THESIS

INVESTIGATION OF THE INHIBITORY EFFECT OF BACILLUS PUMILUS ON NANNOCHLOROPSIS SALINA

Submitted by

Mirna Dheyaa Ayshoa Al Gabara

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Master's committee:

Advisor: Kenneth F. Reardon

Christie Peebles
Cris Argueso

ABSTRACT

INVESTIGATION OF THE INHIBITORY EFFECT OF BACILLUS PUMILUS ON NANNOCHLOROPSIS SALINA

Microalgae have the potential to be a source of a wide range of industrial materials. To provide the biomass for these products, algae are grown in large volumes. Previous research has shown that there are other microbial species living in algal cultivation systems at these scales, but little is known about the interactions among them. Some of the bacteria in algae cultivations have been identified. Some species can inhibit algal growth, while others are growth promoting. In this research, we focused on one algal species, *Nannochloropsis salina,* and a bacterial species, *Bacillus pumilus.* In previous research in our laboratory*, B. pumilus* culture filtrate had inhibitory effects towards *N. salina.* We are using these species as a model system to understand a mechanism of bacterial inhibition of algae. Specifically, we have investigated the nature of the inhibitory molecule that is produced by *B. pumilus* and when it is produced.

Our results indicate that *B. pumilus* produces at least one inhibitory molecule that is probably a protein larger than 30 kD. Since the bacteria produce the highest level of the inhibitory molecule in the presence of marine broth medium (MB), we studied the effects

Of the components of MB to determine whether one of these induced the production of the inhibitor more than others. *B. pumilus* was inoculated in artificial sea water medium (ASW) and several components of MB (peptone, yeast extract and glucose). The filtrate of *B. pumilus* grown in ASW supplemented with peptone or yeast extract had an inhibitory effect on *N. salina*, but the filtrate of *B. pumilus* grown in ASW supplemented with glucose had no inhibitory effect towards the algal species.

The results showed that the molecule was produced regardless of the presence of the algal species and it was more concentrated at the late stationary phase. Also there was a certain algal phase when *N. salina* had more resistance to the inhibition of *B. pumilus* filtrate. The bacterial species showed the ability to grow on the filtrate of *N. salina* without any other added components.

This knowledge about the mechanism by which this bacterial species inhibits an algae species is useful to determine whether other bacteria use the same strategy and to develop an approach to reduce this inhibitory impact.

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1. MICROALGAE AS A SOURCE OF BIOFUEL AND BIOPRODUCTS

Algae belong to a large group ranging from unicellular to multicellular organisms. Algae use energy from photosynthesis using sunlight and inorganic molecules (carbon dioxide and water) to produce oxygen and organic molecules (sugar). The algae fossil record dates to three billion years ago, well into the Precambrian period. Algae are ubiquitous within the biosphere and have generated a significant fraction of the oxygen present in the earth's atmosphere and a large quantity of organic carbon in the form of coal and petroleum. Algae are important for the life in our planet, as they are at the bottom of food chain. The importance of algae has increased with the search for renewable energy sources. Algae can thrive under unfavorable conditions and produce many various byproducts such as lipids (oils), carbohydrates, proteins, and various feedstocks that can be converted into biofuels and other industrial materials (Menetrez et al., 2012).

Algae are the basis of the food web, so they are primary producers. Also, they often live symbiotically with other organisms. The term symbiosis is generally considered to be beneficial, although it was first coined to mean 'living together' and so strictly also includes pathogenic or parasitic relationships (Cooper et al., 2015).

Using algae for fuel, whether using its biomass or the oil that is produced within the cell, has been an important topic in the energy field. Microalgae are of interest for biofuel industry since they can grow less land in comparison to plants (Mata et al., 2009).
In order to use microalgae as an energy source, it has been suggested that we should combine the microalgae fuel production with wastewater treatment processes and co-production of other products to make the process more economical (Bhatt et al., 2014).

Letcher suggested that algae are like terrestrial crops in that their productivity is affected by biotic factors such as weeds, predators, and other microbes. Since the majority of algae pathogens and pests have not been identified and industry pest management standards are at an early stage of development, it is important to look into the nature of these interactions (Letcher et al., 2013).

1.2. CONTAMINATION IN INDUSTRIAL ALGAE SYSTEMS

Even though genetic modification has been done on microalgal species to improve the rate at which they produce desired products, it's important also have genetic modification that address the problem of contamination and resistance to contaminants (Henley et al., 2013; Perrine et al., 2012; Ort et al., 2011).
To do that, we need to understand contamination better. Industrial-scale algae cultivations have faced the challenge of contamination and poor performance; therefore, the field of algae production needs more information about the poor performance of

algae growth at industrial scales. One of the research projects that addressed this question was conducted by Fulbright and colleagues, who monitored contaminating species in poorly performing industrial algal systems (Fulbright et al., 2016). They isolated different bacterial species and tested their effect on the elite algal species, *Nannochloropsis salina. B. pumilus* was found to have an inhibitory effect towards *N. salina.* Weedy algal species that compete with the elite algal species were identified and found to have no growth inhibition by the bacterial species that inhibits the elite algal species and that's one of the main challenges when growing in large scales. There are problems or barriers surrounding large-scale algae production that limit its growth for these scales, and these issues include providing proven, stable, large-scale cultivation methods for appropriate high-oil-content algal strains and an understanding of culture maintenance and pest management strategies (Shurin et al. 2013).

Studies have documented invasion by weedy species, grazers, and pathogens of large-scale cultures by non-elite species in both open-pond and closed photobioreactor systems (Quinn et al., 2012; Gachon et al., 2009; Li., 2011). For this reason, we need culture monitoring, management, identification of the contaminants, and an understanding of the inhibition mechanisms to make an elite species more resistant.

1.3. ALGAL-BACTERIAL INTERACTIONS IN ECOSYSTEMS

Knowledge about the interactions of algae and bacteria should be collected to make conclusions about the possible interactions. These interactions are present in nature for various reasons that might seem unrelated, including development, acclimation, adaptation, and evolution.

Many studies have suggested functional interactions between algae and bacteria showing that algae require a microbiome to grow. The genomes of 326 algae species were surveyed and it was found that 171 need vitamin B12 from outside sources, such as bacteria. Vitamin B12 auxotrophy in some algae most likely occurred because bacteria are major producers of the vitamin, which takes 19 energy-intensive enzymatic steps to make (Croft et al., 2005). In this case, algae do not need to expend energy to make this nutrient as long as it can develop an intimate relationship with bacteria. Many studies have focused on the bacterial-algal relationships, but most of those studies do not draw strong functional conclusions.

The diversity of interactions can be illustrated by two examples:

- The bacterium *Silicibacter sp*. TM 1040 forms a biofilm on the outside of the dinoflagellate, *P. piscidcida.* When the bacterium is removed from the *P. piscidcida* culture, the dinoflagellate struggles to stay alive, which suggest the assumption that this bacterium is a necessary microbiome component for algae

survival since it provides a function that is not encoded or active in the *P. piscidcida* genome.

- Iron is a limiting nutrient for algae in the ocean and is crucial for photosynthesis and respiration. To overcome a lack of iron, algae associate with marine bacteria that have developed siderophores, which are organic molecules that bind iron and enhance the solubility of iron (and therefore its availability to algae). When *Marinobacter* is present, there is a 20× increase in iron assimilation in the dinoflagellate *Scrippsiella trochoidea*. Moreover, *Marinobacter* only grew when the dinoflagellate was present, which suggests a mutualistic relationship (Jermy, 2009).

Additional evidence for the dependence of algae on bacteria in some scenarios comes from studies that have determined that specific bacteria stimulate algae growth through activities including regulation of the amount of available nutrients including iron, nitrogen, and phosphates (Amin et al., 2009; Foster et al., 2011) or releasing phytohormones into the growth environment (De Bashan et al., 2008).

In addition to these functional relationships between algae and bacteria, recent studies have revealed that major groups of algae have assimilated metabolic pathways from bacteria on several occasions in their evolution. Examples include cellulose synthesis in red algae (Collén et al. 2013); the urea cycle in diatoms (Allen et al., 2011) and other Ochrophytes (Allen et al., 2011); and the acquisition of the mannitol cycle by a common ancestor of brown algae and Dictyochophytes (Michel et al., 2010).

All four examples show the importance of bacteria as factors that shaped the evolutionary history of the algae when they co-existed with them. Thus, the entity of algae and the associated 'ecosystem' of microbes could be seen as a 'superorganism' that functions and, to some extent, evolves as a whole. If more studies focused on these interactions, it would not only help the commercialization stakeholders but also help in understanding some of basic but pertinent questions like involvement in endosymbiosis, multicellularity and vital habitats. Analysis of bacteria associated with particular algae has provided evidence for some degree of co-evolution between the algae and its associated bacterial community (Dittami et al., 2012; Ramanan et al., 2015).

One of the factors that control the nature of the algal-bacterial interaction is community complexity (Fulbright, 2015). Ramanan mentioned that when different kinds of algae were co-cultured, they were more resistant to the inhibitory effect of bacteria. This makes for an exciting new era with a paradigm shift from single-species cultivation (Ramanan et al., 2015).

Some bacteria are considered to be algicidal because they have the ability to kill or inhibit algal growth. Broadly, there are two scenarios in which bacteria can detract from algal production systems, a gradual reduction in productivity over extended periods of time or a sudden event that causes cultures to die rapidly ("crash"). Conceptually, bacteria could exert detrimental effects either by directly attaching to algal cells or by

releasing algicidal or inhibitory molecules into the surrounding environment (Fulbright et al., 2014).

The next step in algicidal bacterial research will be to document when the bacteria are killing phytoplankton in nature. To accomplish this goal, we should have the mechanistic understanding of how algicidal bacteria kill their phytoplankton targets. Many studies have shown that proteases may be involved in the killing activity. Also, Mayali and coworkers mentioned that once signature DNA sequences or proteins for the process of algicidal activity have been discovered, they can be used to develop molecular probes unique to those signatures to search for the algicidal process in nature. (Mayali et al., 2004).

Rivas and colleagues mentioned that it is important for the future to demonstrate that the phenomenon of phytoplankton death due to bacteria does indeed occur in the ocean, and that it is a significant process in the marine assemblages under natural conditions. For example, *Acinetobacter sp.* decreased *B. brauni* growth (Rivas et al., 2010). This will need a mechanistic understanding of how algicidal bacteria kill their phytoplankton prey as well as in situ rate measurements. To help constrain the phenomenon in an ecosystem context it is important to identify the biochemical basis of the phenomenon, and the identification of the responsible molecules. It is also interesting and important understanding whether there are energetic restrictions on the proliferation of algicidal bacteria, whether algicidal bacteria are obligate or facultative in

their lifestyle of killing algae, and whether the relevant phenotype is expressed only during algal blooms. (Mayali et al., 2004).

A goal for the future is to know the importance of trophic interactions for the population dynamics of algicidal bacteria in various ecosystems. These are challenging problems, but their resolution is necessary given the considerable interest in understanding the potential role of bacteria in the decline of harmful algal blooms. The knowledge that we gain will also be required to incorporate the consequences of bacteria-algae interactions in our concepts and models of the oceanic carbon cycle (Mayali et al., 2004).

Understanding the nature of the molecules produced by the bacterial species that co-exist with algal species would let us know the algicidal or the beneficial compounds that the bacteria produce when growing with algae. Lee and colleagues were the first to document a dissolved algicidal protease using a combination of genetics and biochemistry. They isolated a 50-kDa serine protease from the filtrate of *Pseudoalteromonas* strain A28 that showed algicidal activity towards the diatom *Skeletonema costatum*. They found that the culture filtrate had high protease activity, whereas that of non-algicidal mutants did not (Lee et al., 2000).

Several other studies have provided evidence for soluble inhibitory/algicidal molecules produced by bacteria:

- Mitsutani found that a stationary culture cell extract of *Pseudoalteromonas* strain A25 showed both algicidal and high protease activities while exponential phase

culture filtrates (as well as both growth phases of a non-algicidal mutant) did not have either of the activities. These results show that at least some algicidal bacteria kill their algal prey using proteases. If proteases are involved in an algicidal activity, we conclude that phytoplankton cell-surface polysaccharides play a role in defense against algicidal bacteria by protecting the cell against proteolytic attack. Interestingly, some algicides are resistant to autoclaving and thus are unlikely to be enzymes (Skerratt et al., 2002). Still, their chemical structures remain uncharacterized (Mayali et al., 2004).

- Fulbright and colleagues determined that *B. pumilus* culture filtrate was capable of inhibiting *Nannochloropsis* sp.*,* suggesting that an inhibitory molecule is released into the culture (Fulbright et al., 2016).
- *Roseobacter* may settle on algae cells, enter sessile phase, and release phytohormones such as indole-3-acetic acid into the growth environment (Geng et al., 2010).
- *Bacillus sp*. was found to release extracellular molecules that lysed two algae species, *M. aeruginosa* and *Chlorella* sp. (Pei et al., 2007).

Based on these reports, it appears that the majority of known, characterized algicidal bacteria inhibit algae growth by releasing molecules into the local aquatic environment (Mayali et al., 2004; Wang et al., 2005; Fulbright et al., 2014).

Sometimes the bacteria reduce algae productivity by competing for these same nutrients (Cole, 1982; Kazamia, 2012). In addition to the nutrient competition, nonlethal bacterial pathogens may inhibit algae productivity by diverting cellular resources from

Algal growth to cell defense. Finally, as mentioned previously, some bacteria can directly kill algae, causing cultures to collapse (Lewin et al., 1997; Fulbright et al., 2014).

In some cases, bacteria can increase or promote algae growth. In one example, a *Rhizobium sp.* increased growth of oil-producing *Botryococcus braunii* by 50% (Rivas et al., 2010). Eight bacteria isolated from a *Chlorella* culture promoted *Chlorella* growth when individually co-cultivated with the alga. In particular, *Brevundimonas* directly adhered to the *Chlorella* cell and increased growth by three times (Park et al., 2008; Fulbright et al., 2016).

1.4. RESEARCH GOALS

In a previous study, Fulbright investigated the ecosystem of *Nannochloropsis salina* and *N. gaditana* cultivations using molecular techniques to identify the algal and bacterial species that co-exist with the elite algal species (Fulbright, 2015). As part of that investigation, Fulbright found a bacterium, identified as *Bacillus pumilus*, which inhibited the growth of both *Nannochloropsis* species by releasing one or more molecules into the culture medium. Fulbright also identified some of the environmental conditions under which the inhibition was more pronounced. However, Fulbright's experiments did not reveal the nature of the inhibitory molecules nor whether the molecules were intracellular (released by lysis) or extracellular.

The goals of this research were to learn more about the inhibitory molecule and how is inhibits the growth of the algal species *N. salina*. An additional goal was to investigate the factors that increase the inhibitory effect, including both factors related to the algae and those related to the bacteria. We also aimed to know more about the factors in the algal ecosystem that are surrounding the two organisms and that increase or promote the inhibitory effect.

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CHAPTER 2

MATERIALS AND METHODS

2.1. CULTURING *BACILLUS PUMILUS*

Bacillus pumilus was grown in solid Marine Agar Petri dishes in an incubator at 30 ℃. The inocula for the solid cultures were taken from –80 ℃ frozen cultures of *Bacillus pumilus*. Liquid cultures were grown in 279110 - BD Difco™ Marine Broth 2216, Liquid cultures were prepared by taking colonies as an inoculum from the plates and adding it to MB in 250- 500 ml Erlenmeyer flasks. Cultures were grown at 30 ℃ with 200 rpm agitation. The time of growth for liquid cultures was based on the experimental requirements. In cases when an inoculum was taken from the liquid culture to inoculate another liquid culture, the bacterial inoculum was taken from 24-hour cultures. An amount of 1 ml of the culture was collected, centrifuged, washed with PBS, centrifuged, and mixed with 5 ml PBS. A 50 ul portion of this suspension was used to inoculate a 1-5 ml culture of algae.

Difco Marine Broth 2216 contains high level of salts and minerals content to imitate the marine environment (where both the algal and bacterial species in this research can grow) and it also has peptone and yeast extract as a source of nitrogen (yeast extract also contains vitamins).

The approximate composition per liter, according to the manufacturer is, peptone 1.0 g, ferric citrate 0.1 g, sodium chloride 19.45 g, magnesium chloride 5.9 g, magnesium sulfate 3.24 g, calcium chloride 1.8 g, potassium chloride 0.55 g, sodium bicarbonate 0.16 g, potassium bromide 0.08 g, strontium chloride 34.0 mg, boric acid 22.0 mg, sodium silicate 4.0 mg, sodium fluoride 2.4 mg, ammonium nitrate 1.6 mg, and disodium phosphate 8.0 mg. An amount of 15 g of Bacto Agar was added for solid Marine Agar preparation. Sterile Petri dishes were used for solid medium preparation. After the medium was prepared and autoclaved inside a bio hood, it was poured into plates and allowed to solidify we covered the plates and sealed them with Parafilm. Liquid MB was made in a glass bottle (500 ml) following the manufacturer's instructions. All growth was heterotrophic.

2.2. CULTURING *NANNOCHLOROPSIS SALINA*

All *N. salina* cultures were grown in an un-buffered artificial sea water medium ASW. The recipe is presented in Table 2.1 with notes and recommendation from Dr. Randor Radakovits, who used it for *N. gaditana.* All cultures were in 250-500 ml Erlenmeyer flasks and were grown phototrophically in a Multitron shaker/incubator with shaking at 120 rpm at room temperature (Fulbright, 2015). The initial inoculum was taken from a liquid culture and scaled up to the volume that was needed.

Table 2.1: The ingredients of 1 liter of ASW medium

Component	Amount	Concentration
Solution #1 (500 ml)		
NaCl	15 g	0.25 M
KNO_3	1.45 g	14.0 mM
KH_2PO_4	0.12 g	0.88 mM
$NaHCO_3$	0.04 g	0.48 mM
$FeCl_3•6H_2O$	0.01 g	0.037 mM
Na_2-EDTA	0.035 g	0.098 mM
3.64 mM $MnCl_2• 4H_2O$	0.25 ml	0.91 mM
Trace metals solution	0.5 ml	
Solution #2 (500 ml)		
$CaCl_2•2H2O$	0.5 g	3.4 mM
$MgSO_4•7H_2O$	6.6 g	26.8 mM
$MgCl_2•6H_2O$	5.6 g	27.6 mM

Trace Metals stock (1 liter) was composed of: 7.8 g Na_2-EDTA and 20 mg $CoCl_2·6H_2O$, 12 mg $Na_2MoO_4·2H_2O$, 44 mg $ZnSO_4·7H_2O$ and 20 mg $CuSO_4·5H2O$. EDTA was dissolved in diH_2O, and each metal was dissolved individually then the pH was adjusted to 7.5.

Autoclaving the solutions combined can lead to precipitation, so solutions1 and 2 were made separately, and then the pH for Solution #1 was adjusted to 7.3 using NaOH, autoclaved, and then combined with the 2_{nd} solution (each solution was autoclaved separately then mixed together). Trace metals were filter sterilized then added.

2.3. BIOASSAY FOR INHIBITION OF *NANNOCHLOROPSIS SALINA*

A bioassay to show growth inhibition of *N. salina* was done in a 24 well plate. Each well usually contained 750 - 1000 µl of *N. salina.* Corning 24-Well Falcon flat bottom plates were more suitable for these experiments because they reduce evaporation of the medium. Minimizing evaporation is important because it takes 4-7 days to see the inhibition of the algal cells. The plates were cultured in a Multitron shaker with light at room temperature and 120 rpm agitation. The plates were sealed with Parafilm to reduce evaporation. In addition, beakers containing chamber air were replaced inside the incubator to increase the humidity. All algal optical densities (O.D) were measured at 750 nm and each O.D value presented in this research is the average of two replicate measurements. An amount of 250 µl of the sample was added to a cuvette and mixed with 750 µl of water since only small samples would be removed. ASW was used as the zero standard.

2.4. PREPARATION OF CULTURE FILTRATE

The filtrate was collected from different stages of bacterial or algal growth based on the requirements of the experiments. Millipore GSTF (catalog # GSTF04700) filters with pore size 0.22 µl were used with a vacuum chamber to separate the cells from the filtrate. Sometimes we substitute the papers by VWR (#28245-501) sterile syringe filters. The filtrate was usually added to the bioassay directly after the filtrate was collected. When filtrate was not used at the same time that it was collected, it was stored in the refrigerator for 24 hours. It starts losing its activity if it stays for a long time under 4 ℃.

2.5. PREPARATION OF PROTEIN SIZE FRACTIONS

Amicon Ultra centrifugal filter units (0.5 ml) from Millipore were used for the purification of macromolecular components found in cell lysates (Figure 2.1). The Amicon Ultra-0.5 device is supplied with two microcentrifuge tubes. During operation, one tube is used to collect filtrate; the other tube is to recover the concentrated sample. These filters can be reversed, which enables recovery of the molecules trapped in the filters. The concentrate volume can be 15-500 µl but in our experiments 500 µl was always used as the sample size and about 100-200 ul of buffer to recover and collect the retained molecule in reverse spinning. It has a concentration factor of 25x to 30x, high sample recovery (greater than 90% of dilute starting solution) and fast processing time.

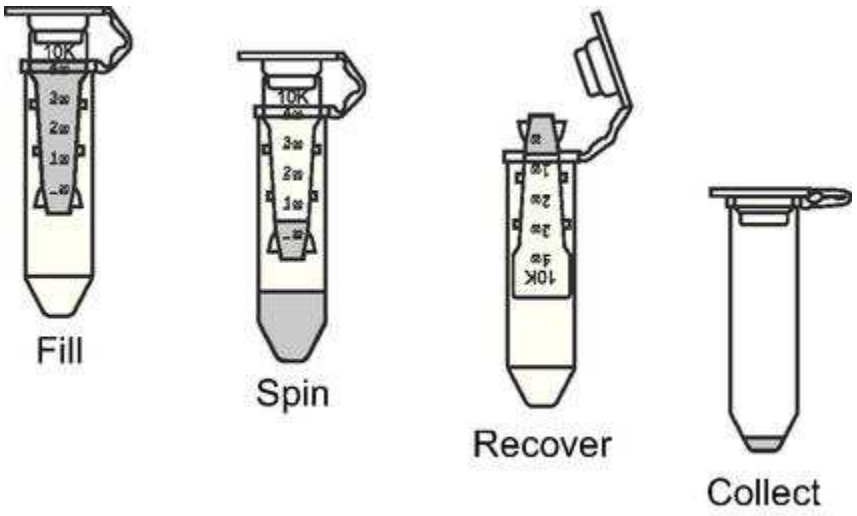

Figure 2.1. Depiction of how the filtrate and the concentrate are collected from Millipore ultra centrifugal filters. Source: http://www.emdmillipore.com/images/large/Amicon05_ReverseSpin-71[83660-ALL].jpg

The speed and time for collecting flow-through from each sample were based on the chart in Figure 2.2. All the filter sizes in this chart were used except for the 50 kD filter.

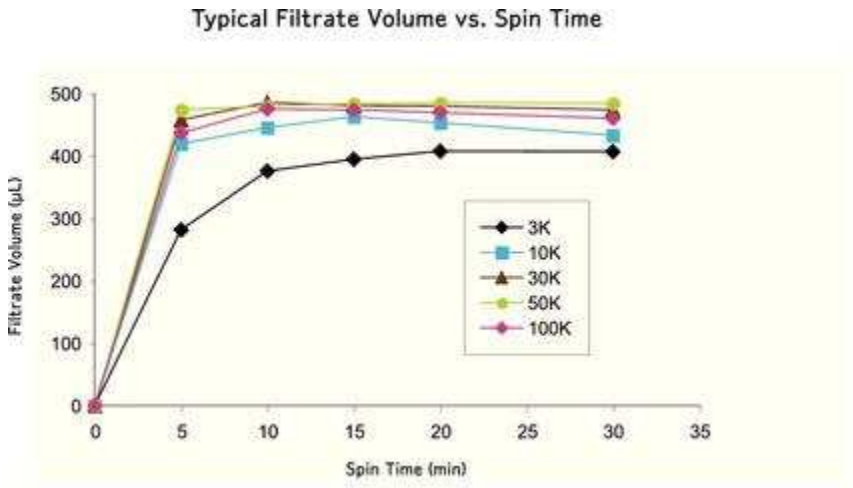

Figure 2.2. The speed and time of centrifugation for each Millipore filter size. Source: http://www.emdmillipore.com/images/large/Amicon05_new-71[84695-ALL].jpg

For the *B. pumilus* cell free filtrate that was concentrated, the volume of buffer was 100-200 µl and the speed was 1000 x for 5 min. For desalting (for the protein purification cartridges steps when we had to have a salt gradient), filters smaller than the expected size of our inhibitory molecule were used then reversed and eluted with 100-200 µl of buffer. PBS or Tris buffer were used depending on the requirement of the experiment (Figure 2.3). For the samples prepared for proteomics, Tris, HEPES and Trizma work well for mass spectrometer proteomics analysis (personal communication from Dr. S. Park). Filters made by a different manufacturer were not useful since the inhibitory molecule was found in neither the filtrate nor the concentrate.

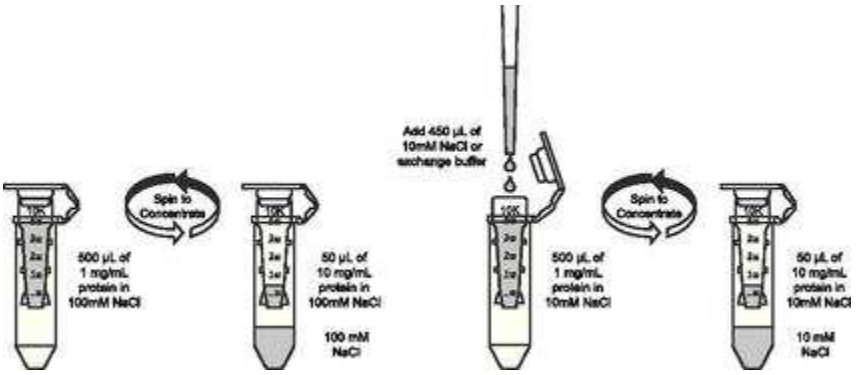

Figure 2.3. Schematic of desalting a sample based on the manual and the website: http://www.emdmillipore.com/images/large/Amicon05_Desalting-71[83650-ALL].jpg

2.6. POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in two different ways based on the suggestions of research scientists who have been using this technique. The results were essentially the same with the two procedures. For the first method, an amount of 37 µl of each sample was added to a PCR tube and an amount of 12 µl of 4x loading dye was added to each tube. To make 10ml of 4x loading dye, we mixed 2.5 ml 1 M Tris-HCl pH 6.8, 1.0 g SDS, 0.8 ml 0.1% Bromophenol Blue and 4 ml 100% glycerol then the final volume was adjusted to 10 ml with ddH20. This protocol is from Cold Spring Harbor Laboratory and it has been used by other researchers in our building.

In the second protocol, we prepared a mix of 950 µl of 2x Laemmli protein sample buffer and 50 µl of 2-mercaptoethanol (ßME) was added to it instead of DTT. A percentage of 1/1 (V/V) of sample to the mixture above was added (Laemmli, 1970). Bio-Rad Mini-Protean TGX 10% precast polyacrylamide gel, 10 well combs, 50 µl/well of each sample, and about 1liter of 10X running buffer was used in this technique.

2.7. PREPARATION OF MEDIA WITH COMPONENTS OF MARINE BROTH

In one experiment, media were prepared with individual major components of marine broth. Three components that make MB different than ASW were chosen and bacteria were grown on each one of them separately. Solutions of peptone (5 g/l); glucose (5 g/l) and yeast extract (5 g/l) were prepared separately. These were autoclaved for 15 min.

To inoculate each fraction that was prepared, a liquid bacterial culture grown in MB was centrifuged, the supernatant was removed, and PBS was added and then the cells were centrifuged again (resuspended in PBS buffer) to remove any remaining MB. After that each fraction of medium (peptone, glucose and yeast extract) was inoculated with these cells.

2.8. PROTEIN FRACTIONATION

A *B. pumilus* culture was grown for 32 h and then cell-free filtrate was collected. An amount of 50 ml of bacterial filtrate was used for each protein purification. The Bio-Scale Mini UNOsphere and Macro-Prep ion exchange cartridges/columns that we used (described more in Chapter 3) were washed with 20% ethanol. The cartridge was reversed when washing with ethanol because that will let all the air bubbles out of the cartridge due to increased pressure. An amount of 15 ml of 50 mM buffer was passed based on the type of the cartridge, TES buffer was used for cation cartridges and Tris for anion cartridges, both chosen to stabilize the pH inside the cartridge (manufacturer's

instructions). After this step, a mixture of the bacterial filtrate and one of the buffers was passed through the column. The first fraction was collected which is the flow-through (2.5 ml of 1 M buffer was added to 48-50 ml of filtrate to get a buffer concentration of 50 mM in the mixture. All were mixed by shaking the 50 ml Falcon tube and then the whole mixture was passed through the column. The flow rate for passing the fractions was 1 ml/min (0.5-3 ml/min is recommended by manufacturer). The cartridge was washed with buffer to get a second fraction of whatever is remaining from the flow-through molecules. We then used three different concentrations of KCl to release the molecules that were attached to the resin in the cartridge. These elute fractions tested on the bioassay that contained KCl, were desalted using Amicon Ultra centrifugal filters. The samples were also tested with salt in them.

KCl concentrations below 3 M did not inhibit *N. salina* growth (it was added as 1 ml of KCl to 1 ml of algae in 24 well plates). Before washing again with ethanol, the column was washed first with 50 mM buffer then with ethanol to avoid direct interaction between ethanol and KCl that might cause precipitation in the column. The column was stored at 4 ℃ to be used later after washing with ethanol as a final step.

Tris, Trizma, MES, and TES Buffers were tested before use for this technique, and none showed growth inhibition on its own except for the MES, so it was not used. Controls were buffer, KCl, MB, 0.22 µm filtrate, 30 kD filtrate, 100 kD filtrate and flow-through, filtrate and buffer mixture before passing through the column. The fractions were stored

at 4 ℃ then the bioassay was done after 12 h by putting 1 ml of *N. salina* in each well of a 24 well plates as shown in Figure 2.4.

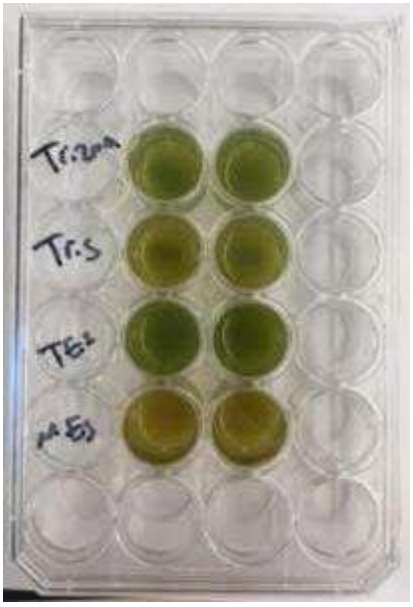

Figure 2.4. Algae growth inhibition bioassay showing the results of the effect of Tris, Trizma, TES, and MES buffers used in ion exchange cartridge purification procedure on *N. salina* growth after seven days.

2.9. PRESERVING GEL BANDS FROM SDS-PAGE FOR PROTEOMICS ANALYSIS

Gel bands were cut with a razor blade then each one was put in an Eppendorf tube, submerged with a solution made of acetonitrile (ACN) 40% and 60% of 50 mM tetraethylammonium bromide (TEAB) to destain the bands. The solution was replaced every 15 min for 3-4 times. The last time the whole solution was taken out of the tubes by pipettes and the gel bands in the tubes were dried in a “speed vac “vacuum

concentrator for 2 h. After that, the samples were frozen in a -80 ℃ freezer to be analyzed by mass spectrometry.

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CHAPTER 3

INVESTIGATING THE NATURE OF THE INHIBITORY MOLECULE PRODUCED BY *BACILLUS PUMILUS*

3.1. INTRODUCTION

In a recent research project, *Bacillus pumilus* was found to have an inhibitory effect on the growth of *Nannochloropsis salina*. The inhibition is caused by molecule(s) released by the bacterium (Fulbright et al., 2016), but the type of molecule was not determined.

Other research about algal-bacterial interactions in marine ecosystems showed that thirteen bacterial species have an algicidal impact on the macroalga *Ulva lactuca.* *Pseudoalteromonas tunicata* is the bacterium that had the strongest effect on this algal species. *P. tunicate* produces an extracellular component with specific activity toward algal spores that is heat-sensitive, polar, and between 3 and 10 kD in size. This biologically active compound was also found to prevent the germination of spores from the red alga *Polysiphonia sp*. and, given the widespread occurrence of *P. tunicata* in a range of marine habitats; this may suggest that it is effective against a variety of marine algae (Egan et al., 2000).

To understand the mechanism of inhibition that *B. pumilus* is using against *N. salina*, we need to know the nature of the biomolecule, whether it is a protein, lipid, nucleic acid, or

other class of molecule. Bacteria commonly secrete proteins to defend against a potential competitor (Tjalsma et al, 2004; Xavier et al., 2004; Skerratt et al, 2002). For this reason, our hypothesis was that the active molecule is a protein. For a protein to be functional, peptides should be folded in three- dimensional conformations; in some cases, several polypeptide chains assemble in a functional complex (Cooper, 2000). Highly acidic or basic conditions affect the folding structure of a protein. Hydrogen and hydroxyl ions interact with the bonds in a protein that maintains the secondary, tertiary, and quaternary structure, causing the protein to unfold and become non-functional. The strategy that in this work was to separate the fraction that has the inhibitory effect. This simplifies the bacterial filtrate and eliminates the proteins that are not related to the inhibitory effect. Also, size separation would be an additional evidence of the type of molecule since nucleic acids are smaller than other molecules.

3.2. RESULTS AND DISCUSSION

3.2.1. Investigating the nature of the inhibitory molecule(s)

To determine whether the molecule(s) was a protein, we added acid (50 ul and 100 ul of 1 M HCl) to 1 ml of *B. pumilus* filtrate from stationary phase. The experiment was repeated 3 times and the pH was below 4. After 15-20 minutes, the pH was adjusted to 7 by adding NaOH and the effect of the acid-treated filtrate was tested in the inhibition bioassay. After five days, there was no inhibition by either of the acid-treated filtrates, demonstrating that the filtrate lost its activity after incubation at low pH. This suggests

that HCl at these levels was enough to denature the inhibitory molecule(s) in the cell-free filtrate of *B. pumilus*. The *B. pumilus* filtrate was also subjected to high temperatures by placing a tube of filtrate in boiling water for 45 minutes or by boiling the filtrate in a glass flask for 30 minutes water. In both cases, the heat-treated filtrate lost the inhibitory effect towards *N. salina*, and the optical density (O.D.) at 750 nm increased from 0.09 for the untreated filtrate to 0.86. The inhibitory molecule is thus heat labile. Higher temperature and/or longer time are required to break down lipid and sugar molecules (table 3.1). We concluded that the inhibitory molecule is a protein.

Table 3.1. Effects of heat and acid on biomolecules showing that heat and acid actually might deactivate other biomolecules but the carbohydrates require more heat to be degraded. (Fagain, et al.,1997; Creighton et al., 1993; Gray et al., 2015; Moshe et al., 2013).

	High temperature (100 ℃)	Acid
Protein	Denatures at 41 ℃ for most proteins	Denatures protein
Lipid	Lipids like canola oil take 7.5 hours of high temperature to break it down while coconut oil takes like a day at 110 ℃.	Acid affects ester linkage
Carbohydrate	Generally unaffected at 100 ℃	Caramelizes sugar molecules
Nucleic acid	DNA in an aqueous solution degrades at 90 ℃	pH can affect nucleic acid stability

Figure 3.1. Algae growth inhibition bioassay results of heat and acid treated *B. pumilus* filtrate. The two wells that remained green are for the filtrate that lost the effect of inhibition after acid and heat treatment versus the well with 0.22 µm untreated filtrate that made algae lose the green color by inhibiting its growth. (ASW is a negative control).

3.2.2. Investigating the size of the inhibitory molecule(s)

To know more about the characteristics of the *B. pumilus* protein inhibitor molecule(s), Amicon ultracentrifugal filters with different molecule weight cutoff (MWCO) sizes were used. The manufacturer's instructions were followed to collect the flow-through filtrate, which was then tested in the algae inhibition bioassay. Filtrate from stationary-phase *B. pumilus* cultures were used since they had the highest level of inhibitory activity. This liquid was first passed through a 0.22 um filter, then through ultracentrifugal filters with 10 kD, 30 kD, or 100 kD MWCO. The filtrate from the 10 kD filter was not inhibitory, but the 30 kD filtrate had the inhibitory effect. Both filtrate and retentate from the 100 kD filter were tested, and both were inhibitory towards *N. salina,* although the retentate had less effect than the filtrate. That suggests that *B. pumilus* may produce more than one inhibitory molecule in the molar mass range that can be separated by the 30-100 kD by the ultracentrifugal filters. Inhibition in the fraction that is smaller than a 100 kD (filtrate of 100k and retentate of 30 kD filters) and the fraction that is bigger than 100 kD

(retentate of 100 kD filter) so that why we think it might be either more than one molecule of different sizes or a molecule that is very close in size to the membrane pores of a 100 kD filters that it is sometimes passed through the filters and sometime was retained above the filter.

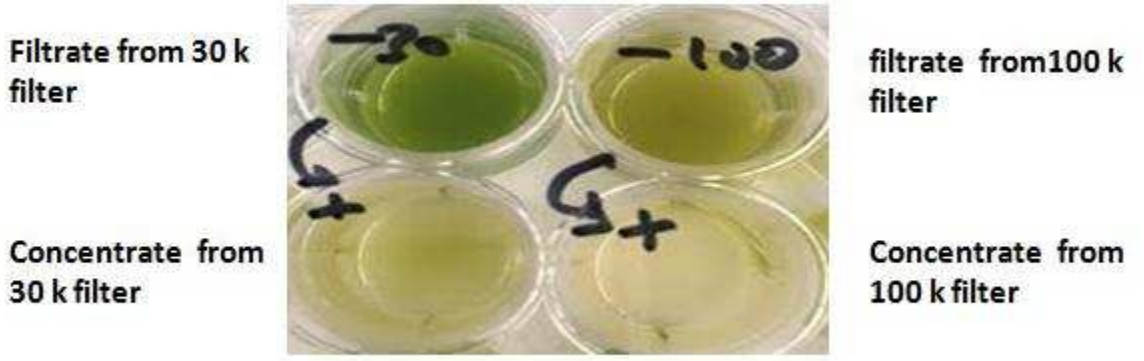

Figure 3.2: Algae growth inhibition bioassay results for the test of different *B. pumilus* filtrate size fractions. The sample of the filtrate from the 100 kD filter resulted in a yellow color due to chlorophyll loss, while the 30 kD filtrate is green. The bottom wells contain the concentrates from these filters. Both have a white to yellow color indicating a high growth inhibition.

3.2.3. Ion-exchange fractionation of the bacterial inhibitory filtrate.

Bio – Scale Mini ion exchange cartridges (Bio-Rad) were used in this experiment to obtain a fraction of the filtrate that has the inhibitory effect. Reducing the complexity of the sample will make the proteomics analysis easier. Since size separation was already done, charge separation was chosen to further narrow the range of the candidate molecule(s). The Bio-Scale Mini cartridges are used for chromatographic separation.

They are designed to be fitted to Luer-Lok syringe. These cartridges contain hydrophilic beads with different ion-exchange properties. Three types of these cartridges were used: UNO Q strong anion, UNO S strong cation and Macro-prep high S. These three were used several times in this study, with similar results except for the last time the UNO Q cartridge was used, (described later in this section). A protein solution is passed through one of these columns, the proteins bind according to their charge and then proteins are eluted by a salt gradient.

Each time these cartridges were used, the inhibitory molecule was found in the eluted fractions, indicating that it was bound to the resin in the cartridge (a hydrophilic molecule) then released by the salt gradient.

Each of the 5 fractions was tested separately in the bioassay. Initially, the experiment was done using the UNO Q strong anion, UNO S strong, and Macro-prep high S without passing the first and second fractions (flow-through and buffer wash) through 30 kD filters (no sample concentration). The bioassay results showed that only the eluted fractions (released by different KCl concentrations) had the inhibitory effect. When the experiment was repeated using the UNO Q cartridge (previously used) but with all fractions passed through the Amicon filters 30 kD, all fractions were found to have the inhibitory effect except for the second fraction (the buffer wash). This indicates that the separation through these columns was not as successful as it was thought based on the first runs. The first and second fractions were not concentrated in the first runs, and this is why we did not see the inhibitory effect. There are two possible explanations: 1) The

first few times the cartridges were used it was new and the separation worked, but after three uses, we couldn't get the same results because the cartridge needed to be cleaned with a strong acid or base; or the first and second fractions (flow-through and buffer wash) actually had the inhibitory effect (at least the flow-through) but because the sample were not concentrated the first two times the inhibition wasn't observed. The first hypothesis is the more probable than this one because the flow-through should have the effect even when it's not concentrated since the filtrate has the effect without being concentrated although the flow-through is diluted with the 5% buffer addition that is not enough dilution to make the bacterial filtrate lose the effect.

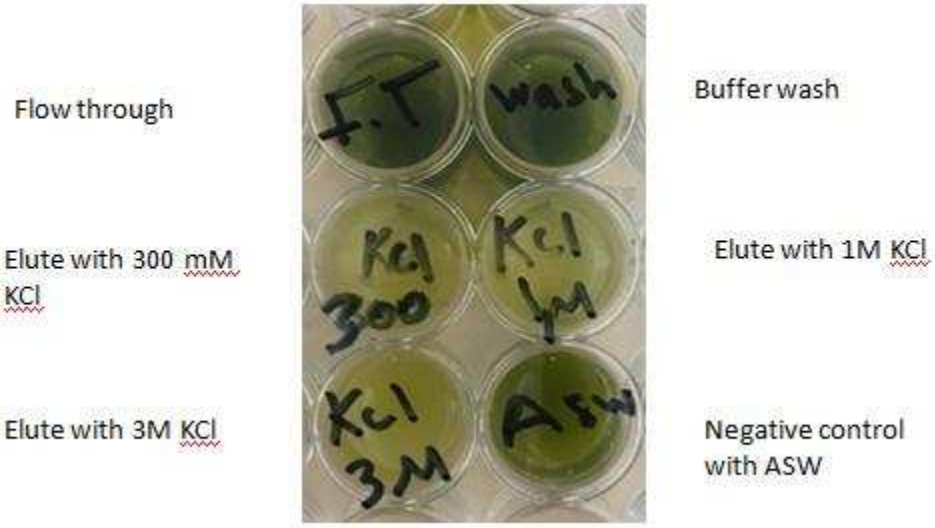

Figure 3.3. Algae growth inhibition bioassay results for ion exchange fractionation cartridges after 7 days. Results are for the first run of the sample through the UNO Q cartridges when showing that the flow-through has no inhibitory effect as well as the wash that never had the effect of inhibition. The other fractions that contained KCl had the inhibitory effect as seen by the yellow color due to chlorophyll loss (KCl fractions were desalted then added to the bioassay).

3.2.4. Using SDS-PAGE to visualize protein bands.

SDS-PAGE was performed twice to evaluate the complexity of the protein fractions. In the first analysis, the lanes were used as follows:

- Lane 0:Bio-Rad Kaleidoscope re-stained SDS-PAGE standards, broad range
- Lane 1: Fraction 1 from UNO S cartridge
- Lane 2: Fraction 1 from UNO Q cartridge
- Lane 3: 0.22 µm filtrate from 32 h culture
- Lane 4: 0.22 µm filtrate from 12 h culture
- Lane 5: concentrate 100 kD 32 h culture
- Lane 6: concentrate 30 kD 32 h culture
- Lane 7: filtrate from 100 kD Amicon filters 32 h culture
- Lane 8: filtrate from 30 kD Amicon filters 32 h culture
- Lane 9: empty

The 5th and 6th lane that had 100 and 30 kD concentrate had two dark bands. That means there was a high concentration of that protein(s) in these two size fractions that had the effect. It does not really mean that this is the right one but it might be a good candidate to start with proteomic analysis.

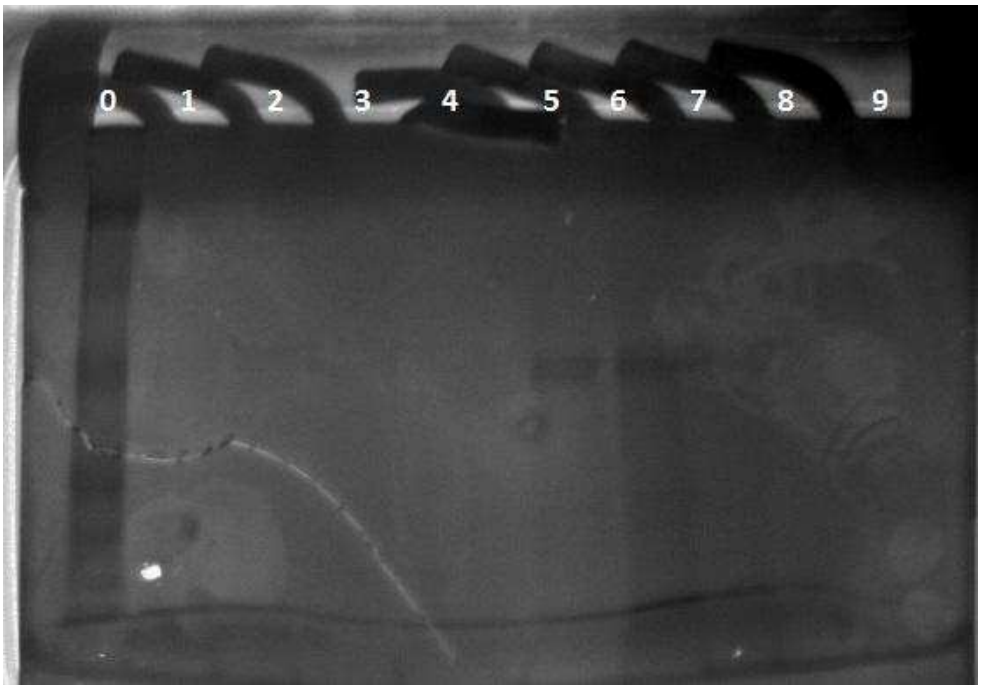

Figure 3.4. Algae growth inhibition bioassay results. Lane 0 contained the protein standard, lane 1: Fraction 1 from UNO S cartridge, 2: Fraction 1 from UNO Q cartridge, 3: Filtrate from 32h grown culture, 4: Filtrate from 12h grown culture, 5: Concentrate +100 kD, 6: Concentrate +30 kD,7: filtrate from -100 kD Amicon filters, 8: filtrate from -30 kD Amicon filters.
In the second SDS-PAGE analysis, the lane assignments (all taken from 32 h cultures):

Lane 0: Standard

Lane 1: 0.22 µm Filtrate

Lane 2: 30 kD concentrate

Lane 3: 100 kD concentrate

Lane 4: UNO Q fraction 1

Lane5: UNO Q fraction 2

Lane 6: UNO Q fraction 3

Lane 7: UNO Q fraction 4

Lane 8: UNO Q fraction 5

In this gel, there were similar bands that appeared in the previous gel, but since all the samples that were run this time were concentrated with 30 kD, the bands were more visible. In Lane 1 (0.22 µm filtrate) there was also a dark band similar to the one found in the 30 and 100 kD concentrates. About 25 bands were cut, chemically treated (as described in Chapter 2) to preserve the proteins, and frozen at -80 ℃ for future proteomics analysis.

Figure 3.5. An SDS-PAGE gel of *B. pumilus* culture filtrate fractions. Lanes 0: the standard, lane 1: 0.22 filtrate, lane 2: +30 kD concentrate, lane 3:+100 kD concentrate, and lanes 4–8 UNO Q fractions 1–5.

3.3. CONCLUSIONS

The conclusion from these experiments is that the inhibitory molecule is probably not a nucleic acid based on the size range determined with the ultracentrifugal filter analysis (micro RNAs are smaller). Some known inhibitory molecules produced by bacterial species are proteins, but there are no reports on a lipid or carbohydrate molecule that has any effect on algae. Some lipids are usually known as endotoxins, but since *B. pumilus* is gram positive bacterium, an endotoxin is not a possibility. In Chapter 4, we show how sonication and breaking the cell open to release all the cell components had no effect on the algal growth while the cell-free filtrate did. Having smaller fractions based on the charge was also possible through the ion exchange cartridges. These will help focus the proteomic analysis.

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CHAPTER 4

EVALUATION OF FACTORS AND CONDITIONS THAT CONTROL THE INHIBITORY EFFECT OF *B. PUMILUS* ON *N. SALINA*

4.1. INTRODUCTION

Bacterial species are abundant in algal cultivation systems (Fulbright, 2015). In one example, two strains of *B. pumilus* were found to have an inhibitory effect on the growth of *N. salina* by secreting one or more molecules (Fulbright et al., 2016). In that study, it was determined that the molecule was produced by the bacterium regardless of the presence of *N. salina*, because when cell-free filtrate of pure cultures of *B. pumilus* was added to *N. salina*, the same inhibitory effect was observed.

The goal of the present work was to determine the growth conditions under which *B. pumilus* produces the inhibitory molecule(s). Experiments were also performed to investigate whether the inhibitory molecule is present in the bacterial culture at all batch growth phases or if it depends on the cell concentration of the culture. A third goal was to determine whether *N. salina* had different degrees of susceptibility to the *B. pumilus* inhibitory molecules at different stages of batch growth.

4.2. RESULTS AND DISCUSSION

4.2.1. The influence of bacterial growth phase on the inhibitory effects

Fulbright et al. (Fulbright et al., 2016) determined that *B. pumilus* inhibited the growth of *N. salina* and *N. gaditana,* both as whole bacterial cells and as cell-free filtrate from *B. pumilus* collected from late stationary phase cultures.

The effect of *B. pumilus* filtrate from different phases was tested on *N. salina*. The cell-free filtrate had a significant inhibitory effect on *N. salina* growth. It decreased the OD_{750} from 0.9 at Day 0 (start of exposure) to 0.1 at Day 6. We were also able to see the effect visually by the color change from green to yellow (Figure 4.1). The longer time required for the inhibition effect of the filtrate versus the whole cells could be caused by a concentration-dependent. Thus, the inhibitory effect increases when the cells are alive and dividing in the culture or if not dividing then constantly secreting the inhibitor. That could explain why we see the effect of the filtrate after several days while we see the effect sooner when the cells of *B. pumilus* are present.

To test the hypothesis that the molecule is present at 12 hours of growth but is not concentrated enough to show the effect, *B. pumilus* was grown for 12 hours (log phase), culture liquid filtered through a 0.22 µm filter (“0.22 µm filtrate”), and that filtrate was concentrated using 30 kD Amicon Ultra centrifugal filters. The concentrate had inhibitory activity when tested with the bioassay; the *N. salina* culture in the wells changed from green to yellow by Day 7 (Figure 4.1). That means the inhibitory effect is

present regardless of the phase or the concentration of the cells while it depends on the concentration of the molecule. The *B. pumilus* cell makes this molecule at all phases of the bacterial culture.

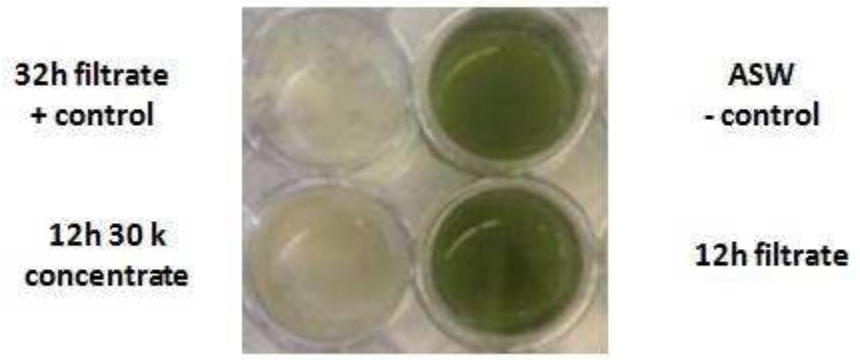


Figure 4.1. Algae growth inhibition bioassay results to test concentrated early culture filtrate. The image shows algae cultures exposed to negative control with only ASW, positive control with 0.22 µm filtrate from 32 hours bacterial culture, 12-h, 0.22 µm filtrate, and the addition of 12-h concentrate from 30 kD Amicon filters.

While Fulbright, 2015 mentioned that there is an active molecule that is released to the culture from *B. pumilus* that is causing the effect, it was unknown whether the molecule is secreted or just released from inside the bacterial cells when some cells lyse in the late stationary phase. To determine whether the molecule is secreted or released when the cell lyses, *N. salina* cells were grown with six different additions (1 ml each) and the results evaluated after 5 days (Figure 4.2):

1. *B. pumilus* whole culture (32 hours) as a positive control. Result: yellow culture with $OD_{750} = 0.54$
2. ASW as a negative control. Result: green culture with $OD_{750} = 0.95$

3. *B. pumilus* (32 hours) 0.22 µm filtrate as a positive control. Result: yellow culture with OD_{750}= 0.48
4. *B. pumilus* (32 hours) culture, ASW-washed, sonicated for 5 min. Result: green culture with OD_{750} = 0.85
5. *B. pumilus* (32 hours) culture, twice-ASW-washed, sonicated for 5 min. Result: green culture with OD_{750} = 0.87
6. *B. pumilus* (32 hours) culture, unwashed, sonicated for 5 min. Result: yellow culture with OD_{750} = 0.54

The results show that the washed, lysed cell suspensions, which contained all of the cell components, did not inhibit the growth of *N. salina*, suggesting that the molecule is not inside the cell but outside as a part of the bacterial secretome. The sonicated cell suspension without washing still exerted the inhibitory effect because it contained whatever was in the culture for 32 h, including the secretome. This test was done in duplicates.

Figure 4.2. Selected results from algae growth inhibition bioassay with duplicate tests after 7 days. Positive controls were whole *B. pumilus* cells and filtrate. Test samples included sonicated MB washed culture and sonicated whole culture of *B. pumilus*.

4.2.2. Investigating the inhibitory effect of *B. pumilus* when MB is not present in the bioassay

The purpose of this experiment was to determine whether *B. pumilus* has an inhibitory effect when MB is not present in the bioassay. The *B. pumilus* was grown for 32 hours in MB as usual under 200 rpm agitation and 30 ℃, then 1 ml of was taken and directly added to 1 ml of *N. salina* in the bioassay 24 well plate. Another 1 ml was taken from the same bacterial culture but it was washed with ASW and added to the algae wells. The results (Figure 4.3) showed that the bacterial inoculum washed of the MB was less inhibitory to *N. salina* growth than the one with MB. That suggests the fact that MB is important for production or secretion of the inhibitory molecule.

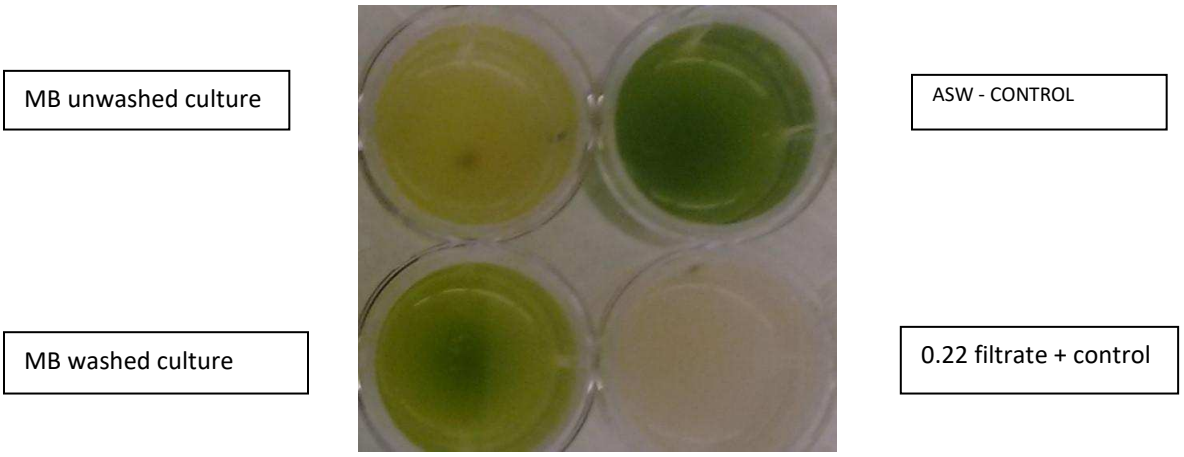


Figure 4.3. Growth inhibition bioassay results from the test of MB effects after 7 days. Wells shown are for *N. salina* grown with bacterial culture bacterial culture washed of MB, positive controls with 32 hours bacterial filtrate, and negative control with ASW medium.

4.2.3. Susceptibility of *N. salina* at different growth stages to the inhibitory effect of *B. pumilus*

To evaluate the relative susceptibility of *N. salina* at different batch growth stages to the inhibitory molecule(s) produced by *B. pumilus*, *N. salina* cultures grown for 2, 3, 4, 5, and 6 weeks were placed into wells in a 24-well plate and inoculated with the filtrate of a 32-h *B. pumilus* culture. After 3-4 days, *N. salina* from older (4-6 week) cultures was still green while the younger cultures were yellow (Figure 4.4) and later died. After 7 days, the older cultures had an OD_{750} of 0.8, while the younger cultures had an OD_{750} of 0.1. About 8-9 days of exposure were required before the effect was visible. Thus, while the filtrate affects *N. salina* at all stages of the growth, the effect occurred sooner in the younger cultures. The same experiment was done using 32 h *B. pumilus* whole cell culture and we had the same results as the filtrate except that the chlorophyll loss was faster as shown in Figure 4.4.

This phenomenon gives us an idea about the mechanism of inhibition of *B. pumilus* towards *N. salina*. The effect of inhibition might be happening by cell wall lysis, based on the time it takes to inhibit the cells in the older culture compared to the younger ones. That would make previous research that showed some algicidal bacterial species were to cause cell lysis of another species of *Nannochloropsis (N. oculata*) (Wang et al, 2014). Since both *N. salina* and *N. oculata* have been proven to be inhibited by *B. pumilus* (Fulbright, 2015), the two species might be inhibited in a similar mechanism.

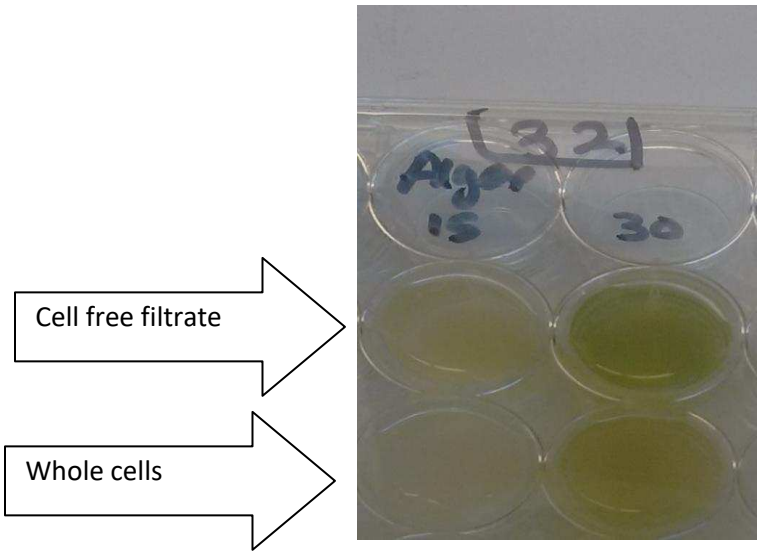


Figure 4.4. Growth inhibition bioassay results for testing the susceptibility of *N. salina* at different growth stages to the inhibitory effect of *B. pumilus*. The picture shows *N. salina* grown for 15 days and another *N. salina* culture grown for 30 days both after 4 days of co-culturing with either *B. pumilus* filtrate or whole cell from 32 h culture.

4.2.4. Essential nutrients for the growth of *B. pumilus* and the production of the inhibitory effect

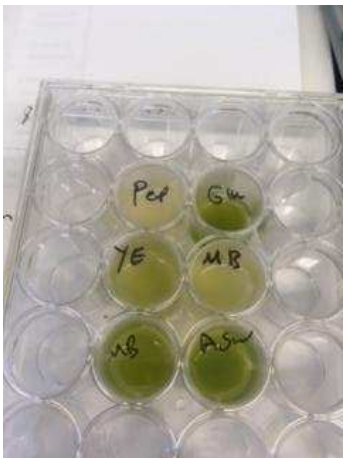
Fulbright, 2015 observed that *B. pumilus* inhibition towards *N. salina* occurred only when MB was present in the co-culture, and adding ASW to *N. salina* cultures did not result in the inhibitory effect of *B. pumilus* towards *N. salina*. Fulbright suggested that the major differences between ASW and MB were the degree of support of bacterial growth. To further investigate this, we need to understand what components of MB are the most essential for growth of *B. pumilus*. Comparison of the compositions of ASW and MB (Chapter 2) shows that there are three primary components of MB that are different: glucose, peptone and yeast extract. *B. pumilus* was separately cultured in each one of these components (dissolved in water) at the same concentration as in the MB recipe.

These MB fractions were inoculated with *B. pumilus* in 1-2 ml cultures and incubated them at 30 ℃ with 200 rpm shaking. After 24 h, bacterial growth at different levels was compared qualitatively by noticing the turbidity change in the culture tubes. Peptone supported growth better than yeast extract, while ASW with glucose did not support growth of *B. pumilus*.

We also tested the inhibitory effect of *B. pumilus* grown in these different media (Figure 4.5). Inhibition by *B. pumilus* grown in peptone was similar to the effect seen when the bacteria were grown in MB. Yeast extract grown *B. pumilus* filtrate also had an inhibitory effect, while as expected the filtrate from the bacteria cultured only in glucose did not

have the effect since cells had not grown. Also, a 100 µl portion of each test tube was taken and grown on an agar MB plate; there was no growth from or the plate inoculated from the glucose-only culture, while there were hundreds of colonies for the other medium fractions.

A



B

Figure 4.5. Growth inhibition bioassay results from the test of the essential nutrients for the growth of *B. pumilus* and the production of the inhibitory effect after 7 days. (A) Negative controls of ASW and MB at the bottom row, filtrate of bacteria grown in yeast extract to the left in the middle row and filtrate of bacteria grown on MB to the right, peptone-grown bacterial filtrate to the left and glucose-grown bacterial filtrate to the right of the top row. (B) *B. pumilus* cultures grown in MB, peptone, yeast extract and glucose are shown from left to right.

4.2.5. Growth of *B. pumilus* on soluble algal products

Algae can produce soluble products that can be used as a carbon source for bacterial growth (Yin et al., 2014). To determine whether *B. pumilus* is capable of growth on the soluble algal materials (SAPs) released by *N. salina* grown in ASW, *B. pumilus* was grown on *N. salina* filtrate (0.22 µm) taken from 2-3 week cultures and from 5 week cultures. Each algae filtrate was inoculated with *B. pumilus* taken from culture grown for 24 hours and was incubated under the same conditions used for standard *B. pumilus* cultures in MB. After 24 hours, there was significant growth of *B. pumilus* in the 5-week *N. salina* filtrate, but less growth in the filtrate collected from the 2-3 week culture. This demonstrates that *B. pumilus* can grow on the SAPs produced by *N. salina* in ASW, and suggests that *B. pumilus* is able to grow in *N. salina* cultures without the presence of

any bacterial medium (Figure 4.6). That helps us understand more about the conditions that are favorable for the growth of inhibitory bacterial species, and to include this in the design and operation of these algae growth systems.

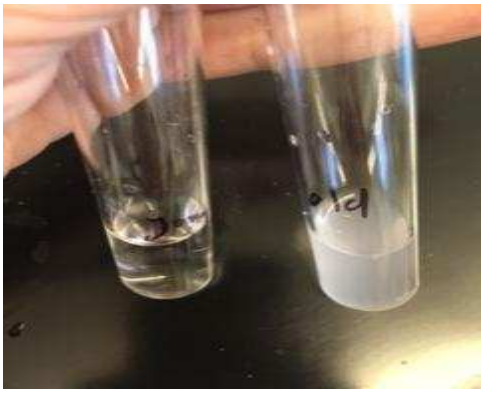


Figure 4.6. Results from test of the growth of *B. pumilus* on soluble algal products. The left side of the picture shows *B. pumilus* grown for 24 h on the filtrate from a 3 week grown *N. salina* culture, while the right side of the picture shows a *B. pumilus* culture grown on an algal filtrate from a 5 week grown *N. salina* culture.

4.2.6. The effect of ASW with different salt concentration on the inhibitory characteristics of *B. pumilus* toward *N. salina*

The goal of this experiment was to determine the range of salinity that is favorable for *N. salina* but not for *B. pumilus*, to minimize the negative effects of contamination by this bacterium. For this purpose, ASW was prepared with four different concentrations of NaCl, 15 g/L (standard ASW concentration), 30 g/L, 40 g/L, and 50 g/L.

Five ml cultures of *N. salina* at log phase were inoculated with *B. pumilus* in small flasks, then grown in a shaker at 200 rpm and 30 ℃. After 30 h, 1 ml of filtrate was

taken from each flask to inoculate 1 ml of *N. salina* in the standard inhibition bioassay. Unfiltered samples of the four *B. pumilus* and *N. salina* co-cultures were tested in the same way. A 100-µl sample was removed from each flask and serially diluted with PBS buffer 1X up to 10^{-6} to determine the bacterial cell concentration.

No inhibition of *N. salina* growth was observed for the filtrate taken from all of these different concentrations. That might be due to the lack of enough nutrients. In the previous experiment it shows bacteria can actually grow if there are enough SAPs after 4-6 weeks of algal growth. This experiment should have been repeated with older bacterial culture or with addition of MB to the bioassay so that the bacteria can actually grow.

Figure 4.7. Growth inhibition bioassay results at Day 7 from the test of the effect of ASW with different salt concentration on the inhibitory characteristics of *B. pumilus* toward *N. salina*. Wells with *N. salina* were inoculated with ASW containing different salt concentration (15, 30, 40 and 50 g/l) with *N. salina* and *B. pumilus* grown together to see if there is a specific salt concentration that decreases the inhibitory effect from happening. No growth of *B. pumilus* was observed, which indicated that there were not enough nutrients in the bioassay to allow the bacteria to grow.

4.2.7. Effects of the filtrate of *N. salina* and *B. pumilus* co-culture on *N. salina* cultures

The goal of this experiment was to show whether *B. pumilus* is more inhibitory when grown in co-cultures with *N. salina* than when it is grown alone. This experiment was done twice, once when the algae and bacteria were both grown in only ASW and the other when they are grown in ASW with the presence of MB. Then, the cell-free filtrate was collected. The filtrate from the culture that lacked MB was not inhibitory (Figure 4.8), suggesting that the bacterial species needs MB to grow and have the inhibitory effect.

Filtrate taken from the co-culture that had MB showed inhibition. However, the inhibition of the bacterial cultures when grown separately on MB then taken to inoculate *N. salina* had more effect than this case. It is possible that the ASW diluted the secretome of the *B. pumilus*, reducing the inhibitory effect. When bacteria were grown on MB, their filtrate has a stronger effect than when there is ASW with the MB.

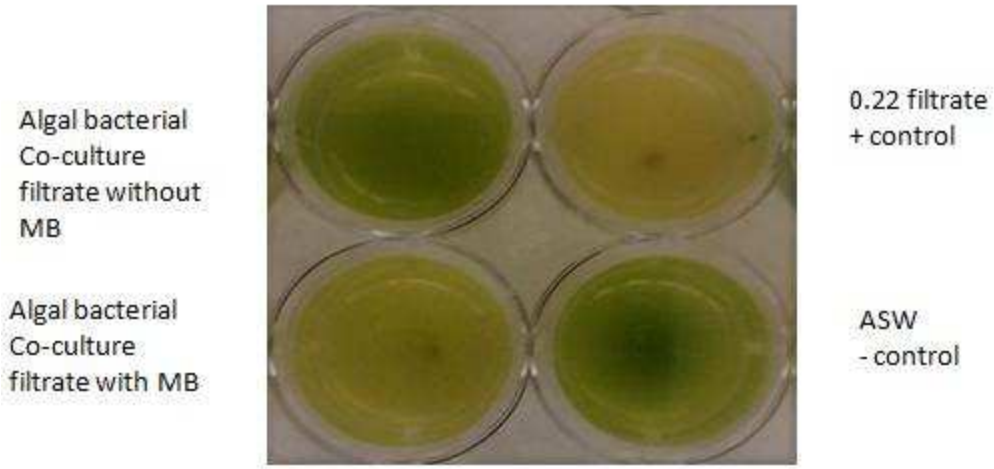


Figure 4.8. Growth inhibition bioassay results from the test of the effects of the filtrate of *N. salina* and *B. pumilus* co-culture on *N. salina* cultures. *N. salina* cultures are shown after 7 days of exposure to the filtrate of algal bacterial co-culture without MB, filtrate of co-culture with ASW and MB in the co-culture, positive control filtrate 0.22 µm of *B. pumilus* in MB, and the negative control (ASW).

4.3 CONCLUSIONS

B. pumilus produced the inhibitory molecule(s) regardless of the bacterial phase or the presence of other species in the ecosystem. The inhibitory molecule (or molecules) was produced in all batch growth phases and became concentrated in the late stationary phase. This bacterial species needed a carbon and a nitrogen source in the algal secretome produced by *N. salina*, similar to the ones in the peptone and yeast extract.

We also observed a phenomenon that the algal cells of *N. salina* become more resistant to the inhibition in older cultures. One reason for this is could be that the cell wall thickness has increased, slowing the entry of inhibitory molecules into the cell. This could be investigated in the future experiments.

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

The process of growing algae for industrial purposes has a promising future, but it will need significant scientific and technical effort to make the cultivation process economically efficient. One of the major problems facing algal growth is contamination. This thesis addressed one type of contamination, in which an algal culture is populated by inhibitory bacterial species. Bacterial inhibitors are abundant in the environment and therefore a common challenge is reducing their presence in algae production systems. The first step, addressed by the work in this thesis, is to investigate and understand the mechanism of inhibition. The inhibition of the alga *Nannochloropsis salina* by the bacterium *Bacillus subtilis* was the specific system investigated.

The experiments reported in Chapter 3 investigated the nature of the inhibitory molecules that *B. pumilus* produces. One focus was on the determination of whether these molecules were proteins, lipids, carbohydrates, or nucleic acids. Based on the results obtained in this work, it is likely that the molecule is a protein. The molecule lost its inhibitor activity at very low pH (acid labile) and after exposure to boiling water (heat labile). In addition the, filtrate was fractionated by size and charges to and eliminate the fractions that do not contain the inhibitory molecule. This will make the future experiments easier to be performed with less effort and shorter period of time. At this point, proteomic analysis followed by more bioassays is the best way to confirm which

molecule is causing the algal growth inhibition. We determined the size range of the inhibitory molecule so that when we have the proteomics analysis, this size fraction that was found to have the inhibitory effect (MWCO between 30-100 kD) will be the fraction to analyze instead of analyzing the whole filtrate. The same strategy for simplifying the filtrate by size was used when the filtrate was passed through the ion exchange resins to get the fraction that has the effect to be able to be separated more based on charge before doing the proteomic analysis. Also, SDS-PAGE was used to visualize the size and charge fraction to confirm based on the molecular weight of the band compared to the standard that the Amicon filters are isolating that size range that they should. In the future, it would be a good idea to use a native gel for these samples so that the proteins are not denatured. Then, each band could be isolated and its effect on *N. salina* tested in a bioassay. Perhaps a native gel could be made with ASW medium in it so that after the bands are visualized and isolated, algae could be grown directly over the gel to and used as an inhibition bioassay. After we know what the protein is, we could express that protein in an organism, purify the protein, and see its effect on our algal species in a bioassay.

It would be a very useful experiment to use an immobilized protease enzyme to deactivate the proteins in the bacterial filtrate then test the effect of the filtrate after protease treatment to see if it still has the inhibitory effect towards the algal species. The molecule would be a protein if the inhibition was lost after adding the protease. Similarly, using RNAase would show whether or not the molecule is an RNA or not.

The research in Chapter 4 focused on different factors in this relationship between *N. salina* and *B. subtilis* that support or prevent the inhibition from happening. It shows that the inhibitory effect was stronger when the filtrate was taken from the late stationary phase. *B. pumilus* secretes the molecules independent of the presence of the affected algal species. Also, as algae cultures are older, their resistance to the inhibitory molecule increases. An interesting finding that will be beneficial for the algal cultivation industry is that we demonstrated that these bacterial species can grow on the culture filtrate of *N. salina*. To minimize algae growth in industrial algae cultures, the medium should be regularly renewed to prevent a favorable bacterial growth environment.

In future work, it would be interesting to evaluate culture parameters like pH and nitrogen (ammonia or nitrate) as *B. pumilus* and *N. salina* are interacting in the bioassay. There may be a pH change or nitrogen depletion that would will have an effect on the resistance of *N. salina* to the inhibition.

Another suggestion for future research, when the inhibitory molecule is known, is to determine the required concentration to produce the effect. It is also important to use microscopy to observe the morphological changes in *N. salina* cells along the time of bioassay (5-7 days) to see what changes are exactly happening, to understand more about how the inhibition occurs, and to learn why the inhibition happened after 5-7 days and not on the first day. As researchers identify the molecule and the surrounding factors that support or prevent the inhibitory effect, it will be easier to think of strategies to prevent this phenomenon from happening, or to eliminate the inhibition by genetically

engineering our elite algal species to resist them. It would be also interesting for future algal researches to search in the algal genome and see if there are any algal cells with immune receptors like the ones in plants. If those exist in an algal species, it might be possible to express that immune gene in an industrially important algal species to make it more resistant to bacterial inhibitors.