Ecology of Verrucomicrobia in a Freshwater Estuary

By

Edna Chiang

Advisor: Dr. Vincent Denef

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Abstract

Verrucomicrobia is a relatively new bacterial phylum that has been detected ubiquitously around the world and participates in a variety of metabolic processes. However, the phylum appears to have been underrepresented in the past due to methodological biases. Although the group was first observed and isolated from freshwater environments, these systems are the least well-studied habitats of Verrucomicrobia. To better understand freshwater lineages of this group, I chose my study site as Muskegon Lake, a mesotrophic freshwater estuary with a stark productivity gradient that connects one of the Great Lakes' largest watersheds with Lake Michigan. Sediment and water samples were collected across the lake at varying depths over the course of three months; DNA was extracted, sequenced, and analyzed from these samples. Sequencing data was complemented with catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) to validate that my employed methods provide a reliable estimate of verrucomicrobial abundance. The relative abundance of the phylum was found to be significantly different only between sediment and water samples. However, the Verrucomicrobia intra-phylum composition was significantly different between samples from different months, limnions, fractions (particle-associated or free-living), and sources (sediment or water). When analyzed with environmental and geochemical factors, verrucomicrobial relative abundance was found to have a negative linear correlation with pH, SO_4, Cl, temperature, and total dissolved solids (TDS) and a positive linear correlation with depth. This thesis represents introductory work in analyzing the drivers of Verrucomicrobia distribution and diversity, and in providing insight into the phylum's ecology and importance in freshwater systems.

Introduction

The field of microbiology has always faced a challenge innate to the creatures of interest: an inability to easily visualize organisms. This difficulty was first overcome by Antonie van Leeuwenhoek whose improvements on the microscope allowed him to observe microorganisms in detail. Since then, developments, such as improved culture techniques, have furthered the ability of microbiologists to observe and learn about these microscopic creatures. However, culture-dependent methods posed a constraint on the field by excluding a vast group of microbes: the uncultured microbial majority [1]. It was only in the recent past that microbiologists overcame this constraint through the advent of molecular techniques [2-5], which allowed them to target not only these elusive organisms, but also whole microbial communities. The consequential expansion in knowledge can be illustrated by the change in bacterial phyla. Bacteria were originally categorized into 11 phyla [6], but that number has now increased to over 70 [5]. One of these newer classifications is Verrucomicrobia, whose name was inspired by the verruca-like, or pedal wart-like, appearance of one of its members, *Verrucomicrobium spinosum* [7]. Verrucomicrobia was first observed in 1933 in freshwater aquariums [8-10] and later isolated from a freshwater pond in 1970 [10]; however, it was not until 1997 that it was recognized as a phylum [11].

Verrucomicrobia has been found in various environments around the world, but the majority of verrucomicrobial studies have been conducted in soil where it appears that the group has been underestimated in the past. A previous meta-analysis of 16s rRNA and 16s rRNA gene libraries reported that Verrucomicrobia constituted, on average, 7% of soil bacterial communities [12]. However, a recent study conducted in 2011 observed that Verrucomicrobia accounted for

an average of 23% of bacterial sequences and was found in 180 out of 181 soils [13]. In fact, in grasslands and subsoil, Verrucomicrobia was often the dominant bacterial phylum [13]. This suggests that verrucomicrobial abundance have been largely overlooked, likely due to primer bias, as common PCR primers fail to target this phylum [13].

Just as Verrucomicrobia is nearly ubiquitous in soil, it is also pervasive in aquatic environments. The group been detected in a variety of marine environments, such as brackish water [14], open ocean [14-17], sediment [18-20], and in association with other marine organisms [20-23]. A recent study found members of the phylum present in 507 out of 517 samples representing a variety of marine environmental conditions [14]. On average, Verrucomicrobia accounted for 2% of the water column and 1.4% of the sediment bacterial communities, and constituted a larger portion of the bacterial community in brackish waters [14]. It is also suggested that verrucomicrobial abundance and distribution may be highly predictable in oceans [17].

Verrucomicrobia has also been detected in a variety of freshwater environments, including suboxic ponds [24], tropical lakes [25], freshwater wetlands [26], and in association with cyanobacterial blooms [27, 28]. A meta-analysis of three 16s rRNA databases of freshwater plankton detected Verrucomicrobia in 9 out of 12 lakes/rivers [29]. Freshwater verrucomicrobial abundance has been reported to be as high as 19% [30] and 88.4% [26, 30]. A recent study of a mesotrophic lake found that Verrucomicrobia accounted for between 0.002% - 1.98% of the particle-associated (PA) bacterial community and between 0.52% - 1.64% of the free-living (FL) bacterial community [31]. Within the PA community, Verrucomicrobia were found to prefer smaller particles in comparison to larger particles [32]. The composition of PA verrucomicrobial communities was found to be distinct from the FL counterpart and influenced by different

environmental factors; verrucomicrobial PA diversity was influenced by phytoplankton richness, rotifer abundance, and inorganic nutrients, while verrucomicrobial FL diversity was more strongly affected by biomass dynamics of phytoplankton classes [31]. The influence of phytoplankton may be in part explained by their utilization of phosphorous, which would decrease the availability of this nutrient for Verrucomicrobia and, in turn, affect its abundance [33]. There have been conflicting reports about nutrient drivers of Verrucomicrobia abundance; some studies found that verrucomicrobial abundance was positively correlated with phosphorous and nitrogen availability [26, 33, 34], whereas a different study argued the exact opposite [30]. Although the phylum was first discovered in freshwater systems, these environments are actually the least well-studied habitats of Verrucomicrobia and microbial ecologists have yet to decipher many of the mysteries surrounding verrucomicrobial freshwater ecology.

Just as Verrucomicrobia is found in a variety of environments, members of the group also participate in a variety of metabolic processes. Some species are acidophilic, aerobic methanotrophs, the first bacteria discovered to oxidize methane outside of the phylum Proteobacteria [35-40]. Members are also capable of degrading diverse polysaccharides [41-43] and fixing nitrogen [44-46]. Because Verrucomicrobia is extremely prevalent and participates in varied metabolic processes, it possesses great potential to play important roles in various global geochemical cycles.

An important player in Michigan geochemical cycles is Muskegon Lake, a freshwater estuary that connects of one of Michigan's largest watersheds with Lake Michigan, one of the Laurentian Great Lakes. This drowned river mouth is ecologically crucial because it provides a habitat for a variety of fish, such as endangered lake sturgeon [47, 48], during important life cycle periods. Additionally, it is a dynamic system that receives inputs of nutrients and organic

matter from both the Muskegon Watershed and Lake Michigan [49]. Muskegon Lake and the surrounding area are also economically valuable, having served in the past as a site for fisheries, lumber mills, foundries, paper mills, and petrochemical storage [48]. As a result of prevalent industrial activity, the lake suffered from repercussions such as habitat loss and degradation, pollution, invasive species introduction, and eutrophication [48, 50]. Consequentially, Muskegon Lake was designed an Area of Concern (AOC) in 1987 [48]. Since then, steps have been taken to evaluate, monitor, and improve the lake's health [48, 50-52]. The Grand Valley State University Robert B. Annis Water Resources Institute (GVSU-AWRI) established a long-term program in 2003 to help monitor the lake's recovery, and although Muskegon Lake remains an AOC today, it has improved significantly.

Both Verrucomicrobia and freshwater estuaries, such as Muskegon Lake, possess great potential as important contributors to various geochemical cycles, making their marriage in my study extremely intriguing. To begin analyzing the ecology of Verrucomicrobia in this lake, I want to answer four questions: (i) do my chosen molecular methods provide a reliable estimate of Verrucomicrobia abundance; (ii) is the phylum differentially distributed temporally and/or spatially in Muskegon Lake; (iii) are there distinct temporal and/or spatial Verrucomicrobia intra-phylum compositions; and (iv) is there a relationship between verrucomicrobial abundance and environmental or geochemical factors? This thesis strives to provide novel insights into the ecology of freshwater lineages of this abundant, yet understudied phylum, and their importance in freshwater systems.

Methods

Field Sampling

Samples were collected on May 13, Jul 22, and Sep 24, 2014 from Muskegon Lake in collaboration with GVSU-AWRI. In May, duplicate samples were collected at three sites (Inlet, Outlet, Deep); in July and September, samples were collected at four sites (Inlet, Bear, Outlet, Deep) (Figure 1). At each site, samples were taken at the epilimnion (0.19 – 0.59 m below the surface), hypolimnion (0.95 – 3.91 m above the bottom), and sediment. Water samples were collected with a Van Dorn bottle and immediately prefiltered through a 210 μm and 20 μm nitex cloth (WildCo., Yulee, FL).

For samples intended for catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH), 20 mL of filtrate was added to 50 mL falcon tubes with 2 mL of 16% paraformaldehyde and stored in a cooler until transported back to the laboratory where they were stored at 4°C until processing. The liquid was processed within 24 hours by filtering onto 0.22 μm express plus membrane filters (47 mm diameter, Millipore, Billerica, MA) according to [53] and stored at -20°C until CARD-FISH was performed. The CARD-FISH samples contain both the particle-associated and free-living fractions (20 – 0.22 μm).

For water samples intended for DNA extraction, within 32 minutes, the filtrate was filtered through a 3 μm isopore membrane filter (TSTP, 47 mm diameter, Millipore, Billerica, MA) to collect the particle-associated fraction (20 – 3 μm), and a 0.22 μm express plus membrane filter (47 mm diameter, Millipore, Billerica, MA) to collect the free-living fraction (3 – 0.22 μm). Filters were stabilized with a 47 mm polycarbonate in-line filter holder (Pall Corporation, Ann Arbor, MI) and sequential in-line filtration was performed using an easy-load

L/S/ peristaltic pump head (Masterflex®, Cole Palmer Instrument Company, Vernon Hills, IL). After filtration, the filters were folded with the biomass side facing in, placed in 2 mL cryovials with RNA*later*® (Life Technologies, Carlsbad, CA), and stored in liquid nitrogen until transported back to the laboratory where they were stored at -80°C until DNA extraction.

For sediment samples, 2 mL cryovials were filled with sediment and stored in liquid nitrogen until transported back to the laboratory where they were stored at -80°C until DNA extraction.

DNA Extraction

Filter DNA was extracted using two different protocols: an optimized version of the AllPrep DNA/RNA Kit (Qiagen), and a protocol that combined the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Limburg) and the AllPrep DNA/RNA Kit (Qiagen). The optimized AllPrep protocol was performed as described in [54] on one set of May replicates. Prior to extraction, whole filters were dipped into phosphate buffered saline (PBS) to remove excess RNA*later*®, blotted on Kimwipes (Kimtech, Irving, TX), and placed onto a clean plate. 125 µL lysozyme at 8 mg/mL was added, and the filters incubated for 5 minutes at 37°C. Afterwards, the filters were transferred into 5 mL tubes filled with 600 µL buffer RLT plus (Qiagen) and 6 µL 2-mercaptoethanol, and shaken for 90 minutes at room temperature in a vortex on setting 5, followed by shaking for 10 minutes in a vortex on the highest setting. Afterwards, the edge of the filter was snapped into the tube cap, and the tubes were centrifuged to remove all liquid from the filter. The liquid was passed through a QIAshredder (Qiagen) column and the remainder of the protocol was performed according to the manufacturer's instructions. DNA was eluted twice with 30 µL buffer EB (Qiagen).

The combined DNeasy/AllPrep protocol was performed on the remaining samples. Prior to extraction, filters were cut in half, dipped into PBS to remove excess RNA*later*®, blotted on Kimwipes (Kimtech, Irving, TX), and placed into 2 mL tubes. Prepared filters that were not immediately extracted were stored at -20°C and processed within 3 days. To begin extraction, 600 µL buffer RLT plus (Qiagen) was added, and the tubes shaken for 90 minutes at room temperature in a vortex on setting 5. The tubes were then shaken for 10 minutes in a vortex on the highest setting. Afterwards, the edge of the filter was snapped into the tube cap, and the tubes centrifuged to remove all liquid from the filter. The liquid was passed through a QIAshredder (Qiagen) column and 300 µL of 100% ethanol was added to the lysate before transferring the liquid to a DNeasy DNA Column (Qiagen). The column was washed with 350 µL buffer AW1, incubated with 80 µL Proteinase K (Qiagen) for 5 minutes, washed with 350 µL buffer AW1, and washed with 500 µL buffer AW2. The column was placed into a clean 1.5 mL tube and DNA was eluted twice, each with 30 µL buffer EB (Qiagen). DNA extracts were stored at 4°C until sequencing. DNA concentration was quantified using the Quant-iT™ Picogreen® dsDNA Assay Kit (Life Technologies).

Sediment DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions.

DNA Sequencing and Processing

DNA extractions were submitted for sequencing at the Microbial Systems Laboratories at the University of Michigan Medical School on two separate runs (Jun 13, 2014 and Feb 27, 2015). Ilumina MiSeq v2 chemistry 2x250 (500 cycles) was performed using dual index-labeled primers that target the V4 region of the 16S rRNA(515F/806R) [55] according to [56]. Data was

analyzed with UPARSE [57] using the Silva database (release 119) for alignment and classification. UPARSE outputs were combined in RStudio using the phyloseq package [58]. Data was normalized by multiplying the relative sequence abundance by the smallest library size (12,159 sequences) and rounding the result [59] in order to account for heteroscedasticity in sequencing depth. Duplicates were combined and averaged to create a dataset of only unique samples.

Statistical Analyses

Statistical analyses were performed in RStudio. Shapiro, ANOVA, and pairwise t-tests were performed using the stats package [60]. Histograms, boxplots, and simple linear regression tests and plots were performed using the graphics package [60]. Bray-Curtis dissimilarity matrices, non-metric multidimensional scaling (NMDS) ordinations, adonis (PERMANOVA), and permutation-based test of multivariate homogeneity of group dispersion (betadisper) were performed using the vegan package [61].

CARD-FISH

CARD-FISH was performed as described in [54] as follows. CARD-FISH was performed according to [62] with the following modifications: during embedding, filters were dipped into 0.1% low-gelling point agarose, placed cell-side down onto a clean microscope slide, and dried for 10 – 30 min at 35 - 40°C; prior to hybridization, filters were incubated in Image-iT Fx Signal Enhancer (Life Technologies) for 30 min at room temperature and then washed twice in PBS; for probe hybridization, a final probe concentration of 5 ng μL^{-1} was used and incubated overnight for up to 15 hours; for signal amplification, a substrate mixture of Alexa Fluor 488 – tyramide

and amplification buffer (Life Technologies) was created based upon the manufacturer's instructions. The probes EUB338 I/II/III [63] [64] were used to tag bacterial cells. Verrucomicrobia was targeted by mixing EUB338 III with the unlabeled competitor probe EUB338 II to minimize non-specific hybridization. Probe NON338 was used as a negative control [65]. All probes were hybridized with 55% formamide. Filters were examined with fluorescent microscopy by taking a photo and counting the number of DAPI-stained and probe-tagged cells within the field; a minimum of 1000 DAPI-stained cells was counted per labeled filter.

Results

I. Evaluating Method Bias

Because it appears that previous studies have underestimated the abundance of Verrucomicrobia, testing was done to determine if my study's molecular methods shared this bias. To evaluate whether this was the case, the Silva TestPrime [66] and the Silva RefNR database were used to evaluate the coverage of Verrucomicrobia by the 515F/806R primers. The primers amplified 80.1% of verrucomicrobial sequences that contained the V4 region of the 16S rRNA. On the phylum level, the primers covered a range of 68.6% to 100% of database sequences, with an average of 84.44 (± 7.06; 95% confidence interval) %.

To further explore the possibility of methodological biases, I compared the average relative abundance of Verrucomicrobia reported by sequencing data and CARD-FISH. I examined samples collected in May that included both PA and FL fractions from the hypolimnion at the Inlet, Outlet, and Deep sites; the following results are described in [54]. The sequencing data identified 14.6 (± 6.0; 95% confidence interval) % of all reads as Verrucomicrobia. Similarly, in CARD-FISH, 17.3 (± 2.8; 95% confidence interval) % of all DAPI-stained cells hybridized to a Verrucomicrobia phylum-specific probe (Figure 2). However, it is important to note that not all the DAPI-stained cells hybridized to the eubacterial probe mixture; in fact, only between 51.2 (± 8.4) and 76.2 (± 6.5) % of the DAPI-stained cells fluoresced. The low detection level of the eubacterial probe mixture may be partially due to an inability to penetrate the Gram-positive wall of Actinobacteria; I obtained no signal when using the Actinobacteria phylum-specific probe AcI-1214 [67] despite using an established protocol. When adjusting the observed relative abundance of Verrucomicrobia to account for this probe

penetration bias, the percentage was even higher. Taken together, these results suggest that although the primers I used do not perfectly cover verrucomicrobial phylum or classes, because the reported relative abundance in CARD-FISH complements that of sequencing data quite well, it is likely that the methods I employ provide a reliable estimate of Verrucomicrobia abundance.

II. Verrucomicrobia Phylum Abundance and Distribution

To begin examining the abundance and distribution of Verrucomicrobia in Muskegon Lake, I first looked at the phylum as a whole. I averaged the verrucomicrobial relative abundance of every sample and found that Verrucomicrobia, on average, constituted 12.18 (± 2.06; 95% confidence interval) % of the Muskegon Lake bacterial community. Compared to the 24 phyla that comprise the Muskegon Lake bacterial community, Verrucomicrobia was the fourth most abundant (Figure 3). However, because Muskegon Lake is a very dynamic system, it is important to further resolve spatiotemporal abundance patterns. There are many environmental factors at play that can have a significant impact on the phylum's relative abundance, such as month (May, July, Sept), site (Inlet, Bear, Outlet, Deep), source (water, sediment), limnion (epilimnion, hypolimnion), and fraction (PA, FL).

To help determine whether Verrucomicrobia distributions differed temporally and/or spatially, I created box-and-whisker plots illustrating the distribution of relative abundances in different groups of samples (Figure 4). However, because this data was not normally distributed (Shapiro test: $W = 0.86$, $p = 0$) (Figure 5A), I performed a log transformation to normalize the data (Shapiro test: $W = 0.99$, $p = 0.79$) (Figure 5B) in order to perform ANOVA and t-tests.

I separated the samples by month and found that the average relative abundance in May samples was 18.63 (± 6.31; 95% confidence interval) %, in July samples was 10.17 (± 2.02; 95%

confidence interval) %, and in September samples was 10.37 (± 1.77; 95% confidence interval) % (Figure 4A). Samples categorized by month did not significantly differ from each other (ANOVA: $F = 1.80$, $p = 0.18$).

In samples classified by site, the average relative abundance in Inlet was 8.50 (± 1.66; 95% confidence interval) %, in Bear was 20.09% (± 2.89; 95% confidence interval) %, in Outlet was 13.77 (± 3.85; 95% confidence interval) %, and in Deep was 15.40 (± 5.41; 95% confidence interval) % (Figure 4B). Samples separated by site did not significantly differ from each other (ANOVA: $F = 1.74$ $p= 0.17$).

When samples were categorized by limnion, the average relative abundance of the epilimnion was 13.00 (± 2.90; 95% confidence interval) % and of the hypolimnion was 15.16 (± 3.41; 95% confidence interval) % (Figure 4C); there was no significant difference between the limnion samples (ANOVA: $F = 1.30$, $p = 0.26$) .

In samples separated by fraction, the average relative abundance in PA was 12.47 (± 2.17; 95% confidence interval) % and in FL was 15.82 (± 3.92; 95% confidence interval) % (Figure 4D); there was no significant difference between the fraction samples (ANOVA: $F = 1.85$, $p = 0.18$).

When samples were categorized by source, water samples had an average relative abundance of 14.10 (± 2.20; 95% confidence interval) % while sediment samples had an average relative abundance of 4.65 (± 0.61; 95% confidence interval) % (Figure 4E). ANOVA reported that water and sediment samples were significantly different from each other ($F = 48.42$, $p = 0$). To further determine which water samples differed from sediment samples, I performed a series of t-tests with Bonferroni correction to compare epilimnion vs. sediment, hypolimnion vs.

sediment, PA vs. sediment, and FL vs. sediment, all of which were significant ($p = 0$) (Figure 4F).

III. Verrucomicrobia Intra-Phylum Composition

After examining the abundance and distribution of Verrucomicrobia as a whole phylum, I wanted to compare the operational taxonomic units (OTUs) within the group itself to determine if Verrucomicrobia had differential intra-phylum composition spatially and/or temporally. To accomplish this, I used a combination of NMDS ordinations (based on Bray-Curtis dissimilarity), PERMANOVAs, and permutation-based test of multivariate homogeneity of group dispersion (betadisper) to examine and compare the abundance of different OTUs within and between samples.

The Verrucomicrobia intra-phylum composition of samples separated by site was not significant (PERMANOVA: Adjusted $R^2 = 0.07$, $p = 0.20$; betadisper: $F = 1.19$, $p = 0.32$) (Figure 6A). However, samples separated by month were significantly different from each other (PERMANOVA: Adjusted $R^2 = 0.19$, $p = 0.00$; betadisper: $F = 0.55$, $p = 0.59$) (Figure 6B). May samples were distinct from both July samples (PERMANOVA: Adjusted $R^2 = 0.19$, $p = 0.00$; betadisper: $F = 0.69$, $p = 0.43$) and September samples (PERMANOVA: Adjusted $R^2 = 0.22$, $p = 0.00$; betadisper: $F = 1.10$, $p = 0.31$). Similarly, July samples were also distinct from September samples (PERMANOVA: Adjusted $R^2 = 0.07$, $p = 0.04$; betadisper: $F = 0.07$, $p = 0.79$).

The verrucomicrobial composition of PA vs. FL samples was also significantly different from each other (PERMANOVA: Adjusted $R^2 = 0.39$, $p = 0.00$) (Figure 6C). However, it was interesting to note that the betadisper test was significant ($F = 4.91$, $p = 0.02$), meaning that the

variances within PA and within FL samples were significantly different from each other; this could lead to a potential false positive in the PERMANOVA test.

Similarly, both PERMANOVA and betadisper were significant when comparing sediment vs. water samples (PERMANOVA: Adjusted $R^2 = 0.32$, $p = 0.00$; betadisper: $F = 8.11$, $p = 0.01$) (Figure 6D). The significant difference between the variances within sediment and within water samples is likely due to the fact that there were fewer sediment samples (11) than water samples (43). Because each group clustered quite distinctly in the NMDS ordination (Figure 6D), it is likely that the Verrucomicrobia intra-phylum compositions of the two groups of samples are, in fact, distinct.

In contrast, when the epilimnion and hypolimnion samples were plotted together on an NMDS ordination, it appeared that the samples were not distinct from each other (Figure 6E); however, PERMANOVA reported a significant difference between the two limnions (Adjusted $R^2 = 0.39$, $p = 0.00$). Interestingly, betadisper was also significant ($F = 4.91$, $p = 0.12$), suggesting that the two groups have very different variances, which may cause a false positive PERMANOVA result.

IV. Correlations between Verrucomicrobia Abundance and Geochemical Data

The temporal and spatial patterns that I examined previously are likely driven in large part by geochemical factors. To begin exploring the relationship between Muskegon Lake geochemistry and Verrucomicrobia, I performed simple linear regressions comparing verrucomicrobial abundance with a variety of geochemical data and environmental factors.

Out of the 13 environmental factors that I examined (Cl, chlorophyll, depth, dissolved oxygen concentration, dissolved oxygen percentage, pH, NH_3, NO_3, SO_4, temperature, total

dissolved solids (TDS), total kjeldahl nitrogen, and total phosphorous), 6 exhibited a linear relationship with verrucomicrobial abundance. pH was slightly negatively correlated with Verrucomicrobia abundance (Coefficient = -0.08, p = 0.01, Adjusted R^2 = 0.12) (Figure 7A). Similarly, SO_4 also appeared to be slightly negatively correlated (Coefficient = -0.01, p = 0.02, Adjusted R^2 = 0.11) (Figure 7B). Cl appeared to have a small negative correlation with Verrucomicrobia abundance (Coefficient = -0.01, p = 0.01, Adjusted R^2 = 0.14) (Figure 7C), as did temperature (Coefficient = -0.01, p = 0.0, Adjusted R^2 = 0.24) (Figure 7E). Total dissolved solids (TDS) had a stronger and larger negative correlation compared to pH, SO_4, Cl, and temperature (Coefficient = -1.62, p = 0.00, Adjusted R^2 = 0.56) (Figure 7D). In contrast to the previous five geochemical factors, depth was slightly positively correlated with verrucomicrobial abundance (Coefficient = 0.002, p = 0.00, Adjusted R^2 = 0.28) (Figure 7F). Regardless of the type of correlation, the linear regressions of each of the six environmental factors had rather low adjusted R^2, indicating that the linear models explain very little of the variation found in the data.

Discussion

Before beginning to examine the ecology of Verrucomicrobia in Muskegon Lake, I first wanted to test whether the methods I used introduced a bias that underestimated the phylum. Although the employed primers did not perfectly cover all of the verrucomicrobial sequences in the Silva RefNR database, the observed relative abundance of Verrucomicrobia in CARD-FISH matched quite well with that reported by the sequencing data; therefore, it is likely that the molecular methods I chose provide a reliable estimate of this phylum.

A future study to complement this work would be to run primers used in previous verrucomicrobial studies through the Silva TestPrime in order to compare the coverage of Verrucomicrobia by different primer sets. I do not know if our primers are significantly better at targeting the phylum compared to other primers, nor to what extent the coverage differs between primer sets.

An additional potential bias is the use of different molecular methods in my study. The samples that were used in the CARD-FISH/molecular data comparison (from the first sequencing run) were extracted slightly differently from the remaining samples (from the second sequencing run). Samples from the first sequencing run included one set of May replicates and were extracted using the optimized AllPrep protocol, whereas those from the second sequencing run were extracted using the hybrid DNeasy/AllPrep protocol. A recent paper reported that different extraction methods and sequencing runs can cause significant differences in the perceived bacterial community composition [54]. The main difference between the two protocols is the use of 2-mercaptoethanol and lysozyme and the extraction of RNA in the optimized AllPrep protocol; however, the addition of lysozyme was not found to have a significant effect on the

observed bacterial community composition [54]. Although the protocols are extremely similar, they remain a potential source of bias in the data. Similarly, the PowerSoil extraction protocol used on the sediment samples was different from those used on the water samples. It is also important to note that I did not perform CARD-FISH on sediment samples and do not know if the PowerSoil extraction contributed to an underestimation of Verrucomicrobia in these samples.

Molecular methods also vary between studies. For example, the previously mentioned study that examined PA and FL Verrucomicrobia extracted DNA as described in [68], amplified 16s rRNA genes using the primers 53F/1492R, and sequenced the genes using the M13F vector primer and ABI BigDye chemistry on an ABI3130x Genetic Analyzer [31]. In contrast, the study which observed 88.4% Verrucomicrobia relative abundance extracted DNA using the E.Z.N.A.® Water DNA kit (Omega Biotek, Norcross, GA), amplified genes using primers 27F/1492R, and constructed clones for sequencing using the pGEM T-easy Vector (Takara Corp, Otsu, Japan) [26]. As one can see, these studies employ very different molecular methods compared to those used my study. This variation can introduce methodological biases that differ between studies and, consequently, differentially influence the perceived bacterial community composition.

One source of bias that I also have not examined is the difference in the representation of Verrucomicrobia between different sequencing databases. Not only would it be interesting to compare the number of verrucomicrobial OTUs in different databases, but it would also be interesting to compare different primer coverage of verrucomicrobial sequences between these different databases. That being said, it is also important to remember that these primers are used to cover databases that have been primarily generated using PCR-amplified sequences; therefore, there is a cycle of potential bias innate to these sequencing-based molecular techniques.

When examining the relative abundance of Verrucomicrobia as a phylum, I found that it is an abundant group and a large constituent of the Muskegon Lake bacterial community. There was no significant difference in the temporal distribution of Verrucomicrobia. The only significant difference in spatial distribution was the relative abundance between sediment and water samples, the former being, on average, three times less abundant than the latter.

However, it is important to remember that many environmental factors can influence verrucomicrobial distribution, and generalizing the samples based upon just one of these characteristics combine many confounding factors that can mask significance. In future work, I plan to separate samples even further to decrease the influence of confounding factors. For example, I will examine not just May samples as a whole, but compare May epilimnion samples from the PA fraction to those from the FL fraction. Just as overgeneralizing environmental factors can drown significance, overgeneralizing bacteria as a phylum can overlook differences found in lower taxonomic-level groups. In the future, I also plan to compare the relative abundances of different classes and orders of Verrucomicrobia.

To begin examining the phylum's constituents, I compared the Verrucomicrobia intra-phylum compositions in different groups of samples. I found that there was a significant difference in phylum composition between samples from May vs. July vs. September, sediment vs. water, PA vs. FL, and epilimnion vs. hypolimnion. It is important to note that an NMDS ordination tries to best represent the dissimilarities between samples in a two-dimensional environment. Biological communities and relationships are very complex, so a two-dimensional space is likely not the best way to visualize these; rather, a three-dimensional ordination would be a much more accurate visualization. This is particularly true for samples that betadisper reported to have significantly different variances, and especially true for the epilimnion vs.

hypolimnion samples, which did not appear to cluster differentially in the NMDS, but was reported to be significantly different in both their dissimilarities and in their variances.

To begin to interpret the relationships between verrucomicrobial abundance and environmental factors such as geochemistry, I performed simple linear regressions. I found that pH, SO_4, Cl, temperature, and TDS were negatively correlated with an increase in Verrucomicrobia abundance whereas depth was positively correlated. However, the adjusted R^2 for each regression was quite low, meaning that the linear models explain very little of the variation in the data. Although a low adjusted R^2 is not synonymous with a poor model fit, it does raise the idea that exploring different models may result in one that is a more accurate representation of the data. For example, although the linear regression of Cl and verrucomicrobial relative abundance reported a significant correlation, a scatterplot of the data (Figure 7C) suggests that a slightly positive quadratic model may be a better fit. Additionally, analyzing the data with other models may help identify a relationship between the data that did not have a significant linear correlation (Figure 8).

Because Muskegon Lake is such a dynamic system, it is possible that there are complex interactions taking place between the biotic and abiotic factors that are difficult to model accurately with correlations. An analysis that I plan to perform in the future is to compare rank similarity matrices between biotic and abiotic variables, as described in [69]. This method will determine what set of abiotic variables best fits and explains the natural biotic ordination.

My thesis just begins to scratch the surface of the freshwater ecology of Verrucomicrobia, but it demonstrates promising results that indicate that this phylum is an abundant and important player in Muskegon Lake, more prevalent in the water column than in the sediment, and has distinct spatial and temporal intra-phylum compositions. In addition to

performing more statistical analyses, I also plan to expand my study to include other Michigan freshwater lakes. These include Lake Michigan and various inland lakes that vary from oligotrophic to eutrophic. By incorporating a variety of lakes, I hope to gain a better understanding of Verrucomicrobia in different freshwater environments. I hope that my thesis and future work will help elucidate the drivers behind Verrucomicrobia abundance, diversity, and distribution in these systems, and also help microbial ecologists understand the ecology and importance of this abundant, yet understudied phylum in freshwater environments.

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Figures

Figure 1: Sampling sites in Muskegon Lake. "Inlet" is the inlet of Muskegon River into Muskegon Lake. "Bear" is the inlet of Bear Lake into Muskegon Lake. "Outlet" is the channel connecting Muskegon Lake to Lake Michigan. "Deep" is the deepest point of the lake. Inlet, Outlet, and Deep were always sampled. Bear was sampled in July and September.

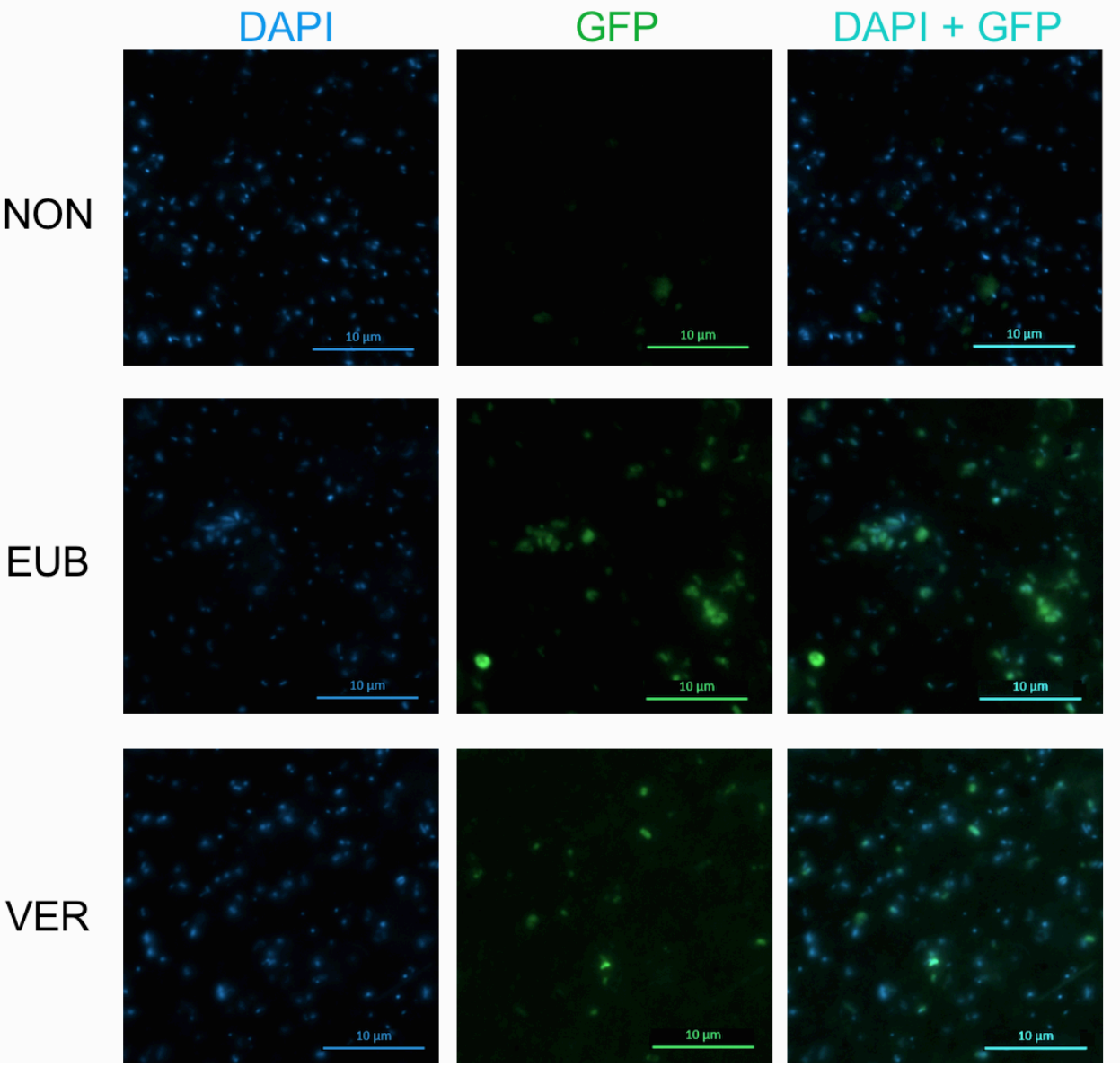

Figure 2: CARD-FISH microscopy images. The columns refer to the fluorescent channel in which the image was taken (DAPI = DAPI-stained cells; GFP = probe-hybridized cells; DAPI + GFP = combined channels). The rows indicate the probe that was used (NON = NON338, negative control; EUB = EUB I/II/III, eubacterial probe; VER = EUB III, Verrucomicrobia phylum-specific probe).

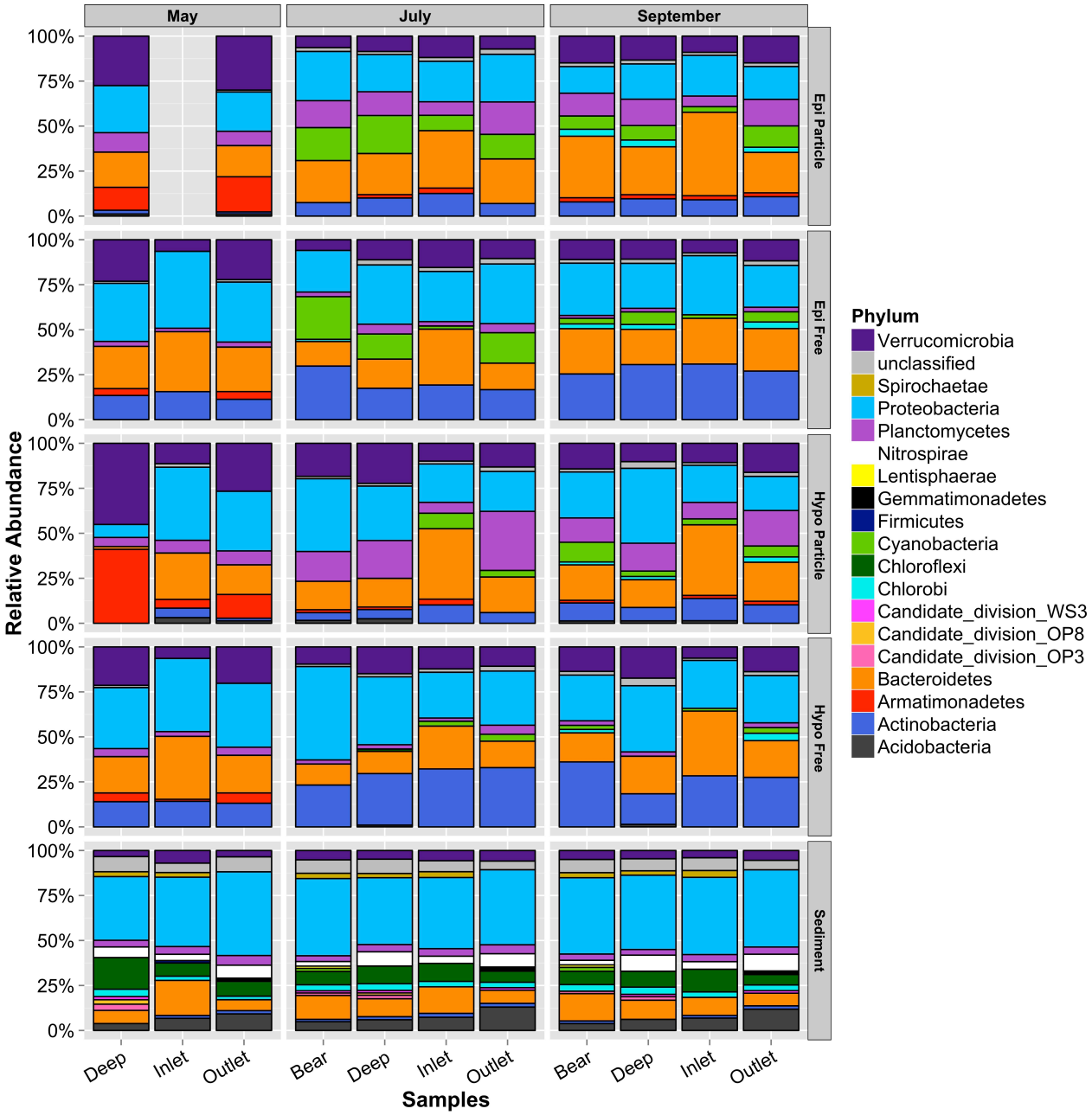

Figure 3: Stacked bar plots illustrating the bacterial community composition as represented by phyla relative abundances in each sample. Samples are separated based upon month (May, July, September), limnion ("epi" for epilimnion, "hypo" for hypolimnion), fraction ("particle" for PA, "free" for FL), and sediment. The relative abundance of Verrucomicrobia is represented by the dark purple fraction at the top of each stacked bar.

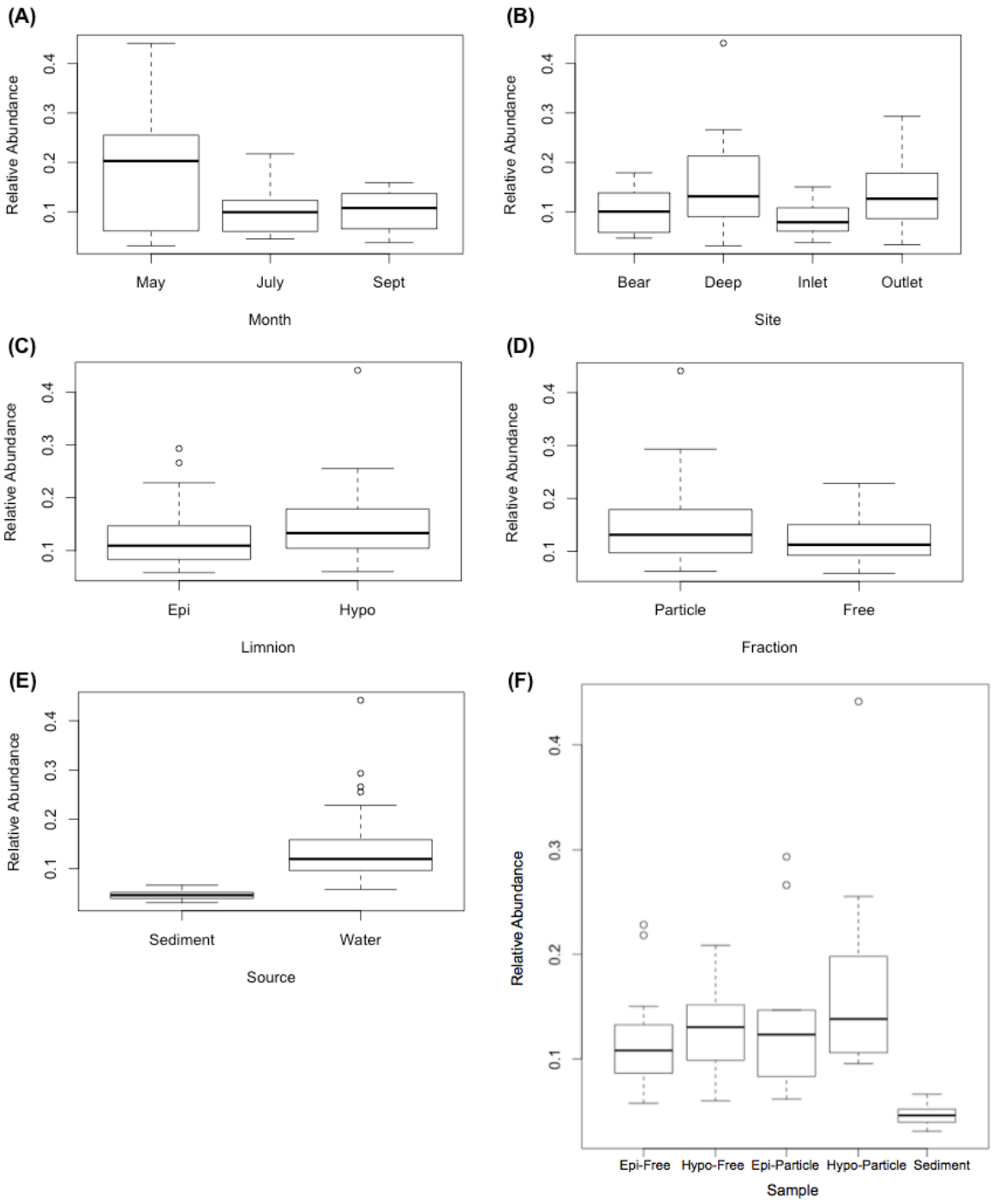

Figure 4: Box-and-whisper plots illustrating the distribution of verrucomicrobial relative abundance in groups of samples categorized by (A) month, (B) site, (C) limnion, (D) fraction, (E) source, and (F) a combination of limnion, fraction, and source.

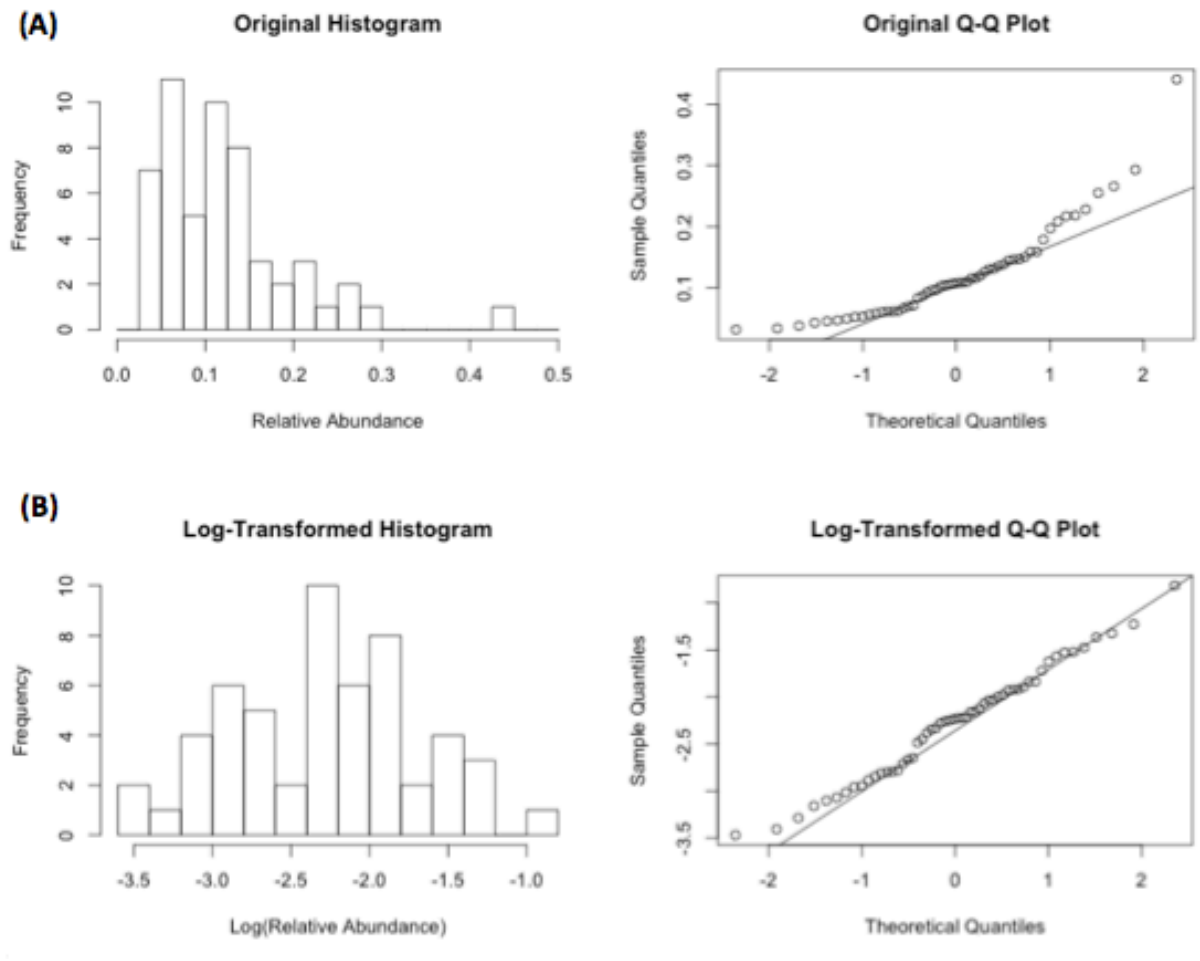

Figure 5: Histograms and Q-Q plots illustrate that (A) the original data was skewed and not normal, whereas (B) the log-transformed data had a normal distribution.

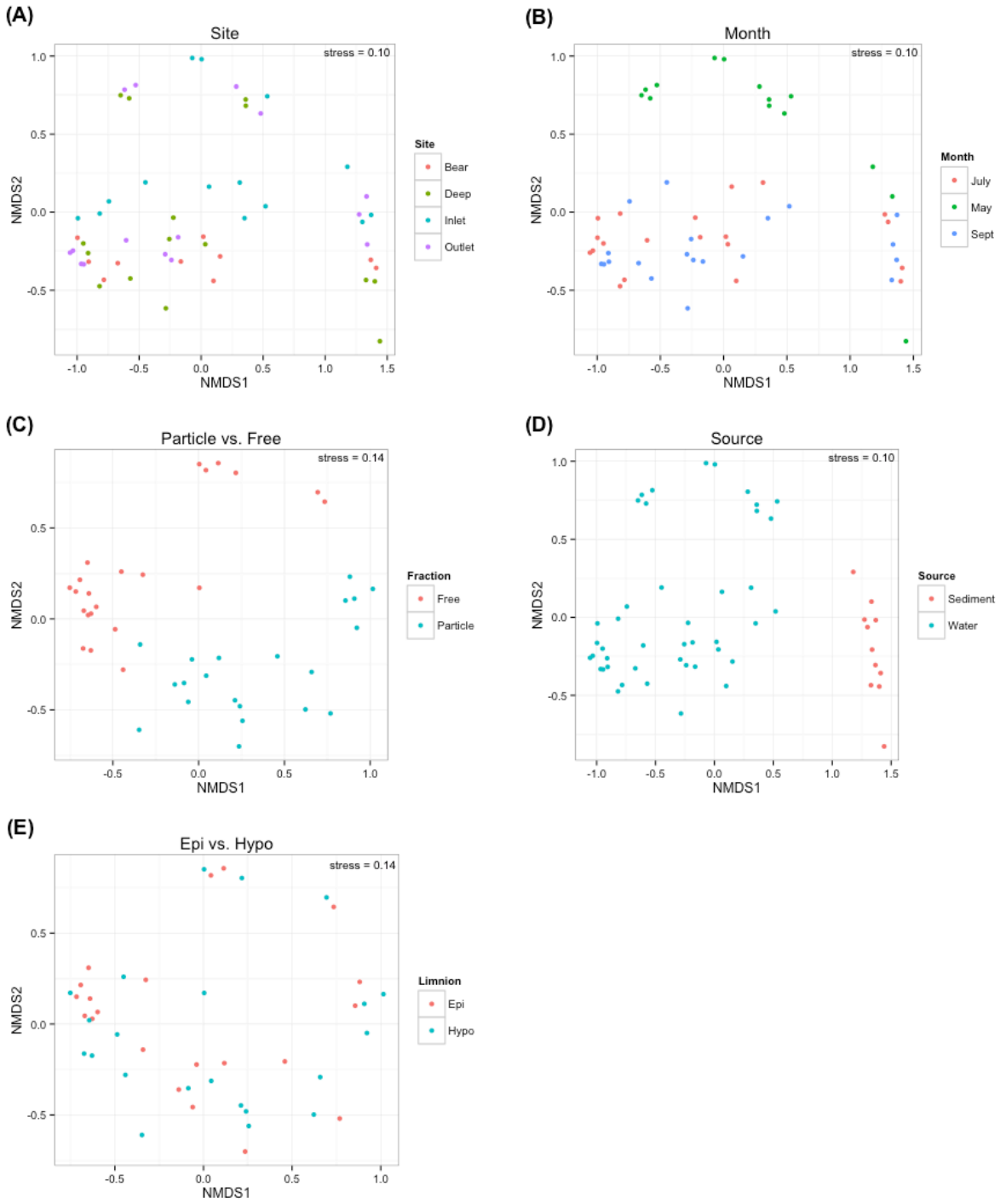

Figure 6: Non-metric multidimensional scaling (NMDS) ordinations based upon Bray-Curtis dissimilarity matrices of samples. (A), (B), and (D) represent the same ordinations colored differently based upon site, month, and source, respectively. (C) and (E) represent the same ordinations colored by fraction and limnion, respectively.

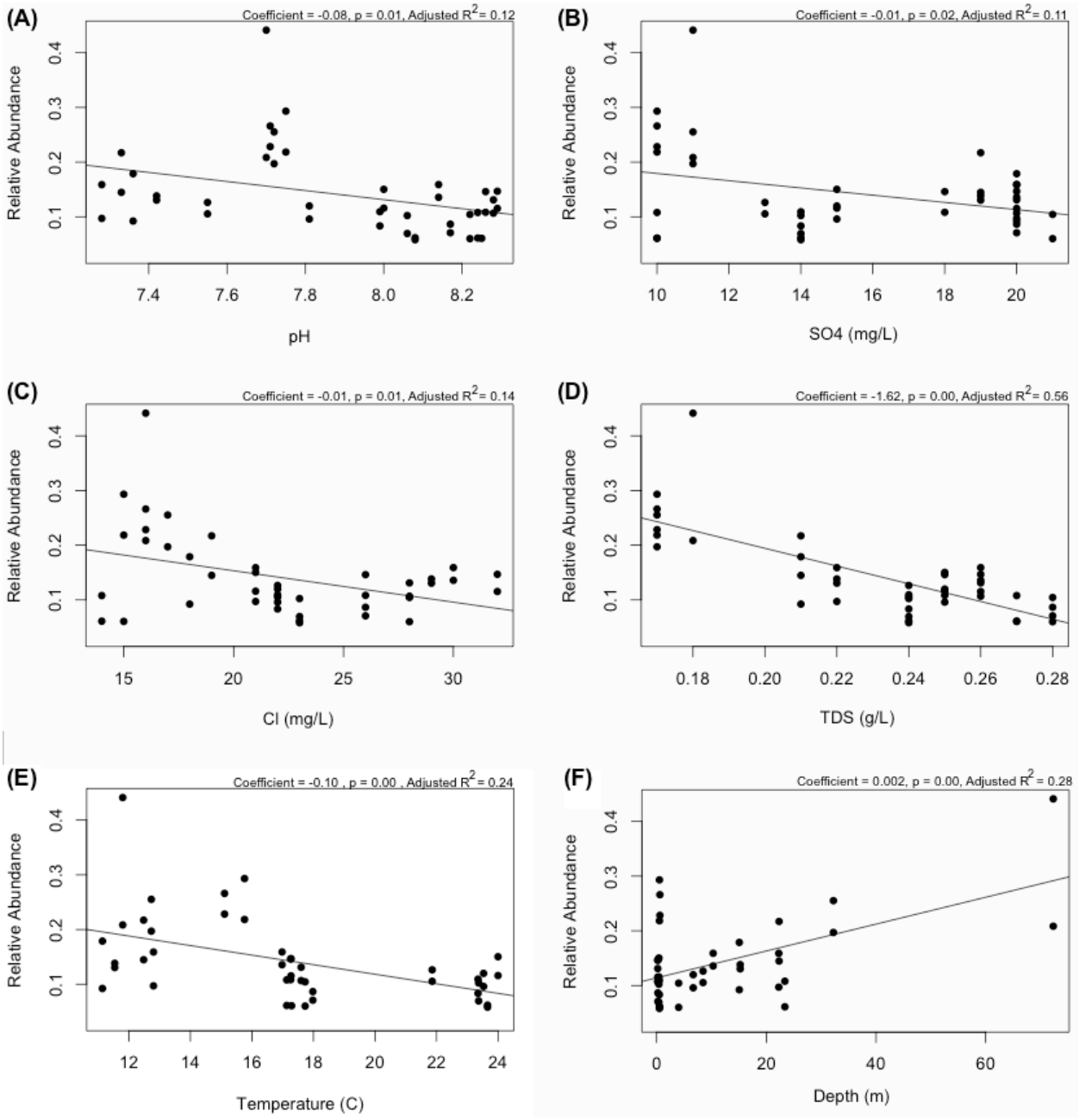

Figure 7: Scatterplots with linear regression lines comparing verrucomicrobial relative abundance to (A) pH, (B) SO_4, (C) Cl, (D) total dissolved solids (TDS), (E) temperature, and (F) depth.

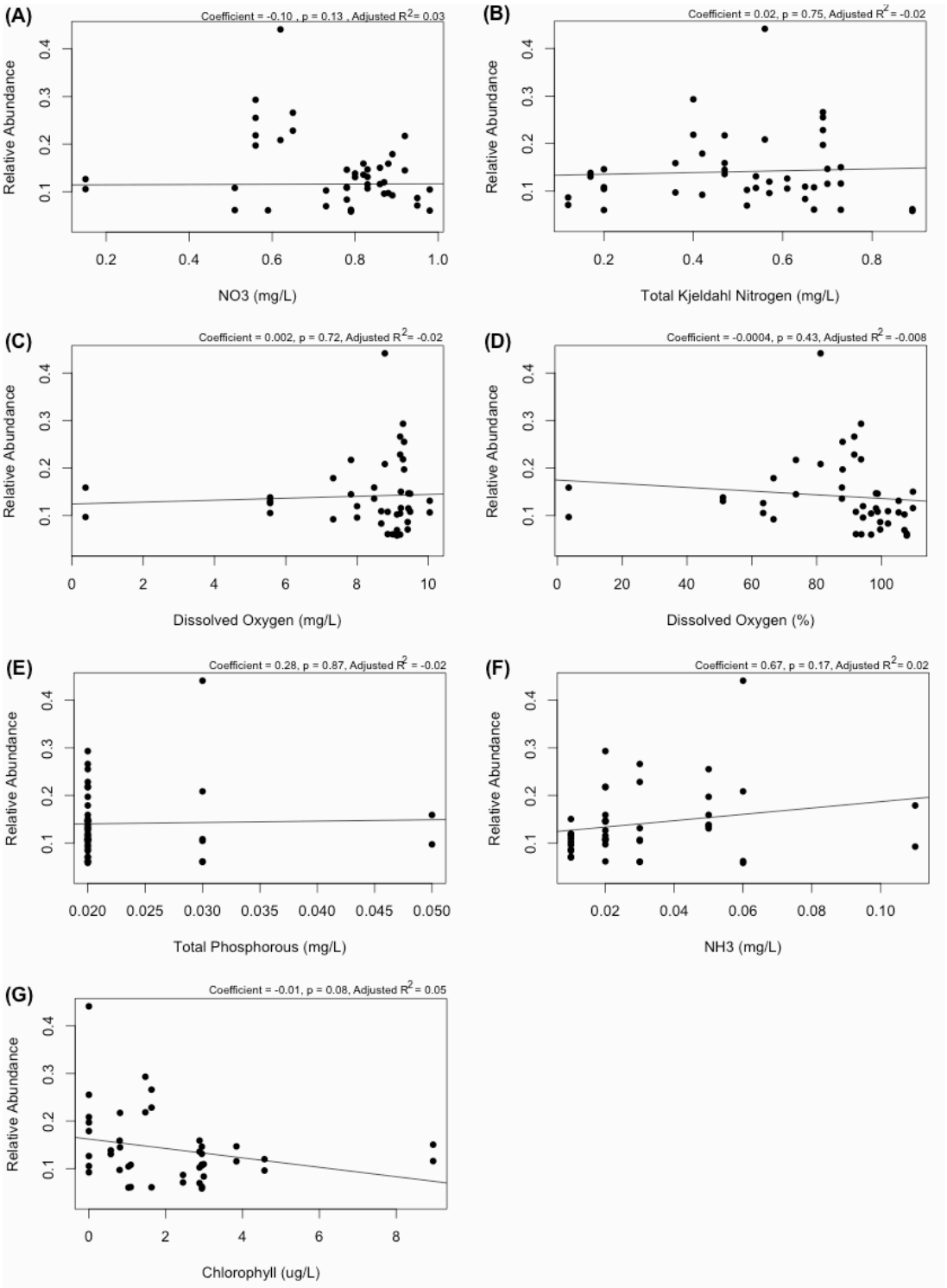

Figure 8: Scatterplots with non-significant linear regression lines comparing verrucomicrobial relative abundance to (A) NO_3, (B) total kjeldahl nitrogen, (C) dissolved oxygen concentration, (D) dissolved oxygen percentage, (E) total phosphorous, (F) NH_3, and (G) chlorophyll.

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