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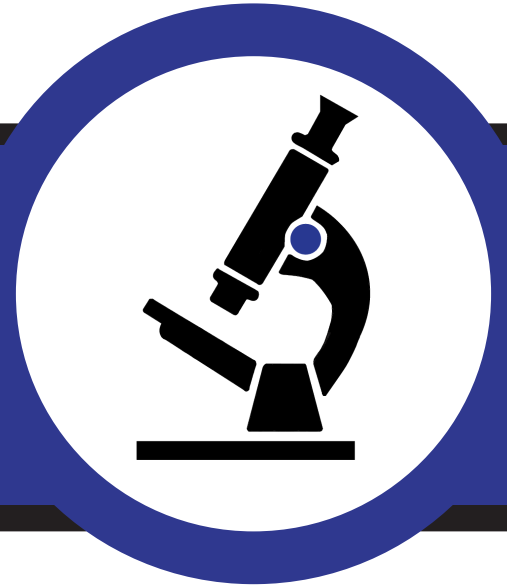

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MISSION

Fine Focus publishes original research by undergraduate students in microbiology. This includes works in all microbiological specialties as well as microbiology education.

SCOPE

We are an international journal dedicated to showcasing undergraduate research in all fields of microbiology. ***Fine Focus*** is managed entirely by undergraduate students from production to print, but utilizes an External Editorial Board of experts for double-blind peer review of manuscripts.

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Objective Lens

Addressing the global antibiotic resistance crisis through a microbiology undergraduate course-based research experience (CURE)

Dr. John McKillip
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Fine Focus has, since our first issue back in 2014, showcased undergraduate research efforts globally. We have grown in many ways and have developed our review team and marketing team activities to continually evolve and stay relevant. Through our recent partnership with the Small World Initiative (www.smallworldinitiative.org), ***Fine Focus*** would like to be a venue to publish novel findings on new antibiotic discovery. Last year, Ball State University became one of the newest partner institutions with the SWI, and I'd like to share with you here how we are in the process of redesigning our majors microbiology course into a format that will allow for crowdsourcing new antibiotic discovery, all the while leading eventually to original research manuscripts publishable in ***Fine Focus***. I invite and welcome other PUIs to do the same and would be happy to answer questions on how your CURE could submit a manuscript to our journal and showcase the work of your undergraduates, or to collaborate with us.

Statement of the Problem:

If no new antibiotics are isolated and made available by 2050, the CDC estimates that 10 million annual deaths will occur globally as a result of this inaction. Yet, surprisingly few have even heard of antibiotic-resistant bacteria or understand the implications for global health. In fact, no new classes of antibiotics have been developed to treat microbial infections in well over 30 years, as pharmaceutical companies have instead pursued research and development of more lucrative drugs for non-infectious diseases. Since this trend is likely to continue into the foreseeable future, this crisis must be addressed using alternative creative approaches. We propose to change our undergraduate microbiology class laboratory's focus from a traditional model to a course-based undergraduate research experience (CURE) design. Specifically, this effort at Ball State University to become a CURE can and must involve collaborations with colleagues in the BSU Department of Chemistry to isolate and identify novel antibiotics from soilborne bacteria and fungi as well as a variety of dairy-relevant natural environments (raw milk, silage, manure, aged cheese, and feedlot samples). These natural environments have not been widely explored in this context before, and yet we will be able to rapidly screen hundreds of isolated bacteria and fungi using standard microbiological methods and well-established chemistry separation protocols used routinely in undergraduate teaching labs. Using resources, expertise, and protocols modified from the Small World Initiative (SWI), our goal is to isolate and identify multiple novel compounds from bacteria. Transforming the undergraduate microbiology curriculum in this way is completely new teaching and research direction for the PI, and will blend pedagogy and original research into an entirely different direction than at present.

Description of CURE Development:

Increasing bacterial resistance spanning over 70 years, combined with a cessation in the discovery of new antibiotics for over a generation has resulted in a global healthcare catastrophe. Crowdsourcing antibiotic discovery is a viable option to meet the challenge of this crisis. The American Society for Microbiology has been instrumental in supporting the Small World Initiative, SWI, (www.smallworldinitiative.org), an international not-for-profit consortium of research and teaching universities in over 17 countries. SWI began in 2012 at Yale University, and now has partner universities, community colleges, and high school partner institutions in 43 U.S. states. The SWI provides a wide range of expertise and physical resources for faculty to transform their research agenda and/or laboratory teaching curricula to systematic discovery of new antibiotics from natural environmental samples, largely soil. This effort will overview our plans to retool our undergraduate microbiology course laboratory's focus to directly align with the SWI mission in order to identify and isolate new types of antibiotics with promise in the global healthcare sector. We will do this by rewriting the class lab to become a course-based undergraduate research experience (CURE). Our hypothesis is that through the CURE model, we will be able to isolate multiple species of naturally occurring bacteria and fungi from raw dairy milk (cow and goat), silage, manure, aged cheese, and feedlot samples, and that at least one novel antimicrobial compound (antibiotic) will be isolated and purified from the host microorganism on a larger scale for promise as a chemotherapeutic agent.

Goal and Objectives:

Our research goal is reflected in our hypothesis. Within this broad goal, we have two smaller objectives that will be addressed over the course of two semesters using a total of 150 undergraduate microbiology students (~75 each semester) in the CURE structure:

Using standard microbiological methods, we will isolate and identify multiple bacteria and/or fungi, at least one of which will produce a secreted compound demonstrating measurable antibacterial activity. Secondly, we will use traditional chemistry separation and analytical methods to purify (on a larger scale) and determine the structure and specific activity of each antibiotic quantified in the first objective. Using a modified course syllabus and student learning outcomes suggested by the American Society for Microbiology (ASM) SWI, we will be able to pilot the CURE for two semesters in order to generate the necessary research results and preliminary assessment data essential to show proof of concept and submit a larger, more collaborative proposal to NSF for external funding in early fall 2021.

Assessment:

A combination of both formative and summative assessments will be used and applied throughout the semester, including but not limited to weekly written quizzes, materials and methods oral self-quizzes (given among the student working groups), and an end-of-the semester capstone poster for each group presented at the spring University Student Research Symposium. Vigorous consultation will occur regularly throughout both semesters with our two science education research faculty, and assessment experts available through the SWI faculty network to ensure measureable data are being collected from these assessments to include in the NSF proposal upon submission in 2021.

Faculty Perspective

The benefits of mentoring undergraduate research students

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Science or history?

I initially chose to pursue a degree in Cell and Molecular Biology as an undergraduate because I loved the subject. I loved learning about cells, how they are shaped, how they function. I loved learning about how organized molecules formed the basis of heredity and inheritance. But always at the back of my mind was the question of "how do we know these things?" I was thus inspired by classic experiments by following the logic presented in impeccable experimental design. The cleverness of scientists like Thomas Hunt Morgan, Louis Pasteur or Hershey and Chase made me appreciate the human endeavor to understand the world around us. These moments of inspiration were always interrupted by a pang of longing, as I perceived the stream of facts being presented as a complete and static collection of knowledge. A history lesson, if you will. The work was done, we knew how the natural world worked. So much intimate detail about life, how could there possibly be more to learn? Thankfully this misconception was shattered through a stint in undergraduate research.

My breakthrough

What I did not realize when I initially chose my degree program was that it was designed for students interested in pursuing graduate school and/or careers in research. Accordingly, the program had strict requirements for participating in undergraduate research. I therefore found myself entering a research laboratory with little to no idea of what to expect. The first small project I was assigned was to clone and sequence a gene involved in organizing microtubules in plant cells. The goal was to understand the evolution of these microtubule organizing centers in the *Arabidopsis* family of plants. My mentor explained it: "There is this oddity in plant cells, they lack the organelles that organize microtubules. Yet they clearly get along just fine. The question we're asking is how have plants evolved independently to accomplish this feat without these seemingly fundamental structures?". There for the first time I was presented with the unknown. It was not only the question that caught me, but the existence of the question itself. I quickly realized that what we know about the natural world is but a mere beginning to a comprehensive understanding. Perhaps even more excited was then being presented with the opportunity to help discover the unknown.

Mentorship is the key

I had amazing research mentors as an undergraduate student: Dr. Regina McClinton at Grand Valley State University and Dr. Aikseng Ooi at the Van Andel Research Institute. They both took a keen interest in fostering the excitement and curiosity I brought to the lab. They not only trained me to be technically proficient, but really spent time to train me as a scientist. In hindsight this went beyond the nuts and bolts of scientific inquiry. I learned other fundamental skills: perseverance, critical thinking, foresight, and imagination just to name a few. This took patience and dedication on their part. Their investments in me paid off when I realized that I was better prepared for graduate school success than peers whose previous research experience was more task-oriented. I excelled in graduate school where others struggled. I attributed this not to my own intellect or ability, but to the mentorship I received as an undergraduate research student.

The payoff

I have had the pleasure of mentoring many undergraduates throughout my career, and the biggest lesson I have learned is that there is not one catch-all approach. Students are motivated and inspired by different ideas or different aspects of the scientific process. One of my primary jobs as a mentor therefore is to listen and be attentive. When asked if they think a proposed experiment is exciting or useful, most will respond “yes” to be polite, but body language and mannerisms often tell otherwise. While there will always be unenjoyable tasks in research, my experience has shown that if these are not punctuated with tasks that excite the student (or myself) will not be doing their best work. I strive to perceive the real experience students are having in the research lab and guide them accordingly.

It is important to me that I bring undergraduates into the scientific process. My ideal goal for each student entering my research lab is for them to achieve a certain degree of independence with the process of scientific inquiry. I view the student as a researcher in the laboratory, not as a producer of data. To truly engage a student in my research requires exposing my thought processes and rationales rather than simply delivering ideas and goals. This is where the real time commitment comes in, but it is also the source of the big payoff for both myself and the student. As students gain experience in research I try to start asking rather than answering the questions: “What do you think we should do next?”, “What do these data mean to you?”, “How would you test that hypothesis?” etc. Watching students develop the skills to critically think about and answer these questions is truly one of the best parts of my career.

But also, reality

These are idealistic circumstances of course. In reality, not every student that participates in our research will be inspired. Most of them may not even pursue a career in research. Occasionally a student realizes quite quickly they want nothing to do with research. Nonetheless, undergraduate research experiences are still valuable. To give a student a glimpse at the reality of research can provide the fundamental understanding that science is a process and not a collection of facts. How science goes far beyond the dense content we present in our courses, how the work of the pioneers I admired is not complete, how scientific knowledge is not static, but dynamic and under constant revision. This seems imperative now more than ever, as I currently sit writing this in my makeshift home office as a result of the ongoing COVID-19 threat. The public response has illuminated many misconceptions about science, both its benefits and limitations. The more students we can expose to the realities of science, the better off we will be.

Factors Affecting Learning Gains among Students in Microbiology Class: A Preliminary Study Between a U.S. Community College and a Canadian Comprehensive University

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Abstract

Though in the past, serious concerns have been raised about students' interest and learning gains in STEM courses, not much research has been done to examine the differences in learning science at community colleges and universities. The purpose of this paper is to close this gap. This paper analyzes the influence of students' demographics, preparedness, major, and attitudes on their learning gains in an introductory microbiology class at a community college vs. a university. Student demographics, information about their preparedness level, major, and attitudes were collected in a questionnaire and students' learning gains were assessed by comparing student performance on a pre- and post-test on four different topics in microbiology. Our results indicate that students' majors and attitudes such as their willingness to actively participate in the classroom discussions and spend time outside the classroom to learn are major factors that enhance their learning. Age and marital status positively impact learning gains while gender, employment status, and citizenship status show no impact on learning gains in students. Our results also indicate that students at the community college who had less exposure to science classes in high school or biology classes in college achieved statistically higher learning gains despite having overall lower scores on two of the four post-tests.

1. Introduction

In recent years, there has been a growing need for students trained in the fields of science, technology, engineering, and mathematics (STEM) in the U.S., while an increased interest in STEM education has steadily been recognized in Canada. STEM's crisis is not as obvious in Canada, however, STEM-related career and job preparedness is more prominent. The Canadian Government has created and put forward many STEM initiatives and educational programs to prepare Canadians for STEM-related careers and jobs (1). Canadian STEM graduates continue to demonstrate disparities between gender and minority representation (2). In the 2011 National Household Survey (NHS), it was found that only 18.6% of adults with a postsecondary certificate, diploma, or degree are qualified in STEM fields of study. Of those, among younger STEM graduates, women held a higher share of university degrees, while men still held the majority of university STEM degrees in older demographics. It seems that overall Canada is better prepared for STEM-related careers and jobs than the U.S. (3,4). Blotnicky et al. demonstrated that overall Atlantic Canada middle school students (7th to 9th graders) from public schools lacked knowledge about STEM career requirements and their findings support the need to create better accessibility of knowledge pertaining to STEM career requirement to facilitate students' understanding of the nature of a STEM career (5). At McGill University, Montreal, Quebec on November 28, 2015 the Canadian Science & Policy Exchange convened a working group composed of STEM students and experts representing the perspectives of academic, government, and private sectors to discuss current challenges and opportunities in Canadian STEM Education (6). Canadian STEM students voiced many challenging issues in regard to the current delivery of their STEM education. These challenges include issues in, one, Curriculum and structure (large class size, lack of applied learning), two, Critical skills development, three, Career exposure, and, four, Metrics and evaluation. After working with experts during the working groups, several solutions to these issues were recommended from the students. The first four solutions at the top of the list were, one, reducing class sizes to improve student engagement, two, developing interdisciplinary courses to expose STEM students to diverse perspectives for problem-solving, three, increasing direct training in the critical skills that employers expect from STEM students, and, four, fostering an early awareness of STEM careers and encouraging students to be proactive in forming a career plan (6). However, there is less research published on STEM education related topics in Canada in relation to the U.S.

Though the number of students pursuing their education in STEM fields in the U.S. has increased, it has not been enough to meet the increasing demands in STEM (7). There has been a shortage of workers and students proficient in math and science (8). The National Math and Science Initiative (NMSI) (http://www.nms.org/) states that the United States is losing its competitive edge in STEM areas (9) and this shortage is appropriately called "The STEM Crisis". In 2012, the President's Council of Advisors on Science and Technology (PCAST) issued a report suggesting an increase in the number of STEM graduates in the next decade to meet projected employment needs (8).

A major reason for "The STEM Crisis" in the U.S. has been a declining trend in the adequate preparation of high school students to be successful in college level courses. According to an NMSI estimate (9), in 2009, only 21% of 12th grade students performed at or above proficient level in science; in the high school class of 2012, of all the students who took an Advanced Placement test, only 19.5% earned a qualifying score; and only 13% of high school graduates in 2013 were ready for college level science. In 2012, U.S. students were ranked 27th in Math and 20th in Science while China, Korea, Japan, and Canada were all ahead of the U.S. Recognizing the STEM crisis, in December 2018, the U.S. federal government has adopted a detailed strategy to make the U.S. a global leader in STEM literacy, innovation, and employment by pursuing three objectives; one, build a strong foundation for STEM literacy, two, increase diversity, equity, and inclusion in STEM, and, three, prepare the STEM workforce for the future (10).

Given the seriousness of issues surrounding science education, it is not surprising that several studies have examined the factors that influence post-secondary students' interest and desire to pursue careers in STEM fields. Astin and Astin (1992), for example, reported a significant positive correlation between institutional traits and background characteristics of students and their interest in studying science and related fields during their post-secondary studies (11). They stated that the level of mathematical preparation in high school is the strongest and most consistent predictor of students' initial interest as well as continuation of their education in STEM fields.

Several studies have examined the impact of background characteristics such as age (12), gender (13, 14), marital status (12), family income (12), academic success (13), financial situation (15), and previous educational experiences (11) on retention of STEM majors into and during post-secondary education. Leppel found that age, marriage, and hours employed have a negative impact on college persistence while family income and GPA have a positive impact on both men's and women's persistence (12). Similarly, Cabrera and La Nasa showed that factors like family income, parental education, gender, ethnicity, and exposure to at–risk factors in lowest socio-economic status place students at a disadvantage and make their path to college extremely difficult (13). Whalen and Shelley showed that underrepresented (female and/or minority) students do not perform as well in STEM majors as traditional white, male students (14).

Academic performance of students has always been a topic of interest, discussion, and research among educators in many disciplines. Much of the earlier research analyzed the relationship between students' demographics, experiences in high school, their entry qualifications, type of institutions and their performance in college classes. Effects of many different demographic factors on student learning in different fields have been examined, for example, age (16, 17, 18, 19, 20, 21), gender (17, 19, 20, 22, 23, 24), race (24), employment status (13, 17), and citizenship (17, 18). In studies where the effects of age and gender on academic performance were examined, mixed results have been reported. In an introductory biochemistry class, Mlambo reported that gender and age did not cause any significant variation in the academic performance (19). Similarly, in a study by Colorado and Eberle, age was not a significant factor affecting academic performance of graduate students enrolled in online classes (16). Jayanthi et al. also reported no significant impact of age on academic performance of post-secondary students (17). Newman-Ford et al. reported minor impact of age and gender on educational achievements in first year undergraduates (20). In a study by Richardson and King (1998), older students exhibited more desirable approaches to learning in terms of their persistence and attainment (21). In some studies, no link was found between gender or race (except for Latinos) and student performance in an introductory college chemistry class (24) and mathematics class (23). In contrast, in other studies, female students were found to outperform male students (17, 22). Few studies reported a correlation between nationality and student performance. A study done in Singapore reported that international students performed better than domestic students (17).

In addition to age, gender, race, and nationality, other factors that influence student achievement in post-secondary education have also been researched. It is widely accepted that well prepared students are more likely to pursue their initial choice of a science major. Astin and Astin (1992) suggested that it may be possible for more students to pursue science majors and careers if the level of overall academic competency could be increased at the secondary level (11). Newman-Ford et al. reported a significant impact of prior educational attainment, and attendance on educational achievement of first year undergraduate students in U.K (20). Along the same lines, Jayanthi et al. reported that intention to pursue higher studies and learning preferences contribute to students' academic performance significantly (17). On the contrary, in a study in an introductory biochemistry class, it was concluded that learning preferences and entry qualifications do not cause any significant variation in the academic performance of students (19).

It is well known that students who have no visible or significant gaps or barriers in learning in high school are better prepared to handle the rigor of college. Tai et al. reported a significant correlation between pedagogical experiences of students during high school class and their performance in introductory college chemistry class (24). They also concluded that demographic predictors like parents' educational level, family income, and affluence of the community were linked to significant differences in how students perform in their college class.

Despite the complexity and the importance of issues relating to student learning in STEM fields, we found very few studies that compared learning gains between two- and four-year educational institutions. The purpose of this paper is to fill this gap. Thus, this paper examined learning gains of students enrolled in an introductory microbiology class at a two-year college in the U.S. and at a four-year university in Canada. This paper compared the student composition (in terms of demographics, level of preparation, major, and attitudes) at the two educational institutions by means of a questionnaire administered at the beginning of the semester. We also analyzed the results of the same pre- and post-tests (details are provided in the methods section) taken by students at both the institutions to test whether their learning gains are linked to the nature of institutions, students' prior exposure to the subject matter, attitudes towards microbiology, and demographics. Further, we have provided several plausible explanations consistent with our findings of differences in students' learning at two types of institutions.

2. Methods

This study was conducted at a U.S. Community College (USCC) and a Canadian University (CU). The microbiology class at USCC was capped at 20 students and there was a mix of traditional (25 or younger) and non-traditional students (above 25 years of age) (72% vs 28%) and the majority of them were pre-nursing majors (88%). At CU, most students were traditional students (98%), majoring in biology/microbiology/pre-med (67%) and the class size was capped at 80 (one big lecture class divided into 5 lab sections with a maximum of 16 students in each lab section). The lab sections met for microbiology experiments (once a week at CU and twice a week at USCC) for a total of 170 minutes per week. At both the institutions, students worked in a group of 2-4 students during each lab period. Though microbiology is a sophomore level class at both the institutions, at USCC it has no prerequisite and almost all the pre-nursing students take it during their freshman year. In contrast, at CU, first year biology and chemistry are the prerequisites to take microbiology, and hence all the students enrolled in microbiology are sophomores. At both the institutions, the traditional lecture discussion format was used in the classroom (lecture followed by discussions from student questions). Despite the differences in class sizes and prerequisites, both the instructors had similar expectations from their students in terms of learning. Both the courses had the same learning objectives and students at both the institutions were given the same pre- and post-tests. To minimize differences in teaching styles and content, both instructors communicated on a weekly basis to discuss the lesson plans and the mode of delivery.

At the beginning of the semester, the purpose as well as the details of this research study were explained to the students. Although, the participation in this study was voluntary and students were asked to sign a consent form, all the students present on day 1 of the semester, chose to participate. Students were also explained that their identities would remain anonymous, the information they provided would always remain confidential, and their names would never be released in any form in the study. The data were not analyzed until after the semester was over and the students' names or identities were not shared between the two instructors (authors of this paper). An IRB approval was obtained at CU before the beginning of the study whereas no IRB approval was required at USCC.

A questionnaire and a test for each of the four selected topics in microbiology were developed (supplemental materials available upon request). The questionnaire consisted of fifteen questions and was administered at the beginning of the semester to collect information on: (a) the demographics of each student, i.e., age, gender, marital status, number of hours working per week, and citizenship status; (b) student preparedness, i.e., number of science courses they completed in high school, other biology courses completed in college before enrolling in microbiology, and their major; (c) students' interest in microbiology and their attitudes, study habits, and behavioral engagement towards learning, i.e., their willingness to actively participate in the classroom, their attitude towards the laboratory participation, and their willingness to spend time learning outside the classroom. All the questions in the tests, administered before (pre-test) and after (post-test) class room discussion, were either selected from the test bank of the microbiology text books (difficulty level 1) or written by the authors at the lower levels of Bloom's taxonomy (knowledge and comprehension). Each question was carefully selected to assess students' basic knowledge and understanding in the following topics:

- Introduction to microbiology
- Cell structure
- Microbial metabolism
- Microbial growth

We assessed the prior knowledge of basic concepts of students enrolled in microbiology class by administering a test before the topic was discussed in class. After the topic was discussed, we administered the same test as a post-test to determine their learning gains by comparing each student's score on post-test to their score on pre-test. Students were not told that they would be taking an identical test for both pre-test and post-test. Each test comprised of a total of 10 questions worth 1 point each, for a maximum of 10 points. The data were collected from both the institutions for statistical analyses after the semester was over. We compared the demographics of students enrolled in the microbiology class at USCC and CU and related them to student learning gains on the post-test versus the pre-test.

Students' responses to different questions on the questionnaire were statistically analyzed and the results are presented in Fig. 1. To statistically analyze the differences in student demographics and their preparedness, a binary classification was used

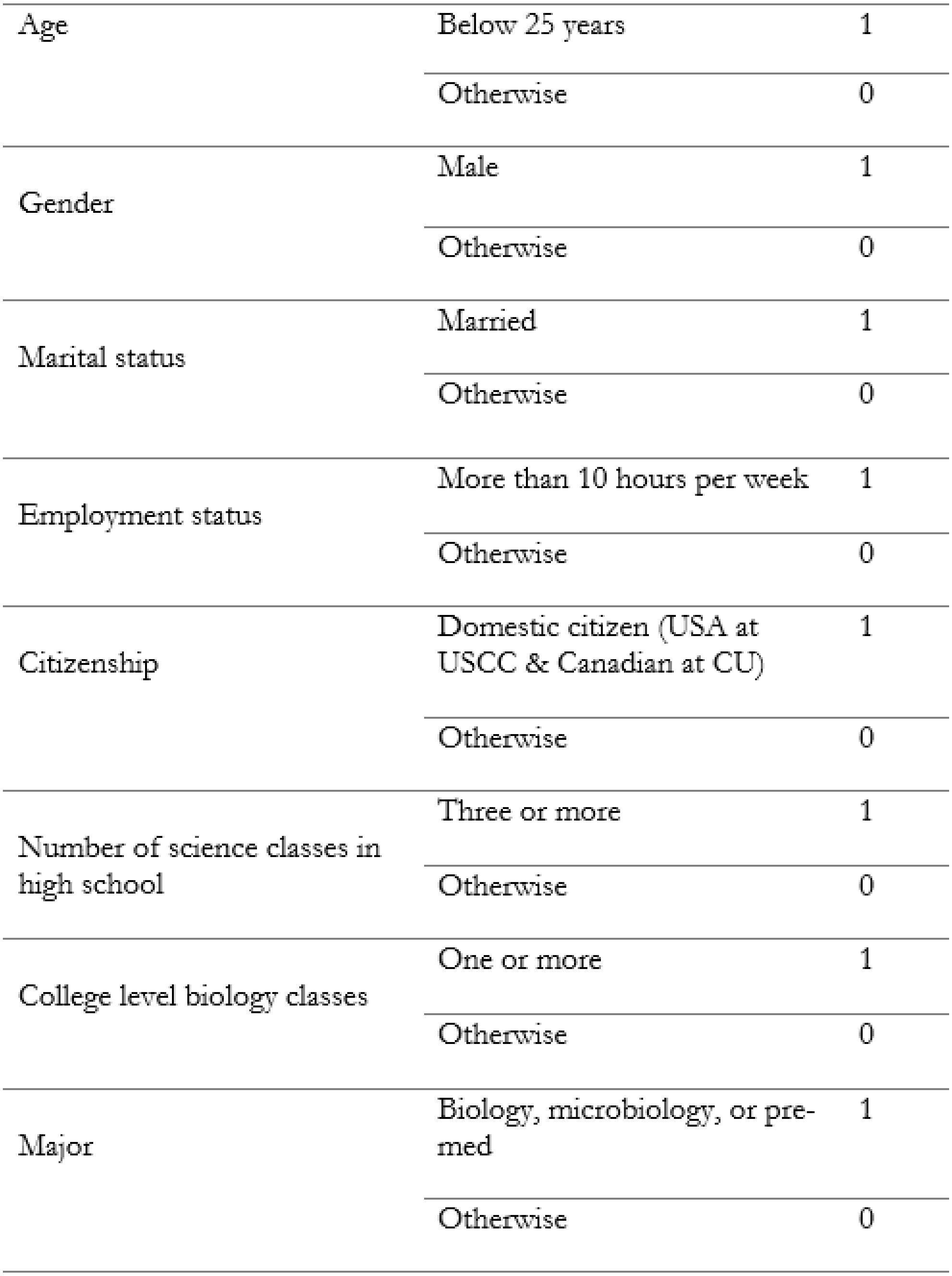
(Table 1).

The data for students' attitudes were collected on a 5-point Likert scale, where 1 represents the least value and 5 represents the highest value (for example, regarding interest in microbiology, 1 represents no interest and 5 represents high interest).

To analyze differences between variables, a test of equality of means was used. We have used 94% level of confidence to accept or reject the null hypothesis. Thus, ***p***-values, based on ***t***-tests, of less than or equal to 0.06 (6%) rejects the null hypothesis of equality of means between variables. EVIEWS software was used for statistical analysis.

3. Results

At the beginning of the semester, on the first day of classes, a questionnaire was administered to collect the basic information of students enrolled in microbiology class at both the institutions. Fig. 1 (A), (B), and (C) summarize the differences between USCC and CU students based on their responses to our questionnaire. Fifteen students at USCC and 70 students at CU completed and submitted the questionnaire. Based on the analysis of our data, Fig. 1 (A) shows that CU had



Age	Below 25 years	1
	Otherwise	0
Gender	Male	1
	Otherwise	0
Marital status	Married	1
	Otherwise	0
Employment status	More than 10 hours per week	1
	Otherwise	0
Citizenship	Domestic citizen (USA at USCC & Canadian at CU)	1
	Otherwise	0
Number of science classes in high school	Three or more	1
	Otherwise	0
College level biology classes	One or more	1
	Otherwise	0
Major	Biology, microbiology, or pre-med	1
	Otherwise	0

Table 1. Binary classification used for the statistical analyses for the variables relating to students' demographics, preparedness, and major.

a statistically significant higher proportion of younger (age 25 or below) and single (based on their marital status) students as compared to USCC. However, our results show no statistically significant difference in student composition in terms of gender, employment status, and nationality.

As shown in Fig. 1 (B), in case of students' preparedness, a statistically significant higher proportion of CU students had completed three or more science classes during high school and had completed one or more college level biology classes before enrolling in microbiology class as compared to USCC students. Thus, the students at CU were academically better prepared to learn microbiology (or any science course) relative to students at USCC. Also, a statistically significant higher proportion of students at CU were biology, microbiology or pre-med majors relative to USCC.

Fig. 1 (C) summarizes the results with respect to students' interest in microbiology and their attitudes towards learning. Our results indicate no significant difference in students' interest in microbiology or their desire to be an active leader during laboratory exercises at both the institutions. However, it is interesting to note that students at USCC were statistically more interested in participating in classroom discussions and in learning course material outside the classroom relative to students at CU.

Next, students' performance in pre-tests and post-tests at USCC and CU was analyzed to assess if mean pre- and post-test scores in the four topics statistically

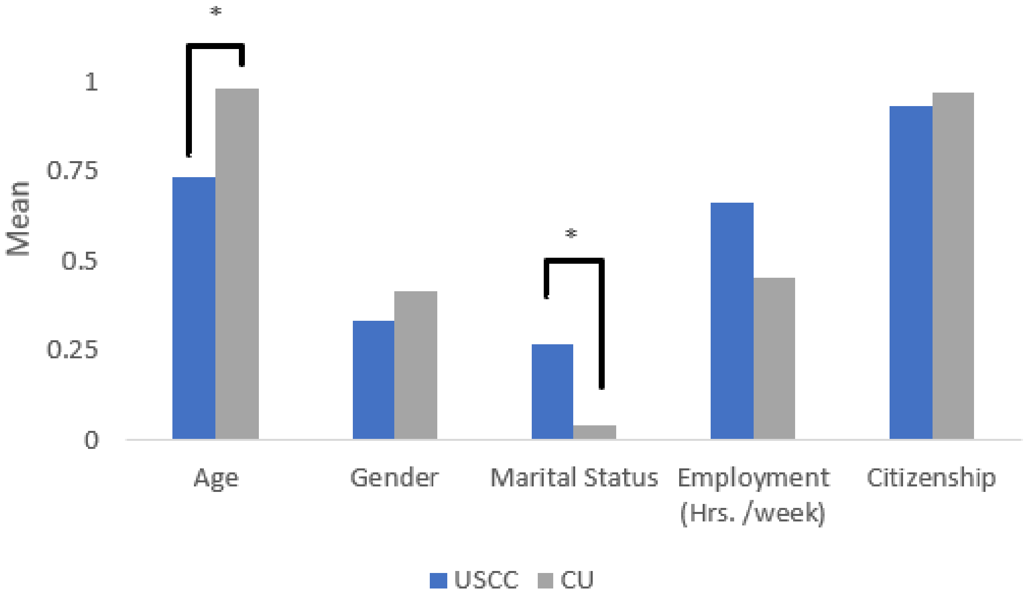


Figure 1A. Comparison of student demographics between USCC (n = 15) and CU (n = 70) based on a survey administered on first day of class. Binary classification of variables (age = 1, if below 25 years; gender = 1, if male; marital status = 1, if married; employment = 1, if hours worked > 10 hours; citizenship = 1, if domestic student and 0 otherwise) was used to test for equality of means between CU and USCC. Results show that age and marital status were statistically significantly different between the students at USCC and CU.

* $p \leq 0.06$.

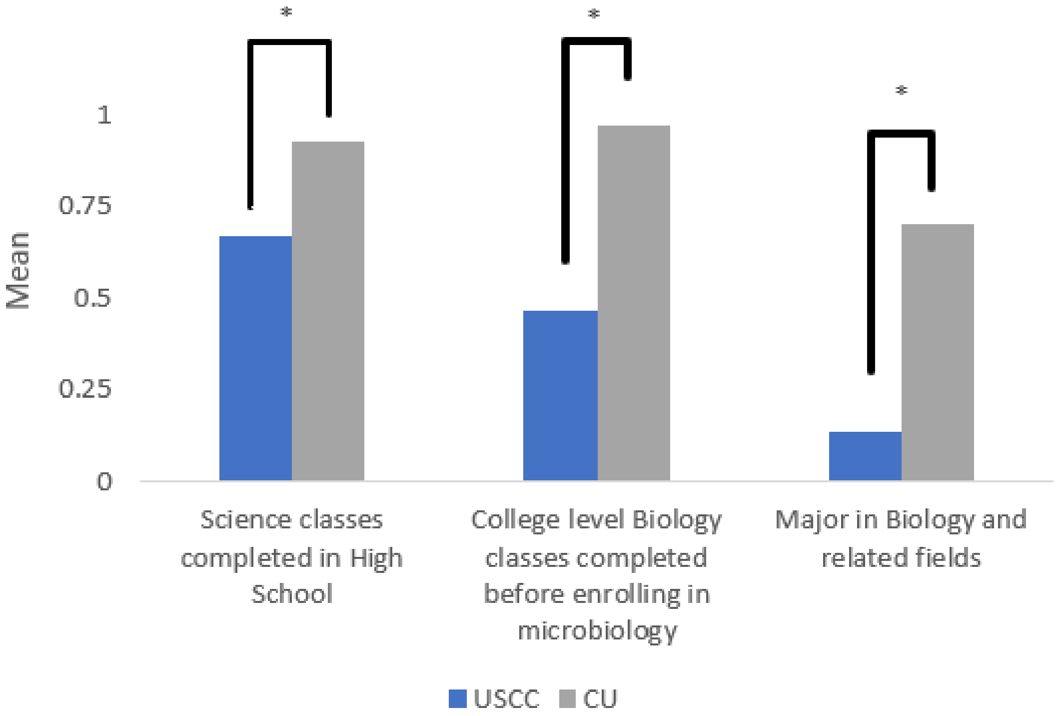


Figure 1B. Comparison of student demographics between USCC (n = 15) and CU (n = 70) based on a survey administered on first day of class. Binary classification (number of science classes completed in high school = 1, if 3 or more; college level biology classes completed before enrolling in microbiology = 1, if 1 or more; major = 1, if biology, microbiology, or pre-med, and 0 otherwise) was used to test for equality of means between CU and USCC. Results show that the number of science classes completed in high school, number of college level biology classes completed before enrolling in microbiology, and students' major were statistically significantly different between the students at USCC and CU.

* $p \leq 0.06$.

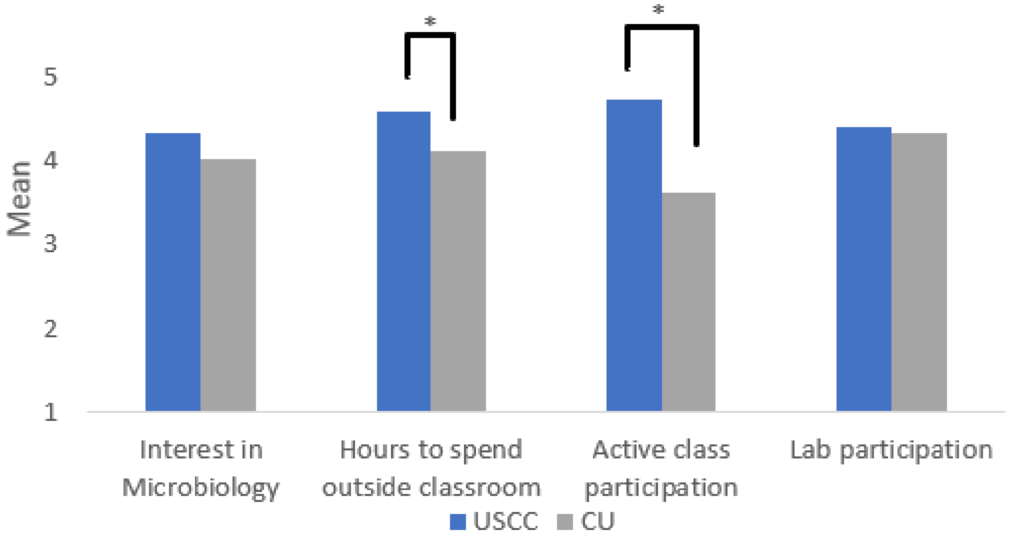


Figure 1C. Comparison of students' interest and attitudes towards learning microbiology between USCC (n = 15) and CU (n = 70) based on a survey administered on first day of class. A 5-point Likert scale was used to compare students' interest and other attitudes towards learning, with 1 representing least interest in microbiology and 5 representing high interest in microbiology; 1 representing less than 1 hour to spend outside the classroom and 5 representing over 5 hours to spend outside the classroom; 1 representing not likely to actively participate in the class and 5 representing very likely to actively participate in class; 1 representing do not care about lab participation and 5 representing taking the lead role in the lab group. Using test of equality of means, the figure show that hours devoted to learning outside the class and willingness for active class participation were statistically significantly higher amongst USCC students relative to CU students.

* $p \leq 0.06$.

vary between the students at two institutions. As shown in Fig. 2, our results indicate that the student scores in pre-tests for introduction, cell structure, microbial metabolism, and microbial growth were statistically significantly higher (at 6% level) at CU relative to USCC, reflecting better prior knowledge about these topics at CU relative to USCC. However, the knowledge gap was closed as indicated by no statistically significant difference in students' mean scores in introduction and microbial metabolism post-tests between the two institutions. In other two topics, cell structure and microbial growth, students at CU had a statistically significant higher post test scores as compared to USCC students.

We further analyzed and compared student performance in pre- and post-tests in different topics at both the institutions and the results are presented in Fig. 3. Our results show that classroom instruction led to significantly higher post-test scores (as compared to their pre-test scores) in all four topics at USCC and there was no significant statistical difference between pre-test and post-test scores in any of the four topics at CU. Thus, based on our analysis, after students were exposed to the topic in the classroom at USCC they had higher learning gains (as reflected by statistically significant higher post-test scores of USCC students in each of the four topics as compared to their pre-test scores).

To analyze learning gains between the students at USCC and CU, the difference in performance of each student before and after a topic was discussed was computed. The means of these differences were compared between the two institutions for each of the four topics. Our analysis shows that students at USCC exhibited statistically significant higher learning gains (at 6% level of significance) in three out of four topics (introduction, cell structure, and microbial metabolism) (Fig. 4). At CU, the students did not exhibit learning gains in any of the four topics.

4. Discussion & Conclusions

This research was conducted to fill the current gap in the literature regarding learning gains of students in a microbiology class at two different types of institutions, a two-year community college in the U.S. and a four-year Canadian university, and their relationship to the demographics, preparedness, attitudes, study habits, and behavioral engagement towards learning. The results of this paper are based on (a) students' responses to a questionnaire on their demographics, preparedness for an introductory course in microbiology, major, and their attitudes and study habits towards learning; and (b) students' test scores in 4 different topics in microbiology, administered before and after the topic was taught in the classroom (pre- and post-tests). Further, all students' responses were compared between 2-year college in the US and 4-year university in Canada.

Our research finds several interesting results: based on our sample results, statistically, students at CU were relatively younger and unmarried when compared to students at USCC. Statistically, students at USCC scored better grades in post-tests as compared to their pre-tests in all the topics. It may be mentioned that our

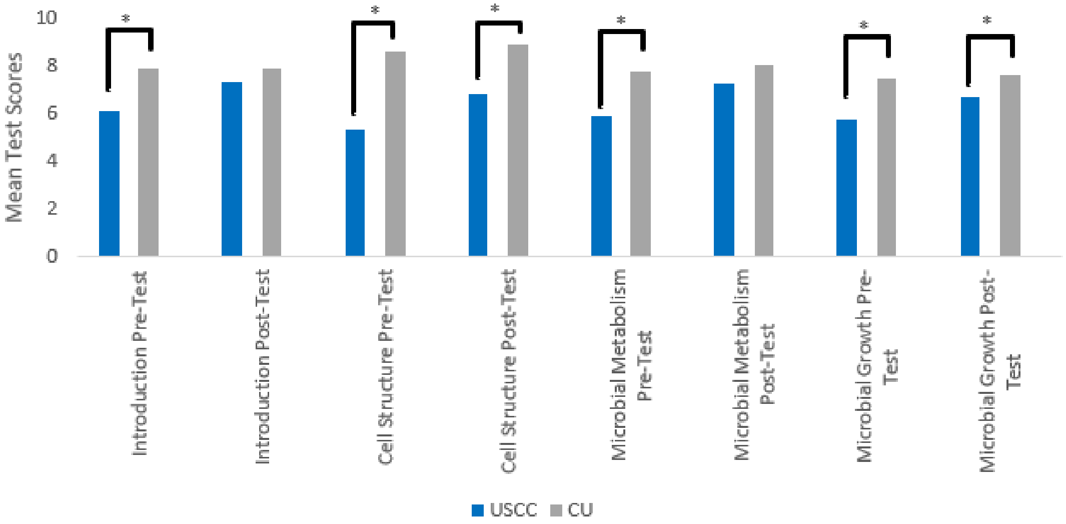


Figure 2. Comparison of mean performance of students in pre- and post-tests at USCC relative to CU. Results from the test of equality of means show that USCC students' mean scores were statistically significantly lower in all four pre-tests relative to CU students. However, performance of USCC students was statistically significantly lower in only 2 out of 4 post-tests (cell structure and microbial growth) relative to CU students.

* $p \leq 0.06$.

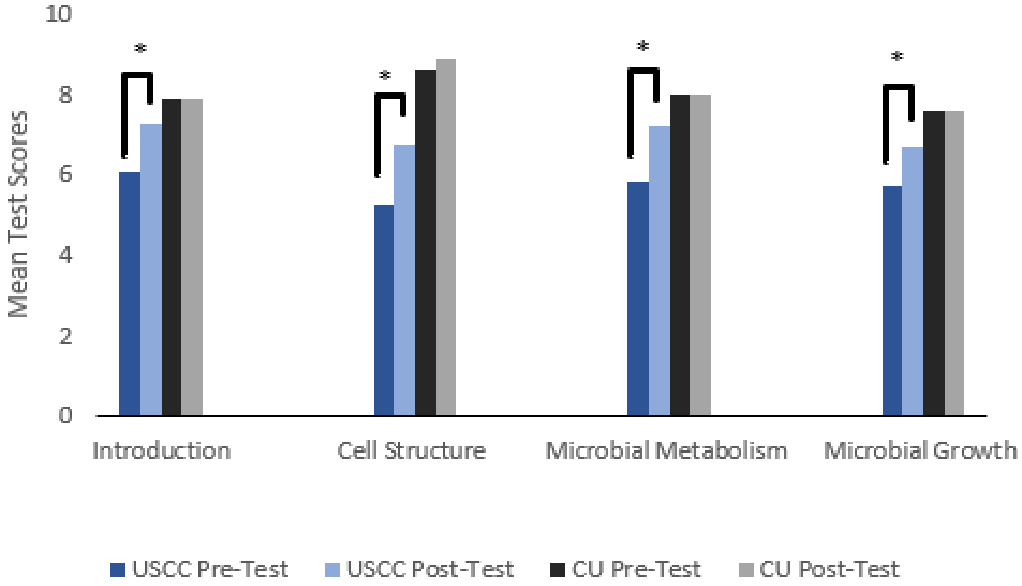


Figure 3. Comparison of means of the pre- and post-test scores of students at USCC and CU. Test of equality of means shows that students at USCC scored statistically significant higher scores in all 4 post-tests relative to pre-tests, reflecting higher learning gains after the topic was discussed in class. However, performance of students at CU was not statistically significantly different between pre- and post-tests in all four topics.

* $p \leq 0.06$.

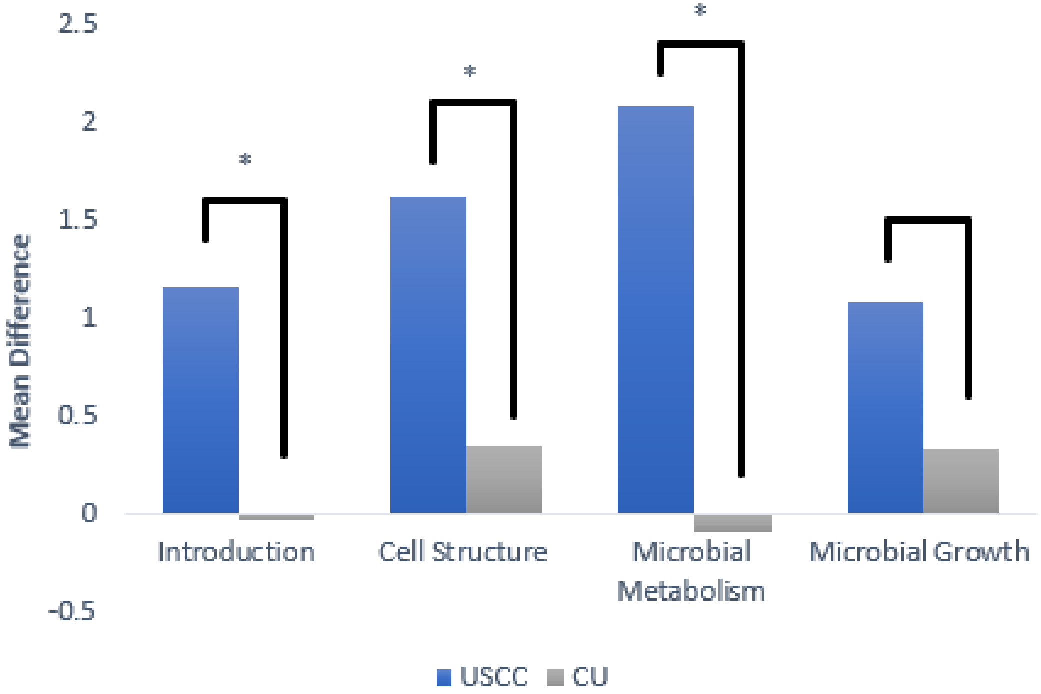


Figure 4. Difference of means between the post-test vs pre-test scores at USCC and CU. Test of equality of means shows that there was a statistically significant difference between the post-test versus pre-test scores amongst USCC students relative to CU students in 3 out of 4 topics (introduction, cell structure, and microbial metabolism).

* $p \leq 0.06$.

results reflect correlations between these variables rather than causality. However, it is interesting to note that Richardson and King concluded that older students tend to exhibit more desirable approaches to learning in terms of both their persistence and attainment (21). Newman-Ford et.al also reported that despite attaining lower results overall, older students achieve a higher proportion of better grades and age had some impact upon educational achievement (20). Our results show no statistical difference between CU and USCC with respect to other three demographic factors, gender, citizenship, and number of students working 10 hours or more per week.

We found that statistically CU students were academically better prepared for introductory microbiology class relative to students at USCC, when preparedness is measured in terms of number of science courses completed during high school and their prior exposure to college level biology classes. A statistically higher proportion of CU students were biology/microbiology majors and had more exposure to science courses in high school and first year of college before taking microbiology class. Our results show that students at CU had statistically significant higher pre-test scores as compared to students at USCC in all four topics studied. Despite this, students at USCC scored statistically significant higher grades in all the post-tests as compared to their pre-tests. These results are contradictory to Newman-Ford et al. who found a strong, statistically significant relationship between prior attainment and subsequent results (20). Our results are consistent with Mlambo who also reported that entry qualifications do not significantly affect academic performance in an introductory biochemistry class (19). Colorado and Eberle also reported that academic performance in online learning environments was not significantly affected by the number of educational degrees attained (16).

We also found that students at USCC were willing to devote more time outside the classroom to study and to actively participate in class to learn microbiology when compared to students at CU. Our results show that students at USCC attained significantly higher post-test scores as compared to their pre-test scores. These results are consistent with the result of a study by Harb and El-Shaarawi that students who participate in class discussion outperform students who do not (22).

We then compared students' scores on post-test with students' scores on pre-test at each of the two institutions separately. Interestingly, we found that the post- test scores of students in all four topics were statistically significantly higher from their pre-test scores at USCC. No such statistical difference between students' performance on pre- and post-tests in the case of CU students was found. It is evident from the questionnaire data that students at USCC were not as well prepared to handle the rigor of college microbiology class (as evident from their lower scores in pre-tests, number of science classes completed at high school, and number of biology courses completed at college before enrolling in microbiology). But after exposure to the topic in the class they achieved statistically higher learning gains as compared to the students at CU. A study by Yager et al. showed that students with or without prior high school science work exhibited similar performance on all assessments after course completion simply because students without high school science program worked extra hard and devoted more time to learning (25).

An interesting question is why students' scores were consistently higher on post–test relative to pre-test in case of USCC and not so in the case of CU. There could be several explanations for this.

The class size was much larger at CU relative to USCC. Though the same lecture-discussion format was used to discuss a topic in the classroom and both the instructors tried to use the class and lab time in a similar manner, due to the smaller class size at USCC, students might have had the advantage of getting more interaction with the instructor and among themselves as compared to the 4-year university where the class size was big. Though, at both the institutions, instructors maintained regular office hours to interact individually with the students and involve them in one-on-one discussion, the students at CU who were mostly biology/microbiology majors with heavier course load and more exposure to college level courses may not have utilized that opportunity.

Based on students' responses, our results indicate that students at CU were better prepared to learn microbiology, as statistically a higher proportion of them had completed three or more science classes during high school and were also exposed to college level biology courses. It is also evident from their significantly higher pre-test scores in all the topics. Despite the lower pre-test scores of USCC students, statistically they were more willing to spend more time outside the classroom to study and actively participate in the classroom to learn when compared to CU students and it is evident from their higher learning gains.

Statistically, at USCC, students were older and married and hence, may be more responsible and committed to learning as compared to more traditional students at CU. As reported by Newman-Ford et al. despite attaining lower results overall, older students achieve a higher proportion of better grades and age had some impact upon educational achievement (20). Richardson and King also concluded that older students tend to exhibit more desirable approaches to learning in terms of both their persistence and attainment (21).

It must be acknowledged that though students at CU did not have any statistically significant learning gains in any of the four topics in microbiology, they still had higher overall scores on the post tests. However, performance of USCC students was statistically significantly lower in only 2 out of 4 post-tests (cell structure and microbial growth) relative to CU students.

In conclusion, our results indicate that if students are willing to spend time to learn, and have the right attitudes towards learning, they can overcome their prior deficiencies and achieve higher learning gains.

This study, though preliminary and exploratory, has created a space for the further investigation of learning gains of major and non-major students in a microbiology class at two different types of institutions with different student demographic. It serves as a starting point of research in such an area.

5. Research Challenges

This research has few limitations. First, it is limited by the differences in class size at the two institutions. CU had a significantly larger class size as compared to USCC. Second, though every effort was made to follow the same type of lecture-discussion format in both the classrooms and both instructors were in frequent communication via phones and email messages about the instructions and worked closely together throughout the project, the individual instructor differences might have caused some differences observed in the results. Third, at USCC, students could enroll in microbiology as freshmen and the course has no prerequisite, whereas at CU it is a sophomore level class with a year of biology and chemistry as prerequisites and, hence, students at CU were academically better prepared for the class.

6. Suggestions for Future Research

This research was done at one 2-year community college and one 4-year university for one semester. It will be interesting and useful to expand it to include more 2-year and 4-year institutions in the U.S. and Canada to increase the sample size and conduct additional statistical investigation to further explain the differences in students' learning gains at different institutions.

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Genetic classification of two *Vibrio* species isolated from Florida Gulf Coast using multi-locus sequence analysis

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Abstract

Prokaryotic species classification has lagged dramatically behind eukaryotic species due to the dependence on the difficult DNA-DNA hybridization technique required for species designation. This is disadvantageous for the general understanding of the diversity of microbial life and unique microorganisms, along with their metabolic abilities, like bioremediation of hazardous waste. This applies to the secondary metabolites microorganisms produce that can be beneficial for humans and other living organisms, such as antibiotics or other industrially important compounds. In an effort to isolate and identify new microbial species, environmental samples were collected from marine sediment environments as marine habitats are under-sampled compared to non-marine habitats. Two marine isolates designated MI-1 and MI-2 were isolated from marine sediment environments off the Gulf Coast of Florida and chosen for further study because of their antibacterial production and unique colony pigmentation. They were genetically analyzed through sequencing of the ***16s rRNA*** gene, shotgun cloning, and an eight-gene multi-locus sequence analysis comparison to 66 other species of the same genus. From these, MI-1 and MI-2 can be classified as members of the *Vibrio* genus most closely related to *Vibrio* ***ruber*** and a distinct strain from ***V. ruber*** type strain VR-1.

Introduction

The goal of biologists and taxonomists for over a century has been to classify organisms into species and taxonomic groups, thus bringing order to the great biodiversity seen in the world. Due to the lack of a broadly applicable species concept (56) and the historical reliance on the time-consuming and labor-intensive DNA-DNA hybridization (DDH) test required for species delineation (6, 28, 41), prokaryotic species classification has lagged far behind that of eukaryotes in achieving this goal. Compared to the number of known eukaryotic species (>1.2 million) (48), the ~12,000 recognized prokaryotic species (48, 67) are lacking, particularly when the number of prokaryotic species on the planet is estimated to be as high as 10^{11}-10^{12} (39, 44). Given the dominance of bacterial diversification as depicted in the recently expanded new tree of life (34), there is a need for increased attention to prokaryotic taxonomy to work towards classifying the remaining 99.999% of unclassified prokaryotic species (44).

Marine environments are a source of microorganisms with important roles including global nutrient cycling where they are responsible for half of the primary production on the planet (5, 14), production of industrially and medically important compounds (3, 10, 15, 19), and essential symbiotic relationships with larger organisms (14, 20, 32). As it is estimated that 91% of species in and around the ocean still await classification and the majority of these are microbial (48), marine environments represent a likely source for the isolation of novel microbes that may have medical or industrial importance. Because the challenges of growth in a marine environment are different from that of terrestrial organisms, marine microorganisms have acquired unique metabolic capabilities that allow for production of compounds not present in terrestrial organisms (14, 24). Antimicrobials are one type of compound that has generated considerable interest for production by marine microorganisms due to both the increased prevalence of pathogens resistant to currently available antibiotics (33, 43) derived from terrestrial organisms and the comparative lack of sampling for antibiotic-producing organisms from marine environments (35, 38). Antibiotic compounds have been successfully purified from marine microorganisms (8, 21, 50), suggesting the marine environment offers an untapped source of antimicrobials that could help combat the impending antibiotic crisis.

One of the most abundant groups of microorganisms in marine environments is the *Vibrio* genus (64). Organisms classified as *Vibrios* are generally small Gram-negative, facultatively anaerobic rods with a single polar flagella that are primarily aquatic and require Na^+ for growth (2, 17, 61, 64). More than 100 *Vibrio* species have been validly described (22). Of these, several have been found to have close relationships with higher organisms, with effects ranging from beneficial symbiosis (32, 47) to harmful pathogenesis (27, 37, 68). *Vibrios* are believed to take part in nutrient cycling (62) and are particularly important for their production of polyunsaturated fatty acids that are vital for the aquatic food web but can only be produced by a handful of organisms (52) and their breakdown of chitin (60). Some *Vibrio* species have also been found to produce antimicrobial compounds (13, 45).

In addition to the *Vibrio* species already characterized, other new candidate *Vibrio* species have been observed but not yet described (22). Species determination in *vibrios* can be complicated as many different species have virtually identical biochemical phenotypes, and some species can have very similar genomes yet still represent evolutionary units in nature (2, 56). Multi-locus sequence analysis (MLSA) can serve as a substitute for the cumbersome DDH test (23, 26). This technique compares multiple housekeeping gene sequences, at least four (22), concantenated together end to end into one larger sequence for phylogenetic analysis. MLSA has been used extensively for species determination in *Vibrio* (55, 61). The genetic classification of two antibiotic-producing *Vibrio* species isolated from the gulf coast of Florida through sequencing of the ***16s rRNA*** gene, shotgun cloning analysis, and MLSA is described here.

Materials and Methods

Isolation of MI-1 and MI-2

MI-1 was isolated from a marine sediment sample aseptically collected from the intertidal zone near a nature park in Pinellas County, FL. The GPS coordinates of the MI-1 collection site were 27°53'19"N, 82°50'22"W. MI-2 was isolated from a salt flat sediment sample aseptically collected from Leffis Key in Bradenton, FL, Manatee County. The GPS coordinates of the MI-2 collection site were 27°27'07"N, 82°41'20"W. One sediment sample was taken from each location. Permission to collect samples was obtained from local authorities in both counties. Both sediment samples were serially diluted in 0.5 M NaCl to 10^{-5} grams soil/ml and grown on starch agar plated supplemented with 0.5 M NaCl (MI-1) or modified potato dextrose agar (PDA) with the following contents (MI-2): tryptone (2 g/l), potato starch (4 g/l), NaCl (0.5 M), glucose (5 mM) filter sterilized and added to the media after autoclaving, and agar (20 g/l). The Neidhardt MOPS salt components were prepared as described (51). To control for fungal growth from environmental samples, 100 μg/1 cycloheximide was added on the initial isolation plates. After four days of growth at 25°C, colonies were isolated and selected for their ability to inhibit the growth of ***Staphylococcus epidermidis***, ***Pseudomonas putida***, and ***Chromohalobacter salexigens***, a marine microorganism, by spread-plating ***S. epidermidis***, ***P. putida***, or ***C. salexigens*** onto the separate starch agar or modified PDA plates supplemented with 0.5 M NaCl and then patch-plating the environmental isolates on top. After initial isolation, MI-1 and MI-2 were maintained on 0.5 M NaCl modified PDA plates and 0.5 M NaCl LB agar plates and stored at 25°C.

Chemical Extraction of Antimicrobial Compound from MI-2

The antimicrobial compound from MI-2 was crudely extracted and tested for activity against five different bacterial species. Isolates of MI-2 were continuously streaked onto 0.5 M NaCl modified PDA plates and incubated at room temperature for seven days. Agar from the plates was cut into cubes and placed into 50 ml media bottles. The bottles were placed in -80°C freezer for 30 minutes. After freezing, 20 ml of ethyl acetate was added to the bottle and shaken on an orbital shaker for 24 hours. The organic ethyl acetate layer was decanted into 20 ml scintillation vials where the ethyl acetate was allowed to evaporate, leaving the extract behind. Extracts were resuspended in 1 ml of ethyl acetate. Four 20 μl portions of the resuspended extract were plated onto a single region on the side of an LB plate and allowed to dry. The same procedure was repeated on the other side of the plate with an ethyl acetate control. A tester strain culture was plated on top of the extract and ethyl acetate control be mixing 100 μl of an overnight culture of ***S. epidermidis***, ***Bacillus subtilis***, ***Citrobacter freundii***, ***C. salexigens***, or ***Enterobacter aerogenes*** in with 10 ml of top agar and poured evenly over the plate. The plates were incubated for 24 hours at 30°C. The zones of inhibition surrounding the chemical extract were measured.

Genus Identification of MI-1 and MI-2

Identification of the genus of MI-1 and MI-2 was completed by PCR amplification of the ***16s rRNA*** gene sequence. The 16S primers 63F (5'- CAG GCC

TAA CAC ATG CAA GTC – 3') and 1387R (5'-GGG CGG WGT GTA CAA GGC – 3') obtained from Integrated DNA Technologies (IDT) were used to amplify the gene along with the MyTaq™ mix from Bioline. The whole colony PCR reaction conditions were run as follows: 1 cycle of 95°C for 10 minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and 1 cycle of 72°C for 5 minutes. Successful PCR amplification was confirmed by agarose gel electrophoresis using a 1% agarose gel in 1X TAE buffer and purified using the Isolate II PCR and Gel Kit from Bioline. Purified PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University. Sequencing results were analyzed by BLAST against the ***16s rRNA*** database of EZtaxon (11).

Genotypic Characterization of MI-1 and MI-2

For all genotypic characterization, genomic DNA was obtained from overnight cultures of MI-1 and MI-2 grown in LB with 4% NaCl using the phenol-chloroform extraction method as described (70). Shotgun cloning of MI-1 and MI-2 was completed. Genomic DNA from MI-1 and MI-2 was digested with Eco-RI and HindIII (New England Biolabs). The resulting fragments were ligated into pGEM7 (Promega) after digestion with the same enzymes. Successful recombinant plasmids were transformed into Escherichia coli DH5α and isolated after overnight growth using the GenElute Plasmid Miniprep Kit (Sigma). Dideoxy-sequencing of the isolated plasmids was performed by Functional Biosciences using the T7 promoter primer. The generated sequences were analyzed using the BLAST (1) feature in the PATRIC, the Bacterial Bioinformatics Resource Center (69), comparing against the genomes of ***V. ruber*** VR1 (listed as DSM 16370 in PATRIC), ***V. rhizosphaerae*** DSM 18581, ***V. gazogenes*** DSM 21264, and V. mangrove CECT 7927. The shotgun clones from MI-2 were deposited in Genbank (9) with the accession numbers shown in Table S1 in the supplemental material.

Whole genome sequencing of MI-1 and MI-2 was completed. After ethanol precipitation of extracted genomic DNA, the sample was checked for integrity by agarose gel electrophoresis and quantified using a NanoDrop 2000 (Thermo Scientific). Whole genome sequencing of the extracted DNA was conducted using HiSeq 2500 technology at Purdue University. The resulting genomic sequence was analyzed using RAST (7).

MLSA of MI-1 and MI-2 along with 66 known *Vibrio* species type strains was completed using eight protein-coding housekeeping genes: ***ftsZ***, ***gapA***, ***mreB***, ***recA***, ***rpoA***, ***rpoD***, ***topA***, and ***toxR***. The gene sequences for all eight genes from MI-1 and MI-2 were obtained from the whole genome sequencing results. Gene sequences for the 66 known *Vibrio* species type strains were obtained from PATRIC (69). Table S2 in the supplemental material contains the locus tag numbers or reference sequence numbers as available for the genes sequences found through PATRIC. All sequences for each gene were aligned using Clustal Omega (29) and trimmed so each gene sequence covered the same aligned region. The aligned regions from each gene were concentrated to create an 8-gene chain. The concatenated sequences were aligned with Clustal Omega and analyzed phylogenetically with MEGA7 (42). Nucleotide percent identity values were acquired using a percent identity matric in Clustal Omega. Maximum likelihood trees with a phylogenetic test of 100 bootstrap replications was created using previously suggested parameters (22) in MEGA7.

Results

Isolation of MI-1 and MI-2

Marine Isolates 1 and 2 (MI-1 and MI-2) were isolated from marine sediment samples collected in intertidal regions of the Gulf coast of Florida in Pinellas County and Manatee County, FL, respectively. These locations were selected because they are undersampled marine environments that do not receive a large amount of daily foot traffic. Each isolate was selected for further study based on its ability to produce antimicrobial compounds that completely inhibit the growth of ***S. epidermidis***, ***P. putida***, and ***C. salexigens***. These organisms were chosen as ***S. epidermidis*** and ***P. putida*** represent safe relatives of ***S. aureus*** and ***P. aeruginosa***, two of the ESKAPE pathogens whose increasing rates of antibiotic resistance represent a great threat to the healthcare field (54). ***C. salexigens*** is a halophilic marine microorganism with a broad range of salinity tolerance (4) that was chosen to see if MI-1 and MI-2 can inhibit halophilic organisms as well as halotolerant ***S. aureus*** and non-halophilic ***P. aeruginosa***. MI-1 was initially isolated on NaCl-supplemented Starch agar where is appeared orange while MI-2 appeared bright pink on the modified-PDA. Different media were used for initial isolation to provide different environments and increase the chances of isolating unique microorganisms. Colonies of MI-1 and MI-2 appear anywhere from pink, red, or orange in pigmentation depending on the type of media on which it is grown.

Fig. 1 shows the antibacterial inhibition and pigmentation of both MI-1 and MI-2. Figure 2 shows the results of chemical extraction of the antimicrobial compound produced by MI-2 against five different bacterial species. The crude chemically extracted antimicrobial compound was capable of inhibiting all organisms it was tested against: two Gram positive species (***S. epidermidis*** and ***B. subtilis***) and three Gram negative species (***C. freundii***, C. ***salexigens***, and ***E. aerogenes***). Its strongest inhibition was against Gram positive species. Similar zones of inhibition were seen for MI-1 (data not shown). A Gram stain of both isolates showed small, slightly curved Gram-negative rods.

Genus Identification of MI-1 and MI-2

PCR amplification and sequencing of the ***16s rRNA*** gene of MI-1 and MI-2 showed both organisms are in the *Vibrio* genus. This confirmed what was previously

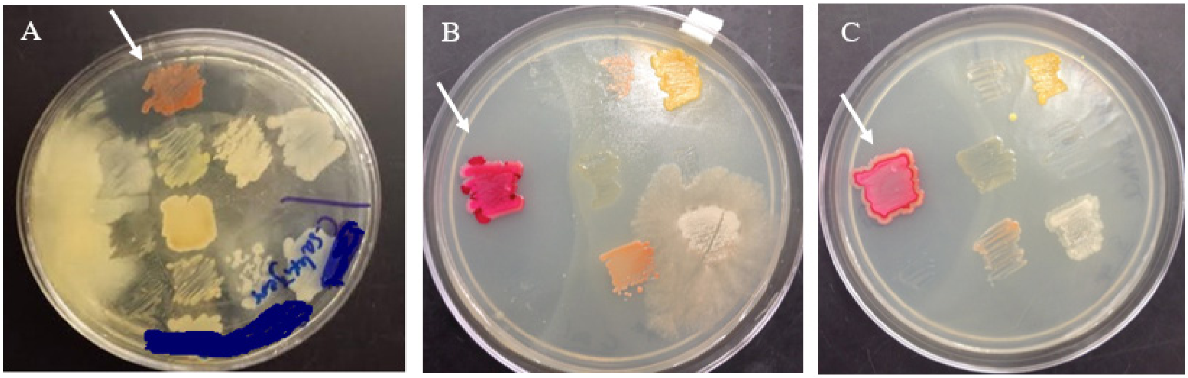
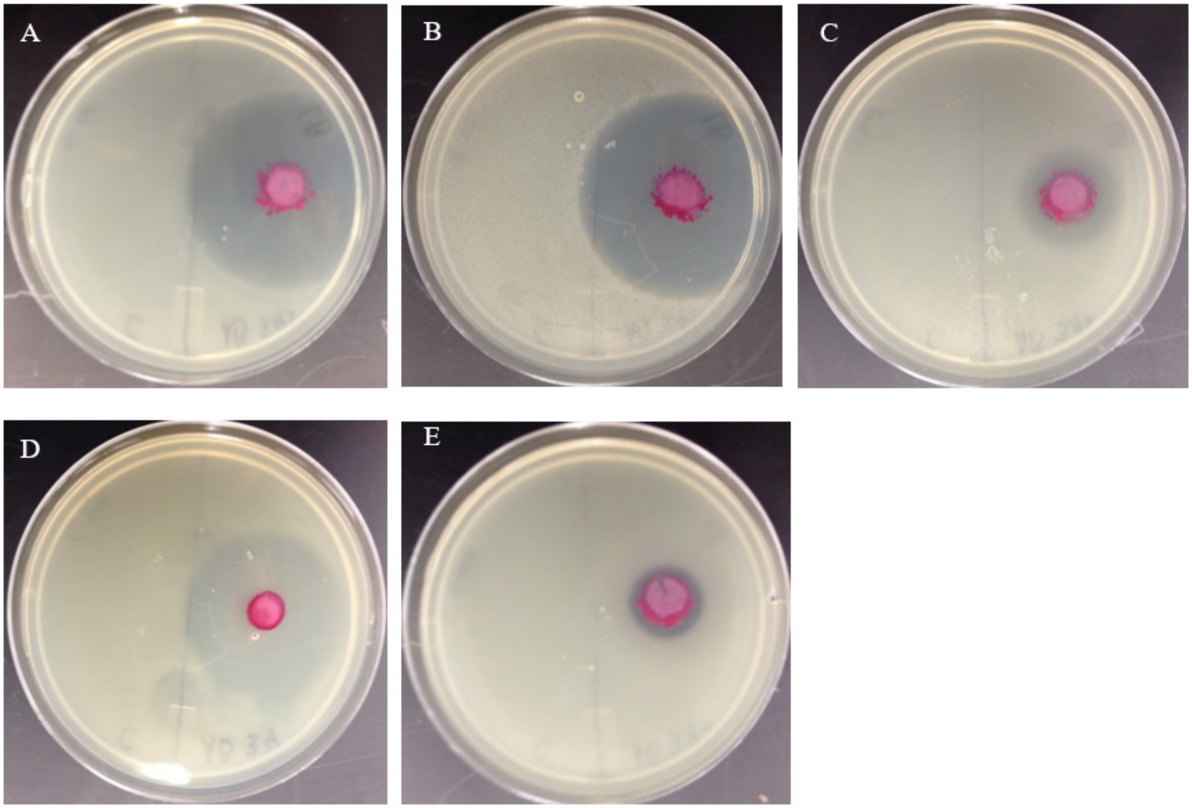
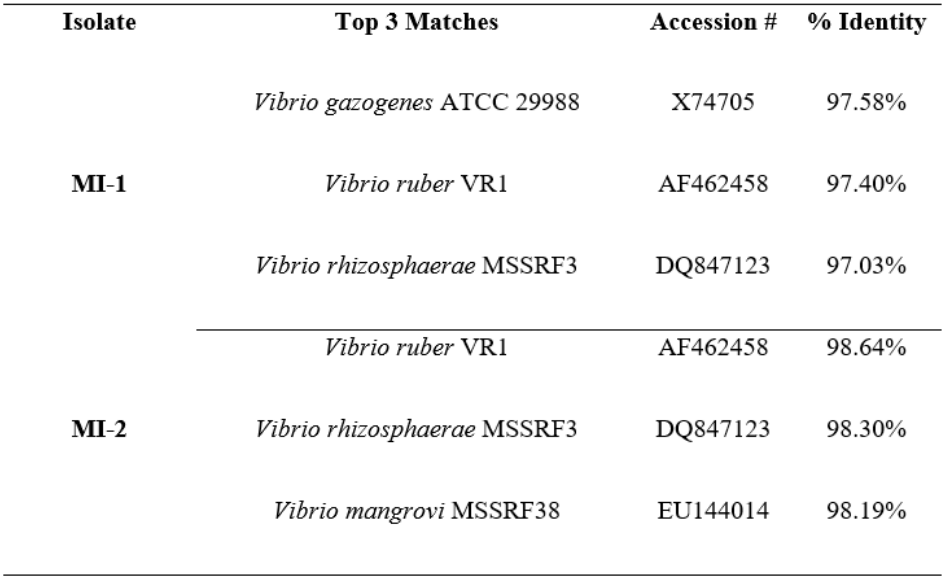


Figure 1. The figure shows the antibiotic production of patch plated MI-1 against C. salexigens (panel A), and patch plated MI-2 against S. epidermidis (panel B) and C. salexigens (panel C). White arrows indicate the location of MI-1 and MI-2 on each plate. Antibiotic production can be seen by the zones of inhibition surrounding the patch plated MI-1 and MI-2. The orange pigment production of MI-1 and pink pigment production of MI-2 can be seen.



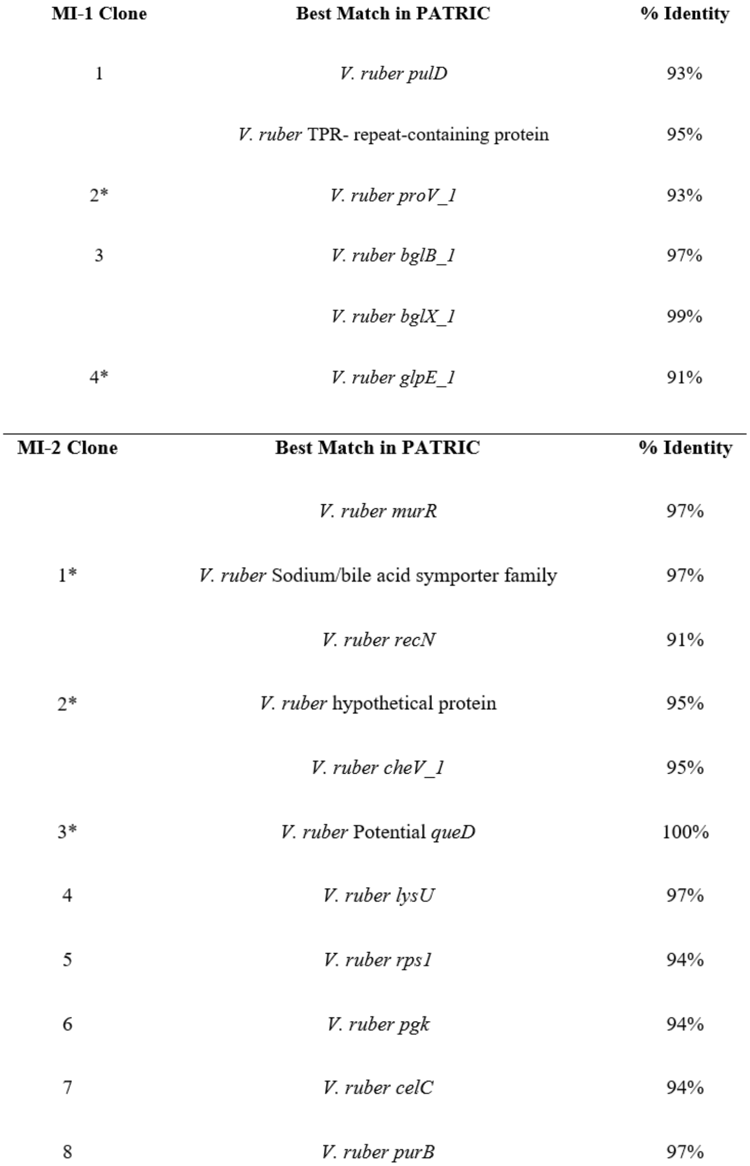
Bacterial Species	***S. epidermidis***	***B. subtilis***	***C. freundii***	***C. salexigens***	***E. aerogenes***
Radius of Zone of Inhibition	24 mm	23 mm	7 mm	19 mm	5 mm

Figure 2. The figure shows the inhibition of the crude chemically extracted antimicrobial compound from MI-2 against *S. epidermidis* (Panel A), *B. subtilis* (Panel B), *C. freundii* (Panel C), *C. salexigens* (Panel D), and *E. aerogenes* (Panel E) which has been recently renamed as *Klebsiella aerogenes*. Zones of inhibition can be seen surrounding the pink-pigmented chemical extract of MI-2. The radius of the zones of inhibition is indicated in the table accompanying the figure. The zones were measured 24 hours after plating.



Isolate	Top 3 Matches	Accession #	% Identity
MI-1	*Vibrio gazogenes* ATCC 29988	X74705	97.58%
	Vibrio ruber VR1	AF462458	97.40%
	Vibrio rhizosphaerae MSSRF3	DQ847123	97.03%
MI-2	*Vibrio ruber* VR1	AF462458	98.64%
	Vibrio rhizosphaerae MSSRF3	DQ847123	98.30%
	Vibrio mangrovi MSSRF38	EU144014	98.19%

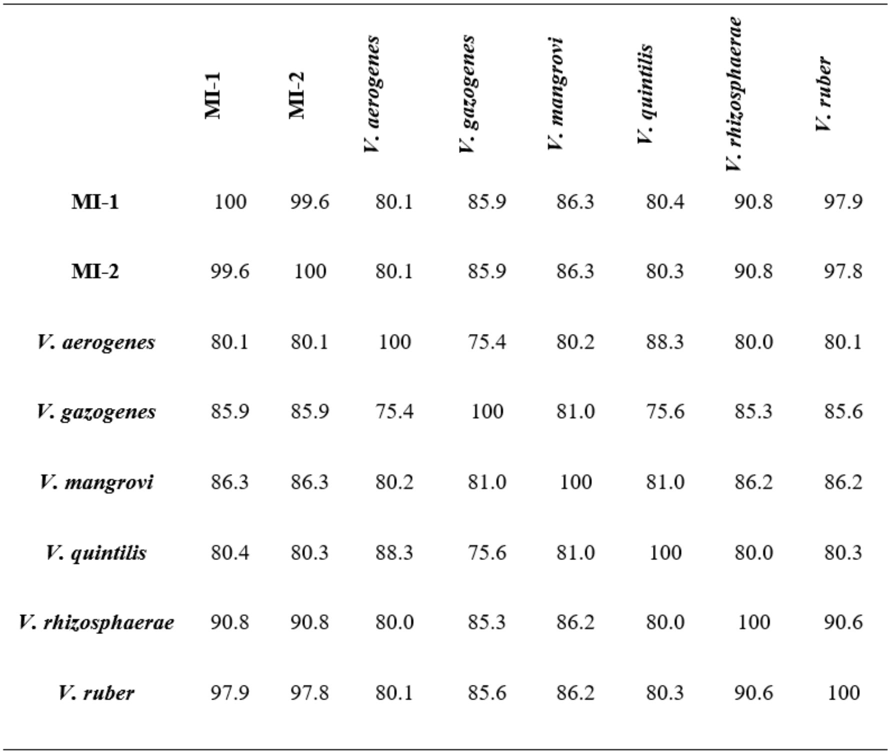
Table 1 – *16s rRNA* Analysis for MI-1 and MI-2. The table shows the three top matches for the ***16s rRNA*** gene sequence of MI-1 and MI-2 after BLASTing the sequence against the ***16s rRNA*** sequence database of EZtaxon. The matches generated by EZtaxon represent the type strains for each of the species.



MI-1 Clone	Best Match in PATRIC	% Identity
1	*V. ruber pulD*	93%
	V. ruber TPR- repeat-containing protein	95%
2*	*V. ruber proV_1*	93%
3	*V. ruber bglB_1*	97%
	V. ruber bglX_1	99%
4*	*V. ruber glpE_1*	91%

MI-2 Clone	Best Match in PATRIC	% Identity
	V. ruber murR	97%
1*	*V. ruber* Sodium/bile acid symporter family	97%
	V. ruber recN	91%
2*	*V. ruber* hypothetical protein	95%
	V. ruber cheV_1	95%
3*	*V. ruber* Potential *queD*	100%
4	*V. ruber lysU*	97%
5	*V. ruber rps1*	94%
6	*V. ruber pgk*	94%
7	*V. ruber celC*	94%
8	*V. ruber purB*	97%

Table 2 – Shotgun Cloning Results for MI-1, MI-2, and *V. ruber*. The table shows the shotgun cloning results for MI-1 and MI-2 when sequences were on a BLAST in the PATRIC database against the type strain genomes of ***V. ruber, V. rhizosphaerae, V. gazogenes,*** and ***V. mangrovi***. The top organism match for each gene present on the shotgun clone is shown along with the information about the gene provided and the percentage identity of the match. * indicates the shotgun clone produced a sequence that contained two genes, each having a separate match.



	MI-1	**MI-2**	***V. aerogenes***	***V. gazogenes***	***V. mangrovi***	***V. quintilis***	***V. rhizosphaerae***	***V. ruber***
MI-1	100	99.6	80.1	85.9	86.3	80.4	90.8	97.9
MI-2	99.6	100	80.1	85.9	86.3	80.3	90.8	97.8
V. aerogenes	80.1	80.1	100	75.4	80.2	88.3	80.0	80.1
V. gazogenes	85.9	85.9	75.4	100	81.0	75.6	85.3	85.6
V. mangrovi	86.3	86.3	80.2	81.0	100	81.0	86.2	86.2
V. quintilis	80.4	80.3	88.3	75.6	81.0	100	80.0	80.3
V. rhizosphaerae	90.8	90.8	80.0	85.3	86.2	80.0	100	90.6
V. ruber	97.9	97.8	80.1	85.6	86.2	80.3	90.6	100

Table 3 – Percent Identity Matrix from MLSA MI-1 and MI-2. The table shows the percent identity matrix for MI-1 and MI-2 and the six other *Vibrio* species from the eight gene MLSA that showed greater than 80.0% identity to MI-1 and MI-2. Percent identity was determined using Clustal Omega.

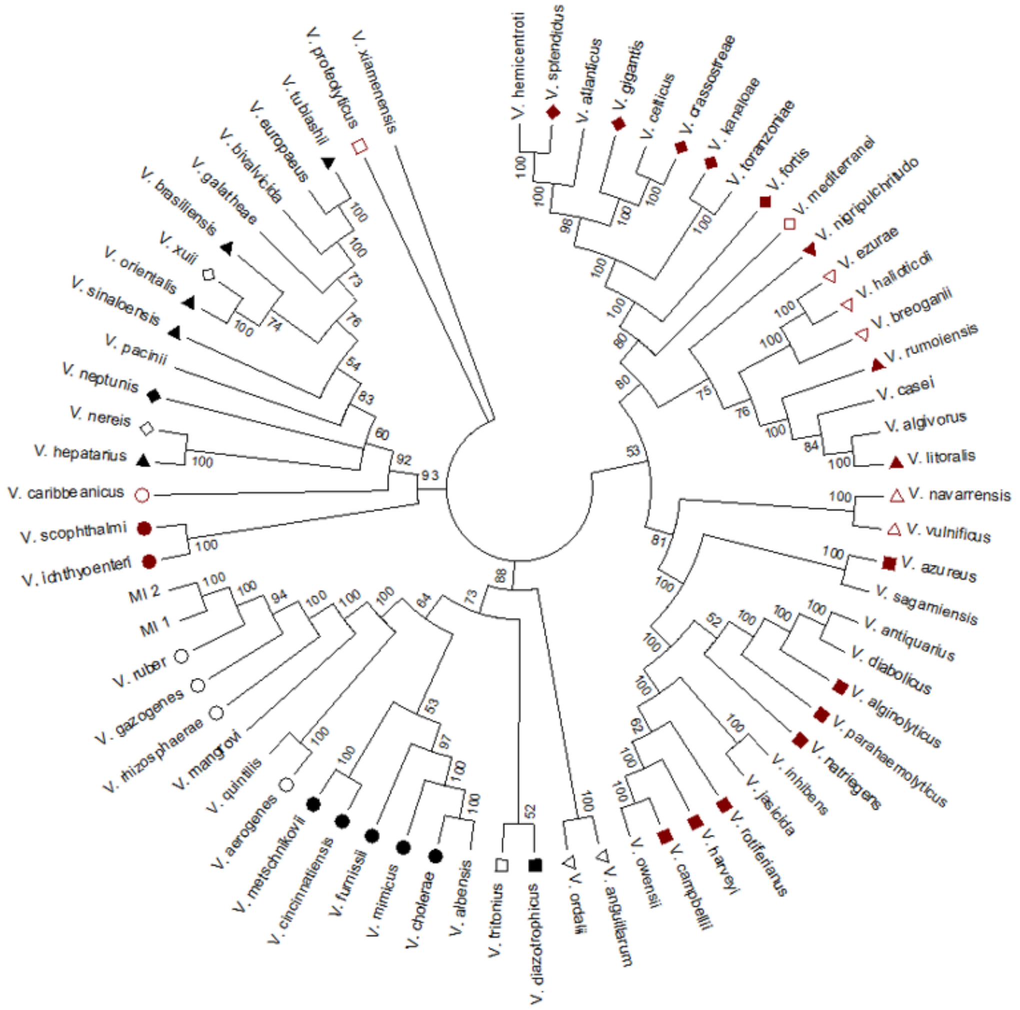


Figure 3 – Molecular Phylogenetic Analysis from MLSA MI-1 and MI-2. The figure shows the phylogenetic tree resulting from the eight gene MLSA of MI-1 and MI-2 with 66 known *Vibrio* species. Symbols indicate the clade identity of each organism as assigned from the eight gene MLSA of Sawabe, 2013; organisms without symbols were not included in this MLSA (61). Evolutionary analyses were conducted in MEGA7 (42). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (40). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (18). All positions containing gaps and missing data were eliminated. There were a total of 9807 positions in the final dataset.

Legend of *Vibrio* Clade Symbols

○ *Gazogenes*	● *Cholerae*	□ *Porteresiae*	■ *Diazotrophicus*
▽ *Anguillarum*	▼ *Orientalis*	◇ *Nereis*	◆ *Coralliilyticus*
○ *Pectinicida*	● *Scophthalmi*	□ *Proteolyticus*	■ *Harveyi*
△ *Vulnificus*	▲ *Rumoiensis*	▽ *Halioticoli*	▼ *Nigripulchritudo*
◇ *Mediterranei*	◆ *Splendidus*	No Symbol – Not included in MLSA of Sawabe, 2013 (61)	

shown for MI-2 (49). The PCR sequencing was completed from three unique colonies with the same results obtained each time. The sequencing results were analyzed using the curated ***16s rRNA*** EZtaxon database. Sequencing results are shown in Table 1. The match producing the highest similarity was *Vibrio* ***ruber*** VR1 for MI-2 at 98.64% while the best match for MI-1 was *Vibrio* ***gazogenes*** ATCC 29988 at 97.58%. Both of these values are below the suggested 98.65% similarity cutoff for species delineation with the ***16s rRNA*** gene sequences (39). As ***V. ruber*** VR1 was the best match for MI-2 and the second match for MI-1, it was obtained from the BCCCM/LMG bacterial culture collection and used as a comparison for genetic characterization for MI-1 and MI-2.

Genotypic Characterization of MI-1 and MI-2

Genotypic characterization began with shotgun cloning to ascertain if MI-1 and MI-2 might be novel enough to warrant a larger investment through whole genome sequencing. Results of the shotgun cloning are showing in Table 2. There were four successful clones for MI-1 that matched up to six unique genes and eight successful clones for MI-2 that matched up to eleven unique genes. The top match for all clones when analyzed by BLAST through PATRIC (69) was ***V. ruber***. The percentage identity ranged from 91-99% with a median of 94% for MI-1 and 86-100% with a median of 95% for MI-2. These values are right around and below the suggested speciation cutoff of 95-96% for average nucleotide identity (ANI) (28, 39, 59), suggesting MI-1 and MI-2 may be novel species and should undergo a more thorough genotypic characterization through MLSA.

An eight gene MLSA of MI-1 and MI-2 was completed with 66 known *Vibrio* type strains with genomes uploaded to the PATRIC database. The eight genes used were ***ftsZ***, ***gapA***, ***mreB***, ***recA***, ***rpoA***, ***rpoD***, ***topA***, and ***toxR***. With the exception of *rpoD* and *toxR*, these genes were previously used for an extensive eight-gene MLSA analysis of the *Vibrio* clades (61). A percent identity matrix for all organisms showing greater than 80% identity to MI-1 and MI-2 from the MLSA is shown in Table 3. MI-1 and MI-2 share 99.6% identity from the MLSA. The closest percent identity match from known *Vibrio* species to MI-1 and MI-2 was ***V. ruber*** at 97.93 and 97.82% identical, respectively. The complete percent identity matrix for all 68 organisms is in Table S3 in the supplemental information. The phylogenetic tree generated from the MLSA data is shown in Figure 3. Organisms with symbols on the figure were included in the eight gene MLSA of Sawabe et al., 2013 (61), and the symbols indicate the *Vibrio* clade to which they were assigned. MI-1 and MI-2 appear most closely related to ***V. ruber*** and belong in the ***Gazogenes*** clade. ***V. mangrovi*** and ***V. quintilis*** were not included in the previous MLSA, but the results from this MLSA suggest they also belong in the ***Gazogenes*** clade along with ***V. ruber***, ***V. gazogenes***, and ***V. rhizosphaerae***.

Discussion

Marine bacterial isolates MI-1 and MI-2 cultured from two locations on the Gulf Coast of Florida showed unique colony pigmentation and production of antibacterial compounds capable of inhibiting both Gram positive and Gram negative organisms. Results of the ***16s rRNA*** gene sequencing indicated MI-1 and MI-2 are in the *Vibrio* genus. ***V. ruber***, ***V. rhizosphaerae***, ***V. mangrovi***, and ***V. gazogenes*** are the four most closely rated species by 16s rRNA sequence homology, however none were above the 98.65% cut-off for species delineation (39). Morphologically, MI-1 and MI-2 are small Gram-negative curved rods. This along with their isolation from marine environments is consistent with all other *Vibrio* species. The four most closely related *Vibrio* species are known to produce red pigments similar to that seen from MI-1 and MI-2 (31, 57, 58, 63). ***V. ruber*** and ***V. gazogenes*** have been experimentally shown to produce the antibacterial compound prodigiosin (31, 46, 63), a chemical compound with red pigmentation responsible for the color produced by the colonies. It is best known for producing the characteristic red pigmentation of ***Serratia marcescens*** (16). ***V. gazogenes*** is also capable of producing cycloprodigiosin, a cyclicized analogue of prodigiosin and fellow member of the prodiginine family of chemical compounds (25) capable of antimicrobial activities. Prodiginine compounds have also been purified from cultures of MI-2 (49). It remains to be experimentally determined if MI-1, ***V. rhizosphaerae*** or ***V. mangrovi*** produce antimicrobial prodiginine compounds, but it is likely they do based on the color of the colony. ***V. gazogenes*** has also been shown to produce the antimicrobial compound magnesidin A (36). In an effort to contribute to the knowledge of marine bacterial species, these organisms were classified genetically through shotgun cloning and an extensive eight-gene MLSA.

Genotypic investigation of MI-1 and MI-2 was completed by performing an eight gene MLSA with 66 known *Vibrio* species whose genomes have been sequenced. ***V. ruber*** showed the closest percent nucleotide sequence identity to MI-1 and MI-2, 97.93 and 97.82%, respectively. Both of these values are below the 98% cutoff suggested by Sawabe et al. as a species boundary (61), suggesting MI-1 and MI-2 may be novel species based on *Vibrio* guidelines, though these values are higher than the accepted 95-96% ANI cutoff value (12, 28, 39, 59). The 99.61% identity between MI-1 and MI-2 indicate they are the same species. The phylogenetic tree generated from the MLSA (Figure 3) showed MI-1 and MI-2 belong in the Gazogenes clad along with ***V. ruber***, ***V. gazogenes***, ***V. rhizosphaerae***, and ***V. aerogenes***, ***V. magrovi***, and ***V. quintilis*** were not included in the original MLSA of Sawabe et al., 2013 (61), but based on their position in the phylogenetic tree, we suggest they be included in the Gazogenes clade as well.

There was general agreement between the clustering of organisms in this MLSA and the clade designations from Sawabe et al., 2013 with the exception of the ***Nereis*** and ***Orientalis*** clades. The ***Nereis*** clade was reported to contain ***V. nereis*** and ***V. xuii*** (61), however this MLSA shows ***V. xuii*** to be more closely aligned with ***V. orientalis*** of the ***Orientalis*** clade and ***V. nereis*** to be more closely aligned with ***V. hepatarius***, also of the Orientalis clade. Reasons for the differences could be explained by the use of ***rpoD*** and ***toxR*** genes in this MLSA in place of ***gyrB*** and ***pyrH*** in Sawabe et al., 2013. The substitution of ***gyrB*** and ***pyrH*** with ***rpoD*** and ***toxR*** occurred based off data from Pascual, 2010 that suggested ***rpoD*** and ***toxR*** are better for reliable species identification of *Vibrio* (53). This study also utilized sequences of the entire gene as opposed to smaller PCR fragments used in Sawabe et al., 2013. ***V. neptunis*** of the ***Coralliilyticus*** clade also aligned closely with ***V. nereis*** and ***V. heptarius***; however the other member of its clade, ***V. coralliilyticus***, was not included in this MLSA, so no conclusions regarding its appropriate clade can be made. Other *Vibrio* species not included in this MLSA of Sawabe et al., 2013 that are closely aligned with members of the ***Orientalis*** clade in this study include ***V. pacinii***, ***V. galatheae***, ***V. bivalvicida***, and ***V. europaeus***.

More clade designation suggestions regarding the *Vibrio* organisms not included in the MLSA of Sawabe et al., 2013 can be made. It appears ***V. albensis*** belongs in the ***Cholerae*** clade, not surprisingly considering it is a Non-O1 serovar of ***V. cholerae*** (30). ***V. owensii***, ***V. jasicida*** and closely related ***V. inhibens*** (66), ***V. diabolicus*** and the closely related ***V. antiquaries*** (65), and ***V. sagamiensis*** closely align with members of the ***Harveyi*** clade; while ***V. algivorus*** and ***V. casei*** appear most closely related to the ***Rumoiensis*** clade. Additionally, ***V. toranzoniae***, ***V. celticus***, ***V. atlanticus***, and ***V. hemicentroti*** appear closely related to members of the ***Splendidus*** clade. ***V. xiamenensis*** appears to be in an orphan clade much like ***V. proteolyticus*** (61).

The genetic analysis has demonstrated that MI-1 and MI-2 are *Vibrio* isolates that can be classified as a strain of ***V. ruber*** distinct from the type strain ***V. ruber*** VR1 used in this study. Genotypically, they are on the threshold of being classified as distinct species. The refinement of the whole genome sequences of MI-1 and MI-2 are currently being completed to fill in the gaps and produce a complete genome sequence that can be confidently compared to ***V. ruber*** by whole genome ANI or digital DDH, an electronic version of the classic DDH mechanism (12). This complete genotypic characterization will be included as a part of a polyphasic approach along with phenotypic and chemotaxonomic characterization (67). Phenotypic characterization will consist of thorough biochemical and growth phenotype testing of MI-1 and MI-2 and comparison with ***V. ruber***. Chemotaxonomic characterization will be performed through analysis of the fatty acid methyl ester (FAME) composition of membrane lipids in MI-1, MI-2, and ***V. ruber***.

Additionally, future research will characterize the antibacterial secondary metabolites produced by MI-1 and MI-2. This will include confirming prodiginine compounds are responsible for pigment production in MI-1 as in MI-2 (49) and determining whether the antibacterial properties of these organisms is solely due to prodigiosin or if they produce additional antibacterial compounds, like ***V. gazogenes*** and its production of magnesidin A (36).

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The prevalence and identification of multidrug-resistant bacteria in adjacent ecological systems in the Hocking Hills region of Appalachia

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Abstract

Multidrug resistance in clinical settings is a major threat to human health, but very little is known regarding the prevalence of multidrug-resistant organisms in the natural environment. Studying antibiotic resistance in the environment is important for understanding the transfer of resistance between environmental microorganisms and those found in healthcare settings. In this study, soil samples from seven adjacent ecological zones were evaluated to determine if there were differences in the amount and types of antibiotic-resistant bacteria present. We hypothesized that we would find antibiotic-resistant bacteria in all ecological zones studied and that these bacteria would be unique to their specific niche. Several resistant organisms from each site were also tested for multidrug resistance and subsequently identified through DNA sequencing of the 16S gene. Antibiotic resistance was discovered in all sites at varying percentages. Some forms of bacteria were present at all sites, but there were differences in types of resistant bacteria found between sites. Six different genera of bacteria were identified, and multidrug resistance was found in all the isolates studied. Our findings indicate that multidrug resistance is prevalent in many different types of environments, including those that have never been directly used for agricultural or urban development.

Introduction

Antibiotic resistance (AR) has been declared a global health epidemic (53). Resistant bacteria currently cause over 2 million infections and 23,000 deaths each year in the USA alone (8). Multidrug resistance (MDR) has amplified this threat by making some infections difficult, if not impossible, to treat with antibiotics (33). The escalation of not only antibiotic-resistant organisms, but multidrug-resistant organisms has been linked to overuse and misuse of antibiotics in clinical and agricultural settings (48). This overuse has led to resistance to every class of antibiotics (8) and consequently, loss of antibiotic efficacy for treating common bacterial infections. In addition, lack of pharmaceutical investment in antibiotic discovery (48) has restricted the availability of novel treatment options. Antibiotics have limited effectiveness as introducing them to bacterial populations creates a selective pressure, allowing resistant bacteria to survive and reproduce. AR can be acquired via a random mutation (52), preexisting efflux pumps, or by horizontal gene transfer (HGT). HGT has been linked to the spread of resistance since AR genes are often carried on mobile genetic elements such as plasmids (28), integrons (51), transposons (45), or bacteriophages (9). Through HGT, these genes can disseminate and proliferate both within a population and between different populations of bacteria.

Most research has studied AR in clinical settings, yet there is evidence that some clinical AR genes have environmental origins (50). Naturally occurring resistance is widely abundant but is still not entirely understood (10, 12, 41). Specifically, it is not well understood if resistance is found only in specific environments or in certain groups of bacteria. Previous studies have found antibiotic-resistant bacteria in pristine environments including remote Alaskan soil (2) and multidrug-resistant bacteria in isolated cave systems (5). A more detailed surveillance of AR in the environment could allow us to determine the relationship between environmental reservoirs and clinical resistance.

The Centers for Disease Control and Prevention (CDC) recommends combatting deadly infections and the spread of resistance by tracking AR patterns in all environments (53). Previous research has also suggested there is a need for thorough environmental surveys, especially of those environments affected by human activities (17, 22, 32). These areas are of interest as urban environments with high population and building density often have higher levels of AR (19, 32), possibly due to antibiotic effluent from hospitals or from farms that pollute the surrounding environment and water bodies (26). People living or working in these areas also acquire resistant bacteria into their own microbiome (42), further spreading the prevalence of AR. To address the CDC's recommendation, a project called the Prevalence of Antibiotic-Resistant Bacteria in the Environment (PARE) has created an infrastructure capable of organizing environmental data collection by large numbers of individual researchers (18). This project coordinates undergraduate and high school students across the USA to collect and compile data using identical methodologies. Eventually, the PARE project will make it possible to track resistance over time, identify areas with high AR, and find relationships between resistance and different environments.

The Merl and Margaret Primmer Outdoor Learning Center (Primmer), is an outdoor education and research property uniquely suited for a study on environmental drug resistance because of its location away from well-developed areas and its ecological diversity. More than 40 plants and 25 animals have been documented (C. Anderson, Capital University, personal communication), and much of this property has never been developed or used for agricultural purposes (Mark Laughlin, Capital University, personal communication). The Primmer research property is located in Appalachia, Ohio, a primarily agricultural region. The Hocking River, adjacent to the property, has been historically targeted by the Environmental Protection Agency (EPA) as it had been contaminated by industrial and sewer discharges, as well as mine drainage and agricultural runoff (39). With its seven different ecological zones located adjacent to one another and in proximity to a water source made this property an ideal and unique setting for quantitatively surveying both the prevalence of antibiotic-resistant bacteria in the different ecological zones and the diversity of these antibiotic-resistant organisms. Select isolates were additionally tested for MDR. We hypothesized that antibiotic-resistant bacteria would be found in all ecological zones studied, and also that these bacteria would be unique to their specific niche. Our findings not only supported our hypothesis by showing that antibiotic-resistant bacteria were found in all seven ecological zones studied, but that multidrug resistance can also be prevalent even in the absence of human development.

Materials and Methods

Sample collection

Soil samples were collected from the Primmer research property, located in Logan, Ohio. Fig. 1 shows a map of Primmer and the locations of the 14 collection sites from the seven ecological zones. The distinct ecological zones that have been identified include a grassland (G), a woodland (WL), a wetland (W), a prairie (F), a spring (S) and a riparian zone (R). Since clinical AR genes have been detected at distances up to 15 km from the discharge point (13), samples were also collected from the Hocking River (HR) to assess possible pollution from upstream locations. Soil samples were collected between May and June 2017. GPS coordinates in decimal degrees for each collection site were recorded (Table 1). The sites were primed by loosening up the soil with a rock or piece of wood from the area. Soil samples were isolated an estimated 2.5 cm below the surface from the upper soil horizon, or topsoil since previous studies have showed that bacterial populations are most abundant in this soil horizon (15). Samples were then transported to the laboratory and stored at 4°C overnight.

Ecological Zone	Site	Latitude	Longitude
Spring	S1	39.544504	-82.443202
	S2	39.544272	-82.442057
Woodland	WL1	39.547797	-82.442072
	WL2	39.548157	-82.441953
Prairie	F1	39.547473	-82.442803
	F2	39.546533	-82.443398
Wetland	W1	39.543284	-82.440598
	W2	39.541241	-82.443711
Hocking River	HR1	39.544660	-82.441914
	HR2	39.544387	-82.442431
Grassland	G1	39.548091	-82.439984
	G2	39.548217	-82.440005
Riparian Woodland	R1	39.547007	-82.441353
	R2	39.546853	-82.442282

Table 1. GPS coordinates of sample collection sites.

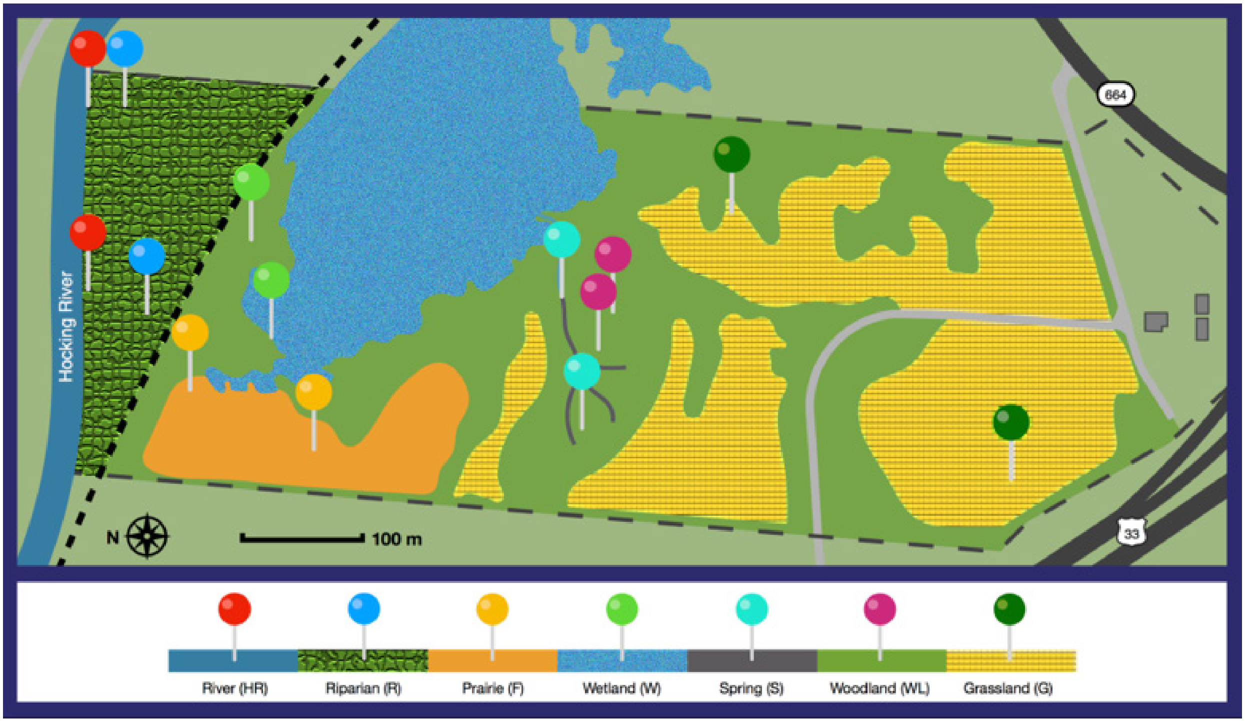


Fig 1. Map of the Primmer Outdoor Learning Center and sample collection sites. Primmer is a 0.3 km^2 private research property with seven unique ecological zones. Modified from the USGS National Map Viewer (2017). Sample collection sites are noted by the pins and correspond to the ecosystems noted in the key.

Determining the prevalence of antibiotic resistance

MacConkey agar and nutrient agar plates without tetracycline (NA), with 3 µg/ml (3tet), or 30 µg/ml (30tet) tetracycline were prepared as per the manufacturer's instructions (BD Biosciences, San Jose, CA). All plates also contained 10 µg/ml amphotericin B to prevent overgrowth on plates by fungi in the soil. One gram of soil was measured, serially diluted for five 1:10 dilutions in sterile distilled water, and 0.2 ml of each dilution were plated on all types of media using sterile glass beads. The plating of bacteria was done in duplicate. Inoculated plates were wrapped in Parafilm and incubated at 28°C for 72 hours. The total number of colony-forming units (CFUs) was determined from countable plates without tetracycline (plates with 30-300 colonies and no overgrowth). The percent tetracycline-resistant colonies were determined by counting CFUs from the plates containing tetracycline and comparing them with the plates lacking tetracycline.

Colony characterization

After incubation, colonies were characterized according to morphological appearances as in Breakwell, ***et al.***, 2007 (7). Colonies of different morphotypes from each ecological zone were selected to test for additional AR. All colonies selected were resistant to either 3 or 30 µg/ml tetracycline.

The Kirby-Bauer test for antibiotic susceptibility was performed as in Hudzicki, ***et al.***, 2017 (24). Tested antibiotic discs (ThermoFisher Scientific, Waltham, MA) contained one of the following: ciprofloxacin (5 µg), ampicillin (10 µg), penicillin (10U), and a tetracycline control (30 µg). These antibiotics were chosen based on their use in human and veterinary medicine (11, 54). The diameter of each zone of inhibition was compared to the standard set by the National Committee of Clinical Laboratory Studies (NCCLS) to determine if the isolate displayed resistance (R), susceptibility (S), or was intermediate (I).

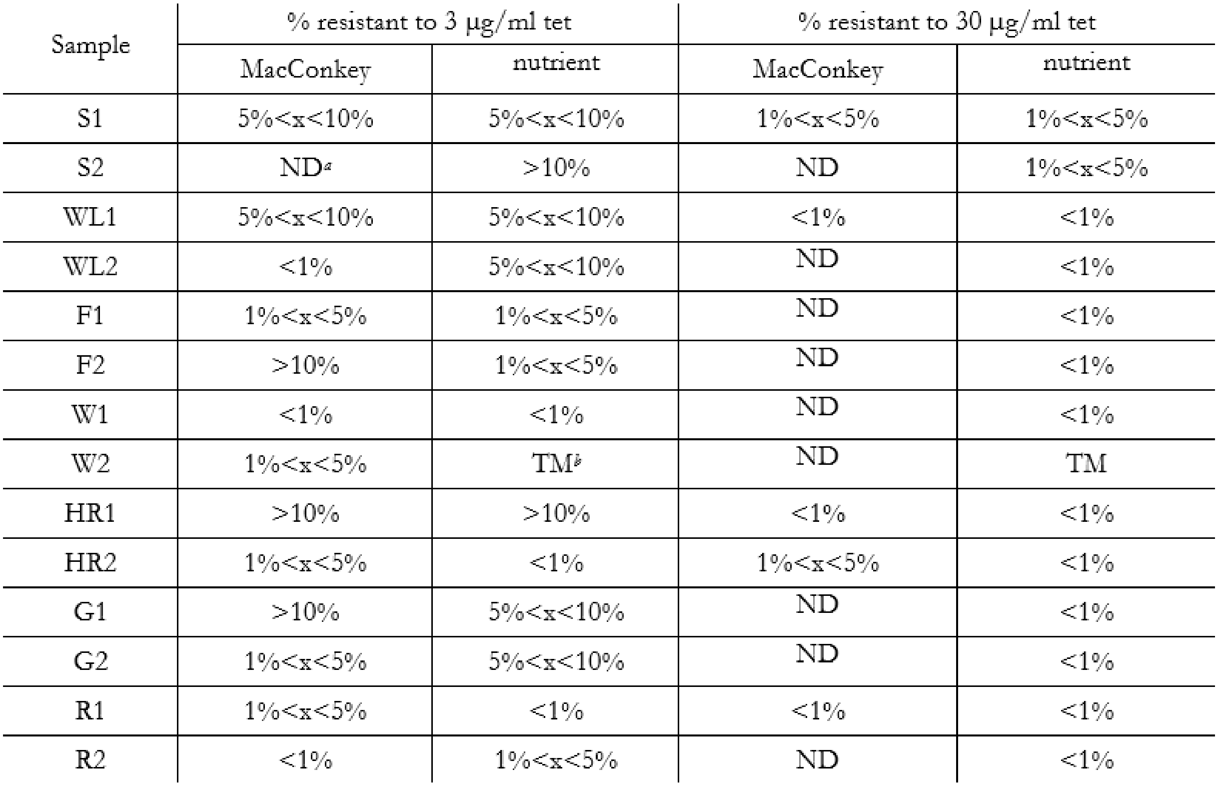
PCR and sequencing

DNA was extracted from isolates by a boiling lysis protocol as found in Queipo-Ortuño ***et al.***, 2008 (37). For each sample, PCR was performed using one of two different primer pairs for part of the 16S rRNA gene. Primer pairs were: (i): Bakt_341F, (CCTACGGGNGGCWGCAG), and Bakt_805R, (GACTACHVGGGTATCTAATCC) (21, 25) and (ii): pA/27F, (AGAGTTTGATCCTGGCTCAG) (14, 27), and 1492R, (TACGGGTACCTTGTTACGACTT) (27, 44). PCR products were purified using the DNA Clean and Concentrator kit (Zymo Research, Irvine, CA) and then sequenced using the forward primer used for amplification by The Ohio State University Comprehensive Cancer Center Genomics Shared Resource facility. Resulting sequences were compared to previously published sequences using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information).

Results

Percentage of antibiotic-resistant bacteria present in soil

To assess the prevalence of AR in the seven different ecosystems, we collected samples from two different sites from each of the seven ecological zones present within the Primmer research property (Fig 1). Soil samples were serially-diluted and plated onto media with or without tetracycline. Two different types of media and two different concentrations (3 or 30 µg/ml) of tetracycline were used. MacConkey agar was used so that a portion of our results could be contributed to the PARE database, allowing future comparisons between additional data collected from other sites. MacConkey agar is the current standard media used by the PARE Project as it commonly results in more uniform colony morphology, which makes colony counting more accurate (18). Nutrient agar was used to better assess the diversity of bacteria present. Of the MacConkey agar data, sites F2, HR1, and G1 exhibited the highest percentage of resistance to 3 µg/ml of tetracycline with the number of resistant colonies at greater than 10% (Table 2). Only five sites (S1, WL1, HR1, HR2, and R1) displayed resistance to the higher concentration of tetracycline (30 µg/ml). With the exception of HR2, these sites showed a decrease in the percentage of resistant colonies to the higher concentration of tetracycline. All but one site (S2) showed resistant colonies at some level of tetracycline concentration.



Sample	% resistant to 3 μg/ml tet		% resistant to 30 μg/ml tet	
	MacConkey	nutrient	MacConkey	nutrient
S1	5%<x<10%	5%<x<10%	1%<x<5%	1%<x<5%
S2	ND[a]	>10%	ND	1%<x<5%
WL1	5%<x<10%	5%<x<10%	<1%	<1%
WL2	<1%	5%<x<10%	ND	<1%
F1	1%<x<5%	1%<x<5%	ND	<1%
F2	>10%	1%<x<5%	ND	<1%
W1	<1%	<1%	ND	<1%
W2	1%<x<5%	TM[b]	ND	TM
HR1	>10%	>10%	<1%	<1%
HR2	1%<x<5%	<1%	1%<x<5%	<1%
G1	>10%	5%<x<10%	ND	<1%
G2	1%<x<5%	5%<x<10%	ND	<1%
R1	1%<x<5%	<1%	<1%	<1%
R2	<1%	1%<x<5%	ND	<1%

Table 2. Percentage of bacterial colomes plated on MacConkey agar and nutrient agar media resistant to 3 μg/ml or 30 μg/ml tetracycline.

[a] ND: Not determined due to low coloy counts (<30) present on plates without tetracyline.

[b] TM: Not determined due to high colony counts on plates with no antibiotic.

As expected, overall colony counts were higher on nutrient agar compared to MacConkey, which facilitated the ability to accurately determine the percent resistance for more samples at more sites (Table 2). Every sample of soil showed some level of resistant bacteria. However, the percent resistance for one of the wetland samples (W2) could not be determined due to high colony counts on plates without antibiotic. Both samples from the groundwater spring zone (S1 and S2) exhibited resistance to both concentrations of tetracycline on nutrient agar. S2 and HR1 had the highest percentage of resistant bacteria to 3 μg/ml tetracycline at greater than 10%. Eleven of the fourteen samples showed less than 1% resistance to 30 μg/ml of tetracycline. When comparing results from both MacConkey and nutrient media, samples S1, WL1, and HR1 had identical ranges for each concentration of tetracycline. All other samples, except F2, showed a slightly higher percent of resistant bacteria on nutrient agar compared with MacConkey. These data suggest that naturally occurring AR is even more prevalent than originally expected.

Identification of isolated antibiotic-resistant bacteria

We next tested our second hypothesis that tet-resistant bacteria would be unique to their particular environment. Tetracycline-resistant colonies on countable nutrient agar plates were analyzed and classified based on their phenotypic characteristics (Table 3). Thirty-six morphotypes were identified from the fourteen collection sites (morphotypes I-XXXVI). Morphotype I, the most common, was found at all 14 collection sites and at percentages ranging from 54.05% (S1) to 98.9% (W2) of the total antibiotic-resistant population. Morphotype I also represented 83.54% of all antibiotic-resistant bacteria. The woodland zone (WL1 and WL2) displayed the highest diversity of morphology with 12 unique morphotypes. These results suggest that, while at low frequencies, adjacent ecological zones contain distinct sub-populations of bacteria.

Morphotype	Form	Elevation	Margin	Size	Color	Site	%[a]
I	Circular	Convex	Entire	Small	White	S1	54
						WL1	75.4
						HR1	73.4
						R1	91.5
						F1	85.9
						F2	96.2
						G1	71
						G2	89.9
						WL2	79.2
						S2*	92.2
						R2	82.1
						HR2*	93
						W1	87.5
						W2	98.9
II	Circular	Convex	Entire	Small	Yellow	S1*	33.8
						WL1	14.8
						HR1*	14.4
						F1	.6
						G1	.8
						WL2	7.8
						R2	.9
						HR2	1.4
III	Circular	Convex	Entire	Small	Orange	G1	3.7
						G2	1.3
						WL2	2.6
						WL1*	2.1
IV	Circular	Convex	Entire	Small	Pink	WL1*	.4

Table 3a. Colony morphology for countable nutrient media plates.

[a] Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

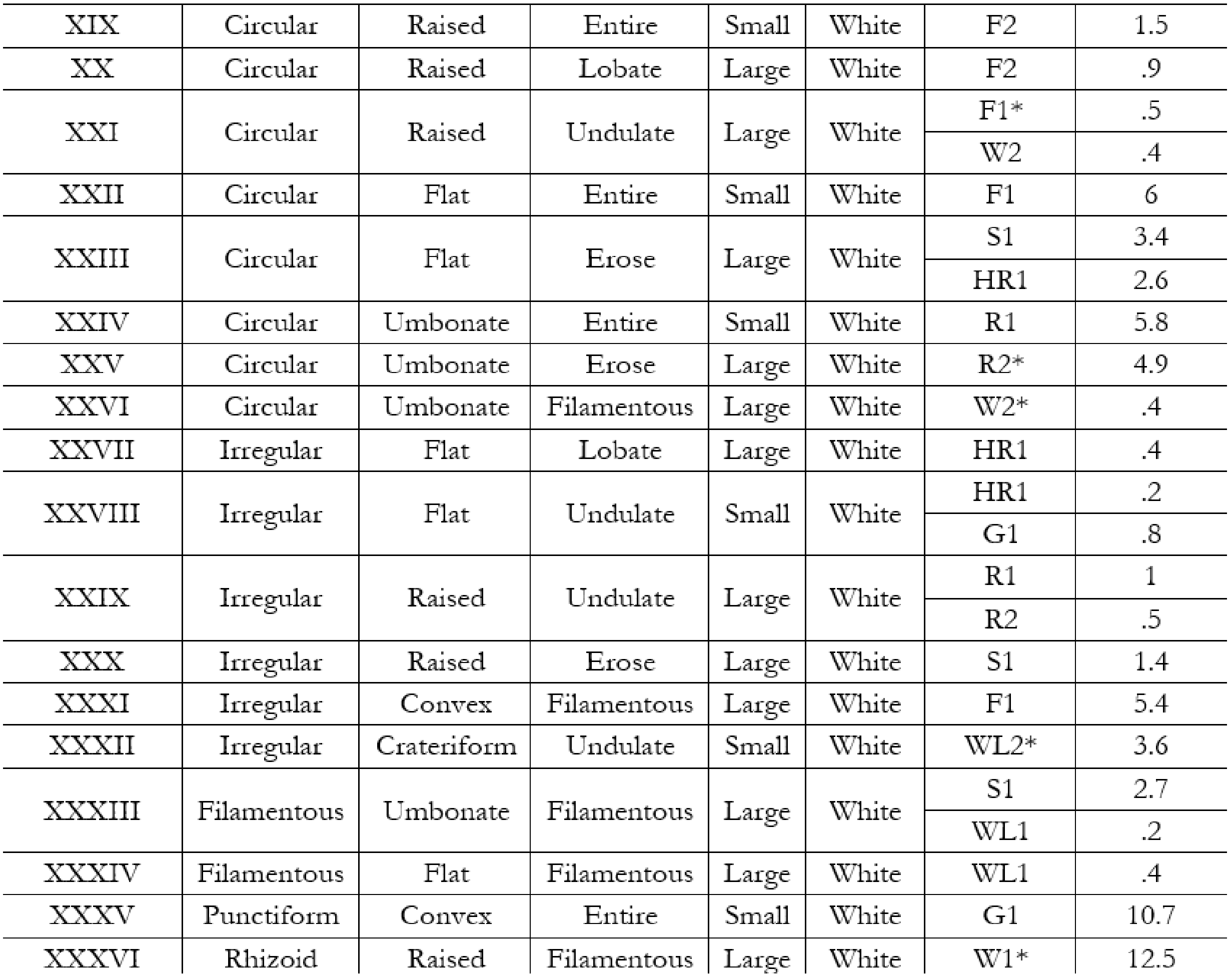
*Selected fror isolation

V	Circular	Convex	Entire	Large	White	S1	2
						HR2	2.1
VI	Circular	Convex	Entire	Large	Orange	S1*	2.7
VII	Circular	Convex	Erose	Large	White	HR1	4.2
						WL1	3.5
VIII	Circular	Convex	Erose	Small	White	HR1	4.9
IX	Circular	Crateriform	Entire	Small	White	G1	2.5
						G2	7.6
						WL2	2.1
						R2	11.7
X	Circular	Crateriform	Entire	Small	Yellow	WL1	1.8
XI	Circular	Crateriform	Entire	Large	White	WL1	.4
						HR2	3.5
XII	Circular	Crateriform	Entire	Large	Yellow	F1	2.2
						W2	.4
XIII	Circular	Crateriform	Erose	Large	White	G2*	1.3
XIV	Circular	Crateriform	Undulate	Small	White	G1*	9.1
						WL2	4.7
						S2	1.7
XV	Circular	Crateriform	Undulate	Large	White	G1	.8
XVI	Circular	Crateriform	Lobate	Large	White	G1	.4
XVII	Circular	Raised	Erose	Large	White	WL1	1
						R1	.3
XVIII	Circular	Raised	Erose	Small	White	R1	1
						F2	.9

Table 3b. Colony morphology for countable nutrient media plates.

[a]Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

*Selected fror isolation



XIX	Circular	Raised	Entire	Small	White	F2	1.5
XX	Circular	Raised	Lobate	Large	White	F2	.9
XXI	Circular	Raised	Undulate	Large	White	F1*	.5
						W2	.4
XXII	Circular	Flat	Entire	Small	White	F1	6
XXIII	Circular	Flat	Erose	Large	White	S1	3.4
						HR1	2.6
XXIV	Circular	Umbonate	Entire	Small	White	R1	5.8
XXV	Circular	Umbonate	Erose	Large	White	R2*	4.9
XXVI	Circular	Umbonate	Filamentous	Large	White	W2*	.4
XXVII	Irregular	Flat	Lobate	Large	White	HR1	.4
XXVIII	Irregular	Flat	Undulate	Small	White	HR1	.2
						G1	.8
XXIX	Irregular	Raised	Undulate	Large	White	R1	1
						R2	.5
XXX	Irregular	Raised	Erose	Large	White	S1	1.4
XXXI	Irregular	Convex	Filamentous	Large	White	F1	5.4
XXXII	Irregular	Crateriform	Undulate	Small	White	WL2*	3.6
XXXIII	Filamentous	Umbonate	Filamentous	Large	White	S1	2.7
						WL1	.2
XXXIV	Filamentous	Flat	Filamentous	Large	White	WL1	.4
XXXV	Punctiform	Convex	Entire	Small	White	G1	10.7
XXXVI	Rhizoid	Raised	Filamentous	Large	White	W1*	12.5

Table 3c. Colony morphology for countable nutrient media plates.

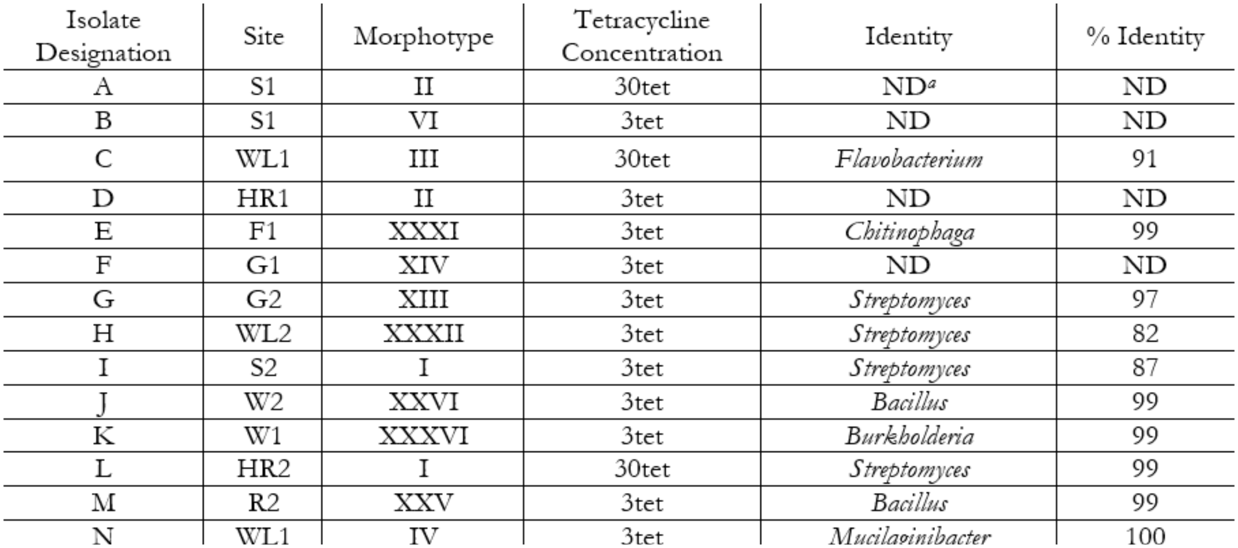
[a]Percentage of antibiotic-resistant colonies with the population at a single soil sample collection site.

*Selected fror isolation

To determine if the resistant bacteria were unique to their ecological zone, we selected a subset of colonies from different ecosystems and of different morphology. Some colonies were selected from 3tet plates while others were selected from 30tet plates. We performed PCR on these isolates using primers designed to amplify the 16S ribosomal RNA gene. Sequence analysis of these genes allowed identification at the genus level. Surprisingly, of the ten bacterial colonies successfully sequenced (Table 4), nearly all of the bacteria identified were of different genera. As expected, ***Streptomyces***, which is prevalent in soil (23)**,** was identified in four different ecological zones (G, WL, S, and HR). Morphotype XXVI from the wetland zone (W2) and morphotype XXV from the riparian zone (R2) were identified as ***Bacillus*** with 99% confidence. The duplicate genera suggest that antibiotic-resistant bacteria are not entirely specific to their ecological zone. These data showed that while some species of antibiotic-resistant bacteria were present in multiple zones, there were some that were specific to different zones, supporting our initial hypothesis. These data also showed that a variety of different bacteria are capable of possessing AR.

Antibiotic susceptibility test

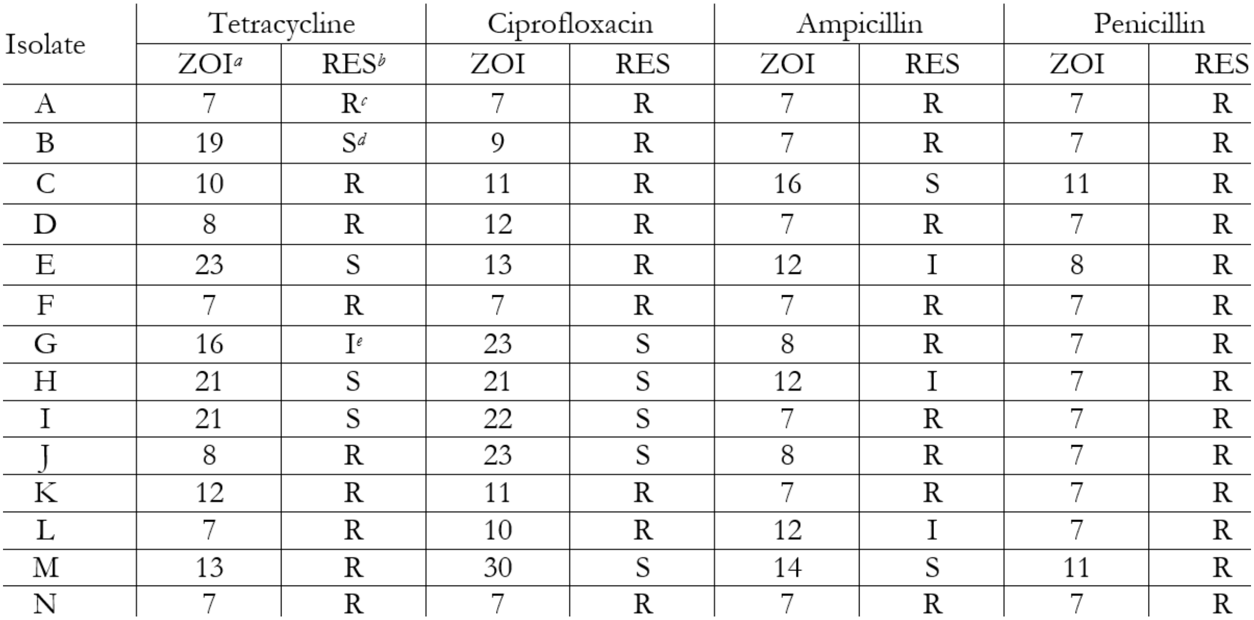
Since all of our isolates were found to be resistant to tetracycline, we wanted to know if they were resistant to other antibiotics. To test this, we performed a Kirby-Bauer disk diffusion test allowing us to test other antibiotics used in human and veterinary medicine. The antibiotics chosen for testing were ciprofloxacin, which has a synthetic origin (49), and ampicillin and penicillin, which are both naturally occurring β-lactam antibiotics (1, 16). Some of our isolates selected from 3tet plates were sensitive to the disk with 30 μg of tetracycline (isolates B, E, H, and I), showing that their resistance is concentration-dependent. Since all isolates were resistant to some concentration of tetracycline and one or more antibiotics, 100% of the isolates were multidrug-resistant (Table 5). Interestingly, every isolate displayed resistance to penicillin. Isolates A, D, F, K, and N (36% of isolates) were resistant to all four antibiotics. Discovery of MDR in each ecological zone indicates that there must be some selective advantage for this, even in naturally occurring environments.



Isolate Designation	Site	Morphotype	Tetracycline Concentration	Identity	% Identity
A	S1	II	30tet	ND[a]	ND
B	S1	VI	3tet	ND	ND
C	WL1	III	30tet	*Flavobacterium*	91
D	HR1	II	3tet	ND	ND
E	F1	XXXI	3tet	*Chitinophaga*	99
F	G1	XIV	3tet	ND	ND
G	G2	XIII	3tet	*Streptomyces*	97
H	WL2	XXXII	3tet	*Streptomyces*	82
I	S2	I	3tet	*Streptomyces*	87
J	W2	XXVI	3tet	*Bacillus*	99
K	W1	XXXVI	3tet	*Burkholderia*	99
L	HR2	I	30tet	*Streptomyces*	99
M	R2	XXV	3tet	*Bacillus*	99
N	WL1	IV	3tet	*Mucilaginibacter*	100

Table 4. Designation for identification

[a] ND: Not determined



Isolate	Tetracycline		Ciprofloxacin		Ampicillin		Penicillin	
	ZOI[a]	RES[b]	ZOI	RES	ZOI	RES	ZOI	RES
A	7	R[c]	7	R	7	R	7	R
B	19	S[d]	9	R	7	R	7	R
C	10	R	11	R	16	S	11	R
D	8	R	12	R	7	R	7	R
E	23	S	13	R	12	I	8	R
F	7	R	7	R	7	R	7	R
G	16	I[e]	23	S	8	R	7	R
H	21	S	21	S	12	I	7	R
I	21	S	22	S	7	R	7	R
J	8	R	23	S	8	R	7	R
K	12	R	11	R	7	R	7	R
L	7	R	10	R	12	I	7	R
M	13	R	30	S	14	S	11	R
N	7	R	7	R	7	R	7	R

Table 5. Zone of inhibition diameter measurements of selected bacterial colonies

[a] Zone of inhibition diameter measured in mm

[b] Resistance determined by diameter standard (CLSI, 2013)

[c] Resistant

[d] Susceptible

[e] Intermediate

Discussion

AR is naturally occurring, even in environments away from anthropogenic selection pressures. Studying this naturally occurring AR is important for understanding and dealing with the growing resistance of pathogenic microorganisms in clinical settings. Previous evidence of natural AR has been found in remote soil and isolated cave systems (2,5). While the Primmer research property is not as isolated as a cave system, our samples were collected from a site that has never been developed. Because resistant organisms were found in each of the ecological zones tested, our results support what previous research has found, that AR occurs naturally in bacterial populations.

Not much is known yet regarding how frequently AR gene transfer takes place in natural environments. MDR can occur through various mechanisms, one of which is efflux of the drugs by membrane transport proteins. Efflux pumps are able to transport specific substrates or expel cytotoxic compounds (6, 29). While there is evidence that efflux pumps are physiologically important (35), they commonly mediate MDR. This has led to the occurrence of microorganisms intrinsically resistant to multiple antibiotics (33). Our data support this claim, as resistance was found to ciprofloxacin, despite a seemingly absence of evolutionary pressures. Interestingly, more of our isolates exhibited resistance to penicillin than ampicillin, yet both are β-lactam antibiotics. This could be attributed to the fact that penicillin has natural origins whereas ampicillin is semi-synthetic. Overall, our results suggest that MDR in a bacterial population is a naturally occurring phenomenon. HGT of naturally occurring resistance genes to pathogens could also be a factor for the acceleration of resistance found in clinical and agricultural settings.

To characterize the diversity of the bacterial populations present in the different ecological zones, we analyzed colony morphology present on our non-selective media plates and selected antibiotic-resistant isolates to be sequenced. Previous research has shown a wide variety of taxonomic groups to have AR and MDR bacteria (31) and also that bacteria are capable of transferring AR genes either between bacteria of the same species or between different species (4). We found a high diversity of colony morphologies (Table 3) and identified six unique bacteria genera (Table 4) from a small subset of total colonies. Since all selected isolates were found to be multidrug-resistant, it is possible that HGT of AR genes occurs naturally at some frequency. These data support our hypothesis that unique bacteria would be found in the different ecological zones, and more importantly, that antibiotic-resistant bacteria can be from a highly diverse group.

Soil is rich in microbial abundance and species diversity. It has been estimated that a single gram of soil can have up to 10^{10} bacterial cells and more than 4×10^{3} different bacteria species (20, 34, 36, 40, 46, 47). In addition, bacteria populations can differ between geography and altitude (30). Using a site with seven adjacent ecological zones gave us the unique ability to directly compare both the prevalence and diversity of antibiotic-resistant organisms in these different ecosystems. We found a large range in the overall percentages of resistant organisms in each of the ecological zones. The highest percent resistant organisms were found in the spring (S), woodland (WL), and river (HR) zones. High percentages in the woodland areas could be due to availability of nutrients around the rhizosphere, an active region around a plant root that microorganisms inhabit (3). The river and spring zones could have high resistance from upstream pollution sources, especially since the Primmer research property is located within an agriculturally focused region. This is not surprising as other bacteria have been previously found to survive in water for a long time, such as those from the genera ***Pseudomonas*** (38, 43).

In conclusion, the differences we found between the adjacent sites show that while there is a relationship between ecology, prevalence of antibiotic-resistant organisms, and types of bacteria present, multidrug-resistance was found in all sites and all types of bacteria tested and is likely more common in the environment than we thought. Additional surveillance of the resistome present in different ecological locations will likely be essential for developing novel antibiotic treatments.

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Loss of protein quality control gene *UBR1* sensitizes *Saccharomyces cerevisiae* to the aminoglycoside hygromycin B

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Abstract

Ubr1 is a conserved ubiquitin ligase involved in the degradation of aberrant proteins in eukaryotic cells. The human enzyme is found mutated in patients with Johanson-Blizzard syndrome. We hypothesized that Ubr1 is necessary for optimal cellular fitness in conditions associated with elevated abundance of aberrant and misfolded proteins. Indeed, we found that loss of Ubr1 in the model eukaryotic microorganism ***Saccharomyces cerevisiae*** strongly sensitizes cells to hygromycin B, which reduces translational fidelity by causing ribosome A site distortion. Our results are consistent with a prominent role for Ubr1 in protein quality control. We speculate that disease manifestations in patients with Johanson-Blizzard syndrome are linked, at least in part, to defects in protein quality control caused by loss of Ubr1 function.

Introduction

The structure and function of the Ubr1 ubiquitin ligase are conserved across diverse eukaryotic organisms (8). In humans, mutation of ***UBR1*** causes Johanson-Blizzard syndrome, a disorder characterized by multi-organ dysfunction, physical malformations, and cognitive impairment (19). Ubr1 has been extensively investigated in the model unicellular eukaryote ***Saccharomyces cerevisiae*** (budding yeast), where it contributes to multiple aspects of protein quality control. Experiments performed with yeast have been foundational in the discovery of molecular mechanisms of quality control conserved among eukaryotes (3). Among other roles, Ubr1 promotes turnover of substrates of the N-end rule (17), endoplasmic reticulum-associated degradation (ERAD) (12, 13), stress-induced homeostatically regulated protein degradation (SHRED) (14), and cytoplasmic quality control (CytoQC) (7, 11) pathways. Yeast lacking Ubr1 exhibit divergent responses to pharmacologic interventions expected to increase the abundance of misfolded proteins. For example, ***ubr1***Δ yeast display enhanced sensitivity to the Hsp90 inhibitor geldanamycin, while they are resistant to the proline analog L-azetidine-2-carboxylic acid (15).

Materials

All yeast strains used in this study are presented in Table 1. These strains have been constructed in previous reports (6, 16). The entire coding sequences of ***UBR1***, ***HRD1***, and ***DOA10*** have been replaced with the ***kanMX4*** allele in gene knockout strains (16). Yeast were cultured in yeast extract-peptone-dextrose medium (1% yeast extract, 2% peptone, 2% glucose, 0.002% adenine, 2% agar) (5) with the indicated concentrations of hygromycin B (Corning).

Methodology and Results

We tested the hypothesis that Ubr1 is necessary for optimal growth in conditions associated with elevated abundance of aberrant proteins. The aminoglycoside hygromycin B reduces translational fidelity by causing ribosome A site distortion and is toxic to yeast at 200 μg/ml (1, 4, 9). We analyzed the growth of yeast lacking Ubr1 in the presence of sublethal doses of hygromycin B, which are expected to increase the cellular concentration of misfolded proteins (**Figure 1**). Wild type yeast, ***ubr1***Δ yeast, and yeast lacking one or both genes encoding the primary ERAD ubiquitin ligases (***HRD1*** and ***DOA10***) (10) were subjected to six-fold serial dilution, beginning with an optical density at 600 nm of 0.2. Each dilution (4 μl) was spotted onto agar plates containing rich yeast growth medium with no drug or increasing concentrations of hygromycin B. Plates were incubated at 30°C and imaged at the indicated times. A detailed explanation of the yeast growth assay procedure can be found in (18). All data were analyzed using Prism software (GraphPad Software Inc., San Diego, CA). Because of highly variable data, values of zero were normalized to 1 to make all data positive and positive data were log-transformed. All means between groups were compared by one-way ANOVA followed by Tukey post-hoc analysis. A ***P*** value less than 0.05 was designated as statistically significant.

In the absence of hygromycin B, all yeast strains exhibited similar growth. Consistent with previous results (2), yeast lacking both ***HRD1*** and ***DOA10*** exhibited a pronounced growth defect in the presence of 75 μg/ml hygromycin B. Individual deletion of ***HRD1*** or ***DOA10*** also impaired growth in the presence of hygromycin B, but to a lesser extent than the double mutant. Finally, loss of ***UBR1*** impaired growth in the presence of the compound more severely than any of the other mutations tested; this is most evident at 50 μg/ml hygromycin B.

This experiment was piloted by undergraduate

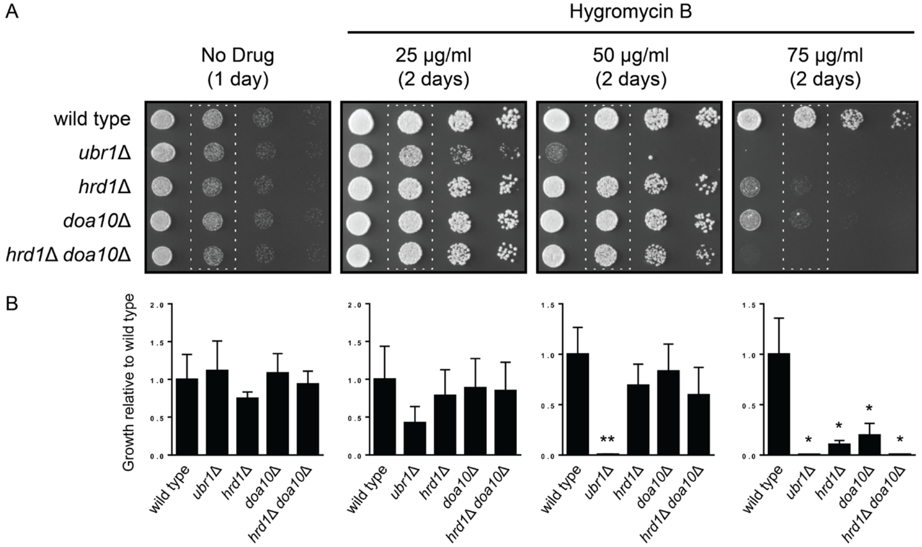

Figure 1. Loss of ***UBR1*** sensitizes yeast to hygromycin B.

(A) Six-fold serial dilutions of yeast of the indicated genotypes were spotted onto agar plates containing rich medium (No Drug) or rich medium containing increasing concentrations of hygromycin B. Plates were imaged after 1-2 days (as indicated) of incubation at 30^{o}C. (B) Growth in the second column of each plate (dashed rectangles) from three replicate experiments was quantified by densitometry. Data were analyzed by one-way ANOVA followed by Tukey post-hoc analysis (*, less than wild type; **, less than wild type, *hrd1*Δ, *doa10*Δ, and *hrd1*Δ *doa10*Δ; $p < 0.05$). Error bars represent standard error of the mean.

students in the Methods in Cell Biology (BIO 315) Course at Ball State University and has been validated by three replicates in the research laboratories of EMR and PJS.

Discussion

As we hypothesized, our results indicate Ubr1 is crucial for optimal growth of yeast in conditions associated with elevated abundance of aberrant proteins, consistent with Ubr1 function in protein quality control. Mutations in ***UBR1*** are found in patients with Johanson-Blizzard syndrome. A previous study demonstrated that homologous mutations also reduce Ubr1 function in yeast (8). We speculate that disease phenotypes present in patients with Johanson-Blizzard syndrome harboring mutations in ***UBR1*** are linked, at least in part, to defects in protein quality control. One limitation of the present work is that hygromycin B is expected to trigger the accumulation of a large and heterogeneous population of aberrant proteins. Therefore, it is difficult to determine which types of protein aberrancies present the most substantial challenge to cellular health in the absence of Ubr1. Future biochemical experiments will be necessary to characterize the substrate range of Ubr1 in yeast and human cells. Such biochemical analyses may also provide insight into the divergent responses of ***ubr1***Δ yeast to different forms of cellular stress.

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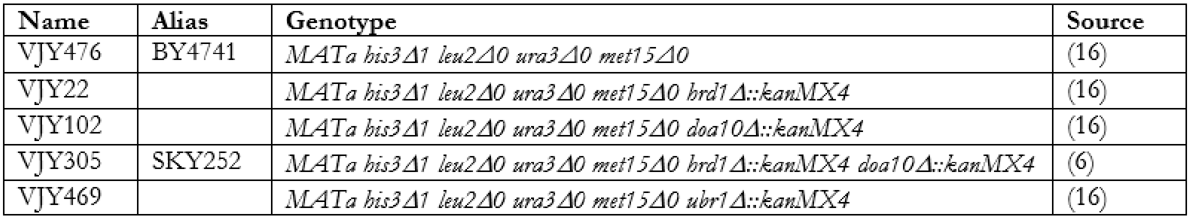

Name	**Alias**	**Genotype**	**Source**
VJY476	BY4741	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(16)
VJY22		*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hrd1Δ::kanMX4*	(16)
VJY102		*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 doa10Δ::kanMX4*	(16)
VJY305	SKY252	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hrd1Δ::kanMX4 doa10Δ::kanMX4*	(6)
VJY469		*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ubr1Δ::kanMX4*	(16)

Table 1. Yeast strains used in this study.

All strains used in this study are congenic with BY4741 (16).

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Enrichment of Microbes Potentially Degrading Polyethylene Using a Microcosm Approach

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Abstract

Plastic pollution is a worldwide phenomenon with concerning effects on the biosphere and particularly on the marine environment. Biodegradation is considered an environmentally friendly alternative to combat the increasing quantities of plastic pollutants where different microbial sources are tested for plastic degradation potential. In this project, a microcosm approach was used as an enrichment method for marine microbes degrading polyethylene. Pieces of low-density polyethylene (LDPE) and high-density polyethylene (HDPE) previously deployed in ocean water have been explored as a source of microbial biomass. This source plastic was added to a synthetic medium containing sterilized pieces of LDPE and HDPE as the sole carbon source and were incubated for extended periods (32-86 days) in the laboratory to promote growth of microbes that can degrade plastic. Biodegradation of polyethylene was confirmed by dry weight measurements and Fourier Transform Infra-Red (FTIR) spectroscopy. For both LDPE and HDPE a significant reduction in dry weight was observed. FTIR analysis showed peaks suggesting oxidative changes in polyethylene's chemical composition. In summary, the microcosm approach can be considered a viable approach for enrichment of plastic-degrading marine microbial populations.

Introduction

Few topics span so many disciplines and touch the public interest as much as the issue of plastic pollution. Plastic is an essential element of our everyday lives, and its introduction in multiple spheres of human activity brought economically and scientifically sensible solutions and innovations. Plastic can be defined as long-chain synthetic polymers created through polymerization of monomers obtained from oil or gas with chemical additives (1). Plastic material is used to package almost every commodity, and it is estimated that one-third of plastic manufactured is used in disposable one-time use products (2). Due to its non-degradable nature, plastic has been accumulating in the environment at exponentially increasing rates since mass production began in the 1940s (1, 3). Some of the plastic accumulating in the environment inevitably makes its way into the ocean environment. Rain, wind, tsunamis, streams, and other phenomena carry plastics into the ocean, where they fragment into microscopic pieces. Additionally, microplastics may enter the marine environment through cleaning scrubbers, abrasive beads for cleaning boats, and deterioration of larger pieces of plastic (4). Plastic litter and contaminants can be found on remote beaches throughout the world constituting not only an eyesore, but also a hazard for the environment. Dangers of plastic include harm to animals (5), ability to transport contaminants around the ocean (6), as well as release of toxic chemicals upon degradation (4, 7, 8).

Diverse abiotic and biotic processes can degrade plastic in marine environments (9–11). Degradation can be defined as a process that leads to a decline of polymer-like properties through multiple steps. Weathering is the physical deterioration of plastic, which occurs in the ocean through wind and waves. Photodegradation is the leading cause for degradation of plastic, as UV-B radiation from sunlight breaks the C-H bonds on the polymer backbone, creating a carbon free radical that most often reacts with oxygen creating a peroxy radical. Olefins, aldehydes, and ketones are the most common products, but C-C crosslinking can also occur (11). Photo-initiated oxidative degradation is the rate determining step of degradation in the marine environment.

Biotic degradation is not as well documented as abiotic processes (12, 13) and it is almost always preceded by abiotic degradation (14). Microbes can excrete extracellular enzymes, which break down and utilize the carbon backbone of most plastics. In the marine environment, conditions are not optimal, so both abiotic and biotic degradation occur slowly on a scale of decades. Potential microbial candidates for biodegradation of plastic have been previously described, including a *Penicillum* species from a landfill (15), *Pseudomonas* (16, 17) *Bacillus* (18) and *Rhodococcus* (19) among others. Recent reviews list a number of marine microbial groups with described or potential plastic degradation ability (20–22).

Polyethylene (PE) is the most common plastic and can be found in two varieties: high-density PE (HDPE) and low-density PE (LDPE). PE represents 64% of all plastic produced and it is the most common floating plastic as well as the most common plastic found on beaches (23). Due to its light weight and hydrophobicity, PE floats in ocean water. Its backbone is composed solely of carbon atoms, which can be the target of microbial activity originating at any terminal methyl group.

Previous studies have described the "plastisphere," a unique microbial ecosystem living on and in floating plastic, containing organisms potentially able to use plastic polymers as a food source (12, 13, 24, 25). An ongoing study exploring the bacterial populations attaching to floating plastic in coastal waters (26) has produced plastic with a rich biomass. We hypothesized that this biomass could be a suitable source for the identification of potentially plastic-degrading microbes.

The microcosm approach, first described in the 19th century (27) has been a debated model to recreate complex ecological systems in a laboratory environment (28–30). A number of authors have used this approach to isolate and identify microbes with the ability to degrade plastic from diverse complex sources (31–33) In this study, we tested a microcosm approach to enrich for marine microbes with the ability to degrade PE. We used PE previously exposed to ocean water as microbial source, and UV-irradiated PE as the sole carbon source in the growth medium. Plastic degradation was tested using chemical methods such as Fourier Transform Infrared Spectroscopy (FTIR) and dry weight measurements. Our results indicate modest degradation by decrease in weight and observed oxidative changes.

Materials and Methods

Microcosm

Figure 1 shows the overall setup of the microcosm experiment to select plastic degrading microbes. For microbial source, PE that had been previously exposed to ocean water (either in the field or in a laboratory setting, see section below) was added to a synthetic medium containing no carbon. As a carbon source, sterilized PE was used. Sterile mason jars were used to contain the medium.

Microbial Source

As source of microbial biomass, PE samples previously incubated in ocean water were employed. These samples are part of a project studying microbial colonization in coastal waters (26). Briefly, plastic squares about 5cm x 5cm were sterilized and placed in sampling cages, which were either attached to the Scripps Oceanography pier in San Diego, CA (GPS coordinates 32°52’00.8”N 117°15’28.1”W, approximately 5m under the water surface), or placed in 5L carboys containing ocean water in the laboratory incubator set at 16 oC. Microbial biomass was collected on three different occasions, after 33 days (pier), 153 days (lab), and 70 days (lab) incubations. Collection times were variable due to both weather conditions and a related educational field trip experience (34). Samples were collected using gloves and sterilized instruments, and placed in sterile pouches. The collected plastic samples with the attached biomass were used as microbial sources for three sets of microcosm experiments. A 3cm x 3cm piece of PE (either HDPE or LDPE), cut with sterile scissors using aseptic technique was added to the microcosms. Control cultures with no microbial source were set up in parallel for the first microcosm experiment.

Source of PE

Sources of high-density PE (HDPE) and low-density PE (LDPE) were clean produce and grocery bags, respectively. The same batches of bags collected from Sprouts and Amazon packaging were used for all experiments. The chemical nature of each polymer was confirmed through FTIR (see section below).

Pretreatment of PE

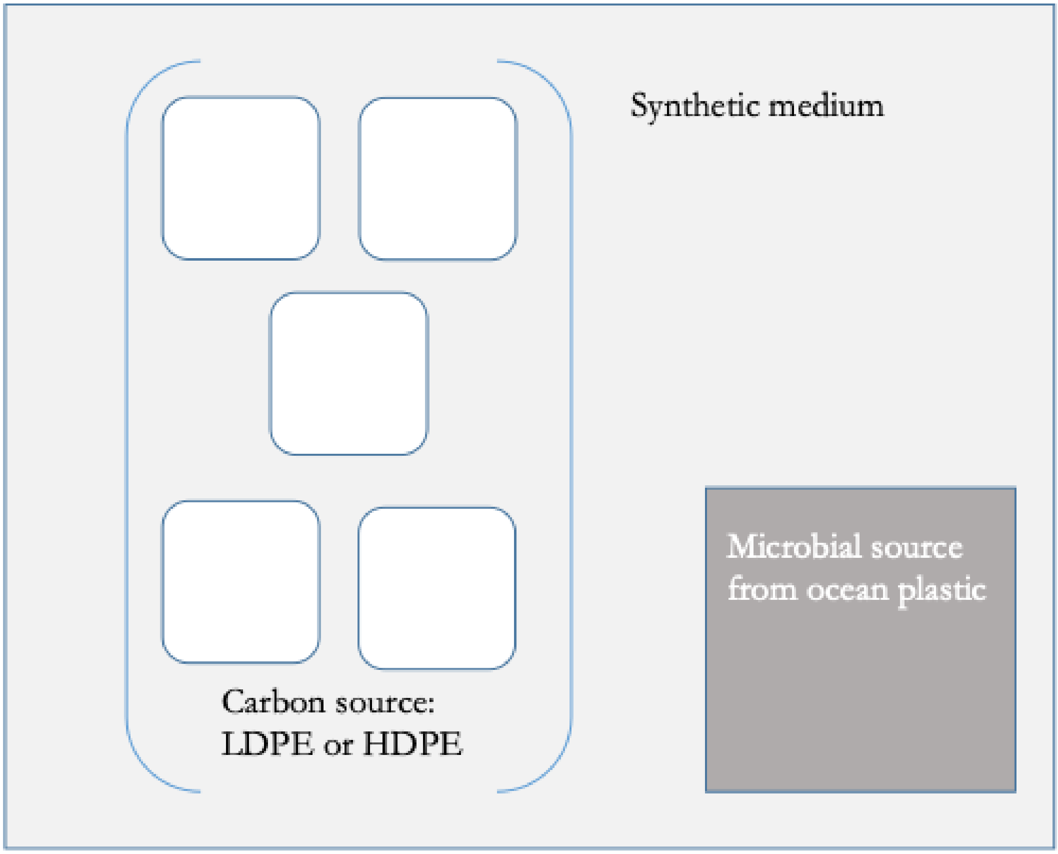

Figure 1. Microcosm setup. PE pieces previously incubated in the ocean were used as source of microbes. The synthetic medium imitated ocean salinity but contained no carbon source. Carbon was provided as sterilized PE fragments (5 per culture).

Prior to placement in medium, the PE used as carbon source was sterilized through serial washes with shaking (40 min in SDS 2%, 15 min in deionized water, 30 min in 70% ethanol), using a method adapted from Das & Kumar (18) . After the last wash, the PE pieces were handled using aseptic technique, stored in sterilized pouches, and left to dry overnight at 60°C. To simulate photo-initiated degradation (naturally occurring in the marine environment by solar radiation), PE samples were incubated under a UVP UV emitter (Model TM-10E, Upland, California, USA). Plastic was cut into 1cm x 1cm squares using aseptic technique and exposed to UV light at 253.7nm for 48 total hours. Five squares per sample were added to each microcosm experiment.

Culture Conditions

For the microcosm experiment, cultures were kept in a previously described synthetic medium (14), at 16°C in the dark. The medium mimicked ocean salinity while containing no carbon sources other than the added plastic. It contained: 1g NH_4NO_3, 1g K_2HPO_4, 0.15g KCL, 0.2g $MgSO_4$, 0.1g $CaCl_2$, 0.1g yeast extract, 1mg $MnSO_4$, 1mg $FeSO_4$, and 1mg $ZnSO_4$ per 1L distilled water. Cultures were set up in 250 mL sterile mason jars. Hundred mL of the medium were supplemented with 5 pieces of 1cm x1cm PE squares and one piece of previously incubated PE as microbial source. Paraffin oil, a non-ionic surfactant, was added at a final concentration of 0.05% v/v to the second and third experimental sets to enhance colonization (17). Three sets of microcosm experiments were completed for a length of 32, 70, and 86 days.

Determination of Dry Weight

Weight of the supplemented PE was recorded after the plastic had been sterilized and left to dry overnight at 60°C, but before it was transferred to the synthetic medium ("before"). After the incubation time concluded, plastic was treated with the same sterilization and drying process and weighed again ("after") on a Mettler Toledo scale (Model XS603S, Switzerland). The scale was previously calibrated according to the manufacturer's protocol and had a sensitivity of ±0.01 mg. Dry weight measurements were compared using a t-test.

FTIR Analysis

FTIR studies were performed on the individual PE pieces at the end of the incubation after the dry weight was recorded. As controls PE fragments sterilized and UV-treated but not incubated were used. FTIR was performed on a Nicolet iZ10 Spectrometer and spectra analyzed using the Omnic Anywhere Cloud Computing system (Thermo Scientific, Waltham, MD). The double bond index (DBI) was calculated based on the relative intensity of the double bond band at 1,650 cm−1 to that of the methylene scissoring band at 1,460 cm−1 (35). The DBI of the control and the experimental samples was compared using a one-way ANOVA. For statistical analyses the GraphPad Prism version 8.0.0 for Mac was used (GraphPad Software, San Diego, CA).

Results

Microcosm experiments

Table 1 shows the details of the microbial sources and incubation times for the three microcosm experiments. Pier and laboratory incubations had been set up in parallel to guarantee samples for an educational project, using the same sampling system and as similar conditions as possible. Laboratory experiments used ocean water from the pier location. Water temperatures at the Scripps pier were averaged based on the daily readings from the incubation

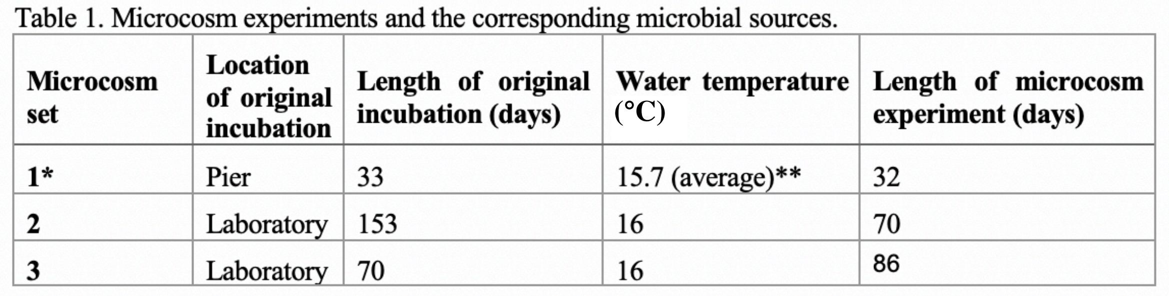

Table 1. Microcosm experiments and the corresponding microbial sources.

Microcosm set	**Location of original incubation**	**Length of original incubation (days)**	**Water temperature (°C)**	**Length of microcosm experiment (days)**
1*	Pier	33	15.7 (average)**	32
2	Laboratory	153	16	70
3	Laboratory	70	16	86

*This set also included a negative control (no microbial source added).

**Data provided by the Shore Stations Program, with current funding provided by the California Department of Parks and Recreation, Division of Boating and Waterways, Award# C1670003. Data are collected by staff aquarists and volunteers with the Birch Aquarium at Scripps.

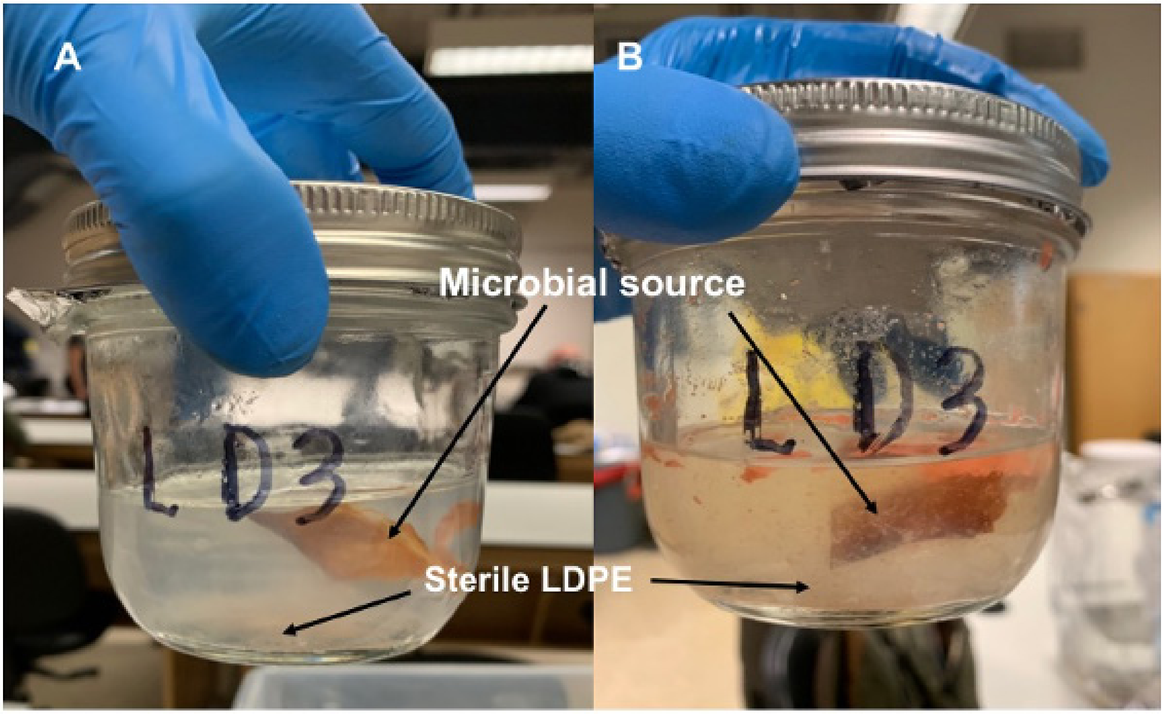

Figure 2. A: a LDPE microcosm at the beginning of incubation. B: the same microcosm at the end of a 70-day incubation.

period, while the temperature was set at 16 oC for the laboratory incubations.

Figure 2 shows a LDPE sample from the set #2 microcosm experiment at day 0 and day 70, respectively. Increased turbidity can be observed in the medium. Overall, microcosm samples consistently showed microbial growth as evidenced by increased turbidity. Uneven loss of volume due to evaporation was observed, preventing measurement of turbidity as a reflection of growth.

Dry weight

Dry weight of the plastic specimens was measured before and after incubation. Due to the extensive manipulation required for the sterilization and drying of the PE samples, some HDPE samples were lost during the process. The negative controls (PE incubated in the microcosm without a microbial source) from set #1 did not show reduction in weight.

Weight loss was recorded in 69% of samples while no change was recorded in 31% of samples. There was no clear relationship between weight loss and length of incubation tested or source of microbial culture. Figure 3 shows the dry weight change in all HDPE and LDPE samples tested before and after the incubation. The average weight for LDPE samples decreased from 11.4 mg (SD=1.35) to 10.1 mg (SD=1.37). Similarly, HDPE weight decreased from 5.5 mg (SD=2.9) to 4.5 mg (SD=2.17). The decrease of dry weight was statistically significant for both LDPE (p=.0133, t=3.074, df=9) and HDPE (p=.0409, t=2.739, df=5).

FTIR

When comparing the FTIR spectra of control (pre-treated but not included in the microcosm) PE with the microcosm LDPE and HDPE samples, peaks indicative of oxidative processes could be observed. Figure 3 shows the FTIR spectra corresponding to representative LDPE and HDPE samples after 70 days. Compared to the control, both LDPE and HDPE show additional peaks corresponding to the range of alkynes (2,200 - 2,300 cm^{-1}) as well as a robust peak corresponding to double bonds such as the carbonyl group (1,640 - 1,680cm^{-1}).

Figure 5. shows the double-bond index (DBI) calculated for each microcosm set. There is no significant difference between the control and the day 30 samples for either LDPE and HDPE. While there seems to be an overall increase in the DBI for both LDPE and HDPE by day 70, only in HDPE was the increase significant from M=0.200 (SD=.0956) to M=0.403 (SD=.07319) (p=.0063). The day 86 samples were not significantly different from the control.

Discussion

The term “great plate count anomaly” was coined by Staley and Konopka in 1985, which described the discrepancy between total microbial counts from a natural environment and colonies isolated by traditional microbiology methods (36). It is estimated that the latter can only recover 1% or less of the bacterial diversity in most environmental samples (37). Therefore it has been a continuing challenge for microbial ecologists to describe complex microbial populations and the key interactions between their components. Culture-independent methodologies, based on DNA analysis using metagenomic approaches are often used to characterize complex populations (38). Such analyses have helped the identification of microbes whose genomes contain

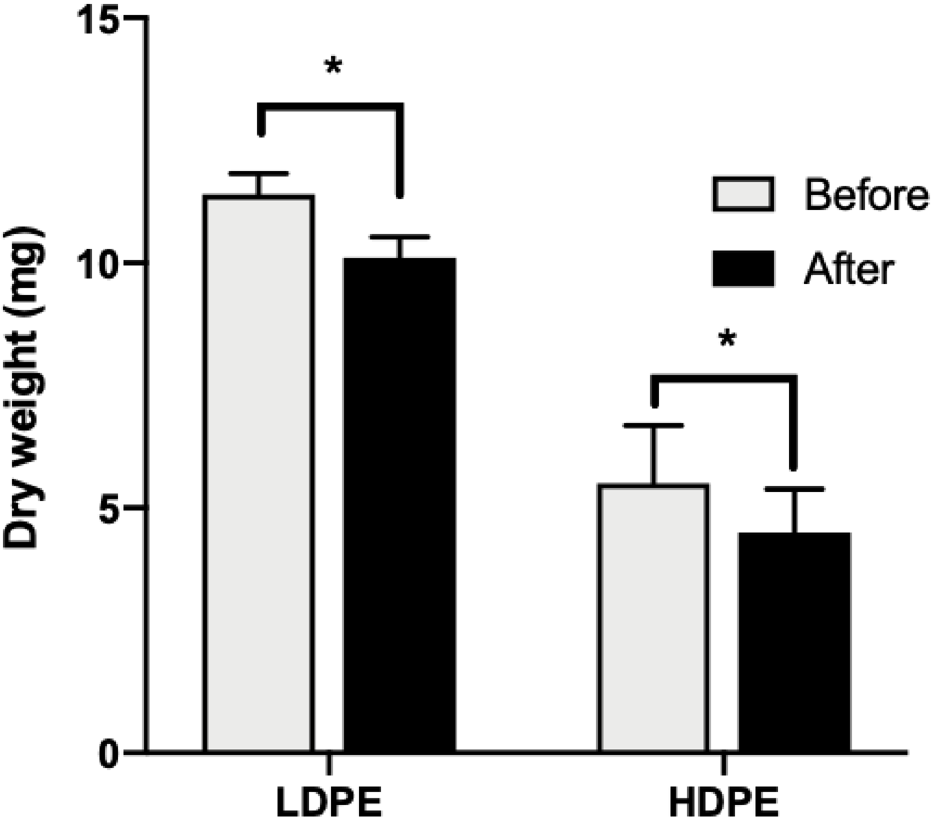

Figure 3. Average dry weight significantly decreased for both LDPE (M=11.4 mg, SD=1.35 to M=10.1 mg, SD=1.37) and HDPE M=5.5 mg, SD= 2.89 to M=4.5 mg, SD= 2.17) after the microcosm incubation. As controls PE fragments sterilized and UV-treated but not incubated were used. The * indicates significant differences of p=.0133 and p=.0409 for LDPE and HDPE, respectively.

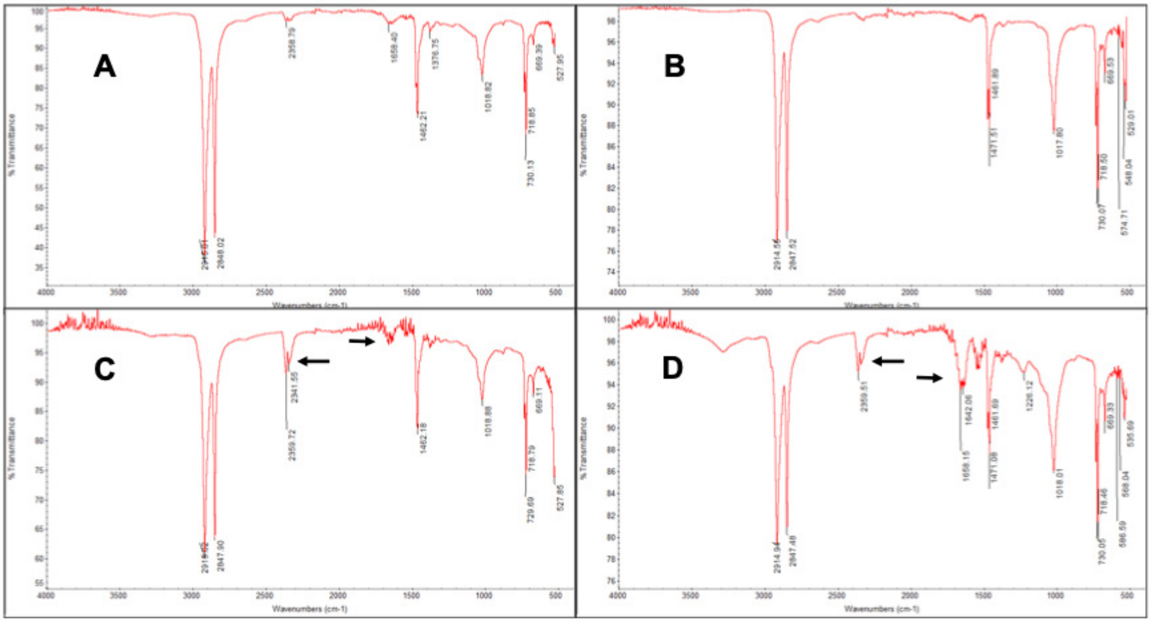

Figure 4. Representative FTIR profiles of LDPE (A-before, C-after) and HDPE (B-before, D-after). Distinct peaks corresponding to alkynes and double bonds are highlighted with arrows.

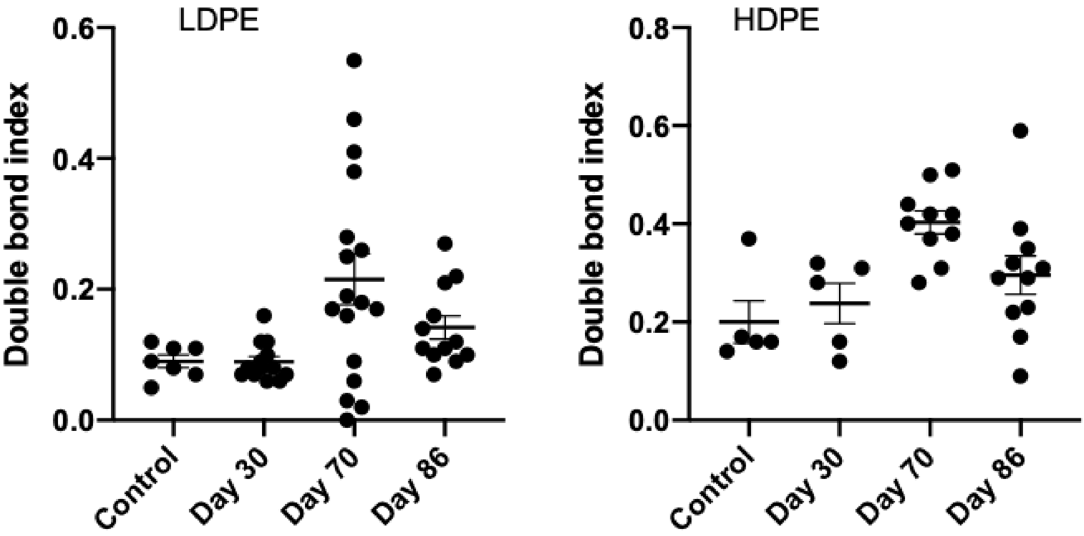

Figure 5. Double bond index values calculated from the samples of the three microcosm experiments using FTIR.

genes for enzymes that may be involved in plastic degradation (12). However, interest for the isolation and characterization of microbes using culture-dependent approaches remains, particularly for marine organisms (39).

A microcosm is a miniature, controlled environment (40) that can act as proxy of complex populations (30, 41). In this study, it served as a "low-tech" approach to select microbial entities with the potential to degrade plastic from a complex biomass. Results showed presence of microbial populations capable of degrading LDPE and HDPE as assessed by decrease in dry weight and chemical changes detected with FTIR.

Previous studies have shown chemical signs of degradation in PE and polypropylene samples deployed in coastal waters (26, 42–44). The microcosm approach of this article was developed to promote the potential biodegradation process in a controlled environment while limiting abiotic processes such as weathering and photodegradation. The setup was conducive to microbial growth, which could be observed as increased turbidity, especially after the addition of paraffin oil to the synthetic medium. Paraffin oil has been described to increase microbial attachment and formation of a biofilm, which ultimately results in enhanced degradation (17). Due to evaporation of culture media in some samples, measurement of absorbance of the culture was deemed not reliable, an aspect that should be improved in future experiments.

Field experiments are notorious for the influence of factors outside the control of researchers. The first set of the microcosm experiments had a microbial source resulting from an incubation in the ocean for 33 days, and itself had a duration of 32 days. Based on the literature and our data showing minimal changes in the FTIR spectra, a decision was made to test microbial sources resulting from laboratory incubations that could be kept for longer times. These incubations were completed at controlled temperatures in large carboys containing ocean water. Sets #2 and #3 of the microcosm experiments, using the laboratory microbial sources, exhibited more growth and stronger signs of degradation according to FTIR, which seems related to the length of the original incubation. It has been described previously that plastic biodegradation is an extremely slow process (9), as also evidenced by our results. While clear FTIR peaks indicative of oxidation were observed after more than 2 months incubation, LDPE and HDPE lost only an average of 11% and 18% of weight, respectively.

FTIR spectroscopy is a commonly used analytical tool to detect changes in chemical composition. Formation of new functional groups as well as the disappearance of others are indicative of significant chemical changes (18). Our results are in agreement with previous authors' findings that show signs of PE oxidation as a result of biodegradation. Interestingly, we did not observe a defined carbonyl stretch peak at 1712 cm^{-1}, a typical finding in UV-treated PE, which is often used as a measure of photodegradation (45). However, the presence of double bonds increased, as shown by both a larger peak in the range of 1640-1680 cm^{-1} and the concurrent changes in double bond index, particularly for HDPE at day 70. This could be explained by microbial activity attacking the carbonyl groups generated by abiotic factors, resulting in unsaturated chains (35).

The fact that only HDPE from the second (70-day) set showed a significant difference compared to the control, while the 86-day third set HDPE did not, is intriguing. However, the second set's microbial source originated from a 153 day incubation, more than double the length of the third set experiment's source (70 days). Enrichment of PE-degrading microbes may have already taken place in the source material of the second set. Previous FTIR analyses of ocean-incubated samples have shown that signs of degradation appear first for HDPE and later for LDPE (manuscript in preparation). If this is due to chemical differences in HDPE and LDPE, or to different physical characteristics is not yet known.

The FTIR results highlight some of the limitations of this study, which are standardization and repeatability. As a side project to a field-based educational project that is highly dependent on weather conditions and logistical issues, these initial samples had microbial sources with variable incubation conditions. However, overall the results seem to suggest that 1) laboratory samples to generate microbial sources are as effective as field samples, and 2) length of the original incubation is a paramount factor.

Metagenomic characterization of the biomass and surrounding water is ongoing. Preliminary results indicate an extensive overlap between plastic and water, which shifts over time; as well as a high prevalence of unknown taxa (Dr. R. Simmons, personal communication). Another ongoing study uses culture-dependent methods by swabbing media plates with the biomass to characterize the resulting colonies via 16S PCR (26).

Future experiments will continue the microcosm approach to enrich in plastic-degrading bacteria using longer incubation times to achieve even higher degradation rates. To isolate and characterize the microbes responsible for degrading PE, standard microbiology dilution techniques combined with selective media will be used, similar to the approach used to isolate a PET-degrading bacterium (46). We plan to use metagenomic sequencing of the original microbial sources and the microcosm cultures to characterize changes in populations. In addition, whole genome sequencing of the population may provide clues to the enzymes involved in the degradation pathway.

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Opinions and attitudes of Serbian high school students regarding usage of antibiotics

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Abstract

Proper usage of antibiotics is a rather important subject that is often overlooked in secondary education, which can provoke misconceptions among youth towards this important topic. The aim of the study was to examine the knowledge and habits regarding the usage of antibiotics in high school population. The research hypothesized that the students would have an acceptable level of basic knowledge on antibiotics and their usage, as students from all study programs are taught equally about antibiotics. The research also examined whether parents' affiliation in healthcare influences their knowledge.

The study has discovered that most of the students have an acceptable knowledge on the matter, with 59% being acquainted with the antibiotics' mechanism of functioning. The largest part (78%) was able to distinguish *Paracetamol* from antibiotics. However, as much as 41% identified that they have taken antibiotics from different time than prescribed and 31% admitted to having used antibiotics to treat viruses. A majority have identified their doctor as their source for advice about treatment (67%).

The research shows that students' knowledge is generally constant among the groups, regardless of the study course or parents' affiliation in healthcare. The only exception is that more students with parents' working in healthcare have taken antibiotics for the time prescribed ($p<0.05$).

Overall, students have demonstrated good theoretical knowledge with habits that should be improved. Students need to be better acquainted with guidelines of proper usage through informative campaigns and school projects.

Introduction

Clinical effectiveness of antibiotics is directly dependent on the manner of their usage [2, 5, 42]. Any improper, irrational use of antibiotics could lead to the treatment failure, adverse side effects, or development of antimicrobial resistance [5, 13, 19, 20, 40, 42]. According to the World Health Organization (WHO), resistance to antibacterial/viral/parasitic/fungal drugs occurs when microorganisms change in ways that render the medication used to cure the infections they cause ineffective [39]. During the first 20 years after Fleming's discovery of penicillin (1928), followed by the discovery of several other classes of antibiotics, it seemed that bacteria-induced diseases were defeated. Unfortunately, since then, almost every type of bacterium had developed resistance to one or more antibiotics in clinical use [3, 7, 30], *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae* being the most reported [14]. It has been estimated that antimicrobial resistance causes 700 000 deaths per year; by 2050 this number may rise to 10 000 000 deaths per year [29]. Bacterial resistance to antibiotics is a global health emergency [33], since there are more and more infections that become harder or even impossible to treat. As in the pre-antibiotic era, the most common infections and minor injuries could become lethal again [3, 11].

Antibiotic misuse can be the consequence of physicians' error, like over-prescription [3]. It can also be associated with consumers' drug use habits and their insufficient/incorrect knowledge of antibiotics indications/contraindications, storage, dosage, possible side effects and duration of therapy [6, 18, 42]. Taking antibiotics without a prescription (self-medication), together with over-prescription, leads to increased antibiotic consumption in the community, which has been positively associated with bacterial resistance [7]. The association is particularly strong in South European countries, including Serbia, where antibiotic consumption is higher than in Northern Europe [7, 30]. Strict guidelines for prescription and antibiotic use resulted in lowest level of resistance in Scandinavian countries, UK, and the Netherlands [4, 18, 19, 21, 36]. Belief that antibiotics can prevent and cure any disease, that all types of infection (including viral or fungal) should be treated with antibiotics, that they can be effective as antipyretics, antitussics, painkillers, are common misconceptions that lead to the development of antibacterial resistance and, consequently, increased morbidity, duration of treatment, side effects, even premature mortality [5, 6, 9, 10, 15, 22, 23, 26, 27, 31, 40].

These worrying data related to antibiotics usage have been the motivation for the conduction of this research, which has encompassed a sample of 130 students of different age groups within a Serbian high school located in the country's third-largest city that features a specialized clinical center [38]. The study's main objective was to examine the overall knowledge, beliefs and habits of Serbian high school students from the sample regarding antibiotics usage. The secondary objective of the study was to determine the influence of the study course as well as the students' family background (i.e. if a person engaged in a medical profession is present in the family) on the students' overall knowledge of antibiotics.

Currently, in the high school education program in the country of study, antibiotics are briefly mentioned in the Chemistry course in the fourth year of the course of study as drugs used to treat common infections of bacterial origin (population of 17 to 18 years of age) [34]. At the time of the study, no participants had completed this chapter. Similar cases of only a small part of the curricula devoted to study of antibiotics have been reported in other European countries, such as Portugal [5]. However, the base of Biology courses in Serbian high schools include a chapter on Bacteria and Viruses, which also includes parts devoted to usage and functioning mechanism of antibiotics. Information reported in this chapter include the division of bacteria into Gram-positive and Gram-negative species. Some examples of bacterial diseases are cited along with the bacterium that causes it, and a short passage is devoted to the advice about treatment of bacterial diseases, emphasizing the importance of medical guidance. In some textbooks [8], a particularly strong emphasis is put on the fact that antibiotics cannot be used to treat viral diseases. During the course of this study, all the participants have already completed the study of this chapter, as it is studied in the first semester of the first year of high school. Therefore, we hypothesize that Serbian high school population will demonstrate knowledge of clear distinction between the two types of diseases, unlike some comparable groups in the region. In addition, we expect that students will demonstrate an acceptable level of basic knowledge on the matter, which would include basic understanding of mechanism of functioning, dosing regimens and importance of medical advice when antibiotics are used.

The importance of the study lies in the fact that the studied age group represents a sample of a future main antibiotic-consumer population. Assessing their current knowledge helps determine their capacity to use the medication in a way that is acceptable for both them and their future generation. Furthermore, the analyzed data on antibiotics usage undoubtedly examines educational system’s attitude towards the very important topic of antibiotics usage. The revealing of possible misconceptions and bad habits regarding the use of antibiotics would enable to update the school curriculum, devise appropriate awareness campaigns and therefore ameliorate the students’ knowledge on such an important topic.

Materials and methods

Prior to any data gathering, the study was approved by the school board of the public high school in Serbia where the study was performed. The main method for gathering data was conducting a questionnaire among the surveyed parties. Students taking part in the survey were between 15 and 18 years of age. In order to comply with legislative norms of the country where the study was performed, all the students under the legal age of 18 were given consent forms for parents to fill in. By signing these forms, parents gave informed consent for their children to participate in the survey by answering the enclosed questionnaire form, and for this information to be processed and studied for research purposes. Students of legal age have signed the consent themselves. Participation in the study was completely voluntary and included no compensation or reward for participating parties.

Participating students were chosen randomly based on the envelope method for each grade of high school. Total number of participants was 130. Class names were inserted in envelopes that were divided into groups in reference to the study course. From each grade and study course, one envelope was chosen, to give a total number of eight surveyed classes. One class was not surveyed due to a low number of students in the class.

Prior to taking the survey, students were asked whether they have ever taken antibiotics, to ensure that students would be able to give answers to the survey. Surveys were answered in a classroom setting. Language of the survey was Serbian.

All the questions referred to the habits of students regarding recent antibiotic usage throughout their

Table 1. *Yes-no questions used in questionnaire*

No	*Question*	*Possible Answer*
1.	Have you ever taken an antibiotic for a longer or shorter time period than prescribed?	Yes/No
2.	Do you consume alcohol when using antibiotics? *	Yes/No
3.	Does any member of your family nucleus work in a healthcare institution?	Yes/No
4.	Have you ever used antibiotics when a virus causes your disease, such as in case of common flu and cold?	Yes/No
5.	Have you ever acquired antibiotics without prescription?	Yes/No
6.	After you consume an antibiotic, do you feel calmer?	Yes/No
7.	Is the Paracetamol** antibiotic?	Yes/No

* Legal age in Serbia for acquiring both cigarettes and alcohol is 18 years of age. However, it is not uncommon that younger individuals have access to these substances.

** *Paracetamol* is a commonly used analgesic or antipyretic drug in Serbia, whose USAN is acetaminophen. This drug is commonly marketed in the USA as **Tylenol**.

Table 2. *Assesment of agreement questions*

No	*Question*	*Possible Answer*
1.	I use antibiotics often on a monthly level for repeated treatments.	1-2-3-4-5
2.	I am well acquainted with antibiotics' method of function.	1-2-3-4-5
3.	I often miss my doses when taking an antibiotic.	1-2-3-4-5
4.	Antibiotics can help us with most diseases (headache, fever, hypertension, flu, etc.)	1-2-3-4-5
5.	Name all the applicable advisors for your treatment when ill:	*Parents in healthcare* *Parents not in healthcare* *Friends* *Media/internet* *Official doctor*

high school studies. The questionnaire distributed among the groups included two main question groups: simple questions requesting Yes/No answers, and questions in which the surveyed individual was to express the level of consent with the phrase given by answering from 1 to 5 on the Likert rating scale, with 5 expressing highest level of agreement. Furthermore, the questionnaire, although fully anonymous, included personal questions that would help process the data among groups for more effective analysis. Design of the questionnaire was based on many previous studies that have encompassed interviewees from different backgrounds [1, 5, 9, 15, 21, 29, 36, and 40].

First question group: yes-no answer

The first question group was designed to be simple in appearance so that quantitative data can be easily obtained. The questions are listed in the Table 1. Questions were designed in a manner so that they can mostly assess multiple points of interest at the same time. Furthermore, the form of the question was laid out in a manner so that it does not in any way resemble a knowledge test, but rather a survey. This was performed so that students do not put much thinking when giving their answer, thus altering the truth in order for their answers to seem more plausible.

Second question group: assessment of agreement – Likert Rating Scale

In the second group of questions, students were asked to assess how much they agree with a given sentence linked to antibiotics usage. These questions were designed with the same idea as the questions in the group one, with an aim to provide slightly more in-depth information about students' habits about antibiotics' usage. The possible answers were numbered from 1 to 5, with number representing levels of agreement, such as: 1 – *I do not agree at all*, 2 – *I mostly do not agree*, 3 – *Unsure/Don't know*, 4 – *I partially agree*, 5 – *I agree completely*, as per the five-degree Likert rating scale. The questions are enumerated in the *Table 2.*

Statistical methods

In order to better assess the results of the questionnaire, all results have been quantified, first represented by the number of students who gave a particular answer to the question, then by percentile, showing the percentage of a particular response in the overall population of students. For the questions where a scale of answers was expected, differently to the simple Yes/No questions, a mode was determined in order to show the most frequently chosen option, similarly to a study conducted on awareness of antibiotics in Norway [36]. In order to determine the statistical importance of data obtained and its connection to the studied factor, a common $\chi 2$-test for independence (Chi-squared test) was used where value $p<0.05$ was considered significant.

Results

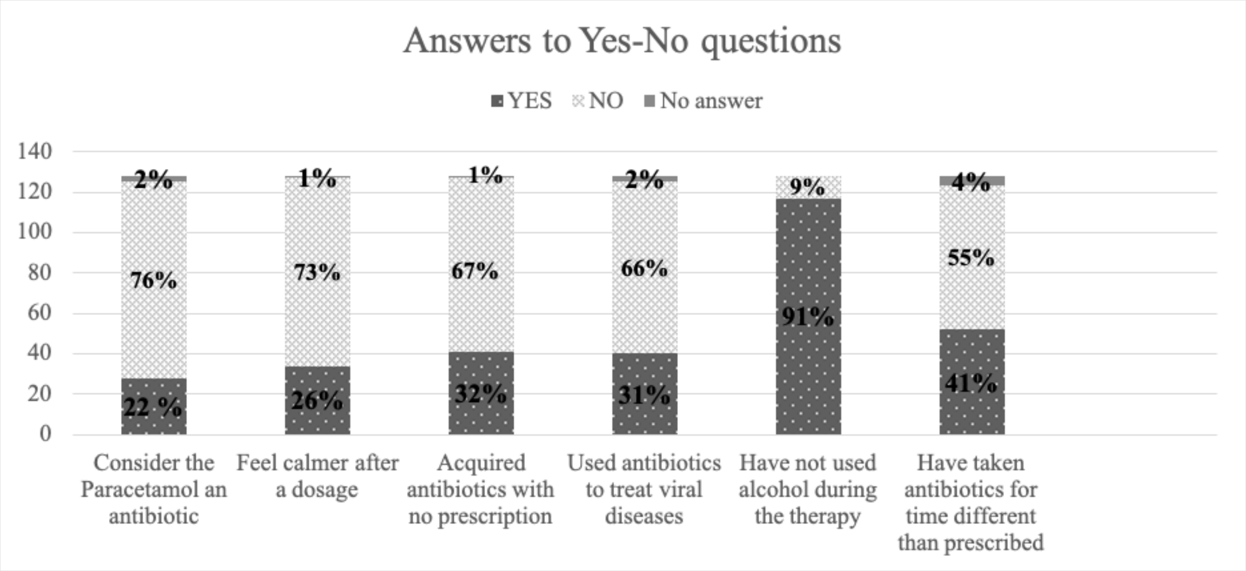
The study was conducted encompassing 130 participants, who have been offered a questionnaire to fill in regarding their habits and knowledge of antibiotics usage. Of 130 participants asked, 128 participants have completed the questionnaire form as they have stated they had previously used antibiotics. As to comply with the legislative norms, all participants aged under 18, 72 of them (56%), provided a parental consent, or a self-signed form if over 18 years of age. The information about the participants of the survey is provided within *Table 3*. Participants in survey are students of the *Science and mathematics* study course (62%), *Languages and*

Table 3. *The number of surveyed individuals*

	Number of students	***Percentage in total population***
All surveyed	128	100%
Students with a medical profession present in the family nucleus	69	54%
Students with no medical profession present in the family nucleus	59	46%
Students of the *Sciences and mathematics* study course	80	62%
Students of the *Language and Humanities* study course	43	34%
Not affiliated in a distinct study course	5	4%

Participants in survey are students` of the *Science and mathematics* study course (62%), *Languages and Humanities* study course (34%) or not affiliated in any of the two main courses of study (4%). Overall, the participants of the study are equally distributed in respect to the presence of member of the family affiliated in a medical profession (54% who have compared to 46% who do not).

Figure 1. *Answers to Yes-No questions*



Humanities study course (34%) or not affiliated in any of the two main courses of study (4%). Overall, the participants of the study are equally distributed in respect to the presence of member of the family affiliated in a medical profession (54% who have compared to 46% who do not).

As seen in the ***Figure 1***, most of the participants of the study are aware of the risks of alcohol usage under a therapy including antibiotics (91%). It is worth noting that of all the participants, 63 (48%) were of the legal drinking age, which is over 18. Only 26% have stated a calming effect of antibiotics. Furthermore, only 22% have incorrectly classified *Paracetamol* as antibiotic. However, as much as 31% have tried to treat viral diseases using antibiotics and as much as 32% have admitted to acquiring antibiotics without an official prescription. Moreover, 41% identified to have taken antibiotics for different time than prescribed.

As seen in the ***Figure 2***, only 18 participants (14%) have admitted to a frequent monthly usage of antibiotics (Mode: 1 – completely disagree). No other question has, however, given such a polarizing answer, where as much as 46% (n=59) of population expressed doubt towards the regularity of their doses (Mode 2 – Partially disagree). Most of the population claims they are aware of the functioning mechanisms of antibiotics (59%, n=76, express a high level of certainty (answers 4, 5; mode 4 – Partially agree), which is slightly opposed to the given opinions whether the antibiotics can be used against most common everyday diseases, such as headaches, high body temperature, hypertension, flu etc. (mode 3 – Unsure/Don't Know, 38%, n=48).

Figure 3 represents the answers to the question asking who the participants' choice is when it comes to health and disease treatment advice. For these questions, participants were asked to select all applicable answers, giving a total of 163 answers. The majority of the surveyed population answered *Official doctor* (86/128 participants or 67%). The next most frequent answer was *Family* (69/128 participants or 54%). Of this number, only slightly more numerous are participants advised by parents not working in healthcare (38/128 participants, 30% of total population or 55% within the answer *Family*). A small number of participants also identified Friends as a source of information and advice (2% of the total population) and *Internet or Media* (3% of the total population).

This study also aimed to compare the answers of participants who have and who do not have a family member affiliated to medical profession, as well as answers of participants following different study courses. For a difference in answers to be considered statistically significant the *p* value must be less than 0.05. No statistically significant difference has been detected between the groups following different study courses ($p>0.05$ constantly) and almost no statistically significant difference was discovered between the groups with a different family composition regarding medical professionals. A statistically significant difference was discovered, however ($p=.045$, $p<0.05$) in answering question about taking the antibiotic for shorter or longer periods than the prescribed time. In the group of students with medical professionals in the family, 23 out of 69 respondents answered that they have done so (35%), while in the group of students without medical professionals in the family, 30 out of 59 students answered affirmatively (52%) with a larger obedience to the therapy period detected in the group whose family members are affiliated to the medical industry.

Discussion

This study aimed to examine the habits of antibiotic

Figure 2. *Answers to questions with assessment of agreement*

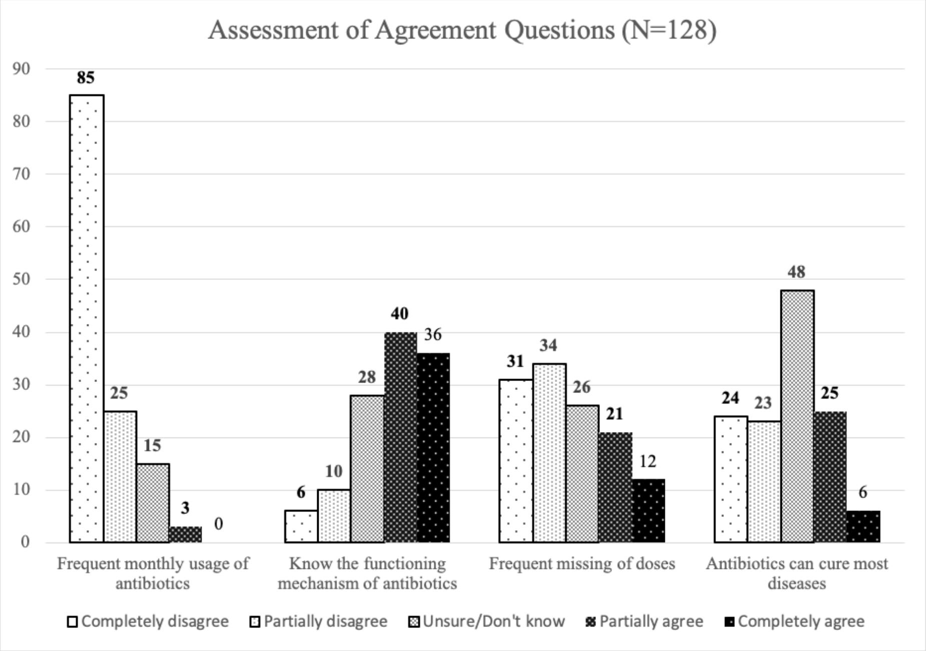
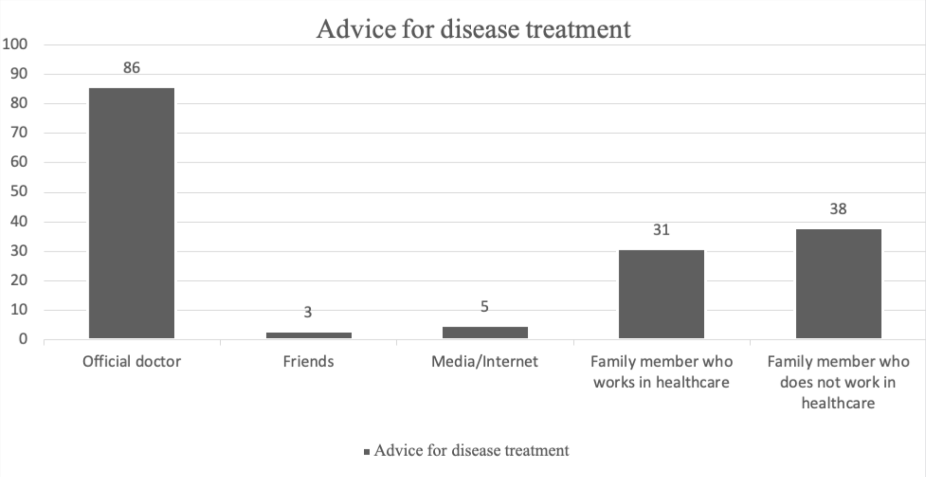


Figure 3. *Sources of advice for disease treatment*



use among students and the knowledge they possess. Furthermore, we wanted to explain the obtained results as well as compare them to similar studies done around the world. Nevertheless, to our knowledge, our study is one of the rare few that bases itself on responses of high-school students [12]. By studying these results, we would be able to observe if there is a widespread problem of misuse and misunderstanding of antibiotics among students that would bring into question the way we currently approach the subject. The results show whether a campaign is necessary to improve students' knowledge on the subject.

The questions were meant to assess: (a) students' knowledge of antibiotics functioning mechanism, (b) students' habits when antibiotics usage is concerned, (c) students' understanding of effects of improper antibiotics usage and (d) students' capacity to distinguish antibiotics from some commonly used medicines. The questions have been designed in compliance with some previous studies in Indonesia [40], Portugal [5], Italy [21] and Oman [15]. The following Table 4 enumerates all the questions in the study with the points that they were meant to assess.

The questionnaire included the usual questions regarding the acquisition of antibiotics, the implementation of a dosing regimen and the usage of alcohol. The question about members of the family nucleus working in health institutions would help assess whether a presence of a medical professional would in any case contribute to a more controlled usage of antibiotics.

Other questions were added in reference to the particular mood regarding usage of medication in Serbia. Being aware of the existence of a belief that antibiotics are extraordinary medicine, able to prevent and cure any disease [40] (representing a "magic bullet", as put by the famous German Nobel prize winner P. Ehrlich), we wanted to examine whether the students would feel calmer believing the antibiotics are bound to help them cure the disease. In reference to this belief, a question about using antibiotics to treat viral diseases was also added. It has been reported that a common practice exists to use additional self-medication even after receiving an official prescription from a medical doctor [9, 15, and 40]. A large study encompassing several developing countries by Pechere et al [27] has also reported that antibiotics have been used to treat viral diseases, which generally include common flu and cold. This is why the students were asked if they had used antibiotics to treat a diagnosed viral disease. A similar tendency was observed in a study in Oman [15] and Indonesia [40], where the latter reported a very high rate of 74% of surveyed parties believing antibiotics are useful in combat against any disease.

Furthermore, the question about Paracetamol was included as this is one of the most commonly used analgoantipyretic drug in Serbia, especially for treating young children. This is one of the medicines that students are most acquainted with from early childhood, and this is why it was important to assess their knowledge on its nature, since some studies reported that antibiotics are being used as antipyretic, antitussives or analgesic drugs [9, 15].

Students from Serbia have demonstrated an acceptable knowledge of antibiotics, as seen in Figure 1. This is similar to the studies on the general public in Indonesia [40] and Norway [36], and unlike studies in general public in Kosovo [41] and a study encompassing Chinese university students [37], which has reported a massive cross-country misuse. A majority of students from our sample (73%) do not report feeling calmer after taking them and do not consume antibiotics on their own (67%). Of the

Table 4. *Study questions with their points*

Statement / Question	Points	*Statement / Question*	Points	*Statement / Question*	Points
Does any member of your family nucleus work in a healthcare institution?	A, C	Have you ever taken an antibiotic for a longer or shorter time period than prescribed?	A, B, C	Do you consume alcohol when using antibiotics?	B, C
After you consume an antibiotic, do you feel calmer?	B, D	Have you ever used antibiotics when a virus causes your disease, such as in case of common flu and cold?	A, B	Have you ever acquired antibiotics without prescription?	B
I am well acquainted with antibiotics' method of function.	A, C	Is the *Paracetamol* an antibiotic?	D	I use antibiotics often on a monthly level.	B, C
Name all the applicable advisors for your treatment when ill.	B	I often miss my doses when taking an antibiotic.	B	Antibiotics can help us with most diseases.	A, C, D

Key. *A – Students' knowledge on antibiotics' mechanism, B – students' habits on usage, C – students' understanding of effects of improper usage, D – students' capacity to distinguish antibiotics from other drugs.*

surveyed students, 78% were able to differentiate analgoantipyretic Paracetamol – acetaminophen from antibiotics, displaying their knowledge of the most commonly used pharmaceutical drug in Serbia that can be acquired without an official prescription. Among surveyed students, 91% do not consume alcohol during treatment. A study conducted in a Serbian hospital [16] reported that 90% of underaged high school students have tried/occasionally used alcohol, with 52.5% stating that they have consumed alcohol in the 30 days preceding the study. Taking these numbers into account, our results could demonstrate awareness of the effects of alcohol usage during treatment of illnesses. In case of certain antibiotics, these include inhibition of the drug's effect or even violent physical reactions with alcohol [32]. Additionally, alcohol usage in general can cause fatigue, lack of energy, and therefore prolong recovery from the illness, as described by the National Health Service of UK (NHS) [24] and by the Mayo Clinic of USA [35].

In general, these numbers reinforce our hypothesis that students have a good basic knowledge of antibiotic use, as they are aware that antibiotics have no calming or analgoantipyretic effect and that alcohol should not be consumed during treatment.

However, they seem to be less aware of the fact that antibiotics are inefficient in treatment of viral infections since 31% of them reported that they had used antibiotics to treat viral diseases. This would indicate that, contrary to our hypothesis, a large number of students are still unaware that antibiotics are not helpful against viruses. In addition, a worrying 41% has reported tampering with the dosing regimen. So, despite their acceptable level of theoretical knowledge, it seems that the students do not apply it to their habits when it comes to consumption of the drugs.

As seen in the Figure 2, 59% express a high level of certainty when it comes to understanding the effects of antibiotic usage, similarly to a study conducted in Indonesia [40]. It seems that the most students consider themselves to be well informed about antibiotics and their function. This is in agreement with the result that only 5% agree completely with the statement that antibiotics cure most diseases. Figure 2 indicates that with only 14% of students reporting frequent usage of antibiotics on a monthly level, it is safe to assume that the overuse of antibiotics is not a major concern. What should be concerning, however, is the fact that almost a half of surveyed students were not aware of the importance of the adherence to dosing regimens. This coincides with the results of a study performed in China in a similar age group where half of the questioned university students admitted ceasing treatment 1-2 days after clinical improvement, without finishing the full course of treatment [17]. It is worth noting that antibiotics often display the desired effect before the entire treatment is complete, as discussed by Bin Zaman et al [42] in reference to the antibiotic resistance, and it is also highlighted in the guidelines by the NHS [25].

Figure 3 shows that 67% of students address their family doctor for medication, but 54% also report that they consult their parents, with no significant difference between those who are and are not affiliated in the healthcare industry. These figures might be explained by students' belief that some everyday ailments are not worth going to the doctor for, and that their family members have enough knowledge and experience to help them. Similar findings were reported in studies done in Turkey [2], Italy [21] and Cameroon [9] while in Kosovo only 0.6% consulted family members [41]. Furthermore, with friends and media/the internet being reported as 2% and 3% respectively, it is clear that students do not consider these sources as relevant when looking for advice concerning treatment. This has reinforced our belief that the education and the family nucleus remain as the most prominent sources of knowledge on antibiotics usage of the surveyed students.

The disparity among the answers of students with and without a family nucleus member affiliated in the healthcare industry was insignificant for most questions. The only significant difference found refers to the adherence to dosing regimen. Students who have a family nucleus member in healthcare have reported fewer disruptions of the prescribed regimen, probably because they were warned (by a family member) that in order to obtain the complete effect of the prescribed medication, the full dose must be taken [1].

It is worth noting that no dedicated health class exists in Serbia. Thus, the only knowledge of the subject that the surveyed students could have acquired through their school studies would come from biology classes, where antibiotics are relatively scarcely covered, [8, 34]. Even though the study course of science-mathematics is oriented towards life sciences and chemistry more than the language-humanities course in terms of time devoted to these subjects, the differences between responses across all questions were minimal. This is likely due to the fact that antibiotics, unlike most topics related to science, are covered equally by the two courses. The subject of bacteria and viruses is studied during first year of biology [8] where the program is identical for both language-humanities and science-mathematics courses. In the time of conducting the study, all the participants had already completed the study of this chapter. Even though the science-mathematics course is geared more towards medicine or pharmacology, these fields are never directly studied based on the official textbook [34].

A group of similar studies on high school students' knowledge on the subject of antibiotics has been conducted in Portugal [4, 5], where the topic is covered once during the biology course. The surveyed students from Serbia have demonstrated an overall better level understanding of the subject of antibiotics and their use than their peers despite the overall similar distribution of the topic in the two curricula. Compared to a large study done on university students in China, the surveyed students in Serbia were significantly less likely to buy antibiotics without a prescription – 3/10 compared to 7/10, as well as tending to consult their doctor more often compared to the university students in China [28].

Conclusion

The results of the study lead to the conclusion that a majority of interviewed high school students understand the basic properties and use of antibiotics. They are able to tell apart the other commonly used analgopyretic Paracetamol from antibiotics and do not consume alcohol during treatment. A majority go to their doctor for advice and prescription. However, many also choose to seek advice from their family, regardless of whether the family member is affiliated with the healthcare industry. The research has shown that a premature interruption of prescribed treatment is common, however it is less frequent among those students whose family is in healthcare as compared to others. Therefore, students need to be better acquainted with the subject through informative campaigns and school projects.

Conflict of interest

The authors have no conflict of interest to declare.

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Undergraduate Student Perspective

A Pre-Med Graduate's Gap Year of Community Immersion: Finding a Greater Purpose

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I. Introduction

My eyes startle awake at the sound of my first alarm. 6:05 a.m. The sky is still dark, but I can already hear the lively bustle of my neighborhood on their way to work below my apartment in Central Los Angeles. I begrudgingly get myself out of bed and prepare for a full day of school. This is a familiar routine. Except this time, I'm not going as a student but as an educator.

Some people seem to have absolute clarity as to what careers they want to pursue while others may feel lost in the sea of possibilities. I belonged to the latter group. While I didn't know the exact passions I wanted to pursue, growing up, I knew the things that I absolutely did NOT want to do – and that was anything involving math and/or science. After barely scraping past basic high school chemistry and biology, I was relieved to know that once I entered college I would not have to meet those formidable foes again. As a lover of music, humanities, and the arts, I imagined that my life and career would be separated from science, which I thought was limited to the world of test tubes, pipettes, and moles. However, I was in for a surprise.

Having a close family member with a severe mental illness, I entered college with some interest in finding a field that would make mental health care more accessible given the extreme shortage of services in my area. In my freshman year, through a mandatory career planning class for psychology majors, I discovered the field of psychiatry. Its ability to holistically treat patients with both psychotherapy and medication immediately piqued my interest, and it became a field I wanted to explore. After realizing that this career would require a medical degree, despite my earlier aversion to science, I decided to take an introductory chemistry course and see how I fared. I was surprised to find that I not only was capable of learning science, but that it also was a field that sparked curiosity and excited me.

By my senior year, I had completed all the necessary coursework and testing to apply for medical school. However, having followed the tight trajectory of a pre-medical track, I felt the need for some space to seriously reflect on this career path, while also permitting myself to explore other interests. Instead of jumping into a medical or graduate program immediately, I decided to take at least one gap year working. While the pressure to start my career was something that initially frightened me, this gap year has been the best gift of time for reflecting, exploring new hobbies, diversifying my professional skill set, and gaining clarity and motivation for a future career in healthcare.

II. A Day in the Life of my City Year

I currently work in the sunny city of Los Angeles with an educational non-profit called City Year. In this national Americorps-sponsored program, thousands of volunteers are placed in high-need public schools across the country to serve as near-peer mentors for students in elementary, middle, and high schools. I chose to serve with City Year because I was drawn to its vision that all children have equal potential for success and deserve a more equal opportunity towards the resources and support necessary for their growth and development. Also, if I wanted to serve low-income populations in urban communities in the future, it was important that I spend time immersing myself in such a community. I am proudly serving at Belmont High School in the Westlake area, where I continuously gain inspiration from my students and am learning the importance and power of community building.

My average day at school begins promptly at 7:15 a.m. After going through the team announcements for the day, we go outside to greet students to get them excited for the school day. This is one of my favorite parts of the day because I love watching students enter school with a smile! Throughout the day we provide in-class support and plan one-on-one teaching activities to help strengthen our students' foundational skills in reading and math. While the focus of our organization is academic, we also serve to support their socio-emotional wellbeing and growth. Thus, we not only assist students with classwork and after school enrichment programs, but oftentimes, we also get to have conversations about their jobs, families, and the stress and joy of being in love for the first time. After a long day, at 5:15pm, we gather as a team to share our triumphs and difficulties of the day before parting and getting ready for the next day.

In the past eight months of service, I have had the honor of getting to know students like Pedro [name has been replaced to protect identity], a vibrant tenth grader. When we first started the year, he refused to work on any assignments during class. After spending more time with him, I learned how he has been living and working in Los Angeles without his parents for the past year. While I can't do much to change his personal situation, I try to make the classroom a welcoming environment so that he feels supported, encouraged, and accountable while at school. I am excited to say that through hours of practice spent in the classroom and after school, we both celebrated him passing all of his classes last semester.

Like Pedro, most of our students at Belmont move to Los Angeles from Central America with the hopes of creating a better life for themselves and their families. Many come here alone to this foreign country where they do not speak the language and are often not accepted by mainstream society. I often feel hesitant sharing stories of my students because I realize that these are not my stories to tell. However, Pedro is more than a story. He is a valuable member of and asset to our community. Acknowledging his history is not to gain sympathy, but rather done with the hope that there is a personal stake in very real, human lives. Taking time to immerse myself in this particular community, I am learning that we are all interconnected. As my service year continues, my goal is to remind my students of their importance and encourage them to become the best advocate for themselves. I hope to be the best cheerleader I can for my students and let them know that they have someone supporting them in their corner.

III. Seeing the Bigger Picture

During my time taking biochemistry at university, I was intrigued by some life lessons it taught me. In the study of metabolism, I learned how the human body is one of the most efficient machines in the entire universe. The interconnectivity of metabolic pathways made me realize the intricate nature of how the body works. It depends on the function of each unique part; nothing can function on its own. My brief tour of the metabolism taught me more than just enzyme names and pathways. It radically changed the way I think about treatment and health delivery. Just like our physical bodies, in order to thrive, human beings must rely on community.

I now see that effective health treatment requires more than just looking at the symptoms and genetic history. It also requires an awareness of how an individual's relationships and living conditions are connected to the community to which they belong. This interconnectivity also made me realize that when one individual struggles, the entire community shares the weight. Thus, it makes sense to acknowledge and advocate for those who may not be given equal resources to care for themselves.

Many times people who go into community service may come from places of privilege with a deficiency-centered outlook where we want to support a "struggling" neighbor. In my time of service, I have had first-hand encounters with injustices in educational practices that favor some neighborhoods while denying others with basic resources. While it is true that my school receives much less support, I am learning that our practice of labeling (e.g., use of deficit-based language like "achievement gaps") significantly undermines student potential and ignores the larger structural issues. I am convinced that how we label others determines how we will treat them. I hope that we shift towards asset-based language (e.g. "opportunity gaps"), which acknowledges the inequities while respecting the dignity of our students and the community. With this shift, I see my community as an asset rather than a burden. If one takes the time to engage, my community offers strength and beauty. When walking around my school community in Westlake, one will find that the streets are full of hardworking individuals who celebrate and share their vibrant culture and strong sense of family values. I have learned that through this year of service, I receive more than I give and am continuously growing through the support in my community.

IV. Gaining a Greater Purpose

The gap year has radically changed my outlook on a future career in healthcare in a number of unexpected ways. One of these realizations is that I need to be intentional about engaging with diverse communities. Living in Los Angeles, I have had the opportunity to be exposed and learn from multiple cultural groups every day. This has been a shift from my previous experiences living in the bubble of my college campus in Indiana. It has taught me that diversity is much more than representation. Diversity is the celebration and acknowledgement of those from all walks of life that may be completely different from my own. The beauty of seeing my students from different countries interact and connect has shown me that diversity teaches us to focus on unity and our need for each other rather than focusing on our differences.

I have also discovered more about what motivates me to do the work I do. I have often heard the phrase, "Knowledge is power." In addition to power, knowledge is also responsibility. In my case, acquiring knowledge requires me to think about how and for whom I will use it. I want to use it for community building, as I learn more about what that means. These thoughts are new realizations that I have been spurred by my unique gap year experience.

I encourage those reading to take time before you commit to a career and try a gap year. Anyone in any field or level can engage in meaningful community work. By taking a non-traditional route and working in education, I have had the opportunity to broaden my perspective and learn that the impact of what we do as researchers, educators, and life-long learners extends far past the domains of our individual sectors.

V. Acknowledgements

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Lisa Ines, my Team Leader, Sarah Perez, and my City Year teammates who have taught me the positive power of collaboration and encourage me to grow into the best mentor I can be for my students. I would also like to extend special thanks to my mother, Chin-Sook Pak, for being the best cheerleader and supporting me every step of the way of this journey. Finally, I would like to thank my beautiful students who have embraced me. Their determination to continuously challenge each day motivates me to push beyond myself and be the best advocate I can for my community.

Undergraduate Student Perspective

STEMinists: An Exploration of the Impact of Female Educators in STEM Fields

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Abstract

With a growing need for skilled STEM workers in the US, it is necessary to examine why minority groups such as women continue to participate in STEM at far lower rates. The historical exclusion of women from education created a system of STEM education that was formed for and by men. Despite changes in the education system that have allowed women to gain traction in almost every other content area, evaluation of the current status of the field reveals STEM subjects remain dominated by men. Additionally, there appears to be a new set of barriers that exclude women at all levels of STEM education. Changes to address these new barriers need to be made — and educators who are at the forefront of this occurrence have a unique opportunity to inspire change for future generations. Specifically, female STEM educators acting as mentors and role models have been shown to help female students overcome the obstacles they face in pursuing a STEM education and career. In order to fully realize the potential of female educators, the treatment of female educators in academia needs to be evaluated to ensure they are properly supported by the schools they work for.

Introduction

Within the United States, there has been a large push to increase the number of skilled Science, Technology, Education, and Mathematics (STEM) workers in order to keep up with the constant advancements being made in these fields. At the current rate, the U.S. is falling behind on the world stage in these areas (Hossain and Robinson, 2012). This lack of STEM workers is likely due to a multitude of factors, including the fact that many minorities, including women, are still represented at low proportionate rates in these fields (Burke and Mattis, 2007, pg 5). Addressing this underrepresentation is not only beneficial in increasing skilled STEM workers, but also in allowing innovation to continue in these fields by providing diverse perspectives.

The incorporation of women in STEM has had a long, complex history. It is this history and the progression into the current era that provides the key to addressing what barriers are preventing female persistence in STEM. Women have been faced with a multitude of barriers to access and success within STEM education and careers. Access to the necessary education is a particularly important factor in ensuring an individual is capable of succeeding in any field, and STEM is no exception. The first step in increasing female participation in STEM careers is ensuring they have the necessary education.

While STEM subjects are generally viewed through the lens of higher education, participation in these subjects begins much earlier. Children are likely first introduced to STEM subjects in elementary school. Developing the talent of these young students and maintaining their interests within the subject is an integral part of growing the STEM field (Hossain and Robinson, 2012). It is with this perspective that it is necessary to evaluate how science is being presented to young learners and how the presentation can be adapted to be more inclusive to girls in and outside of the classroom.

Getting girls involved in STEM from a young age is a good first step but is somewhat arbitrary if the institutions in higher education dissuade women from pursuing careers in these fields. For nearly the entire existence of higher education, women have struggled to gain equal access, especially in the fields of STEM, which were primarily established by and for men. While the outright discrimination within STEM education may not be a major factor in the lack of female participation today, the programs and institutions of colleges and universities still seem to be unwelcoming to female participation.

With all of these issues within STEM education, attention is frequently turned to educators at all levels. Educators exist to facilitate learning and in doing so have the opportunity to serve as role models and supporters to students. It is this potential and their proximity to the issues with gender in STEM that gives educators the opportunity to make a difference. By examining the evolving relationship between women, STEM education, and the role educators play, it is possible to consider options for making improvements in the future.

The Historical Role of Women in STEM

The first step in addressing the lack of women in STEM fields today is to look at past interactions. This approach allows the examination of past establishments that led to inequality, as well as an opportunity to evaluate the success or failure of any actions that may have been taken to address the issue. Historically, STEM education and employment opportunities have been dominated by men (NSB, 2018). This discrepancy has been theorized to have developed due to varying social barriers that prohibited women from being equal contributors to STEM fields. Several of the main contributors to this inequality are traditional gender roles, access to education, and access to equal employment opportunities (Bystydzieński, 2006, pg. 6). While these are not necessarily the only contributors, these factors have had a significant impact on women's ability to be accepted in STEM and, thus, need to be explored.

In order to participate in any field, there are certain requirements for entry. In some cases, those requirements may actually serve as barriers. It is necessary to have the background, knowledge, and training associated with the field. For the majority of jobs within the STEM fields, access to formal education is necessary to gain entry into the workforce. Access to higher education in these fields, like many other fields, has historically been restricted to men. Throughout the journey towards inclusivity, the argument for allowing women access to education has evolved. Initially, the role of mothers in educating their sons was the strongest argument for expanding educational opportunities to women (Solomon, 1985, pg. 2). Over time, educational opportunities, especially at the collegiate level, expanded for women, but these opportunities were frequently unequal to those available to men (Solomon, 1985 pg. 43). Colleges and universities were commonly divided by sex, with 59% of them being open only to men in 1890 (Solomon, 1985, pg. 44). This restriction to the necessary education to join STEM fields acted as one of the largest barriers to inclusion for women. From 1890 to 1928, the number of coeducational, degree-granting institutions increased from 29% to 92% (Solomon, 1985, pg. 44). This huge jump in the availability of education marked a change in attitudes about the abilities of women and their roles in society. This increase shows a marked improvement in educational opportunity for women and is credited in large part to growing feminist movements of the time.

Despite this increase in access to education, there still seemed to be large gaps between men and women in most STEM fields. Going into the 1970s, only 13% of PhDs in life sciences were awarded to females (Ceci and Williams, 2010). These shockingly low numbers show that, while the opportunity to receive the necessary education might be available, there were other factors that were holding women back from contributing fully to these fields. Opportunity varied within STEM fields as well. Bystydzienski notes that engineering schools were particularly resistant to admitting women and were structured in such a way as to be unwelcoming to the few women who did manage to get in (2006, pg. 27).

One reason women were frequently denied access to STEM education was that it was thought to interfere with the traditional roles assigned to women by society. Marriage, raising a family, and the work necessary for success in those endeavors, have traditionally been ascribed to women (Wharton, 2005, pg. 134). Because of this, men were viewed as better suited to filling positions that required a higher time commitment. Family matters were not considered to be an infringement of a man's time. The married family structure does not inherently limit women's career opportunities and, in some cases, can provide a support system for it. Alternatively, these expectations can conflict with women's abilities to commit their full time and attention to such work. Bystydzienski noted this phenomenon, stating that "research and personal memoirs also suggest how positive situations provided support and established safe havens during the sometimes tumultuous early years when a career is established, and, alternatively, how the family and community obligations that women are expected to assume could compromise or even curtail a promising career" (2006, pg. 27). All of this seems to show that women were more welcome in higher education and STEM fields as long as it did not inhibit their ability to fulfill their familial duties.

Looking at the structure of society throughout history, one of the largest barriers to women in STEM is the distinction between male and female work. There has been a longstanding belief that men and women are better suited for different jobs (Wharton, 2005, pg. 167). This institutional ideology has acted as a barrier for women entering many job fields including those associated with STEM. Circumstances such as World War I and II, however, allowed women to fill jobs left by men that they would not have had access to previously. The shift in the workplace demographic resulted in that which "has previously been viewed as quintessentially masculine were suddenly endowed with femininity and glamour for the duration" (Wharton, 2005, pg. 168). The change in the availability of jobs also shifted attitudes about jobs that were suitable for women. Wharton notes, "Masculine jobs that had been filled by men prior to the war were relabeled as appropriate for women" (2005, pg. 168). This subsequent shift in attitudes opened up new opportunities in the lives of women.

Gender cannot be assumed to be the only factor that influenced acceptance into education and, beyond that, into STEM fields. Many other minorities were restricted in their access to education and had compounding effects with the experience of women. Specifically, race has played an interesting role in its relationship with women in STEM. While women overall were kept away from work and discovery opportunities in STEM, there were even more discrepancies between women of different races and ethnicities. Award-winning journalist Rona Cherry offers more of a look into this in her book, ***Woman in the Year 2000***:

> However, in STEM fields, a lower percentage of bachelor's degrees were awarded to females than to males (36 vs. 64 percent). This pattern—in which females received higher percentages of bachelor's degrees overall but lower percentages of bachelor's degrees in STEM fields—was observed across all racial/ethnic groups. The gap between the percentage of STEM bachelor's degrees awarded to males and the percentage awarded to females was largest among White students (33 percentage points), followed by Pacific Islander (28 percentage points), Hispanic (25 percentage points), American Indian/Alaska Native (23 percentage points), Asian students (21 percentage points), and students of Two or more races (21 percentage points). Black students (11 percentage points) had the smallest gap between the percentage of STEM bachelor's degrees awarded to males and the percentage awarded to females.

While gender differences in STEM may be smaller within specific racial groups, these numbers show unequal representation across all listed groups. This is possibly influenced by the fact that household labor is generally more equally shared between men and women in non-white families, allowing for a more equal representation of women in the workforce and in STEM (Wharton, 2005, pg. 134). Additionally, with the combination of both racism and sexism, women of minority race groups were less involved in the STEM work fields and educational fields than even women of white descent (Solomon, 1985, pg. 76). Women of color had to fight even harder to be acknowledged and accepted in the world of STEM with less of a resulting outcome.

These results are contradictory to the statistics on women's pursuit of entering the workforce.

White women listed “balancing work with family responsibilities” as the number one significant issue/challenge female scientists face in their careers (Bystydzieński, pg. 75). However, African American families see more balance and equality when it comes to women entering the workforce and dividing labor between genders.

There were again significant differences that contradicted the participation in the STEM fields, according to an NELS (National Educational Longitudinal Survey) study comparing African American Females to White Females. In 1988, 59% of African American females were looking forward to science classes and a whopping 70% believed science would be useful in their future, compared to 55% and 65% of white females, respectively. However, in 1988, African Americans scored an average 45.12 on their science standardized testing versus White females’ average of 52.73, resulting in an average difference of 7.61 in scores (1990 was even greater with an average difference of 9.08, favoring white females) (NCES, n.d.).

So why is there such an extreme difference between statistics about women in terms of STEM fields and education and breaking down the statistics to different races and ethnicities of women in terms of STEM fields and education? As Bystydzieński expressed, “This [NELS study] examination suggested that simple assumptions about the mismatch between women and science are often based on the experiences of white women. In fact, in the African American community, gender is constructed in a very different way and many of the characteristics that are considered appropriate for females (e.g. high self-esteem, independence, and assertiveness, as well as high educational occupational expectations) are not inconsistent with characteristics that contribute to success in science” (2006, pg. 136).

This follows Bystydzienski’s findings of African American females not finding as high a disparity between work and family responsibilities as white females. Yet despite this desire for more equality in the STEM fields, women of color have been overlooked and ignored, even by those one would assume would be on their side. As Kimberle Crenshaw, lawyer and civil rights advocate, stated in her publication for the Stanford Law Review on intersectionality, “For example, racism as experienced by people of color who are of a particular gender - male - tends to determine the parameters of antiracist strategies, just as sexism as experienced by women who are of a particular race — white — tends to ground the women’s movement. The problem is not simply that both discourses fail women of color by not acknowledging the ‘additional’ issue of race or of patriarchy but that the discourses are often inadequate even to the discrete tasks of articulating the full dimensions of racism and sexism” (1991, pg. 1252).

Women of color have found racism amongst those of the same race or the same sex, meaning that there is even a lack of understanding and support among their peers for their desire to pursue STEM careers, let alone acknowledgment for their efforts to enter said career fields. Women of color and their voices have been erased (Crenshaw, 1991, pg. 1253).

The differences in the numbers cannot be chalked up to or blamed on different attitudes. It comes down to the availability, access, and support different women had when pursuing STEM ideas and education. Yes, white women were not given the same access to STEM fields and acknowledgment for their contributions, but even less opportunity was given to females of minority races and ethnicities. They weren't provided the example of females that looked like them, nor were they held to the same expectations as others in their teachers' eyes. Women want to learn and want to be involved more than society gives them credit for, but without access to support and opportunities, their wants are left abandoned and disregarded and their voices are left erased.

Women in STEM: The Current Picture

Historical inequality between men and women's roles in society has led to a disparity in who was capable of partaking in STEM education. This resulted in a staggering underrepresentation of women in STEM fields. Women had to push past these barriers to show that they were capable of succeeding in these fields. All of this activism has had a clear impact on the involvement of women in STEM education. The number of STEM doctorates awarded to women was six times higher in 1995 than in 1970 (Burke and Mattis, 2007, pg. 29). This marked improvement shows that some barriers that previously existed were overcome and we as a society have made great advancements towards closing this gender gap. While women have made great strides across history in efforts to improve the gender gaps in STEM education, inequality still exists. Despite an increase in collegiate attendance as a whole, women hold a disproportionately low number of undergraduate STEM degrees (Beede et al, 2011). This suggests that while traditional barriers limiting women's access to education may have been overcome or removed, there are still barriers specific to STEM fields that are inhibiting participation.

The discrepancies of female involvement are not limited to education, but rather, also carry forward into the workforce. In a study entitled "Women in STEM: A Gender Gap to Innovation," Beede et al. found that "Although women fill close to half of all jobs in the U.S. economy, they hold less than 25 percent of STEM jobs" (2011). This disparity more clearly shows that the gender gap appears more noticeable within STEM fields than in other areas. Beede et al. went on to show that the disparity in employment in STEM fields exists regardless of education level, with the doctorate level having the closest to even proportions where men are still employed at almost double the rate of women (2011). While access to the necessary education was a major contributor to this gender gap in the past, this study shows that new barriers have emerged to take the place of those that limited women previously. This drastic statistic is explained at least partially by the fact that those women that hold STEM degrees are less likely to obtain jobs within that STEM field than men (Beede et al. 2011). In order to remedy this gap, these new restrictions must be identified and novel solutions must be implemented.

Educational institutions are not the only organizations concerned about the gender discrepancy in STEM. Several private organizations have already taken steps to address the issue. In 2018, Girl Scouts of America released thirty new badges for girls ages five to eighteen to earn and two new "journey" programs for girls to participate in — all of which were STEM-related. The thirty badges are given to girls for their advocacy and exploration in topics such as cybersecurity, robotics, computer science, space exploration, and more (Kelly, 2018). Girls in elementary school can earn badges for designing their own robot, creating algorithms for computer games, creating models of the solar system, and even creating their own home experiments to represent ideas such as static electricity or density. Sylvia Acevedo, CEO of Girl Scouts of America, stated that "Girl Scouts are learning how to proactively address some of the foremost challenges of today while also building skills that will set them up for a lifetime of leadership" (Kelly, 2018). Earning these new badges encourages girls to take charge of their own STEM learning and learn skills, such as problem-solving, the scientific method, computational thinking, and how to receive feedback to expand knowledge and understanding. These badges are the first step into a lifetime of learning, leadership, and STEM appreciation.

As of 2017, 1.7 million girls across the nation are involved in Girl Scouts. For many elementary school girls, it is a rite of passage to belong to this organization — earning badges, swapping stories, creating and exploring the world with their female peers and led by female role models in their community. For over 100 years, Girl Scouts of America has focused on developing young women to be strong ambassadors for their peers, promoting self-confidence, and making the world a better place to live. So why is the addition of thirty new STEM-related badges so significant?

The numbers speak for themselves. According to the Girl Scouts of America, between the years 2012 and 2015, the interest in STEM fields has skyrocketed in their young members. 71% of female participants agree that women can be good engineers as well as 58% disagreeing with the idea that engineering is better suited for men than women. Participants in the Girls STEAM Ahead program across the country (which includes Art with the original Science, Technology, Engineering, and Mathematics acronym) have grown in their appreciation for the subjects. "Between 42% and 56% liked science more and between 39% and 42% liked math more after the program than they had at its start." As girls learn skills associated with the STEM badges and earn and participate in programs that encourage the growth of skills and knowledge, they are understanding the importance of STEM in one's daily lives. With the support of girls and women around them, they are not focused on the gender aspect of STEM careers but are focused more on whether or not they have the tools and drive to reach for STEM careers.

The Girl Scouts of America have seen an increase in interest beyond just the STEM badges and programs they now offer. Girls STEAM Ahead participants are increasingly interested in STEM careers, with 77% to 90% now wanting more information about careers in math, science, and the arts. Between 79% and 83% of those that focused on winning robotics badges are now interested in majoring in a science or engineering-related subject in college. Girls are becoming stronger leaders in their schools and communities, they are getting more involved with STEM in class, and are recognizing that STEM careers are not boring or dull, but filled with advancements, discoveries, and revelations that they could be a part of later in life. With the implementation of these new STEM badges and programs, girls are more likely to want to pursue college careers in these fields.

Girl Scouts of America isn't stopping there. They pledge to "add 2.5 million girls to the STEM pipeline by 2025" (2019). Programs aimed at young girls are helping promote interest in STEM fields. Just these past few years have seen an increase in interest due to these new badges. Girls are joining these programs for the badges but leaving with so much more: an appreciation for the work that comes with STEM as well as a realization that STEM isn't just for men anymore. Girl Scouts of America hope that these kernels of interest, intrigue, support, and fun that are found in programs related to STEM that are designed for young girls will carry with them through high school and into their future careers.

While programs such as the one offered by the Girl Scouts provide an optimistic outlook for the future of women in STEM, female involvement in STEM education tells a different story. The lack of female participation in STEM education is seen at almost all levels: more females than males opt out of science and math courses, some as early as sixth grade; fewer women than men pursue degrees in STEM fields; and higher rates of women switch out of STEM majors than men (Burke and Mattis, 2007). Thus, the interest stops shortly after entering middle school and continues to drop for the next two decades. In fact, although girls at the age of 11 ½ show an interest in STEM subjects, they begin losing interest at age 15 (Trotman, 2017). A study done by Microsoft and KRC Research found that in the Midwest, only 46% of middle school females and 46% of high school females feel powerful doing STEM despite 51% of middle school females and 62% of high school females understanding how to pursue a STEM career (Choney, 2018). And "while the U.S. Bureau of Labor Statistics predicts that technology professionals will experience the highest growth in job numbers between now and 2030, only a fraction of girls and women are likely to pursue degrees that enable them to fulfill these new jobs" (Choney, 2018). These high attrition rates could indicate several things, including a lack of motivation to pursue degrees in these fields or the presence of factors that impact the willingness of females to continue their STEM interests.

The numbers don't add up. Based on the excitement and the stories that girls shared after participation in the new GSA STEM program, there should be an increase in the number of girls seeking STEM careers. Elementary school girls express interest in STEM fields, they promise to pursue STEM-related careers in the future, and they share a belief that women fit the mold for scientists, engineers, and mathematicians as much as men. And still, the numbers are discouraging.

In regard to motivation to join these fields, this lack of participation and high attrition rates are somewhat surprising considering that women in STEM jobs earn 33% more than women in comparable non-STEM positions (Beede et al, 2011). This financial incentive to pursue an education and career in STEM would seem to suggest that more women would be following this path. It also suggests that there are other factors within these areas that are discouraging female participation. All of this culminates in STEM careers that are lacking in the potential contributions of female minds and suggests that today's society has placed new barriers to the inclusion of women within STEM fields. The attention now turns to why the numbers don't increase, but rather decrease as time continues.

The National Survey of Science and Mathematics Education (NSSME) from December 2018 provides information that might lead to an answer. Only 34% of the elementary teachers surveyed have had courses in Earth, life, and physical science. 59% have only had one or two courses in these three areas. In reference to elementary teachers' preparations for mathematics, only 7% have taken courses in the following subject areas: algebra, geometry, number and operations, probability, and statistics. The majority group, 53%, have only had courses in one or two of these subject areas — all subject areas that the National Council of Teachers of Mathematics recommends elementary mathematics teachers take in their college careers.

This is reflected in the preparedness the surveyed elementary teachers have reported in each subject. 77% report feeling very well prepared for Reading/Language Arts subject matter with Mathematics following behind with 73% describing themselves as very well prepared. However, only 31% report feeling very well prepared for teaching Science curriculum. While 58% of high school teachers feel very well prepared to develop students' conceptual understanding of science ideas, only 23% of elementary teachers feel very well prepared and only 9% of elementary teachers feel very well prepared to develop student's awareness of STEM careers. This means that elementary school-aged girls must find their intrigue in STEM-related career fields outside of the school. They are not getting proper support in developing their own understanding of STEM subjects or getting resources about STEM careers from their teachers and from their schools.

If teachers are not properly prepared for teaching STEM subjects at the elementary level, they are less likely to promote the preparation in their students. The lack of understanding when it comes to science and math leads to a lack of conceptual understanding in students and, consequently, leads to a lack of interest in these subject fields further down the line, despite continuous reports of secondary level teachers having more understanding and preparedness in their respective subjects. This could be a major source of the lack in pursuit of STEM field careers by women.

When asked how often elementary teachers spend on science and mathematics instruction, only 17% of K-3 grades taught science all/most days of the week and only 35% of 4-6 grades as well. The majority fall into the category of three or fewer days of the week. The average number of minutes spent on Reading/Language Arts was 82-89 minutes per day. Mathematics averages an hour a day and science takes less than half an hour in grades K-3. Students are clearly not getting a proportional amount of exposure to STEM subjects. This discrepancy could be caused by a multitude of factors. One probable cause is that elementary teachers are feeling unprepared or inadequately prepared for teaching science and mathematics and this is leading to a lack of focus on the subject matter in the daily curriculum.

It could, however, be more than just a lack of teacher preparedness and pursuit of STEM subjects in the classroom. There isn't just one cause and one effect at play here. Media plays a significant role in the lives of young people across the country and how women are presented in various media is a major influencer as well. How women are presented in the media, and the psychological messages it brings, could be a contributing factor.

According to Jocelyn Steinke, "A study focused specifically on television programs likely to be watched by adolescent viewers and broadcast in 2006 found... female scientists characters were outnumbered by male scientist characters by 2 to 1, appeared in fewer scenes, and were less likely to be shown as independent and dominant" (2017). Just a small depiction of women in STEM fields in media, such as television and film, feeds into the stereotypes that male STEM career holders are the heroes while female STEM career holders are less formidable. Take the award-winning television show, ***The Big Bang Theory***, which has been known to showcase one of the most diverse casts in televised scientific history. But even Dr. Amy Farrah Fowler (played by Mayim Bialik, who has a Ph.D. in neuroscience from UCLA) is seen continuously making decisions on what to sacrifice: her work or her personal life, and is often the butt of the jokes on the hit sitcom.

Media is often seen as a reflection of society, of culture, of truth, but "the lens through which we receive these images is not neutral but evinces the power and point of view of the political and economic elites who operate and focus it" (Gamson et al., 1992). It is not a reflection of the truth at all, rather what those behind the camera want the audience to believe is the truth, and their version of the truth leads to the lack of strong, independent, formidable female characters in STEM-related activities or careers. "Thus, exposure throughout childhood and into the adolescent years to media portrayals that support and perpetuate a socially constructed masculine image of these fields may elicit gender biases that directly and indirectly affect adolescent girls' views of who belongs in STEM" (Steinke, 2017).

Sixty percent of girls are more likely to pursue a career in STEM fields if they knew for certain that men and women were equally employed in these professions (Trotman, 2017). The media doesn't portray equal employment in STEM professions and, sadly, this is indeed the truth. Despite women making up half of the college-educated workforce in the United States, only 28% of women make up the science and engineering workforce, including the fields of computer science and physical science (National Girls Collaborative Project, 2018). Further surveys indicate only 9% of the 2015 mechanical engineering workforce were women (NSB, 2018). Despite the significant increase in female careers in STEM fields since the early 1990s, the numbers don't indicate equal employment rates between men and women. This doesn't bode well for the 60% of girls found in Trotman's study.

Unsurprisingly, this workforce gender disparity is seen at highly variable levels across the diverse areas of STEM. By taking a closer look at female participation in these fields, it might be easier to speculate what factors could be causing this inequality to occur. Figure 1 shows females' participation by field as of 2008 (Hill et al, 2010, pg. 14). In order to evaluate this further, these fields have been broken down into three broad categories: life science, computer science, and mathematics and engineering.

Certain STEM areas, such as those within the life sciences, have seen a marked improvement in female participation and, in some cases, approximately equal participation to men. Burke and Mattis found that roughly 53% of bachelor's degrees in Biology are awarded to females (2007). This improvement is a testament to the initiative of past movements to incorporate women into STEM and shows that equality is possible within STEM fields. Having seen the nearly equal proportion of women and men in this area, it is hardly shocking that 57% of females in STEM jobs are in life and physical sciences (Beede et al., 2011). Both of these statistics may be indicative of the importance of representation within the classroom and the field. Despite these relatively high numbers, the rates at which these degrees are being used for field-related jobs are significantly lower. One study found that "Women earned 31.3% of chemistry Ph.D. degrees between 1993 and 2003 but in 2002 were hired for only 21.5% of assistant professorships" (Ceci, Williams, and Barnett, 2009, pg. 218). These statistics show that regardless of academic achievement, there are societal and cultural factors that are impacting women's participation in STEM.

The improvements in life and physical sciences are balanced out by the acute inequality in the proportion of women in fields such as engineering and computer sciences. In the case of engineering, it is "the second-largest STEM occupational group, but only about

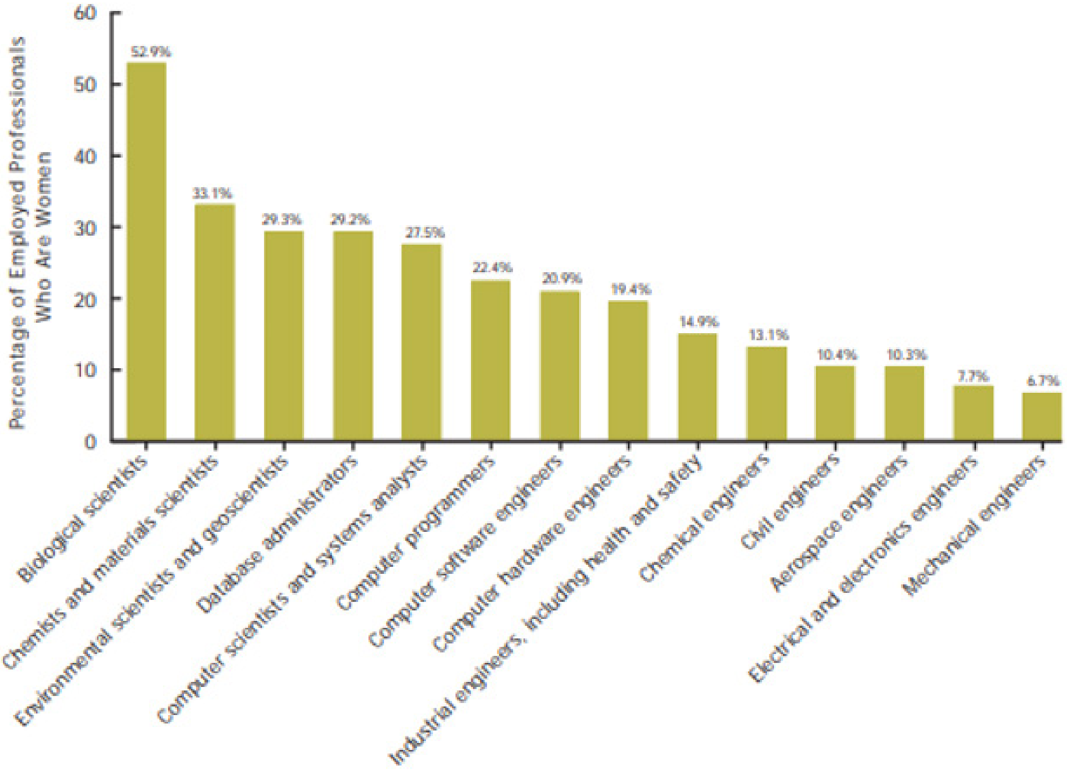

Figure 1. Women in selected STEM Occupations, 2008. All occupations are self-reported. Source: U.S. Department of Labor, Bureau of Labor Statistics, 2009, Women in the labor force: A databook (Report 1018) (Washington, DC), Table 11.

one out of every seven engineers is female" (Beede et al, 2011). One major contributor to this low rate of participation in the workforce is the gap that exists in education. Unlike life sciences, where the percentage of degrees earned is fairly equal between the sexes, only 20% of engineering degrees are earned by women (Burke and Mattis, 2007). Low rates of females earning degrees in engineering seems to indicate that the major issues limiting equal participation in this field stem more from educational systems rather than hiring or workplace institutions.

While these percentages are concerningly low, there has actually been an improvement in engineering degrees awarded to women and participation in the field. In 1970, only 1% of engineering degrees were awarded to women, and this statistic rose to 9% by 1996 (Christie et al. 2017). This continual growth of women within the field provides a basis for a positive outlook for women's participation in engineering in the future. This rise in degrees earned was accompanied by an increase in female educators in the field. In fact, by 2006, the number of female faculty in engineering programs had risen from 1% in 1976 to 16-25% (Ceci, Williams and Barnett, 2009, pg. 218). These improvements indicate that strides towards inclusion are making an impact. They may also indicate a connection between the role of diverse representation among faculty, and minority participation. Despite these vast improvements, the increases in faculty hiring are not proportional to the increase in the pool of female applicants for these positions (Ceci, Williams and Barnett, 2009, pg. 218). This mimics the same patterns that were seen in the life and physical sciences of increases in earned degrees not translating into a movement towards gender equality in hiring.

In computer sciences, female participation has actually been declining in recent years. In 1984, women represented 30% of computer science undergraduates in 1984 but that number decreased to only 20% in 1999 (Burke and Mattis, 2007). This trend does not follow with those established within the life sciences and engineering fields. This decline in female participation is not just limited to education but is seen in the job sector as well. Within the workforce, women are almost twice as likely as men to leave jobs in computer science fields (Burke and Mattis, 2007). This unusual pattern seems to indicate that the barriers to women within these fields may exist in different forms, or present themselves at different levels.

These factors indicate that while traditional barriers, such as the outright barring of women from a formal education, may not be present anymore, there is still something preventing inclusivity in these fields. There are many factors that have been proposed to have contributed to or have caused this inequality. These factors include social and cultural pressures, perceived competence, intrinsic bias, and establishments incorporated in STEM being exclusionary to women. While these factors may vary in their impact on this issue, it is necessary to consider as many contributors as possible in proposing potential solutions.

While past analysis of this gender gap in STEM has heavily considered potential biological differences, experts trying to understand the gender gap are now focusing on the impact of social pressures. Ceci, Williams, and Barnett addressed this shift in their paper "Women's Underrepresentation in Science: Sociocultural and Biological Considerations" by stating that increases in female participation in STEM fields over the past 30 years "are evidence of the strength of cultural factors in determining such outcomes, because biology has not changed over this period" (2009). Instead, it is more likely that these gender differences arise from differing expectations based on biological sex (Ceci, Williams, and Barnett, 2009). Despite the fact that biological differences between men and women have been shown to be fewer and of less magnitude than had been previously assumed, the implications of these assumptions are still present in our society (Wharton, 2005, pg. 24)

Another social factor that may impact women's desire to join STEM fields is the idea of an identity threat. This encompasses the fear of being marginalized within a field or the fear of fulfilling negative stereotypes of being in that field (Cheryan and Plaut, 2010). In this vein, just the possibility of being discriminated against or being perceived negatively acts as a deterrent to those who might otherwise be interested in joining different STEM fields. Even for women who choose to pursue this path, feelings of a social identity threat "can discourage women from persisting in these fields" (Cheryan and Plaut, 2010). Not only does this factor potentially impact enrollment rates, but it could also contribute to the higher attrition rates seen among women in STEM.

Individual cultures may also impact the achievement of women in STEM fields. Studies have found that "large cross-national variation in sex differences... suggests that culture rather than biology is involved because the observed patterns are not otherwise explicable" (Ceci, Williams, and Barnett, 2009, pg. 225). This variation between the achievement of women in STEM fields across cultures hints that this may be a factor that needs to be considered further. Ceci, Williams, and Barnett stated that all of the data regarding sex differences suggests that "culture may play a major, though poorly understood, role in creating proximal differences that lead to differences in STEM fields" (2009, pg. 226).

The institution of education is almost undeniably influenced by these societal constructions of gender. This can be seen in the application of expectations associated with various skills and predispositions. This differentiation between gender can be explained by gender polarization, which states that males and females have distinct roles and interests and the crossing of those boundaries is somehow wrong (Wharton, 2005, pg. 34). These schemas can influence the path individuals choose to pursue. Hill et al stated that "children — girls especially — develop beliefs that they cannot pursue particular occupations because they perceive them as inappropriate for their gender" (2010, pg. 22). In addition to impacting students choices, these roles and expectations can result in differential treatment based on sex. In an educational context, studies have found that teachers in STEM fields "provided boys with more formal and informal reward and support, and a good effective environment in which to learn" (Ceci, Williams, and Barnett, 2009, pg. 228). In comparison "girls were largely ignored" within the same classrooms (Ceci, Williams, and Barnett, 2009, pg. 228). This neglect in the classroom can leave females with a sense they lack belonging in STEM fields and discourage them from continuing within a given field.

Institutional factors such as the gender makeup of the classroom and faculty may also influence participation within a field. In fact, Wharton found that the "sex composition of the faculty and the type of institution a student attends are related (2005, pg. 67). While this refers to colleges and universities as a whole, the same principle can be applied within specific fields such as those in STEM. This lack of representation within collegiate faculty only worsens at higher levels. "Male and female students attending the most elite colleges and universities are least likely to be taught by female faculty members" (Wharton, 2005, pg. 67). This lack of diversity within STEM education can lead to students feeling as though they do not belong in the field. A study by Cheryan and Plaut explores the impact of the perception of the stereotypical student within a field on other's willingness and desire to join that field. They found "the extent to which a person's own perceived traits and attributes overlaps with these academic prototypes are related to improved attitudes toward the field" (2010). Based on this and the knowledge that men have historically dominated STEM fields, it is logical that women may be less inclined to pursue an education and career in these fields.

Many girls choosing their educational path state that they are not interested in pursuing STEM (Hill et al, 2010, pg. 21). This lack of interest is likely impacted by several factors, including a lower perception of their own aptitude for the area. In assessing one's own ability, "girls assess their mathematical ability lower than do boys with equivalent past mathematical achievement" (Hill et al, 2010, pg. 21). This lower assessment of ability may impact females' perceptions of their ability to succeed in STEM and, in turn, diminish their interest in exploring a STEM education. This perception of being capable of succeeding in STEM can be influenced by whether an individual perceives these skills as being learned or innate. Hill et al. found that "when a girl believes that she can become smarter and learn what she needs to know in STEM subjects — opposed to believing that a person is either born with science and math ability or not — she is more likely to succeed in a STEM field" (2010). This information suggests that the way STEM is presented to students matters in their willingness and interest in pursuing it at the higher education level. In a similar vein to underrating their abilities in STEM topics, females also have lower expectations compared to men that they will succeed in STEM as a whole (Cheryan and Plaut, 2010). The undervaluing of their skill and their potential achievement have the power to take a great toll on women's interest in continuing in STEM education or into a STEM career.

As girls venture more and more into what were traditionally male-dominated career fields, they still find barriers placed upon them at every turn. Yes, the stereotypes that were previously attached to STEM careers are starting to fade away as girls and women discover the benefits to pursuits in STEM, but the retention rates are lacking; the numbers still don't match. We must look to the future to see how the number of women in STEM careers can grow - if it's not too late.

"We have to rethink the way we raise our girls," CEO and founder of the program Girls Who Code, Reshma Saujani, states. "Boys are pushed to take risks; girls are not. In fact, they feel like they have to be perfect at everything they do; they see getting a 'B' in math class as bad. We have to teach girls to be imperfect" (Choney, 2018). The way girls approach their school work is much different than boys - on a cultural, social, and cognitive standpoint. Men and women's brains are different, there is no denying, but the problem comes from outside forces as well, not just internal emotions or cognitive development. There needs to be a wall of support surrounding girls in order for them to consider approaching male-dominated career paths, especially STEM.

The Future of Women in STEM

The failure of the current system in STEM education to encourage equal participation of women necessitates actions to diversify the individuals pursuing education in these fields. The ever-present question as to what can be done to mediate this gender inequality within STEM fields is one that has brought about an array of potential causes and solutions. Along this vein, a variety of programs have been enacted in recent years with the hopes of increasing female participation in STEM higher education, as well as increasing retention rates within the programs. These preliminary steps in addressing this issue have had varying degrees of success. Nevertheless, they lay the groundwork for improvements in the future.

The Specialized High School Admissions Test given to high school students in New York City found that ninth-grade girls were on average 4.2 points higher on a 100-point scale than their male counterparts (Barshay, 2019). When looking at exam scores, females taking the entrance exam made up 40 percent of the top 3 percent, meaning they were less than half of those receiving high marks on standardized exams for STEM. Looking at the ninth-grade math and science classes, however, females were half of the grades listed as 95 percent and above (Barshay, 2019). Despite what the admissions test may have led admissions boards to believe, girls were excelling at their classes such as geometry, biology, physical science, and algebra.

This goes completely against former Harvard University president Larry Summers's statement about how "one reason there are relatively few women in top positions in science may be 'issues of intrinsic aptitude'" (Jaschik, 2005). Females are capable of making the cut — the grades and statistics prove it plainly. Maybe one reason there are relatively few women in top positions in science (and technology, and engineering, and math) is that women aren't granted the same amount of access into these programs.

University of Michigan physicist Timothy McKay elaborates that "even the brightest women may not perform at their best when they feel that they are in a stressful environment where women don't traditionally succeed" (Barshay, 2019). Women feel this extra pressure to succeed when it comes to their schooling, especially compared to their male counterparts. There is less room for error in women's mindsets, just as Saujani was stating. Women "have" to be perfect at everything they do, so much so that even a decent grade, an above-average grade, is seen as failing and an embarrassment. Women are in no way suffering from "issues of intrinsic aptitude", but rather this idea that if they do not succeed completely the first time, then they are failing and the pressure to be perfect is winning.

A step to rectifying this inequity would be to further develop the opportunities to encourage women to join STEM programs, jobs, research, and more. The first step to getting women's feet through the door is to create acceptance of women in STEM career paths. However, this is a drastic change that will not happen overnight. The acceptance of new is not something easily accomplished by the typical person, much less an entire professional career integrated into the daily workings of the world. It must start smaller.

Young girls need to see a path forward in STEM. They need to see the opportunities and support that will meet them down the road. As the Microsoft survey found, young girls stated that one of the main reasons for their decision not to join STEM fields was because of a lack of female role models (Choney, 2018). Thus, mentors in universities would help ensure young women already interested can move from academia to career. By fostering the pursuit of a degree in the young women who are already interested in STEM, universities can bridge the gap between men and women receiving STEM degrees.

The relationship between faculty and student can be implemented in institutions of higher education through mentorship programs. Mentorship programs allow women to not only see themselves represented in STEM but also to engage with educators to develop a support system. While these programs can be implemented in many different ways, they allow students to gain greater insight into STEM topics and culture. Lopatto said on this topic "Mentors are teachers, but they are also coaches, gatekeepers to a community of scholars, and conduits for passing on the culture of science" (2010). Programs with the aim of increasing female participation in STEM have been implemented in various universities across the United States, as well as internationally (Barabino et al, 2019). These programs allow students to engage in professional relationships to gain knowledge and skills from female educators throughout their time at the institution. These preliminary programs have had promising levels of success, especially when female students were paired with female educators as mentors. Barabino et al found that "men could not act as role models in the same way women could" (2019). This information, coupled with the fact that the lack of role models serves as a deterrent to women considering STEM education and careers, is evidence of the integral role female educators play in ensuring a future with equal opportunities for all individuals regardless of sex.

The importance of having a mentor who could serve as a role model can be seen in a wide variety of improvements for the female students who took part in these programs. Higher levels of professional success were shown in women who were mentored by female educators in their field rather than male mentors (Barabino et al, 2019). These results suggest that mentorship in the education system carries through into future efforts within the workforce. Mentorship programs offer a promising solution to the women pursuing and obtaining positions within STEM fields, regardless of their degree earned. In addition to higher professional success, female students with mentors were more self-assured in their abilities and skills within their field (Barabino et al. 2019). This is an important finding in addressing the lower evaluation of knowledge and skills seen in female STEM students that have been cited as a contributing factor to the gender gap. Providing female students with female educators as mentors increased the retention rates of these students by providing them with a stronger sense of belonging in STEM (Barabino et al, 2019). This increased sense of belonging could overcome the perception of scientists and mathematicians being primarily male that dissuades some from pursuing these fields in higher education.

With the overwhelming success of mentorship programs within STEM, it is unsurprising that organizations like Achieving XXcellence in Science (AXXS) are considering it as a necessary tool in combating inequality in STEM fields at educational and professional levels (Burke and Mattis, 2007, pg. 38). AXXS even includes mentorship programs in a list of concrete steps professional organizations can take to promote leadership, visibility and recognition of women in STEM (Burke and Mattis, 2007, pg. 39). In regards to mentorship, AXXS calls professional organizations to

1. Establish a national mentorship system for women,
2. Establish mentoring as a core activity of professional societies,
3. Develop effective mentoring programs, and
4. Create a networking website for scientists.

All of these steps, while meant for a professional setting, can also be applied in an educational setting. Establishing mentorship programs and building networks of support for women on a national scale could be essential to overcoming many of the barriers that dissuade women from continuing in STEM education.

As successful as these programs seem to be, they are difficult to implement largely due to the low numbers of female faculty in STEM areas. Currently, females make up only 4% of the full professors and 6% of associate professor positions in U.S. medical schools (Burke and Mattis, 2007, pg. 246). This occurrence is not isolated to the graduate school level. In the top 50 chemistry departments in the U.S., women make up only 12% of tenure track positions (Burke and Mattis, 2007, pg. 246). Clearly the teaching profession is not excluded from the disproportionate representation seen in the rest of the field. Without the female faculty to enact these mentorship programs, the opportunities these programs offer in mediating gender inequality in STEM are lost. Thus, to offer a better educational experience to women in STEM and increase the success of these students, the treatment and hiring of female faculty need to be considered.

While hiring more female faculty seems like a simple solution, the women considering these positions face the same deterrents as students considering STEM studies. To address this, women need to be incentivized to consider these jobs or, better yet, the barriers to pursuing a career as a female STEM educator need to be removed. Smith et al addressed this challenge directly in their attempt to implement a program that addresses the needs of female STEM faculty (2018). They primarily focused on the areas of autonomy, competence, and relatedness as a means to improve overall job satisfaction in those faculty (Smith et al, 2018). Within the timeframe of this study, the percentage of tenure track faculty who were female increased from 32% to 38% largely due to increased job satisfaction (Smith et al, 2018). Not only did the satisfaction of the female faculty increase, but these diversity programs increased the satisfaction of all individuals involved, regardless of whether or not they directly benefited from the program (Smith et al, 2018). The results of this study provide hope for solving issues of gender inequality among STEM faculty. An increase in hiring and retention of female faculty members in STEM has the potential to increase female participation in STEM higher education and in STEM careers.

Implementing programs to increase the experience of female faculty and students in STEM higher education is something all colleges and universities should consider as a means of diversifying participation in these fields. These programs take resources to implement, which is why programs such as ADVANCE are so important. The NSF ADVANCE program seeks to broaden the implementation of evidence-based systemic change strategies that promote equity for STEM faculty in academic workplaces and the academic profession (NSF, 2019). This is accomplished by providing grants for work that is being done in accordance with that goal (NSF, 2019). The existence of funding like this and the strong focus that is being cast on this topic make now the perfect opportunity for higher education institutions to evaluate what they are doing to promote female participation in STEM.

The programs initiated under ADVANCE and other similar initiatives have produced data that give insight into some key characteristics found in successful programs. Responses from the National Conference for Women in STEM Disciplines, which was funded by ADVANCE, clearly outlined several of these factors. The survey found respondents sought more flexible hiring practices for STEM faculty and promoting a healthy work-life balance (Burke and Mattis, 2007, pg. 35). These may seem like broad goals that would benefit all faculty regardless of gender and, in fact, that may be the better approach in these programs. In general, the consensus at this conference was that “these policies should be available to both women and men equally in the department; the best departments have almost no measures targeted solely at women" (Burke and Mattis, 2007, pg. 35). While this may seem counterintuitive, these initiatives act to make STEM faculty positions more accessible to everyone, especially women who have more pressure on them from society to perform housework and raise children. Because of this additional workload, factors such as pausing the time clock for tenure track for women who are on maternity leave are critical for providing women with the same possibility of success in following this career path (Burke and Mattis, 2007, pg. 35). Additionally, they addressed hiring factors that may result in women being perceived as less qualified applicants. The information addresses materials for female applicants such as recommendations where women are seen to be described differently and often less enthusiastically than men with the same level of qualification (Burke and Mattis, 2007, pg. 35). Acknowledging these differences allows faculty selection committees to adjust for them and potentially hire more equally qualified women into positions where they can mentor and inspire future women entering STEM higher education.

Mentorship programs have huge potential in promoting enrollment and retention of women in STEM higher education. It is this potential that causes organizations such as the NSF, Association for Women in Science (AWIS), and Achieving XXcellence in Science (AXXS) all to promote mentorship as a means to combatting the current issues that exist as barriers (Burke and Mattis, 2007; NFS, 2019). In order for these kinds of programs to be feasible, higher education institutions need to prioritize hiring and supporting women educators in STEM. Creating an atmosphere that is inclusive for female educators paves the path for the inclusion and support of the future contributors to STEM.

Unfortunately, it can be hard to change older voices and minds. It may be easier to start young when children are first learning, growing, and discovering themselves. This is why it is equally as important to change how STEM is approached at the elementary level. The rest of this section will be devoted to the changes needed in elementary STEM in order for further growth and inclusion of women in STEM career fields.

The Girl Scouts of America are on the right track, with their STEM pledge to "galvanize a new generation of girls to explore STEM and become confident STEM leaders". They started this initiative with the release of thirty STEM-related badges for girls ages five through eighteen to earn and two new STEM programs that will integrate girls into new STEM projects. This is an initiative that is needed for young girls — an initiative that changes the game and evens out the playing field. It gives girls the opportunities they might be denied later down the line in their careers, or even opportunities not granted by their teachers in their everyday classrooms. As stated before, programs and opportunities like this are encouraging females who want to pursue STEM career fields.

But it's not just the Girl Scouts of America's responsibility to promote new generations of girls as STEM leaders. If they don't have support, their efforts will not amount to anything and the cycle will only continue. STEM needs to be more predominant in the classroom, and this starts with teachers being ready and wanting to include STEM in the everyday classroom.

Teachers don't want to teach the unfamiliar. They want to stick to what they know, a natural human instinct. In order for teachers to want to teach STEM subjects, they must feel confident in their abilities and understanding of said subjects. As stated earlier, the National Survey of Science and Mathematics Education reported that only 34% of teachers have taken collegiate level courses in all three of the recommended sciences: life, physical, and earth. A simple switch would be improving this number and making sure 100% of teachers have taken at least one course of all three recommended science subjects. Just that alone can help improve teachers' confidence in teaching science. The same can be said about mathematics. Universities could follow the NSSME's recommendation of five mathematical courses for teachers: algebra, geometry, number and operations, probability, and statistics. Having at least one course in engineering and another in technology would allow preservice teachers the familiarity of the topics and help improve their confidence in teaching said subjects in their classrooms.

Once teachers are adequately prepared to teach STEM subjects, the next step is for teachers to do just that: teach STEM. However, STEM is an entirely different world than English/Language Arts and thus cannot be approached in the same way. There are two helpful guides to teaching STEM: student-centered instruction and inquiry-based learning.

Student-centered instruction is rooted in the constructivist theory of learning and is a newer idea in the educational world. According to Edglossary.org, student-centered learning includes the following important characteristics:

1. Teaching and learning are "personalized," meaning that it addresses the distinct learning needs, interests, aspirations, or cultural backgrounds of individual students.

2. Students advance in their education when they demonstrate they have learned the knowledge and skills they are expected to learn (for a more detailed discussion, see proficiency-based learning).

3. Students have the flexibility to learn "anytime and anywhere," meaning that student learning can take place outside of a traditional classroom and school-based settings, such as through work-study programs or online courses, or during non-traditional times, such as on nights and weekends.

4. Students are given opportunities to make choices about their own learning and contribute to the design of learning experiences.

Allowing students the flexibility and personalization of learning will give students the opportunity to be more involved with the subjects (particularly STEM subjects as they tend to be more hands-on) as well as feeling a larger responsibility for their success in learning. It also allows teachers the opportunity to understand where each of their students is personally and allows them to develop content and instruction that helps students exactly where they need help. It takes the focus off of the teacher and places it on the students, as the name suggests and as the learning should be.

The second teaching strategy that is important to adopt in STEM subject teaching is inquiry-based teaching. According to the Center for Inspired Teaching, "inquiry-based teaching is a pedagogical approach that invites students to explore academic content by posing, investigating, and answering questions" (pg. 1). Inquiry-based teaching is designed for all subjects at all ages and pairs nicely with student-centered learning as neither includes direct instruction from teachers to students. Inquiry-based teaching allows students to generate their own questions and explore possible answers on their own with guidance from their teacher instead of direct explanation and lecturing. Inquiry-based classrooms are designed to help students develop skills beyond just understanding of instruction. “It teaches students to pose difficult questions and fosters the desire and skills to acquire knowledge about the world. Students are given opportunities to take ownership of their own learning, a skill necessary for one to succeed in college and in most professional settings” (pg. 1).

Having student-centered teaching and inquiry-based learning allows students the opportunity to dig deeper with their learning, to understand the importance of struggling to find a solution, to acknowledge that there isn’t always one right answer, and to feel empowered by their own learning. Developing these approaches to STEM subjects in the classroom will allow students, especially girls, a deeper appreciation of the subjects and what they are learning than they would should they just be lectured and told the answers.

This is exactly what the Girl Scouts of America are encouraging with their STEM badges and programs: the enjoyment of STEM subjects by allowing girls the opportunities to take charge of their own learning. Having student-centered teaching and inquiry-based learning can help support this promotion of girls learning and enjoying STEM beyond just what their textbooks teach them. Having this support both in and out of the elementary classroom can help girls recognize their strengths in the STEM subjects and set them up for a future career in the STEM fields.

Conclusion

Girls deserve to explore STEM. Women deserve to be involved in STEM. The voice is lacking, and the inclusion of women cannot and will not come overnight. But there are many tiny steps that can set up women for success from the beginning and implementing these ideas for girls as early as kindergarten will allow for the future growth of women in STEM career fields. “I was taught that the way of progress was neither swift nor easy,” Marie Curie, first female Nobel prize winner, once said. Women’s voices are missing in all elements of science, technology, education, and mathematics. For now, the wheels of progress for the inclusion of women in STEM education fields are slowly turning.

Women in the past fought and advocated for their inclusion in STEM. The struggle started with a lack of access for women to higher education (Solomon, 1985, pg 44). Yes, more women were allowed into colleges and universities starting in the 1920s but welcomed may not be the right verb to express their inclusion. Despite their attendance in higher education institutions, in the 1970s less than 15% of life science Ph.D.’s were awarded to women (Ceci & Williams, 2010). If that was not enough, marriage and family came into play as a barrier between women and STEM fields, as women were seen better fit for jobs that focused on family life. These were just a few of the issues in history, not to include the major barriers faced by women who were also part of another minority group. Scores in science and mathematics tended to favor white men the most, but even white women were seen to outscore women of color (NCES, n.d.). Women of color and their voices have definitely been erased (Crenshaw, 1991, pg. 1253).

These barriers have shifted and evolved to lead to the current situation of women in STEM education fields. Currently, women are receiving much more recognition for their work than historically speaking, and they are receiving degrees at significantly higher rates than previously seen. Despite this, women hold less than 25% of STEM jobs as careers despite making up over half of those in the current workforce (Beede et al., 2011). This means that men are being employed at double the rate of women in STEM career fields. This imbalance indicates issues within the field that need to be mediated on many levels.

There are current stances to help level out the playing field, especially at a younger age. An in-depth look at the Girl Scouts of America reveals that their thirty new STEM-related badges and two new STEM programs have geared more girls into seeing themselves as future scientists, engineers, mathematicians, and technicians (Kelly, 2018). Up to 90% of girls who participated in one of the new STEM programs agreed that they wanted more information about careers in math, science, and the arts (Girl Scouts of America, 2018).

Despite the efforts currently being made, there is still a lack of female voice in STEM education and careers. Girls as early as sixth grade are losing interest in STEM, with less than half of middle school females and high school females feeling capable of doing STEM (Choney, 2018). On top of the dwindling numbers still occurring, current elementary education teachers are not properly equipped to teach STEM subjects in their general education classrooms, resulting in a lack of time on those subjects. In fact, 93% of teachers have not taken the 5 math classes at the collegiate level that the National Council of Teachers of Mathematics recommends (NSSME, 2018).

Another current issue involving women in STEM career fields is the manner in which female scientists and mathematicians are represented in the media. Female scientists on television and in movies are outnumbered by men 2 to 1 and are seen as more dependent and less capable than their male counterparts (Steinke, 2017). This means that despite efforts of new programs and more acceptance of women at higher educational levels, girls are still not seeing STEM careers as a viable option for themselves, nor are they seeing proper role models already in these fields for them to look up to.

This is why we must look toward the future and take steps to start making changes today. Women's voices are lacking in STEM education and STEM careers. While an immediate solution will not just happen and women will not automatically be accepted and welcomed into STEM fields, as Marie Curie pointed out about a century ago, small steps can be made toward a brighter future.

First, there needs to be the understanding that women are put under more pressure to be perfect (Barshay, 2019). This expectation of perfection leads girls to undervalue their own skills compared to equally qualified boys. The acknowledgment that women do not suffer from "issues of intrinsic aptitude" for this very reason is important as well (Jaschik, 2005). Women are capable of doing the work, they just are not always fully recognized for their successes due to the high pressures placed on them by themselves and others.

Next, there should be more mentoring involved in STEM fields. A large reason behind girls and women not wanting to be a part of the STEM career is the lack of role models they have to look up to. Having mentoring programs in place at the collegiate level and higher educational institutions will help bring women together as students and teachers in recognition that we must all learn from one another. Mentoring programs can lead to higher retention rates, especially women (Beede et al., 2019).

In addition, there should be efforts made to increase the number of female faculty at the collegiate level. This goal can be addressed by improving the working conditions of female faculty, who regularly face the same barriers that affect their students.. As of now, females are only 4% of the full-time professors in medical schools and only 12% of the tenure track (Burke and Mattis, 2007, pg. 256). Having more women on the tenure track and as overall faculty members will help in the retention rate as well as encourage the mentorship programs that should be put into place. It will also help with diversifying colleges and universities and providing strong examples for students, the goal of this being the ultimate diversity of individuals working in STEM fields.

At the elementary level, there needs to be an increase in knowledge for teachers, starting with teachers taking the recommended mathematics and science classes. For example, 100% of the teachers should have all 5 mathematics courses at the collegiate level that are recommended by the National Council of Teachers of Mathematics. Having teachers more knowledgeable on STEM subjects will increase their willingness to teach and confidence in teaching STEM subjects in class. If teachers are more comfortable with the material they will be better able to translate it to their students and hopefully get more students interested in those topics.

After this, teachers must take a student-centered learning approach and inquiries approach to teach STEM at the elementary level. Doing so will help young students, especially girls, feel more comfortable and confident in their abilities while spreading their understanding and knowledge of the topics (Center for Inspired Teaching, pg. 1). Improving girls confidence in their STEM abilities has the potential to increase retention rates in higher levels of STEM education.

Building on society's seeming willingness to change, these are some of the many small steps that must be taken in order to bring more women into STEM. As this world becomes more and more science and technology-based, this goal is more important than ever. As was stated in the research book, ***Women and Education***, "I would like to see more women in science, not only for the sake of the many women who could do talented work but for the sake of science, because it would present a different face to society if the women in it were sufficiently numerous and confident not to follow the male models or definitions" (Maccia, 1975, pg. 158).

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