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MICROPLATE METHODOLOGIES APPLIED TO ENVIRONMENTAL BIOTECHNOLOGY

Farrah Solomon
University of Rhode Island, mythicfs@gmail.com

MICROPLATE METHODOLOGIES APPLIED TO ENVIRONMENTAL BIOTECHNOLOGY

BY

FARRAH SOLOMON

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FARRAH SOLOMON

APPROVED:

Thesis Committee:

Major Professor Vinka Oyanedel-Craver

Leon Thiem

Dawn Cardace

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
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ABSTRACT

Microarray technology is a high-throughput technique that allows a researcher to reduce the amount of reagents for each experiment as well as conduct multiple experiments with varying conditions simultaneously with the capability of analyzing this data quickly. Such microarray techniques have been applied in different fields such as environmental, pharmaceutical, microbiology, and biomedical among others (Kricka 2001).

Chapter 1 provides an introduction to microarray technology with a description of three different techniques: Phenotypic Microarray, DNA Microarray and Protein Microarray, used to study different aspects of microorganisms.

Chapter 2 presents the first manuscript, "A novel application of microarray technology to respiration study of *Escherichia coli"*, with a focus on the development of a respirometric protocol to monitor the respiration of *Escherichia coli* over time.

Chapter 3 presents the second manuscript, "Effect of Dysprosium oxide nanoparticles on *Escherichia coli*", with a focus on the application of the developed respirometric methodology in Chapter 2 to the toxicity effect of dysprosium oxide nanoparticles on *Escherichia coli.*

Chapter 4 presents the third manuscript, "Anaerobic growth of *Acidithiobacillus ferrooxidans* using microarray technology", with a focus on the development of an anaerobic growth methodology for the reduction of iron to be used in future nanotoxicity studies.

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PREFACE

The format utilized for this thesis is the manuscript format instructed by the Graduate School. There will be three manuscripts that encompass the thesis.

Chapter 2 presents the first manuscript entitled, "A novel application of microarray technology to respiration study of *E. coli*".

Chapter 3 presents the second manuscript entitled, "Effect of Dysprosium oxide nanoparticles on *Escherichia coli*" is in preparation by Nelson Anaya, Farrah Solomon, and Vinka Oyanedel-Craver.

Chapter 4 presents the third manuscript entitled, "Anaerobic growth of *Acidithiobacillus ferrooxidans* using microarray technology" is in preparation by Farrah Solomon, Dr. Dawn Cardace, and Vinka Oyanedel-Craver.

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CHAPTER 1

INTRODUCTION

Environmental microbiology techniques used to study growth and respiration processes of microorganisms have been used for as water quality management, nanotoxicology and environmental biogeochemical studies (Fuma et al. 2005; Koster et al. 2003; Giller et al. 1998). Traditional techniques such as growth inhibition using agar plates (Agarwal et al. 2012), respirometric tests with manometric bottles (Tzoris et al. 2005; (Zhang and Oyanedel-Craver 2012) and microcosms constructed to model a niche (Fuma et al. 2005), use large amounts of resources and time. To overcome these challenges, microarray-based techniques can be applied to conduct experiments in an efficient and effective manner.

Microarray techniques use an array (alignment of rows and columns) to analyze multiple conditions in a single run. These arrays can be developed on chips or within microplates (Heller 2002; Howbrook et al. 2003; Jacobsen et al. 2007). Microarray techniques use volumes of reagent in the microliter scale. The high-throughput nature of this technique makes its application possible to a wide variety of fields such as: environmental, pharmaceutical, microbiology, biomedical, and clinical research (Kricka et al. 2001). Examples of microarray-based techniques are phenotype microarray, DNA microarray and protein microarray.

Phenotype microarray (PM) techniques uses a microplate, either commercially or custom made, to analyze the metabolic functions of microorganisms based on different growth conditions (Borglin et al. 2012). Previous studies have demonstrated the ability

to measure cell growth and cell respiration using tetrazolium dye (Bochner 2008; (Bochner et al. 2001). Reduction of the tetrazolium dye can occur at the cellular membrane or intracellular at the mitochondria level, for microorganisms and mammalian cells. When a carbon source is consumed (Figure 1-1), metabolic activity transports electrons from that carbon source to the electron transport chain in the cell membrane or mitochondria where the tetrazolium dye is reduced and as a result it produces a purple color (Bochner et al. 2001; Tremaroli et al. 2009).

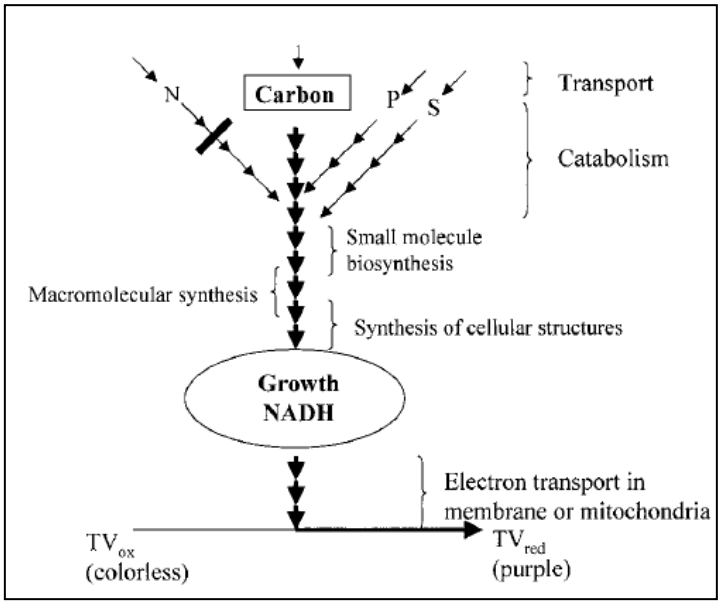

Figure 1- 1 Reduction of Tetrazolium violet by cellular electron transport chain (Bochner et al. 2001).

The purple color can be quantified using a microplate reader at a wavelength of 590nm (Klimek et al. 2007). PM techniques have been primarily developed for growth assays requiring nutrients for the microorganisms to grow (Bochner et al. 2001; Bochner 2008; Borglin et al. 2012). Additionally, this technology has been used to monitor respiration of bacteria for non-growth application that studies metabolic activity only in a stable population of microorganisms under different conditions (Omsland et al. 2011). The isolation of bacterium, *Coxiella burnetti*, from its host

animal cell was placed within a constructed stable environment where microarray technology was used to study non-growth conditions within a microplate to measure the effects of different nutrients through cellular respiration (Omsland et al. 2011).

DNA and protein microarray methodologies are used to analyze gene expression (Howbrook et al. 2003) and protein identification (Templin et al. 2002), respectively, using either a chip and/or microplates. These methodologies can be used for phylogenetic identification of microorganisms in ecological communities (Zhou et al. 2002) and biomedical studies for cancer and drug analysis (Afshari et al. 1999; Liotta et al. 2003). Unlike the phenotype microarray methodologies, DNA and protein microarray methodologies require the extraction of the DNA or protein from the cell type in study or from microorganisms. An example of a DNA microarray based methodology uses a fluorescence compound called SYBR Green I to quantify DNA (Leggate et al. 2006). For protein array based methods the Bradford Assay that uses Coosmassie Blue G-250 and the bicinchoninic acid (BCA) assay both quantify protein (Sapan et al. 1999).

PM, DNA and protein microarray techniques have been used to primarily study aerobic microorganism. However, recent studies have been intended to apply microarray techniques under anaerobic conditions and have reported microarray procedures for anaerobic growth studies of *Desulfovibrio vulgaris* and *Paracoccus denitrificans* (Borglin et al. 2009; Koutny et al. 2005). Studying anaerobic growth is essential to understand some biogeochemical cycles, that could be used for applications such as remediation of acid mine drainage sites or enhanced wastewater treatment (Gadd 2004; Natarajan et al. 2008; Narihiro et al. 2007).

Microarray techniques can be used to study the effect of new materials released in the environment such as nanoparticles (NPs). NPs are defined as particles less than 100nm and can be found in different shapes such as spherical or tubular (Nowack et al. 2007; Handy et al. 2008). They can occur naturally or engineered through various methods. Some examples of naturally occurring NPs are soot, organic colloids or humic acids (Nowack et al. 2007). Naturally occurring NPs can also be found in natural waters (Handy et al. 2008). Naturally inorganic NPs are iron oxides or aerosols such as sea salt (Nowack et al. 2007). Anthropogenic NPs can be manufactured by various methods resulting in different sizes, shapes and surface functionalizations. This includes silver nanoparticles (AgNPs), which are used to eliminate and prevent antimicrobial growth for medical applications because of their antimicrobial properties (Kaler et al. 2010). Also, iron oxide and dysprosium oxide nanoparticles are commonly used as contrasting agents in magnetic resonance images (Hofmann-Amtenbrinka et al. 2010).

Engineered nanoparticles can have unique physiochemical properties not typically compared to naturally occurring NPs. These may include larger surface area, increased purity, specific controllable shapes and surface structure (Nowack et al. 2007; Nel et al. 2006). As a result, these engineered NPs may remain longer in the environment which poses a threat to microorganisms and larger organisms before finally degrading and becoming inert. Therefore, it is very important to study the effects of these engineered NPs on microorganisms to determine potential risks of their release into the environment.

This study aims to develop two microarray methodologies: respirometric assay for the evaluation of dysprosium oxide nanoparticles (nDyO) toxicity effect on *Escherichia coli* (*E. coli*) and anaerobic growth for future nanotoxicity studies of *Acidithiobacillus ferrooxidans* (*A. ferro.*) under anaerobic conditions The second chapter will present a methodology manuscript describing the development of the respirometric methodology for *E. coli*. The third chapter will present the application of the respirometric methodology to conduct toxicity studies in order to evaluate the toxic effect of nDyO on *E. coli*. Lastly, the fourth chapter will present data on development of an anaerobic methodology for the growth of *A. ferro.*

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MANUSCRIPT 1

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A novel application of microarray technology to respiration study of *E. coli*

Farrah Solomon and Vinka Oyanedel-Craver

Corresponding Author

Prof. Vinka Oyanedel-Craver

Department of Civil and Environmental Engineering

University of Rhode Island

Kingston, RI 02881

craver@egr.uri.edu

CHAPTER 2

A novel application of microarray technology to respiration study of *E. coli*

2.1 Abstract

Current advancements in techniques for biotechnology applications has created an ongoing paradigm shift from traditional techniques to effective high-throughput techniques. Microarray technology allows for the analysis of multiple conditions simultaneously. The objective of this study is to develop a non-growth microarray based technique to measure the respiration of *E. coli* for future nanoparticle toxicity studies. Initial non-growth conditions incorporated the use of a tetrazolium dye, that can be reduced through metabolic activity of *E. coli* to produce a quantitative purple color, a PM-MTOX1 plate containing eight carbon substrates to assess different stages of the metabolic process in *E. coli* (concentration measured at Abs_{670nm} at 3.0) and the Biolog OmniLog microplate reader that can measure the dye color change over time at 35^{o}C. Final non-growth conditions used an in house prepared microplate instead of the PM-MTOX1 to test different concentrations of glucose which resulted in the use of glucose concentrations 140 mg/L and below for future nanotoxicity tests since glucose concentration 210 mg/L and above exhibited an inhibitory effect on *E. coli*. Lastly, initial toxicity testing using dysprosium oxide nanoparticles (nDyO) were compared within the Biolog OmniLog and the Synergy^{TM} microplate reader. The Biolog OmniLog uses a colorimetric proprietorial method and the Synergy^{TM} MX microplate reader takes absorbance measurements of the dye. Percent Remaining Respiration (PRR) of the bacteria unaffected by nDyO was calculated for each microplate reader

and similar trends observed. The results were as followed for 0.1 mg/L, 0.5 mg/L and 1.0 mg/L nDyO: Biolog Omnilog PRR was 90.0%, 60.4% and 32.1%, respectively and Synergy^{TM} MX microplate reader PRR was 95.1%, 55.7% and 22.5%, respectively. Both microplate readers showed similar trends and therefore could measure the toxicity of nDyO on *E. coli*, however the Synergy^{TM} MX microplate reader was selected for future nanoparticle toxicity studies because of its highly customizable protocols and different shaker speeds.

2.2 Introduction

Activity of microorganisms can be study using growth and respiration assays (Giller et al. 1998; Koster et al. 2003; Fuma et al. 2005). Examples of some techniques are manometric bottles to assess changes in respiration (Tzoris et al. 2005; Zhang and Oyanedel-Craver 2012), inhibition agar plates for growth of specific microorganisms only or to test the ability of microorganisms to resist certain inhibitors (Agarwal et al. 2012) and microcosms constructed to model a niche for specific impact studies (Fuma et al. 2005). However, it is known that these techniques require the use of large amounts of reagents and time. To overcome these challenges, a novel microarray-based technique has been developed to conduct experiments efficiently and in a cost effective way.

Microarray techniques use an array to analyze multiple conditions simultaneously and only use reagent volumes in a microliter scale compared to the respirometric manometric bottle test (i.e. milliliter volume). The high-throughput nature of this technique has been applied to environmental studies, pharmaceutical, microbiology, biomedical and clinical research (Kricka et al. 2001). Phenotype Microarray (PM) is

an example of a microarray technique used to analyze the metabolic functions of microorganisms based on different growth conditions with the use of a tetrazolium dye indicator (Bochner et al. 2001). The tetrazolium dye is reduced by active cellular respiration (producing a purple color) that can be measured by a microplate reader to assess growth and cell respiration as proven in previous studies (Bochner 2008; Bochner et al. 2001; Tremaroli et al. 2009; Klimek et al. 2007).

PM techniques are primarily based on growth assays that require nutrients for the microorganisms to grow, however this technique has been used to monitor respiration of bacteria within non-growth conditions (Bochner et al. 2001; Borglin et al. 2012). Omsland (2011) studied the isolation of *Coxiella burnetti* from its host animal cell and its placement within a constructed stable environment to measure the effects of different nutrients on cellular respiration using this technique.

This study aims to implement microarray techniques to adapt the traditional respirometric technique to a microarray based technique. The implementation of a non-growth or respiration microarray based technique will allow for initial toxicity testing within two microplate readers, the Biolog OmniLog and the Synergy™ MX microplate reader, for result and capability comparison for future nanotoxicity testing.

2.3 Materials

A nonpathogenic wild strain of *E. coli* purchased from IDEXX laboratory was selected for this study because it has been extensively studied. *E. coli* is a gram negative bacterium that has been found to be metabolically active in saline solution without growth (Doudoroff 1940) also, is a candid microorganism for nanotoxicological studies. Growth media for this bacteria consisted of 10 g/L NaCl, 5

g/L yeast extract, 10 g/L Tryptone (purchased from Sigma Aldrich). A centrifuge (ThemoScientific Sorvall Legend x1R) was used for the collection of *E. coli* at 2000 rpm for 15min.

Dysprosium oxide nanoparticles (nDyO) (99.9%), glucose and NaCl were purchased from Signma Aldrich. The concentration range of nDyO, 0.1 mg/L, 0.5 mg/L and 1.0 mg/L, were used to cover background environmental concentrations while simultaneously covering higher concentrations of nanoparticle exposure for worst case scenarios. Glucose conditions 140 mg/L and 210 mg/L were used as a carbon source for *E. coli*, unless stated differently, to ensure measureable metabolic responses. One water chemistry condition, 85 mg/L NaCl (ionic strength of 1.45 mM), remained constant throughout all preliminary experiments, unless otherwise specified. This concentration was selected because it was based on ionic surface water concentrations (Hoecke et al. 2011).

Tetrazolium dye (Redox Dye Mix A; information about Redox Dye Mix A is available from Biolog, Hayward, CA) purchased from Biolog was used to measure the respiratory activity of *E. coli*. The tetrazolium dye is reduced by active cellular respiration and produces a purple color (Bochner et al. 2001; Tremaroli et al. 2009) measurable by a microplate reader at wavelength of 590nm (Klimek et al. 2007) or by photometric measurements, over time.

Two different microplates were used during the development of the respirometric microarray based methodology: PM-M TOX1 and blank, 96-half area, flat-bottomed, clear polystyrene microplate (Costar^{TM} brand) microplates.

2.4 The development of the respirometric microplate methodology

Initial experiments were performed in the Biolog OmniLog to establish growth and non-growth conditions because this equipment was calibrated to measure changes in the reduced tetrazolium dye. Initial growth and non-growth conditions were established using the PM-MTOX1 microplate. After growth and non-growth conditions were achieved, there was a transition to prepare user made plates instead of PM-MTOX1 so that different concentrations of carbon substrates could be tested. The further improved methodology was tested within two different microplate readers. The Biolog OmniLog uses a colorimetric method and the Synergy™ MX takes absorbance measurements to detect changes within the dye. Preliminary toxicity experiments using nDyO were conducted within each microplate reader and their results compared. Capabilities of the instruments were also taken into consideration before the final selection was made to use for future nanotoxicity experiments.

2.4.1 Growth conditions using a PM-MTOX1 microplate within the Biolog OmniLog

Microplate growth and non-growth conditions used a PM-MTOX1 microplate because it contained eight carbon substrates. In Figure 2-1, the PM-MTOX1 microplate layout is provided. A single carbon substrate in a single row does not vary in concentration. Exact concentrations of each type of carbon source were not provided by the manufacturer; however they are within the range of 5 mM to 10 mM. This plate was selected because it is commonly used for toxicity testing and provides carbon substrates found in different metabolic stages of *E. coli*.

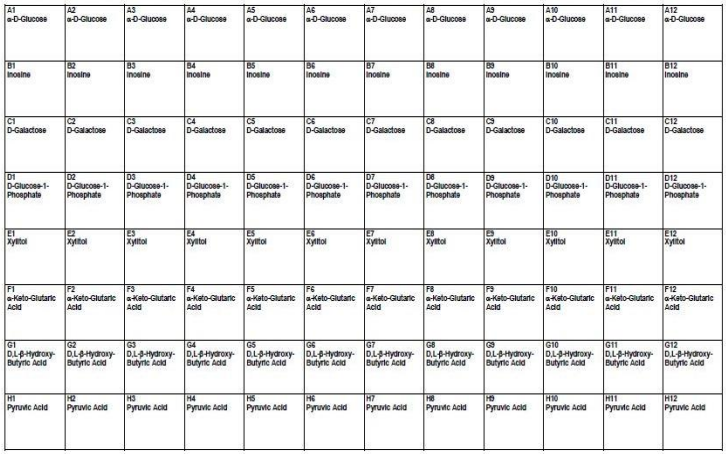

Figure 2- 1 PM-MTOX1 microplate layout consisting of 8 carbon substrates.

Growth conditions within the PM-MTOX1 microplate consisted of a yeast culture media, bacteria solution and 1% dye added (Tremaroli et al. 2009) (Table 2-1). *E. coli* concentration was fixed to OD_{670nm} 0.07 before the addition of dye to the media. Cell solution of 100 µl was evenly inoculated into each well of the microplate (B. Bochner, personal communication, October 17, 2012) and incubated at 35^{o}C.

Table 2- 1 Grow conditions within the Biolog OmniLog

Media	yeast culture media
Carbon Substrate	multiple substrates
Tetrazolium Dye	1%
Microplate Type	PM-M TOX1
Temperature ^{o}C	35
Bacteria (*E. coli*) inoculum	85% Turbidity = Abs_{670nm} 0.07
Time (hrs)	24

Figure 2-2 represents a growth curve produced by the Biolog OmniLog. The typical growth curve stages: Lag phase, Exponential phase, and Stationary phase are identified with arrows in the graph. A control lacking bacteria was used to determine minimal background coloration of the tetrazolium dye and culture media, and to

ensure non-spontaneous reduction of the dye. Expected coloration in the plate due to the growth of *E. coli* did occur and validated use of the tetrazolium dye.

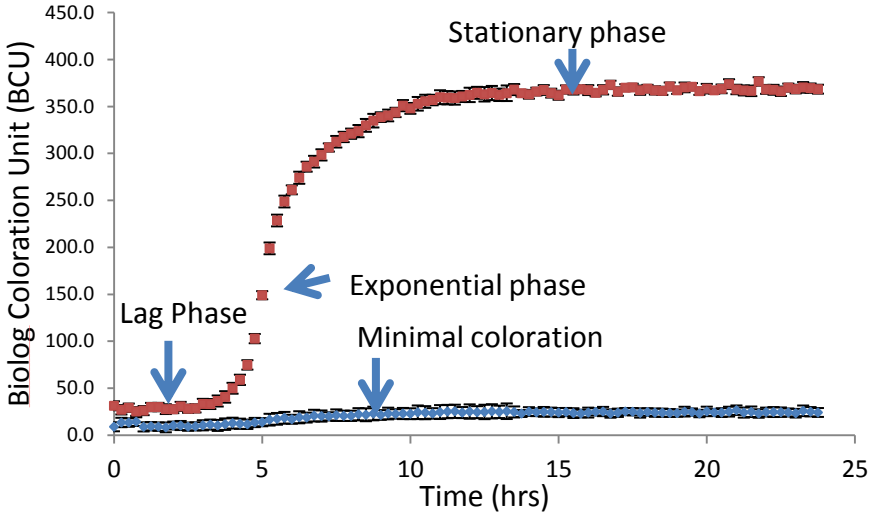

Figure 2- 2 Growth of *E. coli* over 24 hrs using the Biolog OmniLog on α-D-Glucose. Each line consisted of an average of 9 wells.

2.4.2 Non-growth conditions using a PM-MTOX1 microplate within the Biolog OmniLog

Establishing a non-growth methodology allows quantitative information to be gathered on a stable microorganism population in regards to nanoparticle toxicity studies easier than on a growing and constantly changing population of microorganisms. The transition from growth conditions to non-growth conditions using a microplate incorporated the basic methodology used for the conventional manometric respirometric bottle test. A brief summary of the conventional manometric respirometric bottle test is followed by a summary of preliminary test conditions conducted to establish the non-growth conditions within the microplate.

2.4.2.1 Conventional manometric respirometric bottle test

The conventional manometric respirometric bottle tests are used to measure bacterial inactivation batch tests using small bottle that consist of a magnetic stirrer and a small container that fits within the bottle that contains NaOH to absorb the CO_2 produced. An electronic pressure sensor based on the piezoresistive ideology measures kinetic pressure changes equivalent to the O_2 depletion in the attached measuring head. The measurements taken by the measuring head are under sealed and constant temperature conditions which are recorded by the OxiTop controller (OxiTop control system, WTW Weilheim, Germany).

The procedure used by Zhang and Oyanedel-Craver (2012) for toxicity testing of silver nanoparticles (nAg) served as the basis for the developed respirometric microarray based methodology. Briefly, bacteria were incubated at different water chemistry conditions and constant temperature (25^oC) and before the addition of glucose and nAg and the endogenous respiration of the system measured by pressure drop over 2 to 3 hrs. In the next 2 to 3 hrs the oxygen uptake rate (OUR) of the system was measured after the injection of 0.5 ml of glucose followed by the injection of 0.25 ml nAg for the remainder of the experiment. The altered OUR of the system measured after the injection of nAg determined the amount of bacteria reduced. The total duration of the experiment was about 20 hrs. The survival rate was calculated using an equation modified from Tzoris et al. (2005):

$$Survival\ rate\,(\%) = \frac{P_t - P}{P_c - P} \times 100$$

where,

P = Endogenous respiration of bacteria measured as OUR before the addition of carbon source and nAg.

P_c = pressure drop after glucose injected.

P_t = OUR after nAg injected.

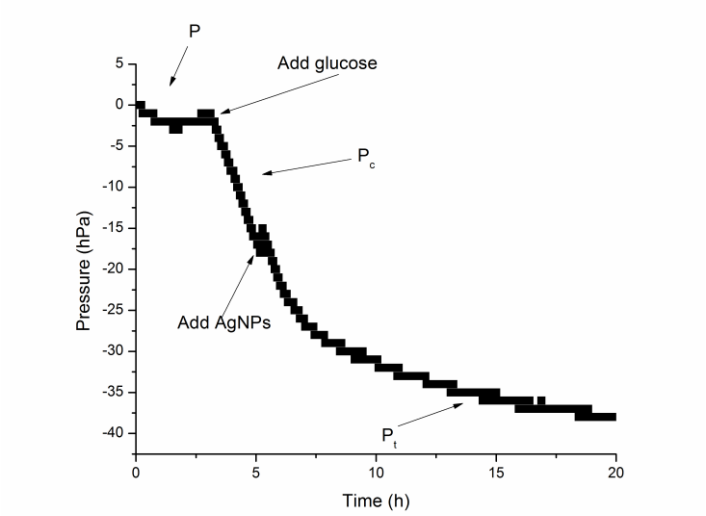

Figure 2- 3 Example bacteria deactivation respiration curve (Zhang and Oyanedel-Craver, 2012).

2.4.2.2 Non-growth conditions within the PM-MTOX1 microplate

The manometric respirometric bottle tests conducted by Zhang and Oyanedel-Craver (2012) served as the basis for the non-growth conditions tested within the PM-MTOX1 microplate. Table 2-2 summarizes the non-growth conditions for both the manometric respirometric bottle test and the conditions tested for the PM-MTOX1 microplate.

Table 2- 2 Non-growth conditions of the manometric respirometric bottle test and the non-growth conditions tested using the PM-MTOX1 microplate.

Respirometric Bottle Conditions		**Microplate Non-Growth Conditions Tested**
Media	NaCl [85 - 8500 mg/L]	NaCl [85 mg/L]
Carbon Substrate	Glucose [70 - 210 mg/L]	**multiple**
Tetrazolium Dye	No	**1%**
Microplate Type	No	PM-M TOX1
Temperature	25^{o}C	**35**
E. Coli	Abs_{670nm} 0.30 ≈ 10^{9} CFU/mL	**Abs_{670nm} 0.03 - 3.0**
Time (hrs)	20	**12 to 48**

Different carbon sources were tested via the PM-MTOX1 microplate to determine which one would be used for future toxicity tests using nanoparticles. A range of *E. coli* concentrations were tested to determine which would provide enough reduction of the tetrazolium dye to produce a quantifiable amount of color change. The temperature of the microplate experiments were conducted at 35^{o}C to ensure the metabolic response was strong enough to reduce the tetrazolium dye and provide enough color change. Lastly, the duration of the experiments initially started at 48 hrs and decreased overtime due to no change in measurable dye over a certain period of time.

One preliminary experiment illustrated in Figure 2-4 show two *E. coli* inoculum concentrations (Abs_{670nm} 0.30 and 3.0) tested on three different carbon substrates over 24 hrs. Based on the curves, it was determined that glucose would be used as a carbon source and an Abs_{670nm} value of 3.0 would be used for *E. coli* concentration for future experiments (Table 2-3).

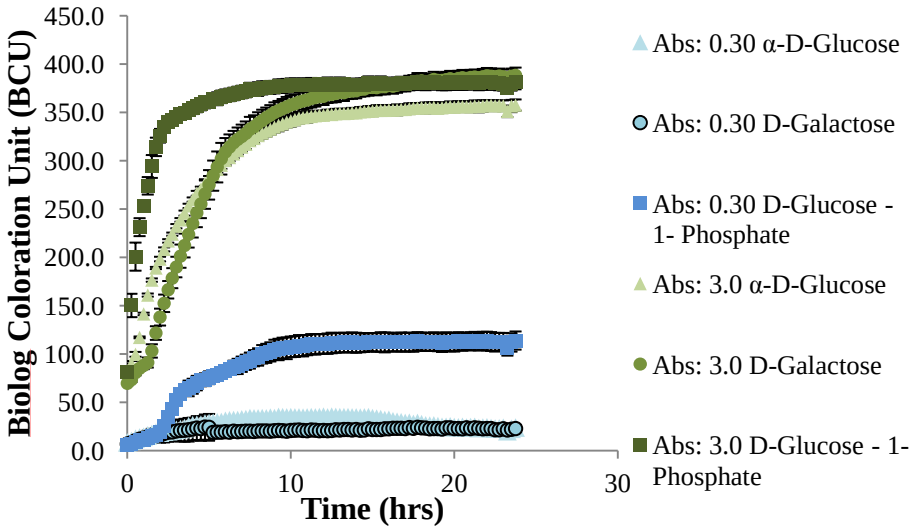

Figure 2-4 Non-growth curves of *E. coli* over 24 hrs using the Biolog OmniLog on α-D-Glucose, D-Galactose and D-Glucose-1-Phosphate. Blue curves indicated an Abs_{590nm} 0.30 and green curves of 3.0. Each curve consisted of an average of 4 wells.

Table 2- 3 Best Non-growth conditions using the PM-MTOX1 microplate.

Best Microplate Non-Growth Conditions	
Media	NaCl [85 mg/L]
Carbon Substrate	α-D-Glucose
Tetrazolium Dye	1%
Microplate Type	PM-M TOX1
Temperature ^{o}C	35
E. coli density w/in microplate	**Abs_{670nm} 3.0**
Time (hrs)	**24**

2.4.3 Non-growth conditions using a in-house made microplate

Non-growth conditions using a PM-MTOX1 microplate provided limited control over using different concentrations of glucose. By using the in-house prepared plate, glucose concentrations could be varied. Also, all materials could now be prepared in a background of 85 mg/L NaCl except the tetrazolium dye. Glucose range of 0 mg/L to 350 mg/L were tested and the results shown in Figure 2-5. It is important to mention

that at this time, *E. coli* solutions were refrigerated at least 30 min prior to inoculation into the microplate. Refrigeration decreased the metabolism of the bacteria and allowed for a homogenous metabolic reaction to the glucose.

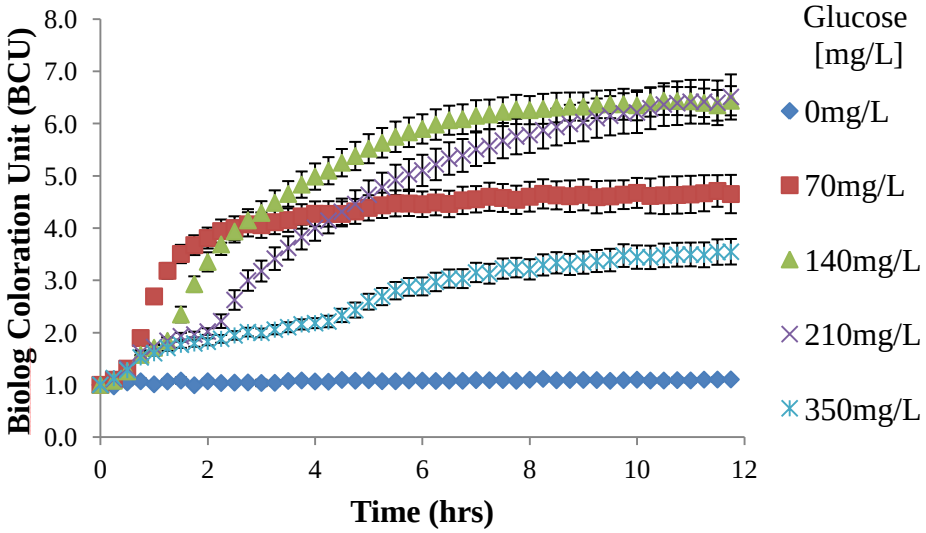

Figure 2- 5 *E. coli* (Abs_{670nm} 0.30) respiration curves on 4 concentrations of glucose with a control of [0mg/L] of glucose. Each curve consisted of an average of 12 normalized wells.

Based on the results in Figure 2-5, optimal glucose concentrations of 140 mg/L and below were chosen for future nanoparticle toxicity tests. Higher glucose concentrations were not considered because our experiments showed that they have an inhibitory effect on metabolic processes on *E. coli*. To ensure changes in metabolic activity were accurately measured, well values were normalized by their initial absorbance values considering metabolic activity is a function of bacteria concentration. The experimental duration was further shortened to 12 hrs since changes in the reduction of the dye remained unchanged.

2.5 Biolog OmniLog vs Synergy^{Tm} Microplate reader

The Biolog OmniLog and the Synergy^{Tm} Microplate reader measure changes in the tetrazolium dye by using two different methods, colorimetrically and by absorbance, respectively. The Biolog OmniLog uses a color camera that takes images from the top of the microplate over time to quantify the reduced dye (Bochner et al. 2001). The Synergy^{Tm} Microplate reader can measure the changes in the reduced dye by measuring the absorbance of light at a particular wavelength (590 nm) over time. To determine if the Synergy^{Tm} Microplate reader could be used for future nanoparticle toxicity studies, a comparison test between the two microplate readers was conducted.

Figure 2-6 (a) and (b) are respiration curves of *E. coli* (Abs_{670nm} 3.0) (70 µl) using three concentrations of nDyO 0.1 mg/L, 0.5 mg/L and 1.0 mg/L (10 µl) with 140 mg/L glucose (10 µl) in 85 mg/L NaCl. 10 µl of tetrazolium dye was added to all wells within both microplates and all wells mixed 10 times using a multichannel pipette. Both tests for each microplate reader were conducted at 35^{o}C and readings were taken after 15 min for a total of 18 hrs. A section of the curve from 1 to 2 hrs was analyzed for their slope values and compared with the control within the microplate which

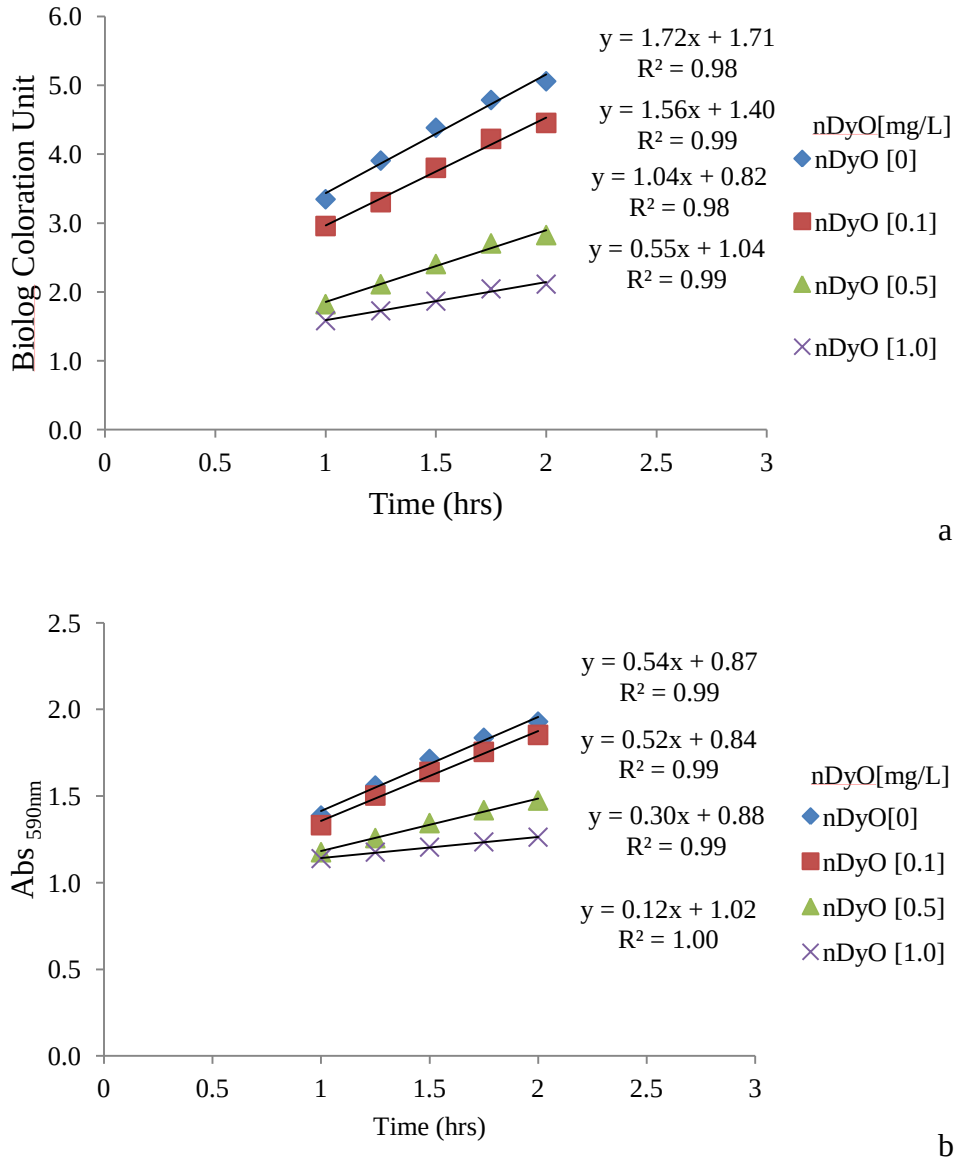

Figure 2- 6 Respiration curves of *E. coli* on three concentrations of nDyO. Each curve produced from the Biolog OmniLog (a) consisted of an average of 6 normalized wells. The Synergy™ MX microplate reader (b) produced curves consisting of an average of 12 normalized wells.

contained no nDyO. Table 2-4 shows the toxicity results of nDyO on *E. coli* between hours 1 and 2. The results show a very similar trend, which suggest that both methods are capable of measuring toxicity effects of nDyO on *E. coli* through respiration or

non-growth conditions. The values variability may be attributed to the difference in instrumental measurements and variation between microplates.

Table 2- 4 Percent Remaining Respiration (PRR) between the Biolog OmniLog and the Synergy™ Mx microplate readers between 1 and 2 hrs.

Biolog OmniLog			Synergy™ MX microplate reader		
Slopes (BCU_{dye}/(hrs•BCU_b))		PRR (%)	Slopes (Abs_{dye}/(hrs•Abs_b))		PRR (%)
Control	1.72		control	0.54	
nDyO [0.1 mg/L]	1.57	90.9	nDyO [0.1 mg/L]	0.52	95.1
nDyO [0.5 mg/L]	1.04	60.4	nDyO [0.5mg/L]	0.30	55.7
nDyO [1.0 mg/L]	0.55	32.1	nDyO [1.0 mg/L]	0.12	22.5

With further investigation into the capabilities of the Biolog OmniLog and the Synergy™ MX microplate reader, the Synergy™ MX microplate reader proved to be more advantageous to future nanoparticles toxicity studies. Observed disadvantages of the Biolog OmniLog were the main mechanism of measuring the tetrazolium dye, lack of shaking capability and the limited ability to customize a protocol for measuring changes in the reduced dye. Unlike the Synergy™ MX microplate reader which uses absorbance to measure changes in the tetrazolium dye, the Biolog OmniLog uses the change of pixel density recorded by a camera (Bochner et al. 2001) making understanding the unit of measurements difficult and incomparable. Furthermore, the Synergy™ MX microplate reader can perform other methods of measurements such as fluorescent for fluorescent assays. The Synergy™ MX microplate reader also has a highly customizable protocol that can even allow the user to inject reagents into each microplate well. In addition, the microplate reader has the capability to shake horizontally from size to size or back to front with different shaker speed settings. The main disadvantage of the Synergy™ MX microplate reader is that the user can only read one microplate at a time, as opposed to the Biolog OmniLog that can read up to

20 microplates at one time.

2.6 Conclusion

The respirometric microarray methodology developed in this study was able to be used for initial toxicity studies of nDyO on *E. coli* using both the Biolog OmniLog and the Synergy™ MX microplate reader. After comparison toxicity tests with nDyO on *E. coli* between the Biolog OmniLog and the Synergy™ MX microplate reader and evaluating each microplate reader's capabilities, it was decided that the Synergy™ MX microplate reader would be used for future nanoparticle toxicity tests. nDyO toxicity results obtained using this respirometric microplate methodology will be further discussed in chapter 3 in relation to the author's contributions in the manuscript, "Effect of Dysprosium oxide nanoparticles on *Escherichia coli*."

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MANUSCRIPT 2

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Effect of Dysprosium oxide nanoparticles on *Escherichia coli*

Nelson Anaya, Farrah Solomon and Vinka Oyanedel-Craver

Corresponding Author

Prof. Vinka Oyanedel-Craver

Department of Civil and Environmental Engineering

University of Rhode Island

Kingston, RI 02881

craver@egr.uri.edu

CHAPTER 3

Effect of Dysprosium oxide nanoparticles on *Escherichia coli*

3.1 Abstract

Rare Earth Element oxide nanoparticles such as dysprosium oxide nanoparticles (nDyO) are increasingly being applied to the biomedical field due to their paramagnetic properties. Appropriate recycling systems for nDyO are thereof deficient and will result in nDyO entering into the environment in high concentrations through various pathways. The objective of this study is to assess the toxicity effects of nDyO on *E. coli*. Standardization of two toxicity array-based methodologies was used to evaluate the physiological and metabolic response of *E. coli* at different environmental conditions: dual staining with fluorescent dyes (Live/Dead BacLight viability kit) and respirometric assays. Toxicology results showed that nDyO is most toxic at high concentrations of 2.0 mg/L after 2 hrs in 85 mg/L NaCl. Dy ions were measured and found to exhibit a sudden increase in concentration from 0 hrs to 0.25 hrs before achieving a plateau in concentration by 2 hrs leading to the possibility of another contributor of nDyO toxicity effect. Respirometric test showed a higher toxic response than membrane permeation assays indicating that metabolic processes are more affected than physical cell structures when exposed to nDyO. The array-based methodologies used in this study could be used to assess the toxicity of different nanoparticles on various microorganisms enabling cost efficient analysis of multiple conditions in a short amount of time.

3.2 Introduction

Nanotechnology and its widespread applications are becoming more prevalent in

the global market. Rare Earth Elements (REEs) are used to manufacture several nanoparticles and nano-based products (EPA 2012). Market applications are found in the glass industry, phosphor, metallurgy and catalysts (Goonan 2011). In addition REE nanoparticles (nREEs) are used in applications related but not limited to: magnets, batteries, automobiles, monitors, contrasting agents for Magnetic Resonance Imaging (MRI), biosensors, drugs delivery, and cancer diagnostic and therapy (Kubik et al. 2005; Kattel et al. 2012; Hofmann-Amtenbrinka et al. 2010; EPA 2012). More specifically, one REE, nDyO, has gained increased application in the biomedical field. nDyO has specific paramagnetic properties that enhance the contrast in MRI images and are small enough to be passed through renal excretion (Kubik et al. 2005; Kattel 2012). Other potential applications of nDyO in the biomedical field consist of the ability to screen new drugs, for cancer research and therapy applications, and as a delivery vehicle for drug application (Goonan 2011; Hofmann-Amtenbrinka et al. 2010).

Due to the lack of appropriate recycling systems, the fate of nanoparticles, especially those containing REEs, and their effects on biological systems within the environment is a growing concern (Valavanidis et al. 2012). It is likely that nDyO will enter into aquatic and land environments through wastewater treatment facility effluent and wastewater sludge due to the inability to recover these nanoparticles completely (Brar et al. 2010). Direct release into aquatic environments could occur through treated wastewater effluent. Mean-while release onto land environments from agricultural application could exposure nanoparticles to surface waters by storm water runoff and to subsurface waters via infiltration through the soil (Batley et al. 2013;

Brar et al. 2010).

In this study we propose the use of microarray techniques to evaluate the effects of nanoparticles on the environment. Successful application of microarray techniques will provide a fast and effective way to perform nanotoxicological studies.

3.3 Materials and methods

A nonpathogenic wild strain of *E. coli* purchased from IDEXX laboratory was selected for this study because it has been extensively studied. *E. coli* is a gram negative bacterium that has been found to be metabolically active in saline solution without growth (Doudoroff 1940) and is a candid microorganism for nanotoxicological studies (Zhang and Oyanedel-Craver 2012; Zhang and Oyandel-Craver 2013). Growth media for this bacteria consisted of 10 g/L NaCl, 5 g/L yeast extract, 10 g/L Tryptone (which can all be purchased from Sigma Aldrich). A centrifuge (ThemoScientific Sorvall Legend x1R) was used for the collection of *E. coli* at 2000 rpm for 15 min.

Different water chemistry conditions were used with NaCl concentrations of 85 mg/L, 850 mg/L and 8500 mg/L (ionic strength 1.45 mM, 14.5 mM and 145 mM respectively). The lowest concentration was based on ionic strength found in surface waters (Hoecke et al. 2011). The highest concentration was based on the live/dead test manufacturer's recommendation of 8500 mg/L NaCl and a middle value was chosen for a three tier comparison.

Tetrazolium dye (Redox Dye Mix A; information about Redox Dye Mix A is available from Biolog, Hayward, CA) purchased from Biolog was used to measure the respiratory responses of *E. coli*. The tetrazolium dye is reduced by active cellular

respiration and produces a purple color (Bochner et al. 2001; Tremaroli et al. 2009) measurable by a microplate reader at wavelength of 590 nm over time (Klimek et al. 2007).

A range of glucose concentrations, 35 mg/L to 140 mg/L, was used to evaluate nDyO toxicity under different aerobic metabolic levels. The glucose concentration of 35 mg/L was the lowest limit of respiratory detection for the microplate reader with the tetrazolium dye under these non-growth conditions.

Three concentrations of non-coated Dy_2O_3 nanoparticles, 0.02 mg/L, 0.2 mg/L and 2.0 mg/L, were used to simulate environmental concentrations and at the same time cover higher concentrations to quantify nanoparticle exposure in worst case scenario. Non-coated Dy_2O_3 nanoparticles were prepared based on the method used by Kattel et al. (2011).

3.3.1 Growth of *E. coli*

A nonpathogenic wild strain of *E. coli* was purchased from IDEXX laboratory and grown for 12 hrs in a culture media consisting of 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone and incubated at 37^{o}C. Growth media was autoclaved before the addition of *E. coli* cells. *E. coli* was harvested during the logarithmic growth phase and centrifuged at 2000 rpm for 15 min. The supernatant was discarded and the pellet re-suspended in NaCl solution (concentrations varied between 85 mg/L and 850 mg/L as needed for the experimental setup).

3.3.2 Microplate preparation

The following was directly inoculated into a blank 96 half area well microplate in the specified order: NaCl (amount varied due to different controls within the

microplate), 10 µl of glucose, 10 µl of nDyO, 10 µl of tetrazolium dye, and 70 ul of cell solution (previously refrigerated). The final volume within each well was 100 ul and mixed thoroughly by pipetting at least 10 times for each well. Glucose, nDyO, and cell solutions were all prepared in the respective saline solution. The tetrazolium dye is added directly into the microplate and is reduced by active cellular respiration which produces a purple color (Bochner et al. 2001; Tremaroli et al. 2009) that can be measured using absorbance (Abs) by the microplate reader at 590 nm. The duration of each experiment lasted for 4 hrs with readings taken every 15 min in between horizontal shaking (medium setting) performed by the microplate reader. The microplates were incubated at 25^{o}C.

A detailed description of the microplate set up is presented in Figure 3-1. The various sections labeled within the microplate, represent the quality control/ quality assurance (QC/QA) measures implemented in each microplate experiment constructed. Sections of the microplate such as, nDyO blank, bacteria blank and background correction, represent sections to detect false positives since no coloration should develop in these wells during the testing period. No coloration in the nDyO blank section indicates no reduction interactions between nDyO and the tetrazolium

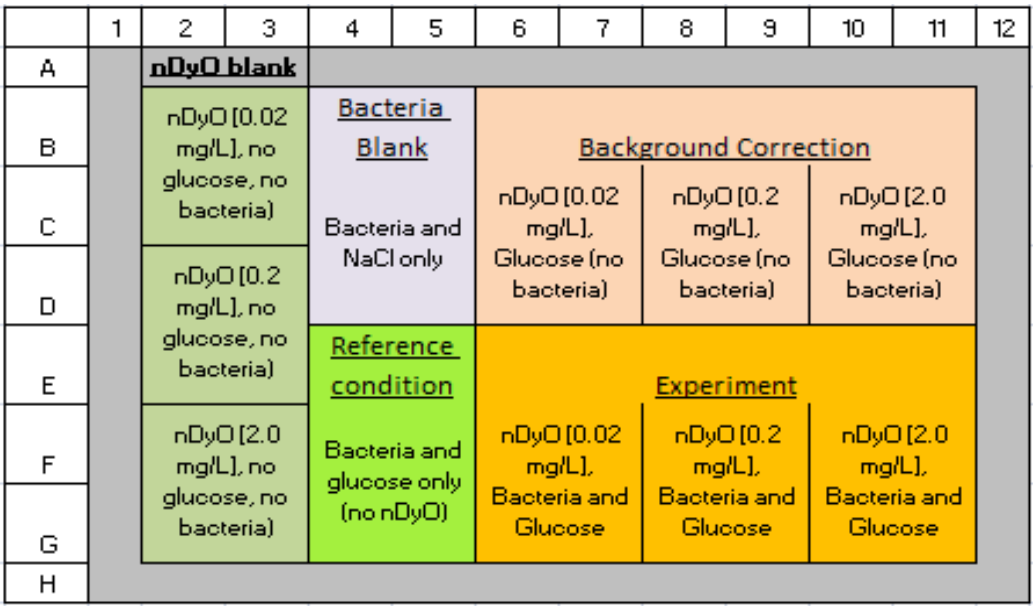


Figure 3-1 Respirometric plate map used to conduct the nDyO toxicity experiments. Each plate tested one water chemistry condition and one concentration of glucose while concentrations of nDyO were varied. All wells contained tetrazolium dye.

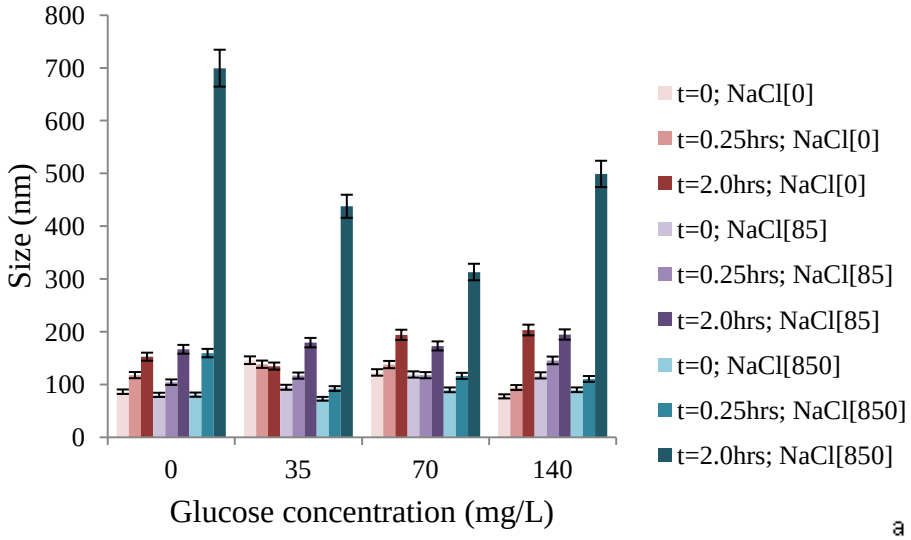
dye that would produce color. The Bacteria Blank section should remain colorless because there is no glucose present therefore not possible electron transfer. To account for background absorbance of nDyO, the Background Correction section was subtracted from the Experiment section and also served as a secondary control to further confirm that experimental conditions had no reducing effect on the tetrazolium dye without the presences of bacteria. Lastly, the Reference condition contained no nDyO and was the experimental control used to compare all experimental values to.

3.4 Results

Physiochemical characterization of nDyO consisted of size and zeta potential measurements in two water chemistry conditions. Due to a problem with 8500 mg/L NaCl which will be explained shortly, experiments associated with that condition were not continued. Ion release experiments were also conducted to determine the amount

of dissolution over time for nDyO.

Figure 3-2 shows the results for size measurements (a) and zeta potential measurements (b) taken at initial conditions, 0.25 hrs and 2.0 hrs for the highest concentration of 2.0 mg/L nDyO. Control measurements were taken in a background of DI water only with no glucose followed by experimental conditions. Experimental conditions for 0 hrs and 0.25 hrs size measurements show nDyO for all water chemistry conditions to be in a range of 75 nm to 150 nm. Size measurements for 2 hrs show an increase in larger nDyO particle size indicating aggregation. This is observed more significantly in water chemistry conditions of 850 mg/L NaCl in all concentrations of glucose. Zeta potential measurements were found to be relatively more stable in all NaCl water chemistry and glucose conditions than in the control conditions of DI water with no glucose present suggesting that the presence of glucose increased the stability of nDyO.



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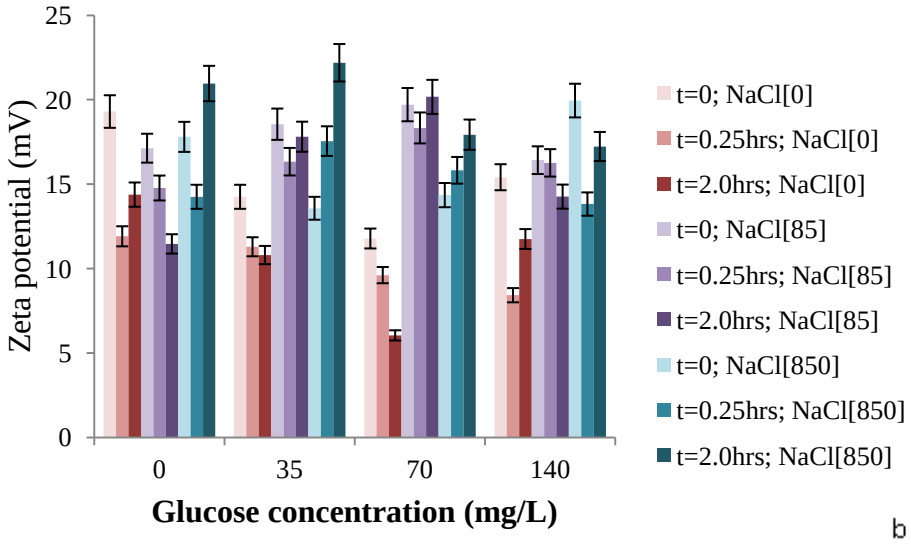


Figure 3- 2 represents physiochemical conditions (size (a) and zeta potential (b)) in DI water (red) and 0 mg/L glucose as the control, and experimental conditions of water chemistry conditions NaCl 85 mg/L (purple) and 850 mg/L (blue), glucose 35 mg/L, 70 mg/L, and 140 mg/L and for 2.0mg/L nDyO. Time measurements were taken at 0 hrs, 0.25 hrs and 2 hrs.

Ion release experiments were conducted by Nelson Anaya for the highest concentration of 2.0mg/L nDyO in all water chemistry and glucose conditions. Table 3-1 shows a sudden increase in Dy ions measured between 0 hrs and 0.25 hrs with a plateau effect occurring at 2.0 hrs.

Table 3-1 Ion release results for 2.0mg/L nDyO in both water chemistry conditions NaCl, 85 mg/L and 850 mg/L, and all glucose concentrations 35 mg/L, 70 mg/L and 140 mg/L. Measurements were taken at 0 hrs, 0.25 hrs and 2.0 hrs.

NaCl	Glucose	Dy ion [mg/L]		
[mg/L]	[mg/L]	t = 0 hrs	t = 0.25 hrs	t = 2 hrs
85	35	0.40	0.65	0.70
	70	0.42	0.68	0.72
	140	0.43	0.69	0.75
850	35	0.47	0.64	0.73
	70	0.48	0.68	0.75
	140	0.49	0.68	0.79

At 8500 mg/L NaCl interaction between the tetrazolium dye and NaCl were

detected. This is suggested by two reasons: the control slope (no nDyO present) also exhibits this decreasing behavior and that *E. coli* is known to survive in higher ionic strengths than present in this study, both short-term and long-term (Vaccaro et al. 1950; Rozen et al. 2001; Arense et al. 2010). The concentration of glucose is also not a factor considering it works well with the other conditions in lower ionic strengths. The inability to analyze this curve beyond 2 hrs lies in the fact the slopes are decreasing which would suggest degradation of the tetrazolium dye, however this is not the case since once the dye is reduced, it remains present within the well (Bochner et al. 2001).

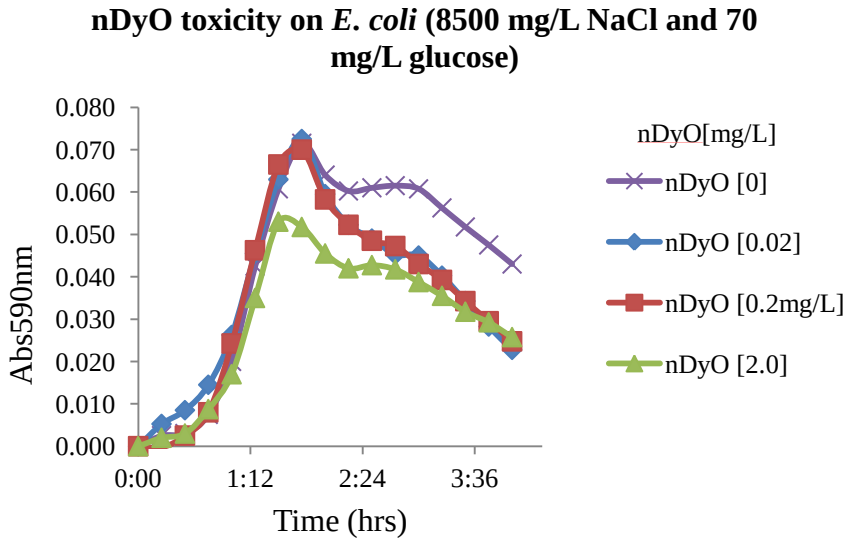
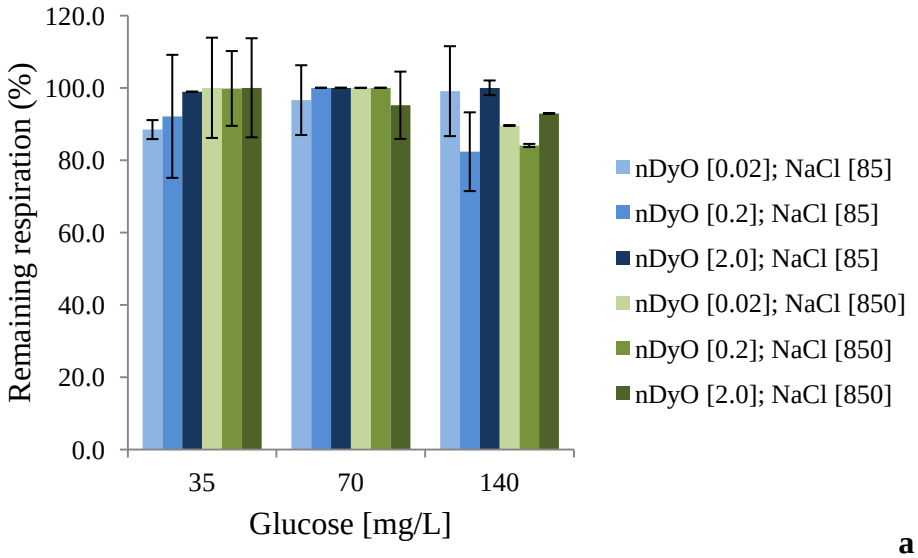


Figure 3- 3 Respirometric results for 8500 mg/L NaCl.

Duplicate plates for the remaining water chemistry conditions were conducted to quantify the Percent of Remaining Respiration (PRR). The PRR compares the slope values representing respiration of the remaining bacteria unaffected by nDyO exposure to the control slope values representing respiration of bacteria not exposed to nDyO through division and then multiples these values by 100 for percent values of

remaining respiration. Figure 3-4 shows the PRR for both water chemistry conditions NaCl, 85 mg/L and 850 mg/L, all three glucose concentrations 35 mg/L, 70 mg/L and 140 mg/L, and all three nDyO concentrations, 0.02 mg/L, 0.2 mg/L and 2.0 mg/L analyzed at 0.25 hrs and 2hrs. PRR time measurements were evaluated for 0.25 hrs and 2 hrs based on the physiochemical characterization of nDyO, before and after aggregation of nDyO. For 0.25 hrs, there was no significant toxicity effect observed for all conditions, however after 2.0 hrs, there was significant toxicity effect observed for certain water chemistry conditions. For the highest concentration of 2.0 mg/L nDyO a greater toxicity effect was observed across all glucose conditions and NaCl water chemistry conditions, with the greatest toxicity effect occurring at 85 mg/L NaCl and 140 mg/L glucose. Toxicity effect was also observed for water chemistry condition 85 mg/L NaCl and 35 mg/L glucose for all three concentration of nDyO.



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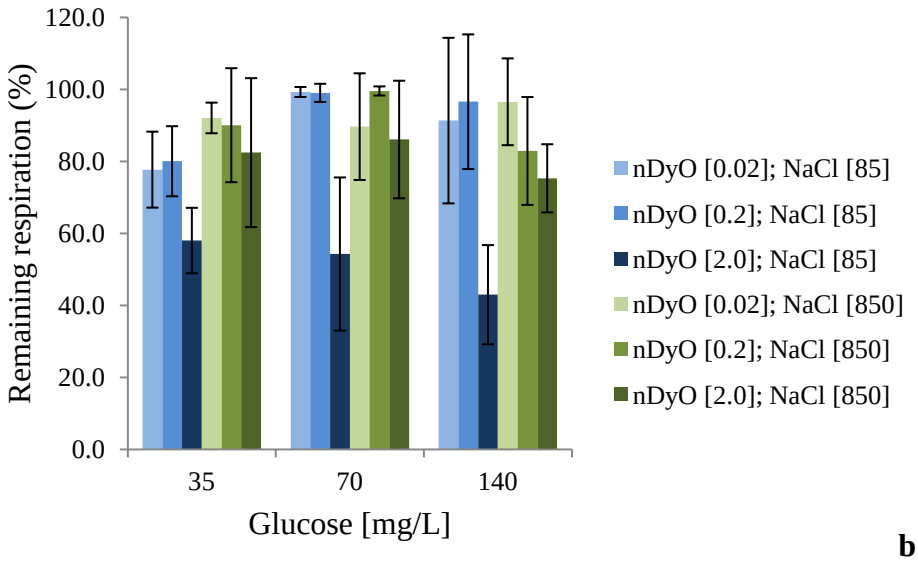

b

Figure 3- 4 represents the percent remaining respiration of *E. coli* under two water chemistry conditions NaCl, 85 mg/L (blue) and 850 mg/L (green) and three concentrations of glucose, 35 mg/L, 70 mg/L and 140 mg/L. Percent remaining respiration values were calculated for three concentrations of nDyO, 0.02 mg/L, 0.2 mg/L and 2.0 mg/L at (a) 0.25 hrs and (b) 2.0 hrs. Each value represents an average of 4 wells from two duplicate plates.

Live/Dead experiments resulted in minimal toxicity effect of nDyO on *E. coli* under the same conditions the respirometric microarray tests were conducted. This test indicates no significant membrane permeation of the cell, thus indicating minimal to no physical damage to *E. coli* cells by nDyO.

3.5 Discussion

Comparison of both methods of measurements, respirometric and Live/Dead tests, which measure the metabolic and physical integrity of *E. coli* respectively, showed significantly different results between the two methods. The respirometric microarray tests showed significantly higher toxicity effect of nDyO on *E. coli* than the Live/Dead test. Results indicate that toxicity effect is concentration dependent with the most significant toxicity occurring with the highest concentration of nDyO in the

lowest 85 mg/L NaCl water chemistry condition. Toxicity effect is also observed to be time dependant and indicates that it can also be attributed to Dy ions. Ion release experiments indicate a sudden increase in Dy ions from 0 hrs to 0.25 hrs before achieving a plateau in concentration by 2 hrs.

To understand the exact contributor of the toxicity effect of nDyO on *E. coli* is not presented in this portion of the thesis.

3.6 Conclusion

Our results show that the implementation of microarray technology to respirometric studies of microorganisms can be used to provide an effective and alternative tool to study nanoparticle toxicity on microorganisms. This study has assessed two methodologies, the Live/Dead test which is primarily used to evaluate toxicity of nanoparticles on microorganisms through membrane permeation and the respirometric microarray tests used to evaluate the metabolic activity of microorganism in response to nanoparticles. The respirometric microarray test proved to be more sensitive compared to the Live/Dead test in measuring nanoparticle toxicity which indicates a need to be selective among toxicity methodologies. For nDyO, toxicity effects are observed through changes in metabolic activity rather than physical integrity, but this could be different for other metal nanoparticles.

By understanding the fate of nanoparticles in the aqueous media, careful selection of appropriate toxicological methodologies will improve the accuracy of future nanotoxicological studies.

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MANUSCRIPT 3

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Anaerobic growth of *Acidithiobacillus ferrooxidans* using microarray technology

Farrah Solomon, Dawn Cardace and Vinka Oyanedel-Craver

Corresponding Author

Prof. Vinka Oyanedel-Craver

Department of Civil and Environmental Engineering

University of Rhode Island

Kingston, RI 02881

craver@egr.uri.edu

CHAPTER 4

Anaerobic growth of *Acidithiobacillus ferrooxidans* using microarray technology

4.1 Abstract

Application of microarray technology as an effective tool to evaluate the growth of microorganisms under anaerobic conditions has shown a good possibility of anaerobic growth of *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) over the course of 12 days with the highest bacteria density of Abs_{500nm} 0.5. Microplates containing *A. ferrooxidans* and nitrogen purged Basal Salt media supplemented with Fe^{3+} were grown under anaerobic conditions consisting of H_2:CO_2 [80:20] atmosphere under 25^{o}C . Proposed methodologies will provide a more robust tool that could be used for future studies in relation anaerobic nanotoxicology studies.

4.2 Introduction

Anaerobic metabolisms and habitats have an important role in biogeochemical cycles such as iron, sulfur and nitrogen cycles (Falkowski et al. 2008). Anaerobic microorganisms can release minerals and nutrients from an inorganic environment making them bioavailable for primary producers, which in combination with CO_2 can synthesize organic matter (Willey et al. 2008). Decomposers, such as fungi, are necessary because they drive the flow of inorganic and organic compounds to maintain a constant supply of materials for primary producers (Daufresne et al. 2001). The importance of studying anaerobic microorganisms are often overlooked due to the extreme environments in which they inhabit and the limitations of maintaining anaerobic environments *in vitr*o.

The objective of this study is to develop a microarray-based procedure to monitor the metabolic activity of *Acidithiobacillus ferrooxidans* in anaerobic conditions. *Acidithiobacillus ferrooxidans* (initially known as *Thiobacillus ferrooxidans* (Dumett et al. 2013)) has gained recent attention due to its metabolic activity that accelerates acid mine drainage, leading to increased heavy metal pollution at mine sites (Mahmoud et al. 2005; Natarajan et al. 2008). However, these bacteria are also useful in bio-mining operations and bioremediation because of their ability to dissolve and mobilize metals within an acidic environment (Gadd 2004; Natarajan et al. 2008).

Acidithiobacillus ferrooxidans has been shown to exhibit both aerobic and anaerobic metabolisms; these bacteria can survive in extreme environments such as the Rio Tinto mining site and its associated Tinto River. The Tinto River extreme environment consists of low pH (0.8 to 3) sulfate-enriched waters that contain high concentrations of ferric iron (up to 30 g/L) resulting from the metabolic activity of *A. ferrooxidans* and *Leptospirillum ferrooxidans* (*L. ferrooxidans*) (Fernandez-Remolar et al. 2004). *A. ferrooxidans* employs metabolic processes including the following: aerobic growth using H_2/O_2 or Fe^{2+} with Oxygen being the electron acceptor, and anaerobic growth using H_2/S_0, H_2/Fe^{3+} or S_0/Fe^{3+} with the electron donors being Hydrogen and Sulfur (Pronk et al. 1992; Ohmura et al. 2002; Hedrich et al. 2013). *A. ferrooxidans* can also fix carbon directly from carbon dioxide (Levican et al. 2008; Gale et al. 1967).

It is expected that the development of a microarray procedure for the anaerobic growth of *A. ferrooxidans* will provide a tool for environmental related studies.

4.3 Materials and Methods

4.3.1 Aerobic growth and medium

A. ferrooxidans was obtained from the American Type Culture Collection: ATCC 19859 and grown aerobically for easier maintenance before transitioning over to anaerobic conditions. In brief, the aerobic growth conditions consisted of Basal Salt Medium (BSM) acidified to a pH of 1.9 with H_2SO_4 and then autoclaved. Basal salt media was used to because it could be autoclaved and the iron components added separately. Fe^{2+} (4 g/L) in the form of $FeSO_4 \bullet 7H_2O$ was added after autoclaving. Growth of *A. ferrooxidans* occurred in 250 ml flasks containing 100 ml to 125 ml of BSM under 25^{o}C. Cells were harvested after 4 days and transferred to fresh BSM containing Fe^{2+} weekly.

Cultures of *A. ferrooxidan*s were harvested after 4 days growth and were filtered to remove visible orange flakes (attributed to iron formation from previous studies (Yates et al. 1987; Wang et al. 2010; Tapia et al. 2013) formed during aerobic growth. Iron and bacteria were centrifuged (ThemoScientific Sorvall Legend x1R) at 4,680 g for 20 min. The supernatant was discarded and the pellet was re-suspended with 0.1 M K- Phosphate Buffer (PBS) and washed two times. Large orange iron flakes were allowed to settle out of the bacteria before transferring it to a new centrifuge tube. Bacteria was allowed to stand for a few minutes while smaller pieces settled out at which point, suspended bacteria was collected from the upper ¾ volume of the centrifuge tube. Collected bacteria were then used for anaerobic growth experiments.

4.3.2 Transition from aerobic to anaerobic experimental growth

The transition to anaerobic growth conditions required the previously collected, aerobically grown *A. ferrooxidans* cells to be placed with an oxygen deficient medium. BSM (300 ml) lacking an iron component, was purged under a positive pressure N_2 environment with N_2 for 30 min to ensure all oxygen was removed (modified from (Bauermeister et al. 2014)). *A. ferrooxidans* cells were re-spun and re-suspended in the nitrogen purged BSM (BSM-N_2). Initial cells concentration were measured to different Abs_{500nm} (0.02, 0.2 and 0.5) and re-suspended in BSM-N_2 containing 1.5 g/L Fe^{3+} (Iron (III) sulfate hydrate). A control of BSM-N_2 containing Fe^{3+} without *A. ferrooxidans* was included.

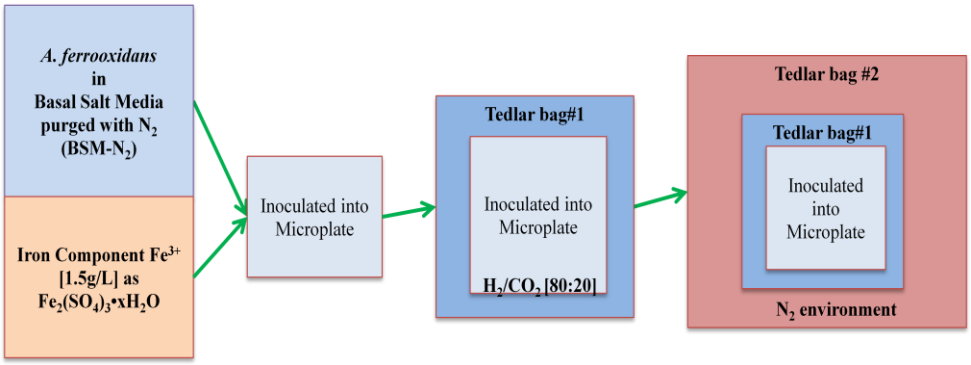

Figure 4- 1 Illustration of anaerobic setup under positive pressure N_2 conditions.

Under a positive pressure N_2 environment (Figure 4-2), 100 µl of cell solution was inoculated into an empty 96-half area, flat-bottomed, clear polystyrene microplate (Costar™ brand). Each microplate was placed within a pre-cut tedlar bag (Zefon International, 0.5L) and heat sealed (8'' Easyway Impulse Heat Sealer). Each tedlar bag containing an inoculated microplate was flushed three times with N_2 before replacing the atmospheric environment with H_2/CO_2 [80:20]. H_2/CO_2 was flushed

through the bag three times to remove all excess N_2. For protective measures, the H_2/CO_2 filled microplate tedlar bags were placed into individual larger tedlar bags (Zefon International, 3 L) and flushed with N_2 three times before filling completely with N_2.

Absorbance values within the microplate were measured and recorded as Day 0. Microplates were incubated at 25^{o}C and taken out for sampling after every 4 days for 12 days. Microplates were carefully removed from the tedlar bags under a fume hood and the Abs_{500nm} was recorded.

4.4 Results

The atmospheric condition chosen for this experiment represent the anaerobic atmosphere this microorganism is capable of growing under using ferric iron. Figure 4-2 shows the anaerobic growth of *A. ferrooxidans* on ferric iron (electron acceptor) and H_2/CO_2 (H_2 as the electron donor) over 12 days within a microplate. Since there was no elemental sulfur added to the media, this is the only redox reaction occurring under these conditions (Ohmura et al. 2002). Three initial concentrations of bacteria were measured outside of the microplate (Abs_{500nm}: 0.5, 0.2 and 0.02) before ferric sulfate hydrate was added to the BSM-N_2 media and inoculated into the plate. The control containing BSM-N_2 media and ferric sulfate hydrate was also included within the plate to account for background measurements of ferric sulfate hydrate. Discrepancy between the initial cell density measured and the absorbance values measured by the plate reader could be attributed to the addition of ferric sulfate hydrate after initial cell measurements were taken. This is indicated by the black line in Figure 4-2 to show all measurements taken within the microplate reader were for

microplate incubated under anaerobic conditions. Each time point was normalized by the control and then an average of 6 microplate wells were taken. Growth of bacteria at Abs_{500nm} concentrations of 0.5 and 0.2 showed after Day 4, with continued signs of increase to Day 12.

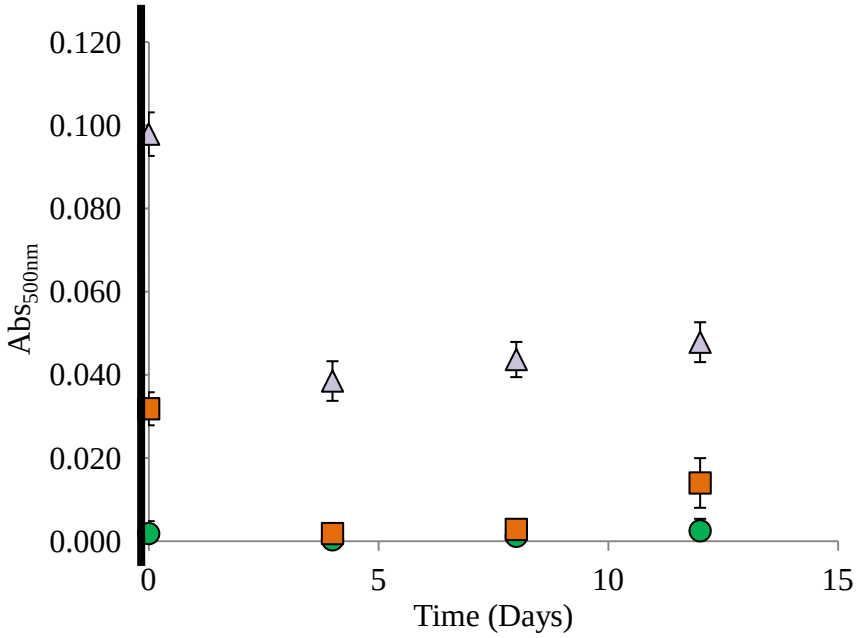

Figure 4- 2 Anaerobic growth of *A. ferrooxidans* on Day 0, 4, 8 and 12. Initial Absorbance values were measured outside of the microplate, under aerobic conditions without ferric iron added as represented by the black line. △ represent initial Abs_{500nm} at 0.5, ▢ at 0.2, and ○ at 0.02. All points were normalized by a control of BSM-N_2 containing ferric iron and consisted of an average of 6 microplate wells.

4.5 Discussion

Previous experiments using similar strains of *A. ferrooxidans* have proven its capability to grow under such anaerobic conditions (Bauermeister et al. 2014; Ohmura et al. 2002). Although not taken into explicit consideration in this study, anaerobic growth of *A. ferrooxidans* can be strongly influenced by the amount of elemental

sulfur present in the media as seen in previous studies however, elemental sulfur does not need to be present for anaerobic growth (Suzuki et al. 1990; Pronk et al. 1992; Ohmura et al. 2002).

The importance of establishing anaerobic growth of *A. ferrooxidans* within a microplate will allow for future studies on the impact of engineered nanoparticles on anaerobic microorganisms. With the growing application of engineered nanoparticles to industrial and consumer products, it is highly likely that these engineered nanoparticles will enter into the environment through various pathways and pose an ecological threat to the environment (Batley et al. 2013; Valavanidis et al. 2012). Before adequately moving forward, it is important to assess the efficiency of this microarray methodology and to adapt other analytical techniques to the microplate to overcome certain limitations of this study.

For more accurate growth assessment using a fluorescent, such as SYBR Green I, can be used to measure the increase of biomass over time. SYBR Green I fluorescent dye is a dye that can be used to quantify DNA due to its cell-permeating nature and its ability to intercalate with DNA (Dragan et al. 2012; Vitova et al. 2005). An example of a DNA microarray based methodology uses this same fluorescent dye to quantify DNA (Leggate et al. 2006). To assess respiratory iron reduction processes, a Ferrozine Assay (Stookey 1970) is proposed to measure increases in reduced iron over time. A modified Ferrozine Assay using hydrochloric acid (HCL) and ferrozine solution to microarray technology has been previously established in a recent study related to the study of potential metabolic processes of *Pelosinus* sp. Strain HCF1 one of which included using ferric iron as an electron acceptor (Beller et al. 2013).

4.6 Conclusions

Preliminary results show a good possibility of anaerobic growth of *A. ferrooxidans* on ferric iron under an experimentally controlled H_2/CO_2 atmosphere over a minimum of 12 days, as observed using microarray technology. The results also indicate that a cell density of Abs_{500nm} 0.5 would be needed to detect significant cellular growth.

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APPENDIX A

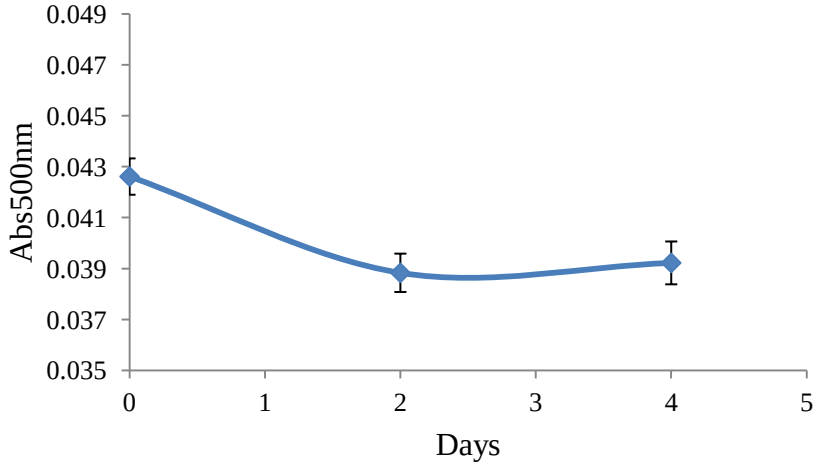

Figure A- 1 First preliminary growth results of *Acidithiobacillus ferrooxidans* over 4 days. *Acidithiobacillus ferrooxidans* was measured to an absorbance (500nm) of 0.216 outside the microplate before the addition of ferric sulfate. Microplates were incubated under H_2:CO_2 (80/20) anaerobic conditions at 25^{o}C.

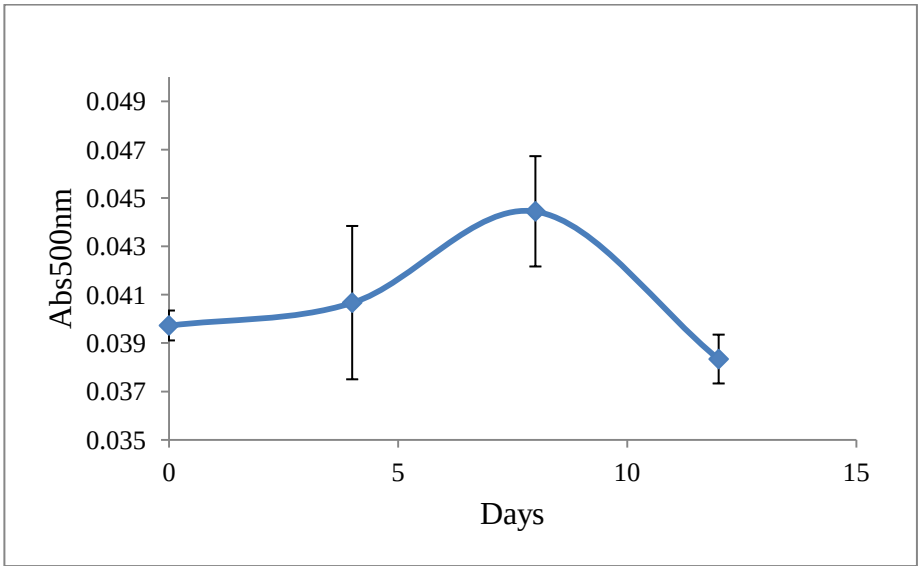

Figure A- 2 Second preliminary growth results of *Acidithiobacillus ferrooxidans* over 12 days. *Acidithiobacillus ferrooxidans* was measured to an absorbance (500nm) of 0.2 outside the microplate before the addition of ferric sulfate. Microplates were incubated under H_2:CO_2 (80/20) anaerobic conditions at 25^{o}C (Possible tedlar bag leakage of anaerobic gas on 12th day).