CLARIFICATION OF RECREATIONAL POOL WATER USING BIOLOGICAL ADDITIVES PRODUCED BY BIOWISH™

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Master of Science in Civil and Environmental Engineering

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ABSTRACT

Clarification of Recreational Pool Water using Biological Additives Produced by BiOWiSH™

Reese Nathaniel Wilson

Effects of commercially available bacterial products were investigated on two common recreational pool contaminants: sunscreen and cyanuric acid (CYA). Microbial products developed by BiOWiSH Technologies, Inc. were tested for enhancing mechanical filtration and water clarification in bench-scale bioreactors, with conditions mimicking those of recreational pool water. Bacterial consortia included proprietary mixes of *Bacillus, Lactobacillus* and *Pseudomonas,* and other genera of bacteria. BiOWiSH products are either fermented on a solid substrate consisting of rice bran and soy meal, or they are mixed with a soluble diluent. Twenty-nine BiOWiSH products were tested throughout forty experiments.

Experiments were carried out to determine both the efficacy of BiOWiSH products for turbidity reduction and the mechanism by which BiOWiSH removes sunscreen from solution. In trials without mechanical filtration, the only product which showed a reduction in turbidity relative to the control, albeit inconsistently, was the solid substrate version of BiOWiSH Aqua FOG™ (Thai FOG). Experiments on BiOWiSH coupled with mechanical filtration showed a 79% average reduction of turbidity in the first 24 hrs. BiOWiSH products containing solid substrate, both active and abiotic, showed an average turbidity reduction of 90% in the first 24 hrs. In the same timeframe, soluble BiOWiSH products

showed a 79% average reduction in turbidity. Thus, the solid substrate provided an additional 11% reduction in turbidity over soluble products and un-amended mechanical filtration. Through experimentation and scanning electron microscopy, it was concluded that the primary mechanism of clarification by the solid substrate is adsorption of sunscreen to the substrate surface.

Further experiments were performed in anaerobic and aerobic environments to determine whether BiOWiSH products can remove cyanuric acid from solution through adsorption or biodegradation. Two measurement methods, turbidimetric and HPLC (high performance liquid chromatography) were used to independently quantify CYA. A reverse-phase HPLC method was developed which utilizes a phosphate buffer and methanol for the separation of cyanuric acid from nitrate and other chemical species. The solid BiOWiSH Aqua FOG product (prod. in Thailand) interfered with the turbidimetric analysis, showing false decreases in CYA. Using HPLC, there was no measureable biodegradation or adsorption of CYA by BiOWiSH products in these bench-scale tests. Significant systematic error in the HPLC analysis prevented conclusive findings; therefore, the ability of BiOWiSH products to reduce CYA from solution remains inconclusive.

Keywords: Bioremediation, BiOWiSH Technologies Inc., Recreational Pool, Swimming Pool, Chlorine, Sunscreen, Turbidity, Cyanuric Acid, HPLC

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1 – INTRODUCTION

The purpose of this research is to investigate the removal of sunscreen and cyanuric acid from swimming pool water by commercially available microbiological products. BiOWiSH Technologies, Inc. provided all bacterial consortia used in these experiments. Based in Cincinnati, Ohio, BiOWiSH is a company dedicated to developing, researching, and manufacturing innovative biological solutions to issues in wastewater, solid waste, agriculture, aquaculture, and recreational pools. Within the recreational pool industry, BiOWiSH products have been used for water clarification and are reported to reduce cyanuric acid levels.

A sufficient chlorine concentration (at least 1-2 mg/L Cl_2 or equivalent) must be maintained in recreational pools to ensure sufficient inhibition of bacteria and protozoa. Hypochlorite (OCl^-) is unstable under UV radiation and must be added in the form of chlorinated isocyanurates. After reacting with oxidizable material, chlorinated isocyanurates release CYA as a stable byproduct. As chlorine is expended and re-dosed, CYA accumulates in swimming pools. While CYA enhances the longevity of residual chlorine, it inhibits the bactericidal effects of chlorine at high concentrations (Shields et al. 2009). Additionally, the only current method for removal of CYA from swimming pools is through draining and dilution with clean water (Pennsylvania Dept. of Public Health 2015). Biodegradation of CYA has been studied extensively, although not in the context of recreational pools.

Leading up to the onset of this research, a third-party pool operator set up a rudimentary test investigating the clarification of sunscreen by the BiOWiSH Aqua FOG product in an aquarium. An observed drop in turbidity led investigators to begin using the product in routine pool maintenance. This research was started validate BiOWiSH products' abilities to clarify swimming pool water. As preliminary research was carried out, the scope and aims of the project evolved into a robust investigation of water clarification. The scope of work was set out as follows:

Specific Tasks

Task I – Determination of Clarification Mechanism

Task II – Efficacy of BiOWiSH Products Clarifying Swimming Pools

Task III – Isolation and I.D. of Microbes Which Survive in Chlorinated Environments

Task IV – Investigation of Biodegradation of Cyanuric Acid

The first task, Task I, sought to determine the mechanism by which BiOWiSH products clarify turbidity from swimming pools. Nine experiments were carried out using heated 10-gallon aquaria filled with chlorinated tap water and fitted with filtration systems to mimic conditions in recreational pools. Sunscreen was used as the sole source of turbidity, as it provided an easily replicable emulsion of oils and carbonaceous material. A wide array of BiOWiSH products were tested in these aquaria against un-amended mechanical filtration. Different combinations of the products' components were tested, including solid substrate products, soluble products, isolated microorganisms, and abiotic solid substrates.

Task II sought to quantify the abilities of various BiOWiSH products at reducing turbidity from chlorinated and non-chlorinated environments without the assistance of mechanical filtration. Nine experiments were carried out to investigate the effects of BiOWiSH products on mechanical filtration of turbidity. Experiments included a UV absorbance calibration curve for sunscreen, determining the extent of chlorine scouring caused by various growth media, determining the rates of chlorine scouring by each BiOWiSH product, testing the influence of settling on turbidity reduction, and investigating the effects of BiOWiSH products on turbidity using dextrose versus sunscreen as carbon sources.

Task III investigated the types and quantity of bacteria present in BiOWiSH products after use in clarification experiments. Samples were plated to investigate the most probable number of Colony Forming Units (CFU), as well as colony morphology, and to isolate individual bacterial species for staining and classification. Due to time constraints, only two experiments were run under this task. One of the experiments became contaminated, leaving just one viable set of data.

Task IV investigated the effects of BiOWiSH products on CYA. This task proved to be the most extensive and challenging, due to highly varied results of CYA measurement from different methods. The first experiment failed due to repeated respirometer malfunctions, and has been omitted. The second through seventh experiments under this task utilized a turbidimetric method of CYA analysis, and a false reduction of CYA was seen due to suspected interference on measurement by solid substrate products. An HPLC method was developed and refined, over seven months, to resolve CYA peaks from those of nitrate and

other chemicals using a phosphate buffer and methanol. HPLC analysis showed no reduction in CYA by any means, including experiments which had previously shown a reduction in CYA through turbidimetric analysis.

CHAPTER 2 – BACKGROUND

2.1 Swimming Pools and Contaminants

There are 14.4 million residential swimming pools and hot tubs and 309,000 public recreational pools in the United States, reported by the CDC in 2013 (CDC 2014, P.K. Data, Inc 2013). Additionally, swimming is the fourth most popular recreational sport, with over 300 million visits to swimming venues nationwide in 2009. There are many health benefits associated with swimming, such as low-impact cardiovascular exercise which is not aggravating to joints, and activity that engages almost every muscle in the body. In order to maintain a healthy environment for swimmers, municipalities have enacted recreational water quality regulations.

Recreational pool water quality is regulated in California by Title 22 Standards (California Code of Regulations 2015). Water clarity is an important indicator of the cleanliness of a recreational pool. Regulated contaminants of swimming pools include dirt, debris, scum, oils, organic and inorganic material (WHO 2006). Almost all recreational pools are fitted with filtration systems which provide predominantly mechanical removal of the above-mentioned contaminants (Linhart 2014). Microbial growth in recreational pool water is slowed by chlorination, preventing significant growth. Microorganisms in the pool water are captured in the filtration system and can potentially biodegrade regulated contaminants within the pool.

In addition to mechanical filtration, chemical control of bacterial contaminants is important for maintaining clear pool water and preventing infection in pool-users. The most common

method of bacterial inhibition in recreational pools is chlorination. Chlorine, when added in the forms of chlorine gas (Cl_2), hypochlorite (OCl^-), or chlorine dioxide (ClO_2), readily oxidizes organic and inorganic contaminants (Cooke 2000).

In California, public swimming pools are governed by Title 22 standards within the California Code of Regulations. Pool operators are required to maintain conditions in which the bottom of the deepest area of the pool can be clearly seen from the pool deck (California Code of Regulations 2015). Public pools must also maintain pH levels between 7.2 and 7.8. Regulated contaminants of swimming pools include dirt, debris, scum, oils, organic and inorganic material (WHO 2006). Heterotrophic bacterial Standard Plate Count is limited to 200 Colony Forming Units (CFU) per mL, and Total Coliform are limited to 2.2 CFU per 100 mL. Disinfectants and their byproducts are also regulated by Title 22.

In the 2008 report on swimming pool water quality compiled by the Centers for Disease Control (CDC), violations were categorized as Serious, Water Quality, or Policy and Management. Disinfectant level violations were given their own sub-category under water quality. Cyanurate violations were compiled with algae and bacterial quality among others under "Other Water Chemistry." Of the 121,000 pools sampled for disinfectant levels by the CDC, 10.7% were in violation. Of the 99,000 pools sampled for Other Water Chemistry issues, 12.5% were in violation (CDC 2010). Since the Other Water Chemistry issues category only required one of nine criteria to fail, it is impossible to discern the prevalence of cyanurate-related violations.

One major contaminant in swimming pools is sunscreen, which is commonly used to protect against skin cancer caused by UV radiation during outdoor activity. Sunscreen is ubiquitous in outdoor swimming pools, and contains many oils and UV-blockers which contribute to water turbidity. The inactive ingredients in sunscreens contribute a large amount of oils to recreational pools, in the forms of ethylhexyl palmitate, Bis-Stearyl Ethylenediamine/Neopentyl Glycol/Stearyl Hydrogenated Dimer Dilinoleate Copolymer, retinyl palmitate, and other fatty acids (DailyMed 2012).

2.1.1 Cyanuric Acid

Cyanuric Acid (CYA, $C_3H_3O_3N_3$), a common chlorine stabilizer against ultraviolet (UV) degradation, is a byproduct which enters outdoor swimming pools in the form of chlorinated isocyanurates. Stabilized chlorine can be obtained in three forms: monochloroisocyanuric acid (monochloro-s-triazinetrione acid), dichloroisocyanuric acid (dichloro-s-triazinetrione acid), and trichloroisocyanuric acid (trichloro-s-triazinetrione). Chlorinated isocyanurates have a high resistance to UV degradation, because their UV wavelength absorbance maxima are below 220 nm, and any UV radiation below 290 nm is absorbed by the atmosphere. Hypochlorite has an absorbance maximum of 290 nm with a spectrum that extends out to around 350 nm. Therefore, hypochlorite is unstable in the presence of UV light, and the introduction of cyanuric acid enhances the longevity of outdoor pools' chlorine residuals.

Although it is an ineffective bactericide, the monochloroisocyanurate ion is beneficial to disinfection. It acts as a reservoir of hypochlorite which can add hypochlorous acid to the

system on-demand (Wojtowicz 2001). It is generally agreed that 25-30 mg/L CYA is required before proper chlorine stabilization is achieved. Alternately, the presence of cyanuric acid has been shown to inhibit the bactericidal effects of. Wojtowicz also noted that there is a strong positive correlation between the concentration ratio of total cyanuric acid to total free available chlorine and the kill time of 99% of a population of *S. faecalis.*

$t_{0.99}$ (pH 7 and 20°C) = 0.119 + 0.0516Cy_T/Cl_T, r^2 = 0.98

At 50 mg/L CYA, hyperchlorination with 20 mg/mL free Cl proved insufficient at obtaining 3-log removal of oocysts in fecal-contaminated swimming pools. After 10 hours of hyperchlorination in the presence of CYA, only 0.7-$\log_{10}$ removal of oocysts was achieved, compared to the 3.7-$\log_{10}$ removal without CYA (Shields et al. 2009). From a disinfection standpoint, the need to manage CYA levels in swimming pools is apparent.

As chlorinated isocyanurates react with biological and other pool contaminants, cyanuric acid is left behind as a very stable byproduct. Title 22 standards call for no more than 100 mg/L cyanuric acid in pools and that the water appear “clean and clear” (California Code of Regulations 2015), because cyanuric acid contributes adversely to turbidity. Every mole of dichloroisocyanuric acid in a pool liberates one mole of cyanurate ions when the released hypochlorite ions react with contaminants. Assuming a daily dose of 1 mg/L hypochlorite, in the form of dichloroisocyanuric acid, and assuming no degradation of cyanuric acid, every swimming pool using dichloroisocyanuric acid will be in violation of the standard after 15 weeks of chlorination. The only viable method of reducing cyanuric acid

concentration is to dump pool water and dilute with clean water (Pennsylvania Dept. of Public Health 2015), which is wasteful and costly to domestic and professional operators.

Cyanuric acid, a white odorless solid, is inactive within the human body passing through the renal system of swimmers without loss of concentration (Zwiener et al. 2007). Concern has arisen regarding the nephrotoxicity (kidney toxicity) of cyanuric acid and melamine (a precursor to cyanuric acid in synthesis) in combination. When ingested together, cyanuric acid and melamine form kidney stones of melamine cyanurate crystals, which can lead to renal failure. In 2007, a crisis emerged in the pet food industry, when a producer added melamine to dog and cat food. Melamine raises the apparent protein content of food samples during analysis, due to its high nitrogen content. Contamination of the melamine with cyanuric acid led to renal failure and the death of up to 36,000 dogs and cats in the United States. The contamination led to the largest FDA recall to date (Rovner 2008).

2.1.2 Clarification Mechanisms

The two main possible pathways of swimming pool clarification are mechanical and biological. Mechanical filtration denotes the filtration mechanism of a pool and incorporates physical separation techniques. Biological filtration includes microbial conversion of contaminants to biomass via metabolism and extracellular enzymatic activity.

2.1.2.1 Mechanical Clarification

There are three main types of pool filters: sand, cartridge, and diatomaceous earth (DE). Sand filters are the cheapest and easiest filters to maintain; however, sand provides the least filtration of water, only removing particles larger than 30 µm (Linhart 2014). Cartridge filters provide good water quality, excluding particles as small as 10 µm (Linhart 2014). They require minimal maintenance and no backwashing, but filter cartridges must be changed out periodically (on the order of 1-3 years). DE filters, provide the best water quality, removing particles as small as to 3-5 µm (Linhart 2014). DE filters require the highest capital investment of the three options, and are the most labor-intensive to maintain.

2.1.2.2 Biological Clarification

Due to the complex composition of commercial sunscreens, there are many possible biodegradation pathways. Inactive ingredients such as sorbitol are readily degraded by many microorganisms (Caspi et al. 2014). The biological degradation of UV blockers in sunscreen is most feasible by fungi such as the white rot fungus *Trameces versicolor* (Badia-Fabregat et al. 2012).

Many papers studying the biodegradation of the s-triazine ring, and CYA, have been published, due to the prevalence of cyclic s-triazine in pesticides. Cyanuric acid is a key intermediate between cyclic s-triazine pesticides and ammonia. CYA is biodegraded via hydrolysis, ultimately producing CO_2 and ammonia. The degradation of CYA produces no primary BOD; however nitrogenous BOD is added in the form of ammonia. Each mole of

metabolized CYA liberates 3 moles of ammonia, by the pathway shown in **Figure 2-1**. It was widely accepted that urea was an intermediate CYA metabolite; however, an extensiveFstudy determined that common analytical methods forced allophanate to decarboxylate into urea which was not present from metabolism (Cheng et al 2005).

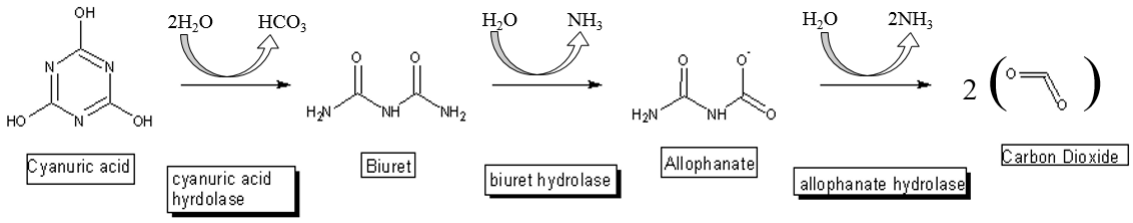


Figure 2-1: Cyanuric Acid Degradation Mechanism (Kotharu 2014)

Biodegradation of cyanuric acid in aqueous systems is possible, especially at low or no dissolved oxygen. While bacteria which degrade CYA proliferate in both aerobic and anaerobic environments, CYA degradation itself only occurs in anaerobic environments (Saldick 1974). Cyanuric acid removal can be obtained at 1-3 mg/L of dissolved oxygen in activated sludge systems with a solids retention time of at least 6 hrs. Systems that have high dissolved oxygen also show CYA reduction, but only in localized anaerobic zones (Saldick 1974). The greatest natural CYA removal occurs in activated sludge systems or mud and muddy creeks. Comparing results from lake water and water containing mud, it is apparent that CYA degradation occurs in the soils and sediment, rather than the water (Saldick 1974). Degradation has also been demonstrated in solutions of 3.5% NaCl. Saldick noted that the addition of glucose speeds up the degradation process. Saldick also noted a lag time of no more than a few minutes between a system turning anaerobic and the resulting increase in biodegradation of CYA. Doubling the concentration of CYA

decreased the rate of degradation of tracer CYA, showing degradation as kinetically not first-order.

2.1.3 Types of Bacteria Used

BiOWiSH Technologies, Inc. produces bacterial consortia with compositions found in **Table 2-1**. Many of the products are proprietary, and only the genera of bacteria are available for publication.

Table 2-1: List of Products Tested

Product Name	Abbreviation	Bacterial Species	Composition
BioCure Microbial Technologies (BMT) Wastewater	BMT WW (BMT WW1 and BMT WW2 are two different batches of BMT WW)	Mix of 6-8 *Bacillus*	*Bacillus* spores, Soluble Diluent
BioCure Microbial Technologies (BMT) Remediate	BMT Remediate (BMT SS)	Mix of *Rhodococcus* and *Arthobacter*	Undisclosed
BiOWiSH Fruit and Vegetable Wash	Fruit Wash	Mix of *Bacillus* and *Lactobacillus*	*Bacillus* spores, Lactobacillus, Soluble Diluent
BiOWiSH Lactic Mix 1	LCM	Mix of *Lactobacillus*	Bacteria, Soluble Diluent
OBAAG-KLB 30	KLB	Single *Bacillus* species	Bacillus spores, Diluent
Microbial Discovery Group (MDG) Waste Water Treatment	MBWWT#1	Mix of *Bacillus*	Salt, *Bacillus* spores

Product Name	Abbreviation	Bacterial Species	Composition
Osprey Biotechnics Waste Water Treatment	OBWWT#1 (Osprey WW)	Mix of *Bacillus*	Undisclosed
BiOWiSH Aqua (Produced in Thailand)	Thai Aqua	Mix of *Bacillus* and *Lactobacillus*	*Bacillus* spores, vegetative bacteria, Rice Bran and Soy meal
BiOWiSH Aqua FOG (Produced in Thailand)	Thai FOG	Mix of *Bacillus* and *Lactobacillus*	*Bacillus* spores, vegetative bacteria, Rice Bran and Soy meal
BiOWiSH Aqua FOG (Produced in Thailand, Irradiated)	Irradiated Thai FOG, (Thai Fog I, IRTF)	Mix of *Bacillus* and *Lactobacillus*, Inactivated by Irradiation	Irradiated *Bacillus* spores, Irradiated bacteria, Rice Bran and Soy meal
Microbial Discovery Group (MDG) Hydrocarbon Remediation Product	MDG Petro	Mix of *Bacillus* and *Pseudomonas*	*Bacillus* spores, vegetative bacteria, soluble diluent
Osprey Biotechnics Hydrocarbon Remediation Product	BPB-100	*Pseudomonas*	Undisclosed
Osprey Biotechnics Hydrocarbon Remediation Product	MPB-5 (Osprey L, Osprey Liq)	Mix of *Bacillus* and *Pseudomonas*	*Bacillus* spores, vegetative bacteria, soluble diluent
BiOWiSH Manure and Odor Treatment, Swine	Manure/ Odor	Undisclosed	1-10% bacteria
Microbial Discovery Group Micronutrient Mix	MDG Micro-N	Abiotic	Undisclosed
BiOWiSH Aqua (Produced in USA)	US Aqua	Mix of *Bacillus* and *Lactobacillus*	*Bacillus* spores, vegetative cells, soluble diluent

Product Name	Abbreviation	Bacterial Species	Composition
BiOWiSH Aqua - Fats, Oils, and Grease (Produced in USA)	US FOG	Mix of *Bacillus* and *Lactobacillus*	*Bacillus* spores, vegetative cells, soluble diluent, emulsifier
BioCure Microbial Technologies Prototype Waste Water Product 001	AP001	Mix of *Bacillus*	*Bacillus* spores, Rice Bran and Soy Meal
BioCure Microbial Technologies Prototype Waste Water Product 002	AP002	Mix of *Bacillus*	*Bacillus* spores, Rice Bran and Soy Meal
BioCure Microbial Technologies Prototype Waste Water Product 003	AP003	Mix of *Bacillus*	*Bacillus* spores, Rice Bran and Soy Meal
BioCure Microbial Technologies Prototype Waste Water Product 004	AP004	Mix of *Bacillus*	*Bacillus* spores, Rice Bran and Soy Meal
Biosource Prototype Lactic Mix 001	BS-AQ-001	Mix of L*actobacillus* and microbial metabolites	Vegetative cells, metabolites, Rice Bran and Soy Meal
Biosource Prototype Lactic Mix 002	BS-AQ-002	Mix of *Lactobacillus* and microbial metabolites	Vegetative cells, metabolites, Rice Bran and Soy Meal
Biosource Prototype Lactic Mix 003	BS-AQ-003	Mix of *Lactobacillus* and microbial metabolites	Vegetative cells, metabolites, Rice Bran and Soy Meal
BiOWiSH Premix (Thailand)	Premix	Mix of *Lactobacillus*	Vegetative cells, Rice Bran, Soy Meal
BiOWiSH Crop	Crop	Undisclosed	Undisclosed

Product Name	Abbreviation	Bacterial Species	Composition
Rice Bran used in Thai FOG Production	Thai Rice Bran	Undefined	Rice Bran
Riceland Rice Bran	US Rice Bran	Undefined	Rice Bran
BiOWiSH Cyanuric Acid Reducer	CAR	Mix of *Bacillus* and *Lactobacillus*	Bacterial spores, vegetative cells, soluble diluent

2.2 Parameters Tested and Quantification Methods

Section 2.2 discusses specific parameters used in this study and the theory behind the parameters' quantification.

2.2.1 Turbidity

Turbidity is a measure of water clarity which denotes the amount of light occluded from passing through a solution by suspended particulate matter. Turbidity measurement does not necessarily measure the amount of solids in a solution. Particle sizes affecting turbidity range from 0.2 µm to 1.0 mm (EPA 2012). The two most common units of turbidity are Formazin Attenuation Units (FAU) or Nephelometric Turbidity Units (NTU), depending on the angle at which the detector sits and the wavelength of the incident beam. NTU is measured perpendicular to the incident light angle with a white light source, whereas FAU is measured in-line with an infrared light source. When compared to a Formazin standard solution, NTU and FAU units are analogous (HACH 2009).

Broad-spectrum sunscreens absorb ultraviolet light from 290-400 nm and higher. Sunscreen can be measured turbidimetrically in aqueous samples without additional sample preparation. No literature was found directly correlating sunscreen to turbidity, so a standard curve was developed as a part of this study based on optical density at 520 nm.

2.2.2 CYA Analysis: Turbidimetric and HPLC

CYA can be tested through turbidimetric and chromatographic analysis. The most common method for CYA analysis by recreational pool operators is turbidimetric, as kits are portable and cheap. Turbidity is induced in a sample from the addition of melamine and the resulting precipitation of melamine cyanurate (HACH 2009). Although it has an upper test limit of 100 mg/L, this method is inhibited by the solubility of melamine in water of 5-10 mg/L. Since a small amount of melamine will remain in solution rather than bond with CYA, analysis is prevented below 10 mg/L and accuracy is limited to +/- 10 mg/L. Inexpensive test kits utilize the "disappearing dot" analysis, similar to that of a Secchi disk, where a plunger is lowered into a sample, and the depth at which the plunger cannot be seen corresponds to a concentration. This method is subject to high variability introduced by user discretion.

More sophisticated turbidimetric methods, such as the one developed by HACH Company, utilize IR absorbance for precise measurement of absorbance from CYA precipitation reactions. HACH Method 8139 for CYA utilizes a mixture of monobasic and dibasic

potassium phosphate, and sodium sulfite to create a white precipitate in the presence of cyanuric acid (HACH 2009). The method has a detection limit of 7.0 mg/L CYA, and a standard deviation of +/- 1.2 mg/L was obtained by one HACH operator (HACH 2009).

An even higher level of precision in CYA measurement can be achieved through High Performance Liquid Chromatography (HPLC). HPLC utilizes selective adsorptive chemistry coupled with UV absorbance to separate, identify, and quantify chemical components within a liquid sample. Tran et al. (2010) achieved reversed-phase separation of melamine and cyanuric acid using a Dionex Acclaim Trinity P1 HPLC column using methanol and ammonium acetate buffer as eluents. In method development for this thesis, multiple iterations of ammonium acetate buffers and potassium phosphate buffers with methanol were investigated. A method was adapted from a paper by Cantú et al. (2001) in which a porous graphitic carbon column was used with 50 mM dibasic potassium phosphate and methanol to resolve the CYA peak from that of nitrate.

2.2.3 Chlorine: Free vs Total Cl Colorimetric

Chlorine is typically measured in three forms in aqueous systems: free chlorine, combined chlorine, and total chlorine. Free chlorine is a combined concentration of chlorine gas (Cl_2), hypochlorous acid (HOCl), and the hypochlorite anion (OCl^-). Combined chlorine is defined as the residual chlorine bound to organic amines and ammonia in the form of chloramines. Combined chlorine is unavailable for disinfection. Total chlorine is the sum of free and combined chlorine (CDC 2009).

Chlorine was measured using a HACH DR/890 Colorimeter and HACH Method 10070. "The combined chlorine oxidizes iodide in the reagent to iodine. The iodine reacts with DPD (N,N-diethyl-p-phenylenediamine) along with free chlorine present in the sample to form a pink color which is proportional in intensity to the total chlorine concentration" (HACH 2009).

2.2.4 Surface Structure SEM

Scanning electron microscopy (SEM) provides insight into a sample's surface topography by focusing a beam of electrons on the sample in a high vacuum system. SEM imaging can attain resolution on the sub-nanometer scale, between 10x and 500,000x magnification.

Biological samples are prepared via chemical fixation or freeze-drying, to prevent the introduction of unwanted moisture to the SEM system. Non-conductive samples are typically sputter-coated with a conductive material such as gold, gold/palladium alloy, platinum, or others. Coating masks the immediate surface structure of a sample, and is not desirable when investigating delicate organic samples. An SEM can be run at a low vacuum of 6-270 Pa (Ou and Duan 2005). At low vacuum conditions, uncoated insulative materials are able to discharge excess electrons to surrounding gas particles, preventing undesirable surface charging and scorching.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Generalized Tests and Experimental Setup

Section 3.1 details the materials and methods for tests used across experiments in this study.

3.1.1 Experimental Setup

Bioreactor General Materials:

- 10-gallon Aquaria
- Aquarium Filter (Tetra Whisper PF10, activated carbon removed)
- Aquarium Thermometer Strips
- Heating Elements
- 250 mL Screw-Top Shaker Flasks
- 500 mL Screw-Top Shaker Flasks
- Shaker Flask Caps With Removable Septum
- Shaker Flask Caps, Silicone, Breathable
- Tap Water
- DI Water With Squirt Nozzle
- Sunscreen (Coppertone Sport SPF 30)
- Bacterial Consortia
- Glass Funnel
- Magnetic Stir Bar

- Magnetic Stir Bar Remover

Growth Media Components:

- Miracle Gro® 20-20-20 Fertilizer
- K_2HPO_4
- KH_2PO_4
- Glucose/Dextrose
- KNO_3
- $FeSO_4$
- $CaCl_2$
- MRS Broth
- Potato Dextrose Broth (PDB)

Bioreactor Inoculation and Sampling:

- 10-25 mL Serological Pipettes
- Plastic Weigh-Boats
- 200 µL Pipette Tips
- 1000 µL Pipette Tips (1 per day of chlorination)
- Clorox Concentrated Bleach
- Electronic Balance
- 100-1000 µL Autopipette
- Light-Duty Chemistry Wipes
- 1000 mL Beaker For Liquid Waste

- 250 mL Beaker for Solid Waste
- Laboratory Notebook

3.1.2 Turbidity

Turbidity levels tested in this study were less than 200 NTU. This was within the acceptable range of 0-1000 NTU for HACH DR/890 Colorimeter using HACH method 8237. No dilution was necessary.

Turbidity Materials:

- DR/890 Colorimeter (1)
- 10-25 mL cylindrical sample cell with cap (2)
- Serological pipette (1 per sample)
- DI Water
- 70% Ethanol in DI Water
- Light-Duty chemistry wipes

Turbidity Procedures:

Before sampling, put on proper Personal Protective Equipment (PPE), including laboratory gloves to prevent smudging of the sample cell.

Zeroing the Instrument:

- A clean sample cell was filled with a DI water blank (DI cell).
- The DR/890 colorimeter was turned on.
- The exterior of the DI cell was wiped clean with a light duty wipe. The DI cell was loaded into the chamber of colorimeter and covered with the lid. The orientation of the DI cell was noted.
- PGRM 95 was entered.
- The ZERO button was pressed, zeroing the instrument, showing 0 NTU before continuing.

Reading Turbidity:

1. The instrument was zeroed to a DI blank, as seen above.
2. The sample cell was filled with 70% ethanol, capped, and shaken vigorously.
3. If a yellow color was noted in the ethanol after shaking, step 2 was repeated.
4. The sample cell was filled with 5-25 mL of DI, capped, and shaken vigorously. Waste was discarded in a proper receptacle.
5. Step 4 was repeated 3 times.
6. The sample cell was filled with 5-25 mL of sample, swirled and discarded.
7. The sample cell was then filled with at least 5 mL of sample to be measured.
8. A light-duty wipe was used to clean the exterior of the sample cell. The sample cell was then loaded into the chamber of colorimeter, and covered with the lid.
9. The READ button was pressed and turbidity was recorded as NTU.
10. Contents of the sample cell were discarded into a proper receptacle.

11. Repeat Steps 2-10 for each sample.
12. At the end of sampling, steps 2-4 were repeated for cleanliness.

3.1.3 Total Chlorine

The HACH DR/890 Colorimeter and HACH method 10070 were used to measure chlorine levels. Total chlorine concentrations tested in this study usually fell between 0 and 8 mg/L; however, some readings exceeded the upper limit of the test (10 mg/L). HACH methods allow for dilution of samples, as long as proper concentration correction is made after reading. All chlorine readings in this study were carried out using a 1:1 dilution. This was achieved by filling sample vials with double the recommended amount of sample before adding the reagent pack. All chlorine readings have been corrected in data tables and the body of this document.

Total Chlorine Materials:

- DR/890 Colorimeter (1)
- DPD Total Chlorine High Concentration reagent pillow packs (1 per sample)
- 10-25 mL cylindrical sample cell with cap (1)
- Serological pipette (1 per sample)
- DI Water
- 70% Ethanol in DI Water
- Light-Duty chemistry wipes

Total Chlorine Procedure:

Before sampling, put on proper PPE, including gloves to prevent smudging of sample cell exterior.

1. A sample cell was filled with 70% ethanol, capped, and shaken vigorously. Ethanol was poured into waste container.
2. If a yellow color was noted in the ethanol after shaking, step 2 was repeated.
3. The sample cell was filled with 5-25 mL of DI, capped, and shaken vigorously. Waste was discarded in a proper receptacle.
4. 5-25 mL of sample were added to the sample cell, swirled and discarded.
5. The sample cell was filled with 10 mL of sample [double the recommended 5 mL].
6. The DR/890 colorimeter was turned on.
7. PGRM 12 was entered.
8. A light-duty wipe was used to clean the exterior of the sample cell. The sample cell was then loaded into the chamber of colorimeter, and covered with the lid.
9. The instrument was zeroed by pressing the ZERO button. Before sampling the screen would read 0.0 mg/L Cl_2.
10. The sample cell was removed from the colorimeter, and add the contents of one HACH DPD Total Chlorine High Range reagent pillow pack were added to the cell.
11. A 3-mintue reaction timer was started.
12. The sample was swirled vigorously until all reagent dissolved. Undissolved reagent does not interfere with the result of the test.

13. After the 3-minute reaction period, the sample cell was loaded back into the sample chamber with the same orientation as when it was zeroed. The sample cell was tapped gently to ensure that there were no bubbles in the sample.
14. The READ button was pressed, and data were recorded as mg/L Cl_2. Note: True Chlorine concentrations are double that of what the machine reads.
15. Waste was discarded into a proper receptacle.
16. Steps 1-15 were repeated for each sample.
17. At the end of sampling, steps 1-3 were repeated for cleanliness.

3.1.4 Cyanuric Acid Turbidimetric Measurement

The materials and methods for the turbidimetric analysis of CYA are outlined below. The method consists of sample cell cleaning, a precipitation reaction between melamine and cyanuric acid, and turbidimetric measurement using a HACH DR/890 Colorimeter.

Cyanuric Acid Materials:

- DR/890 Colorimeter (1)
- Cyanuric Acid 2 Reagent Powder Pillow (1 per sample)
- 25 mL cylindrical sample cell with cap (1)
- Serological pipette (1 per sample)
- DI WATER
- 70% Ethanol in DI Water
- Light-Duty chemistry wipes

Cyanuric Acid Procedure:

Experimenters used proper PPE, including gloves to prevent smudging of sample cell exterior.

1. A sample cell was filled with 70% ethanol, capped, and shaken vigorously. Ethanol was poured into a waste container.
2. If a color or cloudiness was noted in the ethanol after shaking, step 1 was repeated.
3. The sample cell was filled with 5-25 mL of DI, capped, and shaken vigorously. Waste was discarded in a proper receptacle. (repeat 3x)
4. The sample cell was loaded with 5-25 mL of sample, swirled, and discarded.
5. A sample cell was filled with 5-25 mL of sample and diluted to 25 mL. Dilution factors were recorded. (Note: the range of the test is 7-55 mg/L.)
6. The colorimeter was turned on.
7. PGRM 24 was entered.
8. A light-duty wipe was used to clean the exterior of the sample cell. The sample cell was then loaded into the chamber of colorimeter, and covered with the lid.
9. The instrument was zeroed by pressing the ZERO button. Before reading, the screen would read 0 mg/L CYACD.
10. The sample cell was removed, and the contents of one HACH Cyanuric Acid 2 Reagent Powder Pillow were added to the cell.
11. A 3-minute reaction timer was started.
12. The sample was swirled vigorously until all reagent dissolved, then the sample was left to rest for the remainder of the timer.

13. After the 3-minute reaction period, the sample cell was loaded back into the sample chamber with the same orientation as when it was zeroed. The sample cell was tapped gently to ensure that there were no bubbles in the sample.
14. The READ button was then pressed. Data were recorded (as mg/L CYA) and adjusted for dilution.
15. Waste was discarded into a proper receptacle, and the sample cell was quickly rinsed with DI water.
16. The interior of sample cell was scrubbed with a light-duty wipe if a white film formed.
17. Steps 1-16 were repeated for each sample.
18. At the end of sampling, the interiors of any used sample cells were cleaned with light-duty wipes and 70% ethanol followed by 3x DI rinse.

3.1.5 HPLC Analysis of CYA

The HPLC system used to measure CYA was an Agilent 1100 series with the components listed in **Table 3-1**. Chemstation software was used for data collection and analysis.

Table 3-1: HPLC Components

Component	Model Number	Serial Number
Vacuum Degasser	G1322A	JP63205331
Quaternary Pump	G1311A	US70601733
Autosampler	G1313A	US70201655
UV/Vis Detector	G1314A	JP64202932

Two columns used were the Dionex Acclaim Trinity P1 HILIC column, and Waters XBridge C18 column. Column details can be found in **Table 3-2**.

Table 3-2: HPLC Columns

Column	Mode	Particle Size	Column Dimensions	pH Range	Flow Rate	Max Pressure	Suggested Mobile Phase
Acclaim Trinity P1	Reversed Phase/ HILIC	5 µm	150 mm x 3 µm	2.5-7.0	0.3-1.5 mL/min	400 bar	NH_4Ac buffered AcN, or KH_2PO_4 buffered water
Waters XBridge C18	Reversed Phase	5 µm	100 mm x 3 µm	1-12	n/a	400 bar	KH_2PO_4 buffered water and methanol

The Waters XBridge C18 column was used in conjunction with a 2-cm Waters XBridge C18 guard column in the reversed phase mode. A method was developed, **Table 3-3**, which resolves the cyanuric acid peak from other nitrogenous species, specifically nitrate and nitrite.

Table 3-3: HPLC Parameters

Parameter	Value
Column	Waters XBridge C18
Mobile Phase	1% methanol 69.5% 50 mM KH_2PO_4 buffer (pH 5.70) in DI water 29.5% distilled water
Flow Rate	0.300 mL/min
Injection volume	1 uL

Detection wavelength	213 nm
Temperature	18-20^{o}C (ambient)
Sample Run Time	5 minutes

Since the HPLC system does not have a column heater, elution times and peak areas are subject to variations in room-temperature. After discovery of this issue, a four-point calibration curve, including a DI blank, was incorporated at the beginning of each sequence of samples.

Sample Collection and Preparation Materials:

- Serological pipette or graduated transfer pipette (1 per sample)
- 50 mL beaker (1 per sample + 1 additional)
- 3 mL syringe (1)
- Non-sterile syringe filter, 13 mm diameter, 0.2 µm pore-size, PVDF or nylon (1 per sample)
- 12x32 mm (2 mL), clear, crimp-top vial (1 per sample)
- 11 mm aluminum seal with TFE/rubber septum (1 per sample)
- GC/LC 11mm vial crimper
- DI Water
- 70% Ethanol in DI Water
- Permanent Marker

Syringe Cleaning Procedure:

1. A syringe was filled with Alconox soap solution, and the plunger was depressed 3-4 times over a waste container.
2. The syringe was then rinsed with DI water 3x, depressing plunger 3-4 times over waste container.
3. The plunger was drawn back fully, and remaining water was shaken out.
4. The plunger was depressed once more and blotted dry on a paper towel.
5. These methods were repeated after every unique sample.

Sampling Preparation Procedure:

1. Transfer pipettes were rinsed with 70% ethanol and stored in a beaker with tips submerged in ethanol.
2. At least 1.5 mL of sample were pipetted into a 50 mL beaker.
3. A 0.22 µm syringe filter was attached to a cleaned syringe, and at least 0.5 mL of sample were drawn through the filter.
4. The filter was removed and discarded into a waste receptacle.
5. Filtered sample was then loaded into a 2 mL crimp-top vial.
6. The vial was then capped, crimped, and labeled.

Column Flush Procedure:

1. Column flushing was performed before any set of samples was run. Flushing procedure was also run if pressure began drifting upwards, or if the baseline drifted.
2. Eluent was set to 0.7 mL/min; 95% methanol, 5% phosphate buffer for 15 minutes.

3. Eluent was set to 0.7 mL/min; 5% methanol, 95% phosphate buffer for 20 minutes.
4. Eluent was set to 0.7 mL/min; desired operating mobile phase for 15 minutes, or until steady baseline absorbance was reached.

3.1.6 Bacterial Plating

Section 3.1.5 details the materials and methods for bacterial plating and performing plate counting. All bacterial plating was carried out using Plate Count Agar at 35^{o}C. All materials and instruments were autoclaved before use, to ensure aseptic conditions. Since bacterial typically attach to each other and germinate into one indistinguishable colony, bacteria are typically enumerated as Colony Forming Units. The statistical significance of a plate count is between 30 and 300 CFU (Sanders 2012).

Bacterial Plating Materials:

- Petri dishes (about 50 dishes per 1 Liter of agar prepared)
- Dry Agar Mix (quantity varies by type)
- 2 Liter Erlenmeyer Flask
- Aluminum Foil
- Autoclave Tape
- Laboratory Labeling Tape
- Autoclave Tape
- DI Water
- Electronic Balance

- Laminar Flow Hood (optional UV lamp)
- Paraffin Wax

Bacterial Plating Procedures:

1. Dry Plate Count Agar was measured and add to a 2L flask.
2. 1 Liter of DI water was added to flask and swirled to dissolve media.
3. The flask was then covered with aluminum foil, and the foil was taped into place. (A gap was left gas to escape during autoclaving.)
4. The media was autoclaved for 20 minutes at 121^{o}C and 15-20 psig.
5. Petri dishes were placed in a laminar flow hood, and the UV lamp was turned on for 15 minutes.
6. The flask of agar was then placed in a 55^{o}C bath plates were ready to be poured.
7. One Petri dish was partially uncovered, to avoid contamination, and agar was gently poured into the plate until the entire bottom of the plate was covered in agar.
8. The plate was capped and gently moved to the side.
9. Steps 9 and 10 were repeated until agar was expended.
10. Agar was allowed to solidify in the Petri dishes before plating bacteria.
11. If any agar was spilled, it was allowed to solidify before wiping up with a paper towel.
12. Unused plates were refrigerated, inverted.

Performing Bacterial Plate Count:

1. A sterile serological pipette or transfer pipette was used to transfer 1 mL of sample to 9 mL of autoclaved DI water, creating a 10^{-1} dilution.
2. 1 mL of the 10^{-1} dilution was added to 9 mL of autoclaved water creating a 10^{-2} dilution.
3. This method was repeated to prepare dilutions down to 10^{-15} for strong bacterial solutions and 10^{-12} for weak solutions.
4. 20-50 uL was poured onto a sterile agar plate (see plate preparation instructions above).
5. 10-20 sterile glass spreading beads were also added to the plate.
6. The plate was then covered and swirled, so that the beads spread inoculum across the entirety of the plate's surface.
7. The used beads were poured off into a 70% ethanol waste container. (Note: some sample is removed on the surface of the beads; however, this amount is minimal compared to the plated volume.)
8. The agar plate was then capped and labeled, with researcher's name, date, dilution, type of agar, sample source, and expected bacterial strain (if known).
9. Steps 4-8 were repeated for each dilution prepared.
10. Plates were incubated, inverted, for 48 hours at 35ºC.
11. Plates were removed and CFU were enumerated if there were between 30 and 300 individual colonies.

3.1.7 Scanning Electron Microscopy

The SEM used in this study is an SEM FEI Quanta 200, equipped with a Peltier cooling stage, Electron Dispersive Spectroscopy (EDS), and Electron Backscatter Diffraction (EBSD). The Peltier cooling stage prevents samples from being damaged by the electron beam. EDS provides analysis of heavier elements from Boron to Uranium. EBSD allows for the mapping and microstructural analysis of crystalline samples. The EBSD function was used in this study, as surface structure was the only desired attribute. For this study, Dr. Trevor Harding, PhD., operated the SEM in order to expedite the collection of images.

SEM Sample Collection and Preparation:

1. Samples were collected (<0.5g) by scraping a used filter with pipette tip and loaded into 1 mL centrifuge tube.
2. Samples were freeze dried for 24 hours at 100 μmHg in plastic centrifuge tube holder. (Note: Do not use cardboard or other natural/porous material, as it elongates the freeze drying process.)
3. Samples were mounted on the SEM stage by breaking particles up with forceps and placing them on adhesive surface of stage.

Table 3-4: Scanning Electronic Microscope Operating Parameters

Parameter	Value
Electron Beam Voltage	10 kV
Pressure	90 Pa
Spot Size	3.0

3.1.8 UV Absorbance Measurement

A spectrophotometer was used in experiments II-1 and II-1.1 to measure absorbance of samples at specific wavelengths. These experiments were performed before the HACH Colorimeter became available.

UV Absorbance Materials:

- Shimadzu UV-1700 Pharmaspec
- 2 mL Rectangular Cuvette (1 per sample)
- DI Water for Reference Cell
- 5 mL Transfer Pipettes
- Light-Duty Chemistry Wipes

UV Absorbance Methods:

1. The spectrophotometer was turned on, and the UV lamp was allowed to warm up.
2. A sample was collected with 5 mL transfer pipette, and at least 1 mL of sample was loaded into a 2 mL cuvette.
3. The desired mode of measurement was selected (spectrum or single wavelength).
4. The DI reference cell was filled with DI water and loaded into its proper compartment.
5. The sample cuvette was loaded into the spectrophotometer.
6. The machine was then prompted to read absorbance.
7. Absorbance values were recorded, and the sample cell was removed.

8. Steps 5 through 7 were repeated as needed, for each sample.
9. The spectrophotometer was turned off, using the command prompts, not simply the mechanical switch.

3.1.9 Preparing CYA Solution

1. Cyanuric acid was added to DI water at a desired concentration of no more than 3g/L.
2. The solution was heated to 50^{o}C and stirred until all CYA dissolved (5 to 30 minutes, depending on concentration).

3.1.10 Preparing Growth Media

1. Desired media constituents were added to DI water, from highest to lowest concentration.
2. Media was then stirred to mix, and heated if necessary.
3. Growth media was then covered with aluminum foil and weighed.
4. The growth media and additional DI water in a separate container were then autoclaved for 20 minutes at 121^{o}C.
5. Growth media was then re-weighed and any evaporated volume was replenished with autoclaved DI water.

3.1.11 Preparing HPLC Mobile Phase

1. 1 L of DI Water was added to a volumetric flask along with a magnetic stir bar.
2. Desired masses of buffer constituents were added, and the flask was stirred to dissolve.
3. Any residual buffer solution was discarded, and the reagent bottle was rinsed with a small volume of new buffer solution
4. Add new mobile phase to reagent bottle and flush the mobile phase through the HPLC system for at least 20 column volumes.

3.1.12 Dosing Chlorine

Experiments in this study were carried out using household bleach containing NaOCl. The theoretical dose required to obtain the desired mass equivalent of chlorine, measured as Cl_2, can be calculated as follows:

$$V_{bleach} = C_{Cl2} / C_{OCl-} * V_{container} * MM_{OCl} / MM_{Cl2}$$

Where:

V_{bleach} = Volume of bleach to be dosed [mL]

C_{Cl2} = Desired chlorine concentration as Cl_2 [mg/L]

C_{OCl-} = Concentration of OCl^- in the bleach used [mg_{OCl-}/mL_{Bleach}]

$V_{container}$ = Volume of the container being dosed with bleach [L]

MM_{OCl-} = Molar mass of OCl^- [g/mol]

MM_{Cl2} = Molar mass of Cl_2 [g/mol]

1. Desired volume of bleach was pipetted into destination container. When dosing an aquarium, bleach was added to different areas of the tank, including the filter if present.
2. Water within the container was swirled or stirred to mix.

3.1.13 Dosing Sunscreen

1. Desired mass of sunscreen was weighed into a large plastic weigh boat.
2. Approximately 5 mL of DI water was added to the sunscreen.
3. Sunscreen and water were mixed by vigorously stirring with a 200 µL pipette tip to fully homogenize sunscreen with water.
4. Steps 2 and 3 were repeated with increasing volumes of water, two to three times.
5. Diluted sunscreen was then poured into the desired container.
6. A serological pipette was used to draw media from the desired container and rinse residual sunscreen from the weigh boat into the container.
7. The container was then swirled or stirred to mix.

3.1.14 Inoculating Shaker Flasks

1. For liquid cultures, a sterile serological pipette was used to collect desired volume of inoculum.
2. For dry products, weighing-paper was used to measure and transfer product.
3. Products were then added to desired flask containing growth medium.

4. Flasks were then capped and swirled to mix.
5. 70% ethanol was then used to sanitize workspace.

3.1.15 Inoculating Aquaria

1. For liquid cultures, a sterile serological pipette was used to collect desired volume of inoculum.
2. For dry products, a plastic weigh boat was used to measure and transfer inoculums. 20-50 mL of liquid from the destination container were then used to hydrate the sample.
3. When using aquarium filters, hydrated inoculum was added to the interior of the filter, upstream of the filter media bag.
4. When not using aquarium filters, hydrated inoculum was added to the aquarium and a serological pipette was used to mix the solution.
5. After addition of the inoculum, a serological pipette was used to rinse residual inoculum from the weigh boat into the aquarium, using inoculated growth media.
6. The entire volume of aquarium was stirred using a serological pipette.

3.2 Task I - Determination of Clarification Mechanism

3.2.1 Experiment I-1 Effect of Thai FOG on Turbidity, with Mechanical Filtration

A 10-gallon aquarium was washed with bleach and air-dried. The tank was then filled with 30 L of tap water. An aquarium filter was washed with bleach and installed on the aquarium, positioned so that the filter spout was centered on the long side of the tank.

The Tetra Whisper PF10 filter operates with a fibrous "filter bag" containing activated carbon followed by an aeration media. Both the aeration media and the activated carbon were removed from the system, leaving just the filter bag, which more closely mimics pool filter conditions.

A thermometer strip was installed on the tank's exterior, and a heating element was bleached, dried, installed, and set to 30^{o}C. Temperature was allowed to stabilize overnight, before inoculation.

A 3.0-gram sample of Thai FOG was added to the tank. Turbidity was measured using the method outlined above. 3.0 grams of sunscreen were then added to the tank according to the method, as described above. Chlorine was dosed at 3-4 mg/L according to the method outlined above. Total chlorine was measured using the method detailed above, to confirm chlorine residual levels.

Turbidity and total chlorine were measured daily according to methods described above. Chlorine was re-dosed at 3-4 mg/L after each day's measurements according to the methods described above.

All components of the tank, heater, and filter were disassembled and washed in bleach and soap water. Equipment was allowed to air dry before reuse.

3.2.2 Experiment I-2 Effects of BiOWiSH on Turbidity with Sodium Azide Control

Three 10-gallon aquaria were set up identically to the methods of Experiment I-1, with contents detailed in **Table 3-5**. Sodium azide was added at 0.5% as a bacterial inhibitor. The control treatment was not chlorinated, due to incompatibility between sodium azide and chlorine.

Table 3-5: Experiment I-2 Experimental Setup

Treatment	Contents
Thai FOG	100 mg/L Sunscreen, 100 mg/L Thai FOG
US FOG	100 mg/L Sunscreen, 100 mg/L US FOG
Control	100 mg/L Sunscreen, 0.5% w/w Sodium Azide

Turbidity and Total Chlorine were measured daily using methods described above, for each. Chlorine was re-dosed at 3-4 mg/L to the Thai FOG and US FOG tanks daily. Evaporative losses were mitigated by refilling each tank with tap water, daily.

At the end of the experiment, the control tank contents and filter bag were disposed of in a hazardous waste container, due to its sodium azide content.

3.2.3 Experiment I-3 Effects of BiOWiSH on Turbidity with Re-Dosed Sunscreen

Four aquaria were filled with 33 Liters of tap water. The additional water was added in order to prevent splashing of tank contents into other tanks. Filters and heating elements were installed identically to Experiment I-1. **Table 3-6**, below, details the contents of each tank.

Table 3-6: Experiment I-3 Experimental Setup

Treatment	Inoculum
US FOG	33 mg/L US FOG
Thai FOG	100 mg/L Thai FOG
US Rice Bran	100 mg/L US Rice Bran
Control	N/A

Tanks were inoculated with products according to the methods detailed above. Tanks were dosed with 100 mg/L of sunscreen at T=0, T=3, T=7, T=10, and T=16 Days, according to the methods detailed above. The sunscreen dosing schedule was chosen semi-arbitrarily based on the clarity of the control tank.

Similar to Experiment I-2, turbidity and Total Chlorine levels were measured each day, and tanks were refilled and re-chlorinated to 3-4 mg/L Cl_2 after measurement. Tanks were not sampled, refilled, or re-chlorinated on Day 11, due to experimenter oversight. At T=12 Days, the normal daily schedule was resumed.

At the end of the experiment, filter bags were discarded. Tanks and components were cleaned with bleach and soap water.

3.2.4 Experiment I-4 Comparison of Re-Dosed US FOG to Single Dosed Products

Five aquaria were set up as in Experiment I-3, with contents detailed in **Table 3-7**. A sample of Thai FOG was inactivated by gamma irradiation in an external laboratory.

Table 3-7: Experiment I-4 Experimental Setup

Treatment	Inoculum
US FOG Re-Dose	5 mg/L US FOG with each Sunscreen Re-dose
US FOG	50 mg/L US FOG
Thai FOG	100 mg/L Thai FOG
Thai FOG Irradiated	100 mg/L Irradiated Thai FOG
Control	N/A

Treatments were dosed with 100 mg/L of sunscreen at T=0 Days and multiple times thereafter. Due to experimenter oversight and clerical errors, the data tables containing the exact dosing schedule and readings were lost.

Turbidity and Total Chlorine were measured, daily. After each sampling event, treatments were dosed with 3-5 mg/L Total Chlorine and refilled with tap water.

3.2.5 Experiment I-5 Effects of BiOWiSH Products on Re-Dosed Sunscreen

Six aquaria were filled with 33 Liters of tap water and set up identically to Experiment I-3. The contents of each treatment are listed in **Table 3-8**, below.

Table 3-8: Experiment I-5 Experimental Setup

Treatment	Inoculum
US FOG	50 mg/L US FOG
Mix #1	50 mg/L US FOG, 100 mg/L Irradiated Thai FOG
Mix #1	50 mg/L US FOG, 100 mg/L Irradiated Thai FOG
IR Thai FOG	100 mg/L Irradiated Thai FOG
Premix	100 mg/L Premix
BMT SS	100 mg/L BMT Remediate

Sunscreen was added to each aquarium according to the methods above, at T=0, T=2, T=8, and T=16 Days. Treatments were chlorinated, daily, to 6-9 mg/L of Total Chlorine, because residuals dropped to 0 mg/L overnight. Between T=16.1 and T=27 days, the aquaria were not sampled from, chlorinated, or refilled.

3.2.6 Experiment I-6 Investigation of Solid Substrate Products' Effects on Turbidity

Seven aquaria were set up identically to Experiment I-3. Each tank's contents are listed in **Table 3-9**, below.

Table 3-9: Experiment I-6 Experimental Setup

Treatment	Inoculum
Manure/Odor	50 mg/L Manure/Odor Control

Premix	100 mg/L Premix
BS-AQ-001	100 mg/L BS-AQ-001
BS-AQ-002	100 mg/L BS-AQ-002
BS-AQ-003	100 mg/L BS-AQ-003
Thai Rice Bran	100 mg/L Thai Rice Bran
US Rice Bran	100 mg/L US Rice Bran

Sunscreen was dosed to each aquarium according to the methods above, at T=0 and T=1 Days. Daily measurements, chlorination, and refilling were carried out identically to Experiment I-3.

3.2.7 Experiment I-7 Effects of BiOWiSH Products on Re-Dosed Turbidity

Seven aquaria were set up identically to Experiment I-3. The contents of each tank are detailed below in **Table 3-10**. A sample of Thai Rice Bran was inactivated by gamma irradiation in an external laboratory.

Table 3-10: Experiment I-7 Experimental Setup

Treatment	Inoculum
Fruit Wash	50 mg/L Fruit Wash
Premix	100 mg/L Premix
AP 001	100 mg/L AP 001
AP 002	100 mg/L AP 002
AP 003	100 mg/L AP 003
Thai Rice Bran	100 mg/L Thai Rice Bran
Irradiated Thai Rice Bran	100 mg/L Irradiated Thai Rice Bran

Daily measurements, chlorination, and refilling were carried out identically to Experiment I-3.

3.2.8 Experiment I-8 Effects of Thai BiOWiSH Products on Re-Dosed Turbidity

Seven aquaria were set up identically to Experiment I-3. The contents of each aquarium are detailed in **Table 3-11**, below.

Table 3-11: Experiment I-8 Experimental Setup

Treatment	Inoculum
Premix 1	100 mg/L Premix
Premix 2	100 mg/L Premix
Thai FOG 1	100 mg/L Thai FOG
Thai FOG 2	100 mg/L Thai FOG
IR Thai FOG 1	100 mg/L Irradiated Thai FOG
IR Thai FOG 2	100 mg/L Irradiated Thai FOG
Control	N/A

Tanks were dosed with 100 mg/L of sunscreen at T=0, T=3, T=7, T=10, and T=16 Days, according to the methods detailed above. Daily measurements and tank refilling were carried out identically to Experiment I-3. Tanks were chlorinated to at least 5 mg/L, daily, per the above-detailed methods.

At the end of the experiment, samples were taken from Premix 1, Thai FOG 1, IR Thai FOG 1, and the Control for bacterial plating in Experiment III-2. Contents of each tank were then discarded down the sink, and all aquaria and components were cleaned identically to Experiment I-3.

3.2.9 Experiment I-9 Experiment I-9 Effects of Vegetative Bacterial Cultures on Turbidity

Six aquaria were set up identically to Experiment I-3. The contents of each aquarium are detailed in **Table 3-12**, below.

Table 3-12: Experiment I-9 Experimental Setup

Treatment	Sunscreen, re-dosed	CYA	Inoculum	Chlorination
Tank 1	100 mg/L	100 mg/L	Activated CAR	None
Tank 2	100 mg/L	None	Activated CAR	1-5 mg/L
Tank 3	100 mg/L	100 mg/L	Activated CAR	1-5 mg/L
Tank 4	100 mg/L	100 mg/L	Activated CAR	1-5 mg/L
Tank 5	None	100 mg/L	Activated CAR	None
Tank 6	100 mg/L	100 mg/L	Biology from Used Filter Media	None

The Activated CAR product was prepared by incubating OBAAG-KLB, LCM, OBWWT#1, and MBWWT#1 in four separate flasks of modified growth media. LCM was grown in Difco™ Lactobacillus MRS Broth prepared according to the package labeling. OBAAG-KLB, OBWWT#1 and MBWWT#1 were grown in Potato Dextrose Broth (PDB) prepared at half of the suggested concentration with an additional 10 g/L of dextrose. The PDB was prepared at 12 g/L rather than 24 g/L to avoid a pH drop that would inhibit bacterial growth, and the dextrose was added to make up the deficiency in carbon. To prepare the inoculum from a used recreational pool filter, A 105 in^2 segment was collected from a used pleated cartridge filter from a swimming pool and added to 1 L of 12 g/L

PDB/dextrose growth medium, defined previously. All CYA stock solutions were prepared according to the methods detailed above.

The "Activated CAR" product was created by pipetting 10 mL of each stock culture into an Erlenmeyer flask. Once the temperature of each aquarium had stabilized at 30^{o}C, 4 mL of the "Activated CAR" were added to tanks 1 through 5, using the method outlined in previous methods. Tank 6 was inoculated with 4 mL of the used recreational pool filter stock culture using the same dosing method.

Sunscreen was dosed to Tanks 1, 2, 3, and 4 at T=0 Days. The sunscreen dosing schedule was changed at T=1 Day to include dosing to Tank 6. Sunscreen was dosed to tanks listed in **Table 3-12** at T=1, T=4, T=7, and T=12 Days.

Turbidity and Total Chlorine measurements were recorded each day and after re-dosing of sunscreen. Samples were collected at T=1, T=3, T=5, T=6, T=14, and T=15 Days for HPLC analysis of CYA. After sampling, each tank was re-chlorinated and refilled to its original level with tap water. Tank 1 was accidentally chlorinated at T=1.1 Days, and T=4.1 Days.

3.3 Task II - Efficacy of BiOWiSH Clarifying Oils from Swimming Pools

3.3.1 Experiment II-1 Water Clarification by Thai FOG

Aquaria were filled with 30 L at 30^{o}C as in Task I. Mechanical filtration was not used in this experiment, to investigate the clarifying effects of BiOWiSH products, un-agitated. The contents of each tank are listed in **Table 3-13**, below.

Table 3-13: Experiment II-1 Experimental Setup

Tank #	Contents
1	500 mg/L Sunscreen
2	250 mg/L Thai FOG
3	500 mg/L Sunscreen, 250 mg/L Thai FOG
4	Tap Water

Sunscreen was dosed to Tank 1 by direct addition and did not dissolve completely. Before dosing sunscreen to Tank 3, the method in Section 3.2.2.5 was developed. Thai FOG was added, dry, to the surfaces of Tanks 2 and 3.

Since no filtration was used in the experimental setup, tanks were not agitated beyond the convective currents caused by heating elements. During sampling, care was taken not to disturb the settled layer of sunscreen and biomass on the bottom of each tank. Samples for turbidity measurement were collected using 10 mL serological pipettes.

Samples were loaded into test tubes and transferred to 2 mL cuvettes for absorbance measurement in spectrophotometer at 470 nm, relative to a DI blank.

The contents of the aquarium were disposed of down the sink. All components of the tank, heater, and filter were disassembled and washed in bleach and soap water. Equipment was allowed to air dry before reuse.

3.3.2 Experiment II-1.1 Absorbance Calibration Curve for Sunscreen

Serial dilutions of sunscreen dissolved in DI water were created in 25 mL test tubes. Calibration was carried out from 437.5 mg/L down to 10 mg/L.

Samples were and pipetted from the test tubes into 2 mL cuvettes for analysis in the spectrophotometer. Absorbance was read at 520 nm. A calibration curve was created relating absorbance to concentration of sunscreen.

3.3.3 Experiment II-1.2 Investigation of Growth Media Compatibility with Sunscreen

Four autoclaved flat-bottomed boiling flasks were filled with 200 mL of water and growth media, detailed in **Table 3-14**, below.

Table 3-14: Experiment II-1.2 Experimental Setup

Flask #	Contents
1	1 g/L 20-20-20 fertilizer*; 5 g/L dextrose
2	55 g/L MRS Broth
3	1 g/L K_2HPO_4; 1 g/L KH_2PO_4; 1 g/L glucose; 0.1 g/L KNO_3; Trace $FeSO_4$; Trace $CaCl_2$;
4	DI Water

(20% nitrogen, 20% phosphorus, 20% potassium)

Each flask was chlorinated to with 5 mg/L of total chlorine, and covered aerobically. Total chlorine was measured at T=0, T=1, and T=72 hours, according to the method described in Section 3.1.2. Samples for total chlorine tests were collected using non-sterile 5 mL graduated transfer pipettes.

3.3.4 Experiment II-2 Chlorine Decay with BiOWiSH Products

Twelve 500 mL flat-bottomed boiling flasks were cleaned with soap water, rinsed with DI, and autoclaved. Flasks 1 through 8, 11, and 12 were filled with 200 mL of DI water. Flasks 9 and 10 were filled with the minimal growth described in **Table 3-15**.

Table 3-15: Experiment II-2 Minimal Media Composition

Component	Concentration
Glucose	2 g/L
K2HPO4	1.5 g/L
KH2PO4	0.75 g/L
NH4NO3	0.2 g/L
FeSO4	20 mg/L
MnSO4	Trace

Flasks 1 through 8 were dosed with 125 mg/L of the products listed in **Table 3-16**. Flask 10 was dosed with 525 mg/L of sunscreen, and Flask 11 was dosed with 536 mg/L of sunscreen. Sunscreen dosing varied from the methods detailed, due to the small volume of sunscreen being added. Sunscreen was dosed directly to the flasks without dilution. The flasks were then swirled vigorously to fully dissolve sunscreen. Each treatment was then dosed with 3-4 mg/L of total chlorine, and capped aerobically.

Table 3-16: Experiment II-2 Flask Setup

Flask #	Product
1	Osprey MPB 5
2	Osprey BPB 100
3	Osprey Waste Water
4	US Aqua
5	Us FOG
6	MDG Petro
7	MDG Waste Water
8	Thai FOG
9	Minimal Media
10	sunscreen + Media 525 mg/L
11	sunscreen + Water 536 mg/L
12	Tap Water

Total Chlorine was measured at T=0, T=4.5, and T=25 Hours.

3.3.5 Experiment II-3 Turbidity Reduction by BiOWiSH Products

Ten aquaria were set up identically to Experiment II-1 and inoculated with the products listed in **Table 3-17,** below.

Table 3-17: Experiment II-3 Aquarium Contents

Tank	Product
1	Osprey- MPB 5
2	Osprey BPB 100
3	Osprey WW
4	MDG Petro
5	MDG Wastewater
6	BMT Wastewater
7	Thai FOG
8	US FOG
9	Fruit Wash
10	Control – DI Water

Turbidity and total chlorine were measured daily. Chlorine was re-dosed each day to obtain a residual of 0.2 to 0.6 mg/L TC.

3.3.6 Experiment II-4 Agitated vs. Un-Agitated Turbidity Reduction by BiOWiSH Products

Eight tanks were set up identically to Experiment II-1. Each tank's inoculum is listed in **Table 3-18**, below. Tanks 2, 3, 4, 5, 6, and 7 received 100 mg/L of inoculum. Tank 8 was accidentally dosed with 200 mg/L of product.

Table 3-18: Experiment II-4 Experimental Setup

Tank	Contents
1	Control
2	Thai FOG
3	Food Wash I
4	Food Wash II
5	BMT WW1 I
6	BMT WW1 II
7	BMT WW2 I
8	BMT WW2 II

Each tank was dosed to a theoretical TC content of 2.66 mg/L as Cl_2, but readings immediately dropped to between 0.6 and 1.9 mg/L. Thai FOG showed the most chlorine scouring, so it was dosed with additional chlorine, multiple times. After each chlorine dose, TC dropped to around 1.8 mg/L. Chlorination was stopped at a theoretical dose of 3.5 mg/L.

Daily turbidity and TC measurements were taken. Chlorine was then re-dosed to a residual of 2.0 mg/L TC as Cl_2.

3.3.7 Experiment II-5 Turbidity Reduction by BiOWiSH Products

Ten aquaria were prepared identically to Experiment II-3 and dosed with the products listed in **Table 3-19**.

Table 3-19: Experiment II-5 Experimental Setup

Tank	Label	Inoculum [mg/L]
1	Control	0
2	Thai FOG	125
3	LCM 1	125
4	LCM 2	125
5	BMT WW1	125
6	BMT WW2	125
7	BMT KLB Mix I	62.5, and 4.2, respectively
8	BMT KLB Mix II	62.5, and 4.2, respectively
9	KLB I	8.3
10	KLB II	8.3

Daily turbidity and TC were measured daily. Chlorine was re-dosed each day to a residual of 3.0 mg/L TC as Cl_2. Only Tanks 1, 2, 7, and 8 were continued past T=50 Hours, due to time constraints. Treatments were not chlorinated between T=68 Hours and T=113 Hours.

3.3.8 Experiment II-6 Effect of BiOWiSH Products on Turbidity with Dextrose

Each of seventeen 500 mL flat-bottomed shaker flasks were cleaned with bleach water and allowed to air dry. Flasks were filled 300 mL of DI water and 200 mg/L of dextrose as a carbon source. Flasks were then dosed with the products and concentrations detailed in **Table 3-20**.

Table 3-20: Experiment II-6 Experimental Setup

Flask	Concentration
Control	N/A
Osprey MPB-5	100 mg/L
Osprey BPB-100	100 mg/L
Osprey MPB-5 Liq	125 mg/L

BMT WW 1	100 mg/L
BMT WW 2	100 mg/L
MBWWT#1	100 mg/L
MDG Petro	100 mg/L
MDG Micro-N	100 mg/L
Crop	100 mg/L
Fruit Wash	100 mg/L
LCM	100 mg/L
Thai FOG	100 mg/L
Thai Aqua	100 mg/L
US Aqua	100 mg/L
US FOG	100 mg/L
KLB	100 mg/L

Each flask was agitated before daily turbidity and TC measurement. Chlorine was re-dosed each day to a residual of 3-5 mg/L TC as Cl_2. At the end of the experiment, samples were collected from the US FOG, KLB, and Thai FOG treatments for plating in Experiment III-1.

3.3.9 Experiment II-7 Effect of BiOWiSH Products on Turbidity with Sunscreen

Seventeen treatments, identical to those in Experiment II-6, were prepared. Where dextrose was used in Experiment II-6, this experiment substituted 200 mg/L sunscreen. See **Table 3-20**, above, for treatment details.

Each flask was agitated before daily turbidity and TC measurement. Chlorine was re-dosed each day to a residual of 3-5 mg/L TC as Cl_2.

3.4 Task III - Isolation and Identification of Bacteria

3.4.1 Experiment III-1 Bacterial Plating of Experiment II-6

45 PCA plates were prepared according to the method detailed above. Samples were collected from the treatments of MDG Micro Nutrient, Thai FOG, US FOG, and KLB from Experiment II-6. All samples were stored and transported in 25 mL test tubes, covered with paraffin wax. Each sample was plated at dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12}.

A control plate containing no inoculum and a blank plate containing DI water used for dilution were incubated alongside the samples. The control plate was included to indicate the cleanliness of the media and plates used, and the blank plate was included to indicate the cleanliness of plating methods.

All plates were incubated at 35^{o}C for 48 hours before enumeration. After enumeration, all plates were discarded in a waste receptacle designated for Petri dishes.

3.4.2 Experiment III-2 Bacterial Plating of Experiment I-8

PCA plates were prepared according to the method detailed above. Samples were collected from Experiment I-8, Tanks 1, 3, 5, and 7, and stored in 25 mL test tubes, covered with paraffin wax.

Each sample was plated at dilutions of, 10^{-16}, 10^{-20}, and 10^{-28}. Due to experimenter oversight, the water used for dilution was not autoclaved. (This resulted in contamination of almost every plate.) All plates were incubated at 35ºC for 48 hours before enumeration. After enumeration, all plates were discarded in a waste receptacle designated for Petri dishes containing microbiology.

3.5 Task IV - Biodegradation of Cyanuric Acid

3.5.1 Experiment IV-1 CYA Biodegradation in a Respirometer

Experiment IV-1 was developed to investigate CO_2 production by BiOWiSH Thai FOG and US FOG in the presence of glucose and CYA. Due to repeated electrical and mechanical failures, the experiment was omitted from this study. The respirometer was not used in subsequent experiments.

3.5.2 Experiment IV-2 CYA Adsorption to Irradiated Thai FOG

A stock solution of CYA was prepared at 100 mg/L in DI water. The solution was added to each of five autoclaved 250 mL threaded shaker flasks. Flasks were dosed with concentrations of Irradiated Thai FOG shown in **Table 3-21**.

Table 3-21: Experiment IV-2 Irradiated Thai FOG Concentrations

Bottle #	Contents
1	50 mg/L
2	75 mg/L
3	100 mg/L

4	150 mg/L
5	300 mg/L

After inoculation, flasks were capped anaerobically, swirled to mix, and left on the laboratory bench to react at ambient temperature, 18-22^{o}C. Before daily turbidimetric analysis of CYA, each flask was swirled to mix.

3.5.3 Experiment IV-3 Anaerobic Degradation of CYA in DI Water

Ten 250 mL threaded shaker flasks and caps were washed and autoclaved. Each flask was filled with the contents detailed below, in **Table 3-22**.

Table 3-22: Experiment IV-3 Flask Labels and Contents

Flask #	Flask Label	Contents
1	US-200 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 200 mg/L, US Aqua 50 mg/L
2	US-200 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 200 mg/L, US Aqua 50 mg/L
3	US-50 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 50 mg/L, US Aqua 50 mg/L
4	US-50 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 50 mg/L, US Aqua 50 mg/L
5	Thai-200 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 200 mg/L, Thai FOG 50 mg/L
6	Thai-200 Glucose	DI Water 200 mL; CYA 50 mg/L, Glucose 200 mg/L, Thai FOG 50 mg/L

7	Thai-200 Sunscreen	DI Water 200 mL; CYA 50 mg/L, Sunscreen 200 mg/L, Thai FOG 200 mg/L
8	US-200 Sunscreen	DI Water 200 mL; CYA 50 mg/L, Sunscreen 200 mg/L, US Aqua 50 mg/L
9	Cyanuric	DI Water 200 mL, 50 mg/L CYA
10	Water	DI Water 200 mL

Instead of a stock solution, CYA was dosed to each flask, dry. After observing the inconsistencies introduced by this method, addition of CYA via stock solution was adopted as the standard method beginning with Experiment IV-5.

Flasks reacted at ambient temperatures ranging from 18-22^{o}C. Flasks were only agitated when swirled to mix, immediately before daily turbidimetric analysis of CYA.

3.5.4 Experiment IV-4 Anaerobic Degradation of CYA in Minimal Media

Ten treatments were prepared identically to Experiment IV-3 using minimal media, rather than DI Water.

Table 3-23: Experiment IV-4 Flask Labeling and Contents

Flask #	Flask Label	Contents
1	US-200 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 200 mg/L, US Aqua 50 mg/L
2	US-200 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 200 mg/L, US Aqua 50 mg/L

Flask #	Flask Label	Contents
3	US-50 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 50 mg/L, US Aqua 50 mg/L
4	US-50 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 50 mg/L, US Aqua 50 mg/L
5	Thai-200 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 200 mg/L, Thai FOG 50 mg/L
6	Thai-200 Glucose	Growth Media 200 mL; CYA 50 mg/L, Glucose 200 mg/L, Thai FOG 50 mg/L
7	Thai-200 Sunscreen	Growth Media 200 mL; CYA 50 mg/L, Sunscreen 200 mg/L, Thai FOG 200 mg/L
8	US-200 Sunscreen	Growth Media 200 mL; CYA 50 mg/L, Sunscreen 200 mg/L, US Aqua 50 mg/L
9	Cyanuric	DI Water 200 mL, 50 mg/L CYA
10	Water	DI Water 200 mL

Minimal media was prepared with the concentrations in **Table 3-24**. Media was autoclaved before addition to the 250 mL shaker flasks.

Table 3-24: Experiment IV-4 Growth Medium

Constituent	Concentration
K_2HPO_4	1 g/L
KH_2PO_4	1 g/L
Dextrose (Anhydrous)	1 g/L
KNO_3	0.1 g/L
$FeSO_4$	Trace
$CaCl_2$	Trace

CYA was added to each flask in granular form. After addition, each flask was capped and shaken to dissolve CYA. Dry bacterial mixes and additional glucose were then added to each flask. After bacterial inoculation, flasks were loaded into an incubator shaker at 30^{o}C and 75 RPM. Flasks were swirled to mix immediately before daily analysis of CYA. CYA was measured turbidimetrically.

3.5.5 Experiment IV-5 Effect of Activated Thai FOG Supernatant on CYA

Thai FOG was added to 1L of DI water at 750 mg/L and incubated, aerobically, for 24 hours at 30^{o}C and 75 RPM. A stock solution of 62.5 mg/L CYA was prepared. One treatment of 390 mL CYA stock solution was inoculated with 10 mL of the supernatant of the activated Thai FOG. The inoculated treatment was capped anaerobically and incubated at 30^{o}C and 75 RPM. Daily turbidimetric CYA measurements were taken.

3.5.6 Experiment IV-6 Effects of US FOG and Activated Thai FOG on CYA

Six 500 mL threaded shaker flasks were autoclaved then filled with 400 mL of 60 mg/L CYA stock solution. Each flask was dosed with 250 mg/L glucose. CYA was tested turbidimetrically for each flask before the addition of any biological inoculum.

Treatments were set up in triplicate. Three flasks were dosed with 100 mL DI water and 250 mg/L of US FOG. The other three flasks were dosed with 100 mL of supernatant from a solution of 1 g/L Thai FOG. The Thai FOG solution had been activated at 30^{o}C and 75

RPM for 24 hours, inverted and agitated, then allowed to settle for 2 minutes. CYA was tested turbidimetrically for each flask, each day.

3.5.7 Experiment IV-7 Turbidimetric CYA Calibration

Serial dilutions were created from a stock CYA. Each dilution was turbidimetrically, and a linear regression model was fit to the resulting data.

3.5.8 Experiment IV-8 Standard Preparation for HPLC

A stock solution of CYA was prepared. Dilutions were prepared in 2 mL crimp-top vials for HPLC analysis. Concentrations were recorded and written on the exterior of each vial for ease of reference.

3.5.9 Experiment IV-9 HPLC Calibration of CYA

The standard solutions prepared in Experiment IV-8 were tested in the HPLC using the method described in **Table 3-25**, below. The purge valve was maintained partially open throughout HPLC analysis, which heavily influenced elution time and peak area.

Table 3-25: Experiment IV-9 HPLC Operating Parameters

Parameter	Value
Column	Acclaim Trinity P1
Mobile Phase	20% methanol 80% 50 mM K_2HPO_4 buffer
Flow Rate	0.500 mL/min
Injection volume	20 µL

Detection wavelength	213 nm
Temperature	22^{o}C (ambient)
Sample Run Time	12 minutes
Operating Pressure	350 bar

3.5.10 Experiment IV-10 HPLC Calibration using Thai FOG

A stock solution of 50 mg/L CYA was prepared. Thai FOG was added to DI water at 110 mg/L and activated at 30^{o}C and 75 RPM for 24 hours. All samples were filtered through 0.22 µm filters before addition to 2 mL vials at concentrations listed in **Table 3-26**, below. The 40 mg/L standard CYA vial from Experiment IV-8 was also analyzed.

Table 3-26: Experiment IV-10 HPLC Vial Contents

Vial	Contents
1	CYA 50 mg/L
2	Thai FOG 110 mg/L
3	CYA 25 mg/L; Thai FOG 55.3 mg/L
4	40 mg/L CYA Standard

HPLC analysis was carried out with the parameters in **Table 3-27**. The purge valve was maintained partially open, due to high operating pressure.

Table 3-27: Experiment IV-10 HPLC Operating Parameters

Parameter	Value
Column	Acclaim Trinity P1
Mobile Phase	20% methanol 80% 50 mM K_2HPO_4 buffer
Flow Rate	0.500 mL/min
Injection volume	20 uL
Detection wavelength	213 nm
Temperature	21^{o}C (ambient)
Sample Run Time	12 minutes
Operating Pressure	360 bar

3.5.11 Experiment IV-11 Method Development of HPLC using Thai FOG and CYA

A shaker flask was dosed with 100 mg/L Thai FOG and 60 mg/L CYA. The inoculated solution was placed on a heat plate and stirred with a magnetic stir-bar until all CYA had dissolved. The flask was then incubated in a shaker at 30^{o}C and 75 RPM for the duration of the experiment.

Samples were collected for HPLC analysis according to the methods detailed above. HPLC operating parameters were identical to those in Experiment IV-10 and are laid out in **Table 3-27**, above.

3.5.12 Experiment IV-12 Investigation of Products' Effects on CYA

Eight 500 mL shaker flasks were autoclaved and filled with 300 mL of DI water and the contents listed in **Table 3-28**, below. After inoculation, treatments were capped and incubated at 30^{o}C and 75 RPM for the duration of the experiment.

Table 3-28: Experiment IV-12 Experimental Setup

Bottle	Cya [mg/L]	Inoculum	Glucose [mg/L]
1	50	x	x
2	50	Thai FOG	250
3	50	Thai FOG	x
4	50	IR TF	250
5	50	Premix	250
6	50	35 mg Osp Liq	250
7	50	Osp Solid	250
8	50	US FOG	250

Samples were collected daily, for HPLC analysis. HPLC operating parameters were identical to those in Experiments IV-10 and IV-11 and are detailed in **Table 3-27**, above.

3.5.13 Experiment IV-13 HPLC Method Development – Inconclusive Mobile Phase Adjustment

Many mobile phases were tested, and none provided improved separation of CYA peaks from nitrate peaks.

Mobile phases tested include:

90% acetonitrile, 5% methanol, 5% 17.5 mM K_2HPO_4 buffer

95% acetonitrile, 5% 17.5 mM K_2HPO_4 buffer

15% acetonitrile, 85% 50 mM K_2HPO_4 buffer

85% acetonitrile, 15% 20 mM ammonium acetate buffer

3.5.14 Experiment IV-14 HPLC Method Development – High Injection Volume

High injected concentrations of CYA led to column clogging during analysis. No meaningful data were collected in this experiment.

3.5.15 Experiment IV-15 Investigation of C:N:P Ratios on CYA Degradation

A minimal growth medium, with concentrations detailed in **Table 3-29**, was autoclaved.

Table 3-29: Experiment IV-15 Minimal Media Composition

Component	Concentration [mg/L]
Cyanuric Acid	276.4
NH_4NO_3	28.6
K_2HPO_4	56.1

US Aqua and dextrose were combined at a ratio of 44% US Aqua and 56% dextrose. This mixture was added to the minimal media, which was stirred to dissolve additives. Additional dextrose was added to each treatment to obtain desired carbon content. **Table 3-30** details the components of each treatment.

Table 3-30: Experiment IV-15 Carbon, Nitrogen, and Phosphorus Concentrations

Ratio of C:N:P	Product [mg/L]	Dextrose [mg/L]	CYA [mg/L]	NH_4NO_3 [mg/L]	K_2HPO_4 [mg/L]
100:10:01	100	2264.3	276.4	28.6	56.1
60:10:01	100	1264.3	276.4	28.6	56.1
50:10:01	100	1014.3	276.4	28.6	56.1
40:10:01	100	764.3	276.4	28.6	56.1
10:10:01	100	14.3	276.4	28.6	56.1
50:10:01	100	1014.3	276.4	28.6	56.1

After inoculation, treatments 1 through 5 and the control were capped anaerobically. Treatments 6 and 7 were covered with aerobic caps. All treatments were placed in an incubator shaker at 30°C and 75 RPM.

Daily sample collection and preparation for HPLC was carried out according to the methods detailed above. HPLC analysis was performed with the operating parameters laid out in **Table 3-31**, below.

Table 3-31: Experiment IV-15 HPLC Operating Parameters

Parameter	Value
Column	Acclaim Trinity P1
Mobile Phase	95% 17.5mM Phosphate buffer, 5% methanol
Flow Rate	0.400 mL/min
Injection volume	150 uL
Detection wavelength	213 nm
Temperature	22°C (ambient)
Sample Run Time	12 minutes
Operating Pressure	250 bar

3.5.16 Experiment IV-16 Hour-by-Hour Time-Point CYA Degradation

A growth solution containing 500 mg/L CYA, 500 mg/L dextrose, and 100 mg/L ammonium nitrate was autoclaved. Six 500 mL shaker flasks were filled with 300 mL of growth solution. Isolated vegetative bacteria from Thai FOG were provided by BiOWiSH for inoculation in this experiment. Three of the six flasks were inoculated with 100 mg/L of vegetative bacterial suspension.

HPLC analysis was carried out identically to Experiment IV-15, with operating parameters identical to those in **Table 3-31**, above.

3.5.17 Experiment IV-17 Effect of CAR on CYA Concentration

A 200 mg/L stock solution of CYA was prepared. Six 500 mL threaded shaker flasks and caps were autoclaved, and each was filled with 300 mL of CYA solution.

A stock solution of BiOWiSH CAR product was prepared at 10 g/L. Two control flasks were not inoculated. Two flasks were dosed to 5 mg/L of CAR solution. Two flasks were dosed to 50 mg/L of CAR solution. HPLC sample collection, preparation, and analysis were carried out according to the methods and operating parameters detailed earlier.

3.5.18 Experiment IV-18 Effect of CAR on CYA with K_2HPO_4, Varied Glucose

Growth media containing 100 mg/L CYA and trace K_2HPO_4. Before addition of the CYA solution to each of six 200 mL shaker flasks, the stock solution was sparged with N_2 gas to create anaerobic conditions from T=0 hours. Bottles were filled to the brim with between 249 and 257 mL of growth media, CAR product, and additional glucose. Treatments can be found in **Table 3-32**, below.

Table 3-32: Experiment IV-18 Flask Contents

Flask	Volume Growth Media [mL]	CAR Product [mg/L]	Added Glucose [mg/L]	Total Glucose [mg/L]
Control 1	250	0	0	0
Control 2	250	0	0	0
CAR1	249	50.0	0	47
CAR2	253	49.8	0	46.8
CAR+GLU1	250	50.3	50	97.3
CAR+GLU2	257	50.0	50	96.3

After inoculation, shaker flasks were capped anaerobically and incubated at 30^{o}C and 75 RPM.

HPLC sample collection, preparation, and analysis were carried out according to the methods and operating parameters detailed above, using non-sterile graduated transfer pipettes. A 3-point calibration was run at the beginning of HPLC analysis at T= 6, T=8, and T=9 Days, to provide a daily calibration check. This method was used in future experiments to provide accurate conversion of peak area to CYA concentration.

No appreciable bacterial growth was seen within biological treatments after 6 days. To stimulate growth, a solution of 1 g/L CAR in Lactobacillus Broth was activated for 24 hours at 30^{o}C and 75 rpm. The activated product was dosed at 1 mL per flask to all four biological treatments on day 7. HPLC measurement was continued, but at increased intervals.

3.5.19 Experiment IV-19 Effect of Activated CAR on CYA, Varying Dextrose

A stock solution of 50 mg/L CYA was prepared. The activated cultures of OBAAG-KLB, LCM, OBWWT#1, and MBWWT#1 from Experiment I-9 were combined in equal parts to create the "Activated CAR" product. Six 250 mL shaker flasks were filled with 250 mL of CYA solution.

Two control flasks were not inoculated. Two flasks received 15 mL of Activated CAR and 50 mg/L of dextrose, and two flasks received 15 mL of Activated CAR and 287 mg/L of dextrose, as shown in **Table 3-33**. After inoculation, each treatment was capped anaerobically and incubated at 30^{o}C and 75 RPM.

Table 3-33: Experiment IV-19 Experimental Setup

Bottle	Activated CAR [mL]	Additional Dextrose [mg/L]
Control 1	0	0
Control 2	0	0
CYA + Dex 50 ppm 1	15	50
CYA + Dex 50 ppm 2	15	50
CYA + Dex 287 ppm 1	15	287
CYA + Dex 287 ppm 2	15	287

HPLC sample collection, preparation, and analysis were carried out according to the methods and operating parameters detailed above. A four-point calibration was run at the beginning of each analysis event to provide accurate conversion of peak area to CYA concentration.

3.5.20 Experiment IV-20 Effect of Activated CAR and Filter Media on CYA

A minimal growth media was prepared with composition found in **Table 3-34**, below.

Table 3-34: Experiment IV-20 Minimal Media Composition

Component	Concentration
Glucose	2 g/L
K2HPO4	1.5 g/L
KH2PO4	0.75 g/L
NH4NO3	0.2 g/L
FeSO4	20 mg/L
MnSO4	Trace

A stock solution of CYA was prepared at 3.00 g/L. Each flask was dosed with 5 mL stock CYA solution before dilution with DI water or activated culture broth and growth medium. Total volume of each flask was 300 mL, creating a final concentration of 50 mg/L CYA in each treatment.

Three sets of duplicate treatments were prepared in 250 mL Erlenmeyer flasks. Flasks were filled to 300 mL in order to prevent oxygen introduction from an air void volume. 15 mL of stock inoculum were added to each flask, as listed in **Table 3-35,** below.

Table 3-35: Experiment IV-20 Flask Setup

Treatment	Solution	Inoculation
1	DI Water + CYA	None
2	DI Water + CYA	None
3	Minimal Media + CYA	Activated Thai FOG
4	Minimal Media + CYA	Activated Thai FOG

5	Minimal Media + CYA	Activated Pool Filter Media
6	Minimal Media + CYA	Activated Pool Filter Media

After the addition of CYA, growth medium, and inoculum, flasks were capped anaerobically and placed in a shaker incubator at 30^{o}C and 75 RPM to ensure proper mixing. Sampling for HPLC was carried out according to the method and operating parameters outlined above. An additional 1.6 g/L of dextrose were added to Thai FOG 1, Thai FOG 2, Filter Media 1, and Filter Media 2 at T=7 days.

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Task I - Determination of Clarification Mechanism

4.1.1 Experiment I-1 Efficacy of Thai FOG Removing Turbidity, with Mechanical Filtration

At T=0 hours, 100 mg/L of Coppertone Sunscreen was dosed to 30 L of tap water in a 10-gallon aquarium with mechanical filtration. At T=0.25 hours, 100 mg/L of Thai FOG was added to the tank. The tank was chlorinated to with Clorox concentrated bleach to 3-4 mg/L of total chlorine as Cl_2, daily.

After a slight increase in turbidity due to the addition of Thai FOG an 82% decrease in turbidity was observed over 18 hours (**Figure 4-1**). The turbidity reduction continued, at a diminished rate, throughout the experiment.

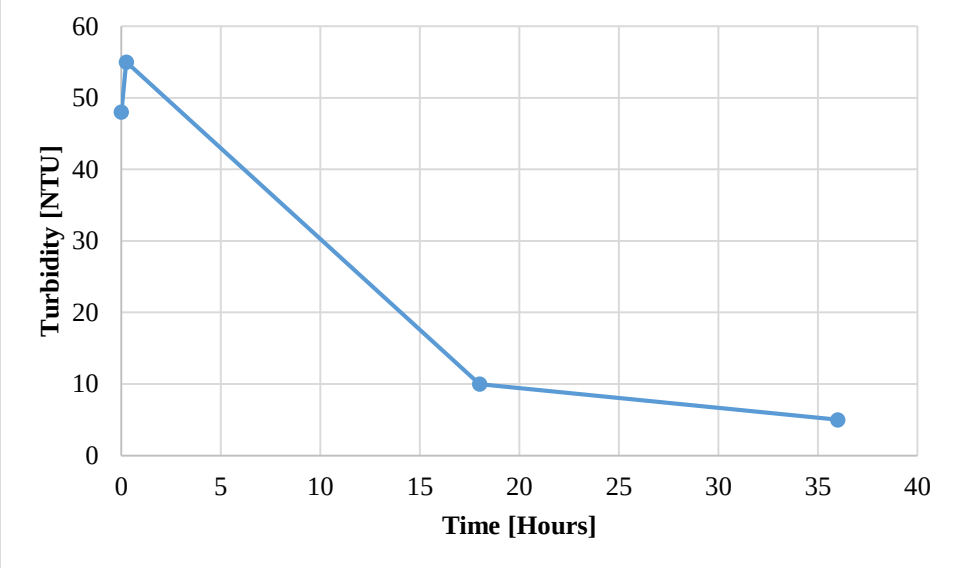


Figure 4-1: Experiment I-1 Turbidity vs. Time, BiOWiSH Thai FOG, Tap Water at 30°C, Mechanical Filtration

The rate of water clarification of water indicated the potential for BiOWiSH to remove turbidity caused by sunscreen. The mechanism by which is unknown, so further research is necessary to determine why turbidity is reduced.

4.1.2 Experiment I-2 Effects of BiOWiSH on Turbidity with Sodium Azide Control

Experiment I-2 replicated the methods of Experiment I-1 with additional treatments including US FOG and an abiotic control. Sodium azide was added to the control tank as a microbial inhibitor.

Each treatment resulted in a sharp decrease in turbidity over the first 18 hours (**Figure 4-2**). Thai FOG, US FOG, and the Control displayed 66%, 77%, and 85% reductions in turbidity, respectively.

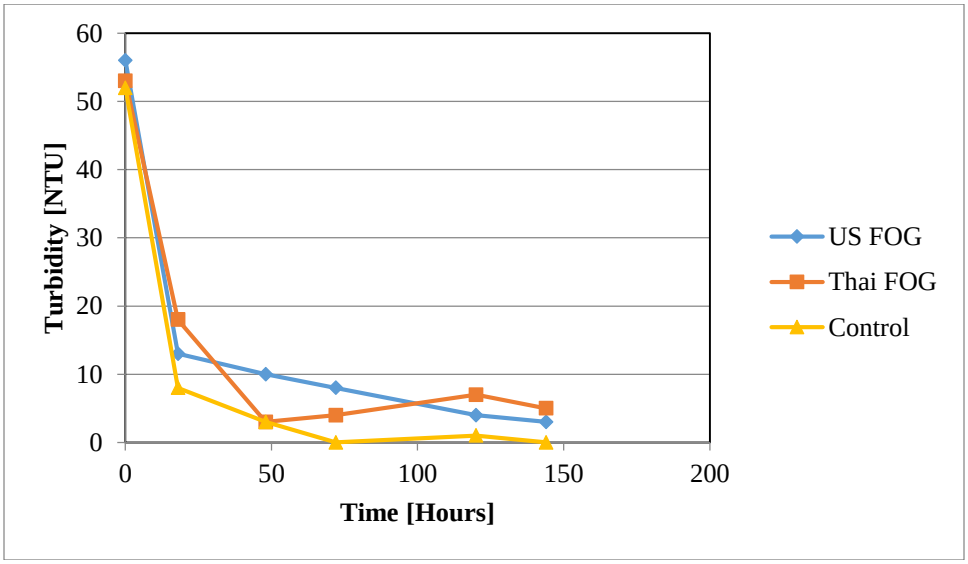


Figure 4-2: Experiment I-2 Turbidity vs. Time, US FOG and Thai FOG, Tap Water at 30°C, Mechanical Filtration

No improvement of clarification over the control treatment was seen by Thai FOG or US FOG. Increased turbidity relative to the control is most likely due to the introduction of particulate matter in each product.

Use of sodium azide as a bacterial inhibitor was discontinued after this experiment due to its high toxicity and splashing caused by filters. To prevent splashing of filters, subsequent treatments were filled with an additional 3 L of water. The additional water provided a gentler re-entry of filter effluent to each tank, minimizing splashing.

4.1.3 Experiment I-3 Effects of BiOWiSH on Turbidity with Re-Dosed Sunscreen

Experiment I-3 was the first experiment in which sunscreen was re-dosed periodically. Additionally a treatment of rice bran was introduced as a control compared to the rice bran used in Thai FOG. Sunscreen was re-dosed at 100 mg/L whenever the turbidity of the control tank leveled out. Residual TC dropped close to zero each day, so each tank was chlorinated to 3-4 mg/L TC as Cl_2, daily.

Each aquarium showed a sharp decrease in turbidity one day after each sunscreen re-dose (**Figure 4-3**). Initial doses of sunscreen were clarified at different rates by each treatment. Thai FOG reduced turbidity by the greatest amount in the first 24 hours, followed by the control and US FOG. Rice bran showed the highest turbidity throughout the experiment, because the fine particles of the substrate did not settle out of suspension, nor were they removed by the filter.

Turbidity reduction of Thai FOG was poor between 1 and 11 days, and turbidity increased after the T=12 Days time-point. This is attributed to an accidental lapse in chlorination at T=11 Days. The *Bacillus* species in Thai FOG were able to proliferate, and continued chlorination did not halt microbial growth. A shock dose of chlorine was considered as an option to stop the bacteria from growing as quickly. For consistency among trials and comparability to future experiments, the regular chlorine dosing schedule was maintained.

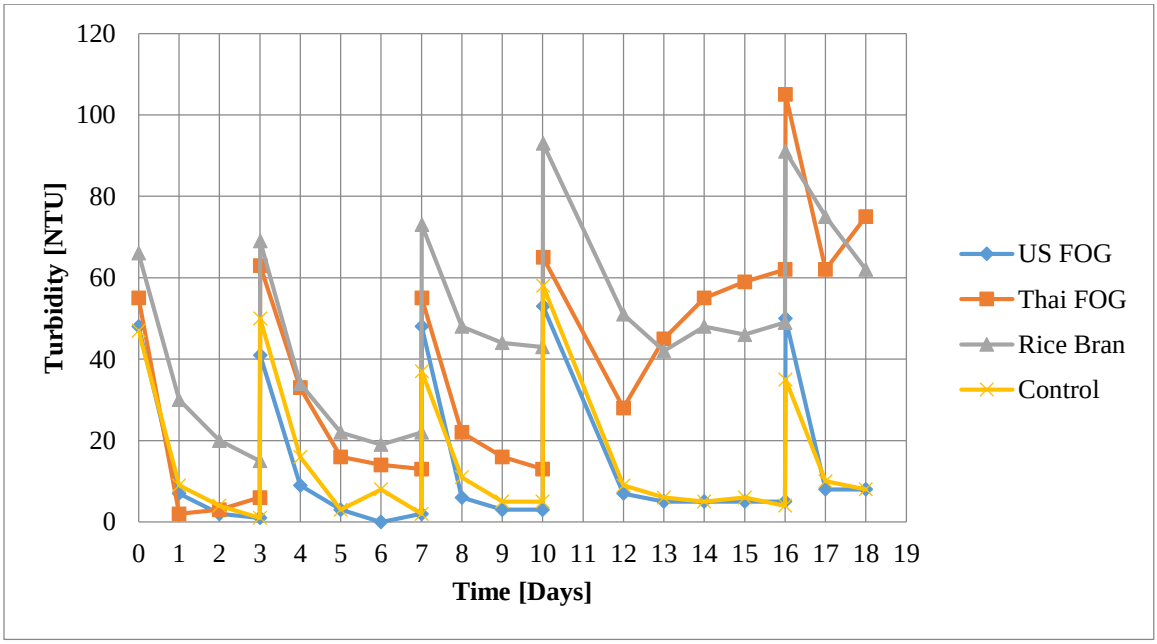


Figure 4-3: Experiment I-3 Turbidity vs. Time, Tap Water at 30°C, Re-dosed Sunscreen, Mechanical Filtration

Thai FOG displayed greater activity than the soluble-diluent US FOG, the abiotic treatments of "Rice Bran" and the Control. Further investigation is necessary to determine whether the microbes in Thai FOG are degrading sunscreen within the first 24 hours, or if the physical properties of the substrate lead to adsorption or improved filtration of sunscreen.

Since the rice bran treatment did not settle out of solution, it cannot be considered analogous to an abiotic form of the substrate of Thai FOG. Experiments I-4, I-5, I-7, and I-8 utilize an irradiated sample of Thai FOG as an abiotic control instead of the rice bran. This control is expected to reveal the effects of the solid substrate without interference by microorganisms.

The results suggest that mechanical removal (either filtration or adsorption) plays a dominant role in the clarification of sunscreen from solution; however, other mechanisms of clarification, such as biodegradation, cannot be ruled out.

4.1.4 Experiment I-5 Effects of BiOWiSH Products on Re-Dosed Sunscreen

Experiment I-5 was designed to compare US FOG and irradiated Thai FOG to a mixture of US FOG and irradiated Thai FOG, among other treatments. Irradiated Thai FOG was used as an abiotic treatment containing solid substrate. US FOG was used as a treatment containing biology and a soluble substrate. Two treatments of US FOG mixed with irradiated Thai FOG were prepared to mimic the bacteria of US FOG mounted to a solid substrate. Proprietary surfactants and the KLB strain of *Bacillus* are added to Thai FOG during production. To observe the effects of a solid substrate, the Thai FOG substrate was used post-fermentation without additives. This product was called Premix. BMT Remediate, called “BMT SS” in this experiment, was tested because it contains a mix of *Rhodococcus* and *Arthobacter* bacteria, rather than the *Bacillus* and *Lactobacillus* of US FOG and Thai FOG.

Chlorine was dosed by adding concentrated bleach between 6 and 9 mg/L TC, which immediately dropped to between 2.1 and 3.0 mg/L in each aquarium. To combat the drop in TC, an additional dose of 6 to 9 mg/L TC was added to each tank. Residuals consistently dropped to around ~1 mg/L Cl_2 overnight. On Day 5, 23 mg/L of CYA were added to each of the tanks in an effort to maintain residual chlorine. However, no change in TC residual was noted. CYA only protects chlorine from being photodegraded by UV radiation, and there are no windows in the lab through which UV radiation could affect chlorine levels in the aquaria.

Sampling and chlorination were discontinued between the final re-dose on Day 16.1 and the final sampling event on Day 27.

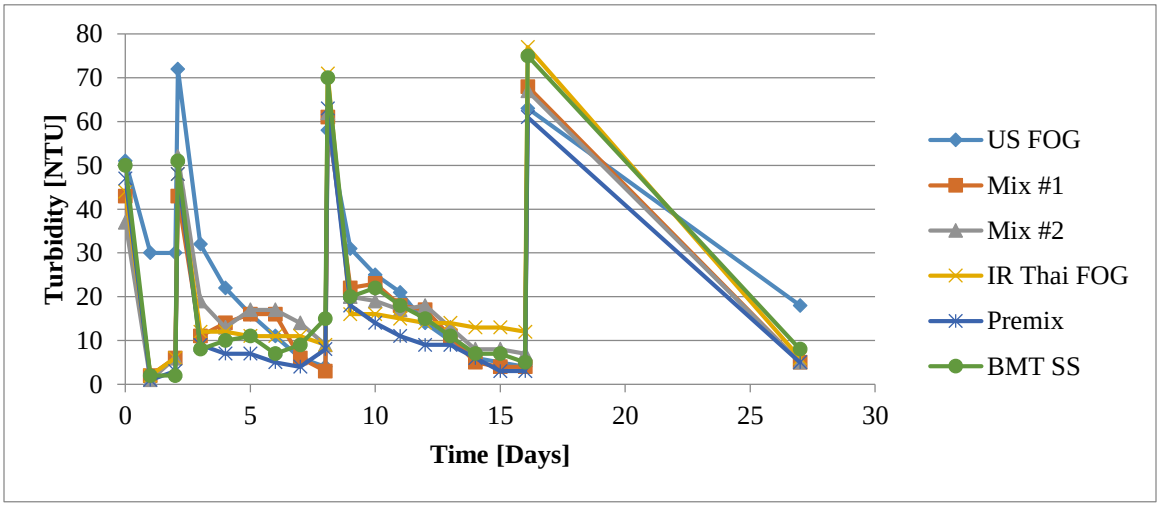


Figure 4-4: Experiment I-5 Turbidity vs. Time, Tap Water at 30°C, Re-dosed Sunscreen, Mechanical Filtration

Between T=0 Days and T=1 Days, the turbidity of US FOG only dropped by 41%, whereas each other treatment reduced turbidity by 95-98%. This is attributed to improper

installation of the filter bag, which caused water to bypass the filter and reduced overall filtration. This mistake shows that without mechanical filtration, clarification is much slower in the first 24 hours of this type of experiment. Additionally, the results of this experiment confirm the results of Experiments I-2 and I-3, which suggest that mechanical filtration is responsible for significantly reduced sunscreen in each treatment, over the first day.

When compared to similar time-points in the control tanks of Experiments I-2 and I-3, "IR Thai FOG" data suggest that the solid substrate does not provide additional removal of sunscreen after re-dosing. This suggests that the majority of clarification during re-doses stems from mechanical filtration, rather than adsorption to the substrate.

4.1.5 Experiment I-4 Comparison of Re-Dosed US FOG to Single Dosed Products

This experiment was designed to compare a treatment which received a single dose of 50 mg/L US FOG to a re-dosed treatment which received 5 mg/L of US FOG each time sunscreen was re-dosed. Additionally, a sample of Thai FOG was irradiated to inhibit biological effects on clarification, if present. The "Irradiated Thai FOG" treatment was compared to a treatment with unaltered Thai FOG.

Due to clerical and experimenter error, the data for T=0 Days through T=23 Days were lost. The trials in this experiment received multiple re-doses of sunscreen before the Day 24 time-point. This experiment highlights each treatment's ability to reduce turbidity after repeated addition of sunscreen.

None of the treatments improved turbidity reduction compared to the control (**Figure 4-6**). The "US FOG Re-Dose" resulted in the highest turbidity until the Day 31 time-point (**Figure 4-7**).

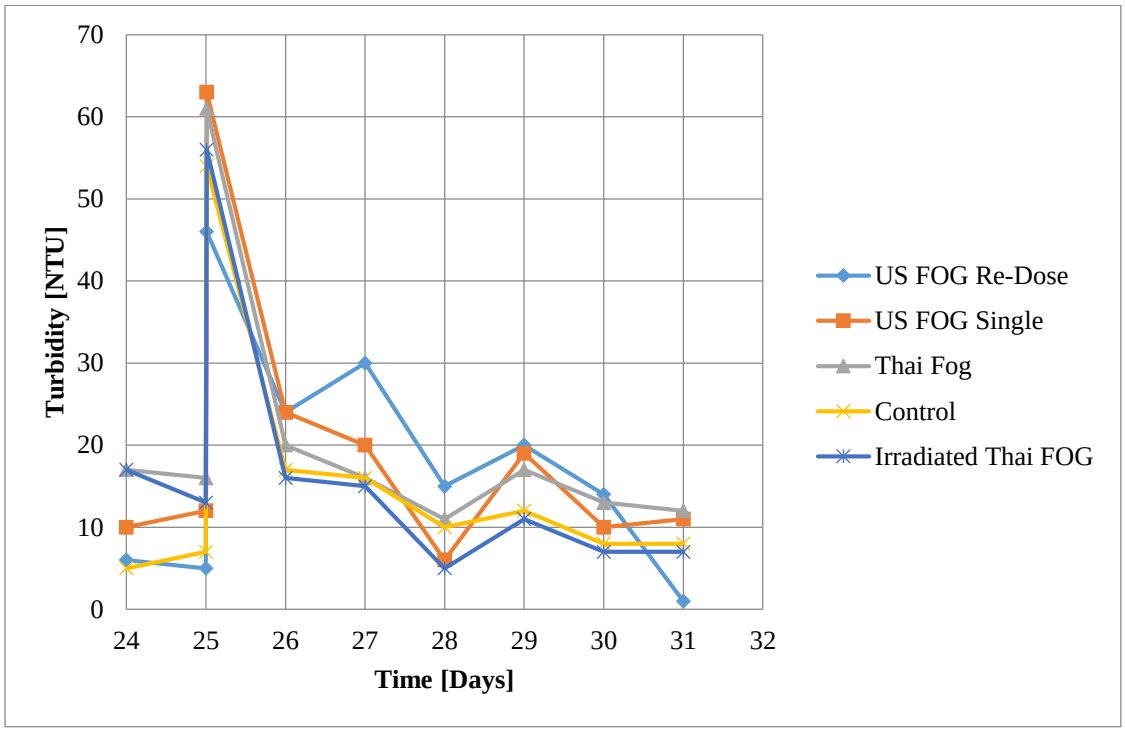


Figure 4-5: Experiment I-4 Turbidity vs. Time, Tap Water at 30°C, Re-dosed Sunscreen Between 24 and 31 Days, Mechanical Filtration

Irradiated Thai FOG showed lower turbidity than the unaltered Thai FOG treatment. This is consistent with the assumption that irradiation deactivated all microbes in the "Irradiated Thai FOG" treatment, and there was minimal bacterial contribution to turbidity in the irradiated product.

By Day 24, the "US FOG Re-Dose" contained a similar quantity of bacteria to "US FOG Single", as microbes in each had been was provided the same amount of substrate in the

form of sunscreen. Results show that re-dosing US FOG is not advantageous over a single dose.

4.1.6 Experiment I-6 Effects of Additional Solid Substrate Products on Turbidity

Building from the findings of Experiment I-5, Experiment I-6 was designed to test a number of additional solid substrate *Bacillus* products. The rice bran which is fermented to create Premix was used in one treatment to investigate the effects of fermentation on the rice bran to remove turbidity from solution. Additionally, the proprietary Manure and Odor Control (Manure/ Odor) product was tested as a treatment containing soluble diluent.

After dosing sunscreen, a significant drop in turbidity was noted in the first day of the experiment for all treatments. Premix showed the greatest drop in turbidity between T=0 and T=1 Days. After the re-dose, Premix again showed the lowest turbidity of all treatments. Beyond T=5 Days, however, Premix showed steadily increasing turbidity.

The prototype BS-AQ products (BS-AQ-001, BS-AQ-002, and BS-AQ-003) performed similarly to each other, showing a decrease in turbidity after the re-dose followed by minimal change in turbidity until T=5 Days. After this time, each product showed a slight increase in turbidity.

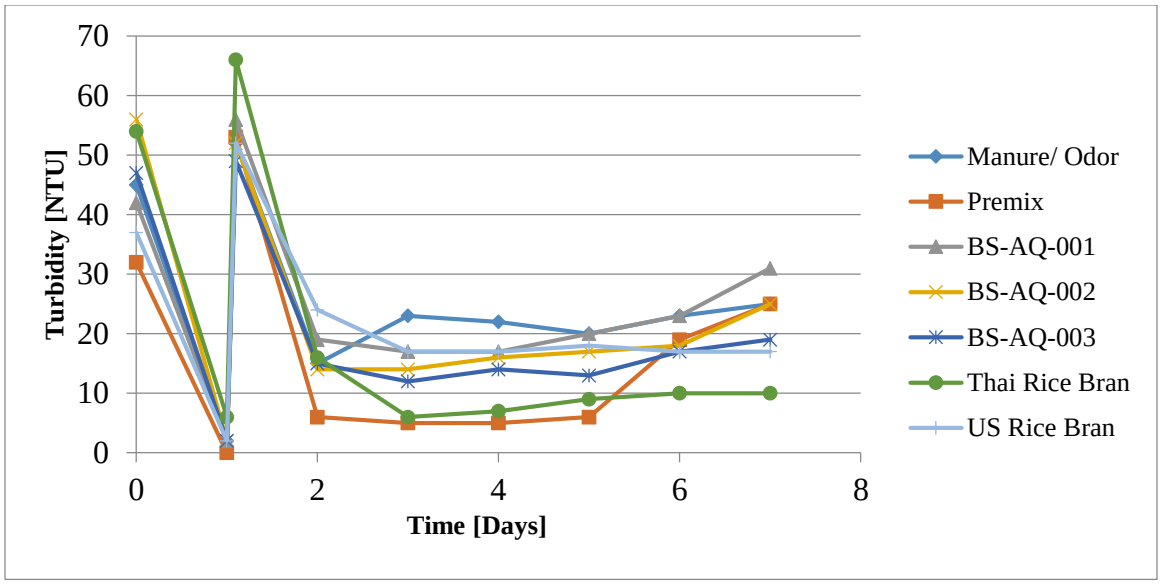


Figure 4-6: Experiment I-6 Turbidity vs. Time, Tap Water at 30°C, Re-dosed Sunscreen, Mechanical Filtration

The Manure/Odor treatment showed an increase in turbidity, relative to the other treatments, starting between T=2 Days and T=3 Days. Premix and Thai rice bran treatments showed the lowest turbidities following the second dose of sunscreen, but apparently bacteria native to the Premix continued to grow, causing an increase in turbidity after T=5 Days. Thai rice bran initially performed similarly to Premix, but Thai rice bran has not been fermented in the presence bacterial starter cultures, so it did not show the same increase in turbidity shown by Premix.

4.1.7 Experiment I-7 Effects of BiOWiSH Products on Re-Dosed Turbidity

Experiment I-7 investigated the effects of various BiOWiSH products, listed in **Table 4-1**, on re-dosed turbidity in the form of sunscreen.

Table 4-1 Experiment I-7 List of Treatments

Tank	Treatment
1	Fruit Wash
2	Premix
3	AP 001
4	AP 002
5	AP 003
6	Thai Rice Bran
7	Irradiated Thai Bran

The greatest reductions in turbidity throughout the experiment were seen by Premix (**Figure 4-7**). No improved reduction of turbidity was seen over time between the solid substrate product, Premix, and the soluble diluent product, Fruit Wash.

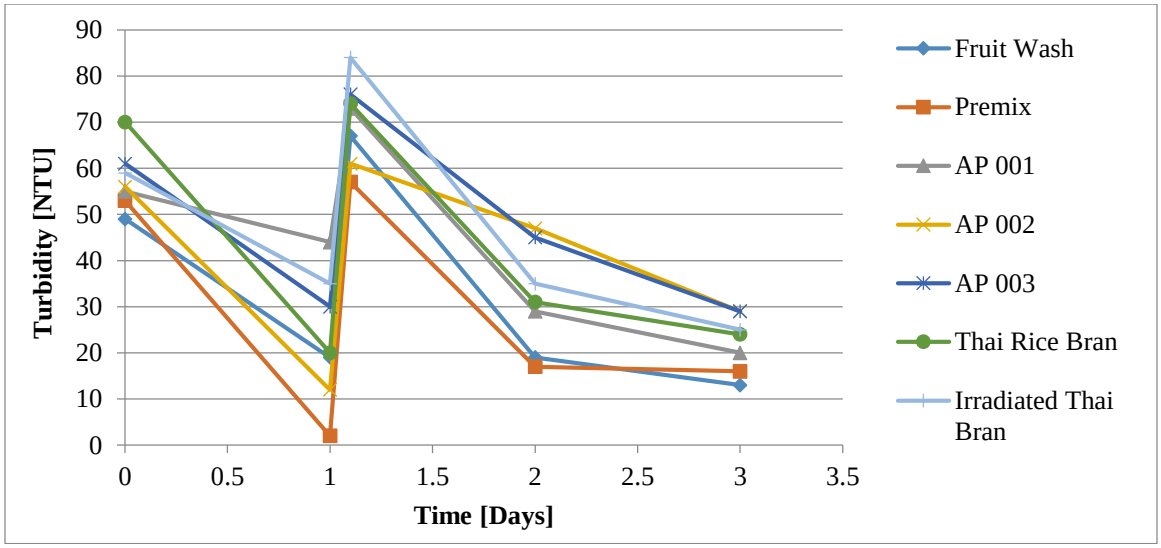


Figure 4-7: Experiment I-7 Turbidity vs. Time, Tap Water at 30°C, Re-dosed Sunscreen, Mechanical Filtration

After the first dose of sunscreen, Thai rice bran reduced turbidity by 71%, whereas Irradiated Thai rice bran reduced turbidity by 40%. This difference is probably not due to the native biology present in Thai rice bran, as no other experiments have shown significant

differences between biological treatments and abiotic treatments in the first 24 hours. The differences in clarification could be due to preexisting differences between the two batches of Thai rice bran, prior to irradiation.

The products AP 001, AP 002, and AP 003 displayed very high turbidity relative to Premix and Fruit Wash, due to particulate matter remaining in suspension. These products were not used in subsequent experiments.

4.1.8 Experiment I-8 Effects of Thai BiOWiSH Products on Re-Dosed Turbidity

Experiment I-8 investigated different Thai products relative to a control tank, including Premix, Thai FOG, and Irradiated Thai FOG. The control treatment was run to confirm the effects of mechanical filtration on sunscreen-induced turbidity without additives. Treatments were chlorinated daily to at least 3 mg/L of total chlorine.

For the first two doses of sunscreen, each product out-performed the control in terms of clarification, as shown in **Figure 4-8**. After the third dose of sunscreen, the best-performing treatment was the control; a trend which continued through to the termination of the experiment. As shown in **Figure 4-8**, the control tank consistently showed lower turbidity than the averages of the treatments after T=2.1 Days.

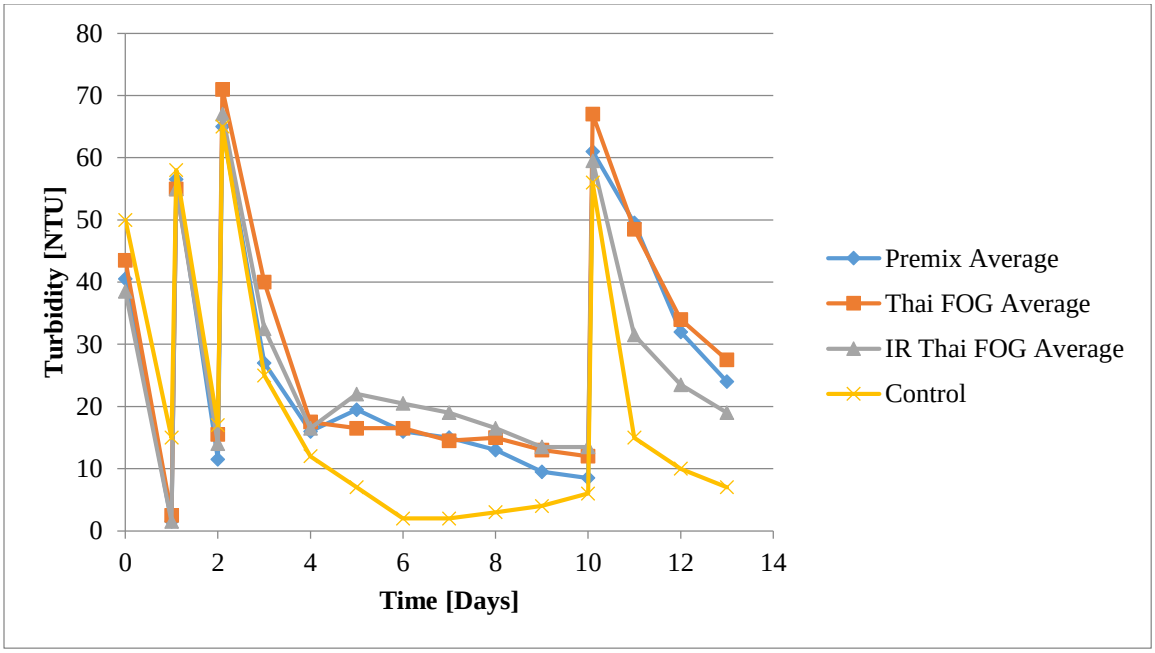


Figure 4-8: Experiment I-8 Averaged Turbidity vs. Time, Thai Products in Duplicate, Tap Water at 30°C, Re-dosed Sunscreen, Mechanical Filtration

The early removal of turbidity by all products confirms previous findings that solid substrate is able to assist in filtration or adsorption of sunscreen, without the assistance of bacteria. As time progressed and more sunscreen was added to each system, results for the treatments suggested that the products added turbidity to the water. The similar results for the various treatments suggest that effects on turbidity were independent of both the bacteria contained in the products and the post-fermentation additives present in Thai FOG and Irradiated Thai FOG.

The long-term results of this experiment, from T=3 Days until the termination of the experiment, coincide with those of Experiment I-3, in which the control consistently showed lower turbidity than treatments containing solid substrate after two doses of

sunscreen. In the short term, the control treatment showed less reduction than solid substrate materials.

There is a high chance that tanks received cross-contamination from each other, considering the length of the experiment and the fact that aquaria were set up side-by-side with open surfaces and filters that splashed when water levels ran low. Additionally, the tanks were dosed with chlorine levels that are low, relative to those needed for complete bacterial inactivation.

It is likely that the solid substrate materials are growing microbes and the turbidity created by the production of bacterial cells masks any biodegradation of sunscreen. The control tank showed improved turbidity reduction over time, relative to the other treatments.

At the end of the experiment, samples were collected and plated for bacterial quantification and colony morphology analysis (see Experiment III-2). Improper plating methods led to contamination and inconclusive results. Solid substrate was also collected from the filters of Premix 1 and Thai FOG 1 to be imaged with Scanning Electron Microscopy.

Products were imaged using SEM before and after use in this experiment. Images were captured between 400x and 20,000x magnification. **Figures 4-9** and **4-10** provide an overview of the differences between the appearances of the two substrates before and after use. Additional SEM images can be found in Appendix D. Prior to use, Thai FOG showed surface structure containing many rounded and jagged particles (**Figure 4-9**, **Left**). The

quantity and size of these particles is similar throughout the field of view, indicating homogeneity of surface structure within the immediate area on the sample. After use in the experiment, Thai FOG showed less distinct surface structure (**Figure 4-9, Right**). Filamentous material can be seen attached to both the flat and the jagged portions of the sample, which may be indicative of sunscreen adsorbing to surfaces of the particles. The Premix material showed a similar coated-looking surface structure, relative to its starting appearance (**Figure 4-10**). The electron microscopy provides more evidence that adsorption of sunscreen to the solid substrate is occurring.

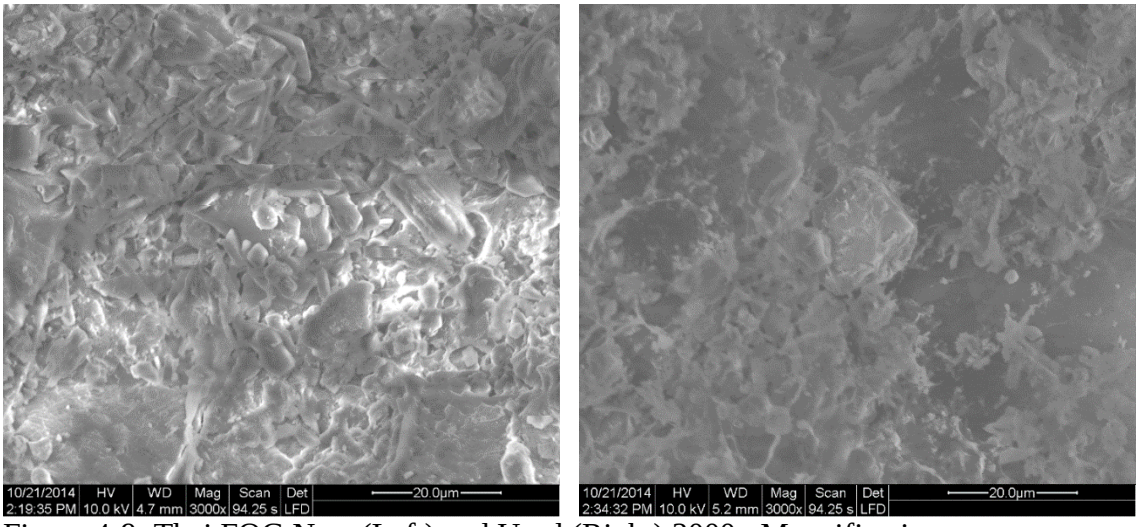


Figure 4-9: Thai FOG New (Left) and Used (Right) 3000x Magnification

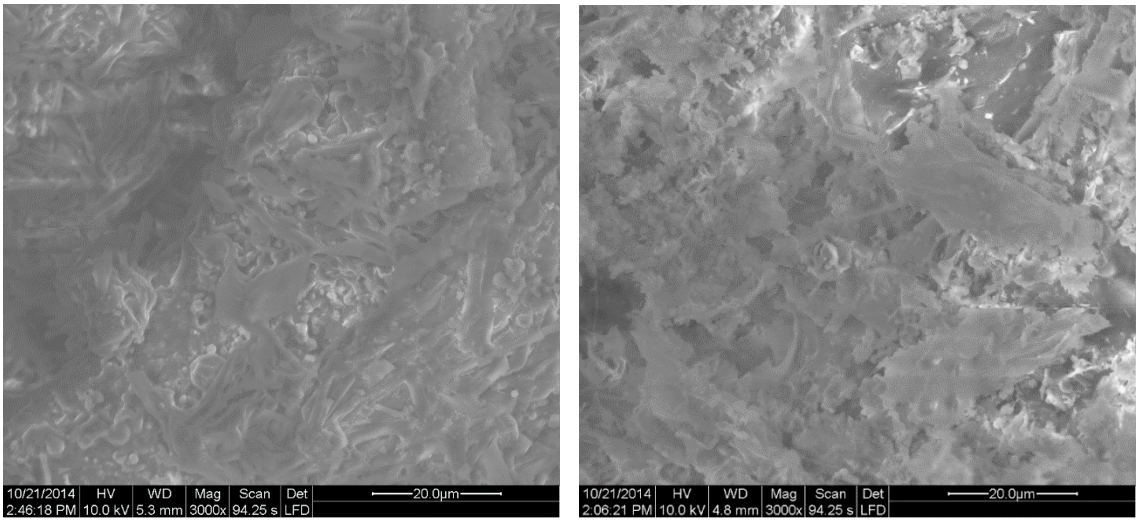


Figure 4-10: Premix New (Left) and Used (Right) 3000x Magnification

4.1.9 Experiment I-9 Effects of Vegetative Bacterial Cultures on Turbidity

Experiment I-9 was developed to investigate the effects of the biological components of BiOWiSH Cyanuric Acid Reducer (CAR) on turbidity with repeated dosing of sunscreen. Treatments were carried out in chlorinated and unchlorinated environments both with and without CYA. Additionally, one treatment was inoculated with a culture of the microflora collected from a spent swimming pool filter.

During incubation of the pool filter and manufactured bacterial cultures, intense gas production and turbidity increases were observed in all cases. This indicates successful activation and proliferation of the microbes.

The most effective and consistent treatment in reducing turbidity was the “Filter Media + CYA” control, as shown in **Figure 4-11**. This treatment was not dosed with sunscreen at T=0, but it was included in all subsequent re-doses. Its efficacy at removing sunscreen from

solution relative to other treatments is attributed to the assumed high biodiversity encountered on the swimming pool filter media. Highly diverse microbial populations will utilize multiple metabolic pathways and will use nutrients more quickly and effectively.

After T=7.1 Days, both of the non-chlorinated treatments which received sunscreen, "CAR + CYA" and "Filter Media + CYA", displayed the greatest reduction in turbidity. This could be attributed to microbial inhibition in chlorinated environments, which could result in slower rates of growth and thus fewer suspended bacteria to contribute to turbidity (Camper and McFeters 1979).

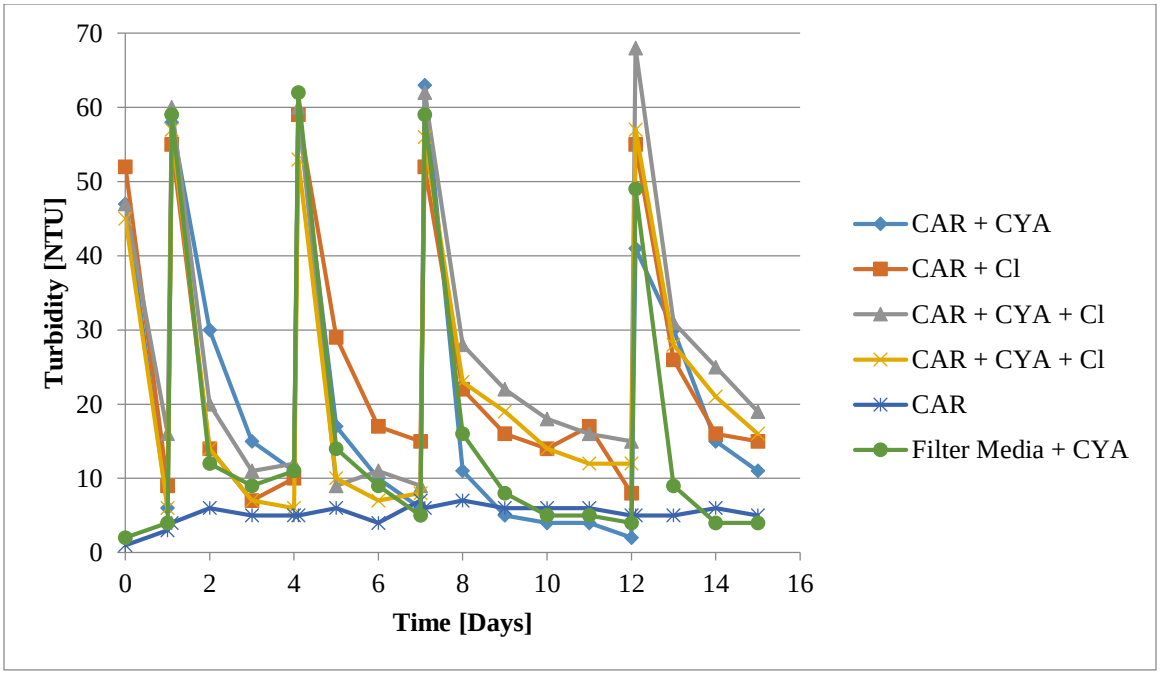


Figure 4-11: Experiment I-9 Turbidity vs. Time, Tap Water at 30ºC, Re-dosed Sunscreen, Mechanical Filtration, with and without CYA

As shown in **Figure 4-11**, the "CAR + CYA" treatment showed less turbidity removal than other treatments during the re-doses at T= 1.1 and T=12.1 Days. At these time-points, the

system was accidentally chlorinated by experimenters. The decreased response to turbidity was not expected, because each other chlorinated treatment showed steep reduction in turbidity at T=1.1 and T=12.1 Days.

Samples were periodically collected from each tank and analyzed with HPLC to track any changes in CYA concentration over time. Each tank containing CYA showed an increase in concentration between T=0 and T=2 Days (**Figure 4-11**). This is attributed to the stock solutions of CYA not being fully dissolved when dosed to each aquarium and the resulting continued dissolution of CYA. Due to the drift in CYA can be seen in **Figure 4-12**, no conclusions were made regarding the degradation or adsorption of CYA.

Two data-points (Tank 2, T=3 Days; and Tank 5, T=5 Days) were omitted from **Figure 4-12**, due to contamination in sampling.

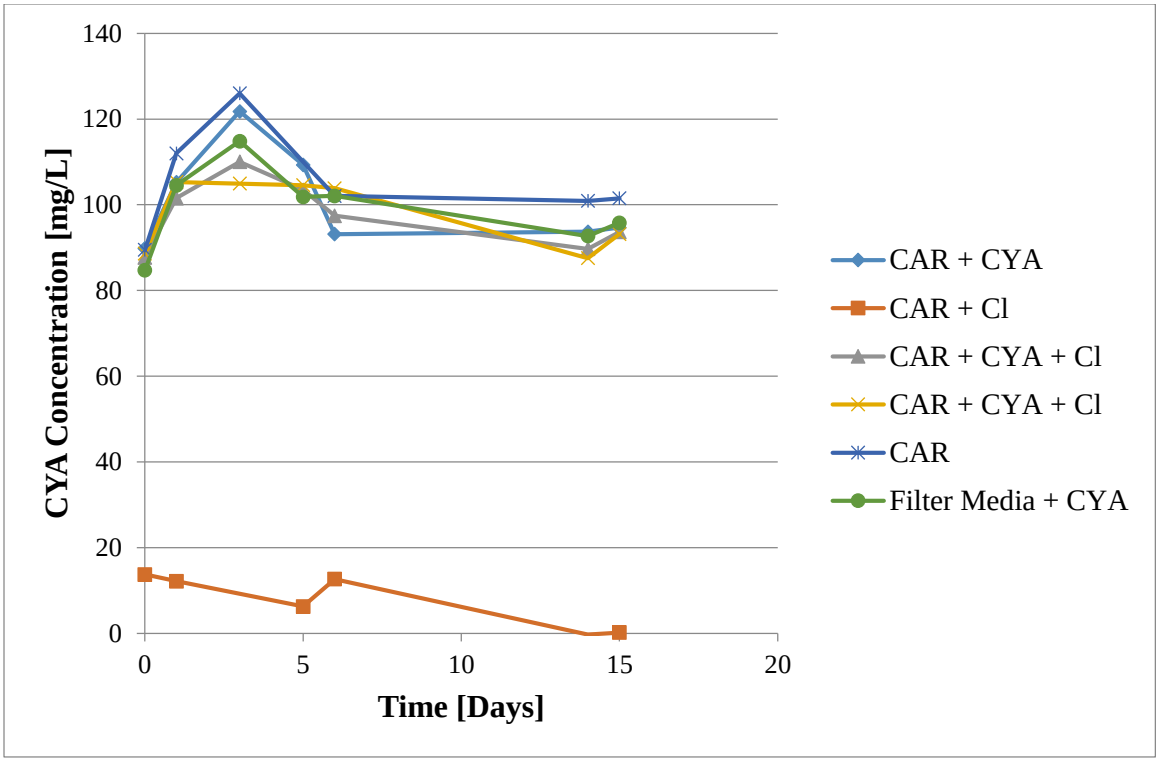


Figure 4-12: Experiment I-9 CYA Concentration vs. Time, Tap Water at 30°C, No CYA in Tank 2, Mechanical Filtration

Total Chlorine residuals were measured between 0.2 to 0.6 mg/L after the addition of CYA. Baseline TC was measured at 0.2 mg/L in the treatment without CYA, so it was determined that the CYA did not contribute significantly to the residual chlorine levels. This is to be expected, because CYA is mainly attributed to protecting chlorine from UV degradation. This experiment was run in a laboratory without windows, so UV radiation from the Sun was not a factor in chlorine reduction. All decreases in chlorine were likely from interaction with oxygen in the atmosphere or with microbes and sunscreen within the aquaria. Subsequent experiments were not dosed with CYA as a means of maintaining chlorine residuals.

4.1.10 Turbidity Reduction for All Experiments at T=1 and T=3 Days

Initial turbidity reduction data were compiled to show the relative effects of each product at T=1 Day in **Figure 4-13**. Early data points can be compared, because each experiment received the same initial dose of sunscreen. The only experiment excluded from this compilation is I-4 because T=0 data were not available.

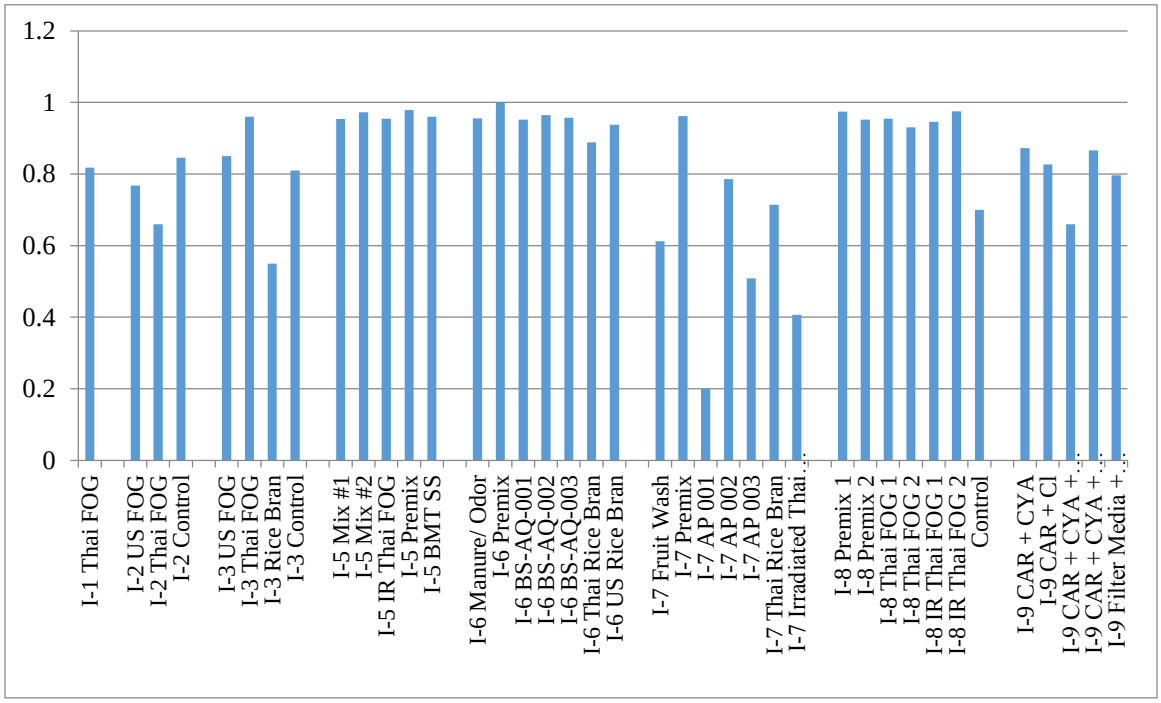


Figure 4-13: Compiled Percent Reduction in Turbidity Across Experiments, One Day after 1st Sunscreen Dose, Mechanical Filtration

Figure 4-13, above, shows that each control tank exhibited at least 70% removal of turbidity in the first day. When the effects of "Irradiated Thai FOG" treatments are compiled and compared to those of the control treatments, the additive effect of solid substrate on clarification can be observed. The average 1-Day percent removal of turbidity by control treatments is 79%, whereas the average 1-Day clarification by solid substrate products is 86%. Excluding the prototype products AP 001, AP002, and AP003, the

average 1-Day turbidity reduction increases to 90%. It can be concluded that the solid substrate of BiOWiSH products provides an additional 11% 1-Day reduction of turbidity over pure mechanical filtration.

The average initial reduction of turbidity by soluble diluent products is 79%. This is identical to the average percent reduction by pure mechanical filtration, indicating that products without a solid substrate do not enhance initial turbidity reduction.

For each experiment including a re-dose, the time-point one day past the second dose of sunscreen was also compiled to compare percent reduction of turbidity by each product tested. The experiments which are included in this comparison are I-3, I-5, I-6, I-7, and I-9, because they were dosed sunscreen on consecutive days at T=0 and T=1.

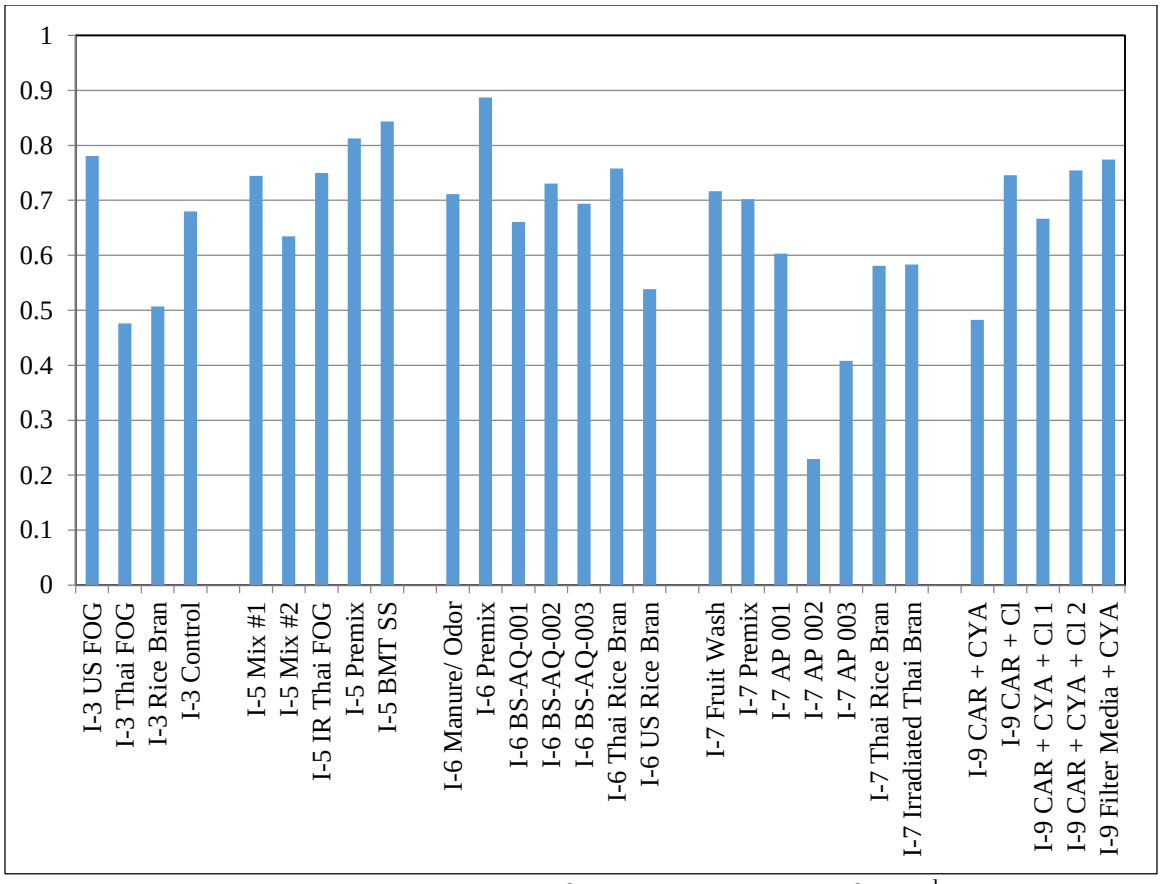


Figure 4-14: Compiled Percent Reduction of Turbidity, One Day after 2nd Sunscreen Dose, Tap Water at 30°C, Mechanical Filtration

The mechanism by which this added reduction occurs is probably mechanical, through adsorption to the substrate or enhanced filtration due to reduced filter pore size. As shown in the SEM images from Experiment I-8 (**Figure 4-9** and **Figure 4-10)** it is apparent that adsorption plays a role in the mechanical removal of sunscreen by the solid substrate. Experiments I-3, I-4, and I-8 showed that solid substrate products did not provide additional clarification after the second dose of sunscreen, possibly due to bacterial growth contributing to turbidity. Adsorption is likely the driving mechanism of additional turbidity removal provided by the solid substrate of BiOWiSH products.

4.2 Task II - Efficacy of BiOWiSH for Clarifying Oils from Swimming Pools

4.2.1 Experiment II-1 Water Clarification by Thai FOG, No Mechanical Filtration

Experiment II-1 was an investigation of water clarification by Thai FOG used to investigate the product's ability to grow in aqueous solution and remove turbidity induced by sunscreen without mechanical filtration. The colorimeter was not available at the time of this experiment, so UV absorbance was measured instead of turbidity. No change was seen in UV absorbance of the Thai FOG + Sunscreen treatment, relative to the Sunscreen treatment without Thai FOG. Additionally, biomass production interfered with optical density readings at 470 nm.

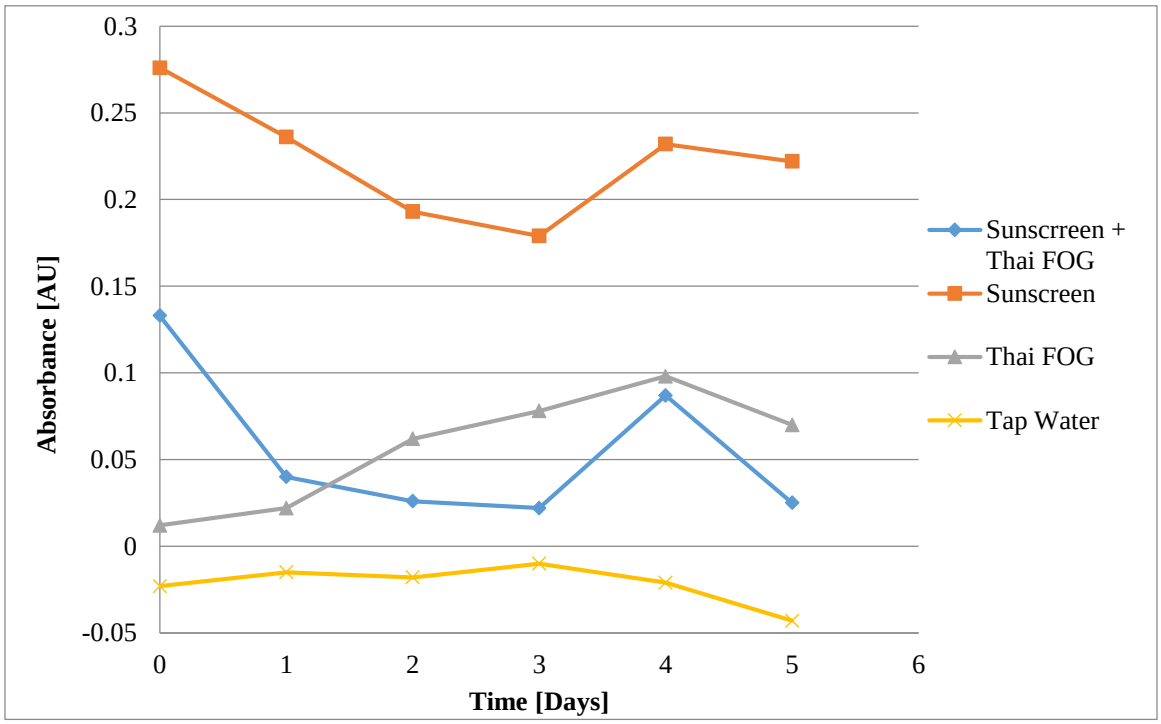


Figure 4-15: Experiment II-1 Absorbance vs. Time at 470 nm, Tap Water at 30ºC, Single Dose of Sunscreen, No Mechanical Filtration

Sunscreen dosed to Tank 1 did not completely dissolve, leading to a much lower starting turbidity than the other tanks. To prevent this issue, Tanks 2 and 3 were dosed sunscreen that had been dissolved into a small volume (10-50 mL) of the respective tank's water within a plastic weigh boat. The aqueous sunscreen mixture showed much greater level of dissolution with the tanks. This method was adopted for all subsequent sunscreen additions.

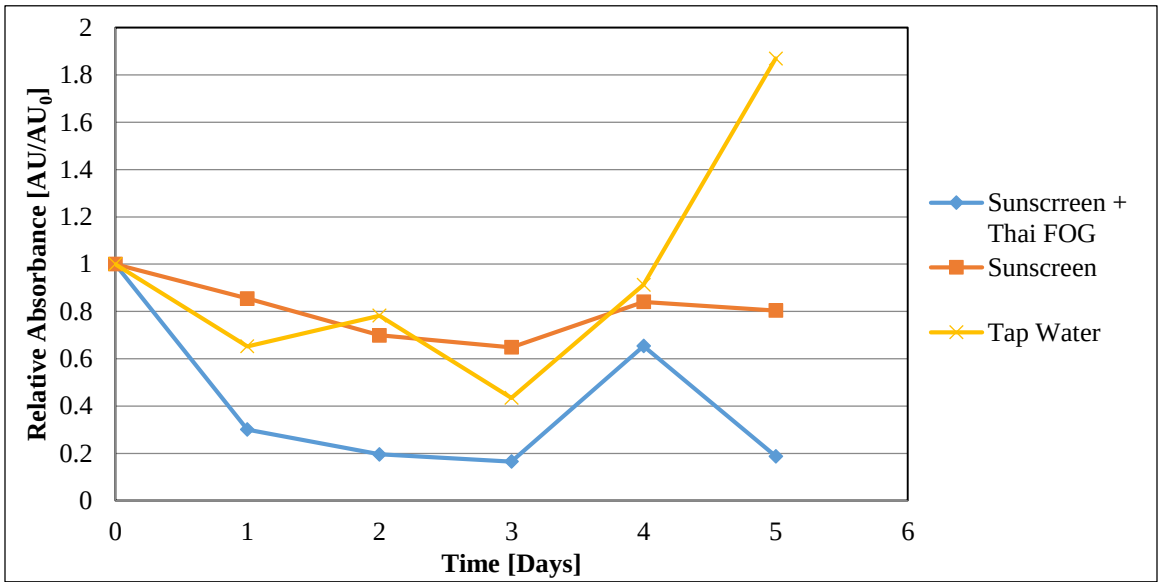


Figure 4-16: Experiment II-1 Normalized Absorbance vs. Time at 470 nm, Excluding Thai FOG, Tap Water at 30°C, Single Dose of Sunscreen, No Mechanical Filtration

Thai FOG showed a change in color and an increase in turbidity. The increased turbidity can probably be attributed to suspended particles in the form of microbial growth. The suspension of substrate was ruled out as a contributor to turbidity, because tanks were not agitated through the duration of the experiment.

An important finding from Experiment II-1 is that the Thai FOG product affects turbidity within the first 24 hours of inoculation. Thai FOG was able to reduce turbidity from sunscreen relative to a treatment of sunscreen without inoculum within the first 24 hours.

This result suggests that either the biology or the substrate of Thai FOG causes reduction of turbidity.

4.2.2 Experiment II-1.1 Absorbance Calibration Curve for Sunscreen

A calibration curve was developed for sunscreen in tap water at 520 nm (**Figure 4-17**). A baseline absorbance of 0.09 AU was observed.

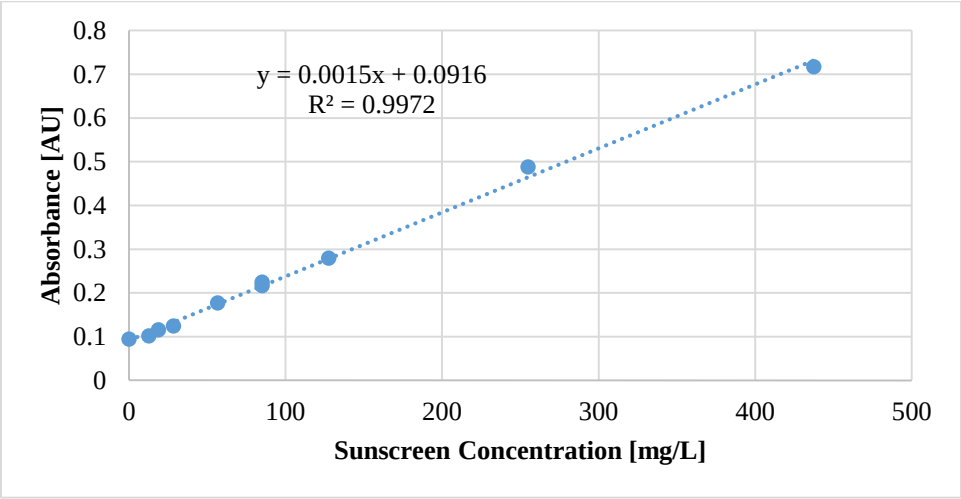


Figure 4-17: Experiment II-1.1 Absorbance vs. Concentration of Sunscreen at 520 nm

Due to the observed interference of BiOWiSH products and bacterial cells on optical density at 520 nm, this calibration curve can only be used to determine concentrations of abiotic trials. Turbidity was determined to be a more valuable indicator of water clarity than absorbance of pure sunscreen. Therefore, this calibration curve was not utilized in any analyses.

4.2.3 Experiment II-1.2 Investigation of Growth Media Compatibility with Chlorine

In order to determine which medium would be best to use in chlorinated experiments, three growth media were tested for compatibility with chlorine alongside tap water. Total chlorine as Cl_2 readings were taken over time. The 20-20-20 Fertilizer and MRS broth were ruled out as viable growth media, due to rapid scouring of chlorine. Based on 72-hour chlorine residual, tap water was the least interfering growth solution (**Figure 4-18**). Minimal media was also determined to be a suitable growth solution in chlorinated environments.

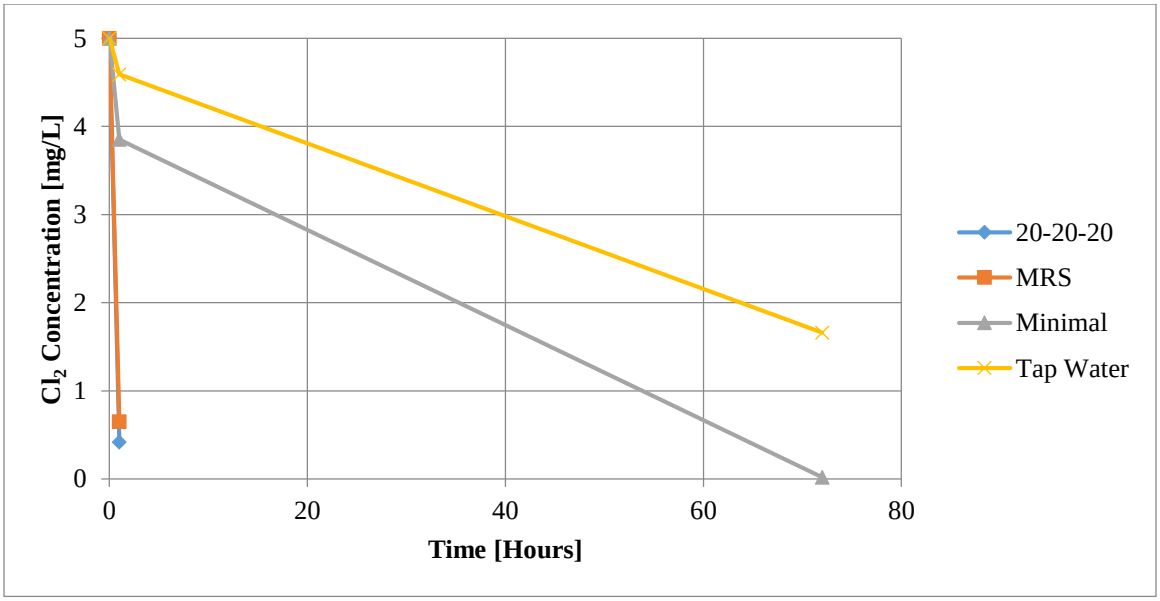


Figure 4-18: Experiment II-1.2 Chlorine Concentration vs. Time in Varying Growth Media, DI Water at 20°C

Hypochlorite is a strong oxidizing agent which readily reacts with organic and nitrogenous material. The fertilizer and MRS broth both have high carbon and nitrogen content, which

interferes with residual hypochlorite. It would be disadvantageous to utilize the fertilizer or MRS broth in chlorinated experiments.

Minimal media showed less interaction with chlorine than fertilizer or MRS broth; however it still caused a substantial decrease in chlorine relative to tap water. Tap water had an original chlorine content of 0.66 mg/L, indicating no chlorine demand and contributing to the higher residual over time. The use of municipal water closely mimics the method of pool filling for many domestic and professional pool operators.

BiOWiSH products contain sufficient glucose and substrate to support bacterial growth when added to tap water. Additional growth media may be advantageous but will not be required in subsequent experiments.

4.2.4 Experiment II-2 Chlorine Decay with BiOWiSH Products

A variety of products were placed in chlorinated environments to determine their effects on chlorine residuals. Total chlorine was measured at T=0, 4.5, 25 hours. Many treatments showed a decrease in chlorine concentration at T=0 after initial dosing of 3-4 mg/L Cl_2. At T=4.5 Hours, the only treatments which had not decreased below 0.5 mg/L were tap water and minimal growth media. At T=25 Hours, all chlorine concentrations had decreased to below 0.5 mg/L.

Every treatment reduced total chlorine concentration by a greater amount than tap water, because hypochlorite reacts with and oxidizes organic material. Minimal media showed the lowest drop in chlorine relative to tap water.

Increases in total chlorine concentrations were observed in several treatments, including those with sunscreen. These increases are attributed to noise in measurements from incomplete cleaning of the sample cell used. Samples containing sunscreen were noted to leave a residue on the interior of the sample cell. Subsequent experiments incorporated a light-duty tissue to wipe away sunscreen residue.

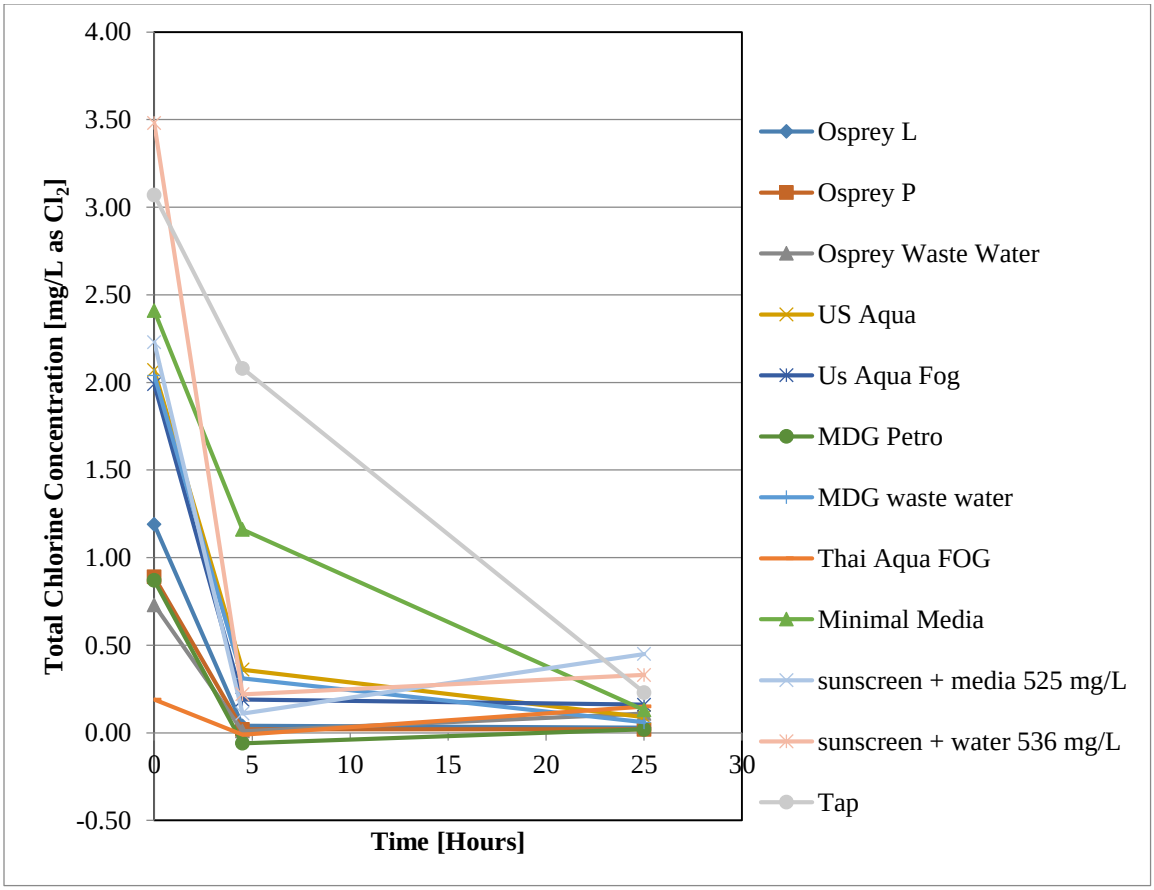


Figure 4-19: Experiment II-2 Chlorine Concentration vs. Time, DI Water at 20°C, Covered with Gas-Permeable Membrane, Single dose of Chlorine

The sharp decrease in chlorine for all treatments indicates that future experiments need to be chlorinated daily if residual chlorine is to be maintained at a certain level. Additionally, trials containing BiOWiSH products may require an initial dose of chlorine that is greater than subsequent daily dosing levels. Further investigation is necessary to determine daily doses of chlorine required for consistent residual levels.

4.2.5 Experiment II-3 Turbidity Reduction by Various BiOWiSH Products without Mechanical Filtration

Ten aquaria, each containing 250 mg/L of sunscreen and 125 mg/L of the BiOWiSH product listed below in **Table 4-2**, were maintained at 30^{o}C for 5 days.

Table 4-2: Experiment II-3 Aquaria Contents

Tank No.	Product
1	Osprey- Liquid
2	Osprey- Powder
3	Osprey- Wastewater Treatment
4	MDG Petro
5	MDG Wastewater
6	BMT WW
7	Thai FOG
8	BUS FOG
9	Fruit Wash
10	Control – DI Water

Each tank, including the control, showed a decrease in turbidity over time (**Figure 4-20**). The greatest turbidity drop relative to starting turbidity, 70%, was observed for the Thai FOG product, which greatly outperformed all other treatments. The control displayed a 37% reduction in turbidity. This was probably due to settling of material and the formation of an oily film on the surface of the tank. Osprey Liquid and Osprey WWT showed a smaller drop in turbidity than the control. BMT WW showed more clarification than the control early in the trial, but at T=114 hours, turbidity of BMT WW rose above that of the control. The T=68 Hours time-point showed a spike in turbidity from the Osprey Wastewater Treatment due to accidental agitation of the settled material at the bottom of the tank.

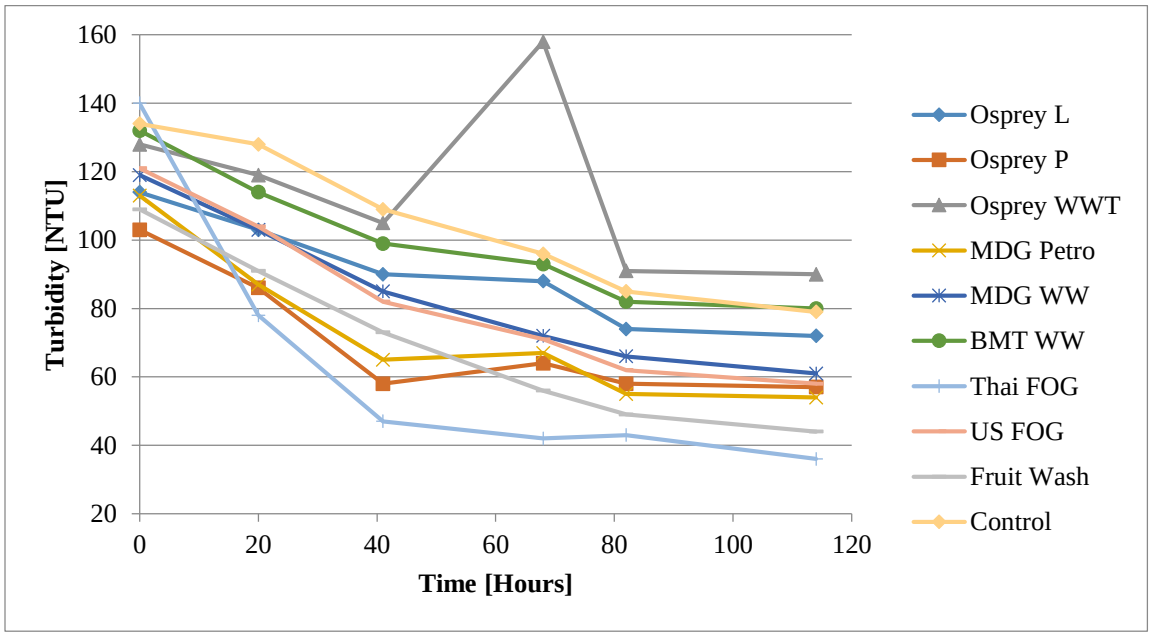


Figure 4-20: Experiment II-3 Turbidity vs. Time, Tap Water at 30°C, Single Dose of 250 mg/L Sunscreen, No Mechanical Filtration

The best-performing treatment was Thai FOG, followed by Fruit Wash, MDG Petro, and US FOG. Thai FOG is being phased out by BiOWiSH. As a result, it will not be considered

as a viable product for clarification of recreational pool waters. Instead, it was used as a positive control to which the performance of other products can be compared.

Every treatment, including control, showed a decrease in turbidity. This can be attributed to the hydrophobic nature of many components of sunscreen and its natural tendency to settle out from solution. The formation of an oily film on the surface of many tanks, coupled with large deposits on the bottom of each tank, indicate that settling plays a large role in clarification.

Chlorine was dosed daily at 0.2 to 0.6 mg/L per tank, which is below the required 1.0 mg/L available hypochlorite found in Title 22 Standards (California Code of Regulations 2015). Since there is no retention agent to provide lasting chlorine residual, all chlorine readings were close to zero by the following day. Previous experiments showed that biomass interferes with turbidity readings if growth is not inhibited by constant chlorination. Therefore, in subsequent experiments, chlorine was dosed at higher concentrations to ensure that a suitable residual is retained overnight.

4.2.6 Experiment II-4 Agitated vs. Un-Agitated Turbidity Reduction by BiOWiSH Products

Replicate treatments of the best-performing treatments from Experiment II-3, Thai FOG and Fruit Wash, were tested in this experiment to provide confirmation of previous results. Additionally, different batches of BMT WW (BMT WW1 and BMT WW2) were compared to Thai FOG and an un-amended control. Duplication of treatments was deemed important

due to potential heterogeneity of each product, the risk of contamination, and variability in day-to-day readings.

Altering methods from Experiment II-3, daily turbidity readings were taken before and after settled material was agitated. This was intended to provide a direct comparison within treatments with and without settling. Measuring turbidity after agitation and re-suspension of settled material showed an increase in turbidity of the Thai FOG treatment and a small decrease in turbidity of other treatments (**Figure 4-22**). No trends in turbidity were observed for any treatment.

Table 4-3: Experiment II-4 Treatments, Duplicated

Tank	Treatment
1	Control
2	Thai FOG
3	Fruit Wash II
4	Fruit Wash II
5	BMT WW1- I
6	BMT WW1- II
7	BMT WW2 - I
8	BMT WW2- II

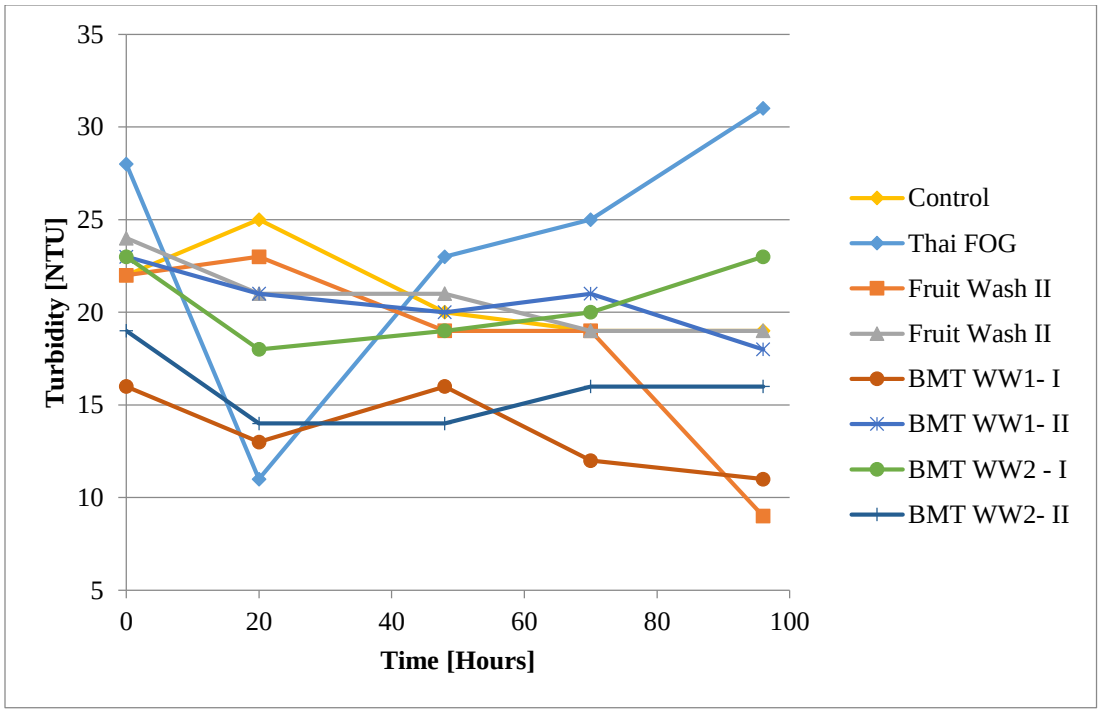


Figure 4-21: Experiment II-4 Turbidity vs. Time, Tap Water at 30ºC, Un-Agitated, Single Dose of Sunscreen, No Mechanical Filtration

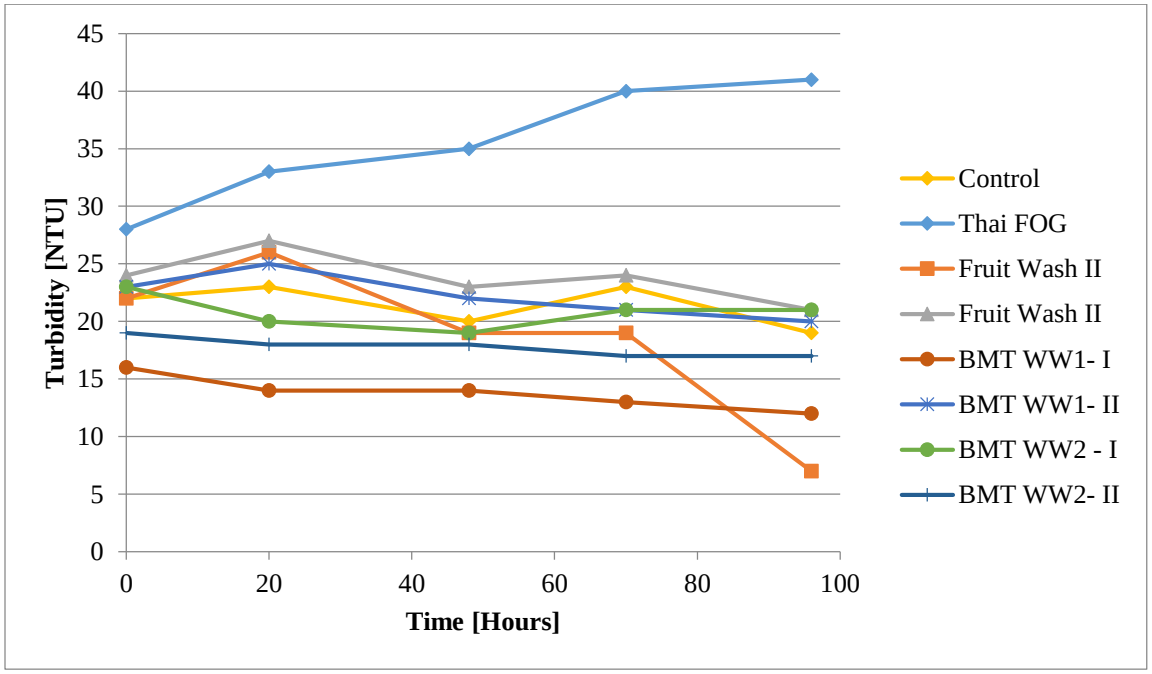


Figure 4-22: Experiment II-4 Turbidity vs. Time, Tap Water at 30ºC, Agitated, Single Dose of Sunscreen, No Mechanical Filtration

Table 4-4 shows the turbidity difference between agitated and un-agitated treatments for each time-point. Positive values indicate an increase in turbidity with agitation. The data show that settling accounts for a larger portion of turbidity reduction early in the trial, at T=20 hours. By T=70 and T=96 hours, agitation only contributes a small amount to turbidity of each treatment. Therefore, throughout the trial, material that can originally be suspended in solution is either removed or converted into material that does not re-suspend in solution. This, along with the slight downward trend in turbidity, may indicate that the biological components of BiOWiSH products are able to convert sunscreen into bacterial cells.

Table 4-4: Experiment II-4 Turbidity Change by Agitation

	Change in Turbidity [NTU]				
Tank	T=0 hours	T=20 hours	T=48 hours	T=70 hours	T=96 hours
Control	0	-2	0	4	0
Thai FOG	0	22	12	15	10
Fruit Wash II	0	3	0	0	-2
Fruit Wash II	0	6	2	5	2
BMT WW1- I	0	1	-2	1	1
BMT WW1- II	0	4	2	0	2
BMT WW2 - I	0	2	0	1	-2
BMT WW2- II	0	4	4	1	1

During initial chlorination, of each tank was dosed to an expected residual of 2.66 mg/L as Cl_2. Readings ranged from of 0.6 to 1.9 mg/L as Cl_2 depending on the level of chlorine scouring displayed by each product. The following day, as tanks were re-dosed with chlorine, enough chlorine was added to reach a 2.0 mg/L minimum in each tank.

The Thai FOG treatment, which displayed the lowest initial chlorine residual, exerts a high chlorine demand. Chlorine was dosed to an expected concentration of 3.5 mg/L as Cl_2, but readings were consistently close to 1.8 mg/L. The low chlorine levels may partially explain why Thai FOG showed a consistent increase in turbidity after the first day.

During turbidity sampling, it was noted that sunscreen builds up on the interior of the sample cell. A DI water rinse was employed between samples in this experiment. It was noted that loading the sample cell with DI water would read as high as 8 NTU, which accounted for between 19.5% and 114% of raw data turbidity readings. The sample cell cleaning method was modified to include 70% ethanol rinse and internal wipe with a light-duty tissue soaked in 70% ethanol, followed by a rinse with DI water.

4.2.7 Experiment II-5 Turbidity Reduction by BiOWiSH Products

Experiment II-5 was performed to investigate the effects on turbidity caused by the Lactic Mix product, BMT WW 1 and 2, pure spores of KLB, and KLB added to the BMT WW product. A positive control was run containing Thai FOG, and a negative control was run containing no inoculum. Due to time constraints, only four treatments were carried out past the T=50 hour time-point: Control, BMT KLB Mix I, BMT KLB Mix II, and Thai FOG.

Thai FOG showed the greatest level of clarification (87%), even after starting with the highest turbidity. The BMT KLB mix treatments returned inconsistent results. One treatment showed a 19% reduction in turbidity, while the other reduced turbidity by 48%.

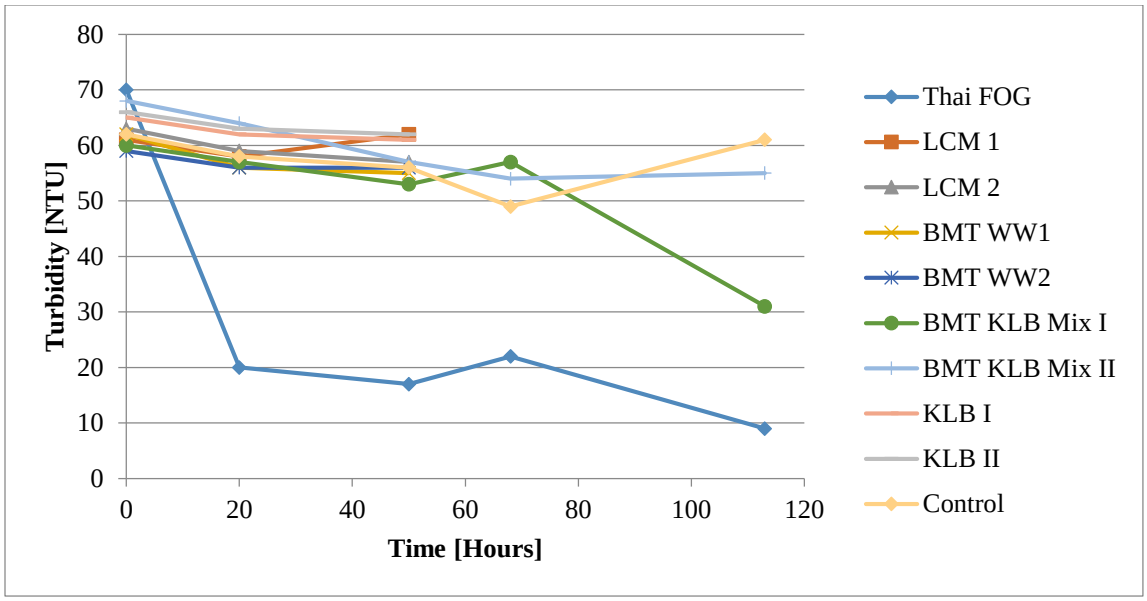


Figure 4-23: Experiment II-5 Turbidity vs. Time, Tap Water at 30°C, Single Dose of Chlorine, Combined Products, No Mechanical Filtration

This experiment showed that KLB mixed with BMT WW does not provide significant reduction of turbidity caused by sunscreen, relative to a control treatment. The treatment of Thai FOG was the only solid substrate-mounted product in the experiment, and it significantly out-performed all other treatments. This is consistent with previous findings, suggesting that biological effects on turbidity are minimal, and that the main effects of BiOWiSH on turbidity are due to solid substrate interacting with the sunscreen.

4.2.8 Experiment II-6 Effect of BiOWiSH Products on Turbidity with Dextrose

Experiment II-6 was performed to investigate the effects of product growth on turbidity. The data showed no trends in turbidity change for any product, relative to a control over 5 days of incubation (**Figures 4-24** and **4-25**). In a chlorinated aerobic environment with

dextrose as a carbon source, no product showed a consistent or significant change in turbidity.

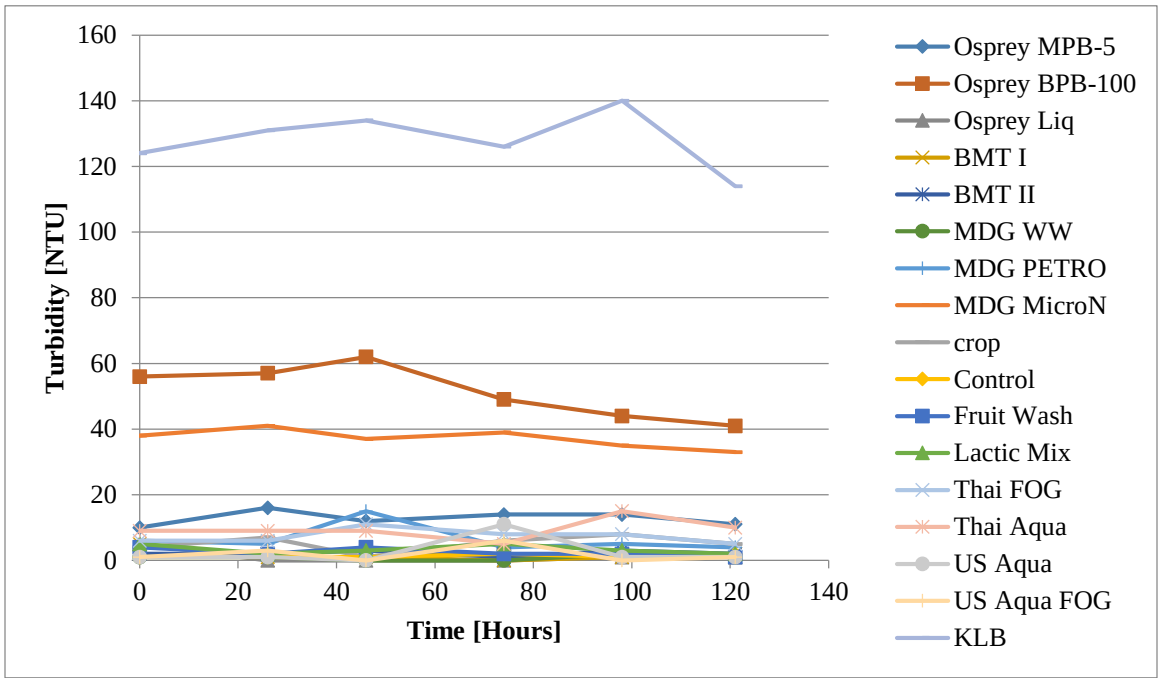


Figure 4-24: Experiment II-6 Turbidity vs. Time, DI Water and Dextrose at 20ºC, No Sunscreen, No Agitation

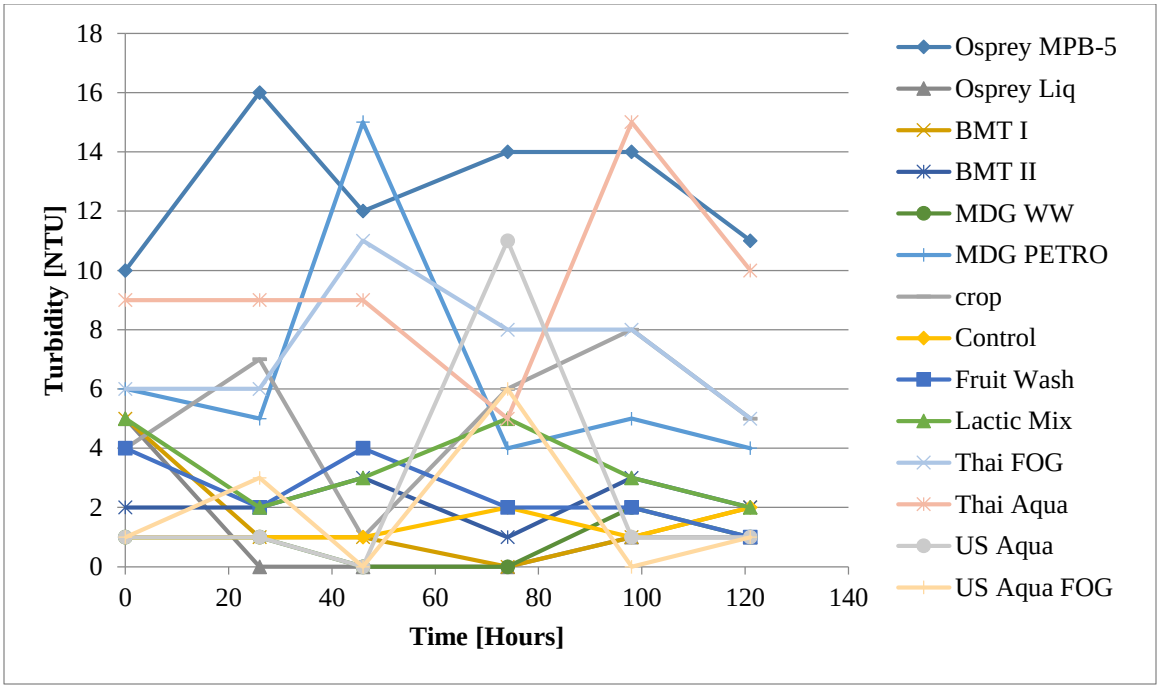


Figure 4-25: Experiment II-6 Turbidity vs. Time Excluding KLB, Osprey BPB-100, and MDG Micro-N, DI Water and Dextrose at 20°C, No Sunscreen, No Agitation

The experiment was carried out in 500 mL shaker flasks with low buffering capacity for fluctuations in temperature and chlorination. Without the nutrients necessary for growth, it is likely that many of the bacterial cultures did not propagate.

Previously, Experiment II-4 showed that the *Bacillus* in Thai FOG can flourish using just the solid substrate that the microbes are mounted on. This growth was not seen in the presence of constant chlorination at 3-5 ppm TC. It was noted that in swimming pools, free chlorine concentrations are usually no higher than 1 mg/L; therefore, this experiment was considered as a worst-case scenario for bacterial survival in chlorinated environments.

Samples from the US FOG, KLB, and Thai FOG treatments were plated for bacterial quantification and colony morphology investigation in Experiment III-1.

4.2.9 Experiment II-7 Effect of BiOWiSH Products on Turbidity with Sunscreen

Experiment II-7 was performed identically to Experiment II-6, but the carbon source (200 mg/L dextrose) was replaced with 200 mg/L sunscreen. **Figure 4-5** shows an increase in turbidity over time for each flask, except the MDG Micro-N. The control showed one of the greatest increases in turbidity, relative to its starting value.

Table 4-5: Experiment II-7 List of Treatments

Flask	Label
1	Control
2	Osprey MPB-5
3	Osprey BPB-100
4	Osprey MPB-5 Liq
5	BMT WW 1
6	BMT WW 2
7	MBWWT#1
8	MDG Petro
9	MDG Micro-N
10	Crop
11	Fruit Wash
12	LCM
13	Thai FOG
14	Thai Aqua
15	US Aqua
16	US Aqua FOG
17	KLB

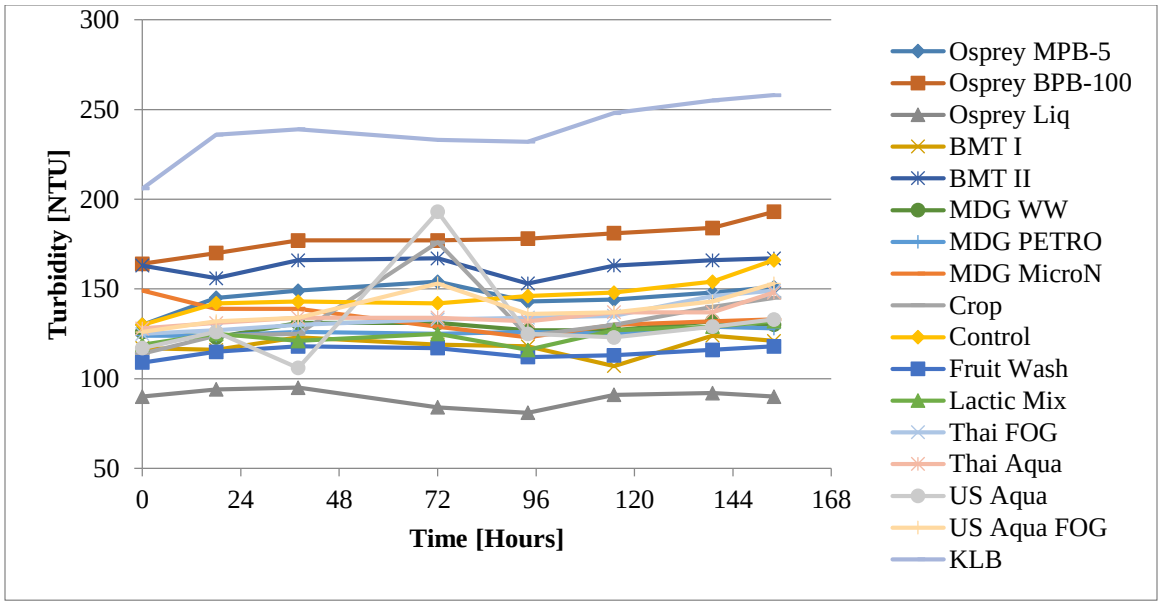


Figure 4-26: Experiment II-7 Turbidity vs. Time, DI Water at 20°C, Single Dose of Sunscreen, Aerobic

Each flask was agitated before sampling to ensure representative collection of samples from each treatment. Components of sunscreen typically separate from solution, but agitation prevented. The increase in turbidity of the control flask is probably due to more complete emulsion and dissolution of sunscreen components over time.

The re-suspension of particulate matter masked settling as a potential mechanism of clarification. Since each treatment showed a smaller increase in turbidity than the control, it is possible that the biology within each flask was able to metabolize sunscreen. The MDG Micro-N treatment showed a decrease in turbidity. The most feasible explanation is that the flask became contaminated during sampling and bacteria encountered the proper nutrients required for degradation of sunscreen without a sizeable increase in turbidity. While contamination is very likely, degradation cannot be confirmed as a mechanism of clarification at this point.

4.3 Task III - Isolation and Identification of Bacteria

4.3.1 Experiment III-1 Bacterial Plating of Experiment II-6

Flasks from Experiment II-6 which demonstrated an increase in turbidity over five days of chlorinated incubation were plated at dilutions ranging from 10^{-1} to 10^{-12}. Plates were enumerated after 48 hours of incubation at 35ºC.

A control plate resulted in zero colonies, denoting uncontaminated agar. One dish was plated with an undiluted sample from the Micro Nutrient flask, which returned $1.4x10^{4}$ CFU per 100 mL. This flask was expected to be abiotic, which raises questions regarding the sterility of chlorination and sampling or the purity of the available Micro Nutrient product.

Table 4-6: Experiment III-1 Plate Count CFU per 100 mL after 48 hours, PCA

	CFU/100 mL at each Dilution							
Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}
Thai FOG	$1.2x10^{6}$	$8.5x10^{5}$	$3.5x10^{6}$	$9.0X10^{7}$	$5.0x10^{9}$	$5.5x10^{11}$	$2.0x10^{13}$	$1.0x10^{15}$
US FOG	$9.5x10^{4}$	$5.6x10^{6}$	$1.5x10^{8}$	$4.3x10^{8}$	$3.1x10^{10}$	$9.9x10^{12}$	$5.8x10^{14}$	$2.5x10^{15}$
KLB	$4.0x10^{4}$	$4.5x10^{5}$	$3.8x10^{7}$	$6.0x10^{7}$	$5.0x10^{9}$	$5.0x10^{11}$	$2.5x10^{13}$	$1.0x10^{15}$

The same two types of bacterial cultures were seen predominantly in each flask, with several anomalous colonies forming. Due to the high biodiversity and unknown composition of Thai FOG, little can be said regarding the bacterial species of each colony

which formed. The fact that the monoculture of KLB showed multiple colonies indicates cross-contamination from other flasks.

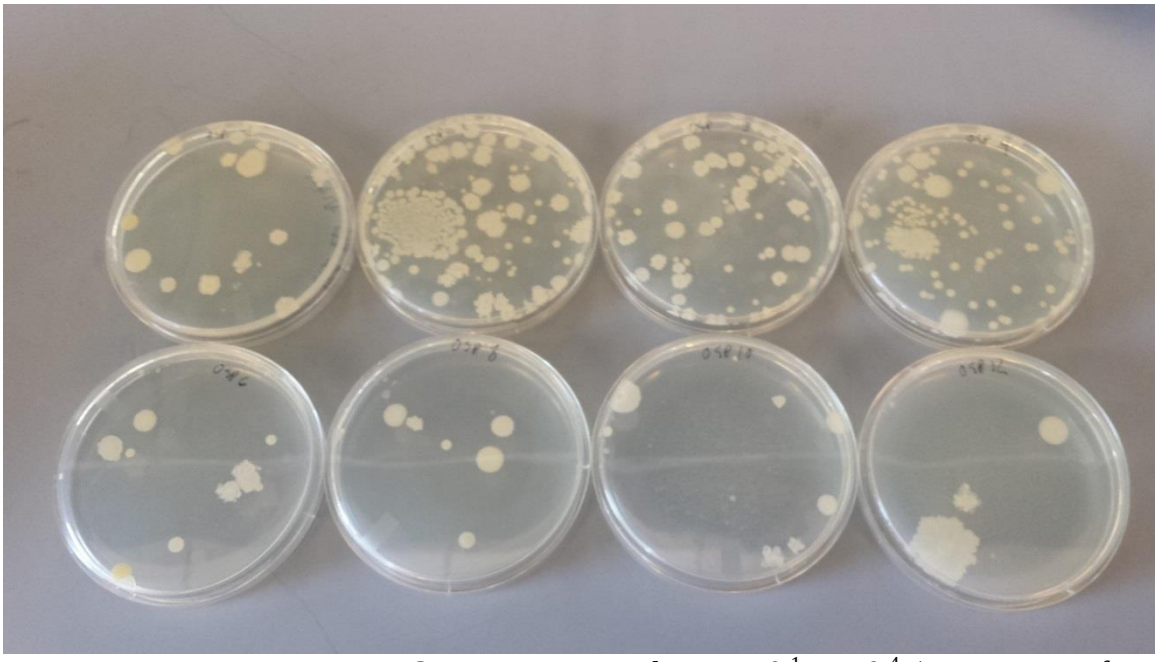


Figure 4-27: Experiment III-1 Osprey MBP-5 Dilutions 10^{-1} to 10^{-4} (Top Row, Left to Right), 10^{-6} to 10^{-12} (Bottom Row, Left to Right)

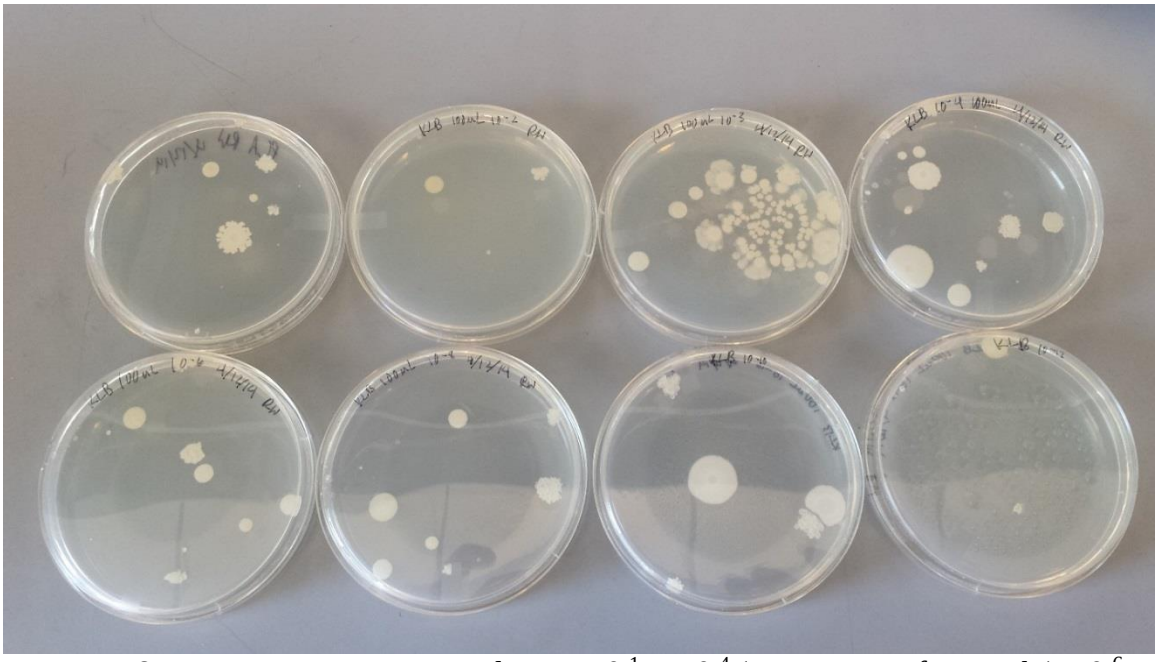


Figure 4-28: Experiment III-1 KLB Dilutions 10^{-1} to 10^{-4} (Top Row, Left to Right), 10^{-6} to 10^{-12} (Bottom Row, Left to Right)

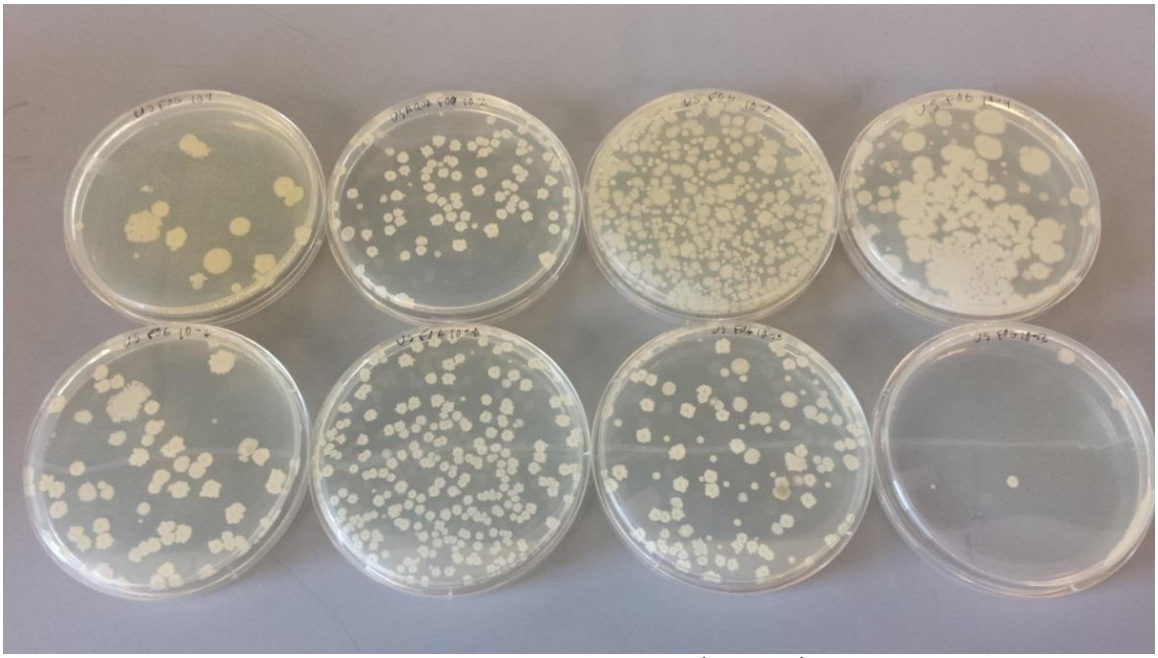


Figure 4-29: Experiment III-1 US FOG Dilutions 10^{-1} to 10^{-4} (Top Row, Left to Right), 10^{-6} to 10^{-12} (Bottom Row, Left to Right)

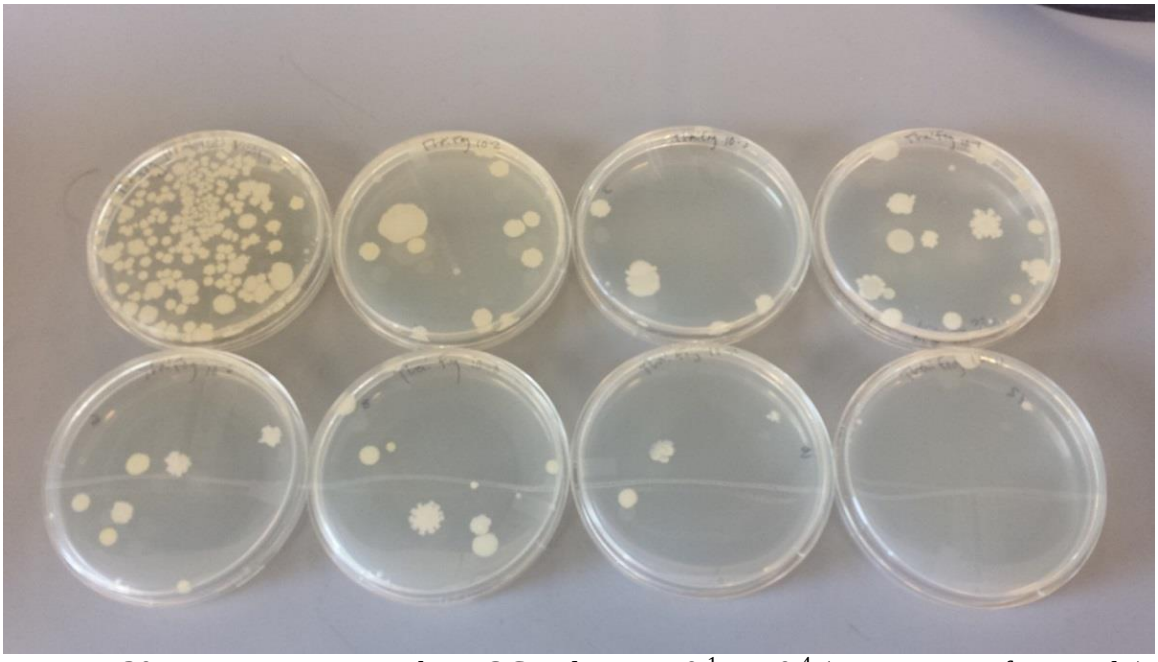


Figure 4-30: Experiment III-1 Thai FOG Dilutions 10^{-1} to 10^{-4} (Top Row, Left to Right), 10^{-6} to 10^{-12} (Bottom Row, Left to Right)

Each plate, including the monoculture of KLB, had two distinct types of colonies growing. **Figure 4-31** shows the Thai FOG 10^{-4} and US FOG 10^{-1} plates side-by-side. The prevalence of multiple bacterial colony types indicates contamination between treatments.

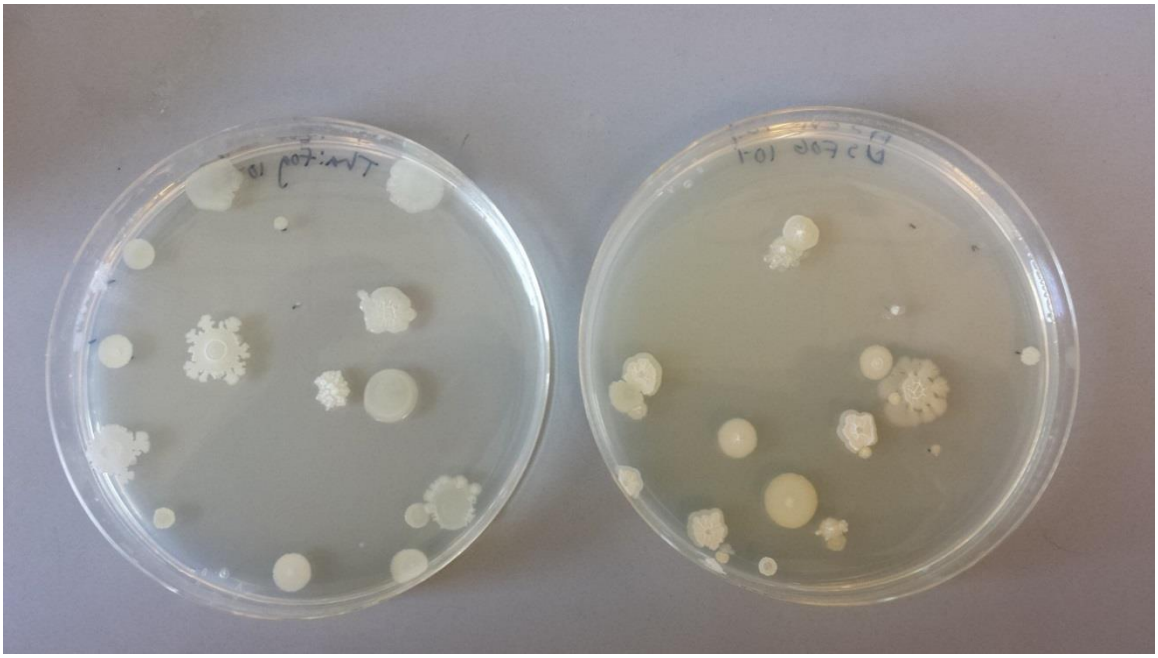


Figure 4-31: Experiment III-1 Comparison of Varied Colony Structure in Thai FOG (Left) and US FOG (Right)

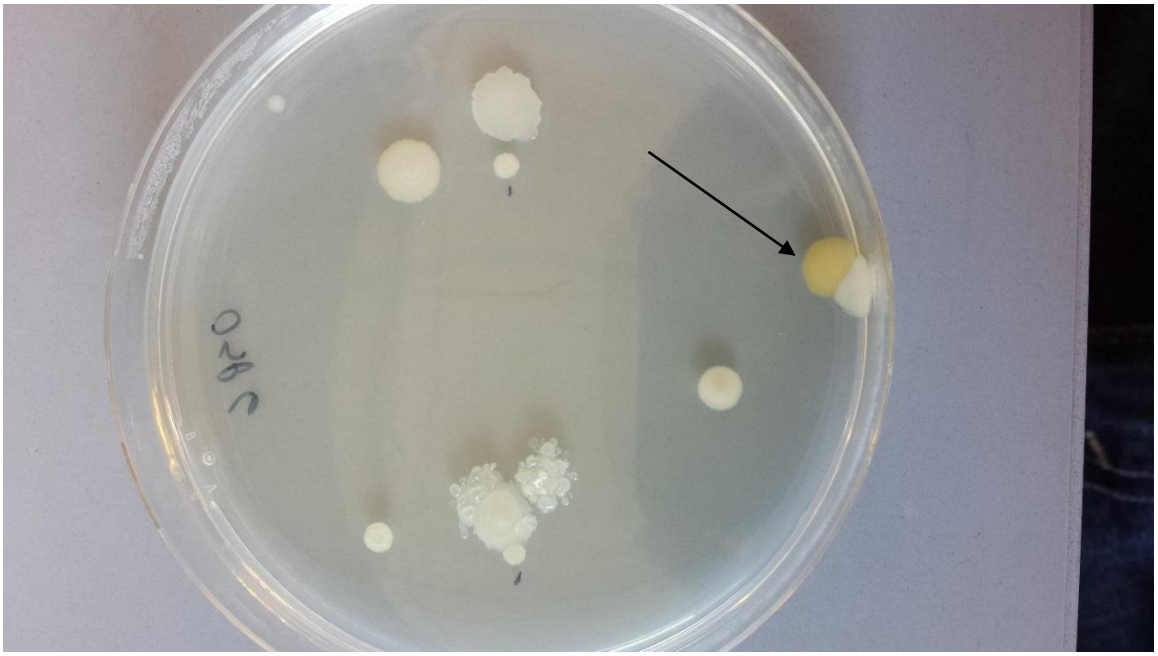


Figure 4-32: Experiment III-1 Orange Colony within the Osprey Product.

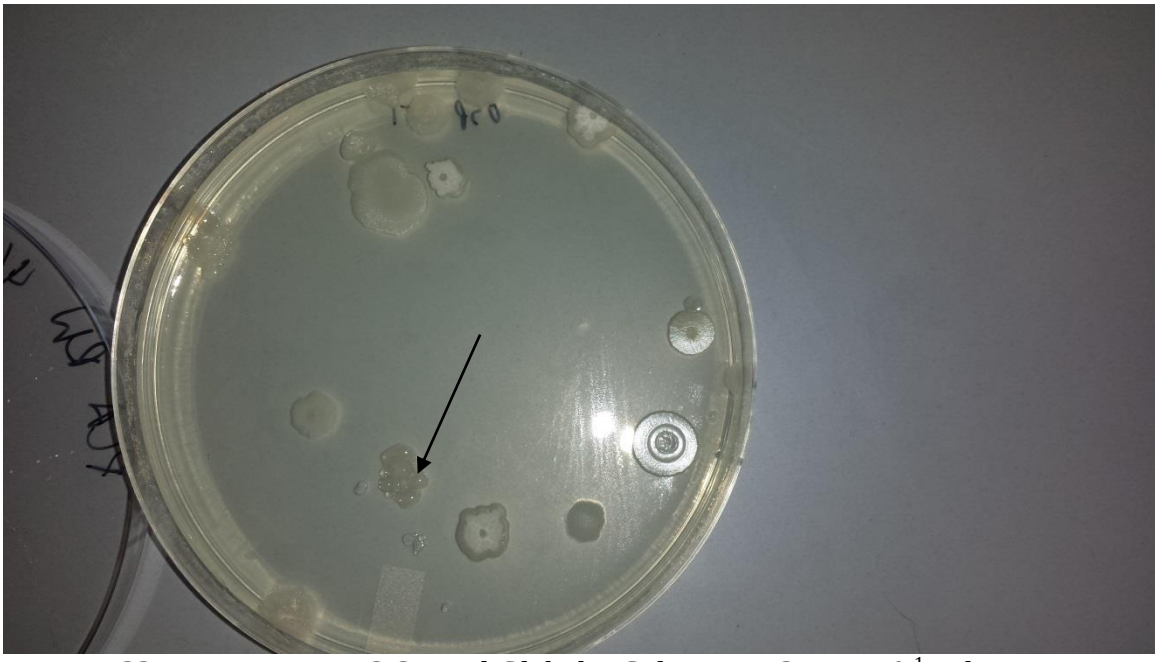


Figure 4-33: Experiment III-3 Several Globular Colonies in Osprey 10^{-1} Dilution

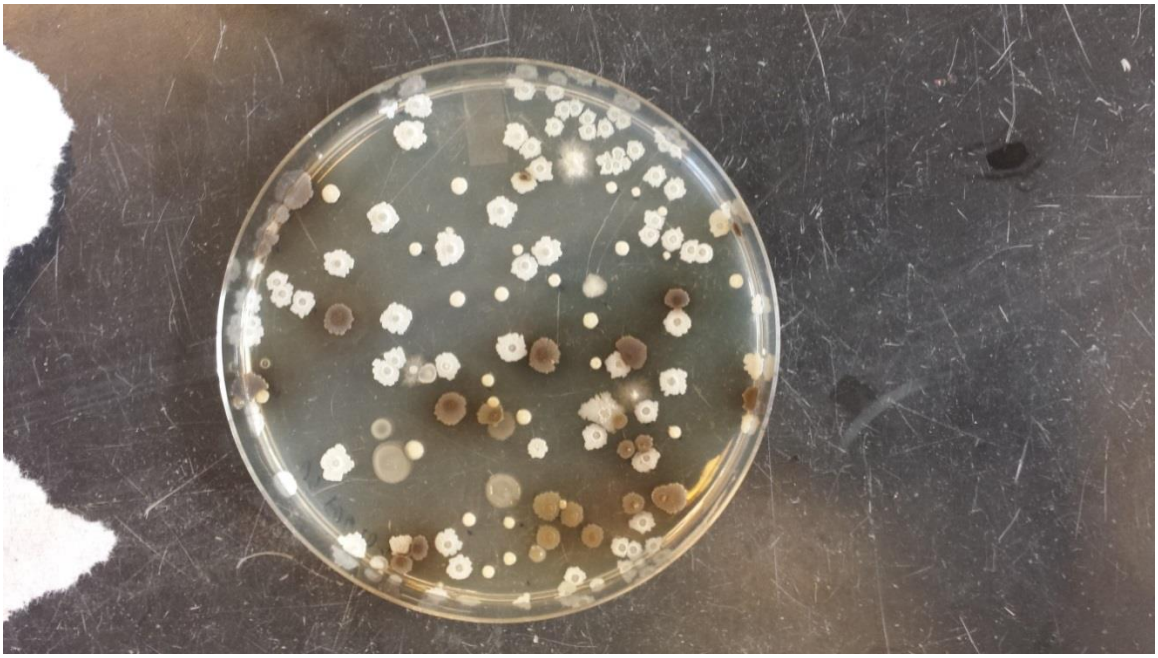


Figure 4-34: Experiment III-1 Black Colonies after 4 Days' Incubation at 35^{o}C

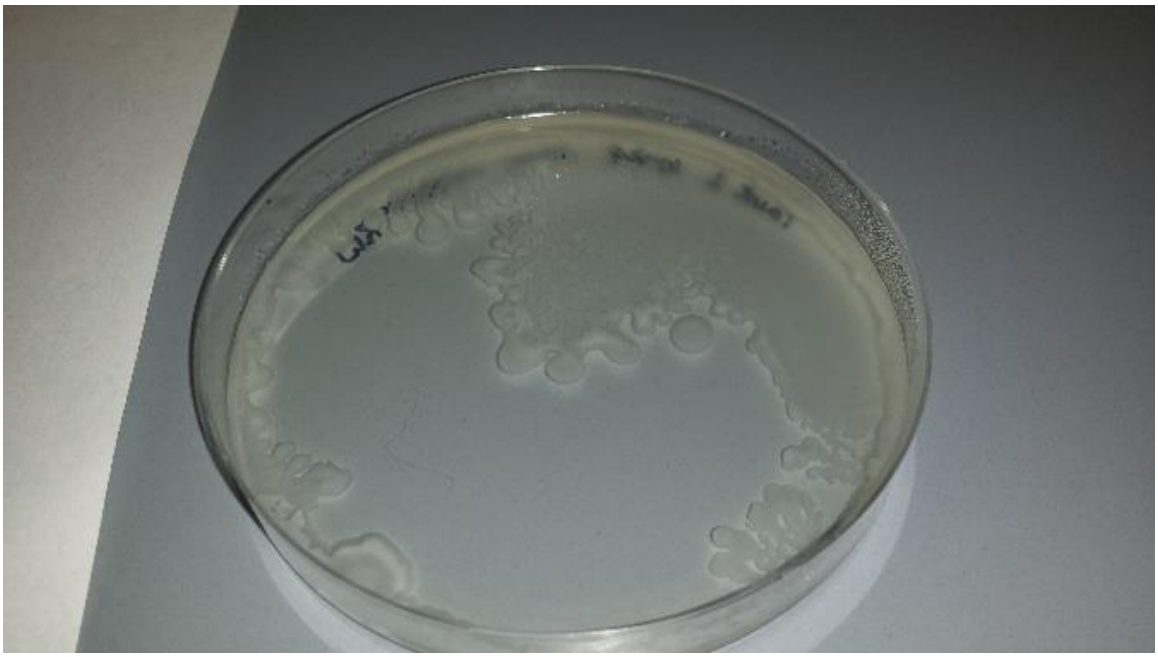
4.3.2 Experiment III-2 Bacterial Plating of Experiment I-8

Samples from treatments of Premix, Thai FOG, Irradiated Thai FOG and Control were plated on PCA and incubated for 48 hours. DI Water used for dilution and plating was not autoclaved. The control plate resulted in $2.5x10^4$ CFU per 100 mL, invalidating the test.

Table 4-7: Experiment III-2 Plate Count Results

	Plate Count		
Tank	10^{-16}	10^{-20}	10^{-28}
1	TNTC	TNTC	TNTC
3	TNTC	TNTC	TNTC
5	3	TNTC	TNTC
7	TNTC	TNTC	TNTC
Control	0	0	0
DI Blank	25		

Figure 4-35: Experiment III-2 Control Treatment, Plate 10-28 Dilution



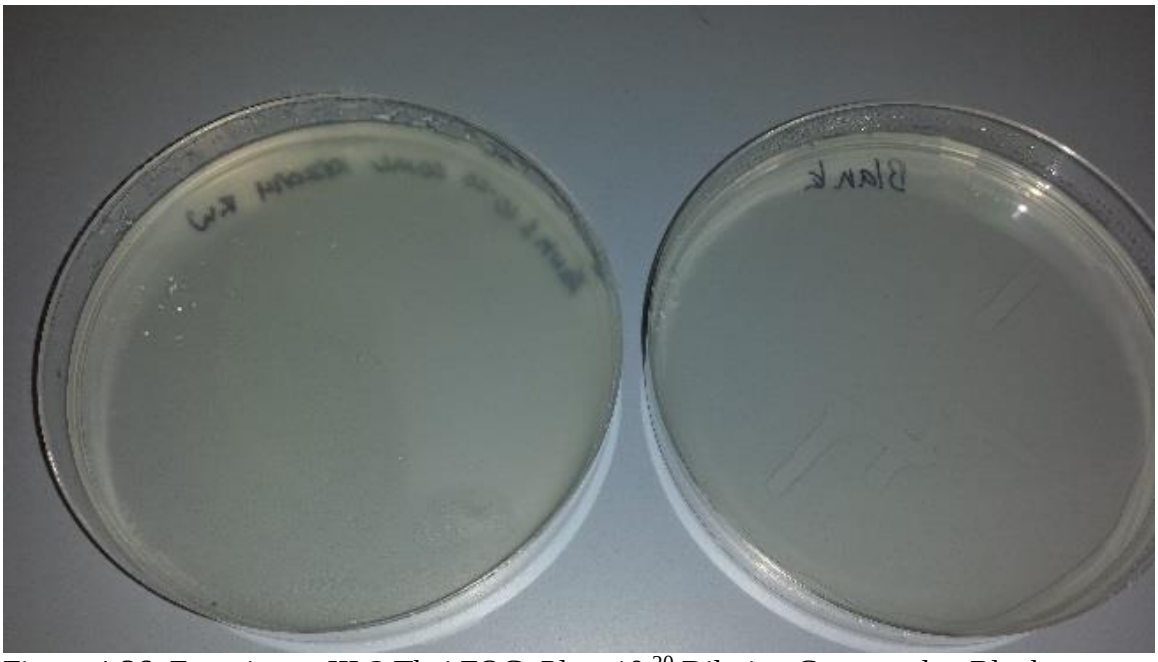


Figure 4-36: Experiment III-2 Thai FOG, Plate 10^{-20} Dilution Compared to Blank

No conclusions were drawn, due to the contamination of the test.

4.4 Task IV - Biodegradation of Cyanuric Acid

4.4.1 Experiment IV-1 CYA Biodegradation in a Respirometer

Experiment IV-1 was developed to investigate CO_2 production by BiOWiSH Thai FOG and US FOG in the presence of glucose and CYA. Due to repeated electrical and mechanical failures, no meaningful data were collected in this experiment. The respirometer was not used in subsequent experiments.

4.4.2 Experiment IV-2 CYA Adsorption to Irradiated Thai FOG

The purpose of Experiment IV-2 was to record the reduction of CYA over time by the substrate of Irradiated Thai FOG, independent of biological interactions. Irradiated Thai FOG was added to each of 5 shaker flasks at concentrations ranging from 50 mg/L to 300 mg/L.

A weak trend in CYA concentrations was observed over 72 hours with respect to irradiated Thai FOG concentration (**Figure 4-37**). The 300 mg/L Thai FOG treatment maintained the lowest CYA concentration among trials, and the 50 mg/L Thai FOG treatment displayed the highest CYA concentration throughout the experiment. An initial drop of CYA was seen in treatments of 100 mg/L and higher, but each treatment (other than 50 mg/L) showed an increase in CYA concentration after the 24-hour time-point.

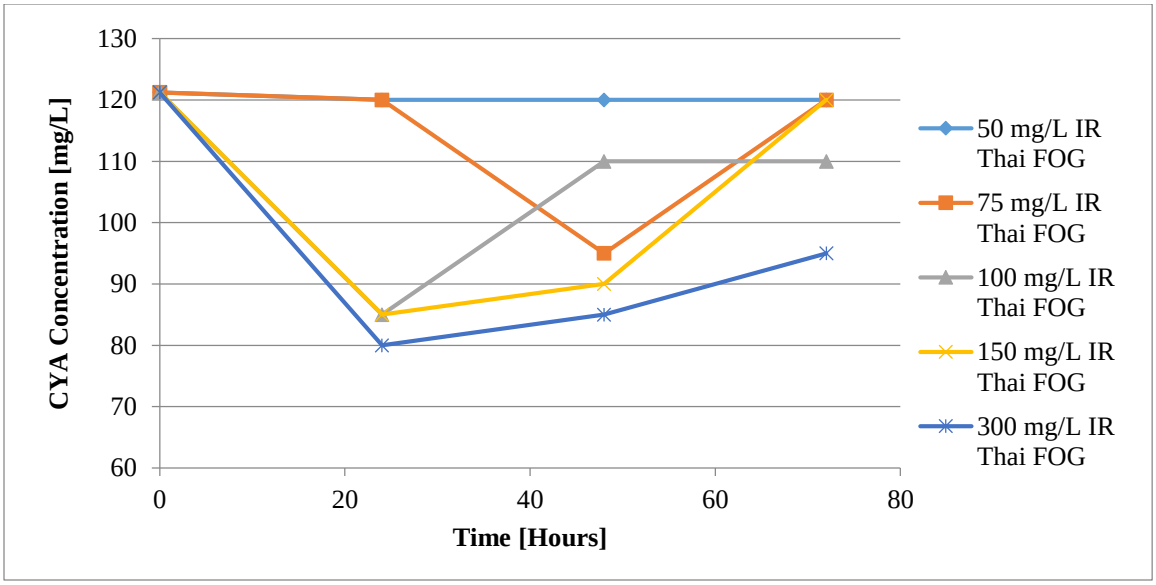


Figure 4-37: Experiment IV-2 CYA Concentration vs. Time, Turbidimetric Measurement, Varied Irradiated Thai FOG Concentrations, DI Water at 18-20°C, No Agitation, Anaerobic

Samples were diluted by a factor of 5 to avoid surpassing the limit of the test (60 mg/L). Variation in CYA readings showcased the inaccuracy of the test when compounding low resolution (+/-1 mg/L) with sample dilution.

The fact that the highest and lowest concentrations of Irradiated Thai FOG showed the lowest and highest CYA concentrations, respectively, indicates some form of concentration-dependent interaction between the two substances. Different methods of CYA measurement would be necessary to differentiate between true adsorption and noise in measurement. At this point, the HPLC system was not functional.

4.4.3 Experiment IV-3 Anaerobic Degradation of CYA in DI Water

Each treatment, excluding the control, was dosed with 50 mg/L of CYA. BiOWiSH products were dosed dry, at 50 mg/L. DI water was used in order to prevent interaction between CYA and residual chlorine found in tap water. **Table 4-8** shows data for one hour post-inoculation and after 9 days. The experiment was carried out anaerobically on a lab bench at ambient temperature which varied between 18^{o}C and 22^{o}C.

Since only two data points were collected for each trial, no definitive conclusions can be drawn from the results. US FOG amended with 200 mg/L glucose showed an average of 66% difference in CYA compared to US FOG with 50 mg/L glucose which showed an average of 32% difference. This implies that glucose, as a carbon source, may act as a limiting factor in the biodegradation of CYA.

Table 4-8: Experiment IV-3 CYA Raw Data

		Cyanuric Acid Concentration [mg/L]	
Flask	Flask Contents	T=0 Days	T=9 Days
1	US FOG, 200 mg/L Glucose	35	7.5
2	US FOG, 200 mg/L Glucose	37.5	17.5
3	US FOG, 50 mg/L Glucose	37.5	25
4	US FOG, 50 mg/L Glucose	40	27.5
5	Thai FOG, 200 mg/L Glucose	19	2.5
6	Thai FOG, 200 mg/L Glucose	46	2.5
7	Thai FOG, 200 mg/L Sunscreen	37.5	30
8	US FOG, 200 mg/L Sunscreen	40	25
9	Cyanuric Acid	65	67.5
10	Water	0	0

Initial CYA measurements varied across treatments, between 19 and 65 mg/L. This is attributed to the low sensitivity of turbidimetric CYA measurement and the inconsistency of individually preparing low-concentration CYA solutions for each treatment. The variation in initial concentrations is not due to adsorption of CYA. Three treatments of Thai FOG showed varying starting values of CYA, even though they contained the same concentrations of Thai FOG. Additionally, the five treatments of US FOG showed reduced levels of CYA without containing the solid substrate that would be responsible for adsorption.

Starting with Experiment IV-5, stock solutions of CYA will be prepared and added to each treatment, providing more consistent initial concentrations and readings.

4.4.4 Experiment IV-4 Anaerobic Degradation of CYA in Minimal Media

Experiment IV-4 replicated Experiment IV-3, but a minimal growth media was used, rather than DI water. Similar to Experiment IV-3, a high degree of variation was observed in initial CYA values. Each treatment was dosed with 15 mg of CYA and shaken, but they were not heated to expedite CYA dissolution. As a result, four of the nine treatments containing CYA showed an increase in CYA concentration over the first day of incubation. This is attributed to the remainder of CYA dissolving during 24 hours at elevated temperature and shaking at 75 rpm. Turbidimetric readings of CYA were taken over 14 days (**Figure 4-38**).

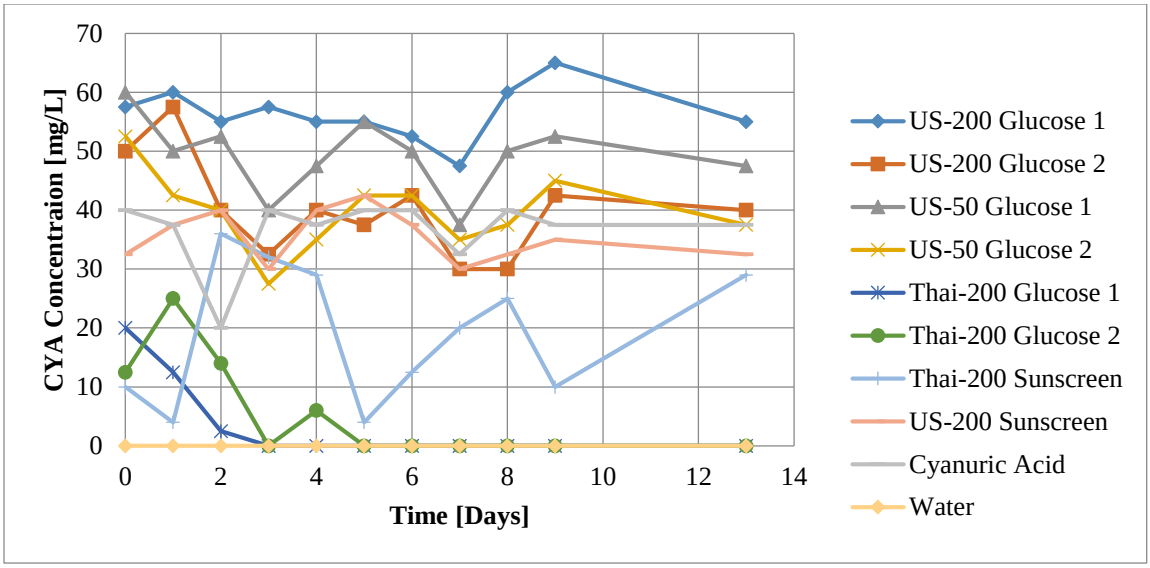


Figure 4-38: Experiment IV-4 CYA Concentration vs. Time, Turbidimetric Measurement, Minimal Media at 30°C and 75 RPM, Anaerobic

Treatments of "US- 50 Glucose" showed 21-29% reduction in CYA from start to finish. Conversely, treatments of "US- 200 Glucose" reduced CYA by 4% and 20%. This contrasts the effect seen in Experiment IV-3, in which US FOG showed greater reduction in CYA with higher glucose concentrations.

The "Thai- 200 Glucose 1" treatment showed a steady decrease in CYA over time and remained at zero after T=3 days. "Thai- 200 Glucose 2" showed similar reduction of CYA but also displayed higher variation across readings. The increase in CYA seen for "Thai-200 Glucose 2" between T=0 and T=1 day is attributed to incomplete dissolution of CYA at initial sampling. These data are in agreement with Experiment IV-3 regarding the efficacy of Thai FOG in removing CYA from solution.

Levels of CYA in the control treatment varied from 32.5 to 40 mg/L, with an outlier of 20 mg/L. The mode of the readings was 40 mg/L. Excluding the individual reading of 20

mg/L, most CYA readings were within 1-2 mg/L prior to correction for dilution. This level of noise in measurement is to be expected, so it was concluded that the concentration of CYA did not change over time in the control treatment.

CYA levels for the "Thai- 200 Sunscreen" treatment varied widely throughout the duration of the experiment. CYA readings for "US- 200 Sunscreen" did not vary as much as those of "Thai- 200 Sunscreen", and CYA readings for "US- 200 Sunscreen" fell in-line with both treatments of "US- 50 Glucose". Therefore, interference of sunscreen with turbidimetric CYA measurement was ruled out as a reason for variation in readings.

4.4.5 Experiment IV-5 Effect of Activated Thai FOG Supernatant on CYA

The supernatant of a solution of Thai FOG (after 24 hours of incubation) was added to 62.5 mg/L of CYA and incubated at 30^{o}C and 75 RPM. No consistent change in CYA was observed over time. Readings varied between 70 and 82.5 mg/L CYA, which can be attributed to the low test resolution magnified by a dilution factor of 2.5 for each reading.

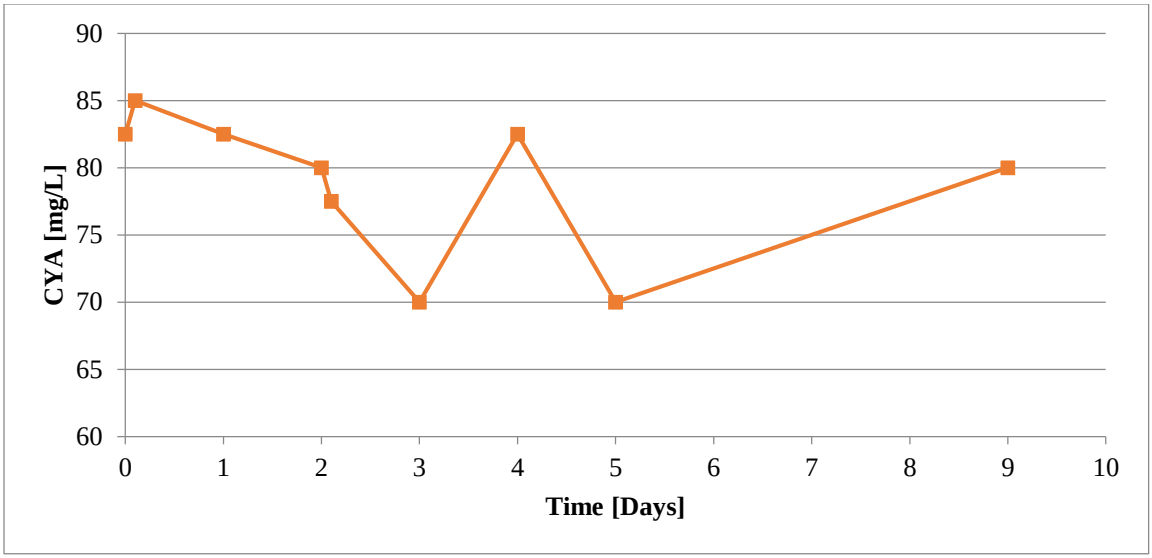


Figure 4-39: Experiment IV-5 CYA Concentration vs. Time, Turbidimetric Measurement, Thai FOG Supernatant in 62.5 mg/L CYA at 30ºC and 75 RPM, Anaerobic

No change was seen in CYA concentration, so it is postulated that the mechanism of CYA reduction by Thai FOG (seen in Experiments IV-2, IV-3, and IV-4) is dependent upon the solid substrate. The fermented rice bran and soy meal which makes up the majority of Thai FOG (98-99%) has a highly porous surface structure as seen in the SEM imagery of Experiment I-8, **Figures 4-9** and **4-10**. The product's porous surface seems to provide a high degree of adsorption of sunscreen and may contribute to adsorption of CYA.

4.4.6 Experiment IV-6 Effects of US FOG and Activated Thai FOG on CYA

Experiment IV-6 was designed to investigate the efficacy of CYA reduction by US FOG and the supernatant from incubated Thai FOG. Treatments were carried out in triplicate to avoid potential inconsistencies in turbidimetric CYA analysis.

Prior to dosing, the flask containing activated Thai FOG was inverted and agitated then allowed to settle for 2 minutes before collecting supernatant material. When collected for dosing, the supernatant was more turbid than before agitation. Increased turbidity indicates that finer particles from the solid substrate of Thai FOG were collected along with vegetative cells.

CYA readings were taken before and after inoculation to capture the initial CYA drop which is characteristic of Thai FOG. Values in **Figure 4-40** have been corrected for dilution caused by the addition of 100 mL of inoculum to each flask. **Figure 4-40** shows a significant drop in CYA by Thai FOG after inoculation. Due to a clerical error, glucose was not added until T=1 Days.

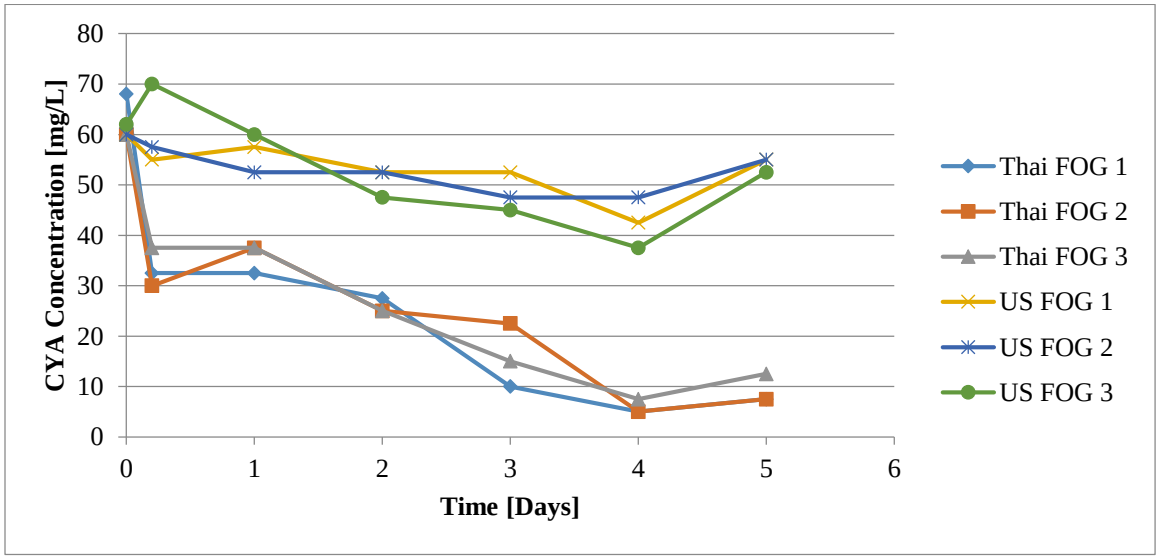


Figure 4-40: Experiment IV-6 CYA Concentration vs. Time, Turbidimetric Measurement, Thai FOG and US FOG in Triplicate, DI Water with Glucose at 30°C and 75 RPM, Anaerobic

Solid substrate material was dosed with the inoculum of each Thai FOG treatment. A 47% drop in CYA was observed at T=0.2 Days by Thai FOG. Results indicate that the Thai

FOG substrate is responsible for the substantial decrease in CYA measurement using turbidimetric measurement.

US FOG showed a 30% reduction of CYA between T=0 Days and T=4 Days. Over the same time-period, Thai FOG showed a 90% reduction in CYA. Discrepancies in dilution methods between experimenters during measurement of CYA caused an increase in CYA at T=5 Days. The consistent grouping of treatments indicates precision in CYA measurement between treatments. Accuracy of measurement was not confirmed, preventing definitive conclusions from being drawn.

Baseline CYA reduction was not confirmed in this experiment, because a control flask was not included in the treatments. Experiments IV-3 and IV-4 showed no change in CYA over time for control treatments, so it is assumed that no change would have been seen in a control flask.

4.4.7 Experiment IV-7 Turbidimetric CYA Calibration

A calibration curve for serial dilutions of CYA was developed using the turbidimetric method. The results show high linearity and are fit to a linear regression model with R^2= 0.916 (**Figure 4-41**).

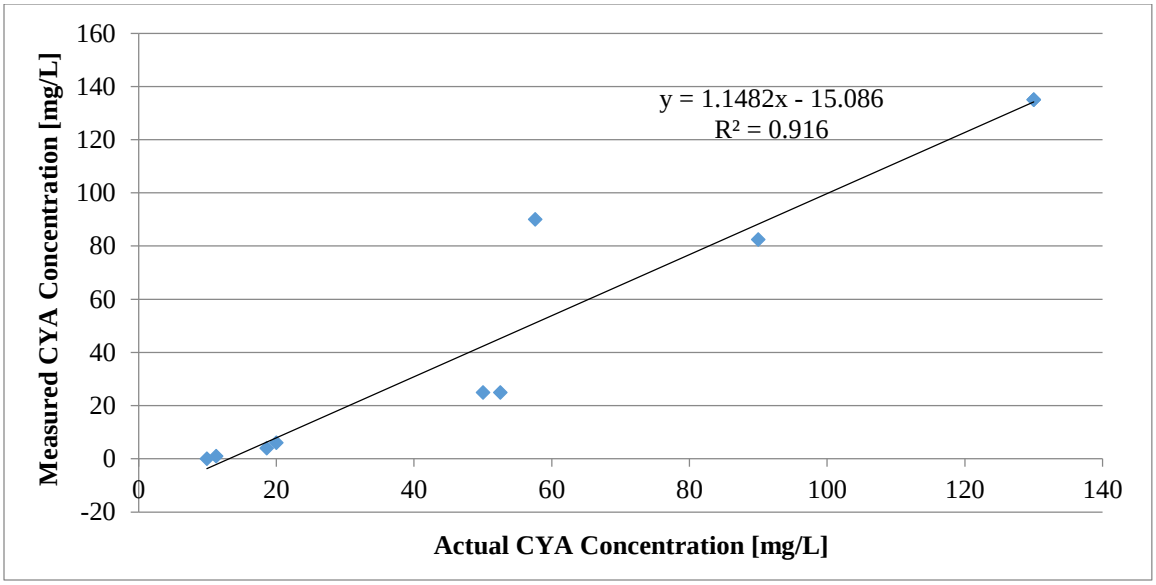


Figure 4-41: Calibration of Turbidimetric Method for CYA Measurement, Turbidimetric Measurement

4.4.8 Experiment IV-8 Standard Preparation for HPLC

Standards of CYA were prepared in 2 mL glass vials. According to the standard method for HPLC analysis of CYA, prepared samples are stable for >69 days at 25°C (Tucker 1994).

4.4.9 Experiment IV-9 HPLC Calibration of CYA

Two HPLC analyses of CYA returned linear calibration curves with highly varied slopes. System pressure was noted to exceed 400 bar when the purge valve was tightened fully. Due to improper experimenter training, the HPLC was run with the purge valve partially open for both standard curves. A portion of the mobile phase flow was allowed to leak from the purge valve. Low pressure corresponded to low eluent flow rate through the

column, wide peaks, and large integrated area. Higher pressure corresponded to higher eluent flow rate, narrower peaks, and smaller integrated area.

Preliminary analysis was run with P= 145 bar. Peaks eluted between 14.8 minutes at low concentrations and 8.1 minutes at high concentrations. Changing elution times indicate inconsistency in analyte retention by the system. To combat this issue, a subsequent analysis was carried out at 350 bar. CYA peaks were much narrower and more uniform than before. The linear regression showed a much shallower slope (**Figure 4-42**). The y-intercept was fixed at zero, and an R^2 value of 0.9988 was acquired, indicating strong linearity.

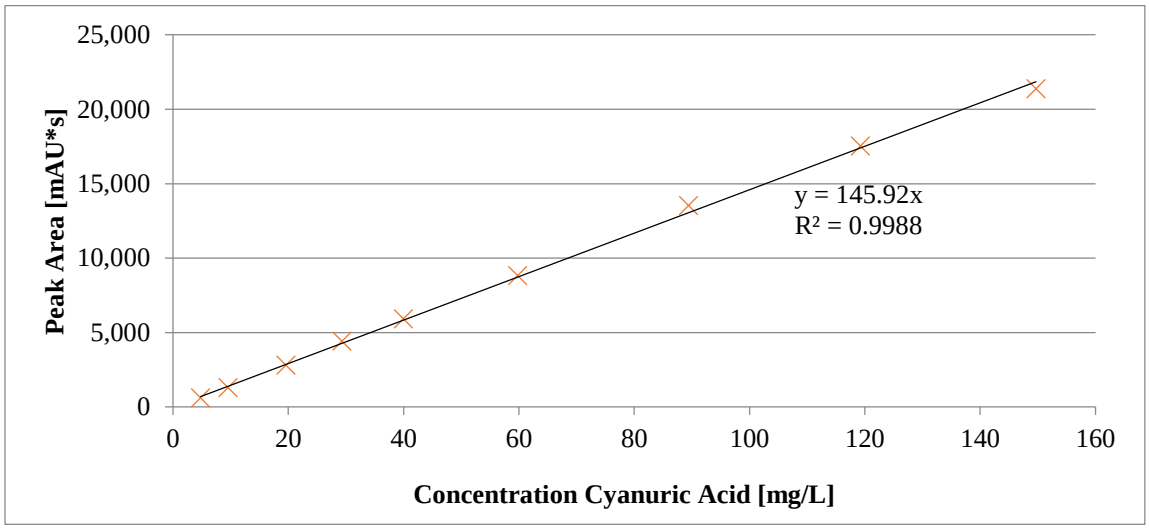


Figure 4-42: Experiment IV-9 Peak Area vs. CYA Concentration Calibration, HPLC Measurement, P=350 bar

System maintenance performed after Experiment IV-12 showed that the purge valve frit had fouled and was occluding mobile phase flow. The problem was not rectified until after Experiment IV-17.

The slope of calibration curve created in this experiment is dependent upon more variables than pressure alone, including temperature, guard column condition, and cleanliness of the purge valve frit. Therefore, results from future experiments cannot be converted to concentration based upon these calibrations.

4.4.10 Experiment IV-10 HPLC Calibration using Thai FOG

The supernatant from an activated Thai FOG solution and a prepared CYA sample were analyzed using HPLC. All samples were filtered through 0.22 µm filter prior to analysis. Peak areas, listed in **Table 4-9**, show that Thai FOG does not interfere with HPLC analysis of CYA.

Table 4-9: Experiment IV-10 CYA and Thai FOG Peak Areas

Vial Contents	Peak Area [mAU*s]	Expected Peak Area Based on 40 mg/L
CYA 50 mg/L	11620	9157.5
Thai FOG 110 mg/L	-128	0
CYA 25 mg/L; Thai FOG 55.3 mg/L	5792	4578.75
40 mg/L CYA Standard	7326	7326

The percent difference between the samples containing CYA at 50 mg/L and 25 mg/L, when corrected for dilution, was 0.3%. This confirms that the HPLC method provides substantially linear results across diluted samples.

Peak areas of samples were also compared to that of the 40 mg/L standard prepared in Experiment IV-8. Percent differences were calculated by comparing observed peak of the prepared CYA samples areas to expected peak areas based on the 40 mg/L standard. The 50 mg/L CYA and 25 mg/L CYA samples were 26.9% and 26.5% different from expected values, respectively, indicating that the CYA solution was diluted accurately, but not prepared accurately.

4.4.11 Experiment IV-11 Method Development of HPLC using Thai FOG and CYA

Thai FOG was added at 100 mg/L to a solution of 60 mg/L CYA. The mixture was heated and stirred until all CYA had dissolved. Peak areas were analyzed over time to investigate relative change in CYA concentration due to adsorption or biodegradation. The turbidimetric method of CYA analysis showed significant decrease of CYA over time by Thai FOG (Experiments IV-5 and IV-6), so a change was expected to be observed. Contrary to previous results, no change in CYA peak area was noted.

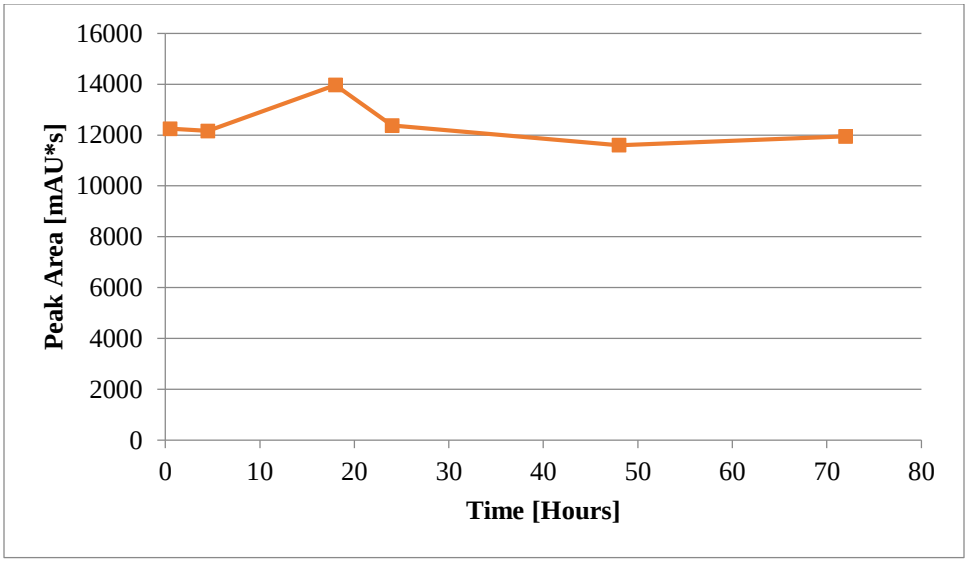


Figure 4-43: Experiment IV-11 CYA Peak Area vs. Time, HPLC Measurement, Thai FOG in 60 mg/L CYA, DI Water at 30^{o}C and 75 RPM, Anaerobic

According to the results, CYA peak area is stable in the presence of Thai FOG, over time.

4.4.12 Experiment IV-12 Investigation of Products' Effects on CYA

Treatments in this experiment each contained 50 mg/L of CYA to observe effects of various BiOWiSH products on CYA in the presence of additional glucose. Inoculums and additional glucose are laid out in Table **4-10**, below.

Table 4-10: Experiment IV-12 Experimental Setup

Bottle	Cya [mg/L]	Inoculum	Glucose [mg/L]
1	50	x	x
2	50	Thai FOG	250
3	50	Thai FOG	x
4	50	IR TF	250
5	50	Premix	250
6	50	35 mg Osp Liq	250
7	50	Osp Solid	250
8	50	US FOG	250

Samples were analyzed using HPLC alongside a standard of 60 ppm CYA, which was used to correct measurements for daily variation in peak elution time. Elution time varied between 2.0 and 2.5 minutes depending on temperature and the amount of eluent diverted by the partially-open purge valve. Glucose was analyzed separately for UV absorbance at 213 nm, and no notable peaks were observed.

In the first 48 hours, all treatments showed a decrease in CYA relative to the standard. The remainder of the experiment displayed a consistent increase in CYA measurement for each trial, with the exception of US FOG.

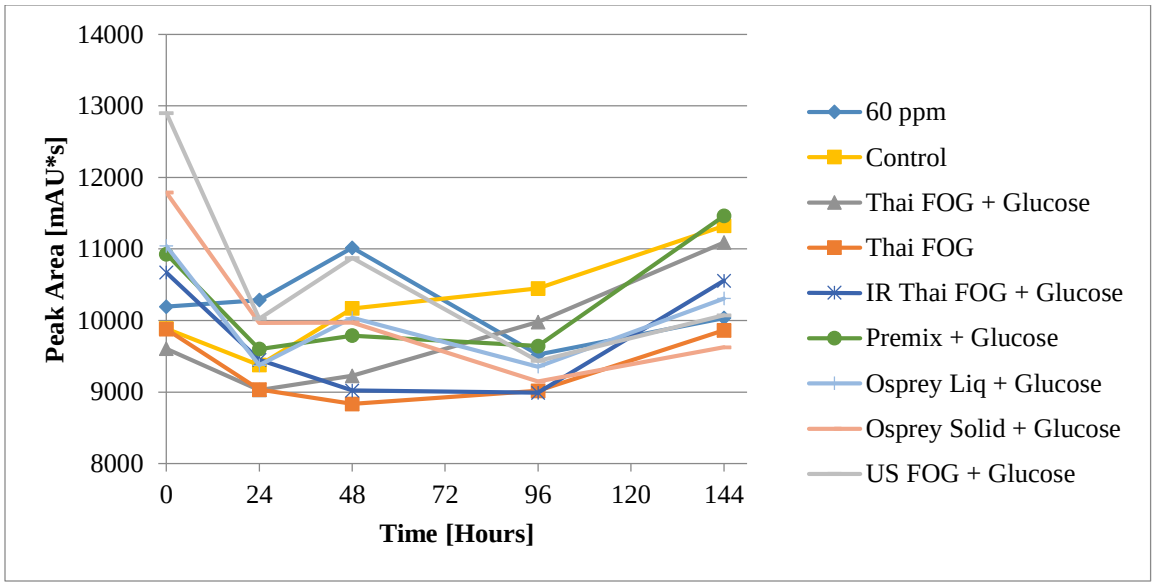


Figure 4-44: Experiment IV-12 Peak Area vs. Time, HPLC Measurement, DI Water at 30°C and 75 RPM, Anaerobic

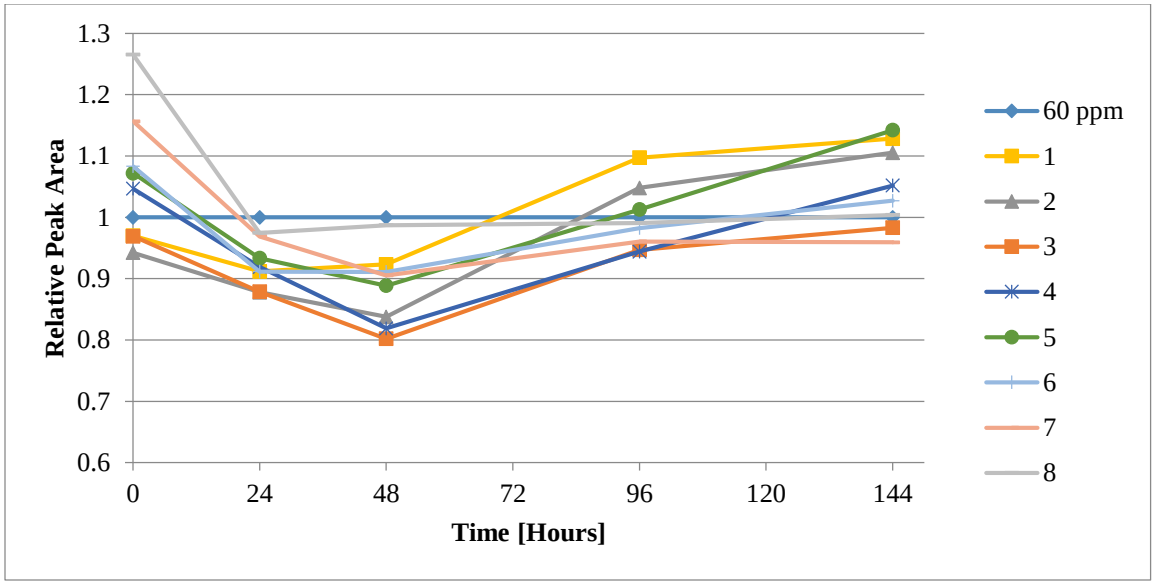


Figure 4-45: Experiment IV-12 Relative Peak Area vs. Time, Corrected to 60 PPM Standard, HPLC Measurement, DI Water at 30°C and 75 RPM, Anaerobic

CYA peaks showed systematic error with all samples changing by a similar amount, relative to control, throughout sampling events. As noted before, the issue stemmed from a clogged purge valve frit which was not discovered until after Experiment IV-16.

4.4.13 Experiment IV-13 HPLC Method Development – Inconclusive Mobile Phase Adjustment

Experiment 4-13 is omitted, due to inconclusive changes in HPLC method. Mobile phase adjustments are summarized in Section 3.5.11.

4.4.14 Experiment IV-14 HPLC Method Development – High Injection Volume

High injected concentrations of CYA led to column clogging during analysis. Peak areas eluted in excess of 4,000 mAU. It was determined that smaller volumes of CYA would be necessary for accurate analysis. No meaningful data were collected in this experiment.

4.4.15 Experiment IV-15 Investigation of C:N:P Ratios Effects on CYA Degradation

Experiment IV-15 was designed to highlight differences in CYA degradation with varying ratios of carbon to nitrogen and phosphorous. The inoculum was a combination of US Aqua and dextrose.

Method development demonstrated that the mobile phase used in this experiment did not separate nitrate from CYA. UV absorbance of nitrate at 213 nm is much stronger than that

of CYA, so ammonium nitrate within the growth media masked any changes in CYA concentration in this experiment.

Table 4-11: Experiment IV-15 Carbon:Nitrogen:Phosphorus Ratios

Bottle	C:N:P Ratio
1	100:10:1
2	60:10:1
3	50:10:1
4	40:10:1
5	10:10:1
6	50:10:1 Aerobic
7	50:10:1 Aerobic + Headspace

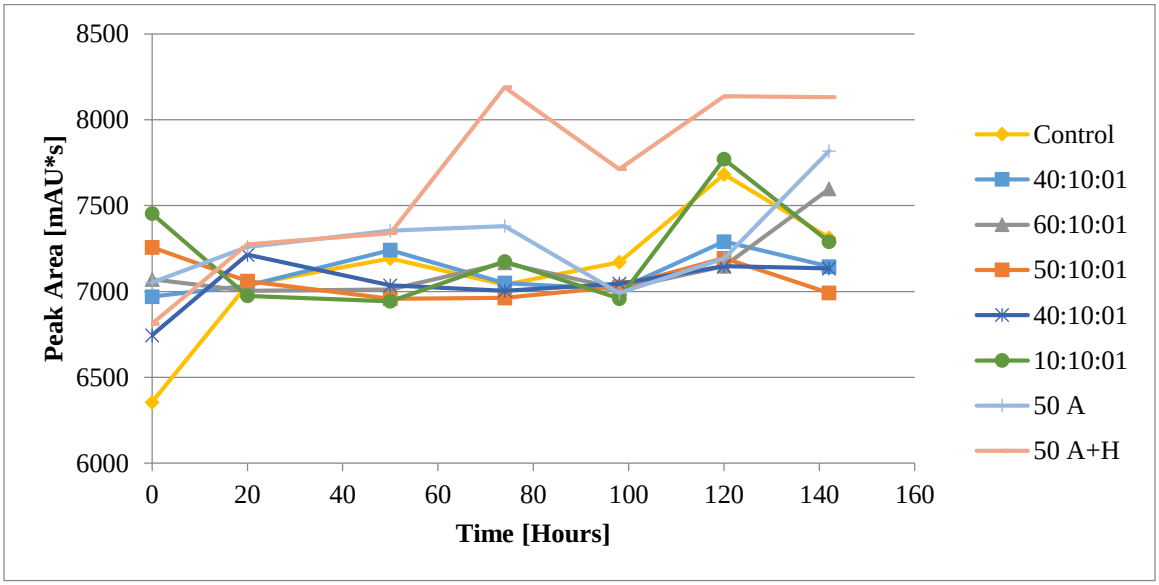


Figure 4-46: Experiment IV-15 Peak Area vs. Time, HPLC Measurement, Minimal Growth Media at 30°C and 75 RPM, Varying C:N:P Ratios, Aerobic and Anaerobic Treatments

Due to the interference of nitrate peaks with those of CYA, data in **Figure 4-46** cannot be utilized for the analysis of CYA concentrations.

4.4.16 Experiment IV-16 Hour-by-Hour Time-Point CYA Degradation

Hour-by-hour time-point CYA degradation was carried out using vegetative cultures suspended in Phosphate Buffered Saline in a growth solution. The HPLC method was not able to resolve nitrate peaks from CYA peaks. Due to the interference of peaks, no meaningful data were obtained.

4.4.17 Experiment IV-17 Effect of Cyanuric Acid Reducer on CYA Concentration

Experiment IV-17 investigated the effects of BiOWiSH Cyanuric Acid Reducer (CAR) product on CYA. The product is primarily composed of dextrose amended with isolated bacterial cells. In order to dose 5 mg/L and 50 mg/L of CAR to 200 mL of growth solution, a stock solution of CAR was used.

Starting at T=19 Hours, a standard of CYA at 97 mg/L and DI water were analyzed with each sampling event as a two-point calibration. An example calibration can be found in Appendix BXX.

All treatments, including the controls, showed drift in peak area with tight grouping after T=2 Hours. This is attributed to diurnal temperature fluctuation. Elution time fluctuations could be prevented with a column heater.

After T=65 hours, low to no bacterial growth was seen. 500 mg/L of glucose was dosed to the non-control flasks to stimulate bacterial growth and potential CYA degradation. Even with the addition of glucose, no consistent trends relative to controls were seen.

Figure 4-47, the raw peak area data, shows strong systematic error in the form of peak area drift between sampling events. This indicates that the developed method does not consistently measure CYA, and needs further refining. Temperature control with a column heater is the first change which could make a significant impact on peak area stability.

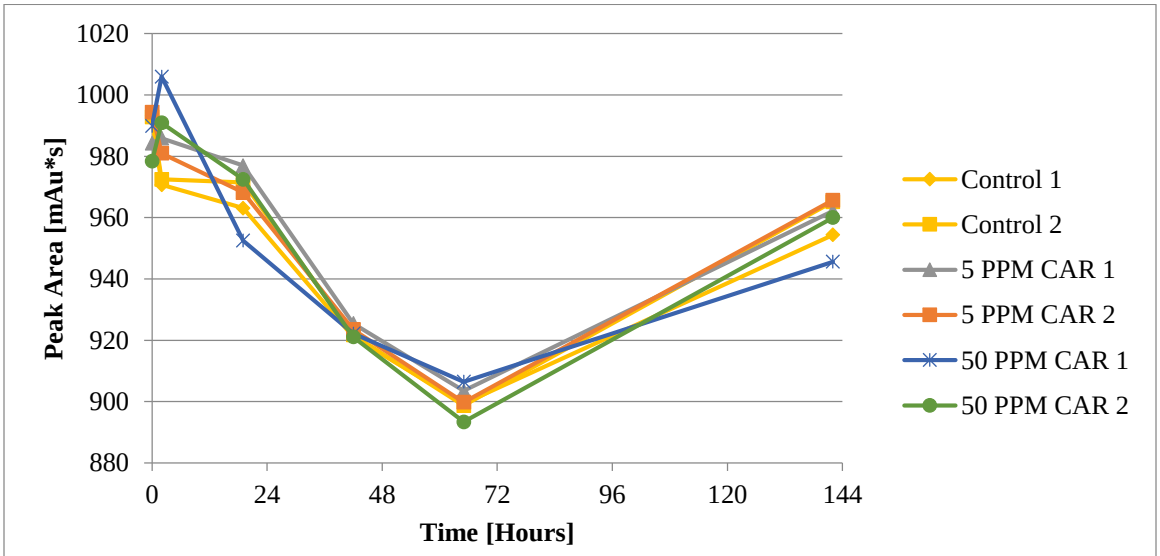


Figure 4-47: Experiment Iv-17 Peak Area vs. Time, HPLC Measurement, CAR Product at 5 mg/L and 50 mg/L, DI Water with Dextrose at 30°C and 75 RPM, Anaerobic

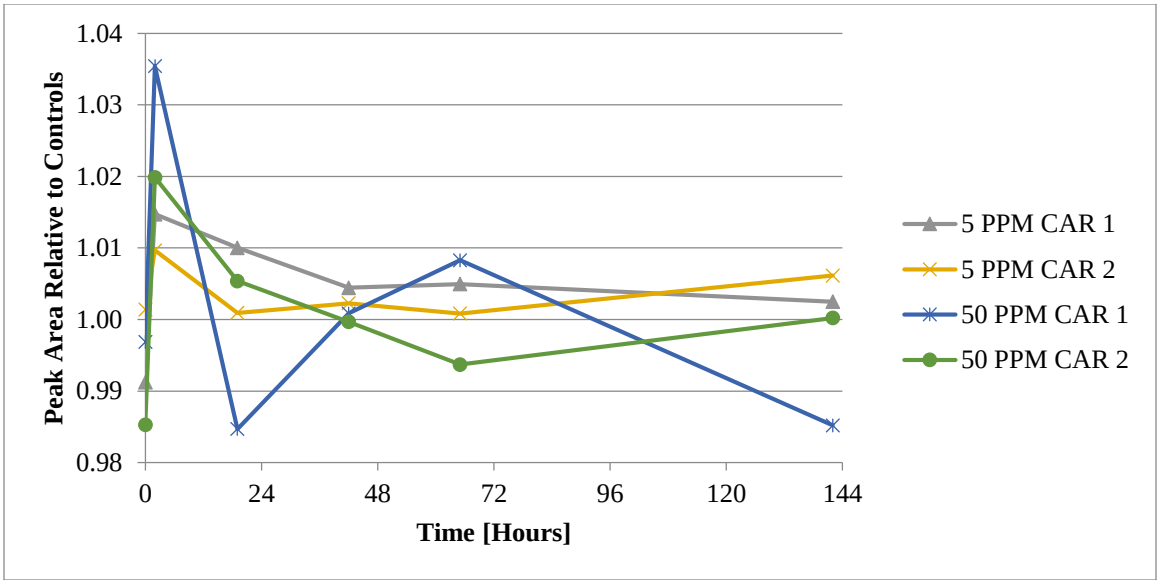


Figure 4-48: Experiment IV-17 Peak Area vs. Time Relative to Averaged Controls, HPLC Measurement, CAR Product at 5 mg/L and 50 mg/L, DI Water with Dextrose and at 30oC and 75 RPM, Anaerobic

Peak area varied from the average of the controls by no more than 4% in either direction throughout the experiment for each treatment **Figure 4-48**. Results show that up to 50 ppm of CAR had no measureable effect on CYA concentration.

4.4.18 Experiment IV-18 Effect of CAR on CYA with K_2HPO_4, Varied Glucose

Experiment IV-18 was developed to investigate the effects of CAR on CYA with varying concentrations of glucose and trace K_2HPO_4. Prior to inoculation, growth media was autoclaved then sparged with N_2 gas in order to remove dissolved oxygen and promote anaerobic metabolism.

After T=1 Days, the HPLC guard column was changed. As a result, operating pressure decreased, peak elution time stabilized marginally, and peak areas dropped significantly.

Figure 4-49 excludes readings from T=0 and T=1 Days, because they are not relatable to the remainder of the data.

Results showed no change in CYA concentrations relative to the controls in the first 6 days. Similarly, no appreciable bacterial growth was seen within biological treatments. A solution of 1 g/L CAR in Lactobacillus Broth was activated for 24 hours at 30°C and 75 rpm. The activated product was then dosed at 1 mL per flask to all four biological treatments at T= 7 Days.

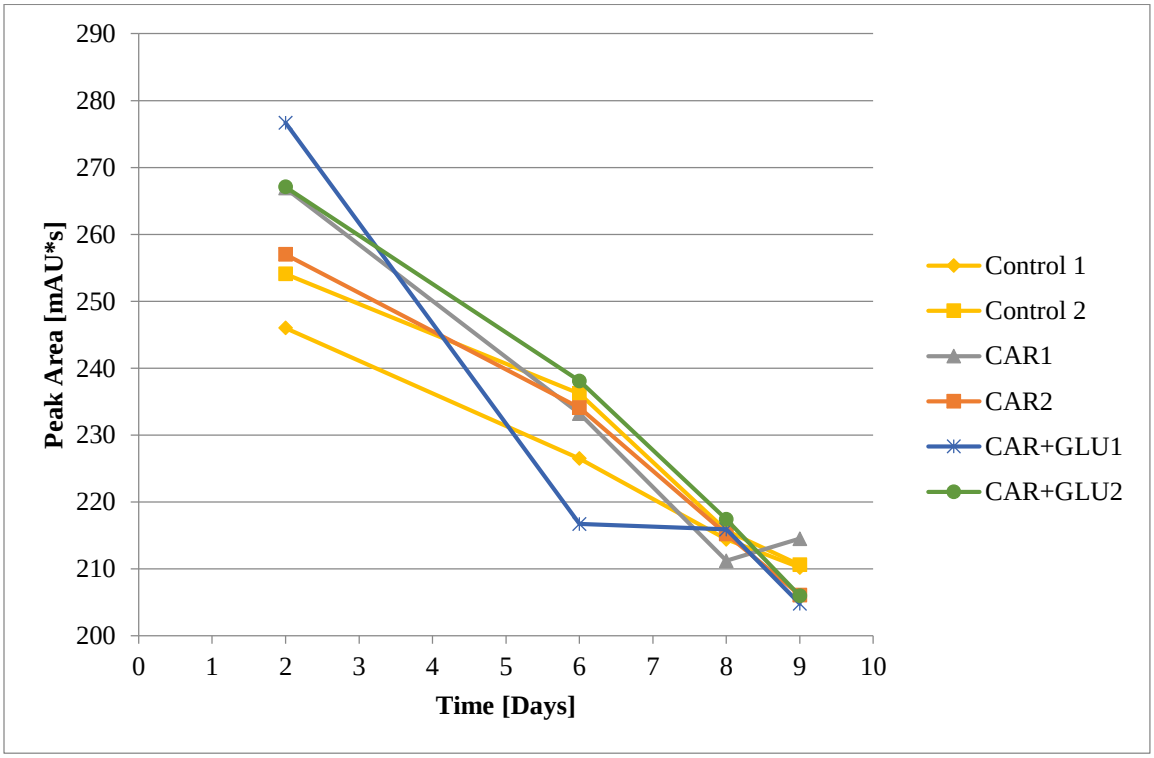


Figure 4-49: Experiment IV-18 Peak Area vs. Time HPLC Measurement, CAR Product and Varied Glucose, DI Water with K_2HPO_4 at 30°C and 75 RPM, Anaerobic

A 3-point calibration was run at the beginning of HPLC analysis at T= 6, T=8, and T=9 Days. An example of the procedure for calibration and conversion of peak area to mg/L of

CYA can be found in Appendix C. The results were not included in this experiment, because it was not used consistently from the onset of the experiment. Subsequent experiments will utilize the three-point or four-point calibration at the beginning of HPLC analyses.

4.4.19 Experiment IV-19 Effect of Activated CAR on CYA, Varying Dextrose

Experiment IV-19 was developed to investigate the effects of the activated bacterial components of BiOWiSH CAR product on CYA, in varying concentrations of dextrose. Prior to inoculation, each bottle of autoclaved CYA solution was sparged with N_2 gas in order to promote anaerobic conditions from the onset of the experiment.

A four-point calibration was run before each sampling event to correct for temperature-dependent peak area variations. A sample calibration can be found in Appendix BXX. It was assumed that the calibration curve created for a sampling event remained valid throughout the sampling event, as temperature did not change significantly during HPLC analysis of each set of samples (maximum of 2 hours). **Figure 4-50** shows minimal change in CYA concentration over time.

After T=3 Days, the HPLC guard column was changed. Starting at T=4 Days, the clean guard column provided much more distinct peaks with less tailing, and CYA readings became much more closely grouped between treatments. In **Figure 4-50**, the final time-point excludes the “Control 2” treatment, as contamination was suspected to have been introduced to the treatment.

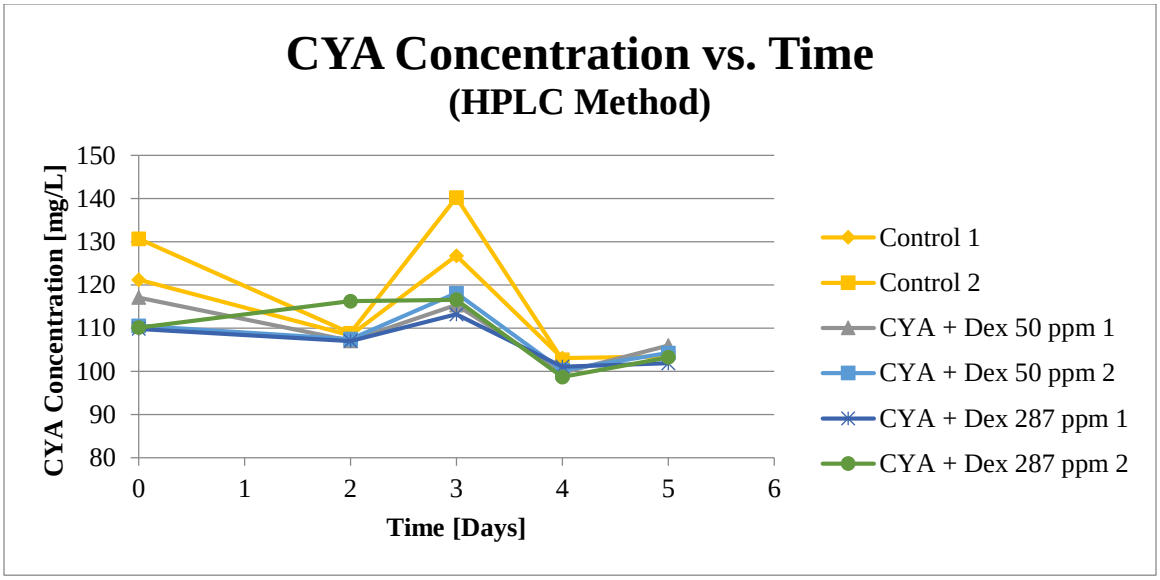


Figure 4-50: Experiment IV-19 CYA Concentration vs. Time, HPLC Measurement, Vegetative Bacterial Inoculums, DI Water with Dextrose at 30°C and 75 RPM, Anaerobic

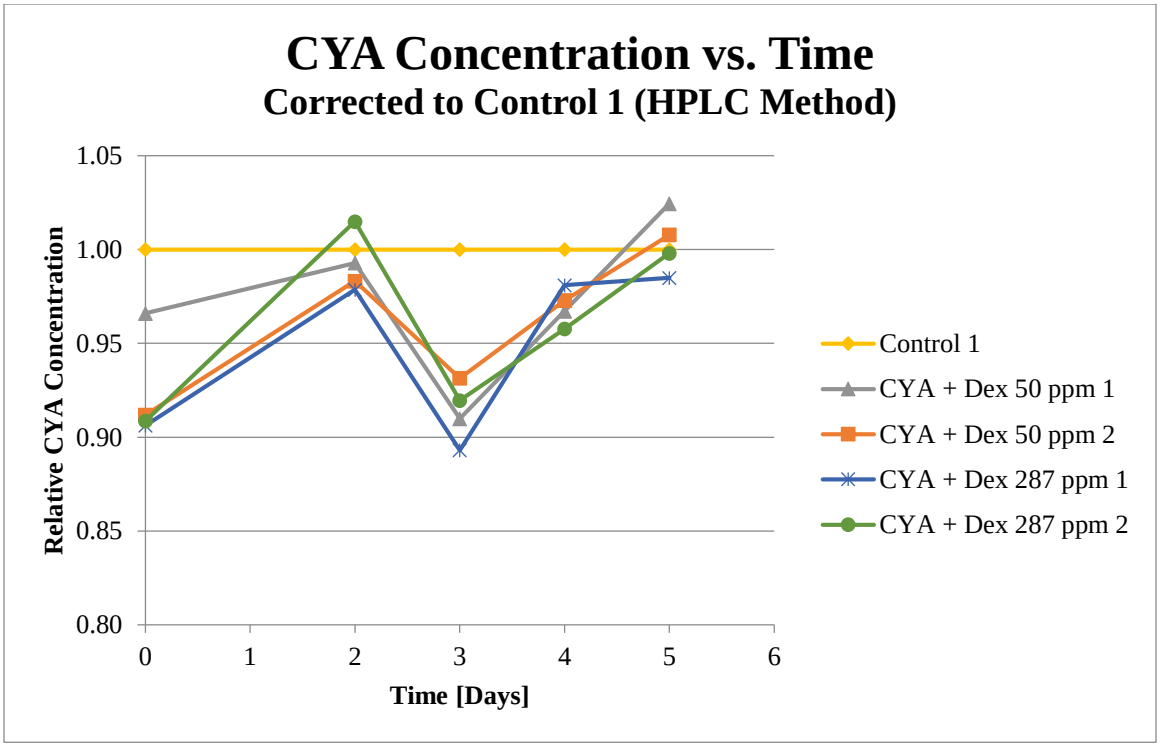


Figure 4-51: Experiment IV-19 CYA Concentration vs. Time Corrected to Control 1, HPLC Measurement, Vegetative Bacterial Inoculums, DI Water with Dextrose at 30°C and 75 RPM, Anaerobic

Data corrected to "Control 1" (**Figure 4-51**) showed systematic error similar to that seen in Experiment IV-17. The systematic error caused the test to be inconclusive; however the data suggest that there is no change in CYA relative to the control treatment. CYA concentrations did not vary by more than 11% from the control before the guard column was changed. After the guard column was changed, CYA concentrations differed from "Control 1" by no more than 5%.

4.4.20 Experiment IV-20 Effect of Activated CAR and Filter Media on CYA

Experiment IV-20 sought to stimulate the degradation of CYA by introducing vegetative Thai FOG supernatant to a minimal growth media containing CYA. Additionally, a section of swimming pool media filter was incubated in growth media, and the activated cultures were added to flasks containing minimal growth media and CYA.

Nitrate in the minimal media caused interference with initial HPLC measurement of CYA, so apparent CYA concentrations at T=0 Days were artificially high (**Figure 4-52**). After three days of anaerobic incubation, it was assumed that all nitrate had been biodegraded from the samples, and that peak areas of CYA were indicative of true concentrations. Chromatograms showed similar levels of absorbance between nitrate and CYA with sufficient separation of peaks (Appendix E).

Significant bacterial growth and gas production were noted within the first three days of incubation. An existing crack in the flask containing the "Thai FOG 2" treatment propagated vertically through the bottle, due to increased pressure. This caused half of the

growth media to be extruded from the flask. It is assumed that air was introduced to the system, preventing a fully anaerobic environment from forming. Therefore, "Thai FOG 2" was not included in the chart of averaged CYA concentrations, **Figure 4-52**.

Gas production by each treatment slowed significantly after T=3 days. An additional 1.6 g/L of dextrose were added to "Thai FOG 1," "Thai FOG 2," "Filter Media 1," and "Filter Media 2" at T=7 days. Gas production continued alongside visible sedimentation/settling of bacterial cells.

When treatments and controls were averaged, no appreciable or consistent change was seen in CYA concentration relative to the control (**Figure 4-53**).

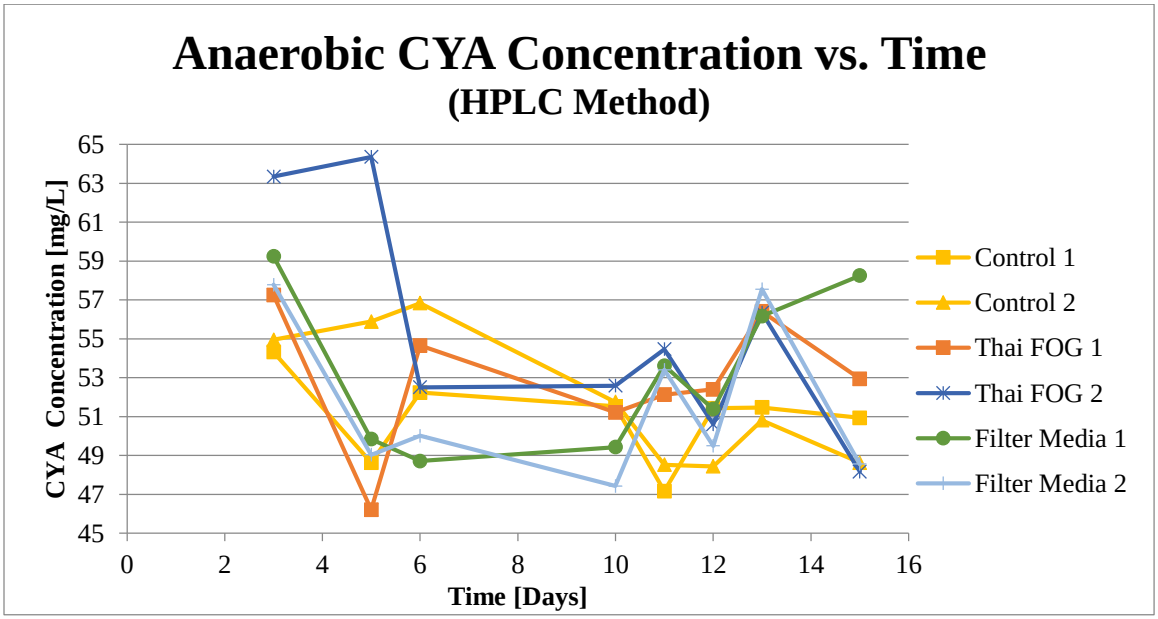


Figure 4-52: Experiment IV-20 Anaerobic CYA Concentration vs. Time, Excluding T=0, Vegetative Bacteria, Minimal Media at 30°C and 75 RPM, Anaerobic

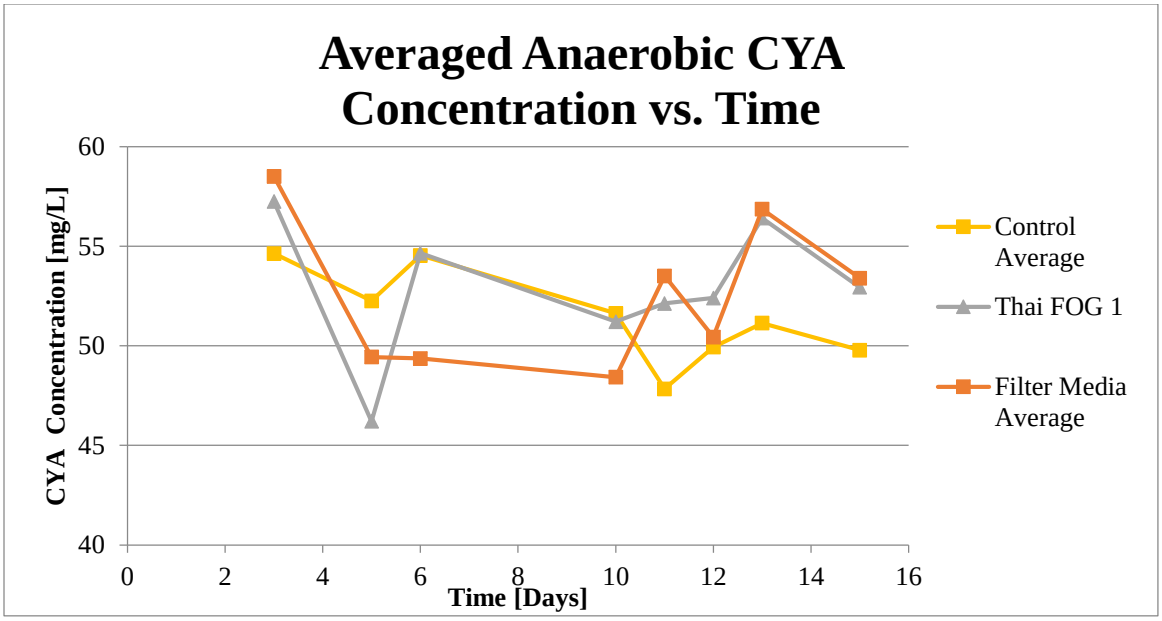


Figure 4-53: Experiment IV-20 Averaged CYA Concentration vs. Time, Excluding T=0, Excluding Thai FOG 2, Vegetative Bacteria, Minimal Media at 30°C and 75 RPM, Anaerobic

Similar levels of variation were seen between all trials in **Figure 4-52**. No degradation of CYA was seen by Thai FOG or the Filter Media treatments using the developed method. The averaged trials in **Figure 4-53** show a relatively high reading of CYA at T=3 Days, which is probably due to continued interference by nitrate. The inability of the method to resolve high nitrate peaks from CYA prevents definitive conclusions from being made.

CHAPTER 5 – CONCLUSIONS

5.1 Results of the Determination of Clarification Mechanism

- Solid substrate products provide enhanced clarification in the first 24 hours, compared to un-amended mechanical filtration. (Section 4.1.10)
- Thai FOG, active or irradiated, provides the greatest enhancement of initial clarification with mechanical filtration, relative to un-amended mechanical filtration. (Section 4.1.10)
- Adsorption is the driving mechanism of additional turbidity removal provided by the solid substrate of BiOWiSH products. (Experiments I-5 and I-8, Section 4.1.10)
- Control tanks with just sunscreen commonly showed levels of turbidity reduction on-par or more effective than BiOWiSH products. (Experiments I-2, I-3, I-4, and I-8)
- Improved turbidity reduction over time is observed in control treatments which became contaminated during long-term clarification experiments. Biodegradation may be the cause of turbidity reduction, but the true mechanism remains unconfirmed. (Experiment I-8)

5.2 Results Regarding the Efficacy of BiOWiSH Clarifying Oils from Swimming Pools

- Without mechanical filtration, Thai FOG reduces turbidity caused by sunscreen within 24 hours of dosing the dry product. (Experiments II-1 and II-5)

- Chlorine is scoured by all BiOWiSH products and growth media. (Experiments II-1.2, II-2, II-3, and II-4)
- Physical separation (floating and settling of material) accounts for a large portion of turbidity reduction. (Experiment II-4)
- In flasks, no reduction of turbidity induced by sunscreen, was seen relative to control treatments. (Experiments II-6 and II-7)

5.3 Results Regarding the Isolation and Identification of Bacteria

- Two predominant types of bacterial colonies are present when samples of used BiOWiSH products are plated. This is most likely due to cross-contamination of treatments. (Experiments III-1 and III-2)

5.4 Results Regarding the Biodegradation of Cyanuric Acid

- Results suggest that there is no measureable change in CYA via biodegradation or adsorption by BiOWiSH products in bench-scale tests, however; the low accuracy of the developed method prevents definitive conclusions from being drawn. (Experiments IV-11, IV-12, IV-15, and IV-17 through IV-20)
- Thai FOG appears to interfere with the turbidimetric precipitation assay of CYA measurement. CYA reduction was observed by the solid substrate of Thai FOG using the turbidimetric method, but not through HPLC. (Experiment IV-2, IV-4, and IV-6)

- Without a column heater, peak areas vary significantly, between analysis sequences. To convert peak area to concentration, a 4-point calibration should be run before each set of samples is analyzed. (Experiments IV-17 through IV-20)
- Using the available Agilent 1100 HPLC system, a method for replicable measurement of CYA was not achieved.
- Separation of CYA from nitrate/nitrite was achieved with the method which follows in **Table 5-1**, below.

Table 5-1: Final HPLC Method for separation of Nitrate/Nitrite from CYA

Parameter	Value
Column	Waters XBridge C18
Mobile Phase	1% methanol 69.5% 50 mM KH_2PO_4 buffer (pH 5.70) in DI water 29.5% distilled water
Flow Rate	0.300 mL/min
Injection volume	1 uL
Detection wavelength	213 nm
Temperature	18-20^{o}C (ambient)
Sample Run Time	5 minutes

- The best resolution of CYA through HPLC analysis was observed at peak areas between 100 and 200 mAU*s, which corresponds with peak heights less than 20 mAU.

5.5 Future Research

- Analyze CYA with HPLC at a constant temperature, to determine whether the systematic error in measurement is indeed temperature-induced.
- Perform an experiment which confirms both the biodegradation of CYA and the ability of HPLC method to measure the reduction of CYA, using a bacteria spp. which has been proven to degrade CYA.
- Clarification with controls in separate laboratory to prevent contamination of the control.
- Test clarification rates in aerated and strictly anaerobic environments in order to gauge the difference in clarification rates.

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APPENDICES

Appendix A: Data Tables

Table 7-1: Experiment I-1 Raw Turbidity Data

	Turbidity [NTU]			
Treatment	T=0 Hours	T=0.25 Hours	T=18 Hours	T=36 Hours
Thai FOG	48	55	10	5

Table 7-2: Experiment I-2 Raw Turbidity Data

	Turbidity [NTU]						
Treatment	T=0 Hours	T=18 Hours	T=48 Hours	T=72 Hours	T=96 Hours	T=120 Hours	T=144 Hours
US FOG	56	13	10	8	0	4	3
Thai FOG	53	18	3	4	0	7	5
Control	52	8	3	0	0	1	0

Table 7-3: Experiment I-3 Raw Turbidity Data

	Turbidity [NTU]			
Day	US FOG	Thai FOG	US Rice Bran	Control
0	48	55	66	47
1	7	2	30	9
2	2	3	20	4
3	1	6	15	1
3.01	41	63	69	50
4	9	33	34	16
5	3	16	22	3
6	0	14	19	8
7	2	13	22	2
7.01	48	55	73	37
8	6	22	48	11
9	3	16	44	5

10	3	13	43	5
10.01	53	65	93	58
12	7	28	51	9
13	5	45	42	6
14	5	55	48	5
15	5	59	46	6
16	5	62	49	4
16.01	50	105	91	35
17	8	62	75	10
18	8	75	62	8

Table 7-4: Experiment I-4 Raw Turbidity Data

	Turbidity (NTU)				
Day	US FOG Re-Dose	US FOG Single	Thai FOG	Thai FOG Irradiated	Control
24	6	10	17	17	5
25	5	12	16	13	7
25.01	46	63	61	56	54
26	24	24	20	16	17
27	30	20	16	15	16
28	15	6	11	5	10
29	20	19	17	11	12
30	14	10	13	7	8
31	1	11	12	7	8

Table 7-5: Experiment I-5 Raw Turbidity Data

	Turbidity [NTU]					
Day	US FOG	Mix #1	Mix #2	IR Thai FOG	Premix	BMT SS
0	51	43	37	44	47	50
1	30	2	1	2	1	2
2	30	6	6	6	3	2
2.1	72	43	52	48	48	51
3	32	11	19	12	9	8
4	22	14	13	12	7	10

5	16	16	17	11	7	11
6	11	16	17	11	5	7
7	6	6	14	11	4	9
8	4	3	9	9	8	15
8.1	58	61	62	71	63	70
9	31	22	20	16	18	20
10	25	23	19	16	14	22
11	21	18	17	15	11	18
12	14	17	18	14	9	15
13	10	11	13	14	9	11
14	6	5	8	13	6	7
15	5	4	8	13	3	7
16	4	4	7	12	3	5
16.1	63	68	67	77	61	75
27	18	5	5	6	5	8

Table 7-6: Experiment I-6 Raw Turbidity Data

	Turbidity (NTU)						
Day	Manure/ Odor	Premix	BS-AQ-001	BS-AQ-002	BS-AQ-003	Thai Rice Bran	US Rice Bran
0	45	32	42	56	47	54	37
1	2	0	2	2	2	6	2
1.1	52	53	56	52	49	66	52
2	15	6	19	14	15	16	24
3	23	5	17	14	12	6	17
4	22	5	17	16	14	7	17
5	20	6	20	17	13	9	18
6	23	19	23	18	17	10	17
7	25	25	31	25	19	10	17

Table 7-7: Experiment I-7 Raw Data Turbidity

Day	Turbidity [NTU]						
	Fruit Wash	Premix	AP 001	AP 002	AP 003	Thai Rice Bran	Irradiated Thai Rice Bran
0	49	53	55	56	61	70	59
1	19	2	44	12	30	20	35
1.1	67	57	73	61	76	74	84
2	19	17	29	47	45	31	35
3	13	16	20	29	29	24	25

Table 7-8: Experiment I-8 Raw Turbidity Data

Day	Turbidity [NTU]						
	Premix 1	Premix 2	Thai FOG 1	Thai FOG 2	IR Thai FOG 1	IR Thai FOG 2	Control
0	39	42	44	43	37	40	50
1	1	2	2	3	2	1	15
1.1	56	57	55	55	58	52	58
2	12	11	15	16	15	13	17
2.1	68	62	71	71	69	65	65
3	27	27	39	41	35	30	25
4	18	14	17	18	17	16	12
5	22	17	16	17	27	17	7
6	18	14	15	18	22	19	2
7	16	14	13	16	20	18	2
8	13	13	15	15	17	16	3
9	9	10	14	12	13	14	4
10	8	9	11	13	12	15	6
10.1	59	63	69	65	55	64	56
11	41	58	54	43	32	31	15
12	26	38	35	33	23	24	10
13	20	28	26	29	20	18	7

Table 7-9: Experiment I-9 Raw Turbidity Data

	Turbidity [NTU]					
Day	CAR + CYA	CAR + Cl	CAR + CYA + Cl	CAR + CYA + Cl	CAR	Filter Media + CYA
0	47	52	47	45	1	2
1	6	9	16	6	3	4
1.1	58	55	60	57	4	59
2	30	14	20	14	6	12
3	15	7	11	7	5	9
4	11	10	12	6	5	11
4.1	59	59	60	53	5	62
5	17	29	9	10	6	14
6	10	17	11	7	4	9
7	6	15	9	8	7	5
7.1	63	52	62	56	6	59
8	11	22	28	23	7	16
9	5	16	22	19	6	8
10	4	14	18	14	6	5
11	4	17	16	12	6	5
12	2	8	15	12	5	4
12.1	41	55	68	57	5	49
13	30	26	31	28	5	9
14	15	16	25	21	6	4
15	11	15	19	16	5	4

Table 7-10: Experiment I-9 CYA Concentration vs. Time

	CYA Concentration [mg/L]						
Tank	T=0 Days	T=1 Days	T=3 Days	T=5 Days	T=6 Days	T=14 Days	T=15 Days
1	90	105	122	109	93	94	95
2	14	12	67	6	13	0	0
3	88	102	110	103	97	90	94
4	89	105	105	105	104	88	93
5	89	112	126	210	102	101	102
6	85	105	115	102	102	93	96

Table 7-11: Compiled Percent Reduction after 1 Day, for Each Experiment

Treatment, Grouped by Experiment	Percent Turbidity Reduction at Day 1
I-1 Thai FOG	82
I-2 US FOG	77
I-2 Thai FOG	66
I-2 Control	85
I-3 US FOG	85
I-3 Thai FOG	96
I-3 Rice Bran	55
I-3 Control	81
I-5 Mix #1	95
I-5 Mix #2	97
I-5 IR Thai FOG	95
I-5 Premix	98
I-5 BMT SS	96
I-6 Manure/ Odor	96
I-6 Premix	100
I-6 BS-AQ-001	95
I-6 BS-AQ-002	96
I-6 BS-AQ-003	96
I-6 Thai Rice Bran	89
I-6 US Rice Bran	94
I-7 Fruit Wash	61
I-7 Premix	96
I-7 AP 001	20
I-7 AP 002	79
I-7 AP 003	51
I-7 Thai Rice Bran	71
I-7 Irradiated Thai Bran	41
I-8 Premix 1	97
I-8 Premix 2	95
I-8 Thai FOG 1	95
I-8 Thai FOG 2	93

Treatment, Grouped by Experiment	Percent Turbidity Reduction at Day 1
I-8 IR Thai FOG 1	95
I-8 IR Thai FOG 2	98
Control	70
I-9 CAR + CYA	87
I-9 CAR + Cl	83
I-9 CAR + CYA + Cl 1	66
I-9 CAR + CYA + Cl 2	87
I-9 Filter Media + CYA	80

Table 7-12: Experiment II-1 Raw Absorbance Data at 470 nm

	Absorbance [AU]			
Day	Sunscreen + Thai FOG	Sunscreen	Thai FOG	Tap Water
0	0.133	0.276	0.012	-0.023
1	0.040	0.236	0.022	-0.015
2	0.026	0.193	0.062	-0.018
3	0.022	0.179	0.078	-0.01
4	0.087	0.232	0.098	-0.021
5	0.025	0.222	0.07	-0.043

Table 7-13: Experiment II-1.2 Chlorine Compatibility Raw Data

	Total Chlorine [mg/L]		
Treatment	T=0 Hours	T=1 Hours	T= 72 Hours
20-20-20	5	0.42	x
MRS	5	0.65	x
Minimal	5	3.85	0.02
Tap Water	5	4.59	1.66

Table 7-14: Experiment II-2 Raw Total Chlorine Data

Product	Chlorine (mg/L)		
	T=0 hours	T=4.5 hours	T=25 hours
Osprey MPB 5	1.19	0.04	0.03
Osprey BPB 100	0.89	0.02	0.02
Osprey Waste Water	0.73	0.01	0.11
US Aqua	2.07	0.36	0.09
Us FOG	1.99	0.19	0.16
MDG Petro	0.87	-0.06	0.02
MDG Waste Water	2.04	0.31	0.06
Thai FOG	0.19	-0.01	0.15
Minimal Media	2.41	1.16	0.13
sunscreen + Media 525 mg/L	2.23	0.11	0.45
sunscreen + Water 536 mg/L	3.48	0.22	0.33
Tap Water	3.07	2.08	0.23

Table 7-15: Experiment II-4 Raw Turbidity Data, Un-Agitated

Tank	Turbidity [NTU]				
	T=0 hours	T=20 hours	T=48 hours	T=70 hours	T=96 hours
Control	22	25	20	19	19
Thai FOG	28	11	23	25	31
Fruit Wash II	22	23	19	19	9
Fruit Wash II	24	21	21	19	19
BMT WW1- I	16	13	16	12	11
BMT WW1- II	23	21	20	21	18
BMT WW2 - I	23	18	19	20	23
BMT WW2- II	19	14	14	16	16

Table 7-16: Experiment II-5 Turbidity Raw Data

	Turbidity [NTU]				
Tank	T=0 Hours	T=20 Hours	T=50 Hours	T=68 Hours	T=113 Hours
Thai FOG	70	20	17	22	9
LCM 1	61	58	62	x	x
LCM 2	63	59	57	x	x
BMT WW1	62	56	55	x	x
BMT WW2	59	56	56	x	x
BMT KLB Mix I	60	57	53	57	31
BMT KLB Mix II	68	64	57	54	55
KLB I	65	62	61	x	x
KLB II	66	63	62	x	x
Control	62	58	56	49	61

Table 7-17: Experiment II-6 Raw Turbidity Data

	Turbidity [NTU]					
Flask	T=0 Hours	T=26 Hours	T=46 Hours	T=74 Hours	T=98 Hours	T=121 Hours
Osprey MPB-5	10	16	12	14	14	11
Osprey BPB-100	56	57	62	49	44	41
Osprey MPB-5 Liq	5	0	0	0	1	1
BMT WW 1	5	1	1	0	1	2
BMT WW 2	2	2	3	1	3	2
MBWWT#1	1	1	0	0	2	1
MDG Petro	6	5	15	4	5	4
MDG Micro-N	38	41	37	39	35	33
Crop	4	7	1	6	8	5
Control	1	1	1	2	1	2
Fruit Wash	4	2	4	2	2	1
LCM	5	2	3	5	3	2
Thai FOG	6	6	11	8	8	5
Thai Aqua	9	9	9	5	15	10
US Aqua	0	1	0	11	1	1
US Aqua FOG	0	3	0	6	0	1
KLB	124	131	134	126	140	114

Table 7-18: Experiment II-7 Raw Data

		Turbidity [NTU]							
#	Flask Label	T=0 Hrs	T=18 Hrs	T=38 Hrs	T=70 Hrs	T=94 Hrs	T=115 Hrs	T=139 Hrs	T=154 Hrs
1	Control	130	142	143	142	146	148	154	166
2	Osprey MPB-5	130	145	149	154	143	144	148	151
3	Osprey BPB-100	164	170	177	177	178	181	184	193
4	Osprey MPB-5 Liq	90	94	95	84	81	91	92	90
5	BMT WW 1	117	116	123	119	118	107	124	121
6	BMT WW 2	163	156	166	167	153	163	166	167
7	MBWWT#1	126	123	131	131	127	127	132	130
8	MDG Petro	124	124	126	125	126	125	129	128
9	MDG Micro-N	149	139	139	129	123	130	132	133
10	Crop	114	124	125	176	124	130	140	145
11	Fruit Wash	109	115	118	117	112	113	116	118
12	LCM	119	125	121	125	116	127	129	132
13	Thai FOG	125	127	130	133	134	135	146	149
14	Thai Aqua	128	131	134	134	132	137	137	148
15	US Aqua	117	126	106	193	125	123	129	133
16	US Aqua FOG	126	132	134	153	136	137	143	153
17	KLB	206	236	239	233	232	248	255	258

Table 7-19: Experiment IV-2 CYA Concentration vs. Time Raw Data

	Cyanuric Acid Concentration mg/L			
Thai FOG [mg/L]	T=0 Hrs	T=24 Hrs	T=48 Hrs	T=72 Hrs
50	121	120	120	120
75	121	120	95	120
100	121	85	110	110
150	121	85	90	120
300	121	80	85	95

Table 7-20: Experiment IV-4 Raw CYA vs. Time Data

	CYA Concentration [mg/L]										
Flask Label	T=0 Day	T=1 Day	T=2 Day	T=3 Day	T=4 Day	T=5 Day	T=6 Day	T=7 Day	T=8 Day	T=9 Day	T=14 Day
US-200 Glucose	57.5	60	55	57.5	55	55	52.5	47.5	60	65	55
US-200 Glucose	50	57.5	40	32.5	40	37.5	42.5	30	30	42.5	40
US-50 Glucose	60	50	52.5	40	47.5	55	50	37.5	50	52.5	47.5
US-50 Glucose	52.5	42.5	40	27.5	35	42.5	42.5	35	37.5	45	37.5
Thai-200 Glucose	20	12.5	22	0	0	0	0	0	0	0	0
Thai-200 Glucose	12.5	25	2.5	0	6	0	0	0	0	0	0
Thai-200 Sunscreen	10	4	36	32	29	4	12.5	20	25	10	29
US-200 Sunscreen	32.5	37.5	40	30	40	42.5	37.5	30	32.5	35	32.5
Cyanuric Acid	40	37.5	20	40	37.5	40	40	32.5	40	37.5	37.5
Water	0	0	0	0	0	0	0	0	0	0	0

Table 7-21: Experiment IV-5 Raw CYA vs. Time Data

Time [Hours]	CYA [mg/L]
0	82.5
0.1	85
1	82.5
2	80
2.1	77.5
3	70
4	82.5
5	70
9	80

Table 7-22: Experiment IV-6 Raw CYA vs. Time Data

	CYA Concentration						
Treatment	T=0 Days	T=0.2 Days	T=1 Days	T=2 Days	T=3 Days	T=4 Days	T=5 Days
Thai FOG 1	68	32.5	32.5	27.5	10	5	7.5
Thai FOG 2	60	30	37.5	25	22.5	5	7.5
Thai FOG 3	60	37.5	37.5	25	15	7.5	12.5
US FOG 1	60	55	57.5	52.5	52.5	42.5	55
US FOG 2	60	57.5	52.5	52.5	47.5	47.5	55
US FOG 3	62	70	60	47.5	45	37.5	52.5

Table 7-23: Experiment IV-11 CYA Peak Area Raw Data

Time (Hours)	Peak Area [mAU*s]
0.5	12251
4.5	12160
18	13966
24	12376
48	11603
72	11947

Table 7-24: Experiment IV-12 CYA Peak Area Raw Data

		Peak Area [mAU*s]				
Flask	Label	T=0 Hours	T=24 Hours	T=48 Hours	T=96 Hours	T=144 Hours
60 ppm	Standard	10194	10286	11017	9523	10035
1	Control	9887	9378	10170	10448	11323
2	Thai FOG	9606	9029	9228	9978	11091
3	Thai FOG, no Glucose	9879	9036	8835	9017	9863
4	Irradiated Thai FOG	10670	9452	9022	8990	10556
5	Premix	10928	9601	9789	9644	11462
6	Osprey Liq	11041	9375	10037	9356	10307
7	Osprey BPB 100	11788	9966	9972	9146	9625
8	US FOG	12899	10025	10874	9436	10072

Table 7-25: Experiment IV-15 CYA Peak Area Raw Data

	Peak Area [mAU*s]							
Flask	T=0 Hours	T=20 Hours	T=50 Hours	T=74 Hours	T=98 Hours	T=120 Hours	T=142 Hours	T=336 Hours
Control	6355.3	7038.9	7191.7	7039.5	7170.7	7682	7313.2	7406.6
40:10:01	6970.3	7033.4	7241.6	7049.8	7013.9	7291.2	7145.4	7423.8
60:10:01	7071.2	7004.2	7010.8	7166.6	7015.7	7147	7597.2	7291.8
50:10:01	7257.3	7060.5	6957.7	6962.9	7027.9	7193.9	6992	7547.6
40:10:01	6744.6	7214.7	7035.3	7004.7	7046	7148.5	7134.3	7622.6
10:10:01	7453.8	6975.3	6942.8	7175.4	6959.2	7770.3	7289.6	8698.9
50 A	7052.2	7259.7	7354.5	7380.8	6992	7192.7	7817.8	7581.9
50 A+H	6811.9	7274.6	7339.9	8189.9	7712.9	8136.4	8132.3	7439.8

Table 7-26: Experiment IV-17 CYA Peak Area Raw Data

	Peak Area [mAU*s]					
Treatment	T=0 Hours	T=2 Hours	T=19 Hours	T=42 Hours	T=65 Hours	T=142 Hours
Control 1	993.2	970.7	963.1	921.1	899.4	954.4
Control 2	992.8	972.5	971.5	921.7	898.7	965.2
5 PPM CAR 1	984.3	985.9	977	925.5	903.5	962.2
5 PPM CAR 2	994.4	981	968.2	923.5	899.8	965.7
50 PPM CAR 1	989.9	1006	952.5	922.2	906.5	945.6
50 PPM CAR 2	978.4	990.9	972.5	921.1	893.4	960
CYA 97	x	x	829.6	784.8	765.1	885.1

Table 7-27: Experiment IV-18 CYA Peak Area Raw Data

	Peak Area [mAU*s]					
Treatment	T=0 Days	T=1 Days	T=2 Days	T=6 Days	T=8 Days	T=9 Days
Control 1	889.6	922.1	246	226.5	214.4	210.2
Control 2	895.4	983	254.1	236.2	215.7	210.6
CAR1	893	961.7	266.9	233.2	211.2	214.5
CAR2	892.4	933.2	257	234.1	215.2	206.1

CAR+GLU1	888.4	926.2	276.7	216.7	215.9	204.8
CAR+GLU2	900.6	929.1	267.1	238.1	217.4	206
CYA 97	788.7	818.5	244	206.7	x	x

Table 7-28: Experiment IV-19 CYA Concentration Raw Data

	CYA Concentration [mg/L]				
Treatment	T=0 Days	T=2 Days	T=3 Days	T=4 Days	T=5 Days
Control 1	121	108	127	103	103
Control 2	131	109	140	103	173
CYA + Dex 50 ppm 1	117	107	115	100	106
CYA + Dex 50 ppm 2	111	107	118	100	104
CYA + Dex 287 ppm 1	110	107	113	101	102
CYA + Dex 287 ppm 2	110	116	117	99	103

Table 7-29: Experiment IV-20 Raw CYA Data

	CYA Concentration [mg/L]								
Treatment	T=0 Days	T=3 Days	T=5 Days	T=6 Days	T=10 Days	T=11 Days	T=12 Days	T=13 Days	T=15 Days
Control 1	46	54	49	52	52	47	51	51	51
Control 2	46	55	56	57	52	49	48	51	49
Thai FOG 1	276*	57	46	55	51	52	52	56	53
Thai FOG 2	281*	63	64	53	53	54	51	56	48
Filter Media 1	269*	59	50	49	49	54	51	56	58
Filter Media 2	271*	58	49	50	47	53	49	58	49

**Data-point excluded from analysis*

Appendix B: Example of Normalization Calculation

Data was normalized to the turbidity level after each dose of sunscreen. **Table 7-30**, below, shows raw and normalized turbidity readings through the duration of Experiment I-3.

US FOG began with 48 NTU at T=0 Days and dropped to 7 NTU at T=1 Days. To normalize the data, the readings from T=0 Days to T=3 Days were each divided by 48 NTU. The Turbidity after the re-dose of sunscreen at T=3.01 Days was 41. Data were normalized from T=3.01 Days to T=7 Days by dividing each value by 41. This method was repeated for each treatment at each re-dose.

Table 7-30: Experiment I-3 Raw and Normalized Turbidity Data

	Raw Data [NTU]				Normalized Data [NTU/NTU_0]			
Day	US FOG	Thai FOG	Rice Bran	Control	US FOG	Thai FOG	Rice Bran	Control
0	48	55	66	47	1.00	1.00	1.00	1.00
1	7	2	30	9	0.15	0.04	0.45	0.19
2	2	3	20	4	0.04	0.05	0.30	0.09
3	1	6	15	1	0.02	0.11	0.23	0.02
3.01	41	63	69	50	1.00	1.00	1.00	1.00
4	9	33	34	16	0.22	0.52	0.49	0.32
5	3	16	22	3	0.07	0.25	0.32	0.06
6	0	14	19	8	0.00	0.22	0.28	0.16
7	2	13	22	2	0.05	0.21	0.32	0.04
7.01	48	55	73	37	1.00	1.00	1.00	1.00
8	6	22	48	11	0.13	0.40	0.66	0.30
9	3	16	44	5	0.06	0.29	0.60	0.14
10	3	13	43	5	0.06	0.24	0.59	0.14
10.01	53	65	93	58	1.00	1.00	1.00	1.00
12	7	28	51	9	0.13	0.43	0.55	0.16
13	5	45	42	6	0.09	0.69	0.45	0.10
14	5	55	48	5	0.09	0.85	0.52	0.09

	Raw Data [NTU]				Normalized Data [NTU/NTU_0]			
Day	US FOG	Thai FOG	Rice Bran	Control	US FOG	Thai FOG	Rice Bran	Control
15	5	59	46	6	0.09	0.91	0.49	0.10
16	5	62	49	4	0.09	0.95	0.53	0.07
16.01	50	105	91	35	1.00	1.00	1.00	1.00
17	8	62	75	10	0.16	0.59	0.82	0.29
18	8	75	62	8	0.16	0.71	0.68	0.23

Appendix C: Example of HPLC Calibration and Conversion of Peak Area to CYA Concentration from Experiment IV-20

Calibration was carried out for experiments IV-17 through IV-20 with increasing complexity, to provide accurate conversion of peak area to concentration of CYA. A four-point calibration was run for Experiment IV-20 using CYA standards of 63, 85, and 105 mg/L, followed by a DI water blank. Calibration and conversion of peak areas to concentration for Day 13 of Experiment IV-20 is shown, below. All peak shapes are representative of duplicate treatments.

Figure 7-1 shows the chromatogram and integration for the 63 mg/L CYA Standard.

Figure 7-2 shows the chromatogram and integration for the "Control 1" treatment.

Figure 7-3 shows the chromatogram and integration for the "Thai FOG 1" treatment.

Figure 7-4 shows the chromatogram and integration for the "Filter Media 2" treatment.

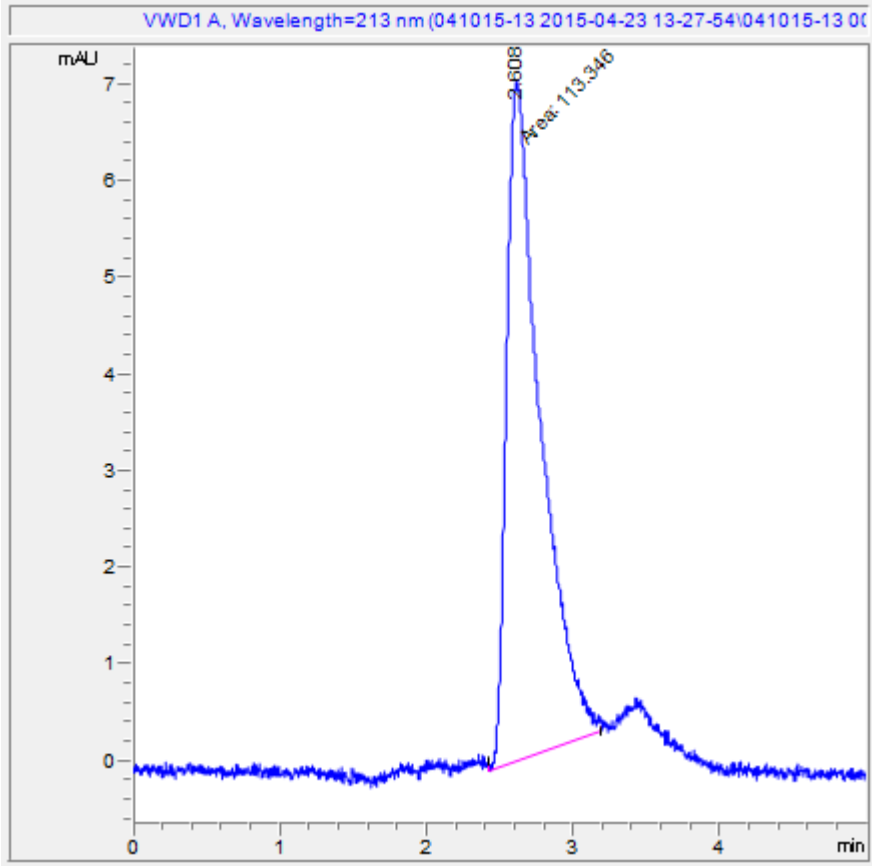


Figure 7-1: Chromatogram for Experiment IV-20, Day 13, 63 mg/L CYA Standard

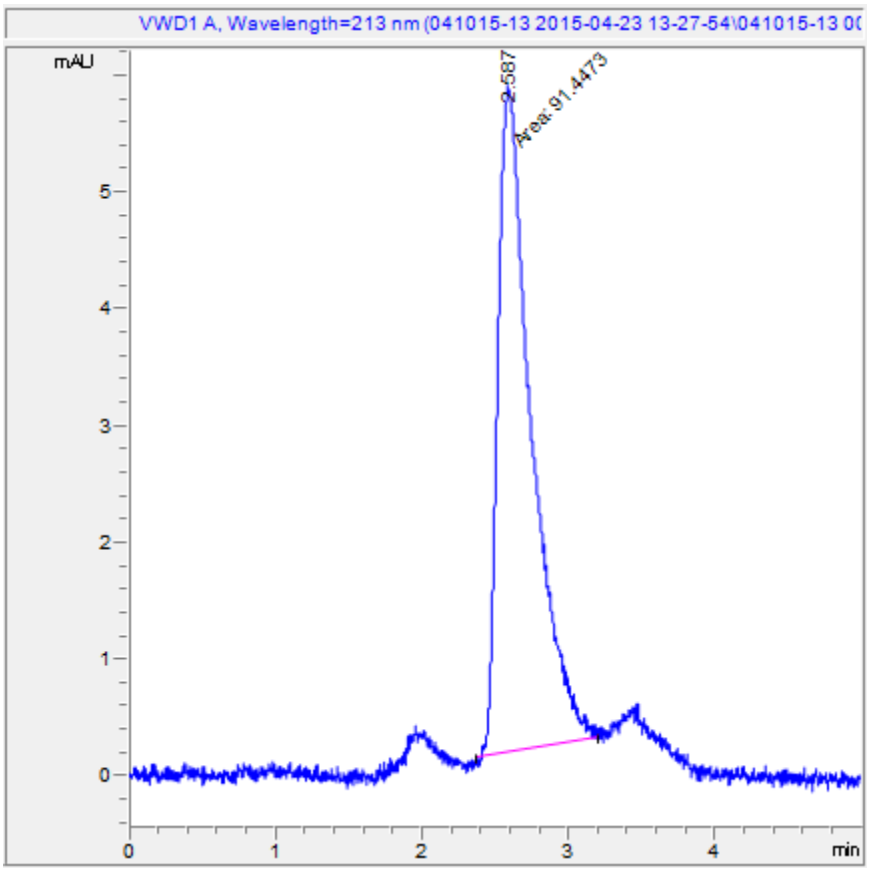


Figure 7-2: Chromatogram for Experiment IV-20, Day 13, “Control 1” Treatment

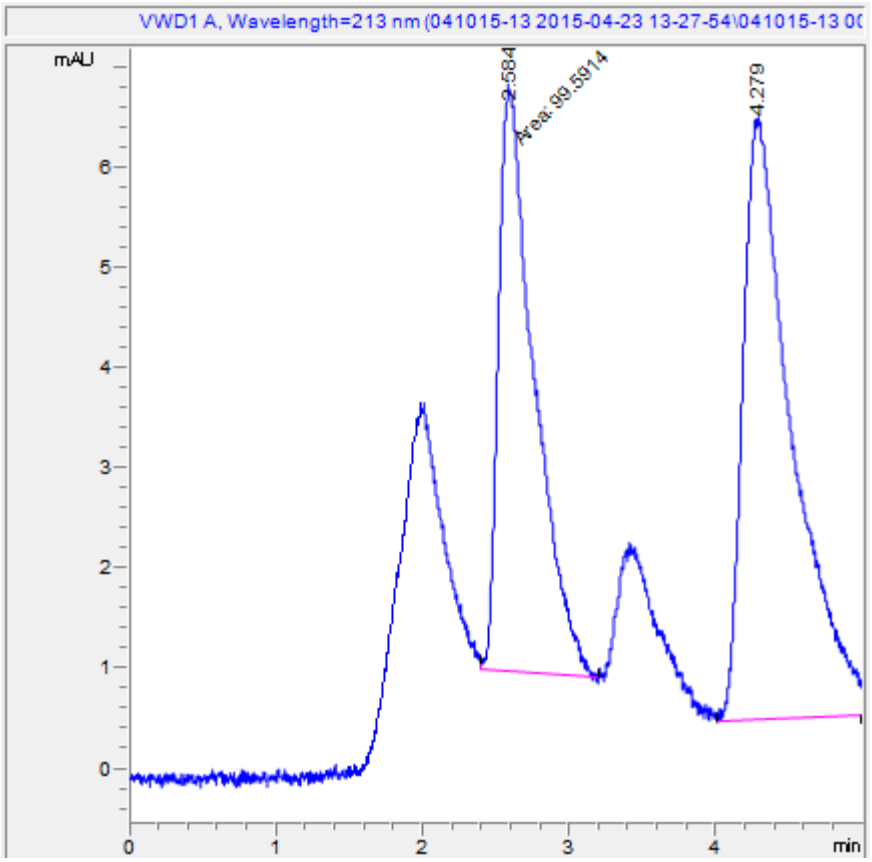


Figure 7-3: Chromatogram for Experiment IV-20, Day 13, “Thai FOG 1” Treatment

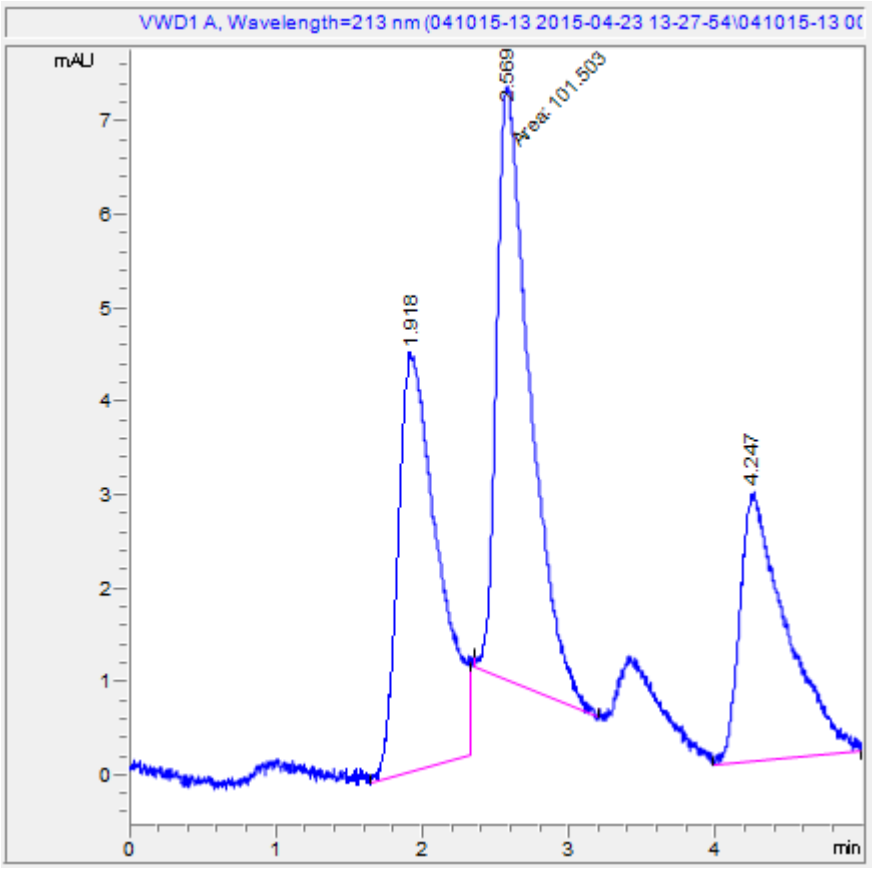


Figure 7-4: Chromatogram for Experiment IV-20, Day 13, "Filter Media 2" Treatment

Peak areas of the standards and blank measured as follows in **Table 7-31**, below.

Table 7-31: Experiment IV-20, Day 13, CYA Standard Curve Peak Areas

CYA Standard mg/L	Peak Area
63	113.3
85	149.5
105	176.6
0	4.4

The resulting peak areas were graphed and fitted with a linear regression model in **Figure 7-5**, below.

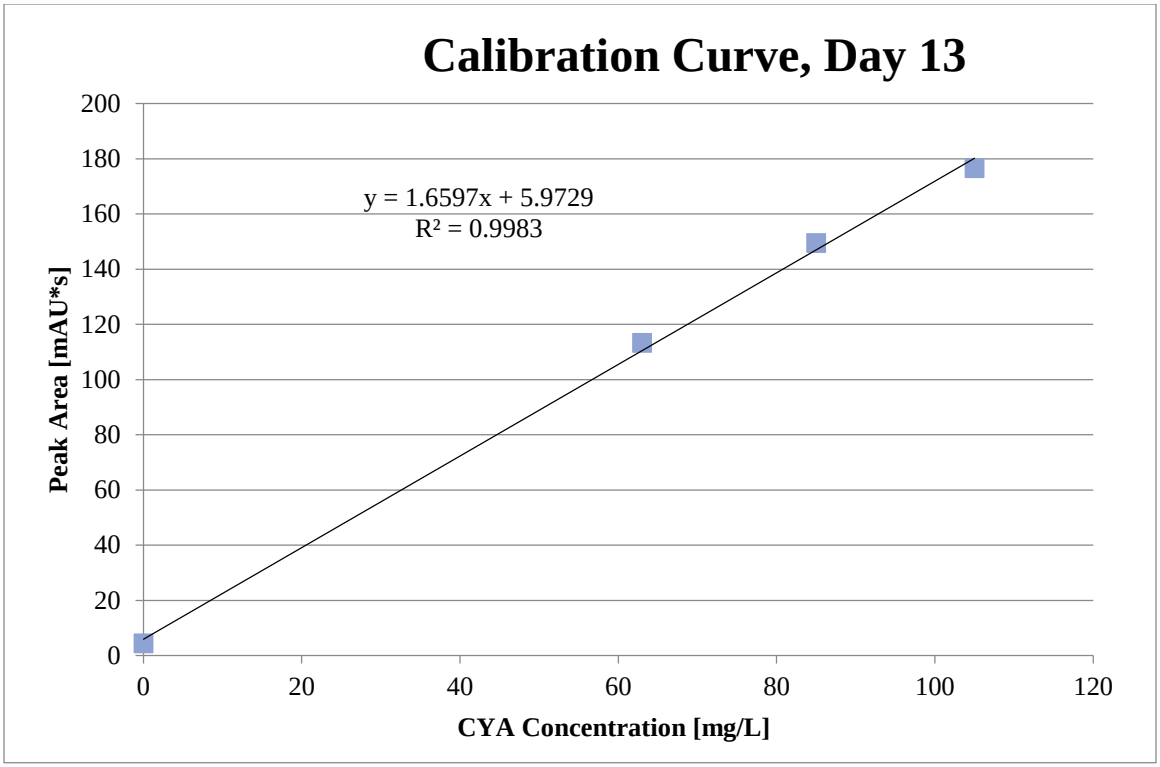


Figure 7-5: Experiment IV-20, Day 13, CYA Calibration Curve

The linear regression returned the equation y = 1.6597x + 5.9729, where "y" represents Peak Area, and "x" represents mg/L CYA. This equation was used to convert peak areas from each chromatogram to mg/L CYA, in **Table 7-32**, below. Concentrations were rounded to the nearest whole number, to match the precision of prepared standards.

Table 7-32: Experiment IV-20, Day 13, Peak Area and CYA Concentration

Treatment	Peak Area [mAU*s]	CYA Concentration [mg/L]
Control 1	91.4	51
Control 2	90.3	48
Thai FOG 1	99.6	52
Thai FOG 2	99.5	51
Filter Media 1	99.2	51
Filter Media 2	101.5	49

Appendix D: SEM Images from Experiment I-8

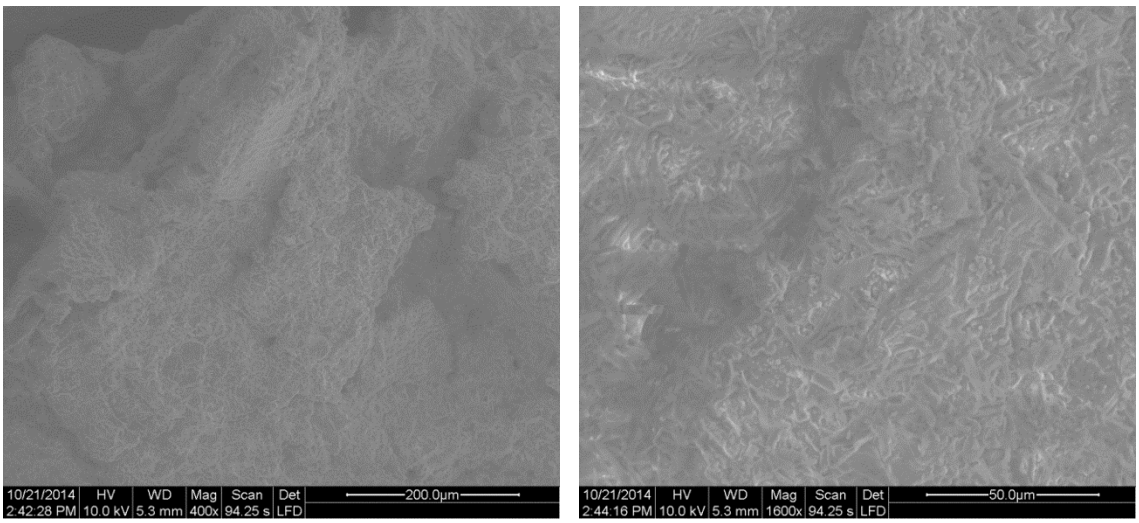


Figure 7-6: Premix New 400x (Left), Premix New 1600x (Right)

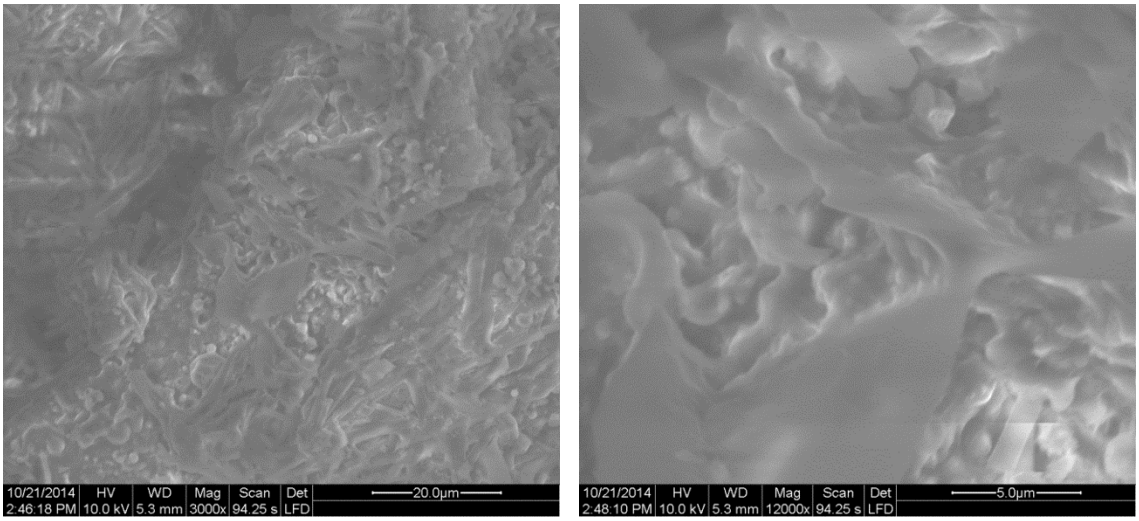


Figure 7-7: Premix New 3000x (Left), Premix New 12000x (Right)

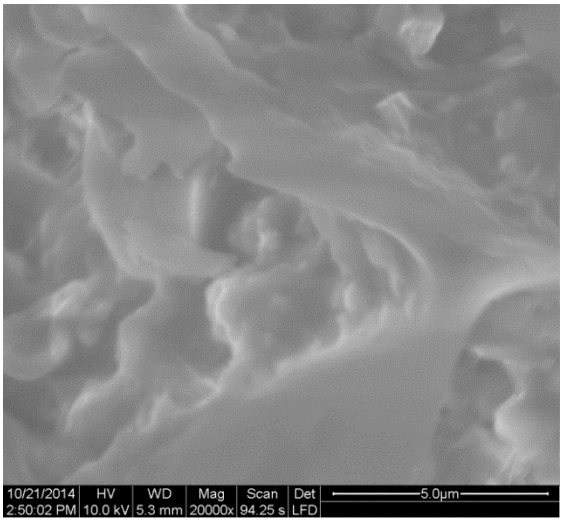
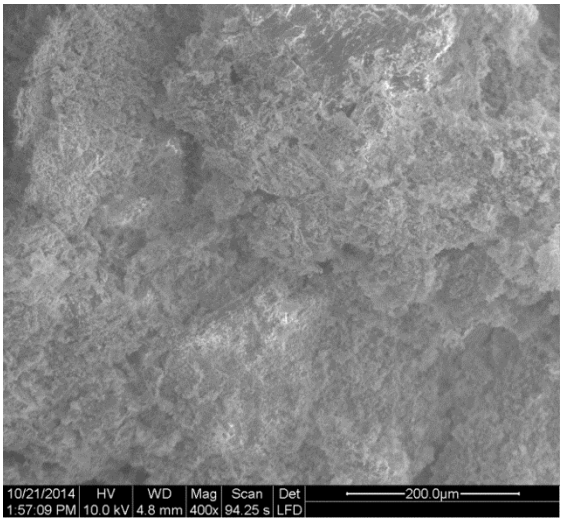


Figure 7-8: Premix New 20000x



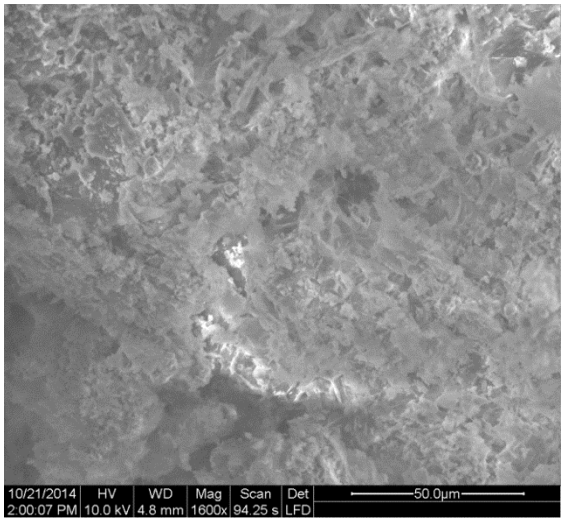


Figure 7-9: Premix Used 400x (Left), Premix Used 1600x (Right)

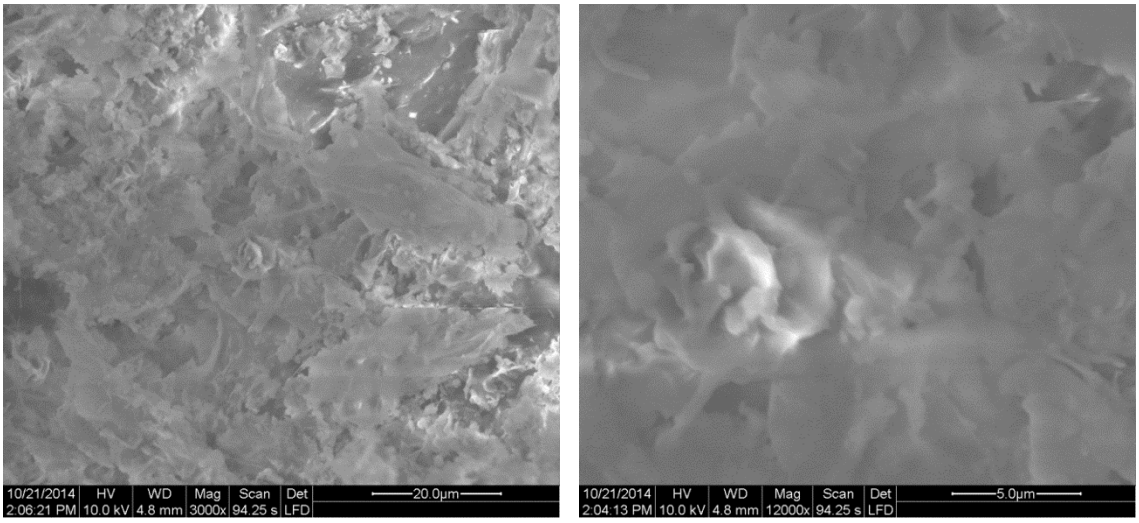


Figure 7-10: Premix Used 3000x (Left), Premix Used 12000x (Right)

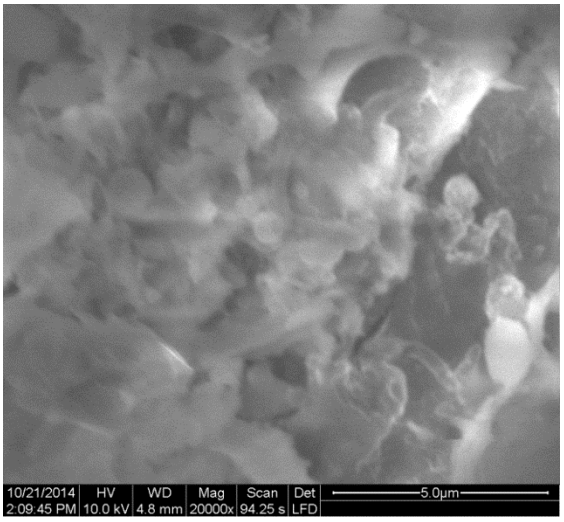


Figure 7-11: Premix Used 20000x

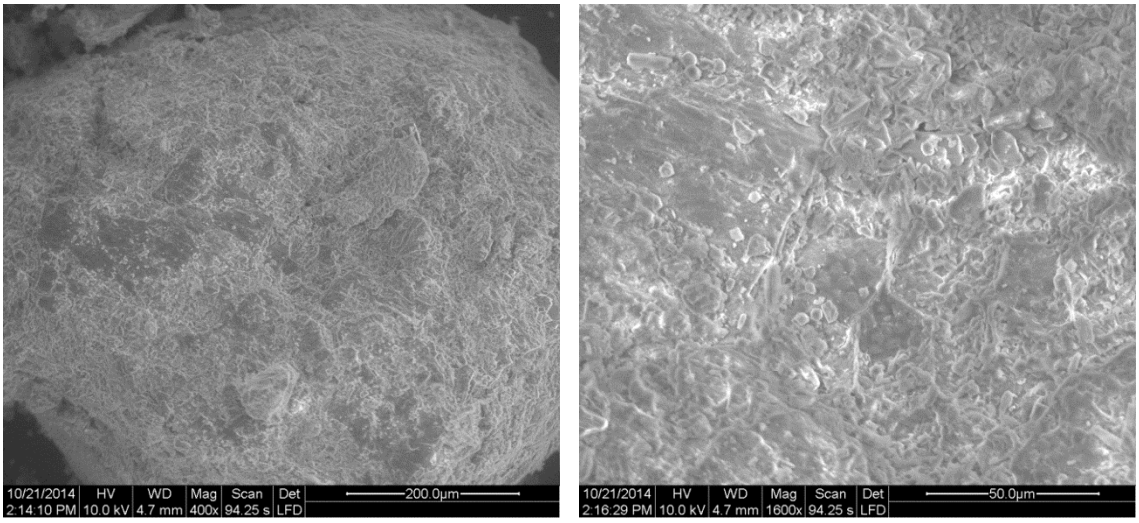


Figure 7-12: Thai FOG New 400x (Left), Thai FOG New 1600x (Right)

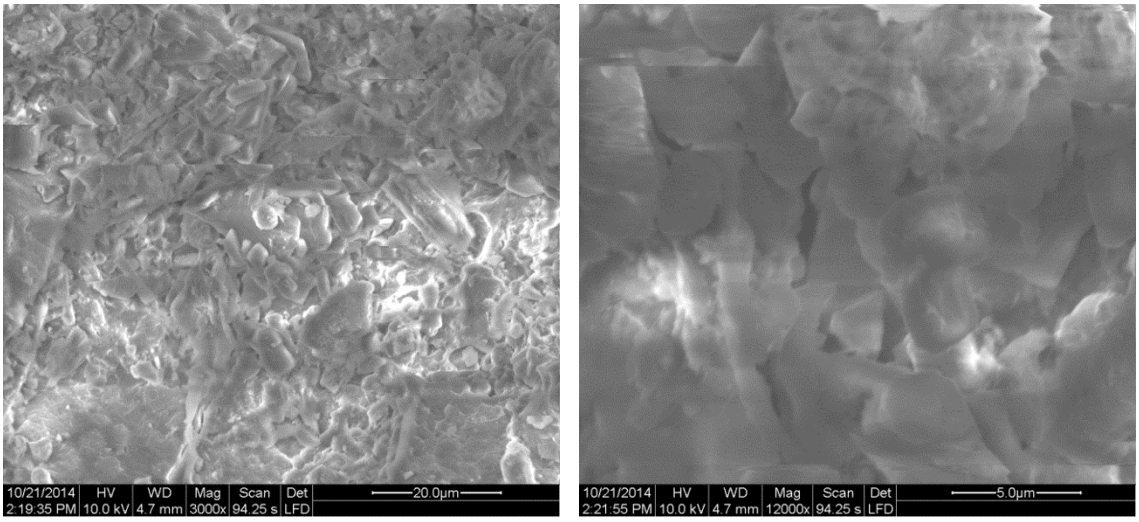


Figure 7-13: Thai FOG New 3000x (Left), Thai FOG New 12000x (Right)

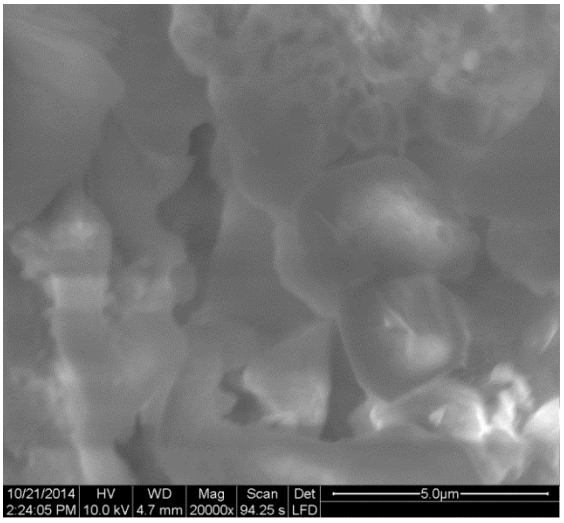
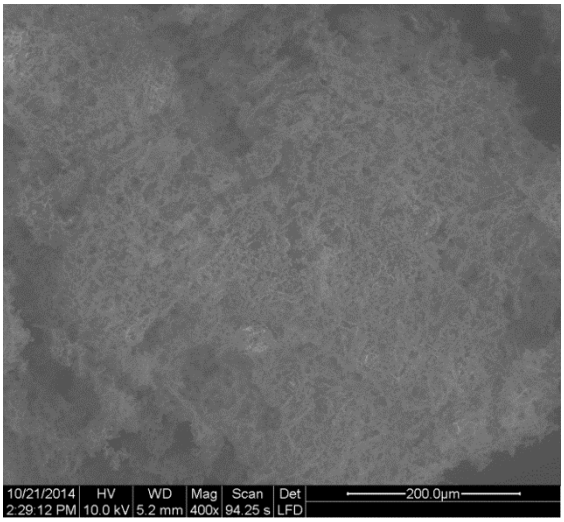


Figure 7-14: Thai FOG New 20000x



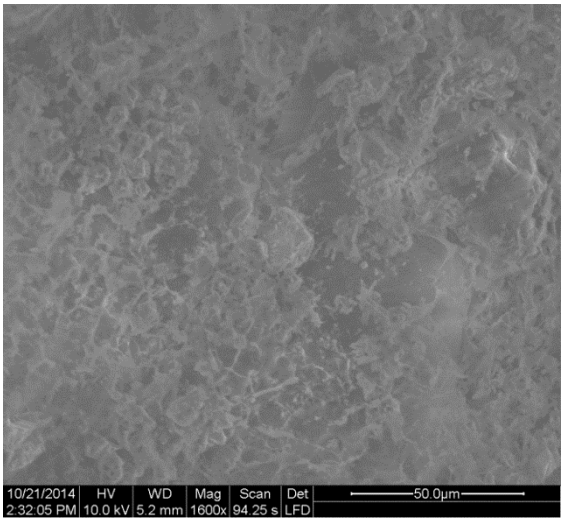


Figure 7-15: Thai FOG Used 400x (Left), Thai FOG Used 1600x (Right)

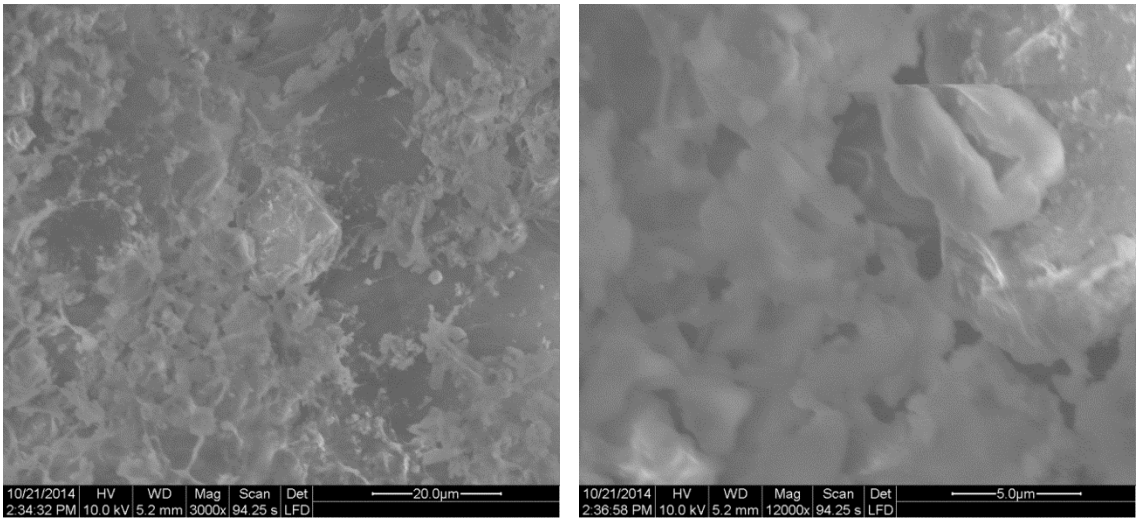


Figure 7-16: Thai FOG Used 3000x (Left), Thai FOG Used 12000x (Right)

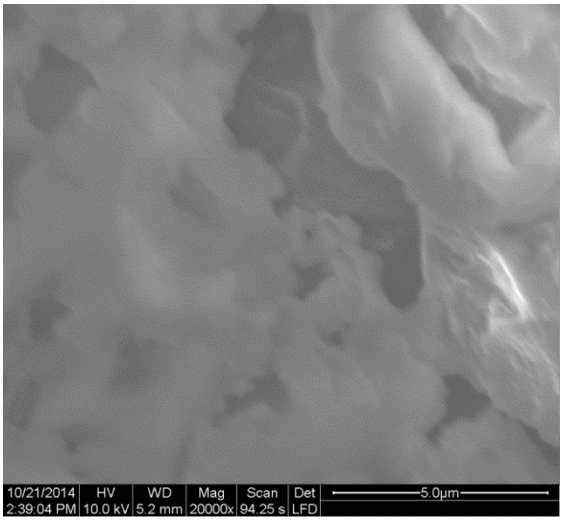


Figure 7-17: Thai FOG Used 20000x

Appendix E: Example HPLC Chromatograms from Experiment IV-20

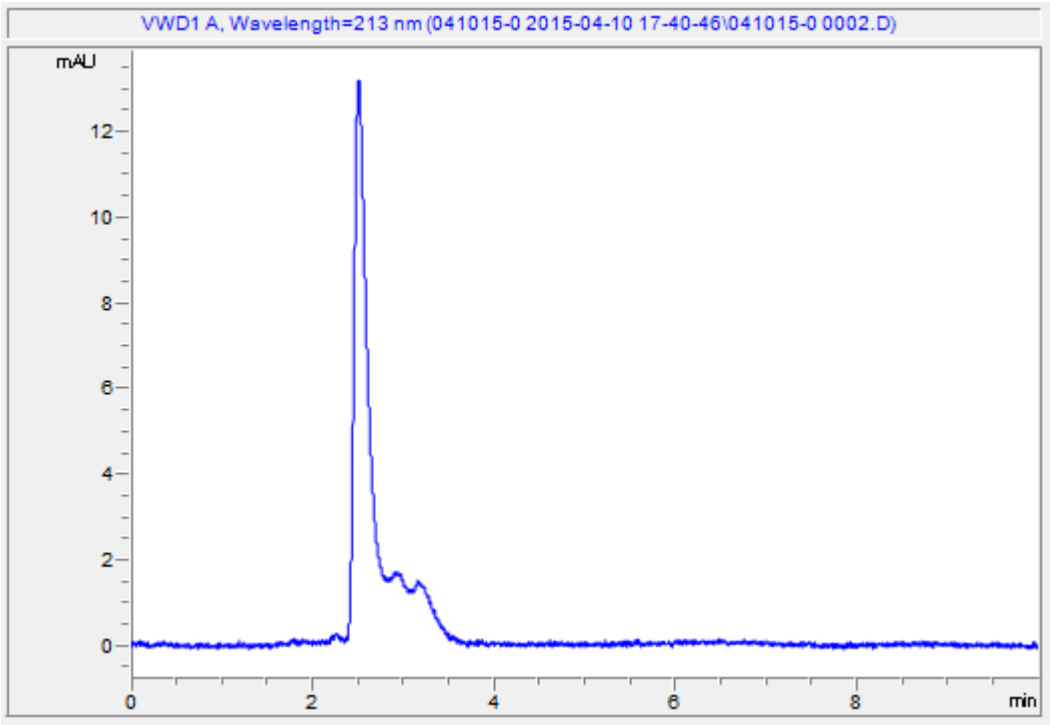


Figure 7-18: Experiment IV-20, 85 mg/L CYA Standard, Day 0

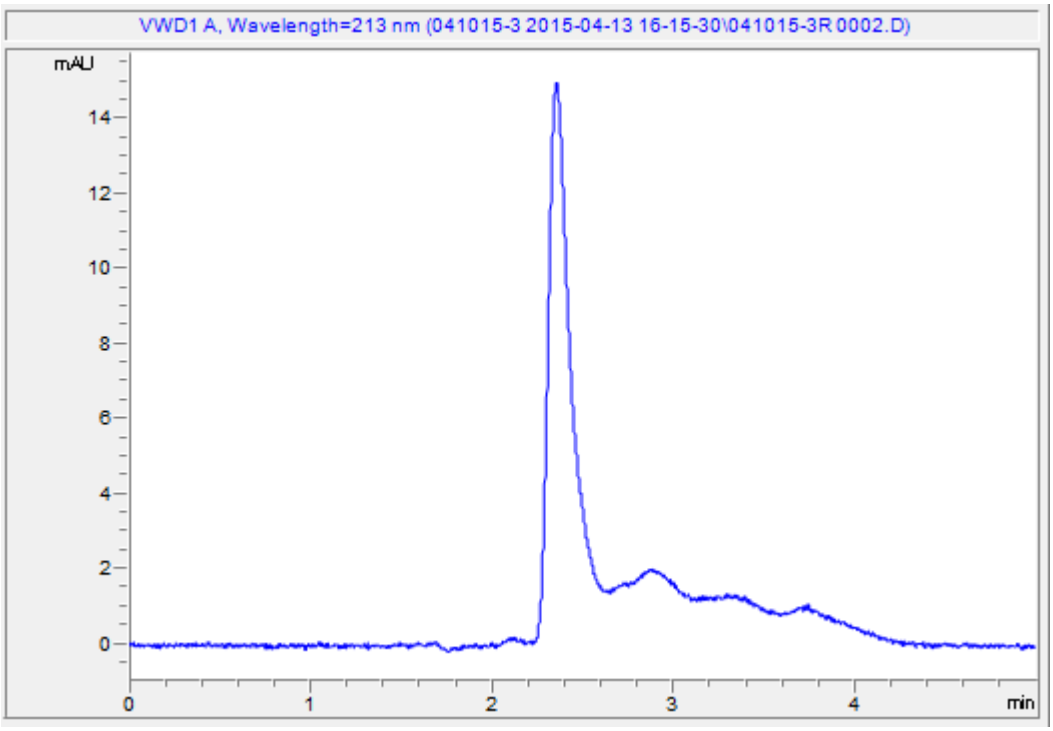


Figure 7-19: Experiment IV-20, 85 mg/L CYA Standard, Day 3

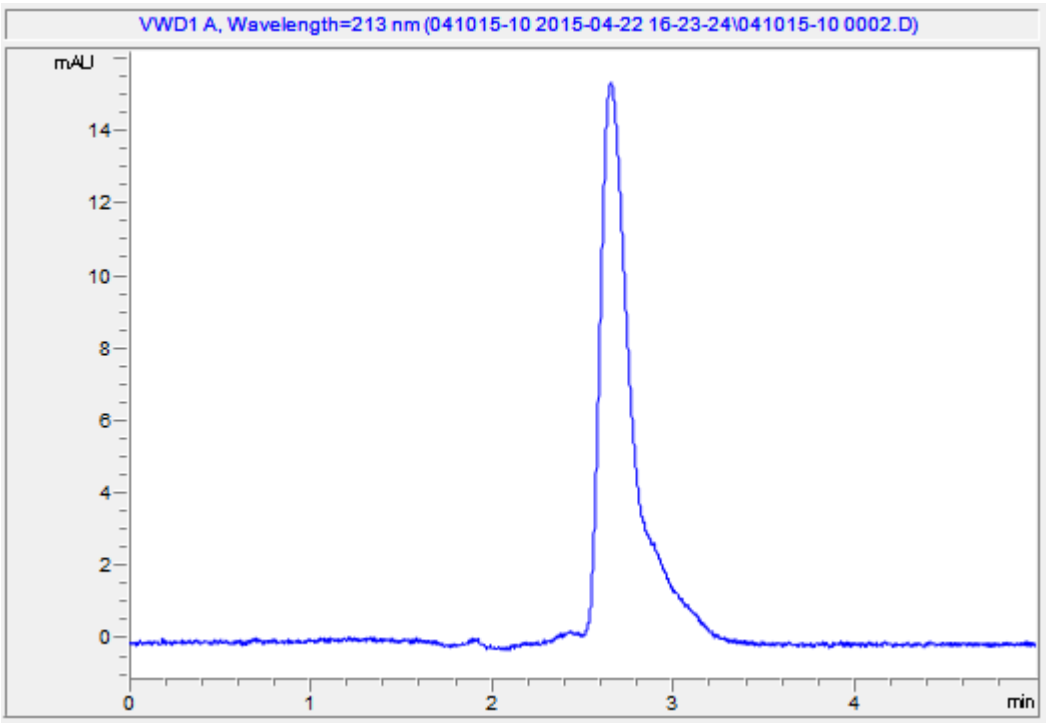


Figure 7-20: Experiment IV-20, 85 mg/L CYA Standard, Day 10

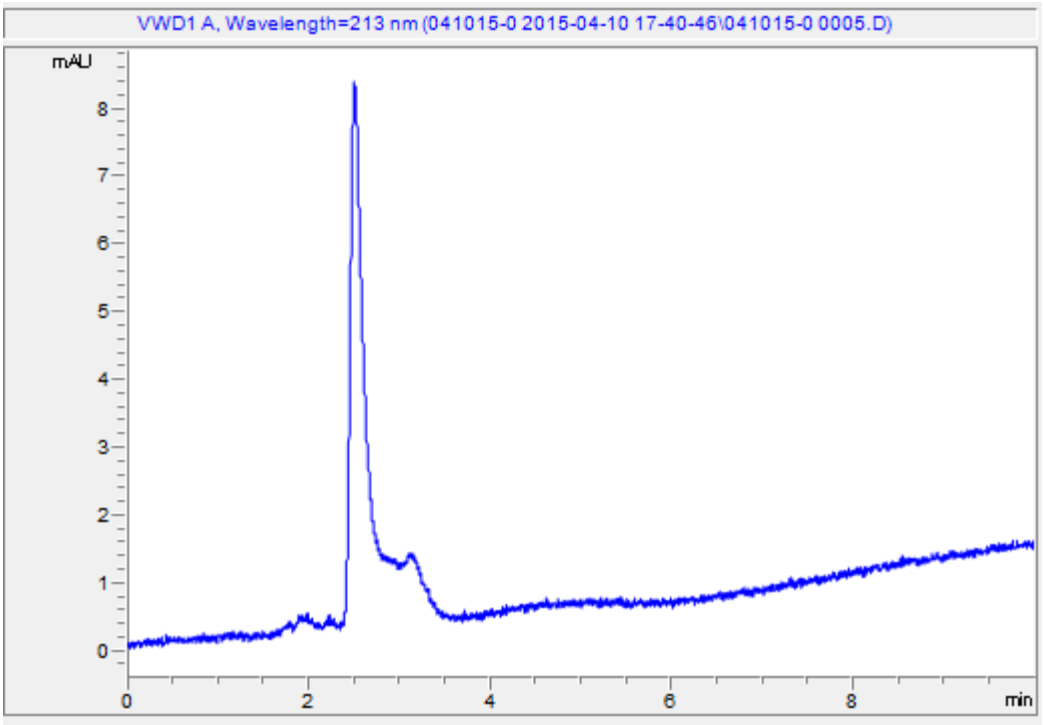


Figure 7-21: Experiment IV-20, 50 mg/L CYA Control, Day 0

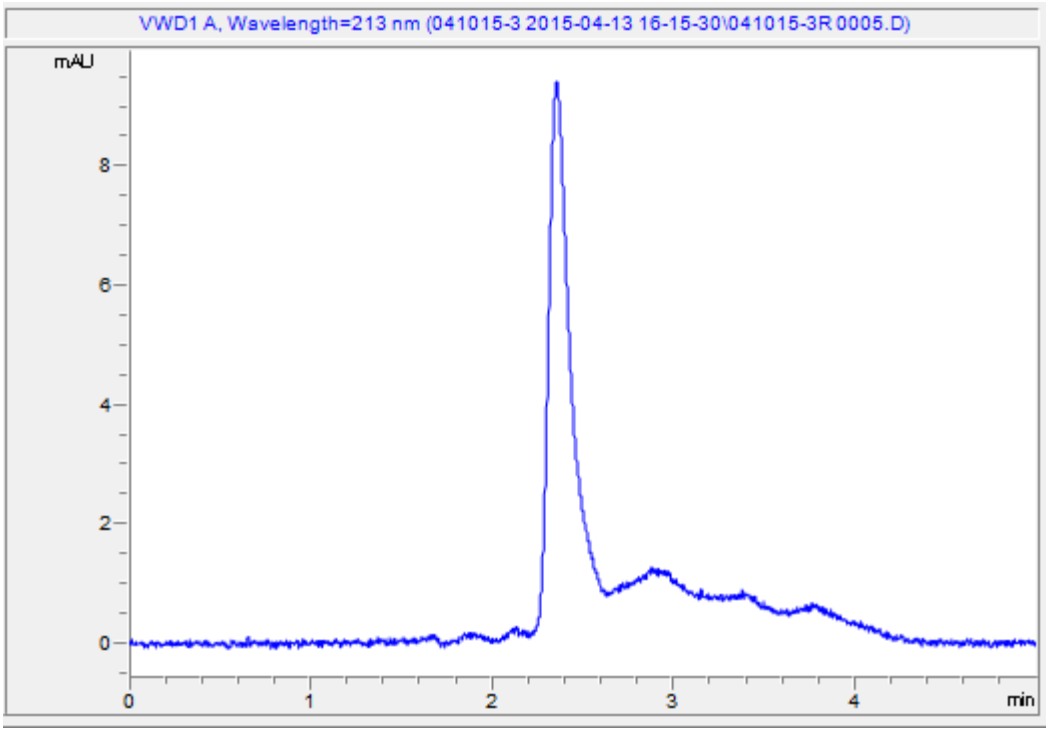


Figure 7-22: Experiment IV-20, 50 mg/L CYA Control, Day 3

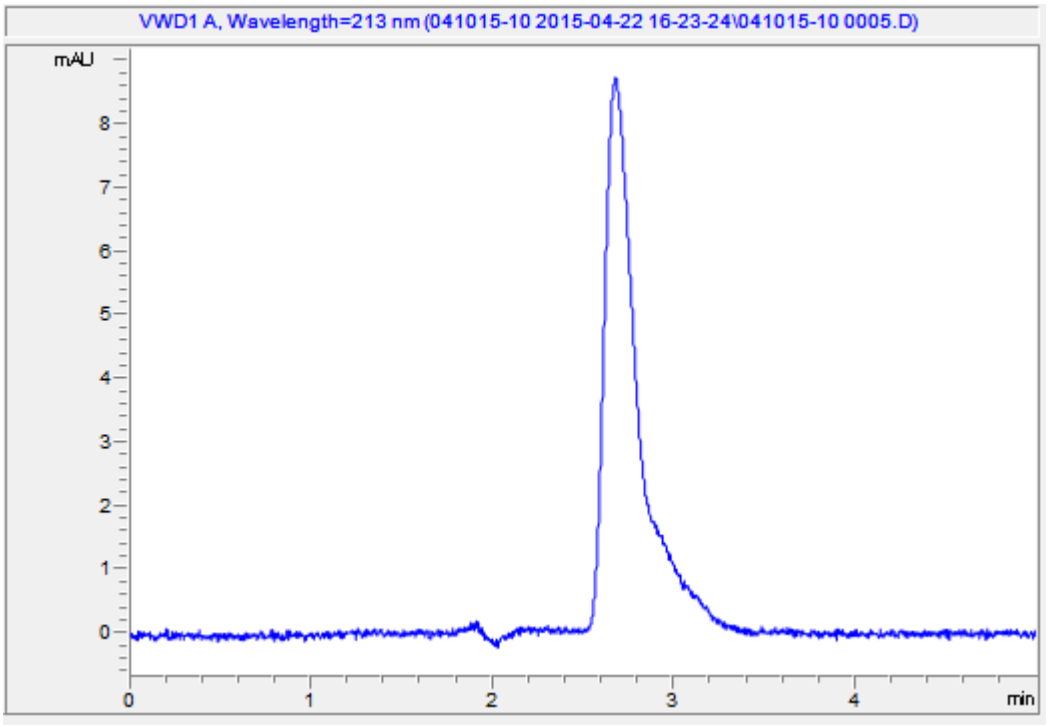


Figure 7-23: Experiment IV-20, 50 mg/L CYA Control, Day 10

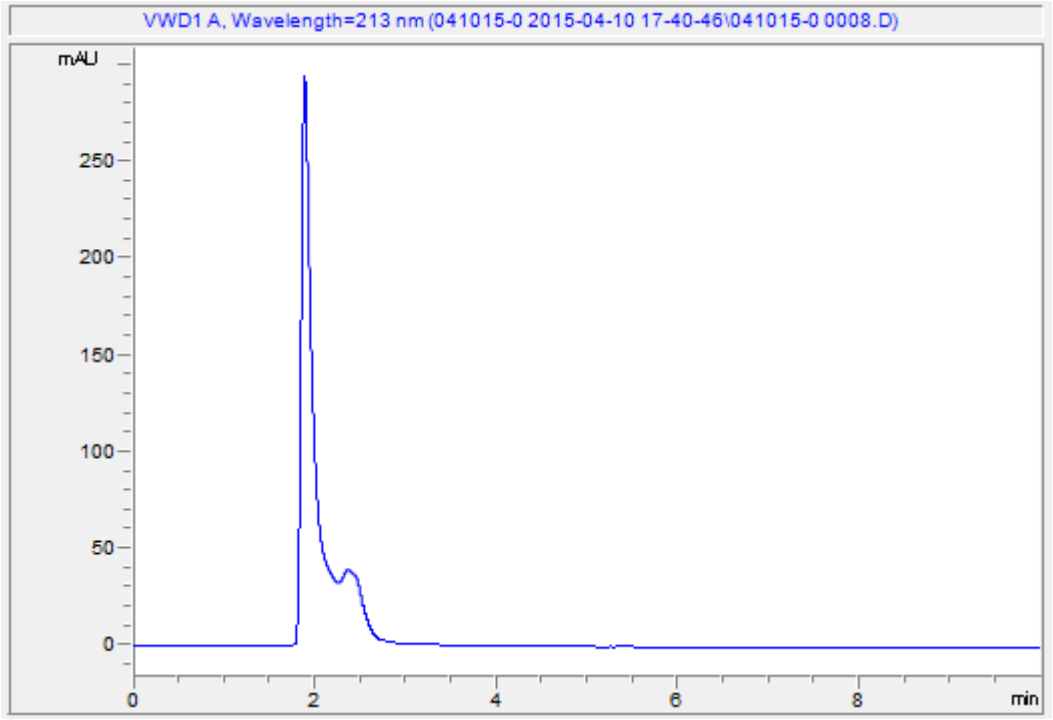


Figure 7-24: Experiment IV-20, Growth Media, Thai FOG with CYA, Day 0

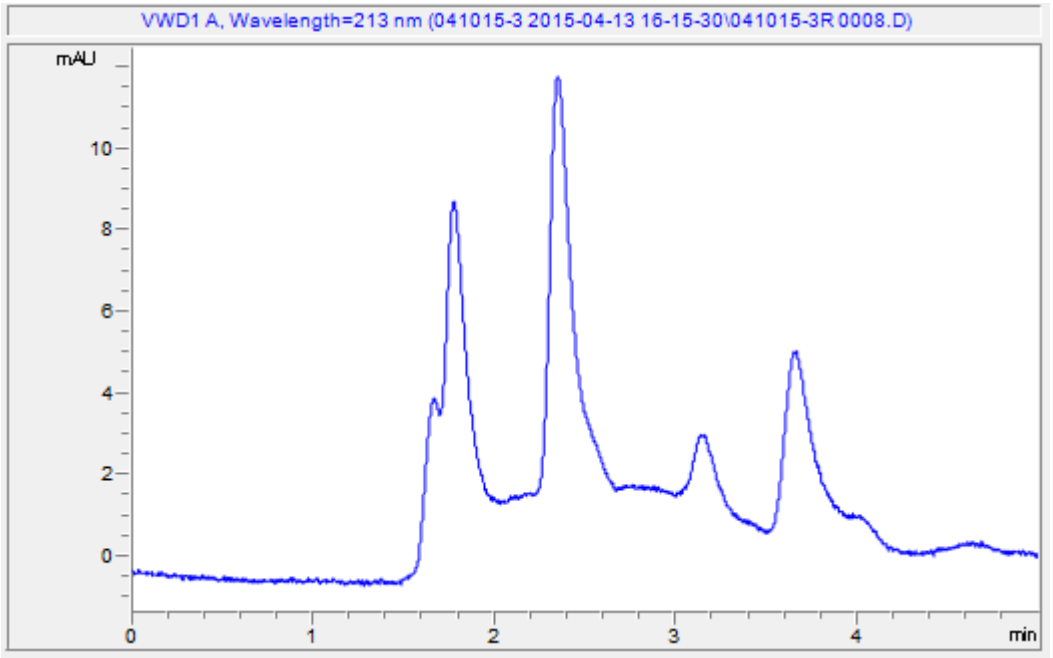


Figure 7-25: Experiment IV-20, Growth Media, Thai FOG with CYA, Day 3

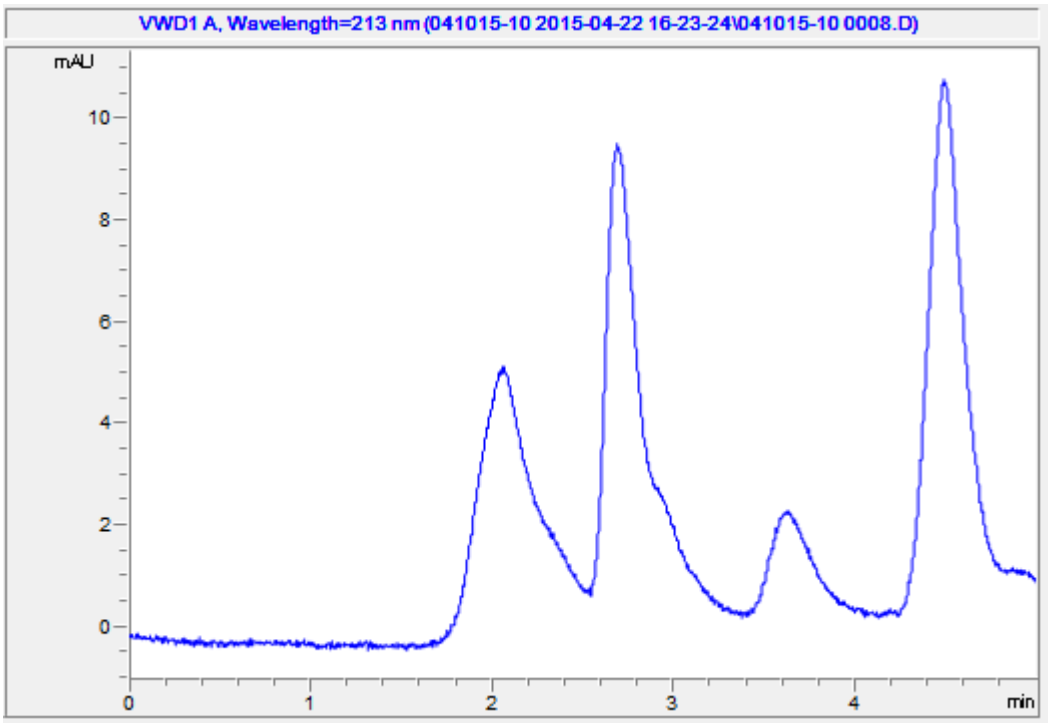


Figure 7-26: Experiment IV-20, Growth Media, Thai FOG with CYA, Day 10

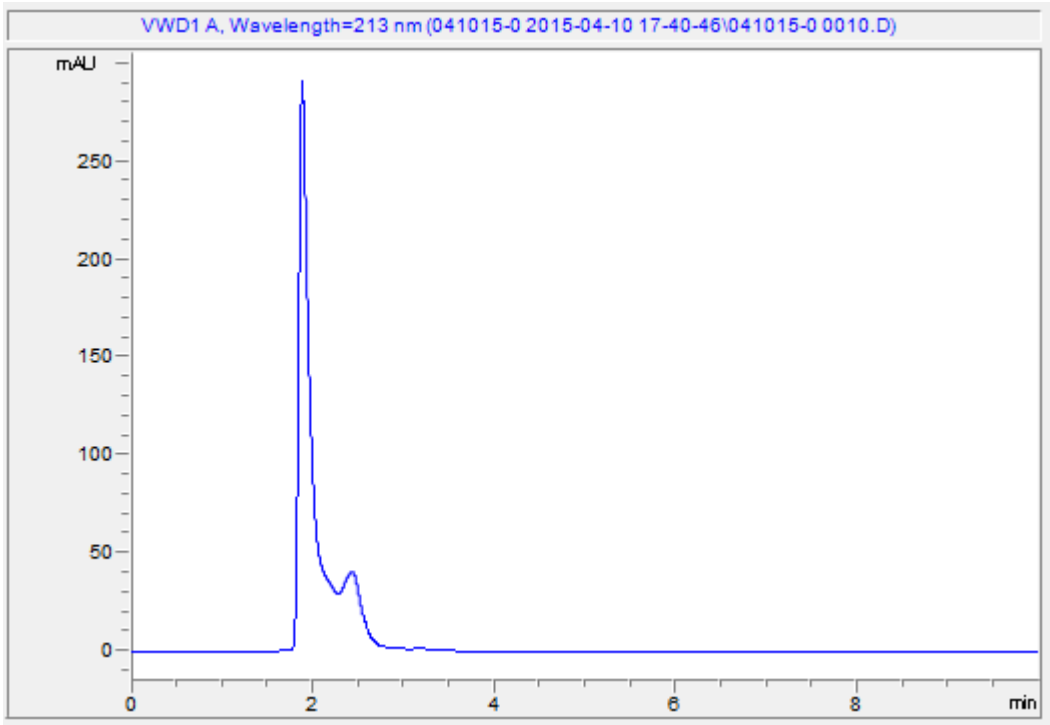


Figure 7-27: Experiment IV-20, Growth Media, Filter Media with CYA, Day 0

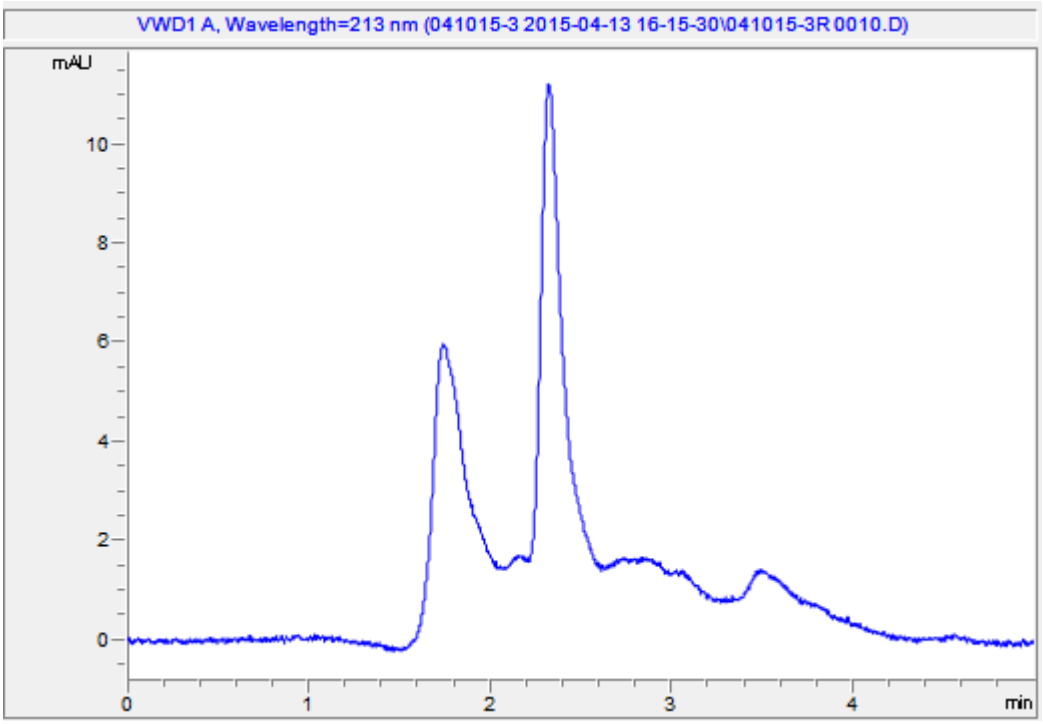


Figure 7-28: Experiment IV-20, Growth Media, Filter Media with CYA, Day 3

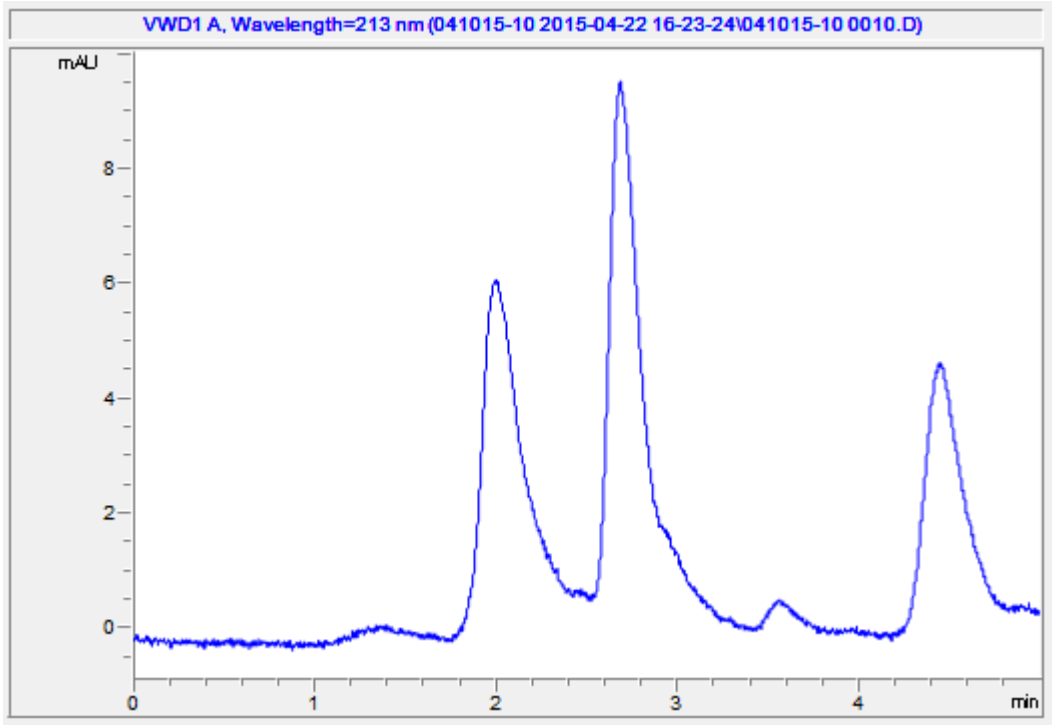


Figure 7-29: Experiment IV-20, Growth Media, Filter Media with CYA, Day 10