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One Strain Solving Two Problems: Removing Harmful Nutrients in Wastewater and Harvesting Lipids for Biofuel with Immobilized Microalgae

Abstract. Microalgae is an applicable solution for the removal of nutrient contaminants (such as nitrogen and phosphorus) that pollute bodies of water. While microalgae can remediate these contaminants when implemented in wastewater treatment, the cultivation of the microalgae is costly. To address this economic obstacle, we maximized the cost-effectiveness and removal efficiency of the microalgae treatment with immobilized *Chlorella pyrenoidosa* and *Scenedesmus quadricauda,* highly efficient and adaptable strains, and the addition of a plant hormone. After nutrient removal, we used the remaining microalgae content to address an additional problem: emissions from conventional transportation fuel. The resulting increase in biomass and lipid yield from the debeaded microalgae has the potential to create a biofuel alternative, and hence, decrease the cost of wastewater treatment. The data we achieved for nitrate-N was promising; the nitrate-N levels in full-strength wastewater decreased from 35.5 mg/L to 3.8 mg/L in 7 days and the nitrate-N content in 25% wastewater (with the same beads/flask and zeatin concentration) decreased from 9.9 mg.L to 1.4 mg/L in 2 days. While the ammonium-N did not reach nutrient starvation, the full-strength wastewater removed ammonium-N from 26.6 mg/L to 5.7 mg/L, reaching

near-starvation levels (< 2 mg/L). The phosphorus removal data is still being processed. The lipid yield data was inconclusive due to the samples not reaching nutrient starvation levels (2 mg/L of nitrate-N, 2 mg/L ammonium-N).

Introduction

Freshwater is a crucial but scarce component of life. As the Earth's temperature is projected to increase by 1.5-2 ºC, an estimated 5 billion people will face water shortages by 2050 (Gerten et. al., 2013). Freshwater is necessary to support economies, with industries reliant on one-third of the global supply of freshwater (Albert et. al., 2021). However, freshwater availability will become insufficient with increasing water demands attributed to rising populations and agricultural development (Gleick & Cooley, 2021). In addition to its use for human consumption, freshwater habitat is vital to some of the most biodiverse ecosystems in the world. Despite representing 0.01% of global water, habitats such as wetlands, lakes, and rivers, are home to 6% of species (Arya, 2021).

Methods of freshwater conservation and reprocessing are essential to sustain global life and promote economic growth. Desalination, while a solution that produces freshwater from saltwater, is energy- and labor-intensive, making the process unaffordable for many nations (March, 2015). A feasible water recycling process that filters out pollutants from agricultural, municipal, and industrial waste are wastewater treatment plants (WWTPs). WWTPs remove solids, organic material, and pathogens in freshwater through a series of filtration processes before discharging the water back into reuse (Rural Community Assistance Partnership, 2015).

Background

Shortcomings of Wastewater Treatment Plants. In the United States, 16,000 WWTPs perform at 81% of their intended capacity, despite providing water treatment for 80% of Americans (ASCE, 2021). Due to this gap, American WWTPs fail to remove a large percentage of critical contaminants for most of its users. For the remediated contaminants, WWTPs require separate chemical and physical processes for the contaminants before discarding them as waste (Rural Community Assistance Partnership, 2015). These energy-intensive treatment processes are why conventional WWTPs contribute to 3% of global greenhouse gas emissions (Ahmed et al., 2022).

Nitrogen and Phosphorus. Two contaminants of concern in WWTPs are inorganic nutrients nitrogen (as ammonium-N or nitrate-N) and phosphorus (as phosphates). While these nutrients are utilized in farming and found in nearly all waste, they are classified as contaminants in excess amounts since they disrupt aquatic life (Chen et. al., 2020). Nitrogen and phosphorus threaten the vitality of aquatic ecosystems due to their high consumption of dissolved oxygen (DO), an index of the total oxygen supply in the water. As these contaminants degrade, their consumption of DO increases— sequestering the overall DO abundance such that aquatic plants and animals cannot access the DO (Chambers et. al., 2006). With this decrease in DO and the over-enrichment of nutrients, the overall water quality diminishes, resulting in eutrophic bodies of water.

Immobilized Microalgae. With microalgae-based remediation, researchers have opted to remove contaminants with immobilized and planktonic microalgae. Immobilized microalgae is constrained from motion and typically encapsulated within beads while planktonic microalgae is free-floating and mobile (De-Bashan & Bashan, 2010). While both forms of microalgae are successful remediators, immobilized microalgae has demonstrated a greater removal efficiency in nitrogen and phosphorus removal. After a 10-day wastewater treatment that utilized *Chlorella vulgaris* microalgae, nitrogen remediation rose from 64% to 89% and phosphorus remediation from 90% to 96% when immobilized microalgae was used over its free-floating counterpart (Solé & Matamoros, 2016). However, the remediation rate varied based on the number of beads per volume of solution.

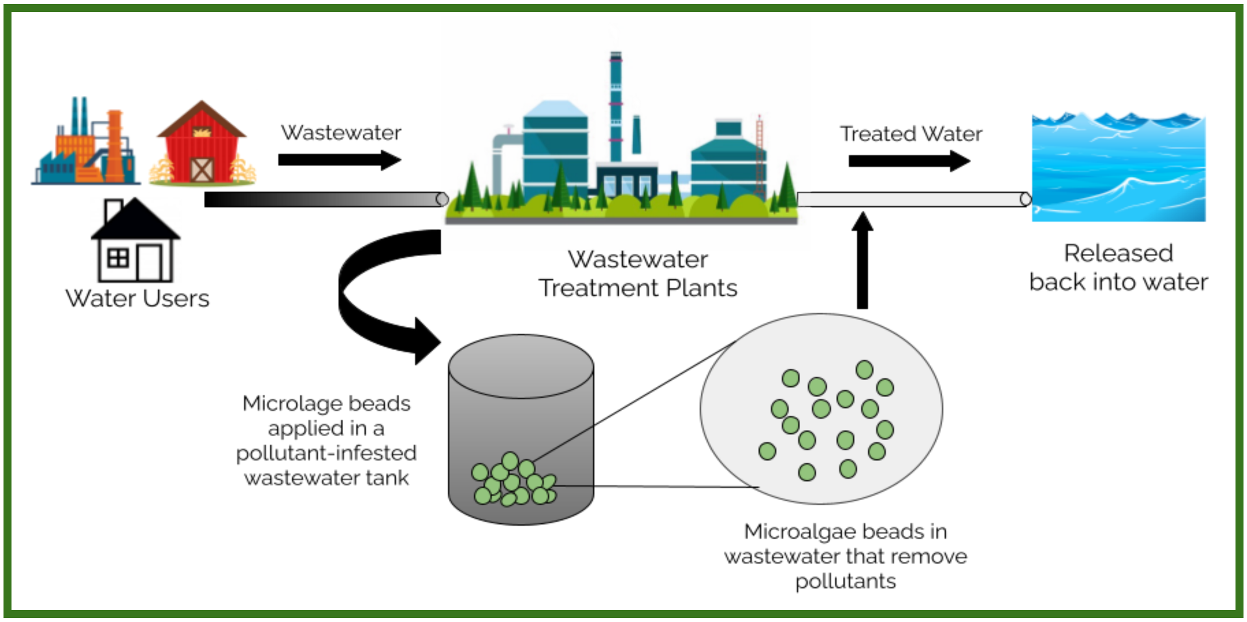


Figure 1: The potential application of microalgae beads in WWTPs.

How to Maximize Microalgal-Based Remediation of Nitrogen and Phosphorus. Due to the opportunity of utilizing microalgae for both their purpose as remediators as well as producers of lipids, studies have used the strategy of extracting lipids from the remaining microalgae (Chen et. al., 2015). This tactic would maximize the resources from microalgal-based wastewater treatment and increase the economic feasibility of remediating nutrients from wastewater using microalgae. The strain of microalgae should be a) compatible with wastewater, b) durable and able to adapt to different conditions, c) able to grow in a short period of time, and d) previously studied in preliminary literature. Both *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* fit these conditions (Sousa et. al., 2022). The cost efficiency of the process should also be considered. Due to the expense of microalgae wastewater remediation, opportunities for reducing that cost must be evaluated, such as harvesting bioproducts from the microalgae post-remediation. To accomplish this, the lipids within the microalgae must be tracked to assess how much biofuel could be produced and how that would impact the cost of remediation. Thus, having species of microalgae that have high lipid content following remediation is necessary, and studies have shown that *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* model this (Anand et. al., 2022; Plöhn et. al., 2021; Soluchana et. al., 2016).

Cost of Biofuel. However, a concern with third-generation biofuels is the investment. Since microalgae production for biofuel requires additional facilities, technology, and energy, this discourages manufacturers from producing at a large scale (Slade et. al., 2013). To increase the efficacy of utilizing microalgae for transportation fuel, the lipid yield of the microalgae must increase. Manufacturing biofuel from microalgae that simultaneously remediate municipal wastewater would decrease the cost, but it could restrict the amount of biofuel produced due to the regional availability of municipal WWTPs (Maliha & Abu-Hijleh, 2022; Gopalakrishnan et. al., 2009).

Research Objectives

To characterize the benefits of using immobilized microalgae as the primary remediation agent, our project used microalgae beads to remove environmentally-relevant concentrations of nitrogen and phosphorus. Once nitrogen and phosphorus concentrations get reduced to "starvation levels", the natural response of microalgae is to stockpile lipids. Nutrient starvation increases the lipid content because nutrient replete conditions decrease microalgal growth, so the microalgae increases its fatty acid synthesis (Ramaya et. al. 2017, Yang et. al. 2018). This metabolic condition and the addition of a plant hormone could boost overall lipid yield and biomass. If plant hormone zeatin increases the biomass and lipid yield of planktonic microalgae as reported in the literature and the immobilized microalgae remediates pollutants effectively, then adding zeatin to immobilized microalgae likewise increases biomass growth of the microalgae inside the beads and lead to higher lipid content in the microalgae, relative to control samples.

Importance and Impact

Microalgae WW Treatment. Studies suggest the application of microalgae in WWTPs is a cost-effective and natural solution to WWTPs inefficiencies for N, P removal (Geremia et. al., 2021).

Microalgae, a microorganism native to water, is an eco-friendly remediation agent that can increase the efficiency of nitrogen and phosphorus removal (Ahmed et. al., 2022). Microalgae-based remediation eliminates the need for additional chemical and physical processes that contaminants undergo in conventional WWTPs. The elimination of these additional processes can reduce the cost of WWTP operation across various sectors such as agriculture, industry, and municipal waste.

Microalgae Biofuel. While making the process of wastewater treatment more economical, creating biofuel from microalgae has additional benefits. The consumption of transportation fuel for personal and commercial use is a crucial problem, with issues of extraction, trade, and emissions (Joshi et. al., 2017). In 2016, 44% of greenhouse gas emissions originated from personal vehicles, but using biofuels, these vehicles would produce 48% less carbon (Ogunkunle & Ahmed, 2021). Biofuels, a transportation fuel extracted from plants, are able to replace fossil-fuel-derived petroleum products; however, first and second-generation biofuels have additional concerns, such as land and resource use as well as availability (Ananthi et. al., 2021). Therefore, third-generation biofuels, from microalgae, which do not utilize necessary land or natural resources, appear as the most reasonable alternative to conventional fuels (Alalwan et. al., 2019). Even microalgae with low oil content can produce around 45 times more biofuel than any other crop (Dewangan, 2018).

Our Experiment's Impact. Our intention is to investigate the combination of these two elements—both microalgae-based nutrient removal and alternative fuel cultivation—to further our strides towards a much greener, sustainable planet.

Materials. Listed here are the materials used to take our data. Immobilized *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* co-culture beads (Algae Research Supply, San Diego, CA), municipal wastewater obtained from a local source (donated), plant hormone Zeatin (Sigma), pipette tips, serological pipettes, and bottle top filtration units. Existing equipment utilized in the project: microalgae bioreactor, media storage bottles with HEPES buffer and ammonium chloride to supplement wastewater media, large Erlenmeyer flasks, Gilson pipetman precision pipettes, Molecular Devices Gemini Fluorescence microplate reader, bioreactor lights, laptop, pH meter, Vernier Nitrate-Nitrogen and Ammonium-Nitrogen probes connected to Vernier LabPro sensor interface, DMSO, Eppendorf tubes, glass vacuum filtration apparatus, and two custom DIY shakers.

Methods. The methods for the experiment are modeled after several published works and previous students' methods at our school. The microalgae beads (a co-culture of *Scenedesmus quadricauda* and *Chlorella pyrenoidosa)* were inoculated into municipal wastewater obtained locally after chlorination or into synthetic WW with nutrient levels comparable to municipal wastewater. All media were supplemented with a 10 mM HEPES buffer at pH 7.2 to keep the pH stable throughout the studies. Microalgae cultures grown in wastewater involve a two-stage approach: the first stage for the remediation of nutrients and the second for the accumulation of lipids. Appropriate control flasks without zeatin were compared with the zeatin-supplemented cultures (at 0.1 nM and 10 nM). After nitrogen depletion, control and variable cultures were evaluated qualitatively for lipid yield using fluorescence microscopy and BODIPY dye that localizes in the microalgae lipid stores.

Growing Conditions. The microalgae flasks remediated nutrients in an existing classroom bioreactor adapted for our purposes that has a controlled lighting and heating environment. *Scenedesmus quadricauda/Chlorella pyrenoidosa* co-culture beads were cultivated in square culture flasks (50 mL volume) at varying beads/flask ratios. Samples were grown at 25 ºC under a 75 W bulb and shaken at a medium speed in an orbital shaker. Microalgae had a photoperiod of 12:12 hours light-to-dark ratio. All work was done as aseptically as possible. This includes wiping everything down surfaces with 70% ethanol solution and using a Bunsen burner to flame tubes and media storage bottles. All media and solutions were sterilized using 0.22-um bottle-top filtration assemblies.

Nutrient Tracking. Nitrogen concentrations in each flask were measured every 2-3 days using Vernier ion selective probes that measure the concentration of ammonium-nitrogen and nitrate-nitrogen. Specific parameters were tracked throughout the studies: microalgal discoloration, degradation of the beads, biomass, pH, and lipid content. Phosphorus concentration data collection is underway currently using standard protocols in the cited literature.

Lipid Tracking. Qualitative data on lipid accumulation was collected after the 7-day remediation through fluorescence labeling with BODIPY (a lipid-selective fluorescent stain) and nucleic acid staining using SYBR Green (provided by Oak Crest scientists). Two beads were removed from each experimental flask and delivered to an Eppendorf tube containing 1 mL of 5% sodium bicarbonate solution; the tubes were agitated by hand over a period of two minutes. Forty microliters of suspended microalgae were delivered to a separate tube, stained with 8 uL of fluorescent dye and covered at room temperature for 5-30 minutes prior to imaging to ensure dye penetration of the lipids or nucleic acids inside the cells. Two microliters of stained microalgal suspension were delivered to a microscope slide and imaged using the EVOS fluorescence imaging microscope made by ThermoFisher. The GFP filter was used and magnification ranged from 10x-60x; acquisition time ranged from 100 ms to 500 ms. Images of the samples stained with SYBR Green were obtained using similar settings.

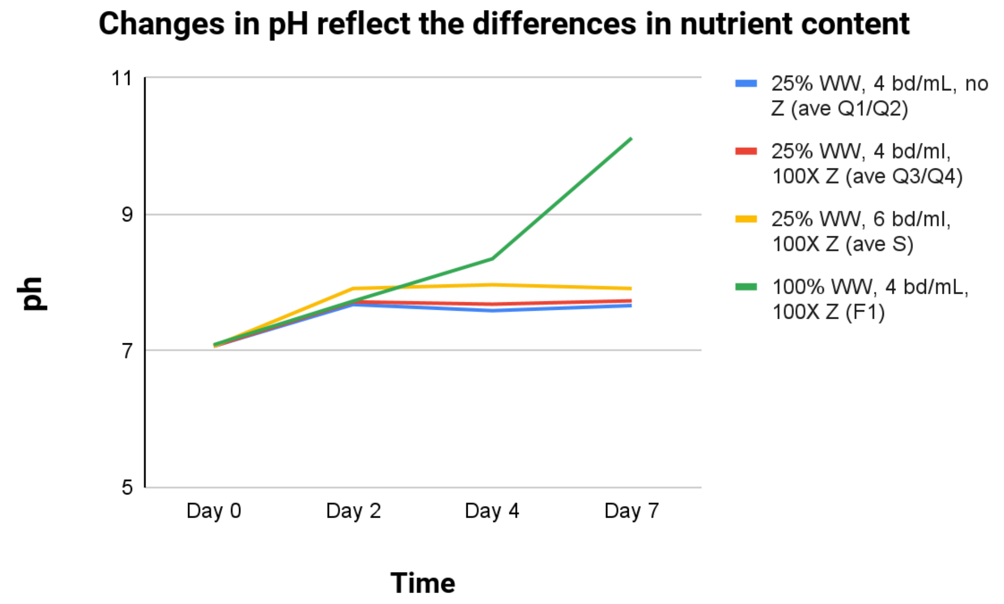
Results

Three major studies were conducted using the same type of beads, media, and growing conditions, and results from the preceding study informed changes in the next study. Nitrogen concentration—both nitrate (NO_3) and ammonium (NH_4-N)–and pH data were collected over a period of 7-10 days. There were control flasks (designated “Q1” and “Q2”), which had a 25% wastewater concentration, 4 beads/mL, and no zeatin, as well as other flasks (designated “Q3” and “Q4”), which were the same except they had 100 nM of zeatin added. Additionally, the two flasks (designated “S”) assessed the effect of a higher bead count, so while the wastewater concentration and zeatin concentration was the same as Q3/Q4, the flasks had 6 beads/mL. Finally, the “F” flask had 100% wastewater concentration, 4 beads/mL, and 10 nM of zeatin. Samples were saved for later phosphorus concentration tracking; those data points are not included here.

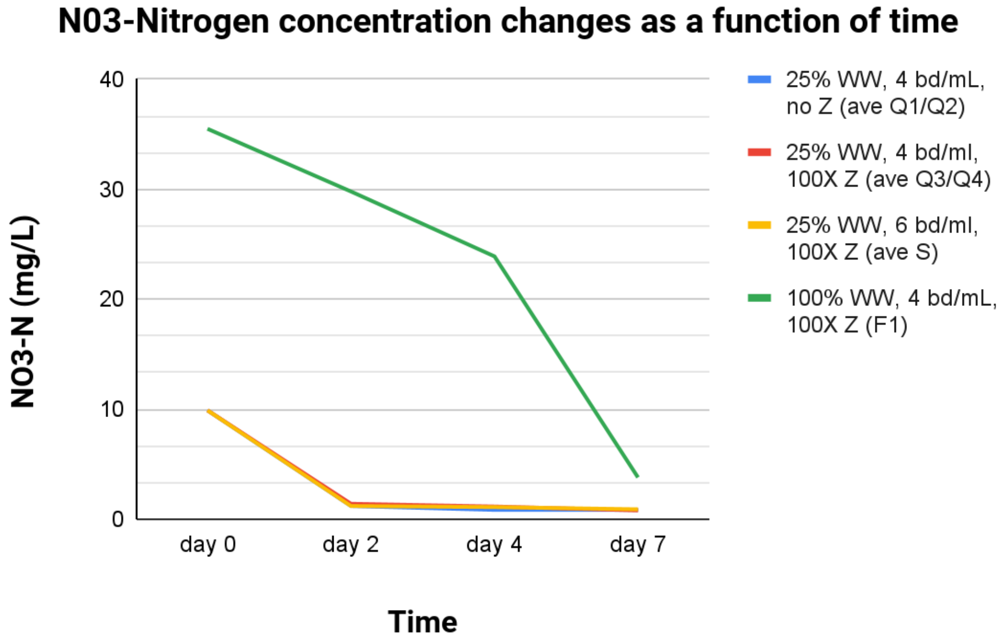
As shown in **Graph 1**, the main factors that affected pH were the initial concentration of nutrients in the wastewater as well as the beads/mL in each flask. This observation of how nutrient concentration increased the pH is especially noticeable when comparing F1 to Q3/Q4, since the only changed variable they have in common is the concentration of the wastewater; this demonstrates that the sample with higher concentrations of nutrients had more photosynthetic activity. Additionally, the S flasks had a higher pH compared to the Q flasks, indicating that the increase in beads/mL corresponded to greater photosynthetic activity, which is to be expected.

Graph 2 demonstrates that all the flasks with 25% WW were able to reach nutrient starvation (see Research Objectives) by Day 2 and the flask with full strength WW had increased remediation from Day 4 to Day 7. This graph successfully demonstrates how decreasing the nutrient levels helped the samples reach nutrient starvation, but that the full-strength wastewater would have a much higher remediation rate because it has more nutrients to remediate. However, the changes in beads/mL and concentrations of zeatin had little impact on nutrient remediation; for example, Q1 and Q2 had marginally lower concentrations of NO3 than Q3 and Q4, their counterparts supplemented with zeatin, until the 7th day.

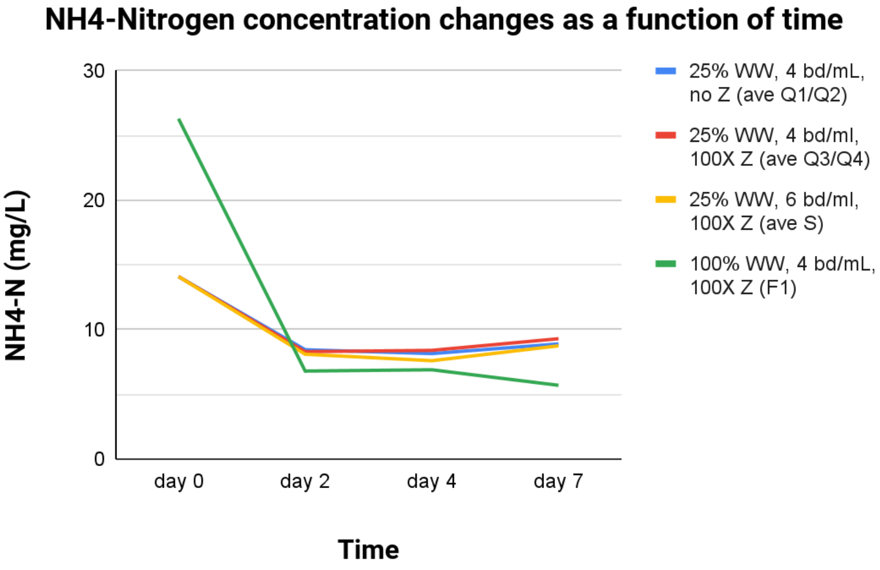
Graph 3 indicates that having a greater concentration of ammonium-nitrate (NH4-N) in the wastewater increased the rate of remediation so greatly that F1 had a lower concentration of NH4-N than the samples that started with less NH4-N. However, the levels of NH4-N in each flask were not low enough to be considered “nutrient starved” and the remediation appears to level out after day 2. While this graph depicted slightly more NH4-N remediated with more beads per flask (on day 4, the average NH4-N concentration for S1/S2 was 7.6 mg/L compared to Q3/Q4 with 8.4 mg/L), the zeatin concentration slightly decreased remediation after day 2.



Graph 1. All microalgae samples experienced an overall increase in their pH. The F1 sample which contained full-strength wastewater experienced the greatest increase with a pH difference of 3 from start to end. This undiluted wastewater signified how having more nutrients greatly increased the photosynthetic activity, hence increasing the pH. The other samples which contained wastewater diluted by 75% experienced a pH increase of less than 1. Other minute differences in data were due to changes in bead amount, with more beads/mL having a higher pH, indicating a higher photosynthetic activity. Additionally, supplementing the samples with zeatin had minute differences in photosynthetic activity; as seen in the difference between Q1/Q2 and Q3/Q4, the sample with zeatin had a slightly higher pH.



Graph 2. All samples obtained from the 25% wastewater concentrations reached nitrate-N starvation after 2 days (initially 9.9 mg/L, then 1.2-1.4 at Day 2), whereas the samples containing 100% wastewater reached near-starvation levels after 7 days (initially 35.5 mg/L, then 3.8 mg/L at Day 7). The nitrate-N levels in the 25% WW samples decreased regardless of beads/mL (4 or 6 beads/mL) and zeatin concentrations (0 or 100 nM zeatin).



Graph 3. All samples from the 25% wastewater concentrations indicated a decrease in ammonium-N content over the duration of 7 days, (initially 14.1 mg/L, then 8.93 at Day 7). However, these samples did not reach the threshold of starvation. All 25% wastewater samples were spiked with ammonium chloride to reach an initial concentration of 14.1 mg/L. The ammonium-N content in all 25% WW concentrations followed the same declining trends with or without the presence of zeatin. The ammonium-N content in the 100% WW decreased at a much quicker rate than the 25% wastewater, and even reached the lowest concentration of ammonium-N by day 2 (initially 26.3 mg/L, then 5.7 mg/L).

Discussion & Conclusion

From our experimental findings, we can derive reasoning for the pH increase, nitrogen reductions, and the implications for different wastewater conditions. All wastewater samples increased in pH— an occurrence that illustrates an effect similar in eutrophic bodies of water. Despite our addition of the buffer to maintain a stable pH range, the presence of nutrients, carbon dioxide, and the bright tent environment supported active photosynthesis in our flasks (Union County, 2016). The deviation in our F1 pH versus all other pH samples is due to the nutrient-rich, undiluted WW concentration in the F1 sample. The F1 WW media provided more nutrients for the microalgae to consume versus the 25% WW samples, therefore the pH is expected to increase due to the greater photosynthetic activity. A drawback of the high pH is that even with a decreased nutrient content after the remediation period, an additional post-remediation step is necessary to neutralize the pH before releasing the water back for reuse.

With our nitrogen remediation results, we can assert that the *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* strains preferred the consumption of nitrate-N over the ammonium-N. Despite diluting the WW so as to draw down the initial nitrate-N in hopes of the 7-day trial period leading to N starvation levels, the ammonium-N content decreased far less than the nitrate-N; the ammonium-N removal stagnated after day 2 and didn't change over the next 5 days. Our nitrate-N samples, however, delivered promising results, which surprised us since previous studies in our lab showed a preferential consumption of ammonium-N over nitrate-N when similar strains were used in nutrient removal studies (*Chlorella sorokiniana, Scenedesmus obliquus, Scenedesmus quadricauda*). Because the nitrate-N reached the starvation threshold by day 2, real WWTPs could cycle out their WW after a 48-hour microalgae remediation period. This approach aligns with a cited experiment method where the replenishment of WW every 24 hours yielded sufficient remediation (Cao et. al., 2022).

Our addition of plant hormone Zeatin did not enhance nitrogen remediation as anticipated, based on the data in **Graphs 2** and **3**. Our attempts to assess changes in lipid content due to the presence of zeatin at two different concentrations were inconclusive; the fluorescence microscopy images were obtained when the N levels had not yet reached the desired "starvation" concentrations that are needed to induce lipid stockpiling by the cells. Perhaps Zeatin was a nonequivalent substitution of our intended plant hormone, 2,4-Dichlorophenoxyacetic acid, which may have delivered more favorable results (Wang et. al., 2021).

Our research confirmed the advantages of microalgae-based technologies with a similar objective to studies prior– microalgae successfully decreases nutrient levels. In future studies, we hope to reach full nutrient starvation to evaluate the benefits of a plant hormone on remediation efficiency and lipid stockpiling. We also wish to implement a strategy that will simultaneously remediate WW while maintaining a neutral pH. Our data was promising and we hope future studies can further demonstrate our hypothesis.

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