Bioremediation of Excess Nutrients in Municipal Wastewater using Immobilized Microalgae Cultures (*Chlorella Pyrenoidosa & Scenedesmus Quadricauda)*

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ABSTRACT

Microalgae are a viable way for removing nutrient contaminants such as nitrogen and phosphorus that, when in excess, can lead to eutrophication of bodies of water. While microalgae can consume these contaminants in laboratory settings, industrial implementation of bioremediation in wastewater treatment facilities poses logistical and financial challenges. To address these obstacles, we explored the use of immobilized microalgae strains as primary remediators of ammonium-nitrogen, nitrate-nitrogen and total phosphorus. Two strains were selected (*Chlorella pyrenoidosa* and *Scenedesmus quadricauda)* and immobilized in the form of alginate beads. The additions of iron and the clay mineral clinoptilolite to the algae beads were also evaluated for possible bioremediation enhancements. Our results showed the fastest removal of ammonium-nitrogen (from 6.4 mg/L to < 1 mg/L in 1 day) by clinoptilolite and by *Scenedesmus quadricauda*. Nitrate-nitrogen concentrations were reduced most significantly (34.5 mg/L to 6 mg/L) by S*cenedesmus quadricauda* alone and by the co-culture (*Chlorella pyrenoidosa* and *Scenedesmus quadricauda)*, although the process took 6 days. Phosphorus removal by the different algae combinations (individual strains vs. co-culture) was comparable after 2 days (1000 ppb to 75 ppb). These results confirm that the microalgae are effective remediators of excess nutrients in wastewater even in the form of alginate beads and that clinoptilolite could enhance the removal efficiency.

INTRODUCTION

Fresh water scarcity represents a growing global challenge, as climate models predict that temperature increases of 1.5-2ºC will result in water shortages affecting 5 billion people by 2050 (Gerten et. al., 2013). The pressure on freshwater resources is significant, with industries consuming one-third of the global freshwater supply (Albert et. al., 2021), while population growth, agricultural expansion, and urbanization continue to drive increased water demands (Gleick & Cooley, 2021). Freshwater ecosystems, despite comprising only 0.01% of global water, support 6% of all known species (Arya, 2021). Although desalination technology offers a potential solution for freshwater production, the high energy and running costs make it out of reach for many nations (March, 2015). Wastewater treatment plants (WWTPs) present a more practical approach. They use multiple filtration processes to remove solids, organic materials, and pathogens from agricultural, municipal, and industrial waste, thereby producing clean water suitable for reuse (Rural Community Assistance Partnership, 2015).

Wastewater treatment plants (WWTPs) in the United States face significant operational problems. While serving 80% of Americans, the nation's 16,000 WWTPs operate at 81% of their capacity (ASCE, 2021), resulting in incomplete removal of contaminants. The treatment process requires separate chemical and physical stages for different contaminants before disposal (Rural Community Assistance Partnership, 2015), contributing to 3% of global greenhouse gas emissions through these energy-intensive processes (Ahmed et al., 2022). Among the most concerning contaminants are inorganic nutrients nitrogen (as ammonium-N or nitrate-N) and phosphorus (as phosphates). Although these nutrients are essential for agriculture and commonly present in waste, their excess poses significant environmental risks (Chen et. al., 2020). The excess nutrient levels lead to eutrophication in large bodies of water, thereby making it a bad problem worse for the environment.

BACKGROUND

Microalgae-based remediation in wastewater treatment plants. The chance to introduce microalgae into WWTP's represents an innovative and cost-effective solution for addressing the traditional inefficiencies in nitrogen and phosphorus removal (Taziki et al, 2015; Geremia et. al., 2021). Microalgae serve as environmentally sustainable remediation agents that can improve nutrient removal while eliminating the need for additional chemical and physical processes, thereby reducing operational costs (Ahmed et. al., 2022). The versatility of microalgae applications across different WWTP sectors has been well-documented (Wollmann et. al., 2019), with three primary removal mechanisms: adsorption, where contaminants attach to the microalgae cell wall (Kaplan, 2013); bioaccumulation, where pollutants enter and are processed within cellular structures (Mustafa, 2021); and biodegradation, where microalgae release compounds that break down contaminants into smaller molecules (Rempel et. al., 2021).

Research has demonstrated that microalgae can be implemented in two forms: immobilized (constrained within beads) and planktonic (free-floating) (De-Bashan & Bashan, 2010). Studies comparing these approaches have shown better performance with immobilized microalgae, particularly in nitrogen and phosphorus removal. For instance, a 10-day treatment using *Chlorella vulgaris* showed increased efficiency when using immobilized versus planktonic forms, with nitrogen removal improving from 64% to 89% and phosphorus removal from 90% to 96% (Solé & Matamoros, 2016). The effectiveness of immobilized microalgae is concentration-dependent, as reported in research using *Tetraselmis sp.* in artificial wastewater, where total nitrogen removal increased from 55.1% at 0.5 beads/mL to 100% at 2.5 beads/mL (Khatoon et. al., 2021).

Two Promising Microalgae Strains. The effectiveness of microalgal remediation in wastewater treatment depends on selecting strains with optimal nutrient removal capabilities. The *Chlorella* genus shows promising removal rates compared to other freshwater strains

(Sousa et. al., 2022). Within this genus, *Chlorella pyrenoidosa* is a naturally occurring algae species with good nutrient removal results (Guo et. al., 2021). Studies have demonstrated *C. pyrenoidosa's* strong performance in both synthetic and natural wastewater environments, achieving over 99% nitrate removal in both conditions, along with phosphate removal rates of 94.2% and 70.1% in collected and synthetic wastewater, respectively (Kumari et. al., 2021). This efficiency comes from the strain's photosynthetic process, which converts nitrate-nitrogen, ammonium-nitrogen, and phosphorus into carbohydrates and amino acids (Plöhn et. al., 2021). The strain's adaptability to various wastewater conditions, including pH and light intensity fluctuations makes it a good candidate for wastewater treatment applications. When immobilized, *C. pyrenoidosa* has demonstrated especially high rates of nitrogen and phosphorus removal through bioaccumulation (Guo et al., 2021).

Research into *Scenedesmus quadricauda* has shown its potential in wastewater bioremediation, with studies reporting that it can remove up to 90% of nitrogen within a 7-day period (Wong et. al., 2015). Further reports have confirmed this microalgae to be a strong performer, particularly at higher concentrations of nitrogen compounds, where *S. quadricauda* achieved removal rates of 97% for ammonium-nitrogen, 95.7% for nitrate-nitrogen, and 93.8% for phosphorus over a 21-day treatment period (Qader & Shekha, 2023). In a 10-day study, this strain efficiently removed phosphorus (85%) and nitrate-nitrogen (94%) (Roychoudhury, 2020).

Iron Supplementation. Iron is the most helpful trace metal cofactor in microalgal growth and lipid production. Iron enhances the photosynthetic process of microalgae, similar to iron as a supplement to the human body which catalyzes the production of hemoglobin and myoglobin (Han et al, 2019). Increasing the iron levels in the culture media of the microalgae increased the biomass and lipid production of the algae. As algae grows, the biosorption of nutrients increases, so there might be a correlation of iron as an accelerant to the bioremediation of nitrogen in its many forms (Han et al, 2021).

LITERATURE GAPS

Existing research has documented the effectiveness of immobilized microalgae strains (*Scenedesmus quadricauda, Chlorella pyrenoidosa*, and their co-cultures) and various clay minerals (including clinoptilolite and other zeolites) in N and P remediation (Murkani et al, 2015). However, there are knowledge gaps that remain. First, no one has explored removal rates if clay minerals are added to the immobilized microalgae. Second, iron is known to improve nutrient processing in some microorganisms, and the effect of iron in these microalgae cultures on N and P remediation has not been evaluated. Third, there is insufficient data on what happens to the pH when multiple remediating agents are added together (clay minerals + microalgae + iron). Lastly, iron might speed up the nitrate-nitrogen removal process but this has not been fully explored.

RESEARCH QUESTION

This study explored the potential benefits of combining clay minerals with immobilized microalgae (*Chlorella pyrenoidosa*, *Scenedesmus quadricauda*, and their co-culture) for enhanced nitrogen and phosphorus removal from wastewater. Additionally, we examined whether the addition of iron (as FeCl3) could serve as a catalyst to improve nitrate-nitrogen removal efficiency in these combined systems.

MATERIALS AND METHODS

Materials. Immobilized algae beads were obtained from Algae Research Supply, San Diego, CA, which contained *Scenedesmus quadricauda, Chlorella pyrenoidosa, and a* co-culture of these two strains. Municipal wastewater was sourced from a local wastewater site within Los Angeles County after the chlorination step. Clinoloptilolite was obtained from the Green River formation in Sweatwater County, Wyoming. Other reagents were purchased from Sigma (HEPES buffer, ammonium chloride, iron chloride).

Media. To prepare the wastewater, air was bubbled through the wastewater for 3 days to remove any volatile gases. HEPES (50 mM) was added to the wastewater to maintain a stable pH during the experiment, achieving a final concentration of 50 mM HEPES (pH 7.2). The microalgae beads were then introduced into the prepared wastewater.

Growing Conditions. The microalgae flasks remediated nutrients. Cultures were grown in square tissue culture flasks containing 30 mL of wastewater media plus supplements; bead concentration was 4 beads per milliliter of media. Flasks were shaken at 80 rpm, and the light:dark cycle was 12:12 inside a temperature-controlled grow tent (T = 24 C) with a 75 W light bulb. Aseptic techniques were followed, including wiping down surfaces with 70% ethanol solution and flaming tubes and media storage bottles with a Bunsen burner. 0.22-um bottle-top filters were used to sterilize media and solutions.

Nutrient Tracking. Aliquots of 4 mL were removed from each culture flask and centrifuged prior to nutrient tracking. Nitrogen concentrations in each flask were measured 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.

Methods. Remediation of nitrogen was determined using Vernier ion selective probes that measure the concentration of ammonium-nitrogen and nitrate-nitrogen. Changes in phosphorus concentrations were tracked using test strips that detected a range of 0-1000 parts per billion (0-1 mg/L). The pH of the cultures were monitored using a handheld pH meter calibrated to pH 4, 7 and 10. Nitrogen content in the form of ammonium-N and nitrate-N was measured using Vernier ion selective probes and Vernier LoggerPro platform.

RESULTS

Three different studies were conducted to measure the changes in N and P concentration over a period of 6 days. The number of experimental flasks was limited to 24 at any given time due to space inside the grow tent. Each experimental group contained 2 replicates and the results for each were averaged. The first study measured changes in pH and nutrient concentrations over 5 days in the presence of C. pyrenoidosa, S. quadricauda, the co-culture (both strains inside the same bead), and alginate beads that contained no microalgae inside. The second study repeated the same experimental conditions and introduced clinoptilolite as another removal agent. The third study again duplicated the experimental conditions and introduced iron as a supplement to fresh cultures containing microalgae beads and clinoptilolite. Select results are discussed below.

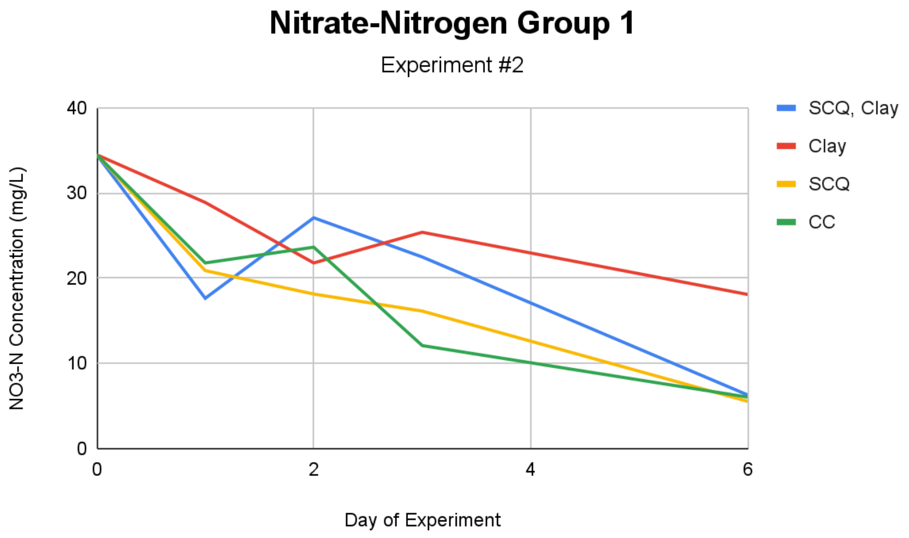


Figure 1: Changes in concentration of nitrate-nitrogen over 6 days in the presence of microalgae (4 beads/mL) and clinoptilolite (5 mg/mL).

Nitrate-Nitrogen removal results (Study #2). The results in Figure 1 show varying removal rates of nitrate-nitrogen (NO3-N) among the four treatments: *Scenedesmus quadricauda* (SCQ), clinoptilolite clay (Clay), *S. quadricauda* and clinoptilolite clay (SCQ, Clay), and a co-culture of *S. quadricauda* and *Chlorella pyrenoidosa* (CC). Initially, all groups started with nitrate-nitrogen concentrations of 34 mg/L. After 1 day, the treatment with SCQ and the clay removed a significant amount of NO3-N, bringing the concentration down to roughly 17 mg/L. Treatments of SQC alone and CC alone also displayed steady removal rates, resulting in concentrations of 21 mg/L and 22 mg/L, respectively. However, by Day 2, the CC treatment and treatment with SCQ and clay caused NO3-N levels to regress to 23 mg/L and 27 mg/L, respectively. Clay and SCQ treatments remained in a steady decline. By Day 3, all treatments but clinoptilolite clay alone, which experienced an increase in NO3-N, showed a return to efficient removal rates. By Day 4, all treatments began to enter a steady decline. Over the six-day experiment, all treatments with the addition of any strain of microalgae resulted in a final NO3-N concentration of 6 mg/L, while clay alone only decreased to 18 mg/L.

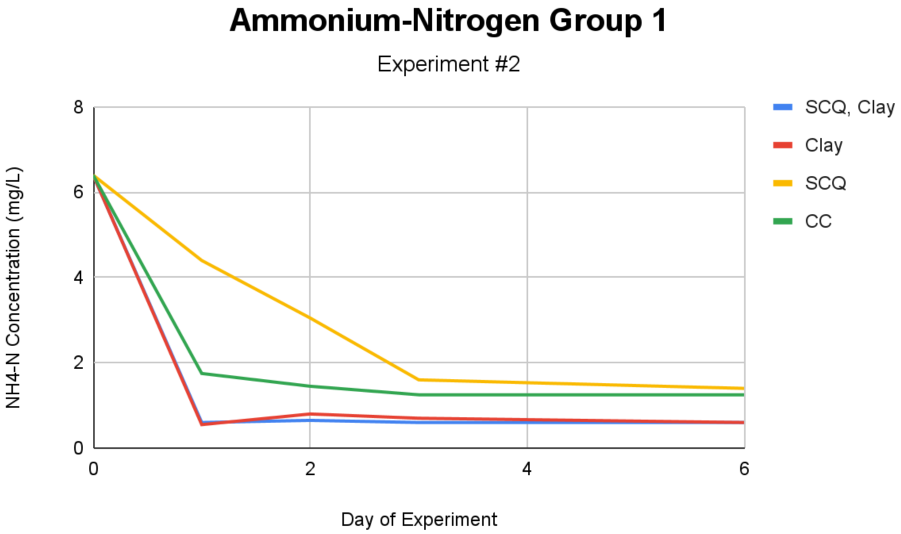
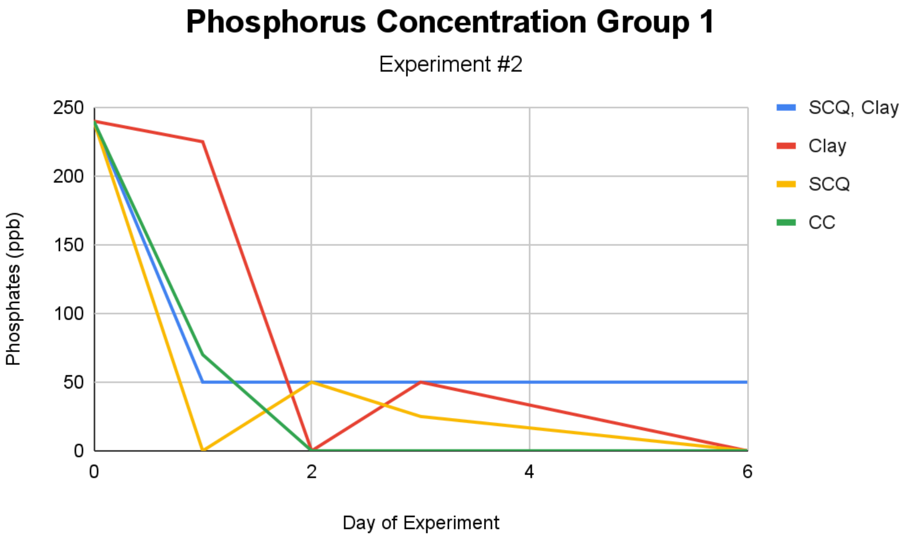


Figure 2: Changes in the concentration of ammonium-nitrogen in the presence of microalgae beads and clinoptilolite over 6 days.

Ammonium-Nitrogen removal results. The results in Figure 2 show the effectiveness of the same four treatments in removing ammonium-nitrogen (NH4-N). Initially, all treatments started with NH4-N concentrations of 6 mg/L. Within the first day, both the clay treatment and SCQ with clay experienced rapid reductions, lowering concentrations to less than 1 mg/L. SCQ alone showed a less pronounced decrease, with levels dropping to about 4.2 mg/L, while the co-culture (CC) dropped to around 1.9 mg/L. By Day 2, all treatments reached near-minimum NH4-N concentrations, except for SCQ, which continued to show a gradual decline. From Day 3 until the end of the experiment, the concentrations for all treatments leveled out, with CC and SCQ maintaining concentrations around 1.3 mg/L and clay alone and SCQ and clay maintaining concentrations around 0.7 mg/L.



Phosphorus Removal. The results in Figure 3 show the effectiveness of the same four treatments in reducing phosphorus concentrations over the span of 6 days. All treatments began with high phosphorus levels of around 250 parts per billion (ppb). Within the first day, there was a significant drop in phosphorus concentrations across all treatments except for clay alone. SCQ and CC treatments lowered concentrations to less than 50 ppb by Day 1. However, the SCQ treatment experienced an increase in phosphorus of around 50 ppb by Day 2. The clay treatment alone followed the same path, decreasing from 225 ppb on Day 1 to 0 ppb on Day 2, but rising back to 50 ppb on Day 3. The CC treatment decreased relatively steadily, reaching 0 ppb by Day 2 and maintaining the concentration throughout the experiment. The treatment of SCQ with clay also maintained phosphorus levels of 50 ppb throughout the experiment after its initial decrease on Day 1.

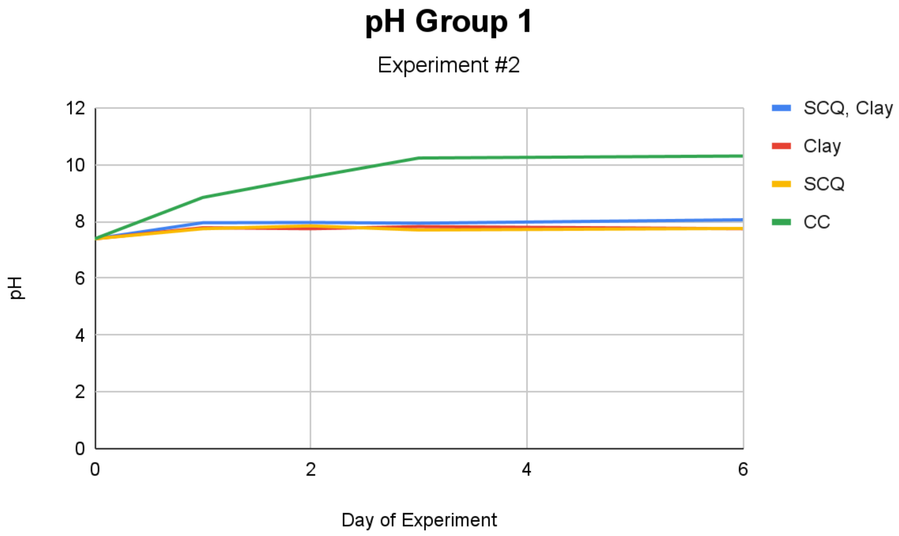
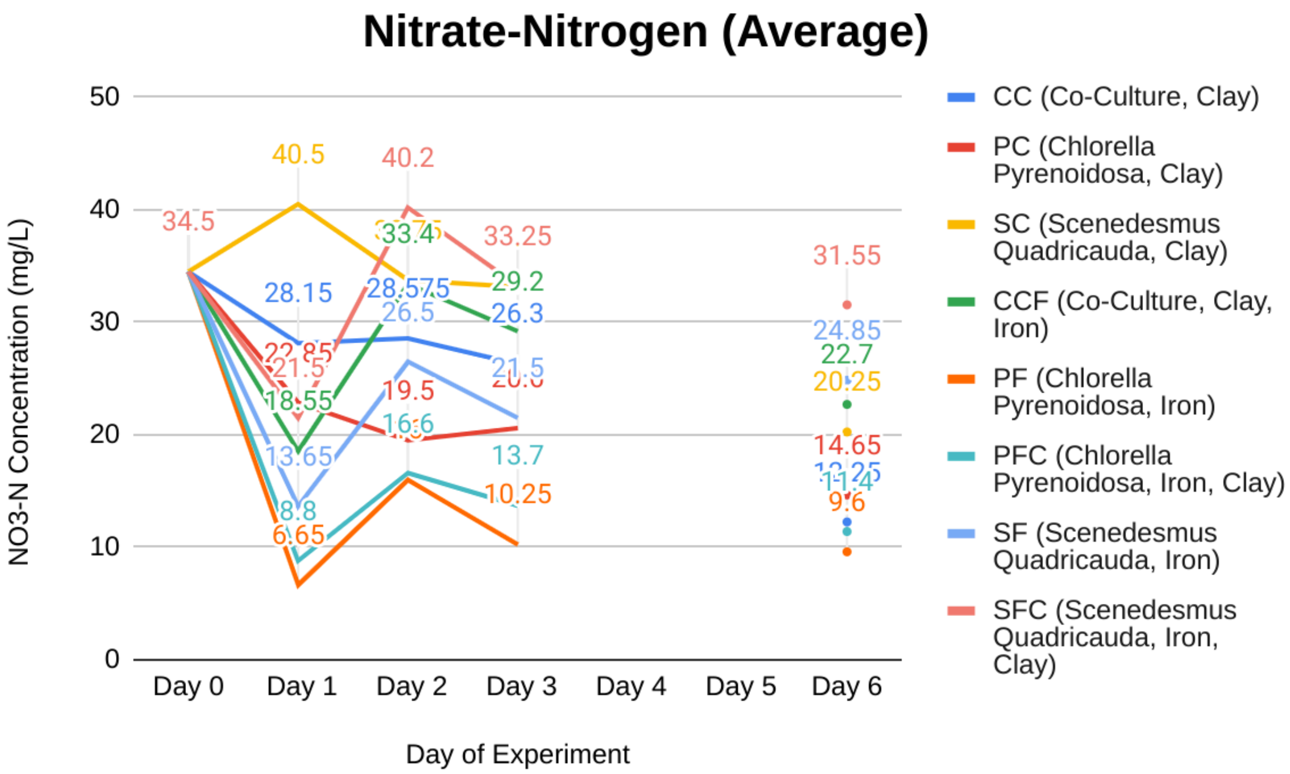


Figure 4: pH changes of the experimental cultures containing 50 mM HEPES (pH 7.2 initially) during the second study.

Changes in pH. The results in **Figure 4** show the pH trends of the same four treatments over the course of six days. All treatments had initial pH values of approximately 7.5. The CC treatment showed a rapid increase in pH, reaching over 10 by Day 3, and then remained constant throughout the experiment. The SCQ with clay treatment showed a moderate increase, stabilizing around pH 8.5 after Day 2. Both the SCQ and clay-only treatments underwent little change in pH, maintaining levels close to the initial pH of 8 throughout the experiment.



Nitrate-Nitrogen removal results. The Algae-supplement combination that remediated the most nitrate-nitrogen was the *C. Pyrenoidosa* with Iron. The *Chlorella* did the best with just the iron, since the media started with 34.5 mg/L of nitrate-nitrogen and was reduced to 9.6 mg/L. Meanwhile, the *Chlorella* with iron and clay came in a close second, remediating to 11.4 mg/L, and clay alone came in fourth at 14.7 mg/L. The co-culture with clay was also significantly useful in remediating the nitrate-nitrogen from 34.5 mg/L to 12.25 mg/L.

Iron Supplementation. The effect of iron on *S. Quadricauda* remediation was minimal (results not shown). Also starting at 34.5 mg/L of nitrate-nitrogen, the *Scenedesmus* with clay maintained levels after Days 2 and 3, (@ 33.15 and 33.75 mg/L, respectively), but decreased to 20.25 mg/L by Day 6. But, The Scenedesmus with Iron had a quick reaction, decreasing to 13.7 mg/L on the first day. However, this sample did not maintain its bioremediation, going back up to 24.85 mg/L on day 6. Finally, the combination of the *Scenedesmus* with iron and clay had a similar initial response, going down to 21.5 mg/L, but then released nitrate-nitrogen on the 2nd day, going up to 40.2 mg/L. By day six, this combination leveled out to near its original concentration of nitrate-nitrogen, 31.55 mg/L.

DISCUSSION and CONCLUSION

The data might be showing a pattern in nitrate-nitrogen removal between different treatment conditions. All treatment groups started with initial NO3-N concentrations of 34 mg/L, but showed different removal trends. The combined clinoptilolite clay and microalgae treatments demonstrated the most rapid initial reduction, bringing concentrations down to approximately 17 mg/L after just 1 day. However, this was followed by an unexpected rebound in NO3-N levels by Day 2, with the co-culture and clay combinations showing increases back to 23-27 mg/L. This suggests an initial rapid adsorption phase by the clay followed by potential desorption or release mechanisms that weren't anticipated. Interestingly, the single-species treatments (SCQ alone and CC alone) showed more gradual but steady declines, maintaining concentrations around 21-22 mg/L through the early phase of treatment.

The pH changes across treatments might show the different biological processes at work. The co-culture (CC) treatment showed a dramatic increase in pH, reaching over 10 by Day 3 and maintaining that elevated level throughout the experiment. This is a sign of a lot of photosynthetic activity, as microalgal CO2 uptake typically raises pH. In contrast, the SCQ with clay treatment showed more moderate pH increases, stabilizing around 8.5 after Day 2. The SCQ and clay-only treatments maintained pH levels close to their initial values around 8. These pH patterns may help explain some of the variation in nutrient removal efficiency, as pH can significantly affect both biological nutrient uptake and chemical sorption processes. The dramatic pH increase in the CC treatment may have actually inhibited optimal nutrient removal by altering the chemical equilibrium of nitrogen species in solution.

The combination of clinoptilolite clay and co-culture worked well at ammonium-nitrogen removal compared to other treatments; complete removal was almost achieved in the first 24 hours. When clay is present with the algae beads, clay might be the main remediator compared to the microalgae. Perhaps only clay is needed for rapid nutrient removal. However, the clay is not easily separated from the wastewater since it settles. The microalgae beads can be easily separated and recycled. These results confirm that the microalgae are effective remediators of excess nutrients in wastewater even in the form of alginate beads and that clinoptilolite could enhance the removal efficiency.

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