INVESTIGATION OF DAIRY WASTEWATER USING BIOWISH™

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ABSTRACT

Investigation of Dairy Wastewater Using BiOWiSH™

Ada Chan

Various bacterial products from BiOWiSH™ Technologies have been tested in dairy wastewater experiments to determine the bacterial mixes' ability for enhanced degradation of biochemical oxygen demand (BOD), solids, and nitrate concentrations. The dairy wastewater was augmented with various bacterial composition obtained from BiOWiSH™. The bacterial mixes experimented were US Aqua, Thai Aqua, BMT, Osprey, Fruit Wash, KLB, LCM1, and MDG. Method development was a crucial process to optimize and test the effects of the BiOWiSH™ Technologies bacterial mixes.

After 5 experiments, the BOD tests showed that the redosage of bacteria helped further drive the BOD concentrations to be lower. With redose, the samples reduced BOD by 10 – 55% to samples that were not redosed. With higher concentrations of redose of 250 ppm and 500 ppm, the BOD levels peaked, however, with the bacterial addition, the BOD levels were further decreased than samples that were not redosed.

For the solids testing, different tests showed either conclusive impacts of bioaugmentation or no effect. For the total solids (TS) and total suspended solids (TSS) tests, both showed about a 10% decrease or increase in solids throughout the experiments. The smaller solids components, total dissolved solids (TDS) and volatile suspended solids (VSS), did demonstrate that the bacterial mixes reduced ions and organic suspended solids more than the control. The bioaugmented samples reduced the

VSS organic material by 5 – 15% compared to the control while TDS particles decreased 5 – 10% with BiOWiSH^{TM}.

Particle size distribution (PSD) tests provided a breakdown of which particle sizes were increasing and decreasing. Those samples bioaugmented with BiOWiSH^{TM} showed that smaller particles (0.7 µm pore size) were getting assimilated by the bacteria which produced more bacteria (larger pore sizes of 5 µm). After the bacteria ran out of food, the sequentially smaller pore size (2.5 µm) increased while the smaller pore sizes (1.6 µm and 0.7 µm) remained low. The rate limiting step was determined to be 1.6 – 2.5 µm where the control's zero rate constant was +1.4 mg/L-day whereas the USA and TA was -1.1 mg/L-day and -1.4 mg/L-day respectively. Thus, the BiOWiSH^{TM} samples decreased TSS in smaller pore size filters by about 10 – 20% more than the control.

Ion chromatography (IC) measured that nitrate levels were clearly reduced by 30 – 50% adding the BiOWiSH^{TM} bacteria compared to the control. Therefore, the additional bacteria further denitrified the nitrate (NO_3-) than if no BiOWiSH^{TM} was added. Denitrification experiments were performed for pure *Bacillus* spores, KLB, that showed a 90% decrease of NO_3- to the control.

Keywords: bioaugmentation, BiOWiSH^{TM}, BOD, dairy wastewater, dissolved solids, ion chromatography, nitrate, particle size distribution, redose, suspended solids, total solids, volatile suspended solids

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Chapter 1
Introduction

1.1 Dairy Wastewater in California

In the United States, approximately 9.2 million cows produce 201 billion pounds of milk in 2013 (USDA, 2013). Although the number of dairy farms have decreased from 3,400 farms in 1975 to 2,000 farms in 2005, the number of cows per farm have increased from about 1,000 cows to about 4,300 cows (CDFA, 2005). California ranks as the number one state when it comes to milk production producing 41.3 billion pounds of milk compared to the second highest milk production state, Wisconsin, of 25.6 billion pounds (USDA, 2014). The five top dairy producing counties in California from most to least is Tulare, Merced, Stanislaus, Kings, and Kern with a combined total of 30.1 million pounds of milk produced in 2013 (CDFA, 2013). Thus, the top five dairy producing counties generate about 73% of the dairy in California, demonstrating the large populations of cows in a concentrated area and the waste that is associated with it.

The large dairy production leads to the topic of dairy waste. Dairy waste consists of the bedding provided for the cows and the number of cows on the dairy farm for milk production. Because the Cal Poly Dairy Unit is a freestall barn, the following statistics will be related since bedding depends on the type of housing. According to the EPA (2012), 0.3 ft^3 per cow per day of chopped straw and 0.2 ft^3 per cow per day is used for bedding for a freestall barn while a dairy farm with more than 150 cows produce a range of 2 – 4 gallons of wastewater per cow per day. (EPA, 2012). For a dairy farm with 1,000 cows, about one million gallons of wastewater would be produced annually.

For solid dairy waste, around 30 million tons of manure are generated each year by the cows in California (EPA, 2013). Depending on the barn layout, there are different types of manure handling systems. The three types of systems used are manual scraping, flush systems, and automatic alley scrapers. The Cal Poly Dairy Unit currently uses a flush system to take care of its manure. Recycled wastewater from the holding tank is flushed into the freestall alleyway and then travels to a solids separation mechanism. The liquid effluent is transported to a holding tank or a lagoon for further solids settling and storage. The solids pile underneath the solids separator, which can be moved for composting or storage purposes (EPA, 2012). The system can be shown in *Figure 1* adapted from the Natural Resources Conservation Service (1992).

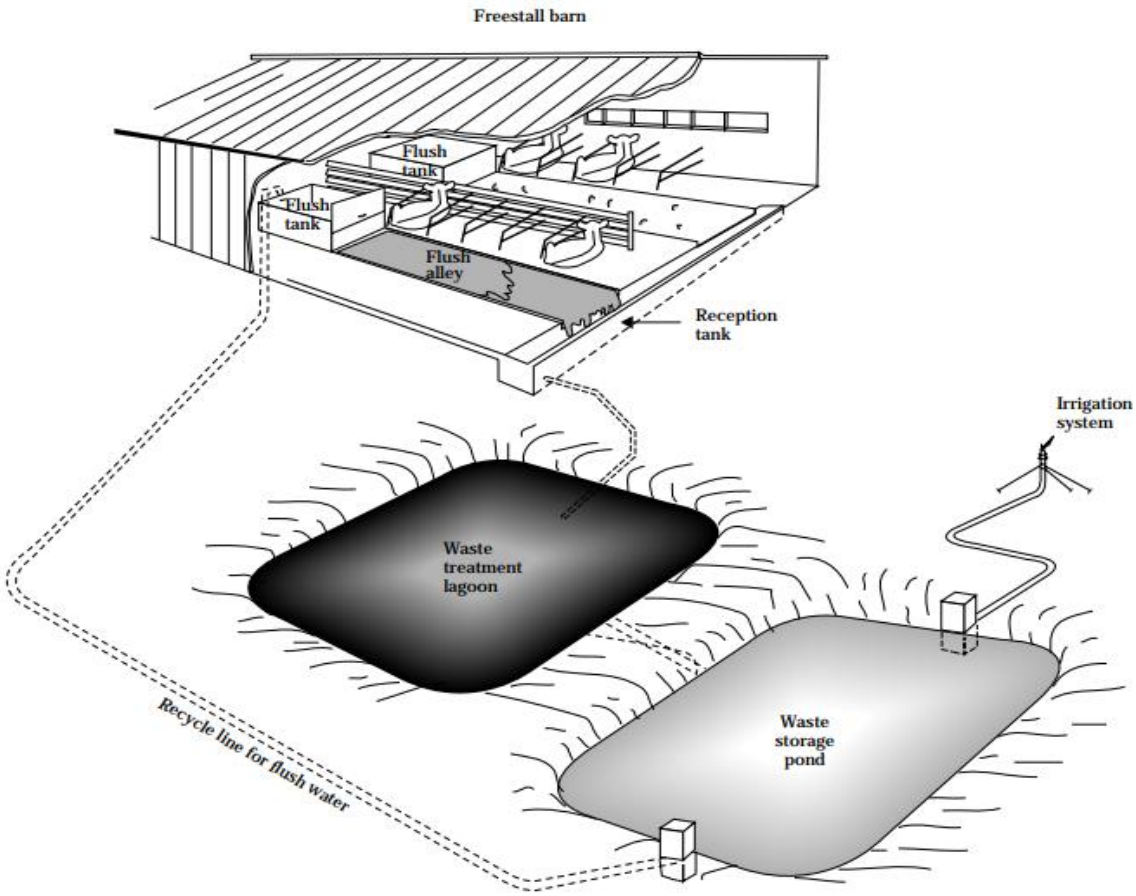


Figure 1. Freestall barn with flushing, lagoon, storage pond, and irrigation system.

The composition of dairy wastewater and the high volume presents significant problems in treatment option. The waste generated from cows have a high biochemical oxygen demand (BOD), suspended solids, nutrients, ammonia-nitrogen, and organic matter. However, the wastewater is biodegradable, excluding the bedding and inert material like gravel, which comes from the lagoon design. Therefore, the preferred system to treat dairy wastewater is a biological system (Mazzucotelli *et al.*, 2014).

1.2 Dairy Wastewater Treatment

With the large production of dairy, dairy wastewater management is an important aspect that needs to be handled efficiently and safely. Dairy wastewater contains a large amount of indigenous bacteria such as *Pseudomonas*, *Achromatium*, *Aquasprillum*, *Desulfobulbus*, and *Clostridium* in circulated wastewater (McGarvey, Miller, Sanchez, Silva, & Whitehand, 2005).

There are three dairy wastewater treatment alternatives: discharge and treatment at a local sewage treatment plant; removal of waste by contractors; or treatment on site (Britz, Hung, & van Schalkwyk, 2005, p. 5). The most cost effective and reliable treatment is treatment on site, specifically aerobic biological treatment (Britz *et al.*, p. 10). Depending on several variables such as land area, budget, and waste composition, different types of aerobic biological systems can be chosen for treatment. It can vary from activated sludge to aerobic filters to sequencing batch reactors (Britz *et al.*, pp. 10–12). *Figure 2*, adapted from the Waste Treatment in the Food Processing Industry (p. 15) by T.J. Britz (2005), shows the current biological treatment of dairy wastewater at the Cal Poly Unit of a treatment lagoon, with the exception of an aerator.

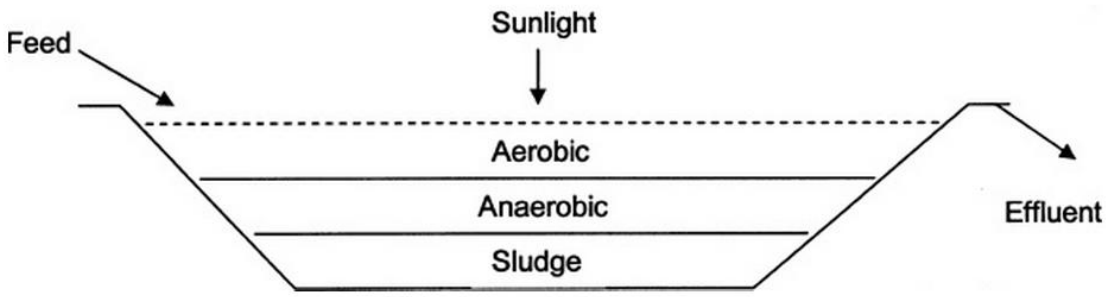


Figure 2. Schematic of an oxidation pond.

Treatment lagoons are typically for dairy farms that have more land available and have a lower budget, since it is the least expensive treatment system of all the available biological treatment systems (Britz *et al.*, pp. 12–13). Also, for farms that do not have a sludge processing facility, the lagoon utilizes the bacteria in the sludge for anaerobic digestion ("Lagoon Systems Can Provide Low-Cost Wastewater Treatment," 1997). The downside of aerated lagoons are the bad odors, large land requirement, possible insect breeding grounds, and potential for groundwater pollution. After the feed reaches a certain hydraulic retention time, the effluent can be used as irrigation for surrounding crops since the effluent is rich in nutrients and low in bacteria (Britz *et al.*, p. 20).

1.3 BiOWiSH™ Technologies

The bacterial mixes experimented on for the dairy wastewater bioaugmentation tests were provided by BiOWiSH™ Technologies Inc. BioWiSH™ is a company that develops, researches, and manufactures innovative products in the Environmental Management, Agri-Business, and Consumer Products fields. Specifically in the Environmental field, BiOWiSH™ focuses on wastewater treatment, waste management, odor and emissions control, and soil and water remediation. Their goal is to develop technology that would provide natural and safe ways to digest organic waste matter,

reduce odors and emissions, enhance soil fertility, and improve water quality (Biowish, 2014).

The products developed by BiOWiSH^{TM}, which were tested during the dairy wastewater bioaugmentation experiments, were BiOWiSH^{TM} Aqua made in the United States and Thailand. The two mixes are differentiated since BiOWiSH^{TM} Aqua was developed in Thailand and the other was developed in the United States, which can be shown in Figure 3. The products accelerate the biological removal of nutrients from wastewater, reduces sludge production and handling, as well as reduces the need for chemical additives. The bacterial mixes are a composite biocatalyst mixture of microorganisms, enzymes, and cofactors that enhances the rate of biochemical reactions

Figure 3. BiOWiSH^{TM} Aqua Thai (l) and USA (r) packets.

in wastewater (Biowish, 2014).

1.4 Using BiOWiSH™ for Dairy Wastewater Management

BiOWiSH™ will be referred to as Biowish moving forward in this paper for simplicity reasons. Adding Biowish bacteria to dairy wastewater to test for further degradation of chemicals is known as a type of bioremediation called bioaugmentation. Bioaugmentation has been implemented in several types of media to determine whether or not adding the non-indigenous bacteria does further reduce different chemical parameter levels in the wastewater. Media such as dairy, municipal, potato chips, starch, and molasses wastewaters were used for bioaugmentation experiments. As Stephenson & Stephenson (1992) listed, a few of the purposes of performing bioaugmentation is to improve BOD, suspended solids, ammonia, and COD removal, reduce sludge production, control foam production, and lower hydrogen sulfide levels.

There have been successful cases of positive results using bioaugmentation, but there have also been inconclusive results and no clear advantage using bioaugmentation. Bioaugmentation involves using bacteria or fungi, which will respectively be discussed in the following paragraphs.

Bacteria has been used for various research projects. Research performed by S. Zhao, Hu, Chen, B. Zhao, and Liang (2009) added the denitrifying bacteria *Bacillus cereus* to determine the amount of organic matter and nitrogen removal to reclaimed wastewater for the purpose of landscape irrigation. Their studies found that the total nitrogen content decreased by 68.6% and the chemical oxygen demand decreased by 71.7% after adding the bacteria powder to the reclaimed water (Zhao *et al*., 2009). In the starch industry wastewater, bioflocculants produced by *Bacillus mucilaginosus* were added to determine if the bioflocculant would increase floc formation and settling in

wastewater treatment (Deng, Bai, Hu, & Luo, 2003). The results demonstrated that the bioflocculant increased the floc formations and the settling of organic materials; thus, after five minutes of settling, the suspended solids decreased by 85.5% and the chemical oxygen demand decreased by 68.5% (Deng *et al.*, 2003). A research study by Tondee and Sirianuntapiboon (2008) used *Lactobacillus plantarum* in molasses wastewater treatment to evaluate the effectiveness of decolorizing the dark brown color, melanoidin pigment, in the wastewater. By adding the bacteria, the researchers found a 68.12% decolorization through gel filtration chromatography at an absorbance of 475 nm (Tondee, T., & Sirianuntaphiboon, S. 2008).

Another study of bioaugmentation using fungi was performed by Mishra, Arora, and Lata (2004), where they analyzed the effect of the fungal culture of *Aspergillus foetidus* and *Aspergillus niger* spore suspension addition in potato chips industry wastewater. The potato chips industry wastewater has high starch and suspended solids, which contribute to high levels of BOD. A 60% decrease of the COD were reduced by the fungus individually, and when both fungi were co-inoculated, COD was reduced by 90% after 60 hours (Mishra *et al.*, 2004). Another research experiment using three fungi, *Aspergillus niger*, *Mucor hiemalis*, and *Galactomyces geotrichum*, in bioaugmentation was performed by Djelal and Amrane (2013) with dairy wastewater. When the three fungi were pre-activated in the dairy wastewater medium and then re-added at the biological tank, the COD values measured at the outlet of the biological tank decreased from 55% to 75% at both the lab scale and industrial scale (Djelal & Amrane, 2013).

Although there are successful research projects demonstrating bioaugmentation applications to different wastewaters, there are research projects that do not show the

same results. A mini review written by Thompson, van der Gast, Ciric, and Singer (2005), discuss the challenges associated with bioaugmentation – ecological considerations, strain identification and selection, and strain survival and activity. A study by Dybas *et al.* (1998) of the *Pseudomonas stutzeri* KC strain, a denitrifying bacteria, in a contaminated aquifer was examined for any denitrification and carbon tetrachloride reduction, specifically transforming carbon tetrachloride to carbon dioxide. The study showed that the carbon tetrachloride levels and denitrification stopped after three weeks with no detection of the bacteria strain. After eliminating an acetate-free water addition to the groundwater, the bacteria was detected and carbon tetrachloride was removed by 60 – 88% (Dybas *et al.*, 1998). Thus, by determining the favorable ecological conditions of the KC strain, the bacteria continued to thrive and reduce chemicals.

1.5 Thesis Outline

Chapter 2 describes the background of the dairy wastewater collected at the Cal Poly Dairy Unit and the Biowish bacteria used for the experiments. The wastewater parameters that were tested – BOD, TS, TSS, TDS, VSS, FSS, particle size distribution, nitrates, nitrites, and bacterial composition – were also further described with equations and purpose.

Chapter 3 first describes the experimental design as a collection, Experiments I – V. Then it breaks down each of the experiments chronologically with their respective tests of the investigation of different Biowish bacteria on dairy wastewater. The materials and procedures are described in more detail following each respective experiment. In Experiment I, only BOD and TSS were tested with four Biowish bacterial mixes – BMT (B), Osprey (O), Thai Aqua (TA), and US Aqua (USA) – and a control in anaerobic

conditions. In Experiment II, BOD, TS, PSD, and IC were performed for two bacterial mixes – TA and USA – and a control in aerobic conditions and with redose. In Experiment III, BOD, TS, PSD, and IC were performed for two bacterial mixes – BMT1 and BMT2 – with a control in aerobic conditions and redose. In Experiment IV, BOD, TS, TSS, TDS, VSS, PSD, IC, and Gram stain microscopy were performed for Fruit Wash (F) and Osprey (O) in aerobic conditions with redose. Lastly, in Experiment V, BOD, TS, TSS, TDS, VSS, and PSD were performed for F and O in aerobic conditions with redose.

Chapter 4 is the section for the experiment results and discussion of the data. The discussion is organized by tests first and then by the experiment number. The tests explained first to last is BOD, TS, TSS, TDS, VSS, PSD, IC, and microscopy.

Chapter 5 concludes and recaps the thesis as a whole with tabulated results and ends with recommendations for future work.

Chapter 6 lists the references used for literature review of prior dairy wastewater research and background information of dairy products and wastewater.

Chapter 7 includes the appendices with various protocols and data that supplement the thesis.

Chapter 2
Background

2.1 Cal Poly Dairy Unit

The diary wastewater collected from the Cal Poly Dairy Unit is from one of two lagoons. The lagoon where the media is collected is an aerated lagoon used primarily for storage. The storage pond has a volume of 19,000 cubic meters (m^3) and is 18 – 20 feet deep. Cal Poly uses a flush system manure handling system for the freestall barns.

The wastes produced by the cows are flushed daily after the evening feeding. The flush leads the waste into a holding tank where it is stored and ultimately pumped up to the solids separator by underground pipes. As shown below in *Figure 4*, when the stalls are flushed, the flow travels downward to the circular holding tank with a wooden top for solid separation. The large grey tank in the back of the barn is the holding tank for the aerated lagoon water, which is used to flush the stalls. It is a continuing cycle.

Figure 4. The Cal Poly Dairy Unit cattle freestall barn.

The sloped screen separates the solids from liquid by conveying the manure over the screen. The solids slide down into a pile underneath the separator while the liquid effluent is sent through another underground pipe into the aerated lagoon. The solids are scooped up by a tractor, as shown in *Figure 5*, into a large solids steel waste container in a holding spot behind the Cal Poly Organic farm for later use as compost on campus ("Waste Management", 2014).

Figure 5. Solids separator.

After the solids and liquid separation, the liquid goes in the aerated lagoon for settling and treatment, then it is pumped for continual recirculation to a large grey holding tank at the top of the hill. The wastewater is also used in the summer to irrigate the corn fields by pumping the water from the lagoon, which is then piped to the fields. The corn rows are furrow irrigated.

When the pond levels are low from irrigation from the summer, the aerator no longer runs during the fall. The wastewater volume in the pond is maintained to be low during the fall quarter in order to prepare for the rainy season and to capture rain runoff.

Figure 6 shows the diary lagoon where the media collection occurs for all the experiments. As well, the aerator is located in the middle of the pond to aerate the water and pull in oxygen into the wastewater after the rainy season, usually from the winter to spring quarter (R. Silacci, personal communication, November 4, 2014).

Figure 6. The aerated dairy lagoon at Cal Poly.

For lab experiments, dairy wastewater was collected in June 2014 and then placed in a Styrofoam box with ice. The wastewater was sent out to Midwest Laboratories to determine the composition of the dairy wastewater using Association of Official Analytical Chemists (AOAC) and American Society for Testing and Materials (ASTM) standard methods. The 1 L bottle was analyzed and had the following characteristics, as shown in *Table 1*.

Table 1. Midwest Lab dairy wastewater composition results.

Sample	Level Found	Units
Moisture (vacuum - 70C)	98.99	%

Fat (acid hydrolysis)	0.3	%
Fiber (neutral detergent)	n.d.	%
Ash	0.59	%
Carbohydrates (calculated)	n.d.	%
Protein (crude)	0.34	%
Aerobic plate count	540000	CFU/g
Bacillus spore forming bacteria	510000	visible spores/g
Nitrogen (total)	0.05	%
Carbon (total)	0.5	%
Carbon Nitrogen Ratio C/N	10:1	

To determine whether the dairy wastewater collected from the Dairy Unit at the surface of the pond was aerobic or anaerobic during the fall season, on October 28, 2014 dairy wastewater was collected into a BOD bottle and transported immediately to the lab to measure the dissolved oxygen levels. Using the YSI dissolved oxygen probe, the probe was given time to equilibrate to a value of 0.25 mg/L. Thus, the dairy wastewater collected at the surface is anaerobic, despite its constant exposure to air.

2.2 Biowish Bacteria Composition

The powder bacterial mix were generously supplied by Biowish for the dairy wastewater experiments, which included bacteria species of *Lactobacillus* and *Bacillus*. The powder mixes were developed to treat wastewater through bioaugmentation processes in the wastewater to further reduce constituents such as BOD and solids, as well as increase the biochemical reaction rates of the treatment process (Biowish Technologies, 2014). The purpose of the performing these experiments were to determine whether bioaugmentation of the dairy wastewater with *Bacillus* and *Lactobacillus* bacterial mixes will produce enhanced reduction of wastewater parameters.

Table 2 lists the various bacteria used for the experiments as well as the species and composition of the mix. For some bacterial mixes, information about its composition was noted as undisclosed due to supplier confidentiality. Some formulations did not have specific percentages, which was also due to the supplier. Instead the main components of the mix were stated.

Table 2. Bacteria mix, nomenclature, species, and composition.

Bacterial Mix Name	**Nomenclature**	**Bacteria Species**	**Composition**
BioCure Microbial Technologies (BMT)	B	*Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Bacillus polymyxa*	4.5×10^9 CFU/g Bacteria Dextrose
Fruit Wash	F	*Bacillus subtilis, Bacillus amyloliquifaciens, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Bacillus polymyxa*	1-10% Bacteria 90-99% Dextrose
Lactic Mix 1	LCM	*Lactobacillus plantarum, Paediococcus acidilactici, Paediococcus pentosaceous*	Bacteria Dextrose
KLB	KLB	*Bacillus subtilis* isolated from the Thai product	Pure Spores
Microbial Discovery Group (MDG)	MDG	*Bacillus licheniformis, Bacillus pumilus, Bacillus amyloliquifaciens*, and *Bacillus subtilis*	Salt Bacteria
Osprey	O	*Pseudomonas*	Undisclosed
Thai Aqua	TA	*Lactobacillus* and *Bacillus*	1% Bacteria 70% Rice Bran 29% Soy Meal
US Aqua	USA	*Lactobacillus* and *Bacillus*	1-10% Bacteria 89-98.5% Dextrose

The brief table was compiled to see the differences and similarities between the various Biowish bacterial mixes. Most of the mixes had *Bacillus* strains, which are rod-

shaped, Gram-positive bacteria that forms endospores and are either aerobes or facultative anaerobes ("Bacillus," 2014). The Thai Aqua and US Aqua had both *Bacillus* and *Lactobacillus* bacteria. *Lactobacillus* are also Gram-positive, rod-shaped bacteria that are aerobic and can survive under low levels of oxygen; however, unlike *Bacillus* bacteria, they do not produce any endospores ("Lactobacillus," 2014). Another difference between the bacterial mixes were that the United States formula contained dextrose as the media while the Thai formula had rice bran or soy meal as the media. All the bacterial mixes were tested at one point during one experiment or multiple times.

2.3 Biochemical Oxygen Demand (BOD)

The BOD_5 test measures the amount of dissolved oxygen required for bacteria to oxidize the organic material in five days. The test determines the amount of biodegradable organic material that has been broken down by the bacteria indirectly by measuring the initial dissolved oxygen levels, when the wastewater sample is diluted with nutrient buffer solution into the BOD bottle, and the final dissolved oxygen level which is measured after an incubation time of five days using Standard Method 5210 (Cleserl *et al.,* 1998). The difference in dissolved oxygen represents the potential oxygen demand that is needed to biodegrade the organic material. BOD is an important wastewater parameter to measure because when the BOD is high, it may deplete the water body of oxygen from other aquatic organisms. To calculate BOD_5, *Equation 1* shows the formula.

Equation 1. BOD_5

$$BOD_5\ \left(\frac{mg}{L}\right) = \frac{\left(DO_i - DO_f\right) - \left(\left(B_i - B_f\right) * (1 - P)\right)}{P}$$

$where$:

$DO\ is\ the\ dissolved\ oxygen\ concentration\ of\ the\ seeded\ dilution$

B *is the dissolved oxygen concentration of the blank*

i *is initial*

f *is final*

P *is* $\frac{mL\ sample}{mL\ total\ volume}$

2.4 Solids

Another important water quality measurement to take for wastewater is solids testing. There are several different solids tests that help characterize the solids in the waste. The encompassing test is the total solids (TS) test, which measures the total weight of the solids in a specified volume of wastewater. Total solids consist of the total suspended solids (TSS) and the total dissolved solids (TDS). TSS are the dry weights of the particulates that get retained in a filter. For the experiments, a 1.6 µm pore size was used, although the American Public Health Association (APHA) Standard Methods 2540 D states 1.5 µm is the filter pore size to use (Cleserl *et al.*, 1998). TDS are the inorganic and organic substances that are dissolved in the liquid, which is simply all the liquid that passes through the filter known as the filtrate. The volatile suspended solids (VSS) test was also performed after TSS to determine the organic material of the suspended solids. Because suspended solids have both organic and inorganic solids, VSS can be calculated by determining the fixed suspended solids (FSS), which are the inorganic solids. Each component of the solids test will be explained further below.

2.4.1 Total Solids (TS)

TS are the total weight of solids in the samples, which is comprised of both total dissolved solids and total suspended solids. TS tests followed Standard Methods 2540 B by Clescerl *et al.* (1998) and calculations are shown in *Equation 2*. The two

subcategories of the solids tests, total dissolved solids (TDS) and total suspended solids (TSS), will be described in more detail below.

Equation 2. Total Solids

$$TS\ (\frac{mg}{L}) = \frac{(post\ weight\ in\ grams) - (pre\ weight\ in\ grams)}{mL\ of\ sample} * 10^6$$

2.4.2 Total Dissolved Solids (TDS)

TDS are inorganic and organic substances that are dissolved in liquid. It is the filtrate that passes through the glass filter when performing the TSS test. For the TSS test, the pore size is arbitrary as long as it is between 1 µm and 2 µm and is recorded. Standard Method 2540 C uses 1.5 µm pore size glass filters for their solids testing; however because there were previously ordered 1.6 µm filters, 1.6 µm filters were used for the solids testing (Cleserl *et al.*, 1998).

To calculate for TDS, the equation used is shown in *Equation 3*. The pre-weight is the weight of the aluminum tray before filtering and the post weight is the weight of the dissolved solids after filtering and being dried in the 180ºC oven with the aluminum tray.

Equation 3. Total Dissolved Solids

$$TDS\ \left(\frac{mg}{L}\right) = \frac{(post\ weight\ in\ grams) - (pre\ weight\ in\ grams)}{mL\ of\ sample} * 10^6$$

2.4.3 Total Suspended Solids (TSS)

TSS is water quality parameter to test with the wastewater samples because the higher the TSS, the less clarity there is in the water which can prevent sunlight from penetrating the water surface and providing energy for the organisms. TSS is the summation of the volatile suspended solids (VSS) and fixed suspended solids (FSS) and

was performed using Standard Methods 2540 D (Cleserl *et al.*, 1998). VSS and FSS will be further explained in the following section. To calculate TSS, it is shown in *Equation 4*.

Equation 4. Total Suspended Solids

$$TSS\ \left(\frac{mg}{L}\right) = \frac{(post\ weight\ in\ grams) - (pre\ weight\ in\ grams)}{mL\ of\ sample} * 10^6$$

2.4.4 Volatile Suspended Solids (VSS)

VSS are organic material that is one of two components in TSS that volatilizes when ignited for 15 minutes in a 550ºC muffle furnace. VSS are the solids that are lost after ignition. The second component is fixed suspended solids (FSS), which are the fixed solids or inorganic component of suspended solids that is retained on the filter after ignition. The tests were done following Standard Methods 2540 E by Cleserl *et al.* (1998). The equations below were used to calculate for VSS and FSS, as shown in *Equation 5* and

Equation 6 respectively.

Equation 5. Volatile Suspended Solids

$$VSS\ \left(\frac{mg}{L}\right) = \frac{(post\ TSS\ in\ grams) - (post\ weight\ in\ grams)}{mL\ of\ sample}$$

Equation 6. Fixed Suspended Solids

$$FSS\ \left(\frac{mg}{L}\right) = TSS\ \left(\frac{mg}{L}\right) - VSS\ \left(\frac{mg}{L}\right)$$

2.5 Particle Size Distribution (PSD)

PSD is a wastewater quality test to determine the concentration of the various sizes of the particles found in the dairy wastewater. A sequential filtration was conducted and the weights of the suspended solids on the filters were weighed in the same manner as the TSS test following Standard Methods 2540 D (Cleserl *et al.*, 1998). The data would show the trends of the varying particle sizes throughout the tests.

Table 3. Particle Size Distribution Filter Characteristics

Pore Size Retention Size (um)	5.0	2.5	1.6	0.7
Brand	ValuSep	Fisher Science (G8)	Fisher Science (G6)	Whatman
Diameter (cm)	4.7	4.25	5.5	4.25

Table 3 shows the brand and the diameter characteristics of the different pore sized filters used for PSD for Experiments II – V.

2.6 Ion Chromatography (IC)

Ion chromatography was performed to determine the nitrate (NO_3-) and nitrite (NO_2-) concentration of the Biowish bacterial samples and control in the dairy wastewater. IC measured NO_3- and NO_2- concentrations by separating the ions based on interactions with a resin. The samples went through a pressurized chromatographic column where ions were absorbed by the column. Then, the eluent ran through the column where the absorbed ions separated from the column. Lastly, the retention time at which it occurred determined which ions were measured and its concentration by area.

The Chromeleon Chromotography Data Software System was used to measure the concentrations and the Dionex DX – 120 Ion Chromatograph Operator's Manual was referenced (Dionex Corporation, 1998). The software measured the NO_3- and NO_2- in

area with the units, µS multiplied by minutes. In order to convert the area to concentration in parts per million (ppm), NO_3- and NO_2- standards were made. Known concentrations of NO_3- and NO_2- were mixed together and added to the test. After the Chromeleon software measured the area of the known standards, a calibration curve was created. Because the NO_3- and NO_2- ppm were known, the curve generated an equation that would use the area and solve for the concentration in ppm for both NO_3- and NO_2-. Thus, the measurements week after each week would produce a change over time graph of the NO_3- and NO_2- concentrations to determine whether denitrification was occurring. *Equation 7* shows the pathway for denitrification.

Equation 7. Denitrification steps.

$$NO_3\text{-} \rightarrow NO_2\text{-} \rightarrow NO + N_2O \rightarrow N_2$$

2.7 Gram Stain Microscopy

The purpose of doing Gram stain microscopy was to obtain a qualitative observation of the different morphologies and Gram stain bacteria in the dairy wastewater. The Gram stain technique was invented by Has Christian Gram in 1884 to characterize the cell walls of various bacteria. There are two groups that are differentiated – Gram-positive and Gram-negative microorganisms (Goldman & Green, 2008).

The Gram stain procedure is straightforward and typically has five steps, which was obtained from the Microbe Library (Hussey & Smith, 2005). The specimen is fixed onto a glass slide under a flame for a couple minutes. Afterward, the crystal violet primary stain is applied for one minute onto the specimen and then rinsed off with water. Next, iodine is added for one minute and washed off with water. A decolorizer, usually alcohol, is applied for 5 – 15 seconds and rinsed with water. Lastly, the counterstain, pink

safranin, is applied for a minute and then rinsed off with water (Goldman & Green, 2008).

If the specimen is Gram-positive, the stain will retain the crystal violet color whereas the Gram-negative microbes will be stained with the pink safranin. Gram-positive microbes have a thicker peptidoglycan layer than Gram-negative microbes, however, Gram-negative microbes have a lipid-protein bilayer which validates the stain they ultimately retain. The thick peptidoglycan entraps the crystal violet stain and prevents the decolorizer from removing the color compared to the Gram-negative microbes where the thin peptidoglycan layer allows the decolorizer to wash off the purple crystal violet stain and retain the pink safranin counterstain (Goldman & Green, 2008).

Chapter 3
Materials and Methods

3.1 Collection of Dairy Wastewater

Dairy wastewater was collected on Day 0 of the experiment at the Cal Poly Dairy farm located on Mount Bishop Road. A 1 gallon (gal) container connected to a polyvinyl chloride (PVC) pipe was used to collect the wastewater liquid from the surface of the lagoon. After collection, the liquid was poured in a 20 L plastic container. The wastewater contained unwanted items such as hay and other plant debris, therefore a sieve with an opening of 850 µm was used to separate the debris from the collected wastewater. After separation of debris from the wastewater, 2 liters (L) of wastewater was measured using a 2 L graduated cylinder and poured to the 2 L Pyrex bottles. The dairy wastewater collection procedure was performed for all experiments.

3.2 Experiments I – V Test Layout

The following sections describe the experiments and their respective test procedures and methodology. In order to view the experiments and their respective tests clearer, *Table 4* shows the different tests that were done for each of the experiments below.

	Tests							
Experiment Number	**BOD**	**TS**	**TSS**	**TDS**	**VSS**	**PSD**	**IC**	**Gram Stain**
I	✓		✓					
II	✓	✓				✓	✓	
III	✓	✓				✓	✓	
IV	✓	✓	✓	✓	✓	✓	✓	✓
V	✓	✓	✓	✓	✓	✓		

Table 4. Experimental layout of tests.

To test the different effects of Biowish's bacterial mixes, a control was implemented with each experiment. Due to limited incubator space and resources, only two bacterial mixes were tested with a control in the experiments since there were duplicates samples for each bacterial mix and bottles would double with redose in two weeks. In *Table 5*, the bacterial mixes that were tested are listed during which experiment number for an overview sight of all of the experiments.

Table 5. Experimental layout of bacterial mixes.

	Bacterial Mix					
Experiment Number	**B1**	**B2**	**F**	**OSP**	**TA**	**USA**
I	✓			✓	✓	✓
II					✓	✓
III	✓	✓				
IV			✓	✓		
V			✓	✓		

3.2.1 Experiment I

The dairy wastewater experiment started on November 1, 2013 and ended on January 12, 2014. The Biowish bacterial mixes that were tested were BMT 1 (B 1), Osprey (O), Thai Aqua (TA), and US Aqua (USA) with one control that had no Biowish bacterial mix – it was only dairy wastewater. The purpose of performing the experiment was to determine the various bacterial mixes' ability to reduce chemical constituents and solids in anaerobic conditions. Duplicates of each of the bacterial mix samples were implemented, excluding the control. Bottle caps were tightened on the bottles to promote anaerobic conditions for the microcosms. BOD_5 and TSS were performed weekly for six weeks and then lastly on the eleventh week. All microcosms were placed in a 30°C temperature regulated Thermo Forma Orbital Shaker, which continually mixed them at 75 rpm.

3.2.2 Experiment II

Experiment II took place from January 30, 2014 to March 11, 2014. The microcosms tested included a control that only contained the bacteria from the dairy wastewater, 250 ppm of Thai Aqua (TA), and 250 ppm of US Aqua (USA). The objective of Experiment II was to test TA and USA effect on dairy wastewater in aerobic conditions and with redose. Aerobic conditions were implemented by sealing an AirOtop filter on the top for air exchange. To compensate for evaporation loss, the bottle weights were weighed on a kilogram scale before placing in the incubator shaker and then reweighed the week after before sampling. Evaporation loss was compensated after determining the weight loss. A 2 L beaker filled with DI water was also placed in the middle of the incubator to help with the evaporation loss. 500 ppm of bacterial mix was added during day 14 for redose. 25 ppm of nitrate (NO_3-) was added to spike the wastewater samples to determine the nitrogen assimilation from NO_3- to nitrite (NO_2-) for IC. To obtain 25 ppm of NO_3-, potassium nitrate (KNO_3) was used and chemistry calculations were performed as shown in *Equation 8*.

Equation 8. Determination of KNO_3 concentration for IC in Experiments II and III.

$$25\ ppm\ NO3- = 25\frac{mg}{L}\ N\ from\ NO3-$$

$$molecular\ weight\ of\ KNO3 = 39 + 14 + 48 = 101\frac{g}{mol}$$

$$\frac{14\frac{g}{mol}}{101\frac{g}{mol}} = \frac{25\ \frac{mg}{L} * 2\ L}{x}$$

$$x = 360\ mg\ of\ KNO3$$

3.2.3 Experiment III

Experiment III took place from March 20, 2014 to April 29, 2014. A control with no additional bacteria and duplicates of BMT, B1 and B2, were tested. The purpose of Experiment III was to determine the bacterial mix's effect on the biochemical and solids component of the dairy wastewater in aerobic conditions and with redose during day 14. 250 ppm of redose was added during day 14. 360 mg of KNO_3 was spiked in the samples from Day 0 for nitrogen assimilation determination in IC testing. The tops were sealed with AirOtops for aerobic conditions when it was incubating in the 30°C shaker.

3.2.4 Experiment IV

Experiment IV was a redose experiment that took place from May 2, 2014 to June 10, 2014. There was one control and two duplicate samples with additional Biowish bacteria supplemented – Fruit Wash (F) and Osprey (O). The objective of Experiment IV was to determine the effect of the bacterial mix on the solids and chemical constituents in the dairy wastewater in aerobic conditions and with redose. Bacterial redose of 250 ppm F and O were executed on day 14. Aerobic conditions were implemented using AirOtops for air exchange when the microcosms were placed in the incubator shakers. 303.6 mg of sodium nitrate ($NNaO_3$) was added for IC tests in order to obtain 25 ppm of N from NO_3. Calculations of the $NNaO_3$ concentration can be shown in *Equation 9*.

Equation 9. Determination of $NNaO_3$ concentration for IC in Experiment IV.

$$25\ ppm = 25\frac{mg}{L} of\ N\ from\ NO3$$

$$molecular\ weight\ of\ NNaO3 = \ 14 + 23 + (16 * 3) = 85\ \frac{g}{mol}$$

$$\frac{14\frac{g}{mol}}{85\frac{g}{mol}} = \frac{25\frac{mg}{L} * 2\ L}{x}$$

$$x = 303.6\, mg\ of\ NaNO3\ /L$$

During this experiment, there were changes to some test procedures throughout the six week course, which will be stated clearly in the next sections. For the mixing, during Day 28, the bottles were no longer shaken by hand. Instead, the bottles were placed on a Fisher Scientific Isotemp stirring plate with a stirrer dropped into the bottle. The stir bar continually mixed the wastewater as aliquots were taken out for tests.

3.2.5 Experiment V

Experiment V started on October 16, 2014 for three weeks and ended on November 6, 2014. It was a repeat experiment of Experiment IV because day 0 measurements were not taken for the TSS, VSS, and TDS tests. The objective of Experiment V was also to perform the tests with as much accuracy to determine the bacterial mixes' effect on dairy wastewater. Fruit Wash and Osprey were the Biowish bacterial mixes tested against the control of only dairy wastewater with no additional Biowish bacteria. Redose of only 10 ppm, instead of 250ppm from previous experiments, of the respective bacteria were added during day 7, which is also a week earlier than the other experiments due to the shorter length of Experiment V. The microcosms were under aerobic conditions using AirOtops.

3.3 BOD_5

BOD_5 test was performed in the dairy waste water with the bacterial mix samples using the Standard Method 5210 (Clescerl *et al.,* 1998). The objective to perform BOD5 was to determine how fast the organic material in the wastewater would degrade. 2 liter bottles were filled with dairy wastewater and a filter on top allowing for air exchange, thus providing an aerobic environment. The apparatus for the experiment were 300

milliliter (mL) BOD bottles, glass stoppers, enhanced seals, YSI Pro20 dissolved oxygen probe and meter, and Hach BOD buffer pillows. The BOD bottles were washed with Alconox soap, rinsed with tap water three times, and rinsed with deionized (DI) water once. Afterwards, the bottles and stoppers were autoclaved with aluminum foil covering the bottle tops and the stoppers placed in a beaker for forty minutes.

During the measurements, the YSI Pro20 probe was turned on and allowed to equilibrate for 20 minutes. Then, the probe was calibrated with readings between 8 and 10 mg/L and 20 – 21°C. The nutrient buffer for the BOD_5 test was made with one HACH nutrient pillow. 4 L of reverse osmosis (RO) water were added for each buffer pillow. A carboy was cleaned and rinsed with RO water before use. The nutrient pillows were added with RO water that filled to the 8 L mark. The carboy was capped and then oxygenated by shaking it vigorously for a couple of minutes.

1, 2, and 3 mL samples from the four bacterial mixes and control were added to the BOD bottles. The BOD bottles were filled halfway with the nutrient buffer and then capped for five seconds of shaking. Then the rest of the bottle was filled with nutrient buffer up until the neck of the bottle. The dissolved oxygen (DO) probe measured the DO in the bottle with the mixer turned on. Bottles were capped with aluminum foil covering the top following measurements. The probe was rinsed with DI water after every measurement. This procedure was continued for the rest of the bottles. After the measurements were taken, the BOD bottles were placed in the 20°C refrigerator for five days.

DO measurements were taken for Day 0 and Day 5 each week. In *Figure 7*, the initial DO measurement was taken by placing the probe into the mixed BOD bottle with a

specified mL of sample with nutrient buffer mixed RO water until the measurement equilibrated on a value.

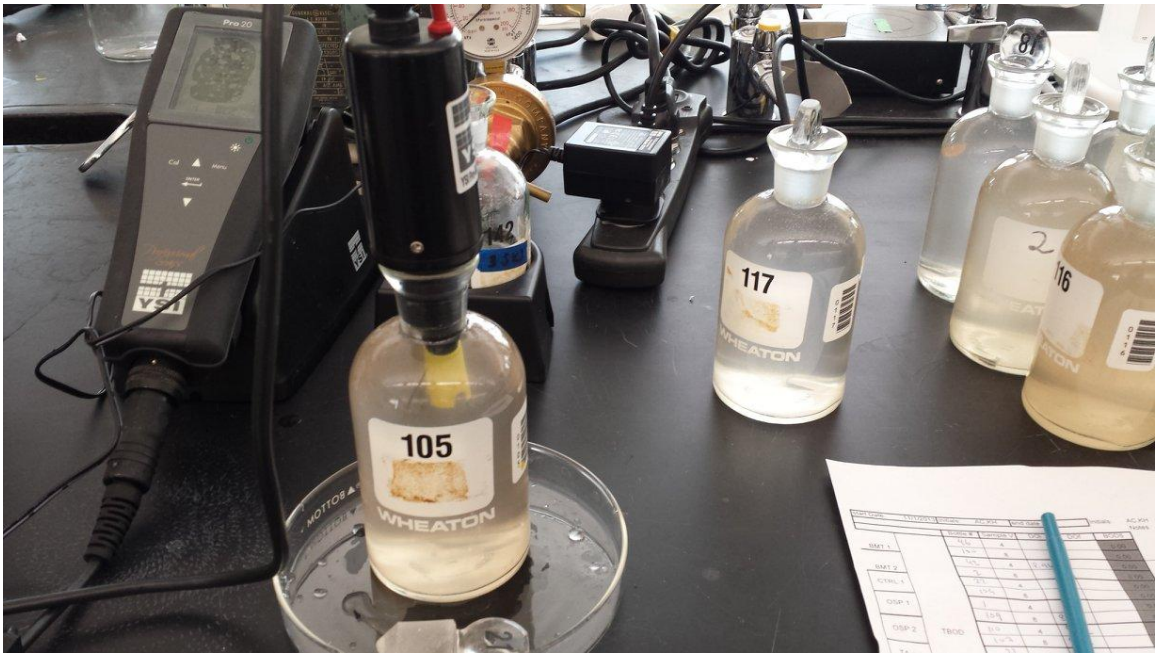


Figure 7. BOD experiment set up.

For Experiment III – V, the BOD_5 preparation was slightly different from the previous experiments with the purchase of buffer nutrient pillows for 300 mL volumes. Rather than adding one nutrient pillow for 4 L of RO water in the carboy, the buffer nutrient pillows were added for 300 mL, the volume of the BOD bottles. Thus, the nutrient pillows were added directly to the BOD bottles. RO water was added halfway in a carboy and shook to aerate. The carboy was filled up to the top and ready for dispensing.

Depending on the weekly sample, the volume of sample varied from 1 mL to 5 mL. The microcosms were shaken for complete mixing conditions. A volume was taken and transported to the respective BOD bottles. One nutrient pillow was cut and distributed to one BOD bottle. RO water was dispensed to fill the BOD bottle halfway, a stopper was secured on the top of the BOD bottle, and it was shaken for the contents to

mix. Then the stopper was taken off to fill the bottle with RO water. DO measurements were taken shortly after for its initial reading and recorded when the DO meter equalized to a value.

3.4 Total Solids (TS)

The TS test was performed using the Standard Method 2540 B to determine the total solids in the wastewater samples and see if the solids reduce over time (Clescerl *et al.*, 1998). 42 mL aluminum trays were used for its larger bottom and top diameter at 5.1 centimeters (cm) and 6.2 cm respectively compared to the smaller 20 mL capacity aluminum trays used for TSS in Experiment I. By having a larger diameter, the solids could evaporate quicker in the oven.

The bottles were rigorously shaken to ensure complete mixing in order for 10 mL aliquots of sample to be taken for the test. The 10 mL samples were dispensed into pre-weighed aluminum trays and stored in the 105°C oven for about two hours or until completely dry. After the liquid had dried, the trays were moved to the desiccator for cooling and then weighed. The difference in weight divided by the volume was the TS measurement. For Experiment III, during Day 28, the aluminum trays were no longer being labeled with Sharpie but rather were labeled by etching as shown in Figure 8.

Figure 8. Total solids.

3.5 Total Dissolved Solids (TDS)

TDS are the solids that can pass through a filter using Standard Method 2540 C, which was performed to determine the salts and ions concentration in the dairy wastewater (Clescerl *et al.*, 1998). TDS testing was performed hand in hand with the TSS testing from Day 7 and test dates forward. From Day 7 until Day 21, the TDS were dried in a 105ºC oven until it all evaporated. From Day 28 to Day 35, the TDS were dried in a 180ºC oven for one to two hours. The TDS tests were performed using the 180ºC oven after it was known that it was available for use. During Experiment V, the oven used for Experiment V for drying the dissolved solids was a Blue M industrial oven that was preheated to 180°C for an hour prior to use. Another procedural change was the use of etching the sample names instead of using a Sharpie to label the trays. In addition to the TSS testing, TDS testing involved using clean glass tubes that were placed inside the filter flask for collection.

The aluminum trays were etched with the sample name and pre-weighed. Then, the TSS test was set up with the vacuum flasks, filter holder, and filters. After the sample was dispensed and the vacuum pump was turned on, the liquid that passed through the filter into the glass tubes were the dissolved solids that were 1.6 µm or smaller. After rinsing the filter with DI water, the liquid in the tube was taken out and poured into the aluminum trays. The tube was rinsed with DI water to collect any leftover liquid and was subsequently poured into the aluminum tray. After all the samples were filtered through and collected, the aluminum trays were placed in a 180ºC oven for about an hour or two to dry. The trays were periodically checked to see the level of evaporation in the trays. After the liquid fully evaporated, the trays were placed in the desiccator to cool for fifteen minutes and then weighed. The TDS test results are shown in *Figure 9*.

Figure 9. TDS test results.

3.6 Total Suspended Solids (TSS)

Fisher Scientific G4 filters, which have a pore size retention size of 1.2 µm, were pre-treated for Experiment I by rinsing with DI water with the vacuum pump on and then being placed in a muffle furnace of 550°C for fifteen minutes. The procedures followed Standard Method 2540 D to determine the concentration of solids larger than 1.2 µm or 1.6 µm for Experiments IV and V (Clescerl *et al.*, 1998). The filters were taken out and allowed to cool in the desiccator for fifteen minutes before use. Aluminum trays, with a

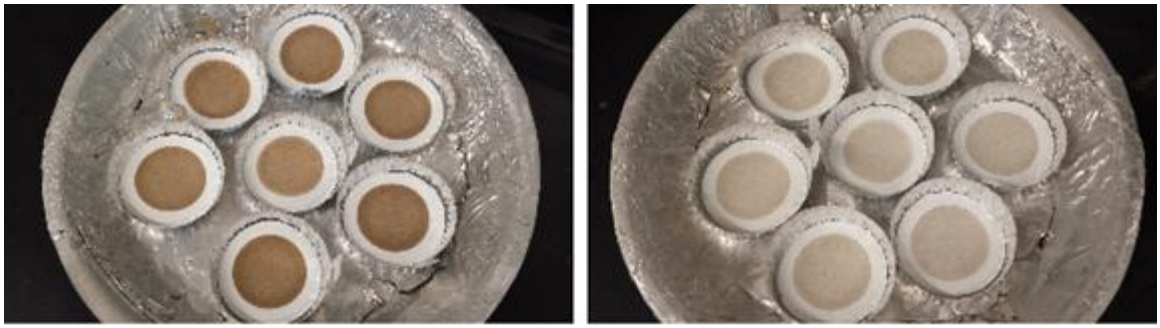
20 mL capacity and a bottom and top diameter of 4.4 centimeter (cm) and 5.2 cm respectively, were etched with the sample name on the tab, and then the pre-treated filters were placed in the trays and pre-weighed on an analytical scale.

After pre-weighing, the filters can be used to filter their respective samples. The 2 L wastewater bottles were shaken rigorously to ensure a completely mixed sample. 4 mL aliquots were retrieved and dispensed into the filter. After the sample drained through and was rinsed with DI water, the filter was taken off the filter holder and placed back in its aluminum tray. The process was repeated for all samples.

After completing all wastewater samples filtering, the trays were put into an oven of 105°C for an hour to two hours to dry. After drying, the trays were cooled in the desiccator for fifteen minutes before measuring its weight. The difference in weight divided by the sample volume was the TSS for the specific day sampling.

3.7 Volatile Suspended Solids (VSS)

VSS are the organic component of the suspended solids that are trapped in the filter, therefore the objective of this test was to determine the concentration of organic suspended solids in the dairy wastewater over time using the Standard Method 2540 E (Clescerl *et al.,* 1998). The TSS test was dried in the oven after filtration and allowed to cool in the desiccator for measurements. After weighing, the same filters were put into the 550ºC muffle furnace for about fifteen minutes and then taken out. The organic materials volatilized and what were left on the filter were the fixed suspended solids (FSS). In *Figure 10*, the image on the left showed the TSS after being dried in the oven and to the right, the image showed FSS, the inorganic material on the filter that did not volatilize. The VSS is the FSS subtracted from the post weight of the TSS.



TSS FSS

Figure 10. VSS test results.

3.8 Particle Size Distribution (PSD)

PSD was performed using Standard Method 2540 D to determine the concentration of particle sizes in the dairy wastewater over time (Clescerl *et al.*, 1998). For Experiment II, the PSD was performed for 91 days. The first 35 days experimented on the original wastewater samples while days 49, 63, 77, and 91 were experimented on only the control and redose samples – TA a and USA a. Also, for day 0 and 7, instead of filtering from pore size 5 µm successively to 0.7 µm, the first filter used was 1 µm in error. Thus, the data acquired from the tests were not included in the graphs and started on day 14.

The apparatus for PSD included glass filters that were pore size 5 µm, 2.5 µm, 1.6 µm, and 0.7 µm, the filter holder set, vacuum pump, desiccator, oven, muffle furnace, and the aluminum trays used to hold the filters. The procedure and methods were similar to TSS except that there were no DI water rinses and all the liquid that passed through the filter was saved to filter through the next set of smaller pore size filters by placing a glass tube within the volumetric flask. The aluminum trays were marked with a sharpie rather than being etched to save time and to see the sample names clearer. The aluminum trays

with their respective filters were pre-weighed to the nearest fourth decimal place and recorded.

All the filters, with the special case of 5 µm, were pre-treated by rinsing about 30 mL of DI water through the filters, placing them in the muffle furnace for 15 minutes to dry, and cooling in the desiccator for another 15 minutes. For the 5 µm pore size filter, the filter was pre-treated like the rest of the filters, however it was noticed that some of the fibers would stick to the filter holder. During day 35 sampling, the 5 µm filter was not pre-treated and simply used from the box. There were no noticeable difference from not pre-treating the filter, thus from Experiment II day 35 to Experiment IV day 35, the 5 µm filter was not pre-treated. As shown in *Figure 11,* the different amounts of particles that remain on the filter vary on the pore size with the most getting trapped in the 2.5 µm filter.

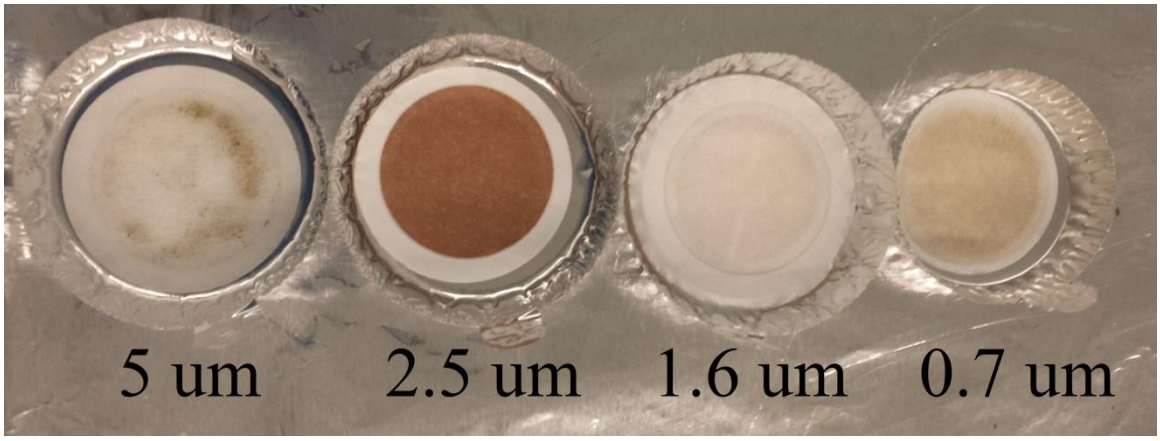


Figure 11. Particle size distribution sequential filters.

After pretreating the filters, a specified aliquot of wastewater sample was taken out of a shaken bottle and filtered through the 5 µm pore size first. A glass tube was inserted into the vacuum flask to collect the filtrate. After filtration, the glass tube that contained filtrate was taken out and a new glass tube was placed inside the flask. The next smaller pore size filter, 2.5 µm, was placed on the filter holder and the filtrate from

the glass tube was poured for filtration. The steps continued with the 1.6 µm and the 0.7 µm filter.

After the successive filtering from the 5 µm filter to the next smaller pore size, the filters were dried in the 105°C oven for an hour to two hours. All the filters were weighed afterwards and recorded for its concentration. Shown in *Figure 12*, the filtrate color becomes lighter as it passes through smaller pore size filters.

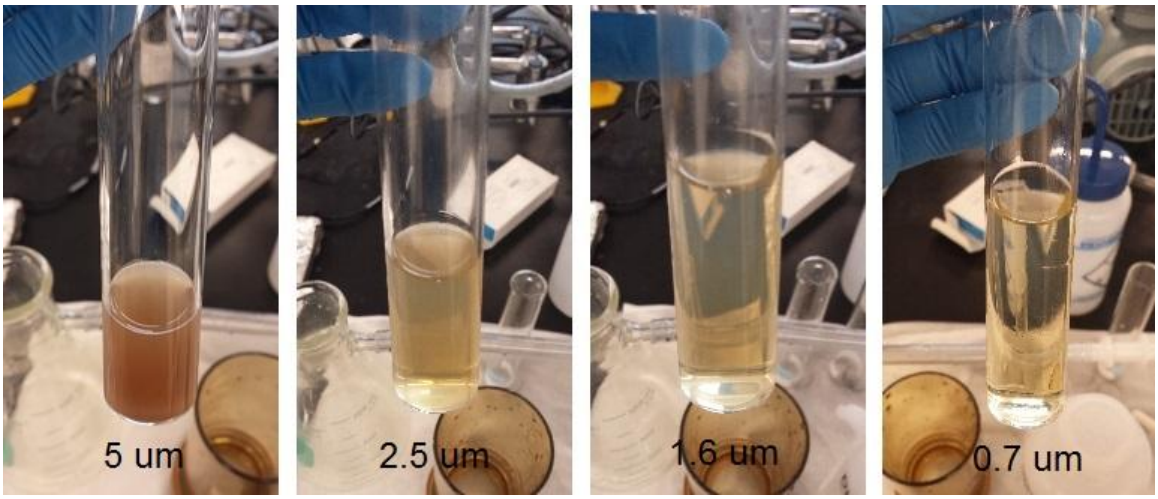


Figure 12. Filtrate from the serial filtration of the dairy wastewater samples.

For Experiment IV, starting from Day 7, after each filtering, a filtrate tube was replaced with a rinse tube. Each filter was rinsed with DI water and the water was collected in the rinse tube to ensure all particles go through the filter. After rinsing, the rinse tube was taken out of the flask and a new tube was placed in flask for the next pore size filtering. The rinse and filtrate were added together and then filtered through the next pore size. The process was repeated with the smaller successive pore sizes. Lastly, the filters were dried in a 105°C oven for about two hours, cooled in desiccator for 15 minutes, and weighed.

Also, during Day 28 of Experiment IV, the 5 µm filter was pre-treated by placing the filter in a 105ºC oven for 1-2 hours instead of using the filters straight from the box.

Prior to not pre-treating the 5 µm, the filters were losing some of its glass filters on the filter holder. Thus, the 5 µm filters did not go through the pretreating process. However, during Day 28, the 5 µm was pre-treated to obtain more accurate results although previous experiments showed no difference for pre-treating the 5 µm.

3.9 Ion Chromatography (IC)

The instrument used for IC was the Dionex D-120 Ion Chromatography (IC) following the Dionex Operator's Manual for procedures to determine the denitrification process occurring in the dairy wastewater by measuring nitrate and nitrite concentrations (Dionex Corporation, 1998). A filter set consisting of a 500 mL Pyrex flask, 1000 mL Pyrex suction flask, vacuum pump, glass microanalysis filter holder, funnel, clamp, and base was used to filter the particles from the fluid. From the filtrate last collected from the 0.7 µm filter in PSD, the 0.22 µm filter was placed on the filter holder and the filtrate was filtered through. After the samples were filtered through the 0.22 µm nitrocellulose filter, three drops of a base – 1 M sodium hydroxide – was added to the filtrate. Then, it was poured into a 5 mL Polyvial, sealed with a filter cap, and aligned on a holder.

Once all the samples had been filtered and collected in Polyvials, the setup of the Dionex instrumentation began. The chromatography data system computer program, Chromeleon, was booted up and the test samples were inputted into the program. The test samples were placed in the holders according to the program with rinses (vials filled with DI water), nitrate and nitrite standards (made by diluting known concentrations), and the shutdown vial, which was also filled with DI water. The holders with the vials were placed in the Dionex AS40 Automated Sampler. The eluent, which was made of sodium bicarbonate and sodium carbonate anhydrous, was pumped with helium for fifteen

minutes. The Dionex instrument pump was warmed up by decreasing and increasing the pressure three times. Lastly, Chromeleon and the instruments were remotely connected and the batch measurements started. Further details can be found in *Appendix 7.1*.

For Experiment IV, from Day 28 to Day 35, unlike the other IC procedures from the previous experiments, the final PSD filtrate that filtered through the 0.7 µm pore size was not used to filter through the 0.22 µm filter since the IC procedure was separate from the PSD test. Thus, a smaller volume of 6 mL of the dairy waste was filtered through various sized pore sizes from 2.5 µm to 0.22 µm. By separating the two tests, there was less clogging of the PSD filters.

3.10 Gram Stain Microscopy

Gram stain microscopy was performed for this experiment in order to obtain a qualitative assessment of the size and type of particles in the dairy wastewater using procedures by Hussey and Smith (2005). The *Bacillus* and *Lactobacillus* bacterial mix from Biowish are Gram positive, thus when performing the Gram stain, the bacteria retains the crystal violet since they have a thick peptidoglycan layer in their cell wall.

The control and only one of each of the duplicate wastewater samples were Gram stained. After shaking the 2 L bottles for mixing, the samples were collected using disposable transfer pipettes. For Day 7 microscopy, one drop of each of the samples was immediately transferred to the glass slide. For Day 35 microscopy, the solids in the sample were allowed time to settle in the pipette. Thus, the solids settled to the bottom of the pipette bulb and the drop of sample was less concentrated. The images were less clouded when observed under the microscope. The glass slides with the samples were

fixed over a flame burned by a Bunsen burner. The heat helped the cells adhere to the glass. After the sample was completely dried, the coloring began.

First, several droplets of crystal violet stain was added to the glass slide. It was stained for about a minute and then rinsed off with water. The next stain was with iodine solution for about a minute. The iodine solution was rinsed off the glass slide with water with any excess water shaken off. Several drops of decolorizer were added until the solution trickled down the slide. After five seconds, the slide was rinsed off to prevent any additional decolorization in the gram positive cells. The last step was the counterstain with safranin solution for about a minute. The stain was gently rinsed off with water and the slide was blotted with a highly absorbent paper.

After the samples were all Gram stained, it was observed under an Olympus CX41 microscope with PlanC N lenses. The visualization attachment was an Infinity2 U-TV0.5XC-3. The slide was first observed using the 10X lens to focus the lens, afterwards, a drop of immersion oil was added to the top of the specimen in order to observe it under a 100X lens.

3.11 Miscellaneous Experiments

There were two miscellaneous experiments performed: a three bacterial mixed BOD_5 test and KLB denitrification tests.

3.11.1 BOD_5 Test with Mixed Bacteria

The BOD_5 test involved three different types of bacterial mixes added into 2 L of dairy wastewater. The three bacterial mixes were the Microbial Discovery Group (MDG), Osprey (O), and Lactic Mix 1 (LCM). Duplicates were performed over a course of 8 days under anaerobic conditions, capped bottles. The CFU count for each bacterial mix was 1

x 10^9. Samples were taken out during day 0, 3, and 8 to see the performance of the mix on the BOD levels.

3.11.2 KLB Denitrification Test

The KLB denitrification test determined the ability of KLB, the *Bacillus subtilis* bacteria isolated from the Biowish Thai product, to denitrify nitrate to nitrite in anaerobic conditions.

3.11.2.1 First KLB Denitrification Experiment

The first KLB denitrification experiment took place on February 10, 2014. There were KLB duplicates and an activation media control. The activation media components are shown in *Table 6*. 0.25 g/L of KLB (2.5 x 10^{10} CFU) was activated in the media with trace amounts of yeast extract for 36 hours at 30°C in an incubator.

After activation, 10 mL of the activated KLB was added to a new 500 mL bottle of activation media. 1 g/L glucose was added to 500 mL bottles as a carbon source for the bacteria and 50 ppm of N (360 mg of KNO_3) was added to spike the test only to the duplicates of KLB. Samples were taken for hours 0, 6, 12, and 24. 5 mL aliquots were taken and filtered through 0.22 µm filters into Polyvials. Two drops of a strong base, potassium hydroxide (KOH), was added to stop any further denitrification processes. Ion chromatography was performed with the Polyvials.

Table 6. KLB denitrification activation media components.

g/L	Chemical
2	Glucose
1	NA_2HPO_4
1	KH_2PO_4
0.25	KNO_3
trace	Yeast extract

3.11.2.2 Second KLB Denitrification Experiment

The second experiment took place February 22, 2014. This experiment corrected for the aerobic conditions in the first experiment by keeping the caps on the 500 mL bottles to create anaerobic conditions. Also, the control for the experiment was water with 30 ppm of NO_3- in order to make sure the bottle was not contaminated with any of the *B. subtilis* bacteria spores.

Duplicates of the KLB samples were activated in activation media with trace amounts of yeast extract for 24 hours at 30°C in an incubator. Afterwards, 10 mL of the activated KLB was transferred to a new 500 mL bottle of activation media. 400 mL of growth media was added to the new bottles, thus 85.7 mg of KNO_3- was added to spike the sample of 30 ppm of NO_3-. The experiment ran for 96 hours with samples collected at hours 0, 6, 20, 46, and 92. 5 mL samples were pipetted and filtered through a 0.22 µm filter into a Polyvial. 2 drops of 10N KOH was added to prevent any further processes from occurring. Afterwards, the Polyvials were tested in the ion chromatography machine for nitrate and nitrite levels.

3.11.2.3 Third KLB Denitrification Experiment

The third denitrification experiment was to repeat the second experiment, but aimed for higher accuracies and to test TA. It began with bacteria reactivation on November 16, 2014 and ended on November 21 with sampling during hour 96. Duplicates of the KLB microcosms were experimented with a water control spiked with 30 ppm of NO_3-, and one sample of TA.

After activating the samples for 30 hours in the activation media, 10 mL of the activated media were added to new activation media in 500 mL bottles. 30 ppm of NO_3-

was added to spike the samples and 0.8 g of glucose was added to the new activation media to act as a carbon source for the bacteria. The control sample did not have glucose added, only DI water and 30 ppm of NO_3- constituted the sample. Bottles were capped for anaerobic conditions and stored in a 30° C incubator to shake. Samples were taken out for hours 0, 6, 18, 48, and 96 hours for NO_3- and NO_2- measurements.

Because the Dionex-120 instrument at the time was not reading any peaks of the solutions spiked with NO_3- and NO_2- during the warm-up exercise, a colorimeter test was performed instead using the Hach DR/890 hand held colorimeter and the procedures in the DR 890 Colorimeter Manual by Hach Company (2013). A colorimeter passes a specific wavelength of light through the solution and measures the difference of light that is absorbed and reflected in order to obtain the concentration of the substance in the solution. The Beer – Lambert's law reflects the proportionality of absorbance and concentration as shown in Equation *10* ("Molecular Spectroscopy," 2014).

Equation 10. Beer - Lambert's Law.

$$A = \varepsilon * l * c$$

where:

ε *is the wavelength dependent molar absorptivity coefficient*

l *is the path length*

c *is the analyte concentration*

To determine the NO_3- levels, a cadmium reduction method using powder pillows for high range NO_3- was performed. To determine NO_2- levels, a ferrous sulfate method was performed for high range NO_2-. A calibration curve was first completed by measuring the known concentrations of NO_3- and NO_2-, then a curve of expected versus

observed concentrations were plotted to obtain an equation for calculating the NO_3- and NO_2- concentrations.

The cadmium reduction method required filling the sample cell with 25 mL of sample and then adding one NitraVer 5 Nitrate reagent Powder Pillow to the prepared sample. A dilution factor was performed if the colorimeter read its maximum limit, thus based on the test, a dilution was done. The sample was capped and shaken vigorously. The sample was given 5 minutes for the color development into an amber color as shown in *Figure 13*. Another cell with 10 mL of DI water, known as the blank, was placed into the cell holder in order to get a zero reading. Afterwards, the prepared sample was placed in the cell holder and the NO_3- concentration was read.

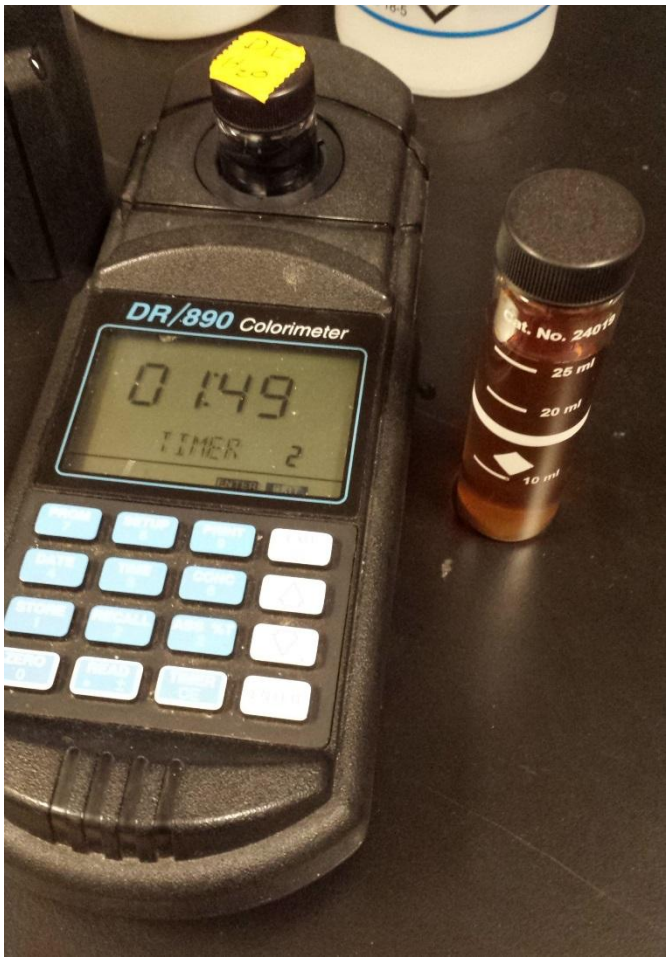


Figure 13. Measuring nitrate concentrations.

The ferrous sulfate method was performed for NO_2- measurements by filling the sample cell with 10 mL of sample with one NitriVer2 Nitrite Reagent Powder Pillow. The cell was capped and inverted 7 times to mix the prepared sample. The prepared sample was allowed 10 minutes to react while another sample cell was filled with 10 mL of the sample as the blank. The blank was placed in the cell holder and zeroed out. Then, the prepared sample was gently inverted twice and then measured in the cell holder.

Chapter 4

Results and Discussion

For the results and discussion section, the layout of the results is organized by the test – BOD, TS, TSS, VSS, PSD, IC, and microscopy – and then the experiment number chronologically. The original data figures and data tables are first analyzed. Afterwards, the normalized data tables follow with an analysis. For PSD, the graphs and results of both the raw and normalized data showing the four pore sizes are displayed following a discussion of the graphs. The normalized graphs are shown in *Appendix 7.4.*

The normalized graphs were normalized to the control by obtaining the ratio from day 0 control to day 0 of the sample for the bacterial mixes without redose. It was multiplied by the value of the sample for each week as shown in *Equation 11*. For the redose normalization, the redose were normalized to the original bacterial mix by obtaining the ratio of the day 14 bacterial mix value divided by the redose day 14 value and multiplied by the redose day 14 value as shown in *Equation 12*.

Equation 11. Original bacterial mix normalized to control.

$$x_{0c} = \left(\frac{c_0}{x_0}\right) * x_0$$

$$x_{7c} = \left(\frac{c_0}{x_0}\right) * x_7$$

$where$:

$x_{0c} = bacterial\ mix\ day\ 0\ value\ normalized\ to\ the\ control$

$c_0 = control\ value\ day\ 0$

$x_0 = bacterial\ mix\ value\ for\ day\ 0$

$x_{7c} = bacterial\ mix\ day\ 7\ value\ normalized\ to\ the\ control$

Equation 12. Redose bacterial data normalized to original bacterial data.

$$r_{14c} = \left(\frac{x_{14}}{r_{14}}\right) * r_{14}$$

$$r_{21c} = \left(\frac{x_{14}}{r_{14}}\right) * r_{21}$$

where:

$r_{14c} = redose\ day\ 14\ value\ normalized\ to\ the\ bacterial\ mix$

$x_{14} = bacterial\ mix\ value\ day\ 14$

$r_{14} = redose\ day\ 14\ value$

$x_{21c} = bacterial\ mix\ value\ for\ day\ 21\ normalized\ to\ the\ bacterial\ mix$

4.1 BOD_5

BOD tests were done for all the experiments with usually a 35 day time period. For Experiment I, the BOD test was done for 35 days every week and then a final testing point at day 57. There was also a miscellaneous 8 day BOD test for a three bacteria mix sample of MDG, LCM1, and Osprey.

4.1.1 Experiment I

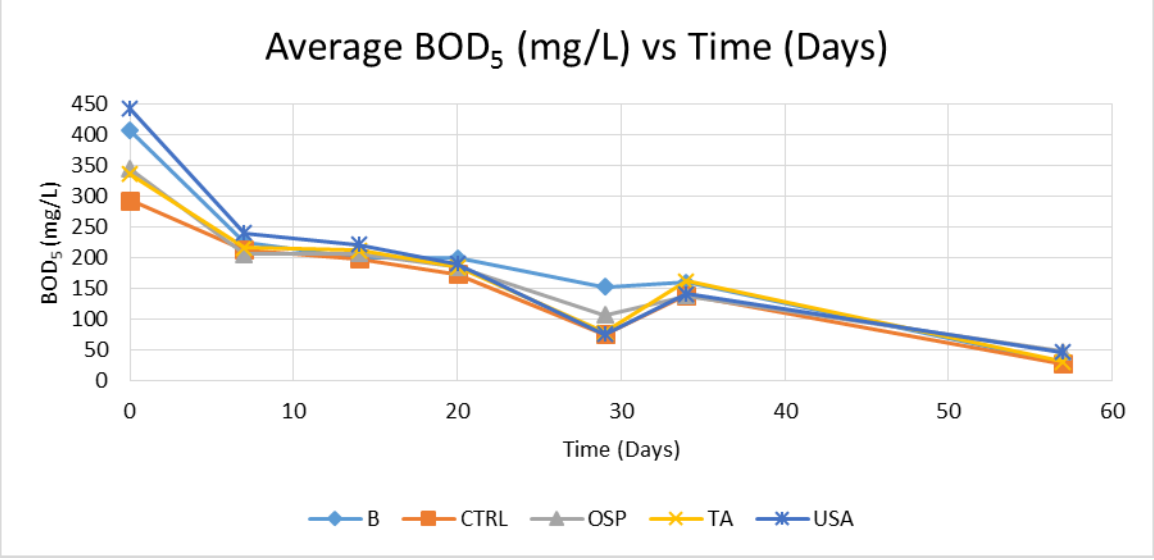


Figure 14. Average BOD_5 graph of the control, B, OSP, TA, and USA.

With the addition of bacteria mix for B, OSP, TA, and USA, the BOD initially also increased. Although the BOD for the bacterial mix addition was higher in the beginning, over time, the BOD_5 levels decreased to about the same as the CTRL. Also, this experiment was closed cap, so there was no air supply to the bacteria.

Table 7. Experiment I BOD Results.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 57 (mg/L)	% Change
CTRL	293.38	27.87	90.5
BMT	406.6	28.8	92.9
OSP	344	47.7	86.1
TA	335.8	31.2	90.7
USA	441.9	46.2	89.6

The BOD levels in this experiment decreased slower than if there was air exchange. BMT had the largest percent change from initial value to final BOD value as shown in *Table 7* with the non-manipulated data.

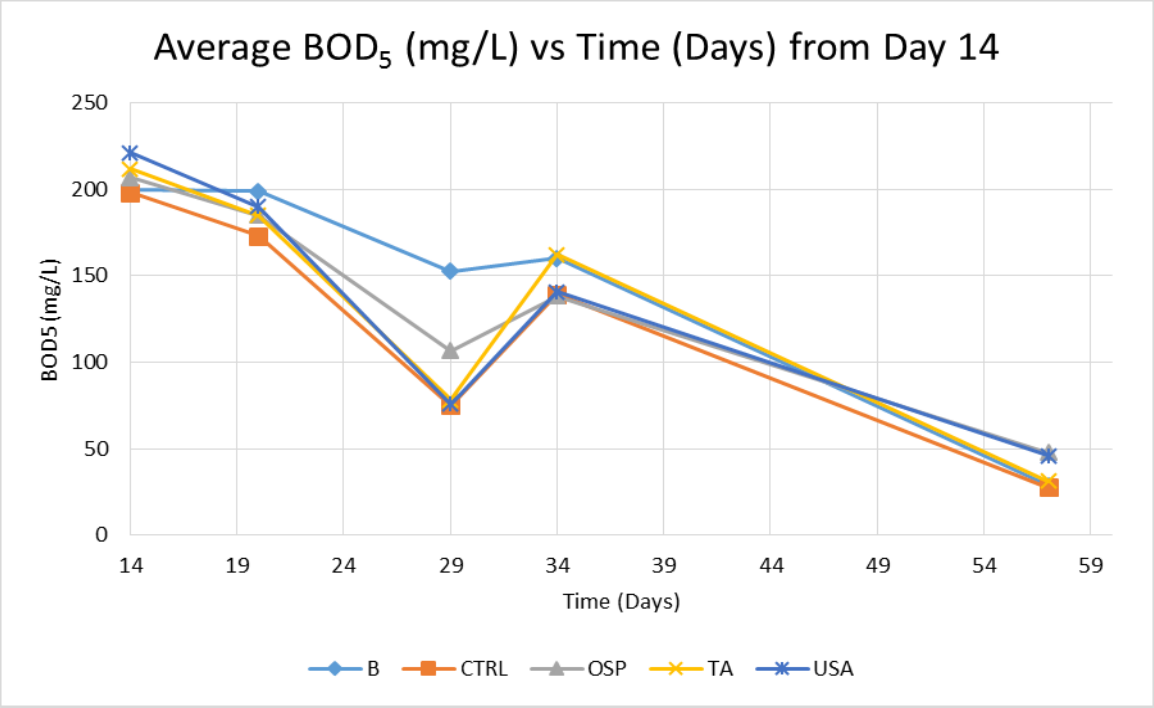


Figure 15. Average BOD results for Experiment I from day 14.

As shown in *Figure 15*, the average data for BOD_5 shows that the USA reduced the BOD faster than most of the other samples. At Day 14, USA lowered the BOD the most from all the samples.

Table 8. Normalized BOD data for Experiment I.

Bacterial Mix	BOD_5 Day 0 (mg/L)	BOD_5 Day 57 (mg/L)
CTRL	293.4	27.9
BMT	293.4	20.8
OSP	293.4	40.7
TA	293.4	27.2
USA	293.4	30.7

H

owever, ultimately, the BMT sample reduced the BOD the lowest with a value of 20.8 mg/L after being normalized to the control as shown in *Table 8*. The control and TA had the next lowest BOD levels at 27.9 mg/L and 27.2 mg/L respectively.

4.1.2 Experiment II

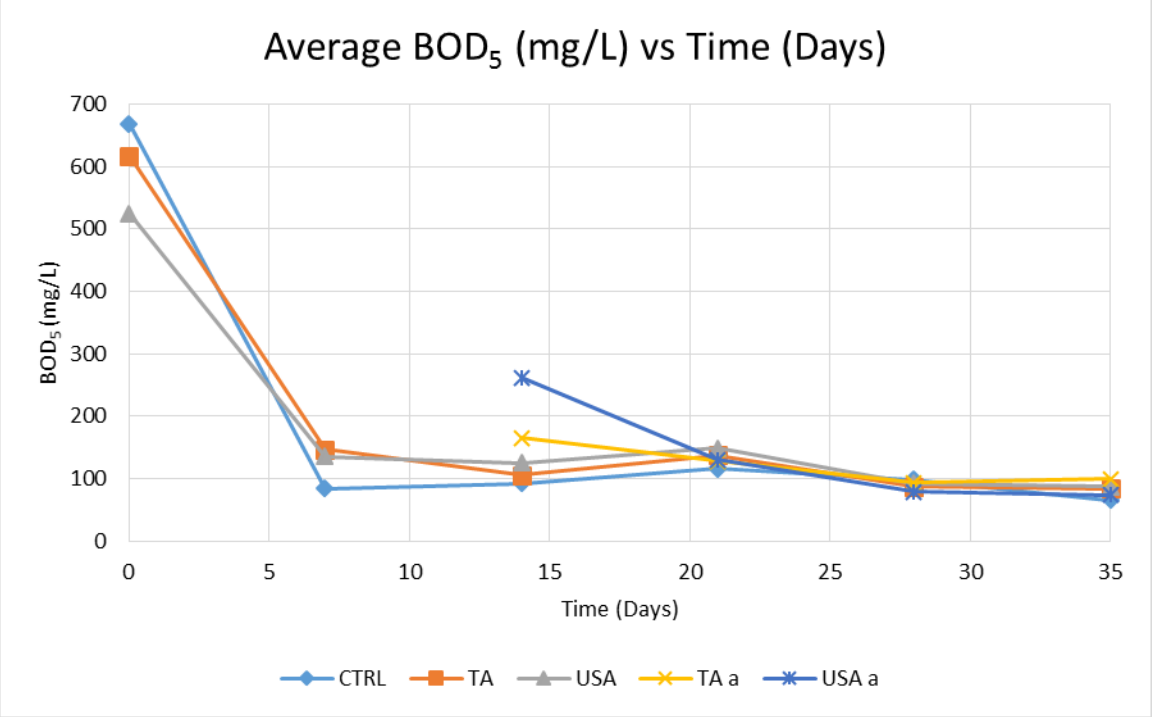


Figure 16. The BOD_5 measurements of the average samples with redose.

The bottles were not capped for this experiment and had a filter that allowed for air exchange, thus the BOD levels decreased quickly from day 0 to day 7 compared to Experiment I. In Experiment I, it took between day 20 and 30 for the BOD levels to reach 100 mg/L while it took only a week for the aerobic conditions to decrease to 100 mg/L as shown in *Figure 16*. The control showed a higher BOD level during day 0, but then it lowered day 7, which may be due to only having one control sample rather than averaging duplicates. It would be expected that control would have lower BOD since there is no additional bacteria added.

Table 9. BOD data for Experiment II.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	668.4	66.0	90.1
TA	616.6	84.6	86.3
USA	524.4	87.3	83.3

In *Table 9*, based on the raw data, the control had a 90.1% BOD reduction compared to TA and USA having a BOD reduction of 86.3% and 83.3% respectively. This could be attributed to the indigenous bacteria in the dairy wastewater to be naturally reducing the BOD while the TA and USA, while also reducing BOD, is not as efficiently.

Table 10. BOD data with redose for Experiment II.

Average	BOD_5 Day 14 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	93.1	66	29.1
TA	105.9	84.6	20.0
USA	125.3	87.3	30.3
TA a	165.8	99.9	39.7
USA a	261.8	73.8	71.8

Table 10 lists the values of BOD for day 14 and day 35 for the samples and shows how redosing during day 14 further reduces BOD levels than if not redosing at all. USA redose showed a 71.8% reduction compared to 30.3% reduction of the USA sample from day 14 to day 35.

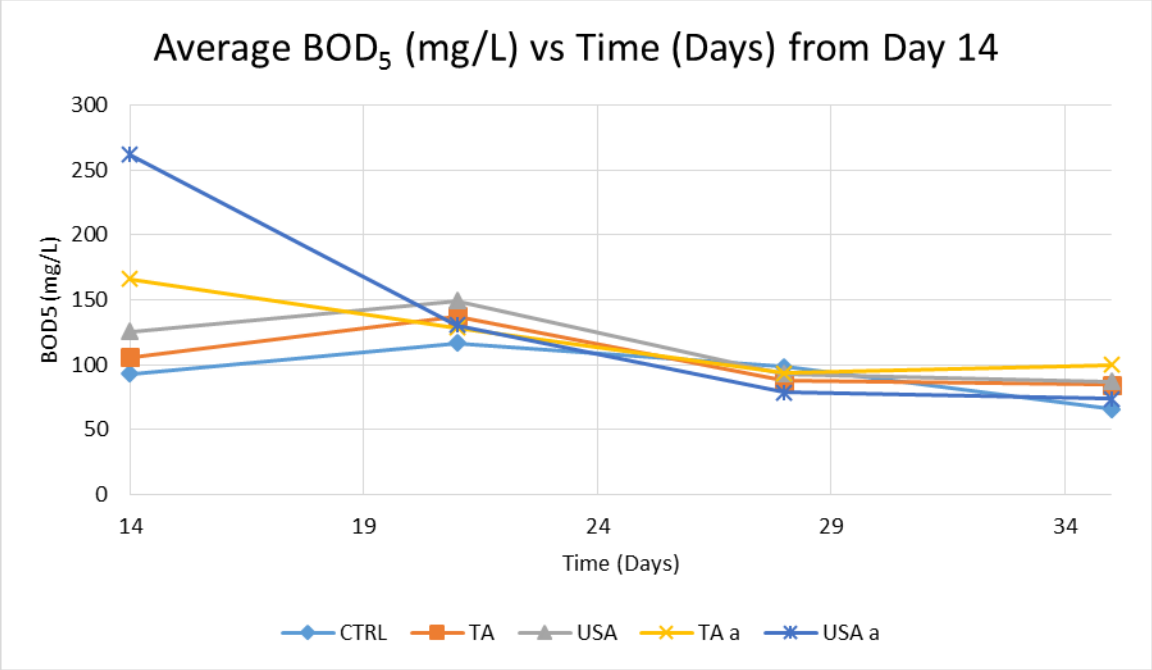


Figure 17. Average BOD graph for Experiment II from day 14.

By observing from day 14, it can be shown in *Figure 17* that the redose bacteria (marked with 'a' after the bacterial mix) does further reduce the BOD levels than if there were no redose at all, since USA a starts at a higher BOD concentration and reaches a final BOD concentration similar to the control.

Table 11. Normalized BOD data for Experiment II.

Average	**BOD_5 Day 0 (mg/L)**	**BOD_5 Day 14 (mg/L)**	**BOD_5 Day 35 (mg/L)**
CTRL	668.4	93.1	66
TA	668.4	114.7	91.7
USA	668.4	159.8	111.3
TA a		114.7	63.8
USA a		159.8	35.3

Table 11 lists the percent difference between the bacterial samples from day 0 to day 14. For the redose samples, they begin on day 14. The table provides the numerical data for the normalized values recorded for BOD. The redose normalized value for TA a and USA a showed the lowest BOD values of 63.8 mg/L and 35.3 mg/L respectively.

4.1.3 Experiment III

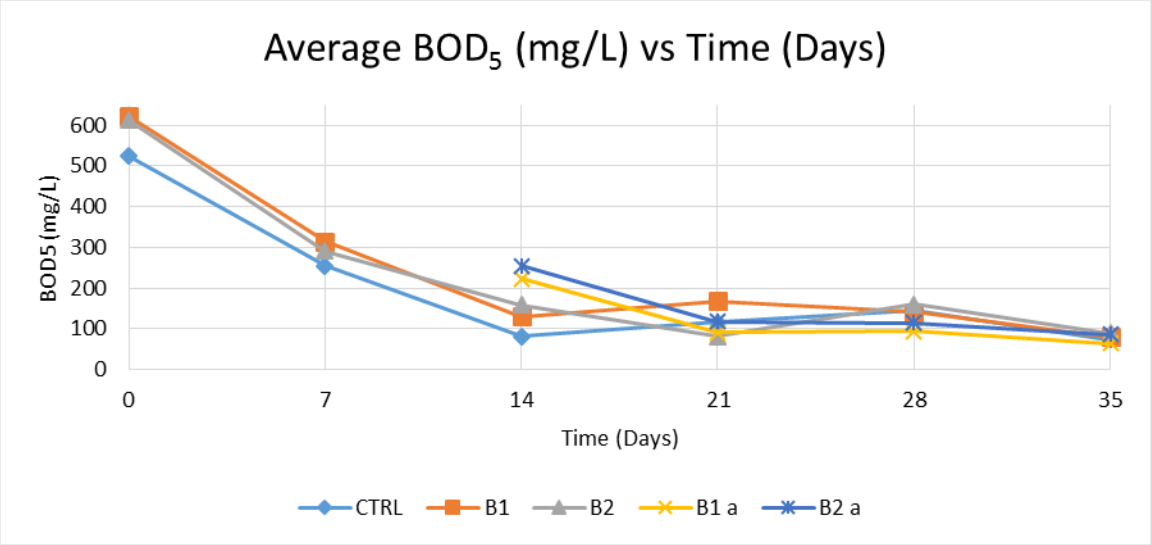


Figure 18. BOD data for Experiment III.

In Experiment III, the bottles were not capped for this experiment and had a filter that allowed for air exchange, thus the BOD levels decreased quickly from day 0 to day 14. The control also showed lower BOD levels throughout the experiment, but it may be due to no bacterial addition. As shown in *Figure 18*, B1 a and B2 a measured higher BOD levels during day 14, but decreased lower than the other samples at day 35.

Table 12. Original BOD data for Experiment III.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	523.6	73.2	86
B1	621.9	79.5	87.2
B2	612.9	89.1	85.5

Table 12 shows close BOD percent difference between the B samples, which are the same bacterial mix but developed in different batches. B1 showed the highest percentage difference from day 0 to day 35 at 87.2%, while the B2 and control trails by a little at 85.5% and 86% respectively.

Table 13. BOD original data results with redose starting day 14 for Experiment III.

Average	BOD_5 Day 14 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	81.37	73.20	10.0
B1	129.37	79.50	38.5
B2	158.62	89.10	43.8
B1 a	222.37	63.60	71.4
B2 a	254.62	85.20	66.5

The redose average BOD values started higher with 250 ppm addition of Biowish bacteria during day 14. By Day 35, the redose BOD values dropped lower than without the redose as shown in *Table 13*. By adding more of the non-indigenous bacteria,

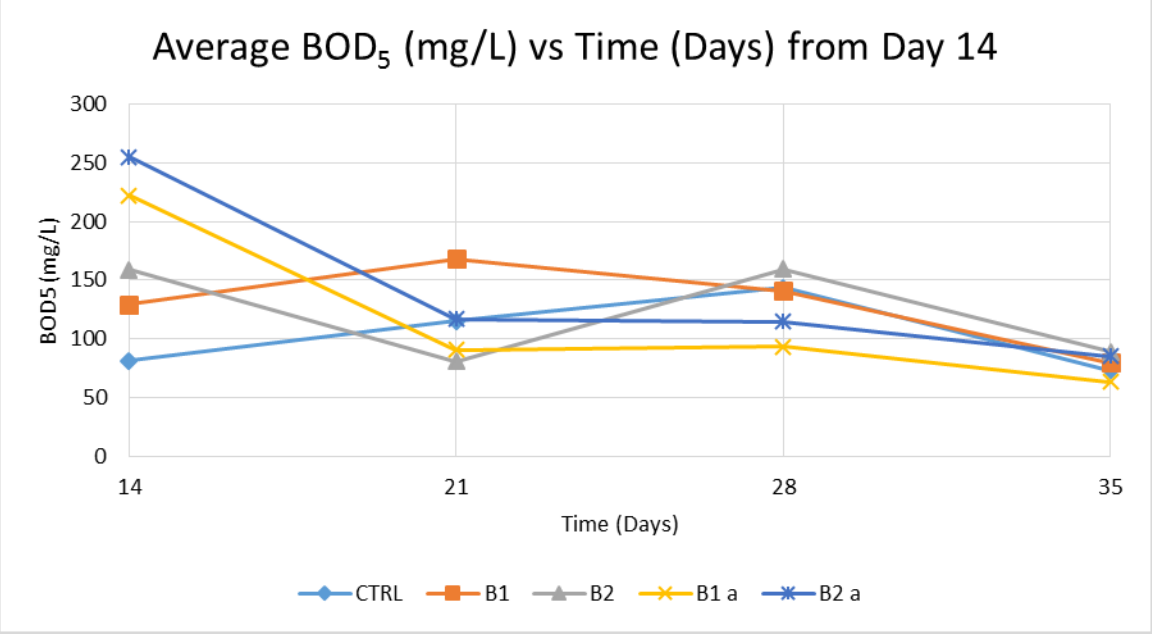


Figure 19. Average data for Experiment III from day 14.

enhanced reduction of BOD is occurring.

During the course of the experiment, from day 14, the redose samples of B1 and B2 showed a lower BOD decrease than without the redose as shown in *Figure 19*.

Table 14. Normalized BOD data for Experiment III.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 14 (mg/L)	BOD_5 Day 35 (mg/L)
CTRL	523.60	81.37	73.2
B1	523.60	109.51	67.0
B2	523.60	135.46	76.1
B1 a		109.51	31.0
B2 a		135.46	45.3

Table 14 shows the normalized BOD values for all the samples during the experiment. The B1 a and B2 a do not have values at day 0 since they are the redose samples that start on day 14. The day 35 values for B1 a and B2 a are lower than the control and the original bacterial mix samples at 31 mg/L and 45.3 mg/L respectively.

4.1.4 Experiment IV

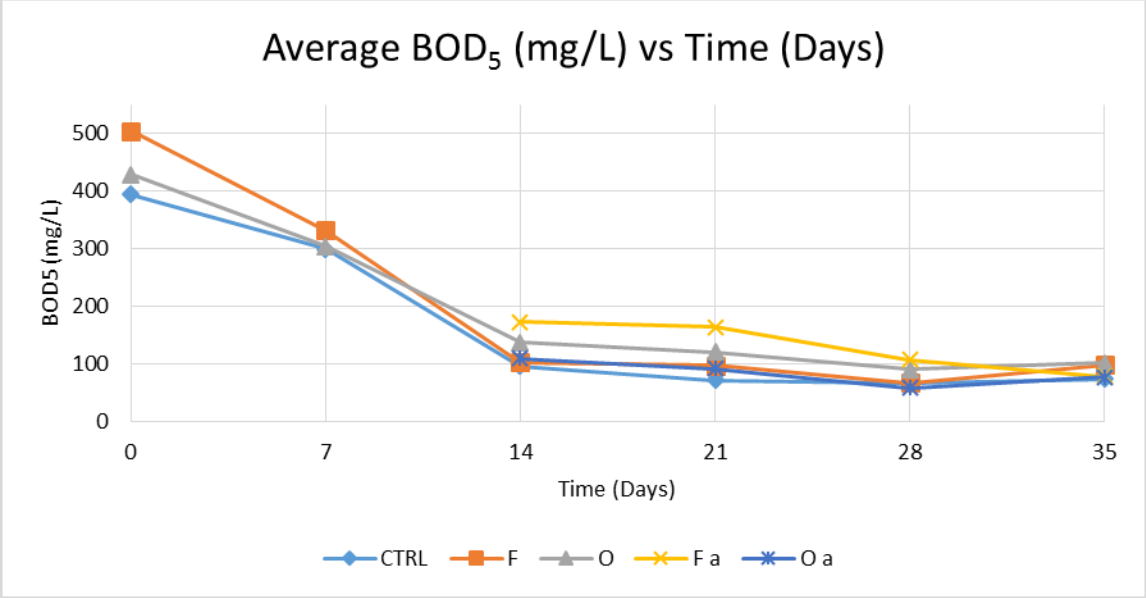


Figure 20. Raw data BOD results of Experiment IV.

In Experiment IV, Fruit Wash and Osprey were tested in aerobic conditions and with redose of 250 ppm of the respective bacterial mixes on day 14. It was surprising to see Osprey around the same level as the bacterial samples that had been around for two weeks prior. It could be that the Osprey bacterial mix of *Pseudomonas* needs special ecological consideration and the dairy wastewater is not a habitable environment for the bacteria to grow as discussed in the research by Thompson *et al.* (2005).

Table 15. BOD raw data for Experiment IV.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	394.76	73.82	81.3
F	504.26	98.04	80.6
O	428.51	101.47	76.3

From *Table 15*, the control showed the highest percent difference of BOD reduction of 81.3%. The Fruitwash and Osprey also had a high percent difference of initial to final BOD of 80.6% and 76.3% removal respectively.

Average	BOD_5 Day 14 (mg/L)	BOD_5 Day 35 (mg/L)	% Difference
CTRL	96.06	73.82	23.2
F	102.81	98.04	4.6
O	136.56	101.47	25.7
F a	172.56	78.11	54.7
O a	109.56	76.40	30.3

Table 16. BOD raw data with redose for Experiment IV.

Table 16 lists the original data for the samples including the redose. The F redose reduced BOD by 54.7% from day 14 until day 35. The control, F, and O showed a smaller change from day 14 to day 35, which may be attributed to the lack of new bacteria to break down the organic material.

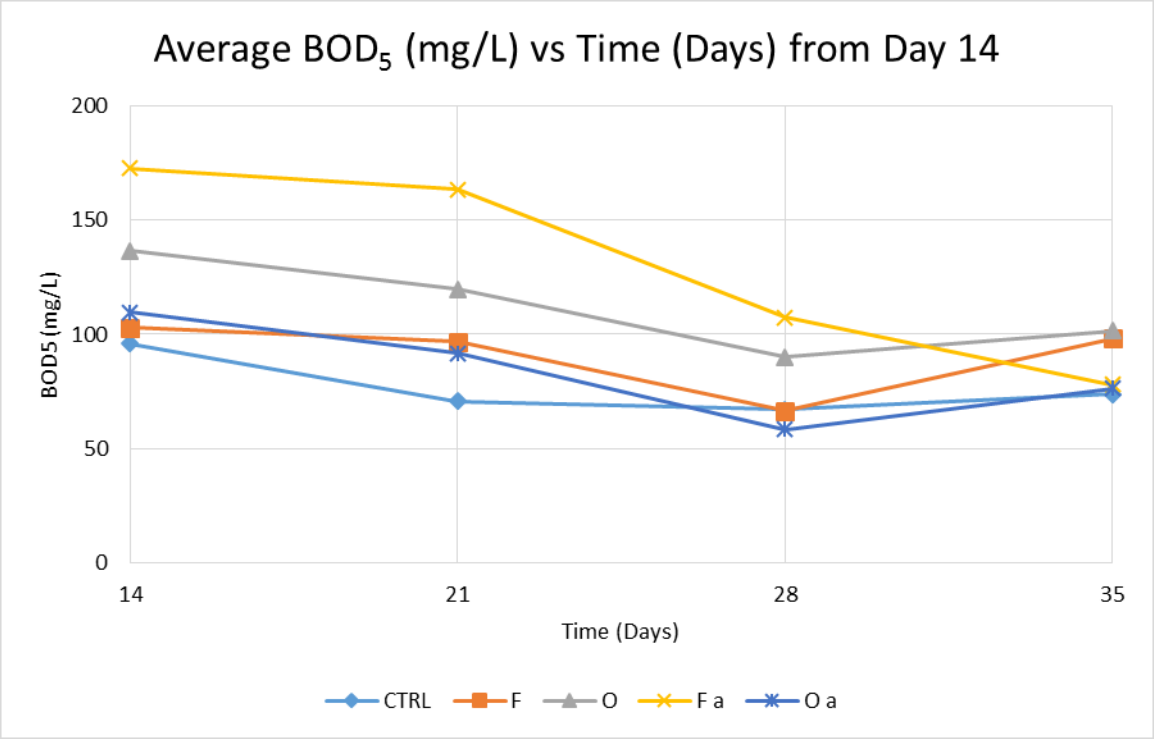


Figure 21. Average BOD data for Experiment IV from day 14.

The average BOD graph from day 14, *Figure 21*, clearly shows that both redose samples continued to reduce BOD levels to the same concentration as the control, even though the control started at lower BOD levels.

Table 17. Normalized BOD data for Experiment IV.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 14 (mg/L)	BOD_5 Day 35 (mg/L)
CTRL	394.76	96.06	73.82
F	394.76	80.49	76.80
O	394.76	125.90	93.59
F a		80.49	36.58
O a		125.90	88.12

Table 17 lists the BOD measurements from day 0, 14, and 35 that is normalized to the control. The F a showed the lowest BOD value for day 35 at 36.58 mg/L whereas the redose for O showed BOD levels close to or higher than those samples that were not redosed.

4.1.5 Experiment V

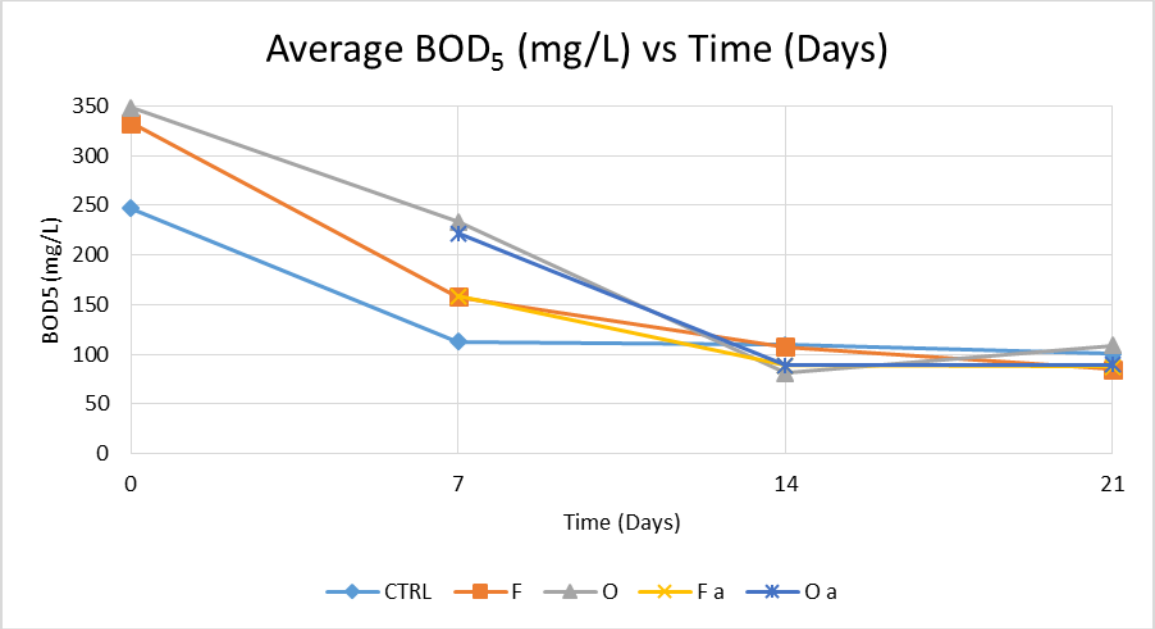


Figure 22. Average BOD concentrations for Experiment V.

For Experiment V, the redose ppm was only 10 ppm, thus from *Figure 22*, there is no peak in BOD during day 7 unlike the previous redose experiments that had 250 ppm or 500 ppm bacterial mix addition. Also, the control starts at a lower BOD concentration since there is no additional bacteria initially compared to the F and O sample where 250 ppm bacteria was added.

Table 18. Average BOD values for Experiment V.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 21 (mg/L)	% Difference
CTRL	246.77	100.23	59.4
F	333.02	84.93	74.5
O	348.02	108.93	68.7

For the original samples, the F sample reduced the BOD concentrations the most at 75% with the O sample following with a 69% BOD reduction as shown in *Table 18*. The control reduced BOD by 60% without any Biowish supplementation. By adding Biowish, the BOD levels get further reduced than without Biowish.

Table 19. Average redose BOD values for Experiment V.

Average	BOD_5 Day 7 (mg/L)	BOD_5 Day 21 (mg/L)	% Difference

CTRL	112.52	100.23	10.9
F	157.52	84.93	46.1
O	233.27	108.93	53.3
F a	158.27	87.63	44.6
O a	222.02	89.43	59.7

When comparing the effects of redose, the BOD levels for day 7, the initial day for redose, to the final day of testing was compared. *Table 19* shows that the original O sample and redose reduced the BOD levels by 53% and 60% respectively. The F a sample did not reduce BOD further than the original sample by 1.5%, which is not too

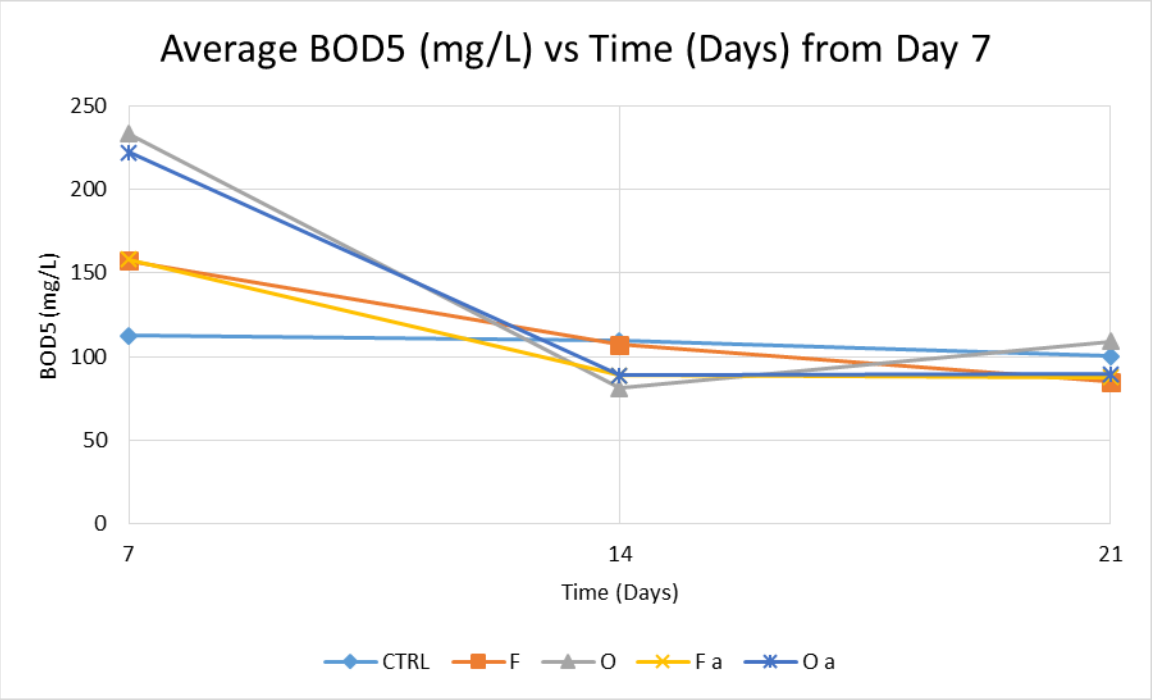


Figure 23. Average BOD results for Experiment V from day 7.

much. It may be due to the low redose concentration.

Figure 23 displays the average BOD graph for all the samples to the control and original samples. The control remained steady after day 7 leveling at about 100 mg/L BOD. The Biowish samples continued reducing BOD to day 14 and leveled at day 21 at around 70 mg/L BOD.

Table 20. Normalized BOD values for Experiment V.

Average	BOD_5 Day 0 (mg/L)	BOD_5 Day 7 (mg/L)	BOD_5 Day 21 (mg/L)
CTRL	246.77	112.52	100.23
F	246.77	122.18	63.83
O	246.77	164.75	77.91
F a		122.18	67.65
O a		164.75	66.36

Experiment V demonstrated that reducing the redose concentration to 10 ppm had an effect on BOD levels. *Table 20* shows that Biowish samples reduced BOD under 100 mg/L with the F sample reducing BOD the lowest to 63.8 mg/L and the Osprey redose following at 66.4%. Thus, in order to prevent BOD peaks from redosing at high concentrations, it is possible to add a smaller concentration of bacterial mix to accomplish a further reduction of BOD.

4.1.6 Miscellaneous Experiment

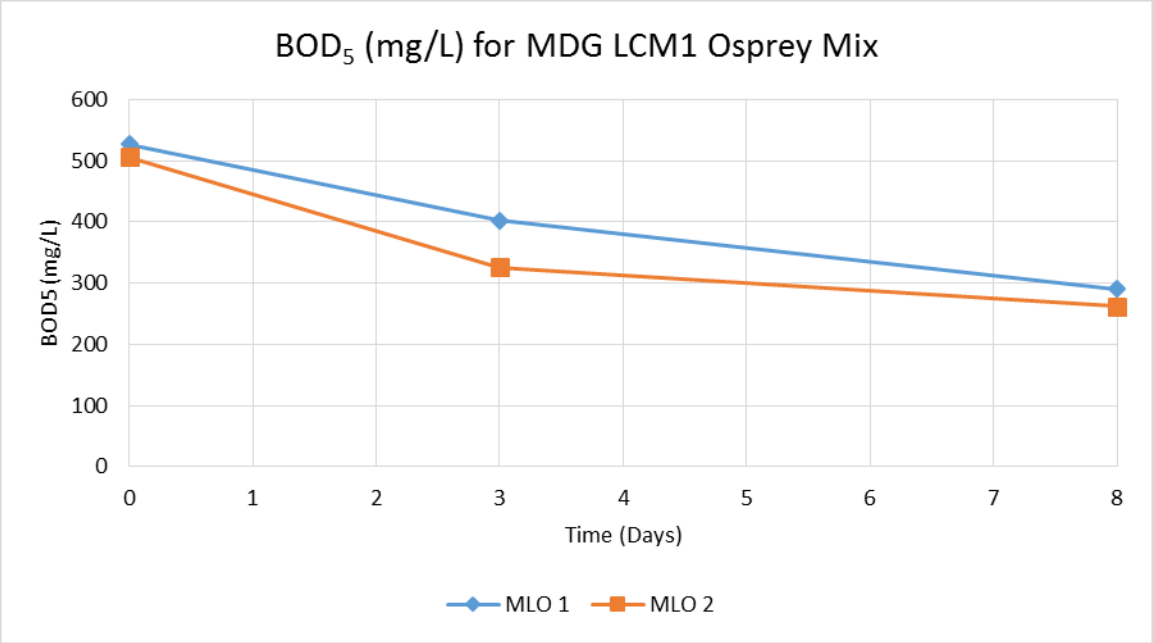


Figure 24. BOD_5 for the bacterial mix of MDG, LCM1, and Osprey over 8 days.

It was an 8 day experiment with capped 2 L bottles under anaerobic conditions with duplicates. *Figure 24* shows the BOD measurements of the duplicates slowly decreasing over time.

Table 21. Percent difference between MLO duplicates.

Bacterial Mix	**Day 0**	**Day 3**	**Day 8**
MLO 1	526.55	401.91	290.41
MLO 2	505.55	325.41	261.91
Change %	4	19	9.8

The trends show similar values as shown in with 4 – 19% difference between the two bacterial mix samples values shown in *Table 21*. The proximity of the values show the test was done with as little error as possible.

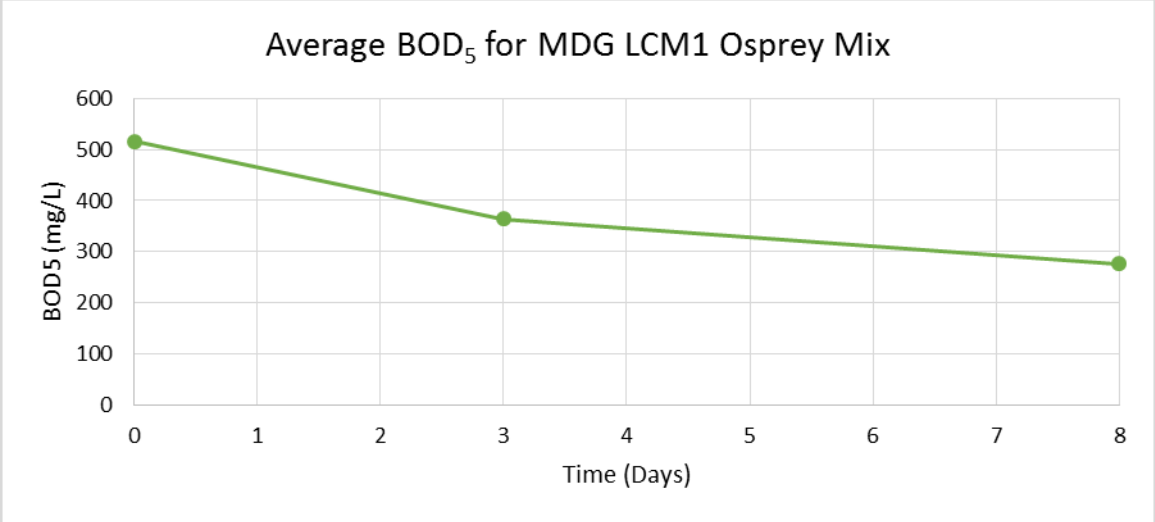


Figure 25. The averaged BOD_5 levels of the MDG, LCM1, and O mix.

In *Figure 25*, the duplicates of MDG, LCM1, and Osprey bacterial mix were averaged to create the graph. 1 x 10^9 CFU of MDG, LCM1, and Osprey were added to the growth media. The bacterial mix of LCM1, MDG, and Osprey showed a slow, but continuous BOD decrease. The slow decrease may be due to the lack of air exchange

since the bottles were capped in this experiment. Anaerobic conditions were implemented for this BOD experiment. BOD_5 was measured for Day 0, 3, and 8 with 2 mL of sample diluted in 300 mL BOD bottles.

Table 22. BOD data results for the miscellaneous experiment.

	Day	**mL of sample**	**BOD_5 (mg/L)**	**Average (mg/L)**
MLO 1	0	2	526.55	516.05
	3	2	401.91	363.66
	8	2	290.41	276.16
MLO 2	0	2	505.55	
	3	2	325.41	
	8	2	261.91	

From *Table 22*, the BOD values for each of the samples are listed with the average calculated to generate the figure of the MDG, Lactic Mix 1, and Osprey bacterial mix. In 8 days, the three bacterial mix was able to decrease the BOD by 46.5%.

4.2 Solids

The solids tests results will be organized by TS, TSS, TDS, VSS, and PSD tests, which is further organized by the chronology of the experiments. For Experiments II – V, TS tests were performed.

4.2.1 Total Solids (TS)

Total solids testing were done for Experiment II – V. The average TS graphs with tables are first analyzed and then the normalized TS graphs and tables are further discussed to analyze the effects of the Biowish bacterial mixes compared to the control.

4.2.1.1 Experiment II

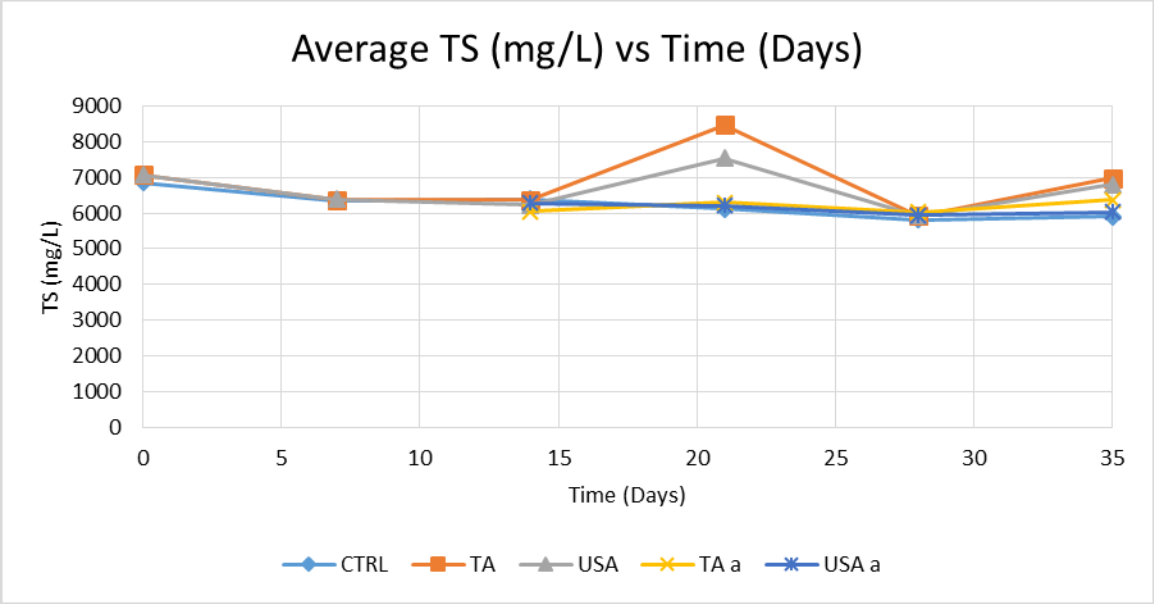


Figure 26. Average TS results for Experiment II.

The TS graph, shown in *Figure 26*, has a steady trend throughout 35 days with a sharp increase during day 21. It may be attributed to that day's sampling methods and lab conditions since the stable trend continues the weeks following. The total solids test also showed the same trend for an aerobic purification of dairy wastewater performed by Carta-Escobar *et al.* (2004) for a single reactor and three-stage reactor experiment with variable COD levels that lasted about 50 days. In *Table 23*, the TS values did not change very much from day 0 to 35.

Table 23. TS data results from Experiment II.

Average	TS Day 0 (mg/L)	TS Day 35 (mg/L)	% Difference
CTRL	6860	5900	14.0
TA	7060	6970	1.3
USA	7075	6795	4.0

Table 24. TS normalized data with redose for Experiment II.

Average	TS Day 0 (mg/L)	TS Day 14 (mg/L)	TS Day 35 (mg/L)

CTRL	6860	6380	5900
TA	6860	6191	6676
USA	6860	6031	6588
TA a		6191	6550
USA a		6031	5791

The normalized TS data for days 0, 14, and 35 are presented in *Table 24* with the USA a sample lowering the TS to 5,791 mg/L from the its initial TS count of 6,031 mg/L at day 14. In three weeks, the TS decreased by about 240 mg/L of solids.

4.2.1.2 Experiment III

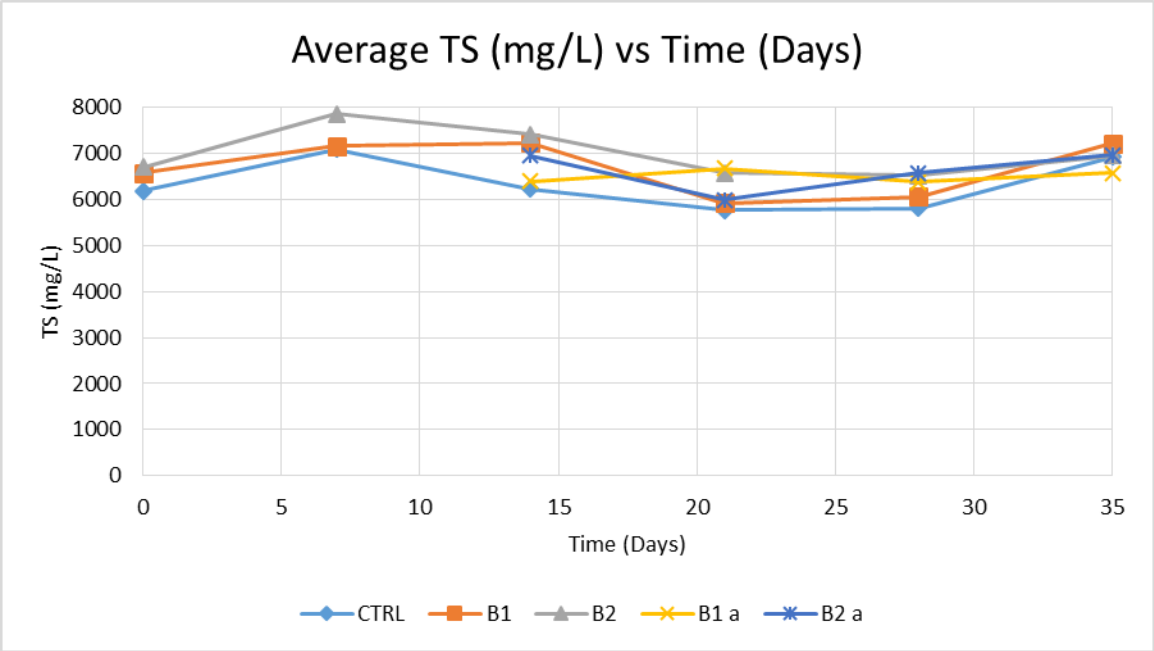


Figure 27. Average TS measurements for Experiment III.

The TS results for Experiment III with the BMT bacterial mix and the control is similar to the results found in Experiment II. *Figure 27* shows fluctuations with the TS data throughout the experiment.

Table 25. TS initial and final results for Experiment III.

Average	TS Day 0 (mg/L)	TS Day 35 (mg/L)	% Change
CTRL	6180	6920	-12.0
B1	6574	7210	-9.7
B2	6690	6945	-3.8

The TS of the control starts lower at 6,180 mg/L as tabulated in *Table 25* since there is no addition of Biowish bacteria that increases the solid content in the other samples. Because of the fluctuations in the data, the difference between the initial and final data points were negative. Laboratory techniques and sampling could have caused the variability and negative values. The levels also rose from day 0 and continued to show an increasing and decreasing trend as the days went by. This unsteady trend can be due to experimental flaws with controlling the moisture in the desiccator or the sampling with wastewater through filters. Overall, the TS for Experiment III shows values from 6,000 mg/L to 7,000 mg/L throughout the 35 day period.

Table 26. TS data with redose for Experiment III.

Average	**TS Day 0 (mg/L)**	**TS Day 14 (mg/L)**	**TS Day 35 (mg/L)**
CTRL	6180	6220	6920
B1	6180	6334	6563
B2	6180	6833	6411
B1 a		6334	6526
B2 a		6833	6813

In *Table 26*, the TS data normalized to the control and the original bacterial samples show that the redose samples did not produce a large difference in total solids reduction. For B2 a, the normalized day 14 value to B2 to its value at day 35 shows a higher TS value of 6,813 mg/L at day 35 compared to B2, which had a TS concentration of 6,411 mg/L.

4.2.1.3 Experiment IV

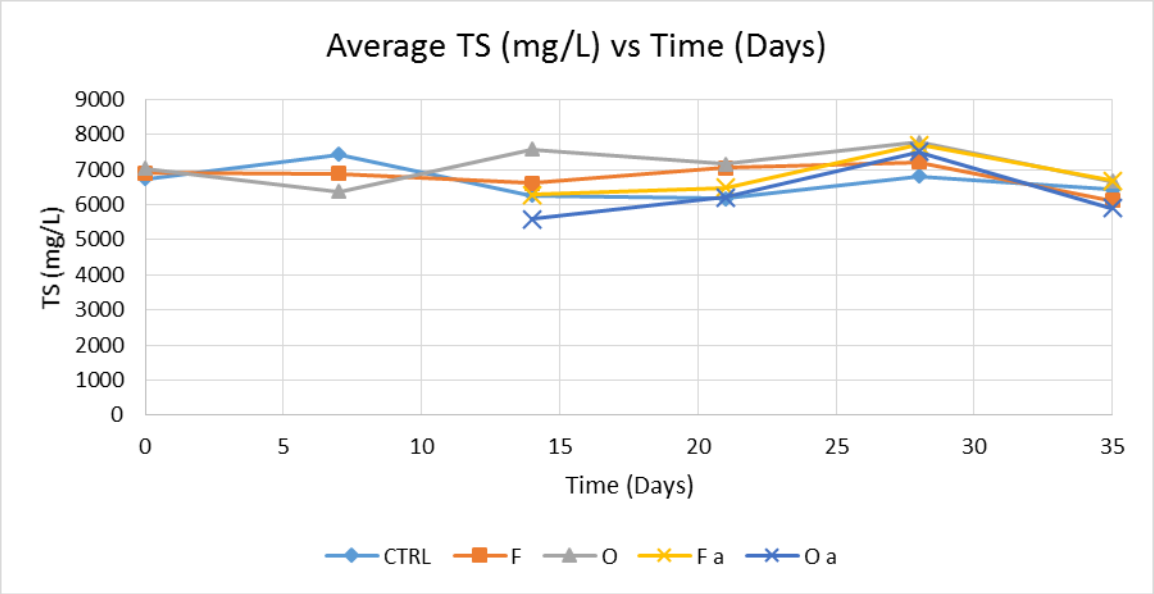


Figure 28. Average TS for Experiment IV.

The TS in Experiment IV, shown in *Figure 28* also does not show any decreasing trends but rather a range of values between 5,500 mg/L – 8,000 mg/L. The total solids in the samples do not change much over 35 days.

Table 27. Initial and final TS data for Experiment IV.

Average	TS Day 0 (mg/L)	TS Day 35 (mg/L)	% Change
CTRL	6740	6420	4.8
F	6895	6125	11.2
O	7025	6670	5.1

As shown in *Table 27*, from day 0 to day 35, the control and Osprey had a low percentage difference of 4.8% and 5.1% respectively. Fruit Wash reduced total solids by 11.2% from day 0 to day 35. The Osprey redose increased higher than all the samples. The F a sample also was higher than the original bacterial mixes and control. This could be that the additional bacterial mix was not needed and contributed to increasing solids rather than further reducing solids.

Table 28. Normalized TS data for Experiment IV.

Average	TS Day 0 (mg/L)	TS Day 14 (mg/L)	TS Day 35 (mg/L)
CTRL	6740	6270	6420
F	6740	6468	5987
O	6740	7359	6426
F a		6468	6864
O a		7359	7772

Based on *Table 28*, the normalized F sample reduced its total solids the most from 6,740 mg/L to 5,987 mg/L compared to the control and O. The redose samples for F and O increased its TS content from day 14 to 35.

4.2.1.4 Experiment V

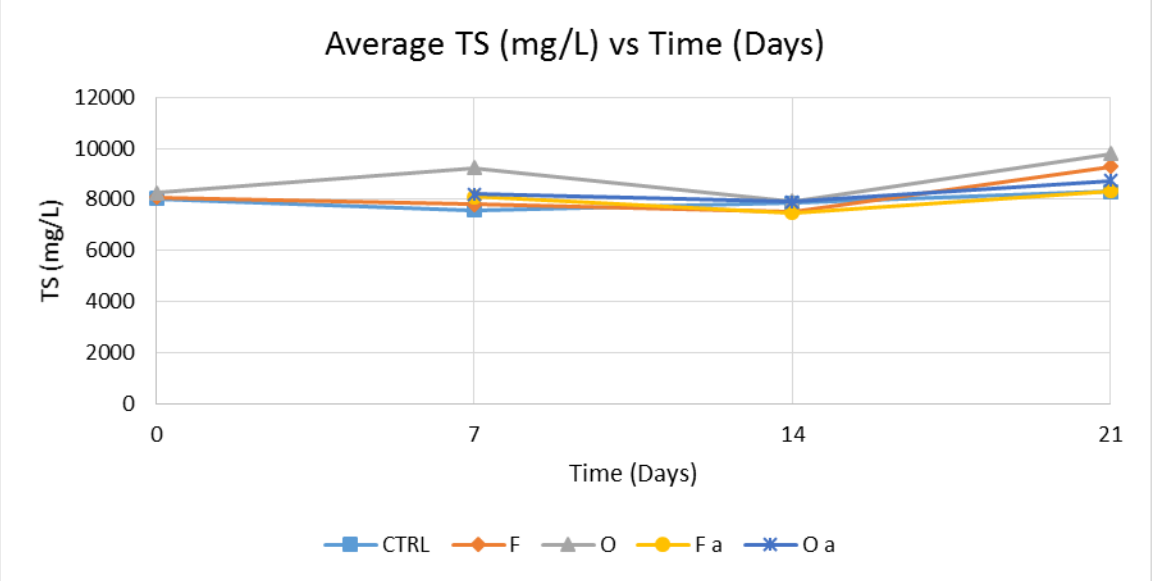


Figure 29. Average TS values for Experiment V.

In Experiment V, the F and O samples were tested again with a day 0 data point. The trend appears steady with periodic increases and decreases in the range of 8000 – 10,000 mg/L TS as shown in *Figure 29.* There was not much change shown in total solids for this experiment.

Table 29. Average TS data for Experiment V.

Average	TS Day 0 (mg/L)	TS Day 21 (mg/L)	% Change
CTRL	8030	8320	-3.6
F	8085	9300	-15.0
O	8255	9800	-18.7

Table 29 tabulates the average values for Experiment V from day 0 to day 21. Because of the increases and decreases throughout the experiment, the percent change is negative. There is not show much change in the TS within the 21 day experiment. During day 21, all samples experienced a slight increase from around 8,000 mg/L of TS to about 9,000 mg/L of TS. Solids change does not occur quickly as demonstrated in this experiment and the previous experiments.

Table 30. Normalized TS data for Experiment V.

Average	TS Day 0 (mg/L)	TS Day 7 (mg/L)	TS Day 21 (mg/L)
CTRL	8030	7570	8320
F	8030	7757	9237
O	8230	8996	9533
F a		7895	8106
O a		7976	8498

Table 30 lists the values for the TS during the initial, final, and redose on day 7. The values are ranged from 7,500 mg/L to 9,000 mg/L TS. As previously stated, the TS trend during the experiment varied from increasing to decreasing and ultimately at the end, increasing.

4.2.2 Total Suspended Solids (TSS)

TSS tests were performed for Experiments I, IV, and V. The following results will be discussed with raw data and normalized graphs with corresponding TSS values in tables based on chronology.

4.2.2.1 Experiment I

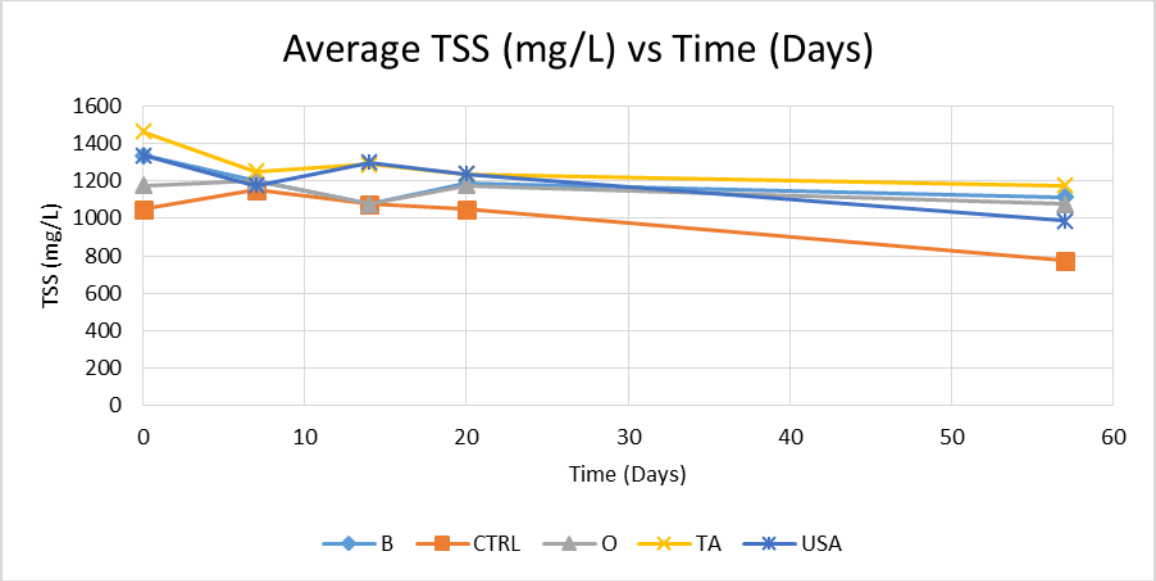


Figure 30. Average TSS data for Experiment I.

For Experiment I, the TSS was tested on three bacterial samples and a control. The TSS was performed weekly from day 0 to day 20, then on day 57. From *Figure 30*, the control had the lowest concentration of TSS compared to the other samples. Rashid and West (2007) showed similar results when they performed TSS for their experiment using effective microbes and duckweed in a dairy wastewater treatment pilot pond. The experiment took about a month and a half in order to see a reduction in TSS in the pond using the effective microbes, duckweed, or a combination of both (Rashid & West, 2007).

Table 31. Average TSS values for Experiment I

Average	TSS Day 0 (mg/L)	TSS Day 57 (mg/L)	% Change
CTRL	1050	775	26.2
B	1337.5	1112.5	16.8
O	1175	1075	8.5
TA	1462.5	1175	19.7
USA	1337.5	987.5	26.2

From Table 31, the control and USA showed the most percentage difference in TSS from day 0 to day 57 of 26.2%. B had the next highest TSS reduction of 20% with TA next reducing TSS by 19.7%. The control and USA have very close low final TSS results around 775 mg/L. The O sample does not show much of a TSS reduction from day 0 to 35. Most of the trend appears to be decreasing over time.

Table 32. Normalized TSS data for Experiment I.

Average	TSS Day 0 (mg/L)	TSS Day 57 (mg/L)
CTRL	1050	775
B	1050	873.4
O	1050	960.6
TA	1050	843.6
USA	1050	775.2

In *Table 32*, the normalized TSS values are shown for all the samples in Experiment I. The control did slightly better at lowering TSS by 0.2 mg/L to the USA. The B and TA sample also resulted in a close TSS reduction of 873.4 mg/L and 843.6 mg/L respectively, while the O sample ultimately reduced its TSS content to 775.2 mg/L.

4.2.2.2 Experiment IV

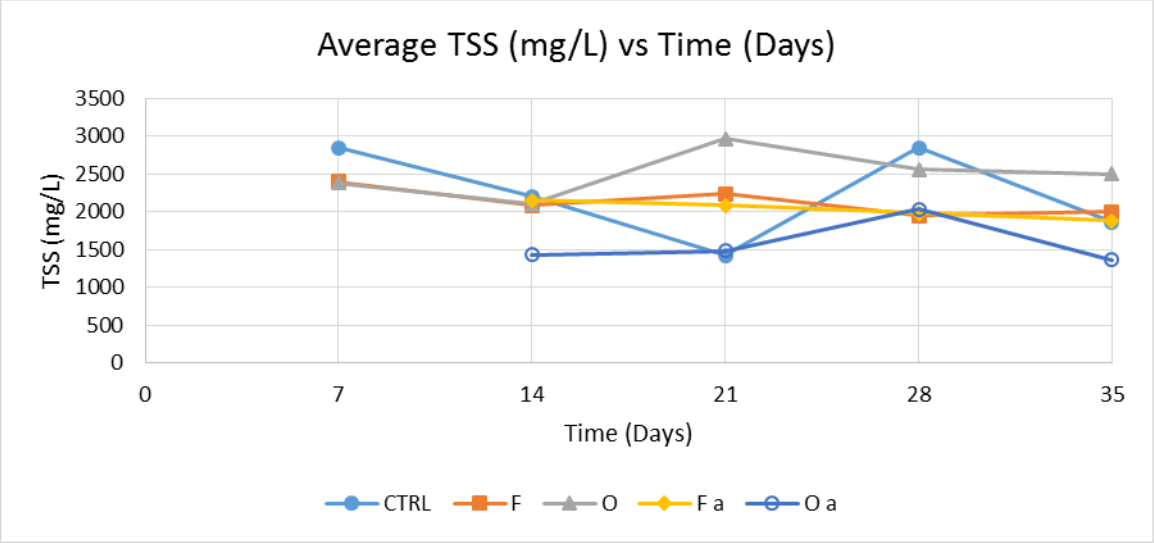


Figure 31. Average TSS results for Experiment IV.

The TSS graph for the average samples in Experiment IV shows varied results, seen in *Figure 31*. The control had higher TSS levels than the Biowish bacterial mixes, however, over time, the control decreased and then increased back to its initial TSS levels. The control showed a lot of variability compared to the other samples.

Average	TSS Day 7 (mg/L)	TSS Day 35 (mg/L)	% Change
CTRL	2850	1866.7	34.5
F	2400	2000	16.7
O	2383.3	2500	-4.9

Table 33. Average TSS data for Experiment IV.

Table 33 shows the average TSS results with the control having the highest percent change from day 7 to day 35. This may be due to its inconsistent data sampling. The F sample has more of a steady trend with a 16.7% change difference while the O sample also shows some variability but with a decreasing trend towards day 35. The control showed the lowest amount of TSS at day 35 with the F a next with the lowest TSS concentrations. As noted before, the inconsistency of the control data means that the data point may not be accurately representative of the sample, as well as not having a day 0 data point.

Table 34. TSS data normalized for Experiment IV.

Average	TSS Day 7 (mg/L)	TSS Day 14 (mg/L)	TSS Day 35 (mg/L)
CTRL	2850	2200	1866.7
F	2850	2474	2375
O	2850	2511.2	2989.5
F a		2474	2167.1
O a		2511.2	2394.4

From *Table 34*, the day 7 normalized TSS data starts at 2,850 mg/L and the lowest TSS concentration after 35 days was the control at 1,866.7 mg/L. The redose

samples showed decreasing TSS values compared to the bacterial samples that did not get redosed.

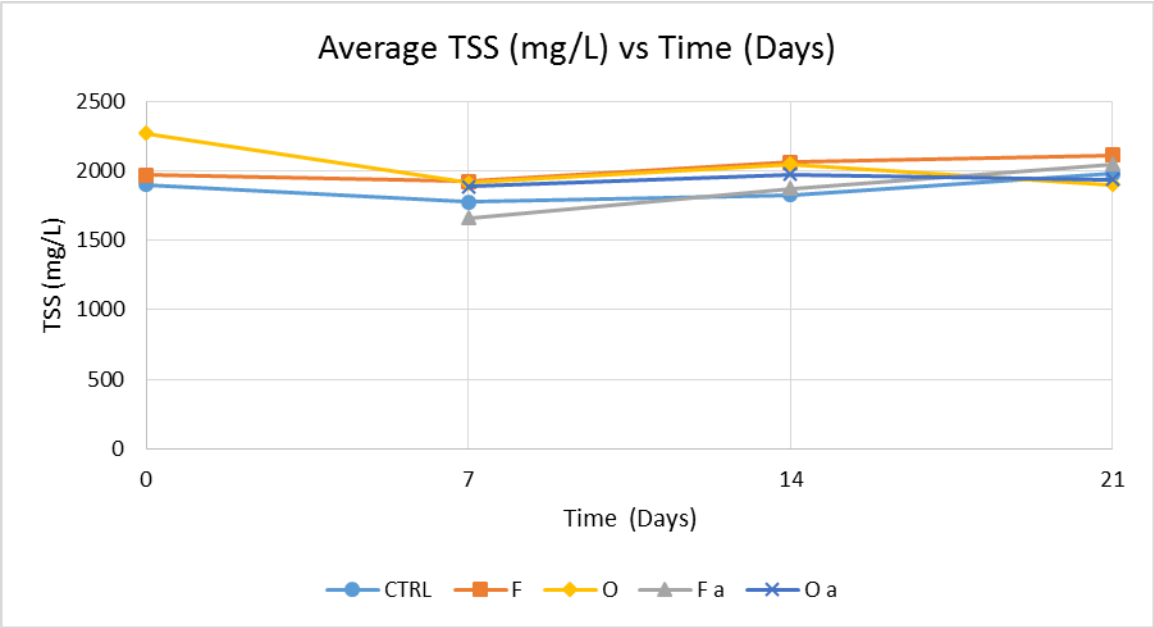


Figure 32. Average TSS values for Experiment V.

4.2.2.3 Experiment V

The TSS levels for F and O showed little change throughout the experiment time period of 21 days as shown in *Figure 32*. The redose sample for Fruitwash showed the lowest TSS value at day 7, but increased throughout the duration of the experiment. The TSS levels maintained constant levels after day 7 with values around 2,000 mg/L.

Table 35. Average TSS data points for Experiment V.

Average	TSS Day 0 (mg/L)	TSS Day 21 (mg/L)	% Change
CTRL	1980	1900	4.0
F	1970	2112	-7.2
O	2270	2113	6.9

The values for the TSS test for Experiment V can be shown in *Table 35*. The O sample had the highest percent decrease of 7% while the F sample increased solids by

7%. The control showed a 4% decrease of TSS. The Fruitwash samples demonstrated higher levels of TSS after 21 days.

Table 36. Normalized TSS date for Experiment V.

Average	TSS Day 0 (mg/L)	TSS Day 7 (mg/L)	TSS Day 21 (mg/L)
CTRL	1980	1825	1900
F	1980	1935	2123
O	1980	1668.2	1842.6
F a		1935	2385.7
O a		1668.2	1712.4

From *Table 36*, the TSS values show that all day 21 points are higher than the previous day 7 values for the redose samples. This may be due to variability from laboratory testing, sampling, and measurements. Osprey shows the largest decrease when normalized to the control with a final TSS concentration of 1,842.6 mg/L whereas the control has a final day 21 concentration of 1,900 mg/L.

4.2.3 Total Dissolved Solids (TDS)

TDS tests were performed for Experiments IV and V to determine the amount of dissolved solids found in the airy wastewater. Average TDS values were graphed with corresponding tables to display values following.

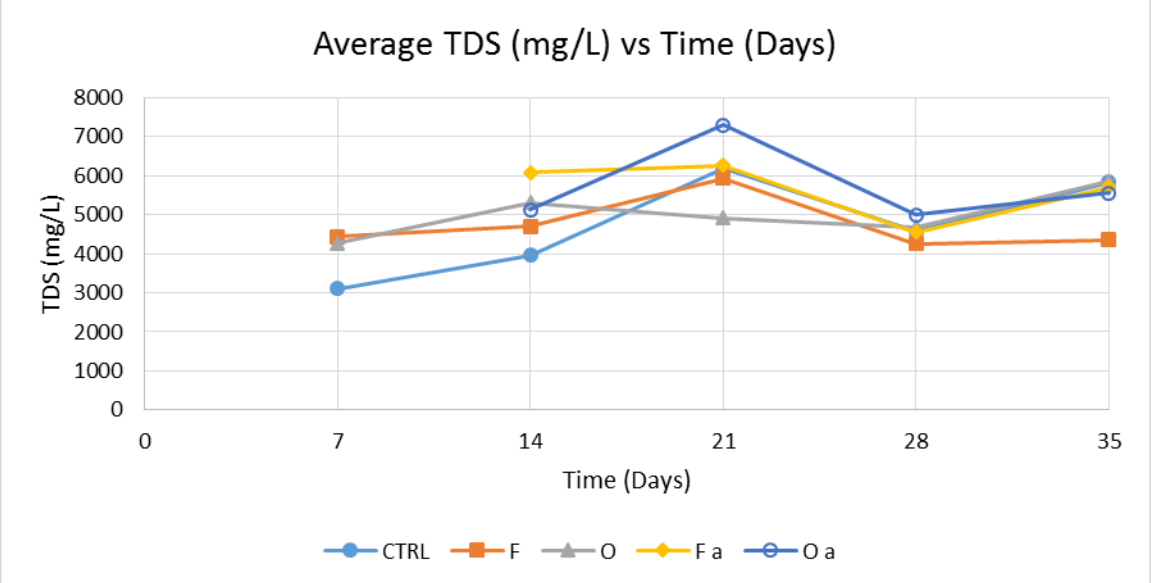


Figure 33. Average TDS results for Experiment IV.

4.2.3.1 Experiment IV

In *Figure 33*, the control appears to have a lower TDS level than the two bacterial mixes. During day 21, all samples, excluding the redose samples, experiences a sudden increase in TDS. This may be due to the change in procedure from day 21 to day 28. The TDS dried in different temperatures - 105°C from day 7 – 21 and 180°C from day 28 – 35.

Table 37. TDS values for Experiment IV.

Average	TDS Day 7 (mg/L)	TDS Day 35 (mg/L)	% Change
CTRL	3100	5833.3	-88.2
F	4433.3	4350	1.9
O	4266.7	5850	-37.1

Because of varying procedural changes throughout the experiment as well as not having the day 0 values, the TDS data shows negative percent changes as shown in *Table 37*. The only sample that showed a decrease in TDS was the Fruitwash sample, but this may be misleading. TDS are particles that are smaller than 1.6 μm and consist of inorganic salts, organic material, and other dissolved particles in the water (Weber – Scannell & Duffy, 2007). Thus, changes do not occur very quickly.

Table 38. Normalized TDS data points for Experiment IV.

Average	TDS Day 7 (mg/L)	TDS Day 14 (mg/L)	TDS Day 35 (mg/L)
CTRL	3100	3966.7	5833.3
F	3100	3286.5	3041.7
O	3100	3850.8	4250.4
F a		3286.5	3079.4
O a		3850.8	4163.3

From *Table 38*, the samples TDS concentrations did not change very much from its initial value during day 7 with the exception of the control, where the TDS increased almost twice its initial. However, because the initial day 0 point was not taken and

procedural changes occurred during the experiment, it may not be reflective of its actual values. Thus, Experiment V was performed to minimize any errors.

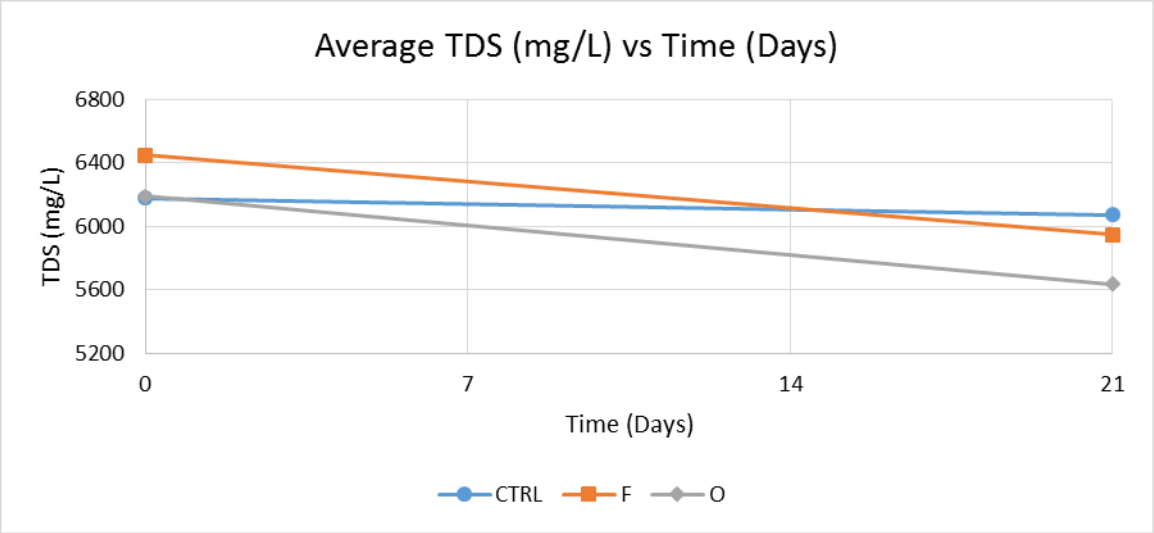


Figure 34. Average TDS concentration for Experiment V.

4.2.3.2 Experiment V

TDS concentrations were measured at day 0 and day 21 since TDS, usually comprised of salts and minerals that filter through a 1.6 µm pore size, do not typically change much in concentration over time. From *Figure 34*, the TDS decreased for all samples from day 0 to day 21.

Table 39. Average TDS concentrations for Experiment V.

Average	TSS Day 0 (mg/L)	TSS Day 21 (mg/L)	% Change
CTRL	6180	6075	1.7
F	6450	5950	7.8
O	6190	5638	8.9

Table 39 shows that the Biowish bacterial mixes of Fruitwash and Osprey decreased TDS levels by 8% and 9% respectively, whereas the control decreased TDS levels by 2%. The control TDS does not reduce any further than 6,000 mg/L while the F and O samples reduce to less than 5,750 mg/L of TDS.

Table 40. TDS normalized concentrations for Experiment V.

Average	TSS Day 0 (mg/L)	TSS Day 21 (mg/L)
CTRL	6180	6075
F	6180	5700.9
O	6180	5628.4

The O and F samples reduced the TDS concentrations to about 5,700 and 5,630 mg/L as shown in *Table 40*. Because the values are normalized to the control, the day 0 value is 6,180 mg/L. The control only reduced TDS to 6,075 mg/L by day 21.

4.2.4 Volatile Suspended Solids (VSS)

Only Experiment IV and V tested for VSS, which are the organic components of the suspended solids which did not pass through the 1.6 µm pore size filters. The bacterial samples that were tested were Fruit Wash and Osprey against the control. The average and normalized VSS graphs were generated with corresponding tables containing values for each sample.

4.2.4.1 Experiment IV

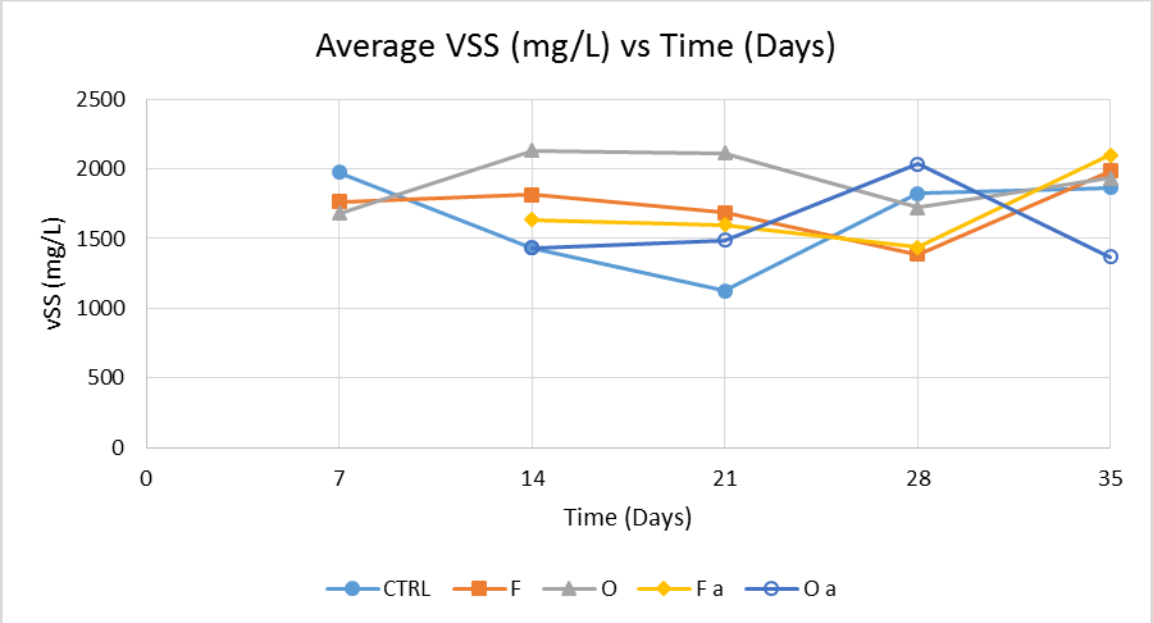


Figure 35. Average VSS values for Experiment IV.

From *Figure 35*, the VSS trends showed to be in the range from about 1500 – 2000 mg/L. The control dropped to its lowest value at 21 days, which is an unexpected low point. Afterwards, the control VSS values increase back to about 2,000 mg/L which may be an experimental error.

Table 41. Average VSS values for Experiment IV.

Average	VSS Day 7 (mg/L)	VSS Day 35 (mg/L)	% Change
CTRL	1975	1866.7	5.5
F	1766.7	1983.3	-12.3
O	1683.3	1933.3	-14.9

Table 41 shows that the only sample that had a positive percent change was the control by 5.5% from day 7 to day 35. The F and O sample had similar negative changes of 12.3% and 14.9% respectively. VSS concentrations increased for the two samples.

Table 42. Normalized VSS data for Experiment IV.

Average	VSS Day 7 (mg/L)	VSS Day 14 (mg/L)	VSS Day 35 (mg/L)
CTRL	1975	1433.3	1866.7
F	1975	2030.9	2217.2
O	1975	2503	2268.3
F a		2030.9	2611.2
O a		2503.0	3595.8

From *Table 42*, the VSS levels for all the samples, excluding the control, increased from day 7 for the original samples and day 14 for the redose samples. As noted, because there were no day 0 data points, the experiment results and effects do not definitively conclude any results. The results showed a trend that may be expected for the next experiment.

4.2.4.2 Experiment V

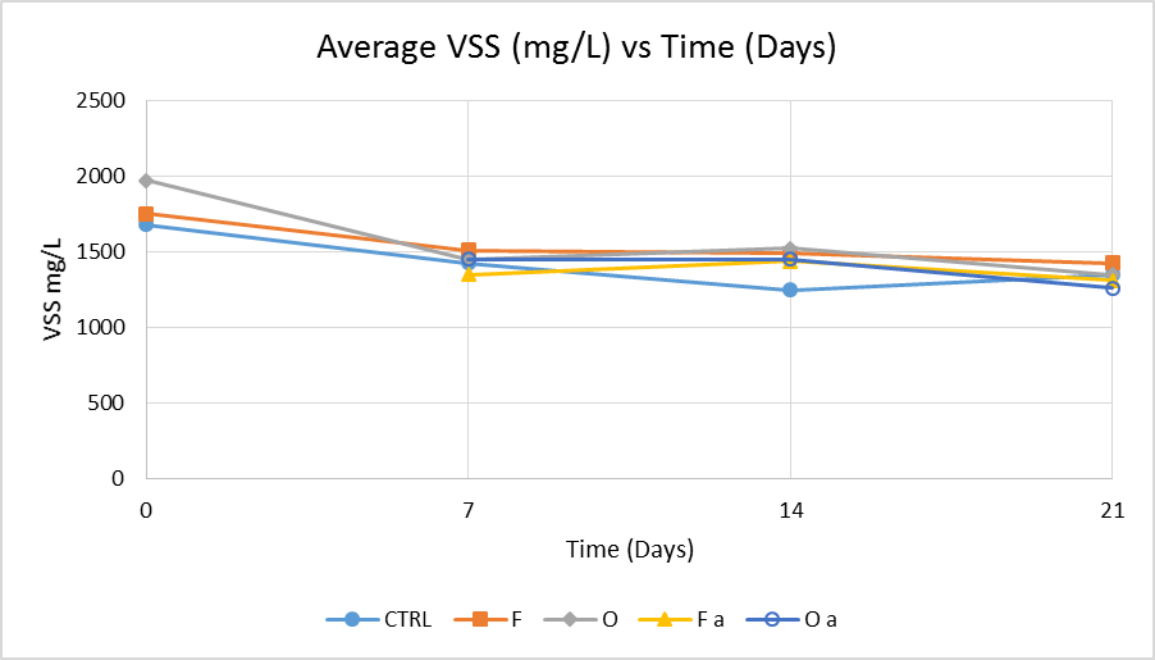


Figure 36. Average VSS concentrations for Experiment V.

VSS results from Experiment V showed a continuous trend of VSS concentration decrease for all samples. The redose samples, since there was only a 10 ppm addition, did not show a large increase during day 7. The O redose had the reduced the VSS concentration the lowest from all the samples.

Table 43. Average VSS concentration for Experiment V.

Average	VSS Day 0 (mg/L)	VSS Day 21 (mg/L)	% Change
CTRL	1680	1350	19.6
F	1750	1312.5	25
O	1970	1262.5	35.9

From *Table 43*, the F and O bacterial mix samples reduced the VSS lower than the control with a 25% and 36% reduction respectively to the control's 20% VSS reduction. It shows the effectiveness of adding Biowish to further reduce the organic material found in the dairy wastewater.

Table 44. Normalized VSS concentrations for Experiment V.

Average	VSS Day 0 (mg/L)	VSS Day 7 (mg/L)	VSS Day 21 (mg/L)
CTRL	1680	1425	1350
F	1680	1452	1368
O	1680	1237	1151.3
F a		1452	1411.7
O a		1236.5	1076.6

The normalized VSS values for the samples are shown in *Table 44*. As previously shown in the graph, the original O and redosed O samples reduced the organic suspended solids to 1,151 and 1077 mg/L respectively. The Fruitwash samples had higher levels of VSS than the control, thus the control reduced the organic suspended solids better than the Fruitwash bacterial sample.

4.3 Particle Size Distribution (PSD)

4.3.1 Experiment II

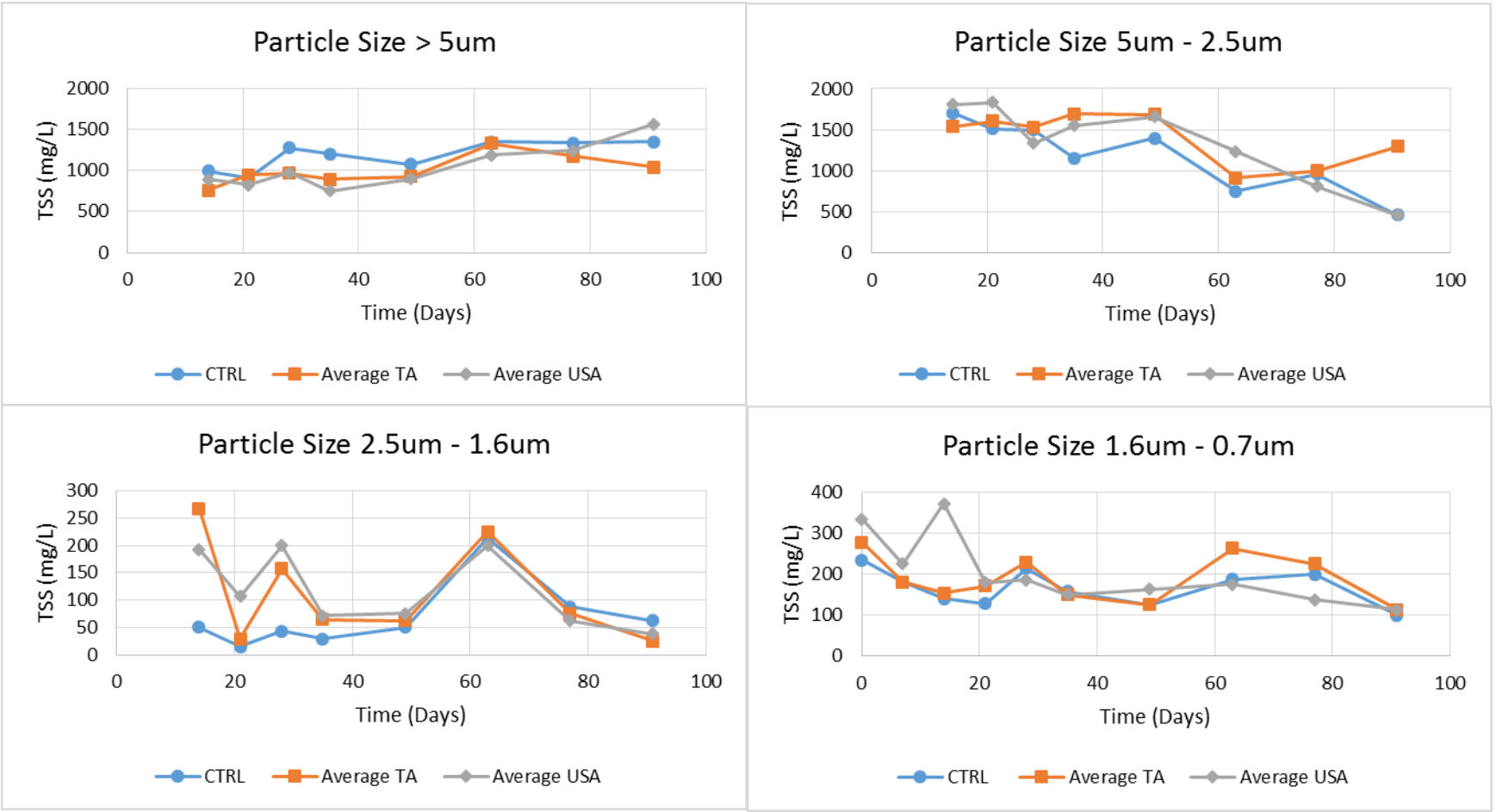


Figure 37. Average PSD for Experiment II.

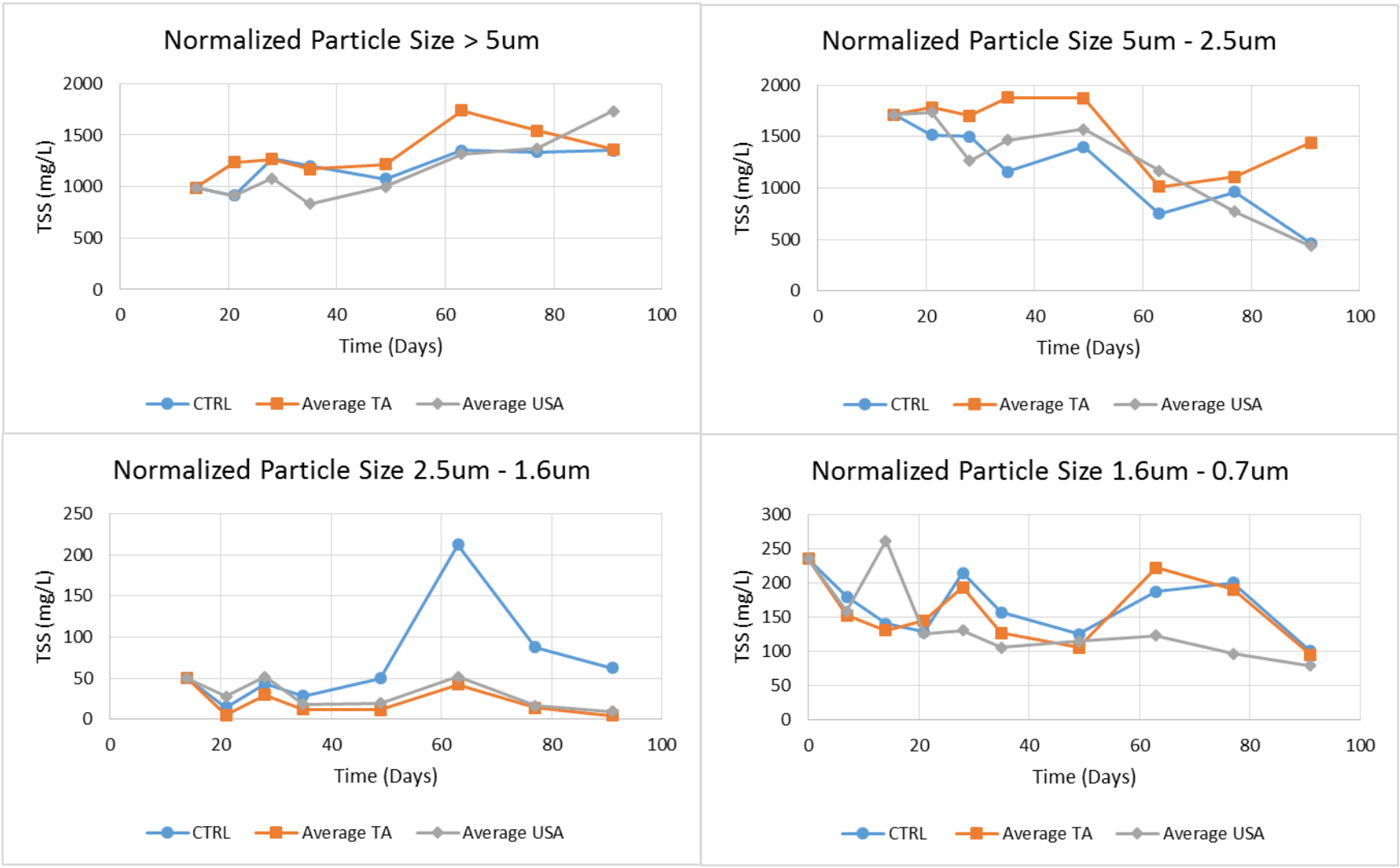


Figure 38. Average normalized PSD results for Experiment II.

Unfortunately, for Experiment II, the 5 µm pore size was not available and the 1 µm pore size was mistakenly used instead. Thus for days 0 – 14, data was not obtainable and only the 0.7 µm was used for days 0 – 14, although it was only particle sizes from 1 – 0.7 µm. As shown in *Figure 37*, the average PSD values were represented in 4 different graphs with various size ranges.

Figure 38 shows the normalized values to the control to compare how the bacterial samples perform to the control. For the particle sizes 2.5 – 1.6 µm, the control is higher than both samples. Both TA and USA decreased TSS at the 1.6 µm pore size by 90.7% and 80.6% respectively while the control increases TSS by 25% as shown in *Table 45*. The smaller particle sizes are further reduced by the bacterial mixes compared to the control. The PSD trends for each sample can be shown in *Appendix 7.2*.

Table 45. PSD average values for Experiment II.

TSS (mg/L)													
		CTRL				**TA**				**USA**			
Pore Size (µm)		**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
Day	**0**	0	0	0	235*	0	0	0	277.5*	0	0	0	333.8*
	14	990	1709.2	50	140	754.5	1541.3	267.5	153.8	892.9	1807.1	192.9	371.4
	91	1350	462.5	62.5	100	1037.5	1300	25	112.5	1562.5	462.5	37.5	112.5
% Change		-36.4^	72.9^	-25.0^	57.4	-37.5^	15.7^	90.7^	59.5	-75.0^	74.4^	80.6^	66.3

* 1 – 0.7 µm particles ^ Percent difference calculated from Day 14 to Day 91

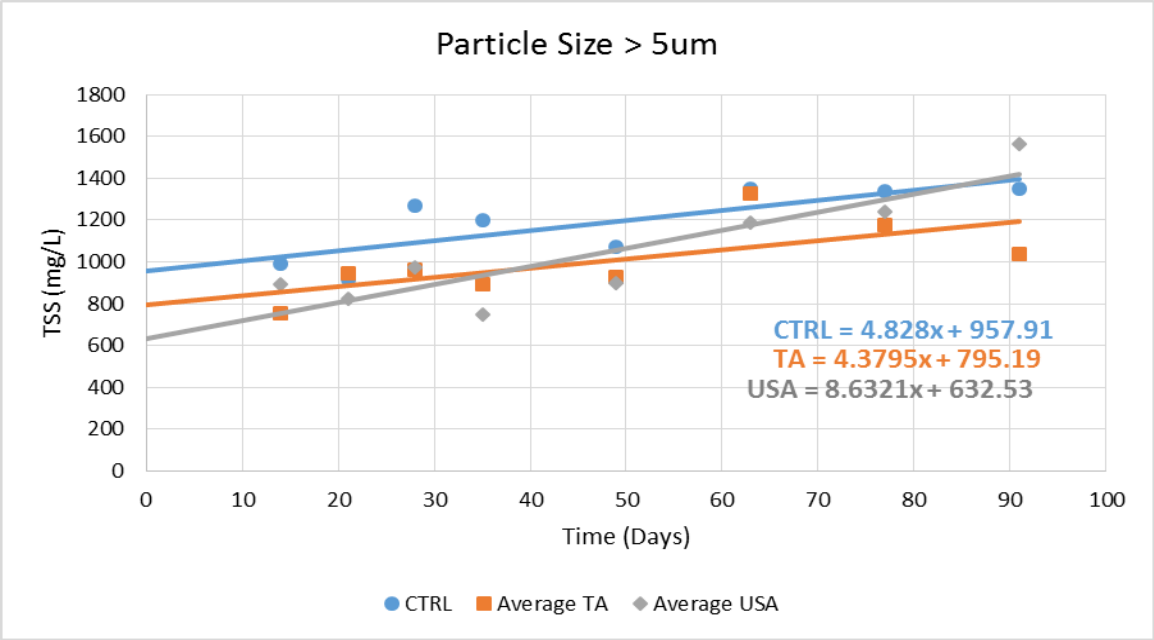


Figure 39. Effect of Biowish on particle sizes greater than 5 µm.

Figure 39 shows the particle sizes larger than 5µm are increasing from day 0 to day 91. This may be due to bacteria that cannot survive and goes to a latent stage forming large lumps as the smaller particles are getting reduced. All three samples show a similar zero rate constant except USA where the rate constant is double the control and TA.

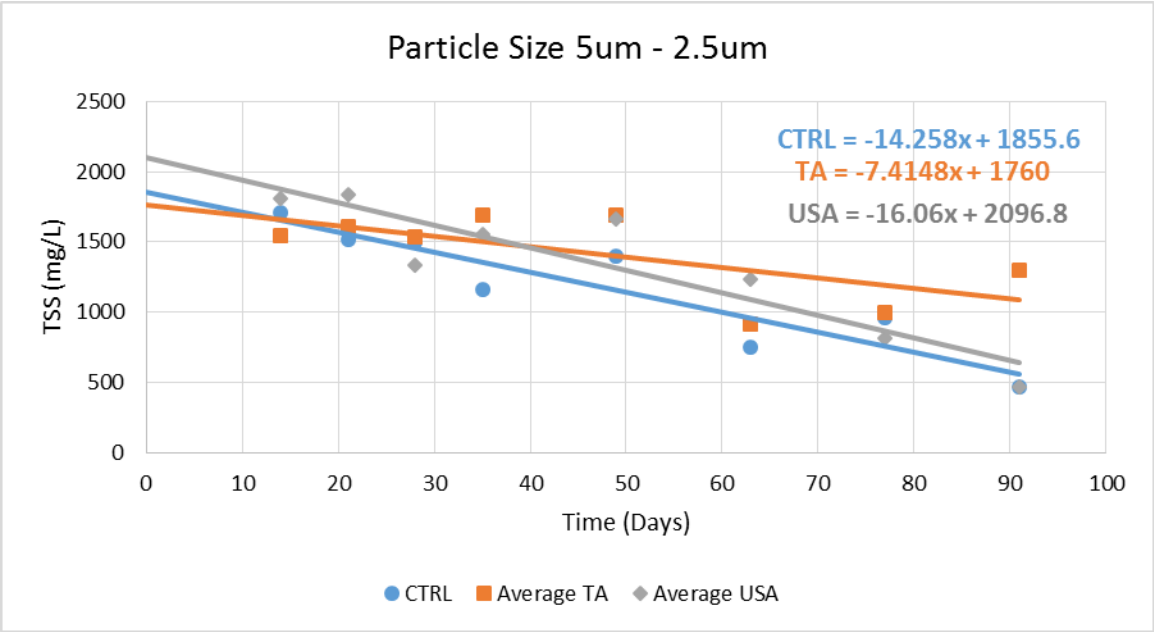


Figure 40. Effect of Biowish on breaking particles 2.5 – 5 µm.

Figure 40 shows the effect that Biowish bacterial samples have on the dairy wastewater for particle sizes 2.5 – 5 µm. BW-1 is the USA sample while BW-2 is the TA sample. For all samples, the particle sizes are being broken down from 14 days to 77

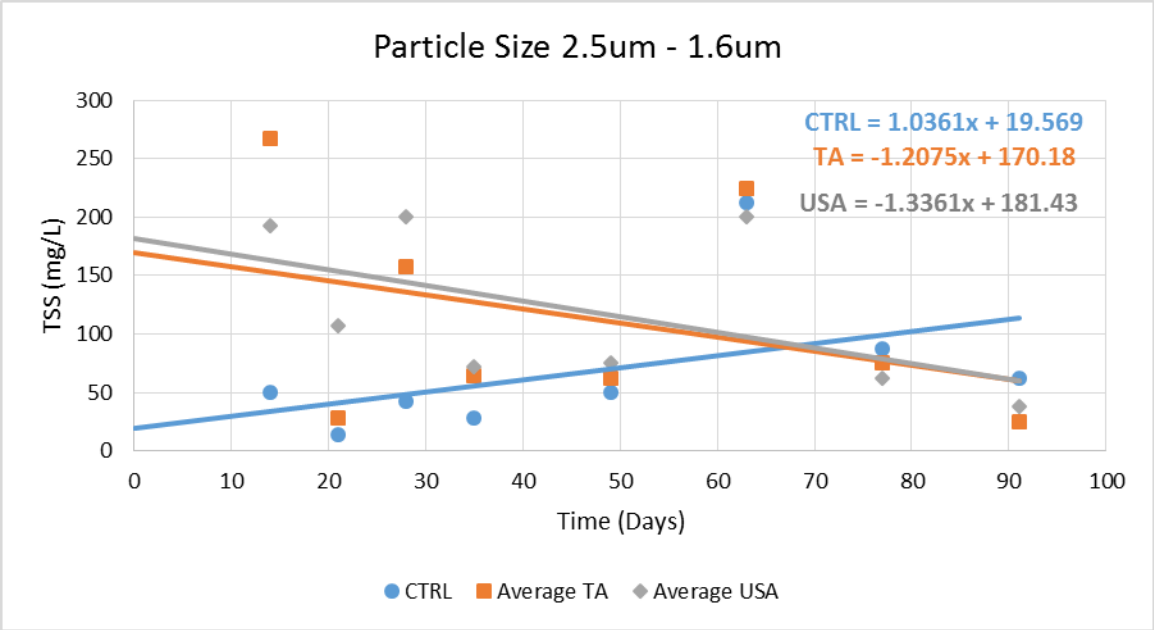


Figure 41. Biowish effect on particle sizes 1.6 - 2.5 µm.
days.

Figure 41 demonstrates that the rate limiting step in the biological attrition process is 1.6 – 2.5 µm. For the control without any Biowish product, the rate constant is +1.03 mg/L-day. Therefore, there is an accumulation of particulates between 1.6 and 2.5 µm. However, for USA and TA, we see a rate constant of -1.3 and -1.2 mg/L-day, respectively. Because of USA and TA, the different enzymes that are produced from the bacterial samples help further degrade the particle size fraction of 1.6 and 2.5 µm compared to the control.

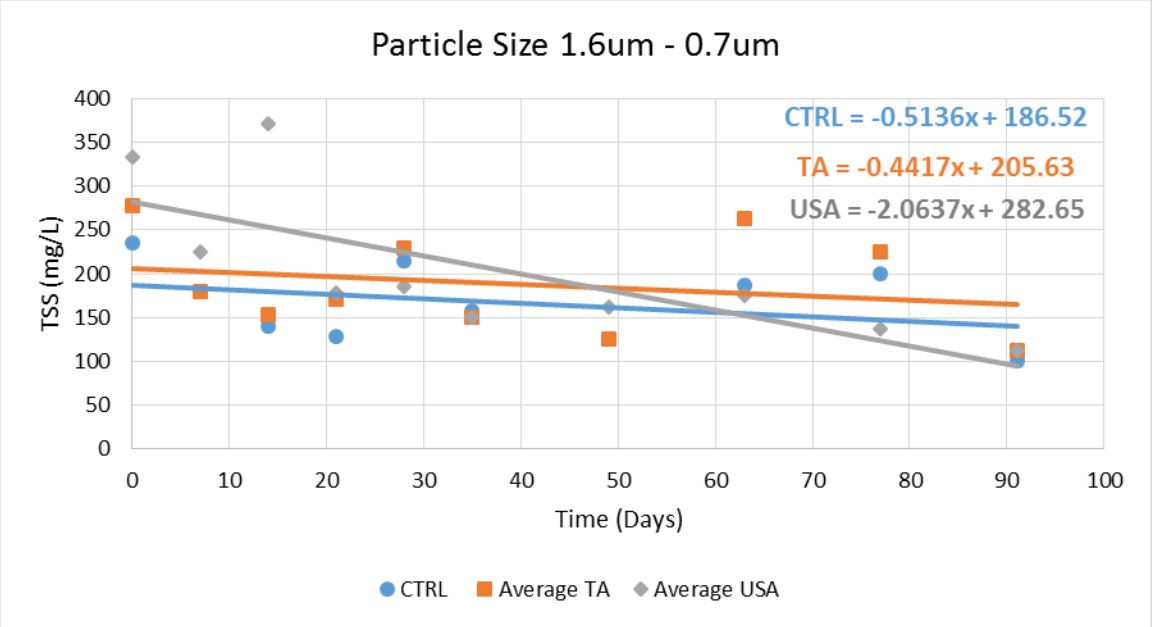


Figure 42. Biowish effect on particle size 0.7 - 1.6 µm.

Figure 42 shows that with USA, the particle sizes 0.7 – 1.6 m are decreasing at a quicker zero rate constant of 2.1 mg/L-day compared to the control where it is decreasing slower at 0.5 mg/L-day. Without the Biowish addition, as shown in the control sample, the finer particles are not degrading any further.

Table 46. Zero rate order constants for Experiment II.

Zero Order Rate Constant (mg/L-day)				
	Pore Size Range (µm)			
Sample	**> 5**	**2.5 - 5**	**1.6 - 2.5**	**0.7 - 1.6**
CTRL	4.8	-14.3	1.04	-0.5
TA	4.4	-7.4	-1.2	-0.4
USA	8.6	-16.1	-1.3	-2.1

In order to view how the rate order constants are different for the various particles size ranges, Table *46* displays the values for comparison.

4.3.2 Experiment III

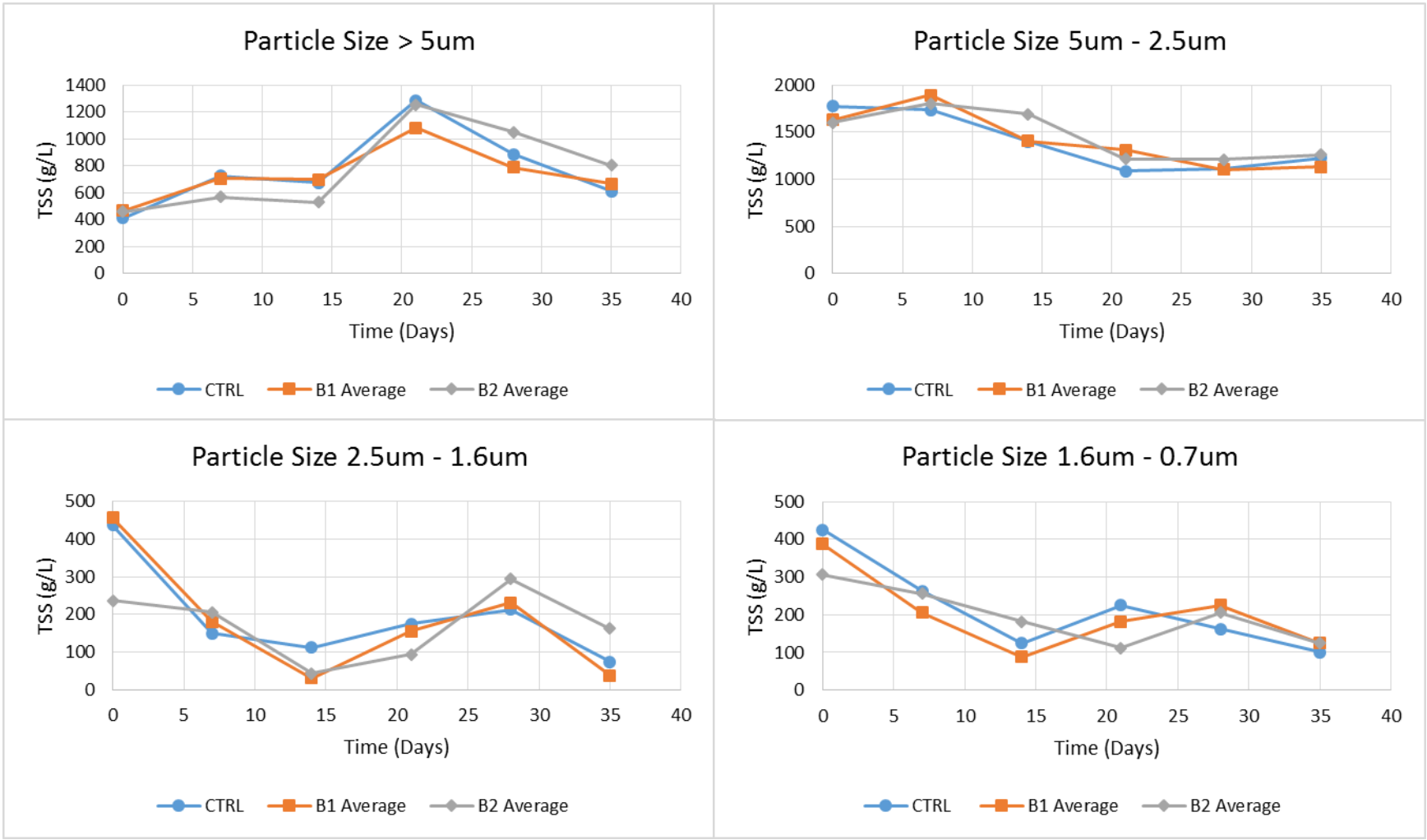


Figure 43. Average PSD trends for Experiment III.

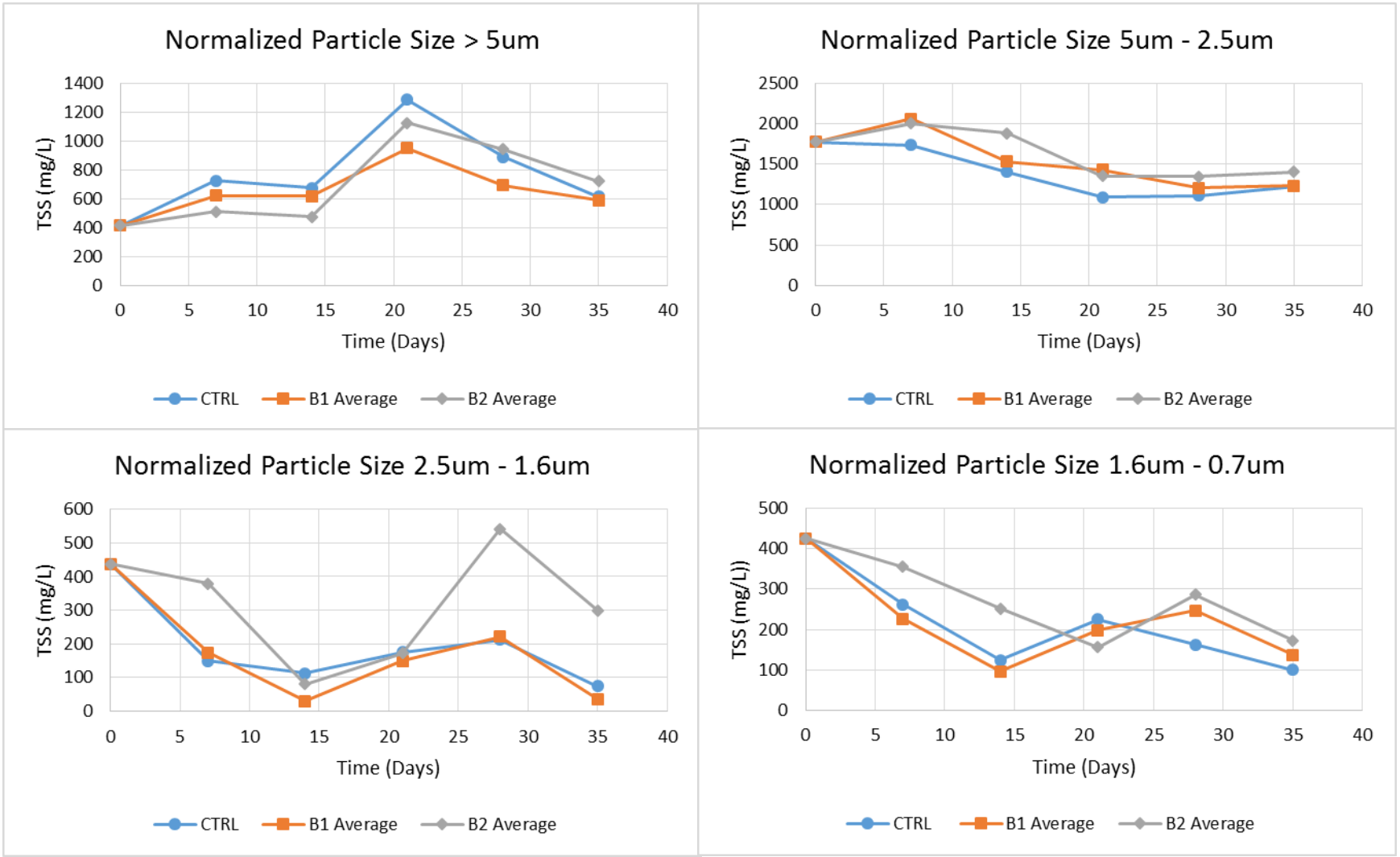


Figure 44. Normalized PSD results for Experiment III.

As shown in *Figure 43*, the trends for particle sizes between 5 – 2.5 µm and 1.6 – 0.7 µm show the TSS decreasing whereas the other two ranges show unexpected increases and decreases. Particle sizes larger than 5 µm increase until day 21 where TSS decrease. Particle sizes 2.5 – 1.6 µm decrease until day 21 where TSS increase until leveling off at day 35.

Figure 44 shows the normalized trends for PSD. The 5 – 2.5 µm particle range has the most in concentration at about 1,800 mg/L and slowly decreasing in TSS over time. All TSS ranges appear to be overall decreasing with the occasional increase shown in the 2.5 – 1.6 µm graph at day 28 and starting day 14 for 1.6 – 0.7 µm and larger than 5 µm. All the samples showed an increase of particle sizes larger than 5 µm while the smallest particle range of 1.6 – 0.7 µm had the largest decrease of TSS by 60 – 77% as shown in *Table 47*.

Table 47. PSD average values for Experiment III.

TSS (mg/L)													
		CTRL				**B1**				**B2**			
Pore Size (µm)		**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
Day	**0**	412.5	1775	437.5	425	468.75	1631.25	456.25	387.5	460	1600	237.5	306.2
	35	612.5	1225.0	75.0	100	668.7	1131.25	37.5	125.0	806.2	1262.5	162.5	125.0
% Change		-48.5	31.0	82.9	76.5	-42.7	30.7	91.8	67.7	-75.3	21.1	31.6	59.2

4.3.3 Experiment IV

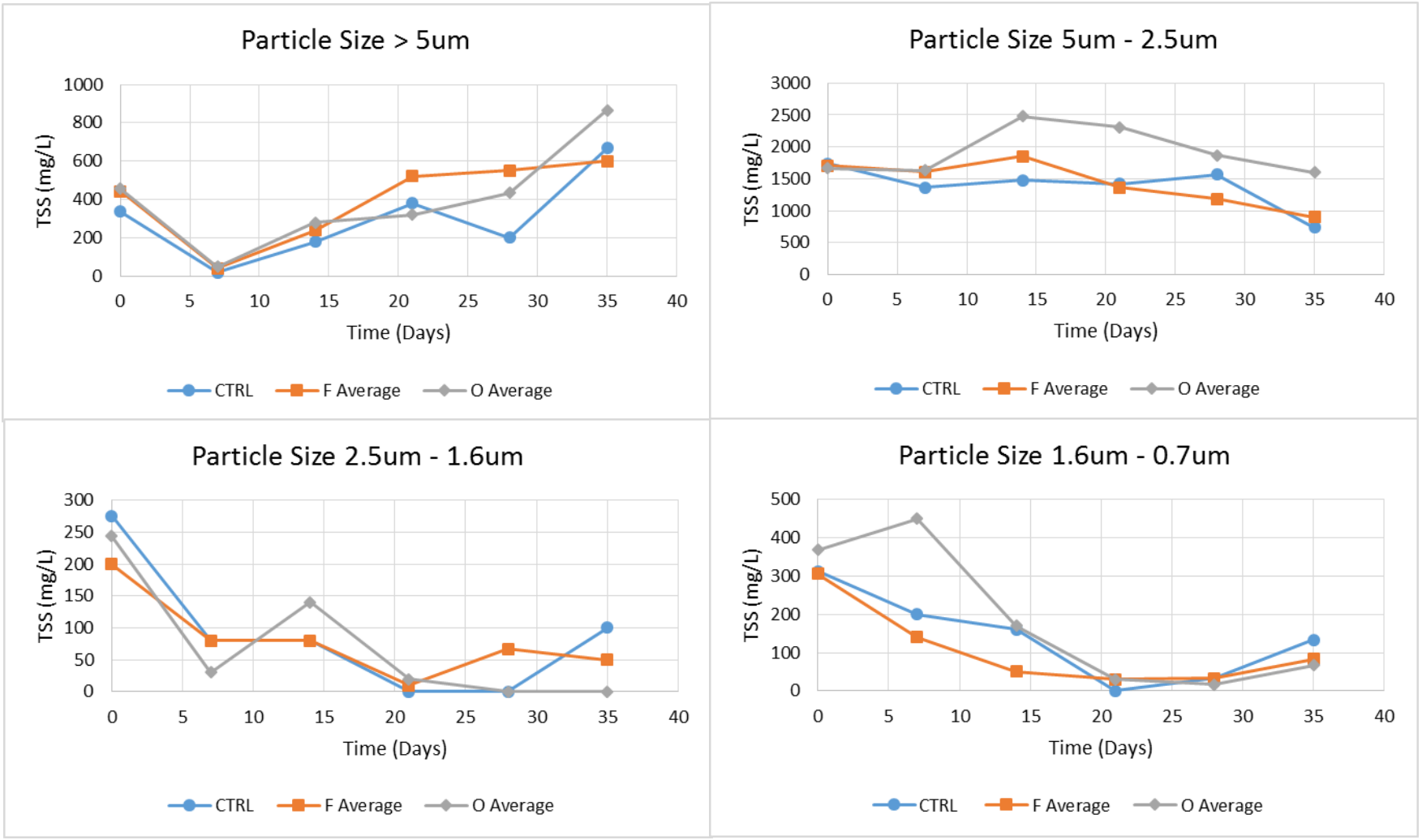


Figure 45. Average PSD results for Experiment IV.

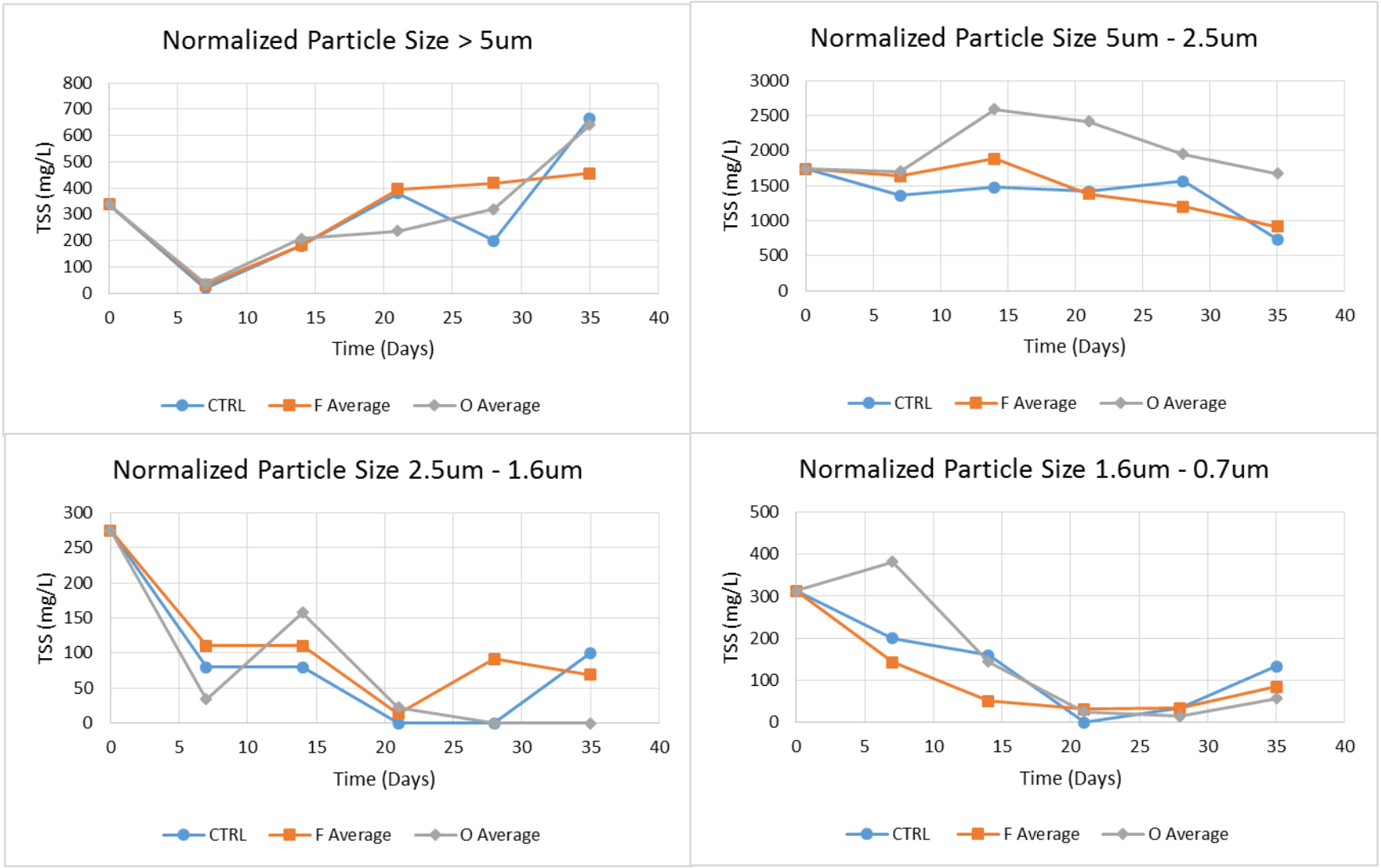


Figure 46. PSD normalized trends for Experiment IV.

During this experiment, it was interesting to note there were times of no TSS difference during day 21 for the control, days 28 and 35 for the O sample, and near 0 mg/L for day 7 for the 5 µm pore size as shown in *Figure 45*. The procedure for measuring PSD changed during day 7 and onwards where DI rinses were performed after filtering the samples through in order to make sure particles were not getting retained in the glass fibers, which may account for the 0 data points.

Figure 46 shows that the 5 µm pore size filter showed increasing trends while the other ranges showed a decreasing trend with occasional increases. The F and O samples show they are breaking down the particle sizes into smaller sizes since the 1.6 – 0.7 µm TSS concentrations are lower than the control. From *Table 48*, there was a 73% and 82% decrease for F and O respectively for the 0.7 µm pore size while the control had a 64% TSS reduction for 0.7 µm.

Table 48. PSD average values for Experiment IV.

TSS (mg/L)													
		CTRL				**F**				**O**			
Pore Size (µm)		**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
Day	**0**	337.5	1737.5	275	312.5	443.7	1883.9	200	306.2	456.3	1662.5	243.7	368.8
	35	666.7	733.3	100	133.3	550	900	50	83.3	866.7	1866.7	0	66.7
% Change		-97.5	57.8	63.6	57.3	-23.9	52.2	75.0	72.8	-90.0	-12.3	100.0	81.9

4.3.4 Experiment V

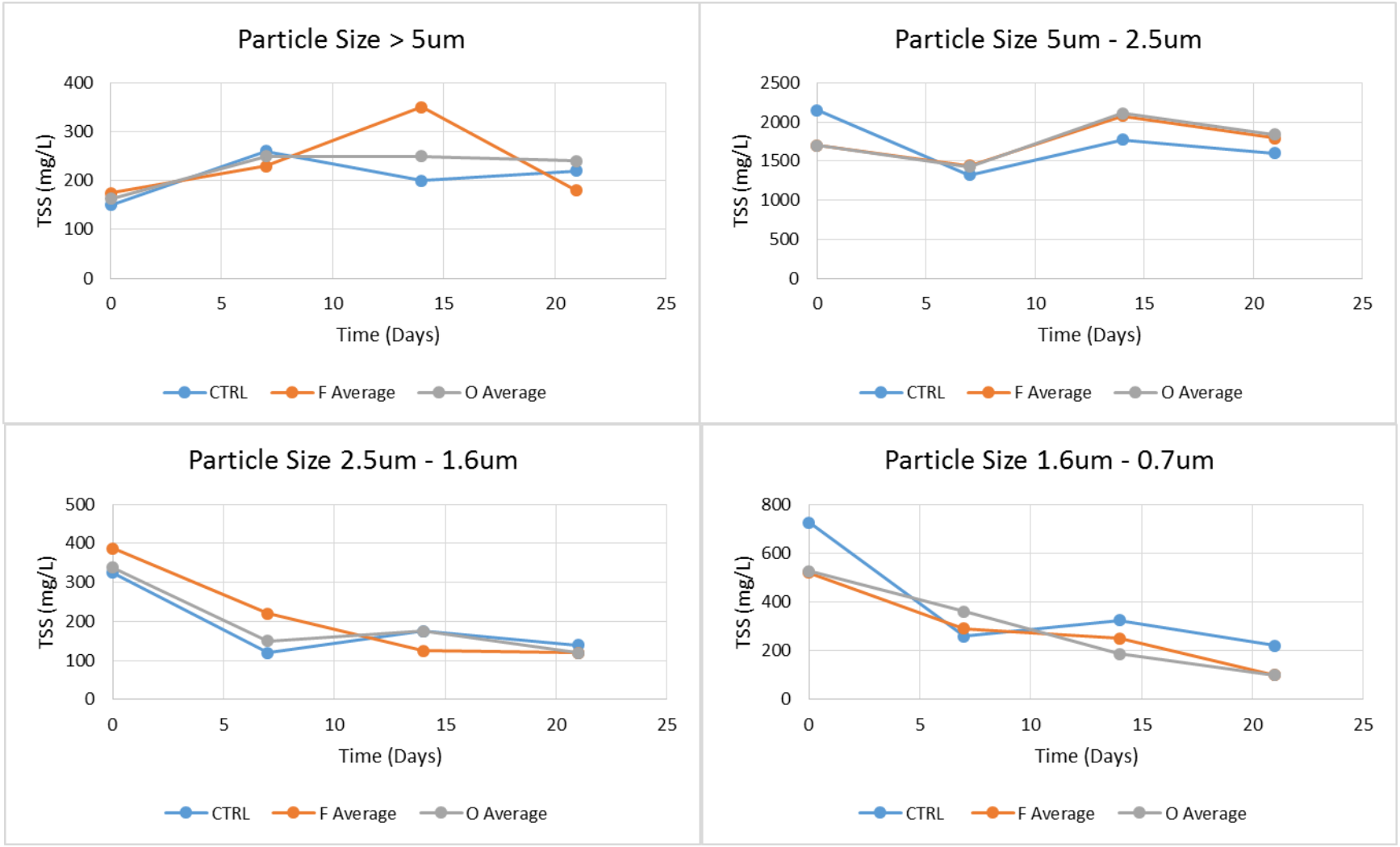


Figure 47. Average PSD values for Experiment V.

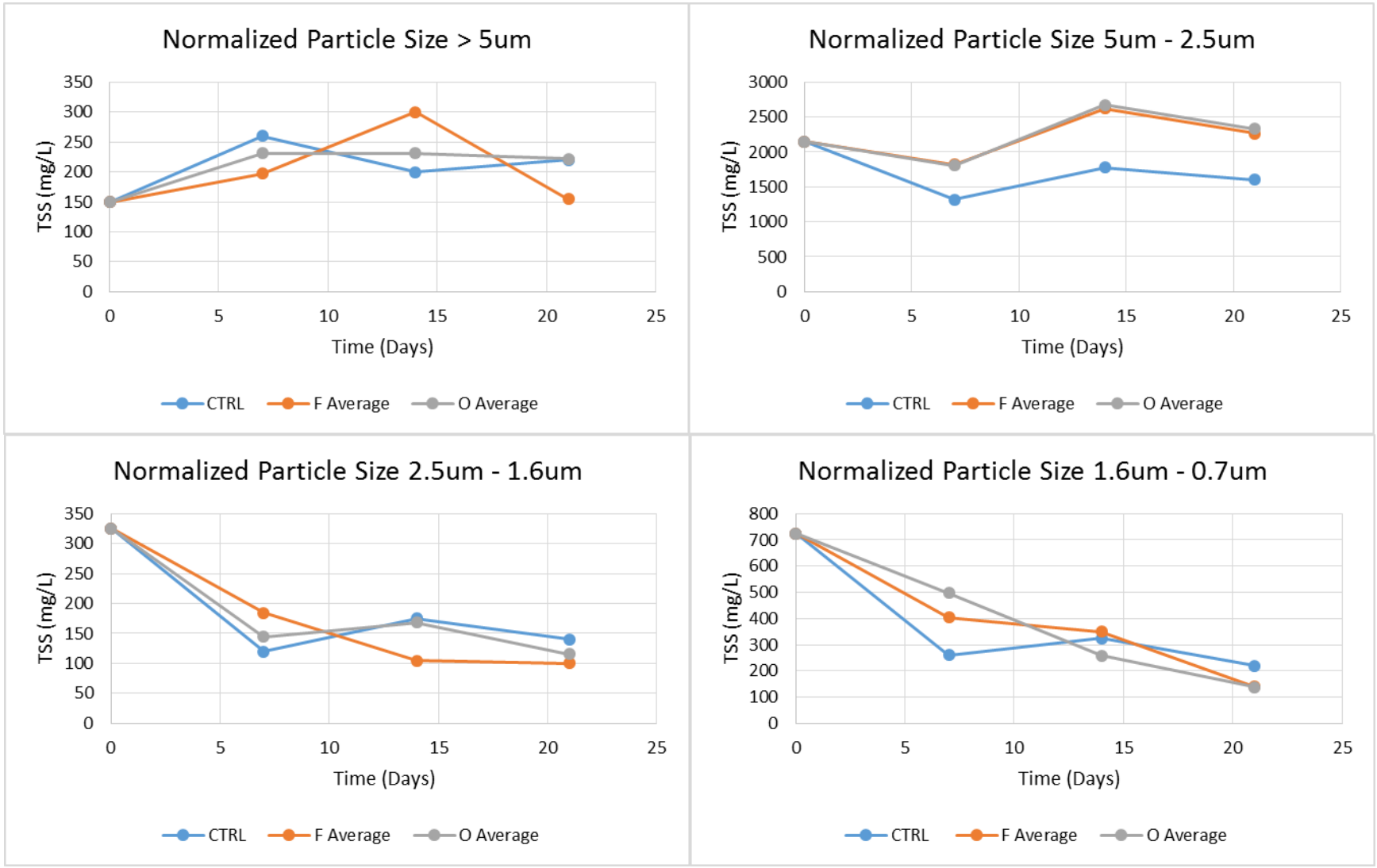


Figure 48. Normalized average PSD values for Experiment V.

Figure 47 shows that the two smaller pore sizes steadily decrease from day 0 to day 21, whereas the two larger pore sizes remain around the same TSS levels and show slight increases in TSS throughout the experiment. The 2.5 µm experienced a dip during day 7, but continued to hover around 2,000 mg/L. It appears that the bacteria are degrading the larger particle sizes to smaller sizes based on the graphs. The pore size 2.5 – 5 µm are the typical range of bacteria. It can be shown in *Figure 48* that the control has less TSS while F and O are maintain its TSS levels. This may show that the bacteria are not dying and rather digesting the larger particle sizes into smaller sizes.

Table 49 lists the values and percent change for each of the pore sizes. All the 5 µm pore sizes had a negative percent change, which may be attributed to not rinsing enough DI water initially to allow smaller particles to pass through and the particles may have been entrapped. As noted, the F and O had higher percentage changes for the smaller pore sizes of 1.6 and 0.7 µm.

Table 49. PSD results for Experiment V.

TSS (mg/L)													
		CTRL				**F**				**O**			
Pore Size (µm)		**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
Day	**0**	150	2150	325	725	175	1700	387.5	520	162.5	1700	337.5	525
	21	220	1600	140	220	180	1790	120	100	240	1840	120	100
% Change		-46.7	25.6	56.9	69.7	-2.9	-5.3	69	80.8	-47.7	-8.2	64.4	81

4.4 Ion Chromatography (IC)

IC was performed for Experiment II – IV to determine the denitrification ability of the bacterial mixes of nitrate (NO_3-) to nitrite (NO_2-). Results are organized by chronology with a graph representing both NO_3- and NO_2- concentrations followed by individual graphs of NO_3- and NO_2- for a closer view of the trends. The normalized IC data graphs are shown in *Appendix 7.3*.

4.4.1 Experiment II

For the dairy wastewater IC tests, 5 mL of wastewater was filtered through a 0.22 µm filter into a Poly vial. The sample was ran in an automated sampler and the peaks were identified at distinct retention times for NO_3- and NO_2-. *Figure 49* shows both the

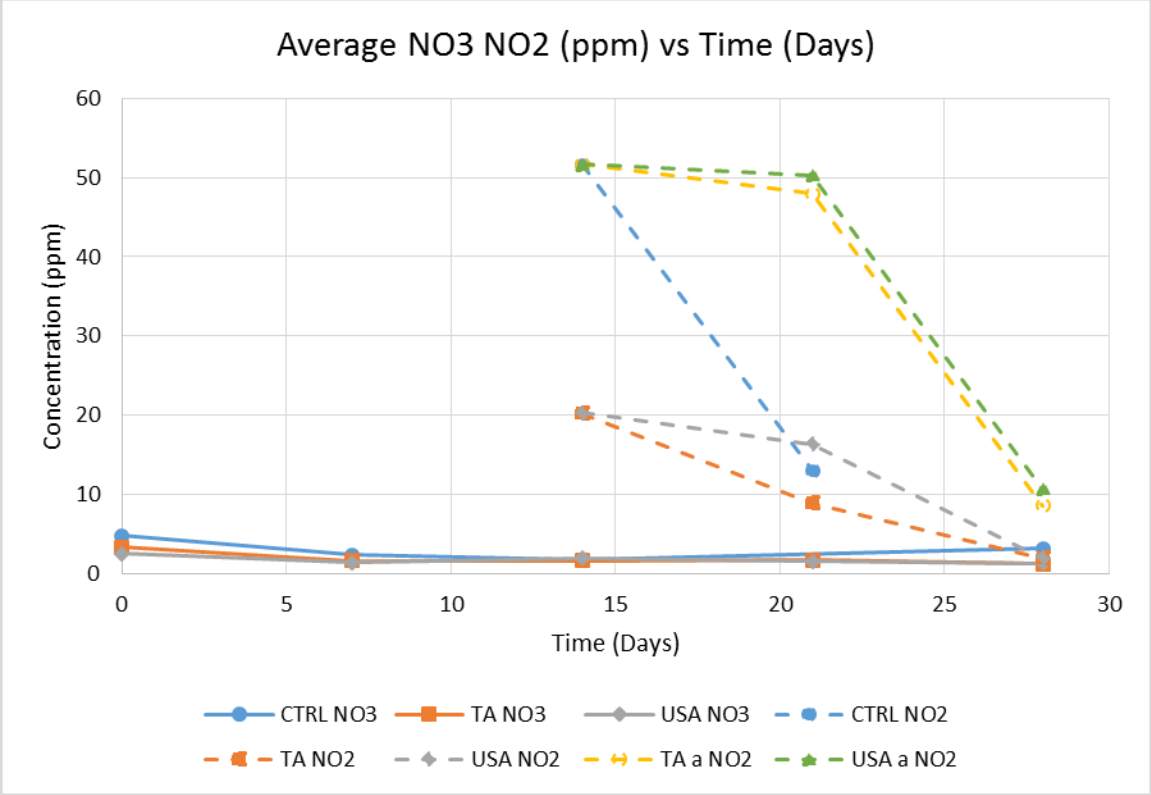


Figure 49. Nitrate and nitrite concentrations for Experiment II.

NO_3- and NO_2- concentrations in ppm. Although the dairy wastewater was spiked with

25 ppm NO_3-, the day 0 measurements from IC range from 3 – 5 ppm. This may be due to the hours in between testing that may have allowed for the bacteria to begin denitrifying the NO_3-. The NO_3- levels remain consistent throughout the experiment while the NO_2- levels show a large decrease. After about 14 days, the IC detected NO_2- at high levels, about 20 – 50 ppm. Throughout the weeks, the NO_2- levels dropped to about 10 ppm. The redose and control showed higher levels of NO_2- while the original bacterial mixes showed lower concentrations of NO_2-.

Figure 50 shows the NO_3- levels from day 0 to day 28. The IC instrument only

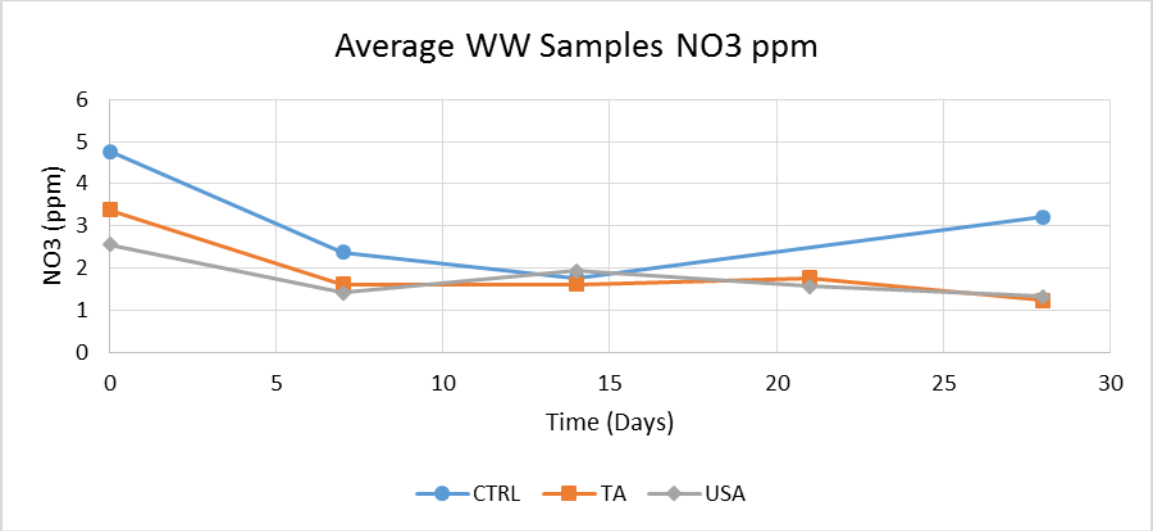


Figure 50. Nitrate concentration for Experiment II.

detected the original samples (no redose) NO_3- levels, where all three samples initially decreased from day 0 to 14, and then the control NO_3- concentration started to increase. The TA and USA samples continued to denitrify and lower NO_3- levels.

Table 50. Nitrate concentration change in Experiment II.

Sample	Initial NO_3- (ppm)	Final NO_3- (ppm)	% change
CTRL	4.8	3.2	32.5
TA	3.4	1.2	63.6
USA	2.5	1.3	48.3

The effectiveness of adding Biowish bacteria can be shown in *Table 50* where the TA and USA had a NO_3- concentration change of 63.6% and 48.3% decrease respectively whereas the control only had a 32.5% decrease.

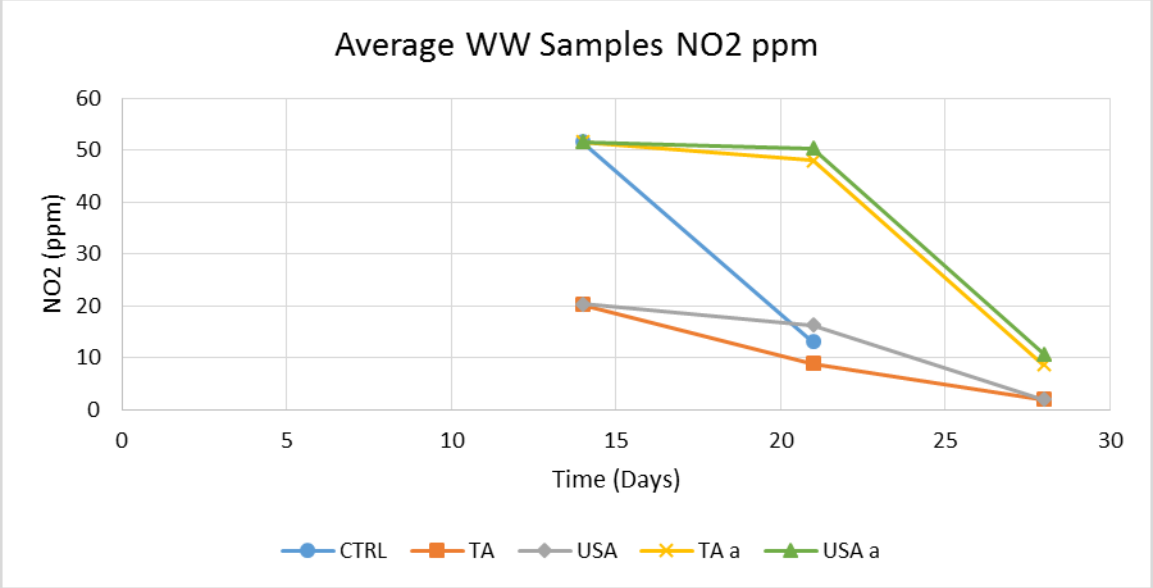


Figure 51. Nitrite concentration of Experiment II.

The NO_2- levels for Experiment II were detected at 14 days, most likely because by day 14, when the samples were split and additional bacteria were added to the redose samples, the bacteria had already denitrified the NO_3- and the additional bacteria denitrified more NO_3- to NO_2-. As shown in *Figure 51*, the redose samples had higher NO_2- levels. The control may not have been representative of the actual NO_2- levels since it decreases by 21 and was not detected afterwards.

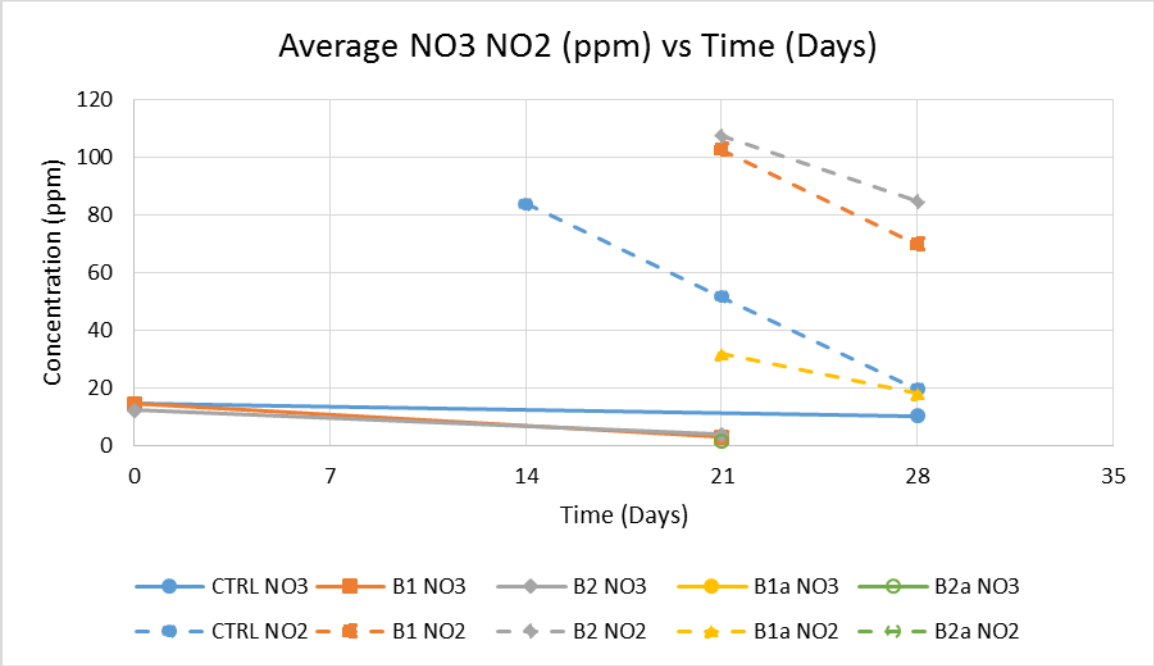


Figure 52. Denitrification results for Experiment III.

4.4.2 Experiment III

Figure 52 shows the NO_3- and NO_2- concentrations that were detected for Experiment III with the BMT samples versus the control. The NO_3- levels decreased from about 20 ppm from day 0 to almost 0 ppm at day 21 for the B samples. NO_2-

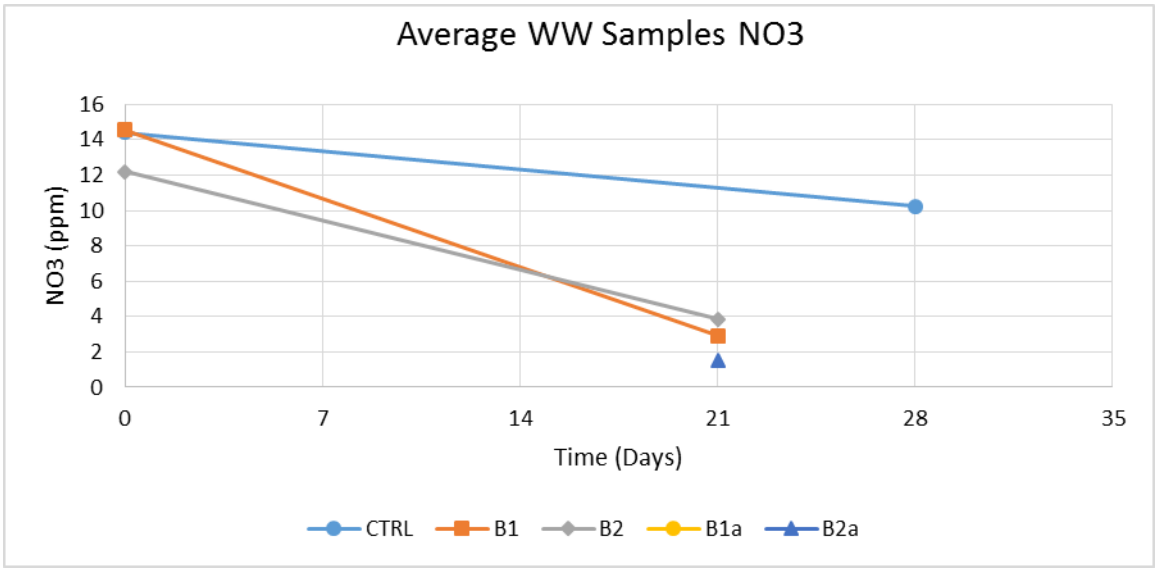


Figure 53. Nitrate concentrations for Experiment III.

concentrations were detected near day 14 and 21 for the samples.

From *Figure 53*, the NO_3- levels decreased further and earlier for the B samples than the control. By day 21, the NO_3- concentrations reached about 3 – 4 ppm compared to the control where by day 28, there was still about 10 ppm of NO_3-detected. The redose sample for B2 showed a NO_3- detection at only day 21.

Table 51. Nitrate levels for Experiment III.

Sample	Initial NO_3- (ppm)	Final NO_3- (ppm)	% change
CTRL	14.4	10.2	29
B1	14.5	2.9	79.9
B2	12.2	3.8	68.4

In order to test the bacteria's effectiveness on the NO_3- reduction, *Table 51* shows the percent reduction that the samples performed. The B1 and B2 samples reduced NO_3- by 80% and 68.4% respectively whereas the control reduced the NO_3- by only 29%.

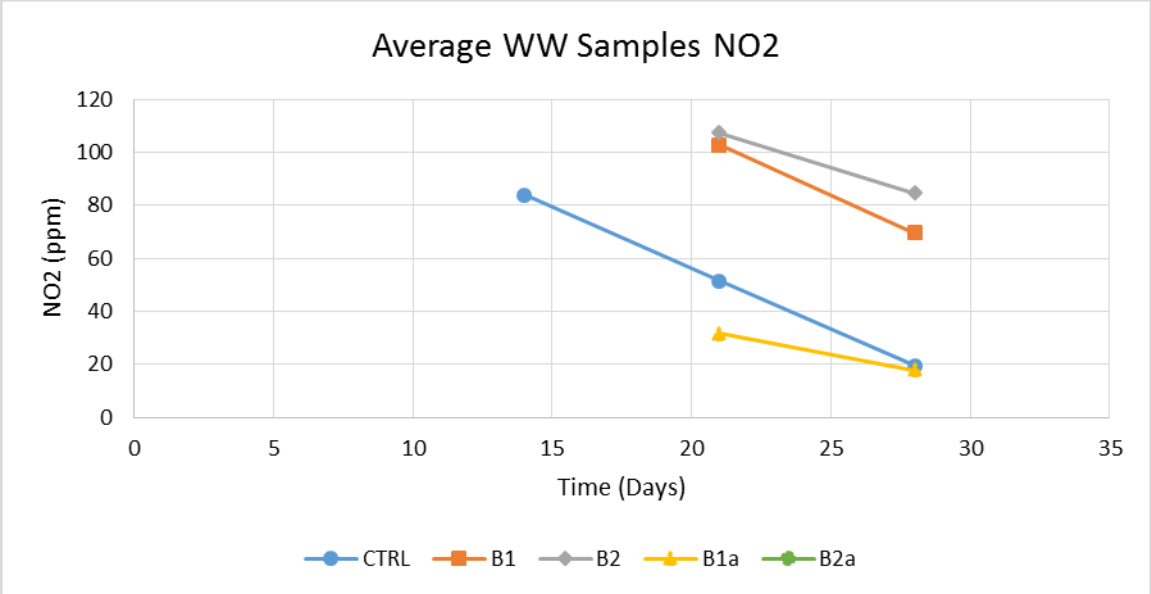


Figure 54. Nitrite levels for Experiment III.

The NO2- levels were measured and showed a spike at day 14 and 21 for each of the samples, excluding B2 a. It appeared there was a high range of NO_2- for the control and original B samples of about 80 ppm and 100 ppm respectively. This may be due to

the bacteria denitrifying the spiked NO_3- and naturally present ammonia (NH_3) in the dairy wastewater. NH_3 may be nitrifying into NO_2- during the course of the experiment. Because NO_3- and NO_2- are measured every week, there may be data points in between that would help identify the surge in NO_2- around day 14.

4.4.3 Experiment IV

The NO_3- and NO_2- results from this experiment were unusual compared to the past experiments because the NO_3- concentrations increased from day 0. There may have been an error measuring the concentrations since day 0 values were less than 1 ppm when the samples were spiked with 25 ppm of NO_3-. Instead of KNO_3- being added to spike the samples, $NaNO_3$- was added. The chemicals may have not been shaken enough for it to disperse in the wastewater. Because of the first data point, the percent change was negative.

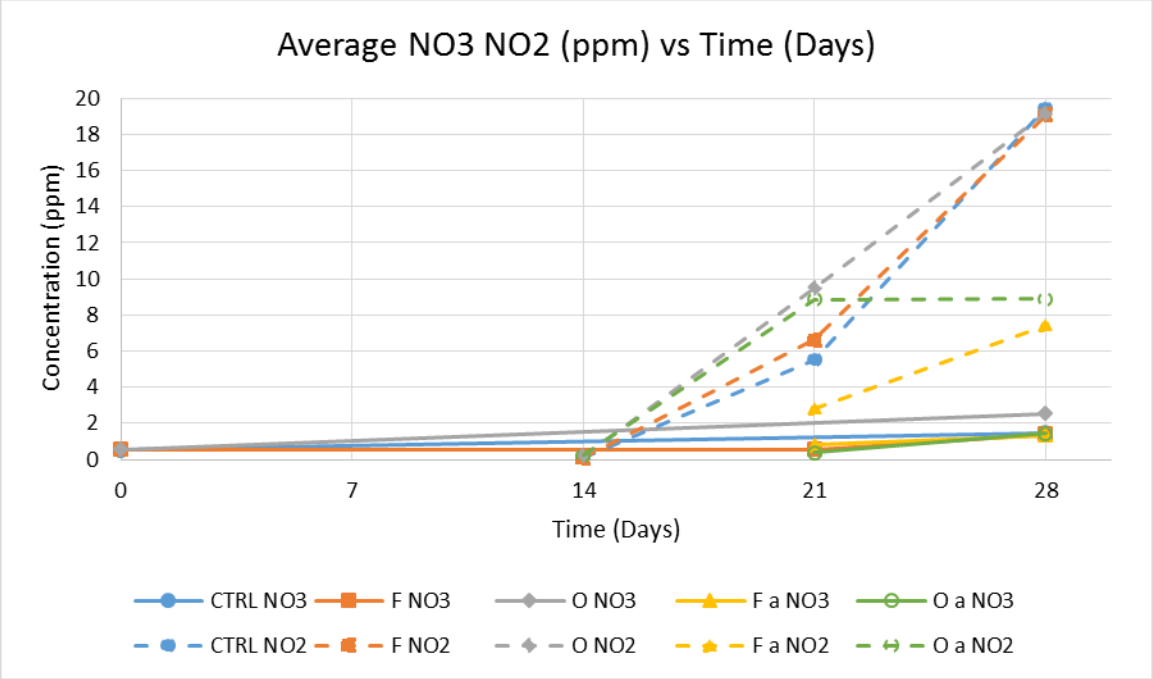


Figure 55. Nitrate and nitrite concentrations for Experiment IV.

From *Figure 55*, the NO_3- levels are increasing from day 0 to 28 while the NO_2- levels are increasing from near 0 ppm at day 14 to about 20 ppm for the original samples. For NO_2- to be increasing, there must be nitrification occurring, which is when naturally

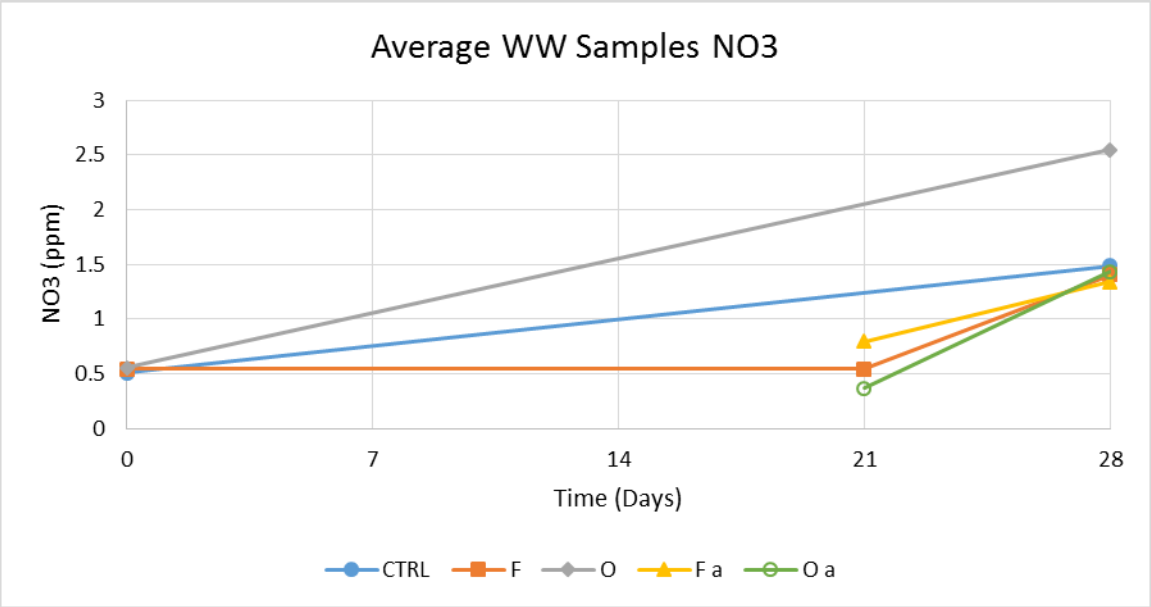


Figure 56. Nitrate levels for Experiment IV.

present NH_3 in the wastewater gets nitrified by nitrifying bacteria to NO_2-.

A closer view of the NO3- concentrations from *Figure 56* show that all samples increased throughout the test. The NO3- concentrations increased the most for the O sample, where it reached about 2.5 ppm while the other samples peaked at about 1.5 ppm. The NO3- concentrations were unusual because it was expected to decrease.

Table 52. Average nitrate concentrations for Experiment IV.

Sample	Initial NO_3- (ppm)	Final NO_3- (ppm)	% change
CTRL	0.5	1.5	-191
F	0.5	1.4	-158.2
O	0.6	2.5	-356.1

Table 52 reveals the NO_3- values for day 0 and day 28. The day 0 data points may be inaccurate because the wastewater samples were spiked with 25 ppm of NO_3-. Thus,

the percent difference shows a negative value due to an increase in NO_3- concentrations. The Osprey sample shows the largest increase in NO_3- whereas the control follows next

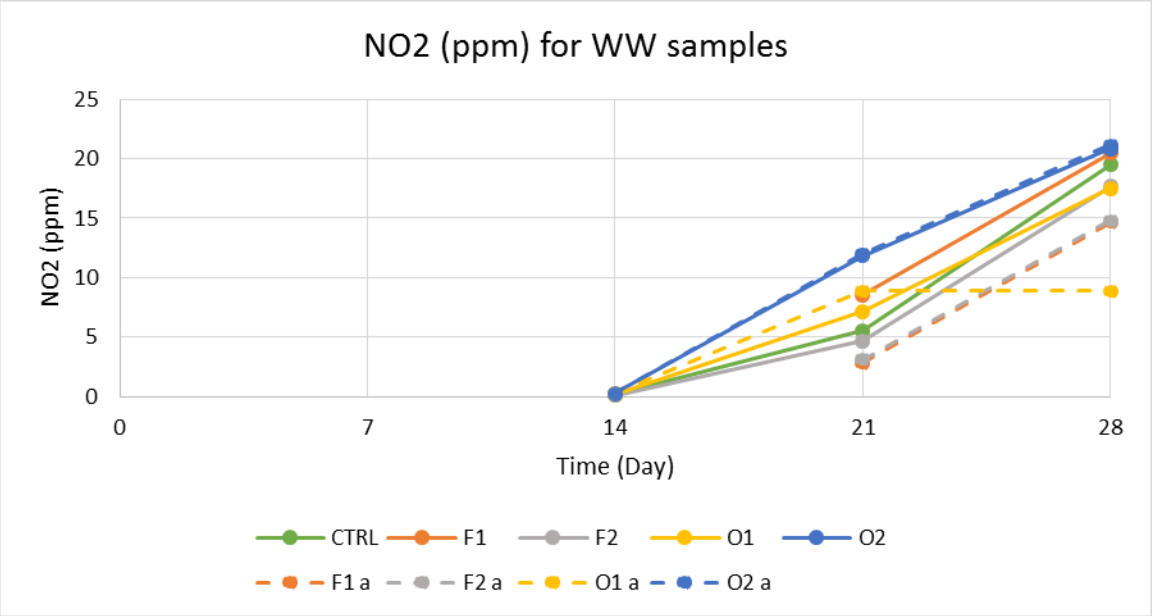


Figure 57. Nitrite concentrations for Experiment IV.

with a -191% difference and lastly, the F sample with a -158% NO_3- reduction.

From day 14 to day 28, the NO_2- levels increased from about 0 ppm to a range of 10 – 20 ppm of NO_2-. Although NO_3- was increasing, the NO_2- concentrations could have been increasing due to nitrification of NH_3 to NO_2- since NH_3 is naturally present in dairy wastewater.

4.4.4 KLB Denitrification Experiments

There were two denitrification tests that were performed with the KLB bacteria to determine its denitrifying abilities. The first test was under aerobic conditions whereas the second test was performed under anaerobic conditions, which is more favorable for KLB bacteria. It can be shown by the following results. Also, the first test was spiked with 50 ppm of NO_3- whereas the second test was spiked with 25 ppm of NO_3-.

4.4.4.1 KLB Test 1

The control showed a decrease in NO_3-, which may be attributed to KLB's spore prominence and thus, cross contamination. The control was the growth media which may have facilitated KLB growth. The KLB curves show denitrification happening with the

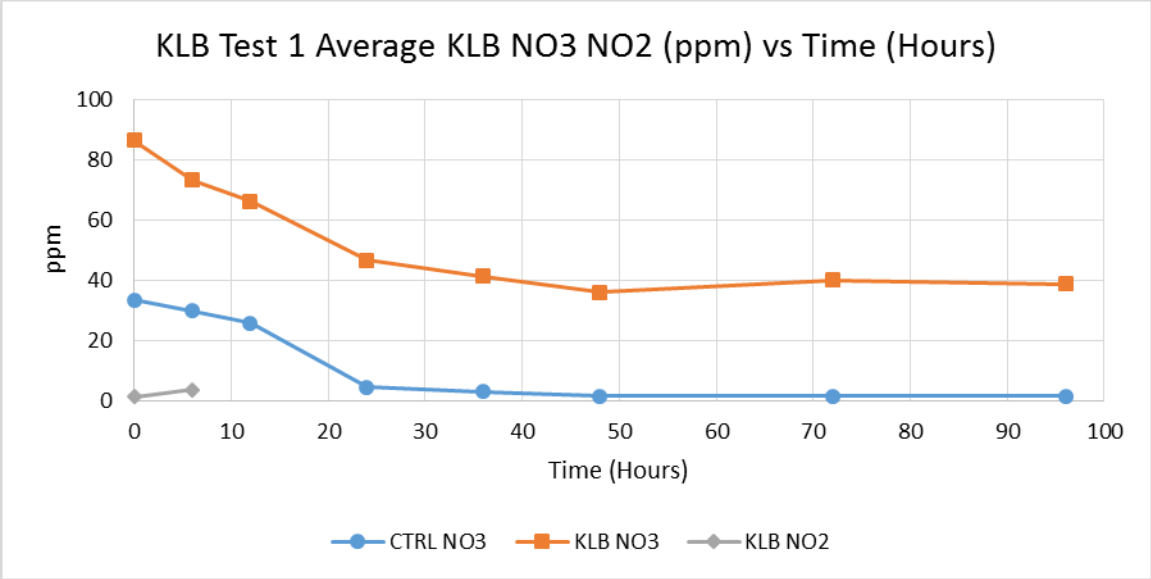


Figure 58. Denitrification results for the first KLB test.

eventual reduction of NO_3- to NO_2-.

With the bottle caps opened, the KLB average NO_3- levels slowly decreased from about 80 ppm to 40 ppm whereas the control decreased from about 35 ppm to almost 0 ppm. NO_2- levels were also slowly increasing as known to denitrification shown in *Figure 58*.

Table 53. Nitrate concentrations for KLB Test 1.

Sample	Initial NO_3- (ppm)	Final NO_3- (ppm)	% change
CTRL	33.3	1.4	95.8
KLB	86.4	38.7	55.2

Because of the possible cross contamination of KLB spores, the control showed a higher NO_3- reduction than the KLB sample with a reduction of 95.8% to the KLB NO_3- reduction of 55.2% as shown in *Table 53*.

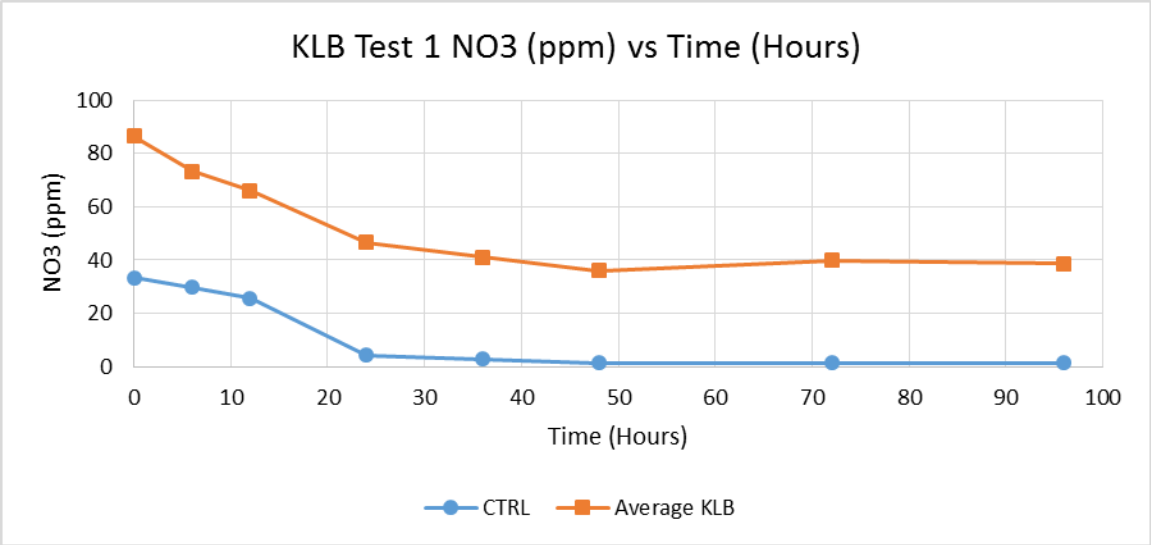


Figure 60. Nitrate levels for KLB Test 1.

The NO_3- levels decreased for both samples, the control with no KLB bacteria added and the sample with KLB. There were duplicates of the KLB, which were averaged to determine the denitrification of the sample. As shown in *Figure 60*, the KLB average decreased nitrate levels twofold. The control also showed a twofold decrease, which may be attributed to possible contamination since the bottles were open and the spores could have been transported to the control.

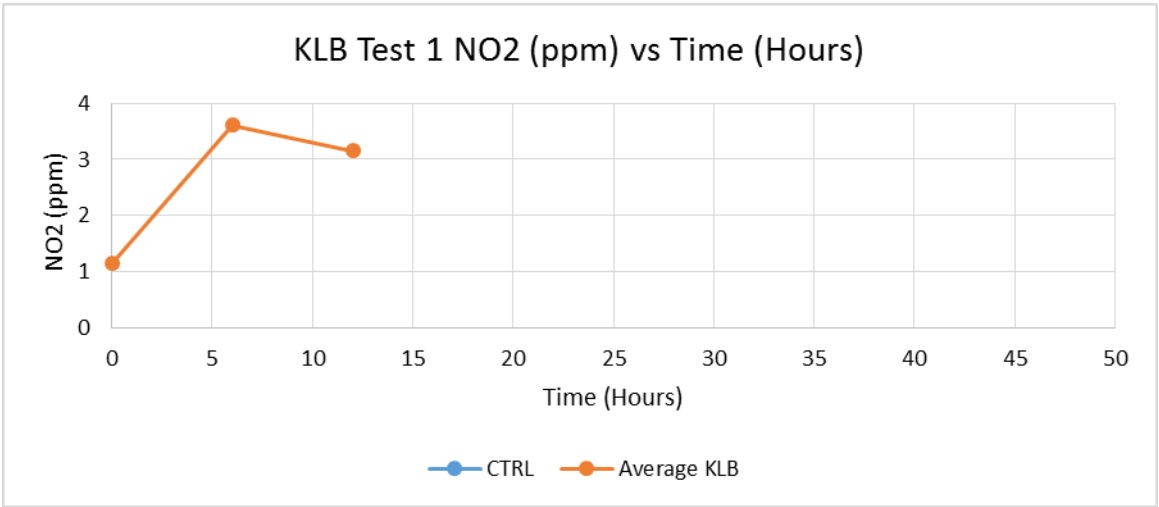


Figure 59. Nitrite levels for KLB Test 1.

Also, from *Figure 59*, a closer view of the NO_2- concentration shows that denitrification was occurring as the NO_2- levels slowly increased to about 3.5 ppm as the NO_3- was being reduced. After 12 hours however, NO_2- was not detected by the IC.

4.4.4.2 KLB Test 2

The second KLB denitrification test was performed to account for the possible cross contamination of the KLB spores into the control. Also, anaerobic conditions were tested rather than aerobic conditions as it was in the first test. The control in this test was DI water instead of growth media and showed no denitrification occurring as expected.

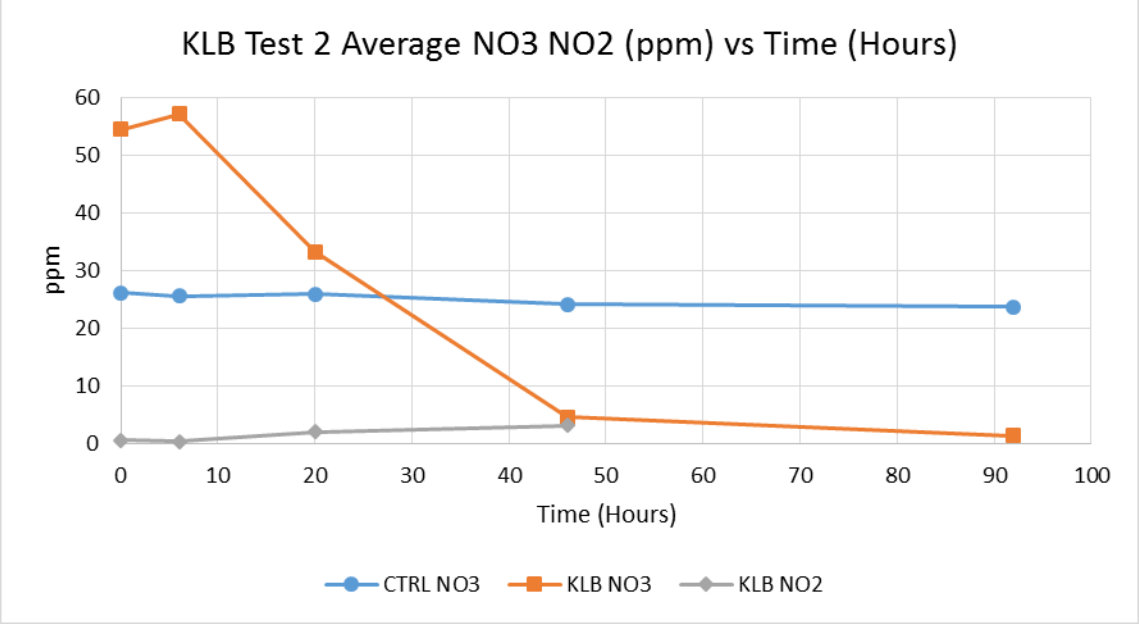


Figure 61. Denitrification results for the second KLB test.

In the span of 96 hours, the NO_3- levels decreased with the activated KLB bacteria while the control, which had no KLB bacteria, remained constant since there was no denitrification occurring as shown in *Figure 61*.

Table 54. Nitrate concentrations for KLB Test 2.

Sample	Initial NO_3- (ppm)	Final NO_3- (ppm)	% change
CTRL	26.2	23.8	9.3
KLB	54.5	1.5	97.3

From *Table 54*, the KLB sample showed a large reduction of NO_3- by 97.3% whereas the control that only had water and NO_3- spiked showed a 9.3% reduction. KLB has the ability to reduce NO_3- by 97.3% in 96 hours.

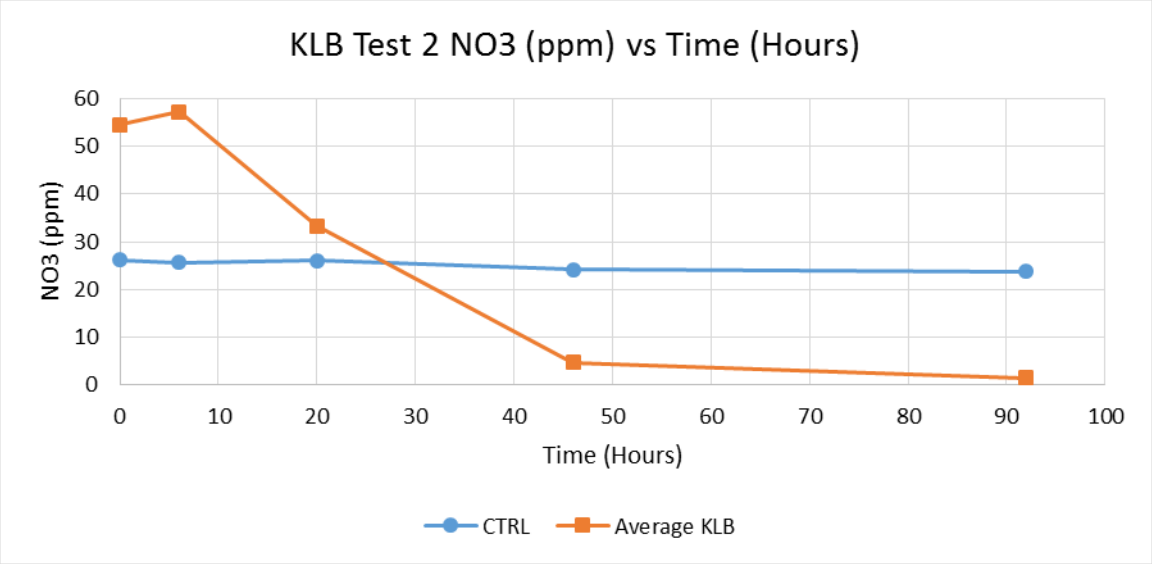


Figure 62. Nitrate concentrations in KLB Test 2.

As shown in *Figure 62*, the average NO_3- levels that had KLB decreased from about 55 ppm to less than 10 ppm within 46 hours. The control, which was spiked with 25 ppm NO_3-, showed no NO_3- reduction throughout the experiment. Thus, KLB shows its prominence of denitrifying under anaerobic conditions with decreasing NO_3- to almost 0 ppm by the end of the 96 hour test period.

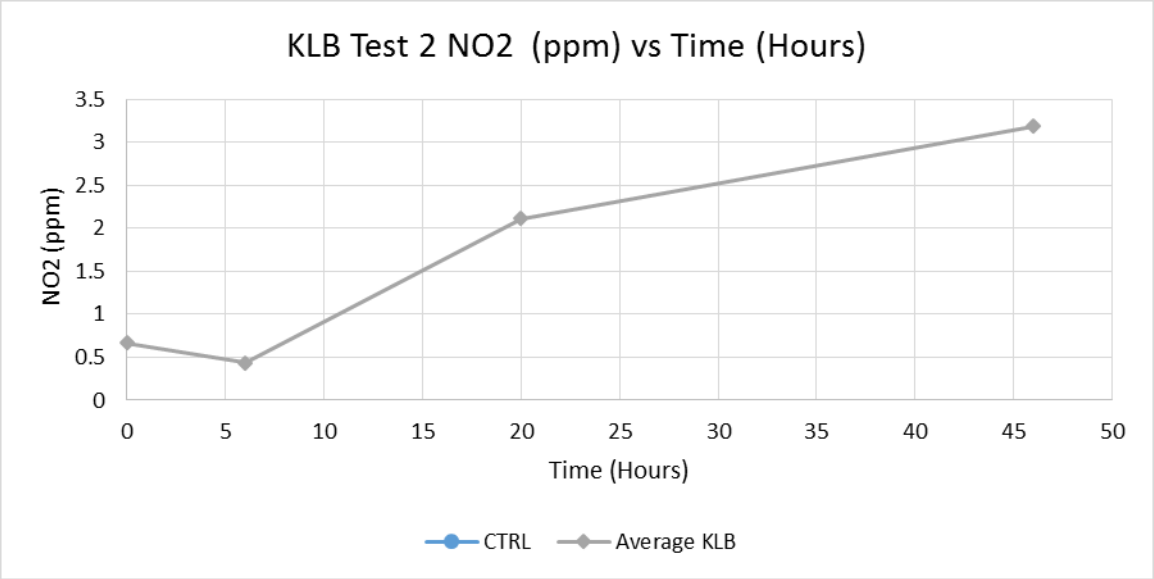


Figure 63. Nitrite levels for KLB Test 2.

From *Figure 63*, the NO_2- levels continually increased from hour 0 to 46. When the KLB bacteria denitrified NO_3- to NO_2-, as the NO_3- concentrations decreased to almost 0 ppm, the NO_2- concentrations increased to about 3 ppm.

4.4.4.3 KLB Test 3

The third denitrification test was performed for the KLB samples to reduce experimentation error from the previous experiment and to also test TA's ability to denitrify NO_3-. A colorimeter was used to measure the NO_3- and NO_2- concentrations over a 96 hour time period.

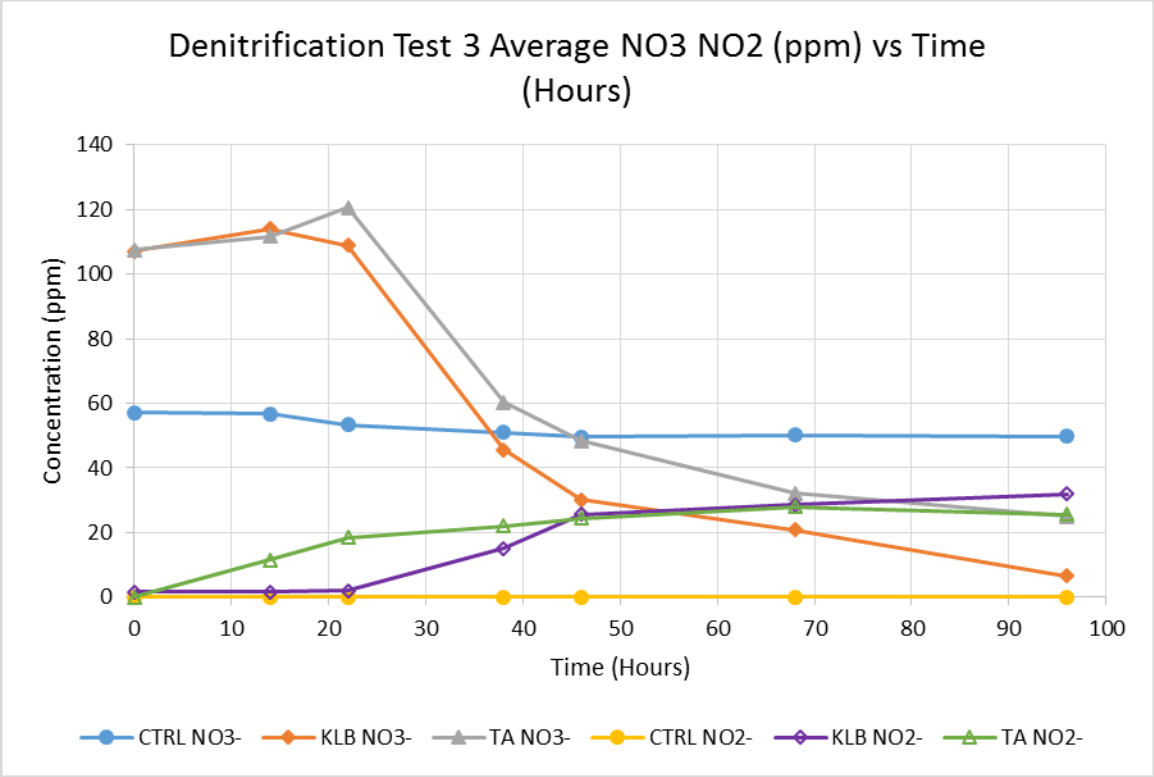


Figure 64. Denitrification test results for Denitrification Test 3.

As shown in Figure 64, all the samples were spiked with 30 ppm with an additional 0.25 g/L $NaNO_3$, about 40 ppm NO_3-, from the new activation media. The control starts at about 60 ppm of NO_3- whereas the KLB and TA start at about 100 ppm NO_3-. The difference in NO_3- concentrations may be attributed to the bacterial mixes' rice bran and soy meal mix.

Table 55. Initial and final nitrate concentrations for Denitrification Test 3.

Sample	Initial NO3- (ppm)	Final NO3- (ppm)	% change
CTRL	57.2	49.7	13.1
KLB	107.1	6.65	93.8
TA	107.45	25	76.7

The control did not show much decrease of NO_3- concentrations as expected since it was only DI water with spiked NO_3-. Table *55* shows that the control decreased NO_3- levels by 13.1% while KLB and TA decreased NO_3- levels by 93.8% and 76.7%

respectively. By adding the Biowish bacteria, NO_3-is being denitrified rapidly in a 96 hour time period.

A closer view of the NO_3- trend for the experiment can be shown in Figure 65

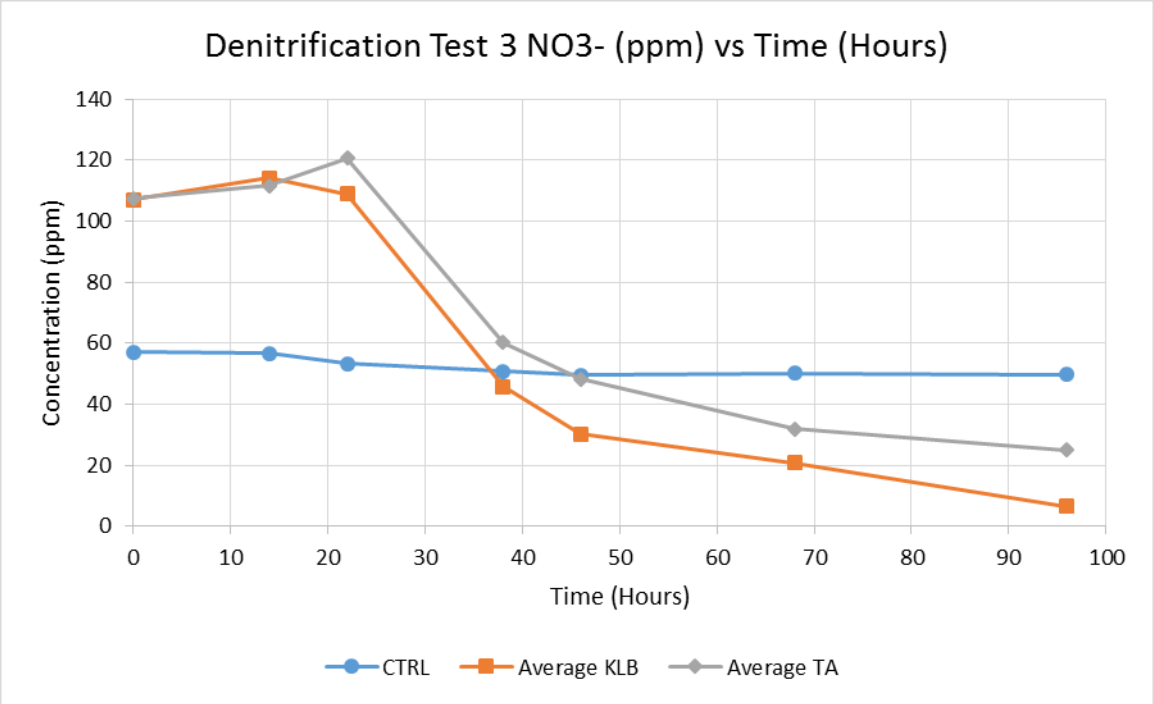


Figure 65. Nitrate trends for denitrification test 3.

with the control being constant at around 60 ppm while the Biowish bacterial samples show a stagnant trend and then decrease of NO_3- by hour 96. The KLB sample further reduces NO_3- concentrations to 6.65 ppm NO_3- compared to the TA sample of 25 ppm NO_3-.

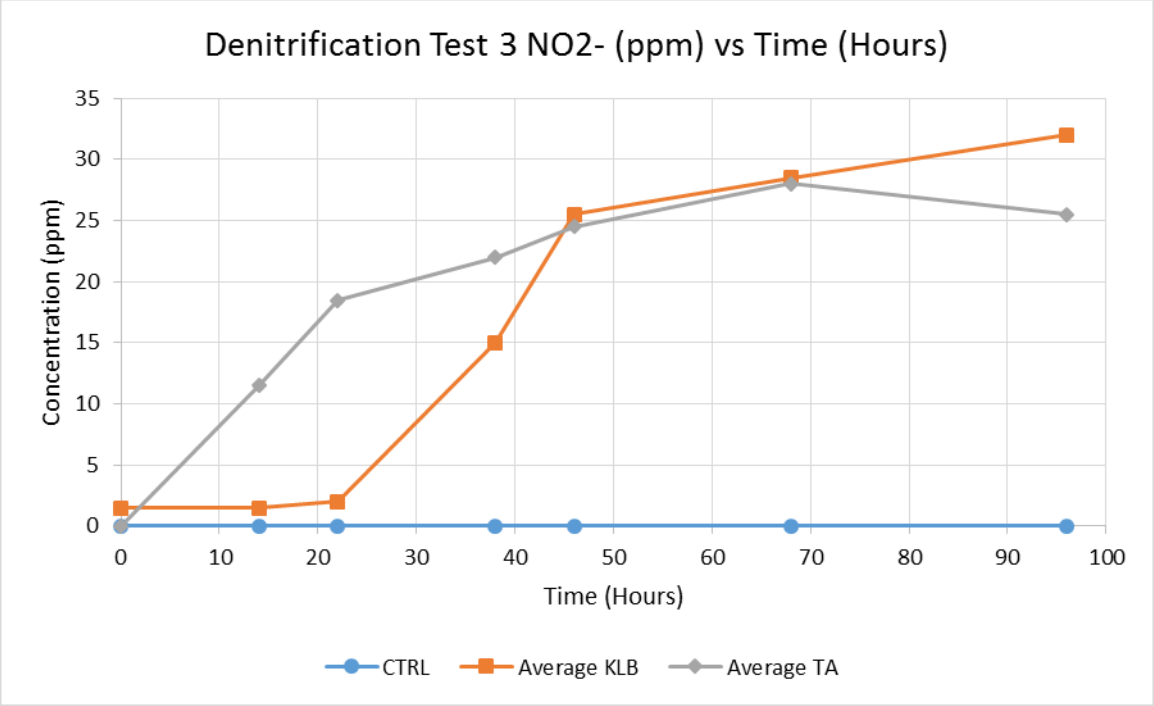


Figure 66. Nitrite levels for denitrification test 3.

Because the control is not denitrifying the NO_3-, the control shows no NO_2- concentrations throughout the 96 hour test shown in Figure 66. The KLB and TA samples do denitrify the NO_3- thus there is an increase in NO_2-concentrations from 20 hours for KLB and 10 hours for TA. Although TA has a faster increase of NO_2- levels, from 70 hours, the NO_2- concentrations steady whereas the KLB samples continually increase to about 33 ppm NO_2- by 96 hours.

4.5 Microscopy

The Gram stain microscopy was performed for the wastewater samples and the images only showed whether there were Gram positive or negative stained bacteria. Dairy wastewater contains thousands of different bacteria, thus, it would be impossible to identify which bacteria were naturally occurring in the water and which were supplemented. Therefore, the purpose of microscopy was to provide a qualitative aspect.

4.5.1 Experiment IV

Bacillus and *Lactobacillus* are Gram-positive bacteria, therefore the bacteria would be stained violet. Also, during the first weeks of performing microscopy, the microcosms were shaken to ensure a mixed culture for collection in the eye dropper. Afterwards, a drop was placed on the glass slide. Since the dairy wastewater was teemed with large populations of bacteria, it was difficult to view specific bacteria, shown in

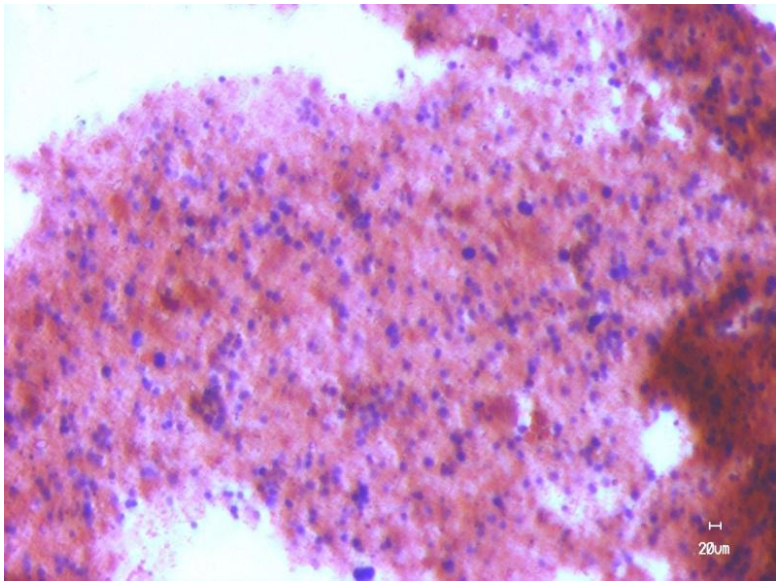


Figure 67. Day 7 control 1000x magnification.

Figure 67.

During Day 35, after learning from the previous week that there were large amounts of bacteria, the dairy wastewater in the eye dropper was given time to settle. The dairy wastewater that dripped onto the glass slide had less solids. From *Figure 68*, the large purple clusters found in the control using 1000x magnification are *staphylococci*, which are Gram-positive bacteria that are from the Bacillales order, which divide at many points creating the grape-like clusters (C. Kitts, personal communication, November 4, 2014).

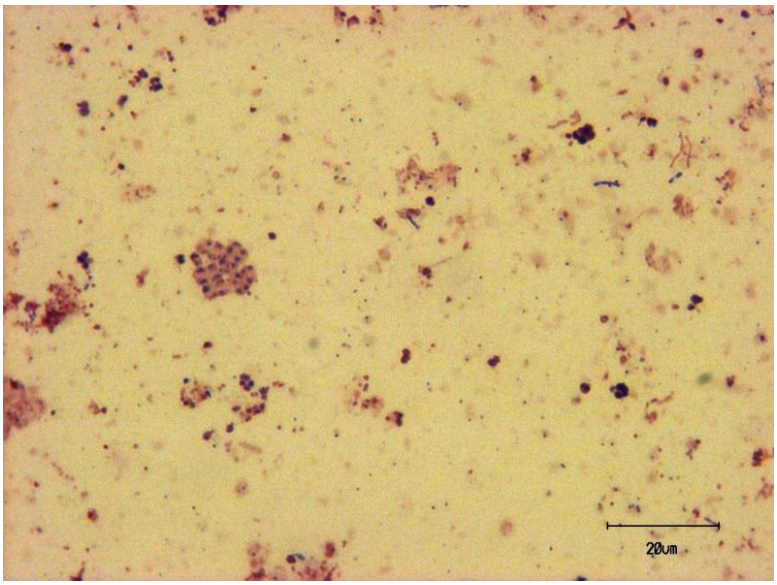


Figure 68. 1000x magnification of the control Day 35.

The microscopy image, shown in *Figure 69*, are from the Fruitwash sample day 35 that revealed the large purple clusters of *staphylococci* as well as *streptococci*. *Streptococci* are circular chains of the order Lactobacillales that divide at one point creating the chain-like structure as shown in the top right. There are also rods that can be shown throughout the image, which are Bacilli, rod shaped bacteria (C. Kitts, personal

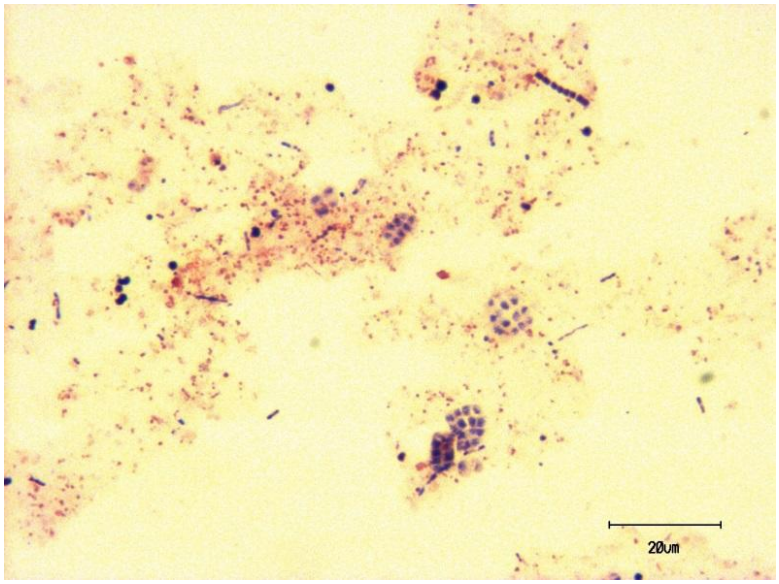


Figure 69. Fruitwash Day 35 1000x magnification.

communication, November 4, 2014).

The Osprey sample microscopy image provides a size of about 9.42 µm for the *staphylococci* shown in *Figure 70*. There is a dense cloud of bacteria in the middle with different types of bacteria morphologies. Rods and *streptococci* are also displayed in the microscopy. The Gram stain microscopy allows for a visual of the dairy wastewater in microscopic view and to decipher Gram-positive bacteria from Gram-negative bacteria. Although there are naturally growing Gram-positive and negative bacteria in the dairy wastewater, the Gram stain allows for quicker identification.

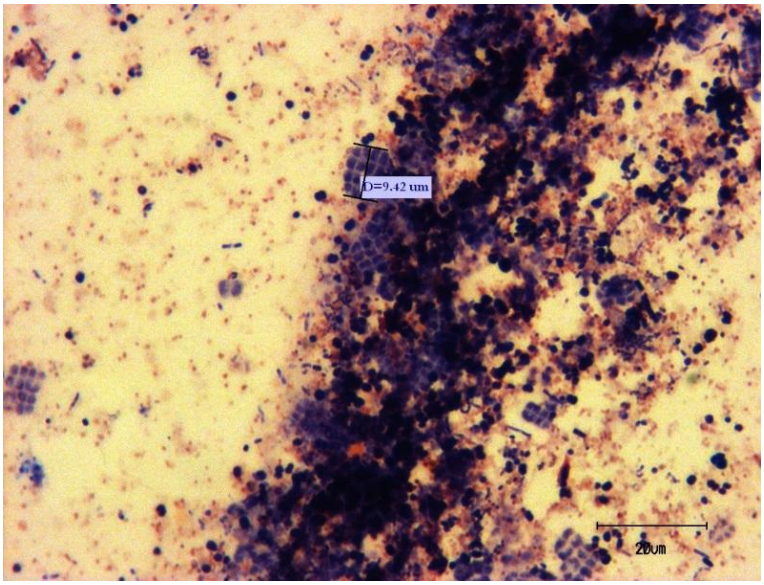


Figure 70. Osprey day 35 at 1000x magnification.

Chapter 5
Conclusions and Recommendations

5.1 Conclusions

After performing several dairy wastewater experiments with different bacterial mixes from Biowish against a control that had no additional bacterial mixes, the results from the many tests ranged from being more effective in reducing chemical constituents and solids to having no clear stimulus on the dairy wastewater.

The BOD reduction for Experiments I – IV did not show a clear distinction of whether or not adding Biowish bacteria helped further reduce BOD concentrations in the dairy wastewater. The values, shown in *Table 56,* demonstrates the control reduces BOD levels near the same as the other samples by the percent change from initial to final. For Experiment V, it was clear that adding Osprey and Fruitwash helped further reduce BOD concentrations with BOD level changes of 69% and 75% respectively to the control's 60% BOD reduction. Another thing to note is the benefit of doing a redose for BOD reduction. By comparing the initial day to the final day for the redose, which is usually day 14 or day 7 for Experiment V, the redose samples show a higher percentage of BOD reduction than without redose. Thus, it was determined that performing redosage to the media helps further degrade BOD.

The TS tests for the experiments did not conclude any definitive results for the effects of supplementing the dairy wastewater with Biowish. Because solids take a longer time period to decrease, the results for a 35 day experiment did not show a straight trend. Instead, there were fluctuations of solids increase and decrease that may be caused by experimental procedures and testing from weighing, filters, storage, and sample volume.

The percent change for the TS tests ranged from -20% - 10% change of TS from initial to final testing days.

For TSS testing, the solids also showed the same steady trend with occasional concentration increases and decreases as the TS tests. TSS tests were performed to determine the various solids components of the dairy wastewater. Because of the fluctuations, some results were negative, which showed an increase in TSS rather than a decrease shown in

TSS Experiment							
I		**IV**			**V**		
Bacteria	**% Change**	**Bacteria**	**% Change**		**Bacteria**	**% Change**	
	Day 0 -57		**Day 7 - 35**	**Day 14 - 35**		**Day 0 - 21**	**Day 7 - 21**
CTRL	14	CTRL	4.8	-2.4	CTRL	4	-4.1
B1	1.3	F	11.2	7.4	F	-7.2	-9.7
O	4	O	5.1	12.7	O	6.9	-10.5
TA		F a		-6.1	F a		-23.3
USA		O a		-5.6	O a		-2.6

Table *62*. Research done by Rashid *et al.* (2007) showed that it took more than five weeks for TSS concentrations to decrease from their duckweed cover and effective microbes addition. Another important procedural adjustment that may have affected the results would be the autofiltration that occurs working with different sample sizes (Tchobanoglous, Burton, & Stensel, 2003). Autofiltration happens when the sample size picked clogs up the filter and the particles itself serve as a filter. Thus, it is important to choose an appropriate sample volume and DI rinse to allow smaller particles to rinse out of the filter.

To continue differentiating and identifying the different types of solids, TDS tests were performed towards the end of the experiments. Dissolved solids are usually

comprised of salts or compounds that disassociate into positive and negative charged ions, which pass through a 1.6 µm filter. TDS has a large fraction of colloidal solids that have a negative charge, which are usually 0.001 – 1 µm in size range (Tchobanoglous, Burton, & Stensel, 2003). It was not expected to change very much throughout the 5 week experiment which can be shown in *Table 63* for Experiment V. Experiment IV contained large percent changes because there was no day 0 data to refer to. Also, the solids were dried in a 105°C oven instead of a 180°C oven as stated in standard methods, which may have caused experimental error. Therefore, Experiment V was performed and showed a decrease of TDS concentrations, 8% and 9% for the F and O samples respectively, whereas the control had a 2% TDS reduction.

VSS tests were performed after measuring TSS to determine how much organic suspended solids decreased throughout the duration. VSS usually characterize organic matter in suspended solids that volatilize in 550°C, however, it may also be inorganic material that ignite at high temperatures or organic material that does not volatilize (Tchobanoglous, Burton, & Stensel, 2003). *Table 64* shows negative values for Experiment IV which may be partly due to the lack of day 0 data as well as testing error performing it for the first time. The Fruitwash and Osprey bacterial mixes in Experiment V further reduced the organic solids by 25% and 36% respectively while the control reduced the VSS concentrations by 20%.

PSD tests were done to distinguish which range of particle sizes were being broken down and whether the concentration was increasing or decreasing. After experimenting with the 5 µm, 2.5 µm, 1.6 µm, and 0.7 µm pore sizes, it was observed that the 2.5 µm pore size filter would retain the most suspended solids. The 5 µm filter

needed to be rinsed thoroughly since particles smaller than 5 µm were getting stuck onto the filter. The percentage difference of initial to final TSS concentration for Experiment II, III, IV, and V are shown in *Table 58*, *Table 59*, *Table 60*, and *Table 61* respectively. Note, for Experiment II, because a 1 µm pore size was first used, the percent difference calculated for the pore sizes prior to 0.7 µm were from day 14 to day 91 rather than day 0.

Lastly, the NO_3- levels were measured by ion chromatography procedures. It was important to measure the NO_3- concentrations to determine the bacteria's ability to denitrify and reduce NO_3- to NO_2-. NO_3- has the potential to contaminate groundwater and surface water by causing eutrophication and depleting oxygen at the water sources. As shown in *Table 65*, Experiments II and III showed a large difference using Biowish bacteria to reduce NO_3- concentrations compared to the control. For Experiment V, the results did not reflect the same conclusions. It may be attributed to the spike of $NaNO_3$- rather than KNO_3- . For Experiment II, the TA and USA reduced NO_3- by 64% and 48% respectively while the control only reduced the NO_3- concentration by 33%. The B1 and B2 samples in Experiment III also showed similar NO_3- level reduction of 80% and 68% respectively whereas the control only reduced NO_3- by 29%.

The experiments performed with the Biowish bacterial mixes to determine whether bioaugmentation would enhance reduction of solids and chemical constituents in dairy wastewater were successful, but also inconclusive for some of the tests. For BOD, although the control and the original samples showed similar BOD concentration reductions, the redosage samples showed that by adding Biowish bacteria, the bacteria were able to enhance BOD reduction concentrations. The solids tests were inconclusive

for TS and TSS. It may be attributed to the short time period since solids need more time to see results. The smaller solids component test such as TDS and VSS did show that that with the Biowish bacteria, there were decreases in organic suspended solids and dissolved solids.

Table 56. BOD Experiment Results.

BOD Experiment													
I		II			III			IV			V		
Bacteria	% Change	Bacteria	% Change		Bacteria	% Change		Bacteria	% Change		Bacteria	% Change	
	Day 0 - 57		Day 0 - 35	Day 14 - 35		Day 0 - 35	Day 14 - 35		Day 0 - 35	Day 14 - 35		Day 0 - 21	Day 7 - 21
CTRL	90.5	CTRL	90.1	29.1	CTRL	86	10	CTRL	81.3	23.2	CTRL	59.4	10.9
B1	92.9	TA	86.3	20	B1	87.2	38.5	F	80.6	4.6	F	74.5	46.1
O	84.1	USA	83.3	30.3	B2	85.5	43.8	O	76.3	25.7	O	68.7	53.3
TA	90.7	TA a		39.7	B1 a		71.4	F a		54.7	F a		44.6
USA	89.6	USA a		71.8	B2 a		66.5	O a		30.3	O a		59.7

Table 57. TS Experiments Results.

TS Experiment										
II			III			IV			V		
Bacteria	% Change		Bacteria	% Change		Bacteria	% Change		Bacteria	% Change	
	Day 0 - 35	Day 14 - 35		Day 0 - 35	Day 14 - 35		Day 0 - 35	Day 14 - 35		Day 0 - 21	Day 7 - 21
CTRL	14	7.5	CTRL	-12	-11.3	CTRL	4.8	-2.4	CTRL	-3.6	-9.9
TA	1.3	-7.8	B1	-9.7	-3.6	F	11.2	7.4	F	-15	-19.1
USA	4	-9.2	B2	-3.8	6.2	O	5.1	12.7	O	-18.7	-6
TA a		-5.8	B1 a		-3	F a		-6.1	F a		-2.7
USA a		4	B2 a		0.3	O a		-5.6	O a		-6.6

Table 58. Experiment II PSD results.

TSS (mg/L)

	CTRL				TA				USA			
Pore Size (µm)	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
% Change	-36.4	72.9	-25.0	57.4	-37.5	15.7	90.7	59.5	-75.0	74.4	80.6	66.3

Table 59. Experiment III PSD results.

TSS (mg/L)												
	CTRL				B1				B2			
Pore Size (µm)	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
% Change	-48.5	31.0	82.9	76.5	-42.7	30.7	91.8	67.7	-75.3	21.1	31.6	59.2

Table 60. Experiment IV PSD results.

TSS (mg/L)												
	CTRL				F				O			
Pore Size (µm)	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**
% Change	-97.5	57.8	63.6	57.3	-23.9	52.2	75.0	72.8	-90.0	-12.3	100.0	81.9

Table 61. Experiment V PSD experiment.

TSS (mg/L)												
	CTRL				F				O			
Pore Size (µm)	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	**0.7**	**5**	**2.5**	**1.6**	0.7
% Change	-46.7	25.6	56.9	69.7	-2.9	-5.3	69	80.8	-47.7	-8.2	64.4	81

Table 62. TSS Experiments Results.

TSS Experiment							
I		IV			V		
Bacteria	% Change	Bacteria	% Change		Bacteria	% Change	
	Day 0 -57		Day 7 - 35	Day 14 - 35		Day 0 - 21	Day 7 - 21
CTRL	14	CTRL	4.8	-2.4	CTRL	4	-4.1
B1	1.3	F	11.2	7.4	F	-7.2	-9.7
O	4	O	5.1	12.7	O	6.9	-10.5
TA		F a		-6.1	F a		-23.3
USA		O a		-5.6	O a		-2.6

Table 63. TDS Experiments Results.

TDS Experiment				
IV			V	
Bacteria	% Change		Bacteria	% Change
	Day 7 - 35	Day 14 - 35		Day 0 - 21
CTRL	-88.2	-47.1	CTRL	1.7
F	1.9	7.4	F	7.8
O	-37.1	-10.4	O	8.9
F a		6.3		
O a		-8.1		

Table 64. VSS Experiments Results

VSS Experiment					
IV			V		
Bacteria	% Change		Bacteria	% Change	
	Day 7 - 35	Day 14 - 35		Day 0 - 21	Day 7 - 21
CTRL	5.5	-30.2	CTRL	19.6	5.3
F	-12.3	-9.2	F	25	5.8
O	-14.9	9.4	O	35.9	6.9
F a		-28.6	F a		2.8
O a		-43.7	O a		12.9

Table 65. IC Experiments Results.

IC Experiment					
II		III		IV	
Bacteria	% Change	Bacteria	% Change	Bacteria	% Change
CTRL	32.5	CTRL	29	CTRL	-191
TA	63.6	BMT1	79.9	F	-158.2
USA	48.3	BMT2	68.4	O	-356.1

5.2 Recommendations for Future Work

After completing five experiments with different changes in variables and tests, there are other possible variations that can be implemented to determine Biowish bacterial mixes' ability to become an effective dairy wastewater management solution. Because dairy wastewater management encompasses many different aspects from physical characteristics of solids to inorganic chemical constituents to organic chemical constituents, there are several recommendations for future work dealing with Biowish in the wastewater. Changes made in bacterial mix concentrations, media characteristics, and tests may offer more definitive results of the bioaugmentation's effect on dairy wastewater.

5.2.1 Bacterial Mix Concentrations

The redose experiments, which added 500 ppm, 250 ppm, and 10 ppm of Biowish bacteria, showed that adding 250 ppm and 500 ppm of bacteria spiked the BOD and solids tests during the first day of redose. Then, the redose samples showed that it further reduced the BOD levels than if there was no redose. A similar reduction also occurred when the redose was lowered to 10 ppm, however it was not further reduced than the original samples.

To provide the most BOD reduction, but at a cost effective stand point, future work can be performed to optimize the redosage concentrations of the Biowish bacteria. Clearly, 250 ppm and 500 ppm are high redosage that spikes the samples' BOD concentrations and would require the client to purchase a lot of bacterial mix. 10 ppm of bacterial redose did not provide the enhanced BOD reduction that was sought after shown in previous experiments. Thus, an optimization experiment to test what redosage

concentration would best be suited for enhanced BOD reduction is a recommendation for future work.

5.2.2 Media Characteristics

Another recommendation for future work would be to alter the dairy wastewater media characteristics by providing dissolved oxygen to the dairy wastewater media during experimentation. Although the Cal Poly Dairy Unit is an aerated dairy lagoon, there is only one aerator in the middle and collection occurs on the side. Thus, the dairy wastewater dissolved oxygen levels, which were recorded in October 2014, showed the diary wastewater to be highly anaerobic.

To determine whether the facultative anaerobic and obligate aerobic bacteria would further reduce the chemicals and solids in the dairy wastewater with added dissolved oxygen, an experiment can be performed by supplementing the media with various concentrations of dissolved oxygen. Experimentation to determine which concentration of dissolved oxygen would better facilitate reduction of solids and chemicals with a control of no dissolved oxygen addition would be the second recommendation for future work.

5.2.3 Dairy Wastewater Tests

Lastly, some tests that were performed were very effective at investigating whether bioaugmentation did enhance chemical and solids reduction and some tests were not. BOD, NO_3-, PSD, TDS, and VSS were tests that helped determine the Biowish bacterial mixes' ability as a dairy wastewater management solution. TS tests provided more of a quality control to calculate whether the TSS and TDS tests were performed as accurately as possible.

In order to produce the most reflective tests for Biowish's bacterial mixes abilities, the following tests – BOD, TDS, VSS, and IC – can continue following Standard Methods by Clesceri (1998) for procedures. PSD could be altered to see changes occur more clearly. Because bacteria sizes are usually 0.2 – 3 µm in size, it would be interesting to see the depth of what particle sizes are entrapped from 2.5 – 5 µm (Tchobanoglous, Burton, & Stensel, 2003). The only glass fiber filter with borosilicate media without binder that fits in that range would be 3 µm. By adding the 3 µm filter in between the 5 µm and 2.5 µm filter, a closer look of what particle sizes larger than 2.5 µm there are. Another filter that would also help break down the various particle sizes would be 2 µm, since bacteria size ranges between 0.2 µm and 3 µm.

Also, because the 5 µm pore size filters were double layered with borosilicate glass fibers, particles smaller than 5 µm were getting stuck in it. The two separate layers of glass fibers may have accounted for the particles getting stuck. For future work, it would be best to use single layer borosilicate glass fiber filters for 5 µm pore sizes to prevent autofiltration.

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Appendices

Appendix A: Dionex DX-120 Ion Chromatograph (IC) Protocol

1. Filtering
    a. Label 5ml poly vials with its respective names and the line for 5 ml
    b. Rinse filter materials with DI water
    c. Place glass tube into the 500 ml Pyrex flask
    d. Place filter on filter holder
    e. Clamp filter holder to suction flask
    f. Shake sample in 40 ml TOC vial and pour sample into the filter
    g. Remove and throw away filter
    h. Rinse the filter apparatus
    i. Pour filtered sample from glass tube into 5 ml Poly vials, up to the marked line, and cap
2. IC Instrument
    a. Eluent
        i. Fill beaker with ~200-500 ml DI water
        ii. Place on hot plate and turn on
        iii. Weigh 0.954g of Na2CO3
        iv. Weigh 0.235g of NaHCO3
        v. Add to hot DI water
        vi. Pour eluent mix into 2 L plastic Dionex bottle (marked with black line)
        vii. Fill bottle with DI water to 2 L mark
    b. Pour into eluent bottle on top of machine
3. Bubble eluent
    a. Rinse with DI water the tubes connected into the machine into the other bottle holder on top
    b. Rinse helium input tubes with DI water into bottle and place tubes into eluent bottle
    c. Push tape away from bend and un-crimp

d. Adjust the helium tank to moderately bubble the eluent for 15 minutes
e. Rinse tubes into bottle holder and crimp
f. Place tubes back into eluent bottle and twist
g. Turn helium tank to 60 psi

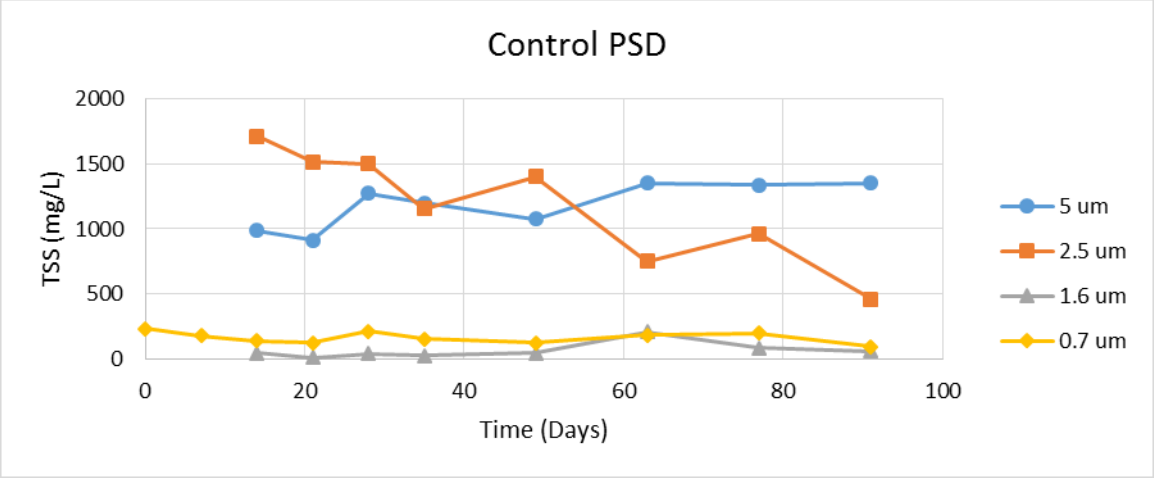
4. Computer
    a. Take holders from Automated Sampler
    b. Make the plan (rinse, standard, sample)
        i. Save as (to get previous plan layout)
        ii. Rinse and blanks get filled with DI water to the mark
        iii. Shutdown vial is provided
        iv. Reuse until the cap is loose at the top
    c. Double check for names
    d. Make sure the Type, Position number, and Program corresponds to the sample
        i. Ex: Rinse, Blank, 1, Di-water Rin
5. IC instrument
    a. Warm the pump
        i. Oscillate the pressure from 500-2000 psi three times
    b. Flow Setting
        i. Make sure the flow rate is 1.2 mL/min
    c. Pressure
        i. Close to 2200 psi and constant
    d. Make sure it is on Local
        i. Press SRS when pressure and flow is constant
        ii. Let air bubbles flow for 3-5 minutes from the Regen A out tube
    e. On the Component On/Off button
        i. The following should be lit
            1. Eluent Pressure
            2. Pump
            3. SRS
            4. Automated Sampler

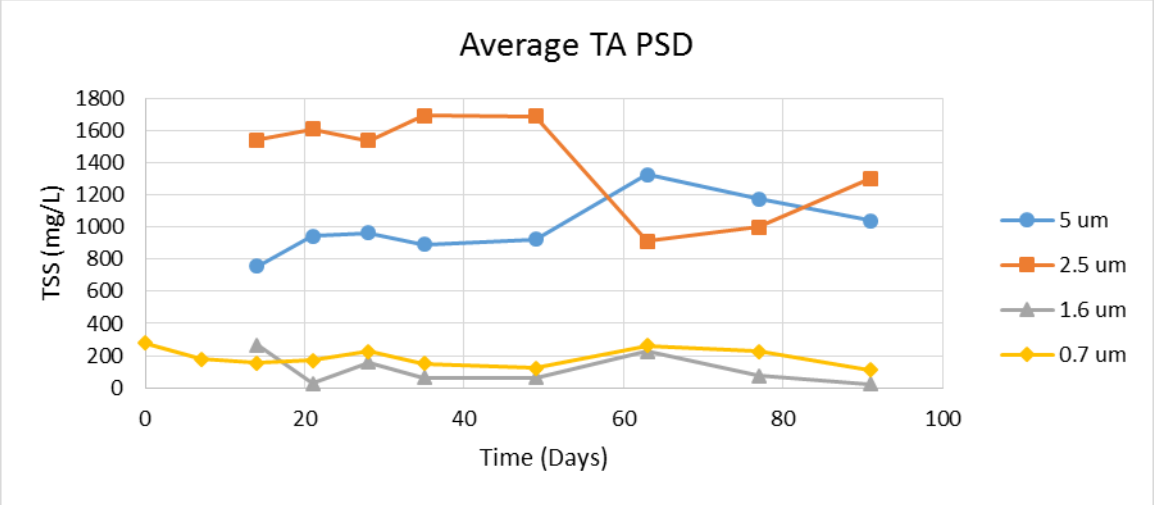
f. Placement
    i. Black dots facing to the right
    ii. Front – beginning of sequence
    iii. End – last sequence

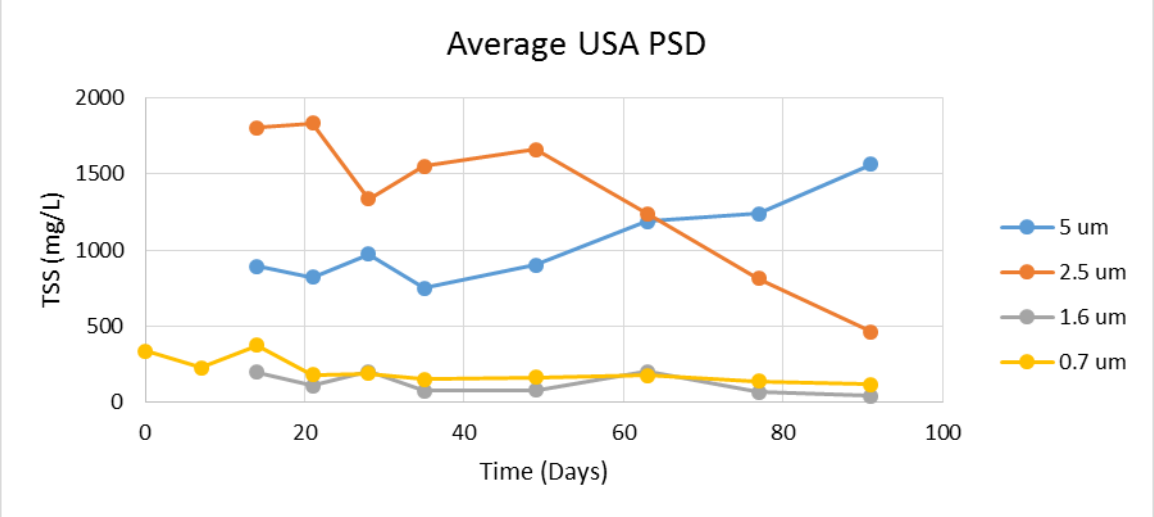
6. IC Instrument
    a. After the air bubbles have been pumping for 3-5 minutes, press remote
    b. Dionex_DX120.pan
    c. Click Connect go back and change to Run on the automated sampler
    d. Batch > Start > Ready Check > Yes > Ready Check was successful > Okay > Start
7. Export Data
    a. Right click on test > Batch Report > Reportsdefault > Export Settings > Location (flashdrive) > File Name Formula > Sample > Sample Number
    b. Excel File format > Next > Only Integration > Okay

Appendix B: PSD Sample Trends

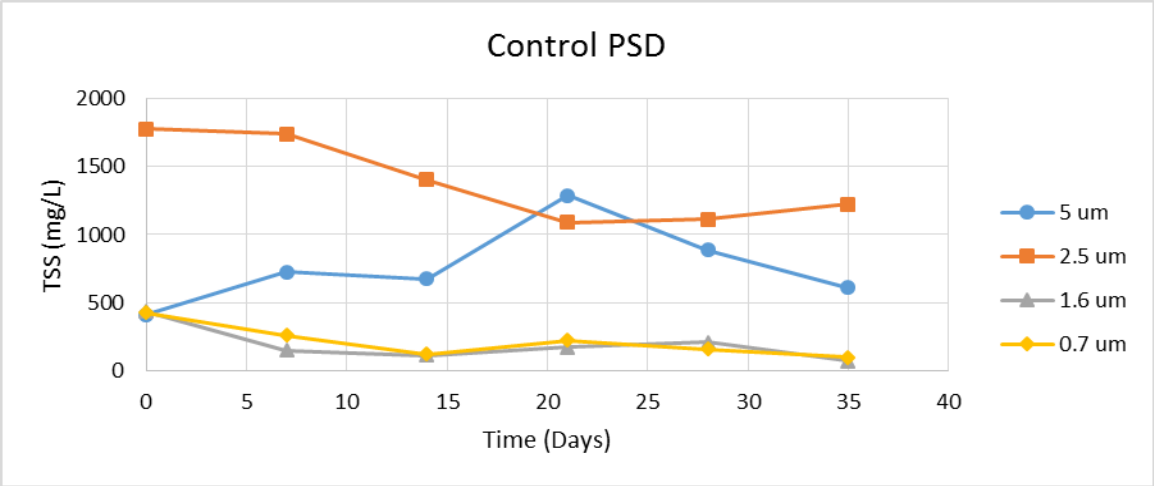
Experiment II

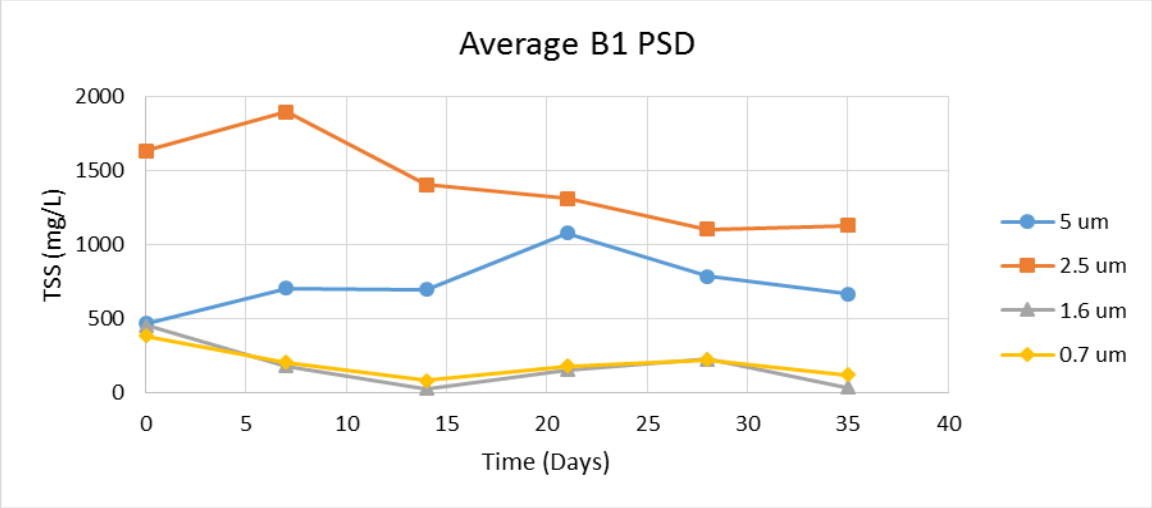


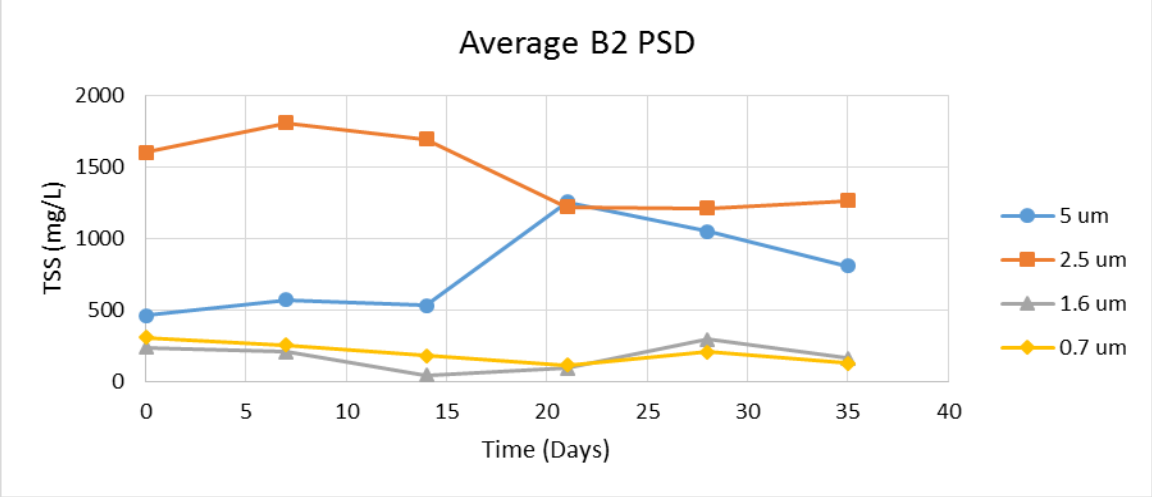




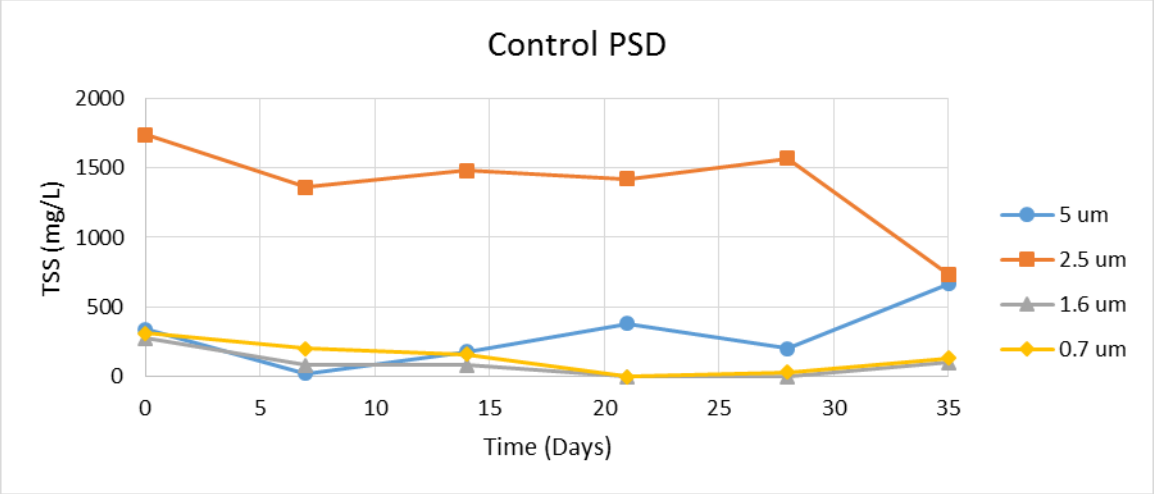
Experiment III

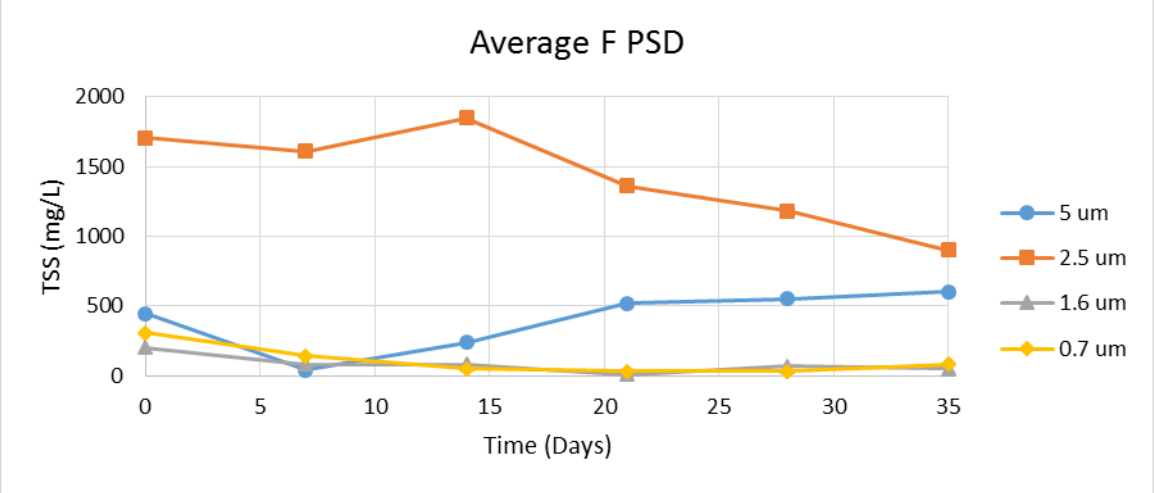


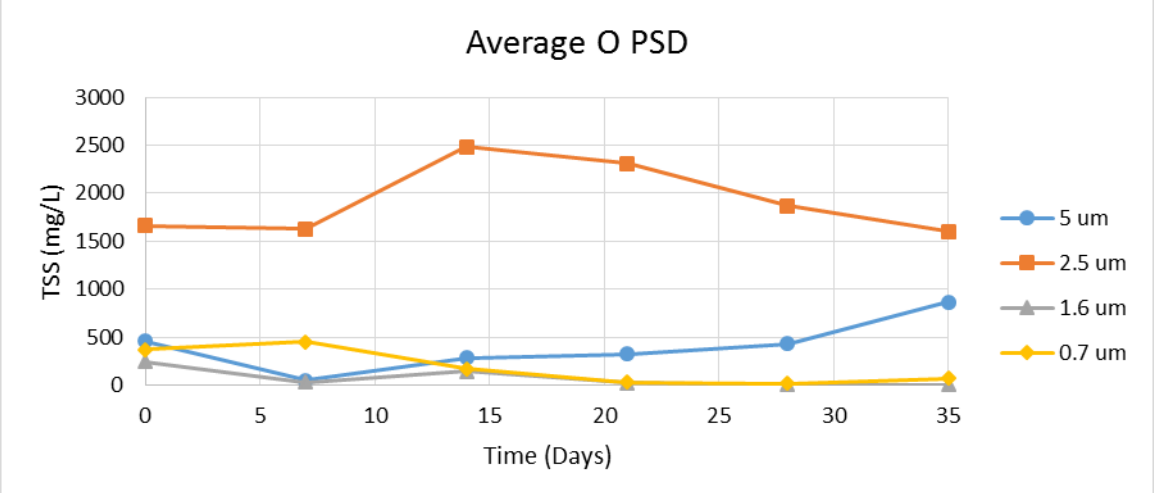




Experiment IV

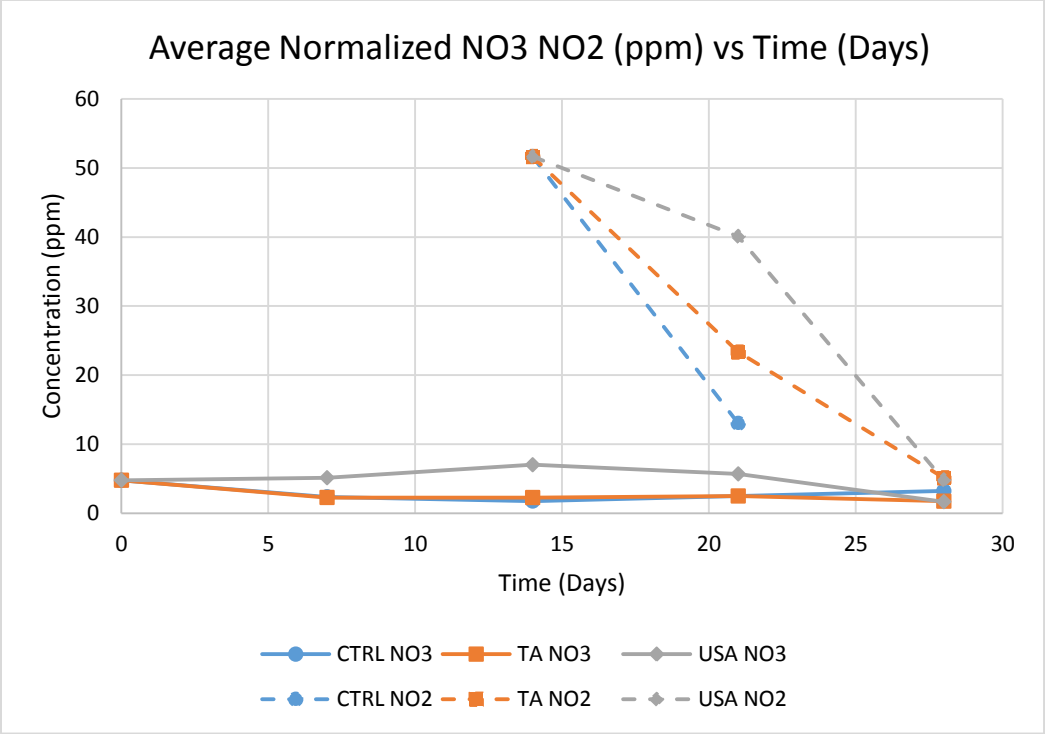


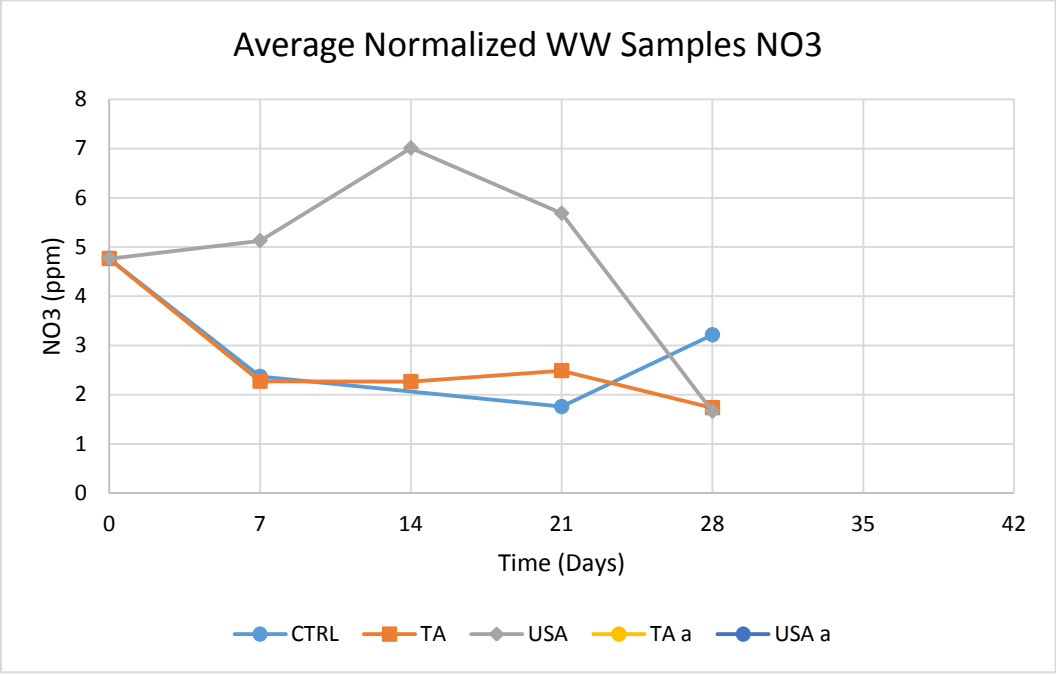


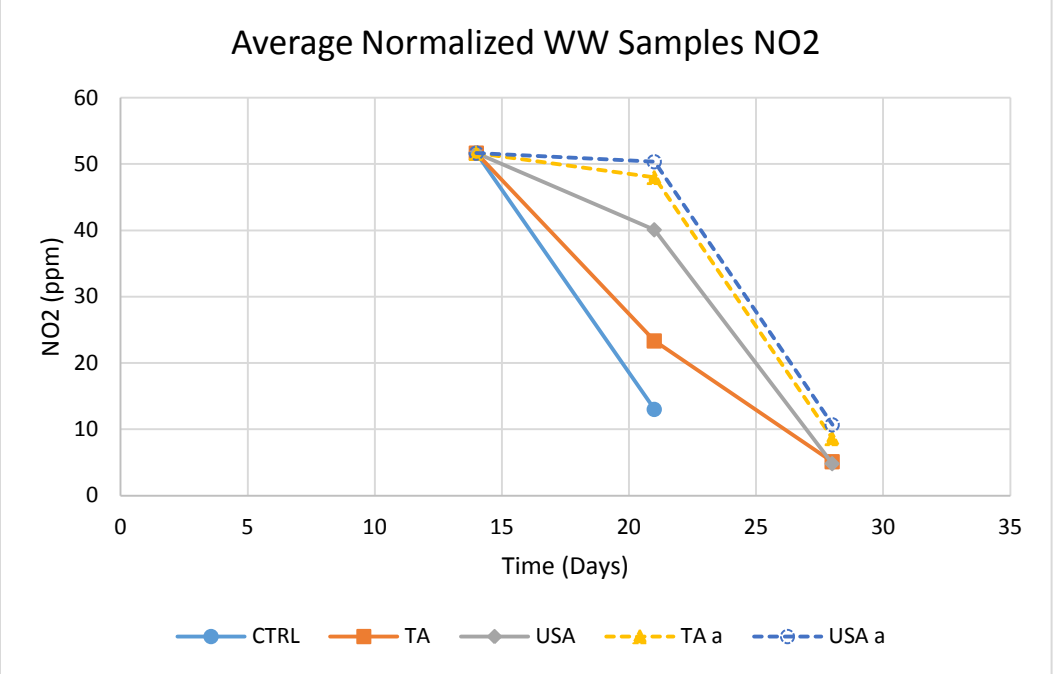


Appendix C: IC Normalized Graphs

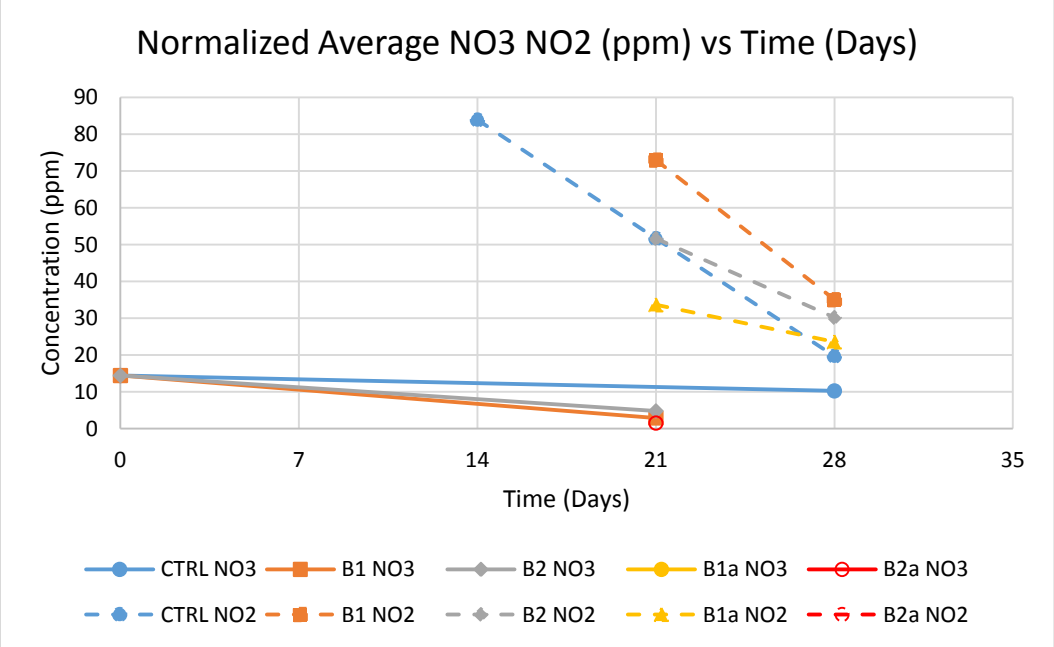
Experiment II

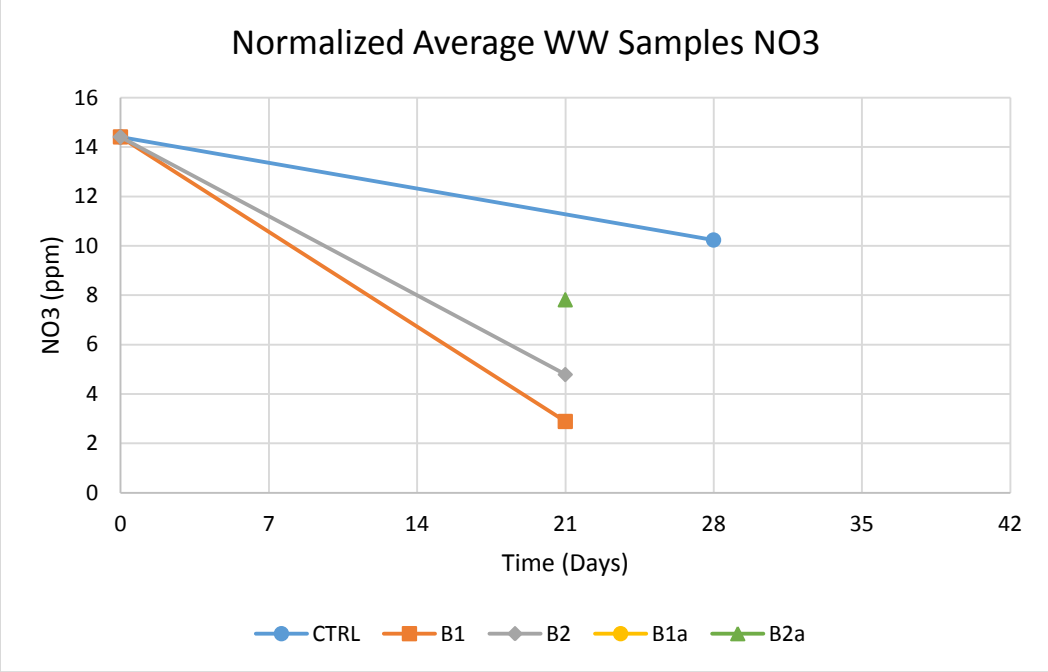


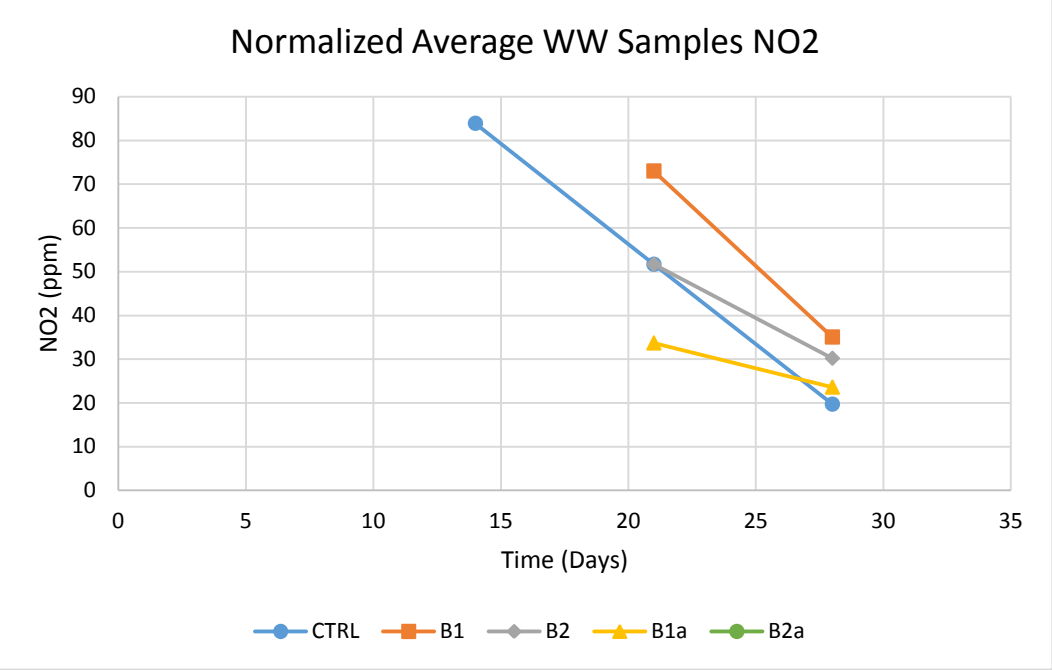




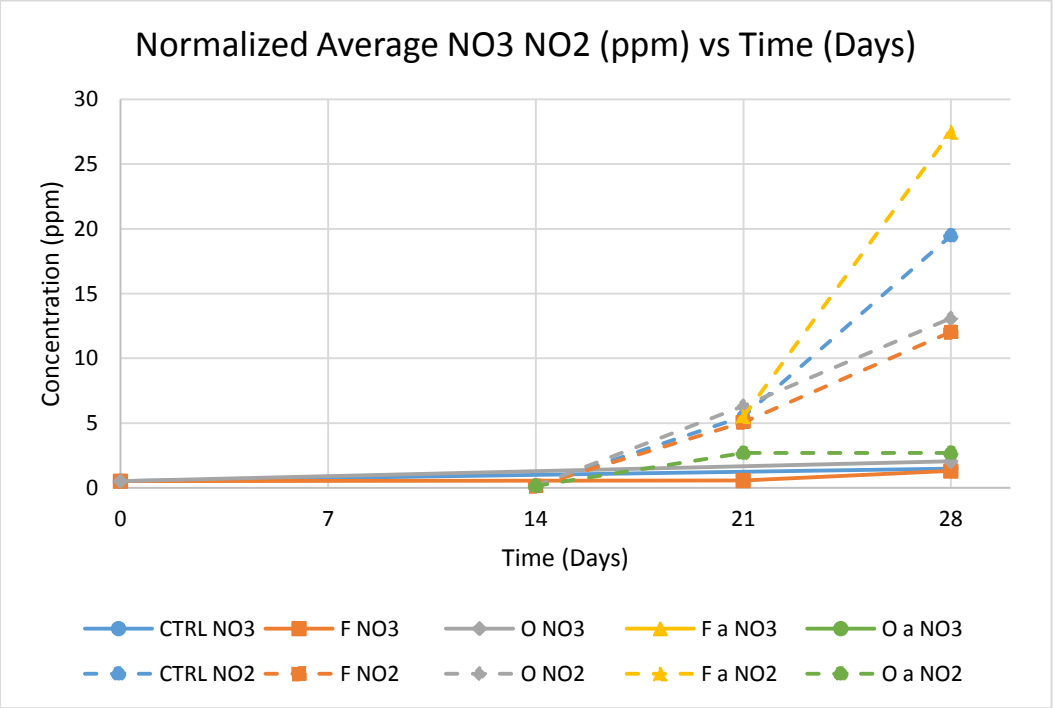
Experiment III

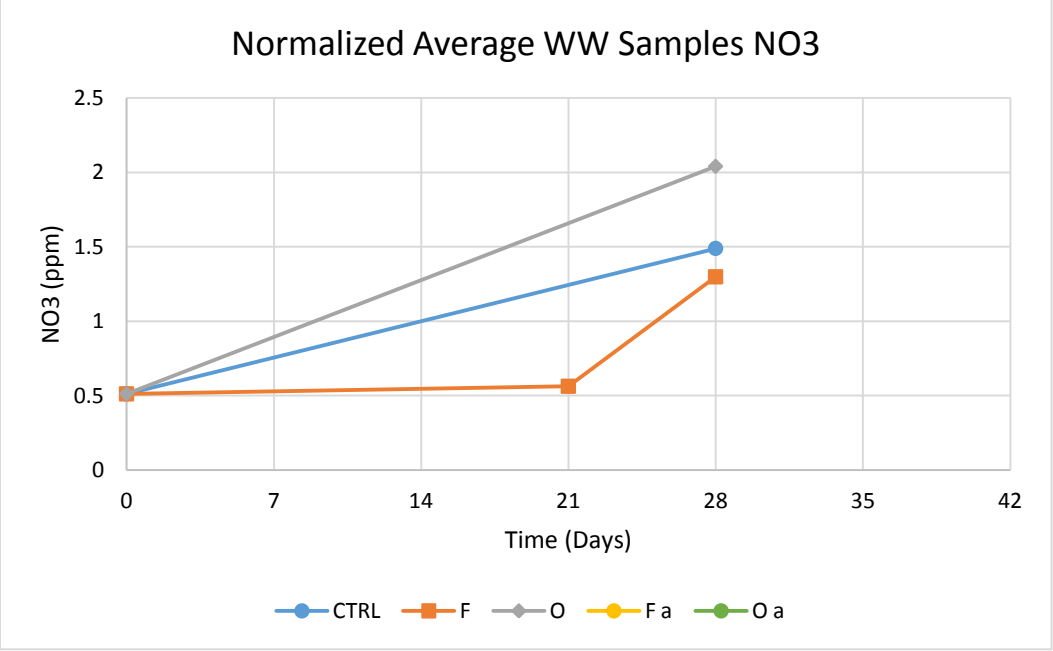


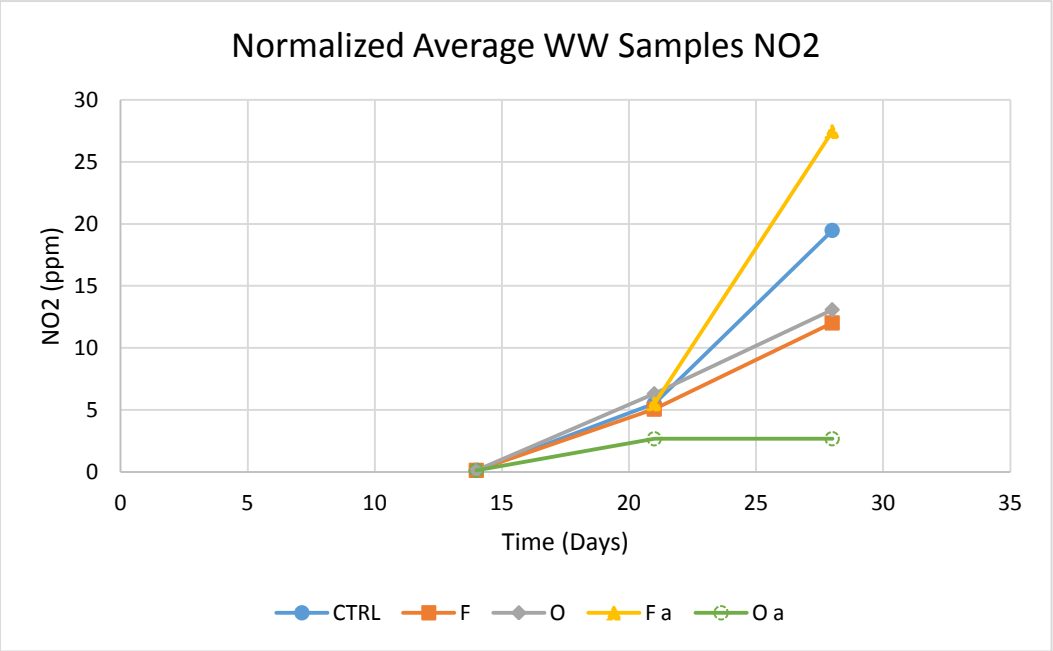

Normalized Average WW Samples NO3
NO3 (ppm)
16
14
12
10
8
6
4
2
0
0
7
14
21
28
35
42
Time (Days)
CTRL
B1
B2
B1a
B2a


Normalized Average WW Samples NO2
NO2 (ppm)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (Days)
CTRL
B1
B2
B1a
B2a

Experiment IV

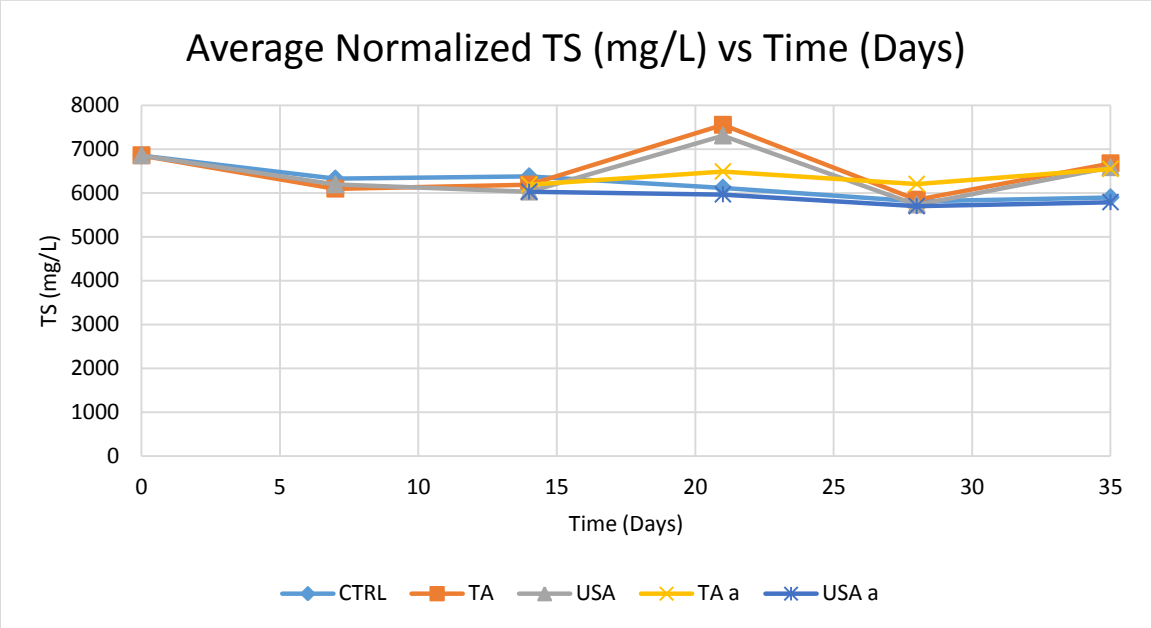




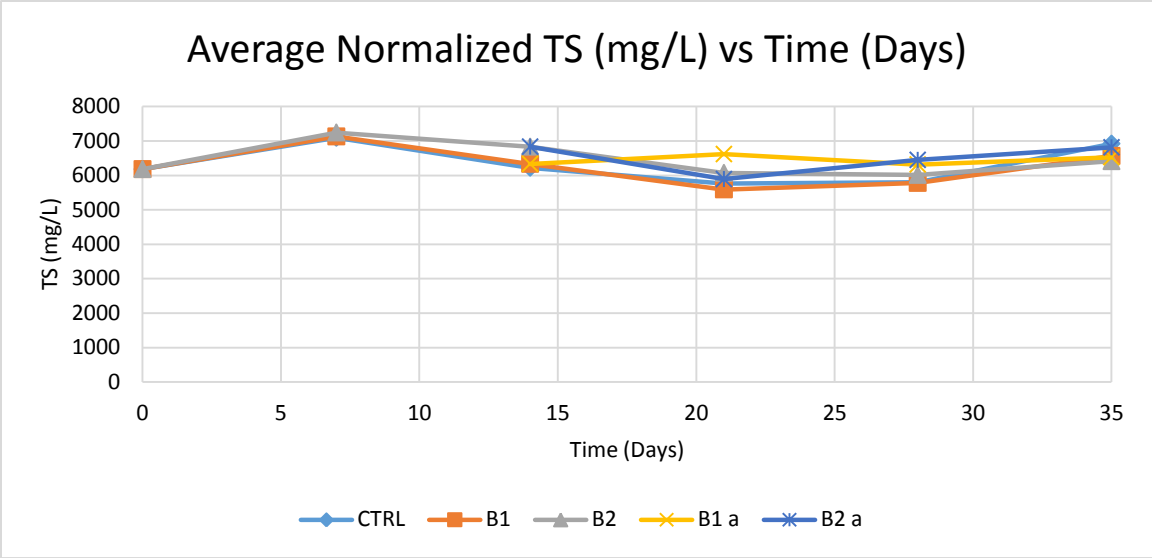


Appendix D: TS Normalized Graphs

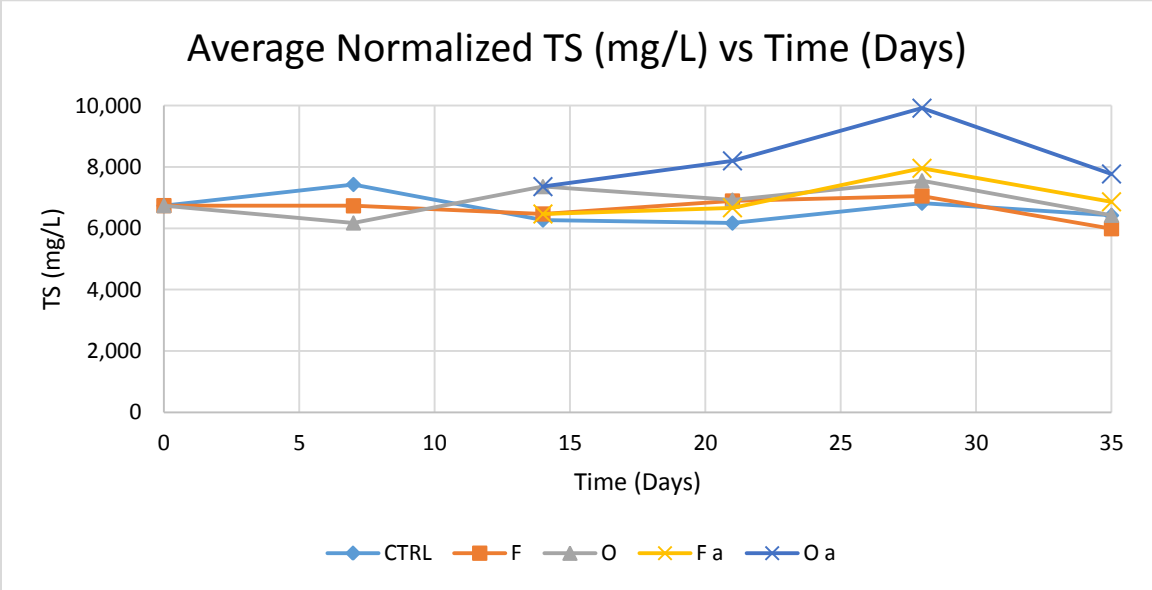
Experiment I



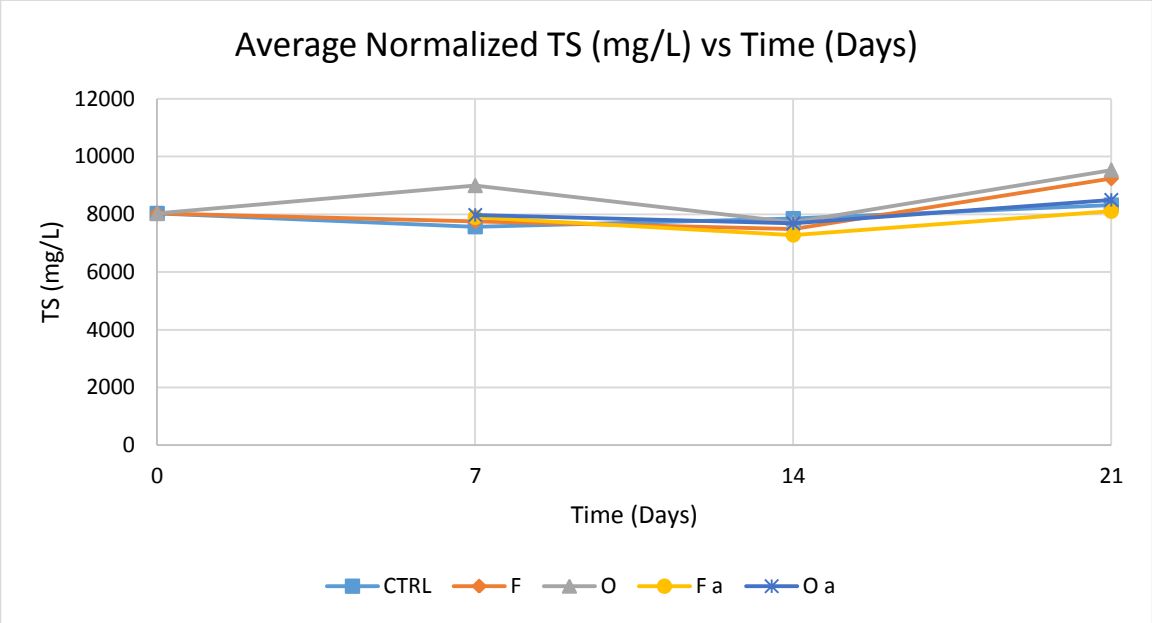
Experiment III



Experiment IV

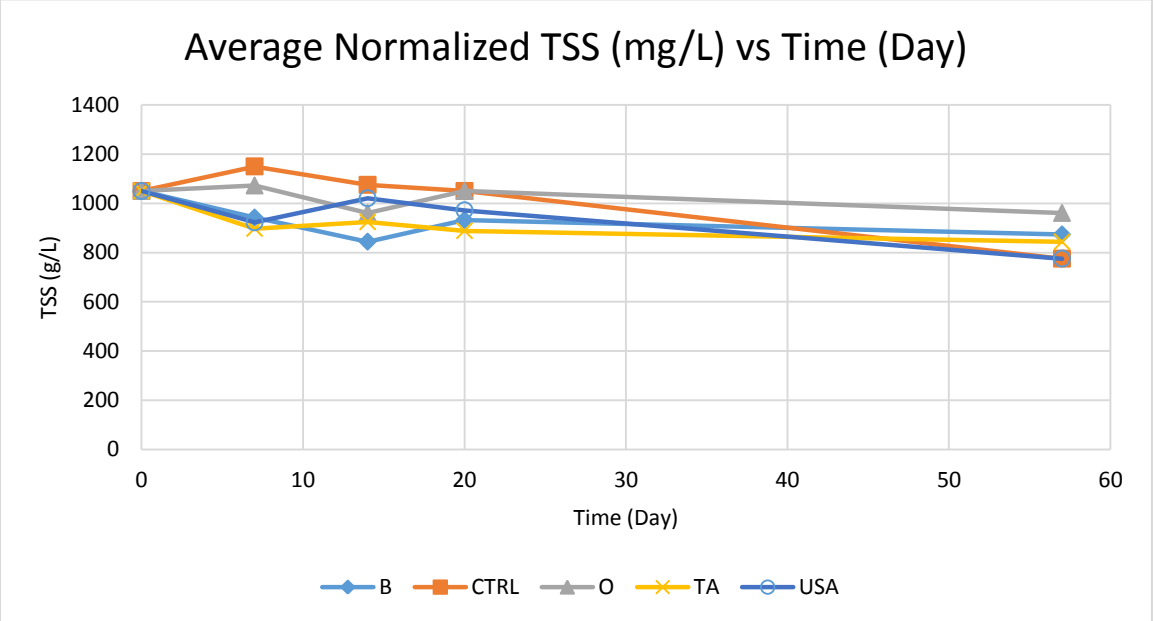


Experiment V

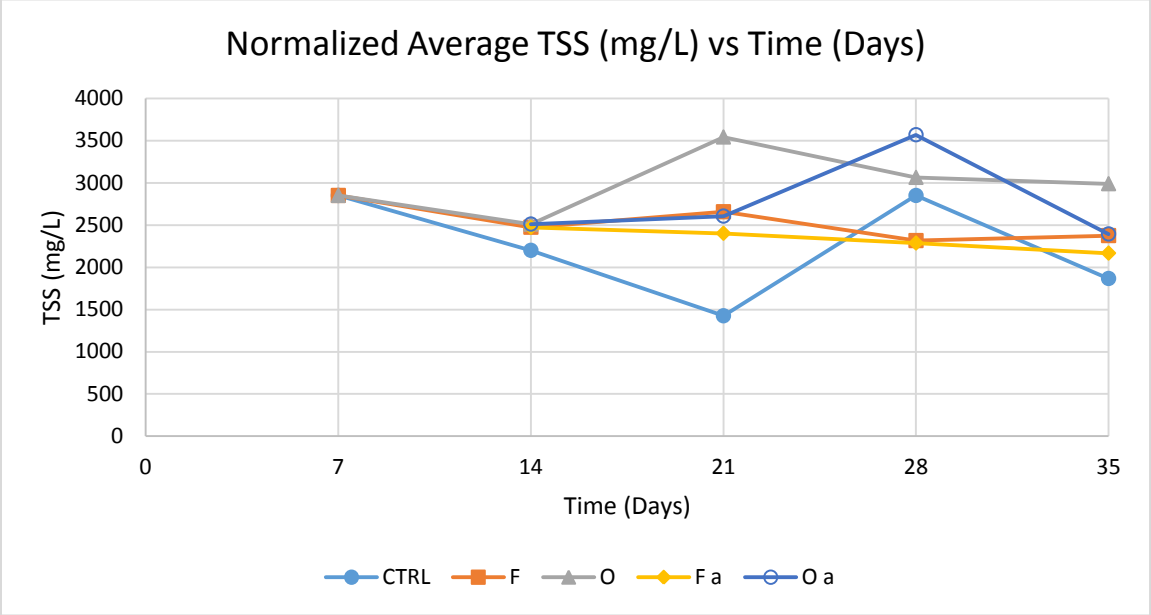


Appendix E: TSS Normalized Graphs

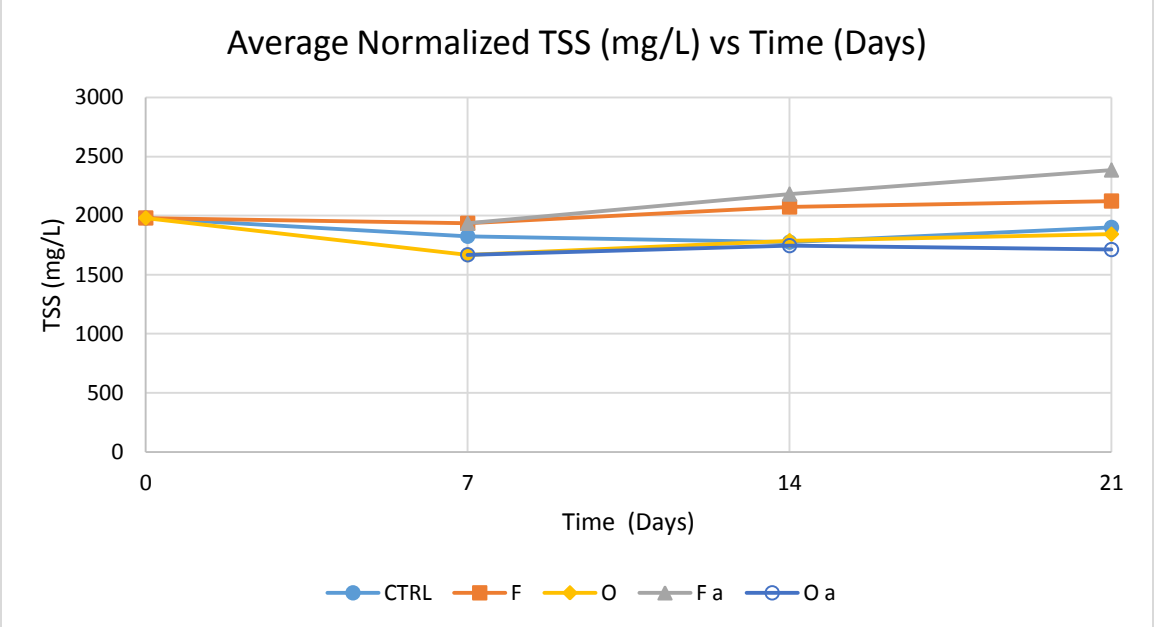
Experiment I



Experiment IV

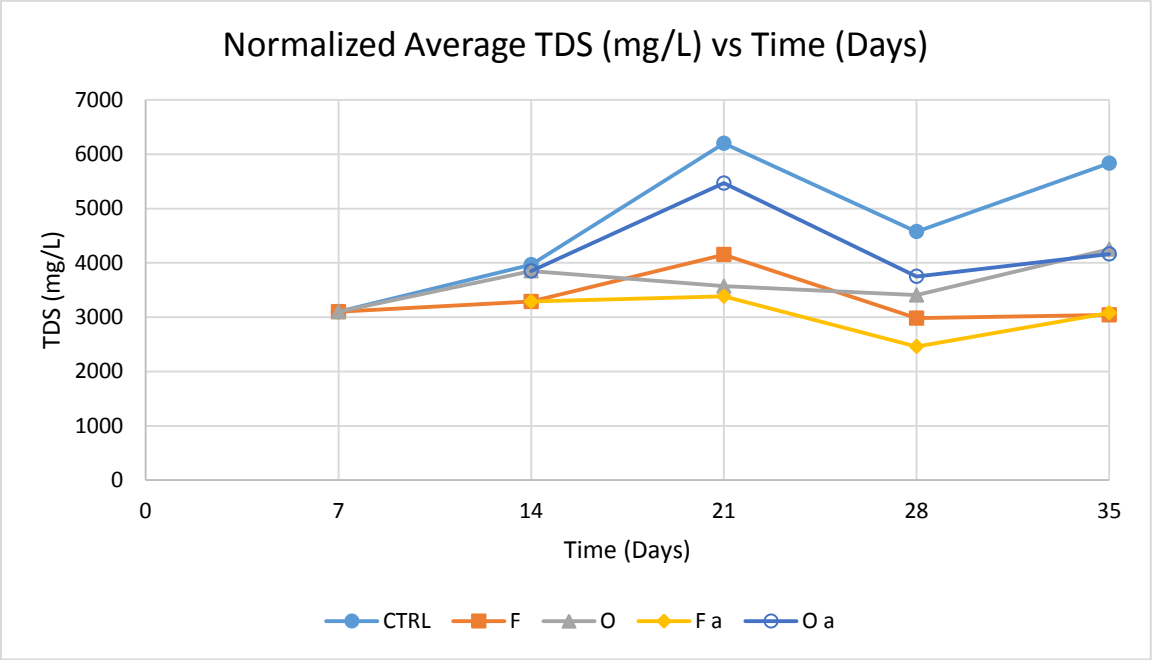


Experiment V

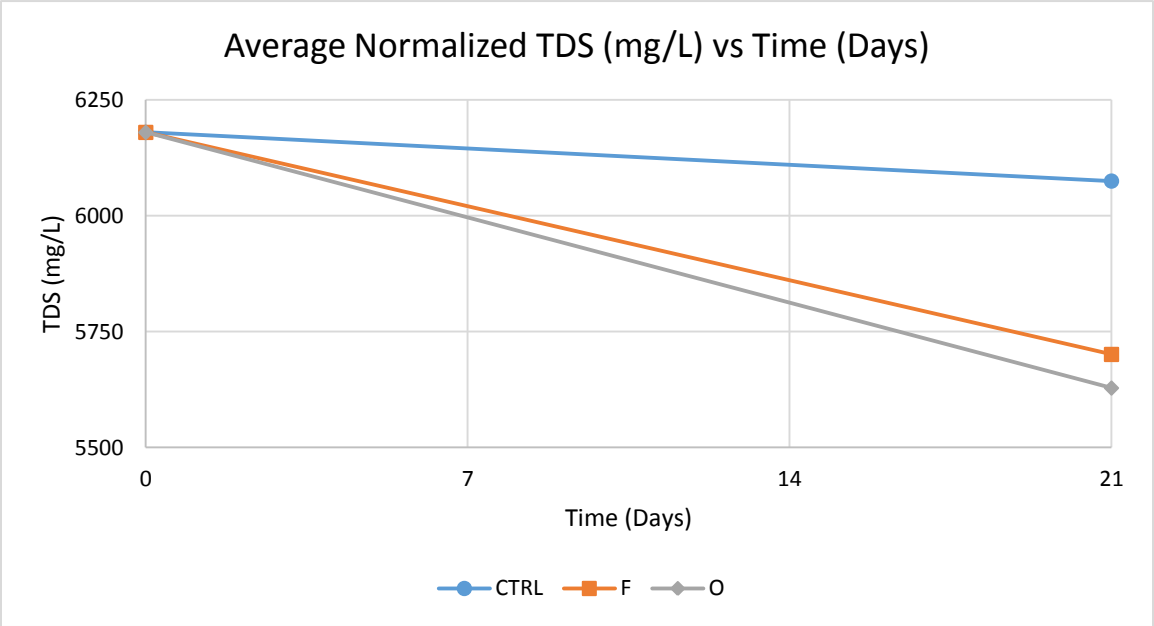


Appendix F: TDS Normalized Graphs

Experiment IV

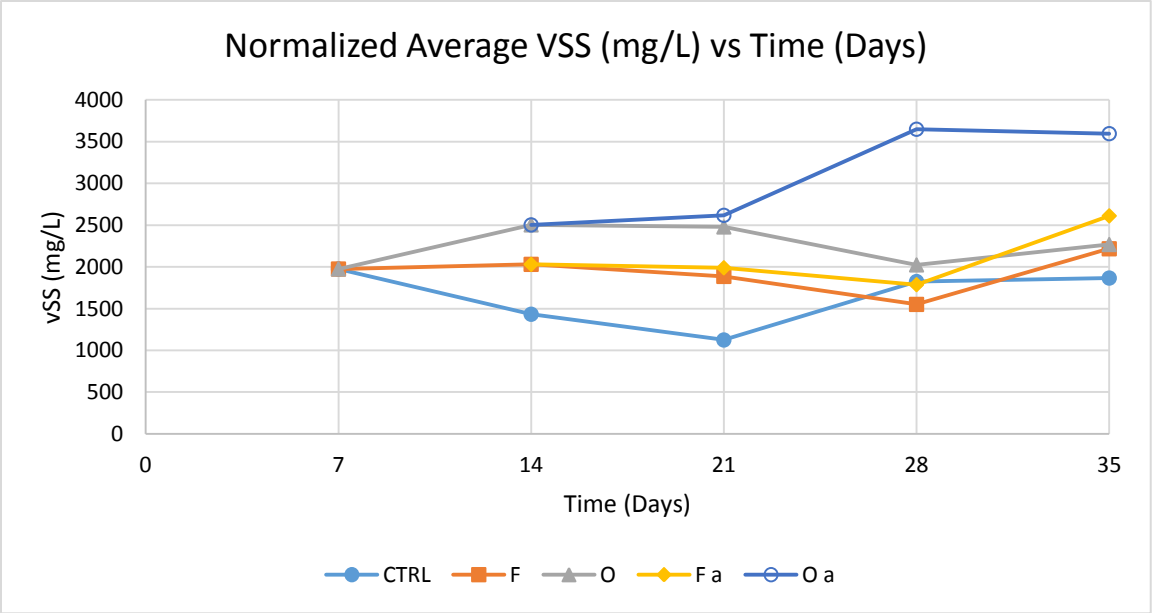


Experiment V



Appendix G: VSS Normalized Graphs

Experiment IV



Experiment V