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

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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, *inv*A gene, PCR, runoff, salmonellae

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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

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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

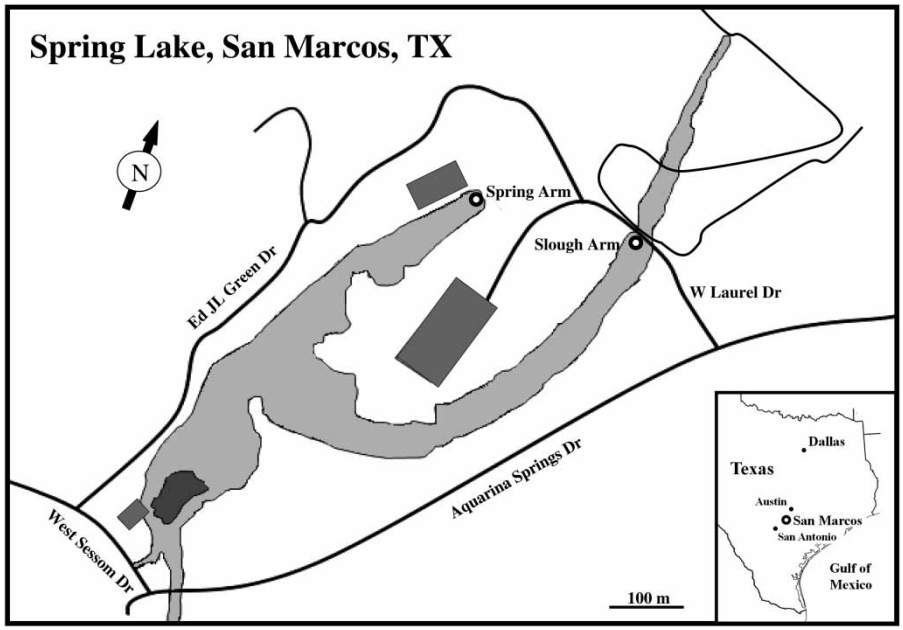

Figure 1 | Schematic presentation of sampling sites (open circles) used for collection of samples to be tested for the presence of *Salmonella* in both the spring and slough arms of Spring Lake, San Marcos, TX, USA (79°53′N, 97°55′W).

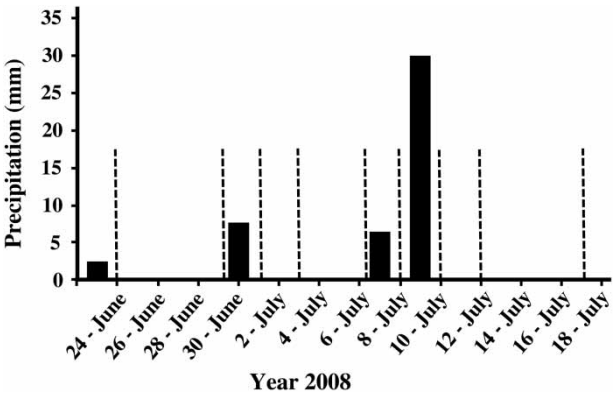

Figure 2 | Precipitation data (dark bars) for San Marcos, TX, USA and sampling dates (dashed lines) used for the collection of samples from Spring Lake.

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-μL) 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-μL 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 μL) 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 μL 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 *inv*A 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′GTGAAATTATCGCCACGTTCGGGCAA3′) and 141 (5′TCATCGCACCGTCAAAGGAACC3′) (Rahn *et al.* 1992) in a final volume of 50 μL containing 1 × PCR buffer (50 mM KCl, 2.5 mM $MgC1_2$, 20 mM Tris/HCI, 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 μL 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 *inv*A 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 × 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 *inv*A 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 *inv*A 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 *inv*A 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 Prevalence[a]	Strain[b]	Slough arm 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	

[a]Prevalence: detection of salmonellae per sample/total number of samples.
[b]Strain assignments are based on distinct rep-PCR profiles (see Figure 3).

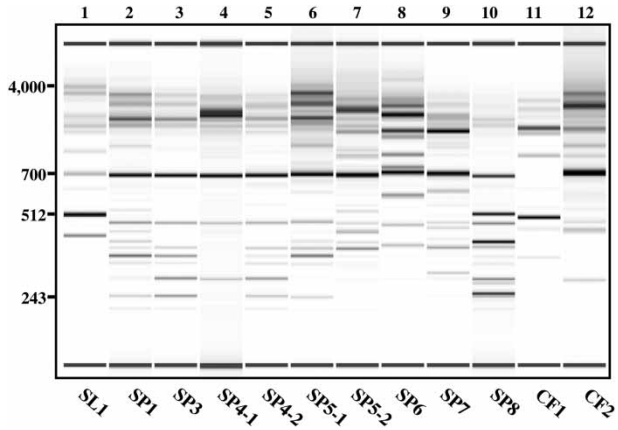

Figure 3 | Representative rep-PCR profiles of *Salmonella* isolates of enrichment cultures from samples taken from water, biofilms and crayfish (*Procambarus clarkia*) (CF) from the slough (SL) and spring (SP) arms of Spring Lake, San Marcos, TX, USA (29°53′N, 97°55′W), and documented using an Agilent 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 *inv*A 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.

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