Entry to the Stockholm Junior Water Prize 2014

Waste to Water:
Biodegrading Naphthenic Acids using Novel Sand Filters

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2. Preliminary Matters

2a. Abstract

This study involved designing, constructing, and testing the effectiveness of slow sand filters (SSFs) newly applied as biofilm bioreactors to the biodegradation of the toxic naphthenic acids (NAs) found in oil sands tailings ponds. After constructing a bench scale SSF bioreactor system, three indigenous tailings bacterial isolates were selected and used in both SSF and equivalent planktonic batch culture (PBC) bioreactors to biodegrade a solution of NAs. Planktonic microbial growth, biofilm development, and NA concentration reductions were monitored to determine the effectiveness of each bioreactor. In one week, the SSF bioreactors reduced the total NA concentrations 2.5 times faster than the PBC bioreactors, achieving concentrations that would almost be safe for fish. In addition, the SSF bioreactors promoted notable biofilm development with seemingly enhanced metabolic capabilities. A sizeable application of these cost-effective and sustainable bioreactors could potentially biodegrade the NAs in all oil sands tailings water in less than 20 years on average (14 times faster than PBC bioreactors). This detoxification of tailings water could prevent additional pollution of the groundwater and surface water resources in the oil sands region.

2b. Table of Contents

2c. Key Words

·Naphthenic Acid ·Slow Sand Filters ·Oil Sands ·Tailings Ponds ·Oil Sands Tailings ·Microbiology ·Bacteria ·*Acidovorax* ·*Pseudomonas* ·*Xanthobacter* ·Biofilm ·Sand ·*Schmutzdecke* ·Bioreactor ·Aerobic ·Planktonic Batch Culture ·Biodegradation ·Bioremediation ·Water Pollution.

2d. Abbreviations and Acronyms

NA-Naphthenic Acid; SSF-Slow Sand Filter; PBC-Planktonic Batch Culture; MBH-Modified Bushnell-Haas; CHCA-Cyclohexanecarboxylic Acid; CHAA-Cyclohexaneacetic Acid; CHBA-Cyclohexanebutyric Acid; CHPeA-Cyclohexanepentanoic Acid; AdCA-1-Adamantanecarboxylic Acid; THNA-5,6,7,8-Tetrahydro-2-Naphthoic Acid; S-Sterile control; A-*Acidovorax* sp.; P-*Pseudomonas* sp.; X-*Xanthobacter* sp.; C-Combination.

2e. Acknowledgements

Lisa Gieg, Ph.D, Assistant Professor, University of Calgary Department of Biological Sciences, and Lindsay Clothier, Masters Candidate, donated and supervised the use of various materials, lab equipment, and lab space for my original project idea.

Shawn Lewenza, Ph.D, Associate Professor, University of Calgary Department of Microbiology, Immunology, and Infectious Diseases, and Dr. Mike Wilton, Ph.D, Postdoctoral Fellow, guided the epifluorescence microscopy.

2f. Biography

Before watching Al Gore's "An Inconvenient Truth" at the age of ten, I never imagined the possibility that humans could significantly affect the planet. The zeal for environmentalism that the film inspired drove me to address these issues in my science fair projects over the next six years. I made my first career decision during this whirlwind of projects on topics ranging from atmospheric haze to solar energy. In eighth grade, I stayed up late to research environmental issues while my parents slept. At that time of night, I should have been tired; instead I felt invigorated. In high school I realized the significance of my scientific passion. I was meant to become a scientist. After all, I couldn't imagine pursuing a better path than one that constantly fascinated me. Unfortunately, my career decisions weren't finished because I still didn't know which scientific discipline to choose. Yet again, I discovered

the answer through science fair. After spending more than two hundred hours working on this project in a microbiology lab and three hundred hours at home, I was still fighting the urge to skip down the hallways. A few months into my research, I realized I'd never enjoyed anything more. Although neither of my parents work in this field, my passion for science has motivated me to rise to the challenge. My future academic goals involve the Ph.D degree program after working alongside Ph.D students in the lab. My passion for science will continue to unlock a world of learning opportunities as I work towards my dream career.

3. Introduction

Tailings ponds are an environmental concern currently facing the rapidly expanding Canadian oil sands industry. During bitumen extraction, an aqueous mixture of fine silts, hydrocarbons, salts, and soluble organic compounds called oil sands tailings is produced. These tailings are acutely toxic to mammals, fish, plants, and all but the most resilient bacteria [1]. In the tailings free water zone, 76% of the acute toxicity is caused by organic compounds called naphthenic acids (NAs) [2]. NAs are a persistent mixture of mono- and polycycloalkane carboxylic acids with aliphatic side chains that are especially difficult to break down due to their hydrophilic and hydrophobic moieties [3]. As a result, they resist degradation and present a long term environmental hazard.

By 2025, the total volume of accumulated tailings is expected to equal one billion m^3 [4]. This large volume of tailings is stored indefinitely in unlined outdoor reservoirs where natural consolidation into a dry trafficable surface would take hundreds of years [2]. During this time, models estimate that 11 to 12.6 million litres of toxic oil sands tailings leaks into the surrounding environment every day [5]. This seepage poses a threat to adjacent boreal forests and freshwater resources because the tailings ponds are located along the shores of the Athabasca River. With oil sands development only expected to increase in the future, there is a clear need for technology addressing the source of the toxicity in the tailings.

Since the primary toxic components of the oil sands tailings (NAs) occur as a variable and uncharacterized mixture, they cannot be effectively treated using physical or chemical methods [6]. The alternative, biodegradation, is the biologically catalyzed alteration of the chemical structure of pollutants that results in less toxic metabolites [7]. Accelerated biodegradation can be accomplished in a bioreactor (an apparatus used to carry out any kind of biological process) [8]. The primary biodegradation of NAs

occurs via β-oxidation of the carboxylic acid functional group [1]. Biological methods are often safe and effective because they involve the chemical breakdown of contaminants instead of storing, evaporating, or diluting them. However, the only NA biodegradation technology that has been attempted (constructed wetlands) was determined to be impractical due to extremely low hydraulic loading rates [4].

Invented in 1804, 'slow' sand filters (SSFs) were the first modern water treatment process [9]. These filters produce potable water by developing a biofilm called a *schmutzdecke* on top of a sand bed which removes any contaminants. A biofilm is the accretion of bacteria embedded in an extracellular polymeric matrix attached to a solid surface [10]. SSF technology has been proven effective over the last two hundred years, with many large European cities still relying on this to clean municipal water [9]. Despite their ability to promote biofilm growth, the potential of SSFs to biodegrade NAs has not been reported in any literature. Instead, SSFs have only ever been used for the treatment of surface or ground water for human consumption. In this project, I investigated the previously undiscovered potential of SSFs when used as bioreactors for the unconventional purpose of biodegrading NAs. The implications of this original scientific research could possibly reveal a new way to treat the NAs in the constantly expanding volume of oil sands tailings.

The purpose of this study was to design, construct, and investigate the use of SSFs newly applied as novel aerobic bioreactors to the microbial degradation of NAs. Specifically, the effectiveness of bench scale SSF bioreactors was assessed relative to equivalent planktonic batch culture (PBC) bioreactors by evaluating their efficiency at reducing the concentration of six increasingly complex NAs through biodegradation. Their success at promoting the planktonic and biofilm microbial growth of three separate indigenous tailings bacterial isolates was also determined. It was hypothesized that if SSFs and PBCs were used as bioreactors to degrade NAs, then the NAs in the SSF bioreactors would consistently undergo the most biodegradation due to the formation of biofilms on the sand particles. Biofilms have frequently been more metabolically efficient than planktonic cells when found in other biological and medical contexts [11]. The *schmutzdecke* that SSFs traditionally develop is a biofilm composed of microorganisms derived from the water supply. Therefore, the SSF bioreactors may promote similar potentially advantageous biofilm growth using the indigenous tailings bacteria.

4. Materials and Methods

4a. Selection of Indigenous Tailings Bacterial Isolates

Before the main experiment started, the three bacterial isolates that would be used in the bioreactors were chosen. Nine triplicate sets of PBC bioreactors containing one of seven available bacterial isolates were created in flasks (Figure 1). My bioreactors contained the following: a Modified Bushnell-Haas (MBH) salts medium to supply inorganic macro- and micronutrients [12], 4 mL of a bacterial isolate liquid culture for inoculation (per every 1 L of MBH salts medium), and 100 mg/L in total of the six NAs [cyclohexanecarboxylic acid (CHCA), cyclohexaneacetic acid (CHAA), cyclohexanebutyric acid (CHBA), cyclohexanepentanoic acid (CHPeA), 1-adamantanecarboxylic acid (AdCA), and 5,6,7,8-tetrahydro-2-naphthoic acid (THNA)]. This is the average concentration of NAs in oil sand tailings ponds [2]. After measuring the microbial growth in these PBC bioreactors over the course of a week using UV-Vis spectrophotometry [13], I determined which bacteria demonstrated the greatest amount of growth despite the highly toxic NAs. Isolates of the bacteria were subjected to DNA extractions [14], PCR amplification [14], agarose gel electrophoresis [15], and PCR product purification [15]. The purified samples were then sent for independent Sanger sequencing [14]. The three bacterial isolates that thrived were identified as *Acidovorax* sp., *Pseudomonas* sp., and *Xanthobacter* sp. via BLAST [15].

Figure 1. The 45 PBC bioreactors built during the isolate selection process. Three replicates of each of the seven bacterial isolates as well as sterile controls were created and monitored.

4b. SSF Bioreactor Trial Designs

In order to conduct the main experiment, an original bench scale SSF bioreactor system was designed, constructed, and tested. Glass 50 mL syringes with a small piece of plastic aquarium foam in the end

were filled with fine white aquarium sand and used as miniature SSF bioreactors. The SSF bioreactors were rinsed with the MBH salts medium [12] and seeded with the selected bacteria via pipette before a solution of the MBH salts medium and a stock solution of the simplest NA (CHCA) was trickled through them daily for three weeks. The amounts of bacterial isolate and NAs added were calculated so that the concentrations would be the same as in the PBC bioreactors. During the first trial design, submerged fountain pumps connected to a tubing system and a digital power bar were used to automate the hydraulic loading of the sand syringes (Figure 2). However, the rapid draining of the supernatant and turbulent disturbance of the cake layer hindered the formation of a *schmutzdecke*. Despite significant troubleshooting over the course of three months, little bacterial growth occurred in these SSF bioreactors. The design was completely revised based on the outcome of this first trial.

Figure 2. The first trial SSF bioreactor design which contained 12 bioreactors. Fountain pump jars were located on the shelf behind a pegboard used to secure the tubing system and the sand syringes.

The second trial design utilized gravity-driven IV bags, IV lines, and binder clips (to open and close the tubing) for manual hydraulic loading (Figure 3). A new set of miniature SSF bioreactors was recreated for the second trial. A diatomaceous earth precoat layer and a U-bend in the outflow tubing were also added to the design to increase hydraulic head loss and successfully maintain the supernatant. After being exposed to the simple NA for three weeks, visible biofilm development and high microbial growth levels (measured via UV-Vis spectrophotometry [13]) indicated that the second trial design was a success and ready to be used for the main experiment.

Figure 3. The second trial SSF bioreactor design which contained 20 new bioreactors. IV bags were held upright in red popcorn tubs above a pegboard used to secure the IV lines and the sand syringes.

4c. Main NA Biodegradation Experiment

In the main experiment, each bioreactor type had five sets consisting of three replicates each. The bioreactor sets were distinguished based on their bacterial content - *Acidovorax* sp. (A), *Pseudomonas* sp. (P), *Xanthobacter* sp. (X), all three (combination - C), or none (sterile controls - S). For the established second trial SSF bioreactors, the five remaining acids were added by weighing the IV bags to find the mass (and thus the volume) of the MBH salts medium remaining after the design stage. The required volumes of stock solutions of the five missing acids were calculated and added to each IV bag via pipette based on the concentrations initially in the PBC bioreactors. Existing PBC bioreactor sets from the bacteria selection process were used since they already contained the selected bacteria and all six of the required acids. Since these bioreactors were established at the same time as the second design trial, weekly microbial growth measurements (via UV-Vis spectrophotometry [13]) had also been taken over the course of three weeks. After the new NA solution had been processed by all the bioreactors for one week, samples were removed via pipette, subjected to liquid-liquid extractions using DCM as a solvent [16], and used for NA concentration measurements via gas chromatography [17].

4d. Assessment of Biofilm Development

After the experiment, the cake layer in the SSF bioreactors was probed and samples were removed with a pipette. These cake layer samples were subjected to epifluorescence microscopy [18] using DAPI (blue), SYTO 9 (green), and propidium iodide (red) dyes to assess biofilm development.

5. Results

Figure 4. During the third week of the second trial SSF bioreactor design, the cake layer in the *Pseudomonas* sp. bioreactors set turned a light pink colour.

Figure 5. After the experiment concluded, probing the cake layers revealed that there was a pink or white elastic pudding-like substance mixed in with the sand.

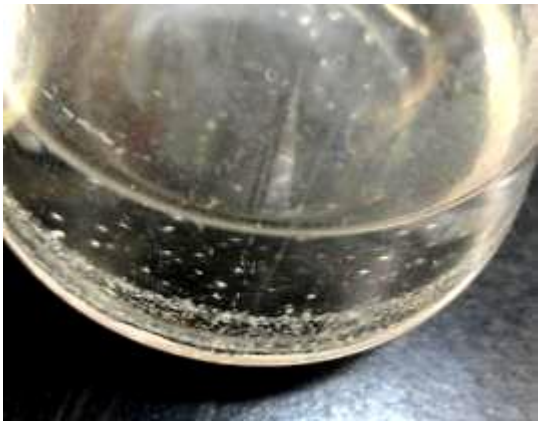
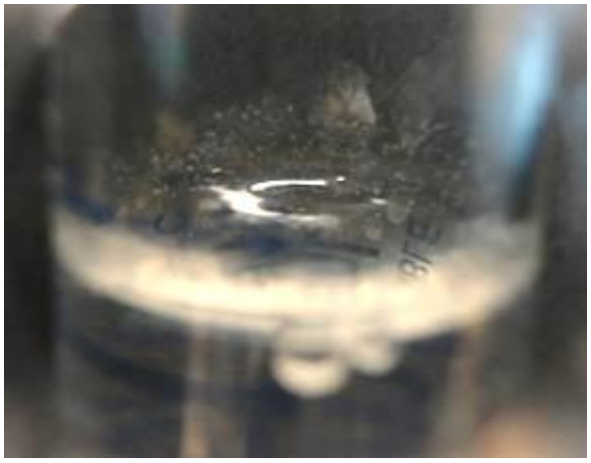


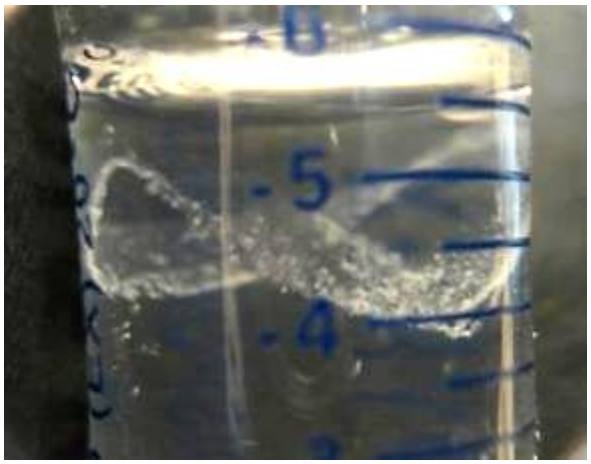
Figure 6. Some of the *Acidovorax* sp. PBC bioreactors developed tiny floating white clump-like

biological growth. Otherwise, no significant biological structures were observed in the PBC bioreactors.

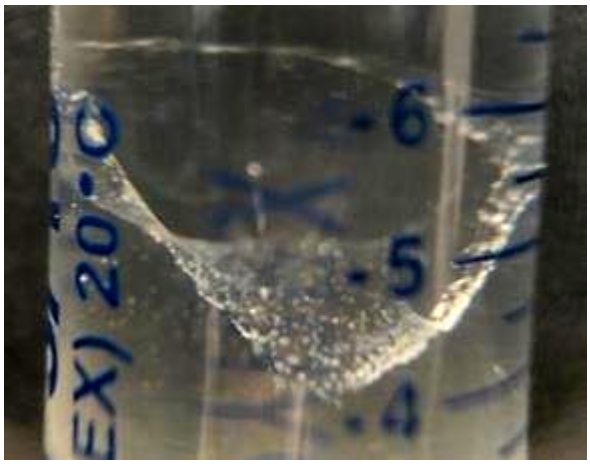


Acidovorax sp.

Pseudomonas sp.

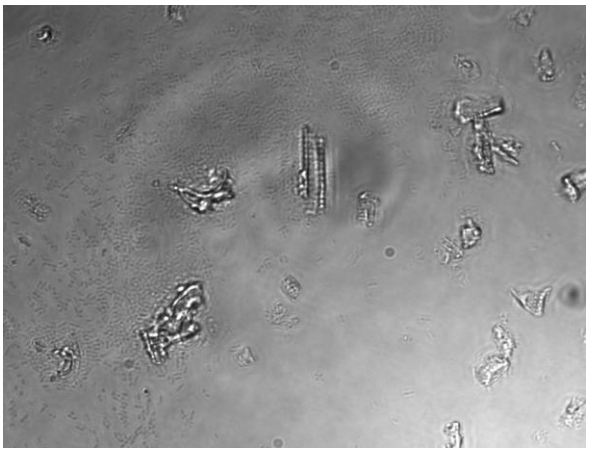
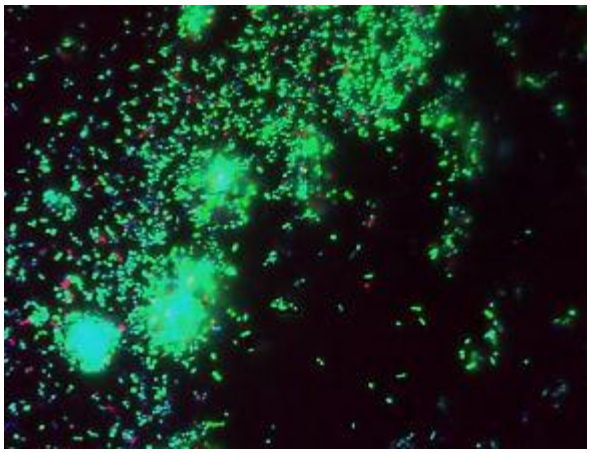
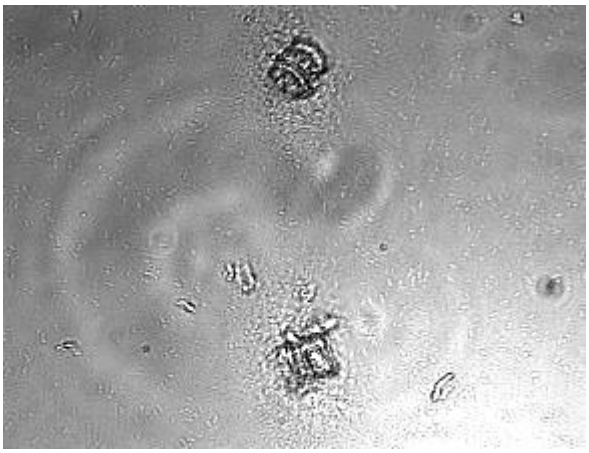
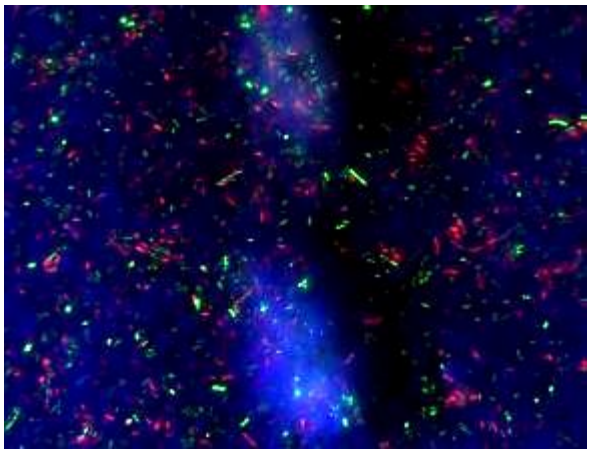
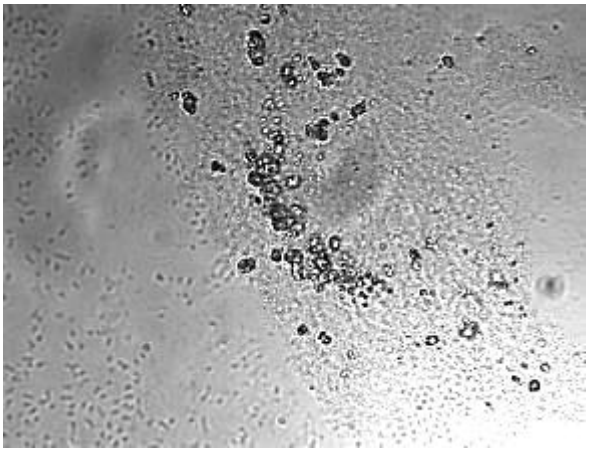
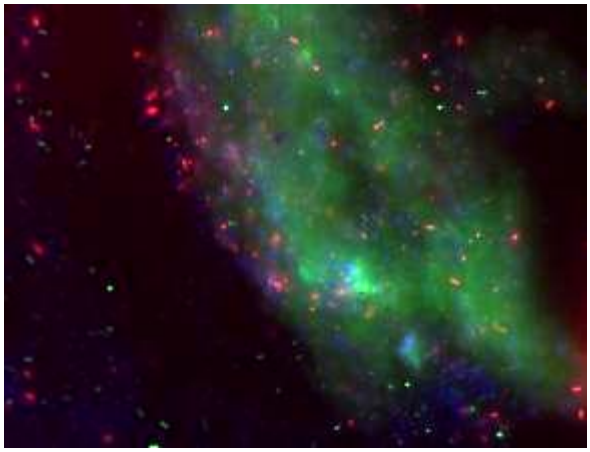


Xanthobacter sp.



Combination

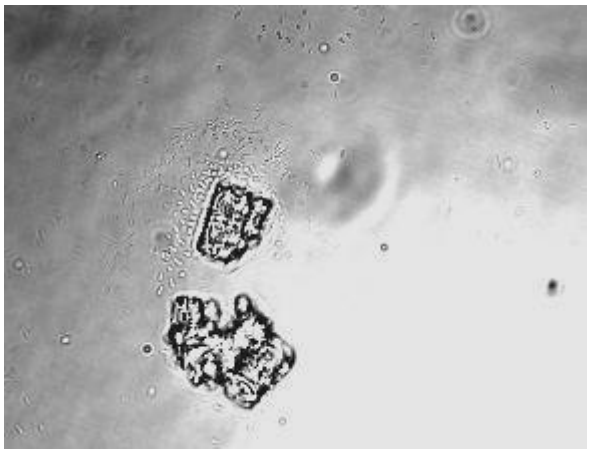
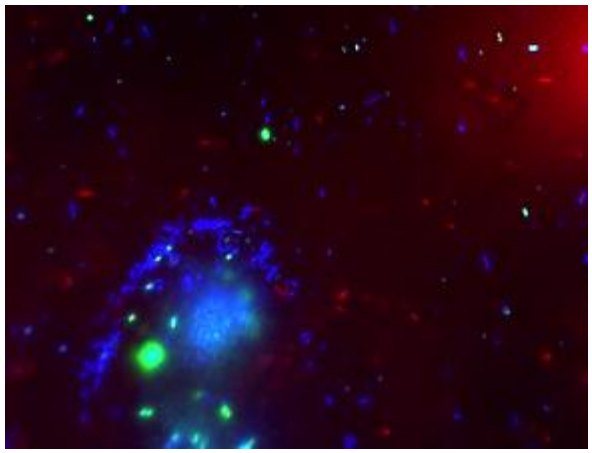
Figure 7. During the third week of the second trial SSF bioreactor design development, white elastic net-like biological structures resembling horizontal cobwebs were found attached inside the glass syringes of several SSF bioreactors. The *Acidovorax* sp. had large speckles on the sides of the syringes instead of large nets.

	Brightfield	Merged
ACIDOVORAX		
PSEUDOMONAS		
XANTHOBACTER		

■ = DAPI dye (stains nucleic acids mostly inside the nucleus)

■ = SYTO 9 dye (stains nucleic acids only outside the nucleus)

■ = Propidium iodide dye (stains the cell membrane)

	Brightfield	Merged
COMBINATION		

■ = DAPI dye (stains nucleic acids mostly inside the nucleus)

■ = SYTO 9 dye (stains nucleic acids only outside the nucleus)

■ = Propidium iodide dye (stains the cell membrane)

Figure 8. The micrographs on the left side are the brightfield images of the samples illustrating the locations of diatomaceous earth and sand particles. In the micrographs on the right side, red indicates only the locations of individual bacterial cells, green indicates only the presence of DNA in the extracellular polymeric matrix of a biofilm, and blue indicates both (cells and biofilms).

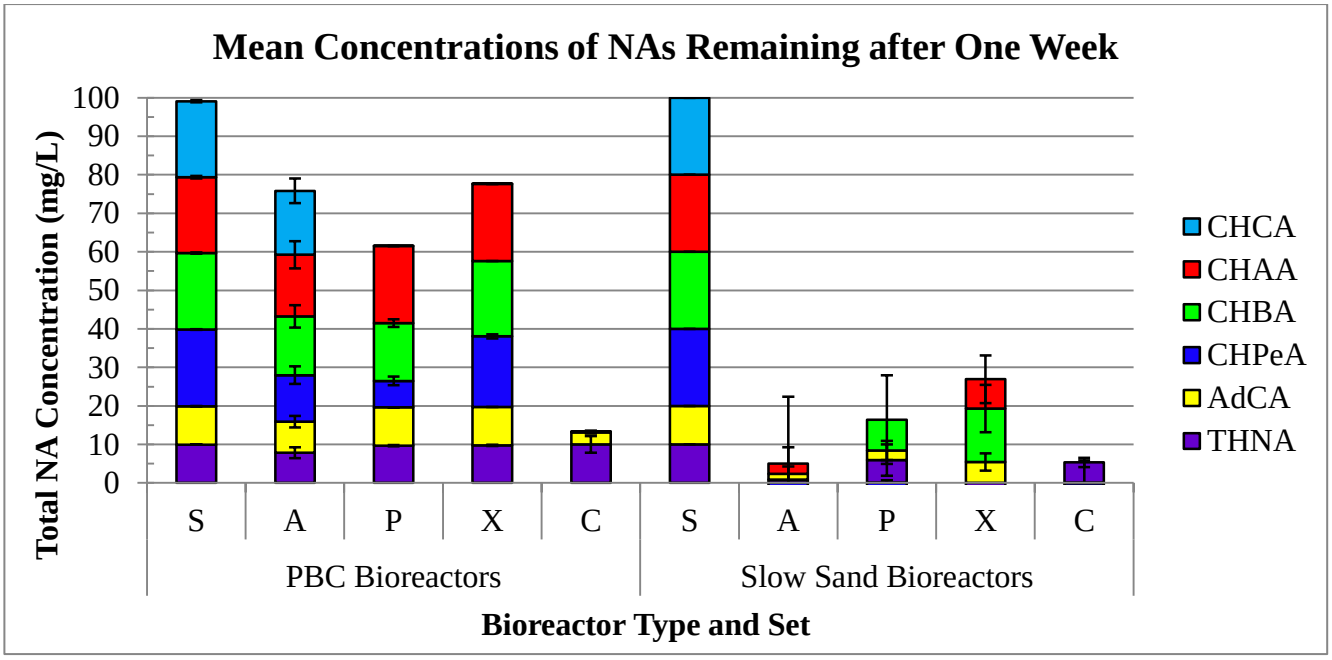


Figure 9. The arithmetic mean concentrations of each NA still present in each bioreactor set after operating for one week (standard error bars).

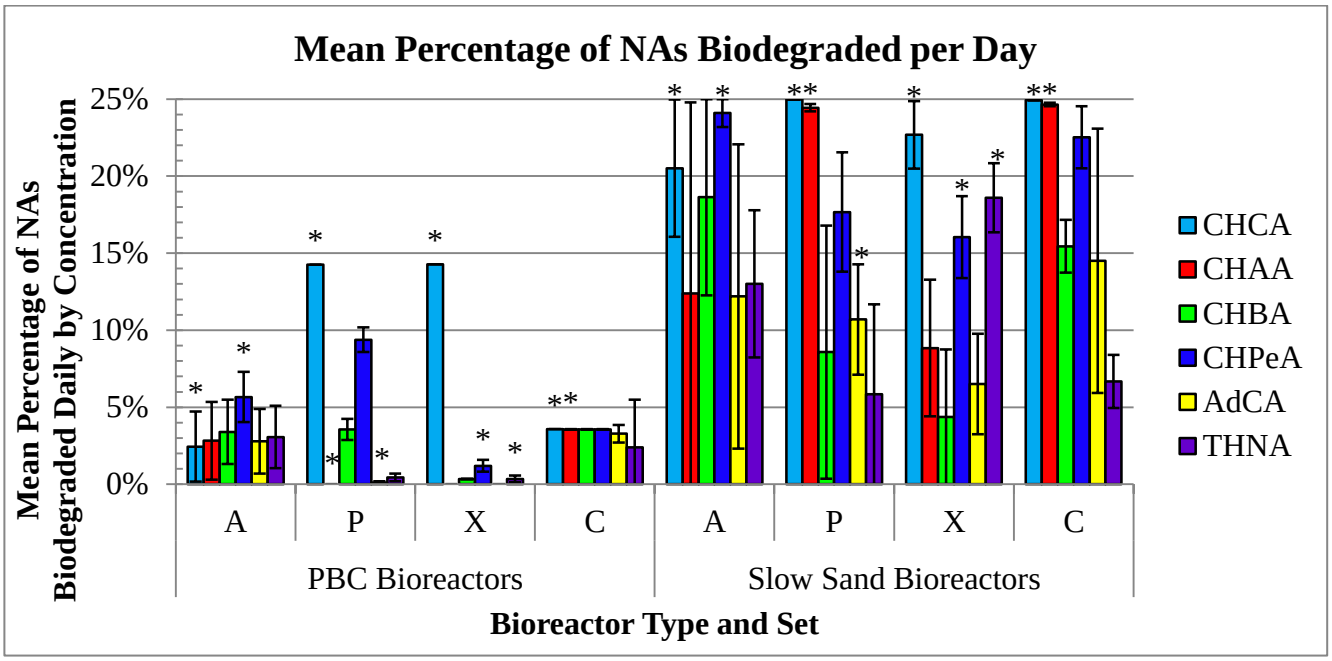


Figure 10. The arithmetic mean percentage of each NA biodegraded per one day of operation, with stoichiometric adjustments made to account for the production of similar metabolites during biodegradation reactions (standard error bars; * indicates $p \leq 0.1$).

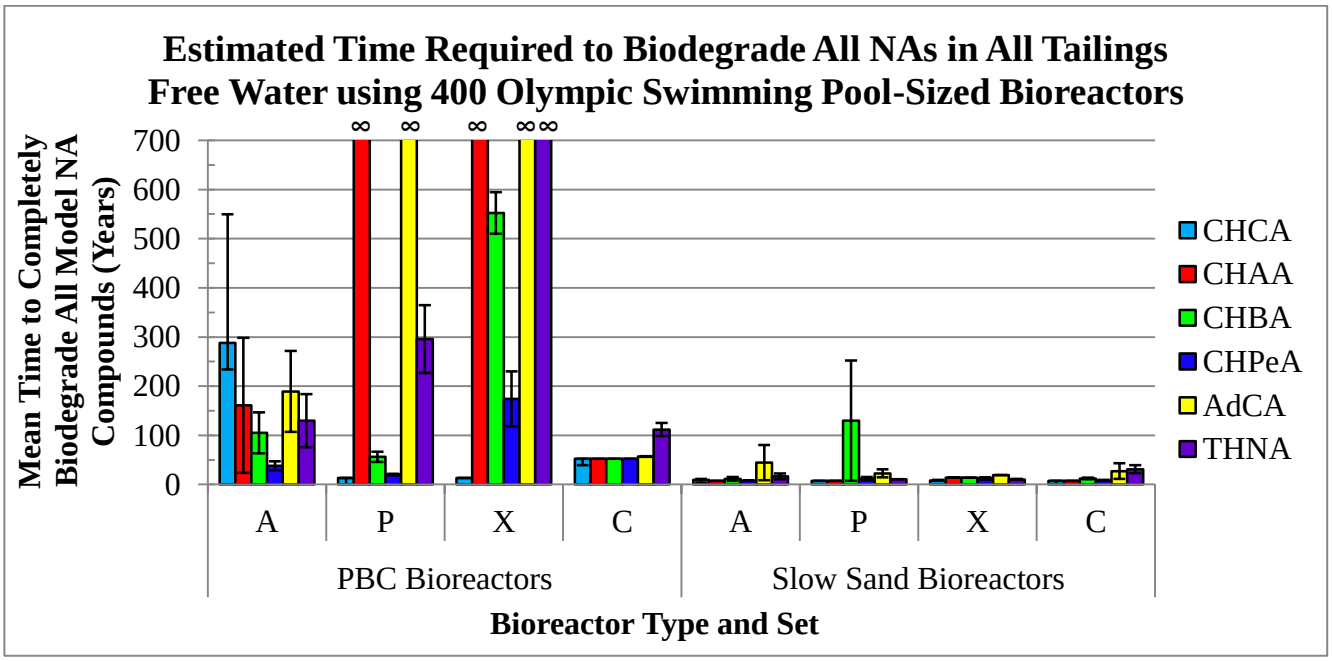


Figure 11. Based on the percentage of each NA biodegraded per one day of operation, the arithmetic mean time required for 400 Olympic swimming pool-sized bioreactors to biodegrade all NAs in all of the tailings free water produced by 2025 [4] was estimated (assuming constant reaction rates comparable to those required to break down the model NA compounds used; standard error bars).

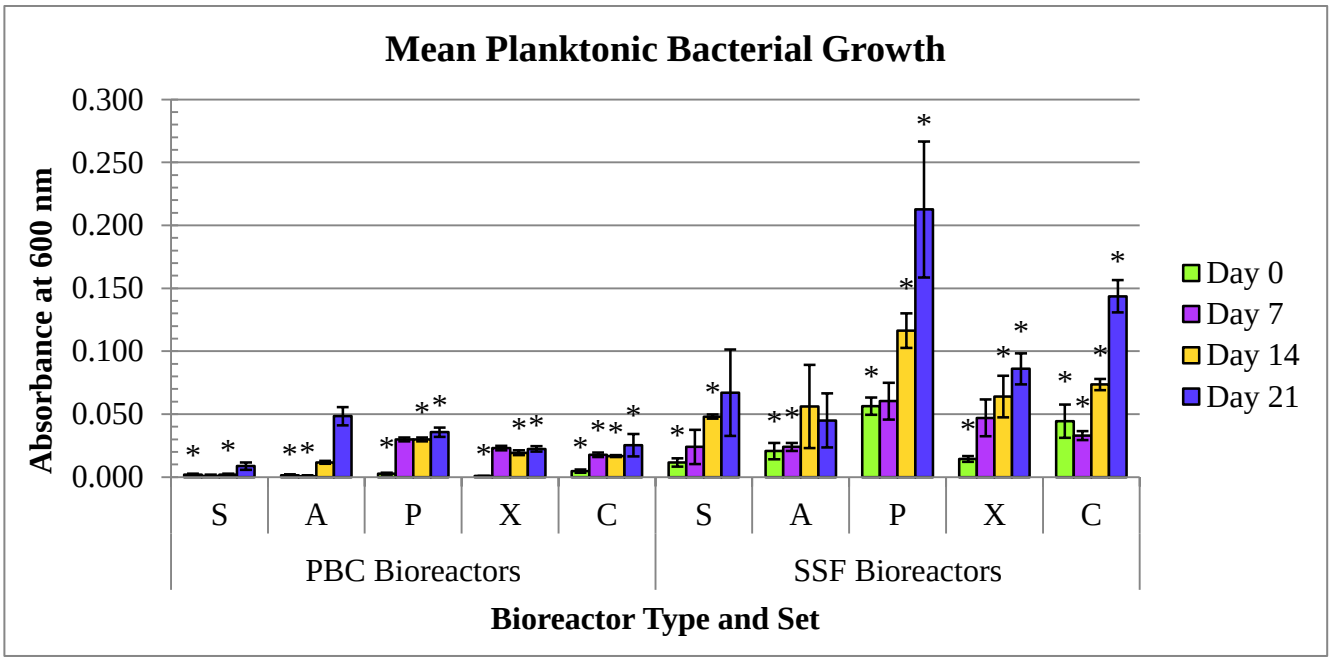


Figure 12. The arithmetic mean absorbance values of each bioreactor set indicative of planktonic microbial growth during the design trial and experimental phases (standard error bars; * indicates $p \leq 0.1$).

6. Discussion

A variety of biological growth was detected in the bioreactor sets. The pink colouring of the SSF bioreactor P set cake layer was likely due to the formation of a *schmutzdecke* (Figure 4). The particular *Pseudomonas* sp. that was used also appeared pink on LB agar plates. The elastic pudding-like substances mixed in with the cake layer sand in the SSF bioreactors (Figure 5) were probably *schmutzdeckes*. Since most bacteria in nature grow as biofilms [11], the tiny white clumps observed in the PBC bioreactor A set likely reflected the bacteria's attempts to form a biofilm (Figure 6). The white elastic net-like biological structures observed in the SSF bioreactors (Figure 7) were identified as streamer biofilms based on their appearance, elastic behavior, and attachment to the glass [19]. Epifluorescence microscopy (Figure 8) revealed that there were high levels of STYO 9 (green) and

DAPI (blue) dyes where sand particles were located in the brightfield images. Since these two dyes bind to nucleic acids and there was a lack of propidium iodide (red) dye (indicating individual cells) in these locations, this strongly suggests that the dyes were identifying the DNA found in the extracellular polymeric matrix of a *schmutzdecke* biofilm on the sand particles.

As shown in Figure 9, the SSF bioreactors consistently outperformed the PBC bioreactors in terms of overall NA level reductions. After one week, the arithmetic mean total NA concentration in the PBC bioreactors was reduced from 100 mg/L to 99.08 mg/L (A), 75.84 mg/L (P), 61.52 mg/L (X), or 13.36 mg/L (C). In the SSF bioreactors, these values were 5.00 mg/L (A), 16.41 mg/L (P), 26.93 mg/L (X), or 5.33 mg/L (C). To put this in perspective, total NA concentrations below 5.00 mg/L are no longer acutely toxic to fish [1] and would be similar to the levels naturally found in the Athabasca River [2]. The arithmetic mean NA concentrations in the sterile control sets (S) were almost identical to the concentrations initially added to the solutions, indicating a lack of abiotic adsorption.

Figure 10 shows that the SSF bioreactors mostly achieved higher biodegradation rates for each specific NA. The arithmetic mean rate of individual NA removal in the PBC bioreactors was 3.37% per day (A), 4.63% per day (P), 2.70% per day (X), or 3.33% per day (C). In the SSF bioreactors, these values were 16.81% per day (A), 15.37% per day (P), 12.84% per day (X), or 18.12% per day (C). These rates were an improvement of five times (except for the P set, which was three times). The SSF bioreactors had the highest arithmetic mean biodegradation rate for every NA in every set of bioreactors. The PBC bioreactor P and X sets were unable to biodegrade NAs like CHAA at all, while the SSF bioreactor sets accomplished notable biodegradation. This difference in metabolic capabilities is possibly due to the conversion from planktonic to biofilm form causing changes in gene expression [11]. However, heteroscedastic two-tailed t-tests indicated that only some of the differences in biodegradation rates were statistically significant. It is possible that the conversion from planktonic to biofilm form only selectively enhanced certain metabolic capabilities.

The estimated time length required to biodegrade the NAs in all of the tailings free water produced by 2025 [4] illustrated the significant difference in bioreactor capabilities. Figure 11 operates on the assumptions that the upper three metres of the average 45 metre deep tailings pond is free water [2] and that the biodegradation rates observed in this experiment would be similar to the reaction rates involving

the uncharacterized NA mixture in oil sands tailings. Based on these assumptions, large PBC bioreactors would take an arithmetic mean time of 151.91 years (A), 427.98 years (P), 413.14 years (X), or 63.03 years (C) to biodegrade most of the NAs. Infinity symbols on Figure 11 indicate that the NAs might never undergo primary biodegradation. The large SSF bioreactors would take an arithmetic mean time of 16.22 years (A), 31.70 years (P), 13.09 years (X), or 15.66 years (C) to biodegrade all of the NAs. This is an improvement of 9 times (A), 14 times (P), 32 times (X), or 4 times (C) for an average improvement of 14 times. The volume of 400 Olympic-sized swimming pools was chosen because it is 1% of the total area of SSFs used for municipal water treatment in London (UK) [9]. This highly practical surface area, 0.007 km^2, would be 0.00004% of the total surface area currently occupied by all oil sands tailings ponds [5].

The SSF bioreactors also outperformed the PBC bioreactors in terms of planktonic bacterial growth. In Figure 12, the arithmetic mean absorbance values of the PBC bioreactor samples ranged between 0.001 to 0.050 during the entire three week time frame (Figure 11). The SSF bioreactor sample values ranged between 0.012 to 0.213, indicating the SSF bioreactors encouraged higher planktonic bacterial growth. Relative to each type of sterile controls, the SSF bioreactors consistently encouraged higher planktonic microbial growth over time. The slightly increasing SSF bioreactor sterile control absorbance values were likely due to slight sand leakage over time.

The significance of the SSF bioreactors' efficiency at biodegrading NAs is the discovery of a new, sustainable, and easily implementable way to reduce the toxicity of the constantly expanding volume of oil sands tailings. Based on the absence of other literature on this subject, this study likely represents the first investigation into the effectiveness of SSFs applied as novel aerobic bioreactors to the microbial degradation of NAs. These results will be useful to companies with oil sands projects because they will help to reduce the environmental impact of the tailings ponds. The SSF bioreactors will also be appealing because they are highly practical and cost-effective. They use gravity instead of electricity, do not introduce potentially invasive species, can be constructed outdoors, require little supervision or maintenance, are made with natural or recycled material, are relatively low cost, and are based on existing historical technology that has been proven effective over centuries. Despite their simple design, SSFs have been used for reliably processing large volumes of water in conventional applications [9] and would likely be similarly reliable in unconventional applications. As long as there is a species of

bacteria capable of bioremediating the pollutant (e.g., hydrocarbons, heavy metals, etc.), this study implies that SSF bioreactors could be used to effectively treat a wide variety of water pollution.

The clean water recovered from the tailings ponds could be reused to reduce the overall freshwater footprint of the oil sands industry. Three m^3 of water are required and four m^3 of tailings are generated for every one m^3 of mined oil sands ore that is processed [4]. The free water in tailings ponds is already recycled several times until it can no longer be efficiently used for the extraction of bitumen. Removing the NAs that have become concentrated in the free water would allow this 'lost' water to re-enter the recycling process. Most importantly, with oil sands development expected to accelerate in the near future, new application of this passive and sustainable technology could decrease the detoxification of the free water seeping from tailings ponds into the environment from centuries to decades.

7. Conclusions

1. The overall results of this experiment supported my hypothesis that the NAs in the novel SSF bioreactors would undergo the most biodegradation due to the formation of biofilms on the sand particles. This was supported by the presence of biofilms and SSF bioreactor total NA reduction rates that were an average of 2.5 times faster than those in the PBC bioreactors.
2. The results regarding individual biodegradation rates also supported the secondary hypothesis that the SSF bioreactors may promote metabolically-efficient biofilm growth. The main difference between the two bioreactor types was the development of a biofilm; therefore the presence of biofilms is likely responsible for the significant difference in metabolic rates.
3. The results regarding individual biodegradation rates provided evidence of potentially enhanced metabolic capabilities. Certain NAs might never be biodegraded by the planktonic cells in the PBC bioreactors; however, all the NAs tested were broken down by the biofilms in the SSF bioreactors.
4. SSF bioreactors are more effective at encouraging biofilm development than PBC bioreactors. Very little visible biological growth was visible in the PBC bioreactors while a large amount of growth was found in the SSF bioreactors (e.g., streamers, high turbidity, *schmutzdecke* biofilms).
5. SSF bioreactors are also better than PBC bioreactors at encouraging planktonic microbial growth, as indicated by UV-Vis spectrophotometry.
6. The SSF bioreactor system I designed is an effective bench scale simulation of existing industrial scale SSF technology.

7. Biodegradation can be an effective and practical way to address the high NA levels in oil sands tailings.
8. Certain *Acidovorax* sp., *Pseudomonas* sp., and *Xanthobacter* sp. are capable of forming biofilms.
9. According to my results, a sizeable set of SSF bioreactors could potentially bioremediate the NAs in all oil sands tailings free water produced by 2025 [4] in less than 20 years on average (14 times faster than equivalent PBC bioreactors).
10. SSF bioreactors present a practical, cost-effective, and sustainable way to significantly detoxify the free water seeping from tailings ponds into the environment, thus preventing the pollution of surrounding groundwater and surface water resources.
11. The clean water recovered from the tailings ponds could be reused to reduce the overall freshwater footprint of the oil sands industry.

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[Untitled photograph of a water drop]. http://www.yaphahdesigns.com/images/water-droplet-header.jpg (accessed Apr 2014).

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