12-2016

The Effect of Varying Environmental Conditions on the Growth of Stenotrophomonas Maltophilia

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THE EFFECT OF VARYING ENVIRONMENTAL CONDITIONS ON THE GROWTH OF *STENOTROPHOMONAS MALTOPHILIA*

by

NORAH ALQAHTANI, BS

A THESIS

Presented to the Faculty of University of the Incarnate Word
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

UNIVERSITY OF THE INCARNATE WORD

December 2016

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ACKNOWLEDGMENTS

First, praise be to Allah to complete and achieve my goal.

I would like to thank my advisor, Dr. Vallor, for always listening to me, supporting, and encouraging me; I really appreciate you. I would also to thank the rest of my committee, Dr. Raymond and Dr. Pierce, for their patience and continual support. The successful completion of my degree would not have been possible without them. I had the additional benefit of serving as teaching assistants for all of them, so it was a pleasure working for them as well. I will not forget to thank Dr. Scott Smith for assisting me in the statistics part and helping me to finish everything in a short period.

I also need to thank all the members of the Department of Biology who helped me in many ways, the UIW International Office, and Office of Research and Graduate Studies.

Finally, I would like to thank my family, especially my parents Mohammed and Mounirah. I would not be where I am today if it were not for them. Thank you, Mom and Dad, for all of your love and support. From an early age, they helped me realize that I can accomplish my goals and dreams. I also would like to recognize my brothers and sisters; I hope that I continue to serve as a role model to you all. Now, I want to thank my brother (Motlaq); he always supported me. He was with me when I started graduate study, but now he was passed away before I completed my courses, I love you so much you are piece of my heart I remember you every times. My family I love you so much, Allah bless you.

THE EFFECT OF VARYING ENVIRONMENTAL CONDITIONS ON THE GROWTH OF *STENOTROPHOMONAS MALTOPHILIA*

NORAH ALQAHTANI

University of the Incarnate Word

Stenotrophomonas maltophilia has emerged as an important opportunistic pathogen in the debilitated host. It possesses the ability to target and colonize respiratory-tract epithelial cells and grow on the surface of medical devices. It is associated with a significant case-fatality ratio in cystic fibrosis patients. *S. maltophilia* species have the ability to grow in bacterial biofilms, which further complicates these infections by providing the opportunity for acquisition of antibiotic resistant genes. This study investigated the effects on the growth of different strains of the same species of *S. maltophilia*, ATCC 51331 and 13637, when the selected environmental factors are varied. Factors studied were temperature and salt concentration in the medium, sodium chloride (NaCl) and ferric chloride (FeCl3). Further, minimal inhibitory concentrations (MICs), against a select panel of antibiotics were assessed for both strains when cultured in the different growth conditions proposed. The expectation of this study is that Ferric Chloride concentrations provided the most significant effects on *S. maltophilia* biofilm growth. Initially Luria Bertani (LB) media (with proper modification to salt content) were used to culture the organisms to visually assess colony growth under these conditions at the following temperatures: 40C, 200C, 370C, 550C and also to conduct MICs. The effects of various experimental conditions were also determined on *S. maltophilia* biofilms formed in the 96 well plate format. Viability of the biofilms have assessed using a previously published metabolic assay.

Our data show that different environmental conditions affect *S. maltophilia* growth. For instance, when grown in different temperatures the morphology of *S. maltophilia* X51331 and X13637 varied during 3 days growth period and the *S. maltophilia* could not grow in the low and high temperatures of 40C and 550C. Furthermore, our data shows the effect of iron and sodium chloride with different concentrations of salt on the growth and metabolic activity of the biofilms of *S. maltophilia* X51331 and X13637 incubated at 300 C and 370 C, as well as *S. maltophilia* growth at varying FeCl3 and NaCl concentrations for 24, 48, and 72 hours. There was a significant increase in the average size of *S. maltophila* X51331 and X13637 colonies when grown in low concentrations of salt 0 μM and 1 μM, and growth was inhibited in the presence of concentrations ≥ 2% NaCl and FeCl3.

Introduction

The opportunistic pathogen *Stenotrophomonas maltophilia* (*S. maltophilia*) was first identified and isolated by Hugh and Ryschenkow in 1961. It is a gram-negative, aerobic, motile, non-fermentative bacterium that has been associated with nosocomial infections in immunocompromised patients (Chang et al., 2015). It belongs to the class Gammaproteobacteria and was initially named as *Bacterium bookeri*. Subsequently it was placed under the genus *Pseudomonas* and named as *Pseudomonas maltophilia*. It was then renamed as *Xanthomonas maltophilia*. Finally, in 1993, *S. maltophilia* was awarded its own genus and became the type species of the *Stenotrophomonas* genus (Palleroni & Bradbury, 1993). *S. maltophilia* is ubiquitous in its natural distribution. It is found in soil, near plant roots as well as aqueous environments (Berg et al., 1999). In the soil it contributes to nitrogen and sulfur cycles (Banerjee & Yesmin, 2002). These organisms are commonly found in water and have been isolated from various animals and fish (Banerjee & Yesmin, 2002).

In the human population, *S. maltophilia* is the second most common organism isolated from clinical specimens. It is an opportunistic pathogen which appears to be the causative agent of secondary infections when the immune system has been compromised due to a previous or current bacterial or viral infections. Von Graevenitz and Bucher reported isolates of the bacteria in the feces of 10.9% of patients reporting diarrheal illnesses (Denton, 1998). Further, the percentage of recovery from human fecal matter increased significantly in cancer patients undergoing chemotherapy and other immunocompromised individuals. Seldom is it able to cause the death of an individual on its own (Denton, 1998).

There are increasing concerns regarding the presence of *S. maltophilia* in the community due to its rapid spread in the community and the targeting of the immunocompromised °

individuals. This increases the challenge of controlling the infection and decreases the success of effective treatment. *S. maltophilia* has the ability to target and cause infection in a variety of different organs of the body, however, its main target appears to be the respiratory tract for a variety of reasons. *S. maltophilia* is a life form of low destructiveness and much of the time colonizes liquids utilized as a part of the healing facility setting (e.g., water system arrangements, intravenous liquids) and patient discharges (e.g., respiratory emissions, urine, wound exudates). *S. maltophilia* typically should sidestep ordinary host protections to bring about human disease. For instance, if a water system arrangement gets to be colonized with this life form, inundating an open injury can bring about colonization or disease of the injury. *S. maltophilia* is generally unequipped for bringing about illness in sound hosts without the help of intrusive medicinal gadget°s that sidestep ordinary host defenses (Araoka., Baba., M, 2010). It can also infect multiple organs but mostly is found in respiratory tract because of the selective habitat properties. There are different mechanisms of colonization and infection found in *S. maltophilia* and they cohabit with the strain of *P. aeruginosa* so respiratory tract is the best area for them to colonize. There is unique cell to cell communication between the two strains hence this mechanism is useful to study and also represents a target for the development of new pharmacological therapies (Székely & Langenheder, 2014). These bacteria are more likely to be obtained from the hospital settings as these are virulent strains. No complete pathogenic mechanism is known but there is evidence to show the likeliness to attach within the respiratory tract than in any other organ.

As stated before *S. maltophilia* commonly infects individuals who have an underlying immune compromising illness, however long term use of antibiotics and/or long stays in the

hospital (intensive care unit in particular) also are contributing factors. Owing to the fact that the bacteria are able to colonize and form biofilms on various catheter surfaces and other tubing (i.e. central lines, ventilator tubes, etc.) used in the hospital settings, patients are also more prone to nosocomial infections caused by antibiotic resistant *S. maltophilia*. One last group of affected individuals is cystic fibrosis patients. Due to the stasis of the thick secretions of these patients, this organism can easily grow and flourish in these secretions (Waters et al., 2011)

According to Brooke (2012), studies revealed that different models showed the pathogenesis of *S. maltophilia* to be caused by the formation of biofilms on nearly every surface. Once introduced into the upper respiratory tract, *S. maltophilia* colonizes and forms biofilm on lung tissue, involving the bronchioles and extracellular matrix. It can also form distinctively different biofilms on biotic and abiotic surfaces as microscopic analysis has demonstrated (Brooke, 2012). It causes bacteremia, most frequently in human hosts. It has been shown to have a contributing role in nosocomial endocarditis and respiratory tract infections. Following neurosurgical procedures, the chances of developing meningitis or subdural abscess caused by this organism is slim but present. There are also increasing reports of its involvement in cases of conjunctivitis, keratitis and corneal ulcers in the patients who wear contact lenses (Brooke, 2012). Finally, *S. maltophilia* has been increasingly found in wounds and skin infections and urinary tract infections but less commonly found in cases of gastrointestinal infection and infection of the bones and joints (Brooke, 2012).

Treatment and antimicrobial resistance

The treatment of this infection has so far been extremely difficult as it shows panresistance to current antibiotics. The only proposed therapy against *S. maltophilia* infection has been the use of bacteriostatic compounds. Currently the compound having the greatest

efficacy against the bacteria is co-Trimethoprim/sulfamethoxazole or TMP-SMX. However, there have been recent reports of the development of resistance to this compound by *S. maltophilia* as well. With the development of resistance against multiple broad spectrum antibiotics, latest strategies have been developed which include the use of high doses levofloxacin. Researchers also report the usage of certain essential plant oils as being effective (Brooke, 2012). Finally, there have also been reports of the ability of *S. maltophilia* to perform horizontal gene transfer of antibiotic resistance to other potential pathogens as well (Brooke, 2012). The *S. maltophilia* genome has been sequenced, and this has supplied insight on certain genes that have been identified to help this bacterium gain antimicrobial resistance (Crossman et al., 2008). These identified genes encode for β-lactamases, as well as other enzymes that support drug resistance (Crossman et al., 2008; Looney et al., 2009).

Morphology and biochemical properties

S. maltophilia are small (0.7 to 1.8μm in length and 0.4 to 0.7μm in breadth) rod shaped bacterium. Their motility is attributed to the presence of a single polar flagella. They are positive for extracellular DNase and catalase, but are negative for oxidase. The oxidase negative property is used as a distinguishing feature to differentiate *S. maltophilia* from other members of the *Stenotrophomonas* genus (Denton, 1998).

Biofilm formation

S. maltophilia is known to form biofilms on plant and human surfaces as well as artificial implants in humans such as catheter tubing. Effective production of biofilm is enabled by the fact that the cells found inside the biofilms have the ability to pass information to each other. According to Horswill et al. (2006), this ability of cells in the biofilms is one of the major factors that enable the production of biofilm. Passing of information within the cells in the

biofilms is referred to as quorum sensing. Quorum sensing is a kind of chemical signaling where the bacteria receive and process cues in their environment so as to keep the cellular density balanced (Cegelski et al., 2008; Horswill et al., 2006).

For one cell to send chemical signals to another, tiny molecules known as auto- inducers must be produced and recognized by the cells in the biofilms. Through a signal transduction path, the auto-inducers are able to regulate all the other processes and also coordinate gene expression in the cell (Cegelski et al., 2008). Individuals suffering from malignancies and those with foreign device implants such as catheters or respiratory aids are also susceptible to *S. maltophilia* infection. As this mode of infection involves biofilm formation, the infections are hard to treat. The high level of multiple drug resistance of these *S. maltophilia* infections can be attributed to the ability to form biofilms. The process of biofilm formation follows the typical pathway where initial adherence to a solid surface is the first step. This adherence can occur to either the mucosal surfaces or to prosthesis (de Abreu et al., 2001). In environmental biofilms this surface is often the surface of plant roots.

S. maltophilia flagella pili are important for adherence of cells to a colonizing surface. The flagella aid the bacteria movement towards a suitable surface. The *S. maltophilia* fimbriae 1 (SMF-1) contain a 17 kDa fimbrin subunit which with large similarity to the N-terminal amino corrosive groupings of a scarce fimbrial adhesions (G, F17, K99 and 20K) present in *Escherichia coli* pathogenic strains and the CupA fimbriae of *Pseudomonas aeruginosa*. The greater part of the clinical *S. maltophilia* confines tried delivered the 17 kDa fimbrin (Oliveira-Garcia, De, et al 2003). SMF1 fimbriae from *S. maltophilia* then help the flagella to adhere to the surface. This bacterium also produces type I pili which helps in adhesion and initial formation of the biofilm matrix. It may also express type IV pili which also aids in adhesion

and aggregate formation in biofilms. To establish a strong biofilm, a thick matrix is needed that protects the bacteria in the biofilms from environmental stresses as well as antimicrobial substances. *S. maltophilia* produces enzymes that may synthesize exopolysaccharides that form this matrix. Once the exopolysaccharide is formed, the biofilm gets established and periodically planktonic bacteria get released from it so that they can move to another suitable location to form new biofilms (Garcia, Carlos, A., et al 2015).

Factors affecting biofilm development

S. maltophilia is commonly found growing as biofilms and signaling factors, such as Ax21 which is regulated by iron, play a significant role in biofilm formation in this bacteria. A deletion of this gene has been associated with reduced biofilm formation, reduced motility and higher susceptibility to aminoglycoside antibiotics (McCarthy et al., 2011). According to Horswill et al. (2006), physical, biological and chemical variation impacts the production, distribution and the stability of the cellular density, thus affecting quorum sensing. The development of bacterial biofilm is also dictated by the varying types of nutrition and temperature around the biofilm cells. For example, the ambiguity of the *S. maltophilia* bacteria helps it to exist and survive in varying temperatures and environments (Rahmati-Bahram et al-1997).

For production, functionality and homeostasis to take place, many cells require iron as it is very essential for the production of various cellular proteins (Reid et al., 2009). Bacterial cells use iron for aerobic respiration because most enzymes function effectively in the presence of iron. Iron is therefore very essential for cell growth and for the development of biofilms. The amount of free iron that a bacterium can access in the human host is very small, because there are other processes, such as the formation of hemoglobin or lactoferrin that use iron thus binding

and reducing the amount of available iron (Berlutti et al., 2005). Sodium chloride is another supplement that has been studied to determine its impact on bacterial contamination and biofilm improvement (Havasi et al., 2008).

Virulence factors

The main mode of virulence of *S. maltophilia* is the formation of biofilms. It often involves the forming of a complex biofilm with other pathogenic bacteria like Pseudomonas aeruginosa. In such cases, *S. maltophilia* biofilms provides P. aeruginosa protection from antimicrobials while it acquires antibiotic resistance associated genes from P. aeruginosa via horizontal gene transfer. *S. maltophilia* has acquired resistant to many broad-spectrum antibiotics such us carbapenems, aminoglycosides, macrolides, tetracycline and chloramphenicol. Two metallo-betalactamase enzymes help the bacteria to degrade the antibiotics. These metallo-beta-lactamases are also believed to have been acquired by horizontal gene transfer. Due to a high level of antibiotic resistance, *S. maltophilia* infection is usually treated by removal of the infected prosthesis. In a majority of cases this is sufficient to control the infection. If antibiotics are essential, then co-trimoxazole and ticarcillin are the drugs of choice. Tigecycline and Polymyxin B are also used limitedly due to potential adverse effects. ß-lactamase is presently considered as the leading reason for resistance among the gram negative bacteria, of ß -lactam in antibiotics, also limited because of resistance. ß -lactam is present in antibiotics like penicillin and carbapenems, and hence the ß -lactamase is a major cause of resistance against these antibiotics. These ß -lactamase enzymes are actually produced by some bacteria and when introduced to ß -lactam through antibiotics, they form resistance against it. The antibiotic is formed by a four atom ring. However, the ß -lactamase opens and breaks the ring and eventually the antibiotic

properties of such ß -lactam are destroyed due to the ß -lactamase function on it. (Chen, Tenover, & Kohler (2004).

Several criteria have been proposed for determination of the ability of *S. maltophilia* to grow and to become more pathogenic. This project investigated the effects of varying environmental conditions, such as different temperatures and different concentrations of salt, on the growth of *S. maltophilia* on solid growth media as well as when grown in RPMI growth conditions that had induced biofilm formation of *Stenotrophomonas maltophilia* 13637 and 51331. The hypothesis is that the metabolic activity of two strains of *Stenotrophomonas maltophilia* will not be differentially affected by temperature but will be inhibited in high concentrations of sodium chloride and ferric chloride. In order to address this the following specific aims are proposed:

1. Determine the effect of the pattern of growth at different temperatures of the two isolates.
2. Determine the effect of the absence or presence of varying concentrations of NaCl and FeCl3 on S.maltophilia isolate growth and biofilm metabolic activity.
3. Observe if under less than optimal growth conditions will antibiotic resistance decrease or increase.

Materials and Methods

Growth and maintenance of S. maltophilia cultures

S. maltophilia isolates used in this study were *S. maltophilia* ATCC 51331 and *S. maltophilia* 13637 (American Type Culture Collection, Manassas, VA). Both isolates were grown and maintained for this study on brain heart infusion (BHI) or Trypticase Soy Blood (TSB) agar at their optimal temperatures: *S. maltophilia* ATCC 51331 (non-type environmental isolate) at 37°C, *S. maltophilia* 13637 (type environmental isolate) at 30°C (Palleroni NJ,

Bradbury JF.1993). *S. maltophilia* species were checked for purity by hemolysis reactions on TSB plates (*S. maltophilia* is gamma hemolytic) and Gram Stain (Gram negative rods) (API 20E QC Set. bioMerieux). For experimental growth studies, Luria Bertani (LB) base media was used with appropriate modification. Ringer's Phosphate Media or RPMI for Biofilm studies was also used.

Growth of *S. maltophilia* isolates on LBA plates delineated the morphology of *S. maltophilia* which were bronzed, wet colonies and appear to be outwards (Figure. 1A). Trypticase Soy Blood Agar at 37°C additionally revealed that *S. maltophilia* isolates also display gamma-hemolysis or partial hemolysis of red blood cells (Figure 1B).

A **B**

Figure 1. Demonstrates colony growth characteristics of *S. maltophilia* on (A.) Luria Bertani agar (LBA) and (B.) Trypticase Soy Blood Agar (TSBA).

Preparation of varying salt concentration in Luria Bertani media

To assess the effects of varying the concentration of the following salts: sodium chloride (NaCl) and ferric chloride ($FeCl_3$) on *S. maltophilia* growth, LB media was modified in the following manner. Luria bertani agar media (LBA) was prepared by mixing 10 grams (g) tryptone, 5 g yeast extract, 15 g of agar and a variable amount of NaCl in grams. Distilled water was added to 1 liter. The amount of salt added to each liter of base media to obtain the desired concentrations in this study were 0 µg/µl, 10.0 µg/µl, 50 µg/µl, 100 µg/µl and 150 µg/µl of NaCl. The standard LBA recipe contains 100 mg\ml NaCl, therefore those plates were considered as control media for normal growth of the organism.

A stock concentration of 100 mM FeCl3 was prepared through filtered sterilization. LB plates containing the following FeCl3 concentrations of 0.25 μM, 2.5μM, 5 μM were made. Due to an unforeseen chemical reaction which affected the solidification of the LBA, FeCl3 concentrations above 5 μM could not be tested when bacterial cultures were grown on solid media.

Assessment of the effects of salt concentrations and temperature on S. maltophilia growth on solid media

LBA plates with varying salt concentration, were inoculated with 100 μl of an overnight culture of *S. maltophilia* 100 μM from the bacteria (approximately 1.5 x 108 CFU) in the center of each plate. Plates were then placed in one of four different temperature incubators: 4° C, 20° C, 37° C, and 55° C. The diameter of growth of the initial colony was measured at 24 hour intervals for 3 days.

Assessment of the effects of salts and temperature on metabolic activity of S. maltophilia biofilms

Assays were conducted according to previously published methods (Pierce, C. G, Uppuluri, P. et. al. 2010) utilizing the reduction of the tetrazolium salt, XXT, as a reporter of metabolic activity with modifications. Overnight cultures of *S. maltophilia* ATCC 51331 and S. maltophilia 13637 were grown in 25ml of BHI broth with constant agitation (180 rpm) at 30° or 37° C depending on the isolate's reported optimal temperature. Cultures were centrifuged at 1000 x g for 10 minutes, cells were washed twice with sterile phosphate buffered saline. Suspensions of each isolate of *S. maltophilia* at a final density of 1.0 × 108 cells /mL in 25ml RPMI 1640 medium buffered with 165 mM morpholinepropanesulfonic acid to pH 7.0. Utilizing 96 well polycarbonate plates, 200 μl of appropriate *S. maltophilia* cells suspension were used to seed wells. Experimental concentrations of NaCl and FeCl3 were added

to wells to assess the effects of the presence of no salt or increasing salt concentration on biofilm metabolic activity. Replicate, seeded microtiter plates were incubated for 24 and 48 hrs at 30°C and 37°C.

At the end of the incubation period, medium was aspirated and plate was manually washed with PBS. XTT tetrazolium salt + menadione solution was added to each well as well as negative control wells. Plates were covered in aluminum foil and incubated in the dark for 2 h at 37°C. Plates were uncovered and 80 µl of the colored/uncolored supernatant from each well was transferred onto a new microtiter plate. Absorbance readings were obtained on the Tecan® plate reader at 490 nm.

Assessment of the effects of salt concentration and temperature on S. maltophilia minimal inhibitory concentrations (MIC) against a panel of selected antibiotics

Minimum inhibitory concentration studies utilizing standard concentration of selected antibiotics were conducted according to the Kirby Bauer technique with modification (Vallor., Enriquez., Pierce. 2016). LB plates were made to contain varying amounts of FeCl3 and NaCl to test the possible effects on *S. maltophilia* ATCC 51331 and *S. maltophilia* 13637 susceptibility or resistance to a panel of antibiotics that included Ticarcillin, Ceftazidime, Cephazolin, Erythromycin, and Amoxicillin (Fisher Scientific, Pittsburgh, PA). These antibiotics were selected due to their regular use to treat common associated infections. Each isolate was grown in LB broth overnight. Purified cultures were diluted with sterile saline until tube turbidity matched 0.5 McFarland standard solution (Approximate cell count 1.5 x 108 CFU). Cultures were then plated onto LB plates made to contain experimental amounts of FeCl3, NaCl agar plates to be tested. Filter disks impregnated with standard concentrations of the antibiotics were placed on the plates. Plates were incubated at 30° C and 37° C for 18 hrs after which the diameter of clear zones of inhibition were measured to assess minimal inhibitory concentrations

and resistance or susceptibility of the organism as denoted by the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Statistical analysis

ANOVA was performed using the EZ package in R. In order to assess statistical significance, factorial ANOVA with a repeated measure was conducted, and interaction plates were created.

Results

Selection of experimental growth temperatures for *S. maltophilia* isolates

Initially *S. maltophilia* isolates were grown at 4 different temperatures to determine a temperature range in which to conduct the studies in this thesis. It was hypothesized that the experiments should be performed at environmental temperatures close to the optimal temperatures of growth as well as to temperatures present in the environments where S. maltophilia may cause infections in human hosts. As demonstrated previously in the literature, growth of both isolates of *S. maltophilia*, were adversely effected by extreme warm (55°C) and cold (4°C) temperatures (Fig. 2). The ideal temperatures in which the isolates grew were 20° C and 37°C, with growth appearing slightly better in the latter temperature. Therefore it was determined that for the rest of the study, we would elevate the temperature from 20°C to 30°C (which was the optimal temperature of *S. maltophilia* 13637, as described by the ATCC, (American Tissue Type collection, Manassas VA).

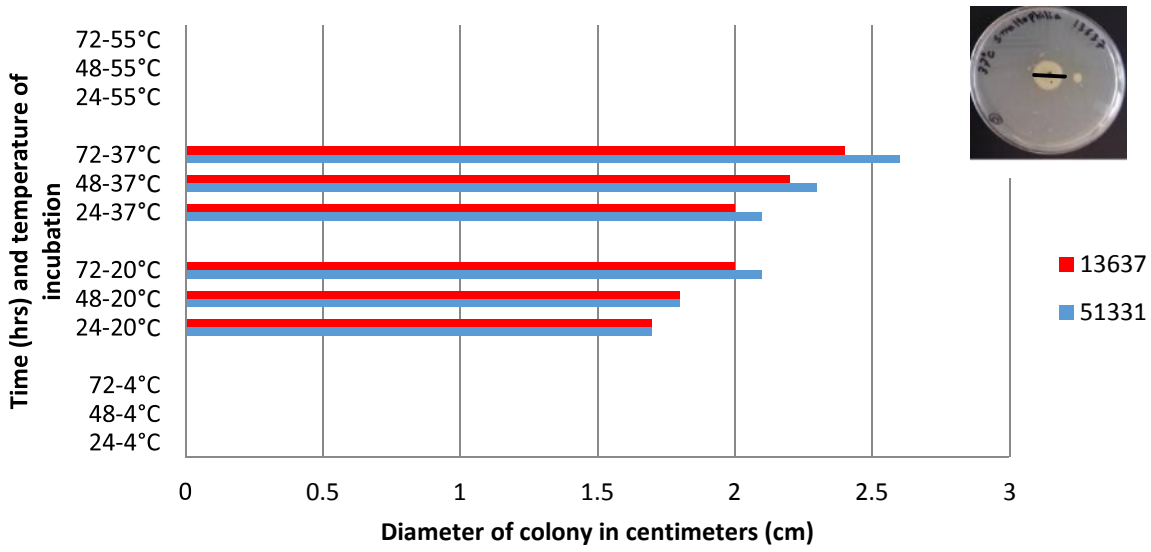


Figure 2. The effect of temperature on the growth of an aliquot of 10^8 CFU of each *S. maltophilia* isolate over 72 hours as assessed by the measurement of colony diameter. Insert illustrates appearance of colony at 48 hrs (n = 3 per condition) and diameter measured.

High concentrations of NaCl in the media adversely affect the growth of S. maltophilia isolates

Sodium chloride has been reported to influence bacterial infection and biofilm development (Havasi et al., 2008). *S. maltophilia* isolates were grown on LBA plates containing NaCl at final concentrations of 0 µg/µl, 10.0 µg/µl , 50 µg/µl, 100 µg/µl and 150 µg/µl at 37° C. As the media increased in salinity, there was a moderate decline of culture growth after 24 hours (Fig. 3, Table 1.) Isolates recovered as time passed (48,72 hours) with no significant difference in growth for 0, 10, 50 and 100 µg/µl NaCl. This is in contrast to isolates grown in the presence of a high amount of salt concentration (150 µg/µl) where no visible growth was detected of either strain of *S. maltophilia*.

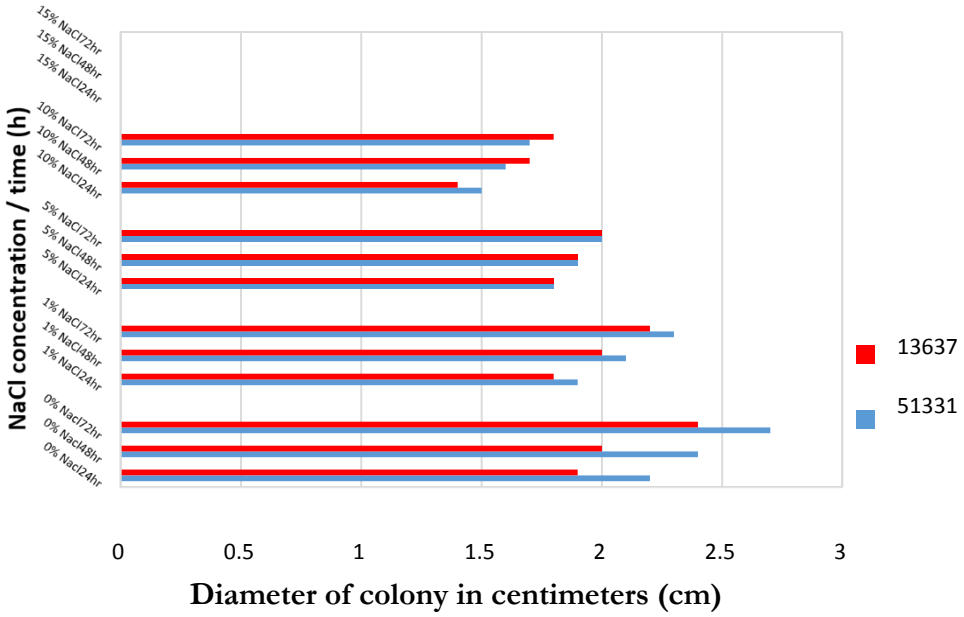


Figure 3. Effect of increasing concentrations of NaCl on *S. maltophilia* 51331 and 13637 grown on LBA plates at 37° C

Table 1. *Diameter of S. maltophilia isolate colonies in response to NaCl concentration*

S. maltophilia isolate Time (h)	NaCl concentration of LB agar Colony diameter in cm (n=3)				
	0	10 µg/µl	50 µg/µl	100 µg/µl	150 µg/µl
51331					
24	2.2	1.9	1.8	1.5	0
48	2.4	2.1	1.9	1.6	0
72	2.7	2.3	2.0	1.6	0
13637					
24	1.9	1.8	1.8	1.4	0
48	2.0	2.0	1.9	1.6	0
72	2.4	2.2	2.0	1.7	0

Varying the amount of iron in the LB media also demonstrated a moderate decrease on *S. maltophilia* colony growth at 37°C as compared to NaCl (Fig. 4 Table 2). The concentrations used of ferric chloride were significantly lower as compared to those used for NaCl due to the

effect of high concentrations of ferric chloride to interfere with the solidification of the agar plates. However as shown by the data, the effects of decreasing the salt concentration in the media aided in mildly improving the growth of *S. maltophilia* isolates on solid agar as shown by the increasingly larger diameter of the colony size when Ferric chloride and NaCl are at a minimal concentration or not present in the media at all (Figures 3 and 4, Tables 1 and 2).

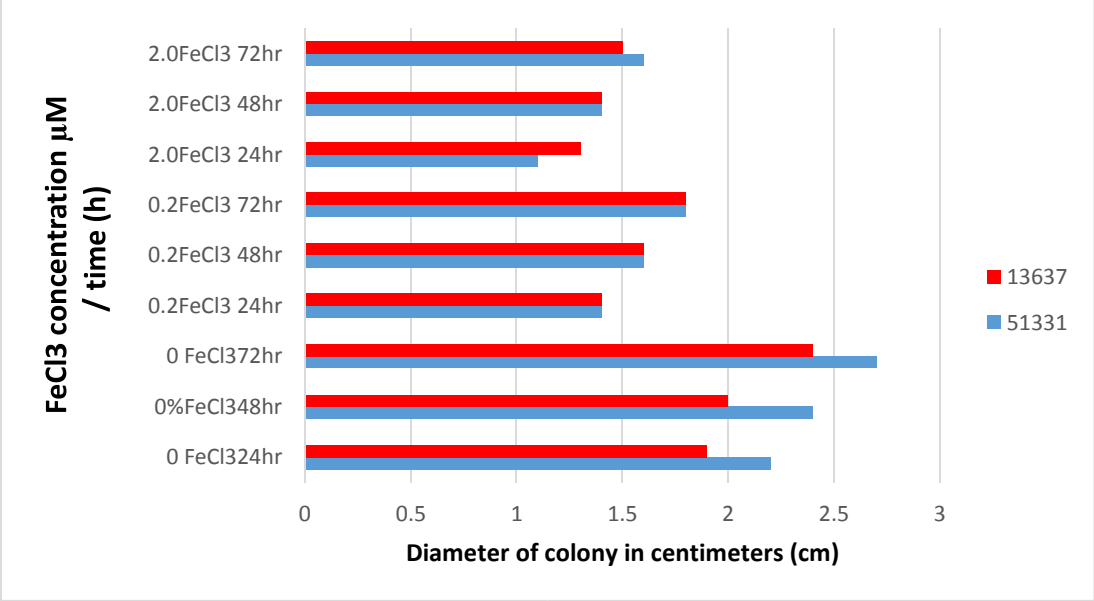


Figure 4. Effect of ferric chloride on *S. maltophilia* X51331 and 13637 growth at 37^0C.

Table 2. *Diameter of S. maltophilia isolate colonies in response to $FeCl_3$*

S. maltophilia isolate Time (h)	$FeCl_3$ concentration of LB agar Colony diameter in cm (n=3)		
	0	0.25 **µM**	2.5 **µM**
51331			
24	2.2	1.4	1.1
48	2.4	1.6	1.4
72	2.7	1.8	1.6
13637			
24	1.9	1.4	1.3
48	2.0	1.6	1.4
72	2.4	1.8	1.5

Assessment of the metabolic activity of S. maltophilia biofilms in response to varying salt concentrations and temperature

In order to obtain quantitative data concerning the growth of *S. maltophilia*, the metabolic activity of growing biofilms in the presence or absence of NaCl and $FeCl_3$. In addition, biofilms were also grown either at 30°C or 37°C.

Biofilms of both isolates grown in the presence of increasing amounts of NaCl for 24 hours displayed no significant differences in terms of metabolic activity at either temperature (Fig. 5 A) and (Fig. 5-C B). Increasing NaCl concentrations in the biofilm growth media did produce a decreased trend in metabolic activity of biofilms at 24 h. At 48 hours of growth, metabolic activity was overall at its highest in the absence and at the lowest concentration of NaCl tested at 37°C for both isolates. At 30°C, metabolic activity of biofilms grown at this temperature remained relatively unchanged after 48 hrs of growth for both isolates as compared to 24 hrs. Interestingly, the activity in conditions of high NaCl showed a decrease on activity. (Fig. 5 B) and (Fig. 5-C B).

When $FeCl_3$ was added to the biofilms, the metabolic activity of the both *S. maltophilia* 51331 and 13637 biofilms was decreased as compared to those grown in the presence of NaCl (Fig. 5A & 6A) at 24 hrs and 48 hrs. However, at 48 hrs, there was a marked decrease in metabolic activity in *S. maltophilia* 13637 in the presence of $FeCl_3$ at 48 hours as compared to 24 hours at 37°C (Fig. 6A, 6B) The same trend was also seen for *S. maltophilia* 51331. Metabolic activity of *S. maltophilia* 13637 biofilms grown at 30°C appeared not to decrease in number but actually maintained their levels of activity or increased at the lower $FeCl_3$ concentrations as compared to those maintained at 37°C at 48 hrs. (Fig. 6B). At 100mg/ml of $FeCl_3$ concentration at 37°C, a substantial decrease in the activity of *S. maltophilia 13637* biofilms was seen that was

not seen when grown at 30°C at 48 hrs. In fact, the activity at 48 hours at this concentration was recorded as less than that obtained at 24 hrs when grown under the same conditions, therefore we can conclude that *S. maltophilia 13637* appears to be more sensitive to growth conditions as compared to *S. maltophilia 51331*.

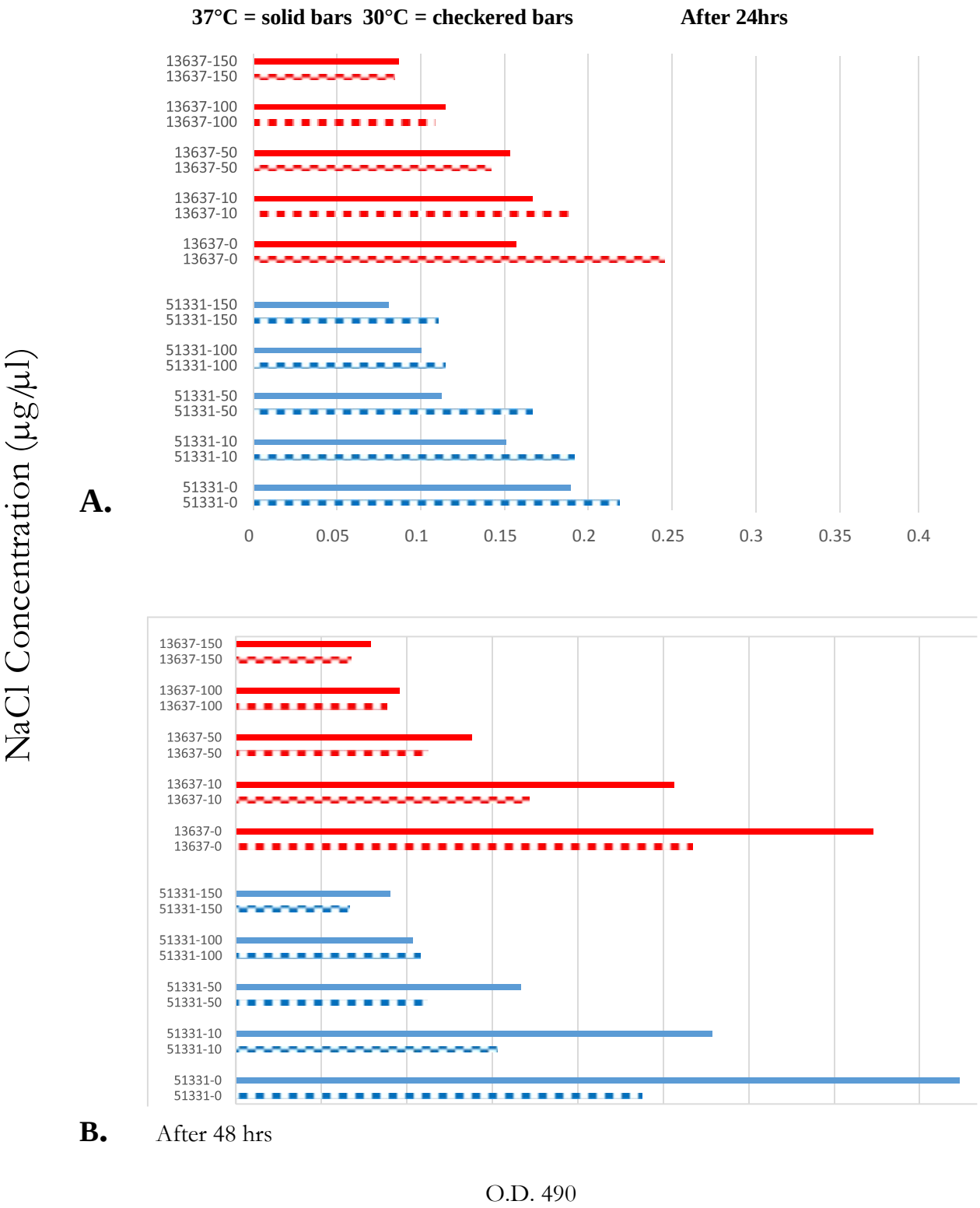


Figure 5. The effect of sodium chloride concentration and temperature on *S. maltophilia* biofilm Metabolic Activity A. 24 hrs and B. 48hrs. (n = 12 for each condition) .

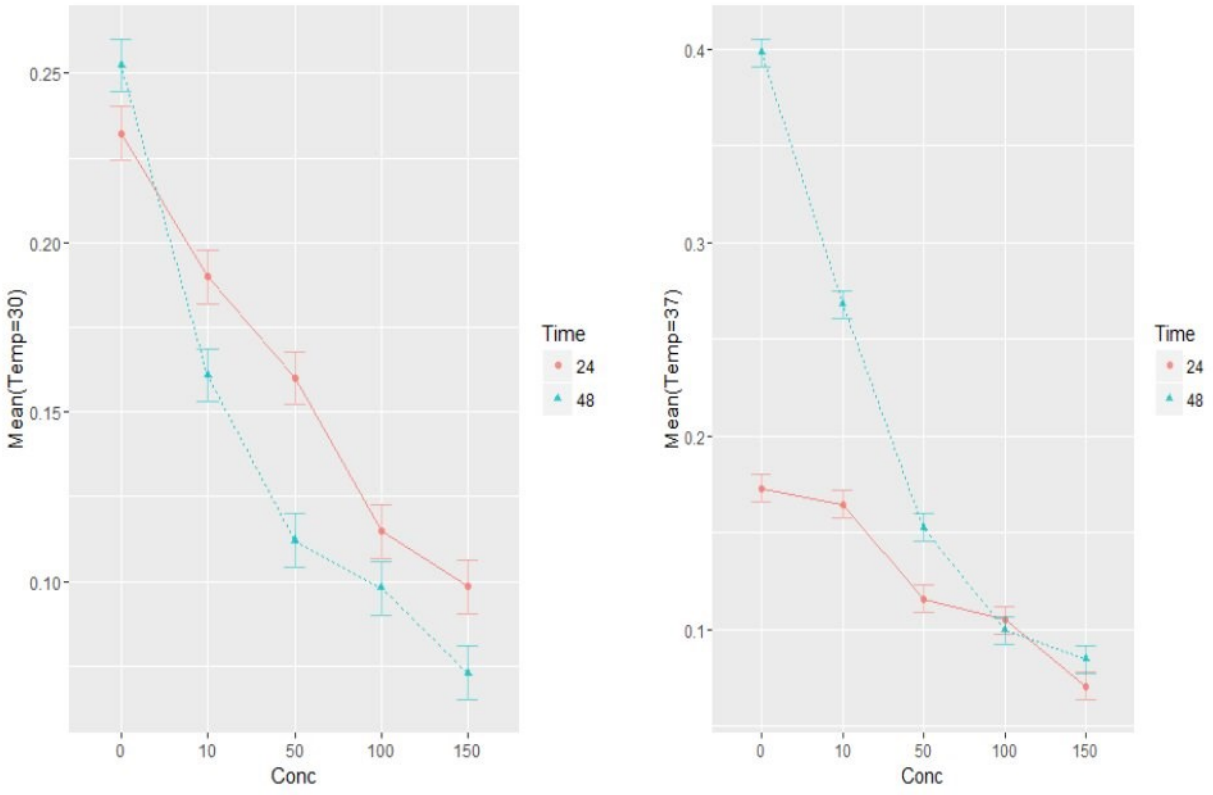


Figure 5-C. NaCl – ANOVA factorial model with measures on *S. maltophilia* biofilm Metabolic Activity with many factors, time, concentrations, temperatures and strains. P value = 1.276060e-01

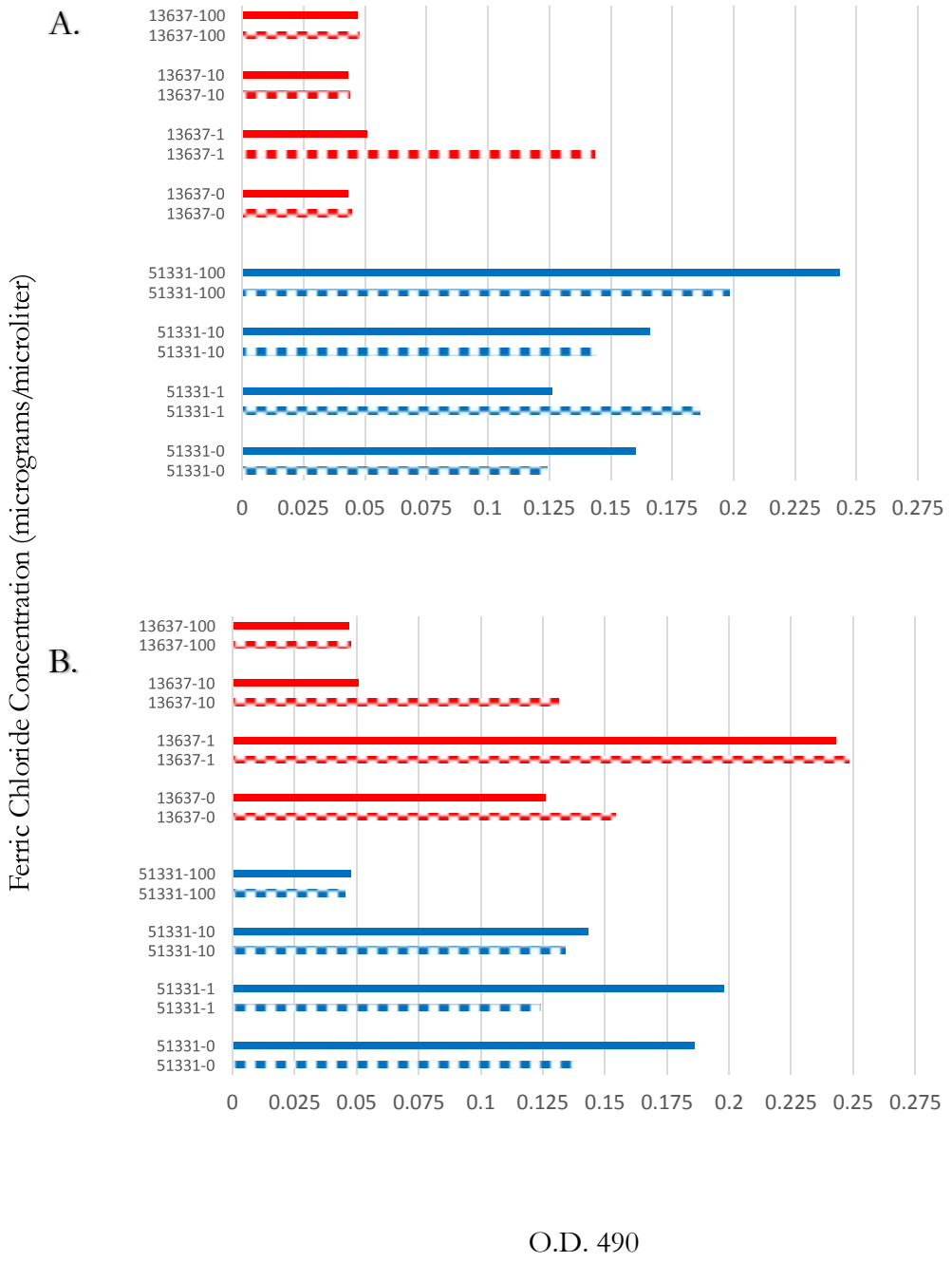
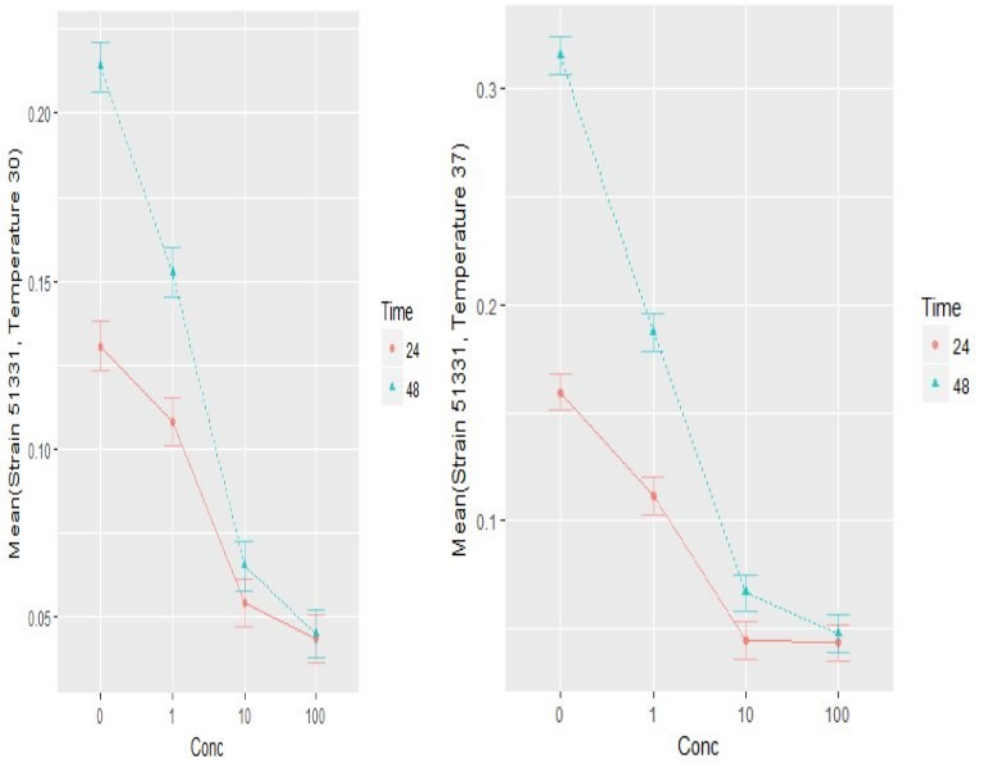
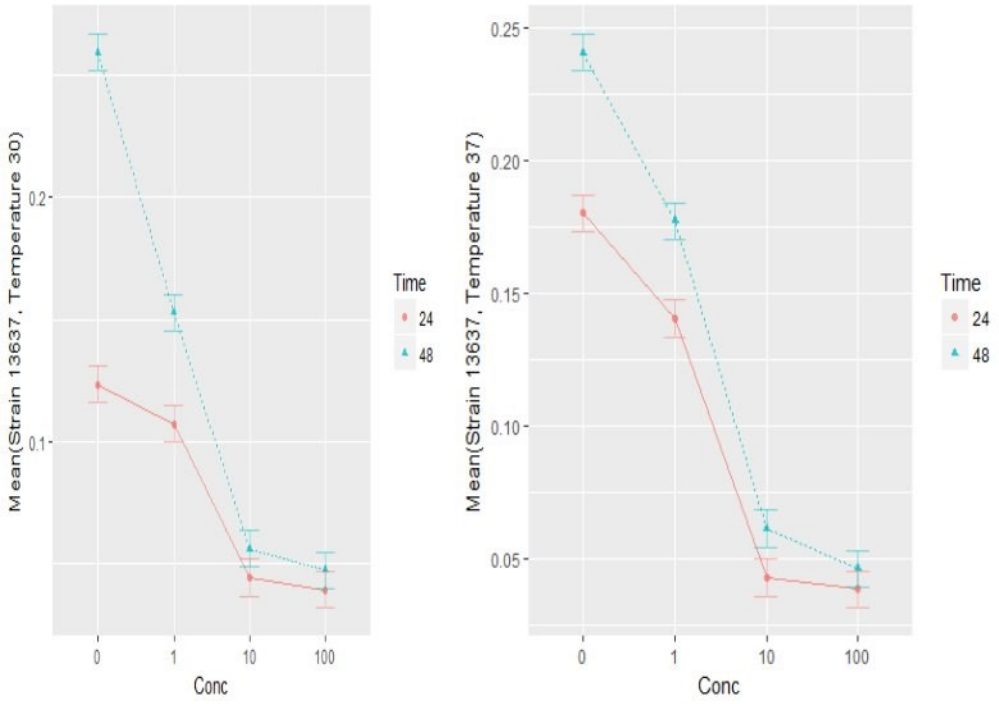


Figure 6-A. The effect of ferric chloride concentration and temperature on *S. maltophilia* biofilm Metabolic Activity. A. 24 hrs and B. 48 hrs incubation Ferric Chloride concentration (µg\ µl) (n=12) for each condition.



P-value = 1.969188e-11



P-value=1.969188e-11

Figure 6-B. $FeCl_3$– ANOVA factorial model with measures Time**,** Concentration, Temperature for the Strain 13637 and Strain 51331 on *S. maltophilia* biofilm Metabolic Activity.

The effect of increasing amounts of salt on S. maltophilia antibiotic drug resistance

With the moderate decrease in colony growth as salt concentration increased, the question arose as to whether the isolates also would become more susceptible to selected antibiotics under adverse conditions. LBA plates were prepared with increasing salt concentration 0-10% and the highest concentration of $FeCl_3$ was used 0.25 µM. Tests were performed and interpreted according to the guidelines of Clinical Laboratory Standards Institute (CLSI).

Both isolates of *S. maltophilia* (51331 and 13637) did not demonstrate any susceptibility to any of the antibiotics tested. It was seen, however, that *S. maltophilia* 51331 demonstrated intermediate resistance to ceftazidime (zones of inhibition, 22.5, 20, and 16mm) when NaCl was absent in the media or present at low concentrations (Table 3). When salt concentrations were increased, full resistance to all the antibiotics was displayed. The same pattern of resistance was also displayed by *S. maltophilia* 13637 when tested under the same conditions as shown in Table 3.

Table 3. *Minimum Inhibitory Concentrations of S. maltophilia isolates as a function of the presence of salt.*

S. maltophilia isolate Antibiotic	Salt concentration of LB agar Zone of clearance diameter in mm (n = 3)				
	0 µg/µl	10 µg/µl	50 µg/µl	100 µg/µl	0.2 mM $FeCl_3$
51331					
Amoxicilin (AMC)	6.5 mm (R)	NZ(R)	NZ(R)	NZ (R)	10 mm (R)
Cephazolin (KZ 30)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
Ceftazidimide(CAZ 30)	22.5 mm (IR)	20 mm (IR)	16 mm (IR)	NZ (R)	NZ (R)
Erythromycin (E15)	13 mm (R)	10 mm (R)	NZ (R)	NZ (R)	NZ (R)
Ticarcillin (TIC75)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
13637					
Amoxicilin (AMC)	6 mm(R)	NZ(R)	NZ(R)	NZ(R)	9.5 mm (R)
Cephazolin (KZ 30)	NZ(R)	NZ(R)	NZ(R)	NZ(R)	NZ(R)
Ceftazidimide(CAZ 30)	22 mm (IR)	18 mm (IR)	15 mm (IR)	NZ (R)	NZ (R)
Erythromycin (E15)	13 mm (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
Ticarcillin (TIC75)	10.5 mm (R)	8.5 mm (R)	NZ (R)	NZ (R)	NZ (R)

Ceftazidime MIC (50) 32 µg/ml, respectively (sensitive ≤ 8 µg/ml and resistant ≥ 32 µg/ml according to CLSI guideline). MIC (75) and MIC (30) for Ticarcillin and Cephazolin respectively (sensitive≥ 20 µg/ml and resistant ≤ 14 µg/ml according to CLSI guideline). Erythromycin "(MIC15), and Amoxicillin (MIC10) respectively (sensitive ≥ 23 µg/ml and resistant ≤ 14 µg/ml for Erythromycin and sensitive ≥ 17 µg/ml and resistant ≤ 13 µg/ml for Amoxicillin according to CLSI guidelines).

Discussion

The urgent need for new innovations in antimicrobial cures is made apparent by the visible rise in community spread and nosocomial infections. Among these infections are ones caused by the bacterium, *S. maltophilia*. *S. maltophilia* is of clinical relevance because of its ability to form biofilms, which is associated with reduced efficiency of cure and enhanced resistance to antimicrobials. Once established, a biofilm must be removed to prevent *S.*

maltophilia disease due to the resistant characteristics of the bacteria and the ability to spread the disease very easily. *S. maltophilia* infections are difficult to cure as previously discussed, especially for patients suffering from cancer and other immunodeficiency.

Biofilm formation is one of the main reasons for making these cures more difficult, hence search is continued for finding novel treatment strategies. This advanced method of avoiding the production of biofilm or lowering the characters of resistance are new and seem to be promising in the medicine and health industry. Hence our laboratory characterized a number of factors that affect the *S. maltophilia* biofilm formation. Our research will possibly help in increasing comprehension of the biofilm production and investigations. This study considered *S. maltophilia* 13637 and 51331 for comparison of these strains in the various growth conditions. While it is simpler to experiment with an individual species of bacteria, a crisis in the health industry is that such infections may be because of various microbial species work together and infected body parts may have many types of microbes. *S. maltophilia* colonizes inside some tissue like the respiratory tract of cystic fibrosis (CF) patients (Denton and Kerr, 2002; DiBonaventura et al., 2007; Nazik et al., 2007). Most of the earlier studies have shown the impact of salt and iron on the growth and biofilm activity it has been characterized for other types of bacteria but not for *S. maltophilia*. This study has shown how these elements impact the growth of *S. maltophilia* and also the biofilm production of this bacteria. Many studies have recommended iron to be a potential cure for biofilms produced by bacteria and investigating the impact of ferric chloride on *S. maltophilia* biofilm activity represents a possible remedy for the biofilm of these bacteria.

Temperature Range and Culture Growth of *S. maltophilia*

The first part of our hypothesis was that the metabolic activity of two strains of

Stenotrophomonas maltophilia would not be differentially affected by temperatures. This hypothesis was not supported. *S. maltophilia* could not grow at the high and low degrees of temperatures. This thesis observed that *S. maltophilia* grows below its perfect temperature range between 20°-37°C (its optimum temperatures), and above temperatures of 40°C the growth is very slow (its minimum temperature) as the metabolic enzymes are not working well for more activity for maximum growth and plasma membranes have more rigidity and also the transport nutrients are affected at 55°C. Similarly, in case a microbe is grown over its perfect temperatures growth range, the metabolic enzymes are disrupted and cannot function properly that lowers the growth and eventually kills the microbe as the heat is increased (Denton, M., & Kerr 1998). On the other hand, this study proved that *S. maltophilia* enhance to grow on the low concentrations of salt but it inhabited at the high concentration of salt for both of strains 13637 and 51331. In statistical analysis the P-value is actually not a significant different in average growth size between the two strains at 300-370 C.

Salt and S. maltophilia biofilms

The second part of hypothesis was that the metabolic activity of two strains of Stenotrophomonas maltophilia will be inhibited in high concentrations of sodium chloride and ferric chloride. This hypothesis was supported by data gotten in this study. This suggests that there is actually not a significant difference in average size between the two strains, but there is a significant effect for the other predictors. The relationship between NaCl concentrations and temperatures (300-370 C) also the relationship between the concentrations and time (after 24-48 hrs.) have large significant effect. According of statistics data analysis, there were differenced averages of the metabolic activity at 0 µg/µl and 10 µg/µl of NaCl. On contrary, there were inhibited at the high concentrations (50 µg/µl, 100 µg/µl, 150 µg/µl) at

temperatures 300-370 C with P-value = 1.799019e-54 and also at the time (24 and 48 hrs.) with p-value = 6.928106e-10. The data observed that a drop in average size between 24 and 48 hrs at 300 C while a large increase in average size between 24 and 48 hrs at 370 C. In brief, at 300C it takes little salt to cause shrinking during the second day and at 37°C it takes between 50 μM and 100 μM of NaCl to see shrinking occur.

In the presence of ferric chloride, while the strain itself does not have a significant effect on the average size of the biofilm, some of the interaction terms are highly significant, so we will keep it in the model this time.

Interestingly, temperature and time were not shown to have a significant overall effect but the interaction between those two factors is significantly different between the two strains. The effect of the strain on the interaction between concentration and time is primarily seen at concentrations of 0 μg/μl and 1 μg/μl for both of salt, NaCl and FeCl3, there is more growth during the second day for strain 51331 than there is for strain 13637. However, they act similarly at concentrations of 10 μg/μl and 100. μg/μl The effect of the strain on the interaction between concentration and temperature is primarily seen at concentrations of 0 and 1, there is a greater difference in average biofilm size for strain 51331 between 30°C and 37°C than there is for strain 13637. However, they act similarly at concentrations of 10 μg/μl and 100 μg/μl for both concentrations of salt. Overall (disregarding FeCl3 concentration), we see that for strain 51331 there is little effect on average biofilm size at 24 hours between temperatures of 30°C and 37°C , but the average biofilm size at 48 hours is significantly higher at 37°C than at 30°C. This trend is actually reversed in strain 13637: we see significantly higher average biofilm size at 24 hours at 37°C, but by 48 hours the average size at 30°C has closed the gap. Finally, the effect of temperature on the interaction between concentration and time is seen to be more

pronounced for strain 13637 than for strain 51331 with P-value 1.969188e-11.

Conclusion

The results of this study showed that the growth of *S. maltophilia* strains X13637 and X51331 were inhibited at the low and high temperatures and the optimal range of growth was at 37°C. This is also the normal temperature of human body, which is consistent with the fact that this bacterium can grow and cause disease in the human body. *S. maltophilia* X13637 and X51331 growth was inhibited at the high concentrations of salt, but it can grow in the low concentrations of salt.

Higher concentrations of salt and antibiotics can have a greater impact on the decrease of metabolic activity of both bacterial strains. Biofilms grown at low concentrations of salt have more metabolic activity with different significance, on the contrary at the high concentrations of salt there was no significant inhibition of metabolic activity.

Future Directions

This thesis study shows that three factors of environmental conditions that effect growth and metabolic activity and biofilm development in *S. maltophilia*. Considering this thesis examined the biofilms after they were fully developed (24-48 hours), one of the most logical next steps to the experiment presented here would be to exam the effects at earlier stages of the biofilm formation (around 12 or 18 hours). Additionally, the effect of these environmental conditions on biofilm formation could be further characterized by determining the biofilm mass versus the metabolic activity at the different temperatures and salt concentrations. It may be possible that the metabolic activity of the biofilms is not a direct correlation to the number of bacterial cells that are present in the biofilms (biofilm mass).

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