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Marsh Soils as Potential Sinks for *Bacteroides* Fecal Indicator Bacteria, Waccamaw National Wildlife Refuge, Georgetown, SC, USA

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Abstract A soil core collected in a tidal freshwater marsh in the Waccamaw National Wildlife Refuge (Georgetown, SC) exuded a particularly strong odor of cow manure upon extrusion. In order to test for manure and determine its provenance, we carried out microbial source tracking using DNA markers for *Bacteroides*, a noncoliform, anaerobic bacterial group that represents a large proportion spectrum of the fecal population. Three core sections from 0–3 cm, 9–12 cm, and 30–33 cm were analyzed for the presence of *Bacteroides*. The ages of core sediments were estimated using ^{210}Pb and ^{137}Cs dating. All three core sections tested positive for *Bacteroides* DNA markers related to cow or deer feces. Because cow manure is stockpiled, used as fertilizer, and a source of direct contamination in the Great Pee Dee River/Winyah Bay watershed, it is very likely the source of the *Bacteroides* that was deposited on the marsh. The mid-points of the core sections were dated as follows: 0–3 cm, 2009; 9–12 cm, 1999, and 30–33 cm, 1961. The presence of *Bacteroides* at different depths/ages in the soil profile indicates that soils in tidal freshwater marshes are, at the least, capable of being short-term sinks for *Bacteroides* and, may have the potential to be long-term sinks of stable, naturalized populations.

Keywords *Bacteroides* · Fecal indicator bacteria · Microbial source tracking · Tidal freshwater marsh

Abbreviations

Allbac	All sources of *Bacteroides*
Bacteroides HF183	Human-derived sources of *Bacteroides*
BoBac	Bovine-ruminant derived *Bacteroides*
MST	Microbial source tracking

1 Introduction

Fecal contamination of watersheds via nonpoint source pollution has long been assessed by monitoring concentrations of *Escherichia coli* (*E. coli*) and fecal coliform in receiving waters (U.S. Environmental Protection Agency 2005). Bacteria in the genus *Bacteroides* have been identified as an alternative and potentially more accurate fecal indicator than fecal coliform or *Enterococcus* groups because they make up a larger proportion

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of the fecal population and have a high degree of host specificity that can be discerned using molecular techniques such as microbial source tracking (MST) (Fogerty and Voytek 2005; Layton et al. 2006). MST is a molecular technique that can be carried out with both library-dependent methods, which rely on the matching of cultured isolates with a "library" of previously identified sources, and library-independent methods, which constitute a more direct and efficient approach requiring only the detection of a highly specific genetic marker extracted with polymerase chain reaction (PCR) techniques (Stoeckel and Harwood 2007).

Microbial source tracking has recently been applied to the vexing problem of pinpointing sources of fecal pollution in aquatic systems. At Lover's Point on the Monterey Peninsula of California, Yamahara et al. (2007) used MST to show possible contamination of sand by human sewage due to the presence of human-associated *Bacteroides* and *Enterococcus faecium* esp gene markers. In a Florida lake, MST approaches for the *Enterococcus faecium* (*E. faecium*) esp gene, human-associated *Bacteroides* (HF183), and human polyomaviruses were used to determine that human sources in stormwater runoff were a significant component of fecal contamination (Staley et al. 2012). In a Lake Superior harbor, MST approaches for total enterococci, all sources of *Bacteroides* (AllBac), and *Bacteroides* HF183 were employed to show that effluent was likely the main source of fecal contamination in the water column. In addition, the greater abundance of AllBac and enterococci in sand/sediments relative to the water column indicated that lake sediments may serve as sinks for fecal bacteria (Eichmiller et al. 2013). To date, such techniques have not been applied in natural wetlands, even though their position at the intersection of land and water makes them likely recipients of contaminants from the watershed and their anaerobic soils may provide ideal conditions for the growth of anaerobic microbes including fecal bacteria.

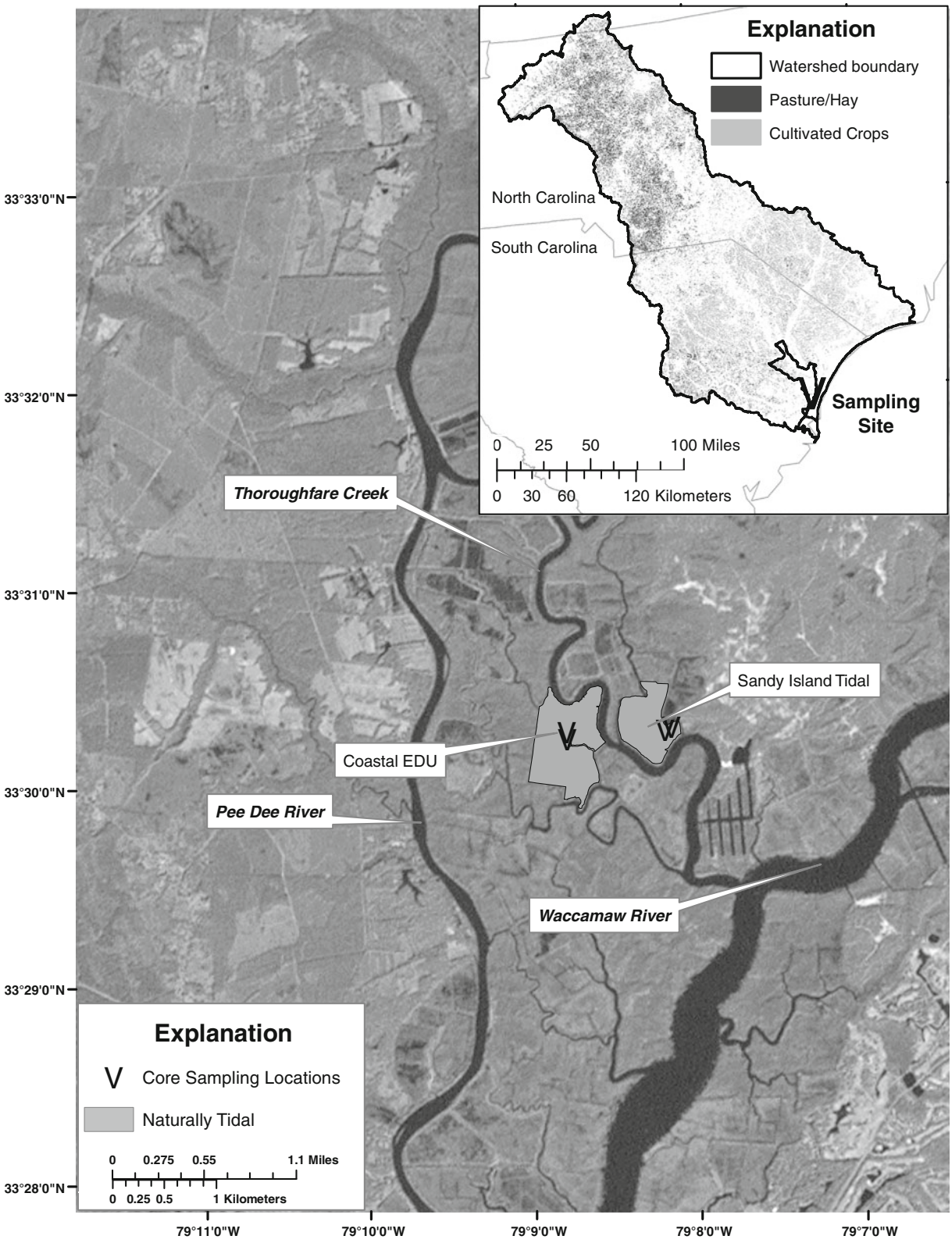
Here, we report a pilot study in which library-independent MST was used to determine whether a soil core from a tidal freshwater marsh, which exhibited a strong cow manure odor, actually contained fecal bacteria from a bovine source. PCR assays targeting the *Bacteroides* 16S rRNA gene were used to identify the presence of bovine-ruminant derived *Bacteroides* (BoBac) in the core (Layton et al. 2006; Duris et al. 2011). We also carried out ^{210}Pb and ^{137}Cs analyses in order to date the sediments and determine whether the potential exists for marsh soils to be long-term sinks for populations of fecal bacteria.

2 Material and Methods

2.1 Study Site

The field work for this pilot study was conducted in marshes in the Waccamaw National Wildlife Refuge (~10,924 ha; WNWR) near Georgetown, SC (Fig. 1). The marshes in the WNWR are tidal freshwater ecosystems dominated by cattail (*Typha latifolia* L.), giant cut grass (*Zizaniopsis miliacea* (Michx.) Döll & Asch.), soft-stemmed bulrush (*Schoenoplectus tabernaemontani* (C.C. Gmel.) Palla), various smartweeds (*Polygonum* spp.) and bulltongue (*Sagittaria lancifolia* L.). The WNWR is located just north of the confluence of the Waccamaw and Pee Dee Rivers. The Pee Dee River flows directly into Thoroughfare Creek, which winds through the WNWR. The Pee Dee (or Great Pee Dee) River basin contains 27 watersheds and covers 8,870 km^2 in South Carolina (South Carolina Department of Health and Environmental Control (SCDHEC) 2005) (Fig. 1). The WNWR is located in the Great Pee Dee River/Winyah Bay watershed, which covers approximately 104,909 ha (259,235 acres) of which 14 % is agricultural (SCDHEC 2013a). The Pee Dee River basin has been listed as "impaired" for fecal coliform under section 303(d) of the Clean Water Act (U.S. Environmental Protection Agency 2013, SCDHEC 2013b). Specific causes of fecal coliform contamination in the Pee Dee River include high deer density, uncovered waste stockpiles from dairy cattle operations, frequent land application of manure as fertilizer, and direct access to creeks by cattle (SCDHEC 2005). In 2005, total maximum daily loads for fecal coliform were established for the entire Pee Dee River basin in order to meet water quality standards (SCDHEC 2005).

Fig. 1 The location of the tidal freshwater marshes Sandy Island Tidal and Coastal EDU along Thoroughfare Creek in the Waccamaw National Wildlife Refuge (WNWR), SC. The areas in which soil cores were collected are depicted by *black triangles*. The small inset map shows the location of the study sites within the Great Pee Dee River/Winyah Bay watershed (104,909 ha), which is part of the larger Pee Dee River basin (8,870 km^2). *Black* and *gray shading* represent pasture and cultivated crops, respectively


Explanation
Watershed boundary
Pasture/Hay
Cultivated Crops
North Carolina
South Carolina
Sampling Site
0 25 50 100 Miles
0 30 60 120 Kilometers
Thoroughfare Creek
Sandy Island Tidal
Coastal EDU
Pee Dee River
Waccamaw River
Explanation
Core Sampling Locations
Naturally Tidal
0 0.275 0.55 1.1 Miles
0 0.25 0.5 1 Kilometers
33°33'0"N
33°32'0"N
33°31'0"N
33°30'0"N
33°29'0"N
33°28'0"N
79°11'0"W
79°10'0"W
79°9'0"W
79°8'0"W
79°7'0"W

2.2 Field Sampling

Soil cores were collected using pre-cleaned acrylic collection tubes attached to a razor-edged piston corer (15-cm diameter) from two tidal freshwater marshes, Coastal EDU and Sandy Island Tidal (Fig. 1), in areas dominated by *Schoenoplectus* spp. The cores were collected as part of a larger study on carbon storage (Drexler et al. 2013). Core tubes were kept out of contact with the marsh surface until coring. After collection, cores were immediately sealed air-tight with clean rubber seals on both ends of the acrylic collection tubes to avoid any contamination from human or marsh sources, laid horizontally on ice for transport, and stored under refrigeration until being shipped overnight to U.S. Geological Survey laboratories in Sacramento, CA, USA for further processing.

2.3 Laboratory Analysis

In the laboratory, cores were extruded from their collection tubes, sectioned into 3-cm intervals, and cut in half longitudinally. Subsequent to extrusion, two cores emitted a manure odor. One core, Sandy Island Tidal core III, had a particularly strong smell and was chosen to be tested for fecal contamination. Longitudinal halves from sections at 0–3 cm, 9–12 cm, and 30–33 cm of depth were sent to the U.S. Geological Survey Michigan Water Science Center in Lansing, MI for library-independent, culture-independent MST analysis.

Total chromosomal DNA was extracted from 0.25 g of core samples from each of the three depths in duplicate using the MoBio Power Soil Kit (MoBio Laboratories, Carlsbad, CA). Replicate 1-μl aliquots of the resulting DNA extracts, with concentrations of DNA ranging from 6 to 25 ng/μl, were tested using a polymerase chain reaction (PCR) assay targeting the 16S rRNA gene in bovine-ruminant derived *Bacteroides* (BoBac) (Layton et al. 2006; Duris et al. 2011). Resulting PCR products were separated by agarose-gel electrophoresis using pre-stained 2.2 % agarose gel cassettes and visualized using UV transillumination (FlashGel® System; Lonza Group Ltd., Basel, Switzerland). Positive and negative controls and inhibition-spike assays were run with the core samples. The positive control was the target sequence cloned into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Sterile nuclease-free water (Fisher Scientific, Hampton, NH) was used as the no-template negative control. The two positive controls yielded a PCR product of the appropriate size and the two negative controls produced no visible PCR products. Core samples spiked with 10^5 copies of positive control yielded a positive PCR result indicating a lack of PCR inhibition in these DNA extracts.

Longitudinal halves from each 3-cm section of Sandy Island Tidal core III were weighed wet, dried at 80 °C, and weighed again. Bulk density was determined for the dried samples using the dry weight and volume of each section. Samples were analyzed at the U.S. Geological Survey in Menlo Park, CA, for ^{210}Pb, ^{226}Ra, and ^{137}Cs to assign estimated dates to core profiles. Both ^{137}Cs and ^{210}Pb have been used successfully for dating lake sediments and wetland soils (Armentano and Woodwell 1975; Appleby et al. 1997). Activities of total ^{210}Pb, ^{226}Ra, and ^{137}Cs were measured simultaneously by gamma spectrometry. The age–depth relationship in Sandy Island Tidal Core III was estimated using the constant rate of supply model (CRS) (Appleby and Oldfield 1978, 1983) and uncertainty analysis was conducted following Van Metre and Fuller (2009). Further information on ^{210}Pb and ^{137}Cs analyses and application of the CRS model is provided in Drexler et al. (2013).

3 Results and Discussion

The three core sections at 0–3 cm, 9–12 cm, and 30–33 cm in depth from Sandy Island Tidal core III were found to contain *Bacteroides* DNA markers related to bovine-ruminant feces. The BoBac DNA marker can be derived from deer, cows, or other ruminants as current MST techniques cannot distinguish clearly between these sources (Duris et al. 2011). The main ruminants in the Great Pee Dee River/Winyah Bay watershed are deer and cattle. It is unlikely that deer could have contributed so much manure as to create a discernible odor and a traceable presence in marsh sediments that extends to different layers of a soil profile. It is more likely that cow manure, which is applied in great quantities as fertilizer or stockpiled at dairy cattle operations (SCDHEC 2005), was washed into a channel during a storm and, subsequently, transported downstream via Thoroughfare Creek to Sandy Island Tidal marsh (Fig. 1). Cow manure could also have entered a channel upstream from the marsh via direct fecal contamination because cattle are routinely given access to creeks bordering agricultural areas within the study region (SCHHEC 2005).

In the top 46 cm of Sandy Island Tidal core III, which covers a period of ~100 years as dated by ^{210}Pb, bulk densities ranged from 0.08 to 1.7 g cm^{-3} and % organic carbon content ranged from 35 % at the surface down to 15 % at the bottom (Table 1). The mid-points (e.g., mid-point of 0–3 cm is 1.5 cm) of each soil section found to contain BoBac were dated as follows: 0–3 cm, 2009; 9–12 cm, 1999; and 30–33 cm, 1961 (Table 1). These results indicate that these soils may be a potential sink for repeated influxes of bacterial contamination from the watershed. It is also possible, however, that the manure was deposited recently and that, after deposition, there was migration of the manure and associated microbial communities down core due to tidal fluctuations or perturbation of the sediment through physical or biological means. Regardless, these results show that marsh soils, similar to lake sediments (Eichmiller et al. 2013) are, at the very least, a short-term sink for *Bacteroides* fecal bacteria.

The fact that fecal contamination is present in the Great Pee Dee River/Winyah Bay watershed is not surprising, because the entire Pee Dee River basin of which it is part has been listed as "impaired" for fecal coliform under section 303(d) of the Clean Water Act (SCDHEC 2013b). What is surprising, however, is that tidal freshwater wetlands, downstream from agricultural areas, can receive *as well as* store this contamination. The viability of *Bacteroides* was not determined in this study; however, detection with PCR does indicate that organisms containing the target sequence were historically present in Sandy Island Tidal marsh. Future work should focus on using methods that can quantify as well as determine viability of *Bacteroides* and other microbial contaminants in marsh soils (e.g., the propidium monoazide-qPCR method; Bae and Wuertz (2012)). Such approaches could be used to establish whether or not *Bacteroides* and other microbial contaminants originating from agricultural operations upstream are capable of forming long-term naturalized populations subsequent to deposition in downstream marshes. If so, it would be of great importance to determine whether or not such sources of bacteria can be re-mobilized during storms or tidal fluctuations. Transport of such viable bacterial populations could have major implications for

Table 1 Core characteristics for Sandy Island Tidal core III

Mid-interval depth below surface (cm)	Bulk density (g cm^{-3})	Percent (%) organic carbon	Total ^{210}Pb (dpm g^{-1})	Total ^{210}Pb error (dpm g^{-1})	Excess ^{210}Pb (dpm g^{-1})	Excess ^{210}Pb error (dpm g^{-1})	^{137}Cs (pCi g^{-1})	^{137}Cs error (pCi g^{-1})	CRS age estimate based on ^{210}Pb	Uncertainty in mid-interval CRS date (±year)
1.5	0.08	37	8.51	0.61	7.11	0.62	0.226	0.039	2009.0	0.1
4.5	0.08	34	9.40	0.56	8.20	0.57	0.272	0.036	2005.9	0.3
7.5	0.06	37	8.07	0.61	6.99	0.63	0.257	0.042	2003.1	0.3
10.5	0.09	34	9.07	0.65	7.82	0.66	0.193	0.042	1999.9	0.3
13.5	0.07	35	9.33	0.64	8.15	0.65	0.249	0.043	1995.7	0.4
16.5	0.10	29	6.61	0.43	5.61	0.43	0.414	0.039	1991.3	0.5
19.5	0.12	32	5.68	0.47	4.45	0.48	0.679	0.058	1986.5	0.6
22.5	0.10	34	5.77	0.51	4.80	0.53	0.675	0.060	1981.5	0.7
25.5	0.10	30	6.18	0.39	5.03	0.39	1.448	0.101	1975.8	0.8
28.5	0.09	30	5.71	0.45	4.59	0.46	3.825	0.259	1969.4	1.1
31.5	0.13	21	5.70	0.42	3.70	0.43	**4.874**	0.326	1961.4	1.7
34.5	0.12	17	4.71	0.46	2.92	0.48	2.922	0.201	1951.4	3.0
37.5	0.14	18	3.96	0.48	2.25	0.49	1.220	0.094	1940.0	4.8
40.5	0.17	16	3.58	0.39	1.38	0.40	0.836	0.065	1926.5	8.5
43.5	0.19	14	3.56	0.27	0.98	0.28	0.313	0.028	1909.7	16.4
46.5	*0.17*	*15*	*3.62*	*0.32*	*0.82*	*0.33*	*0.117*	*0.022*	*1884.9*	*50.3*

The section containing the 1963 peak for ^{137}Cs is shown in *bold*. The age limit beyond which uncertainty is greater than the section age interval covered by dating is shown in *italics*. The uncertainty limit is also the cutoff for inclusion in the table, so the full 54 cm of core characteristics are not shown

meeting current TMDL requirements as tidal resuspension of bacterial sediment reservoirs has been demonstrated to cause failure of water quality standards elsewhere (Yamahara et al. 2007).

Besides concerns about fecal contamination, this study also raises a number of important questions about the function of wetlands in the landscape. Although wetlands are valued for their ability to store carbon in their soils (Reddy and DeLaune 2008; Mitsch et al. 2012), this paper demonstrates that the contents of such carbon sinks may not always be benign. Because tidal freshwater marshes provide ideal conditions for bacterial growth (i.e., abundant moisture, high carbon availability, good sources of nutrients, and a variety of both aerobic and anaerobic niches depending on depth in the soil profile; Craft 2001), it is possible that other microbial communities, including pathogens, are being transported to and taking residence in marsh soils. Bacteria populations including *E. coli*, *Salmonella*, and *Clostridium perfringens* have been identified in estuarine mud flats (Berthe et al. 2008), but little is known about the presence of such fecally derived pathogens in tidal marsh soils. Clearly, more study is needed to better characterize the microbial community resident in the soil profile of tidal marshes, particularly, in microbially impaired watersheds.

4 Conclusion

In a tidal freshwater marsh in the Great Pee Dee River/Winyah Bay watershed of South Carolina, microbial source tracking using bovine-ruminant derived *Bacteroides* (BoBac) was employed to trace the origin of fecal contamination to deer or cow manure. The likely source of BoBac was cow manure, which probably entered upstream waterways either directly by cattle or was washed into channels after manure application to fields and then deposited on the marsh surface during tidal flooding. Soil sections from 0–3 cm, 9–12 cm, and 30–33 cm in depth containing BoBac were dated to 2009, 1999, and 1961, respectively, using ^{210}Pb dating. Although bacterial populations may not necessarily share the same age as adjacent sediments, these results suggest that tidal freshwater marshes in impaired watersheds may, at the least, be short-term sinks for *Bacteroides* fecal bacteria, and may potentially be long-term sinks of repeated bacterial contamination.

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