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Effectiveness of Titanium and Iron Nanoparticles in Treating M. aeruginosa for Harmful Algal Bloom Remediation

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Abstract

Harmful Algal Blooms (HABs) are a growing issue worldwide, posing harm to both aquatic ecosystems and drinking water quality. This issue could be potentially mitigated using nanoparticle (NP) treatment, simultaneously removing cyanobacteria and associated cyanotoxins in HABs. This research seeks to discern the effectiveness of using titanium dioxide and iron (III) oxide NP treatment at removing cyanobacteria via flocculation and sedimentation. Each NP at 25 mg/L and 50 mg/L were used to treat suspended culture of *Microcystis aeruginosa*, the representative cyanobacteria, up to 72 hours. Cell concentration and morphology in the supernatant were measured via a Coulter counter and light microscopy. The decreasing cell concentration in the supernatant showed that both NP can flocculate *M. aeruginosa* and allow subsequent sedimentation. High concentration NP treatments were more effective than low concentration NP treatments, removing a higher percentage of cells in the same amount of time.

Background

Drinking water treatment processes serve to remove contaminants that could potentially harm individuals in the community. The Environmental Protection Agency (EPA) regulates a selection of contaminants such as solids, bacteria, heavy metals, and disinfection byproducts in the Primary Drinking Water Standard, and also publishes a list of unregulated contaminants of interest every five years under the Safe Drinking Water Act. The most recent list expressed great concerns over cyanotoxins ("Fact sheet"; 2016). Cyanotoxins are often associated with the presence of harmful algal blooms (HABs) containing cyanobacteria (Paerl et al., 2016). The most common of toxin-producing cyanobacteria is *Microcystis aeruginosa*, forming the hepatoxin microcystin, which is a liver toxin in mammals (Hodgson, 2012).

HABs form in the presence of excess nutrients in aquatic ecosystems, often due to agriculture and urban runoff. According to the National Oceanic and Atmospheric Association (NOAA), HABs "occur with colonies of algae… grow out of control and produce toxic or harmful effects on people, fish, shellfish, marine mammals and birds" ("What is a Harmful Algal Bloom?"; 2016). The presence of HABs in drinking water sources has greatly increased greatly over the past 40 years, thus increasing the presence of cyanobacteria and associated toxins in drinking water sources world-wide (Duan et al., 2017). Reports of algal blooms containing *M. aeruginosa* date back to 1878 and have since been reported on all continents except Antarctica (Hodgson, 2012). Beyond increasing levels of cyanotoxins, HABs also have detrimental environmental effects via eutrophication. These combined processes lead to the illness and fatalities in fish, shellfish, humans, and marine animals, harming aquatic ecosystems as a whole (Anderson et al., 2002). The EPA classifies exposure to HABs with cyanobacteria concentrations ranging between 10^5 cells/mL to 10^7 cells/mL as having a high probability of causing severe human health effects

(D'Anglada, n.d.). It is therefore beneficial to develop a treatment for HABs which removes cyanobacteria and resulting cyanotoxins. This research seeks to do so in aquatic ecosystems that serve as drinking water sources, effectively treating cyanotoxins and cyanobacteria prior to entering the water treatment plant and reducing the environmental consequences of HABs.

Reactive and catalytic NP materials have been investigated for both the inactivation of HAB-causing cyanobacteria and the degradation of cyanotoxins separately. Previous studies have focused on TiO_2 and Fe-based nanomaterials because of their low environmental and human health impacts. These studies show that TiO_2 and Fe-based NPs effectively cause deactivation of HAB cyanobacteria in a fully-established bloom while simultaneously reducing the total nitrogen and phosphorus levels of the water, likely due to the intake of nutrients from the algal bloom (Bessa da Silva et al., 2016; Comotto et al., 2014; Kim & Lee, 2005; Lee et al., 2013; Sharma et al., 2016; Wang et al., 2015). Reported mechanisms of HAB deactivation include bloom reduction via flocculation and settlement, cell growth inhibition, cell membrane damage, loss of photosynthetic activity, and cell destruction (Bessa da Silva et al., 2016; Comotto et al., 2014; Kim & Lee, 2005; Lee et al., 2013; Sharma et al., 2016; Wang et al., 2015). Although bloom deactivation is the goal, TiO_2 toxicity is a potential negative impact of concern (Bessa da Silva et al., 2016).

Similar studies have shown that Fe-based NPs are effective at cyanobacteria deactivation by the disruption of normal cellular function with minimal toxicity towards desirable aquatic species (Marsalek et al., 2012; Sharma et al., 2016). For both TiO_2 and Fe NPs, the toxicity level varies depending on the organism evaluated and the parameters of exposure (Blaise et al., 2011). These small, consequential risks could be potentially remedied by the strategic spatial placement of the

NPs in the bloom, rather than homogeneously dispersed particles in the ecosystem, and by NP removal following treatment.

With regards to cyanotoxin degradation, TiO_2 and Fe are the primary NP catalysts under investigation thus far. When activated by light, TiO_2 produces hydroxyl radical species that oxidatively degrade cyanotoxins to reduce their toxicity. Hydroxyl radicals are non-specific and, therefore, react with any compound that is near the catalyst. Similarly, the Fe-based NPs produce broad-spectrum reactants that are naturally reactive in aquatic ecosystems, including the oxidative hydroxyl radical and reductants such as hydrogen. The advantage of using a non-specific reactant is that it will likely react with any set of cyanotoxins present in the water; the disadvantages include the need for the target toxins to be close to the catalyst surface and non-productive side reactions with other organics such as natural organic matter. Therefore, it can be hypothesized that the use of TiO_2 and Fe-based NPs will be effective in the mitigation of both present and future HABs and their toxins.

In situ mitigation of HABs and the resulting cyanotoxins remains a challenge. While it will also be important for water treatment plants to address cyanotoxins present in pumped drinking water sources, successful and efficient treatment of HABs and cyanotoxins at the source would provide an initial barrier and immediate mitigation strategy. *In situ* mitigation would reduce cyanotoxin load on treatment plants, prevent unpredicted spikes in cyanotoxin contamination, and reduce the risk of cyanotoxins in finished drinking water while simultaneously remediating the impacts of HABs in aquatic environments. Most proposed approaches suffer from key issues, including the addition of chemicals, the use of chemicals that are difficult to recover and or remove, and the inability to mitigate both the HAB and the cyanotoxin concurrently. There has, however, been successful demonstration of catalytic NP immobilization on various types of

fibers, including silks, filtration polymers, nonwoven materials, fabrics, and meshes (Li et al., 2002; Liang et al., 2013; Lu et al., 2014; Ma et al., 2012; Zhang & Zhu, 2012). These studies demonstrated that the NPs retain their reactivity when immobilized and provide beneficial functions including antimicrobial activity and degradation of water contaminants (Lu et al., 2014; Ma et al., 2012). For TiO_2 and Fe-based NPs, the primary mechanism is the formation of short-lived, broad-spectrum, powerful oxidants as mentioned above. Studies have evaluated the immobilized stability of TiO_2 and Fe-based NPs and have shown that both can be immobilized and reused to a certain extent (Pavía-Sanders et al., 2013; Yu et al., 2013).

The proposed design for the delivery of NPs to water is a polymer fiber net embedded with catalytic, immobilized nanoparticles (NPs) that can be deployed at the location of HABs and retrieved after treatment, minimizing harm to beneficial aquatic organisms. The NPs used here are TiO_2 and Fe_2O_3. The ultimate goal of this project is to immobilize the selected NPs to a net, which can be efficiently applied and withdrawn from the aquatic environment suffering from HABs, minimizing harm to aquatic ecosystems by removing cyanobacteria and cyanotoxins simultaneously.

Objective and Hypothesis

The main objective for this thesis is to discern the treatment effects of titanium dioxide and iron oxide NPs on *M. aeruginosa* regarding inactivation of cyanobacteria by removing them from water. The hypothesis is NPs can remove cyanobacteria by flocculation and sedimentation.

Materials and Methods

M. aeruginosa growth and preservation

M. aeruginosa (strain #2386) in suspension was obtained from the UTEX algae center at the University of Texas, Austin, and maintained in autoclaved BG-11 medium as instructed. Flasks of *M. aeruginosa* were set near a window, allowing for adequate sunlight. The growth of M. aeruginosa was monitored by measuring the optical density at 680 nm. Fresh BG-11 medium was supplemented into existing culture every 21 days to maintain algal growth (UTEX Culture Collection of Algae, 2009).

Experimental Protocol

NP treatment impacts were discerned by adding different concentrations of TiO_2 and Fe_2O_3 to *M. aeruginosa* suspended cell solutions. Both NPs were prepared by Dr. Greenlee's lab at the University of Arkansas and the stock solution of 1 mg/mL concentration were used. Prior to each experiment, cell morphology and concentration were assessed using a Nikon NiE upright light microscope and a Beckman Multisizer 4 Coulter counter, respectively.

10 mL samples were prepared in 15 mL centrifuge tubes with *M. aeruginosa* diluted in phosphate buffer saline (PBS) at a 1:10 ratio. After samples were prepared, centrifuge tubes were gently vortexed to encourage even cell distribution throughout the PBS. Samples of the supernatant were taken for cell concentration measurement prior to NP addition, measuring initial concentrations of *M. aeruginosa* in cells/mL with diameters ranging from 2.5 to 4 μm. All cell concentration measurements were taken using the Coulter counter, prepared in 20 mL accuvettes with 20 μL added of supernatant to 10 mL of Isoton III Diluent as the electrolyte. Prior to being added to the accuvette, the electrolyte was filtered using a 0.22 μm syringe filter. The 20 μm aperture tube was used for Coulter counter readings, and the coulter counter was

operated using the volumetric operating mechanism for the preliminary experiment and the time operating mechanism for the final experiment. After initial cell concentrations were recorded in all samples, NPs were vortexed to evenly mix them. Treatment amounts of NPs were then added to each tube, with no NPs added to the two control tubes (Table 1 and Table 2). Cell concentration was again measured three hours following NP addition and once every 24 hours for three days after NP addition using the Coulter counter. Throughout the experiment, samples were left sitting upright in a 15 mL centrifuge holder near the window.

Table 1. Preliminary experimental design for NP treatment on M. aeruginosa

Treatment	Concentration of NPs (mg/L)	Tube Number
Titanium Dioxide – low concentration	25	P1, P2
Iron (III) Oxide – low concentration	25	P3, P4
Control	0	P5, P6

Table 2. Final experimental design for NP treatment on M. aeruginosa

Treatment	Concentration NPs (mg/L)	Tube Number
Titanium Dioxide – low concentration	25	F1, F2
Iron (III) Oxide – low concentration	25	F3, F4
Titanium Dioxide – high concentration	50	F5, F6
Iron (III) Oxide – high concentration	50	F7, F8
Control	0	F9, F10

All experiments were conducted in duplicate. Results were analyzed using Z-score (Equation 1), which considers values greater than an absolute value of 2 to suggest significant differences (alpha = 0.05); because this analysis involved decreasing concentrations over time, z-scores less than -2 were considered significant cell removal. In the z-score calculation, X represents the individual cell concentration of each tube, $\bar{X}$ represents the average cell concentration of all tubes prior to treatment, and S represents the sample standard deviation of all tubes prior to treatment.

Equation 1. Z-Score Calculation

$$Z = \frac{X - \overline{X}}{S}$$

Cell concentration results were also analyzed in JMP using the analysis of covariance (ANCOVA) in conjunction with Dunnett's test compared against the experimental control group to control multiplicity, in which an effective treatment resulted in values above or below the upper and lower decision limits, respectively (alpha = 0.05). In this analysis, the dependent variable was cell concentration, the independent variable was treatment, and the covariant was hours elapsed.

Finally, percent of cells removed was calculated by subtracting the final cell concentration of each tube from the initial average cell concentration of all tubes in the experiment. This value was then divided by the initial average cell concentration of all tubes in the experiment and multiplied by 100. Duplicates of percent cell removal were then averaged to gain percent cell removal of each treatment in each experiment.

Results

Cell Morphology

M. aeruginosa cell morphology were confirmed using 100x oil emersion light microscopy (Figure 1). Cells were moving rapidly throughout the media; only *M. aeruginosa* cells were seen in the sample.

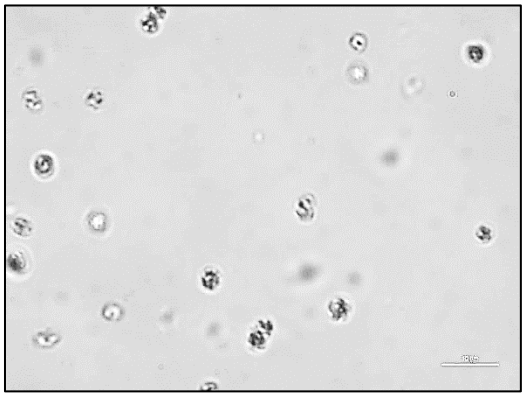

Figure 1. M. aeruginosa cell morphology, 10 µm scale bar

Flocculation

Preliminary experimentation revealed flocculation as the main method of algal removal. This is illustrated in Figure 2, which shows cells prior to treatment (left), after TiO_2 NP treatment (center), and after Fe_2O_3 NP treatment (right). The untreated sample showed cell movement, while the treated samples showed little movement of cells.

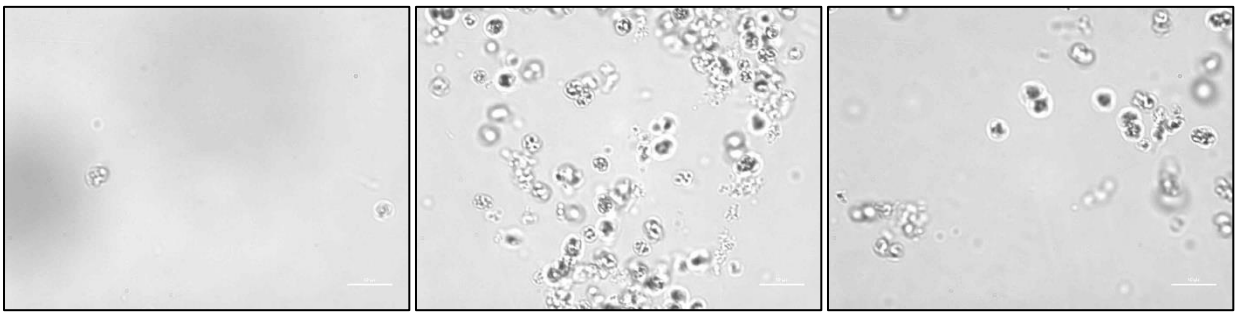

Figure 2. Images of M. aeruginosa prior to dilution in PBS and NP treatment (left), after TiO_2 NP treatment (center), and after Fe_2O_3 treatment (right). The scale bar is 10 µm.

Flocculation was further evident in preliminary experiment results through Coulter counter measurements, showing a decrease in cell concentration of the supernatant as treatment time increased as compare to the average of all initial sample cell concentrations (Figure 3). The z-score here revealed significant reduction in cell concentration in the supernatant for both NP treatment types by 48 hours of treatment (Table 3).

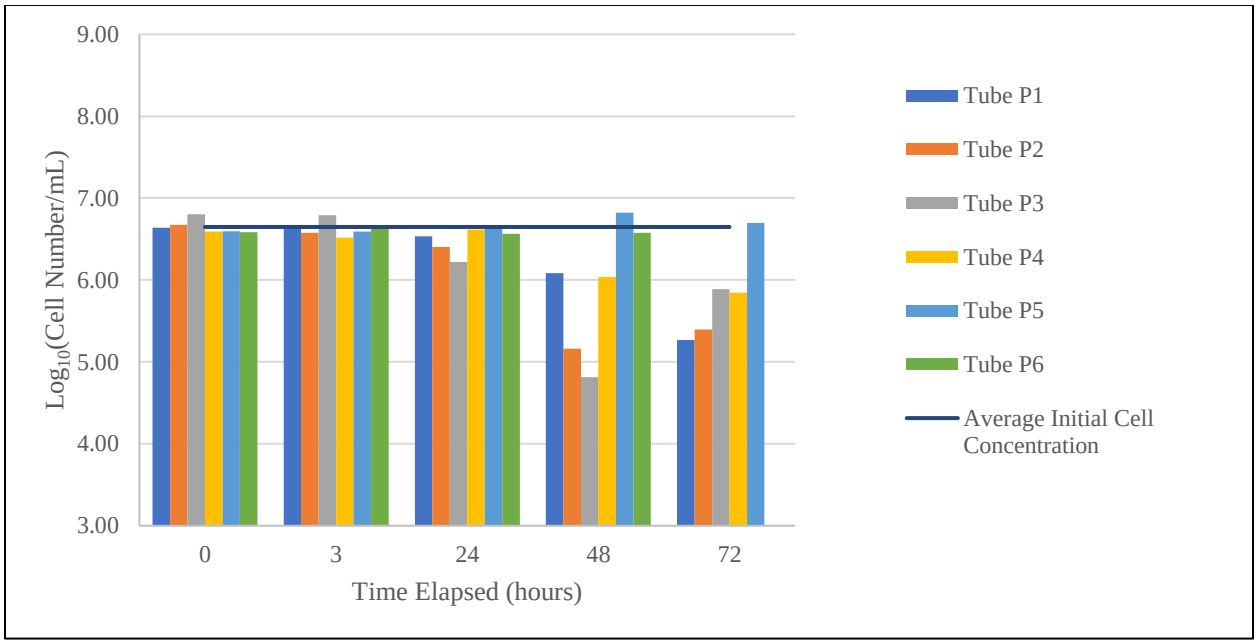

Figure 3. Cell concentration change over the treatment time in the preliminary experiment. Tubes 1 and 2 were treated with the low concentration of TiO_2, tubes P3 and P4 were treated with the low concentration of Fe_2O_3, and tubes P5 and P6 were the control. There is no measurement for Tube P6 at 72 hours because of instrumentation issues.

Table 3. Z-score over cell concentration change after treatment in the preliminary experiment; significant reduction in cell concentration is represented in green. There is no value for Tube P6 at 72 hours because of instrumentation issues.

Treatment:	Hours Elapsed:	3	24	48	72
Titanium Dioxide – low concentration	Tube P1 z-score	-0.27	-1.38	**-6.82**	**-16.67**
	Tube P2 z-score	-0.86	**-2.94**	**-17.95**	**-15.09**
Iron (III) Oxide – low concentration	Tube P3 z-score	1.72	**-5.16**	**-22.15**	**-9.16**
	Tube P4 z-score	-1.57	-0.41	**-7.39**	**-9.69**
Control	Tube P5 z-score	-0.69	0.23	**2.10**	0.59
	Tube P6 z-score	-0.13	-1.01	-0.88	

When *M. aeruginosa* was treated with varying concentrations of NPs, it was revealed that increased concentrations lead to faster flocculation. All four high-concentration NP treatments showed significant reduction in cell concentration by 24 hours, while only one replicate in the low-concentration iron NP treatment achieved the same (Table 4).

Table 4. Z-score over cell concentration change after treatment of varying concentrations; significant reduction in cell concentration is represented in green.

Treatment	Hours Elapsed:	3	24	48	72
Titanium Dioxide – low concentration	Tube F1 z-score	0.49	-0.53	**-2.97**	**-3.11**
	Tube F2 z-score	0.69	0.32	0.47	**-3.11**
Iron (III) Oxide – low concentration	Tube F3 z-score	-0.35	**-2.10**	-1.89	**-2.68**
	Tube F4 z-score	**-6.46**	-0.80	-0.03	-1.84
Titanium Dioxide – high concentration	Tube F5 z-score	0.53	**-3.36**	**-2.17**	**-3.04**
	Tube F6 z-score	0.45	**-3.04**	**-2.10**	**-4.30**
Iron (III) Oxide – high concentration	Tube F7 z-score	1.02	**-3.36**	**-3.04**	**-4.11**
	Tube F8 z-score	0.69	**-3.81**	**-3.95**	**-4.30**
Control	Tube F9 z-score	0.45	0.81	0.19	-0.08
	Tube F10 z-score	0.47	6.68	0.26	0.42

When examining the cell concentration over time, the cell concentrations were more variable at zero and three hours than in the preliminary experiment (Figure 4). To ensure this had no significant affect on results, analysis of variance (ANOVA) was run in JMP with the logarithmic transformation of the cell concentration as the dependent variable and the treatment regime as the independent variable. This resulted in p-values of 0.4044 and 0.1465 for the initial data and data after three hours, respectively, showing that the variance of the data was not significant.

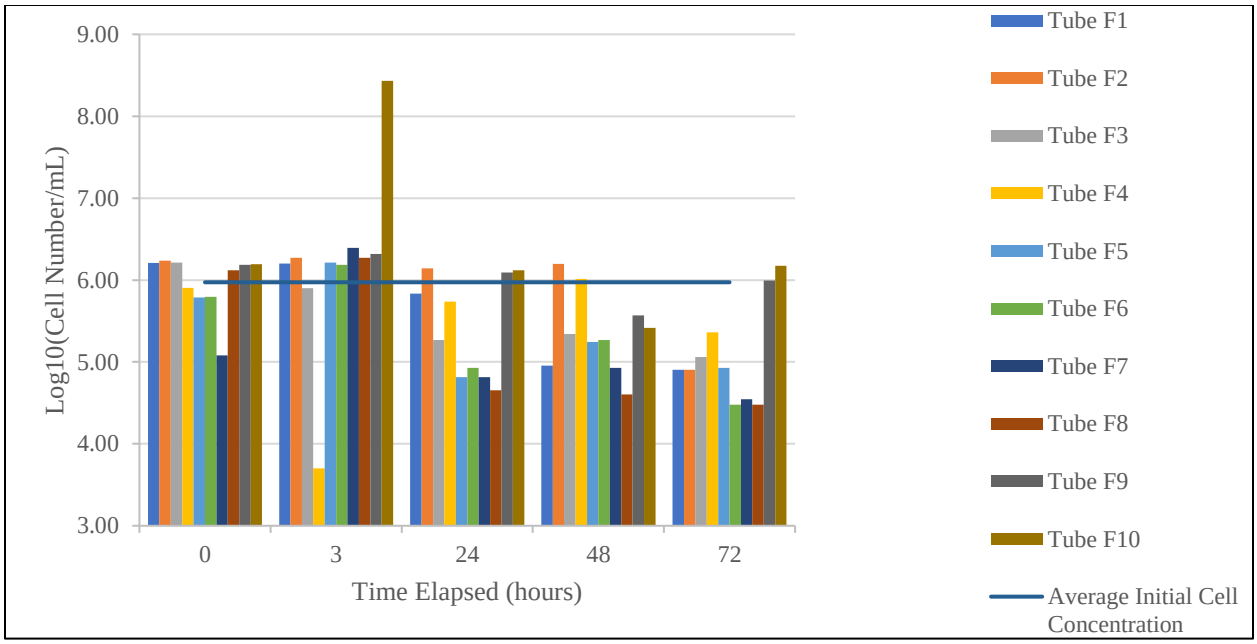

Figure 4. Cell concentration change over the treatment time in the final experiment. Tubes F1 and F2 were treated with the low concentration of TiO_2, tubes F3 and F4 were treated with the low concentration of Fe_2O_3, tubes F5 and F6 were treated with the high concentration of TiO_2, tubes F7 and F8 were treated with the low concentration of Fe_2O_3, and tubes F9 and F10 were the control.

ANCOVA and Dunnett's Test Results

Dunnett's test sets upper and lower decision limits (UDL and LDL, respectively) based on control values; if a treatment value exceeds either limit, it is considered significantly different. A significant reduction of both high-concentration treatments of the ANCOVA and Dunnett's test here was revealed (Table 6. Dunnett's test results for final experiment (Table 6). This was expected because of the evident flocculation occurring throughout the treatment process.

It was also revealed that low concentration treatments are inconsistent in treatment effectiveness, for the preliminary experiment showed only the titanium NP treatment as effective, whereas the final experiment showed only the iron NP treatment as effective (Table 5, Table 6). This difference is likely due to different initial concentrations, which were on average 4.25×10^6 and 1.16×10^6 cells/mL for the preliminary and final experiments, respectively.

Table 5. Dunnett's test results for preliminary experiment

Treatment	Lower Limit	Estimate	Upper Limit	Limit Exceeded
Titanium Dioxide – low concentration	6.178516	6.154286	7.015536	Lower
Iron (III) Oxide – low concentration	6.178516	6.22953	7.015536	Neither

Table 6. Dunnett's test results for final experiment

Treatment	Lower Limit	Estimate	Upper Limit	Limit Exceeded
Titanium Dioxide – low concentration	5.581461	5.785244	6.917054	Neither
Iron (III) Oxide – low concentration	5.581461	5.44993	6.917054	Lower
Titanium Dioxide – high concentration	5.581461	5.364479	6.917054	Lower
Iron (III) Oxide – high concentration	5.581461	5.188172	6.917054	Lower

Because ANCOVA considered both hours elapsed as a covariant to treatment method, ineffective treatments could likely be improved by increasing experimental timespan, or by increasing the NP concentration. Considering the goal of this research is to create a NP-embedded net to be deployed in HABs and later retrieved, increased NP concentration is the preferred method of improving performance of treatment, decreasing the amount of time the net could cause harm to beneficial aquatic life.

Discussion

Flocculation of *M. aeruginosa* was expected because Bessa da Silva et al. (2016) reported 1 g/L TiO_2 NP treatment "enhancing the formation of aggregates and their rapid settlement, thus reducing the algal bloom." According to Sanyano et al. (2013), microalgae carries a negative charge; for this reason, inorganic multivalent metal salts are often use as flocculants. Although TiO_2 and Fe_2O_3 are not salts, they might still have some effect on the charge of microalgae, causing flocculation.

The differences between the low-concentration treatment effectiveness between the preliminary and final experiment was not expected. As stated, the difference in treatment effectiveness is likely due to initial cell concentration. Further differences in the data between the preliminary and final experiment could be in the Coulter counter operating method. Although both methods take accurate readings, the volumetric operating mechanism analyzes the algae over a longer time period than the time operating mechanism. The length of time used for the final experiment was 15 seconds, as compared to approximately 90 seconds in the preliminary experiment. The preliminary experiment had much cleaner data than the final experiment, possibly showing that a longer run time could be an effective method of getting cleaner data.

Concerning the effectiveness of the NP treatments, percent cells removed at 72 hours for each treatment is listed in Table 7. This shows similar removal efficiencies between low-concentration treatments across the preliminary experiment and the final experiment, despite differences in initial cell concentration.

Table 7. Percent algae removed from each treatment

Treatment	Percent algae removed
Titanium Dioxide – low concentration, preliminary experiment	95.2%
Iron (III) Oxide – low concentration, preliminary experiment	84.9%
Titanium Dioxide – low concentration, final experiment	93.1%
Iron (III) Oxide – low concentration, final experiment	85.1%
Titanium Dioxide – high concentration	95.0%
Iron (III) Oxide – high concentration	97.2%

Despite these high removal rates, no samples achieved enough algae removal for concentration to fall below 20,000 cells/mL, which is the threshold the EPA specifies as low probability of human health risk (D'Anglada, n.d.). Cell removal could likely be improved by increasing concentration or amount of time treated.

Conclusion

Although flocculation did occur in all treatment methods by 72 hours after NP addition, the ANCOVA analysis revealed flocculation was not consistently significant in low concentration NP treatments, while high concentration NP treatments resulted in significant cell concentration reductions in *M. aeruginosa* by flocculation and sedimentation. NP treatment removed 84.9% to 97.2% of cells, depending on treatment; concentrations, however, failed to fall below the low health risk threshold. Further experiments should consider increased dosage levels, possible synergistic effects of titanium and iron-based NPs when used simultaneously in treatment, and effectiveness of treatment when NPs are embedded in fibers.

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