ASSESSING FECAL CONTAMINATION AT BRIDGES IN DIFFERENT STREAMS IN CENTRAL TEXAS WITH AND WITHOUT POTENTIAL RUNOFF

by

Jacqueline Hernandez, B.S.

A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
December 2014

Committee Members:

Dittmar Hahn, Chair

Robert J C McLean

Ivan Castro-Arellano

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

DEDICATION

This thesis is dedicated to my mom and dad. They have supported me through every step of my education, and this could not have been done without their support. They have always taught me that education is the most valuable asset. Thank you for the valuable lessons you have taught me and being there for me through it all. I love you!

ACKNOWLEDGEMENTS

I would like thank Dr. Hahn, chair of my committee, for allowing me the opportunity to further my education and skills. He first helped me gain valuable skills through undergraduate research and then helped guide me through my thesis research. Without him, I wouldn't be accomplishing my degree.

I would also like to thank Dr. McLean for all of his support and guidance through both my undergraduate and graduate studies, as well as for being part of my committee. He has given me the opportunity to be an instructional assistant for him, through which I have learned a lot.

I would also like to thank Dr. Castro-Arellano for sitting on my committee and helping with my thesis work. He has given me great advice on my studies and on my research. He has also helped me gain valuable field work skills and experience.

I would like to thank Dr. Green for all of his help with getting my research started out in the field. He has also helped us with field work when it was needed.

I would like to also thank Dr. Weckerly for all of his help with the statistical analysis work. He was a great statistics professor, and he helped me when I was not in his class.

I would like to thank Lorissa DiGiacomo for all of her help out in the field and for being my field buddy. Without her, my field work would not have been as pleasant or done in a timely manner. We had scary moments but we were always there for each other and had great laughs afterward. She helped me identify the different types of birds present at

the bridges and helped watch out for snakes.

I would also like to thank Kyle Winkler for all of his help out in the field. He was my backup field buddy when Lorissa was unavailable or when we needed extra help. He helped me in tough field conditions without any complaints, and I am very grateful. He also supported me throughout my undergraduate and graduate studies. He's always been there for me and he's always been a great friend.

I would like to thank Suvi Samant for all her help both in the lab and out in the field. She has been great, helping me get started in the lab and teaching me new protocols. She has also helped me with field work when no one else could, and we have many stories to share. She's always been there when I needed her knowledge and expertise. I am very grateful to have her not only as a labmate but also a very good friend.

I would like to thank Lorissa, Kyle and Suvi for being there for me during my near drowning experiences. As we have learned the hard way, water can be very dangerous after a rain event.

I would also like to thank Trina Guerra for all of her help in the lab. She first started helping me with my lab work and getting it optimized. Then she was always there with her expertise, ready to help, even during those brain stumping moments. She has also for been there for me during the emotional times. I am very lucky to have her as a labmate and as a very good friend.

I would like to thank all my labmates and friends for their support. Not only for their knowledge but also for their emotional support. They have been there through my

most stressful moments and given me great advice.

I would like to thank the Texas Department of Transportation for their funding. They have been generous enough to finance my research and my education for the extent of the project.

I would also like to thank my family for all of their support. They have stood by me through it all and have given me great advice. I love you all very much and am very grateful to have you in my life.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

ASSESSING FECAL CONTAMINATION AT BRIDGES IN DIFFERENT STREAMS IN CENTRAL TEXAS WITH AND WITHOUT POTENTIAL RUNOFF

Introduction

The potential contamination of surface waters by pathogens is important to human health due to the recreational uses that these waters provide. The quality of surface waters is impacted by a variety of sources, which includes humans, domestic animals, wildlife, and environmental factors such as precipitation and agriculture (1). Contamination levels might be affected by the location of these waters, and the severity associated with the type of pathogens present. One of the primary sources of microbial pollution in agricultural watersheds is livestock (2). In these watersheds some common point sources may be storage facilities and feedlots, while non-point sources comprise of grazed pastures and rangelands (2). Even in rural watersheds not all microbial pollution comes from agricultural sources; other potential sources include failing septic systems and wildlife (2). Bridges and other man-made structures could play an important role in the contamination risk of surface waters by aiding in the presence of animals and humans in these waters, or providing physical structures that accumulate and direct surface runoff into surface waters after large precipitation events.

Bridges have been known to provide habitats for roosting animals and these man-made habitats have contributed to the range expansion of some birds such as Cliff Swallows (*Petrochelidon pyrrhonota*) (3). Cliff Swallows nest in colonies that may

comprise of up to 3,500 active nests. Fecal deposition from birds has been considered as a point-source of pollution contributing to the contamination of surface waters (3, 4). Fecal contamination may also occur in the soil adjacent to surface waters due to nests being present above these soils, and as a consequence of defecating birds leaving their nests. Fecal loads may then be washed into the surface waters after significant rainfall and add to the present contamination (5). Bird feces have been shown to be sources of fecal indicator bacteria (FIB) and pathogens that include *Escherichia coli* (*E. coli*), *Salmonella* sp., *Campylobacter* sp., *Cryptosporidium* sp. or *Giardia* sp. which -once released with feces- can contaminate surface waters or add to present contamination (4, 6, 7, 8). Although bird feces are sources of pathogens, these feces have been considered to be less harmful than those from other animals (e.g. cattle) and humans (9). These pathogens may persist in soil and surface waters, however, survival or viability is often reduced significantly over time (10). The survival of *E. coli* outside of its habitat, i.e. the intestine, for example, is generally low due to the scarce nutrient resources, temperature changes and competition present in secondary environments such as water and soils (11). The persistence of *E. coli* in water, sediment, and soil expressed in net half-life is about 1 day, 1.5 days and 3 days, respectively (11). Therefore, *E. coli* populations found in these environments are usually considered contaminants released into the environment from their habitat (i.e. lower intestinal tract of warm blooded animals) (12).

The most used method for the assessment of fecal contamination is by enumeration of FIB, which includes fecal coliforms, *E. coli*, and enterococci. All are used as indicator organisms for the presence of enteric pathogens in recreational waters. Enumeration is generally performed by growth dependent techniques which result in numbers of target

organisms or Colony Forming Units (CFU) as the unit of quantification (13, 14). According to the Environmental Protection Agency (EPA), numbers of *E. coli* higher than 126 CFU/100 mL of water, and those of enterococci higher than 33 CFU/100 mL in water indicate fecal contamination in these waters (15). While these microorganisms are commonly present in large numbers in both human and animal feces, and thus are easy to detect in assessments of fecal contamination, other microorganisms that are specific for feces of individual animal species are needed for microbial source tracking. Microbial source tracking is a term used to describe the methods used to trace the origin of fecal contamination (16). Instead of monitoring indicator organisms, determination of the presence of pathogens would provide a direct and probably more accurate measure for health risks associated with contamination (16). Because pathogens might be present in very low numbers in environmental samples such as waters, direct detection might be difficult without prior enrichment; however, many of these pathogens have a low infectious dose, and thus fast and accurate quantification of even low level might be advisable (16). Preliminary studies in our lab have indicated that *E. coli* is present only in very small numbers in Cliff Swallow feces, and consequently cannot be used for source tracking. Therefore, studies on the contribution of bird feces to surface water contamination, for example, need to focus on other measures; these might include the comparative quantification of all bacteria present in water samples up- and down-stream of bridges with birds assuming that the difference (i.e. higher numbers down-stream) are contributions of fecal contamination by birds. These contributions might be affected by runoff bridges or adjacent soil after strong precipitation events.

Growth-dependent enumeration of indicator organisms is currently the basis for the assessment of contamination of environmental samples. For *E. coli* that is commonly used as indicator organism, 2 basic methods are currently in use, both of which rely on the activity of the enzyme β-glucuronidase that is specific for *E. coli* (17). One method used to detect the presence of *E. coli* is the Colilert system (IDEXX Laboratories, Inc., Westbrook, USA). This system detects coliforms by relying on *β*-galactosidase activity to metabolize ONPG (o-nitrophenyl-β–D-galactopyranoside to o-nitrophenol) which produces a yellow color (18). The system also targets *E. coli* based on the ability to use *β*-glucuronidase to metabolize MUG (4-methyl-umbelliferyl-β-D-glucuronide to 4-methyl-umbelliferone) and in turn create fluorescence (18). The samples are then enumerated using a Most Probable Number (MPN) method relating cell numbers of *E. coli* to their presence in 100 mL of water sample. The second method is using Tryptone Bile X-Glucuronide Medium (TBX) agar (Oxoid, Basingstoke, UK). TBX agar is selective for *E. coli*, based on glucuronidase activity, in which *E. coli* turn blue due to the chromogenic agent, X-glucuronide (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). The blue colonies are then counted and the CFU per 100 mL are determined. Unfortunately, both methods might underestimate total numbers of *E. coli* in the samples, because some pathogenic *E. coli* have been shown to not express the *uid*A gene that encodes the glucoronidase. Consequently, these *E. coli* will not produce blue-stained metabolites and cells or colonies remain white and thus undetected.

The use of quantitative Polymerase Chain Reaction (*q*PCR) allows researchers to analyze the sample for the presence bacteria without cultivation steps and reliance on gene expression (14). Unlike end-point PCR where only absence or presence can be detected, *q*PCR can also quantify the starting material in a sample. The quantification of the sample

can be done through the use of DNA binding dyes (i.e. SYBR Green) or through the use of probes, which release a fluorescent dye that accumulates and can be quantified. The fluorescent signal has to go above a threshold (cycle threshold, C_t) and the time at which this is reached, the target is quantified (19). Thus, the more starting material present in a sample the earlier the threshold will be reached (i.e. after few cycles in the PCR); for the detection of low quantities of starting material more cycles would be needed and thus quantification performed at a higher C_t (19). A standard curve with known concentrations of DNA or numbers of cells is then used as basis for quantification.

SYBR Green is the most commonly used DNA binding dye in *q*PCR methods; it binds to double-stranded DNA and emits a fluorescence only when it is bound to DNA (20). The binding that occurs with this dye is non-specific, but this method uses a high resolution melting curve to distinguish between the desired product and potential undesired products such as primer dimers (21). The use of hydrolysis (TaqMan) probes provides specific binding to the target amplification, due to it being sequence specific. The probe is labeled with a reporter dye on the $^{5'}$end and a quencher dye on the $^{3'}$end (19). When the probe is intact the reporter dye does not fluoresce since it is still close to the quencher dye, even when it binds to the target sequence during the annealing step. During the extension step the probe is then cleaved by the DNA polymerase (*Taq*), the reporter dye released and once cleaved off and not close to the quencher dye anymore, emits a fluorescent signal (19). This method is highly specific but much more expensive. The signals produced by both the SYBR Green method and the hydrolysis probe method are then used to quantify the starting material in the sample.

*q*PCR can be used a variety of applications in food microbiology or environmental microbiology. One example of *q*PCR used in food microbiology is for the detection and enumeration of yeast in wine (22). This study used a SYBR Green based *q*PCR to enumerate and detect yeast in wine samples that were naturally fermented and samples that were inoculated with known organisms (22). This was done to show the effectiveness of using *q*PCR, due to its high specificity, sensitivity, and fast detection, in food control. *q*PCR is also used to detect and quantify fermenting microorganisms or probiotics as well as pathogens in food (23). However, one of the main uses of *q*PCR methodology has been in environmental microbiology. It has been used mainly to detect and quantify the presence or absence of specific organisms in order to monitor environmental samples. Especially, it has been helpful to move away from culture dependent methods due to the difficulties that are present with non-culturable organisms or the unhealthy state of the target organism to grow efficiently. In some cases it is imperative to use *q*PCR over growth dependent methods due to the difficulty in isolating and enumerating organisms like nontuberculosis mycobacteria due to the over competitiveness of other non-target organisms (24). Both SYBR Green and TaqMan probes were used to detect and quantify *Mycobacterium* spp. in large volume water samples to bypass growth dependent steps (24). Foremost, *q*PCR is the preferred monitoring source in environmental microbiology due to the ability to bypass cultivation steps.

*q*PCR has been designated as a reliable microbial source tracking method because of its speed, sensitivity, and potentially high specificity (25). The use of host specific molecular markers provides for the ability to bypass culturing steps, reduce bias from culturing techniques, and to determine a specific source for the pollution (16). Studies on

the performance of species specific *q*PCR for microbial source tracking have used spiked water samples with known concentrations of organisms, using sewage, wastewater, and contaminated environmental water samples, as well as samples spiked with feces from specific animals (14, 26, 25, 8). One study has shown the limits of detection with the use of species specific markers for microbial source tracking with *q*PCR on sewage spiked samples (14). The study showed that using a marker specific to human feces was able to detect the presence of these organisms in a sewage spiked sample that was comparable to a contaminated water sample (14). It also showed that the use of *q*PCR microbial source tracking would be able to detect contamination at levels that would help protect human health before higher contamination levels would be present (14).

Objective

This study focused on the assessment of surface water contamination at five different bridges located in Central Texas. This assessment was done via the use of two growth dependent *E. coli* enumeration methods. The two methods performed were a most-probable-number (MPN) based enumeration, which is a Texas Commission on Environmental Quality (TCEQ)-accredited procedure performed by the Edwards Aquifer Research & Data Center (EARDC), and a CFU-based enumeration using Tryptone Bile X-Glucuronide Medium (TBX) agar. Both of these methods were compared against each other to determine the reliability of both methods. These two methods were also used to determine the potential effects of runoff in the surface water contamination. Results from both growth-dependent analysis methods were then compared to *q*PCR based microbial

source tracking that is unbiased by the limitations of growth. *q*PCR was also used to assess contamination present in surface water as well as to determine the potential effects of runoff. The expected results were that the growth dependent methods will be comparable in detecting any contamination present. Also that the potential effects of runoff would be significant to the presence of contamination in the surface waters. The use of *q*PCR based microbial source tracking was also expected to determine the contamination levels in the surface water as well as being able to provide a specific source of contamination with the use of target specific primers and determine the effects of runoff on the contamination levels.

Materials and Methods

Sampling. Sampling efforts focused on five bridges (B1 – B5) in Central Texas with bodies of water underneath and with birds nesting on the concrete structures (Fig 1). Water samples were collected about 30 m up-stream and 5 m down-stream of each bridge. Samples were taken in triplicate for analyses using TBX and *q*PCR methods, and in sets of five for the EARDC analyses. Samples were collected in sterile 500 ml glass bottles for TBX and *q*PCR analyses, and in sterile 100 ml plastic bottles for the EARDC analyses. Once the samples were collected, they were kept on ice and transported to their respective location (i.e. EARDC laboratory or the laboratory at the Department of Biology). All samples were collected, delivered on ice and processed within an 8 hour (h) period. Samplings were conducted at 4 times during the season coinciding with different bird abundances (i.e. the absence of birds, their arrival, nesting, and the fledgling of birds). At

each time of the season, attempts were made to sample before and after a precipitation event (>1 inch rainfall, i.e. >26 mm). The sampling phases were repeated in two consecutive years.

Fecal sampling was also conducted for analyses of microbial composition. Samples were collected as "fresh" as possible from known animals where available. Samples were processed as soon as possible to maintain the integrity of the sample.

Sample processing. Water samples were concentrated by centrifuging 100 mL of water (4,000 rpm for 15 min.) and the resulting pellet was re-suspended in 1 mL of sterile distilled H_2O (diH_2O). The 1 mL sample was then subdivided into 500 µL and 300 µL to be used for further analyses, i.e. DNA extraction and *E. coli* enumeration on TBX agar, respectively. Fecal samples were processed by weighing out 200 mg (wet weight) for DNA extraction and weighing out 100 mg and suspending it into 1 mL of diH_2O, that was used as basis for serial dilutions (1:10) for the *E. coli* enumeration on TBX agar.

***E. coli* enumeration.** Assessment of growth dependent *E. coli* enumeration of fecal and environmental samples from different sources (i.e. horse, bird, amphibian, reptile, bat, ruminant, water and sediment) were done on TBX agar. This procedure was done in triplicate for each sample assessed, 100 µL of each sample was plated on TBX agar and were then incubated at 37°C for 12 h. The blue colonies were then counted and the CFU/100 mL of water or 100 mg of feces or sediment was determined. The EARDC analyses was done using the Colilert 18h system. This system targets *E. coli* based on the ability to use β-glucuronidase to metabolize MUG (4-methyl-umbelliferyl to 4-methyl-umbelliferone) and in turn create fluorescence. The samples were then enumerated using a Most Probable Number (MPN) per 100 mL.

DNA extraction. Sodium hydroxide (NaOH) was used to lyse bacterial cells for the DNA extraction of the water samples. The extraction was done on the 500 µL taken from the 1 mL sample concentrations. 500 µL were centrifuged at 13,000 rpm for 2 min, the supernatant was discarded and the pellet was re-suspended in 50 µL 50 mM NaOH. This was then incubated at 65°C for 30 min in order to lyse the cells. After the incubation time, the samples were then centrifuged at 13,000 rpm for 2 min. The supernatant was transferred to a new sterile microfuge tube and the pellet was discarded. The DNA was then stored at -20°C until needed. The fecal samples were extracted using the E.Z.N.A® Stool DNA Kit (Omega Bio-Tek, Norcross, USA), following the manufacturer's instructions. The extractions were eluted into 100 µL buffer which were then stored at -20°C until needed.

***q*PCR.** *q*PCR quantification was performed targeting the *uidA* gene for the detection of *E. coli*. This quantification was performed on fecal and environmental samples in triplicate. The quantification was used to determine the amount of *E. coli* present in the samples and to compare it with the enumeration performed on TBX agar and by the Colilert system. In addition, *q*PCR-based source tracking was done using five different primer sets. These were used for the quantification of all bacteria (Bact2), all Bacteroidales (all Bac), *Helicobacter* spp. (GDF), ruminant Bacteroidales (BacR), and *Salmonella* (Table 1). *q*PCR was performed using the Eco Real-Time PCR system, using both SYBR Green (BioRad, Hercules, USA) and SsoFast Probes Supermix (BioRad, Hercules, USA) based reactions. Reactions were carried out in 10 µL for both detection systems. Each reaction consisted of 1 µL DNA template, 5 µL SYBR Green or SsoFast Probes Supermix (with probe or without probe, respectively), 1 µL forward primer, 1 µL reverse primer, 1 or 2 µL nuclease free

diH_2O (with probe or without probe, respectively), and 1 µL probe if applicable. All reaction cycle conditions started with a polymerase activation step. Following the initial conditions, each reaction was carried out using the optimized cycle conditions for 40 cycles. SYBR Green reactions included a melt curve analysis at the end for specificity determination. Table 2 shows the amplification conditions for specific primer combinations and the primer concentrations. All DNA extraction samples were analyzed using the NanoDrop 1000 Spectrophotomer (Thermo Scientific, Wilmington, USA) to determine DNA concentrations (ng) and potential contamination by proteins (260 nm/280 nm) and salts (260 nm/230 nm) prior to *q*PCR analysis in order to determine whether dilutions were needed to prevent or counter PCR inhibition.

Standard curve. All samples were quantified using a standard curve during the *q*PCR analyses. *E. coli* and *Salmonella* standard curves were based on 10-fold dilutions of cells quantified after *in situ* hybridization by epifluorescence microscopy. The *in situ* hybridization was performed on a pure culture of the respective microorganism with an OD of 1; this OD was achieved by growing the culture in 5 mL LB overnight at 37°C with shaking. These cells were then fixed and counted, and the cell counts then expressed as cells/µL. The 1 mL of culture was divided into 100 µL aliquots, and 1 mL of culture was stored in glycerol at -80°C. The 100 µL aliquots were lysed using 50 mM NaOH and stored at -20°C. One aliquot was then serially diluted (1:10) in TE buffer to create the standard curves. For the primer set targeting all bacteria (Bact2), the *E. coli* standard curve was used for the *q*PCR analysis. All Bac, GDF, BacR standard curves were created from PCR products amplified from fecal samples respective to the specific target. All PCR products were cleaned using the UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories, Inc.,

Carlsbad, USA). These were then analyzed using the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA) to determine concentration of DNA. The PCR products were then serially diluted (1:10) in TE buffer to create the standard curve. All standards were stored at -20°C until needed.

Impact of season and run-off. The impact of bird season, presence and absence, as well as the density of Cliff Swallows on fecal contamination was evaluated using the enumeration performed. The enumeration from these seasons and densities were compared to estimate their impact. In addition, the potential impact of run-off (i.e. large precipitation events) to contamination was evaluated in the same manner.

Statistical Analysis. The R software version 2.15.1 was used to perform the statistical analyses. A linear mixed effect (lme) model was used to analyze the data collected and then an ANOVA was performed on the lme model. SigmaPlot 11 (Systat Software Inc., San Jose, USA) was used to create the graphs shown.

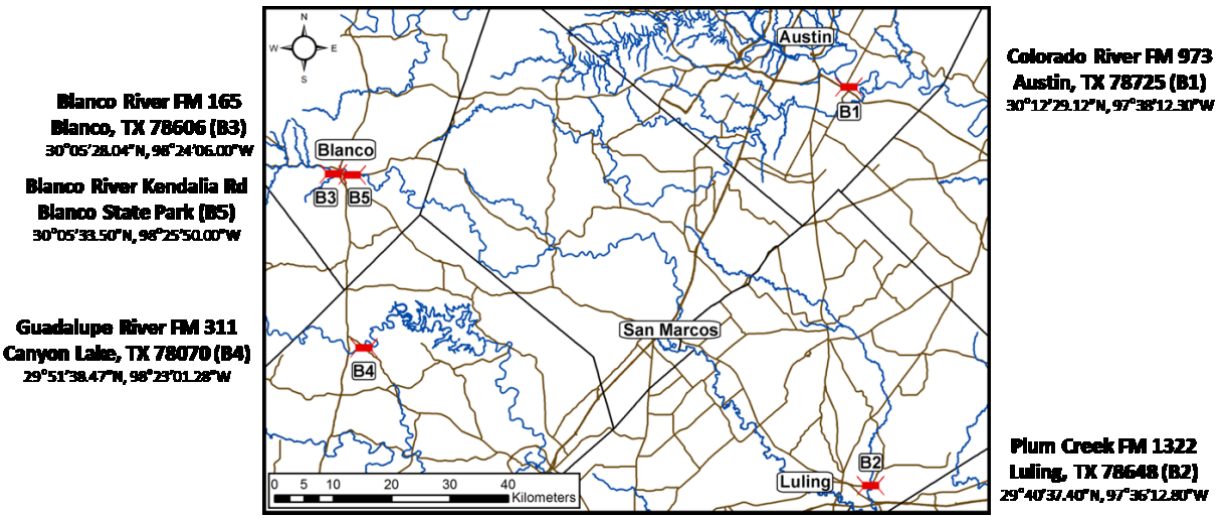

Fig. 1. Selection of bridges in Central Texas used to analyze effects of nesting Cliff Swallows on fecal contamination in surface waters.

Table 1. Primer sets used for target specific source tracking and *E. coli* and *Salmonella* detection.

Target Feces	Primers/Probe	Sequence	Tm (°C)	Size (bp)	Reference
All Bacteria (Bact 2)	Bact 1369f	5' CGG TGA ATA CGT TCC CGG	60	142	10
	Prok1492r	5' TAC GGC TAC CTT GTT ACG ACT T			
	TM1389f	5' FAM-CTT GTA CAC ACC GCC GCC CGT C-(NFQ-MGB)			
All Bacteroidales (all Bac)	AllBac296f	5' GAG AGG AAG GTC CCC CAC	60	106	10
	AllBac467r	5' CGC TAC TTG GCT GGT TCA G			
	AllBac375Bhqr	5' FAM-CCA TTG ACC AAT ATT CCT CAC TGC TGC T-BHQ-1			
Bird (GFD) unclassified *Helicobacter* spp.	GDFf	5' TCG GCTGAG CAC TCT AGG G	57	123	8
	GDFr	5' GCG TCT CTT TGT ACA TCC CA			
E. coli	uidAf	5’ CAA TGG TGA TGT CAG CGT T	58	163	27
	uidAr	5’ ACA CTC TGT CCG GCT TTT G			
Ruminants (BacR)	BacR_f	5' GCG TAT CCA ACC TTC CCG	60	100	10
	BacR_r	5' CAT CCC CAT CCG TTA CCG			
	BacR_p	5' FAM-CTT CCG AAA GGG AGA TT-(NFQ-MGB)			
Salmonella	139	5’ GTG AAA TTA TCG CCA CGT TCG GGC AA	60	284	28
	141	5’ TCA TCG CAC CGT CAA AGG AAC C			

Table 2. Primer specific *q*PCR conditions and primer concentrations.

Target Feces	Primers/Probe	Concentration (nmol)	Polymerase Activation	1 cycle for 40 cycles	Melt Curve
All Bacteria (Bact 2)	Bact 1369f	200	95°C, 5 min	95°C, 30 sec 60°C, 30 sec	NA
	Prok1492r	200			
	TM1389f	250			
All Bacteroidales (All Bac)	AllBac296f	200	95°C, 5 min	95°C, 15 sec 60°C, 30 sec	NA
	AllBac467r	200			
	AllBac375Bhqr	100			
Bird (GFD) unclassified *Helicobacter* spp.	GDFf	100	95°C, 2 min	95°C, 15 sec 59°C, 32 sec	95°C, 15 sec 60°C, 15 sec 95°C, 15 sec
	GDFr	100			
E. coli	uidAf	200	95°C, 5 min	95°C, 30 sec 58°C, 30 sec 72°C, 30 sec	95°C, 15 sec 60°C, 15 sec 95°C, 15 sec
	uidAr	200			
Ruminants (BacR)	BacR_f	100	95°C, 10 min	95°C, 30 sec 60°C, 30 sec	NA
	BacR_r	200			
	BacR_p	200			
Salmonella	141	100	95°C, 10 min	95°C, 30 sec 60°C, 30 sec	95°C, 15 sec 55°C, 15 sec 95°C, 15 sec
	139	100			

NA = not applicable

Results

E. coli enumeration using either the CFU or the MPN methods showed comparable results even though samples were independently taken from each other (Table 3 and 4). For both methods, levels of *E. coli* were below the established threshold for contamination (126 CFU/100 mL) at most of the bridges (B1, B3 – B5) at the first sampling time, i.e. in the absence of Cliff Swallows and prior to precipitation events (Fig. 2a & 2b). Bridge 2 (B2) had a slightly higher number of *E. coli* at this time which can be attributed to it being a creek where water is not constantly flowing at a fast rate thus the water may be stagnant at times. After precipitation a significant increase in the number of *E. coli* was found for all bridges, with numbers above the contamination threshold for all bridges (Fig. 2a & 2b). For both sampling times, i.e. before and after precipitation events, there was no significant difference in numbers of *E. coli* between up- and down-stream sampling locations. At the second sampling time, i.e. in the presence of arriving Cliff Swallows, *E. coli* enumeration before precipitation again showed numbers below the threshold used to describe contamination for bridges 1 and 2 (Fig. 3a) with the CFU method and for all bridges (Fig. 3b) for the MPN method. Again, numbers of *E. coli* were significantly higher and far above the contamination threshold after precipitation events. No significant differences were obtained in numbers between up- and down-stream locations at each sampling time, i.e. before and after precipitation. Thus, the presence of birds did not change the basic pattern of occurrence of *E. coli*, with numbers of *E. coli* below the contamination threshold before

precipitation events, and numbers far above this threshold after precipitation. There were also no differences between up- or down-stream sampling locations.

In the presence of Cliff Swallow nestlings numbers of *E. coli* in samples obtained before precipitation were again below the contamination threshold, except for samples from bridge 2 (Fig. 4a & 4b). This could be attributed to the type of watershed (creek) and due to the fact that it there was a low amount of rain a few days before the sampling occurred. *E. coli* enumeration after precipitation was significantly higher only for bridge 3. Due to high water flow after precipitation and the resulting safety concerns, samples were not obtained for bridges 1 and 5. Bridge 2 and 4 did not have higher numbers of *E. coli* after precipitation. Although this did impact the amount of *E. coli* found, there were no significant differences between locations. Generally, *E. coli* numbers showed the same pattern seen at the previous two sampling time. The fourth and last sampling was done when Cliff Swallow fledglings were present (Fig. 5a & 5b). Numbers of *E coli* in water samples before precipitation showed levels below the contamination threshold. At this time, only bridge 2 could be sampled after a precipitation event. None of the other sites received any precipitation. Again, the enumeration showed significantly higher abundance of *E. coli* after precipitation than before. At both sampling events, no significant differences were obtained between up- and down-stream locations confirming the patterns obtained for the previous three sampling times.

All sampling events were repeated for a second year using the same conditions with respect to absence or presence of Cliff Swallows, with and without precipitation and up- and down-stream sampling, as in the first year. The results of the second year generally confirmed those of the first year. Briefly, numbers of *E. coli* in the absence of Cliff

Swallows were below threshold levels for all bridges before precipitation events, while those for samples obtained after precipitation were far above the threshold level, with no significant differences between up- and down-stream locations (Fig. 6a & 6b). In the presence of Cliff Swallows, abundance of *E. coli* were below threshold levels at all bridges except for bridge 1 (Fig. 7a & 7b). This high number of *E. coli* could be contributed to anthropogenic effects around the river, since vegetation was removed and moved for development purposes. Again, numbers of *E. coli* were significantly higher after precipitation at all bridges, with no significant differences between up- and down-stream locations as in the previous year. The third sampling event again confirmed data from the first year, with levels of *E. coli* before precipitation being below threshold values at all five bridges, and far higher levels after precipitation and no significant differences between up- and down-stream locations (Fig. 8a & 8b). For the last sampling, i.e. in the presence of Cliff Swallow fledglings and without precipitation, water samples from all five bridges contained *E. coli* in numbers below the threshold for contamination (Fig. 9a & 9b). After-precipitation-samples could not be obtained from any of the bridges due to the lack of any rainfall.

CFU-based enumeration of *E. coli* was also performed on different animal feces to assess potential contributions to *E. coli*-based contamination assessments (Table 5). The results demonstrated highly variable numbers of *E. coli* present in different animal feces, with very low numbers of *E. coli* in Cliff Swallow feces. Additional analyses using *q*PCR on white colonies (i.e. colonies not expressing the enzyme glucuronidase) allowed us to detect the *uid*A gene encoding this enzyme, in some of them. Thus, our MPN and CFU-based quantifications might have resulted in an underestimation of actual contamination

depending on the source of the feces. At the same time, no additional *E. coli* colonies were identified in feces of Cliff Swallows that had already very small numbers of *E. coli* based on the MPN and CFU-based analyses.

The use of *q*PCR allowed for the detection of specific bacterial groups by targeting specific gene fragments to be amplified (Table 6). This was done to detect and enumerate Bact2, *E. coli*, GDF, BacR, all Bac, and *Salmonella* spp. in both feces samples from different animals as well as from water samples. Targeting all bacteria allowed us to differentiate samples before and after precipitation. *E. coli* was found in a few samples and was not found to be present in all species. *Helicobacter*, ruminant Bacteroidales and all Bacteroidales were found only in feces specific to those species. *Salmonella* was found in only one of the samples but was not specific to an animal species.

Results using *q*PCR microbial source tracking for the determination of the effects of runoff before and after a significant a rainfall event have been successful in showing that there is an increase in the bacterial loads due to runoff independent of presence or densities of Cliff Swallows (Table 7 and Table 8). These have been the same results for both years' at all five bridges. This also shows that in some instances, usually associated with after rain events, ruminant enumeration was possible. All Bacteroidales were present in most of the water samples taken during both years, with a usual increase in numbers after rain events. The ANOVA results are shown in Table 9.

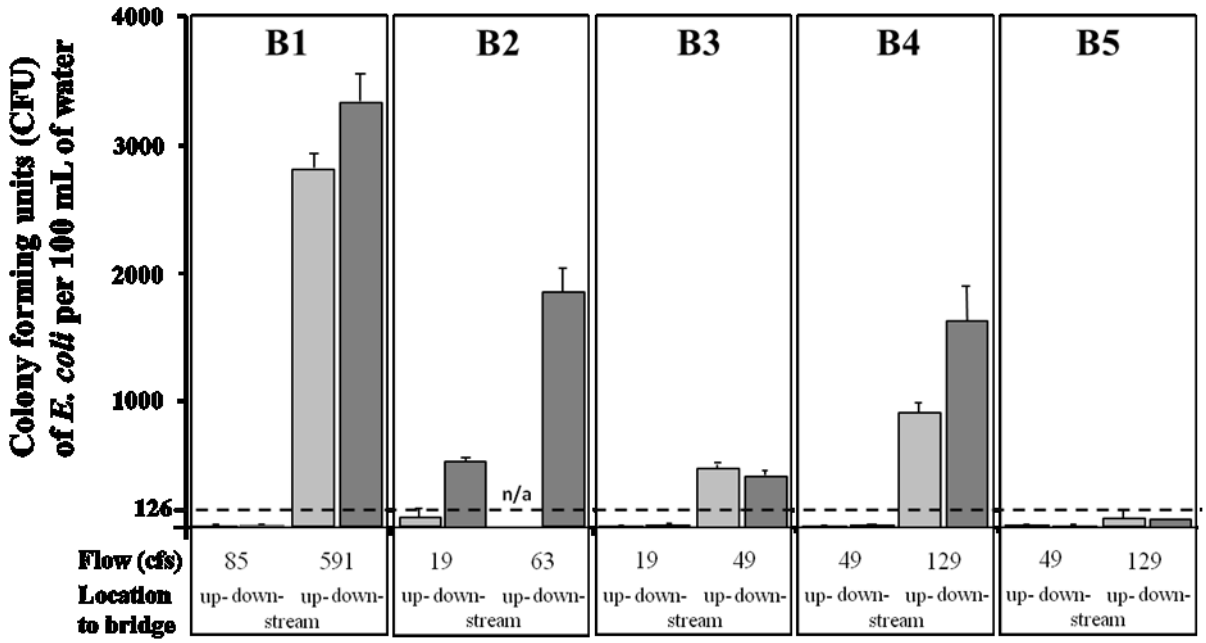

Fig. 2a. CFU enumeration of *E. coli* in the absence of Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (November 2012) and after precipitation (January 10, 2013, as indicated by enhanced water flow), in the absence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

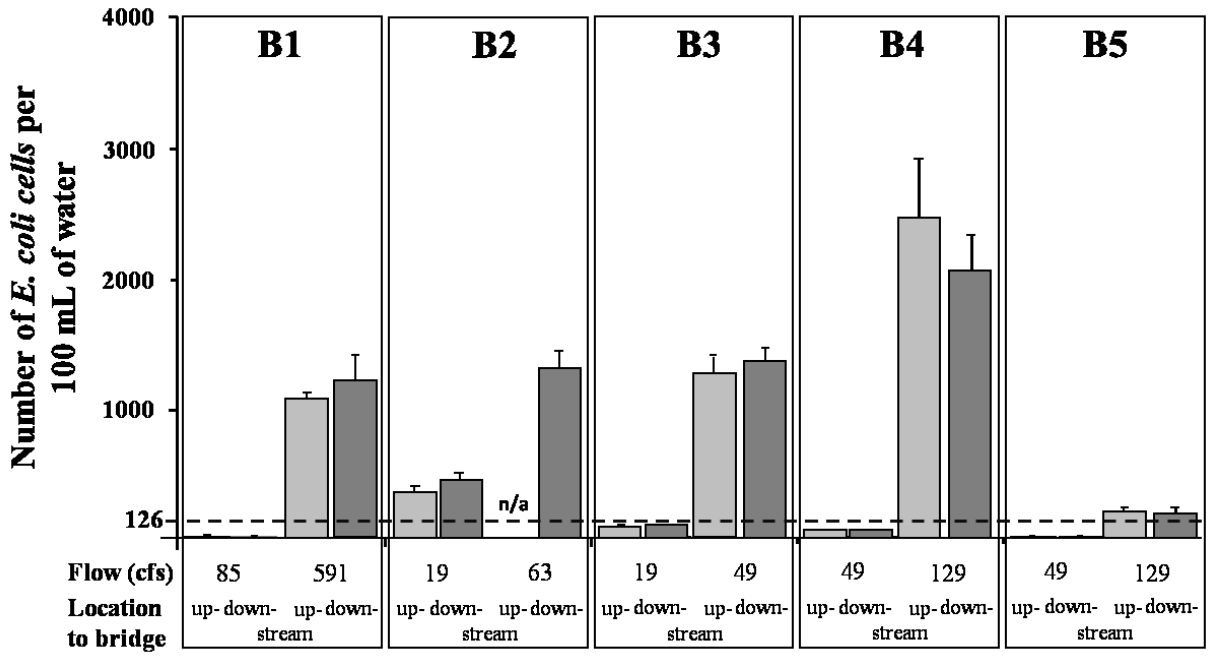

Fig. 2b. MPN enumeration of *E. coli* in the absence of Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (November 2012) and after precipitation (January 10, 2013, as indicated by enhanced water flow), in the absence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

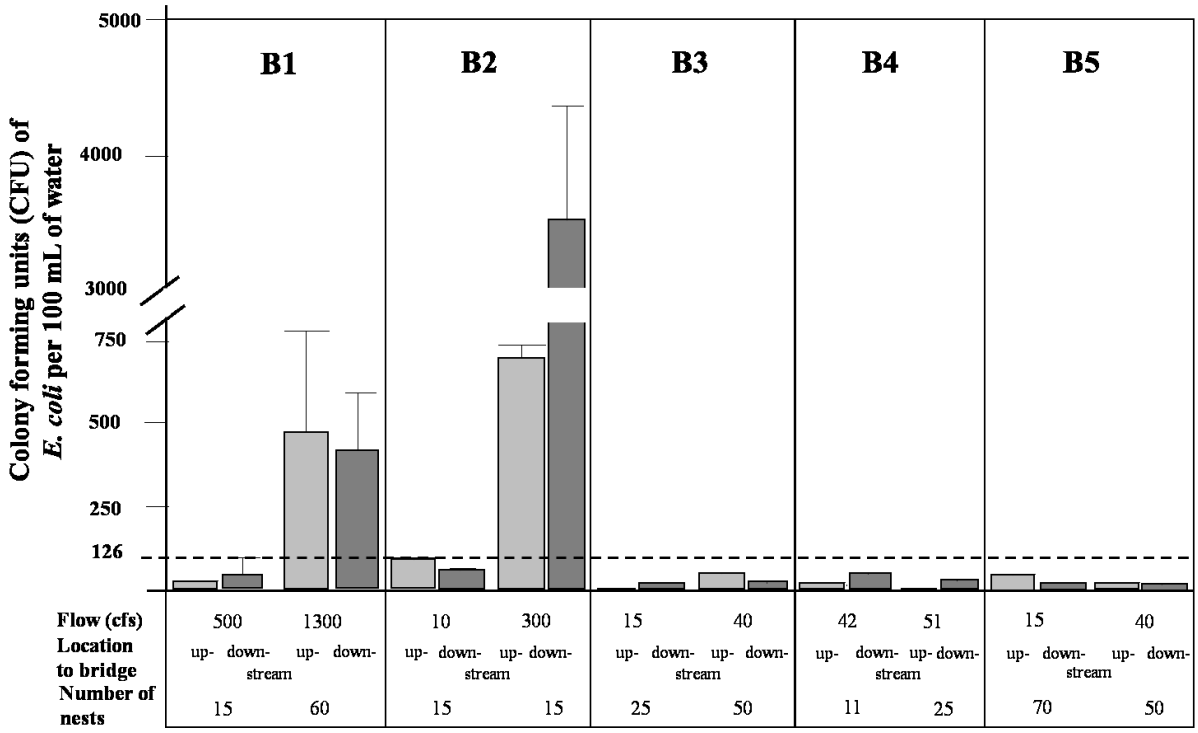

Fig. 3a. CFU enumeration of *E. coli* in the presence of Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (3/27, 2013, 4/15/2013 to 4/17/2014) and after precipitation (4/3/2013 to 4/4/2013); as indicated by enhanced water flow, in the presence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

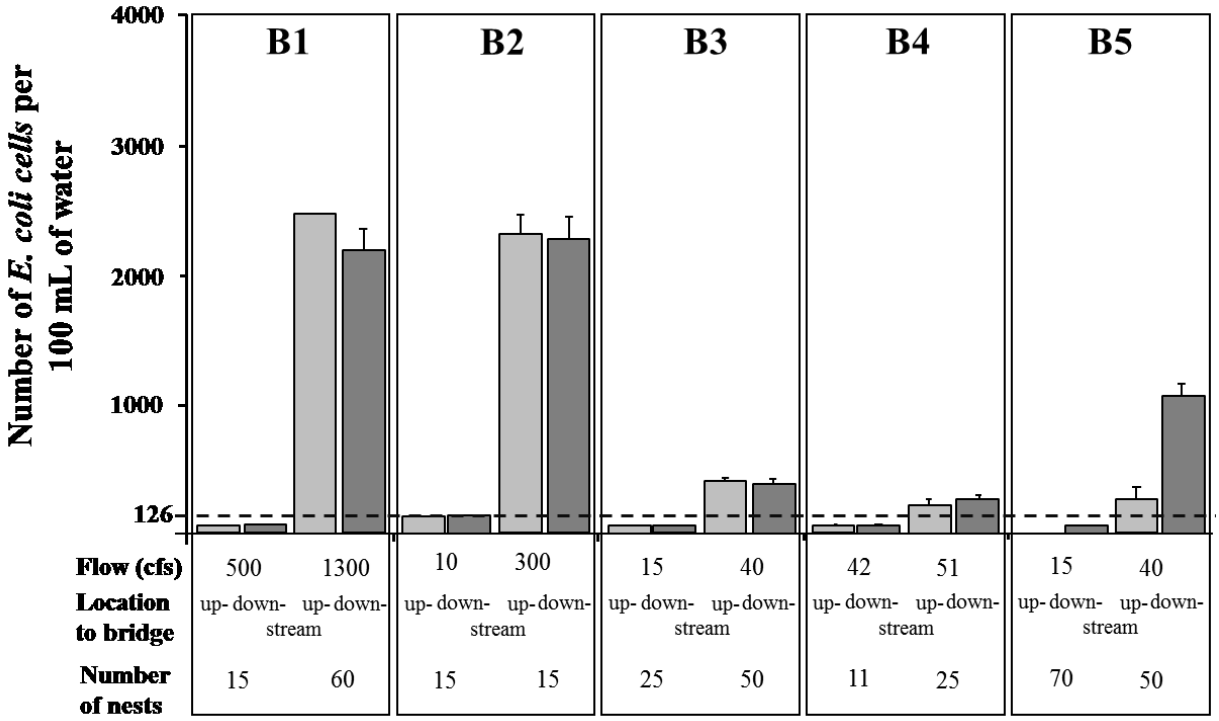

Fig. 3b. MPN enumeration of *E. coli* in the presence of Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (3/27, 2013, 4/15/2013 to 4/17/2014) and after precipitation (4/3/2013 to 4/4/2013; as indicated by enhanced water flow), in the presence of Cliff Swallows just occupying nests. The 126 CFU mark represents the threshold for contamination.

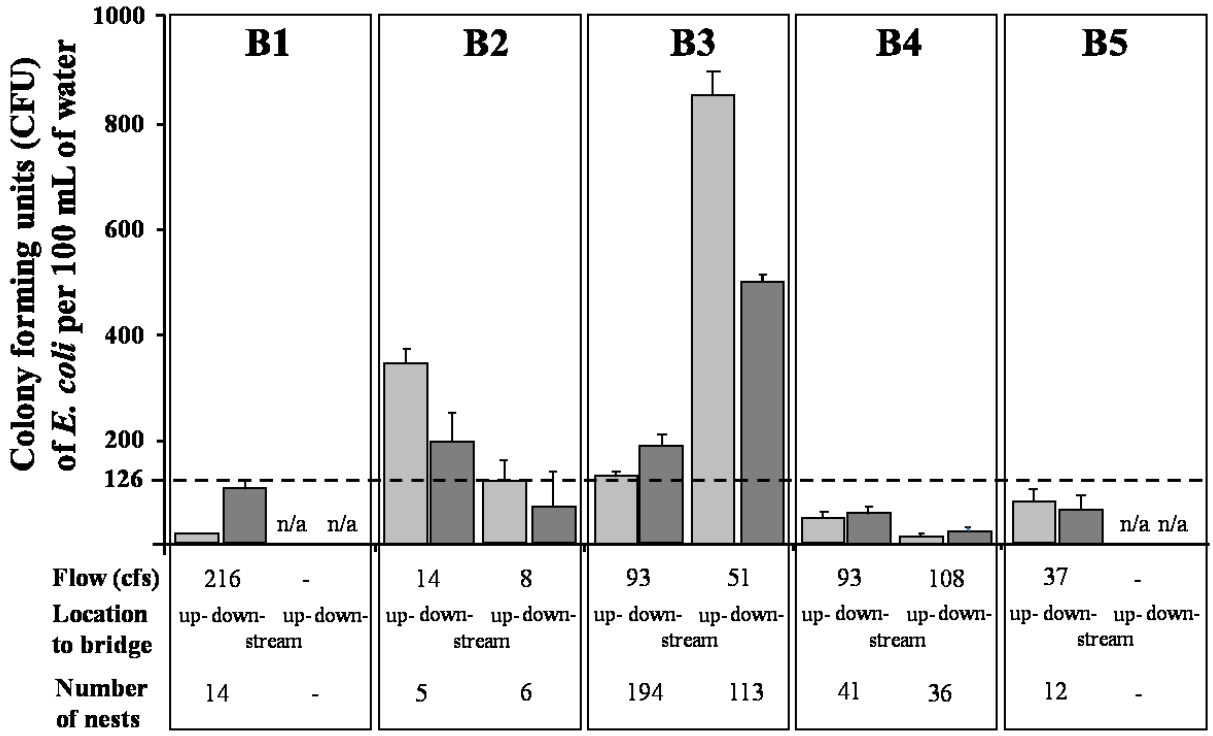

Fig. 4a. CFU enumeration of *E. coli* in the presence of nestling Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (5/28/2013 to 6/4/2013) and after precipitation (5/16/2013), 2013; as indicated by enhanced water flow), in the presence of nestling Cliff Swallows. The 126 CFU mark represents the threshold for contamination. (n/a: no samples were obtained)

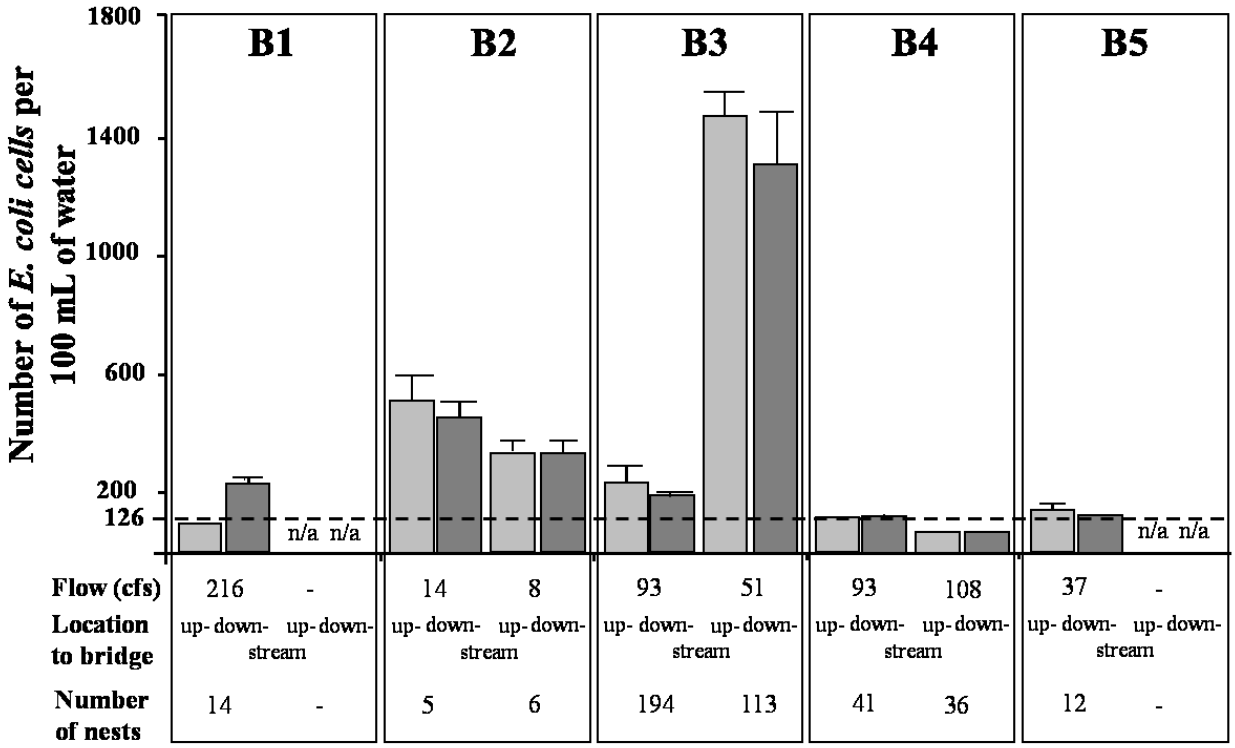

Fig. 4b. MPN enumeration of *E. coli* in the presence of nestling Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using the Colilert MPN method before (5/28/2013 to 6/4/2013) and after precipitation (5/16/2013), 2013; as indicated by enhanced water flow), in the presence of nestling Cliff Swallows. The 126 CFU mark represents the threshold for contamination. (n/a: no samples were obtained)

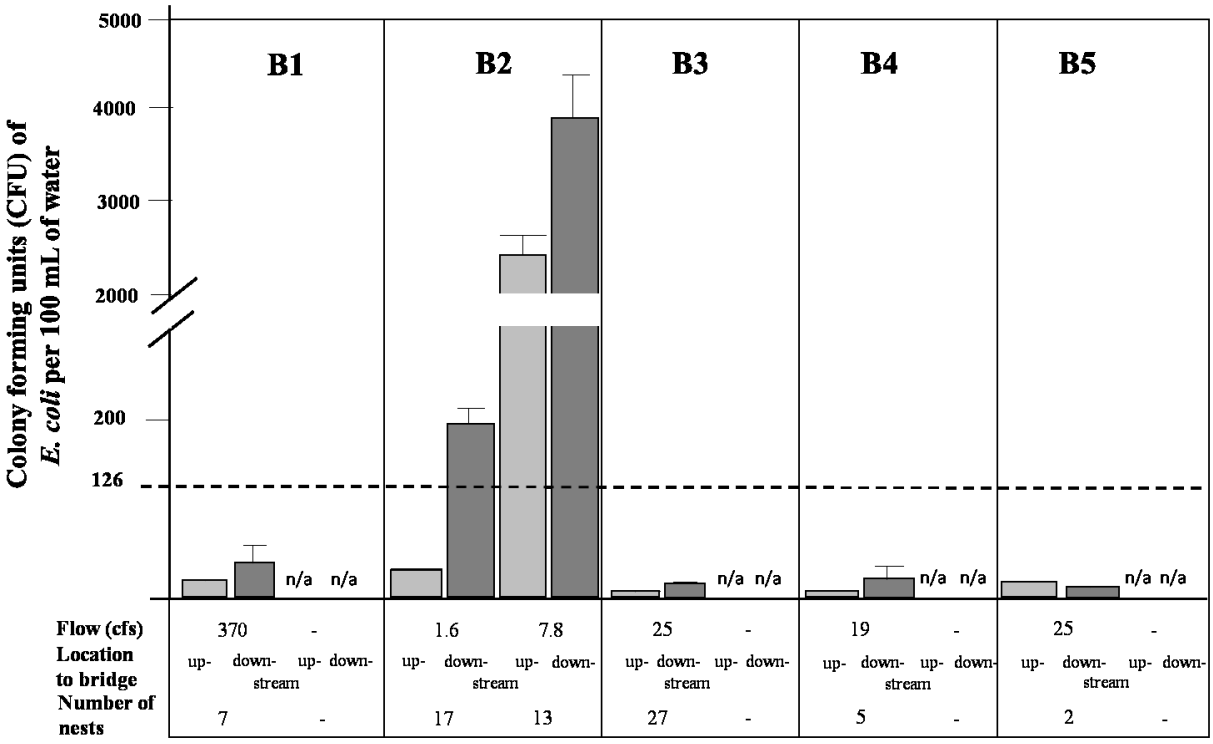

Fig. 5a. CFU enumeration of *E. coli* in the presence of fledgling Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (7/8/2013 to 7/9/2013) and after precipitation (7/22/2013) (after precipitation was not collected for B1, B3-B5); as indicated by enhanced water flow, in the presence of fledgling Cliff Swallows. The 126 CFU mark represents the borderline for contamination. (n/a: no samples were obtained)

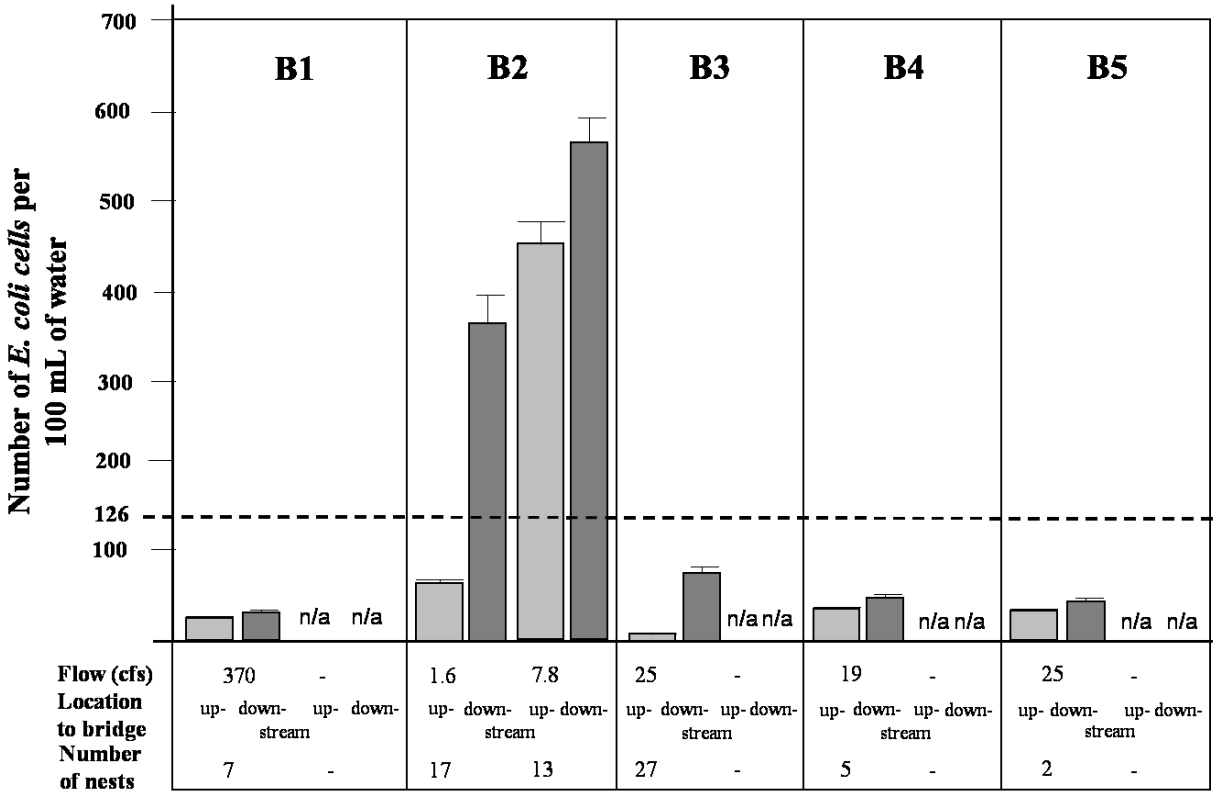

Fig. 5b. MPN enumeration of *E. coli* in the presence of fledgling Cliff Swallows. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (7/8/2013 to 7/9/2013) and after (7/22/2013) precipitation (after precipitation was not collected for B1, B3-B5); as indicated by enhanced water flow, in the presence of fledgling Cliff Swallows. The 126 CFU mark represents the threshold for contamination. (n/a: no samples were obtained)

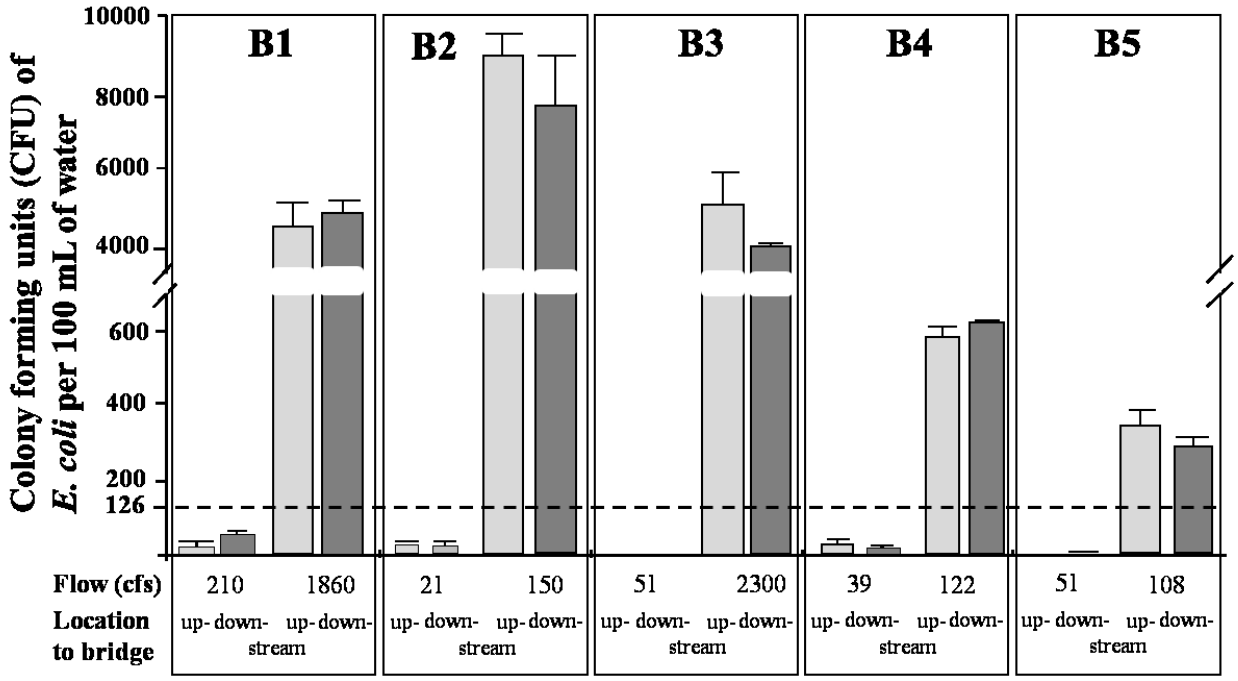

Fig. 6a. CFU enumeration of *E. coli* in the absence of Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (11/14/13 to 12/12/13) and after precipitation (9/21/13 to 10/16/13) (as indicated by enhanced water flow), in the absence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

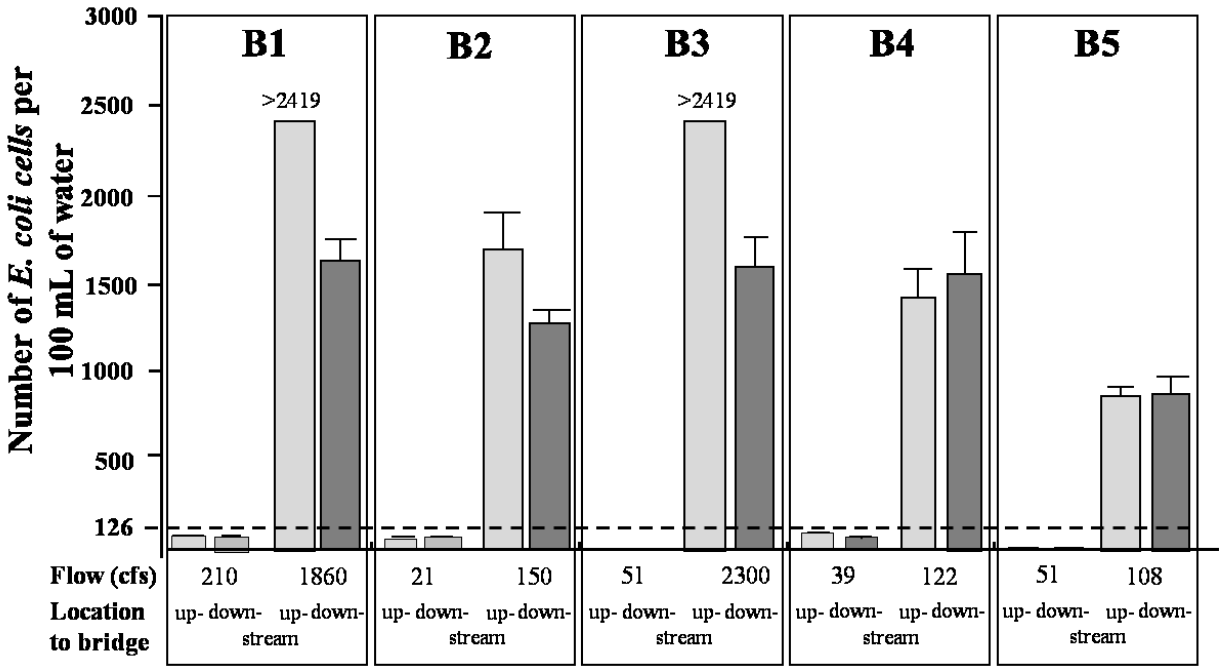

Fig. 6b. MPN enumeration of *E. coli* in the absence of Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (11/14/13 to 12/12/13) and after precipitation (9/21/13 to 10/16/13), as indicated by enhanced water flow, in the absence Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

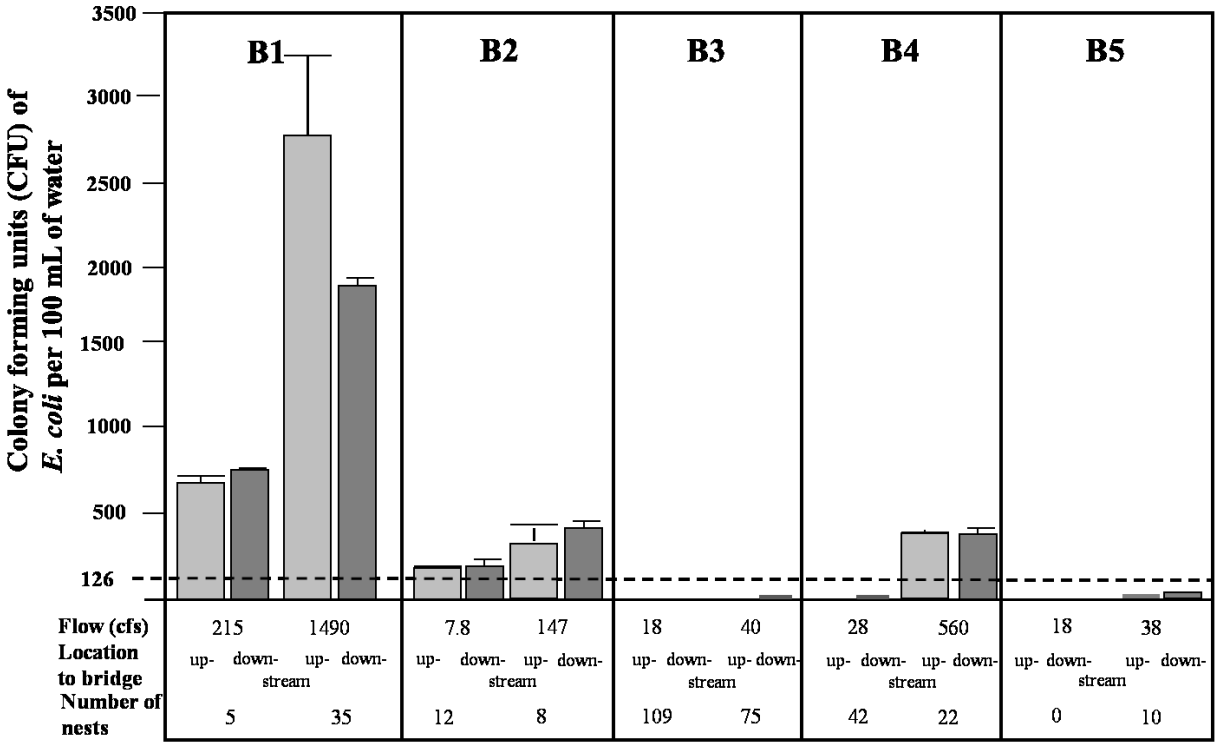

Fig. 7a. CFU enumeration of *E. coli* in the presence of Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (4/7/2014 to 4/15/2014) and after precipitation (5/13/2014 to 5/14/2014); as indicated by enhanced water flow, in the presence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

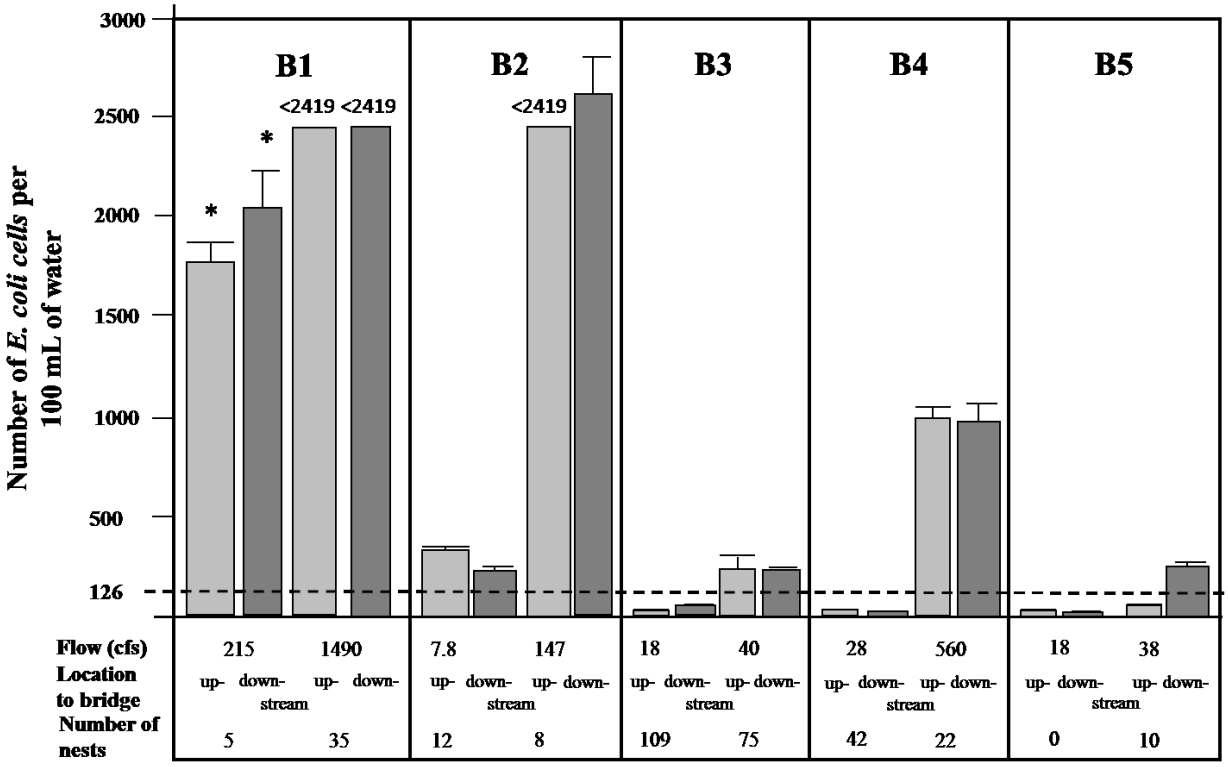

Fig. 7b. MPN enumeration of *E. coli* in the presence of Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (4/7/2014 to 4/15/2014) and after precipitation (5/13/2014 to 5/14/2014); as indicated by enhanced water flow, in the presence of Cliff Swallows. The 126 CFU mark represents the threshold for contamination. * High contamination before rain may be due to anthropogenic effects around bridge (i.e. construction)

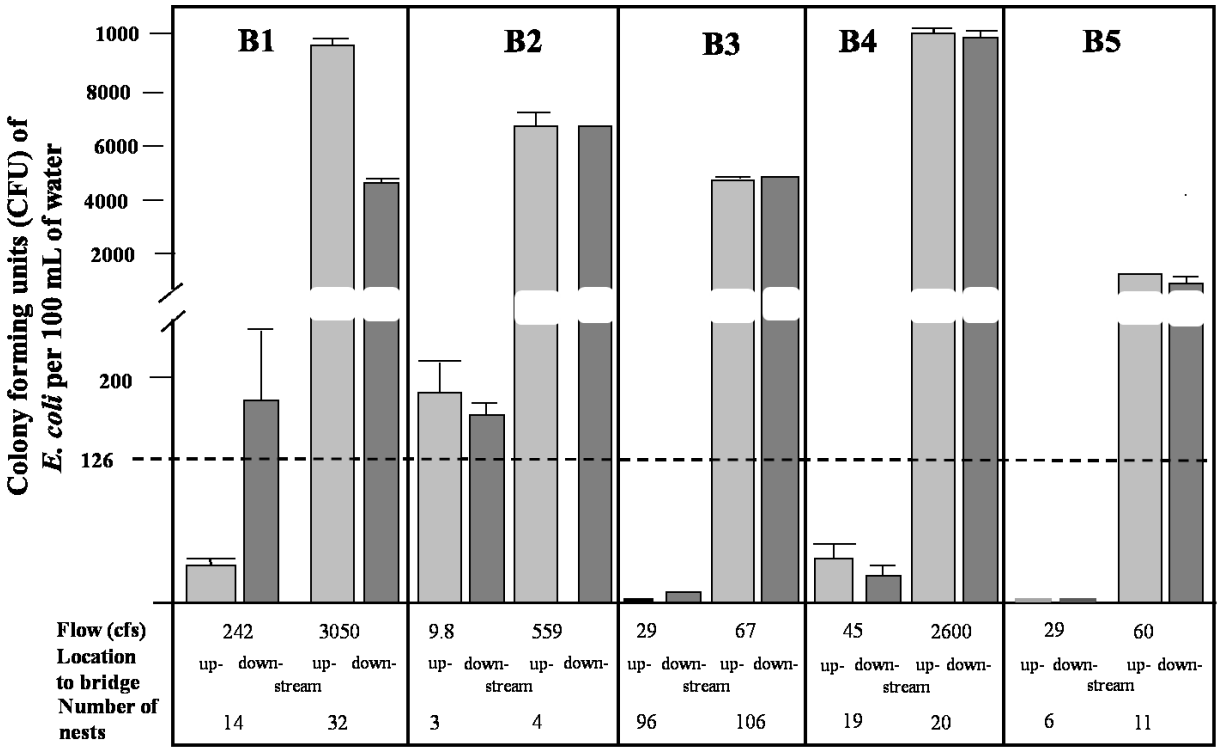

Fig. 8a. CFU enumeration of *E. coli* in the presence of nestling Cliff Swallows year 2. Enumeration of *Escherichia coli cells* in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (6/4/2014 to 6/18/2014) and after (5/26/2014 to 5/27/2014) precipitation; as indicated by enhanced water flow, in the presence of nestling Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

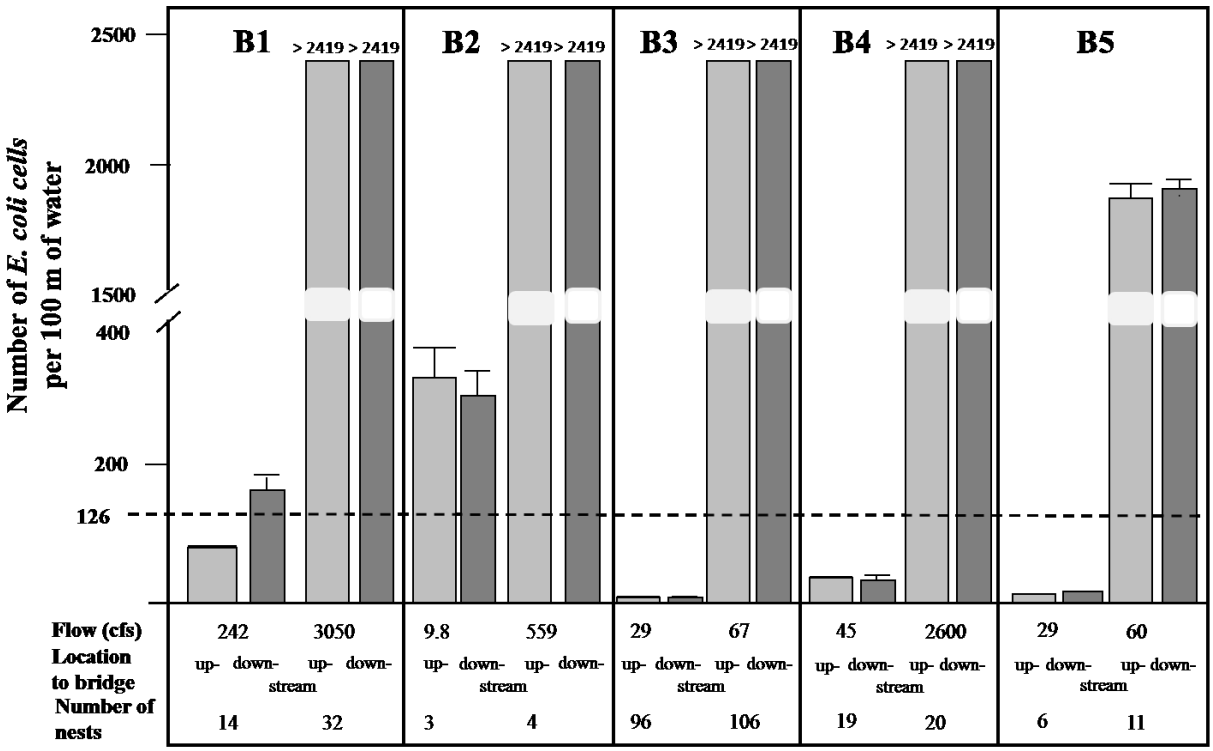

Fig. 8b. MPN enumeration of *E. coli* in the presence of nestling Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (6/4/2014 to 6/18/2014) and after (5/26/2014 to 5/27/2014) precipitation; as indicated by enhanced water flow, in the presence of nestling Cliff Swallows. The 126 CFU mark represents the threshold for contamination.

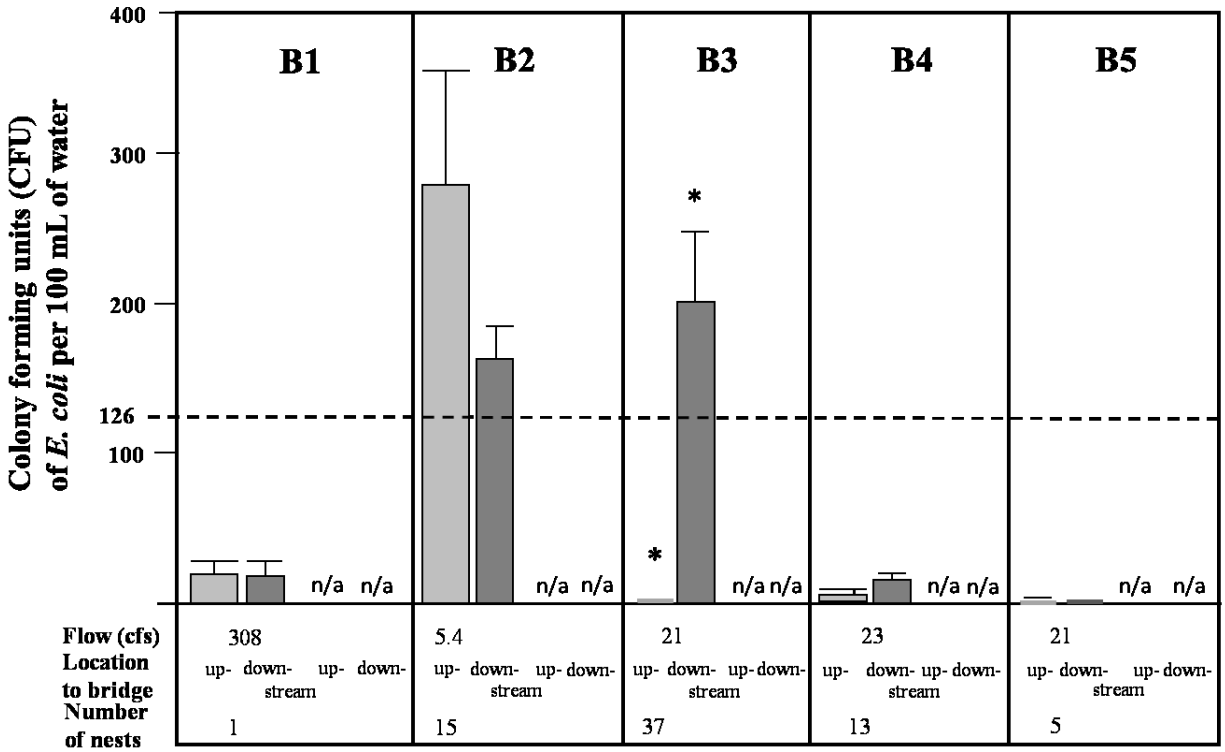

Fig. 9a. CFU enumeration of *E. coli* in the presence of fledgling Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=3) up- and down-stream of each bridge B1 – B5 using the plate spread method on TBX agar before (6/30/2014 to 7/2/2014) (after precipitation was not collected); as indicated by enhanced water flow, in the presence of fledgling Cliff Swallows. The 126 CFU mark represents the threshold for contamination. (n/a: no samples were obtained) * Differences may be contributed to varying water conditions due to drought conditions.

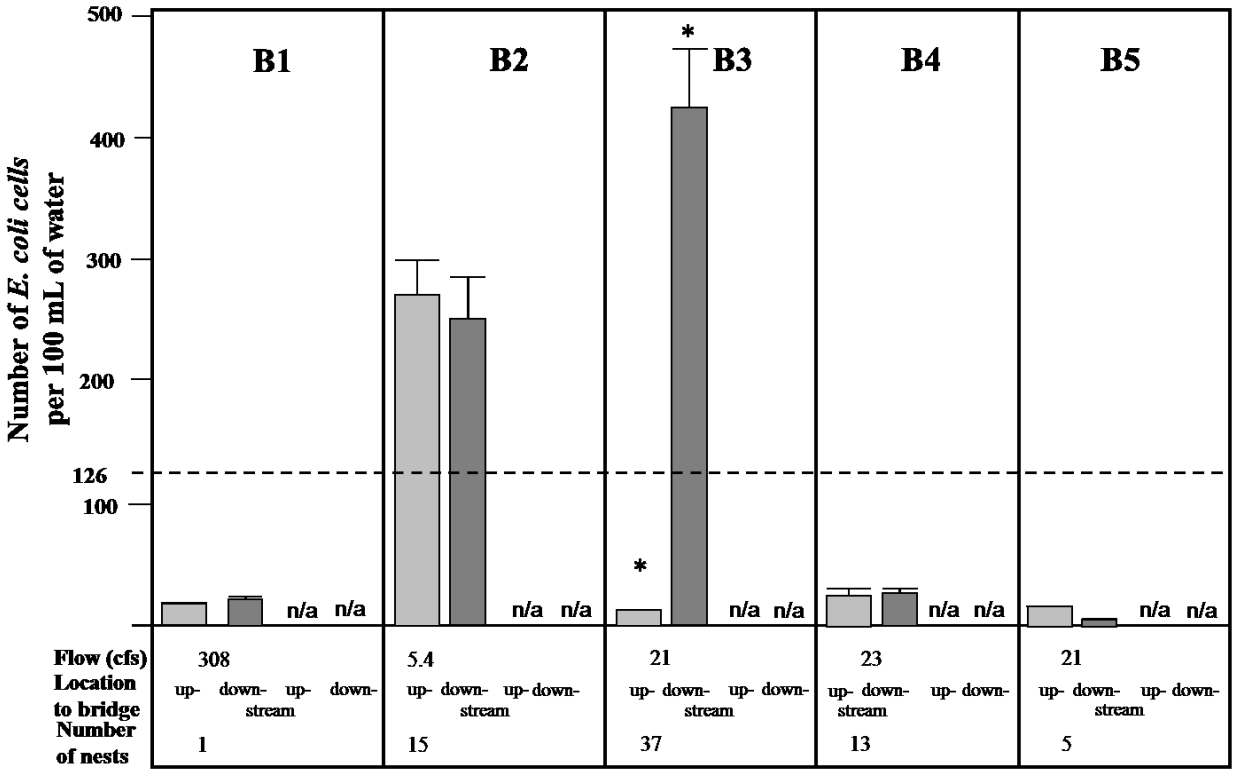

Fig. 9b. MPN enumeration of *E. coli* in the presence of fledgling Cliff Swallows year 2. Enumeration of *Escherichia coli* cells in 100 ml of water (n=5) up- and down-stream of each bridge B1 – B5 using an MPN method with the Colilert system (EARDC laboratories, Texas State University) before (6/30/2014 to 7/2/2014) (after precipitation was not collected); as indicated by enhanced water flow, in the presence of fledgling Cliff Swallows. The 126 CFU mark represents the threshold for contamination. (n/a: no samples were obtained). * Difference may be contributed to varying water conditions due to drought condition.

Table 3. ANOVA statistical summary for growth dependent enumeration for sampling year 1.

Phenology	Method	Factor	p-value	F-ratio	df_n	df_d
Without Swallows	EARDC	Rain	<0.0001	58.43	1	11
		Loc	0.85	0.04	1	11
		Rain:Loc	0.72	0.14	1	11
	TBX	Rain	<0.0001	164.86	1	48
		Loc	0.86	0.03	1	48
		Rain:Loc	0.49	0.50	1	48
Swallows Present	EARDC	Rain	<0.0001	70.25	1	12
		Loc	0.39	0.80	1	12
		Rain:Loc	0.99	0.0003	1	12
	TBX	Rain	0.10	2.77	1	52
		Loc	0.39	0.75	1	52
		Rain:Loc	0.77	0.09	1	52
Swallow Nestlings Present	EARDC	Rain	0.11	3.23	1	8
		Loc	0.93	0.01	1	8
		Rain:Loc	0.90	0.02	1	8
	TBX	Rain	0.04	4.29	1	40
		Loc	0.30	1.12	1	40
		Rain:Loc	0.19	1.74	1	40
Swallow Fledglings Present	EARDC	Rain	Statistical analyses not done due to missing after rain data			
		Loc				
		Rain:Loc				
	TBX	Rain	Statistical analyses not done due to missing after rain data			
		Loc				
		Rain:Loc				

Rain – before/after rain event
Loc – up-/down- stream location
Rain:Loc – interaction between rain event and location

Table 4. ANOVA statistical summary for growth dependent enumeration for sampling year 2.

Phenology	Method	Factor	p-value	F-ratio	df_n	df_d
Without Swallows	EARDC	Rain	<0.0001	109.79	1	12
		Loc	0.21	1.78	1	12
		Rain:Loc	0.22	1.69	1	12
	TBX	Rain	<0.0001	68.27	1	52
		Loc	0.64	0.22	1	52
		Rain:Loc	0.64	0.22	1	52
Swallows Present	EARDC	Rain	0.0038	12.76	1	12
		Loc	0.82	0.05	1	12
		Rain:Loc	0.98	0.001	1	12
	TBX	Rain	0.0001	18.77	1	52
		Loc	0.43	0.63	1	52
		Rain:Loc	0.36	0.85	1	52
Swallow Nestlings Present	EARDC	Rain	<0.0001	1327.68	1	12
		Loc	0.87	0.03	1	12
		Rain:Loc	0.94	0.007	1	12
	TBX	Rain	<0.0001	150.32	1	52
		Loc	0.26	1.32	1	52
		Rain:Loc	0.24	1.44	1	52
Swallow Fledglings Present	EARDC	Rain	Statistical analyses not done due to missing after rain data			
		Loc				
		Rain:Loc				
	TBX	Rain	Statistical analyses not done due to missing after rain data			
		Loc				
		Rain:Loc				

Rain – before/after rain event
Loc – up-/down- stream location
Rain:Loc – interaction between rain event and location

Table 5. Enumeration of *E. coli* in 1 g of fecal samples using TBX agar.

Organism	Enumeration of *E. coli* based upon the activity of glucoronidase[1]	Additional detection of *E. coli* without glucoronidase, but containing the gene[2]
Horse (*Equus ferus caballus*)	5×10^6	yes
Inca dove (*Columbina inca*)	1×10^6	no
Guinea fowl (*Numida meleagris*)	3×10^6	no
Mexican free-tailed bat (*Tadarida brasiliensis*)	3×10^5	yes
Gulf coast toad (*Bufo valliceps*)[3]	10-100	no
Cliff Swallow (*Petrochelidon pyrrhonota*)[3]		
Location Lisa Lane	10-100	no
Location Alkek Garage	10-100	no
Location Plum Creek	10-100	no

[1]Enumeration is based on serial dilutions of feces plated on TBX Agar (Tryptone Bile X-glucuronide Agar), with the activity of the *E. coli* specific glucuronidase resulting in a blue staining of colonies

[2]Detection is based on the PCR-based amplification of the *uid*A gene encoding the *E. coli* specific glucuronidase in unstained white colonies

[3]Enumeration was estimated at low dilution (i.e. 100 mg feces suspended in 1 ml solution, and 100 µl plated, with a few blue spots in a lawn of white cells)

Table 6. Quantification of bacterial load in 1 g of feces using *q*PCR (Jacqueline Hernandez, Biology Department, Texas State University). (x 10^3) (± standard error).

Organism	All Bacteria		All Bacteroidales		Ruminants (BacR)		*Escherichia coli*		*Helicobacter spp.*		*Salmonella*	
	Quantification	%	Quantification	%	Quantification	%	Quantification	%	Quantification	%	Quantification	%
Chicken (*Gallus gallus domesticus*)												
	400000 (30000)	100	7000 (400)	1.9	ND		70 (3)	0.02	ND		ND	
Cliff swallow (*Petrochelidon pyrrhonota*)												
1	20000 (900)	100	ND		ND		ND		ND		ND	
2	2000 (60)	100	ND		ND		ND		ND		ND	
3	20000 (1000)	100	ND		ND		40 (6)	0.17	ND		ND	
4	5000 (100)	100	ND		ND		ND		ND		ND	
5	9000 (1000)	100	500 (20)	5.2	ND		ND		ND		ND	
6	400 (30)	100	ND		ND		ND		ND		ND	
7	6000 (200)	100	ND		ND		ND		ND		ND	
Cave Swallow (*Petrochelidon fulva*)												
1	2000 (80)	100	ND		ND		100 (20)	5.28	ND		ND	
2	30000 (1000)	100	ND		ND		100 (10)	0.32	ND		ND	
Inca dove (*Columbina inca*)												
1	7000 (400)	100	10 (2)	0.14	ND		ND		ND		ND	
2	300000 (10000)	100	50000 (2000)	17.7	ND		60 (8)	0.02	ND		ND	
Gulf coast toad (*Bufo nebulifer*)												
1	100000 (5000)	100	30000 (1000)	24.5	ND		ND		ND		20000 (400)	18.6
2	70000 (4000)	100	500 (40)	0.73	ND		80 (9)	0.12	ND		ND	
3	6000 (200)	100	300 (10)	4.8	ND		ND		ND		ND	
Mexican free-tailed bat (*Tadarida brasiliensis*)												
	600000 (20000)	100	2000 (20)	0.26	ND		100 (7)	0.02	ND		ND	
White-Tailed Deer (*Odocoileus virginianus*)												
	$2x10^6$ ($5x10^4$)	100	500000 (10000)	30.3	700000 (20000)	40.8	90 (4)	0.01	ND		ND	

Table 6, Continued

Cow (*Bos primigenius*) *											
1	10000 (900)	100	7000 (200)	52	600 (4)	4.62	ND		ND		ND
2	10000 (300)	100	8000 (300)	70.1	800 (30)	7.46	ND		ND		ND
3	20000 (700)	100	8000 (200)	51.4	900 (30)	5.63	0.8 (0.07)	0.01	ND		ND
Duck (*Anatidae anseriformes*) *											
1	30000 (400)	100	20000 (400)	65.5	ND		ND		60 (1)	0.65	ND
2	1000 (400)	100	4 (0.5)	0.3	ND		70 (1)	4.97	400 (4)	27.2	ND
Horse (*Equus ferus caballus*) *											
	10000 (400)	100	4000 (300)	34.3	ND		0.4 (0.007)	0.003	ND		ND
Sheep (*Ovis aries*) *											
Ram	30000(2000)	100	9000 (300)	28.3	3000 (100)	11	3 (0.2)	0.01	ND		ND
Ewe	40000 (2000)	100	10000 (500)	26.8	3000 (200)	7.69	ND		ND		ND
Lamb	10000 (1000)	100	3000 (300)	23.6	2000 (200)	14	10 (2)	0.11	ND		ND
Cotton Rat (*Sigmodon*) *											
	3000 (70)	100	2000 (50)	65.4	ND	ND	4 (0.3)	0.14	ND		ND
Guineafowl (*Numididae*) *											
	60000 (1000)	100	20000 (500)	27.8	ND	ND	1 (0.03)	0.02	ND		ND
Pig (*Sus scrofa*) *											
Hog	3000 (200)	100	200 (2)	5.9	ND	ND	70 (7)	1.89	ND		ND
Pig	2000 (60)	100	400 (9)	19.3	ND	ND	0.7 (0.05)	0.03	ND		ND

* (x 10^6) (± standard error)
ND is non-detectable.

Table 7. Quantification of target-specific organisms in 100 mL water before and after a significant rainfall event at Plum Creek at different Cliff Swallow densities for year 1. (x 10^4) (± standard error).

Cliff Swallows	Event	Location	All Bacteria	%	All Bacteroidales	%	Ruminants (BacR)	%	*E. coli* %	*Helicobacter* %	*Salmonella* %
Absent	Before Rain	Up	40 (6)	100	2 (0.5)	4.87	ND		ND	ND	ND
		Down	30 (5)	100	3 (0.5)	8.44	ND		ND	ND	ND
	After Rain	Up	NS		NS		NS		NS	NS	NS
		Down	200 (90)	100	4 (0.5)	1.59	0.3 (0.02)	0.14	ND	ND	ND
Present	Before Rain	Up	7000 (900)	100	70 (10)	0.94	3 (0.2)	0.04	ND	ND	ND
		Down	4000 (700)	100	20 (7)	0.48	2 (0.2)	0.04	ND	ND	ND
	After Rain	Up	90000 (6000)	100	3000 (200)	2.96	10 (1)	0.01	ND	ND	ND
		Down	50000 (7000)	100	1000 (200)	2.43	20 (5)	0.05	ND	ND	ND
Nestlings	Before Rain	Up	2000 (100)	100	50 (5)	2.66	ND		ND	ND	ND
		Down	2000 (100)	100	30 (9)	1.43	ND		ND	ND	ND
	After Rain	Up	3000 (300)	100	8 (0.5)	0.31	0.2 (0.02)	0.01	ND	ND	ND
		Down	5000 (2000)	100	8 (0.6)	0.14	0.4 (0.09)	0.01	ND	ND	ND
Fledglings	Before Rain	Up	2000 (100)	100	50 (3)	2.19	ND		ND	ND	ND
		Down	2000 (200)	100	70 (6)	3.00	ND		ND	ND	ND
	After Rain	Up	200 (20)	100	10 (2)	8.65	ND		ND	ND	ND
		Down	200 (20)	100	20 (1)	8.73	ND		ND	ND	ND

ND – not detectable
NS – not sampled

Table 8. Quantification of target-specific organisms in 100 mL water before and after a significant rainfall event at Plum Creek at different Cliff Swallow densities for year 2. (x 10^4) (± standard error).

Cliff Swallows	Event	Location	All Bacteria	%	All Bacteroidales	%	Ruminants (BacR)	%	*E. coli* %	*Helicobacter* %	*Salmonella* %
Absent	Before Rain	Up	1000 (200)	100	30 (3)	1.88	1 (0.2)	0.07	ND	ND	ND
		Down	1000 (90)	100	10 (2)	1.16	0.3 (0.05)	0.02	ND	ND	ND
	After Rain	Up	300 (30)	100	9 (4)	2.79	ND		ND	ND	ND
		Down	500 (40)	100	9 (0.9)	1.95	ND		ND	ND	ND
Present	Before Rain	Up	700 (40)	100	10 (0.4)	1.39	0.3 (0.8)	0.04	ND	ND	ND
		Down	300 (50)	100	3 (0.3)	1.01	ND		ND	ND	ND
	After Rain	Up	2000 (100)	100	30 (1)	1.63	ND		ND	ND	ND
		Down	2000 (100)	100	30 (1)	1.41	ND		ND	ND	ND
Nestlings	Before Rain	Up	400 (30)	100	2 (0.2)	0.61	ND		ND	ND	ND
		Down	800 (100)	100	3 (0.1)	0.31	ND		ND	ND	ND
	After Rain	Up	10000 (1000)	100	100 (8)	0.92	20 (3)	0.15	ND	ND	ND
		Down	10000 (1000)	100	200 (8)	1.87	30 (3)	0.23	ND	ND	ND
Fledglings	Before Rain	Up	1000 (200)	100	5 (3)	0.37	0.6 (0.1)	0.04	ND	ND	ND
		Down	1000 (100)	100	4 (3)	0.36	0.4 (0.06)	0.03	ND	ND	ND
	After Rain	Up	NS		NS		NS		NS	NS	NS
		Down	NS		NS		NS		NS	NS	NS

ND – not detectable
NS – not sampled

Table 9. *q*PCR ANOVA results for all bacteria quantification.

Phenology	Year	Factor	p-value	F-ratio	df_n	df_d
Without Swallows	1	Rain	0.03	5.96	1	11
		Loc	0.64	0.23	1	11
		Rain:Loc	0.33	1.02	1	11
	2	Rain	0.02	7.50	1	12
		Loc	0.76	0.10	1	12
		Rain:Loc	0.72	0.14	1	12
Swallows Present	1	Rain	0.03	5.73	1	12
		Loc	0.65	0.21	1	12
		Rain:Loc	0.72	0.14	1	12
	2	Rain	0.04	5.26	1	12
		Loc	0.97	0.001	1	12
		Rain:Loc	0.99	0.0003	1	12
Swallow Nestlings Present	1	Rain	0.14	2.76	1	8
		Loc	0.38	0.86	1	8
		Rain:Loc	0.27	1.42	1	8
	2	Rain	0.0008	19.80	1	12
		Loc	0.60	0.29	1	12
		Rain:Loc	0.39	0.79	1	12
Swallow Fledglings Present	1	Rain	<0.0001	379.37	1	4
		Loc	0.08	5.51	1	4
		Rain:Loc	0.51	0.53	1	4
	2	Loc	0.38	0.95	1	4

Discussion and Future Prospects

Current assessments of fecal contamination rely on growth-dependent methods such as the MPN-based Colilert system or CFU-based plate spread methods on selective media such as TBX agar. In Texas, the Colilert system is generally used in laboratories accredited by the Texas Commission of Environmental Quality (TCEQ). In this study, we demonstrate that results obtained by the Colilert system in an accredited laboratory (EARDC) could be reliably reproduced in our laboratory using TBX agar. Small differences with respect to enumeration were obtained though; these differences most likely represent methodological differences (e.g. direct use of serial dilutions in the Colilert system compared to centrifuged samples with subsequent serial dilution in our system) as well as variable numbers in samples that were different for each of the analysis methods. The sensitivity of both systems allowed us to detect *E. coli* as an indicator organism for fecal contamination in low numbers (<10 cells in 100 ml of water) that were far below the threshold limit for contamination (>126 cells in 100 ml of water).

A drawback of these growth-dependent methods is that they fail to detect *E. coli* that do not express the enzyme but harbor the encoding *uid*A gene for the glucuronidase enzyme, and thus enumeration by these methods might underestimate total numbers of *E. coli* present. In feces of different animals, however, only small numbers of these *E. coli*, if at all, were encountered and thus the potential underestimation was either non-significant or specific for the source of contamination. These results support previous studies in which 3-4% of *E. coli* have been estimated to not express the glucuronidase enzyme (30), and thus cannot be detected by the Colilert system or TBX plating.

Both quantification methods for *E. coli* allowed us to distinguish numbers of *E. coli* in waters before and after precipitation events with numbers before precipitation events usually below contamination thresholds, and those after precipitation often 2 orders of magnitude higher (i.e. up to 2,500 cells in 100 ml of water). These results indicate significant effects of precipitation, likely as a consequence of run-off from terrestrial sites, on the abundance of *E. coli* and thus on the intensity of fecal contamination. The impact of run-off on *E. coli* enumeration is similar throughout all sampling periods with the differing densities of Cliff Swallows with the p-value lower than 0.05 for all of the sampling periods. The effect of run-off has previously been shown to significantly increase the microbial loadings in water (30).

Since samples were usually obtained a day after precipitation, it was not surprising, however, that effects of bridges, i.e. differences in numbers of *E. coli* up- and down-stream of bridges, were not detected at any of the sampling events during the 2-year sampling period. This was also true for samples without precipitation at all sampling events indicating that absence or presence of birds had no effect on the enumeration of *E. coli*. In contrast to our results, previous studies showed differences between up- and down-stream sites of bridges and a positive correlation of fecal contamination levels to Cliff Swallow densities (4).

While additional fecal contamination could be expected in the presence of birds, the extent of their contribution to fecal contamination is likely following different scenarios depending on the density of the birds. Returning birds that start to rebuild their nests likely defecate while away or flying away from nests, while nestlings drop feces directly from the nests and thus at high amounts. The failure to retrieve different numbers of *E. coli* up-

and down-stream and use the difference to assess the contribution of birds to fecal contamination, demonstrates limitations of the use *E. coli* for source tracking in our study. While our results can in part be explained by the potential dilution of fecal droppings in water and thus the dilution to below the detection limit, additional studies on feces demonstrated an extremely low abundance of *E. coli*, both by growth-dependent and molecular detection methods. These results demonstrate that *E. coli* is not an appropriate target and indicator organism to assess the contribution of Cliff swallows to fecal contamination in water samples under bridges.

Molecular methods are often presented as faster and more sensitive alternatives to culture dependent techniques, and thus should provide contamination data rapidly and allow for intervention to occur in recreational waters (14). A recurring argument in these presentations is that many environmental bacteria are adapting to the presumably adverse environmental conditions by the formation of dormant cells that will not become easily culturable and thus not be detected by growth dependent methods (31). Our study demonstrated the opposite, i.e. a much higher sensitivity for *E. coli* of growth-dependent detection methods than of molecular tools. This result can easily be explained by the differences in detection procedures: growth-dependent methods like the Colilert system use an entire 100 ml water sample to detect and quantify *E. coli* which results in reliable enumeration of even small numbers (e.g. below 10 cells for 100 ml of water). Molecular detection like *q*PCR targeting *uid*A relies on the extraction of nucleic acid from the cell pellet of 100 ml. Extraction and purification of nucleic acids is now commonly done with commercial kits that often lose up to 90% of the nucleic acids during the extraction and purification process. In addition, extracts are often not clean enough to allow unaffected

PCR amplification, and thus extracts are diluted further to out-dilute potential contaminants. Since initial DNA extracts are usually eluted into a 100 µL volume from which 1 µL is used for the PCR amplification, molecular detection already starts with a 10^4-fold dilution compared to growth-dependent detection such as with the Colilert system. It is therefore not surprising that even high numbers of *E. coli* such as 2,500 cells in 100 ml of water, detected and quantified by the Colilert system, cannot be detected by *q*PCR-based analysis. Thus, while in theory *q*PCR-based detection and quantification is supposed to be faster and more sensitive than culture dependent techniques and therefore should provide contamination data rapidly and allow for intervention to occur in recreational waters, the reality seems to be different even though some studies have shown quantification by *q*PCR to be comparable to growth dependent methods (14, 32). These studies that focused on drinking water that had been spiked with clinical *E. coli* strains, however, do not consider environmental factors in surface waters that bind and therefore reduce yields of DNA extracted from the samples or inhibit amplification. The factors are present in clean surface waters already, but are even more pronounced in runoff or upwelling waters after significant precipitation. A similar reasoning was provided for the differences in detection of *Campylobacter* from beach sands, where growth dependent methods had a lower detection limit than *q*PCR (33). As a consequence, growth-dependent analyses of fecal contamination using *E. coli* as an indicator organism is superior over molecular detection methods, and thus should be the method of choice for this analysis.

Attempts to use more specific bacteria or bacterial groups for microbial source tracking were affected by the same problem as indicated for E. coli, i.e. the large dilution factor, but also by specificity problems. For example, the use of *q*PCR-based microbial

source tracking targeting *Helicobacter* sp. as specific target for birds resulted in amplification products from duck feces, but failed to detect these bacteria in feces from other avian species including Cliff Swallows (8). The use of the ruminant Bacteroidales was shown to be specific, however, the use was restricted because it detected its target organisms in highly contaminated waters only. Quantification of all bacteria could be used to demonstrate effects of runoff, however, it was not useful for microbial source tracking. Similar results were obtained for the detection of all Bacteroidales.

Quantification using qPCR-based methods is further impacted by the need for a conversion factor required to relate a signal to cell numbers. Many molecular detection tools (i.e. all bacteria, all bac, and bacR) use 16S rRNA gene sequences as target for detection, and thus accurate quantification is effected by the number of gene copies per cell. While this might not be a big problem for highly specific targets (e.g. members of the species *Salmonella enterica* or *E. coli* that all are supposed to have 7 copies of the 16S rRNA gene), it definitely is for large groups of organisms (34). For all bacteria, for example, rRNA operons with copy numbers from one to as many as 15 copies have been detected in currently available whole genome sequences (35, 36). Quantification of all bacteria by qPCR uses an average 16S rRNA gene copy number per bacterial cell of 4.2 (36). Therefore, depending on the bacterial community composition in water, the average 16S rRNA gene copy number per bacterial cell might be different from the 4.2 copies. Bacterial communities developing from the same source under different environmental conditions, for example, produced different average copy numbers, i.e. average copy numbers of 1.4, 2.7, 5.4, or 5.5 (37).

In conclusion, both growth dependent methods were reliable in assessing the effects of runoff. The varying Cliff Swallow densities were shown to have no significant impact on the presence of *E. coli* load which is not surprising considering low to non-existing numbers of *E. coli* present in Cliff Swallow feces. The avian specific *Helicobacter* spp. were also not able to be used as indicator organisms in the Cliff Swallow feces, and therefore this target was not useful for detecting the contribution of these birds. Other target organisms that are specific to birds or specifically Cliff Swallows would have to be used in order to determine the contribution, if any, that these birds may have on fecal contamination of surface waters. The *E. coli* loads present in up- and down-stream locations did not have significant differences. The different bridges all followed similar patterns in *E. coli* loads throughout all sampling events. The use of *q*PCR based microbial source tracking has proven ineffective for monitoring contamination in natural water samples, although some results did corroborate with the results found by growth dependent methods on runoff effects.

LITERATURE CITED

1. Whitman, R.L, Nevers, M.B., Przybyla-Kelly, K., and Byappanahalli, M.N. 2011. Physical and biological factors influencing environmental sources of fecal indicator bacteria in surface water. In: The Fecal Bacteria (Sadowsky, M.J., Whitman, R.L., Eds.) ASM Press, Washington, DC, pp.111-134.

2. Jamieson, R., Gordon, R., Joy, D., and Lee, H. 2004. Assessing microbial pollution of rural surface waters: A review of current watershed scale modeling approaches. Agricultural Water Management 70:1-17.

3. Brown, C.R. and Brown, M.B. 1995. Cliff swallow (*Petrochelidon pyrrhonota*), the birds of North America online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online

4. Sejkora, P., Kirisits, M.J., and Barrett, M. 2011. Colonies of Cliff swallows on highway bridges: a source of *Escherichia coli* in surface waters. Journal of the American Water Resources Association 47.6:1275-1284.

5. Kinzelman, J., McLellan, S. L., Daniels, A. D., Cashin, S., Singh, A., Gradus, S., and Bagley, R. 2004. Non-point source pollution: determination of replication versus persistence of *Escherichia coli* in surface water and sediments with correlation of levels to readily measureable environmental parameters. Journal of Water and Health 2.2:103-114.

6. Graczyk, T.K., Majewska, A.C., and Schwab, K.J. 2008. The role of birds in dissemination of human waterborne enteropathogens. Trends in Parasitology 24.2:55-59.

7. Fallacara, D.M., Monahan, C.M., Morishita, T.Y., and Wack, R.F. 2001. Fecal shedding and antimicrobial susceptibility of selected bacterial pathogens and a survey of intestinal parasites in free-living waterfowl. Avian Diseases 45.1:128-135.

8. Green, H.C., Dick, L.K., Gilpin, B., Samadpour, M., and Field, K.G. 2012. Genetic markers for rapid PCR-based identification of gull, Canada goose, duck, and chicken fecal contamination in water. Applied and Environmental Microbiology 78.2:503-510.

9. Soller, J.A., Schoen, M.E., Bartrand, T., Ravenscroft, J.E., and Ashbolt, N.J. 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of fecal contamination. Water Research 44:4674-4691.

10. Mieszkin, S., Furet, J., Corthier, G., and Gourmelon, M. 2009. Estimation of pig fecal contamination in a river catchment by Real-Time PCR using two pig-specific *Bacteroidales* 16S rRNA genetic markers. Applied and Environmental Microbiology 75.10:3045-3054.

11. Winfield, M.D. and Groisman, E.A. 2003. Role of non-host environments in the lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environmental Microbiology 69.7:3687-3694.

12. Savageau, M. 1983. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. The American Naturalist 122.6:732-744.

13. Chase, E., Hunting, J., Staley, C., and Harwood, V.J. 2012. Microbial source tracking to identify human and ruminant sources of fecal pollution in an ephemeral Florida river. Journal of Applied Microbiology 113:1396-1406.

14. Staley, C., Gordon, K.V., Schoen, M.E., and Harwood, V.J. 2012. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. Applied and Environmental Microbiology 78.20:7317-7326.

15. Centers for Disease Control and Prevention (CDC). 2011. Surveillance for waterborne disease outbreaks and other health events associated with recreational water – United States, 2007 – 2008. MMWR 60:1-37.

16. Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., and Lukasik, J. 2002. Microbial source tracking: current methodology and future directions. Applied and Environmental Microbiology 68.12:5796-5803.

17. Rice, E. W., Allen, M. J., and Edberg, S. C. 1990. Efficacy of β-glucuronidase assay for identification of *Escherichia coli* by the defined-substrate technology. Applied and Environmental Microbiology 56.5:1203-1205.

18. Eckner, K. F. 1998. Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and enterococci used in drinking and bathing water quality monitoring in southern Sweden. Applied and Environmental Microbiology 64.8:3079-3085.

19. Wong, M. L. and Medrano, J. F. 2005. Real-time PCR for mRNA quantification. BioTechniques 39:75-85.

20. Gudnason, H., Dufva, M., Bang, D. D., and Wolff, A. 2007. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Research 35.19:e127.

21. Ramos-Payán, R., Aguilar-Median, M., Estrada-Parra, S., Gonzáles-y-Merchand, A., Favila-Castillo, L., Monroy-Ostria, A., and Estrada-Garcia, I. C. E. 2003. Quantification of cytokine gene expression using an economical Real-Time Polymerase Chain Reaction method based on SYBR® Green I. Scandinavian Journal of Immunology 57:439-445

22. Hiero, N., Esteve-Zarzoso, B., González, A., Mas, A., and Guillamón, J. M. 2006. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. Applied and Environmental Microbiology 72.11:7148-7155.

23. Postollec, F., Falentin, H., Pavan, S., Combrisson, J., and Sohier, D. 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiology 28:848-861.

24. Radomski, N., Lucas, F.S., Moilleron, R., Cambau, E., Haenn, S., and Moulin, L. 2010. Development of a Real-Time *q*PCR method for detection and enumeration of *Mycobacterium* spp. in surface water. Applied and Environmental Microbiology 76.21:7348-7351.

25. Fremaux, B., Boa, T., and Yost, C. K. 2010. Quantitative Real-Time PCR assays for sensitive detection of Canada goose-specific fecal pollution in water sources. Applied and Environmental Microbiology 76.14:4886-4889.

26. Gómez-Doñate, M., Ballesté, E., Muniesa, M., and Blanch, A. 2012. New molecular quantitative PCR assay for detection of host-specific Bifidobacteriaceae suitable for microbial source tracking. Applied and Environmental Microbiology 78.16:5788-5795.

27. Srinivasan, S., Aslan, A., Xagoraraki, I., and Alocilja, E. 2011. *Escherichia coli*, enterococci, and *Bacteroides thetaiotaomicron q*PCR signals through wastewater and septage treatment. Water Research 45:2561-2572.

28. Sha, Q., Vattem, DA., Forstner, MR., and Hahn, D. 2013. Quantifying *Salmonella* population dynamics in water and biofilms. Microbial Ecology 65:60-67.

29. Ratnam, S., March, S. B., Ahmed, R., Bezanson, G. S., and Kasatiya, S. 1988. Characterization of *Escherichia coli* serotype O157:H7. Journal of Clinical Microbiology 26.10:2006-2012.

30. Kistemann, T., Claßen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V., and Exner, M. 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Applied and Environmental Microbiology 68.5: 2188-2197.

31. Kinzelman, J., McLellan S. L., Amick, A., Preedit, J., Scopel, C. O., Olapade, O., Gradus, S., Singh, A., and Sedmak, G. 2008. Identification of human enteric pathogens in gull feces at Southwestern Lake Michigan bathing beaches. Canadian Journal of Microbiology 54:1006-1015.

32. Bukh, A. S., Hansen, N. E., Roslev, P. 2012. Detection and Persistence of Clinical *Escherichia coli* in drinking water evaluated by a rapid enzyme assay and *q*PCR. Advances in Microbiology 2:252-262.

33. Yamahara, K. M., Sassoubre, L. M., Goodwin, K. D., and Boehm, A. B. 2012. Occurrence and persistence of bacterial pathogens and indicator organisms in beach sand along the California coast. Applied and Environmental Microbiology 78.6:1733-1745.

34. Pei, A. Y., Oberdorf, W. E., Nossa, C. W., Agarwal, A., Chokshi, P., Gerz, E. A., Jin, Z., Lee, P., Yang, L., Poles, M., Brown, S. M., Sotero, S., DeSantis, T., Brodie, E., Nelson, K., and Pei, Z. 2010. Diversity of 16S rRNA genes within individual prokaryotic genomes. Applied and Environmental Microbiology 76.12:3886-3897.

35. Klappenbach, J. A., Saxman, P. R., Cole, J. R., and Schmidt, T. M. 2001. rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Research 29.1:181-184.

36. Větrovský, T., and Baldrian, P. 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS ONE 8.2:e57923.

37. Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M. 2000. rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology 66.4:1328-1333.