

1 Effects of Fecal Source Input, Environmental Conditions, and Environmental Sources on
2 Enterococci Concentrations in a Coastal Ecosystem

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10 Running Head: Fecal Source Influence on Enterococci Concentrations

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21 **ABSTRACT**

22 Fecal pollution at coastal beaches in the Northeast, USA requires management efforts to address
23 public health and economic concerns. Concentrations of fecal-borne bacteria are influenced by
24 different fecal sources, environmental conditions, and ecosystem reservoirs, making their public
25 health significance convoluted. In this study, we sought to delineate the influences of these
26 factors on enterococci concentrations in southern Maine coastal recreational waters. Weekly
27 water samples and water quality measurements were conducted at freshwater, estuarine, and
28 marine beach sites from June through September 2016. Samples were analyzed for total and
29 particle-associated enterococci concentrations, total suspended solids, and microbial source
30 tracking markers for multiple sources. Water, soil, sediment, and marine sediment samples were
31 also subjected to 16S rRNA sequencing and SourceTracker analysis to determine the influence
32 from these environmental reservoirs on water sample microbial communities. Enterococci and
33 particle-associated enterococci concentrations were elevated in freshwater, but suspended solids
34 concentrations were relatively similar. Mammal fecal contamination was significantly elevated
35 in the estuary, with human and bird fecal contaminant levels similar between sites. A partial least
36 squares regression model indicated particle-associated enterococci and mammal marker
37 concentrations had the most significant positive relationships with enterococci concentrations
38 across marine, estuary, and freshwater environments. Freshwater microbial communities were
39 significantly influenced by underlying sediment while estuarine/marine beach communities were
40 influenced by freshwater, high tide height, and estuarine sediment. We found elevated
41 enterococci levels are reflective of a combination of increased fecal source input, environmental

42 sources, and environmental conditions, highlighting the need for encompassing MST approaches
43 for managing water quality issues.

44 **IMPORTANCE**

45 Enterococci have long been the federal standard in determining water quality at estuarine and
46 marine environments. Although enterococci are highly abundant in the fecal tracts of many
47 animals they are not exclusive to that environment and can persist and grow outside of fecal
48 tracts. This presents a management problem for areas that are largely impaired by non-point
49 source contamination, as fecal sources might not be the root cause of contamination. This study
50 employed different microbial source tracking methods to delineate influences from fecal source
51 input, environmental sources, and environmental conditions to determine which combination of
52 variables are influencing enterococci concentrations in recreational waters at a historically
53 impaired coastal town. Results showed that fecal source input, environmental sources and
54 conditions all play a role in influencing enterococci concentrations. This highlights the need to
55 include an encompassing microbial source tracking approach to assess the effects of all
56 important variables on enterococci concentrations.

57

58 **INTRODUCTION**

59 Fecal contamination of coastal recreational waters is a significant public health concern, as fecal
60 material, often from nonpoint sources, can harbor an array of different pathogens. The US EPA
61 has established regulations based on enterococci bacteria as the indicator of fecal-borne pollution
62 to help manage water quality at estuarine and marine beaches (1). These organisms correlated
63 well with predicted public health outcomes in several epidemiological studies that served as the

64 basis for their adoption as the regulatory water quality indicator (2–5). The presence of human
65 feces can present an elevated public health risk in recreational waters compared to non-human
66 sources due to the lack of an “inter-species barrier” for diseases and the higher density of human
67 pathogens that humans can carry (6–8). Although human pollution represents the greatest public
68 health risk, other fecal sources that contain enterococci and possibly human pathogens can be
69 chronic or intermittent sources of both, making beach water quality management and
70 remediation efforts more complex.

71

72 The need to differentiate fecal sources in recreational waters led to the emergence of microbial
73 source tracking (MST) methods in the early 2000s, most notably the PCR-based assays that
74 target the 16S rRNA gene in *Bacteroides* spp. (9, 10). There are a wide range of species-specific
75 genetic markers designed to identify human fecal sources and various domestic and wildlife fecal
76 sources. These assays have been in use for well over a decade and are supported by numerous
77 and rigorous laboratory evaluations and field applications (11–17). Initial field studies
78 investigated the relationship between MST markers and FIB concentrations in recreational
79 waters to better elucidate potential sources of fecal pollution. Some studies have found strong
80 relationships between the MST markers and enterococci (12, 18) while other studies have found
81 either weak or no relationships (19–21), many of which are discussed in a review by Harwood et
82 al. (22). One main factor affecting the relationship between enterococci and the relative strength
83 of different sources of fecal contamination is that enterococci can persist and grow in the
84 environment, which can significantly influence their concentrations in recreational water (23).

85

86 Due to the pervasiveness of enterococci in natural ecosystems, recent studies have been
87 conducted to not only elucidate environmental parameters controlling their growth, but also to
88 identify naturalized niches that can act as reservoirs for enterococci and the associated influence
89 on water quality measurements. Specifically, enterococci have been shown to persist in fresh
90 water sediments (24–26) and marine sediments (24, 27), and in some cases their relative
91 concentrations in sediments are several orders of magnitude higher than the overlying water (24,
92 28–30). In addition, enterococci persist in soils affected by anthropogenic activities (31) as well
93 as more natural soil environments (32–34). Thus, soil can act as a significant reservoir of
94 enterococci that can, if eroded, confound concentrations observed in recreational waters.
95 Evaluating the influence of sediment and or soil on water quality has, in some studies, been
96 conducted by measuring total suspended solids as a surrogate for sediment-associated
97 enterococci (27, 35, 36), however this non-specific approach does not indicate the specific type
98 of source(s) of the suspended solids. With the advent of next generation sequencing, sources of
99 sediment or soil bacteria can be fingerprinted via 16S rRNA sequencing, and programs like
100 SourceTracker can then determine relative fractions of source-specific 16S fingerprints within a
101 water sample (37).

102
103 This study examined the coastal and estuarine beaches of Wells, ME where there has been
104 historically elevated enterococci levels, as reported by the Maine Healthy Beaches Program (38).
105 Prior to this study, only a ribotyping-based MST study (39) that also involved other indicator
106 tracking work had been conducted in this area. In that study, the two major freshwater inputs,
107 the Webhannet River and Depot Brook were found to be the major influences on water quality
108 related to an array of fecal contamination sources. To investigate potential sources of enterococci

109 we measured three major categories of variables (fecal source input, environmental conditions,
110 and environmental sources) and then used a partial least squares regression model approach to
111 determine the most significant influences on the enterococci concentrations in water samples.

112

113 **RESULTS**

114 **Total and particle-associated enterococci concentrations and total suspended solids in**
115 **water.** During this study, total enterococci concentrations were highest in freshwater sites, with
116 concentrations significantly decreasing from there to the estuary and then the marine beach areas
117 (Figure 2). The geometric mean enterococci concentrations were 197 and 40 CFU/100 ml at the
118 Depot and Webhannet sites, respectively, with 71% of samples exceeding 104 CFU/100 ml at the
119 Depot site compared to 21% at the Webhannet site. In contrast, the geometric mean enterococci
120 concentrations at the other sites were all <15 CFU/100 ml and samples exceeded 104 CFU/100
121 ml 0% (at Wells Beach) to 25% of the time. In addition to measuring enterococci concentrations
122 in water samples, particle-associated enterococci and suspended solid concentrations were
123 measured to better understand the potential mode of transport of these bacteria within this coastal
124 watershed. Throughout the study period (June-September 2016), levels of total and particle-
125 associated enterococci varied by site. Concentrations were lowest at the marine beach (Wells
126 Beach) compared to other sites, with levels significantly higher in all estuary sites (W11-W15)
127 and freshwater sites (Depot & Webhannet; Figure 2).

128

129 Both total and particle-associated enterococci geometric mean concentrations were statistically
130 similar at the estuary beach (W11, W12, W13) and estuary (W14, W15) sites. Freshwater sites

131 (Webhannet and Depot) however, had statistically higher enterococci concentrations than other
132 sites (Figure 2; $p < 0.05$). The ratio of total to particle-associated enterococci varied throughout
133 the season, with an average of 36.3% ($SD \pm 30$) across all sites. Sites within the estuary beach
134 showed the highest ratio (41%, $SD \pm 32$), however there were no significant differences observed
135 between sites or types of sites. Average TSS concentrations were relatively low and similar for
136 most sites, with an overall average of 2.9 mg TSS/L ($SD \pm 1.2$). The Webhannet freshwater site,
137 however, had a significantly lower average TSS concentration (1.2 mg/L $\pm 1.0SD$, $p < 0.05$)
138 (Figure 2), despite, as previously mentioned, having higher enterococci concentrations. The
139 relationship between particle-associated enterococci and TSS was not significant ($r^2 = 0.0011$),
140 and significant rainfall events were seldom and sparse with only one greater than 1 in 48 h prior
141 to sampling. Overall, this study showed enterococci concentrations were significantly different
142 by site and were ubiquitously associated with particles, which was independent of suspended
143 solids concentrations.

144

145 **Presence of fecal sources in fresh, estuarine, and marine waters.** The concentration of fecal
146 pollution in this study area was determined using both PCR and quantitative PCR MST assays to
147 identify and quantify predominant sources of fecal contamination present in the water. The
148 mammal fecal marker (Bac32) was detected via PCR at all sites 100% of the time throughout the
149 study period. (Supplementary Material 1E). The human fecal marker (HF183) was detected in
150 51% of all water samples, with the highest detection rate in fresh water (56%) and the lowest
151 detection rate in marine beach water (46%). Differences in the percent detection of the gull fecal
152 marker (Gull2) were most pronounced between freshwater (10%) and all other sites (>77%). The
153 dog fecal marker (DF475) detection rate was highest in the estuary beach water (10/44 = 23%),

154 however 8 of the 10 positive samples were detected in July (8/13 = 61%). For all other sites, an
155 increase in the detection of dog fecal marker also occurred during July, with 44% (16/36)
156 detection, compared to 0% for August and September and <1% for June. Thus, most of the dog
157 contamination at all sites was associated with unknown dog-related conditions during July.

158

159 **Concentrations of mammal, human, and bird fecal sources.** We used qPCR to provide
160 relative quantitative measures of mammal, human and bird fecal contamination levels. Water at
161 estuary and estuary beach sites contained significantly higher levels of mammal (AllBac) fecal
162 marker copies, with an average of 1.54×10^7 compared to 2.62×10^6 in freshwater and 3.9×10^6
163 copies/100 ml in marine beach ($p < 0.05$). Average concentrations of human (HF183) and bird
164 (GFD) fecal markers were not statistically different between sites, however, concentrations of the
165 human marker in individual samples varied from $0 - 2.04 \times 10^4$ copies/100 ml (Figure 3), while
166 bird fecal marker concentrations were relatively stable across all sites. No significant temporal
167 trends were observed for any of the quantitative fecal marker levels. Compared with
168 presence/absence detection of fecal sources, quantitative measurements also did not show strong
169 spatial patterns, except mammal marker levels showed significant increases at estuary and
170 estuary beach sites compared to marine and freshwater sites.

171

172 **Differences between water, soil, and sediment bacterial community compositions.** 16S
173 amplicon sequencing was used to characterize the microbial community present in water and
174 other sample matrices (soil, sediment, and marine sediment), which was the nexus for ensuing
175 SourceTracker analysis. A total of 3,276,196 reads and 7,706 unique OTUs were obtained from

176 the 177 samples of fresh, estuary, estuary beach and marine beach water and soil, sediment, and
177 marine sediment. The number of OTUs assigned and the Shannon diversity index were
178 significantly higher for soil, sediment, and marine sediment when compared to water samples
179 (Figure 4, $p < 0.05$). Most taxa in the estuary and marine beach water samples were identified as
180 Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria classes, which together
181 accounted for 84% of the total assigned taxa. Cyanobacteria accounted for 34% of the taxa in
182 marine sediment, and Betaproteobacteria was one of the top three most abundant taxa in fresh
183 water, soil and sediment (Figure 4). A Non-Metric Multi-Dimensional Scaling (NMDS)
184 ordination was used to determine if the bacterial communities from water and other matrices
185 (soil and sediments) differed based on their taxonomic composition. Bacterial communities from
186 the marine beach and estuary (All Estuary) waters were similar, but were statistically different
187 from fresh water (Figure 5, $p < 0.05$). The bacterial communities associated with soil, sediment
188 and marine sediment were all distinct when compared to each other and water samples,
189 indicating unique groups of OTUs (Figure 5, $p < 0.05$). Samples taken from different areas
190 within the watershed (soil, estuarine water, freshwater, etc.) contained unique bacterial
191 compositions, allowing for downstream analysis with the SourceTracker software to discern
192 relative contributions of these different communities to the make-up of microbial communities in
193 the different types of water samples.

194

195 **Environmental source contribution to water samples.** The fraction of freshwater, sediment,
196 soil, estuarine sediment, and marine beach water source bacterial communities within estuary
197 and estuary beaches water samples were calculated using the Bayesian mixing model
198 SourceTracker. Freshwater sample analysis showed a high probability of taxa originating from

199 underlying sediment (74%) and much lower probability of taxa originating from soil (2.6%).
200 Initial results for the estuary and estuary beach indicated that marine beach water was the
201 dominant source of bacteria (Table 1). However, given that likely fecal sources are coming from
202 the watershed, we excluded marine beach water as a potential source and included it as a sink
203 then re-analyzed the data. These second results showed that freshwater taxa had a high
204 probability of being a significant fraction of estuary (73%), estuary beach (66%) and marine
205 beach (35%) water communities, with a significantly higher percentage for the estuary locations
206 compared to the marine beach (Table 1, $p < 0.05$), which is more influenced by ocean microbial
207 taxa. Despite the significant percentage of freshwater taxa assignments in the estuary, estuary
208 beach, and marine beach waters there were no freshwater sediment or soil taxa assignments for
209 these sites. The data for the percent of unidentifiable taxa showed the opposite trend compared
210 to percent of assigned freshwater taxa. Unidentifiable taxa in the marine beach were significantly
211 higher (46%; $p < 0.05$), which is not surprising given that marine beach water community would
212 likely be most influenced by non-terrestrial sources. Estuarine sediment was the highest likely
213 identified source in the water from the marine beach site (19%), and it was significantly higher
214 than percentages calculated for all estuary sites ($p < 0.05$). Overall results showed that freshwater
215 source-related taxa were a pervasive source throughout the estuary and marine beach, and while
216 sediment source-related taxa were highly abundant in the freshwater they were not observed
217 within the estuary or marine beach.

218

219 **Relationships between environmental conditions, fecal source concentrations,**
220 **environmental sources and enterococci concentrations.** Two PLSR models were created to
221 determine relationships between enterococci and fecal source concentrations, environmental

222 sources, and environmental conditions (outlined in the Methods). The first 'freshwater' PLSR
223 model indicated particle-associated enterococci concentration, concentration of mammal fecal
224 marker, TSS concentration, percent of sediment source, percent of unknown source, and salinity
225 were important variables ($VIP > 0.8$) in resolving variation in enterococci concentrations (Table
226 1). A one-factor (single PLSR regression) model was deemed optimal (root mean PRESS =
227 0.735), and showed that all variables (except salinity) had positive associations with enterococci
228 concentrations. Values for model performance ($R^2Y = 0.6$, $R^2X = 0.5$, and $Q^2 = 0.4$) indicated
229 that the model fit the data moderately well ($R^2X \geq 0.5$) but had poor predictive capability of
230 enterococci concentrations ($Q^2 < 0.5$; Supplementary Material 3). Out of all the important
231 variables, particle-associated enterococci (Particle ENT) concentrations showed the strongest
232 relationship to total enterococci concentrations (Table 2). The second PLSR model, a two-
233 factor/two PLSR regressions model, was the best fit (root mean PRESS = 0.744) from the PLSR
234 constructed for the estuary, estuary beach, and marine beach sites. The analysis identified
235 particle-associated enterococci concentration, mammal fecal source concentration, percent of
236 freshwater, unidentified and estuarine sediment sources, water temperature, and high tide height
237 as significantly related to enterococci concentrations. Factor one showed that all variables were
238 positively associated, except for the percent unidentified and marine sediment sources. The
239 second factor showed mammal fecal sources, freshwater sources, and water temperatures were
240 negatively related to enterococci concentrations, which was the opposite of their associations for
241 factor one. The high tide height and marine sediment were positively related to enterococci
242 concentrations for factor 2 of the PLSR (Table 2). Together both factors explained 61.8% in the
243 variation observed in enterococci concentrations, and model performance ($R^2Y = 0.6$, $R^2X = 0.5$,
244 and $Q^2 = 0.6$) indicated better predictive ability with a similar fit to the data compared to the

245 freshwater model (Supplementary Table 3). Out of all the potential variables measured (19 total)
246 across three categories (fecal source input, environmental source contribution, and environmental
247 conditions), particle-associated enterococci and mammal fecal marker concentrations had the
248 most significant relationships to enterococci concentrations. The relationships between other
249 variables and enterococci concentrations were specific to freshwater and estuary/marine beach
250 models, indicating ecosystem specific relationships. However, the joint relationship of particle-
251 associated and mammal fecal marker across freshwater and estuary/marine environments
252 indicate their overarching importance in determining enterococci concentrations.

253

254 **4 Discussion:**

255 Geometric mean enterococci concentrations at the marine beach, estuary, and estuary beach
256 sampling sites were all less than the State of Maine water quality standard of 35 CFU/100 ml and
257 the majority of concentrations were less than the 104 CFU/100 ml single sample standard,
258 indicating the water quality was typically considered acceptable for recreational use. Previous
259 monitoring by the Maine Healthy Beaches Program in 2014 had shown the Wells Beach area
260 was one of 7 beaches in Maine that had a greater than 20% exceedance rate, with suspicion that
261 freshwater inputs are a significant source of contamination (38). Our findings confirmed that
262 enterococci concentrations were statistically higher at both major freshwater tributaries to the
263 estuary, especially at the Depot Brook site where levels were regularly above the 104 CFU/100
264 ml single sample standard. The Depot Brook site is located in a watershed with a higher fraction
265 of developed land (0.27-0.50) and more people per km² (325-2,650 people) compared to the
266 Webhannet site watershed that has a lower developed fraction (0.13-0.25) and 150-325 people
267 per km²; 40). This could help explain the difference in enterococci concentrations between

268 freshwater sites as a more urbanized watershed can increase transport of more pollution from the
269 watershed to the freshwater tributary. However, the summer of 2016 was especially dry in this
270 region (41) with just one event with >1 inch of rain (1.73 in., 6/28/16) 48 h prior to the sampling
271 time. This overall dry condition likely contributed to less fecal contamination transport (via
272 freshwater discharge) from the watershed to the estuary and marine beach. This suggests that
273 more typical rainfall conditions would probably have resulted in more freshwater discharge and
274 higher enterococci concentrations than what we observed.

275

276 Enterococci were significantly associated with suspended particles of >3.0 μm diameter ($R^2 =$
277 0.96, $p < 0.05$). On average, 36% ($SD \pm 30$) of the total enterococci concentrations were
278 associated with particles, which suggests particles as a potentially important transport
279 mechanism. Other studies conducted in estuary and storm waters have found similar fractions of
280 particle associated enterococci, but they noted enterococci demonstrated a preference for a larger
281 particle size of >30 μm (42–44). The large standard deviation for particle-associated enterococci
282 could be attributed to the complex nature of particle interactions (sedimentation rate,
283 electrostatic, hydrophobic, and other surface-surface interactions) and hydrogeological dynamics
284 (salinity-driven turbidity maximum) (45). The mechanisms underlying enterococci-particle
285 interactions may also be related to ionic strength in surface waters, as *Enterococcus faecalis* is
286 negatively charged over a broad pH range (2-8 pH units) and in the presence of different ion
287 concentrations (46). Results for this study indicate that TSS and particle-associated enterococci
288 had no linear relationship, indicating particle-associated enterococci were not dependent on the
289 total amount of suspended material and thus the association is likely due to other factors
290 influencing cell-particle interactions.

291

292 Quantitative PCR assessment of several fecal sources is a potentially useful strategy to determine
293 the relative significance of the different sources in a single sample and over time at sites of
294 interest. PCR detection showed a chronic presence of mammalian fecal source(s) (100% of
295 samples) with human fecal source(s) detected in approximately half of all samples, so qPCR
296 analysis is useful for bringing context to the significance of these findings. For example, Mayer
297 et al. (47) showed that wastewater effluent contains about 10^8 copies/100 ml of the AllBac
298 mammal fecal marker, Sowah et al. (48) found that streams impacted by septic systems could
299 contain $10^5 - 10^7$ copies/100 ml depending on the season, and Bushon et al. (49) determined that
300 under storm flow conditions in an urban watershed mammal marker copy numbers could exceed
301 10^8 copies/100 ml. Results for this study ranged from 10^5 to 8.6×10^7 copies/100 ml, values that
302 are within previously reported ranges and likely a concentration reflective of a predominantly
303 non-urbanized watershed and intermediate mammal source loading. The estuary and estuary
304 beach area showed a statistically higher concentration of the mammal marker, however, there
305 was no responsive increase in the concentrations of the human associated fecal marker (HF183),
306 which may indicate that humans are not the primary mammalian source for the increased fecal
307 contamination.

308

309 The average concentration of the human marker was 1,500 copies/100 ml across all sites
310 (geometric mean 167 copies/100 ml), with the highest concentration being 20,364 copies/100ml
311 (Webhannet 6/22/16). Boehm et al. (50) showed that 4,200 copies/100 ml of HF183 is the cutoff
312 for where GI illnesses exceed the EPA acceptable risk level of approximately 30/1000 for
313 swimmers (1). On average, sites in this study did not exceed this benchmark level, however,

314 there were 10 occasions when sites were above the 4,200/100ml threshold (7 different sites
315 across 4 sampling dates), indicating that sporadic events or conditions can cause elevated human
316 fecal contamination and potential public health concerns (Supplementary 4). Boehm et al. also
317 showed that at the LOQ for most assays, 500 copies/100ml or 1000 copies/100ml, there is still a
318 predicted GI illness of 4 or 8 cases per 1000 swimmers, suggesting positive detection at the LOQ
319 is indicative of low level health risk (50). For this study, the LOQ was 250 copies/100ml for the
320 HF183 assay and 67 of 117 samples (57%) tested positive at or above this limit, suggesting that
321 over half of collected water samples indicated the presence of a low-level health risk. Although
322 there were no statistical differences between sites for human fecal contamination, W11 did
323 contain the highest geometric mean (493 copies/100 ml; Supplementary 4). This could be
324 reflective of the location of the site as it's where drainage from the Webhannet and Depot
325 watershed meets and is also directly downstream from a boat marina with the harbor sewage
326 pump station, which could be a possible point source of contamination. Nonetheless, even
327 though sites on average were below published thresholds, detection of human contamination
328 even at low concentrations is a concern.

329

330 Although human fecal sources are the greatest public health concern (6, 7, 22, 51) we did not
331 observe any relationship between human fecal contamination and enterococci concentrations,
332 suggesting other mammalian fecal sources are more influential in explaining the variation
333 observed in this study. Interestingly gull fecal sources were detected in 77% or more of the
334 samples in the estuary and marine beach area, however only 10% of the samples were positive
335 within the fresh water (Supplementary Material 1), despite there being no decrease in the bird
336 fecal marker concentration, suggesting the presence of different bird sources in these areas.

337 Anecdotally, Canada geese were observed upstream of both the Webhannet and Depot
338 freshwater sites periodically throughout the season, which could be a significant source of bird
339 fecal contamination in the fresh water locations (52).

340

341 One of the unique findings of this study was the relative contribution of different sources to the
342 bacterial community in the estuarine water. The bacterial community in estuarine water primarily
343 originated (>90%) from marine beach water, which is not surprising for a well-flushed estuary
344 like the study site. Because the study period was minimally influenced by rainfall and associated
345 runoff of freshwater, we expected that the influence of freshwater sources would be low. In
346 ensuing analyses, we chose not to include marine beach water as a potential source for a variety
347 of reasons. First, the samples were always collected during low tide before the ebb when the
348 estuary water was draining and water was moving from the watershed towards the marine beach.
349 Secondly, we had already shown that the OTU compositions for the marine beach and estuary
350 samples were very similar, increasing the possibility of a type I error (false positive) for
351 identifying marine beach as the likely source of enterococci. Lastly, fecal pollution sources most
352 likely come from the watersheds and not from marine water, so excluding marine beach water
353 helps to enhance the determination of watershed influences. Our second analysis (marine beach
354 source excluded) showed that freshwater was a significant source of bacteria to the estuary
355 (>65% assignment) compared to soil, sediment, and estuarine sediment. This implicates
356 freshwater as a major conduit for bacterial transport, as well as the major source of enterococci
357 to the estuary. Overall this finding highlights the importance of freshwater discharge as a
358 controlling factor in transporting contamination from the watershed to the coast. The specific

359 percent assignment of freshwater source could be an over-estimate, however the trend observed
360 is a likely scenario given the rational discussed.

361

362 Analysis of environmental reservoirs of enterococci (soil, sediment, etc.) and their presence
363 within water samples using SourceTracker revealed a variety of source contributions to
364 freshwater, estuary and marine waters. To date there have been limited studies using
365 SourceTracker to identify soil and sediment-associated taxa within water samples, and none of
366 these studies have focused on a coastal watershed with the potential for freshwater, estuarine and
367 marine sources. One study conducted in the upper Mississippi River identified up to 14% of
368 sediment and 1.4% of soil sources of the taxa within the river water (53). This study, however
369 showed that the sediment source was much more abundant in freshwater (74%), indicating a
370 greater degree of mixing between the freshwater and underlying sediment communities. The
371 amount of sediment and soil sources within water samples may be related to site specific
372 characteristics such as relief or soil texture, which has been shown with TSS fluxes on a global
373 scale (54). Thus, the degree to which the underlying sediment community mixes with the
374 overlaying water is likely site specific. Interestingly, even though freshwater contained a
375 significant amount of sediment source taxa, no sediment source was observed at the estuary and
376 marine beach sites through the SourceTracker analysis. This difference could indicate that rapid
377 sedimentation happens during transit to and within the estuary and at the estuarine turbidity
378 maximum zone (55). TSS concentrations and the ratio of particle-associated to total enterococci
379 concentrations, however, showed no differences between freshwater and estuary/marine sites.
380 This could be related to the separate and quite different hydrodynamics within these different
381 water systems. The percent of sediment source in the freshwater samples observed here might

382 also be an over-estimate/over fit from SourceTracker given the limited number of potential
383 sources used, but results consistently showed an elevated presence of sediment in all freshwater
384 samples in this study. SourceTracker analysis also revealed that the freshwater source was
385 significant (35% or more) in estuary and marine beach water samples, suggesting that fresh
386 water is a significant conduit for microbial, and fecal contamination, transport from the
387 watershed to the estuary and marine beach.

388

389 The use of predictive models for water quality has been a focus in the field in parallel with the
390 adoption of bacterial indicator organisms as the gold standard for water quality determination.
391 The goal of this research was to identify significant influences on enterococci concentrations by
392 measuring a wide variety of variables. To distill this information, we used a PSLR model, which
393 has been shown to out-perform similar multiple linear regression and principle components
394 regression analyses (56) and has gained popularity in the water quality field (57, 58). Results
395 from the PLSR analysis in this study showed that particle-associated enterococci and
396 concentrations of mammal fecal sources were the driving force behind variation in enterococci
397 concentrations, as described by both PLSR models constructed. Other factors were found to
398 influence enterococci concentrations, however, these differed between the freshwater and
399 estuary/marine beach models. For example, TSS concentration as well as the percent of both
400 freshwater sediment and unknown sources positively influenced enterococci concentrations at
401 freshwater sites. This indicates that sediment is a likely source of enterococci that influences
402 concentrations measured in the water. Positive influences from the unidentified source taxa
403 suggests that there is either an alternative source (not measured in this study) within the
404 watershed that also influences enterococci concentrations or that SourceTracker could simply not

405 resolve all the potential sources we used. This finding is not surprising given the vast number of
406 potential sources of fecal pollution within a watershed and that fecal sources were not a part of
407 the SourceTracker analysis. Results from the estuary and marine beach model returned a two-
408 factor regression, with each factor essentially being the inverse of each other. Specifically, it
409 highlighted freshwater being a major conduit for microbial transport to and through the estuary.
410 Negative influences from the unknown source reaffirms this finding, along with positive
411 influences from the previous high tide height. The second factor explained approximately 15% of
412 the variation in enterococci concentration, therefore its importance must be weighed
413 proportionately to factor one, which explained almost 50% of the variation. However, positive
414 loadings from previous high tide height and percent of estuarine sediment indicate estuarine
415 sediment could be a source of enterococci whose influence is dependent on tide height. The
416 negative loadings from mammal fecal source(s) may indicate that enterococci originating from
417 the estuarine sediment are not from mammal fecal sources.

418

419 Overall, the results from this study demonstrated that concentrations of enterococci in the coastal
420 estuarine/marine beach study area were largely controlled by particle-associated enterococci and
421 mammal fecal source input. The influence of these factors is likely universal across freshwater
422 and estuarine environments, however other ecosystem factors likely play a role as well. For
423 freshwater portions of the coastal watershed, sediment may act as a significant enterococci
424 reservoir that is frequently re-suspended within the water column. Freshwater itself could act as a
425 major conduit for bacterial transport to an estuary and marine beach area where other
426 environmental factors (water temperature and high tide height) can influence enterococci
427 concentrations as well. These findings highlight the dynamic nature of enterococci in natural

428 aquatic ecosystems outside of the mammalian fecal tract, and that concentrations within fresh
429 water and estuary/marine beach water are influenced by a variety of factors.

430

431 **Materials and Methods:**

432 **Site description.** This study was conducted in Wells, Maine, USA (Figure 1). Eight different
433 sites were used to monitor water quality (n = 2 freshwater, n = 2 estuary, n = 3 estuary beaches, n
434 = 1 marine beach) as well as twelve soil, twelve fresh-water sediment and four estuarine
435 sediment sampling sites. Data for air temperature and rainfall amount for the 48 h prior to
436 sampling were obtained from Weather Underground
437 (<https://www.wunderground.com/cgi-bin/findweather/getForecast?query=Wells,%20ME>) and
438 characteristics of tides during sampling were obtained from US Harbors
439 (www.meusharbors.com).

440

441 **Water sampling.** Surface water samples were collected weekly from June to September 2017 (n
442 = 117). Sampling started two hours before low tide to maximize the potential impacts of
443 freshwater pollution sources, and samples from all estuary and marine beach sites were collected
444 before the slack tide. Water samples were collected in autoclaved 1L Nalgene™ Wide-Mouth
445 Lab Quality PPCO bottles (Thermo Fisher Scientific, Waltham, MA, USA), and environmental
446 parameters were measured with a YSI Pro2030® dissolved oxygen, conductivity, and salinity
447 Instrument (YSI Incorporated, Yellow Springs, Ohio, USA). A field replicate was collected at a
448 different site for each sampling event.

449

450 **Soil, sediment, and marine sediment collection.** Environmental sources were collected twice
451 throughout the sampling season to build source libraries that were “finger-printed” with 16S
452 sequencing and SourceTracker analysis. Six soil and sediment samples were collected upstream
453 of both freshwater sites (Webhannet and Depot; Figure 1). Soil samples were collected at the
454 crest of the stream embankment, where a 10 x 10 cm a plastic square template was placed down
455 and all soil (O-horizon) within the template at a 2 cm depth was collected. Samples were sieved
456 (USA Standard No. 5) to remove any loose-leaf litter and roots to only sample smaller soil
457 particles and their microbes. Underlying stream sediments were collected using a Van Veen
458 sediment sampler from depositional sites chosen based on the presence of fine grain sediments.
459 One grab sample was collected for each site and then the top 2 cm of sediment was subsampled
460 for analysis. Sediments were sieved (USA Standard No. 45) to remove coarse grain and gravel
461 size particles. Estuarine sediments were collected during low tide when intertidal sediments were
462 exposed using the Van Veen sampler, and the top 2 cm were again collected for analysis.

463

464 **Enterococci and total suspended solids quantification.** Total and particle-associated
465 enterococci were enumerated using the EPA Method 1600 membrane filtration protocol (59) and
466 particle-associated enterococci were determined via filtration through a 0.47 mm diameter 3.0
467 μm pore size polycarbonate filter (MilliporeTM, Darmstadt, Germany) as first reported by Crump
468 et al. (60). The filters were rolled onto plates containing mEI agar and incubated at $41^\circ\text{C} \pm$
469 0.5°C ; representative colonies were counted in 24 ± 2 hours. Total suspended solids (TSS) were
470 measured using EPA method 160-2, where 500 ml of the water sample was used to determine
471 TSS concentrations (61).

472 **DNA extractions.** DNA extraction from all matrices was performed with the PowerSoils® DNA
473 Extraction Kits (MO BIO Laboratories, Carlsbad, CA, USA), with modifications to the
474 manufacture's protocol needed to optimize the extraction from water sample filters. For water
475 samples, 500 ml collected water sample was filtered through 0.47 mm diameter 0.45 µm pore
476 size polycarbonate filter (Millipore™, Darmstadt, Germany), which was stored in a sterile 2 ml
477 cryotube at -80°C for at least 24 h. Prior to DNA extraction, frozen filters were crushed into
478 small pieces with an ethanol sterilized razor blade, a practice commonly used to maximize DNA
479 recovery (62–64). To minimize additional DNA loss during the extraction process solutions C2
480 and C3 (from manufacturer's protocol) were halved in volume and combined into a single step.
481 DNA extraction from soil, freshwater sediment, and marine sediment were conducted per the
482 manufacture's protocol.

483

484 **Microbial source tracking (MST) PCR and qPCR assays.** MST PCR assays that target
485 Mammals (Bac32; 65), Humans (HF183; 9), Gulls (Gull2; 66), Dogs (DF475; 10) and
486 Ruminants (CF128; 9) were used to determine the presence of fecal sources in water samples.
487 Positive control plasmids were created for each PCR assay from fresh fecal samples that came
488 from each target organism (Human, Gull, Dog, and Cow). The TOPO™ TA™ Cloning Kit was
489 used (Invitrogen, Carlsbad, CA, USA), with a blue/white screen of *E. coli* transformants on
490 kanamycin (50 µg/mL) selective TSA plates. Positive *E. coli* colonies were screened with their
491 respective PCR assay, and PCR positive colonies were then grown in TSB and extracted with the
492 PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA). PCR assays were run
493 on a T100™ Thermal Cycler (BioRad, Hercules, CA, USA) with the GoTaq® Green MasterMix
494 (Promega, Madison, WI, USA). Cycling conditions and amplification protocols for each assay

495 targeted the different source specific markers and followed protocols delineated by different
496 studies: Bac32 (67) and HF183 (67), CF128 (68), DF475 (69), and Gull2 (66). Quantitative PCR
497 assays were also run to determine fecal source strength for Mammals (AllBac; 70), Humans
498 (HF183; 71), and Birds (GFD; 72). All qPCR assays were run on a Mx3000P cycler (Agilent
499 Technologies, Santa Clara, CA, USA), TaqMan assays used the PerfecCTa[®] FastMix[®] II
500 (QuantaBio, Beverly, MA, USA) master mix and the SYBR green assay used the FastSYBR[™]
501 Green Master Mix (Applied Biosystems, Foster City, CA, USA). A standard curve ranging from
502 10⁶-10² copies (Mammal assay) or 10⁵-10¹ copies (Human & Bird assay) was also run for each
503 experimental run with the limit of quantification (LOQ) being 100 copies (Mammal) or 10 copies
504 (Human & Bird) per PCR. The Ct values, amplification efficiency, slope, and R² values for each
505 standard curve were compared to previously run standard curves, to ensure satisfactory
506 performance before being used to calculate copy numbers for that run. Each environmental
507 sample was diluted 1:10 and run in triplicate and the reaction volume (25 µl) contained a final
508 concentration of 0.2 mg/ml BSA. Amplification/cycling conditions were preformed per
509 published protocols for AllBac (73), HF183 (73), and GFD (16). TaqMan assays were run with
510 an internal amplification control (74) with a down-shift of 1 cycle considered inhibition. Samples
511 spiked with a plasmid containing 10⁴ copies of GFD amplicon were used as inhibition controls
512 for the SYBR assay, with a recovery of less than 10⁴ copies (100%) considered inhibition. For a
513 list of primers, probes, and standard curve performance, see Supplementary Material 1.

514

515 **16S library preparation.** The V4 region of the 16S rRNA gene, using the 515F-806R primer-
516 barcode pairs, was used for amplicon sequencing (75). The Earth Microbiome Project protocol
517 was used for amplification and pooling of samples, with minor modifications (76). The Qubit[®]

518 dsDNA HS assay was used to quantify sample concentrations, and 500 ng of DNA was pooled
519 per sample. The pool was then run on a 1.2 % low-melt agarose gel to separate primer-dimers
520 from acceptable product, and bands between 300-350 bps were cut and extracted as described
521 above. The final DNA sample was then run on the Agilent Technologies 2200 TapeStation
522 system (Santa Clara, CA, USA) to determine final size, quality, and purity of sample. Each
523 library was sent to the Hubbard Center for Genome Studies at the University of New Hampshire
524 to be sequenced (2 x 250 bp) on the Illumina HiSeq 2500 (San Diego, CA, USA).

525

526 **Quality filtering and Operational Taxonomic Unit (OTU) picking.** QIIME 1.9.1 was used to
527 perform all major quality filtering, and OTU picking (77). Forward and reversed reads were
528 quality trimmed (μ P25) and removed of Illumina adapters via Trimmomatic (78). Any reads that
529 were less than 200 bps were discarded, and reads were merged with the QIIME
530 joined_paired_ends.py, using a minimum overlap of 10 bps and a maximum percent difference
531 of 10%. Paired-end data were analyzed using the QIIME open-reference OTU picking strategy
532 with UCLUST for *de novo* picking and the Greengenes 13_8 database (79) for taxonomic
533 assignment. Alternative OTU picking strategies were also tested to determine best workflow, for
534 performance of difference strategies refer to Supplementary Material 2. Data for all sequenced
535 samples are publicly available through NCBI BioProject
536 (<http://www.ncbi.nlm.nih.gov/bioproject/431501>).

537

538 **SourceTracker analysis.** Samples from 4 source types (fresh water, soil, sediment, and marine
539 sediment) and 4 sink types (fresh water, estuary water, estuary beach water, and marine beach

540 water) were analyzed by the open-source software SourceTracker v1.0 (37). Default parameters
541 were used (rarefaction depth 1000, burn-in 100, restart 10, alpha (0.001) and beta (0.01) dirichlet
542 hyperparameters) in accordance with previously published literature (53, 80). A ‘leave one out’
543 cross validation was performed to assess the general performance of the model and source
544 samples were iteratively assigned as sinks to assess how well a known sink would be assigned
545 (i.e. source = soil and sink = soil). The percent assignments from SourceTracker are the result of
546 the Gibbs Sampler assigning OTUs from an unknown sample to sources in a random and
547 iterative fashion, and then calculating likelihood of that OTU originating from said source. The
548 final output can be interpreted as the percent (or likelihood) of OTUs present in an unknown
549 sample originating from the sources used in the analysis

550

551 **Partial least squares regression model.** A partial least squares regression (PLSR) model was
552 used to determine the most important and significant variables affecting enterococci
553 concentrations (81). Two models were created, one for the estuary, estuary beach, and marine
554 beach sites, and one for the freshwater sites. Particle-associated enterococci, environment
555 variables (water temperature, air temperature, dissolved oxygen, salinity, height of previous high
556 tide, rainfall in previous 48 h), fecal source strength (mammal, human, and bird), and percent of
557 environmental source (fresh water, soil, sediment, and marine sediment) were used as
558 explanatory variables for the non-freshwater model. The same parameters, except height of
559 previous high tide and percent of freshwater source, were used for the freshwater model. All data
560 except the percent assignments from SourceTracker were log (x+1) transformed before
561 performing the analysis. A KFold cross validation (K=7) with the NIPALS method was used to
562 determine optimal factors and variable importance (VIP > 0.8) for each model. Models were then

563 re-run with only explanatory variables that were determined to be significant. To see model
564 validation and diagnostic plots, refer to Supplementary Material 3.

565 **Routine statistical analysis and data visualizations.** All routine statistical analyses were
566 performed in R v3.4.0, Python 3.6.1, or JMP Pro13, while multivariate analyses were performed
567 with PC-ORD v6. Graphing was performed in IPython notebook with matplotlib, seaborn,
568 pandas, and numpy packages. All pairwise comparisons were done using the Kruskal-Wallis
569 nonparametric method, with Dunn's nonparametric multiple comparisons run *post hoc* using a
570 Bonferroni correction.

571

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580

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857

858 **Tables**

Environmental Microbial Community Source (Including Marine Beach Source)

| Water Sample Type | Marine Beach | Freshwater | Estuarine Sediment | Sediment | Soil |
|----------------------|--------------|------------|-----------------------|----------|--------|
| Estuary Beach | 97% | <0.01% | 0.4% | <0.01% | <0.01% |
| Estuary | 94% | 2.9% | 0.2% | 0.02% | <0.01% |
| Freshwater | <0.01% | N/A | <0.01% | 74% | 2.6% |

Environmental Microbial Community Source (Excluding Marine Beach Source)

| Water Sample Type | Marine Beach | Freshwater | Estuarine Sediment | Sediment | Soil |
|----------------------|--------------|------------|-----------------------|----------|--------|
| Estuary Beach | N/A | 66% | 12% | <0.01% | <0.01% |
| Estuary | N/A | 74% | 7.6% | 0.02% | <0.01% |
| Marine Beach | N/A | 35% | 19% | <0.01% | <0.01% |
| Freshwater | N/A | N/A | 0% | 74% | 2.6% |

859

860 **Table 1. The relative contribution of different sources to the microbial communities in**
861 **estuarine and marine water.** SourceTracker was run with two different configurations, one
862 where Marine Beach water was included as a potential source (top) and a second run where
863 Marine Beach water was excluded as a potential source (bottom).

864

865

866

867

| Freshwater | | Estuary, Estuary Beach & Marine Beach | | | |
|---------------------|---------|---------------------------------------|---------|--------------------------|---------|
| PLSR 1 | | PLSR 1 | | PLSR 2 | |
| X Variable | Loading | X Variable | Loading | X Variable | Loading |
| Particle ENT | 0.501 | Particle ENT | 0.456 | Particle ENT | 0.420 |
| qPCR Mammal | 0.352 | qPCR Mammal | 0.438 | qPCR Mammal | -0.337 |
| TSS | 0.408 | % Freshwater | 0.408 | % Freshwater | -0.418 |
| % Sediment | 0.336 | % Unknown | -0.457 | % Unknown | 0.389 |
| % Unknown | 0.476 | Water Temp (C) | 0.302 | Water Temp (C) | -0.123 |
| Salinity | -0.344 | Hightide (ft) | 0.170 | Hightide (ft) | 0.456 |
| Total Y Variance | 60.1% | % Estuarine Sediment | -0.294 | % Estuarine Sediment | 0.401 |
| | | Total Y Variance | 47.2% | Cumulative Y Variance | 61.8% |

868

869 **Table 2. Most Significant Relationships/Contributions for All Factors to Enterococci**

870 **Concentrations. Shown is the output from a partial least squares regression for a**
 871 **freshwater and estuary/marine model.** All variables shown have significant relationships for
 872 each model ($VIP > 0.8$), and loadings are derived from re-running models with only variables
 873 deemed significant. Model loadings are specific weights on a multivariate regression axis,
 874 positive and negative loadings refer to positive or negative relationships to enterococci
 875 concentrations. Negative loadings in the model are designated with a – before the number.

876

877

878

879 **Figure Legends**

880 **Figure 1: Wells Maine Study area and sampling sites.** All water collection sites are marked
881 with a dark grey circle. Sites that correspond to fresh water are indicated with a (1), estuary (2),
882 estuary beach (3), and marine beach (4).

883

884 **Figure 2: Geometric Mean Concentrations of Total and Particle Associated Enterococci**
885 **and Average Total Suspended Solids Concentrations at the Eight Study Sites.** (A) Total
886 enterococci concentrations are represented with the blue bar, and particle associated enterococci
887 concentrations correspond to the green bar. Error bars are derived from variation from each site
888 across the entire study. (B) Violin plots were used to represent TSS concentrations, and the color
889 corresponds to the type of site including marine beach (red), estuary beach (purple), estuary
890 (green), or fresh water (blue). Horizontal lines go through the median of each violin plot.

891

892 **Figure 3: Relative Levels of Mammal, Human, and Bird Fecal Source at the Different**
893 **Types of Study Sites.** Box plots represent levels of microbial source tracking markers at marine
894 beach (Wells Beach), estuary beach (W11, W12, W13), estuary (W14 & W15), and fresh water
895 (Webhannet & Depot). Outlier data are represented with a black diamond.

896

897 **Figure 4: 16S Taxa Profiles and the Top Three Most Abundant Bacterial Classes in All**
898 **Source and Sink Samples.** Stacked bar plots represent percentages of the class level
899 composition of the microbial communities. Source corresponds to environmental sources that

900 were finger-printed with the SourceTracker program, and then used to determine their presence
901 within water (sink) samples. The table represents the top three classes for each group of samples
902 and * corresponds to phylum level. For a complete list of all taxa assignments refer to
903 Supplementary material 4.

904

905 **Figure 5. Differences Between Microbial Communities from Different Source Materials.**

906 Samples are color-coded based on sample matrix (i.e. soil, fresh water, etc.). Percent of variation
907 explained are displayed on the x and y axis and the minimum stress of the ordination is shown in
908 the top left corner.

909

W14 (2)

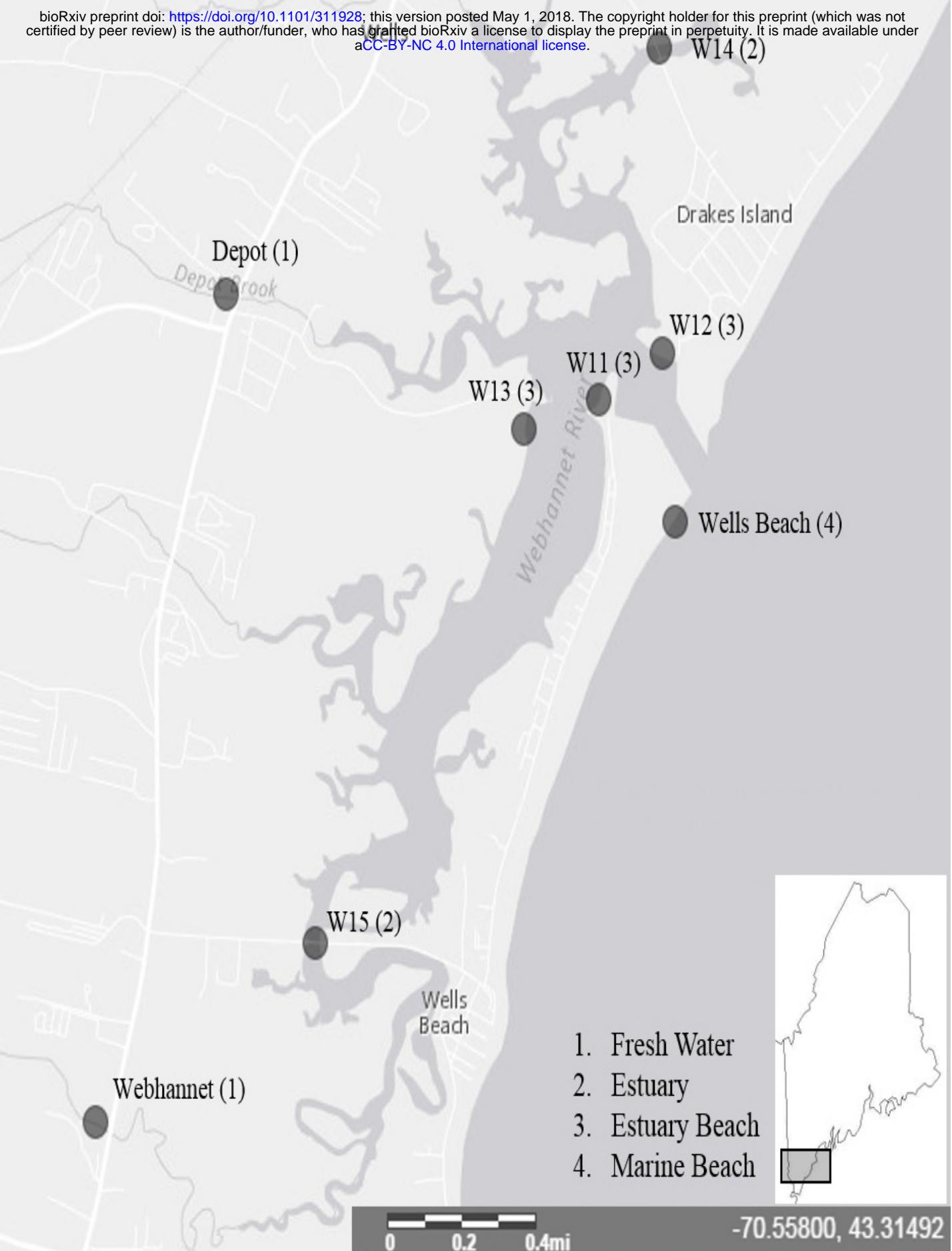


Figure 1: Wells Maine Study area and sampling sites. All water collection sites are marked with a dark grey circle. Sites that correspond to fresh water are indicated with a (1), estuary (2), estuary beach (3), and marine beach (4).

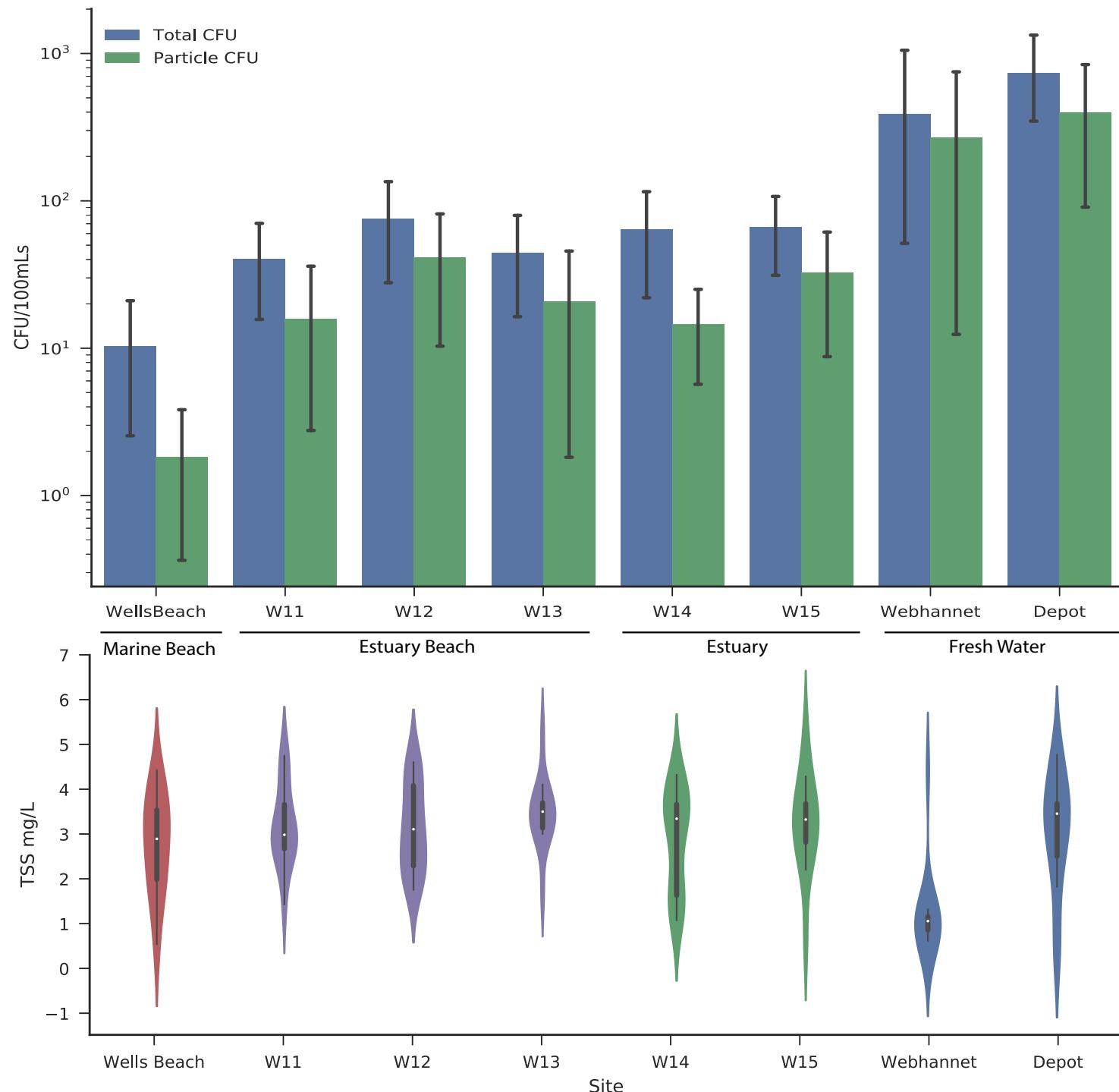


Figure 2: Geometric Mean Concentrations of Total and Particle Associated Enterococci and Average Total Suspended Solids Concentrations at the Eight Study Sites. (A) Total enterococci concentrations are represented with the blue bar, and particle associated enterococci concentrations correspond to the green bar. Error bars are derived from variation from each site across the entire study. (B) Violin plots were used to represent TSS concentrations, and the color corresponds to the type of site including marine beach (red), estuary beach (purple), estuary (green), or fresh water (blue). Horizontal lines go through the median of each violin plot.

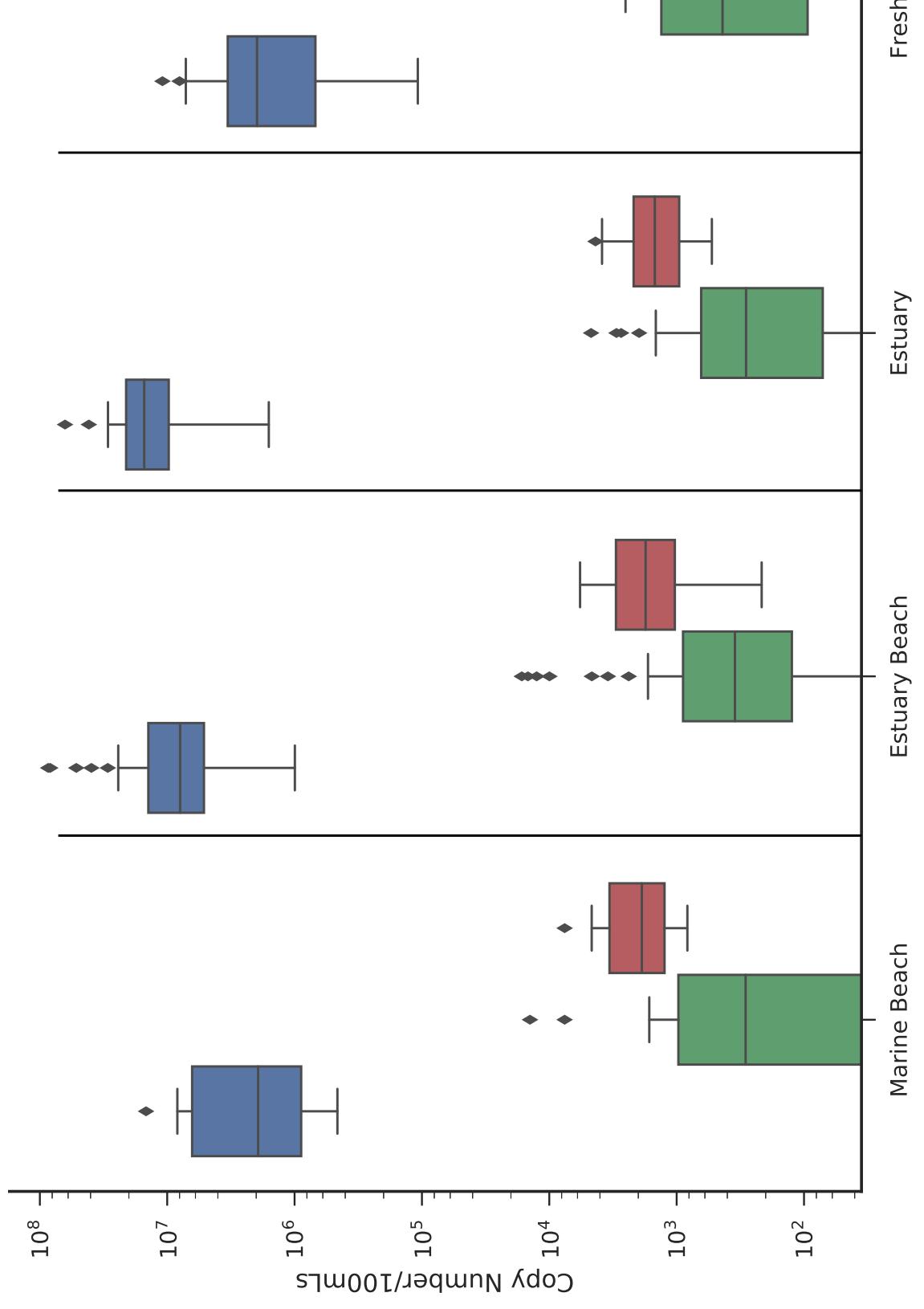


Figure 3: Relative Levels of Mammal, Human, and Bird Fecal Source at the Different Types of Study Sites. Box plots show microbial source tracking markers at marine beach (Wells Beach), estuary beach (W11, W12, W13), estuary (W14, W15, W16), and freshwater (Webhannet & Depot). Outlier data are represented with a black diamond.

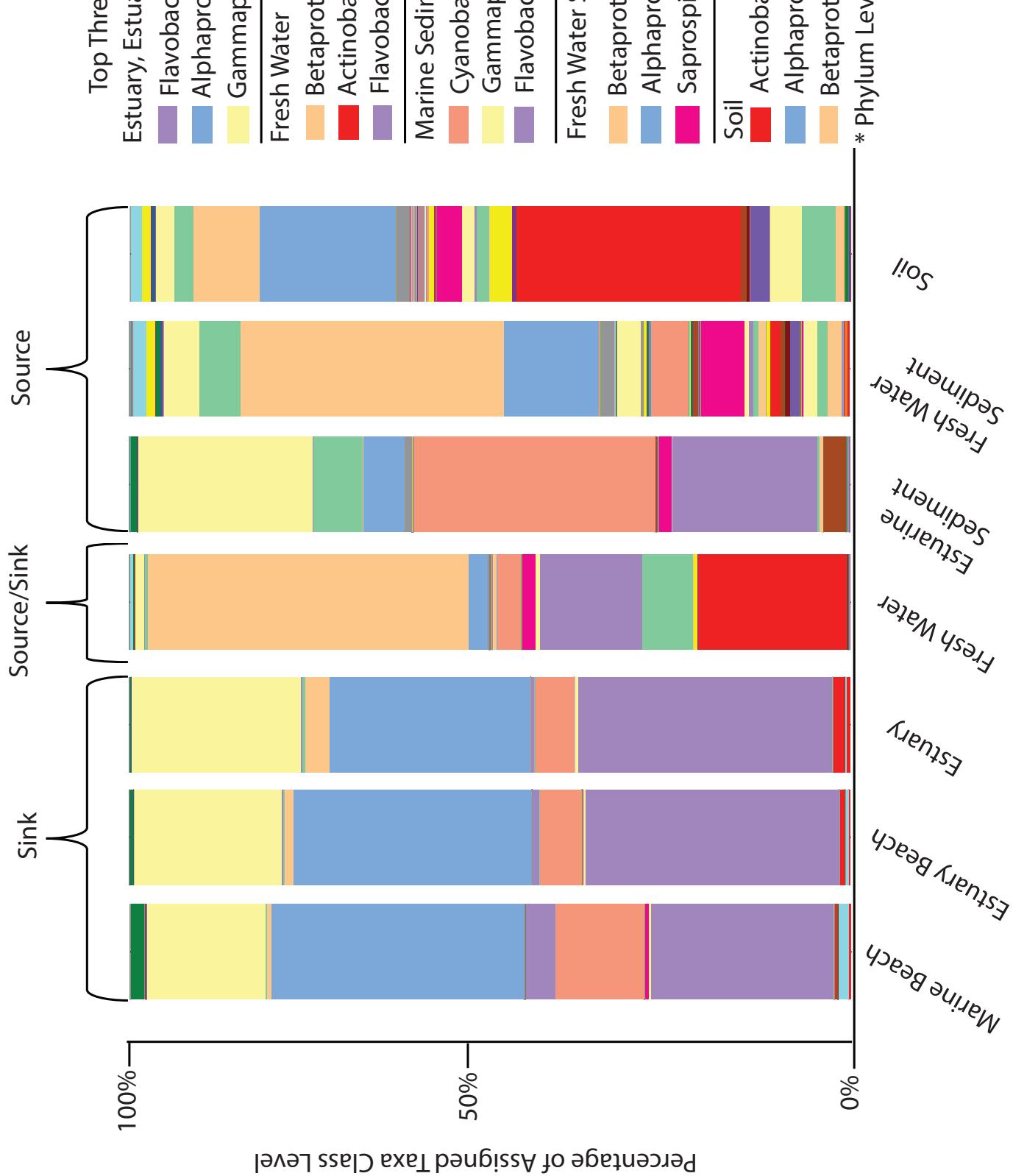


Figure 4: 16S Taxa Profiles and the Top Three Most Abundant Bacterial Classes in All Source and Sink Samples. The bars represent percentages of the class level composition of the microbial communities. Source corresponds to environments where finger-printed with the SourceTracker program, and then used to determine their presence within water samples. Sink represents the top three classes for each group of samples and * corresponds to phylum level. For a complete refer to Supplementary material 4.

NMDS

Min Stress = 8.127
(Monte Carlo – 250 runs)

- △ Freshwater
- △ All Estuary
- △ Marine Beach
- △ Freshwater Sediment
- △ Freshwater Soil
- △ Estuarine Sediment

Axis 2 13.4%

Axis 1 63.9%

Figure 5. Differences Between Microbial Communities from Different Source Materials. Samples are color-coded based on sample matrix (i.e. soil, fresh water, etc.). Percent of variation explained are displayed on the x and y axis and the minimum stress of the ordination is shown in the top left corner.