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ANAEROBIC AND AEROBIC BIODEGRADATION OF THE OIL DISPERSANT COMPONENTS 1,2-PROPANEDIOL AND 2-BUTOXYETHANOL IN SEAWATER

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ANAEROBIC AND AEROBIC BIODEGRADATION OF THE OIL DISPERSANT COMPONENTS 1,2-PROPANEDIOL AND 2-BUTOXYETHANOL IN SEAWATER

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ABSTRACT

Oil spills are a recurring issue associated with fossil fuel consumption. The largest accidental oil spill in the history of the petroleum industry was the *Deepwater Horizon* explosion and seafloor well blowout, where the *Deepwater Horizon* oil rig exploded and sank, allowing the well to gush uncontrolled from April 20, 2010, until it was capped on July 15, 2010, releasing an estimated 210,000,000 gallons of oil. Oil dispersants were used in unprecedented quantities during the cleanup response to the spill with a total of 1,840,000 gallons of the dispersant COREXIT being applied.

The goal of this research was to evaluate the biodegradation of the oil dispersant components 1,2-propanediol (1,2-PD) and 2-butoxyethanol (2-BE) in seawater and under anaerobic conditions, such as those found in near-shore estuarine environments of the Gulf of Mexico. Relatively little is known about the biodegradability of these two compounds in seawater. The specific research objectives were: 1) to evaluate the terminal electron acceptor (TEA) conditions under which 2-BE was biodegraded in microcosms and enrichment cultures based on the stoichiometry of electron acceptor and 2-BE utilization; and 2) to develop enrichment cultures capable of using 2-BE as a growth substrate under anaerobic and aerobic conditions and evaluate the role of sulfate-reducing bacteria through the use of molybdate as an inhibitor.

The work conducted and described herein showed that: 1) Analytical methods were successfully developed for quantification of 1,2-PD, 2-BE, nitrate, ferrous iron, and sulfide in the high ionic strength matrix of seawater; 2) 1,2-PD biodegraded in seawater under both aerobic and anaerobic conditions in microcosms constructed with seawater

and sediment from Bay Jimmy, Louisiana, which appears to be the first report of 1,2-PD biodegradation in a high salt environment. Because of the high level of biodegradable organic matter in the microcosms, it was not possible to associate the anaerobic consumption of 1,2-PD to a specific terminal electron acceptor; 3) 2-BE biodegraded in seawater under both aerobic and anaerobic conditions in microcosms constructed with seawater and sediment from Bay Jimmy, Louisiana, which appears to be the first report of 2-BE biodegradation in a high salt environment. Because of the high level of biodegradable organic matter in the microcosms, it was not possible to associate the anaerobic consumption of 2-BE to a specific terminal electron acceptor; and 4) Biodegradation of 2-BE was sustained in aerobic and anaerobic enrichment cultures, through two transfers (1% v/v). Nitrate, Fe(III) and sulfate did not appear to be used as the TEAs under anaerobic conditions. Molybdate slowed but did not stop consumption of 2-BE. Sulfide was detected, but at levels well below the amount expected if complete mineralization occurred with sulfate as the TEA. Based on these results, the presumptive pathway for anaerobic biodegradation of 2-BE in the study was via fermentation. Products were not measured, although 2-butoxy acetic acid and hydrogen are likely intermediates.

The results of the study definitively show that 1,2-PD and 2-BE biodegraded in microcosms constructed using Bay Jimmy sediment and transfer enrichment cultures. Analytical methods for the measurement of 1,2-PD, 2-BE, Fe(II), nitrate, and sulfide were developed, which has laid the foundation from which future students will

investigate the biodegradation of 1,2-PD, 2-BE, and other COREXIT compounds in seawater and in the presence of petroleum hydrocarbons.

DEDICATION

To my parents, Martin and Madeleine Rhiner, for their support and encouragement.

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LIST OF ABBREVIATIONS

1,2-PD	1,2-propanediol
2-BE	2-butoxyethanol
DDI	distilled, deionized
EDTA	ethylenediaminetetraacetic acid
FID	flame ionization detector
GC	gas chromatograph
TCD	thermal conductivity detector
TEA	terminal electron acceptor
TRC	Thermodynamics Research Center
WC	water controls

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Oil spills are a recurring issue associated with fossil fuel extraction. The most recent large oil spill in the United States, the LA Pipeline spill, occurred on May 15, 2014, near Los Angeles, in Atwater Village, CA, with an estimated 10,000 gallons of oil spilled (1). The most recent large oil spill on a body of water in the U.S. was the BP Lake Michigan spill on March 24, 2014, with an estimated 1,638 gallons of oil spilled (2). The most recent large marine oil spill in the U.S. was the Houston Ship Channel oil spill that occurred on March 22, 2014, near Galveston, TX, with an estimated 168,000 gallons of oil spilled (3). Oil spills are a relatively common form of pollution associated with fossil fuel consumption and pose a significant environmental threat.

The largest accidental oil spill in the history of the petroleum industry was the *Deepwater Horizon* explosion and seafloor well blowout, where the *Deepwater Horizon* oil rig exploded and sank allowing the well to gush uncontrolled from April 20, 2010, until it was capped on July 15, 2010 (4). It was declared sealed on September 19, 2010 (5). An estimated 4.9 million barrels (206 million gallons) of oil were released into the Gulf of Mexico (6). Ironically, the oil rig sank on Earth Day, April 22, 2010.

The largest marine oil spill in the United States prior to the *Deepwater Horizon* blowout was the *Exxon Valdez* oil tanker spill on March 24, 1989, in Prince William Sound, AK, with an estimated 11,000,000 gallons of oil spilled (7). The largest marine oil

spill prior to the *Deepwater Horizon* well blowout occurred about 1,000 km south and three decades earlier, when an exploratory deep sea oil rig, *Ixtoc I,* operated by Petróleos Mexicanos, suffered a well blowout and spilled an estimated 118,000,000 gallons of oil from June 3, 1979 to March 23, 1980 (8).

The oil spill cleanup process can involve various measures including the use of booms to control oil slicks, burning of the oil, and the application of dispersants. Dispersants are mixtures of surfactants and solvents that serve to break up the oil and allow it to disperse into colloids. Dispersants are used to limit the surface impact of an oil spill, although dispersant use increases the impact on the aquatic environment, and can increase the mobility of oil in the environment with the potential to increase its subsurface transport. Dispersant use involves a trade-off; in exchange for protecting shorelines and beaches, the pollution is instead dispersed throughout the water column.

Oil spill contamination can impact the water column, seafloor, and near-shore estuarine areas. The use of dispersants increases the environmental implications of the spill as the dispersants themselves contain toxic compounds (9-11).

Dispersants act as surfactants to break the hydrophobic petroleum into colloids called micelles. These small droplets are easily dispersed throughout a water body. The droplets increase the available surface area of the oil and may speed its natural biodegradation by indigenous microorganisms. However, there is concern that toxic components of petroleum, such as benzene, toluene, ethylbenzene, and xylenes, are able to be transported farther via the highly mobile micelles (12). The increased mobility may spread the reach of the oil spill into areas where the oil and dispersant mixture will

biodegrade under anaerobic conditions, such as deeper into coastal mud flats, which then results in slower degradation than would occur had the oil remained near the surface where degradation can proceed aerobically (13). Taking the oil away from the surface, where it naturally degrades under aerobic conditions, into underwater and subsurface areas where the conditions may be anaerobic, may result in slower rates of degradation and alter the degradation products formed.

Two types of dispersants were used during the *Deepwater Horizon* spill: COREXIT 9527 AND 9500. A variety of compounds are present in COREXIT 9527, including 2-butoxyethanol (BE), organic sulfonic acid salt, and 1,2-propanediol (PD). COREXIT 9500, used later in the *Deepwater Horizon* blowout response, does not contain 2-BE (14) . In total, 1,840,000 gallons of COREXIT were applied during the response, with 771,000 gallons released at the wellhead (6). The toxicity of COREXIT 9500 together with Macondo crude oil was evaluated by Rico-Martínez et al. (9) using *Brachionus plicatilis* species complex (Rotifera). They determined that the dispersant and oil mixture actually has a synergistic toxicological effect, increasing the toxicity of the spill 52-fold.

COREXIT containing 2-BE was also applied during the *Exxon Valdez* tanker spill and during several other marine oil spills (15) . 2-BE was also present in the oleophilic fertilizer Inipol EAP22 that was applied after the *Exxon Valdez* spill in an effort to accelerate natural microbial degradation of the stranded oil (16). It has been found that hydrocarbon-degrading bacteria can be inhibited by COREXIT 9500 and 9527 and that

their use could potentially diminish the capacity of microbial communities to bioremediate the spill (17-19).

In addition to its use in COREXIT, 2-BE is also a component of hydraulic fracturing fluids, which contain high levels of salts and form a brine during and after pumping of the well (20, 21). Degradation of 2-BE will thus occur in an anaerobic, high salt environment, making the results of this thesis relevant to the biodegration of 2-BE in the context of hydraulic fracturing.

Relatively little in general is known about the biodegradability of 2-BE, under aerobic or anaerobic conditions, and particularly in seawater. In the open ocean, sulfate will serve as the predominant electron acceptor under anaerobic conditions. In estuaries and other near-shore areas where groundwater and surface waters discharge, nitrate and ferric iron may be available. No information was found in the literature on biodegradation of 2-BE under nitrate-reducing, sulfate-reducing, or methanogenic conditions. Furthermore, no information was found in the literature on how components of crude oil may influence biodegradation of 2-BE. Information about the fate of 2-BE in seawater is needed to assess the overall environmental fate of this component of COREXIT 9527.

1,2-PD, also known as propylene glycol, is a minor component of COREXIT. In comparison to 2-BE, it is considered to be a readily biodegradable compound, is nontoxic, and is found in a wide range of consumer products ranging from processed foods, to cosmetics, to electronic cigarettes. 1,2-PD is a key ingredient in aircraft deicing fluids (22). In this context, biodegradation 1,2-PD has been evaluated under various

conditions (23-26). Runoff from airport runways have caused airports to exceed National Pollution Discharge Elimination System permits due to high chemical oxygen demand exerted by the 1,2-PD present in aircraft deicing fluids. When the available oxygen is consumed, conditions are quickly driven anaerobic. Studies of the subsurface areas adjacent to airport runways have been analyzed for their ability to attenuate the spilled aircraft deicing fluids. Soil adjacent to runways tended to undergo a decrease in soil redox potential due to the high chemical oxygen demand exerted by the 1,2-PD in aircraft deicing fluids , which will eventually favor the development of methanogenic conditions (24-26) .

The ability of 1,2-PD to serve as a microbial growth substrate has been exploited in the investigation of the anaerobic biodegradation of other environmentally relevant compounds. Adrian and Arnett (26) used 1,2-PD and ethanol to amend microcosms in the investigation of the anaerobic degradation of military explosives. Ethanol and 1,2-PD served as electron donors, providing syntrophically produced hydrogen for stimulating the anaerobic biodegradation of explosives in contaminated soil.

Nevertheless, the biodegradability of 1,2-PD under anaerobic conditions in seawater has not been investigated. This information is needed to assess the overall environmental fate of this component of COREXIT, especially in near-shore estuarine environments where anaerobic conditions may prevail.

1.2 Objectives

The goal of this research is to evaluate the biodegradation of 1,2-PD and 2-BE in seawater and under anaerobic conditions, such as those found in near-shore estuarine

environments of the Gulf of Mexico. Nitrate reducing conditions may occur, with nitrate being introduced from groundwater or from the Mississippi River carrying a high nitrogen concentration from agricultural fertilizers. Ferric iron reducing conditions may occur, with iron being introduced from groundwater and estuarine sediments. Sulfate reducing conditions may occur as sulfate is naturally present in seawater at 28 mM. Sulfate is predicted to be the dominant anaerobic electron acceptor. Fermentative and methanogenic conditions may occur when other more thermodynamically favorable reducing conditions have been exhausted by high TEA demand, which is exerted by the dispersed petroleum and is also found in sediment with a high organic matter content, such as that in near shore estuaries.

The biodegradability of 1,2-PD and 2-BE in seawater under aerobic, nitrate reducing, ferric iron reducing, and sulfate reducing conditions was evaluated in microcosms amended with sediment from the Gulf that was exposed to both oil contamination and dispersant. Sediment from Bay Jimmy, LA, an area impacted by the *Deepwater Horizon* blowout that received COREXIT, was used to construct microcosms in synthetic seawater. Biodegradation of 1,2-PD and 2-BE under anaerobic conditions was observed. Enrichment cultures were then developed for 2-BE (another student developed enrichments for 1,2-PD); dilution of the background organic matter contributed by the Bay Jimmy sediment made it possible to evaluate which TEA was associated with 2-BE biodegradation.

The specific research objectives were:

1. To evaluate the TEA conditions under which 2-BE was biodegraded in microcosms based on the stoichiometry of electron acceptor and 2-BE utilization.
2. To develop enrichment cultures capable of using 2-BE as a growth substrate under anaerobic and aerobic conditions and evaluate the role of sulfate-reducing bacteria through the use of molybdate as an inhibitor.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals, Sediment Samples, and Medium

1,2-PD (99.5%) and 2-BE (99%) were obtained from Alfa Aesar. Instant Ocean® was obtained from Aquarium Systems, Inc., to prepare synthetic seawater (34.9 g/L distilled deionized [DDI] water). Resazurin (1 mg/L) was added to serve as a redox indicator; the color is pink above -110 mV and clear below this level. Methanol (99.99%) was obtained from Burdick & Jackson. Dichloromethane (99.9%) was obtained from EMD Millipore. N,N-dimethyl-p-phenylenediamine sulfate (99%) and sodium sulfide nonahydrate ("extra pure") were obtained from Acros Organics. ENVI-Carb Plus cartridges were obtained from Supelco/Sigma Aldrich. Nitraver 5 cadmium reduction pillows were obtained from Hach. All other chemicals used were reagent grade, unless indicated otherwise.

A 1 M solution of poorly crystalline amorphic ferric iron oxyhydroxide, designed to mimic the naturally occurring mineral ferrihydrite, was made by adding, dropwise, 8 M NaOH to a 40 mM solution of $FeCl_3$. Once the pH reached 7, the solution was allowed to settle in the beaker. This solution was transferred to 8 150 mL centrifuge tubes and centrifuged for 15 min at 10,000 rpm (Evolution RC, Sorvall®). The supernatant was discarded and the centrifuge pellets were resuspended in DDI water. This resuspended solution was centrifuged again and rinsed with DDI. This centrifugation and resuspension procedure was repeated eight times to remove the majority of the chloride, giving an

Fe(III) solution of approximately 1 M (27). This solution is referred to as the Fe(III) gel in the remainder of this thesis.

Sediment from Bay Jimmy, LA, an area impacted by the *Deepwater Horizon* oil spill that received COREXIT, was used to construct the microcosms. The sediment was collected by Dr. Vijai Elango at Louisiana State University on June 26, 2013. The GPS coordinates of the sampling location are N 29.44 and W 89.89. The sediment was stored at room temperature until being shipped to Clemson University on July 17, 2013. The samples were stored at 4 °C until the microcosms were prepared on December 21, 2013.

The organic matter content of the sediment was not measured. However, the total organic content of sediment in Bay Jimmy South, with GPS coordinates 29.445 and 89.891, is an average of 9.06% TOC throughout the sediment column to a depth of 30 cm, based on 10 measurements at 3 cm increments (28).

2.2 Microcosms and Enrichment Cultures

Microcosms were prepared in sterilized 250 mL Wheaton media bottles outfitted with grey butyl rubber septa. The anaerobic treatments were prepared in an anaerobic chamber; water controls (WCs), autoclave controls, and aerobic treatments were prepared on the bench top. Sediment (75 g wet weight) from Bay Jimmy was added to each bottle. Instant Ocean® (150 mL) was filter-sterilized (0.22 µm, Millipore) and added to each bottle. The anaerobic microcosms were removed from the anaerobic chamber and the headspaces were sparged for 2 min with high purity N_2 (passed through a solution of titanium III citrate, to remove trace levels of oxygen). This was necessary to remove the

hydrogen present in the atmosphere of the anaerobic chamber, since residual hydrogen would increase the electron acceptor demand.

A stock solution of 1,2-PD was used to spike the microcosms to an initial concentration of 15 mg/L. This concentration was selected on the basis of being high enough to be measured but not so high that it is not consistent with the concentration that might be expected away from the point of application. The treatments consisted of three WCs, three aerobic bottles, three to which nitrate was added, three to which chelated ferric iron was added, three to which a ferric iron gel was added, and three unamended and presumably sulfate reducing bottles. Stock solutions of sodium nitrate, iron gel, and Fe(III)-EDTA (ethylenediaminetetraacetic acid) were added to the appropriate anaerobic bottles. The autoclave control bottles were autoclaved three times for 60 min, on three consecutive days; no other compounds were added.

The amounts of nitrate and Fe(III) added were based on the calculated TEA demand assuming complete oxidation of 1,2-PD, ignoring cell synthesis, and including a safety factor of 1.5. The calculations for 1,2-PD are included in Appendix A.

The microcosms were monitored for 1,2-PD. As the 1,2-PD was consumed, more was added. Monitoring required removal of 7 mL of settled liquid; the anaerobic bottles were opened in the anaerobic chamber while the aerobic bottles were opened on the bench top. The anaerobic bottles were sparged with N_2 after removal from the chamber, as described above; the aerobic bottles were sparged with room air. With each new addition of 1,2-PD, more nitrate and iron were added.

2-BE was added to the microcosms subsequent to 1,2-PD evaluation. After confirming the biodegradability of 2-BE, transfer enrichments were made. The first transfer was 1% by volume (1.5 mL from the microcosms to the new transfer enrichment bottles). The amounts of nitrate and Fe(III) added were based on the calculated TEA demand, assuming complete oxidation of 2-BE, ignoring cell synthesis, and including a safety factor of 1.5 These calculations are included in Appendix B. The first transfers were monitored for 2-BE and terminal electron acceptor (TEA) use. The enrichments that showed 2-BE biodegradation and TEA consumption were used to construct the second round of transfer enrichments, also at 1% by volume (1.5 mL from the first transfer bottle to the second transfer bottle). The second round of transfer bottles were monitored for 2-BE.

Molybdate was used as an inhibitor for sulfate reducing bacteria. The unamended first round transfer enrichments were presumed to be sulfate reducing, and received molybdate in two of the bottles after the inoculum for the second transfer had been removed. The second round transfer treatments (in triplicate) consisted of unamended bottles spiked with 20 mM molybdate (29-32), unamended without molybdate, aerobic bottles, autoclave controls, and WCs.

2.3 Sample Preparation for Analysis of 1,2-PD and 2-BE

The concentrations of 1,2-PD and 2-BE in seawater were determined by gas chromatographic analysis. Samples were first prepared by solid phase extraction using ENVI-Carb Plus cartridges (Supelco Catalog # 54812-U). The cartridges were prepared by adding 1 mL of dichloromethane and pulling it through with a vacuum (5.08 cm Hg),

followed by 2 mL of methanol, 2 mL more methanol, and 3 mL DDI water. The seawater sample (5 mL) was then added and also pulled through by vacuum; 1,2-PD and 2-BE adsorb to the carbon in the cartridge. The cartridge was then dried by applying a vacuum (25.4 cm Hg) for 10 min and the 1,2-PD and 2-BE were eluted by reversing the cartridge and pulling a 2 mL mixture (50/50 v/v) of dichloromethane and methanol through the cartridge. The 2 mL of eluent was collected in a glass centrifuge vial (14 mL), centrifuged (3000 rpm in a Fisher Scientific Centrific® Centrifuge, 5 min), transferred to an autosampler vial (using a 2 mL Pasteur pipet) and sealed with a Teflon-faced septum and cap. Vials were refrigerated at 4 °C until analysis by gas chromatography. Additional details are provided in Appendix C.

2.4 GC-FID Analysis for 1,2-PD and 2-BE

Samples were analyzed on an HP 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector (FID). The column used was a capillary ZB-FFAP Phenomenex© GC column with dimensions of 30 m x 0.25 mm I.D. and 0.25 μm film thickness containing a nitroterephthalic acid modified polyethylene glycol phase. The GC oven temperature started at 50 ºC (2.5 min), ramped at 8 ºC/min to 200 ºC, and held for 5 min. The injector and detector were set to 220 ºC. The injection volume was 1 μL in splitless mode. The carrier gas was hydrogen at a flow rate of 1.8 mL/min (33). Additional details are provided in Appendix D.

The percent recovery for 1,2-PD from the SPE cartridges was determined to be 80%, as follows. Four samples of 30 mg/L 1,2-PD in Instant Ocean® were treated with

the SPE method. The resulting four extracts were analyzed on the GC, resulting in peak areas of 86,537; 85,175; 114,258; and 107,434.

The "constructed" sample was made by adding 25 µL of a 6 g/L 1,2-PD stock solution to a 2 mL GC vial containing 80% methanol and 20% DCM. The stock solution was made by adding 8 mL methanol, 2 mL DCM, and 57.7 µL 1,2-PD to a scintillation vial and mixing. The resulting mass of 1,2-PD present in the 80/20 mixture of methanol and DCM represented the mass that would be present if there was no loss of 1,2-PD during the extraction process. Analysis of the "constructed" sample on the GC yielded a peak area of 123,333. The percent recovery was calculated by dividing the peak areas from the SPE samples by the peak area for the constructed sample. The average recovery was 80%, indicating a 20% loss of 1,2-PD occurred during the extraction process.

A percent recovery was not measured for 2-BE.

A standard recovery of 1,2-PD was evaluated with a sample from a preliminary microcosm (75 g of sediment + 150 mL synthetic seawater). The microcosm was amended with 30 mg/L of 1,2-PD, extracted and analyzed on the GC. A 50/50 mixture (v/v) of the sample and a standard (also 30 mg/L) was extracted and analyzed on the GC. Then, a 50/50 mixture of the standard and DDI water was extracted and analyzed on the GC. Based on the peak areas obtained for these three samples, the percent recovery for 1,2-PD was 113%.

2.5 Oxygen

The oxygen concentration in the headspace of the aerobic microcosms and transfer enrichment bottles was measured using an HP 5890 Series II GC equipped with a

3.2 mm x 3.2 m stainless steel column packed with 100/120 Carbosieve S-II (Supelco) and a thermal conductivity detector (TCD). Room air was used as a standard (21% O_2), for a one point calibration. Nitrogen was used as the carrier gas. The detector was set on high sensitivity. A headspace sample (0.5 mL) was injected onto the GC. Additional details are provided in Appendix E.

2.6 Nitrate in Seawater

The concentration of nitrate in seawater was determined by a cadmium reduction colorimetric method. Seawater samples (1 mL) were filtered (0.45 µm) and diluted with Instant Ocean® containing 1 mg/L resazurin, in a Hach colorimeter vial (catalog # 24019), so that the final concentration was within the calibration range of 1-30 mg/L NO_3^--N. The contents of a Hach NitraVer 5 Nitrate Reagent Pillow were added to the vial. The vial was capped, shaken, allowed to react, and the absorbance at 520 nm was measured in a Hach calorimeter (DR 890). A calibration curve was used to convert absorbance readings to concentrations, which is necessary since the default calibration curve in the DR-890 was constructed with fresh water rather than seawater. Details of the procedure are outlined in Appendix F.

2.7 Ferrous Iron in Seawater

The concentration of ferrous iron in seawater was determined by the ferrozine assay (34). A seawater sample (100 µL) was added to 4.9 mL 0.5 N HCl in an anaerobic chamber and then transferred to the bench top. An aliquot (100 µL) of the acidified sample was added to 4.9 mL of ferrozine solution, prepared as outlined in Appendix G. The absorbance of this solution was measured at 562 nm. Standards were prepared and a

calibration curve was constructed. A response factor was determined from the calibration curve and used to determine the ferrous iron in the samples.

2.8 Sulfide Measurement in Seawater

The concentration of sulfide in seawater was determined spectrophotometrically after the reaction of sulfide in 6 N HCl containing N,N-dimethyl-p-phenylenediamine sulfate and ferric chloride (35). A calibration curve was constructed using standards made from sodium sulfide nonahydrous. The detailed procedure is provided in Appendix H.

2.9 Methane

Methane in the headspace of the microcosms and transfer enrichment bottles was measured using a GC (5890 Series II) equipped with a 2.44 m x 3.175 mm column packed with 1% SP-1000 on 60/80 Carbopack B (Supelco) and an FID (36). Methane standards were constructed using 250 mL Wheaton media bottles, identical to the ones used for the microcosms and transfer enrichments. Standards were constructed by adding methane to the headspace of these bottles containing 150 mL of DDI water. A headspace sample (0.5 mL) was injected onto the GC. The peak areas from the chromatograms of the standards were used to construct a calibration curve which was used to calculate the moles of the methane in the 165 mL headspace of the sample bottles. The detailed procedure is provided in Appendix I.

CHAPTER THREE

RESULTS

3.1 Microcosm Results

3.1.1 1,2-PD

1,2-PD was consumed to below the detection limit within 17 days in all of the microcosms (Figures 3.1-3.5). While this was expected for aerobic conditions, the biodegradability of 1,2-PD in seawater under anaerobic conditions has not previously been described. Samples were not taken frequently enough to make any conclusions about rates; however, it does appear that the fifth addition of 1,2-PD was consumed at a higher rate (i.e., within two days) compared to the previous additions, which would be expected as the population of 1,2-PD degrading microbes increased. The longer gap between the third and fourth additions was a consequence of not resampling the bottles soon enough after day 36

The aerobic microcosms consumed considerably more oxygen than could be attributed solely to the biodegradation of 1,2-PD (i.e., 4 mol O_2/mol 1,2-PD, ignoring cell synthesis). In fact, oxygen consumption continued even after all the 1,2-PD had been consumed; frequently, oxygen levels fell to below detection before more was provided by sparging with room air. Oxygen consumption in excess of that needed for 1,2-PD can be attributed to biodegradation of the high concentration of readily biodegradable organic matter in the Bay Jimmy sediment (28, 37).

After adding resazurin to the seawater used to prepare the microcosms, the color was blue, indicating a redox level well above -110 mV. When the bottles were shaken,

the suspended solids from the sediment obscured the color. After allowing the solids to settle for approximately 6 h, it was possible to discern the color. All of the anaerobic treatments were clear after only 1 day of incubation. The aerobic microcosms were also clear within several hours of sparging with room air to restore the oxygen supply, indicating that the redox potential quickly fell below -110 mV. This was unexpected, since the headspace of the bottles was sparged prior to each new addition of 1,2-PD. The results suggested that the organic matter in the sediment created a high oxygen demand that was not satisfied by the additions of air. Thus, the possibility that 1,2-PD was being consumed anaerobically in the aerobic microcosms cannot be completely ruled out.

Nitrate was not measured in the microcosms. However, it may be inferred that the nitrate was consumed based on the color of the resazurin, i.e., if nitrate had persisted, the color of the seawater would have remained pink. As was the case with the aerobic microcosms, the nitrate demand created by the organic matter in the sediment apparently exceeded the amount of nitrate added. Consumption of the nitrate would have allowed the redox level to drop below -110 mV. Thus, it is unclear from the microcosm results whether or not 1,2-PD consumption was associated with the use of nitrate as a TEA.

In the treatment with Fe(III)-EDTA added, Fe(II) accumulated to 11.0 mM on day 82, the last day when all 1,2-PD had been consumed (Figure 3.3b). The observed stoichiometry was 11 mol Fe(II)/mol of 1,2-PD. The expected stoichiometry is 16 mol Fe(II)/mol 1,2-PD, ignoring cell synthesis. Assuming a yield of 0.4 electron equivalents of biomass per electron equivalent of 1,2-PD, the expected yield becomes 9.6 mol Fe(II)/mol of 1,2-PD, which is in close agreement with the observed stoichiometry.

However, a higher accumulation of Fe(II) was expected given the results for the aerobic and nitrate-amended treatments, i.e., readily biodegradable organic matter in the sediment should have increased the TEA consumed above what may have been used for 1,2-PD biodegradation. It is possible that more Fe(II) was formed than measured, if some was precipitated with sulfide formed via sulfate reduction, which would have been evident from the accumulation of black precipitates. However, because of the high concentration of suspended solids in the microcosms, it was not apparent if iron sulfides formed. Thus, it is unclear from the microcosm results whether or not 1,2-PD consumption was associated with the use of chelated Fe(III) as a TEA.

In the treatment with Fe(III) gel added, there was much less accumulation of Fe(II) (Figure 3.4b) in comparison to the treatment with Fe(III)-EDTA added. The expected stoichiometry is the same as with Fe(III)-EDTA, i.e., 9.6 mol Fe(II)/mol of 1,2-PD, assuming a yield of 0.4 electron equivalents of biomass per electron equivalent of 1,2-PD. The observed stoichiometry after the first addition of 1,2-PD was consumed, made on day 17, was 11.7 mol Fe(II)/mol of 1,2-PD, which is in reasonably close agreement. However, Fe(II) decreased while the next four additions of 1,2-PD were consumed; it was non-detectable by day 120. As mentioned above, it is possible that Fe(II) was not detected due to precipitation with sulfide. However, because of the high concentration of suspended solids in the microcosms, it was not apparent if iron sulfides formed. Thus, it is unclear from the microcosm results whether or not 1,2-PD consumption was associated with the use of Fe(III) gel as a TEA.

In the unamended treatment, the only TEA was sulfate. Because of the high concentration of sulfate in seawater (28 mM), it was not feasible to use sulfate measurements to ascertain if it was being used in association with biodegradation of 1,2-PD. For example, if all of the 1,2-PD consumed in the unamended microcosms was associated with sulfate reduction, the concentration would have decreased by only 1.97 mM (ignoring cell synthesis), which would have been difficult to quantify in the presence of such a high background level. Nevertheless, whenever any of the anaerobic treatments were sparged with N_2, a strong odor of hydrogen sulfide was detected, indicative of sulfate reduction. Sulfide was also detected in the aerobic bottles when these were sparged with air to replenish oxygen. The pervasive odor of sulfide suggested that sulfate was the predominant electron acceptor in the microcosms, but the high TEA demand of the organic-rich Bay Jimmy sediment precluded making a stoichiometric link between the TEA added and the consumption of the 1,2-PD.

The cumulative amounts of 1,2-PD consumed for all treatments is shown in Figure 3.6. The similar patterns suggests that the same TEA was used in all treatments, and is consistent with the fact that sulfide was noticed in the off-gas stream when the bottles were sparged. Nevertheless, the high background level of organic matter in addition to 1,2-PD precluded establishing a definitive link between 1,2-PD biodegradation and the TEA involved.

3.1.2 2-BE

After consuming five additions of 1,2-PD, 2-BE was added to all of the microcosms on day 98 (Figure 3.1-3.5). Unlike 1,2-PD, there were differences in the

rates of 2-BE consumption among the treatments. Figure 3.7 shows the rate of cumulative 2-BE consumption, which was fastest in the treatment with Fe(III) gel added, followed by the aerobic microcosms. Biodegradation lagged in the other anaerobic treatments, although consumption was complete after nine days of incubation in all of the treatments. No attempts were made to link the amount of TEA consumed with the amount of 2-BE consumed, based on the high electron acceptor demand that continued to be exerted by the organic rich sediment. Evaluation of this issue was pursued in the transfer cultures, in which the concentration of sediment was substantially decreased.

3.2 2-BE, First Transfer

The first transfer enrichments were constructed using a 1% inoculum (v/v) from the microcosms, after 118 days of incubation. The transfer enrichments also contained 150 mL of Instant Ocean® seawater. Photographs showing the substantial difference in seawater clarity between the microcosms and first transfer enrichments are shown in Appendix J. The lower background level of suspended solids made it easier to track the color of resazurin that was added to the seawater. The focus of these transfers was to evaluate biodegradation of 2-BE; further work on 1,2-PD was undertaken by another student.

All of the first transfer treatments consumed the first addition of 11.9 mg/L 2-BE after 19 days (Figures 3.8-3.12). Differences in the rates of 2-BE biodegradation began to develop with the second addition of 2-BE. On day 28, all of the 2-BE was consumed in the unamended treatment. On the same day, the Fe(III) gel amended treatment bottles contained an average of 1.7 mg/L 2-BE, the nitrate amended treatments contained an

average of 2.9 mg/L 2-BE, the aerobic treatments contained an average of 7.3 mg/L 2-BE, and the 2-BE level in the Fe(III)-EDTA treatment had not decreased at all. Cumulative amounts of 2-BE consumption are shown in Figure 3.13. The highest rates of consumption were observed in the nitrate-amended and unamended treatments, followed by the treatment with Fe(III) gel added and the aerobic bottles. After consuming the first addition of 2-BE, activity in the treatment amended with Fe(III)-EDTA stopped.

The oxygen level in the headspace of the aerobic transfer enrichments was monitored and compared to a control set of triplicate aerobic transfer enrichments that received no 2-BE (Figure 3.8b). The difference in oxygen consumption is presumably associated with the aerobic biodegradation of 2-BE; oxygen consumption in the treatment without 2-BE added is a consequence of residual organic matter carried over from the microcosms. Incubation on a shaker table was initiated on day 35 to ensure an adequate rate of oxygen diffusion from the headspace into the seawater. After shaking was initiated, a difference in oxygen consumption between the aerobic enrichment and the control became apparent. This result established an association between oxygen consumption and 2-BE biodegradation in seawater. Over the 55 days of incubation, the net consumption of oxygen was 1.22 mmol per bottle, compared to 0.0588 mmol of 2-BE consumed, or a ratio of 21 mol O_2 per mol 2-BE, which is more than twice as high as the expected stoichiometry of 8 mol O_2 per mol 2-BE, ignoring cell synthesis. The excessive consumption of oxygen suggests that the inoculum contained residual biodegradable organic matter from the microcosms, which contributed to the oxygen demand. Unlike

the microcosms, the color of the first transfer aerobic bottles remained pink at all times, indicating the redox level never fell below -110 mV.

Nitrate levels in the first transfer enrichments did not decrease as more 2-BE was consumed; in fact, the measured nitrate levels increased (Table 1). Measured nitrate levels were compared with the nitrate concentration expected if nitrate was not consumed, i.e., behaved as a conservative compound. The calculation took into account the nitrate removed when liquid was removed for sampling. On days 28, 32, and 39, the measured and calculated concentrations were in reasonable agreement, indicating that nitrate was not being consumed in association with the disappearance of 2-BE. On days 23, 48, 51, and 57, the amount of nitrate measured was actually higher than what was expected based on a lack of nitrate consumption. The discrepancies between the calculated and measured nitrate levels at the higher concentrations may be related to the higher level of dilution required. Regardless, the fact that no nitrate was consumed during the biodegradation of 2-BE indicated this process was not linked to nitrate as the TEA. The fact that the resazurin was clear in the nitrate-amended bottles, rather than pink, is consistent with the assessment that the redox potential was below -110 mV, which is well below what is expected when nitrate serves as the TEA. Furthermore, the similar degradation rate of 2-BE in the nitrate and unamended enrichments (Figure 3.13) suggests that the same TEA was used.

To further confirm the high levels of nitrate measured in the nitrate-amended bottles, standard additions were made for one set of triplicate samples on day 28. The average nitrate concentration in the microcosms was 35.8 mg/L as N. Samples were then

combined in equal volumes with the 10 mg/L nitrate standard used to construct the calibration curve. Based on the responses, the calculated recovery of the standard addition was 98%, which indicated that there was no matrix interference when measuring nitrate, building confidence in the accuracy of the nitrate levels in the samples.

Biodegradation of the first addition of 2-BE in the Fe(III)-EDTA amended bottles was accompanied by an increase in Fe(II) to 4.4 mM (Figure 3.10). This stoichiometry is far above the level expected, even ignoring cell synthesis. It suggests that a considerable amount of electron donor was carried over with the inoculum from the microcosms. Regardless, the second addition of 2-BE was not consumed and there was no further increase in Fe(II). The results suggest that Fe(III) reduction may have been related to 2-BE biodegradation, but without further consumption of 2-BE, it was not possible to verify.

In the first transfer bottles amended with the Fe(III) gel, the net accumulation of Fe(II) was zero (Figure 3.11). Fe(II) was detected on only day 39 (2.3 mM). The resazurin in these bottles was clear, indicating that the redox potential was below -110 mV. At this level, it is possible that ferric iron reduction and sulfate reduction both occurred, which is consistent with the fact that the solids in these bottles were darker, as would occur if iron sulfide precipitates were accumulating.

To further confirm the accuracy of the Fe(II) measurements, standard additions were made for one set of triplicate samples from the Fe(III) gel bottles. The average Fe(II) concentration in the microcosms at the end of the incubation period was non-detectable. Samples were then combined in equal volumes with the 0.8 mM Fe(II)

standard used to construct the calibration curve. Based on the responses, the calculated recovery of the standard addition was 100%, which indicated that there was no matrix interference when measuring Fe(II), building confidence in the accuracy of the Fe(II) levels in the samples.

The highest rate of 2-BE consumption occurred in the unamended anaerobic bottles; results for each are shown in Figure 3.12. The amount of 2-BE added was gradually increased to 59 mg/L to enhance the enrichment process. On day 50, 20 mM molybdate was added to two of the bottles. Molybdate is an inhibitor specific to sulfate reducing bacteria. The introduction of 20 mM molybdate had no effect on the rate of 2-BE biodegradation. On day 60, another dose of 20 mM molybdate was added. Samples (0.1 mL, which was diluted with 19.9 mL Instant Ocean®, providing a 1:200 dilution) removed on day 80 were analyzed for molybdate at Clemson University's Agricultural Services Laboratory; the level was 39 mM, indicating that essentially all of the molybdate added was in solution. At this concentration, molybdate had no impact on 2-BE biodegradation in one of the bottles (#3) and slowed the rate in the other (#2). These results suggest that sulfate reducers are not directly involved in anaerobic biodegradation of 2-BE; the slowing of 2-BE consumption in one of the bottles suggests that sulfate reducers may be playing a secondary role.

Sulfide levels were measured in the unamended first transfer enrichment bottles on day 83. Bottles #2 and #3 showed no sulfide, consistent with the inhibition of sulfate reduction by addition of molybdate. In bottle #1, the sulfide concentration was 0.67 mM. The expected sulfide concentration assuming that all the 2-BE was mineralized by sulfate

reduction is 9.6 mM. The fact that the sulfide level was less than 10% of expected for mineralization of 2-BE under sulfate reducing conditions is consistent with the hypothesis that the role of sulfate reducers is secondary, i.e., the initial biodegradation of 2-BE is carried out by fermentative microbes and the resulting products are consumed by sulfate reducers.

Methane levels in the unamended first transfer enrichment bottles were measured beginning on day 55 (Figure 3.14). None was detected in bottles #1 and #2, whereas accumulation was observed in bottle #3 (which also received 40 mM molybdate). The maximum amount measured (104 µmol) represents only 4.9% of the 8.9 mg of 2-BE biodegraded since day 69, when bottle #3 was last sparged, assuming complete mineralization. These results suggest that methanogens may have replaced the secondary role that sulfate reducers play during 2-BE biodegradation, i.e., consumption of products released by fermentative microbes that carry out the initial biodegradation activity.

In the two unamended bottles that received molybdate (Figure 3.12), an unknown peak appeared in the GC chromatograms at 5.1 min, compared to a retention time of 9.2 mion for 2-BE. This peak was present in all bottles, but at a very low level in comparison to 2-BE. However, in the two molybdate amended bottles, this unknown increased significantly after the first addition of molybdate, to a peak area of 114,00 to 319,000. These areas compare to a peak area of approximately 320,000 for 60 mg/L of 2-BE.

3.3 2-BE, Second Transfer

Second transfer enrichments were prepared with inoculum (1% v/v) from the first transfer bottles after 55 days of incubation and consumption of five additions of 2-BE.

The treatments consisted of aerobic, unamended anaerobic, unamended anaerobic + 20 mM molybdate, WCs, and autoclaved controls. All bottles were initially spiked with 60 mg/L 2-BE. No further enrichment was attempted with nitrate amended bottles, based on evidence that nitrate was not being used as the TEA. Likewise, no further enrichment was attempted with treatments that received Fe(III).

Results for the controls are shown in Figure 3.15. The decrease in 2-BE after 23 to 28 days of incubation was minor in comparison to the live treatments. After 23 days of incubation, 2-BE was completely consumed in the aerobic treatment bottles, along with 4.0 mg of O_2 (Figure 3.16). The resulting stoichiometry was 1.6 mmol O_2 per mmol 2-BE, which is within the maximum ratio of 8 when ignoring cell synthesis, which indicates that the second transfer enrichment did not carry over any significant organic matter from the microcosms, and the consumption of 2-BE was clearly linked to the consumption of oxygen.

The rate of 2-BE biodegradation in the unamended anaerobic bottles was higher, with complete consumption in no more than 7 days (Figure 3.17). Two subsequent additions were consumed at similar or higher rates. The presence of 20 mM molybdate (this time added at the start of the incubation, when biomass levels were at their lowest) slowed but did not stop anaerobic biodegradation of 2-BE (Figure 3.18). There was also more variability among the bottles during degradation of the first addition of 2-BE, which explains the larger error bars. Sulfide was measured in the six live anaerobic bottles and none was detected, which indicated that the consumption of 2-BE in these bottles was not

associated with sulfate reduction. Furthermore, methane did not accumulate in any of these bottles during the 28 day incubation period.

Several days after the transfers were prepared, a white precipitate formed in the bottles with molybdate added, as seen in a picture in Appendix J. No such precipitate formed in the bottles without molybdate added. There was no evidence of a similar precipitate in the first transfer bottles to which molybdate was added. Samples (10 mL) from the second transfer bottles were removed on day 24 and analyzed for molybdate by Clemson University's Agricultural Services Laboratory; the result was 9.7 mM, or approximately one half of what was added. This suggested the precipitate that formed included molybdate. The lower aqueous phase concentration of molybdate may have prevented complete inhibition of sulfate reduction. Regardless, the lack of sulfide accumulation indicates that sulfate reduction did not occur in the bottles with or without molybdate added.

The lower than expected concentration of molybdate was not consistent with an analysis of its solubility made using Visual MINTEQ (Appendix K), which predicted that all of the molybdate would stay in solution. Additional research is needed to resolve the difference between the measured and modeled concentration of molybdate in the second transfer enrichment bottles.

The unknown peak at 5.1 min on the GC chromatograms (mentioned above with respect to the molybdate-amended first transfer bottles, Figure 3.12) appeared in all six of the live anaerobic second transfer bottles (i.e., with or without molybdate added). The

maximum peak area was 511,000, even higher than with the first transfer bottles. It did not appear in the aerobic bottles.

CHAPTER FOUR

DISCUSSION

The biodegradability of 1,2-PD and 2-BE in seawater under various conditions was confirmed in microcosms in the presence of sediment from a part of the Gulf coast that was impacted by the *Deepwater Horizon* oil spill. Both compounds were consumed under aerobic and anaerobic conditions, which is noteworthy, since 1,2-PD and 2-BE are components of COREXIT, the dispersant that was released in unprecedented quantities during the *Deepwater Horizon* spill. The fate of both compounds in seawater had not previously been evaluated. Anaerobic treatments included amendments with nitrate and ferric iron. However, due to the high background concentration of organic material from the sediment relative to the amount of 1,2-PD and 2-BE consumed, it was not possible to link the disappearance of these compounds to a specific anaerobic TEA.

Further insight into the biodegradation of 2-BE in seawater was sought by making a transfer of the microcosms (1% v/v) into seawater. Aerobic and anaerobic biodegradation of 2-BE was again confirmed. In the anaerobic treatments amended with nitrate and Fe(III) gel, and in the unamended treatments, the rates of 2-BE biodegradation were similar. This pattern suggests that the same TEA may have been used in all of the enrichments. Nitrate measurements indicated it was not consumed, ruling out its role as a TEA. 2-BE biodegradation ceased in the treatment amended with Fe(III)-EDTA after the first dose was consumed (Figure 3.10), suggesting that EDTA may have been inhibitory. Li et al. (38) observed that Fe(II)-EDTA levels as low as 5.7 mM have inhibitory effects on the iron-reducing bacterium *Escherichia coli* FR-2. The lack of Fe(II) accumulation in

the treatment with Fe(III) gel added indicated Fe(III) was not used as a TEA for 2-BE biodegradation.

In contrast, repeated additions of 2-BE were consumed in the first transfer treatment with sulfate as the only available anaerobic TEA (Figure 3.12). Given the high background concentration of sulfate in seawater (28 mM) and the relatively small amount that would have been consumed for 2-BE oxidation (0.8 mM per 14 mg/L of sulfate, ignoring cell synthesis), quantification of sulfate was not attempted. When the headspace of bottles was flushed with N_2 after opening them in the anaerobic chamber to remove samples, a sulfide odor was readily detectable in the off gas. However, sulfide measurements indicated that sulfate reduction did not occur or was minor relative to the amount expected if the 2-BE was completely oxidized. The sulfide measurement suggested that sulfate reduction was likely playing a secondary role in the biodegradation of 2-BE, consistent with the observation that molybdate had some impact, but did not completely stop consumption. A molybdate dose of 20 mM has been used in a number of previous studies to completely stop sulfate reduction in seawater (29-32). While the intent of adding molybdate is to interfere with sulfate reduction, it is also known to cause non-targeted inhibition, e.g., by causing the population of butyrate-utilizing acetogens to decrease by 27% in a bench scale anaerobic digester seeded with organic municipal solid waste (39). The fact that methane increased in one of the bottles (Figure 3.14) suggests methanogens play a similar secondary role as sulfate reducers.

The second transfer with 2-BE as the sole source of carbon and energy included only unamended treatments, based on the lack of evidence for nitrate and Fe(III) as

TEAs. Results were consistent with the behavior of the first transfer treatments, i.e., molybdate slowed but did not stop anaerobic biodegradation of 2-BE. Sulfide was not detected, nor was methane. However, the incubation period for the second transfer treatments was shorter, and consequently less total 2-BE was consumed. With additional consumption of 2-BE, it is conceivable that sulfide and/or methane would have become detectable.

Ruling out direct involvement of a TEA during anaerobic biodegradation of 2-BE in seawater suggests fermentation was involved. A proposed pathway is shown in Figure 4.1. It is based on the fermentation pathways observed with many alcohols, i.e., a stepwise oxidation of the alcohol to an aldehyde and then a carboxylic acid (40, 41). The intermediate product would be 2-butoxy acetic acid (2-BAA). Each oxidation step would yield 2 electron equivalents per mole of 2-BE, which would potentially be released as hydrogen. The net proposed reaction is:

$$C_6H_{14}O_2 + H_2O \rightarrow C_6H_{12}O_3 + 2H_2 \quad (4.1)$$

Under standard conditions (all reactants and products at 1 M or 1 atm) adjusted to pH 7, the Gibbs free energy change for this reaction is calculated as follows:

$$\Delta G^{\circ\prime} = (\Delta G_{f,2-BAA} + 2\Delta G_{f,H_2}) - (\Delta G_{f,2-BE} + \Delta G_{f,H_2O}) \quad (4.2)$$

where ΔG_f is the free energy of formation for each compound, indicated by the subscript. $\Delta G_{f,H2}$ is zero and $\Delta G_{f,H2O}$ is -237.17 kJ/mol (42). According to *The Yaws' Handbook of Thermodynamic Properties for Hydrocarbons and Chemicals* (43), the

$\Delta G_{f,2\text{-}BE}$ in the gaseous state is -240 kJ/mol. Using a Henry's law constant of 1.6 $x10^{-3}$ atm·L/mol for 2-BE (44), a universal gas constant (R) value of 8.314 x 10^{-3} L·atm/(mol K), and a temperature of 298 K, the gas phase value ($\Delta G_{f,2\text{-}BE(g)}$) was adjusted to an aqueous phase value ($\Delta G_{f,2\text{-}BE(aq)}$) of -256 kJ/mol based on the following equation:

$$\Delta G^{\circ}_{f(aq)} = \Delta G^{\circ}_{f(g)} + RTln(H) \qquad (4.3)$$

A value for $\Delta G_{f,2\text{-}BAA}$ was sought from a variety of sources, including *TRC Thermodynamic Tables* by Texas A&M, *The Yaws' Handbook of Thermodynamic Properties for Hydrocarbons and Chemicals*, *CRC Handbook of Chemistry and Physics*, and *Perry's Chemical Engineers' Handbook*; however, none of these sources reported a value. It is possible that measurement of $\Delta G_{f,2\text{-}BAA}$ has not yet been made. Without this value, it is not possible to determine a value for $\Delta G^{o'}$ (equation 4.2). In the absence of such a value, one was estimated by comparing the difference in free energies of formation for four alcohols and their corresponding carboxylic acids (Appendix L). Based on these, the difference between an alcohol and carboxylic acid is approximately -180 KJ/mol, such that the value of $\Delta G_{f,2\text{-}BAA}$ would be approximately -436 kJ/mol. Using this value, the Gibbs free energy change according to equation 4.2 is +57 kJ/mol, making the predicted reaction in equation 4.1 endergonic, which is typical for fermentation reactions that yield hydrogen as a product (40, 42). To account for the likelihood that the concentration of hydrogen is lower than 1 atm during fermentation of 2-BE in seawater, the following equation was used:

$$\Delta G = \Delta G^{\circ\prime} + RTln\left(\frac{[2-BAA][H_2]^2}{[2-BE]}\right) \quad (4.4)$$

Assuming the molar concentrations of 2-BE and 2-BAA are the same, equation 4.4 simplifies to:

$$\Delta G = \Delta G^{\circ\prime} + RTln[H_2]^2 \quad (4.5)$$

Setting this equation to zero (i.e., the highest possible value in order for the reaction to be exergonic) and solving for $[H_2]$ yields a value of 10^{-5} atm. Thus, for fermentation of 2-BE to be feasible, the concentration of hydrogen needs to be maintained at a low concentration.

While these calculations are highly speculative in the absence of a reliable value for $\Delta G_{f,2\text{-}BAA}$, they illustrate the potential importance of a low partial pressure of hydrogen during fermentation of 2-BE. This, too, is consistent with fermentation of alcohols to their corresponding carboxylic acids (40, 41). The proposed pathway and thermodynamic approximations are consistent with a secondary role for sulfidogens and methanogens in fermentation of 2-BE, i.e., maintaining a low partial pressure of hydrogen. This role may also be fulfilled by acetogens, i.e., converting the H_2 and CO_2 into acetic acid.

Nevertheless, additional research is needed to validate that fermentation of 2-BE is indeed the pathway involved in its biodegradation in seawater. Validation would include measurements of 2-BAA and hydrogen, as well as addition of hydrogen to determine if that slows or stops 2-BE consumption. The unknown peak that appeared in

the GC chromatograms at 5.1 min for the first transfers with molybdate added and for all anaerobic bottles for the second transfer may be 2-BAA, but this needs to be confirmed. If 2-BAA is the product, its toxicity must also be considered when assessing the fate of 2-BE in sweater. 2-BAA has been studied for carcinogenic effects. Udden (45) exposed both human and rat red blood cells to 2 mM 2-BAA in plasma. The human red blood cells showed no effect, but the rat red blood cells underwent morphologic changes and hemolysis (lysis of red blood cells). 2-BE exposure causes increased liver hemangiosarcomas (a rapid growing, highly invasive form of cancer generally found in animals) in laboratory mice and although the mechanism for selective induction of hemangiosarcomas is unknown, Corthals (46) shows that 2-BE and 2-BAA do not cause hemangiosarcomas through a route of DNA damage.

If Figure 4.1 accurately represents the fermentation pathway for 2-BE, the fate of 2-BAA should be assessed as well. Figure 4.1 shows the hypothesis of one such fate, i.e., breaking of the ether bond to yield butanol and acetic acid. Such a reaction is predicated on an anaerobic pathway that has been reported for methyl *tert*-butyl ether, yielding *tert*-butyl alcohol and acetic acid (the latter via the tetrahydrofolate pathway) (47). The fate of 2-BAA is speculative and needs to be evaluated experimentally, if indeed it is an intermediate.

In both the first and second transfer enrichments, anaerobic biodegradation was faster than aerobic biodegradation, which was somewhat surprising since, in general, higher rates of biodegradation for organic compounds are expected under aerobic conditions (48). The reason is not yet known but may relate to the likely pathways. The

fermentation pathway is discussed above. Under aerobic conditions, biodegradation of ethers typically involves a monooxygenase attack on the compound, yielding potentially toxic metabolites that may slow the rate of biodegradation. Further research is needed to evaluate the aerobic pathway for biodegradation of 2-BE to better understand the impacts on kinetics.

The results of this research provide compelling evidence for the biodegradation of 1,2-PD and 2-BE in seawater, under aerobic and anaerobic conditions. Nevertheless, it cannot yet be concluded that this activity is representative of what occurs in the field. In particular, the effect of petroleum hydrocarbons needs to be assessed. It is not yet known if the dispersed hydrocarbons will inhibit, enhance, or have no impact on biodegradation of 1,2-PD and 2-BE. Polycyclic aromatic hydrocarbons (PAHs) may be expected to exhibit at least some level of inhibition (19). PAHs are abundant in crude oil and have varying degrees of toxicity. The *Deepwater Horizon* blowout resulted in a massive influx of PAHs to the Gulf and the introduction of COREXIT at the wellhead ensured the dispersion and indelible togetherness of the PAHs and COREXIT chemicals of interest.

In the construction and sampling of the microcosms, it became clear that the sediment collected in Bay Jimmy, LA did in fact contain some degree of petroleum, as evidenced by an oil sheen on top of the water in the laboratory sink after cleaning glassware containing the sediment. The hydrocarbons were not quantified, but the oil sheen provides a visible cue that the microcosms and enrichments contain microorganisms that were exposed to petroleum. Additional research is needed to

determine the concentration at which petroleum hydrocarbons affect the biodegradability of 1,2-PD and 2-BE.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. 1,2-PD biodegraded in seawater under both aerobic and anaerobic conditions in microcosms constructed with seawater and sediment from Bay Jimmy, Louisiana. This thesis appears to be the first report of 1,2-PD biodegradation in a high salt environment. Because of the high level of biodegradable organic matter in the microcosms, it was not possible to associate the anaerobic consumption of 1,2-PD to a specific TEA.
2. 2-BE biodegraded in seawater under both aerobic and anaerobic conditions in microcosms constructed with seawater and sediment from Bay Jimmy, Louisiana. This thesis appears to be the first report of 2-BE biodegradation in a high salt environment. Because of the high level of biodegradable organic matter in the microcosms, it was not possible to associate the anaerobic consumption of 2-BE to a specific TEA.
3. Biodegradation of 2-BE was sustained in aerobic and anaerobic enrichment cultures, through two transfers (1% v/v). Nitrate, Fe(III) and sulfate did not appear to be used as the TEAs under anaerobic conditions. Molybdate slowed but did not stop consumption of 2-BE. Sulfide was detected, but at levels well below the amount expected if complete mineralization occurred with sulfate as the TEA. Based on these results, the presumptive pathway for anaerobic biodegradation of

2-BE in this study was via fermentation. Products were not measured, although 2-BAA and hydrogen are likely intermediates.

5.2 Recommendations

As the investigation into the biodegradation of 1,2-PD and 2-BE continues, the following recommendations are suggested:

1. During biodegradation of 2-BE in the enrichment culture, hydrogen levels should be measured, as well as the presence of any 2-BAA (and other potential metabolites).
2. The role of sulfate-reducing bacteria in the enrichments should be established. The sulfate-reducers may be consuming hydrogen or low molecular weight organic acids, such as acetic acid, formed in the fermentation of 2-BE.
3. An investigation into the biodegradation of other COREXIT compounds should be undertaken, which will be more difficult as these heavier weight molecules, such as TWEEN and SPAN, are more difficult to quantify. The solid phase extraction and GC-FID method outlined in this thesis is not an option.
4. The effect of petroleum hydrocarbons on the biodegradation of 1,2-PD, 2-BE, and, later, the other compounds in COREXIT, should be evaluated to further evaluate the impact of the unprecedented release of these compounds into the marine environment.
5. The microbial community responsible for the degradation of 1,2-PD and 2-BE should be characterized.

TABLE

Table 3.1 Nitrate added vs nitrate measured in the nitrate amended first transfer enrichments

	Measured Nitrate		Calculated NO_3^--N (mg/L) [b]
Time (d)	**NO_3^--N (mg/L)[a]**	**Dilution Factor**	
23	30±12	none	18
28	36±8.5	none	33
32	35±9.4	none	33
39	54±4.4	none	63
48	70±6.6	none	122
51	155±17	5	122
57	199±29	10	122

[a] Average of triplicates ± standard deviation.
[b] Calculated based on a mass balance, assuming nitrate behaved conservatively.

FIGURES

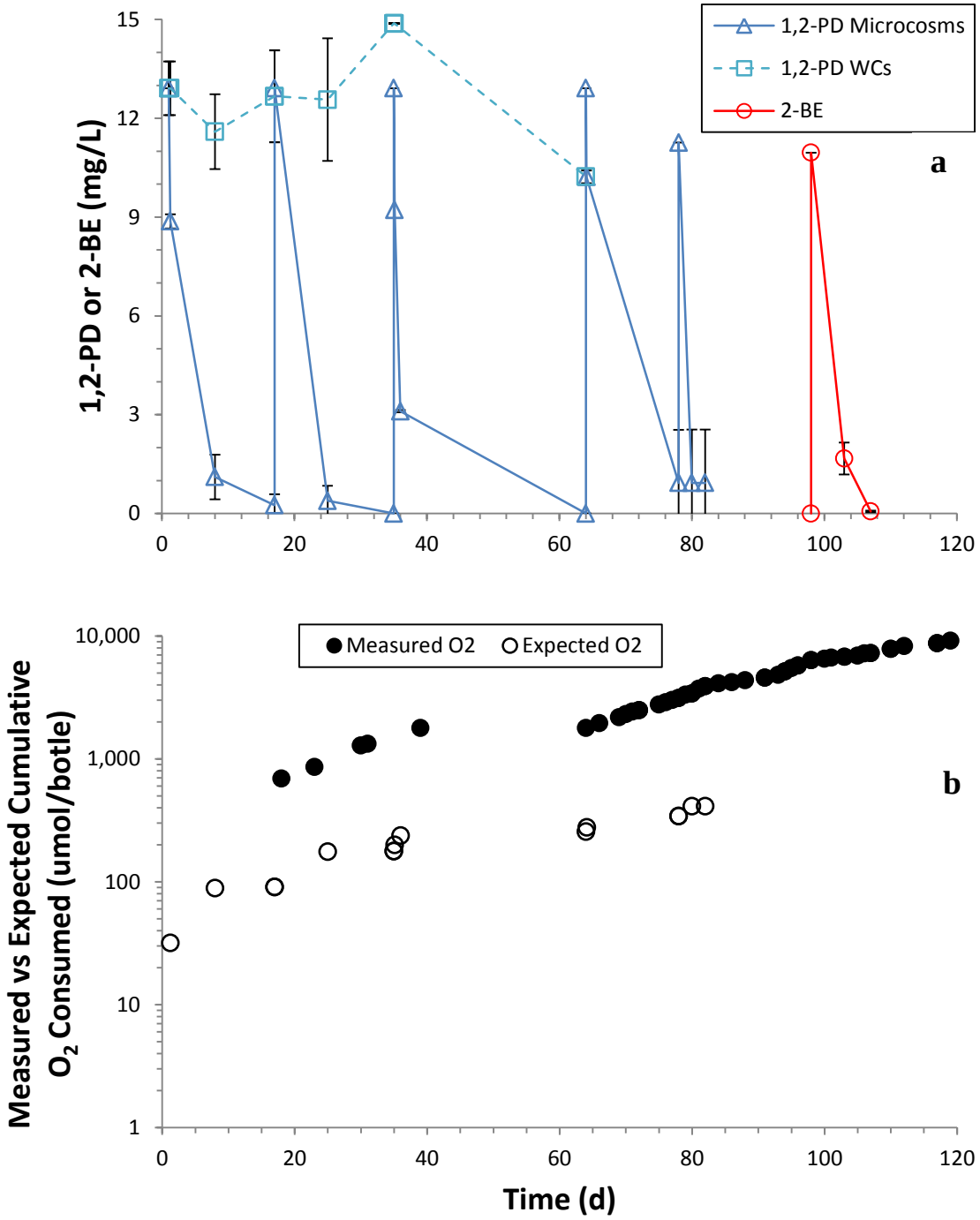

Figure 3.1 a) Average 1,2-PD and 2-BE levels in triplicate aerobic microcosms and WCs for 1,2-PD. Error bars represent standard deviations. **b)** Cumulative oxygen consumption (average of triplicates); solid circles indicate measured values, hollow circles indicate the oxygen consumption expected based on the stoichiometry of 1,2-PD consumed.

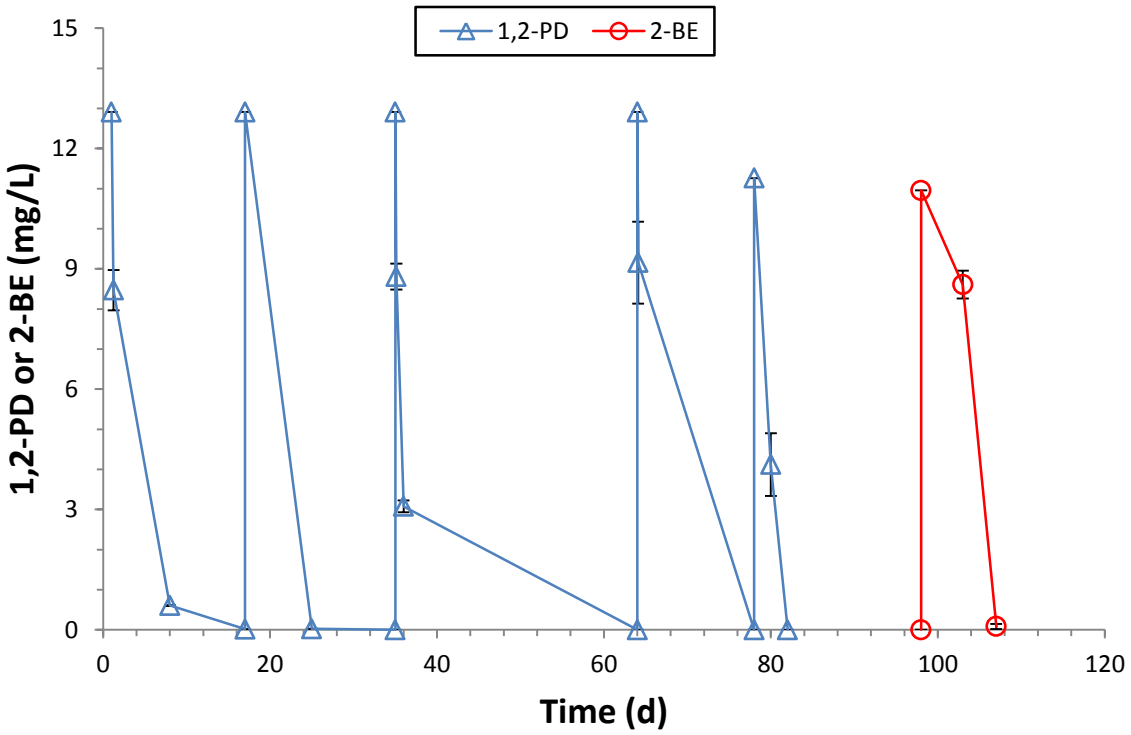

Figure 3.2 Average results for 1,2-PD and 2-BE levels in triplicate nitrate amended microcosms. Error bars represent standard deviations.

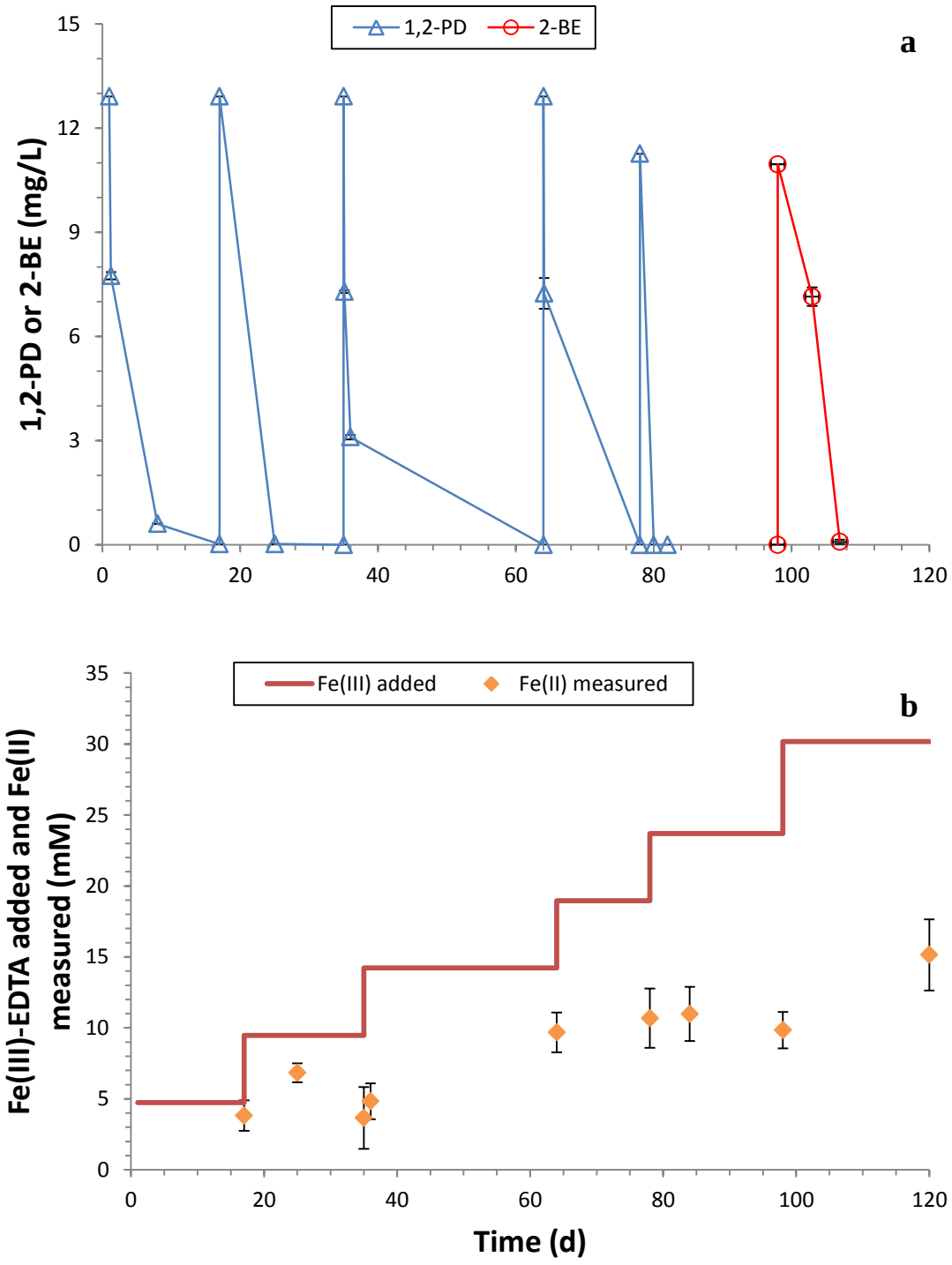

Figure 3.3 Average results for Fe(III)-EDTA amended microcosms; **a**) 1,2-PD and 2-BE, and **b**) Fe(III) added as Fe(III)-EDTA and Fe(II) levels. Error bars represent standard deviations.

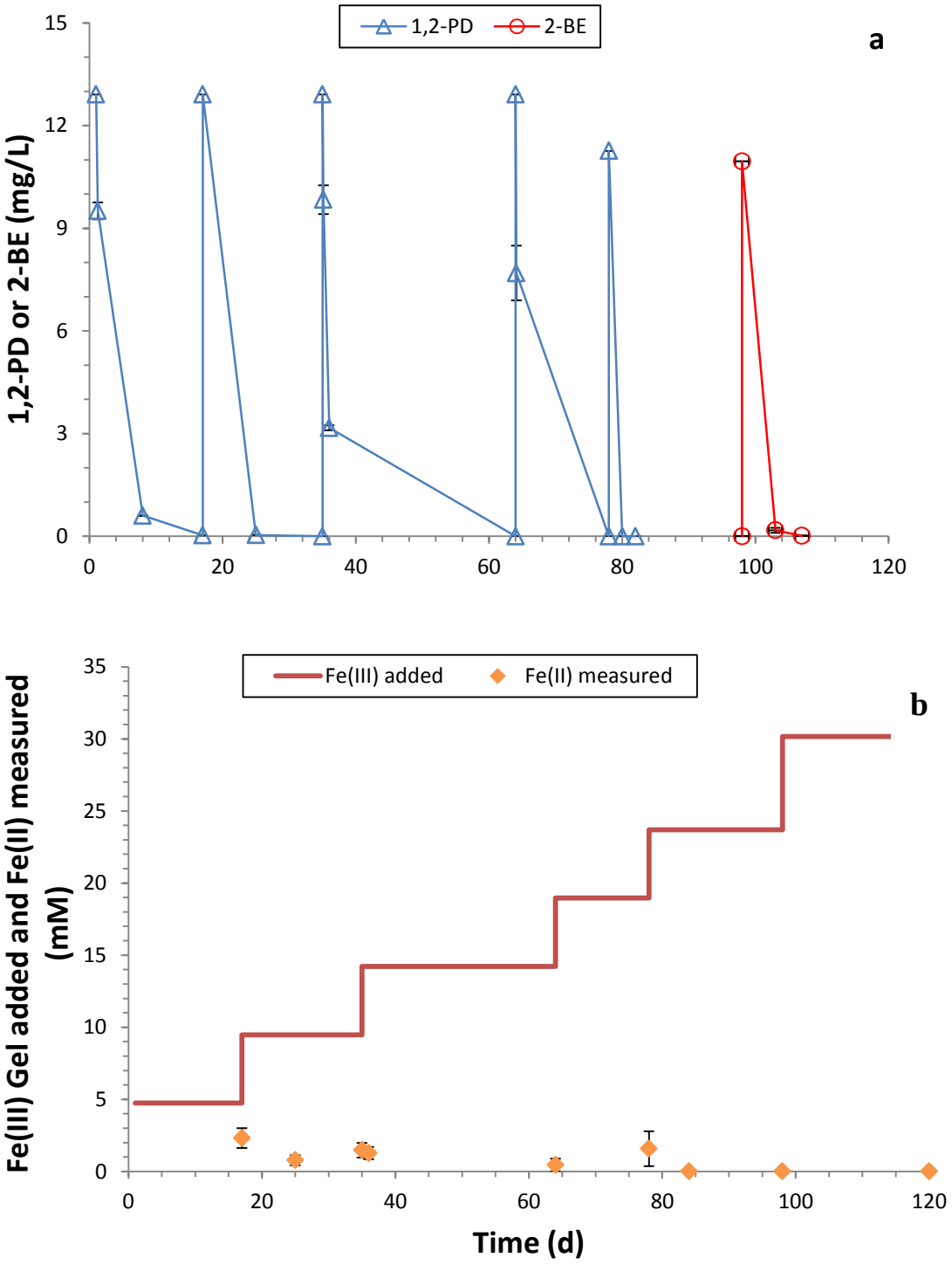

Figure 3.4 Average results for Fe(III) gel amended microcosms; **a**) 1,2-PD and 2-BE, and **b**) Fe(III) added as Fe(III) gel and Fe(II) levels. Error bars represent standard deviations.

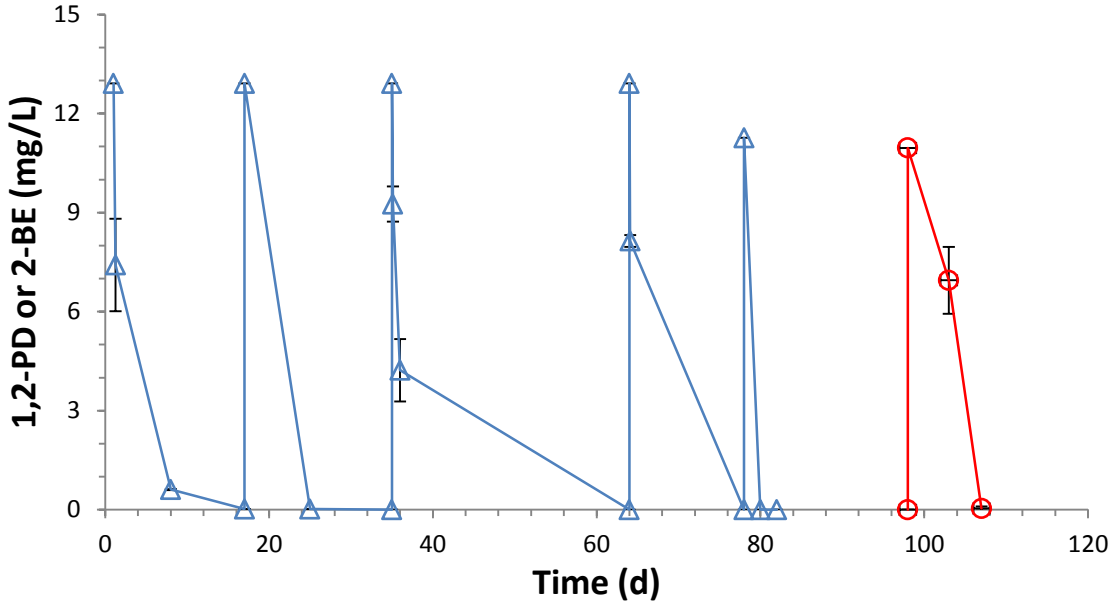

Figure 3.5 Average results for 1,2-PD and 2-BE levels in triplicate unamended anaerobic microcosms. Error bars represent standard deviations.

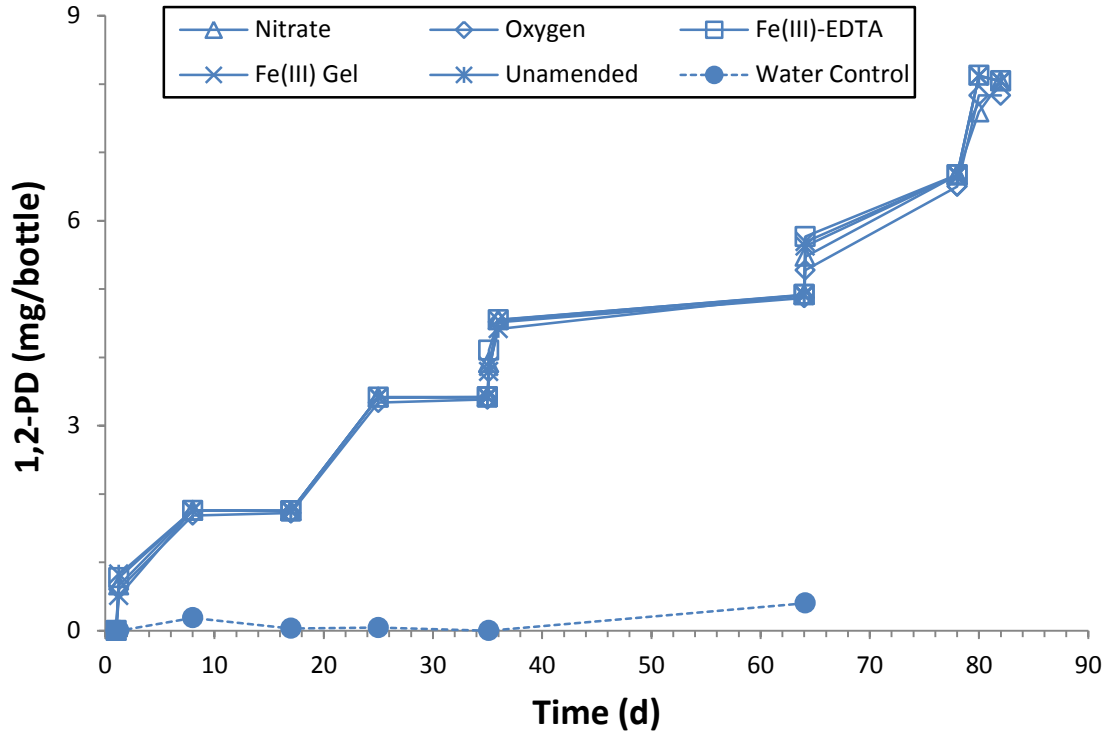

Figure 3.6 Cumulative 1,2-PD consumption in all microcosm treatments.

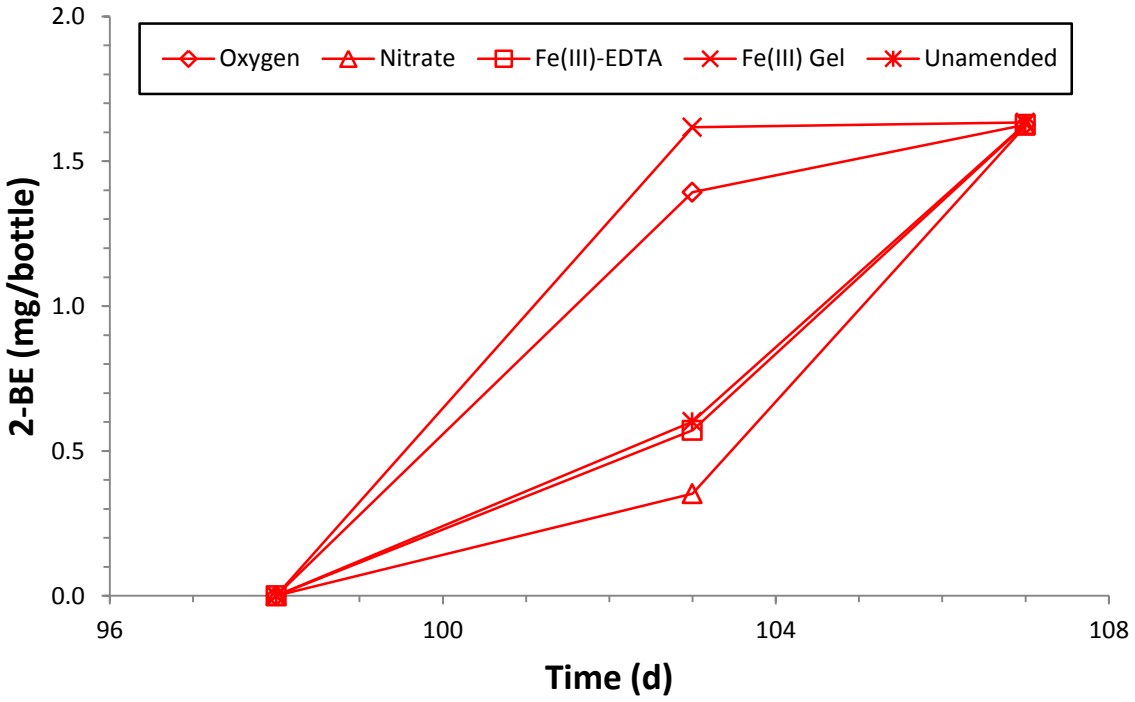

Figure 3.7 Cumulative 2-BE consumption in all microcosm treatments.

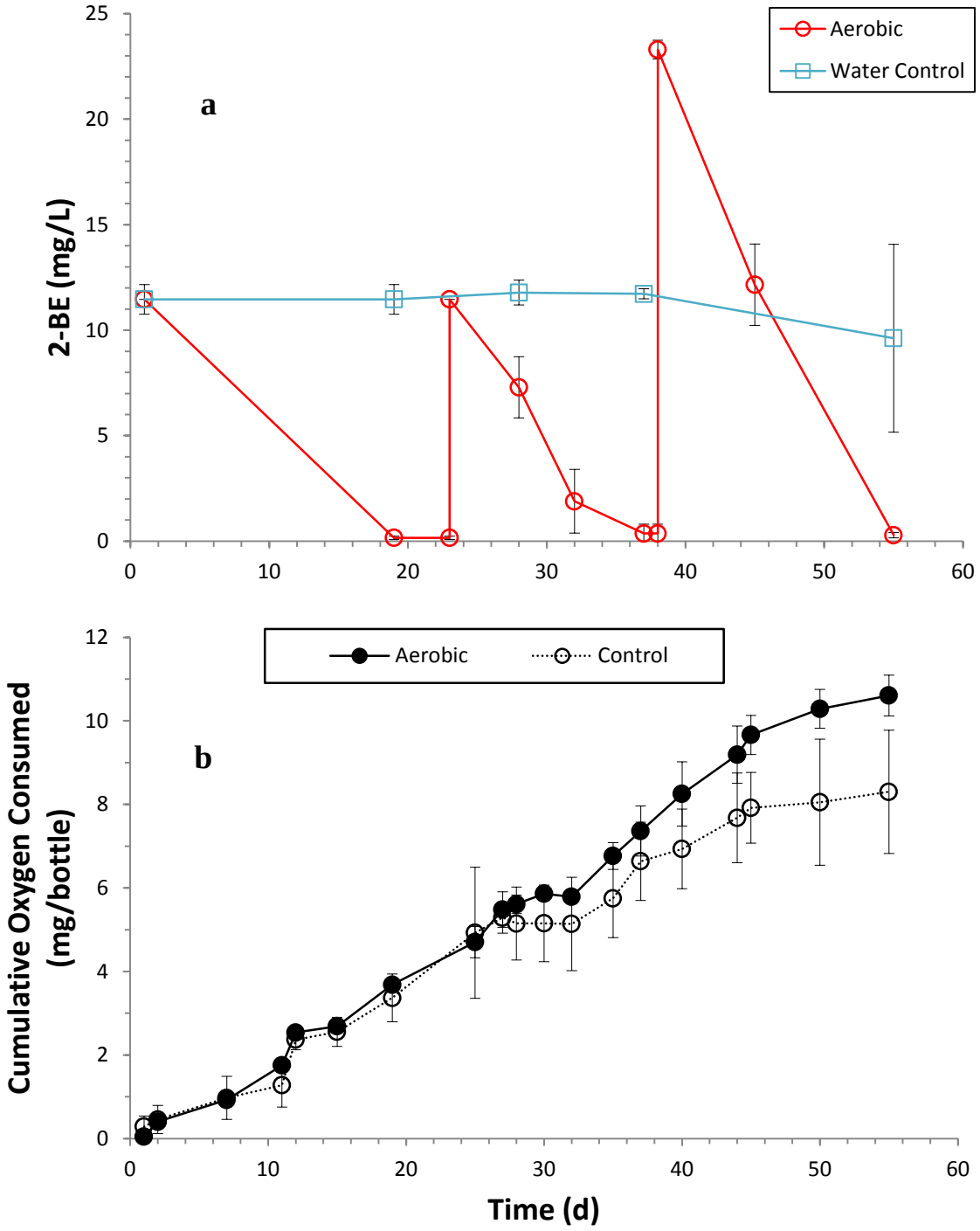

Figure 3.8 **a)** Average results for 2-BE levels in triplicate aerobic first transfer enrichments and WCs. **b)** Cumulative oxygen consumption for triplicate aerobic first transfer enrichments and first transfer aerobic controls (no 2-BE added). Error bars represent standard deviations.

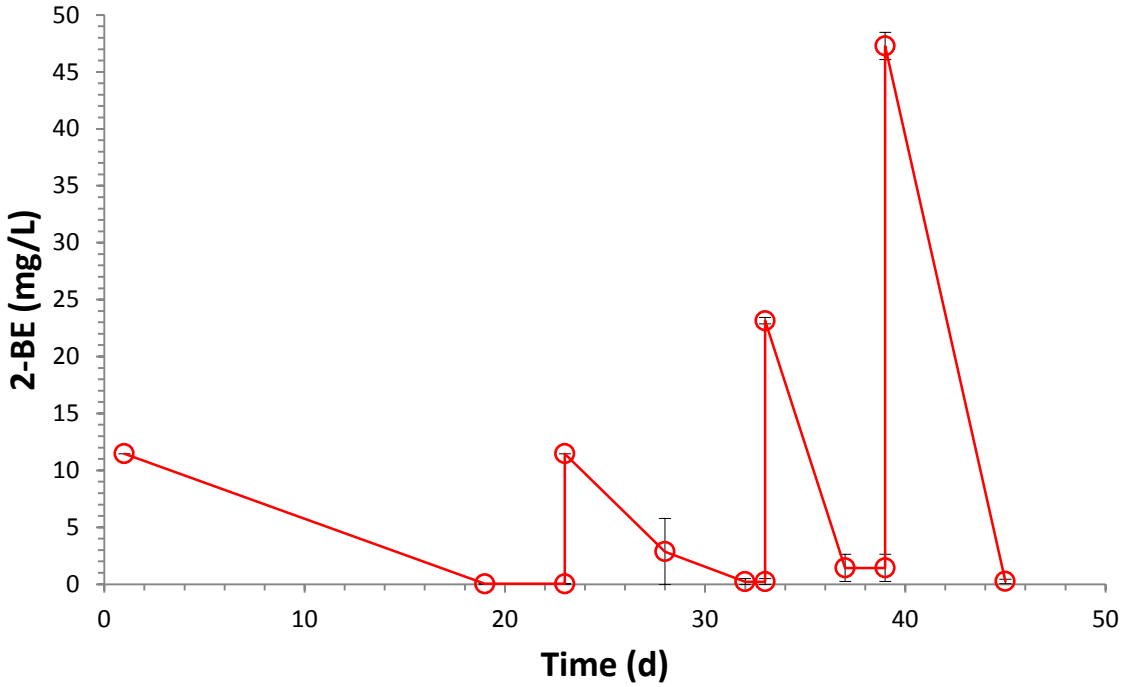

Figure 3.9 Average results for 2-BE levels in triplicate nitrate amended first transfer enrichments. Error bars represent standard deviations.

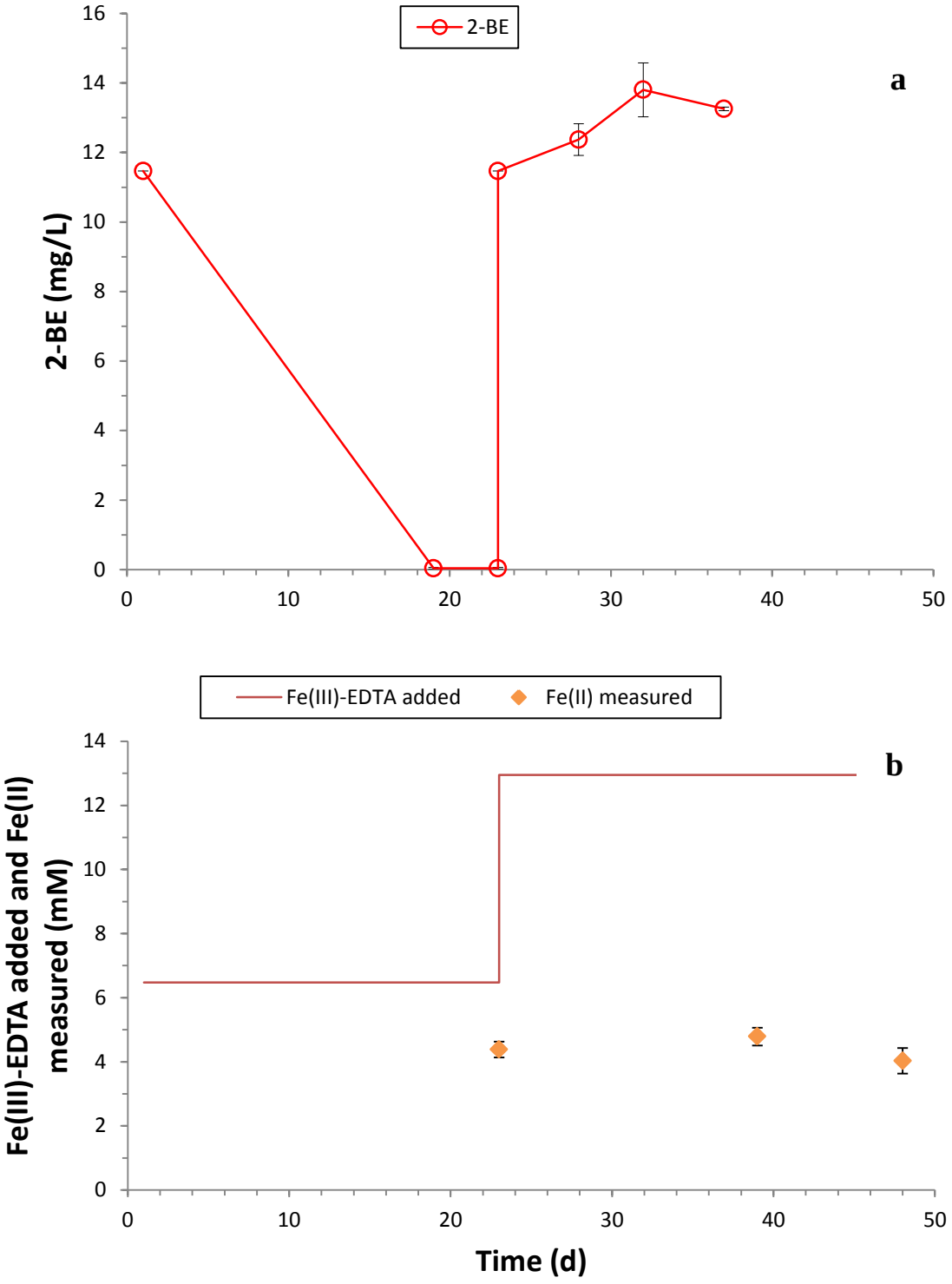

Figure 3.10 Average results for Fe(III)-EDTA amended first transfer enrichments; **a**) 2-BE, and **b**) Fe(III) added as Fe(III)-EDTA and Fe(II) levels. Error bars represent standard deviations.

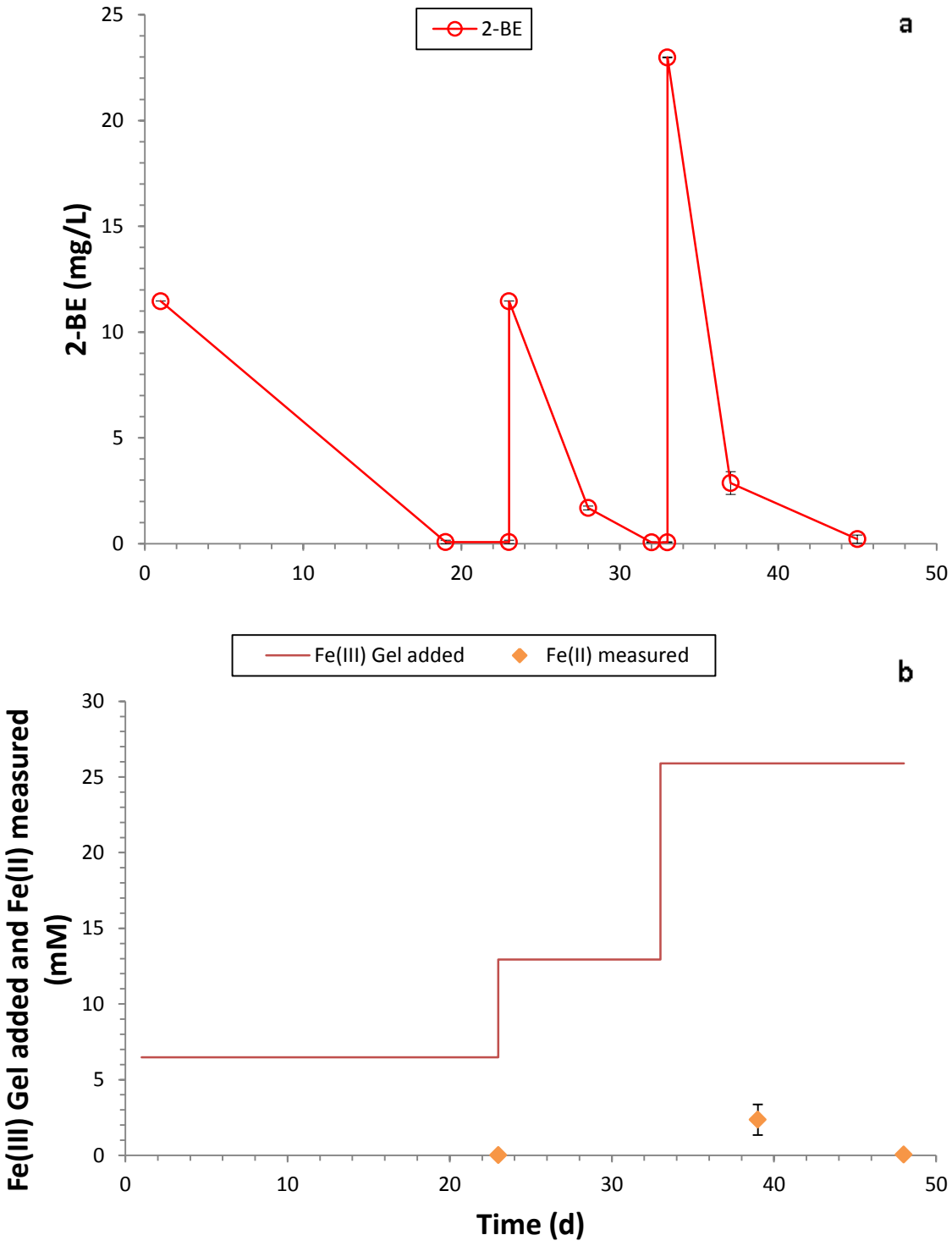

Figure 3.11 Average results for Fe(III) gel amended first transfer enrichments; **a**) 2-BE, and **b**) Fe(III) added as Fe(III) gel and Fe(II) levels. Error bars represent standard deviations.

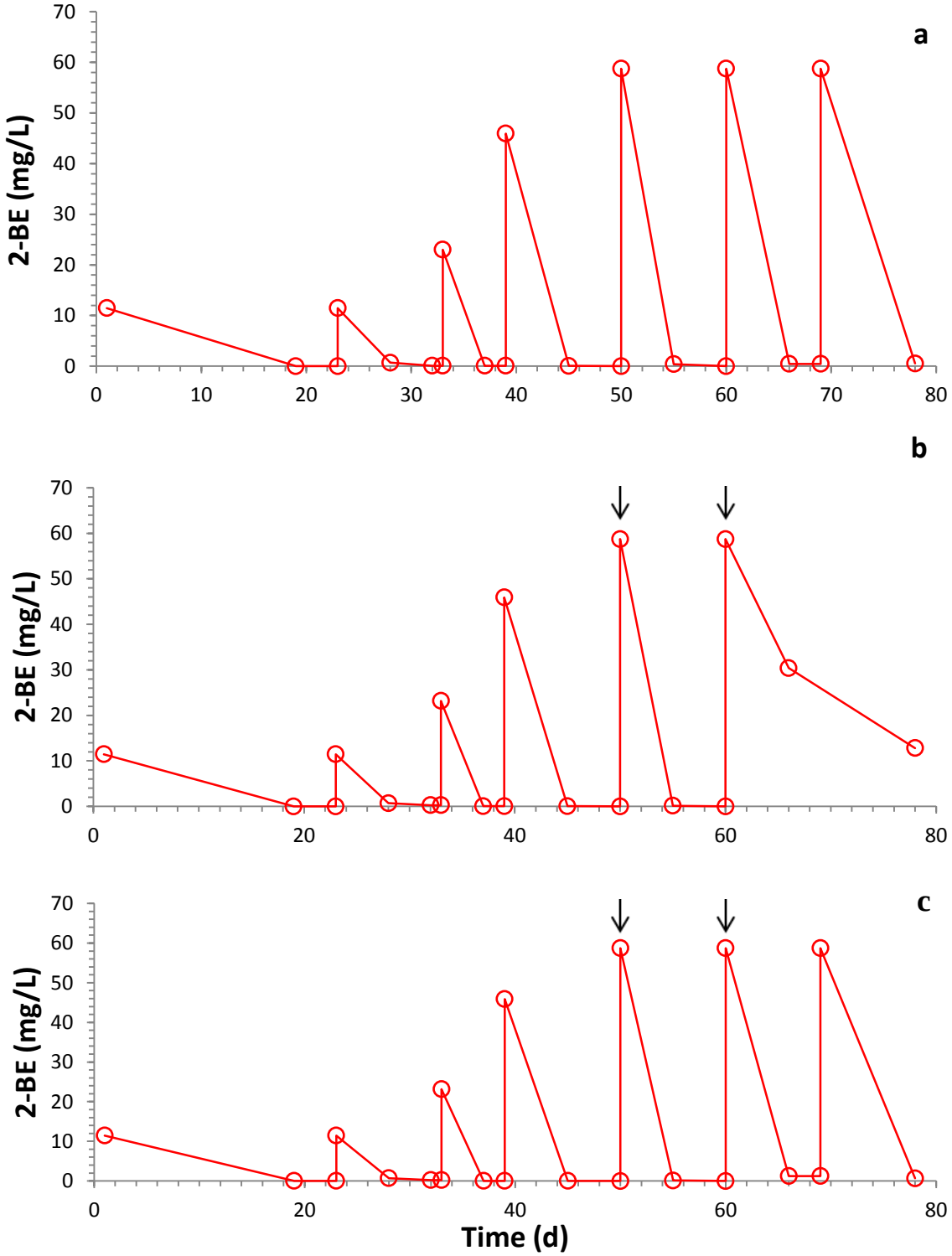

Figure 3.12 2-BE levels in unamended first transfer enrichments; **a**) bottle 1, **b**) bottle 2, and **c**) bottle 3. Arrows indicate addition of molybdate (20 mM).

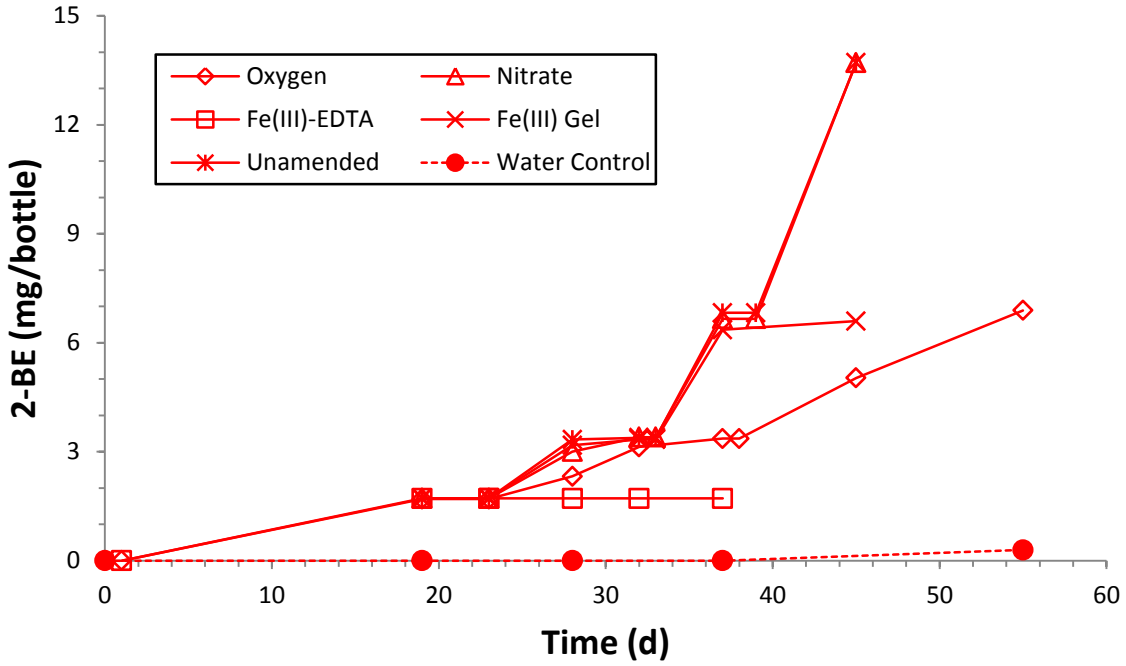

Figure 3.13 Cumulative 2-BE consumption shown as the average of triplicates of all first transfer enrichments.

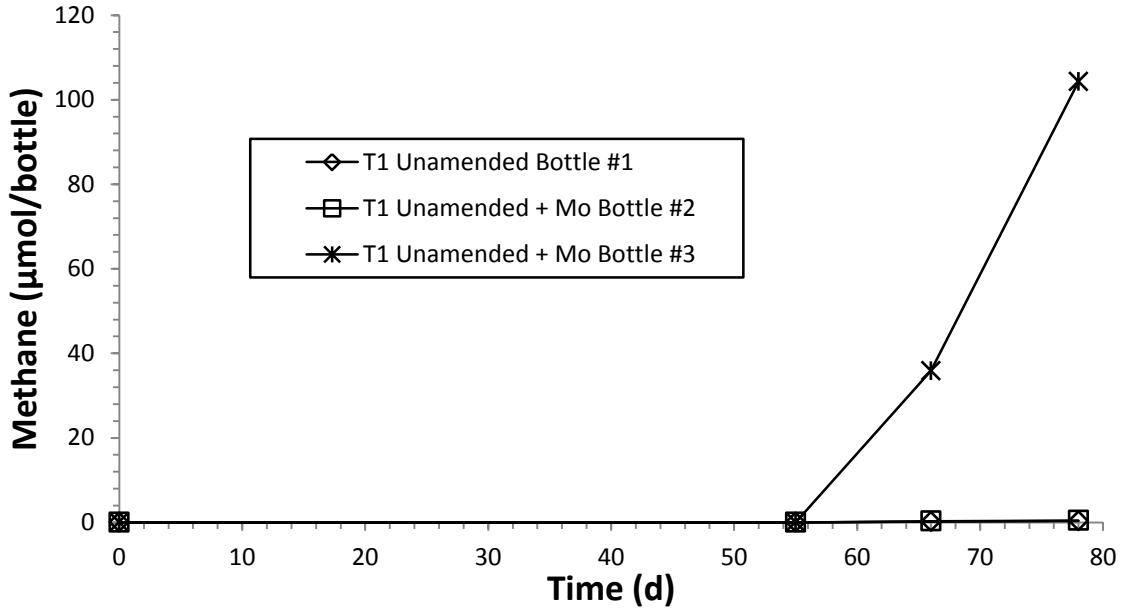

Figure 3.14 Methane levels in unamended first transfer enrichments. Bottles 2 and 3 were spiked with 40 mM molybdate.

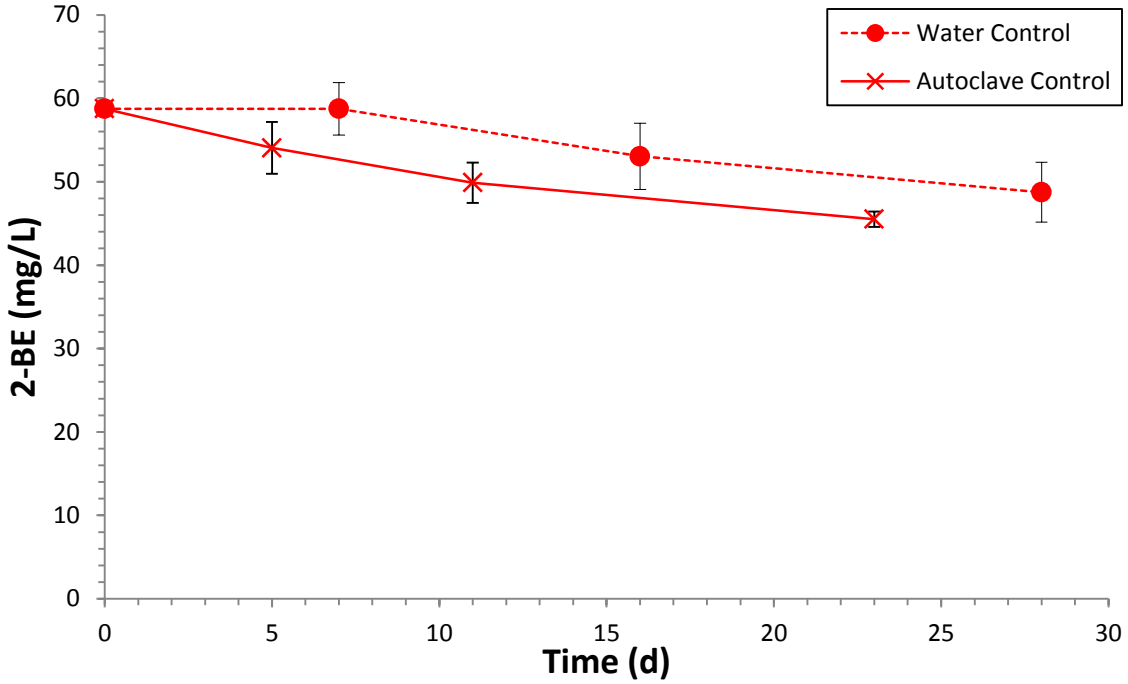

Figure 3.15 Average 2-BE levels in triplicates for WCs and autoclave controls prepared with the second transfer enrichments. Error bars represent standard deviations.

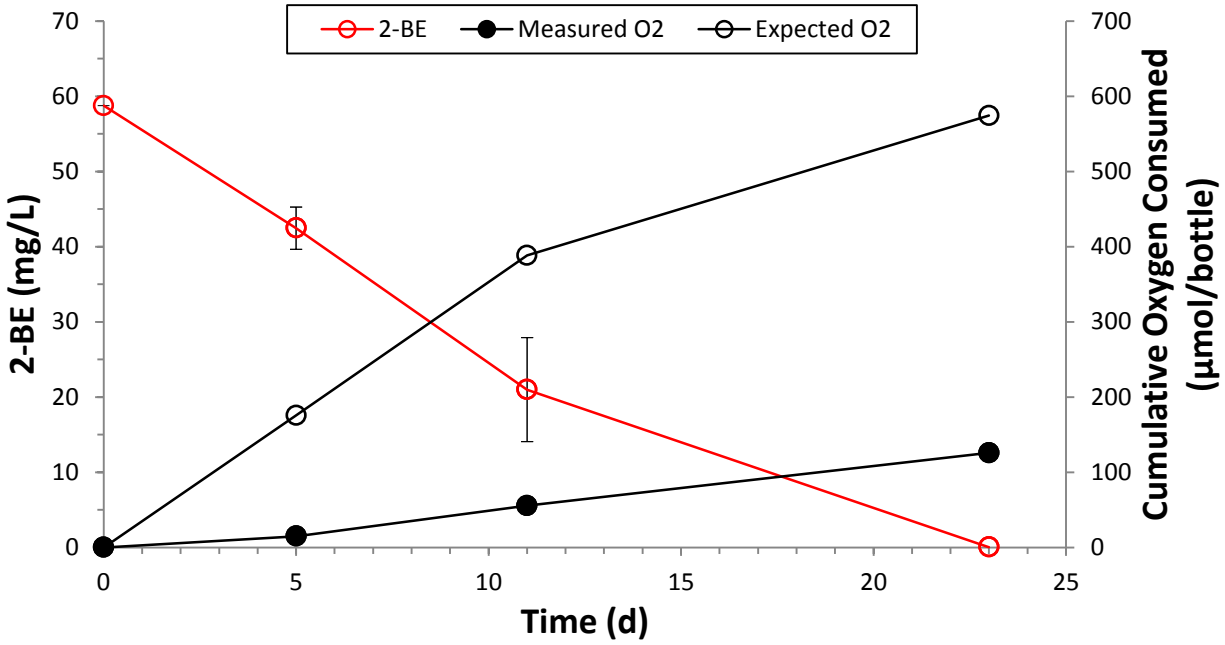

Figure 3.16 Average results for 2-BE levels in triplicate second transfer enrichment aerobic amendments. Error bars represent standard deviations.

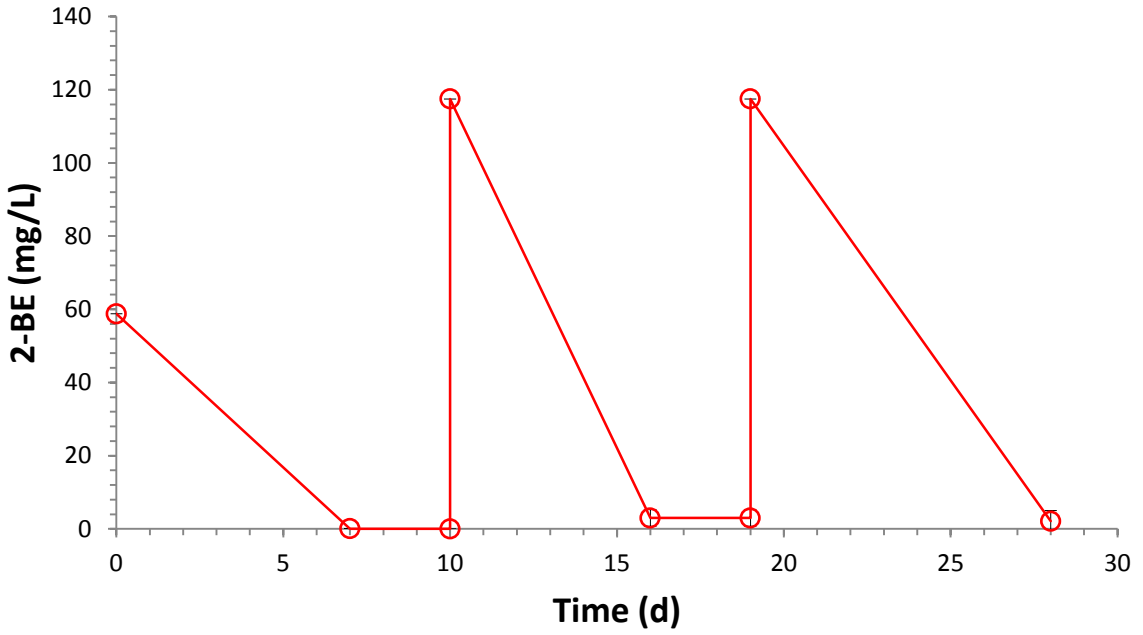

Figure 3.17 Average results for 2-BE levels in triplicate unamended second transfer enrichments. Error bars represent standard deviations.

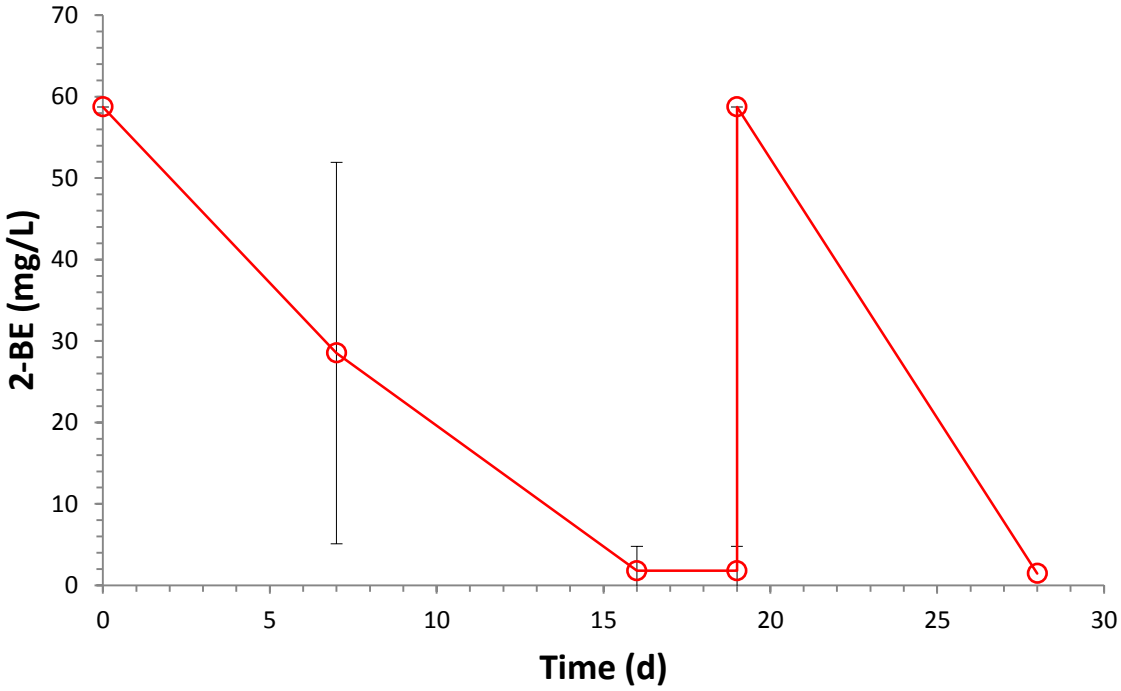

Figure 3.18 Average results for 2-BE levels in triplicate unamended second transfer enrichments spiked with 20 mM molybdate. Error bars represent standard deviations.

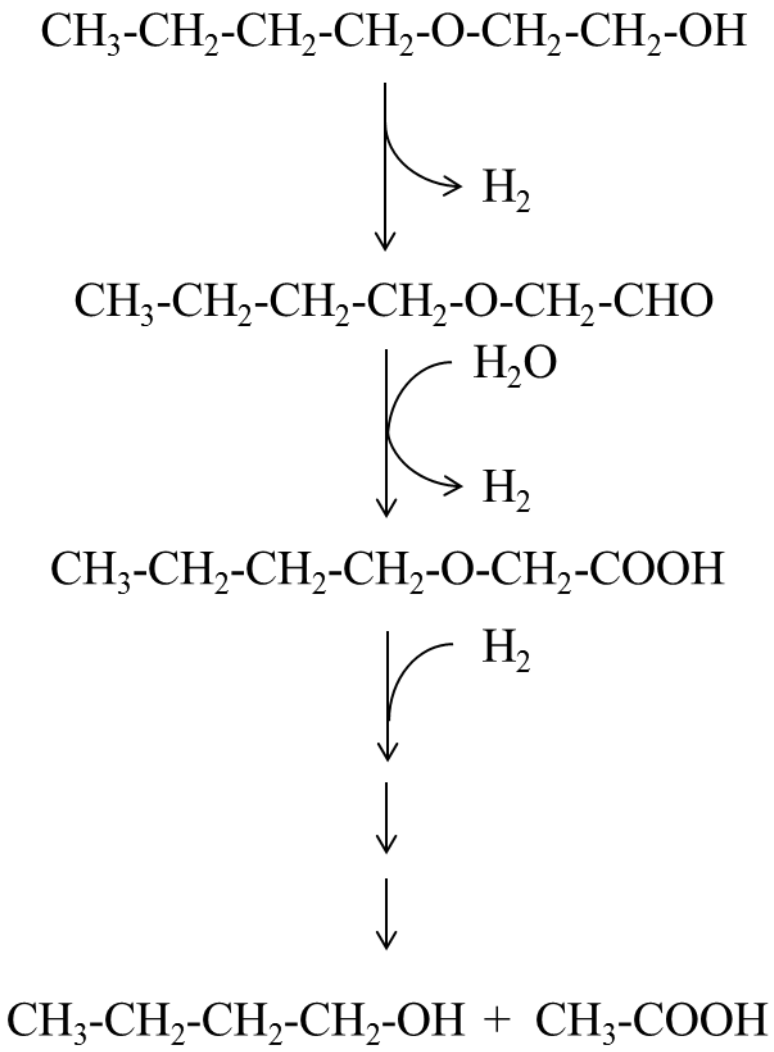

Figure 4.1 Proposed biodegradation pathway for 2-BE starting with stepwise oxidation to 2-butoxy acetic acid and followed by cleavage of the ether.

APPENDICES

APPENDIX A: *TEA Demand for 1,2-PD*

The target microcosm concentration is 15 mg/L:

$$15\,\frac{mg1{,}2PD}{L} * \frac{0.15L}{bottle} = \frac{2.25mg1{,}2PD}{bottle}$$

$$\frac{2.25mg1{,}2PD}{bottle} * \frac{1mmol1{,}2PD}{76mg1{,}2PD} = \frac{0.0296mmol\ 1{,}2PD}{bottle}$$

$$4H_2O + C_3H_8O_2 \rightarrow 3CO_2 + 16e^- + 16H^+$$

Oxidation of 1 mole of 1,2-PD yields 16 electron equivalents.

$$\frac{0.0296mmol1{,}2PD}{bottle} * \frac{16meeq}{mmol1{,}2PD} = \frac{0.474meeq}{bottle}$$

Therefore, the TEA demand per bottle is 0.474 milli-electron equivalents.

Oxygen

Oxygen is reduced to H_2O:

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O$$

Reduction of 1 mole of oxygen consumes 4 electron equivalents.

$$\frac{0.474meeq}{bottle} * \frac{1mmol\ oxygen}{4meeq} = 0.1185\,\frac{mmol\ oxygen}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.1185\ mmol\ oxygen}{bottle} * 1.5 = 0.1778\,\frac{mmol\ oxygen}{bottle}$$

Mass of oxygen consumed to mineralize 1,2-PD:

$$\frac{0.1778\ mmol\ oxygen}{bottle} * \frac{32\ mg\ O2}{mmol\ O2} = 5.69\,\frac{mg\ O2}{bottle}$$

Nitrate

Nitrate is reduced to N_2(g):

$$12H^+ + 10e^- + 2NO_3^- \rightarrow N_2 + 6H_2O$$

Reduction of 1 mole of nitrate consumes 5 electron equivalents.

$$\frac{0.474meeq}{bottle} * \frac{1mmol\ nitrate}{5meeq} = \frac{0.0947mmol\ nitrate}{bottle}$$

Using a safety factor of 1.5: $\frac{0.0947mmol\ nitrate}{bottle} * 1.5 = \frac{0.142mmol\ nitrate}{bottle}$

Concentration of stock solution needed if the volume of stock injected per bottle is 0.5 mL:

$$\frac{0.142mmol\ nitrate}{bottle} * \frac{1bottle}{0.5mlstocksol} = \frac{0.284mmol\ nitrate}{ml} * \frac{1mol}{1000mmol} * \frac{1000ml}{L} = \frac{0.284mol\ nitrate}{L}$$

Mass of sodium nitrate needed to mix with DDI water to make 100 mL stock solution:

$$\frac{0.284mol\ NaNO3}{L} * \frac{0.1L}{stock} * \frac{85gNaNO3}{mol} = 2.414g\ NaNO3$$

Fe(III)-EDTA

TEA demand per bottle is 0.474 milli-electron equivalents.

Fe(III) is reduced to Fe(II):

$$1e^- + Fe^{3+} \rightarrow Fe^{2+}$$

Reduction of 1 mole of Fe(III) consumes 1 electron equivalent.

$$\frac{0.474meeq}{bottle} * \frac{1mmol\ Fe(III)}{1meeq} = \frac{0.474mmol\ Fe(III)}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.474mmol\ Fe(III)}{bottle} * 1.5 = \frac{0.711mmol\ Fe(III)}{bottle}$$

Mass of Fe(III)-EDTA powder to be quantitatively transferred into microcosm or transfer enrichment bottle:

$$\frac{0.711mmol\ Fe(III)}{bottle} * \frac{367.05\ mg\ Fe(III) - EDTA}{mmol\ Fe(III) - EDTA} = \frac{261\ mg\ Fe(III) - EDTA}{bottle}$$

Fe(III) Gel

TEA demand per bottle is 0.474 milli-electron equivalents.

Fe(III) is reduced to Fe(II):

$$1e^- + Fe^{3+} \rightarrow Fe^{2+}$$

Reduction of 1 mole of Fe(III) consumes 1 electron equivalent.

$$\frac{0.474 meeq}{bottle} * \frac{1 mmol\ Fe(III)}{1 meeq} = \frac{0.474 mmol\ Fe(III)}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.474 mmol\ Fe(III)}{bottle} * 1.5 = \frac{0.711 mmol\ Fe(III)}{bottle}$$

Volume of 1M Fe(III) gel to add per bottle:

$$\frac{0.711 mmol\ Fe(III)}{bottle} * \frac{1 mL\ Fe(III)\ gel}{1 mmol\ Fe(III)} = \frac{0.711 mL\ Fe(III) gel}{bottle}$$

Sulfate

TEA demand per bottle is 0.474 milli-electron equivalents.

Sulfate is reduced to hydrogen sulfide.

$$10H^+ + 8e^- + SO_4^{2-} \rightarrow H_2S + 4H_2O$$

Reduction of 1 mole of sulfate consumes 8 electron equivalents.

$$\frac{0.474 meeq}{bottle} * \frac{1 mmol\ sulfate}{8 meeq} = \frac{0.0593 mmol\ sulfate}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.0593 mmol\ sulfate}{bottle} * 1.5 = \frac{0.0890 mmol\ sulfate}{bottle}$$

The concentration of sulfate in Instant Ocean® is 23 mmol/L (49).

The sulfate present in each bottle:

$$\frac{0.15 L\ Instant\ Ocean}{bottle} * \frac{23\ mmol\ sulfate}{1 L\ instant\ ocean} = \frac{3.45\ mmol\ sulfate}{bottle}$$

There is an abundance of sulfate to be used at the TEA for 1,2-PD degradation as 3.45 mmol >> 0.089 mmol

The sulfide concentration expected after the mineralization of 1,2-PD:

$$\frac{0.474 mmol\ 1{,}2-PD}{bottle} * \frac{8\ mmol\ sulfate}{1\ mmol\ 1{,}2-PD} * \frac{1 mmol\ sulfide}{1 mmol\ sulfate} * \frac{bottle}{0.15 L} = 25.28\ mM\ sulfide$$

Methanogenic

TEA demand per bottle is 0.474 milli-electron equivalents.

$$8H^+ + 8e^- + CO_2 \rightarrow CH_4 + 2H_2O$$

Production of 1 mole of methane consumes 8 electron equivalents. The methane expected in the headspace after the mineralization of 1,2-PD:

$$\frac{0.474\ meeq}{bottle} * \frac{1 mmol\ methane}{8\ meeq} * \frac{1{,}000\ \mu mol}{1\ mmol} = \frac{59.3\ \mu mol\ methane}{bottle}$$

APPENDIX B: *TEA Demand for 2-BE*

The target microcosm concentration is 15 mg/L:

$$15\frac{mg\ 2-BE}{L}*\frac{0.15L}{bottle}=2.25\frac{mg\ 2-Butoxyethanol}{bottle}$$

$$\frac{2.25\ mg\ 2-BE}{bottle}*\frac{1\ mmol\ 2-BE}{118.2\ mg\ 2-BE}=0.0190\frac{mmol\ 2-BE}{bottle}$$

$$10H_2O + C_6H_{14}O_2 \rightarrow 6CO_2 + 34e^- + 34H^+$$

Oxidation of 1 mole of 2-BE yields 34 electron equivalents.

$$\frac{0.0190mmol\ 2-BE}{bottle}*\frac{34\ meeq}{mmol\ 2-BE}=0.647\frac{meeq}{bottle}$$

Therefore, the TEA demand per bottle is 0.647 milli-electron equivalents.

Oxygen

Oxygen is reduced to H_2O:

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O$$

Reduction of 1 mole of oxygen consumes 4 electron equivalents.

$$\frac{0.647meeq}{bottle}*\frac{1mmol\ oxygen}{4meeq}=0.1618\frac{mmol\ oxygen}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.1618\ mmol\ oxygen}{bottle}*1.5=0.2426\frac{mmol\ oxygen}{bottle}$$

Mass of oxygen consumed to mineralize 2-BE:

$$\frac{0.2426\ mmol\ oxygen}{bottle}*\frac{32\ mg\ O2}{mmol\ O2}=7.8\frac{mg\ O2}{bottle}$$

Nitrate

Nitrate is reduced to N_2(g):

$$12H^+ + 10e^- + 2NO_3^- \rightarrow N_2 + 6H_2O$$

Reduction of 1 mole of nitrate consumes 5 electron equivalents.

$$\frac{0.647 meeq}{bottle} * \frac{1 mmol\ nitrate}{5 meeq} = 0.129 \frac{mmol\ nitrate}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.129\ mmol\ nitrate}{bottle} * 1.5 = 0.194 \frac{mmol\ nitrate}{bottle}$$

Concentration of stock solution needed if the volume of stock injected per bottle is 0.5 mL:

$$\frac{0.194\ mmol\ nitrate}{bottle} * \frac{1 bottle}{0.5 ml stocksol} = \frac{0.388\ mmol\ nitrate}{ml} * \frac{1 mol}{1000 mmol} * \frac{1000 ml}{L} = 0.388 \frac{mol\ nitrate}{L}$$

Mass of sodium nitrate needed to mix with DDI water to make 100 mL stock solution:

$$\frac{0.388\ mol\ nitrate}{L} * \frac{0.1 L}{stock} * \frac{85 g NaNO3}{mol} = 3.30\ g\ NaNO3$$

Fe(III)-EDTA

TEA demand per bottle is 0.647 milli-electron equivalents.

Fe(III) is reduced to Fe(II):

$$1e^- + Fe^{3+} \rightarrow Fe^{2+}$$

Reduction of 1 mole of Fe(III) consumes 1 electron equivalent.

$$\frac{0.647 meeq}{bottle} * \frac{1 mmol\ Fe(III)}{1 meeq} = \frac{0.647 mmol\ Fe(III)}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.647 mmol\ Fe(III)}{bottle} * 1.5 = \frac{0.971 mmol\ Fe(III)}{bottle}$$

Mass of Fe(III)-EDTA powder to be quantitatively transferred into microcosm or transfer enrichment bottle:

$$\frac{0.971 mmol\ Fe(III)}{bottle} * \frac{367.05\ mg\ Fe(III)-EDTA}{mmol\ Fe(III)-EDTA} = \frac{356\ mg\ Fe(III)-EDTA}{bottle}$$

Fe(III)

TEA demand per bottle is 0.647 milli-electron equivalents.

Fe(III) is reduced to Fe(II):

$$1e^- + Fe^{3+} \rightarrow Fe^{2+}$$

Reduction of 1 mole of Fe(III) consumes 1 electron equivalent.

$$\frac{0.647 meeq}{bottle} * \frac{1 mmol\ Fe(III)}{1 meeq} = \frac{0.647\ mmol\ Fe(III)}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.647 mmol\ Fe(III)}{bottle} * 1.5 = 0.971 \frac{mmol\ Fe(III)}{bottle}$$

Volume of 1M Fe(III) gel to add per bottle:

$$\frac{0.971\ mmol\ Fe(III)}{bottle} * \frac{1 mL\ Fe(III)\ gel}{1 mmol\ Fe(III)} = \frac{0.971\ mL\ Fe(III) gel}{bottle}$$

Sulfate

TEA demand per bottle is 0.647 milli-electron equivalents.

Sulfate is reduced to hydrogen sulfide.

$$8e^- + SO_4^{2-} \rightarrow H_2S$$

Reduction of 1 mole of sulfate consumes 8 electron equivalents.

$$\frac{0.647 meeq}{bottle} * \frac{1 mmol\ sulfate}{8 meeq} = \frac{0.0809\ mmol\ sulfate}{bottle}$$

Using a safety factor of 1.5:

$$\frac{0.0809\ mmol\ sulfate}{bottle} * 1.5 = \frac{0.121 mmol\ sulfate}{bottle}$$

The concentration of sulfate in Instant Ocean® is 23 mmol/L (49).

The sulfate present in each bottle:

$$\frac{0.15 L\ Instant\ Ocean}{bottle} * \frac{23\ mmol\ sulfate}{1 L\ instant\ ocean} = \frac{3.45\ mmol\ sulfate}{bottle}$$

There is an abundance of sulfate to be used at the TEA for 2-BE degradation as 3.45 mmol >> 0.121 mmol

The sulfide concentration expected after the mineralization of 2-BE:

$$\frac{0.647\ mmol\ 2-BE}{bottle} * \frac{8\ mmol\ sulfate}{1\ mmol\ 2-BE} * \frac{1 mmol\ sulfide}{1 mmol\ sulfate} * \frac{bottle}{0.15\ L} = 34.5\ mM\ sulfide$$

Methanogenic

TEA demand per bottle is 0.647 milli-electron equivalents.

$$8H^+ + 8e^- + CO_2 \rightarrow CH_4 + 2H_2O$$

Production of 1 mole of methane consumes 8 electron equivalents. The methane expected in the headspace after the mineralization of 2-BE:

$$\frac{0.647\ meeq}{bottle} * \frac{1 mmol\ methane}{8\ meeq} * \frac{1{,}000\ \mu mol}{1\ mmol} = \frac{80.9\ \mu mol\ methane}{bottle}$$

APPENDIX C: *Protocol for the Analysis of 2-BE and 1,2-PD in Seawater using ENVI-Carb Plus*

Solid phase extraction cartridges were obtained from Sigma-Aldrich Supelco, product number 54812-U.

Solid Phase Extraction Method, as outlined by Sidisky, et al. (33)

- Insert ENVI-Carb Plus reversible cartridge into the port of vacuum manifold.
- Attach an empty tube or syringe barrel to top of reversible cartridge to serve as reservoir as shown in Figure C.1.

Figure C.1 Reservoir, cartridge, vacuum manifold set-up

- Pull 1 mL methylene chloride through cartridge
- Pull 2 mL methanol through cartridge
- Pull an additional 2 mL methanol through cartridge
- Pull 3 mL deionized water through cartridge
- Pull 5 mL sample (seawater + 2-BE or 1,2-PD) through cartridge using 5 in Hg

vacuum

- Discard cartridge effluent
- Pull air with 10 in Hg vacuum for 10 min to dry the cartridge.
- Place clean effluent receptacle inside vacuum manifold under cartridge.
- Remove the reversible cartridge and reattach upside-down using fittings as shown in Figure C.2

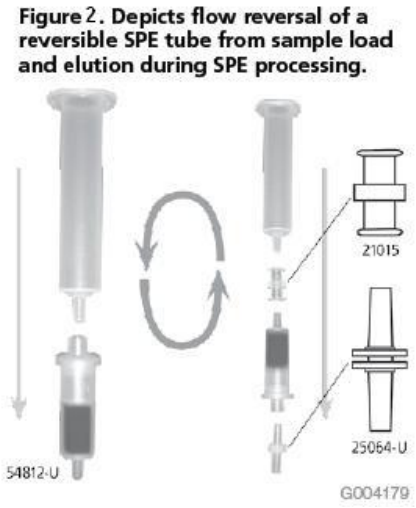

Figure C.2 Diagram of cartridge reversal

- Using 2 mL of an 80:20 methanol:dichloromethane solution, soak cartridge for 1 minute prior to pulling through
- Remove effluent receptacle (now containing the eluted 2-BE and/or 1,2-PD) from vacuum manifold
- This eluent will have a precipitate and must be centrifuged at 3,000 rpm for 5 minutes.

- The centrate is transferred to a 1.8 mL GC autosampler vial using a disposable glass Pasteur pipette. The vial is capped with a Teflon lined septa. The vial is refrigerated until the GC is available.

Other Peaks

After 66 days of incubating the first transfer enrichments for 2-BE, and 16 days of incubating the second transfer enrichments, a new peak was observed on the chromatogram with a retention time of 5 min (compared to a retention time of 9.2 min for 2-BE). The peak area ranged widely from about 455 in bottle 2 of the second transfer aerobic treatment to 283,931 in bottle 1 of the second transfer unamended treatment. This compares to a peak area of 88,409 for 16.5 mg/L of 2-BE, indicating the likelihood that the unknown peak represented a metabolite. The identity of this peak was not determined.

APPENDIX D: *GC-FID Analysis Method*

- The method outlined by Sidisky, et al. (33) uses a Supelco SPB – 1000 GC column containing a bonded, acid-modified poly(ethylene glycol) phase. The dimensions are 30 m x 0.25 mm I.D. x 0.25 μm. Supelco Cat. # 24313
- The column used for the experiments discussed in this thesis is a Phenomenex ZB-FFAP GC column containing a nitroterephthalic acid modified polyethylene glycol phase. The dimensions are 30 m x 0.25 mm I.D. x 0.25 μm. Phenomenex Cat. # 7HG-G009-11
- The GC oven temperature program starts at 50°C for 2.5 min, increases at 8°C / min until reaching 200°C, and held at 200°C for 12 min. The total time is 33.25 min. See the temperature program plot in Figure D.1.

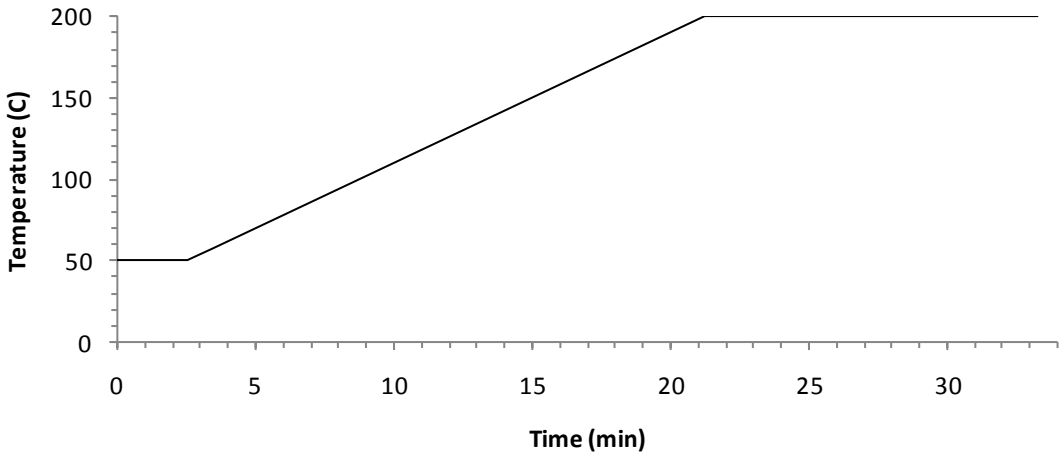

Figure D.1 GC temperature program

- The injector and FID temperatures are both set to 220 °C.
- The GC injection volume is 1 µL in splitless mode.
- The GC liner used was a FocusLiner with 4 mm I.D. with taper.
- Sidisky et al, (33) call for a helium carrier gas at a flowrate of 1.5 mL/min.
- We used hydrogen as the carrier gas at a flowrate of 1.8 mL/min.
- A chromatogram showing the retention times of 2-BE and 1,2-PD is shown in Figure D.2

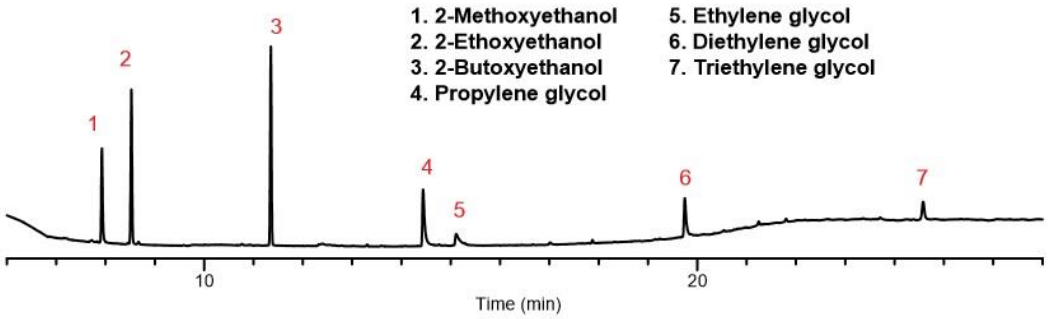

Figure D.2 A chromatogram showing the retention times of glycols and glycol ethers extracted from seawater spiked at 2 mg/L using the ENVI-Carb Plus SPE method and GC-FID method outlined above (33).

APPENDIX E: *Standard Operating Procedure for Measuring O_2 Gas*

- In Lab 112, on the GC computer, open the Unichrom software.
- Load the method "HOLD" on the GC named "Ethel" if it's not already in HOLD.
    - Do this by opening the program "HOLD" by going to File > Open > Desktop > HOLD
    - Click the "Instrument" button and double click "Ethel"
- The GC named "Greta" should be running in the HOLD method. Close this method by selecting it, and then clicking the small "x" in the top right corner of the screen to close the HOLD method. If "Greta" is not running a method, no action is necessary.
- Load the method "Oxygen TCD Greta" on GC "Greta"
    - File > Open > Desktop > Ben Rhiner > Oxygen TCD Greta
- Open the side-door of the GC named "Greta" and open the green valve. This is nitrogen gas. Note that the gas MUST be flowing before the TCD can be turned on! If the TCD is on while no gas is flowing the TCD will be damaged.
- Move the aluminum foil from injector A to injector B.
- Using the buttons on the front of GC "Greta" turn on the TCD detector, which is detector A
    - Press button "DET"
    - Press button "On"
- Using the buttons on the front of "Greta" set the signal to read from detector A
    - Press button "SIG 1"

    - Press button “A”
    - Press button “Enter”
    - Press button “SIG 2”
    - Press button “A”
    - Press button “Enter”
- Using the buttons on the front of “Greta” make sure that detector A is set to high sensitivity
    - Press the small blank button that is a different color (orange-brown). This is the function button, and allows the next button pushed to function as the description given next to it.
    - Press the button labeled “DET” and described as “TCD SENS” and ensure that “High Sensitivity” appears in the display.
- Using the buttons on the front of “Greta” the signal can be read. It will be negative.
    - Press the button labeled “SIG 1” or “SIG 2”
- Inject 0.5 mL of air
- Immediately press the “Start” button after the injection.
    - Using the buttons on the front of the GC, press the “START” button. It is the button that has had its label worn off.
- After the run is complete, inject 0.5 mL of the headspace gas from your sample microcosm.

- The retention time of oxygen is about 3.4 minutes. Integrate the peaks by hand. The peak area of the air injection will be around 7. This corresponds to 21% oxygen. The peak area corresponds to the percent oxygen and is used to find the percent oxygen of the sample gas injected.
- Using the peak area of your sample, solve for x to find the percent oxygen:

$$\frac{0.21}{PA_{air}} = \frac{x}{PA_{sample}}$$

- When finished, using the buttons on the front of "Greta" turn of the TCD
    - Press button "DET"
    - Press button "OFF"
- Set the signal back to reading from detector B
    - Press button "SIG 1"
    - Press button "B"
    - Press button "Enter"
    - Press button "SIG 2"
    - Press button "B"
    - Press button "Enter"
- Open the side-door of "Greta" and close the green valve. This is nitrogen gas.
- Close the "Oxygen TCD Greta" method. Do this by selecting the small "x" in the top right corner of the screen to close the method. Select Yes, save, when asked to save.
- Load the HOLD method again for "Greta".

APPENDIX F: *Protocol for Measuring NO_3^- in Seawater Using Hach DR890 Colorimeter Method 8039 (λ=520nm)*

- In the anaerobic chamber, collect 3 mL of seawater into a centrifuge vial for each nitrate bottle.
- Remove bottles and vials from anaerobic chamber.
- Sparge headspace of nitrate bottles with N_2 gas.
- Using sodium nitrate, make nitrate standards in Instant Ocean containing 1mg/L resazurin to achieve concentrations of: 0, 1, 5, 10, 15, 20, 25, 30 mg/L NO3- N.
- Use 2 mL of each sample to rinse a 0.45 µm syringe filter and put the remaining 1 mL into a Hach colorimeter vial.
- Filter 9 mL of Instant Ocean® using a 0.45 µm syringe filter and add to the 1 mL of sample in colorimeter vial. This provides a 1:10 dilution to bring the nitrate concentration within the range of 1 – 30 mg/L nitrate as N.
- Filter 10 mL of each of the prepared standards using a 0.45 µm syringe filter, and put into colorimeter vials.
- Turn on colorimeter and press PRGM; press 51 ENTER.
- Add NitraVer 5 Nitrate Reagent Pillow (containing Cd) to each colorimeter vial; press TIMER, ENTER. A one-minute reaction period will begin. Be sure the caps are on tight and shake the vials vigorously until the timer beeps.
- After the timer beeps, the display will show: 5:00 TIMER 2 Press: ENTER A five-minute reaction period will begin. Note: A deposit will remain after the

reagent dissolves and will not affect test results. Note: An amber color will develop if nitrate nitrogen is present

- Use the 0mg/L standard as the blank. Place the blank into the cell holder. Tightly cover the sample cell with the instrument cap.
- When the timer beeps, press ZERO. The cursor will move to the right, then the display will show: 0.0 mg/L NO3-N. However, because we want absorbance, press the ABS button. This will display the absorbance, which we will use to make our calibration curve.
- Rinse vials immediately to remove Cd. Put Cd waste into Cd hazardous waste container.
- Determine nitrate concentration using response factor from calibration curve of the standards and accounting for the 1:10 dilution.

APPENDIX G: *Ferrozine Assay*

Iron analysis (ferrozine assay)

The ferrozine assay is specific for Fe(II), or ferrous iron, and does not directly quantify Fe(III), or ferric iron (34). Both measurements are required to assess the extent of Fe(III) reduction, and the concentration of bioavailable iron that remains in subsurface aquifer material. Fe(II) can be measured directly in filtered or unfiltered, acidified groundwater, or in acid extracts of aquifer material that has been digested with HCl. Fe(III) as an aquifer solid can be quantified as the difference between initial Fe(II) and final Fe(II) after reacting the iron extract with a strong reducing compound. The following describes the general procedure first, followed by the ferrozine assay as applied to solid aquifer material for quantifying total iron.

I REAGENT PREPARATION

1.1 Ferrozine Solution Preparation

Reagents: 11.62 g/L HEPES buffer and 1 g/L ferrozine.

STEP 1: Clean all the glassware that will be used for the preparation of the solution (use 0.5N HCl to remove any Iron present in the glass).

STEP 2: Add HEPES to ca. 800 mL water, stir into solution.

STEP 3: Add ferrozine and stir into solution.

STEP 4: Bring pH to 7.0 with 5-10N NaOH. Bring volume to 1 L, then pour it into a plastic container with a cap and label it (chemical name, initials and date). Wrap the container with aluminum foil to avoid light react with the ferrozine. Use at room temperature (you may use a water bath if it's not) but store at 4 °C (in the refrigerator).

1.2 0.5N HCl Solution Preparation

Reagents: 42.1 mL HCl.

STEP 1: Add the HCl into a volumetric flask with 800 mL of water (do not add water to HCl), shake it, and bring volume to 1 L, then pour it to a plastic container with a cap and label it (chemical name, initials and date). Use it at room temperature.

II STANDARD CURVE PREPARATION

2.1 Stock Solutions Preparation

Fe(II) standard are prepared in 0.5N HCl with ferrous ethylene diammonium sulfate (which is stored at 4 °C, in the refrigerator and **USED ONLY INSIDE THE GLOVE BOX**). The following concentration stocks are to be prepared: 1.0mM; 5.0mM; 10.0mM; 20.0mM; and 40.0mM. Preparations should be made in new or acid washed glass serum bottles. The concentrated stock solutions can be kept for up to **six months** at room temperature.

STEP 1: Take the ferrous ethylene diammonium sulfate, five 100mL serum bottles, 0.5N HCl solution, graduate cylinder, blue septa, aluminum caps, crimper, and put them in the glove box (make sure the side you are working on has a digital balance).

STEP 2: Weight 1.53 g of ferrous ethylene diammonium sulfate, and put it into 100mL of 0.5N HCl in one serum bottle, this will be the highest standard (40.0mM).

STEP 3: Take 20mL of standard 40.0mM and put it into a serum bottle with 20mL of 0.5N HCl, this will be standard 20.0mM.

STEP 4: Take 10mL of standard 40.0mM and put it into a serum bottle with 30mL of 0.5N HCl, this will be standard 10.0mM.

STEP 5: Take 5mL of standard 40.0mM and put it into a serum bottle with 35mL of 0.5N HCl, this will be standard 5.0mM.

STEP 6: Take 1mL of standard 40.0mM and put it into a serum bottle with 39mL of 0.5N HCl, this will be standard 1.0mM.

STEP 7: Put the blue septa on each serum bottle, the aluminum cap and crimp them. Finally take them out from the glove box.

2.2 Diluted Stock Solution Preparation

Each standard should be diluted 1:50 in 0.5N HCl for final concentrations of 0.02mM; 0.10mM; 0.2mM; 0.4mM; 0.8mM.

Material: 5 scintillation vials (no caps needed), 0.5N HCl, 5mL Eppendorf pipet, 100µL Eppendorf pipet, ferrozine solution, 5 plastic syringes of 1mL, 5 syringe needles, nitrogen gas line.

STEP 1: If the vials are reused, wash them with 0.5N HCl to ensure the complete removal of any Fe(II) sticking onto the glass.

STEP 2: Pour 4.9mL of 0.5N HCl into the 5 vials.

STEP 3: Acclimate the plastic syringe (with the needle on) with nitrogen gas by sucking it several times and leaving 0.1mL of gas inside the syringe.

STEP 4: Once acclimated the syringe, rapidly (avoiding oxygen transfer into the syringe) to the stock solution, add the 0.1mL of nitrogen gas and then suck 0.1mL of stock solution.

STEP 5: Take the 0.1mL stock solution into the scintillation vial, submerge the needle and release the solution into 0.5N HCl. This will prevent from Fe(II) being oxidized. Throw away the syringe into the respective container. Repeat the same process with other stock solutions using different syringes.

These standards can be kept and used for up to **two weeks** at room temperature. These diluted standards will be used to prepare the standard curve. Standards should be diluted and stored in Teflon-lined plastic scintillation vials or a similar container. They should always be made as dilutions of the concentrated stock solutions, rather than directly from the ferrous salts. This is to ensure that standards and samples are handled and sampled in a similar manner with regard to the initial dilution (aliquot) into 0.5N HCl.

STEP 6: Go to Lab 114 and prepare the spectrophotometer (in front of the GC "instrument 2") by opening the software "*Simple Reads*".

STEP 7: Click on "*Setup ...*" and on the next window, on the read mode, click on "*Read at Wavelength*" and type at the box 562 and press OK (Figure 1).

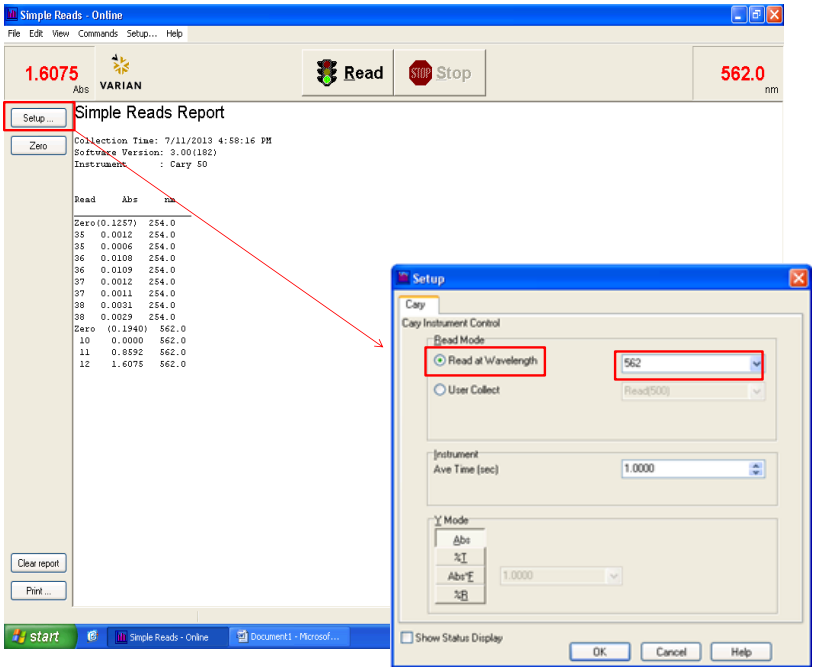

Figure G.1 Setting up the spectrophotometer for total iron.

Ferrozine solution should be used at room temperature (remove from refrigerator at least one hour prior to use).

STEP 8: Add 100µL of stock solution to 4900µL ferrozine solution in a scintillation vial (previously washed with 0.5N HCl). After this step the assay becomes time critical. The color change will become more intense in light at room temperature over time. Absorbance should be measured within five minutes of adding Fe(II) to the ferrozine solution (whether it is a standard or a sample).

STEP 9: Add each standard to a 4.0 mL cuvette and another one with only ferrozine, which will be our blank.

STEP 10: Once the samples are prepared, put the cuvette (the side that has the narrow square goes at the sides, the wider one in front facing towards you) with the ferrozine

only in the spectrophotometer and close the cap. Press on "*Zero*". You should see the word Zero on the report (Figure 2). Press on "*Read*" to see the confirmation of the calibration (you should see at the absorbance column "*0.0000*").

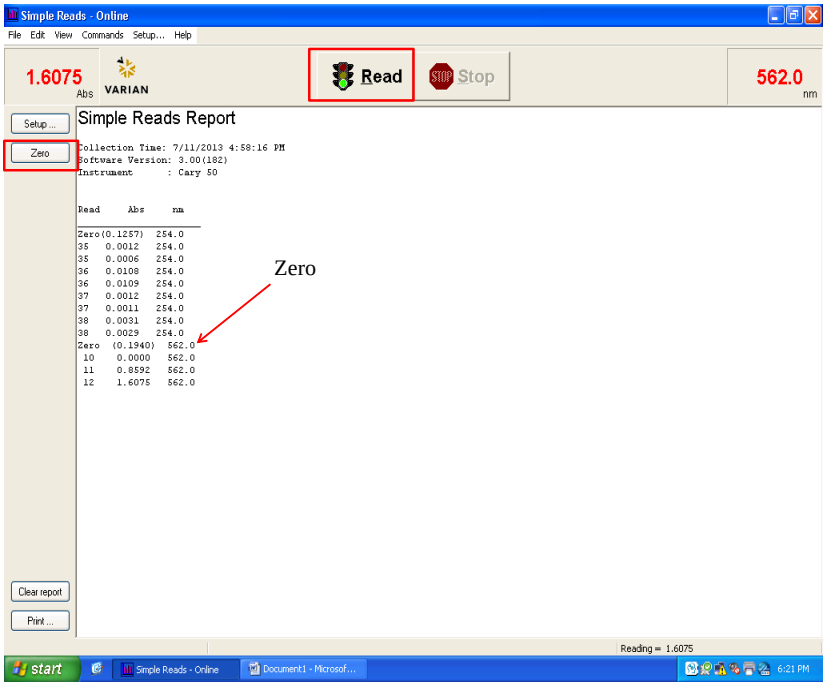

Figure G.2 Calibrating the spectrophotometer.

STEP 11: Introduce now the second cuvette, close the cap and click on "*Read*", do the same with the rest of the cuvettes. The data that we need is from the second cuvette. The standard used to get the slope is the next:

Table G.1 Ferrozine Standards Example.

Fe (II) Standard (mM)	Abs
0.02	0.0239
0.1	0.0583
0.2	0.1145
0.4	0.2298
0.8	0.4295

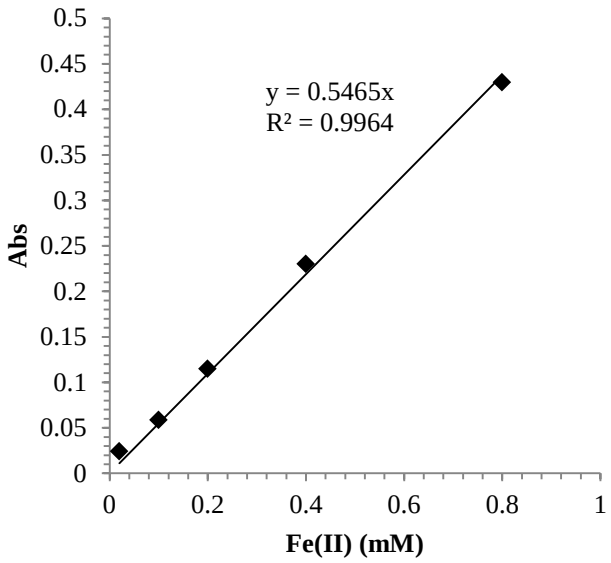

Figure G.3 Plot of the absorbances of the standards.

STEP 12: The slope of the standard curve should fall between 0.45 and 0.55; the r^2 value should be no less than 0.980.

STEP 13: Save the standard curve for sample analysis (automated measurement feature on most spectrophotometers).

III SAMPLE ANALYSIS

STEP 1: Add 100µL of unfiltered sample to 4900µL of 0.5N HCl in a Teflon-lined scintillation vial or similar container. Fe(II) is stable in 0.5N HCl; however, the analysis should proceed as quickly as possible.

STEP 2: Add 100µL of the acidified sample to 4,900µL of ferrozine solution. If there is Fe(II) in solution then there will be a visible color change that will be very light to very dark purple, depending on the Fe(II) concentration.

STEP 3: Measure the sample versus the standard curve prepared earlier at 562nm.

STEP 4: If the measured Fe(II) concentration exceeds the high standard (0.8mM), then the sample (acidified water) should be diluted in 0.5N HCl to bring the measurement within the standard curve. This dilution is very important because for the ferrozine solution absorbance levels-off quickly after the high standard, and measurements can be easily underestimated.

STEP 5: Quantify the Fe(II) via the following equation:

Measured Fe(II) x 50 (from 1:50 dilution in acid) x dilution factor = mM Fe(II)

This final number is only the Fe(II), not total dissolved iron.

STEP 6: Add 200µL of 6N hydroxylamine solution to the acid extract and mix gently.

STEP 7: Allow the acid extract to react with hydroxylamine for one hour.

STEP 8: Add 100µL of acid extract to 4,900µL of ferrozine solution.

STEP 9: Measure the absorbance at 562nm. This is the total HCl-extractable iron fraction of the agar. The Fe(II) fraction of the agar is the final Fe(II) minus the initial Fe(II).

STEP 10: Quantify Fe(II) by the following equation:

[[Measured Fe(II) x 0.005 x dilution factor]/agar wet weight] = mmol Fe(II) per gram agar

Use the equation for initial Fe(II) and final Fe(II) - as above the Fe(II) is the final minus the initial Fe(II).

STEP 11: Quantify the "percent reduced" or "% Fe(II)" associated with the agar fraction by the following equation:

Initial Fe(II)/Total HCl-extractable iron

STEP 12: The "percent reduced" is the proportion of the available Fe(II) that has been reduced by microbial respiration (normalized value that is independent of the actual concentration of iron in the agar).

APPENDIX H: *Procedure for Measuring Sulfide in Seawater*

- The sulfide range that this procedure is designed for is 250 – 1,000 sulfide (µM) (35).
- Dissolve 20 g of N,N-dimethyl-p-phenylenediamine sulfate and 30 g ferric chloride ($FeCl_3*6H_20$) in 500 mL of cool 50% (v/v) reagent grade hydrochloric acid. Store in brown glass bottle in fridge.
- Make enough Instant Ocean® to have 50 mL per standard or sample, and 100 mL for the sodium sulfide stock solution. Transfer the Instant Ocean® to a container that fits inside the vacuum chamber. Put stir bar into container, place the container into the right-hand anaerobic chamber, and stir on the stir plate for several hours before using.
- Take a crystal of Na_2S*9H_20 out of container in fridge. Do this under the fume hood and wear gloves. Rinse the crystal with DDI water to remove oxidized reagent. Then blot dry with a Kimwipe®. Break it into smaller pieces using a ball-peen hammer. Using these pieces, weigh out 2.4 g Na_2S*9H_20 into a scintillation vial. Engage the threads on the vial cap, put it into the vacuum chamber, and put it into the anaerobic chamber. Using Instant Ocean®, quantitatively transfer the 2.4 g Na_2S*9H_20 into a 100 mL volumetric flask, seal with Parafilm©, and mix. This makes a 100,000 µM or 24,000 mg/L sodium sulfide nonahydrate stock solution.
- Using this stock solution, transfer the following volumes into serum bottles containing 50 mL Instant Ocean®, cap with septum and mix:

Table H.1 Preparation of Sodium Sulfide Nonahydrate Standards using 100,000 µM Stock Solution

S (µM)	Na2S*9H2O (µg/L)	Dilution	Receiving Flask (mL)	Stock (mg/L)	Na2S*9H2O (mg/L)
1,000	240,000	0.010	50	24,000	240
800	192,000	0.008	50	24,000	192
600	144,000	0.006	50	24,000	144
400	96,000	0.004	50	24,000	96
200	48,000	0.004	50	24,000	48
0	0	0.000	50	24,000	0

- In the anaerobic chamber, collect 1 mL of sample. Add this sample into a serum bottle containing 9 mL Instant Ocean®, cap with septum and mix. This provides a 1:10 dilution.
- Add 0.8 mL of the mixed diamine reagent to each serum bottle, and mix gently.
- Allow 20 min to react.
- Remove from anaerobic chamber.
- Collect 100µL from each serum bottle and add to a scintillation vial containing 4.9 mL DDI water. This provides the 1:50 dilution called for by Cline for the 250 – 1,000 µM S range (35).
- Fill a 1 cm cuvette with this solution.
- In Lab 114, set the UV-Vis Spectrophotometer to a wavelength of 670 nm. Do not zero the instrument. Instead simply measure the absorbance of each standard and sample.
- Make standard curve to obtain the response factor. The concentration is given by:
  - $C_{\sum S} = F\left(A - A_b\right)$

- Where C is the concentration of sulfide, A is the absorbance at 670 nm, and A_b is the blank absorbance. F is 1/m in y=mx+b, if x is sulfide concentration and y is the absorbance.

APPENDIX I: *Protocol for Measuring CH_4 in Headspace Using GC-FID "Greta"*

- Remove bottles of interest from anaerobic chamber. (36)
- Make standards in 250 mL Wheaton media bottles. Add 150 mL DDI water, cap, and inject 0.1, 0.5, 0.75, 1 mL methane gas.
  - Put tubing onto nozzle of methane tank, and feed the tubing into a container in the fume-hood containing water so that gas bubbles can be seen, providing a visual cue that gas is in fact flowing.
  - Using a 1 mL locking gas syringe, push needle through latex tubing as close as possible to the tank nozzle. Flush syringe several times with methane. Then pull desired volume. Inject into standard bottle.
  - Put standards on shaker table for 1 hour to serve as an equilibration time.
  - Analyze the standards on GC "Greta" using the method "Gossett2".
  - The retention time of methane is ~0.4 min.
- In Lab 112, on the GC computer, open the Unichrom software.
- Load the method "HOLD" on GC "Ethel" if it's not already in HOLD.
  - Do this by opening the program "HOLD" by going to File > Open > Desktop > HOLD
  - Click the "Instrument" button and double click "Ethel"
- The GC named "Greta" should be running in the HOLD method. Close this method by selecting it, and then clicking the small "x" in the top right corner of the screen to close the HOLD method. If "Greta" is not running a method, no action is necessary.
- Load the method "Gossett2" on GC "Greta"

- File > Open > Desktop > Ben Rhiner > Gossett2
- Open valves on compressed air and high purity hydrogen tanks. Open small regulator valves.
- Ignite FID
- Using the buttons on the front of "Greta" make sure that the signal is about 14. It takes several minutes for the signal to stabilize.
- Press the button labeled "SIG 1" or "SIG 2"
- Inject 0.5 mL of air
- Immediately press the "Start" button after the injection.
- Using the buttons on the front of the GC, press the button that has had "START" worn off
- After the run is complete, inject 0.5 mL of the headspace gas from your sample microcosm.
- The retention time of the methane is about 0.35 min.
- Close valves on compressed air and high purity hydrogen tanks. Close small vials on regulator.
- Close the "Gossett2" method for the GC named "Greta." Do this by selecting the small "x" in the top right corner of the screen to close the method. Select Yes, save, when asked to save.
- Load the HOLD method again for "Greta."
- Close the small regulator valves and the gas tank valves on compressed air and high purity hydrogen tanks.

APPENDIX J: *Photographs of Microcosms and Transfer Enrichments*

Figure J.1 Photograph showing an aerobic microcosm on day 80 (right), and an aerobic first transfer enrichment on day 2 (left).

Figure J.2 Photograph showing the unamended first transfer enrichments on day 67. Bottle #1 is on the far left; it contains no molybdate. Bottles #2 & #3 are center and far right, respectively; bottles #2 & #3 contain 40 mM molybdate.

Figure J.3 Photograph showing the precipitate which formed a hazy crust on the glass surface in the 20 mM molybdate spiked second transfer enrichment (left), and the clear unamended second transfer enrichment (right) on day 17.

APPENDIX K: *Visual MINTEQ Output Showing Molybdate Species Present in Instant Ocean® Spiked with 20 mM Molybdate*

Table K.1 Species containing molybdenum

Component	% of Total Concentration	Species Name
MoO4-2	32.1	MoO4-2
	5.0	CaMoO4(aq)
	0.1	HMoO4-
	1.4	AlMo6O21-3
	61.4	MgMoO4(aq)

Table K.2 Species concentrations showing distribution of molybdate

Species	Concentration (mol/L)	Activity	Log activity
AlMo6O21-3	4.516E-05	2.85E-06	-5.55
CaMoO4(aq)	9.948E-04	1.13E-03	-2.95
H2Mo6O21-4	7.713E-14	5.69E-16	-15.25
H3Mo8O28-5	2.699E-18	1.26E-21	-20.90
HMo7O24-5	3.302E-14	1.54E-17	-16.81
HMoO4-	2.438E-05	1.79E-05	-4.75
MgMoO4(aq)	1.228E-02	1.39E-02	-1.86
Mo7O24-6	3.358E-12	5.35E-17	-16.27
Mo8O26-4	4.237E-21	3.12E-23	-22.51
MoO3(H2O)3(aq)	8.661E-08	9.83E-08	-7.01
MoO4-2	6.429E-03	1.88E-03	-2.73

Table K.3 Minerals containing molybdenum and their saturation indices

Mineral	log IAP	Sat. index
CaMoO4(s)	-5.517	2.433
MgMoO4(s)	-4.886	-3.036
MoO3(s)	-15.239	-7.239
Na2Mo2O7(s)	-19.006	-2.409
Na2MoO4(s)	-3.767	-5.257
Na2MoO4:2H2O(s)	-3.783	-5.007

APPENDIX L: *Thermodynamic Calculations*

Table L.1 Difference in Gibbs Free Energy of Formation between alcohols and acids

	ΔG_f° (KJ/mol)		
Alcohol/Acid	Alcohol[a]	Acid[a]	Δ
Ethanol/Acetate	-181.75	-369.41	-187.66
Propanol/Propionate	-175.81	-361.08	-185.27
Butanol/Butyrate	-171.81	-352.63	-180.82
Methanol/Formate	-175.39	-351.04	-175.65

[a]Values from Madigan et al (42)

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