2019

Response and recovery of syntrophic and methanogenic activity to saltwater intrusion in a tidal freshwater marsh soil

David J. Berrier Jr.
Virginia Commonwealth University

Response and recovery of syntrophic and methanogenic activity to saltwater intrusion in a tidal freshwater marsh soil

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

DAVID JAMES BERRIER JR.
Bachelor of Science
B.S., Virginia Commonwealth University, 2009

Major Professors:
RIMA B. FRANKLIN, PH.D.
Associate Professor, Department of Biology

SCOTT C. NEUBAUER, PH.D.
Associate Professor, Department of Biology

Virginia Commonwealth University
Richmond, Virginia
December 2019

Acknowledgments

I would like to acknowledge the continued encouragement and support I received from my family, friends, colleagues, and advisors during this graduate research. This includes Dr. Rima Franklin, Dr. Scott Neubauer, Joseph Morina, Dr. Joseph Battistelli, Dr. Ember Morrissey, Mary Coughter, Aoife Mahaney, Rana Mehr, and Chansothery Dang. They were always willing to offer feedback, guidance, and assistance. This work was also made possible with additional support from VCU's Rice Center, the National Science Foundation, and VCU's generous Graduate Teaching Assistantship.

Table of Contents

Page

List of Figures

Page

Abstract

RESPONSE AND RECOVERYOF SYNTROPHIC AND METHANOGENIC ACTIVITY TO SALTWATER INTRUSION IN A TIDAL FRESHWATER MARSH SOIL

David J. Berrier, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2019.

Major Professors:
RIMA B. FRANKLIN, PH.D.
Associate Professor, Department of Biology

SCOTT C. NEUBAUER, PH.D.
Associate Professor, Department of Biology

Tidal freshwater wetland soils contain large amounts of organic carbon, some of which is mineralized to carbon dioxide (CO_2) and methane (CH_4) by a diverse consortium of anaerobic microorganisms that includes fermenters, syntrophs, and methanogens (MG). These microbial groups are tightly linked and often rely on cooperative interspecies metabolisms (i.e., syntrophy) to survive. Environmental perturbations can disrupt these interactions and thus alter the rates and pathways of carbon cycling. One environmental change of particular concern in coastal wetlands is sea level rise, which can result in increased episodic saltwater intrusion events into these ecosystems. These events cause an influx of sulfate (SO_4^{-2}) to the soils and may stimulate sulfate-reducing bacteria (SRB), which can directly compete with syntrophs for energy sources (e.g., fermentation products such as butyrate). Since syntroph metabolism generates byproducts that serve as the energy source for many MG, this competition can have indirect negative effects on methanogenesis. In addition, SRB can directly compete with MG for these byproducts,

particularly formate, H_2, and/or acetate. The goal of this study was to understand how both MG and syntroph-MG consortia respond to and recover from SRB competition during an episodic saltwater intrusion event. To achieve this, microcosms containing soil slurry from a freshwater wetland were subjected to simulated saltwater intrusion, and metabolic inhibitors were used to isolate the activity of the various functional groups. This study focused on the breakdown of butyrate, which is a key energy source in syntroph-MG consortia metabolisms. The observed changes in butyrate breakdown rates and byproduct accumulation during butyrate degradation assays confirmed that butyrate breakdown was mediated through syntroph-MG consortia, and that formate, rather than H_2, was likely used as an electron carrier during syntrophic activity. Additions of SO_4^{-2} (as Na_2SO_4) to the freshwater microcosms stimulated SRB activity and shifted the MG community to favor acetoclastic members. These changes were accompanied by a 24% increase in CO_2 production and an 80% decrease in CH_4 production. Interestingly, when NaCl was added to achieve similar ionic strength, CH_4 production decreased by ~32%, suggesting SRB competition is not the only factor affecting methanogenesis. Butyrate degradation rates demonstrated that while SRB were strong competitors for butyrate, concurrent syntrophic metabolism was possible. Further, data show that SRB were poor competitors for acetate, which could explain the increase in acetoclastic MG. Following removal of SRB competition, CH_4 production recovered but only by ~50% after 28 days, which suggests that some MG communities in tidal freshwater wetlands may not be resilient to saltwater intrusion events. Over this same time, rates of syntrophic butyrate breakdown largely recovered, but butyrate breakdown resulted in the production of less CH_4 and acetate and more CO_2 and formate, indicating saltwater intrusion events may

lead to persistent changes in the byproducts and pathways of carbon breakdown in tidal freshwater wetlands.

Introduction

Wetlands are globally important ecosystems in terms of climate change because they play a large role in the cycling of carbon and greenhouse gases. For example, wetlands are estimated to store 15-25% of global soil carbon (Matthews and Fung 1987, Mitra et al., 2005, Conrad 2009, Neubauer 2013a). This soil carbon is decomposed by microbes, resulting in the production of carbon dioxide (CO_2) and methane (CH_4). Methane is of particular interest because it is a potent greenhouse gas, with 45 times the sustained-flux global warming potential of CO_2 over a time scale of 100 years (Neubauer and Megonigal 2015). Wetlands produce ~23% of the world's CH_4 (Conrad 2009), though rates vary considerably across wetland types. For instance, saltmarshes have relatively low rates of methanogenesis compared to freshwater wetlands (Odum 1988; Poffenbarger et al., 2011), a phenomenon that results from each environment supporting different microbial functional groups. In saltmarshes, sulfate (SO_4^{2-}) is abundant and can be used by sulfate reducing bacteria (SRB) as the terminal electron acceptor for anaerobic decomposition. Freshwater systems, in contrast, have a limited abundance of terminal electron acceptors, so decomposition generally proceeds more slowly via fermentation and methanogenesis.

Many coastal freshwater wetlands are experiencing increased saltwater intrusion events due to sea level rise induced by climate change. These saltwater intrusion events may shift the soil microbial communities and, consequently, change both the rates of methanogenesis and carbon cycling (Chambers et al., 2011; Weston et al., 2011; Neubauer et al., 2013; Helton et al., 2014). The majority of saltwater intrusion research has focused on the changes in tidal freshwater wetlands (TFW) during an intrusion event,

but has rarely explored the recovery of these systems after fresh conditions have returned. Incorporating recovery into our understanding of saltwater intrusion's effects on microbial communities and their carbon cycling is important as these systems will likely not experience sea level rise as just an overall increase in salinity, but as an increase in the frequency and duration of episodic saltwater intrusion events (Neubauer and Craft 2009; Moftakhari et al., 2015; Tully et al., 2019).

In freshwater wetland soils, a complex interacting consortium of microorganisms mediates decomposition across several steps. Primary fermentation breaks down large organic molecules into a variety of low molecular weight acids and alcohols. These primary fermentation products then serve as the substrates for secondary fermentation and methanogenesis through a series of reactions that are often thermodynamically interdependent. For example, the breakdown of many primary fermentation products (e.g., butyrate and propionate) is only made possible through the cooperative method of interspecies electron transfer between syntrophic bacteria and H_2/formate consuming (sometimes called "hydrogenotrophic") methanogens (McInerney et al., 2009; Stams and Plugge 2009). Specifically, syntrophic bacteria oxidize fermentation products by transferring electrons to an electron-carrier molecule (e.g., by reducing H^+ to H_2 and/or CO_2 to formate (HCO_2^-)), which is then consumed to low concentrations by hydrogenotrophic methanogens (Thiele and Zeikus 1988; De Bok et al., 2004; Stams et al., 2006; Stams and Plugge 2009) (Fig. 1). Syntrophic bacteria and hydrogenotrophic methanogens often form aggregates (De Bok et al. 2004; Stams and Plugge 2009) where the close proximity between microbes allows for the electron-carrier molecules to be rapidly consumed to low enough concentrations that the metabolism of the syntrophs

remains energetically favorable (Sieber et al., 2012, Krylova and Conrad 1998, Hoehler et al., 2001). This syntroph-methanogen interaction is thought to be an important process in the regulation of methanogenesis (Conrad et al., 1989; Bae and McCarty 1993; Stefanie et al., 1994; McInerney et al., 2009) and an integral component of organic matter decomposition in anaerobic environments (McInerney et al., 2009) like those found in freshwater wetland soils.

Most of our knowledge of syntroph-methanogen interactions comes from simpler systems such as mixed cultures (Dwyer et al. 1988) and bioreactors (De Bok et al. 2004, McInerney et al. 2008). We have less information about how these groups actually interact in wetlands systems (Conrad 1999), and we know almost nothing about how they respond to and recover from saltwater intrusion events. One syntrophic metabolism most likely to be affected by saltwater intrusion events is butyrate breakdown. In both freshwater and saline environments, many of the organic matter decomposition pathways include butyrate as an intermediate (Parkes et al. 1989; Rothfuss and Conrad 1992; Glissmann and Conrad 2000; Chauhan et al., 2006, Galand et al., 2010). In freshwater wetlands, the only known genera capable of syntrophic butyrate breakdown, *Syntrophomonas* and *Syntrophus*, are both obligate syntrophs (McInerney et al., 2008; Plugge et al. 2011). However, in saline environments where sulfate (SO_4^{-2}) is naturally occurring, SRB can thrive and may outcompete butyrate-consuming syntrophs, thereby affecting rates of methanogenesis and the relative production of CO_2 vs. CH_4. In addition to competition for butyrate with syntrophic fermenters, SRB can directly compete with methanogens for compounds like H_2, formate, and acetate (Stams 1994; Muyzer and Stams 2008; Chambers et al., 2011) (Fig. 1). These competitive interactions may develop

in TFW soils following a saltwater intrusion event and, since saltwater intrusion may be an episodic phenomenon (Neubauer and Craft 2009), the resiliency of syntroph–methanogen relationships to this competitive stress may be important in determining rates and pathways of carbon cycling once freshwater conditions have returned.

The goals of this study were to: (i) understand how both methanogens and the syntroph-methanogen consortia respond to SRB competition during an episodic saltwater intrusion event, and (ii) to determine whether syntroph-methanogen activity and carbon mineralization rates recovered after SRB competition was removed. These objectives were addressed in a microcosm study where additions of SO_4^{-2} to freshwater soil slurries were used to stimulate SRB activity. Metabolic inhibitors were used to selectively limit the activities of SRB, methanogens, and/or syntrophs, and linkages between each functional group were tracked by monitoring CH_4 and CO_2 production rates and shifts in methanogen abundance. In addition, butyrate degradation assays were performed in which the rate of butyrate breakdown and the accumulation of the products acetate and formate were used to assess syntrophic associations. After ~1 month of exposure to elevated SO_4^{-2}, microcosms were amended with molybdate (MoO_4^{-2}) to suppress SRB activity, allowing us to evaluate the potential recovery of syntroph-methanogen linkages and carbon cycling following an episodic saltwater intrusion event.

Methods

Microcosm setup

The soil used for this experiment was obtained from Cumberland Marsh, a tidal freshwater wetland located on the Pamunkey River in Virginia (latitude: 37.55723 ° N, longitude: 76.97277 ° W). The Cumberland Marsh plant community is dominated by obligate freshwater macrophytes such as *Peltandra virginica* and *Pontederia cordata*. Soil was collected on October 1, 2015, during low tide by first pushing aside the consolidated layer of plant debris and then carefully transferring the top 5 cm to an airtight plastic bag. A total of ~3 kg of soil was collected from several locations across two 40-m transects. The soil had a gravimetric moisture content of ~85%, a redox potential of -130 mV, and an organic matter content of 35%. The salinity of the porewater was <0.1 PSU (conductivity <0.2 mS cm^{-1}) and soil bulk density was ~0.2 g cm^{-3}.

Upon return to the lab, soil samples were combined and ~9 L of bulk slurry (30 g wet soil per 100 ml water) was made using deoxygenated (1 hr with N_2) site porewater. The slurry was manually homogenized, filtered through a 2.38 mm sieve to remove roots, and then aliquoted (100 ml) into glass serum bottles (170 ml). The serum bottles were then sealed with snap-on natural red rubber septa (13 x 20 mm, Wheaton Industries, Millville, New Jersey, USA) crimped with an aluminum seal. These microcosms were pre-incubated in the dark at 25 °C for 22 days prior to the "initial" sampling event (Fig.

2) to allow for the microbial communities to stabilize. All microcosm preparations took place inside an anaerobic chamber that was continually flushed with N_2.

Experimental design

During the "initial" sampling event, a subset of microcosms was destructively sampled to study soil properties, carbon mineralization rates, and the archaea abundance (details in the "Sampling of Bulk Soil Slurries" section below). A separate set of microcosms was used to study butyrate degradation pathways (Fig. 1)(details in the "Butyrate Assays" section). The remaining microcosms were randomly assigned to one of three treatments: fresh control (no change), SO_4^{-2} treatment, and NaCl treatment (Fig. 2). The SO_4^{-2} treatment was designed to stimulate SRB activity in order to study the competitive interactions between SRB, methanogens, and syntrophic bacteria. Using Na_2SO_4, the concentration of SO_4^{-2} was brought to 4 mM (versus <0.04 mM in fresh control microcosms) to mimic SO_4^{-2} availability in oligohaline waters (0.5 - 5 PSU; 1 - 9 mS cm^{-1}) (Weston et al. 2011). The NaCl treatment was designed to help determine whether any differences observed in the SO_4^{-2} treatment microcosms were due to the effects of SRB activity or due to the changes in ionic strength caused by adding Na_2SO_4. In these microcosms, the concentration of NaCl was brought to 12 mM to generate the same ionic strength as the Na_2SO_4 additions to the SO_4^{-2} treatment microcosms. All microcosms were then incubated for 25 days until the "intrusion" sampling event (Fig. 2), when a subset of microcosms from each treatment was removed for sampling and butyrate assays. Immediately following the "intrusion" sampling event, half of the remaining SO_4^{-2} treatment microcosms was supplemented with Na_2MoO_4 (final

concentration of 2.5 mM MoO_4^{-2}) to create a "recovery" treatment. Molybdate is well established as an inhibitor of SRB (Elshahed and McInerney 2001) and its addition to SO_4^{-2} treatment microcosms allowed us to study how methanogens and syntrophic bacteria recovered once SRB competition was removed. The remaining microcosms were allowed to incubate for an additional 28 days until the "recovery" sampling event (Fig. 1). Sufficient microcosms were established at the start of the experiment to allow five replicates for soil analysis for each treatment and time point, and three replicates for each inhibitor addition in the corresponding butyrate assays.

Sampling of bulk soil slurries

At each sampling event, five microcosms from each treatment were destructively sampled to determine carbon mineralization rates (CO_2 and CH_4 production), soil pH, salinity, and archaea abundance. These microcosms are referred to as bulk soil slurries throughout the paper to distinguish them from microcosms used in butyrate assays (described below). Rates of CH_4 and CO_2 production were measured using methods similar to Neubauer et al. (2005). First, each microcosm was shaken and the headspace flushed with N_2 gas for 30 minutes. Gas samples were collected 4-5 times over the next ~48 hr by injecting 8 ml of N_2 gas and immediately withdrawing an equal volume from the headspace with a needle and air-tight syringe. Gas samples were stored in 3 ml Labco Exetainers® (Lampeter, Ceredigion, United Kingdom) and later analyzed on a Shimadzu GC-2014 gas chromatograph (Shimadzu Scientific Instruments, Columbia, Maryland, USA); CH_4 was measured with a flame ionization detector and CO_2 was measured with a

thermal conductivity detector (Shimalite Q column, He carrier; Shinwa Chemical Industries Ltd., Fushimi-ku, Kyoto Japan).

After the gas sampling was complete, the microcosms were opened and the contents were transferred to a sterile plastic bag. The pH and conductivity of the soil slurries were then measured using a SevenGo Duo pro Model SG78 meter (Mettler Toledo, Columbus, Ohio, USA). Soil slurries were immediately transferred to a -80 °C freezer for storage until DNA extraction could be performed using the MoBio PowerSoil DNA Isolation Kit following manufacturer's instructions (Carlsbad, California, USA).

These DNA extracts were analyzed using quantitative PCR (qPCR) targeting conserved regions of the *16S rRNA* gene. Using the primer pair Arch967F (5'AAT TGG CGG GGG AGC AC 3') and Arch1060R (5' GGC CAT GCA CCW CCT CTC 3')(Karlson et al., 2012), we targeted total archaea, which is the domain where all methanogen species are located (Ferry 2010); we considered abundance of this gene as a proxy for methanogen abundance. We also measured the relative abundance of *Methanosaetaceae* (MST), which encompass all known obligate acetoclastic methanogens (Ferry 2010), using Mst702F (5' TAA TCC TYG ARG GAC CAC CA 3') and Mst862R (5' CCT ACG GCA CCR ACM AC 3')(Yu et al. 2005). The ratio of MST 1*6S rRNA* genes copies to archaea *16S rRNA* gene copies was then used as an estimation of the fraction of the methanogen community that are not involved in syntrophy. All qPCR reactions (15 µl, using 4 ng template DNA) were run in triplicate using SsoAdvanced SYBR Green qPCR Supermix (BioRad, Hercules, California, USA) and a Bio-Rad CFX384™Real-Time System C1000 thermal cycler; data were analyzed using Bio-Rad CFX Manager 3.1. Standard curves were constructed with environmental clones

(whose sequences had been confirmed) generated from amplicons using the above primers and a pGEM®-T Easy Vector System II (Promega; Madison, Wisconsin, USA). Reaction mixtures targeting total archaea included 0.3 μM of each primer; the thermal cycling conditions were: 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C (efficiency = 101 %, r^2 = 0.99). Reaction mixtures targeting MST included 0.5 μM of each primer and used the following thermal cycler conditions: 95 °C for 10 min followed by 45 cycles of 10 s at 94 °C and 30 s at 60 °C (efficiency = 90 %, r^2 = 0.99). In all instances, products were confirmed by examining the melt curve.

Butyrate assays

To investigate the breakdown of butyrate and its byproducts (Fig. 1), an additional subset of microcosms was selected at each sampling event to receive butyrate additions to a final concentration of 2.5 mM (Fig. 2). These microcosms were then divided into groups (n=3 in each) to study the activity of various microbial functional groups using metabolic inhibitors: methanogens were inhibited using 50 mM BESA (Liu et al. 2011), SRB were inhibited using 2.5 mM Na_2MoO_4 (Elshahed and McInerney 2001), and syntrophs were inhibited by adding H_2 every other day to a partial pressure greater than 1.5 kPa (Dwyer et al. 1988). The concentrations and incubation times needed for effective metabolic inhibition via BESA and MoO_4^{-2} were determined experimentally (*data not shown*). BESA was added ~12 days prior and MoO_4^{-2} was added 12 hours prior to the start of the butyrate degradation assays to allow the inhibition to take effect.

After butyrate and inhibitor additions, gas samples were taken from the headspace approximately every other day for ~7 days using the methods described above. After each headspace gas sample was taken, 1 ml of slurry was sampled using a needle and syringe.

The pH of the slurry sample was determined and used for the calculation of total inorganic carbon concentrations (details below). The concentrations of butyrate, acetate, formate, and SO_4^{-2} were determined from the soil slurry samples after filtration (0.22 μm pore size) using a Dionex ICS-5000+ ion chromatograph (Thermo Scientific Inc.; Waltham, Massachusetts, USA) equipped with a Dionex IonPac™ AS11-HC analytical column (2 × 250 mm) with the following elution gradient: 1 mM KOH from 0-8 min, a ramp from 8-30 mM KOH from 8-28 min, then a ramp from 30-60 mM KOH from 28-35 min. The ion chromatography results were interpreted using Chromeleon® Chromatography Data System version 7.2.0.3765.

Calculations and statistics

Treatment effects for bulk soil slurries

For each microcosm at each sampling point, total CH_4 production was examined as the sum of gaseous and dissolved CH_4. The latter parameter was determined using the measured CH_4 partial pressure and Henry's law. Total CO_2 production rates were calculated in a similar way using measured slurry pH to account for speciation between dissolved carbonate and bicarbonate. Rates of total CH_4 production and total CO_2 production were then calculated using linear regression; samples for which $r^2 < 0.85$ were excluded from the final dataset. Total carbon mineralization rate was the sum of the CH_4 production rate and the CO_2 production rate. The CH_4 portion of the total carbon mineralization rate was considered methanogenesis' contribution to total carbon mineralization.

Analysis of variance (ANOVA) was used to test for treatment effects on total (gaseous + dissolved) CH_4 and CO_2 production rates, methanogenesis' contribution to total carbon mineralization, methanogen community abundances, and salinities. Because preliminary comparisons of individual treatments across sampling events (e.g., CO_2 production rates for the fresh control at the "intrusion" and "recovery" sampling events) revealed few significant differences, data from the "intrusion" and "recovery" sampling events were combined for this analysis. Whenever a significant ANOVA results was obtained ($\alpha = 0.05$), Tukey's HSD test was used for post hoc comparisons. All statistical analyses were performed using JMP® Pro, version 12.2.0 (SAS Institute Inc., Cary, North Carolina, USA), with the exception of the principal component analysis described below.

Butyrate assays

Butyrate additions were performed and the accumulation of acetate, formate, CO_2, and CH_4 was tracked for seven days. The use of metabolic inhibitors allowed us to monitor product formation associated with the various microbial functional groups (Fig. 1). Results are presented as the proportion of carbon found in each form (butyrate, acetate, formate, CO_2, and CH_4) on each sampling day, relative to the total measured at start of the assay. The rate of butyrate degradation during the butyrate assays was calculated using linear regressions; samples for which $r^2 < 0.85$ were excluded from the final dataset. Data from day 7 were further analyzed to compare accumulation of each product across the various treatments/inhibitors using a one-factor ANOVA and Tukey's HSD post hoc tests. Principal component analysis (PCA) was used to visualize these

results based on the overall changes in the distribution of the major carbon compounds across treatments. Separate PCAs were performed for the "intrusion" and "recovery" sampling events using the PAST statistical package (Version 3, Hammer et al. 2001); calculations were performed using the normalized variance-covariance matrix.

Given that butyrate is broken down to acetate, which itself can be further broken down to CO_2 and CH_4 (Fig. 1), the amount of acetate in each microcosm reflects the balance between rates of butyrate breakdown and acetate consumption/utilization. The percent of generated acetate that was consumed was calculated from the concentrations of acetate in the microcosms (measured) and the expected production of acetate (based on the measured loss of butyrate, with all concentrations in molar units):

$$Acetate\ Consumed\ \% = \left(1 - \frac{Acetate\ Present}{Butyrate\ Loss * 2}\right) \times 100\ \%$$

Results

Initial sampling event

At the "initial" sampling event, the CH_4 production rate (0.12 ± 0.02 μmol hr^{-1}, mean ± standard error) in the microcosms was ~5.6% of the total carbon mineralization rate (2.2 ± 0.1 μmol hr^{-1}). There was no measurable butyrate and negligible concentrations of acetate (3.6 ± 0.3 μM) and formate (2.8 ± 0.2 μM). The soil was slightly acidic (6.5 ± 0.1 pH) with a salinity of 0.28 ± 0.03 PSU (conductivity of 0.6 ± 0.1

mS cm^{-1}), and a low sulfate concentration in the porewater (40.9 ± 0.8 μM). The abundance of archaea *16S rRNA* genes was 2.8 (± 0.2) × 10^5 copies per ng of DNA and the MST:Archaea ratio was 0.75 ± 0.05.

For the butyrate assays performed at this initial sampling event, the major breakdown products were acetate, formate, CO_2, and CH_4, with the rate of butyrate breakdown and the distributions of the end products varying depending on which inhibitor was added. When no inhibitor was added (Fig. 3A), the rate of butyrate loss was fairly linear with 9.1 ± 0.1 % of the original measured carbon lost per day. After 7 days of incubation, 29% of the original carbon remained as butyrate with the balance forming acetate (20%), formate (3%), CO_2 (18%), and CH_4 (19%). There was a fraction of the added butyrate carbon unaccounted for (11%), which likely remained in microbial biomass. In contrast to the microcosms with no inhibitor, the addition of BESA to inhibit methanogenesis led to much slower non-linear butyrate breakdown with only 82% remaining at the end of the assay (Fig. 3B). Most of the lost butyrate accumulated as acetate (7%) and CO_2 (9%), with negligible amounts as formate and CH_4 (~0.5% each). When H_2 additions were used to inhibit syntrophic bacteria (Fig. 3C), the rate of butyrate loss (8.8 ± 0.2 % day^{-1}) was similar to what was observed in the absence of any inhibitor (Fig. 3A). At the end of the 7 days of incubation, 31% of the original carbon measured remained as butyrate, while the other measurable amounts remained as acetate (20%), formate (2%), CO_2 (9%), or CH_4 (17%). Although formate was always a small fraction of the total carbon in the microcosms, significantly more formate accumulated when rates of butyrate breakdown were high (no inhibitor and H_2 addition microcosms) than when methanogenesis was inhibited using BESA (Fig. 4A).

Intrusion and recovery in bulk soil slurries

Measurements of the bulk soil slurries during the "intrusion" and "recovery" sampling events were pooled to analyze the effect of SRB and to determine the ability of the soil slurries to recover in terms of gas production and archaea abundance (Fig. 5). During this time, slurry salinity in the fresh controls (0.3 PSU, 0.6 mS cm^{-1}) was significantly (ANOVA $p < 0.05$) lower compared to the other treatments (SO_4^{-2} treatment: 0.5 PSU, 1.0 mS cm^{-1}; recovery and NaCl treatments: 0.6 PSU, 1.2 mS cm^{-1};) with little variability across sampling events or between replicate microcosms (all standard errors <0.01 PSU, < 0.01 mS cm^{-1}).

Methane production was suppressed in all three treatments relative to the fresh control microcosms (Fig. 5A; $p < 0.05$). The effect was much greater when SO_4^{-2} was added to stimulate SRB activity (~80% decrease) compared to the NaCl additions (32%), where ionic strength but not SO_4^{2-} availability was altered. In microcosms recovering from stimulated SRB activity, the CH_4 production remained reduced by ~50% compared to fresh control microcosms and reduced by ~22% compared to NaCl treatment microcosms (Fig. 5A; $p= 0.09$). The contribution of methanogenesis to the rate of total carbon mineralization in recovery microcosms (4.0%) did increase compared to SO_4^{-2} treatment microcosms (1.5%). However, the contribution of methanogenesis to the rate of total carbon mineralization remained depressed compared to the fresh and NaCl microcosms (8.5% and 7.1 % respectively; $p < 0.05$).

Treatment effects on CO_2 production rates were more modest (Fig. 5A). The addition of SO_4^{-2} increased CO_2 production by 24% relative to the fresh control

microcosms, whereas changes in ionic strength associated with NaCl addition decreased CO_2 production but the effect was not statistically significant. As with CH_4 production, partial recovery of CO_2 production was evident. Rates decreased to a level between that of the microcosms with stimulated SRB activity and the fresh controls, and were not significantly different from either.

The abundance of archaeal *16S rRNA* genes did not vary significantly across treatments (ANOVA p=0.47; Fig. 5B). However, the relative abundance of acetoclastic methanogens, represented using the MST:Archaea ratio, increased with stimulated SRB activity (SO_4^{-2} treatment: 0.78 ± 0.05 versus fresh control: 0.57 ± 0.04; $p<0.05$). After SRB were inhibited for 28 days, the ratio (recovery treatment: 0.66 ± 0.03) decreased some but was not significantly different from any of the other treatments (Fig. 5B).

Butyrate breakdown during intrusion sampling event

There were three experimental treatments at the time of the "intrusion" sampling event: fresh control, NaCl treatment, and SO_4^{-2} treatment (Fig. 2). Butyrate was added to triplicate microcosms of each type, and the relative abundance of the various carbon compounds was tracked for 7 days (Fig. 6A, 6C, and 6D). In the SO_4^{-2} treatment, where SRB activity had been stimulated, the rate of butyrate loss increased by 60% (SO_4^{-2} treatment: 13.1 ± 0.1 % loss of original measured C day^{-1}) compared to all microcosms without stimulated SRB (8.2 ± 0.1% day^{-1}; ANOVA, $p<0.05$). In addition, stimulating SRB activity resulted in a third less CH_4 production (SO_4^{-2} treatment: 9%; fresh control 13%; Fig. 6A & D) and three times more CO_2 production (SO_4^{-2} treatment: 55%; fresh control: 19%) relative to the fresh control microcosms (ANOVA, $p<0.05$). The portion of

formate in the microcosms (Fig. 4B) with increased SRB showed a pattern similar to the fresh controls until ~day 7 when it dropped to <0.7% (attributed to butyrate depletion), while the percent acetate removal tended to be similar to the fresh control throughout the incubation (Fig. 7A).

For the SO_4^{-2} treatment, two additional butyrate assays were set up to help disentangle the relative activity of methanogens (inhibited by BESA addition, Fig. 6F) and SRB (inhibited by MoO_4^{-2}, Fig. 6E). The inhibition of methanogens caused the rate of butyrate loss to decrease 22% (to 10.1 ± 0.4% day^{-1}; ANOVA, $p<0.05$), and production of CH_4 to essentially stop (SO_4^{-2} treatment & BESA: <0.2%; Fig. 6F). The inhibition of methanogens also resulted in a decrease in CO_2 production by approximately half (SO_4^{-2} treatment & BESA: 25%; SO_4^{-2} treatment: 54%; Fig. 6D & F), and dramatically less formate accumulated throughout the incubation (SO_4^{-2} treatment & BESA < 0.6 %; ANOVA, $p<0.05$; Fig. 4B). The MoO_4^{-2} inhibition of SRB in the SO_4^{-2} treatment during the butyrate assays caused butyrate loss to decrease by 40% (SO_4^{-2} treatment: 13.1 ± 0.1 % loss of original measured C day^{-1}; SO_4^{-2} treatment & MoO_4^{-2}: 7.6 ± 0.1 % loss of original measured C day^{-1}). The inhibition of SRB also resulted in a decrease in CO_2 by approximately half (SO_4^{-2} treatment & MoO_4^{-2}: 28%; SO_4^{-2} treatment: 54%; Fig. 6D & E) but no change in CH_4 accumulation (SO_4^{-2} treatment & MoO_4^{-2}: 10%; SO_4^{-2} treatment: 9%).

Similar additions of MoO_4^{-2} to microcosms from the fresh control treatment (Fig. 6B) yielded few differences in the profile of carbon compounds produced compared to the butyrate assays for either the fresh control (Fig. 6A) or the NaCl treatment (Fig. 6C).

This helps confirm that the changes brought on by the MoO_4^{-2} addition were directly related to a change in activity of SRB.

Butyrate breakdown during recovery sampling event

At the "recovery" sampling event, the results of the butyrate assays for the fresh control (Fig. 8A), NaCl treatment (Fig. 8D), and SO_4^{-2} treatment (Fig. 8C) were mostly similar to what was observed at the "intrusion" sampling (Fig. 6A, 6C, and 6D respectively). The rate of butyrate loss in microcosms with stimulated SRB activity remained high (SO_4^{-2} treatment: 11.7 ± 0.1 % day^{-1}) compared to microcosms that had never received a SO_4^{-2} addition (fresh control and NaCl treatment: 8.2 ± 0.1% day^{-1}; ANOVA, $p<0.05$). Stimulated SRB activity still resulted in less CH_4 production (SO_4^{-2} treatment: 9 %; fresh control 15%) and more CO_2 production (SO_4^{-2} treatment: 51 %; fresh control 22%) than in the fresh control microcosms (ANOVA, $p < 0.05$). However, by the time of the recovery sampling event, stimulated SRB activity had resulted in ~50% less formate (SO_4^{-2} treatment: 1.2 %; fresh control 2.2%; Fig. 4C) and a smaller percent acetate removal (SO_4^{-2} treatment: 63 %; fresh control: 73%; Fig. 7B) than in the fresh control microcosms (ANOVA, $p<0.05$).

In microcosms recovering from stimulated SRB activity (i.e., “recovery” treatment, in which SRB were exposed to MoO_4^{-2} for 28 days), the rate of butyrate loss was ~8% less than microcosms that had never received a SO_4^{-2} addition (Recovery treatment: 7.5 ± 0.2 % day^{-1}, fresh control and NaCl treatment: 8.1 ± 0.1% day^{-1}; ANOVA, $p<0.06$). The accumulation of carbon components in this treatment (Fig. 8B) differed from both the microcosms with stimulated SRB activity (Fig. 8C) and those

without (fresh control and NaCl microcosms; Fig. 8A and 8D). Microcosms recovering from stimulated SRB activity had increased percent acetate removal (Fig. 7B) and higher rates of formate accumulation (Fig. 4C) compared to all other treatments. Microcosms recovering from stimulated SRB activity did not produce significantly more or less CH_4 than either microcosms with stimulated SRB activity or fresh controls (Fig. 8). However, microcosms recovering from SRB activity did produce more CO_2 than fresh control microcosms and less than microcosms with stimulated SRB activity.

The short and long-term recovery of syntroph-methanogen activity from stimulated SRB activity can be examined by comparing the recovery treatment (Fig. 8B) to the SO_4^{-2} treatment to which MoO_4^{-2} was added 12 hours before butyrate assays (Fig. 8E). Both showed a similar rate of butyrate loss (recovery treatment: 7.5 ± 0.2 % day^{-1}, SO_4^{-2} treatment & MoO_4^{-2}: 7.5 ± 0.1 % day^{-1}) and no significant differences in the accumulation of carbon components (i.e., acetate, formate, CH_4, and CO_2; ANOVA, $p > 0.05$). Further, these two sets of microcosms had similarly high rates of formate production (Fig. 4C) and acetate consumption (Fig. 7B).

Principal component analysis

The PCA was used to help visualize the differences in the distribution of carbon compounds across the different microcosms at the end of each butyrate assay. For the intrusion sampling event (Fig. 9A, 84% of total variance explained), microcosms clustered into three distinct groups. Microcosms without active SRB were all characterized by greater butyrate, formate, and CH_4. Microcosms with active SRB but inhibited methanogens (BESA addition) comprised another group with decreased CH_4

production and increased acetate accumulation. Microcosms with active SRB and no inhibitor comprised the third group, which had the highest levels of butyrate breakdown and the greatest accumulation of CO_2.

For the recovery sampling, microcosms also separated into three different groups (Fig. 9B, 93% of total variance explained). Microcosms that did not experience SO_4^{-2} additions (fresh control and NaCl treatment) form a cluster that is characterized by greater CH_4 and less CO_2 accumulation. Microcosms that had experienced SO_4^{-2} additions but whose SRB community were inactive (the "recovery" treatment and the "SO_4^{-2} treatment" to which MoO_4^{-2} was added as part of the butyrate assay) formed a second cluster based on their relatively lower rate of butyrate degradation, greater accumulation of formate and CO_2 and their decreased accumulation of CH_4 and acetate. The third group was comprised of microcosms with active SRB communities and was characterized by less butyrate, formate, and CH_4; and more acetate and CO_2.

Discussion

The current framework for understanding how saltwater intrusion affects methanogenesis in TFWs mainly considers how increased SRB activity will impact methanogens based on substrate free energy yields, and largely neglects how SRB activity may affect broader carbon mineralization pathways and disrupt the tightly coupled microbial interactions that govern methane production (e.g., syntroph-methanogen interactions and competition for fatty-acids). This study begins to address these undetermined effects and demonstrates that SRB competition partially disturbs syntroph-methanogen breakdown of butyrate (i.e., a common fermentation product) in

our TFW system. This is also reflected in an increase in the proportion of the methanogenic community that does not participate in tightly coupled syntrophy (i.e., acetoclastic methanogens).

In addition, this work examines the ability of methanogenesis and carbon mineralization pathways to recover after saltwater intrusion has receded, which is relevant given TFW systems will likely experience sea level rise as an increase in the frequency and duration of saltwater intrusion events rather than a simple steady increase in salinity. Although we found syntrophic butyrate breakdown largely recovered following the removal of SRB competition, there was an alteration to the accumulation of byproducts from syntrophic butyrate breakdown and incomplete recovery in terms of methanogenesis' contribution to total carbon mineralization. These results indicate that, even after increased SRB activity has abated, intrusion events may have lasting effects on carbon mineralization pathways.

Syntrophy in TFWs

Syntrophic bacteria are able to metabolize fatty acids like butyrate because the concentration of breakdown products, specifically formate or H_2, is kept below inhibitory concentrations via methanogen consumption. This syntroph-methanogen cooperation appears to be very important in our study system. When methanogens were inhibited, only a small fraction of added butyrate was metabolized (Fig. 3B), whereas slurries with active methanogens readily consumed butyrate (Fig. 3A). Interestingly, addition of excess H_2 to the slurries with active methanogens did not suppress syntrophic butyrate breakdown (Fig. 3C), which suggests H_2 was not the main electron carrier for these

syntrophic associations. Instead, this syntrophic metabolism appeared to be mediated through the consumption of formate. If that were the case, we would expect to see little to no formate accumulate in the presence of an intact syntroph-methanogen consortia, and accumulation of formate at inhibitory levels in microcosms without active methanogens. Our data show the opposite pattern – with greater accumulation of formate in both treatments with active methanogens compared to the soils with a suppressed methanogen community (Fig. 4A) – and it is puzzling why the higher levels of formate accumulation did not limit butyrate consumption.

One possibility for these counterintuitive results is that methanogenic consumption of formate took place rapidly in microbial aggregates, which shielded syntrophs from inhibitory formate accumulation in the immediate microenvironment. Similarly, Krylova and Conrad (1998) observed limited inhibition of propionate breakdown when both formate concentrations and H_2 additions were measured well above inhibitory levels (ΔG values of +60 kJ mol^{-1}) and also hypothesized that microbial aggregates shielded syntrophic propionate degraders from inhibitory concentrations.

Response to saltwater intrusion in bulk soil slurries

Additions of SO_4^{-2} stimulated SRB activity and increased CO_2 production by ~ 25% while decreasing CH_4 production by ~80% (Fig. 5A), which is consistent with previous studies on TFWs (Weston et al., 2006; Neubauer et al., 2013a; Neubauer 2013b). One commonly considered explanation for the decrease in CH_4 production is that methanogen activity was suppressed due to direct competition with SRB for substrates (i.e., H_2, formate, and acetate), a hypothesis that is consistent with thermodynamic

predictions based on the relative free energy yields of the two processes (Muyzer and Stams, 2008). Additionally, the decrease in CH_4 production could also be an indirect response of the methanogens to SO_4^{-2} additions that is controlled by competition between syntrophs and SRB. If SRB outcompete syntrophs for fermentation products, syntrophic production of H_2/formate will be limited and therefore restrict the availability of these electron donors to methanogens.

Our results suggest that short-term saltwater intrusion events do not necessarily alter methanogen abundance, but shift the composition of these communities towards acetoclastic genera (Fig. 5B). Acetoclastic methanogens do not participate in interspecies electron transfer and instead convert acetate directly to CH_4 and CO_2 without benefiting syntrophs. The increased dominance of these organisms after the SO_4^{-2} addition may represent a diversion of fermentation products away from syntrophic-methanogen consortia to SRB, and a disruption of syntroph-methanogen interactions. Additionally, the increase in the relative abundance of MST may be because acetoclastic methanogens in the family MST are more successful competitors with SRB than H_2/formate utilizing methanogens. Methanogens in the MST family have a high affinity for acetate and have a low minimum acetate concentration threshold required for growth (Smith and Ingram-Smith, 2007) making them well suited to compete with SRB (Omil et al., 1998; Stefanie et al., 1994). In contrast, SRB can quickly outcompete hydrogenotrophic methanogens for H_2/formate (Stefanie et al., 1994) or indirectly limit methanogens access because SRB do not produce H_2/formate when consuming fermentation byproducts if SO_4^{-2} is available (Martins and Pereira, 2013). However, these competition dynamics may change over longer exposure periods (i.e., multiple months to a year). For example, Dang et al. (2019)

found that when fresh water marsh soils were transplanted into a saltwater marsh, CH_4 production recovered significantly over 1 year and was correlated with the relative abundance of 3 orders of hydrogenotrophic bacteria that were not prevalent in either the fresh or salt marsh soils.

This study shows that increased ionic strength can also contribute to decreases in CH_4 production (Fig. 5A), though to a much lesser extent than competition with SRB. The salinity in the study site from which our soils were collected is consistently low (<0.8 mS cm^{-1} over the last 2 years, unpublished data), and it may be that the genera in the methanogen community were incapable of acclimating to the induced salinity stress. Other studies have observed either a decrease in methanogenesis (Chambers et al., 2011) or no response (Baldwin et al., 2006) to similar NaCl additions to freshwater wetland soils. The range of methanogen salinity tolerance is large (Patel and Roth 1977), and selective pressure from regular saltwater intrusion events may determine the resistance of the initial methanogen community to salinity stress.

Saltwater intrusion effects on syntrophy

Under freshwater conditions, there was significant breakdown of butyrate via the syntroph-methanogen consortia (see dark blues bars in Fig. 3A, 7A, and 8A). When SO_4^{-2} was added to stimulate SRB, the rate of potential butyrate breakdown increased by ~50% (Fig. 6D and 8C). Although stimulated SRB activity did greatly diminish the role of syntroph-methanogen consortia in butyrate breakdown, syntroph-methanogen consortia still remained active in the face of competition from SRB, accounting for 22% of butyrate breakdown (compare butyrate loss in Fig. 6D, when syntroph-methanogen consortia were

active, and Fig. 6F, when syntroph-methanogen consortia are inhibited). The diminished role of syntrophy in microcosms with stimulated SRB is also evident when tracking the concentration of the putative syntroph electron carrier formate; within seven days of butyrate additions the concentration was significantly less than in those microcosms without stimulated SRB activity (Fig. 4B & C). The observed contribution of the syntrophy-methanogen consortia may be slightly exaggerated in these assays because the excess available butyrate may have decreased the competitive pressure from SRB compared to *in situ.* The diminished but persistent activity of syntrophs in the face of SRB competition is an especially important finding in respect to the resilience of butyrate metabolisms to saltwater intrusion events in TFW soils. This is because *Syntrophomonas* and *Syntrophus*, the only known genera capable of syntrophic butyrate breakdown, are both obligate syntrophs (McInerney et al., 2008; Plugge et al. 2011) and must either successfully compete with SRB or go dormant during saltwater intrusion events. In contrast, other syntrophic metabolisms, such as propionate breakdown, can be performed by SRB in the genus *Syntrophobacter*, who are capable of switching from SO_4^{-2} reduction to a syntrophic metabolism when SO_4^{-2} is absent (McInerney et al., 2008; Plugge et al., 2011); this metabolic flexibility likely provides functional stability in response to saltwater intrusion events.

In contrast with how stimulated SRB competed with syntrophs for butyrate, stimulated SRB did not appear to compete as aggressively with methanogens for acetate. In fact, stimulating SRB activity increased the concentration of acetate (Fig. 9), as has been seen in other freshwater wetland soils where SRB were not associated with acetate consumption (Achtnich et al., 1995). This increase in acetate availability during

stimulated SRB activity may partially explain the relative increase in obligate acetoclastic methanogens (MST) in the bulk soil slurries in this experiment (Fig. 5B). In addition, when methanogens were inhibited in microcosms with stimulated SRB, the removal of acetate was significantly lower on day 3 (SO_4^{-2} treatment: 98%; SO_4^{-2} treatment & BESA: 82%; ANOVA $p < 0.05$; Fig. 7A) and was ~ 50% less by ~ day 11 (data not shown) suggesting methanogens were important consumers of acetate. Given these observations, acetoclastic rather than H_2/formate-utilizing methanogens may be more resistant to saltwater intrusion events. This hypothesis is consistent with the results from Omil et al. (1998), who showed persistence of acetoclastic methanogens in bioreactors during stimulated SRB activity. Even after 250 days of excess SO_4^{-2} additions, methanogens were still responsible for ~50% of acetate consumption. The lack of proclivity for acetate utilization by SRB in this and other studies may be related to the relatively lower energy yield per unit SO_4^{-2} for acetate when compared to other substrates ($\Delta G^{\circ\prime}$ kJ mol^{-1} of SO_4^{-2} for acetate: -47.6; propionate: -50.26; butyrate: -55.6; formate: -144.4; H_2: -151.9; lactate: -160.2; $\Delta G^{\circ\prime}$ values obtained from Muyzer and Stams (2008) except formate, which was calculated by Omil et al. (1998)).

Recovery following saltwater intrusion

In our system, the effects of stimulated SRB activity on carbon dynamics remained evident even after the 28-day recovery period. Specifically, rates of methanogenesis in recovery microcosms remained reduced by 50% compared to fresh controls and by ~ 22% compared to the NaCl treatment (Fig. 5A, $p = 0.09$). The persistent effect of stimulated SRB activity was also evident on methanogenesis's

contribution to total carbon mineralization. Methanogenesis only accounted for 4% of total carbon mineralization in recovery microcosms compared to 7-8% in the fresh control and NaCl microcosms

The recovery of carbon dynamics from saltwater intrusion events in freshwater wetlands is poorly understood and rarely examined. The limited data available give contradictory results. For example, Helton et al. (2014) and Dorwick et al. (2006) found that methanogenesis can recover from the effects on elevated SO_4^{-2} within only a few months, whereas Gauci et al. (2005) reported incomplete recovery two years after the end of SO_4^{-2} exposure. Part of this discrepancy could be due to the duration (e.g., 4 weeks for Dorwick et al. (2006) versus 1 year for Gauci et al. (2005)), magnitude (e.g., 4 mM SO_4^{-2} in this study versus ~0.6 mM in Helton et al. (2014)), or frequency of exposure (Neubauer and Craft 2009). Regular saltwater intrusion events of low magnitude may select for syntroph and methanogen communities capable of acclimating to both increased salinity and SRB competition, while sites that experience more intermittent intrusion events may be less resilient. The Cumberland TFW syntroph and methanogen communities had not experienced a saltwater intrusion event of the magnitude simulated in this experiment for 2 years or more (salinity was consistently measured <0.8 mS cm^{-1}), which may explain why, after SRB competition was removed for ~28 days, the rate of methanogenesis (Fig. 5A) and the contribution of methanogenesis to total carbon mineralization did not fully recover and suggests that intrusion events into more pristine freshwater wetlands systems may have a persistent effect on carbon dynamics.

In contrast to our findings for methane production, the rate of potential syntrophic butyrate breakdown mostly recovered from stimulated SRB activity; butyrate loss rates

were 7.5 ± 0.2 % day^{-1} in the recovery treatment compared to 8.1 ± 0.1% day^{-1} in the fresh control and NaCl treatments. The resilience of butyrate-utilizing syntrophs in TFW environments is plausible given that syntrophic-methanogen consortia appeared to remain active when SRB activity was stimulated (discussed above). Interestingly, despite seeing near complete recovery of butyrate breakdown rates after SRB activity was removed, pronounced changes in the accumulation and production of butyrate-breakdown *byproducts* remained. This is clearly visualized by the distinct grouping of recovery treatments from fresh control microcosms on the PCA ordination (Fig. 9B), and indicates that SRB competition may have lasting effects on how the microbial community utilizes the byproducts of fatty acid breakdown. One possible explanation for the greater accumulation of formate and the more depleted acetate concentrations in recovery microcosms is persistence of the shift towards acetoclastic metabolisms observed during the saltwater intrusion event. However, the MST:Archaea ratios (Fig. 5B) did not remain significantly elevated. This may reflect that DNA-based assays do not distinguish between active and dormant organisms; targeting RNA to better capture active methanogens may elucidate significant changes in methanogen community structure. Given the confined trophic nature of methanogens (i.e., the limited use of substrates) and the conservation of methanogenic metabolisms to a monophyletic group of organisms (Garcia et al., 2000), the methanogen community likely has little functional redundancy and the contribution of individual species may be important to overall ecosystem function and warrant more detailed examination using higher resolution techniques, such as *16s rRNA* Illumina sequencing, in understanding the effects of saltwater intrusion events

(Allison and Martiny 2008; McGuire and Treseder 2010; Morrissey et al., 2014, Dang et al., 2019).

Conclusions

This work informs the current understanding of how sea level rise may affect methanogenesis in freshwater wetlands, and provides novel insight into the potential ecosystem-scale effects of substrate competition between SRB, methanogens, and syntrophic bacteria. The failure of methanogenesis to fully recover from SRB activity in this microcosm experiment provides evidence that TFWs may not all be resilient to saltwater intrusion events and may only slowly recover over months. However, these results should be verified in an *in situ* experiment, as microcosm experiments do not allow for the re-introduction of methanogens from the surrounding environment. The work also shows that at SO_4^{-2} concentrations typical of the oligohaline zone, syntrophic bacteria and SRB can break down butyrate concurrently. Based on butyrate additions and the ratio of MST:Archaea 16s *rRNA* gene abundance, there is evidence that acetoclastic methanogens may be more suited to persist in environments experiencing increased saltwater intrusion events and that these events may alter carbon breakdown pathways. Syntrophy, or "obligately mutualistic metabolism" as coined by Morris et al. (2013), is not confined to microbial metabolisms resulting in methane, but is used throughout the microbial community to survive in resource-limited environments. When examining the effects of saltwater intrusion, it is important to not focus on microbial functional groups as isolated entities but as part of a cooperative microbial metabolism that can determine larger carbon and nutrient mineralization rates.

References

Achtnich, C., Schuhmann, A., Wind, T., & Conrad, R. (1995). Role of interspecies H_2 transfer to sulfate and ferric iron-reducing bacteria in acetate consumption in anoxic paddy soil. *FEMS Microbiology Ecology*, *16*(1), 61-69.

Allison, S. D., & Martiny, J. B. (2008). Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences*, *105*(Supplement 1), 11512-11519.

Bae, J., & McCarty, P. L. (1993). Inhibition of butyrate oxidation by formate during methanogenesis. *Applied and Environmental Microbiology*, *59*(2), 628-630.

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Rídl, J., Vlček, Č., & Voříšková, J. (2012). Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. *The ISME Journal*, *6*(2), 248-258.

Baldwin, D. S., Rees, G. N., Mitchell, A. M., Watson, G., & Williams, J. (2006). The short-term effects of salinization on anaerobic nutrient cycling and microbial community structure in sediment from a freshwater wetland. *Wetlands*, *26*(2), 455-464.

Batstone, D. J., Picioreanu, C., & Van Loosdrecht, M. C. M. (2006). Multidimensional

modeling to investigate interspecies hydrogen transfer in anaerobic biofilms. *Water Research, 40*(16), 3099-3108.

Chambers, L. G., Reddy, K. R., & Osborne, T. Z. (2011). Short-term response of carbon cycling to salinity pulses in a freshwater wetland. *Soil Science Society of America Journal*, *75*(5), 2000-2007.

Chauhan, A., Ogram, A., & Reddy, K. R. (2004). Syntrophic-methanogenic associations along a nutrient gradient in the Florida Everglades. *Applied and Environmental Microbiology*, *70*(6), 3475-3484.

Chaun. A., Reddy, K. R., & Orgam, A. V. (2006). Syntrophic-archaeal associations in a nutrient-impacted freshwater marsh. *Journal of Applied Microbiology, 100*(1), 73-84.

Conrad, R., Mayer, H. P., & Wüst, M. (1989). Temporal change of gas metabolism by hydrogen-syntrophic methanogenic bacterial associations in anoxic paddy soil. *FEMS Microbiology Ecology*, *5*(4), 265-273.

Conrad, R. (1999). Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiology Ecology*, *28*(3), 193-202.

Conrad, R. (2009). The global methane cycle: recent advances in understanding the microbial processes involved. *Environmental Microbiology Reports*, *1*(5), 285-292.

Dang, C., Morrissey, E. M., Neubauer, S. C., & Franklin, R. B. (2019). Novel microbial community composition and carbon biogeochemistry emerge over time following saltwater intrusion in wetlands. *Global Change Biology*, *25*(2), 549-561.

De Bok, F. A. M., Plugge, C. M., & Stams, A. J. M. (2004). Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Research*, *38*(6), 1368-1375.

Dowrick, D. J., Freeman, C., Lock, M. A., & Reynolds, B. (2006). Sulphate reduction and the suppression of peatland methane emissions following summer drought. *Geoderma*, *132*(3), 384-390.

Dwyer, D. F., Weeg-Aerssens, E., Shelton, D. R., & Tiedje, J. M. (1988). Bioenergetic conditions of butyrate metabolism by a syntrophic, anaerobic bacterium in coculture with hydrogen-oxidizing methanogenic and sulfidogenic bacteria. *Applied and Environmental Microbiology, 54*(6), 1354-1359.

Elshahed, M. S., & McInerney, M. J. (2001). Is interspecies hydrogen transfer needed for

toluene degradation under sulfate-reducing conditions? *FEMS Microbiology Ecology, 35*(2), 163-169.

Ferry, J. G. (2010). How to make a living by exhaling methane. *Annual Review of Microbiology*, 64, 453-473.

Galand, P., Yrjälä, K., & Conrad, R. (2010). Stable carbon isotope fractionation during methanogenesis in three boreal peatland ecosystems. *Biogeosciences*, 7, 3893-3900.

Garcia, J. L., Patel, B. K., & Ollivier, B. (2000). Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. *Anaerobe*, *6*(4), 205-226.

Gauci, V., Dise, N., & Blake, S. (2005). Long‐term suppression of wetland methane flux following a pulse of simulated acid rain. *Geophysical Research Letters*, *32*(12).

Glissmann, K., & Conrad, R. (2000). Fermentation pattern of methanogenic degradation of rice straw in anoxic paddy soil. *FEMS Microbiology Ecology*, *31*(2), 117-126.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica 4*(1), 9.

Helton, A. M., Bernhardt, E. S., & Fedders, A. (2014). Biogeochemical regime shifts in

coastal landscapes: the contrasting effects of saltwater incursion and agricultural pollution on greenhouse gas emissions from a freshwater wetland. *Biogeochemistry, 120*(1-3), 133-147.

Hoehler, T. M., Alperin, M. J., Albert, D. B., and Martens, C. S. (2001). Apparent minimum free energy requirements for methanogenic archaea and sulfate-reducing bacteria in an anoxic marine sediment. *FEMS Microbiology Ecology*, *38*(1), 33-41.

Karlsson, A. E., Johansson, T., & Bengtson, P. (2012). Archaeal abundance in relation to root and fungal exudation rates. *FEMS Microbiology Ecology, 80*(2), 305-311.

Krylova, N. I., & Conrad, R. (1998). Thermodynamics of propionate degradation in methanogenic paddy soil. *FEMS Microbiology Ecology*, *26*(4), 281-288.

Liu, H., Wang, J., Wang, A., & Chen, J. (2011). Chemical inhibitors of methanogenesis and putative applications. *Applied Microbiology and Biotechnology*, *89*(5), 1333-1340.

Martins, M., and Pereira, I. A. (2013). Sulfate-reducing bacteria as new microorganisms for biological hydrogen production. *International Journal of Hydrogen Energy*, *38*(28), 12294-12301.

Matthews, E., and Fung, I. (1987). Methane emission from natural wetlands: global distribution, area, and environmental characteristics of sources. *Global Biogeochemical Cycles*, *1*(1), 61-86.

McGuire, K. L., & Treseder, K. K. (2010). Microbial communities and their relevance for ecosystem models: decomposition as a case study. *Soil Biology and Biochemistry*, *42*(4), 529-535.

McInerney, M. J., Struchtemeyer, C. G., Sieber, J., Mouttaki, H., Stams, A. J., Schink, B., Rohlin, L., & Gunsalus, R. P. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Annals of the New York Academy of Sciences*, *1125*(1), 58-72.

McInerney, M. J., Sieber, J. R., & Gunsalus, R. P. (2009). Syntrophy in anaerobic global carbon cycles. *Current Opinion in Biotechnology*, *20*(6), 623-632.

Mitra, S., Wassmann, R., and Vlek, P. L. (2005). An appraisal of global wetland area and its organic carbon stock. *Current Science*, *88*(1), 25-35.

Moftakhari, H. R., AghaKouchak, A., Sanders, B. F., Feldman, D. L., Sweet, W., Matthew, R. A., & Luke, A. (2015). Increased nuisance flooding along the coasts of the United States due to sea level rise: Past and future. *Geophysical Research Letters*, *42*(22), 9846-9852.

Morris, B. E., Henneberger, R., Huber, H., & Moissl-Eichinger, C. (2013). Microbial syntrophy: interaction for the common good. *FEMS Microbiology Reviews*, *37*(3), 384-406.

Morrissey, E. M., Berrier, D. J., Neubauer, S. C., & Franklin, R. B. (2014). Using microbial communities and extracellular enzymes to link soil organic matter characteristics to greenhouse gas production in a tidal freshwater wetland. *Biogeochemistry*, *117*(2-3), 473-490.

Muyzer, G., & Stams, A. J. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews Microbiology*, *6*(6), 441-454.

Neubauer, S. C., Givler, K., Valentine, S., & Megonigal, J. P. (2005). Seasonal patterns and plant-mediated controls of subsurface wetland biogeochemistry. *Ecology, 86*(12), 3334-3344.

Neubauer, S. C., & Craft, C. B. (2009). Global change and tidal freshwater wetlands: scenarios and impacts. *Tidal Freshwater Wetlands*, 353-366.

Neubauer, S. C., Franklin, R. B., & Berrier, D. J. (2013). Saltwater intrusion into tidal freshwater marshes alters the biogeochemical processing of organic carbon. *Biogeosciences*, *10*(12), 8171-8183.

Neubauer, S.C. (2013a). Carbon sequestration in wetland soils: Importance, mechanisms, and future prospects. *Society of Wetland Scientists Research Brief*. Oct 2013-0001. Retrieved from http://www.sws.org/category/sws-research-briefs.html

Neubauer, S. C.(2013b). Ecosystem responses of a tidal freshwater marsh experiencing saltwater intrusion and altered hydrology. *Estuaries and Coasts*, *36*(3), 491-507.

Neubauer, S. C., & Megonigal, J. P. (2015). Moving beyond global warming potentials to quantify the climatic role of ecosystems. *Ecosystems*, *18*(6), 1000-1013.

Odum, W. E. (1988). Comparative ecology of tidal freshwater and salt marshes. *Annual Review of Ecology and Systematics*, 147-176.

Omil, F., Lens, P., Visser, A., Hulshoff Pol, L. W., & Lettinga, G. (1998). Long‐term competition between sulfate reducing and methanogenic bacteria in UASB reactors treating volatile fatty acids. *Biotechnology and Bioengineering*, *57*(6), 676-685.

Parkes, R. J., Gibson, G. R., Mueller-Harvey, I., Buckingham, W. J., & Herbert, R. A.

(1989). Determination of the Substrates for sulphate-reducing bacteria within marine and esturaine sediments with different rates of sulphate reduction. *Microbiology*, *135*(1), 175-187.

Patel, G. B., & Roth, L. A. (1977). Effect of sodium chloride on growth and methane production of methanogens. *Canadian Journal of Microbiology*, *23*(7), 893-897.

Plugge, C. M., Zhang, W., Scholten, J. C., & Stams, A. J. (2011). Metabolic flexibility of sulfate-reducing bacteria. *The Microbial Sulfur Cycle*, 127.

Poffenbarger, H. J., Needelman, B. A., & Megonigal, J. P. (2011). Salinity influence on methane emissions from tidal marshes. *Wetlands*, *31*(5), 831-842.

Rothfuss, F., & Conrad, R. (1992). Vertical profiles of CH_4 concentrations, dissolved substrates and processes involved in CH_4 production in a flooded Italian rice field. *Biogeochemistry*, *18*(3), 137-152.

Sieber, J. R., McInerney, M. J., & Gunsalus, R. P. (2012). Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Annual Review of Microbiology*, *66*, 429-452.

Stams, A. J. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek*, *66*(1-3), 271-294.

Stams, A. J., De Bok, F. A., Plugge, C. M., Eekert, V., Miriam, H. A., Dolfing, J., & Schraa, G. (2006). Exocellular electron transfer in anaerobic microbial communities. *Environmental Microbiology*, *8*(3), 371-382.

Stams, A. J., & Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology*, *7*(8), 568-577.

Stefanie, J. O. E., Visser, A., Pol, L. W. H., & Stams, A. J. (1994). Sulfate reduction in methanogenic bioreactors. *FEMS Microbiology Reviews*, *15*(2-3), 119-136.

Thiele, J. H., & Zeikus, J. G. (1988). Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Applied and Environmental Microbiology*, *54*(1), 20-29.

Tully, K., Gedan, K., Epanchin-Niell, R., Strong, A., Bernhardt, E. S., BenDor, T., Mitchell, M., Kominoski, J., Jordan, T. E., Neubauer, S. C., & Weston, N. B. (2019). The invisible flood: the chemistry, ecology, and social implications of coastal saltwater intrusion. *BioScience*, *69*(5), 368-378.

Weston, N. B., Dixon, R. E., & Joye, S. B. (2006). Ramifications of increased salinity in

tidal freshwater sediments: geochemistry and microbial pathways of organic matter mineralization. *Journal of Geophysical Research: Biogeosciences, 111*(G01009).

Weston, N. B., Vile, M. A., Neubauer, S. C., & Velinsky, D. J. (2011). Accelerated microbial organic matter mineralization following salt-water intrusion into tidal freshwater marsh soils. *Biogeochemistry*, *102*(1-3), 135-151.

Yu, Y., Lee, C., Kim, J., & Hwang, S. (2005). Group‐specific primer and probe sets to detect methanogenic communities using quantitative real‐time polymerase chain reaction. *Biotechnology and Bioengineering*, *89*(6), 670-679.

Figures

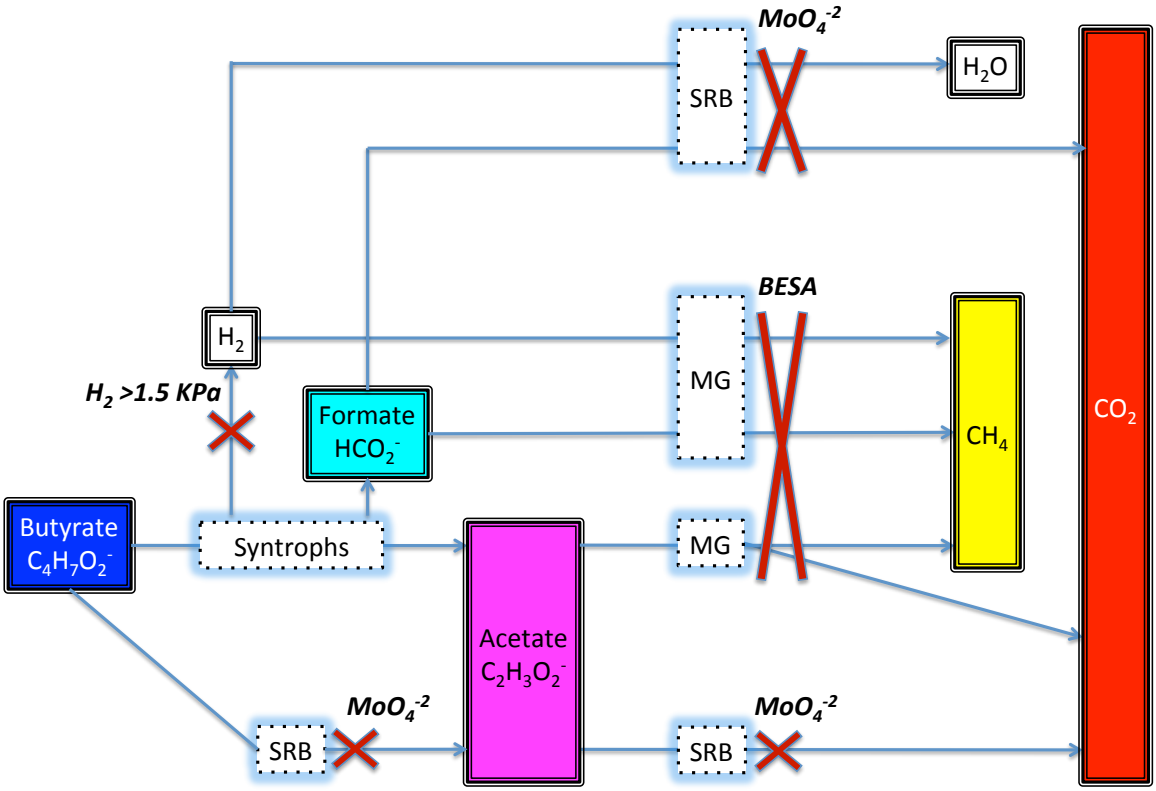

Figure 1. Pathways of butyrate breakdown in anaerobic environments, the microbial groups responsible (white boxes with dashed lines), and the steps affected by the inhibitors BESA, MoO_4^{-2}, and H_2 (red Xs). Methanogens are "MG," syntrophic bacteria are "Syntrophs," and SO_4^{-2} reducing bacteria are "SRB." The CO_2 consumption by formate formation and hydrogenotrophic methanogenesis are not included in the diagram. The production and consumption of H_2O is also not completely represented.

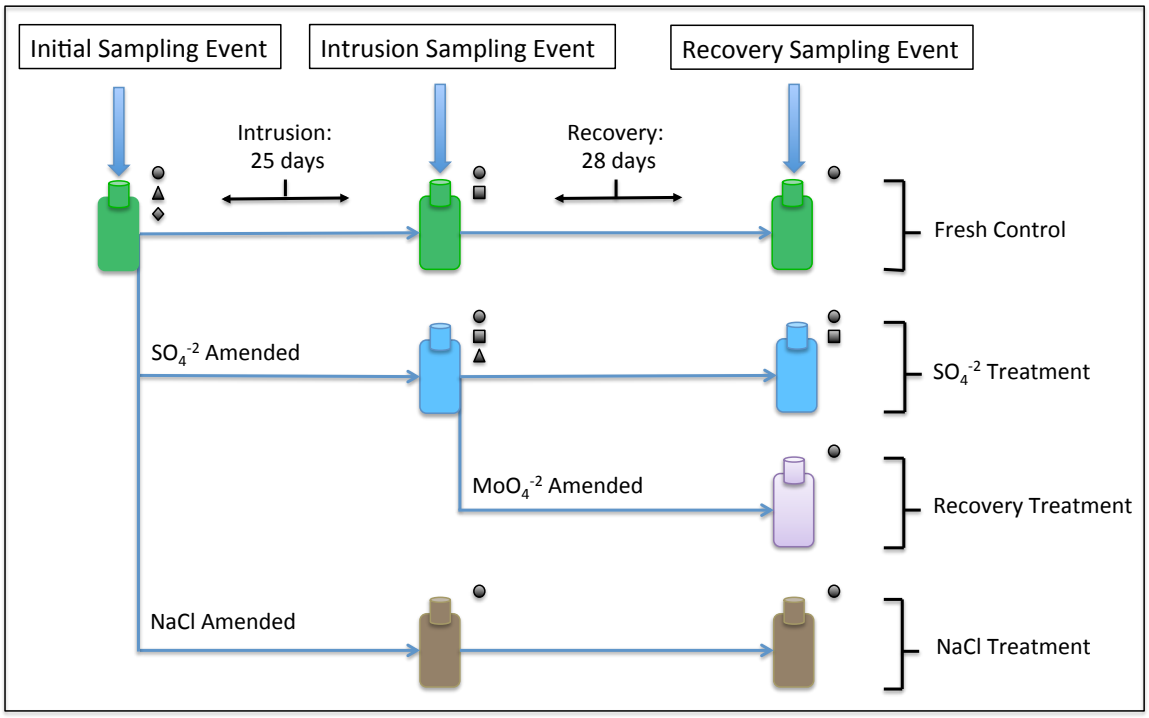

Figure 2. Summary of the treatments, incubation times, and sampling events for this experiment. Different colored bottles correspond to different treatments: Green = "Fresh control" (no amendment), Blue = "SO_4^{-2} treatment" (SO_4^{-2} amendments to stimulate SRB), Purple = "Recovery Treatment" (SO_4^{-2} amendments to stimulate SRB, followed by MoO_4^{-2} to inhibit them), and Brown = "NaCl Treatment" (NaCl amendments to mimic the ionic strength increase due to the SO_4^{-2} addition). At each sampling event, measurements were made of carbon mineralization rates (CO_2 and CH_4 production), soil pH, salinity, and archaea abundance. In addition, butyrate degradation assays were performed for each treatment group. The use of inhibitors during the butyrate assay is indicated next to each bottle: a "●" indicates an assay performed with no inhibitor (note: the "Recovery Treatment" received no inhibitor during the butyrate assay, though they did receive MoO_4^{-2} amendments 28 days earlier), a "▲" indicates an assay performed with BESA to inhibit methanogens, a "◆"indicates an assay performed with H_2 to inhibit syntrophs, and a "■" indicates an assay performed with MoO_4^{-2} to inhibit SRB.

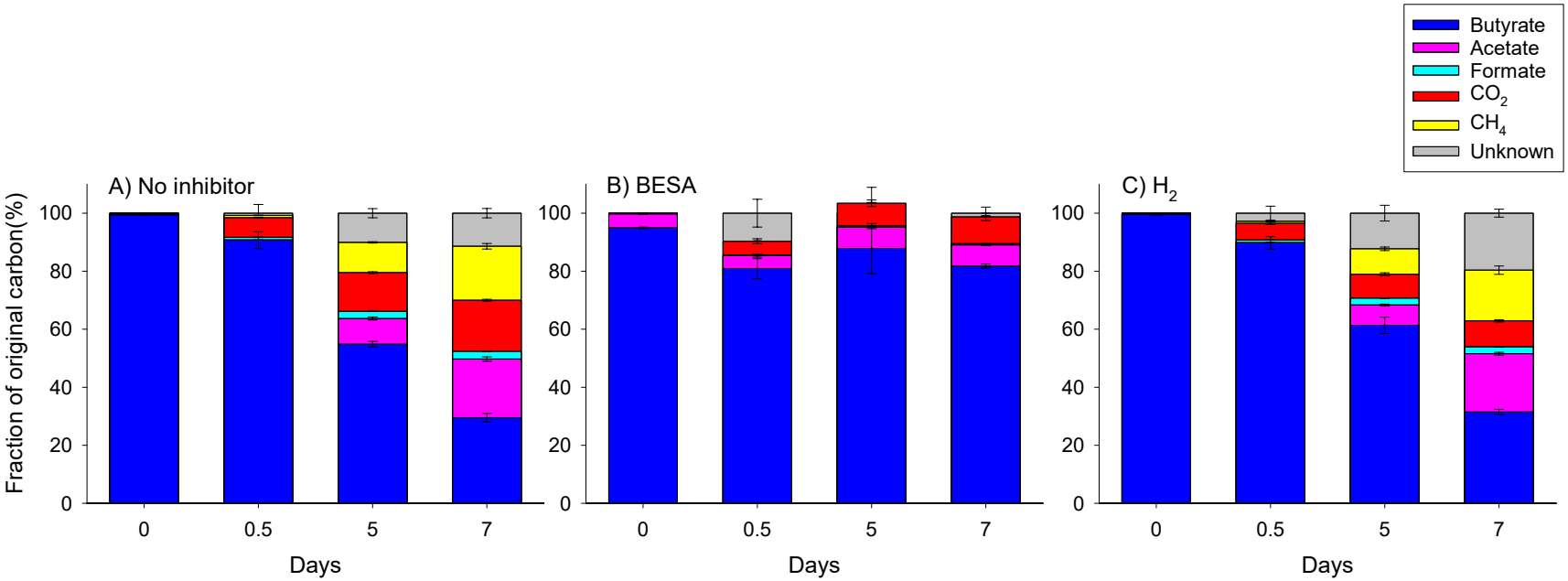

Figure 3. Results of the butyrate degradation assay for the "Fresh Control" treatment from the initial sampling event. Different colored bars represent the mean relative abundance (± standard error) of each carbon compound as a fraction of the original carbon measured at the beginning of the assay. At the start of the assay (day 0), microcosms were amended with butyrate and then incubated with either no inhibitor (A), BESA to inhibit MG activity (B), or H_2 to inhibit syntrophic bacteria (C).

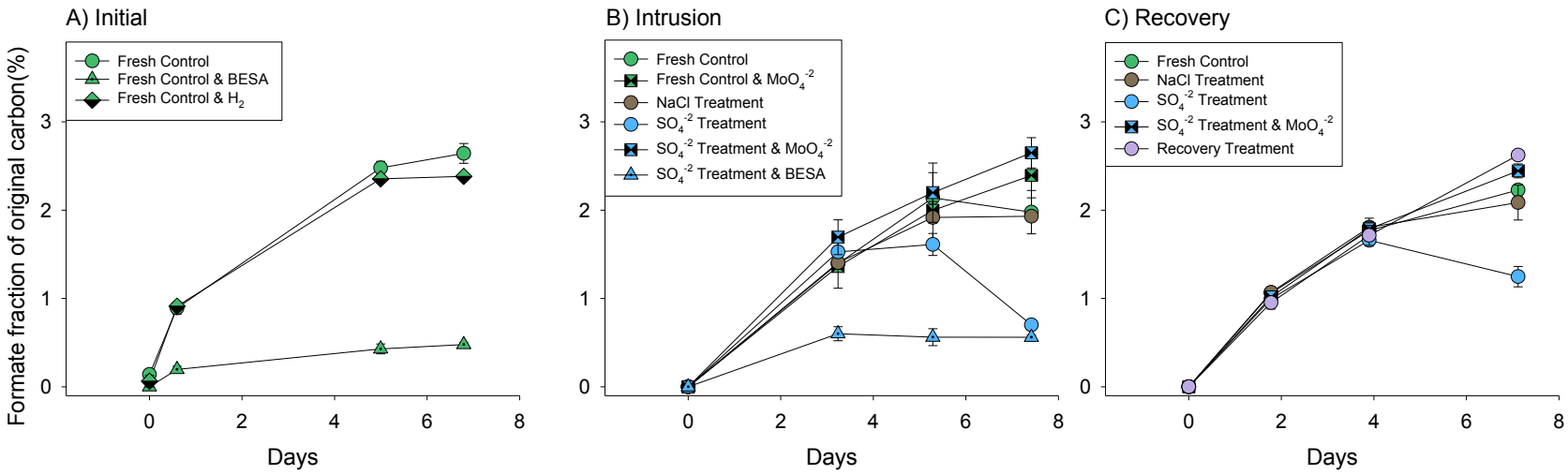

Figure 4. Mean (± standard error) of formate accumulation as a percent of the original carbon measured at the beginning of the butyrate breakdown assays performed during the initial (A), intrusion (B), and recovery sampling events (C).

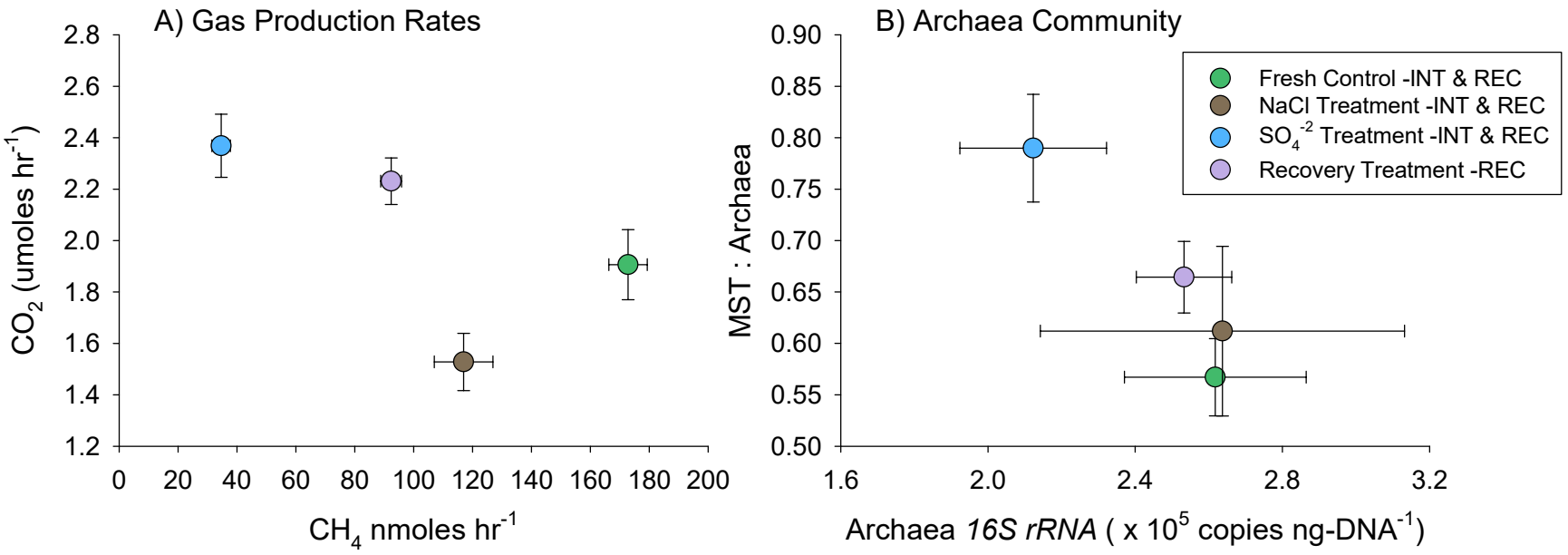

Figure 5. Mean (± standard error) gas production rates (A) and Archaea community data (B) pooled for the intrusion ("INT") and recovery ("REC") sampling events.

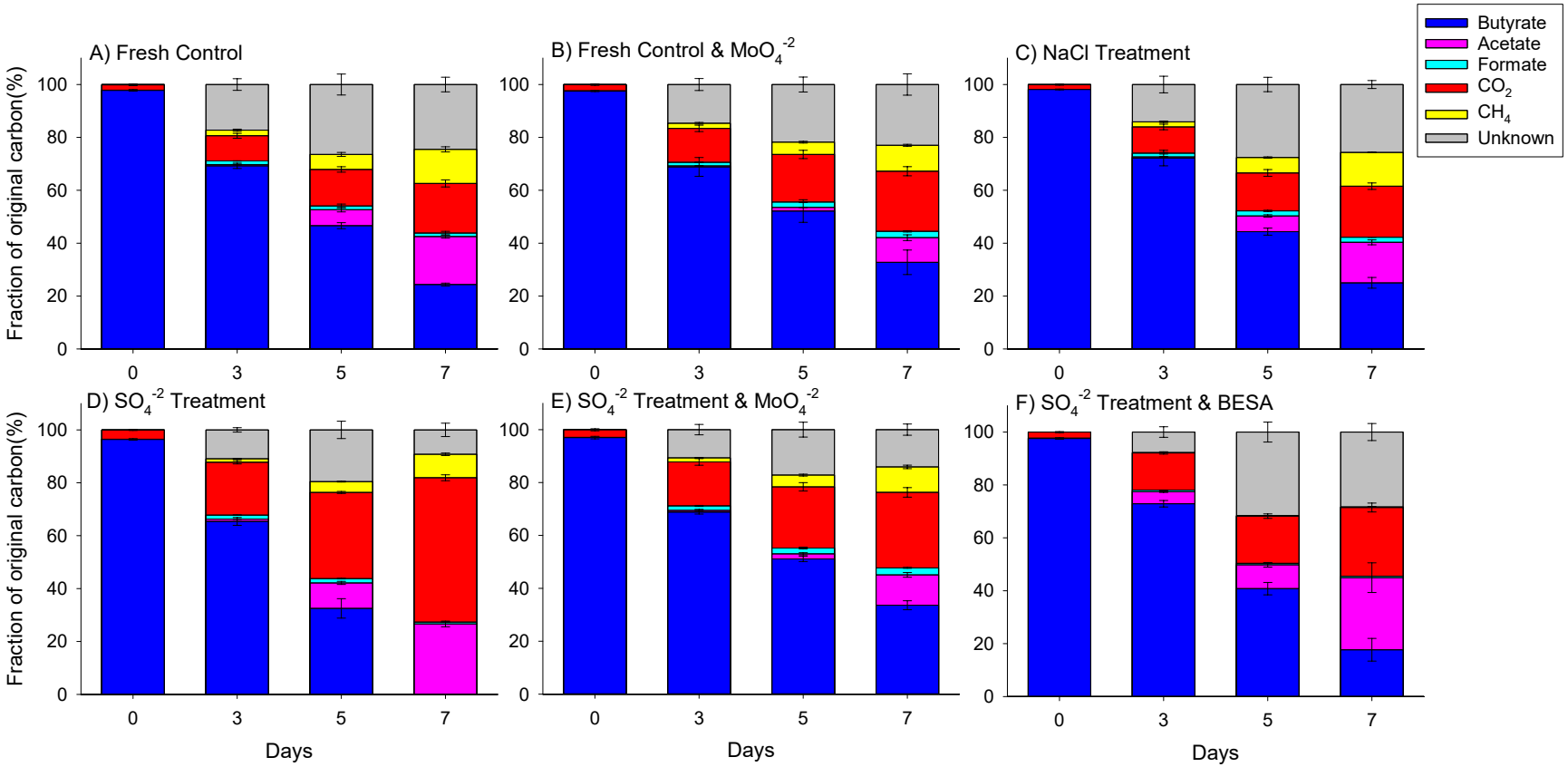

Figure 6. Results of the butyrate breakdown assays for the intrusion sampling event. Different colored bars represent the mean relative abundance (± standard error) of each carbon compound as a fraction of the original carbon measured at the beginning of the assay. At the start of the assay (day 0), microcosms were amended with butyrate and then incubated with either no inhibitor (A, C, D), MoO_4^{-2} to inhibit SRB activity (B, E), or BESA to inhibit MG activity (F).

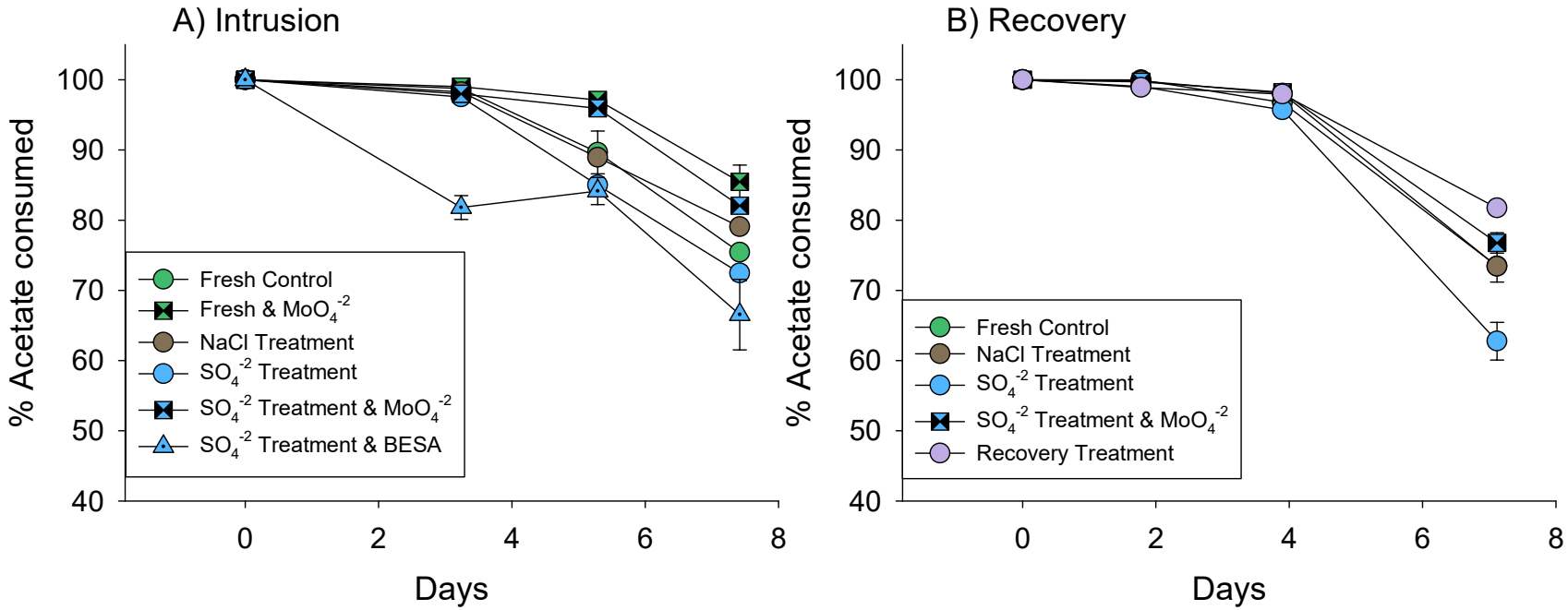

Figure 7. Mean (± standard error) of the percent acetate consumed in the butyrate breakdown assays during the intrusion sampling event (A) and the recovery sampling event (B).

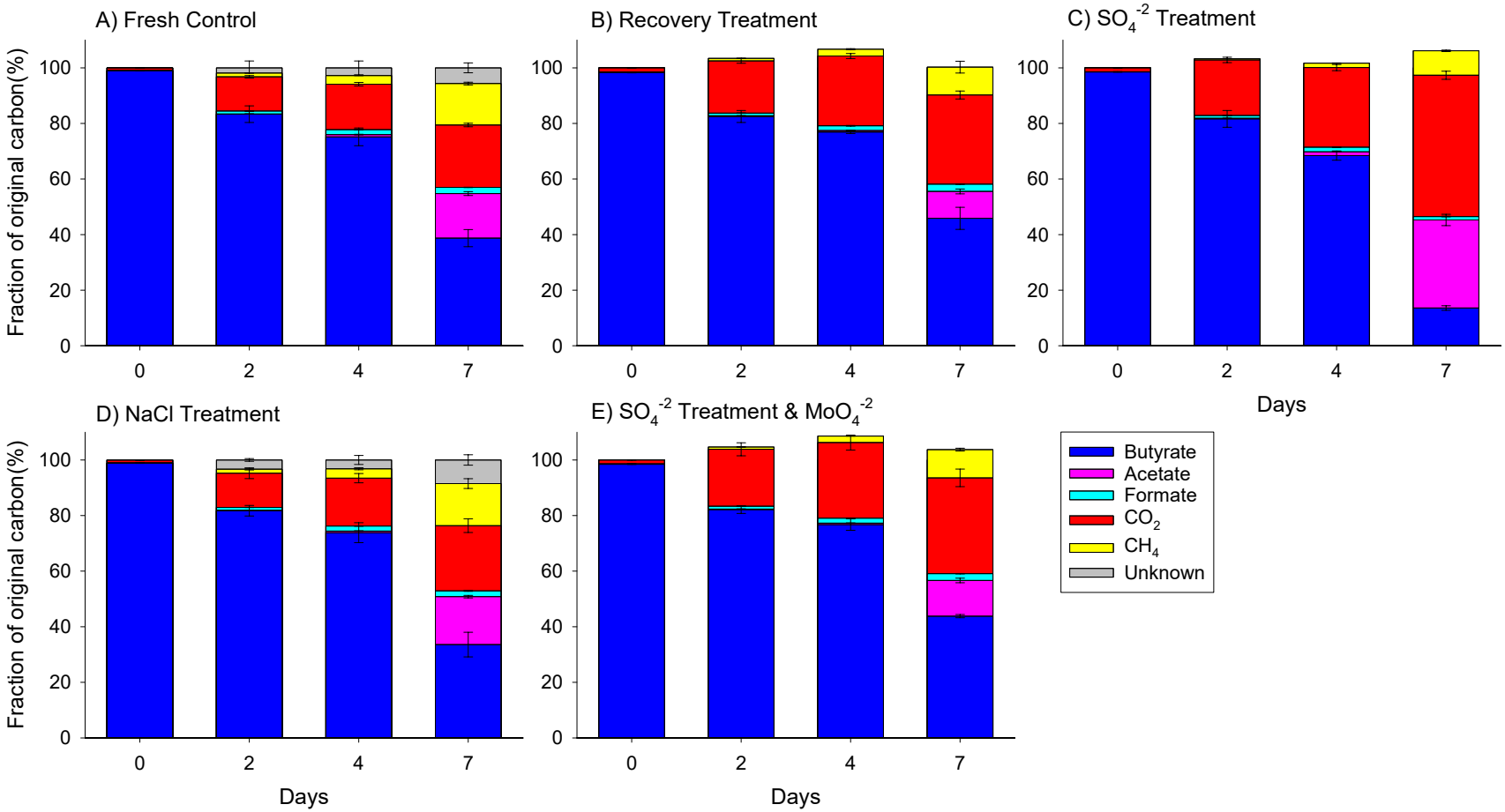

Figure 8. Results of the butyrate breakdown assays for the recovery sampling event. Different colored bars represent the mean relative abundance (± standard error) of each carbon compound as a fraction of the original carbon measured at the beginning of the assay. At the start of the assay (day 0), microcosms were amended with butyrate and then either no inhibitor (A, B, C, D), or MoO_4^{-2} to inhibit SRB activity (E). Note that the "Recovery Treatment" (B) had been incubated with the inhibitor MoO_4^{-2} for 28 days prior to the butyrate assay.

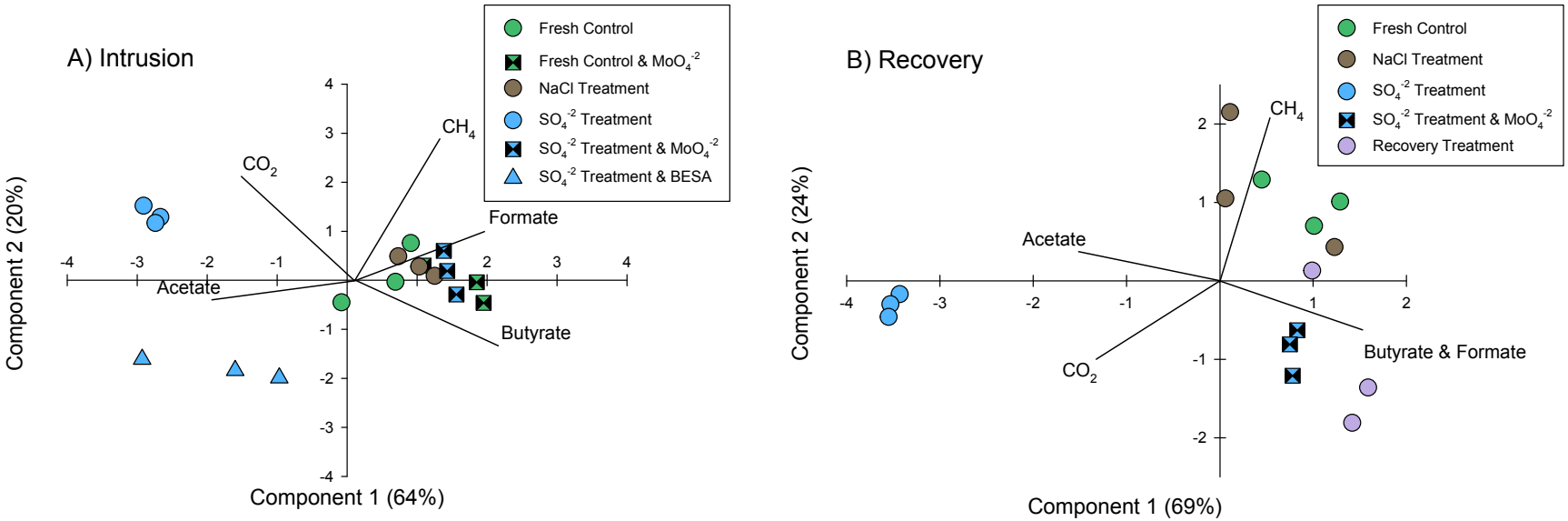

Figure 9. Principal components analysis applied to the distribution of carbon substrates (butyrate, acetate, formate, CO_2, and CH_4) in microcosms on day 7 of butyrate degradation assays performed during the intrusion sampling event (A) and the recovery sampling event (B).

Vita

David James Berrier Jr. was born on March, 7, 1987 in Norfolk, Virginia. He graduated *Magna Cum Laude* with a Bachelor of Science degree in Environmental Studies with a minor in Biology from Virginia Commonwealth University in 2009. From 2009 to 2014, David worked as a research specialist in Dr. Rima Franklin's lab studying the role of wetland microbial communities in biogeochemical cycling as well as water quality in the James River. This work resulted in multiple publications on the role of microbial communities in wetlands. During this time, David also worked as a laboratory instructor for the undergraduate Medical Microbiology lab at Virginia Commonwealth University. In 2014, he began his graduate research with advising from Dr. Rima Franklin and Dr. Scott Neubauer. He was awarded a Graduate Teaching Assistantship and acted as the Biology Capstone laboratory instructor from 2014 to 2016.