Journal of Aquatic Animal Health
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uahh20

Salmonellae in Fish Feces Analyzed by In Situ Hybridization and Quantitative Polymerase Chain Reaction

Qiong Sha [a], Michael R. J. Forstner [a], Timothy H. Bonner [a] & Dittmar Hahn [a]
[a] Department of Biology, Texas State University-San Marcos, 601 University Drive, San Marcos, Texas, 78666, USA
Published online: 24 Jul 2013.

To cite this article: Qiong Sha, Michael R. J. Forstner, Timothy H. Bonner & Dittmar Hahn (2013) Salmonellae in Fish Feces Analyzed by In Situ Hybridization and Quantitative Polymerase Chain Reaction, Journal of Aquatic Animal Health, 25:3, 184-190

To link to this article: http://dx.doi.org/10.1080/08997659.2013.790854

Journal of Aquatic Animal Health 25:184–190, 2013

ISSN: 0899-7659 print / 1548-8667 online
DOI: 10.1080/08997659.2013.790854

ARTICLE

Salmonellae in Fish Feces Analyzed by In Situ Hybridization and Quantitative Polymerase Chain Reaction

Qiong Sha, Michael R. J. Forstner, Timothy H. Bonner, and Dittmar Hahn*
Department of Biology, Texas State University–San Marcos, 601 University Drive, San Marcos, Texas 78666, USA

Abstract

The potential of fish to transfer salmonellae from heterogeneous aquatic biofilms into feces was assessed in controlled aquarium studies with Suckermouth Catfish *Hypostomus plecostomus* and with biofilms inoculated with salmonellae. Neither the presence of catfish nor inoculation with salmonellae had detectable effects on the abundance of the microbial community. Densities of the microbial community were about 10^5 cells/mL in the water during a 1-week period, whereas densities of the microbial community increased 10-fold (10^6 to 10^7 cells/mg) in catfish feces during the same period. Salmonellae were detected by both quantitative polymerase chain reaction (*q*PCR) and situ hybridization in water samples immediately after inoculation, in numbers of about 10^4 cells/mL, representing up to 20% of the cells of the microbial community. Numbers decreased by three orders of magnitude within the first 3 d of the study, which represented only 0.01% of the community, and became undetectable after day 5. In catfish feces, numbers of *Salmonella* initially increased to up to 6% of the cells of the community but then declined. These results suggest that *Salmonella* are not biomagnified during gut passage, and thus, fish only provide a means for the translocation of this pathogen.

Members of the genus *Salmonella* represent important zoonotic pathogens (Humphrey 2000) that have been detected in a broad range of animal reservoirs including invertebrates, reptiles, birds, and mammals (Beach et al. 2002; Refsum et al. 2002; Hahn et al. 2007; Gaertner et al. 2011). Intestinal tracts of vertebrates are assumed to be the native habitat of salmonellae, and terrestrial or aquatic environments are contaminated by the release of salmonellae through feces (Woodward et al. 1997; Gopinath et al. 2012). Salmonellae persist not only in soil and water but also in plants and biofilms for extended periods (Murray 1991; Baloda et al. 2001; Côté and Quessy 2005; Ishii et al. 2006; Byappanahalli et al. 2009). In biofilms, for example, we detected salmonellae even in habitats of exceptional water quality, such as spring-fed Spring Lake and the upper reach of the San Marcos River, Texas (Hahn et al. 2007; Gaertner et al. 2008b, 2011; Sha et al. 2011). Salmonellae were present in natural biofilms in Spring Lake with a significant microheterogeneity and with differences in diversity of viable strains (Sha et al. 2011). In the laboratory, specific isolates remained pathogenic, persistent, and viable in biofilm and the water column for up to 28 d (Sha et al. 2013).

In the upper reach of the San Marcos River, salmonellae were detected in the intestine of four trophically diverse fishes, i.e., piscivorous Largemouth Bass *Micropterus salmoides*, omnivorous Channel Catfish *Ictalurus punctatus*, invertivorous and detritivorous Common Carp *Cyprinus carpio*, and algivorous and detritivorous Suckermouth Catfish *Hypostomus plecostomus*, and up to 33% of the fish analyzed were positive for salmonellae, and serovars were highly variable among individuals (Gaertner et al. 2008c). Salmonellae are not considered to be part of the normal intestinal flora of fish (Janssen and Meyers 1968; Pal and Dasgupta 1991), even though they were detectable for up to 30 d in Channel Catfish artificially exposed to salmonellae (Lewis 1975). Thus, fish exposed to salmonellae could become asymptomatic carriers of this pathogen (Heuschmann-Brunner 1974; Bocek et al. 1992).

*Corresponding author: dh49@txstate.edu
Received November 25, 2012; accepted March 25, 2013

Consequently, fish potentially constitute an important factor in the dissemination and persistence of salmonellae in aquatic environments (Lawton and Morse 1980).

The aim of our study was to determine whether fish would consume salmonellae from natural biofilms and return them to the environment through fecal matter, ultimately enhancing the abundance or persistence of salmonellae in aquatic environments. In this study, we used the same design as in our previous studies on the fate of salmonellae in biofilms, which were conducted as controlled aquarium studies using biofilms on tiles inoculated with salmonellae (Sha et al. 2013). Suckermouth Catfish was selected to assess the role of fish in the transfer of salmonellae from biofilms into feces, because they consume algae and amorphous detritus from benthos of the San Marcos River (Pound et al. 2011). Quantification of salmonellae was achieved at selected sampling times during 1 week using quantitative polymerase chain reaction (*q*PCR) and in situ hybridization, and data were related to shifts in abundance of the entire microbial communities in time.

METHODS

Heterogeneous aquatic biofilms were grown on ceramic tiles (2.2 × 2.2 cm, nonglazed) in a stream channel adjacent to the Freeman Aquatic Biology Building at Texas State University–San Marcos with running spring water for 12 months. Previous studies using more than 120 tiles with biofilms demonstrated the absence of salmonellae (Sha et al. 2013), and therefore biofilms from only 10 haphazardly selected tiles were checked for salmonellae by PCR after semiselective enrichment in Rappaport–Vassiliadis enrichment broth (RVS) (Gaertner et al. 2009; Sha et al. 2011). Since these controls remained negative for salmonellae, all remaining biofilms were assumed to be free of salmonellae as well. Tiles with biofilms were then used in three treatments with three replicates each and established in 36-L aquaria in the laboratory. Treatments 1 and 2 each contained 200 tiles with biofilms free of salmonellae that were placed on the bottom of each aquarium. For treatment 3, tiles with biofilms were covered with 10 L of water in aquaria. This water was inoculated with *Salmonella* strain S11 serovar Thompson with pulsed-field gel electrophoresis (PFGE) pattern XB-SLTH-096 [01] determined at the Texas Department of State Health Services. This strain was previously isolated from biofilms (Sha et al. 2011) and known to be pathogenic in feeding studies with the nematode *Caenorhabditis elegans* (Sha et al. 2013). Strain S11 was grown in Luria–Bertani (LB) medium for 16 h, washed with municipal tap water twice, and inoculated to a final density of approximately 10^6 cells/mL estimated from the reading at an optical density of 564 nm. At 16 h after inoculation, tiles were transferred to three *Salmonella*-free aquaria. Biofilms on these tiles harbored approximately $6.0 \pm 1.4 \times 10^6$ (mean ± SE) *Salmonella* cells per tile as demonstrated by *q*PCR analysis for nine haphazardly selected tiles (Sha et al. 2013). All aquaria were then filled with spring water and aerated through air stones (3 cm^3). Aquaria for treatments 2 and 3 received one large or up to six small Suckermouth Catfish, taken from Spring Lake by grappling. All treatments were kept at room temperature (i.e., 25°C) and artificial light conditions at a 16 h light : 8 h dark photoperiod for 7 d.

Water samples were collected directly after setup, whereas additional water samples and fish feces samples were obtained in 12-h intervals (i.e., 12, 24, 36, 48, 60, and 72 h after setup), followed by 24-h intervals (i.e., 4, 5, 6, and 7 d after setup). Water samples (500 mL) were filtered through 0.2-μm Whatman Nuclepore Track-Etched membranes, and the filter placed into 50-mL graduated plastic tubes (Falcon) containing 20 mL of phosphate-buffered saline (PBS) (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO4, pH 7.2). Cells were released from filters by sonication in a Fisher sonic cleaner (2QT; Fisher Scientific, Pittsburgh, Pennsylvania) for 10 min. Filters were removed afterwards, and released cells were collected after centrifugation at 4,400 × *g* for 15 min (Sha et al. 2013). Fish feces (40 mL) were collected with a syringe from the bottom of each aquarium and concentrated by centrifugation at 4,400 × *g* for 15 min. Cell pellets from water and feces were resuspended in 1 mL of sterile distilled water, and each three subsamples of 100 μL were then used for quantification by in situ hybridization or *q*PCR. After 3 and 7 d, additional 100-μL samples were used for semiselective enrichment and characterization of *Salmonella* isolates by repetitive sequence-based PCR (rep-PCR) (Hahn et al. 2007).

At the end of the study after 7 d, fish were euthanized by pithing, intestines were removed, and intestinal lining and contents were exposed by a longitudinal incision. Intestines from fish of the same treatment were pooled and transferred to 1 mL of distilled water in an Eppendorf microcentrifuge tube, which was then shaken by hand for 20 s to release and disperse the contents of the intestines. The intestines were then removed from the tube, and distilled water was added to the remaining liquid to final volume of 1 mL. Each three 100-μL subsamples were then used for quantification of salmonellae by in situ hybridization and *q*PCR, and for semiselective enrichment and subsequent analysis by end-point PCR.

For quantification of salmonellae by in situ hybridization, the subsamples of water, feces, and fish intestine content were fixed in 4% paraformaldehyde in PBS at 4°C for 16 h (Amann et al. 1990). Afterwards, samples were washed in PBS and stored in a final volume of 500 μL of 50% ethanol in PBS at −20°C until further use (Amann et al. 1990). Samples were spotted on gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$], dried at 42°C for 15 min, and subsequently dehydrated in 50, 70, and finally 95% ethanol for 3 min each. Hybridizations were carried out with probe Sal3 (5′AAT CAC TTC ACC TAC GTG, *Escherichia coli* position 1713–1730) (Nordentoft et al. 1997) that binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (excepting only subspecies IIIa), but should not detect *S. bongori* (Fang et al. 2003). Reactions were performed in 9 μL of hybridization buffer (0.9 M NaCl, 20 mM tris/HCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate [SDS], pH 7.2)

containing 10% formamide, to which 1 μL of probe (25 ng/mL) that included 4′6-diamidino-2-phenylindole (DAPI) at a final concentration of 200 ng/mL was added, at 42°C for 2 h. After hybridization, the slides were washed with hybridization buffer at room temperature for 15 min, rinsed with distilled water, and air-dried. Slides were mounted with Citifluor AF1 solution (Citifluor, London, UK) and examined with an Eclipse 80i microscope (Nikon, Lewisville, Texas), fitted for epifluorescence microscopy with a mercury lamp (X-Cite 120; Nikon) and filter cubes UV-2E/C (EX340–380, DM400, BA4435-485, for DAPI detection; Nikon) and CY3 HYQ (EX535/50, DM565, BA610/75, for Cy3 detection; Nikon), respectively. Bacteria were counted at 1,000 × magnification in 25 fields, selected at random, covering an area of 0.01 mm^2. Detection of DAPI and Cy3 were determined from the same image (using the respective filter cubes)taken with a cooled CCD camera (CoolSNAP *ES*^{2}; Photometrics, Tucson, Arizona), and Nikon's NIS Elements imaging software (version 3). Treatment effects in the number of DAPI-stained cells in water and feces across time intervals were tested with a one-factor ANOVA ($\alpha = 0.05$), and Tukey's honestly significant difference (HSD) was used to test differences between treatments. Analyses were conducted in the software package R, version 2.11.1 (www.R-project.org).

For the quantification of salmonellae by *q*PCR, cells in the subsamples of water, feces, and fish intestine content were lysed in a final volume of 200 μL of 50-mM NaOH at 65°C for 30 min. Detection and quantification of salmonellae was achieved using lysates or 10-fold dilutions as a template in a *q*PCR based on the nucleic acid stain SYBR Green and performed in triplicate in a total volume of 20 μL containing 10 μL of Quanta Mix (Quanta BioSciences, Gaithersburg, Maryland), 0.2 μL of each primer 139 (5′ GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 (5′ TCA TCG CAC CGT CAA AGG AAC C) (100 ng/μL) and 1 μL of DNA template in an Eppendorf Mastercycler (ep realplex2; Eppendorf, Hauppauge, New York) (Sha et al. 2013). Conditions included an initial denaturation at 96°C for 3 min, and 35 cycles of denaturation at 96°C, annealing at 64°C, and extension at 72°C, each for 30 s. Amplification was followed by a melting curve analysis. Quantification was based on a standard curve generated from serial dilutions of ethanol-fixed cells of *Salmonella typhimurium* ATCC 14028 quantified by epifluorescence microscopy (Eclipse 80i; Nikon) after DAPI staining.

Semiselective enrichment of salmonellae was used for their detection in intestine samples by end-point PCR, and for the characterization of isolates in intestine samples, and in water and feces samples collected on days 3 and 7 by rep-PCR. For enrichment, each 100 μL subsample was transferred to a 2-mL cryotube containing 1 mL of buffered peptone water (BPW) (10 g/L peptone, 5 g/L NaCl, 9 g/L Na_2HPO4, 1.5 g/L KH_2PO_4, pH 7.2) (ISO 1993) and incubated at 37°C. After 24 h of incubation, 100 μL of each of these samples were transferred to a 2-mL cryotube containing 1 mL of RVS (4.5 g/L peptone [soymeal], 29 g/L $MgCl_2 \cdot 7\ H_2O$, 8 g/L NaCl, 0.4 g/L KH_2PO_4, 0.036 g/L malachite green, pH 5.2) and incubated at 37°C for 24 h (Vassiliadis et al. 1981). Subsamples (100 μL) of this semispecific enrichment for salmonellae were transferred to new tubes with RVS, and salmonellae were enriched a second time as described above (Gaertner et al. 2008a).

For end-point PCR analyses of intestine contents, 100-μL samples of this second enrichment were transferred to a sterile, 1.5-mL Eppendorf microcentrifuge tube, and cells were pelleted by centrifugation at 14,000 × *g* for 2 min. The cell pellet was washed with 500 μL of sterile distilled water once and subsequently lysed in 100 μL of 50-mM NaOH by incubation at 65°C for 15 min with shaking. Lysed cells were kept at −20°C until use. End-point PCR was performed in a PTC-200 thermocycler (MJ Research, Waltham, Massachusetts) in a total volume of 50 μL containing 10 × PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM tris/HCl, 0.1% Triton 100, pH 8.4), 1 μL dNTPs (each 10 mM in 10 mM tris/HCl, pH 7.5), 0.2 μL *Taq* polymerase (5 U/μL), and 1 μL of each primer 139 and 141 (100 ng/μL) and 1 μL of the cell lysates (Hahn et al. 2007), with an initial denaturation at 96°C for 2 min, followed by 35 rounds of temperature cycling with denaturation at 96°C, primer annealing at 64°C, and elongation at 72°C, each for 30 s (Malorny et al. 2003). *Salmonella typhimurium* ATCC 14028 was used as a positive control. The PCR products were analyzed by gel electrophoresis on 2% agarose gels in tris-acetate–EDTA buffer after staining with ethidium bromide (0.5 μg/mL) (Sambrook et al. 1989).

For the characterization of salmonellae in water, feces, and the intestine samples, 100-μL subsamples of the second enrichments were plated on RVS agar (RVS solidified with 15 g agar/L). After incubation at 37°C for 16 h, 10 colonies were chosen haphazardly from each sample and incubated in LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C for 7 h (Sha et al. 2011). Cells from 100-μL subsamples as well as from a culture of the inoculated *Salmonella* strain S11 were pelleted by centrifugation and lysed in 100 μL of 50-mM NaOH as described above. End-point PCR as described above was used to identify isolates representing salmonellae, which were then further characterized by rep-PCR. Rep-PCR was performed in a total volume of 25 μL with primer BoxA1R (5′ CTA CGG CAA GGC GAC GCT GAC G) and 2 μL of lysate as described in Hahn et al. (2007). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989) and compared with that obtained with lysed cells of *Salmonella* strain S11.

All chemicals used in this study were purchased from Fisher Scientific (Pittsburgh, Pennsylvania) if not indicated otherwise.

RESULTS AND DISCUSSION

The number of DAPI-stained cells did not differ among treatments in water ($F_{2,6} = 4.7$, $P = 0.06$) or in feces ($F_{1,4} = 7.0$, $P = 0.94$). Across treatments, the number of DAPI-stained cells ranged between 0.4 and 3.8×10^5 cells/mL in water

TABLE 1. Mean (±SE in parentheses) number of DAPI-stained cells ($\times 10^2$) in 1 mL of water or 1 mg of Suckermouth Catfish feces (dry weight).

	Time										
	Hours							Days			
Medium	0	12	24	36	48	60	72	4	5	6	7
Treatment 1 (biofilm)											
Water	1,541	1,016	2,865	913	444	582	609	1,333	777	936	983
	(1,370)	(1,322)	(2,594)	(1,057)	(242)	(242)	(52)	(1,002)	(58)	(374)	(641)
Treatment 2 (biofilm, fish)											
Water	2,228	2,400	2,892	2,134	1,348	872	374	986	1,603	3,450	4,028
	(770)	(174)	(203)	(1,810)	(1,403)	(260)	(312)	(577)	(575)	(2,358)	(3,056)
Feces		23,376	31,627	21,961	46,627	34,150	31,544	45,955	46,276	41,233	153,610
		(25,407)	(22,492)	(2,666)	(28,794)	(33,694)	(24,068)	(9,229)	(14,799)	(31,914)	(51,476)
Treatment 3 (biofilm, fish, salmonellae)											
Water	979	3,813	3,130	3,367	987	1,293	3,092	1,870[a]	2,127[a]	2,865	1,681
	(140)	(2,717)	(2,064)	(1,976)	(278)	(483)	(1,665)	(722)	(1,613)	(373)	(968)
Feces		16,061	21,240	42,626	36,537	23,967	32,478	72,474	67,432	82,964	106,223
		(15,746)	(10,029)	(8,066)	(16,969)	(12,718)	(12,388)	(26,992)	(312)	(17,704)	(41,769)

[a]Values obtained from two aquariums instead of three (the aquariums with dead fish were excluded).

and between 1.6 and 10.6 $\times$ 10^6 cells/mg in feces (Table 1). While results for water samples demonstrated that neither fish nor inoculation noticeably affected the microbial community during the experiment, the interpretation of results for feces was more ambiguous since the accuracy of the results is affected by several methodological issues. Since samples from water and feces were not dispersed before application to slides (e.g., in 0.1% pyrophosphate buffer by sonication: Zarda et al. 1997) to avoid dilution of low numbers of salmonellae, accumulations of large numbers of cells on particulate material were observed (Figure 1). These affected within-sample variability during enumeration and thus resulted in large standard errors. This issue was more pronounced in feces samples where accurate enumeration was also affected by the small amounts of feces collected and the associated difficulties to accurately determine dry weights at different times, potentially resulting in an overestimation of cell numbers towards the end of the study. We were also unable to completely remove feces at each sampling, which could have resulted in growth of organisms in aging feces and thus in the detection of higher cell numbers of microbes towards the end of the study. As a consequence, we are unable to state whether the increase in numbers in feces through time is accurate or affected by our experimental setup and analyses.

In situ hybridization with probe Sal3 allowed us to visualize salmonellae in both water and feces samples from treatment 3 where *Salmonella* strain S11 was inoculated (Figure 1). Salmonellae could not be detected in intestine samples from fish harvested at the end of the study from treatment 3, and also not in any samples from treatments 1 and 2 that did not receive salmonellae (data not shown). Detection of salmonellae in samples from treatment 3 was achieved without any pretreatments to enhance cell permeability for probes (Zarda et al. 1997) or the addition of blocking reagents to reduce potential interference of background material (Hahn et al. 1997). However, due to the small number of *Salmonella* cells present, the analyses depended on our ability to concentrate cells from the original samples (i.e., cells from 500 mL of water concentrated in 1 mL of sample) and to avoid any further dilution during sample preparation for

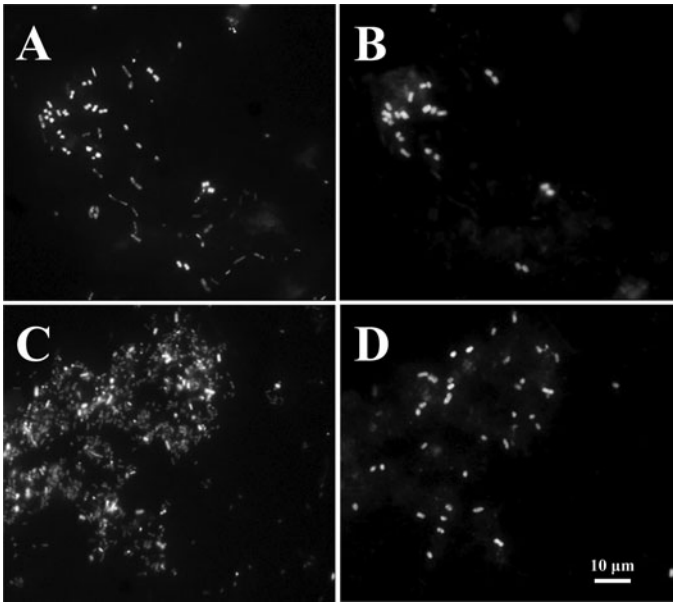

FIGURE 1. Detection of microbes (i.e., DAPI-stained cells) (left panel) and salmonellae (right panel) in (**A** and **B**) water and (**C** and **D**) Suckermouth Catfish feces samples by epifluorescence microscopy.

TABLE 2. Mean (±SE in parentheses) number of salmonellae ($\times 10^2$) in 1 mL of water or 1 mg of Suckermouth Catfish feces (dry weight). A dash (–) indicates cell number <100 cells.

	Time										
	Hours							Days			
Medium	0	12	24	36	48	60	72	4	5	6	7
Detection of salmonellae by in situ hybridization											
Water	107 (59)	52 (30)	14 (9)	16 (7)	8 (9)	6 (0)	1 (2)	5 (6)	4 (5)	–	–
Feces	[a]	221 (199)	543 (575)	2,608 (894)	753 (379)	523 (525)	161 (151)	278 (102)	72 (124)	77 (35)	37 (63)
Detection of salmonellae by *q*PCR											
Water	216 (100)	209 (81)	65 (28)	44 (24)	–	–	–	–	–	–	–
Feces	[a]	364 (120)	741 (845)	1,563 (2,303)	975 (1,001)	33 (51)	170 (265)	16 (25)	–	27 (40)	50 (84)

[a]Not sampled.

hybridization. Salmonellae were detected in water samples directly after setup, in numbers of about 10^4 cells/mL. Numbers decreased by two orders of magnitude within the first 72 h of the study and became undetectable after day 5 (Table 2).

In feces samples, numbers of salmonellae increased 10-fold during the first 36 h of the experiment from 2 to 26 $\times$ 10^4 cells/mg feces and then decreased gradually to about 100-fold at day 7 (Table 2). These findings were similar to our previous studies (Sha et al. 2013) and studies of others (Liang et al. 1982; Klein and Alexander 1986). Basic *Salmonella* population dynamic profiles obtained by in situ hybridization were similar to profiles obtained by *q*PCR analysis (Table 2) in water (r = 0.92) and feces (r = 0.89). Thus, while cell numbers of the entire microbial community were either stable or slightly increased during the experiment in water and feces, respectively, numbers of *Salmonella* decreased rapidly in time. In water samples, *Salmonella* cells were initially abundant, comprising up to 20% of the microbial community, but decreased in relative abundance by one order of magnitude within 12 and 24 h (Figure 2). In feces, *Salmonella* cells comprised up to 6% of the microbial community within the first 48 h before decreasing within 60 h (Figure 2). These results suggest the selective removal of *Salmonella* from these samples, which may be a function of predation as indicated in previous studies on salmonellae inoculated into natural or sterilized lake water (Liang et al. 1982) or other bacteria such as *E. coli*, *Pseudomonas*, or *Klebsiella pneumoniae* (Scheuerman et al. 1988).

Neither in situ hybridization nor *q*PCR analysis did detect salmonellae in intestine samples of fish harvested at the end of the study. However, end-point PCR after semiselective enrichment of salmonellae detected them in intestine samples of all fish from treatment 3 that had received tiles with biofilms inoculated with *Salmonella* strain S11. Rep-PCR patterns of all isolates obtained from these intestines, and also from water and feces samples collected at days 3 and 7, resembled that of strain S11 indicating that this strain was consumed and shed by the catfish. Intestine contents from fish of treatment 2, which had received tiles with biofilms free of salmonellae, were all negative for the *invA* gene. In addition, salmonellae

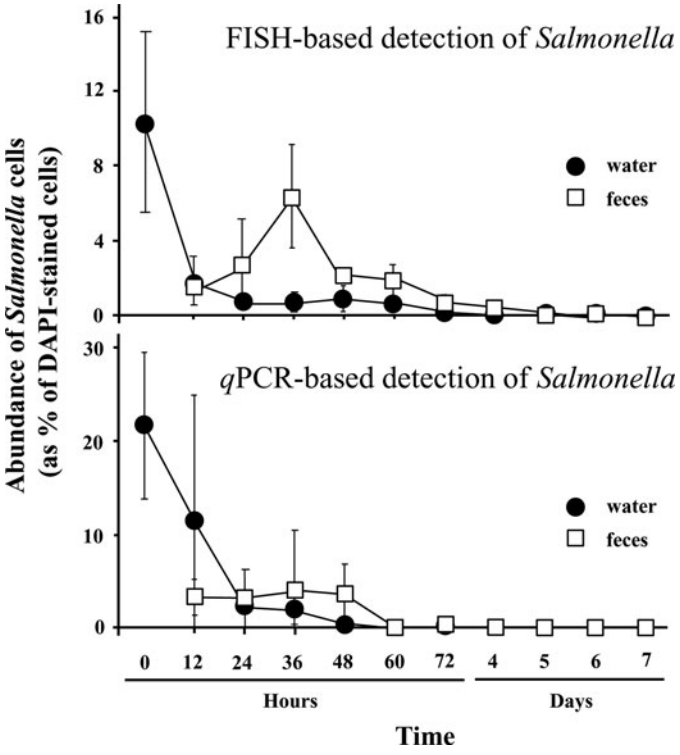

FIGURE 2. Change in abundance of salmonellae, as percentage of all organisms (i.e., DAPI-stained cells), in water and Suckermouth Catfish feces samples from treatment 3, analyzed by in situ hybridization (i.e., FISH-based detection) and by *q*PCR (i.e., *q*PCR-based detection) in time.

could not be isolated from the intestines, or from water and feces samples. These results are in agreement with those of our previous study (Gaertner et al. 2008b), where we had shown that salmonellae in the intestine of fish were normally associated with particulate material in highly variable numbers. This suggests that salmonellae are not components of the indigenous microbial community in fish intestines but are taken up with particulate material including biofilms. While our experimental setup demonstrates that *Salmonella* can be taken up with food sources such as biofilms and be released into feces, it does not allow us to make strong statements about quantitative aspects of this transfer. *Salmonella* in feces could also originate in part from biofilms with cells becoming detached from the biofilms as demonstrated in other studies (Ksoll et al. 2007), released into water, and then adsorbed onto fecal material.

Fish and other aquatic organisms have been documented as potential vectors for human pathogens for many years (Metz 1980; Minette 1986; Chattopadhyay 2000; Fell et al. 2000; Hansen et al. 2008). Infections with salmonellae were generally related to the consumption of fish (Novotny et al. 2004), but they could also come from the environment contaminated by fish. Aquarium water, for example, was the source of salmonellosis in a child (Senanayake et al. 2004). Persistence and dissemination of salmonellae in fish were dependent on the number of salmonellae administered to the fish with high numbers required for their detection in intestines or muscles of the fish 4 weeks after administration (Buras et al. 1985; Nesse et al. 2005). In our previous study (Sha et al. 2013), we demonstrated a fast decline of salmonellae in biofilms in time, which could be the basis for low percentages of salmonellae in both water and feces samples towards the end of the study, and also could explain the necessity to enrich for salmonellae cells so that they can be detected in low numbers in the intestine. Although fish seem to be able to take up salmonellae through their food resources and shed them through their feces into the environment, numbers of salmonellae after gut passage depend on their abundance in the original food resources and are not biomagnified during passage, and thus, fish only provide a means for translocation of this pathogen. Interestingly, our findings are consistent with another pathogen (i.e., *E. coli*) that is consumed and transferred by fish but not biomagnified within the intestine (Hansen et al. 2008). Consequently, fish are vectors for some microbial pathogens but are not contributing to biomagnification of pathogens within the aquatic communities.

ACKNOWLEDGMENTS

The authors are grateful for support from Texas State University, Department of Biology, Alexander–Stone Endowment, National Science Foundation (IOB-0615762; administered by D. Garcia, Texas State University), and the state of Texas through the American Recovery and Reinvestment Act. The study was performed in compliance to the rules overseen by the Texas State Institutional Animal Care and Use Committee (permits 0721-0530-7 and 05-05C38ADFDB) and Texas Parks and Wildlife Department (permit SPR-0601-159).

REFERENCES

Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology 172:762–770.

Baloda, S. B., L. Christensen, and S. Trajcevska. 2001. Persistence of a *Salmonella enterica* serovar Typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. Applied and Environmental Microbiology 67:2859–2862.

Beach, J. C., E. A. Murano, and G. R. Acuff. 2002. Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. Journal of Food Protection 65:1687–1693.

Bocek, A. J., Y. J. Brady, and W. A. Rogers. 1992. Exposure of Silver Carp, *Hypophthalmichthys molitrix*, to *Salmonella typhimurium*. Aquaculture 103:9–16.

Buras, N., L. Duek, and S. Niv. 1985. Reactions of fish to microorganisms in wastewater. Applied and Environmental Microbiology 50:989–995.

Byappanahalli, M. N., R. Sawdey, S. Ishii, D. A. Shively, J. A. Ferguson, R. L. Whitman, and M. J. Sadowsky. 2009. Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. Water Research 43:806–814.

Chattopadhyay, P. 2000. Fish: catching and handling. Pages 806–813 *in* R. K. Robinson, editor. Encyclopedia of food microbiology. Academic Press, New York.

Côté, C., and S. Quessy. 2005. Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. Journal of Food Protection 68:900–905.

Fang, Q., S. Brockmann, K. Botzenhart, and A. Wiedenmann. 2003. Improved detection of *Salmonella* spp. in foods by fluorescent *in situ* hybridization with 23S rRNA probes: a comparison with conventional culture methods. Journal of Food Protection 66:723–731.

Fell, G., O. Hamouda, R. Lindner, S. Rehmet, A. Liesegang, R. Prager, B. Gericke, and L. Petersen. 2000. An outbreak of *Salmonella blockley* infections following smoked eel consumption in Germany. Epidemiology and Infection 125:9–12.

Gaertner, J. P., T. Garres, J. C. Becker, M. L. Jimenez, M. R. J. Forstner, and D. Hahn. 2009. Temporal analyses of Salmonellae in a headwater spring ecosystem reveals the effects of precipitation and runoff events. Journal of Water and Health 7:115–121.

Gaertner, J. P., D. Hahn, J. Jackson, M. R. J. Forstner, and F. L. Rose. 2008a. Detection of Salmonellae in captive and free-ranging turtles using enrichment culture and polymerase chain reaction. Journal of Herpetology 42:223–231.

Gaertner, J. P., D. Hahn, F. L. Rose, and M. R. J. Forstner. 2008b. Detection of Salmonellae in different turtle species within a headwater spring ecosystem. Journal of Wildlife Diseases 44:519–526.

Gaertner, J. P., J. A. Mendoza, M. R. J. Forstner, and D. Hahn. 2011. Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. Journal of Water and Health 9:458–466.

Gaertner, J. P., P. E. Wheeler, S. Obafemi, J. Valdez, M. R. J. Forstner, T. H. Bonner, and D. Hahn. 2008c. Detection of Salmonellae from fish in a natural river system. Journal of Aquatic Animal Health 20:150–157.

Gopinath, S., S. Carden, and D. Monack. 2012. Shedding light on *Salmonella* carriers. Trends in Microbiology 20:320–327.

Hahn, D., J. P. Gaertner, M. R. J. Forstner, and F. L. Rose. 2007. High-resolution analysis of Salmonellae from turtles within a headwater spring ecosystem. FEMS Microbiology Ecology 60:148–155.

Hahn, D., K. Zepp, and J. Zeyer. 1997. Whole cell hybridization as a tool to study *Frankia* populations in root nodules. Physiologia Plantarum 99:696–706.

Hansen, D. L., J. J. Clark, S. Ishii, M. J. Sadowsky, and R. E. Hicks. 2008. Sources and sinks of *Escherichia coli* in benthic and pelagic fish. Journal of Great Lakes Research 34:228–234.

Heuschmann-Brunner, G. H. 1974. [Experiments on the possibilities and course of infections with *Salmonella enteritidis* and *Salmonella typhimurium* in fresh water fish]. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, Serie B 158:412–431. (In German.)

Humphrey, T. 2000. Public health aspects of *Salmonella* infection. Pages 245–263 *in* C. Wray and A. Wray, editors. Salmonella in domestic animals. CABI Publishing, Wallingford, UK.

Ishii, S., T. Yan, D. A. Shively, M. N. Byappanahalli, R. L. Whitman, and M. J. Sadowsky. 2006. *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. Applied and Environmental Microbiology 72:4545–4553.

ISO (International Organization for Standardization). 1993. ISO 6579: detection of Salmonellae (reference method). ISO, Geneva, Switzerland.

Janssen, W. A., and C. D. Meyers. 1968. Fish: serologic evidence of infection with human pathogens. Science 159:547–548.

Klein, T. M., and M. Alexander. 1986. Bacterial inhibitors in lake water. Applied and Environmental Microbiology 52:114–118.

Ksoll, W. B., S. Ishii, M. J. Sadowsky, and R. E. Hicks. 2007. Presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior. Applied and Environmental Microbiology 73:3771–3778.

Lawton, R. L., and E. V. Morse. 1980. *Salmonella* survival in freshwater and experimental infections in Goldfish (*Carassius auratus*). Journal of Environmental Science and Health 15:339–358.

Lewis, D. H. 1975. Retention of *Salmonella typhimurium* by certain species of fish and shrimp. Journal of the American Veterinary Medical Association 167:551–552.

Liang, L. N., J. L. Sinclair, L. M. Mallory, and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Applied and Environmental Microbiology 44:708–714.

Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth. 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Applied and Environmental Microbiology 69:290–296.

Metz, H. 1980. [Water as a vector of infection: waterborne bacteria]. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, Serie BB 172:255–274. (In German.)

Minette, H. P. 1986. Salmonellosis in the marine environment: a review and commentary. International Journal of Zoonoses 13:71–75.

Murray, C. J. 1991. Salmonellae in the environment. Revue Scientifique et Technique Office International des Epizooties 10:765–785.

Nesse, L. L., T. Løvold, B. Bergsjø, K. Nordby, C. Wallace, and G. Holstad. 2005. Persistence of orally administered *Salmonella enterica* serovars Agona and Montevideo in Atlantic Salmon (*Salmo salar* L.). Journal of Food Protection 68:1336–1339.

Nordentoft, S., H. Christensen, and H. C. Wegener. 1997. Evaluation of a fluorescence-labelled oligonucleotide probe targeting 23S rRNA for in situ detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears. Journal of Clinical Microbiology 35:2642–2648.

Novotny, L., L. Dvorska, A. Lorencova, V. Beran, and I. Pavlik. 2004. Fish: a potential source of bacterial pathogens for human beings. Veterinarni Medicina (Prague) 49:343–358.

Pal, D., and C. K. Dasgupta. 1991. Interaction of some city sewage bacteria with an Indian major carp, *Cirrhinus mrigala*. Journal of Aquatic Animal Health 3:124–129.

Pound, K. L., W. H. Nowlin, D. G. Huffman, and T. H. Bonner. 2011. Trophic ecology of a nonnative population of Suckermouth Catfish (*Hypostomus plecostomus*) in a central Texas spring-fed stream. Environmental Biology of Fishes 90:277–285.

Refsum, T., K. Handeland, D. L. Baggesen, G. Holstad, and G. Kapperud. 2002. Salmonellae in avian wildlife in Norway from 1969 to 2000. Applied and Environmental Microbiology 68:5595–5599.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scheuerman, P. R., J. P. Schmidt, and M. Alexander. 1988. Factors affecting the survival and growth of bacteria introduced into lake water. Archives of Microbiology 150:320–325.

Senanayake, S. N., M. J. Ferson, S. J. Botham, and R. T. Belinfante. 2004. A child with *Salmonella enterica* serotype Paratyphi B infection acquired from a fish tank. Medical Journal of Australia 180:250.

Sha, Q., A. Gunathilake, M. R. J. Forstner, and D. Hahn. 2011. Temporal analyses of the distribution and diversity of *Salmonella* in natural biofilms. Systematic and Applied Microbiology 34:353–359.

Sha, Q., D. A. Vattem, M. R. J. Forstner, and D. Hahn. 2013. Quantifying *Salmonella* population dynamics in water and biofilms. Microbial Ecology 65:60–67.

Vassiliadis, P., V. Kalapothaki, D. Trichopoulos, C. Mavrommatti, and C. Serie. 1981. Improved isolation of Salmonellae from naturally contaminated meat products by using Rappaport-Vassiliadis enrichment broth. Applied and Environmental Microbiology 42:615–618.

Woodward, D. L., R. Khakhria, and W. M. Johnson. 1997. Human salmonellosis associated with exotic pets. Journal of Clinical Microbiology 35:2786–2790.

Zarda, B., D. Hahn, A. Chatzinotas, W. Schönhuber, A. Neef, R. I. Amann, and J. Zeyer. 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Archives of Microbiology 168:185–192.