INTRODUCTION

Salmonellosis is one of the most important public health disease problems worldwide with up to 1.3 billion cases reported annually (Pang *et al.* 1995). In the United States it affects 1.4 million people, causing 16,000 hospitalizations and over 500 deaths annually (Mead *et al.* 1999). The majority of illnesses result from exposure to undercooked animal products or cross-contamination with foods consumed raw (Tauxe 1997; WHO 2002); however, salmonellosis can also result from direct contact with contaminated water (Foltz 1969; Harvey *et al.* 1969) or infected animals (Sanyal *et al.* 1997; Wells *et al.* 2004; Nakadai *et al.* 2005). The intestinal tract of vertebrates is generally assumed to be the native habitat of salmonellae (Woodward *et al.* 1997) from which the feces then contaminate environments such as fresh- or marine waters, estuarine environments, vegetables, compost, or soils and sediments

(Thomason *et al.* 1975; Polo *et al.* 1998; Refsum *et al.* 2002; Tavechio *et al.* 2002; Martinez-Urtaza *et al.* 2004). The occurrence of salmonellae in these environments is therefore frequently linked to environmental contamination through, e.g., manure or wastewater discharges (Polo *et al.* 1998; Martinez-Urtaza *et al.* 2004). However, salmonellae have also been detected in pristine aquatic systems (Fair & Morrison 1967; Hendrick & Morrison 1967) and shown to persist in the environment for extended periods of time without significant impact from terrestrial animals (Hendrick 1971; Chao *et al.* 1987). Salmonellae have even been suggested to be part of the natural flora present in some aquatic ecosystems (Jimenez *et al.* 1989). Interactions with animals (Reilly & Twiddy 1992) and associations with biofilms (Armon *et al.* 1997; Barker & Bloomfield 2000) or sediments (Hendrick 1971; Marsh *et al.* 1998) are assumed

to help salmonellae survive in the environment. Despite the evidence that salmonellae have complex interactions with the non-host environment, little is known about the fate of these organisms outside of host organisms.

Recent studies in our laboratory frequently detected salmonellae in water, sediments, animals (i.e. fish, turtles) and biofilms even in supposedly clean habitats such as Spring Lake, the spring-fed headwaters of the San Marcos River in San Marcos, Texas (Hahn *et al.* 2007; Gaertner *et al.* 2008a, b, 2009). Spring Lake is a small reservoir (6 ha) that is considered one of the most pristine waters in Texas (Slattery & Fahlquist 1997). It consists of two arms, the Spring arm, characterized by relatively constant environmental conditions in depth and throughout the year due to the permanent supply of water through numerous springs from the Edwards Aquifer, and the slough arm that represents a more lentic environment with slow flow, large seasonal changes in temperature and redox conditions, and large deposition of organic material (Groeger *et al.* 1997). While the spring arm is bordered at one side by steep slopes of forested limestone and on the other by landscaped parkland, the entire slough arm is surrounded by the Texas State University Golf Course, and connected to the discharge area of Sink Creek. The lake is not affected by point sources of pollution such as sewage treatment facilities which are common sources of impact to Texas Rivers (Groeger *et al.* 1997), however, due to its location within the urban-rural interface of San Marcos, could be affected by several potential non-point pollution sources for salmonellae that include cattle ranching operations as well as wildlife habitat associated with the adjacent golf course. In our previous studies, detection of salmonellae in water and sediments was more pronounced directly after rainfall events (Gaertner *et al.* 2009), and thus likely a consequence of contamination through livestock or wildlife fecal droppings transported into the aquatic system by strong rainfall events and associated runoff as suggested for other systems (Kinzelman *et al.* 2004; Arnone & Perdek Walling 2007). The detection of salmonellae in the intestine of turtles and fish, and especially in biofilms on the carapace of turtles, however, could not be linked to runoff, and thus, opens the door for speculations on the dissemination and on the fate of salmonellae with respect to short- and long-term population establishment in aquatic ecosystems.

The aim of our study was to monitor the presence of salmonellae before and after rainfall events in water and sediment samples, to investigate their potential establishment in biofilms, and to assess the potential for their transfer from biofilms into the food chain. The assumption was that biofilms are used as food resource by herbivorous or omnivorous grazers like snails, and these are preyed upon by carnivorous animals such as crayfish (Nystrom 2002). Both snails (Bartlett & Trust 1976; Moore *et al.* 2003; Tezcan-Merdol *et al.* 2004) and crayfish (Lovell & Barkate 1969) have been shown to be potential hosts for salmonellae, and are abundant in Spring Lake, the headwaters of the San Marcos River, San Marcos, Texas.

MATERIALS AND METHODS

Sample collection and preparation

Samples were taken from two locations, one each in the spring and slough arms of Spring Lake (Figure 1) at different time intervals distributed before and after four separate rainfall events that happened on June 23 (2.5 mm), June 30 (7.6 mm), July 7 (6.4 mm), and July 9 (30 mm) (Figure 2). Samples collected on each date consisted of four water samples, eight biofilm samples, and 10 snails of the species *Tarebia granifera* from each of the spring and slough arms of Spring Lake with the exception of June 24 in which only six biofilm samples were collected from each the spring and slough arm and only two snails were found in the slough arm. Water samples were collected into 50 mL tubes from just below the surface, cells pelleted by centrifugation at 3,000 rpm for 15 min and transferred to 2 mL cryotubes containing 1 mL buffered peptone water (BPW) (L^{-1} : 10 g peptone, 5 g NaCl, 9 g Na_2HPO_4 , 1.5 g KH_2PO_4 , pH 7.2) (Thomason *et al.* 1977; International Organization for Standardization 1993). Biofilm samples were taken by scraping films off of the surface of rocks from within each site directly into 2 mL cryotubes containing 1 mL of BPW. Snails (*T. granifera*) were collected by hand and homogenized in 2 mL cryotubes containing 1 mL of BPW.

Independent of precipitation events, 21 crayfish (*Procambarus clarkia*) were captured by hand prior to June 23rd in both the spring and slough arms of Spring

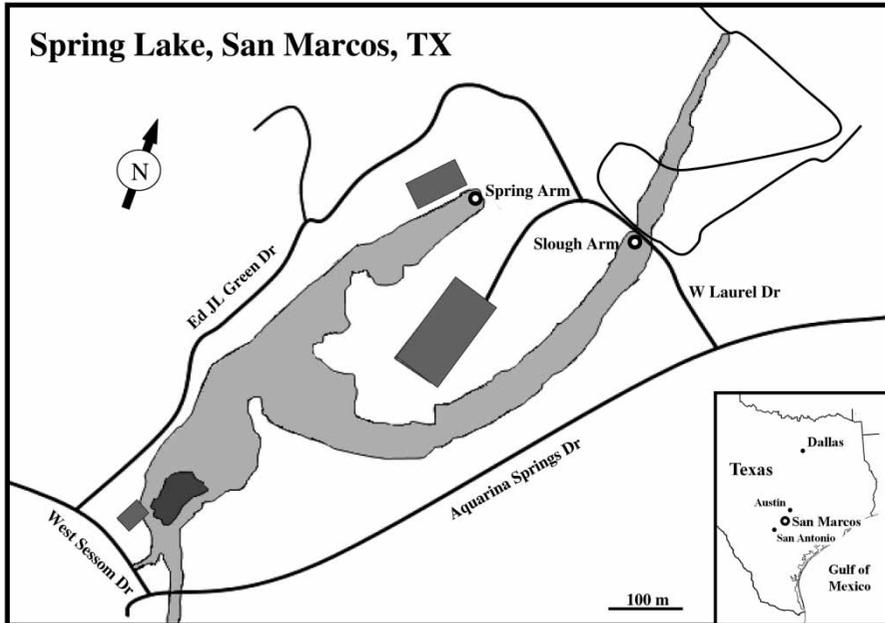


Figure 1 | Schematic presentation of sampling sites (open circles) used for collection of samples to be tested for the presence of *Salmonella* in both the spring and slough arms of Spring Lake, San Marcos, TX, USA (79° 53' N, 97° 55' W).

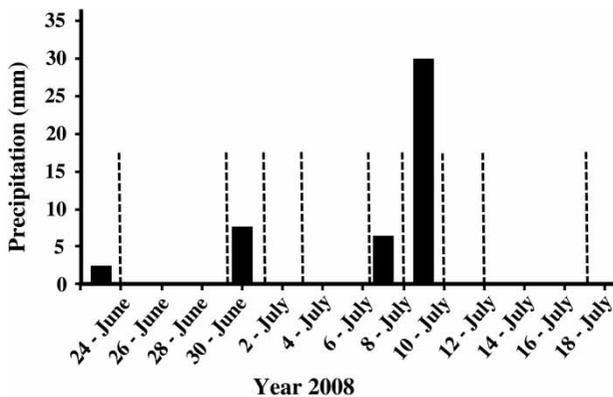


Figure 2 | Precipitation data (dark bars) for San Marcos, TX, USA and sampling dates (dashed lines) used for the collection of samples from Spring Lake.

Lake, and samples from the stomach and intestine separated into 2 mL cryotubes containing 1 mL of BPW.

Enrichment of salmonellae

All samples were incubated in BPW at 37 °C for 16–20 h (International Organization for Standardization 1993). Aliquots (100- μ L) of these cultures were transferred to 2 mL cryotubes containing 1 mL of Rappaport-Vassiliadis Broth (RVS) (L^{-1} : 4.5 g peptone (soymeal), 29 g $MgCl_2 \times 7 H_2O$,

8 g NaCl, 0.4 g KH_2PO_4 , 0.036 g malachite-green, pH 5.2) (Vassiliadis *et al.* 1981). After incubation at 37 °C for 24 h (Vassiliadis *et al.* 1981), 100- μ L sub-samples were transferred to new cryotubes containing RVS media and, again, incubated at 37 °C for 24 h (Gaertner *et al.* 2009). Aliquots (100 μ L) of the final RVS enrichment culture were prepared for polymerase chain reaction (PCR)-assisted detection of *Salmonella*, and the remaining samples were mixed with 600 μ L of 60% glycerol and stored at -80 °C until further use.

PCR-based detection of salmonellae

The presence of *Salmonella* was detected utilizing the amplification of a 284-bp-fragment of the *invA* gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by *Salmonella* (Suárez & Rüssmann 1998; Khan *et al.* 2000). This protocol has been acknowledged as the international standard diagnostic method for quality assurance laboratories in epidemiological studies on all *Salmonella enterica* subspecies as well as in *Salmonella bongori* (Malorny *et al.* 2003). Aliquots of the final enrichment were centrifuged at 14,000 rpm for 2 min, the bacterial pellets washed once in sterile distilled water, and

the bacteria lysed in 100 μL of 50 mM NaOH by incubation at 65 °C for 15 min (Hahn *et al.* 2007). One microliter of lysate was used as template for PCR amplification with primers 139 (5'GTGAAATTATCGCCACGTTTCGGGCAA3') and 141 (5'TCATCGCACCGTCAAAGGAACC3') (Rahn *et al.* 1992) in a final volume of 50 μL containing 1 \times PCR buffer (50 mM KCl, 2.5 mM MgCl_2 , 20 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 0.2 mM dNTPs, 1 U of *Taq* polymerase, and 100 ng of each primer. After an initial 2-min denaturation at 96 °C, 35 rounds of temperature cycling were performed in a PTC-200 Thermocycler (BioRad, Hercules, CA) with denaturation at 96 °C, primer annealing at 64 °C, and elongation at 72 °C, each for 30 s, followed by a final incubation at 72 °C for 7 min (Malorny *et al.* 2003). Lysates of *Salmonella typhimurium* ATCC 14028 and sterilized distilled water were used as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gels in Tris-acetate-EDTA (TAE) buffer after staining with ethidium bromide (0.5 $\mu\text{L mL}^{-1}$) (Sambrook *et al.* 1989).

Rep-PCR

Bacteria from the second enrichment of samples positive for salmonellae were plated onto RVS Agar (i.e. RVS Broth solidified with 15 g agar L^{-1}) and incubated at 37 °C for 24–48 h. Ten colonies from each plate were sub-cultured in LB medium and checked for the presence of the *invA* gene by PCR as described above. All positive isolates were further analyzed by rep-PCR using the BoxA1R primer (5'CTACGGCAAGGCGACGCTGACG3') (Versalovic *et al.* 1998). A total volume of 25 μL was used for each reaction using 2 μL of lysate (Dombek *et al.* 2000), 300 ng of primer, 1 \times Gitschier buffer, 5 mM dNTPs, 10% di-methyl-sulfoxide, 0.0002 μL bovine serum albumin, and 2 U *Taq* polymerase (Rademaker & de Bruijn 1997). Thermocycler conditions consisted of an initial denaturation step of 95 °C for 2 min followed by 30 rounds of temperature cycling with denaturation at 94 °C for 3 s and subsequent 92 °C for 30 s, primer annealing at 50 °C for 1 min, and elongation at 65 °C for 8 min. This was followed by incubation at 65 °C for 8 min (Rademaker & de Bruijn 1997; Dombek *et al.* 2000). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook *et al.* 1989), and

representative profiles documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA).

RESULTS

PCR-based detection of the *invA* gene was achieved in 13 out of 426 samples (72 water, 140 biofilm, 172 snail, and 42 crayfish samples). While none of the snail samples tested positive for salmonellae, amplification of *invA* gene fragments was obtained for 1 water, 10 biofilm and 2 crayfish samples. The positive water sample was collected from the slough arm on July 1, a day after rainfall (7.6 mm), while 8 of the positive biofilm samples were collected from the spring arm and 2 from the slough arm. Both positive biofilm samples from the slough arm were collected on July 10, one day after a precipitation event. Positive samples from the spring arm were collected on five separate dates including three sampling efforts occurring one day after precipitation (June 24 – 2 positives, July 1 – 3 positives, and July 8 – 1 positive) and two sampling efforts occurring 7 days after precipitation (July 7 – 1 positive and July 17 – 1 positive). The two crayfish samples testing positive for salmonellae were collected from the spring arm of Spring Lake on June 12 and were from the intestine of one crayfish and the stomach of the other.

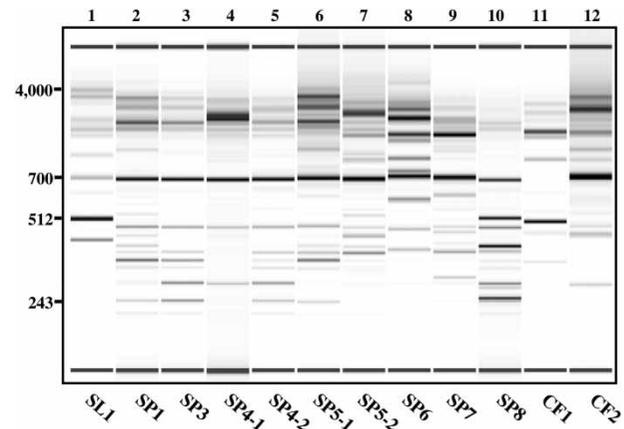
Isolates of *Salmonella* were obtained from all 13 positive samples, with all 10 colonies picked at random for each sample being positive for the *invA* gene, except in the case of a biofilm sample collected from the spring arm on July 1 which yielded only 8 positive colonies. All *Salmonella* colonies for each sample displayed identical rep-PCR profiles, except for salmonellae from 4 biofilm samples taken from the spring arm in which two profiles were obtained (July 8, July 17 and two samples on July 1) (Table 1). Overall, 12 distinct profiles were obtained (Figure 3). Several profiles were shared among isolates from samples collected during the study (Table 1); however, none of them was identical to any of those obtained in previous studies (data not shown). All *Salmonella* isolates from the three positive samples collected from the slough arm of Spring Lake had identical rep-PCR profiles, i.e., profile SL1. These samples included a water sample collected on July 1 and two biofilm samples collected

Table 1 | Prevalence of salmonellae and strain identification in samples collected from water, biofilm and snails (*Tarebia granifera*) from both the spring and slough arms of Spring Lake

Sampling date sample	Spring arm		Slough arm		
	Prevalence ^a	Strain ^b	Prevalence ^a	Strain ^b	
June 24 (last precipitation: June 23 [2.5 mm])					
Water	0/4		0/4		
Biofilm	2/6	SP1	0/6		
Snails	0/10		0/10		
June 30 (last precipitation: June 23 [2.5 mm])					
Water	0/4		0/4		
Biofilm	0/8		0/9		
Snails	0/10		0/10		
July 1 (last precipitation: June 30 [7.6 mm])					
Water	0/4		1/4	SL1	
Biofilm	3/8	SP3, SP4-1, SP4-2, SP5-1, SP5-2	0/8		
Snails	0/10		0/10		
July 3 (last precipitation: June 30 [7.6 mm])					
Water	0/4		0/4		
Biofilm	0/8		0/8		
Snails	0/10		0/10		
July 7 (last precipitation: June 30 [7.6 mm])					
Water	0/4		0/4		
Biofilm	1/8	SP6	0/8		
Snails	0/10		0/10		
July 8 (last precipitation: July 7 [6.4 mm])					
Water	0/4		0/4		
Biofilm	1/8	SP7, CF2	0/8		
Snails	0/10		0/10		
July 10 (last precipitation: July 9 [30 mm])					
Water	0/4		0/4		
Biofilm	0/8		2/8	SL1	
Snails	0/10		0/10		
July 14 (last precipitation: July 9 [30 mm])					
Water	0/4		0/4		
Biofilm	0/8		0/8		
Snails	0/10		0/10		
July 17 (last precipitation: July 9 [30 mm])					
Water	0/4		0/4		
Biofilm	1/8	SP8, SP1	0/8		
Snails	0/10		0/10		

^aPrevalence: detection of salmonellae per sample/total number of samples.

^bStrain assignments are based on distinct rep-PCR profiles (see Figure 3).

**Figure 3** | Representative rep-PCR profiles of *Salmonella* isolates of enrichment cultures from samples taken from water, biofilms and crayfish (*Procambarus clarkia*) (CF) from the slough (SL) and spring (SP) arms of Spring Lake, San Marcos, TX, USA (29° 53' N, 97° 55' W), and documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA). Profile sources and dates are as follows: Lane 1, water July 1st (SL1) and 2 biofilms July 10th (both SL1); Lane 2, 2 biofilms June 24th (both SP1) and July 17th (SP1); Lanes 3–7, biofilms July 1st (SP#3, SP4-1, SP4-2, SP5-1 and SP5-2); Lane 8, biofilm July 7th (SP6); Lane 9, biofilm July 8th (SP7); Lane 10, biofilm July 17th (SP8); Lane 11 crayfish June 12th (CF1); Lane 12, crayfish June 12th (CF2) and biofilm July 8th (CF2).

on July 10. Isolates from two biofilm samples collected from the spring arm on June 24 shared an identical rep-PCR profile with isolates from a biofilm sample collected 23 days later on July 17 (profile SP1). Finally, profiles from isolates from a crayfish sample collected on June 12 matched those of a biofilm sample collected on July 8, both collected from the spring arm (Figure 3, profile CF2).

DISCUSSION

Salmonella were detected in water, biofilm and crayfish samples from Spring Lake, San Marcos, TX, after semi-selective enrichment and subsequent detection on *invA* gene fragments by PCR as described previously (Hahn et al. 2007). Although only about 3% of all samples were found to be positive for salmonellae, their detection is of importance since Spring Lake is generally considered one of the most pristine waters in Texas (Slattery & Fahlquist 1997). Most samples testing positive for salmonellae were obtained a day after rainfall events (i.e. 9 out of 13) suggesting runoff from terrestrial systems or upwelling as potential sources of contamination despite the rainfall events being relatively

weak with 5–30 mm per day. However, in contrast to previous studies that reported significant contamination of water and sediment samples in Spring Lake after extreme flashfloods with up to 740 mm precipitation per day (Gaertner *et al.* 2009), the current rainfall events resulted in only 1 water sample being contaminated with salmonellae indicating lessened effects of runoff or upwelling. The short residence time of salmonellae in water indicated by the detection of salmonellae in samples obtained a day after rainfall events was also evident in our previous study (Gaertner *et al.* 2009), as well as in those of others (Baudart *et al.* 2000; Haley *et al.* 2009; Jokinen *et al.* 2009) indicating the transient nature of the contamination.

Most samples testing positive for salmonellae in this study represented biofilms in which salmonellae were detected 1 day after rainfall, but also in samples taken up to 7 days after rainfall. Since the location of the sampling sites, i.e., rocks collected near the headwaters of both the spring arm and slough arm of Spring Lake, San Marcos, TX, excludes potential contamination from upstream water, runoff from rainfall events (Gaertner *et al.* 2009; Haley *et al.* 2009), in addition to potential small scale contamination by animals, is the most likely source for potential contamination (Geldreich 1996). Since rainfall events were small and detection of salmonellae low in water, it is unlikely that these salmonellae were detected as a consequence of runoff or upwelling. It is more likely that salmonellae from the terrestrial environment are infrequently moved into the aquatic environment by runoff from precipitation and subsequently take up residency in naturally occurring biofilms (Byappanahalli *et al.* 2009).

While salmonellae have the ability to form biofilms on highly diverse surfaces such as those of plastic, cement, stainless steel and even Teflon (Austin *et al.* 1998; Joseph *et al.* 2001), the simple detection of salmonellae in biofilms does not provide sufficient information to assess whether salmonellae are growing as or in biofilms as suggested for *Escherichia coli* (Byappanahalli *et al.* 2003) or are just adhering to the biofilm. Biofilms in aquatic systems represent highly active and heterogeneous communities of auto- and heterotrophic microorganisms that include diatoms, green algae, protozoa, fungi and bacteria, attached to rocks or concrete lining the streambed (Geesey *et al.* 1978). Microorganisms are embedded in an extracellular matrix (Sabater &

Admiraal 2005) that has been shown to increase their survival by providing protection from toxic compounds, and reducing thermal stress and predation pressure (Johnson 2008). Biofilms are also hot spots of rapidly available carbon resources for heterotrophic organisms (Geesey *et al.* 1978; Augspurger *et al.* 2008), created by retaining dissolved organic matter from the water (Fischer *et al.* 2002; Romani *et al.* 2004) or by releasing labile carbon exudates produced by autotrophic organisms such as diatoms or green algae (Sundh & Bell 1992). Enteric pathogens such as *Salmonella* or *E. coli* have been shown to survive for extended periods of time in different habitats (Domingo *et al.* 1989; Smith *et al.* 1994; Ishii *et al.* 2006a; Semenov *et al.* 2009), including biofilms (Ksoll *et al.* 2007) and algal mats (Ishii *et al.* 2006b; Englebert *et al.* 2008; Byappanahalli *et al.* 2009). Biofilms might therefore provide habitats suitable for long term survival of salmonellae in aquatic systems, as discussed for other pathogens (Watnick & Kolter 1999; Yildiz & Schoolnik 1999), or even growth as suggested for other environments such as soil (Topp *et al.* 2003; You *et al.* 2006). While the first assumption is supported by the detection of salmonellae with identical rep-PCR profiles in water samples taken on July 1 and two separate biofilm samples taken 9 days later, as well as of samples of biofilms taken 23 days apart, the latter suggestion remains highly speculative and requires additional quantitative studies on the development of populations of salmonellae in biofilms through time.

Independent of salmonellae persisting or growing in biofilms, the long term survival of salmonellae in biofilms provides additional human health concerns. Since the densities of pathogens in biofilms can be much higher than in water (Hall-Stoodley & Lappin-Scott 1998; September *et al.* 2007), the release of pathogens from biofilms can effectively increase the infective dose in the environment and thus increase the incidence of disease in humans with contact to contaminated water (Purevdorj 2002; Marsollier *et al.* 2004). Biofilms might also provide a potential avenue for transfer of this pathogen through the food chain. Although *Salmonella* was not detected in snails in this study, snails have been shown to be potential reservoirs of the pathogen (Bartlett & Trust 1976; Obi & Nzeako 1980). *Salmonella* ingested during grazing on biofilms by snails could then be transferred to crayfish or fish feeding on

snails as indicated by matching rep-PCR profiles of salmonellae isolated from crayfish collected on June 12 and a biofilm sample collected on July 8. This potential route of contamination, however, needs to be confirmed in more controlled settings in which defined strains of salmonellae are systematically followed as they move through a representative food chain when inoculated into water, establishing in biofilms, taken up by snails and then transferred to crayfish or fish prior to any opportunity of consumption by humans.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from Texas State University, Department of Biology, the National Science Foundation (GK-12 grant No. 0742306), and the State of Texas through the American Recovery and Reinvestment Act (ARRA).

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.
- Arnone, R. D. & Perdek Walling, J. 2007 Waterborne pathogens in urban watersheds. *J. Water Health* **5**, 149–162.
- Augsburger, C., Gleixner, G., Kramer, C. & Küsel, K. 2008 Tracking carbon flow in a 2-week-old and 6-week-old stream biofilm food web. *Limnol. Oceanogr.* **53**, 642–650.
- Austin, J. W., Sanders, G., Kay, W. W. & Collinson, S. K. 1998 Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. *FEMS Microbiol. Lett.* **162**, 295–230.
- Barker, J. & Bloomfield, S. F. 2000 Survival of *Salmonella* in bathrooms and toilets in domestic homes following salmonellosis. *J. Appl. Microbiol.* **89**, 137–144.
- Bartlett, K. H. & Trust, T. J. 1976 Isolation of salmonellae and other potential pathogens from freshwater aquarium snail *Ampullaria*. *Appl. Environ. Microbiol.* **31**, 635–639.
- Baudart, J., Grabulos, J., Barousseau, J. P. & Lebaron, P. 2000 *Salmonella* spp. and fecal coliform loads in coastal waters from a point vs. nonpoint source of pollution. *J. Environ. Qual.* **29**, 241–250.
- Byappanahalli, M. N., Shively, D. A., Nevers, M. B., Sadowsky, M. J. & Whitman, R. L. 2003 Growth and survival of *Escherichia coli* and enterococci populations in the macroalga *Cladophora* (Chlorophyta). *FEMS Microbiol. Ecol.* **46**, 203–211.
- Byappanahalli, M. N., Sawdey, R., Ishii, S., Shively, D. A., Ferguson, J. A., Whitman, R. L. & Sadowsky, M. J. 2009 Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Res.* **43**, 806–808.
- Chao, W. L., Ding, R. J. & Chen, R. S. 1987 Survival of pathogenic bacteria in environmental microcosms. *Chin. J. Microb. Immunol.* **20**, 339–348.
- Dombek, P. E., Johnson, L. K., Zimmerley, S. T. & Sadowsky, M. J. 2000 Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* **66**, 2572–2577.
- Domingo, J. W. S., Fuentes, F. A. & Hazen, T. C. 1989 Survival and activity of *Streptococcus faecalis* and *Escherichia coli* in petroleum contaminated tropical marine waters. *Environ. Poll.* **56**, 263–281.
- Englebert, E. T., McDermott, C. & Kleinheinz, G. T. 2008 Impact of the alga *Cladophora* on the survival of *E. coli*, *Salmonella*, and *Shigella* in laboratory microcosm. *J. Great Lakes Res.* **34**, 377–382.
- Fair, J. F. & Morrison, S. M. 1967 Recovery of bacterial pathogens from high quality surface water. *Water Resour. Res.* **3**, 799–803.
- Fischer, H., Sachse, A., Steinberg, C. E. W. & Pusch, M. 2002 Differential retention and utilization of dissolved organic carbon by bacteria in river sediments. *Limnol. Oceanogr.* **47**, 1702–1711.
- Foltz, V. D. 1969 *Salmonella* ecology. *J. Am. Oil Chem. Soc.* **46**, 222–224.
- Gaertner, J. P., Forstner, M. R. J., Rose, F. L. & Hahn, D. 2008a Detection of salmonellae in different turtle species within a headwater spring ecosystem. *J. Wildl. Dis.* **44**, 519–526.
- Gaertner, J. P., Wheeler, P. E., Obafemi, S., Valdez, J., Forstner, M. R. J., Bonner, T. H. & Hahn, D. 2008b Detection of salmonellae in fish in a natural river system. *J. Aqua. Animal Health* **20**, 150–157.
- Gaertner, J. P., Garres, T., Becker, J. C., Jimenez, M. L., Forstner, M. R. J. & Hahn, D. 2009 Analyses of salmonellae in a headwater spring ecosystem. *J. Water Health* **7**, 115–112.
- Geesey, G. G., Mutch, R., Costerton, J. W. & Green, R. B. 1978 Sessile bacteria – important component of microbial population in small mountain streams. *Limnol. Oceanogr.* **23**, 1214–1223.
- Geldreich, E. E. 1996 Pathogenic agents in freshwater resources. *Hydrol. Proc.* **10**, 315–333.
- Groeger, A. W., Brown, P. F., Tietjen, T. E. & Kelsey, T. C. 1997 Water quality of the San Marcos River. *Texas J. Sci.* **49**, 279–294.
- Hahn, D., Gaertner, J., Forstner, M. R. J. & Rose, F. L. 2007 High resolution analysis of salmonellae from turtles within a headwater spring ecosystem. *FEMS Microbiol. Ecol.* **60**, 148–155.
- Haley, B. J., Cole, D. J. & Lipp, E. K. 2009 Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Appl. Environ. Microbiol.* **75**, 1248–1255.

- Hall-Stoodley, L. & Lappin-Scott, H. 1998 Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*. *FEMS Microbiol. Lett.* **168**, 77–84.
- Harvey, R. W. S., Price, T. H., Foster, D. W. & Griffith, W. 1969 *Salmonellas* in sewage. A study in latent human infection. *J. Hygiene* **67**, 517–523.
- Hendrick, C. W. 1971 Increased recovery rate of salmonellae from stream bottom sediments versus surface waters. *Appl. Microbiol.* **21**, 379–380.
- Hendrick, C. W. & Morrison, S. M. 1967 Multiplication and growth of selected enteric bacteria in clear mountain stream water. *Water Res.* **1**, 567–576.
- International Organization for Standardization 1993 Detection of salmonellae (reference method). International Organization for Standardization, ISO 6579.
- Ishii, S., Ksol, W. B., Hicks, R. E. & Sadowsky, M. J. 2006a Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. *Appl. Environ. Microbiol.* **72**, 612–621.
- Ishii, S., Yan, T., Shively, D. A., Byappanahalli, M. N., Whitman, R. L. & Sadowsky, M. J. 2006b *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Appl. Environ. Microbiol.* **72**, 4545–4553.
- Jimenez, L., Muniz, I., Toranzos, G. A. & Hazen, T. C. 1989 Survival and activity of *Salmonella typhimurium* and *Escherichia coli* in tropical fresh water. *J. Appl. Bacteriol.* **67**, 61–69.
- Johnson, L. R. 2008 Microcolony and biofilm formation as a survival strategy for bacteria. *J. Theor. Biol.* **251**, 24–34.
- Jokinen, C. C., Schreier, H., Mauro, W., Taboada, E., Isaac-Renton, J. L., Topp, E., Edge, T., Thomas, J. E. & Gannon, V. P. J. 2009 The occurrence and sources of *Campylobacter* spp., *Salmonella enterica* and *Escherichia coli* O157:H7 in the Salmon River, British Columbia, Canada. *J. Water Health.* **8**, 374–386.
- Joseph, B., Otta, S. K. & Karunasagar, I. 2001 Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int. J. Food Microbiol.* **64**, 367–372.
- Khan, A. A., Nawaz, M. S., Khan, S. A. & Cerniglia, C. E. 2000 Detection of multidrug-resistant *Salmonella* Typhimurium DT104 by multiplex polymerase chain reaction. *FEMS Microbiol. Lett.* **182**, 355–360.
- Kinzelman, J., McLellan, S. L., Daniels, A. D., Cashin, S., Singh, A., Gradus, S. & Bagley, R. 2004 Non-point source pollution: determination of replication versus persistence of *Escherichia coli* in surface water and sediments with correlation of levels to readily measurable environmental parameters. *J. Water Health.* **2**, 103–114.
- Ksol, W. B., Ishii, S., Sadowsky, M. J. & Hicks, R. E. 2007 Presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior. *Appl. Environ. Microbiol.* **73**, 3771–3778.
- Lovell, R. T. & Barkate, J. A. 1969 Incidence and growth of some health-related bacteria in commercial freshwater crayfish (Genus *Procambarus*). *J. Food Sci.* **34**, 268–271.
- Malorny, B., Hoorfar, J., Bunge, C. & Helmuth, R. 2003 Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl. Environ. Microbiol.* **69**, 290–296.
- Marsh, P., Morris, N. Z. & Wellington, E. M. H. 1998 Quantitative molecular detection of *Salmonella typhimurium* in soil and demonstration of persistence of an active but non-culturable population. *FEMS Microbiol. Ecol.* **27**, 351–363.
- Marsollier, L., Stinear, T., Aubry, J., Saint Andre, J. P., Robert, R., Legras, P., Manceau, A. L., Audrain, C., Bourdon, S., Kouakou, H. & Carbonnelle, B. 2004 Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. *Appl. Environ. Microbiol.* **70**, 1097–1103.
- Martinez-Urtaza, J., Saco, M., de Novoa, J., Perez-Pineiro, P., Peiteado, J., Lozano-Leon, A. & Garcia-Martin, O. 2004 Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Appl. Environ. Microbiol.* **70**, 2089–2097.
- Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. 1999 Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**, 607–625.
- Moore, B. C., Martinez, E., Gray, J. M. & Rice, D. H. 2003 Survival of *Salmonella enterica* in freshwater sediments and transmission by the aquatic midge *Chironomus tentans* (Chironomidae: Diptera). *Appl. Environ. Microbiol.* **69**, 4556–4560.
- Nakadai, A., Kuroki, T., Kato, Y., Suzuki, R., Yamai, S., Yaginuma, C., Shiotani, R., Yamanouchi, A. & Hayashidani, H. 2005 Prevalence of *Salmonella* spp. in pet reptiles in Japan. *J. Vet. Med. Sci.* **67**, 97–101.
- Nystrom, P. 2002 Ecology. In: *Biology of Freshwater Crayfish* (D. M. Holdich, ed.). Iowa State University Press Ames, IA, pp. 192–235.
- Obi, S. K. C. & Nzeako, B. C. 1980 *Salmonella* Arizona, *Shigella* and *Aeromonas* isolated from the snail *Achatina achatina* in Nigeria. *Antonie van Leeuwenhoek.* **46**, 475–481.
- Pang, T., Bhutta, Z. A., Finlay, B. B. & Altwegg, M. 1995 Typhoid fever and other salmonellosis – a continuing challenge. *Trends Microbiol.* **3**, 253–255.
- Polo, F., Figueras, M. J., Inza, I., Sala, J., Fleisher, J. M. & Guarro, J. 1998 Relationship between presence of *Salmonella* and indicators of fecal pollution in aquatic habitats. *FEMS Microbiol. Lett.* **160**, 253–256.
- Purevdorj, B. 2002 Hydrodynamic considerations of biofilm structure and behavior. In: *Microbial Biofilms* (M. A. Ghannoum & G. O'Toole, eds.). ASM Press, Washington, DC, pp. 160–173.
- Rademaker, J. L. W. & de Bruijn, F. J. 1997 Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In: *DNA Markers: Protocols, Applications, and Overviews* (G. Caetano-Anolles & P. M. Gresshoff, eds.). John Wiley & Sons, New York, NY, pp. 151–171.

- Rahn, K., Degrandis, S. A., Clarke, R. C., McEwen, S. A., Galan, J. E., Ginocchio, C., Curtiss, R. & Gyles, C. L. 1992 Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain-reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes*. **6**, 271–279.
- Refsum, T., Heir, E., Kapperud, G., Vardund, T. & Holstad, G. 2002 Molecular epidemiology of *Salmonella enterica* serovar Typhimurium isolates determined by pulsed-field gel electrophoresis: comparison of isolates from avian wildlife, domestic animals and the environment in Norway. *Appl. Environ. Microbiol.* **68**, 5600–5606.
- Reilly, P. J. A. & Twiddy, D. R. 1992 *Salmonella* and *Vibrio cholerae* in brackish water cultured tropical prawns. *Int. J. Food Microbiol.* **16**, 293–301.
- Romani, A. M., Guasch, H., Munoz, I., Ruana, J., Vilalta, E., Schwartz, T., Emtiazi, F. & Sabater, S. 2004 Biofilm structure and function and possible implications for riverine DOC dynamics. *Microb. Ecol.* **47**, 316–328.
- Sabater, S. & Admiraal, W. 2005 Periphyton as biological indicators in managed aquatic ecosystems. In: *Periphyton: Ecology, Exploitation and Management* (M. E. Azim, M. C. J. Verdegem & A. A. van Dam & M. C. M. Beveridge, eds.). CABI Publishing, Cambridge, MA, pp. 159–177.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanyal, D., Douglas, T. & Roberts, R. 1997 *Salmonella* infection acquired from reptilian pets. *Arch. Dis. Childhood*. **77**, 345–346.
- Semenov, A. V., van Overbeek, L. & van Bruggen, A. H. 2009 Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Appl. Environ. Microbiol.* **75**, 3206–3215.
- September, S. M., Els, F. A., Venter, S. N. & Brozel, V. S. 2007 Prevalence of bacterial pathogens in biofilms of drinking water distribution systems. *J. Water Health*. **5**, 219–227.
- Slattery, R. N. & Fahlquist, L. 1997 *Water-quality Summary of the San Marcos Springs Riverine System, San Marcos, Texas, July-August 1994: U.S. Geological Survey Fact Sheet FS-059-97*. Available from: <http://pubs.usgs.gov/fs/Fs05997> (accessed 14 June 2011).
- Smith, J. J., Howington, J. P. & McFeters, G. A. 1994 Survival, physiological-response, and recovery of enteric bacteria exposed to a polar marine environment. *Appl. Environ. Microbiol.* **60**, 2977–2984.
- Suárez, M. & Rüssmann, H. 1998 Molecular mechanisms of *Salmonella* invasion: the type III secretion system of the pathogenicity island 1. *Int. Microbiol.* **1**, 197–204.
- Sundh, I. & Bell, R. T. 1992 Extracellular dissolved organic carbon released from phytoplankton as a source of carbon for heterotrophic bacteria in lakes of different humic content. *Hydrobiologia*. **229**, 93–106.
- Tauxe, R. V. 1997 Emerging foodborne diseases: an evolving public health challenge. *Emer. Infect. Dis.* **3**, 425–434.
- Tavechio, A. T., Ghilardi, A. C. R., Peresi, J. T. M., Fuzihara, T. O., Yonamine, E. K., Jakabi, M. & Fernandes, S. A. 2002 Serotypes isolated from nonhuman sources in Sao Paulo, Brazil, from 1996 through 2000. *J. Food Prot.* **65**, 1041–1044.
- Tezcan-Merdol, D., Ljungstrom, M., Winiacka-Krusnell, J., Linder, E., Engstrand, L. & Rhen, M. 2004 Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhysodes*. *Appl. Environ. Microbiol.* **70**, 3706–3714.
- Thomason, B. M., Biddle, J. W. & Cherry, W. B. 1975 Detection of salmonellae in the environment. *Appl. Microbiol.* **30**, 764–767.
- Thomason, B. M., Dodd, D. J. & Cherry, W. B. 1977 Increased recovery of salmonellae from environmental samples enriched with buffered peptone water. *Appl. Environ. Microbiol.* **34**, 270–273.
- Topp, E., Welsh, M., Tien, Y. C., Dang, A., Lazarovits, G., Conn, K. & Zhu, H. 2003 Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiol. Ecol.* **44**, 303–308.
- Vassiliadis, P., Kalapothaki, V., Trichopoulos, D., Mavrommatti, C. & Serie, C. 1981 Improved isolation of salmonellae from naturally contaminated meat-products by using Rappaport-Vassiliadis enrichment broth. *Appl. Environ. Microbiol.* **42**, 615–618.
- Versalovic, J., de Bruijn, F. J. & Lupski, J. R. 1998 Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. In: *Bacterial Genomes: Physical Structure and Analysis* (F. J. de Bruijn, J. R. Lupski & G. M. Weinstock, eds.). Chapman & Hall, New York, N.Y., pp. 437–454.
- Watnick, P. I. & Kolter, R. 1999 Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol.* **34**, 586–595.
- Wells, E. V. M., Boulton, M., Hall, W. & Bidol, S. A. 2004 Reptile-associated salmonellosis in preschool-aged children in Michigan, January 2001–June 2003. *Clin. Infect. Dis.* **39**, 687–691.
- Woodward, D. L., Khakhira, R. & Johnson, W. M. 1997 Human salmonellosis associated with exotic pets. *J. Clin. Microbiol.* **35**, 2786–2790.
- World Health Organization 2002 World Health Report. Reducing Risks, Promoting Healthy Life. World Health Organization, Geneva, Switzerland.
- Yildiz, F. H. & Schoolnik, G. K. 1999 *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. USA*. **96**, 4028–4033.
- You, Y., Rankin, S. C., Aceto, H. W., Benson, C. E., Toth, J. D. & Dou, Z. 2006 Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl. Environ. Microbiol.* **72**, 5777–5783.