

NOVEL PERSPECTIVES ON CARBON CYCLING IN EXTREME MARINE
ENVIRONMENTS

by

ANDREW MATTHEW MONTGOMERY

(Under the Direction of Samantha B. Joye)

ABSTRACT

This dissertation explores the microbial ecology and biogeochemistry of carbon cycling in extreme marine environments using novel approaches. The Guaymas Basin is a young ocean basin where thermocatalytic alteration of organic matter and hydrothermal fluid flow impact biogeochemical regimes. Methane, a product of hydrothermalism, is actively metabolized in benthic habitats. *In situ* methane concentrations exhibited strong spatiotemporal variability. High methane concentrations (46 mmol L⁻¹) were measured using Jumbo OsmoSamplers, underscoring the need to make *in situ* measurements to constrain their dynamics. Methane concentrations varied substantially over a 3-month time series reflecting the dynamic nature of the system. Energy-laden hydrothermal fluids mix with cold bottom water to form buoyant plumes enriched with labile organic compounds. Heterotrophic metabolism of methane, methanol and acetate was evaluated in a hydrothermal plume. Organic carbon assimilation rates were high, accounting for more than 50% of microbial uptake in the plume, underscoring the importance of heterotrophy in hydrothermally-impacted waters.

In pelagic environments impacted by hypoxia, pelagic organic carbon cycling is poorly documented. Microbially-mediated cycling of methanol and methane were examined across the Gulf of Mexico hypoxic zone; Ammonia Oxidizing Archaea (AOA) are abundant in these habitats. Oxidation of methane was low compared to previous measurements and methanol oxidation and assimilation were important. Constraining the metabolic capabilities and environmental regulation of AOA, among the most abundant microbes in the ocean, is necessary to document their role in ocean biogeochemistry. AOA oxidized methane and incorporated leucine, extending their metabolic portfolio and suggesting additional roles for the AOA in ocean carbon and nitrogen cycling. Collectively, these studies advance our understanding of microbial carbon cycling in several extreme environments.

INDEX WORDS: carbon cycle, methane, methanol, methane oxidation, hydrothermal plume, hypoxia, ammonia oxidizing archaea

NOVEL PERSPECTIVES ON CARBON CYCLING IN EXTREME MARINE
ENVIRONMENTS

by

ANDREW MATTHEW MONTGOMERY

BS, James Madison University, 2013

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2021

© 2021

Andrew Matthew Montgomery

All Rights Reserved

NOVEL PERSPECTIVES ON CARBON CYCLING IN EXTREME MARINE
ENVIRONMENTS

by

ANDREW MATTHEW MONTGOMERY

Major Professor:	Samantha B. Joye
Committee:	Patricia Medeiros
	William B. Whitman
	Elizabeth Ottesen
	Joel Kostka

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2021

DEDICATION

I dedicate this work to my grandfathers and biggest role models:

Erville “Gramps” Koehler & Dr. Richard “Papa” Montgomery

Thank you for always pushing me to be my best.

Love u man

ACKNOWLEDGEMENTS

First and foremost, I want to thank my supervisor, Mandy Joye. I am especially grateful for her unwavering support and guidance during my time in Athens. Through the highs and lows of my graduate career, her mentorship instilled an ability to think creatively and critically in science and life. Her infectious passion for science nurtured my intellectual pursuits and this work would not have been possible without her. I would also like to thank each of my committee members, Patricia Medeiros, Barny Whitman, Liz Ottesen, and Joel Kostka, for their supportive comments and feedback.

My time in the Joye Lab has been a rewarding experience and I appreciate the many connections I have made here. I am forever indebted to Kim Hunter for her constant guidance and support. Her knowledge was indispensable as I navigated both menial and challenging hurdles in the lab. I would also like to thank Hannah Choi, Mary Kate Rogener, Rachael Storo, Tito Peña-Montenegro, Ryan Sibert, Lauren Carroll, Zac Marinelli, Vladimir Samarkin, and many other former Joye Lab members for their enduring support. Many thanks for the encouragement, lengthy discussions, and good times at sea.

Additionally, I would not be in the position I am today without the guidance of Guang-Chao Zhuang. My gratitude cannot be fully expressed for the countless contributions he made throughout the entirety of my graduate career. I am so thankful for

your friendship, and I look forward to continuing our work together in the future. I hope to one day be as qualified as you are.

During my time in the Joye Lab, I have had many opportunities for field and sea-going experience. The data presented here could not have been produced without the support of many chief scientists, collaborators, and ship crew. I would like to thank Joe Montoya, Nancy Rabalais, Rick Petersen, and Andreas Teske for their support and for allowing me to sail on their cruises. This work was made possible by the crew and support staff of the R/V Endeavor, R/V Atlantis, R/V Falkor, R/V Pelican, and R/V Point Sur. Special thanks to the pilots and crew of HOV *Alvin* (AT42-05) and ROV *SuBastian* (FK190211) for their dedication to science and collecting precious samples.

To Laura Lapham, thank you for supplying the OsmoSamplers, and for your aid in sample processing. Thanks to Tim Hollibaugh, Julian Damashek, and Barbara Bayer (and collaborators) for supplying cultures that were instrumental to this work. I am also grateful for Mary Kate Rogener and Laura Bristow who did initial experiments that inspired the culture experiments and dead zone work.

I have been fortunate to have the support of many great friends near and far including Trey, Mustafa, Kelsey, Jacob, JPB, and many others. Shout out to Chris Franklin, Ben Boward, Collin Preston, and Josh Storo who didn't do a damn thing, I just love'em a lot.

I thank my Mom, Dad, Katie, Mike, Bob, Thomas, Grandpa & Grandma Ery, Grandma & Papa Mo, and the rest of my extended family. Their unconditional love and generosity were crucial to my success. I could not have done this without them. I love you all.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW	1
2 <i>IN SITU</i> SURVEYS REVEAL SPATIAL AND TEMPORAL HETEROGENEITY IN METHANE DISTRIBUTIONS IN HYDROTHERMALLY-IMPACTED SEDIMENTS OF THE GUAYMAS BASIN.....	#
3 HYDROTHERMAL PLUMES SUPPORT HIGH RATES OF ORGANIC CARBON CYCLING IN THE GUAYMAS BASIN.....	#
4 METABOLIC FLEXIBILITY IN TWO MARINE STRAINS OF AMMONIA-OXIDIZING ARCHAEA.....	#
5 INVESTIGATION OF FACTORS DRIVING METHANE AND METHANOL CYCLING IN THE GULF OF MEXICO HYPOXIC ZONE.....	#
6 CONCLUSIONS.....	#

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Microbially-mediated processes in the oceans drive biogeochemical cycles that impact Earth's climate and habitability. This suite of studies presented in this dissertation employed interdisciplinary approaches to investigate microbial activity in the context of environmental geochemistry to elucidate the factors driving organic carbon cycling in two extreme marine environments. This work documents: spatiotemporal dynamics of methane concentrations in hydrothermally-impacted sediments of Guaymas Basin; heterotrophic production in a deep-sea hydrothermal plume in Guaymas Basin; the metabolic flexibility of two strains of marine ammonia-oxidizing archaea; and methanotrophy and methylotrophy in the coastal Gulf of Mexico hypoxic zone. The oceanic carbon cycle has been studied extensively (Eppley and Peterson, 1979; Field *et al.*, 1998; Azam and Malfatti, 2007; Armbrust, 2009; Worden *et al.*, 2015), but important aspects of carbon biogeochemistry await discovery.

The ocean is the largest above-ground reservoir of carbon on Earth. So, constraining the factors that regulate carbon cycling in the ocean is imperative for understanding global carbon cycling in the context of global change (Falkowski *et al.*, 2000). Extreme marine environments are characterized by a wide range of biogeochemical and hydrographic conditions, such as high temperatures or low-oxygen concentrations (Rothschild and Mancinelli, 2001). These environments exhibit dynamic biogeochemical

cycling and thus offer a unique opportunity to investigate marine carbon cycling across a range of dynamics. In particular, this work presents new perspectives on carbon cycling in a hydrothermal system, a coastal hypoxic zone, and pure cultures advancing the understanding of methane and low molecular weight (LMW) carbon (*e.g.*, methanol) cycling in these habitats.

Methane is a potent greenhouse gas with a warming potential nearly 30 times greater than carbon dioxide (IPCC, 2019). In the marine environment, methane is a critical component of the carbon cycle, impacting microbial ecology, nutrient cycling, and other biogeochemical processes. Methane production occurs via microbially-mediated methanogenesis as the terminal step of organic matter degradation, from various abiotic/thermogenic processes (*e.g.*, water-rock interactions) in anoxic marine sediments (Jørgensen, 2000; Reeburgh, 2007), or microbial cleavage of methylphosphonate (Karl *et al.*, 2008). Most of this methane-carbon remains in the sediments (~ 90%) after oxidation via anaerobic oxidation of methane (AOM) (Reeburgh, 2007; Knittel and Boetius, 2009).

However, in areas of high methane concentration and dynamic flow (*e.g.*, cold seeps and hydrothermal systems), high concentrations of methane are discharged into the water column. Methane oxidation rates can be elevated in regions of high methane (*e.g.*, in the aftermath of the Deepwater Horizon oil spill), but the extent to which the water column acts as a methane biofilter and the biogeochemical factors that control methane oxidation are not fully understood (Mau *et al.*, 2013; Crespo-Medina *et al.*, 2014; Steinle *et al.*, 2015, 2016; Rogener *et al.*, 2018, 2020).

In contrast, much less research effort has examined the dynamics of LMW carbon compounds. These compounds, which include, for example, methanol, acetate, and methylamine, are especially important in marine sediments where they can serve as substrates for energy conservation and non-competitive methanogenesis (Parkes *et al.*, 2002; Zhuang *et al.*, 2016; Zhuang, Montgomery, *et al.*, 2018). In pelagic environments, these LMW compounds can drive the microbial carbon cycle and atmospheric exchange (Van Neste *et al.*, 1987; Singh *et al.*, 2001; Heikes *et al.*, 2002; Mincer and Aicher, 2016). Relatively few measurements of LMW carbon concentrations exist in marine waters. Methanol and methylamine concentrations are usually less than 1 μmol (Naqvi *et al.*, 2005; Dixon *et al.*, 2011; Yang *et al.*, 2013; Avery *et al.*, 2016) while acetate can be as high as $\sim 10 \mu\text{mol L}^{-1}$ (Lyu *et al.*, 2017). Fast turnover times (~ 5 d) of LMW organics have been observed, suggesting they are important carbon and energy substrates for marine microbes (Dixon and Nightingale, 2012; Dixon *et al.*, 2013; Zhuang, Peña-Montenegro, *et al.*, 2018, 2019). However, the distribution of LMW compounds and the factors controlling their metabolisms are largely unconstrained.

In **Chapter 2** hydrothermally altered sediments were surveyed to assess *in situ* methane dynamics in the Guaymas Basin hydrothermal system. The Guaymas Basin in the Gulf of California is a marginal rift basin, which hosts an end-member hydrothermal system. Thick sediment packages (up to ~ 400 m thick) contain massive quantities of organic carbon that undergo thermocatalytic alterations by deeply-sourced hot fluids that migrate along basaltic sill intrusions (Einsele *et al.*, 1980; Teske *et al.*, 2016). Sediment

temperatures well above 100°C in the top 20 cmbsf are common (Teske *et al.*, 2016). As a result, microbial activity is elevated, and shallow sediments are extremely sulfidic (up to ~ 10 mmol L⁻¹) due to the coupling of sulfate reduction (SR) to organic matter oxidation (Jørgensen *et al.*, 1990, 2019; Kallmeyer and Boetius, 2004).

Methane is the primary product of thermocatalytic alteration in Guaymas Basin sediments (Welhan, 1988), and in surficial sediments, methane is involved and drives a range of biogeochemical processes (Teske *et al.*, 2016). For example, AOM rates have been measured extensively and are elevated in hydrothermal sediments, up to 3.5 μmol cm⁻³ d⁻¹ (Kallmeyer and Boetius, 2004; Biddle *et al.*, 2012; Dowell *et al.*, 2016; Bowles *et al.*, 2019). AOM is generally coupled to SR, which has been commonly observed in Guaymas Basin (Boetius *et al.*, 2000; Kallmeyer and Boetius, 2004), however, extensive decoupling has been shown, indicating other processes (*e.g.*, denitrification) may be coupled to AOM (Bowles *et al.*, 2012, 2019). Methanogenesis via non-competitive substrates also occurs in shallow hydrothermal sediments (Zhuang, Montgomery, *et al.*, 2019). Methane concentrations reach up to ~ 28 mmol L⁻¹ and dynamic fluid flow regimes lead to intense spatial variability (McKay *et al.*, 2012, 2016).

To assess this heterogeneity and quantify *in situ* methane concentrations osmotically driven pumps, OsmoSamplers, were deployed in a range of hydrothermally impacted sediments. OsmoSamplers have been used in numerous environments to continuously measure trends of methane and other geochemical parameters *in situ* on the timescales of several months (Wheat *et al.*, 2000; Jannasch *et al.*, 2004; Lapham *et al.*,

2013; Gelesh *et al.*, 2016; Orcutt *et al.*, 2017; McIntosh Marcek *et al.*, 2021). In this work, OsmoSamplers were deployed similarly to generate a 3-month time series of methane and sulfate concentrations. Additionally, novel Jumbo OsmoSamplers were used for short-term deployments to measure *in situ* methane concentrations for the first time in hydrothermal sediments. The data from this chapter highlight local variations in methane concentrations.

In **Chapter 3**, carbon dynamics were further investigated in a hydrothermal plume in the Guaymas Basin. The advective flow and subsequent venting of hydrothermal fluids deliver elevated concentrations of methane, complex hydrocarbons, low molecular weight organics, and other biologically active compounds into the deep sea (Von Damm *et al.*, 1985; Kawka and Simoneit, 1987; Welhan and Lupton, 1987; Bazylnski *et al.*, 1988; Welhan, 1988; Martens, 1990; Simoneit *et al.*, 1990; Peter *et al.*, 1991; Lang *et al.*, 2010; Lin *et al.*, 2017). Upon discharge, these fluids fuel vibrant deep-sea communities via chemosynthetic ecosystems at the seabed (Reysenbach *et al.*, 2000; Reysenbach and Shock, 2002; Perner *et al.*, 2013). When mixed with cold seawater, hydrothermal fluids produce plumes characterized by strong temperature and turbidity anomalies (Campbell and Gieskes, 1984; Von Damm *et al.*, 1985).

Hydrothermal fluids in the Guaymas Basin are organic-rich, but surprisingly, no studies to date have assessed organic carbon cycling in these plumes. Chemosynthesis, the fixation of inorganic carbon in the dark, is a well-defined feature of hydrothermal systems and supports elevated oxidation rates of inorganic substrates in plumes (Karl *et al.*, 1980; Wirsen *et al.*, 1993; Lam, 2004; Dick *et al.*, 2009; Amend *et al.*, 2011; Beman *et al.*, 2012).

In recent years, several studies have demonstrated that heterotrophic microbes are active in plumes, indicating organic carbon cycling is important (Li *et al.*, 2015; Meier *et al.*, 2016; Zhou *et al.*, 2020). However, the magnitude and geochemical drivers of heterotrophic activity in hydrothermal plumes have not been considered.

Several previous studies examined methane dynamics in hydrothermal plumes. Methane concentrations and oxidation rates have been documented (Simoneit *et al.*, 1990; de Angelis *et al.*, 1993; Cowen *et al.*, 2002), but the factors regulating methanotrophy are largely unknown. Similarly, elevated concentrations of acetate and other organics have been documented (Lang *et al.*, 2010; Lin *et al.*, 2017), but heterotrophic metabolisms in hydrothermal plumes are not constrained. The fate of other compounds, such as methanol and hydrocarbons, has not been explored in any capacity. Microbial cycling of methane, methanol, acetate, methylamine, and two hydrocarbons was measured directly in and out of a hydrothermal plume to investigate the heterotrophic capacity of plume communities and to elucidate potential drivers of organic carbon metabolisms.

In **Chapter 4**, the metabolic flexibility of two marine ammonia-oxidizing archaea (AOA) was evaluated. For many years, *Archaea* were considered to be obligate extremophiles, proliferating only in harsh high-temperature, anoxic, hypersaline, or acidic environments. The discovery of *Archaea* outside of these extreme habitats more than two decades ago opened a new frontier for exploration, assessing the environmental importance of these organisms in more moderate environments (DeLong, 1992). Subsequently, the identification of widespread crenarchaeal genes, establishment of the phylum

Thaumarchaeota, and eventual isolation of AOA represented a major shift in our understanding of marine carbon and nitrogen cycling (Karner *et al.*, 2001; Venter *et al.*, 2004; Könneke *et al.*, 2005; Wuchter *et al.*, 2006).

AOA are among the most abundant microbes in the ocean. They mediate the first step of nitrification (ammonia oxidation) and autotrophically fix carbon via the 3-hydroxypropionate/4-hydroxybutyrate pathway (Berg *et al.*, 2007; Walker *et al.*, 2010; Könneke *et al.*, 2014). The widespread distribution of AOA is due in part to their high affinity for ammonia, allowing them to thrive in even the most oligotrophic environments (Martens-Habben *et al.*, 2009). Since the first isolation of *Nitrosopumilus maritimus* SCM1, nearly 40 strains have been isolated and used to explore the metabolic repertoires of AOA (*e.g.*, Hatzenpichler *et al.*, 2008; Lehtovirta-Morley *et al.*, 2011; Qin *et al.*, 2018).

The physiological capabilities of AOA extend far beyond ammonia oxidation and inorganic carbon fixation. For example, different AOA can conserve energy from urea and cyanate oxidation (*e.g.*, Bayer *et al.*, 2016; Santoro & Casciotti, 2011), some may utilize organic carbon (Tourna *et al.*, 2011; Qin *et al.*, 2014), and some strains produce methylphosphonate (Metcalf *et al.*, 2012) and cobalamin (Heal *et al.*, 2017). The capacity for alternative metabolisms reflects surprisingly broad physiological capabilities for organisms once considered obligate ammonia oxidizers; this flexibility allows them to proliferate in a wide range of habitats (Biller *et al.*, 2012; Lehtovirta-Morley *et al.*, 2014; Carini *et al.*, 2018; Qin *et al.*, 2018). The capacity of two AOA strains (*Nitrosopumilus*

adriaticus NF5 and *Nitrosopumilus piranensis* D3C) were assessed in pure culture using radiotracer techniques.

In **Chapter 5**, I explored the microbial cycling of methane and methanol during the annual summer hypoxic zone in the northern Gulf of Mexico. Each summer, a combination of high nutrient loading from the Mississippi and Atchafalaya rivers drive eutrophication (Nixon, 1995) near the river mouths (Turner *et al.*, 2007). The rapid oxidation of labile organic matter driven by high temperatures in the stratified water column leads to a decrease in dissolved oxygen concentrations (Redalje *et al.*, 1994; Chen *et al.*, 2003; Wang *et al.*, 2004). When stratification persists, hypoxic conditions develop where dissolved oxygen concentrations fall below 2 mg L⁻¹ (63 µmol L⁻¹; Rabalais *et al.*, 2002, 2007).

Coastal eutrophication is linked to anthropogenic nutrient sources (*e.g.*, agricultural nitrate in the case of the Mississippi River). Hypoxic zones are associated with dynamic microbial communities that contribute significantly to marine carbon and nitrogen cycling (Mason *et al.*, 2016; Padilla *et al.*, 2016; Thrash *et al.*, 2017; Gillies Campbell *et al.*, 2019). Many studies have documented nitrogen cycling in the Gulf hypoxic zone, with a specific focus on nitrification rates and the associated microbial community (Stewart *et al.*, 2012; Gillies *et al.*, 2015; Kitzinger *et al.*, 2019). In contrast, almost no studies have investigated organic carbon cycling in the Gulf of Mexico hypoxic zone. Methane oxidation has been documented twice and rates were observed up to 192 nmol L⁻¹ d⁻¹ during hypoxic conditions (Rogener *et al.*, 2021). However, the biological and chemical drivers of methane and other organic carbon metabolisms in hypoxic waters are largely unconstrained.

Given the known sources of methane and methanol, the production and utilization of these compounds should be widespread in eutrophic coastal hypoxic systems. I surveyed the distribution of methane, methanol, and ethanol across the Louisiana shelf during the annual hypoxic event to assess the dynamics of these organic compounds. Additionally, an integrated approach was used to constrain metabolic rates of methane and methanol uptake and to experimentally assess potential drivers of these metabolisms in the Gulf of Mexico hypoxic zone.

Chapter 6 provides a summary of the findings of this dissertation.

REFERENCES

- Amend, J.P., McCollom, T.M., Hentscher, M., and Bach, W. (2011) Catabolic and anabolic energy for chemolithoautotrophs in deep-sea hydrothermal systems hosted in different rock types. *Geochim. Cosmochim. Acta* **75**: 5736–5748.
- de Angelis, M.A., Lilley, M.D., and Baross, J.A. (1993) Methane oxidation in deep-sea hydrothermal plumes of the endeavour segment of the Juan de Fuca Ridge. *Deep. Res. Part I* **40**: 1169–1186.
- Armbrust, E.V. (2009) The life of diatoms in the world's oceans. *Nature* **459**: 185–192.
- Avery, G.B., Foley, L., Carroll, A.L., Roebuck, J.A., Guy, A., Mead, R.N., et al. (2016) Surface waters as a sink and source of atmospheric gas phase ethanol. *Chemosphere* **144**: 360–365.

- Azam, F. and Malfatti, F. (2007) Microbial structuring of marine ecosystems. *Nat. Rev. Microbiol.* **5**: 782–791.
- Bayer, B., Vojvoda, J., Offre, P., Alves, R.J.E., Elisabeth, N.H., Garcia, J.A.L., et al. (2016) Physiological and genomic characterization of two novel marine thaumarchaeal strains indicates niche differentiation. *ISME J.* **10**: 1051–1063.
- Bazylnski, D.A., Farrington, J.W., and Jannasch, H.W. (1988) Hydrocarbons in surface sediments from a Guaymas Basin hydrothermal vent site. *Org. Geochem.* **12**: 547–558.
- Beman, J.M., Popp, B.N., and Alford, S.E. (2012) Quantification of ammonia oxidation rates and ammonia-oxidizing archaea and bacteria at high resolution in the Gulf of California and eastern tropical North Pacific Ocean. *Limnol. Oceanogr.* **57**: 711–726.
- Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) Autotrophic Carbon Dioxide Assimilation Pathway in Archaea. *Science (80-.)*. **318**: 1782–1787.
- Biddle, J.F., Cardman, Z., Mendlovitz, H., Albert, D.B., Lloyd, K.G., Boetius, A., and Teske, A. (2012) Anaerobic oxidation of methane at different temperature regimes in Guaymas Basin hydrothermal sediments. *ISME J.* **6**: 1018–1031.
- Biller, S.J., Mosier, A.C., Wells, G.F., and Francis, C.A. (2012) Global biodiversity of aquatic ammonia-oxidizing archaea: Is partitioned by habitat. *Front. Microbiol.* **3**: 1–15.
- Boetius, A., Ravensschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A., et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. **407**: 623–626.

- Bowles, M.W., Nigro, L.M., Andreas, P., and Joye, S.B. (2012) Denitrification and environmental factors influencing nitrate removal in Guaymas Basin hydrothermally altered sediments. *Front. Microbiol.* **3**: 1–11.
- Bowles, M.W., Samarkin, V.A., Hunter, K.S., Finke, N., Teske, A.P., Girguis, P.R., and Joye, S.B. (2019) Remarkable Capacity for Anaerobic Oxidation of Methane at High Methane Concentration. *Geophys. Res. Lett.* **46**: 12192–12201.
- Campbell, A.C. and Gieskes, J.M. (1984) Water column anomalies associated with hydrothermal activity in the Guaymas Basin, Gulf of California. *Earth Planet. Sci. Lett.* **68**: 57–72.
- Carini, P., Dupont, C.L., and Santoro, A.E. (2018) Patterns of thaumarchaeal gene expression in culture and diverse marine environments. *Environ. Microbiol.* **20**: 2112–2124.
- Chen, N., Bianchi, T.S., and Bland, J.M. (2003) Implications for the role of pre- versus post-depositional transformation of chlorophyll-a in the Lower Mississippi River and Louisiana shelf. *Mar. Chem.* **81**: 37–55.
- Cowen, J.P., Wen, X., and Popp, B.N. (2002) Methane in aging hydrothermal plumes. *Geochim. Cosmochim. Acta* **66**: 3563–3571.
- Crespo-Medina, M., Meile, C.D., Hunter, K.S., Diercks, A.R., Asper, V.L., Orphan, V.J., et al. (2014) The rise and fall of methanotrophy following a deepwater oil-well blowout. *Nat. Geosci.* **7**: 423–427.
- Von Damm, K.L., Edmond, J.M., Measures, C.I., and Grant, B. (1985) Chemistry of submarine hydrothermal solutions at Guaymas Basin, Gulf of California. *Geochim. Cosmochim. Acta* **49**: 2221–2237.

- DeLong, E.F. (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci U.S.A.* **89**: 5685–5689.
- Dick, G.J., Clement, B.G., Webb, S.M., Fodrie, F.J., Bargar, J.R., and Tebo, B.M. (2009) Enzymatic microbial Mn(II) oxidation and Mn biooxide production in the Guaymas Basin deep-sea hydrothermal plume. *Geochim. Cosmochim. Acta* **73**: 6517–6530.
- Dixon, J.L., Beale, R., and Nightingale, P.D. (2011) Microbial methanol uptake in northeast Atlantic waters. *ISME J.* **5**: 704–716.
- Dixon, J.L. and Nightingale, P.D. (2012) Fine-scale variability in methanol uptake and oxidation: From the microlayer to 1000 m. *Biogeosciences* **9**: 2961–2972.
- Dixon, J.L., Sargeant, S., Nightingale, P.D., and Colin Murrell, J. (2013) Gradients in microbial methanol uptake: Productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean. *ISME J.* **7**: 568–580.
- Dowell, F., Cardman, Z., Dasarathy, S., Kellermann, M.Y., Lipp, J.S., Ruff, S.E., et al. (2016) Microbial communities in methane- and short chain alkane-rich hydrothermal sediments of Guaymas Basin. *Front. Microbiol.* **7**:
- Einsele, G., Gieskes, J.M., Curray, J., Moore, D.M., Aguayo, E., Aubry, M.-P., et al. (1980) Intrusion of basaltic sills into highly porous sediments, and resulting hydrothermal activity. *Nature* **283**: 441–445.
- Eppley, R.W. and Peterson, B.J. (1979) Particulate organic matter flux and planktonic new production in the deep ocean. *Nature* **282**: 677–680.
- Falkowski, P., Scholes, R.J., Boyle, E., Canadell, J., Canfield, D., Elser, J., et al. (2000) The global carbon cycle: A test of our knowledge of earth as a system. *Science* (80-.). **290**: 291–296.

- Field, C.B., Behrenfeld, M.J., Randerson, J.T., and Falkowski, P. (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science* (80-.). **281**: 237–240.
- Gelesh, L., Marshall, K., Boicourt, W., and Lapham, L. (2016) Methane concentrations increase in bottom waters during summertime anoxia in the highly eutrophic estuary, Chesapeake Bay, U.S.A. *Limnol. Oceanogr.* **61**: S253–S266.
- Gillies Campbell, L., Thrash, J.C., Rabalais, N.N., and Mason, O.U. (2019) Extent of the annual Gulf of Mexico hypoxic zone influences microbial community structure. *PLoS One* **14**: 1–23.
- Gillies, L.E., Thrash, J.C., deRada, S., Rabalais, N.N., and Mason, O.U. (2015) Archaeal enrichment in the hypoxic zone in the northern Gulf of Mexico. *Environ. Microbiol.* **17**: 3847–3856.
- Hatzenpichler, R., Lebedeva, E. V., Spieck, E., Stoecker, K., Richter, A., Daims, H., and Wagner, M. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 2134–2139.
- Heal, K.R., Qin, W., Ribalet, F., Bertagnolli, A.D., Coyote-Maestas, W., Hmelo, L.R., et al. (2017) Two distinct pools of B12 analogs reveal community interdependencies in the ocean. *Proc. Natl. Acad. Sci. U. S. A.* **114**: 364–369.
- Heikes, B.G., Chang, W., Pilson, M.E.Q., Swift, E., Singh, H.B., Guenther, A., et al. (2002) Atmospheric methanol budget and ocean implication. *Global Biogeochem. Cycles* **16**: 80-1-80–13.
- IPCC (2019) Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and

- greenhouse gas fluxes in terrestrial ecosystems Shukla, P.R., Skea, J., Calvo Buendia, E., Masson-Delmotte, V., Pörtner, H.-O., Roberts, D.C., Zhai, P., Slade, R., Connors, S., van Diemen, R., Ferrat, M., Haughey, E., Luz, S., Neogi, S., Pathak, M., Petzold, J., Portugal Pereira, J., Vyas, P., Huntley, E., Kissick, K., Belkacemi, M., and Malley, J. (eds).
- Jannasch, H.W., Wheat, C.G., Plant, J.N., Kastner, M., and Stakes, D.S. (2004) Continuous chemical monitoring with osmotically pumped water samplers: OsmoSampler design and applications. *Limnol. Oceanogr. Methods* **2**: 102–113.
- Jørgensen, B.B. (2000) Bacteria and Marine Biogeochemistry. In, *Marine Geochemistry*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 173–207.
- Jørgensen, B.B., Findlay, A.J., and Pellerin, A. (2019) The biogeochemical sulfur cycle of marine sediments. *Front. Microbiol.* **10**: 1–27.
- Jørgensen, B.B., Zawacki, L.X., and Jannasch, H.W. (1990) Thermophilic bacterial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent site (Gulf of California). *Deep Sea Res. Part A. Oceanogr. Res. Pap.* **37**: 695–710.
- Kallmeyer, J. and Boetius, A. (2004) Effects of Temperature and Pressure on Sulfate Reduction and Anaerobic Oxidation of Methane in Hydrothermal Sediments of Guaymas Basin. *Appl. Environ. Microbiol.* **70**: 1231–1233.
- Karl, D.M., Beversdorf, L., Björkman, K.M., Church, M.J., Martinez, A., and Delong, E.F. (2008) Aerobic production of methane in the sea. *Nat. Geosci.* **1**: 473–478.
- Karl, D.M., Wirsén, C.O., and Jannasch, H.W. (1980) Deep-Sea Primary Production at the Galápagos Hydrothermal Vents. *Science* (80-.). **207**: 1345–1347.

- Karner, M.B., Delong, E.F., and Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nat. Lett.* **409**: 507–510.
- Kawka, O.E., and Simoneit, B.R.T. (1987) Survey of hydrothermally-generated petroleums from the Guaymas Basin spreading center. *Org. Geochem.* **11**: 311–328.
- Kitzinger, K., Padilla, C.C., Marchant, H.K., Hach, P.F., Herbold, C.W., Kidane, A.T., et al. (2019) Cyanate and urea are substrates for nitrification by Thaumarchaeota in the marine environment. *Nat. Microbiol.* **4**:
- Knittel, K. and Boetius, A. (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu. Rev. Microbiol.* **63**: 311–334.
- Könneke, M., Bernhard, A.E., De La Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.
- Konneke, M., Schubert, D.M., Brown, P.C., Hugler, M., Standfest, S., Schwander, T., et al. (2014) Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO₂ fixation. *Proc. Natl. Acad. Sci.* **111**: 8239–8244.
- Lam, P. (2004) Microbial ammonia oxidation in deep-sea hydrothermal plumes.
- Lang, S.Q., Butterfield, D.A., Schulte, M., Kelley, D.S., and Lilley, M.D. (2010) Elevated concentrations of formate, acetate, and dissolved organic carbon found at the Lost City hydrothermal field. *Geochim. Cosmochim. Acta* **74**: 941–952.
- Lapham, L., Wilson, R., Riedel, M., Paull, C.K., and Holmes, M.E. (2013) Temporal variability of in situ methane concentrations in gas hydrate-bearing sediments near Bullseye Vent, Northern Cascadia Margin. *Geochemistry, Geophys. Geosystems* **14**: 2445–2459.

- Lehtovirta-Morley, L.E., Ge, C., Ross, J., Yao, H., Nicol, G.W., and Prosser, J.I. (2014) Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds. *FEMS Microbiol. Ecol.* **89**: 542–552.
- Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I., and Nicol, G.W. (2011) Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc. Natl. Acad. Sci.* **108**: 15892–15897.
- Li, M., Baker, B.J., Anantharaman, K., Jain, S., Breier, J.A., and Dick, G.J. (2015) Genomic and transcriptomic evidence for scavenging of diverse organic compounds by widespread deep-sea archaea. *Nat. Commun.* **6**:.
- Lin, Y.S., Koch, B.P., Feseker, T., Ziervogel, K., Goldhammer, T., Schmidt, F., et al. (2017) Near-surface heating of young rift sediment causes mass production and discharge of reactive dissolved organic matter. *Sci. Rep.* **7**: 1–10.
- Lyu, L.N., Sun, C., Jin, H., Liu, Z.L., He, Y.H., Yu, L.M., et al. (2017) Annual variation of low-molecular-weight organic acids in the surface seawater of the Jiaozhou Bay. *Mar. Chem.* **194**: 43–54.
- Martens-Habbena, W., Torre, R. De, Stahl, D.A., Berube, P.M., and Urakawa, H. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nat. Lett.* **461**:.
- Martens, C.S. (1990) Generation of short chain acid anions in hydrothermally altered sediments of the Guaymas Basin, Gulf of California. *Appl. Geochemistry* **5**: 71–76.

- Mason, O.U., Canter, E.J., Gillies, L.E., Paisie, T.K., and Roberts, B.J. (2016) Mississippi river plume enriches microbial diversity in the northern Gulf of Mexico. *Front. Microbiol.* **7**:
- Mau, S., Blees, J., Helmke, E., Niemann, H., and Damm, E. (2013) Vertical distribution of methane oxidation and methanotrophic response to elevated methane concentrations in stratified waters of the Arctic fjord Storfjorden (Svalbard, Norway). *Biogeosciences* **10**: 6267–6268.
- McIntosh Marcek, H.A., Lesack, L.F.W., Orcutt, B.N., Wheat, C.G., Dallimore, S.R., Geeves, K., and Lapham, L. (2021) Continuous Dynamics of Dissolved Methane Over 2 Years and its Carbon Isotopes ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) in a Small Arctic Lake in the Mackenzie Delta. *J. Geophys. Res. Biogeosciences* **126**: 1–23.
- McKay, L., Klokman, V.W., Mendlovitz, H.P., Larowe, D.E., Hoer, D.R., Albert, D., et al. (2016) Thermal and geochemical influences on microbial biogeography in the hydrothermal sediments of Guaymas Basin, Gulf of California. *Environ. Microbiol. Rep.* **8**: 150–161.
- McKay, L., MacGregor, B.J., Biddle, J.F., Albert, D.B., Mendlovitz, H.P., Hoer, D.R., et al. (2012) Spatial heterogeneity and underlying geochemistry of phylogenetically diverse orange and white Beggiatoa mats in Guaymas Basin hydrothermal sediments. *Deep. Res. Part I Oceanogr. Res. Pap.* **67**: 21–31.
- Meier, D. V., Bach, W., Girguis, P.R., Gruber-Vodicka, H.R., Reeves, E.P., Richter, M., et al. (2016) Heterotrophic Proteobacteria in the vicinity of diffuse hydrothermal venting. *Environ. Microbiol.* **18**: 4348–4368.

- Metcalf, W.W., Griffin, B.M., Cicchillo, R.M., Gao, J., Janga, S.C., Cooke, H.A., et al. (2012) Synthesis of Methylphosphonic Acid by Marine Microbes: A Source for Methane in the Aerobic Ocean. *Science* (80-.).
- Mincer, T.J. and Aicher, A.C. (2016) Methanol production by a broad phylogenetic array of marine phytoplankton. *PLoS One* **11**: 1–17.
- Naqvi, S.W.A., Bange, H.W., Gibb, S.W., Goyet, C., Hatton, A.D., and Upstill-Goddard, R.C. (2005) Biogeochemical ocean-atmosphere transfers in the Arabian Sea. *Prog. Oceanogr.* **65**: 116–144.
- Van Neste, A., Duce, R.A., and Lee, C. (1987) Methylamines in the marine atmosphere. *Geophys. Res. Lett.* **14**: 711–714.
- Nixon, S.W. (1995) Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia* **41**: 199–219.
- Orcutt, B.N., Lapham, L., Delaney, J., Sarode, N., Marshall, K.S., Whaley-Martin, K.J., et al. (2017) Microbial response to oil enrichment in Gulf of Mexico sediment measured using a novel long-term benthic lander system. *Elementa* **5**:
- Padilla, C.C., Bristow, L.A., Sarode, N., Garcia-Robledo, E., Gómez Ramírez, E., Benson, C.R., et al. (2016) NC10 bacteria in marine oxygen minimum zones. *ISME J.* **10**: 2067–2071.
- Parkes, J., Cragg, B., and Wellsbury, P. (2002) Recent studies on bacterial populations and processes in subseafloor sediments: A review. *Hydrogeol. J.* **10**: 346–346.
- Perner, M., Gonnella, G., Hourdez, S., Böhnke, S., Kurtz, S., and Girguis, P. (2013) In situ chemistry and microbial community compositions in five deep-sea

- hydrothermal fluid samples from Irina II in the Logatchev field. *Environ. Microbiol.* **15**: 1551–1560.
- Peter, J.M., Peltonen, P., Scott, S.D., Simoneit, B.R.T., and Kawka, O.E. (1991) 14 C ages of hydrothermal petroleum and carbonate in Guaymas Basin, Gulf of California: implications for oil generation, expulsion, and migration. *Geology* **19**: 253–256.
- Qin, W., Amin, S.A., Martens-Habbena, W., Walker, C.B., Urakawa, H., Devol, A.H., et al. (2014) Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 12504–12509.
- Qin, W., Heal, K.R., Ramdasi, R., Kobelt, J.N., Martens-habbena, W., Bertagnolli, A.D., et al. (2018) Nitrosopumilus maritimus gen . nov ., sp . nov ., Nitrosopumilus cobalamin genes sp . nov ., Nitrosopumilus oxycinae sp . nov ., and Nitrosopumilus ureiphilus sp . nov ., four marine ammonia- oxidizing archaea of the phylum Thaumarchaeota. *Int. J. Syst. Evolutionary Microbiol.* 5067–5079.
- Rabalais, N.N., Turner, R.E., Sen Gupta, B.K., Boesch, D.F., Chapman, P., and Murrell, M.C. (2007) Hypoxia in the northern Gulf of Mexico: Does the science support the plan to reduce, mitigate, and control hypoxia? *Estuaries and Coasts* **30**: 753–772.
- Rabalais, N.N., Turner, R.E., and Wiseman, W.J. (2002) Gulf of Mexico Hypoxia, A.K.A. “The Dead Zone.” *Annu. Rev. Ecol. Syst.* **33**: 235–263.
- Redalje, D.G., Lohrenz, S.E., and Fahnenstiel, G.L.. (1994) The Relationship between Primary Production and the Vertical Export of Particulate Organic Matter in a River-Impacted Coastal Ecosystem. *Estuaries* **17**: 829–838.
- Reeburgh, W.S. (2007) Oceanic methane biogeochemistry. *Chem. Rev.* **107**: 486–513.

- Reysenbach, A.-L., Banta, A.B., Boone, D.R., Cary, S.C., and Luther, G.W. (2000) Microbial essentials at hydrothermal vents. *Nature* **404**.
- Reysenbach, A.L., and Shock, E. (2002) Merging genomes with geochemistry in hydrothermal ecosystems. *Science* (80-.). **296**: 1077–1082.
- Rogener, M.K., Bracco, A., Hunter, K.S., Saxton, M.A., and Joye, S.B. (2018) Long-term impact of the Deepwater Horizon oil well blowout on methane oxidation dynamics in the northern Gulf of Mexico. *Elementa* **6**: 1–17.
- Rogener, M.K., Hunter, K., Rabalais, N.N., Roberts, B.J., Bracco, A., Stewart, F.J., and Joye, S.B. (2021) Pelagic denitrification and methane oxidation in oxygen- depleted waters of the Louisiana shelf. *Biogeochemistry* **8**:
- Rogener, M.K., Sipler, R.E., Hunter, K.S., Bronk, D.A., and Joye, S.B. (2020) Pelagic methane oxidation in the northern Chukchi Sea. *Limnol. Oceanogr.* **65**: 96–110.
- Rothschild, L.J., and Mancinelli, R.L. (2001) Life in extreme environments. *Nature* **409**: 1092–1101.
- Santoro, A.E. and Casciotti, K.L. (2011) Enrichment and characterization of ammonia-oxidizing archaea from the open ocean: Phylogeny, physiology and stable isotope fractionation. *ISME J.* **5**: 1796–1808.
- Simoneit, B.R.T., Lonsdale, P.F., Edmond, J.M., and Shanks, W.C. (1990) Deep-water hydrocarbon seeps in Guaymas Basin, Gulf of California. *Appl. Geochemistry* **5**: 41–49.
- Singh, H., Chen, Y., Staudt, A., Jacob, D., Blake, D., Heikes, B., and Snow, J. (2001) Evidence from the pacific troposphere for large global sources of oxygenated organic compounds. *Nature* **410**: 1078–1081.

- Steinle, L., Graves, C.A., Treude, T., Ferré, B., Biastoch, A., Bussmann, I., et al. (2015) Water column methanotrophy controlled by a rapid oceanographic switch. *Nat. Geosci.* **8**: 378–382.
- Steinle, L., Schmidt, M., Bryant, L., Haeckel, M., Linke, P., Sommer, S., et al. (2016) Linked sediment and water-column methanotrophy at a man-made gas blowout in the North Sea: Implications for methane budgeting in seasonally stratified shallow seas. *Limnol. Oceanogr.* **61**: S367–S386.
- Stewart, F.J., Ulloa, O., and Delong, E.F. (2012) Microbial metatranscriptomics in a permanent marine oxygen minimum zone. *Environ. Microbiol.* **14**: 23–40.
- Teske, A., De Beer, D., McKay, L., Tivey, M.K., Biddle, J.F., Hoer, D., et al. (2016) The Guaymas Basin hiking guide to hydrothermal mounds, chimneys, and microbial mats: Complex seafloor expressions of subsurface hydrothermal circulation. *Front. Microbiol.* **7**.
- Thrash, J.C., Seitz, K.W., Baker, B.J., Temperton, B., Gillies, L.E., Rabalais, N.N., et al. (2017) Metabolic roles of uncultivated bacterioplankton lineages in the northern Gulf of Mexico “dead zone.” *MBio* **8**: 1–20.
- Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Urich, T., et al. (2011) Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 8420–8425.
- Turner, R.E., Rabalais, N.N., Alexander, R.B., McIsaac, G., and Howarth, R.W. (2007) Characterization of Nutrient, Organic Carbon, and Sediment Loads and Concentrations from the Mississippi River into the Northern Gulf of Mexico. *Estuaries and coasts* **30**: 773–790.

- Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J. a, et al. (2004) Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science* **304**: 66–74.
- Walker, C.B., De La Torre, J.R., Klotz, M.G., Urakawa, H., Pinel, N., Arp, D.J., et al. (2010) Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 8818–8823.
- Wang, X.C., Chen, R.F., and Gardner, G.B. (2004) Sources and transport of dissolved and particulate organic carbon in the Mississippi River estuary and adjacent coastal waters of the northern Gulf of Mexico. *Mar. Chem.* **89**: 241–256.
- Welhan, J.A. (1988) Origins of methane in hydrothermal systems. *Chem. Geol.* **71**: 183–198.
- Welhan, J.A., and Lupton, J.E. (1987) Light hydrocarbon gases in Guaymas Basin hydrothermal fluids: thermogenic versus abiogenic origin. *Am. Assoc. Pet. Geol. Bull.* **71**: 215–223.
- Wheat, C.G., Jannasch, H.W., Plant, J.N., Moyer, C.L., Sansone, F.J., and McMurtry, G.M. (2000) Continuous sampling of hydrothermal fluids from Loihi Seamount after the 1996 event. *J. Geophys. Res. Solid Earth* **105**: 19353–19367.
- Wirsén, C.O., Jannasch, H.W., and Molyneaux, S.J. (1993) Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. *J. Geophys. Res.* **98**: 9693–9703.

- Worden, A.Z., Follows, M.J., Giovannoni, S.J., Wilken, S., Zimmerman, A.E., and Keeling, P.J. (2015) Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes. *Science* (80-.). **347**..
- Wuchter, C., Abbas, B., Coolen, M.J.L., Herfort, L., Van Bleijswijk, J., Timmers, P., et al. (2006) Archaeal nitrification in the ocean. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 12317–12322.
- Yang, M., Nightingale, P.D., Beale, R., Liss, P.S., Blomquist, B., and Fairall, C. (2013) Atmospheric deposition of methanol over the Atlantic Ocean. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 20034–20039.
- Zhou, Z., Liu, Y., Pan, J., Cron, B.R., Toner, B.M., Anantharaman, K., et al. (2020) Gammaproteobacteria mediating utilization of methyl-, sulfur- and petroleum organic compounds in deep ocean hydrothermal plumes. *ISME J.*
- Zhuang, G., Elling, F.J., Nigro, L.M., Samarkin, V., Joye, S.B., Teske, A., and Hinrichs, K. (2016) Multiple evidence for methylotrophic methanogenesis as the dominant methanogenic pathway in hypersaline sediments from the Orca Basin, Gulf of Mexico. *Geochim. Cosmochim. Acta* **187**: 1–20.
- Zhuang, G., Montgomery, A., Samarkin, V.A., Song, M., Liu, J., Schubotz, F., et al. (2019) Generation and Utilization of Volatile Fatty Acids and Alcohols in Hydrothermally Altered Sediments in the Guaymas Basin, Gulf of California. *Geophys. Res. Lett.* **46**: 2637–2646.
- Zhuang, G., Montgomery, A., Sibert, R.J., Rogener, M., Samarkin, V.A., and Joye, S.B. (2018) Effects of pressure, methane concentration, sulfate reduction activity, and

temperature on methane production in surface sediments of the Gulf of Mexico.

Limnol. Oceanogr. **63**: 2080–2092.

Zhuang, G., Peña-Montenegro, T.D., Montgomery, A., Hunter, K.S., and Joye, S.B.

(2018) Microbial metabolism of methanol and methylamine in the Gulf of Mexico :

insight into marine carbon and nitrogen cycling. *Environ. Microbiol.* **20**: 4543–4554.

Zhuang, G., Peña-Montenegro, T.D., Montgomery, A., Montoya, J.P., and Joye, S.B.

(2019) Significance of Acetate as a Microbial Carbon and Energy Source in the

Water Column of Gulf of Mexico: Implications for Marine Carbon Cycling. *Global*

Biogeochem. Cycles **33**: 223–235.

CHAPTER 2

IN SITU SURVEYS REVEAL SPATIAL AND TEMPORAL HETEROGENEITY IN METHANE DISTRIBUTIONS IN HYDROTHERMALLY-IMPACTED SEDIMENTS OF THE GUAYMAS BASIN¹

¹Montgomery, A.M., R. C. Storo, G.C. Zhuang, K.S. Hunter, A.P. Teske, L. L. Lapham, S.B. Joye. A modified version to be submitted to *Geophysical Research Letters*

ABSTRACT

Methane is an important greenhouse gas with a warming potential nearly 30 times greater than carbon dioxide. In marine sediments, methane is produced via several mechanisms and in deep-sea hydrothermal systems, such as the Guaymas Basin, concentrations can be elevated well above saturation. Methane is an important metabolite in the anaerobic carbon cycle, and methane dynamics are controlled by a range of physical and biogeochemical factors. However, many of these controls are poorly constrained, and accurate *in situ* concentrations of methane are lacking in most environments. Here, we surveyed several hydrothermally influenced environments in the Guaymas Basin to assess spatiotemporal patterns of methane dynamics. We used osmotically driven pumps (OsmoSamplers) to collect samples at *in situ* pressures and documented methane concentrations up to 46 mmol L⁻¹. Variability in results between sampling methods underscores the spatial heterogeneity of these hydrothermal sediments. Continuous sampling during a three-month time-series revealed spatiotemporal variation in methane availability from changing fluid flow regimes and indicating dynamic microbial activity. These results provide an important advance in documenting and understanding methane distributions in deep-sea hydrothermal systems.

Keywords: Methane, Hydrothermal System, Guaymas Basin, Anaerobic oxidation, Sulfate Reduction

INTRODUCTION

Deep-sea hydrothermal systems are dynamic environments that operate as a conduit between seafloor biogeochemical processes and the global marine environment. The Guaymas Basin, a marginal rift basin in the Gulf of California, is characterized by intense hydrothermal activity coincident with rapid seafloor spreading in the basement overlain by organic-rich sediment packages up to 400 m thick (Calvert, 1966; Merewether *et al.*, 1985; Lizarralde *et al.*, 2011). Subsurface doleritic sill intrusions instigate thermocatalytic alterations of sedimentary organic matter, producing methane and other low molecular weight carbon substrates like acetate (Einsele *et al.*, 1980; Von Damm *et al.*, 1985; Kawka and Simoneit, 1987; Bazylinski *et al.*, 1988). Advective flow through the sediment column distributes these organic rich fluids throughout the basin, where they mix with seawater and fuel diverse biogeochemical processes (Lonsdale and Becker, 1985; Gundersen *et al.*, 1992).

As a dominant component of hydrothermal fluids (Welhan, 1988), methane has received substantial attention in Guaymas Basin sediments. Methane is produced primarily in the deep subsurface, and methane stable isotope data ($\delta^{13}\text{CH}_4 = -30\text{‰}$ to -70‰) suggest a mixed thermogenic and biogenic origin (Paull *et al.*, 2007; Lizarralde *et al.*, 2011; Biddle *et al.*, 2012; McKay *et al.*, 2012; Teske *et al.*, 2019). As a result, sediments are inundated with methane, with surficial concentrations reaching up to 28 mmol L^{-1} (McKay *et al.*, 2016). During fluid migration through such sediments, methane may be trapped in gas hydrate if *in situ* temperatures are cool enough (Paull *et al.*, 2007). Gas hydrate has been observed at off-axis areas of low-temperature fluid discharge, such as Octopus Mound and

Ring Vent (Teske et al. 2019) or along the Sonoran Margin. While gas hydrate formation is possible in Guaymas Basin, the steep thermal gradients imply that hydrate stability could be possible, but it is likely rare (Sloan, 1998). The formation of gas hydrate can also alter local biogeochemical profiles via ion exclusion (Hesse and Harrison, 1981; Ussler and Paull, 1995; Torres *et al.*, 2004). Alternatively, buoyant hydrothermal fluids eventually reach the surface where they are rapidly discharged through hydrothermal chimneys or vented diffusively at the sediment-water interface (Merewether *et al.*, 1985; Simoneit *et al.*, 1990; Magenheim and Gieskes, 1992).

In addition to these abiotic regulators of Guaymas Basin methane dynamics, microbially-mediated processes also play a dominant role. In shallow sediments of Guaymas Basin, microbial methanogenesis occurs (Zhuang, Montgomery, Samarkin, *et al.*, 2019), but rates of the anaerobic oxidation of methane (AOM) greatly exceeds methanogenesis rates (Kallmeyer and Boetius, 2004; Dowell *et al.*, 2016). Elevated AOM rates, up to $3.5 \mu\text{mol cm}^3 \text{d}^{-1}$, are substantial enough to alter methane isotopic composition (*i.e.*, heavier in shallower sediments) and significantly alters the pool size (Biddle *et al.*, 2012; Bowles *et al.*, 2019). In Guaymas Basin, while some AOM is coupled to sulfate reduction (SR) (Boetius *et al.*, 2000) generating a firm connection between sulfate and methane cycling, AOM is also likely coupled to other processes (Bowles et al. 2019).

The discharge of hydrothermal fluids provides energy-rich substrates that support dynamic and vibrant benthic biological communities (Dubilier *et al.*, 2008; Teske *et al.*, 2014, 2016; Dombrowski *et al.*, 2018). In particular, the boundary of oxygenated waters

and anoxic sediments supports surface microbial mats colonized by large bacteria of the genus *Beggiatoa* (Nelson *et al.*, 1989; Teske and Nelson, 2006). These mats mark areas of hydrothermal inputs of reduced substrates, like hydrogen sulfide, and are associated with elevated methane concentrations (McKay *et al.*, 2016; Teske *et al.*, 2016). Furthermore, the type of *Beggiatoa* may indicate spatial variability in methane concentrations on scales less than one meter (Sassen *et al.*, 1994; McKay *et al.*, 2012)

Methane dynamics are influenced by physical, chemical, and biological processes in Guaymas Basin sediments. The extent to which spatiotemporal variability and changes in methane, and to a lesser extent sulfate, reflect or influence sediment biogeochemistry remains unconstrained. We surveyed *in situ* methane concentrations with Jumbo OsmoSamplers across diverse sites in the Guaymas Basin to extend our understanding of the spatial variability of hydrothermal sediments. We also, for the first time, made long-term (three months) continuous measurements of methane and sulfate concentrations in a hydrothermally influenced *Beggiatoa* microbial mat. The data presented here provide further insight into the spatiotemporal heterogeneity of methane cycling in deep-sea hydrothermal sediments.

METHODS

Study sites

We surveyed sediments and overlying waters from several sites in the Guaymas Basin in the Gulf of California (Fig. 1). Two of the sites, AcetoBalsamico and Cathedral Hill, are hydrothermally influenced and have been characterized extensively. These sites

are adjacent to active hydrothermal vents, exhibit strong thermal gradients in the sediments (up to 120°C at 35 cmbsf), and are overlain with diverse microbial mats typically dominated by the family *Beggiatoa* (Teske *et al.*, 2016). Geochemistry samples were collected from within numerous *Beggiatoa* mats and from overlying bottom waters, with methods described in detail below. Additionally, samples were retrieved from a gas hydrate mound near a cold seep along the Sonora Margin (Fig. 1), a transform fault approximately 65 km to the north of Guaymas Basin (Simoneit *et al.*, 1990). These samples were collected during two cruises on board the RV *Atlantis* (November 2018) and RV *Falkor* (February-March 2019) using the human-operated vehicle (HOV) *Alvin* and the remotely operated vehicle (ROV) *SuBastian*, respectively.

OsmoSampler overview and configurations

OsmoSamplers employ an osmotically driven pump to collect water samples at fixed depths continuously thus revealing temporal dynamics of *in situ* fluid geochemistry and with adaptation, microbial community composition (Jannasch *et al.*, 2004; Robidart *et al.*, 2013; Orcutt *et al.*, 2017). The pump is powered by the osmotic gradient created between two chambers separated by a semi-permeable membrane (Alzet Osmotic Pumps, Model 2ML1, Cupertino, CA, USA). The chamber on the outlet side of the membrane is filled with a supersaturated sodium chloride solution, while the inlet side is filled with fresh deionized water. As freshwater diffuses across the membrane, “sample” fluid is continuously drawn into a sample coil for collection and subsequent analysis. The flow rate of this pump dictates the temporal resolution of sampling and can range from 0.1 mL d⁻¹ to > 5 mL d⁻¹ depending on the design of the pumps and environmental conditions.

Specifically, the size and type of membrane and *in situ* temperature influence the flow rate. In the deep sea, bottom water temperatures do not fluctuate significantly ($\sim 4^{\circ}\text{C}$), thus we assumed pumping rates were constant on the seafloor (Wheat *et al.*, 2000; Jannasch *et al.*, 2004). Generally, OsmoSamplers are advantageous because they allow for continuous long-term (*i.e.*, weeks to years) measurements (Wheat *et al.*, 2000; Gelesh *et al.*, 2016; McIntosh Marcek *et al.*, 2021). One of the most important aspects of OsmoSamplers is that they maintain gas samples at *in situ* pressure (Lapham *et al.*, 2008, 2013; Orcutt *et al.*, 2013), making it possible to accurately quantify *in situ* gas concentrations in deep-sea sediments.

Two OsmoSampler configurations were used to characterize methane and sulfate dynamics in Guaymas Basin sediments. The first configuration was designed to measure *in situ* methane concentrations over a relatively short deployment. These so-called Jumbo OsmoSamplers were fitted with a 24-membrane pump which collected sufficient fluid for geochemical analyses during short-term deployments, as short as 36 hours. The inlet (sampling) end of each pump was connected to a Rhizon Filter A (Rhizosphere Research Products, 0.15 μm pore diameter, Wageningen, NLD) to exclude particulates and preclude living microbes that could alter the pool sizes. The copper sample coil was plumbed to the OsmoSampler pumping chamber with PEEK tubing. Rhizon filters and PEEK tubing each having working temperatures above 90°C , which should be sufficient for surficial Guaymas Basin sediments. Equipment was inspected after each recovery and no thermal damage was evident. Pumps were grouped in triplicate arrays and attached to a T-handle for submersible manipulation and to facilitate the generation of depth profiles (Lapham *et*

al., 2008, 2013). Rhizon filters were attached to the T-handle array so that samples from the seawater interface (SWI), 4 cm, and 8 cm depths were sampled. Flow rates were determined before and after deployments and ranged from 5.8 to 18.1 mL d⁻¹ during the 2018 cruise and from 4.1 to 8.7 mL d⁻¹ during the 2019 cruise. Sample collection coils consisted of 5-m long copper coils (1.6 mm O.D. and 0.8 mm I.D.); copper tubing prevented diffusive loss of gases. Each array was outfitted with a high-pressure valve that was closed on the seabed at the point of recovery; shutting this valve maintained *in situ* pressure and prevented degassing. Deployment information is summarized in Table 1, but briefly, sites were chosen based on the presence of steep thermal gradients, *Beggiatoa* microbial mats, or a gas hydrate mound.

The second configuration was used to investigate longer-term temporal dynamics of methane and sulfate in Guaymas Basin sediments. Four 8-membrane OsmoSamplers were deployed in pairs to monitor the SWI and 8 cm depth with the same array described above for the Jumbo OsmoSamplers. The pumping rates were 0.5 ± 0.07 mL d⁻¹ based on measurements taken after the deployment. The sample coils were the same as above (1.6 mm O.D. and 0.8 mm I.D.) but were 300 m in length. High-pressure valves were not available for long-term deployments. The long-term OsmoSamplers were deployed by HOV *Alvin* (Dive 5000) on 25 November 2018 near Ace's Spire (27° 0.384 N, 111° 24.270 W). During HOV *Alvin* Dive 5000, a “fried-egg” microbial mat was observed (Fig. 13A) and chosen for the OsmoSampler deployment. They were retrieved on 27 February 2019 by the ROV *SuBastian* (Dive 231) at which time remnants of the microbial mat were present, but much of the mat had disappeared (Fig. 13B).

Upon recovery of the OsmoSamplers on deck, each copper coil was immediately sealed by crimping and it was then detached from the pump. A small section of each coil (~ 3 cm) was removed, and the contents were used to check salinity to ensure adequate pumping (i.e., the fluid was seawater not freshwater). The sample coils were stored at 4°C for subsequent shore-based analysis.

Subsampling of copper coils

Coils were processed using methods described previously (Jannasch *et al.*, 2004; Lapham *et al.*, 2013; Gelesh *et al.*, 2016). The sample coils from the long-term deployment between cruises were cut into equal sections based on the measured 0.5 mL d⁻¹ pumping rate. Coils from each pump were sectioned into equal lengths (4.5 – 5 m) which gave temporal resolution ranging from 5-15 days per section. Small subsamples were taken from each coil section to measure salinity with a handheld Extech RF20 refractometer. The transition from seawater salinity to freshwater designated the end of the sampling period. Water from each coil section was extracted with a benchtop roller (McIntosh Marcek *et al.*, 2021). Approximately 2 mL of sample was collected under gas-tight conditions into 13.5 mL glass serum vials that were pre-flushed with ultra-zero air (Airgas) and sealed with a butyl rubber stopper and aluminum crimp cap. After sample addition, each vial was over-pressurized with 8 mL of ultra-zero air and these samples were used to determine methane concentration. Additionally, to measure anions (sulfate and chloride) 200 µL of sample fluid was collected into a 2 mL microcentrifuge tube and acidified with 20 µL of 0.1 mol L⁻¹ phosphoric acid.

Coils from the short-term Jumbo OsmoSampler deployments were processed in nearly the same fashion, with a few minor differences. First, sample coils were not subsectioned, rather each coil was considered a discrete sample. Copper sample coils were squeezed into a plastic gas-tight syringe. Subsamples for anions (200 μL) were collected as described above but were acidified with 40 μL of 0.1 mol L^{-1} phosphoric acid. The remaining volume (~ 2 mL) from each coil was collected for methane analysis, as described above.

Paired Discrete Samples

Push cores were collected directly adjacent to each Jumbo OsmoSampler deployment at the beginning and end of the long-term deployment. Paired push cores were collected as near to the OsmoSampler T-handle array as possible (typically within ~ 30 cm) using either HOV *Alvin* or ROV *SuBastian* (See Fig. 13B for an example). Push cores were collected and sectioned for geochemical characterization using methods described previously (Joye *et al.*, 2004; Zhuang, Montgomery, Samarkin, *et al.*, 2019). For each depth horizon, a 3 mL sediment sample was collected to determine methane concentration. This sample was collected using a cut-end syringe and transferred into a 20 mL serum vial pre-filled with 2 mL of 2 M of He-purged NaOH, which served to halt biological activity. Directly after the transfer of sediment into the vial, it was sealed with a blue rubber butyl stopper and aluminum crimp seal, and vortexed to homogenize the sample and NaOH kill solution. Porewater was extracted from sediments by centrifugation (15 min at 4000 rpm) and subsequently filtered through a pre-rinsed Target[®] filter (0.2 μm). After filtration, sulfate/chloride samples were preserved in trace metal grade nitric acid (Sigma; 10 μL acid

per mL sample). Methane and acidified samples were stored at room temperature until analysis in the shore-based lab.

Analytical Measurements

Methane, sulfate, and chloride concentrations from OsmoSampler coils were determined as described recently (Gelesh *et al.*, 2016; McIntosh Marcek *et al.*, 2021). Briefly, for porewater methane concentration, a diluted headspace sub-sample was injected into a gas chromatograph (SRI 8610C) equipped with a flame ionizing detector (GC-FID), HaySep D column (1.83 m, 3.2 mm I.D.), and a 1 mL sample loop. Several samples had to be diluted serially due to high concentrations. High purity gas standards (Airgas) were utilized to generate a standard curve that was used to calculate concentrations. Corrections were made for porosity and air dilution. Anions (sulfate and chloride) were determined after dilution on a Dionex ICS 1000 ion chromatograph (IonPac AG22 4 x 3 50 mm guard column, IonPac AS22 4 x 250 mm analytical column, and ASRS 300 4 mm suppressor) with an AS40 Autosampler. Standards curves were prepared from IAPSO seawater standards.

These same geochemical parameters were measured in the paired push core samples either shipboard or in the laboratory (Joye *et al.*, 2004). Sediment methane concentrations were determined shipboard in 2018 and back at UGA in 2019. All samples were analyzed on a GC-FID (SRI, 8610C) equipped with a HaySep D packed column by direct injection of 250 μ L of sample headspace. Sediment and gaseous standards prepared from high purity standards of known concentrations (Airgas) were used to determine

methane concentration. Anion concentrations (sulfate and chloride) were measured on a Dionex® Ion Chromatograph and were quantified based on analytical IAPSO seawater standards.

Time-series AOM and SR estimates

We used the time-series methane and sulfate concentrations to estimate temporal changes in anaerobic methane oxidation (AOM) and sulfate reduction (SR) rates. These estimations were based on extensive rate measurements of AOM and SR made during the 2018 and 2019 research expeditions and during previous expeditions (data not shown). The results of these rate assessments are presented separately (Joye *et al.*, in preparation), but a general overview of the methods used to generate the rate constants are given below.

The methods to quantify AOM and SR (*i.e.*, sample collection, processing, and analysis) in marine sediments using radiotracer approaches have been described extensively (Joye *et al.*, 2004, 2010; Bowles, Samarkin, and Joye, 2011; Zhuang, Montgomery, and Joye, 2019; Zhuang, Montgomery, Samarkin, *et al.*, 2019). These methods employ radiotracers to independently track the conversion of $^{14}\text{CH}_4$ to $\text{H}^{14}\text{CO}_3^-$ and $^{35}\text{SO}_4^{2-}$ to H^{35}S^- according to Eq 1:



Processing commenced by transferring ~ 3 ml of sediment from discrete depths to modified Hungate tubes in triplicate under an argon stream to preserve anoxic conditions.

Each tube was capped headspace free and was injected with 100 μL of aqueous tracer. The total activity of tracer injected was ~ 20 and ~ 40 kBq for AOM and SR, respectively. The specific activity of the radiotracers (American Radiolabeled Chemicals Inc.) was 2 GBq mmol^{-1} for $^{14}\text{CH}_4$ and 84 kBq for $^{35}\text{SO}_4^{2-}$. Unlabeled methane (UHP) was added (final concentration of ~ 15 mmol L^{-1}) to account for degassing of methane during sampling. Samples were incubated at near *in situ* pressure and temperature using hydraulic pressure vessels (Bowles, Samarkin, and Joye, 2011) for ~ 2 days. Biological activity was terminated by the addition of 2 mL of 2 M NaOH (AOM) or Zinc Acetate (SR). Killed controls were processed exactly as live replicates but were amended with kill solution prior to tracer injection.

Subsequently, AOM samples were processed via acid digestion and SR samples were processed via hot chromium reduction as described previously (Joye *et al.*, 2004). Rates (Eq. (2)) and turnover constants (Eq. (3)) are calculated below and are in units of $\text{nmol cm}^{-3} \text{ d}^{-1}$ and d^{-1} , respectively:

$$\text{Rate} = [\text{substrate}] \times (\text{DPM}_{\text{product}} / \text{DPM}_{\text{substrate}}) \times (\alpha / t) \quad \text{Eq (2)}$$

$$\text{Turnover constant} = \text{Rate} / [\text{substrate}] \quad \text{Eq (3)}$$

In Eq (2), the substrate concentration is the wet sediment concentration of methane or sulfate, α is a fractionation factor unique to each isotope (Jørgensen, 1978; Alperin *et al.*, 1988) and t is the incubation length. Additionally, the DPM values are the radioactivity

of the products and reactants (after distillation) from Eq (1) determined by liquid scintillation analyzer (Perkin Elmer; Tricarb 2910TR). The turnover constant (Eq (3)) is calculated the same as the rate but doesn't include the substrate concentration.

Temporal estimates of AOM and SR were determined by first calculating the average and 95% confidence intervals of turnover constants for AOM and SR from our Guaymas Basin measurements, which were estimated with jackknife resampling. Then, these turnover constants were multiplied by the methane and sulfate concentrations determined from OsmoSampler deployments in the rate equation. Eq (3) was rearranged to calculate potential rates using Eq (4).

$$\text{Potential rate} = \text{Turnover Constant} * [\text{substrate}] \quad \text{Eq (4)}$$

Here, turnover constant is the average turnover constants from our Guaymas Basin measurements and the substrate concentration is the methane and sulfate concentrations determined from the OsmoSamplers (final units of $\text{nmol cm}^{-3} \text{ d}^{-1}$). Depth integrated rates were calculated by trapezoidal integration and are presented in units of $\text{mmol m}^{-2} \text{ d}^{-1}$.

Statistical analyses

Statistical tests were performed in R (version 4.0.5). Mean comparisons of geochemical parameters (methane, sulfate, and chloride) were made with the Wilcoxon Rank Sum Test after a log transformation. Relationships between data from

OsmoSampler and discrete samples were assessed with Kendall's Tau correlation coefficients. For each test, a p -value of < 0.05 was considered statistically significant.

RESULTS

Jumbo Osmosamplers

Jumbo Osmosampler deployments were made in sediments of the Guaymas Basin to measure sulfate and chloride concentrations, and to quantify *in situ* methane concentrations. For the Jumbo Osmosamplers, depth profiles were generated over depth by probing the surface water interface (SWI), and the 4 and 8 cm depth horizons. T-handle probes were inserted into the sediments associated with several *Beggiatoa* microbial mats and a gas hydrate mound. Push cores were collected in parallel to Jumbo OsmoSampler probes. Additionally, two Jumbo OsmoSampler probes were deployed on the sediment surface directly adjacent to a venting edifice.

Sulfate and methane concentrations were surveyed at the AcetoBalsamico site from four depth profiles; three deployments were in yellow microbial mats, while the fourth was at a reference (Fig. 2). Sediment temperatures were probed with a deployable temperature probe, and the maximum temperature was $\sim 10^{\circ}\text{C}$ at 10 cmbsf. Porewater sulfate concentrations were generally highest in surficial sediments and measurements from all depths ranged from 1.89 to 30.18 mmol L⁻¹. Sulfate concentrations determined from the Jumbo Osmosamplers were not below ~ 13 mmol L⁻¹, while sulfate concentrations determined from push cores taken from a yellow mat and the reference core were below 5 mmol L⁻¹ 8 cmbsf (Fig. 2B & D). Methane concentrations generally increased with depth

and ranged from $3.1 \mu\text{mol L}^{-1}$ to 22.6 mmol L^{-1} . The two cores with depleted sulfate concentrations had sediment methane concentrations up to $\sim 2 \text{ mmol L}^{-1}$.

At the Cathedral Hill site, three white microbial mats and one reference area were sampled to generate four additional depth profiles. Temperatures acquired by the submersible temperature probe at 10 cmbsf ranged from 4°C to 24°C (Fig. 3). Sulfate concentrations were higher at the SWI and generally decreased with depth. Measurements obtained from the Jumbo OsmoSamplers and the discrete samples did not agree, but, in general, sulfate decreased in both profiles. Sulfate concentrations from the OsmoSampler in the 24°C white mat decreased between SWI and 4 cmbsf to 1 mmol L^{-1} but increased again at 8 cmbsf (Fig. 3B). Methane concentrations at Cathedral Hill ranged from $3.1 \mu\text{mol L}^{-1}$ to 46.1 mmol L^{-1} , the highest value presented in this study. This methane concentration was observed at 4 cmbsf and coincided with the depleted sulfate concentration in the 24°C white mat.

Sediments from two additional sites were surveyed with the Jumbo OsmoSamplers, one at gas hydrate mound on the Sonoran Margin and the other directly adjacent to the Falkor Fountain hydrothermal vent, which is near Cathedral Hill (Fig. 4 & 5). Sediment temperatures were 10°C at 10 cmbsf for both profiles at the base of Falkor Fountain; the Sonoran Margin is not influenced by hydrothermal activity and sediments were roughly 4°C . Sulfate concentrations at these sites were mildly depleted ($< 20 \text{ mmol L}^{-1}$) at 8 cmbsf, except the reference site, which showed no sulfate depletion. The white mat exhibited a lens of sulfate depletion at 4 cmbsf ($< 10 \text{ mmol L}^{-1}$; Fig. 5B) from the Jumbo

OsmoSamplers, similar to Cathedral Hill. Methane concentrations ranged up to 4.3 and 6.5 mmol L⁻¹ at the Sonoran Margin and Falkor Fountain sites, respectively. At Falkor Fountain, methane exhibited similar depth distributions between the Jumbo OsmoSampler and push core samples, despite discrepancies in concentrations (Fig. 5).

In addition to the sediment surveys, the Jumbo Osmosamplers were deployed with the T-handle probes on the seafloor to sample water overlying the sediment (Fig. 6 & 12). The Jumbo OsmoSamplers were deployed directly adjacent to a venting edifice named Alvin Spire near Cathedral Hill, which was discovered with HOV *Alvin* (dive 4991) on the RV *Atlantis*. Temperature probes near the venting orifice at the time of discovery documented sediment temperatures up to 70°C at 10 cmbsf. However, temperature measurements were not taken at Alvin Spire at the time of deployment, but because the T-handle probes were deployed on the seafloor we assumed temperatures were near 4°C. The Jumbo OsmoSamplers were deployed to sample near the base (seafloor) and directly adjacent to the spire. Seawater sulfate concentrations were generally ~28 mmol L⁻¹, reflective of typical seawater. Although sulfate was depleted to 13.57 mmol L⁻¹ next to Alvin Spire (Fig. 12A). Methane concentrations were quite variable in these overlying waters, ranging from ~2 µmol L⁻¹ near the spire to 140 µmol L⁻¹ at the seafloor (Fig. 6A).

Long-term OsmoSamplers

Sulfate and methane concentrations were monitored in sediments near the Ace's Spire edifice for 3 months between December 2018 and February 2019 (Table 1). Two T-handle arrays connected to OsmoSamplers continuously collected pore fluids during this

time period at the SWI and 8 cm horizon. At the time of deployment, a robust fried-egg *Beggiatoa* mat was observed, and a probe was inserted into the orange and white mat (Fig. 13A). Sediment temperatures were measured with the HOV *Alvin* temperature probe and were up to 20°C and 44°C at 10 cmbsf for the white and orange mat, respectively. Upon recovery in February 2019, the microbial mat had disappeared and only a few remnants remained (Fig. 13B).

Methane concentrations at the SWI from the white mat were largely stagnant during the deployment, averaging $3.1 \pm 0.2 \mu\text{mol L}^{-1}$ (Fig. 7A). In contrast, methane concentrations in the orange mat, from the same depth, increased by several orders of magnitude over the course of 3 months. A substantial spike in the methane occurred in January 2019, where concentrations increased from $9.2 \mu\text{mol L}^{-1}$ to 1.6 mmol L^{-1} (Fig. 8A). Discrete methane samples were collected from push cores at the deployment and retrieval. For the white mat, methane concentrations determined discretely from push cores were between 13 and ~300 times higher than those obtained from the OsmoSamplers.

At the 8 cmbsf sediment horizon, methane concentrations were dynamic in both profiles and increased below the orange mat and varied minimally below the white mat. For example, methane concentrations in the white mat were initially $82.9 \mu\text{mol L}^{-1}$ and ultimately increased to $191.2 \mu\text{mol L}^{-1}$. However, an initial decline was observed during December 2018 (Fig. 7B). In the sediments below the orange mat, methane concentrations increased substantially between December 2018 and the end of January 2019 from 0.87 mmol L^{-1} to 4.16 mmol L^{-1} (Fig. 8B). Subsequently, a sharp decline was observed, and the

final methane concentration was 2.8 mmol L^{-1} . This pattern was consistent with, but also slightly preceded, similar trends observed at the SWI (Fig. 8).

We also monitored sulfate and chloride concentrations at both depths to assess the temporal dynamics of microbial activity underlying these microbial mats (Fig. 7 & 8). Sulfate concentrations in the SWI sample from the white mat were $\sim 29 \text{ mmol L}^{-1}$ throughout the entire deployment. Furthermore, the sulfate concentrations from the initial and final paired push cores were also comparable. The orange mat surface sulfate concentrations were also largely consistent through time, but a minor decrease was observed in early January (20 mmol L^{-1}). In contrast, sulfate concentrations were more dynamic at 8 cmbsf. Sulfate concentrations in sediments underlying each mat decreased between deployment and January 2019 and this trend was more substantial in the orange mat where the concentration decreased to $\sim 20 \text{ mmol L}^{-1}$. Chloride concentrations were also measured from these pore fluids and chloride/sulfate molar ratios are presented (Fig. 7 & 8). The average $\text{Cl}^- : \text{SO}_4^{2-}$ ratio was 21.1 ± 0.5 , consistent with ratios of typical seawater. At 8 cmbsf, when sulfate concentrations increased above 30 mmol L^{-1} , the $\text{Cl}^- : \text{SO}_4^{2-}$ ratio remained constant because the chloride concentrations also increased. For example, below the orange mat, chloride increased to $724.6 \text{ mmol L}^{-1}$ in late January 2019, coincident with the major increase in sulfate concentrations there (Fig. 8B).

Temporal shifts in potential microbial activity

We estimated average anaerobic methane oxidation (AOM) and sulfate reduction (SR) rates in Guaymas Basin sediments based on extensive *ex situ* measurements.

Specifically, replicated estimates of turnover constants averaged 0.05 ± 0.03 and $0.02 \pm .008 \text{ d}^{-1}$ for AOM and SR, respectively. These turnover constants were used to estimate the temporal dynamics of microbially mediated AOM and SR for the first time in hydrothermally impacted sediments. Estimated SR rates were generally consistent for each probe and depth and ranged from 267.4 to 657.8 $\text{nmol cm}^{-3} \text{ d}^{-1}$. Potential AOM rates were less variable than SR estimates, ranging from less than 0.1 $\text{nmol cm}^{-3} \text{ d}^{-1}$ to 244 $\text{nmol cm}^{-3} \text{ d}^{-1}$. This maximum potential AOM was observed at 8 cmbsf at the orange mat locale in late January 2019 and decreased to 162 $\text{nmol cm}^{-3} \text{ d}^{-1}$ by the end of the deployment (Fig. 10B). These potential estimates were used to calculate depth integrated AOM and SR rates from each mat locale. Average depth integrated rates of AOM and SR were 2.4 ± 0.86 and $32.3 \pm 1.03 \text{ mmol m}^{-2} \text{ d}^{-1}$ (Fig. 11), respectively. Depth integrated SR was largely constant during the entire deployment, while AOM from both sites increased after December 2018.

DISCUSSION

Hydrothermal activity in Guaymas Basin sediments supports diverse and erratic biogeochemical regimes. We probed these sediments to investigate this variability and quantify *in situ* methane concentrations, revealing high spatiotemporal differences in methane availability. Sulfate concentrations were monitored in parallel, to assess the coupling between methane and sulfur cycling. These profiles were paired with discrete push-cores to assess spatial heterogeneity. Temporal shifts of methane and sulfate concentrations coincident with the disappearance of a *Beggiatoa* microbial mat suggested synergistic changes in geochemistry and microbial populations and activity. Together,

these data underscore the dynamic heterogeneity of hydrothermally impacted sediments of the Guaymas Basin.

OsmoSampler Assessment

OsmoSamplers have been used extensively to measure a number of analytes in a wide range of ecosystems, including the deep sea (Tryon *et al.*, 2002; Jannasch *et al.*, 2004; Lapham *et al.*, 2008, 2013; Solomon *et al.*, 2008; Wheat *et al.*, 2011; Robidart *et al.*, 2013; Gelesh *et al.*, 2016; Orcutt *et al.*, 2017; Brankovits *et al.*, 2018; McIntosh Marcek *et al.*, 2021). The Jumbo OsmoSamplers deployed here, while similar in principle to previous iterations, had significantly higher pumping rates (up to 18 mL d⁻¹ compared to ~ 2 mL d⁻¹, previously) and employed high-pressure valves exposed to the local environment (Lapham *et al.*, 2013). Elevated pumping rates were necessary to make short-term *in situ* measurements, but the pumping rate varied substantially, from ~ 4 – 18 mL d⁻¹, which could have caused inconsistencies in sampling. Different pumping rates between two pumps would mean that those fluids would migrate through the sample coil at different rates and would not be temporally matched, despite the relatively short deployment duration. Furthermore, osmotic pumping rates are influenced by temperature (Jannasch *et al.*, 2004), and while bottom water temperatures are relatively stable, intense heating of Guaymas Basin sediments could have influenced pumping rates of the Jumbo OsmoSamplers. A diminished pumping rate would be apparent in the chloride concentrations – if concentrations were lower than typical seawater values (~550 mmol L⁻¹). These issues were likely exacerbated in Guaymas Basin sediments where spatiotemporal variability is high (Teske *et al.*, 2016). No damage was observed for any of the pump units

during post-deployment inspections, however, chemical weathering (*i.e.*, iron sulfides) was present, underscoring that they were exposed to caustic hydrothermal fluids. Temperature probes attached to the pumps and T-handle probes would benefit future surveys of hydrothermal sediments with osmotically driven pumps.

During post-deployment inspections, corrosion was regularly observed on the high-pressure valves. The wide range of methane concentrations (up to 46 mmol L⁻¹) was generally comparable to previous deep-sea deployments (Lapham *et al.*, 2013; Orcutt *et al.*, 2017), suggesting that *in situ* samples were captured. However, chloride concentrations were low in several coils (Table 4; < 500 mmol L⁻¹) which could be due to leaking, diminished pumping rates (as described above), or sampling of low salinity hydrothermal fluid discharge.

Biogeochemical setting

Microbial mats from two sites (Cathedral Hill and AcetoBalsamico) were each sampled several times to investigate *in situ* methane concentrations. These sites have been described in depth (Teske *et al.*, 2016) and have distinct geochemical signatures. Temperatures at Cathedral Hill have generally been warmer than AcetoBalsamico (up to 120°C and 30°C at 40 cmbsf, respectively), in line with our observations (Table 5). Both sites are methane rich, but while Cathedral Hill was sulfidic, the AcetoBalsamico site is depleted in sulfate and sulfide (Teske *et al.*, 2016). Furthermore, high concentrations of DOC including acetate (up to ~ 1 mmol L⁻¹ at AcetoBalsamico) and methanol can support elevated microbial activity (Zhuang, Montgomery, Samarkin, *et al.*, 2019). The Sonora

Margin site, situated to the north of Cathedral Hill/AcetoBalsamico was also sampled with Jumbo OsmoSamplers. It is not directly influenced by hydrothermal activity, but cold seeps are present (Simoneit *et al.*, 1990). These sediments exhibit bottom water temperatures ($\sim 4^{\circ}\text{C}$) and are rich in methane, including gas hydrates, as observed in this study (Vigneron *et al.*, 2013).

Methane concentrations measured from Jumbo OsmoSampler coils ranged from $2.1\ \mu\text{mol L}^{-1}$ to $46.1\ \text{mmol L}^{-1}$ (Fig. 2-5). Most observations were well below the saturation level for methane at *in situ* pressures of Guaymas Basin ($> 100\ \text{mmol L}^{-1}$), regardless of temperature (Duan and Mao, 2006). Discrete measurements of methane concentrations were generally comparable to several previous measurements (Welhan, 1988; Paull *et al.*, 2007; Biddle *et al.*, 2012), and marginally less than the highest concentration, up to $\sim 28\ \text{mmol L}^{-1}$, observed previously in discrete samples (McKay *et al.*, 2016). In contrast, methane concentrations quantified from the Jumbo OsmoSampler coils were roughly two times higher than previous measurements, extending the upper limit of methane concentrations observed in Guaymas Basin sediments. Osmotically driven pumps have been used to measure methane concentrations from other deep-sea gas rich environments, which ranged up to $\sim 85\ \text{mmol L}^{-1}$ (Lapham *et al.*, 2008; Orcutt *et al.*, 2017). Our observations (up to $46\ \text{mmol L}^{-1}$) reflect the unique biogeochemical signature of the Guaymas Basin.

A myriad of biogeochemical factors influence *in situ* methane concentrations in anoxic marine sediments and both biological and abiotic sources and biological sinks of

methane have been studied in the Guaymas Basin. The isotopic composition of methane ($\delta^{13}\text{C}$ ranging from -30‰ to -70‰) is considered to be of mixed biological and thermogenic origin (Simoneit *et al.*, 1979; Welhan and Lupton, 1987; Paull *et al.*, 2007; Lizarralde *et al.*, 2011; Biddle *et al.*, 2012; McKay *et al.*, 2012; Teske *et al.*, 2019). Biological methanogenesis has been detected in surficial sediments at rates up to $\sim 300 \text{ pmol cm}^{-3} \text{ d}^{-1}$ and production is likely important below the sulfate-methane transition zone (SMTZ), where methanogenesis is energetically favorable (Whiticar, 1999; Zhuang, Montgomery, Samarkin, *et al.*, 2019). The extent to which biological methanogenesis is a significant source of methane in Guaymas Basin sediments is unclear. In contrast, high rates of AOM, up to $\sim 3.6 \text{ } \mu\text{mol cm}^{-3} \text{ d}^{-1}$ which exceeds surficial methanogenesis rates by orders of magnitude higher, suggest microbial activity is a net sink of methane in Guaymas Basin sediments (Kallmeyer and Boetius, 2004; Biddle *et al.*, 2012; Bowles *et al.*, 2019).

In some parts of the Gulf of California, methane is concentrated in solid gas hydrate, creating a dynamic waystation for methane. Gas hydrate occurs in areas of high gas flux, low temperature, and high pressure; some methane-rich habitats in this region lie within the range of gas hydrate stability (*i.e.*, $<15^{\circ}\text{C}$ at a water depth of $\sim 2000 \text{ m}$) (Sloan, 1998; Paull *et al.*, 2007), as evidenced by the observed gas hydrate mound along the Sonoran Margin Site (this study) and previously in the Ringvent area (Paull *et al.*, 2007; Núñez-Useche *et al.*, 2018; Teske *et al.*, 2019). Dissolved methane concentrations were one or two orders of magnitude higher adjacent to the gas hydrate mound relative to sediment a few meters away (Fig. 4). Hydrate associated sediments can be enriched with dissolved methane, thus the higher concentration in the hydrate mound would be expected

(Joye *et al.*, 2004; Lapham *et al.*, 2008, 2013). No gas hydrate was observed near the OsmoSampler sites adjacent to the Guaymas Basin hydrothermal area, which is not surprising given that hydrothermally induced temperature spikes disrupt and preclude gas hydrate formation.

In heated hydrothermal sediments, it is more likely that sub-surface fluid flow dynamics would influence methane concentrations. Dynamic advective and diffusive flow are characteristic of the hydrothermal sediments, but our understanding of flow patterns is insufficient to predict methane availability (Cardoso and Cartwright, 2016; Teske *et al.*, 2016). In the Gulf of Mexico cold seep environments carbonate precipitation dictates fluid discharge and thus methane flow (Macelloni *et al.*, 2013; Wilson *et al.*, 2014). Carbonates have been observed in Guaymas Basin sediments (Koski *et al.*, 1985; Paull *et al.*, 2007), but the extent to which they influence fluid flow in heated sediments is unknown.

In situ methane concentrations

Microbial mats at AcetoBalsamico and Cathedral Hill were sampled simultaneously using triplicate with Jumbo OsmoSamplers. Patterns in methane and sulfate concentrations were inconsistent between Jumbo OsmoSampler coils and paired push cores. Push cores were retrieved as close to the Jumbo OsmoSampler T-handle probe as possible (*e.g.*, Fig. 13B), and the distance between paired samples was generally less than ~ 50 cm. Despite the relative proximity in sampling, methane concentrations were significantly different between the two collection methods (Wilcoxon Signed Rank Test, p -value < 0.05, Table 3) and were not necessarily higher in the *in situ* OsmoSampler

samples. Methane concentrations determined from push cores were expected to be lower than those from the Jumbo OsmoSamplers due to degassing that is known to occur during ascent from the seafloor due to the decrease in pressure (Bowles et al. 2019). On several occasions, the *in situ* methane concentration ranged from 22 – 46 mmol L⁻¹, indicating that previous pore fluid methane concentrations measurements substantially underestimated *in situ* concentrations (*e.g.*, Welhan, 1988). The Jumbo OsmoSamplers were designed to maintain the high gas concentrations expected from high pressure, gas-rich environments (Lapham *et al.*, 2013), so, we predicted that methane concentration would be higher than or at least similar to concentrations determined from discrete sediment core samples. Surprisingly, this trend was not observed commonly, and in several instances, methane concentrations determined from push cores were more than an order of magnitude higher than those from the Jumbo OsmoSampler (*e.g.*, Fig. 2B, 3A, or 5B).

Higher concentrations of methane in push core samples relative to Jumbo OsmoSamplers was unexpected, but not improbable. OsmoSamplers characteristically have low pumping rates and during these deployments were outfitted with Rhizon filters (see methods). As a result, only pore-fluid was pumped into the sample coil and thus these methane concentrations exclusively reflect the dissolved aqueous pool (Gelesh *et al.*, 2016). This also implies that these methane concentrations would not account for methane associated with gas bubbles or methane adsorbed to sediment particles, both of which are captured to some degree in discrete sediment samples. In particular, sorption of methane to sediment particles can account for nearly 10% of the total methane concentration in sulfate reducing sediments (Ertefai *et al.*, 2010). However, a more substantial offset in the

methane concentration due to sorption was required to account for the discrepancies observed between paired samples (Table 4).

Instead, sediment heterogeneity generated by dynamic physical and biogeochemical factors likely drives the observed differences in methane concentrations. Biogeochemical regimes can change dramatically over small scales in Guaymas Basin sediments (McKay *et al.*, 2012, 2016; Teske *et al.*, 2016), in line with the data presented here. Advective flow, a fundamental feature of hydrothermal systems, generates channelized flow in the pore-fluid, promoting sediment heterogeneity and influencing microbial activity (Torres *et al.*, 2002; Mahadevan *et al.*, 2012; Wankel *et al.*, 2012). This flow reduces the methane residence times in sediments and individual channels may bypass specific locales causing sub meter-scale discrepancies in observed methane concentrations. Additionally, the high AOM rates (*i.e.*, methane sink) in the Guaymas Basin could contribute to local differences in methane concentrations (Bowles *et al.*, 2019). However, the extent to which microbial activity influences the biogeochemical heterogeneity of hydrothermal sediments is unclear (Wankel *et al.*, 2012; McKay *et al.*, 2016). Although we do not have AOM rates directly associated with these samples, the depletion of sulfate in several depth profiles (*e.g.*, Fig. 2B or 3C) and negative correlation of methane and sulfate concentrations (Table 2, p -value < 0.05), suggests that microbial activity (*i.e.*, AOM coupled to SR) plays a role in determining the spatial distribution of methane. Furthermore, the relative differences in sulfate concentrations between paired samples further exemplify the spatial heterogeneity of Guaymas Basin sediments.

In addition to methane measurements in sediments, we deployed two Jumbo OsmoSampler T-handle probes on the seafloor surface directly at the base (within 2 m) of Ace's Spire and below a venting hydrothermal orifice atop the edifice (Fig. 12). Ace's Spire is an active hydrothermal vent approximately 0.5 km south of Cathedral Hill and 0.3 km east of AcetoBalsamico (Teske *et al.*, 2016). *In situ* methane concentrations were ~ 50 times higher near the vent mound compared to at the base of the edifice and sulfate was only 13.5 mmol L⁻¹ on the mound (Fig. 6). Guaymas Basin hydrothermal fluids are depleted of sulfate (Von Damm *et al.*, 1985), which likely influenced sulfate concentrations near the venting orifice. Methane concentrations can be elevated up to ~ 380 µmol L⁻¹ (Chapter 1 of this dissertation) in fluids near a venting edifice, which is comparable to the concentration observed here (140 µmol L⁻¹) given that the Jumbo OsmoSampler probe was deployed several meters from discharging orifice. The relatively lower methane concentrations at the base of the spire likely reflect lower discharge at the base of the feature. In contrast, below the mound, diffusive flow probably supported the higher methane concentrations there. This idea is reinforced by the presence of several *Beggiatoa* mats in the vicinity of the mound. However, Ace's Spire is ~ 0.5 km east of the southern sill, and whether sub-surface circulation patterns could support fluid flow that far, and thus the observed methane concentrations, remains unclear (Lonsdale and Becker, 1985; Fisher and Becker, 1991; Teske *et al.*, 2016).

Temporal patterns of methane dynamics

The *Beggiatoa* mats from Guaymas mat come in a variety of shapes, colors, and sizes. So-called “fried-egg” mats have orange *Beggiatoa* in the center and white *Beggiatoa*

along the edge. A large (~ 2 m across) fried egg mat was selected for long term observation of *in situ* methane, sulfate, and chloride concentrations over approximately three months. The data presented here are the first temporal analysis of a hydrothermal system in two decades (Wheat *et al.*, 2000) and the only continuous geochemical measurements available for the Guaymas Basin. These data demonstrate the temporal and spatial dynamics of methane and sulfate concentrations and underscore the importance of microbial activity in hydrothermally impacted sediments.

Methane concentrations in the surficial sediments from each locale (*i.e.*, white and orange mats; less than 50 cm apart) were both initially less than 10 $\mu\text{mol L}^{-1}$. However, the orange mat site supported a rapid increase in methane at the SWI reaching > 1 mmol L^{-1} by mid-January 2019 (Fig. 8B). Similarly, at 8 cmbsf, methane concentrations increased substantially below the orange mat, while the white mat locale did not change as much. Local microbial methanogenesis is unlikely to support the observed increase in methane due to the high sulfate concentrations, unless it proceeded with non-competitive substrates at rates that exceeded available data for methanogenesis by orders of magnitude. For example, recent measurements of rates of methanogenesis from non-competitive substrates were ~ 240 times too low to support the observed influx of methane, suggesting that it was sourced from deeper sediment (Zhuang, Montgomery, Samarkin, *et al.*, 2019). Assuming a deeper source for methane, the relative contributions of biotic and abiotic processes influencing its production are unclear due to complicated carbon isotopic signatures for methane (Paull *et al.*, 2007; Lizarralde *et al.*, 2011).

We estimated temporal shifts in AOM and SR reduction rates by averaging rate constants for these processes across sites and depths within Guaymas Basin. Rates of AOM and SR in the environment are considered to be first order, as evidence by the rate equation employed here (Eq. 1). Because of this, rates are directly proportional to environmental concentrations of the relevant substrate (*i.e.*, methane and sulfate). AOM is generally considered to be coupled to SR in anoxic marine sediments, but sulfate is an important electron acceptor for many other metabolisms and AOM can be coupled to other processes as well. Estimated potential AOM rates were generally lower than potential SR during the time series (Fig. 9 & 10). However, 95% jackknifed confidence intervals (see methods) of turnover rate constants indicated that decoupling between the two processes may have occurred between late January and early February 2019, when AOM could have exceeded SR (Bowles *et al.*, 2019). At the white mat locale, SR was consistently two or three orders of magnitude higher than AOM implying a strong dependence of AOM on SR. However, elevated SR rates underscore the importance of sulfate reduction as supporting general rates of microbial metabolism in these sediments (Elsgaard *et al.*, 1994; Zhuang, Montgomery, and Joye, 2019; Zhuang, Montgomery, Samarkin, *et al.*, 2019). In contrast, at the orange mat locale, AOM increased considerably and was more tightly coupled with SR, a clear distinction from the largely stagnant activity beneath the white mat. The influx of methane starting in January 2019 may have induced a biogeochemical shift that favored AOM (Cruaud *et al.*, 2017; Bowles *et al.*, 2019).

To further constrain spatial and temporal patterns of AOM and SR, we calculated potential depth integrated rates (Fig. 11). The consistency of depth integrated SR rates

indicated that temporal shifts were minimal, presumably due to the organic rich nature of these sediments. Integrated SR rates reported here were comparable to previous rates reported for Guaymas Basin ($\sim 40 \text{ mmol m}^{-2} \text{ d}^{-1}$ at 20°C) (Elsgaard *et al.*, 1994). These rates were also similar to those documented from cold seep and gas hydrate environments (Treude *et al.*, 2003; Joye *et al.*, 2004; Bowles, Samarkin, Bowles, *et al.*, 2011). Consistently rapid SR rates would be expected because high sulfide concentrations in pore fluids are the norm for Guaymas Basin sediments and this sulfide fuels the *Beggiatoa* mats observed in the system.

These data are the first temporal estimates of AOM rates from gas rich sediments. At the white mat locale, integrated AOM rates were relatively low (0.09 to $0.92 \text{ mmol m}^{-2} \text{ d}^{-1}$) over the entire monitoring period (Fig. 11A). In contrast, beneath the orange mat, integrated AOM rates increased during January 2019 up to $13.8 \text{ mmol m}^{-2} \text{ d}^{-1}$. This observation was similar to previous reports from the Juan de Fuca Ridge, which documented integrated AOM rates up to $19.1 \text{ mmol m}^{-2} \text{ d}^{-1}$ (Wankel *et al.*, 2012). AOM rates would increase in parallel with the methane concentrations, which began to increase in January 2019 and ultimately exceeding the half saturation constant of local methanotrophs ($\sim 8 \text{ mmol L}^{-1}$) (Bowles *et al.*, 2019). Elevated rates could be supported by archaeal anaerobic methanotrophs (ANME) which are abundant under *Beggiatoa* mats in Guaymas Basin (McKay *et al.*, 2016). Collectively, the marked differences over a microbial mat during a two-month period (Dec 2018 to Feb 2019) underscores the spatial variability of methane dynamics in Guaymas Basin sediments.

We surveyed *in situ* geochemistry from hydrothermally altered sediments of the Guaymas Basin to explore the spatiotemporal dynamics of methane and sulfate. Sulfate concentrations were near seawater concentration (28 mmol L⁻¹) at the SWI and often decreased in deeper sediments indicative of active SR. These data represent the first *in situ* measurements of methane in hydrothermal sediments, and concentrations were elevated up to 46 mmol L⁻¹. Surprisingly, comparisons of paired Jumbo OsmoSampler and push core data demonstrated a remarkable degree of variability in methane concentrations. Local variations, on the scale of tens of centimeters, in porewater sulfate and methane concentrations underscored the intense spatial heterogeneity of this unique ecosystem. We also made the first continuous measurements of methane and sulfate in a hydrothermal system. An influx of > 4 mmol L⁻¹ methane during a three-month deployment was indicative of the robust biogeochemical processes of the Guaymas Basin. Furthermore, these shifts in methane and sulfate revealed a temporal variability that further emphasizes the spatial heterogeneity of the Guaymas Basin. Taken together, these data underscore the dynamic and heterogeneous nature of Guaymas Basin sediments and reveal the importance of further constraining the biogeochemistry there.

REFERENCES

- Alperin, M.J., Reeburgh, W.S., and Whiticar, M.J. (1988) Carbon and hydrogen isotope fractionation resulting from anaerobic methane oxidation. *Global Biogeochem. Cycles* **2**: 279–288.
- Bazylinski, D.A., Farrington, J.W., and Jannasch, H.W. (1988) Hydrocarbons in surface sediments from a Guaymas Basin hydrothermal vent site. *Org. Geochem.* **12**: 547–558.
- Biddle, J.F., Cardman, Z., Mendlovitz, H., Albert, D.B., Lloyd, K.G., Boetius, A., and Teske, A. (2012) Anaerobic oxidation of methane at different temperature regimes in Guaymas Basin hydrothermal sediments. *ISME J.* **6**: 1018–1031.
- Boetius, A., Ravensschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A., et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *407*: 623–626.
- Bowles, M.W., Samarkin, V.A., Bowles, K.M., and Joye, S.B. (2011) Weak coupling between sulfate reduction and the anaerobic oxidation of methane in methane-rich seafloor sediments during ex situ incubation. *Geochim. Cosmochim. Acta* **75**: 500–519.
- Bowles, M.W., Samarkin, V.A., Hunter, K.S., Finke, N., Teske, A.P., Girguis, P.R., and Joye, S.B. (2019) Remarkable Capacity for Anaerobic Oxidation of Methane at High Methane Concentration. *Geophys. Res. Lett.* **46**: 12192–12201.
- Bowles, M.W., Samarkin, V.A., and Joye, S.B. (2011) Improved measurement of microbial activity in deep-sea sediments at in situ pressure and methane concentration. *Limnol. Oceanogr. Methods* **9**: 499–506.

- Brankovits, D., Pohlman, J.W., Ganju, N.K., Iliffe, T.M., Lowell, N., Roth, E., et al. (2018) Hydrologic Controls of Methane Dynamics in Karst Subterranean Estuaries. *Global Biogeochem. Cycles* **32**: 1759–1775.
- Calvert, S.E. (1966) Origin of Diatom-Rich, Varved Sediments from the Gulf of California. *J. Geol.* **74**: 546–565.
- Cardoso, S.S.S. and Cartwright, J.H.E. (2016) Increased methane emissions from deep osmotic and buoyant convection beneath submarine seeps as climate warms. *Nat. Commun.* **7**: 1–6.
- Cruaud, P., Vigneron, A., Pignet, P., Caprais, J.C., Lesongeur, F., Toffin, L., et al. (2017) Comparative study of Guaymas Basin microbiomes: Cold seeps vs. hydrothermal vents sediments. *Front. Mar. Sci.* **4**: 1–15.
- Von Damm, K.L., Edmond, J.M., Measures, C.I., and Grant, B. (1985) Chemistry of submarine hydrothermal solutions at Guaymas Basin, Gulf of California. *Geochim. Cosmochim. Acta* **49**: 2221–2237.
- Dombrowski, N., Teske, A.P., and Baker, B.J. (2018) Expansive microbial metabolic versatility and biodiversity in dynamic Guaymas Basin hydrothermal sediments. *Nat. Commun.* **9**.
- Dowell, F., Cardman, Z., Dasarathy, S., Kellermann, M.Y., Lipp, J.S., Ruff, S.E., et al. (2016) Microbial communities in methane- and short chain alkane-rich hydrothermal sediments of Guaymas Basin. *Front. Microbiol.* **7**.
- Duan, Z. and Mao, S. (2006) A thermodynamic model for calculating methane solubility, density and gas phase composition of methane-bearing aqueous fluids from 273 to 523 K and from 1 to 2000 bar. *Geochim. Cosmochim. Acta* **70**: 3369–3386.

- Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: The art of harnessing chemosynthesis. *Nat. Rev. Microbiol.* **6**: 725–740.
- Einsele, G., Gieskes, J.M., Curray, J., Moore, D.M., Aguayo, E., Aubry, M.-P., et al. (1980) Intrusion of basaltic sills into highly porous sediments, and resulting hydrothermal activity. *Nature* **283**: 441–445.
- Elsgaard, L., Isaksen, M.F., Jørgensen, B.B., Alayse, A.M., and Jannasch, H.W. (1994) Microbial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent area: Influence of temperature and substrates. *Geochim. Cosmochim. Acta* **58**: 3335–3343.
- Ertefai, T.F., Heuer, V.B., Prieto-Mollar, X., Vogt, C., Sylva, S.P., Seewald, J., and Hinrichs, K.U. (2010) The biogeochemistry of sorbed methane in marine sediments. *Geochim. Cosmochim. Acta* **74**: 6033–6048.
- Fisher, A.T. and Becker, K. (1991) Heat flow, hydrothermal circulation and basalt intrusions in the Guaymas Basin, Gulf of California. *Earth Planet. Sci. Lett.* **103**: 84–99.
- Gelesh, L., Marshall, K., Boicourt, W., and Lapham, L. (2016) Methane concentrations increase in bottom waters during summertime anoxia in the highly eutrophic estuary, Chesapeake Bay, U.S.A. *Limnol. Oceanogr.* **61**: S253–S266.
- Gundersen, J.K., Jørgensen, B.B., Larsen, E., and Jannasch, H.W. (1992) Mats of giant sulphur bacteria on deep-sea sediments due to fluctuating hydrothermal flow. *Nature* **360**: 454–.

- Hesse, R. and Harrison, W.E. (1981) Gas hydrates (clathrates) causing pore-water freshening and oxygen isotope fractionation in deep-water sedimentary sections of terrigenous continental margins. *Earth Planet. Sci. Lett.* **55**: 453–462.
- Jannasch, H.W., Wheat, C.G., Plant, J.N., Kastner, M., and Stakes, D.S. (2004) Continuous chemical monitoring with osmotically pumped water samplers: OsmoSampler design and applications. *Limnol. Oceanogr. Methods* **2**: 102–113.
- Jørgensen, B.B. (1978) A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments: I. measurement with radiotracer techniques. *Geomicrobiol. J.* **1**: 11–27.
- Joye, S.B., Boetius, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J., and Lugo, S.K. (2004) The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chem. Geol.* **205**: 219–238.
- Joye, S.B., Bowles, M.W., Samarkin, V.A., Hunter, K.S., and Niemann, H. (2010) Biogeochemical signatures and microbial activity of different cold-seep habitats along the Gulf of Mexico deep slope. *Deep. Res. Part II Top. Stud. Oceanogr.* **57**: 1990–2001.
- Kallmeyer, J. and Boetius, A. (2004) Effects of Temperature and Pressure on Sulfate Reduction and Anaerobic Oxidation of Methane in Hydrothermal Sediments of Guaymas Basin. *Appl. Environ. Microbiol.* **70**: 1231–1233.
- Kawka, O.E., and Simoneit, B.R.T. (1987) Survey of hydrothermally-generated petroleums from the Guaymas Basin spreading center. *Org. Geochem.* **11**: 311–328.
- Koski, R.A., Lonsdale, P.F., Shanks, W.C., Berndt, M.E., and Howe, S.S. (1985) Mineralogy and geochemistry of a sediment-hosted hydrothermal sulfide deposit

- from the Southern Trough of Guaymas Basin, Gulf of California. *J. Geophys. Res.* **90**: 6695–6707.
- Lapham, L., Chanton, J.P., Martens, C.S., Higley, P.D., Jannasch, H.W., and Woolsey, J.R. (2008) Measuring temporal variability in pore-fluid chemistry to assess gas hydrate stability: Development of a continuous pore-fluid array. *Environ. Sci. Technol.* **42**: 7368–7373.
- Lapham, L., Wilson, R., Riedel, M., Paull, C.K., and Holmes, M.E. (2013) Temporal variability of in situ methane concentrations in gas hydrate-bearing sediments near Bullseye Vent, Northern Cascadia Margin. *Geochemistry, Geophys. Geosystems* **14**: 2445–2459.
- Lizarralde, D., Soule, S.A., Seewald, J.S., and Proskurowski, G. (2011) Carbon release by off-axis magmatism in a young sedimented spreading center. *Nat. Geosci.* **4**: 50–54.
- Lonsdale, P. and Becker, K. (1985) Hydrothermal plumes, hot springs, and conductive heat flow in the Southern Trough of Guaymas Basin. *Earth Planet. Sci. Lett.* **73**: 211–225.
- Macelloni, L., Brunner, C.A., Caruso, S., Lutken, C.B., D’Emidio, M., and Lapham, L.L. (2013) Spatial distribution of seafloor bio-geological and geochemical processes as proxies of fluid flux regime and evolution of a carbonate/hydrates mound, northern Gulf of Mexico. *Deep. Res. Part I Oceanogr. Res. Pap.* **74**: 25–38.
- Magenheim, A.J. and Gieskes, J.M. (1992) Hydrothermal discharge and alteration in near-surface sediments from the Guaymas Basin, Gulf of California. *Geochim. Cosmochim. Acta* **56**: 2329–2338.

- Mahadevan, A., Orpe, A. V., Kudrolli, A., and Mahadevan, L. (2012) Flow-induced channelization in a porous medium. *Epl* **98**: 0–6.
- McIntosh Marcek, H.A., Lesack, L.F.W., Orcutt, B.N., Wheat, C.G., Dallimore, S.R., Geeves, K., and Lapham, L. (2021) Continuous Dynamics of Dissolved Methane Over 2 Years and its Carbon Isotopes ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) in a Small Arctic Lake in the Mackenzie Delta. *J. Geophys. Res. Biogeosciences* **126**: 1–23.
- McKay, L., Klokman, V.W., Mendlovitz, H.P., Larowe, D.E., Hoer, D.R., Albert, D., et al. (2016) Thermal and geochemical influences on microbial biogeography in the hydrothermal sediments of Guaymas Basin, Gulf of California. *Environ. Microbiol. Rep.* **8**: 150–161.
- McKay, L., MacGregor, B.J., Biddle, J.F., Albert, D.B., Mendlovitz, H.P., Hoer, D.R., et al. (2012) Spatial heterogeneity and underlying geochemistry of phylogenetically diverse orange and white Beggiatoa mats in Guaymas Basin hydrothermal sediments. *Deep. Res. Part I Oceanogr. Res. Pap.* **67**: 21–31.
- Merewether, R., Olsson, M.S., and Lonsdale, P. (1985) Acoustically detected hydrocarbon plumes rising from 2-km depths in Guaymas Basin, Gulf of California. *J. Geophys. Res.* **90**: 3075.
- Nelson, D.C., Wirsén, C.O., and Jannasch, H.W. (1989) Characterization of Large, Autotrophic Beggiatoa spp. Abundant at Hydrothermal Vents of the Guaymas Basin. *Appl. Environ. Microbiol.* **55**: 2909–2917.
- Núñez-Useche, F., Canet, C., Liebetrau, V., Puig, T.P., Ponciano, A.C., Alfonso, P., et al. (2018) Redox conditions and authigenic mineralization related to cold seeps in central Guaymas Basin, Gulf of California. *Mar. Pet. Geol.* **95**: 1–15.

- Orcutt, B.N., Lapham, L., Delaney, J., Sarode, N., Marshall, K.S., Whaley-Martin, K.J., et al. (2017) Microbial response to oil enrichment in Gulf of Mexico sediment measured using a novel long-term benthic lander system. *Elementa* **5**.
- Orcutt, B.N., LaRowe, D.E., Biddle, J.F., Colwell, F.S., Glazer, B.T., Reese, B.K., et al. (2013) Microbial activity in the marine deep biosphere: Progress and prospects. *Front. Microbiol.* **4**: 1–15.
- Paull, C.K., Ussler, W., Peltzer, E.T., Brewer, P.G., Keaten, R., Mitts, P.J., et al. (2007) Authigenic carbon entombed in methane-soaked sediments from the northeastern transform margin of the Guaymas Basin, Gulf of California. *Deep. Res. Part II Top. Stud. Oceanogr.* **54**: 1240–1267.
- Robidart, J., Callister, S.J., Song, P., Nicora, C.D., Wheat, C.G., and Girguis, P.R. (2013) Characterizing microbial community and geochemical dynamics at hydrothermal vents using osmotically driven continuous fluid samplers. *Environ. Sci. Technol.* **47**: 4399–4407.
- Sassen, R., MacDonald, I.R., Requejo, A.G., Guinasso, N.L., Kennicutt, M.C., Sweet, S.T., and Brooks, J.M. (1994) Organic geochemistry of sediments from chemosynthetic communities, Gulf of Mexico slope. *Geo-Marine Lett.* **14**: 110–119.
- Simoneit, B.R.T., Lonsdale, P.F., Edmond, J.M., and Shanks, W.C. (1990) Deep-water hydrocarbon seeps in Guaymas Basin, Gulf of California. *Appl. Geochemistry* **5**: 41–49.
- Simoneit, B.R.T., Mazurek, M.A., Brenner, S., Crisp, P.T., and Kaplan, I.R. (1979) Organic geochemistry of recent sediments from Guaymas Basin, Gulf of California. *Deep Sea Res. Part A, Oceanogr. Res. Pap.* **26**: 879–891.

- Sloan, E.D. (1998) Clathrate Hydrates of Natural Gases Dekker, M. (ed) New York.
- Solomon, E.A., Kastner, M., Jannasch, H., Robertson, G., and Weinstein, Y. (2008) Dynamic fluid flow and chemical fluxes associated with a seafloor gas hydrate deposit on the northern Gulf of Mexico slope. *Earth Planet. Sci. Lett.* **270**: 95–105.
- Teske, A., De Beer, D., McKay, L., Tivey, M.K., Biddle, J.F., Hoer, D., et al. (2016) The Guaymas Basin hiking guide to hydrothermal mounds, chimneys, and microbial mats: Complex seafloor expressions of subsurface hydrothermal circulation. *Front. Microbiol.* **7**.
- Teske, A., Callaghan, A. V., and LaRowe, D.E. (2014) Biosphere frontiers of subsurface life in the sedimented hydrothermal system of Guaymas Basin. *Front. Microbiol.* **5**: 1–11.
- Teske, A., Mckay, L., Ravelo, A.C., Aiello, I., Mortera, C., Núñez-useche, F., et al. (2019) Characteristics and Evolution of sill- driven off-axis hydrothermalism in Guaymas Basin – the Ringvent site. *Sci. Rep.* 1–16.
- Teske, A. and Nelson, D.C. (2006) The Genera *Beggiatoa* and *Thioploca*. *The Prokaryotes* 784–810.
- Torres, M.E., McManus, J., Hammond, D.E., de Angelis, M.A., Heeschen, K.U., Colbert, S.L., et al. (2002) Fluid and chemical flux in and out of sediments hosting methane hydrate deposits on Hydrate Ridge, OR, II: Hydrological processes. *Earth Planet. Sci. Lett.* **201**: 541–557.
- Torres, M.E., Wallmann, K., Tréhu, A.M., Bohrmann, G., Borowski, W.S., and Tomaru, H. (2004) Gas hydrate growth, methane transport, and chloride enrichment at the

- southern summit of Hydrate Ridge, Cascadia margin off Oregon. *Earth Planet. Sci. Lett.* **226**: 225–241.
- Treude, T., Boetius, A., Knittel, K., Wallmann, K., and Jørgensen, B.B. (2003) Anaerobic oxidation of methane above gas hydrates at Hydrate Ridge, NE Pacific Ocean. *Mar. Ecol. Prog. Ser.* **264**: 1–14.
- Tryon, M.D., Brown, K.M., and Torres, M.E. (2002) Fluid and chemical flux in and out of sediments hosting methane hydrate deposits on Hydrate Ridge, OR, II: Hydrological processes. *Earth Planet. Sci. Lett.* **201**: 541–557.
- Ussler, W. and Paull, C.K. (1995) Effects of ion exclusion and isotopic fractionation on pore water geochemistry during gas hydrate formation and decomposition. *Geo-Marine Lett.* **15**: 37–44.
- Vigneron, A., Cruaud, P., Pignet, P., Caprais, J.C., Cambon-Bonavita, M.A., Godfroy, A., and Toffin, L. (2013) Archaeal and anaerobic methane oxidizer communities in the Sonora Margin cold seeps, Guaymas Basin (Gulf of California). *ISME J.* **7**: 1595–1608.
- Wankel, S.D., Adams, M.M., Johnston, D.T., Hansel, C.M., Joye, S.B., and Girguis, P.R. (2012) Anaerobic methane oxidation in metalliferous hydrothermal sediments: Influence on carbon flux and decoupling from sulfate reduction. *Environ. Microbiol.* **14**: 2726–2740.
- Welhan, J.A. (1988) Origins of methane in hydrothermal systems. *Chem. Geol.* **71**: 183–198.

- Welhan, J.A., and Lupton, J.E. (1987) Light hydrocarbon gases in Guaymas Basin hydrothermal fluids: thermogenic versus abiogenic origin. *Am. Assoc. Pet. Geol. Bull.* **71**: 215–223.
- Wheat, C.G., Jannasch, H.W., Kastner, M., Hulme, S., Cowen, J., Edwards, K.J., et al. (2011) Fluid sampling from oceanic borehole observatories: design and methods for CORK activities (1990-2010). **327**:.
- Wheat, C.G., Jannasch, H.W., Plant, J.N., Moyer, C.L., Sansone, F.J., and McMurtry, G.M. (2000) Continuous sampling of hydrothermal fluids from Loihi Seamount after the 1996 event. *J. Geophys. Res. Solid Earth* **105**: 19353–19367.
- Whiticar, M.J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **161**: 291–314.
- Wilson, R.M., Macelloni, L., Simonetti, A., Lapham, L., Lutken, C., Sleeper, K., et al. (2014) Subsurface methane sources and migration pathways within a gas hydrate mound system, Gulf of Mexico. *Geochemistry, Geophys. Geosystems* **15**: 89–107.
- Zhuang, G., Montgomery, A., and Joye, S.B. (2019) Heterotrophic metabolism of C1 and C2 low molecular weight compounds in northern Gulf of Mexico sediments : Controlling factors and implications for organic carbon degradation. *Geochim. Cosmochim. Acta* **247**: 243–260.
- Zhuang, G., Montgomery, A., Samarkin, V.A., Song, M., Liu, J., Schubotz, F., et al. (2019) Generation and Utilization of Volatile Fatty Acids and Alcohols in Hydrothermally Altered Sediments in the Guaymas Basin, Gulf of California. *Geophys. Res. Lett.* **46**: 2637–2646

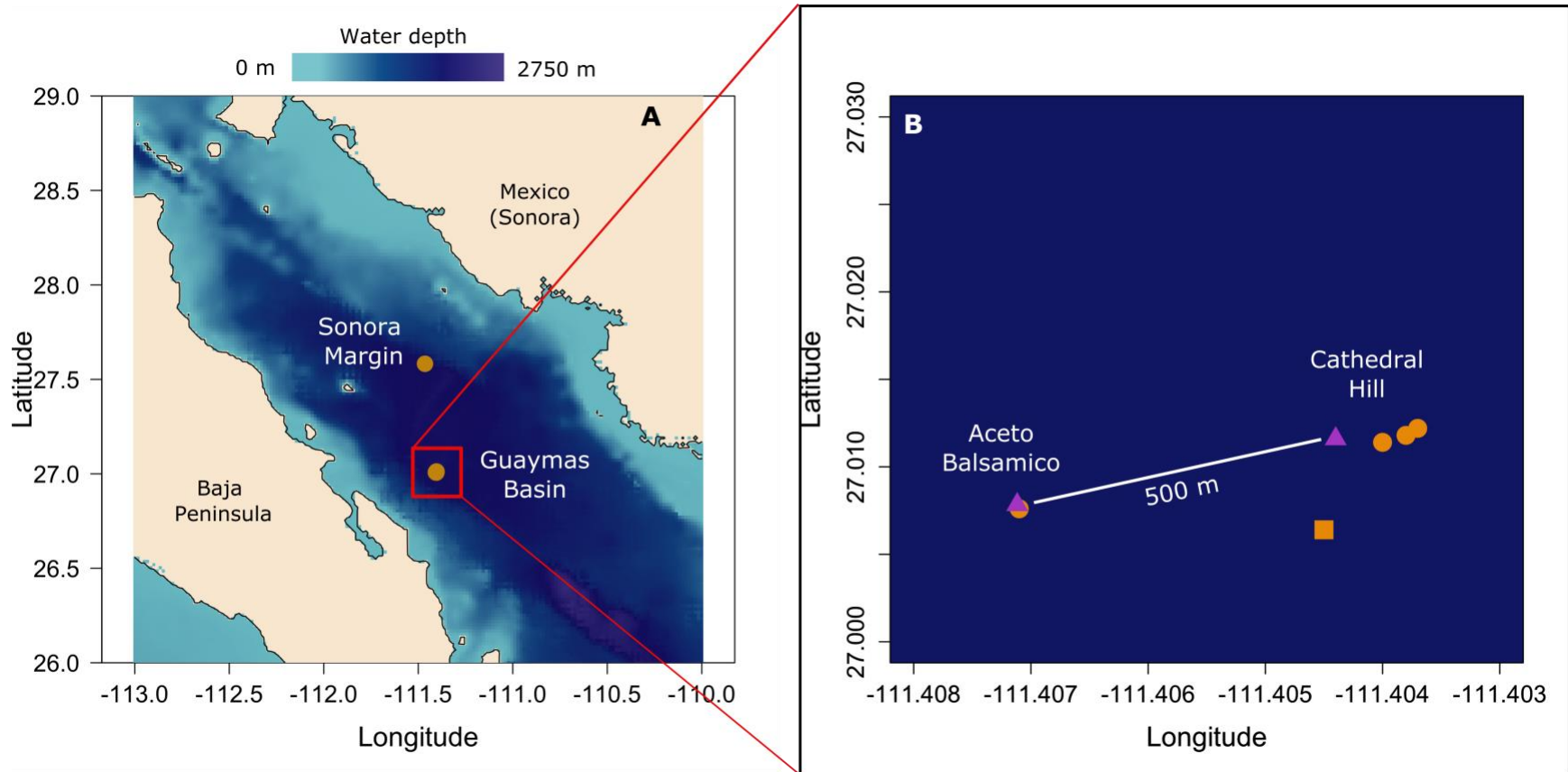


Figure 2.1. Map of the Gulf of California (A) showing the Guaymas Basin and Sonora Margin Basins. (B) is a zoomed in map of the Guaymas Basin showing each OsmoSampler sampling station. Yellow circles represent short-term deployment locations, the yellow square is the site of the long-term deployment. Purple triangles are the stations for AcetoBalsamico mat and Cathedral Hill as defined by Teske *et al.*, 2016.

Table 2.1. Summary of Jumbo Osmosampler deployment and recovery.

Cruise	Station	Latitude	Longitude	OsmoSampler Box #	Depth (m)	Deployment			Recovery		
						Date	Time (Zulu)	Dive	Date	Time (Zulu)	Dive
AT4205	Cathedral Hill	27.0118	-111.4038	1	2005	2018-11-20	17:28	4994	2018-11-22	21:15	4997
AT4205	Cathedral Hill	27.0118	-111.4038	2	2005	2018-11-20	17:52	4994	2018-11-22	20:05	4997
AT4205	Cathedral Hill	27.0118	-111.4038	3	2005	2018-11-20	18:12	4994	2018-11-22	18:59	4997
AT4205	Cathedral Hill	27.0118	-111.4038	4	2005	2018-11-20	17:01	4994	2018-11-22	21:00	4997
AT4205	Aceto Balsamico	27.0076	-111.4071	1	2009	2018-11-23	17:36	4998	2018-11-24	17:47	4999
AT4205	Aceto Balsamico	27.0074	-111.4072	2	2009	2018-11-23	19:05	4998	2018-11-24	19:38	4999
AT4205	Aceto Balsamico	27.0076	-111.4073	3	2009	2018-11-23	18:23	4998	2018-11-24	18:33	4999
AT4205	Aceto Balsamico	27.0074	-111.4075	4	2009	2018-11-23	20:12	4998	2018-11-24	19:59	4999
AT4205/FK190211	Aces Spire (Long Term)	27.0064	-111.4045	1 & 3	2002	2018-11-25	16:02	5000	2019-02-27	19:01	231
FK190211	Sonora Margin	27.5827	-111.4645	1	2015	2019-03-04	23:00	237	2019-03-09	23:59	242
FK190211	Sonora Margin	27.5827	-111.4645	4	2015	2019-03-04	22:50	237	2019-03-09	23:59	242
FK190211	Cathedral Hill (Alvin Spire)	27.0122	-111.4037	2	2005	2019-03-06	15:13	239	2019-03-07	23:25	240
FK190211	Cathedral Hill (Alvin Spire)	27.0122	-111.4037	3	2005	2019-03-06	15:35	239	2019-03-07	23:17	240
FK190211	Falkor Fountain	27.0114	-111.4040	2	2005	2019-03-08	17:10	241	2019-03-10	12:30	244
FK190211	Falkor Fountain	27.0114	-111.4040	3	2005	2019-03-08	17:10	241	2019-03-10	12:30	244

Dive numbers are for HOV *Alvin* (AT4205) and ROV *SuBastian* (FK190211).

AcetoBalsamico

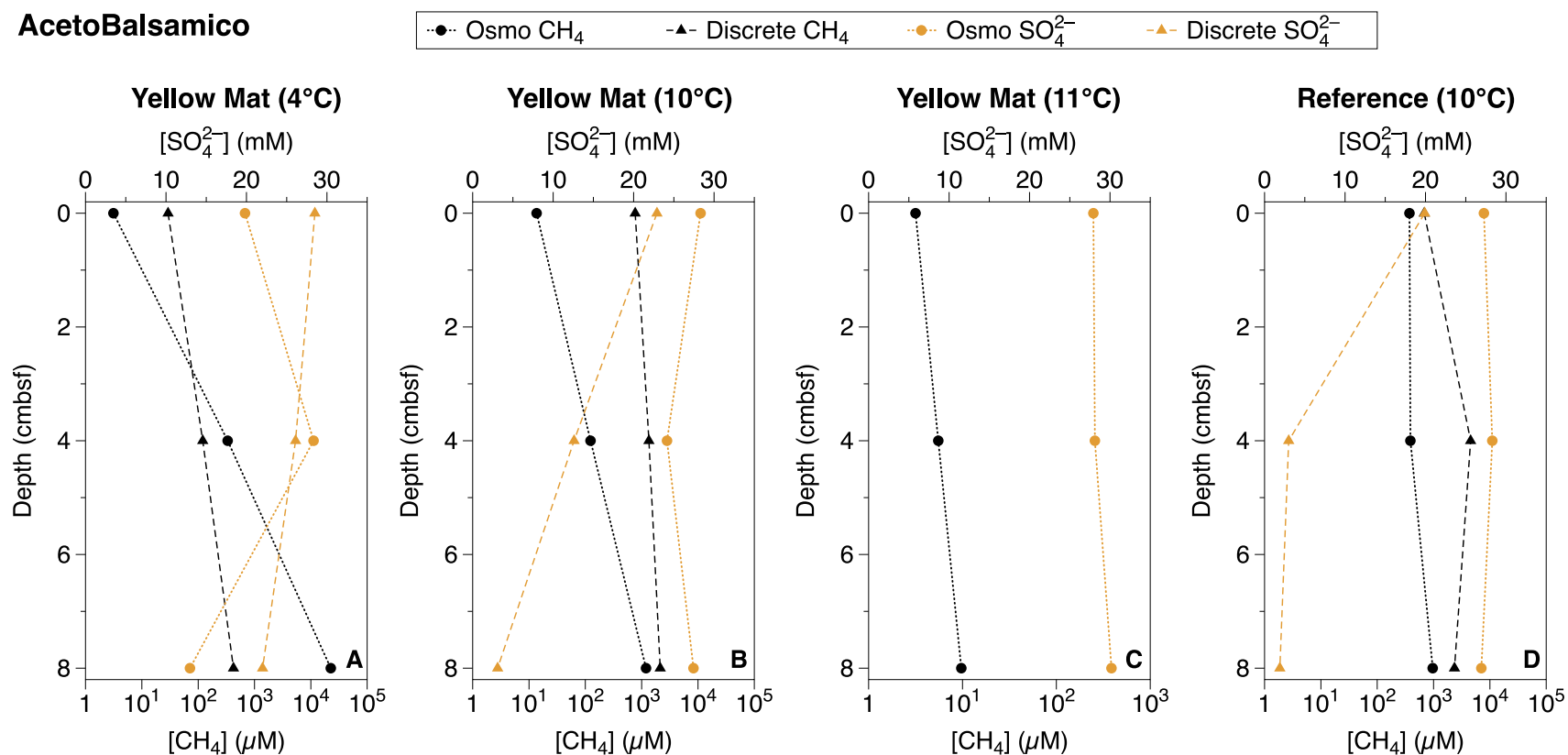


Fig. 2.2. Depth profiles of methane (CH_4) and sulfate (SO_4^{2-}) concentrations at AcetoBalsamico from yellow microbial mats at 4°C (A), 10°C (B), and 11°C (C), and adjacent reference sediment (D). Circles denote samples from Jumbo OsmoSamplers, and triangles denote discrete push core samples. Temperatures were taken at 10 cmbsf. Note differences in scales.

Cathedral Hill

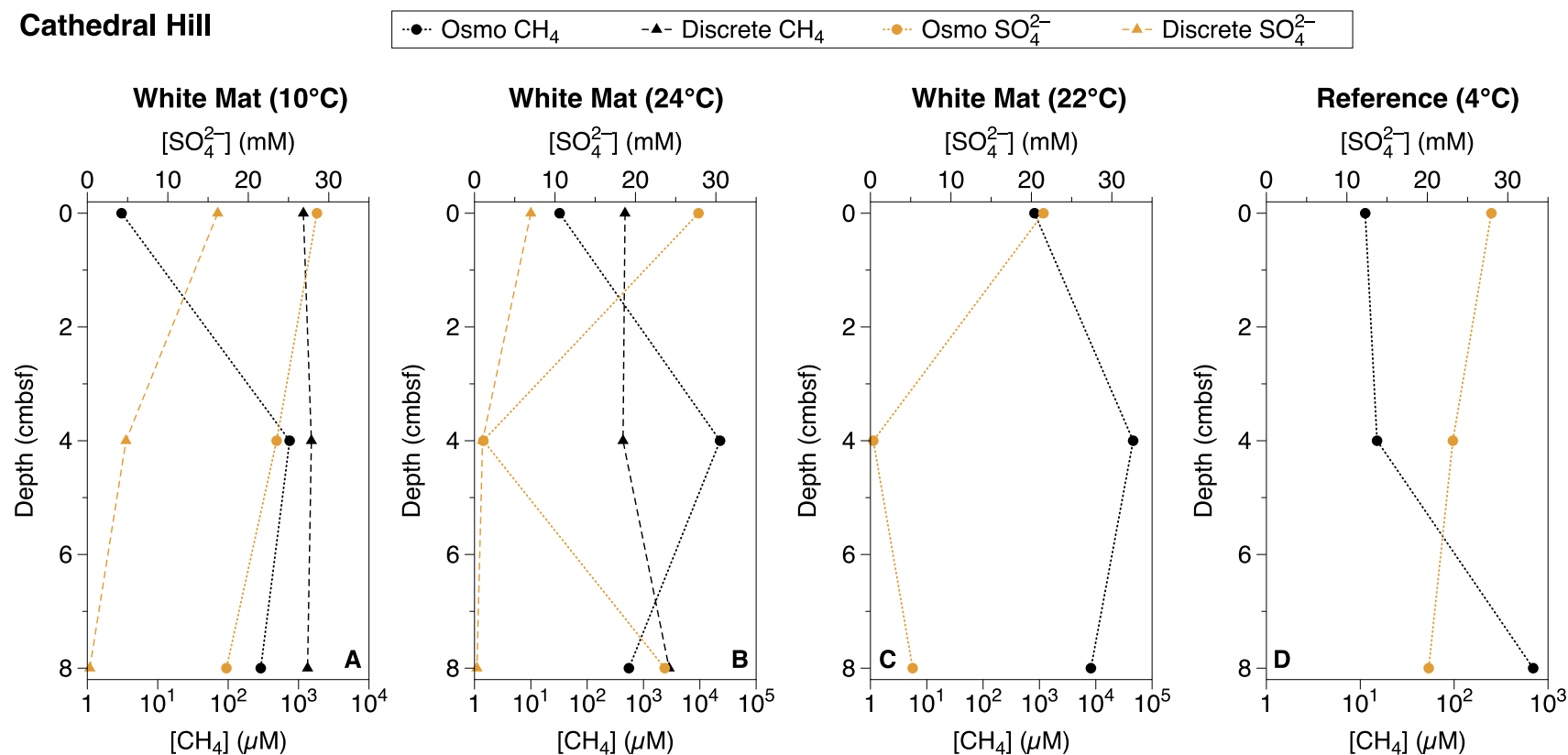


Figure 2.3. Depth profiles of methane (CH_4) and sulfate (SO_4^{2-}) concentrations at Cathedral Hill from white microbial mats at 10°C (A), 24°C (B), and 22°C (C), and adjacent reference sediment (D). Circles denote samples from Jumbo OsmoSamplers, and triangles denote discrete push core samples. Temperatures were taken at 10 cmbsf. Note differences in scales.

Sonora Margin

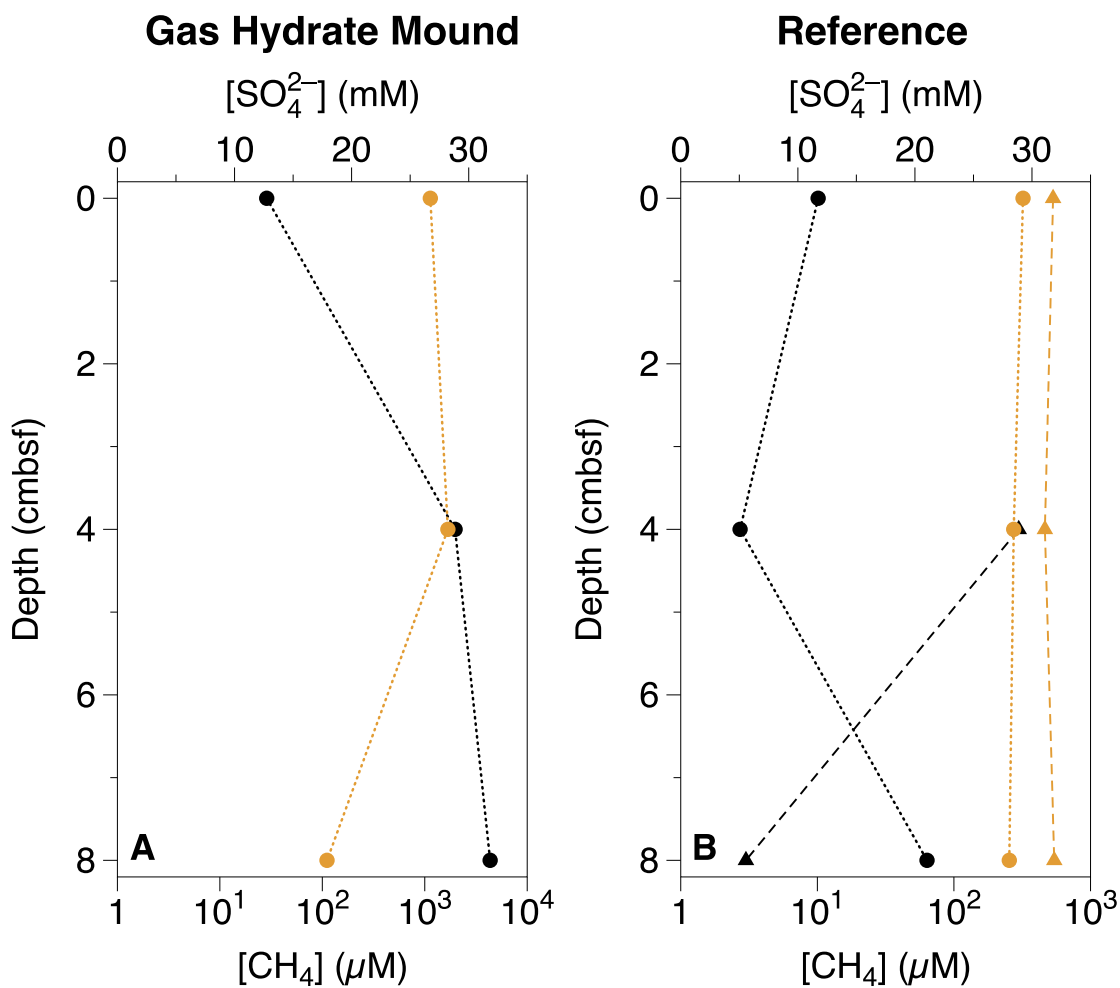


Figure 2.4. Depth profiles of methane (CH₄) and sulfate (SO₄²⁻) concentrations at Sonora Margin from a gas hydrate mound (A) and adjacent reference sediment (B). Circles denote samples from Jumbo OsmoSamplers, and triangles denote discrete push core samples. Temperatures were taken at 10 cmbsf. No paired discrete core was taken for the gas hydrate mound and the SWI CH₄ sample was not determined. Note differences in scales.

Falkor Fountain

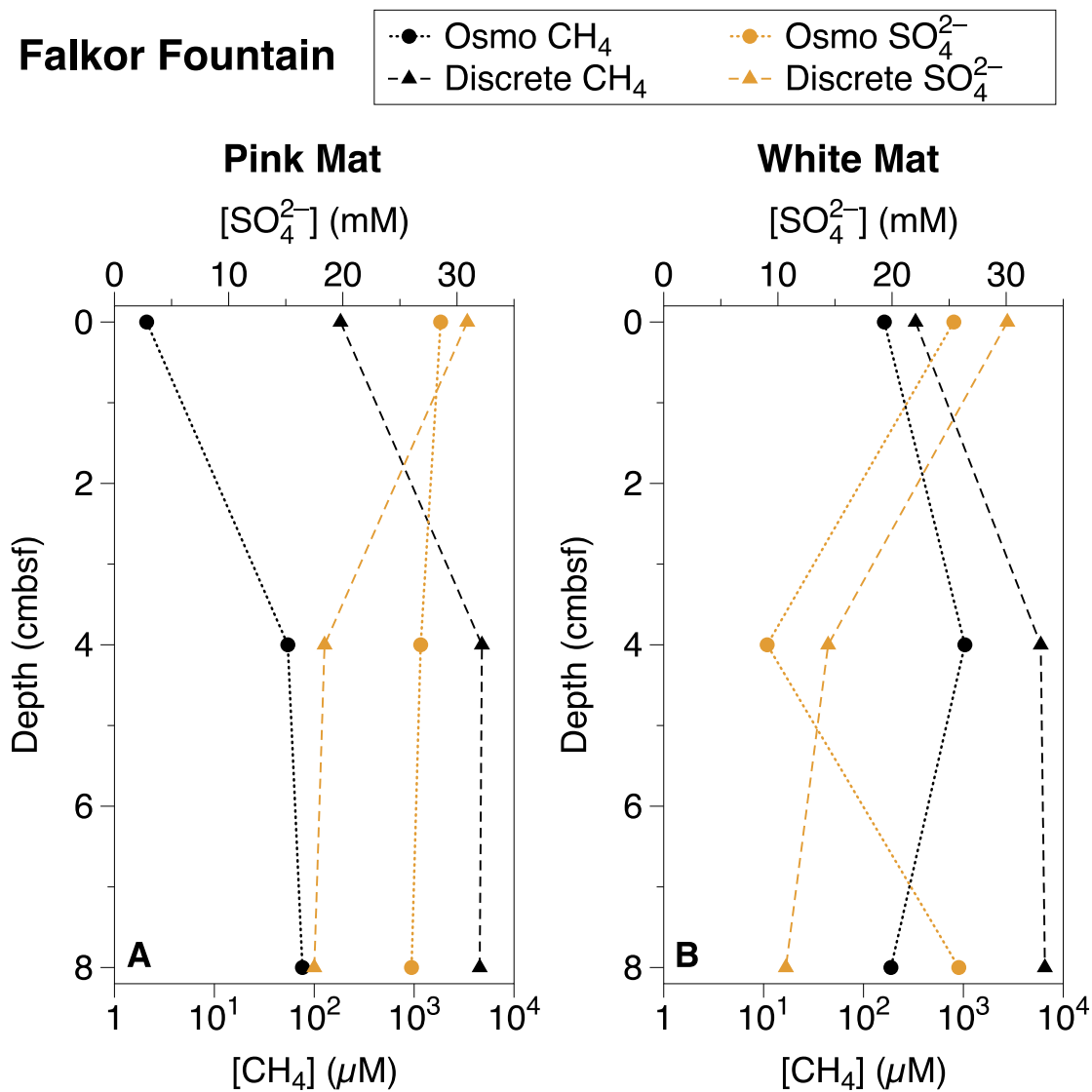


Figure 2.5. Depth profiles of methane (CH₄) and sulfate (SO₄²⁻) concentrations at Falkor Fountain (Cathedral Hill) from pink (A) and white (B) microbial mat. Circles denote samples from Jumbo OsmoSamplers, and triangles denote discrete push core samples. Temperatures were 10°C at 10 cmbsf for both cores. Note differences in scales.

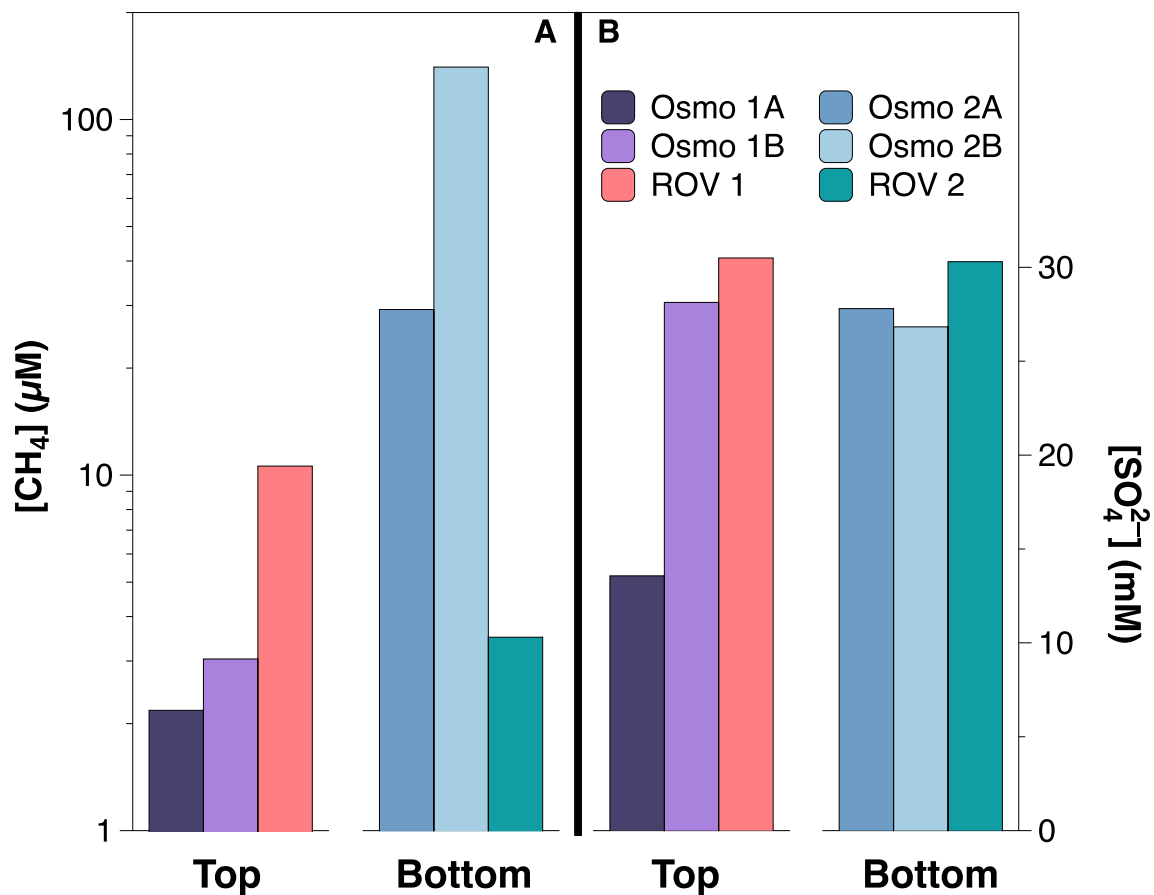


Figure 2.6. Methane (A; CH_4) and sulfate (B; SO_4^{2-}) concentrations at on the top and bottom of Alvin Spire near Cathedral Hill. The Jumbo OsmoSamplers were deployed on the seabed not inserted into sediments. The “Osmo” designation refers to samples collected with Jumbo OsmoSamplers and ROV refers to discrete samples collected with Niskin bottles attached to the ROV. Note differences in scales.

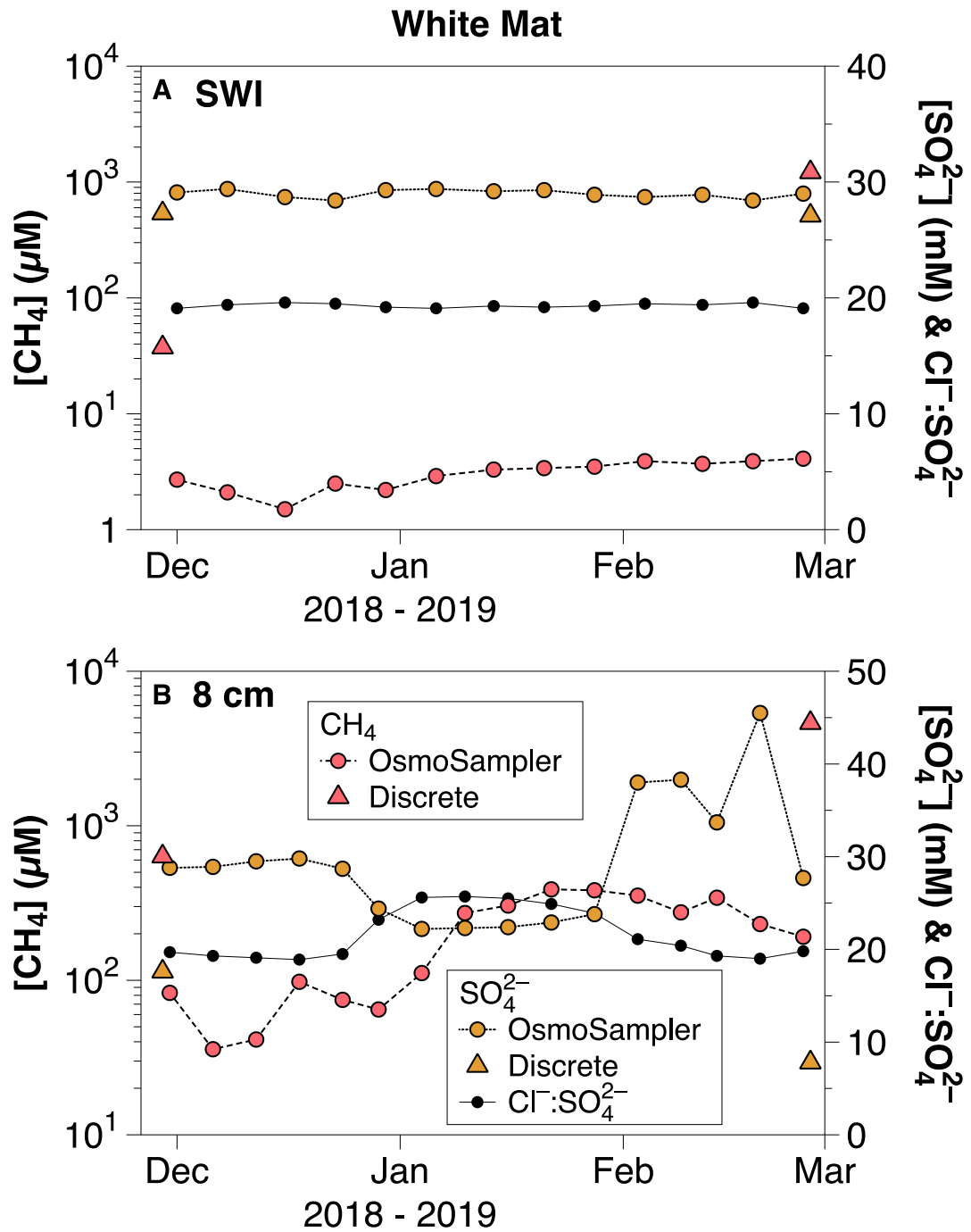


Figure 2.7. Temporal patterns in the white mat locale of methane (CH_4) and sulfate (SO_4^{2-}) concentrations, and $\text{Cl}^-:\text{SO}_4^{2-}$ ratios at the sediment water interface (A) and at the 8 cm horizon (B). Note differences in scales.

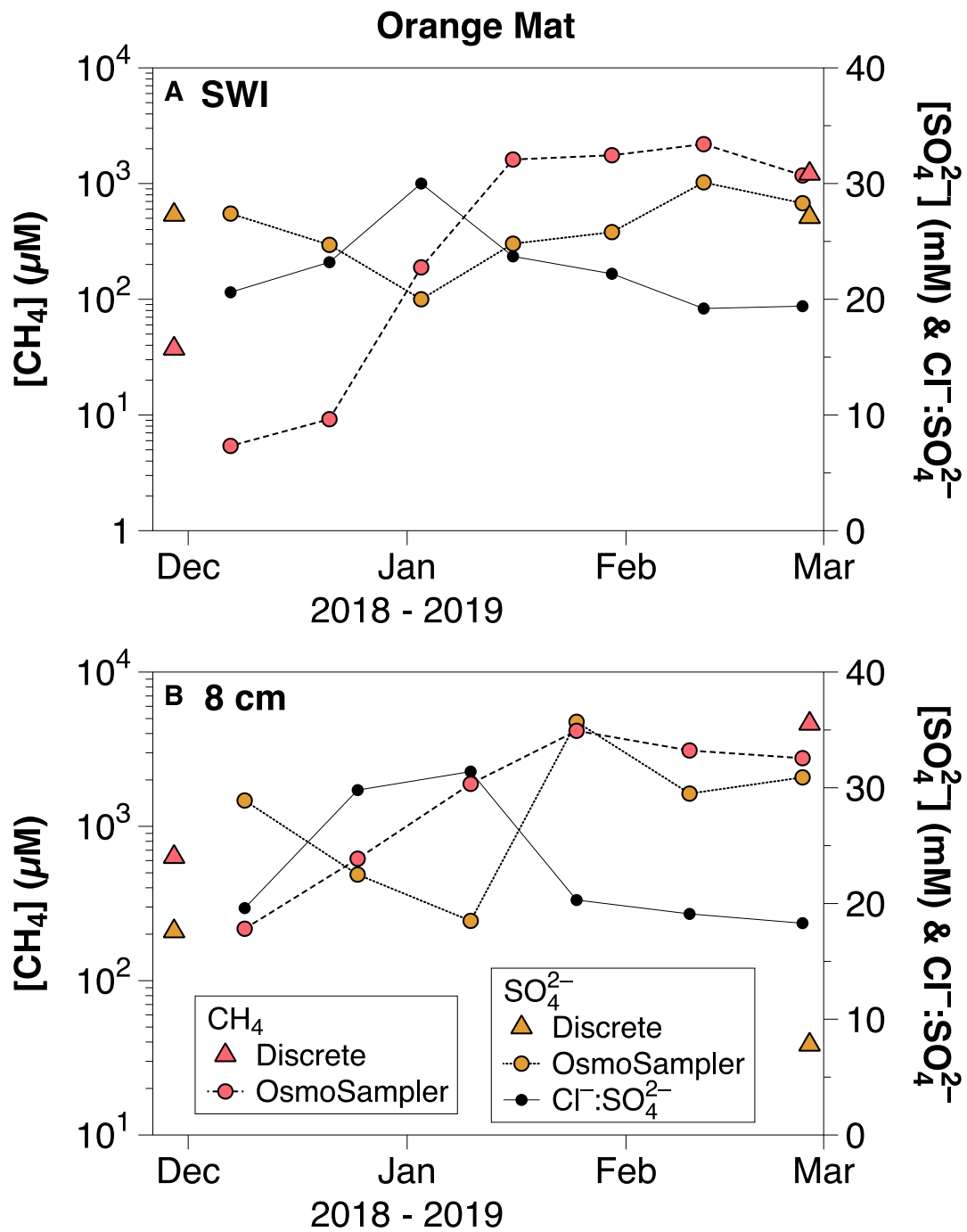


Figure 2.8. Temporal patterns in the orange mat locale of methane (CH_4) and sulfate (SO_4^{2-}) concentrations, and $\text{Cl}^-:\text{SO}_4^{2-}$ ratios at the sediment water interface (A) and at the 8 cm horizon (B). Note differences in scales.

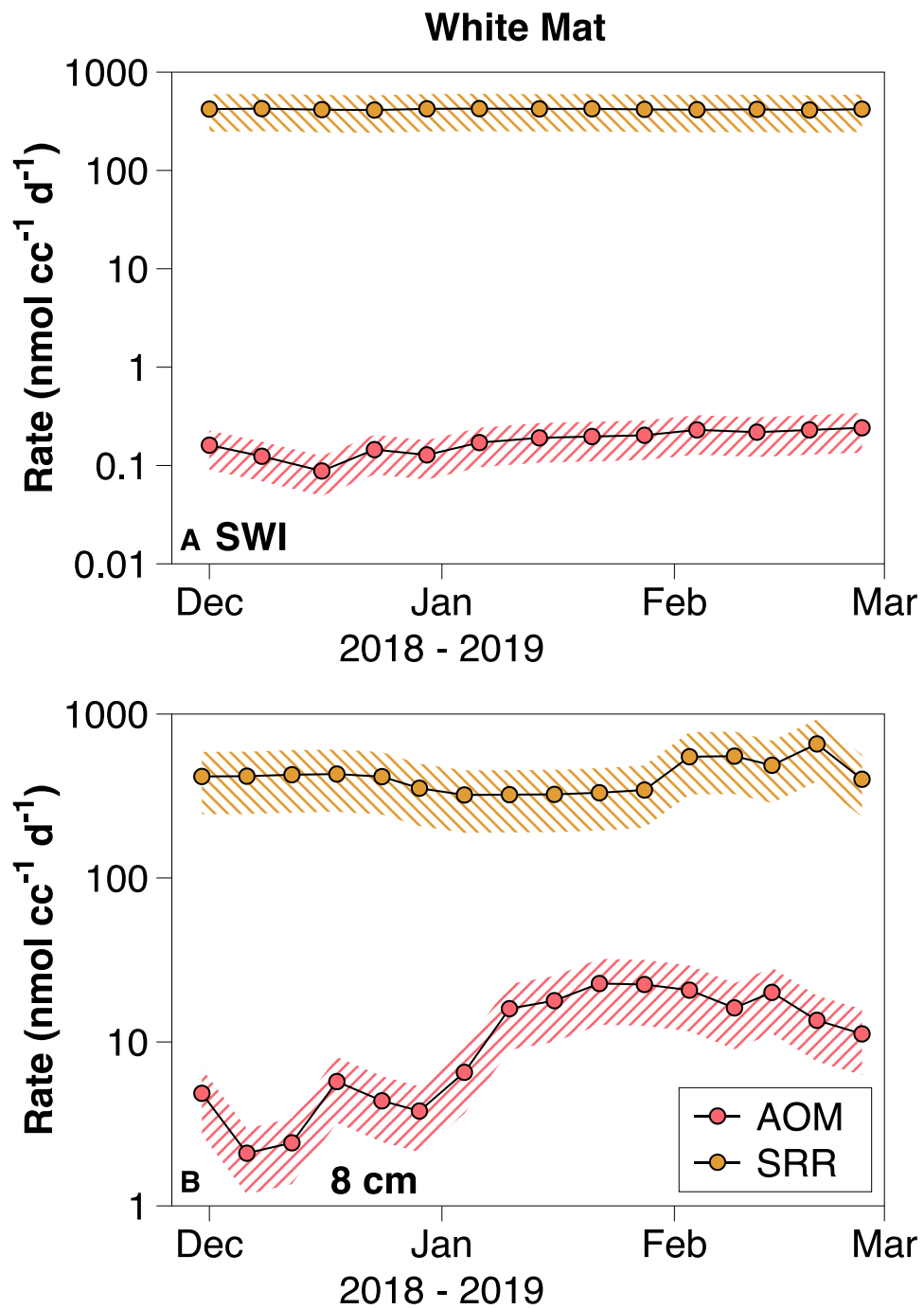


Figure 2.9. Temporal patterns in the white mat locale of potential anaerobic methane oxidation (AOM) and sulfate reduction (SRR) rates at the sediment water interface (A) and at the 8 cm horizon (B). Shaded regions represent resampled 95% confidence intervals of rates. Note differences in scales.

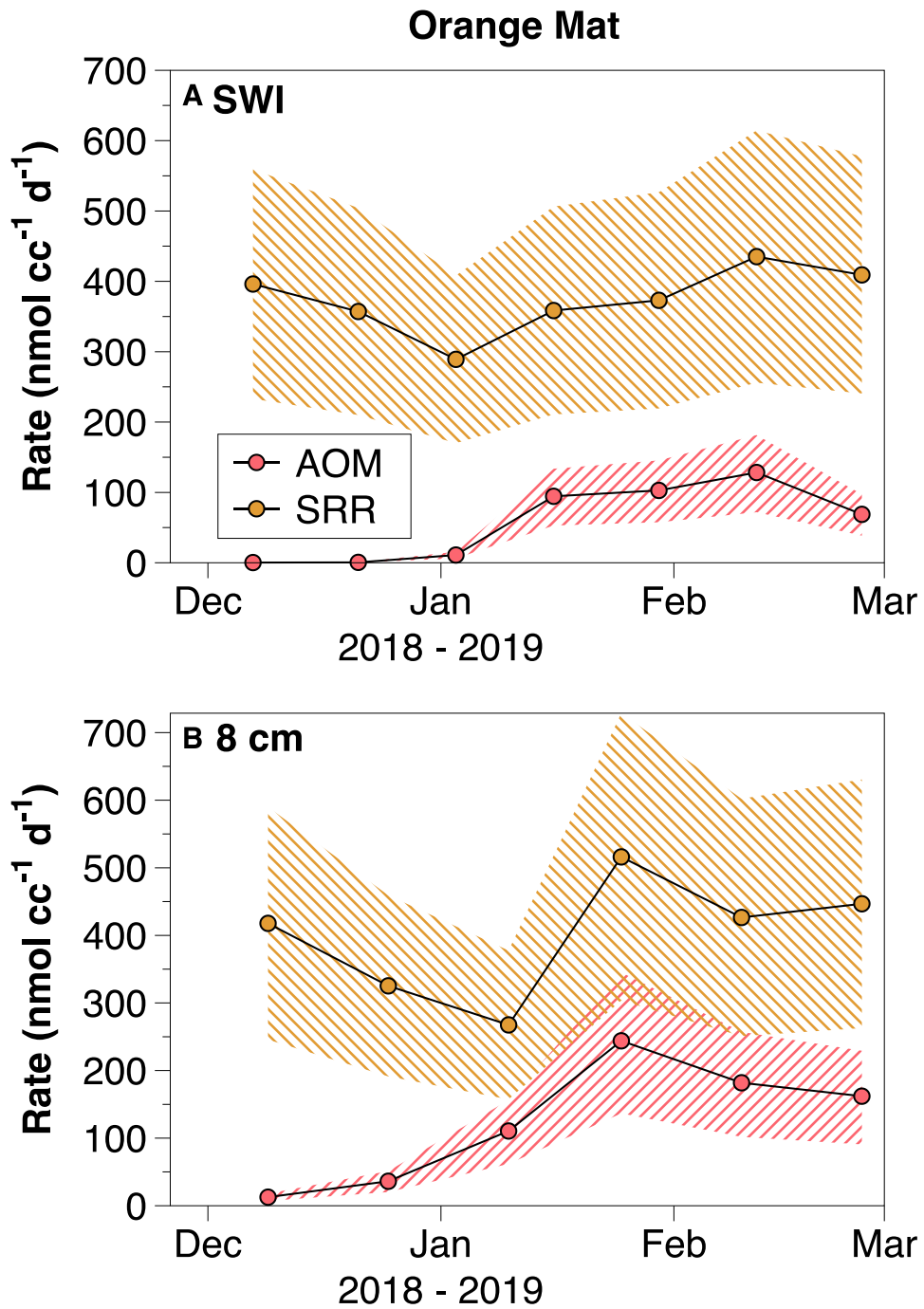


Figure 2.10. Temporal patterns at the orange mat locale of potential anaerobic methane oxidation (AOM) and sulfate reduction (SRR) rates at the sediment water interface (A) and at the 8 cm horizon (B). Shaded regions represent resampled 95% confidence intervals of rates. Note differences in scales.

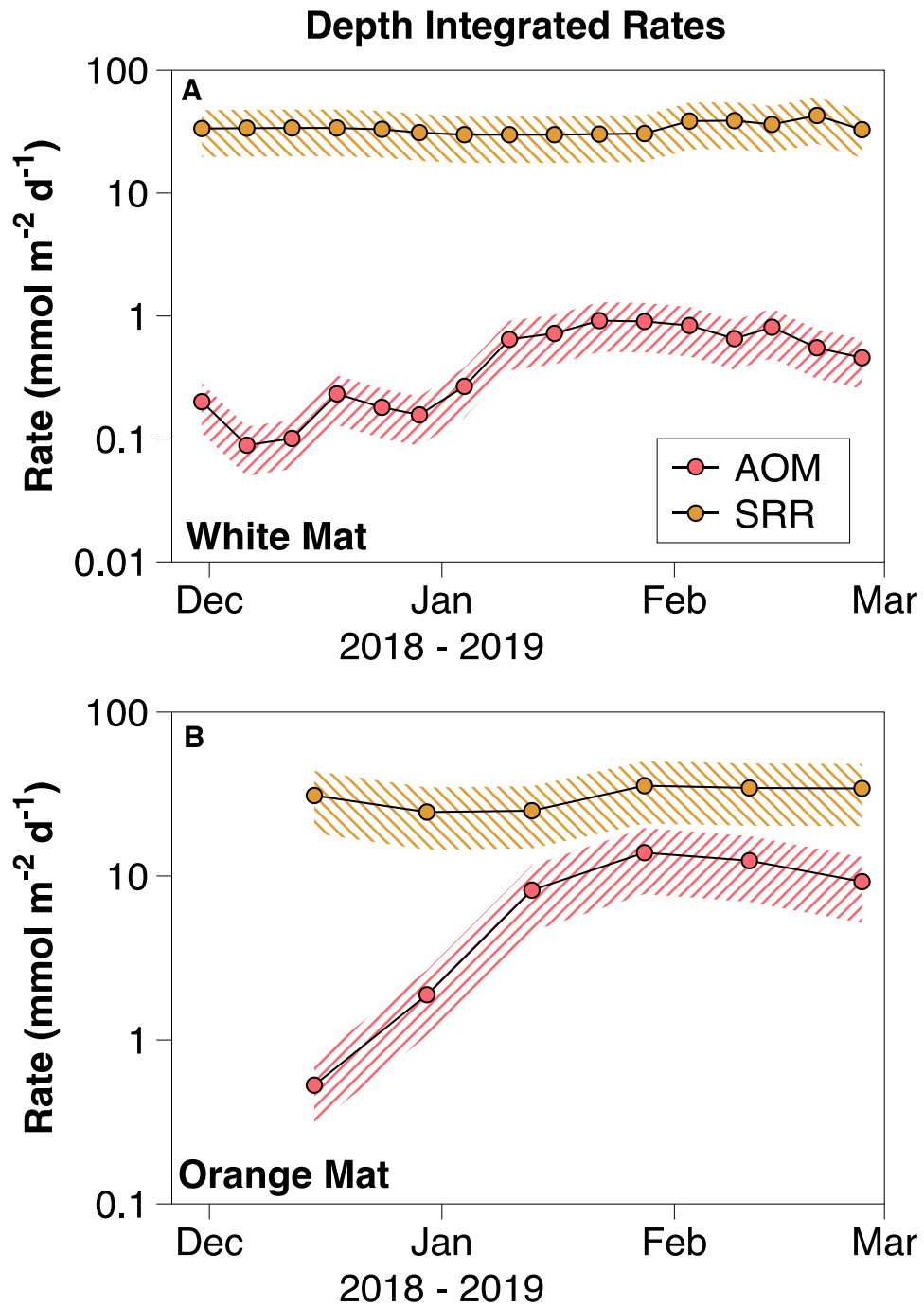


Figure 2.11. Temporal patterns at the orange mat locale of potential anaerobic methane oxidation (AOM) and sulfate reduction (SRR) rates at the sediment water interface (A) and at the 8 cm horizon (B). Shaded regions represent resampled 95% confidence intervals of depth integrated rates. Note differences in scales.

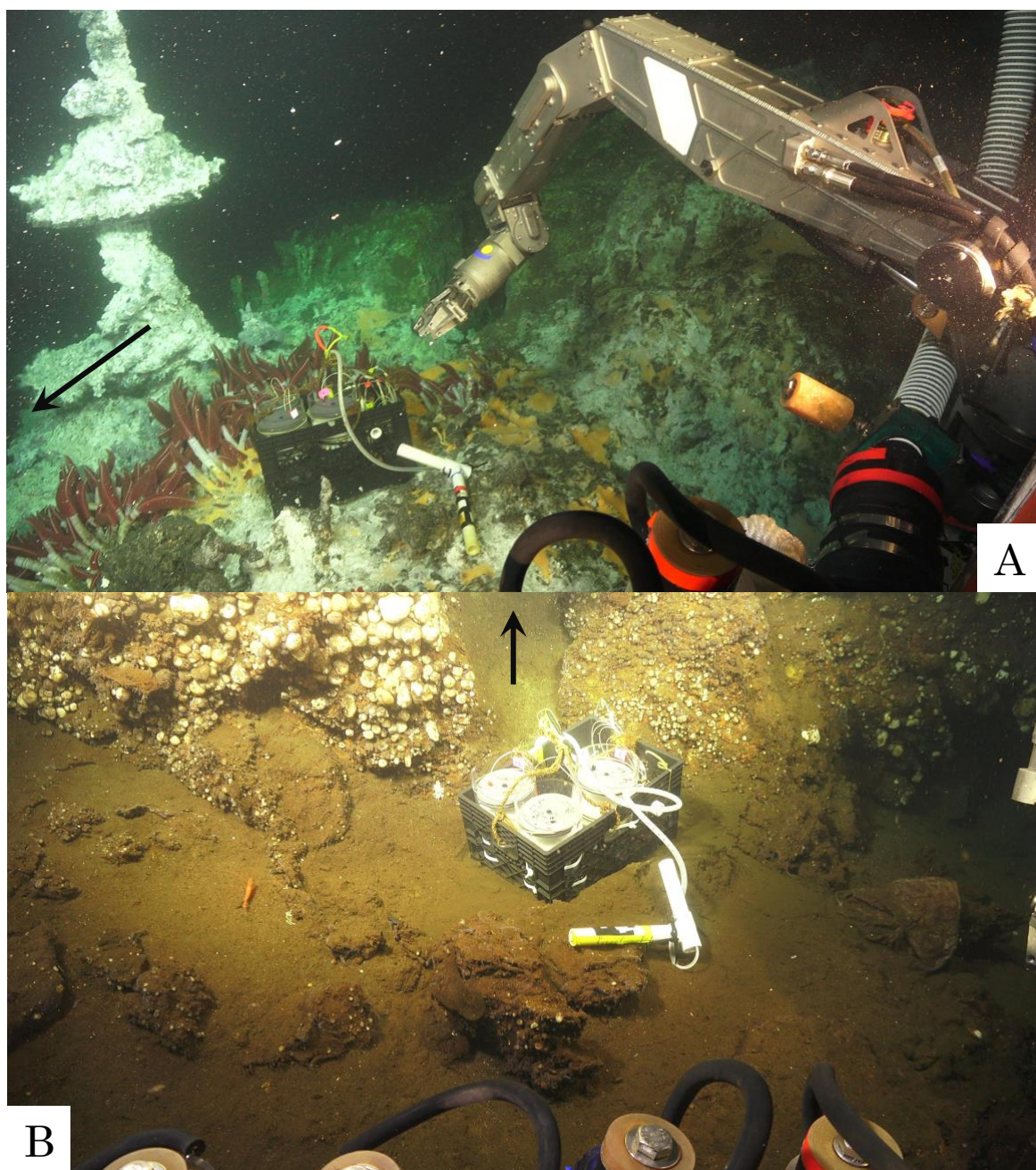


Figure 2.12. Pictures during deployment of Jumbo OsmoSamplers on the seafloor adjacent to (A) and below (B) Alvin Spire. Arrows point in the direction of the other sampling locale and Jumbo OsmoSampler. The approximate distance between the two was 5 m.

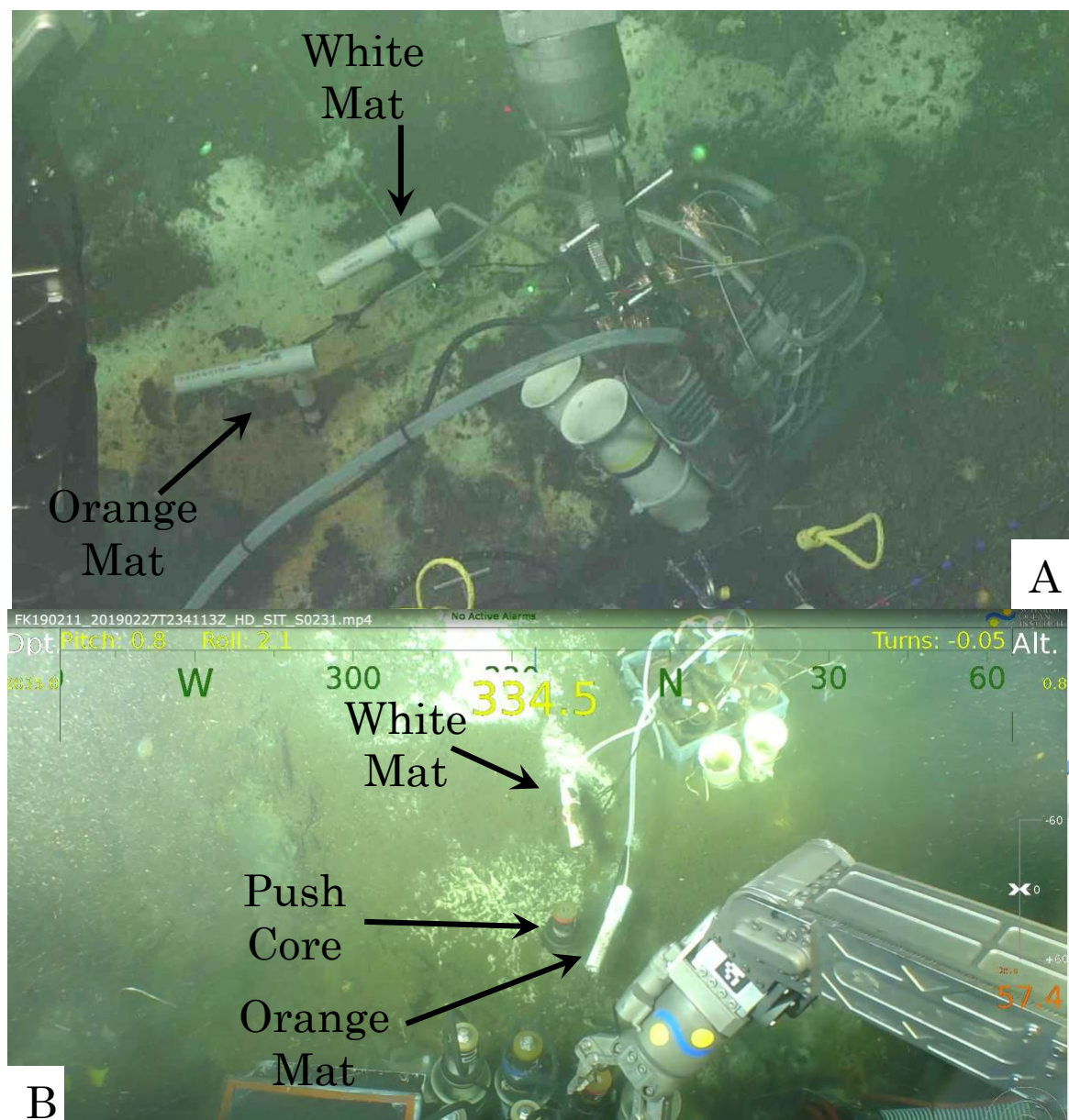


Figure 2.13. Pictures during deployment (A) and recovery (B) of the long-term OsmoSamplers. The observed pristine “fried-egg” mat present during deployment was decimated when we arrived for retrieval. OsmoSamplers were deployed on 25 November 2018 during HOV *Alvin* Dive 5000 and recovered on 27 February 2019 during ROV *SuBastian* Dive 231.

Table 2.2. Summary of Kendall's Tau non-parametric correlation coefficients of relationships between Jumbo OsmoSampler and push core data

Analyte 1	Analyte 2	Kendall's Tau	p-value
CH ₄ Osmo	CH ₄ Discrete	0.225	0.104
SO ₄ ²⁻ Osmo	SO ₄ ²⁻ Discrete	0.352	0.016*
Cl ⁻ Osmo	Cl ⁻ Discrete	0.113	0.441
SO ₄ ²⁻ Osmo	CH ₄ Osmo	-0.507	<0.001*
SO ₄ ²⁻ Osmo	CH ₄ Discrete	-0.17	0.271
SO ₄ ²⁻ Discrete	CH ₄ Osmo	-0.439	0.003*
SO ₄ ²⁻ Discrete	CH ₄ Discrete	-0.475	0.002*

p-values < 0.05 were considered significantly correlated and are denoted by *

Osmo refers to Jumbo OsmoSamplers and Discrete refers to push cores

Table 2.3. Summary of mean comparison between Jumbo OsmoSampler and push core data

Analyte	<i>p</i>-value
CH ₄	0.009*
SO ₄ ²⁻	0.046*
Cl ⁻	<0.001*

p-values < 0.05 were considered significantly different and are denoted by *

Table 2.4. Summary of geochemistry from Jumbo OsmoSamplers and paired push-cores.

Cruise	Recovery Dive #	Site Name	Locale	Temperature (°C)	Depth (cmbsf)	Jumbo OsmoSamplers				Discrete			
						CH ₄ (μM)	Cl ⁻ (mM)	SO ₄ ²⁻ (mM)	Cl:SO ₄ ²⁻	CH ₄ (μM)	Cl ⁻ (mM)	SO ₄ ²⁻ (mM)	Cl:SO ₄ ²⁻
AT4205	4996	Cathedral Hill	Reference	4	0	11.29	535.08	27.96	19.14	ND	ND	ND	ND
AT4205	4996	Cathedral Hill	Reference	4	4	15.09	525.04	23.17	22.66	ND	ND	ND	ND
AT4205	4996	Cathedral Hill	Reference	4	8	694.81	537.13	20.17	26.62	ND	ND	ND	ND
AT4205	4996	Cathedral Hill	White Mat	20	0	3.06	536.37	28.53	18.80	1168.2	539	16.2	33.30
AT4205	4996	Cathedral Hill	White Mat	20	4	746.02	547.73	23.52	23.29	1518.9	513	4.8	106.44
AT4205	4996	Cathedral Hill	White Mat	20	8	290.13	533.96	17.28	30.91	1342.2	540	0.3	1757.17
AT4205	4996	Cathedral Hill	White Mat	24	0	32.40	524.91	27.84	18.85	469.6	557	7.0	79.62
AT4205	4996	Cathedral Hill	White Mat	24	4	22867.19	551.13	1.10	499.89	433.1	580	1.0	557.84
AT4205	4996	Cathedral Hill	White Mat	24	8	550.37	527.58	23.64	22.32	2851.8	565	0.3	1837.11
AT4205	4996	Cathedral Hill	White Mat	22	0	817.41	525.51	21.50	24.44	ND	ND	ND	ND
AT4205	4996	Cathedral Hill	White Mat	22	4	46104.30	590.27	0.36	1627.02	ND	ND	ND	ND
AT4205	4996	Cathedral Hill	White Mat	22	8	8199.59	581.01	5.25	110.57	ND	ND	ND	ND
AT4205	4999	AcetoBalsamico	Yellow Mat	11	0	3.16	529.60	27.93	18.96	ND	ND	ND	ND
AT4205	4999	AcetoBalsamico	Yellow Mat	11	4	5.52	529.54	28.12	18.83	ND	ND	ND	ND
AT4205	4999	AcetoBalsamico	Yellow Mat	11	8	9.71	534.65	30.18	17.71	ND	ND	ND	ND
AT4205	4999	AcetoBalsamico	Yellow Mat	10	0	3.13	375.84	19.82	18.96	29.2	552	28.5	19.36
AT4205	4999	AcetoBalsamico	Yellow Mat	10	4	333.55	535.86	28.34	18.91	120.4	548	26.1	21.02
AT4205	4999	AcetoBalsamico	Yellow Mat	10	8	22573.63	289.26	12.98	22.29	423.0	544	22.0	24.69
AT4205	4999	AcetoBalsamico	Reference	4	0	13.66	535.35	28.32	18.91	767.1	562	22.9	24.57
AT4205	4999	AcetoBalsamico	Reference	4	4	123.79	467.87	24.15	19.38	1344.7	547	12.6	43.32
AT4205	4999	AcetoBalsamico	Reference	4	8	1195.95	531.47	27.44	19.37	2134.6	552	3.1	175.69
AT4205	4999	AcetoBalsamico	Yellow Mat	4	0	373.52	514.82	27.26	18.89	684.9	552	19.9	27.77
AT4205	4999	AcetoBalsamico	Yellow Mat	4	4	388.40	530.02	28.30	18.73	4551.0	569	3.0	187.66
AT4205	4999	AcetoBalsamico	Yellow Mat	4	8	966.73	511.85	26.94	19.00	2351.6	570	1.9	300.79
FK190211	240	Alvin Spire	WC	4	0	2.18	250.59	13.57	18.46	10.6	557	30.5	18.29
FK190211	240	Alvin Spire	WC	4	4	2.19	141.27	7.65	18.45	ND	ND	ND	ND
FK190211	240	Alvin Spire	WC	4	8	3.04	530.19	28.13	18.85	ND	ND	ND	ND
FK190211	240	Alvin Spire	WC	4	0	29.23	525.90	27.80	18.92	1.2	547	28.8	18.98
FK190211	240	Alvin Spire	WC	4	4	4.39	519.49	26.83	19.36	5.7	554	31.7	17.46

FK190211	240	Alvin Spire	WC	4	8	140.44	72.55	4.18	17.36	ND	ND	ND	ND
FK190211	242	Sonoran Margin	Reference	4	0	10.14	554.18	29.23	18.96	ND	558	31.8	17.56
FK190211	242	Sonoran Margin	Reference	4	4	2.72	537.41	28.44	18.89	298	555	31.1	17.81
FK190211	242	Sonoran Margin	Reference	4	8	63.54	534.03	28.06	19.03	3.0	589	31.9	18.48
FK190211	242	Sonoran Margin	Hydrate Mound	4	0	28.63	505.23	26.72	18.91	ND	ND	ND	ND
FK190211	242	Sonoran Margin	Hydrate Mound	4	4	1975.09	550.10	28.22	19.49	ND	ND	ND	ND
FK190211	242	Sonoran Margin	Hydrate Mound	4	8	4342.78	540.17	17.90	30.18	ND	ND	ND	ND
FK190211	244	Alvin Spire	Pink Mat	10	0	2.10	538.46	28.57	18.85	183	554	30.9	17.94
FK190211	244	Alvin Spire	Pink Mat	10	4	54.32	535.49	26.82	19.96	4765	567	18.4	30.80
FK190211	244	Alvin Spire	Pink Mat	10	8	76.04	542.77	26.02	20.86	4530	571	17.5	32.72
FK190211	244	Alvin Spire	White Mat	10	0	161.66	537.15	25.41	21.14	332	556	30.1	18.47
FK190211	244	Alvin Spire	White Mat	10	4	1034.93	564.97	9.06	62.36	5951	553	14.4	38.38
FK190211	244	Alvin Spire	White Mat	10	8	188.17	541.15	25.85	20.94	6546	587	10.7	54.85

cmbsf = cm below seafloor; WC = Overlying water; ND = Not determined

CHAPTER 3

HYDROTHERMAL PLUMES SUPPORT HIGH RATES OF ORGANIC CARBON CYCLING IN THE GUAYMAS BASIN²

² Montgomery, A.M., G.C. Zhuang, Z.M. Marinelli, H.L.Y. Choi, K.S. Hunter, K. Anantharaman, A.P. Teske, S.B. Joye. To be submitted to *Environmental Microbiology*

ABSTRACT

Our understanding of deep-sea microbial ecology and biogeochemical cycling is influenced by the dynamic ecosystems that are hydrothermal plumes. Many studies have focused on chemoautotrophic metabolisms of reduced compounds such as sulfur, hydrogen, manganese, and ammonia and have examined the microbial communities involved. More recently the utilization of organic carbon and heterotrophy has been investigated, however, the biogeochemical fate of organic carbon in hydrothermally impacted waters has remained largely unconstrained. Here, we measured the microbial metabolism of methane, methanol, acetate, hexadecane, and naphthalene in the water column of the Guaymas Basin to track the influence of hydrothermal inputs on marine carbon cycling and to elucidate their relative importance as carbon and energy sources. Elevated plume signatures and the observed high rates of microbial activity indicate that microbial communities respond quickly to hydrothermal inputs. Methane oxidation rates were up to $31.4 \pm 0.6 \mu\text{mol L}^{-1} \text{d}^{-1}$ near an active vent, and are the highest rates recorded in marine waters. Degradation of naphthalene and hexadecane, two representative components of crude oil, was substantial in and out of plume. Methanol and acetate were enriched in the plume and both were utilized as a carbon and energy source. Several metabolisms were significantly higher in the plume, demonstrating the influence of hydrothermal inputs on deep-sea microbial activity. Interestingly, in-plume assimilation of acetate and methanol accounted for more than 50% of total uptake in many cases, elucidating the importance of these compounds as carbon sources. This is the first direct account of heterotrophic activity in hydrothermal plumes. Our results demonstrate that organic carbon is rapidly metabolized in hydrothermal plumes, emphasizing the

importance of heterotrophy and challenging the idea that these systems are dominated by chemoautotrophic processes.

Keywords: Guaymas Basin, hydrothermal plume, organic carbon, methane, methanol, heterotrophy

INTRODUCTION

Hydrothermal venting occurs along tectonic plate boundaries throughout the global ocean system. These unique biogeochemical hotspots impact marine environments locally and globally. Hot, deeply sourced fluids flow through geologic strata driving geochemical reactions and creating site- or regionally-specific chemical signatures. The Guaymas Basin in the Gulf of California is an end-member hydrothermal system where sill intrusions propagate hydrothermal heat through thick layers of overlying diatomaceous sediments, creating unique and intense discharges of super-heated fluids into the water column (Teske *et al.*, 2016). Upon discharge, these fluids mix with overlying seawater creating a dynamic chemical niche that fuels vibrant microbial systems (Reysenbach *et al.*, 2000; Reysenbach and Shock, 2002; Perner *et al.*, 2013). The conditions within hydrothermal plumes promote the development of rich microbial communities with diverse and dynamic metabolic capabilities, which can improve our understanding of deep-sea biogeochemistry (Dick *et al.*, 2013).

Hydrothermal fluids discharging from vents are dominated by energy-rich compounds including metals, hydrogen, ammonium, sulfides, and organics. In the Gulf of

California, discharging hydrothermal fluids are enriched with methane and higher alkanes, other low-molecular weight organic carbon compounds (*e.g.*, acetate, methanol, methylamine, etc.), and complex hydrocarbons including petroleum that can support diverse microbial metabolisms (Merewether *et al.*, 1985; Kawka and Simoneit, 1987; Welhan and Lupton, 1987; Martens, 1990; Peter *et al.*, 1991; Cowen *et al.*, 2002; Lin *et al.*, 2017; Zhuang, Montgomery, *et al.*, 2019). Recent studies have pointed to the microbes responsible for organic matter cycling in these hydrothermal plumes through meta-omics approaches (Li, Jain, *et al.*, 2014; Li *et al.*, 2015, 2016; Wang *et al.*, 2020; Zhou *et al.*, 2020). However, to fully understand the influence of hydrothermal plumes on deep-sea microbiology and carbon cycling, genomics data must be merged with *in situ* geochemical and microbial activity data.

Chemoautotrophic metabolism is a hallmark of hydrothermal systems; fixation of inorganic carbon coupled to the oxidation of reduced energy-rich substrates is the base of the food web, driving proliferation of free-living microbial communities (Kadko *et al.*, 1990) and animals via symbiosis (*e.g.s*, Dubilier *et al.*, 2008; Childress and Girguis, 2011; Petersen *et al.*, 2011). Akin to light-driven photoautotrophy, chemoautotrophy enables dark carbon fixation in hydrothermal plumes (Karl *et al.*, 1980; Wirsén *et al.*, 1993; Amend *et al.*, 2011) at rates that rival those of the surface ocean (Prakash *et al.*, 1991; Markager, 1998). High oxidation rates of inorganic substrates, such as ammonia and manganese (II), exist in Guaymas Basin hydrothermal plumes, illustrating the potential importance of chemoautotrophy (Lam, 2004; Dick *et al.*, 2009; Beman *et al.*, 2012). Elevated methane concentrations (Simoneit *et al.*, 1990; Cowen *et al.*, 2002; Gharib *et al.*, 2005; Holden *et*

al., 2012) and methanotrophic activity (de Angelis *et al.*, 1993) in hydrothermal plumes are also documented.

In contrast, heterotrophic metabolism has received much less attention. Previous studies demonstrated that heterotrophic bacteria (e.g. *Alpha-*, *Delta-*, and *Gammaproteobacteria*) are transcriptionally active in plumes and carbohydrates are hydrolyzed enzymatically in Guaymas Basin (Li *et al.*, 2015; Meier *et al.*, 2016; Wang *et al.*, 2020; Ziervogel and Arnosti, 2020). Given the abundance of potential organic carbon substrates available and diversity of hydrothermal plume microbial communities, heterotrophic organic matter cycling, and bacterial production is likely to be important. Since chemoautotrophic and heterotrophic processes can occur simultaneously, and potentially collaboratively, assessment of both suites of processes offers a new perspective on the microbial ecology of deep-sea hydrothermal plumes. Documenting how plume microbial communities respond to the rapidly changing geochemical regimes in hydrothermal plumes will shed light on the effects of hydrothermal vents.

A number of -omics based studies demonstrated active cycling of sulfur, carbon, hydrogen, and trace metals by a metabolically versatile microbial community in hydrothermal plumes (Baker *et al.*, 2012; Lesniewski *et al.*, 2012; Anantharaman *et al.*, 2013, 2014; Dick *et al.*, 2013; Li, Toner, *et al.*, 2014; Fortunato and Huber, 2016; Fortunato *et al.*, 2018; Zhou *et al.*, 2020). For example, *Gammaproteobacteria* in hydrothermal plumes actively transcribe a number of genes associated with the metabolism of organic sulfur, C-1 compounds, and hydrocarbons (Zhou *et al.*, 2020). Similarly,

metatranscriptomic data from a Guaymas Basin plume showed high transcriptional activity of diverse taxa, including SUP05, Marine Group I, and SAR324 (Lesniewski *et al.*, 2012). Surprisingly, parallel assessment of metabolic activity and omics characterizations in hydrothermal plumes are rare, thus limiting our understanding of plume geochemical cycling.

We used an interdisciplinary approach to investigate the influence of a hydrothermal vent on carbon cycling in a plume in the Guaymas Basin. We sampled the water column above a hydrothermally active area and captured the signature of a rising hydrothermal plume. To obtain a dynamic functional understanding of the hydrothermal plume, we described the chemical regime, constrained microbial activity using radiotracer techniques, and paired these data with metagenomic data. Here we present the first direct measurements of organic carbon cycling in hydrothermal plumes, demonstrating the dynamic nature of these systems. Our results show that organic carbon is rapidly oxidized and assimilated in plumes illuminating the importance of heterotrophic metabolisms in hydrothermally impacted waters and these metabolisms contribute significantly to deep-sea carbon cycling.

METHODS

Sample collection

We collected seawater samples during RV *Atlantis* expedition AT42-05 to the Guaymas Basin in November 2018. The data presented here derive from samples collected from two CTD casts and Human Operated Vehicle (HOV) *Alvin* dive 5000 near the

Cathedral Hill site (Fig. 1; 27°00.696 N, 111°24.265 W) (Teske *et al.*, 2016). One cast collected fluids directly above a venting edifice at Cathedral Hill (in-plume) and the other cast targeted a site approximately 200 m to the southeast of the primary vent, acting as an off-site comparison (non-plume). Hydrographic profiles obtained with a Seabird CTD-rosette system revealed the presence of a hydrothermal plume at the in-plume site, but not at the southeastern site. HOV *Alvin* was used to collect a fluid sample directly at the vent orifice using a Niskin sampler. Samples for chemical analysis, rate measurements, and molecular surveys were collected with 20 L Niskin bottles attached to a rosette (water column profiles) or with 5 L Niskin bottles mounted on the HOV *Alvin* (vent orifice sample).

Upon return to the deck, Niskin bottles were sub-sampled immediately for dissolved methane concentration by filling a He-purged 160 mL serum vial headspace free (Magen *et al.*, 2014). Each serum vial contained 10 g of NaCl and 3 NaOH pellets, was filled with seawater from the Niskin, and then capped with a blue butyl rubber stopper and sealed with an aluminum crimp seal (Joye *et al.*, 2011). Directly after collecting dissolved methane samples, an acid washed 1 L polyethylene terephthalate glycol (PETG) bottle was filled with water from the same Niskin bottle for chemical analysis and/or rate assays and capped headspace free. The remaining water was collected in sterile 5 L PETG bottles for filtration and subsequent genomic analysis. Bottles for chemistry, biogeochemical rates, and molecular analysis were stored at 4°C until processing. Chemistry samples for subsequent nutrient, dissolved organic carbon (DOC), acetate, and methanol analyses were processed within 6 hours on board the ship by passing through a 0.2 µm pore-size filter

(Target[®] filter) as previously described (Joye *et al.*, 2004). Samples were either processed shipboard (see below) or frozen at -20°C for subsequent shore-based analysis, except methanol samples which were stored at -80°C (Zhuang *et al.*, 2014).

Chemical measurements

Ammonium samples (5 mL, 0.2 μm filtered) were preserved with phenol and stored at 4°C until analysis. Ammonium concentrations were measured on the ship within two days of collection using the phenol hypochlorite method; standards were prepared from ACS reagent grade NH_4Cl (Sigma) and the detection limit was 0.1 μM (Solórzano, 1969). In the UGA lab, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) concentrations were quantified by high-temperature combustion using a Shimadzu Instruments TOC-V TNM, as described previously (Joye *et al.*, 2004). Standards were prepared from potassium hydrogen phthalate and the detection limit was 2 μM . Other chemical parameters such as nitrate, nitrite, and phosphates were measured as previously described (Joye *et al.*, 2004).

Samples for methanol and ethanol quantification were collected in combusted 38 mL serum vials, stoppered with a blue butyl rubber stopper, and crimp sealed. Concentrations were determined using a GC-FID (SRI) equipped with a purge and trap system and an MXT[®]-Q-PLOT column (30 m x 0.52 mm I.D., Restek) (Zhuang *et al.*, 2014, 2018). Aqueous standards in a matching chemical matrix were made using analytical grade methanol and ethanol (Sigma); the detection limit was 200 nM. Acetate concentrations were determined by derivatization with nitrophenyl hydrazine and

quantified using high-performance liquid chromatography (HPLC, Fisher) as previously described (Albert and Martens, 1997). Standards were prepared from analytical grade sodium acetate (Sigma) and the detection limit was 1 μM . To quantify methane concentration, a headspace of 60 mL (in plume and vent orifice samples) or 10 mL (out of plume samples) was created in each vial by replacing an equal volume of brined sample with ultra-high purity (UHP) N_2 gas through the stopper with a needle and syringe as processed as previously described (Magen *et al.*, 2014). Headspace methane was determined by injecting 800 μL of gas into a 200 μL sample looped plumbed to a GC-FID (Shimadzu, model 8610C) equipped with an Alltech Carbosphere 80/100 column (Part No. C-5000). Final methane concentrations were calculated using aqueous standards of known concentration produced from gaseous methane standards (Airgas), as described previously (Magen *et al.*, 2014; Rogener *et al.*, 2018). The detection limit was ~ 0.75 nM methane.

Rate measurements

Rates of metabolism for a range of carbon substrates were determined in discrete water samples using radiotracer techniques. Seawater was transferred to incubation vials using an open bore 60 mL syringe fitted with a piece of silicone tubing to minimize disruption of particle-associated microorganisms (Rogener *et al.*, 2018). Incubation vials were sealed headspace free immediately after filling with three volumes to avoid loss of dissolved gases. For each discrete sample, 3 live replicates and a killed control were injected with a radiotracer (American Radiolabeled Chemicals), either a ^{14}C -substrate (1.85 – 2.22 GBq mmol^{-1} of methanol, acetate) or tritiated methane (C^3H_4 ; 700 GBq mmol^{-1}), incubated at *in situ* temperature for 49 to 60 hours and then killed and preserved for shore-

based analysis (Sibert *et al.*, 2016; Rogener *et al.*, 2018). Killed controls were amended with a solution to halt biological activity prior to radiotracer injection (see below). Microbial oxidation and/or assimilation rates were quantified by tracking the conversion of radiolabeled substrate to $^{14}\text{CO}_2$, ^{14}C - biomass, or tritiated water ($^3\text{H}_2\text{O}$; for methane oxidation).

Seawater samples to measure oxidation or assimilation of methanol, methylamine, or acetate were injected with 100 μL of ^{14}C -labeled aqueous radiotracer, corresponding to final radioactivities of 700-766 Bq, 600-616 Bq, and 666-833 Bq, respectively. In 38 mL serum vials that were stoppered with a blue butyl rubber stopper, crimp-sealed, and incubated as previously described (Zhuang *et al.*, 2018; Zhuang *et al.*, 2019). After incubation (~ 2 d), a 20 mL subsample to measure assimilation into biomass was removed from the sealed serum vial using a syringe and needle (displaced with lab air) to a clean 50 mL centrifuge tube and biological activity was immediately terminated by adding 1 mL of 100% trichloroacetic acid (5% final concentration, TCA). The remaining 18 mL sample volume (for oxidation) in the sealed vial (for oxidation rates) was immediately amended with 1 mL of 100% TCA to halt biological activity. Back in the lab, the oxidation of these three substrates to $^{14}\text{CO}_2$ was measured quantitatively using the methods described by Zhuang *et al.* (2018 & 2019). The evolved $^{14}\text{CO}_2$ was quantitatively trapped ($>90\%$ recovery) in 1.5 mL of 3-methoxypropylamine (MilliporeSigma) in 4.5 mL of scintillation fluid (Biosafe II, Research Products International, Mount Prospect, IL) and counted using a Tricarb 2910TR liquid scintillation analyzer (Perkin Elmer). Radiolabeled methanol, methylamine, or acetate assimilated into biomass was quantified by filtering samples

through a 0.2 μm polyethersulfone filter (MilliporeSigma), rinsing three times with 0.5% TCA in 35 ppt NaCl solution (pH $\sim$ 3) to remove any residual ^{14}C -bicarbonate or unused substrate, and counting the filter in 6 mL of scintillation fluid. Incubations to measure dark bicarbonate fixation were carried out using a previously described method (Galand *et al.*, 2010). Briefly, seawater was subsampled into a 38 mL serum vial with no headspace and stoppered with gray chlorobutyl septa, crimp sealed, and injected with 100 μL of aqueous ^{14}C -bicarbonate tracer (5×10^5 Bq). Samples were incubated for 48 to 60 hr. Biological activity was terminated with formaldehyde (3.7% final concentration) and samples were processed the same as the assimilation samples described above.

Methane oxidation rates were measured by quantifying the microbial conversion of tritiated methane (C^3H_4) to tritiated water ($^3\text{H}_2\text{O}$), as previously described (Rogener *et al.*, 2018). Samples were collected in 16 mL Hungate tubes and sealed headspace free with a gray chlorobutyl septum and sealed with a screw cap. After incubation, biological activity was halted with 1.6 mL of 37% formaldehyde. Each sample vial was injected with 25 μL of gaseous tritiated methane gas (N_2 balance, >20 kBq) before incubating for $\sim$ 48 hr. Following the incubation, 100 μL aliquots were collected from each sample vial and counted in 2 mL of scintillation fluid headspace free to confirm aqueous tracer activity. Samples were then transferred to a 50 mL centrifuge tube with 37% formaldehyde to halt biological activity. Each sample was purged with hydrated air within a few hours of termination to removed residual tritiated methane tracer. Subsequently, 5 mL of sample was counted in 15 mL of scintillation fluid on the scintillation counter.

Hydrocarbon (naphthalene and hexadecane) oxidation rates were measured as previously described (Kleindienst *et al.*, 2015; Sibert *et al.*, 2016). Briefly, samples were collected in 7 mL scintillation vials and sealed headspace free with PTFE lined septa to prevent adsorption of tracer. Samples were injected with 20 μ L of tracer (8.3 – 16.6 kBq, dissolved in 200 proof ethanol) using a glass syringe to avoid tracer loss and incubated for $\sim$ 1 d. Incubations were terminated by transferring samples to 15 mL centrifuge tubes with 1 mL of 2 M NaOH to trap $^{14}\text{CO}_2$ and halt biological activity. Back in the lab, samples were shaken with activated charcoal to irreversibly trap unused tracer and then distilled to quantify biological conversion of hydrocarbons to $^{14}\text{CO}_2$ by acid digestion (5 mL H_3PO_4 >85% wt.). Volatilized $^{14}\text{CO}_2$ was quantitatively trapped (>90% recovery) in 7 mL scintillation vials containing $\frac{1}{4}$ of a glass fiber filter and 1.5 mL of 3-methoxypropylamine. After a 6 hr distillation, samples were amended with 4.5 mL of scintillation fluid and counted on the scintillation counter.

Rate Calculations

Turnover constants, k (d^{-1}), were calculated as the ratio (f) of recovered ^{14}C (i.e. $^{14}\text{CO}_2$ for organic substrate oxidation or ^{14}C -biomass for assimilation) or ^3H (i.e. $^3\text{H}_2\text{O}$ for methane oxidation) product counts to the activity of the substrate initially added divided by incubation time (t):

$$\text{Turnover constant} = k = f / t$$

$$f = \text{DPM-product} / \text{DPM-substrate}$$

Turnover times (d) were defined as the inverse of turnover constants ($1/k$). Killed controls were used to correct for background tracer turnover and were typically comparable to instrument background counts. Rates were calculated by multiplying *in situ* substrate concentrations by k :

$$\text{Oxidation or Assimilation Rate} = [\text{substrate}] * f$$

If substrate concentrations were not measured (i.e. hydrocarbons and methylamine) potential rates were estimated based on substrate concentration from tracer amendments.

Statistical analysis

Statistical analyses were performed either in JMP Pro 15 or in R (version 3.4.4). Means of methane and methanol concentrations, turnover constants, and rates were compared using the Tukey-Kramer HSD test. Data were log-transformed prior to statistical analyses and p -values of < 0.05 were considered statistically significant. Correlations between hydrographic data, geochemical data, rate constants, and rate data were assessed non-parametrically using Spearman's rank correlation with the `cor.test()` function in R (version 3.4.4).

RESULTS

Guaymas Basin Hydrography and Geochemistry

Biogeochemical profiles directly in and above a hydrothermal plume and an adjacent out-of-plume site at Cathedral Hill in the Guaymas Basin (depth ~2000 m) were

compared. At both sites, chlorophyll concentrations in shallow waters (30 m) were greater than $1 \mu\text{g L}^{-1}$ but were near or below detection at other depths (Fig. 2 & Supplementary Fig. S1). Guaymas Basin waters exhibit a unique dissolved oxygen profile that we observed in both casts. In the shallowest, ~40 m dissolved oxygen was at atmospheric equilibrium (*i.e.*, $\sim 6 \text{ mg L}^{-1}$), but deeper, the dissolved oxygen concentration rapidly declined to anoxic at $\sim 350 \text{ m}$ water depth (Fig. 2). The anoxic layer was hundreds of meters thick, reaching a depth of $\sim 950 \text{ m}$. The dissolved oxygen concentration then steadily increased (*i.e.*, hypoxic, $< 2 \text{ mg L}^{-1}$) until it reached $\sim 0.7 \text{ mg L}^{-1}$ at 1500 m water depth (Fig. 2). Temperature profiles were nearly identical at both sites ranging from 22°C near the surface to $< 4^\circ\text{C}$ below 1000 m (Fig. 2 & Supplementary Fig. S1). A temperature anomaly of $\sim 0.5^\circ\text{C}$ was documented approximately 12 m above the seafloor at the in-plume site, reflecting sampling of the hydrothermal plume; no temperature anomaly was observed in the off-site cast (Fig. 2).

The fluid discharging from the vent orifice was hot (*i.e.*, $> 300^\circ\text{C}$) and visibly shimmering as observed during HOV *Alvin* dive 5000. The water temperature near the collection site (vent orifice) was $\sim 40^\circ\text{C}$, illustrating mixing with overlying bottom water (data not shown). Temperature and turbidity were used to assess the presence of a hydrothermal plume in real-time (*i.e.*, during the CTD cast). Large anomalies in temperature and beam transmission in the CTD data indicated the presence of a hydrothermal plume extending nearly 300 m off the seafloor (Fig. 2). No such anomalies were observed in the off-vent cast.

Chemical signatures were generally similar in the upper water column for both casts but varied below ~1700 m (Fig. S2 and Fig. S5). Dissolved organic carbon (DOC) was elevated near the surface relative to the midwater in both casts and was similar to deep water in the hydrothermal plume. DOC from deep waters in the off-vent cast was ~50 μM , which was comparable to mid-water depths (Fig. S5). The highest DOC concentration, 119 μM , occurred in conjunction with a temperature and turbidity spike ~12 m above the seafloor in the hydrothermal plume (Fig. 2 and Fig. 3D), while concentrations near the vent orifice sample were slightly lower (96 μM). Ammonium concentrations ranged from below detection to 25 μM at the vent orifice. In the hydrothermal plume, ammonium was mildly elevated, from 0.6 to 6.3 μM (Fig. 3D). In contrast, ammonium concentrations did not exceed 0.4 μM in the southeastern off-vent cast (Fig. S5). DOC and ammonium concentrations were significantly higher in the plume than in the control cast (Table 1; p -value < 0.05). Other chemical factors including nitrate, organic nitrogen, phosphates exhibited typical seawater profiles in both casts (Table S1). Dissolved sulfide could not be detected in our samples.

Methane dynamics and methane oxidation in the Guaymas Basin

Methane concentrations in off-vent control waters reflected typical seawater, generally below 5 nM (Fig. S2 and Fig S5). In the bottom water (~4 m above the seafloor) of the control cast, the methane concentration was 1 nM and was less than 5 nM throughout the deep water (up to ~1500 m off seafloor). In contrast, methane concentrations near the vent orifice and in the hydrothermal plume were elevated significantly (Table 1; p -value < 0.05). The highest methane concentrations were observed in the deepest samples and

steadily decreased upwards away from the core of the plume until typical background values were detected (~1300 m above the seafloor; Fig. 3). Near the vent orifice, the methane concentration was 384 μM and was 20-fold higher than the methane concentration in the plume 5 m above the seafloor (18.4 μM ; Fig. 3C). Methane concentrations were statistically higher in the hydrothermal plume (including the vent orifice sample) relative to the control site (Table 1; p -value, <0.05).

Microbial methane oxidation was detected from all depths in the Guaymas Basin in tritiated methane (C^3H_4) incubations. The highest turnover constants were observed in the plume at 25 and 308 m above the seafloor, with average values of 0.12 and 0.11 d^{-1} , respectively, corresponding to turnover times less than 10 d (Fig. 4A). In the plume, methane turnover at other depths was also elevated (including at the vent orifice) but was surprisingly variable, being an order of magnitude lower (0.01 d^{-1}) 5 m above the seafloor. In contrast, at the non-plume site, methane oxidation turnover constants were significantly lower (Table 1; p -value <0.05). At both sites, in the anoxic layer, methane turnover constants were much lower than deep waters (Fig. S3 and Fig. S6).

We used *in situ* methane concentrations and turnover constants to quantify rates of methane oxidation in the Gulf of California. Microbial methane oxidation rates ranged from below detection limit (0.05 $\mu\text{mol L}^{-1} \text{d}^{-1}$) in the anoxic layer to near or above 1 $\mu\text{mol L}^{-1} \text{d}^{-1}$ in the hydrothermal plume (Fig. S3A). The highest rates measured were $31.48 \pm 0.57 \mu\text{mol L}^{-1} \text{d}^{-1}$ near the vent orifice and $0.38 \pm 0.01 \mu\text{mol L}^{-1} \text{d}^{-1}$ approximately 12 m above the seafloor in the hydrothermal plume (Fig. S2). Methane oxidation rates in and

above the plume were generally a few orders of magnitude higher than in the control cast adjacent to the plume. Elevated methane oxidation rates were detected in the hydrothermal plume more than 300 m above the seafloor relative to the rates at similar depths from the control cast (Fig. S3A and Fig. S6A). Methane oxidation rates in deep samples (>1600 m depth) in the plume were significantly higher than out of the plume (Table 1; p -value <0.05).

Dynamics of LMW organics in the Guaymas Basin

Seawater methanol concentrations ranged from below detection in and out of the plume to 8.4 μM in the plume (Fig. 3C). Methanol concentrations varied with respect to depth, but higher concentrations were generally observed in deeper water. The highest concentration was observed in the hydrothermal plume ~12 m above the seafloor (8.4 μM). The methanol concentration at the vent orifice was 0.43 μM , which was similar to the concentration ~10 m above the seafloor at the control site (Fig. 3C and Fig. S5). Despite the high concentration detected in the hydrothermal plume, methanol concentrations were not statistically different between sites.

Using ^{14}C -labeled substrates, methanol and acetate oxidation and assimilation and methylamine oxidation were observed at nearly every depth sampled in the Guaymas Basin. Generally, the vent orifice supported the highest rate constants for LMW carbon metabolism (Fig. 4 and Fig. S3). Methanol oxidation and assimilation at the vent orifice averaged 0.25 ± 0.009 and $0.03 \pm 0.002 \text{ d}^{-1}$, respectively (Fig. 4C and Fig. 5C). These turnover constants corresponded to methanol turnover times of 4 and 40 d. Acetate

oxidation and assimilation were also highest, 0.06 ± 0.017 and $0.10 \pm 0.003 \text{ d}^{-1}$, respectively, at the vent orifice (Fig. 4B and Fig. 5B). In contrast, the highest methylamine oxidation ($0.07 \pm 0.003 \text{ d}^{-1}$; Fig. 4D) occurred near the surface (30 m). However, methylamine rate assays were not measured at the vent orifice. Methylamine assimilation was below our detection limit for all samples collected (Table S3).

In the plume, methanol oxidation turnover constants were at least two orders of magnitude lower than the vent orifice and were comparable to constants out of the plume. These turnover constants ranged from $0.0006 \pm 0.0001 \text{ d}^{-1}$ 12 m above the seafloor in the plume to 0.004 at several depths in and out of the plume (Fig. S3 and Fig. S6). Methanol assimilation constants followed a similar pattern to oxidation and did not differ significantly between sites (Table 1; p -value >0.05). Excluding the vent orifice sample, assimilation constants were generally less than 0.035 d^{-1} . Methanol assimilation was below detection in the deepest in-plume sample (5 m above the seafloor; Fig. 5C). Around 700 m depth in-plume and non-plume sites, methanol assimilation constants were elevated to 0.016 ± 0.007 and $0.013 \pm 0.004 \text{ d}^{-1}$, respectively (Fig. S4 and Fig. S5). Methanol oxidation was also elevated at 30 m water depth.

Methanol oxidation and assimilation rates ranged from less than 0.05 nM d^{-1} to 108 nM d^{-1} and from below detection limit to 30.4 nM d^{-1} , respectively (Table S2 and Table S4). Methanol oxidation was generally higher in deeper waters, with the exception of the shallowest depths measured. These values were approximately an order of magnitude higher than rates in and above in the anoxic layer. The highest rate of methanol oxidation,

$108.9 \pm 12.7 \text{ nM d}^{-1}$, was observed at the vent orifice (Fig. 4C). Methanol oxidation rates were $2.5 \pm 0.4 \text{ nM d}^{-1}$ and $5.4 \pm 2.5 \text{ nM d}^{-1}$ approximately 10 m above the seafloor at non-plume and plume sites, respectively (Fig. 4C and Fig. S6). There was no significant difference between methanol oxidation rates at the two sites (Table 1), despite the elevated rate observed at the vent orifice. Methanol assimilation rates were also generally higher in deeper waters. The highest rates were 12 m above the seafloor in the plume and at the vent orifice, $30.4 \pm 3.8 \text{ nM d}^{-1}$ and $10.8 \pm 0.85 \text{ nM d}^{-1}$, respectively (Fig. 5C). Directly between the two highest measured assimilation rates (~ 5 m above seafloor), methanol assimilation was not detected (Fig. 5C). In shallower water, the rates were at least an order of magnitude lower and ranged from 0.01 to 0.60 nM d^{-1} (Fig. S4 and Fig. S7). When considering all depths, methanol assimilation rates were significantly higher in plume relative to the control site (data not shown). However, there was no statistical difference between sites when considering plume influenced depths (below 1600 m) only (Table 1).

Patterns of acetate oxidation turnover constants were similar to those observed for methanol. The highest turnovers were detected at the vent orifice and were generally an order of magnitude lower at other depths. In other samples, acetate oxidation turnover constants ranged from $0.002 \pm 0.001 \text{ d}^{-1}$ at 700 m in the non-plume cast to $0.01 \pm 0.001 \text{ d}^{-1}$ at 710 m in-plume (Fig. S3 and Fig. S6). Deep samples at both sites (i.e. deeper than 1600 m) were all $< 0.06 \text{ d}^{-1}$ (Table S3). Acetate assimilation turnover constants differed significantly between sites and were higher in the hydrothermal plume. The assimilation rate constant was $0.10 \pm 0.003 \text{ d}^{-1}$ (Fig. S4B) at the vent orifice and was at least an order of magnitude higher than all other depths. Two samples collected from the in-plume cast

(250 and 700 m depth) were comparatively high relative to other depths, however, this corresponded to relatively elevated methanol uptake and acetate oxidation also observed at 700 m.

Methylamine oxidation was detected at both sites and most depths, but active assimilation was not detected. The highest oxidation turnover constants were 0.07 ± 0.003 and $0.06 \pm 0.002 \text{ d}^{-1}$ at 30 m of the in-plume and non-plume cast, respectively (Fig. S3 and Fig. S6). All other samples were at least an order of magnitude lower. At both sites, ~10 m above the seafloor, there was an observed dip in activity that corresponded with lower detected activity for methanol oxidation. No vent orifice sample was available for methylamine uptake rates due to volume limitations. There was not a substantial difference between in and out of plume samples. Methylamine assimilation was below detection limit in all samples (Table S5).

Dark bicarbonate fixation

We measured dark autotrophic production in the Guaymas Basin water column by tracking the fixation of ^{14}C -labeled bicarbonate into biomass. At the vent orifice, bicarbonate was fixed at a rate of $1,561.7 \text{ nM d}^{-1}$, which equaled $18.8 \mu\text{g C L}^{-1} \text{ d}^{-1}$ and was at least two orders of magnitude higher than any other depth (Fig S4A). Generally, more bicarbonate was fixed in shallower water, while deep samples (in and out of plume) were less than 3 nM d^{-1} (Fig. S4 and Fig. S7). There was no apparent difference between in-plume and non-plume samples, with the exception of the vent orifice (Table 1).

Hydrocarbon oxidation

We examined potential hydrocarbon metabolisms by tracking oxidation of ^{14}C -labeled hexadecane and naphthalene to $^{14}\text{CO}_2$, and in general, activity was detected most depths in both casts. At the vent orifice, turnover constants were 0.031 ± 0.003 and 0.007 ± 0.001 for hexadecane and naphthalene, respectively. The fastest turnover measured for both rates was hexadecane oxidation at 30 m depth from the in-plume cast, corresponding to a turnover time of ~ 29 d. This was comparable to the turnover time detected for hexadecane oxidation at the vent orifice (31 d). Generally, hexadecane turned over more rapidly than naphthalene, although hexadecane oxidation was below detection in the deepest 15 m at the hydrothermal plume site where naphthalene oxidation was active. There were no discernable differences between sites for either substrate.

Comparisons of sites and correlations

We compared observations made between the in-plume and non-plume control sites to elucidate the influence of hydrothermal fluids on plume biogeochemistry. Several rate measurements including multiple turnover constants (*i.e.*, methanol, acetate, hexadecane, and naphthalene oxidation and DIC fixation), methane oxidation rates, and DIC fixation rates were highest at the vent orifice compared to all other depths measured. Furthermore, a few turnover constants, such as methane and methanol oxidation and acetate assimilation were significantly higher at the in-plume site than the non-plume site (Table 1; p -value < 0.05). This coincided with similarly significant (Table 1; p -value < 0.05) differences that were observed for ammonium, methane, and DOC concentrations between the two sites.

We compared trends in geochemical signatures with metabolic rates and turnover constants to examine potential environmental controls on organic metabolisms in hydrothermally impacted waters. Spearman rank correlation coefficients were used to assess significant relationships (with significance set at a p -value < 0.05) between these parameters. Dissolved methane concentrations were positively correlated with DOC and ammonium concentrations (Fig. 6) while methanol concentrations were positively correlated with phosphate concentrations (data not shown). Methane oxidation rates were positively correlated with dissolved oxygen, ammonium, dissolved inorganic nitrogen (DIN; nitrate + nitrite + ammonium), and DOC concentrations (Fig 6.). Methane oxidation rate constants were correlated with dissolved oxygen and DIN concentrations, depth, and temperature. Methanol oxidation turnover constants were correlated with methanol and nitrate concentrations and the metabolism of multiple carbon substrates (*i.e.*, DIC, acetate, hexadecane, methylamine; Fig. 6). Methanol assimilation turnover constants were correlated with a similar suite of rates and methanol oxidation turnover. Significant correlations exist between a rate measurement and its corresponding substrate concentration and/or rate constant, which is driven by the use of these values in the rate calculation.

DISCUSSION

We studied carbon cycling in a hydrothermal plume in the Guaymas Basin by constraining metabolic rates and community structure in and out of a hydrothermal plume and examining variations to understand drivers of microbial carbon cycling. The data presented here demonstrate distinct differences in carbon metabolism within the plume,

illuminate taxa driving deep-sea carbon cycling, and underscore the importance of heterotrophic carbon metabolism in hydrothermal plumes. This is the first study to directly assess organic carbon metabolism by conducting *ex-situ* rate measurements in hydrothermal plumes.

The hydrothermal plume sampled in this study was near Cathedral Hill (Teske *et al.*, 2016) in the Guaymas Basin and was biogeochemically distinct from surrounding waters less than 200 m to the southeast (Table S1). A comparison of chemistry and metabolic rates demonstrated that ammonium and DOC concentrations, methane and methanol oxidation, and acetate assimilation were significantly higher in the plume, highlighting intense rates of carbon cycling in hydrothermal plumes (Table 1).

Hydrothermal vents deliver a diverse suite of reduced compounds to overlying waters, enriching the deep sea with labile substrates. Observed concentrations of DOC, methane, methanol, and acetate were significantly enriched in the sampled hydrothermal plume waters in the Guaymas Basin. DOC has been determined previously in the Guaymas Basin hydrothermal plumes, and our measurements were generally in agreement with previously reported values (Merewether *et al.*, 1985; Simoneit *et al.*, 1990; Lin *et al.*, 2017). Methane concentrations (Fig. 3C) were more than an order of magnitude higher than previously reported values for the Guaymas Basin (Lam, 2004), suggesting we sampled directly in-plume. In contrast, concentrations of LMW organics, including methanol and acetate, have not been reported in Guaymas Basin waters. Acetate concentrations in the Guaymas Basin plume were enriched relative to background seawater and were

comparable to values observed at the Lost City hydrothermal field and were much higher than surface waters at cold seep sites in the Gulf of Mexico (Foustoukos and Seyfried, 2004; Lang *et al.*, 2010; Zhuang, Montenegro, *et al.*, 2019). Similarly, methanol was also enriched in the Guaymas Basin, up to $\sim 8 \mu\text{M}$ (Fig. 3C), and these data represent the first published measurements of methanol in hydrothermal plumes. Intense heating in Guaymas Basin sediments and associated microbial cycling of organic matter supplies overlying waters with labile carbon (Von Damm *et al.*, 1985; Campbell *et al.*, 1988; Lin *et al.*, 2017; Zhuang, Montgomery, *et al.*, 2019). This carbon could fuel microbial metabolisms in hydrothermal plumes; however, the extent and magnitude of organic matter cycling have remained largely unconstrained.

In the Guaymas Basin water column, uptake of multiple carbon substrates was rapid, showing that labile carbon acts as a carbon and energy sources for the plume microbial community. The highest metabolic rates were generally observed near the vent orifice, where hydrothermal fluids are discharged and decreased up through the water column with the rising plume. The maximum rate constant observed in the plume was methanol oxidation (0.25 d^{-1} at the vent orifice; Table S3) and all other metabolisms, with the exception of methane oxidation, were at least an order of magnitude lower. Interestingly, methane oxidation turnover constants increased up through the water column, with a maximum turnover constant $\sim 300 \text{ m}$ above the seafloor.

Methane oxidation rates and turnover constants were elevated in the hydrothermal plume relative to non-plume control waters in Guaymas Basin (Table 1). The maximum *in*

situ rates and turnover constants in the plume were more than 10,000 times higher and 5 times faster, respectively, than those from the non-plume control site. Methanotrophy at the non-plume site exhibited very little variability with depth, although both measurements were slightly elevated in deeper waters relative to shallower waters (Fig. S6). In contrast, at the in-plume site, methane oxidation rates and turnover constants varied dramatically with depth (Fig S3). At the deepest depths and at the vent orifice, the methane oxidation rate was highest and decreased up through the plume. Meanwhile, the turnover constants were slightly lower in deeper waters and were highest 25 and 300 m above the seafloor in the plume (Fig 4A). These patterns of methanotrophy could be connected to biogeochemical and physical factors that distinguish the rising in the plume from the buoyant plume (Bennett *et al.*, 2013; Reed *et al.*, 2015; Fortunato *et al.*, 2018). In particular, the interplay of chemical inputs (*i.e.*, methane, metals, nitrogen, etc.) and deep-sea biomass will be affected by physical mixing and transport of hydrothermally altered fluids up through the water column. Methanotrophs, and microbial populations in general, that become entrained in the rising plume respond to the intense input of hydrothermal fluid as evidenced by the high rates near the vent orifice. However, the extent of their growth is likely limited in the rising plume because residence times are relatively short (Jiang and Breier, 2014). The doubling time of methanotrophs is expected to be much longer than the residence time in the rising plume, suggesting methanotrophic activity will be higher in the buoyant plume (Reed *et al.*, 2015). This was corroborated by our observation of high turnover constants higher in the plume (Fig 4). Furthermore, methanotrophic biomass likely accumulates in the buoyant plume, thus driving the elevated turnover constants there (Rogener *et al.*, 2018).

Methanotrophic activity observed in the hydrothermal plume in this study was comparable to values for other methane rich environments. For example, in-plume methane turnover constants ranged up to 0.12 d^{-1} and 0.15 d^{-1} in Guaymas Basin (Fig. 4) and Juan de Fuca Ridge (de Angelis *et al.*, 1993) hydrothermal systems, respectively. However, the methane oxidation rates were much higher in the Guaymas Basin (up to $31.5 \mu\text{M d}^{-1}$) than the Juan de Fuca Ridge, with the difference presumably driven by the nearly 700 times higher methane concentrations in the Guaymas Basin. Methane oxidation is a first-order reaction and thus the calculation (see methods) depends directly on the *in-situ* methane concentration. In the aftermath of the Deepwater Horizon disaster, where methane concentrations reached $381 \mu\text{M}$, peak methane turnover constants (up to 0.21 d^{-1}) were comparable to those observed in this study (Crespo-Medina *et al.*, 2014; Rogener *et al.*, 2018). However, methane oxidation rates were still lower ($\sim 6 \mu\text{M d}^{-1}$), which could be due to differences in methane or metal concentrations of the two systems.

Methane turnover constants reflect the abundance of methanotrophic biomass and the inherent capacity of the community to oxidize methane. In contrast to previous studies, methane turnover constants were not significantly correlated with methane concentration in the Guaymas Basin (Scranton and Brewer, 1978; Ward *et al.*, 1987; de Angelis *et al.*, 1993; Valentine *et al.*, 2001). In deep in-plume waters (i.e., deeper than 1690 m) the highest turnover constants were observed at 1690 and 1976 m. At these depths, methane concentrations were still enriched relative to background seawater but were significantly lower than deeper waters. The lack of a linear correlation (Fig. 6) suggests methanotrophs

are initially responding to inputs of metals, inorganic nitrogen (*e.g.*, NH_4^+), or metals near the vent while they become primed for metabolism higher in the plume.

Low molecular weight organics were actively metabolized in hydrothermally impacted waters of Guaymas Basin. At the in-plume site, methanol turnover constants ranged from 0.001 to 0.25 d^{-1} and below detection to 0.03 d^{-1} for oxidation and assimilation, respectively (Fig. 4 and Fig. 5). These values were comparable to values previously reported in the Atlantic Ocean and Gulf of Mexico (Dixon *et al.*, 2011, 2013; Sargeant *et al.*, 2018; Zhuang *et al.*, 2018). Acetate turnover constants were generally comparable to those of methanol in the Guaymas Basin. In the plume, acetate oxidation and assimilation each spanned two orders of magnitude from 0.001 to 0.1 d^{-1} (Fig. 4 and Fig. 5). The acetate oxidation and assimilation turnovers presented here were relatively lower than values reported from the Gulf of Mexico (Zhuang, Montenegro, *et al.*, 2019). However, the measurements from Zhuang *et al.*, were made in surface waters where warmer temperatures and differences in microbial community composition may have also contributed to the observed differences in acetate metabolisms. The highest turnover constants for both substrates were measured at the vent orifice, which is the most chemically enriched sample presented here. Methanol uptake (oxidation plus assimilation) has been shown to be stimulated by nutrient addition (Zhuang *et al.*, 2018), while acetate utilization was not enhanced with nutrient amendment (Zhuang, Montenegro, *et al.*, 2019). Oxidation turnover constants for both compounds were significantly correlated with nitrate concentration, but not with ammonium or phosphate concentration (Fig. 6). This suggests that these metabolisms are not nutrient limited and hydrothermally supplied ammonium is primarily

oxidized as previously observed (Lam *et al.*, 2004; Baker *et al.*, 2012). Additionally, the input of organics from the subsurface and temperature anomalies near the vent orifice likely contribute to maximal turnover constants observed here.

We observed oxidation of two primary oil components, hexadecane and naphthalene, a mid-chain alkane and polycyclic aromatic hydrocarbon (PAH), respectively. These and other hydrocarbons including crude oil are found in the Guaymas Basin (Kawka and Simoneit, 1987; Peter *et al.*, 1991), which likely influences their degradation. The rates of hexadecane and naphthalene oxidation reported here are the first direct measurements of their cycling in a hydrothermal plume. The turnover constants of these compounds were generally comparable to other substrates measured here, despite being largely more recalcitrant. The turnover constants for hexadecane and naphthalene (up to 0.03 ± 0.003 and $0.0087 \pm 0.004 \text{ d}^{-1}$, respectively; Fig. 7 and Table S3) were comparable generally comparable to oil contaminated seawater from the Gulf of Mexico (Kleindienst *et al.*, 2015; Harrison and Joye, 2017). Reports of degradation rates are quite limited, likely due in part to the difficulty of quantifying *in situ* concentrations of hexadecane and naphthalene, however, a few studies have estimated potential oxidation rates of these compounds. For example, potential hexadecane oxidation rates could be up to 30 nmol and 4 nmol $\text{L}^{-1} \text{ d}^{-1}$ at an anthropogenically impacted site in the northern Gulf of Mexico or in the South China Sea, respectively. Potential naphthalene oxidation rates were up to $\sim 2 \text{ nmol L}^{-1} \text{ d}^{-1}$ in both systems (Harrison and Joye, 2017; Peng *et al.*, 2020). Since we did not quantify *in situ* concentrations of hexadecane and naphthalene in this study, we estimated potential oxidation rates based on added tracer concentrations. Based on the

turnover constants quantified here, potential hexadecane and naphthalene oxidation ranged from 4.1 to 38.3 and 1.6 to 8.7 nmol L⁻¹ d⁻¹, respectively. However, these values represent crude estimates, and the solubility of hydrocarbons may vary significantly (Shiu *et al.*, 1990; Marche *et al.*, 2004; Lin *et al.*, 2017), thereby altering oxidation rates. Therefore, *in situ* concentrations are necessary to better quantify oxidation rates of these compounds in Guaymas Basin hydrothermal plumes.

The potential degradation of oil compounds could be important for deep-sea carbon cycling, even in the absence of *in situ* concentrations. For example, deep-sea microbial communities exposed to regular hydrocarbon inputs are expected to readily oxidize oil as has been suggested for Gulf of Mexico cold seeps (Joye *et al.*, 2014). In the Guaymas Basin, the persistent hydrothermal input of hydrocarbons likely primes plume microbial communities to metabolize oil compounds such as hexadecane and naphthalene. Interestingly, the turnover constants of naphthalene and hexadecane were comparable across all depths (with a few exceptions) at both sites, suggesting that microbial communities capable of oil degradation are present throughout the basin. This extensive activity could be a result of more widespread input (i.e., diffuse flow from oil saturated sediments) or preferential metabolism of simpler hydrocarbons or other labile organic matter (Kleindienst *et al.*, 2016; Teske *et al.*, 2016; Hu *et al.*, 2017). In either case, these hydrocarbons could be likely to be distributed beyond the plume where they could be utilized on a basin level scale, as evidenced by increased turnover constants higher in the water column and at the control site (Table S3). Furthermore, the degradation of oil can

produce metabolic intermediates, such as salicylic acid, that can serve as more labile carbon sources by the general microbial community (Liu *et al.*, 2020).

The collection of metabolic rates presented here represents an initial investigation into the direct influence of hydrothermal plumes on deep-sea carbon cycling. Relatively few studies have attempted to constrain plume metabolisms from an elemental cycling perspective and thus the extent to which plume microbial communities mediate organic carbon cycling remains poorly understood. The plume was presumably enriched with typical hydrothermal constituents (*e.g.*, metals) in addition to the observed significant enrichment (Table 2) of DOC (*e.g.*, methanol) and methane (Von Damm *et al.*, 1985). Additionally, the geochemical signature of the plume can influence microbial community composition (Anantharaman *et al.*, 2016; Meier *et al.*, 2017) and transcriptional activity (Lesniewski *et al.*, 2012). Our results expand on this, illustrating that carbon is differentially cycled within the plume. For example, methane oxidation rates were on average ~17,000 times higher in-plume than out of plume and other organic metabolisms were in some cases more than an order of magnitude higher (*e.g.*, methanol oxidation). These differences, with the exception of methane oxidation (which were more drastic), were similar to comparisons of plume and non-plume ammonia and manganese oxidation (Lam *et al.*, 2004, 2008; Dick *et al.*, 2009; Lesniewski *et al.*, 2012). Collectively, these metabolic rates indicate that microbial communities are influenced by hydrothermal inputs and are more active in the plume.

The discharge of hydrothermal fluids from vents directly connects the subsurface with plumes, delivering elevated concentrations of DOC and other biologically relevant elements to deep waters (Lin *et al.*, 2017). This discharge influences biogeochemical cycling and shapes the microbial community of the deep sea (Dick, 2019). The microbial population that responds to these inputs and ultimately becomes entrained in the plume originates from the water column rather than the seafloor (Lesniewski *et al.*, 2012) meaning they respond to hydrothermal inputs after discharge from the vent. The microbial response time must be very quick because the rising velocity of hydrothermal fluids is very rapid causing dilution of the hydrothermal signature (Converse *et al.*, 1984) and residence times in the rising plume may be less than 1 hour (Reed *et al.*, 2015). However, our data are in contrast to previous models, which suggest microbial activity in the plume is insignificant (Jiang and Breier, 2014; Reed *et al.*, 2015). Our results demonstrate the microbial community in the deep-sea responds quickly to the input of DOC, methanol, and acetate from hydrothermal vents (Fig. 4 and Fig. 5). This response feeds deep-sea microbes and drives the high rates observed near the vent orifice and in the rising plume. Taken together, this suggests the microbial community near the vent orifice rapidly responding to the input of organics drive the high rates observed in this study, as has been previously described for deep-sea microbial communities (Sebastián *et al.*, 2019).

The total uptake constants of acetate and methanol in the hydrothermal plume ranged from 0.005 to 0.16 and 0.001 to 0.25 d⁻¹, respectively (Table 2). These values were comparable to values previously reported. For example, methanol uptake in the Gulf of Mexico surface waters ranged from 0.02 to 0.29 d⁻¹ (Zhuang, Montenegro, *et al.*, 2019).

Interestingly, the total methanol uptake at the vent orifice was more similar to a coastal site with high riverine input. Similarly, total acetate uptake rates generally fell within previously reported ranges, but the maximum rates were considerably lower than maximum rates reported at other sites. The maximum total acetate uptake constants in the Gulf of Mexico, Long Island Sound, and Cariaco Basin were 0.34, 0.89, and 1.0 d⁻¹, respectively (Wu *et al.*, 1997; Ho *et al.*, 2002; Zhuang, Montenegro, *et al.*, 2019). This difference could result from the abundant suite of organic molecules available in the Guaymas Basin plume, thereby decreasing dependence on acetate.

In general, methanol and acetate were more readily used as an energy source than carbon source in the Guaymas Basin plume in line with the chemoautotrophic nature of hydrothermal systems. Although, surprisingly, acetate assimilation was higher than oxidation at the vent orifice, revealing a novel importance of heterotrophic metabolisms in very hot hydrothermal fluids. Previous studies have demonstrated that heterotrophs are present and actively oxidize carbon in hydrothermal plumes (Li *et al.*, 2016; Meier *et al.*, 2016; Wang *et al.*, 2020; Ziervogel and Arnosti, 2020). However, this is the first direct measurement of organic carbon assimilation in hydrothermal plume samples. Given the relatively high assimilation activity and known presence of heterotrophic microbes, we sought to constrain the contributions of LMW carbon compounds as carbon and energy sources.

We estimated the importance of acetate and methanol as carbon and energy sources in a hydrothermal plume based on proportional relationships of oxidation and assimilation.

At the in-plume site, methanol and acetate assimilation accounted for on average 42.1% and 35.0%, and a maximum of 84.9% and 59.7% of total substrate uptake, respectively. At the non-plume site, acetate was hardly assimilated, but the observed portion of acetate assimilation is comparable to previously reported values (Zhuang, Montenegro, *et al.*, 2019). In contrast, methanol was assimilated significantly more in the plume relative to the Gulf of Mexico where it was predominantly used as an energy source (Zhuang *et al.*, 2018). Interestingly, the ratios of oxidation to assimilation were distinct with respect to substrate and depth. The maximum percentage of assimilated carbon from acetate (60%) was observed at the vent orifice, while that of methanol occurred 25 m above the seafloor (Table 2). Differential metabolisms of LMW organics across multiple depths in the hydrothermal plume is evidence of the diverse nature of these systems (Perner *et al.*, 2013). The rapid uptake of acetate and methanol in the rising plume in combination with the associated microbial community indicates a novel importance of hydrothermal systems in deep-sea carbon cycling. This dataset begins to fill a long-standing gap in our understanding of carbon cycling from a geochemical perspective in hydrothermal plumes.

We conducted an interdisciplinary biogeochemical study in the Guaymas Basin to explore microbial carbon cycling in a hydrothermal plume and implicate the microbial community responsible. Elevated concentrations of organic carbon including methane (up to 380 μM), methanol, (up to 8 μM), and acetate were detected in the plume. Hydrothermal vents supply the deep sea with these and other energy rich substrates that act as a microbial energy source as evidenced by the high oxidation rates observed here, underscoring its importance in hydrothermal plumes. Chemoautrophic rates of DIC fixation were elevated

in the plume as is characteristic of hydrothermal vent ecosystems. The organic carbon sources studied here were generally used as energy sources, however, assimilatory activity accounted for a significant portion of uptake (up to 85%). This dataset represents the first direct documentation of heterotrophic metabolisms in a hydrothermal plume. Taken together, our data suggest hydrothermal vents have a profound impact on local carbon cycling within plumes and likely contribute significantly to oceanic biogeochemical cycling.

REFERENCES

- Albert, D.B. and Martens, C.S. (1997) Determination of low-molecular-weight organic acid concentrations in seawater and pore-water samples via HPLC. *Mar. Chem.* **56**: 27–37.
- Amend, J.P., McCollom, T.M., Hentscher, M., and Bach, W. (2011) Catabolic and anabolic energy for chemolithoautotrophs in deep-sea hydrothermal systems hosted in different rock types. *Geochim. Cosmochim. Acta* **75**: 5736–5748.
- Anantharaman, K., Breier, J.A., and Dick, G.J. (2016) Metagenomic resolution of microbial functions in deep-sea hydrothermal plumes across the Eastern Lau Spreading Center. *ISME J.* **10**: 225–239.
- Anantharaman, K., Breier, J.A., Sheik, C.S., and Dick, G.J. (2013) Evidence for hydrogen oxidation and metabolic plasticity in widespread deep-sea sulfur-oxidizing bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 330–335.

- Anantharaman, K., Duhaime, M.B., Breier, J.A., Wendt, K.A., Toner, B.M., and Dick, G.J. (2014) Sulphur Oxidation Genes in Diverse Deep-Sea Viruses. *Science* (80-.). **344**: 757–760.
- de Angelis, M.A., Lilley, M.D., and Baross, J.A. (1993) Methane oxidation in deep-sea hydrothermal plumes of the endeavour segment of the Juan de Fuca Ridge. *Deep. Res. Part I* **40**: 1169–1186.
- Baker, B.J., Lesniewski, R.A., and Dick, G.J. (2012) Genome-enabled transcriptomics reveals archaeal populations that drive nitrification in a deep-sea hydrothermal plume. *ISME J.* **6**: 2269–2279.
- Beman, J.M., Popp, B.N., and Alford, S.E. (2012) Quantification of ammonia oxidation rates and ammonia-oxidizing archaea and bacteria at high resolution in the Gulf of California and eastern tropical North Pacific Ocean. *Limnol. Oceanogr.* **57**: 711–726.
- Bennett, S.A., Coleman, M., Huber, J.A., Reddington, E., Kinsey, J.C., McIntyre, C., et al. (2013) Trophic regions of a hydrothermal plume dispersing away from an ultramafic-hosted vent-system: Von Damm vent-site, Mid-Cayman Rise. *Geochemistry, Geophys. Geosystems* **14**: 317–327.
- Berg, R.D., Solomon, E.A., and Teng, F.-Z. (2019) The role of marine sediment diagenesis in the modern oceanic magnesium cycle. *Nat. Commun.* **10**.
- Campbell, A.C., Bowers, T.S., Measures, C.I., Falkner, K.K., Khadem, M., and Edmond, J.M. (1988) A time series of vent fluid compositions from 21°N, East Pacific Rise (1979, 1981, 1985), and the Guaymas Basin, Gulf of California (1982, 1985). *J. Geophys. Res.* **93**: 4537–4549.

- Childress, J.J. and Girguis, P.R. (2011) The metabolic demands of endosymbiotic chemoautotrophic metabolism on host physiological capacities. *J. Exp. Biol.* **214**: 312–325.
- Converse, D.R., Holland, H.D., and Edmond, J.M. (1984) Flow rates in the axial hot springs of the East Pacific Rise (21°N): implications for the heat budget and the formation of massive sulfide deposits. *Earth Planet. Sci. Lett.* **69**: 159–175.
- Cowen, J.P., Wen, X., and Popp, B.N. (2002) Methane in aging hydrothermal plumes. *Geochim. Cosmochim. Acta* **66**: 3563–3571.
- Crespo-Medina, M., Meile, C.D., Hunter, K.S., Diercks, A.R., Asper, V.L., Orphan, V.J., et al. (2014) The rise and fall of methanotrophy following a deepwater oil-well blowout. *Nat. Geosci.* **7**: 423–427.
- Von Damm, K.L., Edmond, J.M., Measures, C.I., and Grant, B. (1985) Chemistry of submarine hydrothermal solutions at Guaymas Basin, Gulf of California. *Geochim. Cosmochim. Acta* **49**: 2221–2237.
- Dick, G.J. (2019) The microbiomes of deep-sea hydrothermal vents: distributed globally, shaped locally. *Nat. Rev. Microbiol.* **17**: 271–283.
- Dick, G.J., Anantharaman, K., Baker, B.J., Li, M., Reed, D.C., and Sheik, C.S. (2013) The microbiology of deep-sea hydrothermal vent plumes: Ecological and biogeographic linkages to seafloor and water column habitats. *Front. Microbiol.* **4**: 1–16.
- Dick, G.J., Clement, B.G., Webb, S.M., Fodrie, F.J., Bargar, J.R., and Tebo, B.M. (2009) Enzymatic microbial Mn(II) oxidation and Mn biooxide production in the Guaymas Basin deep-sea hydrothermal plume. *Geochim. Cosmochim. Acta* **73**: 6517–6530.

- Dixon, J.L., Beale, R., and Nightingale, P.D. (2011) Rapid biological oxidation of methanol in the tropical Atlantic: Significance as a microbial carbon source. *Biogeosciences* **8**: 2707–2716.
- Dixon, J.L., Sargeant, S., Nightingale, P.D., and Colin Murrell, J. (2013) Gradients in microbial methanol uptake: Productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean. *ISME J.* **7**: 568–580.
- Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: The art of harnessing chemosynthesis. *Nat. Rev. Microbiol.* **6**: 725–740.
- Fortunato, C.S. and Huber, J.A. (2016) Coupled RNA-SIP and metatranscriptomics of active chemolithoautotrophic communities at a deep-sea hydrothermal vent. *ISME J.* **10**: 1925–1938.
- Fortunato, C.S., Larson, B., Butterfield, D.A., and Huber, J.A. (2018) Spatially distinct, temporally stable microbial populations mediate biogeochemical cycling at and below the seafloor in hydrothermal vent fluids. *Environ. Microbiol.* **20**: 769–784.
- Foustoukos, D.I. and Seyfried, W.E. (2004) Hydrocarbons in Hydrothermal Vent Fluids : The Role of Chromium-Bearing Catalysts. *Science (80-.).* **304**: 1002–1005.
- Galand, P.E., Casamayor, E.O., Pedro, C., Alonso-sa, L., Bertilsson, S., and Blanes, D. (2010) High bicarbonate assimilation in the dark by Arctic bacteria. *ISME J.* **4**: 1581–1590.
- Hansell, D.A. (1993) Results and observations from the measurement of DOC and DON in seawater using a high-temperature catalytic oxidation technique. *Mar. Chem.* **41**: 195–202.

- Harrison, S. and Joye, S.B. (2017) Lessons from the Taylor Energy oil spill: History , seasonality, and nutrient limitation.
- Ho, T.Y., Scranton, M.I., Taylor, G.T., Varela, R., Thunell, R.C., and Muller-Karger, F. (2002) Acetate cycling in the water column of the Cariaco Basin: Seasonal and vertical variability and implication for carbon cycling. *Limnol. Oceanogr.* **47**: 1119–1128.
- Hu, P., Dubinsky, E.A., Probst, A.J., Wang, J., Sieber, C.M.K., Tom, L.M., et al. (2017) Simulation of Deepwater Horizon oil plume reveals substrate specialization within a complex community of hydrocarbon degraders. *Proc. Natl. Acad. Sci. U. S. A.* **114**: 7432–7437.
- Jiang, H. and Breier, J.A. (2014) Physical controls on mixing and transport within rising submarine hydrothermal plumes: A numerical simulation study. *Deep. Res. Part I Oceanogr. Res. Pap.* **92**: 41–55.
- Joye, S.B., Boetius, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J., and Lugo, S.K. (2004) The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chem. Geol.* **205**: 219–238.
- Joye, S.B., MacDonald, I.R., Leifer, I., and Asper, V. (2011) Magnitude and oxidation potential of hydrocarbon gases released from the BP oil well blowout. *Nat. Geosci.* **4**: 160–164.
- Joye, S.B., Teske, A.P., and Kostka, J.E. (2014) Microbial dynamics following the macondo oil well blowout across gulf of Mexico environments. *Bioscience* **64**: 766–777.

- Kadko, D.C., Rosenberg, N.D., Lupton, J.E., Collier, R.W., and Lilley, M.D. (1990) Chemical reaction rates and entrainment within the Endeavour Ridge hydrothermal plume. *Earth Planet. Sci. Lett.* **99**: 315–335.
- Karl, D.M., Wirsén, C.O., and Jannasch, H.W. (1980) Deep-Sea Primary Production at the Galápagos Hydrothermal Vents. *Science* (80-.). **207**: 1345–1347.
- Kawka, O.E. and Simoneit, B.R.T. (1987) Survey of hydrothermally-generated petroleums from the Guaymas Basin spreading center. *Org. Geochem.* **11**: 311–328.
- Kleindienst, S., Grim, S., Sogin, M., Bracco, A., Crespo-Medina, M., and Joye, S.B. (2016) Diverse, rare microbial taxa responded to the Deepwater Horizon deep-sea hydrocarbon plume. *ISME J.* **10**: 400–415.
- Kleindienst, S., Seidel, M., Ziervogel, K., Grim, S., Loftis, K., Harrison, S., et al. (2015) Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *Proc. Natl. Acad. Sci.* **112**: 14900–14905.
- Lam, P. (2004) Microbial ammonia oxidation in deep-sea hydrothermal plumes.
- Lam, P., Cowen, J.P., and Jones, R.D. (2004) Autotrophic ammonia oxidation in a deep-sea hydrothermal plume. *FEMS Microbiol. Ecol.* **47**: 191–206.
- Lam, P., Cowen, J.P., Popp, B.N., and Jones, R.D. (2008) Microbial ammonia oxidation and enhanced nitrogen cycling in the Endeavour hydrothermal plume. *Geochim. Cosmochim. Acta* **72**: 2268–2286.
- Lang, S.Q., Butterfield, D.A., Schulte, M., Kelley, D.S., and Lilley, M.D. (2010) Elevated concentrations of formate, acetate, and dissolved organic carbon found at the Lost City hydrothermal field. *Geochim. Cosmochim. Acta* **74**: 941–952.

- Lesniewski, R.A., Jain, S., Anantharaman, K., Schloss, P.D., and Dick, G.J. (2012) The metatranscriptome of a deep-sea hydrothermal plume is dominated by water column methanotrophs and lithotrophs. *ISME J.* **6**: 2257–2268.
- Li, M., Baker, B.J., Anantharaman, K., Jain, S., Breier, J.A., and Dick, G.J. (2015) Genomic and transcriptomic evidence for scavenging of diverse organic compounds by widespread deep-sea archaea. *Nat. Commun.* **6**:.
- Li, M., Jain, S., Baker, B.J., Taylor, C., and Dick, G.J. (2014) Novel hydrocarbon monooxygenase genes in the metatranscriptome of a natural deep-sea hydrocarbon plume. *Environ. Microbiol.* **16**: 60–71.
- Li, M., Jain, S., and Dick, G.J. (2016) Genomic and transcriptomic resolution of organic matter utilization among deep-sea bacteria in Guaymas basin hydrothermal plumes. *Front. Microbiol.* **7**: 1–13.
- Li, M., Toner, B.M., Baker, B.J., Breier, J.A., Sheik, C.S., and Dick, G.J. (2014) Microbial iron uptake as a mechanism for dispersing iron from deep-sea hydrothermal vents. *Nat. Commun.* **5**: 1–8.
- Lin, Y.S., Koch, B.P., Feseker, T., Ziervogel, K., Goldhammer, T., Schmidt, F., et al. (2017) Near-surface heating of young rift sediment causes mass production and discharge of reactive dissolved organic matter. *Sci. Rep.* **7**: 1–10.
- Liu, Y., White, H.K., Simister, R.L., Waite, D., Lyons, S.L., and Kujawinski, E.B. (2020) Probing the Chemical Transformation of Seawater-Soluble Crude Oil Components during Microbial Oxidation. *ACS Earth Sp. Chem.* **4**: 690–701.

- Magen, C., Lapham, L.L., Pohlman, J.W., Marshall, K., Bosman, S., Casso, M., and Chanton, J.P. (2014) A simple headspace equilibration method for measuring dissolved methane. *Limnol. Oceanogr. Methods* **12**: 637–650.
- Marche, C., Ferronato, C., and Jose, J. (2004) Solubilities of alkylcyclohexanes in water from 30 °C to 180 °C. *J. Chem. Eng. Data* **49**: 937–940.
- Markager, S. (1998) Dark uptake of inorganic ¹⁴C in oligotrophic oceanic waters. *J. Plankton Res.* **20**: 1813–1836.
- Martens, C.S. (1990) Generation of short chain acid anions in hydrothermally altered sediments of the Guaymas Basin, Gulf of California. *Appl. Geochemistry* **5**: 71–76.
- Meier, D. V., Bach, W., Girguis, P.R., Gruber-Vodicka, H.R., Reeves, E.P., Richter, M., et al. (2016) Heterotrophic Proteobacteria in the vicinity of diffuse hydrothermal venting. *Environ. Microbiol.* **18**: 4348–4368.
- Meier, D. V., Pjevac, P., Bach, W., Hourdez, S., Girguis, P.R., Vidoudez, C., et al. (2017) Niche partitioning of diverse sulfur-oxidizing bacteria at hydrothermal vents. *ISME J.* **11**: 1545–1558.
- Merewether, R., Olsson, M.S., and Lonsdale, P. (1985) Acoustically detected hydrocarbon plumes rising from 2-km depths in Guaymas Basin, Gulf of California. *J. Geophys. Res.* **90**: 3075.
- Peng, C., Tang, Y., Yang, H., He, Y., Liu, Y., Liu, D., et al. (2020) Time- and compound-dependent microbial community compositions and oil hydrocarbon degrading activities in seawater near the Chinese Zhoushan Archipelago. *Mar. Pollut. Bull.* **152**..

- Perner, M., Gonnella, G., Hourdez, S., Böhnke, S., Kurtz, S., and Girguis, P. (2013) In situ chemistry and microbial community compositions in five deep-sea hydrothermal fluid samples from Irina II in the Logatchev field. *Environ. Microbiol.* **15**: 1551–1560.
- Peter, J.M., Peltonen, P., Scott, S.D., Simoneit, B.R.T., and Kawka, O.E. (1991) 14 C ages of hydrothermal petroleum and carbonate in Guaymas Basin, Gulf of California: implications for oil generation, expulsion, and migration. *Geology* **19**: 253–256.
- Petersen, J.M., Zielinski, F.U., Pape, T., Seifert, R., Moraru, C., Amann, R., et al. (2011) Hydrogen is an energy source for hydrothermal vent symbioses. *Nature* **476**: 176–180.
- Prakash, A., Sheldon, R.W., and Sutcliffe, W.H. (1991) Geographic variation of oceanic 14C dark uptake. *Limnol. Oceanogr.* **36**: 30–39.
- Reed, D.C., Breier, J.A., Jiang, H., Anantharaman, K., Klausmeier, C.A., Toner, B.M., et al. (2015) Predicting the response of the deep-ocean microbiome to geochemical perturbations by hydrothermal vents. *ISME J.* **9**: 1857–1869.
- Reysenbach, A.-L., Banta, A.B., Boone, D.R., Cary, S.C., and Luther, G.W. (2000) Microbial essentials at hydrothermal vents. *Nature* **404**:
- Reysenbach, A.L. and Shock, E. (2002) Merging genomes with geochemistry in hydrothermal ecosystems. *Science (80-.)*. **296**: 1077–1082.
- Rogener, M.K., Bracco, A., Hunter, K.S., Saxton, M.A., and Joye, S.B. (2018) Long-term impact of the Deepwater Horizon oil well blowout on methane oxidation dynamics in the northern Gulf of Mexico. *Elementa* **6**: 1–17.

- Rogener, M.K., Sipler, R.E., Hunter, K.S., Bronk, D.A., and Joye, S.B. (2019) Pelagic methane oxidation in the northern Chukchi Sea. *Limnol. Oceanogr.* 1–15.
- Sargeant, S.L., Murrell, J.C., Nightingale, P.D., and Dixon, J.L. (2018) Basin-scale variability of microbial methanol uptake in the Atlantic Ocean. 1–28.
- Scranton, M.I. and Brewer, P.G. (1978) Consumption in the deep ocean1. *Sci. York* **23**: 1207–1213.
- Sebastián, M., Estrany, M., Ruiz-González, C., Forn, I., Montserrat Sala, M., Gasol, J.M., and Marrasé, C. (2019) High growth potential of long-term starved deep ocean opportunistic heterotrophic bacteria. *Front. Microbiol.* **10**: 1–12.
- Sharp, J.H., Carlson, C.A., Peltzer, E.T., Castle-Ward, D.M., Savidge, K.B., and Rinker, K.R. (2002) Final dissolved organic carbon broad community intercalibration and preliminary use of DOC reference materials. *Mar. Chem.* **77**: 239–253.
- Shiu, W.Y., Bobra, M., Bobra, A.M., Maijanen, A., Suntio, L., and Mackay, D. (1990) The water solubility of crude oils and petroleum products. *Oil Chem. Pollut.* **7**: 57–84.
- Sibert, R., Harrison, S., and Joye, S. (2016) Protocols for Radiotracer Estimation of Primary Hydrocarbon Oxidation in Oxygenated Seawater.
- Simoneit, B.R.T., Lonsdale, P.F., Edmond, J.M., and Shanks, W.C. (1990) Deep-water hydrocarbon seeps in Guaymas Basin, Gulf of California. *Appl. Geochemistry* **5**: 41–49.
- Teske, A., De Beer, D., McKay, L.J., Tivey, M.K., Biddle, J.F., Hoer, D., et al. (2016) The Guaymas Basin hiking guide to hydrothermal mounds, chimneys, and microbial

- mats: Complex seafloor expressions of subsurface hydrothermal circulation. *Front. Microbiol.* **7**:.
- Valentine, D.L., Blanton, D.C., Reeburgh, W.S., and Kastner, M. (2001) Water column methane oxidation adjacent to an area of active hydrate dissociation, Eel River Basin. *Geochim. Cosmochim. Acta* **65**: 2633–2640.
- Wang, W., Li, Z., Zeng, L., Dong, C., and Shao, Z. (2020) The oxidation of hydrocarbons by diverse heterotrophic and mixotrophic bacteria that inhabit deep-sea hydrothermal ecosystems. *ISME J.* 1–13.
- Ward, B.B., Kilpatrick, K.A., Novelli, P.C., and Scranton, M.I. (1987) Methane oxidation and methane fluxes in the ocean surface layer and deep anoxic waters. *Lett. to Nat.*
- Welhan, J.A. and Lupton, J.E. (1987) Light hydrocarbon gases in Guaymas Basin hydrothermal fluids: thermogenic versus abiogenic origin. *Am. Assoc. Pet. Geol. Bull.* **71**: 215–223.
- Wirsén, C.O., Jannasch, H.W., and Molyneux, S.J. (1993) Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. *J. Geophys. Res.* **98**: 9693–9703.
- Wu, H., Green, M., and Scranton, M.I. (1997) Acetate cycling in the water column and surface sediment of Long Island Sound following a bloom. *Limnol. Oceanogr.* **42**: 705–713.
- Zhou, Z., Liu, Y., Pan, J., Cron, B.R., Toner, B.M., Anantharaman, K., et al. (2020) Gammaproteobacteria mediating utilization of methyl-, sulfur- and petroleum organic compounds in deep ocean hydrothermal plumes. *ISME J.*

- Zhuang, G.-C., Montenegro, T.D.P., Montgomery, A., Montoya, J., and Joye, S.B. (2019) Significance of Acetate as a Microbial Carbon and Energy Source in the Water Column of Gulf of Mexico : Implications for Marine Carbon Cycling Global Biogeochemical Cycles. *Global Biogeochem. Cycles* 223–235.
- Zhuang, G.-C., Montgomery, A., Samarkin, V.A., Song, M., Liu, J., Schubotz, F., et al. (2019) Generation and Utilization of Volatile Fatty Acids and Alcohols in Hydrothermally Altered Sediments in the Guaymas Basin, Gulf of California. *Geophys. Res. Lett.* **46**: 2637–2646.
- Zhuang, G., Lin, Y., Elvert, M., Heuer, V.B., and Hinrichs, K. (2014) Gas chromatographic analysis of methanol and ethanol in marine sediment pore waters : Validation and implementation of three pretreatment techniques. *Mar. Chem.* **160**: 82–90.
- Zhuang, G., Peña-montenegro, T.D., Montgomery, A., Hunter, K.S., and Joye, S.B. (2018) Microbial metabolism of methanol and methylamine in the Gulf of Mexico : insight into marine carbon and nitrogen cycling. *Environ. Microbiol.* **20**: 4543–4554.
- Ziervogel, K. and Arnosti, C. (2020) Substantial Carbohydrate Hydrolase Activities in the Water Column of the Guaymas Basin (Gulf of California). *Front. Mar. Sci.* **6**: 1–8.

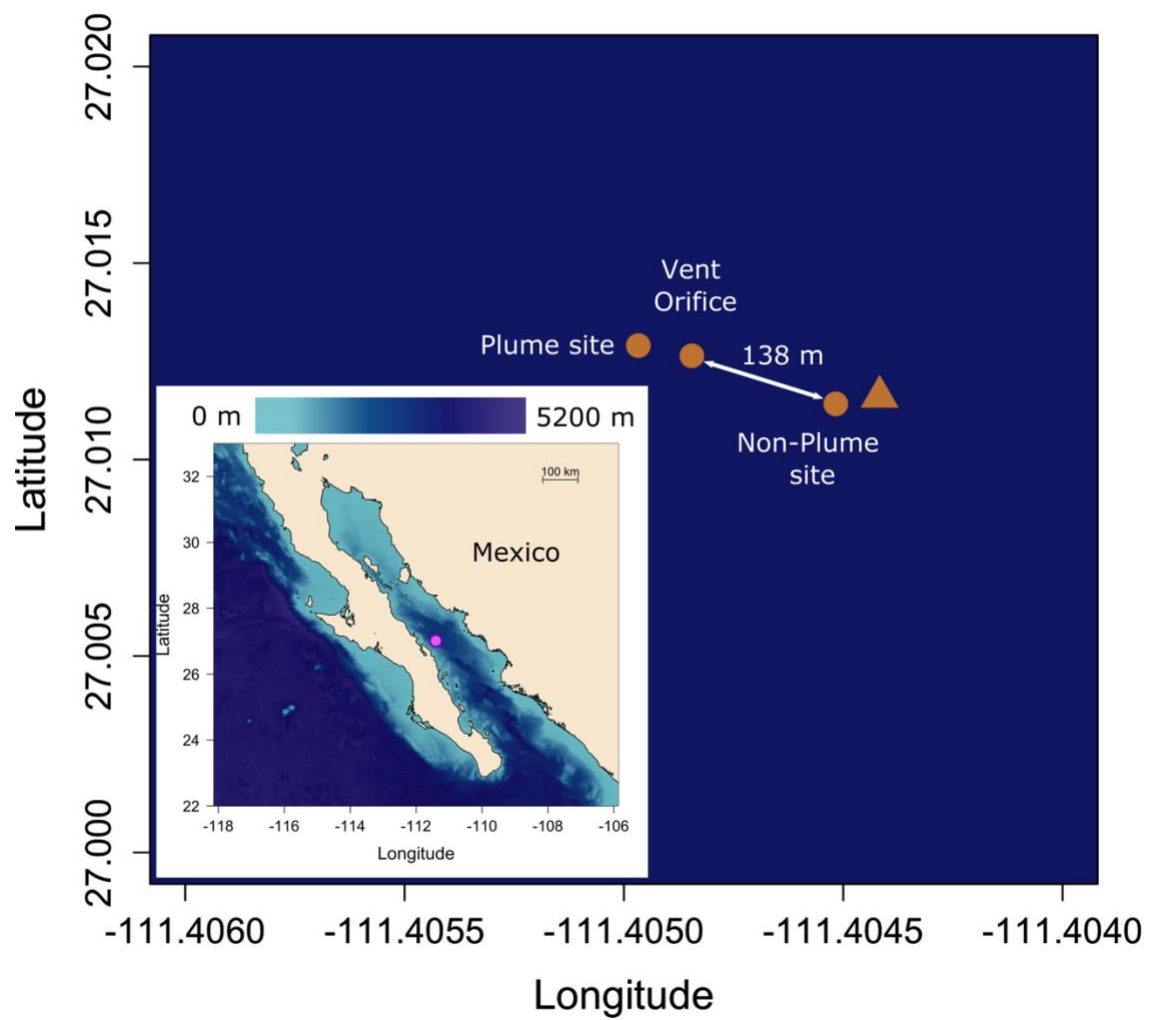


Figure 3.1. Site map of sample collection sites in the Guaymas Basin. Orange circles represent sample collection sites and the triangle is the Cathedral Hill site from Teske *et al.*, 2016. The inset shows a regional map of the Gulf of California and the Eastern Tropical North Pacific.

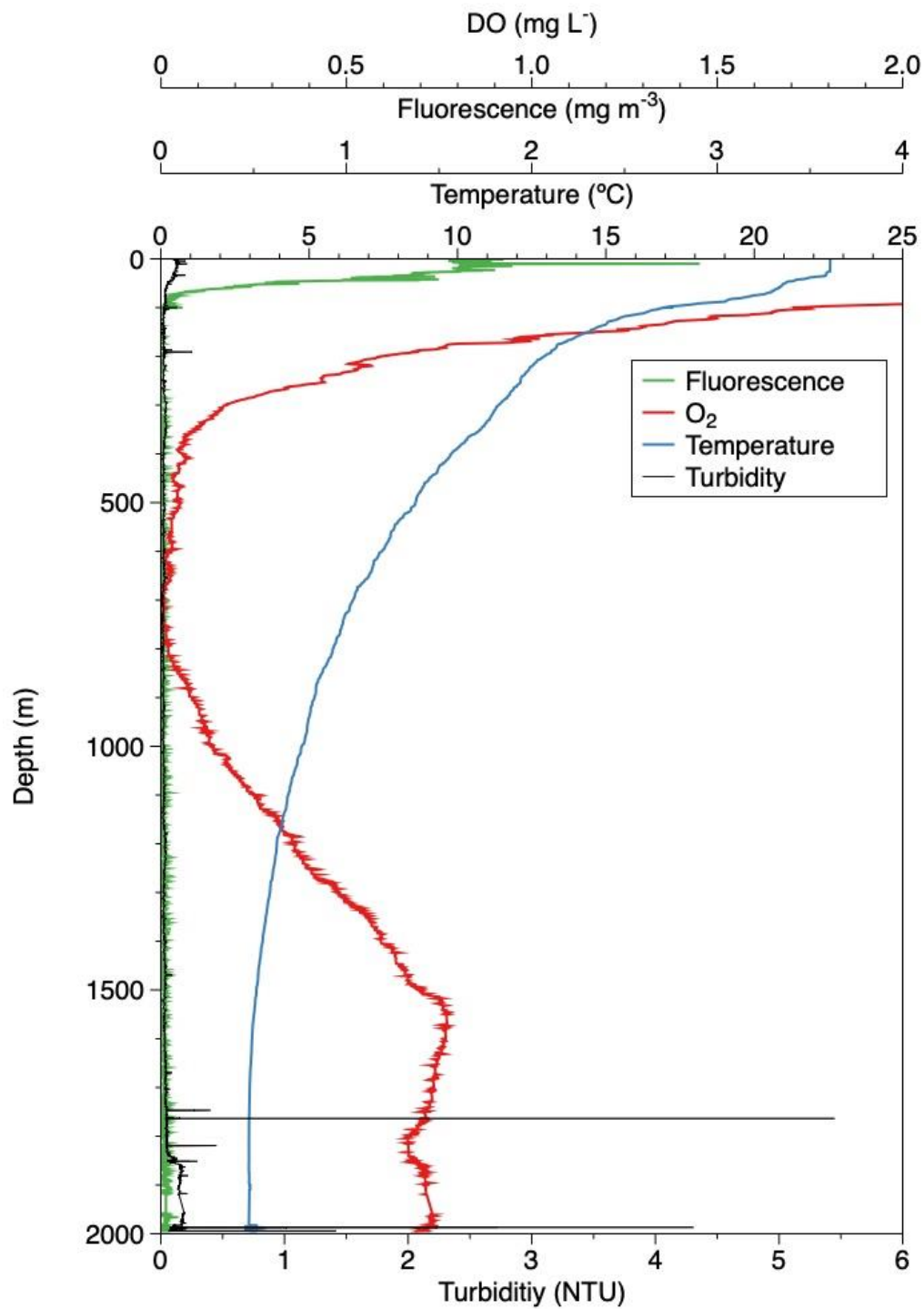


Figure 3.2. Hydrographic profiles of temperature (blue), turbidity (black), oxygen (red), and fluorescence (green) from the in-plume site in the Guaymas Basin.

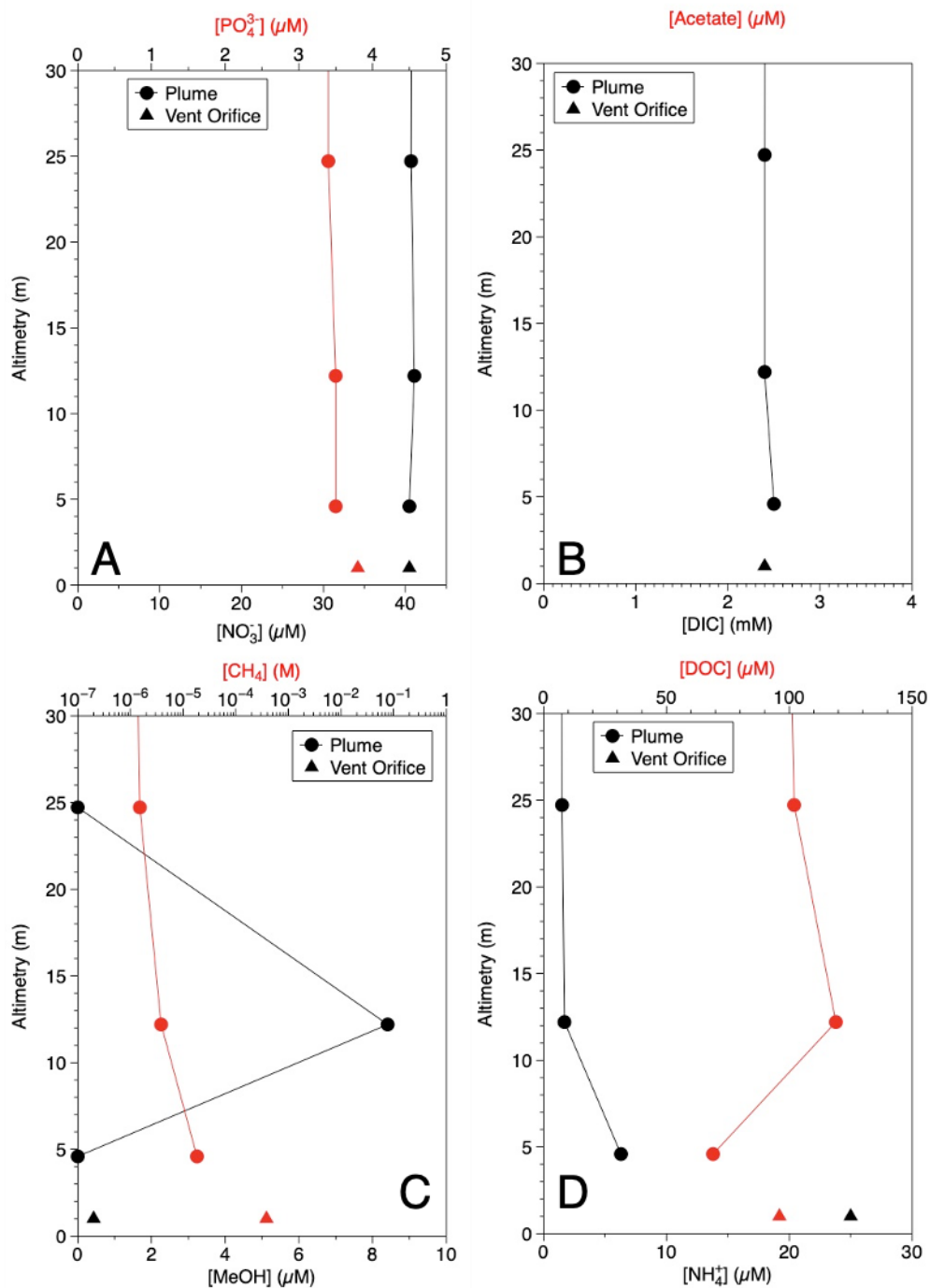


Figure 3.3. In-plume depth profiles of select geochemical parameters: (A) phosphate and nitrate, (B) acetate and DIC, (C) methane and methanol, and (D) DOC and ammonium. Circles denote samples collected from the CTD and triangles were samples collected at the vent orifice.

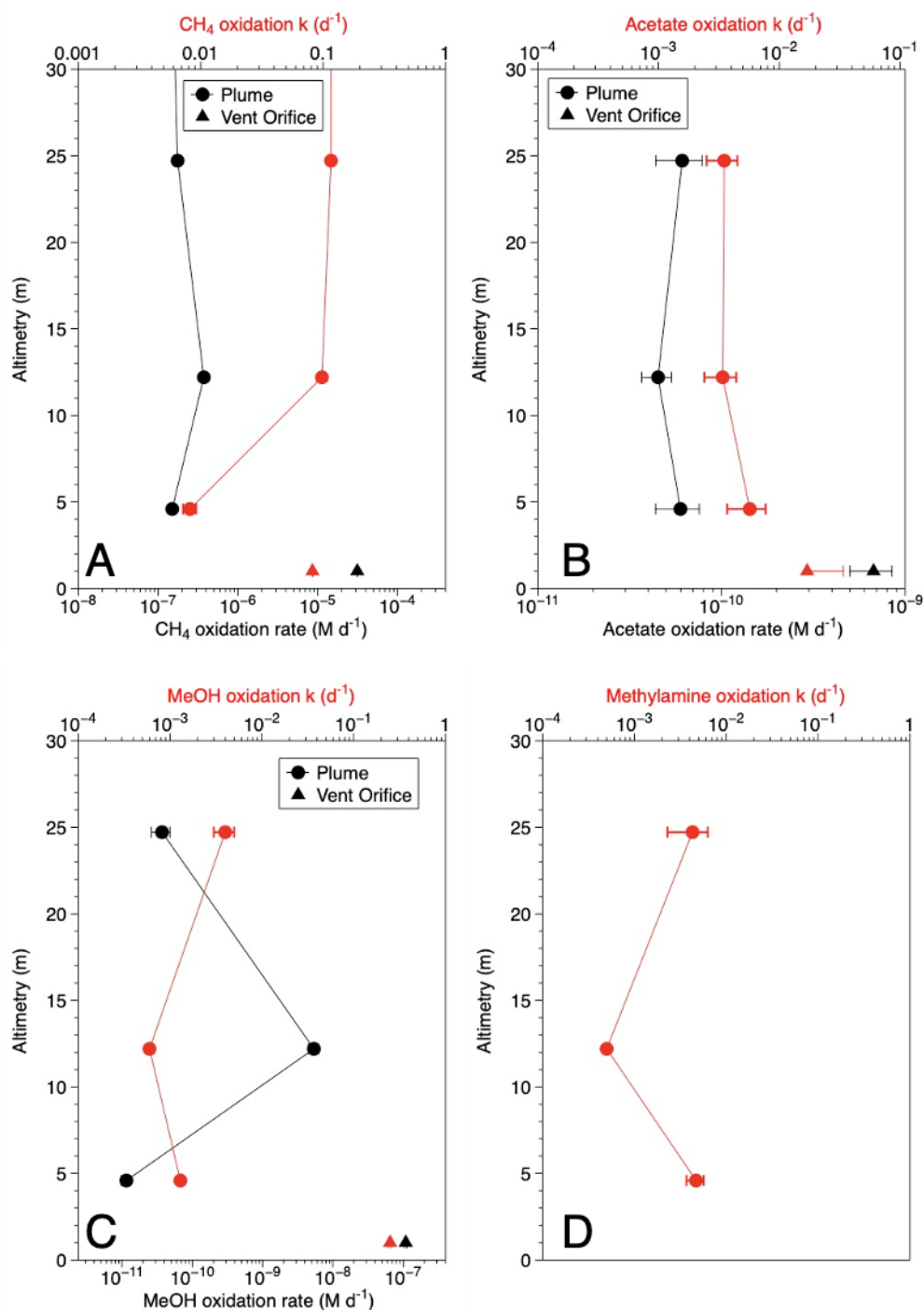


Figure 3.4. In plume depth profiles of oxidation rates (black) and turnover constants (red) of (A) methane, (B) acetate, (C) methanol, (D) methylamine. Rates could not be calculated for methylamine because *in situ* concentrations were not measured. Circles denote samples collected from the CTD and triangles were samples collected at the vent orifice. Error bars represent standard error, some of which are not visible due to small error. Note differences in scales.

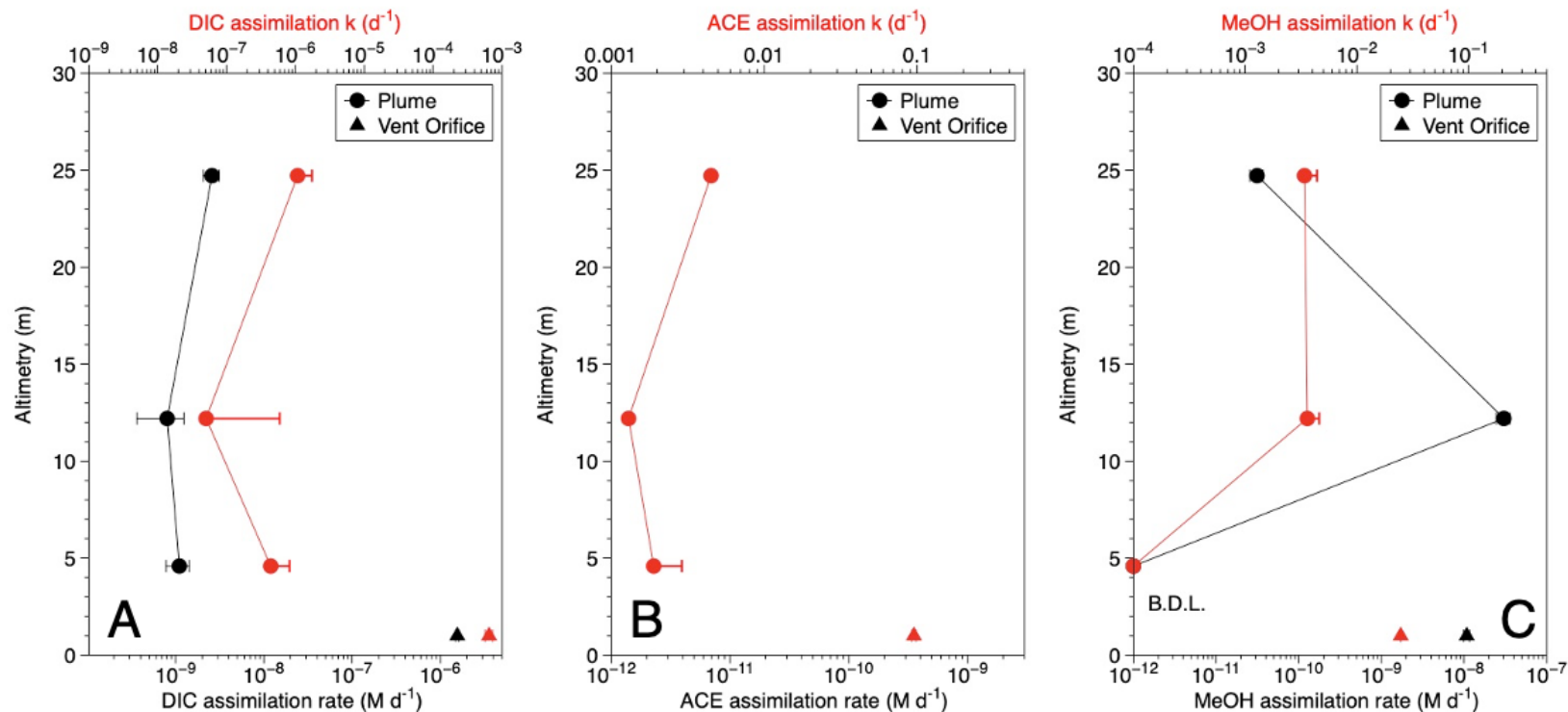


Figure 3.5. In-plume depth profiles of assimilation rates (black) and turnover constants (red) of (A) DIC, (B) acetate, (C) methanol. Circles denote samples collected from the CTD and triangles were samples collected at the vent orifice. Error bars represent standard error, some of which are not visible due to small error. Note differences in scales. B.D.L. = Below detection limit.

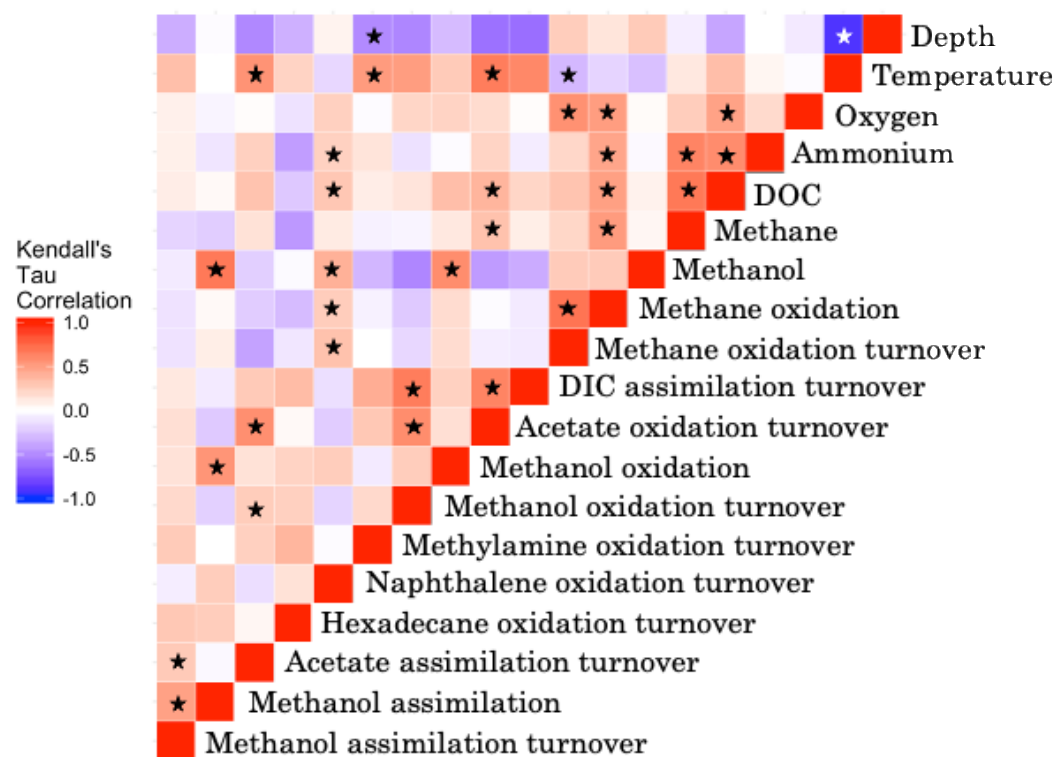


Figure 3.6. Spearman's Rank Correlation of relationships between biogeochemical parameters and rate measurements. Stars represent relationships that were statistically significant (p -value < 0.05). Turnover refers to turnover constants.

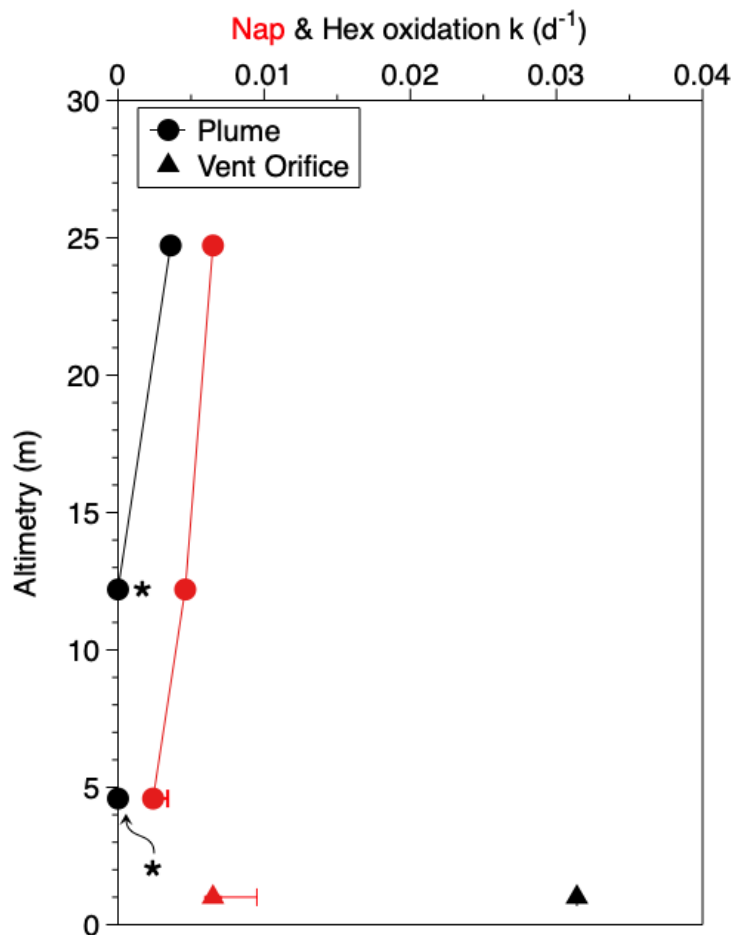


Figure 3.7. In-plume depth profiles of hydrocarbon oxidation turnover constants for hexadecane (black) and naphthalene (red). Circles denote samples collected from the CTD and triangles were samples collected at the vent orifice. Error bars represent standard error, some of which are not visible due to small error. * = Below detection limit.

Table 3.1. Tukey-Kramer HSD mean comparison between sites

	Plume and Vent Orifice	Plume only
Ammonium	0.018	0.027
Dissolved organic carbon (DOC)	0.016	0.029
Methane	0.016	0.029
Methanol	N.D.	N.D.
Methane oxidation rate	<0.0001	<0.0001
Methane oxidation turnover constant	0.013	0.039
Hexadecane oxidation turnover constant	N.D.	N.D.
Naphthalene oxidation turnover constant	N.D.	N.D.
Acetate oxidation turnover constant	0.088*	N.D.
MeOH oxidation rate	N.D.	N.D.
MeOH oxidation turnover constant	N.D.	0.012
Methylamine oxidation turnover constant	N.D.	N.D.
DIC assimilation rate	N.D.	N.D.
DIC assimilation turnover constant	N.D.	N.D.
Acetate assimilation turnover constant	0.009	0.018
MeOH assimilation rate	N.D.	N.D.
MeOH assimilation turnover constant	N.D.	N.D.

Note: mean comparisons were considered significant if p -values were < 0.05.

* = p -value < 0.1, N.D. = No difference between means.

Table 3.2. Relative contributions of oxidation and assimilation to acetate and methanol uptake.

Site	Depth (m)	Altimetry (m)	Acetate oxidation (d ⁻¹)	Acetate assimilation (d ⁻¹)	Acetate uptake (d ⁻¹)	Fraction oxidation (%)	Fraction assimilation (%)	Methanol oxidation (d ⁻¹)	Methanol assimilation (d ⁻¹)	Methanol uptake (d ⁻¹)	Fraction oxidation (%)	Fraction assimilation (%)
NP	30	1982.79	0.0520	0.0140	0.0661	78.77	21.23	0.0917	0.0086	0.1002	91.46	8.54
NP	300	1712.79	0.0380	0.0004	0.0384	98.97	1.03	0.0049	0.0013	0.0062	78.95	21.05
NP	600	1412.79	0.0036	0.0003	0.0039	92.71	7.29	0.0013	0.0030	0.0043	29.18	70.82
NP	710	1302.79	0.0021	0.0000	0.0022	98.11	1.89	0.0024	0.0128	0.0152	15.91	84.09
NP	810	1202.79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1030	982.79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1690	322.79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1998	14.79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	2003	9.79	0.0019	0.0001	0.0020	96.23	3.77	0.0037	0.0020	0.0058	64.62	35.38
NP	2009	3.79	0.0023	0.0000	0.0023	100.00	0.00	0.0042	0.0022	0.0064	65.62	34.38
IP	30	1968.59	0.0418	0.0061	0.0479	87.31	12.69	0.0592	0.0078	0.0670	88.30	11.70
IP	250	1750.72	0.0354	0.0361	0.0714	49.51	50.49	0.0095	0.0026	0.0121	78.76	21.24
IP	700	1299.2	0.0116	0.0106	0.0222	52.11	47.89	0.0048	0.0159	0.0207	23.16	76.84
IP	1690	308.59	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
IP	1976	24.72	0.0035	0.0045	0.0080	44.22	55.78	0.0040	0.0034	0.0075	54.12	45.88
IP	1987	12.2	0.0034	0.0013	0.0047	72.48	27.52	0.0006	0.0036	0.0042	15.03	84.97
IP	1994	4.59	0.0057	0.0019	0.0076	74.56	25.44	0.0013	0.0000	0.0013	100.00	0.00
VO	2000	1	0.0642	0.0951	0.1593	40.28	59.72	0.2498	0.0247	0.2745	90.99	9.01

Note: Uptake is the sum of oxidation and assimilation. Percent contributions were calculated as fraction of total uptake (assimilation or oxidation turnover constant/uptake *100. NP = non-plume cast, IP = in-plume cast, VO = vent orifice sample, N.D. = Not determined.

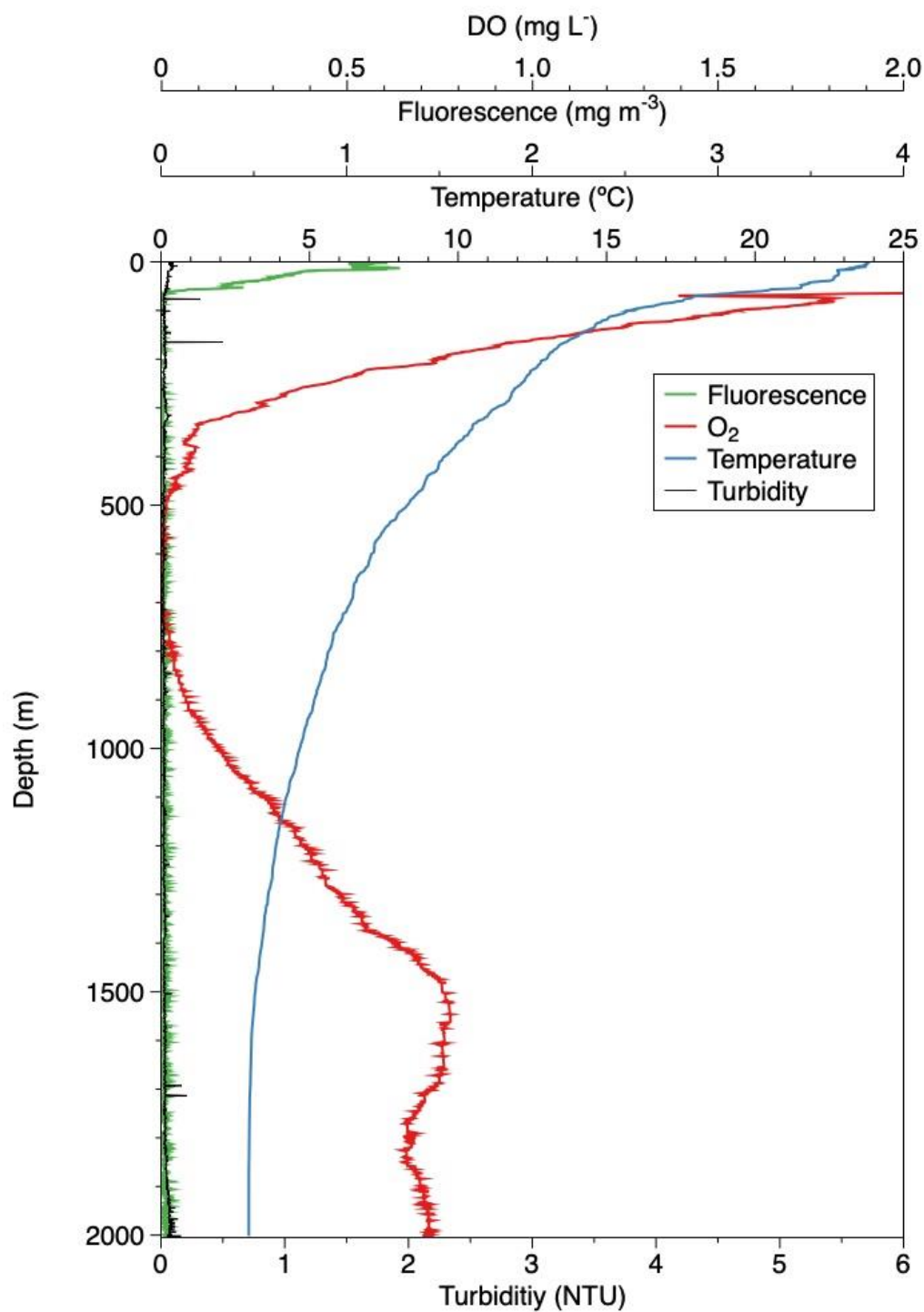


Figure 3.S1. Hydrographic profiles of temperature (blue), turbidity (black), oxygen (red), and fluorescence (green) from the non-plume control site in the Guaymas Basin.

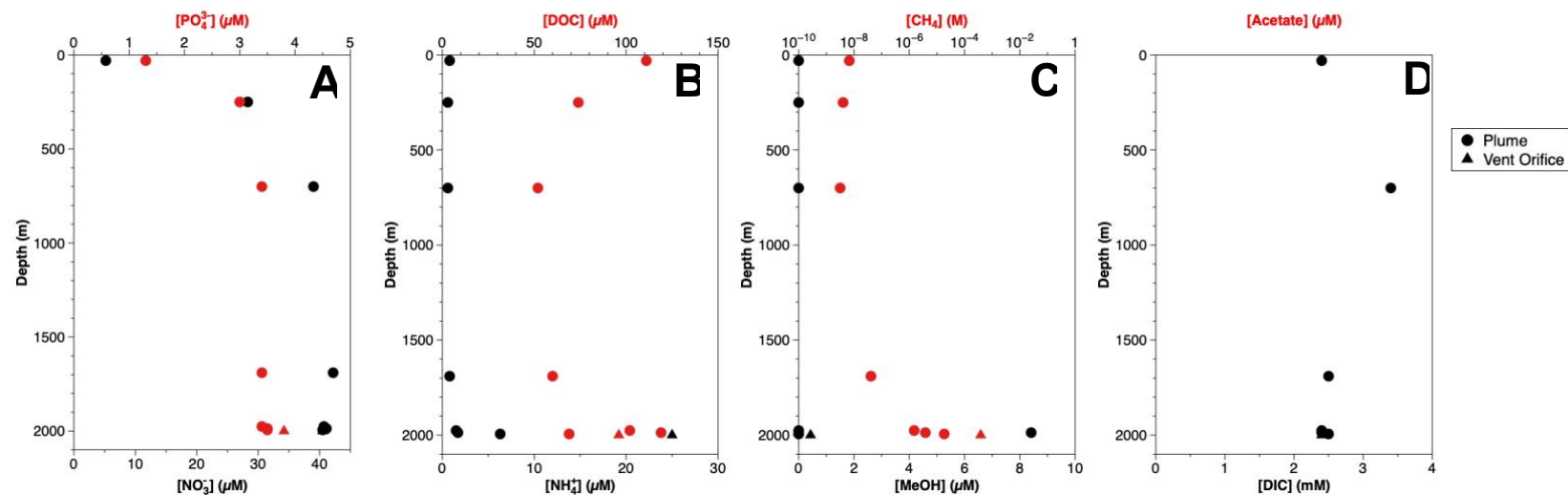


Figure 3.S2. In-plume depth profiles of phosphate and nitrate (A), DOC and ammonium (B), methane and methanol (C), and acetate and DIC (D). Circles were collected from the in-plume CTD cast and triangles were collected from the vent orifice during *HOV Alvin* dive 5000.

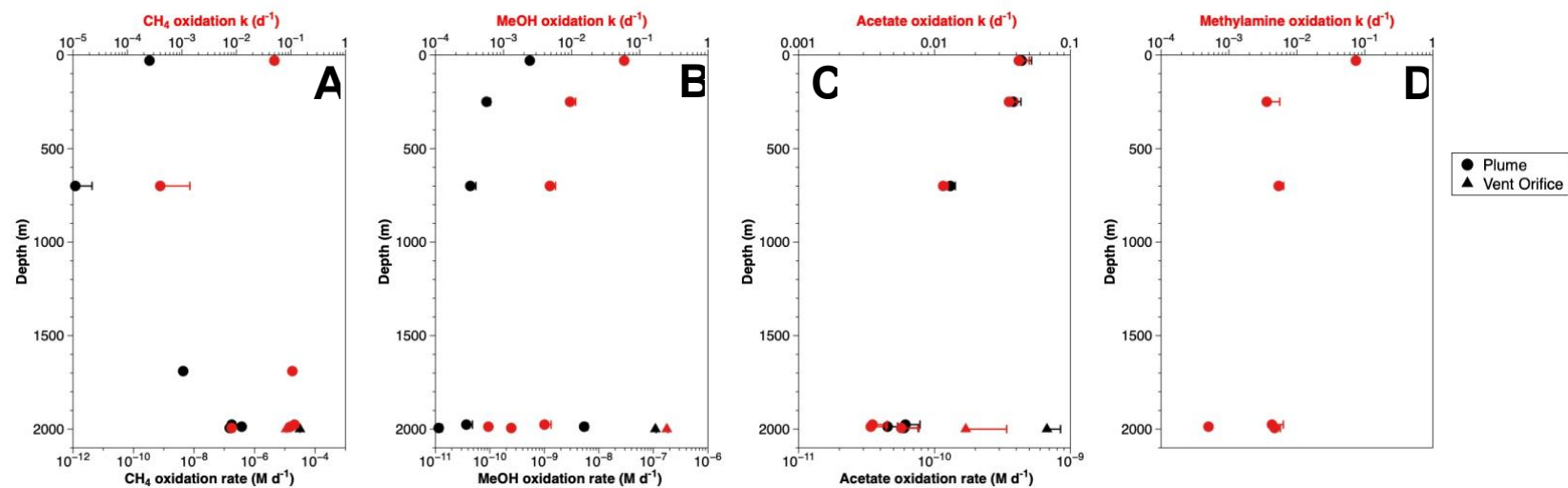


Figure 3.S3. In-plume depth profiles of rates and turnover constants for methane oxidation (A), methanol oxidation (B), acetate oxidation (C), and methylamine oxidation (D). Circles were collected from the in-plume CTD cast and triangles were collected from the vent orifice during *HOV Alvin* dive 5000.

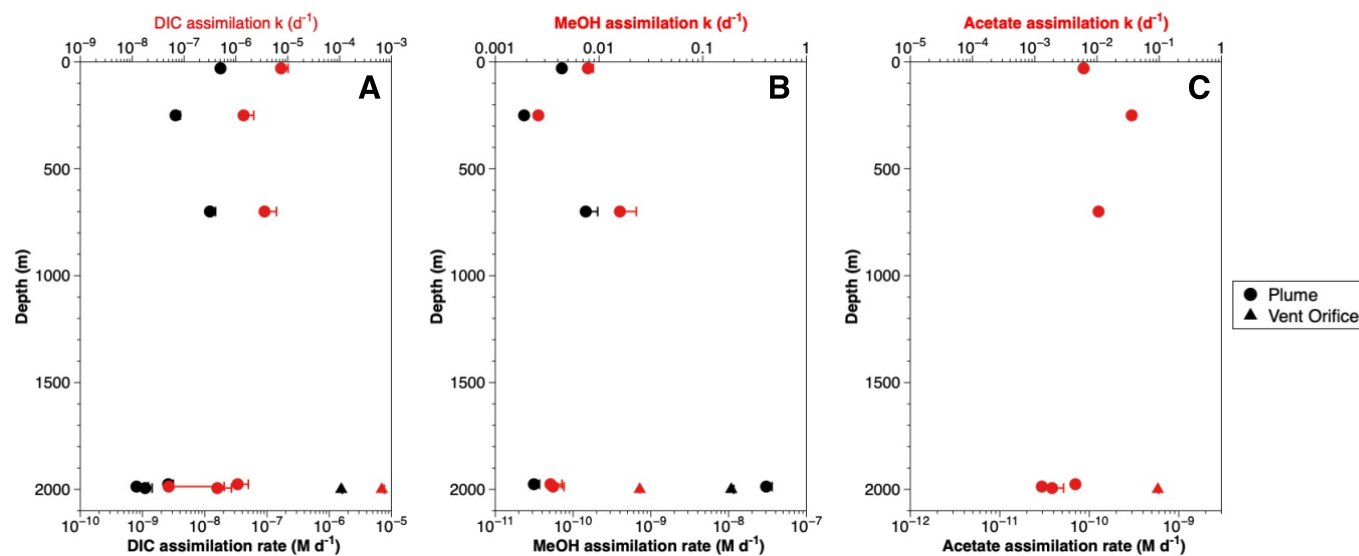


Figure 3.S4. In-plume depth profiles of rates and turnover constants for DIC assimilation (A), methanol assimilation (B), acetate assimilation (C). Circles were collected from the in-plume CTD cast and triangles were collected from the vent orifice during *HOV Alvin* dive 5000.

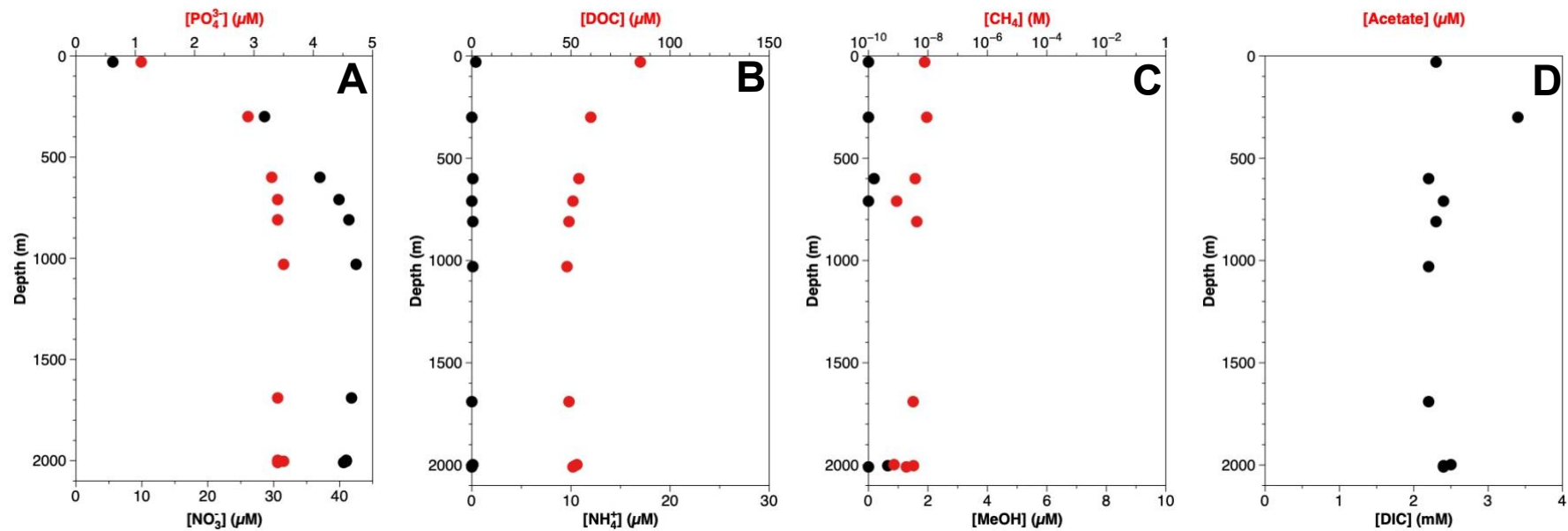


Figure 3.S5. Non-plume depth profiles of phosphate and nitrate (A), DOC and ammonium (B), methane and methanol (C), and acetate and DIC (D).

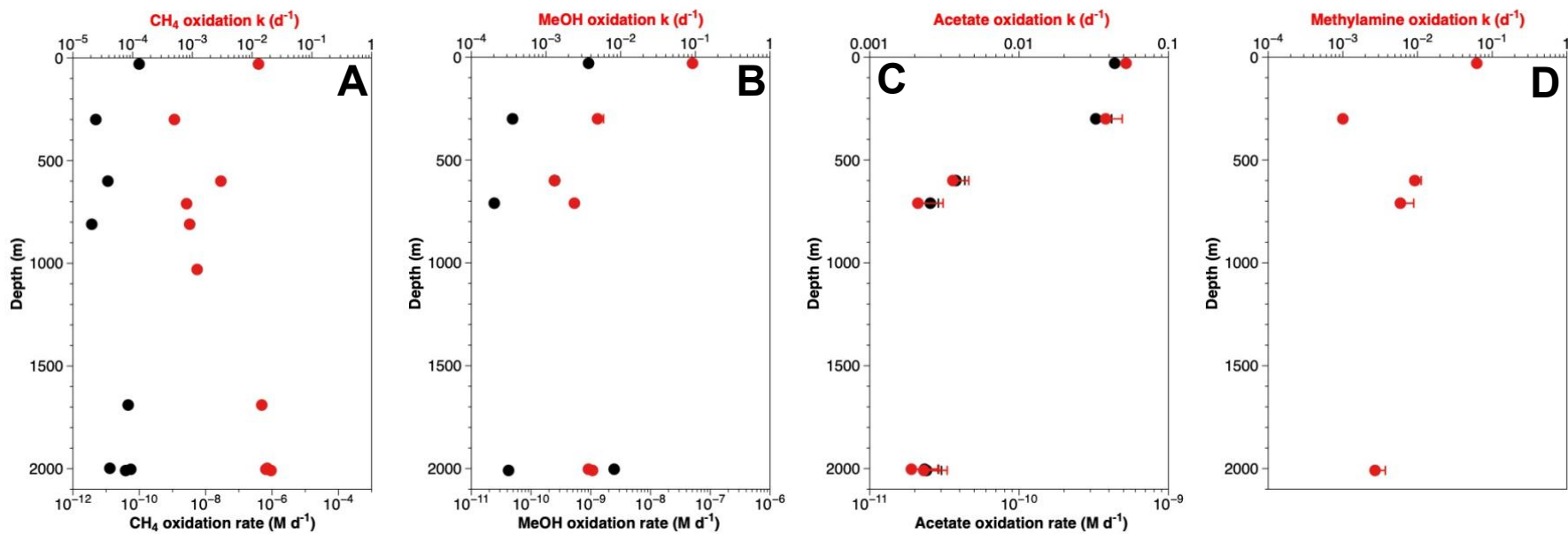


Figure 3.S6. Non-plume depth profiles of rates and turnover constants for methane oxidation (A), methanol oxidation (B), acetate oxidation (C), and methylamine oxidation (D).

Table 3.S1. Overview of Sampling depths and geochemistry in the Guaymas Basin.

Site	Depth m	Altimetry m	pH	Salinity	O ₂ mg L ⁻¹	Temperature °C	Chlorophyll mg m ⁻³	NO ₃ ⁻ μM	NO ₂ ⁻ μM	NH ₄ ⁺ μM	DOC μM	DIC mM	Methanol μM	Methane nM
NP	30	1983	8.13	35.3	5.506	22.94	1.125	5.6	0.2	0.4	85	2.3	B.D.L.	7.7
NP	300	1713	7.65	34.7	0.26	11.40	0.015	28.6	0.0	0	60	3.4	B.D.L.	9.1
NP	600	1413	7.56	34.5	0	7.11	0.004	37	0.0	0.1	54	2.2	0.188	3.7
NP	710	1303	7.55	34.5	3E-04	6.24	0.017	39.9	0.0	0	51	2.4	B.D.L.	0.9
NP	810	1203	7.55	34.5	0.027	5.60	0.008	41.4	0.0	0.1	49	2.3	N.D.	4.2
NP	1030	983	7.91	34.5	0.179	4.57	0.024	42.5	0.0	0.1	48	2.2	N.D.	0.0
NP	1690	323	7.56	34.6	0.751	3.00	0.022	41.8	0.0	0	49	2.2	N.D.	3.2
NP	1998	15	7.55	34.6	0.724	2.95	0.032	41	0.0	0.1	53	2.5	N.D.	0.7
NP	2003	10	7.53	34.6	0.724	2.96	0.030	41	0.0	0	52	2.4	0.65	3.3
NP	2009	4	7.53	34.6	0.723	2.96	0.026	40.6	0.0	0	51	2.4	B.D.L.	1.9
P	30	1969	7.58	35.3	5.985	22.35	1.467	5.2	0.0	0.8	111	2.4	B.D.L.	6.8
P	250	1751	7.09	34.7	0.296	11.82	0.001	28.3	0.0	0.6	74	N.D.	B.D.L.	4.0
P	700	1299	7.01	34.5	B.D.L.	6.49	0.004	39	0.0	0.6	52	3.4	B.D.L.	3.2
P	1690	309	7.03	34.6	0.737	2.99	0.028	42.2	0.0	0.8	60	2.5	N.D.	41.2
P	1976	25	6.96	34.6	0.75	2.97	0.029	40.7	0.0	1.5	102	2.4	B.D.L.	1514.5
P	1987	12	7.01	34.6	0.737	3.0	0.023	41.1	0.0	1.7	119	2.4	8.41	3834.4
P	1994	5	7	34.6	0.719	3.07	0.024	40.5	0.0	6.3	69	2.5	B.D.L.	18353.1
VO	2000	1	7.01	34.6	0.719	3.05	0.025	40.5	0	25	96	2.4	0.43	383892.3

Note: NP = non plume cast, P = in-plume cast, VO = vent orifice sample, DOC = dissolved organic carbon, DIC = dissolved inorganic carbon. N.D. = Not determined, B.D.L. = below detection limit.

Table 3.S2. Oxidation rates of methane, acetate, methanol, methylamine, hexadecane, and naphthalene from all sites and depths in the Guaymas Basin

Site	Depth m	Altimetry m	Methane oxidation pmol L ⁻¹ d ⁻¹	SE	Acetate oxidation nmol L ⁻¹ d ⁻¹	SE	Methanol oxidation nmol L ⁻¹ d ⁻¹	SE	Methylamine oxidation nmol L ⁻¹ d ⁻¹	SE	Hexadecane oxidation nmol L ⁻¹ d ⁻¹	SE	Naphthalene oxidation nmol L ⁻¹ d ⁻¹	SE
NP	30	1983	99.1	0.147	0.437	0.009	0.917	0.040	0.500	0.018	1.097	0.143	0.186	0.044
NP	300	1713	4.9	0.220	0.326	0.090	0.049	0.007	0.008	0.001	0.878	0.017	B.D.L.	B.D.L.
NP	600	1413	11.3	0.511	0.038	0.006	0.248	0.034	0.074	0.016	1.368	0.157	0.502	0.034
NP	710	1303	0.7	0.072	0.025	0.003	0.024	0.003	0.047	0.020	1.669	0.523	0.282	0.056
NP	810	1203	3.7	0.141	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1030	983	B.D.L.	B.D.L.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1690	323	46.2	1.504	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1998	15	13	0.944	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	2003	10	55.3	4.902	0.023	0.005	2.457	0.139	B.D.L.	B.D.L.	0.000	0.017	0.916	0.432
NP	2009	4	38.8	1.349	0.024	0.006	0.042	0.002	0.021	0.005	0.498	0.000	0.018	0.004
P	30	1969	337.3	48.515	0.439	0.080	0.540	0.045	0.579	0.026	7.125	0.438	1.046	0.194
P	250	1751	B.D.L.	B.D.L.	0.380	0.053	0.087	0.015	0.028	0.013	1.096	0.270	0.631	0.047
P	700	1299	1.2	3.080	0.131	0.012	0.044	0.012	0.043	0.010	1.754	0.451	0.514	0.083
P	1690	309	4379.5	201.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P	1976	25	176233.3	2787.7	0.061	0.017	0.037	0.011	0.034	0.012	0.762	0.184	1.895	0.513
P	1987	12	375996.2	10360.4	0.045	0.008	5.370	0.866	0.004	0.001	B.D.L.	B.D.L.	0.969	0.236
P	1994	5	151139	15089.6	0.060	0.016	0.011	0.002	0.037	0.007	B.D.L.	B.D.L.	0.511	0.149
VO	2000	1	31480887	568143.6	0.673	0.174	108.934	4.088	N.D.	N.D.	6.593	0.722	1.362	0.245

Note: NP = non plume cast, P = in-plume cast, VO = vent orifice sample. N.D. = Not determined, B.D.L. = below detection limit.

Table 3.S3. Turnover constants for oxidation of methane, acetate, methanol, methylamine, hexadecane, and naphthalene from all sites and depths in the Guaymas Basin

Site	Depth m	Altimetry m	Methane oxidation turnover constant d ⁻¹	SE	Acetate oxidation turnover constant d ⁻¹	SE	Methanol oxidation turnover constant d ⁻¹	SE	Methylamine oxidation turnover constant d ⁻¹	SE	Hexadecane oxidation turnover constant d ⁻¹	SE	Naphthalene oxidation turnover constant d ⁻¹	SE
NP	30	1983	0.0128	0.000	0.0520	0.001	0.0917	0.004	0.0622	0.002	0.0105	0.001	0.0018	0.0004
NP	300	1713	0.0005	0.000	0.0380	0.011	0.0049	0.001	0.0010	0.000	0.0084	0.000	B.D.L.	B.D.L.
NP	600	1413	0.0030	0.000	0.0036	0.001	0.0013	0.000	0.0092	0.002	0.0130	0.001	0.0048	0.0003
NP	710	1303	0.0008	0.000	0.0021	0.001	0.0024	0.000	0.0059	0.003	0.0159	0.005	0.0021	0.0005
NP	810	1203	0.0009	0.000	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1030	983	0.0012	0.000	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1690	323	0.0145	0.000	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1998	15	0.0179	0.001	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	2003	10	0.0170	0.002	0.0019	0.001	0.0037	0.000	B.D.L.	B.D.L.	0.0106	0.0002	0.0087	0.0041
NP	2009	4	0.0207	0.001	0.0023	0.001	0.0042	0.000	0.0027	0.001	0.0047	0.0000	0.0000	0.0001
P	30	1969	0.0497	0.007	0.0418	0.008	0.0592	0.005	0.0739	0.003	0.0340	0.002	0.0050	0.0009
P	250	1751	B.D.L.	B.D.L.	0.0354	0.005	0.0095	0.002	0.0036	0.002	0.0052	0.001	0.0030	0.0002
P	700	1299	0.0004	0.001	0.0116	0.001	0.0048	0.001	0.0054	0.001	0.0084	0.002	0.0015	0.0006
P	1690	309	0.1063	0.005	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P	1976	25	0.1164	0.002	0.0035	0.001	0.0040	0.001	0.0043	0.002	0.0036	0.001	0.0065	0.0023
P	1987	12	0.0981	0.003	0.0034	0.001	0.0006	0.000	0.0005	0.000	B.D.L.	B.D.L.	0.0046	0.0011
P	1994	5	0.0082	0.001	0.0057	0.002	0.0013	0.000	0.0047	0.001	B.D.L.	B.D.L.	0.0024	0.0007
VO	2000	1	0.0820	0.001	0.0642	0.017	0.2498	0.009	N.D.	N.D.	0.0314	0.003	0.0065	0.0012

Note: NP = non plume cast, P = in-plume cast, VO = vent orifice sample. N.D. = Not determined, B.D.L. = below detection limit.

Table 3.S4. Assimilation rates of DIC, acetate, methanol, and methylamine, from all sites and depths in the Guaymas Basin.

Site	Depth m	Altimetry m	DIC assimilation nmol L ⁻¹ d ⁻¹	SE	Acetate assimilation nmol L ⁻¹ d ⁻¹	SE	Methanol assimilation nmol L ⁻¹ d ⁻¹	SE	Methylamine assimilation nmol L ⁻¹ d ⁻¹	SE
NP	30	1983	55.85	1.76	0.1221	0.005	0.0856	0.008	B.D.L.	B.D.L.
NP	300	1713	15.86	2.46	0.0034	0.001	0.0131	0.003	B.D.L.	B.D.L.
NP	600	1413	8.03	2.44	0.0025	0.000	0.6019	0.138	B.D.L.	B.D.L.
NP	710	1303	0.71	0.24	B.D.L.	B.D.L.	0.1278	0.037	B.D.L.	B.D.L.
NP	810	1203	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1030	983	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1690	323	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1998	15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	2003	10	1.01	0.18	0.0006	0.000	1.3455	0.178	B.D.L.	B.D.L.
NP	2009	4	1.22	0.46	0.0000	0.000	0.0220	0.007	B.D.L.	B.D.L.
P	30	1969	17.85	2.18	0.0661	0.003	0.0716	0.007	B.D.L.	B.D.L.
P	250	1751	3.39	0.64	0.3921	0.055	0.0234	0.002	B.D.L.	B.D.L.
P	700	1299	12.14	2.84	0.1158	0.006	0.1454	0.062	B.D.L.	B.D.L.
P	1690	309	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P	1976	25	2.58	0.53	0.0485	0.024	0.0314	0.006	B.D.L.	B.D.L.
P	1987	12	0.80	0.43	0.0141	0.002	30.3620	5.719	B.D.L.	B.D.L.
P	1994	5	1.10	0.32	0.0212	0.009	B.D.L.	B.D.L.	B.D.L.	B.D.L.
VO	2000	1	1561.72	62.69	1.0345	0.037	10.7925	0.849	N.D.	N.D.

Note: NP = non plume cast, P = in-plume cast, VO = vent orifice sample. N.D. = Not determined, B.D.L. = below detection limit.

Table 3.S5. Turnover constants for assimilation of DIC, acetate, methanol, and methylamine, from all sites and depths in the Guaymas Basin.

Site	Depth m	Altimetry m	DIC assimilation turnover constant d ⁻¹	SE	Acetate assimilation turnover constant d ⁻¹	SE	Methanol assimilation turnover constant d ⁻¹	SE	Methylamine assimilation turnover constant d ⁻¹	SE
NP	30	1983	2.43E-05	2.30E-06	0.0140	0.001	0.0086	0.001	B.D.L.	B.D.L.
NP	300	1713	4.66E-06	2.17E-06	0.0004	0.000	0.0013	0.000	B.D.L.	B.D.L.
NP	600	1413	3.65E-06	3.33E-06	0.0003	0.000	0.0030	0.001	B.D.L.	B.D.L.
NP	710	1303	2.94E-07	3.02E-07	B.D.L.	B.D.L.	0.0128	0.004	B.D.L.	B.D.L.
NP	810	1203	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1030	983	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1690	323	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	1998	15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NP	2003	10	4.19E-07	2.21E-07	0.0001	0.000	0.0020	0.000	B.D.L.	B.D.L.
NP	2009	4	5.09E-07	5.77E-07	B.D.L.	B.D.L.	0.0022	0.001	B.D.L.	B.D.L.
P	30	1969	7.44E-06	2.72E-06	0.0061	0.000	0.0078	0.001	B.D.L.	B.D.L.
P	250	1751	1.41E-06	7.98E-07	0.0361	0.005	0.0026	0.000	B.D.L.	B.D.L.
P	700	1299	3.57E-06	2.51E-06	0.0106	0.001	0.0159	0.007	B.D.L.	B.D.L.
P	1690	309	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P	1976	25	1.08E-06	6.66E-07	0.0045	0.002	0.0034	0.001	B.D.L.	B.D.L.
P	1987	12	5.06E-08	5.43E-07	0.0013	0.000	0.0036	0.001	B.D.L.	B.D.L.
P	1994	5	4.39E-07	3.85E-07	0.0019	0.001	B.D.L.	B.D.L.	B.D.L.	B.D.L.
VO	2000	1	6.51E-04	7.84E-05	0.0951	0.003	0.0247	0.002	N.D.	N.D.

Note: NP = non plume cast, P = in-plume cast, VO = vent orifice sample. N.D. = Not determined, B.D.L. = below detection limit.

CHAPTER 4

METABOLIC FLEXIBILITY IN TWO MARINE STRAINS OF AMMONIA-
OXIDIZING ARCHAEA³

³Montgomery, A.M., M.K. Rogener, G.C. Zhuang, J. Damashek, L.A. Bristow, F.J. Stewart, J.T. Hollibaugh, S.B. Joye. A modified version to be submitted to *ISME Journal*.

ABSTRACT

Ammonia oxidizing archaea (AOA) in the phylum *Thaumarchaeota* comprise an abundant and diverse group of microorganisms in the marine environment. Consequently, these microorganisms thrive in a wide range of habitats and play central roles in global carbon and nitrogen cycling. The AOA mediate the first step of nitrification, ammonia oxidation, and they fix carbon autotrophically. Recently, however, several AOA have been implicated in alternative metabolisms. AOA can utilize organic substrates, such as urea, and are involved in the production of methylphosphonate and nitrous oxide, but the full scope of their metabolic capabilities is unknown. Here we assess the metabolic flexibility and ability to oxidize methane and leucine metabolism in two *Thaumarchaeota* strains, *Nitrosopumilus adriaticus* NF5 and *Nitrosopumilus piranensis* D3C. Rates of cell-specific complete methane oxidation to bicarbonate averaged 0.02 ± 0.004 amol cell⁻¹ d⁻¹. In contrast, rates of partial methane oxidation to metabolic intermediates (*e.g.*, methanol) were ~ 20 times higher, averaging 0.44 ± 0.07 amol cell⁻¹ d⁻¹. These AOA contribute predominantly to incomplete methanotrophy. Estimates of the AOA contribution to methane oxidation from these data suggested that they may contribute to 47% or more of pelagic marine methanotrophy in certain environments. Leucine incorporation rates were up to 0.38 amol cell⁻¹ d⁻¹ implying that AOA can utilize this amino acid for biomass production and, as a result, may also contribute to heterotrophic bacterial production in the marine environment. Rates of AOA activity were significantly different with respect to methane and ammonium availability and under low oxygen conditions, highlighting the ability of AOA to respond nimbly to environmental stressors. These results demonstrate the metabolic flexibility of these AOA, underscore the importance of constraining the

ecophysiology of these dynamic organisms, and provide novel perspectives of AOA-mediated carbon cycling.

Keywords: Ammonia-oxidizing archaea, methane, organic carbon, leucine, bacterial production

INTRODUCTION

The discovery of ammonia-oxidizing archaea (AOA) sparked investigations of their diversity, distribution, and ecophysiology, and marked a pivotal transition in our perspective of archaeal biology and the global nitrogen cycle (Könneke *et al.*, 2005; Treusch *et al.*, 2005; Wuchter *et al.*, 2006; Prosser and Nicol, 2008; Stahl and de la Torre, 2012). The AOA are one of the most abundant groups of organisms on Earth, representing up to 40% of marine and 5% of soil microbial populations (Karner *et al.*, 2001; Teira *et al.*, 2004; Francis *et al.*, 2005; Bates *et al.*, 2011). The AOA are found ubiquitously in natural and engineered ecosystems across a wide range of environmental conditions (Francis *et al.*, 2005; Alves *et al.*, 2018; Carini *et al.*, 2018). With their discovery came the realization that they conserve energy by oxidizing ammonia to nitrite in the first and rate-limiting step of nitrification (Könneke *et al.*, 2005; Martens-Habbena *et al.*, 2009). Additionally, AOA autotrophically fix inorganic bicarbonate into biomass and thus play major roles in the global nitrogen and carbon cycles (Wuchter *et al.*, 2003; Konneke *et al.*, 2014).

Nitrosopumilus maritimus SCM1, the first isolated species of AOA, was used to characterize the physiological capabilities of AOA (Könneke *et al.*, 2005). Specifically, SCM1 exhibits an extremely high affinity for ammonia and a low half-saturation constant for ammonia oxidation (Martens-Habben *et al.*, 2009), and it utilizes the ultra-efficient 3-hydroxypropionate/4-hydroxybutyrate pathway to fix carbon (Berg *et al.*, 2007; Walker *et al.*, 2010; Könneke *et al.*, 2014). Subsequent enrichment of AOA generated nearly 40 cultivated strains, further expanding our understanding of their physiology (*e.g.*, Hatzenpichler *et al.*, 2008; Lehtovirta-Morley *et al.*, 2011; Qin *et al.*, 2018).

All known AOA oxidize ammonia and fix carbon via the same pathway as strain SCM1, but we now know their physiological repertoires extend beyond these two metabolisms. For example, several strains have demonstrated the capacity to conserve energy via the oxidation of alternative substrates, such as urea and cyanate (Santoro and Casciotti, 2011; Tourna *et al.*, 2011; Jung *et al.*, 2014, 2016; Bayer *et al.*, 2016; Lehtovirta-Morley, Ross, *et al.*, 2016; Sauder *et al.*, 2017; Abby *et al.*, 2018), with potentially important impacts on dissolved organic nitrogen pools and cycling rates (Palatinszky *et al.*, 2015; Kitzinger *et al.*, 2019). Furthermore, some AOA have exhibited mixotrophic tendencies (Tourna *et al.*, 2011; Qin *et al.*, 2014) and others produce methylphosphonate and cobalamin (Metcalf *et al.*, 2012; Heal *et al.*, 2017). These metabolic capabilities likely enable niche partitioning as a function of environmental conditions in the AOA. For instance, distinct physiologies have been observed amongst phylogenetically similar AOA strains (Lehtovirta-Morley *et al.*, 2014; Bayer *et al.*, 2016; Qin, Heal, *et al.*, 2018). Some metabolic and physiological capacities likely remain undiscovered; more in-depth

exploration of AOA physiology is likely to reveal new insight into their role in carbon and nitrogen cycling.

AOA are members of class *Nitrososphaeria* in the phylum Thaumarchaeota and our understanding of their phylogenetic diversity relies primarily on studies of the ammonia monooxygenase (AMO) genes which encodes for the primary enzyme responsible for ammonia oxidation (Francis *et al.*, 2005; Kerou and Schleper, 2016; Gubry-Rangin *et al.*, 2018). AOA split into 4 evolutionarily distinct sub-lineages within the phylum Thaumarchaeota, and closely related AOA may display distinct ecophysologies (Spang *et al.*, 2010; Alves *et al.*, 2018). The AOA - AMO enzyme is a member of the copper-containing membrane monooxygenase (CuMMOs) superfamily, which also includes enzymes responsible for bacterial ammonia oxidation and aerobic alkane oxidation, including methanotrophy (Tavormina *et al.*, 2011; Simon and Klotz, 2013; Kozłowski *et al.*, 2016; Rochman *et al.*, 2019). The alpha subunit of the AMO enzyme, *amoA*, has been regularly used to investigate the AOA diversity, abundance, and activity in the environment (Biller *et al.*, 2012; Cao *et al.*, 2013; Sintes *et al.*, 2013; Yao *et al.*, 2013; Alves *et al.*, 2018).

Despite the importance of *amoA* in documenting the ecology of AOA, our knowledge of the archaeal AMO is relatively limited. For example, the function of the *amoX* subunit of the archaeal AMO remains unclear (Treusch *et al.*, 2005) and the location of the enzyme's active site is not known (Walker *et al.*, 2010; Lehtovirta-Morley, Sayavedra-Soto, *et al.*, 2016; Lehtovirta-Morley, 2018). In general, CuMMOs are fairly

promiscuous, and substrate plasticity is well known in this protein superfamily (Tavormina *et al.*, 2011; Khadka *et al.*, 2018). For example, *Nitrosococcus oceanus*, a marine nitrifying bacteria, can oxidize methane with its AMO (Ward, 1987, 1990) and the bacterial methanotroph *Methylococcus capsulatus* can oxidize ammonia with its methane monooxygenase (Hutton and Zobell, 1953; Dalton, 1977; Bedard and Knowles, 1989). Furthermore, AMO can oxidize carbon monoxide, polycyclic aromatic hydrocarbons, and chlorinated ethenes, although growth is not sustained by these substrates (Bedard and Knowles, 1989; Rasche *et al.*, 1990; Hooper *et al.*, 1997; Yoon *et al.*, 2011). While the archaeal AMO is evolutionarily divergent from other CuMMOs (Alves *et al.*, 2018; Khadka *et al.*, 2018), the known promiscuity of CuMMOs generally suggests that AOA may be able to oxidize alternative substrates.

AOA proliferate in a diverse range of habitats, including marine and freshwater environments (DeLong, 1992; Preston *et al.*, 1996; Francis *et al.*, 2005; Hollibaugh *et al.*, 2011; Mosier *et al.*, 2012; Park *et al.*, 2014; Santoro *et al.*, 2015; Zhang *et al.*, 2020), soils (Gubry-Rangin *et al.*, 2011; Tourna *et al.*, 2011; Jung *et al.*, 2014; Lehtovirta-Morley *et al.*, 2014; Zhalnina *et al.*, 2014), and hot springs (De La Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008; Zhang *et al.*, 2008; Lebedeva *et al.*, 2013; Daebeler *et al.*, 2018) and their widespread success appears dependent on certain environmental factors. Specific habitat characteristics including differences in ammonium, oxygen, and other nutrient concentrations are associated with niche partitioning of AOA and may drive ecophysiological adaptations (Martens-Habbena *et al.*, 2009; Sintes *et al.*, 2013; Qin, Amin, *et al.*, 2018; Meador *et al.*, 2020; Qin *et al.*, 2020). For example, differences in

ammonia concentration between the tropical Atlantic and coastal Arctic oceans drove differentiation of AOA ecotypes in these habitats (Sintes *et al.*, 2013). Additionally, oxygen availability has been routinely associated with AOA evolution and physiology (Gillies *et al.*, 2015; Hollibaugh, 2017; Qin *et al.*, 2017; Ren *et al.*, 2019). In particular, oxygen limitation influences AOA community structure, niche separation, and rates and modes of nitrification (Berg *et al.*, 2015; Kitzinger *et al.*, 2019, 2020; Lu *et al.*, 2019, 2020). However, the ways in which the metabolic capabilities of AOA are impacted by environmental factors and strain-level differences are not fully known.

Many studies have documented the remarkable physiological and phylogenetic diversity of AOA, and in this study, we continue this endeavor by documenting a role for AOA in methane oxidation and leucine incorporation. We present field data from the Gulf of Mexico hypoxic zone to illustrate a previously unidentified relationship between ammonium and methane dynamics. Subsequently, we maintained two strains of AOA (*Nitrosopumilus adriaticus* NF5 and *Nitrosopumilus piranensis* D3C) in culture under nitrogen limitation in the presence of methane. Rate measurements revealed the capacity of both strains to oxidize methane and incorporate leucine, in addition to their conventional metabolisms, ammonia oxidation, and inorganic carbon fixation. Utilization of these novel substrates, while relatively low, was significantly increased under hypoxic conditions. We conclude that AOA-mediated methane oxidation and leucine incorporation has significant implications for marine carbon and nitrogen cycling, especially in oxygen minimum zones.

METHODS

Field Experiments

Water column samples were collected from the Gulf of Mexico hypoxic zone during a research cruise onboard the R/V Pelican in July 2016 (PE1705). Sample metadata (collection and processing methods) and *in situ* geochemistry and hydrographic data have been described in detail (Rogener *et al.*, 2021). Water was collected from station I3 (29° 17.718N, 92° 44.97W), a site that consistently exhibits summertime bottom water hypoxia (Rabalais *et al.*, 2002) for experimental amendments. Bottom water was collected in 1 L polyethylene terephthalate (PETG) bottles headspace free and amended with ammonium methane, urea, and cyanate (final concentrations 5, 1, 1, and 1 $\mu\text{mol L}^{-1}$, respectively). Samples were stored at 4°C for ~ 24 h before subsamples were aliquoted for metabolic rate measurements. Methane and ammonia oxidation rates were determined as described previously (Bristow *et al.*, 2015; Kitzinger *et al.*, 2019; Rogener *et al.*, 2021)

Nitrosopumilus strains

Two strains of AOA, *Nitrosopumilus adriaticus* NF5 and *Nitrosopumilus piranensis* D3C (hereafter referred to as NF5 and D3C, respectively) were maintained in pure culture as previously described (Bayer *et al.*, 2016). They were grown in Synthetic Crenarchaeote Medium (SCM) (Könneke *et al.*, 2005) in 50 mL polypropylene culture flasks at 29°C. During experimental incubations, growth was monitored daily by measuring ammonium drawdown and nitrite production (Bendschneider and Robinson, 1952; Solórzano, 1969). Each strain was grown to mid-log phase based on nitrite accumulation (~500 – 700 $\mu\text{mol L}^{-1}$ nitrite), prior to establishing experimental treatments.

Media and experimental set-up

A modified Synthetic Crenarchaeota Medium (SCM) was prepared as described previously (Könneke *et al.*, 2005). Briefly, a basic salt solution was prepared by dissolving 26 g NaCl, 5 g MgSO₄•7H₂O, 5 g MgCl₂•6H₂O, 1.5 g CaCl₂•2H₂O, and 0.1 g KBr into 1 L of *MilliQ* water and autoclaving. Subsequently, 980 mL of this solution was supplemented with HEPES buffer with NaOH (final concentration 10 mM and 5 mM), KH₂PO₄ (14.7 µM final concentration), NaHCO₃ (2 mM final concentration), FeNaEDTA (final concentration 7.5 µM), trace element mixture (100 µM HCl, 0.5 µM H₃BO₃, 0.5 µM MnCl₂•4H₂O, 0.8 µM CoCl₂•6H₂O, 0.1 µM NiCl₂•6H₂O, 0.01 µM CuCl₂•2H₂O, 0.5 µM ZnSO₄•7H₂O, and 0.15 µM NaMoO₄•2H₂O, final concentrations), NH₄Cl (final concentration 1 mM), sodium pyruvate (final concentration 200 µmol L⁻¹), and streptomycin and kanamycin (final concentration 50 µg L⁻¹, each). The pH was adjusted to pH 7.2 ± 0.1 with trace-metal grade HCl (Fisher) and all components were sterilized via filtration (0.22 µm polyethersulfone filter).

We reduced the ammonium concentration to 200 or 20 µmol L⁻¹ to mimic nitrogen limitation and further supplemented cultures with dissolved methane concentrations of 0.5 or 2 µmol L⁻¹ (Table 1). The low oxygen (LO2) treatment was purged with UHP N₂ (Airgas) to remove oxygen and these samples were further manipulated to generate hypoxic conditions (*i.e.*, <2 mg L⁻¹ oxygen). Briefly, after sterilization, LO2 treatment bottles were purged aseptically for ~2 hr with UHP N₂ gas (Airgas) to remove dissolved oxygen and sealed with a butyl rubber stopper. Subsequent addition of oxygenated

inoculum through the stopper served as the oxygen addition for these samples. Triplicate 2 L bottles with 1400 mL of modified SCM were inoculated with 400 mL of culture. Media bottles were sealed with black butyl rubber stoppers to maintain the dissolved gas regime. Concentrations of dissolved oxygen and methane were determined regularly (see below). Within 3 hr of transferring, initial subsamples for rate assays and chemistry were collected. Samples were then incubated in the dark at 29 °C. Each following day, nitrite and ammonium concentrations were determined to track growth and monitor nitrogen availability. Once ammonium concentrations were less than 10% of the initial concentration (*e.g.*, 2 µM for the 20 µM treatments), bottles were supplemented with additional ammonium. Typically, the low ammonium treatments were supplemented daily and the high ammonium treatments were supplemented every other day. Once a discernible difference in nitrite production between treatments was observed (D3C, 4 d; NF5, 10 d), the final subsampling commenced.

Chemical analyses

At both timepoints, samples for ammonium, nitrite, methane, oxygen, and cell counts were collected. Additionally, each day during the incubation a 5 mL aliquot was removed from each sample to measure nitrite and ammonium. Each subsample was removed with a sterile needle and syringe, and the volume removed was replaced with an equal volume of 0.2 µm filtered lab air. UHP N₂ was used to balance the LO₂ treatments. This procedure did not alter the concentration of dissolved methane significantly. After the subsample was removed, it was filtered through a pre-rinsed 0.2 µm filter (Target®) for

immediate analysis. Daily nitrite concentrations were used to estimate ammonia oxidation rates (see below).

Filtered subsamples for ammonium determination were collected in 2 mL aliquots and preserved with phenol before measurement via the phenol hypochlorite method (Solórzano, 1969). Nitrite subsamples (2 mL) were also filtered and were measured via the Griess colorimetric method (Bendschneider and Robinson, 1952). Standards were generated using NH_4Cl and NaNO_2 (Reagent Grade; Fisher) in ammonium-free SCM and the detection limits were 100 nM for ammonium and 50 nM for nitrite. Absorbances were measured using a Shimadzu Instruments Spectrophotometer Model UV-1601.

Subsamples for dissolved oxygen measurement were collected at the start and end of the incubation. Briefly, subsamples were collected in a 12 mL exetainer (Labco) and preserved with 100 μL of saturated zinc acetate. Sample vials were sealed headspace free with a gray chlorobutyl rubber septa and plastic screwcap. Oxygen concentrations were measured with a membrane inlet mass spectrometer (MIMS), as previously described (Kana *et al.*, 1994).

Methane concentrations in the media were determined using a modified version of the headspace equilibrium method (Magen *et al.*, 2014). At both timepoints, sample was gently removed from the sealed media bottles with a sterile N_2 purged needle and syringe. The removed liquid was replaced with an equal volume of 0.2 μm filtered lab air or UHP N_2 . Serum vials (57 or 60 mL) were filled headspace free after overflowing with ~ 3

volumes and closed with a blue butyl rubber stopper and crimp sealed. Immediately, 1.5 ml of sample was replaced with an equal volume of 8 M sodium hydroxide through the stopper to halt biological activity. Subsequently, each vial was amended with 15 mL of UHP N₂ replacing an equal volume of culture medium. Samples were stored upside down at room temperature until methane analysis.

Methane concentrations were determined by injecting 250 µL (NF5) or 500 µL (D3C) of sample headspace into a gas chromatograph equipped with a flame ionizing detector (GC-FID). Concentrations of the methane in the NF5 experiments were measured with a Shimadzu® GC-FID (GC-2014) equipped with an Alltech Carbosphere 80/100 column (Part No. C-5000). Concentrations of methane in the D3C experiments were determined on a separate GC-FID (model 8610C, SRI®) equipped with a HaySepD packed column. Final methane concentrations were calculated using aqueous standards prepared analogously to samples. Methane standards were generated using high purity methane standards (Airgas) and the limit of detection was ~0.75 nM.

Subsamples for cell counts were taken at the initial and final timepoint. Nine mL of unfiltered culture medium was amended with 1 mL of sterile formaldehyde (final concentration 3.7%) and frozen for subsequent counting. Cell counts were determined via epifluorescence microscopy as described previously (Lunau *et al.*, 2005). Briefly, 67.5 µL of sample was diluted in 382.5 µL of sterile 35‰ NaCl in *MilliQ* water and collected on a 0.2 µm filter. Cells were stained with SYBR Green I solution (Molecular Probes) diluted 1:15. Cell counts were determined by manual counting on an Olympus BX-40 microscope

at 1000x magnification with a built-in lamp. Cells were enumerated after counting 25 fields.

Rate measurements

Ammonia oxidation rates were determined by calculating the change in nitrite concentration between consecutive days (*e.g.*, t_0 and t_1):

$$\text{Ammonia oxidation rate} = [\text{NO}_2^-]_{x+1} - [\text{NO}_2^-]_x \quad (1)$$

where $[\text{NO}_2^-]_x$ and $[\text{NO}_2^-]_{x+1}$ are the concentrations of nitrite ($\mu\text{mol L}^{-1}$) from two separate days and x is the time in days between sampling. The ammonia oxidation rate was calculated in units of $\mu\text{mol L}^{-1} \text{ d}^{-1}$. Since stoichiometric oxidation of ammonia to nitrite correlates with growth (Könneke *et al.*, 2005), we used nitrite production to estimate growth rates during the incubation.

Methane oxidation rates were measured by incubating sub-samples with $^{14}\text{CH}_4$ and tracking the production of $^{14}\text{CO}_2$. We subsampled each treatment replicate and generated a pooled killed control; each individual sample was transferred to a 16.6 mL Hungate tube. Sample tubes were over-flowed with approximately 2 volumes and capped headspace free with a grey chlorobutyl rubber septum and screw cap. We used an opened bore serological pipette to minimize disruption of particle-associated cells (Rogener *et al.*, 2018). We injected each sample with 100 μL of $^{14}\text{CH}_4$ (American Radiolabeled Chemicals Inc., 1.8 GBq mmol^{-1}) liquid tracer with a sterile needle and syringe. This addition of $^{14}\text{CH}_4$

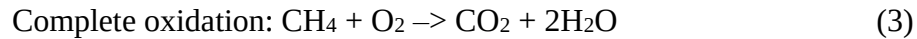
increased the methane concentration by $\sim 0.25 \mu\text{mol L}^{-1}$. The average dissolved activity, 21.6 kBq, was determined by counting 100 μL of sample after the incubation. Before radiotracer injection, killed control samples were amended with 1.5 mL of 2 M sodium hydroxide to halt biological activity.

Samples were incubated in the dark at 29°C for $\sim 2\text{--}4$ d. After incubation, samples were immediately fixed with 1.5 mL of 2 M sodium hydroxide to terminate biological activity and trap $^{14}\text{CO}_2$. Within 2 days, samples were purged for >1 h with lab air to remove unused $^{14}\text{CH}_4$. Subsequently, we distilled the ^{14}C -DIC via acid digestion and trapped the oxidation produced using methods described previously (Joye *et al.*, 2004; Bowles *et al.*, 2011). Briefly, samples were acidified with 5 mL of 30% H_2SO_4 (final pH <2) and shaken in sealed media bottles for >4 h. The bottles were sealed with a modified black rubber butyl stopper that held a glass 7 mL scintillation vial containing a filter wick along with a cocktail of 100 μL of 2 M sodium hydroxide and 1.5 mL of 3-methoxypropylamine (Sigma) that served to trap evolved $^{14}\text{CO}_2$. After distillation, 4.5 mL of Biosafe scintillation cocktail (Research Products International) was added to each sample for liquid scintillation counting (Perkin Elmer, Tricarb 2910TR liquid scintillation analyzer). Samples were counted for 5 min. Recovery efficiency was $>95\%$ based on distillation of ^{14}C -DIC standards prepared analogously to samples. Methane oxidation (MOx) rates were calculated as:

$$\text{MOx rate} = [\text{CH}_4] \times (\alpha_{\text{CH}_4} / t) \times (\text{DPM-}^{14}\text{CO}_2 / \text{DPM-}^{14}\text{CH}_4) \quad (2)$$

where the MO_x rate is in units of pmol L⁻¹ d⁻¹, α is the fractionation factor (1.02; Alperin *et al.*, 1988), [CH₄] is the methane concentration (nmol L⁻¹), t is the incubation time (d), DPM-¹⁴CO₂ is the radioactivity of transformed substrate after accounting for the killed control, and DPM-CH₄ is the radioactivity of dissolved methane available to the microorganisms. Killed controls were typically comparable to instrument backgrounds.

We hypothesized that AOA could oxidize methane similar to the way that nitrifying bacteria oxidize methane. We anticipated that they may not have the enzymatic machinery to oxidize methane completely to inorganic carbon. Therefore, we developed a method to quantify partial methane oxidation by measuring the production of metabolic intermediates (*i.e.*, methanol, formaldehyde, formate) in these ¹⁴CH₄ tracer incubations. Complete and partial methane oxidation reactions were generally defined as:



where complete oxidation represents scenarios where methane is converted completely to carbon dioxide and partial oxidation signifies incomplete metabolism of methane or assimilation into biomass. When partial oxidation occurs, CH_xO represents soluble metabolites including methanol, formaldehyde, and formate, where “x” corresponds to the number of hydrogen atoms bonded to carbon.

To determine partial oxidation, we collected samples in tandem with the methane oxidation rates, described above, and treated them similarly. Incubations were run for 2-4 days. Biological activity was terminated with the addition of 4 mL of 37% formaldehyde (final concentration ~3.7%) and killed controls were fixed prior to radiotracer injection. Subsequently, rate samples were transferred to a 50 mL centrifuge tube and acidified with 1 mL of 100% trichloroacetic acid (TCA; final concentration ~5%; Fisher) to volatilize the ^{14}C -DIC. Residual $^{14}\text{CH}_4$ tracer and ^{14}C - CO_2 product were removed by gentle purging for >1 h with lab air. An aliquot (~10 mL) of each sample was filtered through an 0.2 μm sterile filter to remove any $^{14}\text{CH}_4$ that was assimilated into biomass. The residual radioactivity in the filtrate reflected soluble metabolites derived from ^{14}C -methane. A 1 mL aliquot of this filtrate was transferred to a scintillation vial containing scintillation cocktail and counted as described above. Efficiency controls with known activities of ^{14}C -methanol, $^{14}\text{CH}_4$, or ^{14}C - NaHCO_3 were acidified and purged analogously to samples. We observed >95% recovery for methanol suggesting minimal loss of this compound during processing. Similarly, $^{14}\text{CH}_4$ and ^{14}C - NaHCO_3 controls were similar to instrument background, indicating removal of methane and DIC. Partial methane oxidation rates ($\text{pmol L}^{-1} \text{d}^{-1}$) were calculated as:

$$\text{partial MOx rate} = [\text{CH}_4] \times (\alpha_{\text{CH}_4} / t) \times (\text{DPM-}^{14}\text{C-DOC} / \text{DPM-}^{14}\text{CH}_4) \quad (5)$$

where $\text{DPM-}^{14}\text{C-DOC}$ was the activity of partially oxidized methane after accounting for dilution and killed controls. We were not able to differentiate potential DOC intermediates (*e.g.*, ^{14}C -methanol, ^{14}C -formaldehyde, etc.).

We measured dissolved inorganic carbon (DIC) fixation by tracking the incorporation of ^{14}C -DIC into biomass as described previously (Herndl *et al.*, 2005, Montgomery *et al.*, in preparation). Culture medium was transferred to 10.5 mL serum vials, which were capped headspace free with a gray chlorobutyl rubber septum and crimp sealed. Rate samples were collected in triplicate alongside a killed control (killed with 37% formaldehyde prior to radiotracer injection) and injected with 100 μL of ^{14}C - NaHCO_3 (American Radiolabeled Chemicals Inc., 1.8 GBq mmol^{-1} ; final activity ~ 500 kBq). Incubations were terminated after ~ 2 d by amendment with 37% formaldehyde (final concentration $\sim 3.7\%$). Samples were filtered with an 0.22 μm polyethersulfone filter (Millipore) and rinsed with 0.5% TCA in 35‰ NaCl. Filters were transferred to scintillation cocktail (Research Products International) and counted for 5 min in the liquid scintillation analyzer. The resulting counts were adjusted for killed controls and DIC fixation rates ($\text{nmol L}^{-1} \text{d}^{-1}$) were calculated:

$$\text{DIC fixation rate} = [\text{DIC}] \times (\alpha_{\text{DIC}} / t) \times (\text{DPM-}^{14}\text{C-biomass} / \text{DPM-}^{14}\text{C-DIC})$$

(6)

where [DIC] is the concentration of bicarbonate in the culture medium, $\text{DPM-}^{14}\text{C-biomass}$ is the activity of fixed carbon by AOA, and $\text{DPM-}^{14}\text{C-DIC}$ is the activity of tracer added to each incubation vial.

Cell-specific rates and yield

To assess how these AOA strains responded to the treatment regimens imposed during the incubations, we calculated cell-specific rates for each substrate measured in this study:

$$\text{Cell-specific rate} = \text{metabolic rate} \times \text{cell density} \times 10^6 \quad (7)$$

where the metabolic rate ($\text{pmol L}^{-1} \text{d}^{-1}$) is the rate measurement of interest (*e.g.*, methane oxidation) and the cell density (cells L^{-1}) is the concentration of cells at the time of collection. The cell-specific rates were scaled to units of $\text{amol cell}^{-1} \text{d}^{-1}$. The carbon yield was determined by dividing the cell-specific rate of DIC fixation by the cell-specific rate of ammonia oxidation. For example:

$$\text{Carbon yield} = \text{DIC fixation rate} / \text{NH}_3 \text{ oxidation} \quad (8)$$

Statistical analyses

All statistical analyses were performed in R (version 3.4.4). Means of rates and chemistry data were log-transformed before statistical analysis. Preliminary environmental rates and chemistry, rates, and growth were compared with one-way ANOVA, and mean comparisons between treatments were assessed using Tukey-Kramer HSD. Any *p-value* less than 0.05 was considered statistically significant. Students t-tests were used to compare means between strains and timepoints were compared with paired t-tests. Samples

compared with a t-test were considered significantly different after adjusting for false discovery with the Benjamini-Hochberg method of Bonferroni correction.

RESULTS

Field Data

At site I3 in the northern Gulf of Mexico hypoxic zone, ammonia and methane oxidation rates were elevated relative to other sites sampled. Nitrification (*i.e.*, ammonia oxidation) and methane oxidation (MOx) at 16 m depth were 1709 ± 178.2 and 192 ± 6.7 nmol L⁻¹ d⁻¹, respectively. The methane oxidation rate constant was 0.31 ± 0.04 d⁻¹. These rates served as unamended controls and were compared to parallel MOx and nitrification rates that were measured after amendment with ammonium, methane, urea, or cyanate (Table 2). Ammonia oxidation rates increased with the addition of ammonium (5 μ mol L⁻¹) by a factor of ~ 1.75 relative to the control. This nitrification rate in ammonium amended samples (2984 ± 491 nmol L⁻¹ d⁻¹) was significantly higher than all other rates (Fig. 1A). The addition of methane significantly reduced nitrifying activity by $\sim 60\%$, while urea and cyanate did not alter nitrification rates significantly. Similarly, MOx rates were significantly higher (TukeyHSD, p -value < 0.05) with the addition of 2 μ mol L⁻¹ methane, increasing to 406 ± 17.7 nmol L⁻¹ d⁻¹ (Fig. 1B). In contrast, methane oxidation rate constants decreased with all additions, and methane, urea, and cyanate were significantly lower than the control (Fig. 1C, TukeyHSD, p -value < 0.05).

AOA strain growth and chemistry

The growth of *Nitrosopumilus adriaticus* sp. nov (NF5) and *Nitrosopumilus piranensis* sp. nov. (D3C) were monitored by tracking nitrite production and ammonium drawdown. Average initial nitrite concentrations were 87.8 ± 3.4 and $154.1 \pm 12.0 \mu\text{mol L}^{-1}$ for NF5 and D3C, respectively, and each bottle was amended with either 20 or 200 $\mu\text{mol L}^{-1}$ ammonium (hereafter low and high, respectively; Table 1). Nitrite concentrations were measured daily, and nitrite production was observed in all treatments (Fig. 2D-F and Fig. S1). Ammonium concentrations decreased daily (Fig. 2A-C and Fig. S1), and fresh amendments were made when only 10% of the treatment target concentration was observed. Initial cell abundances ranged from 2×10^6 to $1.1 \times 10^7 \text{ cells mL}^{-1}$ for both strains. In nearly all treatments, cell abundances increased during the incubation, and final cell concentrations were up to $4.68 \times 10^7 \pm 0.26 \times 10^6$. The exception was strain D3C in the hypoxia-like treatment, which exhibited a marginal decrease in cell abundance despite active nitrite production (Fig. 2E). Dissolved oxygen concentrations decreased during the incubation, and high ammonium treatments typically displayed a larger oxygen drawdown. High ammonia treatments became hypoxic ($< 2 \text{ mg L}^{-1} [\text{O}_2]$) during incubation, but no treatment became anoxic (Table S2). Methane concentrations were lower than the target concentration but did not decrease appreciably during incubation (Table S2).

Observed changes in cell abundance coincided with growth rates ranging from 0.044 ± 0.01 to $0.384 \pm 0.04 \text{ d}^{-1}$ (Table 2). Significantly higher (Table S1, TukeyHSD, p -value < 0.05) growth rates were generally observed in ammonium replete treatments (200 $\mu\text{mol L}^{-1}$ ammonium) relative to ammonium limited (20 $\mu\text{mol L}^{-1}$) treatments. Growth rates

were comparable between strains in high ammonium incubations: strain D3C exhibited growth rates up to $0.384 \pm 0.04 \text{ d}^{-1}$, while NF5's growth rate was $0.274 \pm 0.02 \text{ d}^{-1}$ (Table 2). In contrast, strain NF5 exhibited remarkably higher growth rates in low ammonium treatments ($0.165 - 0.187 \text{ d}^{-1}$) than D3C (0.044 d^{-1}). Growth rates under hypoxic conditions were 0.053 ± 0.016 and 0.008 d^{-1} (no replicates because cell abundance did not increase) for NF5 and D3C, respectively, which was significantly lower (Table S1, TukeyHSD, p -value < 0.05) for both strains relative to the other treatments.

AOA oxidation of ammonium and methane

To assess the metabolic capabilities of AOA strains NF5 and D3C, we quantified absolute rates of ammonia oxidation (nitrification). Absolute nitrification rates ranged from ~ 2 to nearly $70 \mu\text{mol L}^{-1} \text{ d}^{-1}$ (Fig. 3). Nitrification rates by strain NF5 spanned a much larger range than strain D3C. At the initial timepoint, strain NF5 in all treatments oxidized ammonia at an average rate of $10.6 \pm 1.7 \mu\text{mol L}^{-1} \text{ d}^{-1}$ (Fig. 3A). Ammonia oxidation increased in the high ammonium treatments, by a factor of ~ 5.5 at the final timepoint and was significantly higher than other NF5 treatments, which reflected cellular growth (Fig. 3A, t-test, p -value < 0.05). After incubation, ammonia oxidation for the low ammonium treatments decreased or was comparable to initial rates, which could be due to relatively smaller increases in cell abundances compared to high ammonium treatments (Table S2). In contrast, strain D3C exhibited much higher initial ammonia oxidation rates (up to $68.8 \pm 6.9 \mu\text{mol L}^{-1} \text{ d}^{-1}$). Nitrification by strain D3C, in high ammonium treatments, did not increase as drastically as NF5, and in some cases, decreased. Generally, ammonia oxidation by D3C was more variable, but no significant differences were observed between

treatments for this strain. In the hypoxic treatment, the nitrification rate by NF5 increased during the incubation, the only low ammonium treatment. In contrast, nitrification by strain D3C decreased by ~ 50% (not significantly different) in the low oxygen treatment, the largest decrease observed for this strain.

We observed methanotrophic activity in both strains across all treatments. Bulk methane oxidation rates ranged from ~100 pmol L⁻¹ d⁻¹ to ~10 nmol L⁻¹ d⁻¹ (Fig. 4), which was at least 3 orders of magnitude lower than the lowest ammonia oxidation rate. We assessed whether these AOA strains could completely oxidize methane to carbon dioxide by tracking complete and partial (*i.e.*, incomplete) methane oxidation (see methods). Complete methane oxidation represented the lower range (up to 358 ± 137 pmol L⁻¹ d⁻¹ by strain D3C) of observed methanotrophy, while partial oxidation was always higher (minimum 570 pmol L⁻¹ d⁻¹ by strain NF5; Fig. 4). Complete oxidation by both strains generally did not vary significantly with respect to treatment and significant differences between timepoints were minimal. Conversely, strain D3C partially oxidized methane at significantly higher rates than NF5 in several treatments, including in the low methane treatments (Fig. 3; t-test; *p*-value < 0.05).

The methane turnover rate constants ranged from below detection to 0.012 d⁻¹ (Fig. 6). Both strains exhibited lower turnover constants across all treatments for complete oxidation relative to partial oxidation. The lowest turnover constants were observed for complete oxidation by strain NF5 (up to 0.0002 ± 0.00004 d⁻¹) irrespective of treatment or timepoint. The turnover rate constants for complete oxidation were typically an order of

magnitude lower than partial oxidation as reflected in the rate measurements. Few differences in these rate constants were observed with respect to time. The exceptions to this were that strain NF5 exhibited significant decreases (Fig. 5B; t-test; p -value < 0.05) in methanotrophy when provided with only 20 $\mu\text{mol L}^{-1}$ ammonium and either low and high methane (0.5 and 2 $\mu\text{mol L}^{-1}$). Initial rate constants of complete methane oxidation were also significantly higher by D3C compared to NF5 under all incubation conditions, except in the hypoxic treatment.

Bicarbonate and leucine uptake by AOA

Autotrophic carbon fixation rates varied with respect to strain, treatment, and time, ranging from 14.7 to 10,616 $\text{nmol L}^{-1} \text{d}^{-1}$ (Fig. 6). Strain D3C showed higher rates of carbon fixation and the low oxygen treatment exhibited the maximal rate of carbon fixation (Fig. 6B). For this strain, DIC fixation increased or remained the same during the incubation, while strain NF5 generally decreased through time. High ammonium treatments generally exhibited higher bulk DIC fixation rates relative to the low ammonium treatments. The notable exception to this was the hypoxic treatments for both strains (Fig. 6A & B).

We observed leucine incorporation in all treatments for both strains, although the rate was usually 3-4 orders of magnitude lower than autotrophic carbon fixation. As a result, the maximum leucine incorporation rate was $0.59 \pm 0.05 \text{ nmol L}^{-1} \text{d}^{-1}$ in the 200 $\mu\text{mol L}^{-1}$ ammonium-only treatment for D3C (Fig. 6D). The incorporation of leucine followed a pattern similar to DIC fixation and was highest in the high ammonium treatments. With the exception of these high ammonium treatments, no substantial

differences were observed through time for either strain. However, strain D3C incorporated significantly more leucine (Fig. 6; t-test; p -value < 0.05) than NF5 with respect to all treatments and times.

Cell yield and specific rates

Maximum cell-specific ammonia oxidation rates of strain NF5 and D3C were comparable and were up to 6.13 ± 1.2 and 6.3 ± 0.9 fmol cell⁻¹ d⁻¹, respectively (Fig. 7). These rates were comparable to all initial treatments and cell-specific ammonia oxidation decreased in all treatments over the course of the incubation. The largest decreases were observed in low ammonium treatments for strain NF5, and the lowest rate observed was 0.15 ± 0.05 fmol cell⁻¹ d⁻¹ by strain NF5 (low ammonium and methane treatment; Fig. 7A). In contrast, strain D3C exhibited the largest decrease in cell-specific nitrification in high ammonium treatments (Fig. 7B). After incubation, cell-specific ammonia oxidation in the hypoxic treatments was 1.65 ± 0.21 and 2.93 ± 0.5 fmol cell⁻¹ d⁻¹ for strain NF5 and D3C, respectively. These treatments did not decrease as drastically for strain NF5 relative to other low ammonium treatments. Strain D3C maintained significantly higher (Fig. 7; t-test; p -value < 0.05) cell-specific nitrification rates at the end of the incubation in low ammonium treatments relative to strain NF5. For example, in the low ammonium high methane treatment, ammonia oxidation by D3C was 4.40 ± 1.9 fmol cell⁻¹ d⁻¹ while the same rate was 0.20 ± 0.1 fmol cell⁻¹ d⁻¹ for NF5.

The maximum cell-specific autotrophic carbon fixation rate for strain NF5 (2.39 ± 0.78 fmol cell⁻¹ d⁻¹) was more than twice as high as that of D3C (0.99 ± 0.45 fmol cell⁻¹ d⁻¹).

¹). Strain NF5 varied considerably between treatments and cell-specific carbon fixation decreased significantly (Fig. 9A; t-test; p -value < 0.05) in most treatments during incubation, except the hypoxic treatment. At the final timepoint, carbon fixation by NF5 in the low oxygen treatment (1.65 ± 0.2 fmol cell⁻¹ d⁻¹) was significantly higher (Fig. 9A; t-test; p -value < 0.05) than the other low ammonium treatments. In contrast, rates of autotrophic carbon fixation were similar with respect to time and treatment for D3C. DIC fixation marginally increased in the hypoxic treatment during the incubation, while high ammonium treatments significantly decreased (Fig. 9B; t-test; p -value < 0.05). Furthermore, at the final timepoint, D3C carbon fixation in the hypoxic treatment was significantly higher than several treatments (Fig. 9B; t-test; p -value < 0.05). A comparison of strains demonstrated that NF5 fixed carbon significantly higher than D3C initially, but by the end of the incubation, cell-specific rates were more comparable (Fig 10A & B).

The cellular yield, defined as the fraction of DIC fixed per unit of ammonia oxidized, ranged from 0.009–1.033 for NF5 and 0.1–0.38 mol C fixed per mol N oxidized for D3C, respectively (Fig. 10). For strain NF5, the initial yield under hypoxic conditions was significantly higher than all other treatments (Fig. 10A). Notably, while all treatments exhibited a decrease in cellular yield after incubation, the yield in the hypoxic treatment did not decrease significantly. In contrast, the yield for strain D3C did not exhibit any significant trends even though the final hypoxic treatment was notably higher ($0.39 \pm .17$ mol C fixed per mol N oxidized; Fig 11B). Interestingly, the initial cellular yield for strain NF5 was significantly higher (Fig 11; t-test; p -value < 0.05) than strain D3C in all treatments.

Cell-specific methane oxidation rates by AOA were significantly lower than nitrification in all treatments. The maximum rate of complete oxidation was 0.08 ± 0.04 amol cell⁻¹ d⁻¹ while partial oxidation reached up to 1.164 amol cell⁻¹ d⁻¹ (Fig. 8). Differences were observed between treatments, strain, and timepoints. For example, cell-specific methane oxidation (complete and partial) for strain NF5 significantly decreased in several treatments (Fig. 8A & B; t-test; p -value < 0.05) during incubation, with the notable exception being the hypoxic treatments. Similarly, strain D3C exhibited significant decreases in cell-specific methane oxidation after prolonged incubation (Fig. 8C & D; t-test; p -value < 0.05) for complete and partial oxidation in several high ammonium treatments. Simultaneously, the hypoxic and low ammonium treatments were similar between timepoints, and in some cases increased (*e.g.*, low ammonium, high methane treatment). In a few instances, complete cell-specific methane oxidation was higher than or comparable to partial oxidation. For instance, strain NF5 complete oxidation in the hypoxic treatment was 0.025 ± 0.006 amol cell⁻¹ d⁻¹ but was 0.019 ± 0.03 amol cell⁻¹ d⁻¹ in the low ammonium, high methane treatment.

Cell-specific leucine incorporation rates were orders of magnitude lower than cell-specific DIC fixation, similar to the observed relationship between methane and ammonia oxidation. Strain NF5 incorporated leucine at a significantly higher per cell rate (up to 0.39 ± 0.06 amol cell⁻¹ d⁻¹) compared to strain D3C (up to 0.021 ± 0.006 amol cell⁻¹ d⁻¹; Fig. 9C & D). Significant differences were observed between strains for per cell leucine incorporation rates, while few differences were observed during incubation. For example, in the hypoxic treatment, leucine incorporation was up to 0.34 ± 0.06 amol cell⁻¹ d⁻¹ for

strain NF5 but was only $0.02 \pm 0.0006 \text{ amol cell}^{-1} \text{ d}^{-1}$ for strain D3C (Fig. 9C & D). This observed value for D3C increased significantly (Fig. 9D; t-test; p -value < 0.05) by a factor of ~ 2 from the initial measurement; the only observed increased for either strain.

Nitrogen Budget

The total nitrogen added was 894 ± 8.9 and $115 \pm 4.2 \text{ } \mu\text{mol NH}_4^+$ for high and low ammonium treatments, respectively (Fig. 11). Similarly, more nitrite was produced in the high ammonium treatments (up to $936 \pm 2.5 \text{ } \mu\text{mol}$ for strain NF5) compared to low ammonium treatments, which ranged up to $197 \pm 43.7 \text{ } \mu\text{mol L}^{-1}$. Strain D3C followed a similar pattern, but overall, less nitrite was produced (up to ~ 650 and $\sim 234 \text{ } \mu\text{mol L}^{-1}$ for high and low ammonium, respectively). We determined the absolute change in ammonium for each treatment by calculating differences in total ammonium added and final ammonium concentration. For strain NF5, ammonium concentrations decreased on average by 678 ± 15.9 and $102 \pm 16.0 \text{ } \mu\text{mol L}^{-1}$ for high and low ammonium concentrations, respectively (Fig. 11A). In contrast, D3C exhibited ammonium drawdowns of 831 ± 14.2 and $78 \pm 1.7 \text{ } \mu\text{mol L}^{-1}$ for high and low ammonium concentrations (Fig. 11B).

Observed differences in nitrite accumulation and this calculated ammonium drawdown revealed discrepancies in ammonia oxidation tendencies for the two strains. For strain NF5, significantly more nitrite was produced than ammonium consumed (Fig. 11A). Specifically, high ammonium treatments exhibited up to $\sim 260 \text{ } \mu\text{mol L}^{-1}$ excess nitrite produced relative to ammonium removed, while low ammonium treatments were up to $\sim 90 \text{ } \mu\text{mol L}^{-1}$. Furthermore, more nitrite was produced than total ammonium added for strain

NF5. In contrast, strain D3C did not exhibit excess nitrite production. Instead, D3C exhibited excess ammonium drawdown. For example, in all high ammonium treatments, ammonium drawdown averaged $831.9 \pm 14.1 \mu\text{mol L}^{-1}$, while nitrite production was approximately half this value (average $472 \pm 11.9 \mu\text{mol L}^{-1}$) over the course of the experiment.

DISCUSSION

Many studies have documented the genomic and physiological characteristics of AOA revealing details of their central role in global carbon and nitrogen cycling. Here we provide evidence that two strains of AOA may play important roles in methanotrophy and in leucine incorporation using radiotracer techniques applied to pure cultures. Ammonia limitation and, in particular, hypoxia influenced the relative metabolism of alternative substrates. Though observed AOA-mediated rates of methane oxidation and leucine incorporation were low relative to rates of primary metabolism, *i.e.*, ammonia oxidation and autotrophic carbon fixation, the abundance of AOA in the environment suggests that they contribute significantly to marine methane oxidation and organic carbon uptake, especially in low oxygen environments.

Methane and ammonia oxidation dynamics in the Gulf of Mexico hypoxic zone

A large hypoxic zone develops in the northern Gulf of Mexico each year, in response to high nutrient loading from the Mississippi and Atchafalaya rivers and subsequent eutrophication (Rabalais *et al.*, 2002). During a research cruise to the Gulf of Mexico hypoxic zone in the summer of 2016, we sampled bottom water in an area

characterized by recurring hypoxia (Rogener *et al.*, 2021). The dissolved oxygen concentrations were $< 63 \mu\text{mol kg}^{-1}$, indicating a hypoxic event and methane concentrations were elevated, reaching 636 nmol L^{-1} (Rogener *et al.*, 2021). Ammonium concentrations can be as high as $\sim 10 \mu\text{mol L}^{-1}$ at some sites in the northern Gulf of Mexico hypoxic zone (Rabalais *et al.*, 2007; Kitzinger *et al.*, 2019), but were only 82 nmol L^{-1} in these samples.

Ammonia oxidation rates in the Gulf of Mexico hypoxic zone (Bristow *et al.*, 2015; Gillies Campbell *et al.*, 2019; Kitzinger *et al.*, 2020) can reach as high as $\sim 3.5 \mu\text{mol L}^{-1} \text{ d}^{-1}$ (Carini *et al.*, 2010). Measured ammonia oxidation rates for the samples from the 2016 expedition were within the previously observed range (up to $1.7 \mu\text{mol L}^{-1} \text{ d}^{-1}$) and were significantly stimulated by the addition of $5 \mu\text{mol L}^{-1}$ ammonium (Fig 1A). AOA are important drivers of ammonia oxidation in hypoxic waters and presumably contributed to the high oxidation rates and drawdown of ammonium we observed (Berg *et al.*, 2015; Bristow *et al.*, 2015; Gillies Campbell *et al.*, 2019).

In contrast, methane cycling has received less attention in hypoxic waters, despite its importance as a greenhouse gas. Methane oxidation rates in the Gulf hypoxic zone were up to $192 \text{ nmol L}^{-1} \text{ d}^{-1}$, the highest rates reported to date for hypoxic waters (Rogener *et al.*, 2021), and activity was stimulated by the addition of $1 \mu\text{mol L}^{-1}$ methane (Fig. 2B), suggesting primary substrate limitation, similar to the case for ammonia oxidation. Ammonia oxidation was significantly lower in samples amended with methane (Fig. 1A, TukeyHSD, $p\text{-value} < 0.05$) relative to the control. Competitive inhibition of

methanotrophy by ammonia is well-known (reviewed by Bedard and Knowles, 1989; Stein *et al.*, 2012), but the inhibition of nitrification by methane was surprising.

Ammonia-oxidizing bacteria (AOB) can oxidize methane to some degree (Ward, 1987), however their affinity for ammonia is not comparable to the AOA and, consequently, their abundance and activity in marine environments is limited (Martens-Habben *et al.*, 2009). This is especially true in coastal oxygen minimum zones, where AOA are considered the primary nitrifiers and significantly outnumber AOB populations by large margins, up to 90,000:1 (Beman *et al.*, 2012; Stewart *et al.*, 2012; Bristow *et al.*, 2015; Lau *et al.*, 2019). Given this, we assumed AOB did not contribute significantly to methanotrophy in the Gulf hypoxic zone. Additionally, canonical methanotrophs are surprisingly absent in the Gulf of Mexico hypoxic zone, despite the elevated methane oxidation rates observed there (Bristow *et al.*, 2015; Gillies *et al.*, 2015; Thrash *et al.*, 2017; Rogener *et al.*, 2021). So, we speculated that AOA may contribute to methanotrophy in the Gulf of Mexico hypoxic zone.

Cellular growth and nitrogen dynamics

Growth was not observed for either AOA strain when cells were incubated in ammonia-free media with a methane headspace (data not shown), indicating these particular AOA strains cannot survive by utilizing methane as the sole energy source. Our next step was to incubate strains NF5 and D3C under low ammonia availability with methane to assess their capacity for alternative physiologies under substrate limitation, as observed previously (Qin, Amin, *et al.*, 2018). Under ammonium replete conditions (*i.e.*,

1 mmol L⁻¹ ammonium) ammonia oxidation by both AOA strains produced nitrite stoichiometrically and is coupled to growth (Könneke *et al.*, 2005; Bayer *et al.*, 2016). When supplemented with 200 µmol L⁻¹ ammonium (hereafter noted as high ammonium), both strains exhibited near exponential growth as evidenced from nitrite production (Fig. 2A). This was also reflected in observed increases in cell abundances (Table S2), although final cell abundances were generally lower than those previously observed for both strains in culture (Bayer *et al.*, 2016). In 20 µmol L⁻¹ ammonium treatments (hereafter low ammonium), significantly less nitrite was produced (Fig. 2). The addition of methane did not significantly alter nitrite production. Cell abundances increased much more in high ammonium treatments relative to low ammonium treatments (Table S2), which corresponded to larger oxygen drawdown and in some cases higher ammonia oxidation rates. For example, strain NF5 exhibited significantly higher ammonia oxidation rates in high ammonium treatments (Fig. 3A; TukeyHSD; *p*-value < 0.05) relative to low ammonium treatments. Strain D3C did not exhibit as much variability in ammonia oxidation; this strain is known to utilize organic nitrogen, such as urea (Bayer *et al.*, 2016). While urea was not available in this media, other organic nitrogen containing-substrates, though more complex, such as antibiotics, could serve as alternative metabolic substrates (further discussion below).

Across all treatments, the patterns in growth rates corresponded largely to differences in ammonia oxidation rates for strain NF5, but not strain D3C (Table 2 and Fig. 2). Observed growth rates (0.008 – 0.384 d⁻¹) were generally comparable to those documented previously for these and other AOA strains (Könneke *et al.*, 2005; Bayer *et*

al., 2019). However, lower growth rates were often observed in ammonium-limited treatments (Table 2). This was somewhat surprising since AOA have a high affinity for ammonium and model strain *N. maritimus* SCM1 can maintain a constant growth rate even at nanomolar ammonium concentrations (Könneke *et al.*, 2005; Nakagawa and Stahl, 2013). Strain D3C seemed to be more impacted by lower ammonium availability, as reflected in its decreased growth rates in the low ammonium treatment (Table 2). In contrast, strain NF5 exhibited much lower bulk ammonia oxidation rates in low ammonium treatments despite higher growth rates, possibly due to its lower initial cell abundances (Table S2 and Fig. 2A). This was clear in the observed comparable cell-specific ammonia oxidation rates between the two strains (Fig. 7).

Cell-specific ammonia oxidation rates from the initial timepoint (Fig. 7) were comparable to those previously reported for strains D3C and NF5 (Bayer *et al.*, 2019). However significant decreases were observed in a number of treatments during the incubations (paired t-test, p -value < 0.05). For example, in low ammonium treatments for strain NF5, cell-specific ammonia oxidation was $< 0.5 \text{ fmol cell}^{-1} \text{ d}^{-1}$ (Fig. 7A), an order of magnitude lower than the initial rates. This observation stood in contrast to observed growth rates (up to 0.187 d^{-1}) in these same treatments. We postulated that this contradiction could be due to a lower nitrogen demand for strain NF5 (*i.e.*, lower nitrogen content per cell) compared to strain D3C, or more likely because of alternative substrate utilization.

Many AOA strains can use urea as an energy source and in some cases use organic compounds as a carbon source (Tourna *et al.*, 2011; Qin *et al.*, 2014). For example, strain D3C can grow with urea as a lone energy source, and, moreover, urea and cyanate both play important roles in nitrification in the environment (Palatinszky *et al.*, 2015; Bayer *et al.*, 2016; Kitzinger *et al.*, 2019). In contrast, strain NF5 appears able to utilize only ammonium and cannot grow or conserve energy from any alternative substrates, including urea. We observed sustained growth rates by strain NF5 in low ammonium treatments, despite low ammonia oxidation rates, suggesting that strain NF5 was harnessing energy from an alternative source. First, growth by strain NF5 in low ammonium treatments was paired with cell-specific DIC fixation rates (up to $1.46 \text{ fmol cell}^{-1} \text{ d}^{-1}$; Fig. 9A) that were comparable to rates in high ammonium treatments and values reported previously for this strain (Bayer *et al.*, 2019). Furthermore, the cellular yield from nitrification ($\text{mol C fixed cell}^{-1} \text{ per mol N fixed cell}^{-1}$) ranged from 0.01 to 0.3 in low ammonium treatments, which was also similar to values reported for strain NF5 and other AOA (Bayer *et al.*, 2019; Meador *et al.*, 2020). This suggests that strain NF5 was harnessing energy from an alternative source. While we did not supplement the culture medium during these experiments with urea or cyanate, other N sources in the media may have fueled strain NF5's growth (see below).

Three compounds in the medium – pyruvate, antibiotics (kanamycin and streptomycin), and ethylenediaminetetraacetic acid (EDTA) – have been documented to be microbially degraded and could serve as potential energy sources for NF5. Of these three compounds, pyruvate is the only compound that has been linked with AOA metabolism.

Specifically, strain SCM1 exhibits higher growth rates in the presence of pyruvate (Qin *et al.*, 2014; Qin, Heal, *et al.*, 2018) and *Nitrosphaera viennensis*, a strain of AOA cultivated from soil, requires pyruvate for growth (Tourna *et al.*, 2011). Pyruvate is involved in peroxide detoxification for AOA, and this may contribute to the increased growth rates when pyruvate is added to the culture medium (Kim *et al.*, 2016; Qin *et al.*, 2017). However, two lines of evidence suggested that pyruvate was not being used by strain NF5 for energy. First, while many strains of AOA, including strain NF5 harbor genes involved in the tricarboxylic acid (TCA) cycle, it remains unclear whether AOA can exploit standard TCA bypass reactions or employ the reverse TCA cycle (Spang *et al.*, 2012; Bayer *et al.*, 2016). Furthermore, strain NF5's growth was not impacted by the addition of pyruvate, unlike strain SCM1. Second, during our incubations, we observed major discrepancies in the nitrogen budget (Fig. 11). In particular, strain NF5 produced excess nitrite in every treatment relative to the amount of ammonium added and ammonia oxidized. For example, in high ammonia treatments, strain NF5 produced nearly 1000 $\mu\text{mol L}^{-1}$ nitrite, but only 800 $\mu\text{mol L}^{-1}$ ammonium was added and the drawn down ammonium (*i.e.*, the sum of ammonium added minus ammonium remaining at end of incubation) was < 750 $\mu\text{mol L}^{-1}$. This discrepancy indicated that an alternative N source was oxidized to nitrite. Pyruvate oxidation cannot explain this observation.

In contrast, EDTA and both antibiotics utilized to minimize bacterial growth are potential sources of organic nitrogen and could be the source of excess nitrite produced. EDTA is an aminopolycarboxylic acid, which consists of four carboxylate groups bound to two central nitrogen atoms that are derived from glycine (Bell, 1977; Bucheli-Witschel

and Egli, 2001). EDTA is a metal chelator and plays a particularly important role in iron complexation. A few studies have demonstrated that EDTA can be microbially degraded (Kaluza *et al.*, 1998), however, it is unclear whether its degradation is limited to artificial environments (*i.e.*, laboratory cultures and wastewater treatment facilities). EDTA can suppress marine phytoplankton growth and is known to be toxic to *Nitrosopumilus maritimus* SCM1 at high concentrations (Muggli and Harrison, 1996; Amin *et al.*, 2013). The primary genes that mediate EDTA oxidation include a nitrilotriacetate monooxygenase (Witschel *et al.*, 1997) that may be distantly related to the archaeal AMO (Jun *et al.*, 2016). To date, no phylogenetic or physiological links exist between ammonia oxidation and EDTA degradation, so this possibility requires further study to be substantiated. Importantly, the final molar concentration of EDTA ($7.5 \mu\text{mol L}^{-1}$) is too low to account for the $\sim 200 \mu\text{mol L}^{-1}$ discrepancy observed for nitrite production (Table S3). This does not preclude EDTA as a source of organic nitrogen for these AOA, however, other substrates are required to satisfy the nitrogen budget within these incubations.

The antibiotics kanamycin and streptomycin in the media are also possible sources of the observed excess nitrite. These compounds are aminoglycoside antibiotics, and little has been done to explore their biodegradation. No evidence for microbial utilization of kanamycin exists, but a limited number of studies have documented streptomycin degradation in bacteria (Klein and Pramer, 1961, 1962; Fenton *et al.*, 1973; Badalucco *et al.*, 1994). These studies demonstrated that ammonium is a product of streptomycin degradation and suggested that a urease could mediate the ammonium release from this antibiotic (Klein and Pramer, 1962). Strain D3C harbors urease-encoding genes (Bayer *et*

al., 2016), although it is not known to metabolize antibiotics. In contrast, strain NF5 does not have any known ureases and cannot use urea as an energy source.

The large excess of nitrite was only observed for strain NF5 implying that this strain has the capacity to metabolize organic nitrogen sources. Ammonification of amine groups in kanamycin or streptomycin could occur via an amidohydrolase in a process analogous to ammonification of urea by AOA strains (Qin *et al.*, 2014; Bayer *et al.*, 2016; Sauder *et al.*, 2017). Strain NF5 harbors four amidohydrolases, two of which have no assigned function, that could potentially serve to generate ammonium from organic nitrogen substrates like these antibiotics. AOA sometimes prefer organic-derived ammonia to inorganic ammonia (Levičnik-Höfferle *et al.*, 2012), suggesting that strain NF5 may have oxidized these antibiotics as the source of surplus nitrite (Fig. 11). However, as with EDTA, this idea is unproven, and additional empirical evidence is required to verify this supposition.

The significant surplus of nitrite that accumulated in all treatments for strain NF5 indicated that it can exploit an alternative and potentially novel source of ammonium for energy. However, the source of excess nitrogen and the mode by which nitrite is produced from organic nitrogen is unclear. We assessed the potential sources and sinks of nitrogen in the culture medium (Table S3) to ensure the excess nitrite production was stoichiometrically feasible. Across treatments, a wide range of nitrite was produced (19-936 $\mu\text{mol L}^{-1}$) during these incubations. Additionally, a relatively small amount of nitrogen should have been assimilated into AOA biomass. Based on limited previous measurements, strain D3C cellular N concentrations were 4.78 fg N cell⁻¹ (Bayer *et al.*, 2019; we assumed

the same cellular N concentration for strain NF5 as no data are available for that strain), which we used to estimate the total amount of ammonium assimilated into biomass. The treatment with the largest increase in cell abundance (*i.e.*, strain D3C; 200 $\mu\text{mol L}^{-1}$ ammonium plus 0.5 $\mu\text{mol L}^{-1}$ methane) would result in $\sim 7 \mu\text{mol L}^{-1}$ of ammonium (or other N sources) removed from solution. In that treatment, 471 $\mu\text{mol L}^{-1}$ nitrite was produced indicating that nitrogen assimilation was inconsequential relative to oxidation. In addition to the free ammonium added, sufficient organic nitrogen in the form of antibiotics was added to satisfy the excess nitrite produced by strain NF5. Since ammonification of antibiotics has been previously observed (see above) and the number of moles of amino nitrogen from these compounds is enough to satisfy the nitrogen budget (Table S3), we postulate that the amino groups of kanamycin and streptomycin could be sources of the observed excess nitrite. It is possible that EDTA and/or HEPES buffer could also account for some if not all the excess nitrite production. However, importantly, there is not enough EDTA in these incubations to produce $\sim 200 \mu\text{mol L}^{-1}$ excess nitrite. Additionally, microbial degradation of HEPES buffer has never been documented, although there would be enough nitrogen supplied from this compound in the media if AOA metabolism of HEPES is possible. The exact mechanism of this non-canonical nitrification is currently unclear, but the observed imbalance in the nitrification budget underscores the importance of alternative substrates for AOA metabolisms.

Leucine incorporation by AOA

The incorporation of leucine into biomass is a widely used approach to measure bacterial production (Kirchman *et al.*, 1985; Kirchman and Hoch, 1988; Kirchman, 2001).

We assessed the capacity of AOA to metabolize leucine to determine whether they could use leucine as an organic carbon and nitrogen source and to assess their potential contribution to measurements of “bacterial production”. Strains NF5 and D3C incorporated leucine at relatively low rates (Fig 6C and D) ranging up to 0.23 ± 0.001 and 0.59 ± 0.05 nmol L⁻¹ d⁻¹, respectively, corresponding to per cell rates that ranged from 0.01 to 0.39 amol cell⁻¹ d⁻¹.

These rates are considerably higher than leucine incorporation rates described previously (0.009 and 0.011 amol cell⁻¹ d⁻¹ for NF5 and D3C, respectively) (Bayer *et al.*, 2019). This was especially true for strain NF5, which incorporated leucine nearly 50 times faster in our experiments compared to the activity levels reported by Bayer *et al.* (2019). These strains of AOA increased metabolism of leucine under ammonium-depleted conditions. Ammonia depletion influences transcriptional activity related to ammonia oxidation and carbon fixation in AOA, and could also prompt a shift in metabolism, as observed in energy-limited habitats (Lever *et al.*, 2015; Qin, Amin, *et al.*, 2018). Similar energy-dependent metabolic switches could occur in the environment where ammonium availability is often limited and easily explain the substantially higher leucine incorporation rates observed in our experiments.

Autotrophic carbon fixation (DIC fixation) was several orders of magnitude higher than leucine incorporation (Fig. 6 and 9), suggesting that neither strain used leucine as its primary carbon source under the experimental conditions. All known AOA use the ultra-efficient 3-hydroxypropionate/4-hydroxybutyrate pathway for DIC fixation and the culture

medium was prepared with 2 mmol L⁻¹ bicarbonate (Konneke *et al.*, 2014). In contrast, no leucine was added to the medium and the sum of labile organic compounds (*i.e.*, pyruvate, antibiotics, and EDTA) was less than 500 µmol L⁻¹ (Könneke *et al.*, 2005). Furthermore, up to 200 µmol L⁻¹ of the N-containing organic substrates appears to have been oxidized as evidenced by the disproportionate nitrite accumulation (Fig. 11). Even though autotrophic DIC fixation dominated, some leucine was assimilated by these AOA (Lehtovirta-Morley *et al.*, 2014; Qin *et al.*, 2014; Bayer *et al.*, 2016).

Despite the observed low activity of leucine incorporation, it could be an important carbon source for AOA under some circumstances. First, the method we used to assess leucine incorporation provides leucine at a low concentration (~10 nmol L⁻¹) and the incubations are terminated after only a few hours (Kirchman *et al.*, 1985; Kirchman, 2001). Under these conditions, it is unlikely that AOA strains could respond quickly enough to synthesize the enzymes necessary to assimilate leucine. Under ammonium and copper depletion, AOA did not respond to substrate input for several hours (Qin, Amin, *et al.*, 2018). Hence, the enzymes for leucine metabolism must have been expressed constitutively. This suggests that if AOA were exposed to higher leucine concentrations or for a longer period of time, incorporation rates may be more substantial (Stahl and de la Torre, 2012; Lehtovirta-Morley *et al.*, 2014). Leucine concentrations in the open ocean are similar to the concentration added during these rate incubations (*e.g.*, Hubberten *et al.*, 1994), but are likely higher in eutrophic environments where AOA thrive (*e.g.*, coastal hypoxic systems).

Leucine incorporation rates are used commonly to estimate heterotrophic bacterial production rates in the environment (Kirchman *et al.*, 1985). Strains NF5 and D3C incorporated leucine at rates up to $0.39 \pm 0.06 \text{ amol cell}^{-1} \text{ d}^{-1}$ (Fig. 9), which were comparable to dark leucine uptake rates by *Prochlorococcus*, the most abundant pelagic marine photoautotroph (Björkman *et al.*, 2015). Dark leucine incorporation by *Prochlorococcus* could account for as much as 60% of euphotic zone leucine uptake. Cell-specific leucine incorporation rates averaged about 0.19 ± 0.04 and $0.48 \pm 0.15 \text{ amol cell}^{-1} \text{ d}^{-1}$ in the Arctic Ocean during winter and summer, respectively (Nikrad *et al.*, 2012) and were approximately $0.5 \text{ amol cell}^{-1} \text{ d}^{-1}$ in more temperate waters (Turley and Stutt, 2000; Longnecker *et al.*, 2006). Additionally, in the North Sea, a majority of cells took up less than $1 \text{ amol leucine cell}^{-1} \text{ d}^{-1}$ during winter (Sintes and Herndl, 2006).

If the leucine incorporation rates by strain NF5 observed here are representative of AOA in the environment, then they are likely major contributors to leucine incorporation in marine settings, given their high abundances. AOA-like organisms have been implicated in amino acid uptake (Ouverney and Fuhrman, 2000; Teira *et al.*, 2004; Herndl *et al.*, 2005) implying that AOA may mediate a significant portion of environmental leucine incorporation. A substantial contribution to leucine incorporation by organisms considered to be autotrophic complicates the interpretation of leucine uptake as an index of purely heterotrophic bacterial production.

Methane oxidation by AOA

Both strains of AOA exhibited the capacity to oxidize methane. Bulk rates of complete methane oxidation (*i.e.*, methane to bicarbonate) were $< 1 \text{ nmol L}^{-1} \text{ d}^{-1}$ (Fig. 4A & C), while partial oxidation often exceeded this value ($>1 \text{ nmol L}^{-1} \text{ d}^{-1}$; Fig. 4B & D). These rates corresponded to cell-specific rates ranging from 0.0015 to 0.08 $\text{amol CH}_4 \text{ cell}^{-1} \text{ d}^{-1}$ for complete oxidation and from below detection to 1.16 $\text{amol CH}_4 \text{ cell}^{-1} \text{ d}^{-1}$ for partial oxidation (Fig. 8). Cell-specific methane oxidation rates up to 4.8 fmol cell d^{-1} are known from methanotrophic cultures (Hanson and Hanson, 1996). Meanwhile, cell-specific methane oxidation by known methanotrophs in the North Atlantic (Steinle *et al.*, 2015) and Mono Lake (an alkaline saline lake; Carini *et al.*, 2005) ranged from 0.002 to 0.384 $\text{fmol cell}^{-1} \text{ d}^{-1}$. Cell-specific methane oxidation by strains NF5 and D3C were several orders of magnitude lower than these previously documented rates, which is unsurprising since methane oxidation is a secondary metabolism (Fig. 8).

Neither AOA strain was able to grow on methane in the absence of ammonia (data not shown). AOA preferentially oxidize ammonia over methane, given their high affinity for ammonia (Martens-Habbena *et al.*, 2009). Therefore, even when small amounts of ammonia are available, ammonia oxidation is the primary metabolism for energy conservation of strains NF5 and D3C. However, the established ubiquity and diversity of AOA and the fact that both AOA strains can oxidize methane means that they likely contribute to methane oxidation in the environment (Karner *et al.*, 2001; Teira *et al.*, 2004; Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Khadka *et al.*, 2018). These data suggest an unanticipated role for the AOA in global methane dynamics.

The archaeal ammonia monooxygenase (AMO) enzyme is abundant in the environment, however, predictions about AMO biochemistry, in general, can be obtained through our knowledge of particulate methane monooxygenase (pMMO) enzyme, which has been characterized extensively (Lehtovirta-Morley, 2018). These two enzymes belong to the diverse copper-containing membrane-bound monooxygenase (Cu-MMO) family. Enzymes in this family can oxidize ammonia, methane, and several short-chain hydrocarbons as primary substrates (Tavormina *et al.*, 2011; Khadka *et al.*, 2018), and in some cases, a given Cu-MMO can mediate metabolism of multiple substrates. For example, nitrifying bacteria can employ their AMO to oxidize methane, and at least some species, *e.g.*, *Nitrosococcus oceanus*, can grow on methane as the sole energy source (Ward, 1987, 1990). Given the diversity of AOA and their widespread niche separation, some AOA, or other members of Thaumarchaeota may be able to grow primarily on methane, as some nitrifying bacteria are able to do (Ward 1987).

Partial methane oxidation rates were always higher than complete oxidation rates (Figs. 4 & 9), implying most methane was metabolized to a soluble intermediate. Canonical methane oxidation begins with the oxidation of methane to methanol and then to formaldehyde. Subsequently, formaldehyde is either oxidized to formate and then bicarbonate or assimilated into biomass via the serine or ribulose monophosphate pathways (Reviewed in: Chistoserdova, 2011; Chistoserdova and Kalyuzhnaya, 2018). Genes encoding methanol dehydrogenase enzymes (MxaFI and XoxF) are transcriptionally upregulated during methanol oxidation (Yu *et al.*, 2017). Strains NF5 and D3C do not

possess these genes but they both harbor numerous alcohol dehydrogenase genes (Bayer *et al.*, 2016).

As with copper monooxygenases, alcohol dehydrogenase enzymes can exhibit substrate promiscuity (Behiry *et al.*, 2018), implying that alternative genes could drive AOA mediated methanol oxidation. Furthermore, a recent *Thaumarchaeota* metagenome assembled genome included a methanol dehydrogenase, indicating that other members of the phylum may be able to metabolize methanol (Reji and Francis, 2020). As with methanol, these AOA do not contain genes encoding canonical enzymes involved for formaldehyde and formate oxidation (*e.g.*, formaldehyde hydrolase and formate dehydrogenase), but they do harbor genes that encode for enzymes of analogous function. Similarly, a cultivated AOA member, *Nitrosocosmicus exaquare* G61, possesses genes associated with formaldehyde oxidation. Hence, other AOA may be able to oxidize methane completely to bicarbonate.

Methane assimilation was not detected in these experiments, indicating these AOA either cannot or do not incorporate formaldehyde into biomass. Canonical methanotrophs assimilate methane-derived carbon via the serine cycle or the ribulose monophosphate cycle (*Verrucomicrobia* are a notable exception) (Hanson and Hanson, 1996). The inability to use formaldehyde is either because the end-product of the reaction is methanol or some other compound. If methanol was the terminal product, eventually methanol would accumulate. We did not detect methanol accumulation, but even the highest methane oxidation rates measured would not have produced enough methanol during the incubation

to detect the product via gas chromatography (limit of detection is $0.2 \mu\text{mol L}^{-1}$) (Zhuang *et al.*, 2014). Formaldehyde is unlikely the final product given its cytotoxicity. Hence, we suspect that methane is partially oxidized to either methanol or formate, or completely oxidized to bicarbonate. Available data demonstrate that AOA are capable of oxidizing methane partially and completely to carbon dioxide, but the mechanism of the reaction remains unconstrained. Describing the biochemical pathway requires further work on AOA mediated methanotrophy.

Preliminary estimates of environmental AOA methanotrophy

We estimated the potential contribution of AOA to marine methanotrophy using the cell-specific rate data from our experiments. Since we observed orders of magnitude differences between complete and partial cell-specific methane oxidation (Fig. 8), we estimated each process separately. Also, because there were significant differences between treatments (t-test, p -values < 0.05), we used minimum and maximum rates to generate a range of potential AOA-mediated methanotrophy in the environment (Table 3). Due to the relatively high abundance of AOA in low oxygen habitats (Gillies *et al.*, 2015; Garcia-Robledo *et al.*, 2017; Kitzinger *et al.*, 2019) and the fact that elevated methane oxidation rates were maintained under hypoxic conditions (Fig. 8), we used rates from these treatments to estimate AOA contribution to methanotrophy in oxygen minimum zones. Additionally, we used available AOA cell abundance data from the open ocean surface and deep (above and below 150 m), coastal environments, and hypoxic zones to calculate the potential contributions of AOA to methane oxidation in these environments.

AOA mediated methane oxidation was estimated up to 157.01 and 942.1 $\text{pmol L}^{-1} \text{d}^{-1}$ in open ocean and hypoxic marine waters, respectively (Table 3). The highest contribution of AOA to methane oxidation could be in the Baltic Sea, where AOA cell abundance was approximately $6.9 \times 10^5 \text{ cells mL}^{-1}$ and potential nitrification rates were nearly $900 \text{ nmol L}^{-1} \text{d}^{-1}$ (Berg *et al.*, 2015), indicating AOA were abundant and active. In the Baltic Sea, methane oxidation rates were measured up to $\sim 2 \text{ nmol L}^{-1} \text{d}^{-1}$ (Steinle *et al.*, 2017) and AOA could account for approximately 47% of methanotrophic activity in this environment. AOA also constitute a large portion of the microbial population in the Gulf of Mexico hypoxic zone (Gillies *et al.*, 2015; Kitzinger *et al.*, 2019) where methane oxidation rates ranged from 10 to $192 \text{ nmol L}^{-1} \text{d}^{-1}$ (Rogener *et al.*, 2021). In this seasonal coastal hypoxic zone, AOA could account for nearly all methanotrophic activity.

Methane oxidation is an important process in permanent marine oxygen minimum zones, such as the eastern tropical north pacific where rates were methane oxidation rates were $4 \text{ nmol L}^{-1} \text{d}^{-1}$ along the oxycline (Pack *et al.*, 2015; Chronopoulou *et al.*, 2017; Thamdrup *et al.*, 2019). AOA constitute $\sim 10\%$ of transcriptional activity there (Garcia-Robledo *et al.*, 2017) indicating they are abundant and active and could account for nearly 25% of methane oxidation. In field samples, a clear interaction between methane availability, nitrification, and methanotrophy was observed (Fig.1), raising important questions about the AOA per cell rate of methane oxidation across a spectrum of ammonium and methane availability in natural habitats.

In the open ocean, potential AOA mediated methane oxidation could be up to 157 pmol L⁻¹ d⁻¹. Despite significant differences in the total and relative abundance of AOA between shallow and deep waters, average methane oxidation rates were only marginally lower in deeper waters (Table 3). Methane oxidation rates in the Gulf of Mexico (pre-oil spill) were less than 1 nmol L⁻¹ d⁻¹ (Rogener *et al.*, 2018) and are often comparable in other oligotrophic waters (Holmes *et al.*, 2000; Valentine *et al.*, 2001). In open ocean waters, ammonia and methane concentrations are typically low, suggesting AOA would preferentially oxidize ammonia due to their high affinity for their primary substrate (Martens-Habben *et al.*, 2009). However, in habitats with high methane input, such as hydrocarbon seeps or hydrothermal vents, persistent methane availability may initiate a switch for where AOA oxidize relatively more methane.

AOA represent a globally dispersed clade and significantly influence carbon and nitrogen cycles. In the Gulf of Mexico hypoxic zone, ammonia oxidation was significantly inhibited by the addition of methane, implying a previously unobserved connection between these two metabolisms. Following this observation and considering the high abundance of AOA in marine hypoxic zones, we hypothesized that AOA could participate in methane oxidation under low-oxygen conditions. Therefore, we assessed the metabolic flexibility of two AOA strains and have demonstrated their ability to utilize methane and leucine. Methane oxidation rates by strains D3C and NF5 were up to 1 amol cell⁻¹ d⁻¹, which while low relative to ammonia oxidation rates (6.3 ± 0.4 fmol cell⁻¹ d⁻¹), were comparable to environmental per cell rates (*e.g.*, Carini *et al.*, 2005). In marine environments where AOA are especially abundant, such as hypoxic zones, we estimated

that AOA could account for more than 45% of methane oxidation activity and in some cases could be the primary methanotrophs. These AOA strains did not grow on methane as the sole energy source (*i.e.*, no ammonium added) indicating a requirement for ammonium to survive. Whether other AOA strains can conserve energy predominantly through methane oxidation is unknown but could have meaningful implications for marine methane cycling. During these experiments, we observed a substantial imbalance in the nitrogen budget, where more than 200 $\mu\text{mol L}^{-1}$ excess nitrite was produced by strain NF5. We postulated that strain NF5 was metabolizing organic nitrogen in the culture medium, such as ethylenediaminetetraacetic acid (EDTA) or antibiotics. We corroborated this observation by measuring leucine incorporation rates which averaged 0.11 ± 0.03 amol $\text{cell}^{-1} \text{d}^{-1}$. While leucine incorporation rates were relatively low compared to autotrophic production, the abundance of AOA in marine waters suggests they could significantly contribute to heterotrophic production. Collectively, we have demonstrated that AOA can utilize alternative substrates as carbon and energy sources, which underscores their dynamic and diverse nature. A role for AOA in the global methane cycle is likely.

REFERENCES

- Abby, S.S., Melcher, M., Kerou, M., Krupovic, M., Stieglmeier, M., Rossel, C., et al. (2018) Candidatus Nitrosocaldus cavascurensis, an ammonia oxidizing, extremely thermophilic archaeon with a highly mobile genome. *Front. Microbiol.* **9**..

- Alperin, M.J., Reeburgh, W.S., and Whiticar, M.J. (1988) Carbon and hydrogen isotope fractionation resulting from anaerobic methane oxidation. *Global Biogeochem. Cycles* **2**: 279–288.
- Alves, R.J.E., Minh, B.Q., Urich, T., Von Haeseler, A., and Schleper, C. (2018) Unifying the global phylogeny and environmental distribution of ammonia-oxidising archaea based on amoA genes. *Nat. Commun.* **9**: 1–17.
- Amin, S.A., Moffett, J.W., Martens-Habbena, W., Jacquot, J.E., Han, Y., Devol, A., et al. (2013) Copper requirements of the ammonia-oxidizing archaeon *Nitrosopumilus maritimus* SCM1 and implications for nitrification in the marine environment. *Limnol. Oceanogr.* **58**: 2037–2045.
- Badalucco, L., Pomaré, F., Grego, S., Landi, L., and Nannipieri, P. (1994) Activity and degradation of streptomycin and cycloheximide in soil. *Biol. Fertil. Soils* **18**: 334–340.
- Bates, S.T., Berg-Lyons, D., Caporaso, J.G., Walters, W.A., Knight, R., and Fierer, N. (2011) Examining the global distribution of dominant archaeal populations in soil. *ISME J.* **5**: 908–917.
- Bayer, B., Vojvoda, J., Offre, P., Alves, R.J.E., Elisabeth, N.H., Garcia, J.A.L., et al. (2016) Physiological and genomic characterization of two novel marine thaumarchaeal strains indicates niche differentiation. *ISME J.* **10**: 1051–1063.
- Bayer, B., Vojvoda, J., Reinthaler, T., Reyes, C., Pinto, M., and Herndl, G.J. (2019) *Nitrosopumilus adriaticus* sp. nov. and *nitrosopumilus piranensis* sp. nov., two ammonia-oxidizing archaea from the adriatic sea and members of the class nitrososphaeria. *Int. J. Syst. Evol. Microbiol.* **69**: 1892–1902.

- Bedard, C. and Knowles, R. (1989) Physiology, Biochemistry, and Specific Inhibitors of CH₄, NH₄⁺, and CO Oxidation by Methanotrophs and Nitrifiers. *Microbiology* **53**: 68–84.
- Behiry, E.M., Ruiz-Pernia, J.J., Luk, L., Tuñón, I., Moliner, V., and Allemann, R.K. (2018) Isotope Substitution of Promiscuous Alcohol Dehydrogenase Reveals the Origin of Substrate Preference in the Transition State. *Angew. Chemie - Int. Ed.* **57**: 3128–3131.
- Bell, C.F. (1977) Principles and Applications of Metal Chelation Clarendon Press, Oxford.
- Beman, J.M., Popp, B.N., and Alford, S.E. (2012) Quantification of ammonia oxidation rates and ammonia-oxidizing archaea and bacteria at high resolution in the Gulf of California and eastern tropical North Pacific Ocean. *Limnol. Oceanogr.* **57**: 711–726.
- Bendschneider, K. and Robinson, R.J. (1952) A New Spectrophotometric Method for the Determination of Nitrite in Sea Water.
- Berg, C., Vandieken, V., Thamdrup, B., and Jürgens, K. (2015) Significance of archaeal nitrification in hypoxic waters of the Baltic Sea. *ISME J.* **9**: 1319–1332.
- Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) Autotrophic Carbon Dioxide Assimilation Pathway in Archaea. *Science (80-.)*. **318**: 1782–1787.
- Biller, S.J., Mosier, A.C., Wells, G.F., and Francis, C.A. (2012) Global biodiversity of aquatic ammonia-oxidizing archaea: Is partitioned by habitat. *Front. Microbiol.* **3**: 1–15.

- Björkman, K.M., Church, M.J., Doggett, J.K., and Karl, D.M. (2015) Differential assimilation of inorganic carbon and leucine by *prochlorococcus* in the oligotrophic north pacific subtropical gyre. *Front. Microbiol.* **6**: 1–14.
- Bowles, M.W., Samarkin, V.A., and Joye, S.B. (2011) Improved measurement of microbial activity in deep-sea sediments at in situ pressure and methane concentration. *Limnol. Oceanogr. Methods* **9**: 499–506.
- Bristow, L.A., Sarode, N., Cartee, J., Caro-Quintero, A., Thamdrup, B., and Stewart, F.J. (2015) Biogeochemical and metagenomic analysis of nitrite accumulation in the Gulf of Mexico hypoxic zone. *Limnol. Oceanogr.* **60**: 1733–1750.
- Bucheli-Witschel, M. and Egli, T. (2001) Environmental fate and microbial degradation of aminopolycarboxylic acids. *FEMS Microbiol. Rev.* **25**: 69–106.
- Cao, H., Auguet, J.C., and Gu, J.D. (2013) Global Ecological Pattern of Ammonia-Oxidizing Archaea. *PLoS One* **8**:
- Carini, P., Dupont, C.L., and Santoro, A.E. (2018) Patterns of thaumarchaeal gene expression in culture and diverse marine environments. *Environ. Microbiol.* **20**: 2112–2124.
- Carini, S., Bano, N., LeClerc, G., and Joye, S.B. (2005) Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ. Microbiol.* **7**: 1127–1138.
- Carini, S.A., McCarthy, M.J., and Gardner, W.S. (2010) An isotope dilution method to measure nitrification rates in the northern Gulf of Mexico and other eutrophic waters. *Cont. Shelf Res.* **30**: 1795–1801.

- Chistoserdova, L. (2011) Modularity of methylotrophy , revisited. *Environ. Microbiol.* **13**: 2603–2622.
- Chistoserdova, L. and Kalyuzhnaya, M.G. (2018) Current Trends in Methylotrophy. *Trends Microbiol.* **xx**: 1–12.
- Chronopoulou, P.M., Shelley, F., Pritchard, W.J., Maanoja, S.T., and Trimmer, M. (2017) Origin and fate of methane in the Eastern Tropical North Pacific oxygen minimum zone. *ISME J.* **11**: 1386–1399.
- Daebeler, A., Herbold, C.W., Vierheilig, J., Sedlacek, C.J., Pjevac, P., Albertsen, M., et al. (2018) Cultivation and Genomic Analysis of “Candidatus Nitrosocaldus islandicus,” an Obligately Thermophilic, Ammonia-Oxidizing Thaumarchaeon from a Hot Spring Biofilm in Graendalur Valley, Iceland. *Front. Microbiol.* **9**: 1–16.
- Dalton, H. (1977) Ammonia oxidation by the methane oxidising bacterium *Methylococcus capsulatus* strain bath. *Arch. Microbiol.* **114**: 273–279.
- DeLong, E.F. (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci U.S.A.* **89**: 5685–5689.
- Fenton, J.J., Harsch, H.H., and Klein, D. (1973) Production of volatile nitrogenous compounds from the degradation of streptomycin by *Pseudomonas maltophilia*. *J. Bacteriol.* **116**: 1267–1272.
- Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., and Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci.* **102**: 14683–14688.

- Garcia-Robledo, E., Padilla, C.C., Aldunate, M., Stewart, F.J., Ulloa, O., Paulmier, A., et al. (2017) Cryptic oxygen cycling in anoxic marine zones. *Proc. Natl. Acad. Sci.* **114**: 201619844.
- Gillies Campbell, L., Thrash, J.C., Rabalais, N.N., and Mason, O.U. (2019) Extent of the annual Gulf of Mexico hypoxic zone influences microbial community structure. *PLoS One* **14**: 1–23.
- Gillies, L.E., Thrash, J.C., deRada, S., Rabalais, N.N., and Mason, O.U. (2015) Archaeal enrichment in the hypoxic zone in the northern Gulf of Mexico. *Environ. Microbiol.* **17**: 3847–3856.
- Gubry-Rangin, C., Hai, B., Quince, C., Engel, M., Thomson, B.C., James, P., et al. (2011) Niche specialization of terrestrial archaeal ammonia oxidizers. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 21206–21211.
- Gubry-Rangin, C., Williams, W., and Prosser, J.I. (2018) Approaches to understanding the ecology and evolution of understudied terrestrial archaeal ammonia-oxidisers. *Emerg. Top. Life Sci.* **2**: 619–628.
- Hanson, R.S. and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol. Rev.* **60**: 439–471.
- Hatzenpichler, R., Lebedeva, E. V., Spieck, E., Stoecker, K., Richter, A., Daims, H., and Wagner, M. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 2134–2139.
- Heal, K.R., Qin, W., Ribalet, F., Bertagnolli, A.D., Coyote-Maestas, W., Hmelo, L.R., et al. (2017) Two distinct pools of B12 analogs reveal community interdependencies in the ocean. *Proc. Natl. Acad. Sci. U. S. A.* **114**: 364–369.

- Herndl, G.J., Reinthaler, T., Teira, E., Aken, H. Van, Veth, C., Pernthaler, A., and Pernthaler, J. (2005) Contribution of Archaea to Total Prokaryotic Production in the Deep Atlantic Ocean. *Appl. Environ. Microbiol.* **71**: 2303–2309.
- Hollibaugh, J.T. (2017) Oxygen and the activity and distribution of marine Thaumarchaeota. *Environ. Microbiol. Rep.* **9**: 186–188.
- Hollibaugh, J.T., Gifford, S., Sharma, S., Bano, N., and Moran, M.A. (2011) Metatranscriptomic analysis of ammonia-oxidizing organisms in an estuarine bacterioplankton assemblage. *ISME J.* **5**: 866–878.
- Holmes, M.E., Sansone, F.J., Rust, T.M., and Popp, B.N. (2000) Methane production, consumption, and air-sea exchange in the open ocean: An evaluation based on carbon isotopic ratios. *Global Biogeochem. Cycles* **14**: 1–10.
- Hooper, A.B., Vannelli, T., Bergmann, D.J., and Arciero, D.M. (1997) Enzymology of the oxidation of ammonia to nitrate by bacteria. *Antonie Van Leeuwenhoek* **71**: 59–67.
- Hubberten, U., Lara, R.J., and Kattner, G. (1994) Amino acid composition of seawater and dissolved humic substances in the Greenland Sea. *Mar. Chem.* **45**: 121–128.
- Hutton, W.E. and Zobell, C.E. (1953) Production of nitrite from ammonia by methane oxidizing bacteria. *J. Bacteriol.* **65**: 216–219.
- Joye, S.B., Boetius, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J., and Lugo, S.K. (2004) The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chem. Geol.* **205**: 219–238.

- Jun, S.-Y., Lewis, K.M., Youna, B., Xun, L., and Kang, C. (2016) Structural and Biochemical Characterization of EDTA Monooxygenase and its Physical Interaction with a Partner Flavin Reductase. *Mol. Microbiol.* **100**: 989–1003.
- Jung, M.Y., Kim, J.G., Sinninghe Damsté, J.S., Rijpstra, W.I.C., Madsen, E.L., Kim, S.J., et al. (2016) A hydrophobic ammonia-oxidizing archaeon of the Nitrosocosmicus clade isolated from coal tar-contaminated sediment. *Environ. Microbiol. Rep.* **8**: 983–992.
- Jung, M.Y., Park, S.J., Kim, S.J., Kim, J.G., Sinninghe Damsté, J.S., Jeon, C.O., and Rhee, S.K. (2014) A mesophilic, autotrophic, ammonia-oxidizing archaeon of thaumarchaeal group I.1a cultivated from a deep oligotrophic soil horizon. *Appl. Environ. Microbiol.* **80**: 3645–3655.
- Kaluza, U., Klingelhöfer, P., and Taeger, K. (1998) Microbial degradation of EDTA in an industrial wastewater treatment plant. *Water Res.* **32**: 2843–2845.
- Kana, T.M., Darkangelo, C., Hunt, M.D., Oldham, J.B., Bennett, G.E., and Cornwell, J.C. (1994) Membrane Inlet Mass Spectrometer for Rapid High-Precision Determination of N₂, O₂, and Ar in Environmental Water Samples. *Anal. Chem.* **66**: 4166–4170.
- Karner, M.B., Delong, E.F., and Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nat. Lett.* **409**: 507–510.
- Kerou, M. and Schleper, C. (2016) Nitrososphaera.
- Khadka, R., Clothier, L., Wang, L., Lim, C.K., Klotz, M.G., and Dunfield, P.F. (2018) Evolutionary History of Copper Membrane Monooxygenases. *Front. Microbiol.* **9**: 1–13.

- Kim, J.G., Park, S.J., Sinninghe Damsté, J.S., Schouten, S., Rijpstra, W.I.C., Jung, M.Y., et al. (2016) Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea. *Proc. Natl. Acad. Sci. U. S. A.* **113**: 7888–7893.
- Kirchman, D. (2001) Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments. In, *Methods in Microbiology*, pp. 227–237.
- Kirchman, D. and Hoch, M. (1988) Bacterial production in the Delaware Bay estuary estimated from thymidine and leucine incorporation rates. *Mar. Ecol. Prog. Ser.* **45**: 169–178.
- Kirchman, D., Nees, E.K., and Hodson, R. (1985) Leucine Incorporation and Its Potential as a Measure of Protein Synthesis by Bacteria in Natural Aquatic Systemst. **49**: 599–607.
- Kitzinger, K., Marchant, H.K., Bristow, L.A., Herbold, C.W., Padilla, C.C., Kidane, A.T., et al. (2020) Single cell analyses reveal contrasting life strategies of the two main nitrifiers in the ocean. *Nat. Commun.* **11**..
- Kitzinger, K., Padilla, C.C., Marchant, H.K., Hach, P.F., Herbold, C.W., Kidane, A.T., et al. (2019) Cyanate and urea are substrates for nitrification by Thaumarchaeota in the marine environment. *Nat. Microbiol.* **4**..
- Klein, D. and Pramer, D. (1961) Bacterial dissimilation of streptomycin. *J. Bacteriol.* **82**: 505–510.
- Klein, D. and Pramer, D. (1962) Some products of the bacterial dissimilation of streptomycin. *J. Bacteriol.* **83**: 309–313.

- Könneke, M., Bernhard, A.E., De La Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.
- Konneke, M., Schubert, D.M., Brown, P.C., Hugler, M., Standfest, S., Schwander, T., et al. (2014) Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO₂ fixation. *Proc. Natl. Acad. Sci.* **111**: 8239–8244.
- Kozlowski, J.A., Stieglmeier, M., Schleper, C., Klotz, M.G., and Stein, L.Y. (2016) Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. *ISME J.* **10**: 1836–1845.
- De La Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M., and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ. Microbiol.* **10**: 810–818.
- Lau, E., Frame, C.H., Nolan, E.J., Stewart, F.J., Dillard, Z.W., Lukich, D.P., et al. (2019) Diversity and relative abundance of ammonia- And nitrite-oxidizing microorganisms in the offshore Namibian hypoxic zone. *PLoS One* **14**: 1–20.
- Lebedeva, E. V., Hatzenpichler, R., Pelletier, E., Schuster, N., Hauzmayer, S., Bulaev, A., et al. (2013) Enrichment and genome sequence of the group I.1a ammonia-oxidizing archaeon “Ca. Nitrosotenuis uzonensis” representing a clade globally distributed in thermal habitats. *PLoS One* **8**: 1–12.
- Lehtovirta-Morley, L.E. (2018) Ammonia oxidation: Ecology, physiology, biochemistry and why they must all come together. *FEMS Microbiol. Lett.* **365**: 1–9.
- Lehtovirta-Morley, L.E., Ge, C., Ross, J., Yao, H., Nicol, G.W., and Prosser, J.I. (2014) Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their

- inhibition and stimulation by organic compounds. *FEMS Microbiol. Ecol.* **89**: 542–552.
- Lehtovirta-Morley, L.E., Ross, J., Hink, L., Weber, E.B., Gubry-Rangin, C., Thion, C., et al. (2016) Isolation of “*Candidatus Nitrosocosmicus franklandus*”, a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS Microbiol. Ecol.* **92**: 1–10.
- Lehtovirta-Morley, L.E., Sayavedra-Soto, L.A., Gallois, N., Schouten, S., Stein, L.Y., Prosser, J.I., and Nicol, G.W. (2016) Identifying potential mechanisms enabling acidophily in the ammonia-oxidizing archaeon “*Candidatus Nitrosotalea devanattera*.” *Appl. Environ. Microbiol.* **82**: 2608–2619.
- Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I., and Nicol, G.W. (2011) Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc. Natl. Acad. Sci.* **108**: 15892–15897.
- Leininger, S., Urich, T., Schlöter, M., Schwark, L., Qi, J., Nicol, G.W., et al. (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**: 806–809.
- Lever, M.A., Rogers, K.L., Lloyd, K.G., Overmann, J., Schink, B., Thauer, R.K., et al. (2015) Life under extreme energy limitation: A synthesis of laboratory- and field-based investigations. *FEMS Microbiol. Rev.* **39**: 688–728.
- Levičnik-Höfferle, Š., Nicol, G.W., Ausec, L., Mandić-Mulec, I., and Prosser, J.I. (2012) Stimulation of thaumarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen. *FEMS Microbiol. Ecol.* **80**: 114–123.

- Longnecker, K., Sherr, B.F., and Sherr, E.B. (2006) Variation in cell-specific rates of leucine and thymidine incorporation by marine bacteria with high and with low nucleic acid content off the Oregon coast. *Aquat. Microb. Ecol.* **43**: 113–125.
- Lu, Y., Cheung, S., Chen, L., Kao, S.J., Xia, X., Gan, J., et al. (2020) New insight to niche partitioning and ecological function of ammonia oxidizing archaea in subtropical estuarine ecosystem. *Biogeosciences* **17**: 6017–6032.
- Lu, Y., Xia, X., Cheung, S., Jing, H., and Liu, H. (2019) Differential distribution and determinants of ammonia oxidizing archaea sublineages in the oxygen minimum zone off Costa Rica. *Microorganisms* **7**:
- Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., and Simon, M. (2005) An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ. Microbiol.* **7**: 961–968.
- Magen, C., Lapham, L., Pohlman, J.W., Marshall, K., Bosman, S., Casso, M., and Chanton, J.P. (2014) A simple headspace equilibration method for measuring dissolved methane. *Limnol. Oceanogr. Methods* **12**: 637–650.
- Martens-Habbena, W., Torre, R. De, Stahl, D.A., Berube, P.M., and Urakawa, H. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nat. Lett.* **461**:
- Meador, T.B., Schoffelen, N., Ferdelman, T.G., Rebello, O., Khachikyan, A., and Könneke, M. (2020) Carbon recycling efficiency and phosphate turnover by marine nitrifying archaea. *Sci. Adv.* **6**:

- Metcalf, W.W., Griffin, B.M., Cicchillo, R.M., Gao, J., Janga, S.C., Cooke, H.A., et al. (2012) Synthesis of Methylphosphonic Acid by Marine Microbes: A Source for Methane in the Aerobic Ocean. *Science* (80-.).
- Mosier, A.C., Lund, M.B., and Francis, C.A. (2012) Ecophysiology of an Ammonia-Oxidizing Archaeon Adapted to Low-Salinity Habitats. *Microb. Ecol.* **64**: 955–963.
- Muggli, D.L. and Harrison, P.J. (1996) EDTA suppresses the growth of oceanic phytoplankton from the Northeast Subarctic Pacific. *J. Exp. Mar. Bio. Ecol.* **205**: 221–227.
- Nakagawa, T. and Stahl, D.A. (2013) Transcriptional response of the archaeal ammonia oxidizer *Nitrosopumilus maritimus* to low and environmentally relevant ammonia concentrations. *Appl. Environ. Microbiol.* **79**: 6911–6916.
- Nikrad, M.P., Cottrell, M.T., and Kirchman, D.L. (2012) Abundance and single-cell activity of heterotrophic bacterial groups in the western Arctic Ocean in summer and winter. *Appl. Environ. Microbiol.* **78**: 2402–2409.
- Ouverney, C.C. and Fuhrman, J.A. (2000) Marine planktonic archaea take up amino acids. *Appl. Environ. Microbiol.* **66**: 4829–4833.
- Pack, M.A., Heintz, M.B., Reeburgh, W.S., Trumbore, S.E., Valentine, D.L., Xu, X., and Druffel, E.R.M. (2015) Methane oxidation in the eastern tropical North Pacific Ocean water column. *J. Geophys. Res. Biogeosciences* **120**: 1078–1092.
- Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., Von Bergen, M., et al. (2015) Cyanate as an energy source for nitrifiers. *Nature* **524**: 105–108.

- Park, S.J., Ghai, R., Martín-Cuadrado, A.B., Rodríguez-Valera, F., Chung, W.H., Kwon, K.K., et al. (2014) Genomes of two new ammonia-oxidizing archaea enriched from deep marine sediments. *PLoS One* **9**: 1–10.
- Preston, C.M., Wu, K.Y., Molinski, T.F., and Delong, E.F. (1996) A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. *Proc. Natl. Acad. Sci. U. S. A.* **93**: 6241–6246.
- Prosser, J.I. and Nicol, G.W. (2008) Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. *Environ. Microbiol.* **10**: 2931–2941.
- Qin, W., Amin, S.A., Lundeen, R.A., Heal, K.R., Martens-Habbena, W., Turkarslan, S., et al. (2018) Stress response of a marine ammonia-oxidizing archaeon informs physiological status of environmental populations. *ISME J.* **12**: 508–519.
- Qin, W., Amin, S.A., Martens-Habbena, W., Walker, C.B., Urakawa, H., Devol, A.H., et al. (2014) Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 12504–12509.
- Qin, W., Heal, K.R., Ramdasi, R., Kobelt, J.N., Martens-habbena, W., Bertagnolli, A.D., et al. (2018) *Nitrosopumilus maritimus* gen. nov., sp. nov., *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxycinae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov., four marine ammonia-oxidizing archaea of the phylum Thaumarchaeota. *Int. J. Syst. Evolutionary Microbiol.* 5067–5079.
- Qin, W., Meinhardt, K.A., Moffett, J.W., Devol, A.H., Virginia Armbrust, E., Ingalls, A.E., and Stahl, D.A. (2017) Influence of oxygen availability on the activities of ammonia-oxidizing archaea. *Environ. Microbiol. Rep.* **9**: 250–256.

- Qin, W., Zheng, Y., Zhao, F., Wang, Y., Urakawa, H., Martens-Habbena, W., et al. (2020) Alternative strategies of nutrient acquisition and energy conservation map to the biogeography of marine ammonia-oxidizing archaea. *ISME J.* **14**: 2595–2609.
- Rabalais, N.N., Turner, R.E., Sen Gupta, B.K., Boesch, D.F., Chapman, P., and Murrell, M.C. (2007) Hypoxia in the northern Gulf of Mexico: Does the science support the plan to reduce, mitigate, and control hypoxia? *Estuaries and Coasts* **30**: 753–772.
- Rabalais, N.N., Turner, R.E., and Wiseman, W.J. (2002) Gulf of Mexico Hypoxia, A.K.A. “The Dead Zone.” *Annu. Rev. Ecol. Syst.* **33**: 235–263.
- Rasche, M.E., Hyman, M.R., and Arp, D.J. (1990) Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. *Appl. Environ. Microbiol.* **56**: 2568–2571.
- Reji, L. and Francis, C.A. (2020) Metagenome-assembled genomes reveal unique metabolic adaptations of a basal marine Thaumarchaeota lineage. *ISME J.* **14**: 2105–2115.
- Ren, M., Feng, X., Huang, Y., Wang, H., Hu, Z., Clingenpeel, S., et al. (2019) Phylogenomics suggests oxygen availability as a driving force in Thaumarchaeota evolution. *ISME J.* **13**: 2150–2161.
- Rochman, F.F., Kwon, M., Khadka, R., Tamas, I., Lopez-Jauregui, A.A., Sheremet, A., et al. (2019) Novel copper-containing membrane monooxygenases (CuMMOs) encoded by alkane-utilizing Betaproteobacteria. *ISME J.*
- Rogener, M.K., Bracco, A., Hunter, K.S., Saxton, M.A., and Joye, S.B. (2018) Long-term impact of the Deepwater Horizon oil well blowout on methane oxidation dynamics in the northern Gulf of Mexico. *Elementa* **6**: 1–17.

- Rogener, M.K., Hunter, K., Rabalais, N.N., Roberts, B.J., Bracco, A., Stewart, F.J., and Joye, S.B. (2021) Pelagic denitrification and methane oxidation in oxygen- depleted waters of the Louisiana shelf. *Biogeochemistry* **8**:
- Santoro, A.E. and Casciotti, K.L. (2011) Enrichment and characterization of ammonia-oxidizing archaea from the open ocean: Phylogeny, physiology and stable isotope fractionation. *ISME J.* **5**: 1796–1808.
- Santoro, A.E., Dupont, C.L., Richter, R.A., Craig, M.T., Carini, P., McIlvin, M.R., et al. (2015) Genomic and proteomic characterization of “Candidatus Nitrosopelagicus brevis”: An ammonia-oxidizing archaeon from the open ocean. *Proc. Natl. Acad. Sci. U. S. A.* **112**: 1173–1178.
- Sauder, L.A., Albertsen, M., Engel, K., Schwarz, J., Nielsen, P.H., Wagner, M., and Neufeld, J.D. (2017) Cultivation and characterization of Candidatus Nitrosocosmicus exaquare, an ammonia-oxidizing archaeon from a municipal wastewater treatment system. *ISME J.* **11**: 1142–1157.
- Simon, J. and Klotz, M.G. (2013) Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochim. Biophys. Acta - Bioenerg.* **1827**: 114–135.
- Sintes, E., Bergauer, K., De Corte, D., Yokokawa, T., and Herndl, G.J. (2013) Archaeal amoA gene diversity points to distinct biogeography of ammonia-oxidizing Crenarchaeota in the ocean. *Environ. Microbiol.* **15**: 1647–1658.
- Sintes, E. and Herndl, G.J. (2006) Quantifying substrate uptake by individual cells of marine bacterioplankton by catalyzed reporter deposition fluorescence in situ

- hybridization combined with microautoradiography. *Appl. Environ. Microbiol.* **72**: 7022–7028.
- Solórzano, L. (1969) Determination of Ammonia in Natural Waters by the Phenolhypochlorite Method. *Limnol. Oceanogr.* **14**: 799–801.
- Spang, A., Hatzenpichler, R., Brochier-Armanet, C., Rattei, T., Tischler, P., Spieck, E., et al. (2010) Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. *Trends Microbiol.* **18**: 331–340.
- Spang, A., Poehlein, A., Offre, P., Zumbärgel, S., Haider, S., Rychlik, N., et al. (2012) The genome of the ammonia-oxidizing candidatus nitrososphaera gargensis: Insights into metabolic versatility and environmental adaptations. *Environ. Microbiol.* **14**: 3122–3145.
- Stahl, D.A. and de la Torre, J.R. (2012) Physiology and Diversity of Ammonia-Oxidizing Archaea. *Annu. Rev. Microbiol.* **66**: 83–101.
- Stein, L.Y., Roy, R., and Dunfield, P.F. (2012) Aerobic Methanotrophy and Nitrification: Processes and Connections. *eLS*.
- Steinle, L., Graves, C.A., Treude, T., Ferré, B., Biastoch, A., Bussmann, I., et al. (2015) Water column methanotrophy controlled by a rapid oceanographic switch. *Nat. Geosci.* **8**: 378–382.
- Steinle, L., Maltby, J., Treude, T., Kock, A., Bange, H.W., Engbersen, N., et al. (2017) Effects of low oxygen concentrations on aerobic methane oxidation in seasonally hypoxic coastal waters. *Biogeosciences* **14**: 1631–1645.
- Stewart, F.J., Ulloa, O., and Delong, E.F. (2012) Microbial metatranscriptomics in a permanent marine oxygen minimum zone. *Environ. Microbiol.* **14**: 23–40.

- Tavormina, P.L., Orphan, V.J., Kalyuzhnaya, M.G., Jetten, M.S.M., and Klotz, M.G. (2011) A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. *Environ. Microbiol. Rep.* **3**: 91–100.
- Teira, E., Reinthaler, T., Pernthaler, A., Pernthaler, J., and Herndl, G.J. (2004) Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. *Appl. Environ. Microbiol.* **70**: 4411–4414.
- Thamdrup, B., Steinsdóttir, H.G.R., Bertagnolli, A.D., Padilla, C.C., Patin, N. V., Garcia-Robledo, E., et al. (2019) Anaerobic methane oxidation is an important sink for methane in the ocean’s largest oxygen minimum zone. *Limnol. Oceanogr.* **64**: 2569–2585.
- Thrash, J.C., Seitz, K.W., Baker, B.J., Temperton, B., Gillies, L.E., Rabalais, N.N., et al. (2017) Metabolic roles of uncultivated bacterioplankton lineages in the northern gulf of Mexico “dead zone.” *MBio* **8**: 1–20.
- Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Urich, T., et al. (2011) Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 8420–8425.
- Treusch, A.H., Leininger, S., Kietzin, A., Schuster, S.C., Klenk, H.P., and Schleper, C. (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* **7**: 1985–1995.

- Turley, C.M. and Stutt, E.D. (2000) Depth-related cell-specific bacterial leucine incorporation rates on particles and its biogeochemical significance in the Northwest Mediterranean. *Limnol. Oceanogr.* **45**: 419–425.
- Valentine, D.L., Blanton, D.C., Reeburgh, W.S., and Kastner, M. (2001) Water column methane oxidation adjacent to an area of active hydrate dissociation, Eel River Basin. *Geochim. Cosmochim. Acta* **65**: 2633–2640.
- Walker, C.B., De La Torre, J.R., Klotz, M.G., Urakawa, H., Pinel, N., Arp, D.J., et al. (2010) Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 8818–8823.
- Ward, B.B. (1987) Kinetic studies on ammonia and methane oxidation by Nitrosococcus oceanus. *Arch. Microbiology* **147**: 126–133.
- Ward, B.B. (1990) Kinetics of ammonia oxidation by a marine nitrifying bacterium: Methane as a substrate analogue. *Microb. Ecol.* **19**: 211–225.
- Witschel, M., Nagel, S., and Egli, T. (1997) Identification and characterization of the two-enzyme system catalyzing oxidation of EDTA in the EDTA-degrading bacterial strain DSM 9103. *J. Bacteriol.* **179**: 6937–6943.
- Wuchter, C., Abbas, B., Coolen, M.J.L., Herfort, L., Van Bleijswijk, J., Timmers, P., et al. (2006) Archaeal nitrification in the ocean. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 12317–12322.
- Wuchter, C., Schouten, S., Boschker, H.T.S., and Sinninghe Damsté, J.S. (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol. Lett.* **219**: 203–207.

- Yao, H., Campbell, C.D., Chapman, S.J., Freitag, T.E., Nicol, G.W., and Singh, B.K. (2013) Multi-factorial drivers of ammonia oxidizer communities: Evidence from a national soil survey. *Environ. Microbiol.* **15**: 2545–2556.
- Yoon, S., Im, J., Bandow, N., Dispirito, A.A., and Semrau, J.D. (2011) Constitutive expression of pMMO by *Methylocystis* strain SB2 when grown on multi-carbon substrates: Implications for biodegradation of chlorinated ethenes. *Environ. Microbiol. Rep.* **3**: 182–188.
- Yu, Z., Beck, D.A.C., and Chistoserdova, L. (2017) Natural Selection in Synthetic Communities Highlights the Roles of *Methylococcaceae* and *Methylophilaceae* and Suggests Differential Roles for Alternative Methanol Dehydrogenases in Methane Consumption. *Front. Microbiol.* **8**: 1–13.
- Zhalnina, K. V., Dias, R., Leonard, M.T., De Quadros, P.D., Camargo, F.A.O., Drew, J.C., et al. (2014) Genome sequence of *Candidatus nitrososphaera evergladensis* from group I.1b enriched from everglades soil reveals novel genomic features of the ammonia-oxidizing archaea. *PLoS One* **9**: 21–23.
- Zhang, C.L., Ye, Q., Huang, Z., Li, W.J., Chen, J., Song, Z., et al. (2008) Global occurrence of archaeal *amoA* genes in terrestrial hot springs. *Appl. Environ. Microbiol.* **74**: 6417–6426.
- Zhang, S., Qin, W., Xia, X., Xia, L., Li, S., Zhang, L., et al. (2020) Ammonia oxidizers in river sediments of the Qinghai-Tibet Plateau and their adaptations to high-elevation conditions. *Water Res.* **173**: 115589.
- Zhuang, G., Lin, Y., Elvert, M., Heuer, V.B., and Hinrichs, K. (2014) Gas chromatographic analysis of methanol and ethanol in marine sediment pore waters :

Validation and implementation of three pretreatment techniques. *Mar. Chem.* **160**: 82–90.

Table 4.1. Experimental conditions of culture experiments.

ID	CH₄ μmol L⁻¹	NH₄⁺ μmol L⁻¹	[O₂]	Strains applied to
LL	0.5	20	atmospheric	NF5 and D3C
LH	2	20	atmospheric	NF5 and D3C
L-	0	20	atmospheric	D3C
HL	0.5	200	atmospheric	NF5 and D3C
HH	2	200	atmospheric	D3C
H-	0	200	atmospheric	NF5 and D3C
LO2	2	20	hypoxic*	NF5 and D3C

Note: * = < 50 mmol L⁻¹ or 2 mg L⁻¹

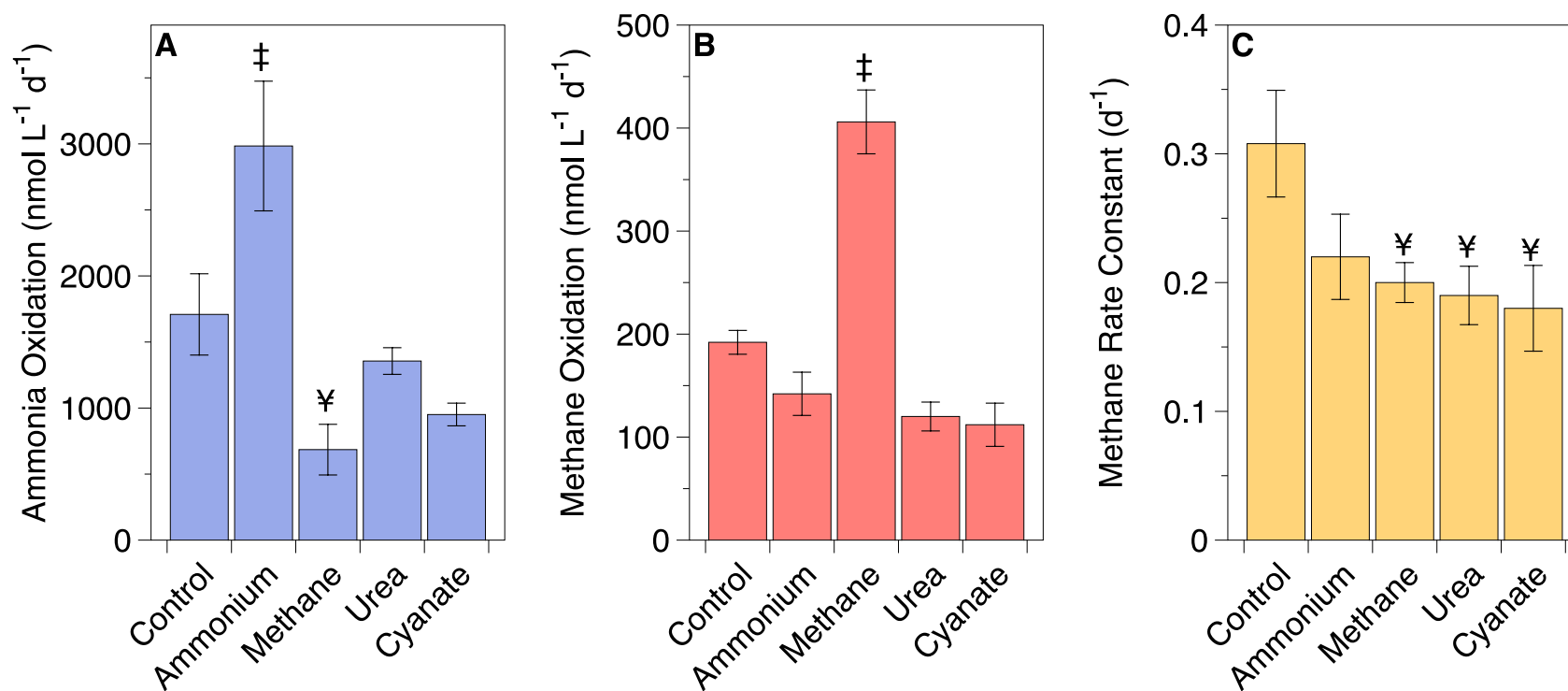


Figure 4.1. Effects of ammonium, methane, urea, and cyanate, on ammonia oxidation (A), methane oxidation (B), and methane oxidation rate constant (C) relative to unamended control at site I3. Statistical difference (based on TukeyHSD) from the control (¥) or from all other treatments and control (‡) are denoted. A p -value of < 0.05 was considered significant. Note: the control methane oxidation and rate constant were previously reported by Rogener et al 2021

Table 4.2. Growth Rates of AOA strains NF5 and D3C under experimental conditions.

Treatment	NF5		D3C	
	Growth Rate d-1	SE	Growth Rate d-1	SE
LL	0.187	0.006	0.044	0.016
LH	0.165	0.040	0.044	0.022
L	N.D.	N.D.	0.155	0.029
HL	0.274	0.021	0.384	0.039
HH	N.D.	N.D.	0.331	0.033
H	0.227	0.010	0.343	0.013
LO2	0.053	0.016	0.008*	N.D.
Average	0.181	0.022	0.225	0.037

Note: SE = standard error N.D. = not determined

* = no replicates due to decrease in cell abundance

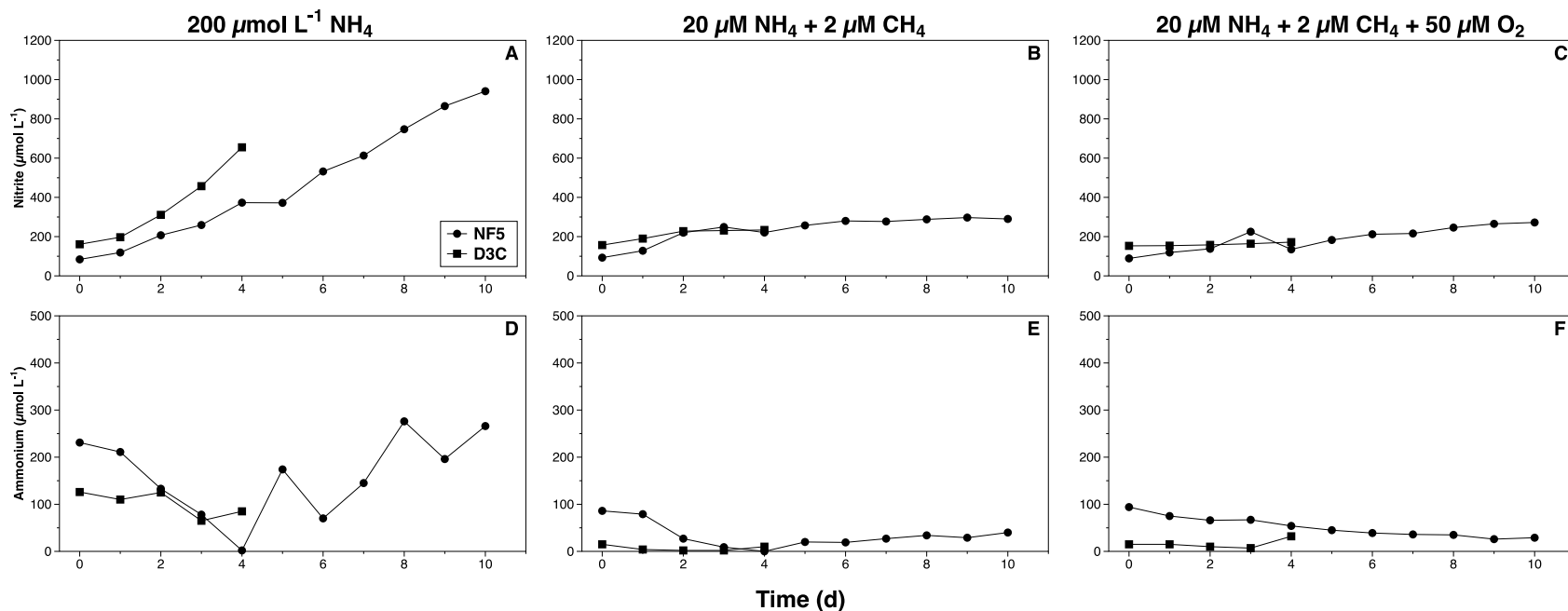


Figure 4.2. Time series of nitrite production (A-C) and ammonium drawdown (D-F). Spikes in $[\text{NH}_4]$ resulted from addition amendments of NH_4 made during incubation. D3C is represented by squares and NF5 by circles. Note the treatment regimes above (not all shown). Note differences in scales between top and bottom row

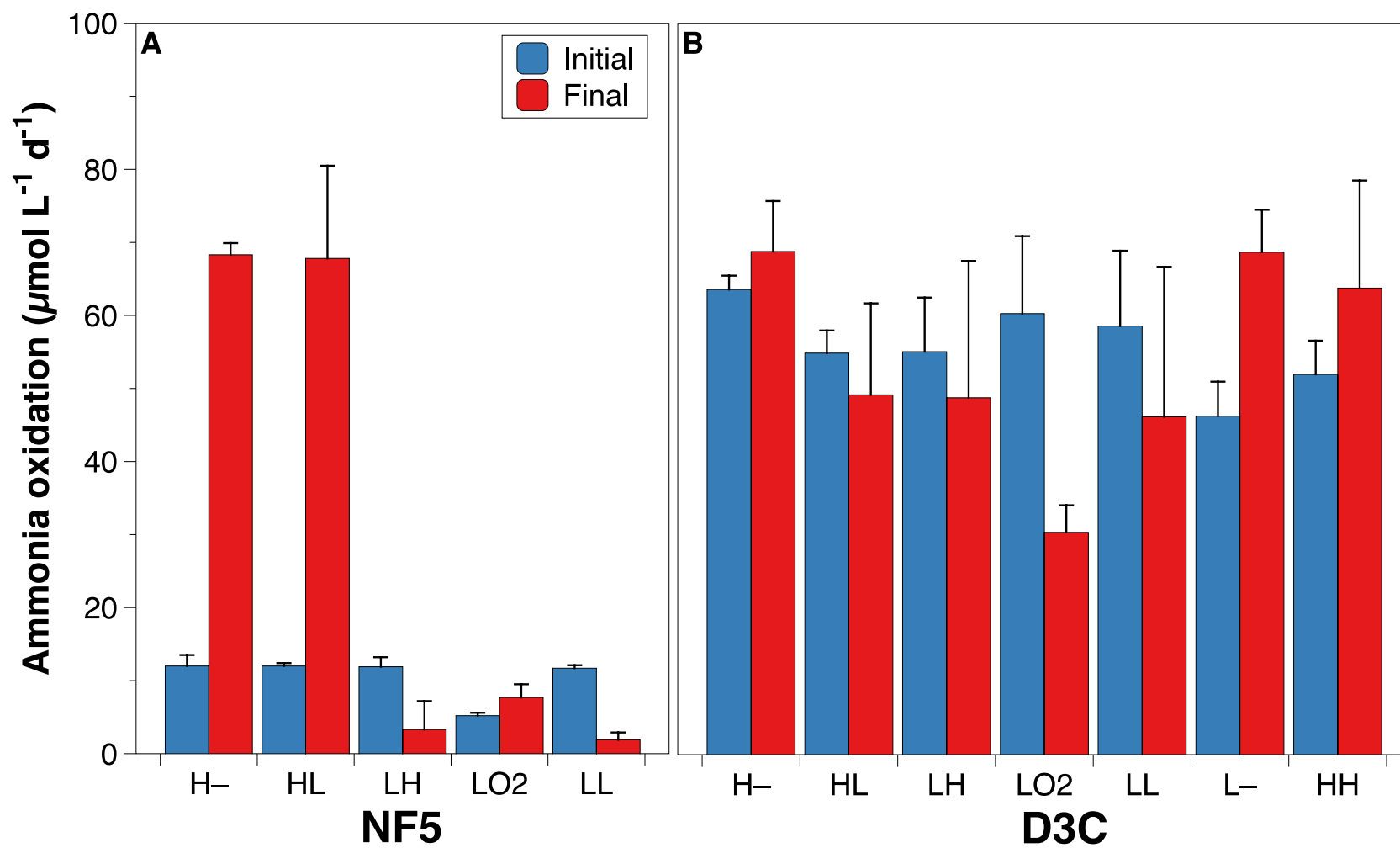


Figure 4.3. Ammonia oxidation rates by NF5 (A) and D3C (B) in all treatments. Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates

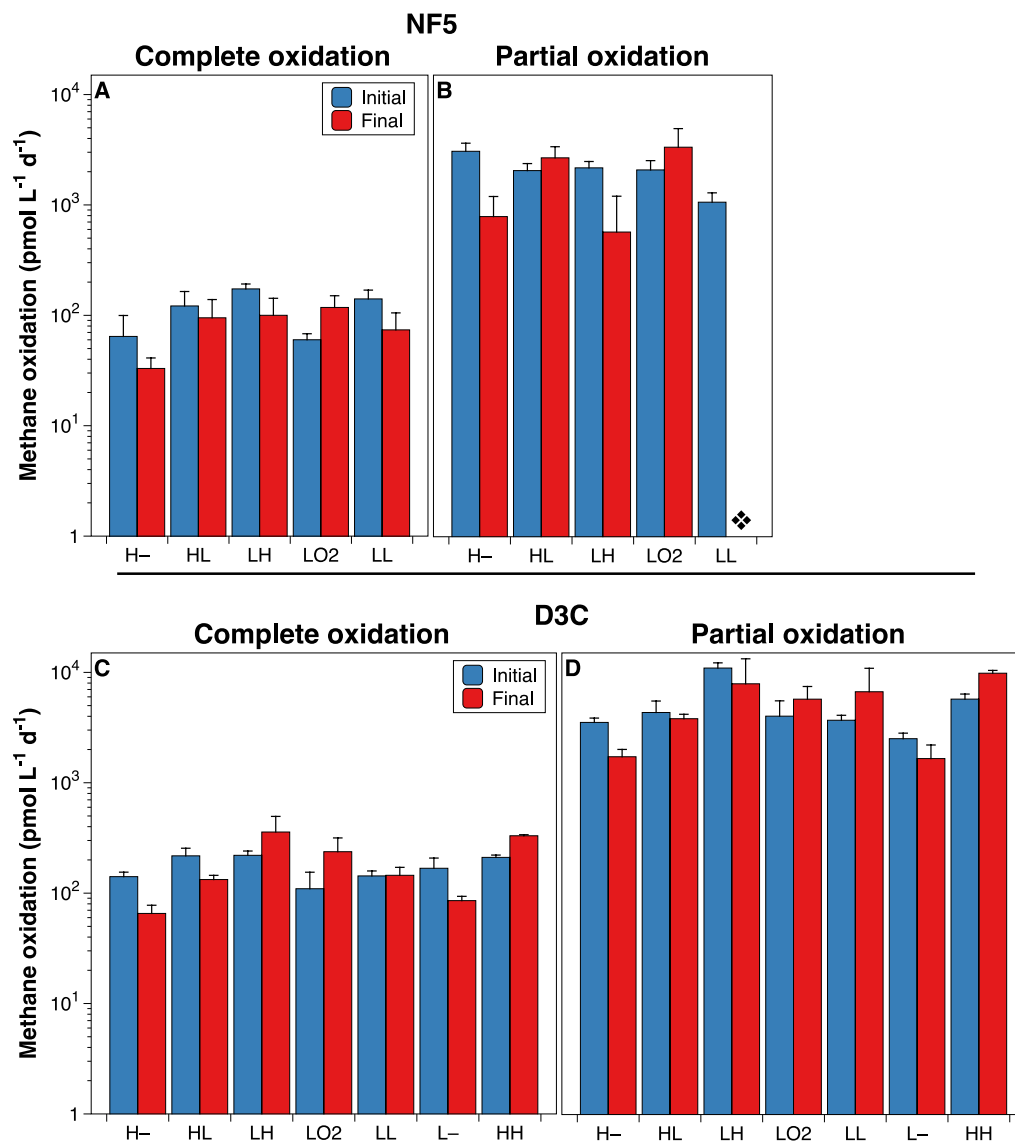


Figure 4.4. Methane oxidation (A & B) and partial methane oxidation (C & D) in each treatment. Note strain NF5 (A & C) and D3C (B & D). Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates. ♦ = Below detection limit.

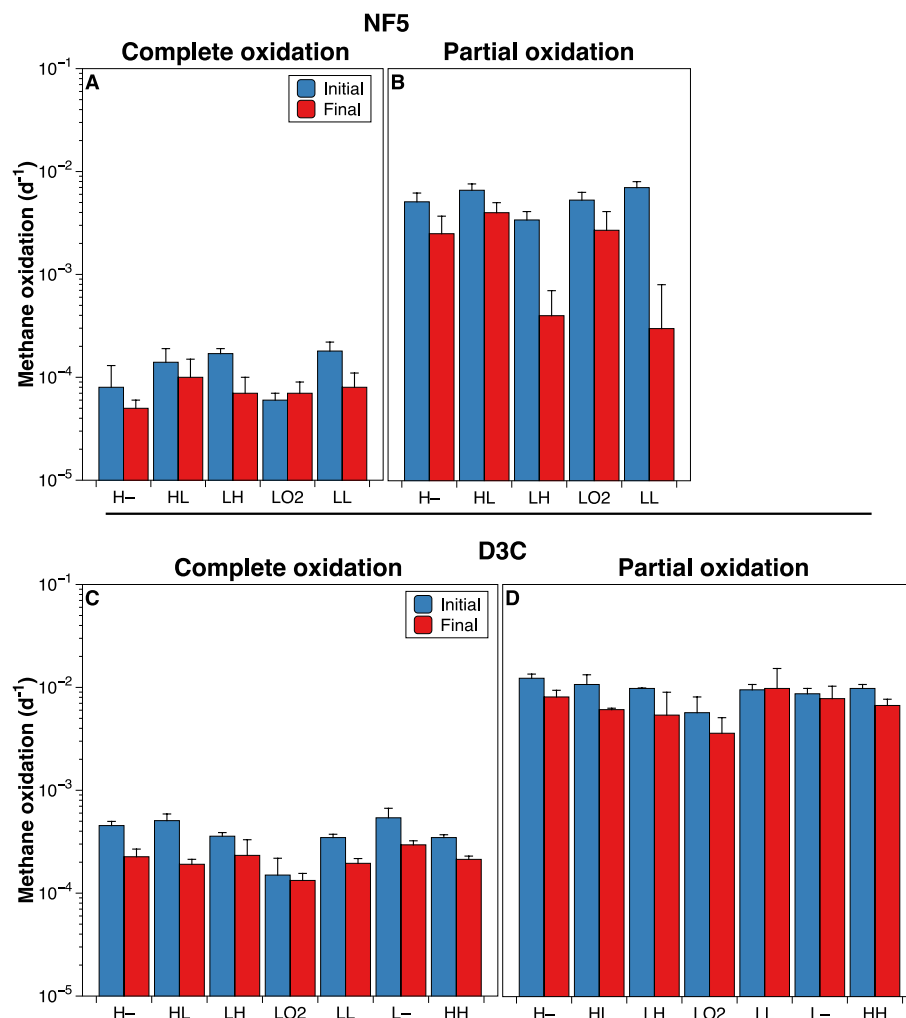


Figure 4.5. Rate constants of methane oxidation (A & B) and partial methane oxidation (C & D) in all treatments. Note strain NF5 (A & C) and D3C (B & D). Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates.

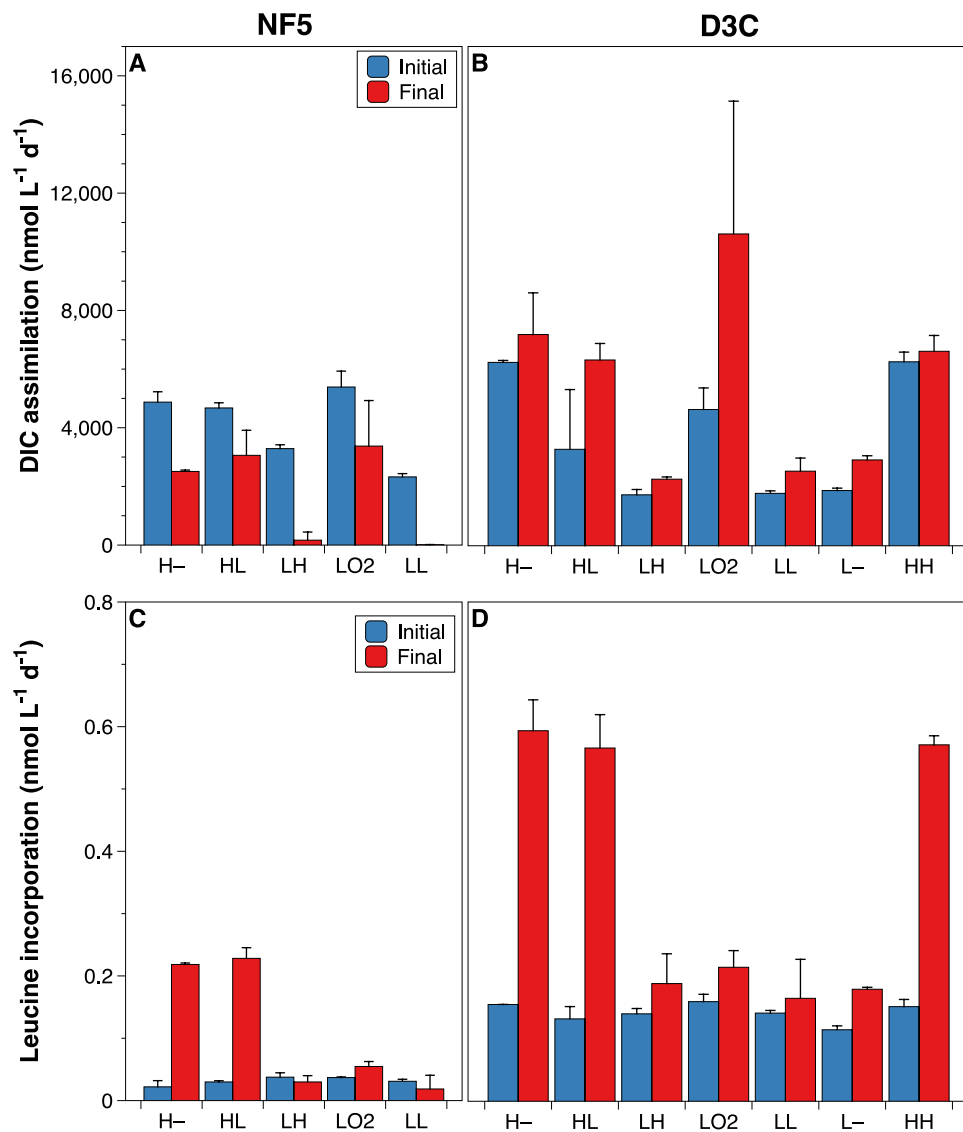


Figure 4.6. DIC Assimilation (A & B) and Leucine incorporation (C & D) in all treatments. Note strain NF5 (A & C) and D3C (B & D). Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates.

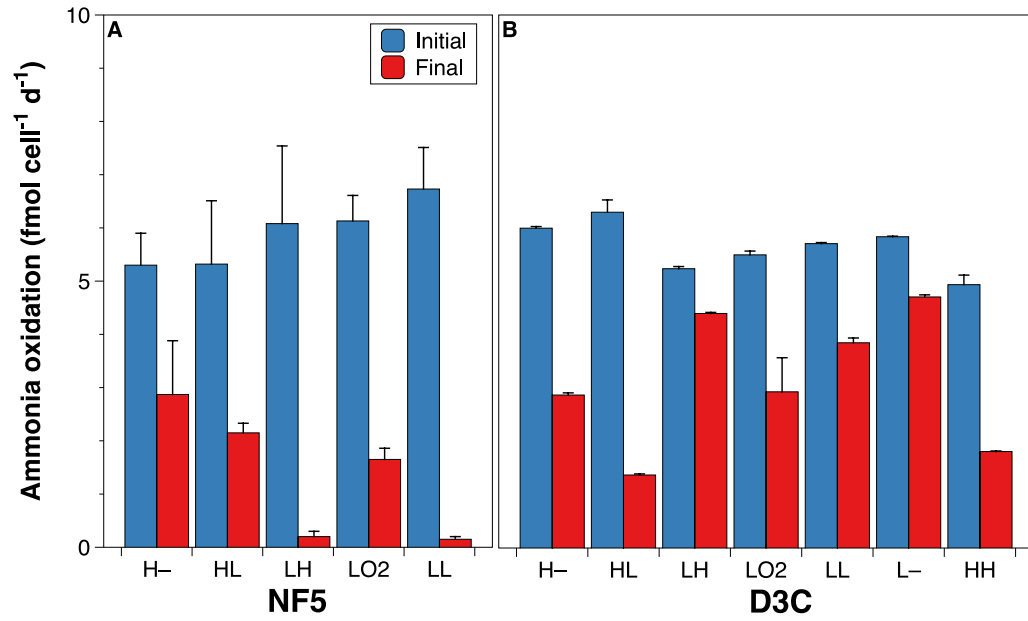


Figure 4.7. Cell specific ammonia oxidation rates by NF5 (A) and D3C (B) in all treatments. Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates.

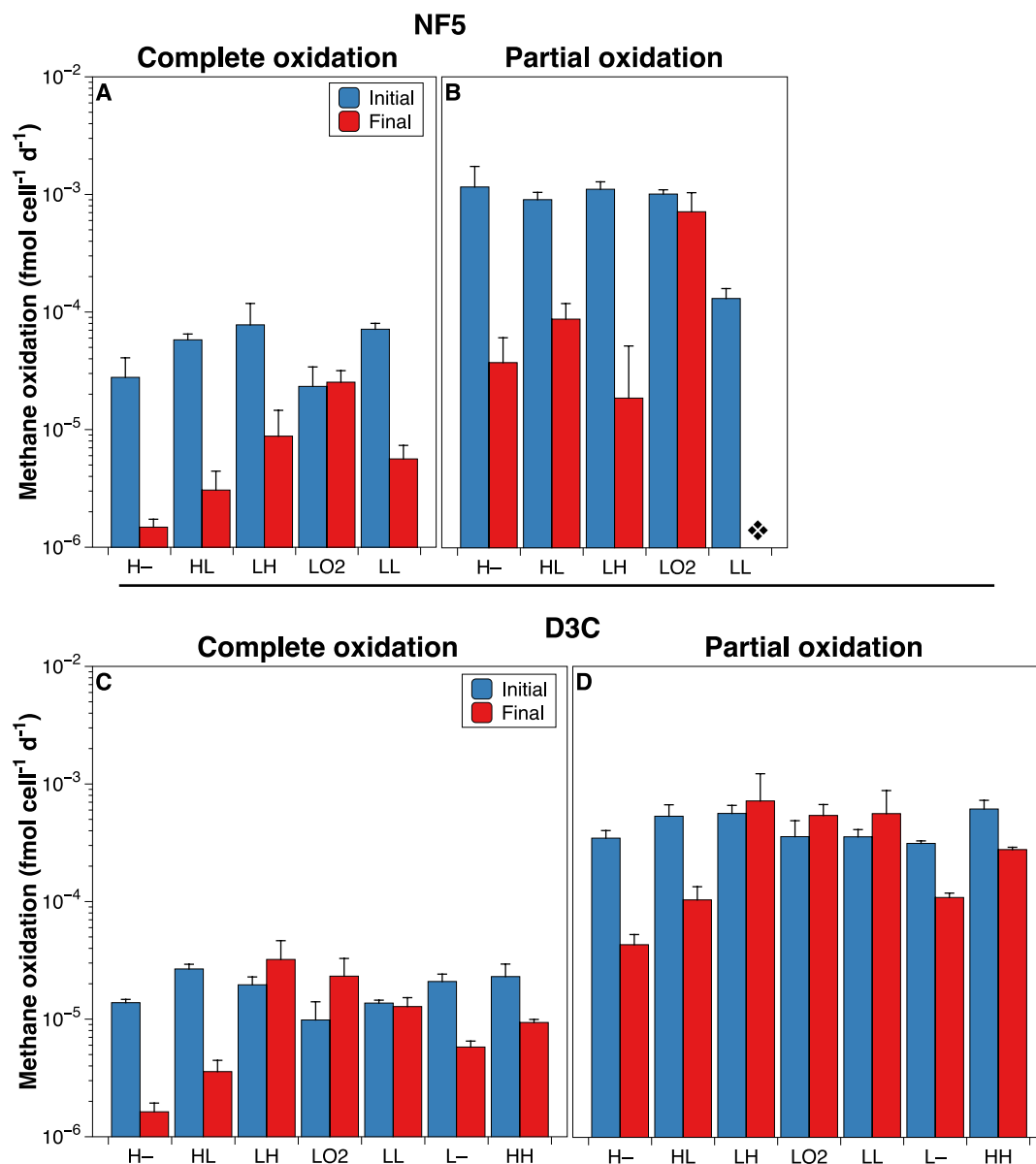


Figure 4.8. Cell specific rates of methane oxidation (A & B) and partial methane oxidation (C & D) in all treatments. Note strain NF5 (A & C) and D3C (B & D). Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates. ❖ = Below detection limit

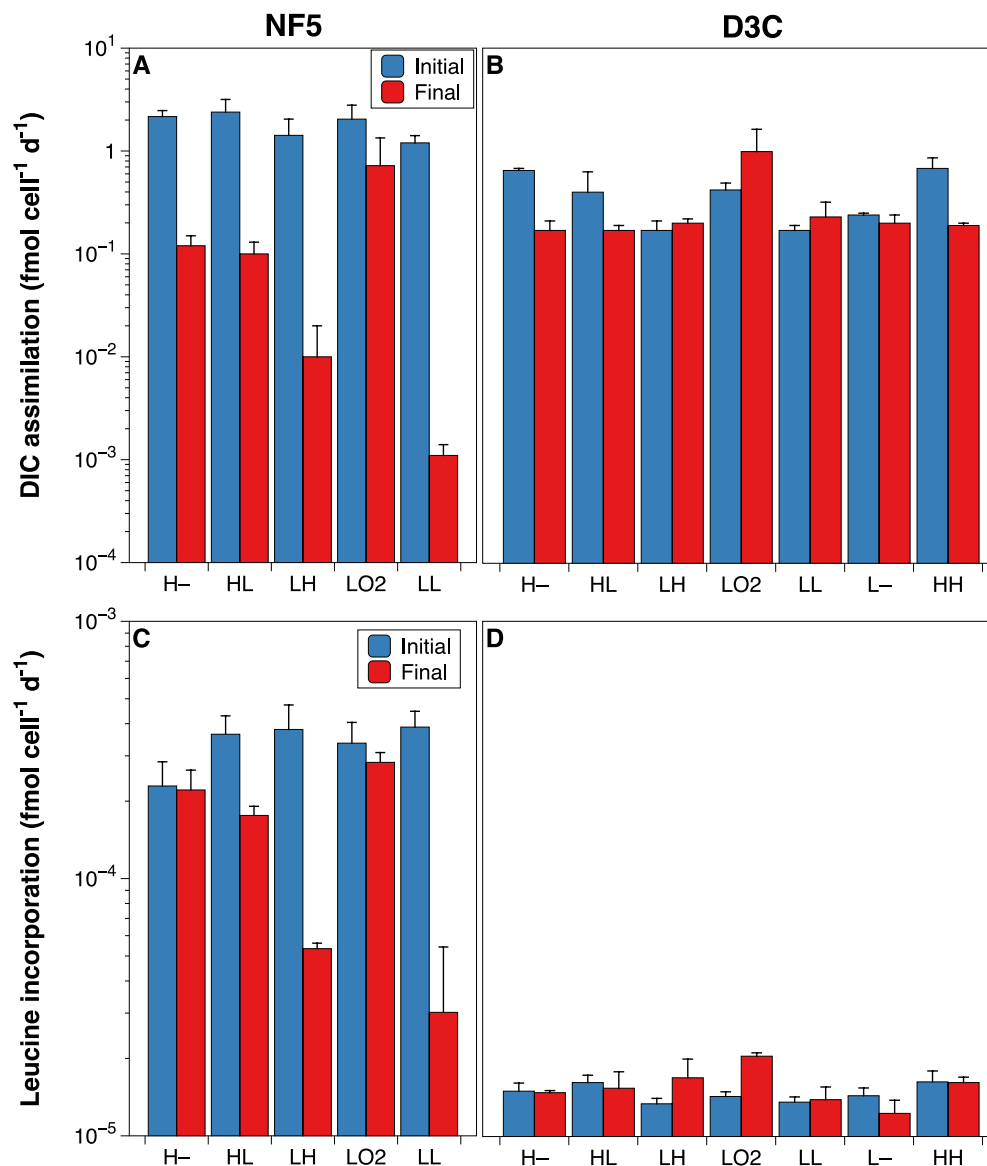


Figure 4.9. Cell specific rates DIC Assimilation (A & B) and Leucine incorporation (C & D) in all treatments. Note strain NF5 (A & C) and D3C (B & D). Measurements were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates.

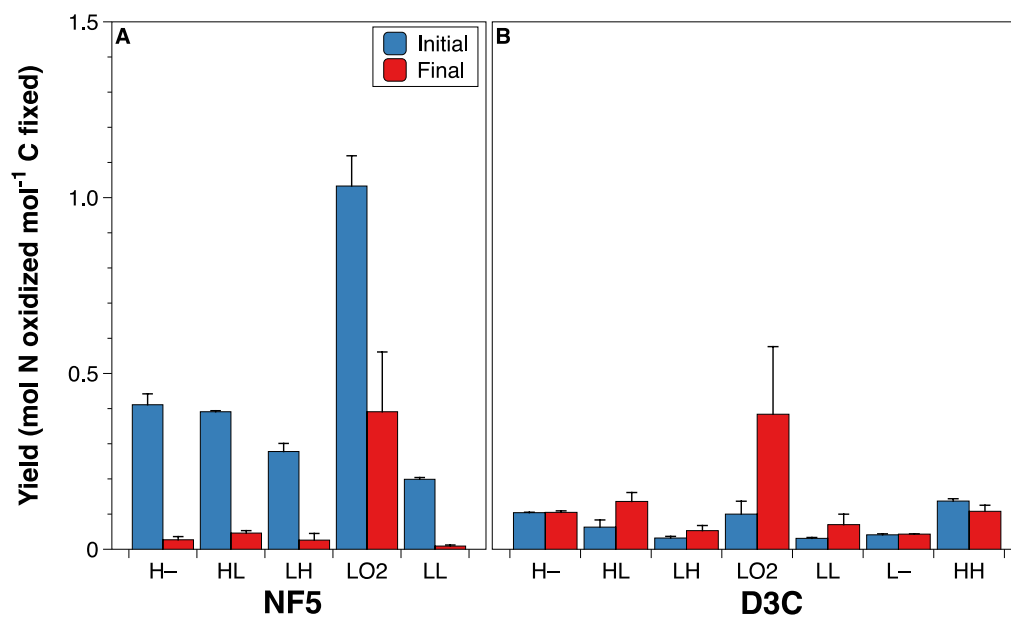


Figure 4.10. Fixed carbon yield from ammonia oxidation by strain NF5 (A) and D3C (B). Measurements used for calculations were made at the start (blue) and end (red) of incubation. Error bars represent standard error of replicates.

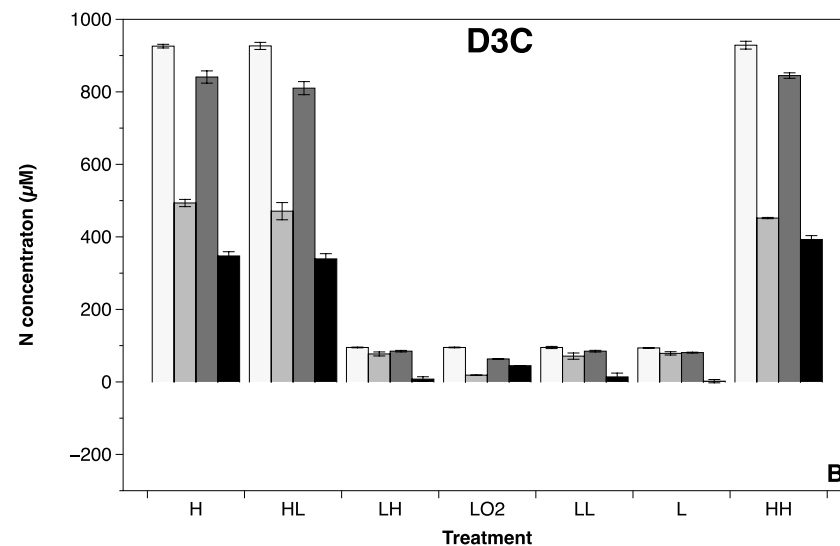
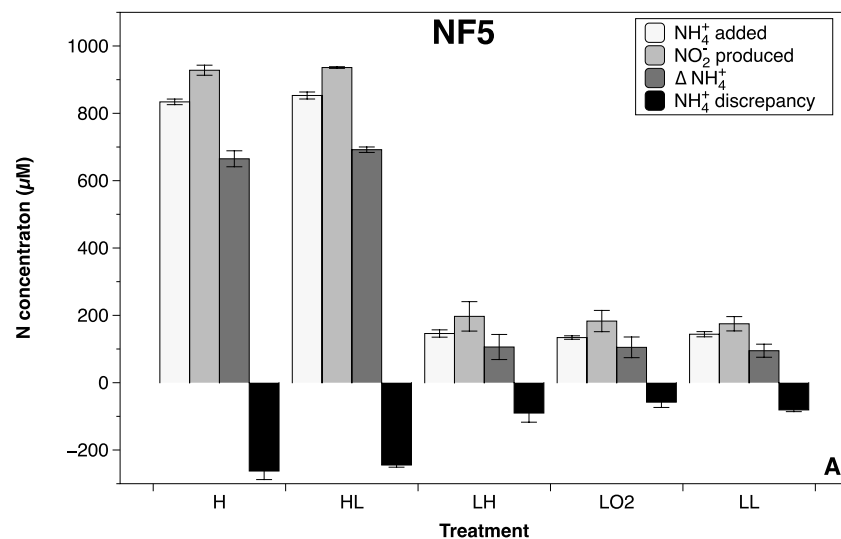


Figure 4.11. Nitrogen balance for each strain and treatment. Ammonium added includes the initial concentration plus all amendments made during incubation. Nitrite produced is the change in nitrite concentration from the initial timepoint to final. ΔNH_4^+ is the difference between total ammonium added and the final ammonium concentration. Ammonium discrepancy is the difference between nitrite produced and ΔNH_4^+ .

Table 4.3. Estimates of AOA contributions to methanotrophy from several pelagic marine environments

Location	Depth	Complete methane oxidation	Average	Partial methane oxidation	Average	Cell counts reference
North Sea/Wadden Sea	All	0.01 - 11.42	0.76 ± 0.02	0.13 - 157.01	17.53 ± 0.30	Wutcher et al., 2006
	< 150 m	0.05 - 4.79	0.91 ± 0.02	0.80 - 65.82	20.86 ± 0.37	
	> 150 m	0.03 - 2.25	0.43 ± 0.01	0.038 - 30.88	9.78 ± 0.20	
North Pacific subtropical gyre	All	0.02 - 1.80	0.34 ± 0.01	0.30 - 24.75	7.84 ± 0.09	Karner et al., 2001
	< 150 m	0.03 - 2.56	0.49 ± 0.01	0.43 - 35.25	11.17 ± 0.17	
	> 150 m	0.01 - 1.07	0.2 ± 0.004	0.18 - 14.79	4.68 ± 0.06	
Gulf of Mexico hypoxic zone	All	0.60 - 17.84	2.99 ± 1.37	24.30 - 785.10	281.32 ± 7.30	Kitzinger et al., 2019; 2020
Baltic Sea hypoxic zone	All	1.50 - 68.49	12.98 ± 1.64	60.75 - 942.10	298.49 ± 18.20	Berg et al., 2015
Southern Ocean	All	0.02 - 2.28	0.43 ± 0.05	0.97 - 31.40	9.95 ± 0.30	Church et al., 2003

All rates are presented in units of $\text{pmol L}^{-1} \text{d}^{-1}$.

Errors represent standard errors of the mean.

Ranges were calculated using the highest and lowest rate and average cell counts.

Table 4.S1. Tukey-Kramer HSD mean comparison of growth rates.

Treatments		D3C	NF5
LL	LH	N.D.	N.D.
LL	L	N.D.	–
LL	HL	<0.0001	N.D.
LL	HH	<0.0001	–
LL	H	<0.0001	N.D.
LL	LO2	N.D.	0.03
LH	L	N.D.	–
LH	HL	<0.0001	0.09
LH	HH	<0.0001	–
LH	H	<0.0001	N.D.
LH	LO2	N.D.	0.08
L	HL	0.003	–
L	HH	0.02	–
L	H	0.01	–
L	LO2	0.03	–
HL	HH	N.D.	–
HL	H	N.D.	N.D.
HL	LO2	<0.0001	0.001
HH	H	N.D.	–
HH	LO2	<0.0001	–
H	LO2	<0.0001	0.007

Note: *p*-values <0.05 were considered significant.

N.D. = no difference between means.

Table 4.S2. Overview of methane, cell and oxygen concentrations for all treatment regimes.

Treatment	Timepoint	[CH ₄] μmol L ⁻¹	Cell Counts cells mL ⁻¹	[O ₂] mg L ⁻¹
NF5				
LL	initial	0.03	1.98E+06	5.53
LH	initial	0.26	2.76E+06	5.50

	H	initial	B.D.L.	2.27E+06	5.53
	HL	initial	0.03	2.08E+06	5.48
	LO2	initial	0.35	2.82E+06	1.48
	LL	final	0.37	1.29E+07	5.01
	LH	final	0.92	1.36E+07	5.17
	H	final	B.D.L.	2.26E+07	1.97
	HL	final	0.38	3.16E+07	1.90
	LO2	final	1.04	4.65E+06	0.72
D3C					
	LL	initial	0.02	1.04E+07	3.57
	LH	initial	0.01	1.05E+07	3.51
	L	initial	B.D.L.	7.98E+06	3.57
	HL	initial	0.01	8.16E+06	3.50
	HH	initial	0.01	9.54E+06	3.43
	H	initial	B.D.L.	1.02E+07	3.33
	LO2	initial	0.08	1.12E+07	0.35
	LL	final	0.05	1.16E+07	3.40
	LH	final	0.07	1.14E+07	3.37
	L	final	B.D.L.	1.51E+07	3.36
	HL	final	0.05	3.82E+07	1.11
	HH	final	0.09	3.54E+07	1.10
	H	final	B.D.L.	4.02E+07	1.20
	LO2	final	0.29	1.04E+07	0.25

Note: B.D.L. = Below detection limit.

Table 4.S3. Quantity of moles of nitrogen removed and added to incubations.

Nitrogen sinks	μmol N ^a	Nitrogen containing compounds	Amino N?	mol N per compound	μmol compound ^a added	μmol N ^a added	μmol amino N ^a added
nitrite	19-936	ammonium	yes	1	94-929	94-929	94-929
biomass	up to 7	EDTA	no	2	7.5	15	NA
		kanamycin	yes	4	103	412	412
		streptomycin	yes	7(4)*	87	609	348
		HEPES	no	2	10,000	20,000	NA
		<i>Total</i>				10,292-11,127	21,036-21,965

^a = μmol added normalized to 1 L of culture medium

* = streptomycin contains 3 “non-amino” nitrogen atoms.

Amino N = ammonium and –NH₂ functional groups.

Ranges refer to different amounts of ammonium added to incubations.

NA = not applicable

CHAPTER 5

INVESTIGATION OF FACTORS DRIVING METHANE AND METHANOL
CYCLING IN THE GULF OF MEXICO HYPOXIC ZONE⁴

⁴Montgomery, A.M., G.C. Zhuang, H.L.Y. Choi, T.D. Peña-Montenegro, K.S. Hunter, N.N. Rabalais, S.B. Joye. A modified version to be submitted to *Global Biogeochemical Cycles*.

ABSTRACT

The Mississippi River delivers high nutrient loads to the northern Gulf of Mexico, which influences marine biogeochemical cycling. These nutrients are anthropogenically sourced and promote eutrophication and hypoxia ($O_2 < 2 \text{ mg L}^{-1}$) in stratified bottom waters. The magnitude and drivers of Gulf of Mexico hypoxia have been surveyed comprehensively and the dynamics of nitrogen cycling have been investigated extensively there. In contrast, organic matter cycling has received significantly less attention, despite its importance for the onset of hypoxia. We documented the distribution of methane, methanol, and ethanol, across the Louisiana shelf hypoxic zone. Methanol and ethanol concentrations were elevated in these hypoxic waters, ranging up to 3.5 and $1.6 \text{ } \mu\text{mol L}^{-1}$, respectively. We made measurements of methane oxidation and methanol transformations to elucidate factors driving their cycling. Oxidation of methane and methanol was widespread, and rates were up to 0.11 and $168.4 \text{ nmol L}^{-1} \text{ d}^{-1}$, respectively, while methanol assimilation was up to $0.18 \text{ nmol L}^{-1} \text{ d}^{-1}$. Chemically amended rates implicate trace metals and the interplay of methane and ammonium, as important drivers of methane cycling. Amendment with methane and ammonium influenced methanol uptake demonstrating the interconnectedness of methane and methanol metabolisms. These data represent the first direct measurements of methanol cycling in a hypoxic zone. The results presented here demonstrate the relative importance of methane and methanol in the Gulf of Mexico hypoxic zone and provide insight into the factors controlling their metabolisms.

Keywords: Gulf of Mexico, hypoxia, methane oxidation, methanol, ethanol, eutrophication, methylotrophy

INTRODUCTION

Marine hypoxia occurs when water column dissolved oxygen (DO) concentrations drop below 2 mg L^{-1} or $63 \text{ } \mu\text{mol L}^{-1}$. The frequency of hypoxic zones is on the rise globally, in line with the general decline of DO in marine environments (Breitburg et al., 2018; Diaz & Rosenberg, 2008). Hypoxic zones vary in area, stratification, and duration (*i.e.*, seasonal, diel, or permanent), but persistent hypoxia can decimate fisheries and is considered a threat to the economies which rely on them (Breitburg et al., 2018; N. N. Rabalais & Turner, 2001). One of the largest coastal hypoxic zones occurs annually along the Louisiana shelf in the northern Gulf of Mexico as a result of intense nutrient loading from the Mississippi and Atchafalaya rivers (N. N. Rabalais et al., 2002, 2007). As the number and extent of marine hypoxic zones continues to rise, a comprehensive understanding of the microbial and chemical factors that drive hypoxia will have important implications for climate change and policy decisions (R. Eugene Turner et al., 2008). In the Gulf of Mexico hypoxic zone, nitrogen cycling has been the focal point of many biogeochemical studies but improving our understanding of carbon cycling is necessary to constrain the factors driving hypoxia there.

Coastal hypoxic zones are formed from a combination of compounding factors. First, nitrogen limited primary production is stimulated when anthropogenically sourced nutrients enter the water column through riverine discharge (Dugdale, 1967; R. Eugene Turner et al., 2007; Vitousek et al., 1997). This can lead to eutrophication when decaying algal biomass is exported in the form of labile organic matter to bottom waters where it becomes available to low oxygen water and sedimentary microbial populations (Chen et

al., 2003; Nixon, 1995; Redalje et al., 1994; Wang et al., 2004). Finally, hypoxic conditions form when aerobic respiration coupled to degradation of this organic matter exceeds the input of DO in stratified bottom waters (N. N. Rabalais et al., 2007). Along the Louisiana shelf, warm spring/summer temperatures co-occur with peak riverine inputs which drive intense eutrophication and stratification in bottom waters, thus giving rise to hypoxic conditions there (Dinnel & Wiseman, 1986; Lohrenz et al., 1999; Wiseman et al., 1997). Despite our understanding that coastal hypoxia arises from the degradation of organic matter, investigations describing the details of organic matter processing are surprisingly rare.

Along the Louisiana Shelf, bottom water hypoxia, nutrient replete conditions, and carbon fluxes lead to diverse microbial communities and high rates of metabolism (Bristow et al., 2015; Gillies Campbell et al., 2019; Mason et al., 2016; Rogener et al., 2021; Thrash et al., 2017). For instance, ammonia oxidizing archaea (AOA) are abundant in hypoxic zones and mediate the first step of nitrification (Gillies et al., 2015; Könneke et al., 2005; Stewart et al., 2012; Tolar et al., 2013). AOA also play an important role in marine carbon cycling, including methane production (Heal et al., 2017; Kitzinger et al., 2019; Könneke et al., 2014; Metcalf et al., 2012) illustrating the potential importance of carbon in hypoxic zones. Furthermore, denitrification may be coupled to methanotrophy in oxygen minimum zones further demonstrating the intertwined nature of carbon and nitrogen cycling (Padilla et al., 2016; Rogener et al., 2021; Ward et al., 2009).

In contrast to the numerous studies documenting nitrogen cycling in the Gulf of Mexico hypoxic zone, carbon cycling has received much less attention. The data presented here were used to assess microbial metabolisms to better constrain methylotrophy and methanotrophy in hypoxic waters. Methanol may be an important substrate in coastal marine environments as it can be sourced from phytoplankton (Mincer & Aicher, 2016) and anthropogenic sources (Singh et al., 2001). Transport models have indicated that the bulk of labile dissolved organic carbon (DOC) is produced *in situ* from phytoplankton activity (Liu & Xue, 2020; Wang et al., 2004) suggesting that methanol could be produced in the water column of the Gulf of Mexico. Furthermore, methanol uptake (assimilation plus oxidation) was stimulated by nutrient addition in marine surface waters (Zhuang, Peña-Montenegro, et al., 2018) implying it could be an important carbon source for coastal pelagic microbial communities.

In comparison, methane dynamics have been studied in coastal hypoxic zones and rates of methanotrophy can approach $200 \text{ pmol L}^{-1} \text{ d}^{-1}$ in the northern Gulf of Mexico (Rogener et al., 2021). Denitrification, inorganic nitrogen availability, and changes in DO may each act as important drivers of methane oxidation after exposure low oxygen conditions however, their roles are not fully understood (Chronopoulou et al., 2017; Kits et al., 2015; Padilla et al., 2016; Steinle et al., 2016). Additionally, the sources of methane in hypoxic zones are unclear. Methanogenesis can be produced anaerobically in the sediments of coastal hypoxic zones (Gelesh et al., 2016; Zhuang, Montgomery, et al., 2018) but may also be produced in the oxygenated water column (Carini et al., 2014; Karl et al., 2008; Metcalf et al., 2012). Methanol is an important substrate for methanogenesis in the

Gulf of Mexico hypoxic zone sediments, demonstrating a potential interplay between benthic and pelagic metabolic processes (Zhuang, Montgomery, et al., 2018).

To better constrain our understanding of how microbes mediate carbon cycling in the Gulf of Mexico hypoxic zone, we quantified potential rates of methanotrophic and methylotrophic metabolisms using several radiotracer approaches. We profiled the chemical regime at several sites routinely impacted by seasonal hypoxia and made amendments to rate assays to assess the factors that influence cycling of these key carbon substrates. We aimed to advance previous studies of methane dynamics on the Louisiana shelf and present methane oxidation rates that build on those previously described (Rogener et al., 2021). Furthermore, we present the first measurements of dissolved methanol concentrations and metabolic rates in hypoxic waters. Our results provide novel insights into low-oxygen carbon cycling while simultaneously building on previous work in the Gulf of Mexico hypoxic zone.

METHODS

Site Descriptions and Sample Collection

Seawater samples were collected from the northern Gulf of Mexico during a research expedition onboard the R/V Pelican in July 2018 (PE1904). The purpose of this expedition was to characterize the extent of hypoxia across the Louisiana shelf, as part of the annual shelfwide monitoring cruise, led by Louisiana Universities Marine Consortium (LUMCON). Samples were collected from series of transects spanning from south of the mouth of the Mississippi River westward to south of the Louisiana-Texas border (Fig. 1).

The samples used to generate dataset presented here were collected from a subset of these stations and are outlined in Table 1. These stations were selected based on a previous study (Rogener et al., 2021) and because several sites (*i.e.*, C6C and I3) have historically exhibited consistent summertime hypoxia (N. N. Rabalais et al., 2002).

Samples were collected in 5 L Niskin bottles attached to a CTD rosette, along with a SBE 911plus conductivity-temperature-depth, and a SBE 43 dissolved oxygen (DO) sensors. Surface (< 1 m) and bottom (< 1 m above the seafloor) samples were collected at each site. At core sites (*i.e.*, A3, A5, C6C, F2A, and I3) mid-water depths were also sampled along the oxycline. Hydrographic profiles, in particular DO data, were used to select mid-water sampling depths. Winkler titrations collected from CTD casts were used to verify SBE DO sensor data and were well correlated ($R^2 \approx 0.99$).

Seawater was retrieved from Niskin bottles immediately upon recovery of the CTD on deck taking particular care to minimize atmospheric contamination. First, samples for dissolved methane concentrations were collected by filling a nitrogen purged 160 mL serum vial gently from the bottom and overflowing with 2-3 volumes to minimize atmospheric contamination. Once full, each serum vial was sealed headspace free with a blue butyl rubber stopper. Immediately after, either a 250 mL or 1 L polyethylene terephthalate glycol (PETG) bottle was filled, similarly, and capped headspace free. This bottle was used for chemical and microbial rate measurement sub-samples. Additional 1 L PETG bottles were collected in the same manner to filter for subsequent DNA sampling and sequencing and/or for amendment experiments. Bottles were stored at 4°C until sample

processing, which commenced within 30 minutes of collection. Between 1 and 2 L of seawater for metagenomic sequencing was passed through a Sterivex® filter using a peristaltic pump. Filters were flash frozen in liquid nitrogen and stored at -80°C until nucleic acid extraction.

Water column methane and chemistry

Seawater was filtered through a 0.2 µm Target® filter that was pre-rinsed with at least 10 mL of sample. Aliquots of filtered seawater were distributed for immediate shipboard analysis or preserved for later processing at the University of Georgia (UGA). Samples for dissolved organic carbon (DOC) and nutrients were collected in a 60 mL HDPE bottle and frozen at -20°C until analysis. Methanol samples were collected in a 38 mL serum vial, sealed with a blue rubber butyl stopper, and initially frozen at -20°C. Upon return to the University of Georgia, methanol samples were stored at -80°C until analysis.

Dissolved ammonium concentrations were quantified using the ortho-phthaldialdehyde (OPA) method optimized for low ammonium concentrations (Holmes et al., 1999). Briefly, 20 mL of working reagent (21 mmol L⁻¹, borate buffer; 0.063 L⁻¹, sodium sulfite; and 29 mmol L⁻¹ OPA; final concentrations) was added to 80 mL subsamples of seawater or aqueous standards. Samples were shaken and incubated in the dark for 2-3 h at ambient temperature. Samples and standards were measured with a Turner Designs fluorometer (Model 7100-000) outfitted with the ammonium optical module (Kit No. 10-303). Batches of ammonium samples were analyzed daily, and duplicate samples were collected to account for background fluorescence and matrix effects. Standards were

prepared from ACS reagent grade ammonium chloride, and the detection limit was ~ 25 nmol L⁻¹.

Methane samples were processed and analyzed following a slightly modified version of a method previously described (Magen et al., 2014). The 160 mL sample serum vials were amended with 1 mL of 8 mol L⁻¹ NaOH solution within an hour of collection to halt biological activity. The NaOH solution was added with a needle and syringe through the rubber stopper and an equal volume of sample was removed. Once the sample was well mixed, a 60 mL aliquot of sample was removed and replaced simultaneously by an equal volume of UHP N₂ (Airgas). Samples were stored upside down at room temperature for subsequent shore-based analysis. Standards were created in parallel to field samples to account for storage artifacts. Samples were analyzed back in the UGA lab within 1 month of collection. Headspace methane concentrations were determined using a gas chromatograph (SRI; model 8610C) equipped with a flame ionization detector (GC-FID) and HaySepD packed column. Injection volumes were 250 μ L for samples and standards. Aqueous and gaseous standards prepared from high purity standard of known concentration (Airgas) were used to calculate recovery and sample concentration. The detection limit of methane was 0.75 nmol L⁻¹.

Back in the UGA lab DOC concentrations were determined using a Shimadzu TOC analyzer after acidification and purging with nitrogen, as described previously (Joye et al., 2004). ACS reagent grade potassium hydrogen phthalate was used to prepare aqueous standards and the detection limit was 300 nmol L⁻¹. The sum of nitrate and nitrite (NO_x)

concentrations were determined by reduction to nitric acid with acidic vanadium (III) and quantification by chemiluminescence using an Antek 7050 nitric oxide detector (Braman & Hendrix, 1989). Nitrite concentrations were determined via the Griess method (Grasshoff et al., 1983). Nitrate concentrations were calculated as the difference in NO_x and nitrite concentrations. Standards were prepared from ACS reagent grade KNO₃ and NaNO₂ and the detection limits were 50 and 150 nmol L⁻¹ for nitrite and NO_x, respectively. Phosphate concentrations were determined with the molybdate blue method (Solórzano & Sharp, 1980). ACS reagent grade potassium orthophosphate was used to generate standard curves and the detection limit was 160 nmol L⁻¹. Dissolved concentrations of methanol and ethanol were quantified as previously described (Zhuang et al., 2014; Zhuang, Peña-Montenegro, et al., 2018). Briefly, dissolved alcohols from 7 mL samples were concentrated by purge and trap. The purge and trap system was plumbed to a GC-FID (SRI) with an MXT[®]-Q-PLOT column (30 m x 0.52 mm I.D., Restek). Analytical standards were prepared from ACS reagent grade ethanol and methanol and the detection limit was 200 nmol L⁻¹.

Rate measurements

We measured rates of methane and methanol oxidation and methanol assimilation using radiotracer techniques. Seawater was transferred from 1 L PETG bottles into vials for rate incubations using an open-bored 60 mL syringe to avoid disruption of aggregated cells (Rogener et al., 2018). Four replicate vials were filled headspace free for each discrete sample, one of which was used for a killed control. We measured methane oxidation rates using two separate radiotracer approaches, one with tritiated methane and the other with

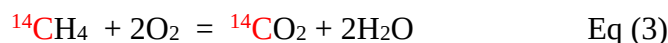
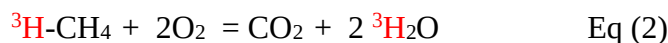
¹⁴C-labeled methane, which were incubated in 16.6 mL hungate tubes and 38 mL serum vials, respectively. Hungate tubes were sealed with a gray chlorobutyl rubber septum (Labco®) and an open top screw cap. The serum vials were sealed with a blue butyl rubber stopper and aluminum crimp-seal. Samples for methanol uptake (oxidation and assimilation) were incubated in 38 mL serum vials analogously to ¹⁴C methane oxidation rate samples. Subsampling was performed shipboard, and samples were stored at 4°C until radiotracer injection and incubation. A portable scientific van for radioisotope isolation was not available for this expedition, therefore samples were processed and incubated back at the UGA lab. Microbial respiration in sample vials may have induced anoxia, so we used previous respiration rate data from the Gulf of Mexico hypoxic zone and temperature coefficient (Q_{10}) to estimate potential DO drawdown during storage based on Equation 1:

$$Q_{10} = (R_2 / R_1)^{10/(T_2 - T_1)} \quad \text{Eq. (1)}$$

Where R_1 and R_2 are the respiration rates at temperature T_1 and T_2 , respectively. Respiration rates from 2013 to 2015 in the Gulf of Mexico hypoxic zone averaged $7.8 \pm 0.9 \mu\text{mol L}^{-1} \text{d}^{-1}$ and summer time temperatures were $\sim 28^\circ\text{C}$ (Rogener et al., 2021). We used a value of 2.5 for Q_{10} as previously reported (Yvon-Durocher et al., 2012). Samples were stored for up to 7 d before radiotracer injection.

For all rate measurements, controls were fixed prior to radiotracer injection (see below for fixation procedure). Samples were incubated in the dark at 28°C for approximately 36 h. Potential methane oxidation rates were quantified by independently

(i.e., individual vials) tracking the conversion of tritiated methane ($^3\text{H-CH}_4$) to $^3\text{H}_2\text{O}$ and $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$ in the following reactions (tracers are highlighted in red):



Tritiated methane is typically used to measure pelagic methanotrophy because it has a high specific activity ($\sim 0.7\text{ TBq mmol}^{-1}$). Tracer injections correspond to the addition of $\sim 2\text{ nmol L}^{-1}$ methane, therefore minimally impacting the *in situ* concentration. Samples were injected with $20\text{ }\mu\text{L}$ of gaseous C^3H_4 which equaled $\sim 33\text{ kBq}$ and a final tracer concentration of $\sim 3\text{ nmol L}^{-1}$. Biological activity was terminated with the addition of 1.5 mL of 37% formaldehyde. At the end of the incubation, the dissolved tracer activity was quantified for each incubation vial by counting a $100\text{ }\mu\text{L}$ sub-sample. This sub-sample was transferred by pipette, directly after opening the hungate tube and immediately prior to incubation termination, to a vial containing scintillation fluid. These sub-samples were counted on a Beckman® 6500 scintillation counter within 30 min of fixation. Simultaneously, the remaining fixed sample was vigorously purged with hydrated lab air for at least 1 h to remove unused C^3H_4 . Then, within two days, 5 mL aliquots of each sample were counted on the scintillation counter to quantify $^3\text{H}_2\text{O}$.

In contrast, $^{14}\text{CH}_4$ has a much lower specific activity ($1.85\text{ GBq mmol}^{-1}$) and thus results in a much higher final radiotracer concentration. Rates for $^{14}\text{CH}_4$ oxidation were amended with $100\text{ }\mu\text{L}$ of aqueous tracer, which equaled 23 kBq and resulted in a final

aqueous $^{14}\text{CH}_4$ concentration of $\sim 340 \text{ nmol L}^{-1}$. In some cases, tracer addition increased the methane pool by more than 10-fold. These incubations were terminated with 1.5 mL of $2 \text{ mol L}^{-1} \text{ NaOH}$, which served to halt microbial activity and trap evolved $^{14}\text{CO}_2$ as dissolved inorganic carbon. After incubation, samples were transferred to 50 mL centrifuge tubes and purged with lab air to remove unused $^{14}\text{CH}_4$. Subsequently, aqueous ^{14}C -inorganic carbon was recovering during a 4-6 h acid distillation, as described previously (Joye et al., 2004). Volatilized $^{14}\text{CO}_2$ was collected in traps containing 1.5 mL of 3-methoxypropylamine, 200 μL of $2 \text{ mol L}^{-1} \text{ NaOH}$, and a glass fiber filter wick while shaking. Traps were amended with 4.5 mL of scintillation fluid and counted on the scintillation counter. Distillation efficiency was determined by distilling ^{14}C -bicarbonate standards of a known activity, and recovery was $> 95\%$.

The biological transformation of ^{14}C -labeled methanol ($1.85 \text{ GBq mmol}^{-1}$) to $^{14}\text{CO}_2$ and microbial biomass was determined, as previously described (Zhuang, Peña-Montenegro, et al., 2018). Each sample was injected with 100 μL of aqueous tracer which resulted in a final activity of $\sim 800 \text{ Bq}$. The final tracer concentration was less than 10 nmol L^{-1} methanol to maintain near *in situ* methanol concentrations. Live replicate incubations were terminated in several steps. First, a 20 mL sub-sample was removed through the stopper by needle and syringe while displacing with air. The sample remaining in the incubation vial was then amended with 1 mL of 100% trichloroacetic acid through the stopper to determine the oxidation rate. The removed sub-sample was transferred to a 50 mL centrifuge tube and killed with 1 mL of 100% TCA to determine the methanol assimilation rate.

The oxidation of ^{14}C -methanol to $^{14}\text{CO}_2$ was determined by the quantitative strip and trap method. Samples were acidified by the addition of TCA and the oxidation product converted to $^{14}\text{CO}_2$ via a gentle stream of nitrogen gas. Each sample vial was connected to several traps in series via gas tight tubing and stripped for 1 h. Two intermediate traps contained 1 mol L^{-1} phosphoric acid which were kept on ice to remove unused tracer while simultaneously permitting the passage of $^{14}\text{CO}_2$. The final trap was 1.5 mL of 3-methoxypropylamine in 4.5 mL of scintillation fluid. Recovery efficiency was checked regularly with known activities of ^{14}C -bicarbonate, and was consistently $> 90\%$. Assimilation activity was determined by passing 20 mL of sample through an $0.2 \mu\text{m}$ polyethersulfone filter. Each filter was rinsed 3 times with 0.5% TCA in 35‰ NaCl solution to remove unused tracer and residual ^{14}C -DIC without rupturing cells. Each filter was collected into a scintillation vial filled with 6 mL of scintillation fluid and counted on the scintillation counter.

Rate calculations

Turnover rate constants (k) were calculated as the fraction of tracer used per unit time.

$$k = f/t \quad \text{Eq (4)}$$

The fraction of tracer used (f) was defined as ^{14}C -product (*i.e.*, $^{14}\text{CO}_2$ or ^{14}C -biomass) or $^3\text{H}_2\text{O}$ measured divided by the total amount of tracer added, initially. Turnover constants

(d⁻¹) were calculated by dividing this value by incubation times (d). The activity of each product was corrected by subtracting the measured activity of killed controls, which were similar to activity in instrument blanks.

The final rate was calculated by multiplying the *in situ* concentration of the relevant substrate (*i.e.*, methane or methanol) by the turnover constant:

$$\text{Rate} = k * [\text{substrate}] \quad \text{Eq (5)}$$

Rates were expressed in units of pmol L⁻¹ d⁻¹ or nmol L⁻¹ d⁻¹ for methane oxidation and methanol uptake, respectively.

Experimental metabolic rate amendments

Previous studies in the Gulf of Mexico hypoxic zone have demonstrated high abundances of ammonia oxidizing archaea (Gillies et al., 2015; Kitzinger et al., 2019, 2020), unique methane and ammonium dynamics, and potential for AOA to oxidize methane (See Chapter 5). Therefore, we made amendments of methane and ammonium to further assess the relationship between these two substrates. We measured methane oxidation and methanol uptake after amendment with methane (final concentrations 0.25 or 2 µmol L⁻¹), ammonium (final concentrations 2 or 20 µmol L⁻¹), and streptomycin and kanamycin (final concentrations 1 mg L⁻¹). These antibiotics are commonly used for enrichment of archaeal nitrifiers (Könneke et al., 2005), which we suspect oxidize methane in the Gulf of Mexico hypoxic zone.

Additionally, we assessed the effect of two trace metals (iron and copper) on the metabolisms of methane and methanol. Final concentrations of either 50 or 500 nmol L⁻¹ were added to rate samples in addition to either 20 µmol L⁻¹ ammonium or 2 µmol L⁻¹ methane. Experimentally amended rate samples were prepared in a 1 L PETG, that was collected as described above. Experimental alterations were made by injecting aqueous amendments through the septum with a needle and syringe. These samples were kept at 4°C for at least 24 h before sub-samples were aliquoted into rate vials as described.

Correlations and statistical analyses

Statistical analyses were performed in R (version 3.4.4). Spearman's correlation coefficients were used to evaluate relationships between rates and relevant chemical constituents. Two-way analysis of variance (ANOVA) and post-hoc Tukey HSD were used to assess differences in metabolic rates with respect to site and depth. Depths were binned as surface (< 1 m depth), bottom (< 1 m above seafloor), and mid (all other depths in between). Comparisons between treatment experiments were made with the pairwise Student's t-test function in R. All values were log-transformed prior to analysis and for pairwise t-tests, *p*-values were adjusted using the Benjamini and Hochberg false discovery rate method (Benjamini & Hochberg, 1995).

RESULTS

Water column chemistry

Seawater samples were collected from several depths at 9 stations in the northern Gulf of Mexico during summer 2018. Hydrographic and chemical data were measured as part of the annual hypoxia monitoring program. Bottom water (< 1 m above seafloor) DO concentrations were less than $63 \mu\text{mol L}^{-1}$ at stations A3, C6C, and F2A, indicating hypoxic conditions at the time of sampling. Nitrate concentrations ranged from below detection limit at site I3 and $13 \mu\text{mol L}^{-1}$ at site A5 (Fig. 2-6). Nitrite concentrations were generally lower than nitrate and ranged from below to detection at several sites to $\sim 2.5 \mu\text{mol L}^{-1}$ at site C6C (Fig. 2-6). Dissolved ammonium concentrations were less than $1.5 \mu\text{mol L}^{-1}$ at all sites and in several cases were below detection (*e.g.*, Fig. 5A). At site C6C, ammonium concentrations were highest, ranging up to $\sim 1.3 \mu\text{mol L}^{-1}$ in the bottom water. Phosphate was detectable at all stations and depths with concentrations reading up to $\sim 1.6 \mu\text{mol L}^{-1}$. Fluorescence, temperature, and salinity were all measured via CTD. Fluorescence ranged from < 0.5 to $3.98 \mu\text{g L}^{-1}$ across all sites. Several sites exhibited a spike in fluorescence between 5 and 10 m water depth (Fig. 3 & 4). Seawater temperatures were generally higher in shallower waters, ranging from 22.7 to 30.5°C. Salinity was generally higher in deeper water, ranging from 27.9 to 36.4. The average temperature and salinity were $26.9 \pm 0.5^\circ\text{C}$ and 34.2 ± 0.4 , respectively across all stations (Table 1).

Distribution of dissolved organic carbon and methane

Dissolved organic carbon (DOC) concentrations in the Gulf of Mexico hypoxic zone ranged from 79 to $1133 \mu\text{mol L}^{-1}$. The DOC concentrations were fairly consistent

across all stations and depths, except the bottom water at station C6C (Fig. 4C), which contained at least ten times more DOC than any other station. Despite the high DOC concentration in the bottom water at C6C, DOC was significantly (Spearman's correlation; p -value < 0.001; Fig. 16) and negatively correlated with depth. Bottom waters at stations D5 (35 m depth) and A3 (16 m depth) exhibited the lowest DOC concentrations (Fig. 2C and Table 2).

Methane concentrations ranged from 23 nmol L⁻¹ in the surface water of station F5 to 132 nmol L⁻¹ in bottom water of station F2A. In surface waters, average methane concentrations were 51 ± 10 nmol L⁻¹, which was significantly lower than mid and bottom water concentrations (Fig. 7A). Methane concentrations in hypoxic waters averaged 104 ± 17.6 nmol L⁻¹ but were not significantly different than other bottom water methane concentrations. Stations exhibiting hypoxia (A3, C6C, and F2A) contained marginally more methane than other stations. However, pairwise comparisons of stations indicated that methane concentrations were not significantly different between sites. We calculated fluxes of methane to the atmosphere, which varied between sites. At station A5, the methane flux was 33.6 $\mu\text{mol m}^{-2} \text{d}^{-1}$, the lowest flux calculated. The largest flux was at station A3 (180 $\mu\text{mol m}^{-2} \text{d}^{-1}$) and the average methane flux was 77.9 ± 16.3 $\mu\text{mol m}^{-2} \text{d}^{-1}$ across all stations sampled during summer of 2018.

Alcohols were detected at several sites and depths in the hypoxic zone. Methanol and ethanol concentrations reached up to 3.4 and 1.6 $\mu\text{mol L}^{-1}$ (Fig. 7B & C), respectively, but most alcohol concentrations were below detection limit (0.2 $\mu\text{mol L}^{-1}$). Of detectable

values, average ethanol concentrations were $0.85 \pm 0.1 \mu\text{mol L}^{-1}$ and average methanol concentrations were $0.73 \pm 0.1 \mu\text{mol L}^{-1}$ (Table 2). Alcohol concentrations did not vary significantly across site or depth or as a function of hypoxia (Fig. 7).

Dynamics of methane and methanol metabolisms

Using radiotracer techniques, we assessed the oxidation of methane and methanol, and the assimilation of methanol in the water column of the Gulf of Mexico hypoxic zone. Methanotrophic activity using tritiated ($^3\text{H-CH}_4$) methane was detected at all sites, and methane oxidation rates ranged from 9.2 ± 2.4 to $112.4 \pm 9.6 \text{ pmol L}^{-1} \text{ d}^{-1}$. Significantly higher methane oxidation rates (ANOVA, p -value < 0.05) were observed in mid and bottom waters relative to in surface waters. Additionally, methane oxidation rates at several sites, in particular, stations A3 and C6C (Fig. 9), were significantly higher (ANOVA, p -value < 0.05) than other sites. Notably, both these sites exhibited bottom water hypoxia at the time of sampling. Turnover constants of methane oxidation ranged from 0.003 to 0.0013 d^{-1} . Similar to methane oxidation rates, turnover constants were lower in surface waters relative to mid and bottom waters (ANOVA, p -value < 0.05 , Fig. 8A). The highest turnover constant was observed at station C6C at 6 m water depth, but comparably high turnovers were observed in the bottom water there ($0.0010 \pm 0.0002 \text{ d}^{-1}$). Virtually no significant differences were observed for turnover constants between sites.

Methane oxidation rates measured with $^{14}\text{CH}_4$ were surveyed at three stations and methanotrophy was confirmed in all samples. Rates of ^{14}C -methane oxidation varied significantly with respect to station and depth (ANOVA, p -value < 0.05 , Fig. 9B). The

highest oxidation rate of 3,454 pmol L⁻¹ d⁻¹ was observed in the surface water at station F2A (Fig. 9B). In contrast, bottom water at station I3 exhibited the minimum rate of 1,012 pmol L⁻¹ d⁻¹. Turnover constants of ¹⁴C-methane oxidation followed similar patterns to the rate. These turnover constants ranged from 0.0023 to 0.008 d⁻¹ (Fig. 8B), corresponding to the minimum and maximum rate observations. Across each station measured for ¹⁴C-methane oxidation, turnover constants were significantly different (ANOVA, *p*-value < 0.05). Significant differences were also observed each depth, except for the surface and bottom waters.

We used ³H and ¹⁴C labeled methane to independently quantify methane oxidation and significant differences were observed between these two approaches. Paired t-tests indicated that turnover constants and rates were significantly different between paired rates (T-test, *p*-value < 0.001). For example, the average tritiated methane oxidation rate (41.84 ± 5.4 pmol L⁻¹ d⁻¹) was ~ 60-fold lower than the average rate determined with the ¹⁴C method (2586.7 ± 343.7 pmol L⁻¹ d⁻¹). Similarly, the average turnover constant for ¹⁴C oxidation (0.006 ± 0.0008 d⁻¹) was an order of magnitude higher than tritiated methane oxidation (0.0006 ± 0.00005 d⁻¹). These differences were further reflected by tritiated methane oxidation constituting up to 36.5% and 3.7 % of ¹⁴C methane oxidation turnover and rates, respectively (Table 2). These two approaches did not exhibit any correlation (Fig. 16).

Similar to methane, active methanol oxidation and assimilation were observed at nearly all sampled stations in the Gulf of Mexico hypoxic zone. The highest rate constant

for methanol oxidation was $0.06 \pm 0.01 \text{ d}^{-1}$ at station I3 in the bottom water (Fig. 10A). The highest assimilation rate constant was observed at station C6C (10 m depth) and was $0.0012 \pm 0.0003 \text{ d}^{-1}$ (Fig. 10B). Methanol oxidation turnover constants were less variable than assimilation turnover constants, which spanned several orders of magnitude, including below detection. Rates of methanol oxidation ranged from $0.05 \text{ nmol L}^{-1} \text{ d}^{-1}$ to $168.5 \text{ nmol L}^{-1} \text{ d}^{-1}$ at in surface waters at site A5 and I3, respectively (Fig. 11A). Assimilation rates spanned from below detection at station I3 to $0.178 \pm 0.01 \text{ nmol L}^{-1} \text{ d}^{-1}$ at station F2A (Fig. 11B). Interestingly, the lowest assimilation rate coincided with the highest oxidation rate at station I3 (Fig. 11). Methanol assimilation represented from 0.4% to 2.3% of methanol oxidation (Table 2).

Amendment experiments

Compared to unamended controls, additions of methane, ammonium, iron, copper and antibiotics altered microbial metabolisms with respect to substrate, station, and depth (Fig. 12, 13 & Fig. S1). Methane oxidation with tritium was significantly stimulated in nearly all treatments (t-test, p -value < 0.05 , Fig. S1A & B) when considering all sites and depth. In particular, rates in methane amended treatments were up to 70 times higher than the control. This trend was exaggerated in at stations I3 and C6C, where $2 \text{ } \mu\text{mol L}^{-1}$ methane additions increased methane oxidation rates by more than 200 times (Fig. 12). Elevated methane turnover constants were observed with the addition of 500 nmol L^{-1} copper plus $20 \text{ } \mu\text{mol L}^{-1}$ ammonium. For example, at stations A5 and F2A turnover constants were up to 3.2 times higher than the controls (Fig. 14). In contrast, methane oxidation rates from ^{14}C -methane were generally inhibited or unaffected by the same

additions. For example, ^{14}C -methane turnover constants and rates were $\sim 30\%$ of unamended controls (Fig. 15). The addition of methane ($200\ \mu\text{mol L}^{-1}$ or $2\ \mu\text{mol L}^{-1}$) significantly increased ^{14}C methane oxidation (t-test, $p\text{-value} < 0.05$, Fig. 13A & B).

Methanol oxidation and assimilation were generally less affected by amendments than methane oxidation. For all sites and depths, methanol oxidation rates were significantly lower than controls in the presence of $20\ \mu\text{mol L}^{-1}$ ammonium plus $0.25\ \mu\text{mol L}^{-1}$ methane (t-test, $p\text{-value} < 0.05$, Fig. S1). In each metal treatment, methanol oxidation rates were diminished by a factor of ~ 4 (Fig. 15D). Methanol assimilation rates were ~ 3 times higher in bottom waters with the addition of $5\ \text{nmol L}^{-1}$ copper plus $2\ \mu\text{mol L}^{-1}$ methane (Fig. 15). In surface waters, $20\ \mu\text{mol L}^{-1}$ ammonium plus $2\ \mu\text{mol L}^{-1}$ methane increased methanol assimilation turnover constants and rates by a factor of 2 and 34, respectively (Fig. 13E & F). In contrast, the addition of antibiotics reduced all methanol assimilation to levels that were below detection (Fig. 13E & F).

Correlations

We evaluated relationships between the metabolic rate measurements and water column chemistry presented here in an effort to constrain environmental controls on methane and methanol cycling in the Gulf of Mexico hypoxic zone. Phosphate, nitrate, nitrite, and chlorophyll concentrations were each correlated with methane oxidation rates and turnover constants (^3H and ^{14}C methods) and methanol assimilation turnover constants, but not methanol oxidation (Fig. 16). Methane oxidation rates and turnover constants were not correlated between methods. Methane concentrations exhibited a positive correlation

with both methane oxidation rates and ^{14}C methane turnover constants. Methanol and methane concentrations were negatively correlated. Methanol oxidation rates were positively correlated with methanol and ethanol concentrations, methanol assimilation rates, but negatively correlated with methanol assimilation turnover and ^3H methane oxidation rates. Methanol was significantly and positively correlated with ethanol, methanol assimilation turnover constants and negatively correlated with chlorophyll and methane concentrations, and methane turnover constants.

DISCUSSION

The northern Gulf of Mexico experiences annual hypoxia as a result of anthropogenic nutrient loading from the Mississippi and Atchafalaya rivers (N. N. Rabalais et al., 2002). Over the last century, the use of fertilizer for agricultural purposes has increased dramatically (Diaz & Rosenberg, 2008) and nutrient-enriched runoff fuels eutrophication in coastal zones (Lohrenz et al., 1999; R. Eugene Turner et al., 2007). The modes of labile DOC metabolism during hypoxic episodes remain unclear. We sampled several historically hypoxic stations across the Louisiana shelf and used water column chemistry data to contextualize methane and methanol metabolisms and investigate the factors controlling their cycling. The results presented here mark the first measurements of methylotrophy in a coastal hypoxic system, provide further insight into pelagic methanotrophy, and underscore the importance of methane and methanol in marine hypoxic zones.

The extent and duration of summer hypoxia across the Louisiana shelf has been well documented for several decades; the hypoxic zone measured 7,040 km² in 2018 (N. N. Rabalais et al., 2002; N. Rabalais & Turner, 2018; R. Eugene Turner et al., 2007). This was one of the smallest annual hypoxic zones in the Gulf of Mexico since monitoring began in 1985. Surprisingly, monitoring in Baton Rouge indicated Mississippi River discharge rates were relatively high (up to 38,200 m³ s⁻¹) and NO_x concentrations were comparable to averages in recent years (116.1 ± 3.2 μmol L⁻¹; United States Geological Survey – National Water Information System). These values correspond to nitrogen loading rates comparable to those previously described (~60 kg N s⁻¹; Rogener *et al.*, 2021). Despite this, there are several factors that could have influenced the observed reduction in the hypoxic zone. The peak discharge rate was observed in March 2018, which is several months earlier than normal. Additionally, cross-shelf winds were observed during summer monitoring, which can drive surface waters offshore and mixing of the water column (N. N. Rabalais et al., 2007).

Dynamics of alcohols and methane

Methanol and ethanol were detected at several sites and depths across the Gulf of Mexico hypoxic zone. Relatively few measurements have been made of these labile carbon sources in pelagic marine environments, but observed concentrations have generally been less than 1 μmol L⁻¹ for both (Avery et al., 2016; R. Beale et al., 2010; Dixon et al., 2011b; Yang et al., 2013), in line with our observations (Fig. 7). However, one exception to this was in the surface waters at station I3, where the methanol concentration was up to ~3.5 μmol L⁻¹, which is approaching the concentration observed in a hydrothermal vent plume

(Chapter 1 of this dissertation). Ethanol and methanol were positively correlated with each other (Fig. 16), as observed previously in marine sediments (Zhuang et al., 2014).

Ethanol concentrations were marginally higher than methanol, which could be due to differences in inputs or removal rates of each alcohol. The magnitude of their exchanges between surface waters and the atmosphere are unclear (Avery et al., 2016; Rachael Beale et al., 2013; Willey et al., 2019; Yang et al., 2013). For example, the use of ethanol as a fuel source has increased its concentration in rainwater, which could be deposited in surface waters (Willey et al., 2019). Furthermore, ethanol is a product of fermentation and can be produced by algae (Eichler & Schink, 1984; Ueno et al., 1998), representing a potential biological source in the water column across the Louisiana shelf. Similarly, atmospheric deposition and marine phytoplankton are potential sources of methanol in the Gulf of Mexico hypoxic zone (Rachael Beale et al., 2013; Heikes et al., 2002; Mincer & Aicher, 2016; Yang et al., 2013). Specifically, methanol was produced by marine algae in culture (Mincer & Aicher, 2016), indicating that the services of phytoplankton could increase methanol availability and stimulate hypoxia formation (N. N. Rabalais et al., 2007). However, methanol concentrations were negatively correlated with chlorophyll concentrations (Fig. 16), suggesting that algal biomass may not have been the primary source of methanol. Furthermore, the strong positive correlation between methanol and ethanol (Fig. 16) could suggest that they were sourced similarly. Taken together, this implies that atmospheric deposition could be an important source of methanol and ethanol to the northern Gulf of Mexico hypoxic zone.

Environmental conditions in the Gulf of Mexico hypoxic zone suggest that alcohols could be removed through atmospheric exchange or microbial metabolisms. The fluxes of methanol and ethanol between surface waters and the atmosphere are not fully understood (Avery et al., 2016; Heikes et al., 2002; Yang et al., 2013), especially on the Louisiana shelf where variable wind speeds (https://www.ndbc.noaa.gov/station_page.php?station=SPLL1) could influence exchange (Rachael Beale et al., 2013; Wanninkhof, 2014). Alternatively, several biological processes could be important sinks of methanol and ethanol in the Gulf of Mexico hypoxic zone. Microbial uptake rates of methanol are discussed in detail below, but ethanol could also be an important substrate for microbial metabolisms. For instance, denitrification can be coupled to ethanol oxidation (Istok et al., 2007), and denitrification was recently shown to be important in hypoxic waters (Rogener et al., 2020). Ethanol can also be oxidized microbially to acetate (Metje & Frenzel, 2005), an important carbon substrate in the pelagic marine environment (Ho et al., 2002; Wu et al., 1997; Zhuang et al., 2019). The conversion of ethanol to acetate is considered to be obligately anaerobic, which could imply a source from anoxic sediments or sinking particles.

Methane dynamics were recently examined in the Gulf of Mexico hypoxic zone and our data were in general agreement with those observations (Rogener et al., 2021). Methanogenesis appears to be an important methane source during the onset of coastal hypoxia as algal biomass is degraded providing labile carbon to methanogens (Bange et al., 2010; Steinle et al., 2017). Additionally, DO consumption and DOC degradation rates are highest near the sediment water interface during coastal hypoxia (Liu & Xue, 2020;

McCarthy et al., 2013) underscoring the importance of organic carbon export to the benthos. If methanol or acetate (from ethanol), produced from decaying phytoplankton biomass accumulated in coastal sediments, they could serve as substrates for methanogenesis. For example, methylotrophic and acetoclastic methanogenesis are important processes in the sediments of the Gulf of Mexico hypoxic zone (Zhuang, Montgomery, et al., 2018).

The methane concentrations presented here (up to 132 nmol L⁻¹) were considerably lower than previously documented in summer 2016 when methane concentrations were > 600 nmol L⁻¹ (Rogener et al., 2021). This discrepancy could be driven by shifts in methanogenic and/or methanotrophic rates, or differences in physical dynamics. For example, persistent winds limited the extent of hypoxia in 2018 (N. Rabalais & Turner, 2018), which would reduce stratification, increase physical mixing, and potentially affect methane dynamics. Specifically, strong winds would drive surface waters offshore, which would induce upwelling of low oxygen bottom water and limit organic matter export to the sediments (N. N. Rabalais et al., 2007). As a result, methanogenesis rates could be inhibited. Additionally, the anaerobic oxidation of methane in anoxic sediments could reduce methane flux into the water column (Reeburgh, 2007). Anaerobic methane oxidation rates have not been measured in sediments of the Gulf of Mexico hypoxic zone. Alternatively, strong winds could increase the flux of gases to the atmosphere, representing another loss of methane from the water column. The calculated methane flux was up to 180 $\mu\text{mol m}^{-2} \text{d}^{-1}$ during our sampling, which is nearly twice as high as the largest flux previously reported for the Louisiana shelf ecosystem (Rogener et al., 2021). The Gulf of

Mexico hypoxic zone surface waters are clearly a source of methane to the atmosphere and the source strength varies temporally and spatially.

Shelfwide methanol metabolism

This dataset is the first report of methanol cycling in the water column of the Gulf of Mexico hypoxic zone. Our results demonstrate widespread methanol metabolisms along the Louisiana shelf and widens the growing number of studies on pelagic methylotrophy. Methanol oxidation and assimilation rates were up to 168.4 ± 19.7 and 0.178 ± 0.012 nmol L⁻¹ d⁻¹ (Fig. 11), respectively and varied across sites and depths. While these data are the first documented rates of methanol cycling in coastal hypoxic waters, our results were largely consistent with previous reports of methanol uptake (Dixon et al., 2011b, 2011a; Dixon & Nightingale, 2012; Sargeant et al., 2018; Zhuang, Peña-Montenegro, et al., 2018). In those studies, several assess dynamics in coastal ecosystems, and rates ranged from less than 1 nmol L⁻¹ d⁻¹ for assimilation to 146.4 nmol L⁻¹ d⁻¹. However, some previous reports did not provide or could not detect methanol. The methanol turnover constants measured here were up to 0.06 and 0.0012 d⁻¹ (Fig. 10) for oxidation and assimilation, respectively, and were generally lower than those previously reported. For example, methanol oxidation and assimilation constants were up to 0.18 and 0.11 d⁻¹ at several coastal sites in the Atlantic ocean (Dixon et al., 2011a, 2013), which was at least an order of magnitude higher than rate constants reported here. Similarly, turnover constants at the nearby Taylor Energy site were greater than 0.1 d⁻¹ for methanol uptake despite methanol concentrations being below the detection limit (Zhuang, Peña-Montenegro, et al., 2018). This contrast is surprising, given the nutrient-replete conditions of the northern Gulf of Mexico, and could

be indicative of physical or biogeochemical constraints on methylotrophy in the hypoxic zone.

Methanol oxidation turnover constants were largely consistent at all sites and ranged from 0.025 d^{-1} at station C6C to 0.064 d^{-1} at station I3 (Fig. 11). Methanol oxidation turnover constants at these stations were significantly different (ANOVA, p -value < 0.05) from each other, but were still an order of magnitude lower than previous reports (*e.g.*, Dixon et al., 2011a). Despite high nutrient loading from Mississippi River discharge (R. Eugene Turner et al., 2007), the size of the hypoxic zone in 2018 was one of the smallest on record (N. Rabalais & Turner, 2018). Persistent winds provoked physical mixing forcing surface waters offshore and to the east, which would inhibit production of DOC produced across the Louisiana Shelf (Feng et al., 2012; N. N. Rabalais et al., 2007; Wiseman et al., 1997). Lowered DOC availability would limit bacterial productivity and could explain the diminished rates of methanol uptake. Furthermore, coastal upwelling could serve to dilute biomass which can influence and alter microbial metabolisms (Behrenfeld, 2010; Haas et al., 2020). Alternatively, C:N ratios indicated that organic matter in the northern Gulf of Mexico hypoxic zone is preferentially degraded based on the carbon source (Liu & Xue, 2020), implying differential metabolisms could be important there. Examining interannual variability in methanol uptake rates, especially in a year of widespread hypoxia, could help elucidate the effects of mixing on methylotrophy and methanol dynamics.

Methanol oxidation (rates and turnover constants) was generally not correlated with any biogeochemical factors (Fig. 16). The high nutrient loading across the Louisiana shelf

(N. N. Rabalais et al., 2002) and lack of correlations imply that methanol oxidation was not nutrient-limited. In contrast, methanol assimilation turnover constants were positively correlated with nitrate, nitrite, phosphate, and chlorophyll concentrations. Because nutrient availability is generally high during the summer (N. N. Rabalais et al., 2002; R. Eugene Turner et al., 2007), it is unlikely that methanol assimilation was nutrient-limited. We postulate that nutrient replete conditions positively influenced cellular growth in accordance with the increased methanol assimilation activity when nutrient availability was higher. The relative contribution of assimilatory activity to total methanol uptake (oxidation plus assimilation) was up to 2.2% (Table 1). In contrast, assimilation accounted for 35% at the nearby Taylor Energy site (Zhuang, Peña-Montenegro, et al., 2018) and 50% in the English Channel (Dixon et al., 2011b), suggesting that methanol is used primarily as an energy source, rather than as a structural carbon source across the Louisiana shelf hypoxic zone. One possible explanation for this discrepancy is that coastal hypoxia supports diverse microbial populations (Gillies Campbell et al., 2019; Mason et al., 2016) which could differentially use methanol. For example, SAR11 *Alphaproteobacteria* are among the most ubiquitous microbes in the pelagic marine environment and they use methanol primarily as an energy source (Sun et al., 2011).

The modularity of methanol metabolism and methylotrophy, in general, may also influence the methanol oxidation patterns observed here (Chistoserdova, 2011). Multiple dehydrogenase enzymes are associated with methanol oxidation (MxaFI and XoxF), but their physiological and ecological roles are not fully understood (Chistoserdova & Kalyuzhnaya, 2018). The lanthanide-dependent XoxF-type enzyme can function as the

lone methanol dehydrogenase enzyme, but MxaFI appears to need multiple enzymes (XoxF or MxaFI pair) to function (Jimenez-Infante et al., 2016; Pol et al., 2014; Skovran et al., 2011). Differential expression of XoxF genes was observed under several experimental conditions using pure cultures (Krause et al., 2017; Vorobev et al., 2013), implying methylotrophs could respond to changes in environmental substrate availability. Furthermore, methylotrophs can fix carbon via the RuMP, Calvin Benson, or serine cycles, and may not always assimilate methanol-derived carbon (Chistoserdova, 2011), which could explain the relatively low assimilation rates.

Distribution of methane oxidation

Methane oxidation varied across the Louisiana Shelf hypoxic zone and significant differences were observed between the two methods used here (Fig. 8 & 9). Tritiated methane oxidation rates were up to $112.4 \pm 9.6 \text{ pmol L}^{-1} \text{ d}^{-1}$ at station C6C and was about 10 times higher than the lowest rate at station I5 ($9.3 \pm 2.4 \text{ pmol L}^{-1} \text{ d}^{-1}$). Corresponding turnover constants ranged from $0.0003 - 0.0012 \text{ d}^{-1}$. We extended our evaluation of methane oxidation across the Louisiana shelf and observed comparable methane oxidation rates for the most part. However, rates observed here were markedly lower than in 2016, which were supported by widespread hypoxia (Rogener et al., 2021). Furthermore, these methane oxidation rates were notably lower than rates in several other coastal studies: Randers Fjord ($15.2 \text{ nmol L}^{-1} \text{ d}^{-1}$), Western Svalbard Margin ($7.1 \text{ nmol L}^{-1} \text{ d}^{-1}$), Arctic fjord Storfjorden ($1.9 \text{ nmol L}^{-1} \text{ d}^{-1}$), the North Sea ($\sim 15 \text{ nmol L}^{-1} \text{ d}^{-1}$), and the Baltic sea ($11.6 \text{ nmol L}^{-1} \text{ d}^{-1}$) (Abril & Iversen, 2002; Gründger et al., 2021; Kelley, 2003; Mau et al., 2013; Osudar et al., 2015; Steinle et al., 2017).

In contrast, methane oxidation rates with ^{14}C -methane were all greater than $1 \text{ nmol L}^{-1} \text{ d}^{-1}$ (Fig. 9), more in line, but generally still lower than previous reports. Observed methane oxidation rates using the ^3H method were on average 2.2% of the rates using ^{14}C -methane oxidation rates (Table 1). A similar pattern was observed in Storfjorden, a stratified brine-influenced fjord, where rates differed between these methods by ~ 10 fold (Mau et al., 2013). Additionally, turnover constants were significantly lower using ^3H compared to ^{14}C , ranging from 3.9% to 36.5% (Table 1).

These differences are most likely explained by methodological differences between these radiotracer techniques. Tritiated methane is preferred for measuring methane oxidation rates because it has a high specific activity (units of radioactivity per mol) which enables higher activity at lower methane concentrations relative to ^{14}C -methane (Bussmann et al., 2015). As a result, our rate incubations were amended with 3.07 and $340.6 \text{ nmol L}^{-1}$ of C^3H_4 and $^{14}\text{CH}_4$, respectively. Thus, *in-situ* methane concentrations (average $71.4 \pm 6.8 \text{ nmol L}^{-1}$) were considerably altered by the addition of ^{14}C -methane, but not ^3H -methane, and the associated increased methane concentration significantly increased ^{14}C oxidation rates since the reaction is first order. The observed differences in turnover constants between radiotracer methods shed light on enzyme kinetics in the hypoxic zone. Methane oxidation follows Michaelis-Menten kinetics (Ward & Kilpatrick, 1990) and the substantial increase in turnover constants between ^{14}C and ^3H implies that methane concentrations were below enzyme saturation in our samples. Additionally, ^3H turnover constants increased after addition with $2 \text{ } \mu\text{mol L}^{-1}$ methane, but ^{14}C did not, indicating that

the half saturation constant for methane oxidation in the Gulf of Mexico hypoxic zone is less than 340 nmol L⁻¹.

Methane oxidation rates across the Louisiana shelf hypoxic zone rates were up to 192 nmol L⁻¹ d⁻¹ in 2016, a year with widespread hypoxia (Rogener et al., 2021), and ~ 57 nmol L⁻¹ d⁻¹ ~ 20 years ago (Kelley, 2003). These methane oxidation rates were at least 500 times higher than the highest value presented here, however, methane oxidation rates in 2013 and 2015 averaged 245.7 ± 47.1 pmol L⁻¹ d⁻¹, more in line with our observations. While methane oxidation rate measurements from these three studies varied with respect to location and methodology (net consumption versus radiotracer techniques), substantial temporal differences were observed. The high methane oxidation rates observed in 2016 were presumably influenced by widespread hypoxia that year (Rogener et al., 2021) consistent with observed negative linear relationships of oxygen with methane oxidation rates and turnover (Fig. 16). In contrast, the northern Gulf of Mexico hypoxic zone was significantly smaller during summer of 2018, which may have reduced the methanotrophic potential there if DO is an important driver of methane oxidation. Annual differences in the magnitude of hypoxia are linked to variations in nutrient loading, eutrophication, and biomass abundance (R. E. Turner & Rabalais, 2013), which could each influence methane oxidation rates. Whether this was a direct result of differing oxygen regimes or there are other compounding factors is unclear. In coastal ecosystems, the extent to which methane oxidation is influenced by DO concentrations (Osudar et al., 2015; Rogener et al., 2021; Steinle et al., 2017) and nitrogen dynamics (Rogener et al., 2021) is not fully understood. Furthermore, intense nitrification across the Gulf of Mexico hypoxic zone (Bristow et al.,

2015; Kitzinger et al., 2019, 2020) could reduce methanotrophic activity there (Stein et al., 2012).

Factors driving methanotrophy and methylotrophy

Methane oxidation rates in samples amended with ammonium, methane, copper, and iron exhibited responses that varied with respect to site and depth (Fig. 13 & 15). Methane oxidation rates were significantly stimulated by the addition of methane, regardless of concentration. Methanotrophic biomass was presumably enriched for in samples amended with methane during the several days of storage between collection and radiotracer amendment (see methods). However, since samples were stored at 4°C until incubation, it is possible that the observed higher rates reflect a physiological response to higher methane concentrations rather than growth of new biomass. Therefore, it is important to consider the turnover constants which are indicative of methanotroph biomass. In particular, in the bottom water at station C6C, methane turnover constants were significantly higher than the unamended control, indicating the emergence of fresh methanotrophic biomass. Whether this observed increase in turnover constants represents new growth of already active methanotrophs or signifies a switch in metabolism of non-canonical methanotrophs is unclear. Taken together, these data suggest that methane oxidation could be substrate limited or certain microbial populations use alternative energy substrates under certain methane thresholds.

To assess if non-canonical methanotrophs contribute to methane oxidation in the hypoxic waters of the Gulf of Mexico, we made amendments aimed at specifically

inhibiting canonical methanotrophs. Unexpectedly, the addition of streptomycin and kanamycin antibiotics plus methane did not inhibit methane oxidation, but in several cases stimulated it. Streptomycin and kanamycin are aminoglycoside antibiotics, which inhibit protein synthesis in gram-negative bacteria, which includes most known methanotrophs. Methanotrophy was completely inhibited by streptomycin in soils (Hanson & Hanson, 1996) and these antibiotics should diminish methane oxidization in hypoxic waters. Critically, these antibiotics were chosen because they are routinely used to cultivate ammonia oxidizing archaea (AOA), which we hypothesized contribute to methane oxidation. They can be used to differentiate AOA from ammonia oxidizing bacteria, which can also contribute to methane oxidation (Bayer et al., 2016; Könneke et al., 2005; Ward, 1990). AOA are among the most abundant microorganisms in the marine environment, especially in low oxygen waters where they mediate ammonia oxidation, the first step of nitrification (Bristow et al., 2015; Gillies et al., 2015; Kitzinger et al., 2020). We recently presented evidence that two strains of AOA (see Chapter 2 of this dissertation) can oxidize methane and AOA in general may contribute significantly to marine methane oxidation. Comparable turnover constants and increased methane oxidation rates with the addition of these antibiotics suggest that AOA could be metabolizing methane in the Gulf of Mexico hypoxic zone.

In contrast, amendment with ammonium did not influence methane oxidation rates compared to methane addition (Fig. 13). Methane oxidation turnover constants were marginally increased with ammonium addition, while the addition of methane plus ammonium increased methane oxidation rates by a factor of approximately two. Elevated

ammonium availability has been associated with saturation of methane monooxygenase (Stein et al., 2012), the enzyme responsible for methane oxidation, so the stimulation of the turnover constants by ammonium was unexpected. However, the persistent availability of ammonium in the Gulf of Mexico hypoxic zone may drive methanotrophs to distinguish methane from ammonium to optimize energy conservation. Further investigations with experimental approaches are necessary to understand the dynamics between ammonium and methane cycling in nutrient rich environments.

Methane oxidation was significantly stimulated by additions of iron and copper (Fig. 14), indicating metal limitation at several sites. At station C6C (bottom water), the addition of 50 nmol L⁻¹ copper stimulated methane oxidation, while 500 nmol L⁻¹ had no effect, suggesting methanotrophs there had a high affinity for copper (Fig. 14C). Iron did not stimulate methanotrophy as extensively as copper, which could be due to the relative importance of iron for phytoplankton. However, methane oxidation coupled to iron(III) reduction during hypoxia was recently described, highlighting it as important alternative electron acceptor for methanotrophy (Zheng et al., 2020). Furthermore, copper and iron are co-factors for methane monooxygenase and formate dehydrogenase, respectively, and thus play essential roles in methanotroph biochemistry and methane oxidation (Jollie & Lipscomb, 1991; Matsen et al., 2013; Park et al., 1992).

The amendment experiments aimed to explore the dynamics of methane oxidation; however, several factors concurrently emerged as important drivers of methanotrophy in the Gulf of Mexico hypoxic zone. In several instances, the addition of methane stimulated

methanol uptake, but in other cases, inhibited it (Fig 13). Methane is oxidized to methanol in the first step of aerobic methanotrophy, thus the metabolisms of these substrates are intimately intertwined (Chistoserdova & Kalyuzhnaya, 2018; Sirajuddin & Rosenzweig, 2015). The stimulation of methanol uptake could result from incomplete methane oxidation after methane addition as was demonstrated for the deep sea methanotroph *M. sedimenti* (Tavormina et al., 2017). This stimulation was remarkably prevalent for methanol assimilation suggesting that methanol accumulation from methane oxidation is an important source for heterotrophic production. In contrast, methanol oxidation was inhibited by methane additions. It is likely this inhibition reflects the preferential oxidation of methane over methanol by methanotrophs. Alternatively, methanol dehydrogenase, the canonical gene for methanol degradation, may have oxidized methane when methane availability increased (Yu et al., 2017).

Methanol oxidation and assimilation were differentially affected by the addition of antibiotics. Oxidation of methanol was largely unaffected, while assimilation was completely inhibited. Since it is understood that different microbial populations use methanol for carbon and energy (*e.g.*, SAR11 only oxidizes methanol), this observation is plausible (Sun et al., 2011). Methyloproteobacteria comprise diverse clades, including *Alphaproteobacteria* and *Gammaproteobacteria*, among others (Howat et al., 2018; Neufeld et al., 2008; Urakami & Komagata, 1987), which would likely be inhibited by the addition of aminoglycoside antibiotics. If primary oxidizers of methanol (*i.e.*, they do not assimilate methanocarbon) were not affected by antibiotic amendment, but organisms that

assimilate methanol were, then assimilatory activity would be inhibited as we observed. This could implicate novel clades in methanol cycling in the Gulf of Mexico hypoxic zone.

We surveyed the waters across the Louisiana shelf to investigate organic carbon dynamics in the Gulf of Mexico hypoxic zone. The first documented methanol and ethanol concentrations in a coastal hypoxic ecosystem ranged up to 3.5 and 1.5 $\mu\text{mol L}^{-1}$, respectively. Methane concentrations were measured up to 132 nmol L^{-1} and were a continuation of our recent investigations. Physical and biogeochemical processes associated with coastal hypoxia such as wind speed, phytoplankton blooms, and nutrient loading likely contribute to the production, distribution, and degradation of these carbon compounds. We also constrained metabolic rates of methane and methanol metabolism to explore carbon cycling during hypoxia. Methanol oxidation and assimilation were documented in hypoxic waters for the first time and were up to 168.5 and 0.18 $\text{nmol L}^{-1} \text{ d}^{-1}$ underscoring the importance of methylotrophy in coastal ecosystems. In particular, methanol was predominantly used as an energy source, which could contribute to hypoxia when coupled to oxygen respiration. Methane oxidation rates were at least 500 times lower than summer 2016, but were comparable to 2013 and 2015 (Rogener et al., 2021), suggesting annual variations in hypoxia could influence microbial activity. Experimentally amended rates revealed methane, ammonium, and metals, as important factors driving methanotrophy and methylotrophy. Furthermore, these results potentially implicate non-canonical clades in metabolisms of methane and methanol. Taken together, these data provide novel insights into marine carbon cycling, shed light on the importance of methane

and methanol in low oxygen systems, and complement our understanding of the Gulf of Mexico hypoxic zone as a dynamic ecosystem.

REFERENCES

- Abril, G., & Iversen, N. (2002). Methane dynamics in a shallow non-tidal estuary (Randers Fjord, Denmark). *Marine Ecology Progress Series*, 230, 171–181.
<https://doi.org/10.3354/meps230171>
- Avery, G. B., Foley, L., Carroll, A. L., Roebuck, J. A., Guy, A., Mead, R. N., et al. (2016). Surface waters as a sink and source of atmospheric gas phase ethanol. *Chemosphere*, 144, 360–365. <https://doi.org/10.1016/j.chemosphere.2015.08.080>
- Bange, H. W., Bergmann, K., Hansen, H. P., Kock, A., Koppe, R., Malien, F., & Ostrau, C. (2010). Dissolved methane during hypoxic events at the Boknis Eck time series station (Eckernförde Bay, SW Baltic Sea). *Biogeosciences*, 7(4), 1279–1284.
<https://doi.org/10.5194/bg-7-1279-2010>
- Bayer, B., Vojvoda, J., Offre, P., Alves, R. J. E., Elisabeth, N. H., Garcia, J. A. L., et al. (2016). Physiological and genomic characterization of two novel marine thaumarchaeal strains indicates niche differentiation. *ISME Journal*, 10(5), 1051–1063. <https://doi.org/10.1038/ismej.2015.200>
- Beale, R., Liss, P. S., & Nightingale, P. D. (2010). First oceanic measurements of ethanol and propanol. *Geophysical Research Letters*, 37(24), 1–5.
<https://doi.org/10.1029/2010GL045534>
- Beale, Rachael, Dixon, J. L., Arnold, S. R., Liss, P. S., & Nightingale, P. D. (2013). Methanol, acetaldehyde, and acetone in the surface waters of the Atlantic Ocean.

- Journal of Geophysical Research: Oceans*, 118(10), 5412–5425.
<https://doi.org/10.1002/jgrc.20322>
- Behrenfeld, M. J. (2010). Abandoning sverdrup's critical depth hypothesis on phytoplankton blooms. *Ecology*, 91(4), 977–989. <https://doi.org/10.1890/09-1207.1>
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)*, 57(1), 289–300.
- Braman, R. S., & Hendrix, S. A. (1989). Nanogram Nitrite and Nitrate Determination in Environmental and Biological Materials by Vanadium(III) Reduction with Chemiluminescence Detection. *Analytical Chemistry*, 61(24), 2715–2718.
<https://doi.org/10.1021/ac00199a007>
- Breitburg, D., Levin, L. A., Oschlies, A., Grégoire, M., Chavez, F. P., Conley, D. J., et al. (2018). Declining oxygen in the global ocean and coastal waters. *Science*, 359(6371). <https://doi.org/10.1126/science.aam7240>
- Bristow, L. A., Sarode, N., Cartee, J., Caro-Quintero, A., Thamdrup, B., & Stewart, F. J. (2015). Biogeochemical and metagenomic analysis of nitrite accumulation in the Gulf of Mexico hypoxic zone. *Limnology and Oceanography*, 60(5), 1733–1750.
<https://doi.org/10.1002/lno.10130>
- Bussmann, I., Matousu, A., Osudar, R., & Mau, S. (2015). Assessment of the radio 3H-CH₄ tracer technique to measure aerobic methane oxidation in the water column. *Limnology and Oceanography: Methods*, 13(6), 312–327.
<https://doi.org/10.1002/lom3.10027>

- Carini, P., White, A. E., Campbell, E. O., & Giovannoni, S. J. (2014). Methane production by phosphate-starved SAR11 chemoheterotrophic marine bacteria. *Nature Communications*, 5, 1–7. <https://doi.org/10.1038/ncomms5346>
- Chen, N., Bianchi, T. S., & Bland, J. M. (2003). Implications for the role of pre- versus post-depositional transformation of chlorophyll-a in the Lower Mississippi River and Louisiana shelf. *Marine Chemistry*, 81(1–2), 37–55. [https://doi.org/10.1016/S0304-4203\(02\)00138-X](https://doi.org/10.1016/S0304-4203(02)00138-X)
- Chistoserdova, L. (2011). Modularity of methylophony , revisited. *Environmental Microbiology*, 13(10), 2603–2622. <https://doi.org/10.1111/j.1462-2920.2011.02464.x>
- Chistoserdova, L., & Kalyuzhnaya, M. G. (2018). Current Trends in Methylophony. *Trends in Microbiology*, xx, 1–12. <https://doi.org/10.1016/j.tim.2018.01.011>
- Chronopoulou, P. M., Shelley, F., Pritchard, W. J., Maanoja, S. T., & Trimmer, M. (2017). Origin and fate of methane in the Eastern Tropical North Pacific oxygen minimum zone. *ISME Journal*, 11(6), 1386–1399. <https://doi.org/10.1038/ismej.2017.6>
- Diaz, R. J., & Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science*, 321(5891), 926–929. <https://doi.org/10.1126/science.1156401>
- Dinnel, S. P., & Wiseman, W. J. (1986). Fresh water on the Louisiana and Texas shelf. *Continental Shelf Research*, 6(6), 765–784. [https://doi.org/10.1016/0278-4343\(86\)90036-1](https://doi.org/10.1016/0278-4343(86)90036-1)

- Dixon, J. L., & Nightingale, P. D. (2012). Fine-scale variability in methanol uptake and oxidation: From the microlayer to 1000 m. *Biogeosciences*, 9(8), 2961–2972.
<https://doi.org/10.5194/bg-9-2961-2012>
- Dixon, J. L., Beale, R., & Nightingale, P. D. (2011a). Microbial methanol uptake in northeast Atlantic waters. *The ISME Journal*, 5(4), 704–716.
<https://doi.org/10.1038/ismej.2010.169>
- Dixon, J. L., Beale, R., & Nightingale, P. D. (2011b). Rapid biological oxidation of methanol in the tropical Atlantic: significance as a microbial carbon source. *Biogeosciences*, 8(9), 2707–2716. <https://doi.org/10.5194/bg-8-2707-2011>
- Dixon, J. L., Sargeant, S., Nightingale, P. D., & Colin Murrell, J. (2013). Gradients in microbial methanol uptake: Productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean. *ISME Journal*, 7(3), 568–580.
<https://doi.org/10.1038/ismej.2012.130>
- Dugdale, R. C. (1967). Nutrient Limitation in the Sea : Dynamics, Identification, and Significance. *Limnology and Oceanography*, 685–695.
- Eichler, B., & Schink, B. (1984). Oxidation of primary aliphatic alcohols by *Acetobacterium carbinolicum* sp. nov., a homoacetogenic anaerobe. *Archives of Microbiology*, 140(2–3), 147–152. <https://doi.org/10.1007/BF00454917>
- Feng, Y., Dimarco, S. F., & Jackson, G. A. (2012). Relative role of wind forcing and riverine nutrient input on the extent of hypoxia in the northern Gulf of Mexico. *Geophysical Research Letters*, 39(9), 1–5. <https://doi.org/10.1029/2012GL051192>
- Gelesh, L., Marshall, K., Boicourt, W., & Lapham, L. (2016). Methane concentrations increase in bottom waters during summertime anoxia in the highly eutrophic estuary,

- Chesapeake Bay, U.S.A. *Limnology and Oceanography*, 61(2011), S253–S266.
<https://doi.org/10.1002/lno.10272>
- Gillies Campbell, L., Thrash, J. C., Rabalais, N. N., & Mason, O. U. (2019). Extent of the annual Gulf of Mexico hypoxic zone influences microbial community structure. *PLoS ONE*, 14(4), 1–23. <https://doi.org/10.1371/journal.pone.0209055>
- Gillies, L. E., Thrash, J. C., deRada, S., Rabalais, N. N., & Mason, O. U. (2015). Archaeal enrichment in the hypoxic zone in the northern Gulf of Mexico. *Environmental Microbiology*, 17(10), 3847–3856. <https://doi.org/10.1111/1462-2920.12853>
- Grasshoff, K., Kremling, K., & Ehrhardt, M. (1983). *Methods of Seawater Analysis: Third, Completely Revised and Extended Edition. Methods of Seawater Analysis: Third, Completely Revised and Extended Edition.* Verlag Chemie.
<https://doi.org/10.1002/9783527613984>
- Gründger, F., Probandt, D., Knittel, K., Carrier, V., Kalenitchenko, D., Silyakova, A., et al. (2021). Seasonal shifts of microbial methane oxidation in Arctic shelf waters above gas seeps. *Limnology and Oceanography*, 1–19.
<https://doi.org/10.1002/lno.11731>
- Haas, S., Robicheau, B. M., Rakshit, S., Tolman, J., Algar, C. K., LaRoche, J., & Wallace, D. W. R. (2020). Physical mixing controls and decouples nitrification via biomass dilution. *Proceedings of the National Academy of Sciences*.
<https://doi.org/10.1073/pnas.2004877118>
- Hanson, R. S., & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiological Reviews*, 60(2), 439–471. <https://doi.org/10.1128/mmbr.60.2.439-471.1996>

- Heal, K. R., Qin, W., Ribalet, F., Bertagnolli, A. D., Coyote-Maestas, W., Hmelo, L. R., et al. (2017). Two distinct pools of B12 analogs reveal community interdependencies in the ocean. *Proceedings of the National Academy of Sciences of the United States of America*, 114(2), 364–369.
<https://doi.org/10.1073/pnas.1608462114>
- Heikes, B. G., Chang, W., Pilson, M. E. Q., Swift, E., Singh, H. B., Guenther, A., et al. (2002). Atmospheric methanol budget and ocean implication. *Global Biogeochemical Cycles*, 16(4), 80-1-80–13. <https://doi.org/10.1029/2002gb001895>
- Ho, T. Y., Scranton, M. I., Taylor, G. T., Varela, R., Thunell, R. C., & Muller-Karger, F. (2002). Acetate cycling in the water column of the Cariaco Basin: Seasonal and vertical variability and implication for carbon cycling. *Limnology and Oceanography*, 47(4), 1119–1128. <https://doi.org/10.4319/lo.2002.47.4.1119>
- Holmes, R. M., Aminot, A., Kerouel, R., Hooker, B. A., & Peterson, B. J. (1999). A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Canadian Journal of Fisheries and Aquatic Sciences*, 56(10), 1801–1808.
- Howat, A. M., Vollmers, J., Taubert, M., Grob, C., Dixon, J. L., Todd, J. D., et al. (2018). Comparative genomics and mutational analysis reveals a novel XoxF-utilizing methylotroph in the roseobacter group isolated from the marine environment. *Frontiers in Microbiology*, 9(APR), 1–12. <https://doi.org/10.3389/fmicb.2018.00766>
- Istok, J. D., Park, M. M., Peacock, A. D., Oostrom, M., & Wietsma, T. W. (2007). An experimental investigation of nitrogen gas produced during denitrification. *Ground Water*, 45(4), 461–467. <https://doi.org/10.1111/j.1745-6584.2007.00319.x>

- Jimenez-Infante, F., Ngugi, D. K., Vinu, M., Alam, I., Kamau, A. A., Blom, J., et al. (2016). Comprehensive Genomic Analyses of the OM43 Clade, Including a Novel Species from the Red Sea, Indicate Ecotype Differentiation among Marine Methylotrophs. *Applied and Environmental Microbiology*, 82(4), 1215–1226. <https://doi.org/10.1128/AEM.02852-15>
- Jollie, D. R., & Lipscomb, J. D. (1991). Formate dehydrogenase from *Methylosinus trichosporium* OB3b: Purification and spectroscopic characterization of the cofactors. *Journal of Biological Chemistry*, 266(32), 21853–21863. [https://doi.org/10.1016/s0021-9258\(18\)54716-5](https://doi.org/10.1016/s0021-9258(18)54716-5)
- Joye, S. B., Boetius, A., Orcutt, B. N., Montoya, J. P., Schulz, H. N., Erickson, M. J., & Lugo, S. K. (2004). The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chemical Geology*, 205(3–4), 219–238. <https://doi.org/10.1016/j.chemgeo.2003.12.019>
- Karl, D. M., Beversdorf, L., Björkman, K. M., Church, M. J., Martinez, A., & Delong, E. F. (2008). Aerobic production of methane in the sea. *Nature Geoscience*, 1(7), 473–478. <https://doi.org/10.1038/ngeo234>
- Kelley, C. (2003). Methane oxidation potential in the water column of two diverse coastal marine sites. *Biogeochemistry*, 65(1), 105–120. <https://doi.org/10.1023/A:1026014008478>
- Kits, K. D., Klotz, M. G., & Stein, L. Y. (2015). Methane oxidation coupled to nitrate reduction under hypoxia by the Gammaproteobacterium *Methylomonas denitrificans*, sp. nov. type strain FJG1. *Environmental Microbiology*, 17(9), 3219–3232. <https://doi.org/10.1111/1462-2920.12772>

- Kitzinger, K., Padilla, C. C., Marchant, H. K., Hach, P. F., Herbold, C. W., Kidane, A. T., et al. (2019). Cyanate and urea are substrates for nitrification by Thaumarchaeota in the marine environment. *Nature Microbiology*, 4(February).
<https://doi.org/10.1038/s41564-018-0316-2>
- Kitzinger, K., Marchant, H. K., Bristow, L. A., Herbold, C. W., Padilla, C. C., Kidane, A. T., et al. (2020). Single cell analyses reveal contrasting life strategies of the two main nitrifiers in the ocean. *Nature Communications*, 11(1).
<https://doi.org/10.1038/s41467-020-14542-3>
- Konneke, M., Schubert, D. M., Brown, P. C., Hugler, M., Standfest, S., Schwander, T., et al. (2014). Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO₂ fixation. *Proceedings of the National Academy of Sciences*, 111(22), 8239–8244. <https://doi.org/10.1073/pnas.1402028111>
- Könneke, M., Bernhard, A. E., De La Torre, J. R., Walker, C. B., Waterbury, J. B., & Stahl, D. A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, 437(7058), 543–546. <https://doi.org/10.1038/nature03911>
- Krause, S. M. B., Johnson, T., Karunaratne, Y. S., Fu, Y., Beck, D. A. C., Chistoserdova, L., & Lidstrom, M. E. (2017). Lanthanide-dependent cross-feeding of methane-derived carbon is linked by microbial community interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 114(2), 358–363.
<https://doi.org/10.1073/pnas.1619871114>
- Liu, Z., & Xue, J. (2020). The Lability and Source of Particulate Organic Matter in the Northern Gulf of Mexico Hypoxic Zone. *Journal of Geophysical Research: Biogeosciences*, 125(9), 1–16. <https://doi.org/10.1029/2020JG005653>

- Lohrenz, S. E., Fahnenstiel, G. L., Redalje, D. G., Lang, G. A., Dagg, M. J., Whitledge, T. E., & Dortch, Q. (1999). Nutrients, irradiance, and mixing as factors regulating primary production in coastal waters impacted by the Mississippi River plume. *Continental Shelf Research*, 19(9), 1113–1141. [https://doi.org/10.1016/S0278-4343\(99\)00012-6](https://doi.org/10.1016/S0278-4343(99)00012-6)
- Magen, C., Lapham, L., Pohlman, J. W., Marshall, K., Bosman, S., Casso, M., & Chanton, J. P. (2014). A simple headspace equilibration method for measuring dissolved methane. *Limnology and Oceanography: Methods*, 12(SEP), 637–650. <https://doi.org/10.4319/lom.2014.12.637>
- Mason, O. U., Canter, E. J., Gillies, L. E., Paisie, T. K., & Roberts, B. J. (2016). Mississippi river plume enriches microbial diversity in the northern gulf of Mexico. *Frontiers in Microbiology*, 7(JUL). <https://doi.org/10.3389/fmicb.2016.01048>
- Matsen, J. B., Yang, S., Stein, L. Y., Beck, D., & Kalyuzhnaya, M. G. (2013). Global molecular analyses of methane metabolism in Methanotrophic alphaproteobacterium, methylosinus trichosporium OB3b. Part I: Transcriptomic study. *Frontiers in Microbiology*, 4(APR), 1–16. <https://doi.org/10.3389/fmicb.2013.00040>
- Mau, S., Blees, J., Helmke, E., Niemann, H., & Damm, E. (2013). Vertical distribution of methane oxidation and methanotrophic response to elevated methane concentrations in stratified waters of the Arctic fjord Storfjorden (Svalbard, Norway). *Biogeosciences*, 10(10), 6267–6268. <https://doi.org/10.5194/bg-10-6267-2013>
- McCarthy, M. J., Carini, S. A., Liu, Z., Ostrom, N. E., & Gardner, W. S. (2013). Oxygen consumption in the water column and sediments of the northern Gulf of Mexico

- hypoxic zone. *Estuarine, Coastal and Shelf Science*, 123, 46–53.
<https://doi.org/10.1016/j.ecss.2013.02.019>
- Metcalf, W. W., Griffin, B. M., Cicchillo, R. M., Gao, J., Janga, S. C., Cooke, H. A., et al. (2012). Synthesis of Methylphosphonic Acid by Marine Microbes: A Source for Methane in the Aerobic Ocean. *Science*, (August).
- Metje, M., & Frenzel, P. (2005). Effect of temperature on anaerobic ethanol oxidation and methanogenesis in acidic peat from a Northern Wetland. *Applied and Environmental Microbiology*, 71(12), 8191–8200.
<https://doi.org/10.1128/AEM.71.12.8191-8200.2005>
- Mincer, T. J., & Aicher, A. C. (2016). Methanol production by a broad phylogenetic array of marine phytoplankton. *PLoS ONE*, 11(3), 1–17.
<https://doi.org/10.1371/journal.pone.0150820>
- Neufeld, J. D., Boden, R., Moussard, H., Schäfer, H., & Murrell, J. C. (2008). Substrate-specific clades of active marine methylotrophs associated with a phytoplankton bloom in a temperate coastal environment. *Applied and Environmental Microbiology*, 74(23), 7321–7328. <https://doi.org/10.1128/AEM.01266-08>
- Nixon, S. W. (1995). Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia*, 41(1), 199–219.
<https://doi.org/10.1080/00785236.1995.10422044>
- Osudar, R., Matoušů, A., Alawi, M., Wagner, D., & Bussmann, I. (2015). Environmental factors affecting methane distribution and bacterial methane oxidation in the German Bight (North Sea). *Estuarine, Coastal and Shelf Science*, 160, 10–21.
<https://doi.org/10.1016/j.ecss.2015.03.028>

- Padilla, C. C., Bristow, L. A., Sarode, N., Garcia-Robledo, E., Gómez Ramírez, E., Benson, C. R., et al. (2016). NC10 bacteria in marine oxygen minimum zones. *ISME Journal*, 10(8), 2067–2071. <https://doi.org/10.1038/ismej.2015.262>
- Park, S., Shah, N. N., Taylor, R. T., & Droege, M. W. (1992). Batch cultivation of *Methylosinus trichosporium* OB3b: II. Production of particulate methane monooxygenase. *Biotechnology and Bioengineering*, 40(1), 151–157. <https://doi.org/10.1002/bit.260400121>
- Pol, A., Barends, T. R. M., Dietl, A., Khadem, A. F., Eygensteyn, J., Jetten, M. S. M., & Op den Camp, H. J. M. (2014). Rare earth metals are essential for methanotrophic life in volcanic mudpots. *Environmental Microbiology*, 16(1), 255–264. <https://doi.org/10.1111/1462-2920.12249>
- Rabalais, N., & Turner, R. E. (2018). Bottom Water Dissolved Oxygen – 2015 Size of bottom-water hypoxia in mid-summer. Retrieved November 25, 2019, from https://gulfhypoxia.net/research/shelfwide-cruise/?y=2018&p=press_release
- Rabalais, N. N., & Turner, R. E. (2001). *Coastal hypoxia: consequences for living resources and ecosystems*. (M. J. Bowman, R. T. Barber, C. N. K. Mooers, & J. A. Raven, Eds.), *Coastal and Estuarine Studies*. Washington, D.C.: American Geophysical Union.
- Rabalais, N. N., Turner, R. E., & Wiseman, W. J. (2002). Gulf of Mexico Hypoxia, A.K.A. “The Dead Zone.” *Annual Review of Ecology and Systematics*, 33(1), 235–263. <https://doi.org/10.1146/annurev.ecolsys.33.010802.150513>
- Rabalais, N. N., Turner, R. E., Sen Gupta, B. K., Boesch, D. F., Chapman, P., & Murrell, M. C. (2007). Hypoxia in the northern Gulf of Mexico: Does the science support the

- plan to reduce, mitigate, and control hypoxia? *Estuaries and Coasts*, 30(5), 753–772.
- Redalje, D. G. ., Lohrenz, S. E. ., & Fahnenstiel, G. L. . (1994). The Relationship between Primary Production and the Vertical Export of Particulate Organic Matter in a River-Impacted Coastal Ecosystem. *Estuaries*, 17(4), 829–838.
- Reeburgh, W. S. (2007). Oceanic methane biogeochemistry. *Chemical Reviews*, 107(2), 486–513. <https://doi.org/10.1021/cr050362v>
- Rogener, M. K., Bracco, A., Hunter, K. S., Saxton, M. A., & Joye, S. B. (2018). Long-term impact of the Deepwater Horizon oil well blowout on methane oxidation dynamics in the northern Gulf of Mexico. *Elementa*, 6, 1–17. <https://doi.org/10.1525/elementa.332>
- Rogener, M. K., Sipler, R. E., Hunter, K. S., Bronk, D. A., & Joye, S. B. (2020). Pelagic methane oxidation in the northern Chukchi Sea. *Limnology and Oceanography*, 65(1), 96–110. <https://doi.org/10.1002/lno.11254>
- Rogener, M. K., Hunter, K., Rabalais, N. N., Roberts, B. J., Bracco, A., Stewart, F. J., & Joye, S. B. (2021). Pelagic denitrification and methane oxidation in oxygen-depleted waters of the Louisiana shelf. *Biogeochemistry*, 8. <https://doi.org/10.1007/s10533-021-00778-8>
- Sargeant, S. L., Murrell, J. C., Nightingale, P. D., & Dixon, J. L. (2018). Basin-scale variability of microbial methanol uptake in the Atlantic Ocean, (February), 1–28.
- Singh, H., Chen, Y., Staudt, A., Jacob, D., Blake, D., Heikes, B., & Snow, J. (2001). Evidence from the pacific troposphere for large global sources of oxygenated

- organic compounds. *Nature*, 410(6832), 1078–1081.
<https://doi.org/10.1038/35074067>
- Sirajuddin, S., & Rosenzweig, A. C. (2015). Enzymatic oxidation of methane. *Biochemistry*, 54(14), 2283–2294. <https://doi.org/10.1021/acs.biochem.5b00198>
- Skovran, E., Palmer, A. D., Rountree, A. M., Good, N. M., & Lidstrom, M. E. (2011). XoxF is required for expression of methanol dehydrogenase in *Methylobacterium extorquens* AM1. *Journal of Bacteriology*, 193(21), 6032–6038.
<https://doi.org/10.1128/JB.05367-11>
- Solórzano, L., & Sharp, J. H. (1980). Determination of total dissolved phosphorus particulate phosphorus in natural waters. *Limnol. Oceanogr.*, 25(4), 754–758.
- Stein, L. Y., Roy, R., & Dunfield, P. F. (2012). Aerobic Methanotrophy and Nitrification: Processes and Connections. *ELS*. <https://doi.org/10.1002/9780470015902.a0022213>
- Steinle, L., Schmidt, M., Bryant, L., Haeckel, M., Linke, P., Sommer, S., et al. (2016). Linked sediment and water-column methanotrophy at a man-made gas blowout in the North Sea: Implications for methane budgeting in seasonally stratified shallow seas. *Limnology and Oceanography*, 61, S367–S386.
<https://doi.org/10.1002/lno.10388>
- Steinle, L., Maltby, J., Treude, T., Kock, A., Bange, H. W., Engbersen, N., et al. (2017). Effects of low oxygen concentrations on aerobic methane oxidation in seasonally hypoxic coastal waters. *Biogeosciences*, 14(6), 1631–1645.
<https://doi.org/10.5194/bg-14-1631-2017>

- Stewart, F. J., Ulloa, O., & Delong, E. F. (2012). Microbial metatranscriptomics in a permanent marine oxygen minimum zone. *Environmental Microbiology*, 14(1), 23–40. <https://doi.org/10.1111/j.1462-2920.2010.02400.x>
- Sun, J., Steindler, L., Thrash, J. C., Halsey, K. H., Smith, D. P., Carter, A. E., et al. (2011). One carbon metabolism in SAR11 pelagic marine bacteria. *PLoS ONE*, 6(8). <https://doi.org/10.1371/journal.pone.0023973>
- Tavormina, P. L., Kellermann, M. Y., Antony, C. P., Tocheva, E. I., Dalleska, N. F., Jensen, A. J., et al. (2017). Starvation and recovery in the deep-sea methanotroph *Methyloprofundus sedimenti*. *Molecular Microbiology*, 103(2), 242–252. <https://doi.org/10.1111/mmi.13553>
- Thrash, J. C., Seitz, K. W., Baker, B. J., Temperton, B., Gillies, L. E., Rabalais, N. N., et al. (2017). Metabolic roles of uncultivated bacterioplankton lineages in the northern gulf of Mexico “dead zone.” *MBio*, 8(5), 1–20. <https://doi.org/10.1128/mBio.01017-17>
- Tolar, B. B., King, G. M., & Hollibaugh, J. T. (2013). An analysis of thaumarchaeota populations from the northern Gulf of Mexico. *Frontiers in Microbiology*, 4(APR). <https://doi.org/10.3389/fmicb.2013.00072>
- Turner, R. E., & Rabalais, N. N. (2013). Nitrogen and phosphorus phytoplankton growth limitation in the northern Gulf of Mexico. *Aquatic Microbial Ecology*, 68(2), 159–169. <https://doi.org/10.3354/ame01607>
- Turner, R. Eugene, Rabalais, N. N., Alexander, R. B., McIsaac, G., & Howarth, R. W. (2007). Characterization of Nutrient, Organic Carbon, and Sediment Loads and

- Concentrations from the Mississippi River into the Northern Gulf of Mexico. *Estuaries and Coasts*, 30(5), 773–790.
- Turner, R. Eugene, Rabalais, N. N., & Justic, D. (2008). Gulf of Mexico hypoxia: Alternate states and a legacy. *Environmental Science and Technology*, 42(7), 2323–2327. <https://doi.org/10.1021/es071617k>
- Ueno, Y., Kurano, N., & Miyachi, S. (1998). Ethanol production by dark fermentation in the marine green alga, *Chlorococcum littorale*. *Journal of Fermentation and Bioengineering*, 86(1), 38–43. [https://doi.org/10.1016/S0922-338X\(98\)80031-7](https://doi.org/10.1016/S0922-338X(98)80031-7)
- Urakami, T., & Komagata, K. (1987). Characterization of Species of Marine Methylophages of the Genus *Methylophaga*. *International Journal of Systematic Bacteriology*, 37(4), 402–406. <https://doi.org/10.1099/00207713-37-4-402>
- Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., et al. (1997). Human alteration of the global nitrogen cycle: Sources and consequences. *Ecological Applications*, 7(3), 737–750. [https://doi.org/10.1890/1051-0761\(1997\)007\[0737:HAOTGN\]2.0.CO;2](https://doi.org/10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2)
- Vorobev, A., Beck, D. A. C., Kalyuzhnaya, M. G., Lidstrom, M. E., & Chistoserdova, L. (2013). Comparative transcriptomics in three Methylophilaceae species uncover different strategies for environmental adaptation. *PeerJ*, 2013(1). <https://doi.org/10.7717/peerj.115>
- Wang, X. C., Chen, R. F., & Gardner, G. B. (2004). Sources and transport of dissolved and particulate organic carbon in the Mississippi River estuary and adjacent coastal waters of the northern Gulf of Mexico. *Marine Chemistry*, 89(1–4), 241–256. <https://doi.org/10.1016/j.marchem.2004.02.014>

- Wanninkhof, R. (2014). Relationship between wind speed and gas exchange over the ocean revisited. *Limnology and Oceanography: Methods*, 12(JUN), 351–362.
<https://doi.org/10.4319/lom.2014.12.351>
- Ward, B. B. (1990). Kinetics of ammonia oxidation by a marine nitrifying bacterium: Methane as a substrate analogue. *Microbial Ecology*, 19(3), 211–225.
<https://doi.org/10.1007/BF02017166>
- Ward, B. B., & Kilpatrick, K. A. (1990). Relationship between substrate concentration and oxidation of ammonium and methane in a stratified water column. *Continental Shelf Research*, 10(12), 1193–1208. [https://doi.org/10.1016/0278-4343\(90\)90016-F](https://doi.org/10.1016/0278-4343(90)90016-F)
- Ward, B. B., Devol, A. H., Rich, J. J., Chang, B. X., Bulow, S. E., Naik, H., et al. (2009). Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature*, 461(7260), 78–81. <https://doi.org/10.1038/nature08276>
- Willey, J. D., Avery, G. B., Felix, J. D., Kieber, R. J., Mead, R. N., & Shimizu, M. S. (2019). Rapidly increasing ethanol concentrations in rainwater and air. *Npj Climate and Atmospheric Science*, 2(1), 1–5. <https://doi.org/10.1038/s41612-018-0059-z>
- Wiseman, W. J., Rabalais, N. N., Turner, R. E., Dinnel, S. P., & Macnaughton, A. (1997). Seasonal and interannual variability within the Louisiana coastal current: Stratification and hypoxia. *Journal of Marine Systems*, 12(1–4), 237–248.
[https://doi.org/10.1016/S0924-7963\(96\)00100-5](https://doi.org/10.1016/S0924-7963(96)00100-5)
- Wu, H., Green, M., & Scranton, M. I. (1997). Acetate cycling in the water column and surface sediment of Long Island Sound following a bloom. *Limnology and Oceanography*, 42(4), 705–713. <https://doi.org/10.4319/lo.1997.42.4.0705>

- Yang, M., Nightingale, P. D., Beale, R., Liss, P. S., Blomquist, B., & Fairall, C. (2013). Atmospheric deposition of methanol over the Atlantic Ocean. *Proceedings of the National Academy of Sciences of the United States of America*, 110(50), 20034–20039. <https://doi.org/10.1073/pnas.1317840110>
- Yu, Z., Beck, D. A. C., & Chistoserdova, L. (2017). Natural Selection in Synthetic Communities Highlights the Roles of Methylococcaceae and Methylophilaceae and Suggests Differential Roles for Alternative Methanol Dehydrogenases in Methane Consumption. *Frontiers in Microbiology*, 8(December), 1–13. <https://doi.org/10.3389/fmicb.2017.02392>
- Yvon-Durocher, G., Caffrey, J. M., Cescatti, A., Dossena, M., Giorgio, P. Del, Gasol, J. M., et al. (2012). Reconciling the temperature dependence of respiration across timescales and ecosystem types. *Nature*, 487(7408), 472–476. <https://doi.org/10.1038/nature11205>
- Zheng, Y., Wang, H., Liu, Y., Zhu, B., Li, J., Yang, Y., et al. (2020). Methane-Dependent Mineral Reduction by Aerobic Methanotrophs under Hypoxia. *Environmental Science & Technology Letters*. <https://doi.org/10.1021/acs.estlett.0c00436>
- Zhuang, G., Lin, Y., Elvert, M., Heuer, V. B., & Hinrichs, K. (2014). Gas chromatographic analysis of methanol and ethanol in marine sediment pore waters : Validation and implementation of three pretreatment techniques. *Marine Chemistry*, 160, 82–90. <https://doi.org/10.1016/j.marchem.2014.01.011>
- Zhuang, G., Montgomery, A., Sibert, R. J., Rogener, M., Samarkin, V. A., & Joye, S. B. (2018). Effects of pressure, methane concentration, sulfate reduction activity, and

temperature on methane production in surface sediments of the Gulf of Mexico.

Limnology and Oceanography, 63, 2080–2092. <https://doi.org/10.1002/lno.10925>

Zhuang, G., Peña-Montenegro, T. D., Montgomery, A., Hunter, K. S., & Joye, S. B.

(2018). Microbial metabolism of methanol and methylamine in the Gulf of Mexico : insight into marine carbon and nitrogen cycling. *Environmental Microbiology*, 20(12), 4543–4554. <https://doi.org/10.1111/1462-2920.14406>

Zhuang, G., Peña-Montenegro, T. D., Montgomery, A., Montoya, J. P., & Joye, S. B.

(2019). Significance of Acetate as a Microbial Carbon and Energy Source in the Water Column of Gulf of Mexico: Implications for Marine Carbon Cycling. *Global Biogeochemical Cycles*, 33(2), 223–235. <https://doi.org/10.1029/2018GB006129>

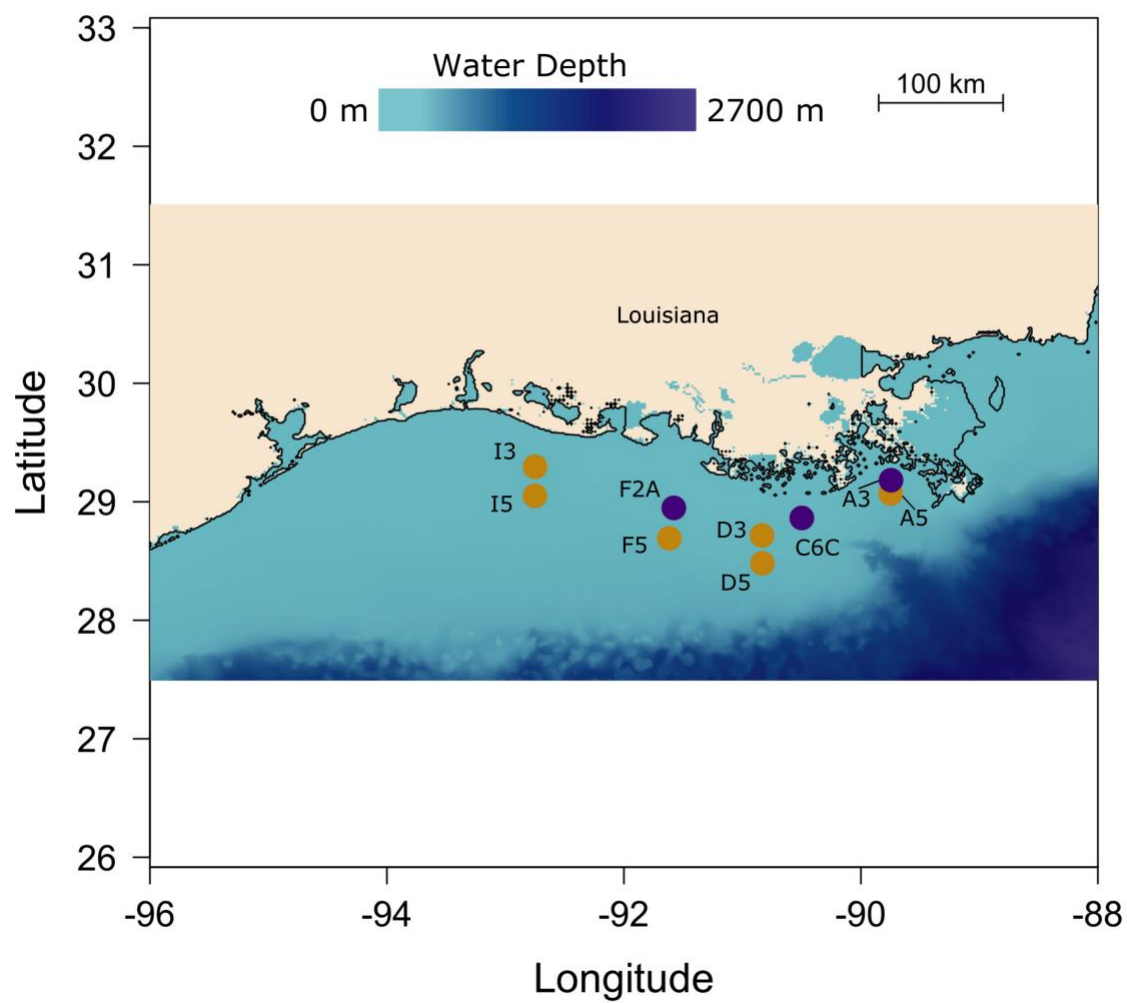


Figure 5.1. Station map of samples collected from the Gulf of Mexico hypoxic zone in July 2018. Purple circles depict stations with bottom water hypoxia (<2 mg L⁻¹) at the time of collection, while yellow circles were not hypoxic at the time of sampling.

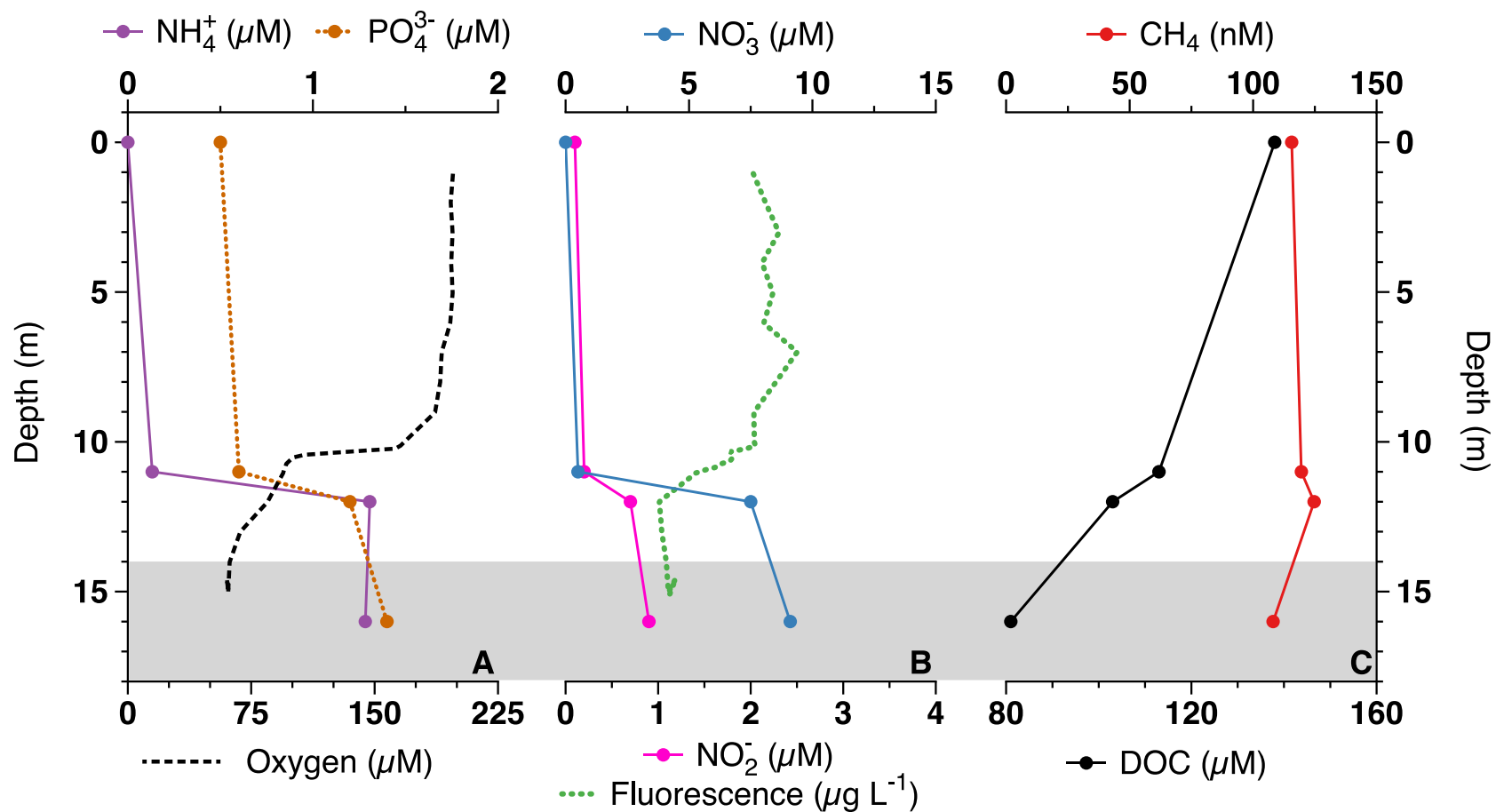


Figure 5.2. Depth profiles of ammonium, phosphate, and oxygen (A), nitrate, nitrite, and fluorescence (B), and methane and dissolved organic carbon (C) at site A3 in the water column of the Gulf of Mexico hypoxic zone.

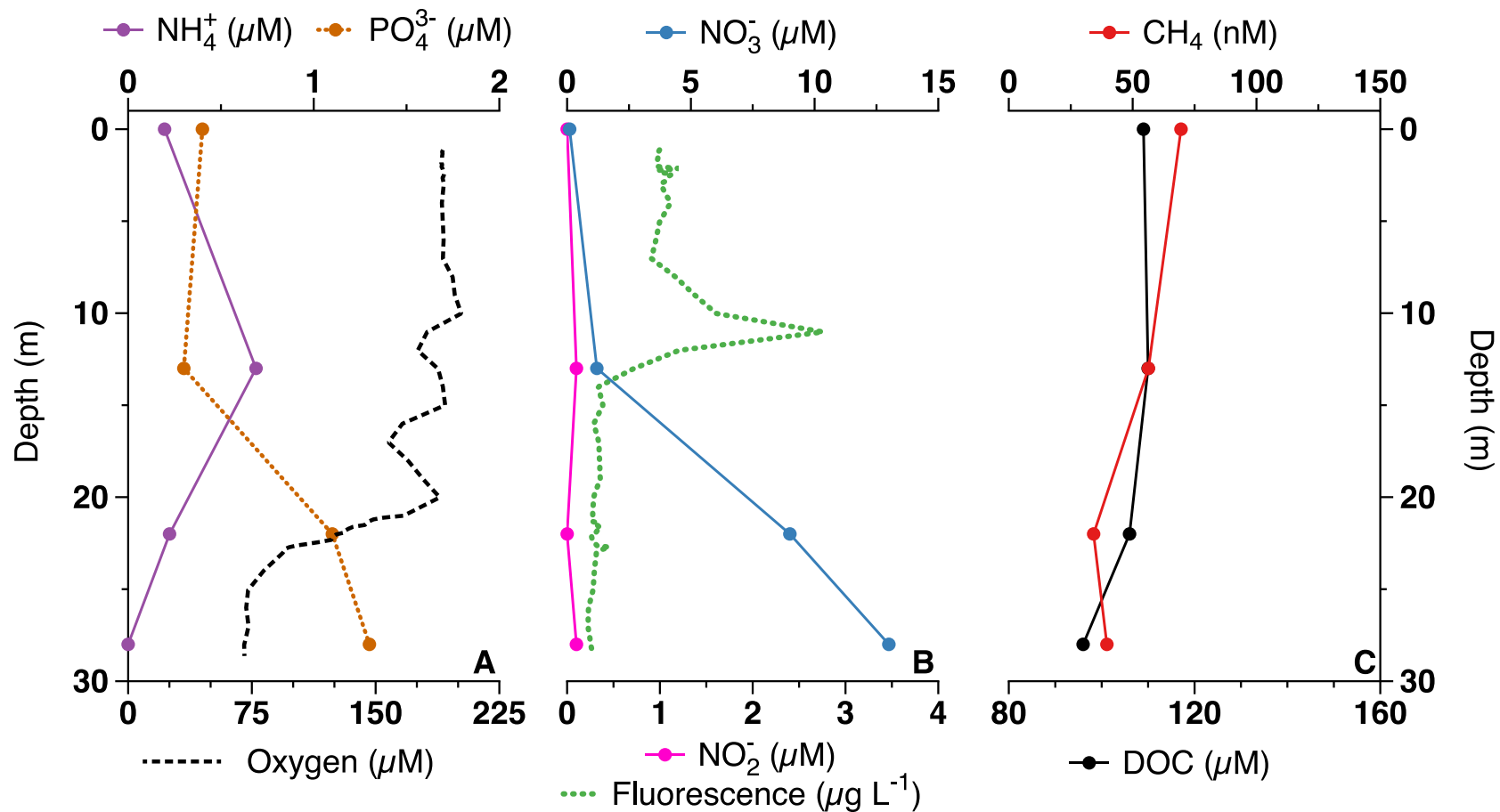


Figure 5.3. Depth profiles of ammonium, phosphate and oxygen (A), nitrate, nitrite, and fluorescence (B), and methane and dissolved organic carbon (C) at site A5 in the water column of the Gulf of Mexico hypoxic zone.

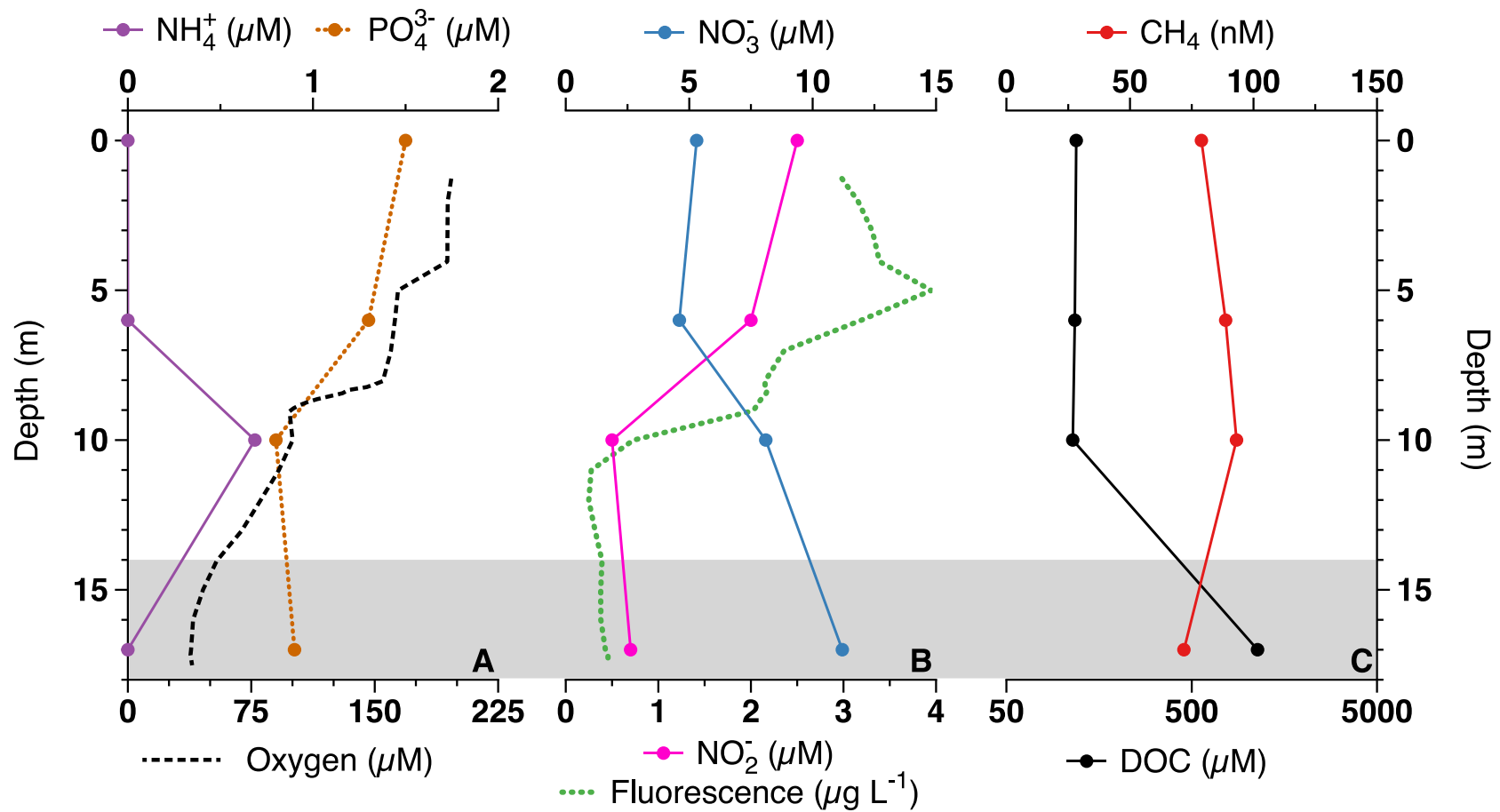


Figure 5.4. Depth profiles of ammonium, phosphate and oxygen (A), nitrate, nitrite, and fluorescence (B), and methane and dissolved organic carbon (C) at site C6C in the water column of the Gulf of Mexico hypoxic zone.

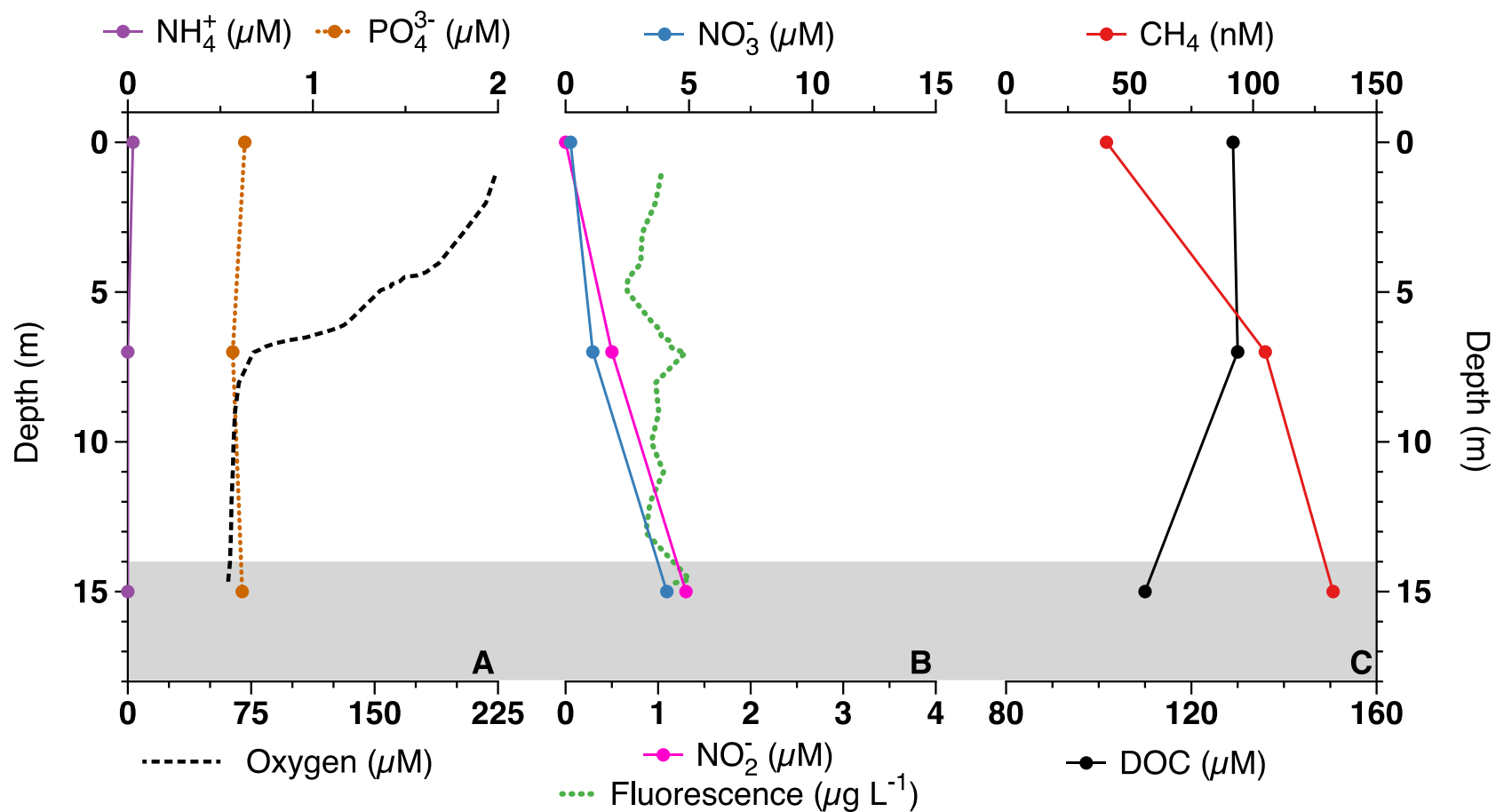


Figure 5.5. Depth profiles of ammonium, phosphate and oxygen (A), nitrate, nitrite, and fluorescence (B), and methane and dissolved organic carbon (C) at site F2A in the water column of the Gulf of Mexico hypoxic zone

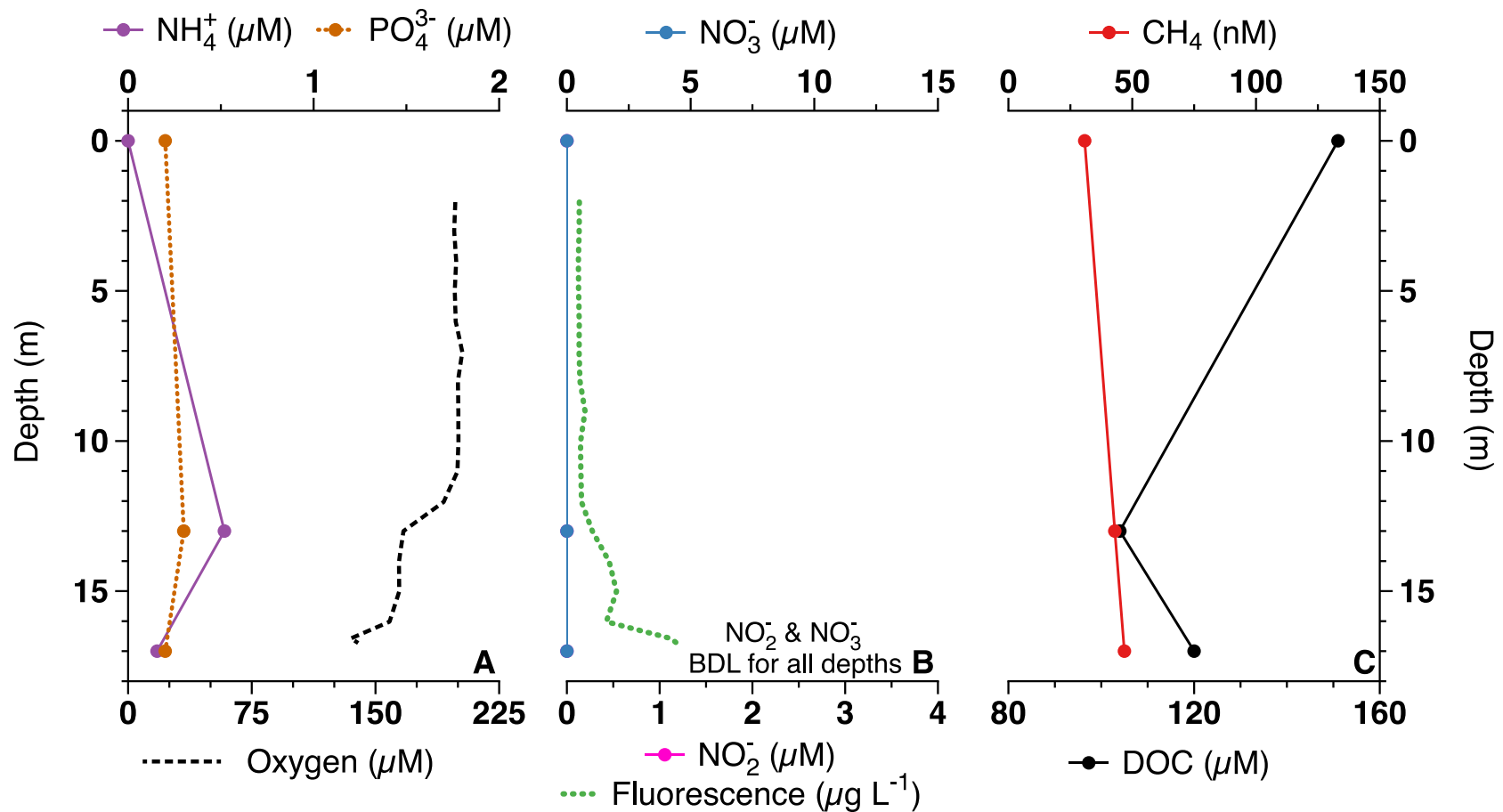
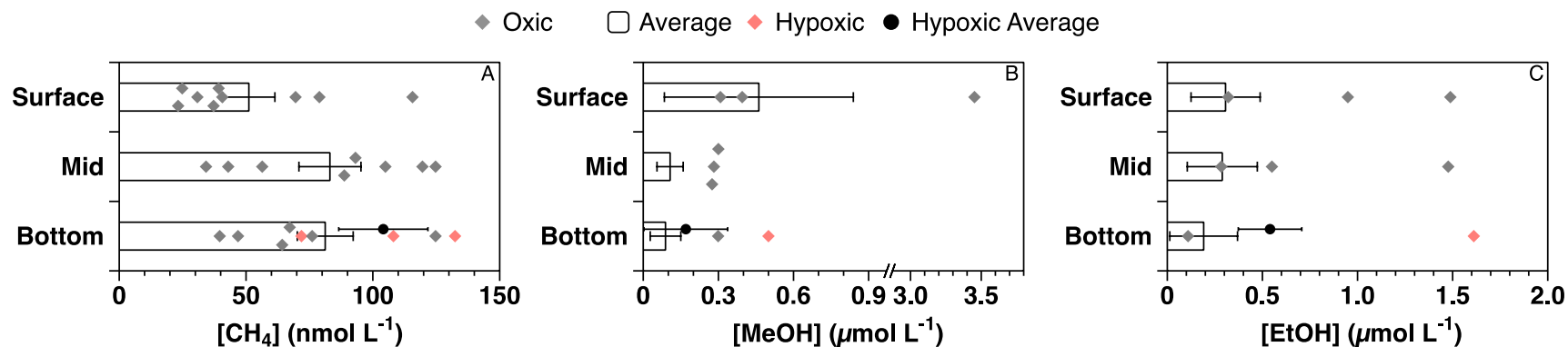


Figure 5.6. Depth profiles of ammonium, phosphate and oxygen (A), nitrate, nitrite, and fluorescence (B), and methane and dissolved organic carbon (C) at site I3 in the water column of the Gulf of Mexico hypoxic zone. BDL = Below detection limit



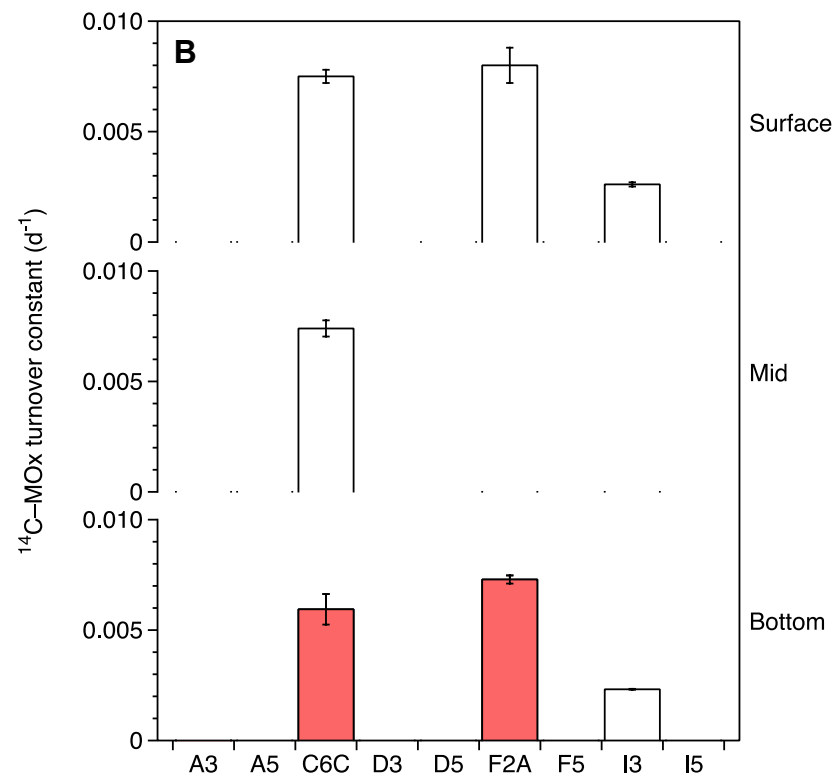
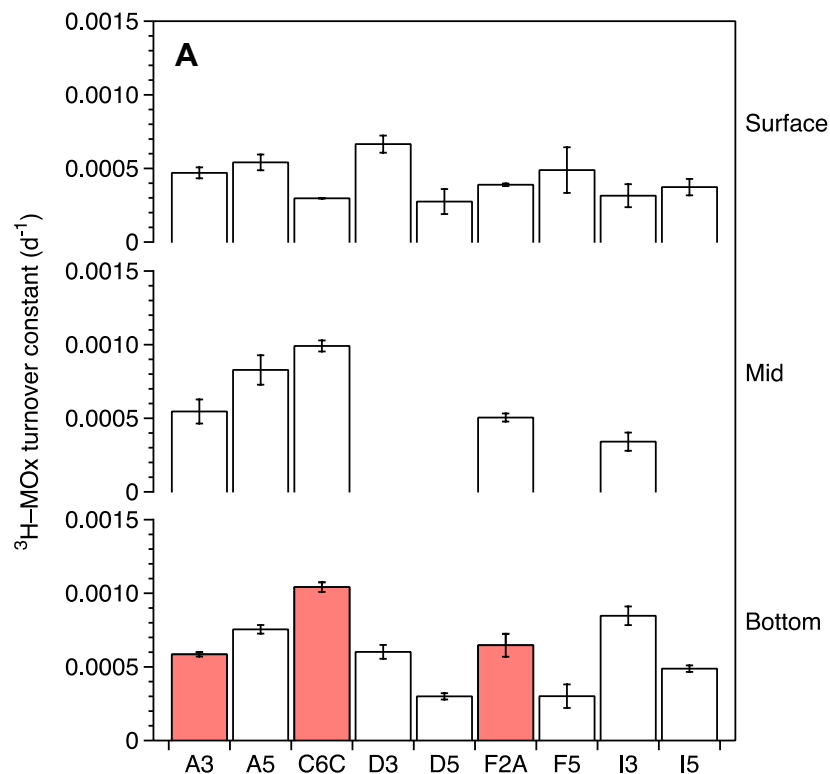


Figure 5.8. Potential methane oxidation turnover constants using the ^3H (A) and ^{14}C (B) radiotracer methods at all sites separated by depth. Surface samples were less than 2 m deep, mid samples were between 2 m water depth and 2 m above the seafloor, and bottom samples were less than 2 m above the seafloor. Depth and altimetry data were based on CTD data. Samples highlighted in red were hypoxic at the time of sampling. Note differences in scales.

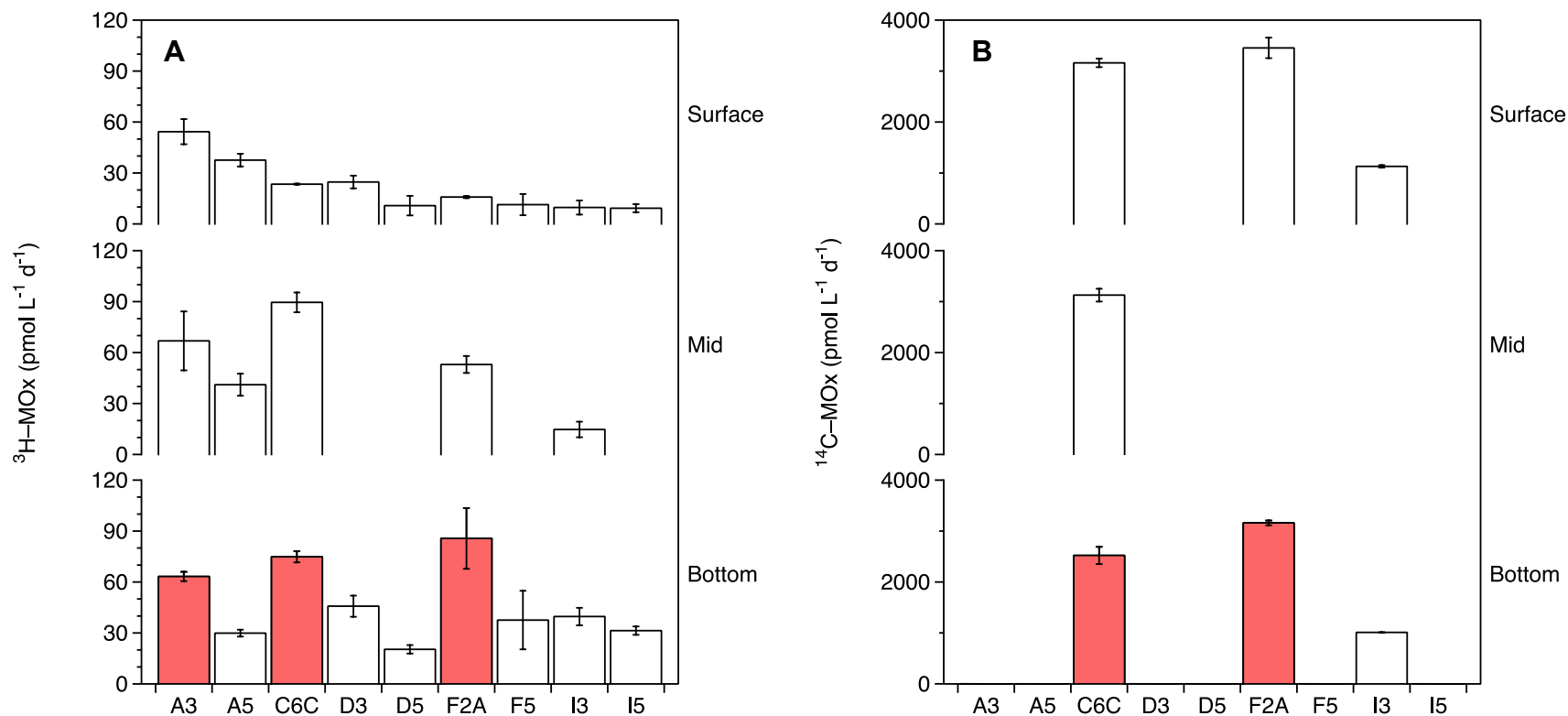


Figure 5.9. Potential methane oxidation rates using the ^3H (A) and ^{14}C (B) radiotracer methods at all sites separated by depth. Surface samples were less than 2 m deep, mid samples were between 2 m water depth and 2 m above the seafloor, and bottom samples were less than 2 m above the seafloor. Depth and altimetry data were based on CTD data. Samples highlighted in red were hypoxic at the time of sampling. Note differences in scales.

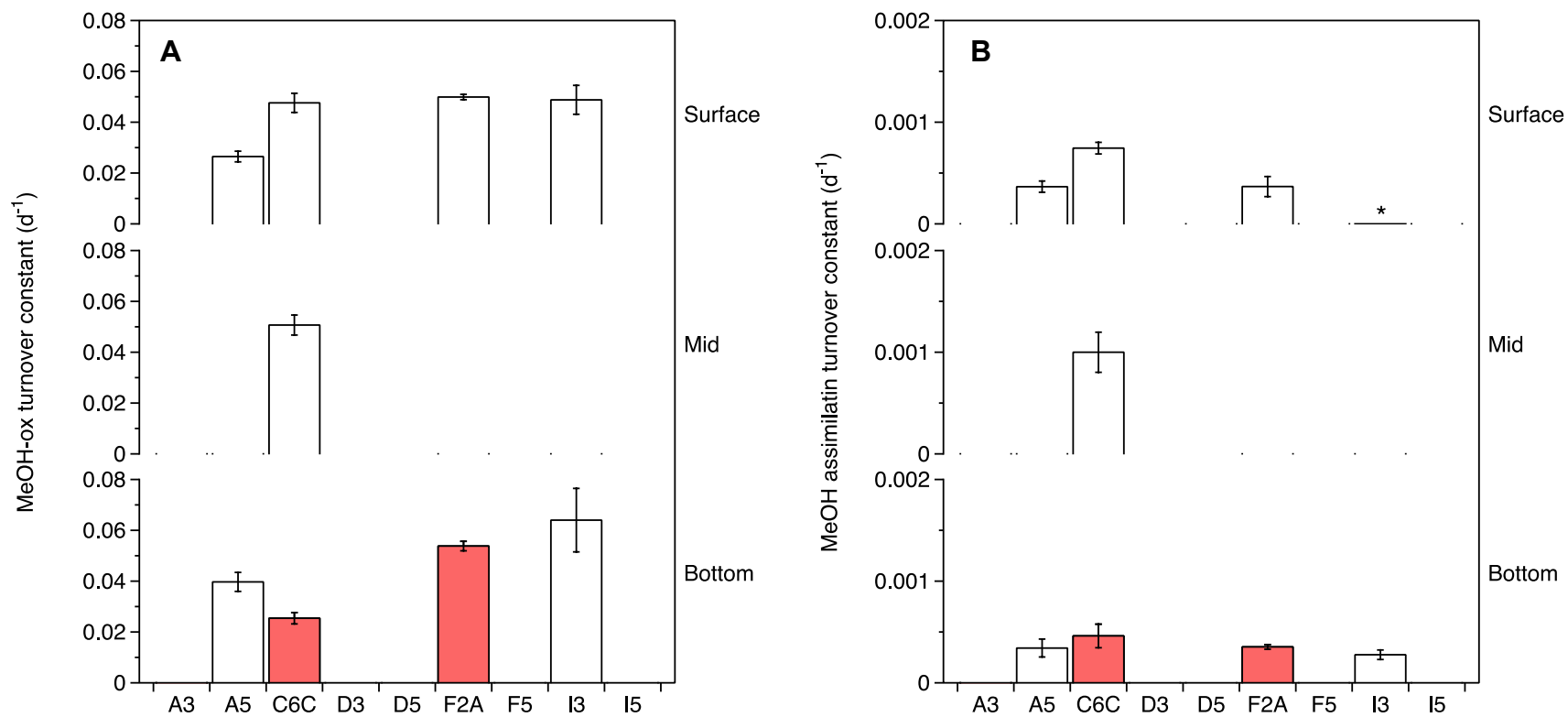


Figure 5.10. Potential methanol oxidation (A) and assimilation (B) turnover constants at all sites separated by depth. Surface samples were less than 2 m deep, mid samples were between 2 m water depth and 2 m above the seafloor, and bottom samples were less than 2 m above the seafloor. Depth and altimetry data were based on CTD data. Samples highlighted in red were hypoxic at the time of sampling. Note differences in scales. * indicates sample was below detection

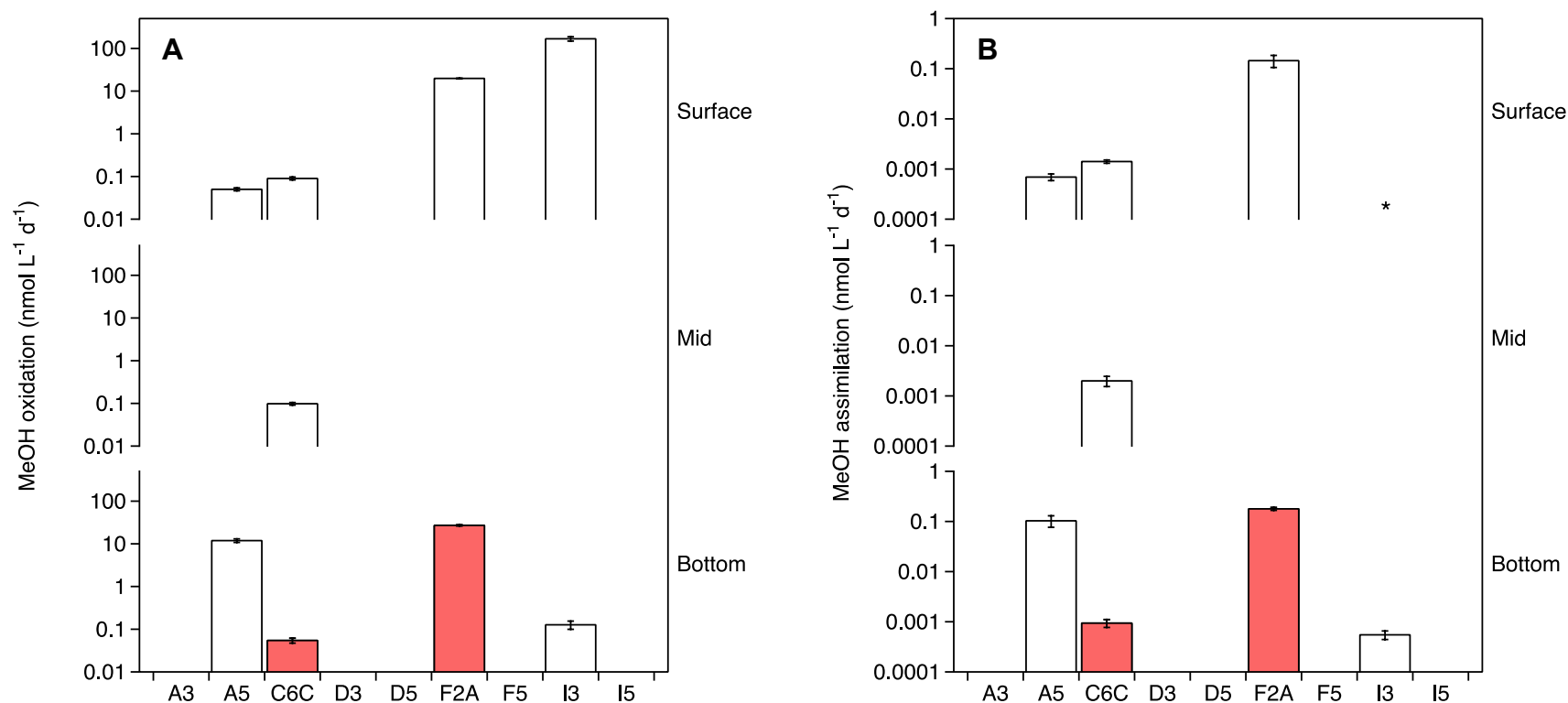


Figure 5.11. Potential methanol oxidation (A) and assimilation (B) rates at all sites separated by depth. Surface samples were less than 2 m deep, mid samples were between 2 m water depth and 2 m above the seafloor, and bottom samples were less than 2 m above the seafloor. Depth and altimetry data were based on CTD data. Samples highlighted in red were hypoxic at the time of sampling. Note differences in scales and log scales. * indicates sample was below detection

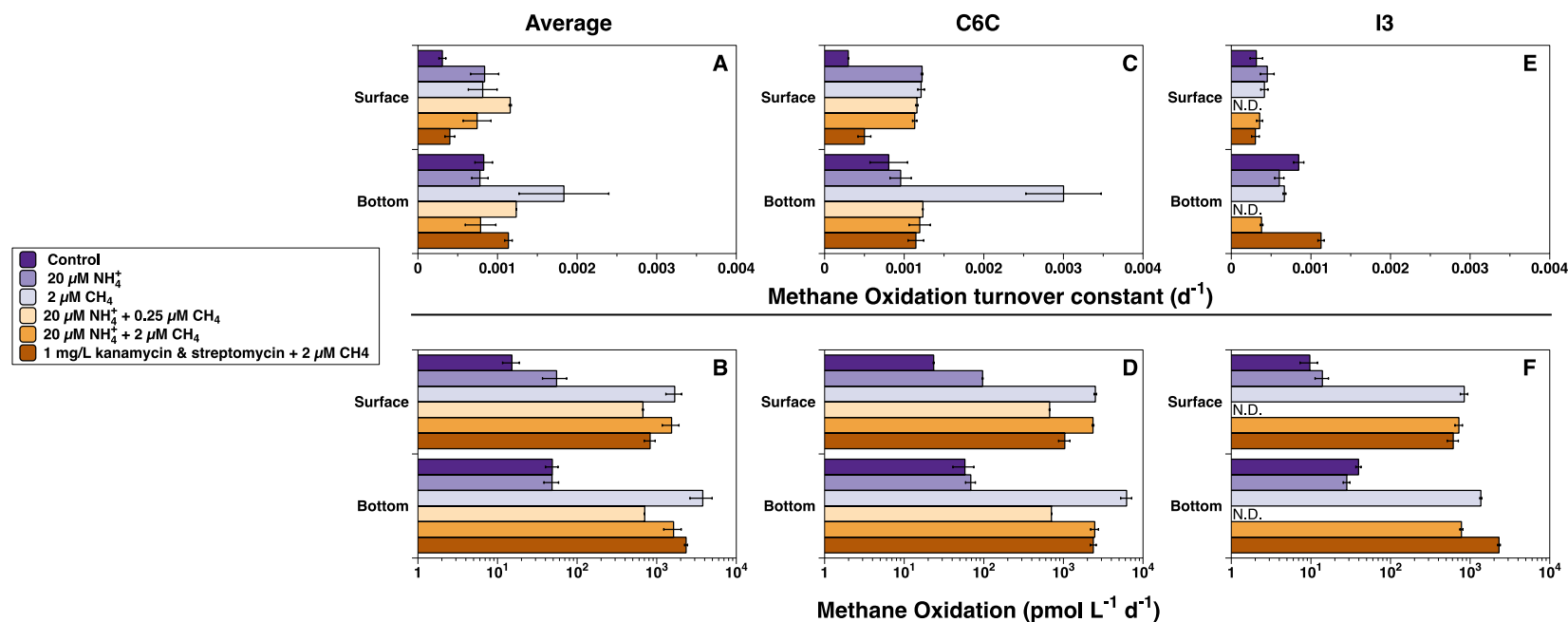


Figure 5.12. Effects of amendments on turnover constants (A, C, E) and potential rates (B, D, F) of ^3H methane oxidation from stations C6C, and I3. Surface samples were less than 2 m deep and bottom samples were less than 2 m above the seafloor based on CTD data. N.D. = not determined; B.D.L. = below detection limit; Note differences in scales and units.

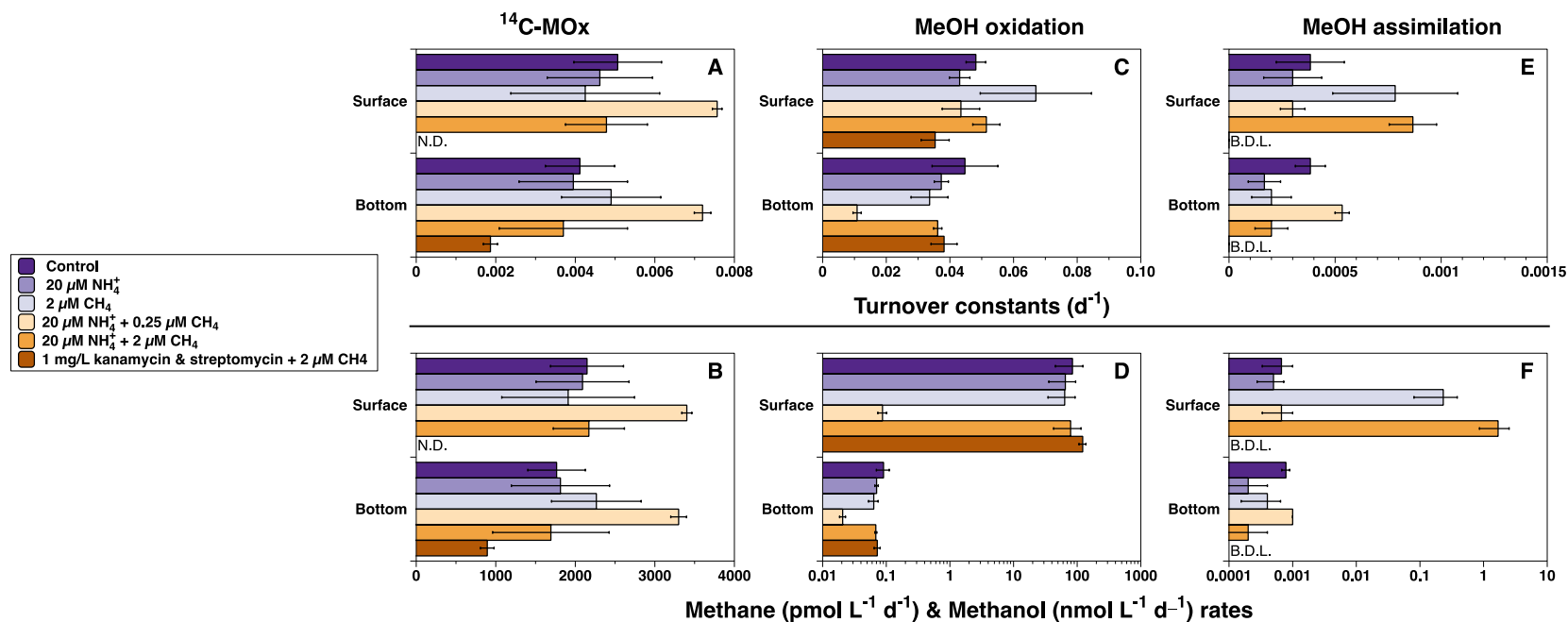


Figure 5.13. Average effects of amendments on turnover constants (A, C, E) and potential rates (B, D, F) of ^{14}C methane oxidation, methanol oxidation, and methanol assimilation from sites A5, C6C, and I3. Surface samples were less than 2 m deep and bottom samples were less than 2 m above the seafloor based on CTD data. N.D. = not determined; B.D.L. = below detection limit; Note differences in scales and units.

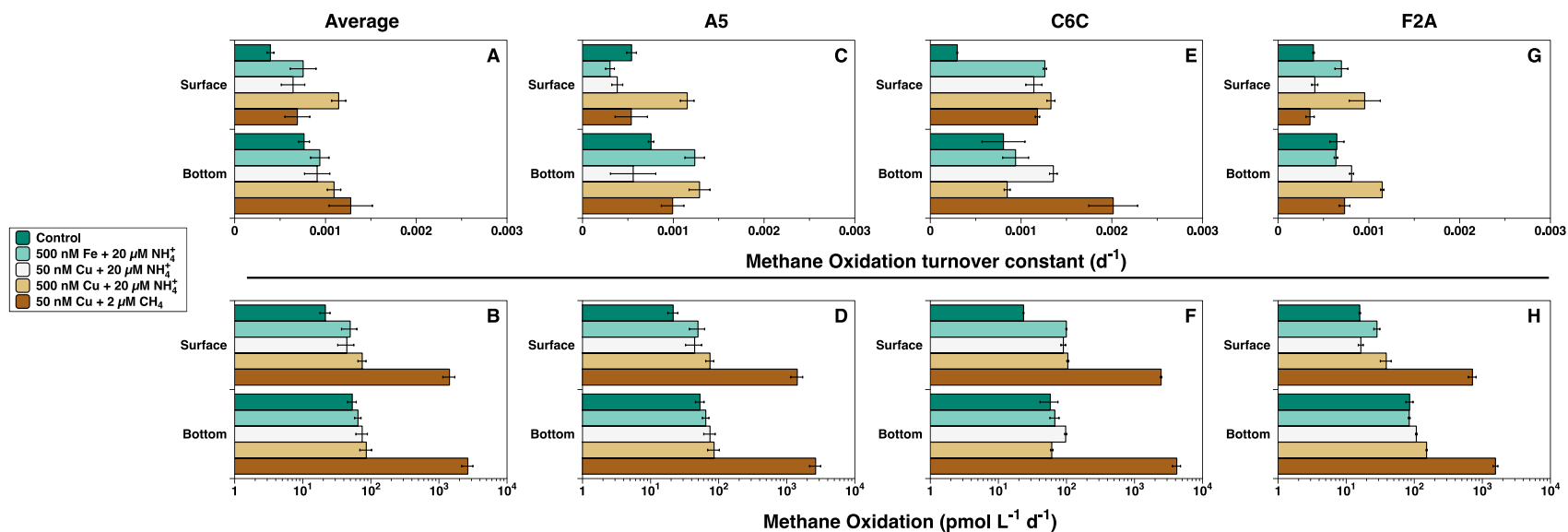


Figure 5.14. Effects of amendments on turnover constants (A, C, E, G) and potential rates (B, D, F, H) of ^3H methane oxidation from sites A5, F2A, and combined average. Surface samples were less than 2 m deep and bottom samples were less than 2 m above the seafloor based on CTD data.

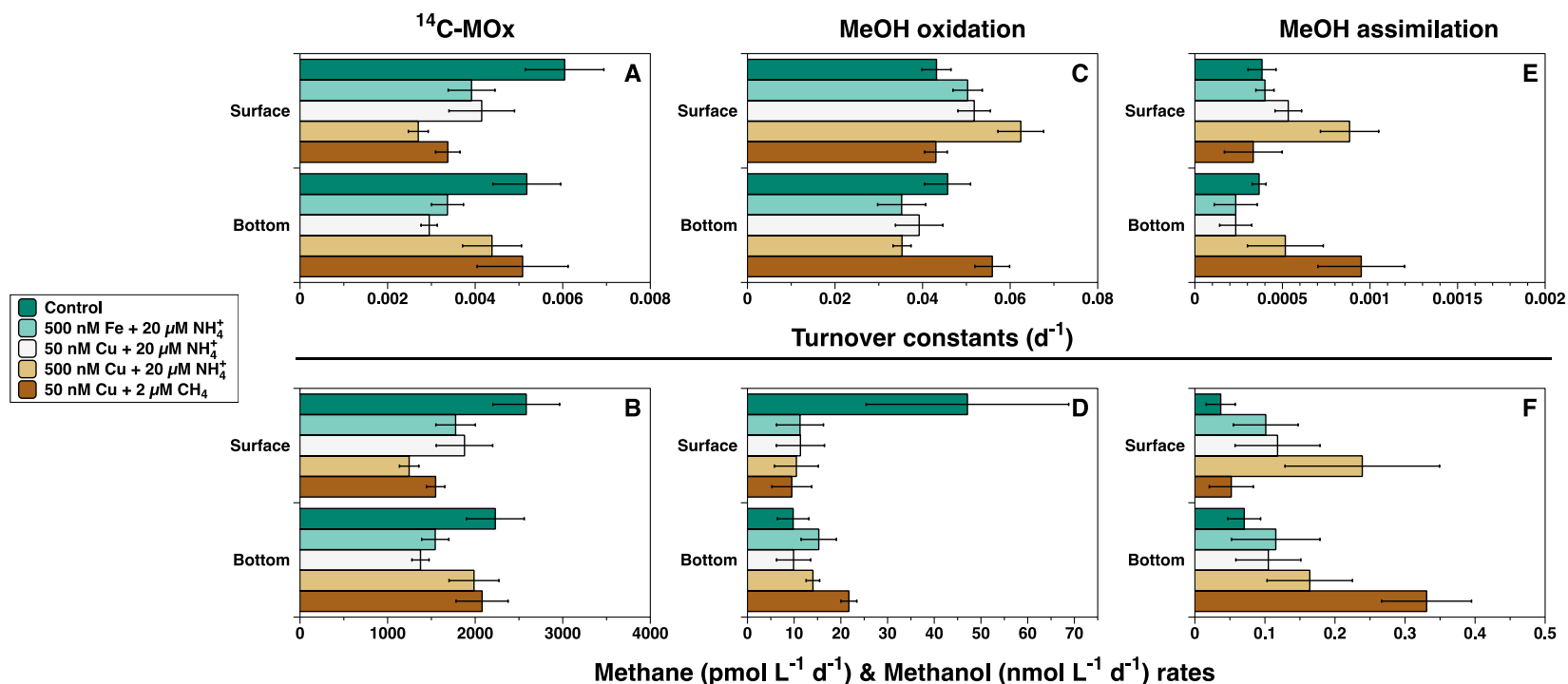


Figure 5.15. Average effects of amendments on turnover constants (A, C, E) and potential rates (B, D, F) of ^{14}C methane oxidation, methanol oxidation, and methanol assimilation from sites A5, F2A, and combined average. Surface samples were less than 2 m deep and bottom samples were less than 2 m above the seafloor based on CTD data. Note differences in scales and units.

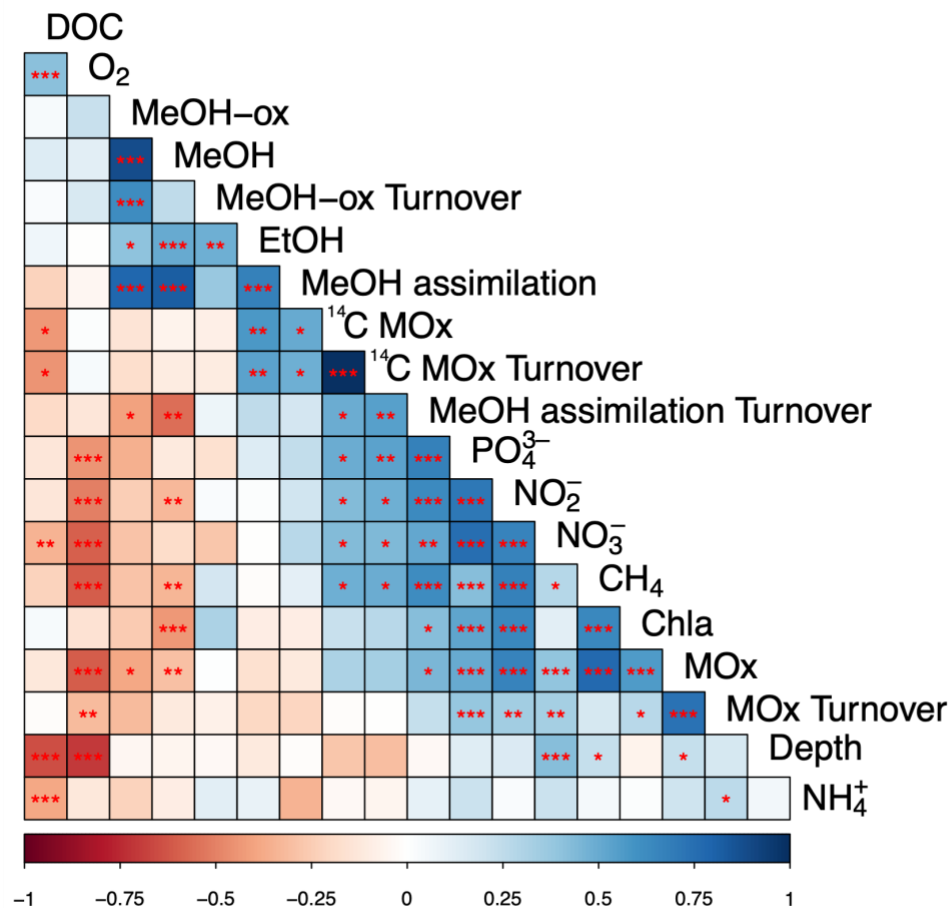


Figure 5.16. Heatmap of Spearman's correlation coefficients between rates and geochemical factors in the Gulf of Mexico hypoxic zone. Color intensity is indicative of strength of correlation; red is negative, and blue is positive. Statistically significant (p -value < 0.05) relationships are denoted by red *.

* $.01 \leq p < 0.05$ ** $0.001 \leq p < 0.01$ *** $p < 0.001$

Table 5.1. Summary of ratios of selected rates at certain stations and depths

Station	Depth	³ H MOx : ¹⁴ C MOx		MeOH Assimilation : MeOH Oxidation
		Turnover	Rate	
A5	Surface	N.D.	N.D.	0.014
A5	Bottom	N.D.	N.D.	0.009
C6C	Surface	0.040	0.007	0.016
C6C	Mid	0.175	0.037	0.018
C6C	Mid	0.095	0.021	0.022
C6C	Bottom	0.176	0.030	0.018
F2A	Surface	0.049	0.005	0.007
F2A	Bottom	0.089	0.027	0.007
I3	Surface	0.120	0.009	N.D.
I3	Bottom	0.365	0.039	0.004
	Average	0.138	0.022	0.013

MOx = methane oxidation; MeOH = methanol; Turnover = turnover constants

Ratios are presented as: tritium over ¹⁴C & MeOH assimilation over MeOH oxidation

N.D. = not determined

Table 5.S1. Sampling stations from the northern Gulf of Mexico hypoxic zone and associated sampling information

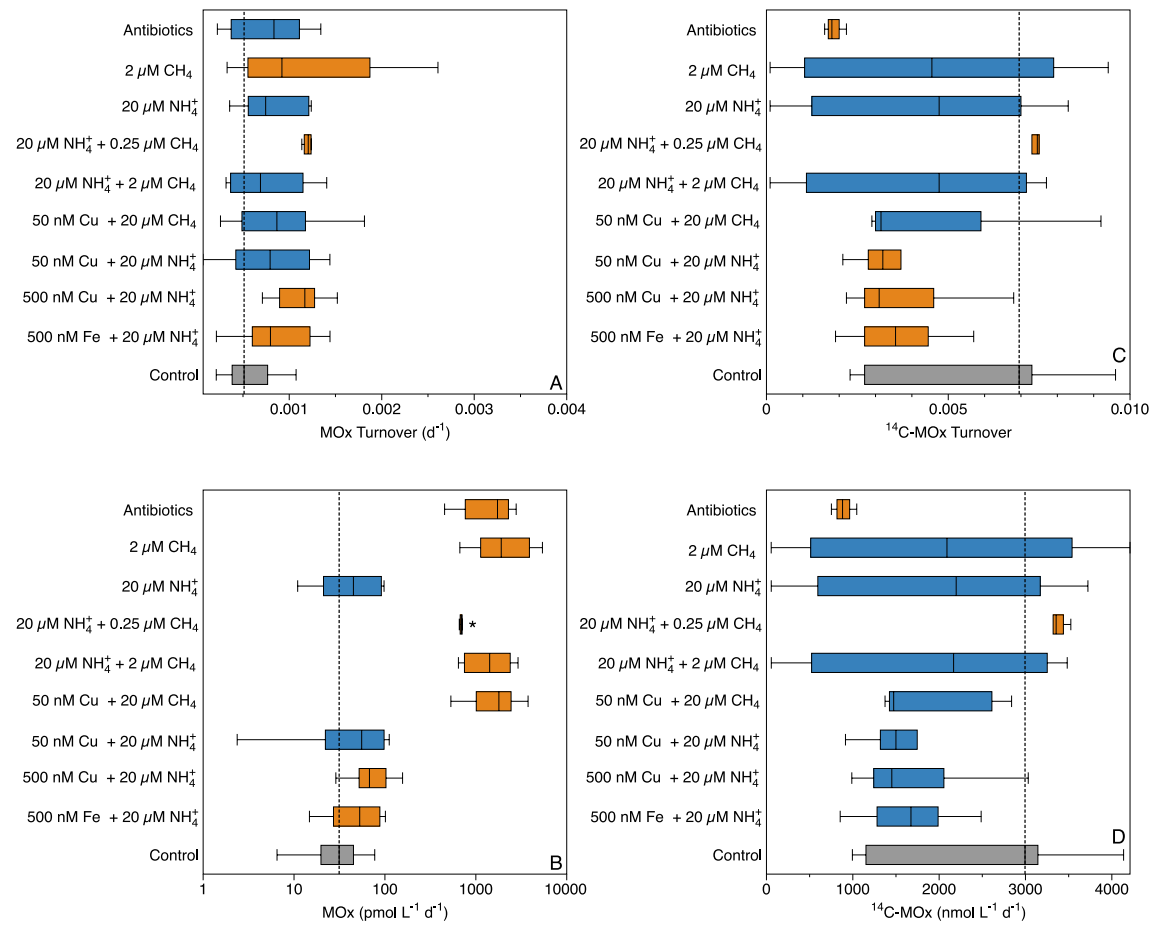
Station Name	Latitude	Longitude	Date Collected	Depth (m)	
A5	29.0701	-89.7489	23/7/18	0	Surface
A5	29.0701	-89.7489	23/7/18	13	Mid
A5	29.0701	-89.7489	23/7/18	22	Mid
A5	29.0701	-89.7489	23/7/18	30	Bottom
A3	29.1821	-89.7457	23/7/18	0	Surface
A3	29.1821	-89.7457	23/7/18	11	Mid
A3	29.1821	-89.7457	23/7/18	12	Mid
A3	29.1821	-89.7457	23/7/18	16	Bottom
C6C	28.8635	-90.4986	24/7/18	0	Surface
C6C	28.8635	-90.4986	24/7/18	6	Mid
C6C	28.8635	-90.4986	24/7/18	10	Mid
C6C	28.8635	-90.4986	24/7/18	17	Bottom
D5	28.4817	-90.8309	25/7/18	0	Surface
D5	28.4817	-90.8309	25/7/18	35	Bottom
D3	28.7163	-90.8342	25/7/18	0	Surface
D3	28.7163	-90.8342	25/7/18	16	Bottom
F5	28.6916	-91.6166	25/7/18	0	Surface
F5	28.6916	-91.6166	25/7/18	29	Bottom
F2A	28.9483	-91.5789	25/7/18	0	Surface
F2A	28.9483	-91.5789	25/7/18	7	Mid
F2A	28.9483	-91.5789	25/7/18	14	Bottom
I3	29.2953	-92.7495	27/7/18	0	Surface
I3	29.2953	-92.7495	27/7/18	13	Mid
I3	29.2953	-92.7495	27/7/18	17	Bottom
I5	29.0501	-92.7503	27/7/18	0	Surface
I5	29.0501	-92.7503	27/7/18	24	Bottom

Table 5.S2. Student's T-test comparing amended rates with unamended controls.

		500 nM Fe + 20 μM NH4		50 nM Cu + 20 μM NH4		500 nM Cu + 20 μM NH4		50 nM Cu + 2 μM CH4		20 μM NH4 + 2 μM CH4		20 μM NH4		2 μM CH4		20 μM NH4 + 0.25 μM CH4		1 mg/L kanamycin & streptomycin + 2 μM CH4	
Site(s)	Depth	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate	Turnover	Rate
³ H- Methane Oxidation																			
A5	Surface	0.0302	N.S.	N.S.	N.S.	0.0033	0.0043	N.S.	0.0077	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
A5	Bottom	0.0360	0.0395	N.S.	N.S.	0.0342	0.0308	N.S.	N.S.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
C6C	Surface	0.0002	<0.0001	0.0102	0.0033	0.0016	0.0002	0.0005	<0.0001	0.0008	<0.0001	<0.0001	<0.0001	0.0018	<0.0001	<0.0001	<0.0001	N.S.	0.0019
C6C	Bottom	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.0276	0.0039	N.S.	0.0057	N.S.	N.S.	0.0177	0.0025	N.S.	0.0202	N.S.	0.0074
F2A	Surface	0.0456	0.0242	N.S.	N.S.	N.S.	0.0365	N.S.	0.0008	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
F2A	Bottom	N.S.	N.S.	N.S.	N.S.	0.0209	0.0437	N.S.	0.0002	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
I3	Surface	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.S.	0.0008	N.S.	N.S.	N.S.	0.0005	N.A.	N.A.	N.S.	0.0003
I3	Bottom	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0125	<0.0001	0.0463	N.S.	N.S.	<0.0001	N.A.	N.A.	0.0279	<0.0001
All	Both	0.0171	0.0274	N.S.	N.S.	<0.0001	<0.0001	N.S.	0.0001	N.S.	<0.0001	N.S.	N.S.	0.0395	<0.0001	<0.0001	<0.0001	N.S.	<0.0001
¹⁴ C- Methane Oxidation																			
All	Surface	N.S.	N.S.	N.S.	N.S.	0.0052	0.0103	0.0215	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.A.	N.A.
All	Bottom	N.S.	N.S.	0.0231	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.0342	0.0218	0.0019	0.0023
All	Both	0.0098	N.S.	0.0208	N.S.	0.0241	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.0071	0.0023	<0.0001	0.0002
Methanol oxidation																			
All	Surface	N.S.	N.S.	N.S.	N.S.	0.0074	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.7817	N.S.	N.S.	N.S.	0.0118	N.S.	0.0023
All	Bottom	N.S.	0.0172	N.S.	N.S.	N.S.	0.0138	N.S.	0.0050	N.S.	0.0053	N.S.	0.0055	N.S.	0.0039	<0.0001	0.0007	N.S.	0.0058
All	Both	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	<0.0001	N.S.	N.S.
Methanol assimilation																			
All	Surface	N.S.	N.S.	N.S.	N.S.	0.0261	N.S.	N.S.	0.6045	0.0043	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.0005	N.S.
All	Bottom	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.0003	N.S.	0.0064	0.0493	0.0063	N.S.	0.0065	0.0106	0.0085	<0.0001	0.0055
All	Both	N.S.	N.S.	N.S.	N.S.	0.0478	N.S.	N.S.	0.0236	N.S.	N.S.	N.S.	0.0010	N.S.	N.S.	N.S.	0.0014	<0.0001	0.0007

Mean comparisons with p -values < 0.05 were determined to be significantly different from unamended controls and are shown.

N.A., samples not collected. N.S., no significant differences between means.



(Two-page figure)

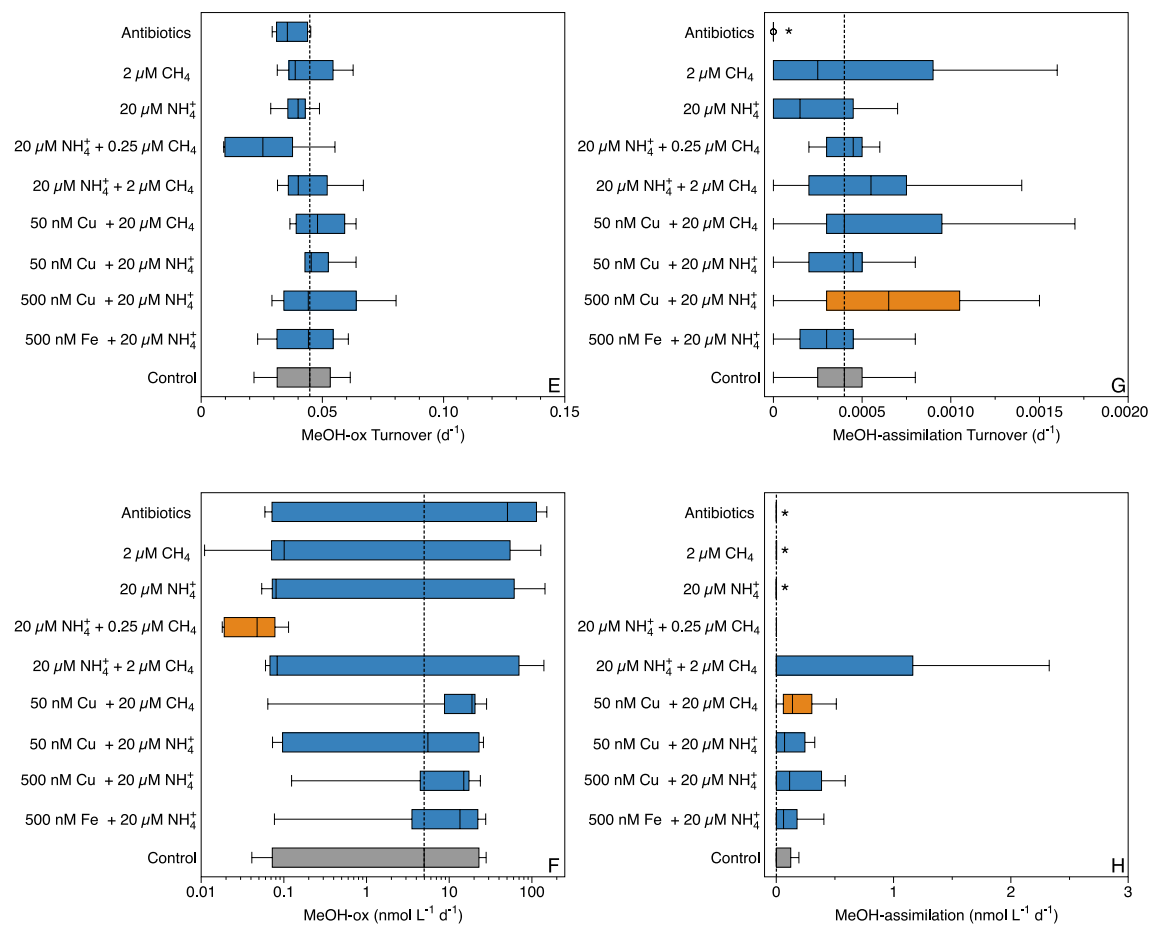


Figure. 5.S1. Box plots depicting effects of amendments on ³H methane oxidation (A, B), ¹⁴C methane oxidation (C, D), methanol oxidation (E, F), and methanol assimilation (G, H) turnover constants and rates across all sites and depths. Treatments that were significantly different than unamended controls (grey) are colored in yellow and in some cases labeled with * for clarity. Blue boxes were not statistically different from controls. Vertical dashed line represents the median of the control. Note differences in scales. Antibiotics = streptomycin and kanamycin (1 mg L⁻¹, each), and 2 μmol L⁻¹ methane

CHAPTER 6

CONCLUSIONS

In marine environments, carbon cycling is influenced by biogeochemical and physical forcing functions. Today, anthropogenic forcing also impacts the carbon cycle, and together these forces modulate carbon cycling with profound repercussions for Earth's climate and sustainability. Systematic knowledge of marine carbon cycling is necessary to understand global environmental change. Studying carbon cycling in extreme marine environments, where microbial life is pushed to its limits, can provide critical insight into the connections between biogeochemical drivers and cycling rates and pathways. In this dissertation, marine carbon dynamics were assessed from four distinct perspectives. The research investigated carbon cycling in the context of two extreme environments, specifically, the Guaymas Basin deep-sea hydrothermal system and the Gulf of Mexico hypoxic zone. The overarching goal was to evaluate microbial and biogeochemical drivers of organic carbon cycling in the marine environment and this goal was achieved using a suite of multidisciplinary approaches.

The scope of the four chapters comprising this dissertation is broad, but taken together, the body of work provides novel insights into marine carbon dynamics and advances our understanding of organic carbon cycling. The spatiotemporal variability of hydrothermal sediments (**Chapter 2**) underscored the influence of sedimentary processes in deep-sea ecosystems. Biogeochemical processes in marine sediments (*e.g.*, anaerobic

oxidation of methane) help regulate the flux of carbon from the benthos to overlying waters. Hydrothermal vents in the Guaymas Basin serve as a conduit to deliver significant quantities of energy-rich substrates to the deep sea, directly connecting sedimentary and water column reservoirs and processes. This work demonstrated, for the first time, the importance of this connection for organic carbon cycling in this deep-sea extreme environment (**Chapter 3**). The data presented here indicated the unrecognized importance of heterotrophic processes in hydrothermal plumes and imply that hydrothermal systems are an important source of labile organic carbon to deep-sea ecosystems. Unlike the typical deep ocean, which is largely depleted in bio-available organic carbon, hydrothermal plumes are oases of deep-sea organic matter cycling and heterotrophic production, revising our understanding of these systems as areas predominantly characterized by chemosynthetic production.

The deep water in the Guaymas Basin is hypoxic ($O_2 < 2 \text{ mg L}^{-1}$), a phenomenon that is also observed in the northern Gulf of Mexico due to excessive nutrient loading from the Mississippi River. In **Chapter 4**, the impacts of this coastal hypoxia on methane and methanol cycling were explored. Deoxygenation of marine environments is predicted to become more pervasive in the coming decades, which will have significant influences on marine biogeochemical cycles and the global economy via impacts on marine fisheries. Elevated rates of microbial activity were observed in low-oxygen environments suggesting that hypoxic systems may serve to reduce the flux of volatile organics and greenhouse gases (*e.g.*, methane and methanol) to the atmosphere. As the extent of low oxygen environments increases across the globe, understanding the microbial drivers of carbon

cycling will provide crucial insight into how marine environments are responding. Constraining microbial dynamics from multiple environments will aid in this endeavor.

Ammonia-oxidizing archaea (AOA) from the phylum Thaumarchaeota are abundant and active in Guaymas Basin plumes, the Gulf of Mexico hypoxic zone, and other low-oxygen habitats. In **Chapter 5**, the capacity for AOA to metabolize methane and leucine was explored, revealing unconventional metabolisms for these unique and globally abundant microbes. Given the abundance of AOA, they may contribute significantly to organic carbon cycling (*e.g.*, methanotrophy, heterotrophic production) in marine environments. Furthermore, AOA are known to adapt to changing environmental conditions, so it is likely that we are only at the cusp of understanding the ecophysiology of these microbes in the context of global change.

The investigations presented herein give rise to future questions that will help advance the understanding of marine carbon cycling. Given the spatiotemporal variability of hydrothermal systems, a more comprehensive survey of sedimentary organic dynamics will shed light on the physical, chemical, and biological factors that control carbon cycling there. Likewise, a more robust sampling of hydrothermal plumes is necessary to constrain the importance of heterotrophic metabolisms in these systems. End-member activity measurements, higher-resolution sampling, and -omics-based approaches should be combined and leveraged to explore carbon dynamics within hydrothermal plume ecosystems. Future expeditions should also couple sediment surveys (*i.e.*, with OsmoSamplers) with paired water column measurements to connect these dynamic deep-

sea habitats. Elevated methane oxidation rates were observed during bottom water hypoxia, implying a connection between the sediments and overlying waters of coastal hypoxic systems. Constraining sedimentary fluxes in areas of high bottom water microbial activity (*i.e.*, hypoxic waters) might help clarify the biogeochemical drivers of carbon cycling during bottom water hypoxia. Furthermore, recognizing the metabolic flexibility of AOA to metabolize methane and leucine suggests that there are other substrates that these intriguing microbes can use as carbon and energy sources. *In situ* experiments and enrichments are necessary to constrain the environmental capacity of AOA to mediate alternative metabolisms. Genomic, transcriptomic, and proteomic evidence is also necessary to elucidate the pathway(s) by which AOA can metabolize methane and leucine in culture and the environment. Ultimately, the continued use of multidisciplinary approaches in many habitat types is necessary to continue advancing our understanding and fully constrain the biogeochemical drivers of organic carbon cycling in marine environments.