Microbial Ecology (2019) 78:348–360
https://doi.org/10.1007/s00248-018-1295-9

ENVIRONMENTAL MICROBIOLOGY

Comparative Analysis of Anuran Amphibian Skin Microbiomes Across Inland and Coastal Wetlands

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Received: 6 July 2018 / Accepted: 15 November 2018 / Published online: 8 December 2018

Abstract

Amphibians host a community of microbes on their skin that helps resist infectious disease via the dual influence of anti-pathogenic microbial species and emergent community dynamics. Many frogs rely on freshwater habitats, but salinization is rapidly increasing saltwater concentrations in wetlands around the globe, increasing the likelihood that frogs will come into contact with salt-contaminated habitats. Currently, we know little about how increased salt exposure will affect the symbiotic relationship between the skin microbes and frog hosts. To better understand how salt exposure in a natural context affects the frog skin microbiome, we use *Hyla cinerea*, a North American treefrog species that can inhabit brackish wetlands, to explore three questions. First, we determine the extent that microbial communities in the environment and on frog skin are similar across populations. Second, we assess the microbial species richness and relative abundance on frogs from habitats with different salinity levels to determine how salinity affects the microbiome. Third, we test whether the relative abundances of putatively pathogen-resistant bacterial species differ between frogs from inland and coastal environments. We found that the frog microbiome is more similar among frogs than to the microbial communities found in surface water and soil, but there is overlap between frog skin and the environmental samples. Skin microbial community richness did not differ among populations, but the relative abundances of microbes were different across populations and salinities. We found no differences in the relative abundances of the anti-fungal bacteria *Janthinobacterium lividum*, the genus *Pseudomonas*, and *Serratia marcescens*, suggesting that environmental exposure to saltwater has a limited influence on these putatively beneficial bacterial taxa.

Keywords Microbiome · Anuran amphibian · Frog · Mutualism · Skin · Pathogen resistance · Bacteria · Secondary salinization

Introduction

Global climate change and anthropogenic activities are dramatically modifying ecosystems around the world at an accelerating pace [1, 2]. One way that ecosystem changes may impact biodiversity is by disrupting important symbiotic relationships among organisms [3, 4]. In fact, mismatched phenology [5] and other alterations to symbiont communities [6, 7] from climate change have already been documented in multiple systems.

Amphibians have an important symbiotic relationship with the microbial community inhabiting their skin. The thin epidermis of amphibians plays a key role in respiration, thermoregulation, osmoregulation, pigmentation, and protection from predators and pathogens [8, 9]. Amphibian skin is stippled with an array of mucus and granular glands that secrete oils and other substances that protect against desiccation and predation [8, 10]. These glandular secretions, in conjunction with the morphology of the skin, provide a matrix that supports a community of microbes that can synthesize vitamins and anti-microbial agents that aid in pathogen resistance [11–13]. For example, some cutaneous microbes provide resistance to infections from the fungal pathogen, *Batrachochytrium dendrobatidis*, which has been linked to global declines in amphibian populations [14–17]. Specifically, the bacterium, *Janthinobacterium lividum*, produces the anti-fungal metabolite compound, violacein [18, 19], which can reduce host mortality from *B. dendrobatidis* [19, 20]. Other microbial species have also been shown to inhibit *B. dendrobatidis* growth in vitro [21].

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While the presence of specific anti-fungal species appears to contribute strongly to host defense against *B. dendrobatidis*, in some instances, anti-microbial properties emerge as a result of the interactions among diverse members of the skin microbial community. For example, in *J. lividum*, the production of anti-fungal metabolites is triggered by competition between microbial species [22]. Moreover, established microbial communities can be more resistant to invading colonists, including infectious pathogens [23–25]. Indeed, ecological dynamics likely explains why individuals experimentally inoculated with beneficial species can still have high *B. dendrobatidis* infection rates [20] and why individuals whose microbiomes were experimentally disturbed had higher rates of infection [26, 27]. Therefore, determining the factors that impact both community assembly and the presence of potentially beneficial species is important to understanding how a changing environment will impact the structure and function of the skin microbiome.

Because the skin microbial community is situated at the boundary between the host and the environment, a variety of biotic and abiotic processes can impact community assembly and structure. During initial colonization, microbes may be shared between hosts via horizontal (e.g., fighting or breeding) or vertical (e.g., parent to offspring) transfer [13, 28–30]. Horizontal and vertical transfer via host-to-host contact is likely to produce similar communities of microbes on individuals within a population, which may facilitate host microbe symbioses and promote the integrity of beneficial microbiome assemblages, such as the presence of anti-pathogenic species [31]. Alternatively, microbes may be transferred onto hosts following contact with the environment (e.g., water, soil, vegetation) [32–34]. Microbial communities that are primarily sourced from the environment are likely to be less deterministic in their structure and composition than those transferred via host-to-host contact, instead reflecting stochastic processes and idiosyncratic differences in abiotic and biotic environments at different locations within the population [35, 36]. Determining the degree that environment or horizontal transfer among hosts contribute to the cutaneous microbiome community may have important implications for the ecology and persistence of host populations faced with exposure to pathogens or other environmental stressors.

Following initial contact and colonization, factors intrinsic to both the hosts and the microbes may influence the assembly of the microbiome through time [37–39]. For example, the physical and chemical properties of the host's skin (e.g., mucus secretions) may facilitate or inhibit the establishment and persistence of certain microbes over others [40]. Likewise, the microbes themselves may have different abilities to compete for and utilize available niche space (i.e., niche appropriation theory) [41–43]. For example, a rare but highly competitive microbe species may eventually outcompete and outnumber a more abundant colonist that is a poor competitor.

Environmental characteristics that make up the host's habitat can significantly alter the outcomes of the interactions and dynamics that occur during and after colonization, which may in turn affect the ability of the microbiome to function optimally [44]. Indeed, a changing environment may alter the microhabitat of the host's skin directly or indirectly by changing host physiology (e.g., dehydration may increase the amount of mucus produced). Similarly, the environment may alter or disrupt ecological dynamics occurring among microbial species, thus impacting the ability of some microbes to establish populations.

An increasingly common abiotic environmental factor that impacts both host physiology and the structure and stability of the microbiome is the presence of salt in the environment [45–47]. Secondary salinization, hereafter referred to as salinization, is a growing environmental concern in which concentrations of soluble salts increase at rates far exceeding natural levels [48]. Coastal areas are particularly vulnerable to salinization due to increased saltwater incursion/flooding from sea level rise, greater frequency and magnitude of storm surges, frequent dredging of canals, and saltwater intrusion into freshwater aquifers from over extraction [49–52].

Anuran amphibians (e.g., frogs and toads) are considered to be salt-sensitive, freshwater species not typically associated with salt-intruded or brackish habitats [53]. Currently, only 2% of frog species are known to use saline habitats [54]. However, given present rates of salinization, it is likely that frogs will increasingly come into contact with water with elevated salt concentrations [48]. The direct impact of salinization on anurans has been studied extensively [53, 54], and it is generally assumed that the abundance and richness of frogs will decrease in salinized wetlands, primarily because high osmotic stress is toxic to amphibians [53, 54]. However, at low concentrations, salt may not be lethal but still impose indirect impacts, such as affecting the symbiotic relationship between the microbes and their anuran hosts.

Salinity may affect the skin-associated microbial communities and their functionality by reducing the abundance of beneficial microbial species or disrupting community assembly and thereby impairing the health of the host. However, it is also possible that exposure to high salinity may ultimately benefit hosts by increasing the abundance of salt-tolerant microbial species that have beneficial properties. For instance, *Saprolegnia* water fungus and *B. dendrobatidis* loads on amphibian hosts are decreased in brackish environments, which suggests that saline wetlands may provide some protection to amphibians against fungal pathogens [55–57]. This has been termed the "salt-refuge hypothesis." However, it is unclear whether alterations to the microbiome contribute to the protection resulting from salt exposure or whether it is simply that

the pathogen species are unable to persist in saline habitats [55].

Because little is known about the effects of the environment on the amphibian skin microbiome, it is difficult to predict how saltwater exposure will affect the symbiotic relationship between microbes and amphibians. This study explores these questions in three ways by comparing populations of the American green treefrog (*Hyla cinerea*) inhabiting freshwater or brackish water. First, to determine the extent that microbial communities in the environment and on frog skin are similar across coastal and inland populations, we compare the microbial community collected from treefrog skin to the microbial community collected from the environment. Second, we determine whether microbial skin community richness and relative abundance differ among treefrogs collected from inland (freshwater) or coastal (brackish) wetlands to understand how inhabiting coastal wetlands affects the skin microbiome. Third, we test whether the relative abundances of certain microbial taxa that are known to produce beneficial secondary metabolites differ between treefrogs that inhabit inland or coastal wetlands to explore whether differential abundance of these species may be associated with the "salt-refuge hypothesis."

Methods

We studied *Hyla cinerea*, the American green treefrog, a species that can tolerate moderate amounts of salinity [53, 58, 59]. All life stages of *H. cinerea* have been observed in brackish marshes in North Carolina, in some cases in salinities exceeding 20 parts per thousand (ppt) [53]. We focused on *H. cinerea* due to their fairly unique ability among anurans to inhabit brackish and freshwater wetlands [53], yet it is important to note that this species has low *B. dendrobatidis* prevalence and intensity across their natural range [60, 61].

Experimental Design and Field Collection of Samples

We sampled adult individuals across multiple populations from brackish wetlands along the outer and inner banks of North Carolina (NC) and compared the microbiome on these individuals to individuals in inland freshwater wetlands. Specifically, we sampled 5 adult *H. cinerea* from each of 4 geographically discrete populations from freshwater wetlands around Greenville, NC (hereafter called "inland" frogs) and 5 adult *H. cinerea* from each of 4 discrete populations from coastal, brackish wetlands (hereafter called "coastal" frogs) ($N = 40$ frogs; 20 from coastal populations, 20 from inland populations) (Fig. 1). Frogs were collected using new, sterile nitrile gloves for each frog, and individuals were placed into sterile Whirl-Pak® bags (Nasco) following capture. All frogs were swabbed within 30 min of capture. Prior to swabbing, each individual frog was rinsed with ~ 50 mL of sterile, reverse osmosis water to remove dirt and ephemeral bacteria. The cutaneous surface of each individual was then swabbed with a sterile swab (Medical Wire Dryswab™) in a standardized procedure [62]. The ventral surface of each frog was swabbed 10 times (up and down is one swab), each thigh swabbed five times in a single direction, and each hind foot five times in a single direction. Swabs were then placed into sterile, labeled 2 mL centrifuge tube and immediately placed on ice and stored in − 80 °C until extractions. Following swabbing, each frog was weighed, snout-vent length (SVL) was measured, and individuals were released at the site of capture. All protocols were approved by the East Carolina University IACUC (AUP #D302).

Environmental samples were three swabs of the surface water and the soil near the pond margin from which we collected the frogs (similar to [62]). To collect water swab samples, three random locations were chosen within 10 m of the location that frogs had been collected, and a sterile swab was stirred in the water for 5 s at a depth of 1 cm. Likewise, we selected three random points along the bank of the pond near the population of collection and swabbed soils for 5 s each. We also used a YSI Professional Plus multi-parameter meter (Xylem, Inc., Yellow Springs, OH) to record the temperature, salinity, conductivity, and pH of the water. See Table 1 for detailed site information.

DNA Extraction, Amplification, and Sequencing

Frog samples were sequenced individually, but environmental samples were pooled by type (e.g., water, soil) for each population to gain the broadest view possible of what was present in the environment [62]. We randomly selected two populations for soil sequencing and sequenced all four water sample populations resulting in a total of 12 environmental samples (two pooled soil samples and four pooled water samples from coastal and inland locations). DNA was extracted from the swabs (both frog and environmental swabs) using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol, with the initial lysozyme incubation step for 1 h at 37 °C for Gram-positive bacteria. For 16S rRNA gene amplicon sequencing, the V4 region of the DNA was amplified with primers 515F and 806R [63]. The individually barcoded reverse PCR primers had a 12-base error-correcting Golay code to allow for sample multiplexing. PCR reactions were run in triplicate, and each 25 μL reaction contained 13 μL molecular-grade PCR water, 10 μL 5 Prime Hot Master Mix, 0.5 μL each of the forward and reverse primers (10 μM final concentration), and 1.0 μL genomic DNA. PCR conditions were the following: denaturation step for 3 min at 94 °C, amplification step at 35 cycles for 45 s at 94 °C, annealing for 60 s at 50 °C, extension for 90 s at 72 °C, and a final extension of 10 min at 72 °C. Triplicate reaction products

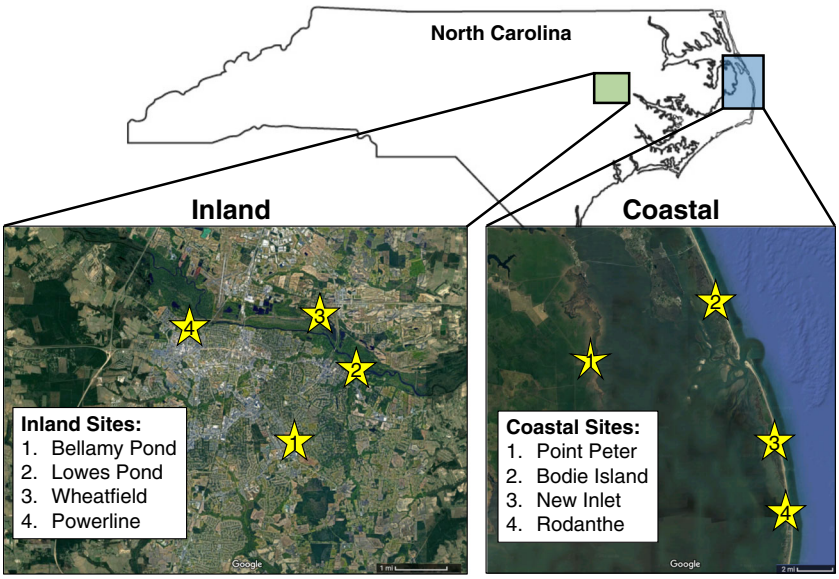

Fig. 1 Map of the four study populations across inland and coastal locations in North Carolina. Inland and coastal locations are separated by approximately 190 km. Stars denote the location of ponds that microbial samples were collected. We sampled *Hyla cinerea* (frog) skin, surface pond water, and pond soils from each location (Table 1). Satellite images are 2018 GoogleMaps ® images

were pooled and checked for integrity by running on a 1% agarose gel, and the amount of DNA in each sample was quantified with a Qubit® 2.0 Flourometer and dsDNA HS assay kit (Life Technologies). Equal amounts (200 ng/sample) of the final PCR products were pooled into a single sample and cleaned using a QIAquick PCR purification kit (Qiagen). The sample was sequenced with a 250 single-end strategy on an Illumina Mi-Seq using the 300 cycle v2 kit (MS-102-2002) at the Dana-Farber Cancer Research Institute at Harvard University, Boston, MA, with 10% PhiX added to increase base diversity.

Microbial Community Data Processing

The raw Illumina 16S rRNA gene amplicon data were processed and quality-filtered using the QIIME 1.9 pipeline [64]. The raw Illumina 16S rRNA gene amplicon data produced 14,030,629 total reads. We used the default settings for demultiplexing of the forward reads except that we allowed for no errors in the barcode and we set the required phred score at 20. After quality-filtering, we maintained 8,444,412 reads. In Geneious (Biomatters, Ltd.), we rapidly mapped the reads to the PhiX genome to remove any remaining PhiX, and from the non-PhiX reads, we extracted all 251 bp sequences for further analysis. These sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence similarity using the UCLUST method [65] in QIIME. We used the most abundant sequence from each cluster to represent the OTU. These representative sequences were aligned to the Greengenes 13_8 reference database [66] using PyNAST [67]. Taxonomy was assigned using the RDP classifier [68]. From the resulting OTU table, we filtered out OTUs that were

Table 1 Sampling populations, dates, and information for coastal and inland *Hyla cinerea* used for these analyses. Average snout/vent length measurements and average weight are presented for each population. Group indicates the populations in the freshwater group, low-salinity group, and high-salinity group (Fig. 3)

Collection date	Location	Specific location	Average snout/vent length (mm) (± SD)	Average mass (g) (± SD)	Group	Water temp (°C)	Water pH	Salinity (ppt)	Conductivity (μs/cm)
7/23/2015	Inland	Bellamy's Pond	42.22 (± 2.7)	4.38 (± 1.09)	Fresh	25.8	7.7	0.02	50.1
8/1/2015	Inland	Lowe's Pond	46.02 (± 1.12)	5.11 (± 0.65)	Fresh	29.5	7.42	0.01	32.7
7/23/2015	Inland	Powerline Cut	42.23 (± 2.45)	4.38 (± 0.68)	Fresh	25.8	7.69	0.03	75
8/1/2015	Inland	Wheat Field	42.3 (± 3.1)	4.11 (± 0.89)	Fresh	29.3	7.36	0.05	115.6
7/24/2015	Coastal	New Inlet's Pond	43.13 (± 1.48)	5.08 (± 0.37)	Low	27.5	7.58	1.75	3486
7/24/2015	Coastal	Bodie Lighthouse	42.44 (± 1.6)	4.39 (± 0.79)	High	28.8	7.7	14.9	26,384
7/24/2015	Coastal	Point Peter	39.86 (± 2.45)	3.21 (± 0.56)	Low	24	7.6	3.35	6045
7/24/2015	Coastal	Rodanthe	42.29 (± 3.09)	4.45 (± 0.92)	Fresh	26	7.98	0.98	1968

classified as mitochondrial or chloroplast sequences or were unassigned to any taxa and then removed those with fewer than 0.01% (< 741 reads) of the total number of reads [69]. We rarefied all samples to a sequencing depth of 28,000. Rarefication resulted in the loss of seven samples from the dataset that had read counts < 8500 (5 frog samples and 2 water samples).

Statistical Analysis

All analyses were conducted in the R statistical programing environment [70] using packages vegan [71], reshape [72], lme4 [73], ggplot2 [74], and betareg [75]. To characterize the diversity of the microbial communities, we first calculated four different alpha diversity metrics: species richness, Shannon's diversity index (H′), Simpson's diversity index (D), and Faith's phylogenetic diversity. Each of these metrics provides a different but complementary perspective on OTU richness and abundance among individuals. Species richness is the number of OTUs in the sample. Shannon's index takes both richness and relative abundance into account, with larger values typically signifying increases in both richness and relative abundance. Simpson's index (often referred to as a dominance index) includes both richness and evenness, with more common species weighed more heavily than the Shannon's index. Faith's phylogenetic diversity assesses taxonomic diversity conveyed as the number of tree units present within each sample [76]. To test whether the skin microbial communities differed by location (i.e., coastal or inland), we used linear mixed-effects models to test each diversity index (i.e., species richness, H′, D) against collection location, with the specific collection population as a random effect to control for population-specific variation. We assumed a log-normal error distribution for species richness and Shannon-Weaver data [77, 78]. Simpson's diversity values, in contrast, are bound between 0 and 1; so, we assumed beta-distributed errors for these analyses.

To analyze the differences in the relative abundances of microbial species in the community (ß-diversity) between inland and coastal populations, we used the function Adonis() in vegan to conduct Permutational MultiVariate Analysis of Variance (PERMANOVA) on the Bray-Curtis dissimilarity values. For this analysis, populations were nested within locations so that the permutations were stratified appropriately for comparisons of microbial abundance across both population and location [71]. As a complement to the PERMANOVA, we use betadispr() function in vegan to test for homogeneity [71]. A significant beta dispersion indicates that differences revealed by the PERMANOVA may be not driven by differences in microbiome composition *between* locations but rather by differences in the dispersion of species *within* each location. We visualized ß-diversity using principal coordinates analysis (PCoA).

To further understand how salinity (and not just location) affects the relative abundance of bacteria, we grouped each population according to salinity (< 1 ppt = freshwater, 1 to 5 ppt = low salinity, and > 5 ppt = high salinity; Table 1) instead of location and tested for differences using PERMANOVA and betadispr. The species contributing the most to overall differences among freshwater, low, and high salinity populations were identified by filtering the relative OTU abundance data using multipatt() in the package "indicspecies" to the level of $p = 0.05$ [79]. To visualize these differences, we plotted the log-ratio of relative OTU abundance of indicator taxa between low salinity:freshwater populations and high salinity:freshwater populations. To test whether the microbial community from the frogs overlapped with the microbial community present in their environment (e.g., soils and water), we again used PERMANOVA and betadispr and visualized the data using PCoA.

For our targeted analyses, we focused on three bacterial taxa with known in vitro anti-fungal properties that have been isolated from amphibian skin [21] and tested for differences in relative abundances on frogs from coastal and inland habitats. Specifically, we chose *Janthinobacterium lividum* [18, 19], the genus *Pseudomonas* [80], and *Serratia marcescens* [81]. For these analyses, we limited the dataset to just the 16s rRNA gene amplicon sequences that were assigned as these three taxa. Because there were several OTUs taxonomically identified as *Pseudomonas* spp. and *J. lividum*, we used PERMANOVA and betadispr to test for differences in the relative abundance and dispersion of these taxa across location and population. There was only one OTU that was identified as *S. marcescens*; so, we used a generalized linear mixed-effects model assuming a Poisson error distribution and a random effect of collection population, to analyze differences in *S. marcescens* among coastal and inland locations.

Results

The final rarefied dataset of OTU relative abundances consisted of 692 OTUs across 45 samples ($N = 35$ frog samples (19 coastal samples and 16 inland samples) and 10 environmental samples (coastal: 4 water samples, 2 soil samples; inland: 2 water samples, 2 soil samples)), with a range of 175–376 OTUs/sample. Microbial OTUs from 15 phyla were observed on frogs sampled across coastal and inland population (Fig. 2). We observed phylum Parvarchaeota (Domain: Archaea) on coastal frog skin but did not observe any representatives within this domain on inland frogs (Fig. 2). When samples were classified according to salinity (i.e., high, low, freshwater), we found significant differences in relative abundance of microbial taxa among salinities (pseudo $F_2 = 3.14$, $p = 0.002$) (Fig. 3), with no significant differences in beta dispersion among salinities (pseudo $F_2 = 1.64$, $p = 0.2$).

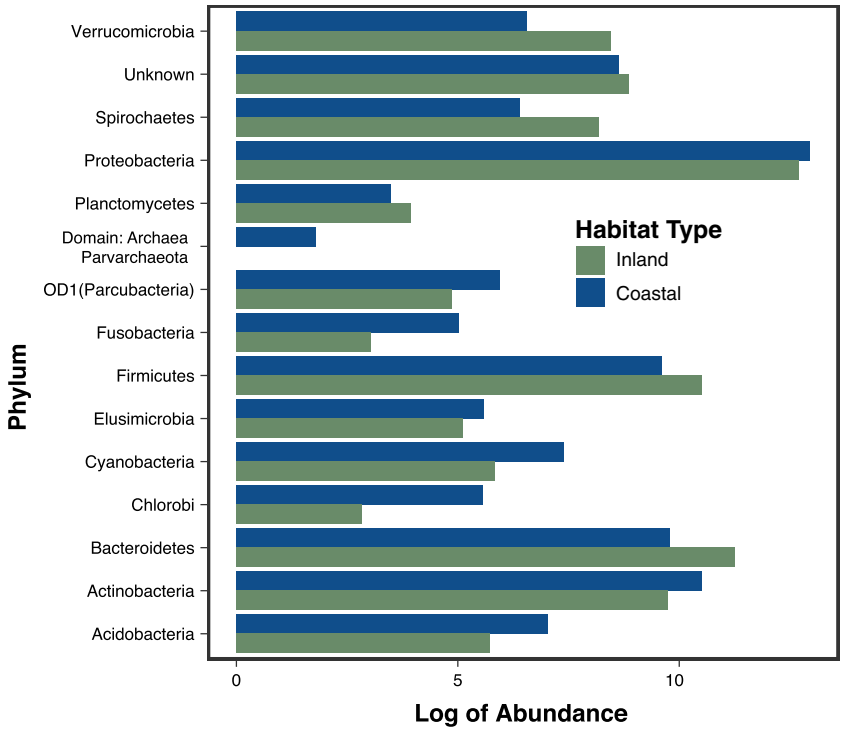

Fig. 2 The log of the relative abundance of each skin-associated microbial phyla present on the skin of *Hyla cinerea* frogs. Green bars denote the relative abundance of each phylum present on inland frogs, while blue bars denote the relative abundance of each phylum on coastal frogs

Indicator species analysis revealed that out of 692 total OTUs, 176 emerged as significant ($p < 0.05$) drivers of differences among groups. These 176 OTUs occur within 49 different microbial families (Fig. 3). When contrasted against the abundances observed on freshwater frogs, we found that members of the Comamonadaceae, Rhodobacteraceae, and Chromatidaceae families increased the most in abundance in low- and high-salinity populations, while those of Methylobacteriaceae, Lachnospiraceae, and Bacteroidaceae decreased the most in low- and high-salinity populations (Fig. 3).

When compared against the environmental samples, the microbial communities on frog skin differed from the microbial communities found in the water and soil samples (pseudo $F_2 = 4.97$, $p = 0.001$) (Fig. 4). There were also significant differences in the beta dispersion (pseudo $F_2 = 26.83$, $p = 0.001$). Out of the 593 different OTUs observed on inland frogs, 344 were also observed in the water and 411 were observed in the soils (Fig. 5). Coastal frogs host 585 different OTUs, and we observed 496 of those same OTUs in the water samples and 376 in the soil samples (Fig. 5). We found that 210 OTUs out of 692 total OTUs were shared across both inland and coastal locations and across frog, water, and soil samples (Fig. 5).

The community structure of microbial OTUs on frog skin differed between coastal and inland wetlands (pseudo $F_1 = 3.46$, $p = 0.007$) and among collecting populations (pseudo $F_6 = 3.02$, $p = 0.001$) (Fig. 3), with no clear separation in beta dispersion between coastal and inland locations (pseudo $F_1 = 0.88$, $p = 0.39$) (Fig. 6). However, there were no differences in the mean species richness of the skin microbial communities among populations or between inland and coastal locations (population: $\chi^2_{10} = 11.55$, $p = 0.08$; location: $\chi^2_4 = 0.02$, $p = 0.88$) (Fig. 6a). Likewise, there were no differences in Shannon-Weaver's diversity, Simpson's diversity, or Faith's phylogenetic diversity across populations or locations (Shannon-Weaver's population: $\chi^2_{10} = 7.94$, $p = 0.24$; Shannon-Weaver's location: $\chi^2_4 = 1.58$, $p = 0.21$, Fig. 6b; Simpson's diversity population: $\chi^2_2 = 11.90$, $p = 0.10$; Simpson's diversity location: $\chi^2_2 = 1.99$, $p = 0.16$, Fig. 6c; Faith's PD population: $\chi^2_{10} = 9.45$, $p = 0.15$; Faith's PD location: $\chi^2_4 = 0.69$, $p = 0.95$, Fig. 6d).

For the targeted analysis of the three bacterial taxa with suspected anti-Bd properties, we found no differences in the relative abundance or beta dispersion of *Janthinobacterium lividum* (PERMANOVA: pseudo $F_1 = 0.88$, $p = 0.341$; dispersion: pseudo $F_1 = 0.25$, $p = 0.65$) (Fig. 7a), *Pseudomonas* OTUs (PERMANOVA: pseudo $F_1 = 0.24$, $p = 0.85$; dispersion: pseudo $F_1 = 1.82$, $p = 0.16$) (Fig. 7b), or *Serratia marcescens* ($Z = -0.48$, $p = 0.64$) (Fig. 7c) in the skin microbial communities of frogs across coastal and inland wetlands.

Discussion

Saltwater is considered to be a strong abiotic filter affecting microbial communities on organisms [45–47]; yet, we found that skin microbial species richness did not differ between

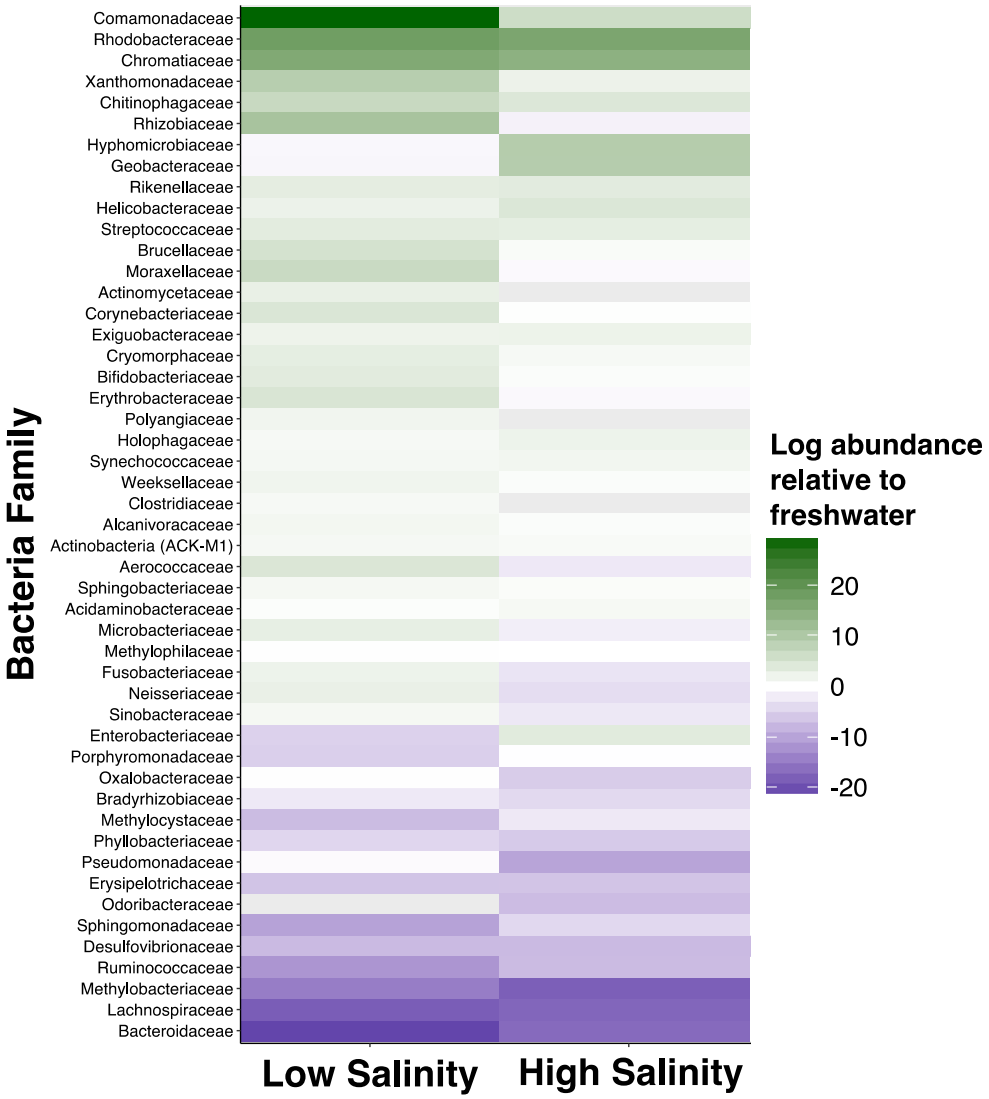

Fig. 3 Log-ratio showing differences in abundance of skin-associated microbial families in low-salinity and high-salinity populations relative to freshwater populations. This dataset has been filtered to display the 49 microbial families that emerged as dominant drivers of differences between salinity groups. Green indicates that abundance is enhanced between groups, white indicates no change in relative abundance, and purple indicates a reduction in relative abundance between groups. See Table 1 for information about salinity groups

coastal and inland populations in any of the four diversity metrics (Shannon-Weaver, Simpson's index, OTU richness, and Faith's phylogenetic distance). However, we found that the community structure of microbes on frogs differed between coastal and inland frog populations, as well as along a salinity gradient. Several factors could explain these results. First, it could be that the salinities in the wetlands that coastal populations inhabit are not high enough to affect diversity yet are sufficiently high to disrupt or alter assembly and population dynamics, causing shifts in relative abundance of the microbes present across locations. A study examining how saltwater affected euryhaline fish gill microbiota found slight differences in the microbiome in 0 and 5 ppt salinity treatments but greater shifts in microbe abundances in 18 and 30 ppt treatments [45]. Adult green treefrogs spend the majority of their time out of water and only enter the ponds to breed and rehydrate. Because we sampled during the breeding season, it is possible that occasional contact with brackish/saline water in coastal populations disrupts or alters microbial community dynamics; yet, the exposure is brief or mild enough that species diversity is not heavily impacted. It is also possible that the microbiome may be resistant to minor disturbances like occasional saltwater submersion and regular skin shedding. Indeed, recent efforts to inoculate frogs with probiotic microbial species as a defense against chytridiomycosis reported that frogs often did not sustain the new species through time, suggesting that the microbial community is resistant to invading species [82]. Moreover, there are likely wetland properties, in addition to salinity, that affect the microbiome, such as pH and temperature. For example, despite having a low salinity, the microbiomes on coastal frogs from the Point Peter population were the most dissimilar to other coastal populations, even though Bodie Island was the most saline population (Fig. 4). Interestingly, the salinity,

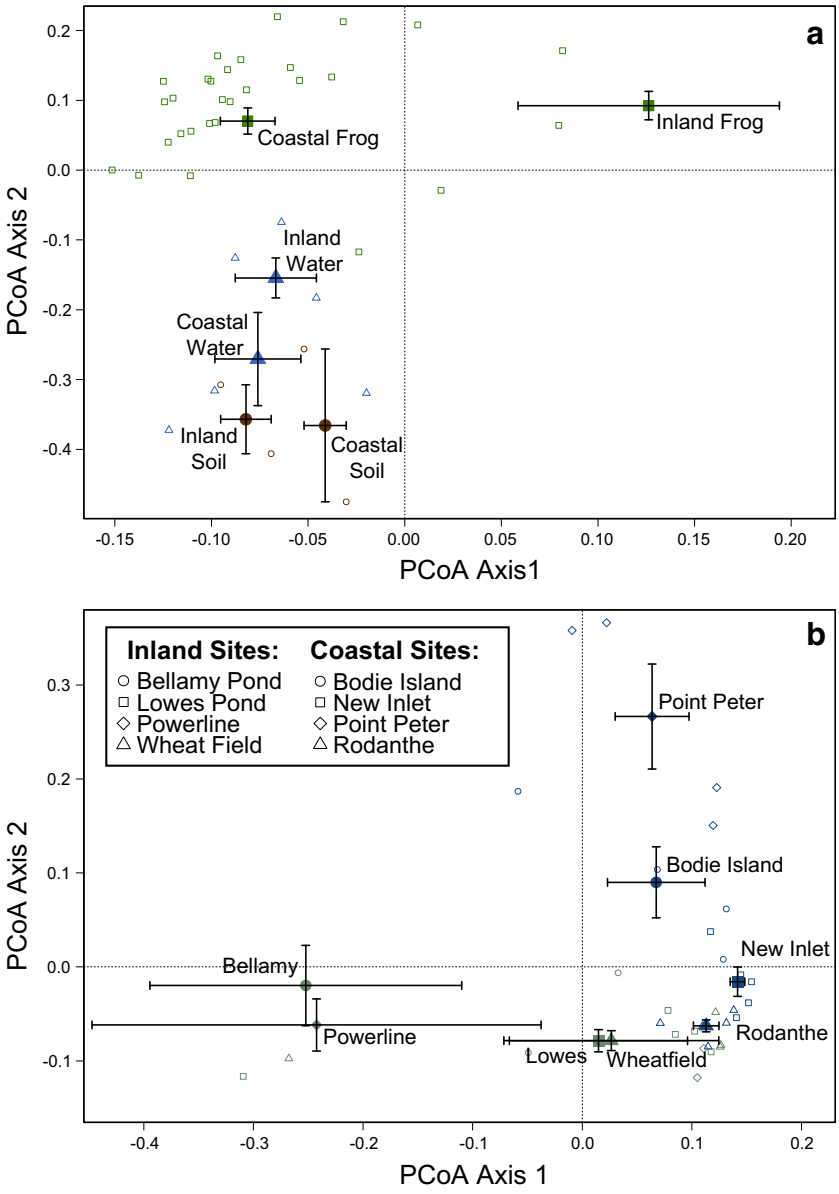

Fig. 4 Principal coordinates analysis showing the microbial communities from frog, water, and soil samples (**a**) and the frog skin-associated microbial communities according to sampling location (**b**). Filled points (with standard error) represent the average microbial sample collected, with open points showing individual samples. Panel a represents the microbiome sampled from coastal and inland *Hyla cinerea* populations (green squares), the surface water (blue triangles), and pond soil (brown circles). Panel b shows the skin-associated microbiome sampled from *Hyla cinerea* integument that inhabit either coastal (blue) environments or inland (green) environments, with population specified by shape. In panel a, axis 1 explains 52.1% of the variation, while axis 2 explains 51.2% of the variation. For panel b, axis 1 explains 53.8% of the variation, while axis 2 explains 53.7% of the variation

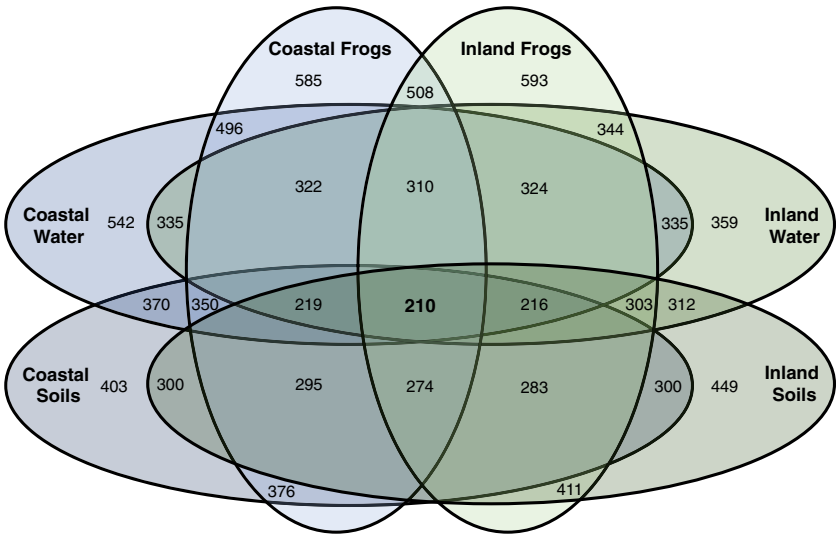

Fig.5 A Venn diagram showing the number of OTUs that are shared among water samples, soil samples, and frog samples collected from coastal and inland locations

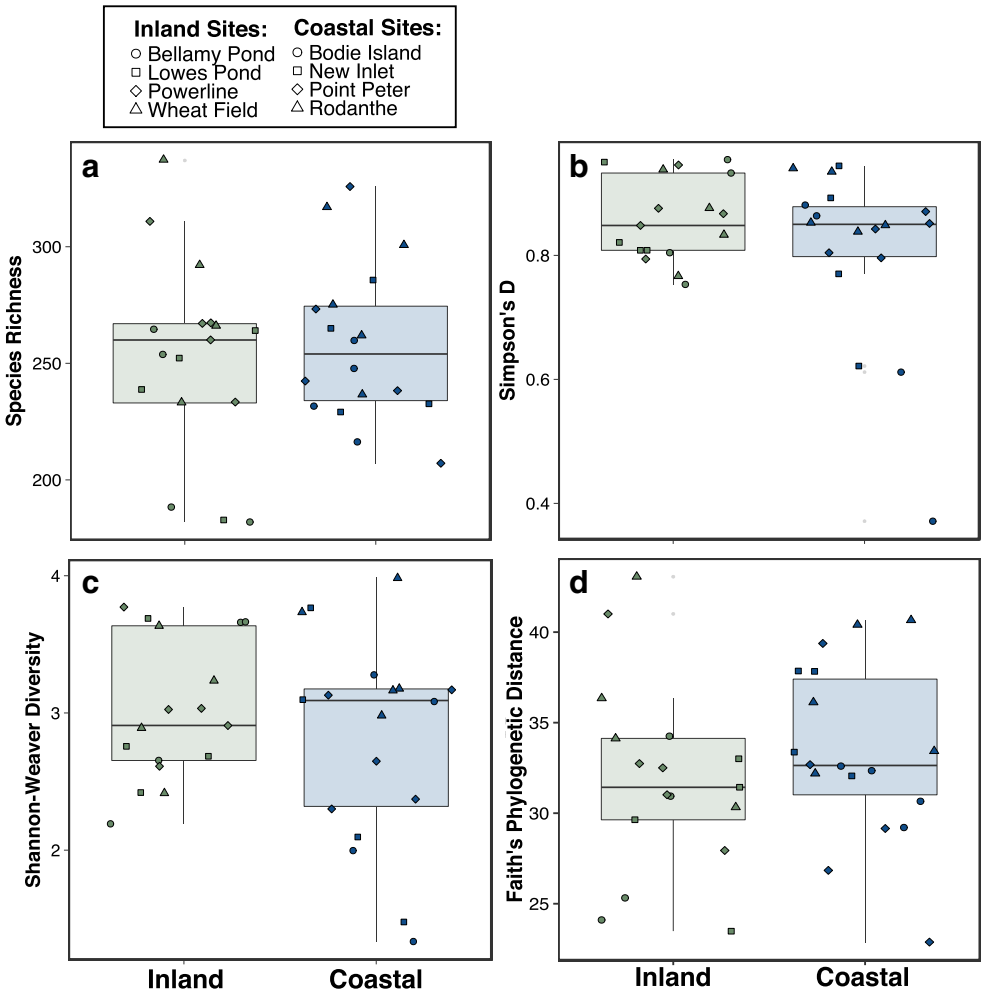

Fig. 6 Diversity of the skin-associated microbial communities on *Hyla cinerea* from sampling locations that were either coastal (blue) or inland (green) environments. Panel **a** is the microbial species richness, panel **b** is the Simpson's diversity index, panel **c** is Shannon-Weaver's diversity index, and panel **d** is Faith's phylogenetic distance. Each point is the diversity on individual frogs with the shape corresponding to the sampling population (see Table 1 for additional population information). There are no statistical differences in any diversity metrics between inland and coastal populations

conductivity, temperature, and pH of Point Peter were consistent with other coastal populations; so, we are unsure what may be driving the dissimilarity (Table 1).

Within the 49 microbial families that emerged as significant drivers of differences among salinity groups, the families with the largest reductions in relative abundance were observed in the Methylobacteriaceae, Lachnospiraceae, and Bacteroidaceae families. The families that increased the most in relative abundance were Comamonadaceae, Rhodobacteraceae, and Chromatiaceae. Interestingly, we observed phylum Parvarchaeota (Domain: Archaea) on coastal frog skin but did not observe this phylum on inland frogs (Fig. 1). Parvarchaeota is notable for its occupation of harsh environments, including marshes, soils, and the ocean [83] and may explain why coastal frog samples overlap with coastal soil and water along axis 1 in Fig. 4.

The microbial community among green tree frog individuals is different from the environmental microbial community in surrounding water and soil, although there is overlap (Figs. 4 and 5). If the microbes found in the environment strongly influenced the microbes that persist on anuran microbiomes, we expected the frog microbiomes to align with differences in the microbial communities found in the environmental samples across populations [35, 42, 43, 45]. Frog microbiomes tend to be more similar among conspecific individuals, but the environment does influence the skin-associated microbiome in this species. This result supports findings of other studies that while frog microbiomes are similar across hosts, they do use environmental source pools to maintain the skin microbiome [37, 38, 62].

In general, the similarities among the microbial communities on frog skin may also indicate the possibility of shared functions among *H. cinerea*, resulting in reduced vulnerability to pathogens or environmental stressors (e.g., via herd immunity) [31, 84, 85]. The similarities in the skin-associated microbiome among individual frogs are likely due to both host-specific factors (e.g., differences with integument morphology) and different competitive abilities of microbes; yet, we were unable to determine the relative contribution of each factor in microbiome composition in this study.

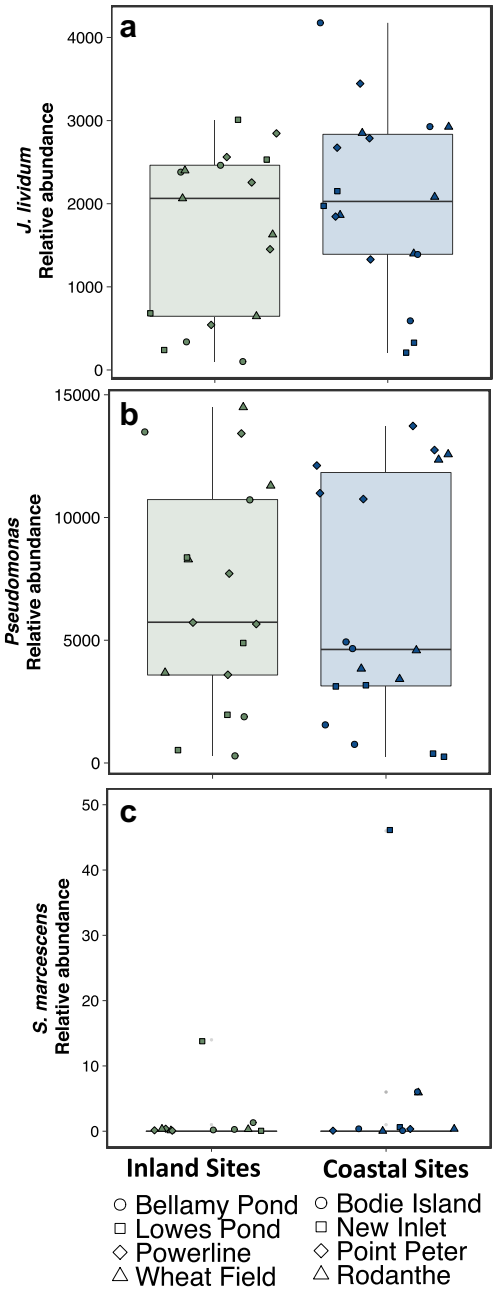

Fig. 7 The relative abundance (read counts/28,000 total per sample) of the OTUs classified as *Janthinobacterium lividum* (**a**), *Pseudomonas* sp. (**b**), and *Serratia marcescens* (**c**) present on *Hyla cinerea* skin across populations sampled. Each point is the abundance on each individual frog sampled with specific population denoted by the shape. We observed no differences in the abundance of these three isolates between inland and coastal populations. In panel c, we have removed the outlier individual from the Lowes (inland) populations on which 336 *S. marcescens* were detected

Chytrid fungal loads are lower in salt-exposed hosts [55], and clinical literature suggests that a salt bath can be used to treat infected frogs [57, 86, 87]. One hypothesis for the beneficial effects of salt baths is that it facilitates the representation of anti-fungal microbes in the skin microbiome. However, we did not find differences in the relative abundances of taxa that are thought to inhibit *B. dendrobatidis* in vitro, which suggests that any therapeutic benefits provided by saltwater to infected amphibians may not operate solely by increasing the relative abundances of anti-pathogenic isolates, although many different taxa in these communities can likely produce anti-fungal metabolites [88]. Rather, it may be that the saltwater has a direct influence on the pathogen itself. However, this hypothesis is speculative, as we considered only a few taxa and did not culture and test them directly.

Our study suggests that increased exposure to saline habitats could alter the symbiotic relationship between frogs and the cutaneous microbiome. Additionally, the differences observed across populations suggest that there are wetland-specific characteristics, other than salinity, that influence the skin-microbiome (Fig. 4). The differences we observed in community turnover between coastal and inland populations may indicate differential vulnerability to infection, but we do not have enough data to infer whether these differences increase or decrease susceptibility to infectious disease. On one hand, disruptions in community assembly may leave niche space open for infectious colonists [25]. Yet, it is also possible that the observed differences in the relative abundances of some species could influence important symbiotic interactions. For example, certain microbes interact with mucus on frog skin to boost the *B. dendrobatidis*-killing properties of the mucus [89]. Future work should use experimental and in vitro approaches to improve our understanding of how salinity directly and indirectly affects the skin-associated microbiome and chytridiomycosis.

Conclusions

In this study, we investigated how the skin-associated microbial community on frogs differs along a salinity gradient, examined the extent that the microbes found on frog skin overlap with microbes in the soil and water, and tested how the relative abundances of potential anti-fungal isolates on frog skin differed according to location. We found that the skin-associated frog microbiome was more similar to other frogs compared to the microbial communities found in surface water and soil, but there was overlap between frog skin and the environmental samples. Skin microbial community richness was not different between coastal and inland frogs, but the relative abundances of microbes differed according to population and the salinity of the wetland. Finally, we found no differences in the relative abundances of *Janthinobacterium lividum*, the genus *Pseudomonas*, and *Serratia marcescens* between coastal and inland frogs.

Collectively, our findings suggest that rising salinities due to anthropogenic salinization of wetlands will affect the skin-associated amphibian microbiome; yet, exposure to saltwater may have a limited influence on some anti-fungal microbial taxa. So, it is uncertain whether an altered microbiome will ultimately affect the survival or fitness of frog hosts.

Acknowledgements The authors would like to thank members of the K. McCoy and M. McCoy lab groups at East Carolina University for thoughtful insights in the development of this project. We thank Laila Kirkpatrick at Virginia Tech for laboratory assistance as well as Zach Herbert and the staff at the Dana-Farber Cancer Institute's Molecular Biology Core Lab at Harvard University for Illumina sequencing. Finally, we thank two anonymous reviewers whose reviews helped improve this manuscript.

Author Contributions MA and MM conceived the study; MA and MM performed the field sampling; LB analyzed the samples; and MA, MM, and LB analyzed the data. MA, MM, and LB contributed to the writing of this manuscript.

Funding Information Funding for this study was provided by the NSF grant DEB 1136640 awarded to Lisa Belden and the North Carolina Sea Grant (Project No. 2014-R/14-HCE-3) awarded to Michael W. McCoy and Molly A. Albecker.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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