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TABLE OF CONTENTS

PERSPECTIVE

OBJECTIVE LENS

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UG: HEADING FOR AN ACADEMIC CAREER?

Undergraduate students: do you want to teach someday in an academic position? Have you spoken with your advisor or research mentor about what you could be doing now to prepare yourself for a tenure-track position in academia? Now is the time to begin documenting your achievements and prioritizing how you spend your time in research, volunteerism, and teaching/mentoring opportunities. For research, presenting and publishing your undergraduate research results is vital. These presentations and publications (for example, in *Fine Focus*) should be meticulously updated on your CV. You may have to ask, but most colleges and universities have undergraduate research mini-grant opportunities, so work with your research mentor to develop and submit at least one of these before you graduate. Even if your proposal is not funded, you may still put this on your CV as relevant grantsmanship experience.

Step out of your comfort zone and opt to give oral presentations when offered at local, regional, and national conferences, as this will give you confidence and hone your teaching abilities as well. As your college career progresses into your M.S. thesis or Ph.D. dissertation research, be mindful of the importance of publishing, as this is one of the principal criteria that selection committees will be looking at later – how many coauthored papers do you have, in what journals, and in what specific area(s) of research? Try to get at least one coauthored paper from your undergraduate research, one or two from your M.S. (if you go that route), and at least three from your Ph.D. dissertation.

Be selective about the extracurricular activities in which you spend time participating. Try and keep your involvement to one or two service/volunteer-oriented activities, and one or two disciplinary commitments (such as an officer position in an ASM Chapter). If possible, these activities should reveal your leadership qualities and accomplishments through long-term involvement, rather than simply membership. For example, as the President of your collegiate ASM Chapter, you could indicate that you invited and hosted outside speakers to your department, organized and managed a fundraiser for a well-recognized charity, or led a field trip to tour a pharmaceutical company, turning this into a career development opportunity for your members. Selective and committed involvement in activities such as these carries much more credibility than overextending yourself in many organizations, where you have little time investment and even less impact.

Lastly, but certainly no less importantly, during your graduate school years, seek out teaching opportunities if you are not already assigned as a TA. You may be somewhat dissuaded from doing this if you are in an RA position, but the fact is, one day you will not only be expected to know how to teach well, but also to have interesting ideas for novel pedagogical approaches for lecture and lab courses. So when exactly were you supposed to have learned how to be an effective teacher? The answer is that this begins in graduate school. Thus, keep all of your teaching evaluations, and if none are mandated by the department, then elect to administer your own, and file them by semester and class for future reference. Written comments from your students are particularly valuable, especially if these address specific questions about your use of technology or innovative and interactive approaches to presenting material. During this time, seek advice from trusted science educators who value teaching. Ask them to sit in on several of your classes or labs

as the semesters progress, to document the development of your teaching style, and to offer valuable feedback.

Likewise, as a postdoc, make a special effort to teach when at all possible. Document the topics you cover, size of each class, and keep written feedback. Even at the bench during your postdoctoral years, opt to mentor a couple of undergraduate students on their respective projects as well. Engage them in journal clubs, lab meeting presentations, and guide them into presenting at conferences, just as you did a few years prior. This certainly counts towards teaching experience in more ways than you might think. Their progress in research reflects your progress as a mentor. If you are in the mindset of teaching through research, and have a true passion for teaching undergraduates, you will soon find these things to become second nature. Good luck!

–JLM

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APPLICATION OF MOLECULAR TECHNIQUES TO BETTER UNDERSTAND THE ROLES OF RUMEN MICROBIOTA IN CATTLE FEED EFFICIENCY

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ABSTRACT

Feed efficiency, simply expressed as less feed inputs versus animal production outputs, can be measured in several ways, such as feed conversion ratio (FCR) and residual feed intake (RFI). FCR is a common measurement in beef cattle operations, and is the ratio of feed intake to live-weight gain. RFI is defined as the difference between actual and predicted feed intake after taking into account variability in maintenance and growth requirements. Rumen microbiota, which inludes bacteria, archaea, protozoa, and fungi, play an essential role in the digestion of lignocellulosic plant biomass, and can provide more than 70% of the host ruminants energy requirements *via* the production of volatile fatty acids (VFAs). Methane, a potent greenhouse gas (GHG), is produced in large quantities by the rumen microbiota, and is a known contributor to the global increase in GHG emissions. Studies have shown a negative relationship between methane emission and feed efficiency. Therefore, there is a need to study the feed efficiency from a rumen microbiome perspective and explore the probability of improving feed efficiency and hence reduce methane production in cattle by manipulating the rumen microbiome. The development of high-throughput sequencing technologies incuding metagenomics and metatranscriptomic analyses in the past decade has led to a sharp increase in understanding the rumen microbiota and associated function. As such, this mini-review will focus on the new findings during the last decade in cattle feed efficiency and the rumen microbiome.

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KEYWORDS

- RFI
- Rumen microbiota
- Pyrosequencing
- Next Generation Sequencing
- Metagenomics

INTRODUCTION

The term feed efficiency implies a ratio of outputs to inputs. Therefore, feed efficiency of beef cattle is the relative ability of the animal to turn feed nutrients into animal products (8). Feed efficiency in cattle is important since it is directly associated with economic profit and enteric greenhouse gas emission from agriculture sectors. For farm owners, 66% of costs in calf management are spent in feed, rising to 77% in yearling finishing systems (1). Fox *et al.* (11) estimated that while a 10% improvement

in daily gain would increase profitability by 18%, and improving growth efficiency by 10% could increase profits by 43% in feedlot cattle. Another study demonstrated that improvement in feed efficiency has 7–8 times greater economic impact than similar improvements in daily gain (27). Other than the economic effect, improving feed efficiency can reduce GHG emission, as cattle with higher feed efficiency are reported to produce 20% to 30% less methane than inefficient ones under the same conditions (33). Given its importance in production systems, measuring the feed efficiency trait in cattle is important. Several different measurements of feed efficiency have been developed and used by industry, such as feed conversion ratio (FCR) and residual feed intake (RFI). FCR is a common measurement in beef cattle operations, which is the ratio of feed intake to live-weight gain (8). While FCR is useful for evaluating management, feed quality, and environment on efficiency in growing and finishing cattle, it has limited value with genetic improvement. FCR has a strong correlation with growth traits, meaning that the selection by lower FCR will increase cow mature size, rather than reduce feed inputs (8). Nowadays, researchers use RFI more generally than FCR for feed efficiency measuring in selecting beef cattle. The concept of RFI is defined as the difference between actual and predicted feed intake after taking into account variability in maintenance requirement and growth; therefore, when cattle consume less feed than expected for their body size and rate of gain, they are considered to have a negative RFI, which means a higher feed efficiency status as compared to positive RFI (17). Compared to FCR, selection by RFI would produce efficient offspring in all segments, because the progeny would be similar to their low RFI parents in yearling weight and average daily gain after almost two generations, but the progeny have been reported to have a lower feed intake (4). The moderate heritability of RFI indicated that selection from the low RFI herd will result in progeny that consume less feed than the high RFI herd (3).

Feed efficiency in cattle is influenced by multiple factors, such as variation in breeding, feed formulation and the rumen microbiota. This review will focus on the role of rumen microbiota in cattle feed efficiency, and will explore the feasibility of improving feed efficiency in beef cattle by modifying the rumen microbiota.

RUMEN FUNCTION

The cattle rumen is the largest of the four compartments of the stomach; with the other three being reticulum, omasum and abomasum. The rumen is a fermentation chamber where fibers are broken down into smaller digestible components by symbiotic microbiota (28). Rumen epithelium can efficiently absorb lactic acid, electrolytes, water and volatile fatty acids (VFAs). VFAs produced by rumen fermentation can meet more than 70% of the ruminant's energy requirement, and are absorbed across the ruminal epithelium for metabolism in the liver (28).

RUMEN MICROBIOTA

The rumen microbiota, comprised of bacteria, archaea, protozoa and fungi, play an essential role in the digestion of recalcitrant lignocellulosic plant matter. Consequentially, examination of this microbiota has been of interest for many years. Wilson and Briggs applied a counting based method in 1955 by obtaining material from diluted rumen content and found that there were about 10^8–10^{10} microorganisms per gram in rumen contents (36). Wilson and Briggs results were similar to a more recent study, in which the mean population densities of bacteria, Archaea, protozoa, and fungi were reported as 10^{10}–10^{11}, 10^7–10^9, 10^4–10^6, and 10^3–10^6 (cells/ml rumen conten), respectively (33). This population, however, can be affected by many other factors, such as the time of the day, host, and diet. The microbes of the rumen may be separated into three distinct populational niches: solid adhered, free in the fluid, and attached to the epithelium wall (16). Bacteria are the most diverse microbes in rumen content, and are largely involved in digesting lignocellulosic feed and producing VFAs for host maintenance and growth, most notably acetate, propionate and butyrate (33). *Fibrobacter succinogenes, Ruminococcus flavefaciens*, and *Ruminococcus albus* are some of the main species involved in cellulose and hemicellulose digestion (32). Recently, Henderson *et al.* (13) studied rumen microbiota composition in ruminants from 35 countries using a deep sequencing approach, and found that the dominant microbes in rumen change with diets, host species, and geography. Despite this, their results also showed the existence of a core rumen microbiota, with the 30 most abundant bacterial groups present in over 90% of the samples, regardless of the factors mentioned above. The most abundant bacterial groups in all samples included *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales*.

Methanogenic archaea are also established members of the rumen microbial community, with the phylum *Euryarchaeota* dominating (33). Henderson *et al.* (13) found that rumen archaea are much less diverse than rumen bacteria because the two largest groups, *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* clades, were found in almost all samples, accounting for 74% of all archaea. Together with a *Methanosphaera* sp. and two *Methanomassiliicoccaceae*–affiliated groups, the five dominant methanogen groups comprised 89.2% of the archaeal communities.

Protozoan species of the rumen microbiota are in lower abundance than the prokaryotes, but may account for as much as 50% of the microbial biomass, due to their larger size (34). Protozoa exist in close association with other microbial groups by scavenging oxygen, transferring nitrogen from bacteria to the host, and regulating microbial population through predation (33). The majority of known rumen protozoa have been assigned to one of 12 genera (13). The rumen protozoal community structure has strong host individuality (35).

Anaerobic fungi in the rumen are also significant players in plant fiber digestion, degrading the lignocellulosic biomass by invasive rhizoidal growth and production of polysaccharide–degrading enzymes (18).

Rumen microbes are different in functions, but they also interact with each other by digestion. Fungi can assist bacteria and other microbes by the initial colonization of fiber. Bacteria, fungi and protozoa break down the indigestible lignocellulosic and release hydrogen, which archaea can utilize. Other fermentation end-products, carbon dioxide, formate and methyl-containing compounds are important substrates for methanogenesis by archaea (9).

RUMEN MICROBIOTA AND FEED EFFICIENCY

Because of the importance of rumen microbiota, there are emerging studies focused on understanding its role in feed efficiency in cattle. The first attempt to link rumen microbiota and cattle efficiency was reported by Guan and colleagues, who showed that bacterial profiles detected by fingerprint of L-RFI animals were grouped together, which was distinct from H-RFI animals (12). Guan *et al.*'s study indicated that specific bacterial groups may only inhabit efficient steers and host genetics may play an important role in rumen microbial structure. Hernandez *et al.* and Zhou *et al.* confirmed the difference in rumen bacteria and methanogens between H- and L- RFI beef steers under both low and high energy diets (14,15,37,38). Hernandez *et al.* found the abundance *Eubacterium* spp. was significantly ($P<0.05$) different between RFI groups that were only on the high-energy diet and observed correlations between the abundance *Robinsoniella* sp. and RFI ($P<0.05$) for H-RFI animals (15). Zhou *et al.* found *Methanobrevibacter gottschalkii* was linked to the low-energy diet, whereas *Methanobrevibacter smithii* and *Methanobrevibacter* sp. AbM4 were associated with the high-energy diet (38). For RFI groups in Zhou *et al.*'s study, *Methanosphaera stadtmanae* was detected more frequently in L-RFI animals, and *Methanobrevibacter ruminantium* more likely to appear in H-RFI animals with *Methanobrevibacter smithii* was observed only for H-RFI animals (38). A later study by Carberry *et al.* (6) showed that *Prevotella* abundance was higher ($P<0.0001$) in inefficient animals and other bacterial populations had relationship with different diets.

Archaea in rumen are responsible for methane production by phylum Euryarchaeota, which is usually the only phylum found in rumen (33). Studies have showed that methanogens were greatly affected by different diets (high energy vs low energy), as well as feed efficiency (L-RFI vs H-RFI) (36). Carberry et al. (7) also conducted a study that focused on the rumen methanogen microbiota of cattle divergent for phenotypic RFI across two contrasting diets (high forage vs high grain). Results showed that *Methanobrevibacter* spp. was the dominant methanogens in rumen, with *Methanobrevibacter smithii* being the most abundant species. The abundance of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were detected from the low forage diet group; but irrespective of diet, *Methanobrevibacter smithii* was different between H-RFI and L-RFI animals, which was significantly overrepresented in H-RFI animals.

Other than bacteria and rchaea, researchers have also tried to examine the relationship between cattle rumen feed efficiency and fungi or protozoa. In Carberry *et al.*'s study (6), there was no evidence that the total abundance of fungi could be influenced by feed efficiency or diet, but a positive association between the abundance of fungi and CH_4 emission was observed in the study. Carberry *et al.* (7) also observed a negative relationship between protozoa and propionate concentration and positive relationships between protozoa and butyrate, isobutyrate, and acetate propionate (A:P) ratios. Since decreased A:P ratio is associated with decreased methane emissions, and propionate provides most energy requirement for weight gain as a major contributor to gluconeogenesis (5), improving feed efficiency in cattle may be implemented by removing protozoa (defaunation), thereby increasing propionate concentrations and reducing A:P ratio. However, Newbold *et al.* (26) studied the role of protozoa in rumen and the results suggested that the main drawbacks of defaunation is decreasing feed digestibility, since defaunation could limit the feed intake and feed utilization efficiency.

With the understanding of relation between rumen microbiota and feed efficiency, improving feed efficiency may be implemented by regulating the rumen microbiota. Since cattle with higher feed efficiencies are reported to produce 20% to 30% less CH_4 (33), it was thought that reducing methanogen populations in rumen would lead to the improvement of feed efficiency. However, Zhou *et al.* (38) found that total methanogen population did not correlate with differences in feed efficiency, diet, or metabolic measurements. Li *et al.* (20) tried to reduce the methane production during the fermentation in an *in vitro* continuous culture system (Rusitec) with *Eremophila glabra*, a native Australian shrub. After 33 days fermentation, the results showed that the total gas production, methane and volatile fatty acid concentrations were significantly reduced with the addition of *E. glabra*. The overall methane reduction was 32% and 45% with 150g/kg DM and 250g/kg DM respectively, compared to the control group. Though the total bacterial numbers did not change, the total methanogen population decreased by up to 42.1% (with 400g/kg DM) when compared to the control group. This suggests that reducing methane emission by changing the fibrous substrate is feasible. However, studies to date have shown a trend of short term effectiveness of dietary intervention, and there are practical impediments to on-farm use of ingredients that may be hard to obtain in some regions.

TECHNOLOGY USED FOR STUDYING RUMEN MICROBIOTA

The evolution of next generation sequencing (NGS) technology over the last 10 years has led to a sharp increase in studying the gastrointestinal microbiota in production animals, without the need for time-consuming cultivation studies. The first of the "next generation" sequencing technologies to emerge was 454, commercialized by Roche and based on pyrosequencing mechanism (22). Pyrosequencing can detect the pyrophosphate release upon nucleotide incorporation in real time, the pyrosequencing relies on the

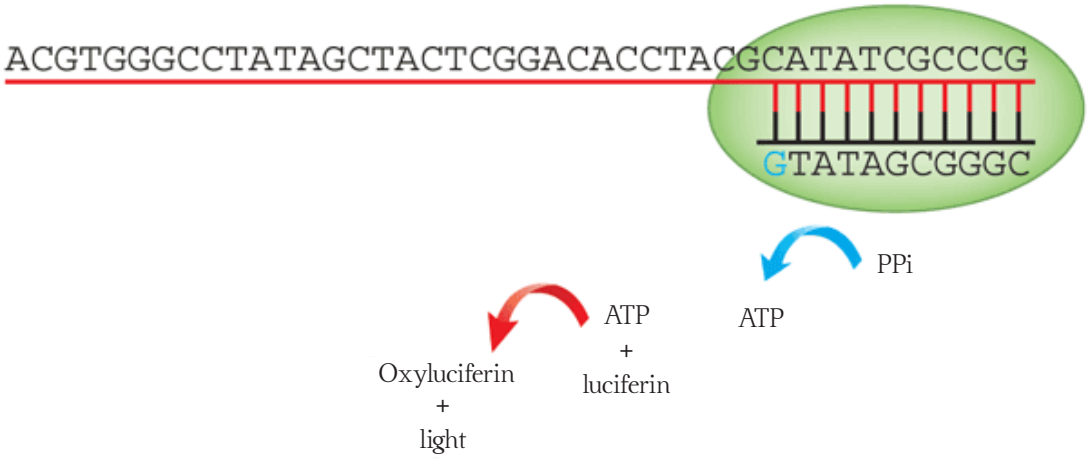

Figure 1. The template strand is represented in red, the annealed primer is shown in black and the DNA polymerase is shown as the green oval. Incorporation of the complementary base (the blue "G") generates inorganic pyrophosphate (PPi), which is converted to ATP by the sulfurylase (blue arrow). Luciferase (red arrow) uses the ATP to convert luciferin to oxyluciferin, producing light (32).

cooperation of four different enzymatic reactions (Fig. 1) (2,32). Pyrosequencing was initially used for single-nucleotide polymorphism (SNP) based genotyping, rather than standard DNA sequencing, because of the short read-lengths (31). 454 sequencing is a parallelized version of pyrosequencing, which was the first NGS available as a commercial product. Such technologies have dramatically increased the throughput of microbiota studies, as hundreds of samples may be multiplexed on a single sequencing run. For example, the latest version of 454 sequencing named "GS FLX Titanium XL+" can provide up to 1,000 base pairs (bps) read length with 700 Mbps throughput in a single 23-hour run, compared to Sanger Sequencing which can only provide 1000-1200 bps each time with the limitation of electrophoresis (24,29). McCann *et al.* (23) reviewed the recent bovine rumen metagenomic publications and found that the Roche 454 FLX platform is commonly used today because of the longer read lengths. However, the increased throughput and lowered cost of Illumina platforms (MiSeq, HiSeq), has led to increases in their use. Illumina platforms also allow for paired end sequencing, as an alternative to the expensive long-read system employed by 454. Fouts *et al.* (10) pointed out that next generation sequencing technologies provide promises to help us better understand how rumen microbial community structure and function affects ruminant feed efficiency, biofuel production, and environmental impact.

Metagenome is the DNA sequence information of a community as a whole (21), which is commonly used today to study rumen microbiota. The complex nature of the rumen environment is difficult to replicate in the laboratory. In determining an accurate rumen microbiome, a whole microbial community database would be the most promising option (25), therefore, a metagenome system is essential in the study. Ross *et al.* (30) developed a reference metagenome to compare rumen metagenomic profiles for individual cattle. When the reads from the study were aligned to a rumen metagenome

reference, rumen metagenome profiles were repeatable (P <0.00001) within sample regardless of the location of sampling rumen fluid. Consequently, determining the accurate microbiome by metagenomics analysis strategy can help researchers study the role of particular groups of microbiome in the whole community and the connection with feed efficiency.

Additionally, metagenomic studies also allow for elucidation of the functional potential of the rumen microbes. While function can be predicted from amplicon studies using bioinformatic tools like PICRUSt, metagenomic investigations will yield definitive data on the fibrolytic enzymes encoded by the ruminal metagenome (19). However, while amplicon sequencing has decreased sharply in cost in recent years, metagenomic studies remain, for many people, prohibitively expensive due to the depth of sequencing required.

CONCLUSION

In summary, with the evolution of new molecular biology techniques, researchers can determine more about rumen microbiota composition, function, and its relationship with feed efficiency, as well as attempting to improve feed efficiency by manipulation of rumen microbiome. However, it is still hard to permanently change the rumen microbiome.

While this review has focused solely on literature of the rumen microbiota and its relationship with feed efficiency, future studies should examine microbiota throughout the GIT, as intestinal microbiota also play important roles in feed efficiency. The interaction between the host and resident microbes also warrants further study in terms of feed efficiency. Also, studies of beef cattle rumen microbiota mainly focused on feedlot cattle, the differences between feedlot cattle and free grazing cattle rumen microbiota are still unknown.

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GRAM-NEGATIVE, OXIDASE-POSITIVE BACTERIA IN RAINWATER AND WIND SAMPLES

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ABSTRACT

Gram–negative, rod–shaped, oxidase–positive bacteria, such as *Aeromonas* and *Pseduomonas*, are widespread in the environment. *Aeromonas* are emerging human pathogens associated with extraintestinal and opportunistic infections. Though there are various biological particles known in the atmosphere, these microbial communities are poorly characterized. Bacteria have the ability to remain suspended in the air for prolonged periods of time and can be transmitted through both airborne and droplet means. This study aimed to isolate *Aeromonas* and other similar bacteria from samples from the troposphere in order to learn more about the distribution of these organisms. Eleven precipitation and wind samples were aseptically collected in Abilene, Texas, and plated onto *Aeromonas* Blue Medium plates with and without ampicillin. The 16S rDNA sequences were amplified from 28 Gram–negative, oxidase–positive isolates. The analyzed sequences showed that none of the isolates belonged to the genus *Aeromonas*, but did include *Pseudomonas*, *Sphingomonas*, *Massilia*, *Naxibacter*, *Paracoccus*, *Novosphingobium*, and *Mesorhizobium*, giving clues to the distribution of these organisms. Furthermore, six isolates appeared to be novel species of bacteria, and several more were uncultured before this study.

INTRODUCTION

Aerobiology is the study of the occurrence, dispersion, and passive transport of airborne biological particles, which could be viable or nonviable, and are distributed in and throughout the air (11). A seasonal difference has been found among the amount of culturable bacteria from air samples; the highest concentrations were shown during the summer months (29). Microbial presence in air is an important factor that contributes to the transmission of infectious diseases, as evidence from previous studies has illustrated that pathogenic bacteria in the air have the ability to travel over long distances and come into contact (through inhalation and ingestion) with individuals who had no previous contact with the source of the infectious disease (8). Although hundreds of thousands of individual microbial cells can exist in a cubic meter of air, the diversity, interactions, and distribution of these organisms are poorly understood (4).

There are constant human–microbe interactions, both pathogenic as well as beneficial (17). Various bacteria have the ability to remain suspended in the air for prolonged periods of time and can be

transmitted through both aerosolized or airborne and droplet means (8). Aerosols in the atmosphere have the ability to influence cloud formation and precipitation development, impacting the earth's climate, water cycle, and atmospheric reactions (22,26). There is evidence suggesting that bacteria may be a major factor within the biological aerosols found in the atmosphere (33). The atmosphere has been described as "one of the last frontiers of biological exploration on Earth," which further supports the importance to investigate its impact on microbial life (34).

The water cycle describes the continuous movement of water around and through the earth as its physical state is altered between liquid, gas, and ice. Three percent of the earth's water is found in the atmosphere (22). The lowest part of earth's atmosphere, known as the troposphere, contains microbial communities that are poorly characterized at high elevations and in the air masses above the ocean (6). The poor characterization of these microbes is due to the difficulty in acquiring samples of adequate biomass, which leads to further challenges of finding sufficient DNA to be used for analysis (6).

Rainwater harvesting is becoming an increasingly popular drinking water source. However, the consumption of rainwater could be dangerous due to the potential health risks of chemical and microbiological contamination (1). Though there have been countless studies performed concerning chemical contamination of rainwater, microbial contamination continues to be somewhat unknown due to the difficulty involved in detecting pathogens (16). However, a small number of recent studies have emerged that do confirm the presence of waterborne pathogens in rainwater and air samples, but there is still much to learn (3,16,20).

Little is known about the occurrence and movement of particular genera of bacteria, such as *Aeromonas*, in the troposphere. *Aeromonas* cells are Gram-negative, oxidase-positive, non-spore forming, rod-shaped bacteria that are found in various aquatic habitats. This genus is a pathogen found in fish and other poikilothermic organisms, and is also regarded as an emerging pathogen in humans causing bacteremia, gastroenteritis, cellulitis, and sepsis (21). Our first hypothesis was that we would isolate *Aeromonas* from wind, rainwater, and hail samples because of its ubiquity in aquatic and other environments. However, *Aeromonas* was not found, so our second hypothesis was that we would isolate other unique bacteria with characteristics similar to *Aeromonas*. The second hypothesis was supported in the successful isolation of other Gram-negative, oxidase-positive bacteria such as *Pseudomonas*, *Aurantimonas*, and *Paracoccus*, which could potentially hold more importance than *Aeromonas* and give insight into the microbial communities of the troposphere.

MATERIALS AND METHODS

SAMPLING, MEDIA, AND GROWTH CONDITIONS

Rainwater, wind, and hail samples were collected in Abilene, Texas, from May to June of 2014 and from January to March of 2015. The rainwater and hail were collected using

sterile sampling jars that were set outside away from runoff of roofs and trees. Hail was allowed to melt in the lab at room temperature before processing. The wind was collected using plates of *Aeromonas* Blue Medium (ABM) (14) by standing outside in an open field, without buildings in the way to ensure as pure of a collection as possible, with the plate open against the direction of the wind for five minutes. The temperature, time, wind direction, wind speed, humidity, and dew point were recorded for each day of wind collection. ABM was made since it is not available commercially, and consisted of 4.0 g of soluble starch (Mallinckrodt, St. Louis, MO), 0.25 g of NH^4Cl (Mallinckrodt, Paris, KY), 1.0 g of tryptone (Sigma–Aldrich, St. Louis, MO), 0.5 g of yeast extract (Sigma–Aldrich, St. Louis, MO), 0.04 g of bromothymol blue (Ward's Natural Science, Rochester, NY) 15.0 g of agar powder (Alfa Aesar, Ward Hill, MA), 1 L of distilled water and was adjusted to a pH of 8.0 using 1M KOH. After autoclaving and cooling, 0.1 g of sodium desoxycholate (Sigma Chemical Company, St. Louis, MO), 5.0 mL of 0.41% L–tryptophan, and 5.0 mL of 0.99% L–phenylalanine were added and mixed. ABM was made with and without 32 µg/ml ampicillin (GBiosciences, St. Louis, MO). Ampicillin was added to the plates in later collection dates in order to select for *Aeromonas* and against *Pseudomonas* since many *Aeromonas* strains have chromosomally encoded β–lactamases and pseudomonads typically do not (14).

After collection of wind–generated samples, the ABM plates were incubated aerobically at 30°C for 24 to 72 hours for colony growth. The rainwater and melted hail were diluted in 0.85% NaCl to obtain countable colonies and spread onto ABM plates with and without ampicillin and also incubated at 30°C for 24 to 72 hours, depending on how quickly colony growth occurred.

After growth on the ABM plates, individual colonies were chosen and subcultured onto 0.2X Tryptic Soy Agar (TSA) plates and incubated at 30°C for 24 to 48 hours. 0.2X TSA plates consisted of 8.0 g of TSA (Carolina, Burlington, NC) and an additional 12.0 g agar per liter of medium. Cultures were subcultured until pure cultures were obtained.

SCREENING AND AMPLIFICATION OF 16S RDNA FROM SELECTED ISOLATES.

Each presumptive aeromonad colony was tested for a positive oxidase reaction using OxiStrips (Hardy Diagnostics, Santa Monica, CA). Each colony that was oxidase–positive was kept for further testing, since *Aeromonas* species have cytochrome c oxidase in their electron transport chains. Then, they were Gram stained. Gram–negative, oxidase–positive rods were selected for amplification by colony PCR and sequencing of the 16S rRNA gene. The protocol is as follows: 5.0 µl of 10X Standard *Taq* Reaction Buffer (New England BioLabs Inc.), 1.0 µl of 10 mM dNTPs, 1.0 µl of 10 µM 27Fm Forward Primer 5'–AGAGTTTGATYMTGGCTCAG–3' (9) (Invitrogen, Carlsbad, CA), 1.0 µl of 10 µM 1492R Reverse Primer, 5'–TACCTTGTTACGACTT–3' (9) (Invitrogen, Carlsbad, CA), 0.25 µl of NEB *Taq* DNA Polymerase (New England BioLabs Inc.), 41.75 µl of Nuclease–free water (Invitrogen; Grand Island, New York) and template in the form of a colony, which totaled to 50 µl per reaction. Several of the genes from isolates could not be amplified using the colony PCR protocol, so pure DNA was extracted as previously described (30). The thermocycling conditions are as follows: initial denaturation: 95°C for 5 minutes,

30 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, 68˚C for 1 minute 30 seconds followed by a final extension of 68˚C for 5 minutes. The products were visualized on a 0.8% agarose (Invitrogen; Carlsbad, CA) gel containing ethidium bromide. Products of 1.5 kb were purified using DNA Clean & Concentrator (Zymo Research Corporation, Irvine, CA) and sent to DNA Analysis Facility on Science Hill at Yale University (New Haven, CT) for sequencing.

DNA SEQUENCE ANALYSIS.

The National Center for Biotechnology Information (NCBI) BLASTn tool and Ribosomal Database Project (RDP) SeqMatch tool were used to search nucleotide databases and to analyze and interpret the results of each gene that was sequenced. Two databases were used in order to maximize the chance of finding accurate species identifications. Of the NCBI BLASTn tools, the following information was recorded for each sequenced sample: genus, species, strength (e-value), and identification number (2). Of the RDP SeqMatch tool, the following information was also recorded for each sequenced sample: genus, species and the strength (S_ab score) (19).

RESULTS

Table 1. Weather parameters at the time of rainwater sample collections. A "/" between NCBI and RDP genus names is shown when the two databases gave conflicting identifications.

Date	Genera Isolated	Low Temperature	High Temperature	Total Rainfall
2014-05-13	*Pseudomonas*	17.2ºC	8.9ºC	0.05 cm
2014-05-23	*Paracoccus, Pseudomonas*	28.3ºC	18.9ºC	0.61 cm
2014-05-25	*Massilia/Naxibacter, Pseudomonas*	23.3ºC	17.2ºC	2.39 cm
2015-01-21	None	0.6ºC	9.4ºC	7.20 cm
2015-01-31	*Agrobacterium/Aurantimonas, Mesorhizobium, Pseudomonas*	6.7ºC	12.2ºC	3.60 cm

The rainfall on the days rainwater was collected varied from 0.05 cm to 7.20 cm, with temperatures ranging from 0.6 ºC to 28.3 ºC (Table 1). Wind samples were collected only in May in winds ranging from 4.8 kph to 22.5 kph in temperatures from 22.8 ºC to 28.3 ºC. Wind direction, humidity, and dew point were also recorded and shown in Table 2.

After isolating, subculturing, testing for oxidase, and Gram staining, 28 DNA sequences from presumptive aeromonads were analyzed. None of these isolates were obtained from the hail sample, as the sample did not yield any bacteria with the phenotypes of interest. As shown in Table 3, of those 28 sequences, no isolates belong to the genus *Aeromonas*. However,

Table 2. Weather parameters at the time of wind sample collections. A "/" between NCBI and RDP genus names is shown when the two databases gave conflicting identifications.

Date	Genera Isolated	Time	Temperature	Wind Direction	Wind Speed	Humidity	Dew Point
2014-05-20	*Novosphingobium*/ *Sphingomonas*, *Pseudomonas*	11:00	26.1ºC	South	22.5 kph	48%	14.4ºC
2014-05-22	none	11:00	26.7ºC	South	19.3 kph	56%	16.1ºC
2014-05-23	*Novosphingobium*/ *Sphingomonas*	11:00	22.8ºC	South	20.9 kph	68%	16.7ºC
2014-05-26	none	16:00	28.3ºC	South-Southwest	4.8 kph	44%	15.0ºC
2014-05-27	none	11:00	23.3ºC	North	4.8 kph	68%	17.2ºC
2014-05-28	none	11:00	24.4ºC	North	22.5 kph	58%	16.1ºC

the NCBI BLAST database resulted in the following number and identities of bacteria: 14 *Pseudomonas*, 6 various bacteria that were previously uncultured and only identified through environmental sequencing methods, and 8 other bacteria: *Massilia varians*, 2 strains; *Paracoccus* 'Mali 27', 1 strain; *Novosphingobium* clone SeqSEEZ199, 1 strain; *Mesorhizobium opportunistum*, 2 strains; *Agrobacterium* H13-3, 1 strain; and *Paracoccus* QUEBA07, 1 strain. As a comparison, analysis of the sample using the RDP database resulted in the following number and identities of bacteria: 16 *Pseudomonas*, 4 various previously uncultured bacteria, and 8 other bacteria: *Naxibacter* 6981, 2 strains; *Sphingomonas* 44/40, 3 strains; *Aurantimonas ureilytica*, 2 strains; and *Rhizobium* Gls-4, 1 strain. The highest S_ab score for the collection of sequences was 1.00 while the lowest was 0.520 (for Isolate 6 and Isolate 23). The S_ab signifies that the sequences in this study had unique 7-base oligomers shared between the sample sequence and a given RDP sequence (which was then divided by the lowest number of unique oligonucleotides in either of the two sequences).

Data were analyzed based on date of sampling, source of isolation, and species identification (Fig. 1). *Pseudomonas* species were found both in rainwater and wind and both in May and January. Paracocuss and *Massilia/Naxibacter* and *Novosphingobium/ Sphingomonas* were only isolated in May, while *Agrobacterium/Rhizobium* and *Mesorhizobium/Aurantimonas* were isolated in January.

Table 3 (opposite page). NCBI BLAST Database and RDP Database Comparison for Sequence Samples. Isolates KEP1-KEP13 were isolated on *Aeromonas* blue medium without ampicillin and isolates KEP24-KEP28 were isolated on *Aeromonas* blue medium supplemented with 32 µg/ml ampicillin. All strength (E-value) scores were ≤ 2 × 10-165. Isolates with a star are those thought to be novel species. Uncultured refers to strains that were previously identified through environmental sequencing methods only.

	NCBI BLAST Database		RDP Database	
Isolate	Genus and Species	Identity	Genus and Species	Strength (S_abscore)
KEP1: Wind	*Pseudomonas putida*	99%	*Pseudomonas putida*	0.969
KEP2: Wind	Uncultured *Novosphingobium* clone *SeqSEEZ199*	99%	*Sphingomonas* 44/40	0.949
KEP3: Wind	Uncultured *Novosphingobium* clone *SeqSEEZ199*	99%	*Sphingomonas* 44/40	0.942
KEP4: Wind	Uncultured *Novosphingobium* clone *SeqSEEZ199*	99%	Uncultured *Sphingomonadaceae*	0.942
KEP5: Rain	Uncultured *Pseudomonas*	100%	*Pseudomonas fulva*	0.98
KEP6: Rain*	*Pseudomonas plecoglossicida*	89%	*Pseudomonas* CPA30	0.520
KEP7: Rain	*Pseudomonas* MC83	99%	*Pseudomonas* MC83	0.974
KEP8: Rain	*Massilia varians*	97%	*Naxibacter* 6981	0.870
KEP9: Wind	*Pseudomonas* JSM 2215099	100%	*Pseudomonas fulva*	0.997
KEP10: Rain	*Pseudomonas fulva*	100%	*Pseudomonas* 471-1	0.997
KEP11: Rain	*Pseudomonas* 9DLP	99%	*Pseudomonas* PSB1	0.992
KEP12: Rain	*Paracoccus* QUEBA07	99%	Uncultured Proteobacterium	0.974
KEP13: Rain	*Paracoccus* 'Mali 27'	99%	Uncultured Proteobacterium	0.962
KEP14: Wind	*Novosphingobium* clone SeqSEEZ199	99%	*Sphingomonas* 40/40	0.949
KEP15: Wind	*Pseudomonas putida*	99%	*Pseudomonas putida*	0.969
KEP16: Wind	Uncultured *Novosphingobium* clone SeqSEEZ199	99%	Uncultured *Sphingomonadaceae*	0.942
KEP17: Rain	*Pseudomonas* MC83	99%	*Pseudomonas* MC83	0.974
KEP18: Rain	*Massilia varians*	97%	*Naxibacter* 6981	0.870
KEP19: Rain	*Pseudomonas* JSM 2215099	100%	*Pseudomonas fulva*	0.997
KEP20: Rain	*Pseudomonas fulva*	100%	*Pseudomonas* U471-1	0.997
KEP21: Rain	*Pseudomonas* 9DLP	99%	*Pseudomonas* PSB1	0.992
KEP22: Rain	Uncultured *Pseudomonas*	100%	*Pseudomonas fulva*	0.983
KEP23: Rain*	*Pseudomonas plecoglossicida*	89%	*Pseudomonas* CPA30	0.520
KEP24: Rain*	*Mesorhizobium opportunistum*	94%	*Aurantimonas ureilytica*	0.911
KEP25: Rain*	*Mesorhizobium opportunistum*	94%	*Aurantimonas ureilytica*	0.916
KEP26: Rain	*Agrobacterium* H13-3	99%	*Rhizobium* Gls-4	0.972
KEP27: Rain*	*Pseudomonas syringae*	96%	*Pseudomonas fluorescens*	0.876
KEP28: Rain*	*Pseudomonas syringae*	93%	*Pseudomonas* S1(2011b)	0.552

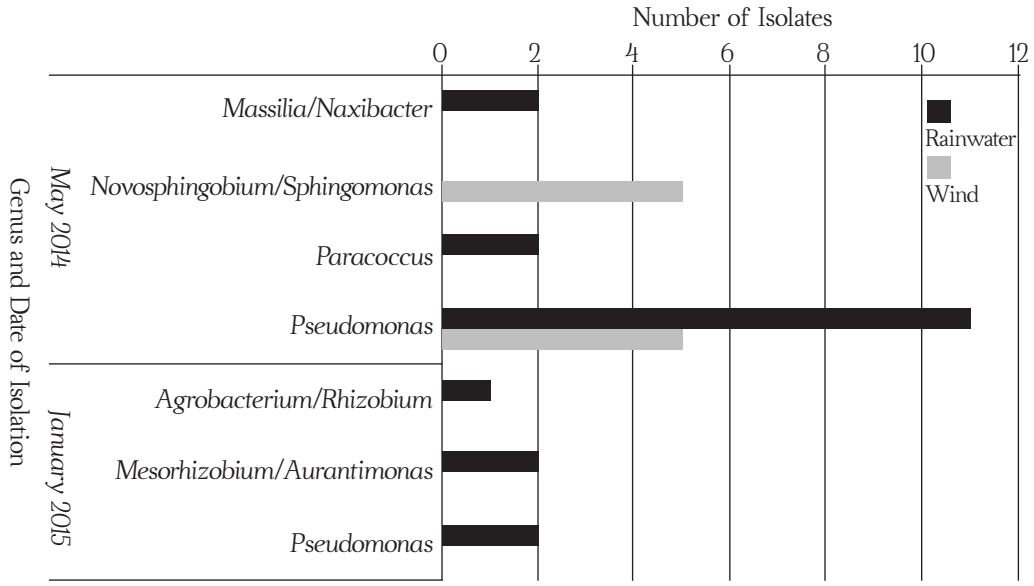

Figure 1. Number of isolates with regard to date of sampling, source of isolation, and identification. Only isolates with the phenotypes of interest that were further characterized are included in this graph. A "/" between NCBI and RDP genus names is shown when the two databases gave conflicting identifications.

Both databases resulted in identification of previously uncultured bacteria. Thus, this study resulted in successfully culturing those bacteria. For the NCBI BLAST database identifications, three of the six total previously uncultured bacteria were *Novosphingobium* (Table 3).

Of the samples that were sequenced, the identities of six of the samples appear to be new species (or at least type strains) of bacteria. According to the NCBI BLAST Database, these presumptive new species ranged from 89% to 96% identity to known species (Table 3). These isolates could be representatives of previously unknown species since their species identities are less than 97% identical to known species (9).

DISCUSSION & CONCLUSIONS

There are many reasons why *Aeromonas* may not have been found in the samples: 1) the lack of a proximal lake or stream as an initial source of *Aeromonas* prevents detection of these types of cells, 2) *Aeromonas* may not be able to survive in the atmospheric or wind conditions of the study, 3) *Aeromonas* may be present in the precipitation, but the methods were not sensitive enough to detect them, and finally, 4) the microbe may not be present at all in precipitation.

Even though we did not find *Aeromonas* as we had hypothesized we would, we did find other interesting strains with a

diversity of implications. The majority of the isolates found were from the genus *Pseudomonas*, including *P. putida* (Isolate 1), *P. plecoglossicida* (Isolate 6, Isolate 23), *P. fulva* (Isolate 10, Isolate 19, Isolate 20, Isolate 22), *P. syringae* (Isolate 27, Isolate 28), and *P. fluorescens* (Isolate 27) (Table 3). Members of the genus *Pseudomonas* are often found in water and soil ecosystems, so their abundance among the samples collected in this study is not surprising (23,24). However, since members of this genus have also been associated with the transmission of diseases in humans, animals, and plants, the possibility of coming into contact with pathogens through rainwater or wind exists. (7).

Another significant characteristic of pseudomonads is that they are thought to play an important role in the atmosphere. *P. syringae* is associated with bioprecipitation. Bioprecipitation is a feedback cycle in which land plants produce airborne particles or aerosols containing microorganisms that impact the formation of clouds by their ice nucleation ability. This feedback cycle leads to rainfall that benefits both plant and microbial growth (25). The microorganisms that are encapsulated within these airborne particles are found in the center of snow and rain on five continents, with many of these microbes appearing in agricultural regions that are increasingly farmed. It is thought that the bacterium *P. syringae* is "the most prolific ice nucleator" because it can act within a set of warm atmospheric temperatures and may also cause other nucleators to lose their abilities (5). Furthermore, as a plant pathogen, *P. syringae* damages certain agricultural crops through the utilization of its ice making capabilities (5).

As stated before, pseudomonads are found in both soil and water. Since wind speeds ranged from 4.8 kph to 22.2 kph when the wind samples were collected (Table 2), it is possible for particulates from the ground to be swept up and included in the samples. Many of the sequenced isolates include genera often found in soil environments. Some of these isolates were identified as genera previously known to fix nitrogen, such as a *Pseudomonas* (12). Another bacterial isolate found that is also important in the nitrogen cycle was *Paracoccus*, which has previously been reported to reduce nitrate (13). As mentioned before, there were several bacteria whose closest sequence matches were previously identified through environmental sequencing but were not cultured until this study. Interestingly, two of these strains have previously been reported to fix nitrogen, a member of the order Rhizobiales and *Novosphingobium* (15,28). *Sphingomonas* was also found and is a genus known for its diversity of nitrogen-fixing capabilities as well as its association with plants and their roots (27).

Other soil bacteria of the genus *Naxibacter* and *Massilia* were also found among the isolates (35). Members of the *Massilia* genus are not only closely related to members of the *Naxibacter* genus, but both genera have been isolated from air samples in another study (32).

It was also no surprise that *Aurantimonas* was also found, since species within this genus have been isolated from air samples collected in the Republic of Korea (31). Finding *Aurantimonas*, *Naxibacter*, and *Massilia* suggests that there may be an important ecological role of these genera in air. Other isolates not usually isolated from air that were found in our study belong to the genus *Sphingomonas*. Members of this genus form biofilms within rainwater harvesting tanks that act as a bio-control agent and natural filter by removing

contaminants and bacteria from rainwater (18). These explanations illustrate that the isolates discovered in this study have unique capabilities and can survive amongst various environmental pressures.

Expanding both frequencies of collection and diversity of seasons will improve future research in this area. Wind samples were only taken in the summer. With samples taken throughout the year, this could increase the diversity of microbes found. As there are different forms of precipitation such as ice, snow, hail, sleet, and fog, collecting these forms of water will improve future research and allow for comparison between the microbes found in each state of water.

This study revealed the presence of interesting bacterial strains present in the troposphere. We found organisms important in bioprecipitation as well as those that have the potential to be important in the nitrogen cycle that are typically found in soil. In this study, we easily isolated previously uncultured bacterial strains as well as up to six novel species. This indicates that there is much to discover about the troposphere and how microbes are moved from environment to environment via transpiration and through updrafts of particulate matter from the terrestrial environment.

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BACTERICIDAL EFFECTS OF LOW-IRRADIANCE LOW LEVEL LIGHT THERAPY ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS IN VITRO*

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ABSTRACT

Low Level Light Therapy (LLLT) within the visible blue spectrum (400–470 nanometers) is a well-documented therapeutic alternative to combat multidrug resistant organism infections through the generation of reactive oxygen species (ROS). However, one shortcoming of LLLT is that many studies deliver therapy through high powered lasers and lamps. High powered light sources not only require specialized staff to operate, but they also deliver the total light dose (fluence) at an exceptionally high intensity, or irradiance, which could consequently deplete the oxygen supplies required to promote LLLT's bactericidal properties. To overcome these faults, low-irradiance LLLT, or delivering the same total fluence of LLLT over an extended period of time with decreased irradiance was evaluated *in vitro*. To further explore this alternative approach, the bactericidal effects of low-irradiance (10.44 mW/cm^2) LLLT using wavelengths of 405-nm, 422-nm and 470-nm were studied on methicillin-resistant *Staphylococcus aureus* (MRSA) cultures. Among these wavelengths, it was determined that 405-nm LLLT provided the most effective reduction of bacterial load at the lowest total fluence (75 J/cm^2) (94.50% reduction). The bactericidal effects of 405-nm low-irradiance LLLT were then further studied by treating MRSA cultures to 75 J/cm^2 LLLT while using irradiances of 5.22 mW/cm^2 and 3.48 mW/cm^2. It was concluded that there was a greater reduction of MRSA bacterial load when samples were exposed to irradiances of 5.22 mW/cm^2 (95.71% reduction) and 3.48 mW/cm^2 (99.63% reduction). This study validates the bactericidal properties of low-irradiance LLLT on MRSA, and subsequent studies should be completed to optimize its full therapeutic potential.

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KEYWORDS

- Photoinactivation
- bactericidal
- *MRSA*
- Low Level Light Therapy

INTRODUCTION

The plight of antibiotic resistance, partially due to the medical community's overreliance of antibiotics, has led to efforts by healthcare systems to determine alternative strategies for antimicrobial treatments. In the United States, there are more than 2 million cases of hospital-acquired infections (HAIs) annually, resulting in annual costs of $20 billion and nearly 100,000 deaths (16,26). Methicillin-resistant *Staphylococcus aureus*

(*MRSA*) is one of the prominent pathogens involved with both HAIs and community acquired infections (CAIs). The prevalence of an individual within the intensive care unit who develops either a HAI or CAI due to *MRSA* are greater than 55% and 59%, respectively (10). *MRSA* has been able to ascend to its present status as a predominant infective agent due to its ability to develop high levels of resistance to several classes of antibiotics through different pathways, including mutation, conjugation, transduction, and transformation (2).

There are two distinct sources of *MRSA*: (1) hospital-associated *MRSA* (HA-*MRSA*), which are limited to clinical settings, and (2) community-associated *MRSA* (CA-*MRSA*), which are found in a multitude of environments and settings, including beaches, computer keyboards, schools, gyms, athletic fields, and locker rooms. (5-7). *MRSA*, an opportunistic pathogen, is not only responsible for localized trauma or postoperative infections, but can also be life-threatening, causing bacteremia, endocarditis, sepsis or toxic-shock syndrome (11,13,17). Research also suggests *MRSA* is one of the primary pathogens responsible for failure of surgical implants or prosthetics due to biofilm formation at the soft-tissue-implant interface, which compromises patients' activities of daily living (ADLs), as well as causes re-implantation to eliminate the bacterial load (28).

A long-term solution to better manage multidrug-resistant organism infections is paramount. Low Level Light Therapy (LLLT), also referred to as "biostimulation," is the process of illuminating tissues with a precise wavelength of light over a specific period of time. More specifically, the use of high energy (lower wavelength) waves in the ultraviolet (UV) and visible blue spectra for LLLT have shown more consistent results when it comes to bactericidal effects and reduction of bioburden. The capabilities of UV (100-400nm) LLLT to sterilize wound surfaces has been well-documented (3,8-9,15). Dai *et al.* investigated the potential of prophylactic UV (200-280nm) light treatment for infections developing in superficial cutaneous mouse wounds contaminated with both *MRSA* and *Pseudomonas aeruginosa* (9). For both bacterial infections, UV LLLT significantly reduced the bacterial burden in comparison to untreated wounds, while also increasing the survival rate of *P. aeruginosa* infected mice (58%) and wound-healing rate of *MRSA* infected mice (31%) (9). However, a limitation to UV LLLT is that it is toxic and carcinogenic in tissues with extended exposure (3,8-9,15).

In order to overcome the collateral damage associated with UV LLLT, the use of wavelengths in the visible blue spectrum (400-470-nm) have been proven to be efficacious for both their bactericidal and wound healing capabilities *in vitro* and *in vivo* (1,10,19-20,23,27). While UV LLLT's bactericidal properties are induced through DNA damage, the mechanism of visible blue light involves the photoexcitation of endogenous porphyrins within bacterial species, which is much less detrimental to healthy mammalian cells (1,10,19-20,23, 27). Subsequently, this excitation initiates the formation of reactive oxygen species (ROS), which are toxic to bacterial cells and biofilms, without compromising the DNA of adjacent cells. Maclean *et al.* investigated the bactericidal effects of visible (405-nm) light using a high-intensity xenon lamp, and illustrated that the 405-nm array had a phototoxic effect on a variety of bacteria that are highly prevalent in CAIs and HAIs, including Gram-positive bacteria: *MRSA*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Clostridium perfringens*, and

Gram-negative bacteria: *Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumonia* (19).

The first underlying objective of this study was to compare the bactericidal efficacy of LLLT on cultures of *MRSA* using three specific wavelengths (405-nm, 422-nm and 470-nm) within the visible blue spectrum. Additionally, the objective of this study was to evaluate the efficacy of low-irradiance LLLT, or the concept of delivering the same total light dose, or fluence, of LLLT at a lower intensity, or irradiance, over an extended period of time. This concept contradicts standard LLLT where therapy is administered at a considerably high irradiance (>40 mW/cm^2) and delivered over a period of seconds to 15 minutes (10, 14).

Based on these objectives, this *in vitro* study was conducted in two separate phases. In phase 1, the bactericidal properties of 405-nm, 422-nm and 470-nm LLLT were evaluated to determine which wavelength provided the greatest bacterial load reduction at the lowest possible fluence. MRSA samples were exposed to these three wavelengths at an irradiance of 10.44 mW/cm^2, which is substantially lower than precedent studies (10, 14). After determining the optimal wavelength and the precise fluence, phase 2 was conducted by evaluating the efficacy of low-irradiance LLLT at the optimal wavelength, but at irradiances of 5.22mW/cm^2 and 3.48 mW/cm^2, and durations of 4 and 6 hours. Given the recent advancement of implantable and portable LLLT devices (4, 25), the process of administering LLLT over a period of multiple hours has the potential to be a cost-effective yet clinically feasible alternative to conventional LLLT.

Despite the fact LLLT's bactericidal effects have been well documented within all wavelengths of the visible blue spectrum, it is hypothesized that 405-nm LLLT will provide the greatest bactericidal response, due to the higher energy levels associated with the shorter wavelength photons. Furthermore, it is expected that low-irradiance LLLT will provide a greater bactericidal rate when compared to delivering the same LLLT fluence at a higher-irradiance, because of the fact that visible blue LLLT is dependent upon oxygen supplies for ROS formation. Consequently, low-irradiance LLLT will not deplete oxygen supplies as rapidly and therefore provide greater treatment efficacy.

MATERIALS AND METHODS

STUDY DESIGN:

During phase 1, cultures of *MRSA* were exposed to three separate wavelengths of visible blue light (405-nm, 422-nm and 470-nm) at an irradiance of 10.44 mW/cm^2. The cultures were exposed to LLLT for a total of 8 hours. However, aliquots from each of the exposed cultures were taken in two-hour intervals (t=2,4,6,8 hours), which was the equivalent of LLLT doses of 75 J/cm^2. These aliquots were then quantified to determine the number of colony forming units per milliliter (CFU/mL) through a standard plate counting technique using a Whitley Automated Spiral Plater (WASP). Table 1 outlines the total fluence of LLLT each culture received at each of the two-hour intervals.

Upon completion of phase 1, a prospective analysis was conducted to determine which wavelength would provide the greatest bacterial load reduction at the lowest possible fluence. After determining these conditions, phase 2 was initiated to evaluate the effects of maintaining the aforementioned optimal fluence, but further reducing the irradiance and increasing the total exposure time of LLLT to 5.22 mW/cm^2 (4 hour exposure) and 3.48 mW/cm^2 (6 hour exposure).

BACTERIAL ISOLATES:

The *MRSA* strain that was used in this procedure was ATCC(r) BAA-1761 (ATCC, Manassas, VA). A loopful of frozen inoculum from -80°C frozen stocks was used to quadrant streak onto the surface of a tryptic soy agar (TSA) plate. Each plate was then incubated at 37°C and 5% CO_2 for 24 hours to produce isolated and pure cultures of *MRSA* for this study.

BIOSAFETY PRECAUTIONS:

Throughout the course of the procedure outlined within this experimental study, adequate biosafety measures were taken to comply with the fact that *MRSA* is classified under the Biosafety Level 2 Risk Group. In addition to wearing laboratory coats, gloves and eye protection, all facets of this procedure were conducted using aseptic technique within a Class II laminar flow biosafety cabinet.

LIGHT EMITTING DEVICE:

The LED light sources used during Phase 1 of this study were LEDs at 405±5-nm, 422±5-nm and 470±5-nm wavelengths (Visual Communications Company, Poway, CA). The light-emitting device was constructed by assembling LEDs onto an EIC-108 3220 Tie-Points Solderless Breadboard (EIC Laboratories INC., Norwood, MA). A Labnet Power Station 300 power supply (Labnet International, Edison, NJ) was connected to provide the desired voltages to emit the necessary irradiance. 24 LEDs were assembled onto each breadboard, and were then positioned directly beneath one well on a 24-well plate, so each well would be illuminated by a single LED. To ensure that each well was receiving identical irradiances of blue light, the overall irradiance that each well was illuminated by was measured using a THORLABS Optical Power Meter (Thor Laboratories, Newton, NJ).

PHOTOINACTIVATION PROCEDURE:

MRSA was grown for 24 hours at 37°C and 5% CO_2 in tryptic soy broth (TSB). After isolating colonies of *MRSA*, a single colony of *MRSA* was suspended in TSB to grow for 24 hours. Following incubation, aliquots of the cultures were serially diluted in sterile saline to a final dilution factor of 1:10,000 (TSB suspended *MRSA* culture: Saline). This dilution was completed in order to quantify the bacteria within the constraints of the Whitely Automatic Spiral Plating system outlined below. The concentration of *MRSA* in the diluted cultures was confirmed through monitoring their optical density (OD_{600}) at 600-nm.

275.0 µL of the diluted *MRSA* cultures were loaded into each well of a Corning® Costar® 24-Well Flat Bottom Cell Culture Plate with a Total Well Volume of 3.4 mL (Corning Inc, Corning, NY) to receive light exposure. Throughout the course of this experiment, there were four separate trials conducted to ensure consistency, and to evaluate both the intraplate and interplate bactericidal effects of the low-irradiance LLLT. During each individual trial in phase 1, there were four experimental cohorts where *MRSA* cultures received either: 1) no light exposure (control); 2) 405-nm LLLT; 3) 422-nm LLLT; or 4) 470-nm LLLT. For each cohort that received

LLLT, the light was administered at a fixed irradiance of 10.44 mW/cm^2. During phase 2, there were also found experimental cohorts where *MRSA* cultures received either: 1) no light exposure (control); 2) LLLT at 10.44 mW/cm^2 over 2 hours; 3) LLLT at 5.22 mW/cm^2 over 4 hours; or 4) LLLT at 3.48 mW/cm^2 over 6 hours. During phase 2, cultures that received LLLT were treated with the optimal wavelength determined during phase 1. With the exception of the light wavelengths from the LED source used on the experimental groups, each trial was completed at room temperature in total darkness to eliminate confounding variables from external light sources. Additionally, in order to minimize the risk for contamination, the well plates remained covered for the duration of the light treatment. Throughout the course of each trial, fifty-microliter aliquots were taken from four randomly selected wells using aseptic technique, although no well was repeatedly sampled, in each well plate during increments of 2 hours of light exposure. These aliquots were then plated onto TSA using the Whitley Automated Spiral Plating system.

STANDARD PLATE COUNT PROCEDURE:

A Whitley Automated Spiral Plater (WASP) (Don Whitley Scientific Limited, West Yorkshire, UK) was used to spiral plate 50 µL of each aliquot onto the surface of a tryptic soy agar plate in an Archimedes spiral. The plates were incubated for 24 hours at 37°C in 5% CO_2. Colony counts were manually performed three times per plate, and the mean value was subsequently used to calculate the bacterial load in units of CFU/mL.

STATISTICAL ANALYSIS

Data throughout the course of this study was analyzed *post-hoc* using one-way ANOVA and Student *t*-tests. Each illumination condition was compared to their control, as well as wavelength differences between groups. P values < 0.05 were considered to be statistically significant.

RESULTS

As illustrated in Table 1 and Fig. 1, 405-nm low-irradiance LLLT provided the greatest total bacterial load reduction of *MRSA* cultures (99.06% reduction [$p < 0.05$]) in Phase 1, when compared to 422-nm (98.84%) and 470-nm (98.71%) LLLT. Furthermore, 405-nm LLLT was the only wavelength that successfully provided a 2.0-log bacterial load reduction (≥ 99.0% kill rate).

While 405-nm LLLT provided the greatest cumulative bacterial load reduction among the eight-hour light exposure, Table and Fig. 1 also suggest there was a greater bacterial load reduction seen from a fluence of 75 J/cm^2 across the first two hours of 405-nm LLLT treatment (94.50% [$p < 0.0001$]) than what was seen from a fluence of 150 J/cm^2 across the first four hours of 422-nm LLLT (83.47%), as well as 225 J/cm^2 across the first six hours of 470-nm LLLT (93.95%), respectively. Based on these observations, phase 2 of this study was conducted using the optimal LLLT wavelength of 405-nm at a fluence of 75 J/cm^2.

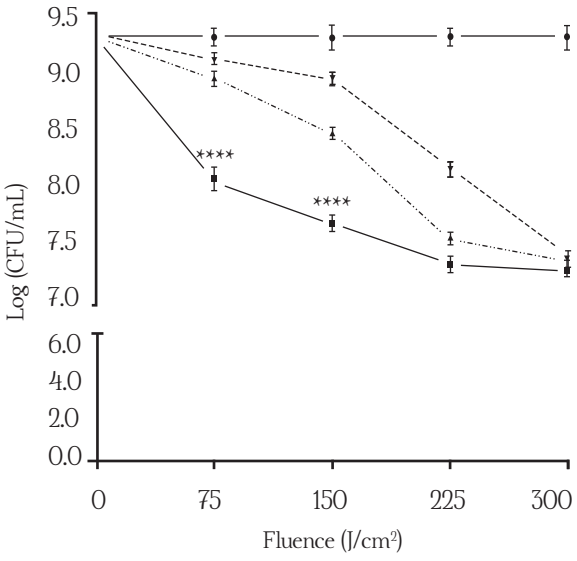

Figure 1. MRSA Colony Forming units (CFUs) Post-Low Level Light Therapy This figure is a graphical depiction of mean bacterial load reductions of MRSA following 405-nm, 422-nm and 470-nm LLLT relative to the total LLLT fluence received. Each experiment was completed at a fixed irradiance of 10.44 mW/cm^2. MRSA samples were collected in increments of 75 J/cm^2 (every 2 hours of the 8-hour treatment) of LLLT. Error bars represent standard error of collected samples. Asterisks (****) signify p-value < 0.0001 following ANOVA to analyze significant differences between experimental wavelengths.

Fluence (J/cm^2)	405-nm		422-nm		470-nm	
	log (± SEM)	Percent Change	log (± SEM)	Percent Change	log (± SEM)	Percent Change
75	8.06 (± 0.03) [p < 0.0001]	94.50%	8.93 (± 0.02)	54.32%	9.10 (± 0.02)	36.87%
150	7.67 (± 0.02) [p < 0.0001]	97.53%	8.46 (± 0.02)	83.47%	8.93 (± 0.03)	53.42%
225	7.31 (± 0.02) [p <0.021]	98.93%	7.54 (± 0.02)	96.92%	8.15 (± 0.02)	93.95%
300	7.26 (± 0.02) [p <0.05]	99.06%	7.36 (± 0.02)	98.84%	7.39 (± 0.02)	98.71%

Table 1. Bacterial Load Reduction of MRSA Samples Following 10.44 mW/cm^2 LLLT: This table indicates the mean bacterial load reductions log(CFU/mL)(± SEM) of MRSA following low-irradiance LLLT at wavelengths of 405-nm, 422-nm and 470-nm. All samples were exposed to light at a constant irradiance of 10.44 mW/cm^2, but received LLLT at varying exposure times and total fluences (J/cm^2). A total of 16 replicates (4 from each experimental trial) of each data point were collected to determine mean bacterial load reductions. P-values signify level of significance differences among the experimental wavelengths using ANOVA. *P*-values < 0.05 are considered significant.

Irradiance (mW/cm^2)	LLLT Exposure Period	Mean Log (± Standard Error)	Mean Bacterial Load Reduction	*P*-value
10.44	2 hours	8.06 (± 0.03)	94.50%	–
5.22	4 hours	7.91 (± 0.04)	95.71%	< 0.023
3.48	6 hours	6.85 (± 0.03)	99.63%	< 0.0001

Table 2: Bacterial Load Reduction of *MRSA* Following Irradiance-Manipulated 405-nm LLLT: This table indicates the mean bacterial load reductions (CFU/mL) of *MRSA* following 75 J/cm^2 of 405-nm low-irradiance LLLT. While all samples received a constant fluence of 75 J/cm^2, the irradiance and exposure times were manipulated from 10.44 mW/cm^2 to 5.22 mW/cm^2 and 3.48 mW/cm^2. A total of 16 replicates (4 from each experimental trial) of each data point were collected to determine mean bacterial load reductions. *P*-values signify level of significance differences among the experimental irradiances using ANOVA. *P*-values < 0.05 are considered significant.

Upon determining the optimal fluence and wavelength of LLLT in Phase 1, Phase 2 was conducted to analyze 405-nm LLLT when delivering the same total fluence of 75 J/cm^2, but through irradiances of 5.22 mW/cm^2 and 3.48 mW/cm^2. By decreasing the irradiances, the duration of each LLLT cycle to receive 75 J/cm^2 was increased from the initial two-hour treatment at 10.44 mW/cm^2 to 4 hours at 5.22 mW/cm^2 and 6 hours at 3.48 mW/cm^2.

Table 2 indicates that there was a correlation associated with a greater bacterial load reduction when an identical fluence of 75 J/cm^2 405-nm LLLT was administered at a lower irradiance and subsequently an increased exposure time. An increased bacterial load reduction was observed when the irradiance was decreased to 5.22 mW/cm^2 (95.71% reduction [$p < 0.023$]) and 3.48 mW/cm^2 (99.63% reduction [$p < 0.0001$]).

DISCUSSION

One significant limitation of conventional LLLT is that the high-intensity light sources that are commonly used to administer therapy are relatively expensive, and require a specialized medical facility and staff to operate and maintain. Consequently, these centers require frequent travel for patients that are looking to receive LLLT, and may not be readily accessible. In order to facilitate introducing LLLT into mainstream medicine, recent studies have analyzed the use of low-power Light Emitting Diodes (LEDs) as an alternative illumination source to the high-powered lasers that are currently used (1, 20, 27).

This *in vitro* study validates the proof of concept of the bactericidal effects of low-irradiance LLLT using visible blue low-powered LEDs on cultures of *MRSA*. Photoinactivation of *MRSA* was achieved through LLLT at wavelengths of 405-nm, 422-nm and 470-nm, as seen in precedent

literature (1,10,19–20,23,27).). However, it was observed that the optimal wavelength for visible blue LLLT was 405–nm ($p < 0.0001$).. The results of this study are consistent with the findings of Bumah *et al.*, which illustrated that 405–nm photoinactivation resulted in a more efficient colony count reduction as opposed to other wavelengths within the visible blue spectrum (6).

While multiple studies have confirmed the efficacy of LLLT at one discrete wavelength or fluence, this study intended to evaluate the photoinactivation rates of multiple wavelengths to determine the optimal wavelength and fluence of LLLT within the visible blue spectrum. As seen in Table 1 and Fig. 1, 405–nm photoinactivation effectively illustrated a more immediate bactericidal effect compared to its 422–nm and 470–nm counterparts. Despite achieving similar bacterial load reductions at each wavelength following the entire 300 J/cm^2 light treatment, 405–nm LLLT resulted in a 94.50% load reduction following its initial 75 J/cm^2 (2 hours) of irradiation. Based on the parameters of this phase 1 study, 75 J/cm^2 of 405–nm blue light was determined to be the optimal blue light dosage, and was therefore held constant throughout phase 2.

Following phase 1, the irradiance (mW/cm^2) at which the light was delivered to the *MRSA* cultures was manipulated during phase 2 to determine if a lower, more continuous light exposure could result in a maximum formation of reactive oxygen species (ROS) through a specific fluence. As illustrated in Table 2, low–irradiance LLLT provided a statistically significant improvement [$p < 0.0001$] in reducing the bacterial load of *MRSA*, and should be considered as a viable, therapeutic alternative to high intensity LLLT, which conversely deliversthe total fluence over short durations, ranging from 60 seconds to 10 minutes. Based on this principle, LLLT illumination using a high irradiance light source could potentially deplete oxygen supplies more rapidly than low–irradiance LLLT, and therefore compromise the efficacy of the intervention (4, 38). Future studies using this low–irradiance strategy will add merit to the growing body of evidence of the efficacy and safety of LLLT.

One of the limitations of this study was the inability of the LED apparatus to administer a uniform irradiance of less than 3.48 mW/cm^2 to each of the 24–wells due to a constraint of the power supply used in this experimental setup. Based upon the parameters of this study, subsequent studies should be completed to evaluate the bactericidal effects of LLLT using irradiances lower than the 3.48 mW/cm^2 to determine if there is a minimum threshold to low–irradiance that would consequently compromise efficacy of treatment. Another limitation of the current study was that the bactericidal effects of low–irradiance LLLT were only evaluated on one strain of *MRSA*. Studies completed by Grinholc *et al.* (13) determined after analyzing over 40 distinct strains of *MRSA* that the bactericidal effect of LLLT was strain–dependent, with bacterial load reduction counts ranging from 0% – >99.9% (3 log–reduction) (13). While this study did analyze the effects of LLLT using a light source in the visible red (624–nm) spectrum, subsequent analyses of the bactericidal effects of 405–nm low–irradiance LLLT should be evaluated among various Gram–positive and Gram–negative organisms, and their respective strains.

In addition to subsequent studies strictly analyzing the efficacy of 405–nm low–irradiance LLLT for its bactericidal and wound healing capabilities, there is a growing body of evidence that LLLT using multiple wavelengths has provided

synergistic effects that benefit the overall therapeutic outcomes when compared to using solely monochromatic LLLT (12, 14, 18, 21–22). Studies from Goldberg *et al.* (12) and Lee *et al.* (18) observed the efficacy of visible blue (415±5 nm) and red (633±6 nm) combination light for the treatment of acne vulgaris in a 27 patient study, and determined that phototherapy using multiple wavelengths was an effective strategy to reduce bacterial load as well as accelerate skin healing (12, 18). Translating the efficacy of combination wavelength treatments to other areas of medicine could possibly reduce the potential of oral tissue infection following oral surgery while simultaneously promoting faster healing of gingival tissue or additional soft tissues within the oral cavity. Furthermore, combination wavelength treatments show promise in dermatologic, surgical, and military environments where infection management, tissue rescue, and tissue healing are directly connected to survival or other favorable outcomes.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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PERSPECTIVE

EXPLOITING AN INTERDISCIPLINARY APPROACH USING UNDERGRADUATE RESEARCH

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INTRODUCTION

Coming out of high school, I chose to pursue a career in chemistry on the basis of it being regarded as the "central science". My logic was that if I wanted to change directions, I could easily go into geology, biology, or physics. I knew from an early age that I wanted to go into science for the purpose of doing research, so going to university to pursue a career in chemistry seemed an obvious choice. As a child, I observed that I tended to learn things best by trying to connect new knowledge to things that I already knew, and topics with a "hands-on" component were particularly appealing. What I did not count on, however, was falling deeply in love with chemistry, which was further cemented by my experiences in research, a stint in the chemical industry, and other opportunities to apply bench craft to new challenges. What came as an even larger surprise was that microbiology turned out to be an exciting outlet that would prove to be every bit as rich and interesting as chemistry and that my background in laboratory work and chemistry would serve as a catalyst to further enhance my appreciation of other disciplines. As an undergraduate, my research experiences in both fields altered my way of thinking and the way in which I approach science. I started my university career in the United States majoring in chemistry at a fairly large institution. Two of my professors strongly encouraged me to seek out undergraduate research opportunities and to get involved in education rather than being a passive element in the process of higher education. Heeding their advice, I sought out other opportunities to further my scientific knowledge which turned out to be one of the best things that I could have done for my scientific career.

One problem in pursuing an education in the sciences is the risk of over-specialization; there is an old adage that states that all problems look the same when you are only given one way of approaching problems. Fortunately, a diversity of experiences in different types of professional labs as an undergraduate has shaped my interdisciplinary approach towards tackling research problems while giving a diverse skill set with which to take on new problems. In my 8 years as an

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undergraduate, I have done stints working in two different research groups in organic chemistry, two groups in microbiology, and a three and a half year stretch working as a lab technician in the chemical industry, as well as two years working in a clinical lab. While this may be quite extreme, I strongly believe that even a few limited interdisciplinary adventures can greatly benefit anyone that is serious about a career in science.

When it comes to enriching your education, there are lots of options, most of which provide excellent opportunities to advance your knowledge, enhance your ability to communicate science, or give back to the community. It has been my experience, both personally and now as someone in charge of supervising the research work of others, that real laboratory experience puts you ahead in terms of laboratory performance (i.e. actually standing at a bench and doing science). Here I will go through some of the lessons that I learned being an undergraduate involved in research and cross-sectorial activities that I feel really enhanced my abilities at the bench as I further explore the intersection of chemistry and microbiology.

PACKING YOUR TOOLBOX USING AN INTERDISCIPLINARY APPROACH

One of the analogies that were frequently used in my undergraduate education in the U.S. was the "toolbox". I used every opportunity to learn as many techniques, whether "soft" skills or more specialized methods, as possible and try to apply them to new situations. This is particularly relevant when you are transferring skills from one field of research to another. Over time, I found that this interdisciplinary approach was exceptionally useful and rewarding. Being able to use old knowledge and techniques to acquire new knowledge and solve problems is incredibly rewarding.

Beyond having an interdisciplinary approach, cross-sectorial training is also a highly valuable experience. There is more than one way to do science and the ultimate focus of that science very much informs the way in which day-to-day operations are approached. Experiencing the way a clinician approaches science is often very different from the way someone working in an industrial field would.

The most often used piece of my kit that I picked up somewhere along the way is a little mental experiment called "the truck test". The truck test posits a simple question: could someone pick up and carry on your work from this given moment if you got run down by a truck? The degree of success to which you can answer this question revolves around several soft skills that I have found often go unaddressed in undergraduate (and often graduate) settings. Traditionalists often tote the utility of the laboratory notebook as a tool for reproducing exactly what you have done. Unfortunately, your experiment is more than just what you were doing; it is highly informed by the matrix in which you were doing it.

WHY DO UNDERGRADUATE RESEARCH?

During my undergraduate work in the U.S., I and many others routinely heard about why we should do undergraduate research. Professors often proclaimed that having research on your resume could make or break an application to a graduate program. While this certainly caught the ears of many students thinking about highly competitive careers as a medical doctor or pharmacologists, selling these opportunities to those bound for careers in science seemed to relegate doing science for the sake of science to the back burner. The traditional rationale for doing undergraduate research often includes benefits such as the following:

- Resume building
- Networking
- Money
- Class credit
- Publications
- Letters of recommendation

All of these are nice, but much of this has very little to do with standing at the bench and doing actual science (which did not deter me from getting involved at the earliest opportunity!). I went into science to do science rather than chasing a career with a large paycheck attached to it. In my mind, the real benefits of doing undergraduate research really have to do with why you are presumably studying science in the first place: to learn to do actual science while being tied to a lab bench. Part of doing science is venturing into the darkness of the unknown and trying to figure out what the room looks like and how stuff works.

I am a firm believer that we learn by doing; thus, the best way to learn how to become a scientist is to do science. Working at the bench with a more accomplished master is a fantastic way to learn techniques, carefully plan, and execute experiments, and interpret the results to answer questions and hopefully ask new questions. Some of the often overlooked reasons to pursue undergraduate opportunities include the following:

- Cycle of science (the "real" scientific method)
- Confidence
- Bench skills
- Quality control and streamlining
- Data and project management
- Innovative problem solving
- Communication of scientific concepts
- Cultivating curiosity

THE CYCLE OF SCIENCE

As it turns out, the daily grind of science is quite different from the pre-programmed laboratory experiments that you are exposed to in your general course work or the nicely displayed algorithms of the scientific method. One of the biggest takeaways from my undergraduate research experiences is mastering the workflow of real laboratory work and that successfully applying the scientific method requires a good deal of

creativity. In many ways, the analogy of a craftsman and an apprentice fits quite well.

In undergraduate laboratory exercises, more often than not in my experience, a lot of the thinking and preparatory work has been done for you. Procedures are often step-by-step and have been fined-tuned to a degree of precision that leaves no room for actual "discovery". In real life, the flow of a research project has a "tick-tock" type rhythm whereby the results of one round of experiments directly informs the design of the next set of experiments.

Furthermore, most if not all of the preparatory work has been done for you in advance: plates have been poured, reagents are ready to use, and instruments are in working order and ready to use. This creates the illusion of ease because in the real world, it is unlikely that things are to be in such a state that you can simply walk into the lab and walk out with publishable results. The amount of preparation that goes into executing actual experiments can often take longer than the experiment itself.

When I started my research in Iceland, no one had touched one of the HPLCs in years and no one knew how to operate the new one. Fortunately, the three and a half years that I spent in the chemical industry as an intern lab technician (tied to the HPLC and GCs) taught me everything that I needed to know to strip down an HPLC and get it running in short order. This gave me a tremendous advantage to carry on with my own research projects while supporting the on-going work of researchers in other groups.

CONFIDENCE

The psychological benefits of being professionally active during your undergraduate training are not to be underestimated. Building up your self-confidence to tackle complex, new, and highly involved research projects is critical to your ability to successfully execute laboratory operations. Furthermore, having a track record including a number of failures is also important as it demonstrates that not only is botching a procedure okay, it is often part and parcel of the day-to-day grind of doing research. Even after only having worked a few months one summer in a research lab radically altered the way that I approached standard coursework. As I have transitioned more towards a teaching role, I continue to find that students that engage in research early on in their careers are often much more capable when it comes to tackling their later coursework efficiently as well as conducting actual work with a high degree of reproducibility.

DATA AND PROJECT MANAGEMENT

One of the lessons that you learn from working on a research project is how to manage the planning and execution of a project as well as keeping data organized in such a way that it is actually useable. Without a lot of prior experience, it is easy to become overwhelmed with the scope and interrelatedness of a series of experiments and end up dropping the ball due to disorganization. While the use of laboratory notebooks receives a tremendous degree of credit, in the electronic age, a lot of your data is captured electronically in Excel worksheets or other formats. At least in my experience, data sets in "real world" research projects tend to be quite large with measurements from multiple instruments over time needing to get kept in order. An extremely useful strategy for dealing with the inflow of data is to design an Excel sheet in parallel while designing the experiment. Furthermore, working with an outline of

the report or manuscript and plugging in data and discussions as you go rather than handling data after the fact can be a very efficient way of working with a data set.

COMMUNICATION OF SCIENTIFIC CONCEPTS

Stepping out of the classroom and into the real world of research showed me that scientific writing comes in more flavors than producing reports and scientific articles. In order to be an effective scientist, writing for different audiences is a skill that must be thoroughly mastered. An often overlooked backdoor route to improving your scientific communication skills is to carefully dissect the work of others. This has the advantage of being something that you can start early in your career before you know much of anything but can still be of use even if it is catching a potentially embarrassing grammatical error. Early in my career, tearing apart manuscripts prior to submission was a favorite past time among those in the research groups with which I was involved. When I transferred my focus more towards the biological, I found that my background in chemistry and having English as a native language was very helpful with improving the manuscripts of other more advanced researchers. At some point, revising the work of others lead to conversations regarding ways to improve experimental designs or upgrading methodology to be higher throughput or more robust which eventually lead to my first few co-author credits.

One of the aspects of real-world science that I found most surprising was the grant writing process. I had the (mis)fortune of becoming involved in writing applications for several ideas that I had while I was still an undergrad. The amount of time that goes into constructing well-thought-out grant proposals can typically run into the hundreds of man hours depending on the size of the application and the number of people involved. Thus, learning how to effectively multitask while managing the contributions of others is absolutely essential. One aspect of communicating science was how to organize your ideas into do-able units which are often referred to as "work packages" consisting of well-defined tasks and subtasks. Writing grant proposals that are reviewed by experts and non-experts alike requires careful balance. My earliest experiences writing grant applications were an abysmal failure: I found it difficult to overcome the formal, highly technical writing style that was drilled into my head. After several rejections and heeding the careful comments of peer-review, I started to have more success as I moved through my undergraduate career. Furthermore, writing up your scientific work to be broadly understood by the public is another highly valuable writing skill.

Another often under-emphasized aspect of communicating your scientific work, either verbally or in writing, to the broader public. Effectively communicating the importance of advanced science to laymen with little or no scientific training presents a tremendous challenge. I have found that a good place to start is by talking to friends about your work in such a way as they do not lose interest. This can often be accomplished by talking about the broader implications and impacts of your work. Understanding the impacts of your own work are also of great importance when going before an opponent critically reviewing your thesis work or when an application for funding is being scrutinized by a panel of experts. Another potential target to practice your communication skills include younger school children. While this often involves a healthy degree of generalization and making analogies to things

with which can be related to everyday life, I have often found that if you can adequately explain a scientific concept to a 5 year old, you can probably effectively communicate it to anyone else.

Learning how to communicate visually with effective figures is often a highly useful skill. While data visualization is a topic all by itself, taking the time to explore a few entry level texts (refer to the Further Reading section) can be rewarding. For instance, showing the interrelationship between tasks with a simple flow chart can add clarity to complex experimental designs and quickly confer the just of what you are doing to a reader.

BENCH SKILLS

Standing (or sitting) at a lab bench and performing tasks is the bread and butter of working in a laboratory in any context. While the individual techniques and practices that you learn may ultimately have value, the real benefit stems from your ability to rapidly assimilate a new methodology and put it into practice. I found that having a broad base of analytical techniques to choose from really enhanced my work.

As you spend more time at the bench, you will observe that laboratory life is like a small ecosystem unto itself and one of the most useful elements that I gained from being involved in professional laboratories was becoming attuned to the heart beat of the lab. In many ways, any lab is like an ecosystem with an inflow of consumables and an outflow of data and waste. Furthermore, each piece of equipment has its own "vital signs" and becoming attuned to the needs of each piece of equipment is a lot like the dating process. Many weekends of being alone in the lab troubleshooting instrument problems became a somewhat regular phenomenon. Learning to listen to equipment's "vital signs" is often a good place to start. Being attuned to what something is "supposed to sound like" can often quickly alert you to something being wrong.

Developing solid troubleshooting skills can save costly visits from instrument specialists as well as greatly reduce unnecessary downtime. During slow moments, I often found it useful to flip through the "troubleshooting" and "maintenance" sections of instrument manuals. Also, learning how to keep equipment in proper working order by following a regular maintenance cycle can often prevent problems, especially since many consumable parts wear out in a predicable fashion with regular usage.

DEVELOPING A STREAMLINED APPROACH AND QUALITY MANAGEMENT

During the "undergraduate era" of my career, which spanned clinical, research, and industrial labs, I noticed that turnover had a profound effect on efficiency. Every time that one of the more senior members of a research group left it was like cutting off an arm. Most of the time, it was just basic routine tasks that suffered most, not to mention the specialized knowledge that seemingly evaporated overnight. Highly efficient enterprises (like multinational corporations) tend to treat people like replaceable cogs and have a system in place to deal with retaining knowledge. In my experience, this is something that research groups do quite poorly. Larger research groups seem better able to maintain some sort of procedural continuing while the loss of a senior member in a small research group is devastating. Re-inventing the wheel can be costly and laboratories performing

the same tests as other labs need to be able to demonstrate that they get comparable and highly reproducible results. In an industrial lab, especially one that operates a quality management system (QMS) such as ISO 17025 or ISO 9001, commonly performed procedures have a high degree of standardization; procedures are written and made centrally available. While QMS topics often illicit groans from academics, I learned firsthand their efficacy which was further confirmed as I put lessons from QMSs into practice in my own research.

While ISO is often considered to be too paper heavy for research groups that need to maintain a high degree of fluidity, some of the major concepts can and should be readily integrated into your daily work. ISO principles include using a process approach, systematic approach to management, striving for continual improvement, and taking a factual approach to decision making. Key concepts within these principles are particularly relevant such as maintaining traceability, good records keeping, document control, and employee training.

Standard operating procedures (SOPs) maintained by one person can easily be disseminated to new hires or visiting researchers. Having a system for routine procedures saves time and often money. I have found that having bottles for individual assays or media components with labels that include dummy-proof directions are a fantastic way to go. Not only does it take the guess work out of preparing reagent solutions, the directions are right in front of you. As it turns out, reproducibility problems are the Achilles heel of experimental results. Thus, developing good quality control practices, even in a research setting, is absolutely vital to ensure that you are producing trustworthy results.

Fortunately, you do not need to have completed any education to start filling the gaps without much experience in quality management. After spending a few years working in the chemical industry, I found that my awareness of these procedural gaps was heightened and all that you really need to do is recognize the need to fill a gap. As someone with experience with a particular method, you can start writing SOPs and specific methods yourself and make them centrally available to your group. I have observed countless times in my work in research labs so-called "procedural drift" whereby a specific procedure is passed down from senior researcher to new inductee, who will then transfer their version of a procedure to someone in the future. This can result in large deviations in practice and often result in disastrous mishaps costing valuable time and deteriorating the quality of the output result.

You can similarly apply the creation of standardized documentation for standard control charts or reagent logbooks. Not only will this make life easier for your supervisor, it will help those that come after you more quickly adapt to their new surroundings while making it easier to catch instrumentation problems or operator errors. An added benefit of taking the initiative writing procedures and creating standardized forms is that you can build your own personal repertoire of methodologies. Over my career to date, I have written dozens upon dozens of such procedures adapted from the scientific literary, crafted de novo, or "borrowed" and adapted to my own liking. This gives you a tremendous degree of power and you can easily pass this knowledge to a colleague in need with a few mouse clicks. A common topic within many QMSs is continuous improvement. As you begin to develop your own SOPs and systematic documents, you

can also improve them based upon flaws or amended ways of doing things.

Aside from the ISO quality management systems, there are many other quality management systems that may be more relevant to the constantly evolving environment you find in research. A background in Good "Anything" practices (GxP) such as Good Manufacturing Processes (GMP), Good Laboratory Practices (GLP), or Good Clinical Practices (GCP), will serve you very well as you move through your coursework, practical training, or first stint in a research lab. Despite having different names, many of the practices in GMP, GLP, and GCP share many commonalities.

INNOVATIVE PROBLEM SOLVING

Innovation is not just a business buzzword; it describes ways of thinking in terms of problem solving with limited resources. Doing research with limited resources, while challenging, is rewarding. The reality of science, at least as I have experienced it thus far in my career, is that you never have "ideal" conditions to do the work that you want. You might be missing a piece of equipment or full funding for a particular project, or extra pairs of hands. Stated more succinctly, you have to make do with what you have or can easily get your fingers on. Fortunately, this is something that time at the bench as an undergraduate can quickly teach you how to deal with using a bit of innovation and problem solving.

Having to deal with equipment on a daily basis, I quickly learned that being well acquainted with instrument manuals and having a supply of texts on specific techniques to be an excellent first line of defense to dealing with problems. Instrumentation, whether in a lab with a chemistry focus or a biological one, is absolutely critical to being able to consistently turn out quality results. Instrument downtime is an absolute killer of efficiency. In order to avoid downtime, not only should you learn the ins and outs of your equipment, you should get into the practice of learning preventative and routine maintenance procedures. I found time and again that maintaining a pool of spare parts, whether it be replacement consumables parts of an HPLC's pumps or on occasion, asking around for spare parts and solutions is a cost-effective solution and can also serve as a catalyst for professional networking. But when all else fails, you must sometimes rely on your wits to solve problems especially when replacement parts are weeks away or there is no money in the lab's budget for a service call.

The basis of all problem solving skills is a combination of highly disciplined and systematic step-by-step investigations into a problem with a bit of innovation thrown in. When I first moved to Iceland, my background was mostly in chemistry. Two of the first major challenges I faced in working in a microbiology lab involved patching together a water distiller with a cracked boiling housing and resuscitating a mistreated HPLC. As both pieces of equipment were absolutely vital to doing any sort of microbiology, I got to work. The cracked boiler housing was a bit of a challenge. I decided to try carefully applying electrical tape, which is reasonably heat resistant. Much to my surprise, it worked and the boiling chamber actually managed to run nearly continuously until the electronics failed a number of years later. Coaxing the HPLC back to working order, on the other hand, required several weeks of methodical troubleshooting. The biggest tell that there was a problem in the first place was incredibly high back pressure (in excess of

40 MPa!) at only 10% of the recommended flow rate. Unfortunately, there was no "log book" detailing the instrument history so I started by patiently unraveling the history of the instrument, who had used it, and when the problem started. I slowly and patiently worked the problem backwards by eliminating one variable at a time only to find out that a series of problems was to blame. Upon the instrument's initial installation, the wrong diameter guard column had been installed and the steel tubing had been kinked by a careless technician. To make matters worse, someone had injected unfiltered biological samples onto the column.

Learning how to maintain, repair, and keep equipment in working order all represent a highly valuable transferable skill. Once you learn how to keep one piece of equipment in order, adding another is less of a mystery. It has been my observation that hiring managers, whether interviewing to fill a research job or a job in the private sector, tend to value these types of transferable skills over an applicant's ability to perform specific procedures. By paying close attention to parallel disciplines, you can often transfer ways of handling science to your own work. Furthermore, by diversifying your work experience by venturing into an industrial or clinical setting, you can gain valuable insights into areas such as quality management which are often neglected in research.

CULTIVATING CURIOSITY THROUGH INVOLVEMENT IN INTERDISCIPLINARY STUDIES

Beyond developing transferable competencies, the opportunity to do research at the undergraduate level also allows you the rare opportunity to explore your own interests beyond the highly pre-programmed nature of the vast majority of undergraduate work. I found that I naturally gravitated towards organic chemistry as a discipline while enjoying related aspects in the fields of biochemistry and microbiology. As it turns out, the intersection of these disciplines is incredibly fascinating and acted as a catalyst for my further learning.

While I was in my initial undergraduate period, I had stumbled across a few papers by George Whitesides in which he used enzymes as catalysts for organic synthesis. This planted the idea in my head and I caught on fire. As a result, I decided to dual major in biology; at that time, green chemistry was up and coming and had not reached the level of awareness that it currently enjoys. While the use of enzymes in niche applications including in food processing, textiles, and organic chemistry, to name a few.

While standing at the bench in a research lab as an undergraduate will often reinforce the lessons taken from your course work, it is also a fantastic opportunity to go beyond the book and expand upon your own intellectual curiosity.

APPRECIATING NATURE'S ORGANIC CHEMISTS

Going through undergraduate coursework, I could not help but stand in awe of the great synthetic masters such as Karl Fisher, Whitesides, and E.J. Corey. Their insights into the way that molecules behave are nothing short of a marvel of our species'f ability to understand. At some point, however, I came across a quotation from Francois Jacob that goes as follows:

Nature is a tinkerer, not an inventor

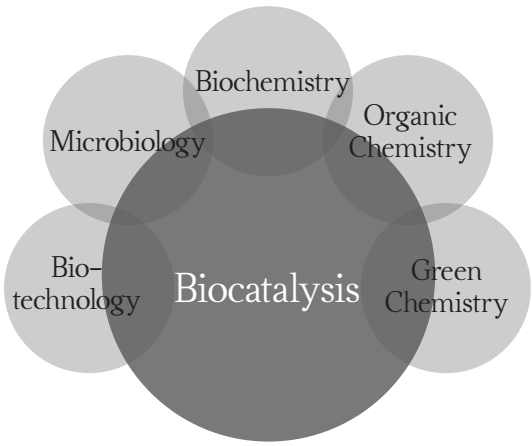
Table 1. Green chemistry (adapted from references 1 & 2)

Green Chemistry Principle	Traditional Synthesis	Biocatalysis
Prevent waste	X	✓
Maximize atom economy	X	✓
Minimize toxicity	X	✓
(preserve functionality/efficacy)	X/✓	✓
Minimize auxiliary substances	X	✓
Minimize energy input	X	✓
Renewable feedstocks	X	✓
Minimize protecting group usage	X/✓	✓
Use catalysts	X/✓	✓
Design for biodegradability	X/✓	✓
Real-time analysis	✓	✓
Minimize accidents	X/✓	✓

As it turns out, nature has been at the synthesis game a lot longer than we have been on the scene. Biochemical reactions have been around on this planet for at least 3.5 billion years, and evolution has spent millions and millions of years fine-tuning numerous reactions to specific conditions ranging from highly saline and icy cold to well beyond the boiling point of water. Not surprisingly, enzymes are highly efficient catalytic systems given this protracted development time. To put their efficiency in perspective, a typical catalyst for a reaction uses between 0.1 to 1 mole percent (2). Enzymes on the other hand, can catalyze reactions in the range of 10^{-3} to 10^{-4} mole percentage! Fortunately, we are not limited to "natural" reactions. Many enzymes will accommodate other substrates, a trait commonly referred to as enzyme promiscuity.

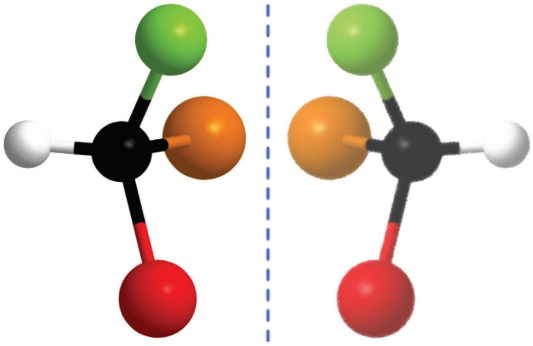
Biocatalysis is a broad and highly-interdisciplinary field (Fig. 1) that looks at the use of living systems, in part or in whole,

Figure 1. Biocatalysis is a multidisciplinary field calling on many other disciplines



to perform reactions. Thus, the whole gamut from cultures of microbes, crude extracts, to purified enzymes can be used in various applications. Biocatalysts have traditionally been touted for their high catalytic efficiency, mild operating conditions, high selectivity (chemo-, stereo-, and regio-selectivity), immobilization, and biodegradability. Their drawbacks, however, are likely the

Figure 2 Enantiomers are moleculer mirror images

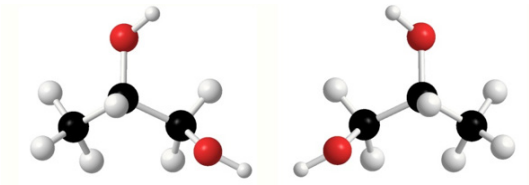


reason for their relegation to specialized roles; enzymes are frequently prone to inhibition phenomena, cofactors, solvent compatibility, lack of commercial availability, and their selectivity. For these reasons, talk of the use of biocatalysts is frequently regarded with suspicion and viewed as a black box due to the complexity of working with living organisms or parts thereof.

With the push for "greener" chemistry and industrial practices, however, the use of biocatalysis in many applications is beginning to gain a foothold. Of the commonly cited twelve principles of green chemistry (Table 1) originally put forth by the EPA, biocatalysts are capable of being compatible with all twelve principles.

Microbes provide a vast library of enzyme chemistries just waiting to be explored. Given their ubiquitous nature and adaptations to broad environmental conditions, they are a potential treasure trove. One particularly interesting aspect of enzymes is their inherent stereopreference opening the doorway for their use as chiral catalysts in synthetic applications.

Figure 3 – 1,2-propanediol and its two enantiomers. Note the difference in configuration at C2



CHIRALITY: THE HANDEDNESS OF LIFE

While taking organic chemistry, I became enamored with the "handedness" of life which I found to be a bit of an abstraction at first. Chirality (Greek for "handed-ness") is of fundamental importance in the biological sciences and poses a number of serious problems in organic synthesis. Enantiomers are stereoisomers that are mirror images of one another (as shown in Fig. 2) whereas diastereomers are stereoisomers that are not mirror images of one another.

Most biomolecules have inherent chirality yet we take this for granted. Sugars typically have multiple chiral centers. At some point during my long undergraduate tenure, I took an interest with 1,2-propanediol (1,2-PD, Figure 3). This three carbon diol has a single stereocenter at C2 and thus exists as two enantiomers. While being an extremely simple molecule, separating the two enantiomers of 1,2-PD is incredibly challenging and caught my attention.

EXPLOITING THE CHIRALITY OF ENZYMES

Enzymes, being proteins, are composed of L-amino acids. As such, enzymes are highly specialized organic chemists that often demonstrate chiral preferences and in many cases can accept non-

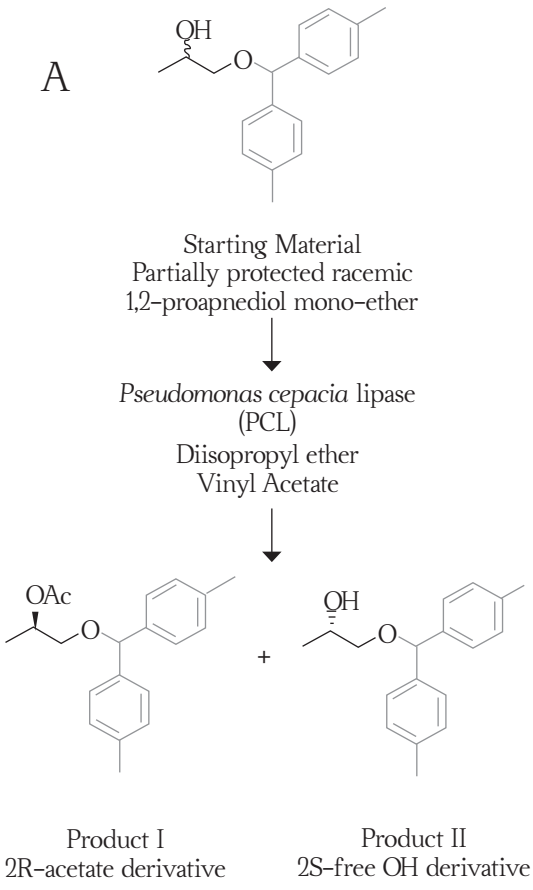
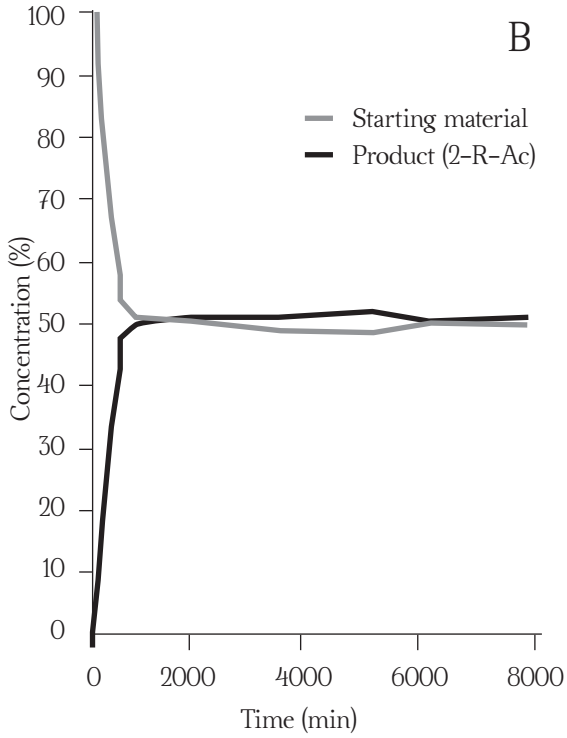


Figure 4. A – *Pseudomonas cepacia* lipase (PCL) catalyzed kinetic resolution of 1-bis(4-methylphenyl)methyl-propan-2-ol; B – Kinetic resolution of racemic 1,2-propandiol derivative using PCL monitored by high performance liquid chromatography



native substrates (a feature often referred to as "catalytic promiscuity"). As I neared graduation, I decided to use the opportunity to combine my love of organic chemistry with my new found appreciation for microbiology. The work I did for my B.Sc. thesis examined the use of lipases from psychrophilic or psychrotolerant microbes, such as *Pseudomonas cepacia (PCL)*, to resolve partially protected 1,2-propanediol derivatives. The approach I chose was two pronged: a regioselective protection using a variety of tin(II) halide catalyzed etherification reactions that had shown to have a high degree of regiopreference in partially protected carbohydrate diol systems followed by an enzymatic resolution of the two enantiomers (Fig. 4).

Given my extensive background with high performance liquid chromatography (HPLC), I decided to monitor the reactions in near-real time to ensure that only one of the enantiomers was indeed reacting. While the PCL-catalyzed reaction portion worked very well, there were issues with the regioselectivity of the initial protection step necessitating an extra derivatization step. While the project as a whole was only partially successful, it gave me crucial insights into the challenges of separating biologically relevant enantiomers and gave me the impetus to pursue other lines of thought to reach my goal of separating a racemic mixture.

BIOPROSPECTING FOR EXTREME ENZYMES AND CHIRAL PRODUCTS

Since finishing my Master's degree (albeit in a project focusing on carbohydrate chemistry), I have had the good fortune to manage a number of projects all of which have given undergraduates research opportunities at the intersection of microbiology and chemistry. The focus of this work has been on two fields: prospecting for enzymes with biotechnological potential and bacteria producing (*R*)– or (S)–1,2–propanediol. The logic behind these two approaches is that it is easier to produce an enantiomerically pure compound such as 1,2–PD rather than trying to separate a mixture of the two enantiomers after the fact and that producing these compounds from enzymatically treated abundant biomass (such as lignocellulose) would be the most sustainable approach.

The early work involved leading a few groups of willing undergraduate into the field to collect samples from Iceland's various geothermal areas (Fig. 5). Once in the lab, the laborious process of enriching samples and isolating pure cultures began and continues to this day (some 3 years later). One of the reasons for going after thermophiles was that it has not been widely reported that they are capable of producing 1,2–PD.

Screening for 1,2–propanediol producing bacteria and enzymes of biotechnological potential has since expanded to new environs fueled by the initiative of a new generation of undergraduate researchers. Watching students enrich and screen isolates from environments and finding new and exciting bugs and expanding the knowledge base has been rewarding in its own right.

Figure 5. Professor Johann Orlygsson (University of Akureyri) demonstrating sampling technique in geothermally heated stream while several undergraduate researchers look on.

PARTING WORDS

Science is very much an active endeavor, and one's ability to succeed can be very much enriched by making connections by different disciplines and moving out of your comfort zone. Once piece of advice I like to give my students is to fervently seek out new opportunities in science, whether at home or abroad, and to strive to create a culture of learning. There are many ways of enriching your undergraduate experiences beyond research including doing science outreach (a great activity for sharpening those communication skills while giving back to the community) as well as teaching, whether it be acting as a teaching assistant for undergraduate course work or tutoring high school students.

ACKNOWLEDGMENTS

I would like to express my gratitude to those that have supported my career including the faculty members at the University of Toledo and the University of Akureyri with a special thank you to Professor Sigthor Petursson who gave me a high degree of freedom to pursue my interests. Additionally, I would like to thank the staff of Perstorp Polyols for the opportunity to work in their quality lab. A special thanks is due to Eva Maria Ingvadottir for editing this manuscript.

FURTHER READING

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ERRATUM

Beilke, M.L, & Fritz, J.D. 2016. Frequency of antibiotic residues in a central Wisconsin dairy. Fine Focus 2:15–21. The following table should be used as a reference in lieu of the existing published Table 1 for this manuscript.

Total # of raw milk samples collected and tested for bulk tank inclusion	264
# of raw milk samples positive for antibiotics	0
# of raw milk samples negative for antibiotics	264

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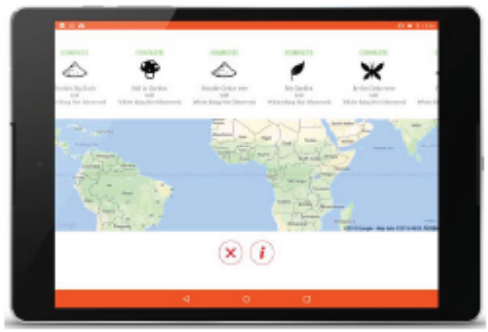
By partnering with universities & pharmaceutical companies, the massive costs for finding new drugs can be diminished when giving access to this platform.

Impact
The coming cost of antimicrobial resistance is 10 million deaths per annum by 2050. This is estimated to cost about 3% of the global GDP. Antibiotic resistance is growing faster than pharmaceutical companies are finding new ones. Corporate Social Responsibility of a pharma company can largely benefit from an educational initiative that promotes growth of young minds in STEM careers in research.

Method
Users test their samples like a crushed bark of a tree in their backyard with the toolkit that contains E.coli bacteria. If the tested tree bark has antimicrobial properties, it will be recognized by the platform and the user is encouraged to submit the original platform. Using gamification, users are incentivized to test different samples, geographical areas, and to use their peers for verifying their findings.



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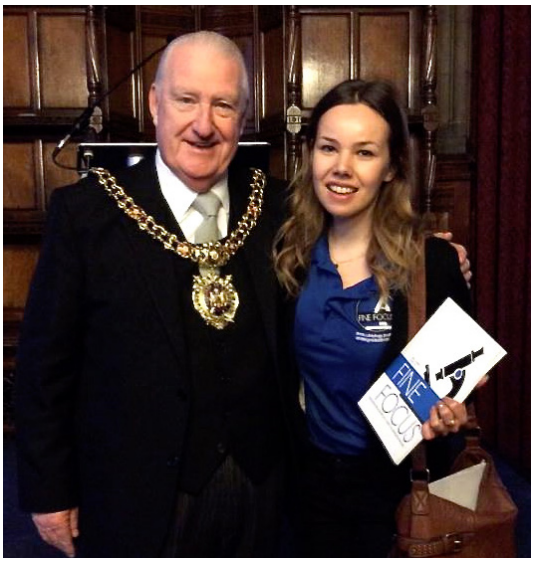
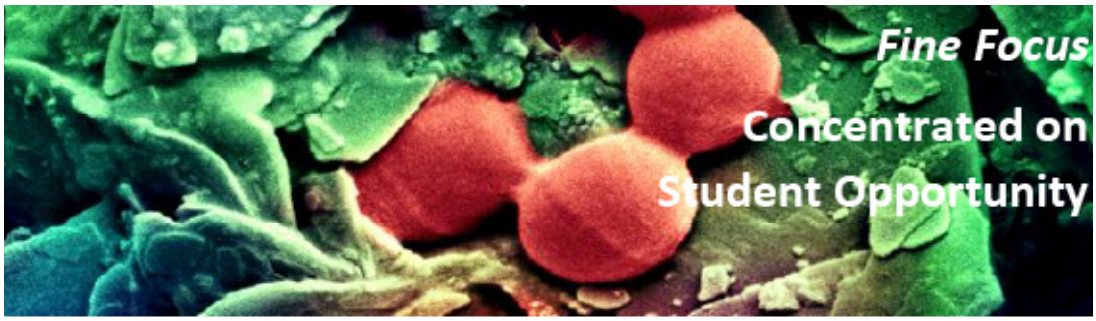


Clockwise from top left: Finnish international marketing student Saara-Maria Helena Kallio presents Fine Focus to Lord Mayor, Manchester (UK) city councillor Paul Murphy OBE at the British Conference of Undergraduate Research (BCUR) in March, held at Manchester University, in order to promote our journal to an audience of European undergraduate microbiology students and their mentors; Ball State University Junior genetics and microbiology major Ellen Wagner shares Fine Focus with Dr. Stefano Bertuzzi, ASM CEO at the Indiana Branch ASM Meeting in Fort Wayne recently; Fine Focus Spring 2016 student group; Fine Focus students tour CS Kern Printers in Muncie, IN, where our bound journals are printed for distribution; Saara-Maria Helena Kallio presents Fine Focus at the British Conference of Undergraduate Research (BCUR) in March. Saara served on the Fine Focus Marketing Team when the journal was managed last autumn from Akureyri, Iceland.



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