Novel Scientific Research in the College Classroom: Identification of Antibiotic Resistant *Pseudomonas* Species from a Wastewater Treatment Plant

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This chapter presents a way in which to bring novel scientific research with broad environmental and societal implications into the college classroom. The project described models real world science as a process of literature exploration, experimental design and execution, data collection, analysis and presentation, and application of what was learned to designing new experimental questions, as well as addressing societal problems. In the laboratory, the students were charged with the task of isolating and properly identifying Pseudomonads from wastewater treatment plant samples taken during a class tour of the local treatment facility. Techniques used by the students included filtration, bacterial culture on selective and differential media, biochemical tests, and polymerase chain reaction to detect 16S rDNA characteristic of Pseudomonads. Once identification was complete, antibiotic resistance testing was performed. This project allowed students enrolled in a course scheduled in the traditional three credit-hour time slot to participate in novel scientific research, to reflect on science as a process, and to discuss possible implications their work might have on society.

Keywords antibiotic resistance; Pseudomonads; scientific literacy; student research; wastewater

1. Introduction

1.1 Research in the classroom

Research involving undergraduates has received much attention over the past decade with the idea that students should be afforded the opportunity to experience science and the nature of scientific inquiry [1]. Inherent goals in research training include learning to work collaboratively, as science as a discipline values and requires, building confidence in a laboratory setting, learning to read and understand scientific literature, content knowledge, laboratory skills, and the ability to present research findings and interact with other scientists [2].

At St. John Fisher College, a small, private liberal arts institution, we have experienced rapid growth, especially in the sciences, over the past five years. As our department adjusts to the increase in student numbers, we are working on ways to satisfy student desire to engage in faculty-guided research. One way we have done this is to incorporate a laboratory component into a three credit-hour upper-division elective course in Microbial Ecology. Pre-requisites for this course included our General Biology course and Microbiology course; both contained laboratory components. Including a research experience into a course is also appealing to students as they are dealing with full schedules as they work toward their undergraduate degrees.

1.2 *Pseudomonas* and human health

Pseudomonas aeruginosa is found ubiquitously in the environment. This species can be found in a free-floating planktonic state or in biofilms as a community of cells. The Gram negative, rod-shaped bacterium *P. aeruginosa* is one of concern to human health today because it is a formidable nosocomial pathogen. *P. aeruginosa* is very common in association with a variety of health ailments including: urinary tract infections, respiratory infections, dermatitis, bone and joint infections, gastrointestinal infections, and systemic infections [3]. This species is the number one cause of death in patients with the genetic disease Cystic Fibrosis (CF). Due to a variety of virulence factors, *P. aeruginosa* has the ability to evade the host defenses. These virulence factors include the ability to produce exotoxin A, which inhibits the elongation factor 2, resulting in the cessation of protein production and evident death. Exoenzymes can be produced which degrade cell membranes of eukaryotes. These are in association with lipopolysaccharides (LPS), which have the ability to change the O-antigen, to evade detection of any host immune system. In addition to these virulence factors, *P. aeruginosa* has an inducible β-lactamase-encoding gene to hydrolyze certain antibiotics (i.e. those classified as β-lactams). Furthermore, the bacteria encompass pump systems that can remove certain antibiotics that may enter the cell. *Pseudomonas* can readily acquire new resistance genes via the uptake of DNA and is able to mutate rapidly in adverse conditions. This hypermutation is found in about 40% of CF patients infected with *P. aeruginosa*, and the use of antibiotics against the bacteria have selected for hypermutants [4]. Due to the widespread importance of *P. aeruginosa*

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in the environment and human health, this organism (along with other Pseudomonads) was selected for study for this project.

1.3 Wastewater treatment and antibiotic resistance

A report released on World Water Day 2010 by the United Nations Environment Program revealed that more people die from polluted water each year than from all forms of violence, including war [5]. Sewage run-off and industrial waste promote the spread of disease and damage ecosystems. In our limited environment in upstate New York, USA, we do not often experience any problems relating to wastewater and the treatment process receives very little community attention simply because of this. Unfortunately, wastewater treatment plants (WWTP), even in areas where wastewater is well managed and seems of little concern, potentially select for antibiotic resistant bacteria that can have detrimental public health effects. Recent studies [6-8] have revealed that wastewater treatment encourages the emergence of antibiotic resistant strains of *Salmonella*, *Escherichia coli* and *Enterococcus*, and *Acinetobacter*, respectively. To date, only one other study regarding *Pseudomonas* in wastewater has been linked with antibiotic resistance [9].

The wastewater treatment plant in Rochester, NY, USA, where samples were taken for this study, operates using an activated sludge system. Indigenous bacteria (protozoa and other microbes are sometimes also involved) form flocs, or small biofilm communities, where they metabolize the organic molecules present in the wastewater thereby cleaning it [10]. Although this method of treatment seems simplistic, there are many variables that can alter the efficacy of this process. For example, there are no means of temperature control for the outdoor tanks. In Rochester, NY we experience all four seasons and a wide range of temperatures throughout the year. In the winter months, salt is often used on the roads to eliminate ice, and this salt becomes incorporated in area run-off and affects the wastewater treatment process. Also, the aeration process must be carefully monitored and varied to accommodate changes in ambient temperature and to control for weather events (rapid snow melt, heavy rains, etc.). An optimal oxygen concentration needs to be maintained to support respiration and growth of the indigenous microbes responsible for the wastewater treatment [10]. After treatment, the final effluent from the plant in Rochester, NY is dispensed into Lake Ontario, a Great Lake bordered by both the United States and Canada.

Due to the high concentration of bacteria in close proximity through collection into flocs during the activated sludge and mixed liquor stages of treatment, horizontal gene transfer is likely to occur frequently. It is well established in the literature that antibiotic resistance can not only result from chromosomally encoded resistance mechanisms [11], but from genetic exchange of plasmid-encoded resistance genes through the processes of conjugation and transformation [8, 12]. Scientists still disagree if the wastewater treatment process itself plays an active role in increasing the antibiotic resistance of bacterial strains [8, 13]. Due to the exceptionally costly processes necessary to remove antibiotics from wastewater, no such step is in place in many treatment plants. It has been shown that after water has passed through wastewater treatment, pharmaceuticals are released directly into the environment [14]. Intensive animal husbandry uses 70% of the antibiotics produced in the United States, which is about 11,500 tons of antibiotics just for animals [14]. This intrinsic relationship has introduced pharmaceuticals into the environment because of water run-off and from the use of animal feces as manure to fertilize crops [15]. Although antibiotic levels are generally 1000-fold less concentrated than levels recommended as minimum inhibitory concentrations in clinical practice, these levels have the potential to select for resistant bacteria [15]. Resistance limits routine therapeutic courses of antibiotic treatment, which limits options for patient care. Unfortunately, this then leads to increases in cost of care, morbidity, and mortality [16].

2. Materials and Methods

2.1 Collection

Samples were collected during a class tour at the Frank E. VanLare treatment plant in Rochester, NY from five points in the wastewater treatment process. One liter samples were collected at the following five points: influent, mixed liquor, activated sludge, primary effluent, and final effluent. Samples were mixed by hand and 25mL were poured into a vacuum filter apparatus. Samples were filtered onto a 0.2 μm sterile filter paper (Millipore, Billerica, MA) using aseptic technique. After filtration, filter paper was transferred to R2A agar (Difco BD, Franklin Lakes, NJ) and incubated at 20-22°C for 72 hours.

2.2 Isolation

From R2A plates, each sample was streak-plated onto Luria Bertani (LB) agar (5g yeast extract, 10g NaCl, 10g tryptone, 15g agar, 1L dH_20) and *Pseudomonas* isolation agar (PIA) (Difco BD), and then incubated at 20-22°C for 48 hours. After incubation, morphologically different isolated colonies were re-struck for pure cultures onto LB and PIA. Stock cultures were prepared using skim milk and glycerol and stored at -80°C.

2.3 Identification

The control strains selected for this study were *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. Gram stains of each isolate were performed using standard protocols. Sulfide indole motility (SIM) deeps, Simmons citrate slants, and MacConkey agar plates (Difco BD) were utilized per manufacturer recommendations. All incubations were carried out at 20-22°C under aerobic conditions. Wet mounts were used as a secondary test for motility and were performed using broth-grown cultures. A test for the presence of the oxidase enzyme was performed using sterile cotton swabs coated with fresh LB-grown bacteria and the oxidase reagent (BD BBL Oxidase). Standard *Taq* DNA polymerase was utilized in colony polymerase chain reaction (PCR) to detect 16S rDNA. Primers used were specific to *Pseudomonas* spp. [17]. PCR Cycle: 35X for the following: 95°C for 30 seconds, 65°C for 1 minute, 72°C for 1 minute. PCR products were visualized under UV light using a 2.5% agarose gel prepared with SYBR Safe Stain (Molecular Probes, Invitrogen, Carlsbad, CA).

2.4 Antibiotic Resistance Profiling

LB grown broth cultures of each isolate were utilized for antibiotic resistance testing. Cultures were incubated in an agitating water bath at 25°C for 16-18 hours prior to plating on Mueller-Hinton agar (Remel, Lenexa, KS). Using sterile cotton swabs, the LB broth cultures were swabbed over the entire surface of 90mm agar plates according to the E-Test strip protocol. E-Test Strips (AB bioMérieux, Durham, NC) Ciprofloxacin (abbreviated CI; fluoroquinolone) and Ceftazidime (abbreviated TZ; β-lactam) were used to test for resistance. After Mueller-Hinton agar plates were inoculated and dried, E-Test strips were applied according to the manufacturer instructions. Results were read 16-20 hours post application and pictures were taken for documentation.

3. Results and Discussion

3.1 Course Goals and Outcomes

At the beginning of the semester, students were assigned to read a set of articles chosen by the instructor to provide a framework for understanding the process of wastewater treatment and the role of naturally occurring microbes in this process. Students were also assigned reading from the primary scientific literature about a similar study in order to demonstrate that scientific research is both cumulative and collaborative. By having students read background literature as well as primary research articles describing the scientific process, we were also able to address scientific literacy objectives set forth by our Biology department.

Students were encouraged to work in self-selected groups to complete a packet of questions designed to gauge their understanding of the assigned reading and to help them identify the hypothesis and experimental plan for this semester-long (15 weeks) project. Prior to the class tour of the WWTP facility, we reviewed the hypotheses and plans and collected materials to use in our initial experiments for isolation as described above in the Materials and Methods section.

The class agreed on the following hypothesis to focus our study: *The wastewater treatment plant will harbor Pseudomonads that are resistant to the commonly prescribed antibiotics Ceftazidime and Ciprofloxacin.* Students predicted from their reading that we would be more likely to find highly antibiotic resistant Pseudomonads in the activated sludge and mixed liquor stages of treatment as these stages rely on flocculation (essentially biofilms). The close proximity of the cells would allow for genetic exchange of resistance genes. Experimental results and conclusions are described in section 3.2 of this chapter.

After collecting data, students compiled their work into scientific posters. These presentations were made to faculty members of the Biology department at St. John Fisher College and to our collaborator, Stephen Bland, M.S., from the WWTP. Students described the semester-long process of engaging in research as part of their class as well as research results, conclusions, and suggestions for future studies. A rubric was used to assess student learning and achievement of the project goals through the design and presentation of the posters. A portion of the rubric including average student scores for each goal is shown below (Table 1).

Table 1 Rubric used to assess student learning using the poster presentation as a final cumulative assessment for the project.

GOALS	SCORING	AVERAGE STUDENT SCORE
Group:		
All required parts present (title, intro/abstract, data, conclusions, references)	0 – missing any one of the listed parts; 1 – contained all required parts	0.7
Data are accurate	0 – inaccuracies in data presented; 1 – typos or accidental inaccuracies; 2 – data are accurate	1.6
All + and – controls included	0 – controls not included or inadequately included; 1 – controls included throughout	0.7
Legends included and correctly articulated	0 – no legends used; 1 – alternate method used; 2 – clear legends used	0.7
Data slides represent data clearly and logically	0 – illogical data organization and representation; 1 – some data represented logically; 2 – all data represented logically	2
Conclusions are appropriate – relate to the experiments and do not overly generalize	0 – conclusions present, yet incomplete or illogical; 1 – conclusions present; 2 – conclusions demonstrate thought and articulation of data	0.7
Individual:		
Presenter was knowledgeable and able to answer questions	0 – could not answer questions; 1 – tried to answer questions, yet answers lacked substance; 2 – completely answered questions and engaged listeners	1.7
Asked meaningful questions of presenters	0 – did not ask meaningful, relevant questions; 1 – asked meaningful, relevant questions	1

Students performed well when dealing with the data they had collected, yet had difficulty drawing relevant conclusions from their data. We see this higher order critical thinking skill to be most troublesome even for our junior and senior students. Often times in laboratory classes the end result is something that is predictable and expected so it is very easy for students to make conclusions. When dealing with novel research results and data, students need to be reminded to reflect back on their original hypothesis about the work while interpreting results and drawing conclusions. After careful questioning by faculty during the poster presentations, which students performed well on (see above, Table 1), it was clear that students were capable of critically analyzing their results and discussing how their project impacted the greater society. Students were genuinely interested in this work, and a final written reflection, due after the poster presentations, on this project revealed the following student comments:

> *"I really enjoyed tackling this project because it has significant tangible relevance for us as a population. I liked the aspect of our individual groups getting to choose what tests, what approaches and in what styles we would tackle uncovering the underlying questions, rather than being told this is what you are going to do and this is how you are going to do it. It made me as a student feel more engaged because my work feels relevant. I liked being able to choose the tests being run and deciding for ourselves what we found. I also like making the poster presentation at the end because it really brought everything together and showed me how much we as a group had accomplished and what exactly we found."* Mark G.

> *"After collecting the samples and bringing them back to the lab I liked that this was new to Dr. Picardo as well and that she did not know what the outcomes of the experiments would be. I also liked that this class did not have a scheduled lab during the week but class time was used as lab. This is a good way to break up the lectures so that we do not spend all of our time in a classroom but doing hands on work as well."* Maureen S.

> *"The laboratory portion of this class allowed everyone to see "how" scientific data is found, and what kind of process it is to conduct real science research. Many of the research projects we do in our classes consist of a lot of reading and writing, whereas with this project we actually got to collect data and do the tests ourselves. It is amazing how much it takes to do all of this."* Jonathan P.

"The research performed has an impact on peoples' lives. After the poster presentation today I got a lot of insight from the visitors that came to ask questions; it really sparked some interests and got me thinking a lot more." Sarah C.

Clearly the students undertaking the research project as part of this upper-division elective in Microbial Ecology have demonstrated learning and increased interest in the subject matter and in scientific research. We plan to continue to improve this research experience for this course and hope to provide a meaningful experience for any student wishing to take part in this course in the future.

3.2 Research Results and Conclusions

Samples from the VanLare WWTP were collected during the first week of February 2010 as described above in the Materials and Methods. Within two days of sample collection, the isolation procedure was begun. From this first collection, three potential *Pseudomonas* strains were identified (Table 2).

Table 2 Biochemical properties for each isolate identified as *Pseudomonas*.

Bacterial Isolate	Gram Character	SIM (H_2S Production)	SIM (Indole Production)	Simmons Citrate (Citrate Utilization)	MacConkey (Lactose fermentation)	Oxidase
P. aeruginosa (ATCC 27853)	-	-	-	+	-	+
Primary Effluent C	-	-	-	+	-	+
Final Effluent B	-	-	-	+	-	+
Final Effluent C	-	-	-	+	-	+

We expected to isolate *Pseudomonas* from all stages of treatment sampled, but as shown above we were not able to definitively identify Pseudomonads in the influent, activated sludge, or mixed liquor samples. Preliminary evidence from a second collection does, in fact, suggest that *Pseudomonas* is present at these stages. We suspect that our filtration procedure will need some modification to successfully culture these species in the future. In addition, we are aware that not all Pseudomonads will adhere to the characteristics shown in Table 2, above.

After identification using the tests described, we utilized PCR as a molecular approach to support our data. Results are shown in Fig. 1 below.

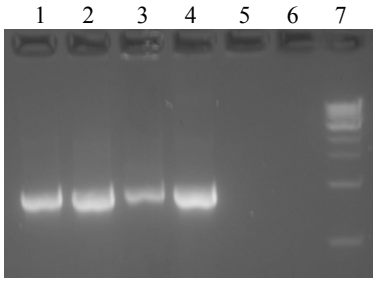

Fig. 1 PCR results support all three strains identified as *Pseudomonas* using biochemical tests are in fact *Pseudomonas.* The primers used for these reactions are specific to Pseudomonad 16S rDNA. Lane 7 is the molecular weight marker, lanes 5 and 6 are empty, lane 4 is the positive control strain *P. aeruginosa* ATCC 27853, lane 3 is final effluent C, lane 2 is final effluent B, and lane 1 is primary effluent C.

After confirmation of Pseudomonad identity, we moved forward with the antibiotic resistance testing using the E-test strips. These strips contain varying concentrations of antibiotic along a single piece of membrane paper. Once laid onto the inoculated agar (as described above in the Materials and Methods), antibiotic can diffuse into the surrounding area. A typical elliptical pattern of inhibition of bacterial growth is used to read the results. Positive control strain *P. aeruginosa* ATCC 27853 and negative control strain *E. coli* ATCC 25922 were included in these studies (Fig. 2 below).

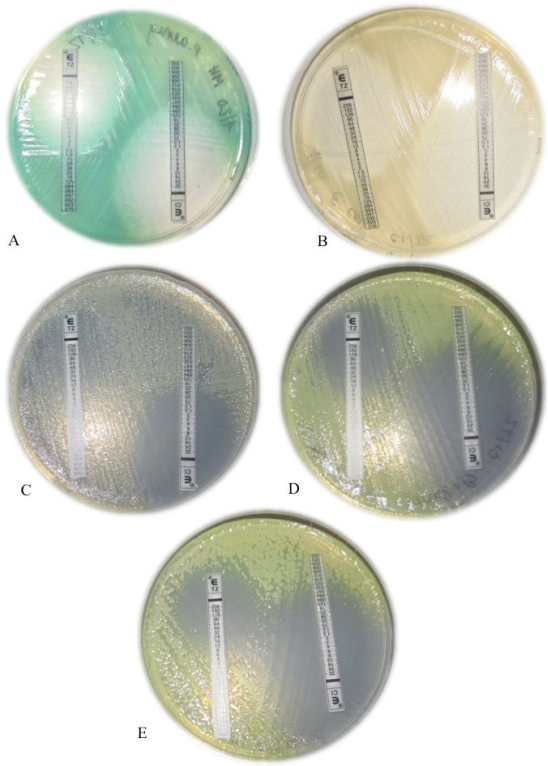

Fig. 2 E-test strips were used to test for resistance to the antibiotics Ceftazidime (abr. TZ, shown on the left-hand side of each plate) and Ciprofloxacin (abr. CI, shown on the right-hand side of each plate). A) *P. aeruginosa* ATCC 27853, B) *E. coli* ATCC 25922, C) Primary Effluent C, D) Final Effluent B, and E) Final Effluent C.

All three experimental isolates showed resistance to the β-lactam antibiotic, Ceftazidime, which supported the students' original hypothesis. In contrast, all three isolates were susceptible to the fluoroquinolone antibiotic, Ciprofloxacin.

In conclusion, this study suggests that *Pseudomonas* species are in fact present during the wastewater process at various stages of treatment. In addition, the isolates studied to date possess resistance mechanisms to a commonly prescribed β-lactam antibiotic, yet remain susceptible to the broad-spectrum fluoroquinolone, Ciprofloxacin. Further studies are warranted and planned as described in section 3.3 of this chapter.

3.3 Future Directions

Studies from a second sample collection are currently underway. In addition, we plan to test for resistance to a third commonly used antibiotic, Tobramycin. A larger goal to be implemented in the coming semesters will be to use generic 16S ribosomal RNA gene-specific primers to begin a sequencing project. Students will be responsible for identifying bacterial species from the original collection of bacterial colonies on R2A agar, obtain 16S rDNA sequence information, and utilize bioinformatic tools freely available on the web to determine the species identities for their collections.

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