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Increased variability of microbial communities in restored salt marshes nearly 30 years after tidal flow restoration

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**INCREASED VARIABILITY OF MICROBIAL COMMUNITIES IN RESTORED SALT
MARSHES NEARLY 30 YEARS AFTER TIDAL FLOW RESTORATION**

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Abstract

We analyzed microbial diversity and community composition from four salt marsh sites that were impounded for 40-50 years and subsequently restored and four unimpounded sites in southeastern Connecticut over one growing season. Community composition and diversity were assessed by terminal restriction fragment length polymorphism (TRFLP) and sequence analysis of 16S ribosomal RNA (rRNA) genes. Our results indicated diverse communities, with sequences representing 14 different bacterial divisions. *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* dominated clone libraries from both restored and unimpounded sites. Multivariate analysis of the TRFLP data suggest significant site, sample date and restoration status effects, but the exact causes of these effects are not clear. Composition of clone libraries and abundance of bacterial 16S rRNA genes were not significantly different between restored sites and unimpounded sites, but restored sites showed greater temporal and spatial variability of bacterial communities based on TRFLP profiles compared to unimpounded sites and variability was greatest at sites more recently restored. In summary, our study suggests there may be long-lasting effects on stability of bacterial communities in restored salt marshes and raises questions about the resilience and ultimate recovery of the communities after chronic disturbance.

65 **Introduction**

66 Many New England salt marshes altered by tidal restrictions and impoundments half a
67 century ago have undergone extensive restoration over the past twenty years. A substantial
68 effort has also gone into monitoring the recovery and evaluating the effectiveness of these
69 restorations (see Warren et al. 2002; Crain et al. 2009). The vast majority of these studies,
70 however, has focused on vegetation (Sinicrope et al. 1990; Roman et al. 2002),
71 macroinvertebrates (Peck et al. 1994; Swamy et al. 2002), birds (Brawley et al. 1998; Benoit and
72 Askins 1999) and fish species (Allen et al. 1994). Very little, if any, attention has been paid to
73 recovery of community structure and diversity of microorganisms.

74 The high productivity of salt marshes supports an active and diverse microbial
75 community, with microorganisms mediating most major nutrient transformations in the salt
76 marsh. Additionally, salt marsh macrophytes have been shown to enhance microbial
77 colonization and organic matter decomposition (Oliveira et al. 2010; Santos et al. 2007) and
78 influence microbial community structure (Ravit et al. 2006; Ravit et al. 2007). Therefore,
79 changes to the marsh are likely to have significant impacts on the microbial communities and the
80 elemental cycles that they mediate. For example, changes in tidal flow to the marsh may reduce
81 the supply of sulfate, and may cause a shift in the dominant carbon cycling process from sulfate
82 reduction to methanogenesis, thus contributing to the accumulation of greenhouse gases
83 (Valentine 2002). Additionally, changes in drainage from the marsh will effect changes in
84 aeration to the sediment (Portnoy and Giblin 1997; LaRiviere et al. 2004), and may impact
85 anaerobic respiration pathways, including sulfate reduction, methanogenesis, and denitrification.
86 Thus, changes that may be brought about by tidal restriction and subsequent tidal flow
87 restoration would likely be reflected in significant changes in the function of the marsh, brought
88 about, in part, by changes in the microbial community structure and diversity.

89 Changes in microbial species composition might also be expected as the salinity changes
90 since salinity can influence the abundance and community structure of bacteria (Bordalo 1993;
91 Revilla et al. 2000). This is not surprising as salinity has been identified as one of the primary

92 drivers of microbial community composition (Bouvier and del Giorgio 2002; Crump et al. 2004;
93 Bernhard et al. 2005a; Bernhard et al. 2005b) and function (Bernhard et al. 2007). However,
94 since many other environmental factors covary with salinity (such as nutrient concentrations), it
95 is often difficult to identify a single variable as the effector of community changes.

96 Because microbes are responsible for much of the nutrient cycling, significant changes in
97 the microbial community may have serious impacts on process rates in an ecosystem (Begon et
98 al. 1990), resulting in changes to nutrient flow through the system. Within in an ecosystem,
99 there are often multiple populations that carry out the same process, known as metabolic
100 redundancy, providing a level of insurance for the ecosystem in the event that some populations
101 are lost due to changes in the environment. Thus, a loss of a particular population may lead to a
102 loss of metabolic redundancy and may leave the community more vulnerable when challenged
103 with environmental stressors. Empirical studies of microbial community stability and function
104 have generally borne out these predictions (Hashsham et al. 2000; Maoz et al. 2003). Thus,
105 microbial community diversity may serve as an indicator of ecosystem function and stability.

106 The focus of our research was to evaluate community structure and diversity of bacteria
107 in restored salt marshes and unimpounded marshes. The research was carried out in the
108 Wequetequock-Pawcatuck tidal marshes (locally referred to as Barn Island marshes) and the
109 Cottrell Marsh of southeastern Connecticut. The Barn Island marsh system is composed of a
110 series of valley marshes, each having undergone different management histories (Miller and
111 Egler 1950). The four valley marshes sampled in this study were impounded by a dike in the
112 1940's to provide habitat for waterfowl. Seawater access to these impoundments has since been
113 restored through a series of culverts, beginning in 1978. Recovery of the vegetation in
114 impounded marshes has been documented, and in most cases, indicates a shift from *Typhus* and
115 *Phragmites*-dominated marshes to *Spartina* spp. (Sinicrope et al. 1990). Many of the
116 macroinvertebrate populations also appear to be recovering and are similar to populations in
117 adjacent unimpounded reference marshes (Swamy et al. 2002). Similar patterns have been
118 reported for fish populations and bird use of the marsh, all indicating a trajectory of recovery for

119 the marshes. Given the data on other communities in the marsh and fast generation times of
120 bacteria, we expected to find little evidence of the impact of the impoundment on the microbial
121 communities. Although our findings suggested little difference in the taxonomic assemblage
122 among restored and unimpounded sites, we detected long-term effects on the stability of the
123 communities, which may have as yet undetermined effects on ecosystem function.

124

125 **Methods**

126 *Site Description.* Four sites that were impounded and subsequently restored were located in the
127 Wequetequock-Pawcatuck tidal marshes, which cover 1.4 km², bounded on the west by
128 Wequetequock Cove, on the east by the Pawcatuck River near the border of Rhode Island, and to
129 the south by Little Narragansett Bay (Figure 1). The marsh is separated from Long Island Sound
130 by a series of bars and islands. Tidal flow was restored to Impoundments 1 (IP1) and 2 (IP2) in
131 1978 by the installation of 1.5 m culverts. In 1982, an additional 2.1-m culvert was created in
132 IP1 to more fully restore tidal access. Tidal flow to Impoundment 4 (IP4) was restored in 1987
133 with a 1.3-m culvert and to Impoundment 3 (IP3) in 1991 with a 1.5-m culvert. Headquarters
134 (HQ) Marsh was selected as one of two reference sites in Barn Island due to its location just
135 seaward of IP1 and IP2. However, HQ has undergone extensive vegetation changes, likely due
136 to sea level rise (Warren and Niering 1993), so Wequetequock Cove (WE) was chosen as an
137 additional reference site within the Barn Island marsh system. Two additional unimpounded
138 sites located in the Cottrell Marsh (CO), 6 km west of the Wequetequock-Pawcatuck marshes,
139 were also selected as reference sites from a nearby marsh that has never been impounded. More
140 extensive descriptions of these marsh sites are found elsewhere (Warren and Niering 1993,
141 Warren et al 2002).

142

143 *Sample collection.* Triplicate sediment cores (6.5 cm diameter) were taken from IP1, IP2, IP3,
144 IP4, HQ, and WE marshes in June, July, and October 2006 and from CO-1 and CO-2 in July and
145 October 2006. Plots in areas dominated (60-100% coverage) by the marsh grass *Spartina patens*

146 were selected at each site. Each plot was designated with a 0.25 m² grid sectioned into 25 x 0.1
147 m squares. A random numbers table was used to select three squares within the grid in which to
148 collect a core. Cores were placed on ice in the dark for transport back to the lab. In the lab, the
149 top 0-2 cm section of each core was homogenized and 0.5 g aliquots were stored at -80°C for
150 DNA processing and an additional 1-2 g of sediment was weighed for dry-weight determination.
151 Wet sediment was dried overnight at 70°C and reweighed. Porewater from each core was
152 obtained from the remaining sediment by centrifugation (5000 x g for 5 minutes) in 50 ml tubes
153 with a 0.45 µm cellulose acetate filter insert (Chrom Tech, Inc., Apple Valley, MN).

154

155 *Physical and chemical sediment characterization.* Salinity was measured from the porewater in
156 each core using a hand-held refractometer, except during June when salinity was measured
157 on site (not from individual cores). pH was also measured from each core using a pH100 meter
158 with a piercing probe (YSI, Yellow Springs, OH). Water content of the sediment was determined
159 by the change in weight of sediment after drying at 70°C overnight.

160

161 *DNA extraction.* We extracted DNA from the 0.5 g aliquots using the PowerSoil™ DNA
162 Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's recommendations. The
163 quantity and quality of the DNA was evaluated by measuring the optical density at 260 and 280
164 nm using a SmartSpec Plus spectrophotometer (BioRad, Hercules, CA) and by gel
165 electrophoresis in a 1% agarose gel with 1 µg/ml of ethidium bromide. Samples that had a
166 260:280 ratio less than 1.5 were thrown out and another aliquot was extracted.

167

168 *Real-time PCR of Bacterial 16S rRNA genes.* Bacterial 16S rRNA genes were quantified using
169 primers GM3 and 338R as described in Könneke et al. (2005). All reactions were run in an
170 iCycler (BioRad) using SYBR Green I master mix (BioRad), 0.5 µM of each primer, and 0.008%
171 BSA with the following amplification cycle parameters: 95°C for 10 minutes followed by 40
172 cycles of 95°C for 15 sec, 55°C for 20 sec, 72°C for 30 sec. Melt curve analysis was performed

173 after each experimental run to confirm the product specificity. Sample amplification was
174 compared to a standard curve generated in each experimental run using five standards ranging in
175 DNA concentration from 1 fg/ μ l to 10 pg/ μ l, which is equivalent to 1.8×10^2 to 1.8×10^6 gene
176 copies/ μ l. Standards were a mix of 29 different purified plasmid DNAs from clones generated
177 from bacterial 16S rRNA genes recovered previously from salt marsh sediments (Nielsen et al.
178 2004) representing 11 different bacterial divisions. Average amplification efficiency was $91.7 \pm$
179 4.5%. Effects of inhibition during PCR were previously tested on the same DNA samples and
180 we determined that a 1:10 dilution was optimal for amplification with minimal inhibition (Moin
181 et al. 2009).

182
183 *TRFLP analysis.* Bacterial 16S rRNA genes were amplified using the Bacterial-specific primer
184 27F (Lane 1991) and the universal primer 1492R (Lane et al. 1985). The forward primer (27F)
185 was fluorescently labeled with 6-FAM (Operon Technologies, Huntsville, AL). Each 20 μ l PCR
186 contained 10 μ l of 2X iQ Supermix (BioRad), 0.5 μ M each primer and 1 μ l of template DNA,
187 diluted 1:10 with water to reduce interference from inhibitors. An iCycler (BioRad) was used for
188 all reactions with the following cycle sequence: 94°C for 10 min., followed by 30 cycles of 95°C
189 for 15 s, 55°C for 20 s, and 72°C for 2 m, with a final 5 minute extension at 72°C. PCR products
190 were evaluated following electrophoresis on 1% agarose gels and comparison of the band
191 intensities and migration distances to a low DNA mass ladder (Invitrogen, Carlsbad, CA).

192 PCR products were digested with 10 units of *MspI* (New England Biolabs, Beverly, MA)
193 overnight at 37°C. Following ethanol precipitation, samples were resuspended in 10 μ l of
194 deionized H₂O. To prepare samples for analysis, 3 μ l of each sample, 0.2 μ l of the internal size
195 standard, GS500-ROX (Applied Biosystems Inc., Fremont, CA), and 10 μ l of Hi-Di Formamide
196 (ABI) were mixed and sent to the Biotechnology Resource Center at Cornell University
197 (<http://cores.lifesciences.cornell.edu/brcinfo/>) for analysis on an Applied BioSystems 3730xl
198 DNA Analyzer. Terminal restriction fragment (TRF) sizes and relative abundances were
199 estimated using GeneMarker software, v.1.4 (SoftGenetics, State College, PA). We determined

200 the range of reliable TRF size estimates to range from 67 to 500 bp based on amount of
201 background and reproducibility among replicates. Peak heights were normalized to account for
202 differences in the amount of DNA analyzed using the method from Dunbar et al. (2000).

203

204 *Clone library construction.* Bacterial 16S rRNA genes were amplified from samples collected in
205 June 2006 from sites IP1, IP2, IP3, IP4, WE, and HQ and from all eight sites in July 2006 using
206 the same primers as above, but the 27F primer was unlabeled. PCR products were cloned using
207 the StrataClone PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA) according
208 to the manufacturer's instructions. Transformants were randomly selected and inoculated into
209 100 μ l LB broth with 100 μ g ml⁻¹ ampicillin in 96-well microtiter plates. All plates were
210 incubated overnight at 37°C. Clones were screened for the presence of correctly-sized inserts by
211 PCR using vector-specific primers T3 and T7. Clones containing the correct insert were
212 sequenced by High Throughput Sequencing Solutions (University of Washington, Department of
213 Genome Sciences, Seattle, WA) using the Bacterial-specific primer 700R (Urbach et al. 2001).

214

215 *Sequence analysis.* Sequences were aligned using the sequence editor and Fast Align in ARB
216 (Ludwig et al. 2004) and checked manually. Phylogenetic affiliations of individual sequences
217 were based initially on analysis by the Ribosomal Database Project (Wang et al. 2007) and were
218 confirmed by phylogenetic tree construction using the neighbor-joining algorithm in ARB. We
219 compared the placement of each sequence in trees constructed using 256 bases at the 5' end and
220 the 3' end of the sequences to identify potential chimeras. Sequences showing evidence of
221 possible chimeric structure were removed from the analysis. A total of 553 sequences was
222 analyzed, ranging from 48-95 sequences per site. We determined predicted TRF sizes for all
223 sequences in silico.

224

225 *Statistical analysis.* All multivariate analyses were performed using PC-Ord version 6 (McCune
226 and Mefford 1999). The relative abundance data were transformed by an arcsine square root

227 function to reduce skew. Non-metric multidimensional scaling (NMS) (Kruskal 1964) was used
228 to ordinate samples in gene fragment space, using the Sørensen's distance measure. The
229 autopilot option was set to the slow and thorough level for all ordinations. Dimensionality (the
230 optimal number of dimensions or axes required to explain a sufficient proportion of the variance)
231 was assessed by choosing the number of axes that minimized final stress and maximized
232 interpretability of the results. Monte Carlo tests were run to confirm that results obtained were
233 significantly better than would be obtained from randomized data. Additionally, the proportion
234 of variance explained by each axis and the cumulative variance explained was determined by
235 calculating the coefficient of determination between distances in ordination space and distances
236 in the original p-dimensional space. Correlation coefficients in the ordination space were
237 determined for each environmental variable and TRF by rotating the ordination to maximize the
238 coefficient on one axis (varimax rotation) in order to facilitate detecting clusters of samples
239 (McCune and Grace 2002).

240 Multi-response permutation procedure (MRPP), a nonparametric test, was used to test
241 for differences between restored and unimpounded sites, among different sites, and among
242 sampling dates. MRPP is a variant of ANOSIM (Analysis of Similarity) and provides a measure
243 of the effect and p value when testing for differences between two or more groups defined by the
244 user (McCune and Grace 2002).

245 Coverage of each clone library was calculated with the equation: $C = 1 - (n/N)$ where $n =$
246 number of singleton sequences and $N =$ total number of sequences analyzed. Differences among
247 libraries were evaluated by β -Libshuff, AMOVA (analysis of molecular variance), and
248 HOMOVA (homogeneity of molecular variance) analyses using the program mothur (Schloss et
249 al. 2009). These three methods provide a complementary analysis to detect differences among
250 communities (Schloss 2008). AMOVA determines whether the genetic diversity among the
251 communities is greater than their pooled genetic diversity. HOMOVA determines whether the
252 amount of genetic diversity within each community is significantly different (Schloss 2008). β -
253 Libshuff is based on the Cramér-von Mises-type statistic and uses a Monte Carlo procedure to

254 compare differences based on pair-wise comparisons (Singleton et al. 2001; Schloss 2008). We
255 ran pair-wise comparisons of clone libraries generated from samples collected in July since we
256 did not sample the Cottrell Marsh in June. To account for experiment-wise error, we applied the
257 Bonferroni correction to the results. For pair-wise comparisons among all 8 libraries, p values
258 less than 0.00092 were considered significant. We also ran a comparison of combined libraries
259 from all four restored sites and all four unimpounded sites. Diversity indices were calculated
260 from TRFLP data (evenness, richness, Shannon-Weiner, and Simpson's) using PC-Ord and from
261 sequence data (Chao1) using mothur.

262

263 Nucleotide sequences for Bacterial 16S rRNA genes can be found under the following Genbank
264 accession numbers: JN684211 - JN684753.

265

266 **Results**

267 Although there were no significant differences between restored and unimpounded sites
268 for salinity, pH, or water content, we identified patterns that corresponded to restoration status
269 (Table 1). For example, among the restored sites, salinity was generally lower at sites more
270 recently restored compared to sites that were restored earlier. Additionally, pH was lower at the
271 unimpounded sites compared to the restored sites in July, but the pattern was not consistent on
272 other sampling dates.

273 Relationships among the environmental variables suggest some co-variation. Percent
274 water content was positively and significantly correlated with pH (Pearson's correlation
275 coefficient, $r = 0.53$, $p < 0.00001$) and salinity ($r = 0.38$, $p = 0.003$), but no significant correlation
276 was detected between salinity and pH ($r = 0.13$, $p = 0.35$).

277 Bacterial 16S rRNA gene abundance ranged from 1.6×10^9 to 2.4×10^{11} copies g
278 sediment (dry weight)⁻¹. However, no significant differences were detected between restored
279 sites and unimpounded sites (Figure 2).

280 Phylogenetic analysis of 553 Bacterial 16S rRNA gene clones revealed sequences
281 affiliated with 14 bacterial divisions (Figure 3, Table 3). Coverage of clone libraries was similar
282 among all libraries (21.6 ± 6.5 and $23.3 \pm 6.4\%$ for restored and unimpounded sites,
283 respectively). The most frequently recovered clones were affiliated with the
284 *Gammaproteobacteria*, *Bacteroidetes*, and *Planctomycetes*. Other frequently encountered
285 divisions included *Acidobacteria*, *Deltaproteobacteria*, *Verrucomicrobia*, and *Chloroflexi*.
286 Interestingly, the taxonomic composition in restored and unimpounded sites was very similar
287 (Figure 3). These results were confirmed by the limited number of significant differences among
288 gene clone libraries based on β -Libshuff, AMOVA, and HOMOVA analyses (Table 2). Most of
289 the significant differences detected involved comparisons of libraries with IP3 and IP4, which
290 are the most recently restored sites.

291 TRFLP analysis was performed on all DNA samples to characterize the bacterial
292 communities. A total of 224 TRFs was detected among all the samples, ranging in size from 67
293 bp to 500 bp. Sequence analysis of bacterial 16S rRNA gene clones was used to identify
294 presumptive phylogenetic affiliations of each TRF. In some cases, sequences of different
295 phylogenetic affiliations represented a single TRF and not all TRFs were represented in the clone
296 libraries. Of the 224 TRFs, 131 (58.4%) matched predicted TRFs from sequenced 16S rRNA
297 gene clones.

298 We applied nonmetric multidimensional scaling (NMS) analysis to identify patterns
299 among the bacterial communities based on the TRFLP profiles. The first two axes of the
300 ordination explained 54.1% of the variability among the communities (Figure 4). Although
301 some bacterial communities found at restored and unimpounded sites appeared to be similar,
302 restored sites were more variable compared to unimpounded sites. The average distance between
303 samples among restored sites was significantly higher compared to samples from unimpounded
304 sites (Student's t-test, $p = 0.01$). When the two outlier samples were removed, the effect was still
305 significant ($p = 0.04$).

306 MRPP (multi-response permutation procedure) confirmed a significant restoration effect,
307 and identified even stronger effects based on site and sampling date (Table 4). Interestingly, at
308 the restored sites, there was a directional and synchronous temporal shift in the communities
309 from June to October, with the exception of two samples from October at sites IP1 and IP4, but
310 no temporal shifts were detected among the unimpounded sites (Figure 5). Within each site,
311 however, there were distinct temporal differences.

312 Measures of diversity based on TRFLP data revealed very little difference among sites,
313 and there were no significant restoration effects on any of the diversity indices (Table 5).
314 However, there were differences in the variability of diversity within the sites. Average
315 coefficients of variation were always higher at restored sites compared to unimpounded sites.
316 Although the differences were not significant, the trends were consistent. Additionally,
317 variability was highest at IP3 and IP4, the most recently restored marshes. Chao1 estimates of
318 diversity based on sequence data also showed no significant difference between restored and
319 unimpounded sites (104.8 ± 33.9 and 118.5 ± 69.4 , respectively).

320

321 **Discussion**

322 Analysis of bacterial communities in restored and unimpounded salt marsh sediments by
323 DNA fingerprints and gene sequences revealed that although the communities were, in some
324 cases, very similar, communities at the restored sites were more variable compared to
325 communities at unimpounded sites. We hypothesized that because recovery in the marsh has
326 been underway for nearly 30 years and given the relatively fast generation times of bacteria we
327 would not detect any significant differences in microbial communities between restored and
328 unimpounded marshes. However, several other studies of recovery in coastal ecosystems have
329 reported significant differences in hydrologic and edaphic conditions (Onaindia et al. 2001) or
330 phosphorus cycling (Herbert and Fourqurean 2008) after 2 decades of recovery. Our data, in
331 combination with these studies, suggest that although the marsh may appear to be recovered and
332 comparable to pre-disturbance conditions based on vegetation and macroorganism populations,

333 the less visible components, such as nutrient cycles and microbial communities, may not be as
334 resilient. Allison and Martiny (2008) reported that most microbial groups are sensitive to
335 disturbance and not immediately resilient, regardless of taxonomic breadth or type of
336 disturbance.

337 In support of our initial hypothesis, taxonomic composition of clone libraries suggest
338 little difference between restored and unimpounded sites. Sequence identification of major
339 groups of bacteria indicated diverse bacterial communities, with few differences in the
340 proportion of clones from represented groups among sites. The dominance of
341 *Gammaproteobacteria* and *Bacteroidetes* was not unexpected, as members of these bacterial
342 groups are common in coastal environments (e.g. Bouvier and del Giorgio 2002; Bernhard et al.
343 2005) and are known to play important roles in carbon mineralization and decomposition. In
344 some cases, members of *Gammaproteobacteria* have been found to account for up to 28% of
345 total sediment DNA (Hardwick et al. 2003). Similarly, *Verrucomicrobia* and *Planctomycetes* are
346 also common members of aquatic communities (Fuerst 1995; Glockner et al. 1999; Urbach et al.
347 2001). Many of the known sulfate-reducing bacteria are affiliated with *Deltaproteobacteria*, and
348 thus would be expected to be a part of salt marsh bacterial communities where sulfate is often
349 abundant. *Acidobacteria* are common inhabitants of soil communities, so their presence in
350 coastal sediment communities should not be surprising. Unfortunately, most of what is known
351 about *Acidobacteria* comes from molecular studies, which provide little insight into their
352 metabolic function or ecology. In general, results from our clone libraries are consistent with the
353 TRFLP data and salt marsh ecology, but offer little insight into specific microbial populations
354 that might be critical players in the return to communities that are comparable to unimpounded
355 marsh communities.

356 We were initially surprised by the highly congruent composition of clone libraries from
357 restored and unimpounded sites. However, our clone library results were corroborated by the
358 highly similar diversity indices based on the TRFLP analyses. In fact, at first glance, it would

359 appear that the microbial communities at restored and unimpounded sites are not different until
360 one considers differences in variability.

361 Increased variability at the restored sites may reflect more variable conditions at these
362 sites, or alternatively, the bacterial communities have not reached a stable state after chronic
363 disturbance (impoundment) and subsequent restoration of tidal inundation. Our data do not
364 indicate significant differences in salinity, pH, or water content of the sediment, but it is certainly
365 possible that the sites may differ in other parameters such as nutrient concentrations or redox
366 potential. Porewater nitrate and ammonium concentrations from some sites and sampling dates
367 suggest no significant differences among sites (Bernhard, unpublished), but the available data on
368 nutrients and redox for these sites are limited.

369 Variability at the restored sites may also be related to differences in landscape patterns
370 brought about by impoundment. For example, Swamy et al. (2002) state that although IP1 is in
371 an advanced stage of recovery after 21 years of restored tidal inundation, there are still some
372 attributes, such as marsh elevation, that differ from marshes that have never been impounded.
373 Zedler and Kercher (2005) argue further that restoration can reverse some degradation, but other
374 damages may be irreversible, particularly attributes such as marsh elevation and slope that can
375 then impact other abiotic and biotic components. For instance, changes in the elevation can
376 significantly impact hydroperiods in the marsh sediment that would impact important
377 biogeochemical functions mediated primarily by microbes, including denitrification, sulfate
378 reduction, and methanogenesis. The further the community shifted from pre-impoundment
379 community composition, the longer it may take for the community to stabilize once salt water
380 flow was restored. Frequent disturbances have been found to reduce recovery potential and
381 increase the variability of the system (Odum 1985; Collins et al. 2001).

382 The high variability of bacterial communities at the restored sites in Barn Island may also
383 indicate a more flexible community that can respond to changing conditions and may represent
384 an alternative state, as described by Denslow (1985), in which interactions of species and
385 responses to disturbances are altered from the pre-disturbance condition. If the post-restoration

386 environment is more variable compared to the unimpounded marsh sites, the less stable
387 microbial communities at the restored sites may actually be more resilient (Holling 1973).
388 Fraterrigo and Rusak (2008) suggest that variability, although not typically used as a recovery
389 metric, may actually be a sensitive metric for disturbance. Ayala del Rio et al (2004) also
390 suggest that some microbial communities are dynamic and never reach a climax community, but
391 rather are "shifting mosaics." McCune and Cottam (1985) also reported this phenomenon for
392 forest communities. More dynamic community structures may lead to more functionally stable
393 communities (Fernandez et al. 2000). Our study, however, measured only community
394 composition, and not function.

395 It has been suggested that regular disturbances can increase community stability (Ayala-
396 Del-Rio et al. 2004; Grman et al. 2010). Therefore, it is possible that the regular disturbance
397 caused by tidal inundation helps to maintain the stable communities observed at the
398 unimpounded sites. Because tidal inundation was disrupted for 40-50 years in the impounded
399 sites, community stability may have been significantly disrupted, and may take much longer to
400 return. Although tidal inundation and impoundment may both be considered disturbances, they
401 operate on very different spatial and temporal scales, so their effects may be reflected in very
402 different community dynamics.

403 Given the significant differences in stability of microbial communities between restored
404 and unimpounded sites in our study, one might predict differences in diversity as well. Studies
405 have reported positive links between microbial diversity and stability (Naeem and Li 1997;
406 Grman et al. 2010). Our results, however, suggest that diversity may recover more quickly than
407 stability, leading to an uncoupling of diversity and stability. Finlay et al. (1997) argue that
408 microbial diversity is unimportant because communities harbor many rare or cryptic species that
409 are just waiting for new niches to open as conditions change. However, it is also possible that
410 community composition is more important than diversity alone in driving community stability
411 after perturbation (Griffiths et al. 2004).

412 Many studies of microbial communities have identified salinity as a major factor in
413 driving microbial diversity and community composition in estuaries (Bouvier and del Giorgio
414 2002; Crump et al. 2004; Bernhard et al. 2005; Bernhard et al. 2005), so we expected salinity to
415 be important in the community composition in the restored marshes. However, salinity patterns
416 did not correlate with restoration status, suggesting that other factors exert greater influence on
417 microbial community structure in these marshes. Swamy et al. (2002) identified salinity as one
418 of the key factors driving the biology of restoration in Barn Island, but found that it did not
419 account for all the differences in recovery rates detected in different marshes in Long Island
420 Sound. Multivariate analysis of bacterial communities in our study indicated differences among
421 the sites, but no single environmental variable was identified as a major driver of the observed
422 differences. Additionally, significant effects of site, sampling date, and restoration status on
423 bacterial community composition were detected by MRPP analysis, making it difficult to isolate
424 specific environmental factors that may contribute to these effects.

425 We find it intriguing that the temporal shifts appear to be directional and synchronous
426 among the restored sites while communities at unimpounded sites appear more temporally stable.
427 The causes of the temporal patterns in restored sites and the lack of such patterns in
428 unimpounded sites are not clear. It is possible that the microbial communities are more sensitive
429 than communities in the unimpounded sites to changes in environmental conditions, such as
430 salinity, pH, or temperature. We did find differences in salinity and pH that correlated with
431 sampling dates. Temporal shifts in communities at restored sites may also help to explain
432 increased variability among samples from these sites compared to those from unimpounded sites.
433 However, in another New England salt marsh, distinct temporal patterns were not detected in
434 control sites or fertilized sites (Bowen et al. 2009), suggesting that salt marsh microbial
435 communities may not generally show pronounced temporal shifts.

436 In conclusion, our results indicate that after nearly 30 years of restored tidal flow,
437 microbial community composition in restored sites was not significantly different from
438 undisturbed sites, but there were significant differences in community stability. Because our

439 study focused on surface sediments from one impounded marsh system, it is uncertain how
440 broadly the effects we detected may be distributed in other marshes. Future studies on marshes
441 undergoing restoration should include a microbial community component to determine the extent
442 of restoration impacts. Furthermore, whether more variable microbial communities lead to
443 changes in microbial processes in the marsh, such as nutrient cycling, has yet to be investigated.
444 We believe this is a critical next step.

445

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447

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453

454

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635 Table 1. Average values (\pm standard deviation) of sediment chemistry parameters from Barn
 636 Island and Cottrell salt marsh sites.
 637

Site	Month	Salinity (psu)	pH	% water content
CO1	Jun	nd	nd	nd
	Jul	21.3 \pm 1.8	5.97 \pm 0.01	0.73 \pm 0.03
	Oct	25.0 \pm 0.0	6.84 \pm 0.05	0.52 \pm 0.09
CO2	Jun	nd	nd	nd
	Jul	30.8 \pm 3.4	5.78 \pm 0.18	0.72 \pm 0.04
	Oct	33.2 \pm 0.3	6.43 \pm 0.07	0.51 \pm 0.01
HQ	Jun	nd	5.66 \pm 0.30	0.67 \pm 0.02
	Jul	23.0 \pm 1.4	5.54 \pm 0.42	0.65 \pm 0.03
	Oct	30.0 \pm 0.0	6.28 \pm 0.02	0.57 \pm 0.02
WE	Jun	10.0 ^a	6.37 \pm 0.02	0.54 \pm 0.10
	Jul	16.7 \pm 1.5	5.50 \pm 0.49	0.75 \pm 0.02
	Oct	25.0 \pm 0.0	6.49 \pm 0.01	0.57 \pm 0.04
IP1	Jun	18.0 ^a	6.22 \pm 0.18	0.57 \pm 0.07
	Jul	24.8 \pm 0.4	5.86 \pm 0.08	0.53 \pm 0.01
	Oct	32.0 \pm 0.7	6.51 \pm 0.04	0.53 \pm 0.04
IP2	Jun	13.0 ^a	6.42 \pm 0.11	0.67 \pm 0.08
	Jul	22.7 \pm 0.6	6.16 \pm 0.58	0.68 \pm 0.03
	Oct	30.8 \pm 1.0	6.43 \pm 0.09	0.49 \pm 0.01
IP3	Jun	10.0 ^a	6.44 \pm 0.02	0.67 \pm 0.02
	Jul	20.7 \pm 1.4	6.15 \pm 0.11	0.73 \pm 0.07
	Oct	25.3 \pm 0.7	6.65 \pm 0.01	0.56 \pm 0.05
IP4	Jun	11.0 ^a	6.10 \pm 0.00	0.60 \pm 0.02
	Jul	16.3 \pm 1.5	6.07 \pm 0.02	0.66 \pm 0.03
	Oct	25.0 \pm 1.0	6.47 \pm 0.04	0.39 \pm 0.10

638 ^aSalinity values in June were taken on site from a single reading in situ.

639 Table 2. Results from AMOVA, β -Libshuff, and HOMOVA analyses of Bacterial 16S rRNA
 640 gene clone libraries. All pairwise comparisons for individual sites were conducted, but only
 641 pairs of individual sites that were significantly different are shown.
 642
 643

Statistical Test	Comparison	P value
AMOVA ^a	IP3 vs IP4	0.001
	IP3 vs WE	<0.001
	IP1 vs IP4	<0.001
	unimpounded vs restored ^b	0.279
β - Libshuff ^c	WE-CO1	0.0005 (XY), 0.0036 (YX)
	IP3-WE	0.0002 (XY), 0.0117 (YX)
	unimpounded vs restored	0.7825 (XY), 0.0009 (YX)
HOMOVA	unimpounded vs restored	0.882

644 ^a $P \leq 0.00178$ were considered significant based on the Bonferroni correction for multiple comparisons.

645 ^b Sequences from the four unimpounded sites and the four restored sites were combined.

646 ^c Only when both comparisons (XY and YX) are significant are the two clone libraries considered to be significantly
 647 different in composition. To be considered significant, $p \leq 0.00092$ based on the Bonferroni correction for multiple
 648 comparisons (this is lower than the cutoff for AMOVA or HOMOVA because β -Libshuff makes two comparisons
 649 for each pair of libraries).

650 Table 3. Percentage of sequences in each taxonomic group recovered from each site.
 651

Taxonomic Group	CO1	CO2	HQ	WE	IP1	IP2	IP3	IP4
Acidobacteria	0.06	0.06	0.06	0.08	0.10	0.07	0.09	0.14
Actinobacteria	0.02	0.00	0.04	0.01	0.00	0.00	0.03	0.06
Alphaproteobacteria	0.06	0.00	0.06	0.07	0.03	0.03	0.04	0.08
Bacteroidetes	0.19	0.08	0.16	0.25	0.17	0.19	0.14	0.14
Betaproteobacteria	0.00	0.00	0.00	0.01	0.03	0.01	0.01	0.04
Chloroflexi	0.06	0.13	0.04	0.04	0.12	0.07	0.09	0.02
Cyanobacteria	0.02	0.15	0.01	0.03	0.06	0.12	0.06	0.04
Deinococcus	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00
Deltaproteobacteria	0.15	0.04	0.01	0.17	0.07	0.09	0.08	0.12
Gammaproteobacteria	0.19	0.25	0.26	0.18	0.16	0.23	0.13	0.20
Gemmamondales	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Nitrospira	0.00	0.00	0.01	0.00	0.03	0.01	0.03	0.01
OP11	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Planctomycetes	0.19	0.25	0.19	0.10	0.19	0.12	0.24	0.08
Plastid	0.02	0.00	0.01	0.00	0.01	0.05	0.03	0.00
TM7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Verrucomicrobia	0.04	0.00	0.07	0.03	0.03	0.01	0.05	0.06
Number of clones	48	48	68	71	69	75	79	95

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655 Table 4. Results from multiresponse permutation procedure (MRPP) based on TRFLP
 656 fingerprints. Grouping variables were used to group the samples according to site, sample date
 657 and restoration status (either restored or unimpounded).
 658

Grouping variable	A ^a	T ^b	Observed δ^c	Expected δ	P value ^d
Site	0.030	-5.42	0.571	0.588	<0.0001
Sample date	0.014	-5.25	0.579	0.588	<0.0001
Restoration status	0.004	-2.14	0.585	0.588	0.032

659 ^a A is the intragroup average distance; when all items are identical within groups, A=1
 660 ^b $T = (\delta - m)/s = (\text{observed} - \text{expected})/s$. dev. of expected, where m and s are the mean and
 661 standard deviation of δ under the null hypothesis
 662 ^c Observed δ is the average of the observed intragroup distances weighted by relative group size
 663 ^d P is the probability of a smaller or equal δ
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 668

669 Table 5. Average diversity indices \pm one standard deviation for restored and unimpounded sites.
 670 Average coefficients of variation \pm one standard deviation are shown parenthetically. Indices
 671 were based on TRFLP data and are averaged over all dates and sites.
 672

Diversity index	Restored sites	Unimpounded sites
Richness ^a	63.6 \pm 1.7 (9.2 \pm 2.2)	63.4 \pm 1.1 (6.8 \pm 1.7)
Evenness ^b	0.984 \pm 0.001 (0.24 \pm 0.08)	0.985 \pm 0.001 (0.22 \pm 0.09)
Shannon-Weiner (H)	4.083 \pm 0.03 (2.4 \pm 0.7)	4.085 \pm 0.02 (1.7 \pm 0.4)
Simpson's (D)	0.982 \pm 0.0007 (0.20 \pm 0.08)	0.982 \pm 0.0005 (0.14 \pm 0.04)

673 ^a the number of terminal restriction fragments detected in a sample

674 ^b $H/\ln(\text{richness})$, where $H = -\sum P_i (\ln P_i)$, where P_i = the proportion of each TRF in a sample

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678 **Figure Captions**

679

680

681 **Figure 1.** Map of sampling sites in the Cottrell (a) and Wequetequock-Pawcatuck (b) marshes in

682 Southeastern Connecticut. IP = Impoundments (numbered 1-4); WE = Wequetequock Marsh;

683 HQ = Headquarters Marsh; CO = Cottrell Marsh (numbered 1-2).

684

685 **Figure 2.** Average abundance (\pm standard error) of Bacterial 16S rRNA genes in marsh

686 sediment samples. Site abbreviations are the same as in Figure 1.

687

688 **Figure 3.** Distribution of Bacterial 16S rRNA gene clones from restored and unimpounded

689 marshes. A total of 235 sequences was analyzed from the unimpounded sites and 318 sequences

690 from the restored sites.

691

692 **Figure 4.** Non-metric multidimensional scaling ordination of samples based on TRFLP analysis

693 of the Bacterial 16S rRNA genes amplified from restored and unimpounded marshes. Site

694 abbreviations are the same as in Figure 1. The ordination was rotated to maximize separation

695 based on restoration status. Open symbols represent restored sites, closed symbols represent

696 unimpounded sites.

697

698 **Figure 5.** Non-metric multidimensional scaling ordination of samples from restored (panel a)

699 and unimpounded (panel b) marshes. The ordination has been rotated slightly from that depicted

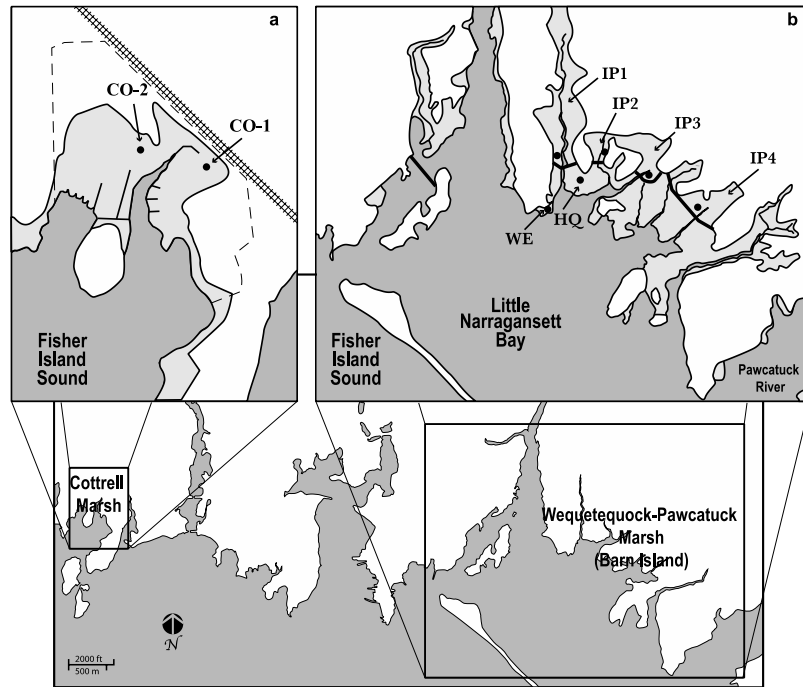
700 in Figure 4 to maximize the separation based on sampling date. Samples collected from each

701 sampling date are circled. Numbers next to the symbols in panel A indicate the Impoundment

702 from which the samples were collected.

703 Figure 1

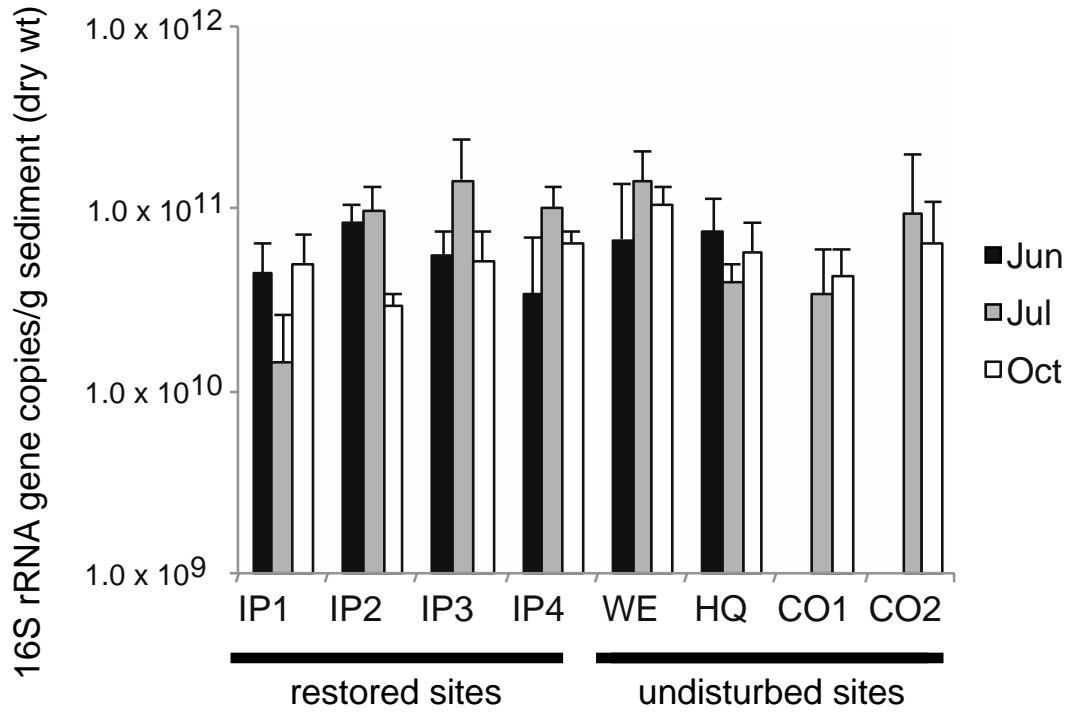
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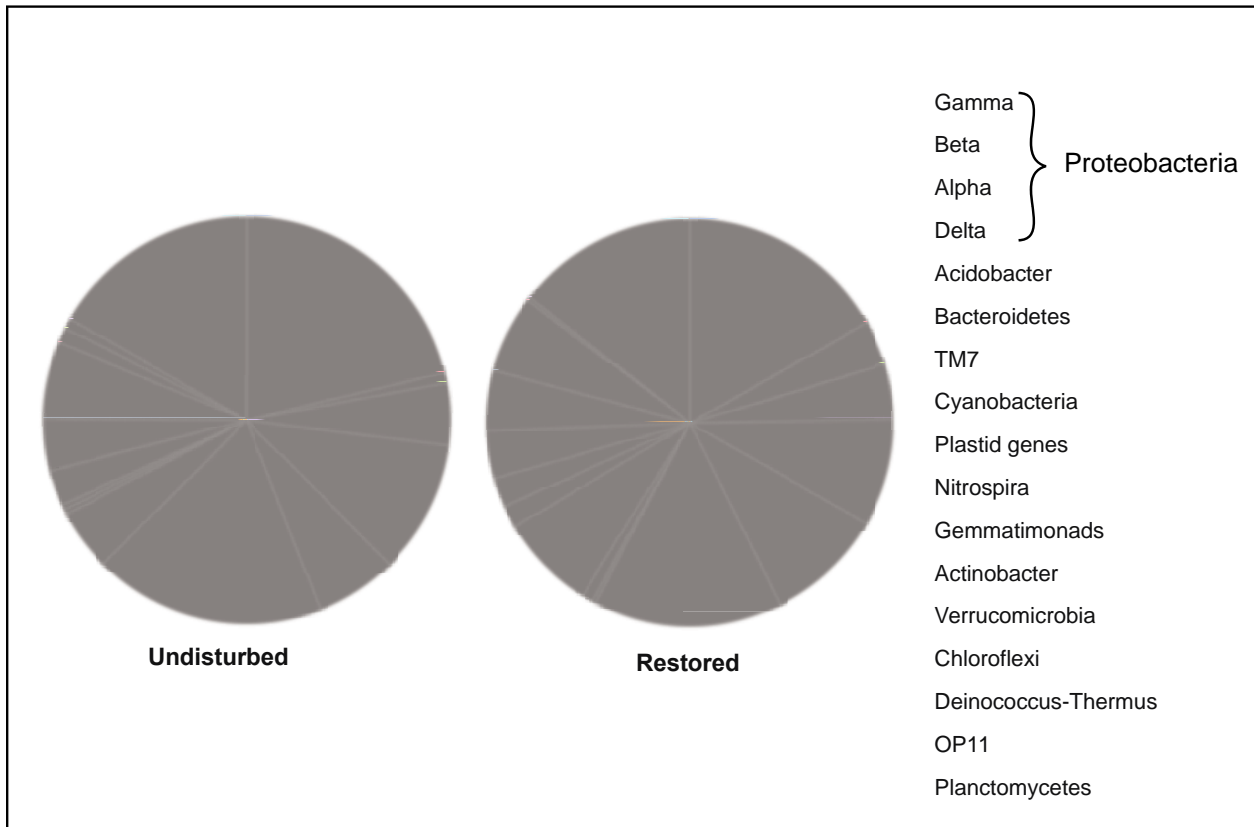
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707 Figure 2
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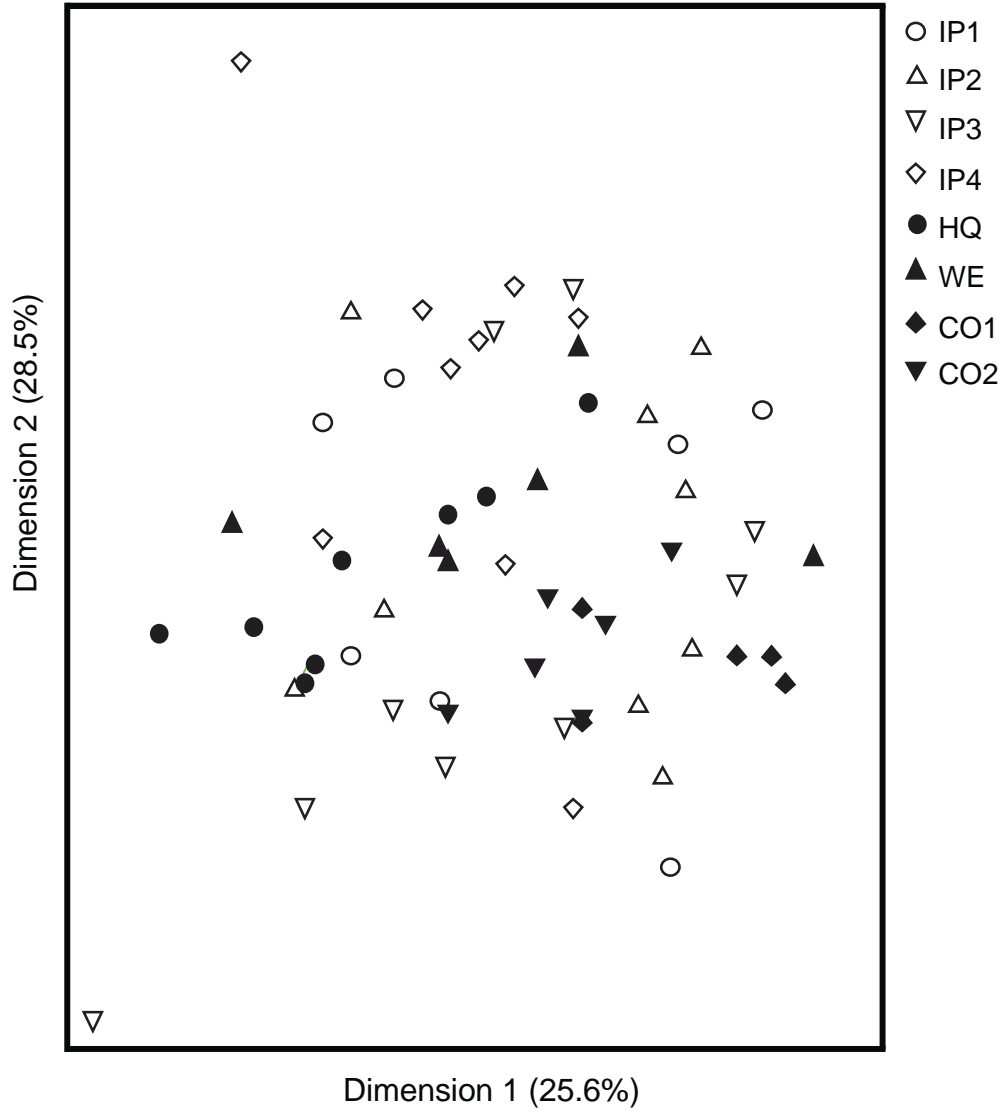
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719 Figure 3
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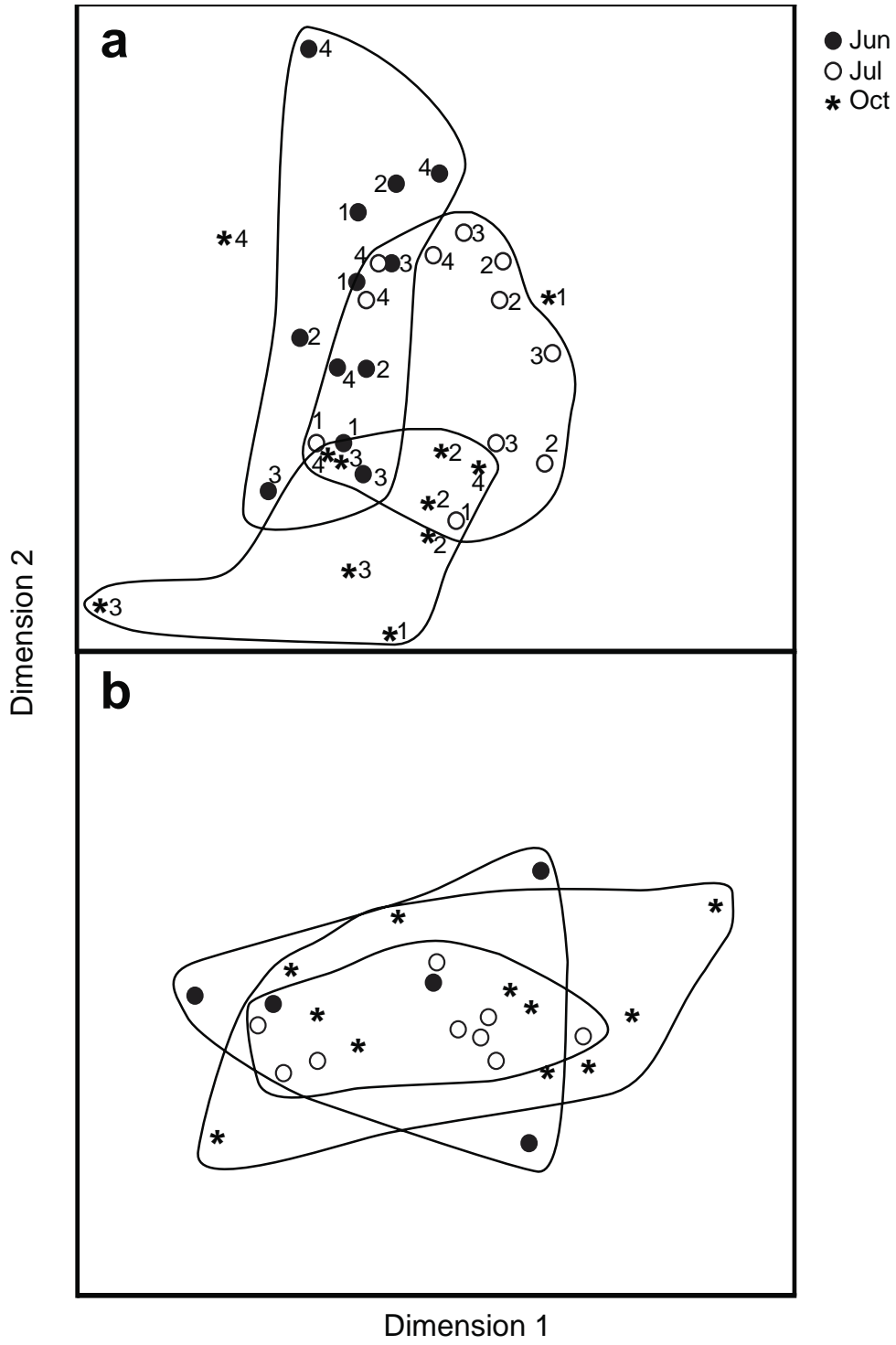
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727 Figure 4
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733 Figure 5
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