EVALUATING THE EFFICACY OF BAQUACIL® AGAINST *Salmonella* sp. IN THE AQUATIC HABITAT OF THE RED-EARED SLIDER (*Trachemys scripta elegans*)

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Abstract:

Turtle-associated salmonellosis (TAS) in humans has been a concern of public health officials since the 1960's. The rising incidence of TAS in young children during the late 1960's and early 1970's eventually led to the implementation of inter- and intrastate regulations on the sale of chelonians less than 10.2-cm in length in the United States of America. Although attempts to eliminate *Salmonella* spp. in chelonians using antibiotics have been made, they have not been successful in reversing the current regulations. Baquacil® is a commercial algistat and microbicide. Fifty-five red-eared slider (RES) turtles (*Trachemys scripta elegans*) were used to evaluate the efficacy of Baquacil® as a method to suppress *Salmonella* sp. in RES habitat. The RES were maintained individually in plastic containers that contained either chlorinated tap water (n=15), dechlorinated tap water and 25 ppm Baquacil®, or dechlorinated tap water and 50 ppm Baquacil®. Water samples were collected from each RES container three times per week for one month and cultured for *Salmonella* sp.. Water samples collected from the RES housed in Baquacil® were less likely to be *Salmonella*-positive than those in the control group (p<0.001). There was no difference in the *Salmonella* status of the water samples between the 25 and 50 ppm treatment groups. At the conclusion of the study, the intestinal tracts of the RES were cultured for *Salmonella* sp.. There was no difference in the *Salmonella* status of the intestinal cultures collected from any of the RES at necropsy (p=0.8). No pathological lesions were found to be associated with swimming RES in 50 ppm Baquacil® for one month. Baquacil® may be used to suppress *Salmonella* sp. in the water column of RES.

Introduction

Salmonella Newport was first isolated from a chelonian, Geochelone giganteas, by McNeil and Hinshaw in 1946. It wasn't until almost seven years later that the first case of turtle-associated salmonellosis (TAS) in a human was reported (Boycott, et al, 1953). The apparent incidence of TAS continued to increase over the next two decades (Williams and Heldson, 1965). The public health concern for TAS during this period was limited, but would change with the increased incidence of cases reported in young children. The first case of TAS in a child was reported in 1963 (Hersey and Mason, 1963). Salmonella Hartford was recovered from a 7-month old infant with diarrhea, vomiting, and fever. An investigation of the infant's environment resulted in the isolation of the same serotype from the family's pet turtle.

The increased incidence of TAS in children during the late 1960's and early 1970's became an important concern to both state and federal health officials. In 1972, the Food and Drug Administration required certification verifying Salmonella-free status for the interstate transport of pet turtles. This program was found to be ineffective. A study conducted by the Center for Disease Control concluded that 38% of the animals certified to be Salmonella-free were contaminated (CDC, 1974).

Therefore, in 1975, the Food and Drug Administration restricted the intra- and interstate sales of all turtle eggs and live turtles with a carapace length less than 10.2-cm. The decision to restrict the sale of turtles with a carapace length less than 10.2-cm was based on the assumption that these animals would be less desirable to young children. Enforcement of this policy resulted in a 77% reduction in the incidence of cases in those states not producing turtles (Cohen, *et al*, 1980).

Exceptions to this 1975 law were made for marine turtles and educational and scientific institutions. Violators of this law are provided a written demand to destroy the animals under FDA supervision within 10 days. Violators are also subject to a fine not more than $1000 and/or imprisonment of not more than one year for each violation. Herpetoculturists that breed exotic chelonians have avoided prosecution by selling animals under the guise of educational animals. Currently, there are several hundred reptile swap meets a year in the U.S. where chelonians with a carapace length less than 10.2-cm can be purchased. In addition, chelonians less than 10.2-cm in length are routinely sold via the internet. The availability of these chelonians, in combination with the apparent public health concern for TAS, suggest that there is a need to develop methods to minimize *Salmonella* spp. in the chelonians or their environment to minimize the health risks associated with pet reptile ownership.

Attempts to reduce or eliminate *Salmonella* spp. in turtles with antimicrobials were initiated after the FDA regulation was implemented in 1975. Treatment of hatchlings with oxytetracycline in their tank water for up to 14 days

alleviated shedding in treated turtles, but did not affect systemic infection (Siebling, *et al*, 1975). Treatment of the freshly laid eggs with oxytetracycline or chloramphenicol with a temperature differential egg dip method was also successful at eliminating *Salmonella* spp. in eggs less than one day old, but did not clear eggs greater than two days old (Siebling, *et al*, 1975). Large-scale experimentation on commercial turtle farms with surface decontamination and pressure or temperature differential treatment of eggs with gentamicin dip solutions for eggs greater than 2 days old, followed by hatching the eggs on *Salmonella*-free bedding, substantially reduced *Salmonella* sp. infections and shedding rates in hatchling turtles (Siebling, *et al*, 1984). Forty percent of the eggs not treated with the gentamicin were found to harbor *Salmonella* sp., whereas only 0.15% of the treated eggs were positive. Legislative implementation of this concurrent method of surface decontamination and gentamicin treatment by the Louisiana Department of Agriculture in 1985 was hailed as victory by turtle farmers.

Unfortunately, the use of gentamicin and the other antimicrobials has led to an even greater concern due to the development and persistence of antimicrobial resistant strains of *Salmonella* sp.. The occurrence of *Salmonella* sp. in red-eared turtles (RES)(*Trachemys scripta elegans*) eggs exported to Canada from four different Louisiana turtle farms in 1988 was examined, and of the 28 lots tested, six (21%) lots from three of four exporters were *Salmonella* sp. positive (D'Aoust, *et al* , 1990). Of the 37 *Salmonella* strains isolated, 30 (81%) were gentamicin resistant (D'Aoust, *et al*, 1990). Similar results have been

reported from samples collected directly from the farms in Louisiana. Shane et al. (1990) collected environmental samples and live hatchlings directly from two Louisiana turtle farms. Isolates of S. Arizonae and *S.* Poona collected from turtles at one of the farms were resistant to erythromycin, gentamicin, tetracycline, and sulfonamide. Pond water samples from both farms showed similar antimicrobial resistant patterns to erythromycin. In 1988, 115 batches of turtle hatchlings were submitted from 28 farms to the Louisiana Department of Agriculture and Forestry for analysis (Shane, *et al*, 1990). Five (4.3%) *Salmonella* isolates were obtained. Four of the organisms were submitted for serotyping; three were *S.* Arizona and one was *S.* Poona. All four isolates were resistant to erythromycin, gentamicin, tetracycline, and sulfonamide. The findings of these studies suggest that the application of a single intervention, such as egg washing, will not be sufficient to suppress or eliminate *Salmonella* sp. from captive bred chelonians.

To successfully manage *Salmonella* sp. in chelonians, a series of interventions will be necessary. A methodical approach comprised of treatment interventions that reduce S*almonella* sp. in adult breeding chelonians, reduce S*almonella* sp. contamination of the egg, reduce *Salmonella* sp. colonization of the hatchling, and reduce S*almonella* sp. dissemination in the environment are required. Each of these management schemes will require extensive research. The purpose of this research was to evaluate a specific method of reducing S*almonella* sp. in the environment of hatchling chelonians.

Polyhexamethylene biguanide is a sanitizing agent that is considered safe for human and animal use. This compound has been used as a mouth rinse for

humans (Rosin, *et al*, 2001), a microbicide for chicken eggs (Cox, *et al*, 1994), and a treatment for protozoal fungal keratitis (Panda, *et al*, 2003). The antimicrobial effect of this compound varies with concentration, being bacteriostatic at low concentrations and bacteriocidal at higher concentrations. There are many different derivatives of polyhexamethylene biguanide. One such derivative, Baquacil® (poly-iminoimidocarbonylimino- hexamethylene hydro chloride)(Avecia Inc., Wilmington, DE 19850 USA), is a commercial swimming pool sanitizer and algistat. This product is used as a safe alternative to chlorine for swimming pools. Because Baquacil® is considered safe for humans, its application as a microbicide may prove useful for captive chelonians.

The primary objective of this study was to determine if Baquacil® would suppress or eliminate *Salmonella* sp. in the aquatic habitat of the RES. The specific hypotheses being tested were:

1. *Salmonella* sp. is less likely to be isolated from water samples collected from RES habitats with Baquacil® than from those without Baquacil®.

2. There will be no difference in the *Salmonella* sp. status of water samples collected from water treated with 25 or 50 ppm Baquacil®.

3. There will be no difference in the prevalence of (intestinal) *Salmonella* sp. between treatment (Baquacil®) and control (no Baquacil®) groups at necropsy.

4. There will be no difference in the frequency of *Salmonella* sp. positive cultures between the original and delayed secondary enrichment samples.

Materials and Methods

This study was conducted in accordance with the regulations specified by the Louisiana State University Institutional Animal Care and Use Committee (03-004). Fifty-five hatchling *Salmonella*-positive RES were used for this study. The RES were housed in individual plastic containers with approximately 1 liter of chlorinated water or a dechlorinated water and Baquacil® (25 or 50 ppm) solution. The RES were randomly assigned to three different treatment groups using a random generator: Group 1) 25 ppm Baquacil® (n=20), Group 2) 50 ppm Baquacil® (n=20), and Group 3) chlorinated tap water (n=15). Baquacil® concentration, water pH, and alkalinity were determined using Aquachek pool and spa test strips (Environmental Test Systems, Inc., Elkhart, IN 46514). The ambient air temperature was maintained between 24.4-26.7^{o}C (76-80°F), and the water temperature maintained between 21.1-22.8 ^{o}C (70-73 ^{o}F). The RES were provided a photoperiod comprised of 12 hours of light and 12 hours of darkness. The pH of the water was adjusted to 7.5 and the alkalinity maintained between 80-120 ppm. The water from the enclosures was changed weekly, and fresh Baquacil® solution was made weekly. The RES were offered a *Salmonella*-free commercial turtle pellet food (Fluker Farms, Port Allen, LA 70767) daily.

Water samples were collected three times a week for four weeks and evaluated for *Salmonella* sp. using standard microbiological techniques. A total of 3-ml of water was collected from the center of the enclosure, where organic material accumulated, and added to 7-ml of selenite enrichment broth. The samples were incubated at 37°C for 48 hours under aerobic conditions. After

incubation, the enriched selenite cultures were mixed on a Vortex agitator for 5 seconds. A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-tergitol agar (XLT-4) (Remel, Lenexa, KS 66215). Streaked plates were incubated at 37°C for 48 hours under aerobic conditions. Presumptive *Salmonella* sp. colonies were evaluated on indicator media including urea, lysine iron agar (LIA), and triple iron agar (TSI). A heat-sterilized bacterial loop was used to streak a portion of a suspect colony onto slants of urea, LIA and TSI agar, and preparations were incubated aerobically at 37°C for 24 hours. The presence of *Salmonella* sp. was denoted by a negative urea test, positive LIA with hydrogen sulfide production (H$_2$S), and alkaline over acid with H$_2$S in the TSI. Presumptive *Salmonella* colonies were further evaluated with API 20E Test Strips® (bioMerieux Vitek, Inc., Hazelwood, MO 63042). A heat-sterilized bacterial loop was used to transfer sample colonies from the TSI slant to 10 ml of 0.85% saline to attain a concentration equivalent to a 0.5 McFarland's equivalence turbidity standard (Remel, Lenexa, KS 66215). Samples were placed into designated receptacles on the test strips in accordance with the manufacturer's directions and incubated aerobically at 37°C for 24 hours. Bacterial reactions were interpreted with the appropriate key compiled by the manufacturer of the test strip.

A 96-hour delayed secondary enrichment (DSE) was performed on all of the samples to increase the likelihood of identifying samples with low concentrations of *Salmonella* sp.. The original selenite samples were placed at room temperature for 96 hours. A 3-ml aliquot of the original selenite sample was

added to 7-ml of selenite enrichment broth and incubated aerobically at 37°C for 48 hours. The samples were processed using the techniques described previously.

At the conclusion of the 4-week trial, the RES were humanely euthanized using an overdose of a barbiturate (0.05 ml/turtle, intracardiac)(Beuthanasia, Schering Plough Animal Health Care, Union NJ 07083). A gross necropsy was performed using sterile techniques. Scissors were used to cut the bridge of the shell and facilitate the removal of the plastron. The gastrointestinal tract was excised, placed into 7-ml of selenite broth, and vortexed to macerate the tissues. The samples were incubated aerobically at 37°C for 48 hours, and the samples processed using the techniques described previously. A 96-hour delayed secondary enrichment was also performed with the necropsy specimens. The carcasses of the RES from the control group and the 50 ppm Baquacil® group were placed into 10% neutral buffered formalin and submitted for histopathology. Tissue samples, including sections of the skin and carapace, the eyes, conjunctiva, and oral mucosa, were taken from six randomly selected RES from the control group and from six turtles in the 50 ppm Baquacil® treatment group. The tissues were processed routinely, sectioned (4 µm thickness), stained with hematoxylin and eosin, and examined microscopically.

The sample size required for this study was calculated under the following assumptions and criteria: that the proportion of *Salmonella*-positive RES in the control group would be > 0.7, and that the proportion of *Salmonella*-positive RES in the Baquacil group would be < 0.2, that the $\alpha = 0.05$, and the power = 0.89.

The 95% binomial confidence intervals (CI) were calculated for each of the proportion estimates. In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by van Belle and Millard (1998). The first hypothesis tested in this study was that turtles housed in a Baquacil® solution would be less likely to be *Salmonella*-positive than those housed in chlorinated tap water (H_0: $\mu_B = \mu_{NB}$; H_1: $\mu_B < \mu_{NB}$). A Cochran's Q test was used to determine if there was a difference within-subjects for the RES. A second hypothesis was tested to determine if there was a difference in the *Salmonella* status of the water samples collected from RES in 25 ppm and 50 ppm Baquacil® (H_0: $\mu_{B50} = \mu_{B25}$; H_1: $\mu_{B50} < \mu_{B25}$). The 95% CI for the two groups were compared by day of week to determine if a difference existed. A third hypothesis was evaluated to determine if *Salmonella* sp. would be detected at a higher frequency from Baquacil® vs. non- Baquacil® turtles at necropsy (H_0: $\mu_B = \mu_{NB}$; H_1: $\mu_B < \mu_{NB}$). Fisher's exact test was used to compare data when an expected cell value was < 5. The final hypothesis tested for this study was that there would be no difference in the frequency of *Salmonella* sp. between the original and DSE cultures (H_0: $\mu_O = \mu_{DSE}$; H_1: $\mu_O < \mu_{DSE}$). McNemar's test was used to determine if there was a difference in the prevalence of *Salmonella* sp. isolation between the original and delayed secondary enrichment cultures. Values of $p < 0.05$ were considered statistically different. Statistical analysis was performed using SPSS 11.0 (SPSS, Inc., Chicago, IL 60606).

Results

Salmonella sp. was detected in the water column from each group at least once during the study (Table 1). Only one RES in the control group was Salmonella sp. positive on every culture. There was a significant within-groups difference over time (Cochran's Q: 138.2, p<0.001). The prevalence of Salmonella sp. in the water column of the control RES sliders was significantly higher than in the treatment groups (Figure 1). There was no difference in the Salmonella-status (95% CI) of RES between the 25 and 50 ppm treatment groups, nor was there an apparent difference based on the day of week (95% CI) the sample was collected. There was no significant difference (p=0.8) in the Salmonella-status of the RES from the three treatment groups at necropsy (Control group: 80%, 95% CI: 60-100; 25 ppm: 70%, 95% CI: 50-90; 50 ppm: 75%, 95% CI: 56-94). There was a significant difference (p=0.001) in the prevalence of Salmonella sp. between the original (19%, 113/594) and DSE (14%, 83/594) cultures. Overall, there was an 11.7% (70/594) disagreement between the two groups. When comparing all samples, 52% (74/141) of the cultures were positive on the original and DSE cultures, while only 36% (51/141) were positive on the original culture and 11% (16/141) positive on the DSE. Interestingly, the frequency of DSE culture-positive only samples was greatest in the 50 (36%, 5/14) and 25 (29%, 5/17) ppm treatment groups.

Histologically, the eyes, corneas, eyelids, tongue, oral mucosa, skin, and carapace of the 50 ppm Baquacil® treated RES were no different than those of the control group. In both groups, the eyelids and corneas were uniformly thick

with normal epithelial cells and no evidence of degeneration, erosion or inflammation. Internal ocular structures were within normal limits. Likewise, the oral mucosa, tongue, skin, and carapace and underlying structures were normal in both groups.

Discussion

The propagation of *Salmonella*-free chelonians is a primary objective for chelonian producers, and is a concern of public health officials. Currently, chelonian eggs are sanitized using a combination of washing with water or sodium hypochlorite solution and temperature or pressure differential treatment using gentamicin. Although the apparent prevalence of *Salmonella* sp. in these eggs is very low, occasional cases of antimicrobial resistant *Salmonella* sp. are reported (D'Aoust, *et al*, 1990; Shane, *et al*, 1990). In addition, once the chelonians are removed from a "clean environment" and disseminated into the pet retail trade, the industry has no control method in place to prevent the re-colonization of a chelonian or suppress/eliminate shedding in *Salmonella*-positive chelonians.

Polyhexamethylene biguanide has been found to be effective at eliminating experimentally inoculated *Salmonella* Typhimurium from the surface of chicken eggs. In a study to evaluate the efficacy of several different microbicides, including quaternary ammonium, peroxygen compounds, hydrogen peroxide, ethylene oxide, phenols, and sodium and potassium hydroxide, only the polyhexamethylene biguanide (0.035%, Cosmocil CQ, ICI Americas, Inc.,

Wilmington, DE 19897) product was 100% effective at eliminating the salmonellae (Cox,*et al*, 1994). The results of this study also indicate that polyhexamethylene biguanide products have some effect against reptile serotypes too.

Because hatchling chelonians serve as the primary source of infection for pet owners, neonates are a logical starting point for programs focused on suppressing or eliminating *Salmonella* sp.. The primary objective of this study was to determine if Baquacil® could be used in a bath to suppress or eliminate *Salmonella* sp. in hatchling RES. The findings of this study suggest that Baquacil® can suppress *Salmonella* sp. in the water column of RES. Although this product was not 100% effective, it did significantly reduce the presence of *Salmonella* sp. in the water column. The use of this product was tested in *Salmonella*-positive turtles and may prove even more beneficial as a method of control in cases where RES are *Salmonella*-free.

The variable prevalence of *Salmonella* sp. in these RES, especially the control group, was not unexpected. Transient shedding of *Salmonella* sp. has been documented previously in reptiles (Mitchell, 2001). Inappropriate environmental conditions and other causes of physiologic stress may increase the rate of shedding. Historically, the management of RES in captivity was unacceptable. The public health concern identified in the 1960's and 1970's was likely the result of a limited understanding of the husbandry requirements of these animals. In the case of this study, even though the RES were housed in simple enclosures, the prevalence of *Salmonella* sp. in the water column never

approached 100%. These finding suggest that, even in the control group, that the risk of contracting TAS is not constant. Providing an appropriate environment and diet, in addition to using a sanitizing agent such as Baquacil®, may reduce the zoonotic health risk associated with these reptiles.

Because the purpose of this study was to determine the prevalence of *Salmonella* sp. in the habitats of RES at given time points, an enrichment broth and DSE were used to increase the likelihood of isolating the organism. Attempts to isolate *Salmonella* sp. without enrichment may result in misclassification (false-negatives). However, enrichment broth may also mask the true risk associated with this organism. In general, approximately 10^3-10^6 *Salmonella* organisms are required to infect a human. There are a number of factors that can affect a human's susceptibility to contracting TAS, including age, previous exposure and immune status. Based on the findings of this study, a large number of the positive isolates identified in the treatment groups were only characterized after DSE, suggesting the actual number of organisms in the water column may be small. Additional research evaluating the numbers of organisms in the treated water samples without enrichment confirms that they are $< 10^3$ (Mitchell, unpublished research).

There was no significant difference in the *Salmonella* status of the RES at necropsy. The similar prevalence of *Salmonella* between the three RES groups suggests that Baquacil® has no effect on the colonization of *Salmonella* in these animals. However, it was interesting to note that a percentage (control: 20%, 25 ppm: 30%, 50 ppm: 25%) of these turtles were *Salmonella*-negative at the time of

the necropsy. This finding may have been the result of using microbiological culture as the method of detection. Mitchell (2001) estimated the sensitivity of microbiological culture as a method of detecting *Salmonella* sp. in green iguanas (*Iguana iguana*) to be approximately 70%. This would suggest that approximately 3/10 positive samples could be misclassified as false negative samples. In addition, if these animals were harboring low numbers of salmonellae, the assay could have been insufficient to detect them. However, DSE was used to increase the likelihood of detecting low numbers of *Salmonella* sp.. Another possible explanation for this finding is that the RES could have self-cleared the *Salmonella* sp.. *Salmonella* sp. infections and carrier states are generally considered to be self-limiting in mammals, and persistent in reptiles. Based on these findings, this may not be the case, and some reptiles may also self-clear or not remain colonized.

There were no significant histologic lesions in the RES treated with 50 ppm Baquacil®. The high dose (50 ppm) used for this study is similar to that recommended for the treatment of swimming pools and is lower than that used to treat experimental fungal keratitis in rabbits (Panda, *et al*, 2003). Because the gastrointestinal tracts were removed from these animals for culture, we were unable to evaluate them for pathologic changes. However, the eyes, palpebrae, oral cavity, skin and shell were evaluated and lacked microscopic evidence of damage. Future studies should evaluate longer-term exposure to Baquacil® and complete necropsies should be performed to determine if there are any pathologic lesions associated with the ingestion of Baquacil® treated water.

Conclusions

The results of this study are promising and serve as an initial step in the development of an intervention plan for the management of *Salmonella*-positive chelonians. Additional long-term studies to evaluate the effects of this compound in different species of chelonians and on specific reptile *Salmonella* serotypes should be pursued. In addition, studies to evaluate the potential for the development of resistance to Baquacil® need to be pursued.

Acknowledgements

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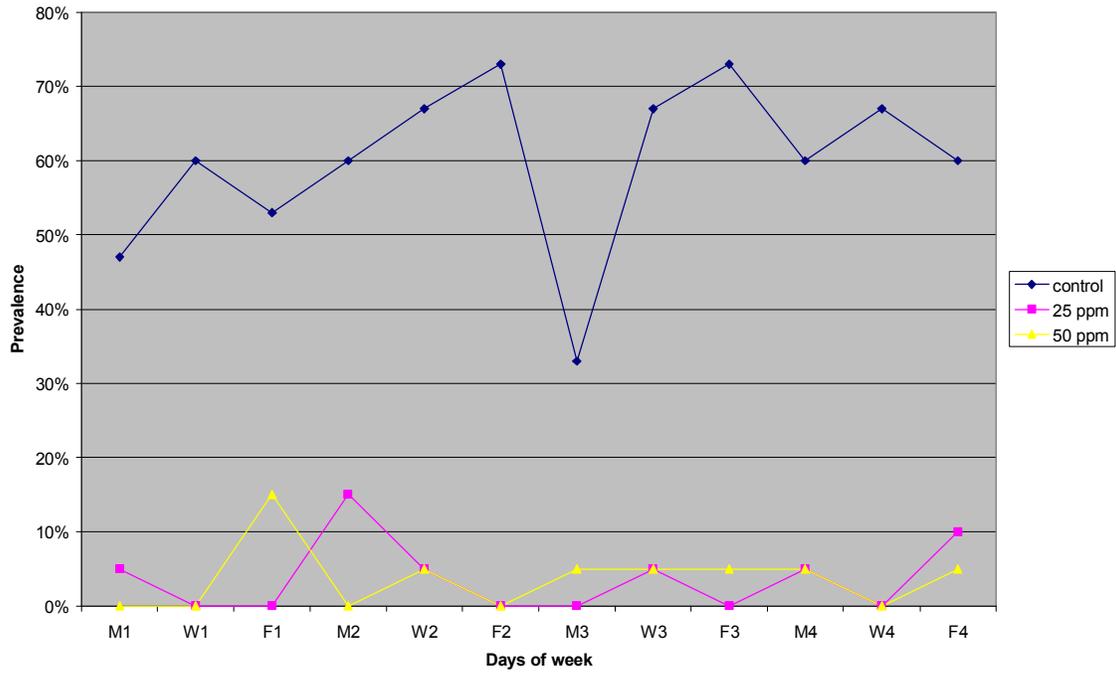**Prevalence of *Salmonella* in RES Habitats**

Figure 1. Prevalence of *Salmonella* sp. in the aquatic habitat of RES. Days of the week are recorded as M, W, and F for Monday, Wednesday and Friday, respectively. The number following each day represents the week of the study (1-4).

Table 1. Prevalence and 95% confidence intervals (in parentheses) of *Salmonella* sp. in the water samples collected from RES habitats during the study.

Week 1	Day 2	Day 4	Day 6
Control	47% (22-73)	60% (35-85)	53% (28-88)
25 ppm	5% (0-14)	0 (0-15)	0 (0-15)
50 ppm	0 (0-15)	0 (0-15)	15% (0-15)
Week 2	**Day 2**	**Day 4**	**Day 6**
Control	60% (35-85)	40% (15-65)	73% (50-96)
25 ppm	15% (0-30)	10% (0-23)	0 (0-15)
50 ppm	0 (0-15)	5% (0-14)	0 (0-15)
Week 3	**Day 2**	**Day 4**	**Day 6**
Control	47% (22-72)	67% (43-91)	67% (43-91)
25 ppm	5% (0-14)	5% (0-14)	0 (0-15)
50 ppm	5% (0-14)	5% (0-14)	5% (0-14)
Week 4	**Day 2**	**Day 4**	**Day 6**
Control	60% (35-85)	67% (43-91)	53% (28-88)
25 ppm	5% (0-14)	0 (0-15)	10% (0-23)
50 ppm	5% (0-14)	0 (0-15)	5% (0-14)