EUREKA!

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Genetic mutations lead to drunker *Drosophila melanogaster* fruit flies

Eureka!

The exclamation "Eureka!" is attributed to the great mathematician Archimedes after he discovered that the volume of water displaced by an object placed in water is equal to the volume of the object itself. When the solution to this problem was revealed in his mind, he exclaimed "EUREKA! EUREKA!" which roughly translates to "I HAVE FOUND IT, I HAVE FOUND IT". As all scientists know, the *moment of discovery* is a cherished event; the prospect of discovery is the reason to get up in the morning, and it is what carries scientists through long nights of struggle and frustration. It is THIS moment we wish to share with students of the natural sciences. "Eureka" is therefore the perfect distillation of the spirit of the natural sciences and an appropriate title for a journal whose goal is to provide a forum in which students can share their Eureka! moments.

To those who reviewed manuscripts for this publication;

Thank you for your time and effort on the behalf of our students. We know the density of your schedule and understand the sacrifice you have made to review our work. This sacrifice is greatly appreciated. The students participating in this research program are enthusiastic, and their work strives to reveal interesting and pertinent things about the world around us. Each manuscript published herein is the result of input from at least three faculty reviewers and the interpretation of this input by the student researchers. We have done our best to address the concerns expressed in each review, and your comments and suggestions have greatly improved the quality of our manuscripts. Please understand that the final manuscripts are the results of the efforts of reviewers, students and mentors, and that not all suggestions may be incorporated, but were certainly considered. We will continue to recruit new students, and therefore hope that this is not the last time we call upon you to review such work.

We sincerely thank you for your help,

Laura Leverton, Ph.D.; Editor-in-Chief

Cover photo credits: Genetic mutations in fruit flies result in alcohol intolerance. Image taken and provided by Erin Doughney, and is associated with the work of Molly Alexander (see pages 1-6).

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Measuring ethanol tolerance in diverse strains of *Drosophila melanogaster* to identify if alcohol tolerance is genetically driven

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Abstract

This research aims to analyze genetic inheritance of ethanol tolerance among diverse strains of *Drosophila melanogaster* (fruit flies*)*. Using three different strains of *D. melanogaster,* researchers were able to quantitatively evaluate ethanol tolerance among mutated fruit flies. Genetic strains of *D. melanogaster* consisted of a control strain and two mutants of the alcohol dehydrogenase gene, Adh^{n1} and Adh^{+}. The data to date indicate flies with the modified alcohol dehydrogenase gene become inebriated faster than the control group, thus suggesting the mutant gene influences alcohol tolerance. Future research intends to continue to collect data on *D. melanogaster's* alcohol tolerance and to become a genome wide association study (GWAS). Further gene mutations can then be explored to assess their genetic influence on alcohol tolerance and be further linked to the human genome in relation to alcohol tolerance.

Introduction

Alcohol tolerance research has traditionally be carried out with fruit flies as test subjects (Reiter et al., 2001). Fruit flies, specifically *Drosophila melanogaster,* are the ideal model organism when it comes to testing alcohol tolerance against genetic variances. Since 1995, *D. melanogaster* flies have been noted being especially important to biology and genetic advancements due to their quick reproduction cycle, affordability, and unique polytene chromosomes (Manning, 2008). NASA has even reportedly used the fruit fly as a test subject to study the effect space travel has on genes (NASA, 2004). Along with the benefits of fruit flies in

the lab, *D. melanogaster* also have a 61% gene overlap with human genes, further linking any research of alcohol tolerance in fruit flies to humans (NASA, 2004).

Previous research into *D. melanogaster*'s alcohol tolerance has highlighted the alcohol dehydrogenase gene. The alcohol dehydrogenase (ADH) gene in *D. melanogaster* flies is essential to alcohol tolerance because of its ability to adapt and withstand exposure to ethanol alcohol (David et al., 1978). The ADH gene in fruit flies is known as a protein coding gene and is involved in the biological processes of alcohol catabolic processes, alcohol metabolic processes, ethanol oxidation, behavioral responses to ethanol, and ethanol metabolic processes (FlyBase, 2017).

This research explores the mutated *D. melanogaster* flies Adh^{n1} and Adh^{+}. Adh^{n1} mutated flies are sensitive to ethanol and have been known to phenotypically show a hyperactive stage that is much lower than control fruit flies when in the presence of ethanol. These mutant strains can acquire higher amounts of ethanol than control types (FlyBase, 2017).

Furthermore, this research aims to statistically analyze if the mutated ADH genes have a significant effect on alcohol tolerance in *Drosophila melanogaster* fruit flies. Our null hypothesis of having no statistical deviations between KOT50s of all three *D. melanogaster* strains will be investigated in this paper. The goal is to make connections between the results and the specific mutated genes in fruit flies in order to expand the research into a genome-wide association study (GWAS) and link genetic alcohol tolerance in humans. A genome-wide association study will compare different gene mutations in fruit flies to their alcohol tolerance and expand the research to a fuller understanding of genetically driven alcohol tolerance.

Materials and Methods

D. melanogaster strains used for this research were received from North Carolina Central University. Strains included W^{1118} (wild-type), and two alcohol dehydrogenase gene mutants Adh^{n1}, and Adh^{+}. Flies were further separated by sex and placed into assay vials of 10 males or 10 females. Flies were then exposed to ethanol by saturating a cotton plug with 500 µL of 99% ethanol alcohol which was then used to seal the assay vials. The data were recorded measuring how long, in minutes (mins), it took for flies to become noticeably impaired in their ability to fly or climb the walls of the tube. Characteristics of impairment were characterized by inability to walk, fly, or crawl vertically up the vials. While not undergoing experiments, *D. melanogaster* were kept in breeding vials at 21 and 22 ^{o}C.

Results

Data were collected in minutes it took for *D. melanogaster* flies to become impaired by ethanol alcohol. Data were statistically analyzed using ANOVA (Figure 1). The time it took for 50% of the flies to become impaired in each assay (KOT50) represented an average time it took for 50% of the flies to become impaired. Mutated strains, Adh^{+} and Adh^{n1} became impaired, on average, between 10-20 mins. Control strain of W^{1118} became impaired, on average, around 23 mins. Boxplots for each strain show an overlap in variance.

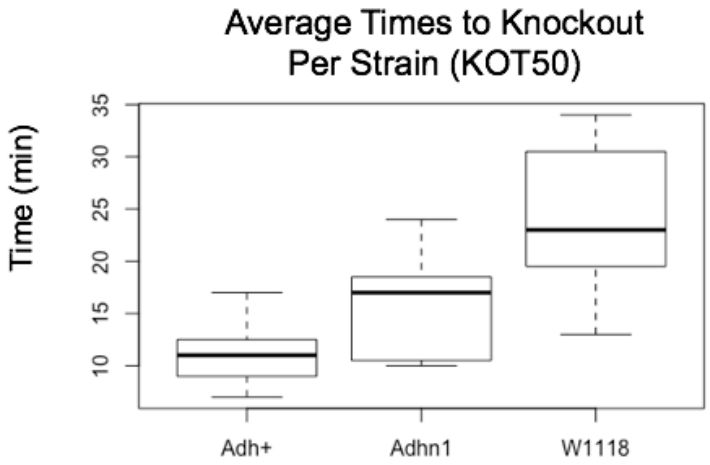


Figure 1: Analysis of variance (ANOVA) yields an F-value of 9.3278 and a P-value of 0.00166.

Tukey analysis was calculated to explore the significance of each strains variance compared to the other strains (Table 1). When comparing Adh^{+} and Adh^{n1}, the P value calculated was 0.347012. When comparing W^{1118} and Adh^{+}, the P value was 0.001347. Lastly, when comparing Adh^{n1} and W^{1118}, the P value was 0.02942. These values represent the significant difference between the KOT50 shown in figure 1.

Table 1: Tukey analysis displays the significant P values between various strains tested.

Tukey Analysis				
	Difference	Lower	Upper	P value
Adh^{n1} VS Adh^{+}	4.4286	-3.47379	12.33093	0.347012
W^{1118} VS Adh^{+}	13.143	5.2405	21.04521	0.001347
W^{1118} VS Adh^{n1}	8.7143	0.811929	16.61664	0.02942

Conclusion

Various strains of *D. melanogaster* flies were successfully analyzed for their ethanol alcohol tolerance in quantifiable data. KOT50 results show a time variance among the different strains of fruit flies with some time overlapping. Tukey analysis analyzed these overlaps and calculated a P value between the Adh^{n1} and the Adh^{+} (0.347012), showing no real significance between the two mutated *D. melanogaster* strains. When comparing the control group (W^{1118}) to the mutated flies (Adh^{+} and Adh^{n1}) a low P value represents a significant difference in the KOT50. These data allow us to reject our null hypothesis of having no statistical deviations between KOT50s of all three *D. melanogaster* strains. This means the gene mutated alcohol dehydrogenase strains of *D. melanogaster* flies became impaired significantly faster than the W^{1118} (control) flies. The alcohol dehydrogenase mutated gene does have an effect on the KOT50.

Potential problems with data collected could have swayed results. These potential risks include unexpected fly death or fly strain bias when recording data. If assay vials were made and kept overnight, flies had the potential to unexpectedly die causing the assay count to be less than 10 flies. Results also showed a trend of the fly strain W^{118} to take longer to become impaired. Knowing this to be a common occurrence, biases can be unintentional when recording the time of fly impairment to favor the other two strains of *D. melanogaster.*

In conclusion, this research hopes to shed light on the genetics of alcoholism in humans. Humans use the alcohol dehydrogenase gene much like the fruit flies, to breakdown toxic alcohol to prevent damage to the nervous system (Templeton, Boerwinkle, & Sing, n.d.).

Next steps for this research include gathering more data on tolerance among the current *D. melanogaster* strains, integrating in more strains of *D. melanogaster,* and constructing a genome wise association study (GWAS). Future plans for this research is to make connections between *D. melanogaster* alcohol tolerance and human alcohol tolerance.

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Detecting emerging antibiotic resistance genes in environmental water samples

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Abstract

This study discusses the findings of extracting bacterial DNA from an aquatic environment to test for the presence of the emerging antibiotic resistance genes NDM-1 and KPC. A secondary goal was development of an efficient water sample collection method to test for antibiotic resistance in aquatic environments. DNA was isolated from water samples collected from a small, man-made drainage system of Goose Creek and PCR reactions run using 16S, Amp-R, NDM-1, and KPC primers. PCR amplified both 16S and Amp-R, but failed to amplify NDM-1 and KPC.

Introduction

Antibiotic resistance genes occur in all natural environments including aquatic ones (Allen et al., 2010; Henriques et al., 2006). To aid in human's future ability to fight infection, investigation regarding antibiotic resistance and its role in aquatic environments is necessary to further insight on the itinerant process of resistance genes and detection in natural samples (Baquero, et al. 2008; MLOT, 2000). Some studies have focused on specific genes altering microbial ecosystems (Keen and Patrick 2013). NDM-1 and KPC have been recently identified in the United States and have demonstrated resistance to virtually all antibiotic drugs in common use (Lascols et al., 2009; Keen and Patrick 2013). This study focuses on the isolation of DNA from aquatic samples to detect NDM-1, KPC, and Amp-R using PCR.

Materials & Methods

DNA extraction

The study site was a small, man-made drainage of Goose Creek, south of the intersection of Market Street and Lee Drive, Baytown, Texas (Figure 1), about 8.37 m wide and vegetated shorelines. Two sterile 50 mL conical tubes were filled with surface water within 1 m of the edge.



Figure 1. Small, man-made drainage of Goose Creek, Baytown, TX (29°44'00.5"N 94°58'47.2"W).

In the lab within 30 minutes of collection, for each 50 mL conical tube, 10 mL of the water sample was transferred to a 15 mL conical tube and centrifuged at 4,400 RPM for 5 minutes. The final pellet was then resuspended in 200 µl 1x PBS and 20 mL was transferred to and stored in a 1.5 mL microtube at -20º F. Isolation of DNA was executed using the Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo Research, CA, U.S.A). For the protocol's step of lysing the cells, a Benchmark Scientific Mortexer™ (Benchmark Scientific Inc., NJ, U.S.A) was used at a medium-high speed for 5 minutes. Following extraction according to instructions by the manufacturer, DNA was transferred to and stored in a 1.5 mL microtube at -29 ^{o}C.

PCR using universal primers

The following procedure focuses on detection using universal 16S PCR primers. PCR amplification was carried out by a Bio-Rad MyCycler™ thermal cycler (Bio-Rad Laboratories) with a 20 µl reaction mixture containing 10 µl 2x GoTaq® DNA Polymerase, 2 µl 16S Forward (5' –AGAGTTTGATCMTGGCTCAG – 3') and 2 µl 16S Reverse (3' –TACGGYTACCTTGTTACGACTT – 5') primers, 4 µl nuclease free H_2O, and 2 µl of extracted metagenomic DNA. Cycle durations and

temperatures consisted of: initial denature at 95 °C for 3 min; 35 cycles of denaturation (95 °C for 30s), priming (60 °C for 30s), and extension (72 °C for 30s); and a final extension at 72 °C for 12 minutes (Jiang *et al*., 2006). PCR products were stored at 4 °C.

PCR using NDM, KPC, and Amp-R primers

The procedure for PCR was the same as used for the universal 16S PCR primers, each consisting of the same number of aliquots: NDM-1 Forward (5' – GGTGCATGCCCGGTGAAATC – 3'), NDM-1 Reverse (3' – ATGCTGGCCTTGGGGAACG – 5'), KPC Forward (5' – CGTCTAGTTCTGCTGTCTTG – 3'), KPC Reverse (3' – CTTGTCATCCTTGTTAGGCG – 5'), Amp-R Forward (5' – AGATCAGTTGGGTGCACCAG – 3'), and Amp-R Reverse (3' – AGTAAGTTGGCCGCAGTGTT – 5').

Gel Electrophoresis

PCR products were evaluated on 1% agarose gel prepared with 1x TBE utilizing Bio-Rad Mini-Sub® Cell GT Cell system (100 V for 30 min.).

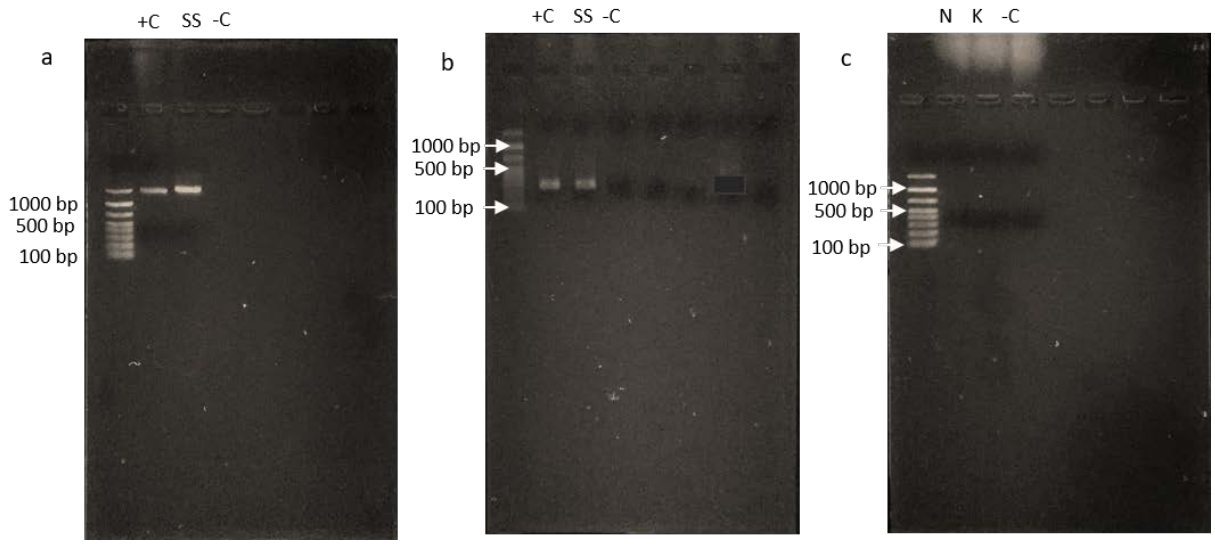


Figure. 2. (a) Detection of 16S (1100 bp) in water sample from study site (SS). (b) Detection of Amp-R (333 bp) in water samples from study site (SS). (c) No detection of NDM-1 (N) or KPC (K) in water sample from study site (no positive control was available for these target sequences).

Both 16S and Amp-R sequences were detected in water samples collected at the study site, however NDM-1 or KPC were not (Figure 2).

Discussion

Extraction of DNA utilizing the method described in this paper was successful based on amplification of 16S and Amp-R sequencing by PCR confirmed by gel electrophoresis. PCR of DNA isolated from water samples collected at the sample site did not detect antibiotic resistance genes NDM-1 or KPC in PCR. Future research will include a wider geographic sampling of other natural water bodies.

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Characterization of an ampicillin-resistant bacterium from an organic garden

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Abstract

Agricultural amelioration of soil alters the chemical environment in which soil bacteria grow and reproduce, possibly leading to the serendipitous acquisition of antibiotic resistance. This study sought to examine the hypothesis that gardens using traditional chemical-based gardening techniques would harbor more ampicillin-resistant bacteria than gardens using organic gardening techniques. Bacteria were extracted from the soil of two gardens, one that used standard chemical fertilizers (Piney Grove Wilbon; PGW) and pesticides and another garden that did not (Stone Crystal; SC). These extracts were used to inoculate solid media containing ampicillin; incubation revealed that the SC garden contained far more ampicillin-resistant bacteria than the PGW garden. The SC ampicillin-resistant population was dominated by a single bacteria species that was identified as *Pseudomonas fluorescens*, a common, beneficial soil bacterium that is known to be naturally resistant to ampicillin. The results of this study did not provide support for the original hypothesis, but do imply that differences in gardening techniques can alter the bacterial community in important ways.

Introduction

Antimicrobial resistant bacteria (ARB) are a widespread health concern (Zaman *et al.*, 2017). Several human activities have contributed to the frightening rise of ARB, including the unnecessary prescription of antimicrobials by physicians, the misuse of antimicrobials by patients, and the widespread and heavy agricultural use of antimicrobials (Ponce de León-Rosales *et al.*, & López-Vidal, 2015). However, antimicrobials also have an ecological role, providing microorganisms which can produce antibiotics (i.e. antimicrobials of natural origin, including penicillin and streptomycin) an advantage over microorganisms which are susceptible

to those antibiotics (Keen & Patrick, 2013). In turn, the organisms which are the targets of these natural antimicrobials have been developing countermeasures for millions of years (Barlow & Hall, 2002). Therefore, ARB is not a new or particularly human-driven phenomenon, but the product of millions of years of natural evolution.

A single gram of soil can harbor 10,000 different microbial species and as many as one billion microbial cells (Ingham). The abundance and diversity of microorganisms in soil provides intense competition for resources and space, and creates a complex chemical environment. Bacteria evolving within this exceedingly complex chemical and biological environment have developed both the genetic means to resist antibiotics, as well as the ability to physically transfer these genes to other cells (Allen *et al.*, 2010). Humans can and have altered the soil environment, providing selection pressure driving the evolution of new microbial characteristics. The agricultural use of manure, chemical fertilizers, herbicides and pesticides further complicates soil chemistry and may drive the serendipitous development of ARB even in the absence of antibiotics (Allen *et al.,* 2010).

Conventional gardening allows for the use of chemical pesticides, herbicides, chemical fertilizers, and nonorganic animal manures. These practices can introduce novel chemicals and organisms that could promote ARB. On the other hand, organic gardening relies on biological pest control and the addition of nutrients through crop rotation, organic animal manure and compost. These practices are likely to change the soil biome and chemistry in different ways, and therefore may be less likely to produce ARB species. Therefore, this study sought to test the hypothesis that gardens maintained using conventional techniques would yield more ARB species than those maintained using organic techniques. The prevalence of ARB

microorganisms was surveyed by inoculation of soil extract on solid, general medium containing ampicillin. Enumeration of the subsequent growth indicated that the organic garden actually harbored more ARB microorganisms than the conventional garden, although this was primarily due to the presence of a single bacterial species. Standard microbiological and molecular analyses identified this bacterial species as *Pseudomonas fluorescens,* an abundant and important soil bacterium which can form mutualistic symbioses with many plant species.

Materials and Methods

Sample collection, extraction and culture

Soil was collected from two gardens in the Fuquay Varina, North Carolina area. The Piney Grove Wilbon garden (PGW) used common modern gardening techniques. Chemical pesticides such as Sevin dust were used when necessary. Chemical fertilizer (10-10-10), horse manure (not organic certified) and fertilizer-coated seeds were used. This soil had an earthy smell and was sandy and dense with a uniformly light brown color, with little to no organic material visible. The Stone Crystal garden (SC) used natural pest control methods (e.g. planting naturally resistant crops, use of diatomaceous earth) organic gardening techniques, such as adding natural sources of nutrients (e.g. leaves and grass clippings, mushroom compost). Only regular, uncoated seeds were planted, although some seedlings and young plants were purchased from hardware stores and nurseries. This soil also had an earthy smell, but was less dense, and had a dark brown to black color with an obviously high organic content due to the presence of visible partially degraded plant material.

Three samples were aseptically collected from each of these two gardens and placed into sterile containers. The soil was transported back to the laboratory, where two grams of each sample were transferred to a sterile test tube using sterile tongue depressors. Sterile phosphate buffered saline (PBS; 5 ml) was then added to each tube and mixed. The tubes were then placed into a refrigerator overnight to allow the soil to settle. The following day, each sample was used to inoculate nutrient agar plates with and without 10 µg/ml Ampicillin. Briefly, 250 µl of a sample was transferred to the surface of a plate, and then spread using a bent, sterile glass rod. Control plates were inoculated with PBS only. Agar plates were usually incubated overnight at room temperature. However, a Muellar Hinton agar plate was inoculated with SC1-1 and incubated overnight at 37 °C. SC1-1 did not grow at this temperature; therefore, a second Muellar Hinton agar plate was inoculated with SC1-1 and incubated overnight at room temperature (approximately 30 °C).

Identification of bacterial isolates

Three morphologically distinct bacterial colonies from the Stone Crystal garden (SC1-1, SC1-2, and SC1-3) were selected for further analysis. Bacteria from these colonies were subcultured onto nutrient agar containing Ampicillin (10 µg/ml) for isolation. Pure subcultures were then used as test material for subsequent microscopic, biochemical and genetic analysis.

Microscopic characteristics

All three isolates were Gram stained and observed under 1000X total magnification. Additionally, preparations of SC1-1 were stained using Congo red (as a negative stain) and Maneval's stain (as a positive counter stain to visualize the bacterial cells) method in order to detect capsules.

Culture and biochemical techniques used to identify SC1-1

Sheep blood agar (tryptic soy agar including 5% sheep red blood cells) is an enriched, general medium used to detect and differentiate between three types of bacterial hemolysis. Alpha hemolysis is the partial lysis of red blood cells, usually accompanied by a green discoloration of the agar. Beta hemolysis is the complete lysis of red blood cells, typically rendering the agar transparent. Gamma hemolysis is the absence of hemolysis. Unless otherwise noted, all agar plates were inoculated with SC1-1 using a standard quadrant streak for isolation technique.

MacConkey agar is a selective agar used to isolate gram negative bacteria that are associated with animal digestive systems. The presence of lactose and the pH indicator neutral red allows for the differentiation between bacteria that can or cannot use lactose in acid fermentation.

Mannitol salt agar is used to isolate bacterial species that are halophilic or halotolerant through the inclusion of 7.5% sodium chloride, and can differentiate between bacteria that can or cannot use mannitol in acid fermentation due to the inclusion of mannitol and the pH indicator phenol red.

Triple sugar iron agar contains the sugars glucose, sucrose and lactose, as well as phenol red, allowing the detection of acidic and gaseous products of sugar fermentation. In addition, the inclusion of iron in this medium allows the detection of bacterial production of hydrogen sulfide gas, which reacts with the iron, forming a black precipitate. Triple sugar iron agar slants were inoculated using a stab and streak technique.

Simmons' citrate agar contains sodium citrate and the pH indicator bromothymol blue. Simmons citrate agar allows for the detection of citrate transport into bacterial cells, which

subsequently use it as a carbon source. Typically, this bacterial process releases alkaline metabolic products, increasing the pH of the medium, leading to a change in color from green (at neutral pH), to blue (at alkaline pH). Simmons citrate agar slants were inoculated using a simple surface streak technique.

The catalase enzyme converts hydrogen peroxide to gaseous oxygen and water, and is present in many organisms living in the presence of oxygen. Bacterial catalase is easily detected by mixing bacterial cells with hydrogen peroxide and looking for bubbles. Briefly, a circle is drawn on a glass slide, into which a drop of hydrogen peroxide is placed. A sterile, disposable loop was used to remove a colony of SC1-1 from an agar plate and to mix these cells into the hydrogen peroxide.

The presence of tryptophanase and cytochrome oxidase are also easily detected. In the presence of water, tryptophanase converts tryptophan into indole, pyruvate and ammonia. Its presence in SC1-1 was examined by collecting a colony from an agar plate on a sterile cotton swab, and then applying a drop of indole reagent (Becton Dickinson DMACA indole reagent) to the bacteria cells. The test is positive for tryptophanase if the swab turns blue. Cytochrome oxidase is the final enzyme in the cellular respiratory chain of many aerobic organisms using oxygen as the terminal electron acceptor. The presence of this enzyme in SC1-1 was examined by collecting a colony from an agar plate on a sterile cotton swab, and then applying a drop of cytochrome oxidase reagent (Becton Dickinson oxidase reagent) to the bacterial cells. The cytochrome oxidase of most bacteria will oxidize this reagent, turning it dark blue.

Brown blotch disease was detected as described by Gandy (Gandy, 1967), with modifications. Briefly, small white button mushrooms were inoculated with either sterile

tryptic soy broth (TSB), *Pseudomonas aeruginosa* growing in TSB, *Escherichia coli* growing in TSB, or SC1-1 growing in TSB. Each treatment was replicated on three mushrooms. Once inoculated, the mushrooms were allowed to incubate at room temperature for six days. A positive result is indicated by brown blotches on the mushroom caps, usually noticeable after 24 hours.

Pectinolytic activity was detected using the method of Gonzalez et al. (Gonzalez *et al.*, 2003). Briefly, Russet potatoes were sliced into 0.25 inch slices with a sterile knife and the slices placed into individual, sterile petri dishes. Individual potato slices were inoculated with 10 µl of TSB in which either SC1-1 or *Pseudomonas aeruginosa* (as a negative control) was grown. Other potato slices were treated with sterile tryptic soy broth only, as a second negative control. Bacterial species secreting pectinolytic enzymes degrade pectin, a major component of plant cell walls, leading to plant cell lysis, allowing the bacteria to use the released organic material. Therefore, brown, liquefied potatoes are indicative of a bacterial species positive for pectinolytic activity.

Genetic analysis of SC1-1 16S ribosomal RNA

A SC1-1 colony was placed into a 0.2 ml PCR tube containing sterile water. The tube containing SC1-1 was centrifuged at 3000 X g to pellet the cells, and the supernatant was removed. The bacterial cell pellet was then incubated at 95 °C for five minutes to kill and lyse the cells, and to release bacterial DNA. Subsequently, 20 µl of a polymerase chain reaction (PCR) solution (containing 10 µl of Promega GoTaq enzyme master mix, 2 µl each of *Pseudomonas* specific 16S forward primer (5'-GACGGGTGAGTAATGCCTA -3') and reverse primer (5'-CACTGGTGTTCCTTCCTATA-3') and 6 µl of sterile water) was added to the bacterial

DNA. After an initial denaturing step of 95 °C for three minutes, this reaction was subjected to 35 cycles of the following PCR program: denaturing at 95 °C for 30 seconds, followed by annealing at 58 °C for 30 seconds, followed by extension at 72 °C for 1 minute, then returning to the denaturing step. Gel electrophoresis of 10 µl of the resulting PCR products revealed a single product of approximately the correct size (1100 bp). A second, larger reaction (50 µl) was then cycled in the same manner, and when gel electrophoresis confirmed successful amplification, 40 µl was delivered to the University of Texas at Austin Center for Biomedical Sciences for sequencing.

Results

Growth on agar plates

All samples from both SC and PGW gardens plated on nutrient agar without Ampicillin yielded colonies that were too numerous to count (TNTC). However, there were clear differences between the gardens when samples were plated on nutrient agar with ampicillin. The PGW garden yielded very few ampicillin-resistant colonies. The morphology of the PGW colonies and microscopic characteristics of the cells following Gram staining resembled those of fungi. These microorganisms were not analyzed further. In contrast, the SC samples yielded ampicillin-resistant growth which was TNTC. The plate also had a distinctive rotten odor. There were three distinct colony morphologies on these plates, but most were small, white and circular (this colony type was named SC1-1, as the colony chosen to represent the type was taken from soil sample SC1). SC1-2 bacteria grew in flat colonies with ridges and were small, Gram negative coccobacilli when Gram stained. SC1-3 bacteria were Gram positive bacilli that grew in flat, umbonate colonies.

The subculture of SC1-1 inoculated onto Mueller Hinton agar and incubated at 37 °C did not grow, however a second subculture on Mueller Hinton agar inoculated the following day and incubated at room temperature grew well, indicating the optimal temperature for SC1-1 is below 37 °C.

Characterization of the SC1-1 bacterium

A streak-for-isolation on nutrient agar of the SC1-1 colony yielded small, white circular colonies with smooth edges. The isolated SC1-1 growth exuded the same distinctive rotten smell as was present from the original sample. Gram staining of SC1-1 revealed gram-negative bacilli which had no arrangement (characterization of SC1-1 is summarized in Table 1). Upon storage of this plate at 4 °C, the colonies became mucoidal, and were sticky when probed with an inoculating loop. A capsule stain revealed the presence of a well-developed capsule surrounding the SC1-1 cells. SC1-1 did not grow at 37 °C on Mueller Hinton, but grew well at 30 °C on this same medium.

Table 1: Morphological and biochemical characterization of bacterial isolate SC1-1

Analysis of SC1-1	Result
Gram stain	Gram negative rod with no arrangement
Capsule stain	Positive for capsule
Sheep blood agar	Heavy growth with beta hemolysis
MacConkey agar	Heavy growth with no lactose fermentation
Mannitol salt agar	No growth
Triple sugar iron agar	Growth; no fermentation or H_2S production
Simmon's citrate	Positive for citrate transport and use
Catalase	Positive
Tryptophanase	Negative
Cytochrome oxidase	Positive
Brown blotch disease	Negative
Pectinolytic activity	Positive

Isolated colonies were also used for biochemical characterization. SC1-1 did not grow on mannitol salt, but grew well on sheep blood agar and was beta hemolytic. SC1-1 also grew well on MacConkey agar, and was negative for lactose fermentation. This was confirmed with triple sugar iron agar, which indicated no fermentation of glucose, sucrose or lactose.

Triple sugar iron agar also revealed SC1-1 did not produce hydrogen sulfide gas. SC1-1 exhibited the ability to transport and utilize citrate when grown on Simmon's citrate medium. SC1-1 was positive for catalase, cytochrome oxidase and pectinolytic activity, but was negative for tryptophanase activity and brown blotch disease.

Genetic Analysis

PCR reactions using a SC1-1 colony as a DNA source yielded a single product of approximately the correct size. Sequencing of this product revealed a sequence that was 100% congruent with the known 16S sequence of *Pseudomonas fluorescens.*

Conclusions

The results of this study do not support the hypothesis that gardens maintained using conventional techniques yield more ARB species than those maintained using organic techniques. In fact, the organic garden yielded far more ARB colonies than the conventional garden, harboring at least three distinct ARB bacterial species. This could be due to several factors. Both gardens have a history of amelioration to promote plant growth and reduce plant pathogens; however, the source and content of the additions vary greatly and most likely resulted in the addition or maintenance of different bacterial species. The chemicals added may have also provided differential selective pressures, leading to alterations in the bacterial

community between gardens. And of course, there were probably natural differences in the normal soil flora between the two gardens.

What is clear from the results of this study is that a single species dominated the microflora from the organic garden. Characterization of representative colonies from the Ampicillin-containing medium revealed a Gram-negative bacillus that grew well on MacConkey agar, implicating a Gram-negative enteric bacterium. The lack of sugar fermentation, and the presence of cytochrome oxidase and catalase indicates this bacterium is an aerobe. These characteristics, added to the fact the bacterium is able to transport and utilize citrate, places this species in the genus *Pseudomonas.* However, there are many different *Pseudomonas* species associated with soil. Because of the addition of mushroom compost, a possible suspect would be *Pseudomonas tolaasii*, a species known to grow on mushrooms and produce brown blotch disease. However, SC1-1 did not produce brown blotch disease on white mushroom caps, making it improbable that it is *P. tolaasii.* Another common soil species is *P. syringae*, a plant pathogen that can cause a disease called “bacterial speck”. However, SC1-1 (cytochrome oxidase positive) is not likely *P. syringae* (cytochrome oxidase negative). Likewise, SC1-1, a pectinase positive species, is unlikely to be either *P. putida* and *P. chloroaphis*, both of which are pectinase negative.

P. aeruginosa and *P. fluorescens* are common members of the soil microflora. Both of these species are beta hemolytic, Simmon’s citrate positive, and can produce capsules. Both species are negative for brown blotch disease, tryptophanase, and hydrogen sulfide production. Some strains of both species are positive for pectinase. Therefore differentiating between *P. aeruginosa* and *P. fluorescens* can be difficult. However, *P. fluorescens* has an optimal

temperature for growth between 25-30 ˚C, whereas the optimal temperature for *P. aeruginosa* is 37 ˚C (Todar, 2012). SC1-1 grew well at room temperature (approximately 25 ˚C), but not at all when incubated at 37 ˚C. While the inability to grow at elevated temperature is strong evidence, sequencing of the 16S ribosomal RNA gene confirmed the identity of SC1-1 as *P. fluorescens.*

This conclusion is very important to the ecology of the soil taken from the Stone Crystal garden. *P. aeruginosa* is a known pathogen of insects, worms and other animals, including humans, but is also pathogenic to plants (Walker *et al*., 2004). On the other hand, *P. fluorescens* is included in the "growth promoting rhizobacteria", which are known to promote plant growth through the inhibition of plant pathogens (bacteria and fungi) by producing secondary metabolites such as antibiotics, siderophores, and hydrogen cyanide (Ganeshan & Kumar, 2005). Therefore, the abundance of *P. fluorescens* in the Stone Crystal garden is a good indicator of healthy soil. Conversely, it appears that the Piney Grove Wilbon bacterial population has relatively fewer *P. fluorescens*, although no attempt was made to identify the dominant bacterial species from this garden. Future studies should therefore examine the effect of alterations in the abundance of *P. fluorescens* on plant growth dynamics.

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Assessment of boiling as a mean of control of waterborne pathogens in drinking water

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Abstract

Contaminated water remains a big public health challenge in many countries, as drinking untreated water increases the risk of infectious diseases. The Center for Disease Control and Prevention (CDC) recommends bringing untreated water to a one-minute rolling boil as a standard method to make drinking water safe. While this treatment will kill many bacteria, it will not eliminate bacteria that can produce endospores, a highly resistant dormant stage produced by some bacteria when faced with adverse conditions. Therefore, this project examined whether a one-minute rolling boil makes water safe to drink. Water samples were collected from ponds on the Scott Northern Wake Campus of Wake Tech Community College. Water samples were boiled for one, two and five minutes and then used to inoculate sterile agar media. Resulting colonies were enumerated, and cells surviving the rolling boil were Gram stained for partial identification. The results revealed most microorganisms were killed, and the surviving microorganisms resembled endospore-forming species. Therefore, while the CDC-recommended method may remove most potentially harmful microorganisms, the potential for infectious disease remains. New methods addressing control of microbial contamination of drinking water (especially contamination by endospore-producing species) could result in significant health benefits in countries plagued by untreated drinking water.

INTRODUCTION

Contaminated water remains a major health risk in many countries. Estimations of microorganisms in contaminated water along with information on risk of diarrheal illnesses can be used to evaluate the performance of water sanitation efforts in reducing pathogen transmission through water source treatment interventions. People living in poor countries, and particularly in formal or informal settlements, often have limited access to safe drinking water (World Health Organization, 2017). The World Health Organization (WHO) estimates 423

million people resort to the use of water from unprotected wells and springs, and 159 million people collect untreated surface water from lakes, ponds, rivers and streams (World Health Organization, 2017). There is a correlation between contaminated water and the transmission of certain diarrheal diseases, such as typhoid fever, cholera, polio and many more. Several cases of such diseases could be prevented by the availability of clean water. Because financial and structural resources remain scarce in low income settings, the Centers for Disease Control and Prevention (CDC) recommends alternatives to providing clean water when communities cannot reach the mandatory governmental standard. The CDC recommends bringing contaminated drinking water to a rolling boil for one minute as an effective means to kill disease-causing microorganisms (CDC, 2009). A rolling boil is when a liquid is boiled rapidly, resulting in lots of bubbling, a process that requires much more energy than simmering. The extreme increase in temperature damages cell membranes and causes protein denaturation, leading to a disruption of the cell's function. The CDC states: "Except for boiling, few of the water treatment methods are 100% effective in removing all pathogens" (CDC, 2009). However, boiling water will not kill a highly resistant dormant stage produced by some bacteria faced with adverse conditions called endospores (Cowan, 2016).

Specific bacterial species, such as those in the genera *Bacillus* and *Clostridium,* can form endospores and can be the source of serious illnesses. For example, *Bacillus anthracis* causes anthrax, and *Clostridium botulinum* causes botulism. Endospores allow some species to survive hostile and extreme conditions, such as high or low temperatures, chemical disinfectants, desiccation and ultraviolet radiation. Ingesting endospores will not always result in a disease state, but it may provide the opportunity for such pathogens to transform from the dormant

phase into the fully vegetative bacterium in the host organism, which is the first step to infection (Abel-Santos, 2015). The presence of such resistant bacterial cells brings into question the efficacy of a one-minute rolling boil to treat drinking water. This research found evidence to support the hypothesis that endospore-forming bacterial species are commonly found in aquatic environments, and that these organisms will survive the CDC recommended treatment of a one-minute rolling boil.

MATERIALS AND METHODS

Sample collection

Samples of water were aseptically collected on two different dates from two ponds on the Scott Northern Wake Campus. The first pond, located near building F, was identified as pond number six and a second pond located by building H was identified as pond number four. These ponds are used for other research projects at Wake Tech, thus the numbering system.

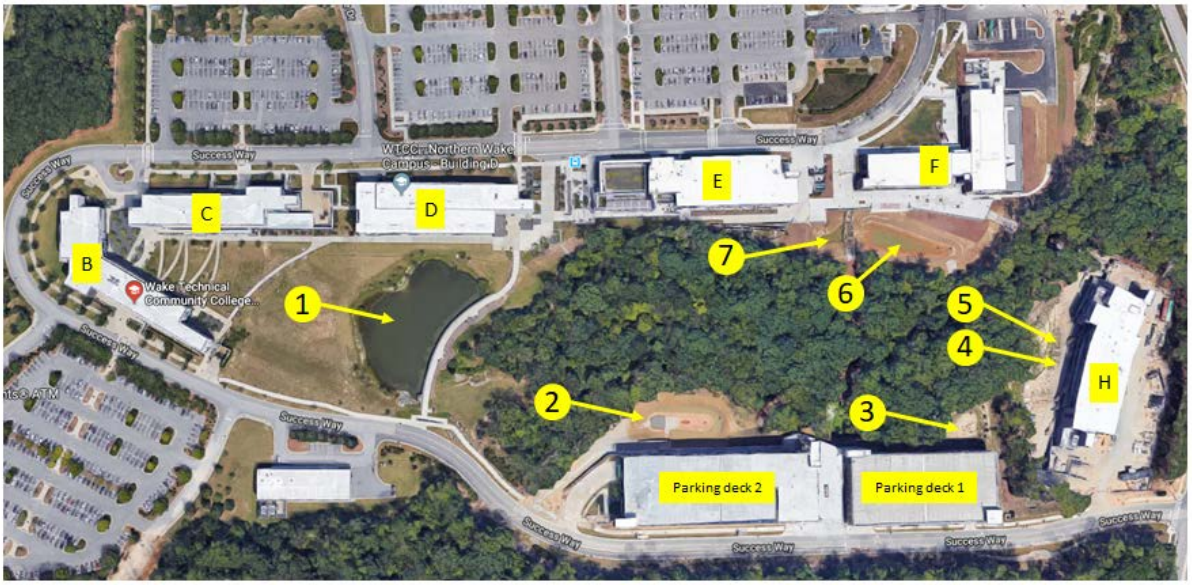


Figure 1: Map of the ponds located on the Scott Northern Wake Campus of Wake Technical Community College. These ponds are used for various research projects, and have been assigned numbers to assist in coordination between such projects. For the current project, it was decided to take samples from ponds four and six due to their proximity to the STEM lab, housed in Building H.

These locations were selected because of their proximity to the laboratory, allowing samples to be collected and transported quickly to minimize environmental, physical and biological changes in the samples. This collection technique also mimics the way water is collected and used in areas where pond/surface water are the primary sources of drinking water (i.e. it's taken from surface water, transported to the community, boiled, used and/or stored for later use). After collection, an identification label was placed on each cup, and the sample was transported to the Scott Northern Wake Campus STEM Laboratory, within five minutes following the collection.

Experiment one

Samples from ponds four and six were collected on the Northern Wake Tech campus on April 9th, 2018 at 1:45 p.m. Aliquots of the untreated samples were used to aseptically inoculate sterile tryptic soy agar (TSA). The remainder of the two samples were transferred into separate sterile glass beakers, placed on hot plates and brought to a rolling boil. Aliquots were then taken at one, two and five minutes and used to aseptically inoculate sterile TSA. Inoculated TSA plates were incubated for 24 hours at room temperature.

Experiment two

Two additional water samples from ponds four and six were collected on April 12th, 2018. Aliquots from both samples were used to aseptically inoculate sterile TSA before and after a one-minute rolling boil treatment. Additionally, an aliquot of the pond four sample was spiked before treatment with *Bacillus thuringiensis*, a species known to produce endospores,

and served as a positive control for endospore growth. Aliquots of the spiked sample were then used to aseptically inoculate sterile TSA before and after one-minute rolling boil treatment.

Gram staining

Gram staining was performed as previously described (Leverton et al., 2018) in order to partially identify bacteria that survived the boiling treatment. Gram-positive bacteria stain purple due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet used in the procedure. Gram-negative bacteria appear pink under the microscope as they do not retain crystal violet well because of a second, outer lipid membrane, but are stained by the safranin also used in the procedure. Slides were observed and photographed at 1000X total magnification under a light microscope.

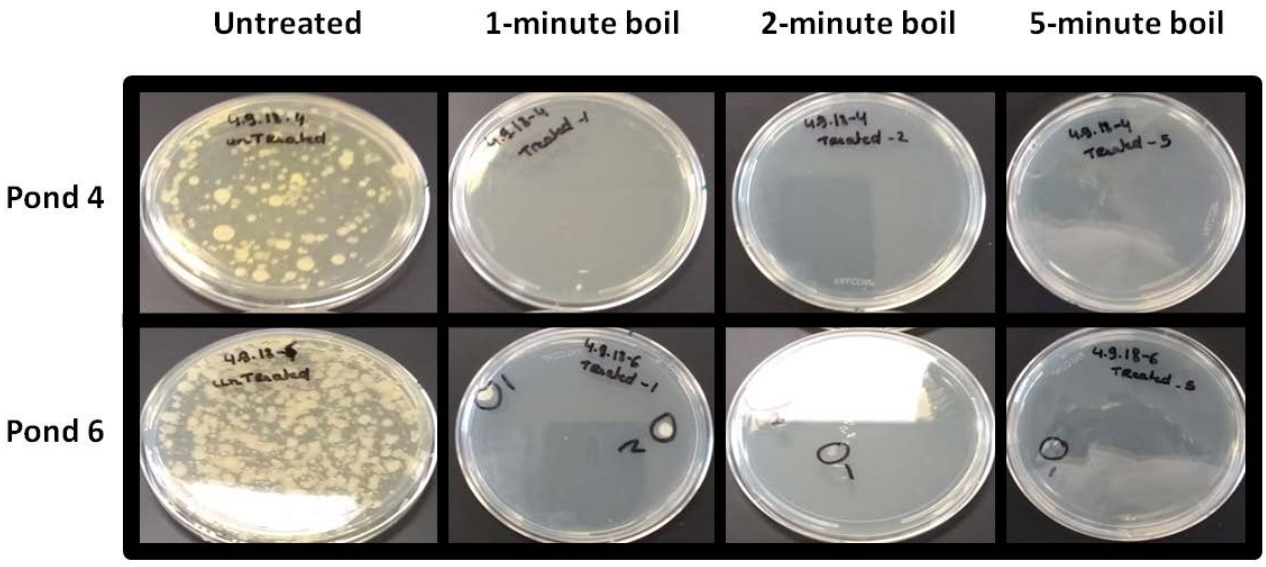


Figure 2: Water was collected from two ponds and subsequently brought to a rolling boil for the indicated times. Aliquots of untreated and boiled water were used to inoculate tryptic soy agar plates, which were incubated at ambient temperatures overnight.

RESULTS

Experiment one: Relationship between boiling time and cell survival.

After a 24 hour incubation, the untreated pond 4 sample showed considerable growth of a number of different bacterial species, based on the morphology of resulting colonies which were too numerous to count (TNTC; Figure 2). No attempt was made to describe these bacterial species at the microscopic level. In contrast, the treated pond 4 samples displayed almost no growth after 24 hours. One colony was seen on the pond 4 sample boiled for one minute; the other treatments resulted in no growth.

The results were very similar for pond 6. The untreated pond 6 sample showed even more bacterial growth after 24-hour incubation than the pond 4 sample, resulting in colonies that were TNTC. The treated pond 6 sample revealed that boiling killed most, but not all, bacterial cells. Colonies grew from pond 6 samples boiled for 1 minute (two colonies), 2 minutes (one colony) and 5 minutes (one colony). All colonies resulting from treated (i.e. boiled) samples were Gram stained for further identification.

Gram staining of bacteria surviving experiment 1 treatment

Gram staining of bacteria from the five colonies which survived boiling (subsequently referred to as species A, B, C, D, and E) revealed that all were bacilli, however there were important differences between species.

Gram staining of species A (the only colony isolated from pond 4) revealed long Gram-negative bacilli with no arrangement, as well as structures that resembled endospores (Figure 3). Species B, isolated from the sample of pond 6 boiled for 1 minute, showed short, thick,

Gram-positive streptobacilli. The Gram stain did not indicate this species produced endospores. Species C was also isolated from the sample of pond 6 boiled for 1 minute, and when Gram stained presented as small, short Gram-positive bacilli with no arrangement. The Gram stain did not indicate this species produced endospores. Species D, which survived after a 2 minute boil, also presented as Gram-positive bacilli with no

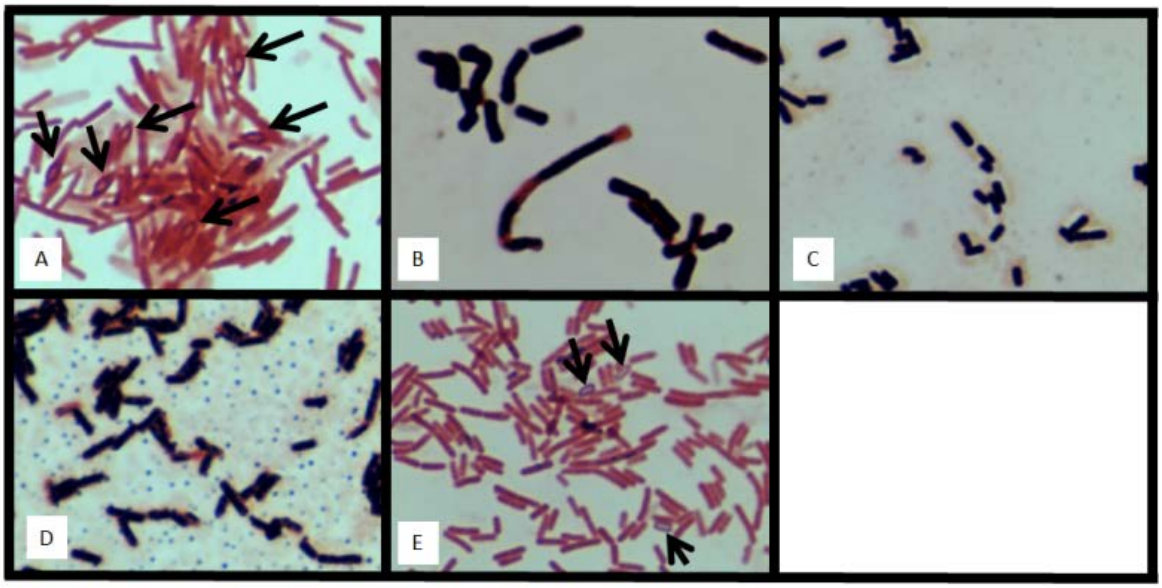


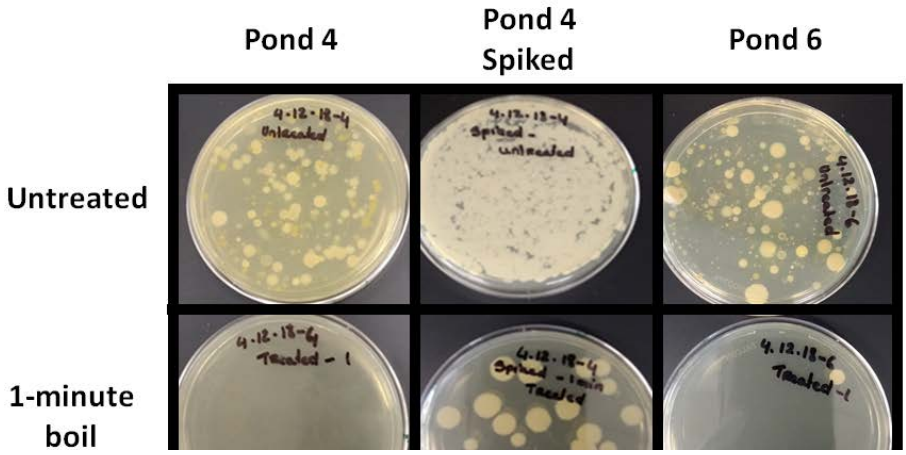
Figure 3: Micrographs of Gram-stained cells isolated from Pond 4 and Pond 6 that survived 1 minute (Species A, B and C), 2 minutes (Species D) and 5 minute (Species E) boiling. Only Species A is from Pond 4. The arrows in the images for Species A and E indicate structures that resemble endospores.

arrangement and did not reveal endospores. Species E survived a 5 minute boil and presented as Gram-negative streptobacilli, with structures that indicated the presence of endospores.

Experiment 2: Second sampling of ponds and spiking sample with *B. thuringiensis*.

After a 24-hour incubation, the untreated spiked plate presented substantial growth of different bacterial species based on colony morphology (Figure 4). As before, we did not attempt to describe any of these species microscopically. The number of colonies were TNTC. The treated spiked sample yielded nineteen colonies which were indistinguishable from each other, and from *B. thuringiensis* colonies, indicating endospore-producing bacterial species can indeed survive a 1-minute boil.

Inoculation of TSA with the unspiked, treated pond samples yielded a single colony, which was from Pond 6. A Gram stain of cells from this colony revealed short, Gram positive bacilli (Species F, Figure 5).



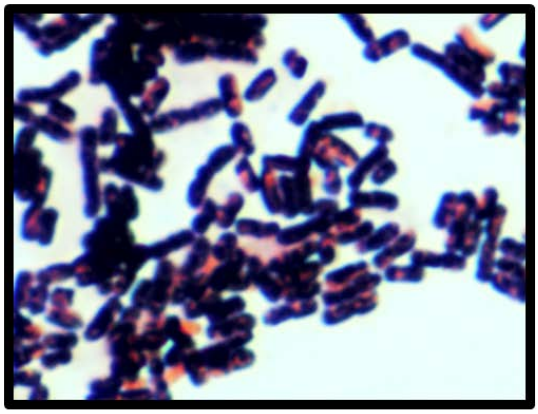


Figure 5: Micrograph of Gram-stained cells isolated from Pond 6, experiment 2, which survived 1 minute boiling.

CONCLUSIONS

The results of this project support the hypothesis that endospore-producing bacterial species will survive the CDC recommended treatment of a 1-minute rolling boil (CDC, 2009). Because a 1-minute boil killed greater than 99% of the bacteria present in the water sampled, this treatment certainly renders water "safer" to drink. Disease-causing bacteria, such as found within the Enterobacteriaceae, Staphylococcaceae, Streptococcaceae, and viruses are known to be killed by the 1-minute boil because they do not produce endospores (Cowan, 2016). However, the current research showed that endospores-producing microorganisms are present in the water samples tested and that endospore-producing bacteria are not killed by a 1-minute rolling boil. Since bacterial endospores are highly resistant, they need stringent conditions (such as autoclaving) to be killed. Therefore, the CDC recommended 1-minute rolling boil may not render water completely safe. The objective of boiling water is to make it potable, meaning ensuring "a minimum microbial hazard" in order to decrease the likelihood of illness. The recommended boiling water standards have a small risk of failure; therefore, it is important to acknowledge the impracticality of eliminating all 100% microorganism in drinking water and render it safe. The treatment clearly reduces the abundance of microorganisms which can reduce the probability of infection but does not eliminate it. Some pathogens have a low infectious dose, meaning they can start an infection with only a few cells. For example, *Bacillus anthracis*, the organism that causes anthrax, has an infectious dose of as low as 8,000 cells (Anthrax, 2018).

Endospores are a dormant phase of bacteria, allowing them to survive without nutrients for long periods of time. The bacteria can return to their vegetative form once the surrounding environment becomes more hospitable. This phenomenon can be particularly important when people collect and boil a large volume of water and save most of it for future use. Endospores that survive the boiling treatment may reactivate and propagate in the water over time. Therefore, there is a possibility that boiling water may make it safer to drink immediately due to reduced microbial load, but the water might become dangerous in the following days. This is a concern, as many people in developing countries do not have access to safe drinking water and must resort to surface water for domestic use. This would also be the case in event of natural disasters which result in poor water quality, or simply an interruption in the supply of normal clean water. Therefore, community education about the distribution and hazards of endospore-forming pathogens, and the long term storage of boiled water may reduce waterborne diseases in at-risk countries even further than the 1-minute boil protocol.

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