DETECTION AND DISTRIBUTION OF SALMONELLAE IN THE INTESTINE OF WARMOUTH (*LEPOMIS GULOSUS*)

THESIS

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by

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DETECTION AND DISTRIBUTION OF SALMONELLAE IN THE INTESTINE OF WARMOUTH (*LEPOMIS GULOSUS*)

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

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Members of the genus *Salmonella* are enteric bacteria that can cause salmonellosis (a disease associated with the gastrointestinal tract, yielding diarrheal symptoms) in both humans and animals. Salmonellosis continues to be a problem for industrialized countries such as the United States where consumption of contaminated, cooked or uncooked food is generally assumed to be the cause of infection with salmonellae. The intestinal tract of vertebrates is generally assumed to be the native habitat of salmonellae from which the feces then contaminate environments such as fresh- or marine waters, estuarine environments, vegetables, compost, or soils and sediments. The occurrence of salmonellae in these environments is therefore frequently linked to environmental

contamination through, e.g., manure or wastewater discharges. Salmonellae, however, have also been detected in water and biofilms in pristine aquatic systems, and in the intestine of animals such as turtles, crayfish and fish living in these systems. 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 this failure 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. In this thesis, I re-evaluated the detection of salmonellae in the intestine of fish, with the specific focus on warmouth (*Lepomis gulosus*). This evaluation was performed as a seasonal study in order to test three hypotheses:

1. Salmonellae can be detected infrequently in the intestine of warmouth, with their detection most likely linked to terrestrial runoff during rainfall events
2. Salmonellae are not part of the indigenous intestinal flora, but their presence is associated to food sources
3. Food sources generally promote bacterial cell growth in the intestine, with fast-growing copiotrophic bacteria (including salmonellae) dominating the flora in the intestine of warmouth.

The study took advantage of the availability of molecular tools, i.e. PCR, and *in situ* hybridization that allowed detection and quantification of salmonellae directly in the environmental samples (i.e. the intestine content). Salmonellae were detected randomly and associated to food sources in the intestine content of individual fish indicating that they were not established members of the indigenous flora, but rather pass through the intestine with the food. Detection of salmonellae was related to precipitation profiles, as

much higher precipitation during fall compared to the remainder of the season resulted in their detection. Cell size of salmonellae associated with food material in the intestine was larger than in pure culture, but reflected cell sizes of the major groups of bacteria present that were dominated by members of the fast-growing copiotrophic Proteobacteria and the CFB phylum.

I. INTRODUCTION

Salmonellosis is a gastrointestinal disease, causing diarrheal symptoms, that is caused by members of the bacterial genus *Salmonella*, and that continues to be a concern for citizens within the United States. It affects 1.4 million people annually, causing 16,000 hospitalizations and over 500 deaths (41). The economic costs associated with these incidents are estimated to be around 3 billion dollars annually (http://www.who.int/mediacentre/factsheets/fs139/en/). In 2006 alone, there were 121 outbreaks, resulting in 3,300 reported cases of illness to the CDC Foodborne Outbreak Reporting System (6). It is well known that uncooked food, cross-contaminated vegetables and ready-to-eat products may serve as an avenue for infection of humans (45). Infection of humans by salmonellae can also result from contact with animals that harbor salmonellae in their intestine (50). One way salmonellae enter the environment from a host is typically through fecal excretion from animals (14). Upon entrance into the environment, they can persist for long periods of time (7). Many salmonellae boast adaptable attributes, thriving in the gut of many animals (22), as well as surviving in other environments such as minute weather pools atop large granite formations, seawater along the shoreline of beaches, within the eggs of infected chickens and even in homemade mayonnaise (22, 24, 47, 60, 61).

Studies have also detected salmonellae within moving freshwater systems (i.e. rivers and streams) in solitary regions free from human fecal contamination (8). Certain

species of salmonellae were found to inhabit the intestines of freshwater fish (16), however, it was speculated that salmonellae were associated to particulate matter ingested by fish, therefore were not justly associated with the autochthonous microflora. Salmonellae are not known to be autochthonous members of the microflora of the intestine of farmed fish, and consequently their detection in these fish indicates a lack of sanitation within the stock tanks (11). It has been suggested that fish might be a potential carrier involved in the dissemination of salmonellae in aquatic environments (34). This suggestion is based on evidence indicating that salmonellae might be capable of reproducing in the intestines of fish, and are subsequently shed into the aquatic system through released feces of the fish (35). Although there is a plethora of studies that have been performed detecting salmonellae in aquacultured, raw fermented, and frozen fish (5, 43, 46), there are only few studies concerning the presence of salmonellae in wild freshwater fish.

Studies analyzing the microbial community structure of both marine and freshwater fish contained in aquaculture, although short in supply, have been performed in the past using molecular tools such as denaturing gradient gel electrophoresis (DGGE) (28, 37, 67, 68, 69, 70). These studies suggest that most of the intestinal microflora of fish belong to the phylum Proteobacteria. Proteobacteria of the α-, β-, and γ-subdivisions as well as those of the CFB phylum are often fast-growing copiotrophic bacteria that are found in nutrient-rich environments (53). All studies presented so far have focused on marine or captive (i.e. farmed) fishes, with little information available on the microbial community in the intestines of wild freshwater fish. Also, all studies generally provide some basic information about bacterial community structure only, without assessments of

abundance or the biomass (e.g. length, volume, etc.) and thus the significance of the bacteria in the intestine.

In order to expand the current knowledge concerning the interaction of salmonellae, the intestinal microbial community and wild fish, I re-evaluated the detection of salmonellae in the intestine of fish, with the specific focus on warmouth (*Lepomis gulosus*) as a seasonal study in order to test three hypotheses:

1. Salmonellae can be detected infrequently in the intestine of warmouth, with their detection most likely linked to terrestrial runoff during rainfall events
2. Food sources generally promote bacterial cell growth in the intestine, with fast-growing copiotrophic bacteria (including salmonellae) dominating the flora in the intestine of warmouth
3. Salmonellae are not part of the autochthonous intestinal flora, but their presence is associated to food sources

The study took advantage of the availability of molecular tools, i.e. PCR, and *in situ* hybridization that allowed detection and quantification of salmonellae directly in the environmental samples (i.e. the intestine content). Preliminary work using these tools has shown the presence of salmonellae in the posterior part of the intestine of three warmouth (*Lepomis gulosus*), caught as bycatch in turtle traps set in the slough arm of Spring Lake, San Marcos, TX, during the Summer of 2008. Warmouth were therefore the fish of choice for the current study. Salmonellae were detected in these three fish only in the posterior part of the intestine suggesting a site-specific interaction. I challenged this finding as artifact, i.e. a by chance detection in a small number of fish, and tested the hypothesis that salmonellae are not part of the normal flora associated with the intestine

of fish, but are rather taken up by chance with the food of the fish. As a consequence, salmonellae should not be distributed evenly in the intestine of the fish or present at a specific site in the intestine only, but should be associated with organic material and pass through the intestine without establishing themselves as a permanent member of the intestine flora. Since uptake of salmonellae with food by fish might be a function of run-off, and thus seeding of the water and sediments after rainfall, the investigations had a seasonal component with analyses of the intestine of fish caught, roughly, once a month starting in December 2009, for a period of one year. These data expanded an already ongoing seasonal sampling scheme that included the four months before (i.e. August to November 2009).

II. METHODS

Fish sampling - All fish were caught in the slough portion of Spring Lake, San Marcos, TX (Nº29.893653', Wº97.927596) (Fig. 1). Two sites were used as indicated by the asterisks; however the site closest to Sink Creek was used for most of the study that was performed in compliance to the rules overseen by the Texas State Institutional Animal Care and Use Committee (IACUC, permits 0721-0530-7 and 05-05C38ADFDB), and with sampling authority from the Texas Parks and Wildlife Department (TPWD, permit SPR-0601-159). All fish were caught using hoop net turtle traps. Roughly, about 5-10 fish were caught every month for one year, which was initiated in December 2009.

Sample preparation - All fish were pithed, and 1-cm segments were taken from the intestine of each fish, starting from the end closest to the stomach. Subsequently, each segment was transferred to 1 mL of double distilled water (ddH_2O) in an Eppendorf tube which was then shaken by hand for 20 seconds to release and disperse the gut content (further referred to as H_2O samples). Washed gut segments were transferred to new tubes containing 1 mL of 0.1% sodium pyrophosphate (further referred to as PYRO samples) and sonicated in a ultrasonic cleaner (FS20, Fisher Scientific, Houston TX) for 5 min. By doing this, we aimed to make a distinction between cells that were bound to the intestinal walls of warmouth (PYRO samples) and those that were just passing through (H_2O samples). Four sub-samples were taken from each sample type (H_2O/PYRO): 400 μL for *in situ* hybridization, 100 μL for semi-selective enrichment followed by end-point PCR,

250 µL for *q*PCR, and a second 250 µL for freezer storage (all in 1.5 mL Eppendorf tubes). For June and July 2010 samples, only one 250 µL sample was taken and used for quantitative polymerase chain reaction (*q*PCR) analyses.

End-point PCR - End-point PCR was performed following the procedure outlined in previous studies (16). Briefly, the 100 µL sub-sample was transferred to a 2 mL cryotube containing 1 mL of Buffered Peptone Water (BPW) (l^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO4, 1.5 g KH_2PO_4, pH 7.2) and incubated at 37ºC. After 24 hs of incubation, 100 µL of these samples were transferred to a 2 mL cryotube containing 1mL of Rappaport-Vassiliadis Enrichment Broth (RVS) (l^{-1}: 4.5 g peptone (soymeal), 29 g $MgCl_2$ x 7 H_2O, 8 g NaCl, 0.4 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) and incubated at 37ºC for 48 hs. Sub-samples (100 µL) of this semi-specific enrichment for salmonellae were transferred to new tubes with RVS, and salmonellae were enriched a second time as stated above.

For PCR analyses, 100 µL samples of this second enrichment was transferred to a sterile 1.5 mL Eppendorf tube, and cells were pelleted by centrifugation for 2 min at 14,000 x g. The cell pellet was washed with 500 µL of sterile ddH_2O once, and subsequently lysed in 100 µL of 50 mM NaOH by incubation at 65ºC for 15 min with shaking. Lysed cells were kept at -20ºC until use.

In situ hybridization - The 400 µL sub-samples of both H_2O and PYRO intestine samples were mixed with 1 mL of 4% paraformaldehyde (PFA) and fixed overnight at 4ºC. Samples were then centrifuged at 14,000 x g for 30 seconds and the supernatant was discarded afterwards. The remaining pellets were washed twice with 500 µL of 1 x

phosphate buffered saline (PBS) (0.13 M NaCl, 7 mM $Na_2H_2PO_4$, pH 7.2) and centrifuged for 30 seconds at 14,000 x g. Cell pellets were resuspended in 100 µL of 50% ethanol in 1 x PBS. Samples were stored at -20ºC until further use.

Quantitative PCR - One of the 250 µL sub-samples taken from the intestine was centrifuged at 14,000 x g for 2 min. After the supernatant was removed, cells were lysed by the addition of 250 µL of 50 mM NaOH and incubation at 65ºC for 15 min with shaking. Samples were stored at -20ºC until needed.

Freezer storage - Aliquoted sub-samples that were not immediately used for the above-mentioned analyses were centrifuged at 14,000 x g for 30 seconds, the supernatants discarded, and the pellets subsequently stored at -80ºC.

Sample analyses - All analyses were performed in the Biology Department at Texas State University-San Marcos, TX. A PTC-200 Thermocycler (Bio-Rad, Hercules, CA) was used for all end-point PCR analyses, a Eppendorf Mastercycler ep Realplex thermocycler was used for all *q*PCR analyses, and a Nikon Eclipse 80i© microscope equipped with a Photometric Cool Snaps ES2© camera was used to view and capture images from all *in situ* hybridization analyses.

End-point PCR - The presence of *Salmonella* spp. was shown using 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 salmonellae (31, 58). Detection of the *inv*A gene has been suggested as the international standard diagnostic method for quality assurance laboratories in epidemiological studies on *Salmonella* spp. (38). A single microliter of lysate was used as template for PCR amplification with

primers 139 (5'-GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 (5'-TCA TCG CAC CGT CAA AGG AAC C) (49) in a final volume of 50 µL containing 10 × PCR buffer (500 mM KCl, 25 mM $MgC1_2$, 200 mM Tris/HCI, pH 8.4, 0.1% Triton 100), 1 µL dNTPs (each 10 mM in 10 mM Tris/HCI, pH 7.5), 0.2 µL *Taq* polymerase (5 U μl^{-1}), and 1 µL of each primer (100 ng μL^{-1}). After an initial 2 minute denaturation at 96°C, 35 rounds of temperature cycling were performed in a PTC-200 thermocycler with denaturation at 96°C, primer annealing at 54°C, and elongation at 72°C, each for 30 seconds (36). Following this step was incubation at 72°C for 7 min (26). Lysates of *Salmonella enterica* Typhimurium (ATCC 14028) as well as sterilized ddH_2O were used as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 µL mL^{-1}) (54).

In situ hybridization - Samples were prepared for analyses as follows: a small amount (1-10 µL) of sample was added to an 8-well, 8 mm HTC Super Cured slide coated with gelatin, and air dried at 42ºC for 20 min. Next, slides underwent 3 minute dehydrations in 50%, 75%, and 95% ethanol in water, respectively, after which they were air-dried. For the detection of salmonellae, samples were hybridized by adding 9 µL of a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) with 15% formamide, 1 µL of a Cy3-labeled oligonucleotide probe Sal3 (25 ng μL^{-1}) (Table 1), and 1 µl of a solution of DAPI in ddH_2O (200 ng μl^{-1}) at 42°C for 2 hs (66). Probe Sal3 ($^{5'}$AAT CAC TTC ACC TAC GTG, *E. coli* position 1713-1730) (44) binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (excepting only subspecies IIIa), but should not detect *S. bongori* (13). Following hybridization, slides were briefly

washed with ddH_2O and placed into a 50 mL Falcon tube with buffer containing 20 mM Tris/HCl, pH 7.2, 10 mM EDTA, 0.01% SDS and 440 mM NaCl for 20 min at room temperature. Slides were briefly washed again with ddH_2O and air-dried. Slides were mounted with Citifluor AF1 solution (Citifluor Ltd., London, UK) and examined with a Nikon Eclipse 80*i* microscope, fitted for epifluorescence microscopy with a mercury lamp (X-Cite™ 120; Nikon) and filter cubes UV-2E/C (Nikon; EX340-380, DM400, BA4435-485, for DAPI detection) and CY3 HYQ (Nikon; EX535/50, DM565, BA610/75, for Cy3 detection), respectively. Bacteria were counted at 1000 x magnification. Forty fields, selected at random, covering an area of 0.01 mm^2 were examined from a sample distributed over eight circular areas of 53 mm^2 each. DAPI and Cy3 counts were obtained from the same image. Pictures were taken from these images using a cooled CCD camera (CoolSNAP *ES*^{2}; Photometrics, Tucson AZ), and Nikon's NIS Elements imaging software (Version 3).

In addition to salmonellae, other bacteria were analyzed using the same procedure described above, except that formamide concentrations were adjusted for each probe targeting a specific bacterial group (Table 1). Bacterial counts and biomass measurements (cylindrical volume, length, and diameter) were taken using the program NIS Elements and figures for distribution were prepared using JMP 8.

Quantitative PCR - Quantitative PCR (*q*PCR) is about 100 x more sensitive than end-point PCR, and was used in an attempt to quantify salmonellae directly in intestine samples without prior enrichment (65). A single microliter of lysate was used as template for *q*PCR amplification, using 0.2 µL of primers 139 and 141 in a final volume of 20 µL containing 10 µL of Perfecta SYBR Green FastMix for iQ (2 x reaction buffer containing

optimized concentrations of $MgCl_2$, dNTPs, AccuFast Taq DNA Polymerase, SYBR Green I dye, 20 nM fluorescein, and stabilizers) (Quanta Biosciences, Foster City, CA), and 8.6 µL of sterile ddH_2O. Following an initial 30 seconds denaturing step at 95ºC, 35 cycles of temperature cycling were performed with denaturation at 95°C, primer annealing at 64°C, and elongation at 72°C, each for 30 seconds. After amplification, a melting curve analysis was performed in order to verify the specificity of the reaction and to determine whether the curve generated was significant.

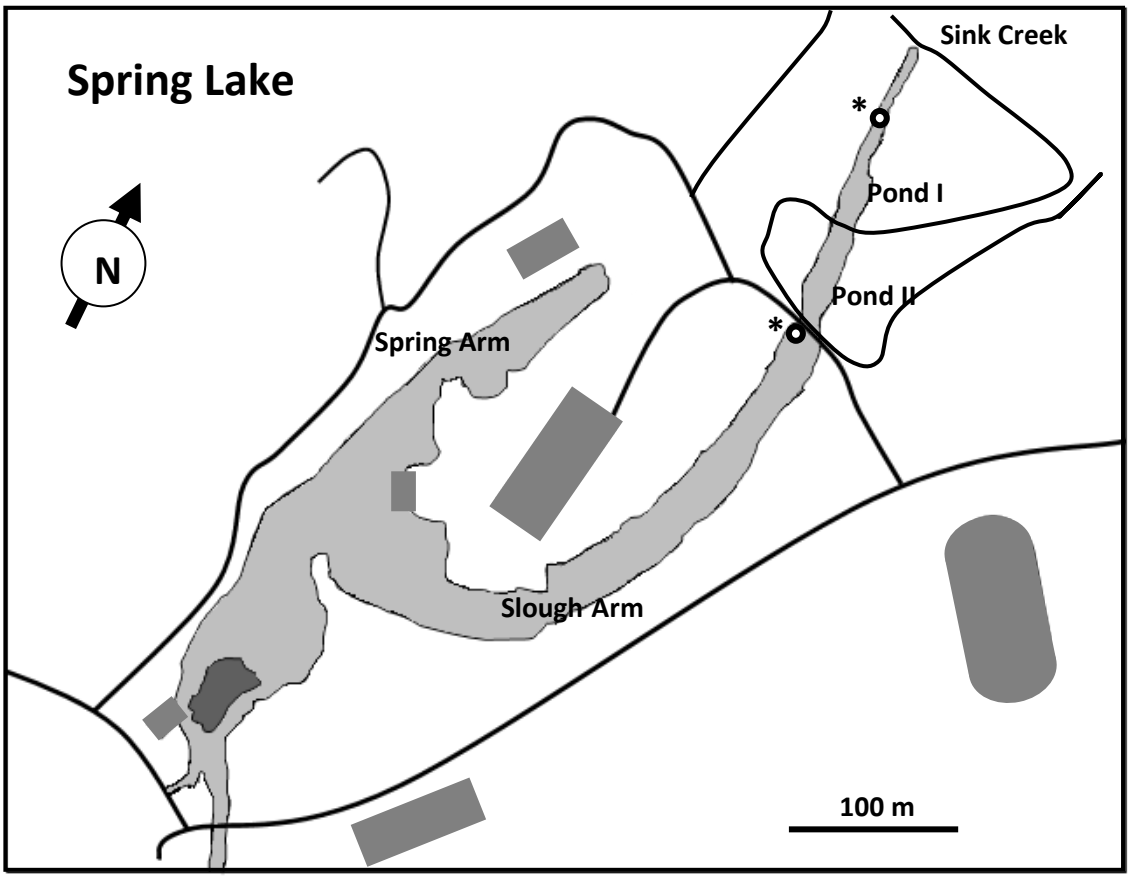

FIGURE. 1. Map of Spring Lake, San Marcos, TX (Nº29.893653', Wº97.927596). Positions with an asterisk (*) indicate areas where fish were caught.

TABLE 1: Oligonucleotide probes used for the classification of the basic community structure of the intestine of freshwater fish.[1]

Sequence (5'-3') Formamide (**%**)	rRNA Target Position	Probe	Specificity	Reference
GCTGCCTCCCGTAGGACT **30**	16S, 338-355	Eub338 I, II, III	Bacteria	Daims et al. 1999
ACCAGACTTGCCCTCC **20**	18S, 502-516	Euk516	Eukarya	Amann et al. 1990
GTGCTCCCCCGCCAATTCCT **20**	16S, 915-934	Arch915	Archaea	Stahl & Amann 1991
CGTTCGYTCTGAGCCAG **10**	16S, 19-35	ALF1b	α-subdivision of Proteobacteria	Manz et al. 1992
GCCTTCCCACTTCGTTT **30**	23S, 1027-1043	BET42a	β- subdivision of Proteobacteria	Manz et al. 1992
GCCTTCCCACATCGTTT **30**	23S, 1027-1043	GAM42a	γ- subdivision of Proteobacteria	Manz et al. 1992
CGGCGTTGCTGCGTCAGG **35**	16S, 385-402	SRBDb	δ- subdivision of Proteobacteria	Rabus et al. 1996
CGGCGTCGCTGCGTCAGG **35**	16S, 385-402	SRB385	δ- subdivision of Proteobacteria	Amann et al. 1990
TATAGTTACCACCGCCGT **20**	23S, 1901-1918	HGC69a	Gram-positive with high-G+C	Roller et al. 1994
YSGAAGATTCCCTACTGC **35**	16S, 354-371	LGCa, b, c	Gram-positive with low-G+C	Meier et al. 1999
TGGTCCGTGTCTCAGTAC **35**	16S, 319-336	CF319a	*Cytophaga-Flavoacterium* cluster of the CFB phylum	Manz et al. 1996
GTGCATCCACTTCACTAA **15**	23S, 1713-1730	Sal3	*Salmonella spp.*	Nordentoft et al. 1997

[1]target site on an insertion not present in *E. coli*; S = G or C; Y = C or T

III. RESULTS

Seasonal detection of salmonellae - Throughout the sampling period of December 2009 - November 2010 (as well as in the samples obtained the previous 4 months), salmonellae were only detected by end-point PCR after semi-selective enrichment, and here only during the months of September – November 2009 and September and October 2010. Figure 2 shows detection of salmonellae was restricted to months with rainfall values exceeding 100 mm, while Figures 3 and 4 show detailed sampling dates corresponding to precipitation within months fish caught were positive for salmonellae in their intestine.

Microbial community analysis - The microbial community structure was analyzed using oligonucleotide probes that detect specific sequences within the 16S or 23S rRNA (Table 1). Using the samples from the first 10 warmouth (i.e. those caught before and after rainfall in August – October 2009, respectively), all organisms that were detected belonged to the Domain Bacteria, while probes targeting the Domains Archaea and Eukarya did not result in any detections. Within the Domain Bacteria, the major bacterial groups were analyzed. All probes detected bacteria in at least one segment sample, however, only 5 of the 9 probes (i.e. EUB338, ALF1b, BET42a, GAM42a, and CF319a) consistently detected bacteria while the others occasionally or rarely resulted in the visualization of the respective target bacteria (Tables 2-6). Overall, between the gut content (H_2O samples) and lining (PYRO samples), there were no discernable differences

in abundance or community structure in the sequence of intestine section samples (Table 2). Bacterial groups seemed to be randomly distributed and not associated to one particular section of the intestine.

Biovolume determination showed no obvious differences in cell size distribution between Bacteria and bacterial groups in different intestine segments of each warmouth, and also not between bacteria from the gut content (Table 5) and the lining (Table 6). Probe EUB338 showed the most diversity with respect to bacterial biovolumes (Fig. 5). Using the probes we were able to observe different size classes among the bacteria ranging from less than 1 μm^3 up to around 5 μm^3, with about 50% being smaller than 1 μm^3 and the majority of the remaining cells being smaller than 2 μm^3. Figure 5 gives examples of the range of bacterial biovolumes observed using some of the probes.

Salmonellae detection - Semi-selective enrichment of salmonellae was required to detect salmonellae in intestine sections of warmouth. Without enrichment, none of the samples analyzed was positive for the *inv*A gene fragments indicative for the presence of salmonellae. Data obtained by end-point PCR after semi-selective enrichment of salmonellae for the first 10 warmouth, i.e. 5 fish caught before and 5 caught after rainfall events (i.e. August – October 2009), demonstrated the presence of salmonellae in the intestine of fish caught after rainfall only, with no detection of salmonellae in fish caught before rainfall. In the intestine of fish caught after rainfall, end-point PCR detected salmonellae in the gut content (i.e. H_2O samples) of up to 75% of the intestine sections of individual fish, with low-end numbers of 33% (Fig. 8). For PYRO samples (i.e. samples representing organisms meant to be attached to the intestine wall), as low as 17% and as high as 100% of the intestine sample sections were positive for salmonellae (Fig. 8). Of

the ten fish caught during September and October 2010, all had at least one segment positive for salmonellae in H_2O samples (i.e. the gut content), and 60% of the fish were positive for salmonellae in at least one PYRO sample. Finally, like the previous year, detection of salmonellae was not observed after November. It should be noted on the 19th of November 2009, one bluegill (*Lepomis macrochirus*) was positive for salmonellae (75% in H_2O samples and 25% in PYRO samples) and all other fish caught after this date were negative for salmonellae.

In situ hybridization on fixed intestine samples of the first 10 warmouth of the study (without prior enrichment) demonstrated the presence of salmonellae in three intestine samples only (i.e. H_2O samples), with no detection in PYRO samples (Fig. 8). Positive detection of salmonellae by *in situ* hybridization matched samples with positive detection by end-point PCR (Fig. 8), however, end-point PCR detected salmonellae in considerably more sections of the intestine. Salmonellae detected by *in situ* hybridization were found associated to particulate material (Fig. 9), and thus exclusively in H_2O samples. Direct detection of salmonellae was only found in 2 of the 10 warmouth with cells ranging on the order of magnitude between 10^5-10^6 segment^{-1}. This number, however, might be largely overestimated, because it is based on the extrapolation of cells counted associated and thus accumulated on particulate material.

Although *q*PCR was meant to be 100 x more sensitive than end-point PCR, direct detection of salmonellae without semi-selective enrichment was not achieved in any of our intestine samples.

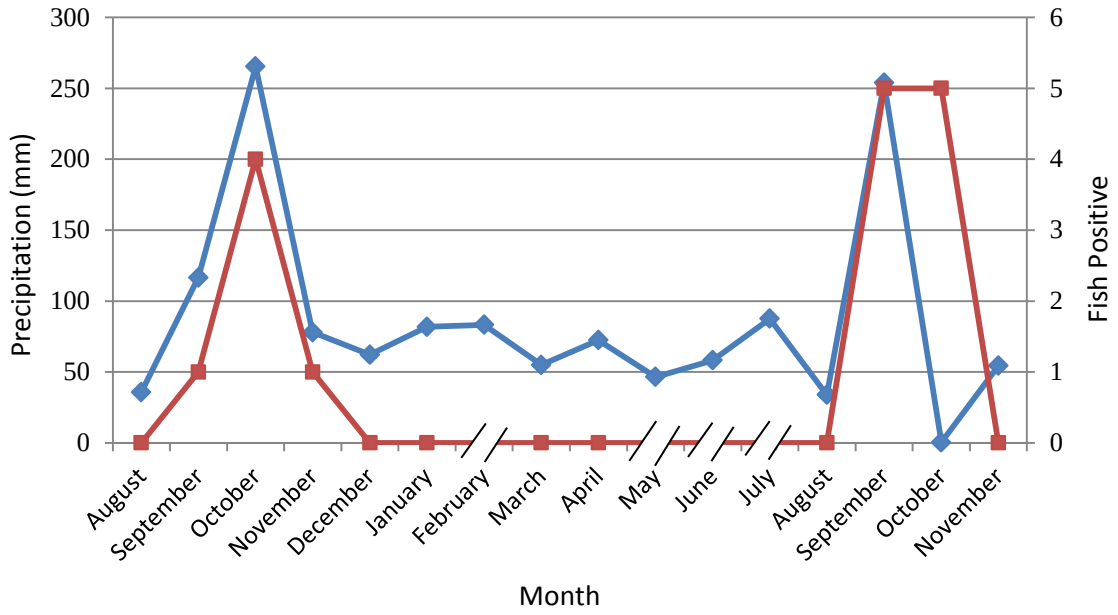

FIGURE. 2. Seasonal analysis (including the first 4 months) of salmonellae, via end-point PCR, in the intestines of freshwater fish from Spring Lake, San Marcos, TX. Precipitation (mm) corresponds to blue points, while the number of fish positive with salmonellae corresponds to red points for each month. Samples were not taken for February and May – July of 2010. Initial analysis of seasonal data revealed detection of salmonellae could be seen increasing with precipitation, however this was not necessarily true upon further inspection of the data. Rain data obtained from the National Climate Data Center and from Weather Underground.

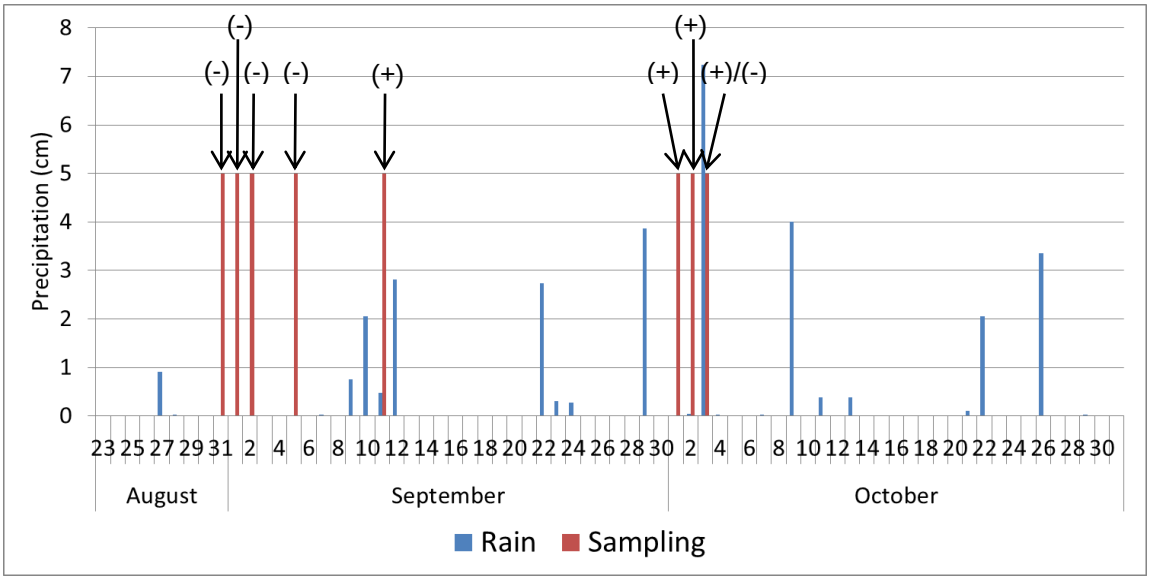

FIGURE 3. Seasonal detection of salmonellae in the first three months of the study (August – October 2009) with precipitation (cm) represented by blue bars and sampling dates represented by red bars with no numerical value. Above each sampling date are the results of end-point PCR analysis with fish being either positive [(+)] or negative [(-)] for salmonellae in their intestine.

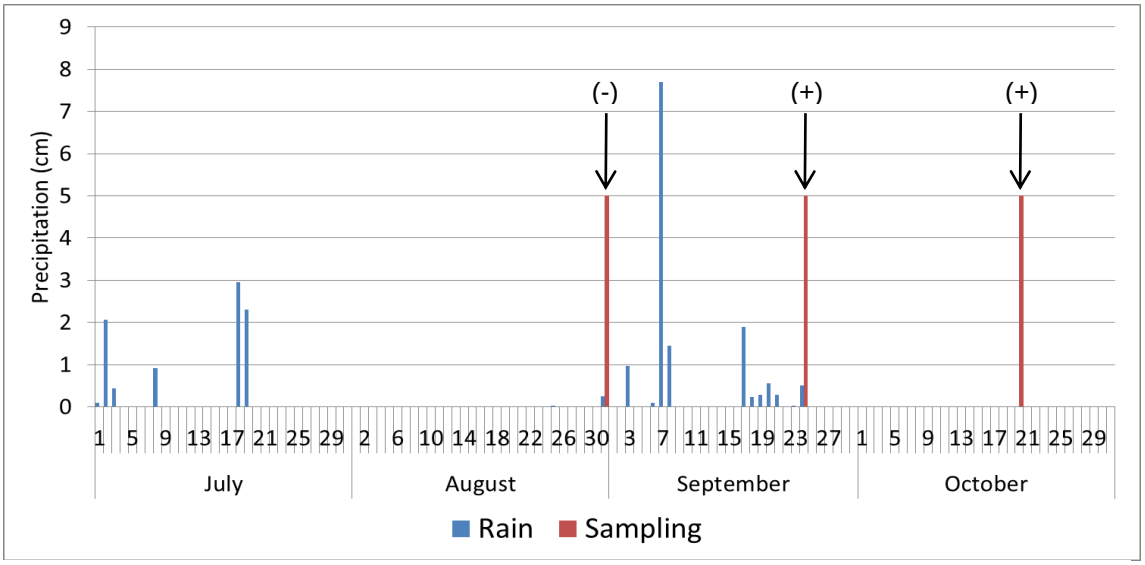

FIGURE 4. Seasonal detection of salmonellae in the last months of the study, excluding November (July – October 2010) with precipitation (cm) represented by blue bars and sampling dates represented by red bars with no numerical value. Above each sampling date are the results of end-point PCR analysis with fish being either positive [(+)] or negative [(-)] for salmonellae in their intestine.

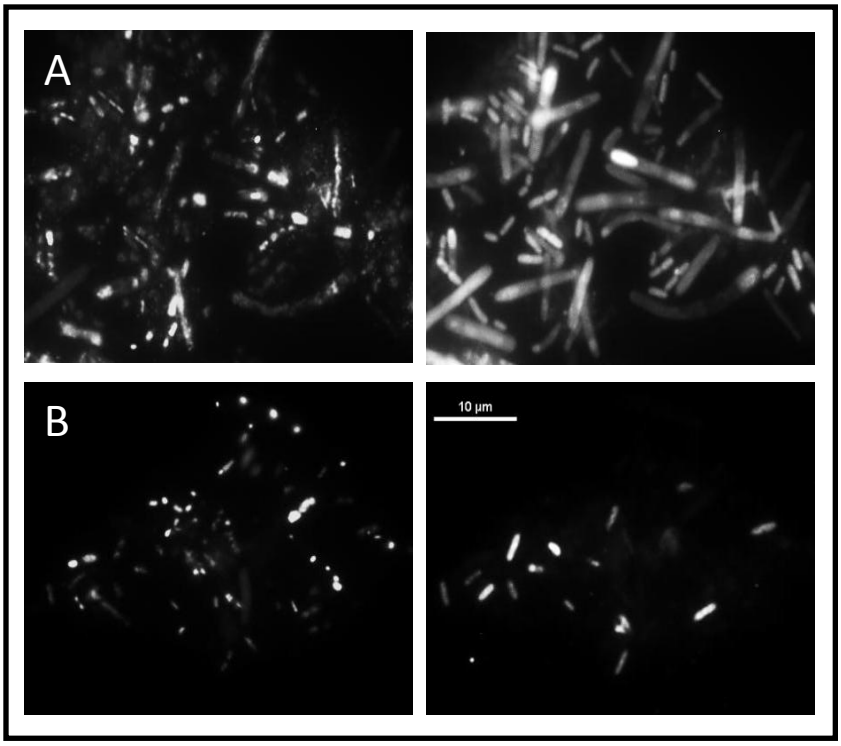

FIGURE 5. Analysis of size variation, via *in situ* hybridization, within the intestinal content of *Lepomis gulosus*. Probes selecting for total DNA content (DAPI; left) and 16S or 23S rRNA (Cy3; right) were used showing cells that were, morphologically, large (A; EUB338) and small (B; GAM42a).

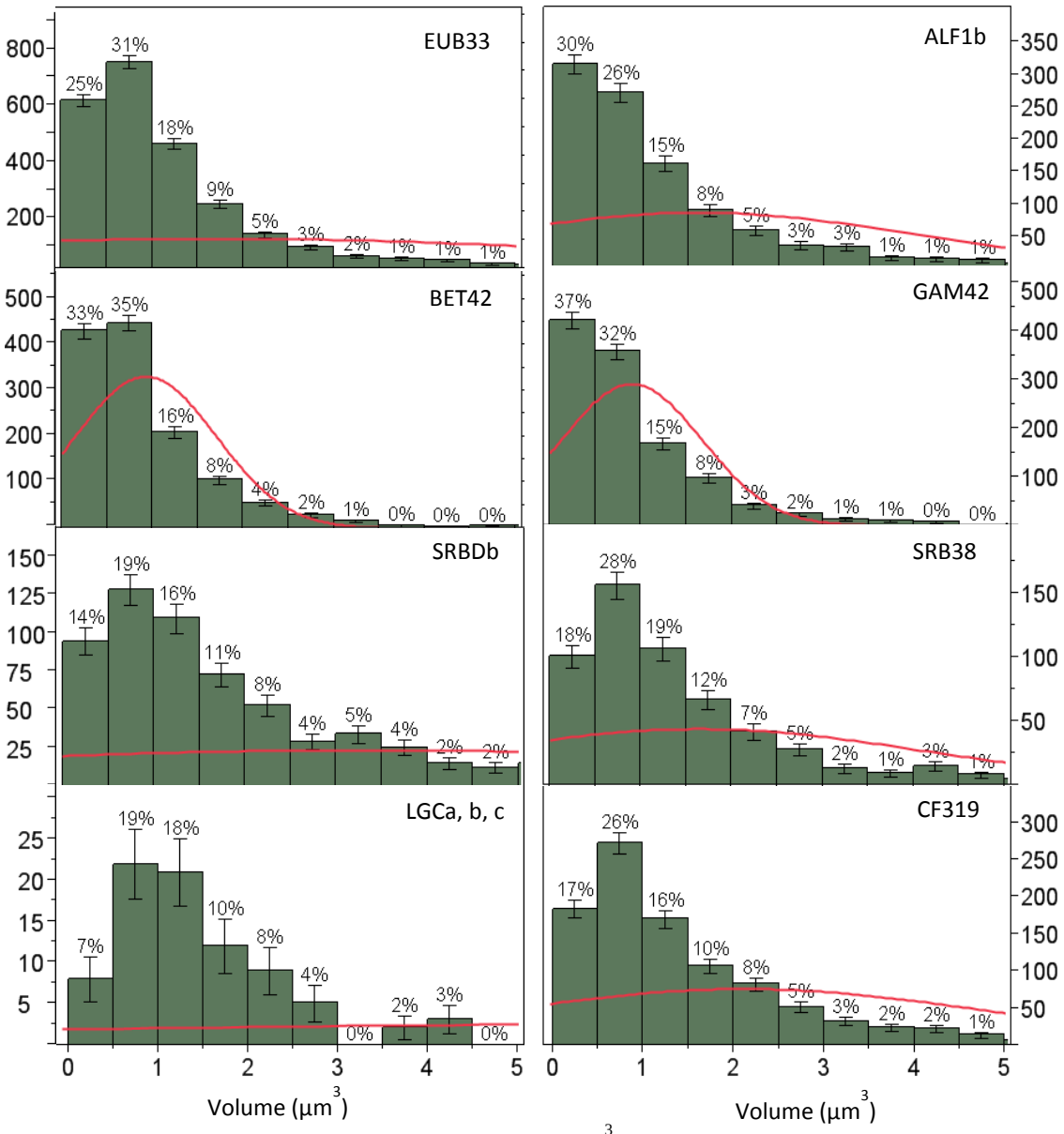

FIGURE 6. The distribution of total bacterial volume (μm^3) accumulated from the intestinal content (H_2O) of the 10 warmouth (*Lepomis gulosus*). Each bar graph represents the distributed the total volumes for each oligonucleotide probe used via *in situ* hybridization with percentages above each bar with standard error bars. The red line represents the normal distribution curve. Graphs whose percentages do not equal 100% (all) show data that represent the most common volumes measured.

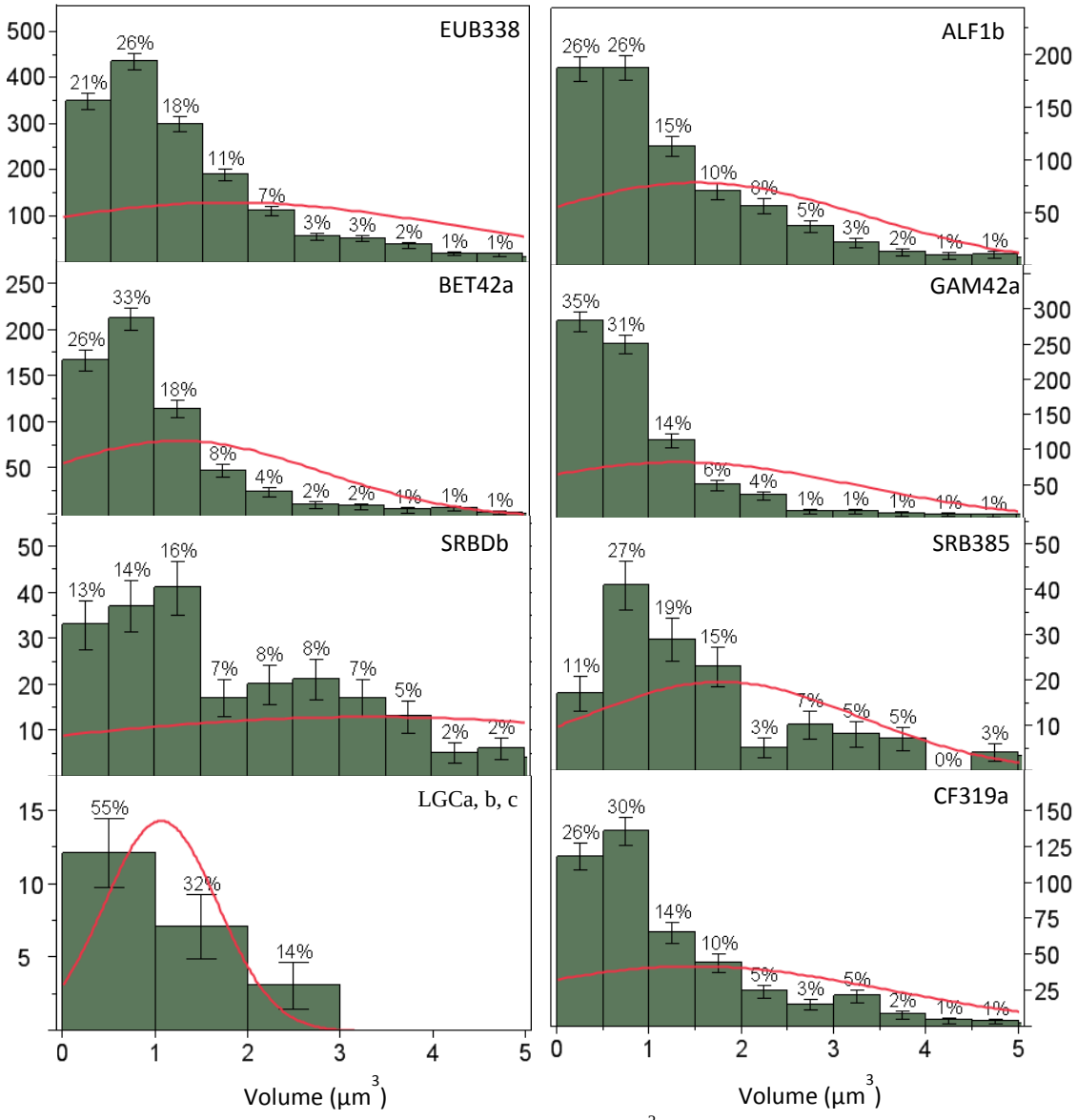

FIGURE 7. The distribution of total bacterial volume (μm^3) accumulated from the intestinal lining (PYRO) of the 10 warmouth (*Lepomis gulosus*). Each bar graph represents the distributed the total volumes for each oligonucleotide probe used via *in situ* hybridization with percentages above each bar with standard error bars. The red line represents the normal distribution curve. Graphs whose percentages do not equal 100% (A, B, C, D, E, F and, H) show data that represent the most common volumes measured.

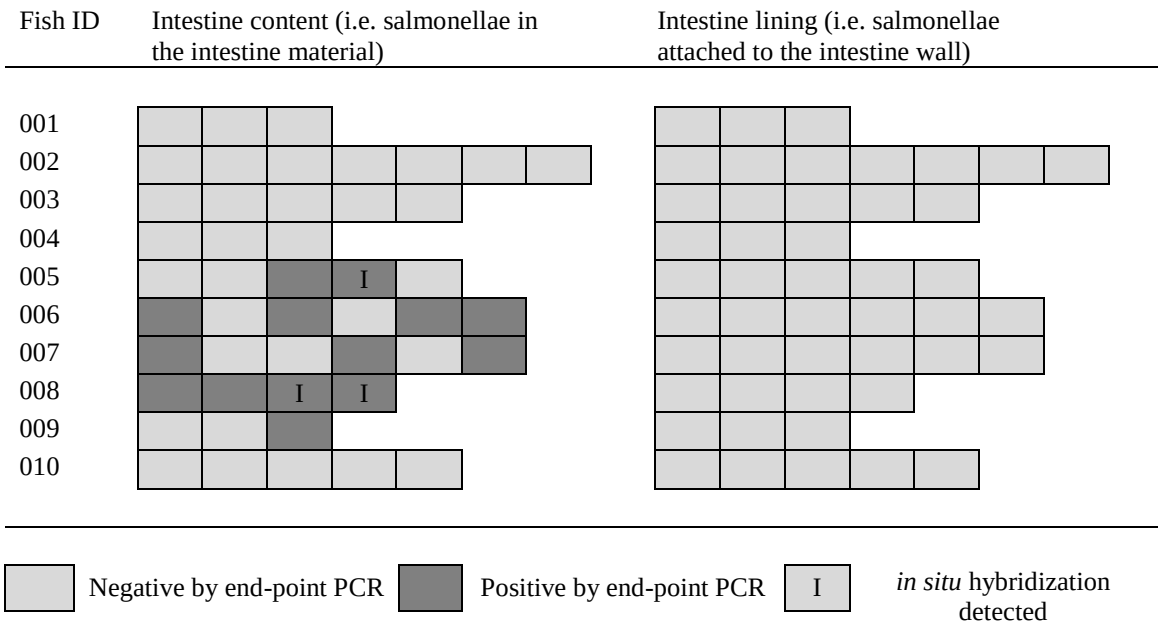

FIGURE 8. Results from end-point PCR and *in situ* hybridization in relation to the intestinal segments (1 cm) acquired from each warmouth (*Lepomis gulosus*). Positive (dark boxes) and negative (light boxes) samples from the content (H_2O) and lining (PYRO) of the intestine are shown for end-point PCR with salmonellae detected by *in situ* hybridization with an "I" in the box.

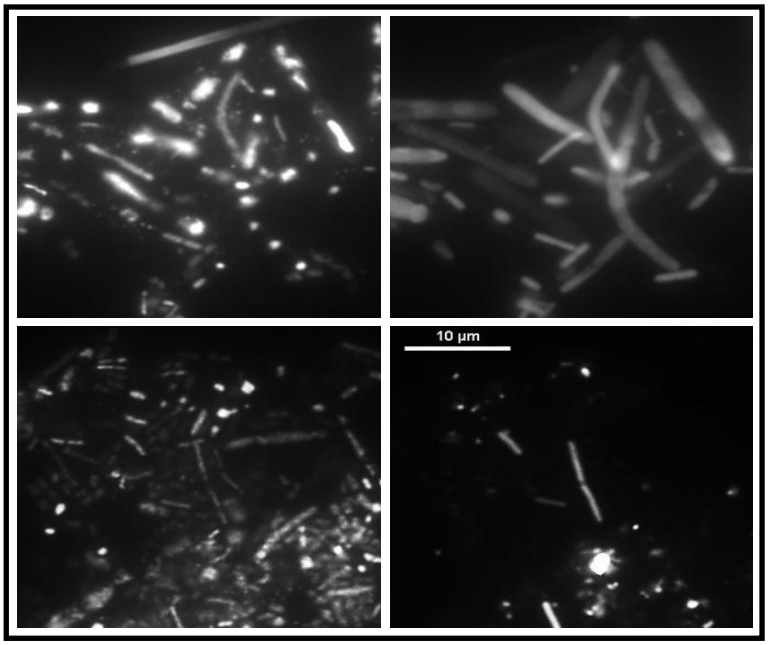

FIGURE 9. Analysis of salmonellae in the intestine of *Lepomis gulosus* via *in situ* hybridization. Probes selecting for total DNA content (DAPI; left) and 23S rRNA (Cy3; right) were used showing a mix of bacteria (A;EUB338) and salmonellae attached to ingested food (B; Sal3).

TABLE 2: Distribution of bacteria complied via *in situ* hybridization within the intestinal content (H_2O) of warmouth (*Lepomis gulosus*). Values under the column "Probe" indicate percent averages taken from 20 trials with standard deviations in parentheses, while values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

Fish ID		n x 10^3[±]	DAPI	Probe				
				EUB338	ALF1b	BET42a	GAM42a	CF319a
0	I	492 [1818]	100	5 (22)	0[1]	0	13 (26)	0
0	II	936 [2682]	100	20 (41)	0	3 (9)	15 (32)	0
1	III	91 [442]	100	15 (37)	0	0	0	0
0	I	87 [428]	100	10 (31)	0	0	0	0
0	II	117 [532]	100	15 (37)	0	5 (22)	0	0
2	III	17 [159]	100	5 (22)	0	0	0	0
	IV	304 [891]	100	0	0	6 (14)	2 (9)	0
	V	145 [588]	100	0	0	7 (19)	0	0
	VI	48 [253]	100	0	0	0	0	0
	VII	131 [516]	100	5 (22)	5 (22)	0	0	2 (8)
0	I	6 [75]	100	0	0	0	0	0
0	II	293 [2010]	100	10 (31)	5 (22)	1 (3)	0	0
3	III	23808 [43902]	100	86 (35)	5 (9)	24 (12)	35 (19)	12 (10)
	IV	10738 [10972]	100	80 (41)	5 (22)	19 (12)	25 (20)	6 (6)
	V	19351 [39949]	100	40 (50)	19 (35)	49 (23)	57 (27)	5 (5)
0	I	34 [253]	100	5 (22)	0	0	0	5 (22)
0	II	863 [1676]	100	50 (51)	5 (22)	38 (43)	39 (41)	0
4	III	4362 [6358]	100	90 (31)	6 (14)	0	0	31 (39)
0	I	310 [1237]	100	10 (31)	0	0	20 (41)	0
0	II	28 [166]	100	0	0	0	0	0
5	III	61 [289]	100	0	0	0	0	0
	IV	5560 [10826]	100	96 (20)	23 (23)	13 (25)	43 (46)	8 (15)
	V	10448 [24451]	100	100 (0.0)	15 (24)	47 (43)	70 (35)	7 (10)

Table 2 Continued

Fish ID		n x 10^3[±]	DAPI	Probe				
				EUB338	ALF1b	BET42a	GAM42a	CF319a
0	I	20591 [17506]	100	95 (22)	10 (7)	0	0	12 (10)
0	II	969 [1355]	100	85 (37)	8 (21)	2 (8)	0	3 (15)
6	III	444 [883]	100	20 (41)	0	0	0	3 (10)
	IV	1972[2750]	100	90 (31)	3 (9)	0	0	6 (11)
	V	32888 [25622]	100	95 (22)	7 (6)	21 (12)	9 (9)	29 (20)
	VI	4488 [4789]	100	95 (22)	6 (2)	4 (12)	1 (3)	11 (17)
0	I	168 [539]	100	15 (37)	5 (22)	0	5 (22)	5 (22)
0	II	0	0	0	0	0	0	0
7	III	0	0	0	0	0	0	0
	IV	0	0	0	0	0	0	0
	V	6 [75]	100	0	0	0	0	0
	VI	89 [429]	100	0	0	0	5 (22)	0
0	I	8828 [20952]	100	55 (51)	1 (3)	57 (37)	37 (47)	1 (2)
0	II	5778 [10809]	100	90 (31)	19 (32)	24 (42)	54 (48)	0
8	III	4186 [4293]	100	80 (41)	3 (6)	0	0	10 (15)
	IV	15944 [15810]	100	95 (22)	0	8 (25)	0	0 (1)
0	I	36 [199]	100	0	5 (22)	0	0	0
0	II	221 [869]	100	30 (47)	0	0	0	0
9	III	nd	nd	nd	nd	nd	nd	nd
0	I	1025 [3019]	100	45 (51)	0	0	0	0
1	II	277 [878]	100	0	0	2 (8)	20 (38)	5 (22)
0	III	796 [1552]	100	30 (47)	0	0	20 (31)	0
	IV	818 [2517]	100	85 (37)	0	0	0	0
	V	nd	nd	nd	nd	nd	nd	nd

[1] All enumerations representing less than 1% of the detectable microbes (i.e., an occasional signal) were assumed to be insignificant for statistical comparison and were counted as “0”

TABLE 3: Distribution of bacteria complied via *in situ* hybridization within the intestinal lining (PYRO) of warmouth (*Lepomis gulosus*). Values under the column "Probe" indicate percent averages taken from 20 trials with standard deviations in parentheses, while values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

Fish ID		n x 10^3 [±]	DAPI	Probe				
				EUB338	ALF1b	BET42a	GAM42a	CF319a
0	I	75 [456]	100	5 (22)	5 (22)	0	0	0
0	II	135 [557]	100	15 (37)	5 (22)	0	0	0
1	III	44 [342]	100	0	0	0	7 (24)	0
0	I	6 [201]	100	5 (22)	3 (11)	0	0	0
0	II	44 [240]	100	10 (31)	0	0	0	0
2	III	13 [150]	100	0	0	0	0	0
	IV	9 [112]	100	5 (22)	5 (22)	0	0	0
	V	72 [415]	100	0	0	0	5 (22)	0
	VI	343 [1219]	100	0	0	0	0	0
	VII	57 [276]	100	0	0	0	0	0
0	I	9 [84]	100	5 (22)	0	0	0	0
0	II	35 [214]	100	0	0	0	0	0
3	III	299 [1055]	100	15 (37)	5 (22)	0	0	3 (11)
	IV	343 [953]	100	15 (37)	0	0	5 (16)	6 (3)
	V	3051 [4100]	100	85 (37)	8 (13)	0	35 (35)	7 (22)
0	I	148 [557]	100	5 (22)	5 (22)		0	0
0	II	512 [1279]	100	35 (49)	5 (22)	5 (16)	0	0
4	III	625 [1284]	100	5 (22)	12 (31)	0	0	5 (22)
0	I	402 [789]	100	30 (47)	5 (22)	0	0	0
0	II	6 [75]	100	0	0	0	0	0
5	III	13 [150]	100	0	0	0	0	0
	IV	1005 [3099]	100	35 (49)	5 (14)	0	0	0
	V	889 [1761]	100	25 (44)	5 (22)	0	0	0

Table 3 Continued

Fish	ID	n x 10^3 [±]	DAPI	Probe				
				EUB338	ALF1b	BET42a	GAM42a	CF319a
0	I	182 [481]	100	20 (41)	0	0	0	0
0	II	189 [559]	100	20 (41)	0	0	0	0
6	III	616 [1399]	100	85 (37)	0	2 (8)	20 (38)	0
	IV	424 [808]	100	10 (31)	0	0	0	0
	V	13105 [11524]	100	95 (22)	15 (19)	2 (5)	58 (26)	14 (13)
	VI	207 [552]	100	10 (31)	0	0	1 (3)	0
0	I	588 [1433]	100	25 (44)	0	13 (31)	0	20 (41)
0	II	16 [112]	100	0	0	0	0	0
7	III	19 [167]	100	0	0	0	0	0
	IV	9 [239]	100	5 (22)	0	0	0	0
	V	13 [129]	100	0	0	0	0	0
	VI	72 [380]	100	5 (22)	0	0	0	0
0	I	280 [730]	100	25 (44)	5 (22)	1 (4)	8 (25)	0
0	II	154 [481]	100	10 (31)	0	5 (22)	0	0
8	III	6482 [7009]	100	90 (31)	20 (32)	21 (19)	0	9 (12)
	IV	3503 [5778]	100	75 (44)	0	2 (8)	4 (17)	0
0	I	16 [135]	100	5 (22)	0	0	3 (15)	0
0	II	0	0	0	0	0	0	0
9	III	82 [373]	100	20 (41)	0	2 (8)	0	0
0	I	60 [366]	100	8 (26)	5 (22)	3 (14)	0	0
1	II	6 [75]	100	5 (22)	0	0	0	0
0	III	6 [75]	100	0	0	0	0	0
	IV	0	0	0	0	0	0	0
	V	22 [154]	100	0	0	3 (15)	3 (11)	0

[1] All enumerations representing less than 1% of the detectable microbes (i.e., an occasional signal) were assumed to be insignificant for statistical comparison and were counted as “0”

TABLE 4: Segments of the distribution of bacteria complied via *in situ* hybridization within the intestinal content (H_2O) of warmouth (*Lepomis gulosus*). These unique segments showed sulfur-reducing bacteria (SRB) and low G+C gram positive bacteria (LGC) can be observed within a given microbial community. Values under the column "Probe" indicate percent averages taken from 20 trials with standard deviations in parentheses, while values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

				Probe		
Fish ID		n x 10^3 [±]	DAPI	SRBDb	SRB385	LGCa,b,c
0 0 5	**V**	10448 [24451]	100	6 (11)	4 (9)	2 (4)
0 0 6	**I**	20591 [17506]	100	18 (12)	2 (2)	0[1]
	III	444 [883]	100	6 (23)	0	0
	IV	1972 [2750]	100	9 (14)	3 (11)	0
	V	32888 [25622]	100	13 (9)	7 (5)	7 (0)
	VI	4488 [4789]	100	2 (3)	2 (5)	0

[1] All enumerations representing less than 1% of the detectable microbes (i.e., an occasional signal) were assumed to be insignificant for statistical comparison and were counted as "0"

TABLE 5: Biomass measurements (averaged) taken from each segment via *in situ* hybridization from the intestinal content (H_2O) of warmouth (*Lepomis gulosus*). The diameter (D; µm), length (L; µm) and cylindrical volume (CV; μm^3) were calculated using NIS Elements with standard deviations in parentheses[1].

Fish ID		Probe														
		EUB338			ALF1b			BET42a			GAM42a			CF319a		
		D	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*
001	I	1 (0)	1 (1)	0.4 (0.3)	-	-	-	1 (0)	2 (0)	0.8 (0.0)	1 (0)	2 (0)	0.7 (0.4)	-	-	-
	II	1 (0)	2 (0)	0.6 (0.4)	-	-	-	1 (0)	1 (0)	0.4 (0.3)	1 (0)	1 (0)	0.3 (0.3)	1 (0)	2 (1)	1 (1)
	III	1 (0)	2 (0)	0.6 (0.3)	2 (1)	2 (1)	0.6 (0.3)	-	-	-	-	-	-	-	-	-
002	I	1 (0)	1 (0)	0.3 (0.3)	-	-	-	1 (0)	2 (1)	1 (0)	-	-	-	1 (1)	2 (1)	1 (2)
	II	1 (0)	1 (0)	0.4 (0.2)	1 (0)	2 (0)	1 (0)	1 (0)	1 (0)	0.4 (0.2)	-	-	-	1 (0)	1 (0)	0.3 (0.1)
	III	1 (0)	2 (1)	1 (1)	-	-	-	1 (0)	2 (0)	0.7 (0.3)	1 (0)	2 (0)	1 (1)	1 (0)	1 (0)	0.5 (0.0)
	IV	1 (0)	1 (0)	0.7 (0.1)	-	-	-	1 (0)	2 (0)	0.6 (0.2)	-	-	-	1 (0)	1 (0)	0.5 (0.1)
	V	1 (0)	2 (1)	1 (1)	-	-	-	2 (0)	2 (0)	2 (1)	1 (0)	2 (0)	1 (0)	1 (0)	2 (0)	0.9 (0.7)
	VI	-	-	-	-	-	-	1 (0)	2 (1)	0.9 (0.5)	2 (0)	2 (0)	1 (1)	-	-	-
	VII	1 (0)	2 (1)	1 (1)	-	-	-	-	-	-	1 (0)	2 (0)	0.9 (0.4)	1 (0)	1 (0)	0.5 (0.3)
003	I	-	-	-	1 (0)	1 (1)	0.3 (0.2)	-	-	-	-	-	-	-	-	-
	II	1 (1)	2 (1)	2 (2)	1 (1)	2 (1)	2 (5)	-	-	-	1 (0)	1 (0)	0.5 (0.7)	-	-	-
	III	1 (1)	2 (1)	1 (2)	1 (0)	1 (1)	1 (1)	1 (0)	2 (1)	1 (1)	1 (0)	1 (0)	0.5 (0.2)	1 (1)	2 (1)	2 (2)
	IV	2 (1)	2 (1)	2 (3)	2 (0)	2 (1)	2 (2)	1 (0)	1 (0)	0.4 (0.3)	1 (0)	2 (0)	0.6 (0.4)	2 (1)	3 (1)	4 (4)
	V	1 (0)	2 (1)	1 (1)	1 (0)	2 (1)	1 (1)	1 (0)	1 (0)	0.4 (0.3)	1 (0)	1 (0)	0.5 (0.2)	2 (1)	3 (2)	7 (11)
004	I	1 (0)	2 (0)	2 (2)	1 (0)	1 (0)	0.3 (0.2)	2 (0)	3 (1)	2 (1)	2 (0)	2 (0)	2 (1)	-	-	-
	II	1 (0)	2 (0)	0.9 (0.7)	1 (0)	2 (0)	0.5 (0.1)	2 (0)	2 (0)	1 (1)	1 (0)	2 (0)	1 (1)	-	-	-
	III	1 (0)	2 (0)	1 (1)	1 (0)	1 (0)	0.5 (0.4)	-	-	-	-	-	-	-	-	-
005	I	1 (0)	2 (0)	0.9 (0.3)	-	-	-	1 (0)	2 (0)	1 (1)	2 (0)	2 (0)	1 (1)	1 (0)	2 (0)	1 (1)
	II	1 (0)	2 (0)	0.9 (0.3)	-	-	-	-	-	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IV	2 (1)	3 (2)	8 (16)	1 (0)	3 (1)	1 (1)	1 (0)	2 (1)	1 (1)	1 (0)	2 (1)	1 (1)	1 (0)	2 (0)	0.9 (0.5)
	V	2 (1)	2 (2)	3 (7)	2 (1)	3 (1)	2 (3)	1 (0)	2 (0)	1 (1)	2 (0)	2 (1)	2 (1)	2 (1)	2 (1)	3 (3)

Table 5 Continued

Fish ID		Probe														
		EUB338			ALF1b			BET42a			GAM42a			CF319a		
		D	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*
006	I	2 (0)	2 (1)	2 (2)	2 (0)	2 (1)	2 (2)	-	-	-	-	-	-	2 (1)	3 (1)	3 (4)
	II	1 (0)	2 (0)	0.8 (0.8)	1 (0)	1 (1)	1 (1)	1 (0)	1 (0)	0.4 (0.1)	-	-	-	1 (0)	2 (1)	0.9 (0.4)
	III	1 (1)	2 (1)	1 (2)	1 (1)	2 (1)	1 (1)	-	-	-	-	-	-	2 (0)	4 (2)	3 (2)
	IV	1 (0)	2 (0)	0.9 (0.7)	2 (1)	3 (2)	3 (3)	-	-	-	-	-	-	2 (1)	3 (2)	3 (4)
	V	1 (0)	2 (1)	1 (1)	2 (1)	2 (1)	3 (4)	1 (0)	1 (0)	0.5 (0.3)	1 (0)	2 (0)	0.7 (0.4)	2 (0)	3 (1)	2 (1)
	VI	1 (0)	2 (0)	0.8 (0.6)	2 (1)	2 (2)	2 (2)	1 (0)	2 (0)	0.7 (0.5)	-	-	-	2 (0)	2 (1)	2 (1)
007	I	2 (0)	2 (1)	2 (1)	2 (1)	5 (3)	3 (3)	1 (0)	2 (0)	1 (1)	2 (0)	3 (0)	2 (1)	2 (0)	2 (0)	2 (0)
	II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	2 (0)	3 (0)	2 (0)	2 (0)	2 (0)	2 (0)	-	-	-
	VI	1 (0)	2 (0)	0.8 (0.6)	1 (0)	1 (0)	0.2 (0.1)	-	-	-	-	-	-	-	-	-
008	I	2 (0)	2 (1)	1 (1)	2 (1)	3 (1)	2 (2)	1 (0)	2 (0)	1 (1)	1 (0)	2 (0)	0.9 (0.5)	1 (0)	1 (1)	1 (1)
	II	2 (0)	2 (1)	2 (1)	2 (1)	3 (2)	3 (3)	1 (0)	2 (0)	0.9 (0.6)	1 (0)	2 (0)	0.9 (0.6)	1 (0)	1 (1)	0.3 (0.2)
	III	2 (1)	3 (2)	4 (8)	2 (1)	3 (2)	3 (4)	-	-	-	-	-	-	2 (1)	3 (2)	3 (3)
	IV	2 (0)	2 (0)	1 (1)	-	-	-	1 (0)	2 (0)	0.9 (0.5)	-	-	-	2 (0)	2 (1)	2 (1)
009	I	1 (0)	2 (0)	0.7 (0.4)	1 (0)	1 (0)	0.1 (0.0)	-	-	-	1 (0)	1 (0)	0.3 (0.1)	-	-	-
	II	1 (0)	2 (1)	0.7 (0.7)	-	-	-	-	-	-	-	-	-	-	-	-
	III	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
010	I	1 (0)	2 (1)	1 (1)	-	-	-	-	-	-	1 (0)	2 (1)	1 (1)	-	-	-
	II	1 (0)	2 (0)	0.7 (0.5)	-	-	-	-	-	-	1 (0)	2 (1)	1 (1)	-	-	-
	III	1 (0)	2 (1)	0.8 (0.6)	2 (0)	2 (1)	2 (0)	-	-	-	1 (0)	2 (1)	0.9 (0.8)	-	-	-
	IV	-	-	-	-	-	-	-	-	-	-	-	-	1 (0)	2 (1)	1 (1)
	V	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[1]All values were calculated by taking initial pixel values and multiplying them by 0.09 in order to obtain units of measurement (volumes were cubed by this value)

TABLE 6: Biomass measurements (averaged) taken from each segment via *in situ* hybridization from the intestinal lining (PYRO) of warmouth (*Lepomis gulosus*). The diameter (D; µm), length (L; µm) and cylindrical volume (CV; μm^3) were calculated using NIS Elements with standard deviations in parentheses[1].

Fish ID		Probe														
		EUB338			ALF1b			BET42a			GAM42a			CF319a		
		D	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*
001	I	2 (0)	2 (0)	2 (1)	1 (0)	2 (1)	0.8 (0.5)	-	-	-	2 (0)	2 (1)	2 (1)	-	-	-
	II	1 (0)	2 (1)	1 (1)	1 (0)	1 (0)	0.4 (0.5)	1 (0)	2 (0)	0.4 (0.1)	-	-	-	-	-	-
	III	2 (0)	2 (1)	2 (1)	-	-	-	-	-	-	1 (0)	2 (0)	0.7 (0.1)	-	-	-
002	I	1 (0)	2 (1)	0.8 (0.8)	1 (0)	2 (1)	0.4 (0.2)	1 (0)	1 (0)	0.4 (0.3)	1 (0)	2 (1)	1 (1)	-	-	-
	II	1 (0)	2 (0)	0.9 (0.6)	1 (0)	2 (0)	0.4 (0.0)	-	-	-	-	-	-	-	-	-
	III	1 (0)	1 (0)	0.5 (0.2)	1 (0)	1 (0)	0.4 (0.6)	-	-	-	-	-	-	-	-	-
	IV	1 (0)	1 (0)	0.5 (0.5)	1 (0)	1 (0)	0.3 (0.1)	-	-	-	-	-	-	-	-	-
	V	2 (0)	2 (0)	3 (1)	-	-	-	-	-	-	-	-	-	-	-	-
	VI	2 (0)	3 (1)	4 (3)	-	-	-	-	-	-	-	-	-	-	-	-
	VII	1 (0)	2 (1)	0.8 (0.6)	-	-	-	2 (0)	2 (0)	1 (0)	1 (0)	1 (0)	0.4 (0.1)	-	-	-
003	I	1 (0)	1 (0)	0.6 (0.5)	-	-	-	-	-	-	-	-	-	-	-	-
	II	1 (0)	2 (1)	1 (1)	-	-	-	-	-	-	-	-	-	-	-	-
	III	2 (1)	2 (1)	2 (2)	2 (0)	4 (1)	3 (2)	-	-	-	1 (0)	2 (1)	1 (1)	2 (0)	2 (1)	2 (2)
	IV	1 (1)	2 (1)	2 (2)	2 (0)	3 (1)	3 (2)	-	-	-	2 (0)	2 (1)	2 (1)	2 (1)	3 (2)	4 (4)
	V	2 (1)	2 (1)	2 (4)	2 (1)	3 (2)	3 (3)	1 (0)	2 (1)	1 (2)	1 (0)	2 (1)	1 (1)	2 (1)	3 (1)	3 (4)
004	I	2 (1)	3 (1)	3 (3)	1 (0)	2 (1)	0.5 (0.4)	2 (0)	3 (1)	2 (2)	2 (0)	3 (0)	5 (1)	-	-	-
	II	2 (1)	3 (2)	5 (6)	1 (0)	2 (1)	0.6 (0.4)	2 (0)	3 (1)	4 (2)	3 (0)	4 (1)	7 (3)	-	-	-
	III	2 (1)	3 (1)	4 (5)	1 (0)	2 (1)	0.8 (0.5)	2 (0)	3 (1)	4 (3)	-	-	-	-	-	-
005	I	2 (0)	3 (1)	4 (3)	2 (0)	2 (1)	1 (0)	-	-	-	-	-	-	-	-	-
	II	-	-	-	2 (0)	4 (1)	2 (1)	-	-	-	-	-	-	-	-	-
	III	2 (0)	3 (1)	0.9 (0.7)	-	-	-	-	-	-	-	-	-	-	-	-
	IV	2 (1)	3 (1)	2 (2)	-	-	-	-	-	-	2 (0)	2 (1)	2 (1)	2 (1)	3 (1)	4 (3)
	V	2 (1)	3 (2)	2 (4)	1 (0)	3 (1)	1 (0)	-	-	-	-	-	-	1 (0)	2 (0)	1 (0)

Table 6 Continued

Fish ID		Probe														
		EUB338			ALF1b			BET42a			GAM42a			CF319a		
		D	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*	*D*	*L*	*CV*
006	I	1 (0)	2 (1)	0.8 (1)	-	-	-	1 (0)	2 (0)	0.6 (0.2)	1 (0)	1 (0)	0.5 (0.3)	-	-	-
	II	1 (0)	2 (1)	1 (1)	-	-	-	-	-	-	1 (0)	2 (0)	0.7 (0.7)	-	-	-
	III	1 (0)	2 (1)	0.9 (0.6)	-	-	-	1 (0)	2 (0)	0.7 (0.3)	1 (0)	2 (0)	0.8 (0.7)	-	-	-
	IV	2 (0)	2 (1)	1 (1)	-	-	-	1 (0)	2 (0)	0.5 (0.1)	1 (0)	1 (0)	0.5 (0.2)	1 (0)	1 (0)	0.5 (0.5)
	V	1 (0)	2 (1)	1 (1)	2 (0)	2 (1)	2 (2)	1 (0)	1 (0)	0.5 (0.3)	1 (0)	2 (0)	0.8 (0.6)	1 (0)	2 (1)	0.5 (0.3)
	VI	1 (0)	2 (0)	0.6 (0.4)	-	-	-	-	-	-	1 (0)	1 (0)	0.4 (0.2)	-	-	-
007	I	2 (0)	3 (1)	1 (1)	2 (1)	5 (3)	3 (3)	1 (0)	2 (1)	1 (1)	-	-	-	1 (0)	2 (1)	0.9 (0.6)
	II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	III	1 (0)	1 (0)	0.8 (0.7)	-	-	-	-	-	-	-	-	-	-	-	-
	IV	1 (0)	1 (0)	0.1 (0.0)	-	-	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	2 (0)	2 (0)	1 (0)	2 (0)	3 (0)	1 (0)	-	-	-
	VI	1 (0)	2 (0)	0.7 (0.3)	1 (0)	1 (0)	0.2 (0.1)	-	-	-	-	-	-	-	-	-
008	I	1 (0)	2 (1)	1 (1)	1 (0)	1 (1)	0.4 (0.4)	1 (0)	2 (1)	1 (1)	1 (0)	1 (0)	0.6 (0.4)	-	-	-
	II	2 (1)	2 (1)	3 (5)	-	-	-	-	-	-	1 (0)	2 (0)	0.8 (0.3)	-	-	-
	III	1 (0)	2 (1)	1 (2)	2 (1)	2 (1)	2 (3)	1 (0)	2 (0)	1 (1)	1 (0)	1 (0)	0.4 (0.3)	1 (0)	2 (1)	2 (2)
	IV	2 (0)	2 (1)	1 (1)	-	-	-	1 (0)	2 (0)	0.8 (0.4)	1 (0)	2 (0)	0.9 (0.7)	-	-	-
009	I	-	-	-	-	-	-	-	-	-	1 (0)	2 (1)	1 (1)	-	-	-
	II	1 (0)	1 (0)	0.4 (0.1)	-	-	-	-	-	-	1 (0)	2 (0)	0.7 (0.2)	-	-	-
	III	1 (0)	2 (1)	0.9 (0.5)	-	-	-	2 (0)	2 (1)	2 (1)	1 (0)	2 (1)	1 (1)	-	-	-
010	I	-	-	-	1 (0)	2 (0)	1 (0)	1 (0)	2 (1)	1 (0)	2 (0)	2 (1)	1 (1)	-	-	-
	II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	III	2 (0)	3 (1)	2 (1)	-	-	-	1 (0)	2 (0)	0.4 (0.0)	-	-	-	-	-	-
	IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]All values were calculated by taking initial pixel values and multiplying them by 0.09 in order to obtain units of measurement (volumes were cubed by this value)

IV. DISCUSSION

Confirming previous studies on the presence of salmonellae in fish from the San Marcos River (16), salmonellae were detected in warmouth in this study. Detection, however, was limited to fish caught in Fall, while fish obtained during the remainder of the year were always negative for salmonellae in their intestine. Because almost all of the fish harboring salmonellae as indicated by end-point PCR were caught after a significant rainfall, terrestrial runoff is suggested as a contributing factor for the entry of salmonellae into the aquatic environment, with their subsequent accumulation in food resources. Previous studies have detected salmonellae in the intestines of freshwater fish (16), however, to date no study has taken precipitation into account when detecting salmonellae in wild, freshwater fish. The potential impact of heavy precipitation on the detectability of salmonellae in the intestine of freshwater fish caught in Fall, i.e. in September and November 2009 and 2010, is further supported by similar detections of salmonellae found in the same time period in 2008 (unpublished data).

The analyses of two sample types (H_2O/PYRO) were meant to provide a differentiation between cells attached to food sources or were free floating (H_2O), and cells that were bound to the linings of the intestinal wall (PYRO) of warmouth. Since one of the hypotheses was that salmonellae were taken up with food and pass through the intestine, we hoped to generate a concise distinction in the abundance of salmonellae between the food ingested (H_2O samples) and intestinal wall (PYRO samples) with more

cells detected on the food compared to the wall, and the random detection of salmonellae in different sections of the intestine. While the latter assumption was supported by our preliminary data, with the detection of salmonellae in different sections of the intestine, a clear pattern favoring the detection of salmonellae in the food is lacking (Fig. 8). However, both data were obtained with a qualitative method, i.e. end-point PCR, and thus need to be supported by quantitative data obtained by *in situ* hybridization and *q*PCR.

The random detection of salmonellae by end-point PCR in the intestine of warmouth suggested two things: [1] salmonellae were not members of the autochthonous intestinal flora in these fish thus had no permanent niche in the intestines of these freshwater fishes, and [2] these freshwater fish could not be considered as a reservoir for salmonellae, rather they ingested food that had salmonellae attached to it within their surrounding environment and pass them asymptomatically. Since analyses of fish intestines for May – July 2010 were entirely based on the *q*PCR method and not on end-point PCR analyses after enrichment, the failure to detect salmonellae in these samples might be a consequence of the limitations in sensitivity of this tool. Detection of salmonellae by *in situ* hybridization and *q*PCR was not as successful as end-point PCR primarily due to the fact that samples designated for end-point PCR were enriched in growth media that were semi-selective for salmonellae, thereby increasing the likelihood for their detection. *In situ* hybridization and *q*PCR depended on direct detection in environmental samples. *In situ* hybridization was therefore giving the operator a visual of the microbial community within a given sample. The rare detection of salmonellae by *in situ* hybridization in about 6% (3 out of 47) of the H_2O samples and the failure to detect any salmonellae in samples from the lining (PYRO) demonstrated this limited sensitivity.

The number of cells enumerated from the 3 segments ranged from 10^5-10^6 segment^{-1} and were also positive by end-point PCR (Fig. 8). *In situ* hybridization, however, allowed us to demonstrate that cells of salmonellae were associated to particulate material, and thus very likely taken up by fish with their food sources.

Salmonella cells visualized in intestinal samples were large (1 μm^3) compared to cell sizes usually encountered in natural environments such as on leaves (0.21 μm^3), in soil (0.13 μm^3), and in aquatic environments (0.19 μm^3) (28, 43, 62). Furthermore, salmonellae grown in pure cultures were no larger than 0.32 μm^3 (unpublished data). These large sizes were likely a consequence of abundant nutrient resources found in particulate material that potentially allowed the associated cells to grow. This speculation is supported by cell size distributions of other bacteria associated to the particulate material, but also those attached to the linings (Fig. 6 and 7). Analyses of the microbial community within wild, freshwater fish have not been established. However, much more is known about the relationship between bacteria, farmed fish, and the environment they share. Past experiments have shown the use of antibiotics and antimicrobials, especially when used extensively, in the stock tanks on fish farms yields antibiotic-resistant bacteria (1, 55). Furthermore, it has been suggested there is a significant relationship between increased bacterial resistance to antimicrobial agents on and around inland fish farms and the antimicrobial agents used at the fish farms (25, 57). The promotion of probiotic bacteria in fish has also been studied, as a method to counteract disease in farm-raised fish suggesting the addition of such probiotic bacteria into feed tends to encourage the proliferation of fish, reduce the incidence of disease and/or mortality, and provides an alternative to the use of antibiotics (18, 19, 21, 29, 32, 64).

Using *in situ* hybridization, we ascertained and generated a general outline of the different bacteria that were ingested by warmouth and those that were meant to belong to the natural intestinal flora of the fish (Table 2). The phylum Proteobacteria, more specifically the α-, β-, γ-, and δ-subclasses, were observed to make up almost half, if not more, of the normal flora of warmouth indicating their importance in the intestine. A study comparing the intestinal flora of marine and freshwater fish is non-existent. However, independent studies classifying the intestinal flora of both environments have been performed extensively suggesting Gram-negative bacteria dominate the intestine of fish with large concentrations of Proteobacteria present as well (37, 67, 68, 69, 70). Bacteria belonging to the Cytophaga-Flavobacteria-Bacteriodes (CFB), or Bacteriodetes, also were found, however, not as frequent as Proteobacteria.

The biovolumes of aquatic bacteria inhabiting different environments such as hypereutrophic ponds (17), simulated freshwater habitats (e.g. aquaria) (43), or intertidal sediments (33) have been shown to be below 1 μm^3. In our study, biovolumes of up to half of the observed cells were larger than 1 μm^3 which is the maximum volume described in previous studies (17, 33, 43). These larger biovolumes may be related to the diet of warmouth. Studies in the past have demonstrated essential biomolecules (e.g. proteins, amino acids, phospholipids, etc.) are crucial in the development and growth of all fish (2, 30, 36, 63). Most sunfish by nature are invertivores and/or carnivores, meaning the basis of their diet are protein-rich prey like insects, snails, crayfish, and other fishes (20). In a recent experiment by Richardson and Nickol (1999) the caeca and intestine of green sunfish (*Lepomis cyanellus*) were shown to consist of high levels of proteins, free amino acids, lipids, proteins, carbohydrates, and bile salts. The high

concentrations of these components, in addition to the naturally moist environment, might explain why the intestines seemed to be an advantageous environment for bacteria to amass large biovolumes in this experiment. In contrast to this, most bacteria (e.g. in aquatic and terrestrial environments) survive in nutrient-limited conditions making it difficult to increase in volume, while some bacteria undergo spore formation and remain dormant until nutrients and/or favorable conditions are made available in the environment (53).

V. FUTURE PERSPECTIVES

Our initial data indicating that the occurrence of salmonellae in intestine samples were related to periods of precipitation in excess of 100 mm, provided a basis for the speculation that the high volume of precipitation washes salmonellae from the terrestrial environment into the aquatic environment through runoff (Fig. 2). However, a more detailed analysis of precipitation events within the month and sampling time revealed the detection of salmonellae in the intestine of fish even in the absence of precipitation. Salmonellae were detected in the intestines of fish for the month of October 2010 after an extended period without precipitation (Figure 4). This indicates salmonellae were likely obtained by fish elsewhere in the aquatic environment. At this time, we can only speculate salmonellae are likely in areas within the aquatic environment (e.g. sediment, biofilms, vegetation, etc.), but probably not in the water column during such dry periods. Thus further studies in the future would need to be conducted in order to clarify these observations.

Even though we could not detect salmonellae in the intestines of fish during most of the seasonal study, recent studies suggest that salmonellae may persist in other areas in the environment even during periods of low precipitation. Sha et al. (2011) found numerous *Salmonella* strains in biofilms formed in both pristine and impacted aquatic environments. Salmonellae were also detected in Spring and Summer of 2009 in Spring Lake, though much less frequently than in samples from Fall. Gaertner et al. (2011)

provided additional evidence detecting salmonellae in the water column and biofilms after precipitation events. Furthermore, they demonstrated some strains of salmonellae can persist in biofilms and sustain their vitality over long periods of time without precipitation. In addition to precipitation, other environmental factors could potentially affect the detection of salmonellae during the season: dissolved oxygen content, vegetation within and on top of the water column, water temperature, potential prey items, local biofilms, and the determination of fecal origin on the terrestrial environment. Analyses of potential effects of these factors on the fate of salmonellae could provide additional evidence for the seasonal occurrence of salmonellae in fish from Spring Lake.

In addition to questions on the seasonal occurrence of salmonellae in fish from Spring Lake, cell size or biovolume estimations require more attention since they might have been impacted by methodological issues. It is possible that the bright intensity of the probes used for in situ hybridization caused us to identify multiple, chain-forming cells as individual cells. Indentions or the beginnings of cleavage furrows may separate dividing cells and individual cells, however the intensity of the probes may mask multiple cells. Counterstaining with DAPI allows one to observe DNA, but discerning between dividing cells again becomes difficult when a microorganism may contain storage compounds such as polyhydroxybutyric acid (PHB). DAPI might not only bind to DNA, but also to PHB, and thus one signal may not accurately relate to one cell. Thus the use of other methods should be employed in order to further distinguish between multiple, chain-forming bacteria and individual cells. Sonication is a technique using ultrasound (high frequency oscillations) to burst open cells, however, at low levels sonication can alter structural and physiological conditions without disrupting the integrity of the cell to the

point of collapse (12). After separation of cell chains into individual cells, *in situ* hybridization could then be used again to determine cell size distributions and verify the biovolumes obtained in our preliminary study.

In this thesis, I have given evidence to suggest salmonellae are not members of the normal flora within the intestines of warmouth and are, likely, detected after precipitation in the intestine of wild, freshwater fish within Spring Lake. Additionally, from this work the intestinal flora of warmouth has been made known via *in situ* hybridization as well as measurements pertaining to their size indicating their potential interaction with nutritious food sources taken up by their host fish.

LITERATURE CITED

1. **Akinbowale, O. L., H. Peng, and M. D. Barton.** 2006. Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. J. Appl. Microbiol. **100:**1103-1113.

2. **Aksnes, A., B. Hope, E. Jonsson, B. T. Bjornsson, and S. Albrektsen.** 2006. Size-fractionated fish hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus mykiss*) fed high plant protein diets. I: Growth, growth regulation and feed utilization. Aquaculture **261:**305-317.

3. **Almeida, C., N. F. Azevedo, R. M. Fernandes, C. W. Keevil, and M. J. Vieira.** 2010. Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for identification of *Salmonella* spp. in a broad spectrum of samples. Appl. Environ. Microbiol. **76:**4476-4485.

4. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

5. **Bernbom, N., Y. Y. Nga, C. Paludan-Müller, and L. Grama.** 2009. Survival and growth of *Salmonella* and *Vibrio* in som-fak, a Thai low-salt garlic containing fermented fish product. Int. J. Food Microbiol. **134:**223-229.

6. **CDC.** 2006. *Salmonella* annual summary 2006. *In* U. D. o. H. a. H. Services (ed.), Atlanta, Georgia.

7. **Chao, W. L., R. J. Ding, and R. S. Chen.** 1987. Survival of pathogenic bacteria in environmental microcosms. Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi **20:**339-348.

8. **Cherry, W. B., J. B. Hanks, B. M. Thomason, A. M. Murlin, J. W. Biddle, and J. M. Croom.** 1972. Salmonellae as an index of pollution of surface waters. Appl. Microbiol. **24:**334-340.

9. **Cho, S. H., J. Y. Jo, and D. S. Kim.** 2001. Effects of variable feed allowance with constant energy and ratio of energy to protein in a diet for constant protein input on the growth of common carp *Cyprinus carpio L*. Aquac Res. **32:**349-356.

10. **Daims, H., A. Brühl, R. Amann, K. H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434-444.

11. **Dalsgaard, A.** 1998. The occurrence of human pathogenic *Vibrio* spp. and *Salmonella* in aquaclture. Int. J. Food Sci. Tech. **33:**127-138.

12. **El'piner, I. E.** 1964. Ultrasound: physical, chemical, and biological effects. Consultants Bureau, New York.

13. **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. J. Food Prot. **66:**723-731.

14. **Foltz, V.** 1969. *Salmonella* ecology. J. Am. Oil Chem. Soc. **46:**222-224.

15. **Gaertner, J., J. A. Mendoza, M. R. J. Forstner, and D. Hahn.** 2011. Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. J. Water Health *in press*.

16. **Gaertner, J., P. E. Wheeler, S. Obafemi, J. Valdez, M. R. Forstner, T. H. Bonner, and D. Hahn.** 2008. Detection of salmonellae from fish in a natural river system. J. Aquac. Animal Health **20:**150-157.

17. **Gattuso, J. P., S. Peduzzi, M. D. Pizay, and M. Tonolla.** 2002. Changes in freshwater bacterial community composition during measurements of microbial and community respiration. J. Plankton Res. **24:**1197-1206.

18. **Gildberg, A., and H. Mikkelsen.** 1998. Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum*. Aquaculture **167:**103-113.

19. **Gildberg, A., H. Mikkelsen, E. Sandaker, and E. Ringø.** 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). Hydrobiologia **352:**279-285.

20. **Goldstein, R. M., and T. P. Simon.** 1999. Toward a united definition of guild structure for feeding ecology of North American freshwater fishes. CRC Press, Boca Raton.

21. **Gomez-Gil, B., A. Roque, and J. F. Turnbull.** 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture **191:**259-270.

22. **Gray, L. D., P. R. Murray, E. J. Baron, M. A. Pfaller, A. F. C. Tenover, and R. H. Yolken.** 1995. *Escherichia, Salmonella, Shigella,* and *Yersina*, 6th ed. ASM Press, Washington D.C.

23. **Groeger, A. W., P. F. Brown, T. E. Tietjen, and T. C. Kelsey.** 1997. Water quality of the San Marcos River, Texas. Texas J. Sci. **49:**279-294.

24. **Guard-Petter, J.** 2001. The chicken, the egg and *Salmonella enteritidis*. Environ. Microbiol. **3:**421-430.

25. **Guardabassi, L., A. Dalsgaard, M. Raffatellu, and J. E. Olsen.** 2000. Increase in the prevalence of oxolinic acid resistant *Acinetobacter* spp. observed in a stream receiving the effluent from a freshwater trout farm following the treatment with oxolinic acid-medicated feed. Aquaculture **188:**205-218.

26. **Hahn, D., J. Gaertner, M. R. Forstner, and F. L. Rose.** 2007. High-resolution analysis of salmonellae from turtles within a headwater spring ecosystem. FEMS Microbiol. Ecol. **60:**148-155.

27. **Holben, W. E., P. Williams, M. Saarinen, L. K. Sarkilahti, and J. H. A. Apajalahti.** 2002. Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. Microbiol. Ecol. **44:**175-185.

28. **Hudson, J. J., J. C. Roff, and B. K. Burnison.** 1992. Bacterial productivity in forested and open streams in southern Ontario. Can. J. Fish Aquac. Sci. **49:**2412-2422.

29. **Irianto, A., and B. Austin.** 2002. Probiotics in aquaculture. J. Fish Dis. **25:**633-642.

30. **Kaushik, S. J., and I. Seiliez.** 2010. Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquac. Res. **41:**322-332.

31. **Khan, A. A., M. S. Nawaz, S. A. Khan, and C. E. Cerniglia.** 2000. Detection of multidrug-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. FEMS Microbiol. Lett. **182:**355-360.

32. **Kim, D. H., and B. Austin.** 2006. Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. Fish Shellfish Immun. **21:**513-524.

33. **Kuwae, T., and Y. Hosokawa.** 1999. Determination of abundance and biovolume of bacteria in sediments by dual staining with 4 ',6-diamidino-2-phenylindole and acridine orange: Relationship to dispersion treatment and sediment characteristics. Appl. Environ. Microbiol. **65:**3407-3412.

34. **Lawton, R. L., and E. V. Morse.** 1980. *Salmonella* survival in freshwater and experimental infections in goldfish (*Carassius auratus*). J. Environ. Sci. Health **4:**339-358.

35. **Lesel, R., and P. Le Gac.** 1983. Transit of Enterobacteria originating from homeotherms in fish living at low temperature. Aquaculture **31:**109-115.

36. **Li, M. H. H., B. C. Peterson, C. L. Janes, and E. H. Robinson.** 2006. Comparison of diets containing various fish meal levels on growth performance, body composition, and insulin-like growth factor-I of juvenile channel catfish *Ictalurus punctatus* of different strains. Aquaculture **253:**628-635.

37. **Liu, Y., Z. Zhou, B. Yao, S. He, L. B. Hølvold, and E. Ringø.** 2008. Effect of intraperitoneal injection of immunostimulatory substances on allochthonous gut microbiota of Atlantic salmon (*Salmo salar L.*) determined using denaturing gradient gel electrophoresis. Aquac. Res. **39:**635-646.

38. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl. Environ. Microbiol. **69:**290-296.

39. **Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer.** 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. Microbiology **142:**1097-1106.

40. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593-600.

41. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607-625.

42. **Meier, H., R. Amann, W. Ludwig, and K. H. Schleifer.** 1999. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+ C content. Syst. Appl. Microbiol. **22:**186-196.

43. **Nakano, S. I., and Z. Kawabata.** 2000. Changes in cell volume of bacteria and heterotrophic nanoflagellates in a hypereutrophic pond. Hydrobiologia **428:**197-203.

44. **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. J. Clin. Microbiol. **35:**2642-2648.

45. **Organization, W. H.** 2002. World health report: reducing risks, promoting healthy life.

46. **Pal, A., and D. L. Marshall.** 2009. Comparison of culture media for enrichment and isolation of *Salmonella* spp. from frozen Channel catfish and Vietnamese basa fillets. Food Microbiol. **26:**317-319.

47. **Polo, F., M. J. Figueras, I. Inza, J. Sala, J. M. Fleisher, and J. Guarro.** 1998. Relationship between presence of *Salmonella* and indicators of fecal pollution in aquatic habitats. FEMS Microbiol. Lett. **160:**253-256.

48. **Rabus, R., M. Fukui, H. Wilkes, and F. Widdle.** 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. **62:**3605-3613.

49. **Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, and R. Curtiss.** 1992. Amplification of an *inv*A gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell Probe **6:**271-279.

50. **Refsum, T., E. Heir, G. Kapperud, T. Vardund, and G. Holstad.** 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.

51. **Richardson, D. J., and B. B. Nickol.** 1999. Physiological attributes of the pyloric caeca and anterior intestine of green sunfish (*Lepomis cyanellus*) potentially influencing microhabitat specificity of *Leptorhynchoides thecatus* (Acanthocephala). Comp. Biochem. Phys. A. **122:**375-384.

52. **Roller, C., M. Wagner, R. Amann, W. Ludwig, and K. H. Schleifer.** 1994. *In situ* probing of Gram-positive bacteria with high DNA G+ C content using 23S rRNA-targeted oligonucleotides. Microbiology **140:**2849-2858.

53. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Mol. Biol. R. **51:**365-379.

54. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

55. **Schmidt, A. S., M. S. Bruun, I. Dalsgaard, K. Pedersen, and J. L. Larsen.** 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. **66:**4908-4915.

56. **Sha, Q., A. Gunathilake, M. R. J. Forstner, and D. Hahn.** 2011. Temporal analysis of the distribution and diversity of *Salmonella* in natural biofilms. Syst. Appl. Microbiol. *in press*.

57. **Spanggaard, B., F. Jørgensen, L. Gram, and H. H. Huss.** 1993. Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture **115:**195-207.

58. **Stahl, D. A., and R. I. Amann.** 1991. Development and application of nucleic acid probes, p. 205-248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

59. **Suarez, M., and H. Russmann.** 1998. Molecular mechanisms of *Salmonella* invasion: the type III secretion system of the pathogenicity island 1. Int. Microbiol. **1:**197-204.

60. **Tavechio, A. T., A. C. R. Ghilardi, J. T. M. Peresi, T. O. Fuzihara, E. K. Yonamine, M. Jakabi, and S. A. Fernandes.** 2002. Serotypes isolated from nonhuman sources in Sao Paulo, Brazil, from 1996 through 2000. J. Food Protect. **65:**1041-1044.

61. **Thomason, B. M., J. W. Biddle, and W. B. Cherry.** 1975. Detection of salmonellae in the environment. Appl. Environ. Microbiol. **30:**764-767.

62. **Tiunov, A. V., and S. Scheu.** 1999. Microbial respiration, biomass, biovolume and nutrient status in burrow walls of *Lumbricus terrestris L*. (Lumbricidae). Soil Biol. Biochem. **31:**2039-2048.

63. **Tocher, D. R., E. A. Bendiksen, P. J. Campbell, and J. G. Bell.** 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture **280:**21-34.

64. **Verschuere, L., G. Rombaut, P. Sorgeloos, and W. Verstraete.** 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. R. **64:**655-671.

65. **Wong, M., and J. Medrano.** 2005. Real-time PCR for mRNA quantitation. Biotechniques **39:**75.

66. **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. Arch. Microbiol. **168:**185-192.

67. **Zhou, Z., S. He, Y. Liu, P. Shi, G. Huang, and B. Yao.** 2009. The effects of dietary yeast culture or short-chain fructo-oligosaccharides on the intestinal autochthonous bacterial communities in juvenile hybrid tilapia, *Oreochromis niloticus*♀×*Oreochromis aureus*♂. J. World Aquacult. Soc. **40:**450-459.

68. **Zhou, Z., Y. Liu, S. He, P. Shi, X. Gao, B. Yao, and E. Ring** 2009. Effects of dietary potassium diformate (KDF) on growth performance, feed conversion and intestinal bacterial community of hybrid tilapia (*Oreochromis niloticus* [♀]× *O. aureus* [♂]). Aquaculture **291:**89-94.

69. **Zhou, Z., Y. Liu, P. Shi, S. He, B. Yao, and E. Ring** 2009. Molecular characterization of the autochthonous microbiota in the gastrointestinal tract of adult yellow grouper (*Epinephelus awoara*) cultured in cages. Aquaculture **286:**184-189.

70. **Zhou, Z., P. Shi, S. He, Y. Liu, G. Huang, B. Yao, and E. Ringø.** 2009. Identification of adherent microbiota in the stomach and intestine of emperor red snapper (*Lutjanus sebae* Cuvier) using 16S rDNA-DGGE. Aquac. Res. **40:**1213-1218.

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