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Drivers of bacterial β-diversity depend on spatial scale

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The factors driving β-diversity (variation in community composition) yield insights into the maintenance of biodiversity on the planet. Here we tested whether the mechanisms that underlie bacterial β-diversity vary over centimeters to continental spatial scales by comparing the composition of ammonia-oxidizing bacteria communities in salt marsh sediments. As observed in studies of macroorganisms, the drivers of salt marsh bacterial β-diversity depend on spatial scale. In contrast to macroorganism studies, however, we found no evidence of evolutionary diversification of ammonia-oxidizing bacteria taxa at the continental scale, despite an overall relationship between geographic distance and community similarity. Our data are consistent with the idea that dispersal limitation at local scales can contribute to β-diversity, even though the 16S rRNA genes of the relatively common taxa are globally distributed. These results highlight the importance of considering multiple spatial scales for understanding microbial biogeography.

microbial composition | distance-decay | Nitrosomonadales | ecological drift

Biodiversity supports the ecosystem processes upon which society depends (1). Understanding the mechanisms that generate and maintain biodiversity is thus key to predicting ecosystem responses to future environmental changes. The decrease in community similarity with geographic distance is a universal biogeographic pattern observed in communities from all domains of life (as in refs. 2–4). Pinpointing the underlying causes of this "distance-decay" pattern continues to be an area of intense research (5–9), as such studies of β-diversity (variation in community composition) yield insights into the maintenance of biodiversity. These studies are still relatively rare for microorganisms, however, and thus our understanding of the mechanisms underlying microbial diversity—most of the tree of life—remains limited.

β-Diversity, and therefore distance-decay patterns, could be driven solely by differences in environmental conditions across space, a hypothesis summed up by microbiologists as, "everything is everywhere—the environmental selects" (10). Under this model, a distance-decay curve is observed because environmental variables tend to be spatially autocorrelated, and organisms with differing niche preferences are selected from the available pool of taxa as the environment changes with distance.

Dispersal limitation can also give rise to β-diversity, as it permits historical contingencies to influence present-day biogeographic patterns. For example, neutral niche models, in which an organism's abundance is not influenced by its environmental preferences, predict a distance-decay curve (8, 11). On relatively short time scales, stochastic births and deaths contribute to a heterogeneous distribution of taxa (ecological drift). On longer time scales, stochastic genetic processes allow for taxon diversification across the landscape (evolutionary drift). If dispersal is limiting, then current environmental or biotic conditions will not fully explain the distance-decay curve, and thus geographic distance will be correlated with community similarity even after controlling for other factors (2).

For macroorganisms, the relative contribution of environmental factors or dispersal limitation to β-diversity depends on spatial scale (12). Fifty-years ago, Preston (13) noted that the turnover rate (rate of change) of bird species composition across space within a continent is lower than that across continents. He attributed the high turnover rate across continents to evolutionary diversification (i.e., speciation) between faunas as a result of dispersal limitation and the lower turnover rates of bird species within continents as a result of environmental variation.

Here we investigate whether the mechanisms underlying β-diversity in bacteria also vary by spatial scale. We chose to focus on the ammonia-oxidizing bacteria (AOB), which along with the ammonia-oxidizing archaea (14), perform the rate-limiting step of nitrification and thus play a key role in nitrogen dynamics. We compared AOB community composition in 106 sediment samples from 12 salt marshes on three continents. A partially nested sampling design achieved a relatively balanced distribution of pairwise distance classes over nine orders of magnitude, from 3 cm to 12,500 km (Fig. 1 and Table S1). We limited our sampling to a monophyletic group of bacteria, the AOB within the β-Proteobacteria, and one habitat, salt marshes primarily dominated by cordgrass (*Spartina* spp.). This approach constrained the pool of total diversity (richness) and kept the environmental and plant variation relatively constant, increasing our ability to identify if dispersal limitation influences AOB composition.

We then asked two questions: (*i*) Does bacterial β-diversity—specifically, the slope of the distance-decay curve—vary over local (within marsh), regional (across marshes within a coast), and continental scales? (*ii*) Do the underlying factors (environmental variation or dispersal limitation) explaining this diversity vary by spatial scale? Because most bacteria are small, abundant, and hardy, we predicted that dispersal limitation would occur primarily across continents, resulting in genetically divergent microbial "provinces" (15). At the same time, we predicted that environmental factors would contribute equally to distance-decay at all scales, resulting in the steepest slope at the continental scale as reported in plant and animal communities (12, 13, 16).

Results and Discussion

We characterized AOB community composition by cloning and Sanger sequencing of 16S rRNA gene regions targeted by two primer sets. Here we focus on the results from a subset of those sequences from the order Nitrosomonadales, generated using primers specific for AOB within the β-Proteobacteria class (17). The second primer set (18) generated longer sequences from

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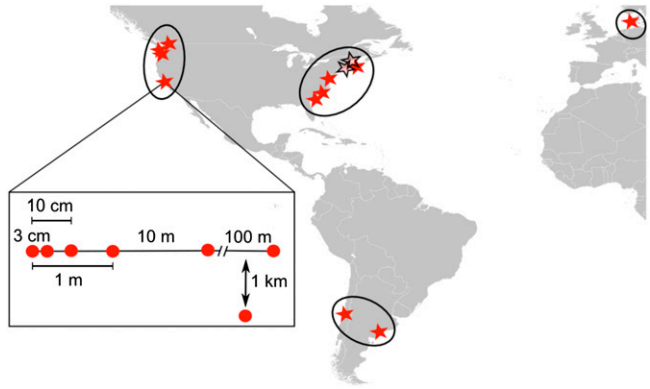

Fig. 1. The 13 marshes sampled (see Table S1 for details). Marshes compared with one another within regions are circled. (*Inset*) The arrangement of sampling points within marshes. Six points were sampled along a 100-m transect, and a seventh point was sampled ~1 km away. Two marshes in the Northeast United States (outlined stars) were sampled more intensively, along four 100-m transects in a grid pattern.

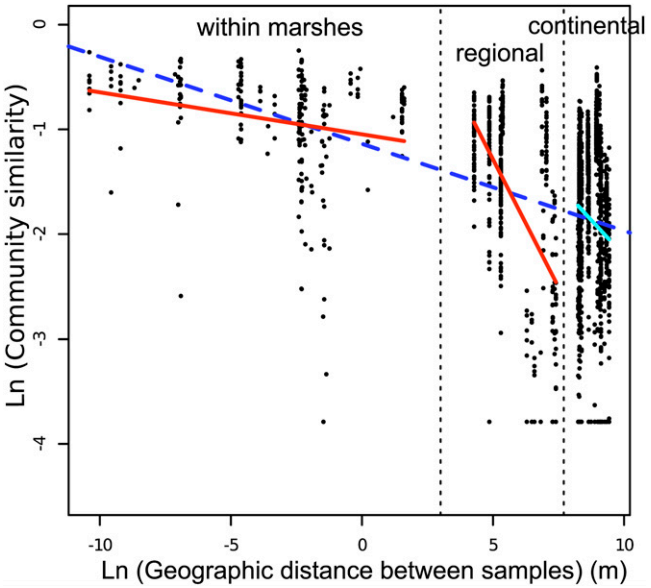

Fig. 2. Distance-decay curves for the Nitrosomadales communities. The dashed, blue line denotes the least-squares linear regression across all spatial scales. The solid lines denote separate regressions within each of the three spatial scales: within marshes, regional (across marshes within regions circled in Fig. 1), and continental (across regions). The slopes of all lines (except the solid light blue line) are significantly less than zero. The slopes of the solid red lines are significantly different from the slope of the all scale (blue dashed) line.

a broader range of Proteobacteria, but yielded similar results (Fig. S1 and Tables S2 and S3).

Across all samples, we identified 4,931 quality Nitrosomadales sequences, which grouped into 176 OTUs (operational taxonomic units) using an arbitrary 99% sequence similarity cutoff. This cutoff retained a high amount of sequence diversity, but minimized the chance of including diversity because of sequencing or PCR errors. Most (95%) of the sequences appear closely related either to the marine *Nitrosospira*-like clade, known to be abundant in estuarine sediments (e.g., ref. 19) or to marine bacterium C-17, classified as *Nitrosomonas* (20) (Fig. S2). Pairwise community similarity between the samples was calculated based on the presence or absence of each OTU using a rarefied Sørensen's index (4). Community similarity using this incidence index was highly correlated with the abundance-based Sørensen index (Mantel test: $\rho = 0.9239$; $P = 0.0001$) (21).

A plot of community similarity versus geographic distance for each pairwise set of samples revealed that the Nitrosomonadales display a significant, negative distance-decay curve (slope = −0.08, $P < 0.0001$) (Fig. 2). Furthermore, the slope of this curve varied significantly among the three spatial scales. The distance-decay slope within marshes was significantly shallower than the overall slope (slope = −0.04; $P < 0.0334$) and steeper across marshes within a region than the overall slope (slope = −0.27, $P < 0.0007$) (Fig. 2). In contrast, at the continental scale, the distance-decay curve did not differ from zero ($P = 0.0953$). Thus, there is no evidence that sampling across continents contributed Nitromonadales OTU diversity in addition to what was already observed at the marsh and regional scales. Furthermore, additional analyses suggest that these results are not driven by a few outlier samples (Fig. S3).

Over all spatial scales, both the environment and dispersal limitation appear to influence Nitrosomonadales β-diversity. Ranked partial Mantel tests revealed that the similarity in Nitrosomonadales community composition between samples was highly correlated with environmental distance ($\rho = -0.5339$; $P = 0.0001$) and geographic distance ($\rho = -0.2803$; $P = 0.0001$), but not plant community similarity ($P = 0.72$) (Table S2).

To further identify the relative importance of factors contributing to these correlations, we used a multiple regression on matrices (MRM). The partial regression coefficients of an MRM model give a measure of the rate of change in community similarity per standardized unit of similarity for the variable of interest; all other explanatory variables are held constant (22). Over all scales, the MRM model explained a large and significant proportion ($R^2 = 46\%$; $P < 0.0001$) of the variability in Nitrosomonadales community similarity. Geographic distance contributed the largest partial regression coefficient ($b = 0.40$, $P < 0.0001$), with sediment moisture, nitrate concentration, plant cover, salinity, and air and water temperature contributing to smaller, but significant, partial regression coefficients ($b = 0.09$–0.17, $P < 0.05$) (Table 1). Because salt marsh bacteria may be dispersing through ocean currents, we also used a global ocean circulation model (23), as applied previously (24), to estimate relative dispersal times of hypothetical microbial cells between each sampling location. Dispersal times between sampling points did not explain more variability in bacterial community similarity (ln dispersal time: $b = 0.06$, $P = -0.0799$; with dispersal $R^2 = 0.47$ vs. without 0.46). Therefore, in the remaining analyses we use geographic distance rather than dispersal time.

As hypothesized, the relative importance of environmental factors versus geographic distance to Nitrosomadales community similarity differed across the three spatial scales. Contrary to our expectations, however, geographic distance had a strong effect on community similarity within salt marshes (partial regression coefficient $b = 0.47$) but no effect at larger scales (Table 1). Furthermore, the relative importance of different environmental variables varied by scale. Sediment moisture, which is likely related to unmeasured variables, such as oxygen availability, was the most important variable explaining community similarity within marshes ($b = 0.63$). In contrast, water temperature ($b = 0.45$) and nitrate concentrations ($b = 0.17$) were more important at the regional and continental scales, respectively.

The varying importance of the environmental parameters at different spatial scales likely reflects differences in their underlying variability at these scales. For example, the MRM model did exceptionally well in explaining variation in Nitrosomadales community similarity at the regional scale ($R^2 = 0.61$) (Table 1). Notably, this spatial scale captures a latitudinal gradient on the east and west coasts of North America, which results in high variability in water temperature. Previous studies in the field and laboratory support the idea that AOB composition is particularly sensitive to temperature (e.g., refs. 25 and 26). Within marshes,

Table 1. Results of the multiple regression on matrices analysis by spatial scale

	All scales R^2 = 0.46 *b*	Within marsh R^2 = 0.27 *b*	Regional R^2 = 0.61 *b*	Continental R^2 = 0.17 *b*
Ln (geographic distance)	0.40***	0.47***		
Ln (plant similarity + 0.005)				
Ln (100 – % sediment moisture)	0.17**	0.63**	0.19*	0.14
Ln (ammonium +1)				
Ln (nitrate +1)	0.10			0.17*
Ln (phosphate +1)			0.11	
pH				
% plant cover	0.13*	0.34***		n.s.
Salinity	0.10		0.16	
Water temperature	0.09	na	0.45***	
Air temperature	0.14**	na	0.24*	0.16*

The variation (R^2) of ln community distance (distance = 1 – similarity) that is explained by the remaining variables and the partial regression coefficients (*b*) of the final model is reported (all values are significant at $P \leq 0.0001$, $n = 62$). If a partial regression coefficient is reported, its significance level (one-way tests) is < 0.0500. *$P \leq 0.0100$, **$P \leq 0.0010$, and ***$P \leq 0.0001$. Water and air temperature were measured for each marsh, and therefore are not included in the within-marsh models.

we found that the percentage of plant cover surrounding each sampling point was important (Table 1), and we hypothesize that this metric could represent differences in sediment surface temperatures because of plant shading.

Why is geographic distance related to Nitrosomonadales community similarity only within marshes? We can think of two nonexclusive explanations for this pattern. First, we may have missed a spatially autocorrelated abiotic or biotic factor that strongly influences AOB composition (e.g., other microbial groups that interact with AOB). Indeed, the parameters that we measured do not explain all of the variability in bacterial community similarity (the unexplained variance is 73% within marshes). To explain our results, the missing parameter would have to be spatially autocorrelated within marshes, but not across marshes, as geographic distance is not related to composition at the larger scales. Although salt marshes are known for steep environmental gradients across relatively small spatial scales (27), we tried to reduce the environmental variation between marshes by selecting for similar plant communities. Thus, it is possible, and perhaps likely, that an unmeasured variable contributes in part to a local distance effect, though not being spatially autocorrelated across marshes.

Second, dispersal limitation may allow for ecological drift of bacterial composition within a marsh. Stochastic births and deaths, along with restricted movement of cells through marsh sediments on ecological time scales, would create patchiness in community composition simply because of parent and offspring proximity (28). This spatial aggregation itself leads to a negative relationship between community similarity and geographic distance (29). In addition, established biofilm assemblages on marsh particles may inhibit subsequent colonization by cells in the porewater, and these priority effects could further strengthen these patterns (30). In larger organisms, ecological drift also appears to contribute to distance-decay patterns (8). For example, Tuomisto et al. (7) found that geographic distance, above and beyond environmental effects, led to steep distance-decay curves for tropical flora at distances less than 80 km. Thus, it seems reasonable that at least some of the distance effect at the local marsh scale may be because of ecological drift.

At the same time, we found no evidence that dispersal limitation affects the β-diversity of Nitrosomadales across regions or continents; geographic distance was not related to community similarity at these spatial scales. The triangular spread of the distance-decay relationship further supports this conclusion. Although the average similarity between two samples tends to decrease with larger distances, some samples 3,000 km apart were as similar as those 70 km apart or from the same marsh. Thus, the pool of AOB taxa, defined at 99% 16S rDNA sequence similarity, appears to be globally distributed.

Although our study is not the first to report a relationship between geographic distance and microbial community similarity (e.g., refs. 6, 31, 32), it demonstrates that an overall relationship between geographic distance and community similarity does not necessarily indicate evolutionary divergent provinces. Rather, it may be driven by ecological drift at local spatial scales. Indeed, a number of studies focusing on smaller spatial scales have found an effect of spatial distance on microbial community composition, where significant evolutionary drift among taxa is unlikely (e.g., refs. 33–35).

Deeper sampling of AOB communities could yet reveal evolutionary drift of 16S rRNA genes. A limitation of the study is that it necessarily focuses on abundant taxa in the targeted groups. Although overall distance-decay patterns are likely robust to this sampling bias (29), it is possible that the factors underlying the β-diversity of rare AOB taxa differ from those of more abundant taxa. For example, rarer taxa may be more susceptible to dispersal limitation, because the number of chances for a propagule to travel a long distance and establish a new population is reduced (15).

In conclusion, salt marsh bacteria may be dispersal limited, even though the 16S rRNA genes of the relatively common taxa may be everywhere. Our results are consistent with the idea that dispersal limitation leads to ecological drift and may be one mechanism that, in concert with measured and unmeasured environmental factors, drives Nitrosomonadales β-diversity at the scale of an individual marsh. In contrast, only environmental factors (and in the case of the broader Proteobacteria, plant composition) (Table S3) appear to determine differences between communities across regional and continental scales. These results do not eliminate the possibility that endemism occurs in the AOB. To the contrary, evidence that these bacteria are dispersal limited at all indicates that evolutionary drift will likely be observed in more rapidly evolving genomic markers; diversification should occur at spatial scales where the dispersal rate among sites is lower than the mutation rate of the genomic marker. As has been pointed out for larger organisms (36, 37), both spatial and temporal scales—and their intertwined nature—are key to investigating the distribution of microbial biodiversity.

Materials and Methods

Bacterial Community Composition. We sampled salt marsh sediments in the summer of 2004 (16 June to 1 September in the northern hemisphere and 11 to 14 December in the southern hemisphere) (Fig. 1 and Table S1). Within

each marsh, we sampled seven points along a transect, with the points ranging from 1 cm to 1 km apart (Fig. 1, *Inset*). Two marshes in the northeast United States were sampled more intensively, along four ~100-m transects in a grid pattern. At each sampling point, a 1-cm diameter sediment core was collected to a depth of 1 cm. The cores were stored on ice in the field, frozen as soon as possible, and stored at −80 °C within 3 d. Community DNA was extracted from the top 0.5 g of the sediment core using the FastDNA spin kit for soils (Qbiogene, Inc.) following the manufacturer's instructions.

We amplified the 16S rRNA gene using two primer sets. The BetaAmo primers were designed to target AOB in the β-Proteobacteria division (18) but are known to amplify many nontarget Proteobacterial sequences in salt marsh sediments (4). The CTO primers were designed to be more specific for β-AOBs but amplify a smaller region (of ~465 bp) of the 16S rRNA gene and require a nested-PCR following amplification with the BetaAmo primers (17). Here we focus on the results from a subset of these CTO sequences. The BetaAmo primers also yielded 5,340 quality sequences (of ~1,170 bp) from a broader range of Proteobacteria but yielded similar results (Fig. S1 and Tables S2 and S3).

Each PCR was performed in triplicate on a 1:10 dilution of the genomic DNA. For the nested PCR, each triplicate from the first PCR was amplified separately for the second round, then combined before cloning. The primer sequences and PCR conditions are reported in Table S4. Pooled amplicons were cloned into *Escherichia coli* using the TOPO-TA cloning kit for sequencing (Invitrogen). Four microliters of PCR product, 1 μL of vector, and 1 μL of salt were combined, and the manufacturer's protocol followed for the remaining steps. For each sample, ~96 clones were sequenced in both directions by Sanger sequencing at the TIGR facility.

The sequences were trimmed, concatenated, and screened for the target length using the Sequencher software. We first aligned the sequences in batches using the GreenGenes webserver (http://greengenes.lbl.gov). We imported these automated alignments into the ARB software (38), inserted these sequences into the Silva Small Subunit rDNA database (39) using the parsimony algorithm, and further curated the alignments manually. The sequences are available from GenBank (HQ271472-HQ276885 and HQ276886-HQ283075), and sequence alignments are available from the authors.

We used numerous procedures to remove chimeric sequences from our data. First, the NAST alignment algorithm used by GreenGenes truncates sequences that cannot be aligned well to any of its curated template sequences. Next, we passed the GreenGenes aligned sequence batches through three detection programs: Bellerophon (40), available through the GreenGenes server, Mallard (41), and Chimera_Check v2.7 (42). We removed any sequence that was indicated by at least two of these three algorithms as a chimera.

To generate an OTU by sample matrix, we created a sequence distance matrix using dnadist in the Phylip software package (43). We then assigned each sequence to an OTU (defined as all sequences ≥ 99% similar) using the cluster function (furthest-neighbor algorithm) in mothur (44).

To create a matrix of pairwise bacterial community similarity by sample, we calculated a rarified similarity matrix (4). This approach controls for unequal sampling (number of sequences) between samples, which influences the number of shared OTUs observed. We calculated average similarity values [Sørensen's incidence- and abundance-based indices (21)] between all pairwise samples using a rarefied subset of drawn sequences (38 sequences for the Nitrosomonadales and 39 for the Proteobacteria) over 10,000 randomizations. Dataset S1 provides the community similarity matrix for the Nitrosomonadales.

Environmental Variables and Plant Community Composition. After the sediment core was taken, plant composition and percent cover were assessed within a 10 cm × 10 cm quadrat centered on the sampling point. Total percentage of plant cover and the fraction of the plant species present in the quadrat was estimated by eye. A similarity matrix for plant genera was calculated using the Bray-Curtis index (45). We measured salinity of the surface porewater with a handheld refractometer by extracting a few drops of porewater with a syringe. Surface pH was measured with a handheld meter pressed slightly (<1 mm deep) into the sediment.

To assess sediment nutrient availability, a 2.6-cm diameter sediment core was collected to a depth of ~2 cm immediately adjacent to the DNA sediment core using a 60-mL syringe with the tip cut off. The cores were stored on ice in the field and frozen in the laboratory until analysis. After defrosting, the sample was sifted through a 1-mm mesh to remove root material. One gram of wet sediment was extracted in a solution of 2 M KCl and 0.5 M NaH_2CO_3. Ammonium, nitrate, phosphate, and sulfate concentrations in the extracts were measured with Hach colorimetric kits, and nutrient concentrations were standardized by the moisture content of the sample. To normalize the values for statistical analyses, we applied a ln transformation to sediment moisture content and a ln (x + 1) transformation to the nutrient concentrations.

Geographic and Ocean Dispersal Distance. To create a geographic distance matrix between each sampling point, we recorded the location and compass direction of each sampling transect with a handheld GPS unit. We used the GPS points, bearing angle, and sample spacing along each transect to calculate the geographic distance between samples within each marsh. We calculated distances between marshes using latitudinal and longitudinal coordinates and the Haversine formula. Dataset S2 provides the geographic distance matrix for the Nitrosomonadales.

To estimate the dispersal time of a hypothetical microbial cell between each sampling location, we used a global ocean circulation model (23) as applied previously in Martiny et al. (24). The model has an approximate horizontal resolution of 3.75° × 3.75° and 29 depth layers. For each marsh, we chose a starting point inside the nearest ocean grid cell at a depth of 5 m. The model calculates the time in years when 10% of a tracer ("cells") from one starting point reaches the nearest ocean grid cell of the other marshes. An ocean dispersal distance matrix was created, using these estimated dispersal times between each pairwise sample. Because the dispersal time from site A to site B may not be equal to the dispersal time from site B to site A, we took the average of the estimates in both directions. Samples within the same marsh, or from marshes with the same nearest ocean grid cell (e.g., Plum Island and Wells Reserve, MA), were assigned a dispersal time of 0.001 y.

Statistical Analysis. The rate of distance-decay of the bacterial communities was calculated as the slope of a linear least squares regression on the relationship between (ln transformed) geographic distance versus (ln transformed) bacterial similarity. Distance-decay curves are traditionally plotted with at least community similarity log transformed (2), as this yields a better linear fit than untransformed values. We chose to transform geographic distance because of our sampling scheme, which purposely samples over many orders of magnitude, otherwise the datapoints would have been highly skewed. In cases where community similarity was zero and therefore undefined on a log scale, we assigned community similarity to be equal to the lowest nonzero similarity observed (to be conservative and not contribute artificially to a negative slope). Because the datapoints (pairwise comparisons) are nonindependent, we used a matrix permutation test to examine the statistical significance of the distance-decay slope. The samples, not the cells of the matrix, were permuted 9,999 times, and the observed slope was compared with the distribution of values in the permuted datasets (22).

We also tested whether the slopes of the distance-decay curve (least squares) at the three a priori-defined spatial scales (1 cm to 5 km; 70 km to 1,350 km; 3,800 m to 12,500 km) were significantly different from zero or different from the slope of the overall distance-decay curve. Again, we used matrix permutations to compare the observed slopes within the three spatial scales to the distribution of slopes observed in those ranges over 9,999 permutations.

To investigate the relationship between bacterial community similarity and plant community similarity, geographic distance, and environmental distance across all spatial scales, we first applied a ranked partial mantel test (which assume a monotonic, but not linear, relationship) in the ecodist R package (46, 47). For the environmental distance matrix, we created a composite environmental distance matrix with a normalized combination of the variables selected by the BEST procedure in PRIMER v6 (48).

To tease apart the relative importance of the environmental variables on bacterial community similarity, we used a modified MRM approach (22). Before applying MRM, we used variable clustering to assess the redundancy of the environmental variables (49). Using the varclus procedure in the Hmisc R package, the highest correlation was between sediment moisture content (percent dryweight) and sulfate concentration (Spearman's ρ^2 = 0.69) (Fig. S4). Water and air temperature were the next most correlated, with only ρ^2 = 0.34. Therefore, we removed sulfate concentration from the MRM analyses, but kept all other variables in the models. We modified the MRM script in the ecodist R package (46, 47) and implemented a matrix randomization procedure (22), standardization of the predictor variables, and one-tailed tests (as we had a priori directional hypotheses). Geographic distance, ocean dispersal distance, bacterial community distance (1-similarity) were ln transformed, and plant community distance (1-similarity) was ln (x + 0.005) transformed. To reduce the effect of spurious relationships between variables, we ran the MRM test, removed the nonsignificant variables, then reran the tests again (49). We report the model results from this second run.

To further examine the relative importance of each predictor variable at the three spatial scales, we investigated scale-specific MRM models. For ex-

ample, within marshes, we calculated the partial regression coefficients, R^2 value, and the F and pseudo t statistics (22) for only those pairwise comparisons between 1 cm and 5 km (includes comparisons between Port Susan Bay and Skagit Bay marshes, which are < 5 km apart). To test the significance of these values, we performed matrix permutations on the entire dataset—all pairwise comparisons included—by permuting the bacterial similarity matrix. After each of 9,999 permutations, we recalculated the MRM parameters for only the within marsh comparisons. We compared the original, observed parameters to the distribution of these permuted values so as to calculate their statistical significance.

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