

Influence of habitat confluence on aquatic microbial assemblages in experimental mesocosms

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ABSTRACT: Bacterioplankton assemblages play crucial ecosystem roles in diverse aquatic habitats. Areas in which 2 distinct aquatic bodies converge provide insight into how bacterial communities respond to dramatic environmental change and assemblage confluence. The tolerance and success of combined assemblage components to mixed conditions, however, is unclear. To address this, freshwater (FW) and seawater (SW) were combined in ratios of entirely SW, 1:10, 1:1, 10:1 and entirely FW in experimental mesocosms on Appledore Island, Gulf of Maine (Shoals Marine Laboratory), to examine the response of microbial abundance and composition to habitat convergence. The relative proportion of operational taxonomic units (OTUs) that increased in each incubation demonstrated that SW OTUs were more capable of success in FW habitats than FW OTUs in SW habitats. Most OTUs from each source that grew under confluent conditions were initially rare (<1% of the total fingerprint amplified fluorescence) in the incubations. These data demonstrate that rarer OTUs may be more responsive to environmental change than abundant OTUs. These data ultimately indicate that habitat confluence imparts changes in microbial assemblages and that mixing zones within areas of habitat confluence (e.g. estuaries and intertidal rock pools) select for new assemblages composed of OTUs that may be adapted to mixed conditions and that are at low abundance in source populations.

KEY WORDS: Microbial community · Mixing · Dispersal · Marine · Operational taxonomic unit · ARISA · Gulf of Maine

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INTRODUCTION

The study of areas in which saltwater and freshwater systems converge is useful for determining what factors influence the structure of microbial communities and how the communities reshape in response to rapid environmental change. Aquatic microbial assemblages are important for the biogeochemistry and ecology of aquatic ecosystems by processing dissolved and particulate organic matter through the microbial loop (Pomeroy 1974, Azam et al. 1983, Ducklow 1983). Despite the overall importance of bacteria across aquatic habitats, the composition of bacterial assemblages in freshwater and saltwater ecosystems is different (for a review, see Logares et al. 2009). Comparison of 16S rRNA libraries of microbial communities from different

aquatic ecosystems reveals a strong clustering of communities from saltwater locations away from freshwater communities, suggesting that salinity is a major determinant of community composition (Lozupone & Knight 2007). Metagenome sequence libraries reflect similar trends, with freshwater libraries appearing distinct from saltwater libraries (Rusch et al. 2007). Even on the level of individual bacterial groups, such as the alphaproteobacterial SAR11 cluster, these groups recovered from marine, brackish and freshwater systems are separated into distinct clades (Zwart et al. 1998, Kan et al. 2008). In addition, phylogenetic groups are more common in one system compared to the other — such is the case with the *Betaproteobacteria*, which are much more dominant in freshwater systems than marine systems (Methe et al. 1998, Bouvier & del Giorgio 2002). These differences suggest that

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over evolutionary timescales, the transition between saltwater and freshwater requires extensive physiological adaptation, and successful transitions are rare events (Logares et al. 2009).

How bacterial communities change through zones of habitat confluence is a common question in microbial ecology. The transition from salt- to freshwater ecosystems is accompanied by specific factors that may influence changes in community composition. These factors include salinity changes (Oren 2001, Logares et al. 2009), introduction of new predators (Lebaron et al. 2001, Bonilla-Findji et al. 2009), competition within converging communities (Logares et al. 2009) and changes in the availability of inorganic and organic nutrients (Cottrell & Kirchman 2000, Bouvier & del Giorgio 2002, Torsvik et al. 2002). Several studies have observed changes in microbial community structure that mirror the sharp changes in biogeochemistry along estuarine gradients (Bouvier & del Giorgio 2002, Herlemann et al. 2011). For example, the prevalence of *Betaproteobacteria* correlated with DOM concentration, although it was noted that all biogeochemical measurements covaried closely with changes in salinity (Bouvier & del

Giorgio 2002). A later study found that longer residence times in an estuary of the Gulf of Maine in the summer and fall allowed a unique estuarine microbial community to develop (Crump et al. 2004), consistent with observations within the Baltic Sea (Andersson et al. 2010). Several studies have observed distinct microbial communities in the marine and freshwater portions of estuaries (Bouvier & del Giorgio 2002, Crump et al. 2004, Herlemann et al. 2011), where typically freshwater bacteria may be present in saltwater assemblages (Kisand et al. 2005, Riemann et al. 2008).

The aim of this study was to examine the impacts of habitat confluence on microbial assemblage structure over time in experimental mesocosms. We used tannin-rich freshwater pools and adjacent marine waters as endmembers for mesocosms under mixed water conditions.

MATERIALS AND METHODS

Sampling location

Appledore Island (Isles of Shoals, Maine, USA) has ca. 1000 supratidal ponds, including 3 larger freshwater habitats that serve as freshwater sources on the fresh to marine spectrum (J. Simonis unpubl. data; Fig. 1). Seawater and freshwater were collected on Appledore Island, adjacent to the Shoals Marine Laboratory, in June 2009. Seawater was collected in an acid-washed and seawater rinsed carboy (20 l) from Babs Cove, a small inlet that at the site of sampling is ~2.5 m deep at mean low water. The tidal range in the cove is ~3 m. The salinity at the time of sampling was ~30 ppt. Freshwater was collected from Old Reservoir (Fig. 1), a small tannic impoundment on the northern side of the island, which has a maximum depth of ~1 m. Old Reservoir water was collected into an acid-washed and sample-rinsed 20 l cubitainer.

Experimental setup

Unfiltered seawater (SW) and freshwater (FW) was mixed in ratios of 10:1, 1:1 or 1:10 FW:SW, with entirely SW and entirely FW incubations as endmember (i.e. source water) controls. The mesocosms were run in duplicate within 4 l cubitainers that were acid-washed and sample-rinsed before the start of the experiment. The incubations were maintained at seawater temperature (~15°C; which was similar to

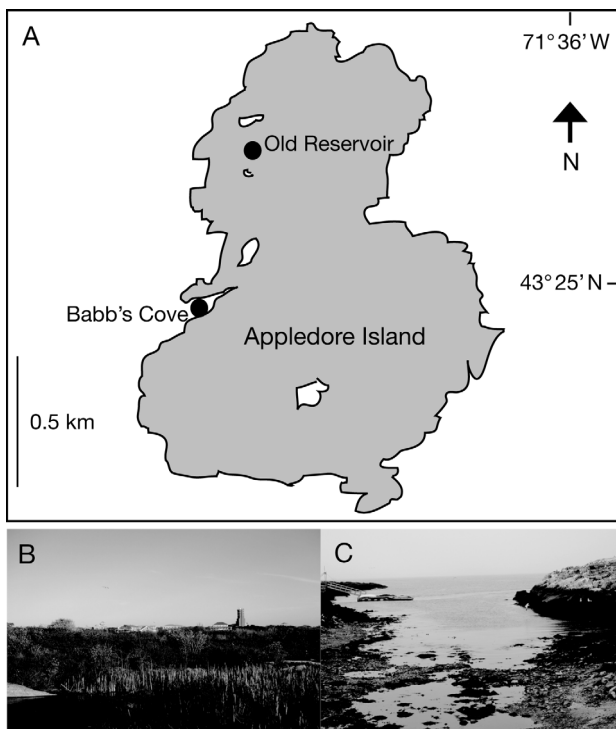


Fig. 1. (A) Map of Appledore Island (42° 59' 17' N, 70° 36' 52' W) showing sampling locations in Old Reservoir (freshwater) and Bab's Cove (seawater) used in preparation of experimental mesocosms; (B) photograph of Old Reservoir and (C) photograph of Bab's Cove

freshwater temperature) in flow-through outdoor aquaria and kept under a neutral-density shade cloth (~25% light attenuation). The incubations were sampled after 0 (T1), 5 (T5) and 7 d (T7) for bacterial composition via automated rRNA intergenic spacer analysis (ARISA), after 0, 1 and 7 d for bacterial and archaeal abundance by epifluorescence microscopy with 4',6-diamidino-2-phenylindole (DAPI) staining and after 0, 1, 5 and 7 d for chlorophyll *a* (chl *a*) concentration via acetone-extracted spectrophotometry. For bacterial composition assessments, 400 ml of incubation water was decanted from incubations at each time point and filtered through 1 μm polycarbonate (Nuclepore) and 0.2 μm Durapore filters (25 mm diameter) using positive pressure filtration driven by a peristaltic pump. The 0.2 μm filter was removed from filter holders and placed into sterile microcentrifuge tubes, which were then immediately frozen on liquid N_2 . For chl *a* analyses, 150 to 500 ml of incubation were filtered through 25 mm GF/F filters (Whatman), which were placed into foil packets and frozen at -20°C until later pigment extraction and quantification. A single sample from each replicate incubation was prepared for chl *a* analysis. Bacterioplankton (*Bacteria* and *Archaea*) abundance samples consisted of 15 ml of incubation samples, which were amended with 2% formalin and kept at 4°C in a refrigerator until processing for microscopic enumeration (within 6 h of collection).

Bacterial enumeration

Bacterioplankton were enumerated by DAPI staining and epifluorescence microscopy (Porter & Feig 1980). Samples (2 ml) were first stained for 2 min with DAPI (1 mg ml^{-1}) and then filtered through 0.22 μm black Isopore (Millipore) polycarbonate filters. The filters were then placed on a glass slide, to which 30 μl immersion oil was added, and covered with a coverslip. Slides were frozen prior to bacterial enumeration at Cornell University. Duplicate slides from each incubation were counted under UV excitation on an Olympus BX-51 epifluorescence microscope. More than 200 cells were counted on each slide in 20 fields.

Chl *a* analysis

Samples for chl *a* analysis were thawed and amended with 10 ml of acetone (90%) in polypropylene centrifuge tubes and allowed to extract at -20°C in the dark overnight. The tubes were cen-

trifuged at $3000 \times g$ for 5 min to pellet filter materials before spectrophotometry. The spectral qualities of extracted pigments in the acetone fraction were measured on a Spectronic 20 (Turner Designs) spectrophotometer at 630, 647, 664 and 750 nm, after which the samples were acidified by addition of 1 drop of 10% HCl and were then re-analyzed at 664 and 750 nm. Chl *a* concentration was calculated according to previously described methods (Parsons et al. 1985).

Automated rRNA intergenic spacer analysis

DNA from filters intended for analysis of bacterial and archaeal assemblages were extracted using a Zymo Soil Mini kit according to the manufacturer's instructions. We chose this extraction kit because it effectively removes PCR inhibitors (tannins were visible in freshwater) that would have interfered with downstream analyses. The composition of bacterioplankton assemblages was assessed using automated rRNA intergenic spacer analysis (ARISA) (Fisher & Triplett 1999), following modifications of Hewson & Fuhrman (2004). Briefly, 10 ng of extracted DNA from each sample was used as a template in the PCR reactions. Each 50 μl reaction contained $1 \times$ PCR Buffer, 2.5 mM MgCl_2 , 1 mM dNTPs (Promega nucleotide mix), 10 pmol each of primers 1392F and 23S-125R-TET (labeled with a 5'-TET fluorochrome) and 0.2 ng μl^{-1} BSA (Sigma 7030). PCR reaction mixes underwent a 5 min heating step at 94°C , followed by 30 cycles of denaturing (94°C for 30 s), annealing (55°C for 30 s) and extension (71°C for 45 s), and following the thermal cycling, a final extension step was conducted at 71°C for 7 min. PCR reactions were purified using Zymo Clean & Concentrator-5 columns, following the manufacturer's recommendations to remove salts and unincorporated nucleotides, and eluted in 10 μl of nuclease-free water. Purified amplified DNA was quantified using PicoGreen fluorescence and diluted to 35 ng μl^{-1} . The products were run on an ABI 377XL capillary sequencer for 4.5 h at 6V, using a custom-made ROX-labeled size standard that contained fragments every 50 base pairs (bp) from 200 to 1200 bp (Bioventures).

Community fingerprint analysis

Outputs from fragment analysis were interrogated using the Peak Scanner 1.0 program (Applied Biosystems). Outputs of relative fluorescence intensity and

size were extracted. ARISA assemblage fingerprints were analyzed according to protocols reported by Hewson & Fuhrman (2006a), following the shifting bin-window technique. Peaks <0.1% of total amplified DNA fluorescence and peaks <400 and >1200 bp were discarded before binning ± 3 bp from 400 to 700 bp, ± 5 bp from 700 to 1000 bp and ± 10 bp from 1000 to 1200 bp (Hewson & Fuhrman 2004). The Whittaker index and Jaccard index were calculated as maxima between 10 bin frames using the XLStat plugin in Microsoft Excel. Agglomerative hierarchical clustering was performed on the matrices of maximum Whittaker and Jaccard indices.

RESULTS AND DISCUSSION

Our data demonstrate that habitat confluence in experimental mesocosms results in changes to the abundance and composition of microbial communi-

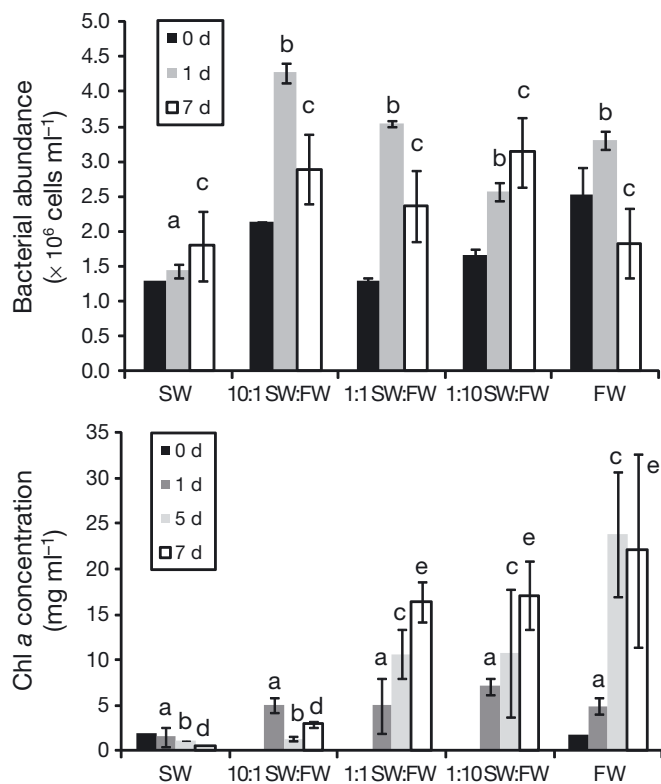


Fig. 2. (a) Bacterial abundance as measured using DAPI staining (Porter & Feig 1980) and (b) chlorophyll a (chl a) as measured using acetone-extracted spectrophotometry in experimental incubations over time. FW: freshwater; SW: seawater. Error bars = SE of replicate incubations ($n = 2$). Letters above bars indicate significant ($p < 0.05$, Student's *t*-test) differences between treatments within each time-point (for timepoints 1, 5, and 7 d)

ties. These impacts, however, are not the same among constitutive assemblage components. Our data provide evidence that when seawater is mixed with a large proportion of freshwater, bacterial OTUs from seawater may be stimulated in relative abundance, while freshwater OTUs did not respond as such when mixed in higher proportion with seawater. Under equal mixing scenarios, more seawater bacterial OTUs were stimulated than freshwater OTUs.

Microbial abundance and biomass

Across incubations, bacterial abundance was greatest initially in freshwaters and least in seawater (Fig. 2). Interestingly, the abundance of bacteria was the same in the 1:1 SW:FW as in the SW-only incubation initially, which may be due to osmotic stress imposed on freshwater bacteria by the addition of salty water. Osmotic stress may cause freshwater cells to burst; hence, they would not appear in microscope counts. The highest bacterial abundance occurred after 1 d in the 10:1 SW:FW incubations. Several incubations experienced sharp increases in bacterial abundance over the first 2 d of the experiment followed by declines, notably the 1:1 SW:FW and the 10:1 SW:FW incubations. After 1 d, all of the incubations containing freshwater had significantly higher abundances than the SW incubation ($p < 0.05$, Student's *t*-test for all pairwise comparisons to SW). Of the incubations containing FW, the 1:10 SW:FW incubation was significantly lower than other incubations after 1 d ($p < 0.05$, Student's *t*-test for all pairwise comparisons to 1:10 SW:FW). After 7 d of incubation, there were no significant differences among the incubations.

Initial chl *a* levels were the same for FW and SW. Mesocosms containing >50% FW had increased phytoplankton biomass over time based upon chl *a* concentration, while chl *a* concentration in mesocosms with <50% FW decreased gradually over time. Significant differences ($p < 0.05$, Student's *t*-test) between chl *a* levels at 5 and 7 d were seen in incubations containing >50% SW.

Bacterioplankton abundance is generally higher in freshwater than in seawater, primarily because of the large concentration of dissolved organic C (DOC) in freshwater (Maranger & Bird 1995). The freshwater used in this experiment was a deep brown color, presumably due to humic materials leached from surrounding soils and vegetation (i.e. allochthonous DOC). It is interesting to note that the large increase

in chl *a* concentration in FW-only incubations did not correspond to elevated bacterial abundance, suggesting that autochthonous DOC inputs are no more labile than allochthonous DOC. However, this may also be due a larger abundance of particle-attached bacteria, which were not included in our analysis because the samples for nucleic acids were pre-filtered to remove particles and larger eukaryotes. The large increases in bacterial abundance in incubations containing $\geq 50\%$ SW suggests that FW-allochthonous DOC is more labile to marine taxa than their freshwater counterparts, consistent with the findings of Stepanauskas et al. (2003). This finding also supports the observations of Rochelle-Newall et al. (2004) and Bonilla-Findji et al. (2009), whose results indicated that parts of terrestrial DOC are labile to marine bacteria.

Comparison of rare and more highly represented OTU dynamics

To examine the OTUs that were stimulated by habitat confluence, OTUs that had ratios of T7:T5 and T5:T1 > 1 were compared to a list of OTUs that were stimulated in either SW- or FW-only treatments. Matching OTUs were discarded from further analyses (i.e. they were defined as stimulated by containment). The mean contribution to ARISA fingerprint fluorescence of increasing OTUs was 1.46 ± 0.89 ($n = 7$) in the 10:1 SW:FW incubations, 0.10 ± 0.02 ($n = 13$) in the 1:1 SW:FW incubations and 0.34 ± 0.14 ($n = 17$) in the 1:10 SW:FW incubations. Of all stimulated OTUs, only one was shared between treatments; however, we acknowledge that others may have been present below the ARISA detection threshold of 0.1% in the other incubations. Only 3 OTUs contributing $> 1\%$ of the total amplified DNA to T1 fingerprints were among the 36 OTUs across all incubations that increased over the course of the experiment in response to treatment.

Our data demonstrate that most bacterial OTUs that are capable of responding rapidly to habitat confluence are rarer taxa. Bacterial communities across a range of habitats are typically dominated by few dominant taxa, with a long tail of rarer taxa in species distribution curves (Giovannoni & Stingl 2005). Abundant taxa in seawater are believed to be mostly *k*-selected organisms (Morris et al. 2002), while rarer components of assemblages are believed to be *r*-selected and capable of rapid response to prevailing conditions (Lamy et al. 2009). Hence, our data support that rarer organisms within communities are

capable of exploiting the new conditions when habitats are confluent. In addition, the emergence of rare taxa over time within these incubations supports previous findings that a unique brackish community exists when marine and freshwater meet over longer residence times (Crump et al. 2004).

Of the 34 OTUs that increased in relative proportion under confluence, 25 were more highly represented in initial SW fingerprints, while only 9 were more highly represented in FW fingerprints. Notably, of the OTUs that increased in the 1:10 SW:FW treatment, 12 were more highly represented in initial SW fingerprints, and only 5 were more highly represented in FW fingerprints. All of the SW OTUs that increased in the 1:10 SW:FW treatment composed $< 0.5\%$ of the initial fingerprints for that treatment. These data indicate that rare seawater OTUs may be more capable of utilizing the allochthonous DOC introduced into SW than freshwater OTUs in mostly SW habitats. Alternatively, mixing may stimulate OTUs that are adapted to brackish conditions that are present at low levels in the SW inoculum. Because seawater taxa may experience a wide range of salinity in coastal zones, there may be a wider suite of taxa adapted to mixed habitat conditions than freshwater bacteria, which do not experience variable salinity under ambient conditions (Logares et al. 2009).

Habitat confluence

Our data provide evidence that microbial assemblages are responsive to confluence and that assemblages receiving a greater proportion of inoculum from one habitat with respect to another may converge in the assemblage structure of that habitat. Assemblage ARISA fingerprints clustered together similarly using both the Whittaker and Jaccard indices ($p < 0.001$, Mantel test, $r = 0.58$), while the mean similarity between all fingerprints was lower based on the Jaccard index than the Whittaker index (data not shown). Clustering using non-metric multi-dimensional scaling revealed that the fingerprints grouped based on SW and FW composition (Fig. 3). The least change (indicated by the length of arrows on Fig. 3) between sampling times occurred in the 100% SW incubations, and the greatest variation between sampling times occurred in the incubations containing $\geq 50\%$ FW. Incubations containing 50% FW and 50% SW initially were more similar to incubations containing $> 50\%$ SW, but after 5 d were more similar to $> 50\%$ FW incubations. The clustering of predominately FW assemblages away from

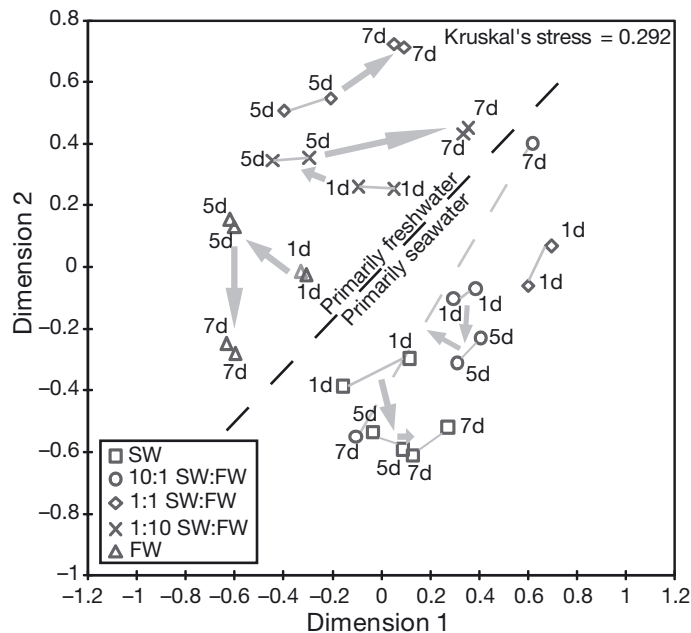


Fig. 3. Similarity of automated rRNA intergenic spacer analysis fingerprints based on the Whittaker index. Fingerprints were clustered by non-metric multidimensional scaling using an absolute model and 5 repetitions. Treatments are coded by symbols. FW: freshwater; SW: seawater. Replicate fingerprints within each treatment and timepoint (1 to 7 d of incubation) are connected by lines. Arrows indicate trajectory between timepoints. The 2 replicates for T7 (i.e. 7 d) 10:1 SW:FW are connected by a broken line for clarity. The trajectory is not indicated for the 1:1 SW:FW incubations between 1 and 5 d. The length of arrows corresponds to the magnitude of change in the composition of the bacterial communities

predominately SW assemblages by the Whittaker index, which considers the relative proportion of amplified DNA associated with each OTU, suggests that dominant bacteria drive much of the similarity based on the endmember inocula.

A key question in microbial ecology is whether similar habitats give rise to similar assemblages of microorganisms (Baas-Becking 1934, Fuhrman 2002). Previous studies have indicated habitat-distinct profiles of bacterial assemblages in particle-attached vs. free-living, depth and estuarine zone comparisons (Moeseneder et al. 2001, Stepanauskas et al. 2003, Hewson & Fuhrman 2004, 2006b, Hewson et al. 2006a). A spatially extensive survey of bacterioplankton composition in the open ocean using distance-decay of similarity approaches found heterogeneity in compositions on spatial scales of 1 to 50 km, which was consistent with physicochemical feature variation (Hewson et al. 2006b), a result confirmed by metagenomic analyses (Rusch et al. 2007). Similarly, assemblages of sediment bacteria were

consistent among habitats sharing gross geological features yet were distinct among habitats (Hewson et al. 2007).

The data from this experiment indicate that observations of mixing zones of assemblages between habitats in rivers, river plumes and estuaries may be temporally dynamic. We found that the majority of taxa that respond favorably to confluence of habitats are initially rare. This demonstrates that potentially rare and weedy taxa are those that may initially respond to changed conditions because they are capable of exploiting resources that are limiting under endmember conditions. Thus, rarer taxa also contribute to much of the variability observed between replicate treatments, which in nature may cause spatial heterogeneity between assemblage compositions. Furthermore, salinity appears to play a key role as a determinant of success under mixed conditions; rarer marine taxa are more capable of success in freshwater habitats than freshwater taxa in marine habitats.

Potential biases of fingerprinting approaches

Our conclusions rely on the microbial assemblage fingerprinting approach ARISA, which characterizes OTUs based on 16S-23S rRNA intergenic spacer length (ITS) heterogeneity. While ITS length is mostly consistent with phylogeny on the genera scale (Brown et al. 2005), there may be overlap among the ITS lengths from disparate taxonomic groups. *Bacteria* may also contain more than a single copy of the rRNA ITS region, which may be of different lengths (Klappenbach et al. 2000). This is particularly the case with fast-growing, less nutrient-limited bacterioplankton taxa; in our experiment, some of the richness observed in freshwater bacterioplankton may have resulted from the presence of taxa with multiple rRNA copies. Because we standardized the template DNA amount and the amount of DNA run per fingerprint, our results do not reflect unequal coverage of bacterial communities. We pre-filtered our samples to eliminate plastids; however, it is possible that microeukaryotes may have made it through our pre-filter and appear as OTUs within fingerprints. *Archaea* are not included in our analyses nor are bacterial taxa with non-syntenuous 16S-23S rRNA, like some *Planctomyces* and *Chloroflexi*. However, these potential biases do not affect our conclusions of the dominance of assemblage components responsive to habitat confluence and greater consistency among faster-growing assemblages.

CONCLUSIONS

The results of this study demonstrate the dynamic nature of microbial assemblages in response to habitat variation. We found that rarer OTUs are more responsive to habitat confluence than dominant components of salt- and freshwater communities, and consequently, over longer timescales, habitat confluence selects for assemblages that are dominated by OTUs that are initially rare. Rare components of marine assemblages, which may be limited by prevailing conditions or represent brackish-adapted taxa at low concentrations of SW inoculum, may respond rapidly to new resource pools and be more tolerant to variable osmotic conditions than freshwater bacteria, despite presumably stronger genome streamlining in marine than freshwater habitats due to greater resource limitation. These data raise interesting questions about the fate of bacteria that are stimulated by confluent conditions within the food web, for example, their consumption by bacterivores and interactions with viral communities of the destination water mass.

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