SIZE PROGRESSION OF OXYGENIC PHOTOGRANULES (OPGs) AND ITS EFFECT ON OPG WASTEWATER TREATMENT

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SIZE PROGRESSION OF OXYGENIC PHOTOGRANULES (OPGs) AND ITS EFFECT ON OPG WASTEWATER TREATMENT

A Dissertation Presented

by

AHMED S.A. ABOUHEND

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2022

Department of Civil and Environmental Engineering
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To my family, for their support and encouragement.
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ABSTRACT

SIZE PROGRESSION OF OXYGENIC PHOTOGRAINULES (OPGs) AND ITS EFFECT ON OPG WASTEWATER TREATMENT

FEBRUARY 2022

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In recent years, the oxygenic photogranule (OPG) process has gained increasing interest because of its potential to treat wastewater without supplemental aeration. Oxygenic photogranules (OPGs) are dense spherical aggregates comprised of phototrophic and nonphototrophic microorganisms. In OPG wastewater treatment reactors, photogranules grow in number as well as in size. The primary goal of this dissertation was to investigate how OPGs grow in size and how the growth affects their structure and functions. We found that OPGs undergo structural changes as they grow bigger in size. As OPGs grow larger, filamentous cyanobacteria become enriched while other phototrophic microbes diminish significantly. OPGs larger than 3 mm in diameter developed a layered structure in which a concentric filamentous cyanobacterial layer encloses noncyanobacterial aggregates. We found that the photogranules’ capability of producing oxygen, the key element in OPG wastewater treatment, is size-dependent.

The results also show that the availability of iron strongly influences the growth and aggregation of filamentous cyanobacteria and thus the size-growth of photogranules in
bioreactors. The selection of filamentous cyanobacteria during the size evolution of OPGs was linked with their ability to utilize the EPS as well as the Fe bound with EPS for their growth. We observed that the aggregation of filamentous cyanobacteria was promoted as both EPS and Fe bound with EPS became limited. Strong negative correlations exist between the abundance of cyanobacteria in OPGs and concentration of biomass-bound EPS as well as between cyanobacteria and concentration of Fe bound with EPS.

The final section of this dissertation focused on the role of shear force in photogranulation. We found that the size of OPGs in reactors is inversely related to hydrodynamic shear. Compared with the OPGs developed at a high shear stress, OPGs produced at low and medium hydrodynamic shear stresses were bigger in size, more spherical, and less hairy. The variations in the particle-size-distribution of OPG biomass in reactors because of shear conditions resulted in significant differences in organic matter and nitrogen removals. The increased hydrodynamic shear forces stimulated tCOD and ammonia removals which could be due to the higher oxygen production capabilities of the smaller OPGs formed at higher shear levels. In contrast, the larger OPGs formed at the lower shear levels achieved a higher total nitrogen removal than the smaller granules formed at the higher shear forces.

Overall, these research findings are expected to enhance the fundamental knowledge on photogranulation phenomenon. Furthermore, engineering the OPG system based on a better understanding of the growth and function of photogranules is expected to advance the development of a new granular technology, which has the potential to treat wastewater without energy-intensive aeration.
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CHAPTER I

EXECUTIVE SUMMARY

Although the activated sludge process (ASP), the most common wastewater treatment process in developed countries, has made a remarkable contribution to wastewater treatment for more than 100 years, it requires high energy expenditure.\textsuperscript{1} The great energy demand for operating ASP is mainly caused by supplying O\textsubscript{2} for organic matter degradation and ammonia nitrification. In the US, mechanical aeration for ASP accounts for 25–60\% of the total energy consumption for wastewater treatment.\textsuperscript{1–3} In addition to the high energy demand, ASP requires large infrastructure investments, making ASP for wastewater sanitation unachievable in many developing countries in the world.

In recent years, there has been a growing interest in the use of phototrophic granular biomass for wastewater treatment.\textsuperscript{4–8} Different from conventional activated sludge (CAS) and aerobic granular sludge (AGS),\textsuperscript{9–14} phototrophic granular biomass contains a significant amount of oxygenic phototrophs. Hence, these phototrophs may autonomously produce O\textsubscript{2} needed for oxidation of organic matter and nitrification, which in the CAS and AGS processes is often provided by energy-intensive aeration. Similar to the AGS process, the phototrophic granular process allows effective biomass separation from water and enhances the system operation in small footprint.\textsuperscript{6,8}

Oxygenic photogranules (OPGs) are a new photogranule type that has been recently developed for wastewater treatment applications.\textsuperscript{15–18} OPGs were first reported from an accidental discovery that activated sludge stored in a closed hydrostatic environment with a source of light transforms into a photogranule.\textsuperscript{17,18} Transformation of activated sludge into a photogranule occurred with the enrichment of phototrophic microorganisms,
including filamentous cyanobacteria, green algae, and diatoms.\textsuperscript{17} Hydrostatically formed photogranules have been reported to be 10–20 mm in diameter.\textsuperscript{17,19,20} When these hydrostatically formed photogranules are seeded into reactors for wastewater treatment, the hydrostatic photogranules disintegrated, and offspring photogranules are generated in completely mixed environments.\textsuperscript{15,17}

The application of OPGs for aeration-free wastewater treatment has been demonstrated with stable and successful reproduction of reactor operation.\textsuperscript{15–17} In OPGs, oxygenic phototrophs live in close proximity to other microbes that possess the essential bioprocesses for wastewater treatment.\textsuperscript{17} OPG process showed effective COD removal and nitrification without aeration.\textsuperscript{15} Due to photoautotrophic assimilation of CO\textsubscript{2}, OPGs showed biomass yields that are 3–4 times greater than activated sludge.\textsuperscript{15} This high yield indicates a potential to recover renewable energy in the form of easily separable biofeedstock, which can be used in downstream processes for energy generation.

Earlier, Abouhend et al.\textsuperscript{15} reported that the photogranules substantially increase in number as well as in size during reactor operation, resulting in an increase in biomass concentration. During quasi-steady state operation, the size of the OPGs in reactors was up to 4.5 mm in diameter.\textsuperscript{15} Previous studies on other granular biotechnologies, such as aerobic granular sludge, showed that the structure of granules changes as the granules grow larger in size.\textsuperscript{21–25} Changes in size and structure influence mass transfer in granules, further causing changes in ecology, physiology, as well as physicochemical properties of granules.\textsuperscript{21–25} It has been also shown in the literature that size and structure of biogranule significantly impact its functions. For example, oxygen diffusion limitation occurring within the larger aerobic granules favors the growth of anaerobic microorganisms, such as
denitrifiers, in their cores.\textsuperscript{26–28} These bigger granules can, therefore, achieve higher nitrogen removal via simultaneous nitrification-denitrification (SND) compared to the smaller granules in which anaerobic conditions may not be established.\textsuperscript{21,26} Hence, granule size is directly related with the characteristics and functions of granules and ultimately determines the performance of bioprocesses. Although the growth of photogranules in size is obvious in OPG reactor operation,\textsuperscript{15–17} the changes in their structure, community, and functions along with size progression remain mainly unknown.

The main objectives of this research were to investigate the effect of size on the structure, community, and performance of oxygenic photogranules (OPGs), and understand the role of iron (Fe) in the size-growth and development of photogranules in reactors. The main research hypothesis was that the size-growth of photogranules will impact their structure and functions and will ultimately determine the performance of OPG process. We further hypothesized that limitation of iron (Fe) selects filamentous cyanobacteria, the key granulating microbial group within OPGs, and promotes the size-growth of OPGs in reactors. To examine these hypotheses and achieve the research objectives, the following three studies were conducted:

1) Growth progression of oxygenic photogranules and its impact on their capability of producing oxygen

This study reports the growth progression of oxygenic photogranules (OPGs) and its impact on their capability of producing oxygen. The study presents that the increase in photogranule’s size occurs with the change in their phototrophic community. The study also reveals the structural changes that photogranules undergo with increasing granule size.
In addition, we report the dynamics of extracellular polymeric substances (EPS) to illustrate how EPS also change along with the photogranule’s size evolution. Finally, this study presents the major impact that the size of photogranules poses on photogranules’ autonomous oxygen production.

2) Investigating the role of iron in the development and size-growth of oxygenic photogranules in bioreactors

In this study, the fate and distribution of Fe in OPG wastewater treatment reactors were investigated. We included manganese (Mn), copper (Cu), and zinc (Zn) in the current study to test whether our hypothesis is true for iron only or other metals as well. We determined the fractions of Fe as well as other metals in the bulk liquid, biomass-bound EPS, and OPG biomass pellet. We studied the changes in the distributions of Fe, Mn, Cu, and Zn with photogranules as they grow larger in size. We discussed the correlations between the levels of metals in OPG biomass and the abundance of phototrophs.

3) The role of hydrodynamic shear in oxygenic photogranule wastewater treatment process

We investigated the effect of shear on photogranulation in three sequencing batch reactors (SBRs) which were operated at different mixing speeds for 250 days to treat wastewater without aeration. Mixing was provided by overhead stirrers equipped with a stainless-steel impeller. Hydrodynamic characteristics of reactors, including Reynolds number (Re), Shear stress (τ), Shear rate (G) and Kolmogorov microscale (η) were determined. The influence of shear on the particle size distribution, cyanobacteria content,
and morphology of OPG biomass was examined. The evolution of EPS in OPG biomass is presented. The study also reports the performance of OPG reactors, including the removal of COD, ammonia, total nitrogen, and the settleability of biomass.

Overall, this doctoral research helps to enhance the fundamental knowledge on photogranulation phenomenon by better understanding the growth progression of oxygenic photogranules in reactor systems, and the impact size evolution on the structure, community, and functions of photogranules. The outcome of this research is expected to help engineer the OPG process by optimizing the process based on granule size and achieve effective wastewater treatment without energy-intensive aeration.
CHAPTER II

LITERATURE REVIEW

2.1. Effect of size on the structure, properties, and performance of aerobic granules

Aerobic granular biomass has been successfully grown in sizes ranging between 0.02 mm and 9 mm,\textsuperscript{27,29} while the size range 0.1–3 mm was the most common.\textsuperscript{30–34} Size is one of the critical characteristics of aerobic granules because it describes the growth and maturation process of granules. The increase in granule’s size can diminish the porosity of granules, leading to the limitation of mass transport within granules.\textsuperscript{10,35–39} Mass transfer limitation in the larger granules could influence the nutrient accessibility and outflow of unfavorable products, affecting the structure of aerobic granules.\textsuperscript{22,24,27,40,41} It would also significantly impact microbial viability and the microstructure of the microbial organization.\textsuperscript{22} For example, if no substrate could penetrate the aerobic granule center, the EPS in the center would be utilized as a potential energy source, and the biomass in the center would undergo microbial decay. These may eventually lead to porous and weak structures of aerobic granules.\textsuperscript{27} A kinetic model simulation revealed the maximum nutrients penetration depth in aerobic granules was 1.25–1.75 mm.\textsuperscript{42} Tay \textit{et al.}\textsuperscript{43} determined the distribution of live and dead cells in aerobic granules and found that granules with a diameter of about 0.6 mm or less entirely consisted of live biomass, while granules larger than 0.6 mm contained some dead biomass.

Oxygen concentration profiles in aerobic granules have shown that oxygen is utilized entirely at the surface layers with a thickness of 0.1–0.7 mm.\textsuperscript{41,44,45} Limited oxygen concentrations in granule center favor the formation of an inner core with anaerobic microbes.\textsuperscript{28} These anaerobic microbes can produce acidic chemicals, decrease the pH of
the inner core and, thus, damage the outer shell and backbone matrix of aerobic granules.\textsuperscript{46,47} Using the fluorescence in situ hybridization (FISH) method, Tay et al.\textsuperscript{48} found an anaerobic layer and a dead cell layer at a depth of 0.8–0.9 mm from the surface of aerobic granules.

Toh et al.\textsuperscript{23} found that the physical properties of aerobic granules also change as they grow in size. The settling velocity and granule density of aerobic granules increase with the size increase while other parameters such as granule strength, specific hydrophobicity, and sludge volume index (SVI) decrease accordingly.\textsuperscript{23} Literature also showed that granule structure and size are critical for the performance of the system. In aerobic granules, simultaneous nitrification and denitrification (SND) during aeration require granules of a certain size. A previous study showed that a maximal N removal was observed in granules with average diameters of 1.3 mm.\textsuperscript{49} An optimal granule size of 0.7–1.9 mm was proposed to achieve sludge stability and higher nitrogen removal.\textsuperscript{40,42,50}

2.2. Size control in aerobic granules

According to the literature, the operational conditions, such as the substrate type, substrate loading rate, shear force, settling time, dissolved oxygen level, and sludge age were found to affect the size-growth of the granules.\textsuperscript{9,10,51,52} To date, several strategies have been proposed to control the size and promote the stability of the aerobic granular sludge process by suppressing the overgrowth of large granules. Removal of large granules during settling\textsuperscript{53,54} and selective discharge of large granules during aeration phases have been successfully used.\textsuperscript{55,56}
Increasing hydrodynamic shear stress has been proposed to suppress the overgrowth of large granules.\textsuperscript{31,46,57} A high hydrodynamic shear stress increases the attrition of granules, suppressing the overgrowth of large granules, whereas a relatively low hydrodynamic shear stress favors the growth of granules. However, high hydrodynamic shear stresses typically require substantial amounts of energy, which increases the cost of industrial applications.\textsuperscript{58}

Lately, new internals, such as baffles, stirring paddles, and tubes, were developed to increase hydrodynamic shear stress in reactors.\textsuperscript{54,59,60} Compared to conventional stirred-tank reactors, airlift reactors have a relatively constant shear stress.\textsuperscript{59} A stronger hydraulic shear stress that favored the granular sludge's structural stability was reported in stirred-tank and airlift reactors.\textsuperscript{54,60} Previous studies showed that the structure of sludge aggregates depends on the given stirring speeds.\textsuperscript{60} However, these internals led to higher flow resistance and was challenging to construct. Moreover, high hydrodynamic shear stresses shift granule size distribution towards smaller sizes, which leads to an unfavorable size distribution for simultaneous nitrification and denitrification (SND) process.\textsuperscript{50,61}

Recently, novel funnel-shaped internals were proposed to optimize granule size distribution under a low superficial upflow air velocity.\textsuperscript{21} This technique showed 68.3±1.4\% efficiency in maintaining the optimal size range (0.7−1.9 µm) in reactors. These funnel-shaped internals effectively suppressed the overgrowth of large granules without requiring additional energy.\textsuperscript{21}
2.3. Effect of hydrodynamic shear force on biogranulation

In traditional aerobic granular sludge systems (i.e., bubble column reactors), hydrodynamic shear force is mainly created by aeration. Hydrodynamic shear force in these reactors can be described by the upflow air velocity. Higher shear forces were reported to favor the formation of more compact and denser aerobic granules. Higher shear forces were also found to stimulate the production of extracellular polysaccharides and enhance microbial activity.\textsuperscript{52,62} Extracellular polysaccharides can facilitate both cohesion and adhesion of cells and help maintain the structural integrity of aerobic granules.\textsuperscript{63} The enhanced production of extracellular polysaccharides at high shear forces can make granule structures more compact and stronger. Aerobic granules can form at different levels of hydrodynamic shear forces. Hence, hydrodynamic shear force is not a primary inducer of aerobic granulation in reactors.\textsuperscript{62} However, the structure of mature aerobic granules is very related to hydrodynamic shear force. High shear in terms of superficial upflow air velocity resulted in more compact, denser, rounder, stronger, and smaller aerobic granules.\textsuperscript{64}

2.4. Iron as an essential micronutrient for phototrophic microorganisms

Iron (Fe) is one of the essential micronutrients required for the growth and survival of phytoplankton.\textsuperscript{65–71} Fe is involved in multiple metabolic pathways for biochemical catalysis and energy production within phytoplankton cells.\textsuperscript{65,72,73} Fe is also required for the production of metalloenzymes and proteins, which are involved in metabolic processes such as photosynthesis, respiration, electron transport, nitrate reduction, and reactive oxygen species detoxification.\textsuperscript{65,66,74–76}
Photosynthetic carbon reduction and nitrogen reduction are considered the two most energy-demanding processes in the phototrophic cell, which are dependent on iron-containing compounds.\textsuperscript{77–81} In photosynthesis, iron is required for the biosynthesis of intermediate electron carriers in photosynthesis, such as cytochromes, ferredoxin, and Fe-S proteins.\textsuperscript{77} These compounds are necessary for the biosynthesis of pigments and the assembly of the photosynthetic apparatus.\textsuperscript{77,82} For nitrogen reduction, iron is required for the synthesis of essential enzymes for nitrogen reduction pathways. Iron is directly involved in the nitrate reduction pathway via nitrite reductase and as a ferredoxin cofactor for nitrate reductase.\textsuperscript{83,84} Iron is also essential in nitrogen fixation metabolism for the synthesis of iron-molybdenum subunits required for the biosynthesis of nitrogen fixation enzyme nitrogenase.\textsuperscript{77,82} It was reported that nitrogen-fixing cyanobacteria require up to 10 times as much iron as required for cyanobacteria growing on nitrate.\textsuperscript{85}

2.5. The impact of iron limitation on cellular processes in phototrophs

Iron limitation influences several metabolic pathways within phytoplankton, mostly photosynthesis.\textsuperscript{65–71} According to literature, low iron levels limit phytoplanktonic growth in up to 30\% of the world’s oceans and many freshwater environments.\textsuperscript{65,86} Iron limitation in phototrophs results in reduced levels of the electron transport intermediates, cytochrome and ferredoxin, causing reduction in the photosynthetic electron flow during photosynthesis.\textsuperscript{82,87} Iron limitation also causes decreased synthesis of photosynthetic pigments, i.e., chlorophyll and phycobilin, resulting in a severe decrease in the net photosynthesis - both in terms of the total carbon fixed and the reductive energy available. Moreover, iron limitation causes an impaired organizational structure of the thylakoid
membrane of phototrophic cells.\textsuperscript{77} Furthermore, Fe limitation has also been shown to impact nitrogen reduction in phototrophs. Iron limitation decreases the level of algal nitrite reductase.\textsuperscript{88} Under Fe-limiting conditions, phytoplankton need to find a mechanism to increase their cellular Fe and lower their Fe requirements.\textsuperscript{65} Both types of adaptation are closely tied into the metabolic pathways of different nutrients within the cell.\textsuperscript{65}

2.6. Scavenging iron by cyanobacteria in iron-limited conditions

Unlike other phototrophs, cyanobacteria can grow in Fe-limited environments as they can acquire and transport iron with inducible siderophore mechanisms.\textsuperscript{85,89–91} Siderophores are low-molecular-weight compounds with an exceptionally high affinity for iron.\textsuperscript{92} Siderophores are widely distributed in bacteria and fungi.\textsuperscript{89,93,94} They are found in marine, freshwater, and terrestrial environments.\textsuperscript{89,93,94} Some eukaryotic algae were also shown to produce siderophores but at much lower levels than cyanobacteria.\textsuperscript{95–97} Typically, siderophores form a strong hexadentate octahedral complex with ferric iron Fe (III).\textsuperscript{98} This complex can then be transported inside the cell. Based on the primary oxygen-donating ligands that bind the Fe, siderophores are generally divided into four types.\textsuperscript{99} These are the hydroxamates, catecholates, carboxylates, and siderophores with mixed types of these functional groups. Cyanobacterial siderophores are either hydroxamates or catecholates. Siderophores are induced under Fe stress and repressed by nutritional Fe.\textsuperscript{89} The ability of cyanobacteria to solubilize and bind Fe by siderophore mechanisms confers a competitive advantage for cyanobacteria over other algae.\textsuperscript{90,91,100} It has been reported that secretion of siderophores in aquatic environments could alter the copper speciation during
cyanobacteria blooms. The free ion activity of copper and thus its toxicity decreases when it binds to hydroxamate siderophores.

2.7. The impact of iron on the growth rate and population dynamics of phototrophic community

The role of Fe in controlling the growth rate and population dynamics of phytoplankton has been widely studied. Sub-nanomolar additions of Fe were found to increase phytoplankton's net specific growth rates in water bodies. de Baar et al. demonstrated a positive relationship between the magnitude of in situ phytoplankton stocks and ambient Fe levels in the South Atlantic. Fe enrichments were found to cause significant increases in chlorophyll a content and, thus, photosynthetic efficiency of phytoplankton.

Several studies have also investigated the influence of light/iron combination on phytoplankton growth. Raven found that the Fe requirements of cyanobacteria and microalgae cells growing under light limitation were 50-fold higher than their Fe requirements under light saturation. Boyd et al. found that the addition of 0.5–3 nM of Fe in the Southern Ocean resulted in an increase in chlorophyll levels in phototrophs up to 1 μg/L regardless of irradiance levels, while a combination of high-light and Fe resulted in an increase in chlorophyll to >2 μg/L. Because of the combined effects of Fe/light, previous studies failed to ascertain the relative contributions of Fe and irradiance to the control of phytoplankton growth.

The impact of Fe on the growth of specific groups of phytoplankton in Ocean water was also studied in several investigations. In situ iron fertilization in Fe-limited
high-nutrient waters led to marine phytoplankton blooms, more than 70% of which were dominated by diatoms.\textsuperscript{70} Fitzwater \textit{et al.}\textsuperscript{70} found that chlorophyll \textit{a} increased from 4% to 68% of the total chlorophyll when 2 nM of Fe was added, mainly due to diatoms growth. Fitzwater \textit{et al.}\textsuperscript{70} also found that the contribution by all the other groups was variable in response to Fe enrichment, either decreased or remained constant. Coale\textsuperscript{108} performed Fe-enrichment experiments in the subarctic Pacific and found that the growth of diatoms, \textit{Pennates} sp., \textit{Nitzschia} sp., and \textit{Pseudonitzschia} sp., increased dramatically with the addition of Fe, making up approximately 42% of the total biomass with the remainder consisting of cyanobacteria and dinoflagellates (<25 μm size class).

Zhao \textit{et al.}\textsuperscript{109} measured the growth rate and photosynthetic activity of the model diatom \textit{Phaeodactylum tricornutum} cultured under different iron concentrations and found that it grew more rapidly and had a much higher photosynthetic efficiency under higher Fe concentrations. A proteomic analysis was conducted to explore the mechanism of the response of diatoms to Fe, and the results indicated that Fe promotes the Calvin cycle of \textit{P. tricornutum}.\textsuperscript{109} Mock \textit{et al.}\textsuperscript{110} has found that Fe may serve as a required cofactor for the silicon metabolism of the marine diatom \textit{Thalassiosira pseudonana}. Marchetti \textit{et al.}\textsuperscript{111} also found that diatoms display a unique transcriptional response to variations in Fe, while the molecular mechanism of the response of diatoms to Fe needs further exploration.

\subsection*{2.8. Effect of iron on aerobic granulation of activated sludge}

Iron salts are widely used in wastewater treatment as a coagulating agent to promote activated sludge flocculation and to remove phosphorous by chemical precipitation.\textsuperscript{112,113} It has been reported that Fe ion can speed up the granulation process \textit{via} neutralizing the
negative charge present on the surface of microbes.\textsuperscript{112,114,115,115,116} Fe was also found to stimulate the secretion of extracellular polymeric substance (EPS), which is advantageous for enhancing granule size.\textsuperscript{117} Yilmaz et al.\textsuperscript{112} reported that adding 1−10 mg/L of Fe during aerobic granulation suppressed filamentous out-growth and favored dense and compact granule formation. The aerobic granular sludge formed in the absence of Fe was fluffy and had a finger-type structure and filamentous out-growth.\textsuperscript{112} Fe addition was also found to enhance the chemical precipitation within the granules.\textsuperscript{112} The amount of Fe precipitates was higher inside the granules than outside.\textsuperscript{112} Agridiotis et al.\textsuperscript{118} also found that adding 30 mg/L of Fe (II) was beneficial for converting filamentous flocs into a compact structure, resulting in significantly improved sludge settleability. Wang et al.\textsuperscript{119} reported that applying a magnetic field during aerobic granulation could promote the accumulation of iron compounds in the sludge, and, thus, enhance biomass aggregation and decrease the granulation time.

Fe addition was also found to promote COD removal in sequencing batch reactors (SBRs) compared to those without Fe.\textsuperscript{117} Iron was also reported to impact nitrogen removal.\textsuperscript{120} Ammonia-oxidizing bacteria (AOB) were found to lose their ability to oxidize ammonia due to a lack of Fe compounds, but they could survive in the aerobic granular sludge.\textsuperscript{120} Limited ferrous iron addition encouraged nitrification.\textsuperscript{120} Liu and Horn\textsuperscript{121} reported that the Fe concentration was linearly correlated with ammonia removal during the nitritation process.
CHAPTER III

GROWTH PROGRESSION OF OXYGENIC PHOTOGRANULES AND ITS IMPACT ON THEIR CAPABILITY OF PRODUCING OXYGEN

Abstract

Oxygenic photogranules (OPGs), spherical aggregates comprised of phototrophic and nonphototrophic microorganisms, treat wastewater without aeration, which currently incurs the highest energy demand in wastewater treatment. In wastewater-treating reactors, photogranules grow in number as well as in size. Currently, it is unknown how the photogranules grow in size and how the growth impacts their properties and performance in wastewater treatment. Here, we present that the photogranules’ growth occurs with changes in phototrophic community and granular morphology. We observed that as the photogranules grow larger, filamentous cyanobacteria become enriched while other phototrophic microbes diminish significantly. The photogranules greater than 3 mm in diameter showed the development of a layered structure in which a concentric filamentous cyanobacterial layer encloses noncyanobacterial aggregates. We observed that the growth of photogranules significantly impacts their capability of producing oxygen, the key element in OPG wastewater treatment. Among seven size classes investigated in this study, photogranules in the 0.5–1 mm size group showed the highest specific oxygen production rate (SOPR), 21.9±1.3 mg O₂/g VSS-h, approximately 75% greater than the SOPR of mixed photogranular biomass. We discuss engineering the OPG process based on photogranules’ size, promoting the stability of the granular process, and enhancing efficiency for self-aerating wastewater treatment.
3.1. Introduction

Photogranulation is a light-driven microbial process in which phototrophic microorganisms and nonphototrophic microorganisms form spherical aggregates. This phenomenon seems to occur ubiquitously since its products, i.e., photogranules, are formed in widely varying environments, including lake water, sea water, melt holes on glacier surfaces (i.e., cryoconite holes), and wastewater treatment systems.

Oxygenic photogranules (OPGs) are a new photogranule type that has been recently developed for wastewater treatment applications. In OPGs, oxygenic phototrophs live in close proximity to other microbes that possess the necessary functional traits for wastewater treatment. Hence, OPGs can remove organic matter and nutrients by using the oxygen generated through photosynthesis, rather than relying on oxygen provided from energy-intensive aeration by mechanical means.

OPGs were first reported from a fortuitous discovery that activated sludge stored in a closed hydrostatic environment with a source of light transforms into a photogranule. This “hydrostatic photogranulation” occurs with the enrichment of phototrophic microorganisms, including green algae, diatoms, and filamentous cyanobacteria, which were initially present at extremely low levels in activated sludge. Among these phototrophs, filamentous cyanobacteria, particularly the order Oscillatoriales, are considered the key granulating microbial group, because not only are they found at the highest population in formed photogranules but also their mat-like layer maintains the photogranule’s structural integrity. Formed out of activated sludge
under hydrostatic conditions, these photogranules have been reported to be 10–20 mm in diameter.\textsuperscript{17,19,20}

The application of OPGs for a flow-based wastewater treatment has been demonstrated with stable and successful reproduction of reactor operation.\textsuperscript{15–17} The OPG process for wastewater treatment starts by seeding a stirred-tank reactor with hydrostatically formed photogranules and operating in sequencing batch mode under cyclic light conditions.\textsuperscript{15–18} Over the first few days, the seed photogranules disintegrate and new photogranular biomass is produced. As reactor operation continues, the photogranules substantially increase in number as well as in size, resulting in an increase in biomass concentration, simultaneously occurring with the treatment of wastewater.\textsuperscript{15–18}

Previous studies on other granular biotechnologies, such as aerobic granular sludge, showed that the structure of granules changes as the granules grow larger.\textsuperscript{21–25} Changes in size and structure influence mass transfer in granules, further causing changes in ecology, physiology, as well as physicochemical properties of granules.\textsuperscript{21–25} Hence, granule size is directly linked with the characteristics and functions of granules and ultimately determines the performance of bioprocesses. Although the size-growth of photogranules is obvious in OPG reactor operation,\textsuperscript{15–17} the structural and community changes during size progression remain mainly unknown. Moreover, since oxygen is produced within the photogranules and not diffused from the bulk liquid by aeration as occurring in aerobic granule sludge,\textsuperscript{24,41,133} the size of the photogranules is expected to impact their oxygen production capability. This key element of the OPG process has remained unexplored until this study.

Here, we report the growth progression of OPGs and its impact on their bioactivity, especially their capability of producing oxygen with removal of oxygen-demanding
matters, enabling aeration-free wastewater treatment. The study presents that the increase in photogranule’s size occurs with the change in their phototrophic community. The study also reveals the structural changes that photogranules undergo with increasing granule size. In addition, we report the dynamics of extracellular polymeric substances (EPS) to illustrate how EPS also change along with the photogranule’s size evolution. Finally, this study presents the significant impact that the size of photogranules poses on photogranules’ autonomous oxygen production. This study is, therefore, expected to help engineer the OPG process by optimizing the process based on granule size and achieve effective wastewater treatment without energy-intensive aeration.

3.2. Materials and Methods

3.2.1. Source of photogranular biomass

Photogranular biomass investigated in this study was collected from laboratory-scale reactors which were seeded with hydrostatically formed photogranules and operated in sequencing batch mode for five months to treat wastewater without aeration. We fed the reactors with primary-effluent wastewater collected from a local wastewater treatment plant (Amherst, MA). Reactors were operated in four 6 h cycles per day. Each 6 h cycle included 2.5 h dark phase, followed by 3.5 h light phase. Light conditions were provided by fluorescent-light bulbs at an approximately photosynthetically active radiation (PAR) of 150 µmol/m²-s on the reactor surface. The illumination provided on the inner surface of reactor under these light conditions was 105 µmol/m²-s. The reactors were mixed at approximately 100 rpm using overhead stirrers equipped with a stainless-steel impeller. Mixing conditions in reactors created an average velocity gradient (G) of 40 s⁻¹ and
Kolmogorov microscale ($\eta$) of 160 μm. Reactors were operated at a hydraulic retention time (HRT) of 0.75–0.9 d and an average solids retention time (SRT) of 30 d. The photogranular biomass was collected from the reactors during the quasi steady-state period. During this period, the size of photogranules was in the range of 0.1–4.5 mm in diameter. Detailed reactor operation and system performance are shown in Abouhend et al.\textsuperscript{15}

### 3.2.2. Size separation of photogranular biomass

The photogranular biomass was classified based on size according to the wet-sieving method.\textsuperscript{134} Briefly, 500 mL of mixed biomass was collected from the reactors and suspended in a glass beaker containing the reactor’s effluent to a total volume of 4 L. The biomass in effluent was then passed through certified testing sieves (Gilson, V200CH) with opening diameters of 0.2 mm, 0.5 mm, 1 mm, 1.7 mm, 2.5 mm, 3.5 mm, and 4.5 mm. The following size classes were then obtained: (1) <0.2 mm; (2) 0.2–0.5 mm; (3) 0.5–1 mm; (4) 1–1.7 mm; (5) 1.7–2.5 mm; (6) 2.5–3.5 mm; and (7) 3.5–4.5 mm. The OPG biomass captured on each sieve was washed three times and transferred into a glass beaker containing reactor effluent with a total volume of 500 mL. To ensure accurate size classification, digital images for each size class in Petri dishes were collected and subjected to particle size analysis using the software ImageJ.\textsuperscript{135}

### 3.2.3. Microscopy

Brightfield and fluorescence microscopy were done following the procedure presented in Milferstedt et al.\textsuperscript{17} Briefly, cross sections of photogranules were created as follows: 1) the entire photogranule was fixed in Tissue-Tek OCT Compound 4583 (Sakura
Finetek, USA); 2) the photogranule was then frozen at −80 °C; 3) the photogranule was removed from the freezer and sectioned at the maximum diameter under a Leica stereomicroscope using a scalpel; and 4) the obtained sectioned photogranules were immediately imaged. The thickness of the cyanobacterial layer and the diameter of noncyanobacterial core biomass in photogranules were determined from the light and phycocyanin autofluorescence images of cross-sectioned photogranules using the software ImageJ.\textsuperscript{135} The volume of noncyanobacterial core biomass was directly calculated from its diameter using an Excel spreadsheet. Phototrophic microbial groups were identified by brightfield microscopy based on morphological information provided in Wehr \textit{et al.}\textsuperscript{136} For autofluorescence microscopy, we used an ET535/50x excitation filter, which is recommended for the detection of cyanobacteria according to the Handbook of Methods in Aquatic Microbial Ecology.\textsuperscript{137}

Photogranules were prepared for SEM following the procedure presented in Milferstedt \textit{et al.}\textsuperscript{17} Briefly, the entire photogranule was suspended in an unbuffered glutaraldehyde solution (1%) and gently shook. After 3–4 h, photogranules were picked from solution and cross-sectioned on wax paper at the maximum diameter under a stereomicroscope. Photogranules and cross-sectioned photogranules were then rinsed with phosphate buffer solution (50 mM Na\textsubscript{2}HPO\textsubscript{4}·2H\textsubscript{2}O; 50 mM NaH\textsubscript{2}PO\textsubscript{4}·H\textsubscript{2}O; pH = 7) three times over 30 min (10 min each). Next, samples were resuspended in osmium tetroxide solution (1%), agitated for 1.5 h at room temperature, and then rinsed with phosphate buffer for 15 min. Samples were then washed with Milli-Q water three times over 40 min. For dehydration, samples were suspended in a graded ethanol series (25%, 50%, 75% and 100%) for 20 min in each grade. The samples were then dried using tertiary butanol
Lastly, photogranule samples were sputter-coated with 1–2 nm gold-palladium using a Polaron E5100 sputter coater. SEM images were taken using a FEI Quanta 200 SEM operated at 15 kV.

3.2.4. Measurements of chlorophylls in photogranules

Extraction and quantification of chlorophylls followed Standard Methods 10200H. Three 10 mL biomass samples were transferred in 50 mL screw-cap centrifuge tubes and then centrifuged at 12,000 rpm for 10 min at 4 °C to separate photogranules from liquid. For chlorophyll extraction, liquid was removed, and the photogranule pellet was resuspended in aqueous acetone solution [one-part saturated magnesium carbonate solution (1%) in nine-parts of acetone] to a volume of 10 mL. Samples were then homogenized at 700 rpm for 30 sec and incubated overnight at 4 °C. Chlorophyll a, b and c concentrations (mg/mL) in the final extracts were determined spectrophotometrically following Standard Methods (10200H). Next, the mass fraction (%) of chlorophyll a, b, and c in each photogranule size class was obtained by dividing chlorophyll concentration (mg/L) by volatile suspended solids (VSS) concentration (mg/L) and then multiplying by 100. Chlorophyll measurements were done on triplicate biomass samples.

3.2.5. Measurement of phycobilin in photogranules

The methods by Bennett and Bogorad and Islam et al. were modified and used to determine phycobiliprotein content in photogranule samples. Briefly, three 10 mL biomass samples were pipetted in 50 mL centrifuge tubes, and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the biomass pellet was resuspended...
in 0.025 M phosphate buffer saline solution (pH 7) to a volume of 10 mL. Biomass samples in phosphate buffer saline solution were then homogenized at 700 rpm for 1 min. After homogenization, samples were sonicated at 20% strength for 2 min, and then centrifuged at 12,000 rpm for 10 min. The absorbance of supernatant was measured spectrophotometrically at the wavelengths 562 nm, 615 nm, and 652 nm. The equations by Bennett and Bogorad\textsuperscript{140} were used to quantify the phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC). Total phycobilin concentration (mg/mL) in samples was calculated as the sum of PC, PE, and APC concentrations. Finally, the mass fraction (%) of phycobilin in each photogranule size class was obtained by dividing phycobilin concentration (mg/L) by volatile suspended solids (VSS) concentration (mg/L) and then multiplying by 100.

3.2.6. Extracellular polymeric substances (EPS) in photogranules

EPS was extracted from the photogranular biomass following the sequential sonication and base extraction method presented in Ansari et al.\textsuperscript{16} Three 10 mL biomass samples were pipetted into 50 mL centrifuge tubes and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was then removed, and the biomass pellet was resuspended in 10 mL phosphate buffer saline (PBS) solution (10 mM NaCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, and 6 mM Na\textsubscript{2}HPO\textsubscript{4}). Samples were then homogenized at 700 rpm for 30 s. Homogenization was followed by sonication at 10% strength for 40 sec, and then centrifugation at 12000 rpm for 10 min. After centrifugation, the supernatant was collected and filtered with 0.45 μm cellulose filter. This supernatant was considered as the “biomass-bound EPS extracted by sonication”.

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The remaining biomass pellets after sonication extraction were resuspended in 10 mL PBS solution. The pH of samples was adjusted to 10.5 using 1 M NaOH. The samples were then shaken at 400 rpm for 2 h at 4 °C. Next, samples were centrifuged at 12000 rpm for 10 min. The supernatant was filtered with a 0.45 μm cellulose filter. This supernatant was considered as the “biomass-bound EPS extracted by base” The total biomass-bound EPS was calculated by taking the sum of biomass-bound EPS extracted by sonication and base. EPS samples were either processed or frozen at −20 °C until the analysis. Polysaccharide and protein concentrations in extracted EPS were measured using Dubois method\textsuperscript{142} and modified Lowry method,\textsuperscript{143} respectively. Finally, EPS protein and polysaccharide content of each photogranule size class (mg/g VSS) was obtained by dividing EPS protein and polysaccharides concentration (mg/L) by volatile suspended solids (VSS) concentration (g/L).

3.2.7. Specific oxygen production rate (SOPR) of photogranules

The oxygen production rate (mg O\textsubscript{2}/L-h) of each photogranule size class was determined in a closed batch system following the procedure shown in Abouhend \textit{et al.}\textsuperscript{15} Briefly, each photogranule size class was suspended in 2x diluted primary-effluent wastewater and then transferred to a 300 mL biochemical oxygen demand (BOD) bottle with no headspace. The initial biomass concentration in all bottles was adjusted to be approximately 1000 mg/L. The BOD bottles were purged with nitrogen, mixed at 100 rpm using a magnetic stirrer, and then exposed to light at photosynthetically active radiation (PAR) of approximately 150 μmol/m\textsuperscript{2}-s. The dissolved oxygen concentration in BOD bottles was monitored over time until dissolved O\textsubscript{2} gets saturated. We plotted the dissolved
oxygen concentration versus time and then determined “the net oxygen production rate (OPR_{net})” of photogranular biomass as the slope of the linear-regression line.

We also measured the initial and final concentrations of chemical oxygen demand (COD) and nitrogen species in the liquid phase. Next, we calculated the theoretical amount of oxygen consumed (OPR_{consumed}) for the oxidation of organic matter and nitrification during the batch reaction.

The total OPR (OPR_{total}) of the OPG biomass was, therefore, obtained based on the following equation:

\[
OPR_{total} = OPR_{net} + OPR_{consumed}
\]  

(1)

where OPR_{total} is the total OPR of OPG biomass, OPR_{net} is the net OPR of OPG biomass, and OPR_{consumed} is the theoretical oxygen consumption rate for organic matter oxidation and nitrification.

The specific OPR (SOPR) of OPG biomass was then obtained using the following equation:

\[
SOPR = OPR_{total} / X_{OPG}
\]  

(2)

where OPR_{total} is the total OPR of OPG biomass and X_{OPG} is the concentration of OPG biomass in the system.

3.2.8. Analytical methods

Measurements of total and volatile suspended solids of the photogranule size classes were conducted according to Standard Methods 2540D/E.\textsuperscript{139} Chemical oxygen demand (COD) was determined following Standard Methods 5220D.\textsuperscript{139} Nitrogen species (ammonia, nitrite, and nitrate) were measured using ion chromatography (Metrohm 830). Zone settling velocity (ZSV) and sludge volume index (SVI) of photogranule size classes were determined following Standard Methods (2710D/E).\textsuperscript{139} The images of OPG biomass
in Petri dishes were collected and subjected to particle size distribution (PSD) and roundness analyses using the software ImageJ.\textsuperscript{135} Roundness values were obtained in the range of 0–1. A perfect spherical granule had a roundness of 1 while a needle-shaped particle had a roundness close to 0.

### 3.2.9. Statistical analysis

The two-samples *t*-test was performed to determine whether there is a statistically significant difference between the variables. We set the default alpha (\(\alpha\)) value at 0.05 so if the p-value is less than 0.05 there is a statistically significant difference between the variables. Regression analysis was also conducted to examine the relationship between the variables.

### 3.3. Results

#### 3.3.1. Source of photogranules and their size separation

The photogranules that we used for the current investigation were generated in lab-scale sequencing batch reactors (SBRs), treating primary-effluent wastewater without aeration.\textsuperscript{15} The mixed photogranular biomass (i.e., biomass in reactors) was obtained from SBRs during the steady-state period between day 100 and day 150. The particle size distribution (PSD) of this biomass is shown in Figure 3.1. The number of photogranules <1 mm was significantly greater than the number of photogranules >1 mm. However, the biovolume of the photogranules <1 mm was less than 10\% of the total biovolume of the mixed photogranular biomass. The largest size of photogranules was approximately 4.5 mm.
Figure 3.1. Number-based and biovolume-based particle size distribution (PSD) of mixed OPG biomass in reactors at the time of sampling.

We used a wet sieving method\textsuperscript{134} to sort photogranules into different size groups. Figure 3.2 shows the images of the photogranules separated into seven size classes used in this study: (1) <0.2; (2) 0.2–0.5; (3) 0.5–1; (4) 1–1.7; (5) 1.7–2.5; (6) 2.5–3.5; and (7) 3.5–4.5 mm.
Figure 3. Reactor photogranules classified into seven size groups. (A) Photogranules less than 0.2 mm in diameter. Parts B–G include photogranules that fall into the following size ranges: (B) 0.2–0.5 mm in diameter. (C) 0.5–1 mm in diameter. (D) 1–1.7 mm in diameter (E) 1.7–2.5 mm in diameter. (F) 2.5–3.5 mm in diameter. (G) 3.5–4.5 mm in diameter. Scale bar for all panels is 1 cm.
3.3.2. Enrichment of filamentous cyanobacteria during photogranulation

We measured chlorophylls \(a\), \(b\), and \(c\) and phycobilin and determined their weight fractions in photogranular biomass. As photogranule size increased from the smallest group \(<0.2\) mm to the class \(0.5–1\) mm in diameter, the fraction of chlorophyll \(a\) in biomass increased from \(0.69\pm0.05\%\) to \(1.14\pm0.08\%\) (Figure 3.3a), indicating enrichment of phototrophs. For the same size classes, however, chlorophyll \(b\) and chlorophyll \(c\) decreased from \(0.13\pm0.01\%\) to \(0.12\pm0.01\%\) and \(0.11\pm0.01\%\) to \(0.08\pm0.01\%\), respectively (Figure 3.3b, Figure 3.3c). These results suggest a decrease in the phototrophic groups producing chlorophyll \(b\) and chlorophyll \(c\), such as green algae and diatoms, respectively. Consequently, the ratios of chlorophyll \(a/b\) and chlorophyll \(a/c\) significantly increased from \(5.2\pm0.2\) to \(9.7\pm0.7\) and \(6.4\pm0.5\) to \(13.6\pm1.8\), respectively (Figure 3.3d, Figure 3.3e).

Similar to chlorophyll \(a\), phycobilin, an accessory photosynthetic pigment in cyanobacteria, increased from \(2.9\pm0.19\%\) to \(6.1\pm0.2\%\), showing 110\% increase, as the photogranule size increased from \(<0.2\) mm to \(0.5–1\) mm (Figure 3.3f). The phycobilin/chlorophyll \(a\) ratio also increased from \(4.2\pm0.3\) to \(5.4\pm0.4\) (Figure 3.3g). There were significant differences for the ratio of phycobilin/chlorophyll \(a\) between the size classes \(<0.2\) mm and \(0.2–0.5\) mm (p-value 0.001) and between \(0.2–0.5\) mm and \(0.5–1\) mm (p-value 0.048). These results suggest that phototrophic enrichment in photogranules up to \(0.5–1\) mm in diameter was mainly due to the growth of phototrophs that contain chlorophyll \(a\) and phycobilin, but not chlorophyll \(b\) and chlorophyll \(c\), i.e., cyanobacteria.
Figure 3.3. Photosynthetic pigment content in photogranules in different size groups. (A–C) The mass fraction of chl $a$, chl $b$, and chl $c$ in OPG biomass. (D–E) The ratio of chl $a$ to $b$ and chl $a$ to $c$. (F) The mass fraction of phycobilin in OPG biomass. (G) The ratio of phycobilin to chl $a$. (H) The relationship between the mass fraction of chl $b$ and chl $c$ in OPG biomass. Error bars represent the standard deviations of triplicate samples.
As the photogranules grew over 0.5–1 mm in diameter, the fraction of chlorophyll \( a \) in the biomass started to decrease with the size. As the photogranule size increased from 0.5–1 mm to 3.5–4.5 mm, the largest size class in this study, chlorophyll \( a \) decreased from 1.14±0.08\% to 0.54±0.07\% (Figure 3.3a), indicating significant reduction in phototrophic population. The fractions of chlorophyll \( b \) and chlorophyll \( c \) continued to decrease over these size classes until they reached 0.03±0.004\% and 0.03±0.007\%, respectively, in the size class 3.5–4.5 mm in diameter (Figure 3.3b, Figure 3.3c). Despite decreases in all chlorophyll contents after class 0.5–1 mm, the chlorophyll \( a/b \) and chlorophyll \( a/c \) ratios still show clear increasing trends (Figure 3.3d, Figure 3.3e). These results indicate that the decrease in overall phototrophic population (seen by chlorophyll \( a \)) as the photogranules grew beyond size class 0.5–1 mm is once again due to continuing decreases in the population of phototrophs producing chlorophyll \( b \) and \( c \).

The fraction of phycobilin continued to increase with photogranule size until it reached 7.3±0.3\% in the size class 1.7–2.5 mm, indicating enrichment of cyanobacteria in photogranules growing up to this size fraction (Figure 3.3f). The phycobilin/chlorophyll \( a \) ratio also increased from 5.4±0.4 to 9.4±0.5 for the same size classes (Figure 3.3g). As photogranules grew above 1.7–2.5 mm in diameter, the weight fraction of phycobilin started to decrease. Phycobilin decreased from 7.3±0.3\% to 4.2±0.1\% as the photogranules’ diameter increased from 1.7–2.5 mm to 3.5–4.5 mm (Figure 3.3f). The fraction of phycobilin in photogranules was significantly different between size classes 1.7–2.5 mm and 2.5–3.5 mm (p-value 0.03) and between 2.5–3.5 mm and 3.5–4.5 mm (p-value 0.0002). These results suggest that the relative population of cyanobacteria in
photogranules also becomes smaller once the photogranules grow above 1.7−2.5 mm, especially above 2.5−3.5 mm in diameter.

Among seven size classes, the size class 0.5−1 mm showed the highest content of chlorophyll \(a\) (1.14±0.08%) while the size class <0.2 mm showed the highest fraction of chlorophyll \(b\) and \(c\) (0.13±0.01% and 0.11±0.01%, respectively). The size class 3.5−4.5 mm showed the lowest content of all chlorophylls. Chlorophyll \(b\) and chlorophyll \(c\) showed a strong positive correlation \((R^2 = 0.97, \text{Figure 3.3h})\). Correlations between chlorophyll \(a\) and chlorophyll \(b\) or between chlorophyll \(a\) and chlorophyll \(c\) were much weaker \((R^2 = 0.31\) and 0.17, respectively). For phycobilin, the size class <0.2 mm showed the lowest fraction \((2.9±0.19%)\) and the size class 1.7−2.5 mm showed the highest fraction \((7.3±0.26%)\). These results suggest that the phototrophic community in photogranules shift from green algae/diatoms in the smaller photogranules to cyanobacteria in the larger photogranules.

3.3.3. Structural development of photogranules

The biomass aggregates in the smallest size class (<0.2 mm in diameter) were not spherical but possessed filamentous cyanobacteria, green algae, diatoms, and bacterial biomass. This size class showed an average roundness of 0.55±0.11 (Figure 3.4). As the size of photogranules increased from <0.2 mm to 3.5−4.5 mm, the roundness increased from 0.55±0.11 to 0.93±0.06, indicating that the photogranular biomass becomes more spherical as it grows larger (Figure 3.4).
As seen with the brightfield and phycocyanin autofluorescence microscopy of cross-sectioned photogranules (Figure 3.5), the photogranules underwent sequential structural changes as they evolved into larger sizes. In photogranules smaller than 3 mm in diameter, filamentous cyanobacteria were spread over the whole body (Figure 3.5a–c, Figure 3.5a'–c').

Scanning electron microscopy (SEM) also showed that high levels of filamentous cyanobacteria are located across the granular body in photogranules <3 mm in diameter (Figure 3.6a–c, Figure 3.7a–c). Photogranules of 3 mm in diameter and above showed a distinct layered structure where a mat-like outer layer of filamentous cyanobacteria encloses noncyanobacterial core biomass (Figure 3.5d–g, Figure 3.5d'–g').
Figure 3.5. Microscopic images showing the structural development of photogranules produced in reactors. (A–G) Cross-sections of photogranules viewed by brightfield light microscopy. (A’–G’) The same cross-sections of photogranules viewed by phycocyanin autofluorescence of cyanobacteria. Scale bar for all panels is 1 mm.
Figure 3.6. Scanning electron microscopy (SEM) of cross-sectioned photogranules of two different sizes. (A) Cross-section of a small photogranule, approximately 1 mm in diameter. (B) Cyanobacterial outer layer of the same photogranule shown in panel (A). (C) The center of the same photogranule shown in panel (A). (D) Cross-section of a large photogranule, approximately 4 mm in diameter. (E) Cyanobacterial outer layer of the same photogranule shown in panel (D). (F) The center of the same photogranule shown in panel (D). Scale bars for panels are (A): 250 μm; (B) and (E): 50 μm; (C) and (F): 25 μm; and (D): 1 mm.

SEM also revealed that filamentous cyanobacteria dominate the outer layer of these large photogranules while the filamentous cyanobacteria are basically absent in the core center of photogranules (Figure 3.6d–f, Figure 3.7d–f). In this core, diatoms, unicellular green algae as well as filamentous bacteria were more readily observed along with EPS-like matter (Figure 3.6f, Figure 3.7d–f). In addition, many fragments of dead diatoms were observed in the center of these large-size photogranules (Figure 3.7e).
The diameter of the noncyanobacterial core increased linearly as the size of photogranules increased from 3 mm to 4.5 mm (Figure 3.8a). The volume fraction of noncyanobacterial core consequently increased from 0.6±0.3% to 10.7±1.9%, suggesting repression of filamentous cyanobacteria in the photogranules greater than 3 mm (Figure 3.8b). These results are consistent with the phycobilin data that showed that the weight fraction of phycobilin starts decreasing from the size class that is 2.5–3.5 mm in diameter. This is also supported by the unchanged depth of the cyanobacterial layer despite the increase in the size of photogranules above 3 mm in diameter (Figure 3.8c).
Figure 3.8. Changes in depth and size of cyanobacterial and noncyanobacterial biomass in photogranules greater than 3 mm in diameter. (A) Changes in the diameter of noncyanobacterial core biomass per the diameter of photogranules. (B) Changes in the volume of noncyanobacterial core biomass in photogranules per the diameter of photogranules. (C) Constant depth of cyanobacterial layer in photogranules greater than 3 mm in diameter. Error bars represent the standard deviations of triplicate measurements.
In photogranules that were 3−4.5 mm in diameter, the thickness of the cyanobacterial layer remained constant at 1.25±0.14 mm. In a spherical structure, this would mean a total depth of 2.5 mm for cyanobacterial growth, which is consistent with Figure 3.5c' showing that in this size of photogranules, filamentous cyanobacteria can be located anywhere in the photogranules.

3.3.4. EPS in photogranules

Concentrations of both EPS proteins and polysaccharides showed a clear decreasing trend as the photogranules grew in size (Figure 3.9). EPS proteins decreased from 150±32 mg/g volatile suspended solids (VSS) to 65±11 mg/g VSS as the photogranules grew from the size class <0.2 mm to the size class 1.7−2.5 mm (Figure 3.9a). For the same size classes, EPS polysaccharides decreased from 49±7 mg/g VSS to 17±2 mg/g VSS (Figure 3.9b). However, both EPS proteins and polysaccharides also increased once the photogranules grew above 1.7−2.5 mm. As the photogranule size increased from 1.7−2.5 mm to 3.5−4.5 mm, EPS proteins and polysaccharides increased to 89±11 mg/g VSS and 32±4 mg/g VSS, respectively (Figure 3.9a, Figure 3.9b). The levels of EPS proteins between size classes 1.7−2.5 mm and 3.5−4.5 mm are likely different (p-value 0.057). Also, statistically significant differences exist between the levels of EPS polysaccharides in size classes 1.7−2.5 mm and 3.5−4.5 mm (p-value 0.01). We found moderate to strong negative relationships between EPS and phycobilin: EPS proteins and phycobilin ($R^2 = 0.75$, Figure 3.9c); EPS polysaccharides and phycobilin ($R^2 = 0.95$, Figure 3.9d).
3.3.5. Oxygen production by photogranules at different sizes

To study how the photogranules’ size affects their oxygen-producing capability, along with the removal of oxygen-demanding matters, we determined the oxygen production rate (OPR) of OPG biomass from different size groups. Figure 3.10a shows the specific oxygen production rate (SOPR) of photogranules in different size classes.
Figure 3. 10. Specific oxygen production rates (SOPR) of different photogranule size classes and their correlation to chlorophyll a content in photogranules. (A) SOPR. (B) Regression analysis showing a linear relationship between SOPR and chlorophyll a in photogranules. Error bars represent the standard deviations of triplicate samples.

SOPR initially increased from 10.9±0.8 to 21.9±1.3 mg O₂/g VSS-h as the photogranule size increased from <0.2 mm to 0.5−1 mm. SOPR then decreased to 8.1±0.5 mg O₂/g VSS-h along with the increase in photogranule size from 0.5−1 mm to 3.5−4.5
mm. The SOPR correlated very strongly with the weight fraction of chlorophyll \( a \) in photogranules \((R^2 = 0.99, \text{Figure 3.10b})\). Among seven size classes, photogranules in the size range of 0.5−1 mm showed the highest SOPR, on average, 21.9±1.3 mg O\(_2\)/g VSS-h. This SOPR was much greater than the SOPR of mixed photogranular biomass collected from the same reactor, which showed, on average, 12.6±2.4 mg O\(_2\)/g VSS-h.

### 3.3.6. Settleability of photogranules

Effective separation of biomass from water is also an advantage of the OPG process for wastewater treatment. We determined ZSV and SVI to study how the photogranules’ growth in size impacts their settling properties (Figure 3.11). The biomass in the smallest size class (<0.2 mm in diameter) showed ZSV and SVI of 3±1.6 m/h and 383±5 mL/g VSS, respectively. These values indicate better biomass settling characteristics of the OPG size class <0.2 mm than the field activated sludge. The activated sludge from the WWTP where we collected wastewater to feed the OPG systems showed ZSV in 0.1−0.2 m/h and SVI in 300−400 mL/g. ZSV of photogranules gradually, and substantially, increased from 3±1.6 m/h to 78.4±4.1 m/h, as the photogranules grew from <0.2 mm to 3.5−4.5 mm (Figure 3.11a). SVI decreased from 383±5 mL/g VSS to 39±2.8 mL/g VSS, as the size class of photogranules increased from <0.2 mm to 1−1.7 mm, which is a strong indication of progression of granulation (Figure 3.11b). The ZSV and SVI of the size class 0.5−1 mm, which showed the highest oxygen production rate, were 24.6±5 m/h and 40.5±2.8 mL/g VSS, respectively. These values are more favorable for operation compared to the values of ZSV and SVI observed for the mixed photogranular biomass, 14.6±0.5 m/h and 53±2 mL/g VSS, respectively.
Figure 3.11. Settling properties of photogranule in different size classes. (A) Zone settling velocity (ZSV). (B) Sludge volume index (SVI). Error bars represent the standard deviations of triplicate samples.
3.4. Discussion

Photogranules, like any other granules, grow in reactor systems via two means: the number and the size. While we previously showed the growth of photogranules from the reactor’s mixed biomass standpoint, how an individual photogranule grows to a larger size and how this change would impact the system performance has remained mainly unknown. The current study focuses on these important matters, by investigating the physicochemical and biological characteristics of photogranules in different sizes and their capability of producing O$_2$, the key property of the OPG process for wastewater treatment.

We found that the increase in photogranule’s size occurs along with the change in phototrophic community, based on the relative change in the levels of photosynthetic pigments in photogranules. In the literature, photosynthetic pigments are commonly used for the detection and quantification of phototrophic community. Chlorophyll $a$ is present in all phototrophic microorganisms including green algae, diatoms, and cyanobacteria. Among phototrophic microorganisms, chlorophyll $b$ occurs only in green algae while chlorophyll $c$ is found only in diatoms and dinoflagellates. Phycobilin is mainly found in cyanobacteria. In the current study, the mass fractions of chlorophyll $b$ and chlorophyll $c$ showed clear decreasing trends with the increase in photogranule size (Figure 3.3b, Figure 3.3c), suggesting a decrease in the abundance of green algae and diatoms, respectively. A strong positive correlation found between chlorophyll $b$ and chlorophyll $c$ (Figure 3.3h) also indicates that this phototrophic community experienced a similar fate along with size progression in photogranules. The decrease in the abundance of green algae and diatoms with increasing photogranule size could be due to the light limitation developed inside larger photogranules. According to Kühl et al., light
limitation progressively increases with depth in phototrophic mats and, thus, phototrophs in deeper layers of mats experience less light.

In contrast to green algae and diatoms, the growth of filamentous cyanobacteria was promoted during the size evolution of photogranules that occurs in reactors. Both chlorophyll \((a, b, c, a/b, \text{ and } a/c)\) and phycobilin data showed that the growth of photogranules up to 1.7–2.5 mm in diameter continued with the enrichment of cyanobacteria. This suggests that cyanobacteria were better adapted to a light-limiting condition than green algae and diatoms. Earlier, Milferstedt et al.\(^{17}\) also reported the presence of high densities of filamentous cyanobacteria in both reactor photogranules and hydrostatically formed photogranules based on MiSeq DNA sequencing analysis. Once the photogranules exceed this size, the photogranules’ cyanobacterial fraction also started to decrease. It is important to note that this decrease coincides with the appearance of a layered structure in the photogranules in which a concentric cyanobacterial layer encloses noncyanobacterial core biomass (Figure 3.5).

We currently postulate that the development of this layered structure is caused by migration of filamentous cyanobacteria towards the photogranule’s surface. Subsection III filamentous cyanobacteria, the main cyanobacterial group enriched in photogranules,\(^{17}\) exhibit phototaxis, which is a movement in response to light.\(^{149–151}\) It can be therefore inferred that the gliding motility of filamentous cyanobacteria gives them the ability to be relocated where light conditions are more favorable, thus thriving in photogranules that increase in size. If the outer layer is formed mainly due to the new growth of cyanobacteria on photogranule’s surface, we would also expect to see significant growth of green algae and diatoms. However, we did not observe that occurrence. The results of EPS also tend to
support the current discussion. It is well known that subsection III filamentous cyanobacteria secret and use EPS for their gliding motility.\textsuperscript{152–154} Significant increases in EPS polysaccharides and proteins, especially the former, within photogranules larger than 2.5 mm (Figure 3.9a, Figure 3.9b) coincide with the development of a layered structure within the photogranules (Figure 3.5). Kuo-Dahab \textit{et al.}\textsuperscript{19} also showed increase in EPS polysaccharides during photogranulation of activated sludge under hydrostatic conditions, and further showed that copious amounts of slimes and sheaths are closely associated with filamentous cyanobacteria in the outer layer of photogranules.

The gliding mobility would, therefore, provide clear advantage to filamentous cyanobacteria over green algae and diatoms during the size evolution of photogranules. Nevertheless, the depth of the cyanobacterial layer remains unchanged although the photogranules continued to grow above 3 mm in diameter (Figure 3.8c). This observation suggests that there is a maximum depth limiting the growth of filamentous cyanobacteria in photogranules. This means as the photogranules continue to grow bigger, the fraction of filamentous cyanobacteria in photogranules becomes smaller, which will ultimately lead to the point where the structure of photogranules may not be sustained. The maximum size of photogranules in this study, however, could have been greater if we had used a lower hydraulic shear force to mix the system. There are several lines of discussion to support this statement. First, the volume fraction of the cyanobacterial layer in photogranules at 4.5 mm in diameter is approximately 90%, which is still substantially greater than the volume fraction of noncyanobacterial aggregates. Furthermore, hydrostatically formed photogranules reported are much larger than the size of photogranules grown in the reactors. Finally, our ongoing reactor study showed that the photogranules growing in
reactors with mixing at 50 rpm (while other operational conditions remain same as the current study) grow up to approximately 5.5 mm in diameter.

The layered structure in photogranules looks similar to that in microbial mats.\textsuperscript{154–157} Nevertheless, photogranules self-organize and grow into a spherical aggregate, whereas mats are formed on a planar solid surface. As discussed by Milferstedt \textit{et al.}\textsuperscript{17} granules similar to photogranules also appear in glacier environments. Cryoconites that form in the melt holes of glacier surfaces are also spherical or sphere-like granules in which filamentous cyanobacteria serve as a key granulating species.\textsuperscript{127–129,158} The layered structure observed in photogranules also appears in cryoconite granules.\textsuperscript{127} The outer layer thickness in cryoconite granules varied among different granules, but is generally about 0.2 mm on average.\textsuperscript{127} This suggests that photogranules and cryoconite granules share the formation mechanism despite extremely different environments in which the growth of these granules occurs. Notably, photogranules are organic rich whereas cryoconite granules are mineral rich\textsuperscript{127–129} and can contain inorganic matter even up to 98\%.\textsuperscript{129} This again suggests that conditions under which these two granule types are generated are substantially different. Knowing the environmental (macro and micro) conditions and biological responses necessary to not only select filamentous cyanobacteria but also induce their physiology leading to a common granular morphology will thus be important to understand the formation of both photogranules and cryoconite granules. We believe this warrants future investigation.

The OPG process is a self-aerating wastewater treatment system where the photogranular biomass produces the oxygen required for the oxidation of organic matter and nitrification.\textsuperscript{15,17} Therefore, the oxygen production capacity of the photogranular
biomass is a key design consideration in the OPG process. In this study, the OPR of photogranules was determined based on the direct measurements of dissolved O$_2$ in closed bottles as well as the theoretical amount of oxygen required for the oxidation of influent COD and ammonia. We assumed that COD that might be released by the growth of OPG biomass in this batch would have been negligible, because Abouhend et al.\textsuperscript{15} demonstrated that autonomous O$_2$ production in an OPG reactor mainly relies on the degradation of influent COD. The generation of dissolved O$_2$ in closed systems indicates the diffusion of O$_2$ from the body of photogranules into bulk liquid. Hann et al.\textsuperscript{159} also showed the gradient of dissolved O$_2$ established within the photogranules where the level of dissolved O$_2$ decreases with depth, suggesting diffusion of oxygen from the outer layer toward the center of photogranules. These outward and inward oxygen diffusions are absent in other microbial granules. For example, in aerobic granule sludge, oxygen is always provided from the external source and diffuses from the bulk liquid into the granule.\textsuperscript{24,41,133}

Our results showed that photogranule size has large influence on the biomass’s capability of producing oxygen and, thus, its treatment potential. Photogranules in the size class 0.5−1 mm showed the highest oxygen production rate, which is 1.4 to 2.7 times greater than that from other size classes. Earlier, Abouhend \textit{et al.}\textsuperscript{15} reported the performance of the OPG systems treating real wastewater along with the evolution of photogranule size over five-months period. During day 50 to day 60, when the majority of photogranular biomass was in the range of 0.5−1 mm with a mean size of 0.75 mm, the reactors showed the efficiency of total COD (tCOD) removal at 88±3%. During the same operation period, effluents tCOD was, on average, 16±5 mg/L, suggesting high effluent quality. This tCOD removal was significantly higher than the removal observed during
earlier or later operation periods when the mean size of biomass, based on both number and biovolume of photogranules in reactors, was smaller than 0.5 mm or larger than 1 mm: 78±7% and 79±5%, respectively. These observations, therefore, suggest that the treatment capacity of the OPG systems will be influenced by size and the oxygenic function of photogranules.

A very strong correlation between SOPR and the fraction of chlorophyll $a$ in the photogranular biomass (Figure 3.10b) indicates that the photogranules’ oxygen production is directly determined by the relative population of phototrophic organisms. The size progression to the size class 0.5−1 mm, showing the highest SOPR among seven size classes, occurs with significant increase in the fraction of phycobilin but decrease in the fraction of chlorophyll $b$ and chlorophyll $c$. Further development to the size class 1−1.7 mm, the group with the second highest SOPR, came along with continuous increase in phycobilin but substantial decrease in chlorophyll $b$ and chlorophyll $c$, indicative of maturation in photogranulation with the enrichment of filamentous cyanobacteria. Hence, photogranules in these two size groups, i.e., photogranules in 0.5−1.7 mm in diameter, would be optimal for the OPG process with respect to both oxygen production (thus, treatment capacity) and the stability or maturity of photogranules. It can also be inferred that the photogranule size greater than 2.5 mm in diameter in which relative cyanobacterial population further decreases and the layered structure starts to develop will not be as effective as smaller photogranules in terms of aerobic treatment. These bigger photogranules may be exploited for nitrogen removal based on simultaneous nitrification and denitrification (SND) based on the formation of stratified structure and the presence of both oxic and anoxic environments within the same biomass. According to Abouhend et
al.,\textsuperscript{15} however, nitrogen removal by SND pathway seems negligible since major nitrogen removal in the OPG systems occurred via bioassimilation and denitrification occurring during the dark cycle (i.e., no light and no oxygen). Nonetheless, the occurrence of SND-based nitrogen removal by different size photogranules may need more investigation.

It can be concluded that the structure and oxygen production capacity of photogranule biomass are both size-dependent. These results are expected to help optimize the OPG process by maintaining the optimal size of the photogranule in the system and, thus, operating the system at high treatment efficiency and stability with respect to granulation. Currently, we speculate that control of the photogranule size can be achieved by using approaches that are already being used in other granular technologies. In aerobic granule sludge systems, for example, an increase in hydrodynamic shear in reactors has been proposed to suppress the overgrowth of large granules.\textsuperscript{21} Thus, the intermittent provision of high-shear conditions may also limit the overgrowth of OPGs in bioreactors. Other approaches include the use of devices that enable the separation of biomass based on size, such as hydrocyclones,\textsuperscript{160–162} which are already being used on an industrial scale.\textsuperscript{160} For this kind of selective biomass removal, however, we may need to consider the growth rates of photogranules and how they may affect the effectiveness of granulation, as the growth rates of photogranules are expected to be different depending on size. Future work will need to establish methods to maintain the optimal sizes of photogranules as well as examine the hypothesis that size control will optimize system performance in reactor operations. Collectively, the engineering of the OPG system based on a better understanding of the growth and function of photogranules is expected to advance the
development of a new granular technology, which has the potential to treat wastewater without energy intensive aeration.
CHAPTER IV

INVESTIGATING THE ROLE OF IRON IN THE DEVELOPMENT AND SIZE-GROWTH OF OXYGENIC PHOTOGRA NULES IN BIOREACTORS

Abstract

In OPG-based wastewater treatment, the granulation process progresses with the increase in the size of photogranules. The evolution of the photogranules’ size occurs with the enrichment of motile filamentous cyanobacteria, the key granulating microbial group leading to the formation of OPGs. To date, the factors that promote the growth and aggregation of filamentous cyanobacteria within photogranules are not clearly understood. The objective of this study was to investigate the role of iron (Fe) in the development and size-growth of oxygenic photogranules (OPGs) in bioreactors treating wastewater without aeration. We found that the enrichment of filamentous cyanobacteria within photogranules as they grew bigger in size occurs simultaneously with significant increases in the concentrations of Fe in the whole biomass as well as in its pellet (the whole biomass minus EPS). This happened along with significant decreases in the concentrations of EPS as well as Fe that is bound with EPS. Strong negative correlations were found between the abundance of cyanobacteria and EPS as well as between cyanobacteria and Fe bound with EPS. These results suggest that cyanobacteria are able to utilize EPS and Fe bound with EPS for their growth, enhancing their dominance among the phototrophic community within OPGs and that the granulation (i.e., increase in OPG size) was promoted as both EPS and Fe bound with EPS became limited. In this study, we also studied the fate and distribution of manganese (Mn), copper (Cu), and zinc (Zn) in the size-growth of OPGs.
In contrast to Fe, other heavy metals did not show a good correlation with the abundance of cyanobacteria. The study also shows that while both Fe and Mn were mainly present in the biomass pellet, Cu and Zn primarily remained in EPS (>86%) regardless of size-growth, suggesting selective metal uptakes occurring in OPGs. The study, therefore, suggests that Fe plays a crucial role in the growth and aggregation of filamentous cyanobacteria and thus the size-growth of photogranules in bioreactors.

4.1. Introduction

Oxygenic photogranules (OPGs) are a new photogranule type recently developed to treat wastewater without supplemental aeration.\textsuperscript{15–17} Oxygenic photogranules (OPGs) are self-immobilized microbial aggregates with dense structures and spherical morphologies, primarily composed of filamentous cyanobacteria, green algae, and nonphototrophic bacteria.\textsuperscript{15,17,163} In OPG reactors, photogranules grow bigger in size, simultaneously occurring with the enrichment of motile filamentous cyanobacteria.\textsuperscript{15,17,163,164} In photogranules with a diameter of 2.5 mm or less, filamentous cyanobacteria are found throughout the granule's body, providing the network for other microorganisms to bind and aggregate.\textsuperscript{163} However, photogranules larger than 2.5 mm in diameter typically showed a distinct layered structure where the outer layer of filamentous cyanobacteria enclosed bacterial biomass.\textsuperscript{163} It seems that the enrichment of filamentous cyanobacteria is necessary for the development and size-growth of photogranules in bioreactors because not only are they the most dominant phototrophs in OPG but also their mat-like layer maintains the structural integrity of the larger photogranules.\textsuperscript{17,163} However,
the factors that promote the growth and aggregation of filamentous cyanobacteria within photogranules along with their size-growth are not well understood.

One of the potential factors that affect the growth and physiology of cyanobacteria can be iron (Fe) availability. Iron (Fe) is an essential micronutrient for phototrophic microorganisms.\textsuperscript{65–71} Fe plays a central role in mediating the growth of phototrophs because it is necessary for the synthesis and activities of key enzymes involved in photosynthesis, electron transport, energy production, and nitrogen transformations (i.e., nitrate and nitrite assimilation, N\textsubscript{2} fixation).\textsuperscript{65,66,74,76} Iron limitation can therefore affect multiple metabolic pathways within phototrophs, most importantly photosynthesis.

Compared to other phototrophs such as eucaryotic microalgae, cyanobacteria require much more iron for their growth due to the higher demand for iron in photosynthesis and the abundance of iron-containing enzymes in the nitrogen-fixation machinery.\textsuperscript{65,68,104,165–167} It has been reported that cyanobacteria require 5–8 times higher cellular Fe : C ratio than eukaryotic algae growing under similar lighting conditions.\textsuperscript{76,85,168} However, excessive free intracellular iron is very toxic to the cyanobacterial cells because it catalyzes the formation of reactive oxygen species (ROS).\textsuperscript{169,170} Effective balance between iron acquisition and metabolism is crucial to maintain the intracellular Fe concentration within nontoxic levels.

To overcome iron-limiting conditions, cyanobacteria have developed highly efficient mechanisms for Fe uptake, including the Fe reductive mechanism,\textsuperscript{171–177} and siderophore-based mechanism.\textsuperscript{178,179} Siderophore-based iron uptake mechanism involves the synthesis and secretion of low-molecular-weight iron chelators that tightly bind iron and form ferri-siderophore complexes for cellular uptake.\textsuperscript{89,167,180,181} The ferri-siderophore
complexes are actively transported across the outer membrane into the cell, where the iron can then be taken up for cellular utilization.\textsuperscript{89,182,183}

In literature, iron limiting conditions were found to provide a competitive advantage to filamentous cyanobacteria over other phototrophs in natural water bodies.\textsuperscript{90,91,100} Iron limitation was also found to induce trichome aggregation and colony formation in cyanobacteria.\textsuperscript{182,184,185} It has been recently reported that that the enrichment of filamentous cyanobacteria during hydrostatic granulation occurred with substantial reduction in the Fe that is bound the EPS of activated sludge inoculum.\textsuperscript{186} This, hence, suggests that the size-growth and aggregation of filamentous cyanobacteria within photogranules in bioreactors are potentially linked with the availability of iron. To date, however, no research has been conducted to investigate the role of iron in the growth and size evolution of photogranules in wastewater treatment reactors.

The objective of this study was to investigate the role of iron in the development and size-growth of photogranules in bioreactors. We hypothesize that the availability of Fe plays a key role in enrichment and aggregation of cyanobacteria within photogranules and, thus, promotes the size-growth of photogranules. To examine the hypothesis, we investigated the fate and distribution of Fe in OPG wastewater treatment reactors. We included manganese (Mn), copper (Cu), and zinc (Zn) in the current study to test whether our hypothesis is true for iron only or other metals as well. We determined the fractions of Fe as well as other metals in the bulk liquid, biomass-bound EPS, and OPG biomass pellet. We studied the changes in the distributions of Fe, Mn, Cu, and Zn with photogranules as they grow larger in size. We discussed the correlations between the levels of metal in OPG biomass and the abundance of phototrophs. The results of this study will be useful to
enhance our knowledge of photogranulation and advance its engineering for aeration-free wastewater treatment.

4.2. Materials and Methods

4.2.1. Generation of seed oxygenic photogranules

The seed photogranules were produced by incubating activated sludge under hydrostatic conditions with illumination following the method in Milferstedt et al.\textsuperscript{17} Briefly, we collected the activated sludge from the aeration basins of a local wastewater treatment plant (Hadley, MA) and pipetted 10 mL into 20 mL glass scintillation vials. We capped the vials with sterile plastic caps and incubated them at constant temperature (20 °C) under hydrostatic conditions with continuous illumination of 150 μmol/m\textsuperscript{2}-s for four to five weeks. The light was provided to vials via LED lights. Photogranules were developed in the vials after 25–35 days of incubation.

4.2.2. Reactor seeding and operation

About 100 hydrostatically formed photogranules were seeded into a glass beaker containing 2 L of primary effluent wastewater collected from a local wastewater treatment plant (Amherst, MA). The reactor was operated in batch mode under light and mixing conditions for 24 h. Light was provided by fluorescent-light bulbs emitting photosynthetically active radiation (PAR) of approximately 150 μmol/m\textsuperscript{2}-s on the reactor surface. The reactor was mixed at about 100 rpm. After two days, the reactor volume was increased to 3 L by adding 1 L of primary effluent wastewater. The OPG mixed biomass was then split into two 1.5 L reactors that were operated in batch mode for 3 days. The two
duplicate reactors were then operated in sequencing batch mode for 130 days. Our sequencing batch reactor (SBR) systems were 2 L flat-bottom glass beakers with a working volume of 1.5 L. Reactors had an inner diameter of 12.2 cm and a working height of 12.9 cm.

During the SBR operation, reactors were operated in four 6 h cycles per day. Each 6 h cycle included 3.5 h of light phase, followed by 2.5 h of dark phase. The light conditions provided to the two reactors during the light phase were the same as those provided to the batch reactors (PAR of approximately 150 µmol/m²-s on the reactor surface). Mixing in reactors was provided by overhead stirrers. Each stirrer was equipped with a stainless-steel flat blade paddle impeller. The paddle-blade impeller had a diameter of 5.2 cm and width 1.9 cm. The impeller off-bottom clearance, the vertical distance from the impeller bottom to the vessel bottom, was set at 4 cm. The mixing speeds in Reactors was set at 100 rpm. Reactors were operated at a hydraulic retention time (HRT) of 0.75 day. The reactors were fed wastewater over 10 min at the beginning of each cycle. The settling time of biomass was set to 10 min with no mixing, followed by 2 min for effluent decanting.

4.2.3. Influent wastewater

We used the primary effluent wastewater of Amherst wastewater treatment plant (Amherst, MA) to feed our OPG reactors. The total COD (tCOD) concentration in influent wastewater ranged between 33 mg/L and 154 mg/L with an average of 65±33 mg/L. The total dissolved nitrogen (TDN) concentration was in the range of 13−35 mg N/L with an average of 20±7 mg/L. Ammonia (NH₄⁺) concentration was in the range of 10−25 mg N/L with an average of 15±4 mg N/L, representing about 75% of TDN.
4.2.4. Measurements of chlorophyll $\alpha$ and phycobilin in photogranules

Extraction and quantification of chlorophyll $\alpha$ in OPG biomass followed Standard Methods 10200H. Three 10 mL biomass samples were transferred in 50 mL screw-cap centrifuge tubes and then centrifuged at 12,000 rpm for 10 min at 4 °C to separate photogranules from the liquid. For chlorophyll extraction, liquid was removed, and the photogranule pellet was resuspended in aqueous acetone solution [one-part saturated magnesium carbonate solution (1%) in nine-parts of acetone] to a volume of 10 mL. Samples were then homogenized at 700 rpm for 30 sec and incubated overnight at 4 °C. Chlorophyll $\alpha$ concentration (mg/mL) in the final extracts was determined spectrophotometrically following Standard Methods (10200H). Next, the mass fraction (%) of chlorophyll $\alpha$ in each photogranule size class was obtained by dividing chlorophyll $\alpha$ concentration (mg/L) by volatile suspended solids (VSS) concentration (mg/L) and then multiplying by 100. Chlorophyll $\alpha$ measurement was done on triplicate biomass samples.

The methods by Bennett and Bogorad and Islam et al. were modified and used to determine phycobiliprotein content in photogranule samples. Briefly, three 10 mL biomass samples were pipetted in 50 mL centrifuge tubes, and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the biomass pellet was resuspended in 0.025 M phosphate buffer saline solution (pH 7) to a volume of 10 mL. Biomass samples in phosphate buffer saline solution were then homogenized at 700 rpm for 1 min. After homogenization, samples were sonicated at 20% strength for 2 min, and then centrifuged at 12,000 rpm for 10 min. The absorbance of supernatant was measured spectrophotometrically at the wavelengths 562 nm, 615 nm, and 652 nm. The equations by Bennett and Bogorad were used to quantify phycocyanin (PC), phycoerythrin (PE), and
allophycocyanin (APC). Total phycobilin concentration (mg/mL) in samples was calculated as the sum of PC, PE, and APC concentrations. Finally, the mass fraction (%) of phycobilin in each photogranule size class was obtained by dividing phycobilin concentration (mg/L) by volatile suspended solids (VSS) concentration (mg/L) and then multiplying by 100.

4.2.5. Extraction of the extracellular polymeric substances (EPS)

EPS was extracted from the photogranular biomass following the sequential sonication and base extraction method presented in Ansari et al.\textsuperscript{16} Briefly, OPG biomass samples were centrifuged at 12,000 rpm for 10 min. The biomass pellets were then used suspended in 10 mL phosphate buffer saline (PBS) solution. Samples were then homogenized, sonicated, and centrifuged at 12000 rpm for 10 min. Next, the supernatant was filtered with 0.45 μm cellulose filter and considered as the “biomass-bound EPS extracted by sonication”. The remaining biomass pellets were resuspended in 10 mL PBS solution. The pH of samples was adjusted to 10.5 using NaOH solution (1 M).\textsuperscript{187} Next, the samples were shaken at 400 rpm for 2 h and then centrifuged at 12000 rpm for 10 min. The supernatant was filtered with a 0.45 μm cellulose filter and considered as the “biomass-bound EPS extracted by base”. The total biomass-bound EPS was calculated by taking the sum of biomass-bound EPS extracted by sonication and base. To measure the polysaccharide and protein concentrations, in the final EPS extract, Dubois method\textsuperscript{142} and modified Lowry method\textsuperscript{143} were used.
4.2.6. Determination of the levels and distribution of heavy metals in OPG biomass

Concentrations of heavy metals in bulk liquid, extracted biomass-bound extracellular polymeric substances (EPS) and whole biomass samples was determined at regular time intervals throughout the study period. In detail, metals in bulk liquid passing through 0.45 μm mixed cellulose ester membrane filter (Fisher Scientific, USA) were considered colloidal/dissolved metals. These 0.45 μm filtrates were immediately acidified with trace metal grade HNO₃ (2% by volume) (Fisher Scientific, USA) and stored in 15 mL falcon tube at 4 °C till analysis. To determine the concentrations of heavy metals in whole biomass, samples were first subjected to acid digestion (Standard Method 3030 E). The acid-digested samples were filtered through a cellulose ester membrane filter (Fisher Scientific, USA) and stored at 4 °C till further analysis. The level of metals that are bound with EPS was determined following acid digestion of EPS obtained via sequential extraction using sonication and base treatment as mentioned above. Prior to metal analysis, the filtered EPS extracts were acidified with concentrated trace metal grade HNO₃ (2% by volume) and stored at 4 °C. Concentrations of heavy metals (mg/L) in acid-digested samples (bulk liquid, EPS extracts, and whole biomass digest) were measured using inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer SCIEX). Metal content in biomass pellet was calculated by subtracting metal concentrations in EPS (<0.45 μm) and bulk-liquid (<0.45 μm) from total metal concentrations in biomass.

4.2.7. Measurements of biomass concentration and effluent quality

The total and volatile suspended solids of the OPG biomass were determined according to Standard Methods 2540D/E. The chemical oxygen demand (COD) of
influent and effluent samples was determined following Standard Methods 5220D.\textsuperscript{139} The total dissolved nitrogen (TDN) concentration in influent and effluent samples were measured by TOC/TN analyzer (TOCVPCH, Shimadzu). In addition, nitrogen species (ammonia, nitrite, and nitrate) were measured using ion chromatography (Metrohm 830).

4.2.8. Statistical analysis

We performed the two-samples \textit{t-test} to determine whether there is a significant difference between the variables. The default alpha (\(\alpha\)) value was set at 0.05 so if the \(p\)-value is less than 0.05 there is a significant difference. We also conducted the regression analysis to examine the relationship between the variables.

4.3. Results

4.3.1. Removal of organic matter and nitrogen in OPG reactors

The influent used to feed our OPG reactors was the primary effluent wastewater of Amherst wastewater treatment plant (Amherst, MA). The total COD (tCOD) concentration in influent wastewater ranged between 33 mg/L and 154 mg/L with an average of 65±33 mg/L (Figure 4.1a). The dissolved organic carbon (DOC) concentration was at an average of 15±7 mg/L, yielding DOC/tCOD at 0.23. Over 130 days of reactor operation, the average concentrations of tCOD and DOC in reactor effluent were 23.5±7 mg/L and 6.2±2 mg/L, respectively (Figure 4.1a). The average removals (%) of tCOD and DOC were 60±11% and 55±10%, respectively (Figure 4.1b).

The total dissolved nitrogen (TDN) concentration ranged between 13 mg N/L and 35 mg N/L with an average of 20±7 mg/L (Figure 4.1a). Ammonia (NH\(_4^+\)) concentration
was 15±4 mg N/L, representing about 75% of TDN (Figure 4.1a). Over the reactor operation period, the average concentrations of TDN and NH$_4^+$ in reactor effluent were 14±3 mg/L and 2.3±4 mg/L, respectively (Figure 4.1a). The average removals (%) of TDN and NH$_4^+$ were 28±9% and 89±19%, respectively (Figure 4.1b).

**Figure 4.1.** Removals (%) of organic matter and nitrogen in OPG reactors. (A) Average concentrations of total COD, DOC, TN, and NH$_4^+$ in the influent wastewater and effluent of OPG reactors. (B) The average removals (%) of organic matter and nitrogen in OPG reactors.
4.3.2. Removal of heavy metals in OPG reactors

Figure 4.2 shows the removal (%) of four heavy metals in OPG reactors. Among the four metals investigated in the current study, iron (Fe) showed the highest concentration in influent wastewater followed by manganese (Mn).

![Diagram showing removal of heavy metals in OPG reactors]

**Figure 4.2.** Removal of heavy metals (%) in OPG reactors. (A) Average concentrations of the soluble and colloidal fraction of Iron (Fe), Manganese (Mn), Copper (Cu), and Zinc (Zn) in the influent and effluent of OPG reactors. (B) Removals (%) of the four metals in OPG reactors.
The concentrations of the soluble-colloidal fractions (<0.45 μm) of Fe, Mn, Cu, and Zn in influent wastewater were in the range of 226−440 µg/L, 71−136 µg/L, 20−48 µg/L, and 39−99 µg/L, respectively, with averages of 317±73 µg/L, 103±17 µg/L, 29±9 µg/L, and 53±18 µg/L, respectively (Figure 4.2a).

Over the reactor operation period, the average concentrations of Fe, Mn, Cu, and Zn in reactor effluent were 106±13 µg/L, 6±5 µg/L, 9±3 µg/L, and 29±7 µg/L, respectively (Figure 4.2a). Among the four heavy metals studied, the removal (%) of Mn was the highest. The removals (%) of Fe, Mn, Cu, and Zn over the entire operation period were 64±8%, 95±5%, 66±13%, and 43±11%, respectively (Figure 4.2b). The estimated removal rate of Fe (95 µg/g biomass-d) was about two times higher than the removal rate of Mn (45 µg/g biomass-d) and about 10 times higher than the removal rates of Cu, and Zn (9.2 µg/g biomass-d, and 11 µg/g biomass-d, respectively).

4.3.3. Concentrations of heavy metals in whole OPG biomass

We measured the concentrations of heavy metals in the whole photogranular biomass categorized into different size classes to investigate the changes in the concentrations of heavy metals within OPGs as they grow bigger in size. Figure 4.3 shows the concentrations of four heavy metals in OPG size classes. As the size of photogranules increased from the smallest group (<0.2 mm) to the class 1.7−2.5 mm in diameter, Fe concentration in the whole OPG biomass increased from 4.0±1 mg/g biomass to 9.6±0.1 mg/g biomass, showing a 140% increase in Fe concentration (Figure 4.3a). As the photogranules grew above 1.7−2.5 mm, the concentration of Fe in the biomass started to decrease with the size. As the size of photogranules increased from 1.7−2.5 mm to 3.5−4.5
mm, the largest size class in this study, Fe concentration decreased from 9.6±0.1 mg/g biomass to 4.4±1 mg/g biomass (Figure 4.3a).

**Figure 4.3.** Concentrations of heavy metals in OPG size classes. (A) Concentration of total Fe in OPG size classes. (B) Concentration of total Mn in OPG size classes. (C) Concentration of total Cu in OPG size classes. (D) Concentration of total Zn in OPG size classes. Error bars represent the standard deviations of triplicate samples.

The concentration of Mn in whole OPG biomass also increased as OPG grew larger in size up to 0.5–1 mm in diameter and then decreased. Mn concentration in the whole biomass increased from 0.3±0.1 mg/g biomass to 1.3±0.3 mg/g biomass, showing a 330% increase in Mn concentration, as the size of photogranules increased from the size class <0.2 mm to the size class 0.5–1 mm. (Figure 4.3b). Mn concentration then decreased from
1.3±0.3 mg/g biomass to 0.4±0.2 mg/g biomass as the size of photogranules increased from 0.5–1 mm to 3.5–4.5 mm (Figure 4.3b).

In contrast to Fe and Mn, the concentrations of Cu and Zn in OPG biomass showed clear decreasing trends with photogranule’s size (Figure 4.3c, Figure 4.3d). Cu concentration in whole OPG biomass decreased from 1.7±0.2 mg/g biomass to 0.3±0.1 mg/g biomass as the photogranules’ size increased from the size class <0.2 mm to the size class 1.7–2.5 mm (Figure 4.3c). For the same size classes, the concentration of Zn decreased from 1.1±0.1 mg/g biomass to 0.3±0.02 mg/g biomass. The concentrations of Cu and Zn increased as the size of photogranules increased above 1.7–2.5 mm. As the size of photogranules increased from 1.7–2.5 to 3.5–4.5 mm, the concentrations of Cu and Zn in biomass increased to 0.9±0.2 mg/g biomass and 0.5±0.1 mg/g biomass, respectively (Figure 4.3c, Figure 4.3d).

Among the four heavy metals studied, Fe was the most abundant metal in all photogranule size classes, while Mn was the second most abundant metal in OPGs. Depending on the size class, Fe concentration in OPG biomass was about 4–12 times higher than Mn concentration and up to 36 times higher than the concentrations of Cu and Zn.

4.3.4. Distribution of heavy metals in OPG biomass

Besides determining the concentrations of heavy metals in the whole biomass of OPG per size class, we also measured their concentrations in the extracted EPS to determine the changes in the distribution of heavy metals within OPGs as they grow larger in size. Figure 4.4 shows the fractions of heavy metals in OPG size classes. In the OPG
size class <0.2 mm, the fraction of Fe bound with EPS represented 27.3% of the total Fe, while the fraction of Fe in the biomass pellet represented 72.7%. As the size of OPGs increased from the size class <0.2 mm to the size class 1.7–2.5 mm, the fraction of Fe bound with EPS substantially decreased (from 27.3% to 1.1%), while Fe in the biomass pellet increased from 72.7% to 98.9%. (Figure 4.4a).

As the size of photogranules increased from 1.7–2.5 to 3.5–4.5 mm, the fraction of Fe in EPS increased back from 1.1% to 12.1% (Figure 4.4a) while the fraction of Fe in biomass pellet decreased from 98.9% to 87.9% (Figure 4.4a). Regardless of the changes in the fractionation of Fe (i.e., Fe bound with EPS and Fe in biomass pellet), the that the majority of Fe (>72% of the total Fe) in all OPG size classes was always present in the biomass pellet (Figure 4.4a).

We observed that fraction of Mn in EPS decreased from 17.5% to 0.8% as the size of photogranules increased from the size class <0.2 mm to the class 0.5–1 mm in diameter (Figure 4.3b) while its fraction in biomass pellet increased from 82.5% to 99.2% (Figure 4.4b). As the size of photogranules increased from 0.5–1 mm to 3.5–4.5 mm, Mn fraction in EPS increased from 0.8% to 6.5%, while Mn in biomass pellet decreased to 87.9%. Like Fe, the majority of Mn (>82% of the total Mn) in all OPG size classes was also present in biomass pellet (Figure 4.4b).
Figure 4.4. Distribution of heavy metals in OPG biomass size classes. (A) Fractions of Fe in the OPG biomass pellet and biomass-bound EPS. (B) Fractions of Mn in the OPG biomass pellet and biomass-bound EPS. (C) Fractions of Cu in the OPG biomass pellet and biomass-bound EPS. (D) Fractions of Zn in the OPG biomass pellet and biomass-bound EPS.

Unlike Fe and Mn, more than 86% of the total Cu and total Zn in OPG size classes were present in EPS, while only 14% or less was present in biomass pellet (Figure 4.4c and Figure 4.4d). As the size of photogranules increased to 0.5–1 mm, the fractions of Cu and Zn in EPS decreased from 97% to 86.5%, while the fractions of Cu and Zn in the biomass pellet increased from 3% to 13.5%. As the size of photogranules increased from 0.5–1 to 3.5–4.5 mm, the fractions of Cu and Zn in EPS increased from 86.5% to 97.4% while the fractions of Cu and Zn in biomass pellet decreased from 13.5% to 2.6% (Figure 4.4c, Figure
These results, therefore, indicate that the size-growth of photogranules occurred with substantial changes in the distribution of Fe and other metals within the biomass. The variations in metal concentrations in the OPG biomass pellet, most likely intracellular metals, suggest that there was selectivity in metal uptake.

4.3.5. Relationship between the concentrations of EPS and heavy metals in OPG biomass

EPS proteins decreased from 183±13 mg/g biomass to 56±12 mg/g biomass as the photogranules grew from the size class <0.2 mm to the size class 1.7–2.5 mm (Figure 4.5a). Likewise, EPS polysaccharides decreased from 55±4 mg/g biomass to 15±3 mg/g biomass for the same size classes (Figure 4.5b). However, as the size of photogranules increased from 1.7–2.5 mm to 3.5–4.5 mm, EPS proteins and polysaccharides increased to 123±15 mg/g biomass and 38±4 mg/g biomass, respectively (Figure 4.5a, Figure 4.5b).

It can be seen that the trend of the concentrations of EPS is the same trend of the concentrations of Fe in EPS (Figure 4.4a). Also, we found strong positive correlations between EPS and the concentrations of Fe in EPS ($R^2 = 0.94−0.96$), demonstrating the strong affinity of EPS for Fe (Figure 4.5c, Figure 4.5d). These results indicate that the EPS and Fe present in EPS underwent significant changes along with the increase in the size of photogranules. It can also be seen that the trends of EPS are the opposite to the trends of Fe in whole OPG biomass and Fe in biomass pellet (Figure 4.3a, Figure 4.4a). We found negative correlations between EPS and Fe in whole biomass ($R^2 = 0.73−0.79$) and between EPS in Fe in biomass pellet ($R^2 = 0.81−0.86$) (Figure 4.6a, Figure 4.6a Figure 4.5c, Figure 4.5c). These results suggest that the EPS and Fe bound with EPS were utilized by the
microbes within OPGs. Compared to Fe, correlations between EPS and Mn in whole biomass were considerably weaker ($R^2 = 0.46−0.48$) (Figure 4.6c, Figure 4.6d). Also, negative correlations were found between EPS and Mn in biomass pellet ($R^2 = 0.72−0.73$) (Figure 4.6e, Figure 4.6f).

In contrast to Fe, the EPS trends are the same trends of concentrations of Cu and Zn in whole biomass (Figure 4.3c, Figure 4.3d). We found strong positive correlations between EPS and Cu in EPS ($R^2 = 0.95−0.96$) as well as between EPS and Zn in EPS ($R^2 = 0.95−0.95$) (Figure 4.5g, Figure 4.5h, Figure 4.5i, Figure 4.5j). Also, strong positive correlations existed between EPS and Cu in whole biomass ($R^2 = 0.96$) as well as between EPS and Zn in whole biomass ($R^2 = 0.94−0.95$) (Figure 4.6e, Figure 4.6f, Figure 4.6g, Figure 4.6h). On the other hand, no correlations were found between EPS and Cu in biomass pellet ($R^2 = 0.02$) as well as between EPS and Zn in biomass pellet ($R^2 = 0.03$) (Figure 4.5g, Figure 4.5h, Figure 4.5i, Figure 4.5j). These results suggest that the changes in the concentrations of Cu and Zn within whole biomass were mainly due to the changes in their concentrations in EPS.
4.3.6. Relationship between the concentrations of phototrophs and heavy metals in OPG biomass

We measured the weight fractions of chlorophyll \(a\) in the OPG size classes to estimate the content of phototrophs in OPG biomass. As the size of photogranules increased from the smallest group (<0.2 mm) to the class 0.5–1 mm in diameter, the fraction of chlorophyll \(a\) in biomass increased from 0.61±0.06% to 1.12±0.14%, indicating enrichment of phototrophs (Figure 4.7a). As photogranules grew above 0.5–1 mm in diameter, the fraction of chlorophyll \(a\) in the biomass started to decrease with the size. As the size of photogranules increased from 0.5–1 mm to 3.5–4.5 mm, chlorophyll \(a\) decreased from 1.12±0.14% to 0.59±0.07%, indicating a significant reduction in phototrophic population (Figure 4.7a).

The trend of chlorophyll \(a\) is the same trend of the concentrations of Mn in whole biomass and Mn in biomass pellet (Figure 4.3b, Figure 4.4b). In addition, strong positive correlations were found between chlorophyll \(a\) and Mn in whole OPG biomass \((R^2 = 0.92)\) as well as between chlorophyll \(a\) and Mn in biomass pellet \((R^2 = 0.74)\) (Figure 4.7c, Figure 4.7g), suggesting that phototrophs utilized Mn for their growth. Although the chlorophyll \(a\) trend appears to be the opposite to the trend of Mn in EPS (Figure 4.4b), linear regression analysis showed weak negative correlations between chlorophyll \(a\) and Mn in EPS \((R^2 = 0.57)\) (Figure 4.7g).

Unlike Mn, very weak positive correlations were found between chlorophyll \(a\) and Fe in whole biomass \((R^2 = 0.26)\) as well as between chlorophyll \(a\) and Fe in biomass pellet \((R^2 = 0.25)\) (Figure 4.7b, Figure 4.7f). We also found weak negative correlations between chlorophyll \(a\) and the concentrations of Cu and Zn in whole biomass \((R^2 = 0.16–0.32)\).
Contrarily, moderate to strong positive correlations were found between chlorophyll $a$ and Cu in biomass pellet ($R^2 = 58$) as well as between chlorophyll $a$ and Zn in biomass pellet ($R^2 = 86$) (Figure 4.7h, Figure 4.7i). These results suggest that chlorophyll $a$ was not strongly linked with Fe, Cu, and Zn.

4.3.7. Relationship between heavy metals and phycobilin in OPGs

We measured the weight fractions of phycobilin in photogranules to estimate the content of cyanobacteria in OPG biomass. The weight fraction of phycobilin increased from 3±0.4% to 9.5±1% as the size of photogranules increased from <0.2 mm to 1.7–2.5 mm (Figure 4.8a), suggesting the enrichment of phototrophs that contain phycobilin, i.e., cyanobacteria. The increase in the weight fraction of phycobilin within OPGs as they grew bigger up to 1.7–2.5 mm in diameter was accompanied with significant increases in the concentrations of Fe in whole OPG biomass and biomass pellet (Figure 4.3a, Figure 4.4a), suggesting that Fe was utilized for cyanobacterial growth.

As photogranules grew above 1.7–2.5 mm in diameter, the weight fraction of phycobilin started to decrease. Phycobilin decreased from 9.5±1% to 4.7±0.7% as the photogranules’ diameter increased from 1.7–2.5 mm to 3.5–4.5 mm, suggesting a 50% decrease in the abundance of cyanobacteria in photogranules (Figure 4.8a). Fe in whole biomass and biomass pellet also showed a decreasing pattern as photogranules grew above 1.7–2.5 mm in diameter (Figure 4.3a, Figure 4.4a). We found strong positive correlations between phycobilin and Fe in whole OPG biomass (R² = 0.89) and between phycobilin and Fe in biomass pellet (R² = 0.94) (Figure 4.8c, Figure 4.9a). Strong negative correlations were found between phycobilin and biomass-bound EPS (R² = 0.93–0.95) as well as between phycobilin and Fe bound with EPS (R² = 0.85) (Figure 4.8b, Figure 4.9a), suggesting that filamentous cyanobacteria utilized the EPS as well as the Fe bound with EPS for their growth within photogranules.
Figure 4.8. The relationship between the mass fraction of phycobilin in OPG biomass, EPS, and heavy metals in whole OPG biomass. (A) The mass fraction of phycobilin in OPG size classes. (B) The relationship between the mass fraction of phycobilin and EPS. (C) The relationship between the mass fraction of phycobilin and Fe in whole OPG biomass. (D) The relationship between the mass fraction of phycobilin and Mn in whole OPG biomass. (E) The relationship between the mass fraction of phycobilin and Cu in whole OPG biomass. (F) The relationship between the mass fraction of phycobilin and Zn in whole OPG biomass.
Compared to Fe, correlations between phycobilin and Mn in whole OPG biomass as well as between phycobilin and Mn in biomass pellet were much weaker ($R^2 = 0.44$ and $R^2 = 0.72$, respectively) (Figure 4.8d, Figure 4.9b). In contrast to Fe, strong negative correlations were found between phycobilin and the concentrations of Cu and Zn in whole OPG biomass ($R^2 = 0.82−0.85$) (Figure 4.8e, Figure 4.8f). No correlations were found neither between phycobilin and Cu concentration in biomass pellet ($R^2 = 0.01$) nor between phycobilin and Zn in biomass pellet ($R^2 = 0.03$) (Figure 4.9c, Figure 4.9d).

![Graphs showing relationships between phycobilin and metal fractions in OPG biomass.](image)

**Figure 4.9.** The relationship between the mass fraction of phycobilin in OPG biomass and the fractions of heavy metals. (A) The relationship between phycobilin and Fe fractions in OPG biomass. (B) The relationship between phycobilin and Mn fractions in OPG biomass. (C) The relationship between phycobilin and Cu fractions in OPG biomass. (D) The relationship between phycobilin and Zn fractions in OPG biomass.
4.4. Discussion

In OPG wastewater treatment systems, photogranules grow in number as well as in size.\textsuperscript{15,17} While we previously showed that the enrichment of filamentous cyanobacteria is necessary for the development and size-growth of photogranules,\textsuperscript{15,17} the factors that promote the growth of filamentous cyanobacteria within photogranules as they grow bigger in size have remained poorly understood. The present study aimed to understand the impact of the availability of Fe on the enrichment and aggregation of cyanobacteria within photogranules in bioreactor systems. Our approach was to investigate the fate and distribution of Fe as well as three other heavy metals (Mn, Cu, and Zn) in OPG wastewater treatment reactors.

Fe concentration in whole OPG biomass increased by nearly 140\% as the size of photogranules increased from the smallest size class <0.2 mm to the size class 1.7−2.5 mm (Figure 4.3a). It is important to note that the increase in Fe concentration in OPGs along with the size increase was mainly due to the increase in Fe residing in the biomass pellet (Figure 4.4a). The detected amounts of Fe in biomass pellet likely include intracellular Fe, Fe bound with unextracted EPS, and Fe precipitates. The increases in Fe concentrations and the size of OPGs occurred simultaneously with 220\% increase in the weight fraction of phycobilin, suggesting that the growth of filamentous cyanobacteria and increased uptake of Fe took place along with the size increase of OPGs or progressive granulation of OPGs (Figure 4.8a). Strong positive correlations between phycobilin and Fe in whole OPG ($R^2 = 0.89$) and Fe in biomass pellet ($R^2 = 0.94$) further suggest that the growth of cyanobacteria was an important cause of the increases in Fe in the OPG growing in size (Figure 4.8c, Figure 4.9a). Importantly, these changes concurred with substantial decreases
(by up to 220%) of both EPS proteins and polysaccharides (Figure 4.5a and Figure 4.5b). Besides strong negative correlations between the phycobilin and EPS in OPG size classes ($R^2 = 0.93−0.95$) (Figure 4.8b), the Fe bound with EPS got nearly depleted (Figure 4.4a).

All these results suggest that filamentous cyanobacteria could utilize the EPS as well as the Fe bound with EPS for their growth within photogranules in bioreactor systems. In the literature, cyanobacteria growing in microbial mats have been shown to degrade and uptake the organic carbon and nitrogen from EPS present within the mat, and scavenge Fe bound with these organic matter. Cyanobacteria growing in mats was also shown to utilize its own extracellular material under light as well as dark conditions. EPS, hence, appears to be a major source of organic carbon, nitrogen, and Fe in OPGs especially for cyanobacteria. Other microorganisms within OPGs (e.g., heterotrophic bacteria) could possibly access a fraction of EPS and Fe as well. However, the contribution from other bacteria to this process is likely to be much smaller than that of the cyanobacteria as filamentous cyanobacteria comprise a major component of the total microbial biomass in OPGs. In natural mats, noncyanobacterial microorganisms represent less than 2% of the total microbial biomass, and their EPS uptake accounts for about 3% of the total EPS utilized within the mat. It has also been reported that cyanobacteria require 10 times higher cellular Fe than non-photosynthetic prokaryotes and 5–8 times higher cellular Fe : C ratio than eukaryotic algae growing under similar lighting conditions. Moreover, Abouhend et al. reported that other phototrophic microorganisms such as unicellular green algae and diatoms diminished significantly during the size evolution of photogranules in bioreactors. These lines of evidence from literature and the results of this study, therefore, suggest that filamentous cyanobacteria are
capable of acquiring carbon, nitrogen, and Fe from EPS, which renders them advantages over other phototrophic microorganisms within OPGs. The light limitation within the larger photogranules could also have contributed to the selection of cyanobacteria within photogranules as cyanobacteria could better adapt to a light limiting conditions than green algae and diatoms.

Although EPS and Fe bound with EPS substantially decreased along with the size-growth of photogranules, filamentous cyanobacteria continued to grow within OPGs (Figure 4.5a, Figure 4.5b, Figure 4.9a). Abouhend et al.,$^{163}$ showed that filamentous cyanobacteria were spread over the whole body of photogranules smaller than 3 mm in diameter. It is important to note that photogranules became more spherical as they grew larger in size.$^{15,163}$ Moreover, larger photogranules were less hairy and have relatively smoother surfaces compared to the smaller photogranules.$^{15,163}$ In addition, negative correlations were found between the content of cyanobacteria indicated by phycobilin and Fe bound with EPS ($R^2 = 0.85$) (Figure 4.9a). These strongly indicate that the granulation (or spherical formation of OPG) with filamentous cyanobacteria became more substantiative as the Fe bound with EPS became limited. It has been reported in the literature the Fe limitation can promote the aggregation of cyanobacteria.$^{182,184,185}$ The filamentous marine cyanobacterium *Trichodesmium* sp., form spherical or fusiform aggregates or colonies under Fe limitation.$^{182,184,185}$ Ansari et al.,$^{186}$ recently reported that Fe limitation plays an important role in selecting cyanobacteria during hydrostatic photogranulation, the transformation of activated sludge into photogranules under hydrostatic batch conditions. Enrichment of filamentous cyanobacteria during hydrostatic photogranulation was reported to occur with decline in the levels of Fe in the bulk liquid,
EPS proteins of activated sludge inoculum, and Fe associated with activated sludge EPS.\textsuperscript{186} This suggests that photogranules grown in reactors and hydrostatically formed photogranules share a common formation mechanism despite extremely different environments in which the growth of OPGs occurs.

This study also showed that Fe concentration in whole OPG biomass decreased by nearly 54% as the size of photogranules increased from 1.7−2.5 mm to 3.5−4.5 mm (Figure 4.3a). For these size classes, Fe in the biomass pellet also decreased by 60% (Figure 4.4a). The decrease in Fe concentration as photogranules grew in these size groups was accompanied with a 50% decrease in the abundance of cyanobacteria indicated by phycobilin, 120% increase in EPS proteins, and 160% increase in EPS polysaccharides (Figure 4.8a, Figure 4.5a, Figure 4.5b). The concentration of Fe bound with EPS also increased by 430%. It can be therefore inferred that the decrease in the relative abundance of filamentous cyanobacteria within photogranules larger than 2.5 mm resulted in a decrease in the utilization of EPS and Fe bound with EPS, leading to the accumulation of both EPS and Fe bound with EPS in OPGs. At this point, it is unclear why the relative abundance of filamentous cyanobacteria within photogranules decreased as they grew larger above 2.5 mm in diameter. The most likely scenario is that filamentous cyanobacteria in the larger photogranules have experienced either nutrient limitation or light limitation. It has been reported that the increase in size can diminish the porosity of biogranules, leading to limitation of mass transport within granules.\textsuperscript{10,35−39} Limitation of mass transfer in the larger aerobic granules could influence the nutrient accessibility and outflow of unfavorable products, significantly impacting microbial viability and the microstructure of the microbial organization.\textsuperscript{22,24,27,40,41} Kühl \textit{et al.},\textsuperscript{148} reported that light
limitation in phototrophic mats increases with depth and thus phototrophs in deeper layers of mats receive less light. It has been also reported that photogranules of 3 mm in diameter and above showed the development of a distinct layered structure where a mat-like outer layer of filamentous cyanobacteria encloses noncyanobacterial core biomass. The development of this layered structure in larger OPGs can be explained by the migration of motile filamentous cyanobacteria toward the photogranule’s surface to overcome light limitation. Filamentous cyanobacteria could use their gliding motility to move the surface of photogranules where light is more available. The increases in EPS observed in the larger granules might be also secreted by cyanobacteria to facilitate their gliding motility. The development of this cyanobacteria layer seems to be playing a key role in maintaining the structural integrity of photogranules.

In the current study, the majority of Mn (> 82%) was present in the biomass pellet (Figure 4.4b). The concentration of Mn in the whole OPG biomass as well as the biomass pellet increased by 300–400% as the size of photogranules increased from <0.2 mm to 0.5–1 mm (Figure 4.3b, Figure 4.4b). The increase in the concentration of Mn in biomass pellet was accompanied with a substantial increase in the weight fraction of chlorophyll a, indicating enrichment of phototrophs, including green algae, cyanobacteria, and diatoms, within OPGs (Figure 4.7a). We found strong positive correlations between chlorophyll a and Mn in whole OPG biomass \( (R^2 = 0.92) \) as well as between chlorophyll a and Mn in biomass pellet \( (R^2 = 0.74) \), suggesting that phototrophs were able to utilize Mn for their growth (Figure 4.7c, Figure 4.7g). According to the literature, Mn is an important cofactor for a number of central enzymes that are involved in oxygen evolution activity in phototrophs. Anoxic photosynthetic microorganisms have also been reported to
use Mn as electron donors for phototrophy.\textsuperscript{193} We also found weak negative correlations between phototrophs indicated by chlorophyll \(a\) and Mn in EPS (\(R^2 = 0.57\)) (Figure 4.7g). It is also important to note weak negative correlations were found between chlorophyll \(a\) and EPS proteins (\(R^2 = 0.24\)) and between chlorophyll \(a\) and EPS polysaccharides (\(R^2 = 0.24\)). These results suggest that the growth of other phototrophs such as green algae and diatoms was not dependent on the EPS as the main source of carbon and Mn. The concentration of Mn in whole OPG biomass and biomass pellet decreased by nearly 65\% as the size of photogranules increased from the size class 0.5–1 mm to the size class 3.5–4.5 mm (Figure 4.3b, Figure 4.4b). The decrease of Mn in photogranules as they grew above 0.5–1 mm in diameter could be due to the decrease in the phototrophic population indicated by chlorophyll \(a\) (Figure 4.7a). The decrease in phototrophic population was due to the decrease in the phototrophic groups producing chlorophyll \(b\) and chlorophyll \(c\), such as green algae and diatoms, respectively.\textsuperscript{163}

Unlike chlorophyll \(a\) which correlates strongly with Mn in whole OPG biomass (\(R^2 = 0.92\)) (Figure 4.7c), phycobilin and Mn in whole OPG biomass did not show good correlation (\(R^2 = 0.44\)) (Figure 4.8d). These observations suggest that the growth of filamentous cyanobacteria with OPGs was not limited by Mn availability. According to the literature, Mn is generally not a limiting factor for the growth of cyanobacteria, although its limitation can affect their oxygen evolution capacity.\textsuperscript{192} On the other hand, the overaccumulation of Mn within cyanobacterial cells causes mismetallation; incorporation of Mn instead of Fe and Mg, and thus inhibiting or deactivating metalloenzymes.\textsuperscript{194,195}

In contrast to Fe and Mn, less than 14\% of Cu and Zn was present in the biomass pellet of the seven size classes while more than 86\% was bound with EPS (Figure 4.4c,
Figure 4.4d). The significant differences in the fractionation among the four metals suggests that there was selectivity in the metal uptake by photogranules (Figure 4.4). Metal selectivity and metal uptake capacity are common in phototrophic and nonphototrophic microorganisms.\textsuperscript{196–199} Weak negative correlations were found between the population of phototrophs, indicated by the concentration of chlorophyll \textit{a}, and the concentrations of Cu and Zn in whole biomass (\(R^2 = 0.16–0.32\)) (Figure 4.7d, Figure 4.7e). Moderate positive correlations were found between chlorophyll \textit{a} and Cu in biomass pellet (\(R^2 = 0.58\)) while strong positive correlations were found between chlorophyll \textit{a} and Zn concentration in biomass pellet (\(R^2 = 0.86\)) (Figure 4.7h, Figure 4.7i). We found strong negative correlations between cyanobacteria indicated by phycobilin and the concentrations of Cu and Zn in whole OPG biomass (\(R^2 = 0.82–0.85\)) (Figure 4.8e, Figure 4.8f). No correlations were found neither between phycobilin and Cu concentration in biomass pellet (\(R^2 = 0.01\)) nor between phycobilin and Zn in biomass pellet (\(R^2 = 0.03\)) (Figure 4.9c, Figure 4.9d). Cu is an essential micronutrient that is required as a cofactor for a number of cuproenzymes in cyanobacteria,\textsuperscript{200,201} while Zn is required for producing carbonic anhydrase; a family of enzymes that catalyze the interconversion between carbon dioxide and water and the dissociated ions of carbonic acid.\textsuperscript{202} Compared to Fe, Mn, Cu and Zn are required at very low concentration for cyanobacterial growth.

The results of this study, therefore, suggest that unlike other studied metals, the availability of Fe has a strong influence on the growth and granulation of filamentous cyanobacteria for OPGs. The above shown results and the literature further suggest that cyanobacteria are able to utilize EPS and Fe bound with EPS, which seems to select filamentous cyanobacteria within photogranules. The limitation of Fe pool seems to be
critical for promoting the granulation of filamentous cyanobacteria. In hydrostatic environments Fe limitation was also found to drive cyanobacteria to granulate during the transformation of activated sludge into photogranules.\textsuperscript{186} Despite extremely different environments in which the growth occurs, the photogranules growing in reactors and hydrostatically formed photogranules seem to share a common formation mechanism. EPS appear to be the major source of organic carbon, nitrogen, and Fe in OPGs. Cyanobacteria’s utilization of EPS for these vital resources enhances our understanding of essential nutrient cycling in OPG reactors. Future research is needed to study the mechanisms of Fe uptake within OPGs which would improve the understanding of the relationship between Fe and photogranulation. Furthermore, studying the influence of Fe addition on microbial communities within photogranules will be useful to enhance our knowledge of photogranulation and advance its engineering for aeration-free wastewater treatment.
CHAPTER V
THE ROLE OF HYDRODYNAMIC SHEAR IN OXYGENIC PHOTOGRA NULE WASTEWATER TREATMENT PROCESS

Abstract
The oxygenic photogranule (OPG) process is a promising biotechnology for wastewater treatment without supplemental aeration. Mixing is essential in OPG process as it promotes homogeneity and facilitates the interaction of photogranules with light. Additionally, the induced momentum exerts shear stresses on microbes growing in the system. The shear force arising from mixing is considered as a core selection pressure for microbial granulation. Currently, little information is available regarding the effect of hydrodynamic shear force on photogranulation. In this study, we investigated the effect of shear on photogranulation in three sequencing batch reactors (SBRs) which were operated for 250 days to treat wastewater without aeration. SBRs were operated at three different mixing speeds (50 rpm, 100 rpm, and 250 rpm) with corresponding shear stresses of 0.015 N m\(^{-2}\), 0.04 N m\(^{-2}\), and 0.14 N m\(^{-2}\) for Reactor 1, Reactor 2, and Reactor 3, respectively. Unlike other granular systems in which hydrodynamic shear is commonly created by airlift or upflow-based mixing, these OPG reactors were operated in stirred-tank reactors in which the hydrodynamic turbulence caused by overhead stirring serves as the source of the main shear force. The increased hydrodynamic shear force stimulated the production of extracellular polymeric substances (EPS) and promoted the formation of stable mature photogranules in the three reactors. We observed significant variations in the particle size distribution and structure of photogranule biomass in the three reactors. Compared with the photogranules developed at a high shear stress, photogranules produced at low and medium
hydrodynamic shear stresses were bigger in size, more spherical, and less hairy. The increased attrition and particle-particle collision at high shear forces seemed to suppress the size-growth of photogranules. However, the relative abundance of cyanobacteria, the key microbial group for OPG granulation, increased as the shear force increased in reactor. Functions of photogranules are size-dependent so that variations in the particle-size-distribution of biomass in the three reactors resulted in significant differences in organic matter and nitrogen removals. Removals (%) of tCOD and NH$_4^+$ in Reactor 3 were higher than Reactor 1 and Reactor 2. In contrast to tCOD and NH$_4^+$, TN removal in Reactor 1 was higher than Reactor 2 and Reactor 3. Hydrodynamic shear can be controlled to drive photogranulation towards a desired size distribution and thus improve the performance of OPG wastewater treatment process.

5.1. Introduction

Mixing is crucial in bioreactor systems as it induces biomass suspension, substrate distribution, and gas transfer in the bulk fluid. While fluid mixing in bioreactors can be achieved in many different ways, the most common mixing techniques are mechanical agitation using an impeller and gas-lift or upflow-based mixing. In a well-mixed system, concentration gradients are negligible and resistance to mass transfer in the bulk liquid is minimal. Mixing in bioreactors additionally exerts a shear stress on cultured cells. The ability of cells to withstand shearing force depends on their individual and aggregate mechanical strength, morphology, the magnitude of shear stress, and the duration of exposure to the shearing force. Individual microbial cells are less susceptible to shear due to their small size relative to the turbulent microscale and protection offered by
the cell wall.\textsuperscript{207–209} While microbial cells can survive high shear, microbial biofilms, aggregates, and granules are affected at much lower shear thresholds.\textsuperscript{207} High shear stresses can damage the aggregate structure of cells or adversely affect their functions.\textsuperscript{204,206,210}

Besides the importance of mixing in promoting flux homogeneity in bioreactors, shear induced by mixing is also known as a core selection pressure for microbial granulation.\textsuperscript{62,211–213} Studies on aerobic granular sludge (AGS) revealed that significant shear forces are required for granule formation.\textsuperscript{62,214–216} Conversely, a number of studies have also reported that AGS granulation cannot occur at low shear forces.\textsuperscript{62,215,216} According to the literature, hydrodynamic shear force promotes microbial attachment and cell-to-cell self-immobilization processes.\textsuperscript{62,215} Liu and Tay\textsuperscript{62} demonstrated that the three-dimensional structure of microbial granules is shaped by hydrodynamic shear. Shear force also influences the structure, morphology, size, and stability of granules.\textsuperscript{62,207,213–217} Relatively high hydrodynamic shear forces can result in the formation of more compact, denser, and smaller granules than the systems with low shear force in which granules tend to become heterogeneous, porous, weaker, and larger in size.\textsuperscript{62,64,214} Hydrodynamic shear force also enhances the production of extracellular polymeric substances (EPS) within granules.\textsuperscript{62,214} EPS are known to promote the compactness and increase the structural strength of granules.\textsuperscript{62,63,218} Moreover, high content of negatively charged amino acids in EPS proteins enhances formation of electrostatic bonds between amino acids and multivalent cations, leading to stabilizing granule structure.\textsuperscript{219}

In most biogranular wastewater treatment systems, hydrodynamic shear is commonly created by airlift or upflow-based mixing.\textsuperscript{64,211–214,217} In these systems, shear force is roughly described by the upflow superficial gas velocity (m/s).\textsuperscript{64,211–214,217}
common approach to quantify shear rate in airlift reactors is based on empirical expressions obtained for bubble column reactors.\textsuperscript{204,220} The average shear rate (G) is calculated as a function of the superficial gas velocity per cross-sectional area of the reactor.\textsuperscript{204,220} This approach, however, has been criticized because it is based on film heat-transfer coefficients at liquid-solid interfaces and neglects the physicochemical and momentum transport properties of the fluid.\textsuperscript{221,222} Therefore, there is still a lack of robust correlations capable of describing the shear field in microbial granule systems.

In recent years, oxygenic photogranules (OPGs) have gained increasing interest due to their ability to treat wastewater without supplemental aeration, substantially reducing the energy demand for wastewater treatment.\textsuperscript{8,15–17,223,224} OPGs are spherical microbial aggregates primarily composed of filamentous cyanobacteria, green algae, and heterotrophic bacteria.\textsuperscript{15,17,19,163} In OPG wastewater treatment systems, oxygen required for the oxidation of organic matter and nitrification can be generated internally by phototrophs and thus sustain self-aeration process.\textsuperscript{15,16} Mixing is essential in OPG reactors to facilitate the interaction of photogranules with light as light availability within reactor is critical for photosynthesis and oxygen generation. Unlike other granules applied in wastewater treatment (e.g., aerobic granular sludge, anammox granules), OPG process is operated in stirred-tank reactors.\textsuperscript{15,17,223} Mixing in OPG reactors is provided by overhead stirrers equipped with flat blade paddle impellers.\textsuperscript{15,17,223} Stirred-tank reactors can offer several advantages to bioprocesses, including good bulk fluid mixing and easy scale-up.\textsuperscript{204,220} The impeller in stirred-tank reactors provides sufficiently rapid agitation to disperse all compounds and achieve an effectively homogeneous concentration inside the bioreactor.\textsuperscript{204} Hydrodynamic turbulence caused by the rotating impeller results in shear stresses within
fluid layers in stirred-tank reactors. Shear rate in stirred-tank reactors is directly correlated with the impeller's rotational speed or power input that depends on impeller speed.\textsuperscript{205,220,225} Shear rate in microbial granular systems is critical because it not only describes the mixing characteristics in bioreactor, but also influences the viability and physiology of microorganisms and thus system performance. Well-established equations are currently used to quantify shear rate in stirred-tank reactors.\textsuperscript{205,217,220,225,226} These equations adapt multiple engineering parameters such as reactor volume, paddle characteristics, and fluid density and viscosity to precisely estimate the shear rate in reactors.\textsuperscript{205,217,220,225,226}

Despite its importance in granule formation, little information is currently available on the role of hydrodynamic shear force in photogranulation. The common effects of hydrodynamic shear force on other microbial granules (e.g., aerobic and anaerobic granular sludge) may not be transferable to photogranules because of the differences in mixing techniques, microbial community, and physiology, and thus granular sensitivity to shear. Compared to other granules primarily consisting of heterotrophic bacteria, OPGs are enriched with phototrophic microbes, primarily filamentous cyanobacteria, which are known for higher shear resistance due to the great thickness of their cell wall (up to 500 nm).\textsuperscript{227–229} Moreover, the mixing intensity in OPG reactors affects the interaction of granules with light substrate and subsequently photosynthesis and oxygen production. It has been known that cyanobacteria have strict light requirements.\textsuperscript{230} Too little light can result in insufficient energy production while too much light can inhibit the growth of cyanobacteria or decrease photosynthesis efficiency.\textsuperscript{231–233} Filamentous cyanobacteria, the key granulating group in OPG, exhibit diverse types of motility.\textsuperscript{152,234} Filamentous cyanobacteria growing in microbial mats often migrate vertically and horizontally within
the mat to optimize their light exposure.\textsuperscript{230} This suggests that the effect of mixing induced hydrodynamic shear on photogranule formation will likely be different from other typical granules.

In this study, we investigated the role of shear force in photogranulation. The study involves three sequencing batch reactors operated at three different mixing speeds. Mixing was provided by overhead stirrers equipped with a stainless-steel impeller. We determined the hydrodynamic characteristics of reactors, including Reynolds number (Re), Shear stress (τ), Shear rate (G) and Kolmogorov microscale (η). The influence of shear on the particle size distribution, cyanobacteria content, and morphology of OPG biomass was examined. The evolution of EPS in OPG biomass is presented. The study also reports the performance of OPG reactors, including the removal of COD, ammonia, total nitrogen, and the settleability of biomass. This work is expected to be useful for a better understanding of the role of shear in photogranulation for future scaleup efforts.

5.2. Materials and Methods

5.2.1. Generation of seed photogranules

The seed photogranules were generated by incubating activated sludge batches under hydrostatic conditions with light following the procedure in Milferstedt \textit{et al.}\textsuperscript{17} Briefly, activated sludge was collected from the aeration basin of a local wastewater treatment plant (Hadley, MA) and 10 mL aliquots were inoculated in 20 mL glass vials. The vials were capped and statically incubated at constant temperature (20 °C) under light conditions. The light conditions provided a photosynthetically active radiation (PAR) of
about 150 μmol/m²-s on the top of vials. Spherical photogranules of 5–10 mm in diameter were produced in the vials after 25–35 days of incubation.

### 5.2.2. Reactors seeding and operation

About 120 hydrostatically formed photogranules were seeded into a glass beaker containing 2 L of primary effluent wastewater collected from a local wastewater treatment plant (Amherst, MA). The reactor was operated in batch mode under light and mixing conditions for 24 h. Light was provided by fluorescent-light bulbs emitting a photosynthetically active radiation (PAR) of approximately 150 μmol/m²-s on the reactor surface. The reactor was mixed at approximately 100 rpm. After 24 h, the reactor volume was increased to 3 L by adding 1 L of primary effluent wastewater. The OPG mixed biomass was then split into three 1 L reactors that were operated in batch mode under three different mixing speeds (50 rpm, 100 rpm, and 250 rpm) for four days. After four days of batch operation, the reactor volume was increased to the final volume of 1.5 L by adding 0.5 L of primary effluent wastewater to each reactor. The three reactors were then operated in sequencing batch mode for 250 days.

During the SBR operation, reactors were operated in four 6 h cycles per day. Each 6 h cycle included 2.5 h of dark phase, followed by 3.5 h of light phase. The light conditions provided to the three reactors during the light phase were the same as those provided to the batch reactors (PAR of approximately 150 μmol/m²-s on the reactor surface). Reactors were operated at a hydraulic retention time (HRT) of 1 d. The reactors were fed wastewater over 10 min at the beginning of each cycle. The settling time of biomass was set to 10 min with no mixing, followed by 2 min for effluent decanting.
5.2.3. Hydrodynamic characteristics of reactors

Our sequencing batch reactor (SBR) systems were 2 L flat-bottom glass beakers with a working volume of 1.5 L. Reactors had an inner diameter of 12.2 cm and a working height of 12.9 cm. Mixing in reactors was provided by overhead stirrers. Each stirrer was equipped with a stainless-steel flat blade paddle impeller. The paddle-blade impeller had a diameter of 5.2 cm and a width of 1.9 cm. The impeller off-bottom clearance, the vertical distance from the impeller bottom to the vessel bottom, was set at 4 cm. Mixing speeds in Reactor 1, Reactor 2, and Reactor 3 were set at 50 rpm, 100 rpm, and 250 rpm, respectively. Hydrodynamic characteristics of reactors including Reynolds number (Re), Shear stress (τ), Shear rate (G), and Kolmogorov microscale (η) were estimated using equations presented in Doran, Furukawa et al.,226 and Wan et al.217

5.2.4. Influent wastewater

The influent used to feed our OPG reactors was the primary effluent wastewater of Amherst wastewater treatment plant (Amherst, MA). The total COD (tCOD) concentration in influent wastewater ranged between 58 mg/L and 204 mg/L with an average of 127±38 mg/L. The total dissolved nitrogen (TDN) concentration was in the range of 20–60 mg N/L with an average of 34±10 mg/L. Ammonia (NH₄⁺) concentration was in the range of 13–43 mg N/L with an average of 27±10 mg N/L, representing about 80% of TDN. Organic nitrogen was at the level of 7±4 mg N/L representing about 20% of the TDN.
5.2.5. Microscopy

Microscopy was regularly performed to monitor the progression of photogranulation in reactors. Brightfield, fluorescence, and scanning electron microscopy (SEM) were performed following the procedure presented in Milferstedt et al.\textsuperscript{17} For the brightfield and fluorescence microscopy, the entire photogranules were fixed in Tissue-Tek OCT Compound 4583 (Sakura Finetek, USA) and then frozen at $-80\, ^\circ$C. The photogranules were then sectioned at the maximum diameter under a stereomicroscope and directly used for imaging by a Leica M205FA stereomicroscope.

For SEM, the entire photogranules were pre-fixed in an unbuffered glutaraldehyde solution (1\%) and then cross-sectioned at the maximum diameter under a stereomicroscope. Photogranules and cross-sectioned photogranules were then rinsed three times with phosphate buffer solution. Next, samples were fixed in osmium tetroxide solution (1\%) and then rinsed three times with phosphate buffer solution and Milli-Q water. Samples were dehydrated in a graded ethanol series. The samples were then dried using tertiary butanol method.\textsuperscript{138} Lastly, photogranule samples were coated with gold-palladium and then imaged by a FEI Quanta 200 Microscope.

5.2.6. Biomass imaging and particle size distribution (PSD) analysis

We regularly collected the images of mixed OPG biomass in Petri dishes (5 cm in diameter) using a digital camera. We determined the particle size of the OPG biomass using the software ImageJ.\textsuperscript{135} Firstly, we set the color threshold range of biomass images. The biomass images were then binarized. The software ImageJ automatically identified the shapes of particles and determined their dimensions. The software also quantified the total
number of OPG particles in the images. This number was used to calculate the total biomass particles in reactor. The violin plots of the particle size distribution (PSD) were created using the software Origin 2019. Finally, we used the Frequency Function in MS Excel 2010 to create the histograms of volume-based particle distributions.

5.2.7. DNA extraction and qPCR analysis

Total microbial DNA of photogranule biomass was extracted using a MoBioPowerSoil DNA Extraction kit following manufacturer instructions. OPG biomass samples were homogenized for 10 sec (IKA T18 basic ULTRATURRAX homogenizer) and then dewatered by centrifuge and pipetting. DNA quantity and quality were determined by Nanodrop. Concentrations of DNA extract that met minimum quality standards (~1.8 for 260 nm/280 nm and ~2 for 260 nm/230 nm) varied between 5 and 50 ng/μL.

The analyses for functional marker genes were performed using Applied Biosystems Step One and MJ Research quantitative PCR system. qPCR reactions were done in 25 μL reactions using 12.5 μL iTaq 2× 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 min followed by 40 cycles consisting of 95 °C for 15 sec, Tannealing for 1 min, and 72 °C for 30 sec, followed by 30 sec at 55 °C and a +0.3 °C ramp up every 15 sec to 95 °C to determine the melting curve for the target gene with the appropriate the annealing temperature listed the primer. Target genes were Cyanobacterial 16S rRNA gene (given shorthand CYAN in this study) – Tannealing = 52 °C, and 16S Universal rDNA – Tannealing = 60 °C.235,236 All reactions were run in triplicate. Standard curves, melting curves and negative controls were run for each qPCR run. The relative
quantification of primer amplification was analyzed using the comparative C_t method using 16S rRNA universal gene as a reference.  

**5.2.8. Extraction and analysis of extracellular polymeric substances (EPS)**

EPS was extracted from the photogranular biomass following the sequential sonication and base extraction method presented in Ansari et al.  

Briefly, OPG biomass samples were centrifuged at 12,000 rpm for 10 min and the biomass pellets were then suspended in 10 mL phosphate buffer saline (PBS) solution (10 mM NaCl, 1.2 mM KH_2PO_4, and 6 mM Na_2HPO_4). Samples were then homogenized at 700 rpm for 30 s. Homogenization was followed by sonication at 10% strength for 40 sec, and then centrifugation at 12000 rpm for 10 min. Next, the supernatant was filtered with a 0.45 μm cellulose filter and considered as the “biomass-bound EPS extracted by sonication.” The remaining biomass pellets were resuspended in 10 mL PBS solution. The pH of samples was adjusted to 10.5 using NaOH solution (1 M) adopting the method shown in Park and Novak.  

Next, the samples were shaken at 400 rpm for 2 h at 4 °C and then centrifuged at 12000 rpm for 10 min. The supernatant was filtered with a 0.45 μm cellulose filter and the filtrate was designated as the “biomass-bound EPS extracted by base.” The total biomass-bound EPS was calculated by taking the sum of biomass-bound EPS extracted by sonication and base. The Dubois method and the modified Lowry method were used to measure the polysaccharide and protein concentrations, respectively, in the final EPS extract.
5.2.9. Analytical methods

The total and volatile suspended solids of the OPG biomass were determined according to Standard Methods 2540D/E.\textsuperscript{139} The chemical oxygen demand (COD) of influent and effluent samples was determined following Standard Methods 5220D.\textsuperscript{139} The total dissolved nitrogen (TDN) concentration in influent and effluent samples were measured by TOC/TN analyzer (TOCVCPH, Shimadzu). Concentrations of dissolved inorganic nitrogen (ammonia, nitrite, and nitrate) were measured using ion chromatography (Metrohm 830). Chlorophyll concentration in the OPG biomass was determined spectrophotometrically following Standard Methods (10200H).\textsuperscript{139} Zone settling velocity (ZSV) and sludge volume index (SVI) of photogranular biomass were determined following Standard Methods (2710D/E).\textsuperscript{139} The linear-regression analysis method shown in Chon \textit{et al.}\textsuperscript{238} was used to determine the observed yield of OPG biomass ($Y_{obs}$) in each reactor as g VSS produced/g tCOD consumed.

5.2.10. Statistical analysis

The two-samples \textit{t-test} were performed to determine whether there is a significant difference between the variables. The default alpha ($\alpha$) value was set at 0.05 so if the p-value is less than 0.05 there is a significant difference. We also conducted the regression analysis to examine the relationship between the variables.
5.3. Results

5.3.1. Hydrodynamic characteristics of OPG reactors

Table 5.1 shows the hydrodynamic characteristics of OPG reactors. The Reynolds numbers (Re) for the 50 rpm, 100 rpm, and 250 rpm mixing speeds in Reactor 1, Reactor 2, and Reactor 3 were 2,253, 4,507, and 11,267, respectively. Mixing in Reactor 1, Reactor 2, and Reactor 3 induced theoretical shear stresses (τ) of 0.015 N m$^{-2}$, 0.04 N m$^{-2}$, and 0.14 N m$^{-2}$, respectively. The rates of shear (G) in Reactor 1, Reactor 2, and Reactor 3 were 15 s$^{-1}$, 40 s$^{-1}$, and 140 s$^{-1}$, respectively. The shear stress and shear rate created in Reactor 3 were almost 4 times higher than those of Reactor 3 and 10 times higher than those of Reactor 1. The estimated Kolmogorov microscales (η) in Reactor 1, Reactor 2, and Reactor 3 were 256 μm, 159 μm, and 85 μm, respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reactor 1</th>
<th>Reactor 2</th>
<th>Reactor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixing Speed (rpm)</td>
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<td>100</td>
<td>250</td>
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<tr>
<td>Reynolds Number, Re</td>
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<td>4,507</td>
<td>11,267</td>
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<td>Shear Stress, τ (Pa, N m$^{-2}$)</td>
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<td>Rate of Shear, G (s$^{-1}$)</td>
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<tr>
<td>Kolmogorov Microscale, η (μm)</td>
<td>256</td>
<td>159</td>
<td>85</td>
</tr>
</tbody>
</table>

5.3.2. Reactor seeding and photogranule formation during reactor startup period

Over the first 24 h of batch operation, seed photogranules were disintegrated, and many small biomass aggregates were released into the bulk liquid (Figure 5.1a1-2). The size of these aggregates was in the range of 50–600 μm with an average diameter of 220±100 μm. Based on microscopy, these biomass aggregates contained detectable amounts of
filamentous cyanobacteria, unicellular green algae, and diatoms (Figure 5.1a1-2). The total number of particles in reactor also increased over 24 h from 120 seed photogranules to 604,800±57,000 bioaggregates. Disintegration of seed photogranules and the formation of numerous new photogranules is likely enhanced by hydrodynamic shear caused by mixing. After 24 h of operation, the reactor volume was increased to 3 L and then split into three 1 L reactors. After splitting biomass, the total number of biomass aggregates and granules in each reactor was about 201,600±19,000, while the biomass concentration was, on average, 525±26 mg/L (Figure 5.1b). For the first 4 days, these three reactors were operated in batch mode, but different mixing speeds were already applied: 50 rpm, 100 rpm, and 250 rpm for Reactor 1, Reactor 2, and Reactor 3, respectively. During this period, the total numbers of biomass aggregates and granules in Reactor 1, Reactor 2, and Reactor 3 increased from 201,600±19,000 to 263,500±21,000, 314,000±53,000, and 884,000±110,000, respectively (Figure 5.1b). The corresponding biomass concentrations in Reactor 1, Reactor 2, and Reactor 3 also increased from 525±26 mg/L to 870±21 mg/L, 940±40 mg/L, and 780±11 mg/L, respectively (Figure 5.1b).

Figure 5.1c shows the evolution of particle size distribution (PSD) in reactors over 4 days of batch operation. On day 1, the size of biomass aggregates in reactors was in the range of 0.05−0.59 mm in diameter with mean and median (D50) particle sizes of 0.22 mm and 0.19 mm, respectively. Large variations were observed in the PSDs of OPG biomass in the three reactors after 4 days of batch operation (Figure 5.1c). On day 4, the sizes of photogranules in Reactor 1, Reactor 2, and Reactor 3 were in the range of 0.05−1.5 mm, 0.02−1.1 mm, and 0.01−0.92 mm, respectively (Figure 5.1c). The mean and median (D50) particle sizes in Reactor 1 were 0.41 and 0.43 mm, respectively. These values were larger
than those of Reactor 2 (0.36 and 0.35 mm) and Reactor 3 (0.25 and 0.23 mm). The mean and median (D50) particle sizes in Reactor 3 were close to those of day 1 (0.22 mm and 0.19 mm).

Figure 5.1. Progression of photogranulation in reactors during the startup period. (A) Brightfield microscopic images of photogranules developed in reactors during the first 4 days of batch operation. Scale bar for the eight panels is 200 μm. (B) Changes in the total suspended solids concentration and photogranules’ number in reactors over four days of batch operation. (C) Evolution of particle size distribution (PSD) of photogranule biomass in reactors over four days of batch operation. Reactor 1, Reactor 2, and Reactor 3 were operated at 50, 100 and 250 rpm, respectively. Error bars represent the standard deviations of triplicate samples.
Based on microscopic observations, filamentous cyanobacteria substantially increased within the photogranule biomass in all three reactors over 4 days of batch operation (Figure 5.1a). On day 4, photogranule biomass in Reactor 1 was more spherical than the biomass in Reactor 2 and Reactor 3 (Figure 5.1a).

5.3.3. Progression of photogranulation in sequencing batch reactors (SBRs)

Over the first two months of SBR operation, photogranules in Reactor 1 and Reactor 2 significantly increased in size, while those in Reactor 3 had minimal change relative to initial conditions. On day 60, the largest photogranule sizes observed in Reactor 1, Reactor 2, and Reactor 3 were 2.5 mm, 2.2 mm, and 0.96 mm with mean photogranule sizes of 0.57 mm, 0.52 mm, and 0.24 mm, respectively (Figure 5.2, Figure 5.3). The maximum and mean photogranule size in Reactor 3 on day 60 (0.96 mm and 0.24 mm, respectively) closely resembled those of day 4 (0.92 mm and 0.25 mm, respectively) (Figure 5.2). On day 120, the largest photogranule sizes observed in Reactor 1, Reactor 2, and Reactor 3 were 3.9 mm, 3.2 mm, and 1.6 mm, respectively (Figure 5.2). On the same day, mean photogranule sizes in Reactor 1, Reactor 2, and Reactor 3 were 1.3 mm, 1 mm, and 0.35 mm, respectively, while the median (D50) photogranule sizes were 1 mm, 0.9 mm, and 0.3 mm, respectively (Figure 5.2).
Figure 5. 2. Violin plots showing the particle size distribution (PSD) of photogranule biomass in reactors over 250 days of operation. (A) Day 4. (B) Day 60. (C) Day 120. (D) Day 250.

Over the remaining operation period, photogranule in the three reactors continued to increase in size. On day 250, the largest photogranule sizes observed in Reactor 1, Reactor 2, and Reactor 3 were 5.5 mm, 3.4 mm, 1.7 mm, respectively (Figure 5.2, Figure 5.3). On the same day, the mean photogranule sizes in Reactor 1, Reactor 2, and Reactor 3 were 0.94±1.2 mm, 0.72±0.8 mm, and 0.36±0.2 mm, respectively, while the median (D50) photogranule sizes were 0.53 mm, 0.32 mm, 0.28 mm, respectively (Figure 5.2). The mean and median (D50) photogranule sizes in Reactor 1 and Reactor 2 on day 250 were much
smaller than those of day 120. The mean and D50 in Reactor 3 on day 250 was almost the same as day 120.

**Figure 5.** Development of the oxygenic photogranules in reactors over 250 days of operation. The petri dish images of mixed OPG biomass produced in reactors over 250 days of SBR operation under different mixing conditions. (A–C) Day 4. (D–F) Day 60. (G–I) Day 120. (J–L) Day 250. Scale bar for the twelve panels is 1 cm.
Figure 5.4 shows the microscopic images of photogranules produced in reactors. Over 250 days of operation, all reactors developed mature photogranules. Photogranules in Reactor 1 and Reactor 2 were more spherical and less hairy than those in Reactor 3. Photogranules in Reactor 3 exhibited outgrowths of filamentous cyanobacteria on the surface (Figure 5.3, Figure 5.4).

**Figure 5.4.** Microscopic images of photogranules produced in reactors. (a, b, g, h, m, and n) Brightfield light microscopy images. (c, d, i, j, o, and p) Autofluorescence of cyanobacterial phycocyanin in photogranules shown in a, b, g, h, m, and n panels. (e, f, k, l, q, and r) Scanning electron microscopy (SEM) of whole and cross-sectioned photogranules produced in reactors. Scale bar is 1000 μm for panels a–f, and 500 μm for panels g–r.

5.3.4. Enrichment of filamentous cyanobacteria within photogranule biomass

We used Log_2 fold change approach to describe the changes in the relative abundance of CYAN (cyanobacterial 16S rRNA) gene in photogranule biomass (Figure 5.5). Figure 5.5a shows the changes in the relative abundance of CYAN gene in photogranules from different size classes collected from the same reactor. This figure shows that the relative abundance of CYAN gene in OPG biomass changed significantly
as photogranules grew bigger in size compared to the smallest photogranule size class (0.2−0.5 mm) in the same reactor (Figure 5.5a). There was greater relative abundance of CYAN gene in photogranule size class 0.5−1 mm compared to this smallest size class (0.2−0.5 mm) of 6.7 log₂ fold, 6 log₂ fold, and 3.2 log₂ fold, for Reactor 1, Reactor 2, and Reactor 3, respectively. There are significant statistical differences in the relative abundance of CYAN gene between the size classes 0.2−0.5 mm and 0.5−1 mm in Reactor 1, Reactor 2, and Reactor 3 (p = 0.03, p = 0.008, and p = 0.0001, respectively). As photogranule size increased from 0.5−1 mm to 1−2.5 mm in Reactor 1 and Reactor 2, log₂ fold change values of CYAN gene increased to 8.1 and 9.2, respectively. Log₂ fold change values of the size classes 0.5−1 mm and 1−2.5 mm in Reactor 1 as well as Reactor 2 are not statistically different (p = 0.35, and p = 0.19, respectively). No data appear for Reactor 3 because the largest photogranule size observed in Reactor 3 was 1.7 mm in diameter. As photogranule size in Reactor 1 and Reactor 2 increased from 1−2.5 mm to 2.5−4 mm, log₂ fold change values of CYAN gene decreased to 3.3 and 1.3, respectively (p = 0.005 and p = 0.02, respectively).

Figure 5.5b shows the changes in the relative abundance of CYAN gene in photogranules from the same size class collected from the three reactors compared to the Reactor 1’s biomass (Figure 5.5b). When comparing the relative abundance of CYAN gene in the size class 0.2−0.5 mm from Reactor 2 and Reactor 3 to the same size class from Reactor 1, there is a greater abundance of CYAN gene in the biomass from Reactor 2 and Reactor 3 (1.1 log₂ fold and 4.8 log₂ fold, respectively). There are significant statistical differences in the relative abundance of CYAN gene between the size class 0.2−0.5 mm from Reactor 1 and Reactor 2 (p = 0.03) as well as between Reactor 1 and Reactor 3 (p =
0.01). For the same size class, log$_2$ fold change of CYAN gene in photogranule biomass from Reactor 3 was substantially higher than Reactor 2 (p = 0.01) (Figure 5.5b).

Similar to size class 0.2−0.5 mm, the relative abundance of CYAN gene in photogranule size class 0.5−1 mm from Reactor 2 and Reactor 3 was higher than Reactor 1 with log$_2$ fold change values of 0.74 and 1.2, respectively (p = 0.7 and p = 0.02, respectively) (Figure 5.5b). The relative abundances of CYAN gene in the size class 0.5−1 mm from Reactor 2 and Reactor 3 are not statistically different (p = 0.2). For the size classes 1−2.5 mm and 2.5−4 mm, the differences in the relative abundances of CYAN gene between Reactor 1 and Reactor 2 are not statistically significant (p = 0.5 and p = 0.2, respectively).

**Figure 5.5.** Changes in the relative abundance of CYAN gene in photogranule biomass. (A) Log$_2$ fold change of CYAN gene in photogranules from different size classes collected from the same reactor using 16S rDNA as a reference and compared to the smallest photogranule size class (0.2−0.5 mm) community. (B) Log$_2$ fold change of CYAN gene in photogranules from the same size class collected from the three different reactors using 16S rDNA as a reference and compared to the Reactor 1 biomass community from the same class. Error bars represent the standard deviations of triplicate samples.
5.3.5. Extracellular polymeric substances (EPS) in photogranule biomass

Figure 5.6 shows the changes in biomass-bound extracellular polymeric substances (EPS) in reactors during the first two months of operation. During the first 10 days of operation, the levels of both EPS proteins and polysaccharides in the biomass from the three reactors showed clear increasing patterns (Figure 5.6a, Figure 5.6b). During this period, concentrations of EPS proteins in the biomass from Reactor 1, Reactor 2, and Reactor 3 increased from 41±8 mg/g VSS, 43±7 mg/g VSS, and 39±9 mg/g VSS to 70±9 mg/g VSS, 104±10 mg/g VSS, and 127±9 mg/g VSS, respectively. EPS PS/PN ratios in the biomass from Reactor 1, Reactor 2, and Reactor 3 also increased during the same period from 0.37, 0.37, and 0.40 to 0.41, 0.44, and 0.44, respectively (Figure 5.6c).

After day 10, EPS proteins and polysaccharides continued to increase in the biomass from Reactor 2 and Reactor 3 while they started decreasing in the biomass from Reactor 1. Between day 10 and day 60, EPS proteins in the OPG biomass from Reactor 1, Reactor 2, and Reactor 3 were at the levels of 38±8 mg/g VSS, 101±7 mg/g VSS, and 165±12 mg/g VSS, respectively (Figure 5.6a and Figure 5.6b). During this period, the average concentrations of EPS polysaccharides in OPG biomass from Reactor 1, Reactor 2, and Reactor 3 were 13±6 mg/g VSS, 55±5 mg/g VSS, and 83±8 mg/g VSS, respectively (Figure 5.6a, Figure 5.6b). Statistically significant differences exist between the concentrations of EPS proteins as well as between EPS polysaccharides in the biomass from the three reactors (p < 0.001).

EPS PS/PN ratios in the biomass from Reactor 1, Reactor 2, and Reactor 3 also continued to increase during this period while started decreasing in the biomass from Reactor 1. Over 2 months, the following pattern was observed for the levels of EPS proteins
and polysaccharides in photogranule biomass: Reactor 3 > Reactor 2 > Reactor 1, suggesting that the shear had a significant influence on the production of EPS.

**Figure 5.6.** Changes in biomass-bound extracellular polymeric substances (EPS) in photogranule biomass during the first two months of operation. (A) EPS proteins. (B) EPS polysaccharides. (C) EPS PS/PN ratio. Error bars represent the standard deviations of triplicate samples.
5.3.6. Biomass concentration, growth yield and settleability

Over the first month of SBR operation, biomass concentrations in Reactor 1, Reactor 2, and Reactor 3 increased from 870 mg/L, 940 mg/L, and 780 mg/L to 3315 mg/L, 3130 mg/L, and 2685 mg/L, respectively (Figure 5.7a). Regular mixed biomass wastage of 20−400 mL with an average of 37 mL/d was undertaken from day 31. Based on this wastage scheme, the monthly solids retention times (SRT) of the three reactors were about 40 d. Beyond the first month, average biomass concentrations in Reactor 1, Reactor 2, and Reactor 3 were 3550±710 mg/L, 4000±750 mg/L, and 3270±490 mg/L with volatile fractions of 87±3%, 88±1%, 89±1%, respectively. Over this remaining operation period, the total numbers of biomass aggregates and granules in Reactor 1, Reactor 2 and Reactor 3 were 112,050±100,200, 166,650±56,271, and 1,122,450±237,100, respectively (Figure 5.7b).

The growth yield of the OPG biomass in reactors was determined using linear regression analysis on the amounts of biomass produced per the amounts of influent tCOD consumed over the entire operation period. According to this analysis, the observed biomass yields in Reactor 1, Reactor 2, and Reactor 3 were 0.71 mg VSS/mg tCOD, 0.79 mg VSS/mg tCOD, and 0.58 mg VSS/mg tCOD, respectively (Figure 5.7c). Figure 5.8 shows the settling characteristics of photogranule biomass, including sludge volume index (SVI) and zone settling velocity (ZSV). On day 1, SVIs of photogranule biomass in Reactor 1, Reactor 2, and Reactor 3 were 108±5 mL/g, 110±4 mL/g, and 109±3 mL/g, respectively, while ZSVs were 1.2±0.3 m/h, 1.3±0.3 m/h, and 1.3±0.3 m/h (Figure 5.8). Over two months of SBR operation, SVIs of OPG biomass in Reactor 1 and Reactor 2 decreased to 43±4 mL/g and 69±6 mL/g, showing characteristics of granular development, while SVI
of biomass in Reactor 3 increased to 122±3 mL/g (Figure 5.8a). By day 250, the SVIs of OPG biomass in reactor R1, R2, and R3 were 58±2 mL/g, 42±2 mL/g, and 75±3 mL/g, respectively (Figure 5.8b). Compared to day 60, SVI of OPG biomass in reactor R1 showed an increasing trend while that of reactor R3 decreased.

Figure 5.7. Biomass concentration and biomass yield in reactors. (A) Dry biomass concentration in reactors. (B) The total number of biomass aggregates and granules in reactors. (C) Biomass yield in reactors.
On the other hand, ZSVs of mixed OPG biomass in Reactor 1 and Reactor 2, increased to 9.5±0.9 m/h and 4.3±0.7 m/h, respectively by day 60, while ZSV of biomass in Reactor 3 decreased to 0.6±0.3 m/h (Figure 5.8b). ZSV increased over the operation period with the mixed OPG biomass in Reactor 1, Reactor 2, and Reactor 3 having ZSVs of 28±2 m/h, 20±2 m/h, and 5.7±1 m/h, respectively on day 250 (Figure 5.8). Compared to day 60, SVI of OPG biomass in Reactor 1 on day 250 was slightly higher, while SVIs of biomass in Reactor 2 and Reactor 3 were much lower.

![Figure 5.8. Settling characteristics of photogranule biomass in reactors. (A) Sludge volume index (SVI). (B) Zone settling velocity (ZSV). Error bars represent the standard deviations of triplicate samples.](image)

### 5.3.7. COD removal

The total COD (tCOD) concentration in influent wastewater ranged between 58 mg/L and 204 mg/L with an average of 127±38 mg/L. The concentration of tCOD in influent wastewater significantly changed over time due to the changes in wastewater strength at the wastewater treatment facility. Over the first 110 days, the average influent
tCOD was 150±34 mg/L. During this period, the tCOD removal (%) in Reactor 1, Reactor 2, and Reactor 3 were 72±11%, 77±11%, and 79±10%, respectively (Figure 5.9a). Concentrations of tCOD in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 39±10 mg/L, 33±9 mg/L, and 29±10 mg/L. Statistically significant differences exist between the concentrations of tCOD in the effluents of Reactor 1 and Reactor 2 (p = 0.02) as well as between Reactor 1 and Reactor 3 (p = 0.001).

![Figure 5.9](image)

**Figure 5.9.** Removal (%) of total COD in reactors. (A) Removal (%) of tCOD during different operation periods. (B) Average removal (%) of tCOD over 250 days of operation.

The concentration of tCOD in influent wastewater significantly decreased between day 110 and day 180. During this period, the average concentration of tCOD in influent wastewater was 89±20 mg/L. The tCOD removal (%) in Reactor 1, Reactor 2, and Reactor 3 during this period were 73±6%, 73.6±5%, and 77±6%, respectively (Figure 5.9a). Concentrations of tCOD in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 23±3 mg/L, 23±2.5 mg/L, and 19±3 mg/L, respectively. Statistically significant differences exist between the concentrations of tCOD in the effluent of Reactor 1 and Reactor 3 (p = 0.005).
No statistical differences exist between the concentrations of tCOD in the effluents of Reactor 1 and Reactor 2 as well as Reactor 2 and Reactor 3 ($p = 0.7$ and $p = 0.1$, respectively).

Over the remaining operation period (days 180−250), the concentration of tCOD in influent wastewater increased back to 124±24 mg/L. The tCOD removal (%) in Reactor 1, Reactor 2, and Reactor 3 during this period also increased to 78±3%, 80±3%, and 84+3%, respectively (Figure 5.9a). Concentrations of tCOD in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 28±6 mg/L, 25±6 mg/L, and 20±3 mg/L, respectively. Statistically significant differences exist between the concentrations of tCOD in the effluents of Reactor 1 and Reactor 3 ($p = 0.0001$) as well as between Reactor 2 and Reactor 3 ($p = 0.005$). Concentrations of tCOD in the effluents of Reactor 1 and Reactor 2 are not statistically different ($p = 0.1$).

Over the entire operation period, the tCOD removal (%) in Reactor 1, Reactor 2, and Reactor 3 were 74±9%, 77±9%, and 80±8%, respectively (Figure 5.9b). The average concentrations of tCOD in the effluents of Reactor 1, Reactor 2, and Reactor 3 during the entire operation period were 32±11 mg/L, 28±8 mg/L, and 24±9 mg/L, respectively. Statistically significant differences exist between the average concentrations of tCOD in the effluents of the three reactors during the entire operation period ($p <0.05$).

5.3.8. Nitrogen removal in OPG reactors

The total dissolved nitrogen (TDN) concentration in influent wastewater was in the range of 20−60 mg N/L with an average of 34±10 mg/L while ammonia ($NH_4^+$) concentration was in the range of 13−43 mg N/L with an average of 27±10 mg N/L,
representing about 80% of TDN. Organic nitrogen was at the level of 7±4 mg N/L representing about 20% of the TDN.

Over the first 110 days of operation, concentrations of ammonia and TDN in influent wastewater were 33±7 mg N/L and 40±10 mg N/L, respectively. During this period, ammonia removal (%) in Reactor 1, Reactor 2, and Reactor 3 were in the range of 5–73%, 14–88%, and 35–90% with an average of 34±18%, 47±22%, and 70±15%, respectively (Figure 5.10a). Statistically significant differences exist between the ammonia removal (%) in the three reactors (p < 0.5). During this period, ammonia concentrations in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 22±7 mg N/L, 18±9 mg N/L, and 10±6 mg N/L, respectively. During the same period, TDN removal (%) in Reactor 1, Reactor 2, and Reactor 3 were 37±16%, 42±12%, and 41±14%, respectively (Figure 5.10c). Levels of TDN in the effluents of Reactor 1, Reactor 2, and Reactor 3 during this period were 25±8 mg N/L, 23±7 mg N/L, and 23±7 mg N/L, respectively. No statistical differences exist neither between the removals (%) nor the levels of TDN in reactor effluents during this period (p > 0.1).

Due to the decrease in wastewater strength at the WWTP between day 110 and day 180, the concentration of ammonia and TDN in influent wastewater decreased to 14±2 mg N/L and 23±2 mg N/L, respectively. During this period, ammonia removals (%) in the three reactors substantially increased to 97% (Figure 5.10a). Concentrations of ammonia of in the effluents of the three reactors decreased to about 0.4 mg N/L. No statistical differences exist neither between the removals (%) nor the concentrations of ammonia in reactor effluents during this period (p > 0.4). During the same period, TDN removals (%) in Reactor 1, Reactor 2, and Reactor 3 were 58±9%, 49±10%, and 27±13%, respectively.
(Figure 5.10c). Concentrations of TDN of in the effluents of Reactor 1, Reactor 2, and Reactor 3 during this period were 9±2 mg N/L, 11±2 mg N/L, and 17±2 mg N/L, respectively. Significant statistical differences exist between the removals (%) as well as the concentrations of TDN in the effluents of the three reactors during this period (p < 0.01).

Figure 5.10. Removal (%) of ammonia (NH4+) and total dissolved nitrogen (TDN) in OPG reactors. (A) Removal (%) of NH4+ during different operation periods. (B) Average removal (%) of NH4+ over 250 days of operation. (C) Removal (%) of TDN during different operation periods. (D) Average removal (%) of TDN over 250 days of operation.
During the remaining operation period (days 180–250), the concentration of ammonia and TDN in influent wastewater increased back to 33±4 mg N/L and 38±3 mg N/L, respectively. During this period, ammonia removal (%) in Reactor 1, Reactor 2, and Reactor 3 decreased to 55±6%, 70±9%, and 86±6%, respectively. Concentrations of ammonia in reactor effluents increased to 15±3 mg N/L, 10±4 mg N/L, and 5±2 mg N/L, respectively. TDN removal (%) in Reactor 1, Reactor 2, and Reactor 3 during the same period were 58±4%, 44±5%, and 28±5%, respectively. Concentrations of TDN in reactor effluents were 16±2 mg N/L, 22±3 mg N/L, and 28±3 mg N/L, respectively. Significant statistical differences exist between the removal efficiencies as well as the concentrations of both ammonia and TDN in the effluents of the three reactors (p < 0.001).

Over the entire operation period, the removal (%) of ammonia in Reactor 1, Reactor 2, and Reactor 3 were 59±30%, 68±27%, and 82±16%, respectively, while the removal efficiencies of TDN were 48±16%, 45±11%, and 33±14%, respectively (Figure 5.10b, Figure 5.10d). Significant statistical differences exist between the removal efficiencies of both ammonia and TDN in Reactor 1 and Reactor 3 as well as between Reactor 2 and Reactor 3 (p < 0.002). No statistical differences exist between the removal efficiencies of both ammonia and TDN in Reactor 1 and Reactor 2 (p = 0.2).

Removals of ammonia and TDN in reactors were accompanied by some levels of nitrate and nitrite appearing in the effluents of reactors, indicating the occurrence of nitrification (Figure 5.11). Over the entire operation period, the average concentration of nitrate in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 3.6±3 mg N/L, 6.5±4 mg N/L, and 15±5 mg N/L, respectively (Figure 5.11a). Concentrations of nitrite in the effluents of Reactor 1, Reactor 2, and Reactor 3 were 0.6±0.5 mg N/L, 1±0.8 mg N/L, and
0.6±0.5 mg N/L, respectively. The concentration of organic nitrogen were 0.7 mg N/L, 0.9 mg N/L, and 1 mg N/L, respectively (Figure 5.11).

Figure 5.11. Average concentrations of nitrate, nitrite, and organic nitrogen in reactors. (A) Nitrate. (B) Nitrite. (C) Organic nitrogen.
5.4. Discussion

Mixing is essential in OPG systems as it promotes bulk homogeneity, induces biomass suspension, and facilitates the interaction of photogranule biomass with light. Additionally, mixing exerts a shear stress on cultured biomass, which can affect their structure and functions.\textsuperscript{203–206} Shear force is considered a core selection pressure for biogranulation.\textsuperscript{62,211–213} The current study focuses on investigating the role of shear force in oxygenic photogranule wastewater treatment process. Three OPG reactors were operated under different hydrodynamic shear environments to treat wastewater without supplemental aeration. Reactor 1 was operated at shear stress and shear rate of 0.015 N m\textsuperscript{-2} and 15.3 s\textsuperscript{-1}, respectively, which were lower than both Reactor 2 (0.04 N m\textsuperscript{-2} and 39.6 s\textsuperscript{-1}) and Reactor 3 (0.14 N m\textsuperscript{-2} and 139.9 s\textsuperscript{-1}) (Table 5.1). The effect of this disparity of shear on photogranule formation was observed at the onset of reactor operation. Already after 4 days of batch operation, the mean and median (D50) particle sizes in Reactor 1 (0.41 and 0.43 mm, respectively) were significantly larger than those of Reactor 2 (0.36 and 0.35 mm, respectively) and Reactor 3 (0.25 and 0.23 mm, respectively). The mean and medium particle sizes on day 4 were higher than the calculated Eddy length scales or Kolmogorov microscales in Reactor 1, Reactor 2, and Reactor 3 (256 μm, 159 μm, and 85 μm, respectively). This growth could be attributed to the resistance of photogranule biomass to shear erosion. The total number of biomass aggregates and granules in Reactor 3 (884,000±110,000) was much greater than Reactor 2 (314,000±53,000) as well as Reactor 1 (263,500±21,000), which could be due to the higher attrition and particle-particle collisions in Reactor 3 compared to both Reactor 2 and Reactor 1 as a result of shear force
(Figure 5.1b). On day 4, photogranule biomass in Reactor 1 was more spherical than the biomass in Reactor 2 and Reactor 3 (Figure 5.1a).

While mature granules developed in all the reactors, large variations were observed in the particle size distributions, cyanobacterial contents, extracellular polymeric substances (EPS), and settleability characteristics of OPG biomass in the three reactors. Over 250 days of SBR operation, the size of photogranules in Reactor 1 and Reactor 2 conspicuously increased while the size of photogranules in Reactor 3 only changed marginally (Figure 5.2, Figure 5.3). The largest and mean photogranule sizes observed in Reactor 1 (5.5 mm and 0.94 mm, respectively) and Reactor 2 (3.4 mm and 0.72 mm, respectively) were higher than those of Reactor 3 (1.7 mm and 0.36 mm, respectively) (Figure 5.2). Although only three data points were used, Figure 5.12a shows strong negative correlations between shear stress and mean photogranule size in reactors both on day 120 ($R^2 = 0.98$) and day 250 ($R^2 = 0.96$). This indicates that hydrodynamic shear stresses in Reactor 1 and Reactor 2 favored the growth of photogranule size while relatively higher hydrodynamic shear stresses in Reactor 3 suppressed the size-growth of photogranules. The decrease in photogranule size with the increase in shear stress could be due to granule surface erosion and sloughing from shear stresses. Moreover, increasing mixing intensity and shear could enhance particle-particle collisions in the bulk liquid, resulting in granule breakup. Photogranules produced in Reactor 1 and Reactor 2 were more spherical and less hairy than photogranules produced in Reactor 3 with the highest shear force (Figure 5.3, Figure 5.4). The increased attrition could also explain why photogranules produced at a high shear stress were smaller, hairy, and higher in numbers compared to photogranules produced at lower shear stresses.
Figure 5. Regression analysis showing the relationships between the shear stress in reactors and the biomass characteristics as well as the reactors' performances. (A) Shear stress vs Mean photogranule size in reactors at Days 120 and 250. (B) Shear stress vs Fold change of CYAN gene in the photogranule size classes 0.2−0.5 mm and 0.5−1 mm. (C) Shear stress vs Quantities of EPS proteins and EPS polysaccharides in photogranule biomass. (D) Shear stress vs SVI and ZSV of photogranule biomass. (E) Shear stress vs COD removal (%). (F) Shear stress vs NH₄⁺ and TN removals (%).
In aerobic granular sludge systems, low shear forces have been reported to result in larger granules but with porous, weak, and unstable structures while high shear resulted in smaller granules but with denser, stronger, and more stable structures.\textsuperscript{9,213,214} Additionally, it has been reported that aerobic granulation did not occur when shear forces were too low.\textsuperscript{62,215,216} However, low shear stresses induced by low flow velocities have been reported to favor biofilm formation.\textsuperscript{239,240} Unlike aerobic granules, oxygenic photogranules can be produced under hydrostatic batch conditions i.e., in the absence of hydrodynamic shear.\textsuperscript{15,17,19} The ability of OPGs to grow under hydrostatic conditions as well as at different levels of hydrodynamic shear suggests that shear is not an essential inducer of photogranulation in SBR. However, the size and structure of mature photogranules are strongly correlated to hydrodynamic shear.

The reasons why photogranulation progresses even at low shear stresses while high levels of hydrodynamic shear are requisites for aerobic granulation are unidentified. This variation in shear-response behavior between OPGs and aerobic granules could be attributed to factors such as differences in the mixing technique (i.e., mechanical stirring using an impeller vs gas-lift or upflow-based mixing), the differences in the microbial community and thus physiology and sensitivity to hydrodynamic shear. The failure of aerobic granulation at very low shear,\textsuperscript{62,215,216} and the formation of porous, weak, and unstable large granules at low shear\textsuperscript{9,213,214} could be due to mass transfer limitation. Doran,\textsuperscript{205} reported that airlift reactors, the conventional reactors for aerobic granules, have lower shear than stirred-tank reactors and do not provide sufficient mixing to allow the cultivation of high-density cultures. The external mass transport coefficient (from the bulk solution into the biofilm) decreased with the decrease in the flow velocity and thus shear
stress in biofilm cultures. Mass transfer limitation to the granular core, could result in microbial decay or lysis, eventually leading to porous and weak structure of aerobic granules. In the current study, OPG process is operated in stirred-tank reactors equipped with flat blade paddle impellers. Stirred-tank reactors offer good bulk fluid mixing and easy scale-up to bioprocesses. The impeller in stirred-tank reactors provides sufficiently rapid agitation to disperse all compounds and achieve an effectively homogeneous concentration inside the bioreactor. Therefore, good mass transfer in OPG reactors could be the cause for promoted photogranulation at low mixing speeds.

Unlike other granules which are primarily composed of nonphototrophic bacteria, oxygenic photogranules are enriched with phototrophs, primarily filamentous cyanobacteria, green algae, and heterotrophic bacteria. Filamentous cyanobacteria are considered the key granulating microbial group in OPGs and their growth is crucial for the growth and stability of oxygenic photogranules under hydrostatic and hydrodynamic conditions. Although the effects of hydrodynamic shear force on biogranules and bacterial biofilms have been extensively studied, information regarding the effects of hydrodynamic shear force on filamentous cyanobacterial biogranules and biofilms is relatively unknown. A few studies have shown that the development of filamentous cyanobacteria biofilms was promoted at low shear forces. Remeu et al. studied biofilm formation by marine filamentous cyanobacteria at different shear forces and found that biofilm formation was higher under low shear conditions while the increased attrition at high shear forces suppressed biofilm growth.
In the current study, the relative abundance of CYAN gene (cyanobacterial 16S rRNA) in OPG biomass produced in Reactor 3 was much higher than biomass produced in Reactors 1 and 2 (Figure 5.5.). A strong positive correlation exists between the shear stress and the relative abundance of CYAN gene in photogranule biomass ($R^2 = 0.99, R^2 = 0.97$) (Figure 5.12b). It seems that the increased shear stress was not a significant obstacle to cyanobacterial growth within photogranules although the size of photogranules was controlled by hydrodynamic shear. Wang et al.\textsuperscript{227} reported that, cyanobacteria (e.g., *Aphanizomenon* sp. and *Spirulina* sp.) have the second greatest tolerance to shear stress among phototrophic microorganisms after unicellular microalgae (e.g., *Chlorella* sp. and *Scenedesmus* sp.). Higher resistance of cyanobacteria to shear force could be attributed to the larger thickness of their cell walls, consisting of 3–4 peptidoglycan layers with a total thickness of 10–35 nm.\textsuperscript{227–229} Cell wall thickness can be 500–700 nm in large filamentous cyanobacteria like *Oscillatoria princeps*.\textsuperscript{228,229} This could potentially explain why photogranulation was maintained in Reactor 3 with the highest shear stress studied. However, high hydrodynamic shear in Reactor 3 limited the size-growth of photogranule biomass and did not allow photogranules to grow larger than 1.7 mm in diameter.

Extracellular polymeric substances (EPS) play a critical role in photogranule formation.\textsuperscript{19,163} In the current investigation, increasing the shear force in OPG reactors stimulated the production of extracellular polymeric substances (EPS) (Figure 5.6). Biomass-bound EPS proteins and EPS polysaccharides increased by 213\% and 282\%, respectively, as the shear stress increased from 0.015 N/m$^2$ in Reactor 1 to 0.14 N/m$^2$ in Reactor 3 (Figure 5.6). Increasing shear had a positive correlation to EPS proteins ($R^2 = 0.88$) as well as EPS polysaccharides ($R^2 = 0.81$) (Figure 5.12c). The production of more
exopolysaccharides could be cells’ physiological response to the external high shear stress. In aerobic granular sludge systems, shear induces changes in surface properties of microbial cells favoring cell attachment. However, the mechanisms by which hydrodynamic shear force stimulates the production of EPS are not yet clear in a biological sense.

EPS are known to promote the cell attachment and increase the structural strength of biogranules. It has been reported that blocking the metabolic pathways of EPS synthesis prevented microbial aggregation. Liu et al. hypothesized that EPS help bridge two neighboring bacterial cells physically to each other as well as with other inert particulates, and settle out as aggregates. The high EPS protein levels in OPG biomass suggest that EPS proteins play a significant role in photogranulation. It has been reported that the high content of negatively charged amino acids in EPS proteins enhances the formation of electrostatic bonds between amino acids and multivalent cations, leading to stabilizing granule structure. Therefore, it is not surprising that photogranules in Reactor 3 grew and maintained their granular form at very high shear stress (0.14 N/m²). However, the PSD in Reactor 3 did not change significantly over the entire operation period even with the continuous increase of EPS fractions (Figure 5.2, Figure 5.3). This might be due to the breakage of photogranule biomass by the strong shear acting as a size barrier.

The sludge volume index (SVI) and zone settling velocity (ZSV) reflect the compactness of biogranules structure, related to particle size distribution, surface characteristics, density, porosity, and permeability. In this study, SVI of OPG biomass in the three reactors decreased over time while ZSV increased, indicating progression of granulation. The SVIs and ZSVs of OPG biomass in the three reactors at any time during
SBR operation were much better than the field activated sludge which had SVI of 300–400 mL/g VSS and ZSV of 0.1–0.2 m/h. Between the three reactors, SVI of OPG biomass increased with the increase in hydrodynamic shear force in reactors while ZSV decreased. By the end of reactors operation, SVI of OPG biomass in Reactor 3 was 24–43% higher than Reactor 1 and Reactor 2. Among the three reactors, Reactor 2 had the lowest SVI value (42.3±2 mL/g VSS). ZSV of OPG biomass in Reactor 1 was 37% higher than Reactor 2 and 385% higher than Reactor 3. A strong positive correlation exists between the shear stress and SVI of OPG biomass in reactors ($R^2 = 0.97$) while a strong negative correlation exists between the shear stress and ZSV of OPG biomass in reactors ($R^2 = 0.96$) (Figure 5.12d). The settling characteristics of OPG biomass changed with the shear stress most likely because of the changes in the biomass structure and particle size distribution (PSD). Low shear stresses may thus be more favorable in OPG reactors for better biomass setting characteristics compared to high shear stresses. These results are in agreement with Liu et al., who reported that a mild shear strength defined by an agitation rate of 400–600 rpm using magnetic stirrer bar resulted in larger sludge particle sizes and lower SVI. The study also reported that granulation deteriorated and SVI increased when the agitation rate was increased to 800 rpm. Several other studies have reported the decrease in SVI with decreasing shear from superficial air upflow velocity up to 4 cm/s for aerobic sludge granules.

Variations in shear force also resulted in differences in the growth yield of OPG biomass in reactors. Due to photoautotrophic assimilation of CO$_2$, OPGs can achieve biomass yields that are 3–4 times greater than those of activated sludge. While the three reactors were operated at the same solids retention times (SRT) (40 d), biomass
concentration and growth yield in Reactor 3 were lower than those of Reactor 1 and Reactor 2 (Figure 5.7). The lower biomass concentration and growth yield in Reactor 3 compared to Reactor 1 and Reactor 2 are likely due to higher catabolic activities (i.e., respiration activities) than anabolic activities in Reactor 3 as a result of the higher shear force. It has been reported that the respiration activities (i.e., the oxygen utilization and CO₂ production rates) in biofilm and aerobic granular sludge increased with the increase in the shear force which led to a decrease in the growth yield. The growth yield in suspended heterotrophic culture is a function of the partitioning of organic carbon channeled into carbon dioxide by catabolism and that converted to biomass through anabolism. These results suggest that photogranule biomass responded to shear stress by regulating metabolic pathways associated with the substrate flux flowing between catabolism and anabolism, and thus more carbon could have been converted to CO₂ production than to new cell synthesis in Reactor 3 compared to Reactors 1 and Reactor 2.

The performance of OPG reactors in terms of tCOD, NH₄⁺, and TN was significantly affected by the hydrodynamic shear force applied in reactors. In photogranules, oxygenic phototrophs, primarily filamentous cyanobacteria and microalgae, live in close proximity with other nonphototrophic microorganisms with key functional traits for wastewater treatment. The oxygen needed for the oxidation of organic matter and nitrification is produced by phototrophs within OPGs through photosynthesis and not supplied in the bulk liquid by mechanical aeration as occurring in aerobic granule sludge. Abouhend et al. demonstrated the existence of a symbiotic loop in a closed OPG system where CO₂ utilized by phototrophs to produce O₂ mainly comes from organic matter oxidation by heterotrophs.
In the current study, the removals (%) of tCOD and NH$_4^+$ increased as the shear increased in reactors. tCOD and NH$_4^+$ removals in Reactor 3 (80±8% and 82±16%, respectively) were higher than Reactor 2 (77±9% and 68±27%, respectively), and Reactor 1 (74±9% and 59±30%, respectively) (Figure 5.9b, Figure 5.10b). Removal of ammonia in reactors was accompanied by some level of nitrite and nitrate appearing in effluents (Figure 5.11), suggesting the occurrence of active nitrification. Strong positive correlations exist between the shear stress and tCOD removal ($R^2 = 0.92$) as well as between the shear stress and NH$_4^+$ removal ($R^2 = 0.95$) (Figure 5.12e, Figure 5.12f). The variations in tCOD and NH$_4^+$ removals between the three reactors could be correlated to differences in the PSD in reactors and thus the oxygen production capabilities. Earlier, Abouhend et al. demonstrated that the oxygen production by OPG biomass is size-dependent, with smaller photogranules producing more oxygen than larger photogranules due to the higher phototrophic content in the smaller granules compared to the larger granules. The oxygen production rates of photogranules between 0.2 mm and 1.7 mm in diameter were higher than those of photogranules above 1.7 mm in diameter. During quasi-steady state, 100% of photogranule biomass in Reactor 3 was less than 1.7 mm in size compared to 2.6% in Reactor 2 and 2.3% in Reactor 1, suggesting more oxygen production by the smaller OPGs in Reactor 3 than Reactor 2 and Reactor 1. Abouhend et al. also reported that the photogranules above 3.5 mm had the lowest oxygen production rate among other size classes. In the current study, about 90% of OPG biomass in Reactor 1 was greater than 3.5 mm in size while all photogranules in Reactor 2 were less than 3.5 mm. This size distribution suggests highest oxygen production by OPGs in Reactor 3 followed by Reactor 2 and lowest in Reactor 1. The increase in the oxygen production capabilities of OPG
biomass with shear could also be explained by the increase in the relative abundance of cyanobacteria, the core phototrophic microorganisms in OPG biomass. As mentioned above, the relative abundance of CYAN gene (cyanobacterial 16S rRNA) in OPG biomass produced at high shear stresses was much higher than biomass produced at medium as well as low shear stresses. It has been also reported that filamentous cyanobacteria *Spirulina platensis* displayed increased O$_2$ production with increase in shear stress up to 0.3 N m$^{-2}$. Between day 110 and day 180 when the concentration of tCOD and NH$_4^+$ in influent wastewater decreased by 40% and 56%, respectively, the removals (%) of tCOD and NH$_4^+$ in the three reactors became very similar, indicating that there was sufficient oxygen for both organic matter oxidation and nitrification. Abouhend *et al.*$^{15}$ reported that nitrifying bacteria can be outcompeted by heterotrophic bacteria for the limited amount of O2 in OPG reactors at high organic loading rates.

In contrast to tCOD and NH$_4^+$, TDN removal in reactors decreased as the shear force increased. It has been reported that nitrification and denitrification are involved in nitrogen removal in OPG systems.$^{15}$ Stauch-White *et al.*$^{249}$ reported the presence of nitrification and denitrification genes, *amoA* and *narG*, respectively, in seed photogranules. In the current study, TDN removal in Reactor 1 (48±16%) was higher than Reactor 2 (45±11%), and Reactor 3 (33±14%) (Figure 5.10d). A strong negative correlation exists between the shear stress and TDN removal in reactors ($R^2 = 0.99$) (Figure 5.12f). The variations in TDN removal between the three reactors might be due to the differences in the PSD in Reactors and thus denitrification capability. Larger OPGs in Reactor 1 and Reactor 2 can promote the growth of denitrifiers due to the limited mass transfer of oxygen in their cores, thus achieving higher DTN removal through denitrification. It has been also
reported that photogranules greater than 3 mm in diameter showed the development of a layered structure in which a concentric filamentous cyanobacterial layer encloses bacterial aggregates, suggesting limited phototrophic growth in their center.\textsuperscript{163} Hann \textit{et al.}\textsuperscript{159} demonstrated that there is the gradient of dissolved O\textsubscript{2} within photogranules where the level of dissolved O\textsubscript{2} decreases with depth. Microbial activity in larger photogranules was found to be limited by oxygen diffusion.\textsuperscript{159} In aerobic granules, simultaneous nitrification/denitrification (SND) during aeration can be achieved at a certain granule size. An average diameter of 1.3 mm was proposed for a maximal N removal\textsuperscript{49} while an optimal granule size of 0.7–1.9 mm was proposed for both sludge stability and nitrogen removal (\%).\textsuperscript{40,42,50} In aerobic granules, oxygen concentration profiles have shown that oxygen is consumed at the surface layers within a thickness of 0.1–0.7 mm.\textsuperscript{41,44,45} Limited oxygen concentrations in large aerobic granules favor the formation of an inner core with anaerobic microbes.\textsuperscript{28}

This study, therefore, concludes that hydrodynamic shear force plays a crucial role in photogranule formation. Hydrodynamic shear may not be the main driver for photogranulation since OPGs can be produced at diverse levels of shear force as well as under hydrostatic batch conditions. However, this study found that the size, structure, and function of mature photogranules are related to hydrodynamic shear imposed in reactors. Low hydrodynamic shear conditions resulted in more spherical, smoother, and larger photogranules while high shear forces favor the formation of smaller and less spherical photogranules with hairy outgrowths. The increased hydrodynamic shear forces stimulated tCOD and ammonia removals which could be due to the higher oxygen production capabilities by smaller photogranules formed at higher shear levels. In contrast, the larger
photogranules formed at the lower shear levels achieved a higher total nitrogen removal than the smaller granules formed at the higher shear forces. The study also suggests the promoted growth of denitrifiers in the oxygen-limited center of large OPGs, thus leading to higher DTN removal through denitrification. Photogranulation can be engineered towards desired size distribution to achieve treatment targets by varying the hydrodynamic shear. Since a high shear environment requires high energy input to achieve high mixing speeds, increasing the energy cost, future research is needed to optimize the operational conditions and energy balance for OPG wastewater treatment process.
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