Fall 2012-2016

The Role of Nitrification and Denitrification in Successful Cultivation of Oxygenic Photogranules for Wastewater Treatment

Kristie Stauch-White

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The Role of Nitrification and Denitrification in Successful Cultivation of Oxygenic Photogranules for Wastewater Treatment

A Masters Project Presented

By

Kristie Stauch-White

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of Masters of Science in Environmental Engineering

December 2016

Civil and Environmental Engineering Department
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Acknowledgements

I’d like to thank my advisor, Dr. Caitlyn Butler, for her support throughout my non-traditional Ph.D/Master’s experience, both academically and as a woman in STEM. I’d also like to thank Dr. Chul Park and Dr. David Reckhow for participating in my committee, investing the time to support my thesis effort and give valuable feedback.

My ambitious class and research schedule would not have been possible without the generous support of my husband, Michael. During my first year, he uncomplainingly took over almost all cooking, shopping, cleaning and evening childcare duties. Additionally, he took our children to all their weekend activities and on special outings to give me time to study and do lab work. In the years after that, although I was able to participate in family activities more, he still shared in the brunt of household and family tasks.

Of course I have to acknowledge my children, who gave up the undivided attention of their mother. I ask myself continually if it was all worth it. The answer came the other day when my daughter, Frannie asked me if I was going to be president and if I went to school with Hilary Clinton.

Academic research skills and inspiration were shared with me generously by Kaoru Ikuma. With her tireless support, I gained many of the microbiology and laboratory skills I needed to complete my research. She additionally imparted a great deal of insight into critical thinking and bolstered my confidence as a woman in STEM.

I would also like to acknowledge George Hamaoui who generously spent his time helping me troubleshoot qPCR issues and advance my laboratory techniques. Of course, I’d also like to thank Dr. Klaus Neusslein for generously allowing me to use equipment in his lab critical to the completion of my DNA analysis.

Finally, I’d like to acknowledge the support of NSF, first through the NSF OPG program, and then through the Graduate Research Fellowship program. I also received generous support from the University of Massachusetts in the form of the Joseph L. Boscov Fellowship and the Raymond A Noga and Karen Murphy Noga fellowships. Without these generous programs, my research would not have been possible.
Abstract

Oxygenic photogranules (OPGs) are compact, spherical, self-immobilizing biofilms in the form of dense aggregates of microorganisms with a predomination of filamentous cyanobacteria cultivated from photo-illuminated activated sludge. In this study, aerobic and anaerobic nitrogen transformation processes occurring simultaneously within OPGs, during both successful and unsuccessful cultivations, were examined. Chemical analyses including ammonium, nitrate, and nitrite concentrations at the beginning and periodically during OPG cultivations indicated nitrogen transformations occurring during successful cultivations. Additionally, qPCR studies revealed that successful cultivations supported a greater relative abundance of cyanobacteria, nitrifying, and denitrifying populations during the cultivation period. Dissolved oxygen microprofiles within a successfully cultivated mature OPG exposed to light revealed steep oxygen gradients that provide ecological niches for both nitrification and denitrification processes. Although OPGs have been cultivated from a variety of wastewater sources worldwide, it is unclear what parameters determine the success of granule growth from one cultivation to the next. In this study, preliminary evidence supporting the necessity of a minimal initial nitrogen concentration was discovered.
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Introduction

Cyanobacterial aggregates form in many environmental conditions on earth and support extremely diverse microcosms of both prokaryotic and eukaryotic life. Cyanobacteria themselves are an extremely diverse phylum, exceedingly adaptive and are major contributors to global carbon, oxygen, and nitrogen cycling (Tamulonis & Kaandorp, 2014), with Prochlorococcus alone contributing an estimated 4 gigatons of fixed carbon each year (Biller, Berube, Lindell, & Chisholm, 2014). Diazotrophic cyanobacteria contribute nitrogen by the fixation of dinitrogen in nitrogen-poor areas, such as in marine environments (Klawonn, Bonaglia, Brüchert, & Ploug, 2015; Severin & Stal, 2010; J. M. Tuomainen, Hietanen, Kuparinen, Martikainen, & Servomaa, 2003). In nitrogen-rich zones, cyanobacteria assimilate nitrogen in the form of ammonium, nitrate, or nitrite and create biomass through growth, making them the base of aquatic food webs at the primary level (Tamulonis & Kaandorp, 2014).

Microbial mats are a type of biofilm with dense communities of microorganisms with integral functional relationships (Severin & Stal, 2010; Stal, 1995). The community members rely on each other for the exchange of chemicals such as oxygen, carbon, and nitrogen species (Abed, 2010; Muro-Pastor, Reyes, & Florencio, 2005; Stal, 1995). In some cases, the communities align themselves along strata corresponding to their functional relationships (Stal, 1995). Within the ecological community, many different species, phyla, and even kingdoms are essential to provide the genes necessary to perform the interconnected biochemical community functions (Cary, Chishold, & National Science Foundation, 2000). Where cyanobacteria are present and exposed to light, they serve to oxygenate their surroundings. Cyanobacteria are also capable of metabolizing during dark periods by fermentation or glycogen metabolism via glycolysis (Embden Meyerhof Pathway). Because of their facultative nature, cyanobacteria are able to exist in multiple layers of a microbial mat, and move throughout the mats by gliding action. They are also known to self-organize into stable population patterns (Tamulonis & Kaandorp, 2014), which may serve to distribute metabolites between strata.

Microbial mats containing cyanobacteria form naturally in marine and freshwater environments and can be expressed as spherical biofilm aggregates or granules. One type of cyanobacteria aggregate, cryoconite
granules, are spherical cyanobacterial biofilms found in glacial potholes (Takeuchi, Kohshima, & Seka, 2016). These aggregates are thought to form from nutrients found in dust, and from debris from birds (Segawa et al., 2014; Takeuchi et al., 2016). Other types of nitrogen-fixing cyanobacterial aggregates formed with algal and bacterial species are found ubiquitously in marine environments, often surrounding fecal pellets (Klawonn et al., 2015; J. M. Tuomainen et al., 2003). Although it is unknown by what mechanism cyanobacterial aggregates sustain strong granule-like formation, studies have pointed to the stickiness of extracellular polymeric substances (Segawa et al., 2014; J. M. Tuomainen et al., 2003; Zarsky et al., 2013), filament entanglement (Weber, Ludwig, Schleifer, & Fried, 2007), gliding actions that form cohesive patterns (Tamulonis & Kaandorp, 2014), and gliding actions that serve as a defense against extreme light conditions or predation by microbial grazers (Fiałkowska & Pajdak-Stós, 2002). It is likely that the community benefit of simple opportunistic sharing of metabolites such as nitrogenous compounds provides the basic mechanism for granule formation. Although little is known about the microbial communities in marine aggregates and their syntrophic relationships, studies have shown limited denitrification activities in these cyanobacterial mats and aggregates (J. Tuomainen, Hietanen, Kuparinen, Martikainen, & Servomaa, 2006; J. M. Tuomainen et al., 2003). This finding is consistent with nitrogen scarcity in the marine environment, incentivizing nitrogen fixation, an energetically expensive process.

Figure 1. OPG at 2X magnification. The exterior layer of an OPG is dominated by an interwoven web of filamentous cyanobacteria. Extracellular polymeric substances (EPS) associated with the cyanobacteria causes bacteria and floating biomass to stick to it.
Oxygenic photogranules (OPGs) produced in the lab from photo-illuminated activated sludge resemble spherical cyanobacterial aggregates found in nature. OPGs are dense, spherical, interwoven microbial biofilms comprised of cyanobacteria and a host of heterotrophs, autotrophs, and algae as in Figure 1. Like many microbial biofilms found in nature, they are striated with layers of communities and functions. Since naturally-occurring microbial mats are able to sustain themselves during extreme environmental fluctuations such as diurnal and seasonal changes and weather events, the capacity of the mat’s community to adapt is critical. This is particularly important in a wastewater treatment setting, where working microbial communities must be resilient during diurnal cycles, seasonal changes, and even extreme events such as flooding, intermittent loading (such as seasonal/weekend residency), or overloading during times of extreme increase of temporary residency. Both cyanobacteria and algae have extremely versatile metabolisms allowing them to live and thrive in an ever-changing environment, syntrophically supporting and being supported by the non-photosynthetic bacteria within the biofilm community. Additionally, their density allows them to be easily separated from the waste stream, allowing a decoupling of liquid and solid retention times, resulting in less costly, more efficient, lower volume wastewater treatment systems (Abouhend, Park, Butler, & El-Moselhy, 2016; Liu, Sheng, & Yu, 2009; C. Park et al., 2016; Van Loosdrecht & Brdjanovic, 2014)

OPG cultivations represent an excellent model system to understand granule evolution, with the intent of developing an improved wastewater treatment approach. OPG wastewater treatment processes have been proposed that are expected to improve energy efficiency of wastewater treatment by reducing requirements for mechanical aeration. When used as a wastewater treatment option, OPGs remove organic nutrients including COD and nitrogenous compounds without external aeration by supporting the symbiotic growth of bacteria and algae (Abouhend et al., 2016; C. Park et al., 2016), saving 25-60% of the operating costs of municipal wastewater treatment, representing a staggering 2-3% of the total annual U.S. energy consumption (U.S. Environmental Protection Agency, 2010). Additionally, effective nitrogen removal processes are now more needed than ever as nitrogenous constituents are becoming more stringently regulated in wastewater. Ammonia and nitrate can cause serious health effects if they enter drinking water and nitrogenous compounds have deleterious effects on the environment, especially in areas where excess
nitrogen stimulates excess plant growth leading to eutrophication. Full treatment of nitrogenous compounds in wastewater includes conversion of ammonia to nitrate or nitrite via nitrification and the conversion of nitrate and nitrite to nitrogen gas via denitrification. Denitrification is a process requiring several intermediaries including nitric oxide and nitrous oxide. Nitrous oxide is a greenhouse gas approximately 300 times more potent than carbon dioxide (IPCC, *Climate Change 2001; The Scientific Basis; Cambridge University Press*, 2001). Therefore, it is important that nitrogen compounds in any proposed wastewater treatment process complete the nitrogen cycle, converting ammonia fully to nitrogen gas.

To engineer OPG granules for wastewater treatment, it is key to understand their symbiotic functionality, versatility, and resilience. In contrast to aggregates found in nature, OPGs produced in our lab originate from wastewater rich in nitrogen relative to the marine environment. Nitrogen transformations during granule cultivation and within the granule’s diverse community is believed to be integral to successful granule growth. It is hypothesized that OPGs contain zones of oxygen super-saturation, saturation, anoxic and oxygen minimum zones (OMZ), much like microbial biofilms, particularly during changing light availability during diurnal cycles. This is significant since “hot spots” of nitrogen transformation occur at oxic-anoxic interfaces (Wright, Konwar, & Hallam, 2012). Critical to our investigation of the cultivation process is the understanding of such factors leading to successful granule growth. The contribution of the initial nitrogen content of the activated sludge growth medium may be an integral factor, as it is hypothesized that nitrification and denitrification processes are active during a successful cultivation. The focus of this study is the analysis of physiological, microscopic, chemical, and biological constraints contributing to the occurrence of nitrogen transformation during granule cultivation.
Materials and Methods

Cultivation

Activated sludge from the Amherst Wastewater Treatment Plant in Amherst, Massachusetts was collected and 10 mL aliquots were pipetted into 20 mL scintillation vials and capped, leaving head space. Vials were kept in static conditions, illuminated under broadband fluorescent lights at approximately 10 klux, 24 hours per day at room temperature. Sampling was done roughly every two days during the first two weeks, and then weekly for 42 days. At day 42 the cultivation was considered complete and a final sample collection was performed. To determine the success of granulation (>50% of vials yielded a granule), a shake test was performed on the remaining vials by using three firm vertical shakes and then observing the vial contents (Figure 2). When a granule remained intact with little to no cloud of particulates in the bulk liquid, granulation was determined to be successful (Figure 2). Five cultivations were performed over the period of one year. Each cultivation consisted of 200 to 300 vials to allow for the destructive sampling of 2-3 vials for each analysis at each sampling point.

Microscopy

Microscopy was performed on an EVOS FL Color AMEFC 4300 light microscope by sectioning granules in a petri dish with a razor blade and then placing sampled sections on a microscope slide. At least 3 selections from each section were inspected, from at least 2 sampled vials during cultivation in which microscopy was performed. Photos of communities were taking at 2X, 40X, and 100X magnification in multiple locations for each sample.

Chemical Analysis

Nitrate, nitrite, phosphate, chloride, sulfate, and ammonium were measured by ion chromatography on a Metrohm chromatograph following 0.2 µm filtration with an acetate fiber filter. Ammonium, total and soluble nitrogen and COD were measured using Hach kits (Hach, Loveland, CO, USA), per standard methods 10023, 10071, 8000. Chlorophyll, TSS and VSS were measured using standard methods 10020, 208E (APHA, 2005). pH was measured using a Fisher-Scientific probe.
Microprofiling was done on two randomly selected vials for each sampling period using a Unisense clark-type dissolved oxygen microsensor with a 0.1 or a 0.25 µm tip and with a Unisense clark-type nitrous oxide microsensor with a 0.1 µm tip. Measurements were taken every 50 - 100 µm in the bulk liquid to approximately 1 cm depth. Measurements were taken in light conditions with nitrogen gas blown over the surface of the bulk liquid during measurements to prevent falsely elevated dissolved oxygen readings.

**DNA Extraction**

DNA extraction testing was performed using 16 different methods (Table A.1), to determine optimal extraction protocol. Results suggested that each method resulted in some downstream bias. From the results of these tests, the MoBio PowerSoil DNA kit was chosen, using the manufacturer’s protocol, except with only 0.8g of granule biomass rather than the prescribed 0.25g, for consistency and repeatability. Total microbial DNA was extracted using a MoBio PowerSoil DNA Extraction kit following kit instructions. DNA was extracted from 3 vials on each sampling day, using 0.08g of granule biomass following 10 s homogenization (IKA T18 basic ULTRA-TURRAX homogenizer) and dewatering by centrifuge and pipette. DNA quantity and quality was determined by Nanodrop, and was between 5 and 50 ng/µL.

**PCR and quantitative PCR**

Functional marker gene analyses were performed on either an Applied Biosystems Step One or an MJ Research quantitative PCR system. qPCR reactions were done in 25 µL reactions using 12.5 µL iTaq 2X Universal SYBR Green Supermix, 0.2 µM each forward and reverse primers and 10 ng template DNA. Real-time amplification was performed at 95°C for 10 mins. followed by 40 cycles consisting of 95°C for 15 seconds, 55°C for 1 min., and 72°C for 30 sec., followed by 30 s at 55°C and a +0.3°C ramp up every 15s to 95°C to determine the melting curve for amoA. The same protocol was followed for each of the target genes in Tables 1 and 2, except with the appropriate the annealing temperature listed for each primer. All samples were tested in triplicate and for two different vials per sampling date. All primers tested and used in this study are listed in Tables 1 and 2 with the annealing temperature used and the product length.
qPCR data was analyzed using the Pfaffl method (Pfaffl, 2001; Schmittgen & Livak, 2008). Three copy numbers per genome were assumed for narG (Kandeler, Deiglmayr, Tscherko, Bru, & Philippot, 2006).

Table 1. Primers used in this study for PCR and qPCR analysis.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Target Description</th>
<th>Primer Name</th>
<th>Sequence (5’ – 3’)</th>
<th>Anneal (°C)</th>
<th>Length (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA</td>
<td>Betaproteobacteria</td>
<td>amoA-1F</td>
<td>GGGGHTTYTACTGGTGTT</td>
<td>52, 55, 58</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ammonia monoxygenase</td>
<td>amoA-2R</td>
<td>CCCCTCKGSAAAGCCTTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>narG</td>
<td>Nitrate reductase</td>
<td>narG-F</td>
<td>TCGCCSATYCCGGCSATGT</td>
<td>60</td>
<td>173</td>
<td>(Bru, Sarr, &amp; Philippot, 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>narG-R</td>
<td>GAGTTGTAACGACGAGYTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYAN</td>
<td>Cyanobacteria</td>
<td>CYAN 108F</td>
<td>ACGGGTGAACGCGAATCCC</td>
<td>52</td>
<td>269</td>
<td>(Martins &amp; Vasconcelos, 2011; Urbach, Robertson, &amp; Chisholm, 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYAN 377R</td>
<td>CCATGGCGGAAAATCCC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16S</td>
<td>Universal 16S</td>
<td>1114</td>
<td>CGGCAACGAGCGCAACCC</td>
<td>60</td>
<td>161</td>
<td>(Denman &amp; McSweeney, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1275</td>
<td>CCATTGTACAGTGTTGAGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*When multiple annealing temperatures are listed, the last one is the one used in this study.
Table 2. Primers used in this study for PCR with either no amplification detected or non-specific binding.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Target Description</th>
<th>Primer Name</th>
<th>Sequence (5’ – 3’)</th>
<th>Anneal (°C)</th>
<th>Length (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA</td>
<td>Betaproteobacteria ammonia monoxygenase</td>
<td>19F A616r48x</td>
<td>GGWGTKCCRGGGRACWGCMA CGCATCCABCKRTANGTCCA</td>
<td>55,60</td>
<td>60</td>
<td>(Leininger et al., 2006; Schauss et al., 2009; Zarsky et al., 2013)</td>
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<tr>
<td>amoA</td>
<td>Nitrosomonas related ammonia monoxygenase</td>
<td>amoA-nitrosomo-SEGF amoA-nitrosomo-SEGR</td>
<td>CGTATCGATGTCATGTGTCGG AAGACGGTGCAATTACAGTGGT</td>
<td>60</td>
<td>60</td>
<td>(Segawa et al., 2014)</td>
</tr>
<tr>
<td>amoA</td>
<td>Nitrosospira-related ammonia monoxygenase</td>
<td>amoA-nitrososp-SEGF amoA-nitrososp-SEGR</td>
<td>CGCATCAACTCTGATTTCCW ARTGCGTGATCATCAGTGGT</td>
<td>60</td>
<td>94</td>
<td>(Segawa et al., 2014)</td>
</tr>
<tr>
<td>nirK</td>
<td>Nitrite Reductase</td>
<td>nirK-875 nirK-1040</td>
<td>ATYGGCCGVCAYGGCGAGCCGTCACTAGRTRTGGT</td>
<td>63</td>
<td>165</td>
<td>(Henry et al., 2004; Segawa et al., 2014)</td>
</tr>
<tr>
<td>niH</td>
<td>Cyanobacterial Nitrogenase</td>
<td>niH-seq-1F niH-seq-1R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>52,57,60</td>
<td>85</td>
<td>(Segawa et al., 2014)</td>
</tr>
<tr>
<td>nifH</td>
<td>Nitrogenase reductase</td>
<td>niH-seq-1 niH-seq-1R</td>
<td>AAAAGGYYGGGWATCQGGAARTCCACCAC TGGGCTTGGTITCRGCGATYGGCATT</td>
<td>57,62,67</td>
<td>400</td>
<td>(Rösch &amp; Bothe, 2005)</td>
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<tr>
<td>narG</td>
<td>Nitrate reductase</td>
<td>narG1960m2f narG2050m2r</td>
<td>TAYGTGSGGCAGGAAACGTG CAGAAAAGAGCTGTTGTTT</td>
<td>63</td>
<td>110</td>
<td>(López-Gutiérrez et al., 2004)</td>
</tr>
<tr>
<td>nosZ</td>
<td>Nitrous oxide reductase</td>
<td>nosZ-1F nosZ-1622R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>60</td>
<td>453</td>
<td>(Kloos, Mergel, Rosch, &amp; Bothe, 2001)</td>
</tr>
<tr>
<td>napA</td>
<td>Nitrate reductase</td>
<td>napA195F napA1658R</td>
<td>TAYTYYYTNSAARRATHATGTAAYGG DATNGGRTGCATYTGNGCCATRRTT</td>
<td>60</td>
<td>414</td>
<td>(Smith, Nedwell, Dong, &amp; Osborn, 2007)</td>
</tr>
<tr>
<td>napA</td>
<td>Nitrate reductase</td>
<td>napA3F napA4R</td>
<td>TGGACVATGGGYTTYAAYC ACRYTCRGCGVCGRTRCRCA</td>
<td>60</td>
<td>152</td>
<td>(Bru et al., 2007; Correa-Galeote et al., 2013; Smith et al., 2007)</td>
</tr>
<tr>
<td>nirS</td>
<td>Nitrite reductase</td>
<td>nirS-3F nirS-1622R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>60</td>
<td>453</td>
<td>(Kloos, Mergel, Rosch, &amp; Bothe, 2001)</td>
</tr>
<tr>
<td>nirS</td>
<td>Nitrite reductase</td>
<td>nirS-3F nirS-1622R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>60</td>
<td>453</td>
<td>(Kloos, Mergel, Rosch, &amp; Bothe, 2001)</td>
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<tr>
<td>nirS</td>
<td>Nitrite reductase</td>
<td>nirS-3F nirS-1622R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>60</td>
<td>453</td>
<td>(Kloos, Mergel, Rosch, &amp; Bothe, 2001)</td>
</tr>
<tr>
<td>16S</td>
<td>Universal 16S</td>
<td>16S-1F 16S-1R</td>
<td>CAGCMGCGCGGGAATTCACTGATCAGCACCATTCGCTAC</td>
<td>51</td>
<td>166</td>
<td>(Baker, Smith, &amp; Cowan, 2003; Wang &amp; Qian, 2009)</td>
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<tr>
<td>nifH</td>
<td>Cyanobacterial nitrogenase</td>
<td>nifH-seq-1F nifH-seq-1R</td>
<td>CGYTGTCTCGACAGCGAGGCCTGATTGGTGA</td>
<td>60</td>
<td>85</td>
<td>(Segawa et al., 2014)</td>
</tr>
</tbody>
</table>

** touchdown protocol from 58 to 63°C over 6 cycles
Results

Of the five cultivations performed, two successfully produced OPGs in over 50% of the vials within 42 days. OPGs produced were between 1 to 2 cm in diameter, consisting of a dense aggregate of microorganisms resembling a multi-layered spherical agglomeration of filamentous cyanobacteria, algae, and bacteria. Results for two cultivations, one successful (January 2016) and one unsuccessful (November 2015) are reported here as representative of the similarities and differences in trends seen overall between cultivations.

Figure 2. 1) A successful OPG cultivation a) On Day 1, vials contain floating biomass, b) The progression towards granulation can be seen on days 2, 14, and 42, c) A granule can be seen intact before and after 3 vigorous shakes. 2) An unsuccessful OPG cultivation on day 1, 2, 14 and 42 shows loose biomass settled on the bottom of the vial. The shake test reveals that even agglomerated biomass on day 42 is easily shaken apart.
indicating nitrification activity. In unsuccessful cultivations, by contrast, the initial concentration of ammonia was low (<1 mg-N/L) and no corresponding increases in nitrate concentration were observed. This trend (higher initial ammonia and total nitrogen concentrations for cultivations that successfully produce OPGs) appears to hold for all five cultivations performed, as can be seen in Figure 4.

![Figure 3 Concentrations of nitrite, nitrate and ammonia as well as pH values during the course of both a successful and an unsuccessful cultivation.](image)

![Figure 4. Total initial nitrogen, ammonia and nitrate concentrations are reported by cultivation date. Stars indicate successful cultivation dates.](image)
Similarly, but not as pronounced, the initial concentration of phosphate in the successful cultivation was nearly 4 times higher (5.9 mg/L as compared to 1.59 mg/L) than in the unsuccessful cultivation (Figure 5). The success of cultivation did not preclude phototrophic growth. During all cultivations, chlorophyll a increased significantly during the first two weeks of the cultivations, reaching a maximum between days 9 and 12 before a slight decline in the final cultivation weeks (Figure 6).

Interestingly, the chlorophyll a concentration was greater during the unsuccessful cultivation. To find out why, chlorophyll b and c concentrations were investigated. It was found that chlorophyll b was
significantly greater during the unsuccessful cultivation. This indicates a greater abundance of chlorophyll b producers such as algae, in the unsuccessful cultivation. In the successful cultivation, this is not the case, indicating a greater abundance of phototrophs that produce chlorophyll a but not chlorophyll b, such as cyanobacteria.

In the successful cultivation performed in January, 2016, the total suspended solids (TSS) increased from $17.17 \pm .3$ g to $21.33 \pm 1.2$ g, an increase of $4.16 \pm 1.5$ g. Volatile suspended solids (VSS) increased from $14.86 \pm .4$ g to $18.1 \pm .9$ g, an increase of $3.24 \pm 1.3$ g over the course of the cultivation (Figure 7). This represents an overall 26.8% increase in total and a 21.8% increase in volatile suspended solids seen during the successful cultivation. In unsuccessful cultivations, on the other hand, an overall decrease in both TSS and VSS were observed, in one case equaling a decrease from $22.4 \pm 0$ g to $18.75 \pm 1.6$ g, an overall decrease of $3.65 \pm 1.6$ g, and a VSS decrease from $19.05 \pm 0.2$ g to $16.75 \pm 1.6$ g, an overall decrease of $2.3 \pm 1.6$ g, a 16.3% decrease in total and a 12.1% decrease in volatile suspended solids. This indicates overall assimilatory metabolic processes in the successful cultivation, such as through carbon fixation during photosynthesis, that is greater than mass loss through the conversion of nitrogenous compounds to dinitrogen gas and other similar metabolic processes resulting in gas production, such as fermentation and

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**Figure 7.** a) Soluble COD concentrations during both a successful and an unsuccessful cultivation, b) Volatile Suspended Solids (VSS) during both a successful and an unsuccessful cultivation.
methanogenesis. Soluble COD increased for both cultivations (Figure 7), but the successful cultivation shown reached a maximum at day 12 and then decreased while the unsuccessful cultivation realized a gradual and continual increase during the entire cultivation period. The day 12 increase corresponds to a steep decline in the phosphate concentrations during the first weeks of each cultivation. This indicates an initial die-off as populations within the communities shift.

The potential for multiple microbial metabolisms was considered in a mature OPG (age = 42 days) by measuring oxygen profiles with respect to depth into the granule. Oxic and anoxic zones were found (Figure 8) in a mature cultivation granule, with the outermost, phototrophic layer of the OPG most saturated, and a steep decline in saturation with increasing depth towards the center of the granule. This is significant as it is commensurate with the presence of simultaneous nitrification and denitrification within a single granule. While denitrifiers require a maximum DO concentration of 2 mg/L to perform denitrification, nitrifiers are not tolerant of low DO concentrations, consuming approximately 4.6 g O₂/g NH₄⁺-N transformed (Rittman & McCarty, 2001). Microprofiling investigation of the DO profile of the loose biomass during an unsuccessful cultivation reveals generally constant DO concentrations circa 8.5 mg/L throughout the cultivation period once phototrophic communities develop (circa day 5-10), with some local areas of increased oxygenation near clumps of concentrated biomass, but no anoxic zones.

![Mature Cultivation Granule Oxygen Profile](image)

Figure 8. A representative example of a DO microprofile depicting a saturated zone just inside the surface of the granule where light penetrates allowing for the greatest amount photosynthesis to occur. As depth increases, light penetration and thus photosynthesis decreases, and the oxygen concentration correspondingly decreases, eventually to zero.
except perhaps in areas of densely settled biomass. We were unable to acquire DO profiles of settled biomass due to danger of breaking the probe on the vial bottom. The bulk liquid was also tested for the presence of nitrous oxide during granule development. These measurements remained below detectable levels.

Quantitative analysis of genomic data revealed that nitrifying and denitrifying bacteria are present in OPG communities during both successful and unsuccessful granule cultivations. In Figure 9, the fold change in the abundance of nitrifiers and denitrifiers in a successful cultivation is compared to those same abundances in an unsuccessful cultivation.

**Figure 9.** a) The relative quantity of *amoA* in a successful vs an unsuccessful cultivation were measured using 16S as the reference gene for both successful and unsuccessful cultivations. Analysis was done using the Pfaffl method. $R^2$ values were >.98 for all efficiency measurements. Error is for 3 replicates per sample and 2 samples per sample date and encompasses vial to vial variation in communities. b) The relative quantity of *narG* in a successful vs an unsuccessful cultivation calculated using the same method.

in an unsuccessful cultivation with 1 representing equal abundances in both types of cultivation. The
community of nitrifiers are represented by the genomic marker amoA for Betaproteobacteria ammonia monoxygenase, and was higher in the successful vs. the unsuccessful cultivation on day 10 with a >9 fold copy number normalized to the 16S community (Figure 9a). This increase interestingly corresponded to a peak in ammonia concentration around the same day. The initial population of denitrifiers, as represented by the genomic marker for narG, nitrate reductase, is significantly greater (>3 fold) in the successful cultivation relative to the unsuccessful cultivation (Figure 9b). This difference in abundance decreases slightly throughout the cultivation period, with, again, an increase around day 10.

The cyanobacterial population follows a similar trend with a nearly 6 fold difference in abundance in the successful over the unsuccessful cultivation on day 10 (Figure 10). This circa day 10 increase in communities of nitrifiers, denitrifiers, and cyanobacteria in the successful cultivation corresponds to an increase in ammonia, COD, and phosphate, also at or near that sampling date, and follows closely on an increase in nitrate around day 7. A conformational change begins to take place during the successful granulations around this time (Figure 2), as the biomass aggregates and begins to conform to a spherical morphology (C. Park et al., 2016). Interestingly, chlorophyll a was greater in the unsuccessful cultivation than it was in the successful cultivation, particularly on day 9 and later (Figure 6). This might indicate a greater abundance of non-cyanobacterial chlorophyll a producers, such as green algae, supported by
chlorophyll b concentrations and further suggesting that a predominance of cyanobacteria contributes greatly to the success of OPG formation.

The potential for nitrogen fixation was also investigated with two nitrogenase reductase (nifH) marker gene primers, but with negative results, indicating either a lack of nitrogen fixation, or poor primer specificity. This line of testing was not pursued further in this study, as the presence and capacity for nitrification and denitrification are more critical to wastewater treatment.
Discussion

The symbiotic growth of oxygenic algae and bacteria are currently used to treat wastewater in some wastewater treatment facilities in the U.S. and worldwide (J. Park, Craggs, & Shilton, 2011), but the effectiveness of this practice is limited by poor settleability, which affects the ability to decouple biomass and liquid retention times. Dense, spherical OPGs used to treat wastewater in the lab have been shown to effectively remove organic nutrients including COD (Abouhend et al., 2016; C. Park et al., 2016). Therefore, the use of OPGs may significantly advance the implementation of microalgal-based wastewater treatment.

Oxygenic photogranules have been under investigation by the Park and Butler Labs at the University of Massachusetts, Amherst since 2012 (C. Park et al., 2016). Since that time, many cultivations have been successfully performed under a variety of conditions and from a variety of waste streams from around the world (C. Park et al., 2016). In some cultivations, granulation occurs quickly and a majority of vials produce granules, while in other cultivations, only disc-shaped sedimentation or loosely clumped algae-based biomass forms. In successful cultivations, a common factor has been the presence of motile, filamentous cyanobacteria such as Oscillatoria, Microcoleus, Pseudoanabaena, and Leptolyngbya (C. Park et al., 2016). The described investigations involving chemical analyses, microsensing, and qPCR population studies were undertaken in an attempt to link factors that might affect successful OPG cultivation.

In all the described population studies involving genomics, such as qPCR studies, the first step is the extraction of DNA. Challenges in making sure extracted DNA was representative of the source communities and free of PCR inhibitors that would bias downstream population analysis results became apparent. Initially a MoBio PowerSoil kit was used, according to the manufacturers protocol. Challenges first presented themselves as PCR inhibitions in the form of poor PCR amplification efficiency, high baselines indicating autofluorescence, non-linear dilution-amplification curves, and non-repeatability from run to run. OPGs contain many potential PCR inhibitors such as humic acids, chlorophyll, and other
pigments. Many of these were persisting in the DNA solution even after inhibition removal steps during the extraction process. Another challenge was finding unlysed algae and cyanobacteria cells in the extraction pellet after the cell lysing stage of the process. In order to meet these challenges, sixteen protocols, including variations on the original protocol, alternate extraction kits, and non-kit protocols were tried (Table A.1). In the end, the best solution proved to be simply the use of the originally-tested MoBio PowerSoil kit, modified to use a severely decreased mass of source material, 0.08g rather than the prescribed 0.25g. This had the desired effect of increasing the stringency of the DNA lysing step and decreasing the concentration of inhibitors present in the resulting eluted DNA solution.

Dissolved oxygen microsensor data indicates that the outer circumference of the photogranules are supersaturated, but DO concentration drops rapidly with respect to depth (Figure 8). The outermost strata is vibrant green, due to photosynthesis and the innermost layer, where light cannot penetrate, is black (Figure 11). These disparate environmental conditions provide ecological niches within the OPG biomass to support diverse microbial community functions and symbiosis, including the potential to support both nitrification and denitrification processes. While the genomic quantification of the nitrate reductase marker gene, narG, tells us there are abundant denitrifiers present during successful cultivations and unsuccessful

![Figure 11 a) A mature cultivated OPG, sliced in half. Strata of algae and bacteria layers can be seen. These correspond to measured oxygenic, oxic and anoxic redox zones within the granule. b) A model depicting the oxygenic, oxic, and anoxic zones within a typical mature OPG, after cultivation.](image_url)
cultivations, denitrifiers themselves are facultative and may not be transforming nitrate to nitrogen gas, especially in the presence of oxygenic conditions. In successful cultivations, the presence of denitrifiers in conjunction with the presence of anoxic redox zones within mature granules is a greater indicator of active denitrification processes. Additional evidence of nitrate transformation is indicated by a decrease in nitrate concentration.

Nitrate and ammonia are also assimilated during algae and cyanobacteria biosynthesis. Approximately one nitrogen is required for every 6 carbon’s assimilated. Nitrate or ammonia can be utilized as the nitrogen source (Ebeling, Timmons, & Bisogni, 2006; J. Park et al., 2011) through the representative chemical reactions (1) and (2) below, that demonstrate the stoichiometry involved.

\[
16 \text{NH}_4^+ + 92 \text{CO}_2 + 92 \text{H}_2\text{O} + 14 \text{HCO}_3^- + \text{HPO}_4^{2-} \rightarrow \text{C}_{106}\text{H}_{263}\text{O}_{110}\text{N}_{16}\text{P} + 106 \text{O}_2 \quad (1)
\]

\[
16\text{NO}_3^- + 124 \text{CO}_2 + 140 \text{H}_2\text{O} + \text{HPO}_4^{2-} \rightarrow \text{C}_{106}\text{H}_{263}\text{O}_{110}\text{N}_{16}\text{P} + 138 \text{O}_2 + 19 \text{HCO}_3^- \quad (2)
\]

Nitrogen assimilation occurs utilizing the glutamine synthatase and glutamate synthase (GS-GOGAT) pathway in the Citric Acid Cycle (Muro-Pastor et al., 2005) after reduction of nitrate to ammonium. Because reduction of nitrate to ammonium is required, ammonium is the thermodynamically preferred nitrogen source. Successful OPG cultivations demonstrate a greater quantity of cyanobacteria throughout the cultivation period (Figure 10), so dependency on initial ammonium concentration makes sense. Diazatrophic growth is also possible, but thermodynamically very costly, as can be seen in equation (3). It is therefore unlikely when alternate sources of nitrogen, specifically nitrate and ammonium available in wastewater, are present. Additionally, while cultivating cyanobacteria to offer stability and structure to OPGs, the simultaneous development of a community rich in nitrifiers and denitrifiers, as is the case in successful cultivations (Figure 9), is beneficial to downstream wastewater treatment goals.

\[
\text{N}_2 + 8\text{Fd}^- + 8\text{H}^+ + 16\text{ATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 8\text{Fd} + 16\text{ADP} + 16\text{Pi} \quad (3)
\]
The successful cultivation and use of OPGs presents the possibility of a phototrophic granular sludge process in which the multiple redox conditions allow for both nitrification and denitrification to occur concurrently (Figures 8, 11). Granular wastewater treatment processes have become possible in recent years through engineering the morphology of microbial communities. These granular processes offer the potential to minimize energy requirements by offering multiple redox conditions within each granule and thus eliminating the need for multiple treatment phases (Van Loosdrecht & Brdjanovic, 2014). Because OPGs harness the energy of the sun to power the transformation of carbon dioxide into much-needed oxygen, energy requirements are further minimalized.

In this study, the cultivation sets were designed to determine the contribution of initial nitrogen concentration in source activated sludge and the subsequent nitrification and denitrification processes that follow. This line of reasoning could suggest how to ensure cultivation success and to cultivate granules with rich communities of nitrifying and denitrifying bacteria necessary for downstream wastewater treatment goals. Furthermore, understanding the environmental conditions within the granules that favor nitrification and denitrification, specifically oxic and anoxic redox zones, is necessary to conclude the presence of these nitrogen transformations (Van Loosdrecht & Brdjanovic, 2014). Of the five cultivations performed, initial nitrogen concentration strongly correlated with the success of OPG cultivation (Figure 4). Additionally, a surge in nitrogen concentrations around day 10 corresponded with increases in COD, chlorophyll a, and the populations of nitrifiers, denitrifiers and cyanobacteria during a successful cultivation.

Of the five cultivations performed, initial nitrogen concentration was strongly correlated with the success of OPG cultivation (Figure 4). Moreover, the presence of concurrent nitrification and denitrification processes is supported by extant oxic and anoxic redox zones within successfully cultured mature granules as they provide ecological niches for both of these processes (Figure 8). To further support the idea that successful cultivations support greater nitrification and denitrification process, qPCR studies of the communities within the granules was undertaken. It was found that there are abundant nitrifiers, denitrifiers and cyanobacteria during successful cultivations (Figures 9, 10), with cyanobacteria being more abundant.
during successful cultivations. While looking for evidence of nitrogen transformations during cultivations, another interesting phenomena was observed to occur only during successful cultivations. A surge in ammonia and nitrate concentrations around day 10 corresponded with increases in COD, chlorophyll a, and the populations of nitrifiers, denitrifiers and cyanobacteria during a successful cultivation. Future research should be aimed at understanding successful OPG cultivation requirements and maximization of bacterial communities most helpful in wastewater treatment and should focus on finding the minimum and optimal initial nitrogen conditions for granule formation.


Wang, Y., & Qian, P. Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design
https://doi.org/10.1371/journal.pone.0007401

https://doi.org/10.1128/AEM.01002-07


https://doi.org/10.1088/1748-9326/8/3/035044
### DNA Extraction Methods Testing Summary

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*Note: Conditions 1 to 10 represent different testing conditions.*