INFLUENCE OF GROWTH RATE ON SILVER NANOPARTICLE ADSORPTION AND SILVER ION TOXICITY TO NITROSOMONAS EUROPAEAE CELLS CONTINUOUSLY CULTURED IN A CHEMOSTAT REACTOR

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by
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Influence of Growth Rate on Silver Nanoparticle Adsorption and Silver Ion
Toxicity to Nitrosomonas Europaea Cells Continuously Cultured in a Chemostat Reactor

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DEDICATION

Dedicated to my Mother, who always gives everything to ensure that her children have a bright future ahead of them.
The only true wisdom is in knowing you know nothing.

--Socrates
ABSTRACT OF THE THESIS

Influence of Growth Rate on Silver Nanoparticle Adsorption and Silver Ion Toxicity to Nitrosomonas Europaea Cells Continuously Cultured in a Chemostat Reactor

by

Kevin Anthony Csupak

Master of Science in Civil Engineering with a Concentration in Environmental Engineering
San Diego State University, 2013

This paper examines the effects of the adsorption of silver nanoparticles (AgNPs) and toxicity of silver ions (Ag⁺) on Nitrosomonas europaea that is continuously cultured within a chemostat reactor. N. europaea, an ammonia oxidizing bacteria (AOB), is used in the removal of nitrogen from industrial and sewage waste through the oxidation of ammonia (NH₃) to nitrite (NO₂⁻). Additionally it is important to note that AgNPs and Ag⁺ are also commonly found in industrial and sewage waste and therefore the study of their adsorption and toxicity to N. europaea bacterium is crucial.

In order to determine the influence of growth rates on AgNPs and Ag⁺ and their potential adsorption and toxicity to N. europaea cells, respectively, a small scale chemostat was built. This device continuously cultivates N. europaea cells inside of a reactor while constantly adding new growth media for the cells and extracting media at the same flow rate to regulate the internal volume of the reactor. Simultaneously, filtered air is bubbled into the system from a standard aquarium pump. Additionally a pH probe was installed to track the internal pH of the reactor. The oxidation of ammonia to nitrite results in the release of protons and a drop in pH. As the pH drops, the pH meter sends a signal to a peristaltic pump to add sodium carbonate to the system until the pH in the reactor is stable, (pH = 7.8). Once the desired pH is reached the pump shuts off. There are also ports on either side of the reactor that allow for the addition or extraction of media via a 30mL syringe. Flow in and out of the reactor is initially 0.3 mL/min for a first round of testing and is increased to 0.6 mL/min for a second round of testing, and the solution is constantly stirred to ensure the proper amount of agitation to prevent mass transfer limitations.

After cells were grown and the contents of the reactor reached steady state conditions, (as determined by constant cell densities and NO₂⁻ concentrations), cells were withdrawn from the reactor for adsorption and toxicity testing. Adsorption testing was completed using AgNPs and toxicity testing was completed using Ag⁺. After the first round of testing the chemostat flow rate was raised and kept constant at 0.6mL/min and brought to steady state. Adsorption and toxicity testing was redone using the new cells.
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CHAPTER 1

INTRODUCTION

The purpose of this project is to determine the effect of growth rate on the adsorption of AgNPs and toxicity of Ag\(^+\) to continuously cultured \textit{N. europaea} cells continuously cultured in a chemostat reactor. \textit{N. europaea} is an important ammonia oxidizing bacteria (AOB) commonly found in the treatment of industrial and sewage waste as well as other sources containing high levels of ammonium (NH\(_4^+\)), such as wastewater treatment plants (WWTPs) (Chain et al. 2003). AOB are vital for the removal of nitrogen from wastewater treatment plants through the oxidation of ammonia (NH\(_3\)) to nitrite (NO\(_2^-\)). This oxidation is the first step for converting NH\(_3\) found in wastewater streams to nitrogen gas (Figure 1.1).

Figure 1.1. Nitrogen cycle in WWTPs.

According to the World Health Organization (WHO), NH\(_3\) has a toxic effect on humans only if the intake into the body becomes greater than the human bodies capacity to detoxify the excess NH\(_3\) (WHO 1996). However, more concern lies in the eutrophication than human toxicity. Eutrophication is caused by the introduction of artificial substances to an aquatic system can lead to hypoxia, the depletion on oxygen in the water, which can lead to certain species of fish dying off (EPA 1993). One such substance of concern are AgNPs, which can be found in a variety of products including cosmetics, socks, food containers, detergents, sprays and a wide range of other products and are the most widely used
nanomaterial in the world (Williams 2013). AgNPs in water and are oxidized to Ag\(^+\) which may be toxic to bacteria (Figure 1.2).

![Figure 1.2. Lysing of bacteria by silver ions (Ag\(^+\)).](image)

Should the levels of AgNPs or Ag\(^+\) in wastewater streams become too high, the AOB found therein may be killed which could lead to the eutrophication of the receiving bodies of water. It is important to understand how long such a process takes. WWTPs have a retention time that is dependent on the size of the basin and the flow rate of influent. The value, known as the Hydraulic Retention Time (HRT) is equal to the volume of the basin divided by the flow rate (Waterwiki 2013). Since WWTPs typically have HRTs of a day (Burton et al. 2002). Because of this one would need to know what the long term effects of AgNPs and Ag\(^+\) would be on bacteria found within the same wastewater if the flow rate, and subsequently growth rate, were altered. Testing these effects on a smaller scale requires a reactor known as a chemostat (Radniecki and Lauchnor 2011), (Figure 1.3).

A chemostat, also known as a continuous growth reactor, provides an environment suited for cultivating bacteria and keeping bacteria levels stable by constantly adding in new growth media while at the same time removing waste effluent to keep the level of media within the chemostat at a constant volume. Additionally a chemostat can achieve a true steady state through the automated adjustment of pH and the continuous supply of oxygen gas (O\(_2\)) (Radniecki and Lauchnor 2011). This paper looks at the stages involved in creating a chemostat and continuously culturing *N. europaea*. After an initial batch growth stage, the
system is operated in continuously culturing mode and comes to a steady state condition in which the growth and activity of the cells has become constant. Once this is accomplished, cells are drawn from the reactor and used for AgNP adsorption and Ag$^+$ toxicity tests.
CHAPTER 2

METHODS

For this experiment several variables had to be considered. Firstly, a reactor needed to be built in order to grow bacteria that can be used for experimentation. Secondly, several different liquid medias needed to be made including growth media for the bacteria to feed on and a base solution to keep the pH in balance. Lastly, after achieving steady state within the reactor, adsorption testing using AgNPs and toxicity testing using Ag⁺ were performed on N. europaea cells grown at two separate flow rates, (0.3mL/min and 0.6mL/min). To determine when steady state conditions were achieved, daily measurements were taken to determine cell and NO₂⁻ concentrations inside the chemostat.

2.1 BUILDING THE REACTOR

Several materials are necessary to make a successful chemostat. A list of materials and their function can be found in Table 2.1. Most of the materials necessary for building a chemostat were purchased through scientific equipment manufacturers including Cole Parmer, (for pumps), and Fischer Scientific, (for media bottles and other essential lab items). However, there are a few items that could not be purchased and instead had to be made to fit the needs of the reactor. For the purposes of this experiment tri-port caps were necessary for placing on top of all bottles containing media, base solution of any other bottles that tubing feeds in or out of. For this project custom made caps were designed and manufactured by a student machine shop. The caps were made from 304 stainless steel and contain three separate ¼-28 tapped holes for tubing fasteners (Figure 2.1). These caps were designed using a combination of hand drafting techniques and solid modeling software.

Once all of the parts for the chemostat had been gathered the assembly was quite simple. The first step performed was the building of a table to put the chemostat on. For the purposes of this project, a spare laboratory cart was used. Next, the placement of the machinery was determined so that the operation of the chemostat could go on uninhibited. The cart used has a top and bottom rack which worked optimally since there are several
### Table 2.1. Materials List

<table>
<thead>
<tr>
<th>Material</th>
<th>Purpose</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L Dual arm Media Bottle</td>
<td>Main Reactor</td>
<td>1</td>
</tr>
<tr>
<td>2 L Media Bottle</td>
<td>Feed/Waste Bottles</td>
<td>4</td>
</tr>
<tr>
<td>1 L Media Bottle</td>
<td>Base Bottle</td>
<td>2</td>
</tr>
<tr>
<td>Steel Tri-port Cap</td>
<td>Funneling liquid/gas in/out of reactor and other bottles</td>
<td>8</td>
</tr>
<tr>
<td>Peristaltic Pump</td>
<td>Pumping media and base in/out of bottles</td>
<td>2 (one for base solution and one for media in/out flow)</td>
</tr>
<tr>
<td>Stir Plate</td>
<td>Mixing solution</td>
<td>1</td>
</tr>
<tr>
<td>pH Probe (7 inches)</td>
<td>Measuring pH</td>
<td>1</td>
</tr>
<tr>
<td>pH Meter</td>
<td>Monitoring pH levels</td>
<td>1</td>
</tr>
<tr>
<td>Aquarium Pump</td>
<td>Pumping in oxygen</td>
<td>1</td>
</tr>
<tr>
<td>30 mL Syringe</td>
<td>Filtering gas and adding/removing media</td>
<td>6</td>
</tr>
<tr>
<td>Polymer Tubing/cutter</td>
<td>Fitted through caps to remove/deposit media/gas</td>
<td>12-1 foot segments 12-6 inch segments</td>
</tr>
<tr>
<td>Flangeless Fittings Kit</td>
<td>Securing polymer tubing to caps and other fittings</td>
<td>1 Kit</td>
</tr>
<tr>
<td>Tubing Adapter Kit</td>
<td>Securing flangeless fittings and polymer tubing to flexible tubing</td>
<td>1 Kit</td>
</tr>
<tr>
<td>Silicon Tubing</td>
<td>Funneling media/base between polymer tubing</td>
<td>4 feet $\frac{1}{8}$” inner Dia. 6 feet $\frac{1}{16}$” inner Dia.</td>
</tr>
<tr>
<td>Peristaltic Pump Tubing</td>
<td>For Flow of media through peristaltic pumps</td>
<td>2-1 foot segments</td>
</tr>
<tr>
<td>Quick-Connect Kit</td>
<td>For fast disconnecting</td>
<td>1 Kit</td>
</tr>
</tbody>
</table>
Figure 2.1. Steel cap design.

pieces of equipment being used. The main reactor was placed on top of a stir plate on the bottom rack of the cart next to a peristaltic pump used for administering base solution to the reactor as well as next to the aquarium pump used to pump oxygen into the reactor. The feed, waste and base bottles were placed on the top rack of the cart along with a second peristaltic pump used for pumping media in and out of the main reactor. Additionally, a pH meter was placed on the top rack alongside the second pump (Figure 2.2). The caps were placed on all open media bottles and fitted with polymer tubing and pieces from the flangeless fittings kit as well as the tubing adapter kit and quick connect kit. Afterwards silicone tubing was used to connect all of the fittings and inserted through the pump heads. The final piece of equipment installed was the pH probe. A small hole was drilled into the cap of the reactor just big enough for the probe to fit through. To ensure a proper seal between the probe and the cap, a rubber O-rings was placed around the outside of the probe and coated with silicon.

The last step was to connect all components and turn on the reactor. To simplify the setup, a power strip was placed directly on the cart in order to get power to the pumps, stir plate and pH meter. Once the assembly of the chemostat was complete the experiment could move on to the second stage.

2.2 DEVELOPING MEDIA AND BASE SOLUTION

Once the chemostat was built, the media that feeds into the reactor, the reactor media itself and the base solution to balance the pH were made. The growth media that is present in
the reactor and the feed bottle is comprised of the same substances, however the feed bottle contains 2 L of fluid whereas the reactor volume is limited to 1.5 L. The base solution is composed of sodium carbonate (Na₂CO₃). The components and amounts of each media can be found in Table 2.2.

Once the batches of media were made up it was necessary to raise the pH of the media going to the reactor from 5.5 to 7.8 using 10 normal sodium hydroxide (10N NaOH). After this had been completed, the media was ready to be sterilized via autoclaving. The setting used on the autoclave for this experiment was fluid setting for 45 min (120 ºC, 15 psi) with no drying time. When placing the media bottles that need to be sterilized in the autoclave, it is important to remember to loosen the caps on the bottles so that pressure does not build up inside and destroy the bottles.

Finally, after the media had been made, properly autoclaved and given time to cool it was time to start with the next stage of the project which entailed growing *N. europaea* cells within the reactor and getting the reactor to steady state.
### Table 2.2. Growth Media and Base Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount Needed (g or mL)</th>
<th>Concentration in Chemostat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
<td><strong>Feed Bottle (2L)</strong></td>
<td><strong>Reactor (1.5L)</strong></td>
</tr>
<tr>
<td>Ammonia Sulfate (NH₄)₂SO₄</td>
<td>6.6g</td>
<td>4.95g</td>
</tr>
<tr>
<td>HEPES</td>
<td>14.3g</td>
<td>10.725g</td>
</tr>
<tr>
<td>Monobasic Potassium Phosphate KH₂PO₄ (40 ppm)</td>
<td>6.00mL</td>
<td>4.50mL</td>
</tr>
<tr>
<td>1M Magnesium Sulfate MgSO₄ 7H₂O</td>
<td>13.14mL</td>
<td>9.855mL</td>
</tr>
<tr>
<td>1M Calcium Chloride CaCl₂ 2H₂O</td>
<td>3.60mL</td>
<td>2.70mL</td>
</tr>
<tr>
<td>30mM Iron Sulfate (pH: 8) FeSO₄ 7H₂O</td>
<td>5.94mL</td>
<td>4.455mL</td>
</tr>
<tr>
<td>50mM Copper Sulfate CuSO₄ 5H₂O</td>
<td>0.234mL</td>
<td>0.1755mL</td>
</tr>
<tr>
<td>Deionized Water (DI)</td>
<td>2L</td>
<td>1.5L</td>
</tr>
<tr>
<td>Sodium Carbonate Na₂CO₃</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.3 Cell Growth

The process of adding *N. europaea* cells to the chemostat reactor was done inside a laminar flow hood to ensure that sterility was maintained. At this stage, the only bottles that were involved were the reactor and the base solution bottle. Along with these two bottles, a sterile 30mL falcon tube, tweezers, a bottle of ethanol, a sterile transfer syringe, a 35mL pipette, a pipette bulb, the pH probe, a small O-ring to fit around the probe and the silicone sealant were placed inside of the hood before sterilizing the entirety with ultraviolet light.
Once the UV light had sterilized the inside environment of the laminar fume hood for 10 minutes the light was turned off and the *N. europaea* cells that have been previously cultivated in batch reactors were brought into the hood for inoculation of the chemostat media. Several steps were carefully followed in order to inoculate the chemostat with *N. europaea* cells. Those steps are as follows:

1. Spray down laminar flow hood with ethanol and wipe down with a clean paper towel.
2. Ensure that all items listed above have been inserted into laminar flow hood and exposed to ultraviolet light for at least 10 minutes.
3. Spray gloves and tweezers with ethanol and remove covering of bottle with cultured cells using tweezers making sure nothing touches your skin.
4. Remove sterile pipette from its packaging and attach pipette bulb to end.
5. Withdraw 30mL of cells from the bottle containing the cells and place into the 35mL sterile falcon tube.
6. Remove pipette bulb, reinsert sterile pipette back into its packaging and use tweezers to place stopper back onto the cultured media bottle.
7. Transfer 30mL of cells from falcon tube into sterile syringe. Attach syringe to quick connect port and insert cultured cells into fresh media inside of reactor.
8. Take pH probe and douse in ethanol. Place O-ring around upper portion of pH probe and insert probe into hole drilled into reactor cap.
9. Secure the cap on the reactor ensuring that the probe is not touching any of the polymer tubing inside of the reactor.
10. Once the cap is in place take the silicone sealant and form a silicone ring around the probe to ensure that there is a good air tight seal.
11. Connect the base solution tubing to the corresponding port on the reactor.
12. Remove the bottles from the laminar flow hood and place them on their appropriate locations on the cart, base bottle on top and reactor on bottom on top of the stir plate.
13. Start the stir plate and make sure the cells are spinning sufficiently. Make sure the aquarium pump is on and adding oxygen to the system as the media is spinning.
14. Hook the base bottle/reactor tubing through the lower peristaltic pump so that the pH meter can automatically adjust the pH to 7.8.

After these steps were taken the cells were grown in a batch phase (with no in or out flow) for 3 to 4 days to allow the cells to grow to a substantial population. After the initial growth period had passed, the feed and waste tubes to the reactor were connected and the flow was turned on. As the pumps flow, there will be a requirement to change out the feed and waste bottles every so often. This replacement time depends on the flow rate of the
pump. For this experiment, the first set of testing required the bottles to be replaced every 4.6 days. The second round of testing saw an increased growth rate at 0.6 mL/min rather than 0.3 mL/min and required replacing the bottles every other day.

The last portion of this stage was monitoring the system until the media and cells in the reactor reached steady state. This point occurs once the numbers of cells and the amount of NO$_2^-$ produced have leveled out within the reactor. In order to determine if the reactor had reached steady state, samples were taken from the reactor every day and run through an UV-vis spectrophotometer. Only 1mL of cells was required for the test. Separate measurements were made for optical density, which gave an idea of how many cells are in the media, and for NO$_2^-$ production. The optical density of the cells was measured at 600nm while the NO$_2^-$ level was determined from taking measurements at absorption levels of 352nm and 400nm. In order to find the NO$_2^-$ value the following equation is used: \[
\frac{(A_{352}-A_{400})}{(0.0225)}\]. Initially, it took 15 days for the reactor to reach steady state. When it did reach steady state, the experiment moved on to the final stage, testing for adsorption and toxicity.

2.4 Adsorption and Toxicity Testing

The final stage of the experiment involved taking the cells that had been acclimated to steady state conditions within the reactor and putting them through two different tests. The first test involved adsorption.

2.4.1 Adsorption

The purpose for testing adsorption was to determine how much of the AgNPs added to the samples would attach to the grown cells and what concentration of AgNPs would be left over in effluent. Additionally, this test showed how the macromolecule Bovine Serum Albumin (BSA) influenced the adsorption of AgNPs to the cells. This test was administered in triplicate with each bottle containing the following: 10mL of continuously cultured cells, 4mL deionized (DI) water, 15µL AgNPs (gives an overall concentration of 1ppm), 600µL BSA 1000 ppm stock (final concentration in bottle of 40 ppm) and 0.286µL 30mM HEPES at pH 7.8.

There was also a triplicate bottle set used for a control. These bottles contained everything that the test bottles did with the exception of cells. In place of cells, sterilized growth media was used. Additionally, two bottles were used for baseline measurements.
These bottles contained the exact contents of the triplicates they represent with the exception of the AgNPs. The purpose of the baselines was to eliminate any background interference that may be had from constituents in the different test media triplicate bottles. The BSA is a model protein that was used to coat the AgNPs to prevent aggregation and helped to simulate normal conditions seen in a WWTP. The adsorption study lasted 3-hours and proceeded as follows:

1. Three individual bottles are to be used as batch reactors containing a total of 15mL of solution, including cells. Three more bottles are used to represent a control for the experiment and contain growth media with no cells.
2. Add DI water and AgNPs and place on a shaker table at 250 rpm for 15 minutes.
3. At a time of -30 minutes, (30 minutes prior to adding cells), add BSA and HEPES solutions and place back on shaker for 30 minutes.
4. After 30 minutes, (time = 0), add the cells to the mixture.
5. Remove 1mL aliquot from each bottle and place in a 1.5mL centrifuge tube.
6. Spin tubes in micro-centrifuge for 1 minute.
7. Remove centrifuge tubes and add 600µL of supernatant into new 1.5mL centrifuge tube using a low volume pipette (20-200µL) with a small orifice on the tip.
8. Remove remainder of supernatant and discard, taking precaution not to remove the cell pellet.
9. Measure the supernatant on the UV-vis spectrophotometer from wavelengths of 300 nm to 700 nm.
10. Repeat steps 5-9 at minutes 45, 90, 135 and 180, making sure to place the bottles back on the shaker table between each reading.

Once these steps were completed, the data was compiled in Excel and the total adsorption of AgNPs onto the cells was calculated by measuring the samples on the UV-vis spectrophotometer. The concentration of AgNPs can be measured using the adsorption reading at 400 nm. Additionally, the cells were spun down to prevent trace metals and macromolecules from remaining in solution and altering the absorbance readings of the samples. From these readings the max peak height of each bottles absorbance between 300 nm and 700 nm was compared to a previously made standard curve. The difference in values between the control and the test bottles over the duration of the test showed the percent adsorption of the AgNPs to the cells based on the decrease in mass of the AgNPs.

Comparisons between the overall absorbance, the Full Width at Half Maximum (FWHM) (National Optical Astronomy Observatory 2012) and the amount of AgNPs in samples all
over a 3-h time span were made. This test was conducted twice for the purposes of this report. The first test commenced after the cells had been grown given a media flow rate of 0.3 mL/min. The second test was conducted after the cells had been growing at double the original growth rate, (0.6 mL/min).

2.4.2 Toxicity

The next set of testing measured how Ag⁺ toxicity varied based on the growth rate of the continuously cultured cells. For this test a larger volume of silver was required so for the sake of being as economically feasible as possible, Ag⁺ will be used in place of AgNPs. There are several differences in preparation of this test from the adsorption study. In this experiment there are triplicate control bottles and triplicate bottles for four different concentrations of Ag⁺ (.05 ppm, .10 ppm, .15 ppm and .20 ppm), taken from a 100 ppm stock solution. Each bottle, excluding the controls, contained cells, (1.241 mL for test 1 and 1.5 mL for test 2), that had been washed and suspended in 30mM HEPES solution (pH = 7.8). The cells were washed to prevent trace metals and other substances from interfering with the test results. Since these cells were centrifuged and suspended in HEPES, the amount to be added to each bottle was determined by taking an OD₆₀₀ reading of 500µL of cells in 15mL of HEPES buffer (pH 7.8) and adjusting the number of cells added to the bottle to an OD₆₀₀ value of 0.072. All test bottles contained 14mL DI water, Ag⁺, cells and 1 mL 15x HEPES solution. The 15x HEPES solution is a 15 times concentrate containing 450 mM HEPES buffer and 37.5 mM (NH₄)₂SO₄. When added to the bottles it resulted in a final concentration of 30 mM HEPES and 2.5 mM (NH₄)₂SO₄. The purpose of the 15x HEPES was to act as a buffer. The procedure for toxicity testing is as follows:

1. This test required triplicate bottles containing a control and individual triplicates for bottles representing 0.05 ppm, 0.10 ppm, 0.15 ppm and 0.20 ppm Ag⁺.
2. Fill control bottles with 14 mL of DI water remembering to fill another bottle with 15 mL of DI water for the original OD₆₀₀ reading. Add 13.925 mL, 13.850 mL, 13.775 mL and 13.700 mL to the 0.05 ppm, 0.10 ppm, 0.15 ppm and 0.20 ppm Ag⁺ triplicates respectively.
3. Add 75 µL, 150 µL, 225 µL and 300 µL of 100 ppm Ag⁺ stock solution to the 0.05 ppm, 0.10 ppm, 0.15 ppm and 0.20 ppm Ag⁺ triplicates respectively. Add 15x HEPES solution and cells to each bottle and place on shaker table at 250 rpm. Each set of triplicates should have the cells added in a staggered order, (one set of triplicates gets cells and put on shaker, the next triplicates gets cells 3 min later and so on).
4. Samples were taken every 45 min for 3-h with the bottles remaining on the shaker table in between readings.

5. The setup for testing involved a number of 1.5 mL microcentrifuge tubes. This test involved 15 bottles and 1 tube is needed for every bottle at every reading interval as well as a main tube for depositing samples in, (75 tubes in total). Additionally, 3 tubes were required as blanks for overall media samples and 6 more were needed as blanks with the test samples. The purpose of the tubes is to house samples to be used in a colorimetric NO$_2^-$ assay.

6. Each tube should be labeled with the number bottle it corresponds to and the time interval that it is for.

7. Place 890 µL of 1% (w/v) sulphanilamide in 1M hydrochloric acid (HCl) and 100 µL of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) into the microcentrifuge tube.

8. Add 10 µL of test solution into the microcentrifuge tube and close tube. Mix by placing on vortex for 10 seconds.

9. Repeat steps 6-8 for every bottle at 45, 90, 135 and 180 min.

10. This should be repeated in the same staggered fashion that the cells were added to the bottles; a new set of triplicates every 3 min.

11. Once microcentrifuge tubes containing samples have sat for 10 min place 200 µL of each sample into a 96 well NO$_2^-$ assay plate.

12. Make sure to add the samples from the blanks as well. These blanks should contain 890 µL sulphanilamide, 100 µL NED and 10 µL DI water.

13. On a separate NO$_2^-$ assay plate place 200 µL of each of the final bottle concentrations as well as three blanks of only DI water.

14. Place the trays into a reader and record at 540 nm ($A_{540}$).

15. Compare results against a pre-existing standard curve. This provides data on NO$_2^-$ production which provides data on the toxicity of Ag$^+$ on the cells.

This experiment was conducted once at a slower growth rate and once at a higher growth rate (0.3 mL/min and 0.6 mL/min). The purpose of testing at two different growth rates is to see if the number of cells produced will have an effect on the overall toxicity of Ag$^+$ to the chemostat media.
CHAPTER 3

TESTING RESULTS

Three areas of testing were performed in this project; cell growth, adsorption and toxicity. Each test was performed using varying flow rates, (0.3 mL/min for the first set of testing and 0.6 mL/min for the second set of testing). All testing was performed on cells extracted directly from the reactor.

3.1 CELL GROWTH

After inoculation, the cells were given four days to come to maturity in batch growth. During this time, the pH meter kept track of the internal pH of the reactor. When the pH dropped below 7.8 the base pump turned on and buffered the system with 50mM Na₂CO₃. This buffer solution was used since it supplies carbon to the reactor for *N. europaea*.

The dropping of the pH was a sign that the cells were growing. As the cells grow they oxidize NH₃ to NO₂⁻ while releasing H⁺ into the reactor, which lowers the pH. Once the cells had grown for four days and oxidized all available NH₃ to NO₂⁻, the feed and waste tubes were attached and the pumps were turned on to get the system to come to steady state. The time necessary for the reactor to reach steady state is based on the HRT of the reactor. This value was found by dividing the reactor volume (1.5L) by the flow rate (0.3 mL/min and 0.6 mL/min) (Waterwiki 2013). Steady state occurs after approximately 3 HRTs have been passed (Jensen 2001). The initial flow rate into and out of the chemostat was 0.3 mL/min which gives a HRT of 3.47 days, equating to steady state after approximately 11 days. At this flow rate, there was a considerable amount of fluctuation in the levels of cells and NO₂⁻ that were detected in the system. After initial adsorption and toxicity testing had been done on cells grown at the initial flow rate of 0.3 mL/min, the flow rate was doubled to a value of 0.6 mL/min. The flow rate of 0.6mL/min had a HRT of 1.74 days, equating to steady state after 5.5 days. Every day, a sample was taken from the reactor and cell densities and NO₂⁻ concentrations were quantified. The results of these sample readings for the duration of cell growth can be found in Figure 3.1 and 3.2.
Figure 3.1. Cell density in the chemostat. Cell absorbance for days 1-14 represents a flow rate of 0.3 mL/min and days 15-23 represent a flow rate of 0.6 mL/min.

Figure 3.2. NO$_2^-$ levels in the chemostat. Readings from days 1-14 represent a flow rate of 0.3 mL/min and days 15-23 represent a flow rate of 0.6 mL/min.

As can be seen from Figure 3.1, (possibly due to plugging of the outflow that occurred during testing), the cells struggled to reach a steady state within the period when the flow rate was 0.3 mL/min., (through day 14). However, once the flow rate was increased to
0.6 mL/min., the cells came to a steady state condition much faster due to the fact that the HRT for 0.6 mL/min was half as long as it was for 0.3 mL/min. From day 15 through day 23, the cell concentration in the reactor reached a consistent steady-state level. Figure 3.2 shows a very similar pattern with NO₂⁻ levels. From the beginning of sampling through day 15 the level of NO₂⁻ was around 50mM with 6-8%. Once the flow rate was altered to 0.6 mL/min the NO₂⁻ levels reached steady state very quickly and maintained approximately 50 mM the entire time exposed to that rate. On average the cell concentration and NO₂⁻ levels were fairly consistent, despite the change in flow rate, (which corresponds to a change in growth rate). This shows that both growth rates were able to produce enough cells to oxidize NH₃ to NO₂⁻. However, the cells produced from an increased growth rate were able to oxidize NH₃ to NO₂⁻ with fewer cells making 0.6 mL/min a more efficient flow rate in terms of oxidation. This shows that as growth rate increases the cells work more efficiently at oxidizing NH₃ to NO₂⁻ and therefore see a drop in concentration.

3.2 ADSORPTION

The purpose of this testing was to determine how *N. europaea*’s growth rate influences the fate and transport of AgNPs once exposed to the cells. This is a modest representation of how AgNPs might be transported through a WWTP. Testing was done on cells cultured at both flow rates.

Adsorption test 1 was conducted with cells cultured at a flow rate of 0.3 mL/min. Sample readings were taken every 45 min starting after the test bottles and control bottles had been on a shaker table for 90 min. The results from the absorbance readings for bottles without and with *N. europaea* cells present can be seen in Figures 3.3 and 3.4, respectively.

Figures 3.3 and 3.4 show that while both sets of triplicate bottles have similar max peaks appearing at 400 nm, the actual absorbance at 400 nm differs. The control bottles have a max absorbance around 0.10 while the bottles with cells show a max absorbance of 0.07. The absorbance is related to the AgNPs still in solution, thus Figures 3.3 and 3.4 show that the lowering of the max absorbance in cell bottles is due to a lowering of the AgNPs in solution. This suggests that the AgNPs are adsorbing to the *N. europaea* cells.

Based off of these graphs, the Full Width at Half Maximum (width of the absorbance peaks at half of the maximum absorbance reading), or FWHM can be made. A constant
Figure 3.3. Absorbance for control bottles with 1 ppm AgNP and no *N. europaea* cells present. The peak absorbance relates to the AgNPs in solution and is seen at just over 400 nm.

Figure 3.4. Absorbance for test bottles containing 1 ppm AgNPs and *N. europaea* cells. The peak absorbance relates to the AgNPs in solution after adsorption to *N. europaea* cells and is seen at just over 400 nm.
FWHM indicates that the AgNPs are not aggregating. However, an increase in FWHM indicates an aggregation of the AgNPs in solution. Since the FWHM values for both sets of triplicates were almost identical (Figure 3.5) it was assumed that there was no aggregation of AgNPs.

![Figure 3.5. FWHM from 3-h adsorption study 1. There is little difference is seen between FWHM values of the different triplicates. This suggests that the loss of AgNPs seen in Figure 11 is due to adsorption to the cells and not to aggregation.](image)

As can be seen from the graph in Figure 3.5 the FWHM values for both the control bottles and the test media bottles are almost identical. Additionally the percent error is so low, (approximately 2%). This suggests that there is no aggregation of AgNPs in the bottles with the cells which implies that any AgNPs lost is attributed to adsorption to the cells. The amount if AgNPs that have been adsorbed onto the *N. europaea* cells can be seen in Figure 3.6.

Adsorption test 2 was conducted using cells continuously cultured at a flow rate of 0.6 mL/min. The same conditions were observed for this adsorption study as were observed in the first study. For this particular test, UV-vis spectrophotometer readings were taken every 45 min starting at a time of 0 min. The absorbance results from both sets of triplicates in adsorption test 2 can be seen in Figures 3.7 and 3.8. As can be seen from both graphs, the peak width of both the control bottles and the test bottles do not differ a great deal. However,
Figure 3.6. Mass of AgNPs during 3-h adsorption study 1 left in solution after adsorbing to the cells. Approximately 30% of the AgNPs adsorbed to *N. europaea*.

Figure 3.7. Absorbance for control bottles from test 2 containing 1 ppm AgNPs and no *N. europaea* cells. The peak absorbance relates to the AgNPs in solution and is seen at just over 400 nm.
Figure 3.8. Absorbance for test bottles from test 2 containing 1 ppm AgNPs and *N. europaea* cells. The peak absorbance relates to the AgNPs in solution and is seen at just over 400 nm.

the peak height for the control bottles is 0.10 whereas the peak height for the test bottles varies from 0.07 to 0.09, showing a drop in the AgNPs left in solution after contact with the cells.

As before, the data from these graphs was utilized to find the FWHM of both sets of bottles, (Figure 3.9). Figure 3.9 shows the FWHM values for the control bottles and the test bottles did not differ a great deal as time progressed in the experiment.

Thus, it can be inferred that the decrease in maximum peak absorbance (Figure 3.8) is due to AgNPs being adsorbed on the cells and not aggregating. Therefore, the drop in AgNPs show in Figure 3.10 is due to the adsorption of AgNPs to *N. europaea*. Figure 3.10 also shows an approximate 30% drop in the concentration of AgNPs which is quite similar to the results seen in adsorption test 1 (Figure 3.6).

### 3.3 Toxicity

The final stage of testing was to determine the toxicity of Ag$^+$ to *N. europaea* cells continuously cultured at flow rates of 0.3 mL/min and 0.6 mL/min. These tests are a modest way of determining how toxic Ag$^+$ may be to *N. europaea* cells found in WWTPs.

Toxicity test 1 was conducted using cells that had been brought to steady state using a flow rate of 0.3 mL/min. This test determined the overall toxicity of the Ag$^+$ to the cells by
Figure 3.9. FWHM from 3-h adsorption study 2 showing that little difference is seen between FWHM values of the different triplicates. This suggests that the loss of AgNPs seen in Figure 3.6 is due to adsorption to the cells and not to aggregation.

Figure 3.10. Mass of AgNPs during 3-h adsorption Study 2 left in solution after adsorbing to the cells. Approximately 30% of the AgNPs adsorbed to *N. europaea*. 
measuring the production of NO$_2^-$ throughout the 3-h test period. All measurements were done in triplicate. Triplicate bottles were used including a control condition (0 ppm Ag$^+$) as well as conditions containing Ag$^+$ concentrations of 0.05 ppm, 0.10 ppm, 0.15 ppm and 0.20 ppm. Samples were taken every 45 min from each bottle and tested for NO$_2^-$ production (Figure 3.11).

![Figure 3.11. NO$_2^-$ production per mg of protein in toxicity test 1. This shows that despite increasing the concentration of Ag$^+$, there is still NO$_2^-$ produced by the cells.](image)

As can be seen in Figures 3.11 and 3.12, increased Ag$^+$ concentrations had little effect on the nitrification activity of the *N. europaea* cells. The readings showing an overall percent nitrification, seen in Figure 3.12, were taken at the end of the 3-h test.

Toxicity test 2 was performed using *N. europaea* cells that had been continuously cultured at a flow rate of 0.6 mL/min. Consequently, the growth rate was increased as the flow rate increased. All other variables were kept constant so that a comparison on how growth rate affects Ag$^+$ toxicity could be determined with as little error as possible. Figures 3.13 and 3.14 show the NO$_2^-$ production and nitrification activity from this test, conducted with *N. europaea* cells continuously cultured at 0.6 mL/min.
Figure 3.12. Nitrification percentages of *N. europaea* cells continuously cultured at 0.3 mL/min with constantly increasing concentrations of Ag$^+$. Even with 0.20 ppm Ag$^+$ present there is still only a 20% drop in nitrification meaning the cells are highly resistant to Ag$^+$ toxicity at the existing concentrations.

Figure 3.13. NO$_2^-$ production per mg of protein in toxicity test 2. This shows that increasing the concentration of Ag$^+$ quickly halted the production of NO$_2^-$ from cells continuously cultured at 0.6 mL/min.
Figure 3.14. Nitrification percentages of *N. europaea* cells continuously cultured at 0.6 mL/min with constantly increasing concentrations of Ag$^+$. Note the 50% drop in %Nitrification after adding only 0.05ppm Ag$^+$. This conclusively shows that growth rate has affected the cells ability to withstand the toxicity of Ag$^+$.

It can clearly be seen that the change in flow rate (and thus growth rate) has drastically affected the degree to which Ag$^+$ are toxic to *N. europaea*. As can be seen from Figure 3.14, even the lowest tested concentration of Ag$^+$ had almost 50% inhibition to the cells, compared to the testing performed on cells grown at 0.3 mL/min which resulted in only 15% inhibition at the lowest Ag$^+$ concentration (Figure 3.12).
CHAPTER 4

SUMMARY AND RECOMMENDATIONS

The main purpose of this research was to determine the optimal conditions to grow *N. europaea* cells in a chemostat, determine how AgNPs adsorption to *N. europaea* cells is influenced by their growth rate and determine how Ag⁺ toxicity is affected by the growth rate of continuously cultured *N. europaea* cells. In testing for these factors the only condition that was altered between testing periods was the flow rate in and out of the reactor.

Cell growth and nitrification activity were the first aspects to be measured in the chemostat and the results from altering the flow rate are clearly evident when observing the trends found in Figures 3.1 and 3.2. The cells were initially grown and brought to steady state conditions at a flow rate of 0.3 mL/min. There was a fairly steady amount of NO₂⁻ being produced at this time, around 50 mM. However, difficulties arose in keeping the cell density stable through day 14. This up and down trend of cell density is believed to be attributed to the fact that an extra diverter valve had originally been placed on the outflowing waste stream. This valve, even though kept open, had a very small opening for waste to get through and as a result the outflow continually became clogged. Once this was discovered the valve was removed and flow within the reactor stabilized. After stabilizing the reactor testing for adsorption and toxicity was conducted. Once this testing was complete the flow rate was doubled to 0.6 mL/min. At this stage the analog peristaltic pump being used to control the flow rate was switched out for a digitally controlled peristaltic pump. With the previous pump installed it seemed very likely that the knob controlling flow rate could be bumped too easily resulting in a completely altered flow rate. This is also a possible explanation as to why there was variation in cell density throughout the first 2 weeks of operation. However, once the flow rate was increased and a more precise pump was installed the cell concentration and NO₂⁻ production stabilized very quickly. This trend was seen from day 14 through the end of testing.

When comparing the overall amount of AgNPs left in solution after completing a 3-h adsorption study at both flow rates, it can be seen that there was approximately a 30%
decrease in AgNPs throughout the tests (Figures 3.6 and 3.10). This would suggest that the flow rate (and thus growth rate) had little to no effect on the adsorption of AgNPs to N. europaea. Additionally, since the FWHM values for the control bottles and test bottles in both adsorption studies were very close to equal it can be inferred that the AgNPs were adsorbed to the cells and did not aggregate. This is most likely due to using BSA to coat the AgNPs in the hope of keeping them from aggregating.

Toxicity testing was the final stage of this experiment. Ag⁺ were used in place of AgNPs for this study and the only factor varying between testing periods was the cells were grown at different rates. When comparing results from both rounds of testing, a drastic difference in the toxicity of Ag⁺ to the cells was seen. From Figures 3.11 and 3.12, which represent data using cells from the lower flow rate, we can see that there was very little inhibition of the cells with increasing Ag⁺ concentration and as a result the cells continued to produce NO₂⁻. However, when comparing these to Figures 3.13 and 3.14 we see a very dramatic difference. The increased flow rate seems to have made the cells much more susceptible to Ag⁺ toxicity. Even at the lowest concentration of Ag⁺ the nitrification is already down to 50%.

It is clear that increasing the flow rate (and thus increasing the growth rate) made the N. europaea cells far more vulnerable to the toxicity of Ag⁺. In theory this may be due to the fact that as the flow rate increases there is more cell activity in the reactor and thus more enzyme production. The increased enzyme production may lead them to be more vulnerable to toxins such as Ag⁺. However, much more testing has to be done in order to determine exactly why cells grown at a faster growth rate are more susceptible to Ag⁺ toxicity.

Seeing as some of the testing showed no change in results from using differently grown cells (adsorption studies) and some of the testing did show change (toxicity studies), it is recommended that many more variations of this testing be done in the future. If growth rate is to be a factor considered in adsorption and toxicity testing then it would be wise to conduct the same testing at even more growth rates such as 0.1 mL/min, 0.45 mL/min or even as high as 0.9 mL/min, (with doubling times of 10.4, 2.31 and 1.15 days respectively) so that more data points can be compared and a better conclusion can be made about how altering flow rate of the chemostat effects the cells. Additionally small modifications could be made to testing such as using AgNPs in toxicity experiments or even seeing how higher
Ag\(^+\) concentrations effect the nitrification ability of even slower grown cells. It may also be beneficial to see how dosing the cells that are still in the reactor would affect this testing, since all of the testing done for this report involved first extracting the cells from the chemostat.

All in all, it can be seen that the building and operating of a small scale chemostat takes a lot of time and many small adjustments to the reactor in order to keep it running at maximum efficiency. With time, the procedures for operating and altering conditions of the chemostat can be optimized to provide the most accurate results in studies similar to the ones performed in this project.

In conclusion, the building of the chemostat and the growing of *N. europaea* cells was quite successful. Testing for adsorption and toxicity proved beneficial to the understanding of how AgNPs or Ag\(^+\) interact with the *N. europaea* and results were obtained with very little error suggesting that the overall project and experiments have room for potential growth in the future.
REFERENCES


