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The Effect of Ammonia on the Germination and Outgrowth of Bacillius globigii

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THESIS

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Abstract

This research investigated the germination and outgrowth of *Bacillus globigii* in the presence of ammonia in laboratory scale experiments. Germination was measured by monitoring the release of dipicolinic acid, an organic compound present in bacterial spores, while outgrowth was measured using phase-bright microscopy and semi-automated counting procedures. The ammonia-N concentrations investigated generally did not cause statistically significant differences in the initial 1-hr germination rates or the average 3-hr outgrowth rates of *Bacillus globigii* spores in batch style experiments. The average 1-hr germination rates observed in the absence of ammonia-N was 0.0258 hr^{-1} for the water and buffer controls and between 0.00732 and 0.0127 hr^{-1} with synthetic feed over a range of ammonia-N concentrations. The 3-hr outgrowth rates were 0.0761 and 0.1821 hr^{-1} for the buffer control and positive control respectively but it cannot be concluded that ammonia-N was responsible for this difference. Ammonia-N was correlated with subtle but statistically significant impacts over a 7-day period. The sodium bicarbonate present in the synthetic feed likely caused inhibition of the germination rates as well as the shape of the 7-day germination profiles. To this author's knowledge, this study is the first to investigate the effect of ammonia-N on the germination and outgrowth of *Bacillus globigii* in concentrations typically found in wastewater treatment plants and in the presence of synthetic feed.

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The author would like to thank the United States Environmental Protection Agency, National Homeland Security Research Center, Water Infrastructure Protection Division and the Defense Environmental Restoration Account for the financial support required for this research. Mrs. Kandace Hunter for preparing the feed required for the experiments and for helping answer many questions on the protocol and procedures of the lab. Dr. Dan Felker for his tireless effort in ensuring the lab equipment worked properly and his advice in the handling of bacteria. Dr. Yun Xing for her expertise in bacterial cultivation, experimental protocol, and answering endless questions on a daily basis. Dr. Xing also spent many hours harvesting the bacteria required for these experiments, as the author could not master the techniques. Dr. Willie Harper for giving me the freedom to make mistakes and choose the direction the research took to reach its endpoint. Dr. Harper also provided a guiding hand to ensure that the research stayed on task. Finally, my wife, for being supportive throughout this endeavor which meant spending a lot of time apart.

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THE EFFECT OF AMMONIA ON THE GERMINATION AND OUTGROWTH OF *BACILLUS GLOBIGII*

I. Introduction

1.1 Background

The introduction of *Bacillus anthracis* into wastewater treatment plants (WWTP) because of intentional contamination or from the washdown water of post-attack decontamination could pose a threat to public safety. This water may reach wastewater treatment plants through runoff or discharge into the wastewater collection system. It is necessary to understand the impact of sending spore-containing wastewater to a municipal WWTP. Recent work has shown evidence of spore germination and outgrowth during short-term exposure (e.g. 12-17 hrs) to activated sludge (Smith, 2016). Germinated spores lead to outgrowth and the increase in the number of viable and potentially dangerous microorganisms introduced into the water cycle.

Germination is the first step in the transformation of endospores into vegetative cells, and it occurs in response to a variety of chemicals, including amino acids, nucleosides, and sugars (Setlow, 2003). In 1984, Preston and Douthit demonstrated that ammonia can increase germination rates for *Bacillus cereus* when L-alanine and inosine were also present. The free ammonia nitrogen (NH_4-N) concentration of wastewater influent can fluctuate between 12 and 50 mg/L (Metcalf & Eddy, 2002).

1.2 Problem Statement

The Department of Defense (DoD) as well as WWTP operators are greatly concerned with scenarios that include the intentional or inadvertent introduction of

biocontaminants into the wastewater collection and treatment system. Decontamination operations require large amounts of water which, in turn, creates large amounts of contaminated waste which will need to be treated (DoD, 2013). Research is required to determine if the NH_4-N concentrations typically found in WWTPs would increase the percentage of *Bacillus anthracis* spores that germinate.

1.3 Research Objective

The purpose of this research is to determine the effect of NH_4-N concentrations on the germination and outgrowth of *Bacillus globigii*.

1.4 Hypothesis

The hypothesis is that the effect of ammonia-N on the outgrowth should be more significant than on the germination of *Bacillus globigii*. The rationale for this is as follows. Germination takes place over a very short period of time, relatively speaking, through a series of irreversible reactions; in contrast, outgrowth takes place over a longer period of time and requires active metabolism. The presence of ammonia-N should serve as a needed source of nitrogen and support the activity of enzymes required for outgrowth.

1.5 Scope and Approach

This research used *Bacillus globigii* as a surrogate for *Bacillus anthracis*. *B. globigii* is a soil-dwelling, non-pathogenic, spore-forming bacteria (Gibbons et al., 2011). When environmental conditions become unfavorable, *B. globigii* cells will form an endospore and remain dormant making it resistant to extreme conditions to include lack of nutrients. Once favorable conditions return, the spores will become active and

outgrowth will occur. Previous research has indicated that *B. globigii* can remain in WWTPs for up to one year (Horan et al., 1991); however, there has been no research on the effects of varying ammonia concentrations on the ability of *B. globigii* cells to germinate and subsequently conduct outgrowth.

Germination and outgrowth experiments were conducted using a combination of sterile de-ionized water, buffer solution (PBS, pH 7.4), L-Alanine (10 mM), heat activation, and three feed solutions. Feed A consisted of de-ionized water (DI) and 44.5 g/L sodium bicarbonate; feed B_1 consisted of DI water, 12.0 g/L of casamino acids, and 2.5 g/L sodium acetate; and feed B_2 consisted of DI water, 4.52 g/L aluminum chloride, 13.72 g/L magnesium chloride, 3.44 g/L calcium chloride, 1.34 g/L potassium dihydrogen phosphate, and 40 ml/L of trace element solution. The ratio of spores which underwent germination was measured by analyzing the absorbance of dipicolinic acid (DPA) at 270 nm. Absorbance was measured using a Biomate^3 Thermo Spectronic Spectrophotometer by ThermoFisher Scientific. This absorbance was then compared to the total DPA absorbance found in a sample of experimental solution which was autoclaved to kill and lyse the spores resulting in the complete release of their stores of DPA. Outgrowth was measured using a Zeiss Axioskop microscope as well as ImageJ software.

II. Literature Review

2.1 Chapter Overview

This chapter will cover three main topics. First, it will review the role of ammonia in wastewater. Second, it will review the fate of biocontaminants in wastewater. Third, it will review the process and factors involving spore germination and outgrowth.

2.2 The Activated Sludge Process

The activated sludge process is the most used suspended growth process in municipal wastewater treatment (Metcalf and Eddy, 2014). The primary objective of the activated sludge process is to remove organic pollution from wastewater. This is accomplished by a diverse group of microorganisms consisting of bacteria, fungi, algae, protozoa, helminths, viruses, and other microscopic plants and animals which use the organic pollution as food (Metcalf and Eddy, 2014). This method was developed circa 1913 and can be modified to remove nitrogen, phosphorous, metals, pharmaceuticals, hormones, as well as other man-made organic pollutants (Metcalf and Eddy, 2014). Figure 1 shows a simple block-flow diagram of the activated sludge process. This can be

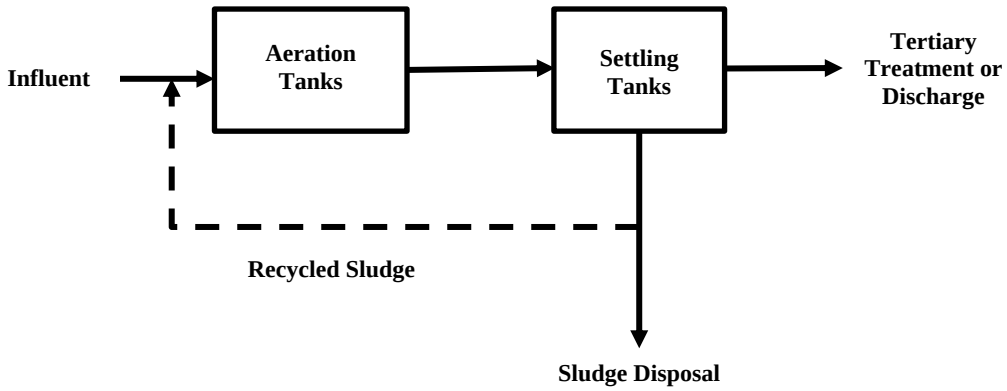

Figure 1: Activated sludge process

augmented with various filtration and disinfection processes to produce water for potable or non-potable reuse (Metcalf and Eddy, 2014).

2.3 Ammonia-N in Wastewater

The concentrations of NH_3-N in municipal WWTPs range from 12 and 50 mg/L (Metcalf & Eddy, 2002). As of 2014, the Ohio EPA limit for ammonia discharged from a wastewater treatment plant based on best available technologies is 1.0 mg/L (30 day) in the summer and 3.0 mg/L (30 day) in the winter (EPA, 2014). There are many processes within the wastewater treatment plant that will reduce the amount of ammonia-N as the wastewater is treated.

2.3.1 *Nitrogen in Influent Wastewater*

The predominant sources of nitrogen in domestic wastewater comes from the bacterial decomposition of proteinaceous matter and the hydrolysis of urea. This results in approximately 60 percent of nitrogen in the organic form and 40 percent in the form of ammonium (Sedlak, 1991). Nitrogen can be found in many forms in wastewater with the most common forms being: ammonia gas (NH_3), ammonium ion (NH_4^+), nitrogen gas (N_2), nitrite ion (NO_2^-), and nitrate ion (NO_3^-) (Metcalf and Eddy, 2014).

2.3.2 *Ammonia-N Transformations*

2.3.2.1 Nitrification

The primary process for removing ammonia from wastewater is through nitrification. Nitrification is a secondary treatment process which occurs in two steps: conversion of ammonia to nitrite by one type of bacteria (i.e., *Nitrosomonas)*, and then

conversion of nitrite to nitrate by another bacterium (i.e., *Nitrobacter*) (Metcalf and Eddy, 2014). Nitrification can occur in single or separate-stage processes as well as by using suspended-growth or attached-growth processes (Metcalf and Eddy, 1991). Separate-stage nitrification uses two reactors to conduct carbon oxidation and nitrification (Metcalf and Eddy, 1991). One advantage to using separate-stage is the reduction of toxic chemicals which may be hazardous to the bacteria involved in nitrification (Metcalf and Eddy, 1991).

2.3.2.2 Biological Assimilation

Microbial growth requires nitrogen so any increase in biomass within an activated sludge system will cause nitrogen removal from the influent. Nitrogen makes up approximately 12.5 percent of microbial dry weight and its removal from wastewater can be defined as:

$$\frac{dNH_3 - N/dt}{dBOD/dt} = (0.125)\frac{dX_v/dt}{dBOD/dt}$$

dNH_3-N/dt = rate of nitrogen removed by assimilation, lb/day

dX_v/dt = rate of active biomass or biological sludge production, lb/day

(Sedlak, 1991).

2.3.2.3 Ammonia-N Volatilization

Ammonia is found in two forms in wastewater: $NH_4^+{}_{aq}$ and $NH_{3\ gas}$. This equilibrium is represented by:

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$$

The ratio of ammonium ion to ammonia gas is dependent on the pH of the wastewater which normally occurs between 6 and 9 (Metcalf and Eddy, 2014). The pK_a of ammonium is 9.4 which means at this range of pH, the amount of ammonium ion will be slightly higher than the amount of ammonia gas. By raising the pH of the wastewater to between 10.5 to 11.5 and providing air-water contact within the solution, the ammonia gas can be stripped from the wastewater (Sedlak, 1991). This can be accomplished in a variety of ways but is not necessarily required. Unaerated ponds kept at a pH of 10.5 have been shown to reduce the ammonia content by 50 percent after 130 hours (Sedlak, 1991).

2.4 Biocontaminants

2.4.1 *Pathogens*

Pathogenic organisms in wastewater include: bacteria, viruses, protozoa, and helminths (Metcalf and Eddy, 2014). The United States Geological Survey (USGS) in partnership with the Ohio Water Development Authority and the City of Delphos, Ohio sampled wastewater in various stages of treatment within five wastewater treatment plants between 2008 and 2010. Three of the treatment plants used a membrane bioreactor (MBR) and two used conventional wastewater treatments. Table 1 contains the median values of five pathogens found in the post-preliminary phase prior to secondary treatment (aeration tank and clarifier for conventional treatment and anoxic tank and aeration tank and membrane for MBR) (USGS, 2011).

Table 1: Median values of pathogens found in five wastewater treatment plants in CFU/100 mL

Plant Number	E. Coli	Enterococci	Fecal Coliforms	Somatic Coliphage	F-specific Coliphage
1 (MBR)	9.5E5	3.4E6	3.4E6	1.8E5	6.25E5
2 (MBR)	2.8E6	2.4E6	5.5E6	2.9E5	1.5E5
3 (Conv.)	4.3E6	1.2E6	3.3E6	3.7E5	7.9E4
4 (MBR)	4.2E6	2.1E6	6.7E6	5.7E5	2.7E5
5 (Conv.)	3.5E6	6.2E5	5.2E6	2.1E5	4.5E5

The concentrations of pathogens found in wastewater can vary depending on the source of that wastewater. Hospital wastewater would be expected to contain a high number of pathogens which will require treatment. One study conducted in Taiwan examined the wastewater from nine hospitals and found the following average concentrations of pathogenic bacteria: *Escherichia coli* (*E. coli*), 10^6 CFU/mL; fecal coliform, 10^5 CFU/mL, fecal streptococci, 10^4 CFU/mL, *Pseudomonas aeruginosa*. 1-100 CFU/mL; and *Salmonella* 1-1000 CFU/mL (Tsai et al., 1998). Most of these organisms are not removed efficiently during primary treatment (settling and prolonged detention) (Payment, Plante, and Cejka, 2001).

2.4.2 *Fate of Pathogens in Activated Sludge Treatment Systems*

Coliform bacteria are referred to as indicator organisms and contains bacteria of the *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebisella* genus (Metcalf and Eddy, 2014). *E. coli* is the most well-known of the *Escherichia* and is entirely of fecal origin (Metcalf and Eddy, 2014). The removal efficiency of these pathogens can vary widely from one wastewater treatment plant to another.

Research conducted on a laboratory scale biological wastewater treatment system by Wen et al. found an average log_{10} removal for *E. coli* of 2.06 (± 0.26); *C. perfringens* of 1.27 (±0.20); 2.37 (±0.41) of total coliforms; and 2.53 (± 0.27) of enterococci (Wen et al., 2009). One study at the Montreal Urban Community wastewater treatment facility reported a removal rate of: 0% for human enteric viruses, 27% for *Cryptosporidium* oocysts, 25% for fecal coliforms, and 12% for *E. coli* (Payment, Plante, and Cejka, 2001). Marin, Goni, Lasheras, and Ormad conducted a study of a WWTP in 2014 using a trickling filter (secondary treatment) and found a 2.34 log removal of *E. coli* in the final effluent (99.5%). Research conducted by Smith in 2017 and Horan et al. in 1991 found that 81 percent and 92 percent (respectively) of *B. globigii* spores had adsorbed to the activated sludge floc. Wiencek et al. demonstrated that *Bacillus* and *Clostridium* spores were much more hydrophobic than their corresponding vegetative cells which may explain this adherence of spores to the sludge floc (Wiencek et al., 1990). This hydrophobicity is further demonstrated by a study conducted in 1998 by Tsai et al. on the hospital wastewater in Taiwan mentioned in the previous section. Tsai et al. reported that the activated sludge contained total coliform concentrations of 5.1E7, 1.4E8, and 3.1E8 CFU/g after 7, 14, and 40 days of thickened then dewatered sludge respectively.

Even if the WWTP is effective at removing pathogens this will not remove the threat of spreading them entirely. Several studies (Ploszaj, Talik, Piotrowska-Seget, and Pastuszka, 2012; Li, Zhou, Zhang, Xu, Dong, and Yao, 2016) have found aerosolized bacteria in and around wastewater treatment plants including human pathogens which pose a threat of infection to operators. This combined with survival times in excess of one

year for *B. globigii* spores (Horan et al., 1991) can result in a continuous hazard even if the WWTP is capable of removing all pathogens from the effluent.

2.5 Germination and Outgrowth of Spores

The life cycle of *Bacillus globigii*, like most bacteria of its genus, consists of three different physiological processes: germination, vegetative growth, and sporulation (Sella, Vandenberghe, and Soccol, 2014). Figure 2 depicts the typical life-cycle of a spore forming bacteria.

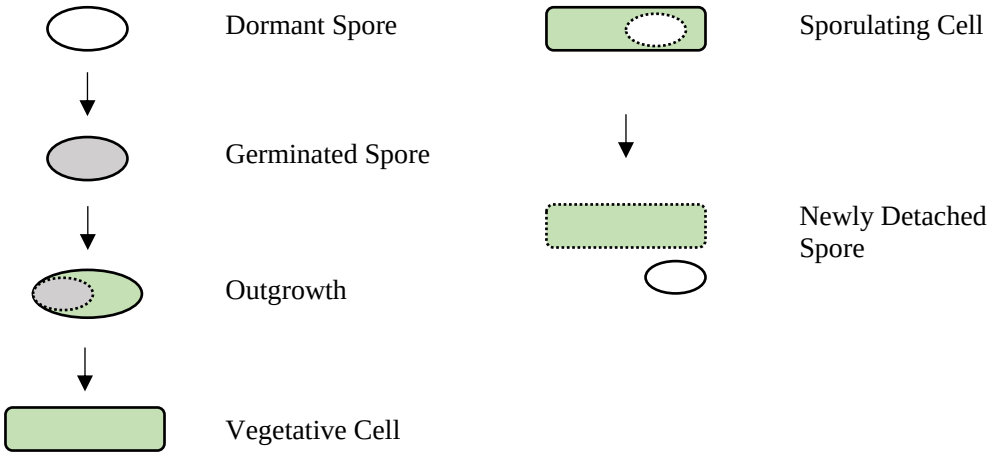

Figure 2: Typical life-cycle of spore forming bacteria

2.5.1 *Germination*

Prior to germination, bacteria exist as spores. Bacterial spores are resistant to heat, extreme pH changes, radiation, desiccation and toxic chemicals (Setlow, 2003). Estimations of the time-frame in which bacterial spores remain viable vary greatly. Studies have shown that spores have been isolated from lake sediments deposited 2750 years ago (Gould, 2006). On the extreme end of this time-frame, a previously unrecognized spore-forming bacterium of the *Bacillus* species was isolated and grown from a 250 million-year-old salt crystal (Vreeland, Rosenzweig, and Powers, 2000).

Whether it is in terms of thousands or millions of years, this extreme longevity is due to "near non-detectable levels of endogenous metabolism" (Gould, 2006). Spores stay dormant until conditions are conducive to cell growth and in response to nutrients which are termed germinants (Setlow, 2003). Figure 3 displays the typical structure of a *Bacillus* spore; however, it should be noted that the exosporium is not present in all species.

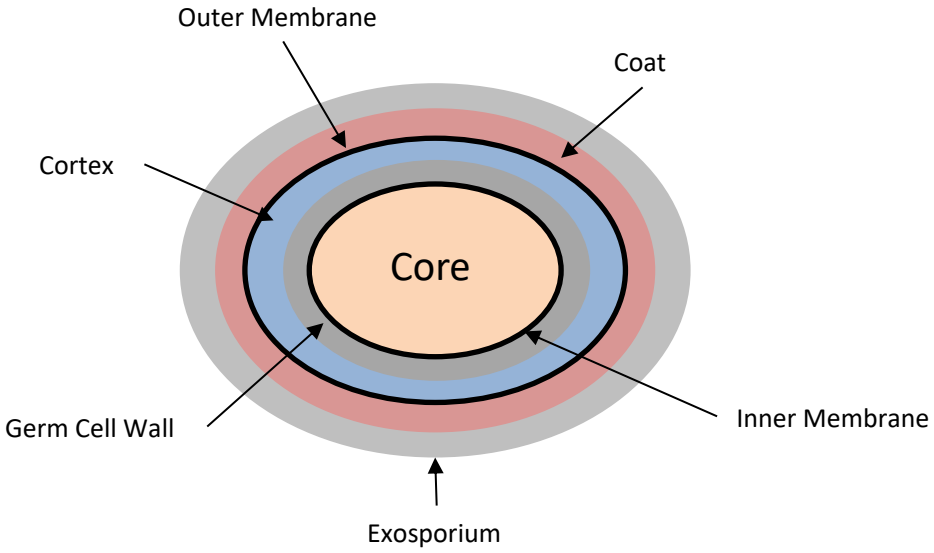

Figure 3: Typical structure of a *Bacillus* spore.

Germination itself consists of three stages: activation, rehydration, and spore coat hydrolysis (Sella et al., 2014). Activation of spores can occur in response to a variety of germinants such as amino acids, sugars, nucleosides, a combination of nutrients, as well as non-nutrients (Setlow, 2003). Germination can also be affected by other factors such as temperature and inoculum size. These two factors will not initiate germination but will increase the percent of spores which germinate. Zhao, Montville, and Schaffner have shown that both inoculum size and temperature have a significant effect on the percent

growth-positive and time-to-detection of samples of *Clostridium botulinum* in separate studies conducted in 2000 and 2001. In 2002, Caipo, Duffy, Zhao, and Schaffner demonstrated that *Bacillus megaterium* displayed similar properties and as the global inoculum size increased, the extent of germination increased, and the time required for germination decreased. Once germination has been initiated, a period prior to vegetative growth occurs which is termed outgrowth. This stage is characterized by the "macromolecular synthesis that converts the germinated spore into a growing cell" (Setlow, 2003).

2.5.2 *Outgrowth*

Spore outgrowth may be initiated prior to the completion of germination events. DNA repair as well as protein synthesis begin during outgrowth; however, at this time the spore does not contain all the proteins required for DNA replication (Paidhungat and Setlow, 2002). Outgrowing spores utilize a different energy source than growing cells. Most of the energy required during outgrowth, such as 3-phosphoglyceric acid and amino acids from the breakdown of spore protein, are endogenous to the spore (Paidhungat and Setlow, 2002). Dormant spores also contain "low-energy" forms (e.g., NAD, ADP) of "high energy" (e.g., NADH, ATP) molecules used in growing cells (Paidhungat and Setlow, 2002). A study conducted in 1976 by Feeherry and Levinson found that both nitrate and ammonium ions can provide a nitrogen source to outgrowing cells of *Bacillus megaterium*.

2.5.2 *Vegetative Growth*

Vegetative growth describes the part of the bacteria life-cycle which occurs between outgrowth and sporulation. Sella, Vandenberghe, and Soccol describe vegetative growth as being "characterized by binary symmetric fission cell growth that occurs when nutrients are available" (Sella et al., 2014). As nutrients remain available for the bacteria to use, the cells will continue to divide creating new daughter cells. These cells "may remain linked together through multiple rounds of binary fission, forming long chains" (Sella et al., 2014). As nutrients are depleted, or other signals such as temperature, high cell density, or pH changes take place, sporulation will occur (Sella et al., 2014). Figure 4 contains examples of both spores and vegetative cells of *Bacillus globigii*.

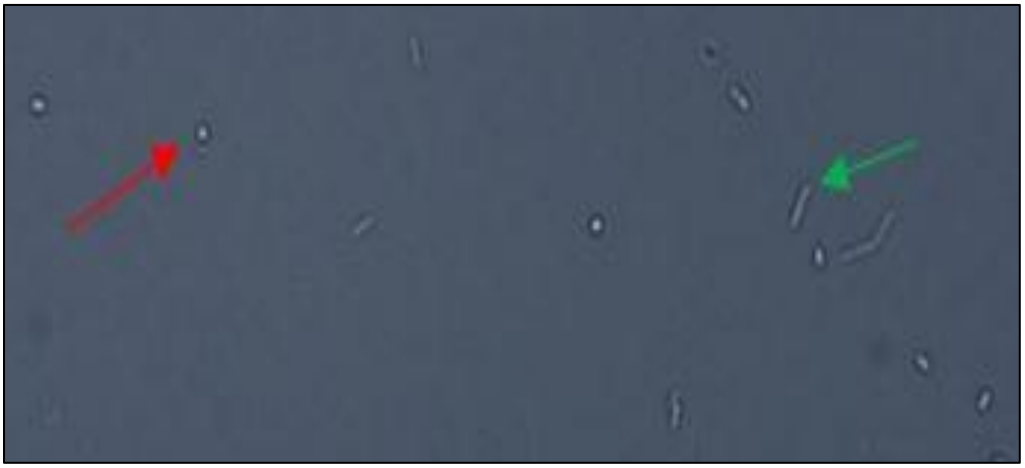

Figure 4: Example of *B. globigii* spores and vegetative cells. The red arrow indicates an ungerminated spore. The green arrow indicates a vegetative cell.

2.5.3 *Sporulation*

During sporulation a forespore is created which the larger mother cell engulfs giving a cell within a cell (Setlow, 2013). This occurs in seven stages and ends with the detachment of the spore from the mother cell (Setlow, 2013; Sella et al., 2014) (see Figure 5).

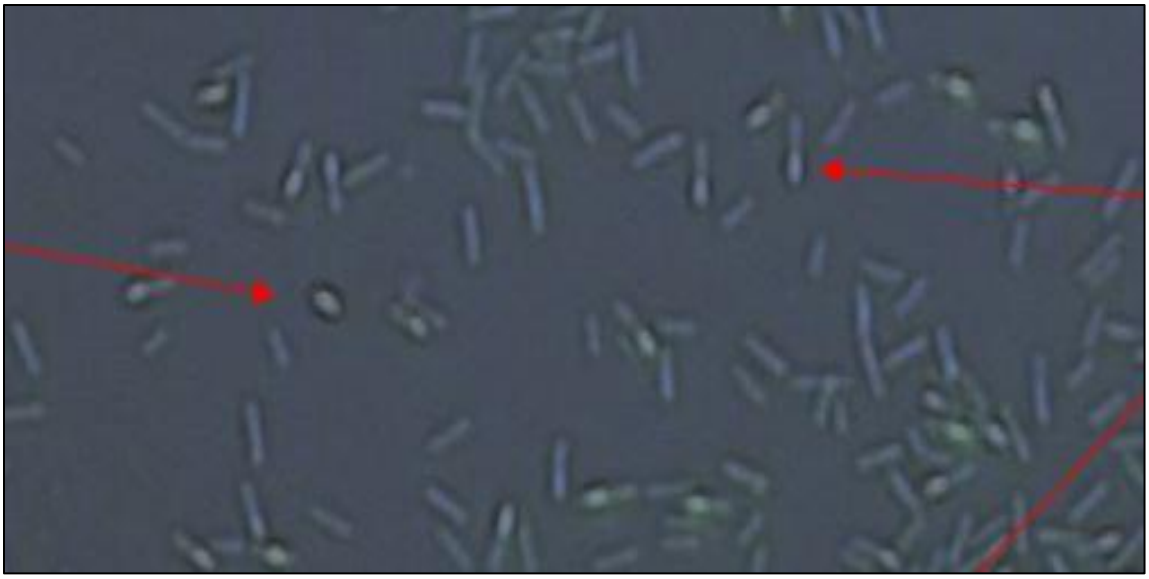

Figure 5: Example of *B. globigii* sporulating cells and detached spores. On the left is a newly detached spore surrounded by both vegetative and sporulating cells. The right arrow indicates a sporulating cell and the left arrow indicates a newly detached spore.

Sporulation ends with the detachment of the newly developed spore which completes the life-cycle of typical *Bacillus* cells.

2.5.4 *Dipicolinic Acid*

Dipicolinic acid (DPA) is the common name for pyridine-2,6-dicarboxylic acid as shown in Figure 6.

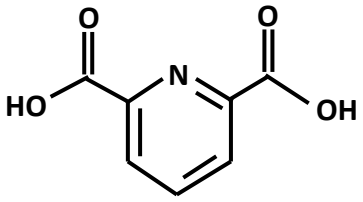

Figure 6: Dipicolinic acid structure

Within the spores of *Bacillus* and *Clostridium* species, DPA forms a complex with cations, predominately Ca^{+}, and makes up 5-15 percent of the spore's dry weight (Setlow, 2005). Shortly after the initiation of germination, the spore will release all the CaDPA

and replace it with water although how the water is taken up by the spore is still unknown (Setlow, 2013). CaDPA released from one spore can initiate germination in other spores as well (Setlow, 2003). Spores containing DPA have been shown to have an increase in wet heat resistance however; it is not clear if this is a result of DPA specifically or the increase in water content or reduction of core mineralization of DPA-less spores (Paidhungat et al., 2000). DPA also aids in the UV-resistance of spores as it acts as a photosensitizer (Setlow, 2006).

Numerous studies have measured the release of DPA to study the germination of spores. For example, Yi and Setlow in 2010 used DPA release measurements to study the effects of heat activation, germinant concentrations, and levels of germinant receptors in spores on the commitment and lag times of *Bacillus* spore germination. Reineke et al., in 2013 used DPA release to study the rate-limiting steps of *Bacillus subtilis* spore inactivation in a high-pressure stabilization process. The release of DPA has also been used to study the germination of *Clostridium sporogenes* (Yang and Ponce, 2009), *Bacillus anthracis* (Zhang et al., 2007), and *Bacillus megaterium* (Scott and Ellar, 1978). The release of DPA is a well-established metric for measuring germination.

III. Methodology

Chapter Overview

This research was conducted in two phases. The first phase involved microscopy with both phase contrast and fluorescence imaging. The second phase switched from microscopy to using spectrophotometry to measure the DPA released by the germinating spores.

3.1 Phase One: Light Microscopy

3.1.1 *Experimental Design*

The initial experimental conditions of the microscopy research varied as there were multiple objectives: familiarization with methods, validating methods, and determining the rate of germination/outgrowth. Timescales and conditions of each experiment varied and are outlined in Table 2.

Table 2: Microscopy experimental parameters

Exp	Buffer	Feed	L-Alanine	Heat Activated	$N\text{-}NH_3$ (mg/L)	Length	Samples Taken
1	X	X	28 mM	-	10.66	7 days	Every 0.5 hr/5 hrs; day/7 days
2	X	X	28 mM	-	10.66	1 day	Every hr/16-22 hrs
3	X	-	-	-	-	7 days	Every 0.5 hr/5 hrs; day/7 days
4	X	-	28 mM	-	-	2 hours	Every 10 mins
5	X	X	28 mM	-	10.66	1 hour	Every 5 mins
6	X	X	28 mM	-	10.66	1 hour	Every 5 mins
7	X	X	28 mM	-	10.66	1 hour	Every 5 mins

Exp	Buffer	Feed	L-Alanine	Heat Activated	N-NH_3 (mg/L)	Length	Samples Taken
8	X	-	-	-	-	7 days	Every 0.5 hr/5 hrs; day/7 days
9	X	-	-	-	-	1 day	Every 0.5 hr/5 hrs; day 1

All experiments were conducted in a phosphate buffered saline (PBS) solution to keep the pH at 7.4. All experiments were incubated at 37 degrees Celsius. The feed used in these experiments was the same which is used in the small batch reactors kept in the lab. This feed mimics food which would be available in a wastewater treatment plant and was adapted from Rauglas et al., (2016). The feed consisted of three solutions which are outlined in Table 3.

Table 3: Makeup and amounts of various feed solutions used

Feed A – 11.4 µL	Feed B_1 – 24.2 µL	Feed B_2 – 24.2 µL
1 L Deionized water	1 L Deionized water	1 L Deionized water
44.6 g Sodium bicarbonate	12.0 g Casamino acids	4.52 g Ammonium chloride
	2.5 g Sodium acetate	13.72 g Magnesium chloride
		3.44 g Calcium chloride
		1.34 g Potassium dihydrogen phosphate
		40 mL of trace element solution

A more thorough description of the feed can be found in Appendix A. Experiments 1 through 6 utilized 1E7 colony forming units (CFU) of *B. globigii* spores to approximate bacterial conditions found in wastewater treatment plants and contamination scenarios (Szabo et al., 2007; Omotade et al., 2014). Experiments 7 through 9 utilized 1E8

CFU/mL of spore solution. This was changed in order to have more cells available per slide for imaging.

The objective of experiments 1 and 2 was to determine the kinetics of key events in the germination and outgrowth of *B. globigii* specifically, germination, sporulation, and detachment of daughter spores. To accomplish this both feed and a germinant were utilized. The objective of experiment 3 was to identify the growth characteristics in a buffered solution with no feed or germinant. The germinant used was L-Alanine at a concentration of 28 millimoles per liter (mmol/L). Experiment 4 was conducted to identify the growth characteristics in a buffered solution with a germinant but no feed. Experiments 5, 6, and 7 were conducted to determine the growth rate of *B. globigii* under favorable conditions. For these experiments both feed and a germinant were added and samples were taken every five minutes for one hour. The objective of experiments 8 and 9 was to identify the growth characteristics in a buffered solution with no feed or germinant. Sampling frequencies varied depending on the objective of experiment in question. During experiments 1, 3, 4, 8, and 9, samples were taken every 30 minutes for the first 5 hours and then every day for 7 days thereafter. During experiment 2, samples were taken every hour between 16 and 22 hours to look more closely at sporulation kinetics. During experiments 5, 6, and 7 samples were taken every 5 minutes for one hour to determine kinetics of germination and outgrowth within the first hour.

3.1.2 *Experimental Protocols*

Prior to the conduct of the experiment the CFU for the spore stock would have to be identified. This was accomplished by adding 500 µL of sterile deionized water to the

spores and then plating diluted samples. The spores were diluted to concentrations of $1{:}10^4$, $1{:}10^6$, and $1{:}10^8$ and nine agar plates (3 for each dilution) were inoculated with 100 µL of each dilution and allowed to grow in an incubator at 37 degrees Celsius for 24 hours. The agar plates were made using 8 g/L of Difco Nutrient Broth and 15 g/L of LB Agar, Miller from Fischer Bioreagents. After 24 hours each plate was counted to identify the CFU of the original 500 µL spore stock solution. Once this was known, the appropriate amount could then be introduced to the experimental solution to achieve as close to 1E7 CFU/mL as possible. For example, if the 500 µL spore stock solution was determined to have 12.8E9 CFU/mL then 16 µL of that stock was added to 20.16 mL of experimental solution to achieve the 1.03E7 CFU/mL required.

$$\frac{1E7\ CFU}{mL} * \frac{mL\ stock}{12.8E9\ CFU} * \frac{1000\ \mu L}{1\ mL} * 20.16\ mL = 15.75\ \mu L$$

$$\frac{12.8E9\ CFU}{mL} * \frac{1\ mL}{1000\ \mu L} * \frac{16\ \mu L}{20.16\ mL} = 1.02E7\ CFU/mL$$

The spores were then washed until all cellular material was removed from solution and stored in a six-degree Celsius refrigerator until they were required. Washing the spores is an important step prior to the conduct of the experiment to ensure that the spore solution contains pure spores with as little cellular or vegetative cells as possible. All experiments consisted of approximately 20.2 mL of experimental solution except for experiment 6 which consisted of 39.94 mL of solution. The sample size for experiment 6 was 2 mL instead of the usual 1 mL sample size taken for all other experiments which required a larger initial experimental solution. Each sample for this experiment was split in two so that 1 mL of sample could be prepped for immediate phase contrast imaging and 1 mL of sample was mixed with Syto9 for fluorescent imaging which will be explained shortly.

For an experiment with a total solution of 20.16 mL, 18 mL of sterile deionized water, 2.0 mL of PBS x10, 80 µL Feed A, 170 µL of Feed B1, 170 µL of Feed B2, and approximately 0.04785 g L-Alanine (28 mM) were added to a sterile 50 mL Erlenmeyer flask. The solution was then inoculated with 16 µL of 12.8E9 CFU/mL spore stock and thoroughly mixed by vigorous shaking. Once the solution was mixed, 500 µL was extracted using a micropipette and placed in a 1.5 mL centrifugation tube as sample T=0. The experimental solution was placed in the incubator at a temperature of 37 degrees Celsius for the duration of the experiment. The 500 µL sample was then centrifuged at 5k rpm for 10 minutes at six degrees Celsius. After centrifugation the solution was washed by decanting 375 µL of the supernatant and replacing it with sterile deionized water to remove feed from the solution. 125 µL of solution had to remain in the centrifugation tube as the concentration of spores was too low to leave a visible pellet. Therefore, more solution had to be left to ensure that spores were not being removed on accident. The spores were washed three times and then placed in a six-degree Celsius refrigerator until imaging could take place. Prior to imaging, a Syto9 labeling solution (0.1 mM) was made by mixing 2 µL of Syto9 (5 mM) with 98 µL of sterile deionized water. 98 µL of the sample solution was then mixed with 2 µL of the Syto9 labeling solution; giving a final Syto9 concentration of 2 µM. This sample tube was then wrapped with aluminum foil to shield the sample from light, placed in a similarly wrapped 40 mL conical tube, and placed on the automatic shaker for no less than 30 minutes. This allowed the Syto9 to attach to the DNA of the bacterial cells and germinated spores. Syto9 won't bind to the DNA of ungerminated spores because of the inner membrane barrier. Once attached to the DNA, the Syto9 would fluoresce and allow differentiation of ungerminated spores

from germinated spores and vegetative cells. The remaining 70 µL of sample solution was placed back in to the six-degree Celsius refrigerator. Once shaking was complete, 10 µL of the solution was placed on a microscope slide and allowed to dry. Once the sample was dry, another 10 µL of the solution was placed on top of the previous placement and allowed to dry. This was repeated until all the imaging solution was used. This drying process is needed to concentrate the spores for easier finding spores under the microscope. One drop of Prolong Diamond Antifade Mount was placed on top of the dry sample and a microscope coverslip was placed on top of the sample. Imaging was conducted utilizing a Zeiss Axioskop microscope equipped with a Zeiss Axiocam camera. Phase contrast images were taken at an exposure time between 150 and 300 ms. Fluorescent imaging was taken at an exposure time between 2500 and 3500 ms. Five phase-contrast and five fluorescent images were taken for each sample point. Images were enhanced using ImageJ software to make counting easier. Of the nine experiments conducted using phase microscopy, only experiments 1 and 8 were used for analysis. Samples from other experiments were deemed not usable either because of 1) contamination of the experimental solution, which resulted in the filtration of experimental solution through a 0.2-micron filter for subsequent experiments prior to inoculation or 2) too dilute concentrations of spores, which made it extremely difficult to find enough spores within reasonable amount of time and resulted in the increase of inoculation concentration in subsequent experiments.

3.2 Phase Two: Absorbance Spectrophotometry

3.2.1 *Experimental Design*

Another method for measuring spore germination is by measuring the amount of DPA released. DPA has a characteristic absorbance peak at 270 nm and thus the absorbance at 270 nm can be used to quantify the DPA contained in the spore solution. This method is quantitative, quick and straightforward, but not as sensitive as fluorescence microscopy; it requires larger sample volume and higher spore concentration. Five experimental conditions were utilized with three experiments for each set of variables resulting in a total of fifteen experiments. Table 4 outlines the various parameters for each experiment. Experiments 10 through 13 were conducted in order to validate the protocol used for the subsequent experiments but were not utilized for the results of this research and thus will not be covered here.

Table 4: Photospectometry experimental parameters

Exp	Buffer	Feed	L-Alanine	Heat Activated	NH_4Cl-N (mg/L)
20	X	X	10 mM	X	10.66
21	X	X	10 mM	X	10.66
22	X	X	10 mM	X	10.66
17	X	-	-	-	-
18	X	-	-	-	-
19	X	-	-	-	-
14	X	X	-	-	0
15	X	X	-	-	0
16	X	X	-	-	0

Exp	Buffer	Feed	L-Alanine	Heat Activated	NH_4Cl-N (mg/L)
23	X	X	-	-	25
24	X	X	-	-	25
25	X	X	-	-	25
26	X	X	-	-	45
27	X	X	-	-	45
28	X	X	-	-	45

All experiments were conducted in a PBS solution to keep the pH of the experimental solution at 7.4 and incubated at 37 degrees Celsius. The feed used in these experiments was the same as was used during the first phase and can be found in appendix A. Feed B2 in its original form was only used for experiments 20, 21, and 22 (triplicates). All other experiments which required feed utilized a modified version of B2 in which the ammonium chloride was omitted. This allowed for a more precise measurement of how much NH_4Cl-N was being introduced to the spores. In experiments 20, 21, and 22 (triplicates) the objective was to identify the germination and outgrowth characteristics of *B. globigii* under the most favorable conditions (with germinant, nutrients from feed, and heat activation). To accomplish this both a germinant and feed were introduced. The germinant used was L-Alanine at a concentration of 10 mM per liter. This concentration has been used in literature for this purpose (Vepachedu and Setlow, 2004). The L-Alanine used in these experiments was from Sigma Aldritch and was ≥98.5% pure. The spores were heat activated for 30 minutes at 75 degrees Celsius prior to inoculation (Yi and Setlow, 2010). This was the only experimental condition which utilized heat

activated spores. Experiments 17, 18, and 19 (triplicates) were conducted as a baseline. The objective of these experiments was to determine the germination and outgrowth characteristics of *B. globigii* in buffered water alone. To accomplish this the spores were added to a solution containing only sterile deionized water with the phosphate buffered saline solution. Experiments 14, 15, and 16 (triplicates) were conducted to determine the germination and outgrowth characteristics of *B. globigii* at 0 mg/L of NH_4Cl-N. These experiments consisted of the same buffer solution and feeds A, B1, and the modified version of B2. Experiments 23, 24, and 25 (triplicates) were conducted to determine the germination and outgrowth characteristics of *B. globigii* at 25 mg/L of NH_4Cl-N. Due to the small volume of experimental solution, an ammonium chloride solution was mixed to alleviate the need for weighing extremely small amounts of ammonium chloride. This was achieved by dissolving 0.14474 g of ammonium chloride in 14 mL of sterile deionized water which resulted in a 0.1933 mmol/mL ammonium chloride solution. The amount of this solution which was required could then be back calculated. For example, experiments 23, 24, and 25 required 25 mg/L of NH_4Cl-N:

$$25\frac{mg}{L}\ N-NH_3 * \frac{53.49\frac{mg}{mmol}NH_4Cl}{14\frac{mg}{mmol}N} * \frac{1\ L}{1000\ mL} * 2.85\ mL = 0.27223\ mg\ NH_4Cl$$

$$0.27223\ mg\ NH_4Cl * \frac{1\ mmol\ NH_4Cl}{53.49\ mg\ NH_4Cl} * \frac{mL}{0.1933\ mmol\ NH_4Cl} * \frac{10^3 \mu L}{1\ mL} = 26.33\ \mu L$$

These experiments also utilized feeds A, B1, and modified B2. Experiments 26, 27, and 28 (triplicates) were conducted to determine the germination and outgrowth characteristics of *B. globigii* at 45 mg/L of NH_4Cl-N. These experiments utilized the

same parameters as the previous six however, 47.4 µL of the ammonium chloride solution was used to achieve the 45 mg/L of NH_4Cl-N which was required.

3.2.2 *Experimental Protocols*

An optical density (OD) at 600 nm of 1.00 was required to achieve a high enough concentration of *B. globigii* spores to facilitate the analysis of DPA released throughout the experiments. Due to the large concentration of spores needed for each experiment, spores were routinely cultured on agar plates using 8 g/L of Difco Nutrient Broth and 15 g/L of LB Agar, Miller from Fischer Bioreagents. The harvesting of spores occurred one week after inoculation. The spores were then washed until all cellular material was removed from solution and stored in a six-degree Celsius refrigerator until they were required. One milliliter of water was placed in the spore stock and various ratios of this spore solution to deionized water were tested until an OD at 600 nm of approximately one was reached. This ratio dictated the amount of spore solution to experimental solution which would be required for each experiment. As an example, an OD 600 of 1.007 was reached with 10 µL of spore solution and 90 µL of deionized water. For an experiment requiring 2.85 mL of total solution, 285 µL of the spore solution would be used. Each spore harvest usually provided enough spore solution for three experiments. Any remaining spores were used to inoculate the next spore cultures. All but one of the experiments had a total experimental solution of 2.85 mL. Experiment 14 had a total experimental solution of 3.66 mL; however, the ratios of feed and buffer were consistent among all experiments. The higher amount of experimental solution in experiment 14 was due to the need of refining the actual amount of solution which would be needed. It

was decided that 2.85 mL was adequate, and all subsequent experiments utilized that amount. Once the ratio of spore solution to experimental solution was determined all constituents were placed in three 1.5 mL centrifugation tubes: 285 µL of PBS x10, 11.4 µL Feed A, 24.2 µL of Feed B1, 24.2 µL of modified Feed B2, 0.1933 mmol/mL ammonium chloride solution (depending on amount required for the experiment) and the remaining sterile deionized water required to achieve 2.85 mL of experimental solution. The amount of sterile deionized water which was required was different for each experiment depending on the ratio of spore solution required as a result of the OD 600 test. The amount of sterile deionized water required was determined by subtracting the spore solution required, feed, and buffer solution from 2.85 mL. Once the buffer, feed, and sterile deionized water was in the centrifugation tubes, it was filtered and transferred to a 20.0 mL conical tube using a 10 mL syringe and 0.2 µm filter. The filter was used to remove any contaminants prior to inoculation. Prior to filtering of the experimental solution, approximately 3 mL of sterile deionized water was pushed through the filter to minimize the amount of experimental solution which would be absorbed by the filter. The experimental solution was then inoculated with the spore solution determined earlier and the time noted as T=0. Immediately upon inoculation, the experimental solution was vortexed to ensure thorough mixing of the spores. Once complete, 125 µL of spore solution was placed in to 19 separate 1.5 mL centrifugation tubes. These tubes corresponded to 18 sample times and one sample for total DPA analysis. Once all samples were in the centrifugation tubes, the tubes were placed on a mechanical mixer inside the incubator at 37 degrees Celsius.

3.3 Analysis

The effect of NH_4Cl-N on the germination and outgrowth of *B. globigii* was analyzed quantitatively by comparing the DPA concentrations found within each sample. Analysis was conducted utilizing a Biomate^3 Thermo Spectronic spectrophotometer at a wavelength of 270 nm. At each sample time, the corresponding 1.5 mL centrifugation tube was removed from the incubator and quickly vortexed to homogenize the solution. The sample was then placed in to a Thermo Scientific Sorvall Legend Micro17 centrifuge at 11.5k rpms for five minutes. Once centrifugation was complete, 100 µL of the experimental solution was decanted and transferred to a cuvette which was placed within the spectrophotometer. Three readings were taken and recorded for each sample point. Once the measurement was complete, the 100 µL of experimental solution from the cuvette was placed in to a clean 1.5 mL centrifugation tube and stored in a minus 60-degree Celsius freezer. The remaining experimental solution which contains the spores was then washed once with 300 µL of sterile deionized water, decanted, and stored in the same freezer. The total DPA contained in each sample was measured by autoclaving the extra T=0 sample prior to the steps outlined above. The high pressure and temperature lysed the cells which released all the DPA contained within the cell. A similar method using boiling water was used in other experiments (Vepachedu and Setlow, 2004). The ratio of the sample absorbance to the total absorbance found in the T=0 sample was used to normalize all sample absorbances. This allowed experimental samples to be compared to each other.

Imaging was conducted utilizing a Zeiss Axioskop microscope equipped with a Zeiss Axiocam camera. Prior to imaging, each sample was prepared utilizing Syto9 and

Prolong Diamond Antifade Mount (ThermoFischer Scientific, P36965). Two µL of Syto9 (5mM) were mixed with 98 µL of sterile deionized water to make the Syto9 labeling solution (0.1 mM). This sample tube was then wrapped with aluminum foil to shield the Syto9 solution from light. Two µL of this solution was then added to a separate 1.5 mL conical sample tube and 98 µL of the sample was then mixed with the 2 µL of Syto9 solution (final Syto9 concentration is now 2 µM). Each sample tube was then wrapped with aluminum foil to shield the sample from light, placed in a similarly wrapped 40 mL conical tube and placed on the automatic shaker. The samples were then shaken for a minimum of 15 minutes.

After 15 minutes, 10 µL of the experimental solution with Syto9 was placed in the center of a frosted microscope slide and allowed to dry. Once dry, one drop of the mounting agent was placed on top of the dried sample and a cover slip was used to facilitate imaging. A minimum of five sets of random images were taken for each sample. Each set consisted of a phase contrast field image and its corresponding fluorescent image. An ungerminated spore is shaped as an oval and appears phase-bright under phase contrast imaging. Germinated spores retain their shape but will lose their phase brightness and have strong Syto9 fluorescence. Ungerminated spores may have some faint fluorescence due to the presence of residual DNA materials from sporulation. Vegetative cells have Syto9 fluorescence and may be found in multiple stages of transition from an oval shaped spore to the characteristic rod-shape of *Bacillus* bacteria. To account for this, a spore which was most likely to be ungerminated due to phase brightness and lack of strong Syto9 fluorescence was located and the exposure time for the fluorescent image was adjusted until it no longer appeared fluorescent in the image.

This exposure time was then used throughout the imaging process. Under the same exposure time, the germinated spores and vegetative cells are expected to appear bright green fluorescent while the ungerminated dormant cells appear non-fluorescent. Randomness was achieved by moving the slide holder left or right and up or down. The images were saved after each set and transferred for analysis.

Analysis began with the counting of each cell type in the images. Once germination occurs the spores, swell, change shape, and lose their bright sheen. Due to the complexity with varying shapes and sizes, the current version of the imaging software was not able to be used and all counting was done by hand, which was not a trivial matter. Prior to counting the spores, the brightness of the fluorescent images was checked again to ensure that spores which looked most likely to be ungerminated were not fluorescing. If one was found to be, the brightness of the image could be adjusted using ImageJ software until it no longer showed on the image overlay. Within each image the number of ungerminated spores, germinated spores, vegetative cells, and sporulating cells were counted. If the image contained too many cells to be counted by hand, the image was split in to 8 or 16 identical sections and the most representative section was counted. Once all cell types were counted the ratio of germinated spores to total spores (germinated and ungerminated), vegetative cells to total cells (total spores plus vegetative cells), and sporulating cells to total vegetative cells were plotted.

Rates of outgrowth in phase one were calculated between 0 and 3 hours. This was conducted by plotting the ratio of vegetative cells to total cells (minus ungerminated spores) on the y-axis and time on the x-axis. A best fit line was then calculated using Microsoft Excel with the slope representing the ratio of outgrowth per hour and will be

discussed in section 4.1. Rates of germination for each experimental condition in phase two were calculated between 0 and 60 minutes, 0 and 300 minutes, and 0 to 6 days. This was conducted for the period from 0 to 60 minutes by subtracting the initial ratio of DPA released from the latter ratio of DPA released which resulted in the total ratio of DPA released per hour. For the period between 0 and 300 minutes and 0 to 6 days, a best fit line was calculated using Microsoft Excel. This resulting slope is the ratio of total DPA released per minute over both time periods and will be discussed in section 4.2. For this analysis the sample points of 0 and 30 minutes for experiment 14 were omitted as all adsorbance values were negative. Negative adsorbance values are not possible unless there was an issue with the cuvette used, rinsing of the cuvette prior to measurement, or the blank used to calibrate the spectrophotometer. Experiment 14 was conducted at the same time as experiment 13 which consisted of different experimental conditions and on a separate occasion than experiments 15 and 16 which were of the same experimental conditions as experiment 14. To correct for this discrepancy at the time of the conduct of experiments 13 and 14, separate cuvettes were used for each sample point of those experiments. All subsequent experimental conditions were conducted together, and each cuvette was rinsed three times with sterile deionized water prior to the measurement of another sample. If any of the rinse water remained inside the cuvette and could not be removed a new cuvette was used and the spectrophotometer was recalibrated using the new cuvette. Additionally, if any measurements were suspicious (i.e. much higher than expected in relation to trends or the other measurements of like experimental conditions) the sample was remeasured using the same techniques. The same sample points from the other two experiments (if already measured) were then measured again to ensure that

they remained the same. This happened a total of four times: exp. 20, T=4.5 hrs, exp. 22 T=5.0 hrs, exp. 23 T=4.5 hrs, and exp 24 T=1.0 hrs.

Statistical analysis was conducted for outgrowth by using the observed data to determine the best-fit-line. For germination, the first moment of area (FrM) was determined for the baseline, 0 mg/L, 25 mg/L, and 45 mg/L of ammonia nitrogen within the first five hours. A comparison of the means was then conducted using a two-tailed student's t-test with an alpha of 0.05. The FrM was also conducted for 0 mg/L, 25 mg/L, and 45 mg/L of ammonia nitrogen within the seven days and a comparison of the means was then conducted using a two-tailed student's t-test with an alpha of 0.05.

IV. Results and Discussion

4.1 The Effect of Ammonia-N on the Outgrowth of *Bacillus globigii*

Concentrations of 0 and 10.66 mg/L of ammonia-N were used to determine its effect on the outgrowth of *Bacillus globigii*. The percentage of outgrowth vs time for both concentrations of ammonia-N are shown in Figure 9. The outgrowth rates of each concentration of ammonia-N are displayed in Figure 9 and are 0.0761 and 0.1821 vegetative cells/hour for 0 mg/L and 10.66 mg/L of NH_4Cl-N respectively. The phase bright images were taken with a 150 ms exposure time and the fluorescent images were taken with a 2000 ms exposure time. The brightness of the fluorescent image was adjusted to differentiate germinated and ungerminated spores. A phase bright spore was selected, and the brightness of the image was lowered until the ungerminated spore no

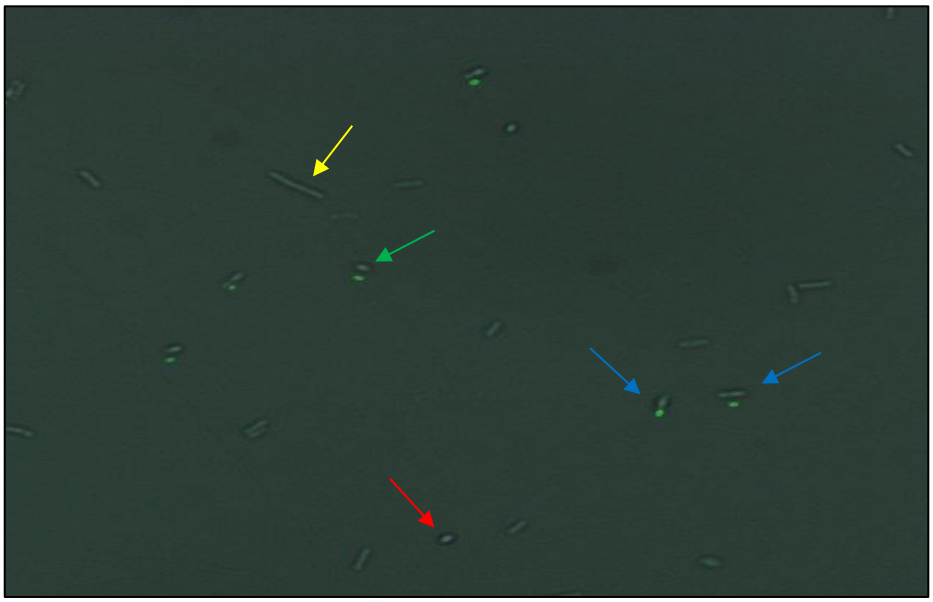

Figure 7: Overlay image of Experiment 8 (water and buffer only) at T=0 hours. The red arrow indicates an ungerminated spore, green arrow indicates a germinated spore, yellow arrow indicates a vegetative cell, and the blue arrows indicate spores conducting outgrowth.

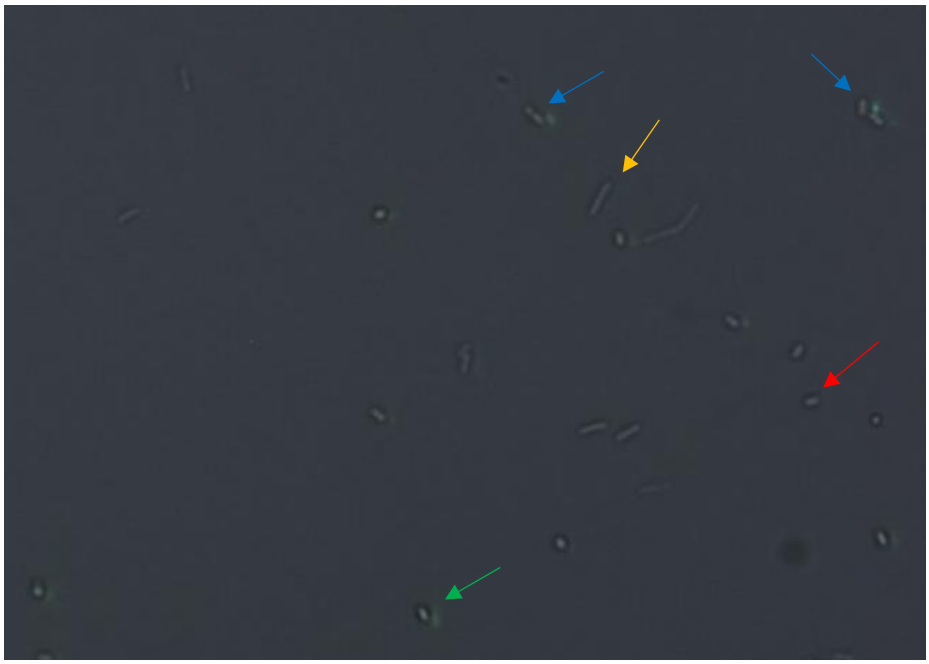

Figure 8: Overlay image of Experiment 1 (Feed, L-Alanine, 10.66 mg/L NH_4Cl-N) at T=0.5 hours. The red arrow indicates an ungerminated spore, green arrow indicates a germinated spore, yellow arrow indicates a vegetative cell, and the blue arrows indicate spores conducting outgrowth.

longer showed fluorescence. This allowed the true identification of germinated and ungerminated spores as well as vegetative cells conducting outgrowth or sporulation. This corrected fluorescent image was then overlaid on to the phase bright image using the ImageJ software to facilitate counting as shown in Figures 7 and 8. Within Figures 7 and 8, the arrows depict an example of each cell type: red is an ungerminated spore, green is a germinated spore, yellow is a vegetative cell, and blue is an outgrowing or sporulating vegetative cell. A sporulating cell can be identified by the fluorescing daughter cell which is linear with the vegetative mother cell (see Figure 5) which would be similar to that seen in a cell conducting outgrowth. As seen in Figure 5, the sporulating cells are close in size to full vegetative cells whereas the cells in Figures 7 and 8, depicted by the blue arrows, are much smaller than full vegetative cells by comparison which lends to the

belief that they are in fact cells in the early stages of outgrowth. Figure 8 is an overlay image at 0.5 hours of experiment one using feed, L-alanine, and 10.66 mg/L of NH_4Cl-N. Based on single images it is difficult to determine which experimental parameters the images came from. The aggregate of all images for each sample point allows for the analysis of outgrowth rates within each experiment. For the determination of outgrowth percentages and subsequent rates, ungerminated spores were not included in the calculations as an ungerminated spore cannot undergo subsequent outgrowth.

The amount of outgrowth seen with water and buffer only was much higher than anticipated. This is mainly because the germination of spores was not expected to occur in an appreciable amount. Factors which may have contributed to germination in a medium consisting of water and buffer only are investigated in section 4.4 and will not be discussed here. This extreme difference in outgrowth rates is expected as the germinated spores in water and buffer only must rely exclusively on energy stores contained within the spore to conduct outgrowth.

There is limited amount of research conducted on the nutritional requirements of germinated spores conducting outgrowth and the feed used in these experiments contained various amounts of amino acids and other nutrients which may have provided the requirements for the outgrowth observed. For this reason, the 10.66 mg/L of NH_4Cl-N cannot be definitively linked as the reason for the increased outgrowth rate.

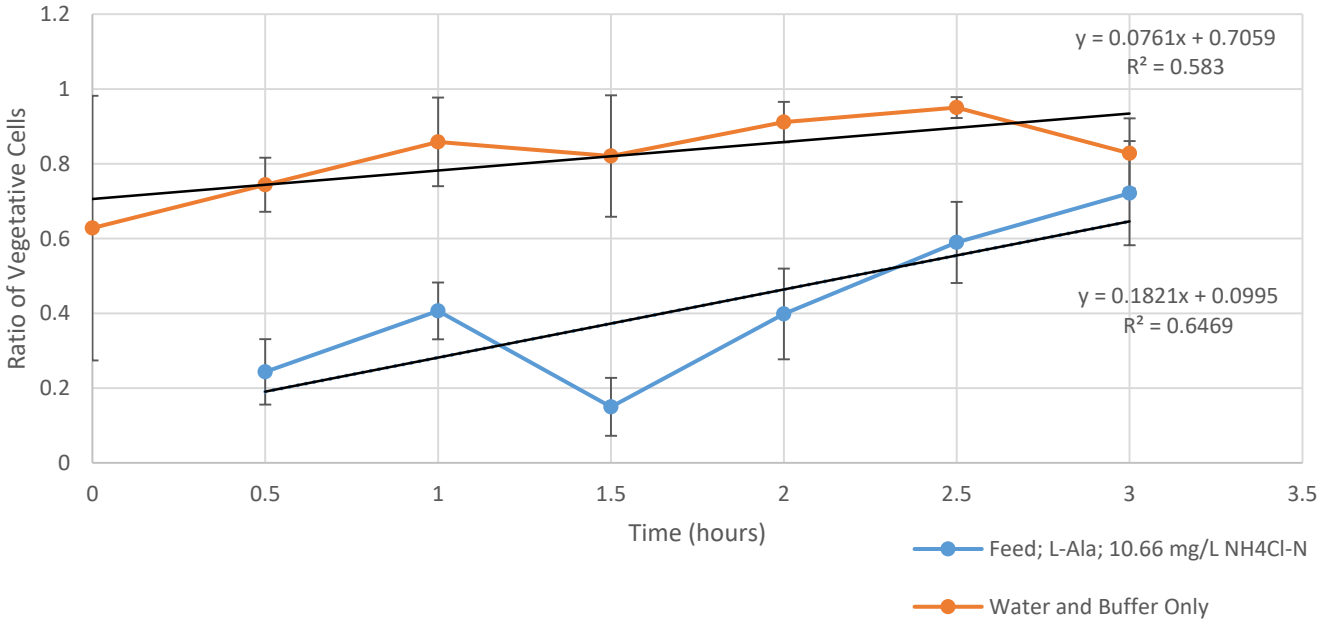

Figure 9: Outgrowth ratio of *Bacillus globigii* between 0 and 3 hours

4.2 The Effect of Ammonia-N on the Germination of *Bacillus globiigi*

The ratio of DPA released at each sample point for each experimental condition of phase two are displayed in Figures 10 and 11. Table 5 lists the rates of the ratio of total DPA released for each experimental condition over one hour, five hours, and six days. Initially it was assumed that the spores which were heat activated and in the presence of

Table 5: Rates of the ratio of total DPA released within defined periods.

	Baseline	**Feed; Heat Act; 10.66 mg/L**	**0 mg/L**	**25 mg/L**	**45 mg/L**
1 hour	0.0258	0.0147	0.0118	0.0127	-0.00732
5 hours	0.00005	0.0001	0.0001	0.0001	0.00005
6 days	0.00002	0.00002	0.00001	0.00001	0.00001

L-alanine would have had a higher germination rate, regardless of the NH_4Cl-N present, as both are known to facilitate this (Setlow, 2014) and are used for this reason in research (Yi and Setlow, 2010; Chesnokova et al., 2009; Ghosh and Setlow, 2009). Similarly, it was assumed that the spores in water and buffer only would result in very little germination. On the contrary, these spores had similar germination rates to those with feed and NH_4Cl-N within the first five hours and then doubled those germination rates after a day to mimic the spores which were heat activated in the presence of a germinant. It is highly unlikely that the sterile deionized water or the buffer used in the baseline experiments contained any kind of germinant. There are two factors which may have attributed to this, inoculum size and temperature, and those will be discussed in section 4.4. NH_4Cl-N has been shown to increase rates of germination in *Bacillus* species (Preston and Douthit, 1984). In their research, they found that NH_4Cl had a rate limiting concentration of 0.5 mM and a saturating concentration of 100 mM. The concentrations

of NH_4Cl used in these experiments were 1.79 mM and 3.21 mM for the 25 mg/L and 45 mg/L of NH_4Cl-N respectively. Therefore, it was expected that those two experimental parameters would show more germination than the spores which were exposed to 0 mg/L NH_4Cl. Qualitatively it is hard to distinguish the experiments which only differed in the concentration of NH_4Cl-N from Figure 10 and although it appears there is a difference in germination rates in the first hour from Table 5 it doesn't follow the pattern which would be expected and most likely may be due to background variability. The germination rates for the range of NH_4Cl-N are very similar from 0 to 5 hours and 0 to 6 days.

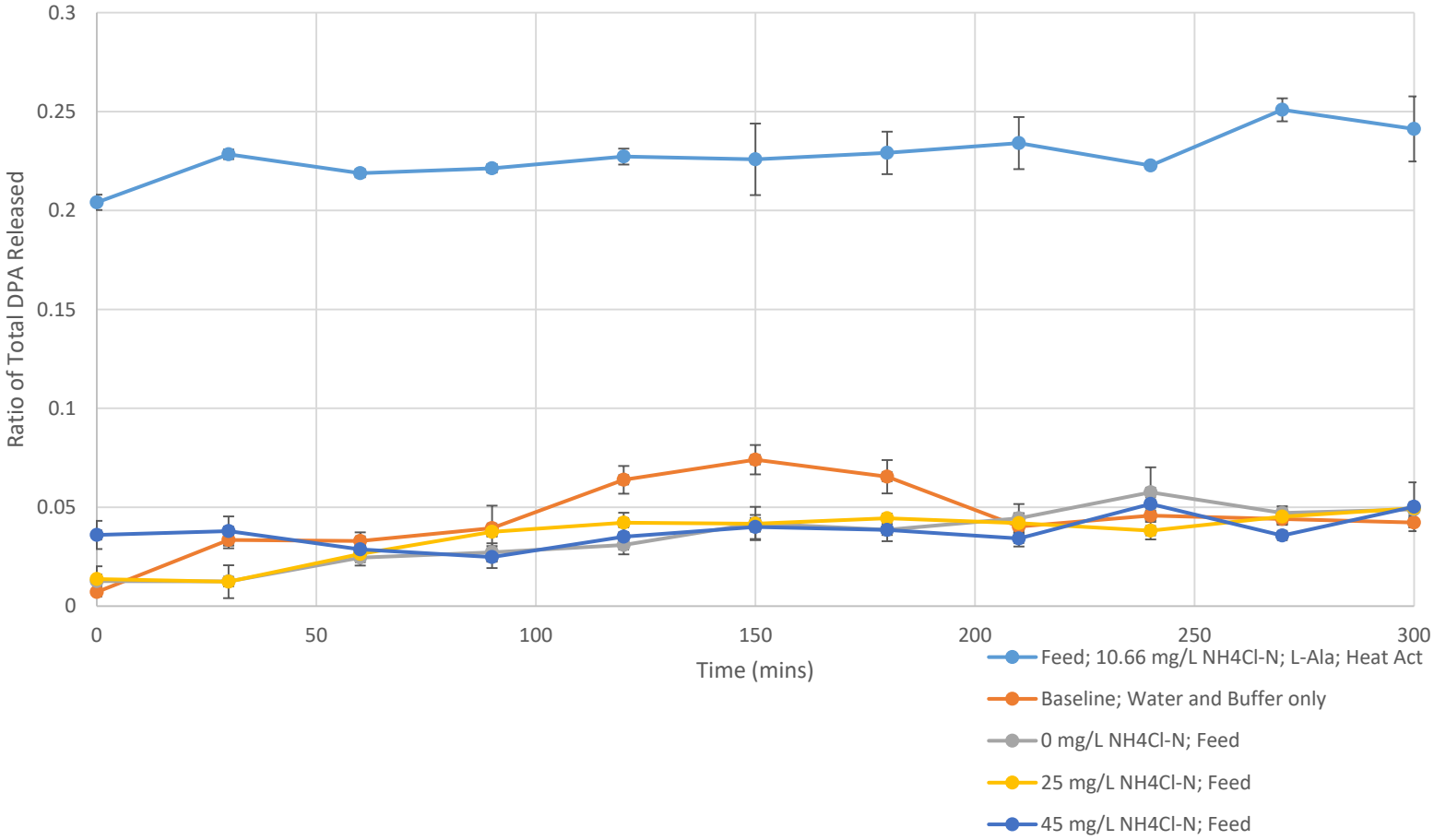

Figure 10: Ratio of total DPA released between 0 and 300 minutes.

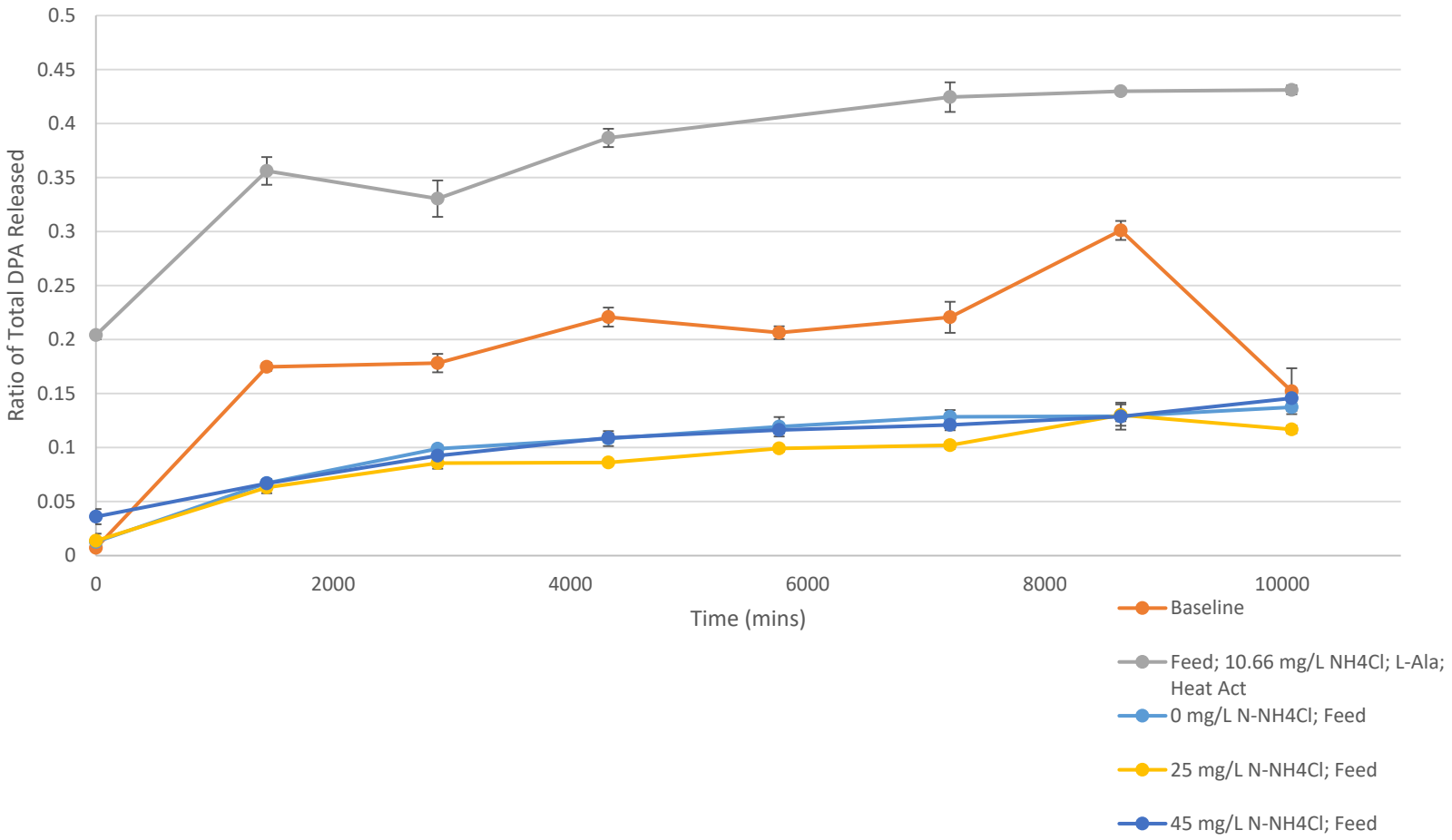

Figure 11: Ratio of total DPA released between 0 and 7 days.

4.3 Evaluation of the Hypothesis

The hypothesis of this research was that the effect of ammonia-N on the outgrowth should be more significant than on the germination of *Bacillus globigii*. This was not confirmed and would require more detailed research.

4.4 Inoculum Size and Temperature Effects

The amount of germination that was seen in the baseline experiments was higher than initially expected and needs to be addressed. As stated in the methods section, the only variables in that experimental condition was sterile deionized water, Fischer Scientific PBSx10 (BP3991), spores at a concentration which gave an OD at 600 nm of 1.006 at 5 µL of spore solution and 95 µL of sterile deionized water. The experiments were then incubated at 37 degrees Celsius for the duration of the experiment. To determine if any of the experimental variables may have had a positive impact on the percent of spores which germinated and consequently released their DPA, each of the variables will be inspected.

4.4.1 *Concentration of Spores*

To conduct DPA analysis it is essential that enough spores are used to release enough DPA that can be analyzed using a spectrophotometer. Other studies analyzing germination by the absorbance of DPA use an OD at 600 nm between 0.5 and 1 (Vepachedu and Setlow, 2004; Yi and Setlow 2010). The CFU inherent in the spores used for these experiments was never determined as it wasn't essential while using DPA release for analysis. However, Nagler and Moeller used an OD at 600 nm of 0.5 for *B. subtilis* in their research and correlated this to 4E07 spores per well (2015). Paidhungat et

al. conducted research using *B. subtilis* as well and correlated the OD 600nm of 1 that they used to approximately 1.5E08 CFU/mL and an OD 600nm of 10 to a concentration of 1.5E09 CFU/mL. One study compared the size of several *Bacillus* strains to *Bacillus anthracis*. This research reported that *B. globigii* and *B. subtilis* spores had mean diameters of 0.65 and 0.48 µm and mean lengths of 1.22 and 1.07 µm respectively (Carerra et al., 2007). Although *B. subtilis* spores are slightly smaller than *B. globigii* the concentration used by Paidhungat et al., at an OD 600 nm of 1 will be used for this research to determine if the concentration of spores may have affected the germination of the baseline experimental condition.

The concentration of spores used in the microscopy phase of this research was approximately 1E07 CFU/mL. This concentration was used as other research studies which examined the scenarios of biocontamination used between 1-2E07 CFU/mL (Omotade et al., 2014). Using a concentration of 1.5E08 contains 15 times more spores than a concentration of 1E07. Multiple studies have shown that higher inoculum sizes will significantly enhance overall germination as well as time to germination for *Bacillus megaterium* and *Clostridium botulinum 56A* (Caipo et al., 2001; Zhao et al., 2000). There is no clear way to compare the inoculum sizes used in those studies directly to the inoculum size which is estimated for this research of 1.5E08 CFU/mL, but each study presented showed that inoculum size was significant in both the percent of germination of spores and the time it took to germinate. Thus, it can be reasoned that with an inoculum size 15 times larger for spectrophotometry and microscopy, an increase in germination would be seen by the former over the latter.

4.4.2 *Incubation temperature of 37 degrees Celsius*

Like inoculum size, research has shown that an increase in temperature will cause time-to-detection to decrease and percent-growth-positive to increase for *Clostridium botulinum* (Zhao et al., 2002). During this study the researchers compared time-to-detection and the percent-growth-positive at temperatures of 15, 22, and 30 degrees Celsius. Similar studies involving *Bacillus* bacteria could not be found. As stated, with an increase in temperature, germination increased; thus, it can be expected that incubating the experiments at 37 degrees Celsius would increase germination versus an experiment which was conducted at room temperature.

It has been shown that the conditions involved in the experimental conduct may have increased germination over what was expected. This begs the question on why the germination decreased in the experiments which used feed and different concentrations of NH_4Cl-N. NH_4Cl is used widely in research as an enhancement to germination and the feed used in this research is that which is supposed to mimic feed which would be found in a wastewater treatment plant.

Due to the positive effects that inoculum size and temperature have on germination, the conclusions drawn from this research only apply to the experimental conditions that were used. Different results may be achieved in experiments using an inoculum size or incubation temperature other than what was used for these experiments.

4.5 The Effect of Various Soluble Compounds on Germination

Appendix A is a complete list of the constituents which make up the feed used in these experiments. There are two well-known inhibitors of germination for *Bacillus*

species, D-Alanine and acetic acid (Yi and Setlow, 2010). Alanine racemase is an enzyme found in multiple species of *Bacillus* bacteria (Stewart and Halvorson, 1952) and is responsible for converting L-Alanine in to D-Alanine. Alanine racemase is produced during the late stages of sporulation in *B. anthracis* and is thought to aid in preventing premature germination (Chesnokova et al., 2009). In 2010 Yi and Setlow found that the addition of acetic acid to a pH of 3.6 effectively inhibited germination. Neither D-alanine nor acetic acid are found in the feed used during these experiments, so the inhibition must be coming from elsewhere.

4.5.1 *Sterile Deionized Water*

The deionized water was collected from the tap which dispensed deionized water to the lab. This water was then sterilized by autoclave. To determine if the water was in fact not deionized a complete analysis would have to be conducted which is not feasible as it would be costly and time intensive. Anything that may have entered the water would be expected to be at too low a concentration to have an impact on germination.

4.5.2 *Fischer Scientific PBSx10*

The buffer used in all experiments was PBS made by Fischer Scientific catalogue number BP3991. This buffer contains 1.37 M NaCl, 0.027 M KCl, and 0.119 M phosphates prior to dilution. After dilution the experimental solution would contain 0.137 M NaCl, 0.0027 M KCl, and 0.0119 M phosphates. Which phosphates are contained are not specified in the Fischer Scientific product description. Katya Nagler and Ralf Moeller conducted research using *Bacillus subtilis* which showed that some salts at low concentrations (0.06 M) slightly enhanced germination (Nagler and Moeller, 2015) such

as NaCl and KCl. This is also true for most phosphates which were used in the study. However, most salts start to lose their enhancement effect and either have no effect or weak to strong inhibition at 0.6 M depending on the specific salt (Nagler and Moeller, 2015). The salt and phosphate concentrations found in the buffer after dilution in the experimental solution could in fact enhance germination over a solution not containing that buffer.

4.5.3 *Salts*

There are many salts contained in the feed which could have an effect on the germination. Nagler and Moeller systematically researched the effect of various concentrations of a number of salts to determine if they either inhibited or promoted germination in *B. subtilius* (Nagler and Moeller, 2015). Table 6 identifies which salts they investigated are found in the feed that was used for this research.

Table 6: Salts contained in feed solutions

Compound	Concentration (moles/L)
NaCl	0.174
$MgCl_2$	0.0012
$CaCl_2$	0.00026
KI	6.14E-08
$FeCl_3$	3.14E-06
$MnCl_2$	3.24E-07
$CoCl_2$	7.85E-08
$NiCl_2$	7.86E-08
KH_2PO_4	8.33E-05

The concentrations which Nagler and Moeller investigated were 0.06 M, 0.6 M, 1.2 M, and 2.4 M. At 0.06 M $FeCl_3$ showed strong inhibition, $MnCl_2$, $NiCl_2$, and $CoCl_2$ showed weak inhibition, $MgCl_2$ and $CaCl_2$ had no notable effect, and NaCl, KH_2PO_4, and KI showed enhancement of germination (Nagler and Moeller, 2015). At 0.6 M $FeCL_3$ showed complete inhibition, KI, $CaCl_2$, and $NiCl_2$ showed strong inhibition, $CoCl_2$ and $MnCl_2$ showed intermediate inhibition, and NaCl, KH_2PO_4, and $MgCl_2$ showed weak inhibition. Unfortunately, their research did not include combinations of salts at concentrations adding to the concentrations studied. Although the research conducted by Nagler and Moeller showed that most salts at concentrations at or above 0.6 M showed inhibition from weak to complete, none of the salts included in the feed that was used are found in concentrations close to 0.6 M. At the concentrations found in the feed, most salts would have an enhancement effect on germination (Nagler and Moeller, 2015).

4.5.4 *Sodium Bicarbonate*

Several studies have investigated the inhibitory effect of sodium bicarbonate on *B. subtilis* and *B. stearothermophilus* (Hachisuka at al., 1956; Cheung et al., 1998). Hachisuka et al. studied the effect of sodium bicarbonate on the germination of *B. subtilis* at 1:80, 1:40, and 1:20 M sodium bicarbonate. These ratios correspond to 0.013, 0.025, and 0.05 M respectively and they found that at these concentrations the rate of germination after two hours was 18, 15, and 11% respectively compared to the 98% germination of the control. Cheung et al. used different w/v% of sodium bicarbonate to determine the inhibitory effect on *B. stearothermophilus*. They found that comparing the control to 0.1% $NaHCO_3$, 80% of the spores were able to germinate after 30 minutes. At

0.3% w/v $NaHCO_3$, 50% of the spores germinated after 30 minutes, and at 1.0% w/v $NaHCO_3$, 30% of the spores were able to germinate after 30 minutes (Cheung et al., 1998). Both Hachisuka et al. and Cheung et al. demonstrated that sodium bicarbonate does inhibit germination of the *Bacillus* bacteria they studied. Feed A used in the current research was made by dissolving 44.6 g of sodium bicarbonate per L of deionized water. This corresponds to a final concentration of sodium bicarbonate in the experimental solution of 0.002124 M or 0.0.1784 w/v%. It is hard to determine exactly how much sodium bicarbonate may have inhibited the *B. globigii* spores, but research has shown that it is very likely to have caused inhibition.

4.6 Retrieval of Kinetic Parameters

The kinetic values described in this research were retrieved using a very simple process. This simple process used took advantage of real data, was easy, and did not require an in-depth knowledge of the mechanisms in which each variable interacted with each other, if at all. The disadvantages are that it ignores any non-linear behavior and the rates produced may not be intrinsic. Ideally, these kinetic values would be modeled using very simple parameters which were introduced one at a time (i.e. NH_4Cl-N, sodium bicarbonate, individual feed compounds). This would allow for the specific determination of each element on the germination and/or outgrowth of the spore.

4.7 First Moment of Area Analysis

To determine if the concentration of N-NH_4Cl had an effect on the ratio of DPA which was released, and effectively the germination, of *B. globigii* the first moment of the area (FrM) was determined for each concentration at 0-300 minutes and 0-7 days.

Figure 10 in section 4.2 showed the ratio of DPA released for each experimental condition between 0-300 minutes. From this graph it is obvious that the total DPA released in the experiment with L-alanine, feed, and heat activation is significantly different than the other experimental conditions. It is not obvious if any of the other experimental conditions are significantly different than each other. In this case the FrM was calculated for these experimental conditions and then compared to each other. Figure 11 in section 4.2 showed the ratio of DPA released for each experimental condition between 0-7 days. From this graph it is obvious that the total DPA released in the experiments with L-alanine, feed, and heat activation and the experiments with just water and buffer are significantly different than the experiments with different concentrations of NH_4Cl-N. In this case the FrM was calculated only for the experiments with different concentrations of N-NH_4Cl and those were compared to each other. Figure 12 is the FrM calculated for 0 to 300 minutes and Figure 13 is the FrM calculated for 0 to 7 days.

The FrM for 0 mg/L NH_4Cl-N and feed from 0 to 300 minutes was 1883 ± 120 ratio of total DPA released-min^2. The FrM for 25 mg/L NH_4Cl-N and feed from 0 to 300 minutes was 1860 ± 108 ratio of total DPA released-min^2. The FrM for 45 mg/L NH_4Cl-N and feed from 0 to 300 minutes was 1767 ± 132 ratio of total DPA released-min^2. The FrM for water and buffer only from 0 to 300 minutes was 2235 ± 63 ratio of total DPA released-min^2. The FrM for each experimental condition was then compared to each other using the students t-test with an alpha of 0.05 to determine significance. Table 7 are the results of the students t-tests.

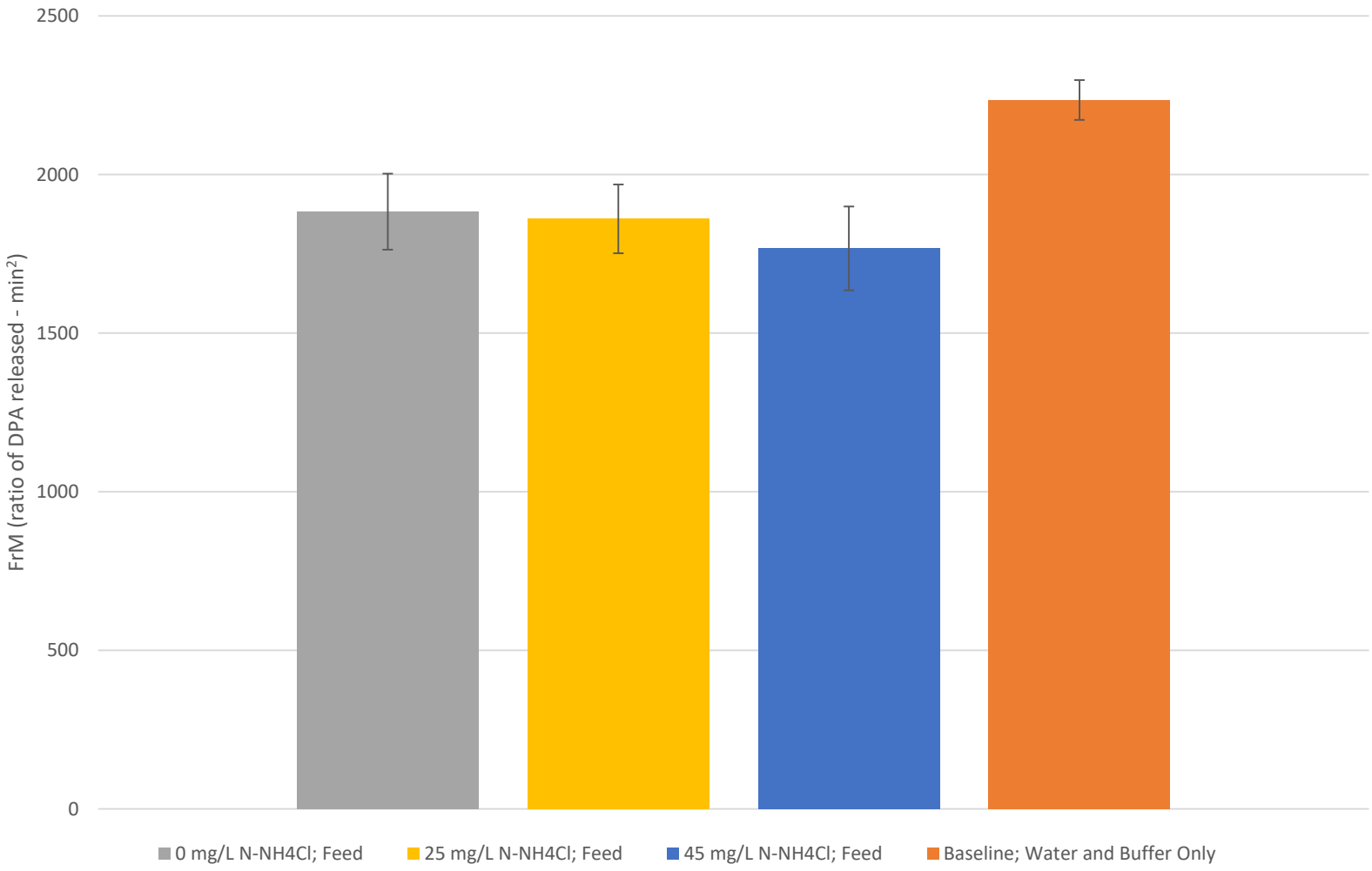

Figure 12: FrM for select experimental conditions from 0 to 300 minutes.

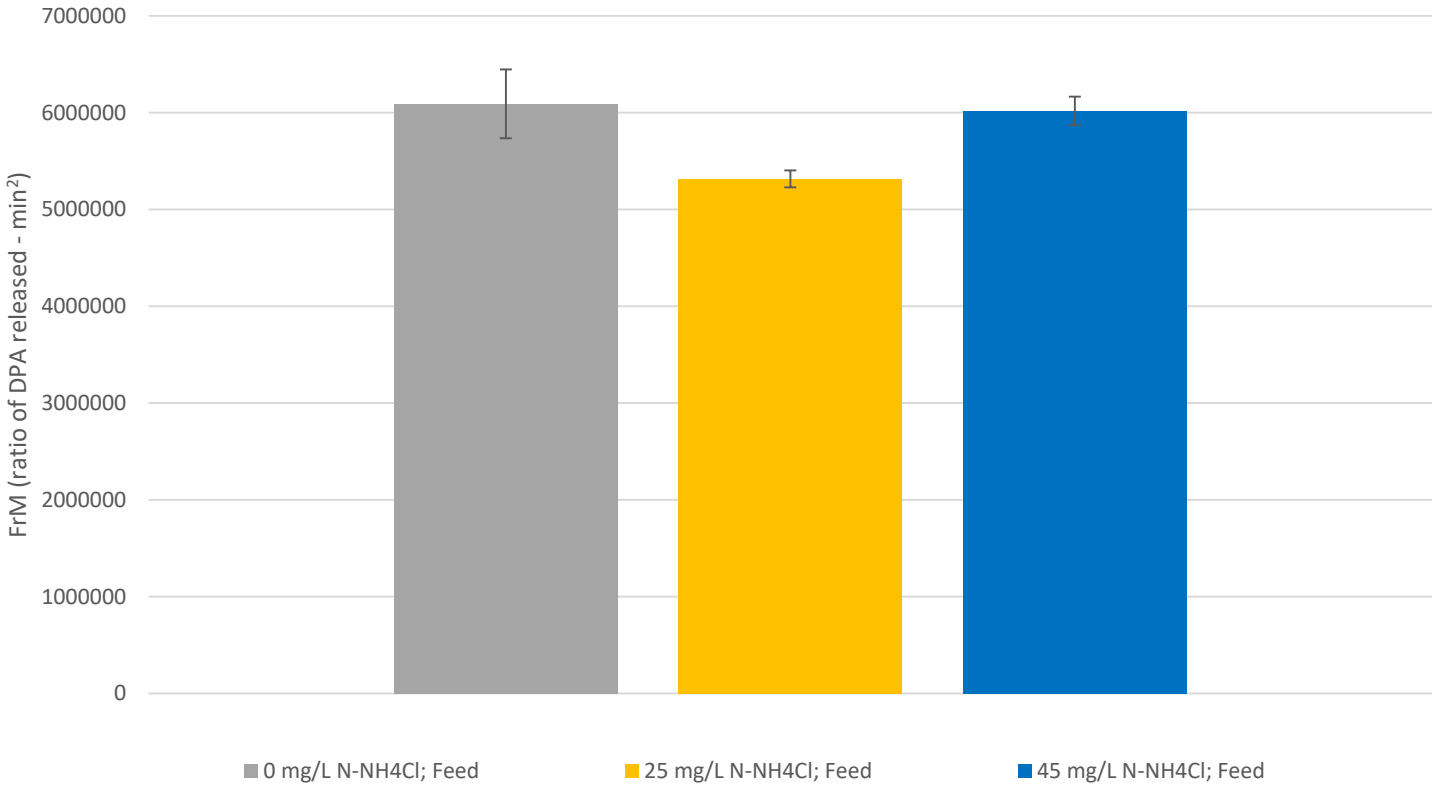

Figure 13: FrM for select experimental conditions from 0 to 7 days

Table 7: Results of student t-tests for FrM from 0 to 300 minutes.

Experiment	p-value
0-25	0.850706
25-45	0.485528
0-45	0.410743
0-Baseline	0.021172
25-Baseline	0.013352
45-Baseline	0.010649
Note: Values shown in red are less than 0.05 (alpha), the 95% confidence threshold	

As Table 7 shows, the FrM of the baseline (water and buffer only) was statistically different than all experiments using NH_4Cl-N with p-values less than 0.05. It also shows that the concentrations of NH_4Cl-N did not result in statistically different FrMs with p-values greater than 0.05.

The FrM for 0 mg/L NH_4Cl-N and feed from 0 to 7 days was 6,091,705 ± 355,714 ratio of total DPA released-min^2. The FrM for 25 mg/L NH_4Cl-N and feed from 0 to 7 days was 5,315,723 ± 87,813 ratio of total DPA released-min^2. The FrM for 45 mg/L NH_4Cl-N and feed from 0 to 7 days was 6,020,055 ± 146,278 ratio of total DPA released-min^2. The FrM for each experimental condition was then compared to each other using the students t-test with an alpha of 0.05 to determine significance. The results of the students t-test are shown in Table 8.

Table 8: Results of student t-tests for FrM from 0 to 7 days.

Experiment	p-value
0-25	0.0401331
25-45	0.0042906
0-45	0.8052148
Note: Values shown in red are less than 0.05 (alpha), the 95% confidence threshold	

As Table 8 shows, the FrM of 25 mg/L NH_4Cl-N and feed was statistically different than both 0 mg/L NH_4Cl-N and feed and 45 NH_4Cl-N and feed with p-values of 0.0401 and 0.0043 respectively. The experiments using 0 mg/l and 45 mg/L of NH_4Cl-N and feed had FrMs which were not statistically different with a p-value greater than 0.05. This indicates that there may be subtle impacts of ammonia nitrogen on germination over a 7-day period.

V. Conclusions

The conclusions of this study are that the NH_4Cl-N concentrations did not cause statistically significant differences in the average 1-hr germination rates in *Bacillus globigii*. The average germination rates observed in the absence of NH_4Cl-N was 0.0258 hr^{-1} for the water and buffer control and between -0.00732 and 0.0127 hr^{-1} with synthetic feed over a range of NH_4Cl-N concentrations. The synthetic feed utilized inhibited germination rates, most likely due to the sodium bicarbonate. The 3-hr outgrowth rates were 0.0761 and 0.1821 hr^{-1} for the buffer control and positive control respectively but it cannot be concluded that NH_4Cl-N was responsible for the difference. The NH_4Cl-N concentration impacted the shape of the germination profiles over a 7-day period. Statistically significant FrM differences between germination profiles occurred between 0 and 25 mg/L NH_4Cl (p-value = 0.04) and 25 and 45 mg/L NH_4Cl-N (p-value = 0.004) suggesting that subtle impacts may be observed over a 7-day period. Water and buffer only controls showed germination rates between 0.00005 and 0.0258 hr^{-1} but were significantly lower and between -0.0732 and 0.0147 in the presence of synthetic feed over a range of NH_4Cl-N concentrations. The inhibition observed in this research is consistent with sodium bicarbonate inhibition documented in previous research.

VI. Recommendations for Future Research

There are several suggestions the author has for future research. It would be beneficial to perform the same experimental conditions using a fluorescing material, such as terbium chloride which complexes with DPA, instead of absorbance as this would give more specific germination data. Conduct baseline experiments, or positive control without feed, with the addition of compounds within the feed to determine if other constituents may be inhibiting germination. Conduct the experimental conditions using real-time microscopy or fluorescence to monitor germination and outgrowth. This would reduce microbial growth during storage and allow for direct observation of germination and outgrowth events. There are two impacts which result from this research. The first is that germination and outgrowth can occur in treated water without the presence of germinants or a food source and those in charge of our water infrastructure must continue to monitor conditions which would not normally be conducive to germination and outgrowth to ensure its safety. The second is that sodium bicarbonate may be a viable buffer solution to prevent germination of biocontaminants.

Appendix A: Feed Solutions

Feed A: Sodium bicarbonate

1 L DI water		
$NaHCO_3$	Sodium bicarbonate	44.6 g

Feed B1: Macronutrients

1 L DI water		
Casamino acids		12.0 g
$C_2H_3NaO_2$	Sodium acetate	2.5 g

Feed B2: Micronutrients

1 L DI water		
NH_4Cl	Ammonium chloride	4.52 g
$MgCl_2$	Magnesium chloride	13.72 g
$CaCl_2$	Calcium chloride	3.44 g
KH_2PO_4	Potassium dihydrogen phosphate	1.335 g
Trace Element Solution		40 mL

Trace Element Solution

1 L DI water		
$C_6H_8O_7$	Citric acid	2.73 g
$C_9H_9NO_3$	Hippuric Acid	2.00 g
$C_6H_6NNa_3O_6 \cdot H_2O$	NTA trisodium salt	0.36 g
$C_{10}H_{12}N_2O_8Na_4 \cdot H_2O$	EDTA tetrasodium salt	0.15 g
H_3BO_3	Boric acid	0.25 g
KI	Potassium chloride	0.03 g
$FeCl_3 \cdot 6H_2O$	Ferric chloride hexahydrate	1.5 g
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate	0.15 g
$MnCl_2 \cdot 4H_2O$	Manganese chloride tetrahydrate	0.12 g

$CuSO_4 \cdot 5H_2O$	Copper (II) sulfate pentahydrate	0.07 g
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate (VI) dihydrate	0.03 g
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride hexahydrate	0.03 g
$NiCl_2 \cdot 6H_2O$	Nickel (II) chloride hexahydrate	0.03 g
$Na_2WO_4 \cdot 2H_2O$	Sodium tungstate dihydrate	0.03 g

Appendix B: Rates of Outgrowth

Positive Control: 10mM L-Ala; 10.66 mg N-NH_4Cl; Feed

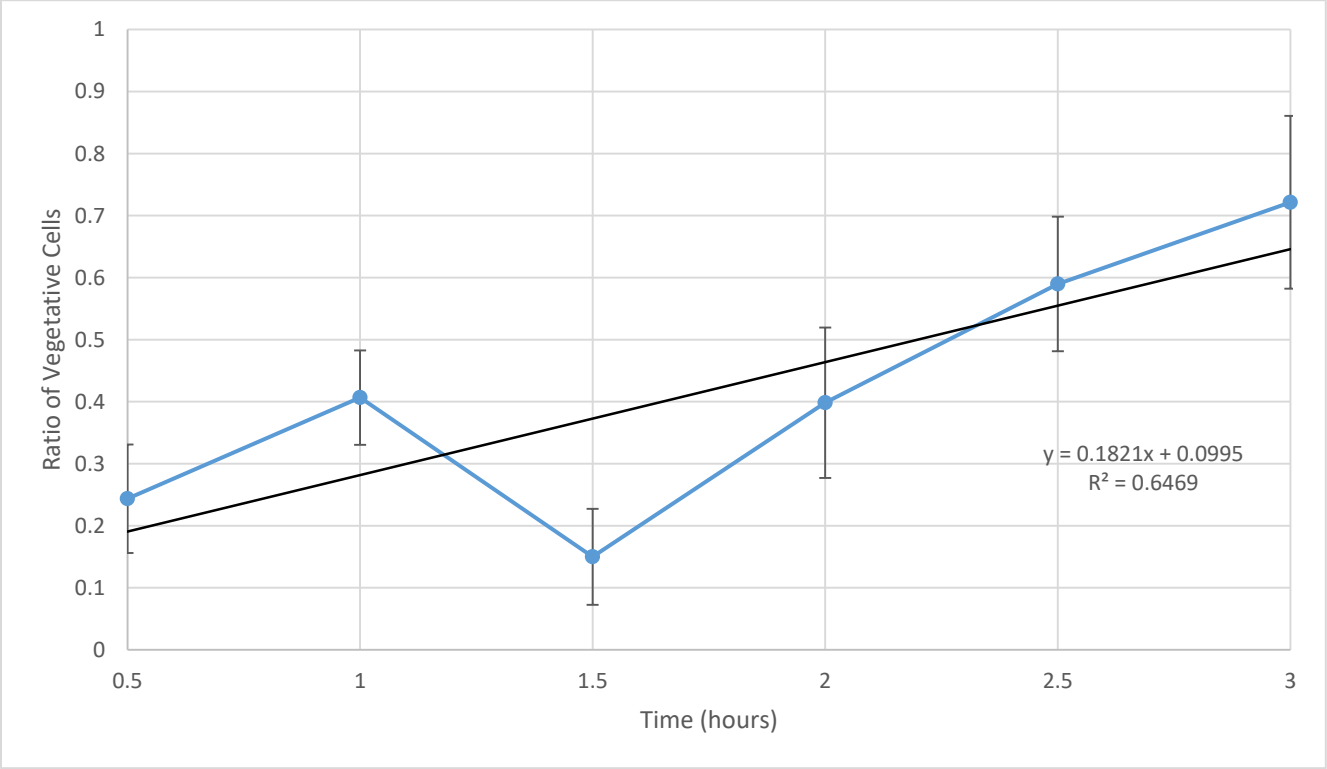

Figure 14: Outgrowth rate of *B. globigii* with feed; L-Ala; 10.66 mg/L NH_4Cl-N

Baseline: Water and Buffer only

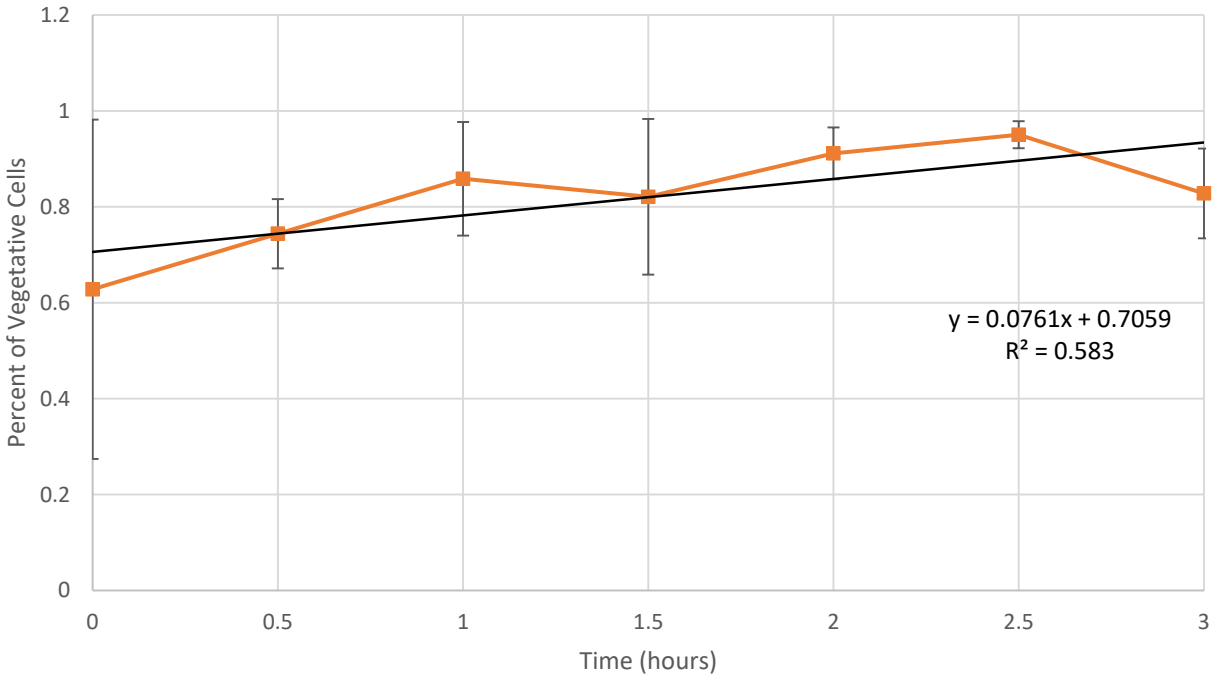

Figure 15: Outgrowth rate of *B. globigii* between 0 and 3 hours with water and buffer only

Appendix C: Ratio of DPA released

Baseline; Water and Buffer Only

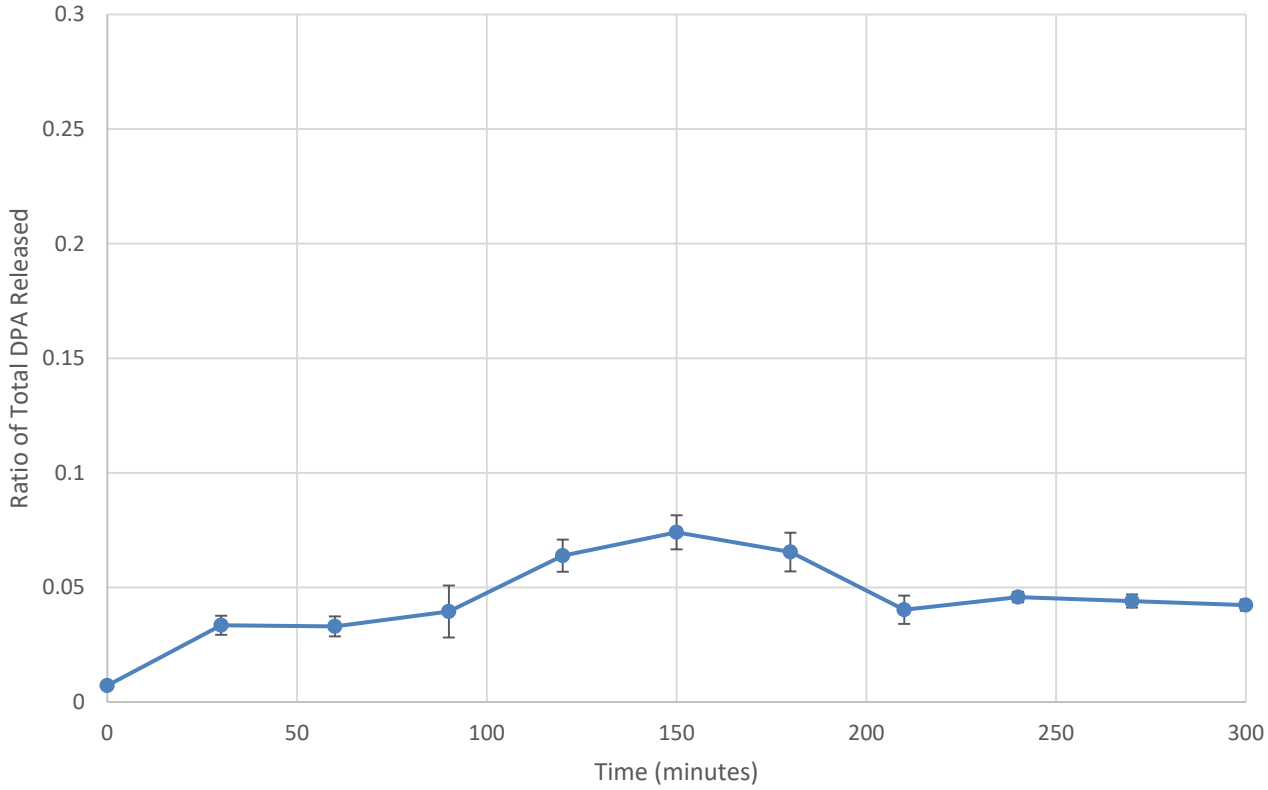

Figure 16: Ratio of DPA released between 0 and 300 minutes for baseline conditions

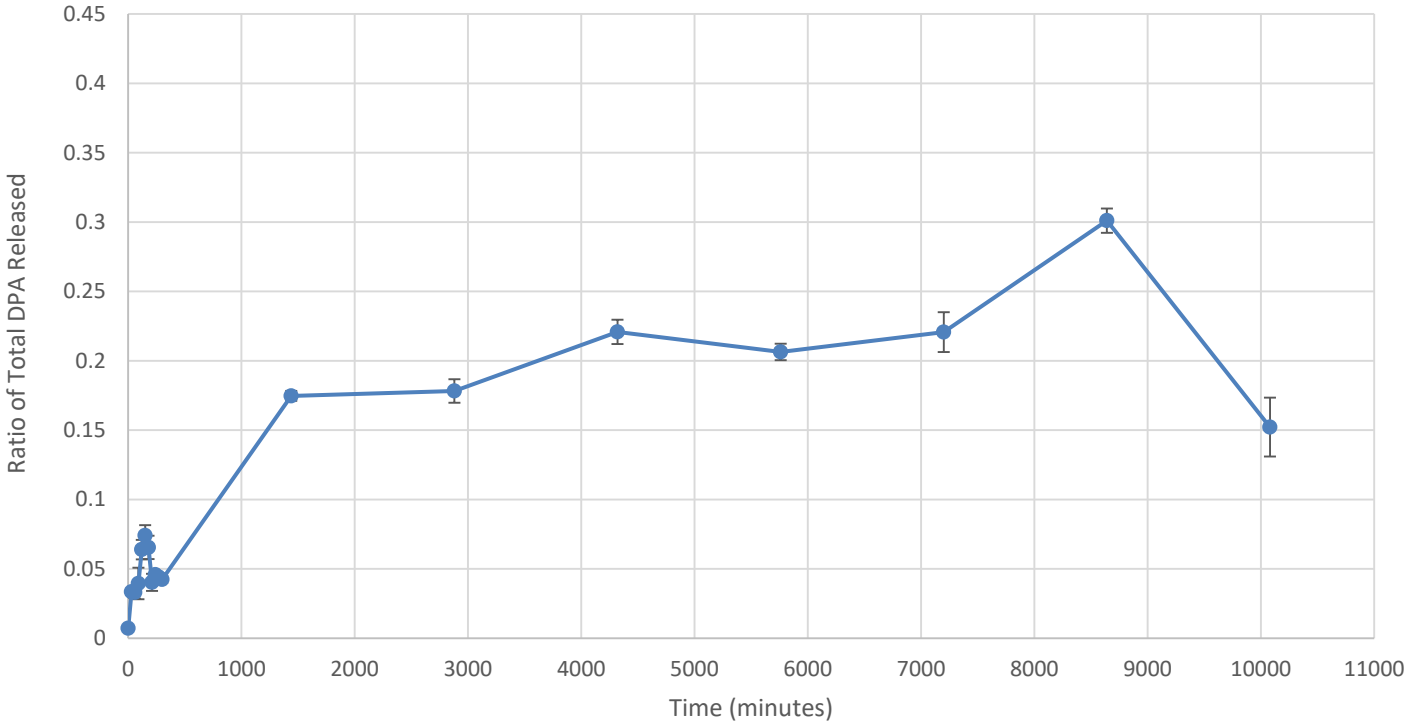

Figure 17: Ratio of total DPA released between 0 and 7 days for baseline conditions

Positive Control: 10mM L-Ala; 10.66 mg N-NH_4Cl; Heat Activated; Feed

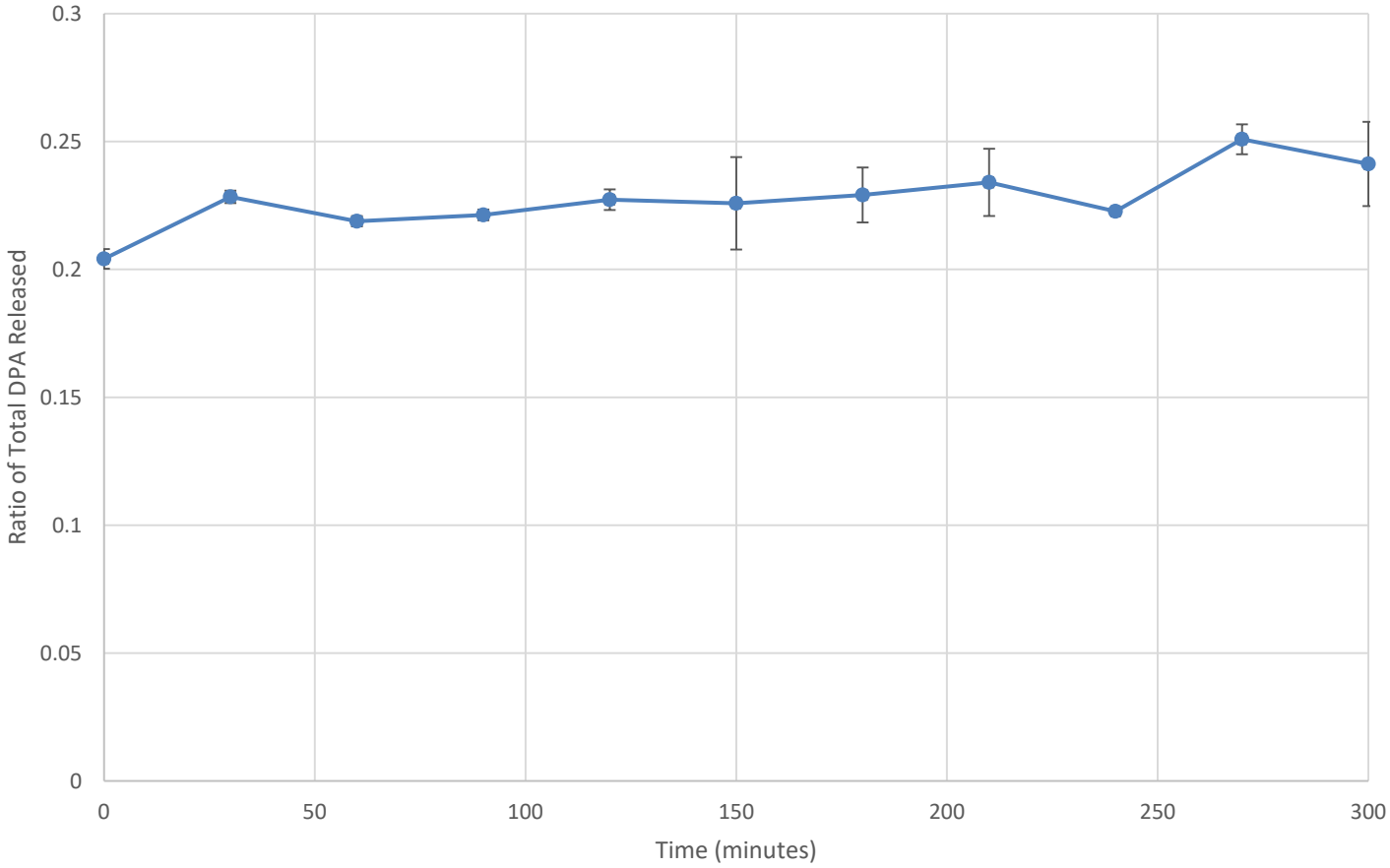

Figure 18: Ratio of DPA released between 0 and 300 minutes for positive control conditions

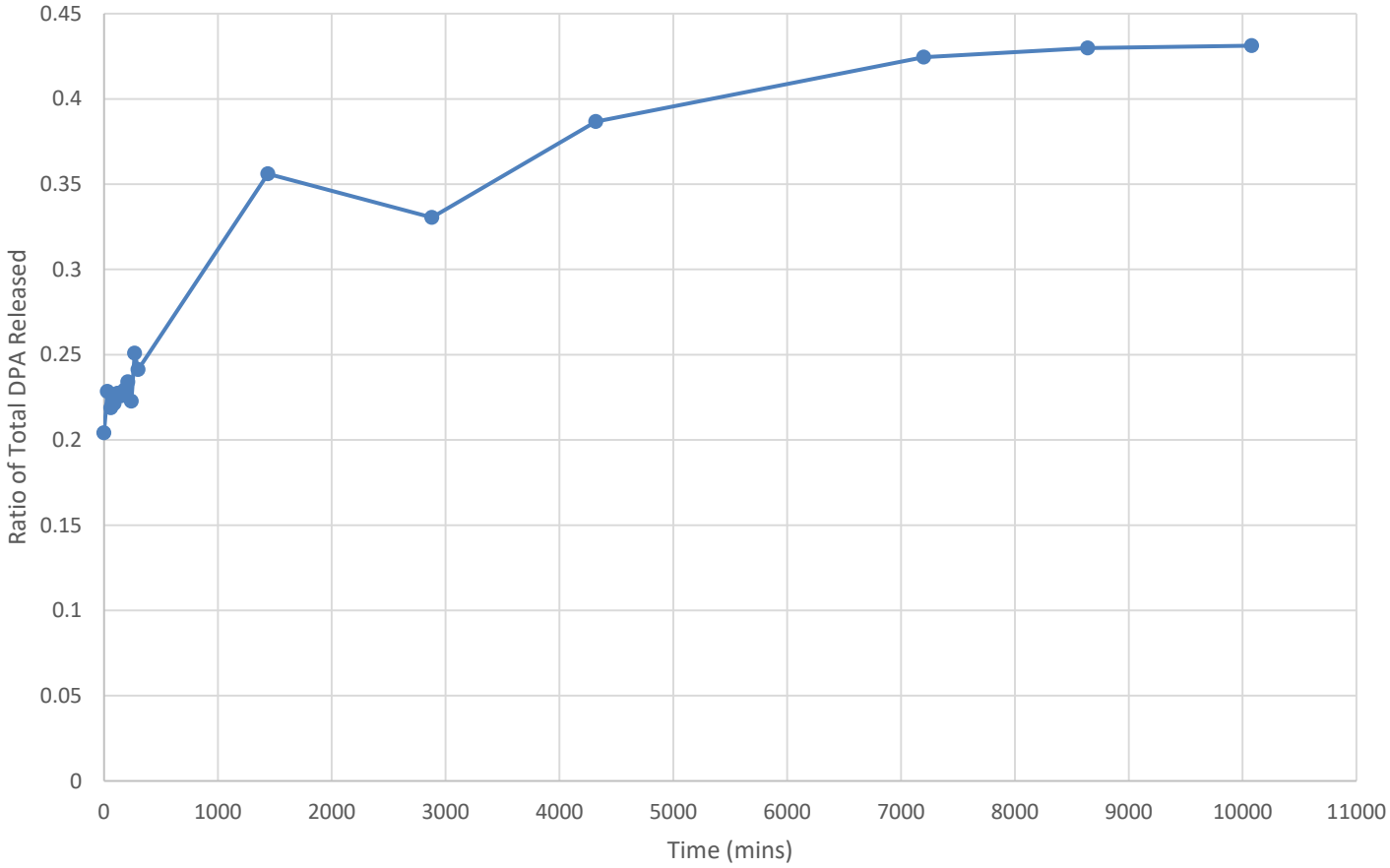

Figure 19: Ratio of total DPA released between 0 and 7 days for positive control conditions

0 mg/L N-NH_4Cl; Feed

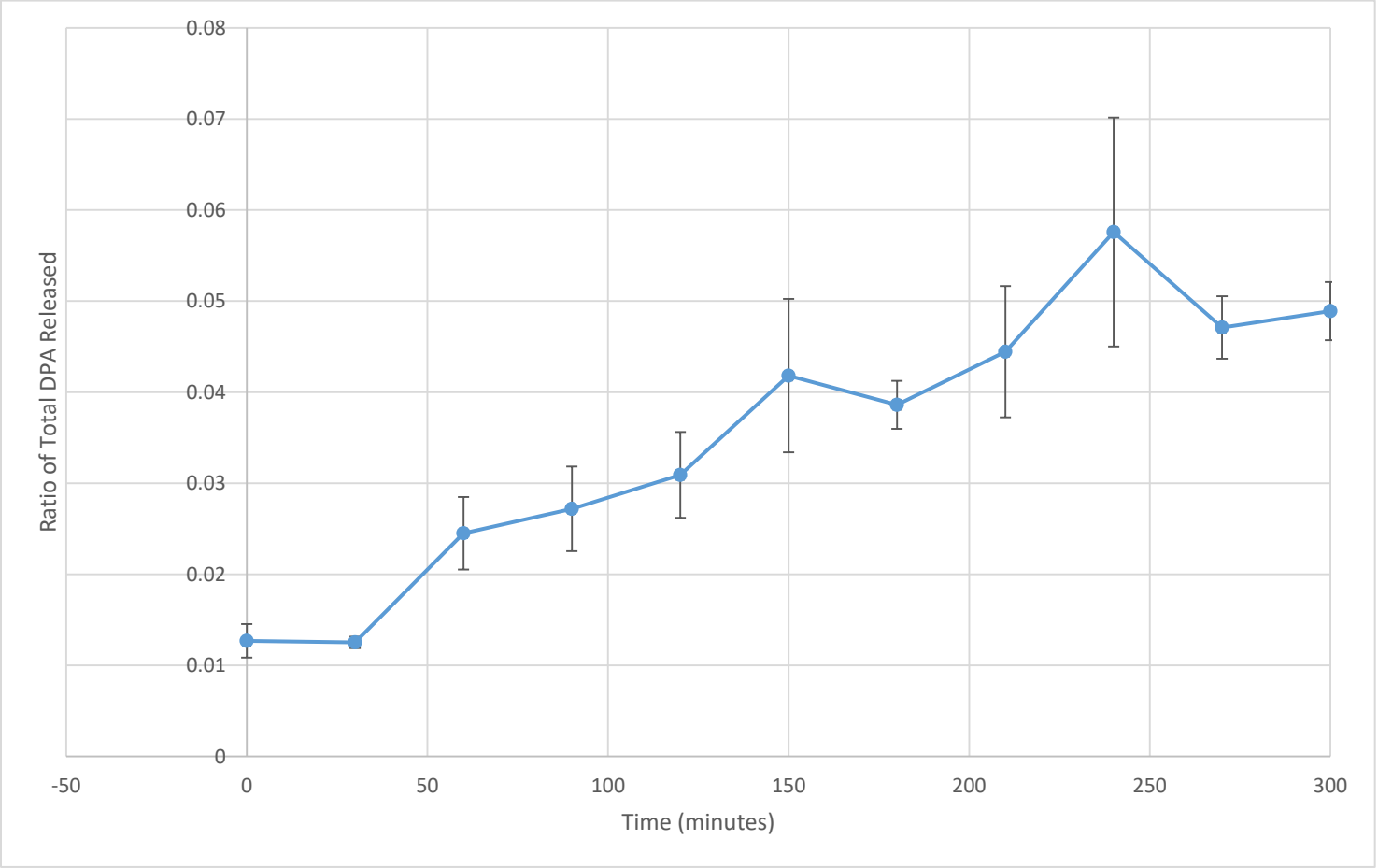

Figure 20: Ratio of DPA released between 0 and 300 minutes for 0 mg/L N-NH4Cl and feed.

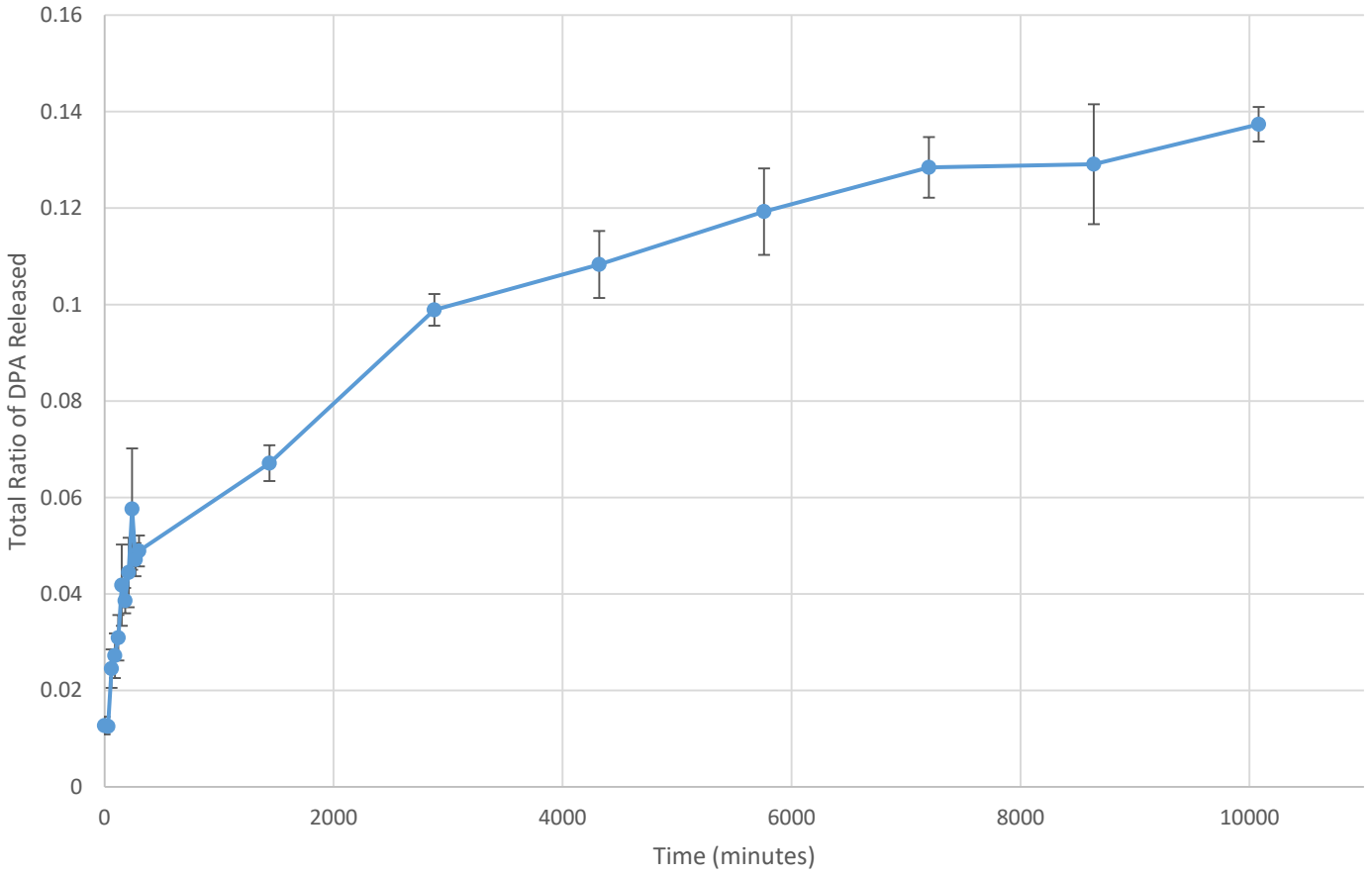

Figure 21: Ratio of total DPA released between 0 and 7 days for 0 mg/L N-NH4Cl and feed.

25 mg/L NH_4Cl; Feed

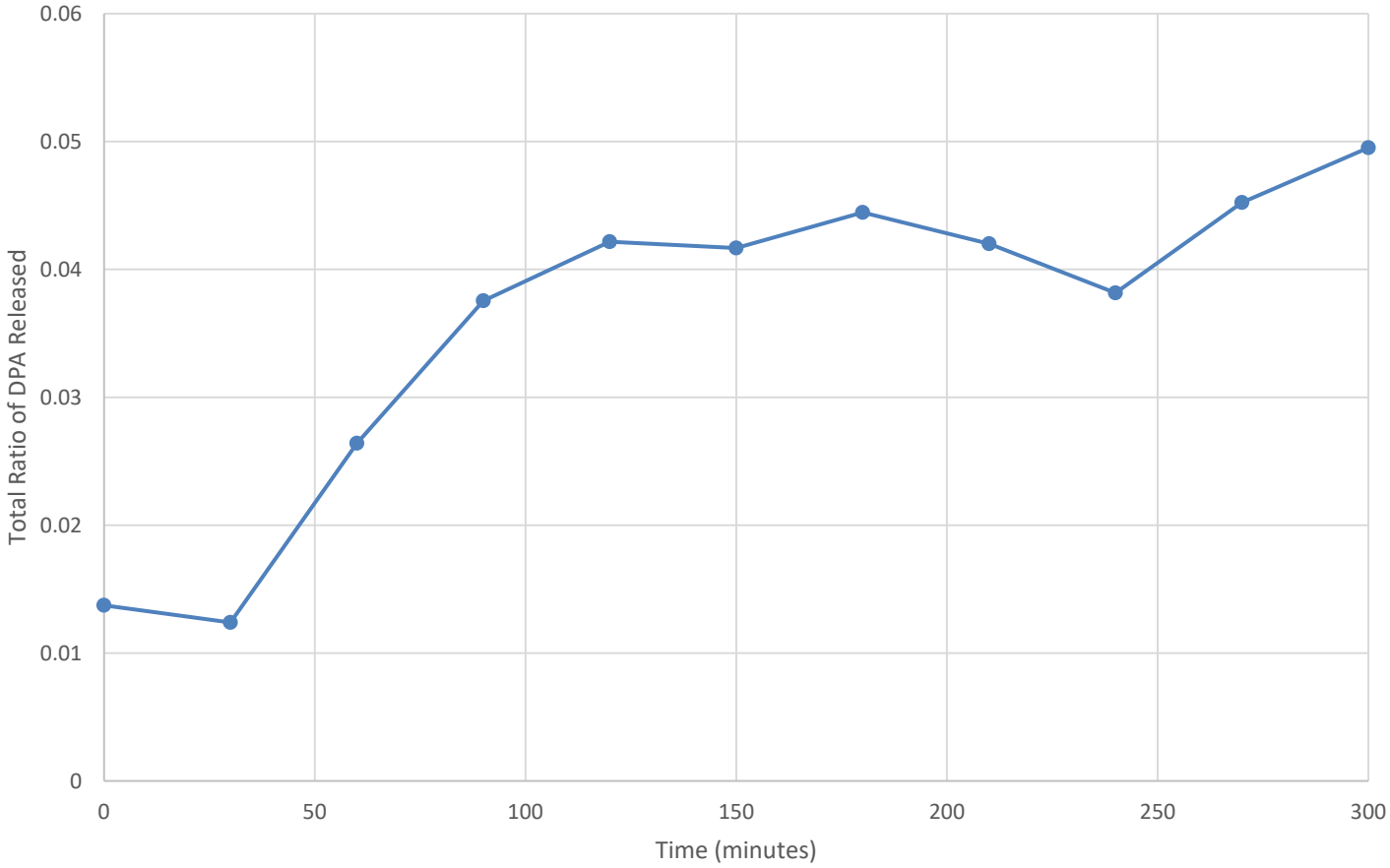

Figure 22: Ratio of DPA released between 0 and 300 minutes for 25 mg/L N-NH4Cl and feed.

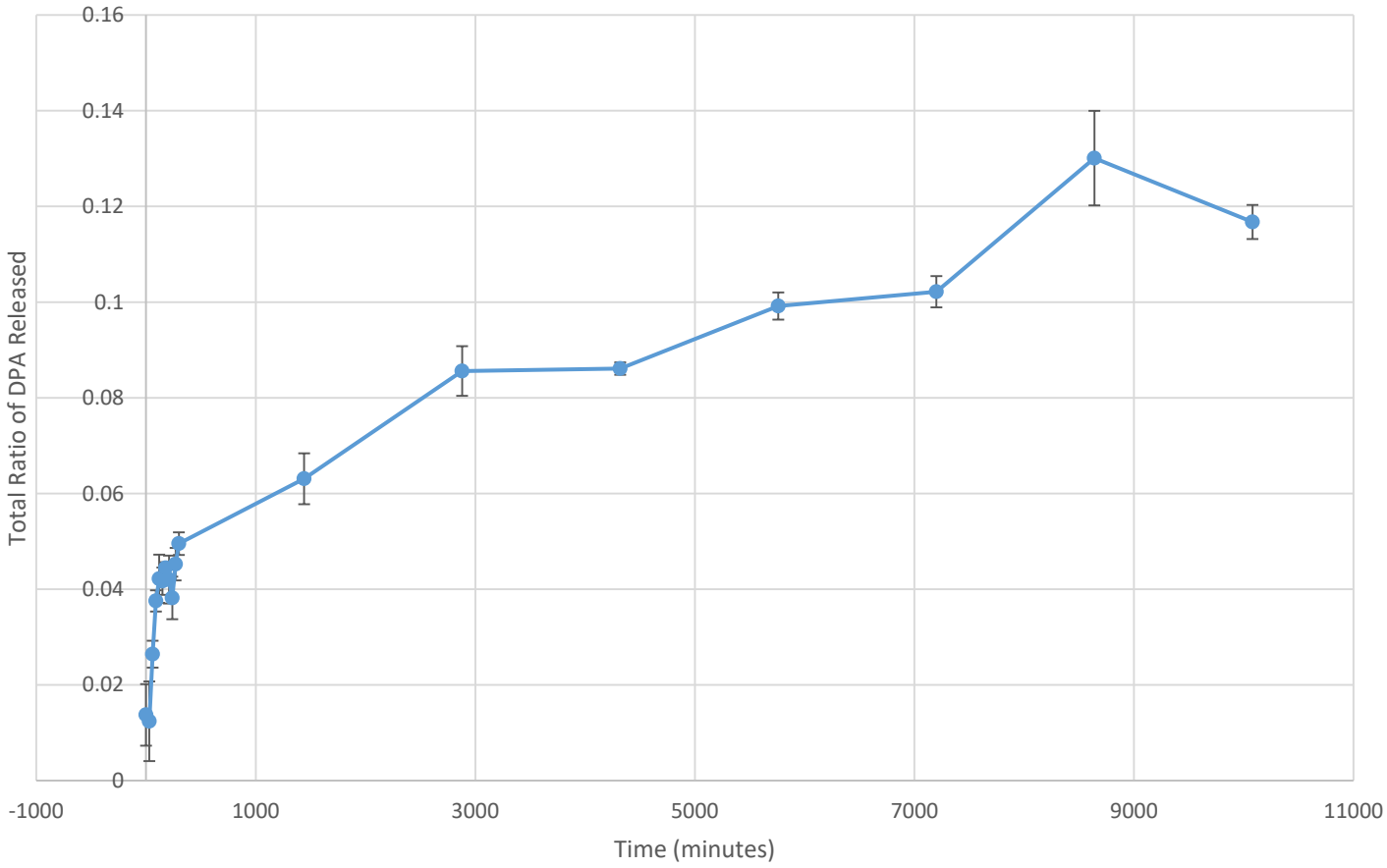

Figure 23: Ratio of total DPA released between 0 and 7 days for 25 mg/L N-NH4Cl and feed.

45 mg/L NH_4Cl; Feed

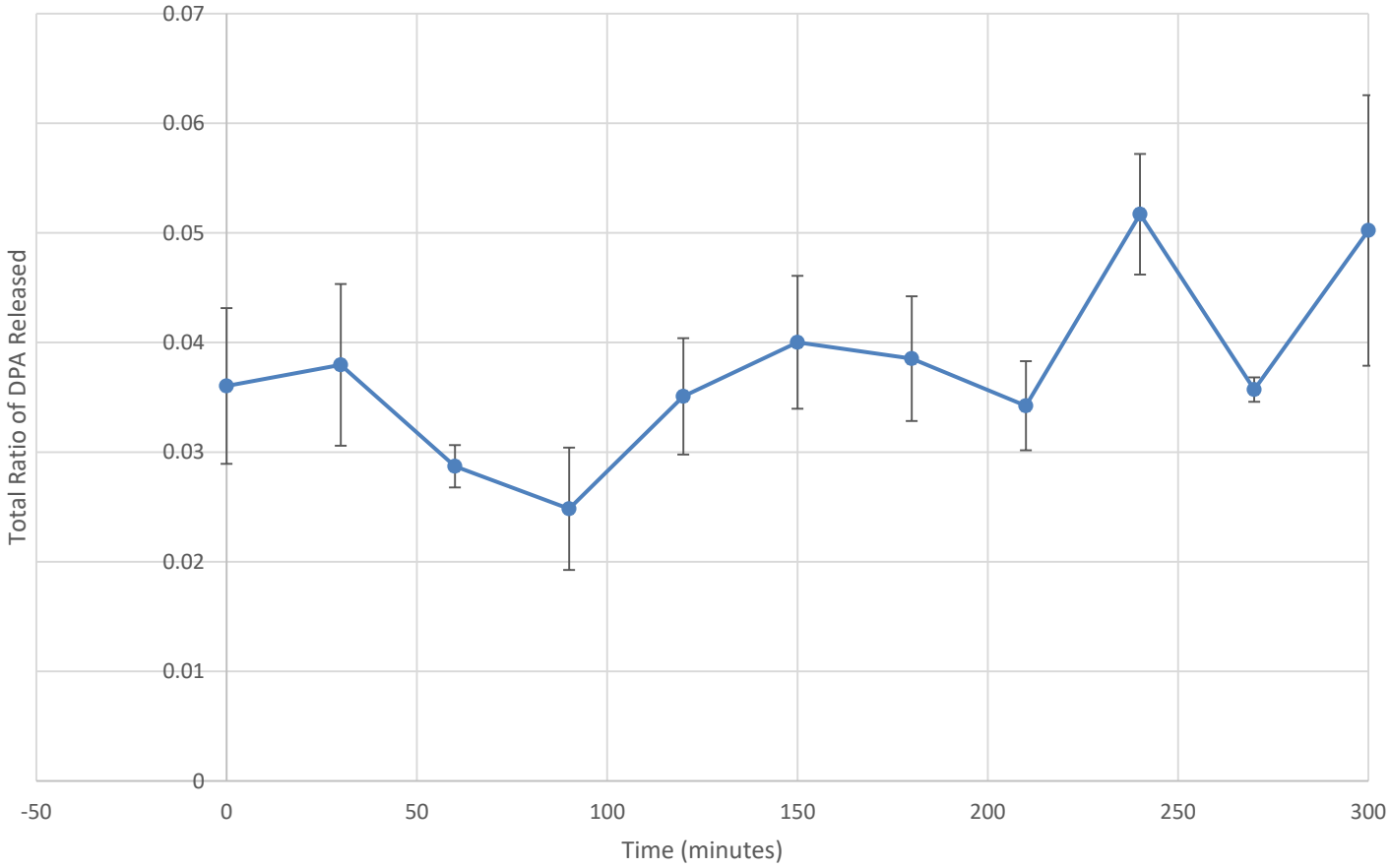

Figure 24: Ratio of DPA released between 0 and 300 minutes for 45 mg/L N-NH4Cl and feed.

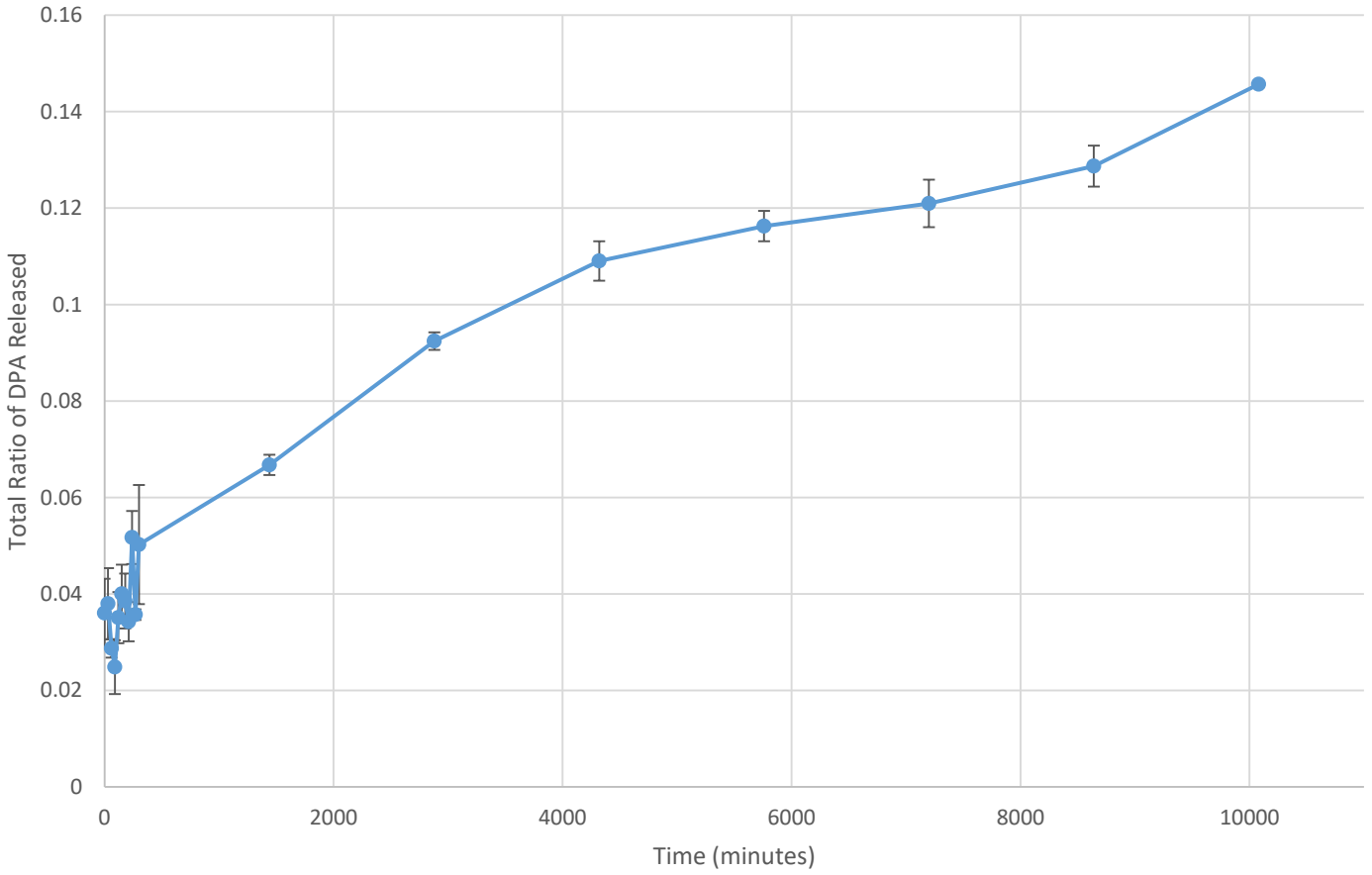

Figure 25: Ratio of total DPA released between 0 and 7 days for 45 mg/L N-NH4Cl and feed.

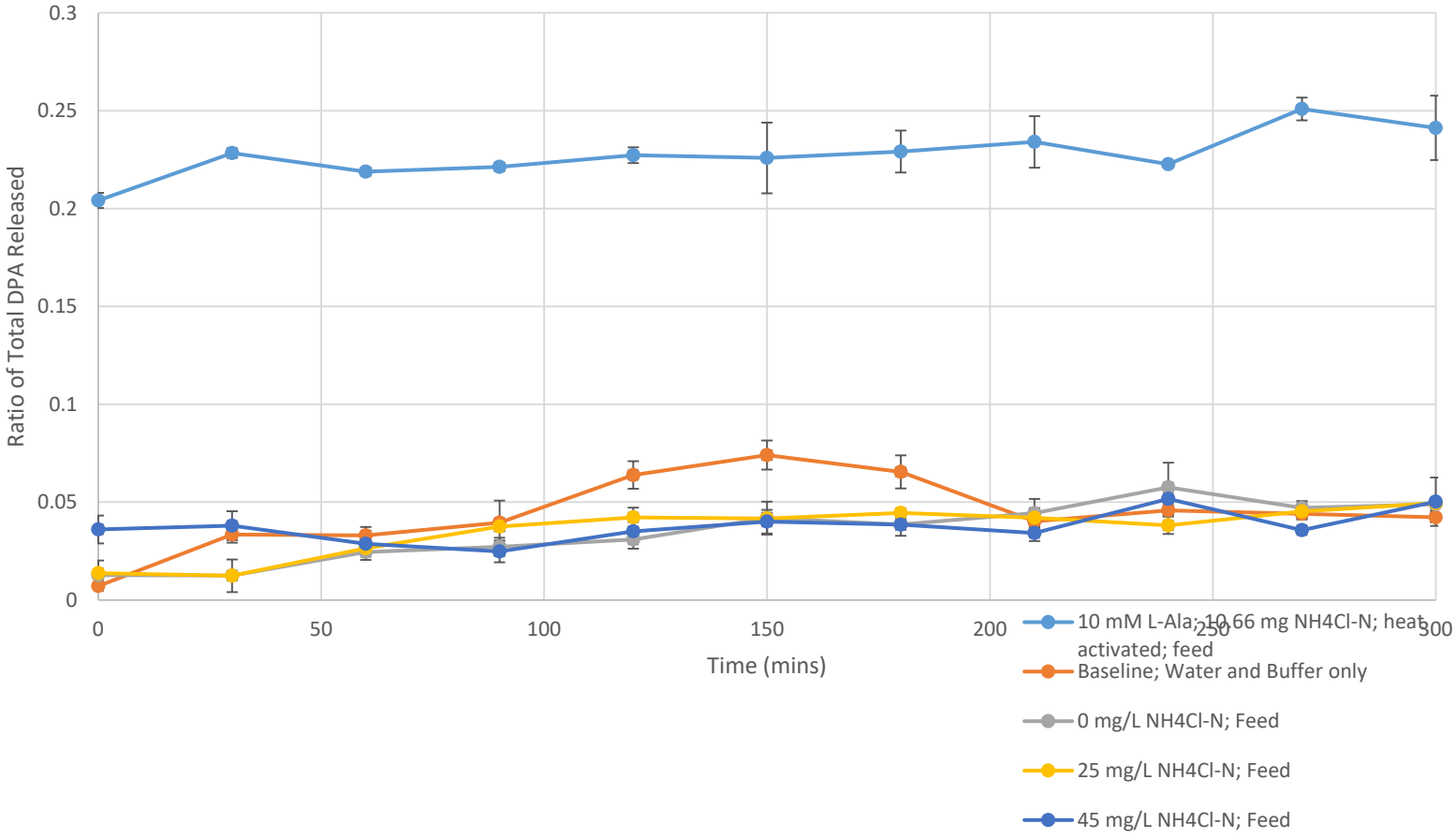

Figure 26: Ratio of total DPA released between 0 and 300 minutes.

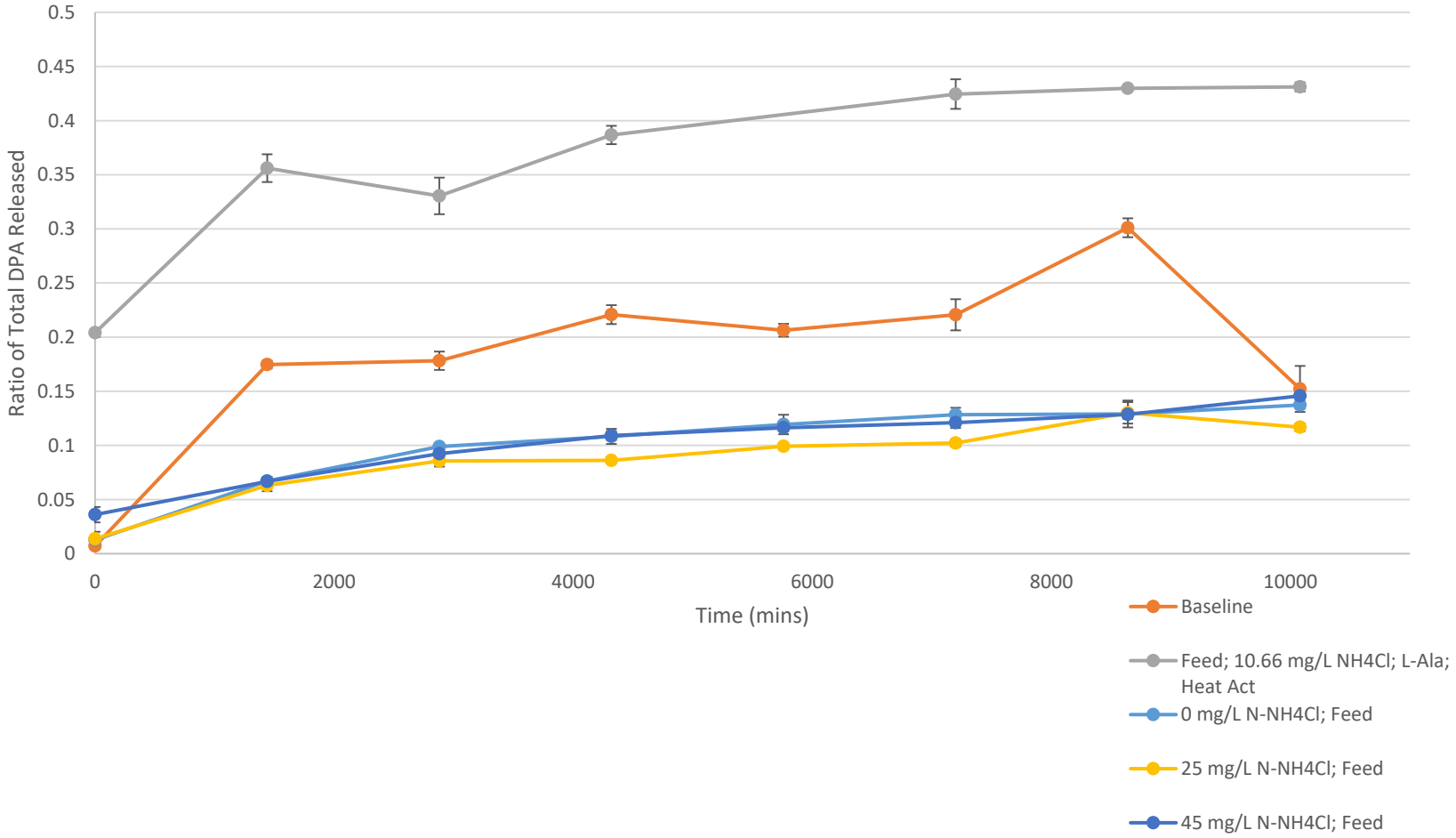

Figure 27: Ratio of total DPA released between 0 and 7 days.

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14. ABSTRACT

This research investigated the germination and outgrowth of *Bacillus globigii* in the presence of ammonia in laboratory scale experiments. Germination was measured by monitoring the release of dipicolinic acid, an organic compound present in bacterial spores, while outgrowth was measured using phase-bright microscopy and semi-automated counting procedures. The ammonia-N concentrations investigated generally did not cause statistically significant differences in the initial 1-hr germination rates or the average 3-hr outgrowth rates of *Bacillus globigii* spores in batch style experiments. The average 1-hr germination rates observed in the absence of ammonia-N was 0.0258 hr^{1} for the water and buffer controls and between 0.00732 and 0.0127 hr^{-1} with synthetic feed over a range of ammonia-N concentrations. The 3-hr outgrowth rates were 0.0761 and 0.1821 hr^{-1} for the buffer control and positive control respectively but it cannot be concluded that ammonia-N was responsible for this difference. Ammonia-N was correlated with subtle but statistically significant impacts over a 7-day period. The sodium bicarbonate present in the synthetic feed likely caused inhibition of the germination rates as well as the shape of the 7-day germination profiles. To this author's knowledge, this study is the first to investigate the effect of ammonia-N on the germination and outgrowth of *Bacillus globigii* in concentrations typically found in wastewater treatment plants and in the presence of synthetic feed.

15. SUBJECT TERMS
Bacillus globigii, spectrophotometry, germination, outgrowth, inhibition, biological warfare

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