Patterns and variability in geochemical signatures and microbial activity within and between diverse cold seep habitats along the lower continental slope, Northern Gulf of Mexico.

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Abstract

We collected 69 sediment cores from distinct ecological and geological settings along the deep slope in the Northern Gulf of Mexico to evaluate whether specific geochemical- or habitat-related factors correlated with rates of microbial processes and geochemical signatures. By collecting replicate cores from distinct habitats across multiple sites, we illustrate and quantify the heterogeneity of cold seep geochemistry and microbial activity. These data also allow documentation of the factors driving unique aspects of the geochemistry of deep slope gas, oil and brine seeps. Surprisingly little variation was observed between replicate ($n=2-5$) cores within site for most analytes (except methane), implying that the common practice of collecting one core for geochemical analysis can capture the signature of a habitat in most cases. Depth-integrated concentrations of methane, dissolved inorganic carbon (DIC), and calcium were the predominant geochemical factors that correlated with a site's ecological or geological settings. Pore fluid methane concentration was related to the phosphate and DIC concentration, as well as to rates of sulfate reduction rates. While distinctions between seep habitats were identified from geochemical signatures, habitat specific geochemistry varied little across sites. The relative concentration of dissolved inorganic nitrogen versus phosphorus suggests that phosphorus availability limits biomass production at cold seeps. Correlations between calcium, chloride, and phosphate concentrations were indicative of brine-associated phosphate transport, suggesting that in addition to the co-migration of methane, dissolved organic carbon, and ammonium with brine, phosphate delivery is also associated with brine advection.

1. Introduction

Cold seeps are highly heterogeneous environments in terms of both their megafaunal, e.g. clams, mussels, urchins, and tubeworms, and microbial inhabitants, and the diversity of geological settings where they occur, e.g. mud or asphalt volcanoes, brine flows, carbonate hard grounds, gas hydrate outcrops, gas/oil vents and chimneys (Levin, 2005, Joye et al., 2010). Cold seeps occur along both active and passive continental margins, and occur widely in the Gulf of Mexico (hereafter, Gulf) along the upper and lower continental slope (Levin, 2005, Aharon and Fu, 2000, Arvidson et al., 2004, Joye et al., 2004, Joye et al., 2010). Joye and coauthors (2010) postulated that microbial activity at cold seeps of the deep slope of the Gulf of Mexico was less than that of the upper slope, and documented differences in activity between the two depth regimes. To further explore this hypothesis, microbial activity and geochemistry in a variety of settings were compared, and characteristic features of these settings were identified (e.g. brines associated with elevated dissolved organic carbon (DOC) and ammonium; Joye et al., 2010). These data showed that: 1) the intensity of seepage, and possibly the depth along the slope, influenced microbial abundance and activity, 2) the composition of seeping fluid directly influenced local geochemistry and biological communities and their activity, and 3) though macro-biology is dependent on seepage and surficial expression, it also overprints geochemical and microbiological features. To date, deciphering the differences and driving factors of these highly interdependent biogeochemical features has been difficult due to variable sampling strategies and different parameters measured in a given research expedition. With replicate sampling across multiple habitats this work sought to identify and explain geochemical and microbial rate variability within and between sites.

In cold seep sediments, biogeochemical gradients can be sharp, both vertically and laterally. These gradients result from the geological setting and/or the megafauna and microbial

communities inhabiting the area. Gradients are apparent on relatively small scales (mm to cm) as well as larger scales (10's of m) (Lloyd et al., 2010, Niemann et al., 2006, Omoregie et al., 2009, Roy et al., 2007). Pronounced lateral gradients in the geochemistry of cold seeps are exemplified within and across sulfur-oxidizing bacterial mats (Lloyd et al., 2010). Sulfate reduction rates (SRRs), sulfate concentrations, and microbial community structure differed in the center of a *Beggiatoa sp.* mat compared to the edge of the mat, which was only 10 cm from bare sediments (Lloyd et al., 2010). The seemingly homogenous structure of the mat upon visual observation makes such differences somewhat counterintuitive, but subtle spatio-temporal variations in seepage generate heterogeneous geochemical and microbiological signatures on strikingly small scales. Niemann et al. (2006) investigated larger scale changes across the Haakon Mosby mud volcano, which included a variety of habitats with mats and tubeworms inhabiting surface sediments. Differences in geochemistry and microbiology were noted in the proximity of the mud volcano as a function of the sediment surface megafaunal biology (Niemann et al., 2006).

The goal of this work was to sample replicate cores from a given habitat as defined by its geologic or megafauna biological signature and to compare different habitats both within and between sites along the deep slope. We hypothesized variation between sites (e.g. brine vs. oil seep) would be greater than variation observed between habitats (e.g. brine flow or megafauna regimes). Rates of microbial sulfate reduction and anaerobic methane oxidation and concentrations of geochemical (gases and dissolved and solid phase) constituents were determined systematically from replicate cores at consistent depth intervals. The sampling strategy allowed the comparison of particular habitats within a given seepage cell, which we define as a broad area (10's to 100's of m²) impacted by seepage of a similar fluid (gas, oil, brine or some combination thereof). The overarching hypothesis was that distinct habitats would generate unique signatures of microbial activity and geochemistry.

2. Methods

2.1 Study Sites and sample types

During July 2007, samples were collected from 9 sites along the lower continental slope (Table 1) of the Gulf of Mexico using the ROV JASON II, which was operated from the R/V *Ron Brown* (National Oceanographic and Atmospheric Administration). Here we provide data from five sites described in Joye and coauthors (2010) (Atwater Valley block 340, hereafter AT340; Green Canyon block 852, hereafter GC852; Walker Ridge block 269/ 270, hereafter WR 269/270; Alaminos Canyon block 645, hereafter AC645; and Alaminos Canyon block 818, hereafter AC818) and four additional sites: Garden Banks block 697, hereafter GB697; Garden Banks block 647, hereafter GB647; Alaminos Canyon block 601, AC601; and the Red Crater. Sediment samples were described by their ecological or environmental signature, as follows: control (background sediment adjacent to seep features, but with no visible signs of active seepage), Siboglinids polychaete worms, bacterial mats (sediments with *Beggiatoa sp.* inhabiting the surface), heart urchins (cores taken from an area with urchin activity based on visible urchins and observable urchin trails), urchin/brine (urchins inhabiting sediments with elevated salinity), brine flow (discrete brine flows), brine pools (collections of brine from a lake or pond-like feature), oil (visibly oil-stained sediments), and mud volcano (sediments characterized by mud flows).

Cores are referenced by their site abbreviation and type (e.g. Atwater Valley block 340 control sediment is denoted AT340-control); a description of all core types and sites is presented in Table 1 with the value representing the number of cores collected at a given site. In total 69 cores are described here, with 2-5 cores collected for each habitat type within a given site. The area of coverage during the repetitive coring in each environment was $>2 \text{ m}^2$.

2.2 Dissolved gases, porewater, and microbial activity sample collection

Cores were retrieved from either the ROV JASON II or the elevator system that was used to transport equipment and cores from the seafloor to the ship during JASON operations. Upon return to the surface, cores were moved immediately to a 4°C cold room and processed within 24 hours of collection. General procedures utilized for porewater collection were described previously (Joye et al. 2010). Each replicate core from a given habitat was sampled for dissolved gases, porewater, solid phase, and microbial activity samples at depths of 1.5, 4.5, 8, and 12.5 cm; an additional overlying water sample was also collected generating 5 samples per core. Upon extrusion of a new interval, a 3 or 5 mL sample was collected for quantification of dissolved methane and hydrogen, respectively. Methane samples were collected with a 3 mL cut-end syringe, then ejected into a 12 mL headspace vial containing 3 mL He-purged 2 M NaOH, crimp sealed with a gray butyl rubber stopper, and vortexed until well mixed. The hydrogen samples were collected with a 3 mL cut-end syringe, then ejected into a 20 mL headspace vial, crimp sealed with a blue butyl rubber stopper, and flushed immediately with He. Next, a 2 cm³ sample was placed into a pre-weighed 7 mL vial with a teflon-coated cap to determine the porosity. Then, a 3 cm³ sediment sample (n=3 per core per assay) for rate assays was collected for sulfate reduction (SR) and the anaerobic oxidation of methane (AOM) into a 5 mL cut-end syringe, which were then sealed with a butyl rubber stopper. Finally, the remaining sediment was collected for porewater extraction using a mechanical press (Joye et al., 2010). The sediment was placed in an Ar-flushed PVC cup and sealed with a PVC piston and cap. Then, porewater was collected into a 30 mL syringe and at least 20 mL of porewater was extracted per horizon. After extraction, the residual sediment was placed into an Ar-purged Whirl Pak bag and stored frozen at -20°C.

2.3 Preservation, storage, and analysis of gases, porewater, and solid phase

During the expedition, samples for dissolved gases and porewater analytes (e.g., ammonium (NH_4^+), nitrate and nitrite (NO_x^-), phosphate (PO_4^{3-}), dissolved organic carbon (DOC), chloride (Cl^-), sulfate (SO_4^{2-}), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+) methane (CH_4), and sulfide (HS^-)) were collected, preserved and analyzed ashore (except for NH_4^+ , which was analyzed shipboard) in the UGA laboratory as described previously (Joye et al., 2004, 2010 and references therein). Hydrogen (H_2) samples were analyzed according to Bowles et al. (2011). Dissolved inorganic carbon (DIC) samples were collected and analyzed shipboard by preserving DIC samples in 20 mL Hungate tubes with butyl rubber stoppers, and then adding 1-mL of saturated CuSO_4 and 1 mL of 56 mM NaMoO_4 that was 10% (v/v) H_3PO_4 . The metal addition precipitated sulfides, preventing interference, while the acid addition converted inorganic carbon into CO_2 (gas). Next the sample was shaken vigorously and passed through an infrared detector (Licor[®]). The precision was 5% based on injection of replicate standards, while the minimum detection limit in porewater was approximately 250 μM . Cations and anions were measured using ion chromatography (Joye et al. 2010). Total carbon (TC) and nitrogen (TN) samples were collected and processed as described previously by Joye and coauthors (2010).

2.4 Microbial activity sample preservation and analysis

Samples for SR and AOM rate measurements were injected, incubated and preserved on board the ship. For SR, the $^{35}\text{SO}_4^{2-}$ tracer solution (0.83 MBq) was injected through the butyl rubber stopper and samples were placed in an N_2 atmosphere for incubation at *in situ* temperature (4 °C) for 24 h. Replicates ($n = 2$) and a killed control were run for each depth horizon in each core assayed. To halt microbial activity, the sample was ejected into a 50 mL centrifuge tube containing 10 mL of 20% (w/v) zinc acetate. To recover the radioactive sulfide produced, samples were rinsed of sulfate by centrifuging and amendment with anoxic seawater (repeated

3x). After the first rinse, a sub-sample was collected to quantify the $^{35}\text{SO}_4^{2-}$ activity. Samples were then stored in ethanol at $-20\text{ }^{\circ}\text{C}$ until distillation. Samples were distilled via a one-step wet-acid reduction and sulfide was trapped in 5% (w/v) zinc acetate (Canfield et al., 1986; Fossing and Jørgensen, 1989). The radioactivity of sulfate and sulfide samples was measured using Scinti-verse BD (Fisher) and Scintisafe Gel (Fisher) scintillation cocktails, respectively. Rates of SR were estimated using the following equation:

$$\text{SR Rate} = [\text{SO}_4^{2-}] \times \alpha / t \times (\text{H}_2^{35}\text{S} / ^{35}\text{SO}_4^{2-} + \text{H}_2^{35}\text{S}) \quad (1)$$

Here, the SR rate ($\text{nmol cm}^{-3} \text{ day}^{-1}$) is the rate of sulfate reduction; $[\text{SO}_4^{2-}]$ is the sediment sulfate concentration (nmol cm^{-3} wet sediment; obtained by multiplying the pore water sulfate concentration times the sediment porosity); α is the fractionation factor (1.06; Jørgensen, 1978), t is the time of incubations (days); H_2^{35}S is the radioactivity of sulfide generated (minus activity in killed controls); and $^{35}\text{SO}_4 + \text{H}_2^{35}\text{S}$ reflects the total tracer activity injected.

Samples for AOM rate determination were amended with dissolved $^{14}\text{CH}_4$ (4.2 kBq) and incubated for 24 h, after which samples were fixed by transferring the sample into a 50 mL centrifuge tube containing 5 mL of 2 M NaOH. Samples were processed via acid distillation in flasks as described in Joye et al. (1999), with a few changes. Namely, the trap used for quantitatively recovering the CO_2 evolved during acidification contained a GFF filter paper (to increase surface area) and 1 mL of CarboSorb E and 1 mL of saturated CuSO_4 was added to the flask to scavenge any evolved sulfide. Samples were distilled for at least 6 h and the recoveries, as determined from $\text{NaH}^{14}\text{CO}_3$ standards, averaged 99%. Rates of AOM were calculated using the following equation:

$$\text{AOM Rate} = [\text{CH}_4] \times \alpha / t \times (^{14}\text{CO}_2 / ^{14}\text{CH}_4) \quad (2)$$

Here, the AOM rate ($\text{nmol cm}^{-3} \text{ day}^{-1}$) is the rate of methane oxidation, $[\text{CH}_4]$ is the concentration of methane (nmol cm^{-3} of wet sediment), α is the fractionation factor (1.018; Alperin et al., 1988), t is the time of incubations (days), $^{14}\text{CO}_2$ is the radioactivity of carbon dioxide generated minus activity from controls, and $^{14}\text{CH}_4$ reflects the ^{14}C tracer radioactivity injected.

We note that these rates were not determined at *in situ* methane concentration, which undoubtedly underestimates *in situ* rates (Bowles et al. 2011, *in prep*). However all rate assays were conducted similarly in this study making the results between habitats directly comparable.

2.4 Principal Component Analysis of Cold Seep Environments

We used principal component analysis (PCA) to elucidate how combinations of geochemical constituents and microbial activity varied between different seeps and between habitats within a given seep. To proceed with principal component analysis, we first condensed data by performing a simple arithmetic average for select characteristics (porosity, SOM, PC, PN, and pH) or a trapezoidal integration for others (CH_4 , DIC, H_2 , HS^- , SO_4^{2-} , Cl^- , NH_4^+ , NO_x^- , PO_4^{3-} , Na^+ , K^+ , Mg^{2+} , Ca^{2+} , DOC, and SR and AOM rates) (see Supplemental Table 1). The trapezoidal integration was applied to the cores to the maximum depth of 15 cm (the deepest sample was from 12-15 cm). Lack of sufficient porewater for one Siboglinid core from WR269 prevented the determination of cations (Na^+ , K^+ , Mg^{2+} , Ca^{2+}), so this core was omitted from further statistical analysis. For some analytes, occasionally no data were obtained due to insufficient volume; this was a rare occurrence (<1% of samples). To continue with the PCA, the missing interval for an analyte was determined by averaging data from surrounding horizons or if in the event the last horizon was missing then the value was estimated from the value in the horizon above it. In the event that HS^- or H_2 was below detection a conservative value of 1 μM or 1 nM, respectively, was assumed to complete data sets. Integrating the profiles allowed us to compare broad-scale

differences using PCA. Sediment characteristics of PC, PN, porosity, SOM, and pH were normally distributed; for other variables, a logarithmic transformation was required. All logarithmic scaling was performed prior to PCA. The PCA was performed in Matlab R2012a.

2.5 Multiple Analyses of Variance and Linear Discriminant Analysis of Cold Seep Environments

Multiple samples from the same habitat at different sites allowed for hypothesis testing regarding a difference between habitats of major ecological or geological features and within given seep locales. The locales were classified as: control, Siboglinid worms, bacterial mat, urchin, brine, flow feature, oily, mud volcano, and gassy (see Table 1 for classifications). The test was accomplished using a multivariable analysis of variance (MANOVA) with a $\alpha = 0.05$ for identifying dimensionality between groups using Matlab version R2012a. The MANOVA was performed on trapezoidal depth integrated totals and averages used in the PCA (see Supplemental Table 1). MANOVA analysis was then used to formulate a linear discriminant analysis, resulting in an identification of settings that were considered unique.

3. Results and Discussion

3.1 Characterizing cold seep locales through ecological, geological, geochemical and/or geophysical signatures.

Individual cold seeps are highly heterogeneous environments and their microbiology and geochemistry are highly interrelated. In general, hydrocarbon seepage supports micro- and macro-biology and specific fauna are associated with varying rates of hydrocarbon flux (Sibuet and Olu, 1998, Fisher et al., 2007). Time-varying geophysical conditions give rise to different seep expressions (Sibuet and Olu, 1998, Joye et al., 2010). For example, brine pools and asphalt volcanoes are derived from different geological processes and have different sedimentological

histories. The challenge lies in how a seep site is defined: Is it best to use geology as a distinguishing feature or the biology inhabiting the setting to define the geochemical and microbiological signatures?

Cold seeps occur within diverse continental slope environments, all of which vary with respect to chemistry, geology, and biology (Levin and Dayton, 2009). Continental slopes include an upper and lower region, which are by convention defined by water depths between 200 and 1000 meters below sea level (mbsl) and 1000 to 2000 mbsl, respectively (Canfield et al., 2005). Lower carbon fluxes from the surface ocean dictate that microbial activity should decrease from the upper to lower slope, and therefore that the related geochemical and microbiological signatures should also change (Bowles et al., 2014 *and references therein*). At cold seeps, however, seepage of reduced carbon from great depths, often in concert with brine flows through fault networks, are largely considered more dependent on fossil carbon than contemporaneous carbon fluxes (Kennicutt et al. 1988; Roberts and Carney 1997; Joye et al. 2004). For cold seeps as well, however, rates of microbial processes decrease from the upper to lower slope, potentially allowing seep sites to be distinguished by depth or the specific nature of the seepage (Joye et al., 2010).

A variety of sites were assessed in this work (Table 1). In addition to biological and geological descriptions included by Joye et al. (2010) we added a number of settings or combination of setting and site (brine pools and edge, flow feature, gray-gassy, oily sediment, Siboglonids and a relict mud volcano). At AC601 we sampled cores within a brine pool and adjacent to pool edge (Crespo-Medina et al., in press). In addition to AT340 brine cores (Joye et al., 2010), we also report data from cores from GB697 for the first time. The sampling strategy, which included a high degree of replication, allowed us to distinguish the variations within and between particular sites and settings.

The extensive sample coverage with respect to habitat at AT340 allowed within habitat variations to be examined within a single site (Fig. 1A-D). Surprisingly, AT340-urchin cores that exhibited significant surface sediment bioturbation were characterized by largely uniform geochemical profiles (Fig. 1A). The AT340-urchin cores were characterized by a slightly elevated average C:N of 13 and high average areal integrated rates of SR and AOM, at 0.21 and 0.012 mmol m⁻²d⁻¹, respectively (Table 2). The AT340-bacterial mat sediments were also relatively uniform with respect to geochemistry (Fig. 1B). These sediments were very typical of bacterial mat inhabited sediments collected elsewhere in the Gulf of Mexico (i.e. elevated concentrations of methane, nitrate/ite, and ammonium and sulfate depletion observed close to the sediment surface; Bowles and Joye 2010). The AT340-brine cores (Fig. 1C) were gas charged and contained high DOC and ammonium concentrations, at 7405 µM and 2706 µM, respectively. This observation aligns well with previous observations of elevated DOC and ammonium in brines (Joye et al., 2010). In AT340-brine cores, despite some variation in concentrations of methane, DOC, and ammonium, they were typically elevated (Fig. 1C). All AT340-control sediments varied little with respect to geochemistry and were typical of control cores observed elsewhere. General trends within the respective habitats were similar to previous observations, but the high level of replication illustrates that the more limited sample collections (i.e., one core for geochemical characterization) that are typically made are sufficient to glean a general understanding of a setting when performing a biogeochemical analysis of sediment.

We can also evaluate the biogeochemical signature of a habitat across sites. The Siboglonid polychaete cores – which were observed and sampled across multiple sites – are a good example (Fig. 2A-C). Little variation was observed within the geochemistry of replicate cores at each site, though we know the activity and abundance of these organisms can cause substantial variation in geochemistry, as both impact the magnitude of bioventilation (de Beer et

al., 2006). These geochemical data, specifically sulfate profiles, suggested different levels of microbial activity at the three sites sampled. Specifically, AC818-Siboglonid (Fig. 2A) cores were characterized by more rapid sulfate depletion, while AC645-Siblogonid (Fig. 2B) and WR269-Siblogonid (Fig. 2B) exhibited lower levels of depletion. Interestingly, when examining average integrated rates of SR and AOM, the rates in AC818-Siblogonid cores were 0.93 and 0.021 mmol m⁻²d⁻¹, respectively, which was much lower than WR269-Siblogonid rates of 2.7 and 0.19 mmol m⁻²d⁻¹, respectively. The apparent disconnect between rates and geochemical profiles can be explained by higher rates of bioirrigation at the WR269 site, which would sustain both higher rates of SR and a reduced magnitude of apparent sulfate depletion.

The sites examined here spanned a range of water depths from 1010 to 2740 mbsf (Table 1) and ranged from brine pools (e.g. AC601) to hydrocarbon seeps (e.g. GB647). Despite clearly different depths and geological settings, no clear difference or clustering was evident in an analysis of principal components (Fig. 3A). These data suggest that although a difference in geochemical signatures and microbial activities were noted between upper and lower slopes (Joye et al. 2010), when comparing a larger expanse of depths on the deep slope, these differences are less pronounced. A variety of geological settings were indistinguishable, suggesting that although the sites have distinct surficial expressions, the site characteristics did not directly reflect the observed geochemistry.

When the data were grouped with respect to the major geochemical or biological identifier the principal component analysis displayed clear trends or clusters (Fig. 3B). The parameters explaining the largest variance experienced for the respective setting were methane and DIC (34 and 33% principal component 1, respectively), and Ca²⁺ (42% principal component 2). Methane is the dominant low molecular weight gaseous hydrocarbon at cold seeps (e.g. Joye et al. 2010, 2011; Treude et al. 2014). The limited solubility of methane at atmospheric pressure versus

pressures experienced *in situ* makes accurate measurements of methane concentration and related processes problematic (Bowles et al., 2011). Multiple studies utilizing methods that better resolve *in situ* methane concentrations observed that methane concentrations exceed 10s of mM concentrations versus typical *ex situ* measurements, which rarely exceed several mM (Wankel et al., 2010; Lapham et al., 2010).

These samples were not collected using pressure vessels, so degassing occurred during return to the surface. Nonetheless, the methane concentrations varied amongst sites with the AC601 brine pool having the highest average methane concentration at 2.6 mM in contrast to control sites at GC852 averaging as low as 1 μ M. In general methane concentration is an indicator of the seepage intensity with high concentrations implying an abundant supply of hydrocarbon and reduced carbon for microbial processes.

Dissolved inorganic carbon is derived from respiratory processes, including the oxidation of methane and oil, and is indicative of overall higher rates of cumulative microbial metabolism. For example, the less active sites such as control sediments exhibited the lowest average DIC concentrations, typically around 2.5 mM (Table 2). Likewise in settings associated with higher rates of sulfate reduction (e.g. oil and the edge of brine pool), DIC concentrations were over 4 mM (Table 2). Calcium drove variability due to its abundance at brine sites. The highest average values of calcium were found in the GB647 brine-influenced oily cores, where concentrations averaged 136 mM, over 10 times typical seawater values, whereas control site values were ~12 mM (Table 1).

The principal components clearly indicate the influence of seepage on methane-carbon, on biology and likewise its relationship to the geological setting; previous studies have hinted at this (Roy et al., 2007, Ritt et al., 2012). Roy and coauthors (2007) performed a principal component analysis and redundancy analysis describing megafauna and geochemical parameters in water or

sediments adjacent (e.g. sulfide, oxygen, pH, and POC) in cold seep sediments inhabited by varying proportional assemblages of mussels and tubeworms off West Africa. Methane explained most of the variation seen in megafaunal biology at sites within a pockmark. In similar work from eastern Mediterranean Sea cold seeps, Ritt and coauthors (2012) found methane concentration to be an important factor for explaining the distribution of megafauna. Megafauna have already been directly implicated in methane cycling at seeps (see Levin, 2005 *and references therein*). Collectively these studies indicate the importance of geochemistry, particularly methane concentration, in influencing the megafaunal community present and its abundance and diversity.

3.2 Driving and limiting factors of geochemical and microbial processes

The identification of carbon, as methane and DIC, as parameters capable of distinguishing geological and biological setting affirms the importance of reductant flux for fueling microbial processes along the lower slope. The fact that methane and DIC are critical geochemical parameters further enforces the premise that carbon delivery at the upper and lower slope drive notable differences in microbial dynamics. The nature of seepage directly influences methane delivery and subsequent DIC production.

The relationship of intensity of methane seepage - measured as methane concentration and which we note is not strictly quantitative due to degassing – documents a relationship with markers of biological activity such as DIC concentrations, rate measurements (SR), and nutrient concentrations (Fig. 4). Specifically, depth integrated logarithmically transformed DIC content versus rates of SR showed a scattered but roughly linear relationship with methane content (Fig. 4A,C). These relationships directly link sulfate reduction to a major electron donor, methane, and the product of the reaction as DIC. DOC content showed no obvious relationship with methane, suggesting that methane is the major component of seepage at these sites, except for brines (Joye et al. 2010) and that it is not strictly related with the migration of other reduced carbon species,

though in this survey only one site had visible oil (Fig. 4B). Additionally, phosphate concentrations were related to methane concentration (Fig. 4D). We postulate that the relationship of phosphate to methane concentrations is largely derived from increased microbial activity in methane rich sediments, wherein increased activity results in the stimulation of endogenous microbial communities that are responsible for the diagenesis of organic matter and production of phosphate (Krom and Berner, 1981). However, phosphate concentrations were more notably enriched in sediment influenced by brine. For example, the AC601 brine pool had an average phosphate concentration of over 40 μM (Table 2) and these data give the first indication that brine may modulate phosphorus fluxes from the deep subsurface.

Nutrients, such as nitrogen and phosphorus, exist as inorganic and organic species in marine sediments and porewaters. In the dissolved inorganic pools measured here with nitrogen (DIN) occurring as nitrate, nitrite, and ammonium and phosphorus (DIP) as phosphate we observed DIN to DIP ratios ranging from 4.8 to 359 (Table 2). The lowest DIN to DIP ratio (4.8) was associated with gassy sediments from GC852, which had relatively low average ammonium concentrations (42 μM). The oily brine-influenced sediments of GB697 maintained the highest ratio (359) resulting from an influence of brine that supplied these sediments with an average ammonium content of ~ 7 mM, while phosphate concentrations were relatively low averaging 19 μM . Bowles and coauthors (2011) report a much smaller range of average DIN to DIP ratios in porewaters (25-65), but the sediments sampled on that cruise were not influenced by brine flows. In mat hosting sediments Joye et al. (2004) reported a DIN:DIP of 7-60 depending on depth and type of sediment (bacterial mat versus control sediment). The larger range reported here is directly related to exceptionally high ammonium concentrations documented in brine-influenced sediments. In terms of a limiting N to P ratio, the value of 9.6 is often cited as the critical threshold separating N and P limitation in prokaryotes, but recently, an archaea grown in pure

culture exhibited a ratio of 20 and 30, which is more typical of values measured here (Duboc et al., 1995; Meador et al., 2014). In total 62% of these sites have an average DIN:DIP ratio outside of the range of 9.6 to 30, with 70% greater than 30. Collectively these data suggest that phosphorus limitation is more common in deep sea cold seep sediment.

3.2 Which geological or ecological habitat was more variable?

The ability to group seep sites in terms of major geological or ecological expressions implies that geochemical features can be generalized amongst these identifiers. To evaluate this we performed an analysis of variance with multiple responses including all geochemical and rate parameters. When considering all sites, a total of 21 site and geological or ecological settings were available with 11 unique geological or ecological settings. The MANOVA identified a total of 13 dimensions ($p = 0.023$, $\alpha = 0.05$ final dimension). The slightly larger number of dimensions compared to unique settings suggests that scatter can exist between the same geological and ecological settings at different sites. Ultimately this further substantiates the findings of the PCA. The heterogeneity of each geological or ecological expression must be evaluated to interpret the internal variation, regardless of site. When geological and ecological settings are used to group sites a total of 9 dimensions ($p = 0.004$, $\alpha = 0.05$ final dimension) are identified. The MANOVA lends itself well to linear discriminant analysis, and when classifying by geological or ecological setting the substitution error during classification of the sample set results in only the incorrect classification of 3 cores. Specifically, mismatches came in the Siboglinids, urchin, and control cores. While not all geological settings or sites could be constrained rigorously, the likely explanation for this is that peripheral environments such as urchin or Siboglinids influenced sediments have very low seepage or as previously mentioned bioturbation masks microbial

imprints on geochemical profiles, leading them to appear geochemically similar to control sediments.

3.3 Brine seepage as a conduit for carbon and nutrients

Brine seepage is known to drive fluxes of DOC and ammonium from the deep subsurface (Joye et al., 2005, Joye et al., 2010). The relationship between brine seepage and phosphate fluxes has not been explored to date. The observation of Ca^{2+} as an important indicator of variation among sites is an additional potential explanatory variable. Gulf of Mexico brines are largely halite brines and relationship between brine-influence and Ca^{2+} was not anticipated. The areal integrated logarithmic transformed concentrations of Cl^- and Ca^{2+} when compared to Na^+ (Fig. 5) suggest that brines were halite derived and that Ca^{2+} enrichment is coincidental as Ca^{2+} is removed from clays as brines migrate through hosting sedimentary packages. Supporting this assertion, areal integrated logarithmic transformed phosphate concentrations were well correlated with Ca^{2+} (Fig. 6A) and Cl^- (Fig. 3D). At elevated Ca^{2+} , indicative of brines, there is a clear relationship with higher phosphate concentrations (Fig. 6C). The similar relationship was noted for ammonium and Ca^{2+} (Fig. 6B, C). Phosphate concentrations were similarly related to Cl^- concentration (Fig. 6D, F), as were ammonium concentrations (Fig. 6E,F). These data corroborate prior observations of the relationship of brine (via Cl^-) and ammonium (Joye et al., 2010). The elevated phosphate is likely derived from similar mechanisms as for ammonium, DOC, and likely Ca^{2+} , i.e., stripping from sediment surfaces by the hypersaline brine as it transits up through the sediment column. During brine migration, the higher salinity mobilizes sorbed phosphate and transports it to the sediment water interface. Together these data imply that brine acts as important conduits for methane, DOC and both ammonium and phosphate.

4. Conclusions

An assortment of 69 cores with systematic geochemical and microbial rate measurements was analyzed to develop an understanding of how these environments differed. In general, we observed that the best distinction of cold seeps was by major biological or geochemical signatures not by geographic location. The key driving or determinant factor of biology or geochemistry was methane, DIC, and Ca^{2+} . These data underscore the importance of the intensity of seepage (as methane) and relevant and resulting microbial processes that produce DIC, in indicating the type of setting. Additionally, the Ca^{2+} concentration was predictive of brine environments. These data indicate that if a seep receives enough carbon-reductant, the most likely limiting inorganic nutrient will be phosphorus. Finally, the data show that brine seepage is potentially responsible for transporting phosphorus, in the form of phosphate, thereby representing a source of nutrients.

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Table Captions

Table 1. Site depths, locations, and number and type of cores collected.

Table 2. Average of geochemical parameters CH_4 (μM), DIC (mM), H_2 (nM), H_2S (mM), SO_4^{2-} (mM), Cl (mM), NH_4^+ (μM), NO_x^- (μM), PO_4^{3-} (μM), Na^+ (mM), K^+ (mM), Ca^{2+} (mM), DOC (μM), DIN:DIP, PC (mg g^{-1}), PN (mg g^{-1}), and C:N, and average areal integrates rates of AOM and SR ($\text{mmol m}^{-2}\text{d}^{-1}$).

Figure Captions

Figure 1. Select geochemical parameters (Cl^- (mM), SO_4^{2-} (mM), H_2S (mM), CH_4 (μM), H_2 (nM), DIC (mM), DOC (mM), NO_x^- (μM), NH_4^+ (μM), PO_4^{3-} (μM)) versus depth (cm) for AT340 (A) Urchin, (B) Bacterial mat, (C) Brine, and (D) Control cores.

Figure 2. Select geochemical parameters (Cl^- (mM), SO_4^{2-} (mM), H_2S (mM), CH_4 (μM), H_2 (nM), DIC (mM), DOC (mM), NO_x^- (μM), NH_4^+ (μM), PO_4^{3-} (μM)) versus depth (cm) for Siboglinids cores from (A) AC818, (B) AC645, and (C) WR269.

Figure 3. Principal component analysis of depth integrated methane, dissolved inorganic carbon, hydrogen, hydrogen sulfide, sulfate, chloride, ammonium, nitrate/ites, phosphate, dissolved organic carbon, calcium, sodium, potassium, SRR, AOM, and average particulate nitrogen, particulate carbon, porosity, percent organic matter, and pH by **A)** site and **B)** ecological characterization.

Figure 4. Logarithmic transformations of depth integrated totals of A) DIC (mol m^{-2}), B) DOC (mmol m^{-2}), C) SR ($\text{mol m}^{-2}\text{d}^{-1}$), and D) PO_4^{3-} (mmol m^{-2}) versus the logarithmic transformation of integrated CH_4 (mmol m^{-2}) by respective geological and/or ecological setting.

Figure 5. Logarithmic transformations of depth integrated totals of PO_4^{3-} (mmol m^{-2}) and Cl^- (open symbol) and Ca^{2+} (closed symbol) (mol m^{-2}), versus NH_4^+ (mmol m^{-2}) versus Na^+ (mol m^{-2}).

Figure 6. Logarithmic transformations of depth integrated totals of A) PO_4^{3-} (mmol m^{-2}) and B) NH_4^+ (mmol m^{-2}) versus Ca^{2+} (mol m^{-2}), and D) PO_4^{3-} (mmol m^{-2}) and E) NH_4^+ (mmol m^{-2}) versus Cl^- (mol m^{-2}). Additionally, PO_4^{3-} (mmol m^{-2}) and NH_4^+ (mmol m^{-2}) as open squares versus C) Ca^{2+} (mol m^{-2}) and F) NH_4^+ (mol m^{-2}) for brine influenced sites alone.

Bowles et al., Fig 1

Cl⁻ (mM) SO₄²⁻ (mM) H₂S (mM) CH₄ (μM) H₂ (nM) DIC (mM) DOC (mM) NO_x⁻ (μM) NH₄⁺ (μM) PO₄³⁻ (μM)

Bowles et al., Fig I Continued

Fig. I. Select geochemical parameters (Cl^- (mM), SO_4^{2-} (mM), H_2S (mM), CH_4 (μM), H_2 (nM), DIC (mM), DOC (mM), NO_x^- (μM), NH_4^+ (μM), PO_4^{3-} (μM)) versus depth (cm) for AT340 (A) Urchin, (B) Bacterial mat, (C) Brine, and (D) Control cores.

Bowles et al., Fig 2

Bowles et al., Fig 2 continued

Fig. 2. Select geochemical parameters (Cl⁻ (mM), SO₄²⁻ (mM), H₂S (mM), CH₄ (μM), H₂ (nM), DIC (mM), DOC (mM), NO_x⁻ (μM), NH₄⁺ (μM), PO₄³⁻ (μM)) versus depth (cm) for Siboglinids cores from (A) AC818, (B) AC645, and (C) VWR269.

Bowles et al., Figure 3

Fig. 3 Principal component analysis of depth integrated methane, dissolved inorganic carbon, hydrogen, hydrogen sulfide, sulfate, chloride, ammonium, nitrate/ites, phosphate, dissolved organic carbon, calcium, sodium, potassium, SRR, AOM, and average particulate nitrogen, particulate carbon, porosity, percent organic matter, and pH by **A)** site and **B)** ecological characterization.

Bowles et al., Table I

Site	Depth (m)	Lat mean	Long mean	Control	Siboglinids	Bacterial mat	Urchin	Brine Edge	Brine Feature	Brine	Oil	Mud Volcano	Gassy
AT340	2216	27.64	-88.36	4		4	4			4			
GC852	1410	27.1	-91.16	2					4				4
GB697	1010	27.31	-92.11							2			
WR269	1950	26.68	-91.66	3	2								
GB647											2		
AC645	2200	26.35	-94.49		4								
AC818	2740	26.18	-94.62	4	4		4						
AC601	2350	27.31	-94.8	5			3	3		3			
Red Crater	2300	26.36	-94.51		2							2	

Table I. Site depths, locations, and number and type of cores collected.

Bowles et al., Table 2

Site	Core Type	CH	DIC	H	H	SO	Cl	NH	NO	PO	Na	K	Mg	Ca	DOC	DIN:DIP	PC	PN	C:N	ΣSRR	ΣAOM
AT340	Urchin	43.3	7.7	46.8	2.9	18.9	529.8	77.6	41.8	14.8	557.7	11.9	48.3	10.3	6597.3	8.1	1.4	0.1	13.4	0.21	0.012
AT340	Control	12.8	2.5	13.6	0.0	28.4	554.4	35.5	46.9	1.4	471.2	9.9	46.7	10.8	3186.0	58.8	1.2	0.1	16.1	0.017	0.0085
AT340	Mats	582.7	8.8	32.2	3.6	20.4	1365.1	741.9	39.4	32.4	1272.2	8.5	48.3	19.9	8216.0	24.1	0.8	0.1	10.4	0.32	0.0093
AT340	Brine	1597.0	10.5	21.7	2.7	4.6	2209.1	2706.2	43.5	16.5	1996.4	7.3	56.2	30.2	7405.5	166.3	1.5	0.2	8.3	4.8	0.026
GC852	Gassy	1166.1	27.5	21.9	34.1	7.6	543.8	42.6	34.8	16.0	472.7	9.9	41.3	6.7	16357.4	4.8	1.2	0.2	8.6	2.4	0.10
GC852	Brine flow	1697.0	22.8	8.2	31.4	5.3	565.8	56.3	33.7	12.9	466.9	9.8	41.9	6.9	15455.6	7.0	1.2	0.1	9.5	1.5	0.077
GC852	Control	0.7	3.7	43.1	1.8	28.1	543.9	65.0	66.8	3.9	516.9	11.1	49.9	12.0	3091.1	33.9	2.0	0.2	8.4	0.21	0.033
GB697	Brine	129.1	5.2	44.5	4.6	20.5	796.8	301.5	62.7	9.0	670.8	9.9	46.1	22.5	4979.9	40.5	1.9	0.2	7.9	2.5	0.010
WR269	Siboglinids	65.5	4.3	17.3	0.0	27.6	560.2	41.1	47.1	4.1	479.9	11.5	44.1	11.1	6172.5	21.3	1.1	0.1	8.6	2.7	0.19
WR269	Control	93.0	5.8	2.4	2.1	28.1	587.7	4.6	45.9	8.3	533.2	11.2	51.1	11.5	3524.9	6.1	0.9	0.1	8.9	0.087	0.066
GB647	Oil	1244.7	7.5	66.1	2.9	11.4	2102.5	6875.0	159.4	19.6	1444.0	130.4	60.7	167.7	5835.8	359.7	7.0	0.2	29.2	15.5	0.023
AC645	Siboglinids	2.9	4.2	4.5	0.4	26.3	539.8	176.4	40.1	6.1	482.7	10.6	48.7	11.1	4301.5	35.8	0.9	0.1	8.5	0.27	0.015
AC818	Siboglinids	91.8	11.0	69.0	11.3	18.0	513.9	114.6	33.5	7.8	468.5	9.8	49.1	9.9	8775.2	19.1	2.0	0.5	8.3	0.93	0.021
AC818	Urchin	53.1	16.6	5.9	12.5	14.9	531.9	206.0	32.7	21.6	470.9	9.6	50.5	8.9	9594.9	11.0	1.9	0.2	8.2	2.0	0.038
AC818	Control	3.4	7.0	8.8	0.2	27.5	542.2	123.5	41.1	4.0	478.0	10.3	46.1	11.2	3243.9	40.6	1.4	0.2	8.7	0.31	0.045
AC601	Brine pool	2386.0	22.7	28.7	0.5	2.2	1615.2	2279.4	36.2	42.8	1277.9	20.7	28.4	67.7	5100.8	54.1	2.2	0.3	8.3	1.0	0.0077
AC601	Edge pool	1509.3	28.1	25.0	11.0	4.4	921.9	2919.0	36.3	17.6	772.8	13.2	38.6	33.3	3952.8	167.6	2.0	0.2	8.6	3.3	0.11
AC601	Urchin	311.1	9.4	34.3	3.6	23.4	654.8	174.1	36.9	9.2	588.5	11.8	55.5	16.0	3377.6	22.9	1.6	0.2	8.7	1.5	0.11
AC601	Control	24.4	2.7	13.0	0.0	28.9	583.5	67.9	46.6	5.7	502.6	10.5	49.1	12.2	3430.9	19.9	1.1	0.1	8.5	0.093	0.041
Red Crater	Siboglinids	1295.3	25.7	31.8	7.1	10.0	649.0	177.8	34.2	19.0	534.5	10.6	47.3	11.8	8491.1	11.2	1.2	0.1	16.0	5.4	0.13
Red Crater	Old MV	1153.8	12.3	11.6	0.1	19.2	630.8	256.7	30.7	16.1	560.0	12.8	50.2	13.6	8211.8	17.9	1.2	0.1	16.7	3.3	0.027

Table 2. Average of geochemical parameters CH₄ (μM), DIC (mM), H₂ (nM), H₂S (mM), SO₄²⁻ (mM), Cl (mM), NH₄⁺ (μM), NO_x⁻ (μM), PO₄³⁻ (μM), Na⁺ (mM), K⁺ (mM), Ca²⁺ (mM), DOC (μM), DIN:DIP, PC (mg g⁻¹), PN (mg g⁻¹), and C:N, and average areal integrates rates of AOM and SR (mmol m⁻²d⁻¹).

Bowles et al., Figure 4

Fig. 4. Logarithmic transformations of depth integrated totals of A) DIC (mol m^{-2}), B) DOC (mmol m^{-2}), C) SR ($\text{mmol m}^{-2}\text{d}^{-1}$), and D) PO_4^{3-} (mmol m^{-2}) versus the logarithmic transformation of integrated CH_4 (mmol m^{-2}) by respective geological and/or ecological setting.

Bowles et al., Figure 5

Fig. 5. Logarithmic transformations of depth integrated totals of PO_4^{3-} (mmol m^{-2}) and Cl^- (open symbol) and Ca^{2+} (closed symbol) (mol m^{-2}), versus NH_4^+ (mmol m^{-2}) versus Na^+ (mol m^{-2}).

Bowles et al., Figure 6

Fig. 6. Logarithmic transformations of depth integrated totals of A) PO_4^{3-} (mmol m⁻²) and B) NH_4^+ (mmol m⁻²) versus Ca^{2+} (mol m⁻²), and D) PO_4^{3-} (mmol m⁻²) and E) NH_4^+ (mmol m⁻²) versus Cl^- (mol m⁻²). Additionally, PO_4^{3-} (mmol m⁻²) and NH_4^+ (mmol m⁻²) as open squares versus C) Ca^{2+} (mol m⁻²) and F) NH_4^+ (mol m⁻²) for brine influenced sites alone.