Comparison of gull feces-specific assays targeting the 16S rRNA gene of *Catellicoccus marimammalium* and *Streptococcus* spp.

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Abstract

Two novel gull-specific qPCR assays were developed using 16S rRNA gene sequences from gull fecal clone libraries: a SYBR-green-based assay targeting *Streptococcus* spp. (gull3) and a TaqMan qPCR assay targeting *Catellicoccus marimammalium* (gull4). The objectives of this study were: to compare the host specificity of a previous *C. marimammalium* qPCR assay (gull2) with that of the new markers; to examine the presence of the three gull markers in environmental water samples from different geographic locations. Of the 255 gull fecal samples tested, 86% were positive for gull2 and gull4, whereas only 28% were positive with gull3. Low prevalence and abundance of tested gull markers were observed in fecal samples from six non-avian species (n = 180), whereas the assays cross-reacted to some extent with other (non-gull) avian fecal samples. The gull3 assay was positive against fecal samples from 11 of 15 avian species including gull, suggesting that it has a potential to be a general avian marker. Of the presumed gull-impacted water samples (n = 349), 86, 59, and 91% were positive for the gull2, the gull3, and the gull4 assays, respectively. Approximately 5% of 239 non-gull impacted waters were positive with the gull2 and the gull4 assays, whereas 21% were positive for the gull3 assay. While the relatively high occurrence of gull markers in waters impacted by gull feces suggests that these assays could be used in environmental monitoring studies, the data also suggest that multiple avian-specific assays will be needed to accurately assess the contribution of different avian sources in recreational waters.

Introduction

Enacted in 2000, the Beaches Environmental Assessment and Coastal Health (BEACH) Act has as a goal to improve the quality of coastal waters designated for recreational uses such as swimming, bathing, surfing, and boating. From a microbiological standpoint, water quality is measured by estimating the levels of fecal pollution using fecal indicator bacteria (FIB). Different fecal sources can contribute to pollution of environmental waters, each of them carrying different health risks. Of all non-human fecal sources, several waterfowl species have been implicated as a source of fecal bacterial indicators in recreational waters (Lévesque *et al*. 2000, Edge and Hill 2007). Specifically, gulls are

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often seen in high numbers in inland and coastal waters, and depending on the geographic location and availability of food sources, gull colonies can be seen all year round near recreational waters, thus chronically contributing to fecal bacterial loadings (McLellan and Salmore 2003, Edge and Hill 2007, Jeter *et al.* 2009). In other cases, gull roosting and nesting are seasonal (Burger 1979) and so is their importance to public health.

Recently, Lu *et al*. (2008) developed a SYBR green-based, qPCR gull-specific assay (i.e., gull2) targeting the 16S rRNA gene of *Catellicoccus marimammalium*. This marker has shown a high level of host specificity and widespread host distribution, and has been detected in waters presumed to be impacted by gull fecal contamination. For example, when 1348 water samples from southern Ontario and around Lake Ontario were tested with the SYBR-green gull assay, 58% of the samples generated positive signals (Lu *et al.* 2011a). A TaqMan-based qPCR has also been developed using the original *C. marimammalium*-specific primers (Sinigalliano *et al.* 2010), but its use has been limited to marine coastal waters. While the aforementioned studies have shown the potential value of the current *C. marimammalium* gull qPCR assays as part of the microbial source-tracking toolbox, some limitations need to be addressed. First, a limited number of fecal samples have been used to test potential cross-amplification with other animals, and therefore further validation is needed. Second, target quantification using SYBR green assays can be hampered by the presence of double-peaks, suggesting secondary amplification products, and therefore significantly limiting its application as presence/absence assays. While Taqman-based assays are an alternative, only scarce information is available on the sequence diversity of the region targeted by Taqman reporter probe and how it affects the sensitivity of the assays. Third, the gull2 marker was designed to target *C. marimammalium* as this species was highly abundant in clone libraries generated using gull fecal samples collected in North America. The relative abundance of *C. marimammalium* in gull species inhabiting other regions may vary considerably; as a result, other bacterial species may potentially be better targets in environmental applications in such cases. Indeed, geographical variability has been documented for other microbial source tracking (MST) markers (Lamendella *et al.* 2007, Stoeckel and Harwood 2007, Fremaux *et al.* 2009) suggesting that multiple methods and approaches may be needed to increase the reliability of accurate source detection (Santo Domingo *et al.* 2007, Lamendella *et al*. 2009).

In this study, we developed a new gull marker (i.e., gull3 assay) targeting *Streptococcus* spp. and a new TaqMan-based assay (gull4) targeting a smaller region of the *C. marimammalium* 16S rRNA gene based on additional sequencing information. Evaluation of gull-specific assays included comparison studies against the original gull2 assay by testing for host distribution and specificity of each assay against a high number of gull and non-gull feces. Finally, the applicability of these assays to environmental water samples was tested by investigating the prevalence of these gull markers in gull and non-gull impacted water samples collected from different geographic locations across North America and Puerto Rico.

Methods

Bacterial Strains and Plasmid Preparation

C. marimammalium DSMZ M35/04/3T (University of Göteborg Culture Collection, Göteborg, Sweden) and *Streptococcus bovis* ATCC 33317 were used for preparing plasmids used as qPCR standards. Briefly, biomass from *C. marimammalium* and *S. bovis* was harvested from Columbia SB agar (Becton Dickinson, Sparks, MD) and heart infusion agar (Becton Dickinson, Sparks, MD) plates, respectively. PCR products generated with relevant primer sets (i.e., gull2 and gull3 assays; Table 1) were cloned into pCR®4 TOPO® vector and transformed to TOPO10 chemically competent *Escherichia coli* cells as described by the manufacturer (Invitrogen, Carlsbad, CA). Individual clones were subcultured at 37°C for 18 hours, and plasmids were extracted and purified using Zyppy Plasmid Miniprep kit (Zymo Research Corp.).

Sample Collection and DNA Extraction

Two gull fecal samples collected in South Africa were used to develop 16S rRNA gene clone libraries and to design the gull3 assay. By using gull samples from a geographically distant location we limited the effect of migration on the overall composition of gull fecal microbial communities as they would be exposed to different regional conditions. For geographic and host distribution of gull markers, 255 fecal samples were collected from a variety of gull

Table 1. Summary of oligonucleotide primers and probes for gull-specific qPCR assays.

Assay	Primer and Probe Sequences (5' to 3')	T_a (°C)*	Size (bp)	Reference
Gull2-SYBR green	Forward: TGCATCGACCTAAAGTTTTGAG	64	412	Lu *et al.* (2008)
	Reverse: GTCAAAGAGCGAGCAGTTACTA			
Gull3-SYBR green	SAG1F: ATTTAACCCATGTTAGATGC	56	319	This study
	SAG1R: CGTCCCTTTCTGGTAAGT			
Gull4-TaqMan	qGull7F: CTTGCATCGACCTAAAGTTTTGAG	60	116	This study
	qGull8R: GGTTCTCTGTATTATGCGGTATTAGCA			
	qGull7P**: 6FAM- ACACGTGGGTAACCTGCCCATCAGA -TAMRA			

* Optimum PCR annealing temperatures were determined using temperature gradients

** FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxytetramethylrhodamine, fluorescence quencher dye

species inhabiting different geographical locations and fecal DNA extracts were tested against all three gull assays. Gull fecal specimens used in this study were collected from California (*Larus californicus*), Ohio (*L. delawarensis*), Alaska (*L. glaucescens)*, Georgia (*L. atricilla*), Delaware (*L. atricilla* and *L. smithsonianus*), and South Africa (*L. cirrocephalus*). Additionally, 429 fecal samples from a variety of non-gull host animals (i.e., 180 fecal samples from domesticated animals and 249 samples from poultry and waterfowl) were used for testing host specificity. The potential value of each assay in environmental applications was tested against environmental water samples presumed to be impacted by gull feces (n = 349) and by fecal sources other than gulls (n = 239) collected from different sites in North America and Puerto Rico. Briefly, gull impacted samples were collected from marine recreational waters in California (Doheny Beach) and Delaware (Tower Beach) and surface waters at five different locations in Alaska, whereas non-gull impacted water samples were collected from an intensive agricultural area in Canada (Sumas watershed, British Columbia), Alaska (three lakes) and Puerto Rico (Rio Grande de Arecibo). Water samples (100 ml) were filtered onto polycarbonate membranes (e.g., 0.4 µm pore size, 47-mm diameter; GE Water and Process Technologies, Trevose, PA). Fecal samples were collected aseptically with sterile spatulas, transferred to sterile tubes, and transported to the laboratory in ice coolers. Filters and fecal samples were shipped overnight on dry ice to the USEPA, Cincinnati, Ohio (OH) and stored at -80°C until further processing. DNA extraction from filters and fecal samples was performed using Mo Bio PowerSoil kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. DNA concentration was measured using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA extracts were stored at -20°C until further processing.

Cloning and Sequencing Analyses

The microbial community composition of the gull fecal samples collected in South Africa was determined as described by Lu and Santo Domingo (2008) with minor modifications. Briefly, PCR was performed using the universal bacterial primer set 8F-787R, and selected PCR products were cloned into pCR®4 TOPO® vector following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Individual clones were subcultured into 300 µl of Luria Broth containing 50 µg/ml ampicillin and screened for inserts using M13 primers. PCR products were sequenced in both directions in the Children's Hospital DNA Core Facility (Cincinnati, OH) using M13 primers and an Applied Biosystems Prism 3730XL DNA analyzer. Raw sequences were processed using Sequencher software (Gene Codes, Ann Arbor, MI). Sequences identified as chimeric structures using Bellerophon (Huber *et al.* 2004) were not included in further analyses. Sequences were submitted to Greengenes for alignment using the nearest alignment space termination algorithm (DeSantis *et al*. 2006a, 2006b). The clone libraries

were compared using Naive Bayesian rRNA Classifier version 2.0 of Ribosomal Database Project (RDP) with 95% confidence threshold (Cole *et al.* 2009). Sequence homology searches were performed using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al*. 1997).

New Assay Development

To develop the gull3 assay we first developed a phylogenetic tree that included sequences generated from South African gull feces and the SILVA database was developed using a neighbor-joining algorithm in ARB (Ludwig *et al.* 2004). Unique phylogenetic clades were identified and candidate primers were then identified for targeting clades using the primer design algorithm in ARB. *In silico* testing of primer specificity was performed against published data and additional avian 16S rRNA gene clone sequences generated in our laboratory (unpublished results). The assay was optimized through temperature gradients and with various concentrations of fecal DNA templates, and tested for host specificity and host and geographic distribution using the fecal samples described above. Additionally, *C. marimammalium* 16S rRNA gene sequences were used to generate a TaqMan assay based on a DNA fragment targeted by the previously designed assay. Primers and a hydrolysis probe for this TaqMan assay were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA; Table 1). This newly developed qPCR assay was also tested for host distribution, host specificity, and presence in environmental waters against the aforementioned set of samples.

Quantitative PCR Assays

Two SYBR green-based qPCR assays (i.e., gull2 and gull3 assays) were tested against fecal and water samples. All water and fecal samples were processed as previously described (Lu *et al*. 2008) with the following modifications. All of the assays were performed with 0.5 - 1 ng μl^{-1} of DNA extracts as templates. Ten-fold dilutions of each DNA extract were used to test for PCR inhibition (Lu *et al.* 2008). Reaction mixtures (25 µl) contained 1× Power SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 0.2 µg/µl bovine serum albumin, and 0.2 µM of each primer (final concentration) and 2 µl of the template. The amplification protocol involved an initial incubation at 50°C for 2 minutes, followed by 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 64°C (gull2) or 56°C (gull3) for 1 minute, followed by melting curve analysis (i.e., from 60 to 90°C in 0.1-degree increments). The qPCR assays were performed using a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems, Foster City, CA, USA). All reaction mixtures were prepared in triplicate in MicroAmp Optical 96-well reaction plates with MicroAmp Optical Caps (Applied Biosystems, Foster City, CA, USA). PCR data were analyzed using ABI's Sequence Detector software (version 2.2.2). PCR signals were recorded as presence/absence data and signal quantity (intensity) values. Four independent standard curves for each qPCR assay were generated by plotting threshold cycle (C_T) values against the number of target copies corresponding to serially diluted plasmid standards.

The target copy numbers (T) were estimated as follows:

$$T = [D/(\mathrm{PL} \times 660)] \times 6.022 \times 10^{23}$$

where D (g/µl) is plasmid DNA concentration and PL (bp) is plasmid length in base pairs.

Each standard curve was generated from at least five 10-fold plasmid dilutions in triplicates. Percent amplification efficiencies were calculated using the following equation per the instrument manufacturer's instructions (Applied Biosystems):

$$\text{Percent Amplification Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$$

where the slope value for each assay is generated from each standard curve.

Two no-template controls per PCR plate were used to check for cross-contamination. Disassociation curves were examined to determine the presence of potential primer-dimers and other non-specific reaction products. Signal intensity values were recorded for those reactions showing one corresponding amplification peak within the disassociation curves. PCR products were also visualized in 1% agarose gels using GelStar as the nucleic acid stain (Lonza, Rockland, ME, USA) to confirm amplification product size.

The Taqman qPCR assay targeting the 16S rRNA gene of *C. marimammalium* (gull4) was performed in 25-µl reaction mixtures containing 1× TaqMan universal PCR master mix with AmpErase uracil-N-glycosylase (Applied Biosystems, Foster City, CA), 0.2 µg/µl bovine serum albumin, 0.2 µM (final concentration) of each primer and FAM-labeled

hydrolysis probe. The amplification protocol involved an initial incubation at 50°C for 2 minutes to activate uracil-N-glycosylase, followed by 10 minutes of incubation at 95°C to activate AmpliTaq Gold enzyme, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR and data analyses were performed as presented above.

Bayesian Statistics

Bayes' theorem was used to estimate the confidence of each assay at detecting gull fecal sources in environmental waters (Kildare *et al.* 2007). To achieve this, the posterior probability was calculated using the following formula:

$$P(A|B) = \frac{P(A) \times P(B|A)}{\{P(A) \times P(B|A) + P(A') \times P(B|A')\}}$$

This involved calculating the posterior probability (P(A|B)) by determining the ratio of true positives (P(B|A)) and false positives (P(B|A')) in fecal samples, and the ratio of water samples that tested positive (i.e., the prior probability or P(A)). The posterior probability was also calculated by varying the prior probability from worst case scenario (i.e., negative signals in all samples or 0) to best case scenario (i.e., positive signals in all samples or 1) as described by Lamendella *et al.* (2009).

Nucleotide Sequence Accession Numbers

Representative sequences were deposited in GenBank under accession numbers JN394017 to JN394075.

Results and Discussion

Phylogenetic Analysis of 16 rRNA Gene Sequences

DNA extracts from gull feces collected in South Africa were used to generate clone libraries in an effort to further understand the diversity of gull fecal microbial communities. A total of 354 clone sequences were analyzed in this study. Excluding sequences unclassified or classified as unknowns, 17 bacterial genera were represented in the clone library (Table 2). The gull bacterial community was mostly composed of populations closely related to *Bacilli* (69.2%), *Gammaproteobacteria* (16.7%), and *Clostridia* (3.1%). No sequences homologous to *Bacteroidetes* were found in this study, which is consistent with the overall low prevalence of members of this phylum in the avian cloacae (Fogarty and Voytek 2005, Lu *et al.* 2008, Jeter *et al*. 2009). Within the *Bacilli*, 116 and 68 sequences were classified as *Streptococcus* spp. (32.8%) and *C. marimammalium* (19.2%) respectively, while the other sequences formed a clade of unclassified *Lactobacillales* (14.1%). In a previous study, *C. marimammalium* 16S rRNA gene sequences constituted 26% of the fecal clones from gull samples collected in North America (Lu *et al*. 2008). The detection of *C. marimammalium* sequences in gulls from geographically distant regions and the high percentage of positive signals detected with the gull2 and gull4 assays (Table 3) further confirm the widespread occurrence of this bacterial species in gulls. In contrast with Lu *et al*. (2008), fewer *Clostridia* sequences were obtained in this study, whereas *Bacilli* were the most dominant bacterial class. Since sequences closely related to *Streptococcus* (i.e., ≥95% identity) were identified as the most dominant species, these sequences were used as potential targets for the development of new gull markers

A total of 24 sequences from genera known to contain species considered human pathogens (i.e., *Campylobacter*, *Shigella,* and *Klebsiella*) were identified in this study, although none of the sequences retrieved were related to pathogenic species. The sequencing effort associated with this study is not deep enough to retrieve sequences from pathogens as they would be considered rare members. However, bacterial pathogens have been isolated from different species of gulls (Girdwood *et al*. 1985, Craven *et al*. 2000, Steele *et al*. 2005). For example, several studies have also documented the presence of pathogenic *Campylobacter* spp. in gull excreta (Whelan *et al*. 1988, Quessy and Messier 1992, Moore *et al*. 2002). On the other hand, Lu *et al*. (2011b) reported high prevalence of campylobacters in California gulls (i.e., 45% positive in 159 fecal samples), but a low occurrence of pathogenic species based on PCR assays and 16S rRNA gene sequences. Based on the latter results, the risk associated with gull fecal pollution has been estimated to be relatively low (Schoen and Ashbolt 2010), suggesting that, compared to human fecal pollution sources, fecal loads from gulls and other waterfowl will have to be high in order for the risks to be significant. The health risks associated with bird fecal pollution sources have been estimated by looking at a limited number of conventional pathogens and using FIB data on a limited number

Table 2. Distribution of 16S rRNA genes in the clone library of gull feces. "--" = not found.

Class or Group	% Total Clones		Genus	No. of Clones	
	This study (n = 354)	Lu *et al*. 2008 (n = 282)		This study	Lu *et al*. 2008
Fusobacteria	3.1	0.7	*Cetobacterium*	11	2
Bacilli	69.2	37.2	*Bacillus*	0	3
			Weissella	5	0
			Lactobacillus	6	9
			Streptococcus	116	0
			Catellicoccus	68	74
			Unclassified *Lactobacillales*	50	7
Clostridia	3.1	17.3	*Dialister*	1	0
			Subdoligranulum	3	0
			Clostridium	3	44
			Unknown	2	2
Erysipelotrichi	0.9	0	*Turicibacter*	1	--
			Unknown	2	
Alphaproteobacteria	0.3	6.7	*Rubellimicrobium*	1	0
			Paracoccus	0	8
Epsilonproteobacteria	0.3	0.4	*Campylobacter*	1	1
Gammaproteobacteria	16.7	11.3	*Acinetobacter*	0	13
			Enterobacter	1	6
			Escherichia	0	6
			Citrobacter	3	0
			Shigella	6	0
			Klebsiella	17	5
			Unknown	32	0
Sphingobacteria	0.6	0	Unknown	2	--
Actinobacteria	0.6	6.4	*Corynebacterium*	0	8
			Unknown	2	3
Mollicutes	0.3	0	*Ureaplasma*	1	--
Bacteroidetes	0	1.1	*Bacteroidetes*	--	1
Betaproteobacteria	0	4.3	*Acidovorax*	--	6
Unknown	5.1	3.2	Unknown	18	9

of waterfowl species. Waterfowl are also believed to be important reservoirs of antibiotic resistant bacteria (Dolejska *et al*. 2009, Bonnedahl *et al*. 2010) and of less studied non-bacterial pathogens such potentially pathogenic protozoa (Graczyk *et al*. 1998, Kassa *et al*. 2004) and influenza viruses (Fouchier *et al*. 2005, Snoeck *et al*. 2011). For example, avian influenza viruses (H5N1) have been detected in poultry and waterfowl such as gulls, geese, ducks, swans, and shorebirds (Olsen *et al*. 2006). While transmission to humans is believed to be relatively low, the mortality rates are relatively high (http://www.who.int/csr/

Table 3. Prevalence of gull markers in various animal feces.

	Animal	Sample Location	No. of Samples	SYBR green		TaqMan
				Gull2	Gull3	Gull4
Gull	California gull (*Larus californicus*)	California	159	138	44	143
	Ring-billed gull (*Larus delawarensis*)	Ohio	2	2	2	2
	Glaucous-winged gull (*Larus glaucescens*)	Alaska	64	53	2	50
	Laughing gull (*Larus atricilla*)	Georgia	5	5	2	2
	Laughing gull (*Larus atricilla*)	Delaware	3	2	3	3
	Herring gull (*Larus smithsonianus*)	Delaware	20	16	16	18
	Grey headed gull (*Larus cirrocephalus*)	South Africa	2	2	2	2
	Total		255	218	71	221
Poultry/ Waterfowl	Chicken (houses)	Puerto Rico	98	12	23	8
	Turkey	Puerto Rico	5	0	5	0
	Duck	Puerto Rico	16	0	16	0
	Pigeon	Puerto Rico	11	1	2	2
	Heron	Puerto Rico	1	0	0	0
	Swan	Puerto Rico	22	0	9	0
	Guineafowl	Puerto Rico	11	0	1	0
	Crane	Nebraska	12	0	2	8
	Snow geese	Nebraska	10	0	0	4
	Pelican	California	10	10	10	10
	Red Knot	Delaware	17	1	0	1
	Turnstone	Delaware	5	2	1	1
	Canada geese	Alaska	25	6	0	4
	Mallard	Alaska	6	0	1	0
	Total		249	32	76	38
Non-Avian Species	Cattle	Puerto Rico	66	0	5	0
	Goat	Puerto Rico	32	0	5	0
	Monkey	Puerto Rico	9	0	0	0
	Fish	Puerto Rico	13	1	0	0
	Horse	Puerto Rico	30	1	3	0
	Pig	Puerto Rico	30	8	13	1
	Total		180	10	27	1

disease/avian_influenza/country/en/). Additionally, the evolutionary rate of influenza viruses is also very high, which explains the widespread occurrence of non-avian reservoirs and reported strains pathogenic to many different mammals (Li *et al.* 2004, Salomon and Webster 2009).

Performance of Quantitative PCR

The range of quantification (ROQ) for the *C. marimammalium* qPCR assays (gull2 and gull4) was 10^1 to 10^6 DNA copies per reaction. For gull3 assay, 10 copies per reaction were below detection limit of the assay, and therefore the ROQ of gull3 assay was determined to range from 10^2 to 10^6 DNA copies. In order to evaluate assay sensitivity, four independent standard curves were used to calculate the percent amplification efficiency average (Table 4). The gull4 assay showed the greatest amplification efficiency, followed by gull2 and gull3, respectively. All of the no-template controls were negative indicating the absence of cross-contamination in the qPCR experiments.

Table 4. qPCR performance characteristics.

Assay	DNA type	Percent Amplification Efficiency (Average ± 1 standard deviation)[a]	R^2	ROQ (copies) for Target DNA[b]
Gull2-SYBR green	Plasmid	85.7 ± 2.5	>0.998	10-106
Gull3-SYBR green	Plasmid	72.8 ± 1.7	>0.990	102-106
Gull4-TaqMan	Plasmid	95.3 ± 0.9	>0.998	10-106

[a] Four independent standard curves were used to calculate the amplification efficiency average, and each standard curve was generated with at least 5 serial dilutions of target plasmids in triplicate.

[b] The range of quantification (ROQ) for the gull3 assay ranged from 10^2-10^6. Ten copies per reaction were below detection limit.

Evaluation of the Gull-Specific Assays

The gull-specific assays designed to target 16S rRNA gene of *C. marimammalium* and *Streptococcus* spp. were tested against individual gull fecal samples (n = 255). Most gull fecal samples were positive for the gull2 (86% or 218/255) and gull4 (87% or 221/255) assays, whereas only 28% (71/255) were positive with the gull3 assay (Table 3). By combining the results from the three assays, the detection levels increased slightly to 88% (225 of 255; Figure 1a). Only one sample was negative with gull2 and gull4 but positive with the gull3 assay, whereas the rest of the gull3 positive samples (n = 70) were positive against both gull2 and gull4 assays. The limit of detection for the gull2 and gull4 assays was one order of magnitude lower than the gull3 assay (Table 4). Interestingly, when compared to samples that were positive to all of the assays, signal intensities of gull2 and gull4 assays were relatively lower in many samples that tested negative by the gull3 assay, but positive to only one of the other assays (Figure 2). These data suggest that while Lactobacillales are the important members of the gull fecal community, their overall abundance is dynamic, possibly due to changes in age, dietary regime, or physiological status of the host.

Markers targeting *Catellicoccus* (gull2 and gull4) were detected in the feces of all gull species tested. Lu *et al*. (2008) reported a 71% detection of the gull2 marker in 58 individual gull fecal samples collected from different geographical locations in North America: Florida (*L. atricilla*), West Virginia (*L. delawarensis*), Ohio (*L. delawarensis*), Georgia (*L. atricilla* and *L. delawarensis*), and Ontario, Canada (*L. delawarensis*). In this study, high prevalence of the gull2 marker was also obtained in additional gull species tested, regardless of the locations at which the samples were collected. The relatively lower prevalence of the gull3 marker was more evident in the Glaucous-winged gulls (*L. glaucescens*) excreta (2 of 64 samples). The prevalence of gull3 was also relatively low in California gulls (*L. californicus*) (i.e., 27%). However, excluding *L. glaucescens* and *L. californicus*, the prevalence of the gull3 marker in other gull species was higher (i.e., about 78%; Table 3). In most gull species the gull3 marker signal had lower intensity than gull2 and gull4, suggesting that *Streptococcus* spp. are not as numerically

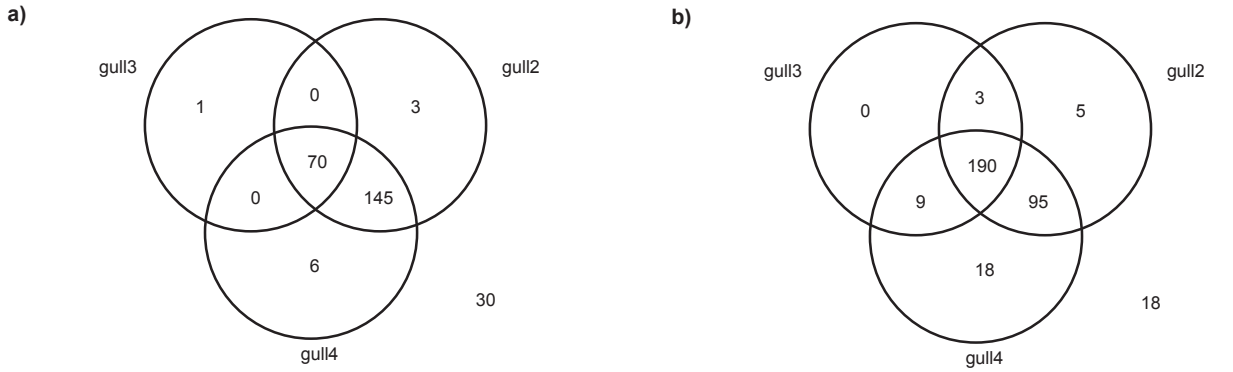

Figure 1. Venn Diagram for gull-specific assay positives against gull feces (a) and water (b) impacted with gull fecal contamination.

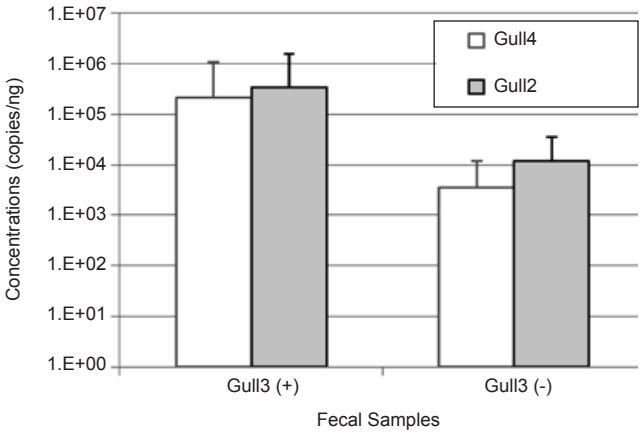

Figure 2. Mean copy number of target markers in gull fecal DNA in samples positive and negative to the gull3 assay. To calculate mean concentrations, below detection limits were treated as zero and double peak values from the gull2 assay were not considered. Error bars represent one standard deviation.

(n = 180), relatively low cross-amplification was observed (i.e., 5.5, 15, and 0.6% for gull2, gull3, and gull4 assays, respectively). However, a considerable number of pig fecal samples were positive with the gull2 and gull3 assays (i.e., 27 and 43%, respectively), although the numbers of DNA copies from pig feces were more than four orders of magnitude lower than those of gull feces (data not shown). The gull4 qPCR assay cross-reacted with only one of the non-avian samples, indicating that it is more specific to gull feces than the gull2 assay.

Host specificity tests against non-gull avian samples revealed some interesting patterns. For example, approximately 13 and 15% of non-gull avian fecal samples were positive for the gull2 and gull4 assays, respectively, whereas twice as many (31%) were positive with the gull3 assay. Specifically, the assays cross-reacted to some extent with chicken, crane, snow geese, and Canada geese, although, in general, the signal intensity was lower than in gull samples (Figure 3). In contrast, not only were all pelican fecal samples (n = 10) positive with all three assays, but abundance of the markers was also relatively high. It should be noted that the prevalence of the gull3 marker in non-gull avian fecal samples was similar to gull feces. Moreover, of the 14 different non-gull avian species tested in this study, 10 of them were positive against the gull3 assay, suggesting that the gull3 assay has a potential to be a general avian marker. However, the value of this marker to detect avian pollution sources may be restricted to waters in which swine and ruminant sources (i.e., application of manure in agricultural areas nearby recreational waters) are not present in light of the cross-amplification signals detected in this study. We also noted a discrepancy between gull2 and gull4 assays and the signals associated with crane and snow geese samples. None of the crane and snow geese fecal samples showed cross-amplification with the gull2 assay, whereas the gull4 marker was detected in 67 and 40% of crane and snow geese fecal samples, respectively (Table 3). This is somewhat puzzling as both gull 2 and gull4 markers were designed using *C. marimammalium* 16S rRNA gene, a bacterium. Sequencing analysis of snow geese and crane fecal clone libraries demonstrated that there is a small number of sequences nearly identical to *C. marimammalium* (i.e., an average of <2%; data not shown). Greater detection limits of the gull4 assay compare to the gull2 assay can potentially explain the differences in detection rates.

Gull PCR-Based Signals in Water Samples

Of the presumed gull impacted water samples tested (n = 349), 86, 59, and 91% were positive for the gull2, the gull3, and the gull4 assays, respectively (Table 5). Most gull impacted water samples tested in this study were collected from California recreational water samples (n = 338). Of those, relatively few samples were negative for all assays (5%) or positive for only one assay (<7%).

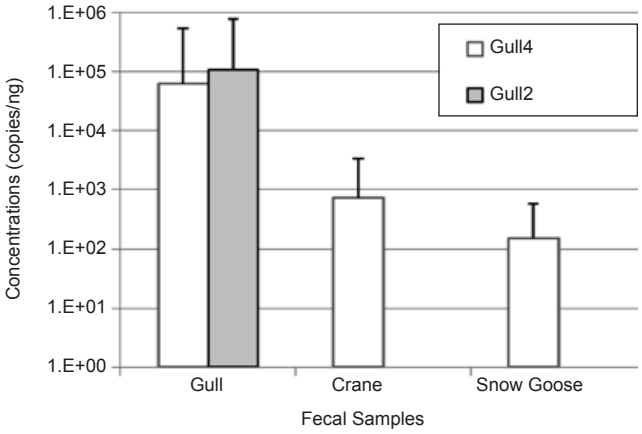

Figure 3. Mean copy number of markers in fecal DNA extracts. To calculate mean concentrations, the below detection limit (BDL) values were treated as zero and double peak values from the gull2 assay were not considered. Error bars represent one standard deviation. The gull2 marker was not detected in any of crane and snow goose fecal samples.

Table 5. Detection of gull markers in water samples by gull-specific assays.

Sampling Location(s)	Sample Type	Sampling Period	No. of Water Samples	No. of Positive Assay Samples			Presumed Primary Source of Fecal Contamination[a]
				Gull2	Gull3	GullTaqMan	
California beach	Freshwater and Seawater	May-September 2008	338	293	202	312	Gull
Delaware beach	Freshwater and Seawater	May 2011	6	5	4	6	Gull
Anchorage, Alaska	Freshwater	October 2010	5	1	1	1	Gull
Anchorage, Alaska	Freshwater	October 2010	3	0	0	0	Unknown
Toronto, Canada	Water Treatment Plant Intake (Lake Ontario offshore)	December 2009	9	0	0	0	Unknown
Toronto, Canada	Sewage Treatment Plant Effluent	December 2009	3	0	0	0	Human, some gull
Toronto, Canada	Sewage Treatment Plant Effluent and CSO Samples[b]	December 2009	6	0	0	0	Human
Sumas Watershed, BC, Canada (agriculture impacted sites)	Freshwater	April 2007-December 2007	64	12	26	10	Chicken, some livestock
Sumas Watershed, BC, Canada (reference site)	Freshwater	April 2007-December 2007	16	1	5	2	Wildlife
Puerto Rico	Freshwater	September 2010-January 2011	138	0	19	0	Domesticated animals (including chicken)

[a] There is historical knowledge that host animals are present at these sites a significant part of the year.

[b] CSO (combined sewer overflow)

Of the other samples, most of them (n = 190) were positive by all three assays (Figure 1b). The gull4 assay showed the highest prevalence possibly due to its higher sensitivity. The amplification efficiency of the gull4 assay ranged from 95 to 98% which was higher than other assays (i.e., less than 90% for the gull2 and the gull3 assays). Of the presumed non-gull impacted waters (n = 239), approximately 5% of the samples were positive with the gull2 and the gull4 assays, whereas 21% were positive for the gull3 assay. Most samples that tested positive for all of the markers were collected from the Sumas watershed in western Canada presumably contaminated by poultry and some livestock fecal sources. Specifically, 26 of 64 (41%) water samples from Sumas watersheds were positive with the gull3 assay (Table 5). Water samples collected from a watershed in Puerto Rico primarily contaminated by human (wastewater and septic tanks) and animal fecal sources (mostly cattle, although birds such as chicken and duck are also present) were tested for the gull assays. Of the 138 water samples tested, 19 were positive by the gull3 assay, although there were no positive signals by the gull2 and the gull4 assays in any samples. These monitoring results are comparable with specificity results showing the relatively higher cross-amplification of avian fecal samples against the gull3 assay.

Since all gull-specific assays showed some level of false positives (cross-amplification with some of the non-gull fecal samples) and false negatives (no signals with some of the gull feces), we conducted Bayesian statistics to determine which assays were more reliable for environmental monitoring. First, the deterministic Bayesian values with gull-impacted water samples examined in this study were estimated as described by Kildare *et al*. (2007; Table 6). Using this approach the predictive positive value of the gull2 and gull4 assays were estimated at 0.98 and 0.99, suggesting a very high confidence level for water samples that tested positive. In contrast, lower confidence levels were determined for the gull3 assay. This is in agreement with the overall higher sensitivity and specificity rates exhibited by the gull2 and gull4 assays compared to the gull3 assay. Bayesian statistical models also showed that the gull2 and the gull4 assays yielded higher confidence in detecting true-positive signals and lower probability of false- negative signals in water samples under a wide range of prior probabilities of gull fecal contamination than the gull3 assay (Figure 4). Altogether, these data indicate that the gull2 and the gull4 assays have more desirable properties for their

Table 6. Bayesian statistics for the three qPCR assays against gull impacted water samples.

Assays	Conditional Probability[a]	Sensitivity[b]	Specificity[c]	Prevailing Rate[d]
Gull2	0.98	0.85	0.9	0.86
Gull3	0.63	0.28	0.76	0.59
Gull4	0.99	0.87	0.91	0.91

[a] The conditional probability (i.e., posterior probability or P(A | B) in the Bayesian formula) was calculated using a Bayesian statistical model.

[b] The sensitivity is the ratio of positive signals in gull fecal samples. It is numerically identical to P(A | B) in the Bayesian formula.

[c] The specificity is the ratio of negative signals in non-gull fecal samples. It is numerically identical to {1- P(A | B')} in the Bayesian formula.

[d] The prevailing rate is the ratio of positive signals in water samples. It is numerically identical to P(A) in the Bayesian formula.

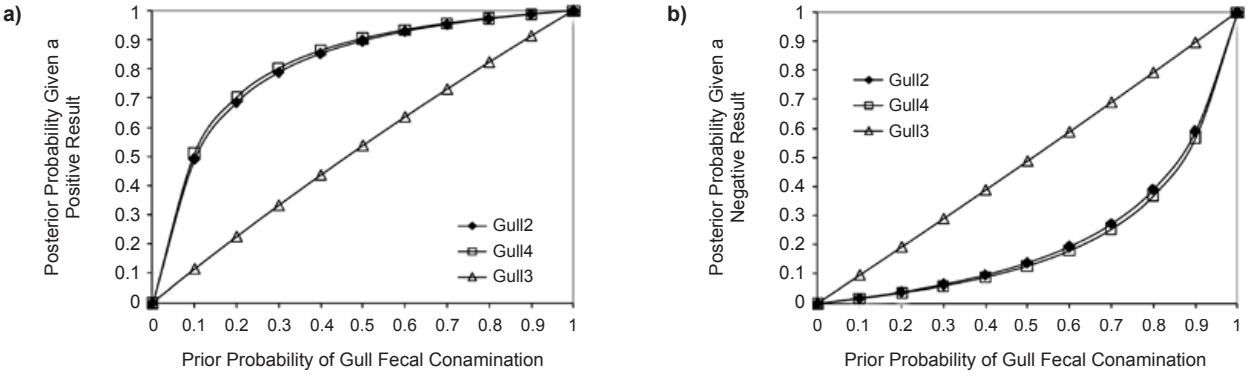

Figure 4. Probability of gull fecal contamination using a Bayesian statistical model. (a) Posterior probability of contamination given a positive qPCR result using three different gull-specific assays over a range of prior probabilities. (b) Posterior probability of contamination given a negative qPCR result using three different gull-specific assays over a range of prior probabilities.

use as MST markers for environmental application than the gull3 assay.

In summary, the gull2 and the gull4 assays exhibited higher specificity to gull feces than the gull3 assay, which cross-amplified with a greater number of non-gull fecal samples. The gull3 assay was originally designed for the detection of gull fecal contamination. However, based on the evaluation regarding host specificity, host distribution, and environmental monitoring potential, the gull3 assay may be used as a general avian marker rather than a new gull contamination marker. Overall, gull-specific assays showed a higher level of cross-amplification with other avian species than non-avian hosts. This suggests that when several waterfowl species are present in recreational waters, multiple waterfowl assays will be needed to accurately assess the contribution of each avian source. However, the relatively high occurrence of gull markers in waters impacted by gull feces suggests that combined these assays could be used in environmental monitoring studies.

Literature Cited

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25:3389-3402.

Bonnedahl, J., P. Drobni, A. Johansson, J. Hernandez, A. Melhus, J. Stedt, B. Olsen and M. Drobni. 2010. Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *Journal of Antimicrobial Chemotherapy* 65:1939-1944.

Burger, J. 1979. Competition and predation: herring gulls versus laughing gulls. *The Condor* 81:269-277.

Cole, J.R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R.J. Farris, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, T. Marsh, G.M. Garrity and J.M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* 37:D141-D145.

Craven, S.E., N.J. Stern, E. Line, J.S. Bailey, N.A. Cox and P. Fedorka-Cray. 2000. Determination of the incidence of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal droppings. *Avian Diseases* 44:715-720.

DeSantis, T.Z., P. Hugenholtz, K. Keller, E.L. Brodie, N. Larsen, Y.M. Piceno, R. Phan and G.L. Andersen. 2006a. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research* 34:W394-W399.

DeSantis, T.Z., P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu and G.L. Andersen. 2006b. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* 72:5069-5072.

Dolejska, M., B. Bierosova, L. Kohoutova, I. Literak and A. Cizek. 2009. Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *Journal of Applied Microbiology* 106:1941-1950.

Edge, T.A. and S. Hill. 2007. Multiple lines of evidence to identify the sources of fecal pollution at a freshwater beach in Hamilton Harbour, Lake Ontario. *Water Research* 41:3585-3594.

Fogarty, L.R. and M.A. Voytek. 2005. Comparison of *Bacteroides-Prevotella* 16S rRNA genetic markers for fecal samples from different animal species. *Applied and Environmental Microbiology* 71:5999-6007.

Fouchier, R.A.M., V. Munster, A. Wallensten, T.M. Bestebroer, S. Herfst, D. Smith, G.F. Rimmelzwaan, B. Olsen and A. Osterhaus. 2005. Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of Virology* 79:2814-2822.

Fremaux, B., J. Gritzfeld, T. Boa and C.K. Yost. 2009. Evaluation of host-specific *Bacteroidales* 16S rRNA gene markers as a complementary tool for detecting fecal pollution in a prairie watershed. *Water Research* 43:4838-4849.

Girdwood, R.W., C.R. Fricker, D. Munro, C.B. Shedden and P. Monaghan. 1985. The incidence and significance of salmonella carriage by gulls (*Larus* spp.) in Scotland. *Journal of Hygiene* 95:229-241.

Graczyk, T.K., R. Fayer, J.M. Trout, E.J. Lewis, C.A. Farley, I. Sulaiman and A.A. Lal. 1998. *Giardia*

sp. cysts and infectious *Cryptosporidium parvum* oocysts in the feces of migratory Canada geese (*Branta canadensis*). *Applied and Environmental Microbiology* 64:2736-2738.

Huber, T., G. Faulkner and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20:2317-2319.

Jeter, S.N., C.M. McDermott, P.A. Bower, J.L. Kinselman, M.J. Bootsma, G.W. Goetz and S.L. McLellan. *Bacteroidales* diversity in ring-billed gulls (*Laurus delawarensis*) residing at Lake Michigan beaches. *Applied and Environmental Microbiology* 75:15251533.

Kassa, H., B.J. Harrington and M.S. Bisesi. 2004. Cryptosporidiosis: a brief literature review and update regarding *Cryptosporidium* in feces of Canada geese (*Branta canadensis*). *Journal of Environmental Health* 66:34-39.

Kildare, B.J., C.M. Leutenegger, B.S. McSwain, D.G. Bambic, V.B. Rajal and S. Wuertz. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. *Water Research* 41:3701-3715.

Lamendella, R., J.W. Domingo, D.B. Oerther, J.R. Vogel and D.M. Stoeckel. 2007. Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of *Bacteroidetes* 16S rRNA gene. *FEMS Microbiology Ecology* 59:651-660.

Lamendella, R., J.W. Santo Domingo, A.C. Yannarell, S. Ghosh, G. Di Giovanni, R.I. Mackie and D.B. Oerther. 2009. Evaluation of swine-specific PCR assays used for fecal source tracking and analysis of molecular diversity of swine-specific *Bacteroidales* populations. *Applied and Environmental Microbiology* 75:5787-5796.

Lévesque, B., P. Brousseau, F. Bernier, E. Dewailly and J. Joly. 2000. Study of the bacterial content of ring-billed gull droppings in relation to recreational water quality. *Water Research* 34:1089-1096.

Li, K.S., Y. Guan, J. Wang, G.J. Smith, K.M. Xu, L.Duan, A.P. Rahardjo, P. Puthavathana, C. Buranathai, T.D. Nguyen, A.T. Estoepangestie, A. Chaisingh, P. Auewarakul, H.T. Long, N.T. Hanh, R.J. Webby, L.L. Poon, H. Chen, K.F. Shortridge, K.Y. Yuen, R.G. Webster and J.S. Peiris. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430:209-213.

Lu, J. and J.W. Santo Domingo. 2008. Turkey fecal microbial community structure and functional gene diversity revealed by 16S rRNA gene and metagenomic sequences. *Journal of Microbiology* 46:469-477.

Lu, J., H. Ryu, S. Hill, M. Schoen, N. Ashbolt, T. Edge and J.W. Santo Domingo. 2011a. Distribution and potential significance of a gull fecal marker in urban coastal and riverine areas of southern Ontario, Canada. *Water Research* 45:3960-3968.

Lu, J., H. Ryu, J.W. Santo Domingo, J. Griffith and N. Ashbolt. 2011b. Molecular detection of *Campylobacter* spp. in California gull (*Larus californicus*) excreta. *Applied and Environmental Microbiology* 77:5034-5039.

Lu, J., J.W. Santo Domingo, R. Lamendella, T. Edge and S. Hill. 2008. Phylogenetic diversity and molecular detection of gull feces. *Applied and Environmental Microbiology* 74:3969-3976.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Heier, I. Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, L. Brettske, S. Gerber, A.W. Ginhart, O. Gross, S. Grumann, R. Hermann, A. Jost, T. König, R. Liss, M. Lübmann, B. May, B. Nonhoff, S. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode and K.-H. Schleifer. 2004. ARB: A software environment for sequence data. *Nucleic Acids Research* 32:1363-1371.

McLellan, S.L. and A.K. Salmore. 2003. Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina. *Water Research* 37:2700-2708.

Moore, J.E., D. Gilpin, E. Crothers, A. Canney, A. Kaneko and M. Matsuda. 2002. Occurrence of *Campylobacter* spp. and *Cryptosporidium* spp. in seagulls (*Larus* spp.). *Vector Borne and Zoonotic Diseases* 2:111-114.

Olsen, B., V.J. Munster, A. Wallensten, J. Waldenstrom, A. Osterhaus and R.A. Fouchier. 2006.

Global patterns of influenza A virus in wild birds. *Science* 312:384-388.

Quessy, S. and S. Messier. 1992. Prevalence of *Salmonella* spp*., Campylobacter* spp. *and Listeria* spp. in ring-billed gulls (*Larus delawarensis).* *Journal of Wildlife Diseases* 28:526-531.

Ryu, H., H. Tran, M. Ware, B. Iker, S. Griffin, T. Edge, N. Newmann, E. Villegas and J. Santo Domingo. 2011. Application of leftover sample material from waterborne protozoa monitoring for the molecular detection of *Bacteroidales* and fecal source tracking markers. *Journal of Microbiological Methods* 86:337-343.

Salomon, R. and R.G. Webster. 2009. The influenza virus enigma. *Cell* 136:402-410.

Santo Domingo, J.W., D.G. Bambic, T.A. Edge and S. Wuertz. 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. *Water Research* 41:3539-3552.

Schoen, M.E. and N.J. Ashbolt. 2010. Assessing pathogen risk to swimmers at non-sewage impacted recreational beaches. *Environmental Science & Technology* 44:2286-2291.

Sinigalliano, C.D., J.M. Fleisher, M.L. Gidley, H.M. Solo-Gabriele, T. Shibata, L.R. Plano, S. M. Elmir, D. Wanless, J. Bartkowiak, R. Boiteau, K. Withum, A.M. Abdelzaher, G. He, C. Ortega, X. Zhu, M.E. Wright, J. Kish, J. Hollenbeck, T. Scott, L.C. Backer and L.E. Fleming. 2010. Traditional and molecular analyses for fecal indicator bacteria in non-point source subtropical recreational marine waters. *Water Research* 44:3763-3772.

Snoeck, C.J., A.T. Adeyanju, S. De Landtsheer, U. Ottosson, S. Manu, W. Hagemeijer, T. Mundkur and C.P. Muller. 2011. Reassortant low-pathogenic avian influenza H5N2 viruses in African wild birds. *Journal of General Virology* 92:1172-1183.

Steele, C.M., R.N. Brown and R.G. Botzler. 2005. Prevalences of zoonotic bacteria among seabirds in rehabilitation centers along the Pacific coast of California and Washington, USA. *Journal of Wildlife Diseases* 41:735-744.

Stoeckel, D.M. and V.J. Harwood. 2007. Performance, design, and analysis in microbial source tracking studies. *Applied and Environmental Microbiology* 73:2405-2415.

Whelan, C.D., P. Monaghan, R.W. Girdwood and C.R. Fricker. 1988. The significance of wild birds (*Larus* sp.) in the epidemiology of *Campylobacter* infections in humans. *Epidemiology & Infection* 101:259-267.

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