PH EFFECTS ON SOLID PHASE EXTRACTABLE DISSOLVED ORGANIC MATTER: EXPANDING THE ANALYTICAL WINDOW

A Thesis

by

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Marine dissolved organic matter (DOM) is the largest exchangeable organic carbon pool, holding comparable amounts of carbon as CO_2 in the atmosphere and exceeding all biomass. DOM constituents are made up of thousands of unique organic compounds with astounding molecular diversity, featuring a wide range of hydrophobicity, size, and acidic or basic properties. Modern high resolution, high accuracy, and fast cycle time mass spectrometers can provide deep molecular insights into complex mixtures but require compatible samples. The inorganic matrix and low DOM concentrations have required organic biogeochemists to rely on Agilent Bond Elut Priority PoLutant (PPL) Solid Phase Extraction (SPE) to isolate and concentrate DOM through hydrophobic interactions. Currently, the standard SPE method has been optimized to maximize recovery? of dissolved organic carbon (DOC) through sample acidification and methanol elution. However, there is a lack of full understanding of the effect of adjusting the sample pH on the extraction efficiency of different DOM compounds.

This study investigated the effects of pH modification on the SPE recoveries and the effects of various procedures on the isolated DOM. This study collected water samples from three sites to represent different marine systems (Lavaca River, Baffin Bay and Gulf of Mexico) with unique sources and signatures of DOM. Samples were acidified to pH 2, kept at natural pH, basified to pH 10 or run sequentially where the permeate was further isolated. Various modified methanol elution solvents were tested, comparing methanol, acidic methanol, basic methanol, and combinations of both. The isolated DOM was chemically characterized in positive mode separated with reverse-phase high performance liquid chromatography (RP-HPLC) and in negative mode with Anion Exchange Ion Chromatography (AEX-IC) on an Orbitrap Fusion

Tribrid mass spectrometer (OT-FTMS) using data dependent acquisition (DDA). The standard SPE procedure with acidification yielded the highest recoveries but showed bias against dissolved organic nitrogen (DON). The samples that were not acidified, including natural pH or pH 10, yielded a different fraction of enigmatic DOM with a higher nitrogen percentage than the DOM isolates using the standard procedure. We found that through isolating DOM from a water sample at natural pH and pH 2, two fractions of DOM can be isolated, including hydrophobic DOM, acidic DOM, and basic DOM. These results suggest to better represent DOM from marine systems, collecting both fractions and analyzing in both positive and negative modes provide a more comprehensive and representative isolate of DOM.

DEDICATION

This is work is dedicated to my parents who have made this all possible. The hard work and sacrifices you have all made will never go unappreciated. Thank you, Dad, Mom, and Joe I could have never gotten here without you guys. I also dedicate this to all my crazy siblings, who are my favorite people in the universe.

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1. INTRODUCTION

1.1 General Biogeochemical Nature of Dissolved Organic Matter

Dissolved organic matter (DOM) makes up the largest global exchangeable organic carbon reservoir holding approximately 680 Gt carbon[1]. On a carbon weight basis, the DOM pool stores more carbon relative to the total of all living biomass[1-3]. DOM serves as the backbone of global organic carbon biogeochemical cycling as it consists of the labile microbial carbon currency that can be quickly utilized for microbial assimilation and catabolism, which mainly demineralize back to CO_2[4]. However, not all the DOM can be seen as a carbon currency for much marine life, as not all organic carbon is quickly shuttled through the biogeochemical carbon cycle. A significant fraction of DOM has been found to accumulate in a pool of 'refractory' carbon that persists for thousands of years[5-11]. The average age of deep ocean DOM is on the timescale of thousands of years, exceeding the thermohaline circulation time of the ocean, highlighting the important roles of stored carbon in the form of DOM can play on the global scale[12]. This justifies the importance of in-depth chemical characterization of DOM and research toward understanding mechanisms that control and regulate the inputs and outputs of carbon within this pool.

Not all organic carbon within the ocean is considered DOM or what is called true dissolved organic compounds. DOM is distinguished from particulate organic matter (POM) based on its ability to pass through a filter (usually from 0.1 μm to 0.7 μm), making it an operational distinction. This distinction excludes all known eukaryotic, most prokaryotic, and archaic organisms from DOM. POM can be sinking or suspended but in a constant state of aggregation (flocculation) and disaggregation processes leading to a shorter water column

residence time compared to DOM[13-15]. DOM may include viruses, colloidal, gel phase matter, aggregates, small particles, and truly dissolved matter.

DOM is ubiquitously found in all aquatic ecosystems like marine, estuaries, rivers, lakes, and groundwater, representing the broadest range of imaginable organic carbon compounds, even being described as the molecular universe[16]. Its immense diversity can be attributed to the many sources of DOM, from food web interactions, anthropogenic runoff, photochemical reactions, geological interactions, and cross reactivity between the DOM pool.

DOM can be produced autochthonously from within marine systems through in-situ primary production mostly from phytoplankton, mangroves, seagrasses, microalgae, marine chemoautotrophs, and heterokonts[17-21]. Not all autochthonous DOM is the direct result of primary production. Effects of ecological interactions like predation and parasitism are expected to release large amounts of organic matter. For example, zooplankton, who feed on phytoplankton, release DOM by feeding behaviors and excretion, resulting in increased levels of bioavailable DOM, like peptides[22]. Below the water column, marine sediments contain DOM in the small pockets of oxic/anoxic water between sediment particles (porewater). The DOM in marine sediments is found in high concentrations enough for continuous diffusion of organic matter into the water column adding approximately 350 Tg C yr^{-1}, a significant flux of DOM to the marine waters[23]. Petroleum seeps also release DOM where ancient 'pre-aged' carbon molecules can enter the sedimentary DOM pool and diffuse into the water column; however, global effects on DOM are poorly understood[24].

Allochthonous DOM is produced/introduced from outside the marine system, such as DOM carried by riverine systems, runoff, groundwater, glacial melt, anthropogenic pollutants, and atmospheric deposition. Compared to marine DOM, terrestrial DOM (tDOM) has notably

different compositions due to the various sources of organic matter (vascular plants) and differing abiotic conditions (like salinity). Anthropogenic activities have forever altered the chemosphere due to the addition of countless plastics, pharmaceuticals, pesticides, manufacturing waste, surfactants, and more. Although predicted to change in composition, anthropogenic DOM will increase with time because of industrial and global population growth[25]. Anthropogenic DOM has a wide range of molecular diversity and studies focusing on specific xenobiotics have highlighted the importance of selective extraction protocols that differ from widely accepted DOM extraction methods[26].

With various DOM sources and biogeochemical reactivity, it is crucial to develop tools to identify and quantify the contribution of various DOM sources and provide a detailed classification of DOM compounds in a wide range of marine environments. Molecular characterization on the structural level is possible only for a fraction of DOM with current analytical techniques. Further expanding the fraction of characterizable DOM will allow us to better understand the carbon biogeochemical cycle and how the carbon cycle interacts with other elements' geochemical cycles like sulfur, nitrogen, phosphorus, and trace metals.

1.2 DOM Reactivity

DOM is classified into different fractions based on reactivity. The scale goes from labile, semi-labile, semi-refractory/recalcitrant, refractory/recalcitrant, and ultra-refractory[27]. Labile DOM (LDOM) is a fraction of DOM of high value to heterotrophic microorganisms, which can be produced and utilized in abundance[27]. Labile and semi-labile DOM add levels of hidden complexity to DOM due to some of these metabolites being produced in such high abundances intracellularly but showing low or undetectable concentrations in seawater[4]. LDOM is a group of transitory molecules commonly substrate and facilitator metabolites including carbohydrates,

carboxylic acids, siderophores, and polyamines [4, 28-31]. LDOM's short lifetime means they are found near their source, like in productive surface waters. Semi-refractory, refractory, and ultra-refractory DOM (SRDOM, RDOM, URDOM) is a fraction of DOM accumulated to existing concentrations by continuously evading microbial and abiotic degradation[5]. Most deep oceans show DOM with average carbon ages of around 2000-6000 years old [11]. Meaning most DOM has persisted longer than the oceans' turnover time, which helps explain the ubiquitous nature of marine DOM and highlights its importance in global long-term carbon storage[32]. Most RDOM is below 800 Da and displays various chemical structures and formulas[2, 27, 33]. It is thought that RDOM could be the degradation products from more bioactive compounds or the 'leftovers' after biotic and abiotic transformations. Arakawa et al. found a collection of compounds that share structural similarities to phytoplankton pigments with C-14 ages around 1500 years[34]. This collection of DOM compounds is thought to be degradation products from pigments that have lost their bioavailability and serve as a model to explain the formation of other RDOM compounds[34].

DOM is also fractionated by size: low molecular weight DOM (LMW-DOM) is any DOM molecule less than 1000 Da and high molecular weight DOM (HMW-DOM) is more than 1000 Da. The HMW-DOM fraction is usually around 35% of DOC, leaving 65% of DOC in the LMW-DOM fraction. The distribution between HMW and LMW DOM was found to be variable based on sampling location and HMW-DOM has been reported to be 20-60% of DOM[35]. Size fractionation holds some biological implications as LMW-DOM can often be transported across cell membranes with general size limitations around 600-1000 Da [36]. HMW-DOM may contain many biologically active sites where they will be desirable energy sources. However, HMW-DOM requires exoenzymatic cleavage to be directly utilized by single and multicellular

organisms. Although size-based fractionation is also an operational distinction, a *size-reactivity continuum* has been observed where the smaller organic matter tends to have longer lifespans than 'larger' organic matter [36, 37].

It is worth noting that RDOM is not as a concrete description as LMW versus HMW DOM. The observed size reactivity continuum is a well-supported observation of DOM but not an absolute description of the DOM reactivity distribution. A significant fraction of LMW-DOM is highly labile, like organic acids and amino acids, leading to a low accumulation rate of these high value LDOM molecules despite their constant presence in marine organisms. This makes labile LMW-DOM cryptic since they can be so short-lived, making them undetected despite being regularly detected intracellularly. HMW-DOM is thought to be relatively more labile but there are some important examples that are resistant to degradation/microbial utilization, like black carbon type molecules, often the products of incomplete combustion of pre-aged carbon[4, 38, 39].

The lifetime and stability of any DOM component depend on the molecule's characteristics and the environment it is in[5, 40]. For example, what is considered RDOM compounds in deep oceans could potentially be degraded/utilized in the right conditions by bacteria with the appropriate enzymatic machinery[41]. Additionally, abiotic factors play important roles where like salinity gradients or water masses having differing levels of photodegradation[40, 42]. Photodegradation is responsible for the remineralization of some DOM to dissolved inorganic carbon (DIC) of the transformation through oxidation and deamination[33, 42-46] Generally, surface waters contain a higher proportion of bioavailable OM produced on modern timescales, which are larger in size on average. The fractions shift towards smaller and older organic molecules deeper in the water column. There is an emphasis on characterizing the enigmatic RDOM

fraction to understand their relationship within the environment and their production. The reasons why some of these organic compounds persist in the environment for such long periods are probably multifactorial, but two major theories exist today.

The first hypothesis attributes the accumulation of RDOM compounds to be due to their structural and chemical characteristics making them intrinsically resistant to degradation. When microbes from surface waters were incubated with various concentrations of deep-sea DOC Barber et al. observed that microbial cultures did not decrease DOC concentrations, suggesting the molecules were resistant to microbial degradation[47]. These findings support the microbial carbon pump (MCP) a contextual framework to describe the production of RDOM[48]. The MCP describes the mechanisms behind the observed *size-reactivity continuum*, where more labile portions of POM and HMW-DOM are utilized first, slowly accumulating semi-labile and refractory LMW-DOM. The second hypothesis to describe RDOM points to DOM's very dilute and diverse nature, the 'dilution hypothesis'. It proposes that some of these long-lived compounds could be in quantities that are so dilute that they are unable to support a microbial niche to utilize them[41]. Arietta et al. tested this hypothesis by incubating marine microbes with deep sea DOM and were able to show that thousands of different components were being microbially degraded when concentrations for those dilute compounds were increased[41]. Supporting aspects of the dilution hypothesis, this study showed that some RDOM compounds were labile/semi-labile once they were in high enough concentrations in the right conditions. Most agree that at least some of the SRDOM and RDOM have persisted because of their dilute nature being unable to overcome the evolutionarily cost of supporting a microbial niche.

With support for both the chemical and dilution hypothesis, both models likely contribute to the natural production of RDOM. Where some long-lived DOM persists due to

their chemical nature and others persist due to their dilute nature. However, studying RDOM production mechanisms has some inherent difficulties due to their long lifespan's diverse structures. One cannot study RDOM production and degradation directly in marine environments since their lifespans often exceed the age of any researcher. However, the characterization of DOM with optimized isolation techniques for LMW-RDOM can provide valuable information about how the ocean stores this carbon for such long periods.

1.3 DOM Characterization with Mass Spectrometry

The need for robust and reproducible DOM characterization is a tall order to fill from the perspective of an analytical chemist. The use of modern high-resolution mass spectrometry (HRMS) in the study of DOM has become the technique of choice by many due to the wide range of detectable analytes, reproducibility, sensitivity, ability to analyze highly complex mixtures, and high throughput. Although not a perfect technique, it has dramatically improved the collective understanding of organic matter in marine, terrestrial and biological settings[49, 50]. HRMS has proved to be an indispensable tool considering all chromatographic techniques, acquisition modes, and data processing variations for characterizing DOM. DOM studies utilizing HRMS allow us to see individual molecular characteristics and track chemical transformations qualitatively and quantitatively. Techniques such as UV-Visible Absorption Spectroscopy, fluorescence spectroscopy, Nuclear Magnetic Resonance spectroscopy(NMR), Fourier Transform Infrared Spectroscopy (FTIR) and DOC analysis do not have the same potential for molecular formula identification and structural elucidation with the same throughput. DOM studies are an analytical challenge as you find tens of thousands of different compounds within one sample, but overall, DOC levels remain dilute[2]. Additionally, a high

concentration of inorganic salts in seawater (at a ratio of 1 part DOM to 750,000 parts inorganic salts) makes direct analysis with mass spectrometry incompatible.

Effective mass spectrometry for molecular characterization of DOM requires high resolution and mass accuracy. These requirements have led to the dominance of Orbitrap (OT-MS) and Fourier Transform Ion Cyclotron Resonance (FTICR) mass analyzers to be the instrumentation of choice for DOM studies. OT-MS and FTICR-MS are unique to many other mass analyzers where ions hitting a detector do not generate the signal and offer some of the highest mass/charge ratio (m/z) resolving powers available.

In an FTICR-MS, the ion cyclotron resonance generates an image current where a Fourier transform can simultaneously produce many m/z values. FTICR-MS offers resolution in the millions (FWHM) with the highest mass accuracy available. This makes FTICR mass spectrometers excellent at assigning the molecular formula of components in a complex mixture, such as DOM. FTICR-MS is ideal for distinguishing isobaric compounds with small mass differences but struggles to distinguish between isomers [51]. Additionally, its lack-luster acquisition speeds inhibit online chromatography coupling and selective fragmentation for structure assignment.

OT-FTMS has become a great alternative high-resolution mass analyzer with arguably a more comprehensive range of abilities. Orbitrap offers resolving powers high enough for molecular formula determination with scan speeds that allow for online chromatography and excellent MS/MS capabilities especially with the Tribrid instrument architecture. The coupling of chromatography techniques to OT-FTMS has tremendous benefits as it can significantly reduce the complexity of DOM by separating the thousands of compounds into more manageable batches, decreasing matrix effects, and ionization suppression[50, 52]. OT-FTMS has lower mass

accuracy than FTICR-MS but has been improved for DOM analysis with 'on-the-fly' lock mass functions[53]. Orbitrap Tribrid instruments offer excellent MS/MS capabilities due to multiple fragmentation methods and most importantly, the addition of a linear ion trap that can collect fragmentation spectra simultaneously to the high-resolution full-scan spectra.

For DOM components to be present on MS spectra, they must enter the gas phase in an ionized state, so the choice of ionization technique determines what type of compounds within the sample end up on the spectra. Electrospray ionization (ESI) has become the primary ionization technique for DOM studies since it can 'softly' ionize a wide range of organic compounds both in size and polarity without fragmenting biologically relavent molecules. ESI operates continuously, which couples well with chromatographic/separation techniques. However, ESI still has its limitations; for example, ESI may struggle to ionize highly non-polar compounds that lack functional groups capable of acid-base chemistry like black carbon. Additionally, ESI has limits in untargeted approaches achieving only semi-quantitative results since signal intensity is a function of both analyte concentration, ion transmission, and ionization efficiency[54, 55]. Ionization efficiency is unequal for all analytes in different solvents and conditions depending on many variables such as sample pH, analyte structure, and sample matrix. For DOM analysis, inorganic ions and salts within seawater can be detrimental to signal intensity by neutralizing charges or physically impeding the capillary preventing proper nebulization and dramatically reducing ionization efficiency[56]. One DOM sample can have hundreds of thousands of different organic compounds, which leads to incomplete compatibility of any single ionization source for the entire DOM pool. [57]

Separation techniques prior to sample introduction to the mass spectrometer are another major factor that can constrain or expand the analytical window. The components within the

mixture need to be adequately separated for identification purposes, and each technique has its strengths and weaknesses. Ultra-high-performance liquid chromatography (UHPLC) is the most common separation technique for DOM analysis. The retention time of analytes adds another dimension of data improving accurate compound identification. However, any given UHPLC method can reduce the analytical window for DOM analysis due to the lack of separation of closely related compounds, poor retention of analytes, and suitability for negative ion mode due to column stability[58]. Reverse-phase C18 columns have maintained popularity due to their remarkable reproducible performance separating light ionic, polar, slightly polar, and nonpolar compounds. However, C18 is not optimized for analyzing highly polar and highly ionic compounds leaving many compounds in this category to elute unseparated early in the run. Hydrophilic interaction liquid chromatography (HILIC) columns have gained popularity by aiming to excel where C18 falls short. HILIC columns utilize highly organic mobile phases with a polar stationary phase that has improved the separation of highly polar and ionic compounds. Both C18 and HILIC columns perform well in acidic conditions but struggle in alkaline conditions. Their stability in acidic conditions has caused many mobile phases to include a small addition of formic acid to mobile phases to help separate and ionize compounds. Positive mode analysis with UHPLC has become an excellent method for identifying and characterizing DOM.

Overall, negative mode analysis compared to positive mode has struggled to provide the same level of quality due to the limitations in UHPLC column integrity in basic conditions. An exciting variation of liquid chromatography separation is ion chromatography, better suited for semi-polar and strongly ionic analytes[59]. The use of anion exchange ion chromatography has proven to be an excellent technique for negative mode analysis where [OH] gradients can shift analyte equilibrium from neutral to an ionized state altering their interactions with the column.

With the addition of an electrochemical suppressor post separation prior to the conductivity detector one can now couple IC to MS easily. One potentially concerning factor of IC is the fact that water requires more heat than organic solvents used in HPLC. This has been greatly improved by adding an acetonitrile lock mass solution directly infused to the eluent prior to the ionization source improving desolvation while greatly increasing mass accuracy. With the addition of anion exchange IC into our workflow, we have greatly increased the spectral depth achievable by unlocking the fraction of organic acids which have been underrepresented in HPLC-MS/MS in both positive and negative modes.

Combining the spectra from UHPLC and IC in positive and negative modes can increase the analytes' spectral coverage within a single DOM sample[53]. With the multiple mass analyzers, extraction techniques, sample storage, separation, and associated parameters, different research groups may get different results when running the same samples with their preferred techniques. Currently, there is no feasible way to analyze every compound within a DOM sample due to the complexity of the mixture, matrix, and instrument capabilities, so it is best practice to alter the methods to shift the analytical window best to tackle the question at hand best. With improvements in instrumentation, small molecule databases, and workflows, many things are moving forward but can all be limited to the DOM isolation stage.

1.4 Extraction/Isolation Methods

DOM isolation is a necessary step in sample processing and a major analytical window determinant. If a compound cannot be isolated from the seawater matrix, it will never make it to the analyzer, regardless of ionization and separation techniques. DOM is found in low concentrations (1-3 mg/L) compared to the relatively high concentrations of salts (20-45 g/L) not to mention the individual concentrations of specific compounds in DOM[60]. The ideal DOM

isolation techniques have a nearly complete recovery of DOM, conserve the distribution and composition of DOM, and remove inorganic ions/salts. Concentrating DOM is an added benefit but can be done post-isolation or incorporated into an extraction technique. Currently, there are 3 main approaches used for DOM isolation: 1) tangential/cross flow ultrafiltration coupled to diafiltration (UF), 2) reverse osmosis coupled to electrodialysis (ROED), and 3) solid-phase extraction (SPE). None of these techniques are truly ideal since each struggles with differing levels of selectivity, recoveries, sample integrity, and salt removal. Additionally, these techniques differ in scalability, cost, and accessibility in the field.

Tangential flow UF with diafiltration is a technique that uses a semi-permeable membrane/filter and hydrostatic pressure that selectively removes compounds that can pass through membrane pores. UF is inherently a size-fractionation technique where the DOM with a larger hydrodynamic diameter found in the polysulfone, polyamide, or cellulose membrane pores are retained as UDOM (ultrafiltration DOM). At the same time, most small molecules (LMW-DOM), inorganic salts, and water molecules are removed. Size fractionation of DOM has come from the use of UF where different membranes are used with a specific nominal molecular weight (NMW) where approximately 90% recovery of larger molecules are retained[1]. Traditional ultra-filtration membranes may become polarized as DOM accumulates, leading to incomplete isolation of HMW-DOM. Membrane fouling is also a concern where particulate deposition, membrane scaling and biofouling may block the pores hindering salt and water removal. Tangential/cross flow or continuous stirring has been implemented to maintain membrane integrity and continuously concentrate DOM while desalinating seawater samples. UF DOC recoveries have been shown to range from 20-71%, where lower salinity is correlated to lower recovery[35, 40, 61]. Even with the addition of tangential or cross flow UF the samples maintain

inorganic salt concentrations high enough to interfere with downstream chemical analysis. Diafiltration post UF is used to remove remaining salts with minimal effects on DOC recovery allowing for MS and NMR analysis[40]. UF generally selectively isolates the HMW fraction of DOM; however, some LMW DOM is retained because of adsorption to membranes, HMW-DOM, and organic metal complexes. The fraction and recovery of DOM collected will depend on membrane material, membrane pore size, flow strength, salinity, pH, ionic strength and more. UDOM has been shown to have C:N ratios consistent with total DOM despite only capturing a small percentage of total organic carbon[35]. As expected, UDOM has younger C-14 ages than total DOM since HMW-DOM tends to have more labile character[62, 63]. UDOM has been found to be enriched in degraded polysaccharides and amino sugars [64].

Like UF, reverse osmosis utilizes a membrane to retain DOM selectively but aims to retain all DOM. Reverse osmosis alone has shown to be effective with freshwater samples; however, it concentrates inorganic salts along with organic matter[65]. RO has shown 60 to 90% DOC recovery but due to the high salt content, DOM characterization becomes severely limited[66]. The coupling of pulsed current electrodialysis allows for the removal of salts by removing anions and cations with a cathode and anode separated by the membranes that retain DOM[66]. RO/ED is conducted on seawater by first using ED to remove salts, RO/ED is used to remove water and prevent inorganic salt precipitation on membranes, then ED is used again to remove remaining salts from the concentrated DOM sample[66]. Small inorganic ions are the first to be removed leaving larger ions such as sulfate accumulating. Pulsing and NaCl addition are thought to improve sulfate removal but have still shown to be a concern with RO-ED DOM. DOM that has adsorbed to the system is rinsed out with clean water and a NaOH solution. RO/ED provides samples with a mass ratio of DOM to inorganic salt from 1:17500 in seawater

to 1:180. DOC recoveries have been reported to average around 75% but are likely around 64% when adjusting for method blank subtraction highlighting issues with carry over[66, 67]. The main concerns regarding RO/ED for DOM isolation include the remaining inorganic salt content, DOC carries over and cost which may prevent RO-ED from gaining widespread popularity. RO-ED is thought to be less selective than UF with support from UV absorbances, C/N ratios and ^{13}C-NMR spectra found to be consistent with the nature of sampling location[68, 69]. Additionally, RO-ED showed strong signals of carboxylic-rich aromatic molecules (CRAM) and are enriched in fatty acids which may be underrepresented in UF or SPE based isolation techniques.

Solid Phase Extraction (SPE) has so far become the most popular technique for DOM isolation. Some sorbents isolate DOM with reverse-phase (RP) interactions where organic compounds are retained with the permeation of water and inorganic salts. Reverse-phase solid-phase extraction exploits the differential affinities of DOM in the mobile phase (seawater) with the sorbent of choice. The retained matter is eluted with organic solvents like methanol (MeOH) that can concentrate samples 500-fold[70]. Further concentration can be achieved by drying techniques to resuspend organic matter in various solvents to desired dilution factors. Due to chemical interaction-based retention, SPE efficiently isolates LMW-DOM with hydrophobic character but has shown some overlap with HMW UDOM[71]. Styrene Divinyl benzene polymer sorbents like PPL have shown to outperform silica-based sorbents such as C18 for seawater with recoveries of 62% and 39% of DOC respectively[72]. Different sampling locations has shown to affect recoveries with open oceans showing slightly lower recoveries averaging around 43% ±13 compared to 62%±11 DOC in estuaries and coastal waters[63, 69, 72]. SPE-DOM isolated from deep water masses have shown to have representative C-14 values of total DOM but skews towards being older in surface waters[63]. PPL SPE-DOM has slight carbon enrichment to bulk DOM

represented by C:N ratios when extracting in acidic conditions[72] [69]. SPE DOM has been shown to be enriched in aromatic and unsaturated compounds like lignin with variability based on sorbent choice [64, 73]. SPE has been shown to achieve competitive yields to other isolation techniques for marine DOM isolation with the added benefits of low cost, ease of use, adjustability, and flexibility. Since isolation is achieved via chemical interactions different sorbents and extraction protocols give researchers great flexibility in what fraction of DOM is retained.

Understanding the selectivity of the isolation method and its effects on the DOM fraction analyzed is a critical aspect of experimental design, especially in hypothesis-generating untargeted studies. In the case of ultrafiltration, it generally preferentially isolates labile HMW-DOM but that is dependent on membrane material and pore sizes used [74]. In comparison, RO/ED seems to isolate more representative DOM fractions but has many drawbacks with carryover, reproducibility and accessibility[75]. Even though the SPE method selectively extracts a specific DOM compound class, this extract represents a major DOC fraction of DOM with high reproducibility. Many attempts try to combine different isolation methods to increase the recovery of DOM isolation. For example, combining RO-ED with SPE was reported to recover 100% based on DOC measurements; however thorough investigation into carry-over and DOM loss was not conducted.[69] A combination of UF and PPL SPE was tested and showed differences in the fractions collected but the study aimed to extract distinct DOM pools by reactivity and did not aim to maximize recovery [63]. Interestingly the study found their DOC recovery for the coupled approach to be lower than SPE-DOM alone, likely due to differences in volume/carbon loading[63]. There is no ideal DOM isolation method due to molecular diversity within DOM and all DOM isolation struggles with some degrees of selectivity. With the ease of use, cost, and high recoveries SPE with PPL resins has become the most prominent technique to isolate DOM

1.5 Solid Phase Extraction of DOM

Solid-phase extraction has become a popular alternative to ultrafiltration and RO-ED as means of inexpensive and easy DOM isolation. Additionally, SPE can help prevent DOM degradation once retained, allowing for easy storage of cartridges, which is especially useful when collecting and processing the samples in the field. With a wide range of commercially available sorbents and flexibility in extraction protocols, SPE can be tailored to be both non-selective for maximum recovery and selective to extract specific targets or fractions[76]. SPE has been used for over 70 years with various uses across many disciplines[76-78]. Many sorbents for DOM studies are hydrophobic to retain the most DOM and greatly decrease matrix effects. Historically XAD and DAX polymer-based resins were the most common, followed by silica-based sorbents (C18) and currently styrene divinylbenzene polymers (PPL) for marine DOM.

To briefly describe a generic SPE protocol, cartridges are activated/conditioned with the elution solvent and washed/equilibrated with water prior to sample loading. Samples can then be eluted either gravimetrically, by pumps or vacuum, where the water permeate is considered waste. Once the sample has passed, it is followed by another washing step to remove salts, followed by complete drying. Dried sorbents are then eluted with an organic solvent, typically methanol, acetonitrile, and ethyl acetate, to elute a major fraction of retained compounds for further analysis. This process can be done manually or using an automated system offline or online with chromatographic systems. No single protocol can be ideal for all compounds due to the immense diversity in chemical structures, which all have different affinities towards the sorbent in different conditions. Ionic compounds may permeate through the sorbent and go undetected, and some hydrophobic molecules (like fatty acids) may retain very strongly, making elution a challenge.

The most common approach to isolating marine DOM has been based on the work of Dittmar et al. 2008 credited with providing the widely used PPL based extraction procedure[72]. To briefly describe this protocol, samples are filtered to remove POM and acidified to pH 2. This acidification will protonate acidic functional groups on DOM to increase their affinity towards the hydrophobic resins. Cartridges are first conditioned with 3 cartridge volumes of methanoland washed with one cartridge volume of Milli-Q water. The acidified sample is then flowed through until the desired volume is met and without drying the cartridge, then it is rinsed with acidified water to help remove most of the remaining salts. The sorbent is then allowed to dry completely before elution with two cartridge volumes of methanol. Samples are then concentrated and ready for mass spectrometer analysis or are resuspended in another solvent if needed. This approach was developed to maximize recovery in a single isolation step and has a bias towards the isolation of hydrophobic and hydrophobic-acidic components of DOM. SPE-DOM from PPL sorbents are enriched in polar functionalized aliphatics which encompass a large fraction of DOM[73]. Some dissolved organic nitrogen (DON) may go undetected, potentially hiding many important organic compounds with 'basic' functional groups. Studies utilizing PPL SPE based approaches to isolate DOM should consider that DON may be more susceptible to misrepresentation utilizing the acidification approach.

It has been recognized that sample pH can be an important factor for the extraction of target compounds with reverse-phase SPE sorbents since it alters the analyte's ionization state. Organic acids or bases can be present in either ionized or neutral states depending on their pKa and sample pH. By shifting sample pH to favor neutral forms of acidic, basic, and amphoteric compounds, one can alter the reverse-phase interactions with PPL sorbents for improved retention. Elution can be aided by shifting elution solvent pH accordingly to shift the equilibrium

towards more ionized states leading to weaker hydrophobic interactions. Studies focusing on the analysis of specific compounds, including antibiotics, herbicides, pharmaceuticals, and pesticides, have shown that sample pH modification may improve or be necessary for the successful extraction of their targets. ***However, the characterization of different PPL-derived SPE-DOM with various sample pH's and elution pH has not been thoroughly investigated.***

By reviewing SPE method development studies on specific targets, we predict different fractions of DOM will be retained with pH modifications. Falco and Legua proposed a method to selectively extract various aliphatic and heterocyclic amines with various RP sorbents through a range of sample pH values and elution techniques. In their study of SPE conditions, they found that many chemical characteristics affected compound retention most notably amine ionization state, polarity, and the presence of carboxylic acid as seen in cephalosporins. When using PPL cartridges, aliphatic amine cadaverine had the best extraction results when the sample was brought up to pH 12 effectively neutralizing amine functional groups and eluted with acid to protonate and ionize amines. C18 cartridges were the main sorbent tested in this study and all non-amphoteric amines had high yields by increasing sample pH with some requiring acidic elution[79].

Hong et al. investigated analytical protocols for the analysis of 121 nitrogenous pharmaceuticals, including antibiotics, cardiovascular drugs, central nervous system drugs, endocrine and family planning drugs, antiparasitic and non-steroidal anti-inflammatory drugs (NSAIDS) from river surface waters. Different sample pH's were tested with values ranging from pH 2 to pH 10 along with the addition of Na_2EDTA, a chelator to release organic matter bound to metals, using Oasis HLB cartridges (polymeric RP sorbent). They determined that surface water split to be extracted at pH 3, pH 6 and pH 9 resulted in a total of 94

pharmaceuticals with sound recovery rates (50%-150%). According to their study pharmaceutical extraction behavior was dependent on analyte stability in solution, metal interactions and ionization state with some overlap between extraction procedures. Although these mentioned examples are not all conducted with PPL sorbents, since PPL, C18 and HLB are reverse phases, it is likely that the ionization state also plays a big role in the recovery of DON with PPL sorbents.

When trying to widen diverse compound classes, like isolating DOM, multiple extraction protocols may be beneficial to get a more comprehensive isolation of DOM. For example, XAD based isolation often includes two sorbents to capture different fractions of DOM. Hydrophobic organic acids (HPOA) are isolated with XAD-8 and transphilic organic acids (TPIA) are isolated with XAD-4. The sorbent material can be packed into columns and run-in series where the sample would pass through both materials[69, 80]. Using just one sorbent, XAD-8 or XAD-4, provided yields that did not exceed 25% DOC recovery, but combining sorbents provided 42% recovery of deep sea DOM, while surface waters had even lower recoveries[69]. However, XAD is outperformed by PPL based SPE, with sample acidification and methanol elution, achieving recoveries of 61% in surface waters and 43% in open ocean water[69]. Swensen et al. proposed a rapid SPE procedure that combined a styrene divinylbenzene guard column (RP-1) stacked on a hyperCarb SPE cartridge (CAR) aiming to achieve higher DOC recovery more efficiently[81]. From the two sampling locations tested, they found that their proposed fast and low volume SPE method achieves similar recoveries where one site was 4% lower than Dittmar's 2008 PPL method and another 12% higher[81].

Few studies have been conducted that specifically investigate the effects of PPL based fractionation of SPE DOM. Chen et al. (2016) monitored changes in the optical properties of

DOM before and after PPL extraction and found decreases in the absorbance indicating the loss of some CDOM and fluorescent DOM (FDOM) thought to be protein-like matter. Wunschet al 2018 also compared the optical properties of SPE-DOM and found they had different properties than the bulk DOM[82]. Li et al. found that PPL-SPE increased compositional similarity between Suwanee River and North Sea samples likely due to the current PPL SPE method's chemical selectivity[83]. Jerusalen-lleo et al. investigated the efficiency and selectivity of PPL-SPE DOM and concluded that the method is selective to hydrophobic LMW carbon that is depleted in nitrogen[84]. The loss of nitrogenous compounds is concerning as many studies do not consider what compounds are being lost and make conclusions about the properties of DOM without a vital portion of DOM. Dissolved organic nitrogen is thought to make up a large portion of LDOM and RDOM when peptides are deaminated[33].These studies have hinted at the need for reconsideration of the current SPE method that would better represent DOM by capturing more of the nitrogenous fraction of DOM.

Investigation of the different fractions of DOM isolated from PPL based SPE with differing sample pH's and elution pH's are not known to date. It has been found that aliphatic amines are underrepresented in C18 SPE-DOM in acidic conditions, so it is reasonable to predict similar behavior in PPL SPE-DOM[69]. We predict that extracting under basic conditions will enrich DON with amines (lower C:N) and have lower O:C ratios. However, it is difficult to predict DON recoveries as many DON compounds such as peptides, are amphoteric, which may require a specific intermediate pH's or are unable to be in the neutral state. Characterization of SPE-DOM fractions differing in the protocol can provide oceanography valuable insight and approaches to expand and shift the selectivity of SPE-DOM. Additionally, the analytical window can be extended when samples can be split for different SPE procedures to uncover previously

hidden fractions of SPE-DOM. With current procedures favoring the extraction of hydrophobic acids SPE procedures that can expand the analytical window or shift it to the research question at hand would be a valuable tool for biogeochemists. In this study I will investigate the effects of sample pH and modified elution solvents on the extraction of DOM using PPL based cartridges. I will test the effects of pH on the loading and elution of DOM and characterize the differences between extracts to increase the versatility of PPL-SPE DOM. This study hopes to isolate previously uncharacterized fractions of DOM to equip biogeochemists tools to to better study the complex enigmatic marine DOM pool with mass spectrometry.

2. METHODS

2.1. Study Sites

Surface water samples have been collected from three different study sites (Baffin Bay, Lavaca River, and coastal Gulf of Mexico) that have various DOM sources (marine and terrestrial DOM) and salinity. Field sampling was scheduled to avoid any previous rain event at least 5 days after rain. All sampling took place between June-August of 2022.

2.1.1. Baffin Bay

Figure 1: Arial view of Baffin Bay sampling location. Red dot indicates exact site of surface water collection.

Baffin Bay is a shallow subtropical bay in the semi-arid coastal plains of Kleberg and Kennedy County in south Texas. Baffin Bay is separated from the Gulf of Mexico by North Padre Island and is an inlet into the larger Laguna Madre. There are no major rivers that feed into Baffin Bay although three creeks feed into the three major branches of the bay. Evaporation rates exceed freshwater inputs and are often considered as a reverse estuary with high salinity, up to

70, and residence times exceeding a year[44, 45, 85]. With little freshwater inputs DOM sources are dominated by autochthonous phytoplankton, microbial, and benthic fluxes rather than terrestrial sources[86]. Notably Baffin Bay is known to have high levels of DON compared to nearby Texas estuaries despite low inorganic nitrogen levels[85, 87].

2.1.2. Lavaca River

Figure 2: Arial view of the Lavaca River sampling location off FM 616 near Lolita Texas from Google maps.

The Lavaca River flows from Gonzales County through Lavaca County and Jackson County, feeding into Lavaca Bay in Calhoun County Texas. The Lavaca River catchments include agricultural land, forest, and oil fields, and flows near several towns but are primarily agricultural and forested. This River is rainwater fed with a consistent flow into Lavaca Bay. Samples were collected approximately 10 miles north of the mouth with brackish waters and are expected to have terrestrial sourced DOM[88].

2.1.3. Gulf of Mexico

The Gulf of Mexico is highly productive water with significant commercial fishery interest and a dynamic region of organic matter cycling[89]. The sampling location was on the northern Gulf of Mexico shelf off the coastal shores of Port Aransas TX. DOM from this area is thought to be primarily autochthonous with natural and anthropogenic allochthonous inputs due to the proximity to the Corpus Christi Ship Channel.

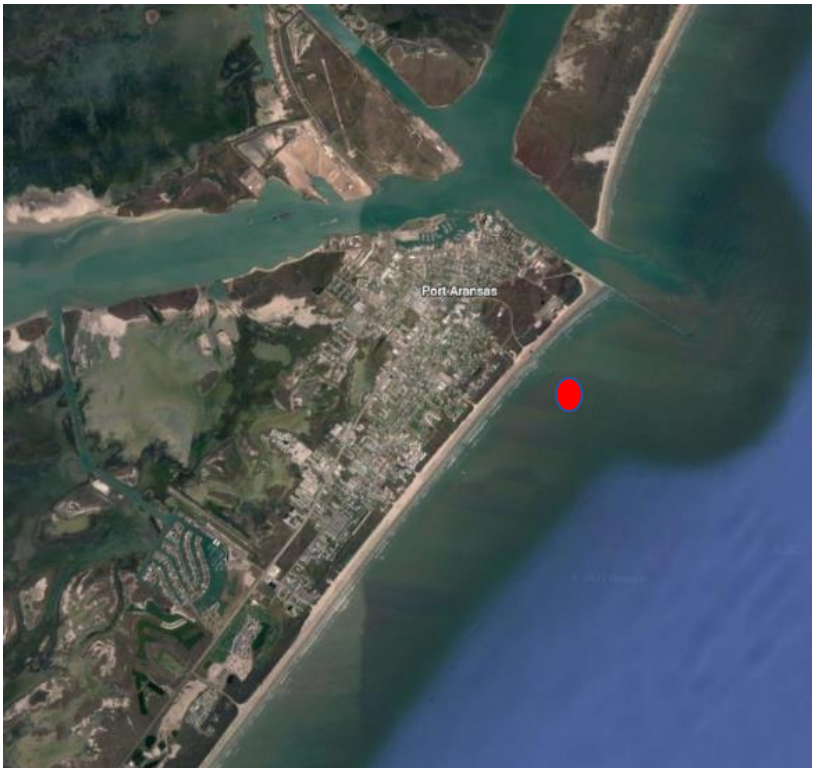


Figure 3: Arial view of the Gulf of Mexico sampling location. Surface water samples collected via bucket at the end of Horace Caldwell Pier Port Aransas Texas.

2.2 Field Sampling

Surface water samples were collected from the three sites in bulk (15 MM 8 L) in one 19 L pre-cleaned carboy from each site between May-June 2022. Before sample collection, all glassware and plasticware were cleaned by soaking in 5% tergazyme solution overnight, then washed with tap water followed by DI water, then soaked in 5% HCl for 12 hr, followed by

cleaning with deionized (DI) and Milli Q–ultrapure grade water. After cleaning, the glassware was oven-dried, combusted at 450 ^{o}C for 12 hrs, and covered with combusted aluminum foil. The same rigorous cleaning process was applied to the polycarbonate plasticware, excluding the combustion. In the field, glassware and plasticware were rinsed several times with water samples before collecting the samples. Field parameters such as salinity were measured using Fisher Scientific Optical Refractometer (Cat. No. FS1394627) and pH were measured using Horiba LAQUA twin pH meter (Model: S010). Upon return to the lab, samples were then immediately sterile filtered with Whatman 0.1µm filters separated and frozen. Thawed samples were stored as 450 ml aliquots.

2.3 Solid-Phase Extraction

Adapted from Dittmar et al. (2008). Agilent bond elute PPL 1 gram 6 ml cartridges were conditioned with 3 cartridge volumes with Optima HPLC grade methanol and washed with 1 cartridge volume of Milli-Q. The water sample was then allowed to pass with vacuum assistance. Samples were washed with either pH 2 Milli-Q with formic acid or pH 10 Milli-Q with NH_4OH. Cartridges were allowed to dry completely prior to elution. Each treatment was conducted in triplicate.

To evaluate the extraction efficiency of Agilent bond elute PPL 1 gram 6 ml cartridges under different combinations of sample adjusted pH and eluate pH conditions. Each subsample was treated with three different pH conditions (see Figure 4)

1) Acidified to pH 2 with 12 M trace metal HCl then either eluted with 1a) 6 ml Optima methanol; 1b) 6ml 1% NH_4OH optimal methanol; or 1c) 3 ml of 1% FA in Optimal methanol followed by 3 ml of 1% NH_4OH optimal methanol.

2) Increase the sample pH to 10 with 12 M NH_4OH then either eluted with 2a) 6ml Optima methanol; 2b) 6 ml 1% NH_4OH optimal methanol; or 3c) 3ml of 1% FA in Optimal methanol followed by 3 ml of 1% NH_4OH in optimal methanol.
3) Kept the sample at its natural pH and then eluted with either 3a) 6 ml Optima methanol, or 2c) 3 ml of 1% FA in Optimal methanol followed by 3 ml of 1% NH_4OH in optimal methanol.
4) Sequential SPE was conducted on one 450 ml water sample where pH was brought down to 2 with HCl and eluted with only 1 cartridge volume methanol The permeate was collected and brought up to pH 10 and a second round of SPE on a new cartridge was performed. Retained DOM is then eluted with 1 cartridge volume of methanol and combined with eluate from the previous extraction.

Samples were then stored in methanol protected from light until concentration with a Labconco Centrivap. Methanol was dried and the SPE-DOM was then reconstituted in Milli-Q with <3 ppb DOC. To reduce DOC measurements being affected by residual methanol, the Milli-Q was dried an extra time and brought to 1 ml. A liquate of 450 ml of Milli-Q blank was treated with 3 different adjected pH conditioned extracted and extracted as blank samples for the extraction procedure. Quality control (QC) pool sample was made by combining an aliquot of 50 μL from all the samples into one vial and used to monitor and correct for the mass spectrometer variability during the entire analysis.

2.4 DOC and TDN Measurements

A Shimadzu TOCL-CPH/CPN was used for DOC analysis by 680C combustion catalytic oxidation method and integrated total dissolved nitrogen (TDN) by 720C catalytic thermal decomposition chemiluminescence methods. For DOC analysis samples were acidified to pH 2

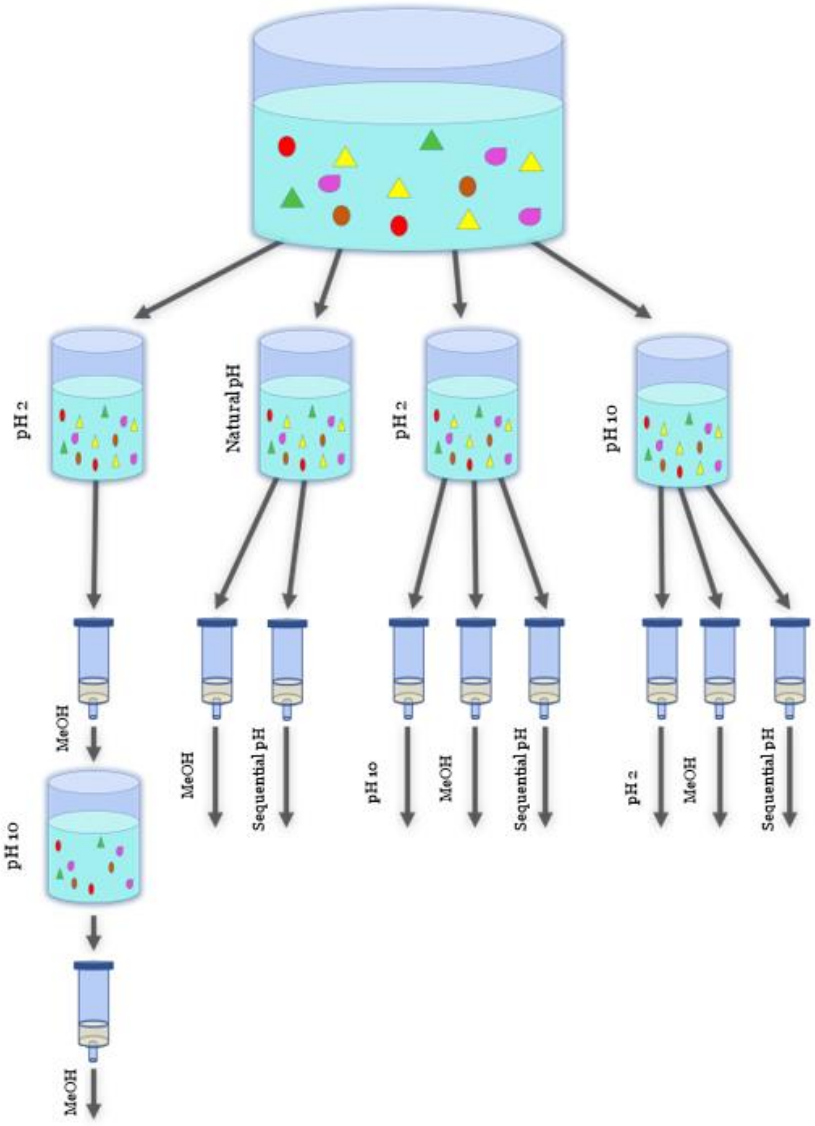


Figure 4: Representation of sample splitting conducted on each surface water sample. Each procedure had 3 replicates per sampling location.

with phosphoric acid to remove DIC. Potassium hydrogen phthalate (KHP) and potassium nitrate (KNO_3) were run from low to high concentrations prior to samples to build a calibration curve. All samples and standards were injected in quintuplets to improve confidence. For comparisons of recoveries DOC and DON were compared and used to generate C:N ratios. DON was calculated by subtracting the contribution of TDN from ammonium and nitrate.

2.5 Ammonium Measurement

Ammonium concentrations were measured via fluorescence using the modified Holmes et al. 1999 ortho-phthalaldehyde (OPA) method with high performance Liquid chromatography (HPLC) [45, 90]. A Thermo Scientific Vanquish HPLC binary pump, autosampler and Ultimate 3000 RS3000 FLD detector were operated with Chromeleon 7.2 for instrument control and quantification. The system was operated with Milli-Q water only at a 1.0 ml/min flow rate. An OPA working solution was created by combining 8 g of sodium tetraborate, 0.008 g of sodium sulfite, and 10 ml of OPA in 210 ml of a 21:1 ratio of water to ethanol. Samples required a 3-hour incubation and were run within 8 hours. An excitation wavelength of 360 nm and emission wavelength of 420 nm were used. Ammonium chloride was prepared for calibration ranging between 0.1-400 µM. The SPE and initial water samples were diluted 5x unless further dilution was necessary.

2.6 Nitrate Quantification

Nitrate was measured via IC-Orbitrap MS. The Dionex ICS-5000+ system was operated in external water mode utilizing EGC 500 KOH cartridges coupled with Dionex AERS 500e Anion Electrolytically Regenerated Suppressors. Thermo Scientific Dionex IonPac AS19-HC 4µm (2000Å, 4 µm x 2 mm x 250 mm) microbore column designed to separate oxyanions. A 0.4 ml/min flow rate used with a multi-step potassium hydroxide [KOH] gradient was used to

separate the major inorganic anions such as chloride and sulfate. A divert valve allowed only the retention time window associated with nitrate to go to the mass spectrometer. Sodium nitrate was used to prepare the calibration curve (from 0.01- 80 µM-N). The mass spectrometer was selected for quantification due to the superior sensitivity compared to conductivity for our samples with m/z detection 61.9878 in negative mode. The H-ESI II parameters optimized specifically for the nitrate signal and were as follows, Aux gas 50 (arb), sheath gas 10 (arb), gas 1 (arb), vaporization temperature 300, ion transfer tube temperature 350, and voltage at 3500 -eV. The Orbitrap Fusion Tribrid was operated in full scan mode with automatic gain control (AGC) set to 3e-5 using a maximum injection time of 50 ms at 120,000 resolutions (FWHM).

2.7 Molecular Characterization

2.7.1. Ultra-High-Performance Liquid Chromatography (UHPLC)

Thermo fisher UHPLC Vanquish system was used with a 1.7µm ACQUITY UPLC BEH C_{18} reversed- phase column by Waters (130 Å, 1.7 µm, 2.1 mm × 150 mm) for separation in positive mode MS. Eluent A, Milli-Q with 0.1% formic acid (FA) and eluent B, acetonitrile (ACN) with 0.1% FA were mixed with a 2.0 ml/min flow rate. Gradient proceeded as follows 95%A Milli-Q and 5% ACN for 2 min, ramp to 65%B for 18 min, ramp to 100%B for 1 min then held at 100%B for 3 min. Labeled proline-$^{13}C_5$,^{15}N (Sigma-Aldrich) was used as the internal locking mass standard, while labeled valine-$^{13}C_5$,^{15}N (Sigma-Aldrich) was used for evaluating the mass locking during the entire run time. The internal standards for the on-the-fly calibration were added in a solution of 96.7% ACN, 3% H_2O and 0.3% formic acid. The locking solution was introduced to the sample via a T-shaped connection post the column separation and before the H-ESI ion source using Dionex AXP-MS metering pump at a flow rate of 0.05 mL/min.

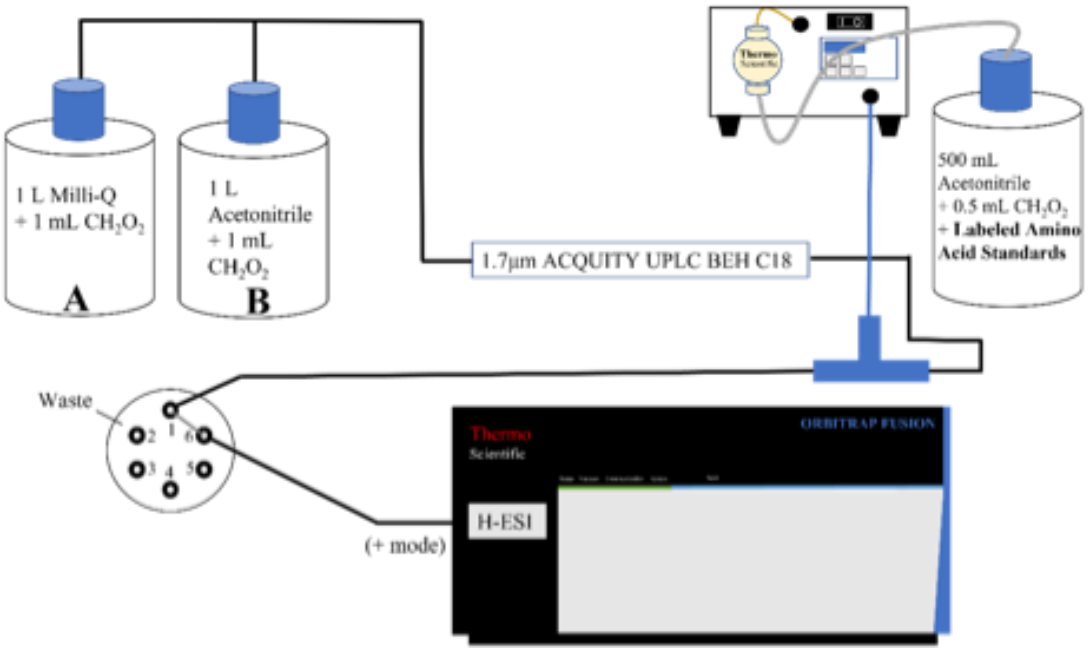


Figure 5: *Instrument diagram for the positive mode analysis of DOM extracts*

2.7.2. Ion Chromatography

Thermo Scientific Dionex ICS-5000$^+$ system was utilized as separation for MS analysis in negative mode. The Dionex ICS-5000+ system was operated in external water mode utilizing EGC 500 KOH cartridges coupled with Dionex AERS 500e Anion Electrolytically Regenerated Suppressors. Thermo Scientific Dionex IonPac AS11-HC 4µm column (2000Å, 4µm x 2mm x 250mm) microbore column designed for the separation of inorganic anions and organic acids was coupled with a Thermo Scientific Dionex IonPac AG11-HC 4 µm microbore guard column (13 µm, 2mm x 50mm). A multi-step gradient was used where [KOH] is set to 1 mM at time 0.1 rising to 4 mM at 5 min, rising to 60 mM at 11 min and 60 mM at 16 min, at 16.10 min [OH] is brought back down to 1 mM at with a total run time of 26 min. Eluent was then passed through the suppressor to remove [OH] prior to conductivity detection. Labeled hippuric acid (ring-$^{13}C_6$, 99%, Cambridge Isotope) was used as the internal locking mass standard, while labeled α-

Ketoisovaleric acid, sodium salt ($^{13}C_5$, 98%, Cambridge Isotope) was used for evaluating the mass lock signal during the run. The internal standards for the on-the-fly calibration were added in a solution of 96.7% ACN, 3% H_2O and 0.3% NH_4OH bottle. The locking solution was introduced to the sample via a T-shaped connection post the column separation and before the H-ESI ion source using Dionex AXP-MS metering pump at a flow rate of 0.200 mL/min.

2.7.3. Mass Spectrometry

Thermofisher Orbitrap Fusion Tribrid was utilized in data-dependent acquisition for both positive and negative modes. In positive mode, HESI was set to 3500v spray voltage, 35 (arb) sheath gas, 0 (arb) sweep gas, and 7 (arb) auxiliary gas. The ion transfer tube temp was set to 300ºC and the vaporization temp to 225ºC. Negative mode HESI was set to 3100v spray voltage with 45 (arb) sheath gas, 15 (arb) sweep gas, and 2 (arb) auxiliary gas. The ion transfer tube was set to 335ºC with a 283ºC vaporization temperature. Needle position and height were determined

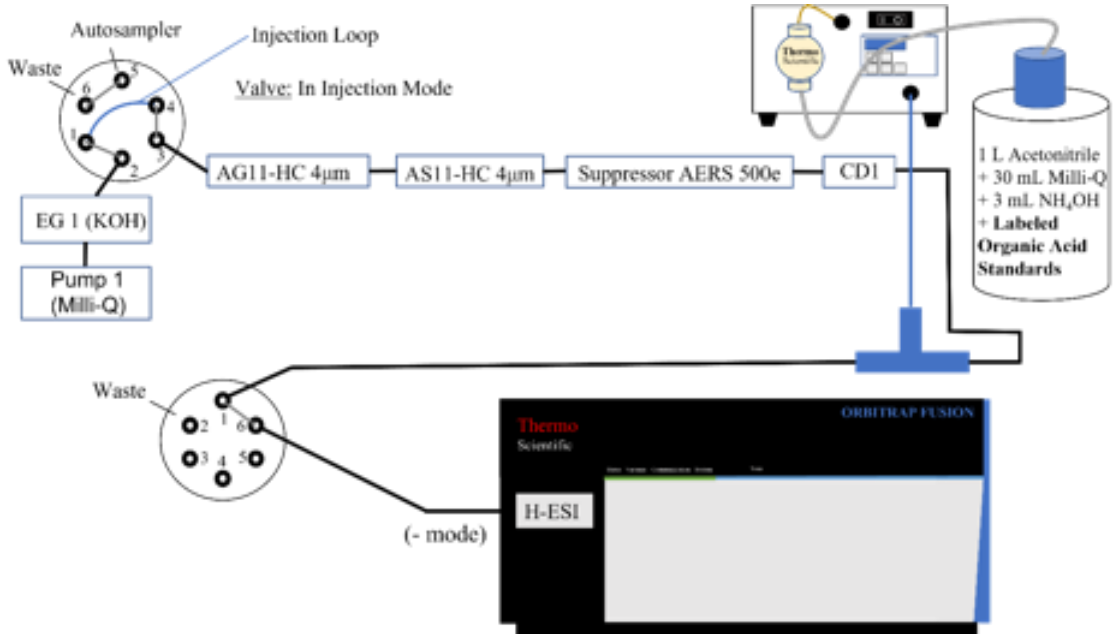


Figure 6: Anion Exchange Chromatography coupled to Orbitrap Fusion Mass spectrometry with Lock mass infusion.

based on locking m/z signal strength and stability. Orbitrap resolution was set to 120,000 (FWHM at m/z 200) for the best resolution and scan speed combination. The scan range was set to 80-700 m/z with a 40% RF lens to focus on the analysis towards LMW DOM compounds. Profile data was collected with an AGC target set to 1.0e6 with maximum injection times of 100 mS. The Dual pressure linear ion trap was used to collect two data-dependent MS^2 spectra (dd-MS^2) with priority on higher-energy collisional dissociation (HCD) followed by collision induced dissociation (CID). Following the full scan two filters will be used for dd-MS^2, Intensity threshold and dynamic exclusion. The intensity threshold was set to 1.0e3 to aid in the quality of dd-MS^2 generated. Dynamic exclusion was utilized to provide wider coverage of features in the full scan by repeatedly preventing fragmentation of the same compounds. Precursors were excluded after 3 dd-MS^2 scan events within a 30 second window. The exclusion duration was set to 60 seconds so that isomers with different retention times would not remain excluded. The tolerance for masses selected was 5 ppm for compounds higher or lower than the measured values. Two fragmentation methods were utilized in two scan type events. Scan event type 1 utilized CID (dd_MS^2 IT CID) using the quadrupole for mass isolation with a 0.7 m/z isolation window. Collision induced dissociation was utilized in assisted energy mode with collision energies at 15%, 30%, and 45% with a 10ms activation time. The ion trap scan rate was set to rapid with an AGC target of 1.0e4 and a maximum injection time of 50 ms for centroid data collection. Scan event 2 utilized HCD (dd_MS2 IT HCD) with priority, also utilizing quadrupole isolation with a 0.7m/z mass window.

2.7.4. Data Analysis

Compound Discoverer 3.2 software (vendor) was used to identify and quantify DOM compounds from both IC (-) and UPLC (+) runs. Retention times will be aligned with an

adaptive curve with up to 2 minutes of shift and 5 ppm mass tolerance. For a compound to be identified, it required a signal to noise ratio above 3, must have at least 5 scans per peak, have at least one corresponding isotope peak, and a peak intensity of 50,000.

For positive mode all available positive adducts were considered:

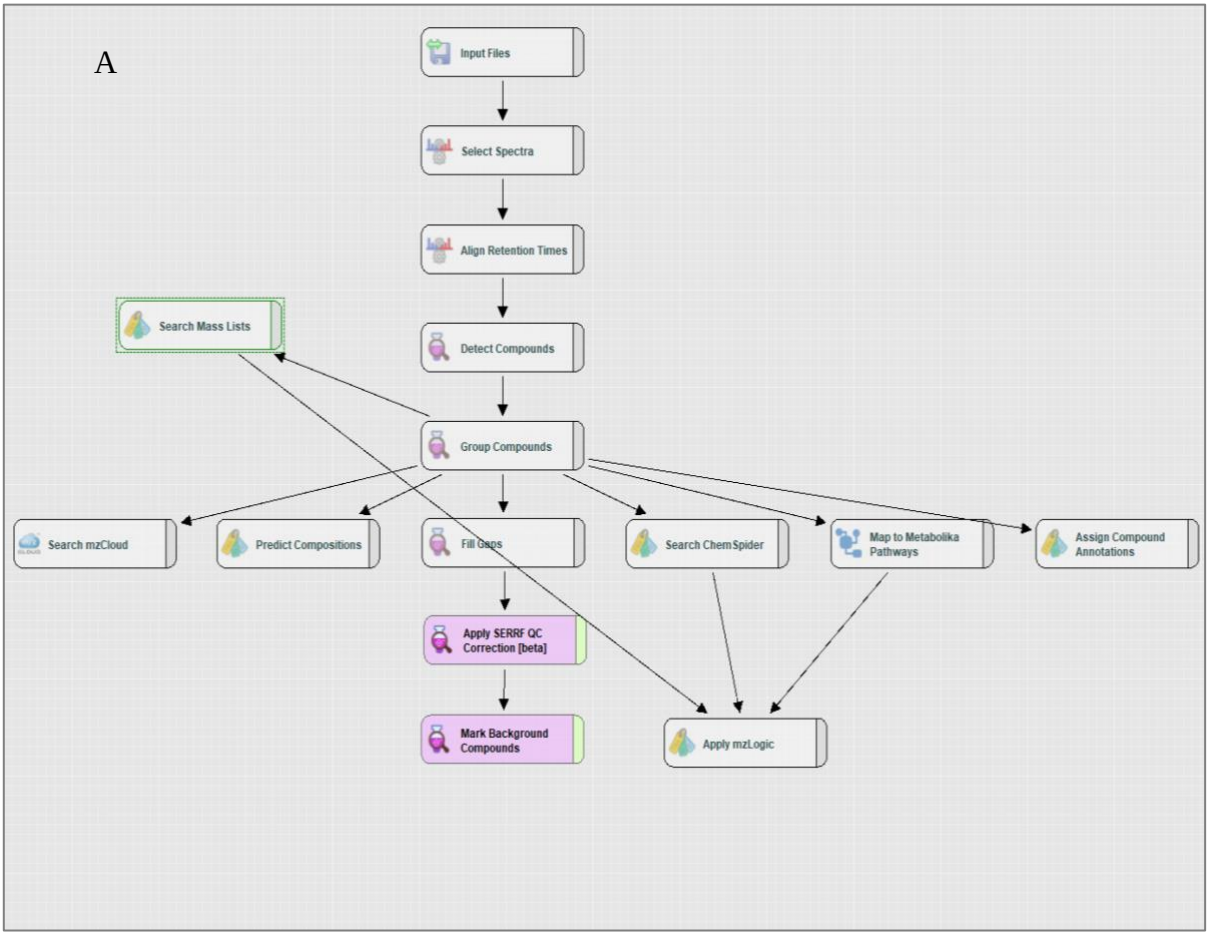
$[M + H]^{+1}$, $[M + K]^{+1}$, $[2M + H]^{+1}$, $[M + Na]^{+1}$, $[M + NH_4]^{+1}$, $[M + ACN + H]^{+1}$, $[M + ACN + Na]^{+1}$, $[M + DMSO + H]^{+1}$, $[M + H + MeOH]^{+1}$, $[M + H\text{-}H_2O]^{+1}$, $[M + H - NH_3]^{+1}$, $[2M + ACN + H]^{+1}$, $[2M + ACN + Na]^{+1}$, $[2M + FA + H]^{+1}$, $[2M + K]^{+1}$, $[2 M + Na]^{+1}$, $[2M + NH_4]^{+1}$, $[M + 2H]^{+2}$, $[M + ACN + 2H]^{+2}$, $[M + H + K]^{+2}$, $[M + H + Na]^{+2}$, $[M + H + NH_4]^{+2}$, $[M + 3H]^{+3}$.

For the negative mode all available negative adducts were considered:

$[2M + FA - H]^{-1}$, $[2M - H]^{-1}$, $[2M - H + HAs]^{-1}$, $[M - Cl]^{-1}$, $[M + FA - H]^{-1}$, $[M - 2 H + K]^{-1}$, $[M - H]^{-1}$, $[M – H + HAs]^{-1}$, $[M – H - TFA]^{-1}$, $[M – H - H_2O]^{-1}$.

Compounds that meet the identification thresholds were then compared to mzCloud, Metabolika and ChemSpider spectral databases for structural identification. Mass lists including structural information are prioritized in identification including lists containing deaminated peptides, short peptides, organic acids, and pesticides. FiSh scoring was utilized to rank proposed structures with a minimum FiSh score of 70 to be accepted. When the structure could not be identified due to identical scores then molecular formula was reported. Predicted compounds were then utilized to assign molecular formulas with a maximum element combination of C_{90} H_{190} N_{10} O_{15} P_1 S_2. Differences in the overall fraction were compared using principal component analysis (PCA) an unsupervised multivariant approach to distinguish any similarities or differences in the extracts analyzed. A volcano plot, a supervised invariant approach, was used to compare direct differences between two sample types.

A



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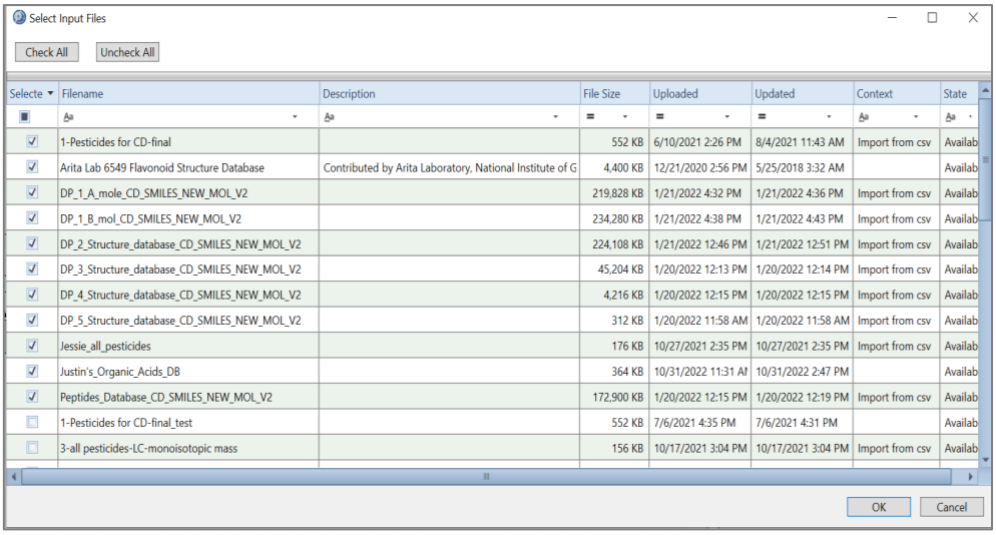

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Figure 7: Data processing within Compound Discoverer. (A) data processing workflow, all value were consistent in negative and positive mode. (B) Mass lists with structures used in both polarity modes**.**

3. RESULTS AND DISCUSSION

3.1. Carbon and Nitrogen Extraction Efficiency

To investigate the recovery of DOM and DON through the PPL bond Elut SPE total non-purgeable organic carbon and dissolved organic nitrogen (DON) were measured for sample extracts and in initial filtered water samples. Non-purgeable organic carbon was equated to DOC via sample acidification to remove inorganic carbon with phosphoric acid. Total dissolved nitrogen was measured via chemiluminescence on the Shimadzu TOC-L analyzer, where all organic and inorganic nitrogen are converted to nitrogen dioxide for detection. Nitrate and ammonium concentrations were subtracted from TDN to estimate DON. Nitrite was not measured as concentrations were expected to have negligible effects on DON concentrations. Each site was selected to represent different types of DOM with various sources and characteristics.

3.1.1. Lavaca River Inorganic parameters, DOC, DON, and SPE Recovery

DOM and DON play large roles within river systems but also play large roles in shaping biogeochemical systems in the estuaries they feed into. The Lavaca River is the main freshwater source feeding into Lavaca Bay, discharging 7.367 $m^{3} \cdot s^{-1}$ of water in 2019 (USGS 2022). The measured DOC concentration for the Lavaca River sample was 623.6 μM. It is consistent with values measured in previous studies where DOC concentrations were higher in summer months[44, 91]. The TDN concentrations were 36.4 μM which was in between values measured in sampling locations above and below our sampling site[44]. Nitrate was measured at 1.2 μM and

ammonium at 0.7 µM leaving a calculated DON of 35.2 µM. Overall the DIN concentrations were lower than previously recorded in sampling sites upstream (Figure 8)[91].

A

Lavaca River	
Salinity	15
pH	7.9
NO_3 (µmol L−1)	1.2
NH_4 (µmol L−1)	0.71
DOC (µmol L−1)	623.6
DON (µmol L−1)	35.2
C:N	17.7

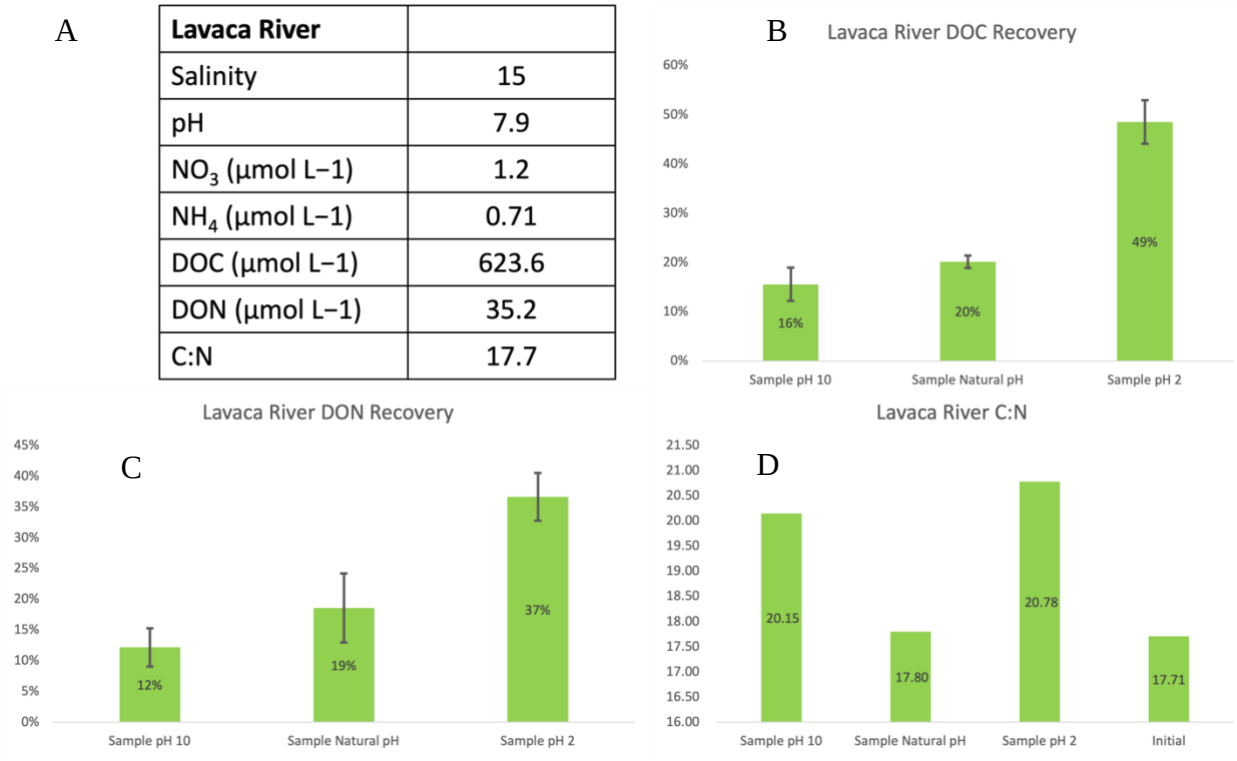

Figure 8: Lavaca River sample site and recovery data. (A) Measured values of salinity, pH, DIN, DOC, and DON (B) DOC recovery for samples with various starting pH's. (D) DON recoveries comparing different starting sample pH's. (E) Comparison of C:N ratios between different starting sample pH.

With elevated salinity levels (salinity 15), I suspect this location could be impacted by high autochthonous DOM production. This could explain the relatively high DON concentrations and relatively lower DIN concentrations and C/N ratio.

Rivers carry approximately 260 tG of DOC to the ocean annually, the largest allochthonous carbon input within the marine DOM pool[13, 92]. Riverine DOM has been shown to shape biogeochemical cycling within estuary systems in various ways, especially across abiotic

gradients. Riverine DOM has been shown to regulate microbial activity and shape microbial communities within highly productive estuarian waters[93]. These rivers also carry many inorganic nutrients that can shape the microbial and phytoplankton communities, modulating DOM composition and concentrations[94]. Some river estuary interfaces have been shown to have DOC that behaves conservatively or nonconservativly where various transformations and/or losses via biotic and abiotic mechanisms occur[44, 45, 92],[40]. These changes can be photochemical reactions where DOM can be directly degraded or indirectly lost via transformations to more labile products [43, 95, 96]. DOM in river systems can vary greatly from open oceans with unique natural and anthropogenic sources with notable composition variation under different flow conditions[97]. Riverine DOM contains large amounts of terrestrially sourced DOM especially from C3 and C4 plants with high C:N ratios which are aged in soil and carried by runoff[92]. This sample site is unique relative to the others (Baffin Bay and Gulf of Mexico) with more terrestrial sourced DOM. This was reflected I the fact that this sampling location provided the highest C:N ratios which is consistent with terrestrially sourced DOM from vascular plants. Many use lignin as a vascular plant biomarker which is thought to be a degradation product that increases in acidic character (oxidation) with time[43]. Similar types of degradation have been observed with DON seen in amino acids and small peptides through photo-deamination and photooxidation[45, 98].

The DOC recoveries for the Lavaca River sample at pH 2, natural pH and sample pH 10 were 49%, 20%, and 16%, respectively (Figure 8 B). Recovery of DON was 37% for sample pH 2 which was the highest measured recovery of DON. Natural pH samples and samples at pH 10 from Lavaca River were 19% and 12%, respectively. DON recovery being the lowest at pH 10 was surprising since most amine functional groups are likely in the neutral state. This could indicate that DON compounds within this sample site have multiple functionalities, maybe with

acidic character which have played a larger role in their isolation. The DON from this sample is likely high in protein/peptide content which is supported by the high DON yields in the acidified and natural pH samples. Additionally, at the time of sampling this location displayed characteristics of an estuarine system with high salinity (relative to river systems) and low DIN despite being 10 miles upstream of Lavaca Bay. I suspect that the organic matter here is labile in nature and combines terrestrial sources and in-situ primary production.

3.1.2. Baffin Bay Inorganic parameters, DOC, DON, and SPE Recovery

Baffin Bay has been described to have eutrophic characteristic supporting "brown tide" blooms. These blooms and eutrophic characteristics have often been associated with dissolved inorganic nitrogen (DIN) inputs but blooms during low freshwater inflow seasons with low DIN concentrations have challenged this model[99]. Although DIN is relatively low, Baffin Bay has exceptionally high DON concentrations and low bulk C:N ratios which serve as an excellent site to investigate methods of DON isolation. DOC concentrations were measured at 841.1 μM and TDN at 94.8 μM. These values were the highest values recorded in this study, but were within the ranges of previously measured/expected values[44]. Salinity was measured at 40 with a natural pH of 8.4 during sampling. Total DIN was very low with nitrate being undetectable and ammonium at 0.8 μM, leaving DON to make up 99.1% of TDN. The high concentrations of DON are reflected by the low C:N ratio (9.0) of the initial bulk DOM pool.

A

Baffin Bay	
Salinity	40
pH	8.4
NO_3 (µmol L−1)	0
NH_4 (µmol L−1)	0.80
DOC (µmol L−1)	841.1
DON (µmol L−1)	94.0
C:N	8.9

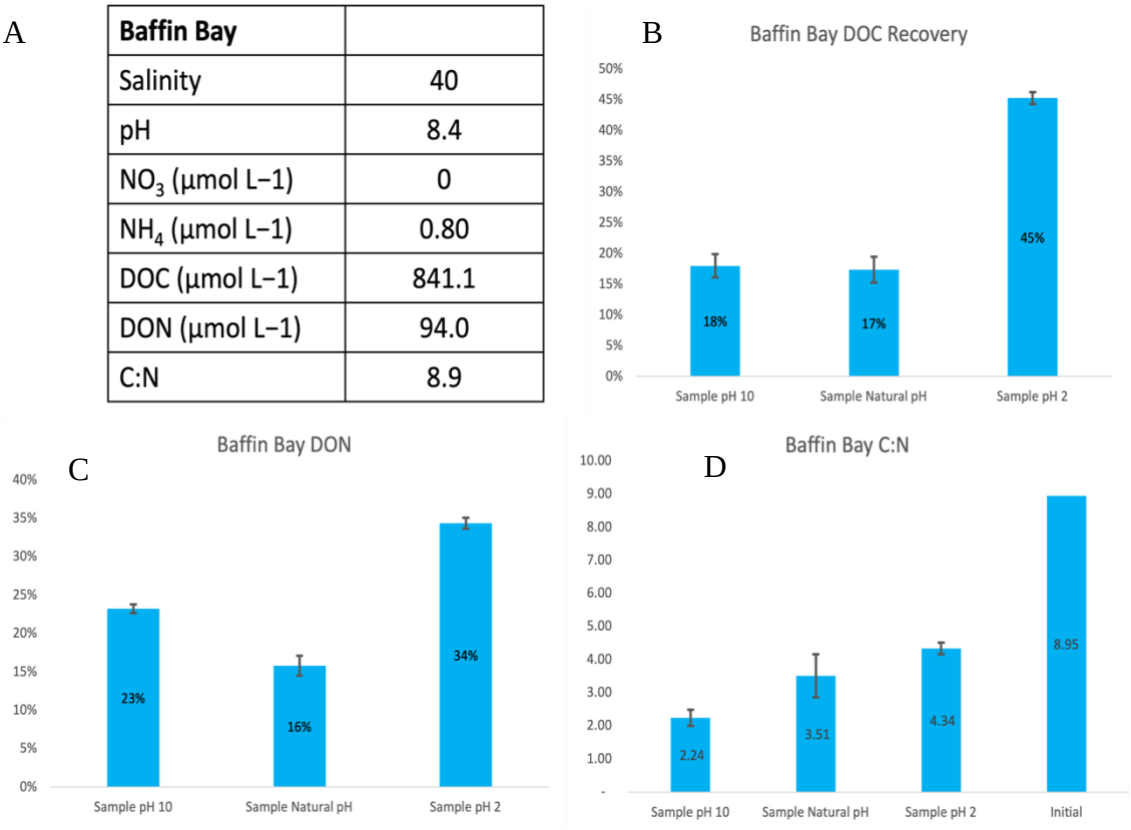

Figure 9: Baffin Bay SPE recoveries. (A) Measured values of salinity, pH, DIN, DOC, and DON (B) DOC recovery for samples with various starting pH's. (C) DON recoveries comparing different starting sample pH's. (D) Comparison of C:N ratios between different starting sample pH.

Estuaries are complex where products of the terrestrial systems blend with the marine systems. Estuarine systems can be nutrient rich and support immense diversity due to the wide range of niches they support with the many environmental gradients they contain. Many estuarine systems like Lavaca Bay are fed freshwater with relatively consistent inputs[44]. However, Baffin Bay is a unique system that lacks consistent river inputs, leading to some unique biogeochemical characteristics[100]. The lack of rivers makes infrequent surges of terrestrial inputs from episodic high flow events, such as tropical storms and hurricanes, to be the largest sources of terrestrial DOM there. Long drought periods in this area have been associated with

seasonal trends where salinity and DOC increase with temperature[99]. This bay is described as an reverse estuary where salinity is highest furthest from the Laguna Madre due to the lack of freshwater inputs in a highly evaporative environment[44]. The trends of DOC concentrations within this bay behave like most estuaries where DOC concentrations decrease approaching the Laguna Madre[40]. Sources of DOC and DON for Baffin Bay are likely from primary production and benthic flux[45]. Benthic flux is a significant source of DOM to the marine systems estimated to contribute comparable amounts of DOC to riverine inputs globally[101].

The samples that were extracted at pH 2 had the highest DOC and DON recoveries at 45% and 34%, respectively. Samples at natural pH and pH 10 both had similar DOC recoveries at 17% and 18%, respectively. DON recovery for pH 10 samples was 23%, notably higher than DON recovery at natural pH, 16%. This could indicate that more DON compounds in Baffin Bay are in the neutral state at pH 10. Acidified samples had the highest DON recovery, which could be represented by nitrogenous compounds that do not have nitrogen with basic characteristics or are in complex molecules like peptides where acidic functional groups are also present. Unlike all other sites, Baffin Bay had a unique characteristic where the initial C:N ratio was higher than seen within all the extracts. This was not expected as, in general, PPL extraction at pH 2 showed bias toward selectively extracting nitrogen-depleted DOM relative to the initial sample[84]. However, the less acidic sample was directly correlated with lower C:N ratios.

3.1.3. Gulf of Mexico Inorganic parameters, DOC, DON, and SPE Recovery

During the sampling event, salinity at the Gulf of Mexico site was 35 with a pH of 8.1 and a nitrate concentration of 0.6 μM and ammonium of 0.71 μM. DOC and DON were measured at 209.8 μM and 16.1 μM respectively, giving a C:N ratio of 13.0. The Gulf of Mexico is a major ocean basin with a 1,550,000 km surface area. Mostly surrounded by land, the Gulf of

Mexico experiences many riverine and estuarine inputs but is also fed water by the gulf stream[102]. From a DOM perspective the Gulf of Mexico largely resembles the open ocean DOM pool with autochthonous sources from in-situ primary production and while also showing characteristics of degraded terrestrial and estuarine DOM profiles[21]. DOC concentrations were the lowest here compared to riverine and estuarine systems which was expected. Due to the proximity to the coast and international ship channel we expected DOC concentrations to be higher here than previously measured DOC concentrations in the open ocean. This was observed for both DOC and DON in our coastal Gulf of Mexico sampling event. Overall, we expected some overlap in DOM sources and more refractory organic matter to make this sample distinct from the other locations. As predicted, the Gulf of Mexico has the lowest DOC and DON concentrations relative to the other two sites (Lavaca River and Baffin Bay). However, its initial C:N ratio is higher than Baffin Bay and lower than Lavaca Bay, which indicates a difference in DOM compositions between the three sites.

DOC recoveries were consistent with the results from Baffin Bay and Lavaca River, where acidified samples had the highest yields, but the Gulf of Mexico samples had the lowest concentration of DOC and recovery at 35%. Natural pH samples had an average DOC recovery of 21% and DON recovery of 15%. Samples extracted at pH 10 had similar DOC recoveries but lower DON recovery at only 7%, the lowest between all sites and sample pH's. In turn these samples (pH 10) had the highest C:N ratios much higher than the initial water sample suggesting a bias against the DON within this sample. All extraction procedures for this site increased the C:N ratios above the initial values where samples at natural pH had the most similar values to the initial DOM pool.

A

Gulf of Mexico	
Salinity	35
pH	8.1
NO_3 (µmol L−1)	0.6
NH_4 (µmol L−1)	0.7
DOC (µmol L−1)	209.8
DON (µmol L−1)	16.1
C:N	13.04

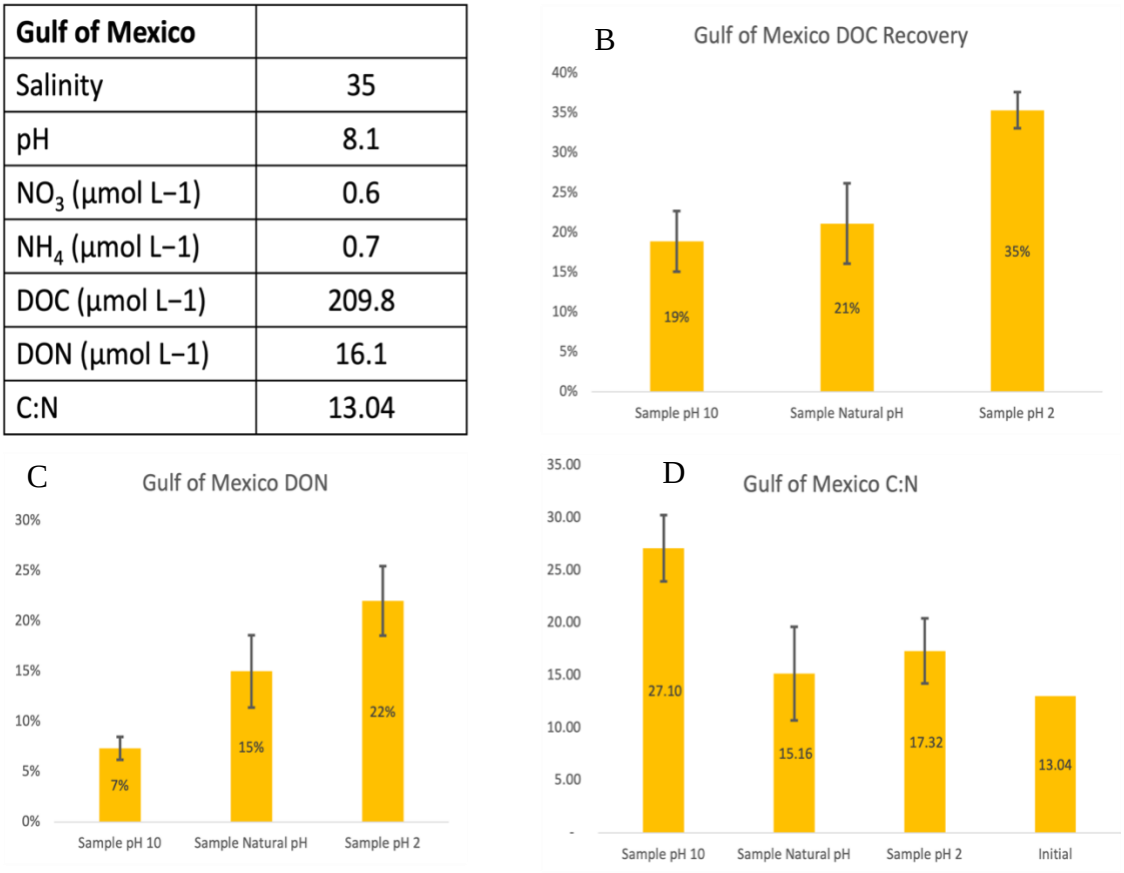

Figure 10: Gulf of Mexico Recovery and sample information (A) Measured values of salinity, pH, DIN, DOC, and DON (B) DOC recovery for samples with various starting pH's. (b) DON recoveries comparing different starting sample pH's. (c) Comparison of C:N ratios between different starting sample pH.

3.1.4. Total Recoveries

DOC recovery was within the expected ranges for all sample sites utilizing the standard acidified sample SPE method, averaging 43% across all replicates and sampling locations. Samples at natural pH and samples at pH 10 had lower DOC recoveries of 19% and 17%, respectively. Acidified samples had DON recoveries of 25% across all sampling locations ranging from 17% to 37%. Natural pH samples had an average TDN recovery of 13% ranging

from 10% to 19%, depending on the sampling location. The pH 10 samples had TDN average recovery of 11% ranging from 7% to 15% depending on the sampling location. Overall, the standard method recovered more total DOC and TDN than the pH adjusted methods, but sample site-specific variation highlighted the variability due to the initial DOM pool. Despite the lower DOC and DON yields in the unacidified samples (natural pH and pH 10), when comparing the C:N ratios of each procedure, there is a trend towards lower C:N ratios indicative of a more nitrogen-rich fraction of DOM collected. Notably, this trend was not seen in all sampling locations, especially in the Gulf of Mexico likely due to differences in the initial DOM collected. These variations in DON recoveries probably highlight structural differences between the major DON constituents between sampling locations. For example, nitrogenous compounds where the nitrogen is a part of heterocycle may not be as pH dependent compared to secondary and tertiary amines which are more electron rich. Samples where pH modified elution solvents were used were not compared in this section as all added carbon and nitrogen in the form of formic acid and ammonium were not accounted for. Using the unadjusted values of DON and DOC recoveries from the pH adjusted methanol elution did not provide reliable recoveries and will be adjusted in future studies..

3.2. Dissolved Organic Matter Characterization Positive Mode

Chemical characterization of SPE-DOM was conducted with Compound Discoverer 3.2 software. Positive and negative mode analyses were processed separately but operated under the same workflow compared to the same spectral databases and mass lists. Pooled quality control samples were incorporated into the sequence to measure instrumental variation over time. In positive mode a total of 5134 hits were identified as unique compounds with 3025 tentative structural identifications and 4322 molecular formulas assigned between all extraction

procedures. In total, only 15.9% of all compounds had neither molecular formula nor structural annotation.

Since SPE retains analytes based on chemical characteristics there were concerns about the extraction method forcing samples from different sites to appear more chemically similar. To investigate this a principal component analysis (PCA) was constructed comparing all different pH treatments SPE-DOM samples from the three sites. The advantage of PCA is a multivariant unsupervised technique that allows us to compare variations in the sample considering all the compounds detected from each sample. Two principal components are plotted on the Y and X axis, Principal Component 1 (PC1) and Principle Component 2 (PC2) respectively. Each

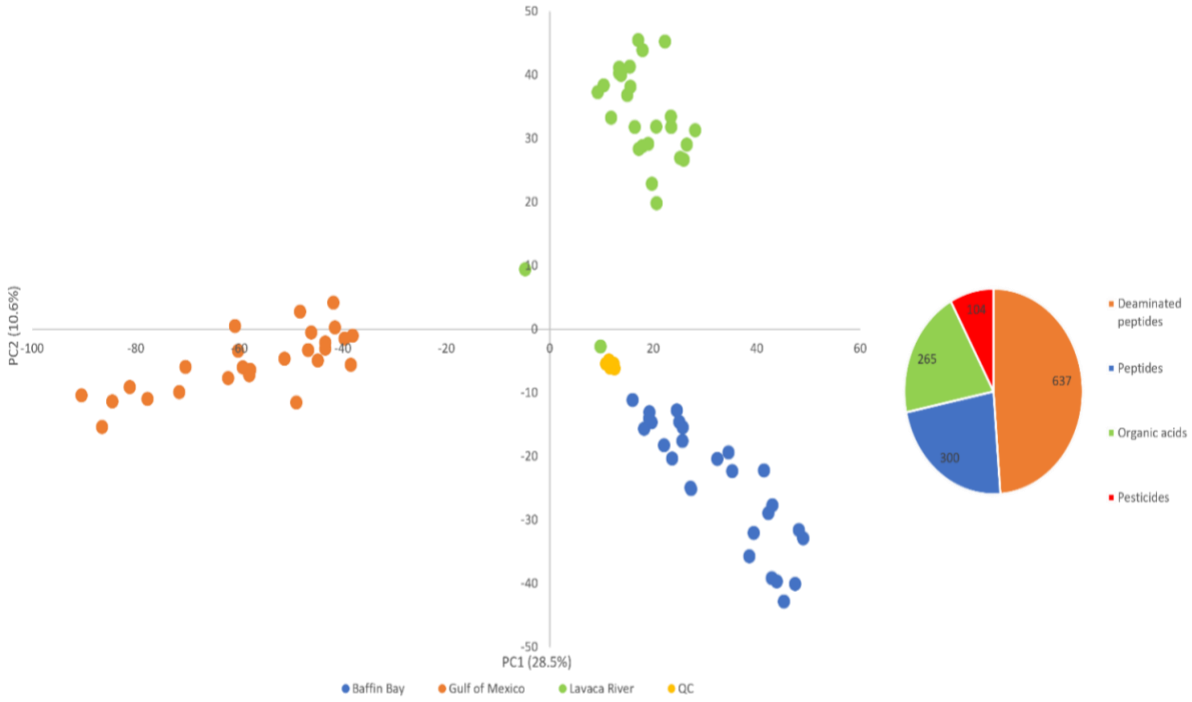

Figure 11: (A) Principal component analysis comparing all injections show clustering based on sample site. Orange represents Lavaca River, green Gulf of Mexico, and blue represents Baffin Bay. (B) Pie chart showing number of mass list matches for compounds principal component is representative of a large group of compounds and their signal intensities so samples that are spatially close on the graph have very similar DOM chemical profiles. Figure

11 shows that despite the extraction procedure sample site was the largest source of variation since all the sample sites clustered together. The pooled quality control clustered nicely towards the middle, a measure of instrument variation and overall data quality. The QC drifted slightly towards the Baffin Bay SPE-DOM cluster, which could be explained by the fact the QC was created via sample volume-based pooling and that Baffin Bay had the highest DOC concentrations.

To investigate differences between the extracts due to pH modifications of the extraction procedure, three sample site specific PCA's were constructed. In Baffin Bay positive mode analysis (Figure 13) samples clustered towards the bottom right of the plot where PC1 is negative and PC2 positive. These were samples that did not receive any sample acidification which included the samples kept at natural pH and brought up to pH 10. A second major cluster can be seen towards to the top left of the graph where PC1 is positive and PC2 is negative. This cluster consisted of all acidified samples and extracts gathered from the sequential SPE procedure. The lack of any distinct clustering based off elution solvent indicated that sample pH is the main determinant of what fraction of DOM is isolated using PPL based SPE for this sample. Since the clearest division of points on this PCA was the sample pH will now refer to each side of the division line included to be either the acidified fraction (lower right corner) and the unacidified fraction (top left corner).

Not all the samples from either acidified or unacidified sample groups clustered closely together for Baffin Bay which suggests that there were some differences in what compounds were within the sample and how much of each was present. The samples extracted at pH 10 and eluted with both acidic and basic methanol ended up close to samples that were extracted in acidic conditions than the other samples extracted at neutral or pH 10 from Baffin Bay. The

proximity of these clusters indicates both extracts that have a similar makeup with overlapping DOM components likely to be hydrophobic compounds which are not pH specific.

In Lavaca River positive mode analysis (Figure 14) samples were in more defined clusters than from Baffin Bay. The acidified fraction, below the red line, was very compact with all samples having a negative PC1 score and positive PC2. This indicated that all samples that were acidified or came from sequential SPE had very similar DOM components at similar quantities despite the different elution solvents used. The unacidified fraction consisting of samples at natural pH and basified samples showed similar clustering patterns with tight clustering where PC1 is positive and PC2 negative.

Overall, the DOM from Lavaca River showed similar responses to variations in the extraction procedure. There are two main fractions one arising from samples that were acidified and the other when samples are not acidified or brought up to pH 10. There were two outliers in the unacidified fraction which were also seen in Figure 11 without a clear cause for such deviation. This could have been due to a small contamination event or have been subject to some carry over from the pooled QC. I suggest the carry over since when looking at Figure 11 these samples had the most similar DOM profile to the QC than any other sample.

In the Gulf of Mexico positive mode analysis (Figure 15) sample clusters were a little less defined than seen in Lavaca River. The samples that were extracted at natural pH and pH 10 were plotted on the top left where PC1 was negative and PC2 was positive (for the most part). Samples that were extracted at pH 2 were clustered together but this time in the top right corner where PC1 is positive and PC2 is positive. Surprisingly the samples that were extracted sequentially with acidic and basic conditions did not cluster closely with the acidified samples as seen in the other sampling locations. Despite the sequential SPE not clustering closely with the

rest of the acidified samples they did still fall on the correct side of the division line drawn in red between the two main fractions.

Why the sequential SPE samples from the Gulf of Mexico were not close to the other acidified samples seen in the other sites are not known. The PC2 values between the sequential SPE and acidified samples were consistent, and it was the differences in PC1 that prevented their clustering. This indicates that these samples were in fact similar to each other but there was some differences in the compounds making up PC1 that were responsible. This is likely a result of differences in the starting DOM pool as all three sites were expected and shown to have a different makeup.

There were varying levels of differences between extraction procedures with two main clusters depending on sample pH across all sampling locations. Overall, samples extracted at natural pH or pH 10 had similar chemical compositions shown by the overlap in the position of those samples. Acidified samples and the sequential SPE procedure did tend to cluster together to make up the 'acidified' fraction. Overall, the elution technique showed no signs of differentiation, indicating the extraction of similar fractions of DOM. The largest determinant on the clustering of samples seems to be the sample pH dependent, where natural pH samples and pH 10 samples represented one fraction while acidified and sequential represented the other fraction. This relationship was most clear in the Lavaca River samples but similar differences were seen in all sites.

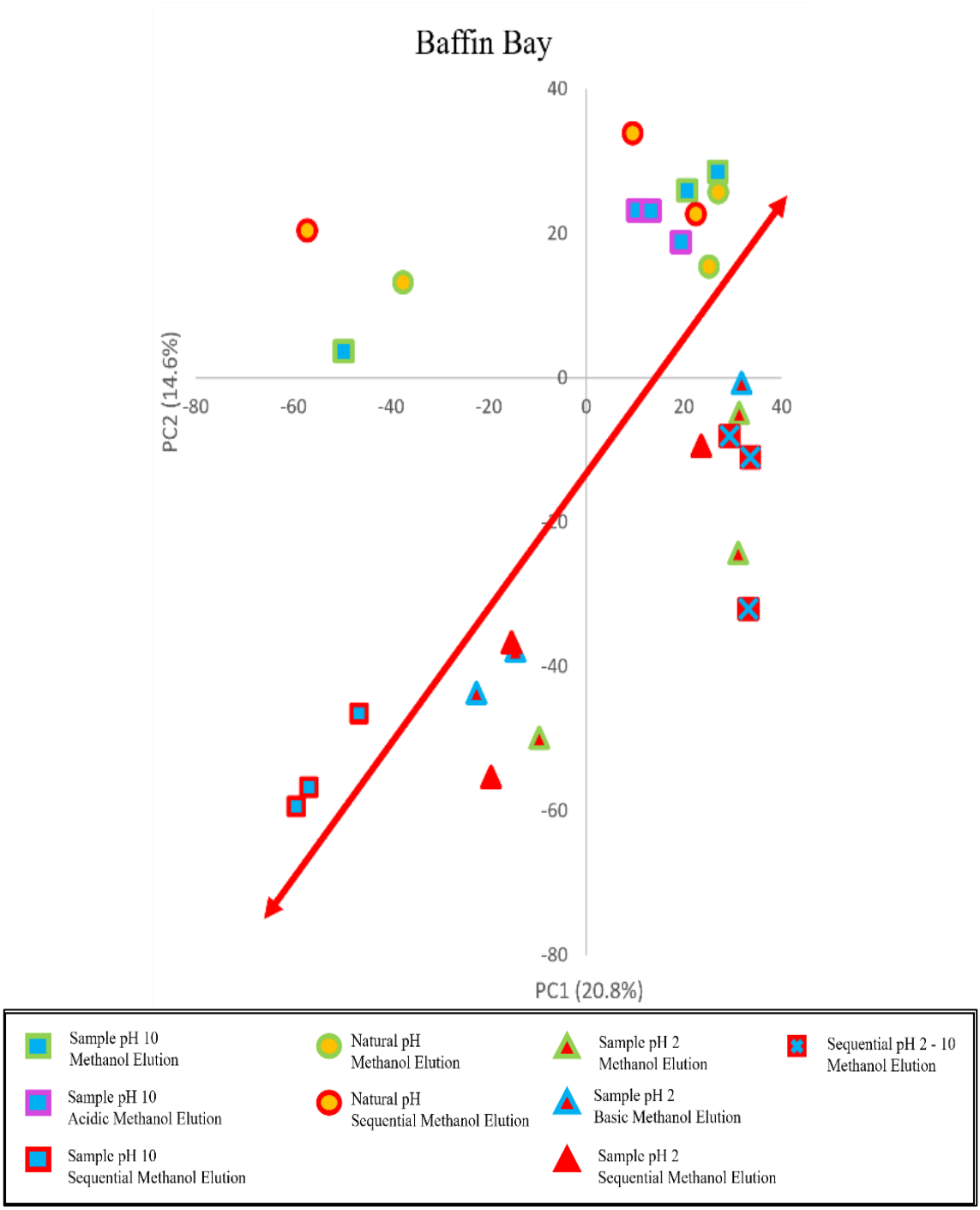

Figure 12: PCA of all SPE procedures from Baffin Bay for positive mode analysis. PC1 accounted for 20.8% of the variation and PC2 accounted for 14.6%. Red line is drawn to show the division between sample types.

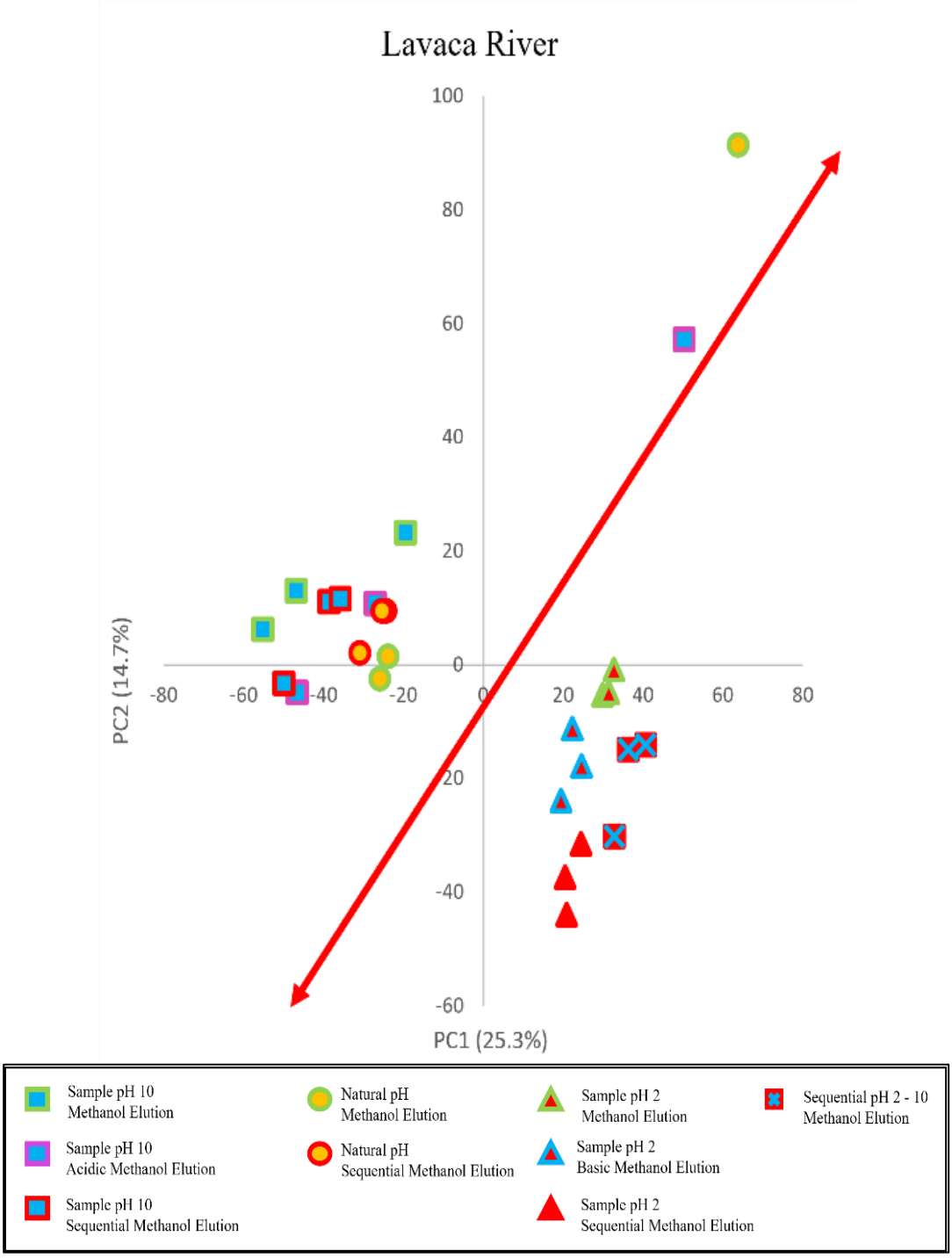

Figure 13: PCA of all SPE procedures from Lavaca River for positive mode analysis. PC1 accounted for 25.3% of the variation and PC2 accounted for 14.7%. Red line is drawn to show the division between sample types.

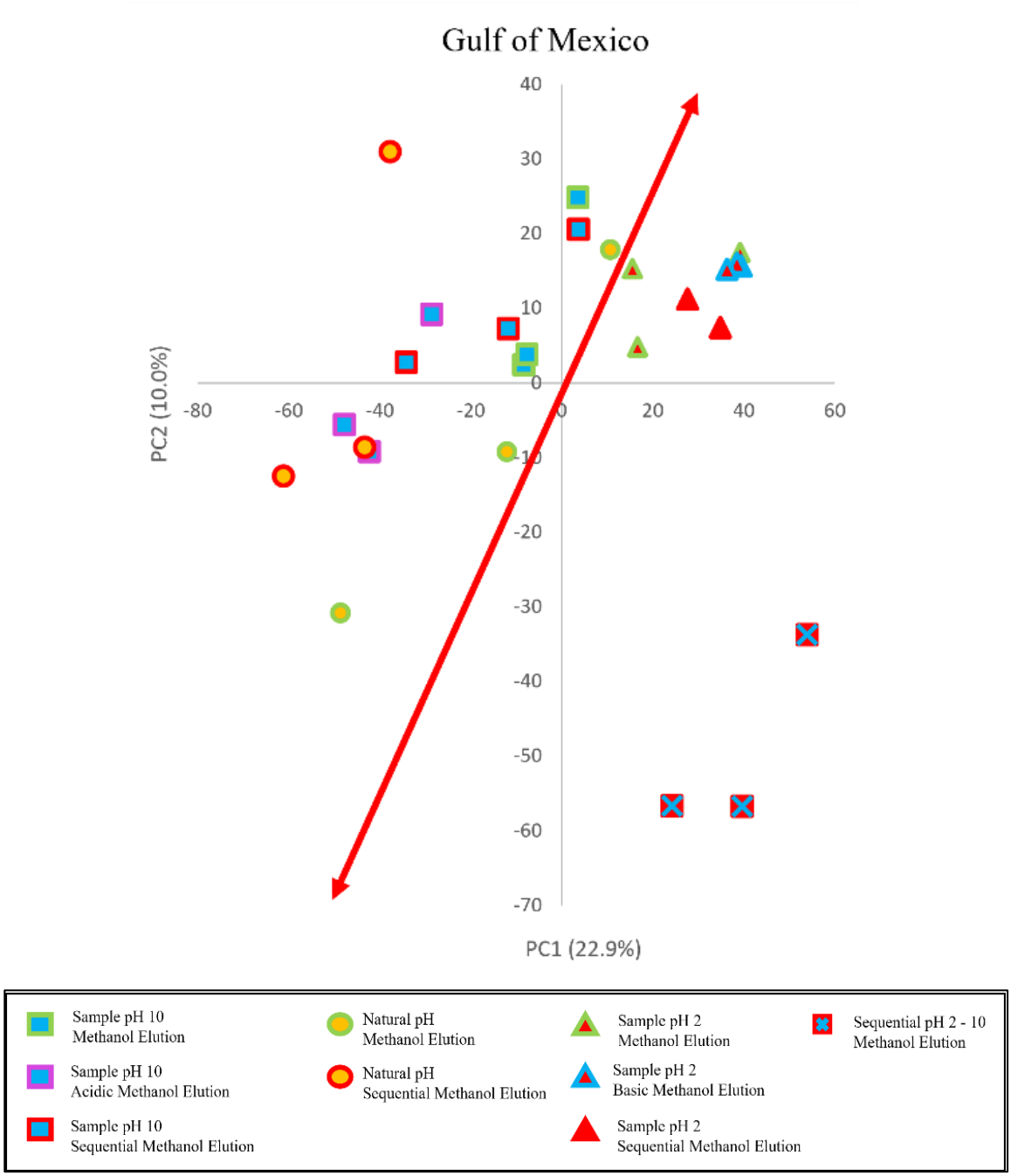

Figure 14: PCA of all SPE procedures from Gulf of Mexico for positive mode analysis. PC1 accounted for 22.9% of the variation and PC2 accounted for 10.0%. Red line is drawn to show the division between sample types.

While PCA can compare many complex relationships simultaneously, volcano plots can show the differences between two sample types while visualizing differences on the individual compound level. Since the PCA plots indicated the extraction of similar fractions between the acidified and nonacidified samples volcano plots comparing the fraction collected between natural pH and pH 2 were constructed (Figures 16-18).

The volcano plot comparing the extraction procedure on the Baffin Bay DOM pool (Figure 16) found a total of 402 compounds that were unique to the sample pH 2 procedure. Out of the unique compounds to acidified samples, only 19 were true unknowns without a molecular formula or structural annotation (4.7% no annotation). This fraction was well characterized with 50 molecular formulas and an additional 334 structural assignments. Samples extracted at natural pH yielded 179 unique compounds which was notably less than the unique compounds that were extracted with sample acidification. The unique compounds from the natural pH fraction were far more enigmatic with 135 unidentified compounds, 18 compounds with molecular formula only and 26 structural assignments (75.4% no annotation).

For Baffin Bay positive mode analysis, the volcano plots show that that acidified samples did lead to the highest number of unique compounds. This was an expected result since the acidified approach had the highest DOC yields. Additionally abiotic mechanisms of DOM transformation could be a cause for such molecular diversity when it comes to DOM with acidic functional groups. Photochemical reactions often lead to more oxidized carbon where the formation of a carboxylic acid functional group can be seen as the last step before DOC is converted to DIC through decaboxylation[46, 96]. Since photochemical reactions are going to be less molecule and position specific than an enzymatically mediated reaction there is an expected increase in molecular diversity due to its lack of specificity which could explain the high number

of unique samples in the acidified samples. The enigmatic nature of the unique compounds identified in the natural pH samples are exciting since they are important compounds that have yet to be identified. Careful isolation and studying of these compounds are necessary especially in systems like Baffin Bay were organic nitrogen and organic matter cycling is unique.

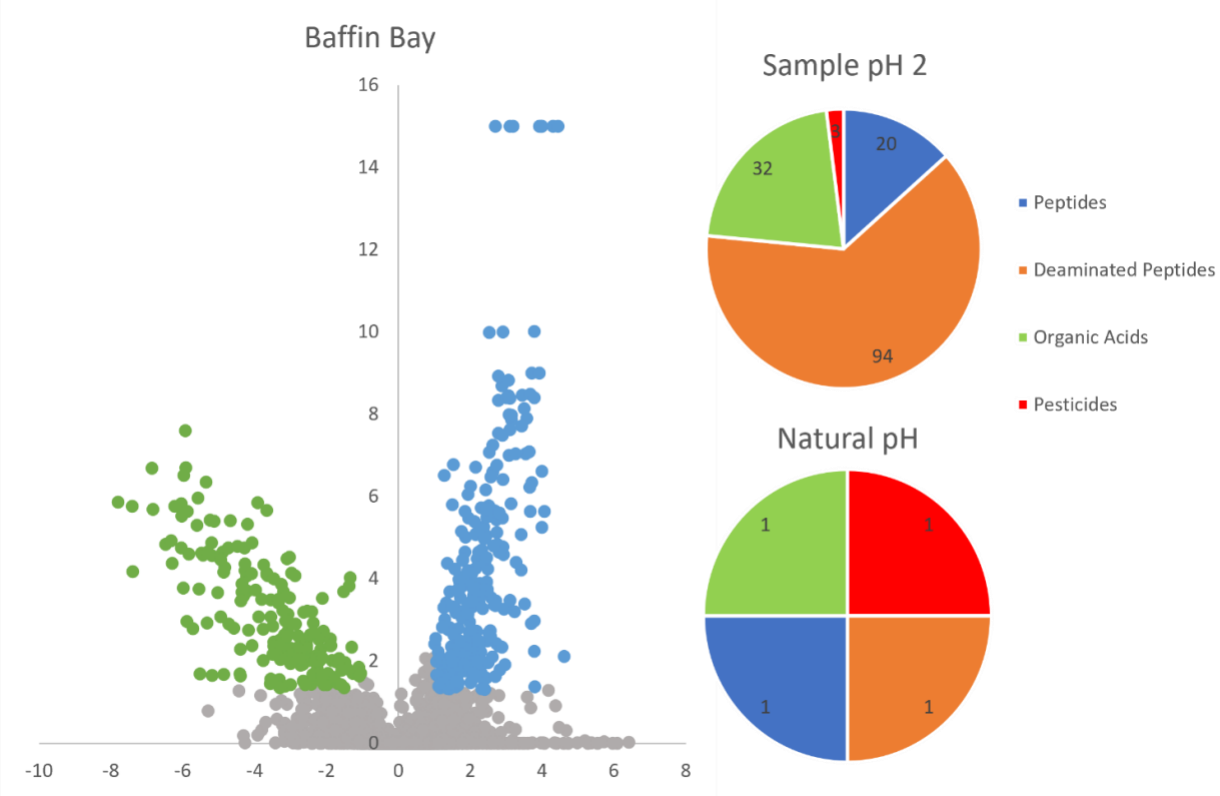

Figure 15: Volcano plot comparing differences between samples analyzed in positive mode at natural pH (left) and samples at pH 2 (right) of Baffin Bay. Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 402 compounds were unique to Sample pH 2 and 179 unique compounds when extracted at natural pH. Pie charts show number of mass list matches.

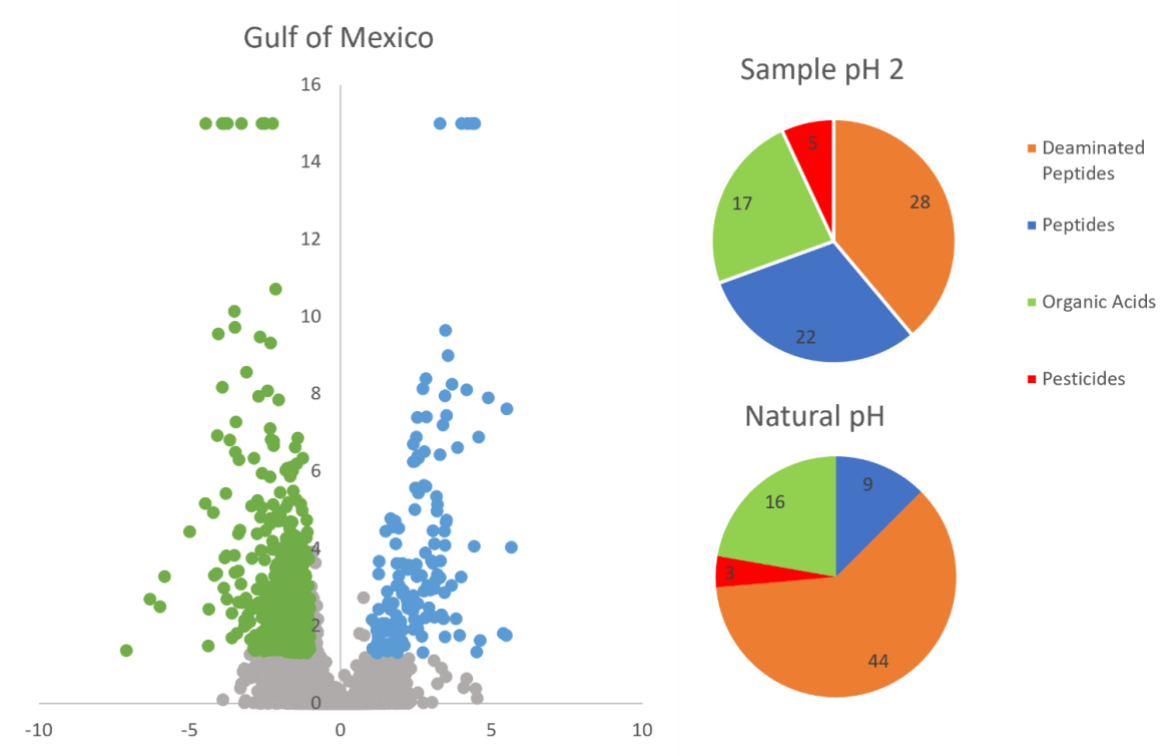

Figure 16: Volcano plot comparing differences between samples at natural pH (left) and samples at pH 2 (right). Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 402 compounds were unique to Sample pH 2 and 179 unique compounds when extracted at natural pH. Pie charts show number of mass list matches.

The Volcano plot comparing the Gulf of Mexico samples extracted at pH 2 and natural pH showed many molecular differences between each sample type (Figure 17). A total of 446 compounds were unique to the sample pH 2 procedure with 78 unknowns without a molecular formula or structural annotation (17.4% no annotation). This fraction was well characterized with 140 molecular formulas and an additional 226 structural assignments. Samples extracted at natural pH yielded 148 unique compounds, less than those extracted with sample acidification. The unique compounds from the natural pH fraction were better characterized compared to

Baffin Bay with only 16 unidentified compounds, 25 compounds with only molecular formula and 106 structural assignments. (10.8% no annotation).

The Gulf of Mexico volcano plot also showed that different sample pH will isolate different compounds from the same DOM pool. The acidified samples had the most unique compounds as well as the highest DOC recovery which likely reflect the highly oxygenated nature of DOM. Both the natural pH samples and acidified samples had a high rate of annotation ranging from 82-89% unlike in Baffin Bay where natural pH samples remained largely unknown. The discrepancy here is likely due to differences in the DOM pools themselves where the Gulf of Mexico contains more organic matter that is better represented within molecular databases used for annotation in this study. These differences likely arise from the different sources of organic matter within these systems.

The Lavaca River DOM pool behaved similarly to Baffin Bay where I found a total of 402 compounds that were unique to the sample pH 2 procedure with only 18 true unknowns without molecular formula or structural annotation (4.4% no annotation). This fraction was well characterized with 50 compounds with only molecular formulas and an additional 334 structural assignments. Samples extracted at natural pH yielded 347 unique compounds, notably higher than the unique compounds for this extraction procedure at the other sampling sites. However, the unique compounds from the natural pH fraction were enigmatic with a total of 216 unidentified compounds, 66 compounds with only molecular formulas and 65 structural assignments. (62.2% no annotation)

The Lavaca River samples really highlighted the potential benefits of using multiple extraction procedures reflected by the high number of unique compounds from each extract compared. Like Baffin Bay the natural pH extract had a low annotation rate showing there are

high amounts of unknown materials that have previously gone undetected and uncharacterized. In comparing the number of hits to our in-house databases represented in the pie charts (Figure 18) a decrease in organic acid detection was observed. With natural pH extracts having a high nitrogen content it raises many questions as to what molecules make up the enigmatic DOM isolated in the unacidified fraction.

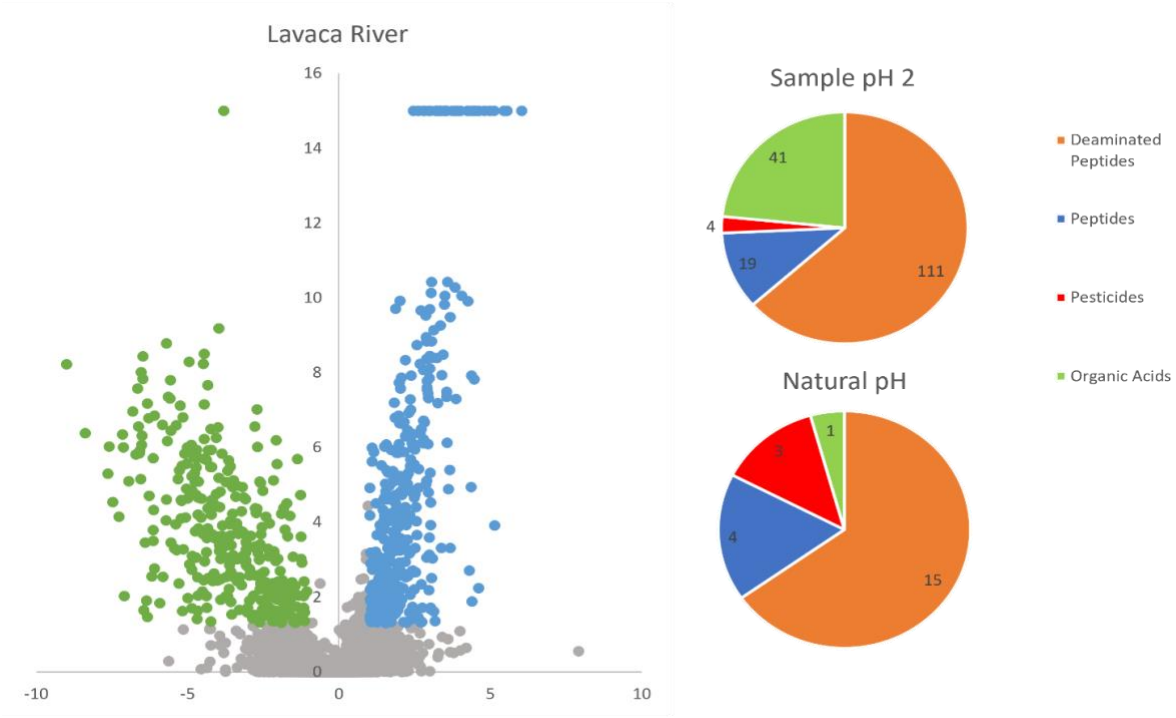

Figure 17: Volcano plot comparing differences between samples at natural pH (left) and samples at pH 2 (right)of Lavaca River. Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 402 compounds were unique to Sample pH 2 and 179 unique compounds when extracted at natural pH. Pie charts show number of mass list matches.

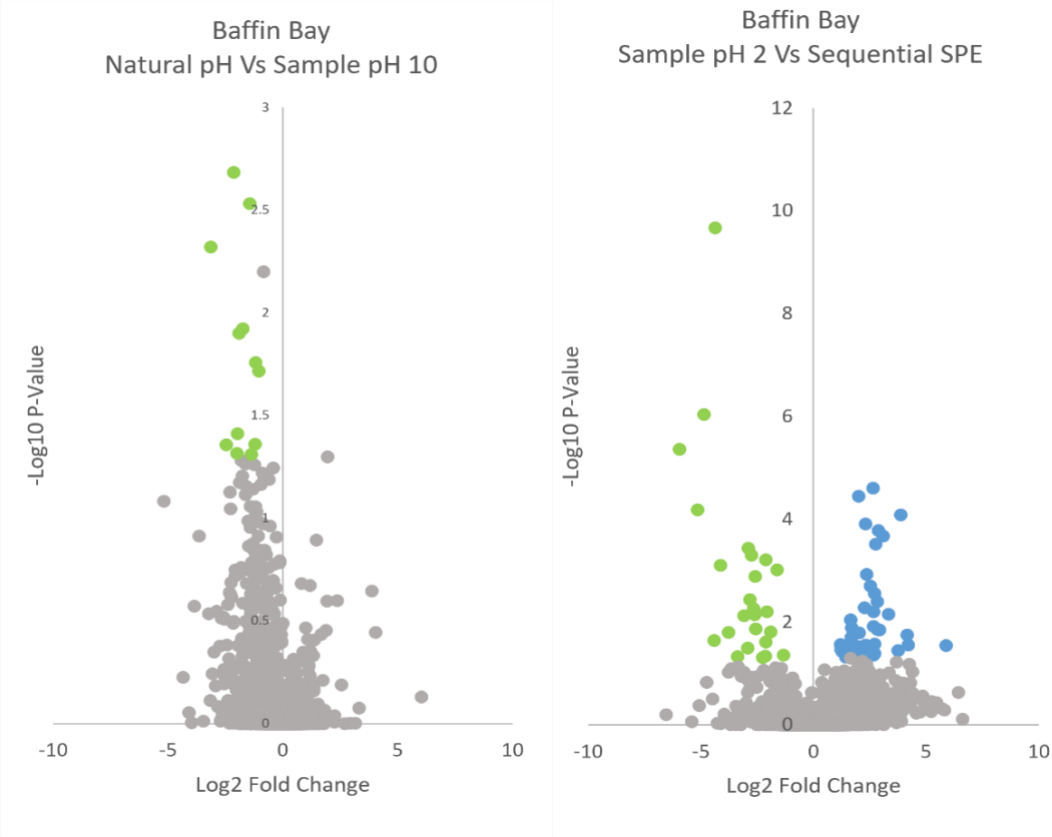

Figure 18: (A.) Volcano plot comparing differences between samples at natural pH (left) and samples at pH 10 (right). Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 12 unique compounds when extracted at natural pH with zero unique from sample pH 10 for Baffin Bay. (B) Volcano plot comparing differences between samples at pH 2 and the sequential SPE.

Comparisons of pH 10 and natural pH samples from Baffin Bay were done (Figure 19) to validate the similarity between the two extracts as suggested in the PCA plot with only 12 unique compounds detected with sample pH 10 and no unique compounds detected with natural pH. Out of the 12 unique compounds, 8 had spectral matches, all with amine functional groups either secondary or tertiary. A comparison of the acidified sample and sequential SPE from Baffin Bay

showed more differences than the comparison of natural pH versus pH 10. Acidified samples yielded 44 unique compounds and the sequential provided 25. In the sequential SPE 6 out of 25 were unknowns and 14 were DON. Very similar trends were observed comparing the same procedures across the other sample sites. These findings suggest that selecting just two representative SPE approaches for each fraction would isolate both fractions of DOM. In hopes to minimize resources necessary and maximize DOM coverage I recommend collecting one extract at natural pH and another at pH 2.

For positive mode analysis of DOM comparing pH effects of PPL derived SPE-DOM two main findings were shared across all sample sites. First is that two fractions of DOM can be isolated and analyzed using PPL through sample pH modification. The acidified fraction consisted of samples extracted at pH 2 or with a sequential SPE utilizing all elution approaches. This led to the highest DOC recoveries and a high number of unique compounds. The unacidified fraction consisted of samples extracted at natural pH ranging from 7.9 to 8.4 which gave rise to a nitrogen rich fraction of DOM isolated. This fraction tended to be more enigmatic with lower annotation rates leaving further investigation into this fraction necessary. In conclusion for positive mode analysis, it would be beneficial to analyze DOM extracted from acidified samples and from samples at natural pH to study DOM and DON.

3.3. Dissolved Organic Matter Characterization in Negative Mode by IC-Orbitrap-MS

negative mode a total of 3443 unique compounds were identified with 1063 tentative structural identifications and 714 molecular formulas assigned between all extraction procedures leaving 1665 unknown compounds (48.5% unknown). Negative mode analysis led to a far lower annotation rate likely due to differences in the overall spectral representation within most databases. A pie chart showing the number of annotations from our selected structural mass lists

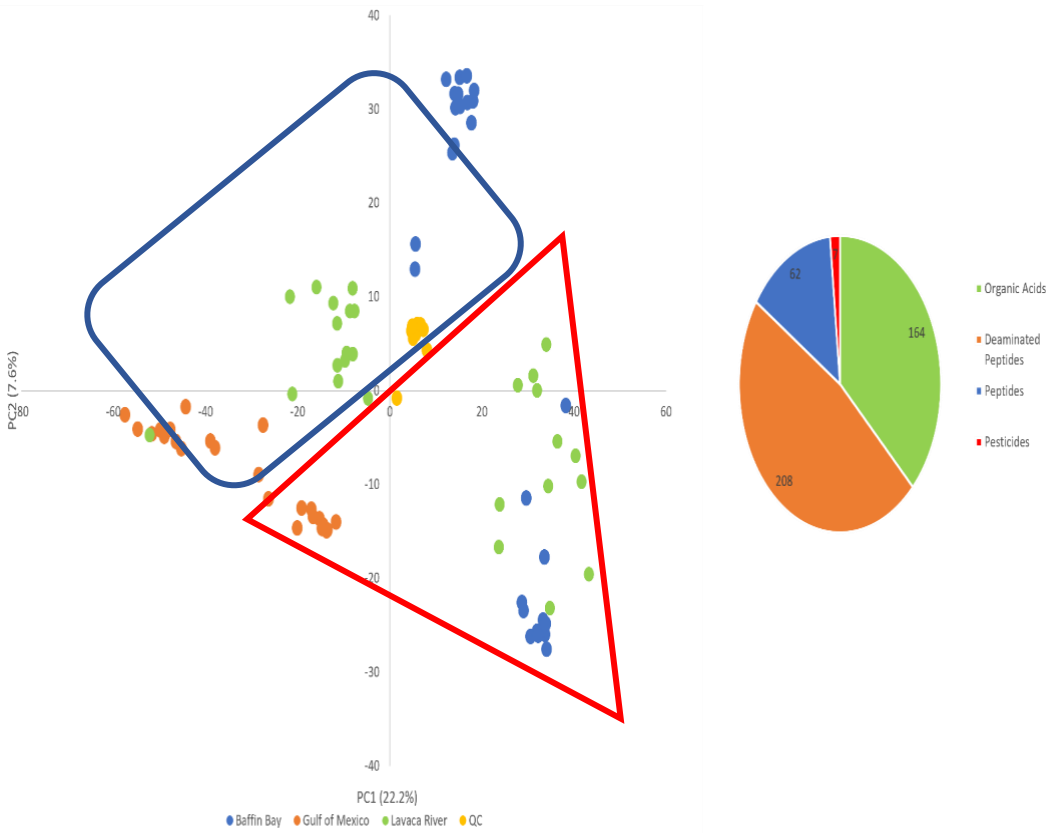

Figure 19: (A) Principal component analysis comparing all injections show clustering based on sample site. (B) Pie chart showing number of mass list matches for compounds identified in negative mode analysis

shows much more organic acids are observed when analyzing in negative mode with IC (Figure 20). A PCA to compare all samples from different sampling locations and SPE procedures was constructed in the same manner but for negative mode analysis. The distinct clustering pattern dictated by sampling location observed in positive mode analysis was not seen here. There was clear clustering of the Gulf of Mexico samples but some overlap within the Baffin Bay and Lavaca River samples. When analyzing the same spread of data but comparing based on sample pH it becomes apparent that sample pH plays a large role in the clustering in addition to the sample site. Samples that are extracted at natural pH or pH 10 are within the blue rectangle shown overlayed on Figure 20. When only considering these samples (natural pH and pH 10),

the sample site was the largest source of variation suggesting that the unacidified fraction between these sites are different. When comparing the samples that were extracted in acidic conditions or with the sequential SPE the Lavaca River and Baffin Bay samples are somewhat overlapping, suggesting similar chemical profiles between these sites. With estuaries and rivers having more-terrestrial inputs, the overlap between these two sites may be related to these systems having similar DOM sources from autochthonous inputs indicated by low C:N ratios.

For each sample site, a PCA was created to compare the overall effects of sample pH and elution solvent pH. Figure 21 shows the PCA for all SPE procedures tested for the Baffin Bay surface water samples. On the right-hand side of the Figure there is a tight cluster of samples that have been acidified to pH 2 with just a few data points that have strayed slightly. This indicates that for all samples that have been acidified or extracted with sequential SPE the DOM profile will come out looking roughly the same regardless of various elution techniques. On the right-hand side of the Figure there is a tight cluster of samples that represent the unacidified fraction. There are three points that are not directly within the tight cluster with differences in PC1.In the Baffin Bay samples these findings suggest that the two fractions identified in positive mode are extractable and analyzable with PPL based SPE. Both the points in the acidified and unacidified fraction that sit outside the tight cluster on either side do not seem to follow any distinct pattern but are differences that arose from PC1. Further investigation into what molecules made the differences PC1 and their potential sources are necessary to theorize why they did not cluster as well as the other points.

For the PCA of negative mode analysis on Lavaca River samples only (Figure 22) a familiar distribution of data can be observed, On the left-hand side, where PC2 is negative, you can find all of the samples that are proposed to extract the unacidified fraction. The samples that

isolate the acidified fraction all had positive PC2 values but were less densly clustered with roughly half having a positive or negative PC1 value. These findings suggest that for the Lavaca River samples that PC2 was responsible for distinguishing which fraction was isolated. Although PC2 can only explain7.5% of the total variation between all samples it is this small collection of molecules that distinguish the fractions. Further investigation into the individual molecules that make up PC2 are necessary to understand what components have previously gone undetected in studies that have only looked at the acidified fraction.

Figure 23 shows the PCA generated from the negative mode analysis of extracts from the Gulf of Mexico. Upon initial glance there are apparent similarities in this PCA to the one generated for Baffin Bay in Figure 21. The acidified samples all had positive PC2 values and mostly negative PC1 values. For the samples extracted at natural pH and pH 10, the unacidified fraction mostly had negative PC2 values with similar PC1 values to the acidified fraction. These findings suggest that PC2 was responsible for the differentiation between the acidified and unacidified fraction which was seen in the previous sampling locations. Further investigation into the specific components within the extract that differentiate these samples are necessary. Additionally, these PCA's show investigation into improving extraction procedure techniques are necessary since in ideal conditions replicates would be identical.

Overall, the PCA's for negative mode analysis uncovered a few important findings. First is that two fractions of DOM can be isolated with SPE with the same divisions as seen in positive mode. However, with PCA the magnitude or details of the individual differences can be lost since it is a dimension reducing technique to highlight the variation between samples. To briefly compare the findings in negative mode to positive mode it appears that PC2 consistently played a large role in differentiating the fraction. The PC2 values in positive mode explain 10-14% of the

variation while PC2 in the negative mode analysis explain 5.1% to 7.5% of the variation. This suggests that the differences in negative mode may not be as dramatic. This is further supported by the fact that in negative mode analysis the separation technique (IC) and polarity is designed to maximize the spectral coverage of organic acids which greatly limits the types of DON that is analyzed. To better understand the magnitude of differences between the two fractions and the effects of collecting both the acidified and unacidified fraction for negative mode analysis are further discussed using volcano plots.

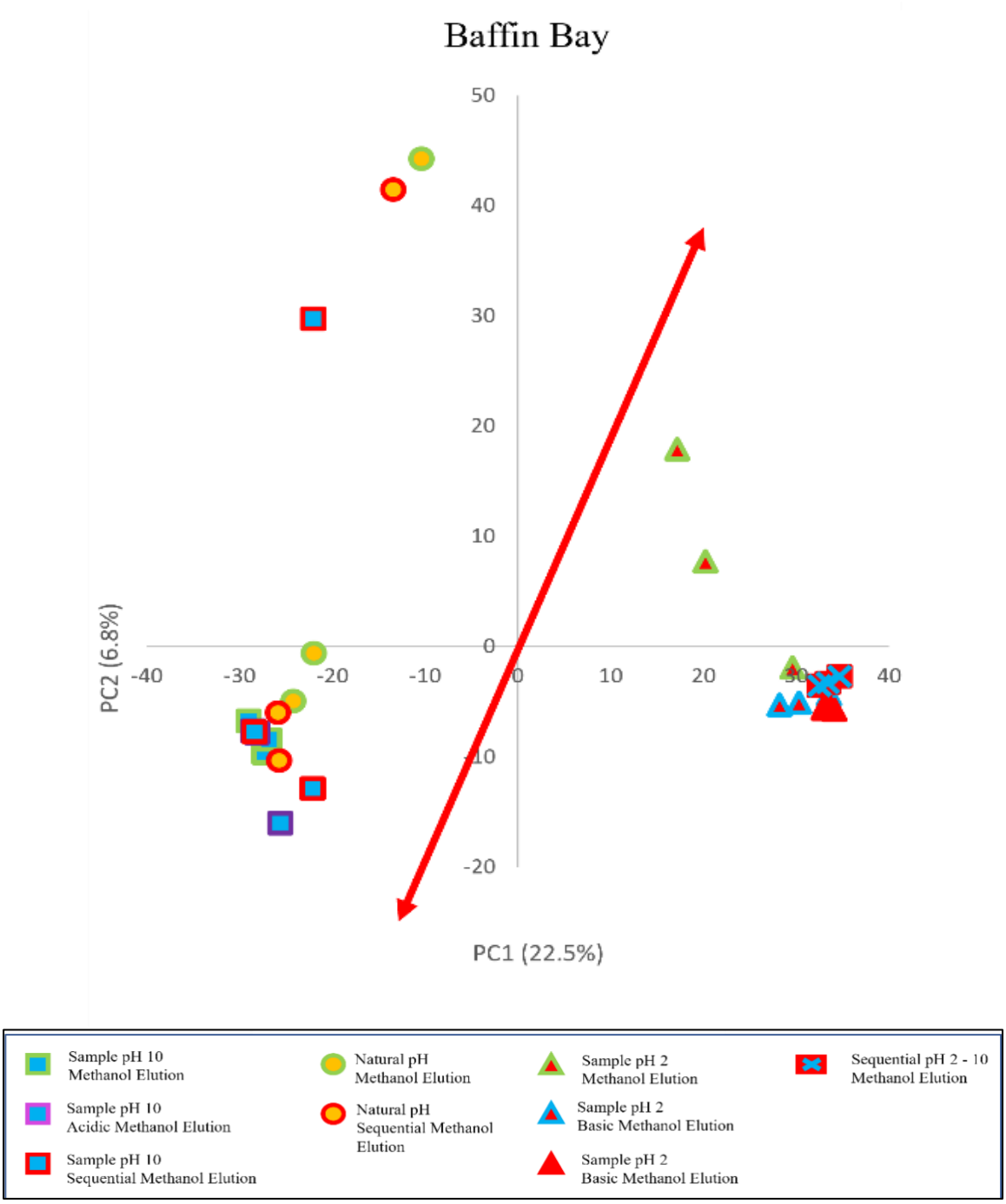

Figure 20: PCA of all SPE procedures from Baffin Bay for Negative mode analysis. PC1 accounted for 22.5% of the variation and PC2 accounted for 6.8%. Red line is drawn to show the division between the two major fractions.

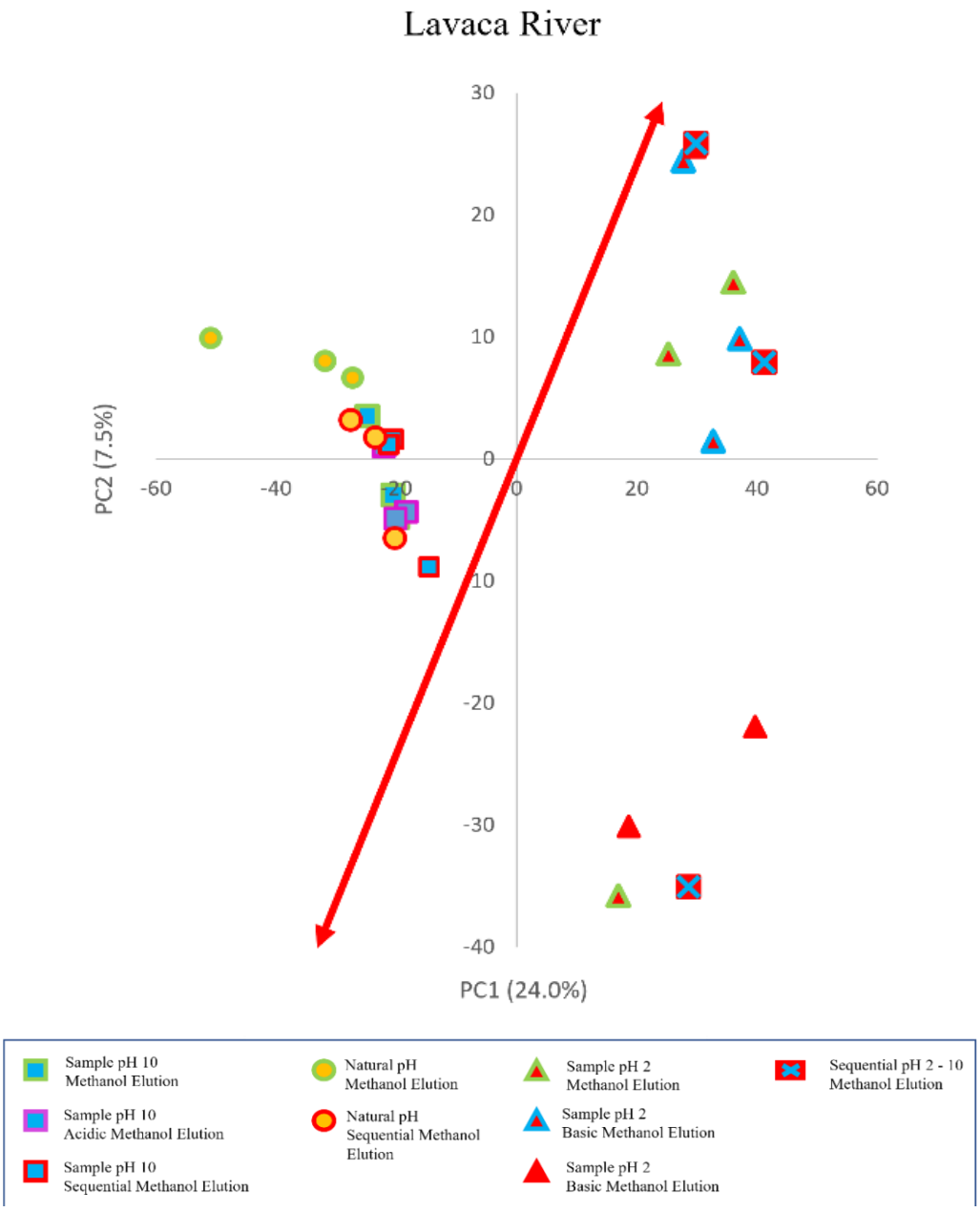

Figure 21: PCA of all SPE procedures from Lavaca River for Negative mode analysis. PC1 accounted for 24.0% of the variation and PC2 accounted for 7.5%. Red line is drawn to show the division between the two major fractions.

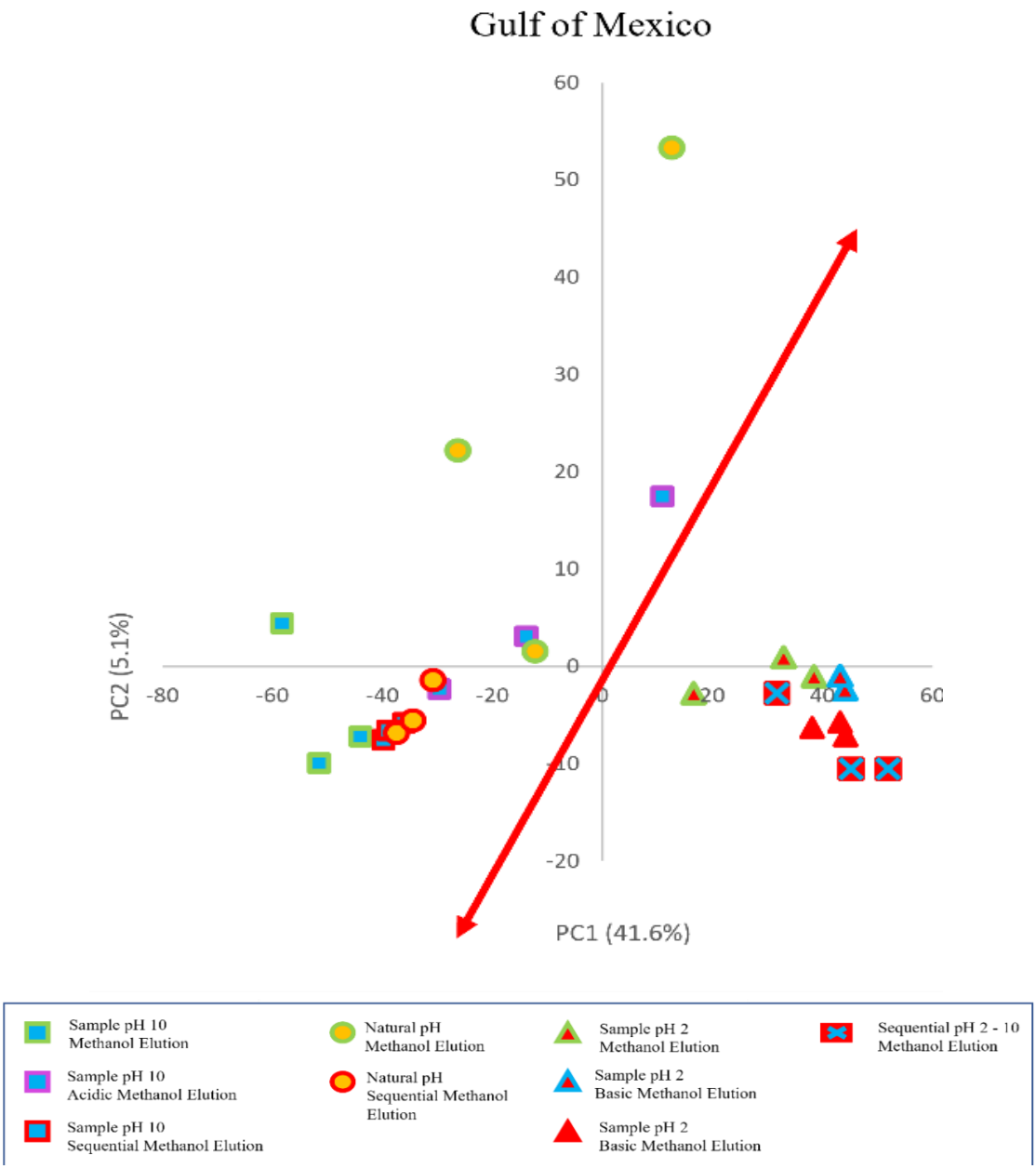

Figure 22: PCA of all SPE procedures from the Gulf of Mexico for negative mode analysis. PC1 accounted for 41.6% of variation and PC2 accounted for 5.1%. Red line is drawn to show the division between sample types.

A volcano plot comparing the extraction procedure on the Baffin Bay DOM pool found a total of 239 compounds that were unique to the sample pH 2 procedure with 117 true unknowns without a molecular formula or structural annotation (48.9% no annotation) (Figure 22). This fraction was poorly characterized with 73 molecular formulae only compounds and 48 structural assignments. Samples extracted at natural pH yielded 127 unique compounds, notably less than those extracted with sample acidification. The unique compounds from the natural pH fraction were moderately annotated with a total of 22 unidentified compounds, 30 compounds with molecular formulas and 74 structural assignments. Unique compounds (17% no annotation) from the natural pH showed more peptides highlighting the capabilities of this fraction study DON.

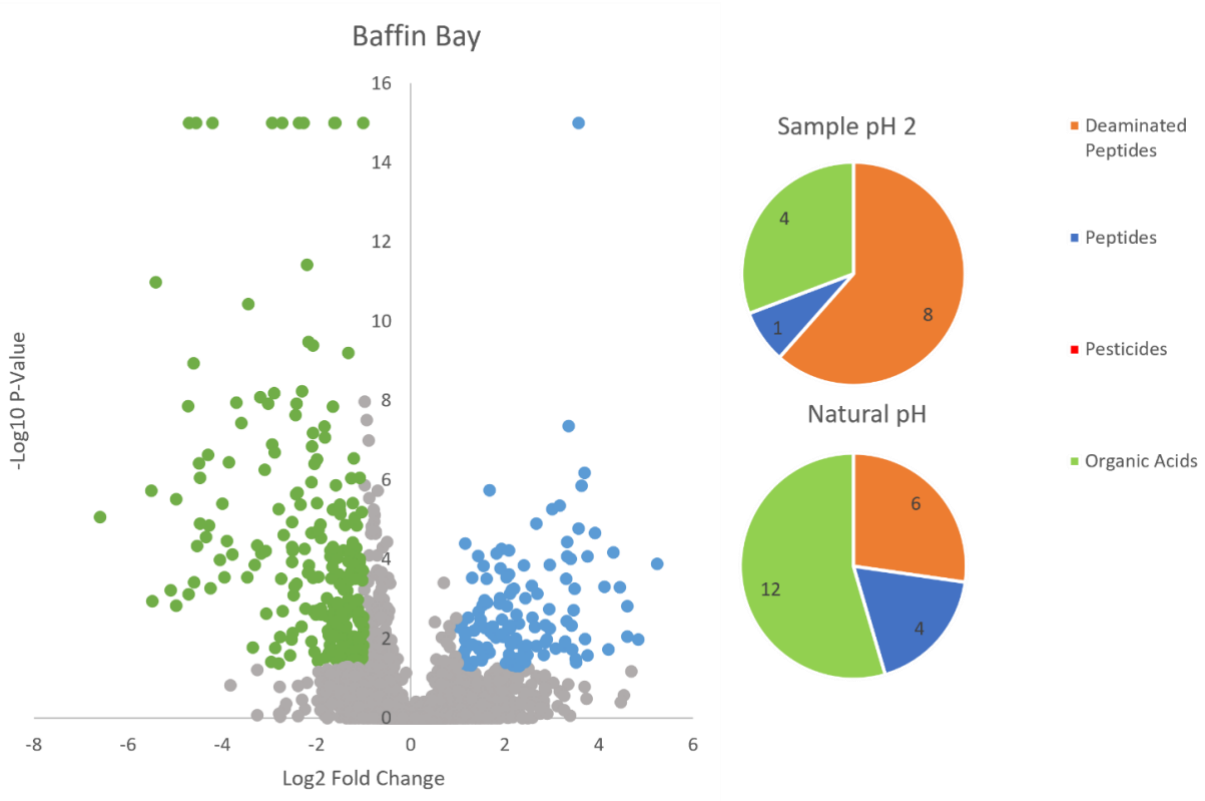

Figure 23: Volcano plot comparing differences between samples at natural pH (right) and samples at pH 2 (left). Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 239 compounds were unique to Sample pH 2 and 127 unique compounds when extracted at natural pH. Pie charts show number of mass list matches.

This Volcano plot comparing natural pH samples to acidified samples from Baffin Bay further support the fact that both procedures are isolating different components within the initial DOM pool at Baffin Bay. Although the number of unique compounds to the natural pH fraction is smaller than what is seen in positive mode analysis the addition of 105 identified compounds could be of great benefit in unlocking the mysteries of DOM. Additionally when comparing the types of compounds that were unique to each fraction the natural pH samples isolated a higher percentage of peptides which are components of DON with acidic character. This finding suggests that for the analysis of small peptides and DON molecules with functional groups capable of negative mode ionization isolating the unacidified fraction is important.

The volcano plot comparing the two major fractions on the Gulf of Mexico DOM pool found a total of 346 compounds were unique to the sample pH 2 procedure with 205 unknowns without a molecular formula or structural annotation (59.2% no annotation). This fraction was poorly characterized by 70 molecular formulas and 70 structural assignments. Samples extracted at natural pH yielded 25 unique compounds, notably less than what unique compounds were extracted with sample acidification. The unique compounds from the natural pH fraction were largely enigmatic with 13 unidentified compounds, 5 compounds with molecular formulas and 7 structural assignments. (52% no annotation).

Unlike what was seen in Baffin Bay negative mode analysis this volcano plot for the Gulf of Mexico shows a similar effect with a differing magnitude of effect. The unacidified fraction isolated from natural pH SPE only provided 25 unique compounds which is dramatically less that what was seen in positive mode analysis. This can be explained by the differences in the initial DOM pool and its characteristics. The Gulf of Mexico had to lowest DON values and the lowest recovery compared to Lavaca River and Baffin Bay. This is likely a result of the different types of DON that are in the Gulf of Mexico compared to the other sites explaining their different behavior.

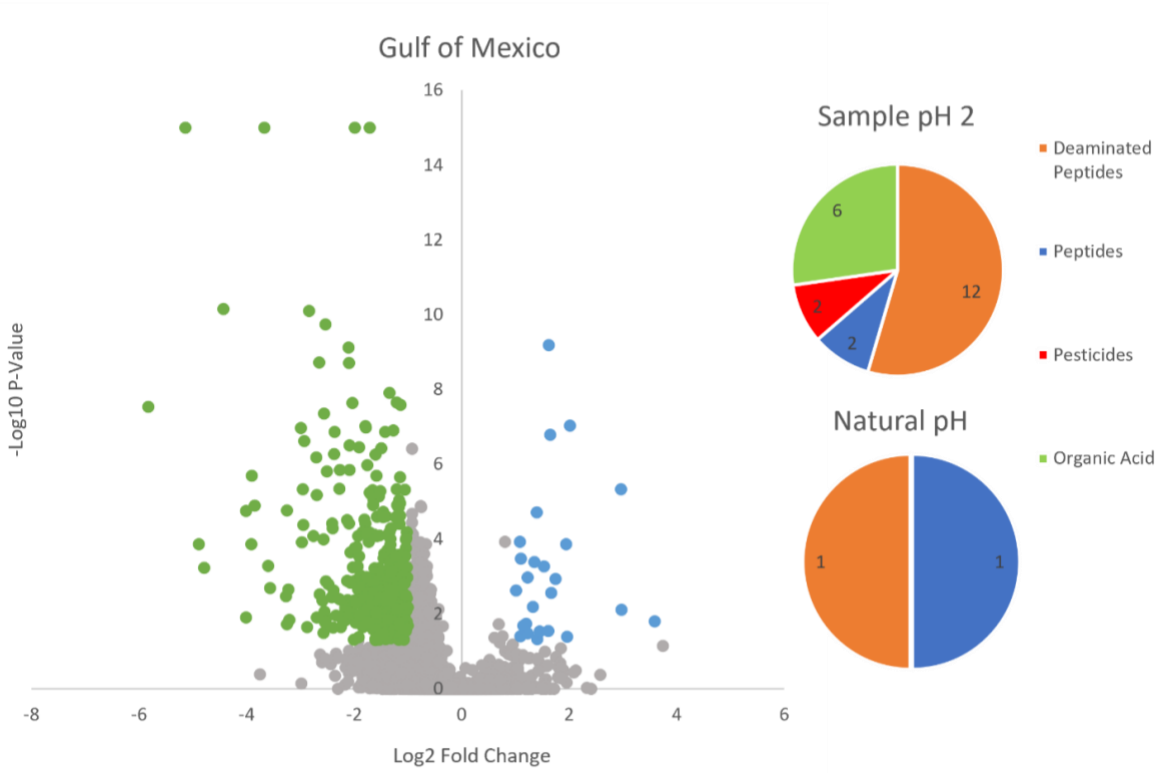

Figure 24: Volcano plot comparing differences between samples at natural pH (right) and samples at pH 2 (left) Gulf of Mexico. Samples above -log10 P-value (y-axis) and Log2 fold change (x-axis) were identified as unique compounds per extraction procedure. 205 compounds were unique to Sample pH 2 and 25 unique compounds when extracted at natural pH. Pie charts show the number of mass list matches.

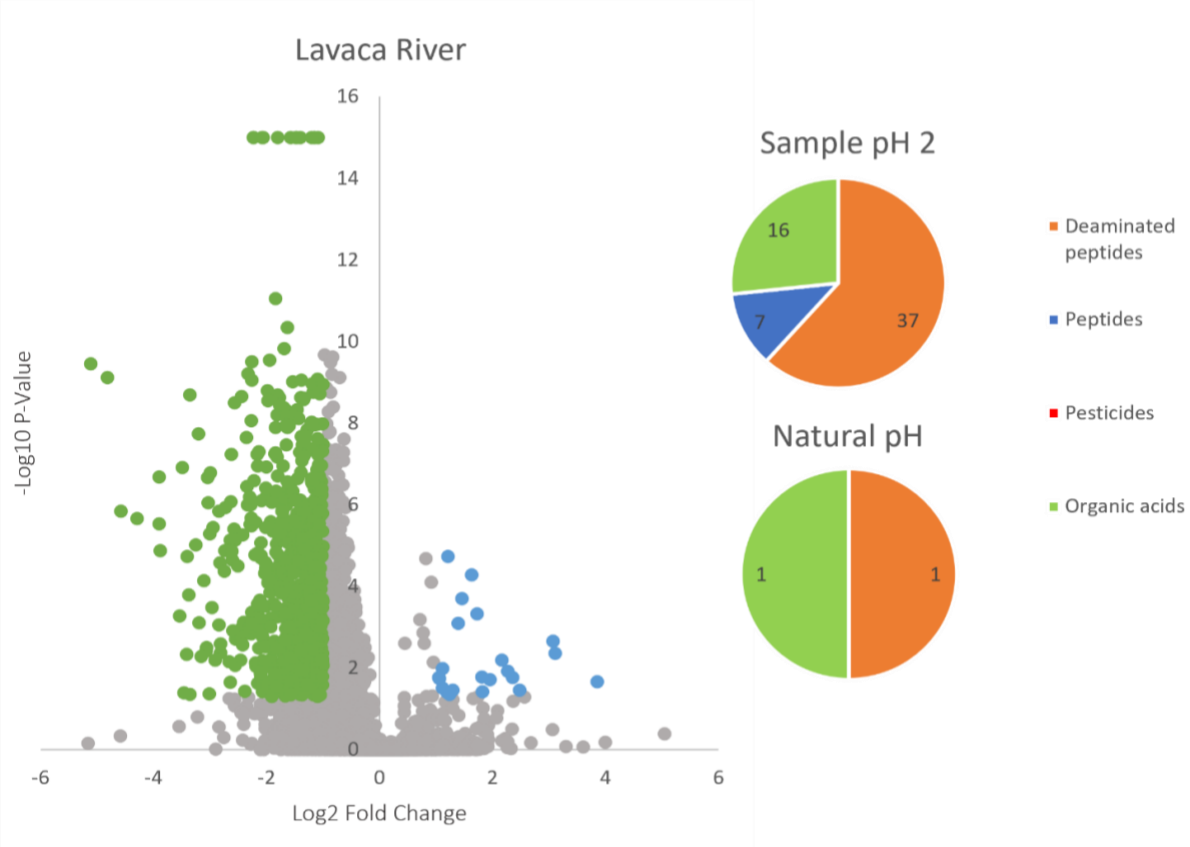

Figure 25: Volcano plot comparing differences between samples at natural pH (right) and samples at pH 2 (left). Samples above -log10 P-value (y axis) and Log2 fold change (x axis) were identified as unique compounds per extraction procedure. 638 compounds were unique to Sample pH 2 and 23 unique compounds when extracted at natural pH. Pie charts show number of mass list matches.

The volcano plot comparing the extraction procedure on the Lavaca River DOM pool found a total of 638 compounds were unique to the sample pH 2 procedure with 323 true unknowns without a molecular formula or structural annotation (50.6% no annotation). This fraction was moderately characterized with 146 molecular formulas and an additional 168 structural assignments. Samples extracted at natural pH yielded 23 unique compounds which

were the least of all extracts that unique compounds were extracted with sample acidification. The unique compounds from the natural pH fraction were enigmatic with a total of 9 unidentified compounds, 4 compounds with molecular formulas and 10 structural assignments (39% no annotation).

For the Lavaca River site this volcano plot shows the limited benefit of targeting the unacidified fraction when preparing for negative mode analysis using anion exchange chromatography. One could speculate different outcomes with negative mode analysis of this fraction using separation technique that is less specific to organic acids.It was shocking to see so many unique compounds to the acidifed fraction in comparison to the other sites. I speculate this could be due to terrestrial sourced DOM like lignin phenols, and CRAM which can be molecularly diverse and unique to this sampling location[97].

Between all the sampling sites the natural pH extraction did not provide a high number of unique compounds for negative mode analysis. This likely reflects the anion exchange chromatography system since it primarily separates compounds based on their negative ionization state. Analytes that do not have acidic characteristics are likely to be unseparated leading to poor spectral coverage since they all reach the MS at once. There was an added benefit of analyzing high nitrogen samples such as samples taken from Baffin Bay which showed an increase in peptide detection. Overall, it seems that in negative mode analysis of the unacidified fraction much of the identified compounds were nonspecific to the extraction procedure. It is likely that the separation seen on the PCA plots are due to large differences in signal intesity and the loss of some acidifed fraction specific compounds that were represented in PC2.

To investigate differences within extraction procedures that target the same fraction of DOM a volcano plot of pH 10 and natural pH samples from the Gulf of Mexico is shown below

(Figure 25). Both extracts showed very little differences between the two indicating they are extracting identical fractions of DOM. This similarity was also observed by comparing the pH 2 samples from Lavaca River to the sequential SPE procedure. Overall, the unique compounds analyzed in negative mode from the unacidified fraction did not provide many additional compounds. This is likely due to the chemical nature of the unacidified fraction, which may separate well within the IC system or be efficiently ionizable in negative mode. The proportion of short peptides identified between all sampling sites and extraction procedures decreased. At the same time, organic acids increased with negative mode analysis highlighting the importance of multiple types of chromatography using both polarities to achieve high coverage of DOM.

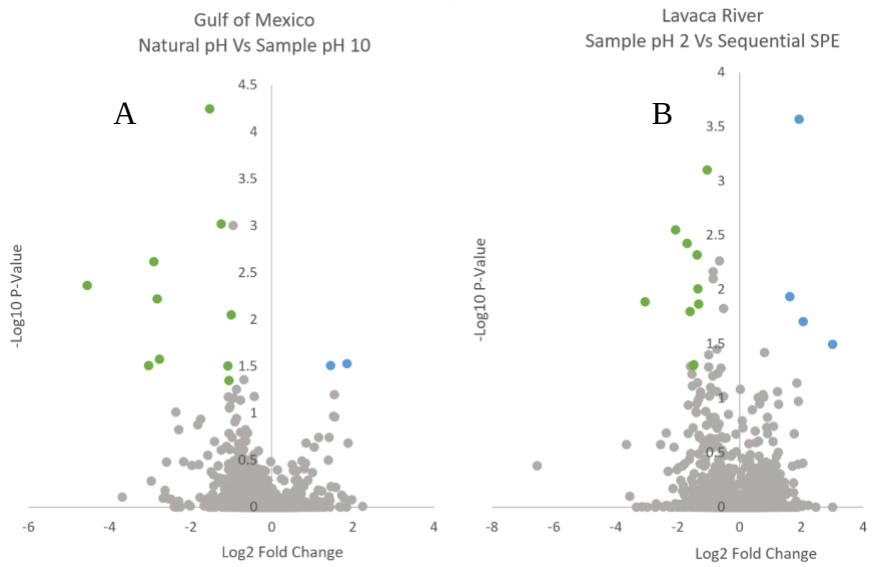

Figure 26: A.) Volcano plot comparing differences between natural pH (right) samples and pH 10 (left) samples. Samples above -log10 P-value (y-axis) and Log2 fold change (x-axis) were identified as unique compounds per extraction procedure. 2 unique compounds when extracted at natural pH with 10 unique from sample pH 10 for Baffin Bay. (B) Volcano plot comparing differences between samples at pH 2 and the sequential SPE. Extracts from pH 2 samples had 4 unique compounds, while the sequential SPE

4. CONCLUSIONS

The ability to characterize and monitor changes in DOM through molecular formula assignment, structural identification, and quantification is ultimately dependent on the sample preparation and the DOM isolation stage. With many possible combinations of chromatography, ionization sources, acquisition modes, and data processing tools, it is critical to evaluate extraction procedures to make well informed conclusions about the data collected and how they represent the actual DOM nature without isolation and extraction bias. After 15 years of DOM research with PPL based sorbents, this study aimed to shift and expand the fraction of DOM isolated through pH modifications.

Sample pH was the largest determinant in the total characteristics of DOM isolated. I propose that through pH modification, one can isolate two major fractions of DOM. The standard SPE procedure initially presented by Dittmar et al. had the highest recovery for both DOC and DON but had the higher C:N ratios than the initial DOM pool. Samples at pH 10 or natural pH had lower overall recoveries but higher C:N ratios with fewer organic acids identified in the extracts. Despite the acidified procedures leading to higher recovery of DON I observed the unacidified approaches extract DON that is unique in composition and likely important molecules of interest. By collecting different fractions of DOM with PPL SPE resins, we are now able to characterize a previously hidden fraction of DOM. Further investigation of the structural differences between the fractions is suggested. Notably, the DOM fractions extracted from the unacidified samples had a higher rate of unknowns in molecular formula assignment and structural elucidation through spectral matching. This fraction was enigmatic in both the positive and negative mode analysis leaving much research to be done in characterization of this DOM. With millions of spectra to compare against the low annotation rate of this fraction raises

questions as to the molecular components and biogeochemical significance. One possibility to explain the lower annotation rate in the unacidified fraction as it contains more diverse compounds that evade our molecular formula predictions and spectral matching since they may include metal complexes or completely novel structures. The molecular formula calculator is constrained by set parameters to control the types and number of the elements to use so combinations that fall outside of the determined parameters will go unassigned to avoid misidentification. Further investigation is necessary to identify and characterize the diverse compounds found in the unacidified fractions in parallel to mass spectrometry.

This study has shown that sample pH is a large determinant in the fraction of DOM isolated when conducting PPL based SPE. Furthermore, using various sample pH's will increase the spectral coverage possible by isolating a wider fraction of DOM. A sequential SPE approach has not been determined to represent both fractions, which could be an artifact of differing size or total contribution to DOC between the hydrophobic acidic and hydrophobic basic fractions. This study has shown that extracting a 1 L seawater sample with two SPE procedures expands the analytical window toward a more comprehensive characterization of DOM. This study also showed the capabilities of an untargeted workflow with optimized extraction and chromatography for positive and negative mode analysis of DOM. When using the standard approach where samples are acidified, and DOM is analyzed with one polarity a large amount of important biogeochemical information is undoubtedly lost. This study provides a simple approach to maximize spectral coverage of DOM through simple modifications to well established SPE procedures. With the analysis of multiple fractions of DOM in both polarities achieved through sample pH modification, I have created tools to expand the analytical window to characterize complex mixtures such as DOM.

5. FUTURE DIRECTIONS

This study has shown that two major fractions of DOM can be collected with PPL based SPE resins through pH modification. By collecting these different fractions via SPE one can now identify more components that make up to total DOM and DON pool. However, expanding the PPL extractable DOM comes at the cost of sample splitting, which increases sample preparation time. Additionally, when analyzing two extracts from the same water sample there are concerns with the hydrophobic DOM that is nonspecific to either technique to be overrepresented. Further development on a sequential extraction technique to combine samples to maximize throughput could benefit biogeochemists. Specifically, we will test an approach where a natural pH sample is isolated and then brought to pH 2 to combine both fractions and minimize sample alteration. I believe our newly adjusted sequential SPE approach can capture the differences between the acidified and unacidified fractions without inflating the proportion of the nonspecific DOM.

However, with increased sample complexity in samples that have both fractions, spectral coverage could be sacrificed as fragmentation spectra collection is time (and cycle time) limited. For samples with combined fractions implementing intelligent acquisition modes like AcquireX could greatly increase spectral coverage and, therefore, structural annotation in highly complex samples, although it increases total analysis time. Using AcquireX can improve spectral coverage by creating inclusion and exclusion lists on the fly aiming to collect the most fragmentation spectra possible with DDA through multiple injections[103].

Across all samples the annotation rate had room to improve in quality and coverage. One way to improve the annotation rate and quality would be to adjust data analysis parameters. Modifying the molecular formula calculations to include a wider variety of elements could provide future directions to investigate more diverse molecular formulas but could increase data

complexity and compromise the quality of the assignment in some cases. Since the overall annotation rates were lower than desired, implementing tools for in-silico structure predictions, molecular networking, and compound classification could improve the amount of information generated from these samples.

Although available spectral databases contain millions of spectra to match our spectra to there is a bias introduced by the compounds that are represented. Better represented types of molecules within these databases will be annotated more and could overshadow important key players within the mixture. General tools to improve the characterization of complex mixtures are desired. To help address this I will develop the largest in-house database for organic acid analysis using anion exchange chromatography which can aid in identifying negatively ionizable components of DOM.

DOM analysis from a true untargeted approach can provide important information about components in a mixture with some capabilities in relative quantification. Improving analysis through a hybrid approach to maximize spectral coverage while specifically targeting key analytes for absolute quantitation is of great interest. To do so, further understanding of individual molecule behavior is necessary through targeted studies to better understand recovery dynamics with a wide range of known compounds. Through the study of representative molecules for a wide variety of compound classes, more insights into structural specifics will improve our understanding DOM and it analysis.

Overall, this study has provided a good starting point in experimental design that could be used to study DOM in various systems. With the increased spectral coverage gained through multiple fraction analysis made possible by this study, transformations in DOM can be monitored in much greater detail than previously possible with PPL based SPE-DOM. I hope this

approach can allow biogeochemists to better understand DOM and uncover many important components of DON that have gone previously unexplored.

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