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Bacteriostatic Effects of Sucralose on Environmental Bacteria

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Bacteriostatic Effects of Sucralose on Environmental Bacteria

By

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

Sucralose is a zero calorie sweetener developed and manufactured by Tate and Lyle Sweetener Company in the 1980's. They sell the sweetener compounded with maltodextrin and dextrose under the brand name Splenda®. Sucralose was developed as a low cost artificial sweetener that is non-metabolizable in humans and can withstand changes in pH and temperature. It is not degraded by the waste water treatment process. Since the molecule can withstand heat, acidification and microbial degradation it is accumulating in the environment, and has been found in waste water, estuaries, rivers and the Gulf Stream. The highest concentration of environmental sucralose detected to date is 300 ng/L (Torres et al., 2009). Our lab has isolated six bacterial species from areas that may have been exposed to sucralose, given that sucralose has been detected throughout the aquatic environment (Mead et al., 2009). These isolates were cultured in the presence of sucralose looking for potential sucralose metabolism or growth acceleration. Sucralose was found to be nonnutritive, and we found bacteriostatic effects on all six isolates. This inhibition was directly proportional to the concentration of sucralose exposure. The amount of the growth inhibition appears to be species specific. The bacteriostatic effect may be due to a decrease in sucrose uptake by bacteria exposed to sucralose. We have determined that sucralose inhibits invertase and sucrose permease. These enzymes cannot catalyze hydrolysis or be effective in transmembrane transport of the sugar substitute. As sucralose builds up in the environment we must consider it a contaminant due to its bacteriostatic effect. Sucralose may also destabilize or shift the compositions of the bacterial communities in microenvironments such as the mammalian gut.

INTRODUCTION

The demand for a non-toxic and highly stable synthetic sweetener came to the attention of the Tate & Lyle Company, based in London in the late 1980s. In 1989 sucralose (Figure 1) was discovered by accident; the Tate and Lyle Company was trying to develop an artificial sweetener using sucrose (common table sugar) as a chemical intermediate (Knight, 1993). Such a sweetener would be relatively easy to produce given sucrose is cheap and readily abundant. Tate and Lyle were in collaboration with the lab of Professor Leslie Hough from King's College of London. Hough's Lab was studying halogenation of sugars. The popular story told is that a graduate student, was instructed to "test" a chlorinated sugar, and instead thought he was told to "taste" the chloro-carbon! This taste test leads to the discovery that chlorinated sugars are sweet; much sweeter than normal sugars (Knight, 1993). Sucralose was the first non-calorie sweetener made from natural sugar.

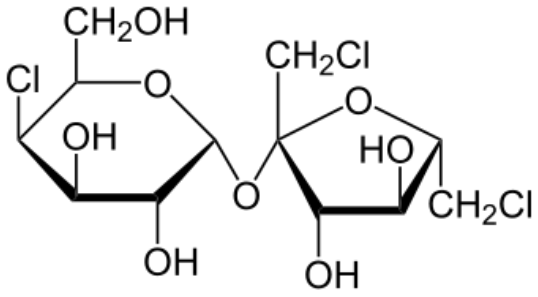

Figure 1. Chemical Structure of Sucralose. (http://drpinna.com/diet-sodas-and-strokes-15640/342px-sucralose-svg) on 12/21/12.

In collaboration Dr. Hough's lab, and Tate and Lyle Research and Development studied many other halo-carbons, including those that were fluorinated. None were as sweet as the chlorinated version of the sucrose molecule (Knight, 1993). It is manufactured by the selective chlorination of sucrose, which substitutes three of the hydroxyl groups with chlorides (Figure 2).

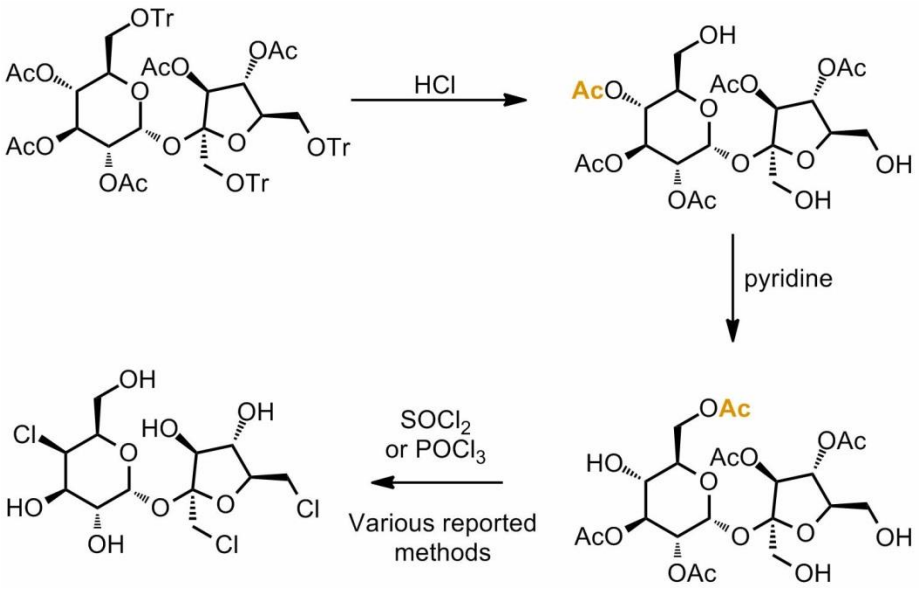

Figure 2. Synthetic pathway for the selective chlorination of sucrose into sucralose. Image provided by http://brsmblog.com/?p=1218 on 12/21/12.

It was found that the chlorines bound the molecule together to foster molecular stability and generate a sweetness that is 600 times sweeter than sugar on the relative sweetness scale, twice as sweet as saccharin, and three times as sweet as aspartame. Generation of a sweet taste comes from the hydrophobic bonding of the taste receptor with electronic bonding of sucralose (Knight 1993).

Sucralose can be found in more than 4,500 food products (Barndt and Jackson, 1990). Maltodextrin is mixed with Sucralose as a 'bulking agent', and is sold internationally under the Splenda brand name (Ma et al., 2010). Sucralose is stable under increased heat and over a broad range of acidic and alkaline conditions. Therefore, it can be used in baking or in products that require a longer shelf life (Ma et al., 2010). Results of a study of carbonated cola at pH 3.1, sweetened with either Splenda or aspartame demonstrated that after one year of storage at 73 °F, 99 % of the Splenda remained unchanged compared to 29 % of the aspartame (Quinlan et al. 1999). Baking studies have determined that sucralose is exceptionally heat-stable. One hundred percent of the sucralose was recovered from cakes, biscuits, and crackers after baking at typical temperatures of 350°F, 410°F, and 450°F, respectively. This seemed to be an excellent artificial sweetener, being that it was so sweet and could handle environmental conditions related to cooking (Barndt and Jackson, 1990).

Sucralose causes exactly zero caloric increase in mammals (Knight 1993). Extensive study on test animals has shown 15% of radiolabel sucralose is excreted via the urine. The other 85% is excreted via the feces. Furthermore, extensive animal testing demonstrated that sucralose is not carcinogenic, neurotoxic, or teratogenic (Goldsmith and Grice, 2000). The United States FDA approved use of sucralose in foods on April 1, 1998. In 1999, FDA approval expanded to classify sucralose as a general purpose sweetener. The compound is approved for use in over 23 countries.

Artificial sweeteners have been considered contaminants by environmental scientists only recently (Scheurer et al., 2011). Due to the human inability to metabolize them, they are

passed on to the environment via human excrement, and the highest concentration (2,800 ± 1,000 ng/L) of combined artificial sweetener contaminants is found in waste-water treatment reservoirs. Artificial sweeteners such as saccharin and cyclamates are found mostly degraded by the waste water treatment process. Sucralose, however, is found in higher concentrations and was degraded minimally (Torres et al., 2011). Degradation only occurs to a limited extent during hydrolysis, ozonation, and microbial processes indicating that breakdown of sucralose will likely be slow and incomplete leading to accumulation of sucralose in surface waters (Soh et al., 2011). Sucralose has been detected in rivers in North Carolina, in the Gulf Stream, and in the waters of the Florida Keys (Mead et al., 2009). Scientists are detecting sucralose in various U.S. inland surface waters, and monitoring its accumulation (Torres et al., 2011).

Most artificial sweeteners are either partially or completely broken down due to the waste water treatment process using high temperatures and changes in pH, and constant filtration. It would seem that the ability of sucralose to withstand drastic pH and temperature changes makes it an exception among artificial sweeteners (Marco et al., 2011). As time passes sucralose may spread to other aquatic and coastal ecosystems, increasing in concentration (Soh et al., 2011).These researchers also speculated that the persistent qualities of sucralose may lead to chronic low-dose exposure with largely unknown consequences for human and environmental health.

To date no study has been published on sucralose's effect on environmental microbes. However, studies of human oral and gut bacteria have shown an inhibition of bacterial growth in the presence of sucralose (Young and Bowen, 1990). In one study the incorporation of 126 mM sucralose into glucose agar medium caused total inhibition of growth of *Streptococcus*

sobrinus 6715-17, *Streptococcus sanguis* 10904, *Streptococcus challis*, *Streptococcus salivarius,* and *Actinomyces viscosus* WVU627 (Young and Bowen, 1990). In a related study rats were infected with *Streptococcus sobrinus,* and following a sucrose water diet, developed dental carries lesions (Bowen and Pearson, 1992). Another group of rats, given the same bacteria but sucralose water instead of sugar water had a significant decrease in carries lesions in their teeth. These researchers concluded that oral bacteria cannot grow on the artificial sweetener hence causing less damage, indicating sucralose is non-cariogenic (Bowen and Pearson, 1992). The same inhibition may be true for environmental microbes.

Since sucralose is increasing in concentration in our waterways, and it has been shown in previous studies to be harmful to oral bacteria, it is proposed that sucralose can negatively affect environmental bacteria as a growth inhibitor. Sucralose may, at higher concentrations, destabilize bacterial communities; the basis for the health of our entire biosphere. These microbes not only provide the basis of the food web of communities, but are also responsible for decomposition in the environment and recycling the nutrients via biogeochemical cycles. Furthermore, if sucralose does inhibit bacterial growth the type of inhibition would need to be identified as either bactericidal (killing the bacteria) or bacteriostatic (slowing bacterial metabolism), and the mechanisms of such inhibition should be elucidated.

Methods and Materials

Summary of methodology

In order to elucidate the effect that sucralose has on bacterial growth environmental sampling of diverse ecosystems is needed. Once bacterial isolates are obtained they should be gene sequenced in order to identify them. In order to validate the gene sequencing, gram staining, colony characters, and cellular and colony morphology shall be inspected. Each bacterium isolated will be surveyed for sucralose metabolism. If sucralose is found to be non-nutritive for the bacterium; the effect on healthy bacterial growth shall be observed via turbidity testing by culturing the bacterial isolates on TSB and amending the media with various concentrations of sucralose. Any inhibition can be typed as either bacteriostatic or bactericidal; this can be determined with a disk diffusion assay and re-culturing. If re-culturing is possible than the effect that sucralose has on the bacteria was bacteriostatic. Finally the mechanism of such inhibitory effect can be identified by enzyme and transport assays, based on the molecular kinetics analysis one could inspect the molecular basis of such inhibition. Transport inhibition and reduction in catalysis could be indicators of competitive inhibition.

Collection and Isolation of Bacteria

Water and soil samples from 7 test sites around Jacksonville, Florida were collected aseptically. Samples were spread plated out onto Tryptic Soy Agar (TSA) (Difco Laboratories, Michigan, USA) within an hour of collection.

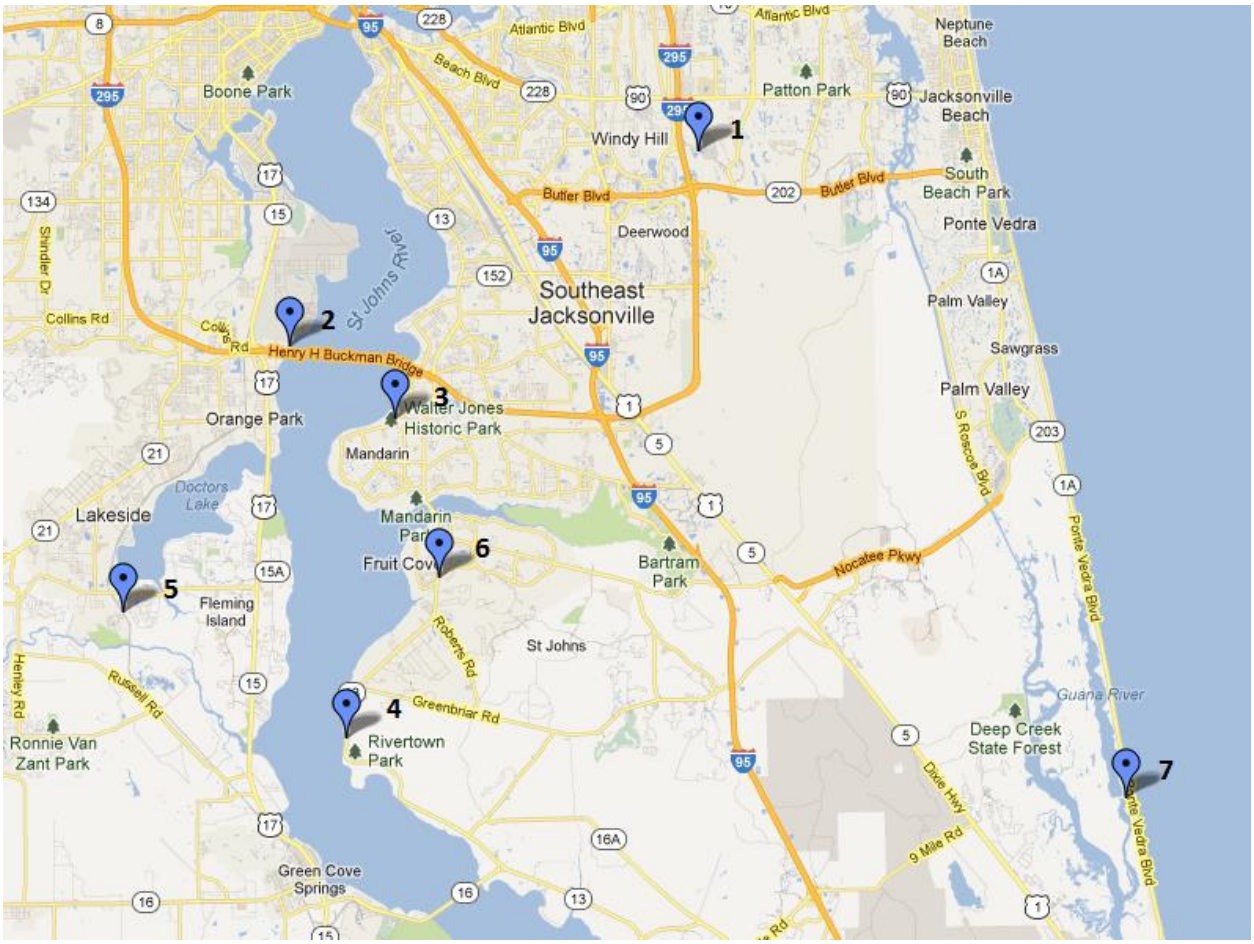

Figure 3. Map of Jacksonville Florida with collection sites indicated. Indicated numbers are referenced in Table 1. https://maps.google.com/maps=34.056179,-118.249669 as of 10/20/12.

Table 1. List of collection sites for environmental sampling.

Sample Number	Location	Sample Type	GPS Coordinate North - West
1	UNF (Lake Oneida)	Water and Soil	30.266912,-81.513347
2	The Rudder Club Dock (St. Johns River)	Water and Soil	30.193071,-81.691266
3	Duval County Dock (St. Johns River)	Surface Water	30.165346,-81.645559
4	St. Johns Parkway Dock (St. Johns River)	Surface Water	30.045679,-81.667192
5	Clay County Waste Water Facility	Nutrient Poor Waste Water	30.093079,-81.764524
6	St. Johns County Waste Facility	Waste Water Product	30.106153,-81.625693
7	Guana River Road (Estuary)	Water and Soil	30 01'23.04-81 19'42.21

Water and soil from samples were serially diluted 3 fold with sterile 0.89% NaCl solution, spread plated on Tryptic Soy Agar, and incubated at 32.7 °C for 48 h. Twenty eight putative bacterial species growing on TSA were isolated into pure cultures based on colony morphology. Isolates were then Gram stained. These isolates were then screened for sucralose metabolism.

Sucralose Metabolism Screening

For each of the original 28 isolates, 0.001 ml of isolate cultures were diluted with 3 ml of 0.89 % NaCl solution. These samples were spread-plated onto M9 agar containing 2% glucose (Technova, Nova Scotia CA) (positive control), M9 agar containing 80 mM sucralose and glucose (experimental), and M9 agar containing no sugars (negative control). The six Isolates which exhibited growth on the M9 agar containing sucralose and glucose were selected for further experimentation.

Identification of Bacterial Species

Selected isolates were then identified via 16SrRNA sequencing. Genomic DNA was extracted from each of the selected bacterial isolates using the Ultraclean Microbial DNA Isolation Kit in accordance with manufacturer protocols (MO BIO Laboratories, California, USA).

The 16S rRNA gene was amplified using the bacterial consensus primers 8F (5′ AGTTGATCCTGGCTCAG 3′) and 1492R (5′ ACCTTGTTACGACTT 3′). The long polymerase chain reactions (PCR) consisted of 41.7 µL dH_2O, 5.0 µL 10x *Taq* buffer, 1.5 µL 50mM $MgCl_2$, 10 µM forward primer, 10 µM reverse primer 0.4 µL 25 mM dNTPs, 0.4 µL 5U/µL *Taq* polymerase, and 1 µL genomic DNA in a final volume of 50 µL. DNA amplification was performed with the following thermocycler regime: 2 min at 98°C followed by 33 cycles of: 98°C for 30 s, 45°C for 60 s, 72°C for 90 s and a single step at 72°C for 10 min. Short PCR amplification consisted of 50 µL reactions with analogous reagents/concentrations to the long PCR, using the additional primers 760R (5′ CTACCAGGGTATCTAAT 3′) and 790F (5′ ATTAGATACCCTGGTAG 3′) with the following thermocycler settings: 25 cycles of 98°C for 30 s, 44°C for 45 s, and 72°C for 90 s.

The short PCR products were cleaned up using the QIAquick PCR Purification Kit following manufacturer protocols (Qiagen, California, USA). The four primers were employed for cycle sequencing on a CEQ 8000 Genetic Analysis System (Beckman Coulter, California, USA) using 1 µL GenomeLab DTCS Quick start master mix, 2 µL primer, 2 µL DNA, and 7 µL dH_2O. Cycle sequencing consisted of 33 cycles at 96°C for 30 s, 37-47°C for 15 s and 60°C for 4 min. Sequencing reactions were performed using each of the amplification primers and internal primers so that each fragment was sequenced in both the forward and reverse directions. Products were cleaned and precipitated according to manufacturer specifications (Beckman Coulter, California, USA).

The obtained sequences were compared to other sequences using the BLAST function through the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences determined 99% certain that the isolates were not new species. Isolates were then identified to the level of species.

Growth/Turbidity Testing

The isolates were cultured in Tryptic Soy Broth (TSB) (Difco Laboratories, Michigan, USA) and incubated at 25°F. The control group consisted of 5ml of TSB amended with additional 0.5 ml growth medium, the experimental groups included 5ml TSB with 0.5 ml of 10, 20, 30 or 40% by volume sucralose added (27.8 mM, 55.7 mM, 83.7mM,111.7 mM). Turbidity of the cultures was inspected spectrophotometrically at 620nm every 24 hours for 9 days.

Individual isolates were also cultured in M9 Broth media and incubated at 25 °C. The control group consisted of 5 ml of M9 broth with no carbon source; the experimental group included 5 ml of M9 broth with sucralose as the only carbon source. Turbidity of the cultures was measured over 9 days using 24 hour time intervals using a Sequoia Turner Spectrophotometer set to 620nm wavelength.

Disk Diffusion Assay and Determination of the Type of Inhibition

Each bacterial isolate was spread-plated onto a TSA media. Disks were prepared by hole punching out filter paper, which were soaked with 1.6 M sucralose. The disks were then placed onto the surface of the media, 3 disks per Petri dish. Samples were incubated over night at 25 °C. Diameters of the zones of inhibition were measured. The zones of inhibition were then swabbed and used to inoculate new TSA media. These re-culture plates were incubated over night at 25 °C and then inspected for growth.

Transport Inhibition Testing

Each isolate was individually cultured onto six M9 agar plates with glucose, and six M9 agar plates with sucrose. Three fold serial dilutions of stock cultures were made and spread out onto the agar plate's surface; then 350 µl of 25.1 mM sucralose was poured onto the surface of each of the sucralose added groups shortly after inoculation. These were incubated at 25 °C for two days. On the third day the plates were inspected and colonies counted. *Streptomyces badius* exhibited the greatest percentage of cell death on the M9 sucrose media compared to M9 glucose media and was selected for transport inhibition testing in order to elucidate an inhibitory mechanism.

A Bradford Coomassie assay was conducted. From this cell culture concentrations were selected in order to yield the appropriate amount of membrane transport proteins. Three test groups were used to measure potential transport inhibition, a 0.1 mM sucrose only group, a 0.1 mM sucrose and 0.1 mM sucralose group, and a 0.1mM sucrose and 0.1mM mannitol group which served as a control to ensure osmotic shock was not occurring during the transport test. Each group contained 700 µl of dilute M9 salt aliquots (64 g Na_2HPO_4 ,15 g KH_2PO_4 , 2.5 g NaCl, 5 g NH_4Cl per 5 liters H_2O), and 0.5µl of ^{14}C radioactive sucrose 0.41 µCi/pmole, exactly 300 µl of cell culture in stationary growth phase was extracted and placed into the mixture and shaken vigorously. The contents of the reaction tubes were incubated at 25 °C for 2 min, filtered onto a 0.45 µm pore size filters, and washed with 2 ml of stop solution (ice cold M9 salt aliquots). The filters were placed into a tube with scintillation fluid and the radioactivity measured via Beckman coulter scintillation counter.

Enzyme Kinetics: Invertase Inhibition Assay

Two test groups were prepared: 1) a sucrose only set, and 2) a sucrose and sucralose set. The sucrose only set had 6 reaction tubes prepared, each reaction tube contained 1 ml 0.3 U/L of invertase, 0.25 ml of benedict's solution, 0.75 ml pH 4 buffer. Each tube contained different amounts of sucrose, 2.5 mM, 5 mM 10 mM, 15 mM, 20 mM, and 25 mM. Reaction tubes were incubated at 75 °C, the absorbance of each tube was measured at 485 nm. At minute 5 the initial velocity was recorded. The sucrose and sucralose set had 6 reaction tubes prepared, each reaction tube was prepared as above, with the addition of 0.55mM sucralose added. Reaction tubes were incubated at 75 °C, the absorbance of each tube was measured at

485 nm after 5 min to record initial velocity. Once these assays were completed, the velocities were analyzed and used to generate an enzyme kinetics plot to determine the type of inhibition sucralose exerts on invertase.

Results

Sucralose Metabolism Validation

The initial environmental sampling yielded 28 different putative bacterial species based on colony morphology. When these isolates were cultured onto 2 % glucose M9 agar laced with 50 mM sucralose, only 6 isolates showed growth (Table 3). The 6 isolates that survived were screened for sucralose metabolism by culturing in M9 media with 20 mM sucralose as the only carbon source. A positive control consisted of M9 media with 20 mM glucose as the sole carbon source and negative control consisted of a "starvation diet" with no carbon source available. There was no difference in the response of the isolates; figure 4 shows the average of all six growth tests. The M9 media with sucralose exhibited no growth and showed a trend that was almost identical to the starvation growth curve.

Table 2. List of bacterial isolates colony morphology, gram character, and identification based on 16S gene sequences.

Organism Identity based on 16srRNA gene	Gram Character	Shape	Colony Morphology
Microbacterium sp. U 13	Gram+	Coccus	Grey pale filamentous flat with filiform margins
Stenotrophomonas sp. I_61	Gram-	Coccus	Yellowish white, circular umbonate form with entire margins
Rhizobium borbori	Gram-	Coccus	Grey, circular convex form, with entire margins
Citrobacter murlinlae	Gram-	Bacillus	Bright white, umbonate form with entire margins
Ensifer arboris	Gram-	Bacillus	Dull white, rhizoid form with filiform margins
Streptomyces badius	Gram+	Bacillus	Bright white, filiform margins

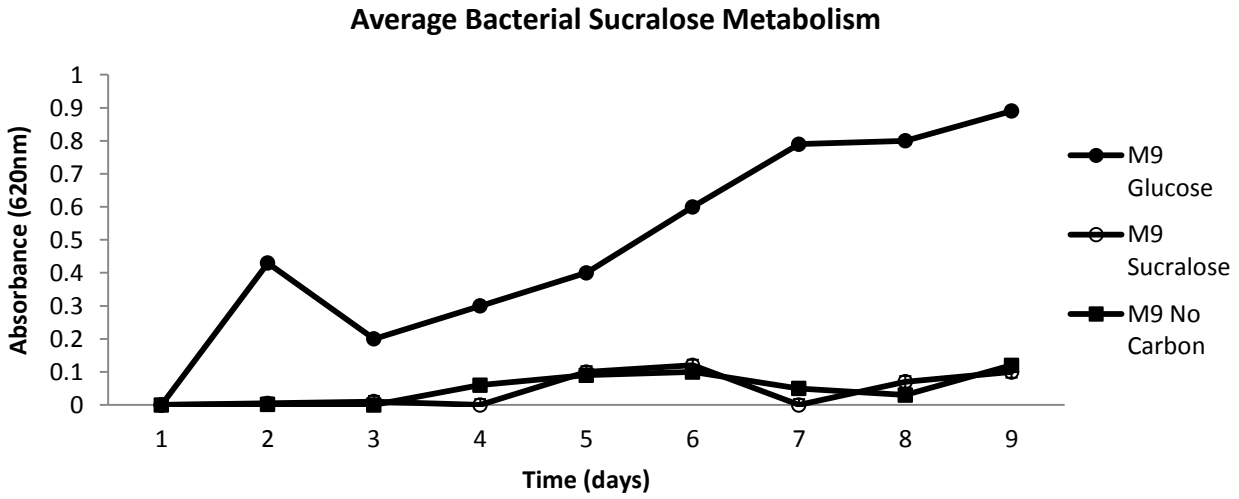

Figure 4. A composite growth curve depicting average bacterial growth with various carbon sources of the 6 isolates. M9 media containing glucose as the only carbon source serves as a positive control, M9 media containing only sucralose as a carbon source was the experimental group, and M9 media containing no carbon source serving as a "starvation diet" or negative control. This was done to indicate the presence, if any, of sucralose metabolism.

Turbidity Testing

To elucidate the effect of sucralose on bacterial growth, turbidity testing was performed. Varying concentrations were utilized to produce a gradient effect graphically. Positive growth effect would display varying concentrations of sucralose groups above the positive control group (a 0mM sucralose group). Figures 5, 6, 7, 8, 9 and 10 show a negative gradient with the varying concentrations of sucralose. Not all concentrations were inhibitory. The least concentrated dilution (28.7mM) showed no inhibitory effects on any of the six bacterial isolates. The 55.7mM sucralose had minor inhibition on the isolates, and was

significantly different for only 2 of the isolates. All six isolates showed inhibited growth; at the 83.7 mM and 111.7 mM concentrations.

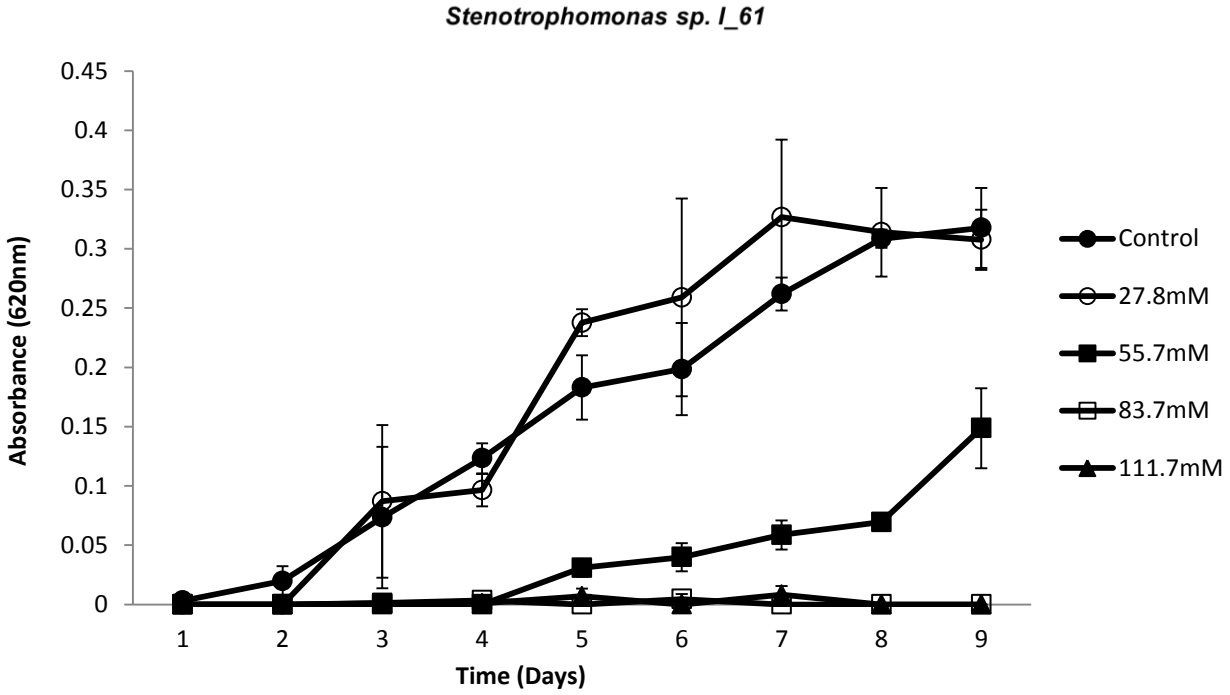

Figure 5. Growth curve for *Stenotrophomonas* sp. I_61. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group, without sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 55.7 mM 83.7 mM and 111.7 mM concentrations were significantly ($p < 0.05$) inhibited compared to the control group.

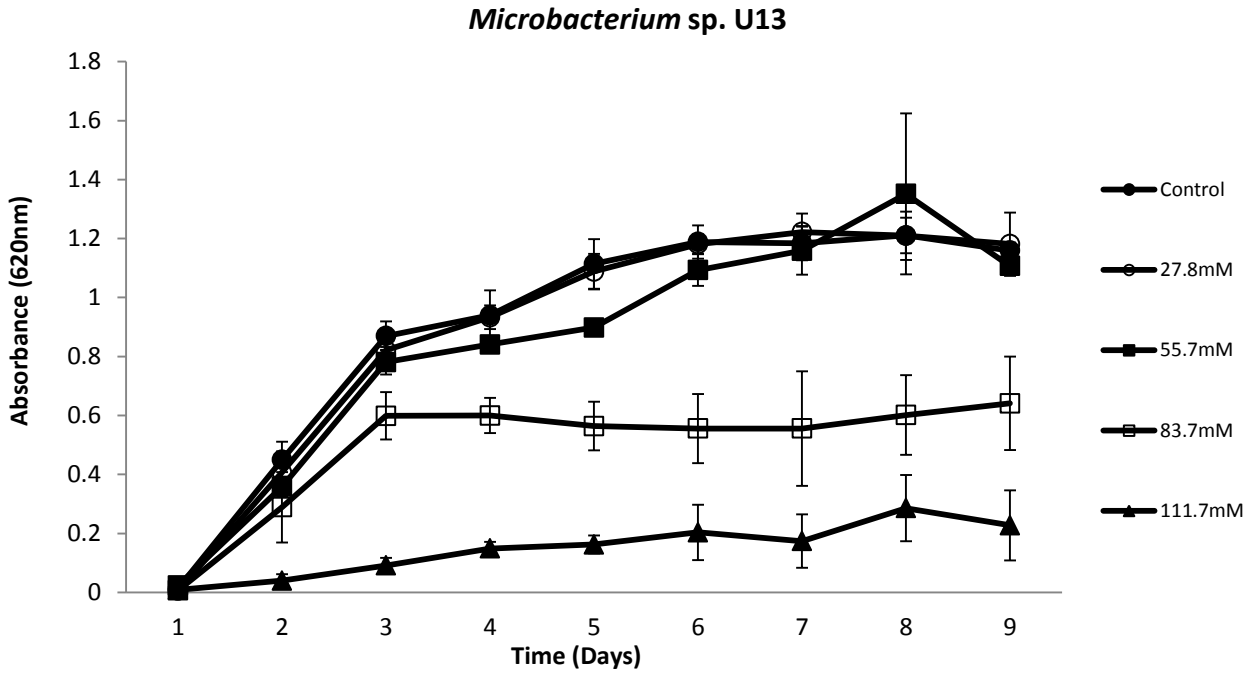

Figure 6: Growth curves for *Microbacterium sp.* U13. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group consisted of no sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 83.7mM and 111.7mM concentrations were significantly ($P< 0.05$) inhibited compared to the control group.

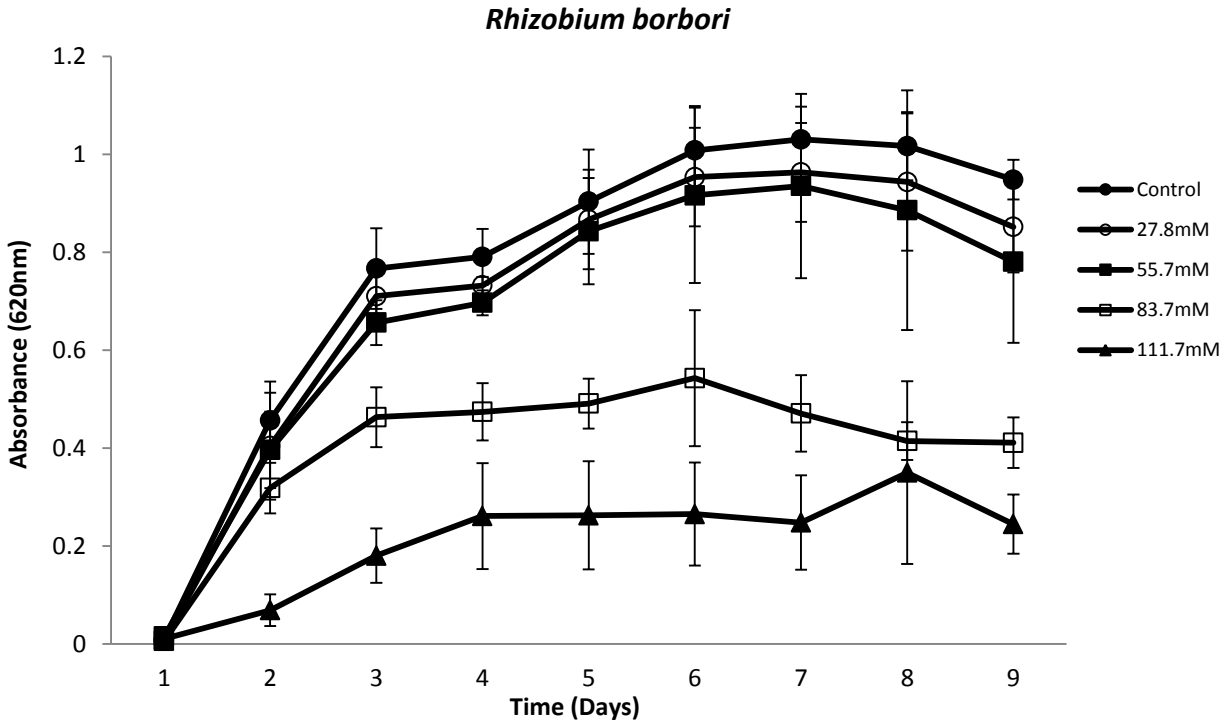

Figure 7: Growth curves for *Rhizobium borbori*. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group consisted of no sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 83.7mM and 111.7mM concentrations were significantly ($p < 0.05$) inhibited compared to the control group.

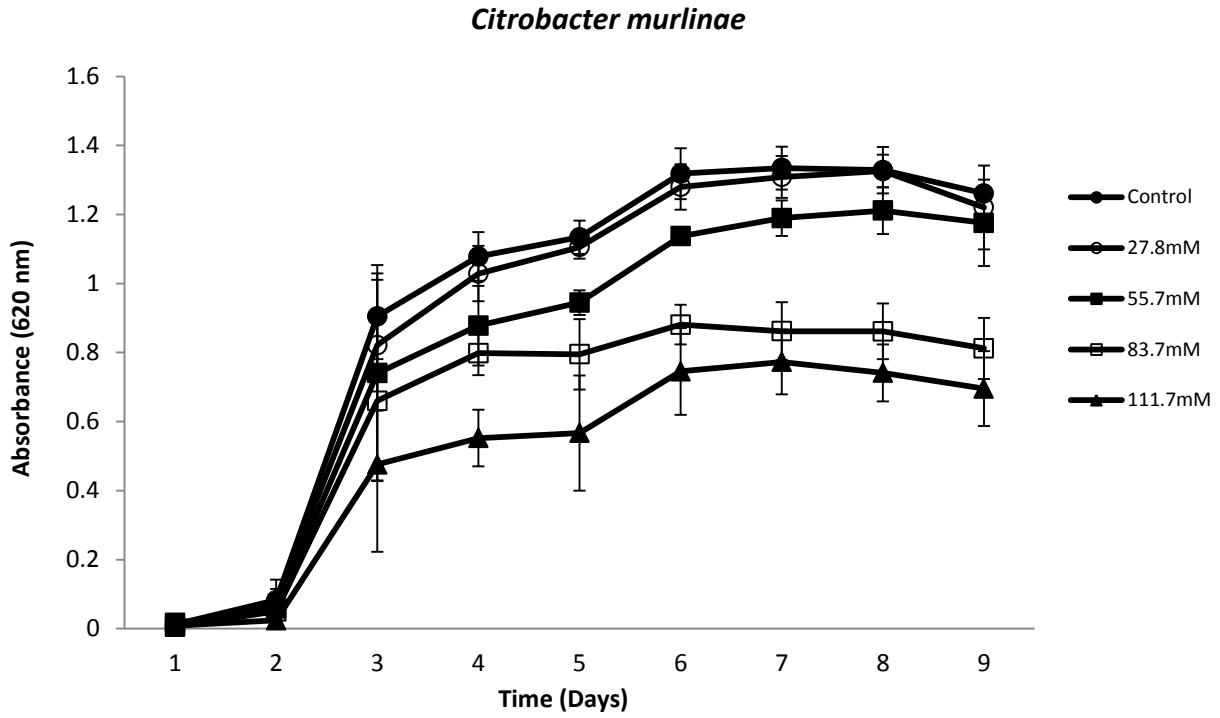

Figure 8: Growth curve for *Citrobacter murlinae*. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group consisted of no sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 83.7mM and 111.7mM concentrations were significantly ($p< 0.05$) inhibited compared to the control group.

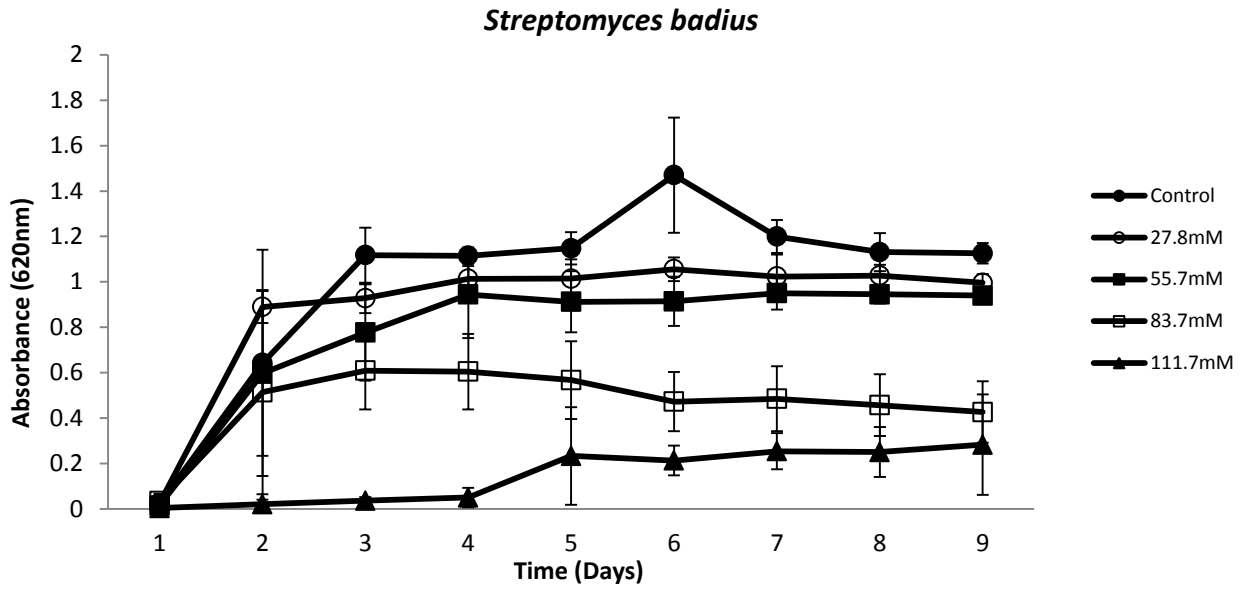

Figure 9: Growth curve for *Streptomyces badius*. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group consisted of no sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 55.7 mM 83.7mM and 111.7mM concentrations were significantly ($p < 0.05$) inhibited compared to the control group.

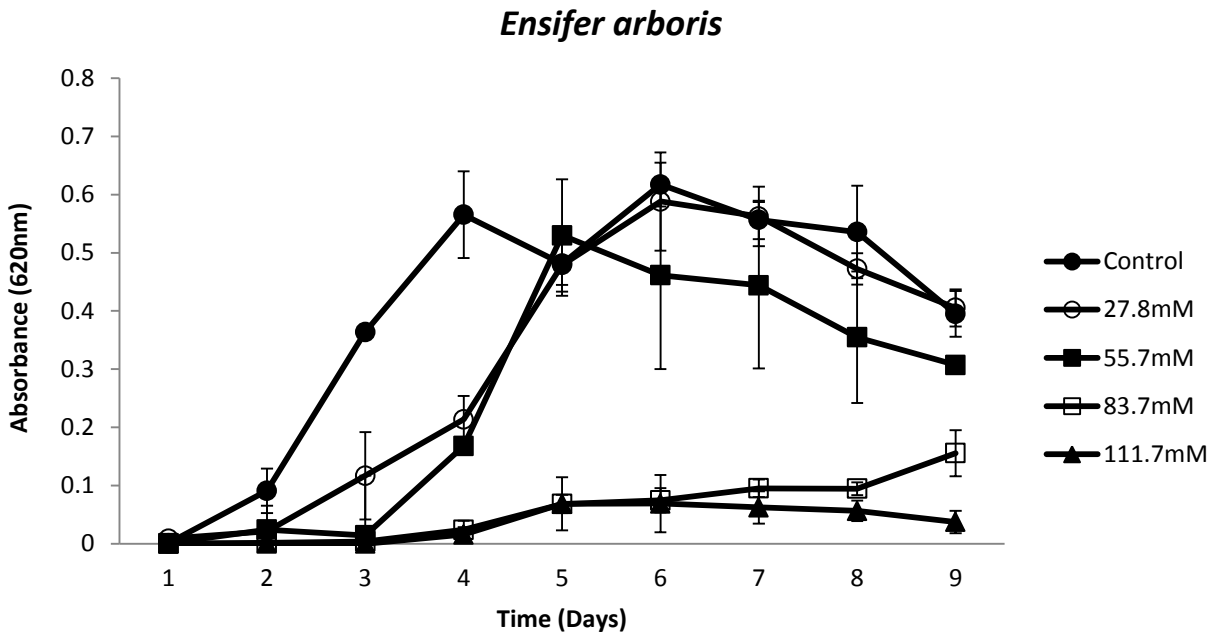

Figure 10: Growth curve for *Ensifer arboris*. The isolate was cultured in TSB amended with varying concentrations of sucralose. The positive control group consisted of no sucralose added to the TSB, in order to ascertain normal growth. This was performed to determine the effect that sucralose had on bacterial growth. The 83.7mM and 111.7mM concentrations were significantly ($p < 0.05$) inhibited compared to the control group.

Inhibition Type Identification

Disk diffusion assays exhibited a wide range of zones of inhibition with species responses being different. Each clear zone (a zone without colonies in it) was then sampled and used to inoculate a fresh culture dish. Regrowth indicated a bacteriostatic effect; with all clear zones sampled yielding growth. Regrowth was of the same colony morphology and gram character as the original culture for each isolate. This result suggests that the sucralose is not a bactericidal agent (Figures 23 and 24).

Table 3: Disk Diffusion assay data, zone of inhibitions are indicated. Regrowth from inhibited zones was tested; regrowth indicated a bacteriostatic inhibition not bactericidal.

Isolate	Average Inhibition (cm)	Regrowth
M. sp. U 13	1.12	yes
S. sp. I_61	1.9	yes
R. borbori	1.07	yes
C. murlinae	1	yes
E. arboris	0.866	yes
S. badius	0.667	yes

Determination of Sucralose effect on Transport Proteins

In order to elucidate the mechanism of the bacteriostatic inhibition further testing was needed. Looking for differential growth effects on normal carbon sources while exposed to sucralose was used to help find such a mechanism. Bacterial isolates were partially inhibited when cultured on glucose M9 agar with sucralose, and on sucrose M9 agar with sucralose. The colony counts for the media containing sucralose were lower than media free of sucralose across the board (Figure 11). *Streptomyces badius* showed greater inhibition on sucralose containing media than other isolates, greater inhibition was observed on sucrose M9 media than on glucose M9 media. Therefore, *Streptomyces badius* was utilized for transport testing (Figure 11). A 0.1 mM mannitol control was used to ensure that the effects of sucralose were not due to osmotic shock. There was a significant decrease in transport of C^{14} labeled sucrose by *Streptomyces badius* when exposed to sucralose (Figure 12).

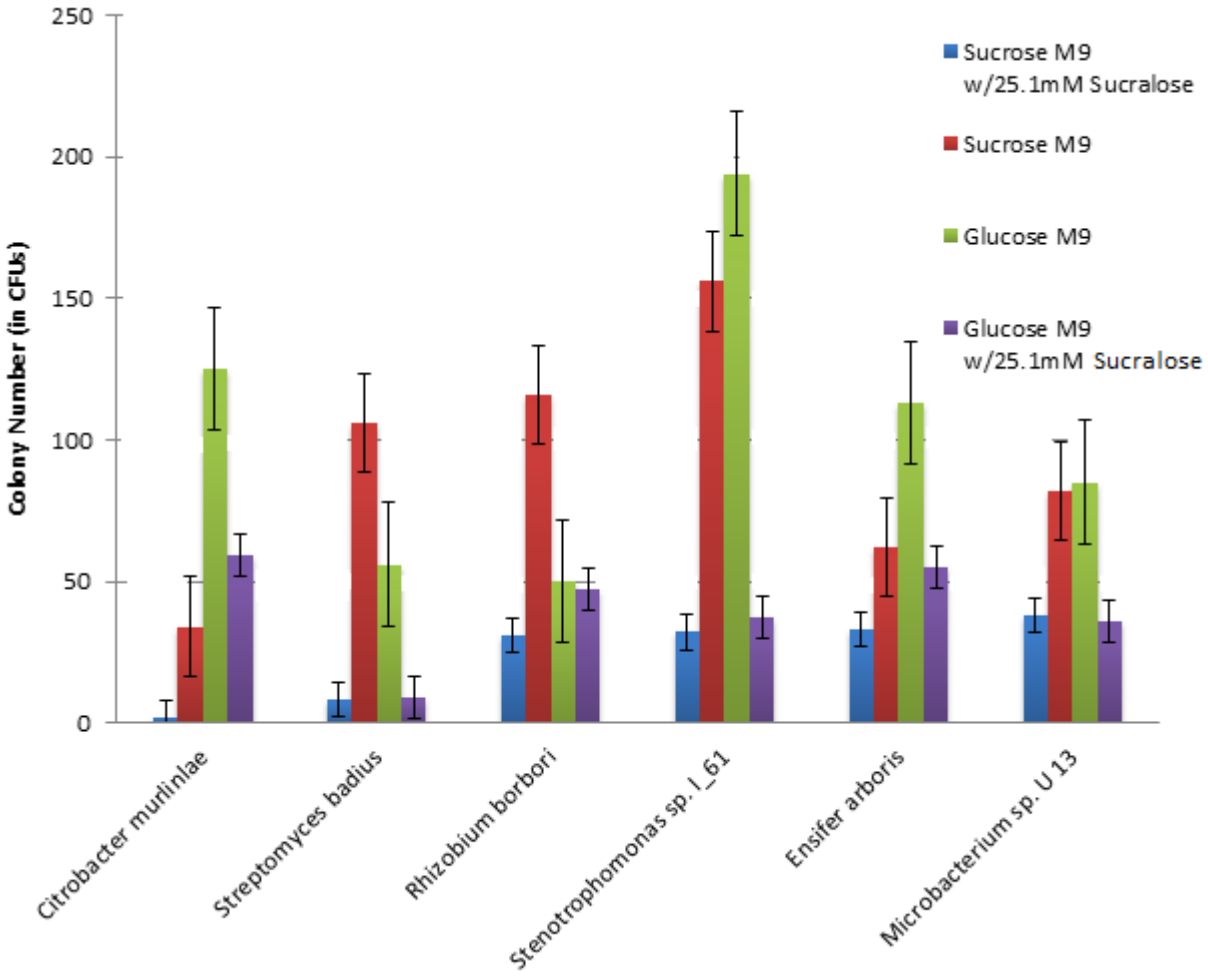

Figure 11: Cell death graph for comparison of inhibition on different carbon source media. Each isolate was cultured in equimolar (111mM) amounts of either sucrose or glucose as their carbon source, with half the samples also containing sucralose. Finally colony counts were performed.

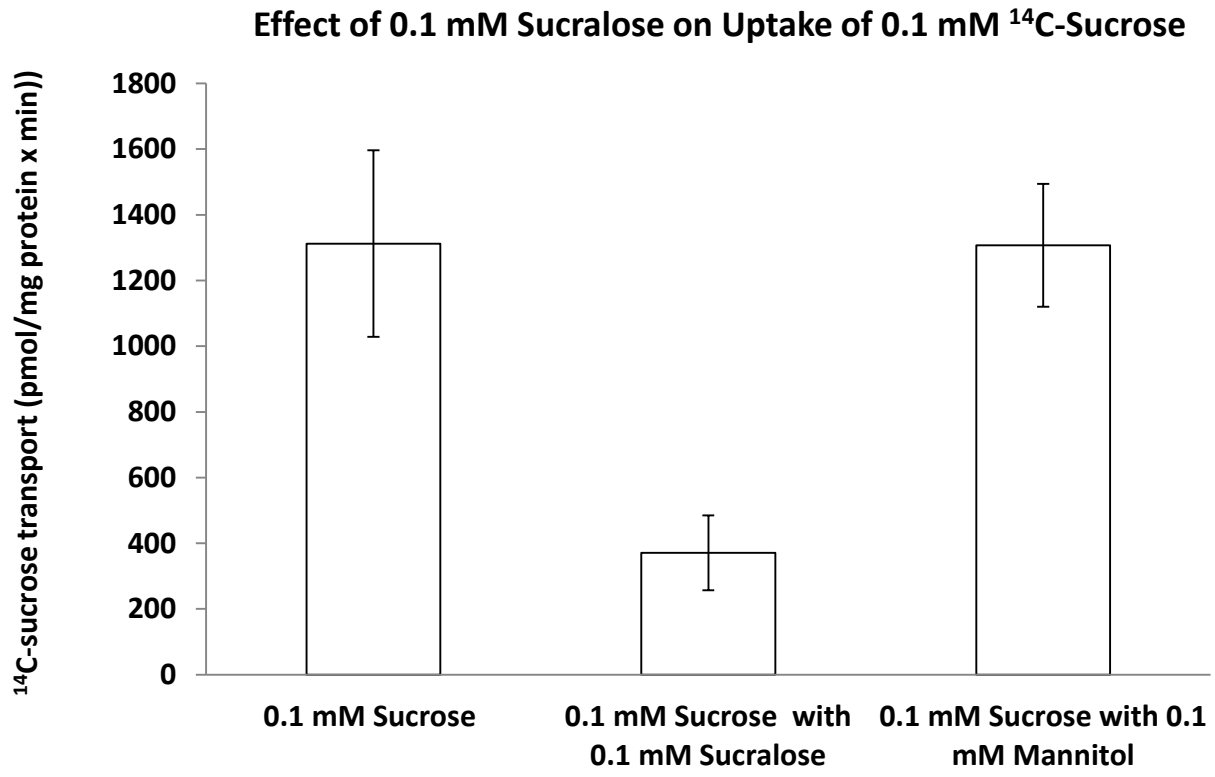

Figure 12: Transport inhibition data: Counts/(min X mg protein) for *Streptomyces badius*. This suggests that sucralose is an inhibitor of sucrose uptake via transport proteins in *S. badius.*

Enzyme Kinetics: Invertase Assay

To further glean a molecular mechanism of inhibition enzyme assays were run using invertase to catalyze sucrose degradation. The initial reaction rate and overall reaction rate of invertase was inhibited when the enzyme was suspended in solutions containing sucralose (Figure 13). This shows that sucralose is an inhibitor of invertase enzymatic activity. The kinetics plot was prepared using the initial velocities of uninhibited reaction with inhibited reactions at equimolar concentrations of sucrose (Figure 13). The results for the kinetics study revealed V_{max} values that were not significantly different ($p > 0.05$), but K_m values for the reactions that were significantly different ($p < 0.05$) (Figure 13; Table 4). This is indicative of competitive inhibition between sucrose and sucralose for binding to invertase.

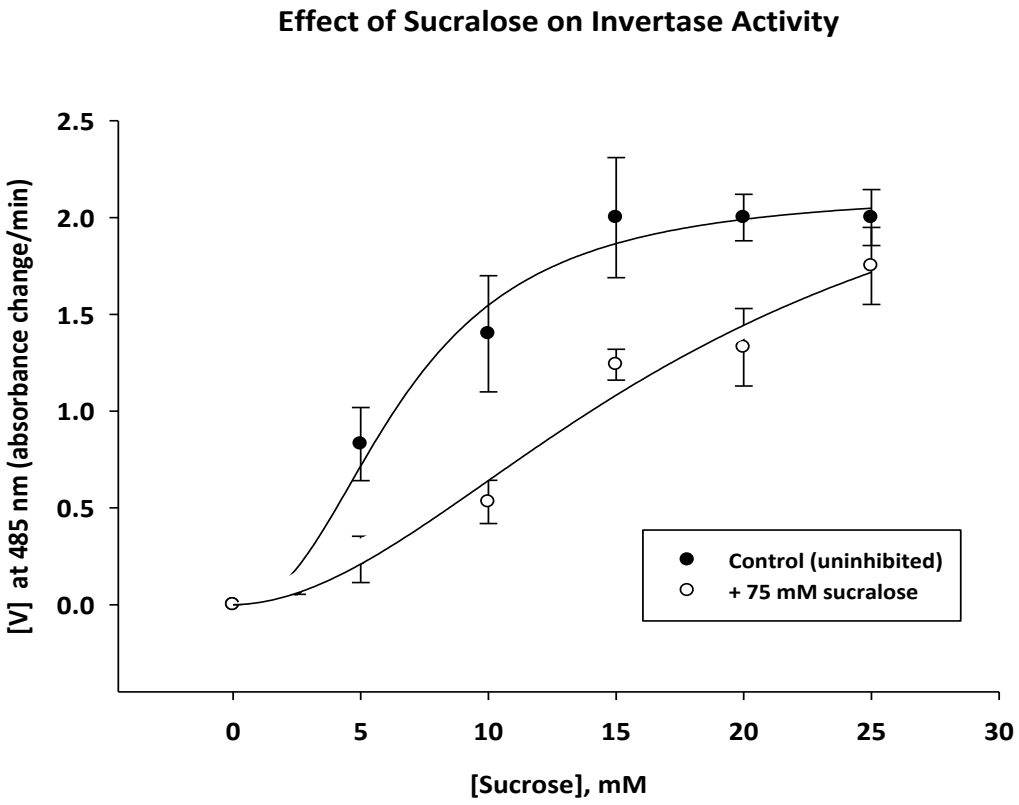

Figure 13. An enzyme kinetics graph the initial velocities of uninhibited invertase reaction and invertase inhibited with sucralose. The overlapping V_{max} values but different K_m values for the reactions indicate competitive inhibition.

Table 4. Invertase reaction rate kinetic constants from Figure 13.

	K_m	V_{max}	Hill coefficient
Positive control	6.66±0.842	2.13±0.16	2.37±0.56
75mM sucralose Added	18.52±9.78	2.69±1.25	1.88±0.65

Discussion

Of the 28 isolates extracted from environmental samples, only 6 had growth on the sucralose laced glucose M9 media. Of the 6 unique bacterial isolates that were obtained, 4 were Gram- and 2 Gram+ (Table 3). They were identified as the bacteria: *Microbacterium* sp. U 13, Stenotrophomonas sp. I_61, *Rhizobium borbori, Citrobacter murlinlae, Ensifer arboris,* and *Streptomyces badius* (Table 3). Isolates that were chosen for gene sequencing and further experimentation were able to withstand culturing on the M9 sucralose and glucose agar.

These 6 isolates had fewer colonies forming units (CFUs) on the media exposed to sucralose than they had on the positive control groups of M9 sucrose and M9 glucose (Figure 11), indicating an inhibitory effect. These organisms were not metabolizing sucralose as shown in Figure 4; no isolate was able to metabolize sucralose indicating that sucralose is non-nutritive for bacteria. The isolates were sub-cultured (n=5) in the presence of 27.8 mM, 55.78 mM, 83.75mM, and 111.7mM sucralose to elucidate effects of sucralose on bacterial growth, with controls consisting of isolates amended with an additional volume of sterile deionized water.

Growth curves showed a decrease in growth with those cultures receiving sucralose addition compared to the control (Figure 5, 6, 7, 8, 9, and 10). Utilization of standard error of the means indicates a significant ($p < 0.001$) difference between control groups and experimental groups amended with 83.75 mM and 111.7 mM sucralose. These results indicate the addition of sucralose is a growth inhibitor for bacteria from diverse genera.

Turbidity testing of the 6 isolates showed there was a gradient in susceptibility, with *Stenotrophophomonas* sp. being the most susceptible to sucralose. The other isolates showed inhibition as well, but not as marked. Statistically significant ($p>0.99$) differences were not observed between control groups and isolates exposed to 25.7 mM sucralose. Of those 6 bacterial isolates not completely inhibited by sucralose, two showed significantly decreased growth ($p<0.05$) response in the presence of 55.78 mM sucralose (Figure 5 and 9). The negative affect that sucralose had on their growth rates was directly proportional to the concentration of sucralose added to the growth media. In summary, the 27.8 mM and 55.78 mM sucralose treatments did not significantly inhibit the growth rates of these isolates, or minimally inhibited the isolates in their growth rates. The 83.75 mM and 111.7 mM treatments did have a rather marked inhibitory effect on bacterial growth across the board. There were species specific differences with the susceptibility to sucralose's inhibitory effect.

For the disk diffusion assays the result was bacteriostatic, 100 % of our disk diffusion assays could be re-cultured from the zone of inhibition (Table 3). This re-culturing was possible despite the rather high concentration of sucralose (1.6 M) that the disks were allowed to soak up. Cell death testing was performed, by lacing various M9 medias with sucralose and comparing the number of colony forming units between those media and media not exposed to sucralose. We used M9 media with glucose and M9 media with sucrose as positive controls, two of our isolates: *Citrobacter murlinae* and *Streptomyces badius* show more drastic inhibition when their carbon source was sucrose, rather than glucose (Figure 11). This indicated possible reduction of sucrose transport by sucralose.

Radiolabeled sucrose transport testing was performed in order to verify this hypothesis. The transport test showed significant ($P < 0.05$) inhibition of transport of sucrose when sucralose was added to the transport solution (Figure 12; Table 4). This reduction was not due to osmotic shock, as indicated by the mannitol control group (Figure 11).

The transport test results suggest that transport proteins were inhibited. All isolates showed growth inhibition regardless of carbon source, knowing the inhibition was bacteriostatic and that it slowed sugar transport in one of our isolates suggested that the mechanism of inhibition was that the bacterial proteins were treating sucralose as if it were sucrose, and then unable to transport it. We proceeded with an enzyme kinetics assay to elucidate if the proposed mechanism affected metabolic enzymes for sugar break down.

Invertase was selected due to its conserved and broad usage in the microbial world, found in bacteria and fungi. It has been found throughout the domain bacteria: in extremophiles, gut flora and environmental bacterial species (Alberto et al., 2004; Yamamoto et al., 1986; Parrent et al., 2009). Invertase is an enzyme that catalyzes the breakdown of sucrose into glucose and fructose.

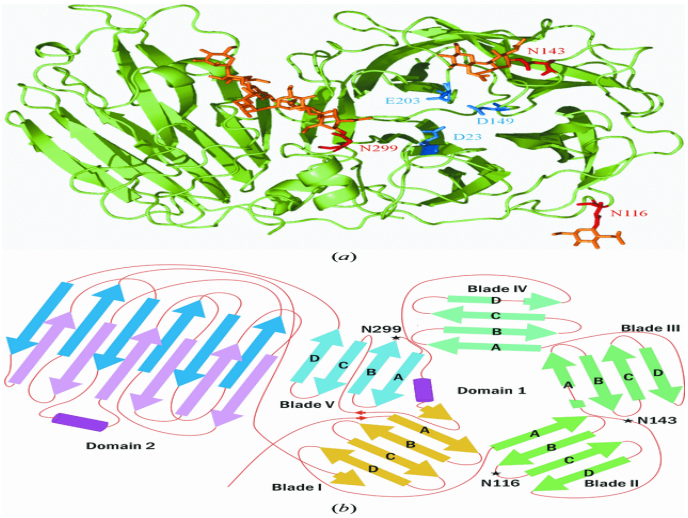

Figure 14. A graphic depicting the 3D structure of invertase, active site is E203, D149, and D23 are colored in blue. (http://www.xtimeline.com/evt/view.aspx?id=131226).

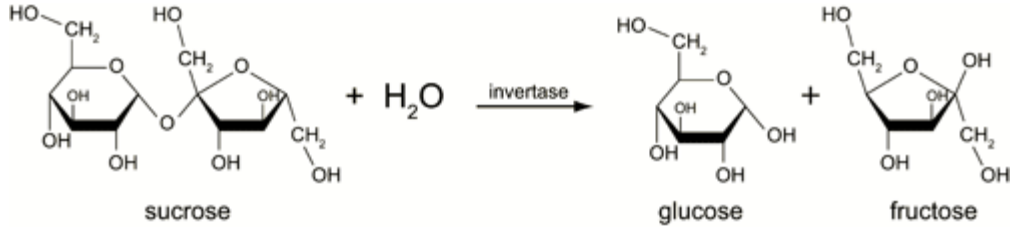

Figure 15. Chemical Reaction showing invertase function (http://nano.cancer.gov/action/news/featurestories/monthly_feature_2005_jun.asp) .

The assay and subsequent data analysis indicated that invertase is inhibited competitively by sucralose (Figure 13; Table 4). The initial and overall reaction rates of invertase are slowed by the addition of sucralose (Figures 13, 24 and 25). The V_{max} values for both inhibited and uninhibited reactions are not statistically significantly different from one another given standard error (Figure 13; Table 4). Together these results indicate that sucralose is a competitive inhibitor of sucrose using invertase. The active site of invertase contains two aspartate and one glutamate residues, these hydrogen bond with sucrose to achieve hydrolysis.

Hydrogen bonding may be sterically hindered by the chlorine groups of the sucralose molecule; preventing invertase from catalyzing sucralose degradation.

Sucralose reducing sucrose uptake and breakdown in bacteria by competing for a binding site serves as a potential mechanism for the bacteriostatic effect observed during growth trials. Also, the fact that sucralose is a competitive inhibitor of invertase, the V_{max} was unchanged while the K_m was significantly different ($P < 0.05$), is another indicator that sucralose is accepted by cellular enzymes that can bind sucrose. While these cellular proteins can accept sucralose into their binding sites they cannot catalyze or transport it.

In previous dental studies sucralose caused oral bacteria to proliferate less frequently, preventing cavity formation (Bowen et al., 1992). These studies also noted that the lab mice given sucralose had less fecal bacteria present, and that gut bacteria were inhibited by sucralose (Abou-Donia, 2008). I propose that the inhibition in previous studies was bacteriostatic, and these oral and gut bacterial tests are in concurrence with the environmental bacterial testing results.

Conclusions

I originally began this study in an attempt to find an organism that could metabolize sucralose. I had hypothesized that bacterial metabolism is diverse, so something must be able to digest sucralose. Looking back, this hypothesis was incorrect; I had to reject my initial hypothesis after performing the sucralose validation many times over (Figure 4). I had failed to find an organism that could metabolize sucralose as a sole carbon source.

It is possible that no microbe can solely metabolize sucralose. The sweetener would probably need to be de-chlorinated before it could be metabolized; the chlorines are what make sucralose hard to handle by invertase and possibly other sugar binding enzymes. Chlorines on the sucralose molecule cause a great deal of steric hindrance and repel negatively charged molecules such as the aspartate and glutamate residues at the active site of invertase (Figure 14). This prevents hydrogen bonding between invertase and sucralose and therefore no hydrolysis. I do not believe an organism could de-chlorinate sucralose. Since our research suggests that sucralose inhibits the function of transport and metabolism proteins involved in sucrose transport and hydrolysis, it is logical to conclude those inhibitory factors are the mechanism of bacteriostatic effects exhibited by sucralose on the environmental bacteria.

The main conclusion that I have come to is that sucralose is an environmental contaminant. It will only accumulate in aquatic environments over time because it is not likely to break down (that would require bacterial metabolism). Previous studies suggest that bacterial consortiums can partially metabolize sucralose into a di-chloro-aldehyde form; however, these studies indicate that the carbon from the sucralose is not incorporated into the

bacterial consortium's biomass (Labare and Alexander, 1993). This means the consortium did not digest the sucralose, these studies also point out that the members of their bacterial consortium could not individually metabolize sucralose as a carbon source (Labare and Alexander, 1995).Contamination of our water ways with sucralose could be similar to dumping antibiotics into our waterways and exposing the bacteria to selective pressures. The current environmental levels of sucralose (around an average of 754.4 nM depending on location) may not have any effect on bacterial growth. Sucralose is, however, increasing in its concentration due to its inability to be degraded by pH and temperature changes (Torres et al., 2011). It is presently in wastewater effluents at levels of several μg/l (ppb), the Swedish Environmental Protection Agency warns that its break down is slow and ecological impact is largely unknown, they emphasize certain concentration levels may lead to damaging arthropod and cyanobacteria communities (Brorstrom-Lunden et al., 2007). Sucralose would at higher concentrations; potentially 55.78mM, hurt the bacterial community. This type of contamination is troubling because the bacterial community is the basis for the health of our entire ecosystem. They provide food for other organisms, and are responsible for decomposition of dead materials in an environment; they also cycle a number of important chemicals.

Sucralose inhibition is bacteriostatic and concentration-based. The present sucralose environmental concentrations are too low to negatively affect bacteria presently living in freshwater or soil systems. The concentration of sucralose in these environments is increasing over time (Torres et al., 2011). Micro-environments could experience inhibition due to sucralose build up. These environments may have limited water volumes. A good example of a micro-environment would be the mammalian gut. Previous studies have shown that normal

sucralose intake over a 12 week period reduces probiotic bacteria in the gut and feces of lab mice (Abou-Donia, 2008). These changes occurred at sucralose concentrations of 1.1-11 mg/kg (the US FDA acceptable daily intake for sucralose is 5 mg/kg). At the end of the treatment period, the numbers of *bifidobacteria*, *lactobacilli*, and *Bacteroides* were significantly decreased; however, there was no significant treatment effect on enterobacteria. Some *Enterobacter* strains have been positively correlated with obesity and gastrointestinal stress (Fei and Zhao 2012). In previous studies lab mice given sucralose were found to be experiencing increased levels of gastrointestinal distress (Kille et al., 2000). The inhibition of positive gut flora may allow negative gut flora to proliferate.

I believe that I have found the mechanism of this phenomenon; the reason why environmental, oral, gut, and fecal bacteria are inhibited by sucralose is because sucralose is structurally similar to sucrose and therefore can be accepted by the transport proteins and enzymes of these bacteria. This is energy intensive because these proteins cannot catalyze or transport sucralose based on the steric hindrances of its chlorine groups; which slows the amount of hydrolysis and transport of normal sucrose.

Higher concentrations of sucralose will damage the bacterial community, which is responsible for nutrient cycling and decomposition of dead materials. It will take a long time for sucralose to accumulate to harmful concentrations in the environment (except for in microenvironments). I recommend that steps be taken to reduce the amount of sucralose being dumped into the environment by our waste water facilities. If we could adapt a consortium to withstand and fully digest sucralose then we could add that consortium to our

waste water bioreactors, this would stem further sucralose contamination of our aquatic ecosystems.

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Appendix

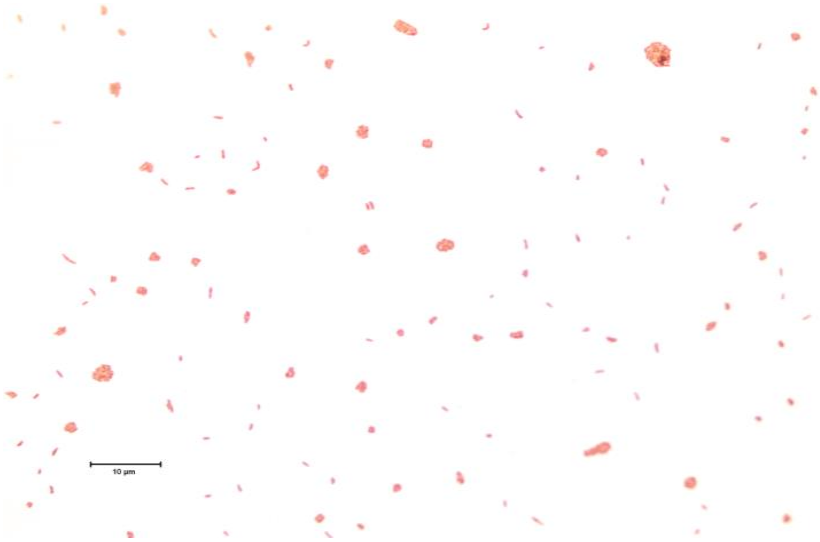

Figure 16. Micrograph of a Gram-stained image of *Stenotrophomonas* sp. I_61 at 100x.

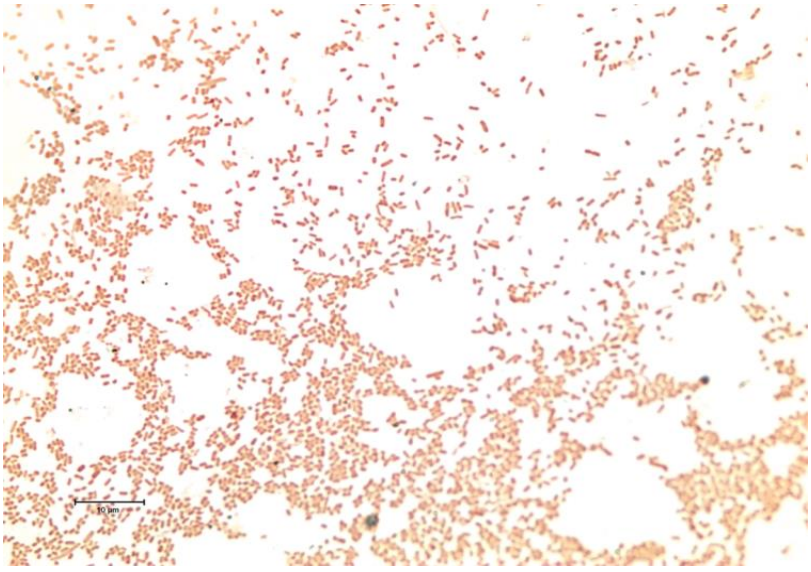

Figure 17. Micrograph of a Gram-stained image of *Rhizobium borbori* at 100x.

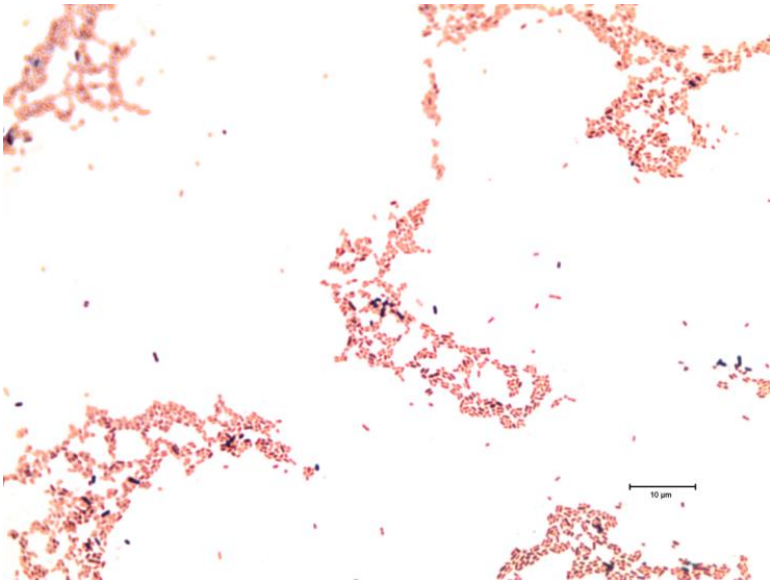

Figure 18. Micrograph of a Gram-stainied image of *Citrobacter murlinlae* at 100x.

Figure 19. Micrograph of a Gram stained image of *Ensifer arboris* at 100x.

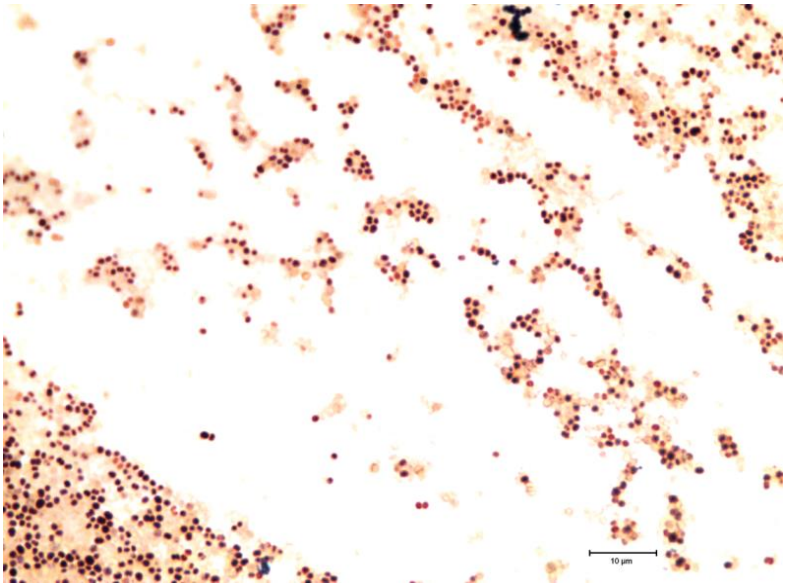

Figure 20. Micrograph of a Gram-stained image of *Microbacterium* sp. U 13 at 100x.

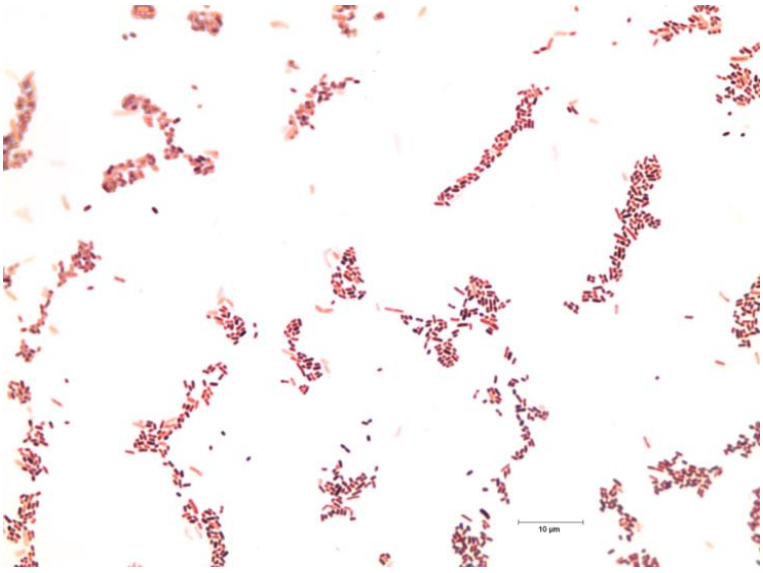

Figure 21. Micrograph of a Gram-stained image of *Streptomyces badius* at 100x.

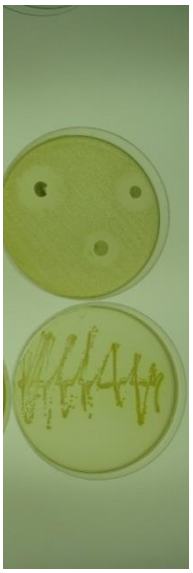

Figure 22. Image of the disk diffusion assay media (top) and the re-cultured media from the zones of inhibition (bottom).

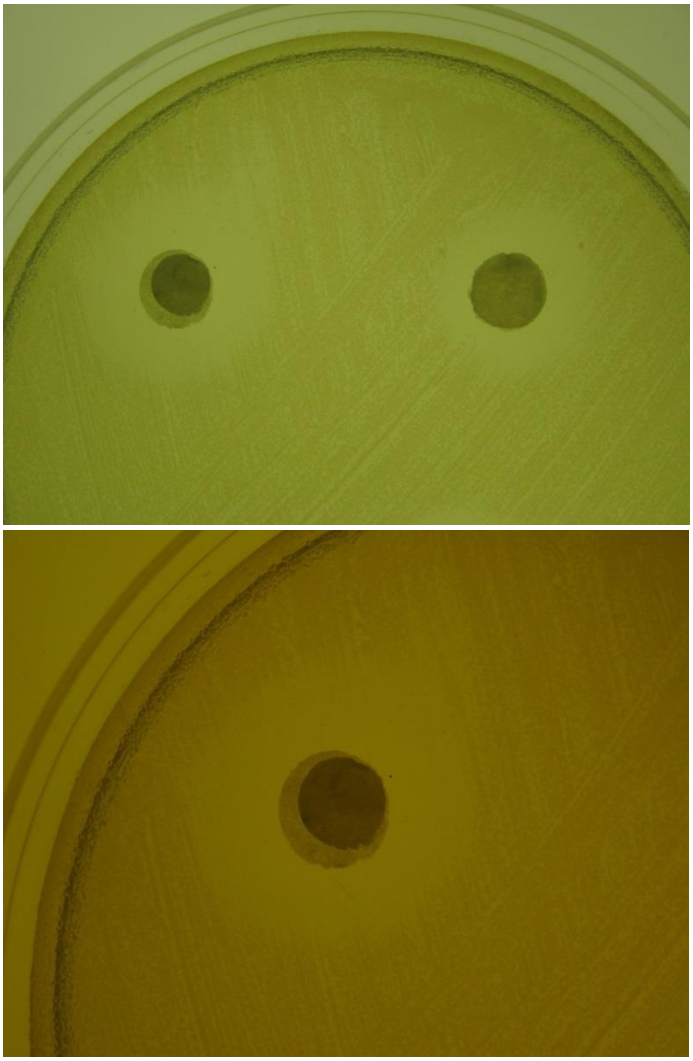

Figure 23. Hi-magnification images of the zones of inhibition from the disk diffusion assays.

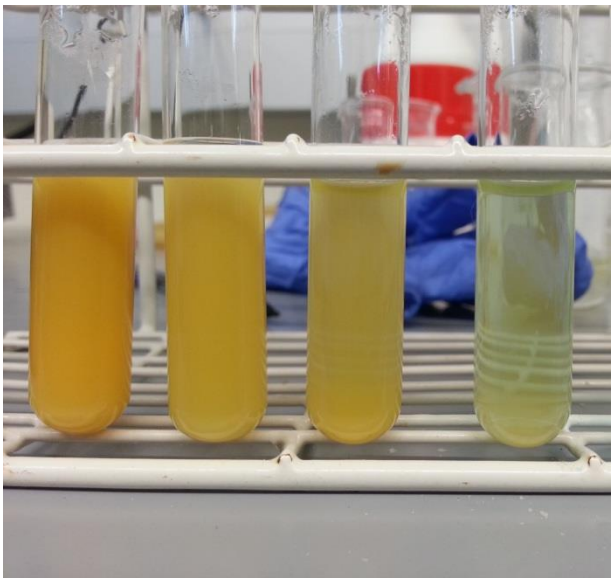

Figure 24. Picture of the sucrose only control group from the enzyme kinetics experiments, in order of decreasing concentration of sucrose from left to right.

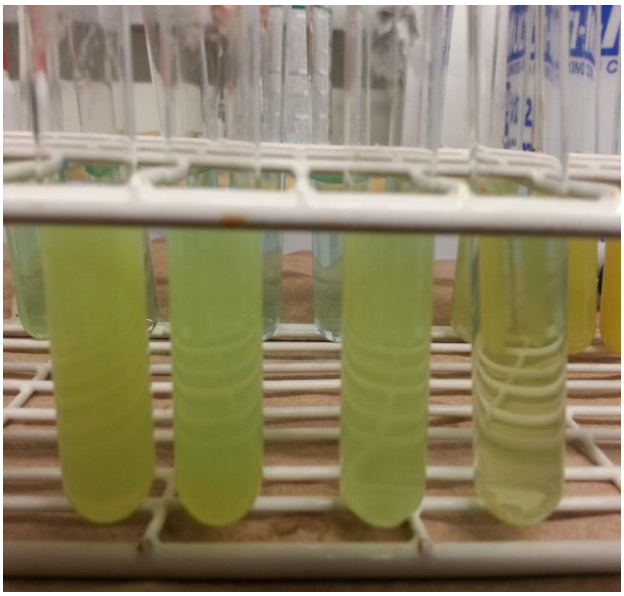

Figure 25. Picture of the sucrose and sucralose experimental group from the enzyme kinetics experiments, in order of decreasing concentration of sucrose from left to right, sucralose concentration does not change from one test tube to another.

Vita

Arthur Omran earned his bachelors of science degree in biology (concentration in microbiology) with a minor in religious studies in the fall term of 2010. He has earned his masters of science degree in biology (track in molecular biology) in the spring term 2013.

He has industrial experience, having worked as a quality control chemist for Health Link inc. (subsidiary of Clorox) from 2008-2010. He has also had experience working in the academic fields as a Graduate Teaching Assistant for the University of North Florida Department of Biology, teaching General Biology 1 Lab, General Biology 2 Lab, and Principles of Biology Lab from 2010- 2013. He has also worked as a chemistry tutor from 2006- 2012 for the University of North Florida Academic Center for Excellence.

He is a member of the American Chemical Society, Phycological Society of America, and Sigma Xi. In the fall term of 2012 he won an award at a microbiology research conference titled: 2012 South Eastern Branch of the American Society of Microbiology Presidents Award: First Place for the Best Oral Presentation Given by a Master's Student. He is currently in entering his works into press with one paper accepted with revisions, and two currently in review. His research focuses on how various chemicals in the aquatic environment affect the microbial communities, having worked with cyanobacteria toxins, artificial sweeteners, and various natural products. He looks forward to continuing his education at the Florida International University to pursue a doctoral degree in the field of biochemistry.