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Multiple microbial guilds mediate soil methane cycling along a wetland salinity gradient

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ABSTRACT Estuarine wetlands harbor considerable carbon stocks, but rising sea levels could affect their ability to sequester soil carbon as well as their potential to emit methane (CH₄). While sulfate loading from seawater intrusion may reduce CH₄ production due to the higher energy yield of microbial sulfate reduction, existing studies suggest other factors are likely at play. Our study of 11 wetland complexes spanning a natural salinity and productivity gradient across the San Francisco Bay and Delta found that while CH₄ fluxes generally declined with salinity, they were highest in oligohaline wetlands (ca. 3-ppt salinity). Methanogens and methanogenesis genes were weakly correlated with CH₄ fluxes but alone did not explain the highest rates observed. Taxonomic and functional gene data suggested that other microbial guilds that influence carbon and nitrogen cycling need to be accounted for to better predict CH₄ fluxes at landscape scales. Higher methane production occurring near the freshwater boundary with slight salinization (and sulfate incursion) might result from increased sulfate-reducing fermenter and syntrophic populations, which can produce substrates used by methanogens. Moreover, higher salinities can solubilize ionically bound ammonium abundant in the lower salinity wetland soils examined here, which could inhibit methanotrophs and potentially contribute to greater CH₄ fluxes observed in oligohaline sediments.

IMPORTANCE Low-level salinity intrusion could increase CH₄ flux in tidal freshwater wetlands, while higher levels of salinization might instead decrease CH₄ fluxes. High CH₄ emissions in oligohaline sites are concerning because seawater intrusion will cause tidal freshwater wetlands to become oligohaline. Methanogenesis genes alone did not account for landscape patterns of CH₄ fluxes, suggesting mechanisms altering methanogenesis, methanotrophy, nitrogen cycling, and ammonium release, and increasing decomposition and syntrophic bacterial populations could contribute to increases in net CH₄ flux at oligohaline salinities. Improved understanding of these influences on net CH₄ emissions could improve restoration efforts and accounting of carbon sequestration in estuarine wetlands. More pristine reference sites may have older and more abundant organic matter with higher carbon:nitrogen compared to wetlands impacted by agricultural activity and may present different interactions between salinity and CH₄. This distinction might be critical for modeling efforts to scale up biogeochemical process interactions in estuarine wetlands.

KEYWORDS methane, methanogenesis, methanotrophs, salinity, sulfate, carbon cycling, decomposition, wetlands

The carbon sequestration potential of vegetated estuarine ecosystems, referred to as blue carbon, has been the subject of considerable interest as a climate mitigation strategy (1–3). Tidal salt and brackish marshes are the most prevalent of the blue

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carbon habitats (salt marshes, mangroves, and seagrass meadows) in the United States and therefore represent key targets for both preservation and restoration. Rates of carbon (C) burial per unit area of tidal wetlands greatly exceed those in all upland terrestrial ecosystems (4); indeed, despite their relatively small area, the total C sequestered annually in tidal wetlands has been estimated to be as high as that in tropical rainforests. Yet the carbon storage potential of estuarine wetlands may be threatened by rising sea levels and consequent inundation and salinization (5, 6). Salinization of estuarine wetlands may result from sea-level rise-driven intrusion of seawater or from decreased freshwater flows during droughts, which may further interact with urbanization and agricultural nutrient loading in many major estuaries (7–12). Salinization may imperil net soil carbon storage in estuarine wetland habitats by both reducing plant primary productivity (i.e., decreasing carbon inputs) and accelerating decomposition of soil carbon stocks (i.e., increasing carbon losses) (10, 13–16).

The impacts of salinity and salinization on production of the potent greenhouse gas methane (CH_4) are less well understood, particularly in tidal freshwater and brackish marshes. Salinity intrusion into freshwaters is hypothesized to suppress methanogenesis (17–19), as the additional sulfate in seawater may promote growth of sulfate-reducing bacteria, which are expected to outcompete hydrogenotrophic and acetoclastic methanogens for hydrogen and acetate based on the thermodynamic favorability of their respiratory pathways (13, 18, 20). However, observational studies and experimental tests in the field and laboratory of salinity effects on CH_4 in estuarine wetlands have yielded inconsistent results (21). Observational studies have generally, but not always, found a decrease in CH_4 flux with increasing salinity (18, 22). Field experiments in estuarine wetlands have found that soil C mineralization and CH_4 flux were suppressed by salinity intrusion (14) or not affected (23). Laboratory experiments with sediment cores from wetlands have shown decreases in CO_2 and CH_4 flux (24), increases in CO_2 flux concomitant with repression of CH_4 flux (25), or even increases in both CO_2 and CH_4 flux with salinity intrusion (16, 21). These mixed results suggest that more complex microbial responses and interactions control the effects of salinization on CH_4 fluxes.

Several studies to date have investigated effects of salinization on methanogen populations (26, 27), microbial communities (28), or microbial control of nutrient cycling (29, 30). However, these studies have largely focused on these dimensions individually, while microbial controls over CH_4 production are influenced by interactions with additional processes including decomposition, fermentation (31), methanotrophy (32), and nitrogen cycling (33, 34). Moreover, efforts to synthesize effects of estuarine salinity gradients and salinization on greenhouse gas fluxes suggest a need to better account for changes in electron acceptors, decomposition rates, alternative methanogenesis pathways (e.g., methyl dismutation and methyl reduction), and nutrient availability, as well as complex interactions among them (10, 13).

We sought to unravel ecosystem-scale relationships between salinity and CH_4 as a function of underlying microbial processes in wetlands spanning the natural salinity gradient across the San Francisco Bay estuary, including freshwater soils in the Sacramento-San Joaquin Delta (the Delta). Using both 16S rRNA gene data and shotgun metagenomic data, we compared microbial community features and CH_4 fluxes across the salinity gradient, including both reference and restored wetlands, which were paired where possible. We obtained soil CO_2 and CH_4 flux data from intact wetland soil cores, which were also used to characterize variation in soil chemistry and microbial community structure and function. Our objectives were to (i) determine patterns in CH_4 fluxes across the salinity gradient, including the influences of salinity and restoration; (ii) identify microbial metabolic pathways and taxa associated with CH_4 production; and (iii) assess interactions among methanogenic and non-methanogenic microbial functional groups (guilds) which might contribute to net CH_4 fluxes, along with the environmental drivers of those interactions. Understanding how microbial communities and biogeochemical processes change across this detailed salinity gradient will help us

predict how salinization will affect these parameters in the near and long terms, with implications for carbon storage and greenhouse gas emissions.

RESULTS

Soil CH₄ fluxes and biogeochemistry along the salinity gradient

Soil methane (CH₄) fluxes increased from freshwater to oligohaline (ca. 2.5-ppt salinity) wetlands but then decreased markedly across the increasing salinity of the San Francisco Bay and Delta following a broadly log-linear relationship (Fig. 1a and b; Fig. S1), with breakpoint regression showing a breakpoint at 1.4 ppt. This trend was driven by the markedly elevated emissions from the oligohaline Mayberry Farms restored wetland complex in the Delta, which were significantly greater than in most other locations. CH₄ flux varied significantly by individual site location [linear mixed effect (LME), *P* < 0.05] but not by wetland status (reference versus restored) (LME, *P* > 0.05). Restored wetlands did, however, have significantly higher CH₄ emissions in the Delta (LME, *P* < 0.05). CH₄ flux and soil respiration (CO₂ flux) were generally only loosely (but significantly) associated across the whole data set (*R*² = 0.25), while at Mayberry only they were more closely coupled (*R*² = 0.88, Fig. 1c). Soil chemistry and physical properties varied significantly along the salinity gradient, including percent soil C, which was highest in freshwater wetlands and broadly decreased with salinity, but also varied within and between wetland complexes (Fig. 1d). In turn, soil C was closely coupled to the relative abundances of nitrogen (N) and phosphorus (P) as expressed by N:P ratios, with increasing N:P in higher C (lower salinity) soils (Fig. 1e). High-C, low-salinity soils also had greater soil

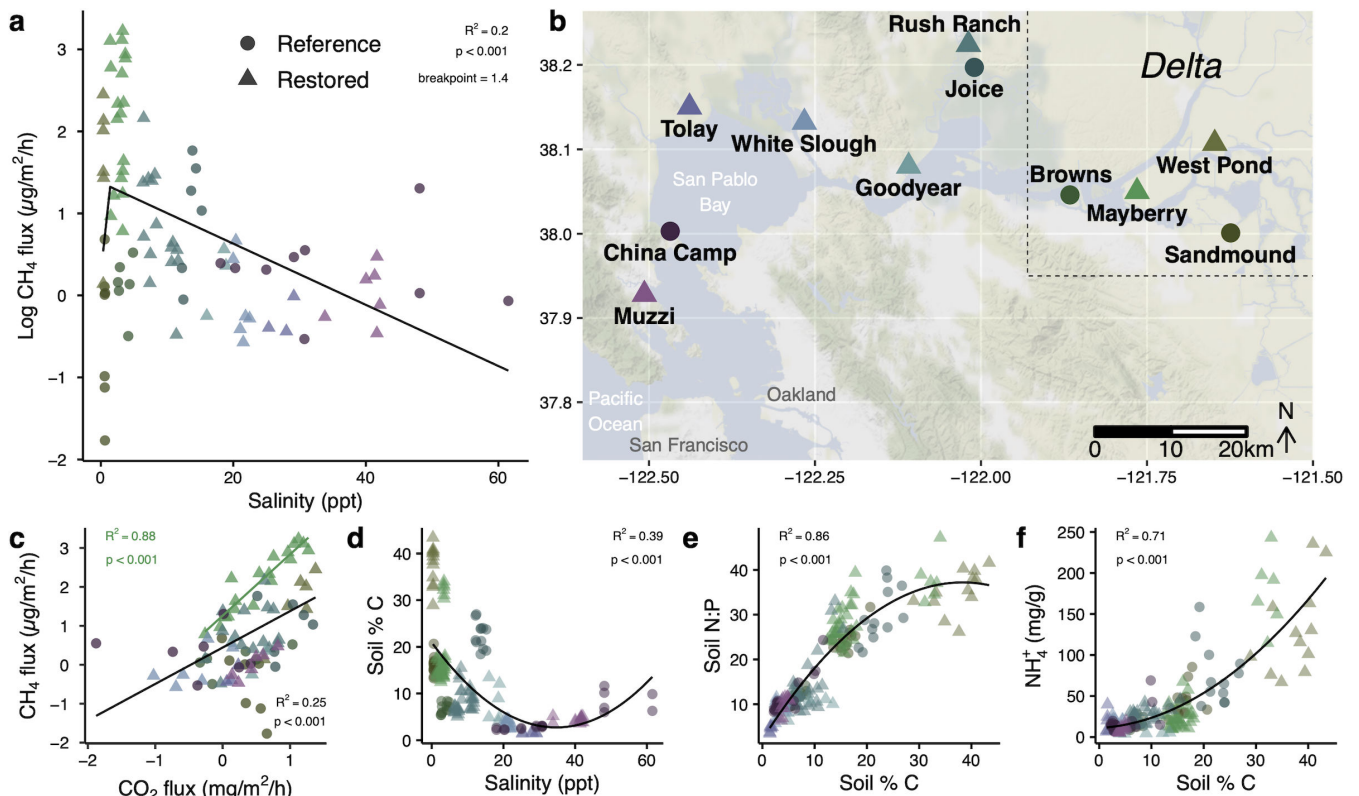


FIG 1 Biogeochemical patterns in greenhouse gas fluxes and soil chemistry in wetland sites along the San Francisco Bay-Delta salinity gradient. (a) Variation in soil methane fluxes with salinity among wetland soils sampled along the gradient, with a segmented (breakpoint = 1.4 ppt) linear regression line (b) spanning the northern San Francisco Bay and Delta. (c) Relationship between soil methane and carbon dioxide fluxes, with linear regression lines shown separately for the Mayberry Farms wetland (green) and for all sites (black). (d) Soil carbon pools varied with salinity, and in turn, (e) soil N:P and (f) soil ammonium varied as a function of soil C in relation to salinity. Points are colored by site as in panel b; circles are reference wetlands and triangles are restored wetlands. Regression lines in panels d-f are from second-order polynomial regressions, which fit the data better than linear regressions.

ammonium pools (Fig. 1f). Soil C, N:P, and ammonium were not significantly affected by depth (LME, $P > 0.05$).

Across all the sites, CH₄ fluxes were most positively correlated with soil CO₂, soil C and N:P, and dissolved organic carbon (DOC) (Table S4) and were negatively correlated with water salinity as well as soil sulfate (SO₄²⁻) and chloride (Cl⁻). However, when considering only Delta sites, CH₄ was not correlated with SO₄ and only weakly (positively) correlated with salinity and chloride concentrations (Table S5). CH₄ fluxes in the Delta were, as across all sites, positively correlated with CO₂, soil N:P, and DOC, and inversely correlated with soil volumetric water-filled pore space, a measure of bulk density and moisture content (Table S5). Among all sites, the strongest combined predictors of CH₄ fluxes based on least absolute shrinkage and selection operator (LASSO) variable importance scores were DOC, CO₂ flux, and soil N:P ratios (Fig. S2a). However, DOC was a poor predictor of CH₄ fluxes in a similar LASSO model using only Delta sites, which had the highest CH₄ emissions and where CO₂, total nitrogen, salinity, and SO₄²⁻ were the strongest *positive* predictors of CH₄ flux (Fig. S2b), implying an increasing influence of seawater was associated with *higher* CH₄ fluxes in the Delta. Volumetric water-filled pore space and soil NO₃:NH₄ ratios were negative predictors of CH₄ fluxes in models for the Delta and among all sites (Fig. S2).

Microbial metabolic genes and wetland salinity

With increasing salinity, we observed general increases in sulfur cycling gene relative abundances and decreases in methanogenesis gene relative abundances (Fig. 2b; Fig. S3). Yet among sulfur cycling genes, only *satA* (catalyzing the first step in both assimilatory and dissimilatory sulfur (S) reduction) was strongly correlated with salinity (Fig. S3). Sulfate reduction genes *dsrAB* (for dissimilatory sulfite reductase, converting sulfite to sulfide) were weakly correlated with salinity and moderately correlated with SO₄ across all of our sites (Fig. 2b; Table S6). However, sulfate reduction genes *aprAB* (APS reductase, downstream of *satA*) were moderately correlated with salinity and, like *satA*, were strongly correlated with SO₄ (Tables S6 and 7). Although several genes for CH₄ metabolism (*mcrABG* and *mtrCDEFG*) were negatively correlated with salinity, these relationships were not especially strong, particularly within Delta wetlands (Fig. 2b; Fig. S3; Tables S6 and S7). Most correlations between nutrient cycling genes and salinity were even stronger among reference wetlands than across all sites (Fig. S3); this was also true for genes for aromatic utilization, denitrification, acetoclastic methanogenesis, and CH₄ oxidation. A notable exception to this trend was the gene *mttB* (methanogenic reduction of trimethylamines), which increased with salinity across all soils (Fig. S3).

Several nitrogen cycling genes also varied with salinity and were more abundant in freshwater reference wetlands than nearby restored sites (Fig. S3), including assimilatory and dissimilatory nitrate reductases (*narBH*, *nasB*, and *napA*), and ammonia oxidation and assimilation genes (*pmoBC*, *hao*, and *aspQ*). However, some nitrate reductases (*narBH* and *nasB*) were, like CH₄ flux, highest in oligohaline sites (Fig. S3), as was the gene for utilization of the compatible solute trehalose (*treA*, Fig. S3).

Metabolic genes linked with CH₄ fluxes

Most element cycling genes were more correlated with CH₄ among the subset of Delta sites than in all sites or only reference wetlands (Fig. 2; Fig. S4), suggesting different forces may govern CH₄ fluxes in lower salinity wetlands where CH₄ fluxes were highest. Although central methanogenesis (*mcrABG* and *mtrCDEFG*) and hydrogenotrophic (*fwdDF* and *mtd*) genes were strongly positively correlated with CH₄ fluxes across all sites, the highest flux sites did not have the greatest relative abundances of these genes (Fig. 2; Fig. S4). Genes for the consumption of CH₄ (*pmoC*) were negatively correlated with CH₄ fluxes (Fig. 2; Fig. S4), although *pmoABC* genes cannot be differentiated from closely related ammonia oxidation genes (*amoABC*) in the Metagenomics Rapid Annotation using Subsystems Technology (MG-RAST) annotations. Using the Tree-based Sensitive and Accurate Phylogenetic Profiler (TreeSAPP) to assign *pmoA-amoA* genes to

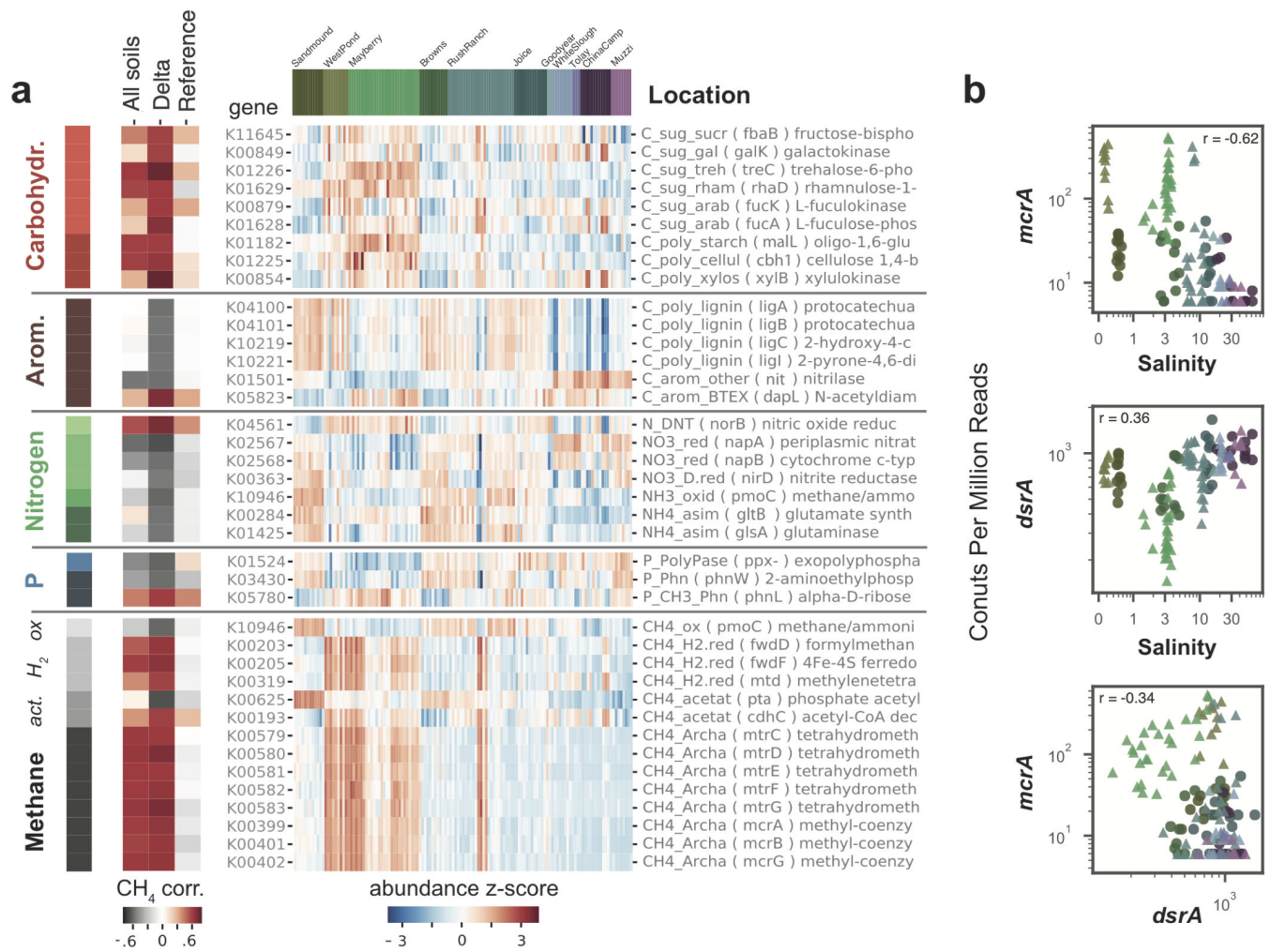


FIG 2 Functional gene relative abundances significantly correlated with CH₄ flux. (a) Relative abundances of element cycling genes, color coded by element cycle and pathway (carbohydrates, aromatics, nitrogen, phosphorus, methane—acetoclastic, hydrogenotrophic, and oxidation), across sites, with site (ordered from low to high salinity) indicated by the bar at the top of the heatmap, which matches colors in the map of locations in Fig. 1b. Correlations with methane fluxes are shown in the leftmost heatmap based on all sites, or subsets of sites corresponding to only sites in the Delta, or to only reference wetland sites. (b) Scatterplots of relative abundances of selected genes for methanogenesis and sulfate reduction vs salinity and vs each other.

methane-oxidizing bacteria, ammonia-oxidizing archaea (AOA), or ammonia-oxidizing bacteria (AOB) and therefore to differentiate *pmoA* and *amoA*, we found that Class IIa MOB *pmoA* relative abundances were negatively associated with CH₄ emissions in the Delta, and AOB *amoA* relative abundances were negatively associated with CH₄ both across the whole data set and within the Delta (Fig. 3). Furthermore, total *pmoA* and total *amoA* relative abundances were negatively associated with each other (Fig. 3). Sulfate reduction genes *dsrAB* were moderately anti-correlated with CH₄ fluxes but poorly correlated with methanogenesis genes *mcrAB*, especially in Delta soils where their relationship appeared positive in restored sites (Fig. 2b; Fig. S5b; Tables S6 and S7). Although *aprAB* genes for an early step in sulfate reduction showed stronger negative relationships with *mcrAB* across all sites than *dsrAB*, these relationships were still weak in Delta soils (Tables S6 and S7). Contrastingly, some genes for S assimilation (*cysC* and *sir*) had stronger negative relationships with methanogenesis genes both among all sites and in the Delta, and these genes were negatively correlated with sulfate reduction genes (Tables S6 and 7), while *sat* genes for the first reaction in sulfate reduction were inversely related to methanogenesis pathways across all sites and to a lesser extent in soils of the Delta (Tables S6 and 7).

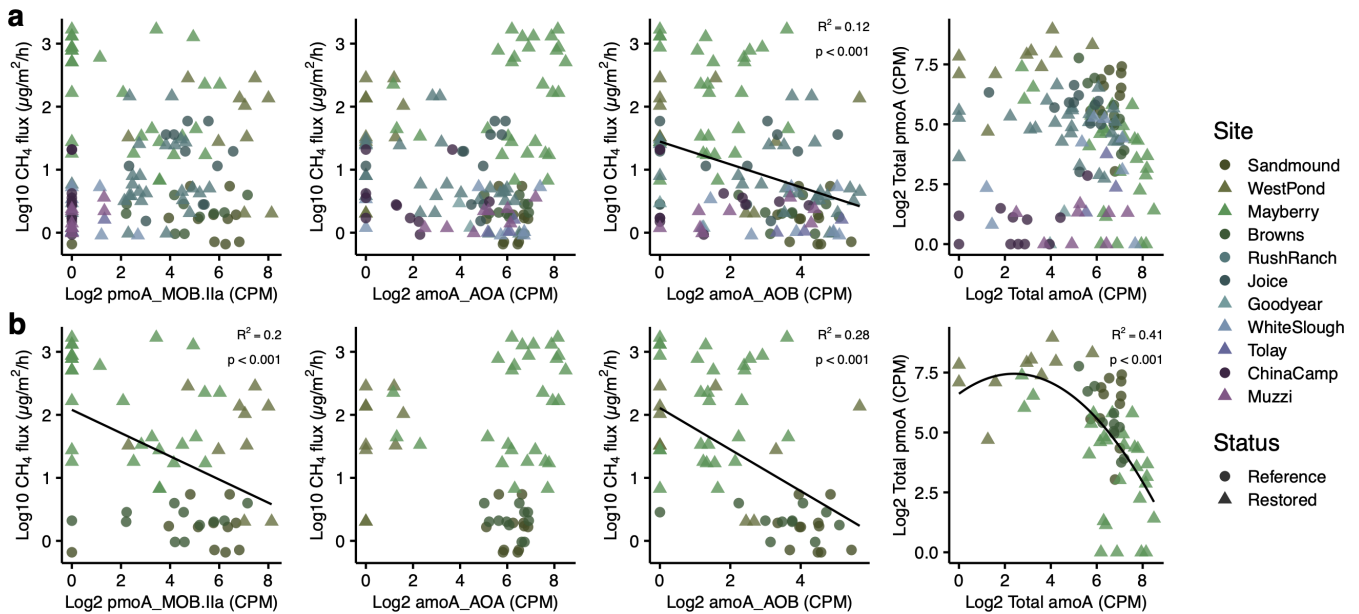


FIG 3 Relationships between CH_4 flux and relative abundance of *pmoA-amoA* genes assigned to Class IIa methane-oxidizing bacteria (*pmoA*_MOB.IIa), ammonia-oxidizing archaea (*amoA*_AOA), or ammonia-oxidizing bacteria (*amoA*_AOB) across the whole data set (a) and in the Delta (b). Also shown are relationships between total *pmoA* relative abundance (*pmoA-amoA* assigned to MOB) and total *amoA* (*pmoA-amoA* assigned to AOA and AOB) relative abundance. Annotation and taxonomic assignment were done with TreeSAPP. Lines are shown when relationships are significant. CPM, counts per million.

Microbial carbon cycling genes revealed marked shifts from utilization of aromatic compounds to carbohydrates as CH_4 fluxes increased, particularly in Delta soils (Fig. 2a; Fig. S4). Lower CH_4 fluxes were associated with genes for lignin degradation (*ligABCL*), while higher fluxes were associated with breakdown of hemicellulose (*xyIB*), cellulose and starches (*cbh1* and *mall*), and sugars including sucrose, galactose, rhamnose, and arabinose (*fbaB*, *galk*, *rhaD*, and *fucAK*, respectively). Trehalose degradation (*treC*) was also associated with CH_4 fluxes in the Delta, although this sugar is also a compatible solute which may reflect osmotic adaptation. Several N cycling genes were negatively correlated with CH_4 in the Delta, including nitrate reductases (*napAB* and *nirD*), and ammonia oxidation (*amoC/pmoC*) and assimilation genes (*glsA* and *gltB*), while nitric oxide reductase (*norB*) was positively correlated with CH_4 (Fig. 2a).

Microbial communities and methane

Across all the wetland soils studied, microbial community composition determined by the 16S rRNA gene (Fig. 4b) was structured by restoration status, salinity, and nutrients (Fig. 4c). Wetland site accounted for much of the variation in microbial community composition [Fig. 4b; permutational multivariate analysis of variance (PERMANOVA), $R^2 = 0.66$, $P = 0.001$], while vegetation type as a second predictor variable had a lesser effect (Fig. S6; PERMANOVA, $R^2 = 0.06$, $P = 0.001$). Several soil features were also closely linked with microbial community composition, including salinity, bulk density, C, and N, among others (Fig. 4c). Bacterial communities were dominated by several classes of Proteobacteria and the phyla Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Firmicutes, and Nitrospirota (Fig. 4b). Archaeal phyla were less abundant, but included Halobacteriota, Crenarchaeota, Euryarchaeota, Aenigmarchaeota, Altiarchaeota, Asgardarchaeota, Nanoarchaeota, and Thermoplasmatota (Table S8).

Soil CH_4 fluxes were positively correlated with the phyla Firmicutes (including several Bacilli and Clostridia taxa) and Spirochaetota (Fig. 4a; Table S8), along with Halobacteriota (including Halobacteriales and Methanosarcinales) and Chloroflexi (particularly class Dehalococcoidia). Within the less correlated Actinobacteriota phylum, the orders Frankiales, Micrococcales, and Pseudonocardiales had several members well correlated

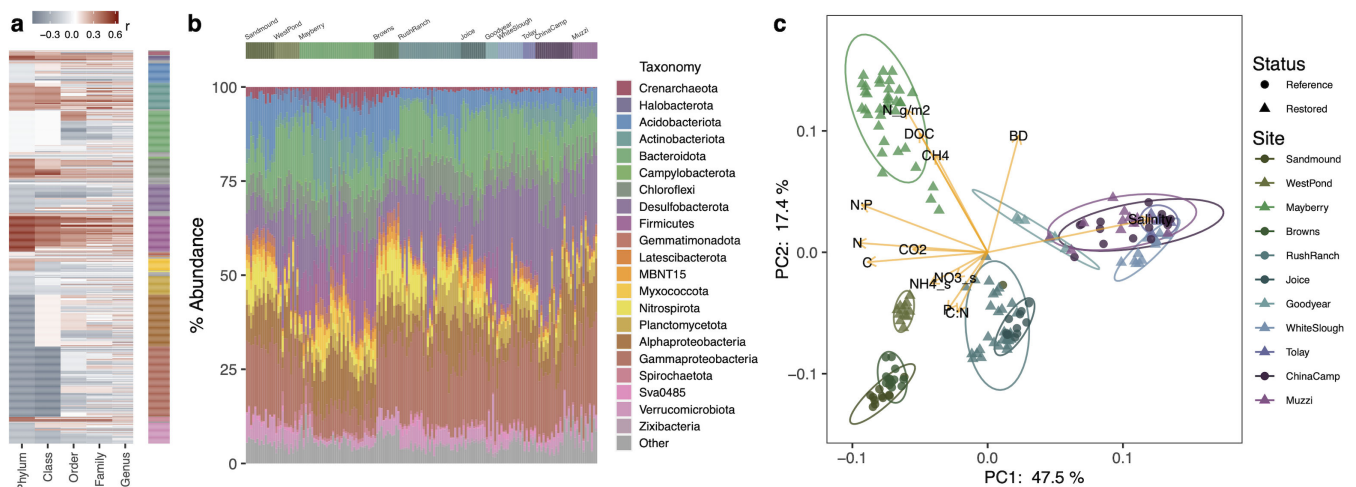


FIG 4 Correlations of microbial taxa with soil methane fluxes (a), microbial community composition based on 16S rRNA gene sequencing (b), and principal component analysis (PCA) of Aitchison distance (c). The heatmap in panel a shows the Pearson correlation coefficient of microbial taxa with soil methane fluxes calculated at each taxonomic rank, with taxonomic groups indicated by the colored bar at right with colors corresponding to the legend in panel b. Details of those results are presented in Table S8. Relative abundances (proportion of total sequence reads after normalization by DESeq2) of major microbial groups (b) are shown for the most abundant phyla (along with *Proteobacterial* classes) for each sample, with study site indicated by the bar at the top, colored to match location data in panel c. PCA ordination in panel c shows clustering of soil microbial communities by wetland location (PERMANOVA, $R^2 = 0.66$), along with projected loadings of environmental chemistry data into the reduced dimensional space. C, N, and P are total elements in soil. Salinity was measured *in situ* in soil coring holes. Ellipses show 95% confidence intervals around the centroid. Abbreviations: BD, bulk density; DOC, dissolved organic carbon.

with CH_4 fluxes. The phyla Verrucomicrobiota and Desulfobacterota were negatively correlated with CH_4 fluxes, as were the phylum Proteobacteria and the order Gammaproteobacteria, though several proteobacterial families (Geminicoccaceae) and genera (*Sphingomonas* and *Ellin6055*) were instead positively correlated with CH_4 fluxes (Fig. 4a; Table S8).

Functional guilds and methane

Functional guilds of microbes involved in carbon, nitrogen, and sulfur cycling were obtained from the taxonomic composition of metagenome sequence reads annotated as encoding the function, or from 16S rRNA gene taxonomy in cases of known vertical inheritance and close relationships between phylogeny and biochemical function. For example, the majority of cellulose degrading *cbh-1* genes were assigned to Firmicutes or Actinobacteriota, explaining the role of these CH_4 -correlated taxa in contributing to this CH_4 -linked degradation pathway (Fig. S7). However, due to the higher statistical resolution and broader site coverage of 16S rRNA data, as well as ambiguities and/or low numeric counts of some genes [beneath quantitative thresholds (35)] in metagenome data, 16S rRNA gene relative abundances were deemed more informative for determining quantitative relationships. Functional guilds from 16S rRNA gene taxonomy were generally consistent with their relative abundance in shotgun sequence-derived annotations using TreeSAPP, although TreeSAPP did not appear to reliably identify *amo* genes from nitrite oxidizers or *pmo* genes from Type IIb CH_4 oxidizers (Fig. S8).

Unlike the patterns across all sites, CH_4 fluxes in the Delta were not strongly positively correlated with any methanogenic genera or genera from any other functional guild. On the other hand, there were several genera from various functional guilds strongly negatively correlated with CH_4 . These include the ammonia-oxidizing archaeal genera *Nitrosarchaeum* and *Cand. Nitrosotenuis*, several genera of ammonia-oxidizing bacteria, and several nitrite-oxidizing bacteria (NOB) (*Nitrospira*, 4–29–1 identified only to class level, and P9 × 2b3D02 identified only to class level) (Fig. S9). Some sulfur-reducing (*Desulfurhabdus*) and sulfur-oxidizing (Thioalkalispiraceae identified to family level) taxa were strongly negatively correlated with CH_4 flux (Fig. S9).

LASSO regression modeling of CH₄ fluxes based on these guild members was used to assess their relative importance in contributing to CH₄ fluxes (LASSO regression model, $R^2 = 0.81$). Guild members most positively associated with CH₄ fluxes in the Delta included the iron oxidizer *Leptolinea*, the methane oxidizer *Methyloceanibacter*, and the sulfur oxidizer *Thiobacillus*, while the most negatively associated genera included the sulfate reducers *Desulfobacca*, *Sva1033* (identified to family level), *Desulfomonile*, and *Desulfatiglans*, the methyloph *Methylotenera*, and the methane oxidizer *Methylocystis* (Fig. S10a). Similarly, when relative abundances of these guilds were considered in aggregate (LASSO regression model, $R^2 = 0.65$), the sulfate reducers and Type I and IIa methanotrophs were the most negatively correlated with CH₄, while iron oxidizers, acetoclastic methanogens, and sulfur-oxidizing bacteria were most positively correlated with CH₄ (Fig. S10b).

Potential interactions among microbial guilds

To assess the influence of multiple microbial guilds correlated with CH₄ on net CH₄ fluxes, we compared the relative abundances of guilds with one another (and known mechanisms) to assess their potential interactions. Strikingly, the relative abundance of methanogens was significantly higher at the freshwater West Pond restored wetland than at the nearby oligohaline Mayberry Farms site (Fig. 5), despite CH₄ fluxes that were up to an order of magnitude higher at Mayberry Farms (Fig. 1a). This was also true of the relative abundance of central methanogenesis genes in shotgun metagenomic data from these sites (Fig. 2a; Fig. S4). However, the relative abundance of methanotrophic bacteria was also significantly higher in West Pond soils, while populations of AOA were significantly higher in Mayberry Farms. While AOA were also present in Sandmound and Brown's Island, reference freshwater and oligohaline Delta wetlands, respectively, these soils also had significantly more abundant AOB and NOB compared to the adjacent restored wetlands (Fig. 5). Methanotrophic genera as a whole declined in relative abundance across the salinity gradient and also shifted in composition, with certain genera more abundant in the Delta (e.g., *Methylocystis* and *Crenothrix*) and others more abundant at the more saline sites (e.g., *Methyloceanibacter*) (Fig. S11).

To evaluate potential effects of guild interactions on net CH₄ fluxes, we constructed a series of structural equation models (SEMs), based on aggregate guild relative abundances derived from 16S rRNA gene taxonomy. The most common mechanistic predictors of CH₄ fluxes in LASSO models were combined acetoclastic and mixotrophic methanogens (CH₄ ac+mix) and Type IIa methanotrophs (MOB IIa), along with CO₂ flux (an indicator of decomposition) and soil bulk density. We then used composite models to incorporate simultaneous predictions of acetoclastic and mixotrophic methanogens and Type IIa methanotrophs into base SEMs. The model with these SEM "branches" was not significant across all sites, but was significant ($P > 0.05$) in the Delta sites (Fig. 6). The final SEM model for the Delta shows acetoclastic and mixotrophic methanogens and CO₂ flux predicting the composite variable "methane generation"; water-filled pore space, AOA, and NOB predicting MOB IIa; MOB IIa and bulk density predicting the composite variable "methane oxidation"; and then methane generation and methane oxidation predicting the observed methane flux (Fig. 6).

DISCUSSION

Estuary-scale patterns in wetland methane fluxes

Our first objective was to understand the patterns and drivers of CH₄ fluxes across an estuarine salinity gradient. CH₄ fluxes exhibited a broadly log-linear relationship with soil salinity across the estuarine gradient (Fig. 1a). However, the highest CH₄ fluxes occurred in oligohaline wetlands, in agreement with ecosystem-scale eddy covariance observations at the same restored Delta wetland sites (22, 36–39) and a previous meta-analysis of tidal marsh soils (18) (Fig. S1). Although the highest CH₄-producing soils in our study were non-tidal restored wetlands, this concordance suggests that maximum

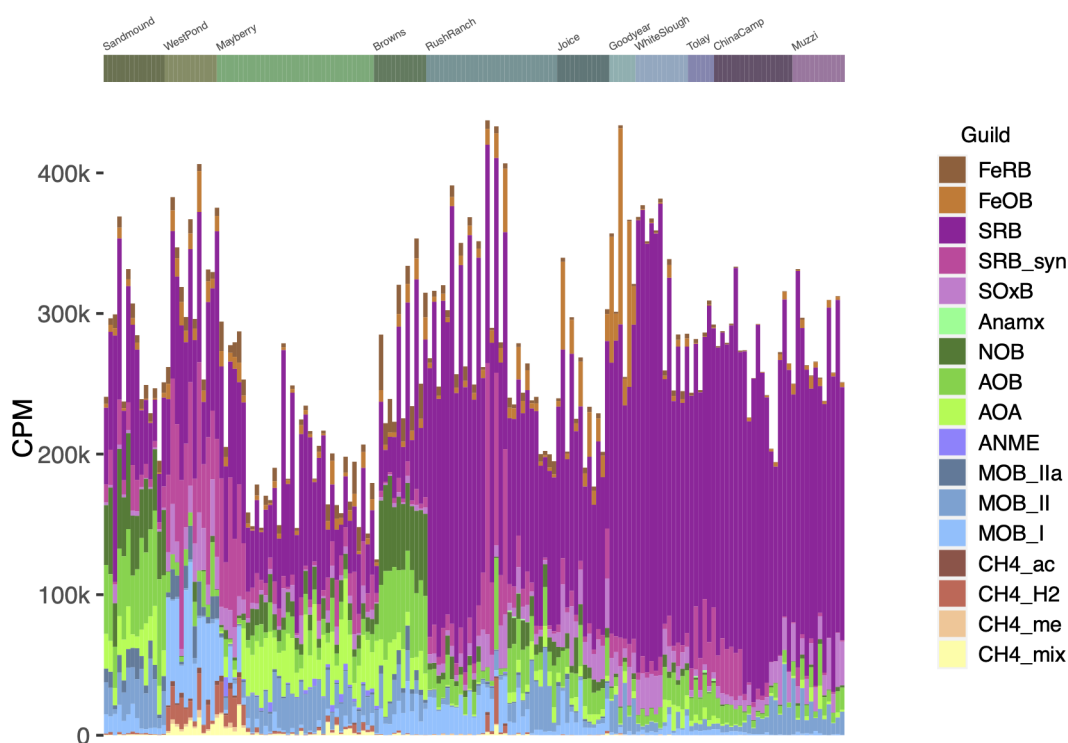


FIG 5 Relative abundance of microbial guilds based on 16S rRNA gene taxonomy. Guild relative abundances are shown as counts per million 16S rRNA gene sequence reads following normalization using DESeq2's variance stabilizing transformation to account for differences in read depth among samples. Guilds were assigned based on taxonomy in published review papers and were iron-reducing and iron-oxidizing bacteria (FeRB and FeOB, respectively); sulfate-reducing bacteria, syntrophs, and sulfur-oxidizing bacteria (SRB, SRB_syn, and SOxB); anaerobic ammonia-oxidizing bacteria (Anamx); nitrite and ammonia-oxidizing bacteria and ammonia-oxidizing archaea (NOB, AOB, and AOA); anaerobic methane-oxidizing archaea (ANME); Type I, II, and IIa methanotrophic bacteria (MOB_I, MOB_II, and MOB_IIa); and acetoclastic, hydrogenotrophic, methyl-reducing, and mixotrophic methanogens (CH4_ac, CH4_H2, CH4_me, and CH4_mix). CPM, counts per million.

net CH₄ fluxes may occur at low but non-zero salinities (40–42), congruent with studies demonstrating an increase in CH₄ following low-level salinization (ca. 5 ppt) of some freshwater wetland soils (16, 21, 43).

These patterns of CH₄ fluxes did not strongly support the hypothesis that competition for carbon substrates from sulfate reducers with increasing seawater influence (increased salinity and sulfate) is the primary factor influencing archaeal methanogenesis and CH₄ fluxes in estuarine wetlands (14, 18, 25, 43). Although CH₄ fluxes were negatively associated with sulfate and salinity across the full salinity range studied (Table S4), in higher CH₄ Delta sites, CH₄ was not correlated with SO₄ and was weakly positively associated with salinity (Table S5). A case in point is the Mayberry Farms location; in the same wetland complex with relatively little spatial variation in salinity (1.4–3.7 ppt), CH₄ emissions varied by over three orders of magnitude (6–1,680 μg/m²/h), indicating that other variables besides sulfate reducer activity exert a strong influence on net CH₄ emissions. CH₄ flux was positively associated with DOC, soil N:P ratios, and CO₂ fluxes across all sites and within the Delta (Tables S4 and 5). Mayberry Farms, in particular, showed a remarkably strong relationship between CH₄ and CO₂ fluxes (Fig. 1c), which could suggest a dominant influence of overall organic carbon decomposition rates on CH₄ production, and/or a lack of CH₄ oxidation at this site, or a greater contribution of methylotrophic or acetoclastic methanogenesis, both of which produce CO₂ (44). 16S and metagenomic data, however, suggest similar correlations between different methanogen guilds and CH₄ emissions in the Delta and the whole data set (Fig. S5). As salinity increases, non-competitive methyl-based substrates such as trimethylamine, which can function as compatible solutes or are degradation products

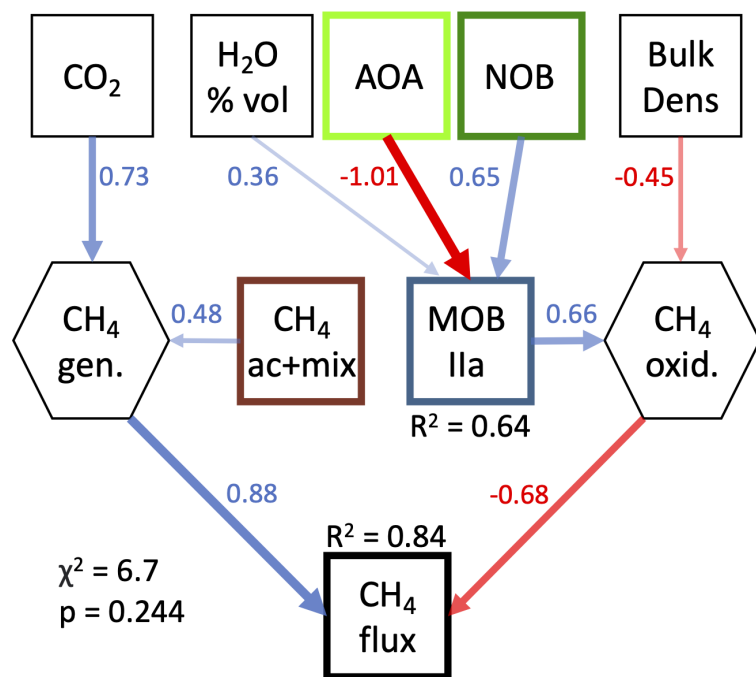


FIG 6 Structural equation model predicting soil methane fluxes as a function of microbial guilds and soil CO_2 , using data from sites from the Delta only. Guild abbreviations and colors match those in Fig. 5, except that here, acetoclastic and mixotrophic methanogens were combined (CH_4 ac+mix). Regression fit coefficients (R^2) indicate the degree of prediction for each predicted feature in the model, and hexagons indicate composite variables. Arrows indicate directional relationships based on known interactions, with blue arrows indicating positive relationships and red arrows indicating negative relationships. Arrow widths are proportionate to the strength of these relationships, also shown by embedded numbers which are scaled model coefficients. Note that here, the P value above 0.05 indicates a significant model.

of compatible solutes (45, 46), may increase and contribute a greater proportion of the CH_4 than in freshwater environments. While the increase in *mttB* relative abundance with salinity partially supports this hypothesis, methyl-reducing and mixotrophic taxa relative abundances did not increase with salinity (Fig. S5). High CH_4 emissions at that site during our sampling may also be attributed to its recent conversion to a restored wetland and the subsequent increase in primary productivity and labile carbon inputs; CH_4 emissions have since declined both there and at West Pond over time (2012–2020) (47). High CH_4 production has also been observed in oligohaline wetlands in the Delaware River estuary where isotopic measurements indicated patterns across the salinity gradient were driven more by a lack of CH_4 consumption than by greater gross CH_4 production (42).

The supply of methanogenic substrates can depend on the overall rate of decomposition in soils, which may in turn be shaped by variation in nutrient availability (13, 48, 49). In our wetland soils, total C pools broadly declined with salinity (Fig. 1d), and lower percent C was associated with lower N:P ratio and NH_4^+ concentrations (Fig. 1e and f; Table S4). These patterns likely arise from lower primary productivity and greater rates of decomposition in more saline wetlands (15, 48, 50), combined with tightly constrained soil C, N, and P stoichiometry (51, 52). Higher soil N:P ratios and inorganic N availability in our freshwater and oligohaline soils (Fig. 1e and f) may further arise from mechanisms promoting increasing N (vs P) limitation with greater salinity in estuaries, including sulfate-driven loss of P sorption (leading to higher P availability) and decreased N availability due to inhibition of N fixation (53–56). Notably, the larger pools of extractable ammonium in our freshwater wetland soils (Fig. 1f) might be susceptible to desorption by ionic exchange with salinity intrusion (16, 42, 57–59), an effect which may depend in part on land use history and agricultural runoff (7).

Microbial taxa, metabolism, and methane fluxes

Within Delta soils, many methanogenic taxa and genes for CH₄ production were more abundant in the lower CH₄-emitting freshwater restored wetland than in the highest CH₄-emitting oligohaline sites (Fig. 2b; Fig. 4), again suggesting other factors contribute to soil CH₄ fluxes. It is important to note, however, that we did not measure gene or protein expression, which could potentially show different patterns than metagenome-based relative abundances; future work using metatranscriptomics and/or metaproteomics would provide valuable additional information. Utilizing both 16S rRNA gene taxonomic data and shotgun metagenomic data, we consider how five factors—CH₄ oxidation, nitrogen cycling, carbon degradation, sulfur cycling, and iron cycling—may contribute to the observed net CH₄ fluxes.

Genes essential to methanotrophy (*pmo*) were negatively correlated with CH₄ fluxes, particularly in the Delta (Fig. 2; Fig. S4d). Yet *pmo* and ammonia monooxygenase (*amo*) gene functions are not differentiated in common gene ontologies or annotation pipelines (60–63) due to their close evolutionary relationship (64–67). Instead, we used phylogenetic placement and taxonomic assignment to distinguish microbes that oxidize CH₄ from those that oxidize ammonia (Fig. 3 and 5; Fig. S5). The relative abundance of *pmoA* genes assigned to Class IIa MOB was negatively correlated with CH₄ flux in the Delta (Fig. 3b; Fig. S5d), as was the relative abundance of *amoA* genes assigned to AOB (Fig. 3; Fig. S5c and d). In the 16S amplicon data, methane-oxidizing bacterial guilds as a whole were negatively correlated with CH₄ fluxes (Fig. S5f), and two CH₄ oxidizing genera were negatively correlated with CH₄ fluxes in the Delta (Fig. S9), supporting the potential for methanotrophs to substantially alter net wetland CH₄ fluxes (68–71). Incongruous patterns of methanogen guilds and CH₄ fluxes, particularly at our highest CH₄ sites (Fig. 5), further suggested the potential importance of methanotrophy in our wetland soils. For example, Mayberry had higher CH₄ emissions than West Pond (Fig. 1a) despite less abundant methanogens (Fig. 5); this may be in part due to more abundant methanotrophs at West Pond consuming much of the CH₄ and leading to less net CH₄ flux relative to Mayberry. Alternatively, soil temperatures at Mayberry in 2013 were generally 2–3°C higher than at West Pond, which could increase methanogenesis rates (72). Detailed soil temperature data are not available for other sites.

If methanotrophy exerts an important influence on net CH₄ flux, it is then also important to consider nitrogen cycling, as studies in agricultural fields, forests, and rice paddies have shown that CH₄ oxidation can be inhibited by excess ammonium and nitrite (33, 34, 73–76). Interactions between inorganic N availability and methanotrophs may depend on both the form of N and the community of microbes present (33). Some methanotrophs (particularly Type IIa MOB) may be sensitive to ammonia due to their inability to detoxify hydroxylamine, the immediate metabolic product of ammonia oxidation (74, 77–81), and may also be inhibited by nitrite accumulation (80–83). Shifts in methanotrophic community dominance from Type II to Type I MOB have been observed with excess inorganic N, including ammonium and nitrite, in several environments (33, 73, 82, 84). The soluble (bioavailable) fraction of ammonium is particularly important for predicting the effects of ammonium on methane oxidation (85).

Indeed, across our sites, nitrogen cycling guild relative abundances generally opposed trends in methanotroph relative abundances, although correlations among those guilds were mixed (Fig. 5; Fig. S5e and f). MOB_I and MOB_IIa were negatively correlated with AOA, particularly in the Delta (Fig. S5e and f). Higher ratios of ammonia oxidizers (generating nitrite) to nitrite oxidizers (consuming nitrate), which may be inhibited at high ammonia concentrations (86), are expected to lead to the accumulation of soil nitrite (NO₂⁻), which could inhibit methanotrophy. Greater ammonia oxidizer (AOA + AOB):NOB ratios were also weakly associated with greater methanogen:methanotroph ratios across all sites ($R^2 = 0.02$, $P = 0.07$) and in the Delta ($R^2 = 0.08$, $P = 0.01$) (Fig. S12). Increases in this ratio have been associated with higher CH₄ flux in previous studies (87, 88), but the weak relationship observed in our data suggests that this exerts only a minor influence on CH₄ flux across our sites.

More specifically, high-CH₄, low-methanotroph Mayberry soils had abundant populations of AOA, unlike the lower-CH₄, high-methanotroph West Pond soils. While the AOA were also prevalent in reference freshwater and oligotrophic wetlands (Sandmound, Brown's Island), at those sites they were accompanied by even greater populations of NOB, suggesting that ammonia at those sites could be fully oxidized to nitrate in contrast to the neighboring restored wetlands (Mayberry, West Pond) in which NOB were nearly absent (Fig. 5). These results linking methanotrophs, ammonia oxidizers, and nitrite oxidizers are broadly consistent with descriptions of inhibition of CH₄ oxidation by excess N (33, 34), and we suggest that this effect might be linked with greater net CH₄ fluxes in our wetland soils, particularly in the Delta (Fig. 1a and f).

Genes reflecting carbon substrate availability were tightly connected to patterns of CH₄ fluxes, particularly in the Delta, as were cellulose degrading taxa. Higher CH₄ fluxes were positively correlated with several genes associated with degradation of plant biomass, including those connected to metabolism of simple sugars, cellulose, and hemicellulose, and inversely correlated with genes for lignin degradation (Fig. 2; Fig. S4a and b). CH₄ fluxes were correlated with most members of the phylum Firmicutes and several members of the Actinobacteriota (Fig. 4A) which were the dominant cellulose degraders in our wetlands (Fig. S7), a function we suggest is linked to measured CH₄ fluxes (Fig. 2). These patterns suggest CH₄ fluxes may be constrained by soil C availability or the overall rate of decomposition in soils (49, 68, 89, 90), both of which are affected by plant productivity and root exudation (68); this is concordant with our finding that CH₄ flux was highly correlated with CO₂ flux in our highest CH₄-producing site (Fig. 1). Although syntrophic bacteria lack a consistent genetic marker (91–94), we found that relative abundances of known syntrophic taxa were also linked to higher CH₄ fluxes (Fig. S5e and f; Fig. S9).

As was the case with the overall patterns in sulfate concentrations, the relative abundances of genes in sulfur cycling pathways showed limited support for the hypothesis that competitive inhibition of methanogens by sulfate-reducing bacteria (SRB) is a key driver of CH₄ fluxes. Overall, neither CH₄ flux nor methanogen relative abundance was strongly negatively correlated with sulfate concentrations or SRB relative abundance (Fig. 2b; Fig. S5a, c, and e), and weak correlations in the Delta sites were actually “positive” (Fig. S5b, d and f). Future work should include hydrogen sulfide (H₂S) measurements to examine the ratio of H₂S to CH₄ as a terminal electron sink.

In addition to sulfate, oxidized iron (Fe³⁺) represents another alternative electron acceptor for microbial metabolism in anaerobic sediments that is more energetically favorable than CO₂. Activity of iron reducers is expected to be negatively correlated with methane concentrations due to competition between iron reducers and methanogens (95). In our data set, the relative abundance of iron reducers was negatively correlated with CH₄ flux only in the Delta (Fig. S5), and this trend was driven primarily by taxa in the Geobacteraceae family (Fig. S10). These findings are in line with another study in the Delta that found higher iron concentrations in alluvium soils were correlated with lower ecosystem-scale CH₄ flux (96). In the context of salinization, previous work has found an initial increase in iron reduction rates and reduced iron (Fe²⁺) concentrations following simulated seawater intrusion, but this effect diminished once the labile pool of iron oxides was consumed (97).

Linking biogeochemistry, functional guilds, and CH₄ fluxes: SEMs

To integrate the interacting effects of multiple microbial guilds on soil CH₄ fluxes, we tested SEMs representing plausible metabolic interactions that determine net CH₄ fluxes (Fig. 6). Significant SEMs were found only for the Delta sites; SEMs for the whole data set were not significant, possibly due to non-linearities in the relationships between biogeochemical and microbial variables across such a broad salinity gradient. In the Delta, CH₄ fluxes were consistently predicted by the relative abundances of acetoclastic and mixotrophic methanogens and Type IIa methanotrophs (dominated by *Methylocystis*), along with soil bulk density and CO₂ fluxes. The predictive value of soil respiration, as

reflected by CO₂ flux, may demonstrate the importance of overall decomposition rates to CH₄ production, consistent with the correlations of carbohydrate-degrading genes (Fig. 2) and taxa (Fig. 4; Fig. S7) with CH₄ flux.

SEM and LASSO regression models indicated a strong influence of acetoclastic methanogens on CH₄ fluxes (Fig. 6; Fig. S10b); this guild was associated with higher CH₄ fluxes elsewhere (98–100) and was previously found to contribute to the majority of CH₄ production in our highest CH₄ site (101). Although acetoclasts were negatively associated with sulfate-reducing bacteria across all sites, these relationships appeared weakly *positive* when considering only freshwater and oligohaline Delta wetlands (Fig. S5d and f). Acetoclasts, along with the other methanogen guilds, were also positively associated with syntrophic bacteria, particularly in the Delta (Fig. S5e and f). This association could contribute to increased CH₄ production under oligohaline conditions if seawater influence were to promote the growth of sulfate-reducing syntrophs, which can produce methanogenic substrates such as acetate and hydrogen (32, 91–94, 102).

Finally, in agreement with our other correlation analyses, integrative SEMs indicated a particular influence of Type IIa methanotrophs (CH₄ oxidizers) on CH₄ fluxes within high CH₄-emitting Delta soils (Fig. 6). These models further indicated effects of the soil structure metrics bulk density and water-filled pore space on CH₄ consumption (Fig. 6). Although these models suggest potential metabolic interactions among microbial guilds linked to CH₄ fluxes, we acknowledge limitations of this statistical approach. Limited sample size impeded development of more complex and comprehensive models, including prediction of soil CO₂ fluxes from taxa or fermentative pathways (32). SEM model stringency also led to the elimination of potentially meaningful factors during model selection due to their covariance with other dominant features. Such factors included hydrogenotrophic methanogens (Fig. 2) which were closely linked to hydrogen production genes (Fig. S5c and d), Type I methanotrophs, and porewater DOC.

Conclusions

Our study of CH₄ fluxes and microbial metabolism across an estuarine wetland salinity gradient found that CH₄ fluxes were not a simple function of salinity or sulfate availability. Although CH₄ fluxes were generally lower as salinity increased, the highest fluxes were observed in non-tidal restored oligohaline wetlands, consistent with a meta-analysis of tidal marsh CH₄ fluxes elsewhere (18). These patterns may suggest that low-level salinity intrusion (i.e., <5 ppt) could increase CH₄ flux in tidal freshwater wetlands, while higher levels of salinization (i.e., >5 ppt) might instead decrease CH₄ fluxes [up until extreme hypersaline conditions (103)]. Our results further indicated that methanogenesis genes alone did not account for landscape patterns of CH₄ fluxes, suggesting mechanisms altering methanogenesis, methanotrophy, nitrogen cycling and ammonium release, and increasing decomposition and syntrophic bacterial populations each could contribute to potential increases in net CH₄ flux as a result of salinity intrusion into freshwater soils. Improved understanding of these influences on net CH₄ emissions could improve restoration efforts and accounting of blue carbon sequestration in estuarine marshes (47).

We suggest that the potential interactions among salinity, mineral N forms, and methanotrophy may merit further investigation in estuarine wetlands, particularly regarding the response of the dominant Type IIa methanotroph *Methylocystis*, and particularly regarding freshwater to oligohaline salinities. We report some of the highest CH₄ emissions in oligohaline sites; this is concerning because seawater intrusion will cause tidal freshwater estuarine wetlands to become oligohaline. More pristine reference sites may have older and more abundant organic matter with higher C:N compared to wetlands impacted by agricultural activity and thus may present different interactions between salinity and CH₄. This distinction might be critical for modeling efforts to scale up biogeochemical process interactions in estuarine wetlands (38, 89), given that agricultural nutrient loading impacts the majority of large estuaries globally. This is particularly important in the context of sea level rise and/or drought conditions, both of

which will increasingly cause estuarine wetlands to experience higher salinities than they have historically.

MATERIALS AND METHODS

Sample collection

Eleven estuarine wetland complexes (16 total sampling sites, Table S1) were sampled throughout the San Francisco Bay and Delta region, with salinities ranging from 0.4 to 61.5 ppt, between 29 August and 14 October 2013 (Fig. 1a). The salinity gradient encompassed sites with below, equal to, and above seawater salinities of ~35 ppt. This sampling time period corresponds to the window of peak methane flux in this region between July and October (72). All samples from the same sampling site were collected on the same day except for White Slough and wetland complexes with multiple sampling sites (Mayberry, Rush Ranch, and China Camp); at these four locations, CH₄ fluxes did not vary significantly by sampling date (analysis of variance, $P > 0.05$). Average daily air temperatures between the sampling dates ranged from 14.6°C to 25.4°C, while 8-cm-deep soil temperature ranged from 12.3°C to 21.0°C, based on data collected at the Mayberry and West Pond sites, which are the only two sites with established flux towers (Ameriflux/FluxNet IDs US-Myb, US-Twi) (72). Site descriptions, soil summary statistics, and sampling details are given in the supplemental text and Table S1. Delta sites range in mean salinity from 0.5 to 3.5 ppt, while Bay sites range in mean salinity from 6.9 to 41.7 ppt.

Wetlands sampled included established reference (undisturbed historic) sites and wetlands restored from former use as agricultural land or as dredged material placement sites. Two of the freshwater and oligohaline restored wetland complexes (West Pond and Mayberry) had been previously characterized for greenhouse gas fluxes (22, 36–38, 104), and one site had been studied for microbe-methane interactions (West Pond) (104). Sampling points were chosen at each site based on dominant vegetation for high and low marsh ecotones (Table S1), and three coring locations (A, B, and C) within plant community type were selected within a 10-m radius at each sampling point. While this sampling scheme likely captures only a fraction of the variation within each site, it enabled us to sample a larger number of sites and span the entire salinity gradient of the estuary at fine increments. Intact soil cores (5-cm diameter, 15 cm deep) were obtained, and following greenhouse gas measurements (see below), they were split into 0- to 5-cm-depth (D1) and 5- to 15-cm-depth (D2) sections. Methane production is known to occur at these depths in wetlands (105, 106). Each section was homogenized and frozen on dry ice in the field, then stored at –80°C prior to DNA extraction and soil geochemical analyses, both of which were conducted on both depths. An additional intact soil core was retrieved adjacent to each of the three DNA soil cores (ca. 50-cm distance) and was transported to the lab at ambient temperature for greenhouse gas analysis. All soils were flooded and surface water was decanted off the cores. Porewater was collected from polyvinyl chloride sampling pipes slotted at 5–10 cm beneath the soil surface, then filtered (0.45 μm) and frozen for subsequent analyses. *In situ* measurements of water pH, temperature, conductivity, dissolved oxygen, reduction-oxidation (redox) potential, and salinity (based on conductivity) were collected from the sediment core holes using a YSI Multi-Parameter Water Quality Sonde (Model 6920-v2; YSI Inc., Yellow Springs, OH, USA).

Greenhouse gas flux analyses

Intact soil cores (at least three per site) were analyzed for greenhouse gas production (CH₄, CO₂, and H₂O) using a Los Gatos Research greenhouse gas analyzer (GGA; Los Gatos Research, Mountain View, CA, USA), which measures CO₂ and CH₄ concentrations at 1 Hz. Cores were closed on the bottom with airtight caps and loaded into a 2-L glass Mason jar fitted with airtight tubing to allow continuous gas exchange with the GGA. Fluxes were determined from the linear slope of gas concentrations over the latter of two

consecutive 500-s intervals, with headspace ventilation for 100 s between cycles. Further details of these methods are given in the supplemental text.

Soil and porewater biogeochemical measurements

Soil carbon content, nutrient concentrations (total N and P, and extractable NH_4^+ , NO_3^- , and PO_4^{2-}), pH, and water content were measured for both the 0- to 5-cm-depth and 5- to 15-cm-depth soil samples at the UC Davis Analytical Lab following protocols listed in Table S2. Additionally, soil diethylenetriaminepentaacetic acid extractable metals (Fe, Cu, Mn, and Al) were measured on 5- to 5-cm horizons by the UC Davis Analytical Lab (Table S2). Detailed descriptions of soil chemical methods are also given in Hartman et al. (107). Filtered soil porewater samples were analyzed for total organic carbon (TIC/TOC analyzer) to determine DOC at the Aqueous Chemistry Laboratory at Lawrence Berkeley National Laboratory as described previously (104). Results from biogeochemical measurements are given in Table S3.

Soil DNA extraction and sequencing

Frozen soil samples were thawed at 4°C and homogenized, and approximately 0.5 g of wet soil sample was removed for DNA extraction from both the 0- to 5-cm (D1) and 5- to 15-cm (D2) soil core strata using the PowerLyzer PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). DNA yield was assessed with the Qubit (v.2.0) fluorometer (Invitrogen, Carlsbad, CA, USA). To determine microbial community composition, we amplified the V4 region of the 16S rRNA gene using barcoded primers 515 F (5'-GTGCCAGCMGCCGCGTAA-3') and 806 R (5'-GGACTACHVGGTTCTAAT-3') established by Caporaso et al. (108). Amplicon sequencing was performed following the JGI's standard protocols (detailed in the supplemental text), where 16S rRNA gene amplicons were diluted to 10 nM, quantified by qPCR, and sequenced on the Illumina MiSeq platform (2 × 300 bp, Reagent Kit v.3; Illumina Inc., Carlsbad, CA, USA).

16S rRNA gene amplicon sequences were analyzed using the iTagger (v.1.1) pipeline (109), which removed Illumina adapters and PhiX sequences, performed paired-end read assembly, read quality filtering, and chimera checking, and clustered reads into operational taxonomic units (OTUs) at 97% similarity. Taxonomic classification of OTUs was achieved using the "assignTaxonomy" function in the *dada2* R package (110) and the SILVA (v.138.1) reference database (111). Microbial sequence reads were further aggregated into functional guilds using taxonomic assignments of 16S rRNA gene reads for groups including acetoclastic, hydrogenotrophic, mixotrophic, and methyl-reducing methanogens, groups of microbes oxidizing CH_4 , ammonia, and nitrite, and sulfate-reducing bacteria. These assignments were based on monophyletic functional groups derived from taxonomic patterns in the literature, described in detail in the supplemental text, along with further details of amplicon sequence data processing methods. Mixotrophic methanogens are taxa that are capable of performing at least two different methanogenesis pathways and include Methanosarcinaceae (contain taxa that can perform one or more of all four pathways) and Methanobacteriaceae (contain taxa that can perform hydrogenotrophic methanogenesis or methyl-reducing methanogenesis) (44, 112, 113). Taxa in the Nitrospirota phylum were all considered to be nitrite-oxidizing bacteria, although some taxa (in the *Nitrospira* genus) have been recently discovered to perform complete ammonia oxidation (114).

Soil metagenomic shotgun sequence data were obtained using a 96-well plate-based DNA library preparation (detailed in the supplemental text) run on an Illumina HiSeq2500 sequencer using HiSeq TruSeq SBS (v.4) sequencing kits (2 × 150 or 2 × 250 run mode) at the Joint Genome Institute. Overall, shotgun sequencing libraries yielded ~5.3 Gbp per sample after contaminant and quality filtering. Unassembled FASTQ-formatted sequencing read data from each sample were submitted to the MG-RAST metagenome annotation server (60, 115), with details of the underlying bioinformatics algorithms described in the supplemental text. MG-RAST and Genomes OnLine Database accession numbers for the metagenomes are presented in Table

S3. Counts of functional annotations organized by the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) (61) were downloaded for each sample from the MG-RAST application programming interface (API) (60) using a custom Python script which merged annotations into a single table of counts for each KO for each sample.

Further annotation of unassembled shotgun sequence reads for specified microbial functional guilds was accomplished using the TreeSAPP (v.0.6.0) pipeline (116), which identifies open reading frames (ORFs), annotates gene function, and assigns taxonomy based on phylogenetic placement relative to reference sequences. FunGene (v.9.5) was used to download reference sequences. This approach was applied to methanogens (*mcrABG*), sulfate reducers (*dsrAB*), ammonia and CH₄ oxidizers (*amo/pmoABC*), and nitrite oxidizers (*nxrAB*), along with single-copy marker genes for DNA replication (*recA*, *rpoB*, and *RPS3A*), as detailed in the supplemental text. Parameters for the TreeSAPP *assign* command are stated in the supplemental text; the TreeSAPP “create” command for identifying sequencing reads homologous to each reference package used the default parameters. This analysis was particularly important for *pmoA-amoA*, which was not annotated by MG-RAST. While none of the other key carbon, nitrogen, phosphorus, or sulfur genes were missed by MG-RAST, it is possible that other genes were not annotated. TreeSAPP also performed better, in terms of correlations with 16S guild relative abundances, than *in silico* PCR, which was tested for *mcrA* and *pmoA* genes (Fig. S13) using the *mlas-mod-F/mcrA-rev-R* primers (117) and the A189-mb661 primers (118), respectively, implemented with the *pcr-seqs* command in the *mothur* software (119).

Statistical analyses

Statistical analyses and data visualizations were conducted using custom scripts developed in Python and R (120), which are publicly available on GitHub (https://github.com/cliffbueno/SF_microbe_methane). Greenhouse gas fluxes and soil chemical data were log-transformed prior to regression analysis (linear, segmented linear, and polynomial) and visualization using *ggplot2* (121) in R. Segmented linear regressions were performed with the “segmented” R package (122). Effects of depth and the nested categorical variables location and wetland status were tested with LME models with the R package “nlme” (123). Gene relative abundance data obtained from MG-RAST were normalized using the “DESeq2” package in R (124), and regressions of log₂ transformed DESeq2-normalized counts with environmental factors were compared while controlling false discovery rate of <0.05. Heatmap summary plots of gene-environment relationships were generated using the Seaborn library in Python. LASSO multivariate selection models (125) for predicting CH₄ fluxes from sets of soil chemical measurements, genes, and taxonomy-based functional guilds were implemented with the Scikit-learn Python package.

16S rRNA counts of OTUs were also normalized with the DESeq2 package in R to analyze taxonomic relative abundance, while center-log-ratio transformation implemented in the “zCompositions” R package (126) was used to analyze composition. Microbial community composition was analyzed with an Aitchison distance matrix calculated with the “compositions” R package (127), and PERMANOVA test implemented with the “vegan” R package (128), and visualized with principal component analysis. Different taxonomic levels were tested for correlations with methane. Finally, directional inter-relationships between CH₄ fluxes, decomposition, and microbial guilds were evaluated by structural equation modeling using the R package “lavaan” (129). Models to test mechanistic hypotheses about environmental and microbial drivers of methane flux were developed with methane generation and methane oxidation as composite variables that drive methane flux. SEMs were run for the entire data set as well as the Delta sites alone.

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Wyatt H. Hartman, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft | Clifton P. Bueno de Mesquita, Data curation, Formal analysis, Investigation, Visualization, Writing – review and editing | Susanna M. Theroux, Conceptualization, Data curation, Investigation, Methodology, Writing – review and editing | Connor Morgan-Lang, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review and editing | Dennis D. Baldocchi, Data curation, Methodology, Validation, Writing – review and editing | Susannah G. Tringe, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review and editing

DATA AVAILABILITY

Raw and processed metagenomic and 16S rRNA gene data and metadata are publicly available; Genomes OnLine Database/Integrated Microbial Genomes, National Center for Biotechnology Information, and Metagenomics Rapid Annotation using Subsystems Technology accession information is presented in Table S3. Biogeochemical data are presented in Table S4. All supplementary tables are also publicly available on Figshare ([10.6084/m9.figshare.24808383](https://doi.org/10.6084/m9.figshare.24808383)). All analysis scripts are available on [GitHub](#).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental Figures (mSystems00936-23-s0001.docx). Figures S1 to S13.

Supplemental Text (mSystems00936-23-s0002.docx). More methodological details.

Supplemental Tables (mSystems00936-23-s0003.xlsx). Tables S1 to S8.

Open Peer Review

PEER REVIEW HISTORY (review-history.pdf). An accounting of the reviewer comments and feedback.

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