

Combating Algae Blooms: Analyzing the Viability of Cecropin A for Anti-Bloom Applications

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ABSTRACT

Microcystis aeruginosa is a common freshwater cyanobacterium that can form toxic algal blooms that harm other species and the environment. This project studied the effects of the antimicrobial peptide Cecropin A on the growth of *M. aeruginosa* to assess Cecropin A's effectiveness as a tool to combat algal blooms and limit their environmental impacts. In this study, different concentrations of Cecropin A were tested on *M. aeruginosa*, the growth of which was then measured using a plate count. Each concentration of Cecropin A tested resulted in a significant decrease in *M. aeruginosa* growth compared to the control group, indicating the effectiveness of this peptide at inhibiting *M. aeruginosa*. Because Cecropin A is a peptide, bacteria can be genetically engineered to produce it for anti-algal applications. This study also analyzed the effects of Cecropin A on the non-pathogenic *E. coli* K12 in order to study development of antibiotic resistance in this bacterium and determine its feasibility for anti-algal applications such as producing or distributing Cecropin A. The effects of Cecropin A were tested on successive generations to determine if this strain of bacterium can build up a resistance to Cecropin A that would make it a suitable candidate to produce large quantities of this peptide. The results over three 24-hour periods of exposure to Cecropin A seem to indicate a development of resistance to Cecropin A by *E. coli* K12, suggesting that this bacterium may be suitable for production and/or distribution of Cecropin A for anti-bloom control efforts.

Introduction

An algal bloom is an event characterized by the rapid population growth of microscopic organisms in water, most iconically characterized by a change in the color of the water's surface. Sometimes these events are benign, but other times they are harmful. In the United States, harmful algal blooms incur costs estimated to be \$100 million per year (Hoagland & Scatasta, 2006). They are responsible for various illnesses and poisonings in humans and other animals, such as various types of shellfish poisoning classified by symptoms (paralytic shellfish poisoning, diarrhetic shellfish poisoning, neurotoxic shellfish poisoning, etc.), ciguatera fish poisoning (or fish poisoning caused by ciguatoxins), and several other illnesses (Berdalet et al., 2016). Additionally, harmful blooms rapidly exhaust supplies of nutrients and oxygen in their environment, which can negatively impact other organisms by leaving them without essential resources (Paerl et al., 2001).

Photosynthetic organisms support aquatic ecosystems. These ecosystems rely on the nitrogen fixation, oxygen production, and biomass production that these organisms provide (Berdalet et al., 2016). Blooms, harmful and otherwise, are the result of the overabundance of these photosynthetic organisms in an aquatic ecosystem. They typically occur when biomass production by these photosynthetic organisms outpaces consumption of these organisms by their predators, resulting in the accumulation of biomass (Paerl et al., 2001). Harmful algal blooms, or HABs, are the result of this kind of overabundance of a few, certain photosynthetic organisms that can, in large numbers, cause harm to humans and other organisms (Berdalet et al., 2016). HABs can be caused by a wide variety of organisms, each with its own characteristics. These include chlorophytes (green algae that are not usually harmful), dinoflagellates (highly motile cells known for causing red tides, a specific type of HAB), cryptophytes (phototrophic algae that, although

they have been connected to water problems when in blooms, are not known to be toxic to humans), chrysophytes (which are known for causing various severe water quality problems and fish deaths via potent toxins), and cyanobacteria (prokaryotes that cause widespread freshwater HABs and are considered the most problematic of the freshwater algae) (Paerl et al., 2001). At least 300 microalgal or cyanobacterial species are known to be involved in the formation of HABs. Of these, more than 100 species are known to produce toxins that can be harmful or even lethal to humans and animals in a variety of ways (Berdalet et al., 2016).

Cyanobacteria are the most notorious freshwater HAB-forming organisms (Paerl et al., 2001). Several genera of cyanobacteria can form HABs, including *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Microcystis*, *Nodularia*, and *Oscillatoria* (Paerl et al., 2001). Cyanobacterial blooms are opportunistic and have a tendency to form in brackish water and nutrient-enriched freshwater (Paerl et al., 2001). This is problematic, as cyanobacterial blooms can cause water quality problems and have been linked to disease in humans (Berdalet et al., 2016). Cyanobacteria are particularly difficult to deal with in HABs because their long evolutionary history has endowed them with a variety of problematic adaptations. These include the ability to fix nitrogen from the atmosphere, excess storage of phosphorus (known as luxury uptake), the ability to colonize fresh, salty, or brackish water, the ability to survive in environments with relatively low levels of phosphorus, a wide array of adaptations to climatic change and geochemical change, and the ability to change their position in the water column by changing their buoyancy (Paerl et al., 2001; Miller et al., 2017; Sanseverino et al., 2016). Cyanobacteria can produce a wide variety of toxic compounds that act by a variety of methods, including microcystins (toxins produced by *Microcystis* species), anatoxin-a, cylindrospermopsin, and saxitoxin. Several of these toxins are known to be dangerous or even fatal to humans (Miller et al., 2017). These toxins usually affect humans through food poisoning or contaminated drinking water (Berdalet et al., 2016). In addition, cyanobacteria vary immensely by species in optimal growth temperature, predator avoidance, colony size, colony shape, colony density, and the ability to produce toxins (Miller et al., 2017). This means that individual species of cyanobacteria have different capabilities as HAB-forming organisms. As such, wide generalizations about the physiology and ecology of cyanobacteria and cyanobacterial blooms are effectively useless. Additionally, attempts to model cyanobacterial bloom behavior on scales relevant to responding to HAB occurrence are usually site-specific (Miller et al., 2017). Because HABs arise due to a complex interplay of physical, chemical, and biological processes, there is no realistic way to consistently prevent them. Additionally, HAB occurrence can be affected by anthropogenic, or human-caused, factors, further increasing unpredictability (Berdalet et al., 2016). There are methods for controlling HABs, however. These methods can be generally categorized as physical methods, chemical methods, or biological methods. Physical methods usually center on physical separation, for example using filters, pumps or barriers to remove the bloom-forming organism from the affected body of water. Physical methods are typically inefficient and difficult to use on a large scale (Zerrifi et al., 2017). Chemical methods typically involve using chemicals to inhibit the growth of or kill algae. While they can be quite effective, chemicals also have the potential to be toxic to aquatic organisms and, because dead algal biomass can cause oxygen depletion and an undesirable release of phosphorus, can encourage HAB recurrence. Additionally, bloom-forming organisms can develop resistance to chemicals such as copper sulfate, which used to be the most commonly used algaecide (Zerrifi et al., 2017). Biological control methods include things like biomaniipulation (the deliberate alteration of an ecosystem through the addition or removal of organisms), and are promising and environmentally friendly. Biological control methods are typically highly specific to the target organism and do not destroy other organisms or directly harm or pollute the environment. However, biological control methods still have the potential to cause imbalances in the food chain of the ecosystem (Zerrifi et al., 2017).

Control methods used specifically to deal with cyanobacterial blooms vary as widely as the organisms themselves. They include algaecides (commonly copper sulfate), nutrient input control, vertical destratification (or mixing), water flushing using the upstream water supply, and various biological control methods. Most of these are limited by other considerations. For example, algaecides are not advisable for use on large bodies of water or water to be used for drinking or recreation, enhanced water flushing requires abundant water supplies, and vertical destratification is mostly useful in shallow waters (Paerl et al., 2001). One common species of cyanobacteria is *Microcystis aeruginosa*, which produces microcystins. Microcystins are toxins that cause non-lethal toxicity in some species of fish (which

can then cause health problems in humans when they ingest these fish), and are genotoxic (damaging to DNA) (Paerl et al., 2001; Rastogi et al., 2015). This study focuses on *Microcystis aeruginosa*, with the goal of contributing data toward the development of new control measures to tackle blooms caused by this organism.

Because modern techniques for controlling HABs all have significant drawbacks, over the past two decades there has been considerable research investigating natural compounds as an alternative to synthetic algaecides (Miller et al., 2017). Compounds isolated from macroalgae, or seaweed, have shown activity against various types of microalgae and cyanobacteria, inhibiting growth or even entirely killing the target cells (Zerrifi et al., 2017). While these compounds have for the most part been used for their antifungal and antibacterial activities pharmaceutically, they also have applications against HABs according to the few studies that have focused on the topic (Zerrifi et al., 2017). In 2012, a compound was extracted from *Phragmites communis* that showed activity against *M. aeruginosa*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris* (Ni et al., 2012). Algicidal marine bacteria often produce types of allelochemicals, or chemicals produced by organisms that are detrimental to the growth of other organisms, that affect various species of microalgae (Kim et al., 2008). A good example of this is the bacterium *Hahella chejuensis*, which produces prodigiosin, a compound toxic to some species of dinoflagellates (Jeong et al., 2005). In addition, use of algicidal marine bacteria has been considered as a possible response to HABs in place of less environmentally friendly methods, such as traditional algicidal chemical treatment (Kim et al., 2008).

Antimicrobial peptides (AMPs) are short, amphipathic peptides that play a role in the innate immune defenses of virtually all organisms, from bacteria to multicellular animals. These AMPs have been found to have antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, eukaryotic parasites, and viruses (Brown & Hancock, 2006). Their cytotoxic mechanism of action involves attaching to and disrupting target membranes, resulting in cell lysis and death (Epand and Vogel, 1999). This class of molecules has not been studied in depth for possible activity against HAB-formers like *M. aeruginosa*.

This study aims to test the effects of a broadly effective AMP called Cecropin A on the common freshwater HAB-forming cyanobacteria *Microcystis aeruginosa*. Cecropin A is known to be effective against multiple types of organisms, including *Acinetobacter baumannii* and *Escherichia coli* (prokaryotic organisms) and *Trypanosoma cruzi* (a eukaryotic organism) (Fiek et al., 2010; Giacometti et al., 2003; Silvestro et al., 2000). If Cecropin A inhibits *M. aeruginosa*, the compound could be a useful tool in the toolkit of modern control methods for HABs and replace older problematic methods.

In addition to studying Cecropin A for inhibitory activity against *M. aeruginosa*, this study aims to test the effects of Cecropin A on the non-pathogenic *E. coli* K12 in order to determine this bacterium's suitability as a method of producing or distributing Cecropin A or related peptides to combat HABs. This study will observe whether resistance to Cecropin A develops in *E. coli*. If *E. coli* K12 is resistant to Cecropin A or quickly develops resistance, it could be useful to produce the peptide commercially as it would be able to produce it and secrete it without falling victim to Cecropin A's toxic effects. If it is not resistant and does not quickly develop resistance, it could be useful to distribute the peptide in ecosystems suffering from blooms, without leading to long-term colonization of those ecosystems by the bacterium itself.

Methods

Microcystis study – Assessing Cecropin A Effectiveness against *Microcystis aeruginosa*.

Materials and Fume Hood Set-Up

Experiments were performed on *Microcystis aeruginosa* purchased from Carolina Biological Supply (<https://www.carolina.com>). Prior to experimentation, *M. aeruginosa* was cultured and subcultured in Alga-Gro® media, also purchased from Carolina Biological Supply, in beakers in a fume hood. This organism was sub-cultured once or twice weekly, where aseptic technique was used to transfer 1 mL of the previous culture into 9 mL fresh sterile

Alga-Gro® media in a new sterile beaker to encourage continued healthy growth. The previous culture was kept for an additional week as a back-up in case of contamination in the new culture. All *M. aeruginosa* cultures were assessed multiple times a week for contamination so only uncontaminated cultures were used in experiments.

During experimentation, *Microcystis aeruginosa* was cultured in Alga-Gro® media inside sterile 1.5 mL test tubes. Transparent tubes without color were chosen for this experiment so that the *M. aeruginosa* cells, being photosynthetic, could receive a known amount of light. These tubes were placed inside a fume hood equipped with a fluorescent light. To maximize the tubes' exposure to light from the fluorescent bulb and minimize the tubes' exposure to light from other sources, the open face of the fume hood was covered with aluminum foil to reflect light inward and block ambient light from the lab windows, allowing a constant amount of light surrounding the tubes.

Some tubes also contained Cecropin A, an antimicrobial peptide purchased from AnaSpec (<https://www.anaspec.com/>) in powder form at >95% purity. The growth of *M. aeruginosa* was measured using a plate count on LB agar plates, poured using pre-sterilized LB agar into sterile 1000 x 15 mm petri plates, both purchased from Carolina Biological Supply. Other equipment, including test tubes, calibrated micropipettes, and micropipette tips were also used, all sterilized before use with an autoclave or 70% ethanol, as appropriate.

Preparation of Cecropin A

A large sterile test tube was filled with 10 mL of Alga-Gro® liquid media. This media was then combined with 1000 µg of Cecropin A and thoroughly mixed using pulses of on a low vortex speed to make a stock solution of Cecropin A at 100 mg/L. This solution was aliquoted into 5 sterile tubes with 2.0 mL solution each. Cecropin A was stored at -20°C until use. Multiple aliquots were made to avoid repeated freeze-thaw cycles between individual experiments, to avoid damage to the peptide.

Preparation of Experimental Samples and Controls

Six 10 mL sterile test tubes were labeled A-F. These were then used to make a positive control, negative control, and serially diluted experimental samples (Table 1). First, 1 mL of Alga-Gro® media was pipetted into each of the tubes. One additional mL of media was pipetted into tube A, which served as the media-only negative control with no Cecropin A peptide or *M. aeruginosa*. For the experimental tubes, 2 mL of 100 mg/L Cecropin A solution (already previously dissolved in Alga-Gro® media) was added to Tube C. Then, 1 mL of the contents of Tube C was pipetted into tube D and thoroughly mixed via an up-and-down pipetting motion, resulting in a concentration of 50 mg/L of Cecropin A in Tube D. This process was repeated for tubes E and F, creating concentrations of 25 mg/L and 12.5 mg/L of Cecropin A, respectively. 1 mL of the contents of tube F was discarded into a container of 10% bleach to ensure that each sample was of equal volume (1 mL each). Then, 1 mL of a *Microcystis aeruginosa* culture was pipetted into tubes B-F, cutting the existing Cecropin A concentrations in half, resulting in final concentrations ranging from 50 mg/L to 6.25 mg/L. Tube B served as the positive control, and tubes C-F served as experimental groups.

Contents of Control and Experimental Tubes in the *M. aeruginosa* Study.

Tube	Type	Cecropin A	<i>M. aeruginosa</i>
A	Negative control	0 mg/L	Absent
B	Positive control	0 mg/L	Present
C	Experimental	50 mg/L	Present
D	Experimental	25 mg/L	Present
E	Experimental	12.5 mg/L	Present
F	Experimental	6.25 mg/L	Present

Table 1. Contents of Control and Experimental Tubes. Six tubes, labeled A – F, were set up with the contents shown here. The “Type” column describes the type of control or experimental combination in the tube. The final concentration of Cecropin A for each tube is also listed, as well as whether *M. aeruginosa* was added to the tube (“present”) or not (“absent”) from a culture that was 24 to 48 hours old.

Experimentation

All tubes were placed inside a fume hood equipped with a fluorescent light bulb. The tubes were left slightly open to allow air to flow into the tubes. The tubes were exposed to a light intensity of approximately 560 lux at all times, measured by a digital photometer. All tubes were kept at room temperature and were stored in the fume hood for 72 hours. After 72 hours, each tube was mixed thoroughly by manual tube inversion. Then the growth of each tube was measured using a plate count on LB agar plates. Each tube was plated three times with 1 mL per plate at 1:1,000,000 dilutions, except for tube A (negative control), from which only one mL undiluted was plated. The 1:1,000,000 dilutions were necessary for the positive control and experimental tubes to prevent growth of lawns and ensure that the number of colony-forming units (CFU) could be counted. All LB plates were left inside the fume hood under the same conditions as the tubes for an additional 24 hours for colonies to grow. The number of CFU on each 1:100,000 dilution plate were then recorded for data analysis. Negative control plates were confirmed to be uncontaminated.

Disposal

Following experimentation, all cultures and plates containing *M. aeruginosa* were autoclaved by the faculty mentor and disposed of in a designated lab trash container. All glassware that had contained *M. aeruginosa* liquid cultures was also sterilized by autoclave following use of 10% bleach to sterilize the liquid cultures.

Data Analysis

Statistical analysis was performed on plate count results with a one-way analysis of variance (ANOVA) test with Tukey post hoc tests (<http://vassarstats.net/anova1u.html>) where differences were significant when $p < 0.05$.

Calculating the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of Cecropin A against *M. aeruginosa* could not be determined experimentally due to limited Cecropin A. Instead, multiple concentrations that inhibited *M. aeruginosa* to varying degrees were tested, with the resulting CFU from plate counts plotted in a Microsoft® EXCEL spreadsheet to calculate a line of best fit for the data, along with an R^2 value indicating the quality of fit of the line for the data set. The square root of the R^2 value was calculated to find Pearson’s r value, then a p -value was calculated for the line of best fit using a Pearson Distribution calculator (<https://www.socscistatistics.com/pvalues/pearsondistribution.aspx>). The line of best fit was then used to calculate a predicted MIC mathematically, by substituting values for ‘ x ’ into the equation for the line of best fit and calculating the resulting ‘ y ’ to obtain the predicted MIC. Values substituted for ‘ x ’ in the equation were 0.1% of the positive control’s average CFU’s and ± 1 SEM of this value from the plate count to predict a specific MIC representing 99.9% inhibition relative to the positive control.

E. coli study – Exploring *E. coli* K12 as a Candidate for Production or Delivery of Cecropin A.

Materials

Experiments were performed on *E. coli* Strain HB101 K-12, a non-pathogenic strain of *E. coli* developed for educational use, purchased from Bio-Rad (<https://www.bio-rad.com/>), and elsewhere abbreviated as *E. coli* K12 in this study. Cultures of this bacteria were maintained at 37°C in nutrient broth until use and were sub-cultured twice weekly to ensure healthy cultures. Cecropin A was also used during experimentation. 1 mg of >95% pure Cecropin A was suspended in Alga-Gro® media and stored at -20°C, as described in the previous section. The growth of *E.*

coli K12 was measured using a plate count on LB agar plates, using pre-sterilized agar and sterile petri plates purchased from Carolina Biological Supply. Other equipment, including test tubes, calibrated micropipettes, and micropipette tips were also used in this study, all sterilized before use with an autoclave or 70% ethanol, as appropriate.

Plate Count Method

Experimentation: Cecropin A was prepared as described in the previous section, using sterile water as the solvent instead of Alga-Gro®, which was only used in the *Microcystis* study to support the growth of *M. aeruginosa*. To perform the plate counts, two 10 mL sterile test tubes were prepared and labeled as control and experimental groups. Then, 1 mL of *E. coli* culture in nutrient broth was pipetted into both tubes. The control tube then received 1 mL of sterile water without Cecropin A while the experimental tube received 1 mL of Cecropin A at 100 mg/L, resulting in a final concentration of 50 mg/L Cecropin A in the experimental tube. Both tubes had a final volume of 2 mL of culture and were placed inside an incubator equipped with temperature control at 37 °C. After 24 hours, each tube was mixed thoroughly by manual inversion. The resulting cultures were named “Generation 1” to indicate that they had been exposed to Cecropin A in the experimental tube for one 24-hour period. The growth of the cultures in each tube were compared using a plate count on LB agar. Each tube was plated several times at diluted concentrations to compare growth between the control and experimental tubes, reserving >1 mL of each culture for use in inoculating the next generation. These plates were left inside the fume hood under the same conditions as the tubes for 24 hours in order for colonies to grow. The number of colonies on each plate was then recorded for data analysis. The entire procedure was then repeated twice, with each 24-hour growth being named as a successive Generation – Generation 2 and then Generation 3 – except each time the *E. coli* K12 inoculum solution was prepared from the plates of the experimental group of the last trial, in order to assess changing levels of resistance in the *E. coli* K12 population over time with continued Cecropin A exposure.

Data Analysis: Because each of the three generations began at a different concentration of cells, there was variation in the CFU counts between generations. Percent inhibition relative to each generation’s positive control was used to analyze data. Statistical analysis of data was performed using an analysis of variance (ANOVA) test with Tukey post hoc test where differences were determined to be significant when $p < 0.05$.

Optical Density Method

Experimentation: Cecropin A was prepared as described in the previous section, using sterile water as the solvent instead of Alga-Gro®, which was only used in the *Microcystis* study to support the growth of *M. aeruginosa*. Using a Cecropin A stock solution starting at 100 mg/L, serial 1:2 dilutions of Cecropin A were created ranging from 50 mg/L to 6.25 mg/L, each with 1 mL total volume. Then, a stock solution of *E. coli* was standardized to a concentration of 1×10^8 cells/mL using a spectrophotometer and 1 mL of the resulting culture was added to a tube with either 100 mg/L Cecropin A (stock solution) or the varying concentrations of Cecropin A created by the described 1:2 serial dilutions, cutting each Cecropin A concentration in half, so that they had final concentrations ranging from 50 mg/L to 3.125 mg/L Cecropin A. These *E. coli* K12 cultures were all incubated at room temperature for 48 hours. The tube with the 50 mg/L concentration of Cecropin A created by mixing the Cecropin A and *E. coli* K12 stock solutions in a 1:1 mixture was set aside. The optical density (cloudiness) of each of the remaining cultures was recorded at 600 nm using a spectrophotometer. More turbid (cloudier) cultures are a result of higher bacterial growth. The *E. coli* K12 tube with the highest concentration of Cecropin A set aside earlier (the tube with *E. coli* K12 incubated for 24 hours with 50 mg/L Cecropin A) was then used to create a new *E. coli* culture to begin the next generation. This entire process was repeated three times, using an *E. coli* culture tube from each iteration to supply the bacteria for the next one, resulting in *E. coli* cells with a longer history of Cecropin A exposure in each subsequent generation.

Data Analysis: Due to limited Cecropin A and the prohibitive cost of obtaining more, no replicates of the groups made for the optical density method were made. As a result, no statistical analysis was performed on the data collected with this method.

Disposal

Following experimentation, all cultures and plates containing *E. coli* K12 were autoclaved by the faculty mentor and disposed of in a designated lab trash container. All glassware that had contained *E. coli* K12 liquid cultures was also sterilized by autoclave following use of 10% bleach to sterilize the liquid cultures.

Results

Microcystis study – Assessing Cecropin A Effectiveness against *Microcystis aeruginosa*.

Cecropin A inhibited the growth of *M. aeruginosa* at all concentrations tested (Figure 1, Figure 2). The negative control plates (Figure 1A), with no *Microcystis* or Cecropin had no colonies, the positive control plates (Figure 1B), with *M. aeruginosa* without Cecropin A had the most colonies, and the experimental plates (Figures 1C – 1F) with concentrations of Cecropin A ranging from 50 mg/L to 6.25 mg/L all have fewer colonies than the positive control plates, indicating that Cecropin A inhibits the growth of *M. aeruginosa* at these concentrations. There was also a trend of more CFU counted as the concentration of Cecropin A decreased.

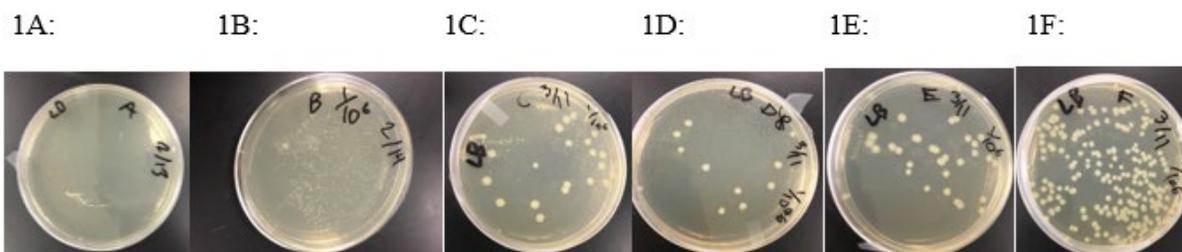


Figure 1: Representative Images of *Microcystis* plate counts with and without Cecropin A.

This experiment used a negative control group with no *Microcystis* or Cecropin A (1A) and a positive control group of *Microcystis* without Cecropin A where numerous colonies are apparent (1B). These colonies were smaller in size than those on plates 1C – 1F because of the much larger number of *M. aeruginosa* colonies in the positive control and is probably a result of density-dependent inhibition. The concentrations of Cecropin A tested were 50 mg/L (1C), 25 mg/L (1D), 12.5 mg/L (1E), and 6.25 mg/L (1F), and all have fewer CFU than the positive control group (1B). Groups B-F were all diluted at a 1:10⁶ dilution, to reduce lawns so growth could be counted in CFU. The negative control plate (1A) is undiluted to ensure no contamination was present in the tubes.

Plate counts from multiple trials (n = 3 to 5 for each Cecropin A concentration tested) were combined to assess the effectiveness of Cecropin A at these concentrations (Figure 2). Statistical significance was determined using a one-way ANOVA test with Tukey post hoc test, where the overall difference across control plates and experimental plates was significant (p < 0.0007), indicating the broad effectiveness of Cecropin A. According to Tukey post hoc tests, the differences between the positive control group and each of the experimental groups were found to be statistically significant (p < 0.05 for each control-experimental comparison). In other words, Cecropin A inhibited *Microcystis* growth at all concentrations tested, with inhibition ranging from approximately 55% for 6.25 mg/L to 95% for

50 mg/L Cecropin A. However, variation between repeats for each concentration tested in successive experiments means that differences in inhibition between different Cecropin A concentrations were not statistically significant by Tukey post hoc test in the one-way ANOVA analysis ($p > 0.05$).

Growth Inhibition of *Microcystis aeruginosa* by Cecropin A

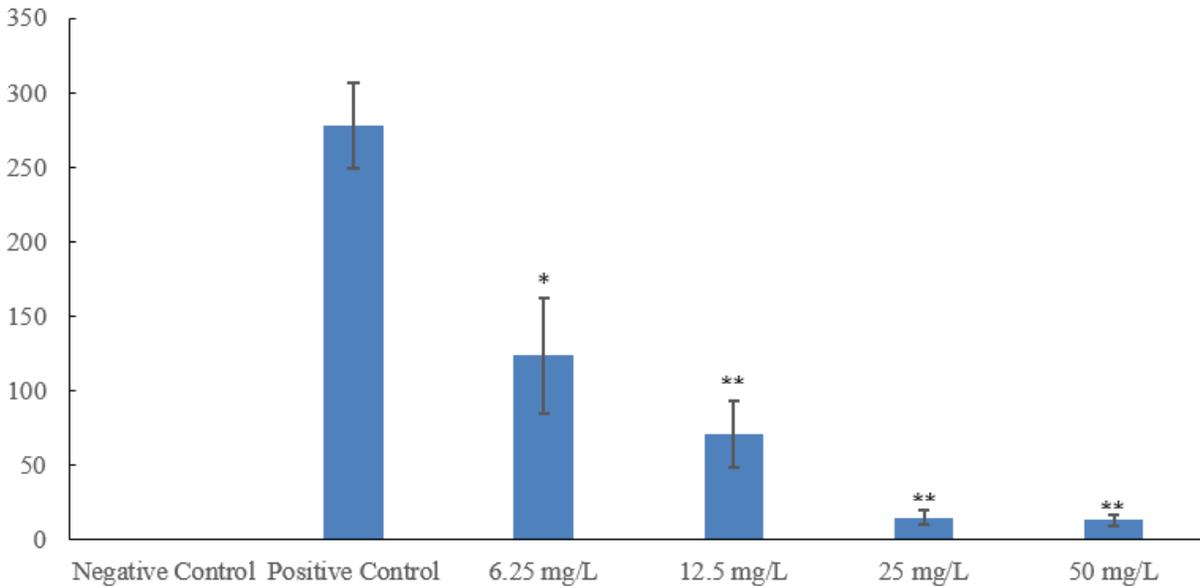


Figure 2. Growth Inhibition of *Microcystis aeruginosa* by Cecropin A.

The negative control plates ($n = 5$) had no colony forming units (CFU) and were plated undiluted. The remaining plates were at a dilution of $1:10^6$ from the sample tubes so growth would be countable as CFU rather than lawns. The positive control plates ($n = 3$) had an average of 278 ± 29 CFU. *M. aeruginosa* treated with Cecropin A at 6.25 mg/L ($n = 4$) had an average of 124 ± 39 CFU (a 55% decrease from the positive control). *M. aeruginosa* treated with Cecropin A at 12.5 mg/L ($n = 4$) had an average of 71 ± 22 CFU per plate (a 74% decrease from the positive control). *M. aeruginosa* treated with Cecropin A at 25 mg/L ($n = 5$) had an average of 15 ± 5 CFU per plate (a 94.5% decrease from the positive control). *M. aeruginosa* treated with Cecropin A at 50 mg/L ($n = 3$) had an average of 13 ± 4 CFU per plate (a 95.2% decrease from the positive control). Error bars represent 1 SEM (a 90% confidence interval). The asterisks denote significant differences between each experimental group and the positive control, calculated by a one-way ANOVA with Tukey post hoc test, where the use of one asterisk (*) corresponds to $p < 0.05$ and the use of two asterisks (**) corresponds to $p < 0.01$ by Tukey post hoc test. The overall p-value for the whole data set was $p < 0.0007$.

The minimum inhibitory concentration (MIC) of Cecropin A against *M. aeruginosa* was not determined experimentally because the highest concentration of Cecropin A tested inhibited only 95.2% of its growth. However, the data collected (Figure 2) was used to calculate a line of best fit that could then be used to predict an MIC for the Cecropin A concentration that would be expected to yield 99.9% inhibition of *M. aeruginosa*. The equation of the calculated line of best fit for the plate count data set is $y = 170.37e^{-0.06x}$ where x = the concentration of Cecropin A and y = the amount of growth (in CFU) expected for a given concentration of Cecropin A (Figure 3). This equation indicates an exponential relationship where an increasing concentration of Cecropin A results in an exponential decrease in growth of *M. aeruginosa*. The calculated R^2 value for this data set was 0.8143, indicating a strong fit of the data to this line, as determined by a Pearson calculator ($p = 0.036$). In other words, the line of best fit is sufficiently strong enough to be used to calculate a predicted MIC.

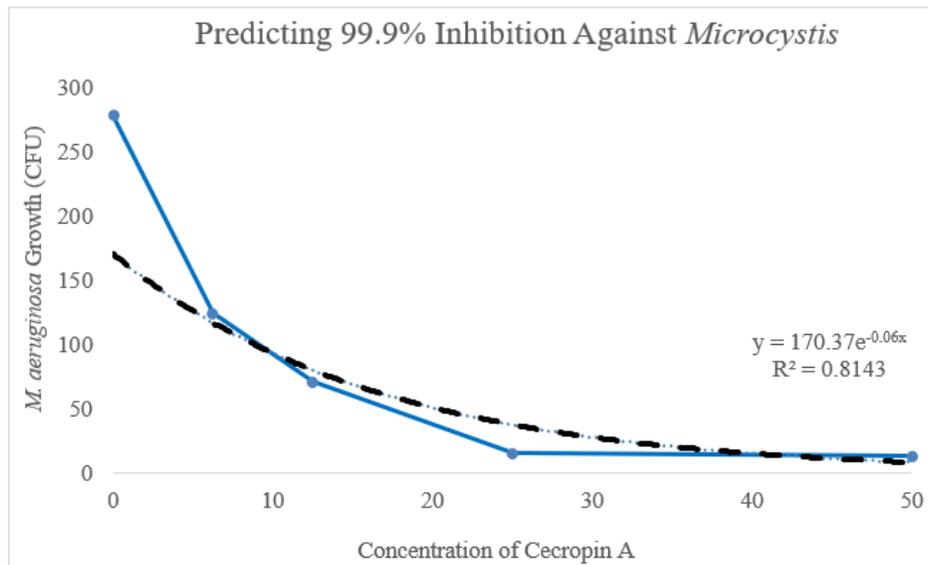


Figure 3: Predicting 99.9% inhibition against *Microcystis*.

A scatterplot was used to predict 99.9% inhibition by Cecropin A. The line of best fit is represented by a dotted black line, with the equation $y=170.37e^{-0.06x}$. The blue solid line shows the actual experimentally collected data points used to calculate the line of best fit. The calculated R^2 value for this data set was 0.8143. A p-value for the correlation of the R^2 value with the data determined by a Pearson calculator is $p = 0.036$.

The equation for the line of best fit was used to calculate a predicted MIC for Cecropin A that would inhibit 99.9% of *M. aeruginosa* growth, with the resulting predicted MIC being 107 mg/L. A most probable range for the MIC was calculated based on 0.1% of values that were +/- 1 SEM from the positive control's average, and found to be between 105 mg/L to 109 mg/L (Table 2), giving a 90% confidence interval for the predicted MIC calculation.

	Positive Control CFU	0.1% of Positive Control CFU	Predicted MIC (mg/L)
Average	278	0.278	107
+ 1 SEM	307	0.307	105
- 1 SEM	249	0.249	109

Table 2. Predicted MIC values based on 99.9% inhibition of *M. aeruginosa* growth.

The numbers in the Positive Control CFU column are the CFU calculated for the positive control plates ($n = 4$) in the plate count experiment (Figure 2), with the average and the average +/- 1 SEM given. To predict an MIC that would inhibit 99.9% of *M. aeruginosa* growth, these numbers were multiplied by 0.001 (0.1%). The resulting values, depicted in the "0.1% of Positive Control CFU" column, were substituted for "y" in the equation of the line of best fit (determined in Figure 3), with the resulting "x" shown in the "Predicted MIC" column.

E. coli study – Exploring E. coli K12 as a Candidate for Delivery of Cecropin A.

Part 1 of the study concluded that Cecropin A is an effective tool to inhibit the growth of *M. aeruginosa* *in vitro*. Next, we consider *E. coli* K12 for its suitability as a candidate to produce or distribute Cecropin A to treat HABs in nature. Specifically, in the remainder of this study, *E. coli* K12 was assessed for its ability to withstand exposure to Cecropin

A, with the reasoning that this bacterium would need to be able to resist the membrane-disrupting effects of the Cecropin A peptide in order for it to produce and disseminate it into an algal bloom as part of anti-bloom control efforts. To this end, *E. coli* K12 was cultured with Cecropin A and plate counts (Figure 4) were conducted to assess its resistance or susceptibility to this peptide. The control plates show more growth than their experimental counterparts, indicating that Cecropin A does inhibit the growth of *E. coli* K12 (Figure 4, Table 3). For each generation, standard deviation and error margins increased (Figure 5), likely due to the random way in which the mutations develop that result in resistance.

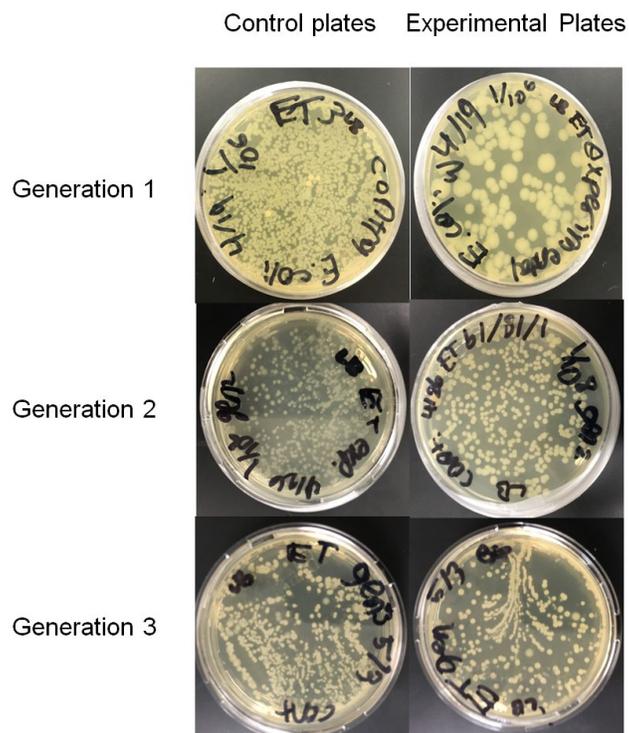


Figure 4: Representative Images of Plate Counts.

This experiment used a control group of *E. coli* K12 that was not exposed to Cecropin A and an experimental group that was exposed to Cecropin A at a concentration of 50 mg/L. Both groups were plated over several generations, with each generation representing 24 hours of exposure to Cecropin A in a liquid culture, followed by 24 hours without any exposure during growth on an LB plate. The colonies depicted in the experimental plates in this figure had been collectively exposed to Cecropin A for 24 hours (Generation 1), 48 hours (Generation 2), and 72 hours (Generation 3). Full data is shown in Table 3.

Plate counts were combined to assess the average % inhibition of *E. coli* for each generation, where the viable cells at the end of the exposure period were considered the first, second, or third generation, corresponding with one, two, or three continuous 24-hour periods exposed to Cecropin A. Differences in CFU between generations vary because colonies were selected at random from the previous generation to inoculate the tubes for the following generation. However, equal amounts of inoculum were used for the control and experimental tubes, allowing for comparison between the control and experimental plates within each generation. For this reason, data is graphed as percent inhibition of the experimental plates relative to the average CFU on the control plates (Figure 5).

Inhibition of *E. coli* K12 by Cecropin A at 50 mg/L was highest in the first generation at 86% and lowest in the third generation at 59% relative to the control cultures for those generations not exposed to Cecropin A. In other words, each generation showed inhibition of *E. coli* K12 by Cecropin A at 50 mg/L, but with a trend towards less inhibition in each generation than the previous one (Table 3, Figure 5), though variation within each generation was

large enough that differences between generations were not significant by one-way ANOVA ($p > 0.05$). The third generation (with the longest exposure to Cecropin A) showed a growing resistance to the peptide, but also had the largest variation, perhaps due to the random nature of mutation, where mutations arise quickly among one lineage and not in others, even within the same culture, resulting in high variation between plates.

Importantly, all inhibition of *E. coli* K12 by 50 mg/L Cecropin A (Table 3) was less than the inhibition of *M. aeruginosa* at the same concentration, which was 95% (Figure 2), indicating that *M. aeruginosa* is more susceptible to Cecropin A at this concentration than *E. coli* K12, suggesting that *E. coli* K12 remains a suitable candidate for Cecropin A production and/or delivery to HABs.

Table 3: Plate Count Data.

Plate Count Data – Percent Inhibition of *E. coli* K12 by Cecropin A in Successive Generations.

	Control CFU	Control Average CFU	Experimental CFU	Percent Inhibition	Average Percent Inhibition
Generation 1	636	727	63	91%	86%
	660		102	86%	
	884		141	81%	
Generation 2	768	683	27	96%	72%
	952		284	58%	
	330		254	63%	
Generation 3	304	187	189	-1%	59%
	63		20	89%	
	195		23	88%	

Control CFU are from control plates of *E. coli* K12 without Cecropin A exposure, while Experimental CFU are from experimental plates where *E. coli* K12 had been previously cultured with Cecropin A at a concentration of 50 mg/L for 24 hours (Generation 1), 48 hours (Generation 2), or 72 hours (Generation 3) in 24-hour increments. Control Average CFU is the average number of CFU for the three control plates of that generation, rounded to the nearest whole number. Percent Inhibition was calculated by dividing each experimental plate's CFU count by the average of the three control plates' CFU counts, then multiplying by 100 and rounding to the nearest whole number. Average percent inhibition is the average of the three percent inhibition numbers for that generation rounded to the nearest whole number.

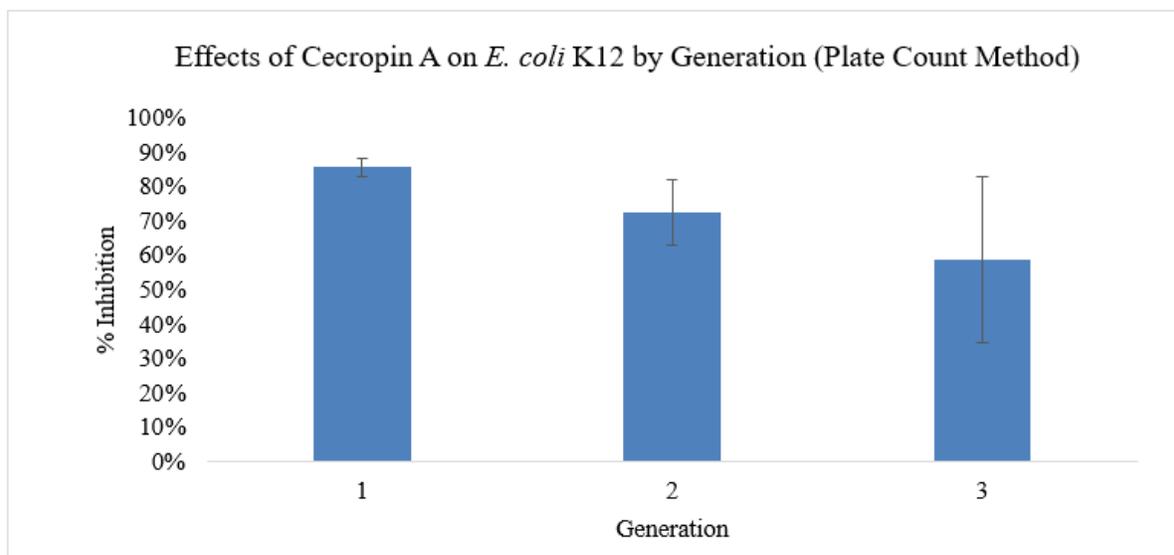


Figure 5: Effects of Cecropin A on *E. coli* K12 by Generation.

The effects of Cecropin A on *E. coli* K12 are represented here as percent inhibition relative to the average of the control plates ($n = 3$) for each generation. Error bars represent 1 SEM (90% confidence interval). The full results are shown in Table 3.

A similar method as with the plate count study was used in the optical density study. In the optical density study, however, four different concentrations of Cecropin A were tested for each generation (25 mg/L, 12.5 mg/L, 6.25 mg/L, and 3.125 mg/L). Due to limitations on the availability of Cecropin A, only one replicate was possible for each group. The resulting indicates a trend of resistance (Table 4, Figure 6) similar to that seen with the plate count method, where there were higher optical densities indicative of more growth, in each subsequent generation at 12.5 mg/L, 6.25 mg/L, and 3.125 mg/L. The combined observations from the plate count and optical density methods are evidence supporting the idea that resistance to Cecropin A may form in *E. coli* K12 over three 24-hour generations.

Table 4: Optical Density Data.

Optical Density Data – OD₆₀₀ of *E. coli* K12 in Successive Generations of Cecropin A Exposure

Concentration of Cecropin A	25 mg/L	12.5 mg/L	6.25 mg/L	3.125 mg/L
Generation 1	0.037	0.012	0.108	0.203
Generation 2	0.021	0.029	0.111	0.228
Generation 3	0.037	0.039	0.164	0.266

An optical density method was used to supplement the plate count method in this study. Optical density was collected by a spectrophotometer at a wavelength of 600 nm (OD₆₀₀). Each column in this table indicates a different concentration of Cecropin A as labeled, while each row contains data for a different generation of *E. coli* K12, with the cells in each generation exposed to their listed concentration of Cecropin A for 24, 48, or 72 hours, respectively, for Generation 1, 2, and 3. Due to limitations on the availability of Cecropin A, the procedure to gather this data was not repeated. As such, no statistical analysis was possible.

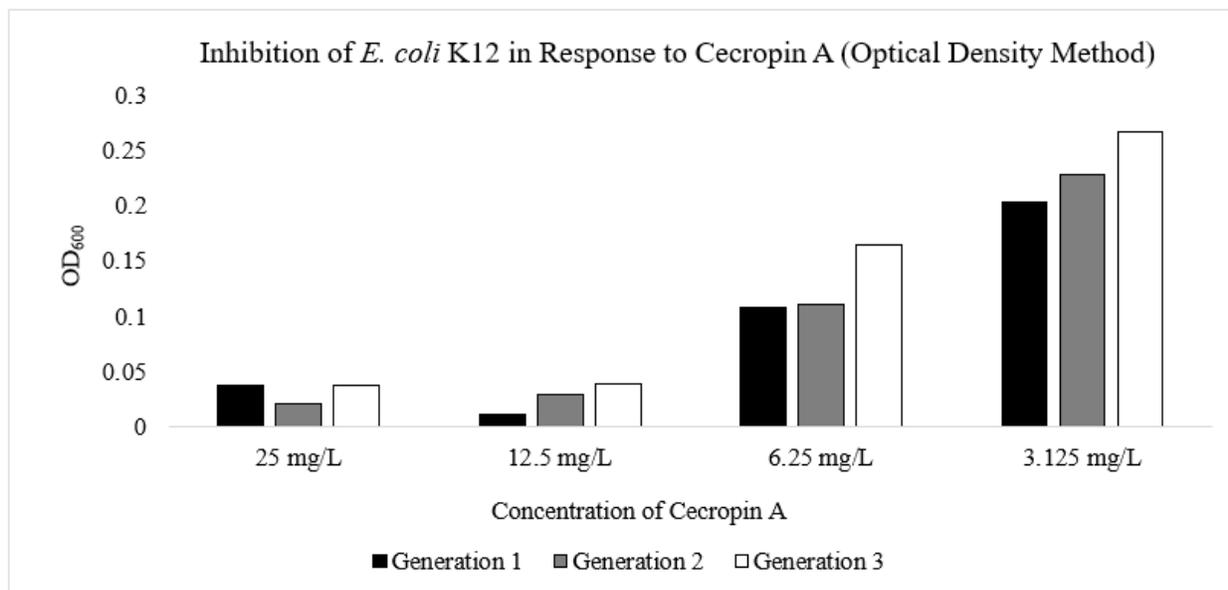


Figure 6: OD₆₀₀ of *E. coli* at Different Concentrations of Cecropin A.

Optical density data is presented for *E. coli* K12 cells cultured with Cecropin A at 25 mg/L, 12.5 mg/L, 6.25 mg/L, or 3.125 mg/L for 24 hours (Generation 1, black), 48 hours (Generation 2, grey), or 72 hours (Generation 3, white).

Discussion and Conclusions

Microcystis study – Assessing Cecropin A Effectiveness against *Microcystis aeruginosa*.

The results of this study indicate that Cecropin A is effective against *Microcystis aeruginosa* with all tested concentrations of the peptide significantly inhibiting *Microcystis* growth. Each culture of *Microcystis* exposed to Cecropin A showed between a 55% to 95% decrease in growth compared to the positive control group, and each was statistically significant. It is perhaps counterintuitive that 6.25 mg/L Cecropin A and 50 mg/L Cecropin A (the lowest and highest concentrations tested) had different percent inhibitions (~55% vs. ~95%) for *Microcystis* but were not significantly different from each other. This lack of significant difference between different Cecropin A concentrations is the result of variation between repeats for each concentration. Additional repeats of the procedure may have decreased variation and resolved the discrepancy, but due to limited Cecropin A stock, it was not feasible to perform additional repeats.

Though no MIC of Cecropin A for *M. aeruginosa* was found experimentally, data-driven mathematical modeling predicted that 107 mg/L should inhibit 99.9% of growth *in vitro*, with a 90% confidence interval of 105 mg/L to 109 mg/L. These numbers could be easily tested experimentally in the future with additional funding for more Cecropin A. Taken together, the data in this section of the study indicate that Cecropin A or other antimicrobial peptides similar in structure, could be used to reduce the severity of HABs, or even reduce their chances of occurring by treating bodies of water that are at risk.

There are several avenues of further study based on this research. Firstly, experimentally determining the exact MIC is a useful next step to understanding more about the interaction between this peptide and *M. aeruginosa*. Secondly, because *M. aeruginosa* is far from the only species of bloom-forming cyanobacteria, testing the effectiveness of Cecropin A against other harmful bloom-formers could afford greater knowledge about the possible uses of this compound for combatting blooms. Likewise, testing other antimicrobial peptides from the Cecropin peptide family may identify other AMPs with even more powerful inhibition of *M. aeruginosa* and other related bloom-forming

cyanobacteria. Lastly, Cecropin A should be tested outside of the lab in an actual algal bloom to determine if it is effective against *M. aeruginosa* and/or other cyanobacteria outside of the *in vitro* environments studied here.

E. coli study – Exploring *E. coli* K12 as a Candidate for Delivery of Cecropin A.

The most obvious and also the most important observation made from this section of the study is that *E. coli* K12 is susceptible to Cecropin A. For the purpose of using genetically engineered organisms to distribute Cecropin A, this is actually a desirable quality. This is because if a strain of this non-pathogenic *E. coli* K12 is genetically modified to produce Cecropin A, while also being susceptible to Cecropin A, the resulting system is self-regulating and thus more environmentally friendly. In other words, once such an engineered strain of this *E. coli* had produced a large enough amount of Cecropin A to reduce the size of the HAB, it would also likely result in feedback control of that strain of *E. coli* disseminated into the body of water with the bloom. In addition to being susceptible to the compound, *E. coli* K12 appeared to begin to develop resistance to the compound. This is not certain, however, because the data indicating this increase in resistance was not statistically significant, although this may be a result of the randomness inherent in mutations that created large variation between replicates. In other words, Cecropin A could have suppressed the growth of some lineages without a resistance mutation while failing to suppress the growth of lineages with such a mutation. The limited amount of Cecropin A available for this study prevented this from becoming clear beyond three generations, so additional study on possible resistance acquisition and potential environmental impacts is warranted before actually deploying such an engineered strain into a body of water with a HAB.

The possible trend towards less inhibition of *E. coli* K12 with each generation also suggests that additional exposure of *E. coli* K12 over more generations could result in a strain of *E. coli* that is more resistant to Cecropin A. Thus, alterations to the bacterium might be necessary in order for it to be practical for large-scale production of Cecropin A, because as it is, it would likely be inefficient at such production compared to a more resistant organism. More research is needed to confirm these findings.

There are several avenues of further study based on this research. Firstly, because of the small sample size as a result of limited funding, repeating this procedure with better funding could produce more precise results, particularly in time frames of more than three generations. Secondly, because *E. coli* is far from the only candidate for production and/or distribution of Cecropin A, evaluating the merit of other microbial candidates for similar biotechnology applications would be a productive line of inquiry. Lastly, the logical extension of this study in genetically engineering this strain of *E. coli* (or another non-pathogenic bacterium with similar qualities) to produce Cecropin A and to secrete it in quantities necessary to control HABs. Development of such genetically modified microbes could result in improved anti-algal bloom applications.

Limitations

There are a handful of limitations that impact this study. First, the cost of purified Cecropin A (>\$700 for 2 mg) was nearly prohibitive for a high school research project, resulting in small sample size in both the *M. aeruginosa* and the *E. coli* K12 trials. Sample size was further reduced by some isolated cases of contamination where some plates could not be counted and had to be excluded from the study. The limited amount of Cecropin A available for this study also made it impossible to test concentrations over 50 mg/L or to assess more than three generations of *E. coli* K12 exposure to Cecropin A.

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