Problem and Research Objectives:

Pathogens and heavy metals are primary threats to water quality around the world. Each year, water-borne diseases cause over four billion episodes of human illness and 1.8 million deaths, mainly due to drinking water contaminated with pathogens and heavy metals[1]. In the Northeast U.S., combined sewer overflows (CSOs) due to aging sewer infrastructure, and rural runoffs take place regularly and are the primary sources that deposit pathogens and heavy metals into surface water[2]. Since surface water, including river water, is the main source of drinking water in the region[2], these contaminant-laden runoffs pose a risk for water-borne diseases and threaten public health.

In algae-based processes, algal biomass could directly or indirectly remove pathogenic microorganisms and heavy metals from wastewater, providing an opportunity to remove the two most potent pollutants together, which cannot be achieved by the activated sludge process. However, ineffective separation of algal biomass from water has limited our ability to use algae-based processes for centralized domestic wastewater treatment or decentralized systems with limited land availability. We recently developed the oxygenic photogranule process in which algae and bacteria make easily-settling granular biomass[3], thus, enabling us to overcome challenges associated with both algae-based and activated sludge processes. During our preliminary research, we recognized a significant potential of photogranules to remove pathogens and heavy metals from wastewater due to unique genetic, physiological, and physicochemical characteristics of algae and bacteria cohabitating in granular biomass.

The main objectives of this research were to investigate the removal of pathogens and heavy metals from wastewater using photogranules and to develop the oxygenic granule process for both central and decentralized systems to treat wastewater. To accomplish our objectives, we convened a research team composed of researchers from Environmental Engineering and Microbiology departments at the University of Massachusetts Amherst. The specific aims of this interdisciplinary research were to:

1) Study the heavy metal removal by the oxygenic photogranule process.
2) Study the efficacy of photogranules in inactivating water-borne pathogenic microorganisms.
3) Investigate continuous wastewater treatment by the oxygenic photogranule process.

Methodology:

1. Operation of the oxygenic photogranule process.

We have operated two oxygenic photogranule systems in the laboratory to treat primary effluent wastewater from a local wastewater treatment plant (WWTP) and to produce photogranule biomass that can be used to study the removal of pathogens and heavy metals. The system was operated in lab-scale glass reactors with a working volume of 1.5 L. The system treated 2 L of wastewater each day corresponding to 0.75 d hydraulic retention time (HRT). There were four cycles of wastewater treatment per day. Each cycle (6 h) consisted of settling, decanting, influent feeding, and reacting periods. Each cycle also had periodic light cycles of 3.5 h/2.5 h light/dark conditions. We regularly characterized photogranule biomass and measured several water quality indicators for both influents and effluents to study the maintenance and performance of the oxygenic photogranule process.

2. Sampling of influent and effluent of the full-scale activated sludge system.

To compare the effectiveness of removing pathogens and heavy metals between the oxygenic photogranule process and conventional activated sludge system, we also conducted regular sampling and analysis of influents and effluents from a local WWTP operating the activated sludge system. Note that we used the primary effluent wastewater to feed the bench-scale oxygenic photogranule system. This

primary effluent is also the feed to the activated sludge system in a full-scale WWTP. Hence, we could compare removal efficiency based on the same source of wastewater treated in the field by the activated sludge process and in the laboratory by the bench-scale oxygenic photogranule process.

3. Enumeration of pathogenic bacteria using culture-based measurement.

Microbial indicators for pathogenic bacteria, including total coliforms, fecal coliforms, and fecal *Streptococci*, were quantified in both influent wastewater and effluent water samples. Samples were collected in 50 mL sterile plastic tubes**.** All samples were brought to the microbiology laboratory and processed in a dark box within one hour of collection. Selective growth media for three different bacterial groups were freshly prepared, poured into sterile petri dishes and left to solidify. Using the membrane filtration technique, 1 mL of serially diluted samples was filtered and placed onto the plate. Plates were incubated at 37 ˚C (for total coliforms, fecal *Streptococci*) and 45 ˚C (for fecal coliforms) for 24-48 h. The number of growing colonies was counted and recorded based on Standard Methods[4].
The total bacterial count (cfu/100 mL) was finally calculated as follows:

$$\text{Bacterial colony /100 ml} = \frac{\text{Bacterial colony counted}}{\text{ml of sample filtered}} \times \frac{\text{Number of colony right in confirmatory test}}{\text{Number of total confirmatory test colony}} \times 100$$

4. Enumeration of pathogenic bacteria using non-culture based technique.

For non-culture based microbial enumeration, we used the quantitative PCR (qPCR) technique during this research project. Both influent and effluent samples were collected and processed through a 0.22 µm PVDF membrane filter. The membranes were re-suspended in sodium chloride Tris EDTA (STE) buffer with 1% SDS. DNA was extracted from each filter by phenol/chloroform extraction procedure. An extra DNA purification step was done using Genomic DNA Clean & Concentrator-10 (Zymo Research). The samples were then diluted for quantitative analysis. qPCR was performed using the various primers (Table 1), and Apex qPCR 2x Master Mix Low ROX. The Mx3005P Real Time PCR system was used and data was analyzed with MxPro QPCR software. The DNA calibration curves were generated with the appropriate sets of DNA (extracted *E. coli* gDNA was used for *E. coli* primers) for each primer set to determine relative amounts within each water sample. The thermocycle was set for 40 cycles of 95 °C for 30 s, 59 °C for 1 m, and 72 °C for 1 m.

Table 1. Various primers used for the qPCR analysis in this research.

Primer			Reference
16S rRNA	F	CCGGATCCGTCGACAGAGTTTGATCITG GCTCAG	Rawlings, 1995[5]
	R	CCAAGCTTCTAGACGG ITACCTTGTTACGACTT	
E. coli uidA	F	ACGCGTGGTTACAGTCTTGCG	Maheux *et al.*, 2009[6]
	R	AAAACGGCAAGAAAAAGCAG	
Enterococcus	F	TCA ACC GGG GAG GGT	Layton *et al.*, 2010[7]
	R	ATT ACT AGC GAT TCC GG	
Enterococcus faecalis SodA	F	ACT TAT GTG ACT AAC TTA ACC	Layton *et al.*, 2010[7]
	R	TAA TGG TGA ATC TTG GTT TGG	

5. Heavy metals determinations

Influent and effluent samples of the oxygenic photogranule reactor were collected three times a week for heavy metals analysis. Water samples were filtered through a 0.45 μm membrane syringe filter and acidified using nitric acid (HNO_3) to adjust to a pH value of 3-4. The samples were stored in acid-washed plastic tubes. Concentrations of heavy metals were measured using Inductively Coupled Plasma Mass Spectrometer system (ELAN ICP-MS, PerkinElmer) as shown in Standard Methods[4].

Principal Findings and Significance:

1. Investigate the oxygenic photogranule system for wastewater treatment.

We evaluated the use of oxygenic photogranules for wastewater treatment. Two sequencing batch reactors were seeded with granules and fed with primary effluent wastewater collected from a local WWTP. Because of oxygenic activity of photogranules, the reactors were operated without external aeration (i.e., mechanical aeration). Dissolved oxygen during the light period ranged between 0.4–3.8 mg/L. Photogranules settled rapidly with an average settling velocity of 6.0±1.6 m/h. This enabled us to operate the system with 15 min of settling, compared to 2–3 h in the activated sludge process, potentially reducing the footprint of wastewater treatment. The combination of rapid biomass settling and continuous generation of photogranules permitted the complete decoupling of hydraulic retention time (0.75 d) and solids retention times (21–42 d), allowing for a high-rate wastewater treatment based on volumetric loads. The reactor operations tested achieved effective wastewater treatment. Despite fluctuation in influent, the average effluent total chemical oxygen demand (COD) was below 30 mg/L, meeting typical requirements for municipal wastewater treatment in THE developed world (Figure 1).

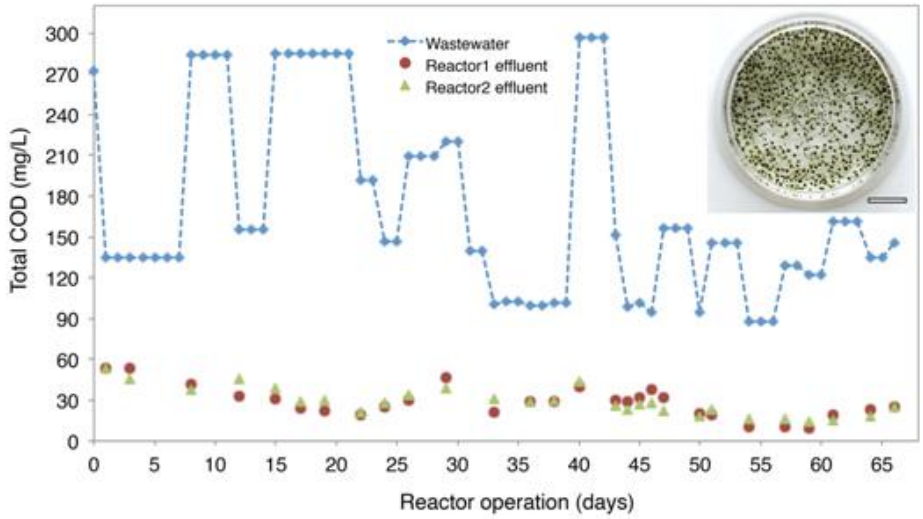

Figure 1. Oxygenic photogranules for wastewater treatment. Removal of chemical oxygen demand (COD) by two bench-scale oxygenic photogranule reactors without aeration. The reactors were operated in four cycles per day with 2.5 h/3.5 h dark and light periods. The HRT was 0.75 d, treating primary effluent wastewater collected from a local wastewater treatment plant. Inserted is the photo of reactor oxygenic photogranules in a petri dish. Scale bar is 1 cm.

2. Removal of pathogenic bacteria

Microbial enumeration based on both standard culture method (Figure 2) and qPCR analysis (Figure 3) showed that the oxygenic photogranule system achieved higher efficiency in the removal of pathogenic bacteria compared to the activated sludge system at the local wastewater treatment plant.

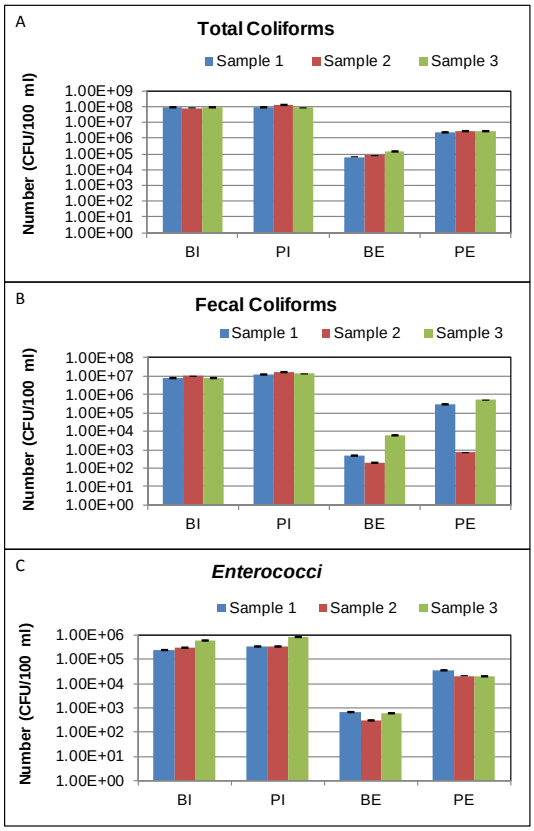

Figure 2. Counts (CFU/100 mL) of total coliforms, fecal coliforms and *Enterococci* bacteria in influent and effluent samples of the oxygenic photogranule system treating real wastewater compared to the activated sludge system at a local wastewater treatment plant. A) Total coliforms. B) Fecal coliforms. C) *Enterococccci* bacteria. Sample 1, Sample 2 and Sample 3 represent three independent sampling points that were taken in three different days during the operation. BI: bench-scale photogranule system influent, BE: bench-scale photogranule system effluent, PI: Plant influent (field activated sludge system), PE: Plant effluent (field activated sludge system).

Based on the culture-based method, the oxygenic photogranule system removed a considerably higher quantity of fecal coliform and *Enterococci* bacteria than the activated sludge process. Particularly for fecal coliform bacteria, the bench-scale oxygenic photogranule system showed removal efficiency greater than 99.99%. This highly effective removal of fecal coliform bacteria by the oxygenic photogranule process is supported by the similar removal efficiency shown by the qPCR analysis for E. *coli*. These results support our hypothesis that the microalgal community in photogranules led to the effective removal of pathogenic bacteria indicated by fecal coliform bacteria. Examples from the literature also support our observations. Araki et al. (2000)[8,9] showed that environmental factors that were favorable for algal growth were unfavorable for the living of coliform bacteria. In addition, extracellular substances secreted by cyanobacteria and algae could inactivate coliform bacteria and certain pathogenic bacteria (Moawad, 1968)[9]. Chlorella, one of the major green algae species present in the photogranule, are also known to produce and release natural antibiotics, termed chlorellin, which exhibit inhibitory activity against certain types of bacteria, including fecal coliform bacteria and human pathogen *Staphylococcus aureus*[11,12]. Our results and examples from the literature, therefore, suggest that the oxygenic photogranule process has greater efficiency of removing pathogenic bacteria compared to the activated sludge system.

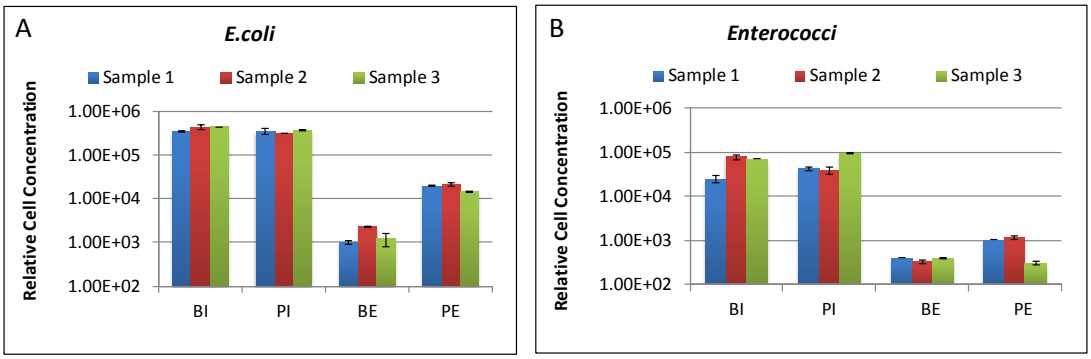

Figure 3. qPCR analysis of influent and effluent samples from the oxygenic photogranule system treating real wastewater compared to activated sludge system at the local WWTP. A) Relative abundance of *E. coli* from each sample. B) Relative abundance of *Enterococcus*. Sample 1, Sample 2 and Sample 3 represent three independent sampling points that were taken in three different days during the operation. BI: bench-scale photogranule system influent, BE: bench-scale photogranule system effluent, PI: Plant influent (field activated sludge system), PE: Plant effluent (field activated sludge system). The relative cell concentration represents a prediction for the number of cells/mL in each sample.

3. Removal of heavy metals by the oxygenic photogranule process

The heavy metal concentration in influent wastewater, to both bench-scale oxygenic photogranule and field activated sludge systems, was on average 171, 152, 64, and 34 µg/L for Fe, Mn, Zn, and Cu, respectively (Figure 4): the ranges of these heavy metals in influent wastewater were 85-274, 100-198, 29-204, and 16-59 µg/L. Although heavy metals are potent inhibitors of enzymes involved in photosynthesis, some species of algae and cyanobacteria are able to sequester them by adsorption and avoid toxic effects of heavy metals (Ginn and Fein, 2008[13]). Our photogranule system showed a higher efficiency of removing heavy metals compared to the activated sludge process. We found that the removal efficiency of the photogranule system for various heavy metals was 94-96% for Mn, 76-80% for Fe, 79-70% for Zn, and 49-54% for Cu, all of which were greater than the removal efficiency shown by the activated sludge system (Figure 4). These results also suggest that the oxygenic photogranule process can solve the current bottleneck in the field of sorption of heavy metals because previous physical and chemical treatment methods are expensive and ineffective for removing low concentrations of heavy metals from wastewater.

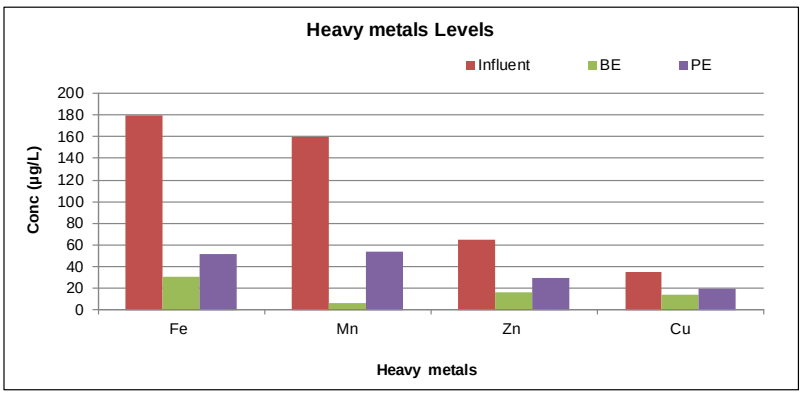

Figure 4. Levels of heavy metals in influent and effluent samples from oxygenic photogranule systems treating real wastewater compared to the activated sludge system at a local wastewater treatment plant. Influent: influent wastewater, BE: effluent of the bench-scale oxygenic photogranule system, PE: Plant effluent (activated sludge).

Overall, the current research has shown promising results that the oxygenic photogranule process can be used for treating wastewater without mechanical aeration, which currently causes the highest energy demand in wastewater treatment. Effective settling and removal of photogranules from water has also enabled us to operate the photogranule process in a high-flowrate wastewater treatment, overcoming the major challenge associated with previous algae-based wastewater treatment processes. This research also conducted two sub-studies designated to investigate the removal of pathogenic bacteria and heavy metals from wastewater using the oxygenic photogranule process. Due to unique physicochemical and biological properties of photogranules, we were able to demonstrate that the photogranule system provides an excellent means to remove these two most potent water pollutants effectively, which cannot be achieved by the traditional activated sludge system. The current research has provided a meaningful foundation for our ongoing and future research on the oxygenic photogranule process for wastewater treatment along with removing potent wastewater pollutants.

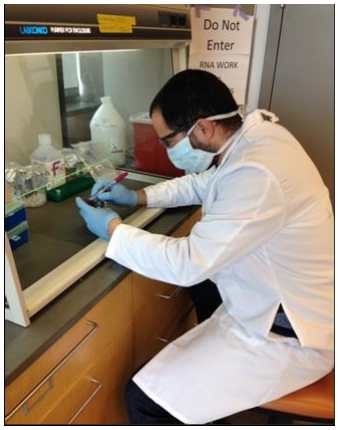

Figure 5: Ahmed Abouhend (Ph.D. student, Civil and Environmental Engineering) preparing microbial enumeration of pathogenic bacteria

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