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As interest in exploiting black shales for hydrocarbon development increases, it becomes increasingly important to understand their microbial community prior and after energy development. Here, we use membrane lipid fatty acids to understand the subsurface microbial community in a Marcellus Shale well and its associated drilling muds. By differentiating the microbial signatures in the shale rock samples from the drilling muds, we are able to provide evidence of rock-indigenous membrane lipid profiles which could be indicative of native deep subsurface microbial life. A good understanding of microbial community of deep surface black shales like the Marcellus Shale, accords enormous opportunities for improving biocides in the shale energy industry, understanding subsurface microbial colonization, and engineering efforts for enhanced gas recovery.

Microbial Lipid Biomarkers Detected in Deep Subsurface Black Shales

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Abstract

Evidence for microbes has been detected in extreme subsurface environments as deep as 2.5 km with temperatures as high as 90˚C, demonstrating that microbes can adapt and survive extreme environmental conditions. Deep subsurface shales are increasingly exploited for their energy applications, thus characterizing the prevalence and role of microbes in these ecosystems essential for understanding biogeochemical cycles and maximizing production from hydrocarbon-bearing formations. Here, we describe the distribution of bacterial ester-linked phospholipid fatty acids (PLFA) and diglyceride fatty acids (DGFA) in sidewall cores retrieved from three distinct geologic horizons collected to 2,275 m below ground surface in a Marcellus Shale well, West Virginia, USA. We examined the abundance and variety of PLFA and DGFA prior to energy development within and above the Marcellus Shale Formation into the overlying Mahantango Formation of the Appalachian Basin. Lipid biomarkers in the cores suggest the presence of microbial communities comprising Gram (+), Gram (-) as well as stress indicative biomarkers. Microbial PLFA and DGFA degradation in the subsurface can be influenced by stressful environmental conditions associated with the subsurface. The PLFA concentration and variety were higher in the transition zone between the extremely low permeability Marcellus Shale Formation and the more permeable Mahantango Formation. In contrast to this distribution, more

abundant and diverse DGFA membrane profiles were associated with the Mahantango Formation. The stress indicative biomarkers like the *trans*-membrane fatty acids, oxiranes, keto-, and dimethyl lipid fatty acids were present in all cores, potentially indicating that the bacterial communities had experienced physiological stress or nutrient deprivation during or after deposition. The DGFA profiles expressed more stress indicative biomarkers as opposed to the PLFA membrane profiles. These findings suggest the probable presence of indigenous microbial communities in the deep subsurface shale and also improves our understanding of microbial survival mechanisms in ancient deep subsurface environments.

Key Words: Deep Subsurface, PLFA, DGFA, Microbial Community Composition, Marcellus Shale

1.0 INTRODUCTION

The ongoing search for microbial life in the subsurface has been a subject of research interest as early as 1926.[1] Subsequent microbiological studies have demonstrated the possibility of microbial adaptation and survival in extreme subsurface environments spanning deep terrestrial and marine ecosystems.[2-18] In addition, it has been suggested that the deep surface ecosystem may account for as much as 30% of the Earth`s biomass.[19-21] Researchers have either examined the microbial survival ability over geological time[22] or have reported the likelihood of potentially indigenous microbial communities in deep rocks.[6-8, 9, 23] Increased interest in energy extraction and environmental applications of deep subsurface shales has further reinforced microbial research in the deep shale ecosystem.[6, 7, 16, 17, 24] The recovery of microbial DNA and biogenic natural gas, [25] as well as the temporal shifts in microbial communities of produced fluids from unconventional wells[26-31] are also evidence of pre- or post-production microbial life in shale systems.

Even though microbes have been shown to exhibit extraordinarily tenacious abilities to survive in extreme environments, more effort still has to be made to understand both viable and non-viable subsurface microbial communities. Questions about the role of microbes in the subsurface remain a scientific challenge. For example, we do not yet know the *in situ* microbial survival mechanisms in the subsurface and how they have been/were affected by the prevailing geologic and environmental conditions. It is also difficult to establish whether the microbes detected in the deep subsurface represent environmental conditions at the time of deposition or if they reflect changes that took place after deposition.[7, 8, 18, 32] This is further complicated by the fact that drilling and fracturing fluids used during development, introduces large quantities of labile carbon, nutrients, and exogenous organisms into the subsurface,[28,30,33, 34] making it unclear whether indigenous communities exist in these shale formations. These challenges highlight the need for a better understanding of subsurface microbial communities and the microbial role in global biogeochemical processes.[24] Examining the role of microbes in these systems before and after shale gas drilling, and the potential influence of geologic and environmental conditions further provides opportunities to better understand the role of deep subsurface microbial communities.

Phospholipids are essential components of microbial cellular membranes which rapidly degrade upon cell death and therefore have been used to provide modern evidence of living microbes in the environment.[15, 35-40] When the microbial cell dies, phospholipase enzymes break the glycerol backbone of the phospholipid fatty acid (PLFA), creating a diglyceride (DGFA) which contains the same fatty acid as the parent phospholipid.[35, 38, 41] DGFAs are the breakdown products of PLFAs and indicate the presence of non-viable microbial communities. Lipid biomarkers in sediments are thus excellent proxies for understanding microbial communities and providing insight into the environmental conditions and post-depositional history.[42-44] However, microbes

can adopt different states of dormancy for survival in the deep subsurface and this could change or modify the biological or chemical definition of "viable" or "non-viable" microbial cells.[45-46] This is more important in the subsurface where the turnover rate of PLFA (removal of a phosphate group) is not known.[8] Generally, the PLFAs have been established as a biomarker for viable microbes while DGFAs as biomarkers for non-viable microbes.[35-39] They also serve as one of the most sensitive and suitable molecular approaches to investigate *in situ* microbial biomass and community.[37, 47]

Here, we utilize the PLFA and DGFA signatures to (i) to evaluate trends in biomarker yield and variety across three geologic horizons, and (ii) to identify signature lipid biomarkers (SLB) and potential microbial groups within the subsurface rock cores. We hypothesized that zones with higher organic carbon content would have higher biomarker composition and variety since organic carbon can serve as both a carbon and energy source for microbial activities in the deep biosphere.[13, 48-51] Based on previous studies we also hypothesized that the transition zone between the extremely low permeability Marcellus Shale and the more permeable Mahantango Formation would have higher lipid abundance and diversity. We thus collected deep subsurface core samples from Marcellus Shale Top, upper Marcellus Shale zones, and the overlying Mahantango Formation to examine PLFA and DGFA membrane lipid profiles across these zones. We also evaluated the differences between the lipid profiles in the cores, drilling mud, and core washes to discern what fraction (if any) was unique to the cores.

GEOLOGIC BACKGROUND

The geologic units sampled in this study all belong to the Hamilton Group and were deposited in the Appalachian Basin during the Acadian Orogeny of the Middle Devonian.[52, 53] The

Marcellus Shale is a thinly-laminated, gray- to black-colored organic-rich shale and is overlain by the Mahantango Formation which is predominantly a gray, thickly laminated, interbedded silty mud, and sandstone unit.[54-55] The Marcellus Shale and the Mahantango Formations are both composed primarily of mud rock, although the rocks of the Marcellus Shale Formation are more organic-rich (Figure 1). Recent studies on the Hamilton Group in this part of the basin have indicated that variations in paleoenvironmental conditions and sources of sediment influx were responsible for the differences in lithological composition, age, mineralogy, and geochemistry of the different formations.[56-60] All these units were deposited in the shallow to the deep marine environment, however, the Marcellus Shale was deposited in relatively deeper anoxic conditions compared to the Mahantango Formation.[59, 61- 62]

MATERIALS AND METHODS

Site Description, Drilling, and Sample Collection Procedures

The study site is the Marcellus Shale Energy and Environment Laboratory (MSEEL), located two miles southeast of Morgantown, West Virginia. The MSEEL site contains four producing wells (MIP 3H, MIP 4H, MIP 5H, MIP 6H) and a scientific well (MIP SW) each penetrating more than 2,250 m into the Marcellus Shale (Figure 1A, B, C). The funding for MSEEL is provided by the Department of Energy's National Energy Technology Laboratory (NETL) with the goal of improving the efficiency of unconventional hydrocarbon production while minimizing adverse environmental impacts. The site is developed and managed by the Department of Geology and Geography at West Virginia University (WVU), in collaboration with Northeast Natural Energy (NNE), NETL and The Ohio State University (OSU). The samples for this study were side wall cores collected from MIP-3H. A total of five formations were targeted for sidewall cores

ranging in depth from 2,175 m to 2,306 m. For this study, sidewall core samples collected at selected intervals from the Mahantango Formation, Marcellus Shale top, and the upper Marcellus Shale zones were chosen for analysis (Figure. 1) and transported to OSU for decontamination and processing. Prior to obtaining field core samples, rigorous decontamination methods and small batch core processing protocols were developed to ensure all core samples were free of exogenous microbial signatures.[63] The cores were subsequently collected in a manner to ensure minimal contamination from the drilling process or sample collection. Specifically, drilling mud tracers (i.e. fluorescent microspheres[64] were added to the drilling mud to track any microbial contamination. In total 2.8×10^{13} particles of Fluoresbrite, Carboxylate YG 0.50 micro microspheres (Polysciences Inc., Warrington, PA) were added to the input drilling muds. Samples of the drilling muds (Drilling Mud 9-03 (2015) and Drilling Mud 8-28 (2015)) were then collected in sterile 1 liter Nalgene bottles (stored on ice) to serve as a control for confirming lipid profiles indigenous to cleaned cores.

In order to remove contamination that may have resulted from drilling, sample collection and/or handling, each core was placed in a sterile saline bath (1.5M NaCl) and the outer portion was abraded with autoclaved steel wool.[63] Cores were next placed in successive saline baths to remove decontamination, with wash fluids retained to evaluate potential contaminants. After the cores were cleaned, the outer portion was flame sterilized. The cores were then crushed with a Plattner mortar and pestle (Humboldt Mfg. Co., Elgin, IL) and then ground in a ceramic mortar and pestle using sterile technique. The ground core samples were passed through a series of autoclave-sterilized brass sieves with mesh sizes of 2000 μm, 1000 μm, and 500 μm (Dual Manufacturing Co., Inc., Franklin Park, IL). Powdered samples were continuously ground until all samples could pass through a 500 μm sieve. Three out of the five cores from each depth were

homogenized and stored in autoclaved wide-mouthed, amber glass jars with Teflon-coated lids at -80°C (Thermo Fischer Scientific, Waltham, MA). To ensure that the lipid biomarkers were representative of the microbial community of the deep subsurface cores and not introduced during drilling, samples of saline decontamination baths from each core depth were also collected for lipid analyses. The samples were stored at -80ºC until extraction.

Lipid Extraction

Lipid extraction and analysis were performed in the Pfiffner lab at the Center for Environmental Biotechnology at the University of Tennessee (Knoxville, TN, USA). A modified summarized scheme of the methodology for the lipid extraction.[65] All glassware were cleaned in a 10% (v/v) micro alkaline cleaning solution (International Products Corporation, Burlington, NJ) and 5 times with distilled water and 5 times with Millipore water before being combusted in a muffle furnace at 550°C for 6 hours. Metal lab ware (forceps, mortar, pestle, and spatulas) were cleaned with tap water and then with distilled water and finally with a solution of 1:1 chloroform: methanol. Teflon-lined caps were cleaned in the same manner as the glass-wares and then solvent-rinsed with acetone. All reagents and solvents used during the extraction and analytic experimental process were of high grade (HPLC, Fisher Optima). The samples were lyophilized and weighed before extraction, which provides better adheres to the solvent ratios used by removing the water in the sample.

Samples were extracted ultrasonically according to the modified Bligh and Dyer procedure[35, 66] with an intact polar lipid (Phosphate Buffer + phosphatidylcholine, POPC) amendment[65]. To make sure the added POPC was not a contaminant in the total lipid concentrations, we subtracted the equivalent concentration of the amended lipids from the PLFA

yield. The amended POPC was meant to improve lipid extraction efficiency in deep subsurface formations characterized by extremely low biomass, complex shale matrices, and mineralogy.[65] Procedural blanks and experimental controls (drilling muds and core washes) were also analyzed to monitor any laboratory contamination and the possibility of contamination during drilling and extraction process. The procedural blanks and experimental controls were prepared and analyzed identically as the samples. Lipid extractions were carried out on 37.5 g of the powdered rock sediments. Solvent extraction mixtures of chloroform-methanol-phosphate buffer, 1:2:0.8 (v/v/v, Chloroform:MeOH:Buffer) were used to suspend the powdered rock sediments in a 250 mL glass centrifuge bottle. Phosphate buffer (0.05 M) was prepared by adding 8.7 g of dibasic potassium phosphate (K_2HPO_4) with 1 liter of HPLC-grade water and neutralized with 1N HCL to pH of 7.4. The buffer was washed with chloroform (5% of buffer volume) by vortexing and storing at room temperature overnight. 50 uL of 50 pmol/ µL of internal standard (1, 2-dinonadecanoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids) was added, and the suspension was shaken and sonicated twice in an ultrasonicator for 30 to 45 seconds with a 30-second interval between sonication cycles to aid in cell lysis. Bottles were shaken vigorously for 15 seconds and vented before incubation overnight in the dark at room temperature. The samples were centrifuged for 30 minutes at 2000 rpm at 4°C after incubation and transferred into a 250 ml glass separatory funnel. Our extraction, separation, and analysis were based on traditional lipid extraction procedures.

Chloroform and water were added to the suspension to achieve a ratio of 1:1:0.9, chloroform:methanol:buffer (v/v/v) and the separatory funnels were shaken vigorously for 15 seconds and let to rest overnight to split phase (upper: aqueous phase, lower: organic phase containing the lipids). The organic phase was collected into a 250 mL round bottom flask and evaporated to near dryness using a rotavap system (Buchi Corporation). The total lipid extract

(TLE) was then transferred quantitatively into test tubes by the use of three washes of 2 mL of chloroform, after which the solvent was evaporated with a N_2 blowdown evaporator at 37°C. The dried TLEs were re-suspended in 2 mL of chloroform and sequentially fractionated on an activated silicic acid column, 100-200 mesh powder (dried at 110°C for 1 hour; Clarkson Chromatography Products, Inc), into fractions of different polarities using hexane, chloroform, acetone, and methanol[67-69.] Silicic acid column chromatography uses solvents of increasing polarity (hexane<chloroform < acetone < methanol) to selectively elute the lipid classes from the silicic acid stationary phase. The active sites on the silicic acid (silanols) contain –OH groups directly bound to the silicon atom which interact with the polar groups of the lipid classes. As the polarity of the solvents increases, the lipid classes are selectively eluted from the silanols, thereby effecting separation. Because the separations are based on polarity, the n-alkanes are eluted from the hexane fraction, the DGFAs, sterols, and respiratory quinones from the chloroform fraction, glycolipids from the acetone fraction, and polar lipids (including phospholipids) from the methanol fraction. The PLFAs were recovered from the methanol fraction while the DGFAs were recovered from the chloroform fraction. The chloroform and methanol fractions were evaporated to dryness before trans methylation into fatty acid methyl esters (FAMEs) using methanolic potassium hydroxide for subsequent analysis with the gas chromatography-mass spectrometer (GC-MS).[35, 39, 41, 70] Because additional lipids like betaine lipids (BLs) are characterized by an ether bond connecting the head group with diacylglycerol (DAG) backbone[71] we do not account for them in the separation. It is also important to understand that ether-linked polar lipids may be recovered in the methanol fraction, however, the extraction is not vigorous enough to sufficiently recover Archaeal lipids.[72] More so, ether lipids do not undergo methylation. Lipid extraction, silicic acid column separation, and methylation are generally performed at room temperature (not to exceed 37°C)

followed by storing the samples in the freeze after each procedure.[38, 73] Mild alkaline methanolysis cleaves the fatty acids from the phospholipid glycerol backbone and replaces the glycerol bonds with methyl groups, creating FAMEs.[38] Reagents for this procedure include chloroform, toluene, hexane, methanol, acids, and bases. The lipid extracts were redissolved in 1 mL toluene: methanol (1:1, v:v) and 1 mL 0.2M methanolic KOH and the mixture vortexed for 5 minutes at temperatures no greater than 37ºC. After the samples cooled to room temperature, 2 mL of hexane:chloroform (4:1 v;v) was added and the sample vortexed prior to being neutralized (pH 6-7) with approximately 200 µL of 1N acetic acid. After neutralization, 2 mL of nanopure distilled water was then added to break phase and the samples vortexed for at least 30 seconds. The phases (upper: organic containing the FAME, lower: aqueous) were separated by centrifugation (5 minutes, approximately 2000 rpm). The upper phase was transferred to a clean test tube and the lower phase re-extracted with 2 mL of hexane, centrifuged, and transferred as above, two more times. The solvent was then removed with the nitrogen gas blow-down and the FAMEs extracts stored in the freezer until separation and quantification.

GC-MS Analysis, Quantification, and Lipid Identification

The lipid samples were then dissolved in 200 µL of hexane containing 50 pmol/uL of external injection standard (docosanoic acid methyl ester, Matreya, Inc) and transferred into GC-MS vials containing 500 µL glass inserts. The external standard was used to quantify the concentration of individual fatty acids in each profile. Aliquots of samples were then injected into an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector (MS) equipped with a non-polar cross-linked methyl silicone column (Restek RTX-1 column 60m, 0.25mm I.D. ×0.25µm film thickness) to be further separated, identified, and quantified. The GC operating conditions were as follows: 60°C for 2 minutes then increased at a rate of 10°C/minute

to 150°C and followed by a second ramp at 3°C/minute to 312°C for a total run time of 65 minutes.[38] The injector temperature was 230°C; the detector temperature was 300°C, and Helium was the carrier gas. The following methyl ester standards (Matreya LLC, State College, Pennsylvania, USA) were included in each sample run to calibrate retention times and assist with peak identification: Bacterial Acid Methyl Ester CP Mixture (BacFAME [1114]), Polyunsaturated FAME Mixture 2 (PUFA-2 [1081]), and Polyunsaturated FAME Mixture 3 (PUFA-3 [1177]). An internal standard curve (1 pmol, 5 pmol, 10 pmol, 20 pmol, 30 pmol, 40 pmol, and 50 pmol/ μL) was prepared and analyzed on the GC-MS to determine the detection limit and to establish the best sample dilution range. The standard curve and the regression analysis had a linear relationship (0.99). The external standard curve of 1 pmol standard peak represented a detection limit of 0.18 ng. Multiple replicates of core samples would be ideal for estimating uncertainty in PLFA or DGFA yield, however, we were restricted in the amount of uncontaminated core available. Because we expected low concentrations of lipids to be present,[63] we prioritized detecting a diverse array of lipids by maintaining the standard protocol for lipid extraction input mass instead of opting for extracting less starting material with multiple replicates. Indeed, further studies would benefit by increased starting core material and additional replicates. Although ionization efficiency can vary between FAMEs, we assume equimolar ionization response for standard FAMEs as described in previous studies.[38, 74-75] Nevertheless, we calculated an average standard deviation based on the BacFAME methyl ester standard (Matreya, Inc.) analyzed in duplicates at the time of sample analysis. The standard contains 26 FAMEs ranging from 11-20 carbons in length and has representative saturates, terminally branched saturates, monounsatruates (including cyclopropyl FAMEs), and polyunsaturates. We examined the results for differences in MS detection of the FAMEs in the Bac FAME standard which showed similar peak area detection across the

FAMEs. The average standard deviation by GC-MS analysis was 0.07%, which implies that variance in GC-MS had a minimal influence on the variability of PLFA and DGFA results.

In some lipid samples, the external standard peak co-eluted with phthalate isomers and was corrected before the lipid concentration was calculated.[65] Identified peaks were confirmed across all samples and validated independently via GC-MS spectra using the Agilent MSD ChemStation Data Analysis Software F.01.00 along with the NIST11 compound library. Lipid identities were as described in[39]. A single-ion monitoring program was also used to scan the base peaks for lipids to validate all identified peaks. The SIM program was based on m/z (43, 55, 74, and 87) common among FAMEs ions. Once peaks were identified, the PLFAs and DGFAs were quantified by integration of the TIC peak areas. This was done by calculating the area of the corresponding peaks in the chromatograms and quantifying them with respect to the peak area and known concentration of the external standard (C22:0). The following calculation was used to obtain the molar or weight amounts per sample and normalized to per gram by using appropriate dilution factors and mass measurements as shown below.

$C_x = (A_x/A_{ISTD}) * C_{ISTD} * D$ where:

C_x is the calculated concentration of compound X (moles or weight per unit volume),

A_x is the GC area of compound X (unitless),

A_{ISTD} is the GC area of the external injected standard

c_{ISTD} is the concentration of the external injection standard, and

D is the appropriate dilution factor

Statistical Analyses

Non-metric multidimensional scaling (NMDS) analysis was used to evaluate variations in the lipid profiles between the formations and the drilling mud samples. NMDS analysis was performed in the R statistical software version 3.2.4 using the 'stats' (version 2.15.3) and 'vegan' (version 2.3-5) packages.[76] Bray-Curtis distances were calculated from absolute lipid (PLFA and DGFA) concentrations (pmol) and the resulting distance matrices were used in the NMDS. A dendrogram was designed from the Bray-Curtis distances and used to sort samples for heat map analysis. Differences in drilling muds, core washes, and core samples were analyzed using one-way Analysis of Variance (ANOVA) tests.

RESULTS

Individual PLFA and DGFA concentrations in pmol/g and relative abundance in mol%, as well as functional group lipid biomarkers, are shown in Tables 1 and 2. Samples from the Mahantango Formation had lower total PLFA concentration (23 pmol/g) than samples from both the Marcellus Shale Top (83 pmol/g) and Upper Marcellus Shale (55 pmol/g) zones (Figure 2). Conversely, samples from the Mahantango (258 pmol/g) had a higher total concentration of DGFA than the Marcellus Shale Top (162 pmol/g) and Upper Marcellus Shale (183 pmol/g) zones (Figure 2). In the drilling mud samples, PLFA concentrations ranged from 287 pmol/g to 476 pmol/g and total DGFA concentrations ranged from 305 pmol/g to 318 pmol/g. Overall, the drilling mud samples had significantly higher ($p=0.01$) lipid biomarker concentrations compared to the core samples for both PLFA and DGFA. The PLFA concentrations for the core wash samples range from 189 pmol/g, 75 pmol/g and 1.7 pmol/g while the DGFA ranged from 222 pmol/g, 210 pmol/g and 157 pmol/g for the Mahantango Wash, Marcellus Top Wash, and Upper Marcellus Shale Wash respectively (Figure 2). Even though the biomarker yield in drilling mud samples was higher, the biomarker profiles detected in the cores and drilling muds were compositionally different (Figures

3, 4), suggesting that individual lipid profiles associated with the cores were derived from the subsurface and not from the drilling muds. We used procedural blanks in each extraction treatment to monitor laboratory contamination. With the exception of the internal and external standard peaks, the blanks did not have any fatty acid peaks (detection limit <0.18 ng), indicating that no fatty acids were introduced during the extraction process. In addition, the absence of fluorescent beads on decontaminated cores was another indication that there was minimal influence of drilling mud contaminants in these samples.

Of the 38 PLFAs detected in the dataset, 20 (52.63%) were detected solely in the drilling mud samples, 6 (15.78%) in the core and core wash samples, 2 (5.26%) solely in the core samples, and 10 (26.31%) were shared across all samples (Figures 3A, 4A). All polyunsaturated and long-chain saturated (>C22) PLFAs were specific to drilling mud samples and were not found in any of the core samples. The PLFAs (9:0-ox-9, 20:1ω9t, 18:1ω9t-ep, 10:0-ox-DME, 10:1-DME, C18:0-10-ox, 10:0-DME, 8:0-DME) which were only present in the core samples and core washes are potential evidence of rock-hosted indigenous microbial lipid signatures. With the exception of Upper Marcellus Wash, the PLFA profiles of core wash samples were more similar to the cores than the drilling muds (Figure 4A). Because the PLFA Upper Marcellus Wash consisted of only two commonly detected fatty acids (C16:0 and C18:0) we determined that the sample was likely compromised during analysis. The low abundance lipid fatty acids (<10% relative abundance) PLFA functional groups such as the dimethyl, keto, and oxiranes were only present in the core samples and exhibited little variability (Figure 5A). The high abundance lipid fatty acids (>10% relative abundance) such as normal saturates and other low abundance fatty acids like monounsaturates and terminally branched were shared across all the samples (Figure 5A). However, individual PLFA biomarkers of the functional groups were not shared across all the

samples (Figure 4A). Lipid biomarkers that were shared across the drilling mud and core samples could be potential contaminants and as such were not considered in the interpretation of lipid microbial community composition. With the exception of one dimethyl lipid fatty acids which was identified near analytical detection limits (<0.18 ng) in Drilling Mud 9-08, dimethyl ester PLFAs were also unique to core samples.

Of the 38 DGFAs detected in the dataset, 29 (76.31%) were detected solely in the core samples and 9 (23.68%) were shared across all samples (Figures 3B, 4B). DGFA profiles like keto-, oxiranes, hydroxyl, branched saturates, and dimethyl lipid fatty acids were present only in the core samples while lipid biomarkers such as normal saturates, monounsaturates, terminally branched, and polyunsaturates were shared across all the samples (Figure 3B, 5B). The high abundance lipid fatty acids (>10% relative abundance) such as normal saturates and monounsauturates were only consistent within the drilling mud samples while other high abundance lipid fatty acids like the keto- and oxiranes were solely present in the core samples (Figure 5B). Like the PLFAs, the individual DGFA biomarkers of the functional groups were not shared across all the samples (Figure 3B, 4B). The profiles of core wash samples were considerably more similar to the cores than the drilling muds (Figure 4B).

As can be seen in figure 6, the distribution of the functional group lipid profiles was also different in the cores and drilling mud samples. The NMDS analysis showed differences in the distribution of PLFA and DGFA functional group patterns across two dimensions (Figures 6A, B). Terminally branched and monounsaturated PLFAs were significantly correlated to lipid distribution in the drilling mud samples, while oxiranes were significantly correlated to lipid distribution in the core samples (Figure 6A). The polyunsaturated and monounsaturated DGFAs were significantly correlated to drilling muds while core samples were predominantly driven by

oxiranes, keto-, and terminally branched DGFAs (Figure 6B). We also estimated variations in ratios of signature lipid biomarkers like the Gram (+): Gram (-) and the saturated:monounsaturated lipid fatty acids between the cores and drilling muds. The ratio of Gram (+): Gram (-) lipid biomarkers were higher in the core samples relative to the drilling mud samples (Figure 7A, B). The core samples also showed higher saturated:monounsaturated ratios compared to the drilling mud samples (Figure 7C, D).

DISCUSSION

Lipid Biomarkers Recovered in Drilling Muds and Core Samples

In accordance with appropriate sampling protocols[63, 64, 77, 78] and to rule out any microbial contamination during sample collection and processing, we compared the yield and diversity of lipids (estimated as the number of individual lipids in each sample) detected in the cores to the drilling muds and core washes. We found considerably higher concentrations of PLFA and DGFA in the drilling mud samples relative to the cores samples (Figure 2). Despite the high lipid concentration in drilling muds, the diversity of PLFA and DGFA profiles in the drilling muds and core samples were substantially different (Figures 3, 4, and 5), a strong indication that the biomarkers were probably sourced from indigenous subsurface microbial community. Based on the average standard deviation (0.07%) calculated from the BacFAME methyl ester standard, the variance in GC detection had minimal influence on the variability between PLFA and DGFA results. Our data further revealed that the polyunsaturates which are indicators of microeukaryotes, [79-81] were prominent in drilling mud samples but were not detected in the core samples (Figures 3, 5). The absence of polyunsaturates in the core samples highlights the fact that the drilling mud components had limited influence on the integrity of the lipid profiles of the core samples. If the

drilling muds were major contributors to the core lipid profiles one would not expect the remarkable differences in the diversity of the profiles between the cores and drilling mud samples. Most of the lipid biomarkers detected in the cores were representatives of Gram (-), Gram (+), as well as stress indicative biomarkers. The iso-, anteiso-, terminally branched, and mid-branched lipid fatty acids have been associated with Gram(+) bacteria,[82-83] while the monounsaturates have been associated with Gram(-) bacteria.[15, 84] The presence of Gram(+) and Gram(-) microbial communities in the drilling muds is consistent with 16S rRNA gene biomarker analysis from Marcellus shale wells[27, 31] as well as studies conducted on drill mud samples collected from boreholes in the Barnett Shale.[85] Similarly we suggest that the lipid profiles in the drilling mud samples could potentially represent a mix of drilling mud and native subsurface microbial signatures.

Lipid Biomarker Distribution and Implications of Subsurface Microbial Life

Lipid biomarkers have been used as essential proxies to monitor the microbial ecology of natural subsurface environment where most bacterial species are uncultured.[86- 89] Membrane lipid biomarkers do not, however, possess the taxonomic specificity of other -omic-based techniques like DNA analysis. Instead of using the membrane lipid fatty acids as chemotaxonomic tools, we have used the concentration and diversity of the individual profiles to understand microbial membrane distribution along the different lithologic gradients. The higher concentration and diversity of PLFA biomarkers in the Marcellus Shale Top (targeted to be the Marcellus/Mahantango interface) compared to the Mahantango Formation and the upper Marcellus Shale zone (Figure 2) could be a result of organic matter content. The organic carbon concentrations in all our core samples exceeded 3.5% (Figure 1D), and though the lability can vary widely between different shale formations,[90-92] this abundant organic matter may have served as

carbon substrate for deposited microorganisms and influenced microbial dynamics before and during diagenesis. The permeability of the Mahantango Formation estimated to be 6 millidarcy (mD), higher than the 2.5 mD in Marcellus Shale Top and the Upper Marcellus Shale, [93] could also have influenced microbial presence and activity. Previous microbial and geochemical investigations in shale/sand interfaces have also demonstrated higher subsurface microbial activity and biomass in the shale/sand contact[48] fractured zones, [6] organic-filled matrix voids[30, 92]and zones of higher permeability, [8, 48, 49]due to increased nutrient diffusion across interfaces.

Unlike the PLFA, the DGFA concentration did not coincide with high organic matter content. We did not expect a similar distribution for PLFA and DGFA biomarkers as other factors like diagenesis, redox conditions, salinity could affect the distribution of the PLFA and DGFA (by affecting the rate of cell death or rate of PLFA to DGFA conversion.[8, 10, 30, 39, 90, 91, 92] PLFA and DGFA therefore represent different microbial communities with DGFAs being more stable and less polar as compared with PLFAs.[5, 8, 15, 39, 45] Although PLFAs are generally known to be fragile, the rate of PLFA to DGFA conversion in subsurface shales has not been characterized. [10], argued that severe water-limited environments could impede dephosphorylation of PLFA, raising uncertainties about PLFA hydrolysis in such geological settings. Because PLFA dephosphorylation requires water, the rate of PLFA to DGFA conversion in deep shale may be considerably lower than shallow terrestrial systems with a higher degree of saturation. Furthermore, under conditions of low permeability and limited nutrient supply, microbes are known to adopt various states of dormancy which can leave the lipid signature from subsurface microbial communities unchanged for several million years.[7, 8, 94-96]

Long-term preservation of PLFAs and DGFAs may also derive from their interactions with the shale.[97-99] Black shales are made up of complex organic matter matrices and clay mineralogy,

capable of interacting with intact phospholipids fatty acids,[100-103] thereby hindering microbial and chemical degradation.[104-106] In addition to preservation, higher DGFA concentrations in subsurface samples could also be related to biological inactivity, long-term sequestration, or nutrient deprivation associated with the deep subsurface,[107-108] although this study did not directly assess these environmental conditions.

Lipid Biomarkers as Indicators of Environmental Conditions

Microbes are able to adjust their cell membranes to adapt for survival under stressful conditions or environmental disturbance associated with natural environments.[5,109-113] For instance, microbes are known to synthesize the more stable *trans*-monoenoic fatty acids, alter the *cis*-fatty acids to their cyclopropyl derivatives or increase levels of unsaturation during starvation.[5,79, 114-116] The *trans*-configured monounsaturates in the core samples (Figures 3, 4) suggest environmentally stressful conditions such as physiological stress or nutrient deprivation.[5,113] As mentioned earlier, some of the PLFA *trans*-membrane lipid profiles were also detected in the drilling mud samples (Figure 4A) and were not considered to be indigenous to the core samples. Among the *trans*-membrane lipid fatty acids in the cores, the highest concentration and diversity occurred within the DGFA profiles as opposed to the PLFA profiles (Figures 3, 4, and 5). A possible explanation for the relatively higher concentration and diversity of the *trans*-lipid biomarkers in the DGFA than the PLFA profiles can be attributed to the fact that the DGFA biomarkers represent non-living microbial signatures, and as such could have experienced more stressful environmental conditions compared to the PLFA biomarkers which represent relatively recent microbial communities. An alternative explanation is that the microbial community responded to environmental stress through cell death resulting in a higher concentration of DGFA stress biomarkers and modifications to cellular membranes.

The PLFA and DGFA profiles of the core samples also contained the oxiranes, keto-, and dimethyl lipid fatty acids which were not present in the drilling mud samples (Figures 3, 4, and 5). The NMDS analyses (Figure 6), further illustrates the functional group differences between the core samples and drill mud samples. The presence of oxiranes has been associated with both salt stress and nutrient limitation.[117-118] Oxiranes are produced from the oxidation of unsaturated fatty acids under stress in the presence of radical oxygen species (ROS) or from lipid monooxygenases.[117] The presence of oxiranes have previously been reported in a deep subsurface gold mine in Witwatersrand Basin in South Africa.[15, 119] Notably, both the Witwatersrand Basin and the Marcellus Formation are chemically reducing environments with little or no molecular oxygen necessary for the monooxygenase mechanism. However, authors[15] suggested that the decay of naturally occurring radioactive material (NORM) could generate ROS needed for the formation of oxiranes. The high NORM levels in the Marcellus Shale samples, [120-122] (Figure 1D), support the possibility of a similar mechanism for the generation of oxiranes. Researchers[118] also argued that microbes in deep subsurface systems could utilize non-radioactive H_2 generated by the radiolysis of water to sustain microbial activities. Like the oxiranes, keto-lipids can also be formed by free radicals and ROS.[123] Their occurrence in the core samples could, therefore, be indicative of microbial response to stress and their mechanism of formation can be explained in a similar mechanism as the oxiranes. Similar to the *trans*-configured lipid fatty acids, the concentration and diversity of oxiranes and keto-lipids were higher in the DGFA than the PLFA biomarkers, further supporting the notion that the dead microbial community represented by the DGFAs experienced relatively more stressful environmental conditions compared to the recent microbial community represented by the PLFA.

Dimethyl lipid features in microbial membranes of thermophiles[124-125] and acidophiles[126] have also been reported. Given the projected Marcellus Formation temperature of ~70ºC, [127] microbes must adapt to survive such extreme conditions. It is suggested that dimethyl lipid fatty acids can covalently link polar membrane glycerol groups[128] spanning across cell membranes [125] thereby reducing membrane permeability and enhancing membrane resistance to heat and acid. Additionally, membrane lipid fatty acids can incorporate an additional methyl group into their structures to congest the hydrophobic region of their bilayers (membrane bulking), thereby limiting ion leakage across the membrane under heat stress, which in turn conserves energy.[129-131] The rationale is that by reducing the permeability, the microbes are able to restrain the diffusion of ions thus conserving energy under elevated temperatures.[132-133] These observations in combination with our results suggest that the dimethyl lipid fatty acids in core samples may be related to physiological adaptation to increasing temperatures, acidity, and perhaps salinity. Similar to other stress indicative biomarkers, we also observed more diversity of dimethyl lipid fatty acids among the DGFA profiles than in the PLFA profiles.

The higher ratio of Gram (+) to Gram (-) biomarkers in the core samples compared to the drilling mud samples (Figure 7A, B), could also be related to the stressful environmental conditions. Gram (+) bacteria have thicker peptidoglycan layer in their cell wall which could aid survival under environmental stress and pressure.[134-135] Many spore-forming bacteria are Gram (+) and are known for their extraordinary ability to persist and survive environmental stresses such as heat, UV radiation, gamma radiation, lack of nutrients, and desiccation. As a result, the high abundance of Gram (+) lipid biomarkers in our samples may be attributed to membrane adaptation to environmental stress. Other physiological adaptations to persist under extreme environmental conditions include the ability for microbes to decrease unsaturation levels in response to elevated

temperatures and lack of nutrients.[114,131,136,137,138] In addition, there is experimental evidence suggesting that the ratio of saturated:unsaturated lipid fatty acids increase with increasing levels of nutrient deprivation in bacteria[5]. By increasing levels of saturation, the lipid membranes are rendered less mobile and less fluidized, thereby hindering unnecessary ion cycling, and thus conserving energy.[138] It is, therefore, possible that the relatively high ratios of saturated: unsaturated lipid biomarkers in our samples (Figure 7C, D), is related to physiological adaptations for limited energy and/or nutrient resources.

Summary

Irrespective of whether microbial lipid signatures in our samples represent dead, dormant, or active microbial communities, any given microbial cell (ancient or modern) must adjust its cell membranes to the *in situ* subsurface conditions. Even though studies have suggested that PLFA and DGFA biomarkers in the deep subsurface shale cores could be associated with sediments at the time of deposition and early diagenesis[6, 8, 10, 64] we are unable to say with certainty if the lipid profiles in these cores were associated with the sediments during deposition or were re-colonized. These indications of indigenous microbial life in deep shale like the Marcellus Shale accords enormous opportunities for the improvement of biocides in the shale energy industry, understanding subsurface microbial colonization, and engineering efforts for enhanced gas recovery.

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Figure Captions

Figure 1. Map showing the approximate location of the Marcellus Shale Energy and Environment Laboratory (MSEEL) in West Virginia, USA (A), MSEEL well design with four producing wells and scientific well (B), sidewall coring locations (C), generalized schematic of stratigraphy, total organic carbon content in the Mahantango, Marcellus Shale Top, and Upper Marcellus Shale zones (D).

Figure 2. PLFA and DGFA yields (detection limit <0.18 ng) in the Mahantango, Marcellus Shale Top, Upper Marcellus Shale, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 9-03 (2015) and Drilling Mud 8-28 (2015) samples.

Figure 3. Venn diagram illustrating the distribution of the individual and functional group lipid biomarkers for the PLFA (A, C) and DGFA (B, D) within the core samples, core washes, and drilling mud samples. The figure shows the lipid fatty acids detected only in the cores, and those shared across the drilling muds and core samples.

Figure 4. Dendrogram and heat map distribution of individual PLFA (A) and DGFA (B) biomarkers in the Mahantango, Marcellus Shale Top, Upper Marcellus Shale, Mahantango Wash, Marcellus Top wash, Upper Marcellus Wash, Drilling Mud 8-28 (2015), and Drilling Mud 9-03 (2015) samples together with common lipid structures. Samples are sorted based on dendrogram groupings calculated from Bray-Curtis dissimilarities.

Figure 5. Relative abundance and distribution of PLFA (A) and DGFA (B) biomarker functional groups for the Mahantango, Marcellus Shale Top, Upper Marcellus Shale zones, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 8-28 (2015) and Drill Mud 9-03 (2015) samples.

Figure 6. Nonmetric multidimensional scaling (NMDS) plot showing PLFA (A) and DGFA (B) functional groups of core samples (circles), drilling muds (triangles), and core washes (squares). NMDS is performed based on Bray-Curtis dissimilarities of the DGFA and PLFA relative abundances. The vectors which correspond to PLFA and DGFA classes and significantly correlate ($p<0.05$) with the samples were plotted from the origin.

Figure 7. Biomass yields showing ratio of Gram(+)/Gram(-) lipid biomarkers and ratio of saturated/unsaturated lipid biomarkers for the PLFA (A and C), DGFA (B and D), in the Mahantango, Marcellus Shale Top, Upper Marcellus Shale, Mahantango Wash, Marcellus Top wash, Upper Marcellus Wash, Drilling Mud 8-28 (2015), and Drilling Mud 9-03 (2015) samples.

Table 1.1 Individual PLFA concentrations (DL<0.18 ng) and relative mole percentages in the Mahantango, Marcellus Shale Top, Upper Marcellus Shale, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 9-03 (2015), and Drilling Mud 8-28 (2015) samples.

	Mah	Mah	Mar Top	Mar Top	U Marc	U Mar	Mahan Wash	Mahan Wash	Ma Top Wash	Ma Top Wash	U Mar Wash	U Mar Wash	D Mud 9.03	D Mud 9.03	D Mud 8.28	D Mud 8.28
PLFA	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**	**pmol/g**	**mol%**
16:0 cyclo	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.4	1.79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
16:1w7t cyclo	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3	20.6	0.4	0.1	N.D.	N.D.
C10:0-DME	0.2	0.7	0.3	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C9:0-DME	1.4	5.9	4.0	4.8	1.6	2.9	5.1	2.7	1.3	1.74	N.D.	N.D.	N.D.	N.D.	0.1	N.D.
C10:0-DME	N.D.	N.D.	0.4	0.5	0.2	0.4	0.6	0.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C10:0-ox-4-DME	0.1	0.6	1.7	2.0	1.1	2.0	3.9	2.1	8.8	11.70	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C18:1ω9t-ep	1.4	6.0	8.4	10.1	6.1	11.0	20.8	11.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C9:0-9ox	0.4	1.7	2.0	2.4	1.5	2.7	5.0	2.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C18:0-10ox	1.2	5.0	6.3	7.6	4.0	7.2	14.0	7.4	6.9	9.13	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C16:1w9c	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.5	0.3	1.1	0.4
C16:1w7t	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	10.6	2.2	6.7	2.3
C18:1ω9t	0.1	0.2	0.1	0.2	0.2	0.4	1.6	0.9	1.3	1.67	N.D.	N.D.	40.6	8.5	24.1	8.4
C18:1ω7t	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	11.1	2.3	5.9	2.0
C20:1ω9c	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.4	0.3	0.6	0.2
C20:4ω6c	0.2	0.9	1.5	1.8	1.0	1.8	3.6	1.9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C12:0	2.1	8.8	3.5	4.2	0.8	1.5	2.5	1.3	N.D.	N.D.	N.D.	N.D.	0.1	0.0	0.1	N.D.
C14:0	0.3	1.4	1.4	1.7	0.9	1.7	3.1	1.7	0.6	0.74	N.D.	N.D.	3.5	0.7	2.3	0.8
C15:0	N.D.	N.D.	0.2	0.2	0.1	0.2	0.6	0.3	N.D.	N.D.	N.D.	N.D.	1.0	0.2	0.7	0.2
C16:0	7.1	30.1	25.1	30.2	17.8	32.2	58.1	30.8	21.7	28.80	0.3	18.8	74.6	15.7	43.6	15.2
C17:0	0.1	0.60	0.7	0.8	0.5	1.0	1.8	1.0	1.1	1.46	N.D.	N.D.	6.8	1.4	4.0	1.4
C18:0	8.6	36.9	25.5	30.7	17.9	32.4	62.6	33.2	30.6	40.51	1.0	60.6	245.4	51.5	148.0	51.5
C20:0	0.2	0.6	1.0	1.2	0.8	1.4	2.8	1.5	0.9	1.22	N.D.	N.D.	11.1	2.3	7.5	2.6
C21:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.9	0.2	0.6	0.2
C23:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3	0.3	1.0	0.3
C24:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.6	1.0	3.3	1.1

C25:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.9	0.2	0.7	0.3
C26:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.8	0.4	1.6	0.6
C16:3ω4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	5.7	1.2	4.9	1.7
C18:2ω6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	18.6	3.9	7.1	2.5
C20:2ω6t	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.2	0.2	0.8	0.3
iC14:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3	0.1	0.2	0.1
iC15:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	14.6	3.1	9.7	3.4
aC15:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	11.0	2.3	7.1	2.5
iC17:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.6	1.0	3.4	1.2
aC17:0	0.2	0.6	0.8	1.0	0.6	1.07	2.0	1.1	0.9	1.25	N.D.	N.D.	2.2	0.5	1.6	0.6
iC18:0	N.D.	N.D.	0.3	0.3	0.2	0.33	0.6	0.3	N.D.	N.D.	N.D.	N.D.	0.5	0.1	0.3	0.1
Sum	**23.4**	**100**	**83.0**	**100**	**55.3**	**100**	**188.9**	**100**	**75.4**	**100**	**1.7**	**100**	**476.1**	**100**	**287.2**	**100**

Table 1.2 Distribution of PLFA functional group biomarkers in the Mahantango, Marcellus Shale top, and upper Marcellus Shale zones, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 9-03 (2015), and Drilling Mud 8-28 (2015) samples

Functional Group	Mah	Mar Top	U Mar	Mahan Wash	Ma To wash	U Mar wash	D. Mud 9.03	D.Mud 8.28
Normal Sats	78.4	69.1	70.3	69.7	72.7	79.4	73.9	74.3
MonoUnsats	0.2	0.2	0.4	0.9	1.7	N.D.	13.7	13.4
PolyUnsats	0.9	1.8	1.8	1.9	N.D.	N.D.	5.4	4.5
Keto	7.2	11.9	11.9	12.1	20.8	N.D.	N.D.	N.D.
Oxirane	6.0	10.1	10.9	11.0	N.D.	N.D.	N.D.	N.D.
TermBr	0.6	1.3	1.4	1.4	1.3	N.D.	7.0	7.8
DME	6.6	5.7	3.2	3.0	1.7	N.D.	N.D.	0.0
Cyclo	N.D.	N.D.	N.D.	N.D.	1.8	20.6	0.1	N.D.

Abbreviations: D.L = Detection Limit, Mah=Mahantango, Mar Top= Marcellus Shale Top, U Mar= Upper Marcellus Shale, Mahan Wash=Mahantango Wash, Ma Top Wash=Marcellus Top Wash, U Mar Wash=Upper Marcellus Wash, D Mud 9-03= Drilling Mud 9-03 (2015), D Mud 8-28= Drilling Mud 8-28 (2015) samples. Normal Sats= Normal Saturated, MonoUnsats= Monounsaturated, PolyUnsats= Polyunsaturated, TermBr= Terminally Branched, DME=Dimethyl ester, Cyclo= Cyclopropane lipid fatty acids.

Table 2.1 Individual DGFA concentrations (DL<0.18 ng) and relative mole percentages in the Mahantango, Marcellus Shale top, Upper Marcellus Shale, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 9-03 (2015), and Drilling Mud 8-28 (2015) samples

	Mahantang	Mahantang	Marce Top	Marc Top	Upper Marce	Upper Marce	Mahan wash	Mahan wash	MarTop Wash	MarcTop Wash	U Marce Wash	U Marce	D Mud 8:28	D Mud 8:28	D Mud 9.03	D Mud 9.03
DGFA	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**	**pmol/g**	**mol %**
c11:0	N.D.	N.D.	N.D.	N.D.	0.7	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.9	3.2	8.7	2.7
19:1ω1	0.6	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10:0-9-ox	0.5	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10:0-10-ox	0.8	0.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
c12:0	27.3	10.8	N.D.	N.D.	18.0	9.8	6.5	2.9	1.9	0.9	N.D.	N.D.	82.1	26.9	70.4	22.1
iC12:0	0.2	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
11:0ω-10-ox	0.5	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
c13:0	0.3	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.2	0.6	N.D.	N.D.	N.D.	N.D.	3.78	1.19
10:0-DME	2.4	1.1	0.1	0.1	1.4	0.8	1.0	0.4	1.0	0.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
c14:0	9.3	3.4	2.6	1.6	5.1	2.8	6.2	2.8	8.2	3.9	6.35	4.03	76.9	25.2	72.6	22.8
11:0-DME	2.1	0.7	0.2	0.1	0.8	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
15:0ω9-OH	1.4	0.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
16:1ω9	0.2	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
i15:0	0.4	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
a15:0	0.6	0.2	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.56	2.5	8.9	2.8
c15:0	2.5	0.8	1.2	0.7	1.07	0.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C16:0	44.2	14.4	36.3	22.4	43.0	23.5	52.4	23.6	61.3	29.1	56.9	36.1	28.4	9.3	40.4	12.7
C18:0	41.6	13.5	41.6	25.6	36.9	20.1	54.4	24.5	68.5	32.6	67.4	42.8	31.3	10.2	36.7	11.5
16:4ω1	0.6	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.0	2.8
iC16:0	0.5	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
16:1ω7t	1.6	0.5	0.2	0.15	0.5	0.26	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
16:1ω7-DME	1.3	0.1	N.D.	N.D.	2.3	1.23	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10:0-4-DME	11.4	3.7	4.2	2.6	4.4	2.42	1.60	0.72	2.54	1.21	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
iC17:0	0.3	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

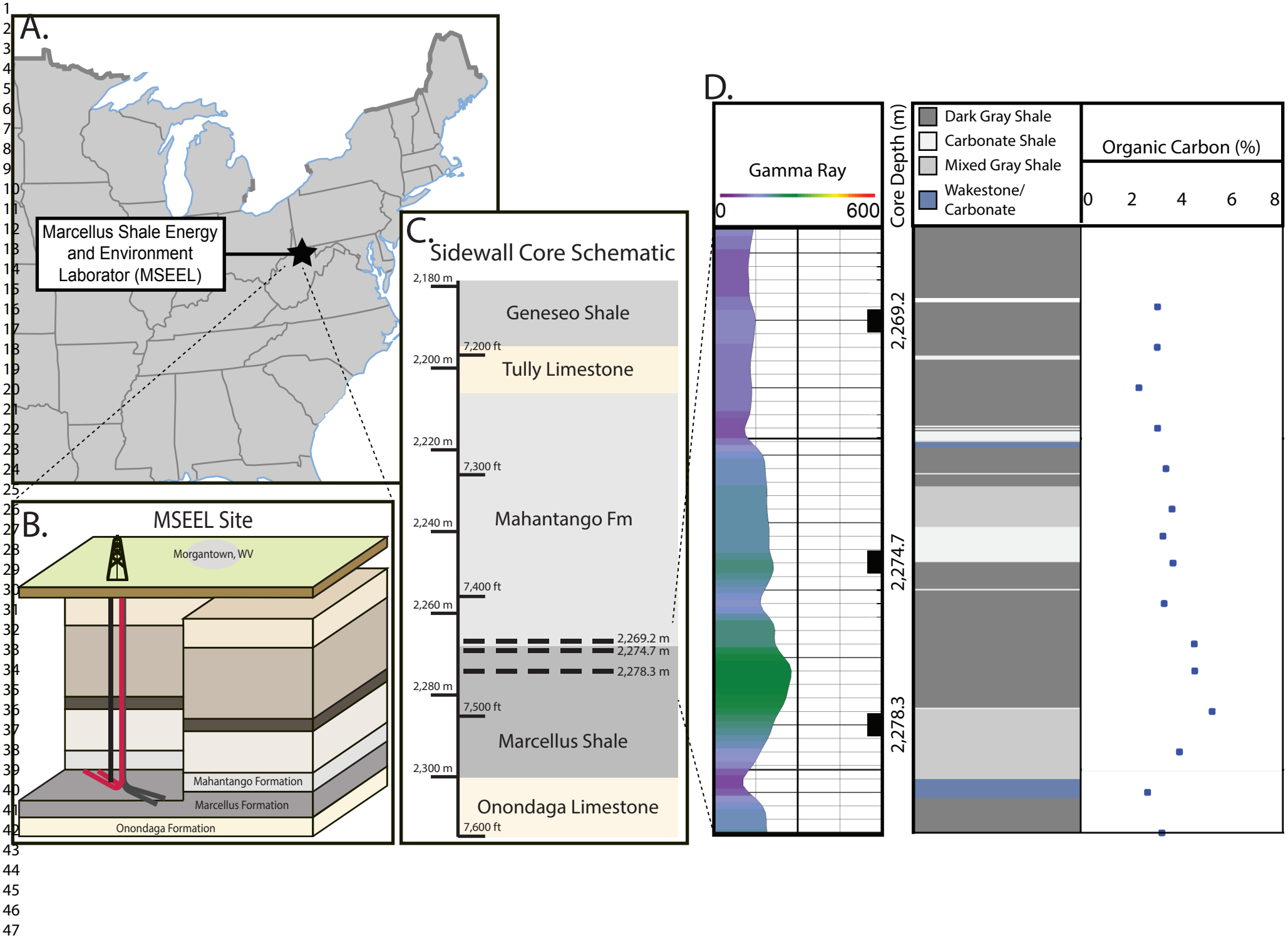
aC17:0	6.2	2.0	0.7	0.4	2.7	1.5	4.1	1.9	3.9	1.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C17:0	5.3	1.7	2.6	1.6	3.0	1.6	8.7	3.9	3.7	1.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:2ω6	1.3	0.4	N.D.	N.D.	0.6	0.3	4.9	2.2	8.4	4.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:1ω9t	4.1	1.3	0.6	0.4	4.0	2.2	29.9	13.4	14.8	7.0	19.0	12.0	69.4	122.7	68.36	21.5
18:1ω7t	0.9	0.3	0.2	0.1	N.D.	N.D.	4.1	1.9	3.6	1.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:2ω11	0.7	0.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10Me18:0	0.5	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:1ω9t-ep	31.7	0.2	31.5	19.4	26.6	14.5	20.1	9.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:1ω6c-ep	0.5	20.0	N.D.	N.D.	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:0ω9-ox	30.0	13.0	30.6	18.9	20.9	11.4	22.4	10.1	27.0	12.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:2ω6t	12.6	4.1	6.2	3.8	4.0	2.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:1ω9t	9.4	3.1	3.5	2.1	5.1	2.8	5.5	2.5	4.6	2.2	8.0	5.1	N.D.	N.D.	N.D.	N.D.
c20:0	4.0	1.3	N.D.	N.D.	1.3	0.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Sum	**258.0**	**100.0**	**162.2**	**100.0**	**183.2**	**100.0**	**221.9**	**100.0**	**210.5**	**100.0**	**157.5**	**100.0**	**305.5**	**100.0**	**318.6**	**100.0**

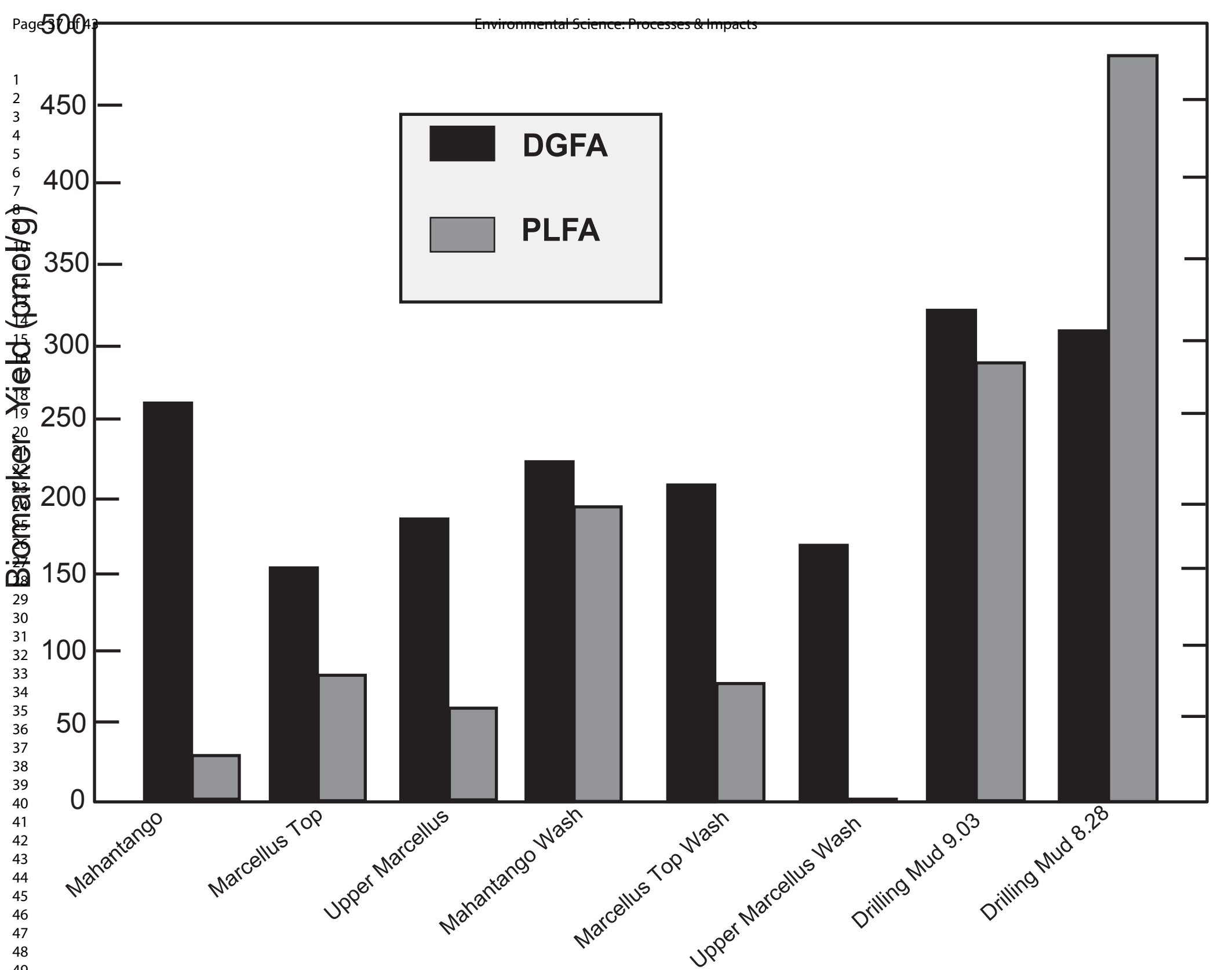
Table 2.1 Distribution of DGFA functional group biomarker in the Mahantango, Marcellus Shale top, upper Marcellus Shale, Mahantango Wash, Marcellus Top Wash, Upper Marcellus Wash, Drilling Mud 9-03 (2015), and Drilling Mud 8-28 (2015) samples.

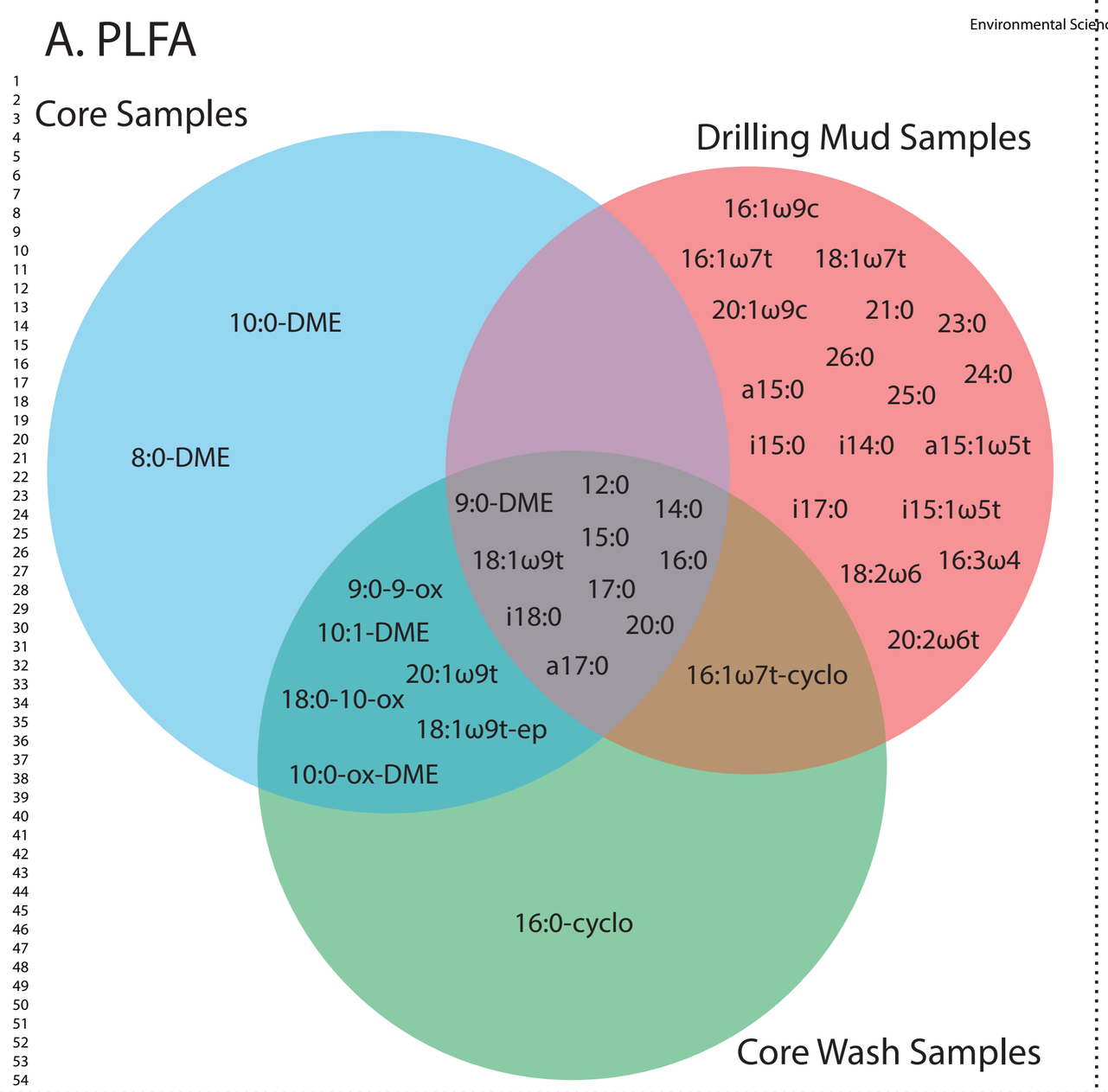
Functional Group	Mah	Mar Top	U Mar	Mahan Wash	Ma To Wash	U Mar Wash	D Mud 8:28	D Mud 9.03
normal sats	46.0	52.0	59.5	57.8	68.8	83.0	79.2	69.8
mono-unsats	5.5	2.8	5.2	17.8	10.9	17.1	17.5	24.0
term branched	2.7	0.4	1.5	1.9	1.8	N.D.	3.4	2.2
Poly unsats	5.3	3.8	3.1	2.2	4.0	N.D.	N.D.	4.0
hydroxy	0.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Oxiranes	20.2	19.4	14.5	9.1	N.D.	N.D.	N.D.	N.D.
Keto	13.6	18.9	11.4	10.1	12.8	N.D.	N.D.	N.D.
Branched Sats	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dimethyl Esters	5.6	2.8	4.9	1.2	1.7	N.D.	N.D.	N.D.

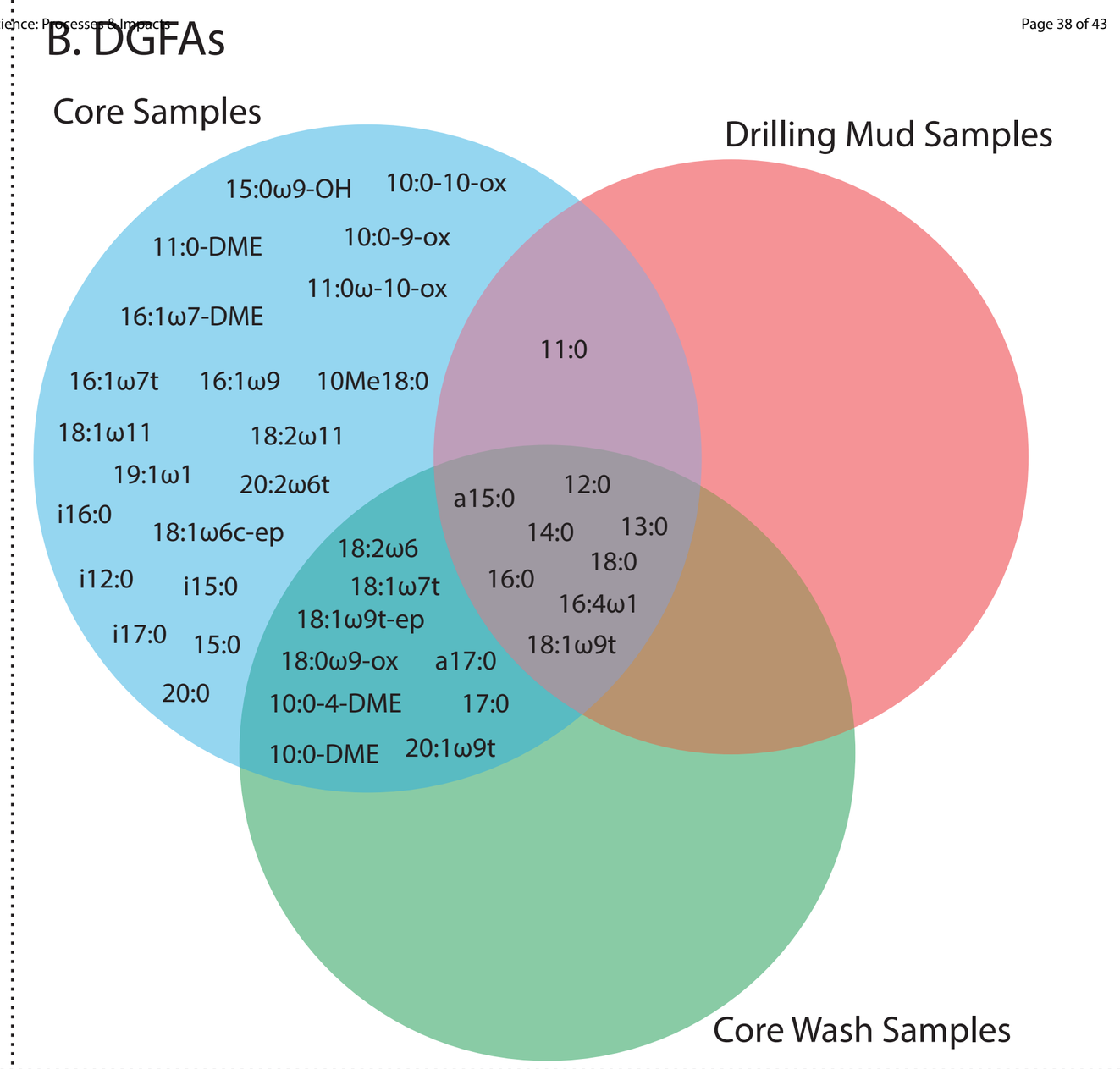
Abbreviations: Mah=Mahantango, Mar Top= Marcellus Shale Top, U Mar= Upper Marcellus Shale, Mahan Wash=Mahantango Wash, Ma Top Wash=Marcellus Top Wash, U Mar Wash=Upper Marcellus Wash, D Mud 8.28= Drilling Mud 8-28 (2015), D Mud 9-03= Drilling Mud 9-03 (2015) samples. Normal Sats= Normal

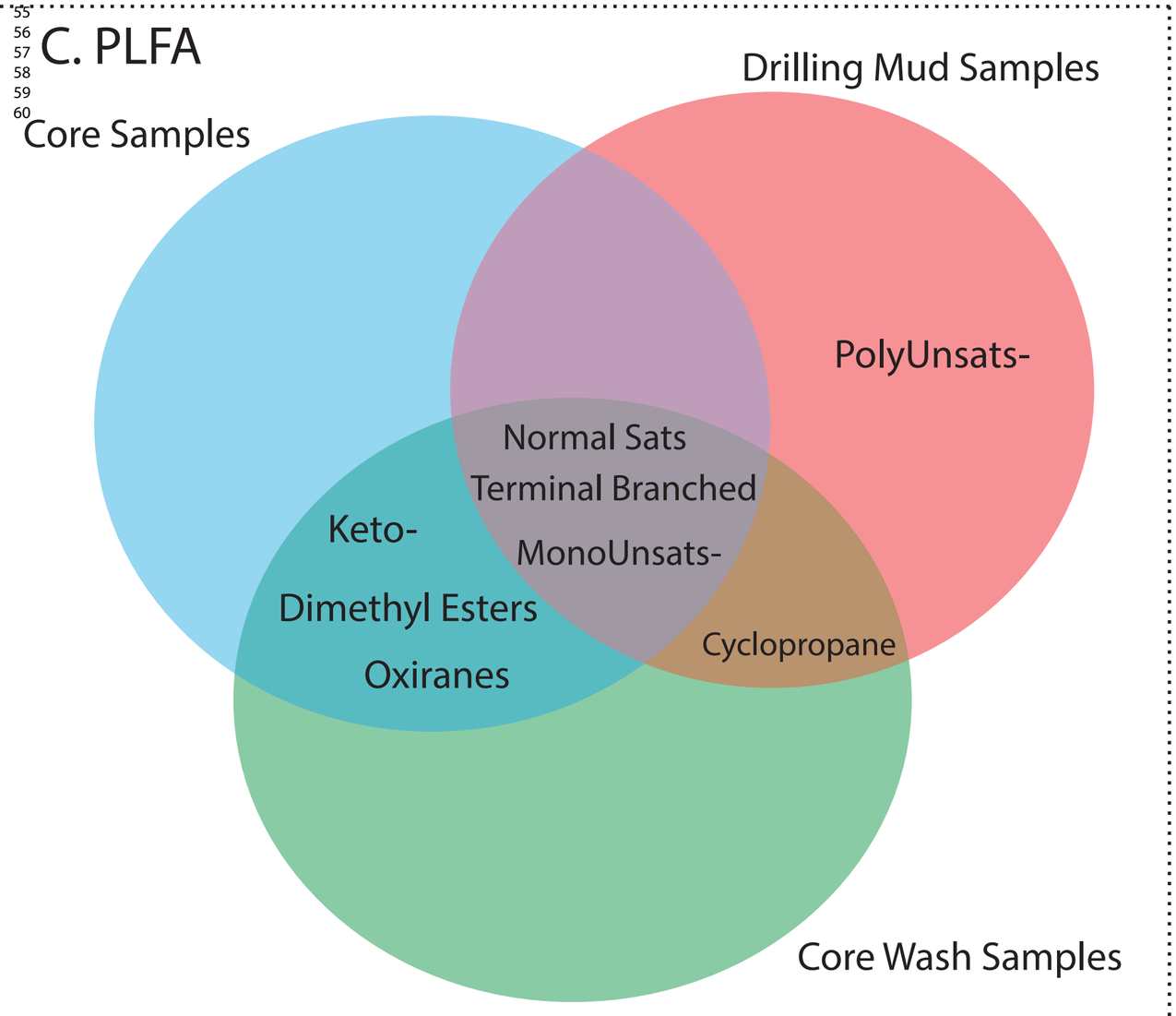
Saturated, MonoUnsats= Monounsaturated, PolyUnsats= Polyunsaturated, TermBr= Terminally Branched, DME=Dimethylester, Cyclo= Cyclopropane, Branched Sats= Branched Saturated, and hydroxyl= hydroxyl lipid fatty acids.

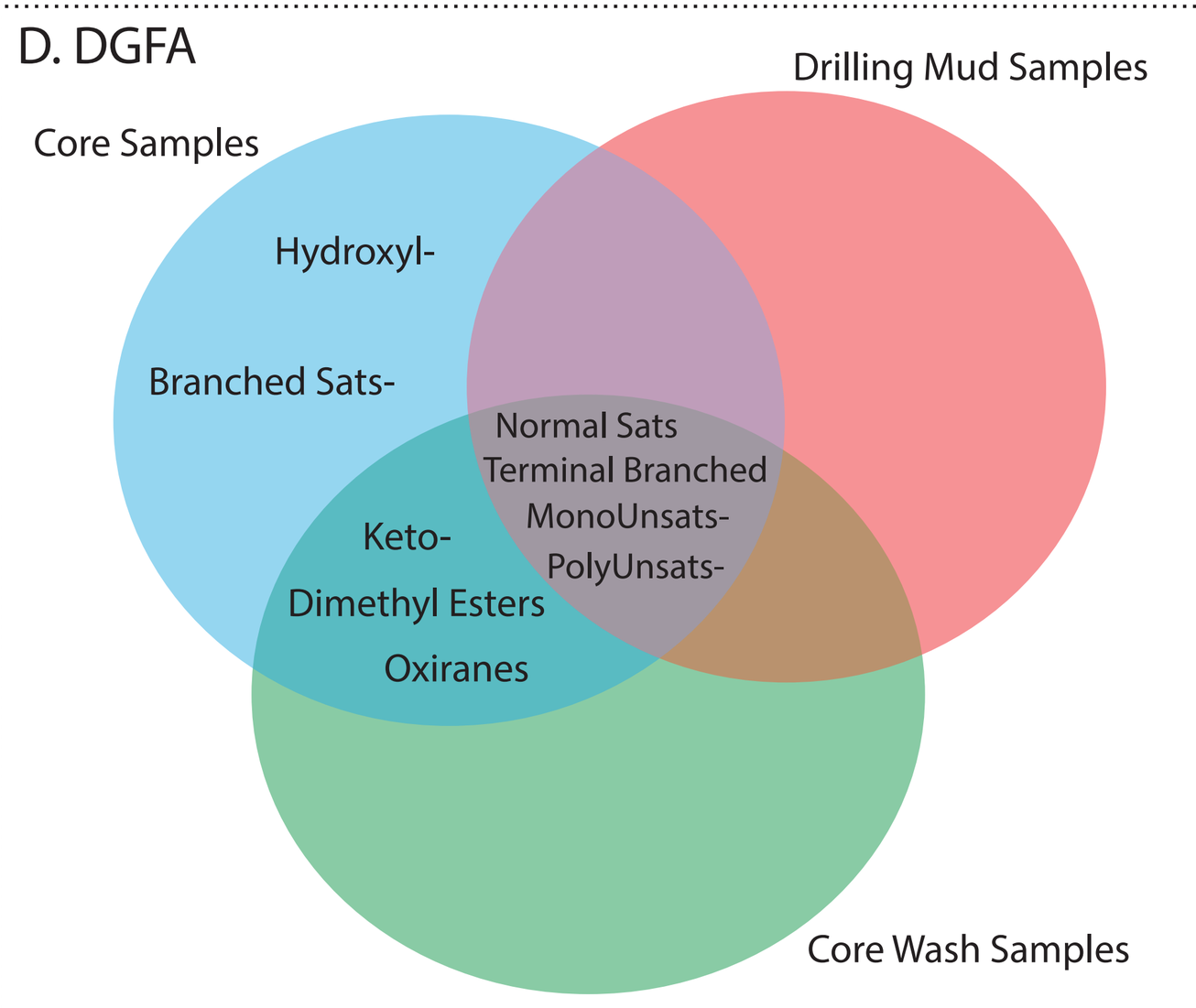

A.
Marcellus Shale Energy and Environment Laborator (MSEEL)
B.
MSEEL Site
Morgantown, WV
Mahantango Formation
Marcellus Formation
Onondaga Formation
C.
Sidewall Core Schematic
2,180 m
Geneseo Shale
7,200 ft
2,200 m
Tully Limestone
2,220 m
7,300 ft
Mahantango Fm
2,240 m
7,400 ft
2,260 m
2,269.2 m
2,274.7 m
2,278.3 m
2,280 m
7,500 ft
Marcellus Shale
2,300 m
Onondaga Limestone
7,600 ft
D.
Gamma Ray
0
600
Core Depth (m)
2,269.2
2,274.7
2,278.3
Dark Gray Shale
Carbonate Shale
Mixed Gray Shale
Wakestone/ Carbonate
Organic Carbon (%)
0
2
4
6
8

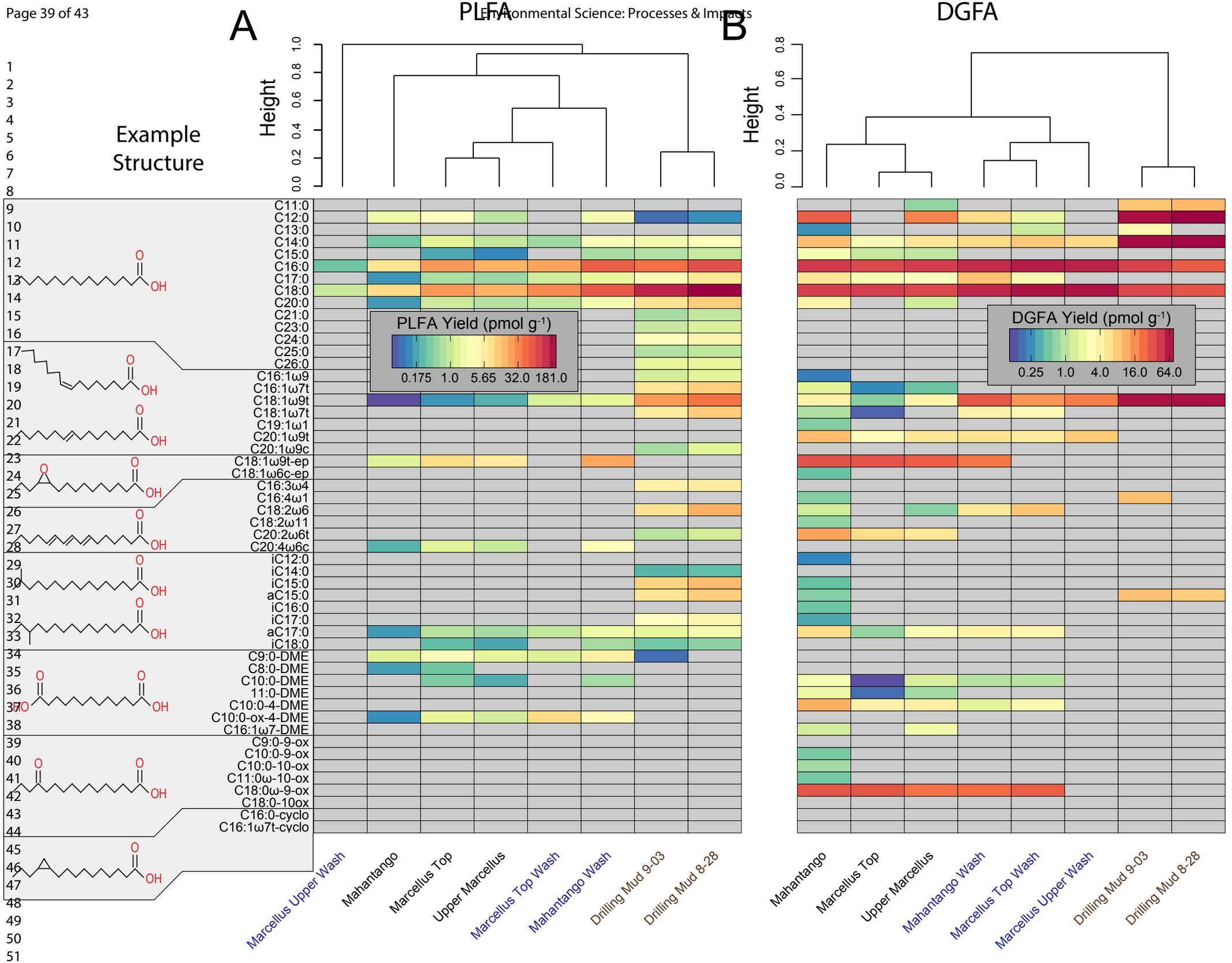

Biomarker Yield (pmol/g)
500
450
400
350
300
250
200
150
100
50
0
DGFA
PLFA
Mahantango
Marcellus Top
Upper Marcellus
Mahantango Wash
Marcellus Top Wash
Upper Marcellus Wash
Drilling Mud 9.03
Drilling Mud 8.28

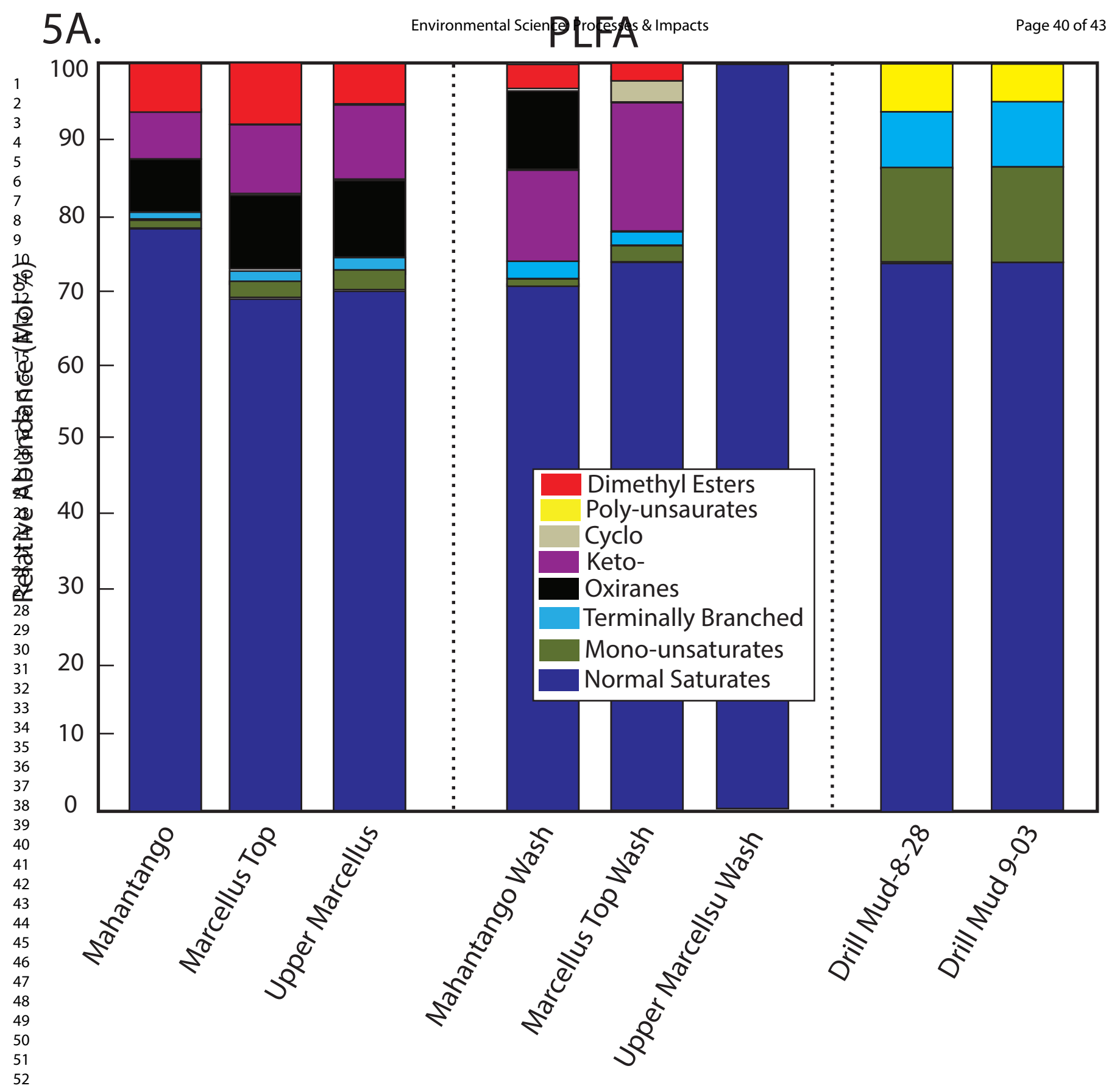

A. PLFA
Core Samples
Drilling Mud Samples
Core Wash Samples
10:0-DME
8:0-DME
9:0-DME
12:0
14:0
15:0
18:1ω9t
16:0
17:0
9:0-9-ox
i18:0
20:0
10:1-DME
a17:0
20:1ω9t
18:0-10-ox
18:1ω9t-ep
10:0-ox-DME
16:1ω7t-cyclo
16:0-cyclo
16:1ω9c
16:1ω7t
18:1ω7t
20:1ω9c
21:0
23:0
26:0
24:0
a15:0
25:0
i15:0
i14:0
a15:1ω5t
i17:0
i15:1ω5t
18:2ω6
16:3ω4
20:2ω6t

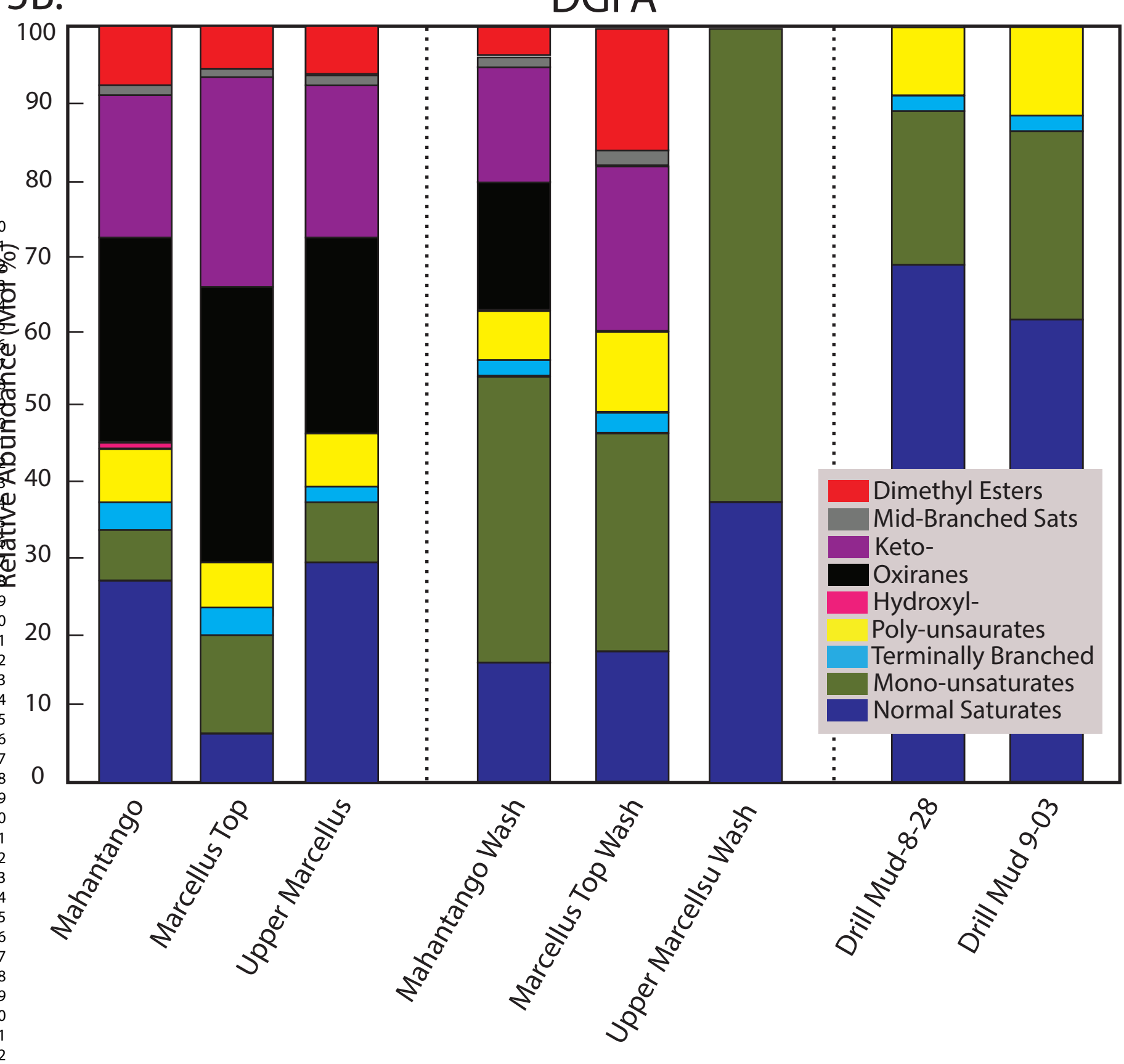

B. DGFAs
Core Samples
Drilling Mud Samples
Core Wash Samples
15:0ω9-OH
10:0-10-ox
11:0-DME
10:0-9-ox
11:0ω-10-ox
16:1ω7-DME
11:0
16:1ω7t
16:1ω9
10Me18:0
18:1ω11
18:2ω11
19:1ω1
20:2ω6t
i16:0
18:1ω6c-ep
i12:0
i15:0
i17:0
15:0
20:0
a15:0
12:0
14:0
13:0
18:0
16:0
16:4ω1
18:2ω6
18:1ω7t
18:1ω9t-ep
18:1ω9t
18:0ω9-ox
a17:0
10:0-4-DME
17:0
10:0-DME
20:1ω9t

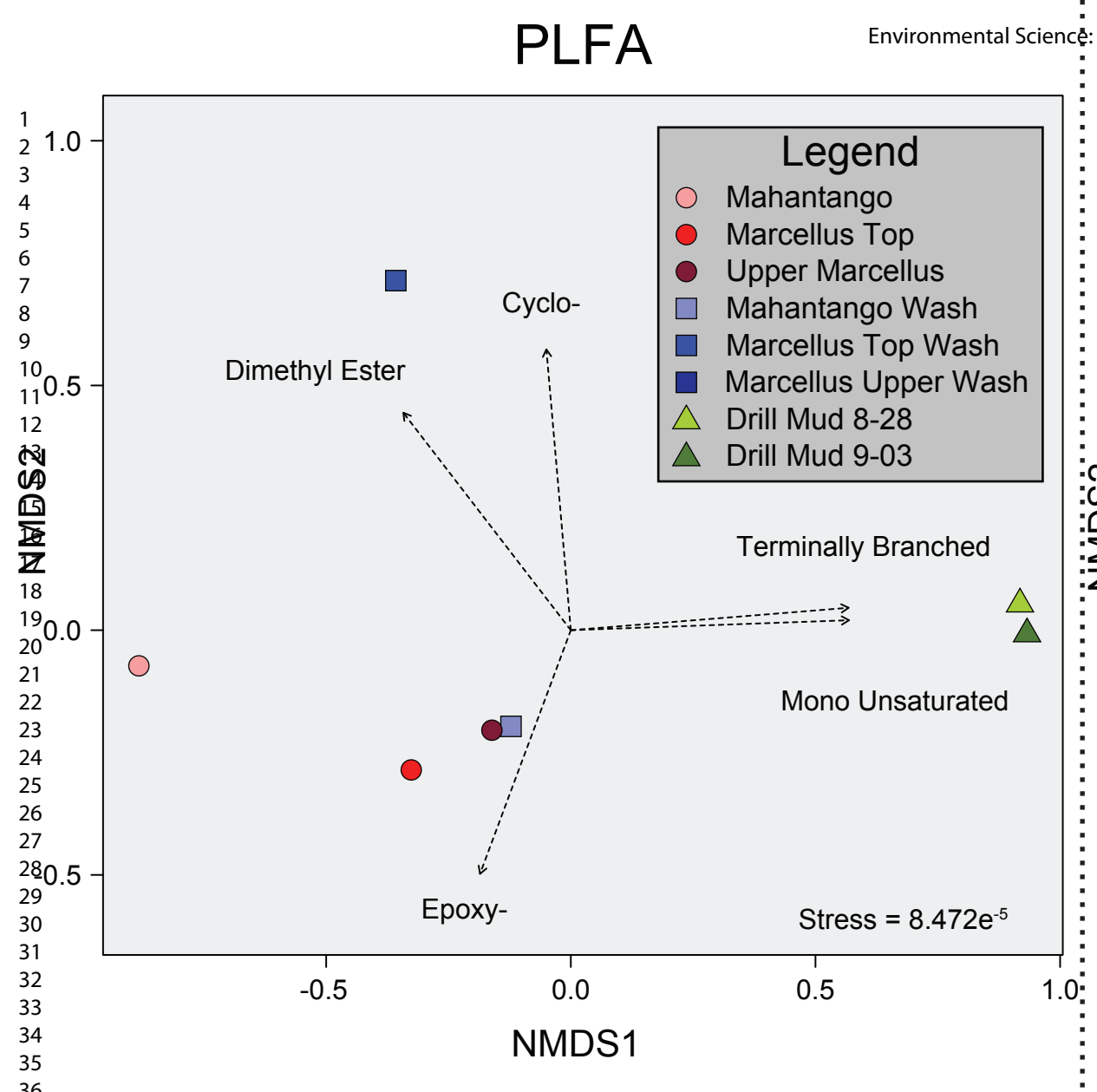

C. PLFA
Core Samples
Drilling Mud Samples
Core Wash Samples
PolyUnsats-
Normal Sats
Terminal Branched
MonoUnsats-
Keto-
Dimethyl Esters
Oxiranes
Cyclopropane

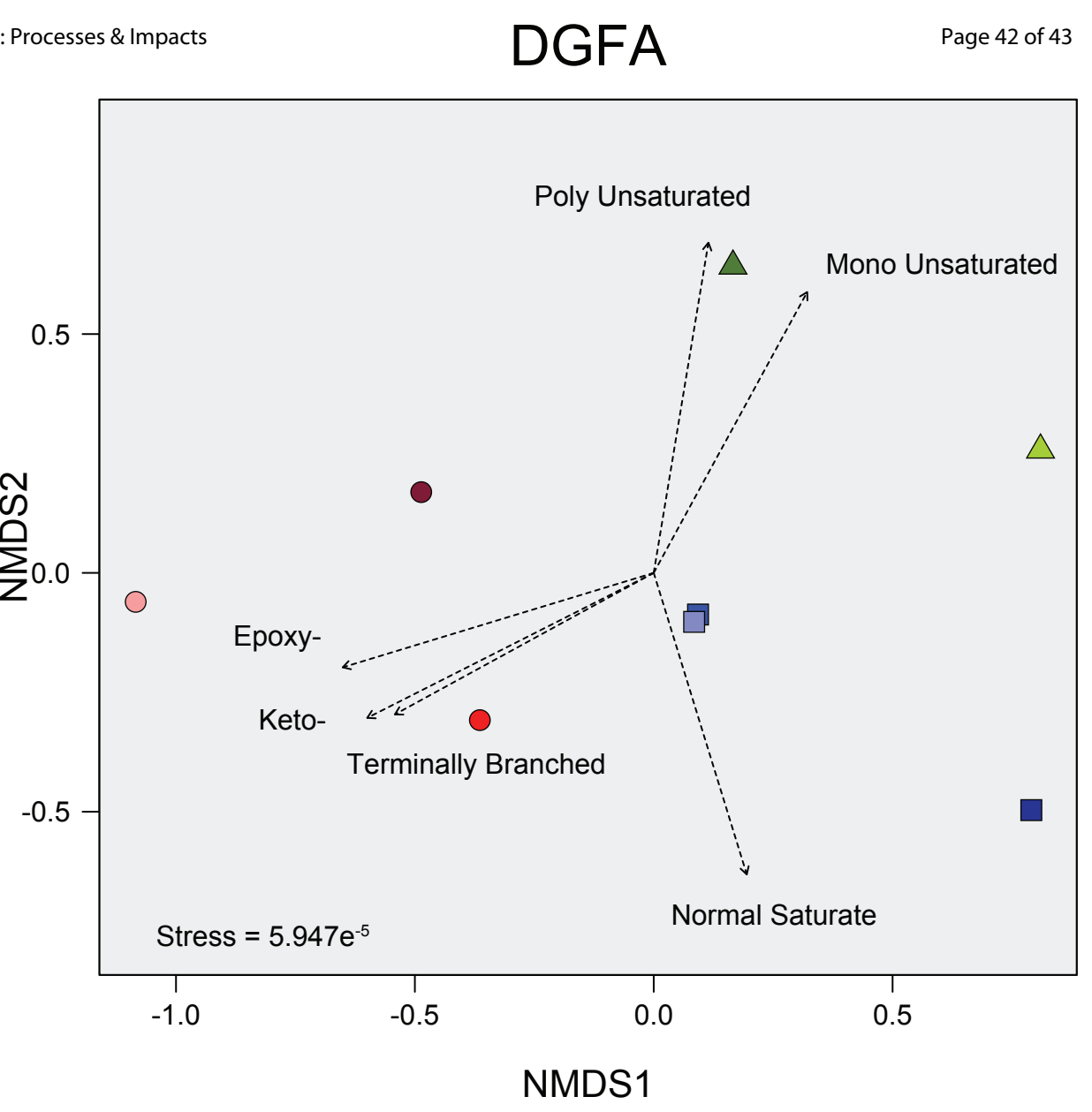

D. DGFA
Core Samples
Drilling Mud Samples
Core Wash Samples
Hydroxyl-
Branched Sats-
Normal Sats
Terminal Branched
MonoUnsats-
PolyUnsats-
Keto-
Dimethyl Esters
Oxiranes

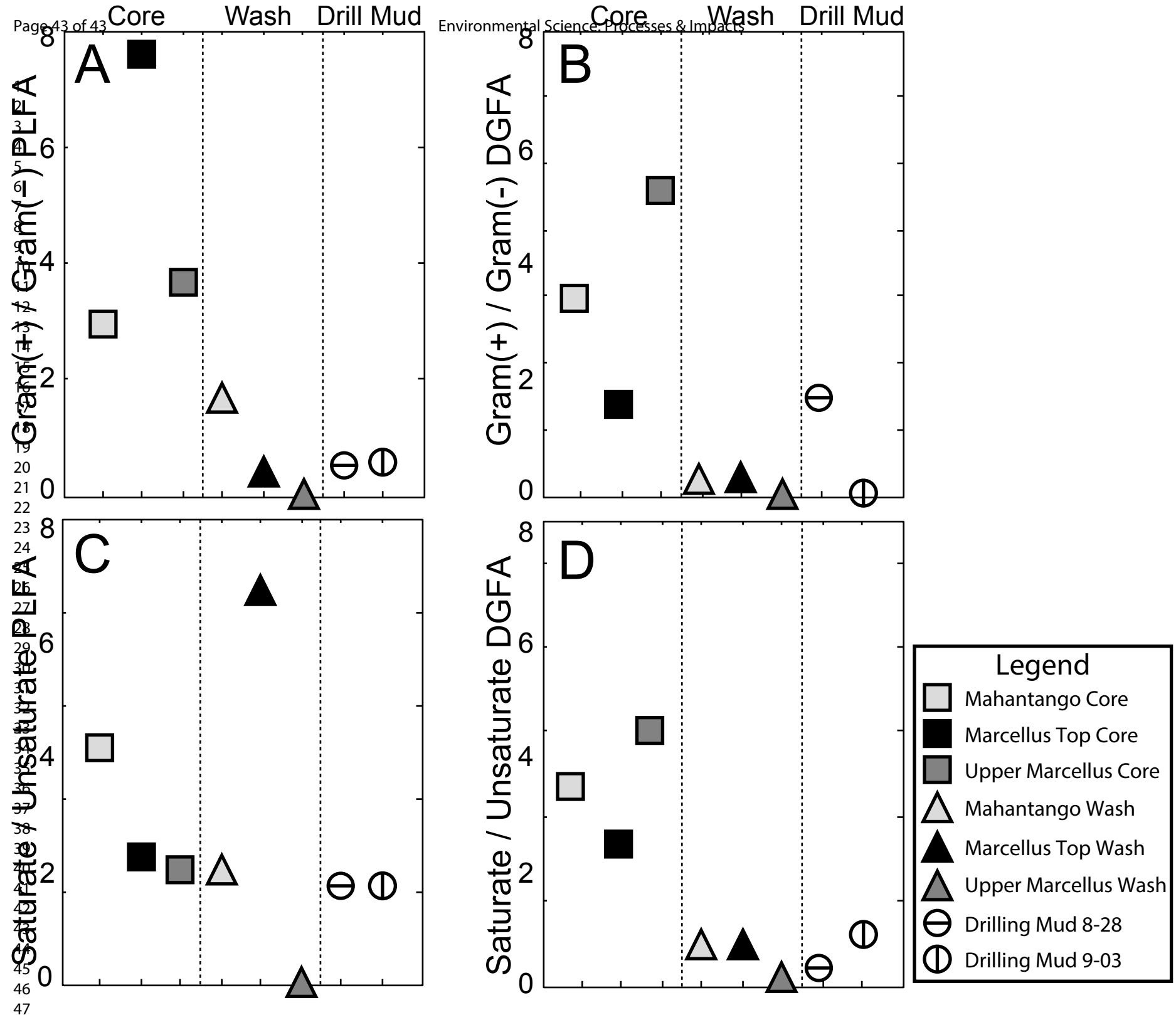

A
PLFA
B
DGFA
Height
Example Structure
PLFA Yield (pmol g-1)
0.175 1.0 5.65 32.0 181.0
DGFA Yield (pmol g-1)
0.25 1.0 4.0 16.0 64.0
C11:0
C12:0
C13:0
C14:0
C15:0
C16:0
C17:0
C18:0
C20:0
C21:0
C23:0
C24:0
C25:0
C26:0
C16:1ω9
C16:1ω7t
C18:1ω9t
C18:1ω7t
C19:1ω1
C20:1ω9t
C20:1ω9c
C18:1ω9t-ep
C18:1ω6c-ep
C16:3ω4
C16:4ω1
C18:2ω6
C18:2ω11
C20:2ω6t
C20:4ω6c
iC12:0
iC14:0
iC15:0
aC15:0
iC16:0
iC17:0
aC17:0
iC18:0
C9:0-DME
C8:0-DME
C10:0-DME
11:0-DME
C10:0-4-DME
C10:0-ox-4-DME
C16:1ω7-DME
C9:0-9-ox
C10:0-9-ox
C10:0-10-ox
C11:0ω-10-ox
C18:0ω-9-ox
C18:0-10ox
C16:0-cyclo
C16:1ω7t-cyclo
Marcellus Upper Wash
Mahantango
Marcellus Top
Upper Marcellus
Marcellus Top Wash
Mahantango Wash
Drilling Mud 9-03
Drilling Mud 8-28
Mahantango
Marcellus Top
Upper Marcellus
Mahantango Wash
Marcellus Top Wash
Marcellus Upper Wash
Drilling Mud 9-03
Drilling Mud 8-28


PLFA
Relative Abundance (Mol %)
100
90
80
70
60
50
40
30
20
10
0
Dimethyl Esters
Poly-unsaurates
Cyclo
Keto-
Oxiranes
Terminally Branched
Mono-unsaturates
Normal Saturates
Mahantango
Marcellus Top
Upper Marcellus
Mahantango Wash
Marcellus Top Wash
Upper Marcellsu Wash
Drill Mud-8-28
Drill Mud 9-03


5B.
DGFA
Relative Abundance (Mol %)
100
90
80
70
60
50
40
30
20
10
0
Dimethyl Esters
Mid-Branched Sats
Keto-
Oxiranes
Hydroxyl-
Poly-unsaurates
Terminally Branched
Mono-unsaturates
Normal Saturates
Mahantango
Marcellus Top
Upper Marcellus
Mahantango Wash
Marcellus Top Wash
Upper Marcellsu Wash
Drill Mud-8-28
Drill Mud 9-03


PLFA
Legend
Mahantango
Marcellus Top
Upper Marcellus
Mahantango Wash
Marcellus Top Wash
Marcellus Upper Wash
Drill Mud 8-28
Drill Mud 9-03
Cyclo-
Dimethyl Ester
Terminally Branched
Mono Unsaturated
Epoxy-
Stress = 8.472e-5
NMDS1
NMDS2
1.0
0.5
0.0
-0.5
-0.5
0.0
0.5
1.0


DGFA
Poly Unsaturated
Mono Unsaturated
Epoxy-
Keto-
Terminally Branched
Normal Saturate
Stress = 5.947e-5
NMDS1
NMDS2
0.5
0.0
-0.5
-1.0
-0.5
0.0
0.5


A
B
C
D
Core
Wash
Drill Mud
Gram(+) / Gram(-) PLFA
Gram(+) / Gram(-) DGFA
Saturate / Unsaturate PLFA
Saturate / Unsaturate DGFA
0
2
4
6
8
Legend
Mahantango Core
Marcellus Top Core
Upper Marcellus Core
Mahantango Wash
Marcellus Top Wash
Upper Marcellus Wash
Drilling Mud 8-28
Drilling Mud 9-03