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Survivin is Essential for Efficient Cell Mobility and Proliferation in U87 and C6 Glioma Cells
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Commentary on Pomfret High School’s Research Program and Student’s Article: “The Effect of Red Light Stimulus on the Foraging Behavior of Drosophila melanogaster Through Measuring the Proboscis Activity”

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Perhaps you too have heard the old line about graduate school and the pursuit of scientific knowledge through research: the training of scientists forces them to learn more and more about less and less. And so it is against that prejudicial backdrop, and the growing need to develop empowered and savvy citizens capable of inventing the work and skill set of tomorrow, that we teach science in the United States. It is a tall order indeed, and many metrics would suggest that we are failing at that task as a country.

At Pomfret School, a coeducational boarding and day school for grades 9-12 located in northeastern Connecticut, we believe that research and investigation are essential tools in building that inquisitive, worldly, engaged, and nimble thinker needed in the world. As a result, the science department here strives to combine inquiry and experimentation with content, choosing to create environments where students can build their own knowledge and receive quality coaching, author and address probing questions, and explore firsthand how the world works.

Hokyung Keum is a product of that climate, and her paper, on the response of fruit flies to visual and olfactory stimuli, can be used to debunk that old line paraphrased above. Enrolled during her junior year in a new course, Directed Science Exploration, Hokyung researched behavioral studies and created a testable question of her own, asking whether \textit{D. melanogaster} can link one positive stimulus to another, effectively testing if fruit flies can learn to associate red light and food. Raising the question is one thing; figuring out how to “ask” the question in a way that yields useful data is another thing all together. Ms. Keum needed to create a protocol for manipulating her flies and how best to assess the level of the response, a process which took several iterations and called upon her to invent, test, modify, and refine her approach. In completing that task, she called upon outside mentors and local experts, brokered relationships and build a supportive network of scientific support, and experienced the non-linear path of science and discovery. This is a far cry from the conformational labs that most students experience in their science classrooms; Hokyung, and to a lesser extent her peers at Pomfret School, knows that she can pursue an answer without knowing the outcome or the pathway in advance.

Science is littered with examples of people building something new to answer a novel question, and it is this very ingenuity and resolve that we should be teaching in the science classes of this country. Kids need practice building solution strategies; they need opportunities to make mistakes, devise data collection methodologies and crunch data, assess its quality and significance, and to determine alternative approaches and the next steps that can further test emerging understanding. Doing so gives students the confidence to discover and piece together meaning—it is the very same problem solving and invention that the 21stcentury skill set demands.

The take home message for Hokyung is not really connected to fruit flies at all. Rather, it is the knowledge and confidence that she can map out and conduct a novel inquiry event and call upon her considerable problem-solving skills to achieve success. What better lesson could we possibly seek in science education?

Commentary on the Jericho High School Science Research Program

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Jericho’s Science Research Program enlivens students to create a new and endearing thirst for learning and achieving mastery in various science disciplines. Course skills Jericho students acquire include: pinpointing a science topic of interest, identifying current trends in the science, create a pivotal question to investigate; established after reading ideas presented in the popular science articles (Science News, Science Daily), complete in depth “dissection” of journal articles (using Pub Med and Scopus), explain each journal and aspects
of the methodology (including experimental tools used), explain the relevance of the results and why the future investigations are needed for each journal read. By the end of a student’s sophomore school calendar year, they will have read upwards of 25-30 journals to understand the current science related to their topic and relate these scientific works to the answering their pivotal question.

The Jericho HS Research students then present synopses’ of journals acquired in PPT presentations and begin writing a skeleton paper which includes an introduction/background information, rationale of the research, brief methodology (referenced and properly cited), and ideas for future investigations learned from literature reviews. Finally they are asked to develop research plans and to conduct “In-House” research experiments for the topic of their choice. Lastly, they are encouraged to conduct wet bench research during the summer at a regulated research institution.

Our students are intellectually curious and often show a keen insight of materials presented. They understand the meaning of hard work and getting the job done in the delegated time frame. In total, our program stimulates the minds of these young adults to identify solutions to the many issues our country is facing today and will face in the future. Our article discusses the implementation of various methods to achieve high-performance water filtration and efficient heavy metal adsorption. Currently, the effects of the global water crisis are becoming increasingly severe, so water purification is a very prominent topic in our society.

Student Led Research at Oregon Episcopal School

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The expectations of today’s high school student are many and varied. As such, it may be easy for a student conducting an independent research project to focus on the end product such as the academic grade and/or potential awards from science fair competitions. Yet, I continue to be impressed with the unique yet relevant studies many students decide to pursue. The study done by Andrea Boorse represents a sincere effort to understand the impact of human endeavors on the natural world. She developed this study soon after the oil spill in the Gulf of Mexico when the topic of the effect of oil dispersants on living species arose. Having just entered the ninth grade, one might think a student would not have the knowledge base or laboratory skills to conduct a research study on the bioconcentration of oil dispersants in oysters. I myself, a physics teacher with a degree in materials science and a background in steelmaking and semiconductor processing did not have the expertise needed to design a study of this nature. Yet, for this student led project and others that I have guided over the past three years, I found myself compelled to try. The end result is inspirational.

Incorporating student led science research projects into the core science curriculum has been a tradition at Oregon Episcopal School for nearly 30 years. It comes with its own challenges for a teacher such as managing 40+ projects a year as well as a reduction in the traditional curriculum taught, but the benefits are immense. This is particularly evident at the annual Aardvark Science Expo, an all day school-wide event where students present their projects before judges, teachers, students, and parents. It is truly a celebration of what these young people are capable of and a hint at what the future holds. Many of our students may not go on to become scientists, doctors or engineers, but having experienced this truly inquiry form of learning, we feel they will have the knowledge base and tools to apply critical thinking to their world. This fills me with a sense of hope and purpose and for that, I am grateful.

The Research Program at Amity Regional High School

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The research program at Amity Regional High School in Woodbridge, CT is an honors level elective course which students take in addition to their regular, traditional course load. Students entering the program must be recommended by their eighth grade science teachers and have outstanding academic grades – particularly in math and science. The program offers an opportunity for students to create and conduct authentic science research and is recommended to be a three or four year commitment. Year 1 is primarily an overview of research design, data collection and analysis as students become familiar with the scientific method. In subsequent years, students secure a mentor outside of the program to assist with design and experimentation of their authentic research.
Student Research at Phillips Andover Academy

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While many departments and programs at Phillips Academy feature faculty members and students who are deeply engaged in research projects, the Division of Natural Sciences has enjoyed an especially robust research program in recent years. Within the last decade, several instructors have joined the science faculty following careers in research, bringing their own special interests and expertise to the courses offered in the Division. As a consequence, Phillips Academy’s Division of Natural Sciences has seen an increase in active research, even beyond the strong existing research programs already in place. The signature course for research in the Division is Biology 600, a dedicated laboratory course normally reserved for seniors (or accelerated 11th graders) who have completed most of the available advanced classroom coursework in biology. Beginning with instruction on model systems and techniques widely used in professional laboratories, students enrolled in Biology 600 embark on independent research projects of their own choosing. In past years, students have explored a wide range of topics, from the roles that specific genes play in the growth of brain cancer cells, to the genetic controls promoting successful regeneration of motor neurons in nematodes, to novel gene targeting strategies for the production of “humanized” proteins in bacteria.

One recent study from our lab, an investigation into potential role(s) for the protein Survivin in glioma cell proliferation and migration (Montana), is presented in this issue. Generally speaking, Biology 600 students spend two to three trimesters working on their projects. Along the way, they become active participants in the broader scientific community, communicating with professional laboratories and companies and learning the refined art of collaboration. Each year, students present their findings before a collection of peers, faculty, staff, alumni and administrators, at a special event that celebrates their efforts and achievements.

The Abbott Independent Scholar Program offers additional support for independent research (in place of standard coursework) here at Andover, and many opportunities to execute experiments on a smaller scale are also presented to students within the framework of existing courses. For example, the focus of the Biology 580 course—the final term of a three-term advanced sequence that builds from cellular and molecular biology to ecology and evolution—is an experimental research project into an ecological topic of the students’ choosing that is conducted over the course of most of the spring term. The current issue of the Journal of Experimental Secondary Science features an article (Howard et al.) that was the product of one such project: an investigation into the effects of soil conditions on thigmotropism (rapid snap-trap closure) in Venus flytrap plants. As testament to the creativity of students involved in these projects, the flytrap experiments were conceived, designed, and executed completely by the students themselves.

Even as future sections of the Biology 580 course will work on student-driven ideas for ecology projects, and Biology 600 still presents opportunities for long-term, in-depth research, students continue to express interest in model systems and experimental work that will push the boundaries of the research offerings at Phillips Academy. Indeed, a trio of students who have taken Biology 570—the human anatomy and physiology course—are currently writing proposals for an upcoming Independent Project that will focus on recording muscle potentials from genetically-modified fruitfly larvae whose motor neurons contain photo-activatable ion channels to allow for remote muscle stimulation.

We expect that, as long as Phillips Academy students remain curious and motivated by the promise of discovery, and faculty members of the Division of Natural Sciences remain committed to their own professional development, as well as to mentorship and the teaching of practical science, we will continue to see expanded opportunities for students to conduct even more diverse projects in various biological disciplines in coming years.

*More information about Independent Research at Phillips Academy is available online:
http://www.andover.edu/Academics/NaturalSciences/IndependentResearch/Pages/default.aspx
Teen Research and Education in Environmental Science (TREES): A Summer Program for High School Students

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—where there's an unexpected, exciting result, it's just the same thrill all over again. You go home, and you think about it when you go to sleep, and you think about it when you wake up in the morning, and you know there is something new in the world. —David Baltimore, Nobel Laureate, from a 2006 interview with Discover Magazine.

The difference between students and researchers is that the former are consumers of knowledge, while the latter are producers of knowledge. The transition from a student to a researcher is usually carried out through mentoring programs. After classroom training, there is a long period of individual mentoring in graduate school and then, in many fields, yet more mentoring in postdoctoral training. Most research scientists cite their first experience in a lab as motivating them to choose a career in research. To provide that first experience to a young group, in the summer of 2007, the Center for Excellence in Environmental Toxicology, Perelman School of Medicine of the University of Pennsylvania launched a community outreach education program for high school students called the Teen Research and Education in Environmental Science (TREES) summer program. The TREES program is a unique hands-on research experience for high school students that introduce them to laboratory science. About seven students are recruited each year from local high schools for the ~five week program. They are taught by graduate student mentors, alumni high school student mentors and faculty members, all of whom volunteer their time to guide the students one-on-one or in the small group.

**TREES Academic program**

There is a daily lecture on an environmental issue. TREES students also watch and discuss movies about environmental issues (e.g. An Inconvenient Truth, Thank You for Smoking). Other activities teach “survival” skills such as laboratory safety, library research, internet research, scientific writing and presentation skills. There is also a college admissions workshop, a career discussion, several responsible conduct of research discussions, a library tour and a Penn campus tour. Twice each week TREES students participate in an activity with undergraduates (who intern in labs through the STEER program), once to hear a member of the Penn faculty give a cutting edge lecture and then again to travel off campus for a field trip. Students also prepare reports on a natural product that originated from an environmentally sensitive region of the world.

**TREES Research program**

Since most students are in a research laboratory for the first time, TREES labwork begins with about two weeks of structured laboratory exercises to teach basic lab techniques such as pipetting, weighing, sterile technique, and several spectrophotometer level assays. The structured laboratories also include safety training and instructions on using common laboratory equipment. This sets the basic training for what is the most unique aspect of the program: an individually guided research project on a topic chosen by the student. The projects are developed in consultation with the mentors and faculty and then executed by the student. Students present their results in a report, a poster and a PowerPoint presentation to the group and invited guests in a student run symposium. The projects cover a range of environmental topics; as the publications at the end indicate, students have worked on CO$_2$ sequestering, food safety and chemical carcinogens, as well as arsenic poisoning, phosphate trapping and biofuels.

**Beyond TREES**

Science does not end with their laboratory experience, as the data collected must be analyzed and presented according to scientific standards. Students work with their mentors after the program to develop projects for public presentations, science fairs and their senior thesis. Others have found creative ways to bring the message of their project to their schools. For example one student who made and tested biodiesel as his project, organized a used cooking oil recycling collection in his school. TREES projects have earned students numerous awards back at school and in local science fairs; several won invitations to national competitions and scholarships. Three, including Jeremy Wortzel, whose paper is published in this issue, published their work in peer reviewed scientific journals. The program is currently funded by a grant from the National Institute of Environmental Health Science (NIEHS).

**Applications to TREES**

Interested high school students from the Philadelphia area should download an application from the website at: http://www.med.upenn.edu/ceet/TREES.shtml. The applications require an essay, a copy of the transcript, and two letters of recommendation. There is no tuition or fees for TREES program. Inquiries: mytrees@mail.med.upenn.edu

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The Effect of Red Light Stimulus on the Foraging Behavior of Drosophila melanogaster Through Measuring the Proboscis Activity

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Abstract

Drosophila melanogaster is commonly used as a model organism for biological research. A behavioral study on the effect of red light on fruit flies was conducted, asking specifically if a positive stimulus, red light, has an amplifying effect on D. melanogaster's craving behaviors. Another question asked in this study was if red light alone can train these organisms to anticipate food and to adopt specific foraging behaviors, even when food is not present. The proposed hypothesis was that when experimental D. melanogaster are conditioned with red light stimulus, the average amplitude of their foraging response would be greater than the average amplitude of the response recorded in control flies when they were presented food. Such a result would indicate that red light has a positive effect on these flies' response to food. According to the two sample T-test, the mean amplitude in the test conducted after the fifth trial in both the control and experimental groups were statistically equal (t-value=0.5169). However, the experimental group had statistically greater net average amplitude of response to food (t-value=-3.50), and the experimental group had statistically faster average response time (t-value=2.037). The data supported the hypothesized positive effect of red light on the flies' foraging behaviors. The data also suggested that the red light alone could not train the flies to successfully associate red light to food.

Introduction

Many of the behavioral studies of Drosophila melanogaster conducted to date have involved a negative stimulus and a positive outcome. Multiple studies that have questioned the ability of D. melanogaster to associate a certain stimulus to the anticipated behavior have been performed to date. In Mery's experiment1, a repugnant chemical was used as a conditioning stimulus. The negative chemical stimulus was used to train the flies to avoid a certain fruit as an ovipositing site1. As a result, a significant number of experimental flies developed a connection between the repugnant chemical stimulus and the correct ovipositing sites, achieving success faster than the control flies.

Unlike Mery, Chabaud used a positive olfactory stimulus to train D. melanogaster2, This study examined a fly's proboscis, an elongated appendage that is positioned on the flies' head, and the flies were used for feeding. Response to the introduction of banana odor was rewarded with sucrose. Using the positive banana odor as the conditioning stimulus, D. melanogaster were trained to exhibit a strong activity in the proboscis, anticipating a food reward2.

Dobzhansky’s experiment on phototaxis and geotaxis in D. melanogaster offered evidence that D. melanogaster responds photopositivity to red light3, thus making it a positive stimulus. Under red light, which had an intensity of 0.2 lux, a photopositive population of flies remained photopositive and the photonegative population became either photopositive or photoneutral3, suggesting that to D. melanogaster, red light is a positive stimulus.

Dobzhansky's experiment1 gave an interesting result that suggested the possible usage of red light as a positive stimulus. The experimental results from Mery and Chabaud2 suggest that D. melanogaster is capable of associating one stimulus to an anticipated behavior such as the extension of the proboscis. Combining the conclusions from the three experiments from Mery, Chabaud and Dobzhansky, the central question of this research became whether a positive stimulus (red light) can magnify D. melanogaster's behavior toward food and whether D. melanogaster can demonstrate a connection between the positive stimulus and a positive outcome. The methodologies applied here to investigate the effect of red light on the foraging behavior in fruit flies follow closely the techniques employed in the three works cited above.

In this study, 18 male vestigial-wing D. melanogaster, conditioned under red light from the time they were in the pre-pupa stage, were collected to serve as the ‘experimental group’2. The experimental flies were starved for six hours before experimentation. For five trials, D. melanogaster were presented with 20 seconds of red light, during which they were also presented with a Q-tip soaked with 0.01 M sucrose. After the fifth trial, a test of D. melanogaster's ability to associate red light with food was conducted - the test involved showing the fly just red light, and measuring its proboscis activity, a foraging behavior. There was also a control group, which again came from various single generations of male vestigial-wing D. melanogaster, and the control flies were also starved for six hours before the experimentation. The control group was presented with a Q-tip soaked with 0.01 M sucrose to the proboscis for five trials, but not red light. After the fifth trial, the feeding response of the control flies was tested by presenting the animals with a clean Q-tip; this same test was conducted for the experimental population, with the Q-tip accompanied by red light. For both groups, the amplitude of response was measured using the 6-point system.

Materials and Methods

Control Group:
A total of 18 male vestigial-wing D. melanogaster were used. After anesthetizing the flies with FlyNap® (Carolina Biological Supply Company), male flies were separated from females. Only two flies were tested at a time. Selected male flies were mounted onto a paraffin wax-wrapped toothpick secured at the base of the wings. By poking a small hole (the width of toothpick) on the polyurethane foam vial cap, the fly-attached toothpick was securely placed into the hole made on the foam cap (Figure 1).
The flies were then kept in the vials for a six-hour starvation period. After 6-hours, the fly, still attached to the toothpick, was placed under the dissecting microscope, proboscis side up. Before beginning each trial, the amplitude of spontaneous movement of the proboscis (initial state of the proboscis) was measured. Then, the amplitude of response to access to food, including the time it took for that specific response, was measured. The amplitude (amplitude of response to access to food – spontaneous response) was measured through the 6-point system (Figure 2). The spontaneous movement of the proboscis was the amplitude of the control response exhibited by the fly when a clean, dry Q-tip was introduced. The Q-tip was placed where the fly's proboscis almost touched the head of the Q-tip. Then, the amplitude of the response to food was judged when a Q-tip soaked with 0.01M sucrose was made available. The flies were given a maximum of 20 seconds to exhibit a response to the sucrose Q-tip. If the fly still did not show any response after 20 seconds, the response time was still recorded as 20 seconds. Sucrose was given as a reward after a positive response. This two-part trial was repeated five times with 5 minute resting intervals between each trial. Continuously testing three flies at a time inevitably caused each fly to have a waiting period. Unless a uniform interval of waiting period was assigned, the waiting period of each fly would be irregular. By setting the waiting period (and calling it interval of resting) to be five minutes, each fly had equal interval of waiting in between each trial, and five minutes was the minimum amount of time needed to finish one trial without any rush. Right after the fifth trial, testing for learned behavior was conducted: first, the natural position (movement) of the proboscis was recorded. Then, a clean Q-tip was introduced near the fly's proboscis, and the amplitude of response was recorded. The purpose of this test was to see if the control flies had associated the Q-tip with food.

**Experimental Group:** Eighteen vestigial-wing male *D. melanogaster* grown under red light since birth were used as the experimental group. Two flies were trained at a time. The flies were mounted on the paraffin waxed-wrapped toothpicks using the same procedures and equipment used to prepare the control group. These experimental flies were also kept in vials containing moist cotton balls. The only difference was that next to the hole on the polyurethane foam, there was another hole to hold the red light. Therefore, experimental flies were constantly exposed to red light from the time of birth to the time of experiment in order to increase the flies' familiarity with the red light. The actual experiment began after 6-hours of starvation time. The dissecting microscope light was not turned on; only the natural lighting coming in through windows was present. Each trial began with measuring the spontaneous response—a clean Q-tip was introduced near the fly's proboscis area. With red light in hand, the fly, under the dissecting microscope, was exposed to red light for 10 seconds. After 10 seconds, a sucrose-soaked Q-tip was introduced to the fly (with red light still shining down on the fly simultaneously). The fly was exposed to red light longer than sucrose soaked Q-tip. The fly grew since the pre-pupa stage feeding in the food vial under the red light. This pre-conditioning the fly received led to the anticipation that 10 seconds of exposure to red light prior to 10 seconds of access to food will aid the fly to better express foraging behavior. If the fly showed response, sucrose was given to the fly as a reward. A total
of five trials were carried out, and there was a five-minute resting period between trials. Immediately after the fifth trial, a learning test was conducted - the fly’s natural position of the proboscis, without the introduction of a clean Q-tip, was recorded. The fly was then exposed to red light for twenty seconds, or less if the fly showed a response. The purpose of this learning test in the experimental group was to see if the flies had associated the red light stimulus with food. The data collected from both the control and experimental group were analyzed through series of statistic tests; two sample t-test with \( \sigma = 0.05 \). A total of four different, two sample t-tests were carried out (Table 1).

### Results

The results from Table 2 describe the control group flies’ response when they were exposed to the clean Q-tip and food, and when they were tested for learned behavior. The results from Table 3 describe the experimental flies’ response when they were exposed to the clean Q-tip and food given while shining red light, and when they were tested for learned behavior.

In order to analyze the data, two sample T-test (\( \sigma = 0.05 \)) was used. A total of four statistic tests were conducted to verify that Q-tip is an ignorable variable in the experiment, the experimental group flies show significantly stronger response to the food source, the experimental group flies show significantly faster response to the food source, and the learning test response in both groups are statistically equal (Table 4).

In both the control and the experimental group, average amplitude of response to the clean Q-tip showed little variance and only a few data points showed significant deviation from the mean (meancontrol=0.5722, meanexperimental=0.4).

Because the data of the average amplitude of response to food in the control group compared to that of the experimental group’s seemed to differ significantly, a graph was created as a visual aid to seeing the data difference (Figure 3).

### Table 1. Hypothesis for four different two sample t-tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>Hypothesis</th>
<th>Symbol</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>The mean initial response in control flies is equal to the mean of initial response in experimental flies</td>
<td>( H_0 )</td>
<td>( H_1 )</td>
</tr>
<tr>
<td>Test 2</td>
<td>The mean amplitude of the feeding response seen in control flies is equal to the mean of amplitude of response in experimental flies</td>
<td>( H_0 )</td>
<td>( H_1 )</td>
</tr>
<tr>
<td>Test 3</td>
<td>The mean response time in control flies is equal to the mean of response time in experimental flies</td>
<td>( H_0 )</td>
<td>( H_1 )</td>
</tr>
<tr>
<td>Test 4</td>
<td>The mean amplitude of the response to the learning test in control flies is equal to the mean of that response in experimental flies</td>
<td>( H_0 )</td>
<td>( H_1 )</td>
</tr>
</tbody>
</table>

### Table 2. Average data of the control group flies conditioned with red light stimulus.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
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<td>24.645.347</td>
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<td>0.914.327</td>
<td>24.645.347</td>
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</tr>
</tbody>
</table>

The values are the average of five trials of spontaneous response, response to access to food and response time. Learning test is not averaged since only 1 trial of the testing was done for each fly. In the case of a -1 response amplitude, initial response (natural position of the proboscis before testing) was greater than the response to the learning test.
Discussion

The result of statistical test 1 (Table 4), on the spontaneous response in both control and experimental groups, failed to reject $H_0$, $t=0.7133$. This indicated that the average spontaneous response in control and experimental flies are equal, or there was no statistically significant difference. The clean Q-tip did not have a significant role in one group over the other. Therefore, Q-tip was not an interfering variable in the experiment—any response to sucrose-soaked Q-tip can be attributed to the food and not to the instrument.

The result of statistical test 2, on the average amplitude of response during training, rejected $H_0$, $t=3.504$. This showed that the experimental group exhibited a greater overall response to food (sucrose). This suggests the positive effect of red light on the flies' response to food—the feeding response is magnified when flies are conditioned with red light. By being conditioned with red light continuously from the pre-pupa stage and through the five trials, the experimental flies may have become familiar with the red light. This familiarity in turn could have stimulated the taste sensors of these flies. The control group did not exhibit response to food, but not as strong and consistent as the experimental group.

The result of statistical test 3 conducted on the average response time in both groups rejected $H_0$, $t=2.037$. Thus, the control group exhibited a slower response than the experimental group. Again, this suggests that red light had a positive effect on the experimental flies' response to food: the flies responded to food more quickly when conditioned with red light. The first 10 seconds of exposure to red light without the introduction of sucrose-soaked Q-tip could have served as an informing stimulus, hence accelerating the experimental flies' response to food. The positive effect of red light on the flies' response to food is easier to see in Figure 3. The graph shows that the majority of the experimental flies have exhibited average response amplitude of above 3. However, for the control group, the majority of the flies exhibited average response amplitude of below 3.

Combining the results of all three statistical tests conducted on the data from Table 4 and Figure 3, the hypothesis, stating that the experimental group's overall amplitude of response will be greater than that of the control group's, was supported by the data of the experiment. By conditioning the flies with red light, the flies showed overall a greater and timelier response to food. Dobzhansky's experiment concluding that *D. melanogaster* favors red light is supported in this experiment: red light not only increased the intensity of the flies' response to food, but it also quickened the flies' response to food. Considering these two conclusions that has been made from the data, conditioning flies with red light effects the flies perhaps mentally. Red light alone (referring to Table 2 and 3 'amplitude of response during the learning test') does not trigger any significant response (Table 4, statistical test 4 - $t=0.7133$), but red light with sucrose amplifies the proboscis activity. This leads to a possibility that the olfactory neurons were stimulated by the red light to amplify the flies' ability to detect the sweet odor of sucrose and thus amplifying the activity of the flies' proboscis and accelerating the flies' response to food upon the introduction of sucrose.

However, whether or not red light directly increased the flies' foraging behavior by stimulating the taste sensors is unknown. The effectiveness of red light as a learning device for *D. melanogaster* is hard to confirm. Statistical test 4, on the average response during the learning test in both groups, showed that there was no significant difference between the two groups' proboscis activity. Therefore, red light was not associated with food nor did it trigger an anticipatory response in the flies, even though significantly larger behavioral responses to food were seen in the experimental group. Thus, it would not be safe to conclude that red light successfully conditioned the flies to anticipate food. Instead, according to statistical test 2 and 3, red light did have an effect on how strongly and how quickly the flies responded to food upon the introduction of sucrose. This suggests the positive effect of red light on the experimental flies' response to food—the feeding response is magnified when flies are conditioned with red light. By being conditioned with red light continuously from the pre-pupa stage and through the five trials, the experimental flies may have become familiar with the red light. This familiarity in turn could have stimulated the taste sensors of these flies. The control group did not exhibit response to food, but not as strong and consistent as the experimental group.

Certainly, there were some errors, which could have been fixed. First, more than five trials should have been conducted for each test subject. Increasing the number of trials could have improved the results of the learning test, possibly altering the conclusion that red light is not an effective learning device. Five trials of conditioning resulted in the amplification of the foraging behavior and the quickening of the flies' response to food. More trials could have resulted in the flies actually associating the red light stimulus to sucrose. Also, using the Q-tip to feed the flies may have been a poor choice because the cotton head was far larger than the flies' very small proboscis. Sometimes the sucrose solution from the Q-tip covered the whole head of the flies, hampering the collection of quality data. Instead, a micropipette could have been used to feed the flies. The sucrose solution would not easily drop to soak the flies with sucrose because the solution is

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**Table 4. Two sample t-test results**

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean control</th>
<th>Mean experimental</th>
<th>T-value</th>
<th>P-value</th>
<th>Result</th>
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<td>0.9</td>
<td>3.504</td>
<td>0.00138</td>
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<td>2</td>
<td>2.812</td>
<td>3.555</td>
<td>2.037</td>
<td>0.0371</td>
<td>Rejected $H_0$</td>
</tr>
<tr>
<td>3</td>
<td>9.479</td>
<td>6.389</td>
<td>0.4824</td>
<td>0.6368</td>
<td>Failed to reject $H_0$</td>
</tr>
<tr>
<td>4</td>
<td>0.611</td>
<td>0.3689</td>
<td>0.4824</td>
<td>0.6368</td>
<td>Failed to reject $H_0$</td>
</tr>
</tbody>
</table>

---

**Figure 3.** This graph is a comparison between the control and experimental groups on the average amplitude of response to food. (N=18; 5 control group data points and 11 experimental group data points appear as if they are not represented in the above graph because these data points overlap with other data points that have the same amplitude of response).
kept in the pipette unless it is squeezed out. The flies can feed on the solution by sinking its proboscis through the small opening of the pipette where the sucrose solution is suspended because of the solution’s surface tension.

Another error that should have been thought out was exposing red light to the experimental group prior to the experimentation. The initial logic was that early exposure to red light was what distinguished the experimental group from the control group. Early exposure to red light had been incorrectly used as a part of the conditioning process of the experimental group for conditioning the experimental flies with red light from the pre-pupa stage created another variable. The increased magnitude of foraging behavior in the experimental group could have been affected by this extra variable.

An inconvenient choice was the use of vestigial-wing flies. Vestigial flies have very small, deteriorated wings, which made mounting these flies onto toothpicks difficult and time consuming. Many times the flies detached itself from the toothpick because it was easy for the flies’ small vestigial wings to rip off from either the toothpick or the flies’ bodies. These flies walked off from the microscope and test subjects were many times lost as a result. As a result, data was ruined, and the ruined data had to be discarded.

Another difficulty was running the risk of hurting the flies with the heated dissecting needle. Because the dissecting needle was not thin enough, sometimes the needle touched the bodies of the flies instead of its wings. Whenever the hot needle touched parts of the flies other than the vestigial wings, the flies’ abdomens curled up and proboscis extended immediately. These behaviors seemed to be signs of pain. Flies that have been burned by the hot dissecting needle were found to die easily, not surviving long enough to conduct the five trials of experiment; this error served to slow down the data collecting process. Using wild type flies would have been more ideal since their wings are large, giving more surface area of attachment onto the toothpick.

In the next experiment, many of these errors will be corrected, and the reason behind why red light amplified and quickened the foraging behavior of the flies will be studied. Also, this research will be redesigned to focus on the effectiveness of red light as a learning device. The ability of the flies’ to associate one stimulus to another will be one of the central questions in hand.

Acknowledgements
Thank you Dr. Chun Geun Lee, MD, PhD, MSc at Yale University for always sparing some time to answer my conceptual question on fruit fly research.

References


The Effect of Soil Nitrogen Levels on Thigmotropic Responses in the Venus flytrap, *Dionaea muscipula*

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Abstract

Venus flytraps are perhaps the world’s most well-known carnivorous plant. They rely on small insects to acquire nitrogen, as the swampy environment that they inhabit in southeastern United States’ Carolina coastal plains is insufficient in nitrogen. We studied the effect that soil nitrogen levels have on the flytrap’s carnivorous behavior. We manipulated soil nitrogen levels to produce nitrogen-sufficient and nitrogen-deficient growth conditions for Venus flytraps. We then compared the closure time of the snap traps of the plants in nitrogen-sufficient and nitrogen-deficient soil, following touch stimulus. We found that flytraps in nitrogen-rich soil exhibited longer closure times. We concluded that, because the plants were grown in a nitrogen-rich environment, they did not require supplemental insect nitrogen, and were possibly conserving energy by responding less vigorously to touch stimulus.

Introduction

Venus flytraps, *Dionaea muscipula* - with their rapidly-closing snap-trap mechanisms for catching their prey - have mesmerized generations of observers, from Charles Darwin to today’s preschool students¹. *Dionaea*’s indigenous habitat - swampy, low-lying regions in the Carolinas of the southeastern United States - contains fairly nitrogen-depleted soil. These conditions led to the evolution of carnivorous behavior for obtaining adequate amounts of nitrogen¹. *Dionaea* captures its prey (small animals and insects) with a highly-sensitive and rapidly-moving snap-trap mechanism. The plant is separated into many leaves, each of which is divided into two parts that close together to form the trap². The inner epidermis of the leaves contains three trigger hairs, that when stimulated, activate the closing of the snap-trap². The stimulation of the trigger hairs generates action potentials that create an electrical charge to stimulate the motor cells of the leaf to close, interlocking the cilia on the edges of the leaves to prevent the prey from escaping².

We investigated whether relatively nitrogen-rich soil conditions might affect the kinetics of this mechanism. We exploited the thigmotropic characteristic of *Dionaea* (its movement in response to touch) to compare the time it took the snap-traps to close in response to consistent stimulus of the trigger hairs of plants in nitrogen-depleted soil versus those in soil treated with fertilizer (to increase the nitrogen content of the soil). We hypothesized that the speed of the thigmotropic response to touch stimuli would be reduced for *Dionaea* snap-traps growing in nitrogen-rich soil, as the high nitrogen content in the soil would satisfy the plant’s need for nitrogen.

Materials and Methods

**Growth plot preparation and maintenance**

Four plastic aquarium containers measuring 23.2 cm long by 15.2 cm wide by 16.8 cm high were each filled with 7 cm of commercially-available topsoil, a mixture of red sedge peat and sand. The topsoil initially measured nitrogen levels of “N0.5”, ~7.5 parts per million (ppm), indicating nitrogen depletion; see below. One Venus flytrap (Carolina Biological Supply Company; Burlington, NC) was placed at the center of each of the four containers with the soil in which it was delivered left intact. Soil tests were performed on both the transplanting topsoil and the soil associated with the flytraps’ roots to ensure that they had the same initial nitrogen concentrations (both N0.5, nitrogen-depleted; see below). Two containers were designated to be fertilized (for nitrogen addition), two designated to be nitrogen depleted. Peat moss (Carolina Biological Supply Company; Burlington, NC) was added to the second set of containers, surrounding each flytrap, to assist in the depletion of the nitrogen from the soil. The containers were fitted with plastic mesh covers and placed indoors by a window to be exposed to constant temperature and a natural light-dark cycle. For preparation of fertilization solution, 10 grams of commercial fertilizer (Carolina Biological Supply Company; Burlington, NC) was dissolved in a gallon of distilled water. Nitrogen-addition containers were watered with 100 mL of fertilizer water daily; nitrogen-depletion containers were watered with 100 mL of distilled water daily. Soil nitrogen level testing: A commercially-available soil testing kit (Model 5880, LaMotte; Chestertown, MD) was used to determine the relative nitrogen concentrations on a scale from N0 to N4. 0.5-g samples of soil were treated with nitrogen extraction solution (dilute HCl; order #5702, LaMotte) and nitrogen indicator powder (N-(1-Naphthyl)ethylene diamine dihydrochloride, and sulfanilamide; order #5703, LaMotte) per the manufacturer’s instructions. Following treatment, colorimetric analysis of the resulting solutions by comparison with standards afforded estimates of soil nitrogen content. N0 indicated nitrogen-depleted soil = 0-7.5 ppm; N1, deficient = 7.5-15 ppm; N2, adequate = 15-22.5 ppm; N3, sufficient = 22.5-30 ppm; and N4, surplus = 30+ ppm. Soil tests were performed every other day, on average, for the duration of the experiment. Measurement of closure times: In order to measure the closure times of the Dionaea snap-traps, each open trap from each plant was mechanically stimulated on each trial day. Only fully-open traps were tested. To initiate the snap-traps’ closure, one cm of graphite was extended from the tip of a mechanical pencil to stimulate the trigger hairs. Timing was started once stimulation began, and was stopped once the leaf of the flytrap stopped moving (reached full closure).
Results

Soil nitrogen levels remained constant throughout testing. Throughout the experiment, we attempted to create and maintain two extreme cases of soil nitrogen levels: sufficient and depleted. We daily provided soluble fertilizer to two of the four containers in which flytraps were grown to increase nitrogen levels; we added peat moss and no fertilizer to the other two containers to deplete soil nitrogen levels. After transplantation of the Dionaea plants, soil testing (see Materials and Methods) was conducted every other day (on average) over 15 days to monitor nitrogen levels.

Colorimetric analysis indicated that each container had an initial soil nitrogen content of N0.5 (~ 7.5 ppm). The two containers treated with soluble fertilizer immediately (on the next testing day; two days later) displayed increased nitrogen levels of N3 (~ 22.5-30 ppm). Over this time, a scatter plot of the nitrogen content (on a 0-4 scale) versus day displayed relatively steep a slope of 1.25 (data not shown). Following this initial jump in soil nitrogen content, we monitored nitrogen levels in the nitrogen-sufficient containers for over one week. We began closure-time testing only after it was determined that the two fertilized containers had attained stable elevated soil nitrogen levels (average of N3; ~ 22.5-30 ppm). We made this determination by plotting measured nitrogen levels (0-4 scale) versus day after transplantation, now excluding the first day of initial low levels. Over a whole week, the slope of the resulting line reached a near-zero slope of -0.042 (p = 0.60, linear regression T-test); we thus deemed the nitrogen levels to be stable. These levels remained stable for the subsequent trials of closure-time testing.

Throughout the experiment, the soil containing peat moss, and watered without fertilizer, maintained a stable nitrogen content of N0.5 (~ 7.5 ppm), and did not deviate from this level. We began experimentation on plants in these containers only once the nitrogen levels in the nitrogen-sufficient containers had been deemed stable.

Dionaea snap-traps in nitrogen-sufficient soil close more slowly than those of Dionaea in nitrogen-deficient soil.

We performed closure-time testing to investigate the effect of soil nitrogen levels on the time it takes the snap-traps to close completely in response to a consistent stimulus. After stable nitrogen levels were achieved in each set of containers, closure-time testing was carried out only on the fully-opened Dionaea heads of each container for four out of five consecutive days. Closure time was recorded as the time from the initiation of the stimulus until the end of snap-traps’ movement.

For calculation of average closure times and comparison of conditions, we pooled data from all tests performed on Dionaea from both of the nitrogen-sufficient containers; we similarly pooled data from both of the nitrogen-deficient containers. For nitrogen-deficient Dionaea, we measured a mean closure time over all trial days of 3.58 + 2.04 seconds (n = 25 trap closures), with a range of 1.68 to 9.09 seconds. Snap-traps of Dionaea grown in nitrogen-sufficient soil exhibited a significantly longer time to complete closure (p < 0.01, two-tailed Mann-Whitney U-test), with a mean of 16.12 + 7.42 seconds (n = 5 trap closures), and a range of 8.9 to 25 seconds (Figure 1). A smaller percentage of nitrogen-sufficient snap traps exhibit closure, as compared with nitrogen-deficient snap traps.

![Figure 1. The closure time of Dionaea in response to a consistent stimulus at different soil nitrogen levels.](image)

In addition to testing the effect of soil nitrogen levels on the closure time of Dionaea snap-traps, we also examined the relationship between soil nitrogen levels and the probability of the snap-traps’ closing. We only performed closure-time testing on the traps open at the time of the experiment. Of these traps, some closed within 60 seconds of stimulation and some did not. Thus, for each day of testing, and for each condition (nitrogen-depleted and –sufficient), we calculated the fraction of snap-traps that closed, out of the total number open at the start of that day’s trials.

We found, first, that the absolute number of open snap-traps decreased over time for the nitrogen-sufficient Dionaea. During the course of the experiment, progressively more previously-stimulated snap-traps from Dionaea plants in nitrogen-sufficient soil remained closed until the next trial (data not shown). We also found that for the nitrogen-sufficient Dionaea, the fraction of evoked snap-trap closures decreased over time, whereas for the nitrogen-deficient Dionaea, the fraction remained relatively stable.
Discussion

Nitrogen deficiency in Dionaea results in longer time to snap-trap closure.

By stimulating the head of the Dionaea and thus simulating the presence of an insect, we caused the leaves of the plant to close. In comparing the closure times between snap-traps of Dionaea in nitrogen-sufficient and nitrogen-deficient soil, we obtained results demonstrating that leaves of Dionaea close more quickly in nitrogen-deficient soil. These results suggest that there is little or no need for the Dionaea to ‘snap’ shut with the presence of nitrogen in the soil, as they did not have a need for prey-derived nitrogen. We inferred that the plants were most likely fully supplied with nitrogen from the fertilizer.

Additionally, we are able to conclude that the closure of the leaves within the nitrogen-deficient soil was not subject to habituation (Figure 2). Though this is a secondary conclusion, and solely based upon four trials, it suggests that Dionaea is not quick to habituate to repeated patterns of stimulation. This result additionally supports the notion that the reduced number of closures in nitrogen-sufficient Dionaea is not a result of habituation.

Possible Sources of Error and Additional Experiments

In order to prevent the plants from ingesting flies or other nitrogen-providing insects, we enclosed each plant in a plastic container with a lid that allowed air exchange through small slits. Very small insects may have been able to access the inside of the growth chamber through these slits, potentially affecting not only the number of open leaves but also the availability of nitrogen to the plants. However, the ventilation slits were sufficiently narrow that the number and size of insects able to enter through these holes would both be very small, and unlikely to have significant effects.

In conducting our experiments, we attempted to simulate the presence of a fly within the leaves of the Dionaea. In order to do so, we applied slight pressure within the leaf. Not knowing precisely where to target, we tried to stimulate lightly the entire leaf of each plant in the same way during each trial. While there was a chance for human error, we attempted to stimulate the leaves in a consistent manner. Future experiments may utilize an automated stimulating device to minimize human error.

Another issue to consider is that the fertilizer provided the Dionaea plants in nitrogen-sufficient soil with additional macronutrients that

Table 1. Fraction of closures of snap-traps.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Fraction of closures; nitrogen-sufficient soil</th>
<th>Fraction of closures; nitrogen-deficient soil</th>
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<tr>
<td>1</td>
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<td>0.75</td>
</tr>
<tr>
<td>4</td>
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<td>0.67</td>
</tr>
<tr>
<td>MEAN</td>
<td>0.21</td>
<td>0.69</td>
</tr>
<tr>
<td>+/-SEM</td>
<td>+/- 0.125</td>
<td>+/- 0.03</td>
</tr>
</tbody>
</table>

Fraction of snap-traps under each condition that closed in response to touch stimulus (on a trial-by-trial basis). A significant difference was detected between the fraction of closures under each condition (n = 4 trials for each condition; p < 0.05, two-tailed Mann-Whitney U-test).

Figure 2. Average closure time, by trial, of the nitrogen-deficient Dionaea. Red points represent the mean closure times (+ SEM) for nitrogen-deficient Dionaea for each trial day. (Between the first and third day, no testing was performed.) No clear upward or downward trend is evident in the data; the line of best fit to the mean points had an equation of y = -0.0083 x + 3.63.
were not present in the soil of those plants with depleted nitrogen levels. Additionally, peat moss in the nitrogen-deficient containers likely absorbed other nutrients besides nitrogen. These factors might have differentially affected the levels of non-nitrogen nutrients in the two soil conditions, introducing additional uncontrolled-for variables. While we are reasonably confident that the observed differences in thigmotropic responses were the result of different nitrogen levels, future experiments would necessarily involve testing for and carefully controlling for the presence of nutrients other than nitrogen.

Finally, testing snap-traps’ closure responses in the presence of actual flies would be a logical extension of these investigations. Future experiments might examine whether plants in nitrogen-depleted soil would in fact prey upon more flies in order to gain nitrogen.

Could increased soil nitrogen levels affect native ecology via altered Dionaea behavior?

We can infer from our results that wild Dionaea growing with the presence of excessive nitrogen in the soil would be less quick to and less likely to ‘snap’ down upon flies.

Dionaea grow in very specific environments, limited to the coastal plains of the Carolinas³, and the plants are already considered a threatened species by the National Red List. Studies have indicated a 400% increase in fertilizer usage in North Carolina since 1945⁴, suggesting a likely increase in soil nutrients, nitrogen included, in those states where Dionaea are most prevalent. As nitrogen levels increase in the Carolina coastal plains, Venus flytraps may become less sensitive to prey. Over time, this could conceivably lead to loss of flytraps’ carnivorous behavior.

A loss of Dionaea’s carnivorous characteristics could affect more species aside from the plant itself. The Venus flytrap’s natural prey - small insects and spiders⁴ - would lose one of the main predators in the local microenvironment, possibly leading to overpopulation. An overabundance of small arthropods, particularly spiders, could upset the predator/prey balance of the microenvironment considerably, as spiders function in many ecosystems as predators themselves⁵. Future studies must be conducted to explore the long-term effects of fertilizers and other forms of human activity on local indigenous species, as an upset in the balance of species’ behavior in established ecosystems could have lasting consequences.

References


Acknowledgements

We thank Melanie Poulin for providing us with technical assistance and necessary supplies during the execution of these experiments. We also thank Matthew Lisa (Phillips Academy Department of Mathematics) for helpful advice and comments on statistical analyses. This work was supported by the Phillips Academy Department of Biology and the Israel Family Fund for Science Research (to G.R.T.).
**IN SCHOOL ARTICLE**

**Hypericum perforatum** Extracts and Hypericin Treatment of a Mouse Mammary Cancer Cell Line Induces Growth Inhibition in a Dose Dependent Manner

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**Abstract**

*Hypericum perforatum*, commonly known as St. John's Wort, has been found to exhibit many medicinal, especially anti-depressant, properties. Hypericin is thought to be the main chemical constituent responsible for *H. perforatum*'s medicinal properties. We report here the ability of *H. perforatum* and hypericin to inhibit the growth of mouse mammary cancer CRL2539 cells. *H. perforatum*, at concentrations of 0.4% and 0.8%, significantly (P<0.05) inhibited cell growth in a concentration dependent manner. Hypericin (purity 80-90%) at a concentration of 0.001% also significantly inhibited cell growth but not to the extent which the *H. perforatum* extracts did. In addition, *H. perforatum* at a concentration of 0.8% inhibited cell growth significantly more than *H. perforatum* at a concentration of 0.4%. Our study shows a promising therapeutic strategy in using the whole *H. perforatum* extract as its own form of treatment to effectively slow the growth rate of cancer cells, and potentially overcome the negative side effects associated with current forms of cancer treatment.

**Introduction**

*Hypericum perforatum* is a yellow flowering plant naturally found in various locations around the world including West Asia, Europe, and North Africa. *Hypericum perforatum* has been studied for its effects in treating depression and attention deficit hyperactivity disorder. Cancer prevention research is one of the new areas that *Hypericum perforatum* and its main chemical constituents are being applied to. Research has shown that the most significant compounds in *Hypericum perforatum* for cancer prevention are hypericin and hyperforin.

Hypericin is used in photodynamic therapy (PDT) as a form of cancer treatment. PDT requires a photosensitizing agent (photosensitizer) and visible light of a wavelength that correlates with the absorption spectrum of the drug. Alone the light and photosensitizer have no therapeutic effect, but when combined produce cytotoxic products which trigger irreversible tumor destruction and cell damage. Out of 36 species of *Hypericum*, 27 held hypericin, the most common being *Hypericum perforatum*.

The cell type in which hypericin is applied dictates the killing efficacy and the cellular distribution of the drug. In colon carcinoma CACo-2 cells for example, hypericin is found to accumulate in the nuclear and plasma membranes. Hypericin mainly targets cell membranes and can affect critical mitochondrial functions in a photodependant manner. Although hypericin does not gather in mitochondria, hypericin's photodynamic action primarily targets these cell sites as shown by the impairments in mitochondria bioenergetics when hypericin is present and combined with a visible light wavelength.

Another form of cancer treatment that involves the use of hypericin is catalytic therapy (CT). CT is a cancer treatment that involves the use of substrate molecules and a catalyst to generate reactive oxygen species (ROS). Hypericin from *Hypericum perforatum* as an active photosensitizer assists in ROS generation in response to light. *Hypericum perforatum* alone has little effect on cell life but when an activation mixture, made up of ascorbic acid, is added, it drastically increases early apoptosis in cells.

These researchers could not find studies delineating the effects of the whole extract as opposed to its component compounds. Although hypericin from *Hypericum perforatum* has had positive effects in cancer treatments so far, when combined with other drugs for in vivo treatment it can cause dangerous side effects such as reduced plasma levels of antiretroviral agents, which increases patients' risk for disease progression. Also, when used as a photosensitizer in photodynamic and catalytic therapy, an increase in dosage of hypericin results in a shift from apoptotic to necrotic death resulting in a harmful inflammatory response in the patient.

Our study tests the effects of a whole extract of *Hypericum perforatum* and one of its chemical constituents, hypericin, on the growth of a mouse mammary cancer cell line. The first hypothesis is that if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, then the cancer cells exposed to the extracts will grow at a slower rate than the control cells. The second hypothesis is that if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, they will slow the cell growth at an equal rate. The third hypothesis is that if *Hypericum perforatum* and hypericin are added in greater concentrations to the mouse mammary cancer cells, then the cells will grow at an increasingly slower rate.

Our study is important because although hypericin has been used before in cancer such as PDT or CT, there are many negative side effects associated with current treatments such as increased risk for disease progression and induction of an inflammatory response. If we could find the optimal dosage of hypericin and *Hypericum perforatum* extract, it could be used on its own as a separate form of cancer treatment.

**Materials and Methods**

CRL2539 mouse mammary cancer cells (American Type Culture Collection) were seeded at concentrations of 1.0 x 104/25cm2.
Cancer inhibitory activity of liquid glycerol based *Hypericum perforatum* extract (0.3% hypericin) (Puritan’s Pride Incorporated) and hypericin (Planta Analytica) were determined by exposing cells to their various concentrations for 96 – 264 hrs. or until control cells reached confluency. Each set of trials were run with three 25 ml control flasks, three 25 ml 0.4% *Hypericum perforatum* flasks, and three 25 ml 0.8% *Hypericum perforatum* flasks or three 25 ml 0.001% hypericin flasks. Seven mL of media were needed for each flask, so media was made in sets of 21 mL with dilutions as follows. 21 mL of DMEM with 10% FBS were made for the control cells. 0.084 mL of *Hypericum perforatum* and 20.916 mL of DMEM with 10% FBS were made for the 0.4% *Hypericum perforatum* flasks of cells. 0.168 mL of *Hypericum perforatum* and 20.832 mL of DMEM with 10% FBS were made for the 0.8% *Hypericum perforatum* flasks of cells. 0.021g of hypericin was dissolved in 1mL of ethanol for the 0.001% hypericin flasks of cells. This was added to 20mL DMEM, 1mL of ethanol was added to 20mL of DMEM for the ethanol flasks of cells. Cells were cultured in a 5% CO2, 95% air, fully humidified incubator at 37°C. Control CRL2539 cells were cultured in DMEM containing 10% FBS. Experimental CRL2539 cells were cultured in DMEM containing 10% FBS and 0.4% *Hypericum perforatum*, 0.8% *Hypericum perforatum*, or 0.001% hypericin. Cell growth was estimated at periods of 24 – 48 hrs. using pictures from the inverted microscope. Three pictures were taken at randomly dispersed locations throughout each flask. The cells in each picture were counted and averaged so that the number of cells per mm² was found. This number was multiplied by the surface area of the flask, 1763 mm², to find the average number of cells in each flask at a certain time. At the end of each trial we found the final cells counts in each flask using a hemocytometer. We performed a hemocytometer count per flask three times and average the number of cells counted to ensure an accurate representation. Statistical evaluation of the results was performed with the Student’s t-test using the Microsoft Excel software. Probability values equal or less than equal to 0.05 were considered significant. Normal distribution graphs were constructed based t-test values using Fathom Dynamic Data software.

**Results**

Graphs comparing the control and experimental trials were made to compare the growth rates of the cells over time. Figures 1 and 2 show the average number of cells that were in each of the three control or experimental flasks over time. After 96 hours for the 0.4% *Hypericum perforatum* and after 72 hours for 0.001% hypericin it is clear that the experimental cells are growing slower than the control cells. The difference in the cell populations between the experimental and control flasks increases as time goes on. Figure 3 shows the average number of cells in each of the three 0.4% or 0.8% *Hypericum perforatum* flasks. The figure shows that the cell population of the 0.8% *Hypericum perforatum* concentration is less than the cell population of the 0.4% concentration over time.

To analyze the cell population data, t-tests were run to compare the mean number of cells at various times between the control and experimental flasks. The purpose of this test is to determine if there is a statistically significant difference between the cell populations at the set times, shown in the resulting p-value. As shown in table 1, p-values of less than 0.05 were observed for

![Figure 1: Hypericum perforatum at 0.4% inhibited the growth of CRL2539 cells.](image1)

![Figure 2: Hypericin at 0.001% inhibited the growth of CRL2539 cells.](image2)

![Figure 3: Hypericum perforatum 0.8% concentration inhibited the growth of CRL2539 cells further than Hypericum perforatum at 0.4% concentration.](image3)
the control compared to the *Hypericum perforatum* (0.4%) at times 96 hours (p-value 0.013) and 144 hours (p-value 0.0015). As shown in table 1, at 24 hours the p-value was not less than 0.05 (p-value 0.45). As shown in table 3, p-values of less than 0.05 were also observed for the control compared to the hypericin (0.001%) at times 72 hours (p-value 0.037) and 120 hours (p-value 0.007). However, at time 48 hours, the p-value for the hypericin trial was not less than 0.05 as shown in table 3. T-tests were also run comparing the cell populations of 0.4% and 0.8% concentrations of *Hypericum perforatum* at certain times. Table 2 shows p-values of less than 0.05 at times 48 hours (p-value 0.04), 96 hours (p-value 0.03), and 120 hours (p-value 9x10^-5). In addition, table 4 shows a p-value of more than 0.05 at time 48 hours comparing the ethanol population to the control population. These statistical tests show that the hypericin and *Hypericum perforatum* concentrations significantly slow the rate at which the cancer cells are growing after certain times. Ethanol does not significantly affect the rates at which the cancer cells are growing. From this, it can be concluded that the anti-cancerous properties exhibited by hypericin are not a result of the ethanol the extract was dissolved in.

Figures 4-6 provide a visual demonstration of the statistically significant difference between the experimental and control means. Because none of the graphs had overlapping error bars, the mean values of the control and experimental flasks were all found to be statistically different.

**Discussion**

The first hypothesis, “If *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, then the cancer cells exposed to the extracts will grow at a slower rate than the control cells” was accepted. It was concluded with confidence that *Hypericum perforatum* and hypericin extracts did significantly slow the growth of the mouse mammary cancer cells after times of 96 hours for the *Hypericum perforatum* extract and 72 hours for the hypericin extract. With time, both extracts decreased cell growth at greater rates as shown in the decreasing p-values over time (0.013 to 0.0015 from times 96hrs to 144hrs for the *Hypericum perforatum* extract and 0.037 to 0.007 from times 72hrs to 129hrs for the hypericin extract). The hypericin did not slow the growth of the cells to as great an extent as the whole *Hypericum perforatum* extract.

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**Table 1. t-Test Two-Sample 24, 96, 144 Hours 0.4% Hypericum perforatum vs. Control**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Mean Number of Control Cells</th>
<th>Mean Number of 0.4% Hypericum perforatum Cells</th>
<th>P-value (two-tail)</th>
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<tbody>
<tr>
<td>24</td>
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<td>144</td>
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<td>0.0015</td>
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</tbody>
</table>

**Table 2. t-Test Two-Sample 48,72, 120 Hours Control vs. 0.001% Hypericin**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Mean Number of Control Cells</th>
<th>Mean Number of 0.001% Hypericin Cells</th>
<th>P-value (two-tail)</th>
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<tbody>
<tr>
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<tr>
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<tr>
<td>120</td>
<td>818000</td>
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</tbody>
</table>

**Table 3. t-Test Two-Sample 48, 96, 120 Hours 0.4% Hypericum perforatum vs. 0.8% Hypericum perforatum**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Mean Number of 0.4% Hypericum perforatum Cells</th>
<th>Mean Number of 0.8% Hypericum perforatum Cells</th>
<th>P-value (two-tail)</th>
</tr>
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<tbody>
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**Table 4. t-Test Two-Sample 48 Hours Control vs. Ethanol**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Mean Number of Control Cells</th>
<th>Mean Number of Ethanol Cells</th>
<th>P-value (two-tail)</th>
</tr>
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<tbody>
<tr>
<td>48</td>
<td>43194</td>
<td>33105</td>
<td>0.26</td>
</tr>
</tbody>
</table>
extract did shown by the cell populations of the hypericin at the same time as the *Hypericum perforatum* populations which were higher. Therefore, the second hypothesis “if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, they will slow the cell growth at an equal rate” was not accepted. This demonstrates that hypericin is not the only active compound responsible for the anti-cancerous properties of *Hypericum perforatum*. The third hypothesis “if *Hypericum perforatum* is added in greater concentrations to the mouse mammary cancer cells, then the cells will grow at an increasingly slower rate” was accepted because the cells with concentration of 0.8% grew at a significantly slower rate than the cells with concentration 0.4%.

This study demonstrated that *Hypericum perforatum* significantly slows the rate at which mouse mammary cancer cells grow. The results indicate that as concentrations of *Hypericum perforatum* increase, the rate at which the mouse mammary cells are growing decreases. This study did not determine if there is an optimum dosage of *Hypericum perforatum* for the cells. This study also indicated that hypericin significantly decreases the growth rate of the mouse mammary cancer cells. However, the hypericin did not slow the growth rate to as great an extent as the 0.4% *Hypericum perforatum* whole extract did, indicating that hypericin is not the only component of *Hypericum perforatum* responsible for its anti-cancerous properties.

The cell populations of the 0.4% and 0.8% concentrations of *Hypericum perforatum* were also found to be statistically significantly different. The cell populations of the flasks with 0.8% *Hypericum perforatum* concentration were significantly lower than the flasks with the 0.4% concentration. Therefore, it has been demonstrated that with an increase in concentration of *Hypericum perforatum* there is a correlating decrease in the rate at which the mouse mammary cancer cells were growing.

In an unpublished study, *Hypericum perforatum* was found to inhibit or denature the proteins aldolase and enolase in *Drosophila melanogaster* flies. Aldolase and enolase both play a major role in glycolysis. Upregulation of glycolysis has been observed in many cancer studies which suggest that inhibition of the glycolytic pathway may decrease or halt cancer progression. Therefore, we will examine the mechanisms behind the inhibition of cancer progression through *Hypericum perforatum* shown in this study looking specifically for the inhibition or denaturation of aldolase and enolase. Further research could also investigate the mitochondrial membrane to determine if the extracts are interfering with its function thus giving more evidence for the mode of action hypericin uses to inhibit cell growth.

![Figure 5: Hypericum perforatum at 0.8% inhibited the growth CRL2539 cells significantly compared to the growth of Hypericum perforatum at 0.4% cells starting at 48 hours.](image)

![Figure 6: Hypericin at 0.001% inhibited the growth CRL2539 cells significantly compared to the growth of control cells starting at 72 hours.](image)
References


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Survivin is Essential for Efficient Cell Mobility and Proliferation in U87 and C6 Glioma Cells

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Abstract
The BIRC5 gene, which codes for Survivin and is a member of the Inhibitor of Apoptosis family, is activated in most cancer cells including gliomas. The BIRC5 gene's role in cancer motility is not well understood. From the functions of similar genes, like those in its family, it was hypothesized that the gene would play an important role in cancer motility as well as cancer proliferation1. The BIRC5 gene's importance in migration and proliferation was studied in rat C6 and human U87 glioma cells. The gene's importance in migration was assessed by observing the migration behaviors of four groups of cells: an experimental group that was transfected with BIRC5 siRNA and permitted to grow for one day after transfection, another experimental group that was permitted to grow for two days after transfection, and two corresponding control groups that did not undergo RNAi of BIRC5 and were permitted to grow for one and two days. The control groups exhibited near-equal levels of migration when a migration assay was performed. Both groups that underwent RNAi of BIRC5 showed impaired migratory ability. When proliferation was analyzed for the same groups, the cells that underwent RNAi showed diminished ability to reproduce and survive, especially when they were plated at lower densities. These results suggest Survivin is a promising drug target in the control of glioma cell motility and proliferation.

Introduction
Gliomas are the most common type of malignant tumors of the human brain and are known for their ability to develop rapidly and migrate quickly throughout the brain. Though, gliomas can differ in many traits- such as migratory abilities, proliferation rates, morphology, and extent of drug resistance- they are highly aggressive especially in later stages. Their migratory abilities permit the glioma cells to embed themselves deep within the brain, grow into large tumors, and elude early diagnosis. By the time symptoms, such as impaired vision and hearing, are evident, these tumors have often already spread and have caused irreparable damage to the brain; in fact, GBM most often goes undetected until the last stage of cancer, stage IV. This migratory characteristic is also what renders many of the tumors they form inoperable and usually lethal2.

Glioblastoma Multiforme (GBM) is the most common type of glioma. The three other types of glioma are: anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma. Most GBM patients have a survival rate of less than two years. There have been cases of patients who have 99% of tumor mass removed, undergo chemotherapy and radiation, but die within a few years because the 1% of cancerous tissue that was not removed was so motile and resilient. In Stage 1 and 2, gliomas have not become extremely motile, and in fact begin to form small clumps of cancerous cells and have improved survival rates compared to other cancers, especially if they are operable. By stage III, tumors begin to grow much faster than before, become extremely aggressive and begin to spread to other parts of the brain. By this point, tumors have grown large enough to kill off healthy tissue and take root near large blood vessels, which support their growth. The tumors begin consuming the healthy tissue on their periphery and siphon nutrients to their edges, weakening the tumor at the center, and accelerating outward growth. The median survival for stage 3 GBM is 4 years while the prognosis for stage 4 is 12 months or less. It is the hope of researchers to develop a treatment to reduce the migratory characteristic of the cancer cells so that radiation, chemotherapy, and surgery can be rendered more effective1.

Irregular function of proteins that control apoptosis is closely tied to increased cancer susceptibility and aggression. p53, for example, serves many functions, but a key role in organisms is to coordinate cell-death by acting on a variety of different proteins that promote apoptosis and regulate the cell cycle. A deformity of this protein that impairs function or the lack of p53 in functional levels often leads to cancer, though not exclusively for its ability to coordinate cell death. Similarly, over-activity or excessive production of proteins that allow cells to bypass cell cycle checkpoints has been found to aid in the development of cancer1. Many recent studies have found that Inhibitors of Apoptosis (IAP family), like XIAP and cIAP are notably up-regulated in most cancer cell lines and have been closely tied with increased tumor aggression and proliferation2. Surprisingly, though most of the IAP family and similar protein have been successfully tied to playing a large role in fostering the aggressive nature of tumors, the importance of these proteins has not been quantified in many cancer cell lines5.

Survivin itself has also been tied to increased resistance to chemotherapy in most cancer cell lines, but we know little of its effects on Glioma proliferation and it is unknown whether this protein affects movement of gliomas which is a serious concern when observing cells that are as motile as gliomas6. It is logical that Survivin, like similar proteins, plays an important role in the ability of cancer cells to be resilient and prolific because Survivin is a key protein in regulating cell death. Cancer cells with elevated Survivin expression have in fact been found to be strongly correlated with shorter remissions and over-all survival when compared to cancer cells that have relatively typical Survivin levels for their cell type. Moreover, elevated Survivin has been so strongly correlated with enhanced proliferation abilities, reduced apoptosis, resistance to chemotherapy, and tumor reoccurrence that Survivin expression has been used as a prognostic tool in many cases.
It is not surprising that Survivin is found to aid in the G2-M phase transition in most cancer cell lines. Its anti-apoptotic effects primarily work to inhibit caspase activation, and so its presence - which is rare in most adult mammalian cells - disrupts the cycle of cell death and permits cells to bypass regulatory checkpoints that would otherwise lead to cell death in the cancer cells. Though its anti-apoptotic expression is cell cycle-dependent in most cancer cell lines, there are some lines that have cell cycle-independent Survivin expression, suggesting other functions beyond its effect in G2-M phase transition. Another way Survivin regulates cellular activities in cancer cells is by aiding in the formation of microtubules and spindle fibers which could affect both proliferation and migration. Cells that are becoming more and more mutated would typically be programmed to die. Tumor cells which exhibit augmented Survivin continue living and accumulating more mutations. In fact, in recent studies, adult rats (which normally do not produce Survivin) suffered from increased cancer rates when given Survivin injections. Researchers believe that if Survivin were knocked down, many cells might be automatically launched into a cycle of programmed death. In a less idealistic result, only the most extensively mutated cells might be destroyed, leaving less mutated and probably less aggressive cells behind and would probably make cells more susceptible to apoptosis outside of Survivin’s G2-M phase function.

Beyond Survivin’s known role in cancer cells, it is known to function in other mitotically active cells, especially those that have not fully differentiated. Elevated levels of the protein have been found not only in mammalian tumors, but also in mammalian fetuses. In mammalian fetuses, brain cells are particularly reliant on the protein to survive, and for this reason, it is thought that the protein might play a particularly important role in gliomas. When this protein is knocked down or knocked out in mammalian fetuses, there is widespread cell death. The greatest rate of cell death in fetuses lacking the protein was found in neurons, pointing to evidence that these sensitive cells may be particularly reliant on this protein to grow, and survive. For this reason, Survivin is of particular interest to researchers of brain cancer. Furthermore, when one considers the long lifespan of cerebral cells, and their sensitivity against mutations, it would be logical that Survivin would be of special importance for cells to bypass regulatory checkpoints.

Survivin has not been found to be expressed in most adult human tissues, though Survivin has been found to be expressed in functional concentrations in a limited number of adult human hematopoietic progenitor cells as well as some immune cells. When studied in a rat model, whose cells also express Survivin in the same tissues as humans, decreased levels of Survivin in developmental stages did decrease the number of peripheral blood T cells, but had no effect on normal thymic function. Moreover, Survivin deletion reduced the total number of total progenitor cells and primitive hematopoietic cells, but had little effect on mature blood cells. The rat model studies that have tested Survivin targeted treatment of tumors showed promising results with either no measurable adverse effects or limited adverse effects on normal tissues. Researchers believe that a likely reason why anti-Survivin treatments showed few adverse effects is the localized nature of anti-Survivin treatments. Because anti-Survivin reagents are administered within tumors, there is probably little dissemination of anti-Survivin reagents to healthy tissues elsewhere in concentrations that could be hazardous. Another proposed explanation is that anti-Survivin therapy may not affect nonproliferating adult tissues because Survivin may only be required for proliferating adult tissues. Overall, research has found that anti-Survivin treatments can affect some healthy cells, but no systemic toxicities have been found in animal models. Of course, organ systems that continually renew themselves will need to be closely monitored in long-term anti-Survivin treatments, but over-all Survivin has been found to be a promising target for clinical intervention.

Though Survivin has been implicated in the resilience, drug resistance, and proliferation of most types of cancer, it is not known whether Survivin plays a large role in the migratory characteristics of many cancers. However, some recent studies have found that Survivin is important in the motile characteristics of melanoma cells. Similar findings have been seen in renal cell carcinomas. The exact mechanism by which Survivin contributes to cell migration is unknown. However, it is known that Survivin is needed for microtubule formation to occur properly in a variety of cancer cell lines, and thus its inactivation might adversely affect the creation of pseudopodia needed for glioma migration. It is the hope of this research to demonstrate whether Survivin is an important protein in permitting cell migration in human and rat glioma cells. This question will be investigated with the aid of RNAi.

RNA Interference (RNAi) is a relatively new, but extremely promising tool in the field of biology. Unlike previous techniques for suppressing gene expression, like those involving the use of antisense RNA, RNAi does not risk triggering the interferon response of cells that shuts down nearly all gene expression. RNAi is selective in its suppression or knockdown of genes. When a cell is infected by a virus, which often injects a dsRNA (Double Stranded RNA) copy of its genome into the cell, a response is triggered within the cell that inhibits only the expression of the corresponding genes of the viral dsRNA. An enzyme called dicer hydrolyzes the dsRNA into shorter sequences called siRNAs that are then unwound. Once unwound, each half of the siRNA is incorporated into a group of proteins to form RISC (RNA-induced Silencing Complex). RISC targets only corresponding mRNA molecules in the cell, both native and invasive. Once a target mRNA is found, it binds to RISC and an enzyme called slicer destroys the mRNA, and RISC moves on to find more target mRNA molecules.

The first model system for this investigation was a rat cell line: the C6 rat glioma cell line. C6 glioma cells are a functional model system for research involving cancer, namely GBM. The cell line is useful as a model system in part because it not only resembles GBM in vitro, but also accurately resembles human GBM when these cells are transplanted into rats’ brains. When the cells are transplanted into rat brains, they very closely resemble GBM in certain strains of rats.

It is important to take into consideration, however, that although C6 glioma cells behave similarly to human glioma cells, the lines are not identical. Although humans and rats share much genetic material, many proteins behave differently in each
organism. Also, if in vivo survival experiments are ever to be conducted in rats using the rat cell line, it would be wise to ensure that the in vitro studies show the protein behaves similarly in both human and rat lines prior to experimentation. For these reasons, human U87 glioma cells were added to the scope of this study.

In this experiment, the importance of Survivin on glioma cells was investigated. Specifically, its importance on the cancer’s ability to proliferate and migrate was observed. By treating rat C6 and human U87 glioma cells with siRNAs that would inhibit Survivin production, the importance of the protein was assessed in comparison to cancer cells that were mock transfected (no exposure to siRNA took place, but all other reagents were used). Proliferation assays were conducted at different times after RNAi in order to measure the importance of the protein in concentration. Migration assays were conducted with cells that had undergone RNAi at the same time intervals.

Materials and Methods

Cell lines and RNAi: C6 rat and U87 human glioma cells were a kind gift from Dr. Peter Canoll (Columbia University). Birc5 Silencer Pre-designed siRNA was purchased from Ambion. Birc5 siRNA sense strand is as follows: 5’ GGAACUGGAAGGCUGGGAA 3’. All cells were maintained at 37°C at 5% CO₂. The incubation period with RNAi reagents was six hours to allow for transfection and interference to occur. The half-life of Survivin is only 30 minutes, as it is a short-lived protein; for this reason we are confident that endogenous Survivin was not allowed to function long after exposure to RNAi reagents. Furthermore, we believe that later generations of cells also displayed knockdown, as one transfection with RNAi reagents at proper concentrations is sufficient to knockdown gene expression in most cancer cell lines for 5-7 days. However, these statements were not verified by testing for mRNA coding for Survivin or Survivin protein directly. Western blotting and quantitative PCR are possible ways of addressing this uncertainty. Proliferation Assays: C6 and U87 glioma cells were first transfected with the BIRC5 siRNA using Lipofectamine 2000 reagent (Invitrogen), in accordance with the manufacturer’s instructions (20 pmol of siRNA were diluted in 50 ul of pure DMEM in one tube. In a separate tube, 20 ul of Lipofectamine 2000 reagent were diluted in 50 ul DMEM. Both tubes were incubated for at least 5 minutes and were then combined and incubated for at least 20 minutes. The mixture was then added to groups of 5,000 and 2,500 C6 and U87 glioma cells that had been plated on poly-L-lysine-coated glass coverslips, prior to transfection. The coverslips were maintained in 0.5 ml of pure DMEM. After the mixture containing the siRNA was added to the DMEM where the cells were, the cells were incubated for six hours. Control groups of C6 and U87 cells at corresponding original densities were treated with the same reagent mixture as the experimental groups, excluding siRNAs. Six hours after exposure to transfection reagents, the pure DMEM solution covering the cells was removed and replaced with DMEM containing the antibiotics penicillin and streptomycin. Cells were then incubated at 5% CO₂ and 37°C. Cells that were plated on coverslips at densities of 5,000 cells were allowed to grow for 24 hours and cells plated at concentrations of 2,500 were allowed to grow for 48 hours. The same procedure was carried out with a group of C6 cells plated at 70,000 and 35,000 cells. This was done in order to determine whether cell density plays an important role in Survivin behavior in this cell line. It was not performed in the human line.

Analysis of Proliferation Assays: After the cells were permitted to grow for the determined amount of time, the DMEM and antibiotic solution was removed and replaced with phosphate buffered saline (PBS). The new saline, which had come into contact with the cells was removed and gently replaced with fresh PBS. This step was performed a total of three times, in order to minimize the DMEM solution left on the cells. The cells were then stained with Hoechst solution (gift from Maria Chiara Manzini) and were plated. For the analysis of the proliferation assays consisting of cells plated at 5,000 and 2,500 cells, eight randomly selected viewing fields of each cover slip were photographed at low magnification using a Hamamatsu digital camera. The cells were then counted individually. To analyze the density of the cells of the group plated at 70,000 and 35,000, nine randomly selected fields were captured for each coverslip, and two random but equally sized subfields of each of the nine fields were counted. After the covers were counted for individual slides in a group, the values of those slides were all averaged. For the U87 line, three coverslips were made for each of the two controls. For the experimental groups, four coverslips (each a separate RNAi trial) were made for the experimental group that grew for 24 hours and three were made for the experimental group that grew for 48 hours after transfection. For the C6 group plated at low densities, four coverslips were made for the control that grew for 24 hours and three were made for the control that grew for 48 hours. For the C6 cells grown at low densities, four coverslips (each a separate RNAi trial) were made for the each of the experimental groups. For the C6 cells plated at high densities, four cover slips were used for all of the controls and four cover slips were made for all of each of the experimental groups. Migration Assays: C6 and U87 glioma cells were first transfected with the Birc5 siRNA using Lipofectamine 2000 reagent, in accordance with the manufacturer’s instructions, in a way similar to that used for the proliferation assays (200 pmol of siRNA were diluted in 50 ul of pure DMEM in one tube. In a separate tube, 200 ul of Lipofectamine 2000 reagent were diluted in 50 ul DMEM. Both tubes were incubated for at least 5 minutes and were then combined and incubated for at least 20 minutes. The mixture was then added to groups of 500,000 C6 and U87 glioma cells that had been plated on 60 mm plates 24 hours before transfection. The plates were filled with 5 ml of pure DMEM. After six hours, the DMEM on the cells was changed for DMEM containing Penicillin and Streptomycen. After the first six hours the cells were placed back in an incubator for at 5% CO₂ and 37°C. Again, two groups were made: one permitted to grow for 24 hours after transfection, the other was permitted to grow for 48 hours after transfection. The cells were trypsinized after 24 and 48 hours, and 500,000 cells were added to the top of a collagen coated transfilter. The top of the filter was filled with 200 ul of pure DMEM (serum –free). The well on which the filter sat was filled with 500 ul of DMEM and a chemoattractant: in this case 10% Fetal Bovine Serum (FBS). The migration assay was permitted to run for 6 hours. Controls were plated at the same original densities and were mock transfected with the same protocol, but excluding the addition of siRNA. Filters for the...
controls were prepared in the same way as filters for the experimental groups following the controls’ mock transfection. **Analysis of Migration Assays:** After the migration assays were performed, the filters were rinsed with PBS much like the coverslips of the proliferation assay. The top of each filter was rinsed with .2ml PBS and was moved to wells filled with .5 ml PBS after every rinse, in order to clean the underside of the filter. A total of three rinses were performed. After the filters were rinsed, the filters were dyed with Hoechst solution and mounted on microscope slides. Under a microscope (Carl Zeiss Axioplan 2 upright fluorescent microscope) four randomly selected viewing fields of the underside of each filter were captured (this would be the end point of the assay as cells were added to the top of the filter). All cells in those fields were counted and the numbers were averaged. Three filters were used for each of the control and experimental groups in both cell lines with the exception of the C6 line that was transfected 24 hours before the migration assay was run. For both the control and experimental groups of C6 cells that were transfected 24 hours before the migration assay was conducted, four filters were used.

**Results**

**Proliferation assays**

When the experiment was performed and the control groups were analyzed, similar numbers of cells were seen on the control groups for the group that was allowed to grow 24 hours and the group that grew for 48 hours. This suggests that the cells in the experimental groups can have their rates of proliferation compared between the 24 and 48 hour groups, as the controls were growing at similar rates. That is, the 24 hour group was plated at 5,000 cells and ended with 10,000 cells while the 48 hour group was plated at 2,500 cells and ended with 10,000 cells also. This information supports the notion that both groups had cells that were doubling roughly every 24 hours.

When the data for the group of U87 cells that were plated at 5,000 cells (and were permitted to grow for 24 hours) was first analyzed, there was a 53±4.8% decline in cell numbers relative to its control. This was interesting because this was a cell population that was doubling every 24 hours, and instead of doubling in number declined to less than one half the end value of the control (below the hypothetical starting value of the experimental group). When the proliferation assay that used cells that were allowed to grow for 48 hours after transfection was compared to its control, a decline of 76±2.9% was observed. Figure 1 shows a graphical representation of these results in terms of cell numbers. As seen in Table 1, when the controls and the experimental groups were compared, highly significant correlation was found in all cases. P<0.0027 for the groups that grew for one day and p< 0.00033 for the group that grew for two days. The two control groups were not significantly correlated as p>0.15; this is not surprising considering their values overlapped.

The group of C6 cells that were originally plated at 5,000 and 2,500 cells was then analyzed. The 5,000 cell experimental group that grew for 24 hours after transfection showed a 31±5.2% decline in cell numbers relative to its control. When the group that grew for 48 hours after transfection was compared to its control, a 61.8 ± 7% decrease was noticed relative to the corresponding control. Figure 2 shows a graphical representation of these results in terms of cell numbers. As seen in Table 2, when the controls and the experimental groups were compared, highly significant correlation was found in all cases. P<0.00012 for the groups that grew for one day and p< 6.06x10⁻⁵ for the group that grew for two days. The two control groups were not significantly correlated as p>0.78; this is not surprising considering their values overlapped.

![Figure 1. U87 5,000/2,500 Cell proliferation assay run for 24 hours and 48 hours.](image)

**Table 1. U87 proliferation Assays**

<table>
<thead>
<tr>
<th></th>
<th>Average Value</th>
<th>% Change relative to the corresponding control</th>
<th>p-value between the control and experimental group</th>
<th>p-value between the two controls</th>
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</thead>
<tbody>
<tr>
<td>24 hour control</td>
<td>183.5</td>
<td>53 +/- 4.8% decrease</td>
<td>p&lt;0.0027</td>
<td>p&gt;0.15</td>
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<tr>
<td>24 hour experimental</td>
<td>86.24</td>
<td>76 +/- 2.9% decline</td>
<td>p&lt;0.00033</td>
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<tr>
<td>48 hour control</td>
<td>194.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>48 hour experimental</td>
<td>46.68</td>
<td></td>
<td>p&lt;0.00033</td>
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</tbody>
</table>
When the data for the group of C6 cells that was plated at 70,000 cells (and was permitted to grow for 24 hours) was first analyzed, there was an 18±3.1% decline in cell number relative to the corresponding control. When the proliferation assay that used cells that grew for 48 hours after transfection was compared to its control, a decline of 36±4.3% was observed. Figure 3 shows a graphical representation of these results in terms of cell numbers. As seen in table 3, when the controls and the experimental groups were compared, highly significant correlation was found in all cases. P<0.00036 for the groups that grew for one day and p< 0.0039 for the group that grew for two days. The two control groups were not significantly correlated as p>0.238.

**Migration Assays**

After the U87 cells were transfected, both the group that was permitted to grow for 48 hours after transfection and the group that was permitted to grow 24 hours after transfection had 500,000 cells counted and placed in a transfilter. When the control of the group that was assessed one day after transfection was compared to the experimental group, a 26±2% decrease in migration of the experimental was observed. When the group that was assessed two days after transfection was compared to its control, a 49±3% decrease in migration of the experimental group was observed. Figure 4 depicts this data in terms of cell numbers. As seen in table 4, when the controls and the experimental groups were compared, highly significant correlation was found in all cases. P<0.00066 for the groups that grew for one day and p< 1.69x10^-5 for the group that grew for two days. The two control groups were statistically uncorrelated as p>0.179; this is not surprising as there was overlap.

After the C6 cells were transfected, the group that was permitted to grow for 48 hours after transfection and the group that was permitted to grow for 24 hours after transfection had 500,000 cells counted and placed in a transfilter. When the controls were compared, they had similar numbers of cells per viewing field- there was only about 4% difference in number between the controls where the group that grew for only one day was slightly more populous. When the control of the group that was assessed one day after transfection was compared to the experimental group, a 23±3.1% decrease in migration in the experimental was observed. When the group that was assessed two days after transfection was compared to its control, a 46±6% decrease in migration of the experimental group was observed. Figure 5 depicts this data in terms of cell numbers. As seen in table 5, when the controls and the experimental groups were compared, highly significant correlation was found in all cases. p<1.78x10^-6 for the groups that grew for one day and p< 0.0012 for the group that grew for two days. The two control groups were statistically uncorrelated as p>0.597.

**Table 2. C6 Proliferation Assays at Low Densities.**

<table>
<thead>
<tr>
<th></th>
<th>Average Value</th>
<th>% change relative to the corresponding control</th>
<th>P value between the control and experimental group</th>
<th>P value between the two controls</th>
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<tr>
<td>24hr. control</td>
<td>279.25</td>
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<td>P=0.78</td>
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<tr>
<td>24hr. experimental</td>
<td>192.7</td>
<td>31±5.3% decline</td>
<td>P&lt;0.00012</td>
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<td>48hr. control</td>
<td>256.33</td>
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<tr>
<td>48hr. experimental</td>
<td>106.6</td>
<td>60±7.2% decline</td>
<td>P&lt;0.06 x 10^-5</td>
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</tbody>
</table>

**Table 3. C6 Proliferation Assays at High Densities.**

<table>
<thead>
<tr>
<th></th>
<th>Average Value</th>
<th>% change relative to the corresponding control</th>
<th>P value between the control and experimental group</th>
<th>P value between the two controls</th>
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<td>338.3</td>
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<td>P=0.238</td>
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<tr>
<td>24hr. experimental</td>
<td>293.56</td>
<td>18±3.1% decline</td>
<td>P&lt;0.00036</td>
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<tr>
<td>48hr. control</td>
<td>339.4</td>
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<td></td>
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<tr>
<td>48hr. experimental</td>
<td>217.22</td>
<td>36±4.2% decline</td>
<td>P&lt;0.0039</td>
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</tbody>
</table>
Discussion

The motility of glioma cells is one of the characteristics that renders this type of cancer so deadly. At the same time, these cells are resilient, and can withstand chemotherapy well, especially because of the blood-brain barrier that prevents many chemicals from entering the brain\(^1\). For this reason, scientists and doctors are looking for non-invasive treatments for GBM and other cancers that arise from glial cells. Survivin is a promising drug target for many researchers because it is not present in most healthy adult cells, and anti-Survivin treatments in animal models have not resulted in over-toxicity in the few adult tissues that do express Survivin (primarily hematopoietic cells and some immune cells)\(^7\).

It was the hope of this study to explore the role Survivin plays in glioma cells. Through this research, five major questions were answered: How significant is Survivin’s effect on viability in glioma cells? How significant is Survivin’s effect on glioma migration? Is the effect on migration a result of an effect on viability? Does cell density alter the importance Survivin has on viability? Does Survivin have comparable effects on viability and motility between C6 rat glioma cells and U87 human glioma cells? For the most part, these questions were answered with extremely high statistically significant results. Based on the assumption that Survivin was knocked down, they hypotheses were supported. However, as Western blot of Survivin has not been performed yet, this assumption cannot be made with absolute confidence, despite the manufacturer’s guarantee of specificity for Survivin.

Glioma cells are, like many other cancer cells heavily mutated and have often bypassed cell cycle checkpoints with the aid of inhibitors of apoptosis. Once such inhibitory molecules are removed, there may be few things preventing...
the cancer cell from undergoing apoptosis. Most Surviving cells would be expected to be compromised, and would probably dependent on other similar proteins which would not be able to protect them beyond a certain point in the life cycle of the constantly mutating cancer cell especially because many inhibitors of apoptosis are cell cycle dependent and have little control over whether a cell bypasses regulatory checkpoints that specific protein is not responsible for regulating\textsuperscript{13}.

Though a lack of Survivin leads to a decline in cell numbers in all groups, when one compares the group of cells plated at high densities (70,000 and 35,000 cells) to the group plated at low densities (5,000 and 2,500 cells) there are differences in the rate of decline, as seen in Figures 2 and 3. While the high density group experienced a decline in cell counts of about 20% within 24 after transfection and a decline of about 40% within 48 hours, the low density group had higher rates or cell death. The low density group’s counts experienced a decline of about 35% within 24 hours of transfection and about 60% after 48 hours. From these findings it is clear that Survivin’s role in cell proliferation is altered by cell density.

It is not surprising that Survivin’s importance is reduced when cell density is high. Though Survivin is important for cancer cells to bypass regulatory checkpoints, other mechanisms can support growth and may partially counteract the lack of Survivin, by signaling growth through other means. One mechanism that controls cell growth when cell density is high, is the release of cytokines into the surrounding medium. It would have been easy for the cells plated at high density to condition their medium and support each other’s growth, but far more difficult for cells plated at a fourteen times lower density to condition a proportionately large volume of medium. This would explain the disparity between the rates of regression between the densely plated and sparsely plated proliferation assay groups, though there are many more reasons that cell density can affect the behavior of cancer cells.

The second set of experiments demonstrated that Survivin is an important contributing factor to the motility of both U87 glioma cells and C6 glioma cells. As is seen in figure 4, between the control and the experimental group assessed one day after transfection in U87 cells there was a decline of roughly 26% in migration. Between the control and the experimental group of U87 cells assessed two days after transfection, there was a decline of roughly 49% in total migration. As can be seen in figure 5, when compared to its control the group assessed one day after transfection in C6 cells showed a reduction of about 23% in migration. Between the control and the experimental group of C6 cells assessed two days after transfection, there was a change of 46% in total migration. Because there was such a large reduction in the number of cells that were able to migrate across the transfilter, the data suggests that Survivin is responsible for aiding migration significantly.

When one couples the reduction in the number of cells due to their death and the decrease in migration, it might be tempting to believe that the reduction in cell numbers that Survivin knockdown causes is at least a partial reason for the reduction in migration. This is because cells could be dying on the way through the filter, while control cells might be reproducing. However, analysis of the data shows that while cells are migrating, cell number changes are only partially responsible for the apparent reduction in migration.

There are two extremely important things to keep in mind when analyzing this data. First, cells in the migration assay only had six hours to pass through the filter before the migration assay was stopped (1/4 the normal life cycle). Second, cells that were treated against Survivin were either not increasing in number or increasing in number more slowly when compared to their control's rate of change. Over a six hour period, a mock-transfected control group in all cell lines will increase in cell number to about 125% of the starting number (because every 24 hours cells increase in number by 100%). In the case of U87 cells, a humble decline of about 1% would be seen in the course of six hours. For C6 cell experimental groups, an increase of as little as 3.75% of the starting value would be seen (This would be the case that most heavily favors the null hypothesis). Because migration was measured relative to the number of control cells at the end of the assay, the difference in reproduction rates could of course largely contribute to the results seen.

Regardless, when cell numbers are adjusted to take cell reproduction into account, drastic decreases in migration still could not be explained. For example, in the U87 cell line, for every 100 cells that started migrating through the filter in the experimental group, 99 cells could be expected to be alive after the six hour long experiment. In contrast, for every 100 cells that were used in the corresponding control, 125 should have been alive at the end. If the null hypothesis were correct, and migratory ability was not impacted by lack of Survivin, then a decline of only 20.8% in migration would be expected after 6 hours. However, this is not the case, as 26% less migration is seen relative to the control. Moreover, in the migration assay of cells that underwent Survivin knockout 48 hours before the start of the assay, a decline in migration of about 49% was seen. This leaves roughly a 5% decline and a 28.2% decline unaccounted for in the 24 and 48 hour groups of U87 cells that underwent RNAi specific for Survivin, respectively.

In the C6 cells, for every 100 mock transfected cells present at the start of the migration assay as many as 125 would have been alive at the end of the assay. For every 100 cells that underwent RNAi against Survivin that entered the migration assay as few as 103.75 might have been alive at the end (this is the situation that would most favorably support the null hypothesis). If the null hypothesis were correct, then only a 17% decline in counts would be seen when the experimental groups are compared to the controls. However, this was not the case as in this cell line a 23% and 46% decline in counts was observed in the groups that grew for 24 and 48 hours after exposure to RNAi reagents, respectively. This leaves a decline of 6% and a decline of 29% unaccounted for in the 24 and 48 hour groups respectively. These results suggest that cells whose ability to survive is not affected by Survivin knockdown, still experience a change in their migratory ability. This suggests that the role Survivin plays in migration could be separate from the role it plays in cell proliferation. This could be supported by recent findings that cytoskeletal integrity might be correlated with Survivin function as well as Survivin’s role in microtubule formation\textsuperscript{7}.
With the findings that compare the effects of Survivin knockdown between C6 and U87 glioma cells hint at another conclusion: The migratory patterns of these two cells are nearly identically affected by Survivin, but their vitality is affected in different ways.

Survivin is a promising new drug target in the fight against cancer in many varieties of cancerous cells and seems an especially promising drug target in gliomas. As in many other cancer cell lines, Survivin is a key component to the vitality of gliomas and is also correlated with the movement of the cells. It is promising also that human adult differentiated tissues express little if any Survivin, making it a potentially noninvasive and possibly side effect free drug target.

It would be interesting to analyze the importance of Survivin in motility when gliomas are placed in an environment that more closely resembles a living brain. It would also be interesting to see the importance of Survivin in gliomas that are living in a live rat's brain. This is because though many cancer cells behave similarly in vitro and in vivo, the behaviors of cells in the two environment are sometimes drastically different. Ultimately, it is in the brain that the cells will be combated, and so it is also important to study the cells in environments that closely resemble the place where they will be faced by scientists and doctors.

References


Acknowledgements

Special thanks is given to Peter Canoll of Columbia University for providing cell lines and expertise concerning the lines used. We also thank Maria Chiara Manzini of Harvard University for providing Hoechst solution.
Carbon Sequestration to Generate Calcium Carbonate: A Practical Approach to Sequester Residential CO2 Exhaust

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Program Advisor: Jeffery Field, PhD, University of Pennsylvania Department, TREES Program
*Correspondence: jdwortzel@gmail.com

Abstract
The sequestration of carbon dioxide (CO2) from fossil fuel exhaust can reduce greenhouse gas emissions that contribute to global warming. Current methods of CO2 sequestration have multiple steps, are expensive, and potentially hazardous. In contrast, lime water sequesters CO2 to generate calcium carbonate (CaCO3) in a one-step process, producing a safe product that has many industrial applications. A laboratory model was designed to explore the potential of lime water to sequester CO2 from residential furnace exhaust. The quantity of CO2 sequestered and CaCO3 generated by lime water was theoretically calculated. Lime water, made from calcium hydroxide, was used to sequester CO2 from dry ice. The average amount of CaCO3 generated was 0.90 g/L lime water, which was statistically similar to the predicted value of 1.05 g/L lime water (p = 0.20). The experimental yield was 86%, and the quantity of CO2 sequestered was 0.40 g/L lime water. The volume of lime water required to sequester CO2 from the average American home furnace was determined, and a household sequestration apparatus was designed. This study supports the potential to utilize lime water as a novel approach to sequester CO2 from home furnace exhaust, and in turn aid in reversing global warming.

Introduction
Our culture is currently dependent on fossil fuel\(^1\), which generates significant quantities of greenhouse gasses upon combustion. Greenhouse gasses have been implicated to be responsible for contemporary global warming. Carbon dioxide (CO2) is the most prominent greenhouse gas generated by man, and the average American home produces 5,298 kg CO2 per year. Future generations may overcome the problems of fossil fuel combustion by implementing sustainable sources of energy. However, until that transition occurs, the detrimental effects of the production of greenhouse gasses needs to be addressed. Attempts to sequester CO2 have been met with difficulty, including the technological and economical problems of storing the sequestered CO2.\(^2\) The use of a simple one-step approach to both simultaneously sequester and store CO2 exhaust would therefore be beneficial. In addition, if the stored CO2 could generate an environmentally safe and useful commodity, an economic incentive might help drive this process.

Carbon sequestration by bivalves has been a natural ecological process for ages. These organisms remove CO2 from seawater and use it to produce calcium carbonate (CaCO3) to establish a hard outer layer on their shells.\(^3\) The sediment from these bivalves then forms limestone on the bottom of the ocean. Man, in turn, then harvests this limestone, and utilizes the CaCO3 as an ingredient for many industrial products, including paper, plastics and paint, as well in the making of cement. The use of modern technology to simulate the ocean’s approach could therefore have a useful application for our current need to sequester and store greenhouses gasses. On a small scale, this process is currently being used to sequester CO2 from industrial exhaust. Ingeniously, a cement company was established by collaborating with a desalination facility and an industrial plant, where industrial exhaust is percolated through ocean water to precipitate CaCO3 and generate calcium-free water. In addition to expanding this technology in the industrial arena, it could also be beneficial in the residential sector. For example, the exhaust from fossil fuels generated by home furnaces, cars, and lawn equipment could be sequestered and the resultant CaCO3 could then productively utilized by industry.

Lime water is created by adding calcium hydroxide (Ca(OH)\(_2\)) to water. When CO2 is percolated through the lime water, the CO2 interacts with the Ca(OH)\(_2\) to generate calcium carbonate (CaCO3) as per the chemical equation: Ca(OH)\(_2\) + CO2 -> H2O + CaCO3. CaCO3 is poorly soluble and rapidly precipitates out of solution. The process is spontaneous, occurs in minutes, and the production of the limewater is inexpensive. Therefore, the use of lime water could be a potentially useful resource to sequester CO2 to generate CaCO3.

In the context of this background information, the hypothesis of my experiment is: If carbon sequestration by lime water can be theoretically quantified, then a laboratory model can reproduce these predicted results and be applied to sequester CO2 from residential furnace exhaust. I therefore devised an experiment to attempt to address this.

Materials and Methods

*Set up of apparatus.* CO2 was generated by placing a 10 g piece of dry ice into 1L of room temperature distilled water within a 2 L Erlenmeyer flask. The CO2 gas then passed through plastic tubing whose end was placed at the bottom of a 250 ml Erlenmeyer flask containing 200ml of lime water. The CO2 bubbled through the limewater for 2 minutes. The CaCO3 precipitated out of solution and was then weighed. Three parameters of this set-up were varied in an attempt to optimize the experiment: lime water preparation, use of controls, and method to remove the precipitated CaCO3.

*Lime water preparation:* Saturated lime water was made by dissolving 1.5g of Ca(OH)\(_2\) (Home Science Tools, CAS # 1305-62-0, lot # AD-10022-2, Billings, MT) into 1L distilled water at room temperature. At various time points (1 min, 40 min, or 24 hr) the lime water preparation was stopped, and excess precipitated Ca(OH)\(_2\) was removed by one of several methods (decanting, filtration through lab grade filter paper, or centrifugation) as per...
the design of each experiment. Experimental controls: Each experiment was internally controlled by weighing each piece of filter paper before and after filtering to account for the variation in weight of each piece of filter paper. Where noted in the experimental design, a negative control was also employed where 200 ml of distilled water, rather than the experimental sample, was poured through filter paper. After drying, the change in weight of this negative control was subtracted from the weight the experimental sample. This negative control was used to account for potential incomplete drying of the experimental sample, and to account for any change in weight of the filter paper by the experiment. Removing precipitated CaCO3: The precipitated CaCO3 was removed from the lime water by one of three methods: filtration with coffee filters, filtration with lab grade filter paper, or by centrifugation at 1000 rpm’s (Beckman, model J6-HC), as per the design of each experiment. When filter paper was used, the filter paper was dried at room temperature for 12 hr prior to determination of yield. Duration of experiment: The duration of the experiment was established by determining the time it took for all of the Ca(OH)2 in the lime water to be transformed into water and CaCO3. As per the chemical reaction Ca(OH)2 + CO2 -> H2O + CaCO3. The pH of lime water is pH 12.5, and the pH of water is pH 7, when the pH of the mixture reached pH 7, this indicated that all of the Ca(OH)2 was used up to form CaCO3. To determine this endpoint, the indicator bromothymol blue (Sigma, No. B-7271, lot # 51H3692, St. Louis, MO) was added to the lime water prior to bubbling through the CO2, as bromothymol blue is blue at pH 7.6 and yellow at a pH of 6.0. It was noted that the lime water turned yellow 1.5 minutes after the CO2 was bubbled through 200ml of lime water. To assure that each subsequent experiment would run to completion, each experiment was stopped after a duration of 2 minutes. Experimental Design (Table 1): In four independent experiments, three parameters of the set-up were varied in an attempt to optimize the experiment: lime water preparation, use of controls, and method to remove the precipitated CaCO3. Experiment #1: Limewater preparation - 24 hr duration, then decanted; Controls - no negative controls; Removing the precipitated CaCO3 - filtration with coffee filters. Experiment #2: Limewater preparation - 2 hr duration, then decanted; Controls - negative controls used; Removing the precipitated CaCO3 - filtration with lab grade filter paper. Experiment #3: Limewater preparation - 24 hr duration, filtration with lab grade filter paper, then filtered again immediately prior to experimentation; Controls - negative controls used; Removing the precipitated CaCO3 - filtration with lab grade filter paper. Experiment #4: Limewater preparation - 1 min or 40 min duration, centrifugation immediately prior to experimentation; Controls - negative controls for filter paper not needed; Removing the precipitated CaCO3 - centrifugation.

### Table 1. Parameters varied in each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lime water preparation</th>
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<th>Removal of CaCO3</th>
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<tbody>
<tr>
<td>1</td>
<td>24 hr, decanted</td>
<td>None</td>
<td>Coffee Filters</td>
</tr>
<tr>
<td>2</td>
<td>24 hr, decanted</td>
<td>Yes</td>
<td>Lab Grade Filter Paper</td>
</tr>
<tr>
<td>3</td>
<td>24 hr, filtered(b)</td>
<td>Yes</td>
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</tr>
<tr>
<td>4</td>
<td>1 min or 40 min</td>
<td>Not needed(d)</td>
<td>Centrifugation</td>
</tr>
</tbody>
</table>

(a) Lime water preparation: At various time points (1 min, 40 min, or 24 hr) the lime water preparation was stopped, and excess precipitated Ca(OH)2 was removed by one of several methods (decanting, filtration through lab grade filter paper, or centrifugation).

(b) Lime water was filtered again immediately prior to experimentation.

(c) Change in weight of control filter paper after saturation with 200ml distillated water then air dried at room temperature for 12 hours.

(d) Control for filter paper not needed as no filter paper was used.

(e) CaCO3 was removed from the lime water by one of three methods: filtration with coffee filters, filtration with lab grade filter paper, or by centrifugation.

Results

Generation of CaCO3 from limewater and CO2.

Four independent experiments were repeated 5 times to generate CaCO3 from 200 ml limewater after CO2 from dry ice was percolated through the limewater. Experiment #1 (Table 2): Limewater was prepared for 24 hr and then decanted. Coffee filters were used to remove the precipitated CaCO3, and the filter paper was weighed before each experiment. The mass of the precipitated CaCO3 was then calculated and reveals that an average of 0.12 g CaCO3 was precipitated from 200 ml limewater (0.60 g CaCO3/L lime water). Experiment #2 (Table 2): Limewater was prepared for 24 hr and then decanted. Lab grade filter paper was used to remove the precipitated CaCO3. The filter paper was weighed before each experiment, and a negative control was used for each experiment to account for possible incomplete drying of the filter paper. The mass of the precipitated CaCO3 was then calculated. An average of 0.14 g CaCO3 was precipitated from 200 ml lime water (0.70 g CaCO3/L lime water). Experiment #3 (Table 2): Limewater was prepared for 24 hr and then filtered with lab grade filter paper, and then filtered again immediately prior to experimentation. Lab grade filter paper was used to remove the precipitated CaCO3. The filter paper was weighed before each experiment, and a negative control was used for each experiment. The mass of the precipitated CaCO3 was then calculated. An average of 0.16 g CaCO3 was precipitated from 200 ml limewater (0.80 g CaCO3/L lime water). Experiment #4 (Table 2): Limewater was prepared for 1 min or 40 min and then immediately centrifuged prior to use. Centrifugation was used to remove the precipitated CaCO3. An average of 0.12 g (1 min prep) and 0.18 g (40 min prep) CaCO3 was precipitated from 200 ml limewater (0.60 g and 0.90 g CaCO3/L lime water, respectively). Comparison of experimental results with predicted results is shown in Figure 1.
Discussion

Limewater sequesters CO2 to generate calcium CaCO3 in a one-step process, and the quantity of CO2 sequestered and CaCO3 generated per liter of lime water can be theoretically calculated. This study designed a laboratory model to match the theoretically predicted results, and attained a yield of 86% of the predicted results. The amount of limewater needed to sequester CO2 from home furnace was calculated and revealed the potential to apply this approach to sequester CO2 from home furnace exhaust.

Four independent experiments were performed to improve the yield and determine the best experimental design. The experimental design was improved upon from Experiment #1 to Experiment #4, as evidenced by increased yield (29% to 86%). When removing the precipitated CaCO3, it was evident that centrifugation (Exp. #4; 40 min) and lab grade filter paper (Exp. #2 and #3) gave the best results, but coffee filters (Exp. #1) were least effective. This most likely is because of increased porosity of the coffee filters as compared to the lab grade filter paper. Regarding the preparation of the lime water, removal of excess Ca(OH)2 by filtration or centrifugation rather than decanting would seem to be beneficial to avoid contaminating the CaCO3 collection, but this was not reflected in the data in Experiments #2 and #3. However, filtration or centrifugation of the limewater prior to use would avoid this variable.

Negative controls were necessary when filter paper was used, as evidenced by the small but consistent weight change of these controls. It is evident that the average yield of this laboratory model was at best 86% of the predicted value. This lower yield could have been due an insufficient duration of the experiment. For example, if the experiment was too short, not all of the CO2 would have been sequestered. If it was too long, the CaCO3 would dissolve in solution as calcium bicarbonate. Although Bromothymol blue identified a ballpark duration of the experiment, it would be helpful to use a pH meter, and stop the experiment exactly when the pH reaches 7. In addition, the range of the data for each experiment is fairly wide, particularly in Experiments #1-3. This may have been due to the use of filter paper to collect the precipitate, which required multiple measurements and multiple controls. This variable was avoided altogether by using centrifugation rather than by filtration (Exp. #4). Lastly, more experimental data points should improve the accuracy of the calculated averages.

Although lime water can be utilized to sequester CO2, it is important to note that CO2 is actually emitted when the Ca(OH)2 that is used to make the lime water is produced by the chemical industry. This is because Ca(OH)2 is commonly made by heating the CaCO3 present in limestone, a process which produces CO2 in addition to the Ca(OH)2. However, Ca(OH)2 can also be manufactured in the laboratory from a calcium salt and an alkali, rather than directly from limestone. As less CO2 is emitted into the atmosphere by this alternative process, using this source of Ca(OH)2 would make CO2 sequestration by lime water more efficient.
This approach of CO2 sequestration has been applied to the removal and storage of CO2 from industrial exhaust. The study described herein explores the application of this approach to the sequestration of CO2 generated by residential fossil fuel combustion, specifically from the home furnace. It is therefore necessary to examine the economic and technical feasibility of this endeavor. The data in this study reveals that 0.90 g CaCO3 is generated from 1 liter of lime water (Exp. #4, 40 min). Therefore, 32.0 kg of CaCO3 would be generated by a residence each day from 35,500 L of lime water. This would translate into 11,680 kg CaCO3 each year. It could therefore be conceivable that such a company, possibly with government incentives, would pay for and establish the CO2 sequestration apparatus in each household, as well as set up curbside CaCO3 collection, similar to the collection of other recycled products (e.g., newspaper, glass, plastics).

The technical logistics for the sequestration apparatus in each household would also have to be practical. 35,500 L/day of lime water is too large of a volume to generate, store, and work with. However, if the lime water can be made more rapidly than every 24 hours, the water could be recycled and less water would be needed. Experiment #4 indicated that 40 min preparation is adequate (86% yield), and 1 min preparation is still effective (57% yield). For example, if lime water could be made every 5 min rather than every 24 hours, as these preliminary experiments suggest, this could reduce the volume of lime water by a factor of 1/288. A household would then only require 123 L of lime water each day, which is certainly more manageable.

A preliminary design for the household set-up is shown in Figure 2. The furnace exhaust would percolate through a 125 L tank of lime water to generate CaCO3 (CaCO3 Gen Tank) for a period of 5 minutes (125 L is roughly the size of a small bathtub). This tank would then be emptied, and the contents would pass through a continuous centrifuge where the precipitated CaCO3 would be removed. The supernatant would then be pumped to a second 125 L tank where it would be resaturated with Ca(OH)2 for a period of 5 minutes to generate lime water (LW Gen Tank). At this same time, lime water already saturated within the LW Gen tank will be pumped into the CaCO3 Gen Tank near the chimney to generate more CaCO3. In this way the two volumes of 125 L will be moved back and forth every 5 minutes between the two 125 L tanks. The centrifuge and LW Gen Tank can be located in the basement to allow for easier access by the homeowner, and the CaCO3 Gen Tank would be located high up in the chimney where the exhaust is at a cooler temperature. A pump would be required to circulate the lime water through this system. The homeowner could take the CaCO3 outside for curbside collection. Although this is a rough scheme, and not detailed from an engineering perspective, it suggests the potential practicality of this set up.

Figure 2. Design for household CO2 sequestration apparatus
(a) CaCO3 generation tank (125 L).
(b) Lime water generation tank (125 L).
(c) Continuous centrifuge removes CaCO3.
(d) Exhaust redirected to the CaCO3 Gen Tank.
Future studies would be necessary to determine the minimal timeframe that limewater could be produced. It could also be explored if other salts, such as magnesium hydroxide, might be added to the limewater to sequester additional CO2, and be simultaneously precipitated with the CaCO3. Lastly, the engineering of the household apparatus needs to be explored in depth. A potential modification of the design might be the use of calcium oxide (CaO) instead of Ca(OH)2 to make the lime water. The potential benefit is that the formation of limewater from CaO generates a large amount of heat which could be potentially harnessed for household heating, and to drive the motors for the centrifuge and the pump.

This study supports that limewater can be an innovative method to sequester CO2 that is safe, practical and economically advantageous, as CaCO3 is used for many industrial products (e.g., paint, paper, plastic, cement). In addition to sequestering CO2 from the home furnace, this approach for CO2 sequestration might also be applicable to other sources of residential CO2 exhaust, such as from automobiles and lawn equipment. Lastly, the application of this approach to industrial CO2 production could have an enormous impact on reducing large quantities of this detrimental greenhouse gas. The ultimate goal of this project is to use this approach of CO2 sequestration to help reverse global warming.

References


Acknowledgements
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Stress Response to Different Concentrations of NaCl: Analysis of Root Length and Protein Expression on Wild Type Arabidopsis thaliana

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Abstract

The purpose of this experiment was to examine the stress response of wild type Arabidopsis thaliana to treatment with different sodium chloride concentrations. Previous research has shown that sodium chloride induces a stress response from plants in both a physical sense and molecular sense. The hypothesis was that the plants would exhibit a number of different stress responses including shorter root length and an increased production in specific stress-responsive proteins. The hypothesis was tested by placing germinated Arabidopsis seedlings onto MS (Murashige and Skoog) agar plates with varying sodium chloride concentrations. While the seedlings grew on the plates with sodium chloride, root length was measured. After root length measurements were completed, specific plants from each concentration were subjected to protein analysis through SDS-PAGE to compare the banding patterns of protein expression for each treatment group versus the control. The importance of this study was to better understand how plants tolerate different levels of sodium chloride. Root length analysis showed that higher concentrations of sodium chloride dramatically inhibited a plant’s growth. SDS-PAGE analysis showed a protein band that was present in the control plants and was not present in the plants that grew in an added concentration of sodium chloride. These results show that the plants have specific stress responses to sodium chloride including shorter root length and a decrease in the production of an unknown protein.

Introduction

Roots are the primary site in plants for water-related stress. Since the roots are where the main source of water absorption takes place, it would make sense that it is the site where water-limiting stress would occur. The problem with this is that the roots help to control the productivity of the entire plant, and when the roots don’t function properly, the rest of the plant suffers because of it. Organisms will often express proteins in response to stress. Sodium chloride, or NaCl, has been shown to induce specific stress responses including a decrease in the plant’s productivity and an increase in the production of certain stress-related proteins in Arabidopsis. Based on the research of previous scientists, an interesting question to study is whether different concentrations of NaCl inflict different stress responses on Arabidopsis thaliana.

In a study by Deyholos and Jiang, the scientists wanted to further understand the plants responses to abiotic stress by completing a microarray analysis on the Arabidopsis roots after an exposure to NaCl. The Arabidopsis plants were grown hydroponically, and after 21 days of germination, they were given a 150mM supplement of NaCl. After analyzing the results, they identified different transcriptional responses in the plant, which indicated the production of new proteins, most likely stress-responsive. By completing this experiment, the hope was to gain further knowledge on salt tolerance in plants to try and possibly improve salt tolerance in other plants. The experiment question stated above would build off of this research by further testing to see how NaCl affects the rest of the plant’s productivity.

In another study by Jiang and other researchers, the scientists were continuing their earlier studies described above, focusing on the production of specific stress-responsive proteins. The purpose was to better understand the proteomic level of NaCl stress responses in Arabidopsis roots. After completing a procedure similar to the one above, instead of completing a microarray analysis, the scientists performed electrophoresis to separate and analyze the production of specific proteins that are commonly stress-responsive. They found that post-transcriptional gene regulation plays an important part in expressing the stress-responsive proteins. This experiment would relate to the proposed research question above because they used electrophoresis to perform a proteomic analysis of stress-responsive protein production in NaCl treated Arabidopsis roots.

In testing how different concentrations of NaCl inflict different stress responses on Arabidopsis thaliana, it is hypothesized that the plants will exhibit a number of different stress responses including shorter root length and an increased production in specific stress-responsive proteins. This hypothesis will be tested by placing germinated Arabidopsis seedlings onto agar plates with different NaCl concentrations. While the seedlings grow on the plates with NaCl, root length will be measured. After allowing the seedlings to grow for a certain amount of time on the agar, a certain number of plants from each concentration will be removed to test for a protein analysis through a SDS-PAGE to compare banding patterns for different concentrations with the control. The importance of this study is to better understand how plants tolerate different levels of NaCl in an attempt to improve salt tolerance in other plants. If we understand how Arabidopsis uses certain proteins to deal with tolerating different levels of salts, those proteins could possibly be isolated and used in other plants to help them deal with salt tolerance too. It is important to understand salt tolerance in Arabidopsis so that we can expand our ability to select and engineer better salt tolerant plant strains.

Materials and Methods

The independent variable in this experiment was the different concentrations of sodium chloride. The dependent variable was the growth of the Arabidopsis roots and the protein production in the Arabidopsis roots. The negative control was 0mM of NaCl, and there was no positive control. Some of the constants were the temperature, the environment, the equipment used, and the...
supplies used. There were five different concentrations. For each concentration there were five plates with five plants on each plate. This gave a total of 25 replications for each concentration. This experiment began by making a total of 600mL of MS agar using MS salts, sucrose, and distilled water. Growing plants on MS agar is a common technique taught in the advanced experimental design class that involves growing the plants on a medium that provides them nutrients and allows me to view their roots for easy analysis. A strip of pH paper was used to make sure the solution was around a pH of six. After heating on a hot plate, the 200mL of MS agar was then poured into a 250mL media bottle. The procedure was then repeated two more times to make a total of 600mL of MS Agar. About 200mL of MS Agar was then made for the following concentrations of sodium chloride: 0mM (negative control), 100mM, 125mM, 150mM, and 200mM. This was done using a similar procedure to making MS agar except adding extra amounts of sodium chloride to the agar solution. These extra values of sodium chloride that were added were determined using an equation. Once all of the agar was made, it was placed into an autoclave and sterilized. About 300 Arabidopsis seeds were then sterilized for culture using autoclaved distilled water and 30% bleach. They were placed in the freezer when they were done. After the agar was autoclaved, it was melted on a hotplate and poured into petri plates using sterile technique. Sixteen MS Agar plates were taken out and each plate had 10-12 Arabidopsis seeds placed on it to grow. The seeds placed on the plates had been sterilized and were placed on the plate one at a time using a micropipet. These plates were taped on the sides and stored vertically in a bin under a light cart to germinate for four days so that the roots would grow directly downwards. The plants were given time to germinate on regular MS agar plates before they were transferred so that they could germinate and grow uninterrupted for the first few days. After the germination period, the plants were transferred to new plates using tweezers so that they could now be exposed to the various added concentrations of sodium chloride in the MS Agar. There were five plates for each of the five concentration. With five plants per plate, this totaled about 125 Arabidopsis seeds that were transferred. The new plates were then rotated 90 degrees, taped on the sides, and placed back into the bins to be stored vertically under a light cart to grow so that the new root growth would grow at a new angle, making it easy to measure. For four days after the plants were transferred, each of the plant's change in root length was measured in millimeters using a ruler. After taking observations, plant root samples from each concentration were crushed in Laemmli protein extraction buffer to create a sample to be run in a SDS-PAGE. Multiple samples from each concentration were taken from different times because of lessons learned regarding the SDS-PAGE gels. These lessons will be explained later in the paper. When the SDS-PAGE samples were not being used, they were stored in a -80°C freezer. The samples were run on SDS-PAGE gels in PAGE buffer at 150V for 45 min- 1 hr. and 30min. Once the gels were run, they were then stained by sitting in Biosafe Coomassie stain for ten minutes and then destained using distilled water. Pictures were then taken to show how the banding patterns appeared. It is important to note that a molecular weight marker was run on each gel to use as a point of comparison for the samples. Since there was extra time, a percent germination portion of the experiment was run. This was done by placing ten sterilized Arabidopsis seeds on each of ten MS agar plates containing various concentrations of NaCl, two plates for each concentration. The percent of germinated seeds was then recorded after four days. After all of the experiment was complete, all equipment and stations that were used were thoroughly cleaned and disinfected using sterile procedure. The data was analyzed in four ways. The mean root lengths for every day observations were taken for each concentration and the negative control was found. The standard deviation for each of the means for each concentration and the negative control were found. T-tests (2 type, 2 tailed) were performed between the control and each of the different concentrations for the final observations. The results from the SDS-PAGE gels were analyzed using a proteomic analysis. The proteomic analysis was completed by looking for the formation or deletion of banding patterns (a difference in the banding patterns) as compared to those of the negative control. These final observations were then analyzed by comparing them with each other to determine if the hypothesis should be accepted or rejected.

Results
The change in root length values for the different concentrations of sodium chloride in this experiment show very interesting trends. Figures 1 shows the change in root length (measured in mm) over the course of four days for the different concentrations of sodium chloride: 0mM (negative control), 100mM, 125mM, 150mM, and 200mM. There are standard deviation bars on all of the bars in the graphs to show the variability for each of the values that were graphed. In this graph, you can see that the change in root length at 0mM NaCl steadily and consistently increased throughout the course of the four day period. Since this was the negative control, these results were expected and the changes in root lengths were very large numbers, since the plants continued to grow in the absence of sodium chloride. The standard deviation bars got larger as the days went on, which makes sense as there was more variation in the change in root lengths the more time the plants had to grow. At concentrations of 100mM NaCl, 125mM NaCl, and 150mM NaCl, all showed similar trends in that the change in root length steadily and consistently increased throughout the course of the four day period but on a very different rate.

One difference to note is that at 125mM and 150mM, the standard deviation bars remained mostly constant for the four days and didn't increase. This just means that the error in the values for those concentrations was about the same all four days. At a concentration 200mM NaCl, you can see that the change in root length increased by very small increments and overall did not grow a great deal throughout the four day period. Overall, it can be observed that with each increased concentration of sodium chloride, the change in plant root lengths got shorter and the increments in which they increased also got smaller.

The percent germination portion of this experiment was very helpful in showing that once again, higher concentrations of NaCl inhibit plant growth. In Figure 2 you can see the percent germination of Arabidopsis seeds on different
concentrations of NaCl after four days. The graph shows that the highest percent germination was for the negative control with 0mM concentration of NaCl and the lowest percent germination was for the 200mM concentration of NaCl. There was once again a steady decrease in the percent germination as the concentration of NaCl increased. The standard deviation remains consistent for the first four concentrations. The 200mM concentration of NaCl, however, happens to have a large standard deviation. The statistical analysis portion of this experiment had very conclusive results (Table 1). A t-test was performed between the control and each of the concentrations for the final day that measurements were recorded (Day 4). I decided to do the tests at this point because I thought it would be interesting to see how the final results compared to the control and how statistically different they were. I also decided to do a t-test between the control and each of the concentrations to see if each of the concentrations really had a statistically significant effect on the plant. To complete the t-test, I compared the 25 values of changes in root length for the negative control

Figure 1. This graph shows the comparison between the changes in plant root lengths over a four day period for the different sodium chloride concentrations.

Figure 2. The average percent germination of *Arabidopsis thaliana* at different concentrations of NaCl after four days. Length for each of the NaCl concentrations.
on day 4 to 25 values of changes in root length for each of the NaCl concentrations. For each of the t-tests that I conducted, I found that the p-value was extremely small, less than 0.0001. Since the p-value is less than 0.05, this means that each of my concentrations compared to my control is significantly different. There is a less than 5% chance that my data sets are the same and the differences between them are most likely not due to random variation. In other words, the results that I got for each of the different NaCl concentrations is significantly different from the control values, showing that the NaCl concentrations had a significant effect on the plants and were not just minor effects. It shows that the changes between the data sets are significant.

The proteomic analysis completed for the different samples showed very interesting results. Figure 3 shows the SDS-PAGE gel using 0mM NaCl (Lanes 1-3), 100mM NaCl (Lanes 4-6). Figure 4 shows the SDS-PAGE gel using 125mM NaCl (Lanes 1-3), 150mM NaCl (Lanes 4-6). These results clearly show a band present in the three control plants that is not present in the other plants samples with NaCl concentrations. This shows that NaCl does in fact inhibit some sort of protein expression in plants. It cannot be determined specifically what kind of proteins are being inhibited, nor can a general size of the inhibited protein be given because the protein size marker is not spread out enough to be measured. The main important thing is that there is a band present in the control plants that is not present in any of the other plants. Something else to mention is that there were no samples from the 200mM concentration in the running of the last gels because the plants had died and were disposed of. It is predicted, however, that the results would have shown up the same with the 200mM concentration plants not having the extra band that was present in the control plant samples.

Discussion

Based on my results, I accept my hypothesis for a number of different reasons. My hypothesis stated that the plants would exhibit a number of different stress responses including shorter root length and an increased production in specific stress-responsive proteins when exposed to different concentrations of sodium chloride. My results did show a shorter root length as the concentrations of NaCl increased. So it would make sense that as a stress response to the NaCl, the plants exhibit a shorter root length. It could also be possible that the sodium chloride actually inhibits the proteins needed for the plant’s roots to grow. This inference could be backed up by the results of the proteomic analysis that showed a protein band in the control plants that was not present in any of the NaCl concentration plants. So maybe instead of the production of specific stress-responsive proteins to help conserve water in the plant, instead is the inhibition of specific growth proteins having to do with energy production. This would make sense because if the plants cannot produce energy, then they cannot grow, thus inhibiting their root lengths. However, even though this proteomic analysis does not show the production of a new protein in the NaCl concentration plants, it is important to note that there was a proteomic response to the NaCl in the plants, whether it be an increase or decrease in the levels of a certain protein. In the future, I would like to run more SDS-PAGE gels so that I can get even clearer results with

| Table 1. P-Values of t-Tests (2 type, 2 tailed) |
| Day 4 Negative Control vs. Day 4 100mM NaCl; p-value = 4.1 x 10^-11 |
| Day 4 Negative Control vs. Day 4 125mM NaCl; p-value = 1.2 x 10^-13 |
| Day 4 Negative Control vs. Day 4 150mM NaCl; p-value = 2.3 x 10^-17 |
| Day 4 Negative Control vs. Day 4 200mM NaCl; p-value = 3.5 x 10^-21 |

Figure 3. Banding patterns produced from the 0mM NaCl and 100mM NaCl samples run on a SDS-PAGE gel. Samples from left to right: Ladder, 0mM NaCl (Lanes 1-3), 100mM NaCl (Lanes 4-6). Unique band shown in red squares.

Figure 4. Banding patterns produced from the 125mM NaCl and 150mM NaCl samples run on a SDS-PAGE gel. Samples from left to right: Ladder, 125mM NaCl (Lanes 1-3), 150mM NaCl (Lanes 4-6). Unique band shown in red squares.
more banding patterns. This could potentially yield seeing a new protein expression in NaCl treated plants. Ultimately, I would accept my hypothesis since my results show that sodium chloride causes a shorter root lengths in plants and also a response on the proteomic level with the decrease in levels of a certain protein.

It is important to account for high levels of variation in the data. Some means had a standard deviation of 3, which is a relatively high number compared to the means. It means that there was a lot of variation in the data. Although in some cases there were high standard deviations, they were most likely caused due to outliers since for the most part, standard deviations were around the number one. To decrease the amount of variation in my data, I could have more replicates in a future experiment.

The results I found differed in light of the findings from my initial background articles. The results of the initial articles found that there with the stress of different sodium chloride concentrations being introduced to the plant there was the production of a specific protein to help manage the stress. However, my results found that with the stress of different sodium chloride concentrations there was inhibition of a specific protein. In other words, I did not find that a new stress-responsive protein was produced. Instead, I found that the production of a specific protein was inhibited.

There are many possible sources of error in this experiment. The SDS-PAGE gels could have not run for long enough or run for too long. This would have caused the banding patterns on the SDS-PAGE gels to look different. For future experiments, I could do more background research on how to properly run a SDS-PAGE. The roots for the Arabidopsis plants intertwined at some points after a few days of growth, and so measurements on the change in root length may have been not accurately measured for some days. This would have affected my results in that I would have had different values. To solve this problem in the future, I could come up with a better method of making sure that the roots don’t tangle such as putting separators into the agar.

There are a number of different ways that I could go on to continue this experiment in the future. I could test even more concentrations of NaCl or focus on a narrower range of concentrations. I could also try testing how different NaCl concentrations affect plants that are grown in soil versus agar, and see if it has a difference. Testing different NaCl concentrations on different types of plants could also provide interesting results. I could add more replicates to reduce the amount of error in my experiment. I could also try testing proteins in different areas of the plant, not just the roots and see if there are other protein chances in response to a NaCl stress response. All in all, there are different things that I could so to further continue this experiment.

References


Bioconcentration of Corexit® Dispersant Surfactant in the Oyster Crassostrea gigas

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Abstract
The oil dispersants Corexit 9527 and 9500 A were used in mass quantities in treatment of the Deep Water Horizon Gulf of Mexico oil spill of 2010. There has been much concern and controversy over the effect and toxicity of these oil dispersants on marine biota. Studies were conducted by EPA in 2010 on the toxicity of various oil dispersants and their oil mixtures. Primary active ingredients in oil dispersants are surfactants. To date, very little research has been conducted on the effect, toxicity or bioaccumulation of these surfactants on marine organisms. This investigation focused on the short term bioconcentration of Dioctyl Sulfosuccinate Sodium Salt (DOSS), the primary surfactant found in Corexit products, in the Pacific oyster Crassostrea gigas. DOSS is water soluble and fat soluble and has the potential to accumulate in the fatty tissues of organisms. Oysters were exposed to approximately 500 μg/L of Corexit 9500 A over an approximate 60 hour period of time. Oyster and water samples were taken at approximately six hour intervals, frozen and shipped to the laboratory for DOSS analysis by liquid chromatography tandem mass spectroscopy (LC/MS/MS). The results of this bioconcentration study are discussed.

Introduction
This bioconcentration study of Dioctyl Sodium Sulfosuccinate (DOSS) in oysters, was conducted due to the extensive use of Corexit on the recent Deep Water Horizon oil spill in the Gulf of Mexico. Bioconcentration studies are important to determine if substances accumulate in organisms. It has been shown that DOSS is toxic to some marine organisms when they are directly exposed to high parts-per million (ppm) levels in water1. If DOSS is found to bioconcentrate, then marine organisms would build up toxic levels of the surfactant when exposed to very low concentration parts-per billion/parts-per trillion (ppb or ppt) levels which are typically found after dilution and turbulent mixing which occurs in the ocean2.

Published studies concerning the bioconcentration of DOSS in marine biota were not found when the review of related research was conducted. Analytical methods for detecting DOSS in sea water and tissue are available and the methods are published. DOSS is an anionic surfactant used in Corexit 9500 A and 9527. Corexit 9500 A was the primary dispersant used in the 2010 Gulf oil spill. Corexit is sprayed over an oil slick and is used to break up the oil into smaller parts in order to speed up the process of eliminating the oil through evaporation and biodegradation. Corexit was also injected directly at the leak site to break up the oil before it reached the surface. The concentration of DOSS in the two Corexit products is about 30%. Around 1.8 million gallons of Corexit were used in the Gulf oil spill.

The question this study attempted to answer is whether DOSS will bioconcentrate in Pacific oysters. It was hypothesized that the DOSS will bioconcentrate in Pacific Oysters due to the fat soluble nature of the compound3. The bioconcentration exposure study was similar to the method described in the Journal of Experimental Marine Biology and Ecology3. The DOSS analysis was completed by Columbia Analytical Services using their standard operating procedure developed in 2010.

The study involved setup of a 20 gallon saltwater aquarium system with bottom filtration and circulation pump. An initial stability study for DOSS was completed in the aquarium water. The tank was then cleaned and setup again. Oysters were added to the tank and allowed acclimate. The tank was then spiked with Corexit 9500 A. Water samples and oysters were taken at various time intervals to be analyzed for DOSS levels. All samples were sent to the lab for LC/MS/MS analysis of DOSS concentrations.

Materials and Methods
A 20 gallon marine aquarium was set up with synthetic sea salt (Instant Ocean) and crushed coral filtration system oxygenated by an air pump circulation system. The density of salt water was set at approximately 1.020 g/mL. The temperature of the water ranged from 2 to 8 degrees C. An initial stability study for DOSS was completed by spiking the tank with 100 μL of Corexit 9500 A then sampling the water at various time intervals over a 75 hour period to determine the degradation rate of DOSS in the aquarium. The tank was then cleaned and setup again. Twenty oysters were added over several days. Once the oysters had acclimated to their environment, samples of water and oysters were taken as blanks. The tank was then spiked with 100 μL of Corexit 9500 A. Water samples and two oyster samples were taken at various time intervals (approximately six hours) to be analyzed for DOSS concentrations. Samples were stored in plastic bags, preserved at -18°C and sent to the lab for LC/MS/MS analysis of DOSS. Seawater samples were collected in 15 mL polypropylene centrifuge tubes. Samples were spiked with 13C4 DOSS (Instrument Internal Standard) and 2D18 DOSS (Surrogate) to monitor ionization suppression and extraction recovery, respectively. Seawater samples were extracted using Phenomenex Strata Solid Phase Extraction (SPE) cartridges. Oysters were shucked and the tissue frozen at -20°C then lyophilized (freeze dried). One gram of freeze dried oyster tissue was spiked with 13C4 DOSS (Instrument Internal Standard) and 2D18 DOSS (Surrogate) to monitor ionization suppression and extraction recovery, respectively. Freeze dried tissue samples were sonicated with 10 mL’s methanol/dichloromethane solvent mix for three hours. A 2 mL subsample was taken to dryness and re-dissolved with 1 mL methanol. Liquid chromatography was performed using Shimadzu Prominence. Mass spectrometry was performed using ABSciex API 5000 operating in electrospray negative selective reaction monitoring (ESI – SRM) mode.
Results
Table 1 shows data for DOSS in water samples taken from an initial stability study ran for DOSS in synthetic seawater with no oysters present. The data show a trend of decreasing concentration of DOSS in the water over the 75 hour period. This decrease may be due to biological degradation from by the added bacteria culture. This study shows that DOSS levels would remain at sufficient levels over the set period of time to complete the bioconcentration study. Figure 1 shows the decreases of the DOSS in the primary seawater study through the 75 hour time period. This study was done to determine if DOSS degraded in sea water prior to addition of the oysters.

Table 1. Primary Seawater Study

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time After Corexit was Added (hours)</th>
<th>DOSS in Seawater ug/L</th>
<th>DOSS in Oyster ug/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 hour</td>
<td>330</td>
<td>290</td>
</tr>
<tr>
<td>2</td>
<td>9 hours</td>
<td>330</td>
<td>270</td>
</tr>
<tr>
<td>3</td>
<td>21 hours</td>
<td>280</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>30 hours</td>
<td>290</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>75 hours</td>
<td>220</td>
<td>210</td>
</tr>
</tbody>
</table>

Figure 1. DOSS concentration over time for the primary seawater study

Table 2 shows the analytical results for DOSS concentrations in the oyster samples and water samples taken during the bioconcentration study. This data is also graphed in Figure 2. The data shows one anomaly, trial 2, with a DOSS concentration of 410 μg/kg, which is 60% higher than the overall average concentration of DOSS found in the oyster samples. This high value may be due to laboratory error. The data shows a parallel decrease in the DOSS concentration for oysters and water samples after 31 hours. This is assumed to be due to biodegradation of DOSS by the common soil bacteria Nitrosomonas/Nitrobacteria that was added to the tank. The bacteria culture was added to break down the toxic ammonia expelled by the oysters as waste. Slight degradation of DOSS was noted in the primary sea water study. This degradation did not affect the bioconcentration study.

Table 2. DOSS Data for Oyster/Seawater

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time After Corexit was Added</th>
<th>DOSS Concentration in Oyster ug/kg</th>
<th>DOSS Sea Water ug/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 hour</td>
<td>290</td>
<td>210</td>
</tr>
<tr>
<td>2</td>
<td>7 hours</td>
<td>410</td>
<td>200</td>
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<td>3</td>
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<tr>
<td>6</td>
<td>43 hours</td>
<td>220</td>
<td>130</td>
</tr>
<tr>
<td>7</td>
<td>48 hours</td>
<td>180</td>
<td>130</td>
</tr>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>67 hours</td>
<td>180</td>
<td>94</td>
</tr>
</tbody>
</table>

Discussion
The results show that Dioctyl Sulfosuccinate Sodium Salt (DOSS) does not bioconcentrate in the Pacific oyster Crassostrea gigas. The data shows that the oyster equilibrated to the DOSS water concentration. Concentrations of DOSS declined in the oysters as concentration declined in the aquarium water. If bioconcentration had occurred the levels of DOSS would have steadily increased in the oysters as the exposure time to the DOSS increased. The Relative Percent Difference (RPD) in the analytical method is 30%. Error bars reflecting this RPD have been added to the graph of the data. All but one of the error bars for the oyster data and their respective aquarium water data overlap. Difference in matrices, sample prep and that the oysters contain approximately 10% solid may influence the oyster data thus shifting the overall DOSS concentration higher than the respective water sample data. The concentration of DOSS decreased in the oysters and water samples over time. It is believed that the decrease is due to biodegradation by the bacterial culture added to the aquarium water. It is unlikely that the oysters metabolized the DOSS being the decrease was noted in the primary seawater degradation study which did not involve oysters. There has been great concern in the Gulf of Mexico by the shellfish industry and government agencies about the effects trace levels of DOSS may have on shellfish. The results of this study are important in that it shows that DOSS does not bioconcentrate in oysters. Bioconcentration could have lead to the oyster’s rapid accumulation of high levels of DOSS that could be toxic to the oyster or affect its physiology in some negative way. Further studies could test bioconcentration in other marine organisms and at higher water temperatures. Corexit crude oil mixes could be tested as well to see if bioconcentration is affected. Other studies could also involve environmental degradation rates of DOSS and DOSS oil mixtures. From my study it could be suggested that DOSS biodegrades relatively fast in the presence of bacteria. This degradation could be increased with temperature and exposure to sunlight as well.
Figure 2. DOSS concentration over time for the oyster and salt water sampling study. The data show that there is no bioconcentration of DOSS occurring in the oysters.

References


Improving Solar Water Disinfection (SODIS) with a Photoreactive TiO$_2$/SWCNT Composite on Plastic PET Bottles

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Abstract

Approximately 1.1 billion people in the world lack access to safe drinking water, an issue that causes two million deaths a year due to waterborne diarrheal diseases. Solar disinfection (SODIS), a point-of-use water treatment method that uses UV radiation in sunlight to kill pathogenic organisms, was discovered by Professor Aftim Acra in the early 1980’s. Because SODIS relies largely on weather conditions, its efficiency varies greatly. The purpose of this study was to improve SODIS by coating plastic PET bottles with a TiO$_2$ and a TiO$_2$/SWCNT composite, a photocatalytic nanomaterial that exhibits strong antibacterial activity. Water samples were collected from the Quinnipiac River in Wallingford, CT and transferred into the bottles to be tested for bacteria at time intervals of 0 min., 180 min., and 360 min. of sun exposure. Two trials were performed with the same bottles to test for bottle reusability, and bacterial concentrations were determined through serial dilutions and plate counting. Results show that the TiO$_2$/SWCNT composite coating was the most effective in meeting EPA standards, preventing bacterial growth in optimal temperatures, and producing strong coatings. The TiO$_2$/SWCNT composite coating did kill more bacteria as hypothesized, but the full 360 min. of sun exposure was required.

Introduction

Water is essential for life, yet an inadequate supply of clean drinking water plagues an estimated 1.1 billion people worldwide. In the 1980’s, Professor Aftim Acra discovered solar disinfection (SODIS), which uses UV radiation in natural sunlight to kill pathogenic organisms$^3$. SODIS requires filling water into plastic polyethylene terephthalate (PET) bottles and leaving them in the sun, making it a simple, environmentally sustainable, and virtually costless technology$^2$. But despite its advantages, SODIS alone still faces certain challenges. Because it relies largely on specific weather conditions, its efficiency varies greatly. Due to widespread poverty and varying weather conditions around the world, many countries that lack clean water are located outside of the boundaries where SODIS is most effective$^3$. SODIS alone also does not provide residual protection against bacterial regrowth, as bacteria quickly grows back if disinfected water is stored for a long time and not immediately consumed$^3$.

To improve SODIS, this study utilizes titanium dioxide (TiO$_2$) as a coating on plastic PET bottles. TiO$_2$ is a metal oxide with many unique properties, including high photocatalytic activity, biological and chemical stability, lack of toxicity, strong oxidizing activity, and low price$^3$. Single-walled carbon nanotubes (SWCNTs) are also used; when combined with TiO$_2$ in a composite, SWCNTs not only provide a larger surface area but also trap electrons transferred from TiO$_2$ further enhancing oxidation and antibacterial potential$^4$.

The independent variable in this study is the type of coating, while the dependent variable is the concentration of bacteria found in the water. It is hypothesized that the TiO$_2$/SWCNT composite coating will enhance the SODIS process by killing more bacteria and decreasing the required sun exposure time. The results from this study will be valuable in the development and application of low-cost, energy-efficient, and environmentally-friendly methods of water purification for developing countries.

Materials and Methods

*Synthesis of TiO$_2$ and Composite Suspensions:* The bottles were coated using a 10% w/v suspension. The TiO$_2$ suspension consisted of 1g Acros Organics titanium (IV) oxide anatase powder and 10mL demineralized water; the composite suspension consisted of 50mg Strem Chemical Inc. single-walled carbon nanotubes (SWCNTs) and 200mL water. 950mg TiO$_2$ powder was then added. The suspension was sonicated and heated until the water evaporated. One gram of dried composite was added to 10mL demineralized water and sonicated for 30 min. *Bottle Preparation and Coating:* The bottles were rinsed, filled with river water, sonicated for 1 hour, and dried for 48 hours at room temp. The suspensions were introduced into the bottles and shaken to obtain a homogenous film over the bottle wall. The bottles dried after 24 hours at room temp, and then they were half-filled with water and shaken for 30 sec. to ensure no coating detached before use. *Exposure to Sun:* Nine bottles were tested: 3 blank controls, 3 TiO$_2$ coating, and 3 TiO$_2$/SWCNT composite. The bottles were filled with river water, placed inside an aluminum foil-covered solar reflector box, and left in the sun. Water samples were collected aseptically at 0, 180, and 360 min. The irradiation test was then repeated using fresh river water. *Dilutions and Plate Counting:* Water samples of 1.5mL were collected aseptically in micro-centrifuge tubes. This was the 0x dilution (original sample). 100uL of this 0x sample were plated onto an agar plate with micropipette, and the cells were spread evenly using a metal cell spreader. Successive dilutions of 900uL sterilized water and 100uL of the previous dilution were also plated. Plates were incubated for 3 days and colony-forming units were counted. *Absorbance Measurements:* Samples were analyzed for absorbance and transmittance with a Vernier LabQuest probe and Red Tide Spectrometer. Spectrometer cuvettes were filled ¼ and light was shined on the samples. Absorbance was converted into % transmittance using an absorbance-%transmittance chart$^5$. *Statistics:* Two-sample t-tests were performed on a TI-83 graphing calculator to compare the bacterial concentrations remaining in the bottles after the 360 min. irradiation test. The t-statistic, p-values, and degrees of freedom are reported.
Results
In trial 1, bacterial concentrations decreased substantially throughout the irradiation test. The blank control, the TiO\textsubscript{2}, and the composite started with average concentrations of 8500 cfu/mL, 34000 cfu/mL, and 13333 cfu/mL, respectively (Figure 1). At 180 min., the TiO\textsubscript{2} and composite bottles decreased to below half of the initial concentration, but the blank control still had roughly 63% of its initial bacterial concentration (Figure 2). At the end of the test, both TiO\textsubscript{2} and the composite had less than 6% of their original concentrations (Figure 2). The control, however, still had roughly 30% of the initial bacteria remaining, demonstrating the benefits of the photocatalysts (Figure 2). Only the composite met the Environmental Protection Agency (EPA) standard of 500 cfu/mL of coliform bacteria in water (Figure 1).

In the second trial there was no continuous decrease in bacterial concentration. The control, the TiO\textsubscript{2}, and the composite bottles started with an average of 3000 cfu/mL, 5500 cfu/mL, and 1800 cfu/mL, respectively (Figure 3). At 180 min., the blank control and TiO\textsubscript{2} reached 250% and 150% of the initial concentration, respectively (Figure 4). The cause of the increase is believed to be the increase in water temperature. The composite bottles, however, prevented growth, averaging just 33% of its original concentration at 180 min. (Figure 4). After 360 min., the blank control bottles still had 93% of its initial concentration, ending essentially where it began (Figure 4). The TiO\textsubscript{2} performed slightly better, but the benefits of the composite were evident as it ended with just 8% of its original concentration (Figure 4). The average bacterial concentration in each type of bottle at the end of 360 min. is graphed in Figure 5. The error bars represent standard error, calculated by dividing the standard deviation by the square root of the number of measurements. Again, only 2-3 measurements were available for the calculation of standard deviation and standard error.
Discussion

The purpose of this study was to improve the SODIS method of water treatment by testing the antibacterial activity of two types of photocatalyst coatings, one of TiO\textsubscript{2} and the other of a TiO\textsubscript{2}/SWCNT composite, on plastic PET bottles. Analysis of the bacterial concentrations of river water contained in the coated bottles throughout two trials of a 360 min. irradiation test yielded the conclusion that both the TiO\textsubscript{2} and the TiO\textsubscript{2}/SWCNT composite coatings were able to kill more bacteria than the blank control bottles alone, but the full 360 min. of sun exposure was needed. Complete sun is not necessary, as there was evidence that the photocatalyst-covered bottles were able to reduce bacterial concentrations even in partly cloudy weather. Generally, higher temperatures aid the disinfection process, but results from the second trial demonstrated that high temperatures may also serve to incubate the bacteria and increase concentrations.

There were many benefits to the TiO\textsubscript{2}/SWCNT composite coating, as opposed to the blank or the TiO\textsubscript{2} coatings. First, only the composite was able to reduce concentrations to below the EPA standard of 500 cfu/mL\textsuperscript{6}. Additionally, the TiO\textsubscript{2}/SWCNT composite coating was able to prevent incubation and increases in bacterial concentration, continually killing bacteria even in temperatures optimal for bacterial growth. Finally, the TiO\textsubscript{2}/SWCNT composite produced a more durable coating that the TiO\textsubscript{2} with Pluronic sulfactant, experiencing little to no coating loss after 360 min. the irradiation test.

In conclusion, the TiO\textsubscript{2}/SWCNT composite coating seems to be the best option in terms of meeting EPA standards, preventing growth in optimal temperatures, and producing a strong coating. More trials are needed to confirm reliability and consistency of data through meaningful statistical analyses. Further investigation is necessary to determine the feasibility of the material in terms of cost and potential toxicity.

Sources of Error/Improvements

The most significant source of error is evident in the wide variation of initial bacterial concentrations in the different types of bottles. One possible explanation is that the nanomaterials themselves, particularly the TiO\textsubscript{2}, were contaminated prior to the irradiation test. In the future, this could be avoided by heat or pressure sterilizing the nanomaterials in an autoclave. However, the sterilization conditions would have to be examined to ensure that the structures of the chemicals were not altered in the sterilization process. It is important to note that despite generally higher starting points, the photocatalyst-coated bottles still killed more bacteria and ended with lower bacterial concentrations than the blank control bottles, demonstrating the effectiveness of such materials. The cost effectiveness as well as the availability of resources for mass production are important considerations for the implementation of the nanomaterials.

It was also difficult to position the bottles within the solar reflector box so that each bottle received the same sun coverage. Air and water temperature could have varied in different parts of the box, suggesting slightly different amounts of sun exposure depending on where in the box each bottle is placed. This inconsistency can be corrected by rotating the bottles within the box at specific time intervals so that each bottle is not always located in the same spot.
Further improvements can be made by testing additional time intervals instead of just 0 min., 180 min., and 360 min. Taking samples more frequently would provide a better comprehensive picture of bacterial concentrations in the water during the 360 min. irradiation test. As always, additional trials would be helpful to gain a better understanding of the mechanisms behind the SODIS process.

Directions for Future Research

There are many materials with antibacterial potential to improve SODIS. Investigation into additional nanomaterials such as antimicrobial peptides, chitosan particles, nano-silver compounds, zinc oxide, and fullerenes could yield promising alternatives for point-of-use water disinfection. Metal doping of titanium has also shown potential in past studies.

It would be helpful in future studies to identify the species of bacteria present in the river water at the start of the irradiation test and the species remaining at the end of the test. Besides certain species of indicator bacteria, perhaps there are certain types of bacteria against which SODIS and the TiO$_2$/SWCNT composite are more or less effective. One test for bacterial identification is the API 20E, an 18-24 hour identification test kit that distinguishes between certain Enterobacteriacae and other gram negative bacteria.

Much is still unknown about the toxicity of these nanomaterials. Research into the effects of these materials on lab animals and ultimately humans is needed before consumption of these materials can be risked. TiO$_2$ and SWCNTs have shown exciting antibacterial activity, but many steps remain before they can be implemented into SODIS or other methods of water disinfection.

References


Analysis of PAN and PEGDA Coated Membranes for Filtering Water with Reduced Fouling and Increased Heavy Metal Adsorption
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Abstract
An increase in United States water pollution has been denoted over the past 30 years. With a rise in water pollution, enhanced filtration and heavy metal remediation methods are imperative. Membrane fouling, the process whereby extraneous particles deposit onto a membrane surface and degrade the membrane’s performance, is a major issue facing ultrafiltration. This study’s goals were to enhance membrane flux and anti-fouling performance with a PEGDA filter and to compare the efficiency of PVAm and PEI for heavy metal adsorption. Electrospinning was employed to create membrane platforms for coating and grafting polyethylene glycol diacrylate (PEGDA), N,N’-Methylenebisacrylamide (MBAA), cellulose nanofibers (CNF), polyvinyl alcohol (PVAm) and polyethyleneimine (PEI). The anti-fouling performance of electrospun PAN membranes was determined by coating the surface with CNF, PEGDA, and MBAA, which were chosen due to their hydrophilic and low-fouling properties. Coating the top layer of the membrane with these hydrophilic polymers achieved a flux that remained constant at the quickest rate, at least 20% quicker than that of polyethersulfone (PES) commercial filters. The membrane containing 0.2% CNF and 0.2% PEGDA exhibited 90% rejection and 99% recovery, indicating that CNF and PEGDA exhibited better fouling resistance than PEI. The high fouling performance of the commercial membranes can be attributed to the highly hydrophobic nature of PES, making it prone to membrane fouling. In order to evaluate heavy metal adsorption membrane performance, electrospun PAN membranes were immersed in concentrations of H₂SO₄ (50-70 wt%) to introduce negative charges creating bonds between PVAm, PEI, and the PAN membrane. The outcomes of static adsorption tests indicated that PVAm adsorbed heavy metals at a rate that was ~8-30 mg/g greater than PEI. The higher adsorption rate can be attributed to the increased surface area resulting from the grafted PVAm. In the future, a combined PEGDA, PAN, and polyvinyl chloride (PVC) membrane should be designed to adsorb heavy metals while reducing fouling. The efficacy of modified periods of extensive filtration should also be evaluated.

Introduction
Although the world population is 6.8 billion, 1.2 billion people do not have clean drinking water access1. It is estimated that one-third of the population will face severe water shortages by 20252. As the world population increases by 80 million annually, water shortages also threaten to reduce the global food supply3. Most desalination plants currently rely on flash evaporation, distillation, and electrodialysis for the removal of contaminants, like salt, from water. However, widespread applications are limited by high-energy costs in many third world countries4.

In parallel, high levels of heavy metal pollution have been detected in various water resources throughout the world5. Over 80% of wastewater in developing countries is discharged without treatment, contaminating coastal areas, rivers, and lakes6. With the alarming increase in industrialization and urbanization, the consumption of Chromium (VI) water has been categorized as a major dilemma across the United States7. Imbibition of this heavy metal causes detrimental and permanent health damage due to its carcinogenic effects. Apart from lung cancer and death, the most common effects of ingestion of Chromium (VI) on humans are respiratory problems, genetic alteration, kidney and liver damage, and weakened immune systems8.

Current day filtration techniques include reverse osmosis, hydroxide precipitation, ion exchange, and solvent evaporation. However, many of these methods require large quantities of time and energy, yet produce minuscule filtration and adsorption capabilities.

Electrospinning, a process that uses an electrical current to draw very fine fibers from a liquid, reduces fabrication time for membranes, while introducing small pore sizes for efficient filtration. The use of electrospinning in creating the fibrous membranes allows for random, yet minute pore sizes to be established onto the substrate9. Introducing small pore sizes on the surface of the membrane increases surface area for enhanced heavy metal ions adsorption.

Polymer grafting produces one or more types of polymer blocks and chains connected to a primary chain of a macromolecule10. The introduction of sulfuric acid etches negative charges on the surface of the membrane for polymer grafting11. These negative charges are complemented by the positively charged polymers, which will present active sites that attract the heavy metal ions onto the membrane surface12.

The use of thin film composite (TFC) membranes, which typically consist of 2-3 thin layers, is a robust and efficient method of purifying water13. Yung et al. (2010) demonstrated a novel type of TFCs, called thin film nanocomposite (TFNC) membranes. The bottom layer of this three-tier membrane is a tough, non-woven fibrous material, polyethylene terephthalate (PET). The mid-layer, which supports the barrier layer, consists of electrospun polycrylonitrile (PAN) fibers, which are typically used for the fabrication of microfiltration (MF) and ultrafiltration (UF) membranes14. The top coating layer, which serves as the barrier between solutes and permeates, is constructed with ultra-
fine cellulose nanofibers (CNF) (Figure 1).

PAN (Polyacrylonitrile) has good mechanical properties, good chemical resistance, and is cost-efficient. However, during filtration of wastewater, its hydrophobic nature can dramatically decrease the water permeability due to the tendency of biomacromolecule fouling. The use of cellulose nanofibers in creating membranes increases surface-to-volume ratio to achieve high flux, high durability and high retention with minimal environmental impact. The hydrophilicity of cellulose, due to its large number of hydroxyl groups, gives cellulose nanofibers their anti-fouling characteristic.

Membrane fouling is a significant problem facing ultrafiltration (UF). Permeate flux substantially and irreversibly decreases due to the growth of biofilms on the membrane surfaces, causing a blockage in its pores. Applying a hydrophilic surface coating layer to UF membranes is a potential method of improving their resistance to fouling and maximizing their lifespan.

Polyethylene glycol (PEG)-based materials have been considered for several coating applications due to their excellent resistance to fouling and macromolecule adhesion, amphiphilic nature, and biocompatibility. By varying the concentration or molecular weight of polyethylene glycol diacrylate (PEGDA), its material properties, including mesh size, bioactivity, and degradation rate, can be tightly regulated and systematically modified. N,N'-Methylenebisacrylamide (MBAA) has a hydrophilic nature as well as the ability to serve as a crosslinking agent, therefore synthesizing membranes during free-radical polymerization while increasing filtration performance.

Essentially, hydrophilicity refers to the hydrogen bonding that occurs between polar water molecules. Membranes coated with hydrophilic polymers like MBAA have nitrogen or oxygen in their backbone structure. Therefore, they contain polar functional groups that bond with molecules of water, reducing the buildup of biofilms on membrane surfaces and improving the membrane's ability to adhere to liquid.

Filtration membranes undergo major pressure differentials, so creating a top coating that is as thin as possible is crucial. A thin top coating would have the ability to withstand the shear force and pressure exerted by the fluid in cross-flow filtration. Due to its solvent-free formulations, high polymerization rate, low energy consumption, ambient temperature operation, and ability to tailor polymer properties, UV crosslinking polymerization is widely used to create barrier layers on filtration membranes.

The crosslinking molecules absorb the photons energy via electronic excitation when exposed to UV light. This selective excitation is used to initiate synthesizing chemical reactions by generating reactive species (free radicals or ions). By using the UV crosslinking process, the creation of a dense and tight coating layer for cellulose nanofibrous membranes could be achieved.

A major goal of this study was to evaluate the anti-fouling properties of thin film nanocomposite membranes synthesized via UV crosslinking. By integrating the cellulose barrier layer with hydrophilic UV-sensitive polymers like PEGDA and MBAA, a novel dense barrier layer that can increase membrane specificity and minimize foulant contact with underlying support layers (without severely compromising membrane flux) can be synthesized.

This study’s goal was also to determine the characterization of the electrospun nanofibers of 8 wt% PAN for heavy metal adsorption. By grafting modified PAN electrospun nanofibers with polyethyleneimine (PEI) and polyvinyl alcohol (PVAm) onto an electrospun membrane, enhanced heavy metal adsorption rates would be achieved.

**Materials and Methods**

**Cellulose Nanofiber Membrane Preparation:** The electrospinning process was used to create the mid-layer PAN nanofibrous scaffold, which was then placed on top of the PET non-woven substrate. The combined PET substrate and PAN scaffold were soaked in hydrochloric (HCl) acidic water (pH = 2) for 1 minute. A 7.62 by 10.16 cm membrane was cut, and its edges were taped to a glass plate. A rubber roller was used to drain the excess water. Roughly 4.0 g of a cellulose nanofiber aqueous suspension (0.5 wt%) was cast onto the PAN/PET support and was evenly dispersed over the membrane with the barrier layer thickness regulated by the layers of tape. A cellulose gel was formed immediately upon contact at the interface between the water and cellulose solution. The cellulose nanofiber was thoroughly washed with water and then dried at 100°C for 20 minutes after coating.

**Cellulose Nanofiber/PEGDA/MBAA Barrier Layer:** PEGDA with...
An anti-fouling test was carried out in order to evaluate the anti-fouling properties of each membrane. Multiple trials to determine a constant water flux were carried out. The water flux was recorded every 5 minutes at 30 psi until it remained the same for 15 minutes, the result of multiple trials that determined a constant water flux. After the water flux became constant, protein feed solution was prepared by dissolving Bovine Serum Albumin (BSA) (Sigma Aldrich) in 0.01M phosphate buffered saline (PBS) (pH=7.4) to produce 1 g/L BSA solution, 50 mL of which was then poured into the dead-end cell. BSA was deployed due to its ability to adhere to the membrane surface, providing a clear indication of the membrane’s anti-fouling performance. In order to stabilize the charges of the protein, PBS was incorporated in the BSA solution. The protein flux was recorded every 5 minutes at 30 psi until steady. After the BSA flux was recorded, the membrane was flushed with DI (deionized) water. In order to determine the recovery ratio of the membrane, the water flux before and after the flushing of the membrane were compared. The flux recovery ratio was determined by Equation 1.

\[
R\% = \frac{C_{\text{feed}} - C_{\text{permeate}}}{C_{\text{feed}}} \times 100\%
\]

**Equation 1.** \(C_{\text{feed}}\) and \(C_{\text{permeate}}\) are the concentrations of the feed solution and permeate solution.

**Table 1.** Membrane Coating Solution parameters for PAN/PET support.

<table>
<thead>
<tr>
<th>Sample</th>
<th>%Wt. of CNF</th>
<th>Wt% PEGDA</th>
<th>Wt% MBAA</th>
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</tbody>
</table>

**Flux Determination:** A Millipore stirred (dead-end) cell (model 8050) with an effective filtration area of 0.00134m² was used in order to evaluate the flux and rejection ratio of each membrane sample. The membrane samples that were placed within the dead-end cell were cut into 40 mm diameter circles. A water permeability test was then carried out. The water flux was measured every 5 minutes at 30 psi. A graduated cylinder was used to measure the amount of permeate. **Rejection Ratio Determination:** The rejection percentage (R%) was used to evaluate the filtration performance of the membranes. The rejection percentage was determined by Equation 1. In order to equilibrate the surface hydrophobicity of the membrane, 50 mL of pure water permeate was collected. Following this equilibration, 50 mL of a 1000 ppm polyethylene glycol (PEG) (Sigma Aldrich) aqueous feed solution was used to test the rejection efficiency. PEG solutions with molecular weights of 4600 kDa and 10000 kDa were prepared. Filtration was carried out at room temperature and the operating pressure was 30-40 psi. A total organic carbon analyzer (TOC-V, Shimadzu Corp.) was employed to determine the molecular weight cut-off (MWCO) of the membranes. The MWCO method was used to infer the membrane pore size based upon the molecular weights of a particular molecule that the membrane was capable of rejecting. By determining the organic carbon concentration of the permeate and feed solutions, the TOC-V can be used to calculate the rejection percentage using Equation 1.

**Anti-Fouling Properties and Flux Recovery:** An anti-fouling test was carried out in order to determine the anti-fouling properties of each membrane. Multiple trials to determine a constant water flux were carried out. The water flux was recorded every 5 minutes at 30 psi until it remained the same for 15 minutes, the result of multiple trials that determined a constant water flux. After the water flux became constant, protein feed solution was prepared by dissolving Bovine Serum Albumin (BSA) (Sigma Aldrich) in 0.01M phosphate buffered saline (PBS) (pH=7.4) to produce 1 g/L BSA solution, 50 mL of which was then poured into the dead-end cell. BSA was deployed due to its ability to adhere to the membrane surface, providing a clear indication of the membrane’s anti-fouling performance. In order to stabilize the charges of the protein, PBS was incorporated in the BSA solution. The protein flux was recorded every 5 minutes at 30 psi until steady. After the BSA flux was recorded, the membrane was flushed with DI (deionized) water. In order to determine the recovery ratio of the membrane, the water flux before and after the flushing of the membrane were compared. The flux recovery ratio was determined by Equation 2.

\[
\text{Recovery \% = } \left( \frac{J_{w1}}{J_{w2}} \right) \times 100\%
\]

**Equation 2.** Flux recovery ratio. \(J_{w1}\) is the steady pure water flux before the BSA test and \(J_{w2}\) is the steady pure water flux after the BSA test.
Electrospun membranes were imaged using a scanning electron microscope (SEM) after gold-sputter (SC7620 Sputter Coater, Quorum Technologies). The fiber diameter was analyzed from the SEM images using Leica software. SEM images were received from Phenom. Results were reported as mean ± standard deviation. FTIR: Degree of hydrolyzation of PAN membrane in sulfuric acid was characterized using Fourier transform infrared spectroscopy (FTIR) qualitatively. Pure Water Flux Test: The pure water flux of the membranes was measured by dead-end filtration at the ambient temperature using Milli Q water. A dead-end filtration cell (Millipore, USA) with an effective filtration area of 3.9 x 10^-4 m² was used for the flux measurements. Heavy Metal Adsorption Test: Dry modified PAN electrospun membrane was weighed (~5 mg) and stirred in 20 mL solutions with different initial Cr(VI) concentrations ranging from 5 ug/mL, 10 ug/mL, 20 ug/mL, 40 ug/mL to 100 ug/mL. The concentration of Cr(VI) in solution was determined with UV-Visible Spectroscopy. The amount adsorbed (mg/g) was calculated in Equation 3, q is the amount adsorbed (mg/g), C₀ is the initial Cr(VI) concentration, and Cₑ is the final Cr(VI) adsorption after 24 hours. V is the solution volume, and M is the weight of the PVAm-PAN membrane used. Saturation adsorption capacity of the membrane was calculated according to the Langmuir model, which is given in Equation 4, where Qₑ is the equilibrium adsorption capacity, Ce is the equilibrium Cr(VI) concentration in solution, Qₘ is the saturation adsorption capacity, and Kᵥ is the adsorption equilibrium constant. The Langmuir model is the most common model used to determine the amount of adsorbate adsorbed on an adsorbent.

\[
q = \frac{(C₀ - Cₑ)V}{M}
\]

Equation 3. The concentration of the Cr(VI) adsorbed (mg/g).

\[
Qₑ = \frac{Cₑ}{Qₘ} + \frac{1}{KᵥQₘ}
\]

Equation 4. Saturation adsorption capacity of the membrane.

Results

Cellulose Nanofiber Membrane Performance

Anti-Fouling Performance

In order to determine the anti-fouling properties of each membrane, an anti-fouling test was carried out. The water flux of the 0.02% CNF + 0.2% PEGDA membrane (experimental membrane) became constant after 45 minutes, maintaining a flux of approximately 10.1 L/m²·h at 30 psi. The water flux of the 0.02% CNF + 0.2% PEGDA membrane reached a plateau after 45 minutes of testing. This indicates the high anti-fouling performance of the PEADA membrane. The Koch HFK 328 membrane exhibited the highest initial flux, attributed to its large pore size (5 kDa). The water flux of the Koch membrane become constant after 55 minutes, approximately 20% longer than the time it took for the PEGDA membrane water flux. Due to the large pore size difference of the Pall membranes, the Pall 5K membrane exhibited an initial flux of 45 L/m²·h (at 30 psi), approximately 25 L/m²·h greater than that of the Pall 3K membrane (Figure 3).

Rejection and Flux Recovery Performance

The rejection ratio was determined in order to evaluate the filtration performance of each membrane. The PEGDA (0.02% CNF + 0.2% PEGDA) membrane exhibited a rejection ratio of 90%, which was higher than that of all commercial membranes tested. The rejection ratio of the Pall 5K membrane was higher than that of the Pall 3K membrane by 9%. Although the Koch 328 membrane exhibited the highest initial flux (Figure 3), its rejection was 35% lower than that of the PEGDA membrane. Concluding from the performance of the MWCO and water permeance, the rejection ratio of the PEGDA membrane was 35%-65% higher than that of the three chosen commercial UF membranes. The higher rejection ratio of PEGDA indicates that the experimental membrane served as a more efficient filter; this can be attributed to the hydrophilic properties of PEGDA and the hydrophobic properties of polyethersulfone (PES) in the

Figure 3. Comparison of water flux (at 30 psi) of PEGDA membrane and commercial membranes (Pall 5K, Pall 3K, Koch HFK 328).
commercial membranes (Figure 4).

The recovery ratio was determined by comparing the water flux before and after flushing the membranes with deionized water. The PEGDA (0.2% CNF and 0.2% PEGDA) membrane exhibited a recovery ratio of 99%, indicative of a decrease in fouling (Figure 5).

**Water Contact Angle Test**

A water contact angle test was performed with the use of an optical contact angle meter (CAM200, KSV Instruments, LTD). The water contact angles of the PEGDA/MBAA and PEGDA membranes were significantly lower than that of the commercial membranes, Koch HFK 328 and Pall 5K, respectively. The PEGDA/MBAA and PEGDA membranes had a higher surface hydrophilicity than the commercial membranes. It is interesting to note that the water droplets placed onto the experimental membranes disappeared within a few seconds, while the water droplets placed onto the commercial membranes remained for 4-5 minutes. This is indicative of the higher surface hydrophilicity of the experimental membranes, meaning that they had better anti-fouling properties than the commercial membranes. The hydrophobicity of the PES integrated in the Koch and Pall membranes caused their surfaces to have higher water contact angles (Figure 6).

**PVAm/PEI Membrane Performance**

**SEM Images of Polymer-Coated Membrane**

Figure 7 shows the effect of sulfuric acid on fiber diameter and thickness of the PAN electrospun membrane after being immersed in sulfuric acid. The size of the fiber did not drastically change and was able to maintain minimal size. PAN electrospun nanofibers are non-charged. However, an electrostatic interaction occurred after introducing negative charges onto the surface of the electrospun PAN membrane. Positively charged grafted polymers were able to bind to the PAN electrospun membrane, enhancing the ability of heavy metals to adsorb to the surface of the membrane. Branching of the polymers also increased with the presence of increasing concentrations of sulfuric acid. The rise in polymer branching increased surface area, enhancing heavy metal adsorption. PVAm in solution was initially water soluble, meaning PVAm would not adhere to the membrane when submerged in water. However, with GA crosslinking, PVAm was able to bind and cling to the membrane, making it insoluble in water.

**Characterization of Functional Groups**

Many different functional groups were introduced onto the surface of the electrospun membrane after being etched with sulfuric acid. As represented in Figure 8, the –CN and –CH2– functional groups were most abundant after using the highest concentration of the sulfuric acid.

PVAm already had a functional group that exhibited heavy metal adsorption, the amine group. The positively charged amine group attracted the Chromium (VI) ions to remove heavy metal from wastewater after being grafted. In Figure 9, a schematic diagram shows the active sites of heavy metal adsorption, amine groups, and the GA crosslinking of the PVAm polymer.

**Comparison of Heavy Metal Adsorption Rates of Polymer-Coated Membranes**

The polymer PVAm adsorbed more heavy metal ions when compared to the polymer PEI. PVAm was more efficient because of its pore size, water retention, and strength of the electrically spun fibers. Increasing the polymer concentration also allowed for an increase in Chromium (VI) adsorption rate. With more polymers coated onto the surface of the membrane, more heavy metal ions are able to adsorb onto the surface of the membrane in order for water filtration to occur. Moreover,
the increase in polymer concentration permits an increase in effective areas found on the membrane. Effective areas indicated the amount of heavy metals to be adsorbed onto the surface of the polymer-grafted membrane. Various areas of the membrane have the capability to adsorb heavy metal ions, rather than having only one spot, or certain areas (Table 2).

**Discussion**

A novel cellulose and PEGDA coated membrane was created for filtering water with reduced fouling and increased water permeance. It was found that the membranes coated with PEGDA exhibited the highest rejection and recovery rates, which resulted from the hydrophilic nature of PEGDA. Since the PEGDA membrane had the highest recovery rate, it was least affected by the symptom of fouling. It was also found that the large pore size of the commercial membranes tested, (Pall 3K, Pall 5K, and Koch 328) affected their ultrafiltration performance by decreasing the water flux.

The use of electrospun PAN membrane also proved to be effective and efficient when adsorbing Chromium (VI) ion particles. The PVAm membrane adsorbed a greater amount of Chromium (VI) ions from the turbid water than the PEI membrane. Concentration of the polymers, PVAm and PEI, also played an effective role as it directly correlated to the adsorption rates of heavy metals ions. Increasing the concentration of the polymers established numerous amounts of polymers onto the surface of the PAN electrospun membrane for efficient heavy metal removal. With the addition of polymer grafting onto the electrospun membranes, the fiber diameter increased and pore size decreased for increased surface area.

This research can also lead to the development of new, fouling-resistant, nanofiltration membranes that can help alleviate worldwide water shortages. The membranes incised with sulfuric acid can be used to help industrial wastewater treatment. These charges etched onto the surface of the membrane bonded with the polymers and attracted heavy metal ions in contaminated water because of the existing amine groups of the polymer. The modified membranes efficiently removed heavy metal from wastewater in a short amount of time.

**Figure 8.** The characterization and functional groups present on the surface of the PAN membrane after being etched with sulfuric acid.

**Figure 9.** Schematic diagram of PVAm polymer with active sites (amine group) and polymer crosslinking.

**Table 2.** Comparison of PAN-PVAm and PAN-PEI nanofiber membrane adsorption rates after being treated varied polymer concentrations.

<table>
<thead>
<tr>
<th>Base material</th>
<th>Grafted functional polymer</th>
<th>Treated solution concentration</th>
<th>Adsorption rate (mg/g)</th>
</tr>
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<tbody>
<tr>
<td>PEI</td>
<td>2 wt%</td>
<td>5.1 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 wt%</td>
<td>20.6 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>PAN electrospun nanofibers</td>
<td>2 wt%</td>
<td>13.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 wt% x 3</td>
<td>23.9 ± 3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 wt%</td>
<td>24.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 wt%</td>
<td>39.0 ± 2.3</td>
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Initial Cr(VI) solution was 10 μg/mL in the adsorption test. Adsorption rate was reported in the result of Cr(VI) amount adsorbed per gram of the whole PVAm-PAN membrane, in which PVAm takes up ~8% in weight.
Future Investigations
It is necessary to repeat these experiments and investigate the integration of other hydrophilic polymers into cellulose nanofiber membranes. Furthermore, to determine the fouling resistance of these membranes, additional testing is required as fouling resistance is critical to the commercial viability of desalination membranes. A dually effective filter of waste that reduces fouling and absorbs heavy metal should also be investigated in the future. If the PAN is removed from the filter and modified, it may cause the filter system to exhibit both beneficial properties when it is restored (anti-fouling and heavy metal adsorption). It is also crucial to investigate the results of long-term running experiments (24 hours, 48 hours, etc). Lastly, dynamic adsorption tests should be conducted to determine the membrane’s versatility. Dynamic adsorption tests will provide a different aspect in filtrating heavy metal water as it is pressurized through a syringe pump. Multiple heavy metals, such as lead, copper, and zinc should also be utilized to help validate the created membrane’s proficiency in removing heavy metal from water.

References

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A Multi-Spacecraft Approach to Studying Auroral Kilometric Radiation Using the Virtual Wave Observatory

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Abstract

According to the analysis of the radio wave spectrograms captured by the IMAGE, Geotail, Polar, Wind, Stereo-A, and Stereo-B spacecraft, the correlation between the auroral kilometric radiation (AKR) intensity and the auroral electrojet (AE) index have been found to be negatively correlated as well as positively correlated. These findings raised questions against the present notion that AKR intensity and the AE index are positively correlated. Although the original objective of this study was to establish a set of stable threshold values in the auroral AE index for AKR detection, the negative correlations found between the AKR intensity and the AE index made these threshold calculations unattainable. Furthermore, this study was the first attempt to establish a set of stable threshold AE index values associated with AKR using a multi-spacecraft approach provided by the Virtual Wave Observatory (VWO). This approach was essential to ensure that any changes observed in the spacecraft data were due solely to the AKR source. The whole spectrum of AKR was analyzed, normally 80-800 kHz in the satellite spectrograms. The threshold value was determined by comparing AE index plots of AKR events to corresponding satellite spectrograms of the events. However, the poor correlations found through this study indicated that there may be other drivers affecting the AKR intensity other than the strength of the auroral electrojet current. Thus, a future multi-parameter magnetosphere investigation using other geometric indices, such as the Dst index or the Kp index in comparison with the AE index and AKR intensity, could explain how unknown drivers of the AKR intensity and the AE index synergistically caused the inconsistent correlations. The understanding of the magnetosphere conditions, as measured by the AE index, which trigger the AKR emission is crucial to planetary research because it provides a mechanism to remotely sense the state of the Jupiter's, Saturn's, Neptune's, and Uranus' magnetospheres.

Introduction

In depth understanding of auroral kilometric radiation (AKR) is very crucial to future planetary research due to its similarities to extraterrestrial non-thermal radiations such as Jupiter's decametric radiation and Saturn's saturnian kilometric radiation. These radiation emissions are similar to AKR but are unique in structure based on the dynamics of their magnetospheres and solar wind environments. In addition, non-thermal radio emissions can be used to remotely sense the magnetosphere conditions of complex planets such as Jupiter and Saturn. This would benefit future spacecraft launches to these planetary systems to observe high level interest moons within Jupiter and Saturn's magnetospheres such as Jupiter's moon, Io, and Saturn's moon, Enceladus, by ensuring that favorable conditions are present for spacecraft hardware and observation. Furthermore, Jupiter and Saturn are gaseous planets, and using non-thermal radiation emissions to remotely gather information to profile them would be more practical since there is no tangible surface to survey.

The sun continually emits a fast-moving stream of plasma known as solar wind. Fortunately, Earth's magnetosphere acts as a shield that protects life on Earth from the solar wind. Interactions between the solar wind and the magnetosphere create disturbances that can lead to the production of intense non-thermal radio emissions such AKR. The source of AKR is broadly beamed and found near the North and South Auroral Zones on the night side of Earth. When solar winds strike the magnetosphere, electrons travel along Earth's magnetic field lines and are then driven down to Earth's auroral electrojet in our high-latitude ionosphere. The influx of electrons causes unstable polarization of the ionosphere which results in AKR emission and aurora borealis generation.

Fig. 1. An AKR radio source in relation to a magnetic field line and Earth. Electrons are accelerated downward towards Earth [Louarn, (2006)].

Fig. 2. The auroral electrojet current in Earth's upper ionosphere is depicted. The points represent the magnetometer stations, NLK, OND, ANT, and RUG are relay stations connecting to the magnetometer stations that process the AE index data. [Space, Telecommunications, and Radioscience Laboratory, (1997)].
Twelve ground data stations, located in Earth’s northern polar regions, measure the strength of the auroral electrojet current and represent the strength of the current with the auroral electrojet (AE) index. As a result, the strength of magnetosphere disturbances due to solar winds in the auroral regions can be measured by the AE index. Voots et al. [1977] concluded that the AKR intensity was a reliable indicator of the AE index through statistical analysis using the IMP-6 satellite. This notion has been adopted since then and applied in research. For example, Kurth and Gurnett [1988] developed a proxy to derive the AE index value from the AKR intensity which led to the development of the “AKR index” still used today, and Kunamoto et al. [2005] established seasonal and solar cycle correlations between the AKR intensity and the AE index for the “AKR index”.

With the notion that the AKR intensity was a reliable indicator of the AE index, the original objective of the study was to establish a set of stable threshold AE values for the AKR emission by using a novel multi-spacecraft approach with datasets, mainly spectrograms, provided by the freeware Virtual Wave Observatory (VWO) (http://vwo.nasa.gov). The threshold value is one of the factors that can be used in sensing magnetosphere conditions needed for AKR emission. In addition, this study was the first attempt to establish a threshold AE value for the AKR emission using the VWO. Before the VWO, the act of gathering and comparing multi-spacecraft datasets of a magnetospheric event was cumbersome due to time scale differences and cross database searching. The study of AKR using more than one satellite required simultaneous download access to multiple satellite databases and data navigation tools. The VWO seeks to solve this problem by connecting to all satellite databases and acting as a data delivery service. By using the VWO, datasets were quickly gathered and compared to the AE index from multi-spacecraft databases with a single search engine. The multi-spacecraft approach ensured that any simultaneous changes seen in the spectrograms were due to changes in the source and rather than the orbit or instrumentation of the particular spacecraft. Furthermore, this approach also greatly improves the spatial coverage of the AKR source. Daily spectrograms from the following spacecraft were analyzed: Geotail, Wind, IMAGE, Polar, Stereo A, Stereo B, and Cluster. Corresponding AE values from the daily spectrograms were plotted to correlate with the observed AKR activity. The approximate threshold of AE value was determined to be below a certain AE value when AKR emission is not observed. Because Green et al. [2004] concluded that the AKR frequency and intensity vary seasonally as a function of dipole tilt of the Earth, seasonal data was recorded. Unexpectedly, the AKR intensity and the AE index were found to be negatively correlated which disputed findings by Voots et al. [1977] and put previous AKR intensity and AE index value studies into question including: Green et al. [2004], Kurth and Gurnett [1988], Kunamoto et al. [2005], Kurth et al. [1998], and Benson and Akasofu [1984]. In addition, the results revealed that there is a great fluctuation in the threshold values.

Materials and Methods
Spacecraft Orbital Alignment: Data was collected from Geotail, Polar, Wind, IMAGE, Stereo A, Stereo B, and Cluster spacecrafts as spectrograms using the VWO Data Query. These spacecraft orbit Earth with elliptical trajectories with Earth as a focus. Geotail was operational from 1992-2007, Wind was operational from 1994-present, Polar was operational from 1996-1997, IMAGE was operational from 2000-2005, and Stereo A and Stereo B were only close enough to Earth to observe AKR from late 2006-early 2007. The freeware SSCWeb (http://sscweb.gsfc.nasa.gov) orbit viewer was used to determine and document at what months the spacecraft had their orbital apogee in the northern hemisphere. The documented months were then searched for spacecraft datasets containing closely similar AKR signatures (Fig. 3 below) during the times when all spacecraft were in the northern hemisphere (to make sure the spacecraft were viewing the same AKR event) and downloaded. Establishing AE Index Threshold Values: Comparisons were made between the AKR intensity and the AE index. All frequencies within the bandwidth of the AKR emission (normally 80-800 kHz) were analyzed. AKR was considered “On” when at least two spectrograms showed an AKR signature and was considered “Off” when at least two spectrograms in our datasets showed no AKR signatures (Fig. 3). Times when AKR was “Off” or “On” was then determined using the spacecraft time scale. Numerical, one minute resolution AE index data from the Kyoto Data Index Service (http://wdc.kugi.kyoto-u.ac.jp/aeidr/index.html) for the day and times in which AKR emission occurred was then downloaded. This AE index data was then plotted and aligned in time with spacecraft spectrograms. Datasets were considered to be positively correlated if the AE index values increased and decreased together with the AKR intensity. Datasets were considered to be negatively correlated if the AE index values increased and the AKR intensity decreased or if the AKR intensity increased and the AE index values decreased. A threshold value, designated by a red line (as shown in Fig. 4, 6, 8, 10), was then determined and recorded only for positively correlated datasets. This threshold was established by locating a point in the AE index plot where AKR no longer emits and connecting that point with another point in the AE index plot where AKR reemits. AKR emission was not expected when the AE index values were below the threshold value. Poor correlations made threshold values indeterminate. The number of magnetometer stations that registered AE index measurements for each dataset was then recorded. To account for the seasonal variation in AKR intensity and frequency discovered in Green et al. [2004] and Kunamoto et al. [2005], the thresholds were organized by season. Statistical Analysis: The results (threshold values) were planned to be standardized and statistically analyzed. However, statistical analysis was not able to be accomplished because the threshold values were found to be unstable due to the unexpected finding associated with poor correlation between the AKR intensity and the AE index.
Results

Although several datasets were analyzed, the two most outstanding datasets of positively correlated AKR intensity and AE index values (Fig. 4,5,6,7) and two datasets of negatively correlated AKR intensity and AE index values (Fig. 8,9,10,11) are presented and discussed in detail. The spectrograms displayed the intensity of AKR emission as a function of frequency (Hz or KHz) and time in hours (Universal Time (UT)). In these spectrograms, AKR exhibited a distinctive oscillating structure. Spectrograms and AE index plots were stacked vertically in time to show changes in the AKR intensity with relation to changes in the AE index, measured in nanoteslas (nT). AKR signatures were defined as “On” and “Off” in each figure and vertical lines show the portions in the AE index plot where the AKR was “On” and “Off”. The threshold was then determined. This was indicated by a red line. In addition to these datasets, several others are also included to further illustrate the negatively correlation between the AKR intensity and AE index. Table 1 documented the dates of other studied cases of positively correlated and negatively correlated datasets with the number magnetometer station detection. Table 2 showed fluctuations in the calculated thresholds for positively correlated datasets. Furthermore, the observations associated with the usage of the multi-spacecraft approach and the VWO are discussed in this study.

The multi-spacecraft approach allowed this study to greatly reduce the spacecraft bias that could have occurred if datasets from only one spacecraft had been used. An example illustrating the strength of this approach was shown in Fig. 4, when Wind observed an AKR gap from 06:00-07:00 UT (red circles in Fig.4). If only the Wind spacecraft dataset was studied, it could have been concluded that from 06:00-07:00 UT, AKR was “Off”. However, there was strong indication of AKR emission observed by both Geotail and Polar during this time window. Reducing spacecraft bias ensured that the changes in spacecraft spectrograms were due to solely the AKR source and not the orbital trajectory or the instrumentnation of the spacecraft. In addition, multi-spacecraft datasets from Geotail, IMAGE, Wind, Polar, Stereo-A, and Stereo-B were quickly retrieved and organized by the VWO for analysis. This study demonstrated the power of the VWO in cross dataset wave data retrieval and the importance of the VWO in future research.

Thresholds of Positively Correlated Datasets. Voots et al. [1977] concluded that the AKR intensity and the AE index were positively correlated. As a result, much research has been based on the notion that AKR intensity was a reliable indicator for the AE index, for example, Green et al. [2004], Kurth and Gurnett [1988], Kunamoto et al. [2005], Kurth et al. [1998], and Benson and Akasofu [1984]. Fig. 4 and Fig. 6 highlighted most prominently a positive correlation between the AKR intensity and the AE index. As a result, threshold AE index values for AKR were effectively established on these datasets. Although the threshold values were effectively established, based on the theory developed by Voots et al. [1977], Table 2 showed that the values varied greatly across multiple data sets, including values within their respective seasons. These fluctuations indicated that there exists other influential driver(s) of the AE index, not just AKR. To further understand these fluctuations, in order to establish a set of stable threshold values, additional studies need to be conducted. A multi-parameter magnetospheric state study is the next step to examine how the magnetosphere as a whole affects AKR emission as well as the auroral electrojet current and therefore the AE index. An additional study of the AE index trends using data from months prior to 09/10/1996 and 06/05/1996 may also help future studies understand to what extent magnetosphere conditions prior to the emission of AKR could affect the AKR emission.

Negatively Correlated Datasets: In contrast to the theory developed by Voots et al. [1977], the analysis of the results also indicated that the AKR intensity was, in many cases, negatively correlated with the AE index. Fig. 8 and Fig. 10 highlighted most prominently how the AKR intensity and AE index were negatively correlated. In some gaps, AKR was “Off” but AE values surpassed the threshold value (Fig. 8). In others, AKR was “On” but AE values remained well below the threshold value (Fig. 10). Voots et al. [1977] concluded that these observed negative correlations were caused by the position of the spacecraft and the lack of magnetometer station spatial coverage, not the AKR source or the AE current. However, the negatively correlated AKR and AE index results challenge these conclusions.

Voots et al. [1977] claimed that large AE index values corresponding with little AKR intensities or “Off” AKR was because the IMP-6 satellite was on the dayside of Earth. Due to a cutoff propagation effect of AKR, Voots et al. [1977] explained that dayside satellites could not detect nighttime AKR at low latitudes which accounted for high AE index values but no AKR. However, the analysis of the data showed that on 06/04/2002, IMAGE and Geotail were on the nightside of Earth when they observed the AKR gap between 02:00-04:30 UT (Fig. 8). On the same day, Wind was at a high latitude (high z coordinate value) and also observed the same AKR gap from 02:00-04:30 UT (Fig. 8, 9). Similar AKR structures in spectrogram plots from IMAGE, Geotail, and Wind confirmed that the AKR gap was due only

Fig.4. Spacecraft observations from Geotail, Wind, and Polar with AE index plot on 9/10/1996 showing positively correlated AKR intensity and AE index values. The indicated threshold is 275 nT. Circles show the advantage of using a multi-spacecraft approach. Wind observes AKR “Off”, but Geotail and Polar observe weak, not disappearance of AKR. Even with positively correlated datasets, minor anomalies for short periods of time such as between 0500-0600 UT exist.
Fig. 5. This is a Cartesian coordinate system representation of spacecraft trajectories on 09/10/1996 from 0000-0600 UT with Earth as the origin (Re is Earth radii and the Sun is in positive X direction). Because the Wind spacecraft is much farther away from Earth than Geotail and Polar, Wind has its own orbital plot to prevent scale distortion. All spacecraft were good positions to observe northern AKR and were most likely viewing the same AKR emission. This was confirmed in Fig. 4 where all spacecraft observed similar AKR structures.

Fig. 6. Spacecraft observations from Geotail, Polar, and Wind with AE index plot on 06/05/1996 showing positively correlated AKR intensity and AE index values. The indicated threshold value is 50 nT.

Fig. 7. This is a Cartesian coordinate system representation of spacecraft trajectories on 06/05/1996 from 1200-1800 UT with Earth as the origin (Re is Earth radii and Sun is in positive X direction). Because the Wind spacecraft is much farther away from Earth than Geotail and Polar, Wind has its own orbital plot to prevent scale distortion. Although Wind is in the southern hemisphere, it was far away enough from Earth to view the northern AKR source. This was confirmed in Fig. 6 where Wind observed similar AKR structures compared to Geotail and Polar which were in the northern hemisphere.

Fig. 8. Spacecraft observations from IMAGE, Wind, and Geotail with AE index plot on 06/04/2002 showing negatively correlated AKR intensity and AE index values. Circles show abnormally high AE index values well after AKR disappeared.
to the source, not the spacecraft. The AE index values during this time interval increased sharply past 300 nT when there was no AKR detected by all spacecraft. This indicated that the explanation from Voots et al. [1977] was incomplete since the high increase in the AE index values with the corresponding lack of AKR emission observed by the spacecraft was not due to a cutoff propagation effect. Instead, it was due to the source of the AKR and the AE current.

Furthermore, Voots et al. [1977] claimed that small AE index values corresponding with high AKR intensities or “On” AKR was attributed to low spatial coverage by magnetometer stations. In the dataset collected on 03/25/1996, 10 out of 12 magnetometer stations recorded AE values of the event, well enough to effectively cover the AKR emission (Table 1). In this dataset, an AKR gap existed between 10:15-11:20 UT (Fig.10). Although only Polar was in the northern hemisphere at this time, Geotail and Wind were close to the equator and far enough away to view the northern AKR. Despite the differences in observation points, the existence of AKR and its structures in Geotail’s and Wind’s spectrograms were very similar to Polar’s, which confirmed that all spacecraft were looking at the northern AKR. In agreement with the theory from Voots et al. [1977], the AE values dropped below the threshold within this time frame. However, the AE values continued to be well below the threshold while all spacecraft observed high levels of AKR from 11:20 UT onward (Fig. 10). This indicated that the explanation from Voots et al. [1977] was incomplete since the low observed values in the AE index corresponding with the high AKR intensities was not due to inadequate magnetometer station coverage. Instead, it was due to the source of AKR and the AE current.

Table 1. Other studied cases with magnetometer station detection information. This ensured that spatial coverage for AE value recording was sufficient to conclude that changes in AE values were due to the AKR source.

<table>
<thead>
<tr>
<th>Dates in which AE and AKR are positively correlated (mm/dd/yyyy)</th>
<th># AE Magnetometer Station detection out of 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fig. 49)/10/1996</td>
<td>10</td>
</tr>
<tr>
<td>10/12/1996</td>
<td>10</td>
</tr>
<tr>
<td>3/6/2002</td>
<td>10</td>
</tr>
<tr>
<td>4/7/2001</td>
<td>11</td>
</tr>
<tr>
<td>6/6/1997 (Fig. 50)/5/1996</td>
<td>11</td>
</tr>
<tr>
<td>1/3/1997</td>
<td>11</td>
</tr>
<tr>
<td>12/23/2006</td>
<td>9</td>
</tr>
</tbody>
</table>
Conclusion

Although this study was not successful in establishing a stable set of threshold AE index values for the AKR emission, it uncovered new findings which raised challenging questions against the notion established by Voots et al. [1977] that the AKR intensity is a reliable indicator for the AE index. As a result, past research that was based on the conclusions from Voots et al. [1977] could be enhanced to account for cases when the AKR intensity and the AE index are not consistently correlated. The use of multi-spacecraft approach provided by the VWO strengthened the results if this study since the source of the AKR was viewed from multiple vantage points, thus reducing spacecraft bias. To explain why the AKR intensity and the AE index were negatively correlated in this study and in Voots et al. [1977], a multi-parameter magnetospheric state study using other geomagnetic indices such as the Planetary K index (Kp) index and Disturbance storm time (Dst) index in comparison with the AE index could explain how other magnetosphere disturbances affect the emission of AKR. In addition, multiple factors such as pre-existing magnetosphere conditions and other unknown AE index drivers other than AKR will need to be investigated.

The Dst index and Kp index differ from the AE index in that they measure the geomagnetic storm levels of Earth on a planetary scale, not just the auroral zones. The Dst index measures the strength of storm time ring current. This is a current of electrons which flows east to west in the equatorial plane. The Dst index values are generated by averaging the magnetic field perturbations measured by multiple ground magnetometer stations located at low latitude locations near the equator. A more negative the Dst index value, measured in nano Tesla (nT), indicates a more severe geomagnetic storm [Space Physics Research Group University of California, Berkeley, (2012)]. The Kp index measures geomagnetic storm levels using multiple ground magnetometer stations located at mid-latitudes. Kp is the unit for this index represents a range of nano Tesla. When the auroral zone expands southward during geomagnetic storms, these stations can record the effects of the auroral electrojet current and the ring current. A higher Kp index value indicates a more severe geomagnetic storm [The Center for Science Education at the UC Berkeley Space Sciences Laboratory, (2010)]. The Kp index has a resolution of three hours while the Dst index has a resolution of one hour.

Although the Kp index is hypothesized to be more reliable geomagnetic index for studying AKR intensity, using the short time period datasets from this study limits its effectiveness. The Kp index’s resolution is too low to study data in the span of few hours. Thus, the Kp index could be more useful when compared to the Dst and AE indices over days or months in future datasets because there would be more data points. The times when AKR is “on” and when AKR is “off” are labeled according to the spectrogram in Fig. 8. However, more datasets will need to be gathered in order to confirm this preliminary finding.

To further this study, additional spacecraft spectrograms provided by the VWO are being analyzed to investigate the other parameters that may affect the AKR emission for the AE index. The development of an algorithmic method to provide a more accurate calculation of the threshold AE index values will also need to be investigated. Hopefully, the findings in this study will help refine the theory by Voots et al. [1977] and further enhance the detection mechanism for AKR emission. With the ability to better sense the AKR emission, this similar mechanism could be adapted for Jupiter’s decametric radiation and the Saturn’s saturnian radiation to remotely diagnose the complex magnetospheres of these planets, thus allowing a more effective research tool for Jupiter’s and Saturn’s highly assorted moons.
References


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Chance favors the prepared mind