RSGC
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The Young Researcher

2020 Volume 4 | Issue 1

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Esha Patel

Recommended Citation
Patel, E. (2020). Chemotaxis of C. reinhardtii Towards AHLs. *The Young Researcher,* 4 (1), 84-93. Retrieved from http://www.theyoungresearcher.com/papers/patel.pdf

ISSN: 2560-9815 (Print) 2560-9823 (Online) Journal homepage: http://www.theyoungresearcher.com

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Chemotaxis of *Chlamydomonas reinhardtii* towards AHLs

Esha Patel

Quorum sensing is a widespread phenomenon in various environments, allowing organisms to display symbiosis, pathogenicity, etc. This mechanism has recently been of great interest with the newfound discovery that quorum sensing not only occurs in prokaryotic organisms like bacteria, but also eukaryotes like the green algae *Chlamydomonas reinhardtii*. With this novel information, the question remains regarding how these eukaryotes and prokaryotes interact amongst one another, potentially enhancing human health, biotechnology, pharmaceuticals, etc. Through a chemotaxis assay using liquid algae cultures and capillary tubing along with pixel density analysis using the image processing program *ImageJ*, the movement of *Chlamydomonas reinhardtii* was tested in response to molecules released by bacteria during quorum sensing called AHLs. Through various assays of AHLs, sucrose, and deionized water, the results were shown to be statistically insignificant. However, the setup of the experiment showed great potential and provides a promising future for the direction of this research.

Keywords: quorum sensing, chemotaxis, *Chlamydomonas reinhardtii*, AHLs, pixel density

Introduction

Many Gram-negative bacteria regulate gene expression through the phenomenon known as quorum sensing (QS) (Palmer et al., 2018). QS is a mechanism that produces change based on the population density of the organism, in which genes are activated that in turn cause changes in phenotype, or the observable characteristics of an organism (2018). QS is responsible for behaviors like bioluminescence and virulence, and is even used in finding nesting sites in some insects (2018). This phenomenon is regulated by autoinducer (signaling) molecules known as *N*-acyl L-homoserine lactones, or AHLs (2018). Studying QS pathways is vital to developing a better understanding of influential bacterial behaviors, such as their ability to evade immune response by activating molecules that enhance their effectiveness called virulence factors (2018).

In the past, it was thought that QS was only exhibited by bacteria, which are prokaryotic, meaning they lack a cell nucleus and many organelles (Teplitski et al., 2004). However, it has recently been discovered that QS is also seen in some eukaryotes, or organisms with membrane-bound nuclei and organelles, through the secretion of substances that mimic AHLs (2004). *Chlamydomonas reinhardtii*, a unicellular freshwater green algae, is currently of great interest in the scientific community for its newfound QS behaviors (2004). It is being widely studied due to its well-known genetic sequence in addition to how easy it is to obtain and manipulate as a model organism in the laboratory (Arrieta et al., 2017). *C. reinhardtii* is also known for its motility, using organelles called flagella to swim in a path known as a helical trajectory (2017). The motility of the organism has also been used with various molecules to study its chemotaxis, which refers to the movement of an organism towards or away

from a stimulus (Choi et al., 2016).

Scientists have yet to establish a clear connection between bacteria and algae in regards to quorum sensing. Finding the relationship between these organisms can create a link between QS in prokaryotes and eukaryotes while also shedding light on the ecological relevance of the organisms' behaviors by understanding its interactions with organisms found in various ecosystems. These connections can potentially create a model system for understanding the QS behaviors of other eukaryotes, which could be integral to biotechnology, human health, etc. These effects may be vital in the development of new pharmaceuticals as well as in the improvement of current techniques in research and clinical medicine. Studies have been conducted that display the phenomenon in both organisms individually, but their interactions through AHLs and motility have not been thoroughly explored, thus presenting a gap in the research. Therefore, the question remains: Does *Chlamydomonas reinhardtii* display movement towards bacterial AHLs within an environment lacking key nutrients?

Literature Review

Quorum Sensing/AHLs

Quorum sensing, a density-dependent mechanism that affects behavior, has long been studied in bacteria, but recently the focus has shifted towards QS in eukaryotic organisms. Researchers have found that AHLs, the molecules released to signal quorum sensing, have an effect on various eukaryotic organisms. Dr. Andrew Palmer (2018) tested the effects of AHLs on the plant *Arabidopsis thaliana* in order to identify the phenotypic effects of these molecules. Using this organism allowed him to find that AHLs primarily affect auxin levels, a hormone involved in all plant development (2018). Thus, this finding can be translated to other plants as well as other eukaryotic organisms.

Conversely, research has also found that eukaryotes themselves can affect bacteria and their ability and efficiency to display quorum sensing behavior. Teplitski et al. (2004) investigated the production of AHL mimic substances by the algae *Chlamydomonas reinhardtii*, using agar plate spreads with suspensions of quorum sensing reporter strains to detect luminescence responses. After learning that luminescence was inhibited by the algae, the authors further tested the substances on proteins in bacteria and found that many of the proteins were altered (2004). This provides evidence of eukaryotes affecting bacteria and their signaling molecules, showing the two-way relationship between eukaryotes and the prokaryotic bacteria in terms of quorum sensing. This statement provides a foundation for this study's inquiry. Further, using mass spectral analysis, Teplitski also found that some compounds in *C. reinhardtii* are very similar to bacterial AHLs structurally, providing evidence of quorum sensing in the algae (2004). Working off of this finding, scientists at the University of California, Davis have found some vitamins, namely riboflavin and its derivative lumichrome, in *Chlamydomonas* that mimic AHL activity and are capable of activating quorum sensing receptors in the common bacteria *Pseudomonas aeruginosa* (Rajamani et al., 2013). This provides concrete evidence that quorum sensing is exhibited by prokaryotes, more specifically in these studies, the green algae *Chlamydomonas reinhardtii*. Thus, firm evidence proves QS in *C. reinhardtii*, setting a foundation for this study.

In addition, researchers from the University of Haifa have also discovered evidence of quorum sensing active in marine organisms in the form of inhibitors that use this mechanism to defend against virulence (Saurav et al., 2017). Through the use of biosensors that react to quorum sensing signals, the authors identified various quorum sensing inhibitor (QSI) molecules while also creating the potential for using these marine sources as a form of treatment, since many display anticancer activity (2017). In fact, these QSIs have the potential to be very useful in many types of drugs and antimicrobial therapy, and many patents have already been published related to their use (Jiang & Li, 2013). Thus, this evidence gives further support on eukaryotic QS and how it has already begun to impact pharmaceuticals.

Therefore, sufficient research has been done regarding the evidence of quorum sensing in various eukaryotic organisms from many different ecosystems as well as their effect on bacterial AHLs and vice-versa. The studies have also highlighted the potential benefits of these relationships and quorum sensing activity, from creating model systems for further studies to potential human treatment.

Structure of C. *reinhardtii*

Chlamydomonas reinhardtii is a freshwater green algae and its physiology is well known (Sasso et al., 2018). In 1833, Christian Gottfried Ehrenberg described the genus *Chlamydomonas*, and in 1888, Pierre Augustin Dangeard described the species *C. reinhardtii* (2018). In the early 1950s, *C. reinhardtii* was developed as a model organism as the first mutants were generated (2018).

The motility structures of *C. reinhardtii* are very well-known. Its flagella, an outside organelle used for movement, is composed of centrioles and basal bodies that help distribute the organism's organelles, which are structures within cells that perform specific functions (Aliouche & Greenwood, 2019). The arrangement in *C. reinhardtii* is called "9+2", as there are nine doublets and two central microtubules, and this overall structure helps produce the motions allowing flagella to propel the organism forward (2019). Aliouche further states that *C. reinhardtii* has four basal bodies, two of which are used in flagella. The structures of these two vary: one is centrin-based fibers, and the other is microtubular roots (2019). The movement of *C. reinhardtii* flagella is an asymmetric movement of the two (*cis* and *trans*) flagella (2019). As is seen in this detailed description, the well-understood anatomy of *C. reinhardtii* and its motility structures makes it an ideal organism for studying in a laboratory, especially when testing movement.

In addition to its motility structures, *C. reinhardtii* is also very useful for genetic investigations due to its well-understood organelles, like the nucleus, mitochondria, and chloroplasts (Haire, 2017). The algae is also photosynthetic, which indicates plant-like behavior, and many strains are also phototactic, meaning they display movement toward light (2017). Additionally, the haploid nature of the organism, which refers to its growth and reproductive style, allows creating mutants to become efficient, as the mutant phenotypes are immediately expressed (Sasso et al., 2018). Thus, the wide variety of strains and the ease with which it can be cultured, or grown, make it an attractive option for many research studies.

Chemotaxis

Chemotaxis is the movement of an organism in response to a chemical stimulus, where positive chemotaxis refers to movement towards the stimulus and negative chemotaxis refers to movement away (Choi et al., 2016). This behavior is used by many organisms to find nutrients as well as avoid toxins and predators (though only in multicellular organisms), and is fundamental to processes including biofilm formation, virulence, and carbon cycling (Ahmed et al., 2010). *Chlamydomonas reinhardtii* has been shown to exhibit chemotaxis towards various chemicals, such as bicarbonate, ammonium, and sucrose, but has yet to be tested for chemotaxis towards bacterial AHLs. For instance, researchers at Korea University used microfluidic assays, which utilize diffusion as a method to test the chemotaxis of *C. reinhardtii* towards bicarbonate (HCO_3^-), a molecule often used as a carbon source in aquatic environments (Choi et al., 2016). They found a positive chemotactic response to bicarbonate, suggesting the importance of chemotaxis and *C. reinhardtii* to carbon utilization. Similarly, in 1992, researchers at Vanderbilt University used capillary tube assays, a method that measures the distance traveled in small tubes, to test the chemotaxis of *C. reinhardtii* towards ammonium (NH_4^+) molecules, and also found that the algae displayed positive chemotaxis (Byrne et al., 1992). Ermilova et al. (1993) used capillary tube assays to discover the relationship between *C. reinhardtii* and sugar molecules, namely sucrose and maltose. Their results showed that *C. reinhardtii* displayed positive chemotaxis towards both maltose and sucrose, providing yet another molecule that interacts with *C. reinhardtii* (1993).

In addition to movement towards chemical stimuli, *C. reinhardtii* also exhibits movement towards light stimuli, known as phototaxis (Arrieta et al., 2017). The ability of the algae to move towards light is an evolutionary characteristic as maximum light exposure allows for more photosynthesis thus more productivity and growth (2017). Scientists at the Mediterranean Institute for Advanced Studies along with at the University of Cambridge tested the movement of *C. reinhardtii* towards light by measuring the change in chlorophyll, which is a pigment used for light absorption to provide energy for photosynthesis (2017). In this way, they were able to create a quantitative connection

between phototaxis and photosynthesis (2017). Additionally, Yu et al. (2019) conducted a study testing *C. reinhardtii* and its phototactic response to the element cadmium, which is known for being a hazardous pollutant in aquatic ecosystems. They found that cadmium inhibited cell growth and photosynthesis, while also providing oxidative stress to the algae, ultimately showing the negative association of phototaxis and cadmium-induced toxicity (2019). These studies provide sound evidence of *C. reinhardtii* behavior towards a different type of stimulus: light, which is yet another type of catalyst causing its movement. The evidence of chemotaxis found throughout these studies provide ample information on many types of molecules; however, there is a gap in the study of chemotaxis towards bacterial AHL molecules. Thus, this study focuses on testing the chemotaxis of *C. reinhardtii* towards these AHL molecules, by creating an environment without some necessary nutrients along with a stimulus that allows the algae to have an opportunity for movement.

Method

This study features a quantitative approach with a true experimental method (Creswell, 2014). This method was conducted in a traditional lab setting, which allows for sterile equipment and environments. This true experimental method was chosen in order to allow one variable to be restricted, so that there was a control group and an experimental group (2014). In this study, the control variables were defined as the water and sucrose trials, and the experimental group was defined as the bacterial AHL trials.

The strain of algae chosen for this experiment is called CR-125 (*C. reinhardtii* strain 125). This specific strain was chosen for its particular sensitivity to light, since many other strains do not react as well to light. The algae were cultured in tris-acetate-phosphate (TAP) media, a liquid medium used to help the algae grow ("TAP"). This contains NH_4^+ (ammonium), which is a nitrogen source used as nutrients; Tris, which is an organic compound used as a buffer for the pH; and other trace elements ("TAP"). This media solution allows an optimal growth environment for CR-125, as it provides all the nutrients needed for it to flourish. This liquid culture was left to grow in an Erlenmeyer (cone-shaped) flask for at least 48 hours on a lab shaker to keep the contents evenly dispersed. Once the algae was properly cultured, it was transferred into a test tube and put into a centrifuge, a machine that spins the liquid at very high speeds, allowing the algae to separate from the used TAP media. Using a centrifuge, the algae was able to solidify at the bottom of the test tube, while the remaining TAP media remained in liquid form at the top of the tube. This excess media was decanted, or poured off, into a general waste container, which allows any toxic materials to be disposed of properly (rather than poured down the sink, which could cause contamination in the water supply). Then, the algae were resuspended in TAP media without acetate (which can act as a nutrient source), and heterogenized in the tube using a vortex mixer, allowing the media and algae to effectively mix together and become a single liquid solution. The solution was then transferred into multiple plates with wells. Each plate had six wells, and using a pipette, each well received 2 mL of the algae solution. **Appendix A** displays an example of a plate used for experimentation. As seen in the image, each circle represents one well, which each acts as a single trial.

A capillary tube cutter was used to cut the glass capillary tubes into precise, small pieces that would fit inside of each well. These tubes were left in a small volume of each variable solution, with the controls being deionized water and sucrose since they should show no chemotaxis and positive chemotaxis, respectively. Through capillary action (the ability of the liquid to flow through a narrow space without external forces) , the tubes filled up with these test liquids. A tube was then placed into each individual well, with an equal amount of wells testing the control and experimental variables. These tubes can be seen in **Appendix A** within each well. The plates were then covered and scanned in an Epson Perfection V500 Photo Scanner and saved to a computer. After scanning, the plates were placed in a dark environment so as to prevent light and phototaxis from being confounding variables. After 48 hours, the plates were scanned again and the image was saved.

The analysis method includes using a photo-analysis software program called *ImageJ*, which uses Java and was developed by the National Institute of Health. This analysis procedure closely mirrors the one used by Margrethe Boyd (2018) in a study featuring the motility of *C. reinhardtii* in regards to unicellularity and multicellularity. The program was used to analyze the pixel density, or the number of red-green-blue pixels in a given area, in each experimental well by scanning

each plate initially and after 48 hours. Once scanned, the images of the plates were opened in *ImageJ* and cropped to each well in order to analyze each individually. Then, by selecting "Edit<Selection<Specify" and setting the width and height of the chosen area to 200 each, a uniform area was chosen for analysis for each well. **Appendix B** provides an image of a single well from the experiment, in which the yellow box represents the area chosen for analysis. In this manner, the software analyzed an identical area of each well, and the same area was then compared 48 hours later. These results provided a numerical representation of pixel density, in which the before and after numbers could be compared to show change in the pixel density which translates to change in the movement of the algae.

Before testing the bacterial AHLs as the experimental molecule in the capillary tubes, sucrose molecules were used first. In 1993, Ermilova et al. found that *C. reinhardtii* displays positive chemotaxis towards sucrose in similar conditions. Thus, this molecule was used on the experimental design to test its effectiveness. In a similar manner, deionized water was also used as a control molecule because the algae should show no movement towards the water. Thereafter, the AHL molecules were used.

In regards to safety protocol, proper protection was used throughout the lab experiment. Gloves and protective eyewear were used at all times to protect the researcher from harmful substances. In addition, the experiment was completed entirely under at least a BSL-1 (biosafety level 1) hood, which is usually used with non-toxic organisms to ensure that all of the equipment and materials remained sterile. Thus, the safety and sterile protocols throughout the experiment displayed ethical practices and removed any extraneous variables and thus potential bias.

Results

Using the "Analyze<Measure" feature of the *ImageJ* software, numerical results were collected that represent the pixel density of each well (refer to **Appendix B**). The tool converts RGB (red, green, blue) pixels into brightness values, of which a number was calculated for each sample. The mean for each test molecule (deionized water, sucrose, AHLs) is shown in **Table 1**.

Sample Molecule	Mean (initial)	Mean (48hr)
Deionized Water	88.177	82.614
Sucrose	74.558	73.007
AHL	147.550	128.146

Table 1: Mean pixel densities of various sample molecules from ImageJ analysis. The table shows the average pixel density of wells for each sample molecule at both the initial and 48 hour points.

From this simple average, it is seen that all three molecules displayed a decrease in pixel density between the initial and 48 hour plates, even though the current literature indicates that water and sucrose should have shown no change and an increase, respectively. Since these results appear inconsistent with the literature, there is room to believe that the methodology in question had some flaws. This suspicion is further justified through a paired t-test for statistical significance. With the 30 samples of AHLs, a paired t-test was performed in which the 30 initial AHL pixel densities were compared to the 30 densities from the 48 hour AHLs. The exact numbers used in this analysis can be found in **Appendix C**. From this t-test, a p-value of 0.999 was obtained. This number shows a great lack of significance in the data. However, the lack of significance in the results can most likely be attributed to methodology errors, as the results from the water and sucrose samples showed that the method did not prove information present in current literature. Additionally, the standard deviations for the initial AHL scans and 48 hour AHL scans were quite high — the standard deviation between the initial wells was 48.86 and between the 48 hour wells was 35.98. Considering the initial and 48 hour AHL plates had mean pixel densities of 147.55 and 128.15 respectively, these standard deviations were very large, which means that the data within the different trials varied greatly. These large standard deviations further decrease the significance of the results.

As mentioned in the literature review and the methods section, *C. reinhardtii* has been shown to display positive chemotaxis towards sucrose molecules. In reference to this study, this would mean the 48 hour samples have a higher pixel density than the initial

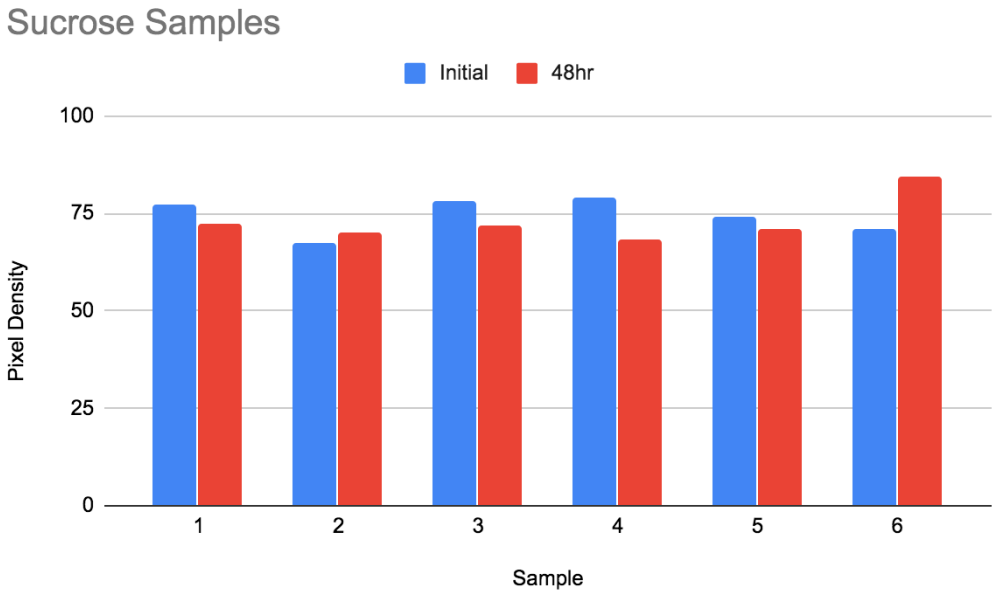

Figure 1: Comparing initial and 48 hour pixel densities of sucrose samples. The bar graph shows the pixel densities of six individual sucrose wells at the initial and 48 hour points.

samples, since the algae should theoretically be more concentrated near the capillary tubes filled with sucrose, which was the area analyzed from the samples. However, looking at six sucrose samples in **Figure 1**, they all showed a miniscule change in density, and in fact many showed a negative change where there should have been a positive change. The mean percent change of these six samples is -1.64%, showing that the change was negative and very small. This demonstrates that the method most likely had an error, as it should have proven the sucrose control to be positive.

Furthermore, a similar result occurred in the water control samples, in which the percent change between initial and 48 hour samples was -6.26%, again a negative change, when as control it should have been a 0% change. The results from both the water and sucrose samples thus prove that there must have been an error that caused results even for the controls to be skewed.

Discussion

These results show that the data does not support the movement of the algae towards the AHL molecules. In fact, it does not support movement of any of the three test molecules. Extensive research has proven that algae display positive chemotaxis towards sucrose and no chemotaxis towards water (Ermilova et al., 1993), yet the results showed slightly negative chemotaxis in both. This suggests that there was most likely a fault in the procedure, and decreases the confidence of the AHL results.

There are a number of possible reasons for skewed data. For instance, the plates were left on the scanner for 48 hours, and from the 48 hour images it seems that most algae moved either to the top or the bottom of each well. This suggests that gravity may have played a role in the algal movement due to uneven ground underneath the plates. Another large factor in the physical setup of the plates that may have af-

fected the results was the placement of the capillary tubes. Although the plates were not moved in between the initial and 48 hour scans, there was not a measure set in place to keep the capillary tubes fixed in place. Thus, the tubes may have moved slightly between scans, which would impact the results during analysis through *ImageJ*. In addition to these physical limitations, an error with the analysis procedure may also explain the results. While scanning, there is the possibility of having a difference in lighting between scans as well as the presence of dust particles. Due to the nature of the *ImageJ* software, it also counts these unwanted pieces in the brightness values. Beyond the specific issues that may have occurred with the plate setup and analysis, the underlying problem may have simply been that the culture of algae used was not concentrated enough, or that 48 hours was not enough time for the algae to display proper movement.

Alongside these unforeseen issues, there were some limitations present from the very beginning that could not be controlled in the scope of the study, including time limits and cropping errors. As mentioned previously, there were only 48 hours after the initial scan for the algae to display movement before another scan was taken. Should this time frame have been longer, the results may have differed. However, with the number of trials that needed to be completed, it was impractical to elongate this period during the experimental phase of the research. Additionally, some aspects of *ImageJ* posed limitations, including the cropping and RBG functions. While cropping images with the software, exact dimensions could not be reached for each well, so the initial and 48 hour images may have been a few pixels off based on human error by cropping. This limitation is similar to the capillary tubes not being able to be fixed in place, as the initial and 48 hour images may have a few pixels shifted. In addition, the software is only capable of measuring a combination of red, blue, and green pixels, rather than just the green pixels that were the target of this research. This means pixels other than green were included in the analysis. However, the scans did not show a significant amount of red or blue coloring, so this most likely did not have a great effect on the analysis measures.

Despite the plethora of potential errors, there is still a possibility that the results obtained were valid and that *C. reinhardtii* performs negative chemotaxis towards bacterial AHL molecules. However, the large p-value of 0.999 obtained from t-testing suggests that the confidence in these results actually being significant are extremely slim. Therefore, the results of the study cannot confidently claim that the algae perform either positive or negative chemotaxis towards AHLs. However, these errors and limitations provide very useful information for future researchers in terms of methodology and analysis techniques.

Future Directions

Despite the errors faced in methodology and analysis, the researcher believes that this procedure using capillary tube assays should be further studied in the future. This present method has been a learning opportunity, and working on setbacks in this procedure will hopefully lead to improvements and significant results in the future. In repeating this study, more improvements may be made in order to eliminate confounding factors. For example, repeating the procedure with a stronger concentration of algae or leaving a longer period of time between scans could reveal better results or simply improve the method, getting closer to the ideal procedure to testing the algal chemotaxis.

Furthermore, addressing the limitations from this study and applying them to another experiment may also advance the direction of this research. Although the researcher believes many of the factors discussed were not a large factor in the results, such as cropping and unwanted dust particles, eliminating any such variables may reap significant data. Therefore, the researcher is confident in this study's implications for improving the process used to find the relationship between AHLs and *C. reinhardtii*.

Conclusion

This study sought to find the relationship between *Chlamydomonas reinhardtii* and bacterial AHLs in order to further understand their interactions under the premise of quorum sensing, a density dependent behavior displayed by bacteria and, as recently discovered, by algae as well. Although the data did not provide significant results, the study provides direc-

tion for future studies in terms of methodology. For example, changes can be made to the concentration of algae and time between scans, as well as the lighting surrounding the scans. In terms of analysis, better methods of cropping and analyzing merely green pixels can be found, in order to eliminate small differences between scans. Also, new mechanisms to improve uniformity must also be found, such as a method of fixing the capillary tubes so as to prevent them from moving between scans.

Overall, this study reveals what changes must be made to the experimental design in the future in order to effectively test the chemotaxis of *C. reinhardtii*. Although this study could have been improved in many ways, it is unique in how it contributes to current work on algal chemotaxis and its novel approach in testing chemotaxis towards AHLs, a scarcely studied area of the topic. It is imperative to continue building off of this study's findings in the future as discovering this algal-bacterial connection could open up new possibilities in human health and pharmaceuticals and provide a greater understanding of various ecosystems. All in all, in light of the new findings on quorum sensing in algae, this study provides a great first step in exploring the interactions of algae with bacterial quorum sensing molecules, paving the way for further research to be conducted on these interactions.

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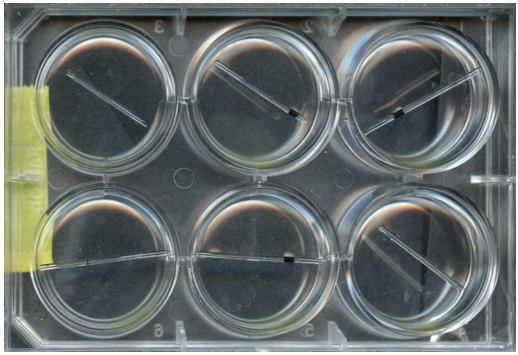
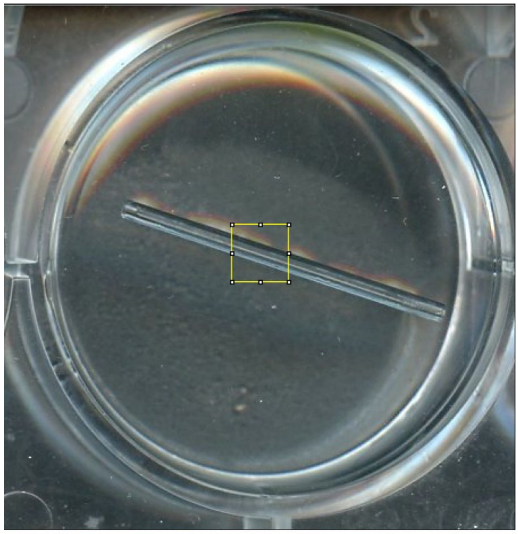
Appendix

Appendix A:
Sample Plate with 6 Individual Wells



Appendix B:
Sample Well Used for ImageJ Analysis



Appendix C:
AHL Pixel Densities (Initial and 48 Hour)

Trial	Initial Density	48hr Density	Trial	Initial Density	48hr Density
AHL1	88.106	76.667	AHL16	187.589	163.339
AHL2	90.494	91.312	AHL17	178.389	159.306
AHL3	96.447	97.817	AHL18	157.226	140.315
AHL4	90.562	95.154	AHL19	189.851	158.742
AHL5	76.429	73.028	AHL20	186.757	156.668
AHL6	105.426	89.234	AHL21	196.897	164.386
AHL7	73.185	89.005	AHL22	186.102	153.249
AHL8	92.127	79.654	AHL23	187.912	156.822
AHL9	87.287	90.867	AHL24	184.127	135.808
AHL10	82.961	89.428	AHL25	194.41	160.973
AHL11	71.974	75.816	AHL26	183.496	155.031
AHL12	134.452	85.06	AHL27	187.759	156.657
AHL13	187.728	159.991	AHL28	177.343	150.788
AHL14	184.125	162.623	AHL29	183.635	152.687
AHL15	206.693	177.196	AHL30	177.01	146.77