MICROBIAL ECOLOGY AND FUNCTIONAL INSIGHTS INTO CONTAMINANT BIOATTENUATION IN ENGINEERED SHALLOW OPEN WATER TREATMENT WETLANDS

by

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A thesis submitted to the Faculty and Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Civil and Environmental Engineering).

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ABSTRACT

Water resources in arid and semi-arid regions globally are experiencing increasing stress from population growth, climate change, and the increasing spread and recognition of contaminants that impair potential water reuse. The adoption of low energy, high volume systems such as the engineered wetlands may contribute to solving this challenge with sustainability advantages over more actively engineered and managed approaches. The objective of this dissertation is to better understand microbial colonization and biological processes involved in trace organic and nutrient attenuation in a novel shallow, open water wetland construct colonized by a benthic photosynthetic biomat receiving nitrified wastewater effluent. The microbial community found within the biomat at both the pilot (400 m$^2$) and demonstration scale (7500 m$^2$) was dominated by the diatom species *Stauriza construens* var. *venter* and an assemblage of bacteria and archaea. This construct allowed for the simultaneous photolytic and biological attenuation of TOrCs offering more consistent and a smaller footprint when compared to vegetated wetlands.

The pilot-scale system which received nitrified wastewater effluent (20 mg/L NO$_3$) also demonstrated robust nitrate removal. Denitrification was the primary mode of removal with an aerial nitrate removal rate faster than 75% of constructed vegetated treatment wetlands. Interestingly, a combination of gene-specific studies coupled to inhibitor and kinetic assays suggested that anammox (anaerobic ammonium oxidation) could be responsible for 15% or more of the nitrate removal. In order to query how anammox could be present in an organic rich system with limited external input of nitrite and ammonium, the geochemical and molecular
inquiry of the biomat was conducted three dimensionally to understand stratification and nitrate attenuation processes. Additional laboratory microcosms, where contributions of sulfur and nitrogen species could be controlled, were used to further query mechanistic insights from the field-scale system. This collection of results demonstrated that sulfide induced dissimilatory nitrate reduction to ammonium was responsible for nitrite and ammonium production, which in turn supported anammox organisms of the Brocadiaceae family.

Due to the success of the pilot-scale system, a larger demonstration-scale system containing three parallel cells was constructed ~350 miles south where natural microbial community colonization and treatment performance were monitored from system establishment through almost 3 years of operation. Despite no form of active intervention in colonization, the shallow cells were dominated by the same species of diatoms. Analysis of phylogenetic 16S rRNA gene sequencing analysis revealed the establishment of an anaerobic community after summer growth and bacterial and archaeal community convergence to one that was highly similar to the established pilot-scale system. Overall the design of the open water unit process cell, notably the shallow water level (20-25 cm) and utilization of a liner to prevent emergent macrophytic growth, select for a similar microbial community and reproducible performance despite geographical separation and different influent properties. The biomat also achieved similar contaminant attenuation rates to those in the mature pilot-scale system. These findings help enable adoption of this system by water entities with a need to treat a variety of water contaminants at a reduced
cost and in doing so increase access to otherwise impaired waters for beneficial
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DEDICATION

This work is dedicated to my parents, Ken and Nancy Jones, who have always loved, encouraged, and supported me in all my endeavors; and to Lucy and Oliver, the best friends a graduate student could have
CHAPTER 1
INTRODUCTORY REMARKS

Natural wetlands provide a multitude of societal benefits including wildlife refuge, flood control, and water purification. Both coastal and inland wetland area has been in decline since it has been monitored. The most recent survey shows an increase in the rate of loss with the largest losses occurring in coastal areas, specifically the Gulf of Mexico due to salt water intrusion and inundation (Dahl and Stedman 2013). In order to help combat these losses, constructed wetlands have shown the dual benefit of restoring habitat and providing passive water treatment. Research on constructed wetlands for water treatment began in Germany in the 1950s (Seidel 1955). North America began research on surface flow constructed wetlands in the late 60’s and 70’s (Odum et al. 1977) and has approximately 1000 constructed wetlands currently in operation (Kadlec and Wallace 2009). The Tres Rios constructed wetland project is often pointed to as the modern constructed wetland success story. In order to avoid water treatment plant upgrade costs of $650 million they opted to build a constructed wetland providing adequate treatment to meet permitting, 700 acres of wildlife habitat, and an estimated savings of $470 million.

Nitrogen is well recognized as a ubiquitous surface and ground water pollutant, and in past decades enhanced monitoring and instrumentation has revealed that freshwater sources across the United States are contaminated with trace organic pollutants (TOrCs) (Diaz and Rosenberg 2008; Kolpin et al. 2002). These pollutants reach streams and lakes via point and non-point sources causing an estimated 2.2 billion dollars in economic damages from eutrophication alone (Dodds et al. 2009). Traditional wastewater and drinking water treatment plants do not treat many of these TOrCs
effectively (Stackelberg et al. 2004) and nitrate regulations allow it to be discharged at concentrations capable of causing eutrophication and dead zones (10 mg/L) (Moffat 1998). Water utilities are actively preparing for more strict proposed regulations for both chemical and nutrient pollution, constructed wetlands could help to prepare for this future challenge in a more sustainable way than traditional engineered water treatment approaches.

To address the need for the attenuation of these ubiquitous water pollutants, a new type of constructed wetland was developed, the shallow, open water unit process wetland cell. This wetland was originally designed for photolytic degradation of TOrCs via hydroxyl radical generation from sunlight and nitrate (Jasper and Sedlak 2013a): in essence a passive advanced oxidation treatment system. The cell bottom is lined with a geotextile or concrete liner to prevent emergent macrophyte growth and a shallow water depth of 20-25 cm allows sunlight penetration throughout the water column. Preventing macrophyte growth also enables more predictable flow patterns and less channeling, or hydraulic short-circuiting, which is a problem typically exasperated by dense vegetation. While not originally considered in the design, the combination of nitrogen rich waters and shallow water depth lead to the formation of a photosynthetic, benthic biomat during pilot-scale implementation. Monitoring and experimentation revealed this biomat’s ability to efficiently attenuate a range of otherwise recalcitrant TOrCs while simultaneously removing nitrate (Jasper et al. 2014b) to an extent that exceeded that achieved by photolysis reactions.

To better understand this system from both a fundamental and applied perspective, the biomat was explored through the lens of environmental engineering
microbiology. Specifically, the objectives of this dissertation focus on aspects of the microbial assemblage found in the open water cell wetland biomat in order to 1) characterize the biomat community and its role in contaminant attenuation, 2) better understand how nitrate removal can occur and potential drivers of perplexing anammox contributions to a system receiving fully nitrified influent, and 3) understand the community shifts in this biomat from establishment through maturity and determine if the design selects for an analogous microbial community and by extension functional processes across locations and scales. As part of the NSF funded engineering research center ReNUWIt, this dissertation and my residence also incorporated strong nontechnical elements targeted at broader impacts and educational and industry interactions. One element of those meaningful experiences is documented in the appendix of this dissertation (A.1), which outlines the content of a three-week undergraduate environmental microbiology laboratory module that I helped to develop in conjunction with my advisor, Dr. Sharp. We delivered this module over the past four years at CSM where I played a particularly important role in the microscopy and molecular biology components of the course.

To begin unraveling the scientific and engineering complexities associated with the microbially-mediated water treatment capabilities of the photosynthetic benthic biomat, we characterized the microbial community of a pilot scale open water unit process wetland and its ability to remove nitrate and TOrCs (Chapter 2). This work was done as an interdisciplinary collaboration and the biogeochemical processes associated with nitrate and TOrC removal have been published in two papers within the journal of Environmental Science and Technology (Jasper et al. 2014a, 2014b). The data presented
in this dissertation focuses on my contributions toward understanding the ecology and potential roles of the microbial community toward attenuation in this unit process wetland; pilot-scale performance, attenuation rates, modeling and broader impacts can be viewed in more detail in the published papers. The shallow, open water unit process wetland was originally developed to enhance the photolytic degradation of recalcitrant emerging organics such as pharmaceuticals and personal care projects in impaired water supplies such as wastewater effluent. Interestingly, transformation rates exceeded those anticipated by photolytic degradation processes suggesting that the bright green benthic biomat that forms on top of the geotextile liner also played an important role in chemical attenuation. Fluorescent and scanning electron microscopy imaging revealed diatoms identified as *Stauriisa construens* var. *venter* as the main photosynthetic primary producing organism within this biomat. Complementary 16S rRNA gene sequencing revealed a diverse associated bacterial community primarily comprised of Proteobacteria, Bacteroidetes, and Verrucomicrobia. To assess the ability of the resident community to degrade a suite of pharmaceuticals found in the wastewater effluent, microcosms experiments were conducted in light and dark conditions utilizing red LEDs to tease out the contributions of photolytic degradation of TOrCs and microbial respiratory processes associated with photosynthesis. Beta blocker bioattenuation occurred more quickly under light conditions while more recalcitrant compounds such as trimethoprim and sulfamethoxazole were transformed faster under dark conditions. In order to assess, nitrate removal capabilities, quantitative PCR was utilized to query for nitrogen removal biomarker genes and then compared to nitrate removal in microcosms and the pilot-scale wetland. Nitrate removal rates were better than 75% of those reported for vegetated
wetlands suggesting the promise for this wetland cell adaptation to rival and potentially surpass vegetated wetlands for one of their primary designed treatment goals. Not surprisingly, denitrification biomarker genes, \textit{nirS} and \textit{nirK} were abundant in association with this process; however, the anammox biomarker gene \textit{hzsA} was unexpectedly found in enriched quantities within the bottom portion of the biomat. Microcosm acetylene block experiments confirmed the importance of anammox within these systems with anammox contributing up to 15\% of inorganic nitrogen removal while denitrification was responsible for the remainder. Overall, the work in Chapter 2 demonstrates that these wetland constructs host a diatomaceous assemblage of diverse microorganisms that are capable of transforming a variety of TOrCs in conjunction with robust nitrate removal capabilities and provided mechanistic explanations for these processes.

The third chapter of this dissertation seeks to understand why anammox bacteria are present in a system receiving fully nitrified wastewater, which lacks the typical anammox precursors, nitrite and ammonium. This body of work has been published in \textit{Applied and Environmental Microbiology} (Jones et al. 2017a). In exploring mechanistic drivers of anammox, 16S rRNA gene sequencing profiles of the biomat within a shallow, open water cell wetland revealed significant microbial community shifts along flow paths and with depth. Notably, there was an increasing abundance of sulfate reducers (Desulfovoccus and other Deltaproteobacteria) and anammox microorganisms (Brocadiaceae) with depth. Pore water profiles demonstrated that nitrate and sulfate concentrations exhibited a commensurate decrease with biomat depth accompanied by the accumulation of ammonium. Quantitative PCR targeting the anammox hydrazine synthase gene, \textit{hzsA}, revealed a 3-fold increase in abundance with biomat depth as well as
a 2-fold increase in the sulfate reductase gene, \textit{dsrA}. These microbial and geochemical trends were most pronounced in proximity to the influent region of the wetland where the biomat was thickest and influent nitrate concentrations were highest. While direct genetic queries for dissimilatory nitrate reduction to ammonium (DNRA) microorganisms proved unsuccessful, which can be explained by technical challenges associate with the requisite primer depth needed for this poorly understood guild, an increasing depth-dependent dominance of Gammaproteobacteria and diatoms that have previously been functionally linked to DNRA was observed. To further explore this potential, a series of microcosms containing field-derived biomat material confirmed the ability of the community to produce sulfide and reduce nitrate; however, significant ammonium production was only observed in the presence of hydrogen sulfide. Collectively, these results suggest that biogenic sulfide induces DNRA, which in turn can explain the requisite coproduction of ammonium and nitrite from nitrified effluent necessary to sustain the anammox community (Figure 1.1).

To explore the dynamics associated with this photosynthetic benthic biomat, we investigated the evolution of the biomat as it established in a newly built, demonstration scale open water treatment wetland (Figure 1.2 Right), and compared the microbial community in this larger scale system (~20 times the size and ~350 miles to the south) to that of the pilot system studied in the previous two chapters (Figure 1.2 Left). This study as articulated in Chapter 4 is currently in peer review. In order to better understand how shallow open water wetland cells self-colonize and evolve, the composition of the microbial community that formed the benthic biomat was monitored for almost 3-years of operation. Monitoring was
Figure 1.1 Depiction of the hypothesized pathway of nitrogen removal within the benthic biomat of a shallow, open-water cell wetland where sulfide production shifts nitrogen cycling toward dissimilatory nitrate reduction to ammonium.

Figure 1.2 Left: The discovery bay wetland design is 20 m x 20 m and includes baffling in order to mitigate short circuiting. Right: An aerial view of the Prado wetlands shows 30 m x 800 m open water wetland cells running in parallel.
conducted in three parallel, demonstration-scale (7500 m² each) constructs at Orange County’s Prado wetland where the cells received water from Santa Ana River (NO₃⁻ = 5.9 ± 0.2 mg/L). Phylogenetic inquiry and microscopy confirmed that diatoms and an associated aerobic bacterial community facilitated early colonization. After approximately nine months of operation and subsequent to warmer and higher sunlight intensity summer months, an anaerobic community emerged with the capability for nitrate attenuation. At this point, the microbial community was comparatively stable for the remaining years of operation and remained highly similar across the operational cells despite different hydraulic residence times that ranged from 1-4 days between these three treatment cells. Furthermore, the microbial communities converged with one that had previously formed in a geographically separated, pilot-scale (400 m²) shallow, open water wetland construct in Discovery Bay that received denitrified (NO₃⁻ = 20.7 mg/L), secondary treated wastewater for 5 years of operation. Establishment of a core microbiome across communities revealed a strong overlap of both aerobic and anaerobic taxa with approximately 50% of the analyzed bacterial sequences shared between the two sites. Additionally, the same species of diatom, Staurocea construens var. venter, was prolific in both systems as the putative dominant primary producer. We conclude that despite geographical separation, operational scale and differences in receiving water properties, the shallow open water wetland design consisting of a geotextile-lined cell of approximately 20-25 cm water depth rapidly selects for an analogous and reproducible self-colonizing benthic, photosynthetic assemblage. This resulting biomat matures over the first growing season to enable the treatment of a wide range of water contaminants including nitrate, trace organic pollutants, and pathogens.
Holistically, this dissertation characterizes the ecological and functional ramifications of a type of microbial assemblage that naturally colonizes these novel shallow, open water constructed wetlands. Insights derived from the pilot-scale studies led to a deeper understanding of nutrient cycling in freshwater systems as a whole and revealed the versatile utility of this managed natural treatment analog. This led to the construction of an optimized demonstration-scale system, which provided the opportunity to study this wetland variant from establishment through maturity and show how system design can lead to the selection of a unique, yet reproducible microbial community. The results presented here contribute to a larger body of research which seeks to understand how to best manage natural systems for water treatment by characterizing microbial communities and their resultant biogeochemical processes at the laboratory, pilot, and demonstration-scale. With respect to student outputs, my residence at Mines has been associated with 6 accepted peer-reviewed publications (two that are not directly associated with this dissertation theme and represented other collaborations where I aided with my expertise in environmental engineering microbiology), another currently in review, preliminary results and insights for future work in this broader theme of treatment wetlands, and the development of an undergraduate environmental microbiology laboratory segment.

The publications associated with my residence at CSM include:


CHAPTER 2
MICROBIAL COMMUNITY CHARACTERIZATION AND CHEMICAL
ATTENUATION IN A PILOT-SCALE OPEN WATER UNIT PROCESS WETLAND

Adapted to reflect the author’s contributions to the following publications in
Environmental Science and Technology*

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Abstract

The shallow, open water unit process wetland was originally developed to enhance the photolytic degradation of recalcitrant emerging organics such as pharmaceuticals and personal care projects in impaired water supplies such as wastewater effluent. Interestingly, transformation rates exceeded those anticipated by photolytic degradation processes suggesting that the bright green benthic biomat that forms on top of the geotextile liner also played an important role in chemical attenuation. Microscopy-focused inquiry using a combination of fluorescent and scanning electron imaging revealed diatoms identified as Staurira construens var. venter as the main photosynthetic primary producing organism within this biomat. Complementary 16S rRNA gene


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sequencing revealed a diverse bacterial community associated with the diatoms dominated by various Proteobacteria, Bacteroidetes, and Verrucomicrobia. To assess the ability of the resident community to degrade a suite of pharmaceuticals found in the waste water effluent in day and night conditions, microcosms experiments were conducted in light and dark conditions utilizing red LEDs to prevent photolytic degradation of TOrCs. Beta blocker removal occurred more quickly under light conditions while more recalcitrant compounds such as trimethoprim and sulfamethoxazole were transformed faster under dark conditions. In order to assess the nitrate removal capabilities, quantitative PCR was utilized to query for nitrogen removal biomarker genes and the wetland removal rate was monitored. As expected, denitrification biomarker genes, \textit{nirS} and \textit{nirK} were abundant; however, the anammox biomarker gene \textit{hzsA} was found in enriched quantities within the bottom portion of the biomat. Nitrate removal rates were better than 75% of reported rates for vegetated wetlands. Microcosm acetylene block experiments confirmed the importance of anammox within these systems with anammox contributing up to 15% of nitrogen removal while denitrification was responsible for the remainder. Overall, this wetland design shows potential at the pilot scale of biologically treating a variety of trace organic contaminants and nitrogen contamination.

2.1 Introduction

The discharge of wastewater effluent and non-point sources of pollution such as agricultural runoff has resulted in the pollution of surface and ground waters with nitrate and “emerging” trace organic contaminants (TOrCs) such as pharmaceutical and personal care products. This pollution threatens public health (Duda 1993), contributes to
eutrophication and large scale dead zones in the ocean (Moffat 1998) and reduces available drinking water (Levin et al. 2002) with further challenges for environmental discharge. While traditional wastewater treatment plants are capable of removing nitrogen from waste streams, this comes at the expense of additional energy and substrate to reach acceptable concentrations prior to discharge (Randall, Barnard, and Stensel 1998) and the presence and attenuation capabilities of TOrCs were largely not considered when designing waste and drinking water treatment plants (Stackelberg et al. 2004). The potential for more stringent nutrient discharge regulations coupled to the need to address the presence and attenuation of TOrCs can be financially challenging to water treatment facilities (Safoniuk 2004).

Constructed wetlands provide a potential solution to these challenges as they offer a cost effective means of treating nitrate and TOrCs (Kadlec and Wallace 2009; Y. Li et al. 2014). The Tres Rios and Prado wetlands are excellent examples of modern wetlands designed for low cost nitrate removal (S. Cole 1998) where attenuation has largely been attributed to denitrifying bacteria (Burgin and Hamilton 2007). Constructed wetlands typically contain emergent vegetation in order to provide a microbial organic carbon source and low oxygen conditions that favor denitrification. However, if the vegetation is not managed properly it can contribute to hydraulic short-circuiting or shunting of significant fractions of the flow, leading to ineffective treatment of targeted contaminants. In addition, we have a limited understanding of how wetlands can be designed to optimize trace organic contaminant removal as only 11% of the total wetland studies before 2010 included a microbial data (Imfeld et al. 2009) despite their vital role in contaminant remediation. The result is pronounced variations in trace organic
contaminant removal efficiencies when contrasting different vegetated wetland systems with limited understanding of the underlying mechanistic selectors involved (Jasper et al. 2013; Y. Li et al. 2014).

In order to address the diverse nature of TOrCs and to minimize short-circuiting, a new treatment wetland design described as a shallow, open water unit process cell was piloted in Discovery Bay, CA. This construct is lined with a barrier composed of concrete or geotextile ground cloth on the bottom to prevent macrophyte growth and utilizes a shallow water depth to allow for full illumination of the water column. Interestingly, while enhanced photolysis was the primary goal of this design, the photosynthetic nature of the biomat formed within this novel system was subsequently deemed to have the potential for further TOrC and nitrate attenuation. A previous study incorporating both laboratory simulated and field removal rates demonstrated the potential of this design for removing photo-labile chemicals (Jasper and Sedlak 2013b), however photolysis alone could not explain the removal of several other contaminants. In addition, monitoring of this operational pilot scale system revealed unexpected, yet significant nitrate removal. As a result, we hypothesized that that the biomat, which formed on the bottom of the wetland cell, was responsible for the unaccounted fraction of TOrC attenuation and the vast majority of nitrate removal. In turn, the purpose of this study was to evaluate and explore underlying mechanisms associated with the ability of a shallow, open-water wetland surface water cell receiving fully nitrified wastewater effluent to remove a suite of wastewater-derived trace organic contaminants as well as investigate nitrogen removal pathways.
2.2 Materials and Methods

2.2.1 Site Characterization

The open water unit process wetland is located in Discovery Bay, CA on site of the town’s municipal waste water treatment plant. The wetland cell is 20 m x 20 m and contains 3 rows of baffling to prevent short circuiting and the bottom is lined with geotextile material and cement to prevent macrophyte growth. The system receives about 10,000 gallons of water per day, has a water depth of 20 cm, and a hydraulic residence time of 1-3 days. The influent water had 20.7 ± 0.7 mg/L-N nitrate, 140 ± 10 mg/L sulfate, 5.9 ± 0.3 mg/L TOC, and a pH of 8.6 ± 0.2.

2.2.2 Sample Collection

Biomat for microcosms and microscopy was collected from the wetland after approximately 3 years of operation in glass amber bottles and transported on ice. Approximately 1 g of biomass was sampled from dark microcosms and microcosms illuminated with visible light (635 nm) at the beginning, middle, and end of biotransformation for molecular analysis. Biomat samples from the wetland for molecular analysis were sampled with a 30ml glass serological pipette in order to sample with depth. Samples were shipped overnight on dry ice and stored at -80° C prior to extraction. Samples for microscopy were collected from the wetland in, chilled, and shipped overnight to preserve structure.

2.2.3 DNA Extraction and Sequencing

DNA was extracted from 0.25g of sample using the Mo Bio PowerBiofilm DNA Isolation Kit per manufacturer’s protocol. Extracted DNA was amplified in triplicate 25
µl reactions without Illumina adaptors or primers pads on a Roche LightCycler 480II. A portion of the 16S rRNA gene was amplified using Phusion Master Mix (New England BioLabs, Inc), 3% final volume DMSO, 0.4x final concentration SYBR Green, 200nM 515F (5’ GTGYCAGCMGCCGCGGTAA 3’), 177 and 12bp Golay barcoded 806R (5’XXXXXXXXXXXXXCCGGACTACHVGGGTWTCTAAT 3’). The amplification program was: 94°C 3 min; 94°C 45 sec, 50°C 10 sec, 72°C 90 sec. The program was stopped after all samples had amplified. Triplicates were pooled and purified using Agencourt AMPure XP and quantified using a Life Sciences Qubit 2.0 Flurometer. Normalized amplicons were sequenced on the Illumina MiSeq platform using NEBNext Ultra DNA Library Prep Kit and a MiSeq Reagent Kits v2 2x250 500 cycle kit. The sets of 250 bp sequences were stitched together using ea-utils fastq-join with a minimum base pair overlap of 100. Stitched sequences were reverse complimented with the fastx toolkit in order to account for sequences that were sequenced in the reverse direction. The resulting sequences were processed in QIIME 1.7 starting with sl_prep_fastq.py to create a S4 barcode .fastq file. The resulting sequence and barcode file were demultiplexed using split_libraries_fastq.py with default parameters, except for “--barcode 12”, to negate error correcting of barcodes, as any sequences with errors would have been filtered out by sl_prep_fastq.py. Otus were piked de novo using Usearch 6.16 and chimeras were filtered out using the Greengenes gold database. Representative sequences were aligned using PyNAST and greengenes 13_5 aligned reference database. Taxonomy was assigned using the RDP classifier and greengenes 13_5 97 otu taxonomy database and the otu table was then rarified to 5050 sequences before further analysis. All phyla with less than 1% relative abundance were filtered out.
2.2.4 Quantitative PCR for Nitrogen Cycling Gene Abundances

Extracted DNA was quantified on a NanoDrop Lite and diluted by a factor of 10 prior to amplification, resulting in concentrations ranging from 10 to 30 ng μL−1. Amplifications for quantitative PCR were performed on a Roche Light Cycler 480 II using Quanta Biosciences PerfeCTa SYBR Green Super Mix in 25 μL reactions using 2 μL of template DNA. Primer sequences and PCR conditions for nirS, nirK, hzs, and 16S rRNA genes can be found in Table 2.1. Each sample was amplified in triplicate with nontemplate and negative extraction controls. Standards were generated from amplicons that were purified using Agencourt AMPure XP magnetic beads and quantified using an Invitrogen Qbit 2.0. No nonspecific amplicon bands were observed when run on an Agilent Bioanalyzer 2100. Standard curves were generated from triplicate 1:10 serial dilutions of their purified standards. Each functional gene was normalized by the total number of 16S gene copies.

2.2.5 Microscopy

Biomat samples (250 μL) for fluorescent microscopy were washed twice by centrifuging and resuspending in phosphate buffer solution, before incubating in SYBR Green (stock solution diluted by a factor of 25 000) for 1.5 h. Fluorescent images were generated using exciting/emission wavelengths of 473/490–540 nm for SYBR Green and 645/664 nm for autofluorescence of chloroplasts (Olympus Fluoview FV10i). Scanning electron microscopy (Hitachi TM-1000 Tabletop Microscope) was conducted on fresh biomass samples dried overnight on foil and placed on carbon tape.
Table 2.1 Primers Information and Thermal Profiles for QPCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence 5'→3'</th>
<th>[Primer] (nM)</th>
<th>Amplicon Size</th>
<th>Target Gene</th>
<th>Thermal Profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cd3aF</td>
<td>GTSAACGTSA</td>
<td>500</td>
<td>425</td>
<td>nirS</td>
<td>25°C 10 min, 8 touchdown cycles [25°C 15 s, 63–58°C (~1°C per cycle) 30 s, 72°C 30 s]</td>
<td>Throbbing et al. 2004</td>
</tr>
<tr>
<td>R3cd</td>
<td>GASTTCGGRT</td>
<td>500</td>
<td></td>
<td></td>
<td>(95°C 15 s, 58°C 30 s, 72°C 30 s)</td>
<td>Henry et al. 2004</td>
</tr>
<tr>
<td>nirK876</td>
<td>ATYGCCCGGA</td>
<td>500</td>
<td>164</td>
<td>nirK</td>
<td>25°C 10 min, 8 touchdown cycles [25°C 15 s, 63–58°C (~1°C per cycle) 30 s, 72°C 30 s]</td>
<td>Henry et al. 2004</td>
</tr>
<tr>
<td>nirK1040</td>
<td>GCTCTGATCA</td>
<td>500</td>
<td></td>
<td></td>
<td>(95°C 15 s, 58°C 30 s, 72°C 30 s)</td>
<td>Henry et al. 2004</td>
</tr>
<tr>
<td>hzsA1597F</td>
<td>WTYGGKTATC</td>
<td>400</td>
<td>260</td>
<td>hzs</td>
<td>95°C 3 min, 40 cycles (95°C 30 s, 55°C 30 s, 72°C 30 s)</td>
<td>Harhangi et al. 2011</td>
</tr>
<tr>
<td>hzsA1857R</td>
<td>AAABGGYYGAA</td>
<td>400</td>
<td></td>
<td></td>
<td>(95°C 3 min, 40 cycles (95°C 30 s, 55°C 30 s, 72°C 30 s)</td>
<td>Harhangi et al. 2011</td>
</tr>
<tr>
<td>EUB338</td>
<td>ACTCTTACCGG</td>
<td>1000</td>
<td>180</td>
<td>16S</td>
<td>95°C 3 min, 40 cycles (95°C 60 s, 53°C 30 s, 72°C 60 s)</td>
<td>Fierer et al., 2005</td>
</tr>
<tr>
<td>EUB518</td>
<td>ATTACCGCGG</td>
<td>1000</td>
<td></td>
<td></td>
<td>(95°C 3 min, 40 cycles (95°C 60 s, 53°C 30 s, 72°C 60 s)</td>
<td>Fierer et al., 2005</td>
</tr>
</tbody>
</table>

2.2.6 Trace Organic Degradation Microcosms

In order to test the biological trace organic attenuation potential of the wetland biomat in day and nighttime conditions, triplicate microcosms containing unbuffered wetland water ([DOC]≈8 mg C L-1; [NO3 -]≈10 mg N L-1) were amended with all six test compounds: atenolol, metoprolol, propranolol, trimethoprim, sulfamethoxazole, and carbamazepine (5 µg L-1) and sampled at daily intervals throughout the experiment. Microcosms were incubated at room temperature (25–30°C) in the dark or under monochromatic visible light (635 nm; 13.5 W red light, GenCom). The light supported photosynthesis without transforming the test compounds via photolysis. Dark microcosms were gently agitated from above using stir bars suspended on nylon string to mimic the effects of mixing in wetlands while minimizing perturbation of the biomat. Illuminated microcosms were mixed via bubbles produced by photosynthesis in the
biomat. 1 mL aqueous samples were filtered through 1 µm glass-fiber Acrodisc syringe filters (Pall Corporation), amended with about 2.5 ng of each isotopically-labeled internal standard, and refrigerated until analysis (within 1 week). Test compounds were extracted from dewatered (centrifuged at 5,000 RPM, 10 minutes), wet biomat samples (about 0.1 g dry weight). Samples were agitated for approximately 8 hours on a rotisserie in 15 mL of methanol amended with about 2.5 ng of each isotopically-labeled internal standard. Methanol extracts were filtered (1 µm glass-fiber; Millipore, Bellerica, MA) and diluted to 1 L with deionized water prior to clean-up and concentration via solid phase extraction (SPE). The SPE media consisted of 50 mg Waters Oasis hydrophilic-lipophilic balance (HLB) in cartridges pretreated with 10 mL of methanol, followed by 10 mL of Milli-Q water. Cartridges were eluted with 12 mL of methanol, dried under a gentle nitrogen stream, and re- suspended in 1 mL of Milli-Q water prior to analysis via HPLC-MS-MS.

2.3 Results and Discussion

2.3.1 Microbial Community Analysis

The microbial community within the biomat in the Discovery Bay wetland consisted of an interspersed assemblage of photosynthetic and heterotrophic microorganisms dominated by a single species of diatom, as evidenced by scanning electron and confocal fluorescent microscopy (Figure 2.1 A,B). It appears that many of the bacteria are closely associated with the diatom aggregates, which is consistent with diatoms hosting symbiotic bacteria based on the observation that the green fluorescence of a general DNA stain was largely in proximity to the autofluorescence of the diatom’s chloroplasts (Figure 2.1A). The diatom species was tentatively identified as Staurosira construens var. venter based on valve morphology (Figure 2.1B) and 16S rRNA gene
sequence analysis of the chloroplasts harbored within these organisms was most similar to that of Stramenopiles (Figure 2.1C). Illumina 16S rRNA gene sequencing indicated that in addition to diatoms (30 ± 3% relative abundance), the associated sequences were dominated by Proteobacteria (37 ± 4%; primarily of the β and γ superclasses), Bacteroidetes (7 ± 1%), and Verrucomicrobia (6 ± 1% relative abundance) (Figure 2.1 C). The remaining phyla and unclassified sequences accounted for less than 15% of the overall relative abundance. Shown that the rates of oxidation of some trace

Figure 2.1 (A) Fluorescent imagery of fresh biomass showed colonization of diatoms (red) and bacteria (green). (B) Scanning electron microscopy illustrated the presence of filamentous diatoms Staurosira construens. (C) Illumina sequencing of microcosm biomass fresh from the wetland (Day 0), and after incubation in the dark (Day 6, dark) or incubation under 635 nm visible light (Day 6, red). Note that Stramenopiles is the phylum of the Staurosira diatom.
2.3.2 Biotransformation of TOrCs

In order to emulate and understand how diurnal cycling of the wetland affects biological TOrC attenuation, microcosms were incubated under red LED light to simulate daytime conditions (i.e., pH 10, supersaturated with dissolved oxygen due to photosynthetic activity) or in the dark to simulate nighttime conditions (i.e., pH ≈ 8.5). As evidenced by 16S rRNA gene phylogenic analysis, microbial community profiles did not change significantly during the course of the experiments, suggesting that the microbes present remained representative of the assemblage in the pilot-scale system (Figure 2.1C). Test compound transformation rates in red light illuminated microcosms without added biomass were negligible (triangles in Figure 2.2), demonstrating that illumination with red light did not cause transformation of test compounds via photolysis, and that biotransformation in the aqueous phase was negligible. This result is expected as a 640 nm wavelength light in not powerful enough to photolytically degrade organic compounds (Lyman, Reehl, and Rosenblatt 1990) yet can still be utilized by diatoms as they contain chlorophyll c which is able to absorb light in this spectrum (Kuczynska, Jemiola-Rzeminska, and Strzalka 2015).

Metoprolol and propranolol biotransformation rates increased by approximately 4 to 8 times in red light microcosms compared to dark microcosms (Figure 2.2). This enhancement may have been attributable to higher concentrations of dissolved oxygen within the biomass: dissolved oxygen concentrations in the irradiated microcosms remained above 15 mg L⁻¹ in the top 1 cm of the biomat, compared to dissolved oxygen concentrations ranging from about 5 mg L⁻¹ at the water–biomat interface to less than 1 mg L⁻¹ deeper than 1 cm in the biomat in the dark microcosms. Previous studies have
organic compounds were enhanced under aerobic conditions in wetlands (Hijosa-Valsero et al. 2010) and in activated sludge wastewater treatment systems (Xue et al. 2010; Lisa et al. 2014). For example, biotransformation of metoprolol was up to 5 times faster under aerobic conditions relative to anoxic conditions in full-scale wastewater treatment plants (i.e., \( k_{aerobic} \approx 1.2 \text{ d}^{-1} \) versus \( k_{anoxic} \approx 0.24 \text{ d}^{-1} \)) (Xue et al. 2010). Biotransformation rates may also have increased upon illumination with visible light due to enhanced microbial activity caused by the release of organic compounds by autotrophic diatoms during photosynthesis (J. J. Cole 1982), as increased labile carbon availability which could enhance TOrC removal rates (Hijosa-Valsero et al. 2010; Matamoros, García, and Bayona 2008).

In contrast, rates of biotransformation of trimethoprim and sulfamethoxazole were more than 5 and 2 times faster, respectively, in the dark microcosms, relative to the illuminated microcosms. This difference in biotransformation rates may have been due to a community metabolic shift caused by anoxic conditions or induced by the absence of primary productivity (Sharp et al. 2007; Patrauchan et al. 2012). Enhanced rates of biotransformation of trimethoprim have been reported at dissolved oxygen conditions below 0.5 mg L\(^{-1}\) in microcosms inoculated with activated sludge (Hollender et al. 2009). Furthermore, the biotransformation of trimethoprim has been suggested to involve certain heterotrophic microorganisms with minimal oxygenase activity, which may be inhibited by high dissolved oxygen concentrations (Khunjar et al. 2011). These results highlight the potential importance of the terminal electron acceptor to rates of transformation of trace organic contaminants. Open-water wetlands exhibit a diurnal fluctuation in redox conditions as well as variations through the vertical profile of the
biomat (i.e., oxygen-reducing at the water biomat interface to sulfate-reducing at the bottom of the biomat), enabling the biotransformation of compounds across terminal electron acceptor gradients. For the removal of compounds that are mainly transformed under anoxic conditions, the use of wetlands with larger anoxic zones (e.g., wetlands with dense macrophytes or subsurface flow wetlands) may be a more effective treatment strategy.

Figure 2.2 Fraction of test compound masses (M/Mo) remaining in microcosms that were: illuminated by red visible light (635 nm) without added biomass (red ▲); in the dark with added biomass (aqueous phase: ●; biomat phase: ○); and illuminated by red visible light (635 nm) with added biomass (aqueous phase: green ◊; biomat phase: green ◊). Mo ≈ 6 μg. Error bars represent ± one standard deviation.
2.3.3 Nitrogen Removal

The open water cell demonstrated faster than anticipated nitrate removal rate, removing greater than 90% of influent nitrate in summer dropping down to 30% removal efficiency in the winter with an approximate 2 day retention time. Quantitative PCR on the top and bottom of the biomat to query for biomarker genes indicative denitrification and anammox showed a clear abundance of the nitrite reductase gene, \textit{nirS} (Figure 2.3). The gene \textit{nirK} is functionally redundant to \textit{nirS} and more commonly found in soil environments while \textit{nirS} is associated with aquatic environments. The presence of \textit{hzsA} confirmed that anammox organisms were present within the wetland biomat (Figure 4) (Harhangi et al. 2011). The normalized abundance of \textit{hzsA} was elevated near the bottom of the wetland where the most anoxic conditions existed and NH$_4^+$ concentrations were highest. The normalized abundance of \textit{hzsA} was similar to the normalized abundances measured in lake riparian zones (up to about 0.0007 copies \textit{hzsA} per 16S gene copy), where anammox accounted for up to 20% of N$_2$ production (Zhu et al. 2013).

Further evidence of anammox activity in the open-water cell biomat was provided by NH$_4^+$ loss in anoxic microcosms containing wetland biomass (Figure 2.4). In microcosms amended with NH$_4^+$ and either NO$_3^-$ or NO$_2^-$, NH$_4^+$ concentrations decreased (up to 12 ± 6 μmol NH$_4^+$ lost; Figure 4, A and B), corresponding to 30–40% of total nitrogen lost due to anammox. In contrast, NH$_4^+$ concentrations did not change significantly in microcosms that were not amended with NO$_3^-$ or NO$_2^-$ (Figure 4 C), demonstrating that volatilization, adsorption, or assimilation were not responsible for NH$_4^+$ loss. In microcosms amended with only NO$_3^-$, NH$_4^+$ concentrations increased by up
Figure 2.3 Abundances of genes involved in denitrification \((nirK \text{ and } nirS)\) and anammox \((hzsA)\) near the inlet of the open-water wetland cell, normalized to 16S gene copies. Measurements were taken near the biomat-water interface (Biomat top) and at the bottom of the biomat (Biomat bottom). Error bars represent ± average of the absolute deviation of duplicate measurements.

to 13 ± 1 μmol, possibly due to ammonification or dissimilatory nitrate reduction to ammonia (Figure 2.4 D). Further experiments using isotopically labeled nitrogen species would be necessary to better estimate the contribution of anammox to nitrogen removal in the open water cell (Thamdrup and Dalsgaard 2002).
Figure 2.4 Concentrations of nitrogen species in anoxic microcosms amended with A: NH$_4^+$ and NO$_3^-$; B: NH$_4^+$ and NO$_2^-$; C: NH$_4^+$; D: NO$_3^-$. Experiment conducted at 22 ± 2°C. Error bars represent ± one standard deviation (n=3).

Anammox bacteria have been identified in wetland sediments (Shipin et al. 2005; Dong and Sun 2007) and have been shown to account for up to 24% of the N$_2$ production in surface-flow wetlands receiving partially nitrified wastewater effluent (Erler, Eyre, and Davison 2008; Davison et al. 2006) and 78% of the N$_2$ production in vegetated wetland microcosms receiving high concentrations of NH$_4^+$ and NO$_3^-$ (i.e., 2–20 mM). Anammox accounted for up to 33% of the N$_2$ production in vertical-flow wetland microcosms inoculated with activated sludge and conditioned at elevated NH$_4^+$ concentrations (i.e., 0.7 mM). In wetlands receiving high concentrations of NH$_4^+$ (e.g., >0.7 mM), nitrification
coupled to anammox may enhance NH₄⁺ removal by reducing the amount of oxygen required to oxidize NH₄⁺ to N₂ by more than half compared to nitrification followed by denitrification (Tao et al. 2012). In wetlands designed to treat municipal wastewater effluent, such as the Discovery Bay wetlands, low concentrations of NH₃ are required to avoid toxicity to mosquitofish (Gambusia sp.) used for vector control (Horne and Fleming-Singer 2005). Under these conditions, anammox could occur following dissimilatory nitrate reduction to ammonia, or if NH₄⁺ is released from decaying organic matter via ammonification as is explored in the subsequent chapter of this dissertation.

2.4 Conclusions

Overall these experiments help enhance our understanding of how these open water cell wetlands biologically remove chemical contaminants such as pharaceuticals and nitrate from polluted water. Results can be utilized to begin better understand the mechanisms responsible for contaminant attenuation, which can then be optimized to better remove pollutants of concern. Periodically removing the biomat might increase photolytic degradation as more water would be illuminated; however, there would be less water contact time in the biomat potentially reducing biological degradation. Biotransformation of compounds that are preferentially transformed in anaerobic zones, such as trimethoprim and sulfamethoxazole, may be especially affected by biomat removal due to a reduced anaerobic zone size. Microcosm and molecular methods demonstrate that denitrification is the primary nitrogen removal mechanism. It has been suggested that denitrification can be enhanced by oscillating oxic and anoxic conditions (Marchant et al. 2017) and combined with a renewable labile carbon source should promote robust nitrate removal.
Additionally the presence of anammox in this system is unexpected as there is negligible influent ammonium. A better understanding of what conditions and processes that contribute to the presence of anammox organisms in this system could also factor into design consideration as well as a better ecological understanding of nitrogen cycling within freshwater systems. This is the topic and focus on the following chapter in this dissertation.
CHAPTER 3
SULFIDE-INDUCED DISSIMILATORY NITRATE REDUCTION TO AMMONIUM SUPPORTS ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) IN AN OPEN-WATER UNIT PROCESS WETLAND

A paper published in *Applied and Environmental Microbiology*

Zackary L. Jones\(^1,2\), Justin T Jasper\(^2,3\), Jonathan O. Sharp\(^1,2\), and David L. Sedlak\(^3,3\).

Abstract

Open-water unit process wetlands host a benthic diatomaceous and bacterial assemblage capable of nitrate removal from treated municipal wastewater with unexpected contributions from anammox processes. In exploring mechanistic drivers of anammox, 16S rRNA gene sequencing profiles of the biomat revealed significant microbial community shifts along the flow path and with depth. Notably there was an increasing abundance of sulfate reducers (*Desulfococcus* and other Deltaproteobacteria) and anammox microorganisms (Brocadiaceae) with depth. Pore water profiles demonstrated that nitrate and sulfate concentrations exhibited a commensurate decrease with biomat depth accompanied by the accumulation of ammonium. Quantitative PCR targeting the anammox hydrazine synthase gene, *hzsA*, revealed a 3-fold increase in

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abundance with biomat depth as well as a 2-fold increase in the sulfate reductase gene, \textit{dsrA}. These microbial and geochemical trends were most pronounced in proximity to the influent region of the wetland where the biomat was thickest and influent nitrate concentrations were highest. While direct genetic queries for dissimilatory nitrate reduction to ammonium (DNRA) microorganisms proved unsuccessful, an increasing depth-dependent dominance of Gammaproteobacteria and diatoms that have previously been functionally linked to DNRA was observed. To further explore this potential, a series of microcosms containing field-derived biomat material confirmed the ability of the community to produce sulfide and reduce nitrate; however, significant ammonium production was only observed in the presence of hydrogen sulfide. Collectively, these results suggest that biogenic sulfide induces DNRA, which in turn can explain the requisite coproduction of ammonium and nitrite from nitrified effluent necessary to sustain the anammox community.

3.1 Introduction

Constructed freshwater wetlands that receive wastewater effluent offer an opportunity to gain insight into biological nutrient cycling process because they are subject to comparatively high nutrient loading rates and optimized for steady state operational treatment. These wetlands have historically been utilized as a comparatively low energy alternative or complement to more traditional wastewater treatment systems in order to mitigate nutrient pollution with the assumption that denitrification is the dominant nitrogen removal process (Vymazal et al. 2011). However, recent findings suggest that anammox, the oxidation of ammonium with nitrite to produce N\textsubscript{2}, may play a more prominent role in these and other wetlands than initially thought (Jasper et al.
More broadly, increased understanding of anammox processes and drivers has led to wastewater treatment innovations (Z. Hu et al. 2013) and a deeper understanding of nutrient cycling as it has been estimated that in the marine environment anammox accounts for an estimated 50% of marine N₂ production (Devol 2003; Capone and Knapp 2007; Hamersley et al. 2007). In some freshwater systems anammox has been estimated to account for up to 35% of nitrate removal (Zhu et al. 2011; X.-R. Yang et al. 2015; Zhu et al. 2013) and while there is evidence for anammox occurrence in natural treatment systems such as vegetated surface flow wetlands and soil infiltration systems (Shipin et al. 2005; Liang and Liu 2008), the environmental parameters supporting anammox, the overall importance of the process to nitrate removal, and potential natural treatment applications are uncertain.

Dissimilatory nitrate reduction to ammonium (DNRA) in terrestrial and freshwater environments is gaining recognition as a significant component of nitrogen cycling (Rütting et al. 2011). In coastal environments it has been estimated that DNRA is responsible for 30% of nitrate reduction (Giblin et al. 2013) with sulfide suggested as a controlling factor (An and Gardner 2002). Sulfide and organic carbon have profound effects on the nitrogen cycle, especially in relation to ammonium. In oxygen minimum zones it was first hypothesized that sulfur cycling was linked to anammox via sulfide driven DNRA, which is a process that converts nitrate to nitrite and subsequently to ammonium (Canfield et al. 2010). Anammox microorganisms were originally found in a sulfidic wastewater treatment bed (Mulder et al. 1995) and DNRA has been demonstrated to support anammox organisms in an enrichment culture where sulfide was used as the electron acceptor for nitrate reduction (Russ et al. 2014). At high enough concentrations,
sulfide can promote DNRA by diverting nitrogen away from the canonical denitrification pathway due to inhibition of NO\textsuperscript{-} and N\textsubscript{2}O\textsuperscript{-} reductases (Brunet and Garcia-Gil 1996) as well as by inhibiting nitrification (Joye and Hollibaugh 1995). Sulfide has been demonstrated to stimulate DNRA in estuaries and in some circumstances may be pronounced enough to outcompete denitrification as the dominant nitrate reduction mechanism (An and Gardner 2002). H\textsubscript{2}S has been shown to inhibit anammox activity at concentrations in excess of 10 - 30 µM in an enriched anammox culture (Carvajal-Arroyo et al. 2013; Russ et al. 2014), however, 2 mM pulses of sulfide to a fluidized anammox bed reactor stimulated ammonium removal, potentially through biological nitrite production from nitrate (van de Graaf et al. 1996). The species *Kuenenia stuttgartiensis* and *Candidatus Scalindua* are capable of self-generating anammox precursors from nitrate, but the overall importance of this process is unknown (Kartal et al. 2007; van de Vossenberg et al. 2013).

The present study focuses on a shallow (20-30 cm) basin that receives nitrified municipal wastewater effluent (Jasper et al. 2014b). As a subclass, shallow open water unit process wetlands were developed as a specialized component of larger engineered wetland systems that receive municipal wastewater effluent and effluent-dominated surface waters. These units were initially designed to enhance the removal of recalcitrant trace organic contaminants such as pharmaceuticals and personal care products (Jasper and Sedlak 2013b; Prasse et al. 2015; Jasper et al. 2014a) but were found to have ancillary benefits for nitrate (Jasper et al. 2014b) and pathogen (Nguyen et al. 2015; Silverman et al. 2015) attenuation through an interplay of photolytic and biological processes that rivaled or even surpassed their vegetated cousins. The open-water unit process wetland is lined with a geotextile fabric in order to prevent the growth of
macrophytes that would shade the water column and contains a benthic photosynthetic biomat that when mature can be up to 10cm thick. Biological activity in the biomat causes diurnal cycling due to competing photosynthetic and heterotrophic processes with oxygen super-saturation in the water column during the day and suboxic conditions at night. The microorganisms that colonized this system were initially investigated in Jasper et al. (Jasper et al. 2014a) and shown to contain a high abundance of a single species of diatom, *Staurosira construens* var. venter, in conjunction with a diverse bacterial community.

A combination of field measurements and microcosms were previously employed to establish denitrification as the primary nitrate removal process; however, anammox organisms were present in the wetland, and ammonium-spiked microcosms suggested that anammox activity was responsible for as much as 25% of total nitrogen attenuation (Jasper et al. 2014b). In the present study, we assess the underlying ecology and mechanisms enabling anammox in these freshwater systems to increase understanding of why and how anammox is occurring in an engineered wetland with limited exogenous contributions of ammonium and nitrite. Specifically, we hypothesized that sulfur cycling plays an important role in the mechanism of nitrate attenuation by providing a shunt to ammonia production with a resultant shift from denitrification to anammox processes. Our investigation was grounded in an operational field-scale system where we synthesized inquiry that targeted nutrient cycling biomarker genes, geochemical analyses and taxonomic and functional microbial profiling. In complement, we utilized bench-scale microcosm experiments containing field-derived material to further explore functional processes. Collectively, our results bring further insight into how geochemical
parameters could potentially be leveraged to impact nutrient cycling and attenuation during the operation of treatment wetlands as well as an increased understanding of the ubiquity of anammox in natural and engineered systems.

3.2 Materials and Methods

3.2.1 Sample Collection and Processing

Samples were obtained from the Discovery Bay, CA pilot scale open-water unit process wetland. This open-water cell receives non-disinfected, nitrified municipal wastewater effluent that typically contains approximately 1.4 mM nitrate, 2 mM sulfate, 0.5 mM total organic carbon and ammonium and nitrite at concentrations below 0.05 mM (Jasper et al. 2014b). The organic matter content of the biomat in the wetland is 32% organic matter and 12% carbon content with little variation throughout the wetland. The wetland cell is 20 m x 20 m and contains baffles to minimize short-circuiting by dividing the cell into 4 runs in series where run 1 receives influent and the end of run 4 is the outlet (Figure A.1). Approximately 20 mL of slurry wetland biomat was collected for pore water and molecular analysis from four different locations during a period of active growth in late spring (May 5, 2014). A depth profile was obtained by slowly sub-sampling in triplicate from the top (proximal to open surface water), middle and bottom (proximal to geotextile membrane) regions of the biomat using a 30 mL pipette. The biomat decreased in thickness from 10 to 2 cm along the water’s flow path and was sampled approximately 2 m, 30 m, 50 m, and 70 m from the inlet along the flow transect. Samples were transported to the laboratory on ice where they were immediately centrifuged at 5000g for 5 minutes to separate water and biomass.
Porewater samples from different depths and reaches within the biomat were analyzed the same day as collected, and biomass samples were frozen at -20°C until processed. Nitrate, chloride, phosphate, and sulfate were analyzed by ion chromatography (Dionex DX-120). NH$_4^+$ was quantified in 1 µm-filtered samples by colorimetric analysis (standard methods 4500-NH$_3$ C) (Eaton et al. 1995). Archived biomat samples were freeze-dried using a Labconco FreeZone. DNA was extracted from 0.025 g of freeze-dried sample using a MoBio Power-Biofilm DNA Isolation Kit according to the manufacturer’s instructions. Extracted DNA was quantified on a Qubit 2.0 and diluted by a factor of 10 prior to amplification, resulting in concentrations ranging from 10 to 30 ng/µL.

3.2.2 Quantitative PCR

Amplification for quantitative PCR was performed with a Roche Light Cycler 480 II using Quanta Biosciences PerfeCTa SYBR Green Super Mix in 25 µL reactions with 2 µL of template DNA. Primer sequences, PCR conditions, and efficiencies for 16S, hydrazine synthase (hzsA), dissimilatory sulfate reductase (dsrA), and nitrite reductase (nrfA) genes are reported in Table 3.1. Samples were amplified in duplicate in parallel with non-template and negative extraction controls. Standards were generated from amplicons that were purified using Beckman Coulter Agencourt AMPure XP magnetic beads (Brea, CA) and quantified using an Invitrogen Qubit 2.0. No non-specific amplicon bands were observed when run on an Agilent Bioanalyzer 2100. Standard curves were generated from triplicate 1:10 serial dilutions of purified standards (Weathers, Higgins, and Sharp 2015). Crossing point values and efficiencies were determined using the Lightcycler 480 II Software v1.5.0 second derivative max method. Gene abundances
were normalized to both 16S copy number and dry weight independently, and significance was determined using students t-test with a p-value of 0.05.

Table 3.1 Primer pairs used for quantitative PCR analysis. EUB (84) primers were used to quantify 16S data in which all following genes were normalize. The following primers were used to show potential for each function: hzsA:anammox (85), dsrA:sulfate reduction (90), nfrA:DNRA (86).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence 5'-'3'</th>
<th>Primer Concentration</th>
<th>Amplicon Size</th>
<th>Target Gene</th>
<th>Thermal Profile</th>
<th>Efficiency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>ACTCTACGGGAA GGCA CAG</td>
<td>1000nM</td>
<td>180</td>
<td>16S</td>
<td>95°C 3 min, 40 cycles (95°C 60 s, 53°C 30 s, 72°C 60 s)</td>
<td>90%</td>
<td>Fierer et al., 2005</td>
</tr>
<tr>
<td>EUB518</td>
<td>ATTACCGGGCT GCTG</td>
<td>10000nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hzsA1597F</td>
<td>WTYGGKTATCAR TAGTATG</td>
<td>400nM</td>
<td>260</td>
<td>hzsA</td>
<td>95°C 3 min, 40 cycles (95°C 30 s, 55°C 30 s, 72°C 30 s)</td>
<td>81%</td>
<td>Harhangi et al., 2011</td>
</tr>
<tr>
<td>hzsA1857F</td>
<td>AAABGGYGAATC ATARTGGC</td>
<td>400nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DsrR</td>
<td>ACCACCTGGGAA CACGGCCG</td>
<td>500nM</td>
<td>221</td>
<td>dsrA</td>
<td>95°C 10 min, 40 cycles (95°C 30 s, 58°C 30 s, 72°C 40 s 80°C quantification)</td>
<td>85%</td>
<td>Spence et al., 2008</td>
</tr>
<tr>
<td>Dsr-F</td>
<td>GTGMRSCGGT GAKRTTGG</td>
<td>500nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfAF2aw</td>
<td>CARTGYCAYGTB GARTA</td>
<td>500nM</td>
<td>269</td>
<td>nrfA</td>
<td>95°C 10 min, 40 cycles (95°C 15 s, 52°C 45 s, 72°C 20 s 80°C quantification)</td>
<td>~50%</td>
<td>Welsh et al., 2014</td>
</tr>
<tr>
<td>nrfAR1</td>
<td>TNWGGCATRTG RCARTC</td>
<td>500nM</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### 3.2.3 16S rRNA Gene Sequencing and Analysis

Amplification, purification, and normalization of samples for 16S rRNA gene sequencing was performed using primers for the V4 region in accordance with published methods with minor modifications to the protocol which exclude the Bioanalyzer steps and include an additional concentration step (Kozich et al. 2013). Briefly, 2 µl of extracted DNA was amplified with dual indexed primers for 30 cycles (Phusion Master Mix, New England Biolabs). Amplicons were normalized and purified with the SequaPrep Normalization Plate Kit. Normalized amplicons were pooled and
concentrated with Amicon Ultra-0.5mL 30K Centrifugal Filter Devices. Pooled concentrates were quantified and sequenced by the Biofrontiers Institute at CU Boulder using the Illumina MiSeq Platform with a v2 2x250 cycle reagent kit.

Resultant 250 base-pair sequences were processed in QIIME 1.9 (Caporaso et al. 2010). Forward and reverse sequences of each sample were joined into contigs with the ‘multiple_join_paired_ends.py’ script with a minimum base pair overlap of 100. Joined sequences were passed through multiple_split_libraries_fastq.py with default quality control parameters. OTUs were parsed using pick_open_reference_otus.py with Usearch 6.1 (Edgar 2010) and chimeras were filtered out using the Greengenes gold database (DeSantis et al. 2006). Representative sequences were aligned using PyNAST (Caporaso et al. 2009) and Greengenes 13_8 aligned reference database, and alignments were filtered using the Lane mask (Lane 1991).

Taxonomy was assigned using the RDP classifier with 0.5 confidence and Greengenes 13_8 OTU taxonomy database. Diatom plastid sequences were removed and the OTU table was rarified to the minimum number of sequences for a sample, 15926, for alpha and beta diversity measurements. OTUs with less than 0.1% relative abundance were filtered out for phylogenetic analysis. The DESeq2 method (Love, Huber, and Anders 2014) was used within QIIME to calculate differential abundance between samples using the filtered taxonomic OTU tables. The principle component diagram was generated in R using the phyloseq package (McMurdie and Holmes 2013). Sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive under accession number SRP069033.
3.2.4 Anaerobic Microcosms

Freshly harvested biomat, collected on January 27, 2015, was shipped overnight on ice and stored refrigerated no more than 2 weeks prior to use. Biomat material was washed 2 times with phosphate buffered saline (PBS) and centrifuged to remove soluble constituents. This process was followed by a final suspension in PBS where 10 mL each of the slurry (0.5 g biomat dry weight) was added to triplicate microcosms containing 70 mL of commercially purchased minimal freshwater diatom DY-V growth media primarily composed of MES buffer, KCl, H$_3$BO$_3$, Na$_2$ b-glycerophosphate, Na$_2$SiO$_3$, CaCl$_2$, trace elements, and f/2 vitamin solution (NCMA at Bigelow Laboratories; omitted subcomponents of the media containing sulfur and nitrogen species: NH$_4$Cl, NaNO$_3$, and MgSO$_4$) and then amended them in accordance with experimental variables where appropriate of nitrate (3.25 mM) and/or hydrogen sulfide at a predicted 1mM aqueous (calculated with a dimensionless $K_{H_2}$ partitioning coefficient of 0.1). Incubations were conducted in 160 mL glass serum bottles purged with N$_2$ gas and sealed with butyl stoppers and shaken at 90 rpm. Control microcosms without additional amendments were also included and all microcosms where incubated in the dark to minimize photosynthesis. Nitrate, nitrite, ammonium, sulfate, and sulfide were monitored with Hach TNTplus kits 835, 839, and 831, SulfaVer 4 Powder Pillows, and Methylene Blue Sulfide Reagents, respectively, using a Hach DR5000 spectrophotometer.

3.3 Results & Discussion

3.3.1 Microbial Ecology of Biomat

Microbial communities in the biomat were significantly different across both the sampling depth ($R^2$ ADONIS = 0.259, $p= <0.001$) and along the wetland flow path ($R^2$
ADONIS = 0.374, p = < 0.001) as interpreted by results of high throughput 16S rRNA
gene sequencing at discrete points (Figure 3.1). The depth trend was most evident in
samples collected closer to the inlet (~2m), where the biomat thickness of approximately
10 cm was most pronounced. In addition to community shifts between sampling locations,
community alpha diversity was found to decrease along flow path (Figure A.2). These
shifts can be explained by nutrient or other resource limitations such as the observed
decrease in nitrate concentration from inlet to outlet.

To better understand the mechanism of nitrate attenuation within the biomat, we
focused on taxonomic distribution as a function of depth at the sampling region nearest to
the wetland inlet, where the communities (Figure 3.1) and water parameters as discussed
later in this paper varied most with depth. Diatom (Stramenopile) plastids accounted for
20.2 ± 3.9% of the total sequences at the top and increased to 31.9 ± 2.5% of the
sequences at the bottom of the biomat suggesting either benthic accumulation or that they
may play a role within the mat beyond that of photosynthesis. Given the large diatom
presence, they were removed from subsequent 16S rRNA gene community analysis in
order to focus on bacterial and archaeal shifts. The majority of the remaining sequences
from the top and bottom of the biomat were assigned to taxonomic classes as listed by
rank (Figure A3). Alphaproteobacteria and Cytophagia were more than twice as abundant
at the biomat top than at the bottom, and Flavobacteria were almost exclusively located
within the top portion of the biomat. In contrast, Anaerolineae, Deltaproteobacteria, and
Bacteroidia roughly doubled and the methanogens Methanomicrobia and
Methanobacteria more than tripled in relative abundance from top to bottom.
Figure 3.1 Microbial community similarity within the biomat as a function of depth (top, middle, and bottom) and distance from inlet. Significant community shifts were seen as both a function of depth and distance from the inlet. Principle coordinate analysis was established using weighted UniFrac 16S rRNA gene sequences where percentage of the variation explained is indicated on the axes.

To better understand functional implications of these shifts, we utilized this differential abundance analysis to focus a literature synthesis of previously reported attributes of bacterial clades primarily at the taxonomic planes of family and genus where putative functionality can be more effectively inferred (Drennan et al. 2015). This approach, with a caveat of imperfect associations between microbial structure and function, enabled us to identify microbial shifts and infer potential associations with carbon, nitrogen and sulfur biogeochemical processes between the top and bottom samples of the biomat in an approximate 10 cm vertical stratification (Figure 3.2 & Table
A.1). Our analysis revealed that the top of the biomat had a relatively higher abundance of phototrophic and aerobic heterotrophic microbial clades that consume simple sugars, which is in line with what would be anticipated in such a photosynthetic mat. The top also contained organisms in the ZB2 and *Hydrogenophaga* clades which are putative aerobic methane (Peura et al. 2012) and hydrogen oxidizers (Willems et al. 1989), respectively.

![Figure 3.2](image)

**Figure 3.2** Differential abundance (bars) between top and bottom expressed as log2 fold change where positive values indicate a greater abundance in the top and vice versa. Putative functionality assigned to clades (Table A.2) supports a shift toward anaerobic respiration with depth. Error bars represent ± 1 standard deviation of triplicate samples. Clades with overlapping error bars were removed for clarity.

Consistent with oxygen consumption with depth, the bottom region of the biomat community was enriched with anaerobic organisms, including the fermentative clades Anaerolineae and Bacteroidales (T. Yamada 2006; Takeshi Yamada and Sekiguchi 2009;
Within this region, methanogens, including *Methanosaeta* and *Candidatus Methanoregula*, which putatively oxidize both acetate and hydrogen/CO$_2$ to produce methane (Smith and Ingram-Smith 2007; Brauer et al. 2011), were more abundant. Anammox organisms from the family Brocadiaceae (Jetten et al. 2015) were enriched 4-fold at the wetland inlet bottom with a total relative abundance of 1.7% versus 0.4% at the top. A similar trend was observed for sulfate-reducers, including Deltaproteobacteria and *Syntrophobacter sp.* (Chen 2005; Krieg et al. 2010) at 1.00% and 0.91% respectively at the bottom of the biomat compared to 0.1% and 0.3% at the top. Another sulfate reducer, *Desulfococcus sp.*, doubled in relative abundance from 2.8% to 5.7% from top to bottom, but was not statistically significant according to the DESeq algorithm. The increased presence of both sulfate reducing bacteria and anammox bacteria suggests a positive relationship between sulfide production and anammox that was explored further.

### 3.3.2 Effect of Sulfide on Ammonium Production

To confirm putative functionality of the organisms observed through sequencing with respect to nitrogen and sulfur biogeochemical processes, a series of biomat microcosms with varying amendments were studied. Microcosms amended with 4 mM sulfate demonstrated the sulfate-reducing potential of the biomat, with conversion of 3.6 ± 0.6 mM sulfate and production of 2.60 ± 0.06 mM sulfide while control microcosms without sulfate had no significant production of sulfide (Figure 3.3). The incomplete stoichiometric recovery of sulfide may be attributed to precipitation of sulfide-containing minerals, adsorption to surfaces or volatilization during analysis.
Figure 3.3 The washed biomat converted sulfate to sulfide at near stoichiometric proportions as evidenced by microcosms amended with (A) sulfate and (B) no amendment, demonstrating a large sulfate reducing capacity. Error bars represent ± 1 standard deviation of triplicate incubations.

To understand the effects of sulfide on nitrogen cycling, specifically nitrate conversion to ammonium, anaerobic microcosms were amended with 3.4 mM nitrate, with or without gaseous additions of hydrogen sulfide equating to 1 mM aqueous concentration. Microcosms containing amendments of both nitrate and sulfide converted 38 ± 1.6% of nitrate to ammonium (700 ± 23 µM) after approximately one day (Figure 3.4a). During this period, 1093 ± 112 µM of sulfide were oxidized and 1844 ± 152 µM of nitrate were reduced implying that DNRA was occurring. The observed sulfide oxidation and ammonium production ratio of 1.5:1 was greater than the theoretical ratio of 1:1.
predicted by eqn. 1 which describes the DNRA reaction. One possible explanation for the
discrepancy between these ratios could be the formation of elemental sulfur from sulfide
oxidation. An analogous experiment by Burnet and colleagues reported a similar ratio of
30% of nitrate converted to ammonia in the presence of elemental sulfur production from
sulfide, however their sulfide consumption to ammonia production ratio was 4 time less
(Brunet and Garcia-Gil 1996) than observed here. Other possible explanations for this
discrepancy include the consumption of produced ammonium due to anammox activity as
well as sulfide association with surfaces in the biomat or precipitation.

\[
\text{HS}^- + \text{NO}_3^- + \text{H}^+ + \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + \text{NH}_4^+ \quad (3.1)
\]

Figure 3.4 Anaerobic microcosms amended with (A) nitrate and sulfide; (B) only nitrate;
and (C) neither nitrate nor sulfide. Microcosms reveal significantly increased ammonium
production in the presence of sulfide. Error bars represent ± 1 standard deviation of
triPLICATE incubations.

Microcosms amended solely with a similar quantity of nitrate (1963 ± 300 µM)
resulted in a much lower yield of 127 ± 7 µM ammonium, which translated to just 6% of
that produced in the sulfide-amended system (Figure 3.4b). In contrast, microcosms
amended with hydrogen sulfide but without supplemental nitrate generated negligible
amounts of ammonium (Figure A.4). Control microcosms that received neither nitrate
nor sulfide produced 210 ± 42 µM ammonium (Figure 3.4c). The comparatively modest
ammonium generation seen in the control and nitrate-only microcosms can be explained by cell death and ammonification associated with the harvesting and washing processes utilized to prepare the biomat. Collectively, these microcosm experiments support the potential of the biomat to facilitate sulfide-induced DNRA.

3.3.3 Spatial Trends in Biogeochemical Processes

Pore water profiles within the biomat from the pilot scale wetland revealed spatial trends further supporting the hypothesis that sulfide-induced DNRA could be an important process near the inlet. Consistent with earlier characterizations of the wetland (Jasper et al. 2014b), concentration dependent nitrate reduction was observed along the horizontal flow path and with depth in the biomat (Figure 3.5a). Approximately 89% of the nitrate was removed between the 2 m and 70 m sample locations assuming that surficial biomat pore water samples, 0.36 mM and 0.04 mM nitrate respectively, were representative of concentrations in the overlaying water. Nitrate concentrations were highest at the inlet surficial regions of the biomat (0.37 ± 0.05 mM) and decreased with depth (0.04 ± 0.016 mM) within the biomat. Mirroring this trend, the highest ammonium concentration was detected at the bottom of the mat near the inlet (2.4 ± 0.76 mM), which was about 6.5 times the concentration of nitrate in the water column indicating a significant accumulation of ammonium. While DNRA is consistent with higher ammonium accumulation within regions fed by higher nitrate concentrations, the accumulation could also be explained by ammonification. If ammonification and nitrification were significant processes in our system, it stands to reason that ammonium concentrations and the relative abundance of anammox microorganisms would be similar at the biomat bottom throughout the cell. However, other sampling locations revealed a
Figure 3.5 Differences in gene abundance and chemical profiles reveal spatial trends for nitrogen and sulfur biochemistry. (A) anammox bio-marker gene (\textit{hzsA}) with ammonium concentration and (B) dissimilatory sulfate reduction gene (\textit{dsrA}) with sulfate concentration. Samples were taken in triplicate at along the horizontal flow path of the wetland cell and discretized by depth (top middle, bottom) and normalized to 16S gene copies. Error bars represent ± 1 standard deviation.

Slight increase in ammonium concentration with depth but concentrations were all less than 0.2 mM (Figure 3.5a) suggesting that ammonification played a lesser role. Aerobic ammonium oxidation is also unlikely as methanogenic communities are observed throughout the bottom layers (Figure 3.2) and it has previously been shown that oxygen penetrates less than 4 cm within this biomat (Jasper et al. 2014b). The accumulation of 1.9 mM ammonium in the bottom pore water was observed approximately 30 m from the
inlet a year prior during the month of April (Jasper et al. 2014b), indicating that ammonium accumulation may not be limited to the inlet portion of the wetland throughout the year. Temperature and sunlight intensity affect nitrate removal rates within open water wetlands (Jasper et al. 2014b), which could lead to seasonal spatial variation in nutrient profiles and explain variability in ammonium accumulation between the two studies.

Sulfate concentrations, like nitrate, decreased with depth within the biomat especially near the inlet. However, unlike for nitrate, net sulfate loss at the biomat / porewater interface was not observed across the wetland transect (Figure 3.5b). Samples closest to the inlet showed the greatest decrease in sulfate concentration with depth (Δ1.5 ± 0.06 mM), with over 77% of the total sulfate present at the biomat surface reduced. The decrease in sulfate concentration with depth was more modest at other sampling locations, with losses of approximately 0.2 mM. The proximal concurrence of nitrate and sulfate reduction in the depth profile combined with the accumulation of ammonium 2 m from the inlet highlights the potential for sulfide-driven DNRA in our system.

In turn, we queried for the abundance of process-relevant genes in association with these trends. The sulfate reductase gene (drrA) exhibited similar trends with depth, but was significantly elevated near the inlet (2 and 30 m sample points) with a maximum of 3.1 ± 0.44% relative abundance (Figure 3.5b and Figure A.5b). Further from the inlet (50 m) abundances were approximately half those within the inlet region. This is consistent with 16S rDNA gene sequencing as the dominant putative sulfate reducer, Desulfococcus sp., harbored a relative abundance of 2.7 - 5.6% closest to the inlet and decreased by about half (1.2% - 2.3%) at the 50 m sampling point. QPCR results
normalized to the 16S gene are approximately a 30% underestimate compared to sequencing relative abundances as the diatom 16S plastid sequences were removed from sequencing analysis.

Identification and enumeration of microorganisms responsible for performing DNRA coupled to sulfide in this system was elusive due to their broad phylogenetic distribution (Mohan et al. 2004). Unfortunately there were limitations in amplification specificity / efficiency of the primers for \textit{nrfA} in our system despite their prior success in an estuarine system (Song, Lisa, and Tobias 2014) (Table 3.1). An octa-heme nitrite reductase, such as those found in anammox organisms (Kartal and Keltjens 2016), could also be responsible for the conversion of nitrite to ammonium. Insights from a recent metagenomic inquiry in estuary sediments that reconstructed genomes of novel organisms involved in carbon, nitrogen, and sulfur cycling help to bridge this gap (Baker et al. 2015). Specifically, this investigation revealed that novel organisms within Gammaproteobacteria have the genes for both sulfide oxidation and nitrate/nitrite reduction. Interestingly, Gammaproteobacteria also exhibited dominance in our open-water wetland cell, with a bacterial population second only to Anaerolineae within the bottom layer of the biomat (Figure A.3). Furthermore, a broad range of diatom species have been reported to perform DNRA in the absence of light (Kamp et al. 2011), which could explain their increased abundance in the bottom of the biomat as described in the prior microbial ecology section. However, without a DNRA biomarker for diatoms and the difficulty of culturing diatoms axenically, it could not be determined if diatoms are directly involved in ammonium production in our wetland biomat.
3.3.4 Distribution of Anammox Bacteria

Preliminary evidence for anammox activity in this engineered wetland was originally documented through the observation of ammonium removal by anaerobic microcosms and the acetylene block method to estimate denitrification and anammox rates. In these experiments, it was estimated that up to 25% of nitrogen was removed via anammox (Jasper et al. 2014b); however this could underestimate the actual contribution as acetylene can also inhibit anammox (M. M. Jensen, Thamdrup, and Dalsgaard 2007) in addition to the targeted denitrification process. In our current investigation, we further queried for potential anammox activity by quantifying the anammox-dependent hydrazine synthase gene, \( \text{hzsA} \), throughout the wetland cell. The abundance of \( \text{hzsA} \) was significantly higher at the bottom and middle of the biomat relative to the top at both the 2 m and 30 m sampling points (Figure 3.5a and Fig A.5a). The maximum relative abundance of \( 0.06 \pm 0.01\% \) \( \text{hzsA} \) decreased to below detection near the outlet (data not shown) where the biomat decreased in thickness and pore water nitrate concentrations were lower. As anammox organisms are oxygen sensitive (Kalvelage et al. 2011; Schmid et al. 2001), this trend with depth could further be explained by oxygen production in the overlying waters in association with diatom primary productivity. Sequencing results also showed enrichment in the anammox family Brocadiaceae at \( \sim 0.6\% \) relative abundance within the biomat bottom (Figure 3.2), which is consistent with other studies showing Brocadiaceae lineages present in soils and freshwater wetlands (Humbert, Zopfi, and Tarnawski 2012; Humbert et al. 2010). Relative abundances of qPCR and 16S sequencing differ by an order of magnitude which is the same result observed by previous
investigations (Lipsewers 2014; Harhangi et al. 2011) possibly due to primer biases (Bale et al. 2014).

Comparatively less sulfate and nitrate reduction may have occurred in the upper portion of the biomat during the day due to oxygen supersaturated conditions resulting from photosynthesis (Jasper et al. 2014b). This would in turn lead to fewer precursors for the anammox process. A similar trend in community distribution has been recently reported in freshwater marshes where a high abundance of anammox organisms was observed near the inlet of a vegetated wetland (Ligi et al. 2014). Though not directly investigated in the current study, nitrite was only detected at trace quantities (<0.05 mM) near the top few centimeters of the studied biomat in a prior investigation (Jasper et al. 2014b). Its absence with depth presumably limits anammox processes as has been documented in mangrove (Meyer, Risgaard-Petersen, and Allen 2005) and marine sediments (Risgaard-Petersen et al. 2003), and provides an explanation for the accumulation of ammonium at the bottom of the biomat. Prior work in our wetland has revealed a significant nitrate gradient along the wetland flow path with approximately 70% of the nitrate removed by the middle of the wetland in summer months (Jasper et al. 2014b); similarly, our present work shows 63% nitrate removal half way through the wetland cell. Building on this theme, the decreasing anammox trend from inlet to outlet could have been due to nitrite and ammonium availability, which would decrease as nitrate decreases.

3.4 Environmental Implications

Our present study provides insights into eutrophic freshwater systems by documenting the interdependence of sulfide and nitrate, as well as the presence of
anammox organisms in this engineered wetland. A recent study investigating nitrogen reduction pathways in estuarine sediments found no correlation between anammox and DNRA rates and sulfide (Plummer, Tobias, and Cady 2015); however, that finding was counter to a study focusing on the same location that found anammox bacterial abundance positively correlated to percent organic carbon and sulfide concentration (Lisa et al. 2014). DNRA has been previously linked to sulfide oxidation in marine environments (Jørgensen and Nelson 2004) and has been directly coupled to anammox in marine sediments and enrichment cultures (Marlene M Jensen et al. 2011; Russ et al. 2014). While hypothesized to be coupled to anammox in terrestrial and freshwater environments (Francis, Beman, and Kuypers 2007), direct evidence in these types of systems has previously proved elusive.

More broadly, our analysis of this wetland biomat supports the importance of linkages between carbon, nitrogen, and sulfur cycling in both engineered and natural environments. The biomat within this wetland has an organic carbon content of 13% (Jasper et al. 2014b). Though its generally accepted that organic-rich environments favor faster reproducing heterotrophs (Tang et al. 2010; van de Graaf et al. 1996), anammox microorganisms have been found in organic-rich sediments in contact with nitrate-rich waters (Schubert et al. 2006; Zhu et al. 2013) as well as periphyton-dominated aerobic sediments (Penton, Devol, and Tiedje 2006) analogous to the open-water wetland cell investigated here. More common surface-flow vegetated treatment wetlands and analogous natural systems, such as wetlands and estuaries, receive nitrate-rich water and often have an active sulfur cycle (Pester 2012; Sturman et al. 2008; Faulwetter et al. 2009) and large soil carbon pools that average 7.7% carbon content (Bridgham et al. 2014b).
These systems may also host DNRA-linked anammox processes, which should be considered when assessing and predicting biogeochemical cycling and water quality. From an applied perspective, the diatomaceous and bacterial photosynthetic biomat formed in open water cells could offer an alternative treatment system to those present in conventional vegetated treatment wetlands (Jasper et al. 2013). Unique and potentially functionally beneficial attributes include depth stratification within the biomat, diurnal cycling of oxygen content and the autotrophic capabilities of the assemblage where organic production via photosynthesis could fuel reductive processes.

The impact of these systems on greenhouse gas emissions is promising but not yet clear. As an autotrophic system, they should represent a net carbon sink, and our microcosms demonstrated that DNRA can achieve similar nitrate removal rates to that of canonical denitrification (Figure 3.4). DNRA linked to anammox has the potential to reduce N\textsubscript{2}O emissions as electrons are shuttled away from N\textsubscript{2}O generation toward ammonium (Zhu et al. 2011; Senga et al. 2006; Freeman et al. 1997), suggesting a potential N\textsubscript{2}O control strategy by utilizing sulfate present in the water supply or adding it as a supplement. However, the biochemistry of organisms that perform DNRA and their potential for N\textsubscript{2}O generation through competing processes such as nitrite/nitric oxide detoxification systems needs further study. There is also the caveat of unintended side effects such as the increased potential for methyl mercury formation associated with sulfide production that should also be considered (King et al. 2002).

Our phylogenetic inquiry also revealed an increasing presence of methanogens in the lower layers of the biomat. As such, it is possible that denitrifying anaerobic methane oxidation (damo) could also be responsible for nitrate reduction or compete with
anammox organism for nitrite, which appears to be a limiting resource in this system. Nitrite dependent methane oxidizers have been co-cultured with anammox bacteria (Luesken et al. 2011) and naturally co-occur in various anaerobic environments (Shen et al. 2014; J. Yang et al. 2012; Wang et al. 2012). Organisms linked to anaerobic methane oxidation with nitrate or nitrite such as ANME or NC10 type organisms (Zhu et al. 2010a; Luesken et al. 2011; Haroon et al. 2013; Vaksmaa et al. 2016) were not identified by 16S sequencing, however, this dynamic needs further investigation in our system.

It has been hypothesized that ammonification or partial nitrification combined with incomplete denitrification at oxic / anoxic interfaces is responsible for providing the precursors for anammox (Zhu et al. 2013; Ligi et al. 2014; Lam et al. 2007; Slickers et al. 2002) and that sulfide production can inhibit the growth of anammox organisms as it prevents nitrification (Zhu et al. 2010a). However, our results provide field evidence for an alternate explanation previously reported in microcosms (Russ et al. 2014) in which anammox organisms increase in abundance in the most reducing zones of the microbial environment and appear to thrive within and be influenced by sulfidic systems due to an increase of DNRA.
CHAPTER 4
CONVERGENCE OF MICROBIAL COMMUNITY STRUCTURE AND
FUNCTIONALITY IN SHALLOW OPEN WATER TREATMENT WETLANDS
ACROSS DISTINCT GEOGRAPHIC LOCATIONS AND SCALES

Modified from a manuscript submitted for publication in Water Research

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Jonathan O. Sharp$^{1,2}$

Abstract
The widespread adoption of engineered wetlands designed for water treatment is hindered by uncertainties in system reliability, resilience and management associated with biological and physical processes. In order to better understand how shallow open water wetland cells self-colonize and evolve, we analyzed the composition of the microbial community in benthic biomats from system establishment through approximately 3 years of operation. Our analysis was conducted across three parallel demonstration-scale (7500 m$^2$) cells located within the Prado constructed wetlands that receive water from Santa Ana River (NO$_3^-$ = 6.1 mg/L). Phylogenetic inquiry and microscopy confirmed that diatoms and an associated aerobic bacterial community facilitated early colonization. After approximately nine months of operation, coinciding with late summer and associated growth, an anaerobic community emerged with the capability for nitrate attenuation. The community was comparatively stable for the

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remaining years of operation and converged with one that had previously formed in a geographically separated pilot-scale (400 m$^2$) wetland in Discovery Bay, California that received denitrified (NO$_3^-$ = 20.7 mg/L), secondary treated wastewater for 5 years of operation. Variations in hydraulic residence time (HRT) from 1 to 4 days across the three cells in Prado resulted in modest changes across systems that were most evident in the outlet regions of the constructs. Establishment of a core microbiome between Prado and Discovery Bay revealed a strong overlap of both aerobic and anaerobic taxa with approximately 50% of the analyzed bacterial sequences shared between the two sites. Additionally the same species of diatom, *Staurira construens* var. venter, was prolific in both systems as the putative dominant primary producer. Our results indicate that despite differences in scale, geographic location and source waters, the shallow open water wetland design selects for a rapid convergence of microbial structure and functionality associated with the self-colonizing benthic biomat. This resulting biomat matures over the first growing season with operational parameters such as HRT further exerting a modest selective bias on community succession.

### 4.1 Introduction

Constructed wetlands designed for water treatment have evolved from traditional vegetated surface flow wetlands to further adapt the natural interplay between macrophytic and microbial processes (Faulwetter et al. 2009; Kadlec and Wallace 2009; Reddy and D’Angelo 1994). Designs incorporate variables including aquatic plants, introduction of geomedia, depth, hydraulic properties, and subsurface flow in order to address regional climate challenges, optimal contaminant removal and other practitioner goals. For example, different plant types in vegetated wetlands have been shown to
promote varying denitrification potential (Bastviken et al. 2003) while substrate amendments such as zeolite have proven to increase ammonium oxidizing microorganisms (Gorra et al. 2007).

Research on wetland biogeochemistry has largely focused around carbon, nitrogen, and sulfur cycling (Reddy and DeLaune 2008) which is still evolving as our understanding of microbial nutrient cycling pathways grows (B. -l. Hu et al. 2014; Jones et al. 2017b; Zhu et al. 2010b). More applied research in engineered systems has investigated the microbially-mediated removal of trace organic contaminants such as pharmaceuticals that are minimally attenuated within traditional wastewater treatment plants (Jasper et al. 2014a; Y. Li et al. 2014; Onesios, Yu, and Bouwer 2009; Verlicchi and Zambello 2014). While the removal of these emerging contaminants is largely attributed to microorganisms, investigations into how environmental conditions (Hijosa-Valsero et al. 2010; Jasper et al. 2014a), removal pathways and microbial diversity impact treatment efficiency are ongoing.

A myriad of factors have been shown to affect microbial community composition in freshwater wetlands including influent properties (Arroyo, Sáenz de Miera, and Ansola 2015), whether the wetland was naturally established or constructed (Ansola, Arroyo, and Sáenz de Miera 2014), sediment properties (R. M. Peralta, Ahn, and Gillevet 2013), previous land use (Hartman et al. 2008; A. L. Peralta, Matthews, and Kent 2010), geographical location and vegetation type (Angeloni et al. 2006; Yarwood et al. 2016; Menon, Jackson, and Holland 2013). This can lead to challenges in design guidelines and inconsistencies in treatment performance and permitting. Hence, the ability to design wetlands and predict the resultant microbial community and functionality would be
beneficial to the increased adoption of constructed wetlands for water treatment. To this end, we have focused research on a novel, macrophyte-free design employing a shallow open water cell with a geotextile liner that is naturally colonized with a photosynthetic, benthic biomat (Jasper et al. 2014a). While the original design was optimized for photolytic degradation or organic pollutants (Jasper and Sedlak 2013b) and pathogen inactivation (Nguyen et al. 2015; Silverman et al. 2015), it became apparent that the microbial community within the self-colonizing biomat was capable of robust organic pollutant biotransformation (Jasper et al. 2014a) and nitrate removal (Jasper et al. 2014b) that rivaled and in many ways exceeded that of more traditional engineered vegetated wetlands.

The study herein focuses on the establishment and colonization of a series of parallel, demonstration-scale, shallow open-water unit process wetlands and their comparison to a smaller (~1/20th area) pilot-scale system that was operational for five years. These engineered treatment wetlands receive effluent-dominated Santa Ana River water and secondary treated, nitrified wastewater respectively. The demonstration-scale wetland cells are located in Prado, CA while the pilot-scale system is located approximately 350 miles to the north in Discovery Bay, CA. While there were pronounced differences in geographic location, scale, and receiving waters, the constructs maintained an analogous design and were operated in a temperate, Mediterranean climate. Both constructs employed a barrier at the ground surface to prevent macrophyte growth, and a shallow water level of 20-25 cm was maintained in order to maximize sunlight penetration of the water column. The objectives of our present study were twofold: (1) to determine if differences in wetland scale and geographic location would
affect the colonization of this benthic biomat and (2) compare the structure and functionality of the microbial communities within the benthic biomat at the different scales and locations. The work was guided by an overarching hypothesis that despite differences in scale and geographic location, similarly designed and constructed shallow open water wetland cells would establish analogous naturally colonized benthic biomats with similar ecological signatures and associated water treatment functionalities.

4.2 Materials and Methods

4.2.1 Site Description

The pilot-scale system was built and operated as a 20 m x 20 m serpentine configuration from 2009 to 2014 in Discovery Bay, CA (Figure B.1). This unit process wetland was constructed as a baffled cell and received nitrified wastewater effluent ($\text{NO}_3^-$ = 20.7 mg/L) directly from a proximal water treatment facility. The demonstration-scale system began operation in December of 2013 and is still in operation with microbial analyses concluding in October of 2016. This larger-scale construct is situated within the Prado, California engineered wetland system and consists of three 30 m x 260 m cells running in parallel that receive effluent dominant river water from the Santa Ana ($\text{NO}_3^-$ = 6.1 mg/L). Further comparison of inlet water parameters and system design can be found in Table 4.1. Discovery Bay is lined half with cement and half with geotextile liner, and had a hydraulic retention time (HRT) of 2 days while Prado is fully lined with a geotextile material and began with a HRTs of approximately 2 days. After the first year of operation, the Prado wetland cells were adjusted to have different HRTs of 1, 2, and 4 days. Additionally, the Prado construct experienced a disturbance when the forebay
equalization pond was reconstructed in January of 2015 during which time flow was cut off and the water was held without flow in the cells for approximately 2 weeks.  

Table 4.1 Comparison between the pilot and demonstration wetlands, Discovery Bay and Prado, respectively. Discovery Bay data was collected from 2012-2013 (Jasper et al. 2014b). Prado influent data was collected from 2014-2016 (Bear et al. In Preparation).

<table>
<thead>
<tr>
<th></th>
<th>Discovery Bay Wetland</th>
<th>Prado Wetland</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>Discovery Bay, CA</td>
<td>Corona, CA</td>
</tr>
<tr>
<td><strong>Latitude, Longitude</strong></td>
<td>37.896, -121.587</td>
<td>33.919, -117.617</td>
</tr>
<tr>
<td><strong>Area (m²)</strong></td>
<td>400</td>
<td>3 cells x 7500</td>
</tr>
<tr>
<td><strong>Operational Start up</strong></td>
<td>2009</td>
<td>December 2013</td>
</tr>
<tr>
<td><strong>Hydraulic Retention Time (d)</strong></td>
<td>1-3</td>
<td>1-4</td>
</tr>
<tr>
<td><strong>Nitrate Removal Rate (mg N m⁻³ y⁻¹)</strong></td>
<td>59.4 ± 6.2</td>
<td>61.7 – 68.1</td>
</tr>
<tr>
<td><strong>Water Depth (cm)</strong></td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td><strong>Influent Parameters:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate (mg/L-N)</td>
<td>20.7 ± 0.7</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Sulfate (mg/L)</td>
<td>140 ± 10</td>
<td>68 ± 3.5</td>
</tr>
<tr>
<td>TOC (mg/L-C)</td>
<td>5.9 ± 0.3</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>pH</td>
<td>8.6 ± 0.2</td>
<td>7.9 ± 0.1</td>
</tr>
</tbody>
</table>

4.2.2 Biomat Collection

Biomat material from the Discovery Bay wetland cell was sampled on May 5th 2014, approximately 5 years after the start of operation. Samples were collected in triplicate in 4 equally spaced locations along the horizontal transect of the wetland. They were further discretized as a function of three different depths (Jones et al. 2017b) for a total of 36 samples. Biomat samples from the three parallel cells in the Prado wetland were collected in triplicate at three different locations (inlet, middle and outlet) approximately monthly after establishment for 2 years (December 2013 – 2015). Four additional sampling events were recorded in August through October 2016. Biomat was collected from the inlet, middle, and outlet regions of each of the 3 parallel cells in 50 ml conical tubes for a total of nine samples per month. An effort was made to sample all
layers of the biomat as it thickened over time. Biomat samples were chilled and then quickly transported to the laboratory where they were centrifuged, decanted and frozen, shipped overnight, and remained frozen until thawed for further processing and analysis.

4.2.3 DNA Extraction and 16S rRNA Gene Sequencing

Archived samples were thawed and then freeze dried in a Labconco FreeZone in order to normalize extraction by dry weight as well as archive samples for future microscopic or other analyses. DNA extractions were performed using the PowerSoil Biofilm DNA extraction kit on 0.0025g of freeze dried biomat. Amplification, purification, and normalization of samples for 16S rRNA gene sequencing was performed in accordance with published methods, using primers for the V4 region with minor modifications to the protocol which exclude the Bioanalyzer steps and include an additional concentration step (Kozich et al. 2013). Briefly, 2 µl of extracted DNA was amplified with dual indexed primers for 30 cycles (Phusion Master Mix, New England BioLabs). Amplicons were normalized and purified with the SequalPrep Normalization Plate Kit. Normalized amplicons were pooled and concentrated with Amicon Ultra-0.5mL 30K Centrifugal Filter Devices. Pooled concentrates were quantified and sequenced by the Biofrontiers Institute at CU Boulder using the Illumina MiSeq Platform with a v2 2x250 cycle reagent kit. Sequences have been deposited in the NCBI SRA database with accession number PRJNA390172.

4.2.4 Bioinformatic Analyses

Processing of demultiplexed raw sequences was performed with QIMME 1.9 as in Jones et al. 2017 (Jones et al. 2017b). After processing and quality filtering the Illumina 16S rRNA gene reads, a total of 7,287,041 paired-end sequences were obtained from 280
samples. Subsequently, 8 samples with fewer than 8000 sequences were dropped from analysis. Sampling depth then ranged from 8,681 to 837,508 sequences per sample.

Unfortunately, 34 samples (5/15/2015 – 8/21/2015 Prado) were identified as compromised due to a thawing event during shipping and prior to DNA extraction. After confirmation of significant community abnormalities from sequencing, these samples were removed from further analysis along with three negative control samples. Two outliers with high and low diversity were also removed from analysis. The remaining analysis was performed on 196 samples from Prado and 36 samples from Discovery Bay. Analyses were based off of a rarefied operation taxonomic unit (OTU) table with 8,681 randomly selected sequences per sample except for differential abundance tests, for which a Negative Binomial model (McMurdie and Holmes 2014) was used to normalize counts for each sample.

To evaluate and visualize compositional changes across time at Prado and compare community structure to Discovery Bay, we utilized a weighted and unweighted UniFrac distance matrix (Lozupone and Knight 2005) followed by principal coordinate analysis and detrended coordinate analysis when temporal data displayed a horse-shoe effect. Statistical differences in weighted UniFrac distances were determined using the ANOSIM test and considered significant when p-values were less than 0.05. Alpha diversity was calculated using both Shannon Diversity and Observed OTU measures to account for both evenness and richness across samples. Differential abundance analyses were used to identify cladal shifts between the inlet and outlet samples at Prado using DESEQ2 (Love, Huber, and Anders 2014). After converting the unrarefied OTU table to a DESEQ object, OTUs were clustered at the family taxonomic level. Families with total
counts across all samples less than 500 were removed to focus on the more abundant families. P-values were generated using the Wald test, adjusted for multiple comparisons using the procedure of Benjamini and Hochberg (Benjamini and Hochberg 1995) and considered significant if less than 0.01. To assess cladal similarities between the mature Prado samples (those taken from August – October 2016) and the Discovery Bay samples, we created core microbiomes defined as the OTUs that were present in at least 90% and 100% of all samples at both locations.

4.2.5 Scanning Electron Microscopy

Freeze dried samples that were used for DNA extractions from both wetlands were placed on carbon tape and gold sputtered with a Hummer IV Sputtering System. Images were taken with a JEOL JSM-700F field emission scanning electron microscope (FESEM). Pictures were adjusted for contrast.

4.3 Results

4.3.1 Colonization and Temporal Evolution of the Prado Biomat

Analysis of community composition at the Prado wetland over the study period of approximately three years (Dec 2013 through Oct 2016) revealed distinct taxa that underwent significant changes in relative abundance until maturation. Within a month of system startup, a green biofilm began to establish on the geotextile liner. Phylogenetic sequencing revealed that the 16S rRNA plastid gene for diatom chlorophyll (stramenopiles) was present within these systems at this early time stage and remained throughout the study period (Figure 4.1). While diatoms initially accounted for 18% of sequences, their relative abundance fluctuated throughout operation with a range of 0.3 %
to 37.6 % (observed in the late summer of 2016). However, it should be noted that this interpretation of diatom abundance links to amplification of 16S plastid sequences harbored within their chlorophyll; hence quantitative interpretation is biased when contrasting to bacterial and archaeal community members and should rather be interpreted as comparative trends and evidence of diatom presence.

Figure 4.1 Diatom relative abundance as tracked by 16S plastid sequence from start of operation until the last sampling point at Prado. Each point is an average of all nine samples from each month and error bars represent 1 standard deviation. The February 2015 sampling event occurred after the hydrologic disturbance in January of 2015. The average relative abundance of the diatoms at Discovery Bay during the month of May is indicated by the dashed line with the shaded region representing + or – one standard deviation.

To better understand system evolution and establishment, the top 20 most abundant bacterial and archaeal families found within the Prado biomat were tracked with time by normalizing the inlet biomat samples derived across the three cells (Figure 4.2). Trends revealed a relative stability for many families within the system including Rhodobacteraceae, Verrucomicrobiaceae, and Flavobacteriaceae. Other lineages
displayed a distinct evolution in relative abundance that took approximately 9 months to maximize and coincided with the September to October 2014 sampling window. Specifically, families containing the strict anaerobes Anaerolinaceae, Methanobacteriaceae, and Methanosarcinales had initial relative abundances of less than 1% but began to increase in abundance as the biomat matured. This trend coincided with increased accumulation of the biomat, which had an average depth of 9.5 cm in the inlet region after 2 years of operation. After this establishment phase and after the first summer growing season, the top 20 most abundant families show little variation for the remaining 25 months of sample collection and analysis.

4.3.2 Prado Community Spatial Association and Effects of Hydraulic Residence

A high degree of similarity was present within the microbial biomats when comparing communities between the three mature Prado wetland cells in the summer of 2016. The last 4 sampling time points enabled us to test if there was a significant difference in communities between the three parallel wetland cells and as a function of horizontal location within a single cell as those dates represent the most established microbial community (Figure B.3). In comparing these different sampling localities within the horizontal flow plane, our analysis revealed no significant difference in community identity between the wetland cells ($p = 0.949, R = -0.008, \text{ANOSIM}$). In order to better understand the relative contribution of temporal community trends to these spatial patterns, a detrended correspondence analysis (Figure 4.3) and principle coordinate analysis (Figure B.4) were performed using a weighted unifrac distance
Figure 4.2 Temporal evolution of microbial community at Prado demonstrates emergence of certain clades during first year of startup. This heat plot depicts the average relative abundance of inlet samples as a function of time (in months) for the 20 most abundant bacterial and archaeal families. The dotted line represents an approximation of community stabilization.

Due to the severe horseshoe effect in the principle coordinate analysis, typically caused by temporal data (Podani and Miklós, 2002), the detrended correspondence analysis was chosen as the better representation of the unifrac distance matrix. This analysis confirmed that despite these spatial trends, time is far more important driver of sample clustering (Figure 4.3; p = 0.001, R = 0.720, ANOSIM).
Figure 4.3 A detrended correspondence analysis reveals that the Prado microbial community becomes similar to mature (spring 2014) Discovery Bay microbial community over time. The dotted line corresponds to approximately 9 months (Aug 2014) after system establishment. Two outlier samples with very high and very low diversity were discarded from analysis.

The Prado wetlands demonstrated some local community variation within cells that was associated with changes in HRT. During the first year of operation at Prado, when the cells all contained the same HRT, community composition did not vary as a function of location between the three parallel cells (ANOSIM: Inlet \( p = 0.914 \), Middle \( p = 0.978 \), Outlet \( p = 0.667 \)). However, when analyzing the mature biomat samples during the late summer of 2016, two years after the HRT across the cells was changed to create a range from 1-4 days, a difference in ecological signatures was found as a function of location (i.e inlet, middle and outlet) within the cells (Figure B.3; \( p = 0.001 \), \( R = 0.155 \), ANOSIM). More specifically, when single locations were discretely compared between the three cells with varying HRTs, neither the inlet or the middle samples varied across cells (Figure B.3; Inlet: \( p = 0.569 \), \( R = -0.034 \), Middle: \( p = 0.221 \), \( R = 0.101 \), ANOSIM);
however, the outlet communities significantly differed across cells as a function of HRT (Figure B.3; p = 0.002, R = 0.405, ANOSIM). By contrasting differential abundance across the cells (Figure B.4), taxa exhibited a higher variance between inlet and outlet locations as the HRT became longer (i.e. the cell with a 1 day HRT was most similar throughout). The families Rhodocyclacea, [Chthoniabacteraceae], Crenotrichacea, were found to be more abundant in the outlets than in the inlet for all 3 different HRTs while Oscillochloridacea, Chlamydomonadacea, Rhodospirillaceae, and Desulfobacteriacea were found to be more abundant in the inlet regions with the cells having 2 and 4 day retention times (Figure B.4).

4.3.3 Comparison of Biomat Between Pilot and Demonstration-Scale Systems

In order to better understand how system design influences microbial colonization and by extension the reproducibility of this managed natural treatment system, biomat samples derived from the pilot-scale open water cell wetland in Discovery Bay collected in May of 2014 were compared to the time series samples collected from the Prado wetland with a detrended correspondence analysis (Figure 4.3) and principle coordinate analysis (Figure B.5) using a weighted UniFrac distance matrix. Consistent with the rapid evolution of the Prado community and its comparative stability within the first year of operation, this biomat evolved into one that was highly similar to that of Discovery Bay. This pilot system operated for ~5 years prior to sampling, is approximately 1/20th the size of and individual Prado cell, and is located 350 miles to the north of the demonstration-scale Prado construct. The Discovery Bay system has previously been studied with respect to seasonal nitrate removal rates and mechanisms (Jasper et al. 2014b) as well as trace organic attenuation (Jasper et al. 2014a).
Functionally, nitrate removal rates between the two systems were highly similar (Table 4.1) highlighting that construction of these two systems resulted in analogous functional nitrate removal (Bear et al. In Preparation) despite differences in latitudinal separation, scales of operation, and in receiving waters.

Specifically, the last 4 biomat sample collections from Prado during the late summer and fall of 2016, significantly clustered with the Discovery Bay samples harvested from a mature biomat in the late spring of 2014 when contrasted with the earlier biomat samples derived from Prado (p = 0.001, R = 0.186, ANOSIM). Taking this into consideration, a core microbiome analysis was performed between these different biomat samples (Figure 4.4). At 100% ubiquity, 36 different OTUs accounting for 38.2% of the total sequences were shared between Prado and Discovery Bay, while at 90% ubiquity 102 OTUs were identified accounting for 47.8% of the total sequences. The most abundant taxonomies in core microbiomes across the sites were diatoms, while Sinobacteraceae, Luteolibacter and various Anerolinaceae were also prominent members. Overall, this core community is more enriched in the mature Prado wetlands as it comprises 42% of the total relative abundance compared to 35% at Discovery Bay. However, the different core community members appear to have relative abundances in analogous ratios generating a microbial “fingerprint.”

The diatoms were also highly similar with respect to both identity and abundance between the two systems. The relative abundance of diatomaceous chloroplast sequences in Discovery Bay in the spring of 2014 was 18.0 ± 4.8 %. While there was considerable temporal variation across the broader Prado sampling time period (Figure 4.1), overall
Figure 4.4 Core microbiome in mature, well-functioning Prado and Discovery Bay wetlands depicted by average relative abundance of OTUs shared across all samples at both Discovery Bay and Prado. The OTU identity is reported at the highest taxonomic classification obtained. Segmented bars represent more than one OTU in that taxonomic classification. A * indicates that two OTUs in the Stramenophiles order exceeded 3% relative abundance (17.4% and 28.5% at Discovery Bay and Prado respectively) and were truncated for visual purposes.

abundance was similar at 15.1 ± 8.4 %. Visual analysis of the biomat samples using FESEM confirmed the ubiquity of diatoms at both wetlands. Importantly, these diatoms appeared to be the same species, *Stauira* *Construens* var. venter, as based on morphological properties relating to frustule and valve structure (Figure 4.5). These diatoms were previously reported to dominate the Discovery Bay system (Jasper et al. 2014a).
Figure 4.5 Diatoms that colonized the different wetland cells are the same as evidenced by scanning electron microscope images from A) Discovery Bay and B) Prado. The morphology and frustule structure is consistent with the taxonomic assignment of *Staurosira construens* var. venter (Jasper et al. 2014a)

4.4 Discussion

Prior research in the Discovery Bay pilot system revealed a wetland unit construct that holds promise for increased attenuation of trace organics, pathogens and nitrate when contrasted with more traditional vegetated engineered wetland systems (Jasper et al. 2014b, 2014a; Nguyen et al. 2015). However, in moving toward broader adoption, it is critical to understand whether this design can be replicated to enable similar microbial ecologic selection and by extension, targeted functionality at a variety of scales, locations, and receiving waters. To this end, the demonstration-scale Prado wetland was constructed with three parallel cells that were orders of magnitude (400 m$^2$ vs 7500 m$^2$) larger than that of the Discovery Bay pilot-scale system. After 1 year of operation, the hydraulic residence within the Prado cells was manipulated to contain three different HRTs of approximately 1, 2 and 4 days for the remaining 2 years of monitoring. The HRT of the single pilot-scale system in Discovery Bay varied from 1-3 days over its 5
years of operation. The Prado system is also located ~350 miles south of the pilot-scale shallow open water cell and received water from the Santa Ana River rather than directly from denitrified treated wastewater effluent resulting in modestly different water properties that were most pronounced for nitrate.

Complementary work into functional treatment attributes has revealed that the pilot and demonstration scale systems exhibit analogous nitrate removal rates despite different influent concentrations (Table 4.1), while utilizing a combination of photolytic and biological pathways to attenuate TOrCs and pathogens (Bear et al. In Preparation). Both wetland constructs, and just as importantly all three parallel cells within the Prado system, rapidly established a benthic biomat containing a photosynthetic microbial surface community underlain by a denitrifying anaerobic layer (Jones et al. 2017b). By following the Prado wetland constructs from their initial establishment through almost 3 years of operation, our ecologically-focused study has the unique capability to explore and understand natural ecological colonization and succession as well as investigate if a similarly designed, larger-scale and geographically distinct system that receives different source waters would host a similar community. As these treatment systems were naturally colonized without any active promotion of inoculation, the work also contributes to our broader understanding of biogeographical principles (Hedlund and Staley 2004) and their relevance to the adoption, design and utility of natural treatment systems.

Our analyses reveal that the Prado open water wetland cell, in which operation and monitoring began in December 2013, was rapidly self-colonized by a photosynthetic, benthic biomat containing a mixture of diatoms, bacteria and sedimentary detritus after
approximately one month of operation. During the initial colonization of the Prado system, the most abundant families of bacteria were characterized as aerobic and included Comamonadaceae (Willems, De Ley, and Kersters 1991), Cyclobacteriaceae, Flavobacteriaceae (Bernardet et al. 2002), Chitinophagaceae (Kampfer, Lodders, and Falsen 2011), Acetobacteraceae (Kersters et al. 2006), and Rhodobacteraceae (Pujalte et al. 2014) (Figure 4.2). After approximately 9 months, members belonging to strictly anaerobic guilds including fermentative microorganisms including Anerolinaceae (T. Yamada 2006) and Caldilineaceae (Sekiguchi 2003) as well as the methanogens Methanosarcinaeae and Methanobacteriaceae (Krieg et al. 2010) increased in presence. This can be explained by vertical stratification associated with biomat growth where a sufficient depth needs to be reached to develop this anaerobic layer within the biomat. In support of this vertical stratification theory, analysis of community shifts as a function of depth within biomat located at the Discovery Bay wetland revealed a pronounced difference between the photosynthesizing surface community and reducing community below capable of supporting denitrification, anammox, sulfate reduction and methanogenesis (Jones et al. 2017b). This is consistent with minimal nitrate removal during this wetland construct’s establishment (Bear et al. In Preparation) as a vertically stratified biomat that contains suboxic zones amenable to denitrification and anammox had not yet established. After this initial colonization period, a predictive model based on seasonality and removal rates for the pilot-scale system closely matched actual nitrate removal rates in demonstration-scale Prado constructs achieving approximately 90% nitrate removal with a 4 day HRT in the summer months (Bear et al. In Preparation).
During the first year of operation of the Prado demonstration-scale system, flow rate and other operating conditions were maintained at similar levels for all three cells. Microbial colonization across cells during this time was similar with minimal differences as a function of location within a cell and across the three different cells (Figure B.3). In the second year of operation, cells operations diverged with respect to (HRT) to better explore contaminant attenuation rates and the potential role of HRT on microbial succession. After changing the HRT to operate at 1, 2 and 4 days between the three different parallel systems, outlet samples diverged as a function of HRT (Figure B.3). In contrast, the inlet community remained similar across all cells. A possible explanation is that differing nutrient gradients or some analogous selection by resource limitation is driving this shift. During the summer months 90% of the nitrate is removed in the cell with a 4 day HRT versus only 65% removal in the cell with a 1 day HRT (Bear et al. In Preparation) suggesting that in addition to optimizing HRT for removal goals, its effect on microbial selection should also be considered.

An even more dramatic perturbation to HRT occurred during the reconstruction of the equalization forebay in January of 2015 where water remained in the three cells for approximately 1 month prior to reestablishing flow. In contrasting prior and subsequent sampling events after this disruption, there was only a slight microbial community shift; however, diatom presence did plummet during that window (Figure 4.1). The first summer drop could be caused by several variables including competition with duckweed and planktonic algae blooms observed in early to mid-summer, bacterial blooms, or predation. The final sampling points in the late summer and fall of 2016 revealed an average diatom relative abundance greater than that at Discovery Bay, suggesting a
complete rebound of the community after the disturbance. Treatment capabilities also remained similar after construction had finished (Bear et al. In Preparation). The stability of this community and its ability to rebound quickly after a hydraulic perturbation is indicative of a robust community and highlights the potential to manage these wetlands to handle intermittent flow events that might result from seasonal disruptions such as storm events. This suggests a potential application toward other types of impaired waters such as storm water runoff, which would be more variable than comparably consistent flows received from wastewater effluent or wastewater effluent-impaired rivers such as the Santa Ana.

In addition to HRT, seasonality played a modest role in the shifts observed during the first few years of operation. Alpha diversity measurements revealed an increase in Shannon diversity and observed richness after 4 months of operation, which coincided with the onset of warmer spring temperatures in April. Both indices appear to drop slightly in the following cooler months; however, a gap in the summer months the following year combined with the wetland community evolving over the first year make it difficult to draw firm conclusions based on season. Other wetland microbial studies have found little seasonal effect on microbial community compositions despite large differences in metabolic rates (Clément, Pinay, and Marmonier 2002; Juottonen et al. 2008) supporting our finding of relative community stability after the biomats first growing season.

In order to understand if system design could select for a similar microbial community in different settings, biomat samples from the pilot scale Discovery Bay wetland after 5 years of operation and the time series of samples collected from Prado
were compared. Correspondence analysis (Figure 4.3) revealed that after the 9-month stabilization period the samples from Prado begin converging with the more mature pilot scale system. This also coincided with the emergence of anaerobic members of the biomat community (Figure 4.2). Though differences were present, established Prado biomat samples from 2014-2016 largely clustered with one another as well as those derived from the Discovery Bay pilot-scale system. To explore this similarity further, a core microbiome was established across these two systems (Figure 4.4). Major guilds identified included strict to obligate aerobic and anaerobic community members as previously documented (Jones et al. 2017b). Interestingly, core microbiomes established for waste water treatment plant reactors running in parallel were found to contain 50-60% of the sequences analyzed (Griffin and Wells 2017; Ibarbalz et al. 2014) which is slightly higher than the percentage of sequences in the core microbiome of the two systems presented here. The open water wetland systems appear able to self-colonize and form communities as similar to each other as ones found in wastewater treatment reactors running in parallel despite being geographically separate.

Diatoms contributed to a large amount of this similarity across systems as they were present within the first month of operation and accounted for the greatest abundance of sequences in both wetland systems. Diatom species *Staurosira construens* var. *venter* was present across these systems and this particular diatom was previously identified as the dominant primary producer at the Discovery Bay wetland (Jasper et al. 2014a). In studies of benthic lake diatoms, pH, trophic status, and water depth have been identified as the three largest driving factors in diatom community composition (Soininen 2007). These factors are systemically embedded in the shallow open water unit process wetland
design. The inlet pH at the Discovery Bay wetland was higher than at Prado, however both are basic and oscillate diurnally (ca. pH 7.5-9.5) in association with photosynthetic oxygen production. While the influent nitrate is about three times higher at Discovery Bay than at Prado (Table 4.1), both wetlands would be classified as eutrophic environments (Richardson and Jørgensen 1996). Water column depth (20 cm vs 25 cm) is similar in both systems and is likely an important factor in selecting for diatom species. Diatoms tend to grow benthically until the water depth reaches 60 cm at which the community favors planktonic growth (Round, Crawford, and Mann 1990). The separation of the biomat from the bottom soil by using some form of liner also seems important for biomat establishment. Anecdotally, this can be observed in areas of the Prado wetland that lack a geotextile liner, yet the water depth and nutrient concentrations are similar to those of the cells where no diatomaceous biomat formation is seen. However, the type of liner material seems less important than just the presence of some sort of ground barrier as the Discovery Bay pilot system was half lined with a geotextile liner and half cement with both regions maintaining similar biomat communities.

Diatoms have co-evolved with bacteria for over 200 million years resulting in close symbiotic relationships. Bacterial families such as Luteolibacter, Rhodobacteraceae, Sinobacteriaceae, and uncultivated C111 are all represented in the core microbiome and all are known to have members that are algal symbionts (Ohshiro et al. 2012; Park et al. 2013; J. Li et al. 2015; Pujalte et al. 2014; Gutierrez et al. 2013). It is likely these aerobic organisms are directly benefiting from the diatom community through their production of extracellular polymeric substance and other metabolite production (Amin, Parker, and Armbrust 2012). The primary production of the diatoms
also indirectly supports the anaerobic community by creating a carbon rich environment through extracellular polymeric substance (Hoagland et al. 1993). This labile carbon source could also be one of the factors promoting higher denitrification rates than vegetated wetlands (Jasper et al. 2014b).

4.5 Conclusions

The shallow open water system design selects for a highly analogous microbial community despite differences in geographic location, size and influent waters when deployed in the Mediterranean California climate. The photosynthetic biomat that forms within this construct develops a vertically stratified and highly analogous community consisting of bacteria and the same species of diatoms in less than one year. This community is presumably selected by the design constraints of water depth (20 to 25 cm) and establishment of a barrier between the ground and water column on which the benthic biomat can colonize. After initial colonization and establishment, which coincides with warmer and higher sunlight intensity summer months and approximately 9 months of operation, community shifts are more modest and start to converge with the community found within a geographically separate open water cell wetland biomat. By looking more holistically at both performance and microbial community, it can be inferred that the photosynthetic communities selected for by this design provide analogous treatment benefits.
Collectively, the work presented here explores the microbial ecology and processes that occur in a novel engineered wetland construct. The objective of our work was to obtain a better understanding of the microbial mechanisms that enhance the performance of this system. The widespread presence of trace organics, nutrients, and pathogens in combination with climate change lowers the availability of clean drinking water, particularly in arid and semi-arid regions, in a time of growing demand (Patz et al. 2000). The adoption of low energy, high volume systems such as the shallow, open water unit process wetland may contribute to solutions to this challenge with sustainable advantages over more actively engineered and managed approaches. By studying this system at the pilot and lab scales and microbial colonization and succession at the demonstration scale, this research demonstrates that this engineered wetland construct reproducibly colonizes a photosynthetic benthic biomat capable of nitrate and trace organic contaminant removal with potential advantages over traditional vegetated wetlands with respect to performance.

In addition to this body of research an undergraduate microbiology lab module was developed for students majoring in environmental engineering (Appendix C). This module focused on understanding how to utilize culture dependent and independent methods to better understand biological systems for contaminant treatment. Students isolated bacterial cultures from the environment and characterized them under a microscope. DNA was also extracted from a mock remediation site in which students amplified the 16S rRNA biomarker gene and were taught how to do database comparisons to identify microorganisms of interest. This module teaches engineers how
microbial data can be utilized to understand and solve bioremediation problems in a manner similar to the work presented here.

While not directly explored here, a further benefit of the open water wetland design in regards to nitrate removal is that it is able to generate its own carbon source for reductive processes such as denitrification. In some cases, influent wastewater does not contain sufficient organic carbon to achieve complete denitrification, necessitating the addition of an external carbon source and associated costs. The organic rich biomat naturally created in unit process wetlands is presumably an underlying factor that contributes to a nitrate removal rate that exceeds 75% of rates reported for vegetated wetlands (Bear et al. In Preparation; Jasper et al. 2014b). Additionally, the high activity of the biomat combined with diurnal cycling and redox stratification contribute to a diverse community capable of breaking down a variety of pharmaceuticals and other recalcitrant anthropogenic compounds.

The success of the pilot scale system in Discovery Bay lead to the construction of a demonstration-scale open water wetland system at the Prado wetlands which treats Santa Ana River water. The microbial community and performance of this system was monitored for 2.5 years resulting in an unprecedented wetlands data set documenting the evolution of the system from start up to maturation. A time series analysis of the data reveals that the initial colonization is dominated by putative aerobic microorganisms with the formation of stratification and emergence of anaerobic microorganisms after a summer’s worth of growth. By comparing ecological fingerprints, it can be seen that the community harbored within the demonstration-scale, Prado system converges with that residence within the pilot scale-system with the selection of the same species of diatom
and highly similar bacterial communities in both locations. Based on this comparison, the
design of the open water cell with its shallow depth selects for a similar microbial
community in these two Mediterranean climatic locations despite geographical separation
and the absence of any form of active inoculation despite different types of influent
waters. In turn, this leads to analogous contaminant removal performance as the pilot
scale system. As a major barrier in a natural system adoption is design guidelines and
system reliability; this study provides water entities, practitioners and researchers with
the understanding necessary to better inform their decision process.

The contribution of anammox to inorganic nitrogen removal in the open water
system was unexpected as anammox processes are typically thought to be limited to low
organic carbon environments, and the influent water within the shallow open water
systems lacked the requisite anammox precursors of ammonium and nitrite. This
dissertation demonstrates that sulfide induced dissimilatory nitrate reduction to
ammonium supports anammox in the pilot scale open water cell. This has broad
ecological implications, as there is a limited understanding of the mechanisms that
support anammox in natural freshwater systems. Anammox has mainly been thought to
exist in boundary suboxic redox zones where ammonium is fixed and nitrate is reduced to
nitrite (Zhu et al. 2013). However, the results in this dissertation demonstrate a selection
for anammox microorganisms in sulfidic reducing regions similar to the oxygen
minimum zones of the oceans where anammox processes are now believed to dominate
nitrogen biogeochemical cycling (Canfield et al. 2010; Hamersley et al. 2007). The
presence of anammox and the potential for anaerobic methane oxidation in this system
could have implications for greenhouse gas emissions with potential promise for strategies that could lead to reductions in natural and engineered wetland systems.

To this end, we have begun inquiry into the generation of greenhouse gases from the shallow, open water unit process wetland cells located at the demonstration-scale Prado site. Wetlands are the largest natural source of methane and contribute 10% of the overall methane flux to the atmosphere (Bartlett and Harriss 1993). Global carbon fixation from wetlands is estimated to be 0.2 to 1.4 t C ha\(^{-1}\) yr\(^{-1}\) (Mitra, Wassmann, and Vlek 2005), however due to emission of CO\(_2\) and methane in the mineralization process, wetlands in general are considered a relatively neutral net source or sink of greenhouse gases (GHGs) (Mitra, Wassmann, and Vlek 2005; Whiting and Chanton 2001). If engineered wetlands can be designed as a greenhouse gas sink, they would provide additional environmental benefit in conjunction to water treatment. To investigate the photosynthetic carbon fixation potential as well as gas release from the biomat, we ran initial laboratory microcosm studies coupled to a two-day field monitoring experiment (methods are described in Appendix C). We hypothesized that different biomat thicknesses will maintain similar photosynthetic surface area but thicker mats will have more anaerobic biomass and therefore produce more methane and CO\(_2\) with an optimal depth that countered formation with scavenging by trophic species. An important consideration was the diurnal flux of CO\(_2\), CH\(_4\) and N\(_2\)O associated with the broad swings in pH and redox that are associated with this photosynthetically-driven system. Preliminary data from the laboratory microcosm studies revealed promise for its ability to query our hypothesis related to how biomat depth can affect the flux of CO\(_2\) and methane (Figure 5.1).
Figure 5.1 CO₂, CH₄, and N₂O, emissions from 1L microcosms as a function of biomat depth (6 cm, 3 cm, and 1 cm) and light over 48 hours. Gray areas indicate when the light was turned off. Negative values indicate a gaseous sink.
Broadly, thicker mats appeared to consume more CO$_2$ but in turn produced more N$_2$O and CH$_4$. However, interpretation of these initial microcosm results was complicated by a phenomenon where the thickest biomat permutation (6 cm) had part of its photosynthesizing surface layer float due to gas generation, causing additional surface area for CH$_4$ and N$_2$O oxidation. While the thinnest layer of biomat had the least robust CO$_2$ fixation, that could be due to a deeper water column and a slightly longer distance from the light source. A minimal amount of N$_2$O was produced consistently at the start and middle of the experiment when nitrate was amended to the microcosms. Depth appears to be a less prominent driver of N$_2$O emission relative to nitrate concentration. Future laboratory studies should address a wider range of biomat depths from 10 cm to 2 cm to better reflect conditions in the wetland, address the potential for an optimal depth for system performance, and to demonstrate more prominent variations in gas flux with depth.

To address limitations from laboratory microcosms and gain more relevance to actual system processes, field greenhouse gas emission fluxes were measured as part of a preliminary field campaign at the Prado demonstration open water wetland cells and the surrounding traditional wetlands in late spring of 2017 (Figure 5.2). The approximate biomat thickness across the flow reach ranged from 12 cm near the inlet to 5 near the middle and 4 near the outlet of this 250 meter reach. CO$_2$ flux demonstrated oscillations between consumption and production though trends were not fully consistent with diurnal cycling. In this initial sampling, the middle reach of the cell is most consistent with our hypothesis and mimics the diurnal effect seen in the microcosms. The biomat is thinnest near the outlet, which supports to observation of less biological contributions to overall
CO₂ flux; however, the negative CO₂ flux (consumption) at night is perplexing. A positive CH₄ flux was seen throughout, with the outlet most representative of what we would hypothesize as it gradually increased during the dark hours as oxygen concentrations dropped. Nitrous oxide fluxes were minimal and suggested a potential correlation with nitrate concentration as decreased from inlet to outlet. This data is highly preliminary and contains some irregularities, notably spikes in CO₂ and CH₄ flux in the inlet region. This can be explained as an artifact of our field sampling methodology; specifically, biomat disturbance in association with sampling as the biomat was thickest in this region and left little room for the submerged portion of the chamber bottom. Another confounding factor was the presence of a planktonic algal bloom, previously characterized as Chlamydomonas, which was concentrated near the inlet in association with the prevalent direction of the Santa Ana winds in the afternoons and re-dispersed at night when these winds died down. Future field efforts to investigate flux should focus on limiting subsurface disturbances of the biomat, potentially by redesigning flux chambers to have less submerged displacement and revisiting chamber deployment techniques. To compare gas fluxes of the shallow open water cells to those in surrounding, more traditionally constructed vegetated wetlands also present at Prado, measurements were taken from the forebay of the wetland (a deeper equalization basin of ~ 30 cm depth with a sandy bottom and no emergent vegetation) and a parallel bypass channel with some vegetation (Figure 5.3). The most notable difference between the shallow, open water cells and these other constructs is in the flux of CO₂ where the overall negative flux of the open water systems stand in contrast to the emission of CO₂ by the surrounding locations. Conversely, methane emissions are higher in the open water cell, likely due to
Figure 5.2 Field gas emission measurements taken over a 24 hour period. Gray areas indicate the time between sunrise and sunset.

higher organic carbon content within the biomat. While not yet measured, it will be interesting to investigate whether the open water cell will have similar methane production values to adjacent vegetated cells. Nitrous oxide emissions are difficult to compare as the bypass channel is not providing much treatment and actually accumulating nitrate as the water flows through it due to water fowl, highlighting yet another field variable that should be considered. However, nearly all locations had emissions less than 1.5 uM/m²/hr which is insignificant compared to the other ghg emissions. Overall, this initial field comparison of gaseous emission rates between
engineered wetlands shows a net carbon sink in the biomat-containing engineered wetland as compared to the more traditional wetlands. This preliminary investigation into the greenhouse gas flux generated from unit process wetlands suggests that they could be a carbon sink and that biomat depth management may increase this environmental benefit.

Figure 5.3 Comparison of gas emissions of the open water cell to surrounding wetland areas including the forebay and parallel bypass channel during sunlit morning hours. Cell 1 is the open water unit process wetland cell with established biomat.
In addition the continuation of investigations into greenhouse gas flux, future studies have begun to explore this system’s potential to treat other types of impaired waters and will continue after my graduation by other researchers in the laboratory group and larger ReNUWIt collaborative. Initial inquiries are focusing on this biomat’s ability to treat nitrogen (both ammonia and nitrate) in the saline waters of brine concentrate found in reverse osmosis reject waters and from produced water sourced from hydraulic fracturing operations. Oxygen generation and assimilative growth associated with the biomat suggests promise for ammonium oxidation. This further broadens the scope of shallow open water treatment wetlands beyond produced water into agricultural runoff treatment and toward applications for nutrient attenuation and reaeration of anaerobic digester effluent. The ability of this novel wetland construct to treat large quantities of water and generate oxygen and biomass with limited operational costs and oversight makes it a promising system for agricultural water treatment applications and for deployment in developing countries, and there could be further sustainability gains in the harvest of this biomat for fertilizer or other applications to offset net operational costs.
REFERENCES


Zhu, Guibing, Shanyun Wang, Xiaojuan Feng, Gaina Fan, Mike S. M. Jetten, and Chengqing Yin. 2011. “Anammox Bacterial Abundance, Biodiversity and

### APPENDIX A

**SUPPLEMENTAL INFORMATION TO CHAPTER 3**

Table A.1 Putative functional assignments for dominant microbial clades in the biomat

<table>
<thead>
<tr>
<th>Clade</th>
<th>Category</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhodobacteraceae (fam.)</strong></td>
<td>Phototroph</td>
<td>(Pujalte et al., 2014)</td>
</tr>
<tr>
<td><strong>Environbacteraceae (fam.)</strong></td>
<td>Phototroph</td>
<td>(Tonon et al., 2014)</td>
</tr>
<tr>
<td>ZB2 (class.)</td>
<td>aerobic methane oxidizer</td>
<td>(Peura et al., 2012)</td>
</tr>
<tr>
<td>Hydrogenophaga (gen.)</td>
<td>hydrogen oxidizer</td>
<td>(Willems et al., 1989)</td>
</tr>
<tr>
<td>Rhodobacter (gen.)</td>
<td>phototroph, hydrogen oxidizer, sulfide oxidizer</td>
<td>(Pujalte et al., 2014)</td>
</tr>
<tr>
<td>Gemmatimonas (gen.)</td>
<td>Phototroph</td>
<td>(Takaichi et al., 2010; Zeng et al., 2015)</td>
</tr>
<tr>
<td>Piscirickettsiaceae (fam.)</td>
<td>sulfide oxidizer, hydrogen oxidizer</td>
<td>(Krieger et al., 2010; Takai, 2006)</td>
</tr>
<tr>
<td>GCA004 (ord.)</td>
<td>Fermenter</td>
<td>(Yamada and Sekiguchi, 2009)</td>
</tr>
<tr>
<td>Bacteroidales (ord.)</td>
<td>Fermenter</td>
<td>(Ontiveros-Valencia et al., 2013)</td>
</tr>
<tr>
<td>Syntrophobacter (gen.)</td>
<td>sulfate reducer</td>
<td>(Chen, 2005; Wallrabenstein et al., 1995)</td>
</tr>
<tr>
<td>Candidatus Brocadiia (gen.)</td>
<td>Anammox</td>
<td>(Strous, 2000)</td>
</tr>
<tr>
<td>SHA-31 (fam.)</td>
<td>Fermenter</td>
<td>(Yamada, 2006)</td>
</tr>
<tr>
<td>envOPOS12 (ord.)</td>
<td>Fermenter</td>
<td>(Yamada, 2006)</td>
</tr>
<tr>
<td>Methanosaeta (gen.)</td>
<td>Methanogen</td>
<td>(Smith and Ingram-Smith, 2007)</td>
</tr>
<tr>
<td>Treponema (gen.)</td>
<td>Fermenter</td>
<td>(Krieger et al., 2010)</td>
</tr>
<tr>
<td>CFB-26 (ord.)</td>
<td>Fermenter</td>
<td>(Yamada and Sekiguchi, 2009)</td>
</tr>
<tr>
<td>WSA2 (fam.)</td>
<td>Methanogen</td>
<td>(Gonzalez-Gil et al., 2014)</td>
</tr>
<tr>
<td>MC6 (class.)</td>
<td>Fermenter</td>
<td>(Kubo et al., 2012)</td>
</tr>
<tr>
<td>Brocadiaceae (fam.)</td>
<td>Anammox</td>
<td>(Strous, 2000)</td>
</tr>
<tr>
<td>Candidatus Methanocorona (gen.)</td>
<td>Methanogen</td>
<td>(Brauer et al., 2011)</td>
</tr>
<tr>
<td>Deltaproteobacteria (class.)</td>
<td>sulfate reducer</td>
<td>(Krieger et al., 2010)</td>
</tr>
<tr>
<td>DHVEG-1 (fam.)</td>
<td>Methanogen</td>
<td>(Iino et al., 2013)</td>
</tr>
</tbody>
</table>
Figure A.1 Rendition of the open-water cell in Discovery Bay, California with approximate sampling locations as they relate to their distance from the inlet flow path. The cell is approximately 20 m by 20 m and contains baffling to divide up the cell into 4 runs in series. Samples were taken from the middle reach of each run except for run 1.
Figure A.2 Rarefaction curves Chao1 (A and C) and Faith’s phylogenetic diversity (PD whole tree) (B and D). Curves show that the diversity of the community was well captured. Both chao1 and Faith’s phylogenetic diversity agree that samples taken closer to the inlet are more diverse while Faith’s phylogenetic diversity suggests that the top is less diverse than the bottom and middle. Error bars represent ±1 standard deviation.
Figure A.3 Rank abundance of the 10 most abundant classes from the 2 m sampling location of (A) top and (B) bottom samples. Errors bars represent ±1 standard deviation of triplicate samples.
Figure A.4 Triplicate microcosms demonstrated that the addition of sulfide alone will not induce ammonium production. Ammonium on average increased by only 50 µM over the course of 6 hours. The increase of ~300 µM of sulfide was likely caused by sulfide compounds in the biomat that were oxidized during biomat preparation and reduced again in the absence of nitrate. Error bars represent ±1 standard deviation of triplicate sample.
Figure A.5 Differences in gene abundance and chemical profiles reveal spatial trends for nitrogen and sulfur biochemistry. (A) anammox bio-marker gene (hzsA) with ammonium concentration and (B) dissimilatory sulfate reduction gene (dsrA) with sulfate concentration. Gene abundance is normalized to grams dry weight where error bars represent ± 1 standard deviation.
Figure B.1 Left: Layout of the 20 m x 20 m baffled open water cell at Discovery Bay, CA. Right: Overview of the Prado open water cells, each 30 m x 260 m, running in parallel.
Figure B.2 Alpha diversity measures of Prado and Discovery Bay (DB) samples. DB samples were taken on 5/5/14. A ‘2’ after the month indicates there were two sampling events in one month.
Figure B.3 When comparing the last 4 sampling events at Prado, samples loosely cluster by location within cell (ie. inlet/middle/outlet) and show correlation to HRT.
Figure B.4 A differential abundance analysis performed on samples as function of HRT collected in the Late summer of 2016, ~1.5 years after the HRT was varied for each cell.
Figure B.5 A principle coordinate analysis comparing the Prado and Discovery Bay samples. The horseshoe effect caused by a time series analysis is evident, however, the correlation between the mature Prado samples and the Discovery Bay Samples remains true.
Laboratory Microcosm Experiments:

2L microcosms were setup under a full spectrum grow light with varying amounts of biomat (1 cm, 3 cm, and 6 cm) in each bottle in order to see the effect of biomat depth on GHG flux. Wetland water was added to each microcosm so the total volume in each bottle was 1.3L. Microcosms were placed on a stir plate and hanging stir bars ensured water column agitation. Gas fluxes were monitored over at least 24 hours with lights cycled on and off every 12 hours. Bottles were sealed with a rubber stopper and measurements were taken every 3 hours with a Picarro G2508 cavity ring-down gas spectrometer through two cannulas which were inserted through the stopper. All fluxes were analyzed with the Picarro Soil Flux Processor.

Field Sampling:

Field sampling was also performed with the Picarro G2508 using the floating chamber method from April 24th to the 26th, 2017. The chambers were deployed approximately 5 m from shore and were connected via tygon tubing to the instrument. Fluxes measures were taken for 10 minutes at each sampling location over a period of 24 hours for the open water cell. Measurements from other locations were collected in the morning and early afternoon and only compared to open water cell measurements taken the same morning.
CEEN 303 Lab 5: Experimental Approach  
Environmental Micro And Molecular Biology  

**Time Required:** 6 laboratory sessions and 4 lectures  
**Dates:** T 4/11, Th 4/13, T 4/18, Th 4/20, T 4/25 (josh in DC), Th 4/27  
**Report Due Date:** Th 5/4

**Instructors and Assistants**

- **Instructors:** Prof Josh Sharp  
- **Assistants:** Zack Jones, Laura Leonard, Michael Vega

**Objectives**

- Familiarity with classification of microorganisms (taxonomy, membrane & functionality)  
- Familiarity with microbial culturing and enrichment techniques  
- Perform microscopy and staining techniques  
- Develop familiarity about diversity and abundance in microbial habitats  
- Understand how to extract DNA from environmental samples and quantify yields  
- Ability to amplify 16S rDNA using PCR and genetic primers  
- Perform analysis with 16S rRNA gene (phylogeny) using electronic databases  
- Develop extrapolation of phylogeny to functional potential relevant to env. engineering

**Module Outline**

- **Lab 5.1** – Collect, culture (30°C) and archive samples - (solids, liquids, plates, etc.)  
  - **Lecture M1** – Microbial Culturing (Josh)  
- **Lab 5.2** – Microscopy and Gram stain  
  - **Lecture M2** – Microscopy (Josh)  
- **Lab 5.3** – DNA extraction and PCR (very full day lab-wise and highly structured)  
- **Lab 5.4** – Capillary Electrophoresis  
  - **Lecture M3** – Molecular Microbiology (Josh or TA)  
- **Lab 5.5** – BLAST of unknown sequences and peer review inquiry  
  - **Lecture M4** – Sequence Analysis (TA)  
- **Lab 5.6** - Synthesis and interpretation  
  - **Discussion** - how to structure sections/subsections of report
Materials & Equipment

Culturing and Visualization

Day 1-Culturing techniques and sample preparation
- Gloves, lab coats, safety glasses
- Tape and pens for labeling
- Lab notebook
- Student cameras
- Sterile cotton swaps
- Liquid LB agar ready to be poured into culture plates
- Sterile plastic petri dishes
- **In class** - Collect microbes and/or source material
- 30°C stationary incubator

Day 2- Culture Visualization and Microscopy
- Gloves, lab coats, safety glasses
- Tape and pens for labeling
- Lab notebook
- Light microscopes
- Bunsen burner
- Slides
- Gram stain supplies, reagents, and setup
- Squirt water bottles
- Sterile toothpicks and/or plastic inoculation loops
- Cameras (students phones)
- 1000ul pipette and tips
- Small tube of DI water

Molecular Biology Techniques

Day 3- DNA extractions and PCR
- Gloves, lab coats, safety glasses
- Tape and pens for labeling
- Lab notebook
- DNA extraction kits (MoBio Powersoil)
- 15mL and 1.5 mL tubes to aliquot reagents
- Microcentrifuge tube racks
- 15mL tube racks
- PCR tube racks- (96 well plates)
- PCR mastermix
- PCR 16S rDNA primers (EUB338 and EUB 518)
- PCR tubes
- Microcentrifuge (borrow from GRL)
- Students’ laptop computers
- Pipets 1000ul, 200ul, 20ul
- Pipette tips 1000ul,200ul,20ul
- Vortexer
- Ice buckets
- Beadbeater
- 1.5 mL Centrifuge tubes

Day 4 - DNA quantification, determination and gel electrophoresis
- Gloves, lab coats, safety glasses
- Tape and pens for labeling
- Lab notebook
- Extracted DNA samples
- PCR samples
- Bioanalyzer (capillary electrophoresis), chips, and reagents
- Nanodrop (borrow from GRL)
- Qubit and kits for DNA
- Gel boxes (gel electrophoresis)
- TAE buffer
- Ethidium bromide
- Loading dye
- DNA ladder
- Transilluminator

Bioinformatics, Data Synthesis, and Paper Preparation

Day 5 – BLAST sequence analysis and scientific literature
- Tape and pens for labeling and lab notebooks
- Students’ laptop computers
- In class focus on literature and experimental synthesis; structure reports
- Downloaded peer-reviewed journal articles relevant to group project

Day 6 – Synthesis, discussion of laboratory reports, and makeup data collection
- Outline of laboratory report and specific questions for instructor
- Lab notebook
- Student computers
- Summary of all laboratory and in silico data
- Interpretation of peer-reviewed journal articles
Tasks

In this first day, you will inoculate LB agar plates from soil and water samples that you collect during the class period (interesting microbe harboring environments and/or soils). In subsequent labs, we will observe and stain the organisms that we cultured, extract DNA from the soils and colonies, amplify this DNA, analyze 16s rDNA products, and interpret those results and their implications.

Lab 5.1: Sample Collection and Microbiology Culturing Techniques

First we will have a demonstration and class members will pour the LB plates for culturing preparation. After an introductory lecture, we will then head out to collect samples and perform a cursory visual inspection of the site (with pictures – Clear Creek Athletic Facility at your own timeframe). You are encouraged to collect soil samples in addition to microbial havens. We will ground the remaining weeks in these samples. Our characterization will tell us more about the presence of microorganisms, their density, and genetic potential to bring about a desired env. remediation outcome.

- Aqueous sample of interest (i.e. MBR, Clear Creek, water fountain)
- LB plate inoculated from interesting location via swab (i.e. fingerprints, phones, faucet, etc.)
- Soil/solid phase (few grams) from the contaminated site on the Mines campus (or fridge)

Procedure:
1. Your TA will prep the LB agar in advance via a procedure using the autoclave at 121°C for 20 min followed by moving the bottled agar into a warm environment.
2. Students will then pour the warm culture media into petri dishes, on a clean bench with aseptic technique followed by allowing the plates to cool and become solid.
3. Once the plates cool, they can be used immediately or stored inverted in a closed container.
4. We will then have a lecture on culturing techniques.
5. Subsequently, we can collect soil samples from contaminated site (+ pictures of site / region). Soil samples can be collected and brought to lab in sterile conical tubes or swabs can be used to collect samples from dirty surfaces. Liquids can also be collected in conical tubes.
6. Use a sterile tool like a cotton swab to dip in an inoculum from sample, and then spread evenly across the petri dish (now hardened) containing solid culture media to perform streaking.
7. Sterile water can be added into a small amount of soil and then streaked onto a plate as the soil settles.
8. Place the petri dish in culturing incubators for 2 days (typically 30°C or 37°C)
9. Keep sufficient soil samples (~5g) for DNA extractions on the next day.

* The lecture on plates / liquid media will provide further details
Lab 5.2: General Microscopy and Observation

Note: This lab requires inoculating a plate during the first lab as well as collecting samples soil during that laboratory. We will now observe and stain the organisms that grew.

I. Light Microscopy Hands-on Tutorial

15 minute tutorial / familiarization with microscopes by TA’s

II. Gram Stain (adapted from http://water.me.vccs.edu/courses/env108/lab2.htm)

Standards – Gram (-) = E. coli; Gram (+) = R. jostii

1. To look at a colony from a plate, first find an isolated colony of the bacteria to be studied and write down a brief description in your lab notebook.
2. Prepare the slide by rinsing it with cleansing solution and blotting it dry with bibulous paper (not necessary if slide is clean).
3. Add a drop of water to the slide. Then use the inoculation loop to transfer a very small amount of bacterial colony into the water droplet. Still using the inoculation loop, spread the solution of bacteria and water out into a thin smear on the surface of the slide. The finished smear should be a circle about the size of a dime. Finally, allow the smear to dry thoroughly before proceeding to flaming the slide.
4. For a liquid sample, simply mix cell suspension well and pipette ~50ul or one drop onto a slide. Spread the drop on the slide using an inoculation loop to about the size of a dime and let air dry before proceeding to flaming the slide.
5. Using a Bunsen burner, lightly flame the bottom of the slide with the dried bacteria. Do this by gently “pulling” the slide through the flame a few times until the glass slide is heated.
6. Cover the slide completely with crystal violet dye (use only a couple drops at most). Let the dye stand on the slide for 20 seconds (or up to 60 seconds if the smear is thick.)
7. Tilt the slide and rinse the smear with water for approximately 5 seconds, being careful to remove only the dye and not the smear. You can either use a plastic water bottle or a slow stream of water from the faucet for this step. After washing, the smear should appear to be blue-violet in color.
8. Cover the smear completely with iodine. Let the iodine stand on the slide for 20 seconds (or up to 60 seconds if the smear is thick.)
9. Rinse the smear with water for approximately 5 seconds. The smear should still appear blue-violet.
10. Add drops of ethanol to the slide so that they run over the smear. This step should be performed quickly, but you must be careful not to add too much ethanol or it will leach the color from gram-positive cells and make them appear to be gram-negative. You should stop dropping ethanol onto the slide when the ethanol running off the smear first
becomes colorless, which should occur within about 20 seconds.

11. Rinse the smear with water for approximately 5 seconds. The smear should now be a paler violet color or clear (depending on whether the bacteria are gram positive or gram negative.)

12. Cover the smear completely with safranin. Allow the dye to stand on the slide for 60 seconds.

13. Rinse the smear with water for approximately 5 seconds. The smear may appear violet or pink.

14. Let the smear dry at room temperature, or by fanning it.

15. Observe the slide under the microscope and determine whether the bacteria are gram-positive (stained purple) or gram-negative (stained red.)

16. Attempt to identify your bacterial species. The flow chart below can be used to identify some species found in wastewater. Is your archived LB e-coli culture still pure compared to other samples?

**Bulking and Foaming**

---

**Fig. 9.4.** Simplified dichotomous key for identification of filamentous microorganisms. Adapted from Jenkins et al. (1984) and Eikelboom (1975).
Lab 5.3: Genomic DNA Extraction and Amplification (note: long lab with important sequential steps)

Use Environmental Samples (culture plates, or soil or MBR) using MoBio Powersoil DNA Isolation Kit

All groups should process the same types of samples

Our first step is to extract DNA from your samples. We will use the Powersoil DNA Isolation Kit (MoBio). Although this kit contains proprietary solutions, which do not list their contents, we have made some assumptions about each step based on previous experience and provide a brief explanation of the key steps (see *italics* throughout the procedure).

I. DNA Extraction Protocol:
Wear gloves at all times *(to minimize the chance of adding your or your normal microbiota’s DNA to the reaction)*

1. To the 2 ml Bead Solution tubes provided, add 0.25 g – 1.0 g of solid material or 250 μL – 1000 μL of liquid (e.g., seawater, sediments, animals, plants, etc.).
2. Add 60 μl of Solution C1 and invert once to mix. *(this solution contains a detergent and buffer to assist in cell lysis)*
3. Place tubes in beadbeater for ≥1 min. *(the longer you beadbeat, the greater the shearing of DNA (= smaller fragments)…however longer beadbeating will also break open cells recalcitrant to lysis – therefore a tradeoff exists)*
4. Centrifuge tubes at 13,000 x rpm for 1 min. *(this step removes sediments and cell debris while nucleic acids remain in solution)*
5. Transfer the supernatant to a sterile microcentrifuge tube. *(it is possible to retain any remaining volume of supernatant and continue purification/recovery if the sample is very valuable, limited, or yield is expected to be low)*
6. Add 250 μl of Solution C2, Vortex 5 seconds. Incubate on ice (~4ºC) for 5 min.
7. Centrifuge the tubes for 1 minute at 13,000 x rpm. *(this step is likely to precipitate proteins out of solution)*
8. Avoiding the pellet *(if there is one)*, transfer up to, but no more than, 600 μl of supernatant to a sterile microcentrifuge tube.
9. Add 200 μl of Solution C3 to the supernatant and vortex briefly. Incubate on ice for 5 min. *(This step helps precipitate humic substances and other PCR inhibitors)*
10. Centrifuge the tubes for 1 minute at 13,000 x rpm.
11. Avoiding the pellet, transfer up to, but no more than, 750 μL of supernatant to a sterile microcentrifuge tube.
12. Add 1200 μL of Solution C4 to the supernatant and vortex for 5 seconds. *(salt solution helps DNA bind to silica membrane)*
13. Load 675 μL onto a spin filter and centrifuge at 13,000 x rpm for 1 minute. Discard flow through. Repeat 3 times.
(binds DNA to silica membrane)

14. Add 500 µl of Solution C5 and centrifuge for 30 seconds at 13,000 x rpm.
(a washing step to remove anything not removed with the initial centrifugation through the membrane. It is possible to repeat this step if sample is particularly “dirty”)
15. Discard the flow through.
16. Centrifuge again for 1 minute.
(this helps remove any residual ethanol)
17. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution C5 onto the spin filter.
18. Add 30-100 µl of Nuclease free molecular grade water to the white filter membrane.
(30 µL will concentrate the sample for downstream PCR applications. Solution C6 can also be used to elute DNA, but you can’t spec DNA concentration accurately due to proprietary compounds)
19. Centrifuge 30 seconds at 13,000 x rpm.
(DNA is eluted from the membrane in this step)
20. Discard spin filter. DNA in the tube is now application ready. No further steps are required.

We recommend storing DNA frozen (-20°C). Solution C6 contains no EDTA (break point if needed).
(EDTA binds divalent cations such as Mg^{2+}; Taq polymerase is an Mg-dependent enzyme, therefore EDTA can inhibit PCR reactions if present)
21. Check DNA concentration on the Nanodrop 1000. Blank Nanodrop with clean water used to elute DNA. Read concentration of extracted DNA that is calculated by Nanodrop. DNA is read at 260 nm and protein is read at 280 nm. The ideal range for a 260/280 ratio is 1.80-1.90. Concentration of DNA should by in the range of 1-100 ng/µL

Now that we have extracted DNA from an environmental sample, we will amplify genes of interest via the polymerase chain reaction (PCR). Amplified DNA can be used for future Sanger sequencing and/or next generation sequencing (i.e. Roche 454 pyrosequencing, Illumina MiSeq).

II. Polymerase Chain Reaction (PCR) – Master Mix Performed by TA or professor for whole

- Because of the sensitivity of PCR, gloves should be worn at all times in order to avoid contaminating your sample.
- Always use a new pipet tip when going into any TUBE

1. **ON ICE** Thaw Promega PCR reagent tube, forward and reverse PCR primers, and template (if not thawed already). The Promega PCR reagent mixture includes Magnesium chloride (1.5 mM), DNTPs, Taq polymerase, and a proprietary buffer.
2. Be sure to mix the Promega PCR Mix and the primer stocks by pipetting up and down before attaining the required volume.
3. Make master mix for all samples according to the table below, leaving out DNA template
4. For a 25ul reaction add 23ul of master mix into a pcr tube. The add 2ul of template DNA. Gently mix the final volume before placing your PCR tube into the thermocycler.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (per rxn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>8.5 µL</td>
</tr>
<tr>
<td>Promega PCR Mix</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Forward primer (338F) (25uM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Reverse primer (518R) (25uM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>DNA template</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

The PCR thermal cycler profile should be set up as follows:

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temp °C</th>
<th>Time</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>2 min</td>
<td>denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>30 s</td>
<td>denaturation</td>
</tr>
<tr>
<td>30**</td>
<td>52*</td>
<td>60 s</td>
<td>annealing</td>
</tr>
<tr>
<td>30**</td>
<td>72</td>
<td>90 s</td>
<td>elongation</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>3 min</td>
<td>final extension</td>
</tr>
</tbody>
</table>

*note*: the optimum annealing temperature is dependent on the primer pair and must be empirically determined. Usually, the annealing temperature should be 5 degrees less than the melting point (tm) of the primer set.

**note**: Generally, fewer PCR cycles is better.

Lab 5.4 Gel electrophoresis using traditional gels versus the Agilent 2100 Bioanalyzer; DNA quantification using the Bioanalyzer, Nanodrop 1000, and Qubit

I. PCR samples: For accurate determination of DNA concentration, the total DNA in sample must be between 0.1–25 ng/μL for the bioanalyzer and 10-750 for the nanodrop. If the concentration of your particular PCR reaction is excessively high, dilute appropriate concentration in water.

A. Agilent 2100 Bioanalyzer (N/A this year – DNA size and quantification)

Preparing the Gel-Dye Mix (this will be done by your TA)
1. Allow DNA dye concentrate (blue) and DNA gel matrix (red) to equilibrate to room temperature for 30 min.
2. Vortex DNA dye concentrate (blue) and add 25 µL of the dye to a DNA gel matrix vial (red).
3. Vortex solution well and spin down. Transfer to spin filter.
4. Centrifuge at 2240 g ± 20 % for 15 min. Protect solution from light. Store at 4 °C.

Loading the Gel-Dye Mix
1. Allow the gel-dye mix equilibrate to room temperature for 30 min before use.
2. Put a new DNA chip on the chip priming station.
3. Pipet 9.0 µL of gel-dye mix in the well marked .
4. Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly 60 s then release clip.
7. Wait for 5 s. Slowly pull back plunger to 1 ml position.
8. Open the chip priming station and pipet 9.0 μL of gel-dye mix in the wells marked ___.

Loading the Markers
1. Pipet 5 μL of marker (green ___) in all 12 sample wells and ladder well. Do not leave
2. any wells empty.

Loading the Ladder and the Samples
1. Pipet 1 μL of DNA ladder (yellow ___) in the well marked ___.
2. In each of the 12 sample wells pipet 1 μL of sample (used wells) or 1 μL of de-ionized water (unused wells).
3. Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting
4. (2400 rpm).
5. Run the chip in the Agilent 2100 bioanalyzer within 5 min.

Sample Processing
1. Save an image of the gel.
2. Quantify DNA in the sample.
3. Is your band representative of 16S rRNA? Why?

B. Gel Electrophoresis (DNA size and visualization)
Note: Steps 1-5 will be prepared by TA before class. Steps 6-9 take ~1 hour
1. Make 100 ml of a 1 % agarose solution (w/v; i.e. 1 g / 100 ml buffer) in 1X TAE. (the volume of the gel can be adjusted depending on the gel box used)
2. Microwave the solution to boil to completely dissolve (stop microwave periodically to swirl dissolving agarose). Make sure solution doesn’t boil over.
3. Allow agarose solution to cool to ~60-65°C (i.e. you can comfortably hold flask)
4. Add 3.0 ml of concentrated Ethidium Bromide stock per 100 ml of agarose gel and gently swirl to mix – generally, a final concentration of 0.3-0.5 ug/ml works well. (Caution: Ethidium Bromide is a carcinogen. Wear gloves when handling the stain and the stained gel.).
5. Pour gel into gel tray (don’t forget the comb). Allow 20 min to solidify.
6. Place gel in running chamber and cover with 1X TAE.
7. Combine 5 ul of PCR product and 3 ul of gel loading dye and load the entire volume into a well in the gel.
8. Run gel (towards red node) at 80-100 mV – or faster if you’re in a hurry.
9. Allow separation of dyes, visualize on transilluminator (with eye protection), take picture.

C. Nanodrop 1000 (DNA quantification)
1. Select nucleic acid program
2. Clean pedestal with 70% ethanol and kim wipe
3. Load 3ul of DI water on pedestal to initiate instrument.
4. Blank instrument with same water loaded
5. Wipe pedestal with Kim Wipe
6. Load 3ul of DNA extraction sample
8. Wipe off pedestal and continue with next samples.

**D. Qubit (DNA quantification)**

1. Prepare dye working solution for High Sensitivity in a plastic tube.
   a. Use 200 µL of buffer for every sample.
   b. Use 1 µL of dye for every sample.
   c. Mix by vortexing.
2. Aliquot 190 µL of Working Solution into two assay tubes for standards
3. Add 10 µL of each Standard to an assay tube and mix by vortexing.
4. Aliquot 198µL of Working Solution into assay tubes for samples.
5. Add 2 µL of each sample to an assay tube and mix by vortexing.
6. Incubate 2 minutes
7. Chose the High Sensitivity program and read the results in the Qubit

**After Gel or Bioanalyzer Run**

- Cut out bands to sequence.
- Separate DNA from gel in column.
- Package to send to sequencing service (in this case, we won’t actually sequence the DNA)

**Lab 5.5: Identification of representative environmental sequences using BLAST**

1. **Identify organisms present in your samples.**
   The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.
   2. Under the Basic BLAST heading select nucleotide blast as you want to compare nucleotide sequences to nucleotide sequences in the database (in FASTA format).
   3. Paste your query sequence into the white box at the top of the page.
   4. Under the Choose Search Set heading select the database to which you would like to compare your sequence – Nucleotide collection (nr/nt)
   5. Leave all the other options as default.
   6. Click the BLAST button at the bottom of the page.
   7. Write down phylogeny (genus species) as well as lineage of microorganism (K P C F G S)
   8. Repeat for additional major sequences.
II. Assign putative function to identified microorganisms
   1. Through the NCBI website, you will be able to link to publications that cite this organism for observed and putative (presumed) functions relating to environmental processes (i.e. reductive dechlorination, iron reduction, etc).
   2. Type in the genus and species into microbial databases such as ATTC and DSMZ to get a better idea of research done and observed applications of microorganisms.
   3. Use Google Scholar to refine your search and find abstracts and key findings for your identified microorganisms.
   4. Download suggested references from BB and identify which are relevant to your sequences.
   5. What types of contaminants or other systems are these microorganisms typically associated with? Is this microbe useful for the remediation of a contaminated site?

Lab 5.6: Project Synthesis
I. Integration of culture dependent and independent techniques
II. Report structure w/ sections and subsections
III. Question / answer about laboratory report content
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Title: Sulfide-Induced Denitrification Nitrates Reduction to Ammonium Supports Anaerobic Ammonium Oxidation (Anammox) in an Open-Water Unit Process Wetland

Author: Zackary L. Jones, Justin T. Jasper, David L. Sedlak et al.

Publication: Applied and Environmental Microbiology

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Date: Aug 1, 2017

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3 messages

Zack Jones <zackjones@gmail.com> Fri, Aug 25, 2017 at 1:52 PM
To: David Sedlak <sedlak@berkeley.edu>, Justin Jasper <justud@gmail.com>

Hi David and Justin,

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A simple email acknowledgment will suffice.

Thanks and have a good weekend,

Zack

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To: Zack Jones <zackjones@gmail.com>

Hey zack

You have my permission.

Best

Justin

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David Sedlak <sedlak@berkeley.edu> Sun, Aug 27, 2017 at 9:12 PM
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David

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