Abstract

HURLEY, FORREST JAMES. Effects of Powdered Kenaf Addition on the Performance of Lab-Scale Activated Sludge Reactors. (Under the direction of Dr. F.L. de los Reyes III.)

The effects on wastewater treatment due to addition to mixed liquor (ML) of a fine lignocellulosic powder made of dried kenaf (plant) were assessed using lab-scale sequencing batch reactors (SBRs) designed for enhanced biological nitrogen and phosphorus removal and treating real municipal wastewater. Removal of organic carbon, nitrogen, phosphorus and suspended solids at various aeration intensities were compared between kenaf treatment and control SBRs. Mixed liquor concentrations, adsorption of heavy metals, sludge settling characteristics and microbial kinetics of the treatment reactor were also compared to those of the control SBR, which received no kenaf.

No significant effects on carbon, phosphorus or suspended solids removal were detected. There was a small increase in mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) with kenaf treatment, possibly more than the accumulated mass of kenaf. Nitrogen removal was unaffected by the treatment when aeration intensity was near optimum, which for these SBRs was an aeration ratio (AR) of roughly 11, where the AR is defined as the mass of O_2 supplied to the SBR each cycle, divided by the mass of COD exerted per cycle. However, the kenaf supplemented ML removed nitrogen significantly (alpha = 0.01) better than the control when the AR was below the ideal range, suggesting that full-scale wastewater treatment plants (WWTPs) supplementing with kenaf might suffer less severe upsets due to perturbations such as a mismatch between aeration intensity and influent strength. A 30% drop in the sludge volume index (SVI) with kenaf treatment provided evidence of a substantial improvement in sludge settling characteristics

which could allow a WWTP to carry a higher MLVSS without overloading the clarifiers or to handle a smaller volume of waste activated sludge (WAS). Arsenic, copper, lead and zinc were found to accumulate significantly (alpha = 0.01, except zinc: alpha = 0.05) in the kenaf treated ML solids, while cadmium, chromium and nickel did not. Selenium and cobalt were significantly (Se: alpha = 0.01, Co: alpha = 0.05) less concentrated in the kenaf sludge solids. Batch reactor testing and inter-cycle sampling of ML provided some evidence of enhanced activity of the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), possibly leading to improved nitrification.

Effects of Powdered Kenaf Addition on the Performance of Lab-Scale Activated Sludge Reactors

by
Forrest James Hurley

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Environmental Engineering

Raleigh, North Carolina

2013

APPROVED BY:

_____	_____
Dr. Detlef Knappe	Dr. Tarek Aziz

_____
Dr. Francis L. de los Reyes III
Chair of Advisory Committee

Dedication

To my wife and sons.

As hard as my going back to school has been for me, it has been much harder for them.

Biography

Forrest James (Jim) Hurley was born in Memphis, TN in 1967. He spent his childhood in the Ozarks of northern Arkansas. In 1989, he graduated *magna cum laude* from Duke University and received a B.A. in Psychology. Jim then worked in film and video production as a freelance gaffer for 17 years, during which time he was married and had three sons. After leaving the film and video industry to take care of his kids, Jim discovered that he was really meant to be an Environmental Engineer and went back to school.

Acknowledgments

I would like to thank the North Carolina Rural Economic Development Center for providing the funding that made this study possible. I would also like to thank my Primary Investigator and advisor Dr. Francis L. de los Reyes III for sharing his deep knowledge base and endless supply of encouragement. I am grateful to the rest of my advisory committee for their patience. Thanks also to the other students, especially Bahareh, who have helped me so many times. Finally, a special thank you goes out to the man who keeps our lab running smoothly and safely, David Black.

Table of Contents

List of Tables

List of Figures

Introduction

Costs associated with waste sludge processing and disposal are a major operating expense for most municipal wastewater treatment plants [1], as are those for energy for aeration needed for carbon removal (expressed as chemical oxygen demand, COD) and nitrification. Additionally, many plants may face penalties for non-compliance with their discharge permits, which have become more restrictive over time. If reduced aeration or enhanced nitrogen and COD removal and improvements in sludge settling characteristics could be accomplished without major changes to the physical plant, operating costs could be lower and compliance with environmental standards more likely.

One common strategy to improve plant performance has been to retrofit suspended growth systems with submerged surface area for attached growth of biofilms. Conventional and enhanced Activated Sludge (AS) systems use complete mixing to maximize contact between suspended microbes and their growth substrates, thus optimizing removal of those substrates. But for some microbes, attached growth may have advantages such as microenvironments that can form within biofilms. For example an anoxic zone in a biofilm exposed to aerated WW may facilitate simultaneous nitrification-denitrification. Close proximity of multiple functional groups within biofilms may also aid interspecies transfer of materials like nitrite, which is both a waste product of ammonia oxidizing bacteria (AOB) and a growth substrate for nitrite oxidizing bacteria (NOB). Hybrid treatment strategies, known as Integrated Fixed Film Activated Sludge (IFAS) systems provide surface area for attached growth within the otherwise suspended growth regime of AS mixed liquor.

IFAS plants may be able to carry a higher biomass concentration in the reactor because the attached fraction of the biomass does not contribute much to clarifier loading. More biomass can allow the plant to support higher organic loading or to achieve more complete removal if it is already overloaded. Because the attached biomass never leaves the reactor, the mean solids retention time (SRT) is longer than that of a hydraulically similar conventional system and so yield is lower and there is less excess sludge production. The increased SRT for attached microbes especially benefits the slow growing nitrifiers. More stable output under varying loads than in conventional systems and improved sludge settling have also been observed in IFAS plants [2]. Plastic media with a rough surface, freely circulating in the mixed liquor can encourage biofilm development, but may necessitate modifications to the plant such as a downstream screen to retain the media. Stationary racks of long fibers for mixed liquor to flow through have also been successful, but require maintenance and may change the plant's hydraulics [3].

Kenaf (*Hibiscus cannabinus* L.) is a sturdy and fast growing annual agricultural crop (Figures 1.1 and 1.2) related to cotton, hemp and okra, which was transplanted to the southern U.S. from Africa and Asia to replace imported Asian jute during WWII. Having been used for thousands of years for cordage fibers and livestock feed [4], kenaf today provides raw materials for many products including paper, cloth, livestock bedding and architectural panels [5]. The stems are also very porous leading to their use as a native material for activated carbon production and as an industrial adsorbent for organic dye removal, heavy metals sequestration and oil field remediation [6, 7, 8, 9; http://www.kengro.com/]. Kenaf has even been grown in wastewater treatment plant (WWTP) effluent as a polishing step for nitrogen and phosphorus removal [10]. One product, a fine porous powder of dried kenaf (Figure 1.3), when added to the mixed liquor of suspended growth activated

sludge systems is reported by its manufacturer to provide freely circulating submerged surface area (7.4 m^2/gram, as measured by BET isotherms) for formation of robust biofilms. The claim is that this results in better COD and nitrogen removal with less aeration as well as enhanced sludge settling and lower sludge disposal volume; and all with no need for media maintenance or recovery or for plant modifications (http://rfwastewater.com/). These claims are based on the company's trials at several full scale WWTPs and have yet to be independently verified in well controlled published studies.

Figure 1.1: Nearly mature kenaf. (businessafrica.net)

Figure 1.2: Harvesting mature kenaf. (Agricultural Marketing Resource Center)

Figure 1.3: Powdered dried kenaf. (Photo by the author)

The objectives of this study were to evaluate the effects of powdered kenaf addition on bulk process parameters of lab scale WWTPs performing biological nitrogen and phosphorus removal. Removal of COD, nitrogen, phosphorus and suspended solids at various aeration intensities were compared between kenaf treated and control bioreactors. Mixed liquor suspended solids and volatile suspended solids (MLSS, MLVSS) were tracked to determine whether changes in influent strength and aeration intensity affected the growth of the microbial cohorts of the systems similarly.

Sludge settling characteristics of the bioreactors were quantified by use of the Sludge Volume Index (SVI) which is calculated by dividing the 30 minute settled sludge volume (SSV30) in mL/L by the MLSS in g/L, resulting in units of mL/g. SVI can be interpreted as the volume occupied by each dry gram of settled sludge solids. Values of SVI greater than 150 mL/g are considered to be indicative of poor sludge settling characteristics while those below 80 mL/g represent sludges with excellent settling characteristics [11]. Distributions of the particle sizes in mixed liquor solids samples were compared in order to elucidate the mechanisms behind any significant differences in SVI.

Differences in the concentrations of heavy metals in dried sludge solids samples from each bioreactor identified elements which tend to adsorb to the kenaf. Lee and Rowell [8] found that of the 5 types of plant fibers they tested, kenaf had the best ability to remove copper, nickel and zinc ions from solution. While removal of heavy metals from the liquid waste stream is desirable, their accumulation in the solids they adsorb to could limit waste sludge disposal options.

The microbial kinetics of each bioreactor's cohort were assessed by batch reactor testing samples of mixed liquor (ML) borrowed from the kenaf treated and control systems, and by intensive *in situ* sampling throughout a single bioreactor treatment cycle.

Materials and Methods

Reactor Design

Laboratory scale sequencing batch reactors (SBRs) were chosen to model the continuous flow systems typical of full scale wastewater treatment plants (WWTPs). While wastewater and biomass encounter different environmental conditions at different locations in continuous systems, in an SBR the same performance can be achieved by changing the conditions over time in a single tank. This is accomplished by operating an SBR in a repeating cycle of phases, each with its own environmental regime. At its simplest, an SBR's cycle consists of a fill phase in which influent wastewater is added to the reactor, a react phase during which biological action transforms problematic constituents in the wastewater, a settle phase to separate solids (including biomass) from the bulk liquid, and a decant phase to remove treated clarified effluent. The react portion of the cycle can be divided into several phases, each with unique environmental conditions and a unique duration. In continuous flow systems the amount of time spent in each treatment stage (for a given flowrate) is determined by tank volume, which is difficult to alter experimentally. However in an SBR, timer settings (which are easy to change) control the duration of each process. The flexibility to alter the length of any phase and even the order of the react phases allows the SBR to simulate the performance of almost any type of WWTP, as long as the systems have comparable mean solids residence times (SRT). [11]

The SBR cycle includes a settle phase that mimics secondary clarification. Because this process occurs within the reactor tank, there is no need to provide a separate clarifier. The cycle also

includes influent and effluent pumping phases. However, the fill, settle and decant phases are not included in calculations of a WWTP's average hydraulic retention time (HRT) or of its SRT, so for comparison to continuous systems one must consider the effective HRT, HRTe, and the effective SRT, SRTe. In either case, the effective value is found by multiplying the base value by the fraction of the cycle time devoted to the react phases. For example, a 10.0 L SBR receiving 10.0 L/day of liquid and operated on a 4 hour cycle which includes 3.5 hours of react phases would have an HRTe of (10.0 L/10.0 L/day) * (3.5 hours/4 hours) = 0.875 days = 21 hours. [11]

Computer Modeling

The treatment cycle for this study was developed with the aid of ASIM modeling software implementing ASM No. 1 in SBR mode. Computer modeling allows many process options to be evaluated quickly. By testing various durations for each treatment phase, the performance of the SBR cycle can be optimized (for a particular wastewater and flow regime). The downside of ASIM (and many computer models) is that it requires inputs of influent constituent species to be in non-overlapping categories or partitions so that the materials mass balances can be solved, rather than using conventional lumped parameters such as MLSS or total COD [12]. Such data is not usually available for a particular wastewater, so it becomes necessary to estimate the ASIM inputs from existing data. A limited dataset (Table 2.1) characterizing the influent wastewater at a local WWTP (one of two which were being considered at the time for wastewater collection for this study's feedstock) served as the basis for estimating the ASIM inputs used for this model. Those influent parameters were then held constant through each model run, rather than varying weekly as the physical bench-top model's feedstock did.

All species concentrations are expressed in terms of grams of COD per cubic meter in ASIM, except nitrogen species which are in grams nitrogen per cubic meter and alkalinity in moles per cubic meter. For this dataset the first step was to estimate total COD (tCOD) from BOD5. According to Grady, Daigger and Lim, tCOD ≈ 2.1 * BOD5 for domestic wastewater, therefore influent tCOD for this model was taken to be equal to 589 g COD/m^3. The biodegradable portion of the total COD was estimated as 1.71 * BOD5 ≈ 480 g COD/m^3 [11]. The inert fraction of tCOD is the difference between the total COD and the biodegradable COD, or 109 g COD/m^3 in this case. Both inert and

biodegradable portions of the tCOD must be further subdivided into solid and soluble fractions based on available solids data.

Table 2.1: Measured influent wastewater characteristics at Triangle WWTP, Durham Co., NC

Date	BOD_5	NH_3	Total N	TSS	Total P
May 2010	324	37.5	54.8	347	8.93
Aug 2010	241.3	31	40.7	248.2	6.93
Nov 2010	278.3	28.7	37	250.5	5.22
Feb 2011	278.5	33.5	44.3	238.2	5.93
Average	280.53	32.68	44.20	270.98	6.75
S.D.	33.85	3.77	7.67	50.96	1.61
C.V.	0.12	0.12	0.17	0.19	0.24

The solids dataset includes only values for TSS, but the wastewater was reported to be higher in fixed suspended solids (FSS) than average (due to some industrial customers), with only about 70% of TSS volatilizing. Therefore the volatile suspended solids (VSS) in COD concentration units was estimated as VSS = 0.7 mg VSS/mg TSS * 1.41 mg COD/ mg VSS * 271 mg TSS /L = 267 mg COD/L. It was assumed that the particulate organic matter (represented by VSS) is 35% to 40% non-biodegradable, and that this inert fraction consists mostly of proteins with 1.5 g COD/g protein [11], in which case the influent inert particulate COD, Xio, is equal to 0.375 mg inert VSS/mg VSS * 0.7 mg VSS/mg TSS * 271 mg TSS /L * 1.5 mg COD/mg inert VSS (protein) = 107 mg COD/L. Then the soluble inert COD, Sio, can be calculated as the inert COD minus Xio, or 3 mg COD/L. The soluble substrate

COD, Sso, was assumed to be approximately 43% of the biodegradable COD, or 206 mg COD/L. The remaining biodegradable COD is particulate, Xso, which is 273 mg COD/L.

Ammonia concentration for this wastewater was converted to ammonia-N concentration by multiplying 33 mg NH_3 by 14.01 mg N/17.03 mg NH_3 = 27 mg N/L. Concentrations of nitrite nitrogen and nitrate nitrogen in municipal wastewater are typically insignificant, so they were assigned a value of zero. The organic nitrogen was then found by subtracting the ammonia nitrogen from the total nitrogen: Norg = TN – NH_3-N = 17 mg N/L. Just as with tCOD, organic nitrogen must be partitioned into particulate and soluble fractions, each of which has inert and substrate components. The particulate inert nitrogen, Xnio, was assumed to be 6% of Xio, for an estimated value of 6.4 mg N/L. The soluble inert organic nitrogen, Snio, was given a value of 1.5 mg N/L [11]. Subtracting the inert components from the organic nitrogen leaves the particulate and soluble substrate organic nitrogen components: Norg – Xnio – Snio = Xnso + Snso = 9.4 mg N/L. The ratio of the slowly available organic nitrogen substrate, Xnso, to the readily available organic nitrogen substrate, Snso, was assumed to be the same as the ratio of slowly available substrate COD, Xso, to readily available substrate COD, Sso. Thus, Xnso = Snso * Xso/Sso = 1.33 Snso, and Xnso + Snso = 1.33 * Snso + Snso = 9.4 mg N/L. Therefore, Snso = 9.4 / 2.33 = 4.0 mg N/L, and Xnso = 9.4 – Snso = 5.4 mg N/L. The soluble organic nitrogen, Sno, is equal to Snio + Snso = 5.5 mg N/L, and the particulate organic nitrogen, Xno = Xnio + Xnso, is 11.8 mg N/L.

Table 2.2: Estimated ASIM input values for influent wastewater

Name	Symbol	Estimate	Units
Soluble Species:			
Oxygen	O2	0	g O_2/m^3
Inert COD	Sio	3	g COD/m^3
Substrate COD	Sso	206	g COD/m^3
Ammonium N	NH3-N	27	g N/m^3
Nitrate N	NO3-N	0	g N/m^3
Organic N	Sno	5.5	g N/m^3
Alkalinity	Alk	4.5	mol/m^3
Particulate Species:			
Inert COD	Xio	107	g COD/m^3
Substrate COD	Xso	273	g COD/m^3
Heterotrophic Biomass	X_H	0	g COD/m^3
Autotrophic Biomass	X_A	0	g COD/m^3
Cellular Debris	X_P	0	g COD/m^3
Organic N	Xno	11.8	g N/m^3

A mean hydraulic residence time of 1 day was chosen to allow time for both nitrification and de-nitrification. An SRT of 10 days was selected to give time for microbial colonization of the kenaf powder. Initial seeding of the bioreactors with activated sludge was assumed to add 800 mg COD/L of heterotrophic biomass, 100 mg COD/L of autotrophic biomass and 450 mg COD/L of cellular debris to the reactor tank prior to the model run. Starting with these concentrations of biomass and debris as the initial conditions of the reactor, and using the values in Table 2.2 for the influent characteristics, a 4 hour treatment cycle was developed that is similar to the 5 Stage Bardenpho

process, except that because SBRs do not permit true internal mixed liquor recycle there is no need for the first anoxic stage. The SBR cycle used in this study is presented in Table 2.3.

Table 2.3: SBR treatment cycle used for both ASIM and benchtop models

Phase	**Duration** (min.s)	**Mixer**	**Volume In/Out**
Fill	5	none	+1500mL WW +167mL DI [+ kenaf]
Anaerobic	50	recirculation	
Aerobic	70	aeration	
Anoxic	85	recirculation	
Air Strip	5	aeration	-167mL biomass waste
Settle	20	none	
Decant	5	none	-1500mL effluent

The short fill time mimics plug flow behavior in that the resident microbes experience high substrate concentration at the beginning of the cycle with declining substrate concentration throughout the remainder of the cycle. However, the small fraction (1/6) of the total volume which is exchanged with each cycle means that the change in substrate concentration from the beginning to the end of the cycle is relatively small, which is more like the behavior of a single constantly stirred tank reactor (CSTR) in which substrate concentration is assumed to be constant throughout the tank. Taken together, the model's performance is between that of a plug flow reactor and a CSTR in order to better simulate the multiple tanks in series arrangement of real multi-stage nutrient removing WWTPs.

The first react phase, which was anaerobic, was designed to facilitate the fermentation of some organic carbon to acetate as well as the ammonification of organic nitrogen. As shown by the ASIM output in Figure 2.1, 50 minutes duration for this phase was just long enough for the ammonia concentration to reach a plateau in the ASIM model. In the physical bench-top model however, most fermentation and ammonification probably occurred in the feedstock tank, before the wastewater entered the reactor. This phase did still function well in the bench-top model as an anaerobic selector benefiting phosphate accumulating organisms (PAOs). Phosphate removal is not supported in ASM No. 1, therefore this process was not modeled in ASIM.

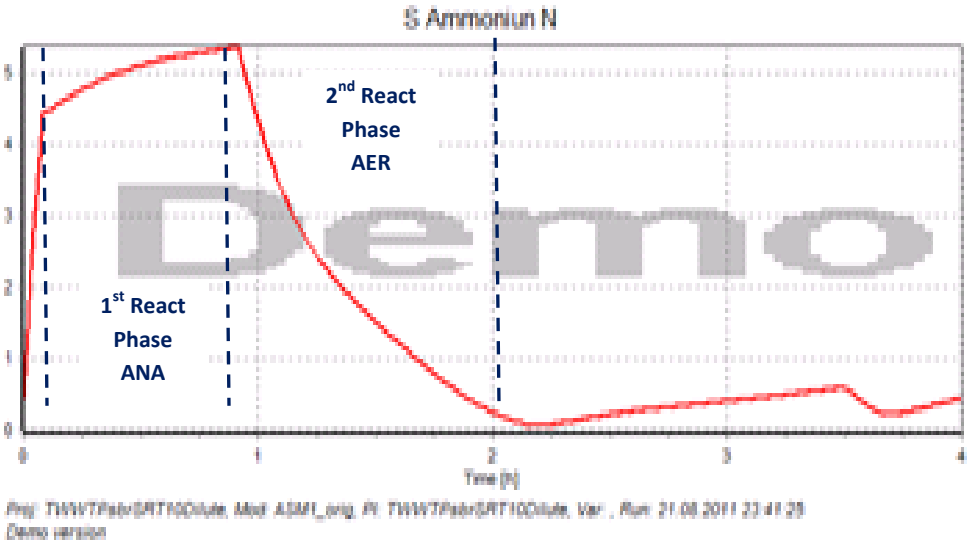


Figure 2.1: Ammonium concentration within reactor through 1 four hour cycle

The second react phase was aerobic. It was designed to last long enough for essentially all of the ammonia to be converted to nitrate by nitrifying bacteria (AOB and NOB) while most of the organic carbon (i.e. COD) is oxidized to carbon dioxide (Figure 2.2). It is important to not allow all of the COD to be consumed during this phase because some will be needed as substrate for the heterotrophic denitrifiers in the subsequent anoxic treatment phase.

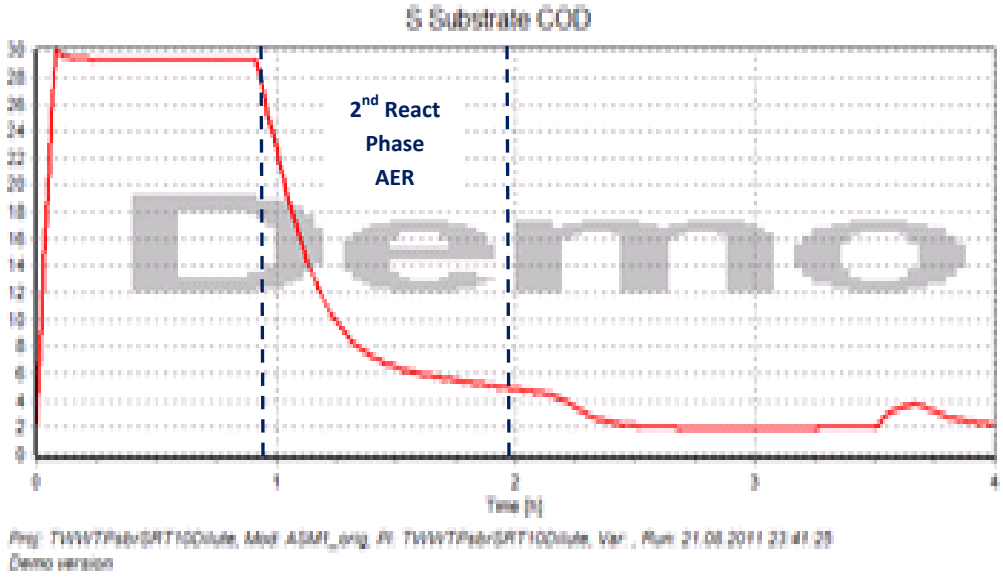


Figure 2.2: Soluble substrate concentration within reactor through 1 four hour cycle

In the ASIM model the dissolved oxygen concentration (DO) was assumed to remain constant at 2.0 mg/L throughout this aerobic phase (and the brief re-aeration phase later in the cycle), while in all non-aerated phases the DO was equal to 0.0 mg/L. In the physical bench-top model, however, the

DO varied continuously, often reaching its peak value only near the end of the primary aeration phase, then declining through the first 10 to 30 minutes of the next phase. Figure 2.3 shows the actual DO concentration in the control reactor through a typical cycle. Dissolved oxygen concentrations in the experimental reactor were similar.

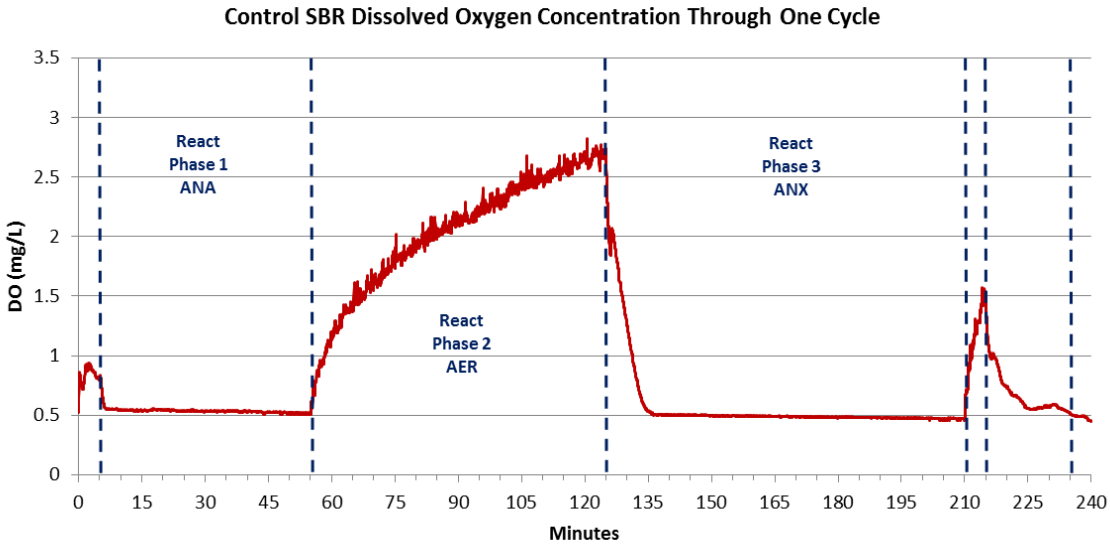


Figure 2.3: Typical actual DO concentration in the reactors

The third react phase was anoxic and had the longest duration in order to allow time for the facultative denitrifiers to convert nitrate from the previous phase to nitrogen gas which then exits the system (Figure 2.4).

The final react phase consisted of 5 minutes of aeration to strip any remaining N_2 from the liquid and to ensure that the DO concentration would be high enough through the settle and decant

phases to inhibit further denitrification (which could otherwise lead to rising sludge during clarification.) Residual DO in the settle and decant phases was also important to prevent the anaerobic conditions that cause PAOs to release all of their stored phosphate back into solution.

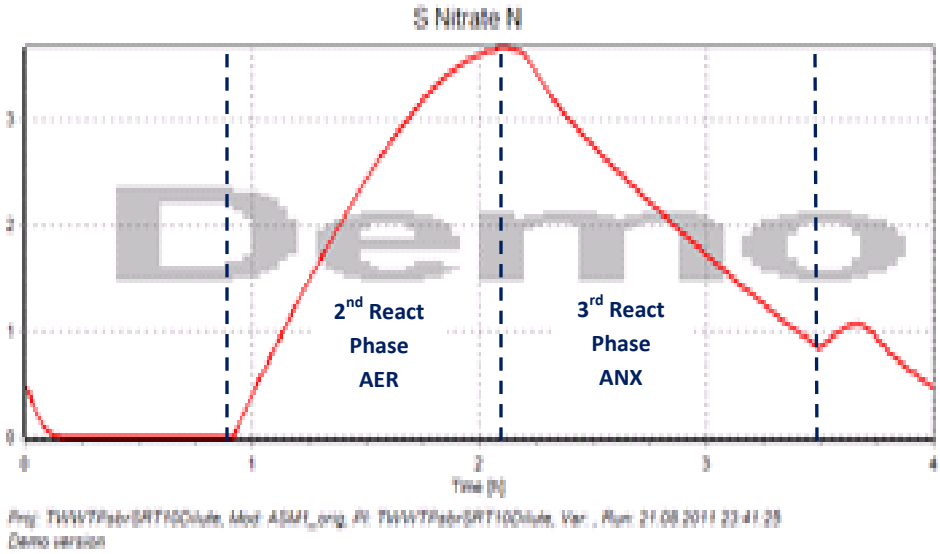


Figure 2.4: Nitrate concentration within reactor through 1 four hour cycle

The outputs from an ASIM model run, like the inputs, also require some interpretation and conversion in order to be able to compare them with common measurements. For instance, the effluent soluble COD can be predicted by aggregating the values for inert and substrate soluble COD: sCOD = Ss + Si. ASIM assumes a perfect clarifier in which no solids remain in the effluent, so the effluent tCOD would be equal to the sCOD. However, in physical reactors and full scale WWTPs,

clarification is never perfect. With no ability to partition the solids into those that settle well and those that do not, there is no way to predict the effluent tCOD from ASIM output. Mixed liquor suspended solids (MLSS, in COD units) can be estimated by summing the particulate substrate and inert COD, Xs and Xi, the heterotrophic and autotrophic biomass, X_H and X_A, and the cellular debris, Xp. The result can be converted to conventional mass units by dividing by 1.2 mg COD/mg TSS. Determining the volatile suspended solids, MLVSS, is more problematic and requires some approximation of the volatile fraction. The approximation used for the influent wastewater would not necessarily be applicable to the output because inert solids, which may be less volatile than biomass or substrate, accumulate in bioreactors.

Lab-Scale Reactors

Two identical 10.0 L reactors were fabricated from clear acrylic which is stronger than Plexiglas and less prone to pitting by attached microbes. Acrylic is also more transparent than plexiglas and therefore permits better observation of the tank's contents. This proved useful in detecting zones of inadequate mixing and in monitoring and removing attached growth from the reactor walls. A dimensioned drawing of the reactors can be found in Appendix 1.

Four 2 channel peristaltic pumps transferred liquids into or out of the reactors. A schematic of the benchtop setup is shown in Appendix 2. Referring to that illustration, all pumps with a letter designation are peristaltic while those with numbers are centrifugal. Pump A was a Cole-Parmer Masterflex L/S model 7554-90 which delivered 167 mL of either DI water (control SBR) or a suspension of kenaf powder in DI water (experimental SBR) to the reactors during the 5 minute Fill phase. Pump B, a Cole-Parmer model 7553-02, was responsible for feeding 1500 mL of wastewater to the reactors in the same 5 minutes. Pump C, a Cole-Parmer model 7554-90, removed 1500 mL of treated effluent in the Decant phase. Biowastage occurred in the re-aeration phase (React Phase 4) when Pump D, a Cole-Parmer model 7553-30, removed 167 mL of mixed liquor in 2 minutes.

Mixing of the wastewater feedstock was accomplished by a gear motor driven stainless steel impeller, beginning 5 minutes before, and continuing through, the Fill phase. A small centrifugal pump (Pump 1, Laing Thermotech, model LMB 10102990, 0.28 A) mixed the kenaf suspension feed tank on the same schedule. Pumps 2 and 3 were larger centrifugal pumps (Little Giant, model 3X-MDX, 0.94 A) which kept the mixed liquor in suspension in the control and experimental reactors during non-aerated react phases. The recirculation flow within the reactors as induced by Pumps 2

and 3 was upward, with return mixed liquor entering near the bottom of the tank then exiting the reactor at the pump intake located approximately 0.1 m below the free surface. Air was supplied to the reactors in the aerated phases (React Phases 2 and 4) by a vacuum/air pump which was connected to Cole-Parmer acrylic variable area airflow meters with direct reading scales from 0.04 to 0.5 LPM and brass needle valve regulators, then anti-siphon check valves and finally to 3 inch disk shaped air stone diffusers placed in the bottom of each reactor.

All pumps and mixers were controlled by 2 ChronTrol XT Series timers, each capable of switching 4 separate 120 v electrical circuits on and off multiple times in each SBR cycle.

The actual benchtop setup is shown in Figure 2.5. For safety reasons, the 25 L WW feedstock tank and the 50 L effluent tank stayed on the floor, and therefore are not shown in this picture.

Figure 2.5: Benchtop setup

Feedstocks

Many lab scale wastewater treatment studies utilize synthetic wastewater as feedstock because its constituents can be fully controlled and characterized analytically, with little or no need for testing, and because it is easy to procure, typically by mixing measured masses of a fairly small number of chemical species with DI water right in the lab as needed. For this study, there was concern that synthetic WW would not adequately model the diversity or the variability of substrates influent to biological treatment at municipal WWTPs, and therefore might select for a substantially less diverse (and less robust) microbial cohort than would be found at a real treatment plant. To more ably apply plant performance and microbial kinetics results from the lab scale reactors to full scale WWTPs, it was decided to feed real municipal wastewater.

Municipal wastewater for feedstock was collected weekly from the North Cary Water Reclamation Facility in North Carolina which treats an average of 7 million gallons a day generated by 60,000 residents and several industries. The plant's reported average influent characteristics for one year (9/1/2010-8/31/2011) are given in Table 2.4.

Wastewater feedstock was transferred from the final tank of the headworks by a submersible centrifugal pump which was placed 0.3 – 0.5 m below the surface. The wastewater in this tank had already undergone primary treatment of band screening and grit and FOG removal. Further physical treatment (necessitated by tubing clogs in the lab) was carried out during the collection procedure by passing the wastewater through a sieve (ASTM No. 16), which probably removed some particulate COD along with the larger inert particles which were targeted for removal by this

process. The feedstock was transported and stored in sealed polypropylene barrels. Any feedstock which would not be used within 48 hours was held at 4°C until needed.

Table 2.4: Annual average influent characteristics at NCWRF

Species	Concentration	
pH	7.44	
VSS	330	mg/L
TSS	360	mg/L
COD	569	mg/L
CBOD	261	mg/L
P	6.53	mg/L
TKN	44.6	mg/L
NH3	31.0	mg/L
NO3	1.70	mg/L
Alk	183	mg/L

Both reactors were seeded with 10.0 L fresh return activated sludge from the North Cary plant. They were initially operated under identical conditions, being fed only 90% wastewater and 10% DI water (no kenaf) until they reached a stable state and were well matched in terms of performance. With a 10 day SRT and influent qualities that changed every 7 days, achieving a true steady state was impossible, just as it is at real WWTPs.

During the subsequent treatment phase of this study, kenaf powder provided by the manufacturer, RFWastewater, was fed to the experimental reactor as a suspension in DI water so that measured amounts could be added in each cycle by peristaltic pump. The volume of the suspension fed per

cycle was constant (1/6 L), but the dry mass of kenaf in that volume was adjusted weekly according to a dosage formula provided by the manufacturer (personal correspondence, Walt Brown, RFWastewater, 2011). The control reactor received the same volume of DI water without kenaf in order to control for dilution.

$$Daily\ Kenaf\ Dose = \frac{\frac{BOD\ load}{R_{BOD}} + \frac{TN\ load}{R_{TN}}}{\frac{SRT}{SA_k}} * Loss\ Factor$$

The daily dose of kenaf required for biofilm development depended on the daily load of BOD (assumed to be 80% of COD) and of total Nitrogen (TN). Loading varied weekly with each new batch of wastewater feedstock. Other dosage formula inputs were held constant. These included: the BOD removal rate (R_{BOD} = 4.5 g/m^{2}day), the total nitrogen removal rate (R_{TN} = 0.125 g/m^{2}day), mean solids retention time (SRT = 10 days), the surface area of kenaf powder (SA_k) which was determined by BET isotherms to be 7.4 m^2/g, and a daily loss factor to account for chemical oxidation and microbial catabolism of the kenaf. Initially the daily loss factor was taken to be 1.25 as recommended by the manufacturer (Kenaf Dose A), but later was adjusted to 1.5 (Kenaf Dose B) and finally to 3.0 (Kenaf Dose C) due to additional losses by adhesion of the kenaf to the feed tank and tubing.

Collection of Samples

In order to test the wastewater feedstock for concentrations of total and volatile suspended solids (TSS and VSS), COD, Total Nitrogen (TN), ammonia nitrogen (NH_3-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N) and Total Phosphate (TPO_4), samples were acquired by thoroughly mixing the wastewater then siphoning approximately 1500 mL into a 2.0 L Erlenmeyer flask. The mouth of the flask was sealed with parafilm. Due to time constraints, not all testing of the sample could be carried out immediately. Any unused sample was held at 4°C until analysis was complete.

Effluent concentrations of the same species as in the wastewater feedstock were also assessed. Samples from each reactor were collected by diverting the appropriate tubing from the waste effluent tank to separate 2.0 L Erlenmeyer flasks. Each flask received the entire 1500 mL of treated effluent which was pumped out of the reactor at the end of a single cycle. Effluent tubing was then returned to the waste barrel, and the flasks were sealed with parafilm. The actual volume of effluent in each flask was observed to ensure that the peristaltic pump was functioning according to design. Analysis of effluent samples was generally performed immediately after collection.

Samples for testing of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were obtained by moving the biowaste tubing from the biowaste collection tanks to individual 500 mL graduated cylinders which then received the entire 167 mL of mixed liquor removed from each reactor during the re-aeration phase of a single cycle. Subsequently, the biowaste tubing was returned to the appropriate collection tanks, and the graduated cylinders were sealed with parafilm. The volume in each graduated cylinder was observed to confirm proper pump function. Solids analysis proceeded immediately after sample collection.

Samples of settled sludge were gathered from each SBR to determine the concentrations of arsenic, cadmium, chromium, cobalt, copper, lead, nickel, selenium and zinc in the sludge solids. Up to two days of biowaste from each reactor was allowed to accumulate in 2.0 L graduated cylinders. Approximately 1800 mL of clear supernatant could then be pumped out of the graduated cylinders leaving 180 to 200 mL of settled sludge which was retained for drying. The sludge was placed in crucibles and dewatered at 90°C for at least 2 days before being dried at 105°C for 1 day. The dry solids were scraped out of the crucibles with a glass rod and put into 15 mL centrifuge tubes. With lids loose, the tubes and their solids were held at 105°C for 1 hour. The lids were then tightened while still warm. The dried solids were stored in the sealed centrifuge tubes in the dark at room temperature until they were analyzed.

Mixed liquor samples for DNA extraction, floc particle size distribution (PSD) measurement and fluorescence in situ hybridization (FISH) were collected using a clean catch technique during the two minutes of regularly scheduled biowaste removal in the re-aeration phase of the SBR cycle. For the first 30 seconds of waste pump run time the mixed liquor was allowed to flow into the usual biowaste collection vessels. This ensured that any liquid which had been retained in the tubing since the previous cycle was ejected so that only fresh mixed liquor would be sampled. Each reactor's biowaste tubing was then diverted to fill a series of four 15 mL centrifuge tubes. The tubes were labeled, tightly capped and placed in slush ice until further processing could be completed. The tubing was then returned to the biowaste collection tanks for normal operation. Three of the filled centrifuge tubes from each SBR were briefly vortexed to re-suspend the settled contents before small amounts of the sample were removed with a pipette in order to adjust the volume to 14.0 mL. These were then centrifuged at 4000 rpm for 8 minutes. The supernatant was discarded and the

biomass pellets frozen at -60°C for future molecular analysis. The fourth tube from each reactor provided ample mixed liquor for fixing of FISH samples and for plating for floc particle size surveys.

Changes in soluble species (sCOD, sTN, sNH_3-N, NO_2-N, NO_3-N) concentrations throughout individual cycles were tracked by intensively sampling the *in situ* ML of the mature reactors. On two consecutive days, 24 samples of 15 mL of mixed liquor were collected from each of the reactors in one 4 hour cycle. Samples were removed from both SBRs simultaneously using 60 mL syringes inserted 2-3 cm below the free surface. Around 25 mL was withdrawn from each tank then 15 mL of that was ejected into 15 mL centrifuge tubes. Whatever ML remained in the syringe was immediately returned to its origin. The centrifuge tubes were sealed, labeled and placed on slush ice for up to 30 minutes until they were centrifuged at 1000 rpm for 10 minutes. The supernatant was retained and stored at -20°C. Prior to analysis of these intra-cycle samples, they were thawed then passed through a 0.45 µm syringe filter to remove any residual solids.

Analysis of Samples

All suspended solids measurements, whether for the wastewater feedstock, the mixed liquor or the effluent, were accomplished by the same method which began by placing an appropriate number of clean crucibles and 55 mm glass microfiber filters in a 550°C furnace for at least 15 minutes. The filters and crucibles were then carefully removed from the furnace with long handled tongs and allowed to cool to room temperature. Only clean gloves or tongs were used to handle the crucibles and filters from this point on to avoid transfer of oils from the skin or other materials from contaminated gloves. Each crucible and filter combination was weighed and the mass recorded. A Buchner funnel was inserted into a side arm flask connected to a vacuum pump. One of the glass fiber filters was placed in the Buchner funnel, the vacuum pump was turned on, and then a small amount of DI water was passed through the filter to secure it to the funnel. A measured volume of the sample was slowly added to the funnel with either a 10 mL pipette or a 60 mL syringe. This volume was recorded.

The volume filtered depended on the concentration of solids in the sample. Only 20 to 30 mL of mixed liquor was required to deposit sufficient solids on the filter, but as much as 300 mL of effluent samples was needed. Standard Methods suggests that the mass of dry solids retained on the filter should be at least 2.5 mg to ensure that results exceed the balance's uncertainty, but no more than 200 mg to avoid damage to the filter from overly long filtration times or trapping water within such a large sample [13]. In practice, this range of solids deposition was generally achieved by adding liquid until the filter was clogged to the point that the final 10 mL of sample took 5 to 10 minutes to pass through the filter. Using forceps, the filter was removed from the funnel, folded and placed in

its crucible. The filtration process was replicated 3 times per sample. One blank in which the filter received only DI water was included in each batch of samples analyzed.

The crucibles and their filters were then dried in a 105°C oven for at least 2 hours, but typically overnight, before being cooled to room temperature and weighed. The masses were recorded. The difference between this mass and the initial empty mass of the crucible and filter, divided by the volume filtered, was the total suspended solids (TSS or MLSS) for the replicate. The mean value for the three replicates was taken to be the TSS for the sample.

The crucibles and their filters were subsequently ignited in a 550°C furnace for at least 30 minutes. They were then cooled to room temperature, weighed and their masses recorded. The mass of the crucible and filter after drying minus its mass after igniting, divided by the volume filtered, was the replicate's volatile suspended solids (VSS). The sample's reported VSS was the average of the replicate values.

The sludge volume index (SVI) is a function of both the MLSS, as determined above, and the 30 minute settled sludge volume (SSV30) which was measured by borrowing 1.0 L of mixed liquor from each reactor 35 to 45 minutes prior to the end of the anoxic phase (React Phase 3). The samples were placed in identical 1.0 L clear graduated cylinders and left undisturbed for exactly 30 minutes. The volume of liquid into which the solids had settled was then read and recorded before the mixed liquor samples were returned to the reactor from which they originated.

Samples of dried sludge solids from each reactor were digested in hot acid then analyzed by a Varian 820 ICP-mass spectrometer to determine their concentrations of arsenic, cadmium, chromium,

cobalt, copper, lead, nickel, selenium and zinc. The procedure used by Kim Hutchison of the EATS laboratory is reported in Appendix 3.

Floc particle size distributions were assessed by first immobilizing small samples of methylene blue stained ML in non-nutritive agarose gel in petri dishes by the method in Appendix 4. In preparation for imaging a group of these petri dishes, they were removed from storage at 4°C, opened and inverted in a portable dehydrator for approximately 20 minutes to equilibrate to room temperature without forming condensation on the gel surface. Droplets of condensation could yield false positive results if they are interpreted as floc particles during image analysis using MetaMorph software. The high resolution black and white CCD camera's power supply and the light box stage were turned on and allowed to warm up. MetaMorph 5.0 r. 7 software running on a Windows 2000 platform controlled the camera. The zoom wheel on the microscope was set to 5X.

One petri dish was placed on the light box in order to test focus on it by eye. Only the left side of the stereo microscope will be functional when it is in "camera" mode. Unfortunately, the camera does not have exactly the same focal plane as the ocular element, so if the eyepiece is in perfect focus, the camera is not. While running "Show Live" in MetaMorph's "Acquire" window, small adjustments were made to the focus until the image looked at least as good as what was observed optically. The petri dish was removed, but the focus left as it was, so that a shading image could be acquired of just the light box, enabling MetaMorph to subtract its variations from all other images in this series by selecting the "Shading Correction -- Image" checkbox in the Acquisition window. The shading image must be saved to the same folder as the images corrected with it.

In order to calibrate distances in the microscope images, a clear ruler with mm marks was placed on the light box, using microscope slides as spacers to position the ruler as close as possible to the original plane of focus so that only small focus adjustments were needed to achieve a sharp image. With “Show Live” still running, the ruler’s position was adjusted such that two demarcations 1 mm apart were centered in the image. An image of the ruler was acquired, named “Ruler” and saved in the same folder as the shading image. The “Measure” tab was selected, then “Calibrate Distances,” then the “Setup” tab in the new window. The “New” option was designated. The ruler image was highlighted and the “Line Region” checkbox was turned on. Units of micrometers were chosen and “1000” entered in the calibration length box. Dragging one end of the calibration bar to the edge of one ruler demarcation, then the other end of the bar to a second point perpendicular to the first on the same side of the other mm mark established the scale of the image of the ruler and therefore of all other images made at the same magnification, enabling MetaMorph to measure particle sizes during Integrated Morphometry Analysis (IMA) of the immobilized floc samples. The calibration was named, saved to file and left active for application to the images which were to be made next.

A petri dish with its sample was then placed on the light box. With “Show Live” running, the framing and focus were adjusted. An image was acquired with shading correction, calibrated, saved and named. Under the “Measure” tab, “Threshold Image” was selected. Thresholding allows the researcher some control over which objects are included in the analysis of particle sizes. By clicking on the lower button to the left of the image, “Auto Threshold for Dark Objects” was activated, and then the bar below that button was dragged to adjust the sensitivity of the threshold so that only a thin grey ring showed around the thresholded objects in green. The thresholded image was saved to the usual folder.

MetaMorph analysis results were saved by exporting the object and summary logs to an excel file. To that end, a new excel file was created in the desktop, named and opened. In the MetaMorph window the "Log" tab was activated, then "Open summary log." The "Log to Dynamic Data Exchange – DDE" option was turned on. In the "Export Log Data" window, "MS Excel 2000" was selected as the application, the name of the new excel file was input as the "Sheet Name" and the desired starting row and column numbers were entered. The object log was opened by the same procedure, taking care to use starting row and column numbers that will not overlap the range of the summary log.

Under the "Measure" tab, "Integrated Morphometry Analysis" was selected. With the Display box in the IMA window reading "Summary," the "Total Area," "Shape Factor," "Equiv. Sphere Radius" and "Equiv. Sphere Volume" were checked for inclusion in the analysis. The "Configure Log" button allowed the same parameters to be checked, as well as "Object #" for inclusion in the final excel data file. The Display box was changed to read "Object" and the "Configure Log" button re-activated. The parameters selected were: Parameter, Count, Average, Standard Deviation, Min, Max and Total. The "Measure" button in the IMA window was activated to populate the logs, then the "Log Data" button exports the object log to excel. The Display box was changed to read "Summary" and the "Log Data" button again selected to export the summary log to excel. After confirming that the excel file contained the desired summary and object data, the summary and object logs were closed. The excel file containing summary statistics and data about the apparent size and shape of every particle in the image was saved. A step-by-step of MetaMorph procedures for surveying particle sizes (with illustrative screen captures) is included with this report as Appendix 5.

The same petri dish previously imaged was repositioned so that a second non-overlapping image could be made and analyzed, and then a third. Three images were thus acquired from each of the three petri dishes which had been prepared from each sample of mixed liquor. For each reactor day sampled, aside from the Shading and Ruler images, a total of 18 images (9 of kenaf ML and 9 of control) were created and analyzed by MetaMorph, which reported that each image contained a few hundred to a few thousand particles. However, the number (and size) of particles detected by MetaMorph in a given target frame was found to be very sensitive to both the camera's focus and the image's threshold setting. The high data density of 9 samples (i.e. images) from just 1.0 mL of mixed liquor was necessitated by the high random variability of this procedure. In some analyses in this report, the random variability was reduced by aggregating the results from all three images from a single petri dish, treating them as a single large sample.

The data MetaMorph exported to excel can be rearranged, filtered or transformed as needed to compare the particle sizes in the control and treatment reactors. For instance, a volumetric particle size distribution (PSD) can be developed using the SUMIFS() command to determine the total equivalent volume of particles within each particle size class, which can then be normalized by the total equivalent volume of all particles in the image as given in the summary statistics. Similarly, a population based PSD can be created using the COUNTIFS() command to find the number of particles in each size class, which can be divided by the total number of particles (from the summary statistics) to determine the fraction of the population that occurs in each size class. Another method for summarizing a distribution of particles is the volume-moment mean which can be found by first generating a "Diameter" column from the equivalent radius, then a column with each diameter raised to the fourth power (D_4) and finally one with each diameter raised to the third

power (D_3). The sum of the D_4 column divided by the sum of the D_3 column is the volume-moment mean or D_{43} [14, 15].

The total and soluble COD, TN, NH_3-N and TPO_4 were measured in the effluents and feedstock using Hach Test-N-Tube kits which were evaluated with a Hach DR/890 colorimeter. Table 2.5 lists the test kit numbers used in this study, as well as their Hach procedure numbers, ranges and reported sensitivities. In some cases dilution of the sample was necessary to prevent measured output from exceeding the test's range.

To differentiate between solid and soluble constituents in effluents and in the feedstock, a portion of these samples was passed through a syringe filter with a 0.45 µm average opening. Measurements obtained from the unfiltered portion were labeled "total" or given a prefix of "t" (e.g. tCOD) while those from the filtered portion were referred to as "soluble" or given the prefix "s" (e.g. sCOD). The fraction of any constituent attributable to the solids was the difference between the total value and the soluble value for that species.

Samples of wastewater and of effluents to be evaluated for nitrite and nitrate concentrations were first filtered through a 0.45 µm IC certified syringe filter, and then frozen at -20°C until analyzed by ion chromatography.

Table 2.5: Hach colorimetric Test-N-Tube kits

Sample Source	Species	Hach Kit Number	Hach Method	Range (mg/L)	Sensitivity (mg/L)	Detection Limit (mg/L)
Wastewater	sCOD	2125815	8000	3-150	± 2	4
Wastewater	tCOD	2125915	8000	20-1500	± 16	30
Wastewater	sTN, tTN	2714100	10072	10.0-150.0	± 3	7
Wastewater	sNH_3-N, tNH_3-N	2606945	10031	0-50	± 5	1
Wastewater	$sTPO_4$, $tTPO_4$	2767245	10127	0-100.0	± 3	7
Effluents	sCOD, tCOD	2125815	8000	3-150	± 2	4
Effluents	sTN, tTN	2672245	10071	0-25.0	± 0.5	2
Effluents	sNH_3-N, tNH_3-N	2606945	10031	0-50	± 5	1
Effluents	$sTPO_4$, $tTPO_4$	2767245	10127	0-100.0	± 3	7

The concentrations of the soluble species sCOD, sTN and sNH_3-N in the intra-cycle samples were measured by the same Hach colorimetric Test-N-Tube kits as were used for effluent testing. The concentrations of NO_2-N and NO_3-N were determined by ion chromatography.

The dissolved oxygen concentration in the reactors was measured intermittently with a portable DO meter (YSI Model 55). In that the only probe available took several minutes to equilibrate with its surroundings each time it was moved from one tank to the other, it was difficult to compare the reactors head-to-head on this sometimes rapidly changing parameter. A data logging bench top DO meter (YSI Model 5000 with Model 5010 BOD Probe) became available late in the study which made it possible to record the DO every second for extended periods of time in one reactor at a time. This still did not allow direct comparison of the DO curves of the reactors in a single cycle. The probe for

this meter also had a tendency to trap bubbles during aeration phases introducing some noise to the DO curves in the form of transient spikes.

Batch tests to compare the ammonia utilization rate of the control SBR's microbial cohort to that of the kenaf supplemented biomass also required borrowing large volumes (500 mL) of mixed liquor from each reactor, repatriating whatever ML was left after batch test sampling. Batch tests were performed both with dissolved oxygen concentrations near saturation and with limited DO. In either case, the samples were placed in identical 1000 mL graduated cylinders and vigorously aerated to saturation for 30 minutes to 2 hours before starting the test. Aeration was provided by a vacuum/air pump connected to small air stone diffusers. This lowers the ammonia concentration in the samples to near zero prior to the addition of a spike of 0.0236 g ammonium sulfate dissolved in 10 mL of DI (for 10 mg N/L in samples) at test time t = 0. The long pre-aeration also exhausts any readily available soluble COD, ensuring that only autotrophs such as the nitrifiers are active. No further aeration was provided in the limited DO condition test (referred to as AmV in lab notes), but vigorous aeration continued throughout the saturation DO condition tests (AmIII and AmVI in lab notes.) In all cases, a timed series of 5.0 mL samples were taken from the 500 mL ML aliquots, filtered through 0.45 µm syringe filters and analyzed for concentrations of ammonia using Hach Test-N-Tube kits and for concentrations of nitrite and nitrate by ion chromatography.

After the two hour pre-aeration, the limited DO condition ammonia utilization test samples were allowed to sit undisturbed except for intermittent gentle stirring for 2.5 hours until the DO was below 30% of the saturation value. A 1.0 L beaker was placed on each of two low speed non-heating stirring plates. A quart size zip-loc baggie was opened inside each beaker. Each baggie received a

nylon covered magnetic stirring bar and a spike of 0.0236 g ammonium sulfate and 0.0123 g sodium nitrite in 10 mL DI. Once baseline samples had been drawn, the aliquots were poured into the baggies which were then sealed while evacuating any air bubbles. Stirring speed was just sufficient to maintain suspension of solids. It was only necessary to temporarily open a small segment of the baggie seal in order to collect the necessary samples using a 5000 µL pipettor.

Samples for future fluorescence *in situ* hybridization (FISH) were fixed by combining 3 mL of fresh mixed liquor with 9 mL of 4% paraformaldehyde solution (prepared according to the procedure in Appendix 6) in a 15 mL centrifuge tube which was sealed and placed on slush ice for 2 hours. The samples were then centrifuged at 2000 rpm for 5 minutes and the supernatant discarded to a hazmat bottle. The pellet was re-suspended by vortexing in 10.0 mL of a commercially available 1X phosphate buffer solution (PBS). The sample was centrifuged a second time as before and re-suspended in 1X PBS. After a third 5 minute centrifugation at 2000 rpm and decanting of the supernatant, the pellet was re-suspended by repeated gentle pipetting in 1.0 mL of a 1:1 solution of 1X PBS and pure ethanol. The entire sample was then transferred by pipette to a 1.5 mL snap-top centrifuge tube for storage at -20°C.

Results

Effluent total and volatile suspended solids

The addition of kenaf seemed to have a weak but significant adverse effect on effluent solids concentrations (VSS, TSS) in this study. Both reactors' effluents exhibited mean TSS values of approximately 30 mg/L and mean VSS concentrations of just over 28 mg/L prior to treatment. During treatment the average suspended solids concentration decreased in both effluents, but the control TSS and VSS dropped more than the kenaf SBR's TSS and VSS did. Kenaf effluent averaged 2.5 mg/L higher TSS and 2 mg/L higher VSS than the control. A paired sample 2-tailed T test failed to reject the null hypothesis that the reactors were undifferentiated prior to treatment, which is evidence that the Pre-Treatment samples from the two reactors could have been taken from a single population or sample source. However, mean effluent TSS and VSS values of the kenaf and control SBRs during the Treatment phase were found to be significantly different at the alpha = 0.05 confidence level, suggesting that treatment lead to a real divergence between the reactors' performances.

Upon review of lab notes, it has been determined that in the period of reactor days 180 through 216 the reactors were being cleaned more frequently due to aggressive attached growth on the SBR walls. During this period only, cleaning was carried out in the final minutes of the mixed (react) phases of the cycle. To avoid cross contamination of kenaf into the control reactor, the control SBR was always cleaned first, kenaf last, but doing this so late in the cycle meant the kenaf reactor had

substantially less time between being disturbed and being sampled than did the control. After this period, cleaning was less frequent and always earlier in the treatment cycle. If this period is rejected from the dataset, there were no statistically significant adverse or beneficial effects on TSS or VSS removal.

Effluent VSS and TSS concentrations are plotted in Figure 3.1, which also shows the influent strength (tCOD) as one possible source of the variability seen in the effluent VSS and TSS. However, no correlation between influent strength and effluent TSS and VSS is evident in this plot which suggests that effluent suspended solids concentration is not a function of influent strength within the range tested. In Figure 3.2 the effluent TSS of both reactors is graphed along with the TSS of the influent wastewater feedstock as another possible source of the variability in effluent suspended solids. Similarly, Figure 3.3 depicts the VSS of both effluents as well as of the influent. No interaction between influent and effluent solids concentrations is apparent in Figures 3.2 and 3.3 which supports the null hypothesis that suspended solids output is not a function of its input concentration.

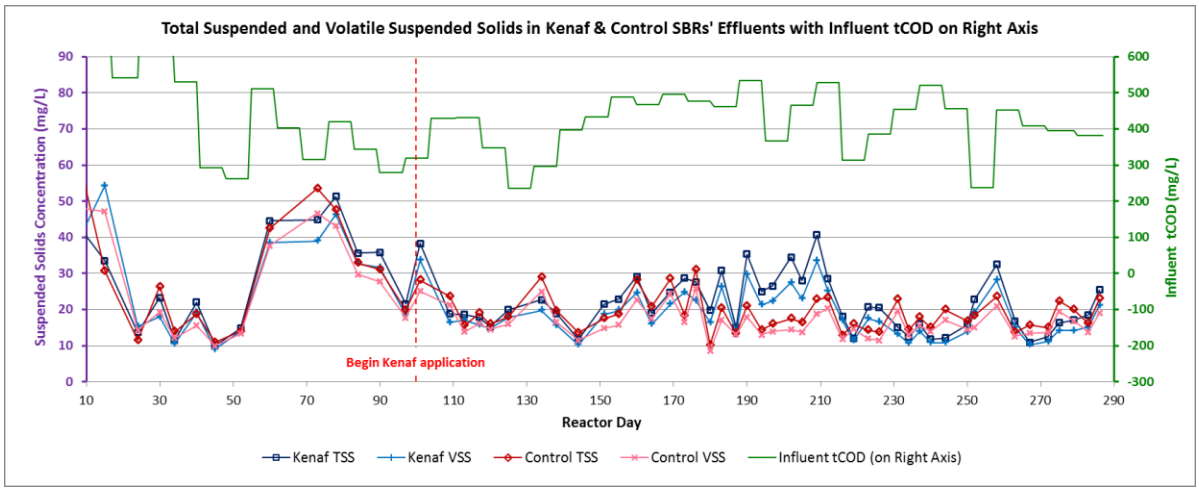


Figure 3.1: Effluent total suspended and volatile suspended solids in the Kenaf (in blue) and Control (in red) SBRs, shown with influent tCOD (in green) plotted on the right side axis

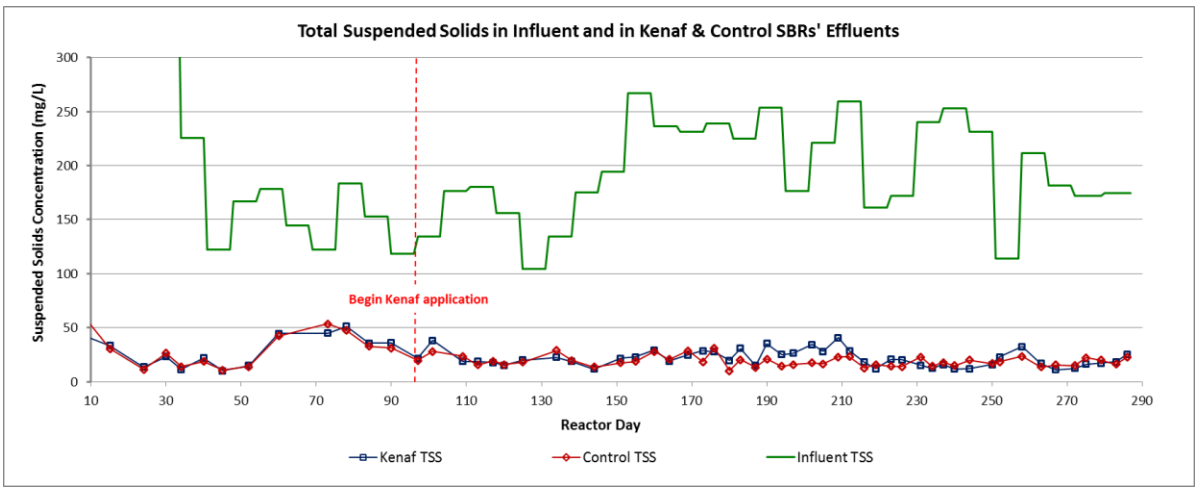


Figure 3.2: Total Suspended Solids concentrations of influent and of kenaf and control effluents

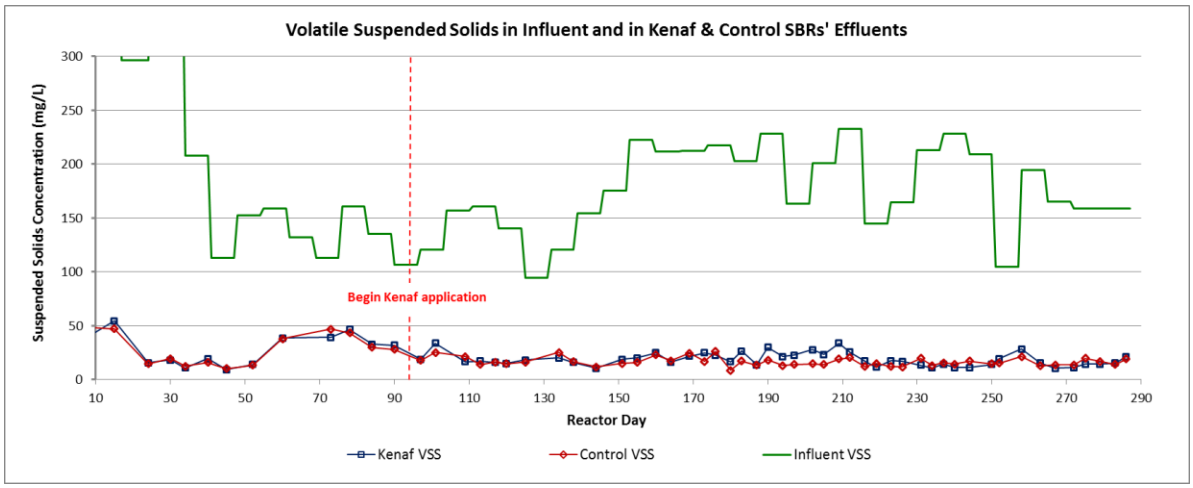


Figure 3.3: Volatile Suspended Solids concentrations of influent and of kenaf and control effluents.

Figure 3.4 compares the percent removal of TSS by the SBRs. Both reactors removed approximately 85% of TSS and VSS in the pre-treatment phase. During treatment the kenaf SBR removed 89% of TSS and VSS on average, while the control SBR removed 90%. A paired sample 2-tailed T test found this difference to be significant at the alpha = 0.05 level. A plot of VSS removal was nearly identical to Figure 3.4 and therefore not included in this report.

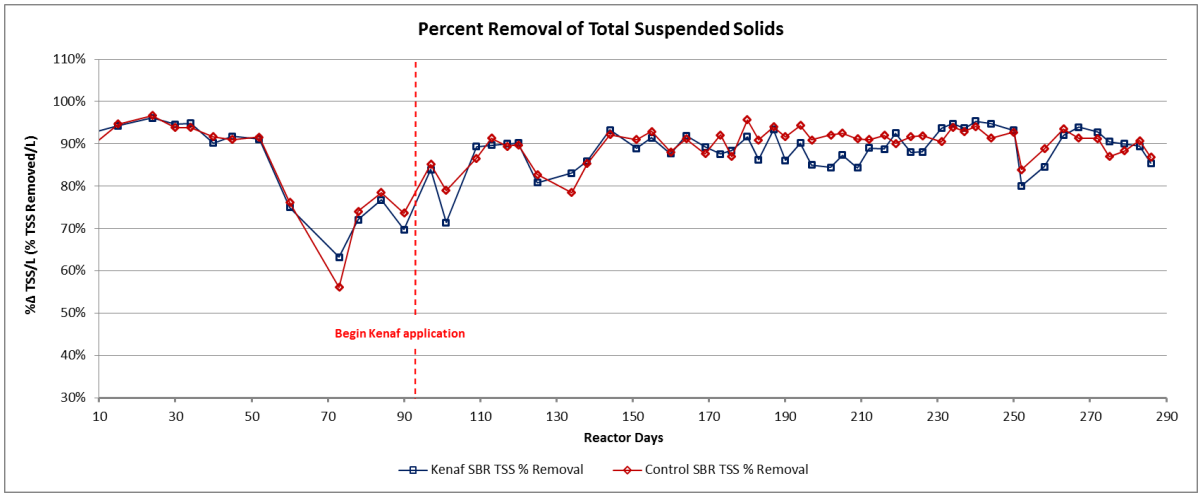


Figure 3.4: Percent removal of TSS

Mixed Liquor Suspended Solids and Volatile Suspended Solids

Kenaf supplementation increased MLSS and MLVSS in the kenaf SBR, but the statistical significance of the increase was difficult to evaluate because the kenaf reactor averaged 5% higher MLSS and 4% higher MLVSS than the control before the treatment phase began. A paired sample 2-tailed T test of the Pre-Treatment phase MLSS and MLVSS data failed to support, at the alpha = 0.05 level, the null hypothesis that the 2 reactors' samples could have come from a single population. The cause of this divergence prior to the Treatment phase is unknown.

During treatment the kenaf reactor's mean MLSS was approximately 11% higher and mean MLVSS 12% higher than the control's as detailed in Table 3.1. The average suspended solids concentration of the control SBR decreased during the treatment phase (relative to pre-treatment) by about 52 mg/L more than the kenaf SBR's MLSS decreased by in the same time, which is a difference of only about one quarter of a standard deviation. This slight difference between the SBRs in the amount of change in their suspended solids from pre-treatment to treatment phases was also approximately equal to the theoretical accumulated concentration of kenaf powder within the kenaf treated reactor (~40 mg/L), suggesting that kenaf adds to the mixed liquor solids rather than replacing a portion of them as has been claimed.

Figure 3.5 plots the MLSS and MLVSS (mg/L) of each reactor, with the influent wastewater strength (tCOD) shown in green and reading on the right side axis. Intuitively this graph suggests that there is some interaction between influent strength and suspended solids concentrations in the SBRs. In Table 3.2 the Pearson Product-Moment Correlation Coefficients support a weak but significant (alpha = 0.05) correlation between influent strength and reactor solids concentrations. This

provides evidence that, as would be expected, some, but not all, of the variability in MLSS and MLVSS is due to the variability of the influent strength.

Table 3.1: Mean suspended solids in mixed liquor (mg SS/L)

	Kenaf SBR MLSS	Kenaf SBR MLVSS	Control SBR MLSS	Control SBR MLVSS
pre-treat mean	1492	1266	1424	1219
pre-treat S.D.	266	192	288	219
treatment mean	1227	1059	1109	948
treatment S.D.	250	207	236	194

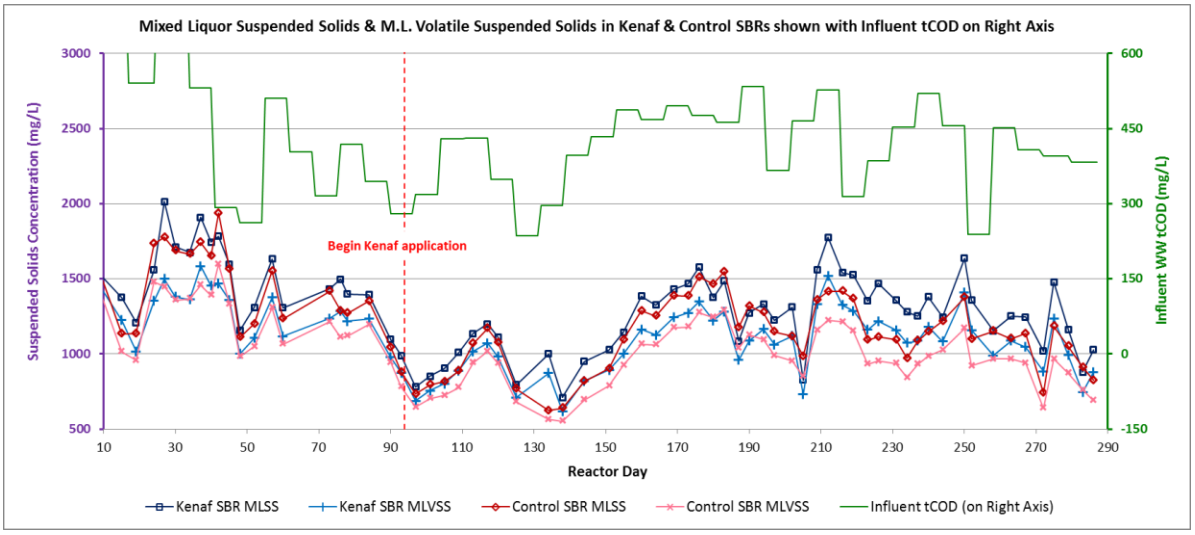


Figure 3.5: Mixed liquor suspended and volatile suspended solids with influent tCOD

Table 3.2: Pearson Product-Moment Correlation Coefficients with 2-tailed critical values (r_{crit})

	Overall	Pre-treatment	Treatment
MLVSS Correlations (r):			
WW tCOD<=>K	**0.468	*0.502	**0.459
WW tCOD<=>C	**0.456	0.364	**0.551
Kenaf<=>Control	**0.913	**0.936	**0.882
MLSS Correlations (r):			
WW tCOD<=>K	**0.467	*0.486	**0.444
WW tCOD<=>C	**0.456	0.363	**0.542
Kenaf<=>Control	**0.911	**0.931	**0.868
	Overall	**Pre-treatment**	**Treatment**
n	68	21	47
df	66	19	45
*>r_{crit} 0.05 =	0.2386	0.433	0.288
**>r_{crit} 0.01 =	0.3104	0.549	0.372

Figures 3.6 and 3.7 compare the reactors' suspended solids concentrations as ordered pairs (kenaf, control = x, y) grouped into Pre-Treatment and Treatment blocks. If the SBRs respond the same way (i.e. with equal changes in MLSS or MLVSS) to the environmental changes they are subjected to (null hypothesis), the slope of the regression line for the data would be equal to one, even if the reactors carry different solids concentrations. Note that for both MLSS and MLVSS the slope of the Pre-Treatment block's regression line (in blue) is approximately one, which supports the null hypothesis that the reactors were similarly reactive prior to the treatment phase despite having had different solids concentrations. The change between the Pre-Treatment and Treatment blocks in the slope of the regression lines indicates that in both MLSS and MLVSS there was more difference between the

SBRs in their responses to environmental changes during Treatment than was evident in Pre-Treatment, in that the slope of the Treatment phase regression line was farther from the null hypothesis's ideal value of 1. If the only difference between the reactors' solids concentrations could be accounted for by the mass of kenaf added, the Treatment phase regression line would be expected to shift to the right of the Pre-Treatment phase regression line but still have a slope of approximately one.

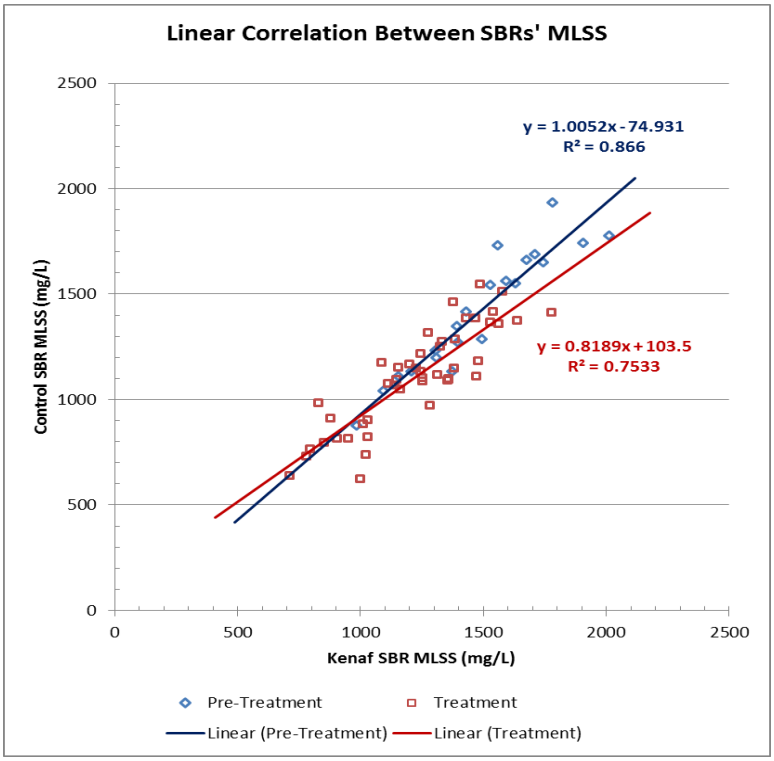


Figure 3.6: Regression on kenaf and control SBRs' mixed liquor suspended solids grouped into pre-treatment and treatment blocks

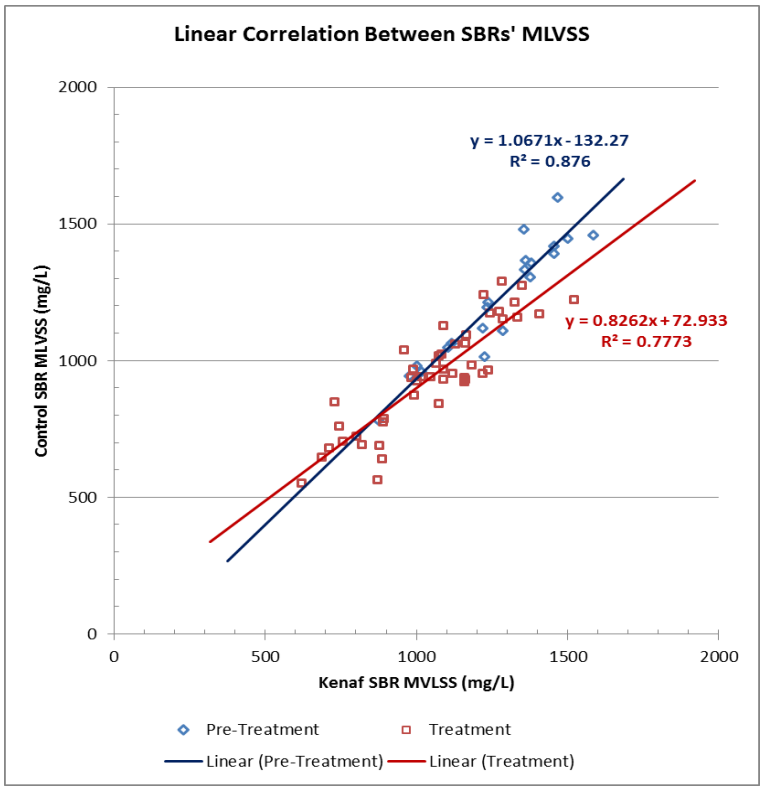


Figure 3.7: Regression on kenaf and control SBRs' mixed liquor volatile suspended solids grouped into pre-treatment and treatment blocks

Sludge Volume Index

The sludge volume index (SVI) was significantly improved by kenaf addition, as shown in Figure 3.8. Both reactors' MLs had average SVIs near 190 mL/g before treatment. A paired sample 2-tailed T test of the reactors' pre-treatment SVIs failed to refute the null hypothesis that the reactors were undifferentiated. While the average SVI of the control SBR's ML did not change during the treatment phase, the kenaf supplemented ML's average SVI dropped to about 130mL/g. As detailed in Table 3.3, the treatment phase average SVIs of the reactors were significantly different at the alpha = 0.01 level, which supports the claim that kenaf supplementation leads to improved sludge settling. All kenaf application rates tested had similar effects on SVI, suggesting that the effect was not highly sensitive to the kenaf dose. Depending on plant design, improved sludge settling could reduce the volume of waste activated sludge (WAS) for processing or disposal, or return more biomass to the beginning of the treatment train allowing the plant to increase its organic loading rate.

Table 3.3: Summary of SVI statistics

	Pre-Treatment		**Treatment**	
Kenaf ML				
mean	192	mL/g	132	mL/g
S.D.	58.0	mL/g	16.55	mL/g
Control ML				
mean	187	mL/g	190	mL/g
S.D.	44.6	mL/g	39.29	mL/g
n	18		46	
T.test p(ϕ) =	0.4024		**6.0E-15	
r	0.9003		0.5129	

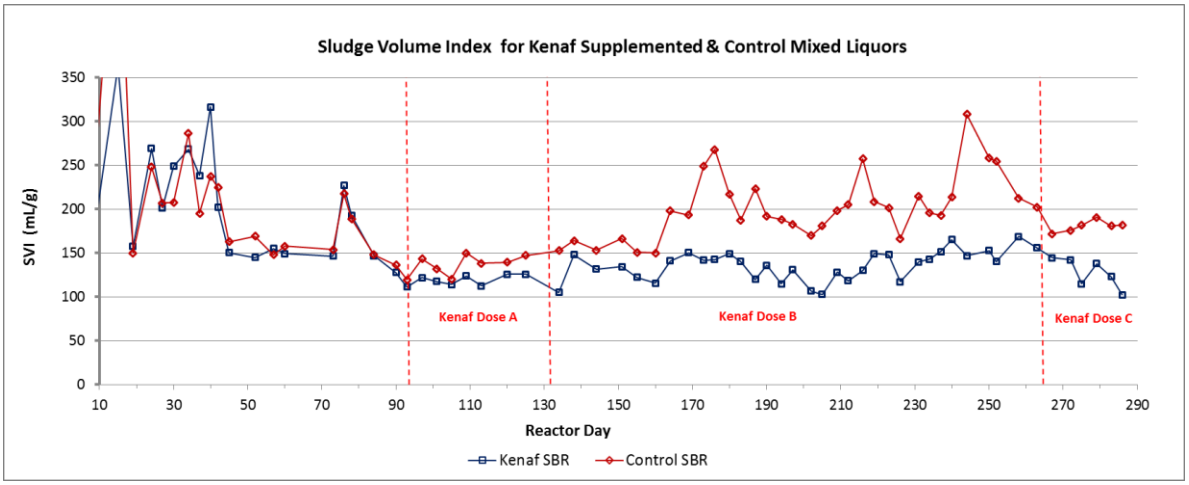


Figure 3.8: Sludge volume index of kenaf and control mixed liquors.

Heavy Metals

Summary statistics for metals concentrations in sludge solids can be found in Table 3.4, which also includes results of paired sample 2-tailed t Tests comparing the kenaf and control sludge solids' mean concentrations of each of the 9 elements analyzed in this study. The t Test values given in Table 3.4 are the probability that the means of two groups of samples, randomly drawn from a single population, would be as different as the means of these particular two groups of samples are. This can be thought of as the probability of getting similar results if the null hypothesis is true, where the null hypothesis is that there is not a significant difference between the two groups of samples. T Test probabilities below 0.05 reject the null hypothesis at the alpha = 0.05 confidence level and are denoted with a single asterisk preceding the value. Probabilities below 0.01 reject the null hypothesis at the alpha = 0.01 level and are denoted with two asterisks preceding the value. Note that Table 3.4 also compares the MLSS of the kenaf and control SBRs on the same days that the solids samples were collected. In this case, the paired sample 2-tailed t Test failed to reject the null hypothesis, from which it can be concluded that for these six samples the suspended solids concentrations of the reactors were not significantly different.

Table 3.4: Mean metals concentrations in 6 samples of dried sludge solids

	SSolids	Arsenic	Cadmium	Cobalt	Chromium	Copper	Nickel	Lead	Selenium	Zinc
	mg/L	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Kenaf ML										
mean	1293	1.06	16.27	1.75	15.12	392.9	30.7	23.62	3.01	1429
S.D.	137	0.26	30.08	0.31	1.05	40.0	24.5	7.06	0.27	401
Control ML										
mean	1232	0.75	19.81	1.99	16.01	267.8	35.7	11.84	3.45	1081
S.D.	149	0.30	46.11	0.40	1.96	37.9	41.2	7.45	0.30	303
t.TEST	0.169	**0.00913	0.653	*0.0343	0.121	**1.18E-05	0.597	**1.23E-04	**0.00808	*0.0324

Figures 3.9, 3.10 and 3.11 indicate that arsenic, copper and lead accumulated significantly (alpha = 0.01, n = 6) more in the kenaf treated ML solids, presumably due to their adsorption to the kenaf. This could potentially limit the disposal options for WAS or require additional testing to ensure that metals concentrations in the WAS stay below compliance thresholds. However, adsorption of these dissolved metals to kenaf powder would also reduce their concentration in the WWTP's effluent; benefitting people, for whom arsenic and lead can be toxic, and aquatic life for which copper can be toxic.

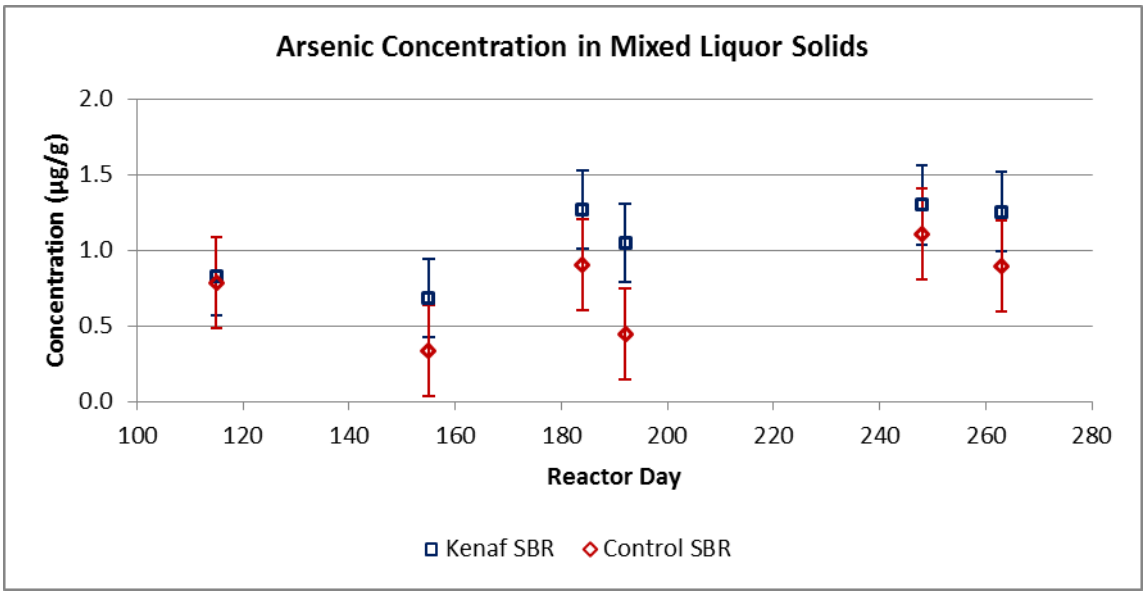


Figure 3.9: Arsenic concentrations in dried sludge solids from kenaf supplemented and control SBRs.

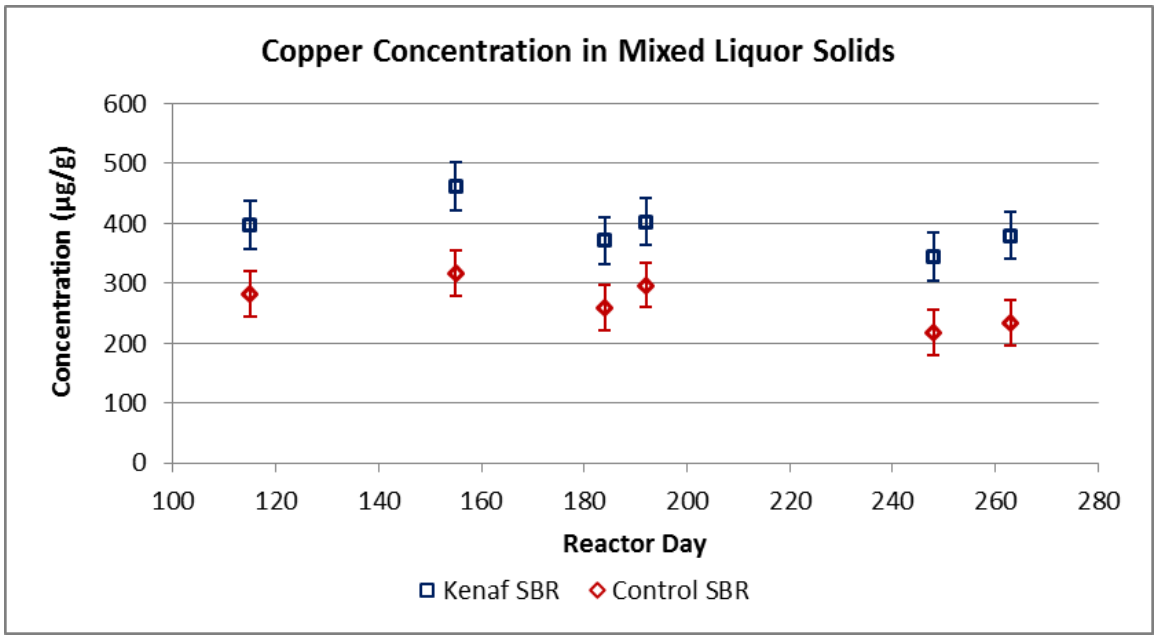


Figure 3.10: Copper concentrations in dried sludge solids from kenaf supplemented and control SBRs.

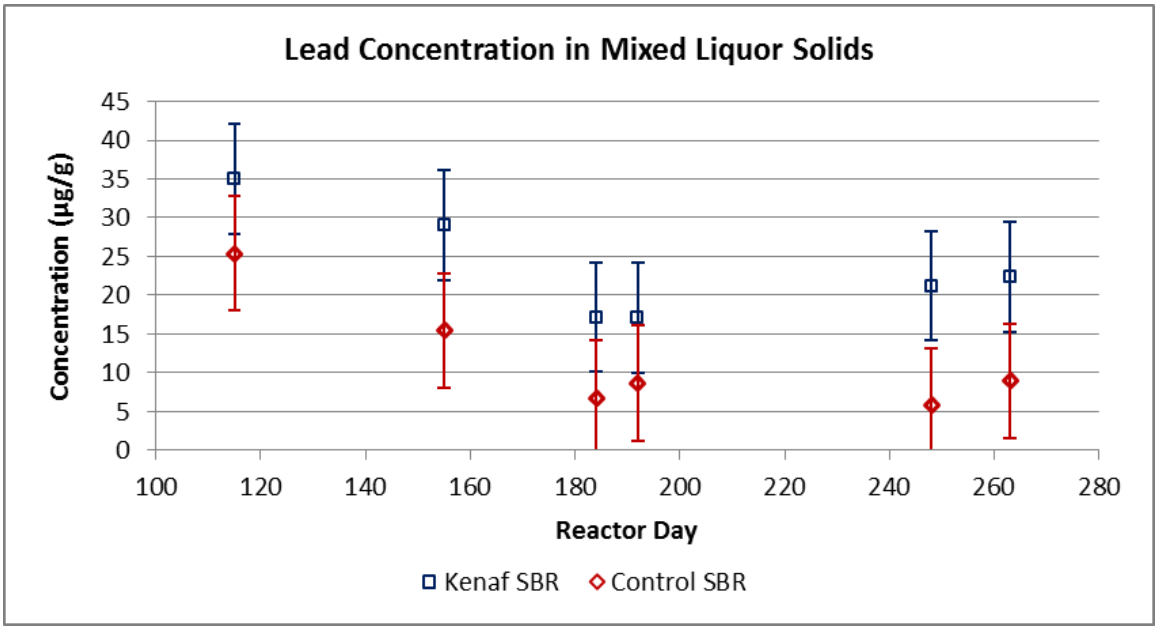


Figure 3.11: Lead concentrations in dried sludge solids from kenaf supplemented and control SBRs.

Increased zinc concentrations in the kenaf supplemented reactor were not apparent from Figure 3.12, and were not significant at the alpha = 0.01 level, but were significant at the alpha = 0.05 level.

Selenium was significantly (alpha = 0.01) less concentrated in the kenaf enriched sludge solids as shown in Figure 3.13. Cobalt (Figure 3.14) was also significantly (alpha = 0.05) less concentrated in the kenaf solids

Figures 3.15 through 3.17 suggest that kenaf did not tend to adsorb cadmium, chromium or nickel.

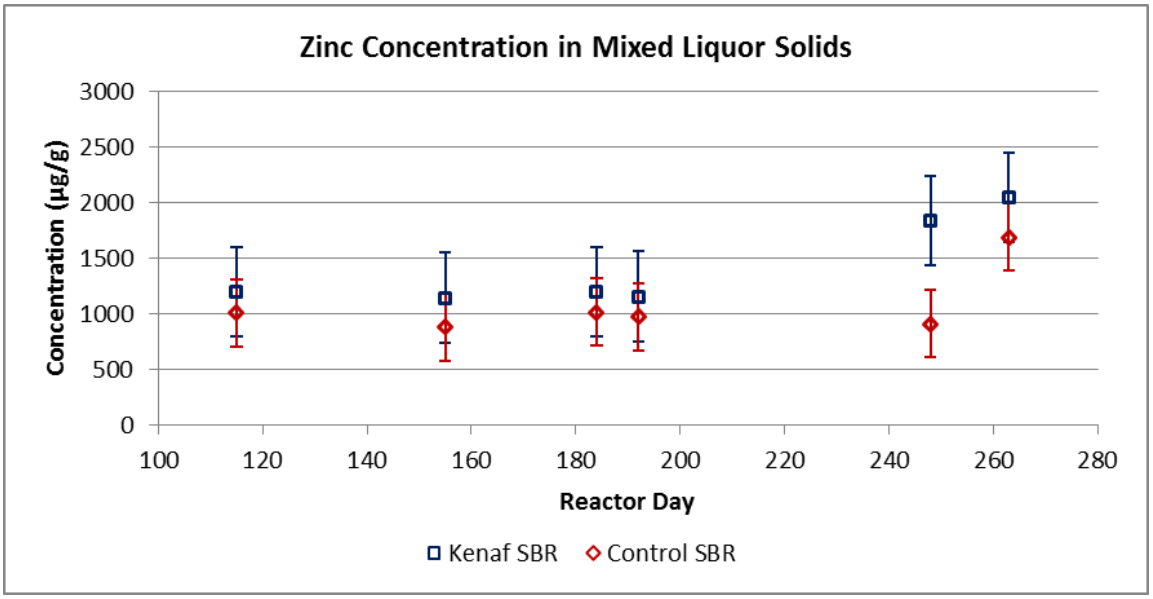


Figure 3.12: Zinc concentrations in dried sludge solids from kenaf supplemented and control SBRs.

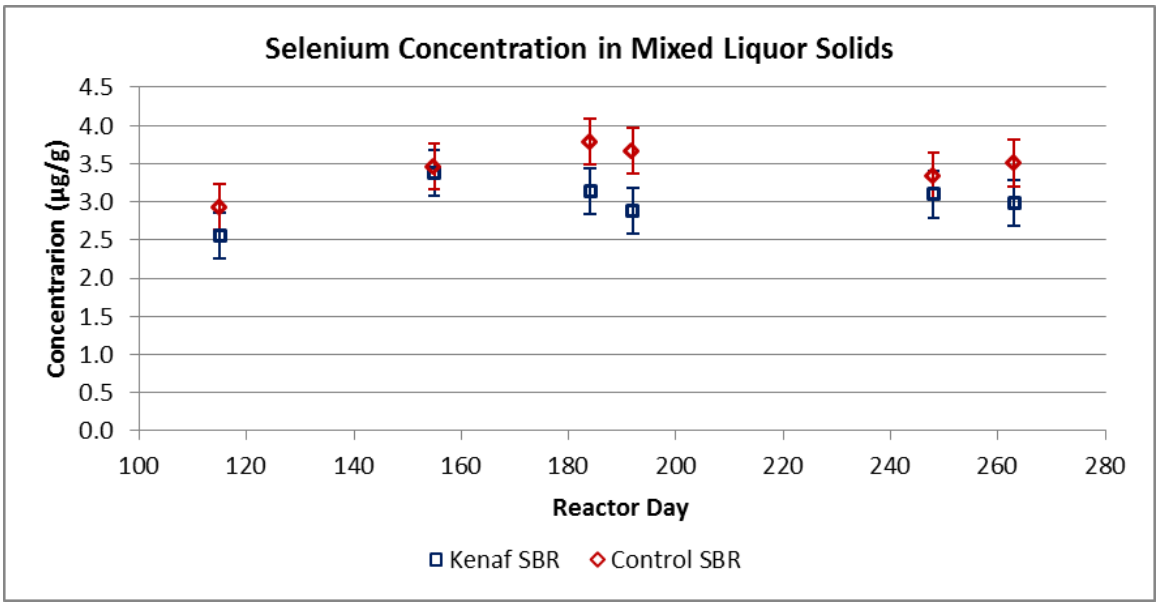


Figure 3.13: Selenium concentrations in dried sludge solids from kenaf supplemented and control SBRs.

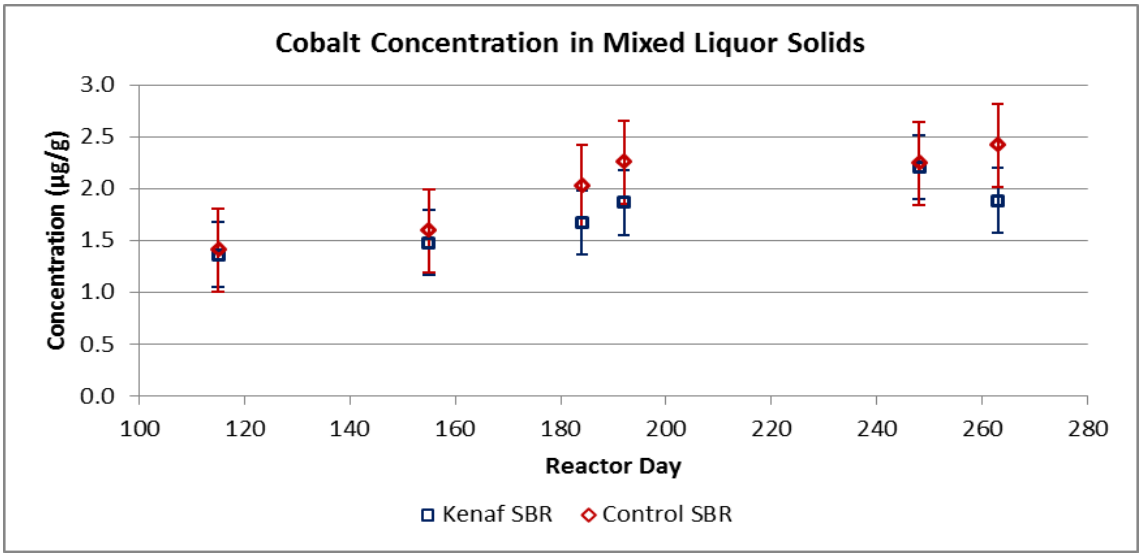


Figure 3.14: Cobalt concentrations in dried sludge solids from kenaf supplemented and control SBRs.

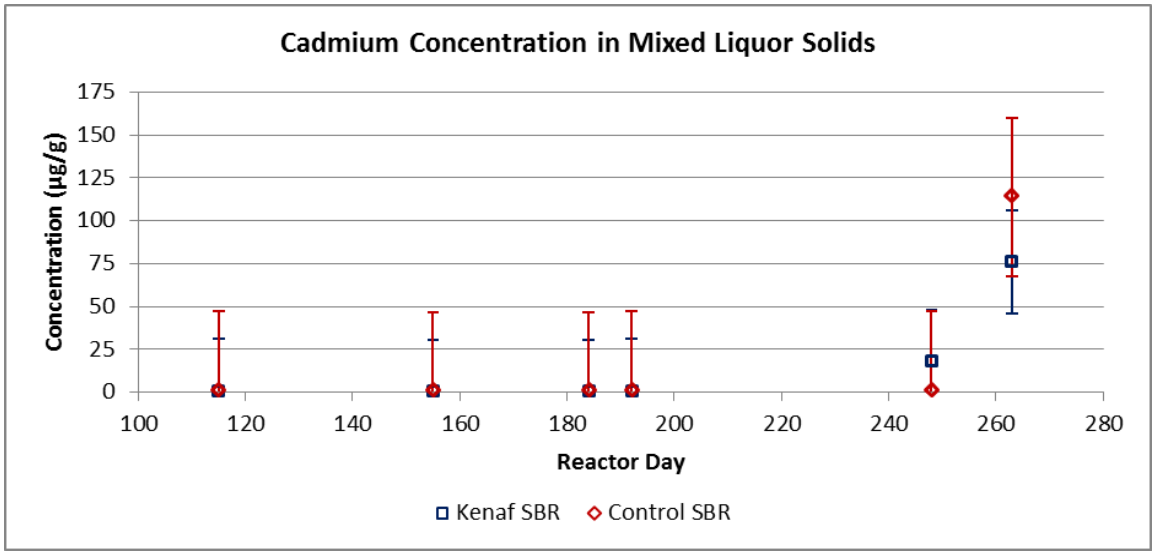


Figure 3.15: Cadmium concentrations in dried sludge solids from kenaf supplemented and control SBRs.

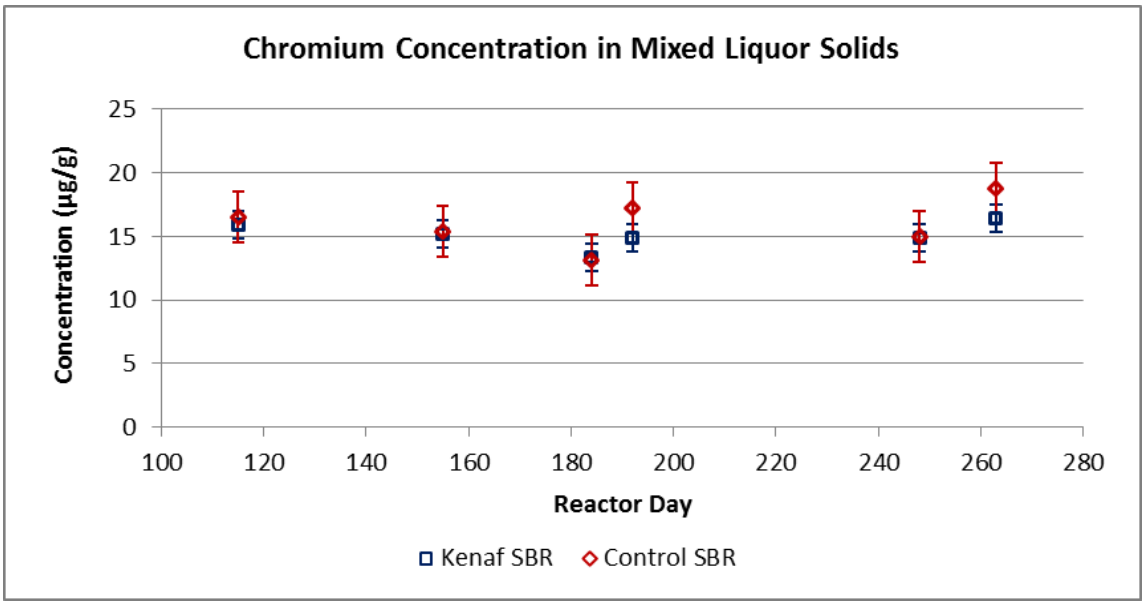


Figure 3.16: Chromium concentrations in dried sludge solids from kenaf supplemented and control SBRs.

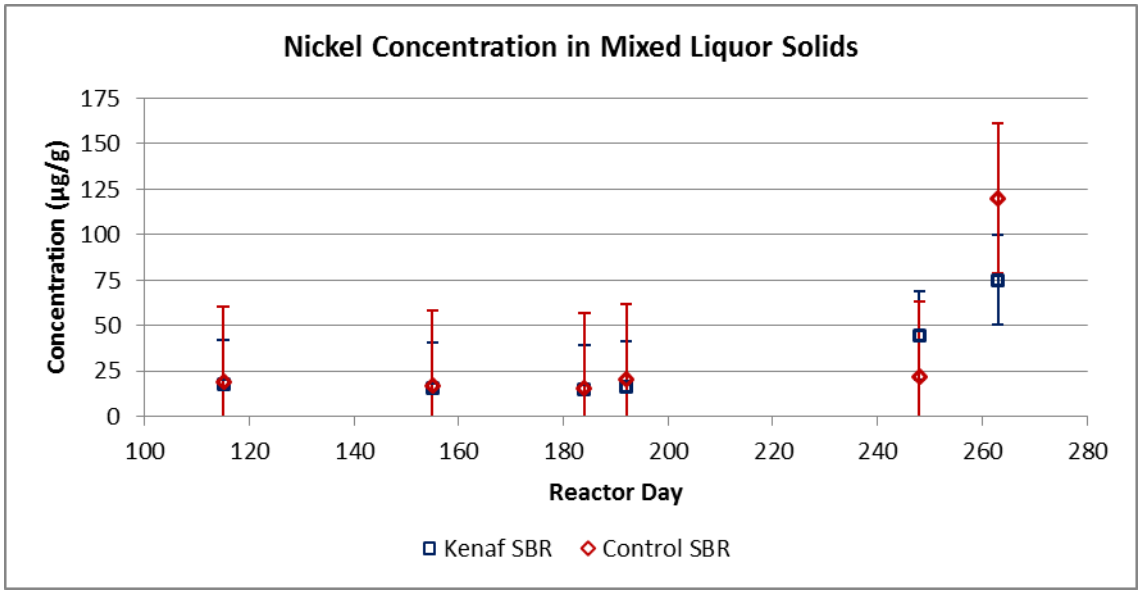


Figure 3.17: Nickel concentrations in dried sludge solids from kenaf supplemented and control SBRs.

Floc size

Kenaf supplemented mixed liquor floc particles, as imaged and analyzed by MetaMorph, tended to be larger and less numerous than those from the control ML. In one pair of ML samples collected early in the treatment phase of this study (12/31/2011, Reactor Day 101) the equivalent radius (MetaMorph output) of the largest particle detected in the nine images of the kenaf ML sample was 210.9 µm, while the maximum size of the control ML particles was 128.4 µm. In Figure 3.18 it can be seen that not only did the control floc have a smaller range of sizes, but a larger fraction of its total equivalent volume (MetaMorph output) was accounted for by the smallest particles. Half of the total volume of floc particles had equivalent radii smaller than approximately 55 µm in the control ML sample and 70% of the volume consisted of particles smaller than 70 µm. However, in the kenaf supplemented floc, half of the volume was particles with equivalent radii smaller than approximately 100 µm and 70% were smaller than 160 µm. MetaMorph detected a total of 8353 floc particles in the images of the control ML sample, but only 6779 in the kenaf ML. Despite having only 81.2% as many particles as control, the sum of equivalent volumes of kenaf ML particles was 157.8% of the control ML sample's measured total equivalent volume. A greater total equivalent volume of immobilized particles does not imply increased settled sludge volume because the larger particles in the kenaf ML will tend to settle better (i.e. into a smaller volume) than the smaller control ML particles can in the same amount of time.

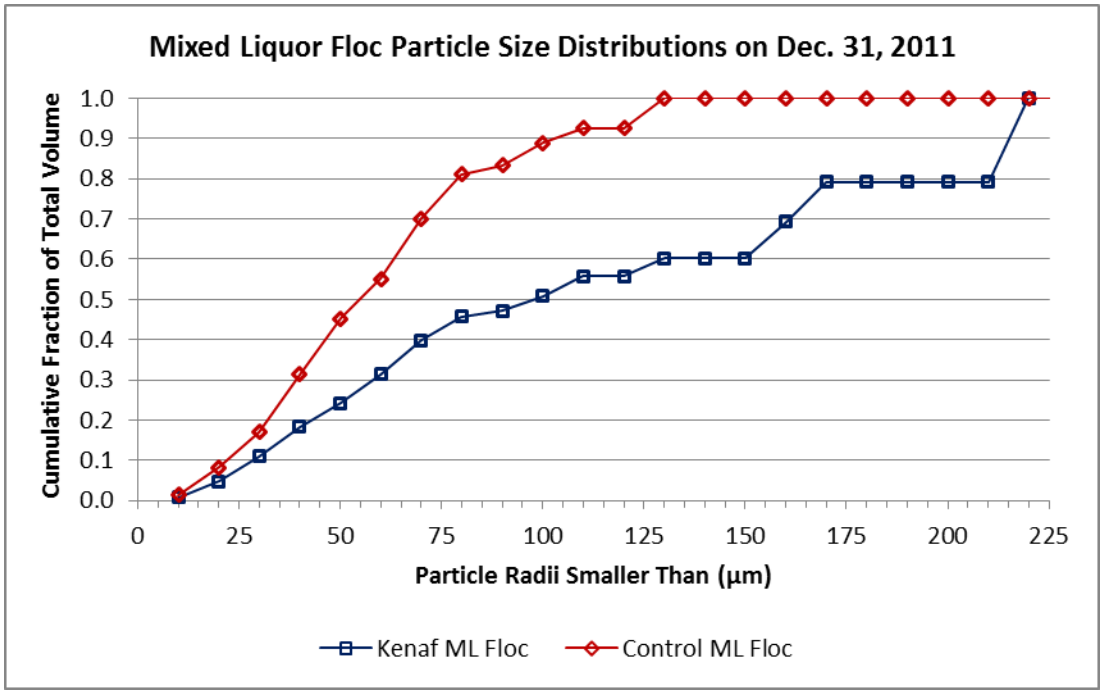


Figure 3.18: Fraction of total volume of particles having equivalent radii smaller than *x* µm on Dec. 31, 2011.

Similar results were obtained from a pair of ML samples collected near the middle of the treatment phase (4/4/2012, Reactor Day 196), as can be seen in Figure 3.19. The largest particle in the control ML sample had an equivalent radius of 108.5 µm. The equivalent radius of the largest floc observed in the kenaf supplemented ML was 168.3 µm. Half of the total equivalent volume of control ML particles had equivalent radii smaller than approximately 48 µm and 70% were smaller than 63 µm. In the kenaf supplemented ML sample half of the floc volume consisted of particles with equivalent radii less than approximately 92 µm while 70% were smaller than 148 µm. MetaMorph detected a total of 13084 floc particles in the control ML sample, but only 4900 in the kenaf ML. Even with only

37.5% as many particles, the kenaf ML had a slightly larger (6%) total equivalent volume than control's.

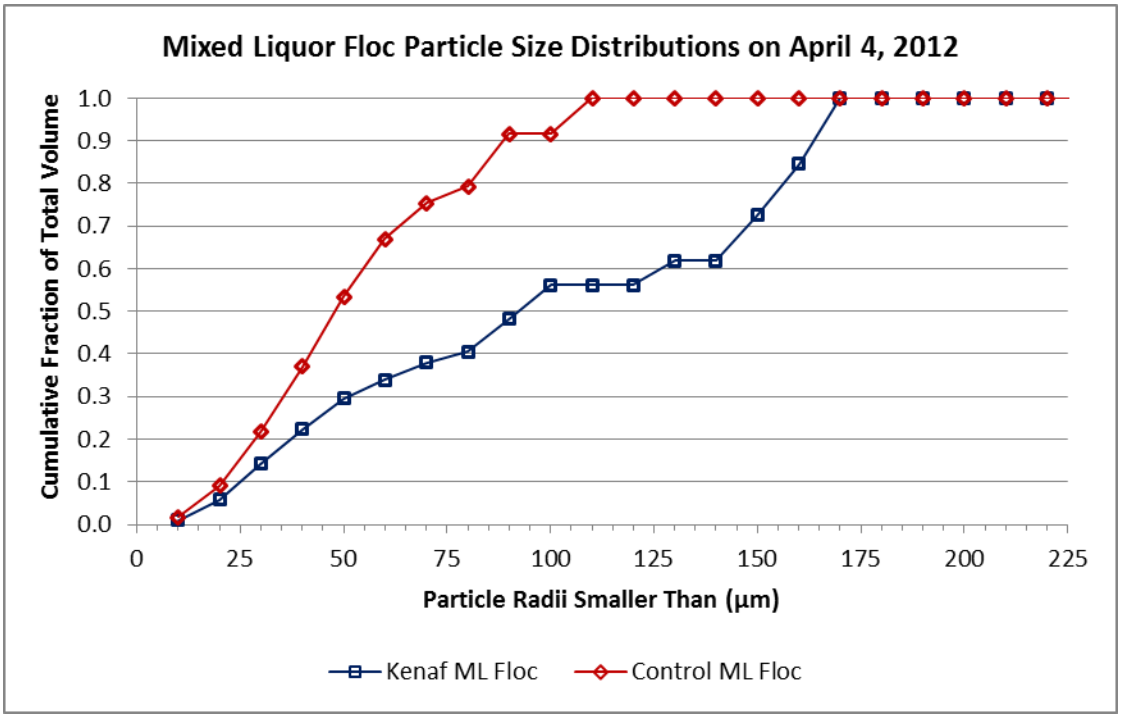


Figure 3.19: Fraction of total volume of particles having equivalent radii smaller than *x* µm on April 4, 2012.

Starting with the same raw particle size data used to generate the PSDs above, as well as that from 8 other pairs of samples, Figure 3.20 summarizes the distributions of particle sizes as their volume-moment means or D_{43}s. For this plot, the Integrated Morphometry Analysis results from the 3 images from each petri dish were aggregated such that each dish was treated as a replicate sample.

The mean D_{43}s of the 3 petri dishes from each ML sample are shown in Figure 20 with error bars at ± one standard deviation. It is evident from Figure 3.20 that the kenaf floc tended to be larger, but also more variable in size than the control floc. A t Test comparing the 10 mean D_{43}s from each reactor rejected the null hypothesis at the alpha = 0.01 confidence level which provides evidence that there is a significant difference between the reactors in the distributions of floc sizes. This difference in floc size distributions could provide an explanatory mechanism for the observed improvements in the kenaf treated SBR's Sludge Volume Index. It is also worth noting that the actual difference between the distributions is underestimated in this analysis in that this method was unable to image and analyze the largest kenaf particles.

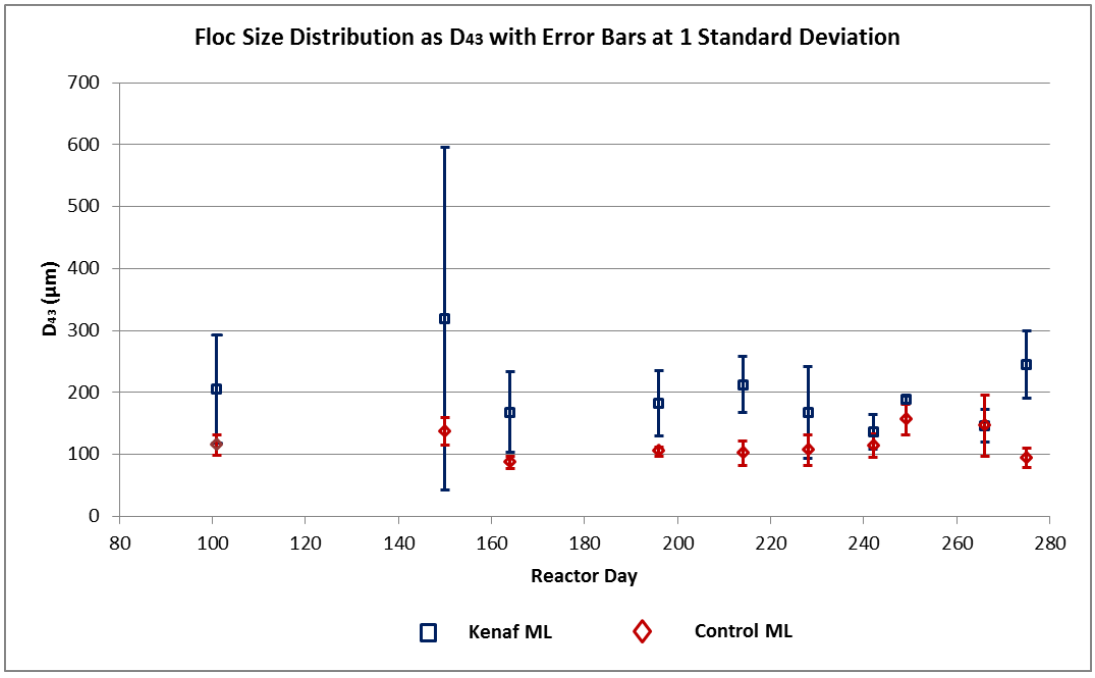


Figure 3.20: Mean D_{43}s for kenaf supplemented and control floc with error bars at ± one standard deviation.

Chemical Oxygen Demand

The control and kenaf supplemented reactors' effluent concentrations of sCOD and tCOD did not diverge significantly in the course of this study, as illustrated by Figure 3.21. Summary statistics are given in Table 3.5, including results of paired sample 2-tailed t Tests comparing the reactors' performances. Note that sCOD and tCOD values were not significantly different (alpha = 0.05) between the SBRs in the Pre-Treatment phase, nor were they in the Treatment phase.

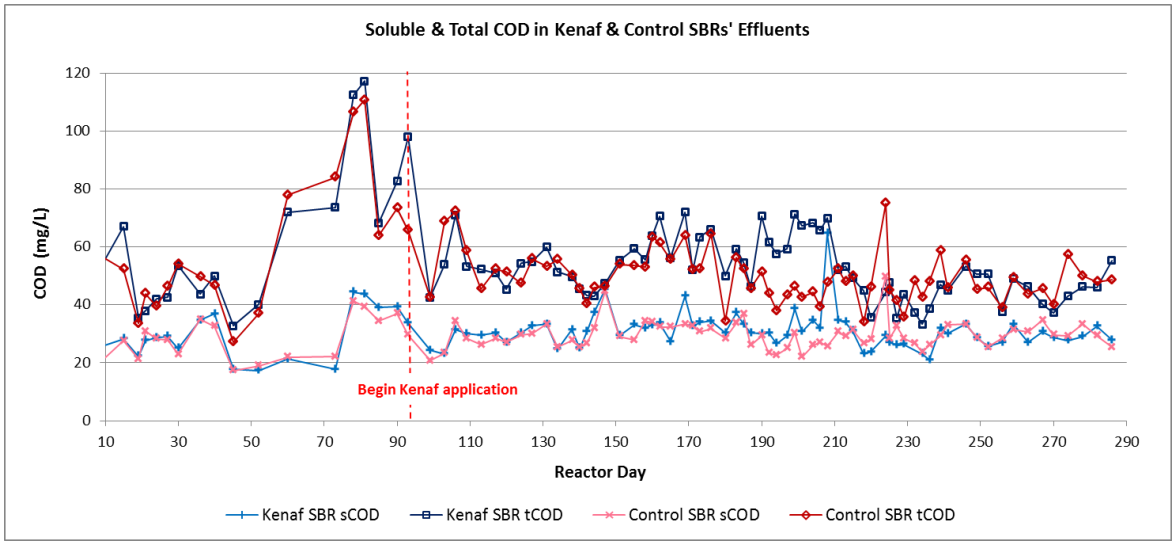


Figure 3.21: Soluble (filtered) and Total (unfiltered) COD concentrations in Kenaf and Control SBRs' effluents.

Table 3.5: Summary of COD statistics

	Pre-Treatment		Treatment	
Kenaf sCOD				
Mean	29.6	mg/L	30.9	mg/L
S.D.	8.7	mg/L	6.2	mg/L
Control sCOD				
Mean	28.3	mg/L	29.6	mg/L
S.D.	7.3	mg/L	4.7	mg/L
n	18		64	
T.TEST(K<=>C)	0.0585		0.104	
Kenaf tCOD				
Mean	62.4	mg/L	52.3	mg/L
S.D.	26.2	mg/L	10.0	mg/L
Control tCOD				
Mean	59.4	mg/L	49.7	mg/L
S.D.	23.5	mg/L	8.4	mg/L
n	18		65	
T.TEST(K<=>C)	0.200		0.050	

In Figure 3.22 the sCOD concentrations of the SBRs are plotted with the influent wastewater's sCOD to look for any apparent interactions between each system's input and its output as a possible source of some of the variability seen in output values. Similarly, in Figure 3.23 effluent tCOD values are shown with influent tCOD. Any effect that influent strength had on effluent strength was not apparent from these graphs. Table 3.6 lists Pearson Product Moment Correlation Coefficients (r) comparing each effluent to the influent strength. In the Pre-Treatment phase there was not a significant correlation between influent strength and either effluent's sCOD or tCOD. The kenaf and

control reactors' effluent sCOD or tCOD values were found to be significantly correlated (alpha = 0.01) to each other, as would be expected. In the Treatment phase the control effluent sCOD and tCOD continued to not correlate significantly with the influent strength. However, there was an unexpected significant (alpha = 0.05) correlation between influent strength and the kenaf SBR's sCOD and tCOD.

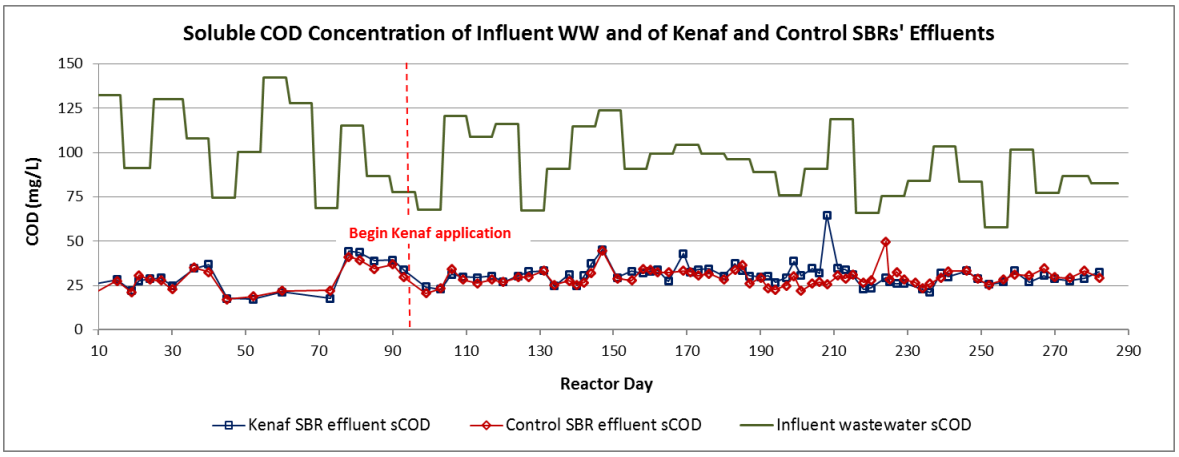


Figure 3.22: Influent and effluent sCOD

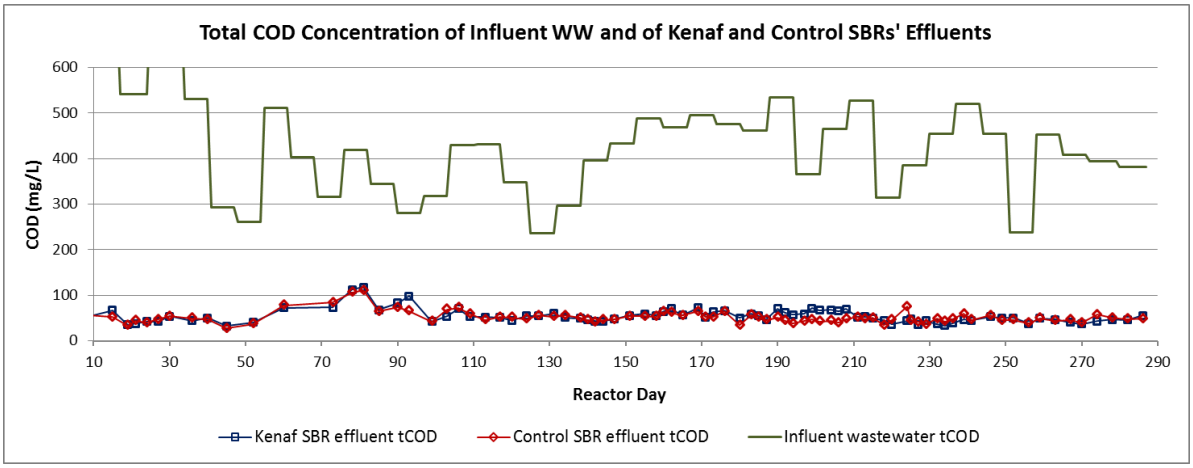


Figure 3.23: Influent and effluent tCOD.

Table 3.6: Linear correlation coefficients

	Pre-Treatment		Treatment	
sCOD				
r(WW<=>K)	0.0544		0.293	*
r(WW<=>C)	-0.0186		0.213	
r(K<=>C)	0.9593	**	0.303	*
n	18		64	
*> r_{crit} 0.05	0.468		0.246	
**> r_{crit} 0.01	0.590		0.320	
tCOD				
r(WW<=>K)	-0.308		0.278	*
r(WW<=>C)	-0.222		0.089	
r(K<=>C)	0.930	**	0.366	**
n	18		64	
*> r_{crit} 0.05	0.468		0.246	
**> r_{crit} 0.01	0.590		0.320	

In Figure 3.24 the data used to generate Figures 3.22 and 3.23 was plotted as percent removal of COD by each reactor. Percent removal of sCOD and tCOD was not found to differ significantly between the reactors during either the Pre-Treatment or Treatment phases of this study. In the Pre-Treatment phase both reactors had mean percent removal values near 71% for sCOD and 85% for tCOD. In the Treatment phase, both SBRs removed approximately 66% of sCOD and 87% of tCOD. Subsequent testing of aliquots of each ML, which had been subjected to extended saturation aeration, suggested that the fraction of sCOD or tCOD removed by the reactors was approximately equal to the biodegradable portion of the influent wastewater.

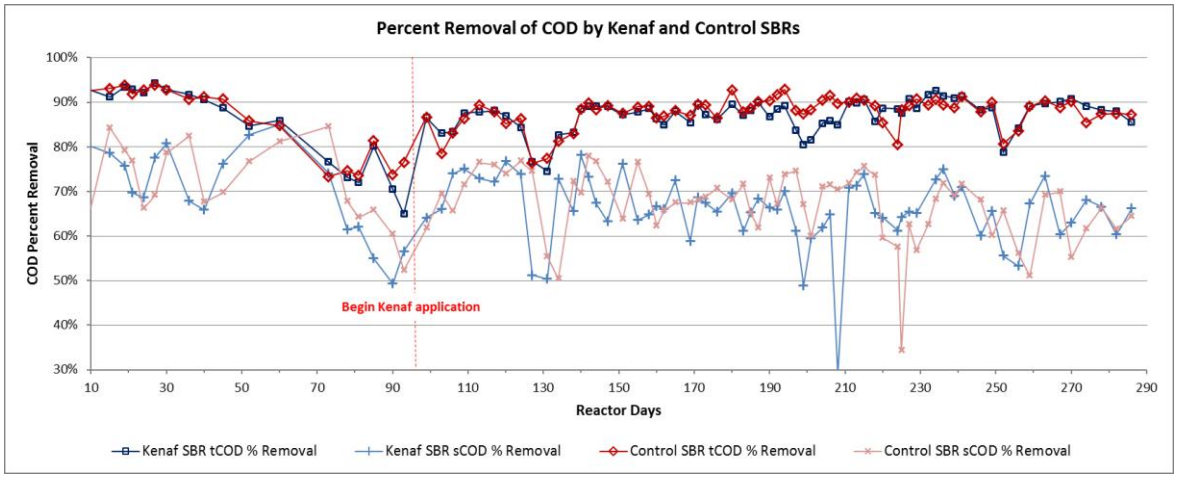


Figure 3.24: Percent removal of sCOD and tCOD by each SBR.

Nitrogen species

Effluent Total Nitrogen (TN) concentrations, both soluble and total, of the reactors are shown in Figure 3.25. Note that in the Pre-Treatment phase, TN concentrations varied considerably over time but were closely matched between the reactors at any given moment. During the Treatment phase there are periods of consistently low TN concentrations in which the SBRs seem to be performing similarly, interspersed with periods of extreme excursions from the typically low TN concentrations during which the control TN concentration increased more than the kenaf treated reactor's did. Summary statistics in Table 3.7 suggest that there was not a significant difference (paired sample 2-tailed t Test, alpha = 0.01) between the SBRs' performances in the Pre-Treatment phase. However the mean TN concentration of the control SBR's effluent in the Treatment phase was approximately 2 mg/L higher than the kenaf treated SBR's, which the two tailed t Test found to be a significant difference at the alpha = 0.01 confidence level.

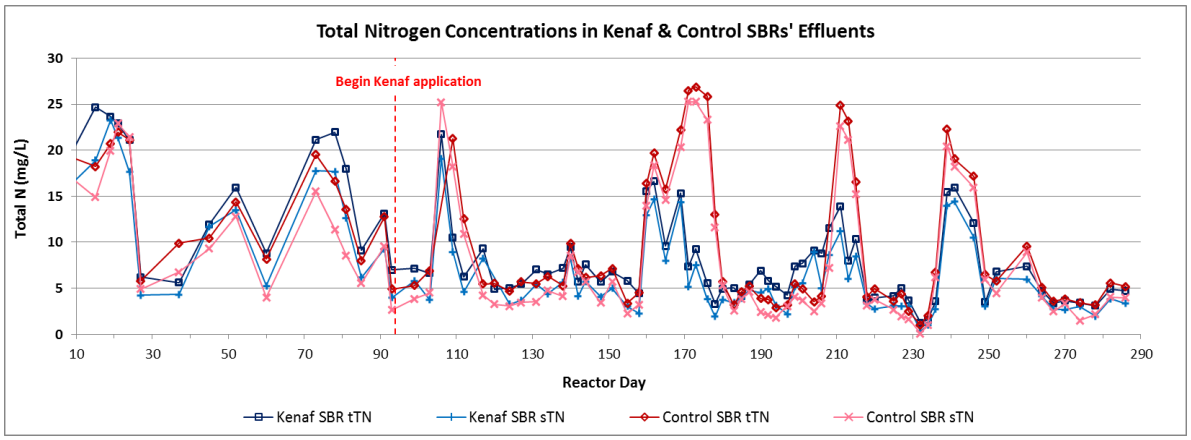


Figure 3.25: Effluent Total Nitrogen concentrations.

Table 3.7: Effluent Total Nitrogen summary statistics

	Pre-Treatment		Treatment	
Kenaf sTN				
Mean	12.8	mg/L	5.7	mg/L
S.D.	6.5	mg/L	3.8	mg/L
Control sTN				
Mean	11.7	mg/L	7.9	mg/L
S.D.	6.4	mg/L	7.3	mg/L
n	16		63	
T.TEST(K<=>C)	0.1307		**0.0021	
Kenaf tTN				
Mean	15.7	mg/L	6.9	mg/L
S.D.	6.8	mg/L	3.6	mg/L
Control tTN				
Mean	14.1	mg/L	8.9	mg/L
S.D.	5.7	mg/L	7.3	mg/L
n	16		62	
T.TEST(K<=>C)	*0.0193		**0.0056	

Plotting the percent of Total Nitrogen removed by the reactors (Figure 3.26) accounts for the variability of the influent concentration, removing it as a possible source of the variability of the system output. Removal rates between 85% and 95% were typical in the Treatment phase. However, the periods of decreased performance are still evident in Figure 3.26 and therefore apparently not the result of changes in the influent concentration. Total Nitrogen percent removal performance in the Treatment and Pre-Treatment phases is summarized in Table 3.8.

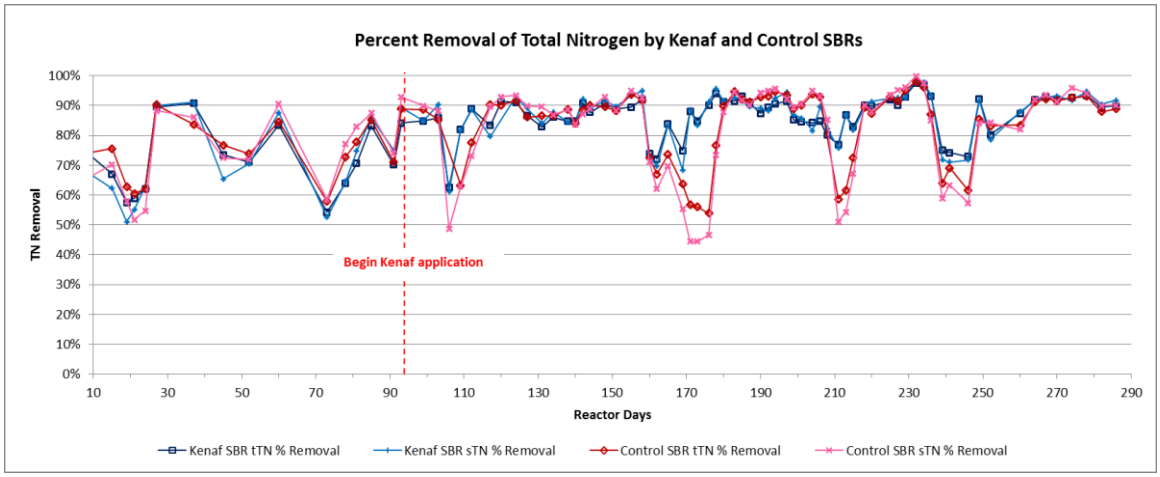


Figure 3.26: Percent removal of Total Nitrogen.

Table 3.8: Percent removal of Total Nitrogen

	Pre-Treatment	Treatment
Kenaf sTN		
Mean	71.5%	87.0%
S.D.	13.8%	7.8%
Control sTN		
Mean	73.9%	82.3%
S.D.	13.4%	15.5%
n	16	63
T.TEST(K<=>C)	*0.1048	**0.0029
Kenaf tTN		
Mean	72.1%	87.3%
S.D.	11.4%	5.9%
Control tTN		
Mean	74.9%	83.9%
S.D.	10.2%	11.9%
n	16	62
T.TEST(K<=>C)	*0.01125	**0.00684

The Aeration Ratio (AR) was developed as an operational parameter to aid in standardizing the aeration intensity to the varying influent strength, but it can also help explain the variability in nitrogen removal performance, as illustrated by Figure 3.27 in which the sTN removal performance can be observed to de-optimize whenever the AR drops below a value of approximately 11. The Aeration Ratio is the mass of oxygen supplied to the mixed liquor by the aeration pump divided by the mass of oxygen utilized by the reactor (i.e. influent tCOD load – effluent tCOD load, or tCOD exerted), and therefore can be considered a unitless value. AR is a function of both the wastewater feedstock's tCOD and of the airflow in liters per minute during the aerobic portions of the treatment cycle.

$$Aeration\ Ratio = \frac{mg\ O_2\ Supplied}{mg\ O_2\ Utilized}$$

$$mg\ O_2\ Supplied = \frac{L\ air}{min} * \frac{75\ min}{cycle} * \frac{0.2095\ L\ O_2}{L\ air} * \frac{1\ atm}{0.08206 \frac{L\ atm}{K\ mol} * 296K} * \frac{31998.8\ mg\ O_2}{mol\ O_2} = \frac{mg\ O_2}{cycle}$$

$$mg\ O_2\ Utilized = \left(\frac{mg\ tCOD_{influent}}{L\ WW} - \frac{mg\ tCOD_{effluent}}{L\ WW} \right) * \frac{1.5\ L\ WW}{cycle} = \frac{mg\ COD_{exerted}}{cycle}$$

The AR does not attempt to estimate the amount of oxygen that transfers from the aeration bubbles to the bulk liquid. The typical ambient temperature of the lab (23°C) was used for all AR calculations. Also, the air is assumed to be at 1.0 atm even though the pressure in the bubbles must have been higher. The optimum range of values for the AR is likely to be very sensitive to factors such as reactor geometry, and it is therefore not meant to be directly generalizable to other plants.

The AR was very useful for operating the reactors, but this ratio can also provide a way to quantify the degree of departure from optimal growth conditions in the SBRs in order to evaluate the impact those departures have on plant performance. Unfortunately, the airflow regulators used for this study demonstrated a tendency to wander from their intended settings. The airflow values recorded were those observed after the reactors had run unattended for several cycles. Because of the instability of the regulators, the SBRs' airflow readings did not always match each other. A 3 day running average of AR values reduced the effect of brief excursions from the intended airflow.

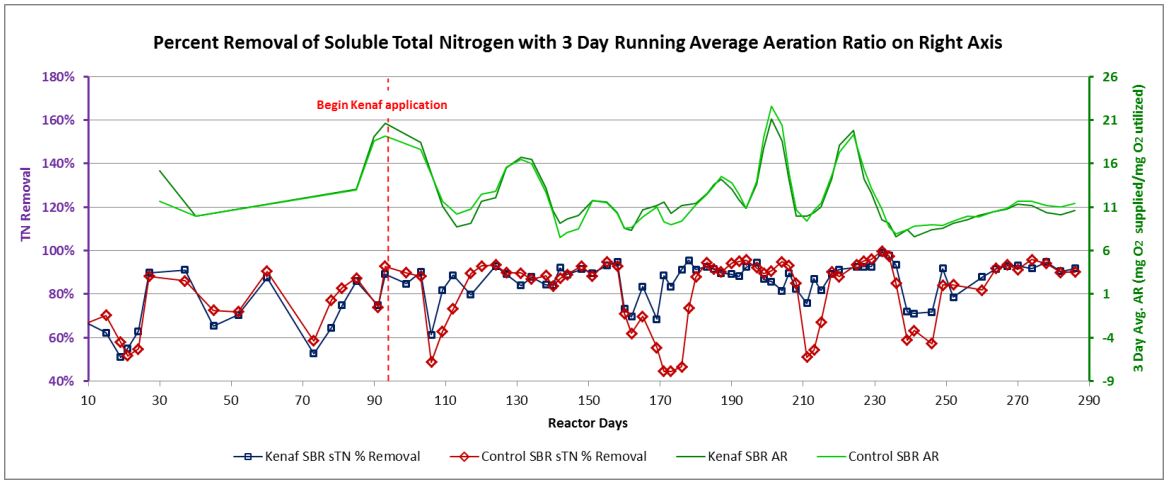


Figure 3.27: Percent removal of TN with 3 day running average AR plotted on right axis.

In order to compare the behavior of the SBRs under optimal and non-optimal conditions, two blocks of data were extracted from the main dataset. One block consisted of AR values and TN concentrations from Treatment phase days on which both reactors were observed to have ARs greater than 11. In the other block, both systems had ARs of less than 11. In the high AR block, the mean AR for both SBRs was 14.3, the mean sTN values were 4.8 mg/L for kenaf and 4.9 mg/L for control and mean tTN concentrations were 6.3 mg/L for kenaf and 6.2 mg/L for control. Both systems removed approximately 88% of sTN and tTN. No significant differences between the plants were detected in this block. By contrast, in the low AR block both reactors had mean ARs of 9.4. The kenaf supplemented SBR's effluent had an average of 7.0 mg sTN/L and 8.5 mg tTN/L, but the control's effluent averaged 10.5 mg sTN/L and 11.8 mg tTN/L, a significant difference at the alpha = 0.01 confidence level. Removal performance was also significantly different (alpha = 0.01) in this block, with control removing approximately 78% of sTN and tTN, while the kenaf SBR was able to remove an average of 85% of sTN and tTN. This suggests that when aeration is below the optimal intensity, as might happen in a full scale plant when a bolus of unusually high COD waste enters the treatment train, nitrogen removal performance of the kenaf supplemented mixed liquor is not as adversely affected as that of untreated ML.

Ammonia-nitrogen was generally the largest component of the Total Nitrogen concentration, as can be seen by comparing the ammonia-nitrogen concentrations in Figure 3.28 to the TN concentrations in Figure 3.25. The influent wastewater's NH_3-N concentration, which was quite variable, is also plotted in Figure 3.28. Note that the filtered and unfiltered effluent NH_3-N concentrations from either reactor were so similar that their plot lines were frequently indistinguishable from each other, suggesting that sNH_3-N and tNH_3-N results could be treated as though they were duplicate samples,

though this analysis maintained soluble and total concentrations as distinct data sets. For clarity, only the results from filtered samples are shown in Figure 3.28. As detailed in Table 3.9, during the Pre-Treatment stage mean sNH_3-N concentrations were 3.8 mg/L for the kenaf reactor and 4.7 mg/L for control which was not a significant difference at the alpha = 0.05 confidence level. Mean tNH_3-N was 4.0 mg/L for the kenaf SBR and 5.5 mg/L for control which was not a significant difference at the alpha = 0.01 level. In the Treatment stage mean sNH_3-N for the kenaf reactor was 2.7 mg/L while control's was significantly different (alpha = 0.01) at 5.3 mg/L. Mean tNH_3-N values were 3.0 mg/L for the kenaf supplemented SBR and 5.8 mg/L for the control, which is a significant difference at the alpha = 0.01 confidence level. Much like Total Nitrogen, the Treatment phase was marked by periods of very low effluent ammonia-nitrogen in both reactors alternating with periods of instability in which the control's effluent NH_3-N peaked at a much higher value than the kenaf supplemented SBR's effluent concentration.

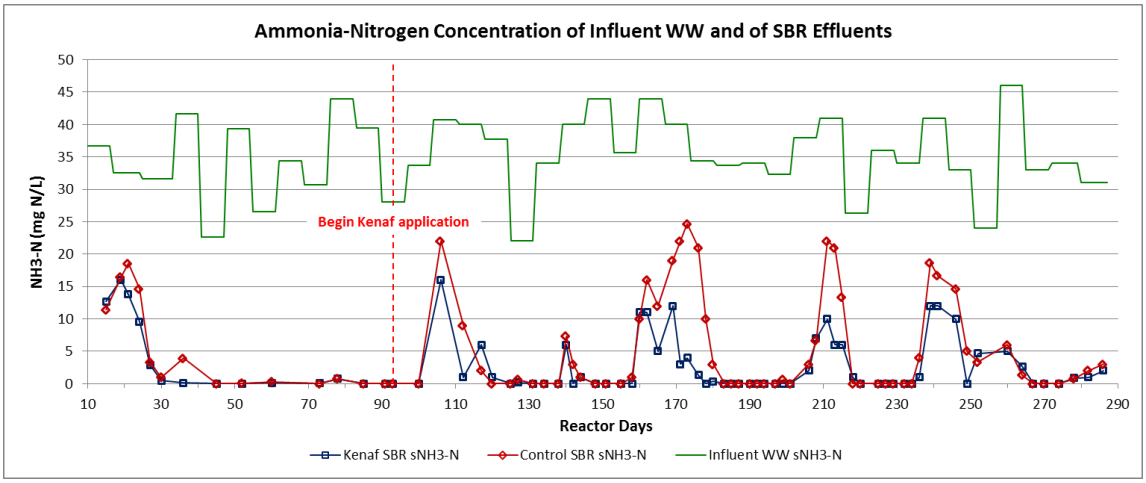


Figure 3.28: Ammonia-Nitrogen concentrations of influent wastewater and of SBRs' effluents.

Table 3.9: Effluent ammonia-nitrogen concentrations

	Pre-Treatment		Treatment	
Kenaf sNH3-N				
Mean	3.8	mg/L	2.7	mg/L
S.D.	5.9	mg/L	4.1	mg/L
Control sNH3-N				
Mean	4.7	mg/L	5.3	mg/L
S.D.	6.9	mg/L	7.6	mg/L
n	15		61	
T.TEST(K<=>C)	0.0817		0.0002	
Kenaf tNH3-N				
Mean	4.0	mg/L	3.0	mg/L
S.D.	6.3	mg/L	4.3	mg/L
Control tNH3-N				
Mean	5.5	mg/L	5.8	mg/L
S.D.	7.9	mg/L	7.6	mg/L
n	15		66	
T.TEST(K<=>C)	0.0286		2.22E-05	

Converting the data from Figure 3.28 to ammonia-nitrogen removal rates removes the influent variability as a source of the peaks in effluent concentration. However, the periods of poor performance were still evident as can be seen in Figure 3.29. During the Pre-Treatment stage the kenaf reactor removed 89% of sNH_3-N, and control removed 86%. The control SBR removed 86% of tNH_3-N while the reactor designated for subsequent kenaf treatment removed 90%. Neither difference was significant at the alpha = 0.01 confidence level according to a paired sample 2-tailed t Test. In the Treatment phase the kenaf supplemented SBR removed 93% of sNH_3-N while the

control continued to remove 86%. The kenaf reactor removed 92% of tNH_3-N to control's 85%. As shown in Table 3.10, the differences between the reactors' NH_3-N removal performances during treatment were significant at the alpha = 0.01 confidence level.

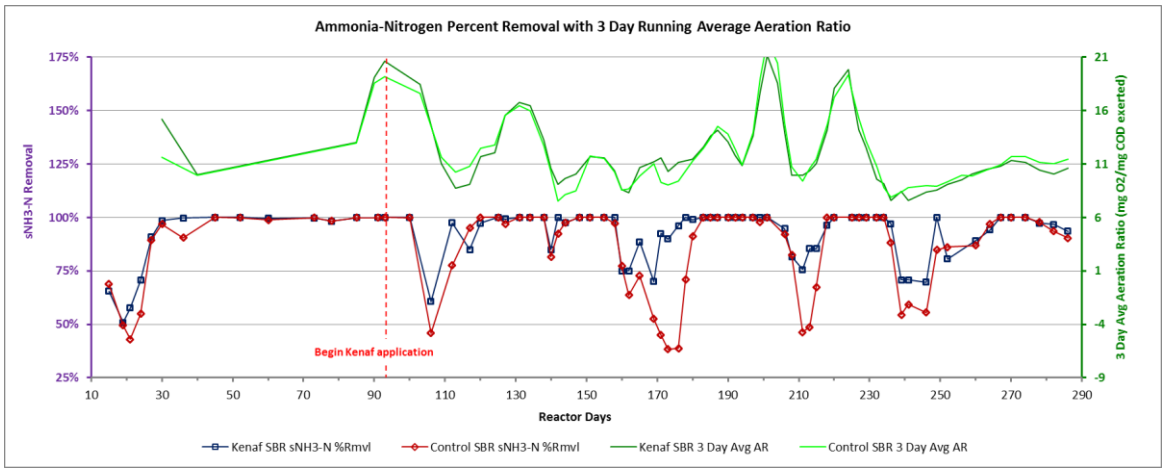


Figure 3.29: Percent of ammonia-nitrogen removed by kenaf and control SBRs with 3 day running average AR.

Table 3.10: Percent of ammonia-nitrogen removed by kenaf and control SBRs

	Pre-Treatment	Treatment
Kenaf %Rmvl sNH3-N		
Mean	88.7%	93.2%
S.D.	17.8%	10.3%
Control %Rmvl sNH3-N		
Mean	86.0%	86.3%
S.D.	20.8%	19.3%
n	15	61
T.TEST(K<=>C)	0.0809	0.0002
Kenaf %Rmvl tNH3-N		
Mean	90.1%	92.2%
S.D.	15.7%	10.9%
Control %Rmvl tNH3-N		
Mean	86.2%	84.6%
S.D.	19.4%	19.6%
n	15	66
T.TEST(K<=>C)	0.0260	2.02E-05

As with Total Nitrogen, it is possible to compare the reactors' Treatment phase performances under optimal and non-optimal aeration conditions by extracting 2 subsets of data, one with both SBRs having AR values greater than 11 and one with both ARs below 11. In the high AR block the kenaf and control reactors had mean ARs of 13.9 and 14.2 respectively, which was not a significant difference at the alpha = 0.01 confidence level. The kenaf SBR removed 98% of NH_3-N leaving an average of 0.87 mg/L sNH_3-N in the effluent. The control removed 96% of NH_3-N leaving 1.53 mg/L sNH_3-N. There were no significant differences in the systems' performances in the high AR block. In the low AR block (mean AR = 9.4) the kenaf treated SBR was able to remove an average of 88% of

sNH_3-N leaving 4.8 mg/L sNH3-N in its effluent. On the other hand, the control reactor removed only 78% of sNH_3-N leaving mean effluent concentrations of 8.6 mg/L sNH_3-N. The differences between the systems' performances in the low AR block were significant at the 0.01 confidence level. This suggests that, as with Total Nitrogen, when aeration is below the optimal intensity, ammonia-nitrogen removal performance of the kenaf supplemented mixed liquor is not as adversely affected as that of untreated ML.

Effluent nitrite-nitrogen and nitrate-nitrogen concentrations are plotted in Figure 3.30 along with the 3 day running average Aeration Ratio. Nitrate was only detected in significant amounts during the startup Pre-Treatment stage. However, small amounts of NO_2-N (less than 5 mg N/L) were common in the effluent whenever the AR was above a value of approximately 10. Nitrite-nitrogen concentrations were strongly positively correlated (alpha= 0.01) with the 3 day running average AR. The mean nitrite-nitrogen concentrations in the kenaf and control SBRs in the Pre-Treatment phase were 2.0 mg/L and 1.7 mg/L respectively, which was not a significant difference at the alpha = 0.05 confidence level according to the paired sample 2-tailed t Test. During the Treatment phase, the kenaf treated reactor's mean NO_2-N was 1.6 mg/L to control's 1.1 mg/L. This difference was significant at the alpha = 0.01 confidence level. Because the reactors ran a nit-denit treatment cycle it is unclear whether this result represents more efficient oxidation of ammonia to nitrite or less effective de-nitrification by the kenaf supplemented mixed liquor. Removal rates for nitrite-nitrogen and nitrate-nitrogen are not meaningful because the influent wastewater generally did not contain either species.

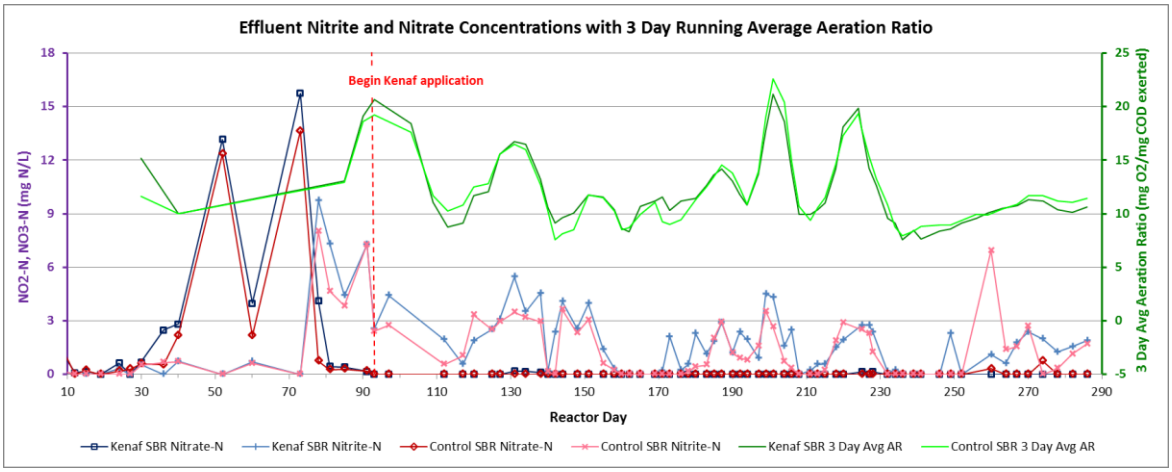


Figure 3.30: Effluent nitrite-nitrogen and nitrate-nitrogen concentrations with 3 day running average Aeration Ratio on right axis.

Total Phosphate

The percent of Total Phosphate removed by each reactor is plotted in Figure 3.31 along with the 3 day running average Aeration Ratio. Optimum phosphate removal appears to occur when the AR is between 8 and 10. Too much aeration led to reduced phosphate removal, probably because residual dissolved oxygen persisted for too long into what would otherwise be the anaerobic selector phase. Too little aeration failed to provide aerobic conditions for growth of Phosphate Accumulating Organisms, which also caused a decrease in phosphate removal. No significant differences in the reactors' performances were detected in either the Pre-Treatment or the Treatment phases.

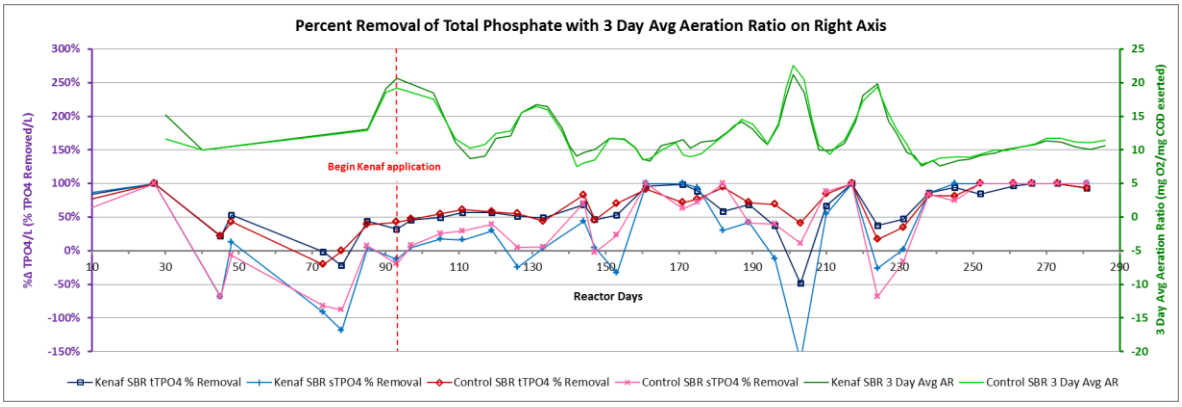


Figure 3.31: Percent of Total Phosphate removed and 3 day running average Aeration Ratio.

Intra-Cycle Samples

Intensive sampling within a single treatment cycle of each reactor's mixed liquor made it possible to track internal changes in the concentration of soluble COD and soluble nitrogen species. Only soluble species were measured because results from unfiltered ML samples would not have been comparable to those generated by this study from unfiltered effluent. A subsequent cycle at the same time the next day was similarly sampled and yielded results nearly identical to those presented here.

Figure 3.32 shows the soluble COD concentrations within the reactors through one 4 hour cycle. Note that the resolution of the sCOD test used was ±2 mg/L, which is not ideal for discerning changes within a range of just over 10 mg/L. A paired sample 2-tailed t Test found no significant differences between the reactors' sCOD concentrations.

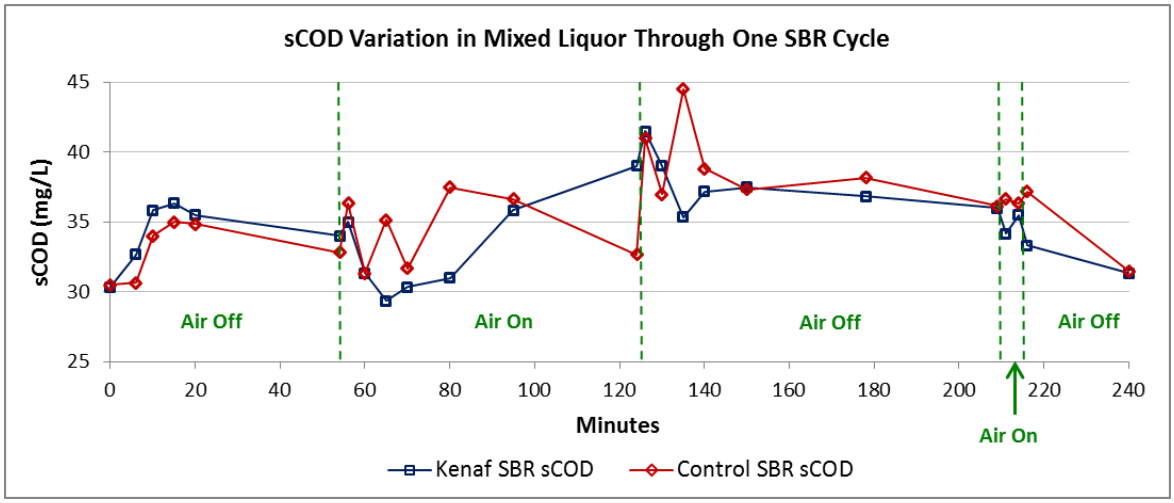


Figure 3.32: Soluble COD in ML through one SBR treatment cycle.

Variations in the concentration of soluble Total Nitrogen through one cycle are plotted in Figure 3.33. A paired sample 2-tailed t Test found that the differences in reactor sTN concentration were significant. However, the magnitude of those differences was less than 2 mg/L. The reported resolution of the Hach TN test is ±0.5 mg/L.

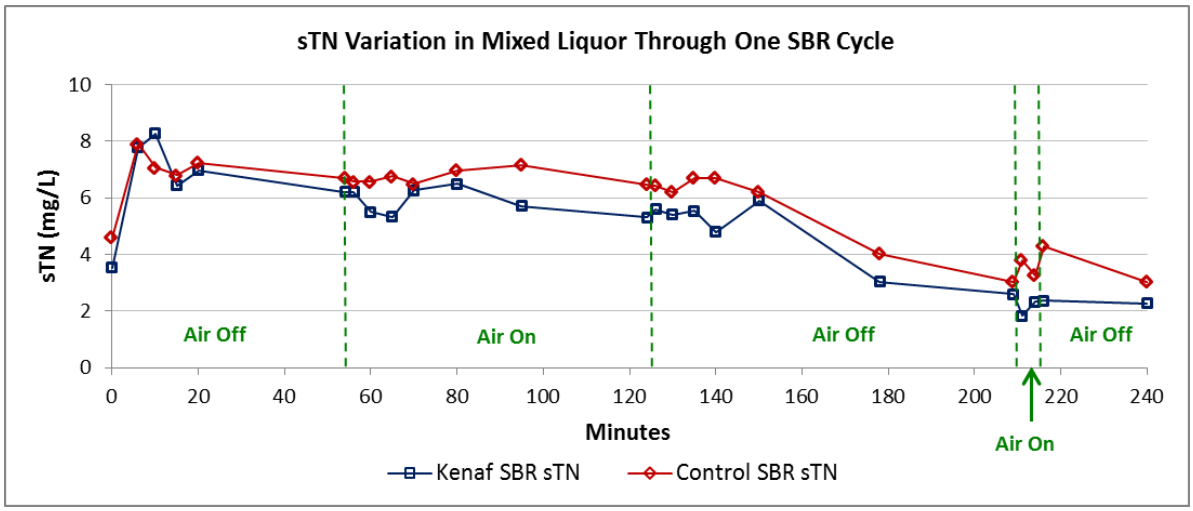


Figure 3.33: Soluble Total Nitrogen in ML through one SBR treatment cycle.

Figure 3.34 shows the ammonia-nitrogen concentrations in the SBRs over one cycle. The sNH3-N concentration in the control reactor was consistently approximately 1 mg/L higher than the kenaf SBR's, a difference which a paired sample 2-tailed t Test found to be significant at the alpha = 0.01 confidence level. However, the reported sensitivity of this test is only ±5 mg/L, bringing into

question the significance of this result. It is worth noting that measurements of standard solutions in our lab were generally within 1 mg/L of the analytically derived concentration.

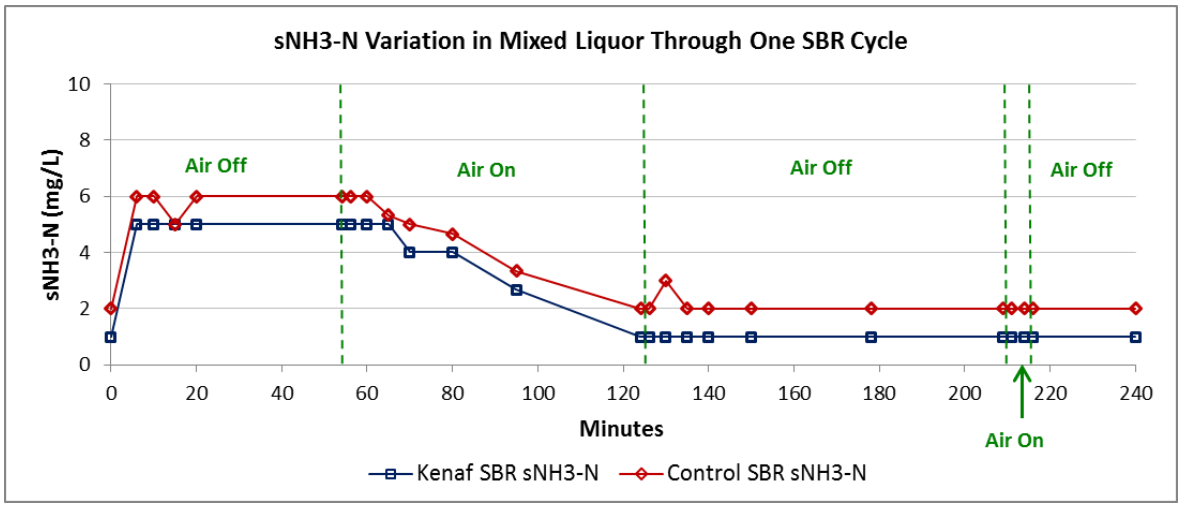


Figure 3.34: Soluble ammonia-nitrogen in ML through one SBR treatment cycle.

As illustrated by Figure 3.35, nitrite-nitrogen concentrations in the reactors were very closely matched, with no significant differences detected.

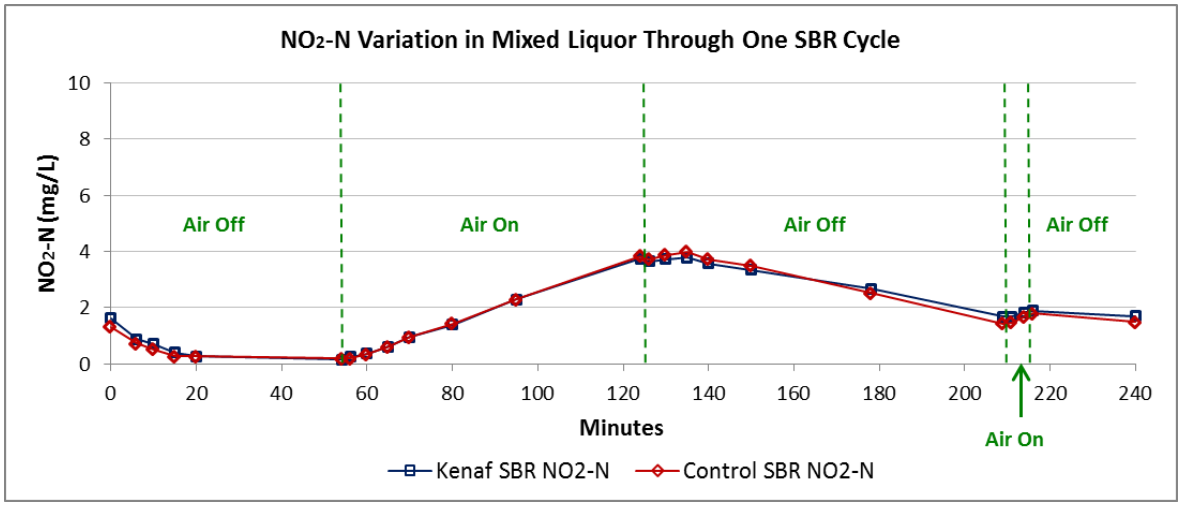


Figure 3.35: Nitrite-nitrogen in ML through one SBR treatment cycle.

Figure 3.36 shows a significant difference (alpha = 0.01) between the reactors in their nitrate-nitrogen concentrations, with the kenaf supplemented SBR's NO_3-N peaking at a level over twice as high as control's highest concentration. It can also be observed that nitrate begins to appear about 10 minutes earlier in the cycle in the kenaf ML than in the control ML. Though the magnitude of the difference in their peak values is only 0.4 mg N/L, in the 10 L tanks that becomes 4 additional mg of nitrogen converted to nitrate by the kenaf mixed liquor than was converted by the control ML.

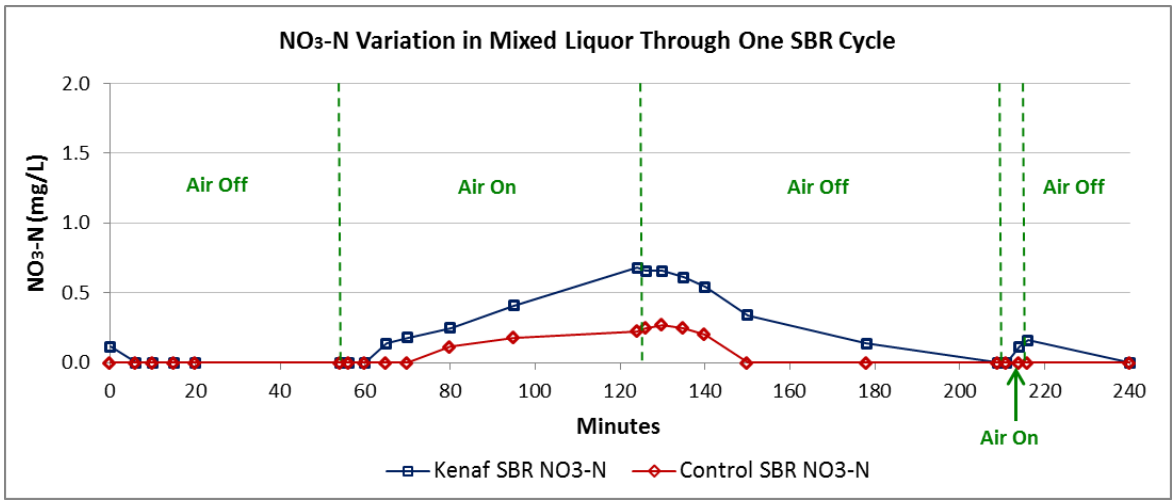


Figure 3.36: Nitrate-nitrogen in ML through one SBR treatment cycle.

One model that could explain the intra-cycle sampling results of reduced ammonia concentration, similar nitrite and increased nitrate concentration in the kenaf supplemented ML would be for the activity of both nitrifying functional groups, the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB), to be enhanced to a similar degree such that there is no net change to the nitrite concentration curve. In other words, ammonia is being converted to nitrite slightly faster, but nitrite is also being converted to nitrate slightly faster, leaving the concentration of the intermediate species in the kenaf reactor almost the same as in the control. Taken together, Figures 3.33 through 3.36 provide evidence that the introduction of kenaf powder to mixed liquor can improve nitrification performance.

Batch reactor tests

Batch testing of ammonia utilization rates revealed that both 500 mL aliquots of mixed liquor converted NH_3-N to other forms at about the same rate ($-7.6X10^{-5}$ mg N/(mg VSS*min)) when saturated with air, as shown in Figure 3.37. A paired sample 2-tailed t Test found no significant difference (alpha = 0.05) between the treatment and control distributions of ammonia-nitrogen concentrations.

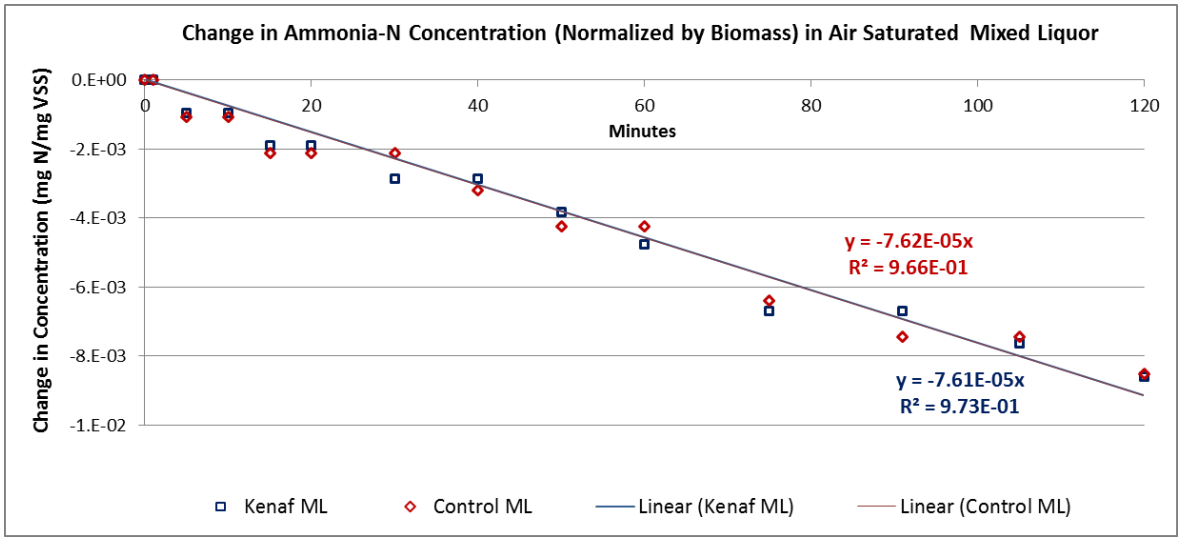


Figure 3.37: Ammonia-nitrogen concentration normalized by biomass.

However, Figure 3.38 indicates that the nitrite concentration increased a bit more quickly in the control ML than in the treatment ML. The nitrite-nitrogen concentration increased at a normalized rate of $6.5X10^{-5}$ mg N/(mg VSS*min) in the control ML, while in the kenaf ML the rate was just $5.8X10^{-5}$ mg N/(mg VSS*min). A paired sample 2-tailed t Test found this difference to be significant at the alpha = 0.01 confidence level. If nitrite production is proportional to ammonia consumption (which was the same for both samples), then the difference in nitrite concentration implies that more nitrite was converted to other forms in the kenaf ML than in the control ML.

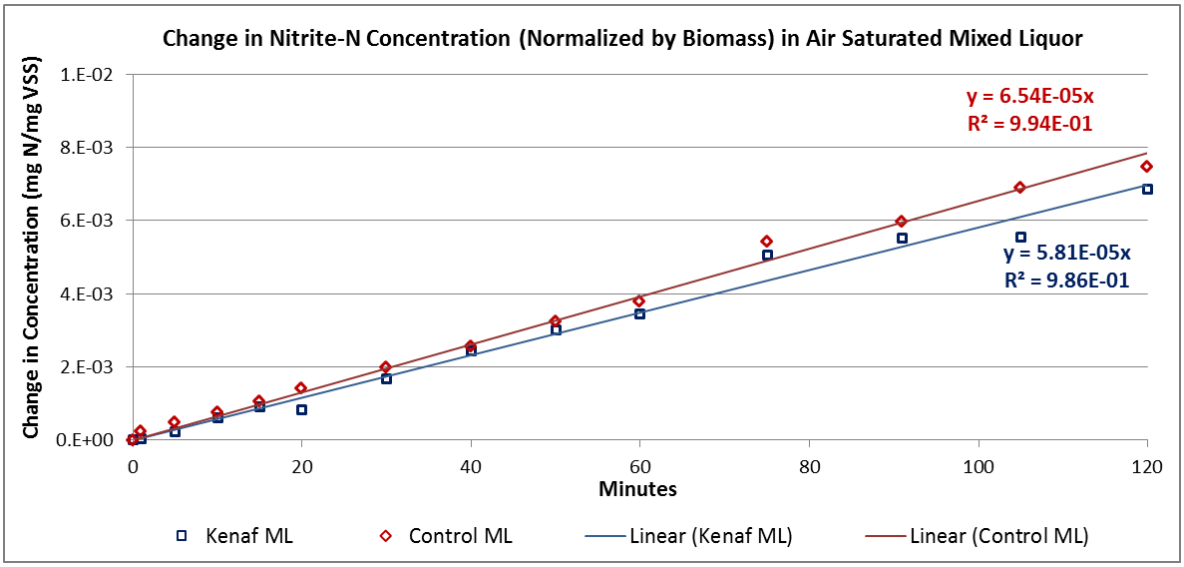


Figure 3.38: Nitrite-nitrogen concentration normalized by biomass.

In Figure 3.39 it can be seen that there was less nitrite in the kenaf supplemented ML at least in part because more of the nitrite had been converted to nitrate in the kenaf ML than in the control. Nitrate-nitrogen concentration increased at a rate of $8.6X10^{-6}$ mg N/(mg VSS*min) in the kenaf supplemented ML, but only $3.3X10^{-6}$ mg N/(mg VSS*min) in the control aliquot. This suggests that the Nitrite Oxidizing Bacteria (NOB) benefit in some way from kenaf addition, which could lead to more complete nitrification at the full scale WWTP.

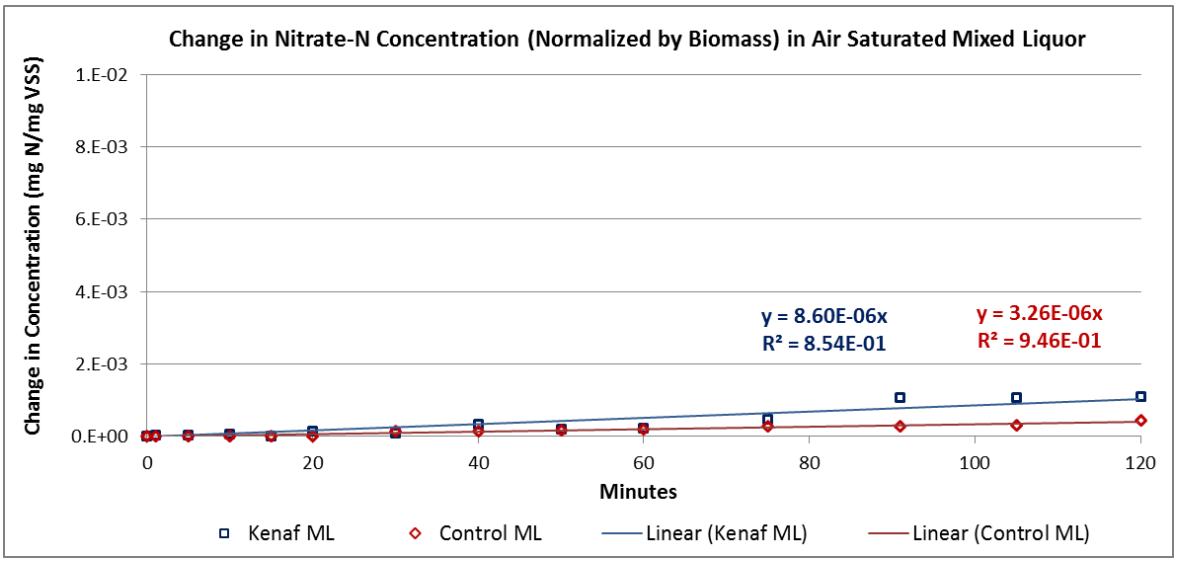


Figure 3.39: Nitrate-nitrogen normalized by biomass.

Conclusions

The treatment reactor's 30% decrease in average SVI during this study provided evidence of a substantial improvement in sludge settling characteristics due to kenaf addition. Floc particles were found to be larger and less numerous in the kenaf treated ML, possibly explaining the improvements in SVI. Mixed liquor suspended solids increased slightly (~50 mg/L), most of which (~40 mg/L) could be accounted for by the accumulated mass of the treatment.

Kenaf addition did not appear to have any significant adverse or beneficial effects on removal of COD, phosphate or suspended solids.

This study has presented evidence of a significantly higher concentration of arsenic, copper, lead and zinc (alpha = 0.01, except zinc: alpha = 0.05) in dried sludge solids from the kenaf treated ML, possibly due to adsorption of the metals to the porous kenaf particles. Kenaf supplementation would be helpful if removal of any of these metals is a goal, but could impact waste sludge disposal options if concentrations exceed compliance thresholds. Cadmium, chromium and nickel did not accumulate differently in the kenaf treated SBR than in the control. Selenium and cobalt were significantly (Se: alpha = 0.01, Co: alpha = 0.05) less concentrated in the kenaf enriched sludge solids.

Kenaf supplementation had no effect on nitrogen removal when aeration was optimal, but when relative aeration intensity was less than optimal, as can happen at a full scale plant when a sudden

increase in influent COD occurs, the nitrogen removal performance of the kenaf supplemented reactor degraded significantly less than that of the control.

Intensive sampling of mixed liquor over a single treatment cycle suggested that the kenaf treated SBR was able to convert more ammonia to nitrite and more nitrite to nitrate each cycle than the control SBR could. This implies a small increase in AOB and NOB activity which could mean more complete nitrification in full scale plants.

Batch reactors of air saturated mixed liquor aliquots from each SBR had similar ammonia utilization rates ($-7.6X10^{-5}$ mg N/(mg VSS*min)), but nitrite-nitrogen concentration increased more slowly in the kenaf supplemented ML ($5.8X10^{-5}$ mg N/(mg VSS*min)) than in the control ($6.5X10^{-5}$ mg N/(mg VSS*min)). This was found to be due at least in part to nitrite being converted to nitrate at a faster rate in the kenaf ML ($8.6X10^{-6}$ mg N/(mg VSS*min)) than in control ($3.3X10^{-6}$ mg N/(mg VSS*min)), which suggests improved NOB activity with kenaf treatment.

Future Work

Molecular analysis techniques such as qPCR could help determine which microbial groups benefit from kenaf addition and to what degree. Kenaf's tendency to adsorb metals raises the question of whether this treatment might also be useful in removal of emerging contaminants such as pharmaceuticals and personal care products. It would also be helpful to study the biological decomposition process of kenaf powder over time in both aerobic and anaerobic conditions in order to better model the contribution of the added kenaf to the MLSS and MLVSS.

References

1. Sajab, Mohd Shaiful, et al., *Citric* Yuansong Wei, *et al*., *Minimization of excess sludge production for biological wastewater treatment*. Water Research 37 (2003), 4453-4467

2. Mostafa Tizghadam, C, Dagot, M. Baudu, *Wastewater treatment in a hybrid activated sludge baffled reactor*. Journal of Hazardous Materials 154 (2008) 550-557

3. U.S. Environmental Protection Agency, *Nutrient Control Design Manual*. EPA/600/R-10/100 August 2010

4. C.L. Webber III, V.K. Bledsoe, "Kenaf Yield Components and Plant Composition," *Trends in New Crops and New Uses*, Janick and Whipkey (ed.s), 2002. ASHS Press, Alexandria, VA

5. LaMahieu, P.J., Oplinger, E.S., Putnam, D.H., *Alternative Field Crops Manual: Kenaf*. (1991) Available from: http://www.hort.purdue.edu/newcrop/afcm/kenaf.html downloaded 11/30/2011

6. *acid modified kenaf core fibers for removal of methylene blue from aqueous solution.* Bioresource Technology 102 (2011) 7237–7243

7. Demirbas, Ayhan, *Heavy metal adsorption onto agro-based waste materials: A review*. Journal of Hazardous Materials 157 (2008) 220–229

8. B.-G. Lee, R.M. Rowell, Removal of Heavy Metal Ions from Aqueous Solutions Using Lignocellulosic Fibers. Journal of Natural Fibers 1 (2004) 97-108

9. Dhiraj Sud, G. Mahajan, M.P. Kaur, *Agricultural waste material as potential adsorbant for sequestering heavy metal ions from aqueous solutions – A review*. Bioresource Technology 99 (2008) 6017-6027

10. Kaoru Abe, Y. Ozaki, *Wastewater treatment by using kenaf in paddy soil and effect of dissolved oxygen concentration on efficiency*. Ecological Engineering 29 (2007) 125-132

11. Grady, Daigger and Lim, *Biological Wastewater Treatment, 2nd Ed.* (1999) CRC Press, Taylor and Francis Group: Boca Raton, FL

12. Gujer and Larsen, *The Implementation of Biokinetics and Conservation Principles in ASIM*. Wat. Sci. Tech. 31 No. 2 (1995) 257-266

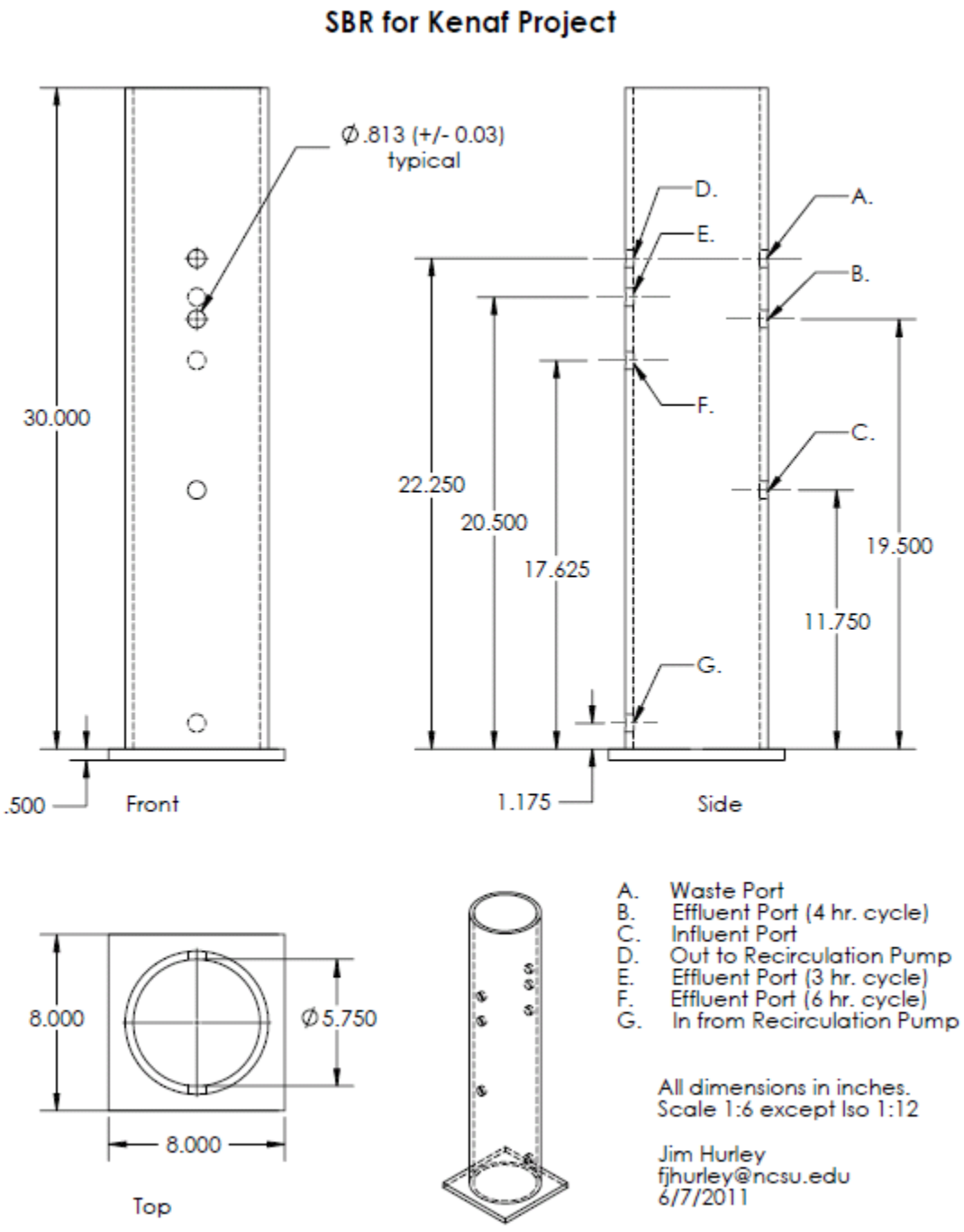
13. Eaton, *et al*. ed.s, *Standard Methods for the Examination of Water and Wastewater, 21st Ed*. (2005) American Public Health Association: Washington, D.C.

14. K. Kaczmarski, J.Ch. Bellot, *Effect of Particle-Size Distribution and Particle Porosity Changes on Mass-Transfer Kinetics*. Acta Chromatographica 13 (2003) 22-37

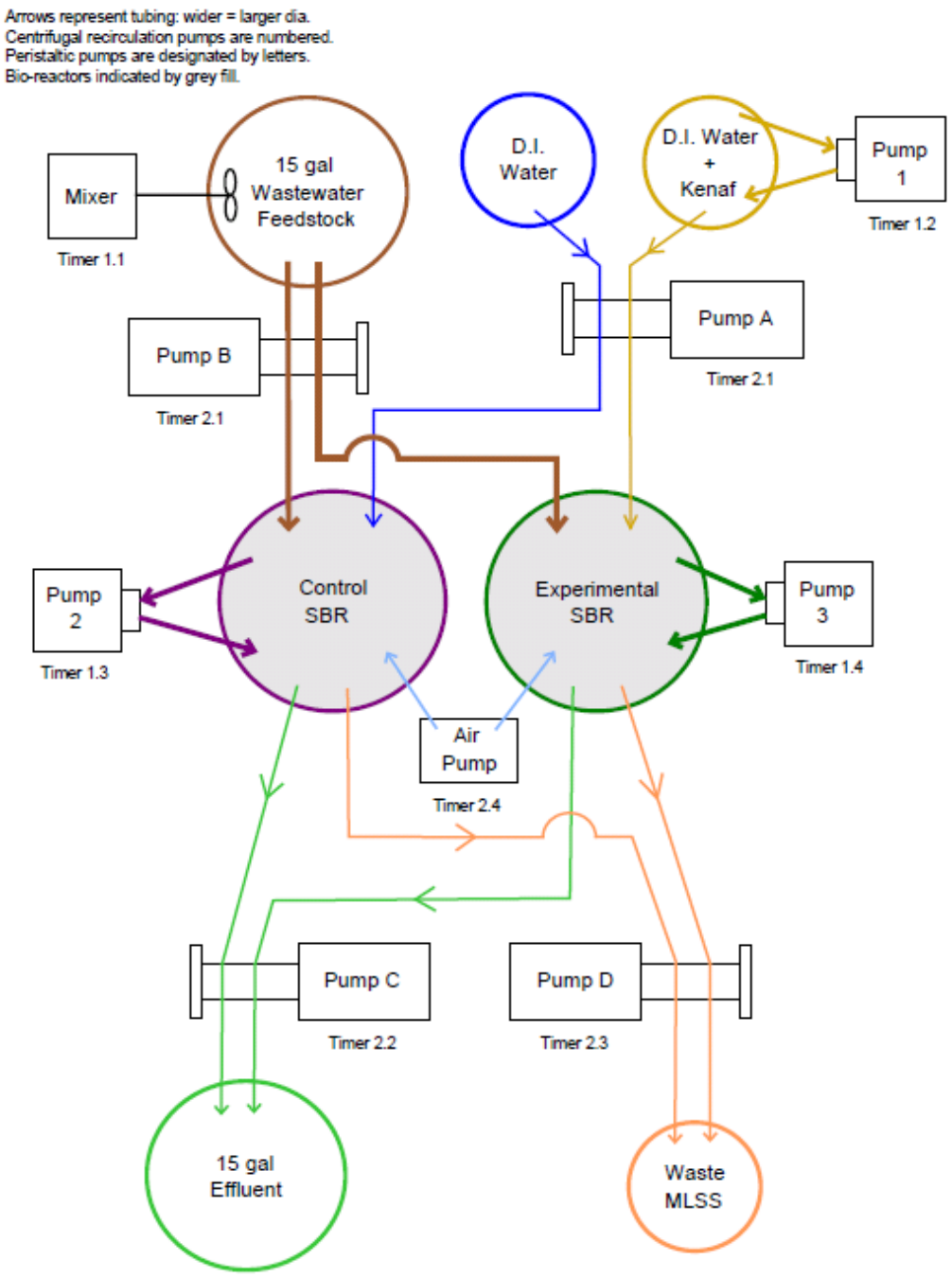
15. Harry G. Brittain, *Particle-Size Distribution, Part 1: Representations of Particle Shape, Size, and Distribution*. Pharmaceutical Technology December (2001) 38-45

Appendices

Appendix 1: Measured Drawing of Reactors



Appendix 2: Schematic of Benchtop Set Up



Appendix 3: Metals Analysis Procedure for Sludge Solids

Reported by Kim Hutchison

Sludge samples were weighed to the nearest 0.0001 g into teflon bottles.

5 mL of conc. optima pure nitric acid was added to each bottle, sitting overnite for 16 h.

Samples were heated (loose caps) for 2 h at 95 degrees C.

Samples were cooled, 1 mL of 30% hydrogen peroxide was added to each bottle and heated, slowly at first to avoid extreme effervescence, and then at 95 degree C for 2 h.

Samples were cooled, and 1 mL of optima pure hydrochloric acid was added to each bottle and heated for 1 h at 95 degrees C.

Samples were brought to 30 mL volume with 18 megaOhm DI water and filtered through a #41 Whatman filter.

Samples were diluted as needed and analyzed on a Varian 820 ICP-mass spectrometer utilizing the Collision Reaction Inteface mode with 75 mL of hydrogen gas per minute to minimize isobaric interferences (molecular species formed in the plasma having the same mass as elements of interest).

Rh was added as an internal standard to samples and standards to correct for small differences in sample transport and uptake.

Standards were acid-matrix matched to the samples.

A method blank was prepared the same as samples, and sample data was 'blank corrected' if needed.

Appendix 4: Procedure to immobilize floc in agar

Constituents sufficient for immobilizing 2 mixed liquor samples (2 X 2.0 mL sample + 6 X 4.0 mL agar):

0.375 g	Bacto-Agar non-nutritive low melting point agarose gel
25 mL	lab demineralized water (DI)
4 drops	methylene blue dye

Equipment:

2	15 mL beakers
1	50 mL beaker
1-2	small nylon covered magnetic stirring bars
6	plastic petri dishes with covers
	Stirring hot plate with low speed settings

Place the stir bar(s) in the 50 mL beaker; add 0.375 g Bacto-Agar, then pour 25 mL lab DI down the side of the beaker. Center the beaker on the stirring hot plate. Adjust the heat to medium high (~6), and set the stirring speed low enough to prevent vortex development, which would otherwise lead to entrainment of air bubbles in the gel. Bubbles can give false positives, reading as floc particles during the Integrated Morphometry Analysis to follow. Both using 2 stir bars in the beaker and moving the beaker off center on the stir plate reduce vortexing by introducing constant random changes to the stir bar's momentum which are transferred in part as changes to the momentum of the fluid surrounding the stir bar. Such random momentum changes do not tend to promote the organization of a vortex.

While monitoring the hot plate, clip approximately 1 mm off of the end of a 1000 µL pipette tip so that its opening is larger, then use it to transfer 2.0 mL of one fresh mixed liquor sample to a 15 mL beaker. Add 2 drops of methylene blue dye and swirl to mix. Reset the pipettor to 333 µL, and use the same tip to place that volume of the stained ML into each of three plastic petri dishes, dividing the dish's sample into three large non-contiguous drops located near the middle of the plate. Using the second 15 mL beaker and a fresh clipped pipette tip, repeat the process with the second 2.0 mL mixed liquor sample and 3 more petri dishes.

As soon as a full, but not rolling boil develops in the agar, turn off the heat element. It may be beneficial to continue stirring until bubbling ceases. Remove the beaker from the hot plate, and recover the stir bar(s). Remove any foamy scum or floaties from the surface with the twisted corner of a kimwipe. Allow the gel to cool until contact between the beaker and the bare forearm is just tolerable. Immediately pour small amounts of the gel into the centers of the petri dishes, portioning it all out approximately equally among the 6 plates. Quickly mix the agar with the stained ML sample by gently swirling the plates, first one direction then the other. Leave the petri dishes undisturbed for a few minutes until the gel sets. Once the contents are firm, invert the open dishes on a clean kimwipe for at least 20 minutes, but not more than 2 hours, while water vapor escapes from the gel. Reposition the dishes whenever the kimwipe becomes moist. Do not allow the tissue to contact the surface of the gel, as this can cause a loss of transparency. Cap the petri dishes, and stack them, taping the three from one sample together. Label the tape with sample source, date and researcher's name. Store petri dishes upside down at 4°C until imaged.

Appendix 5: Using MetaMorph to survey floc particle sizes

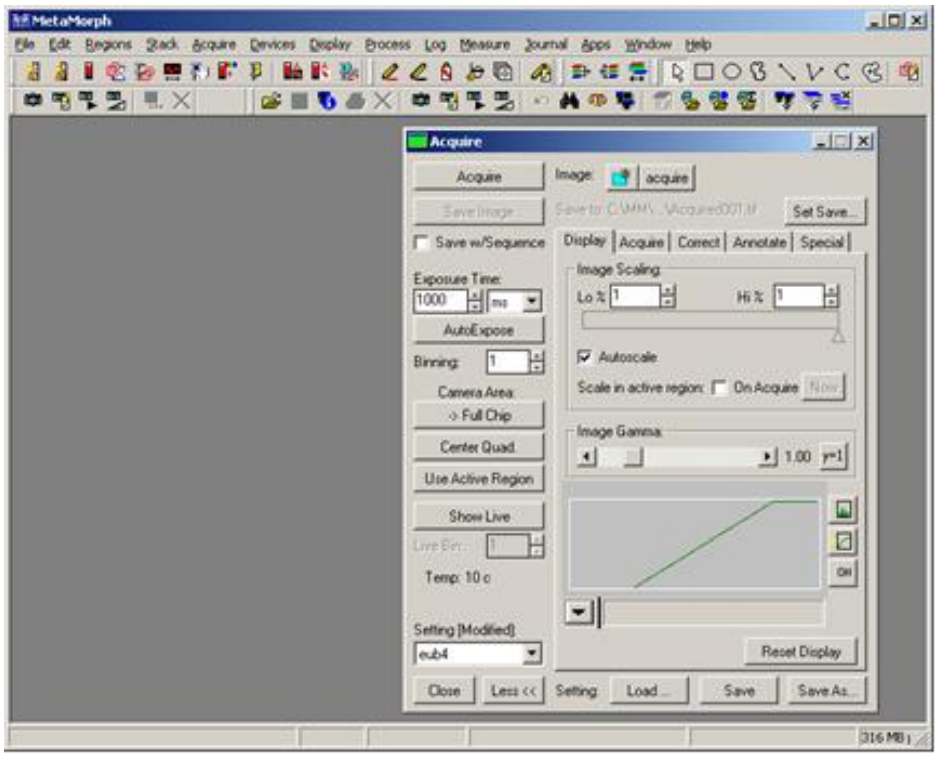
Turn on camera power supply and light box stage.

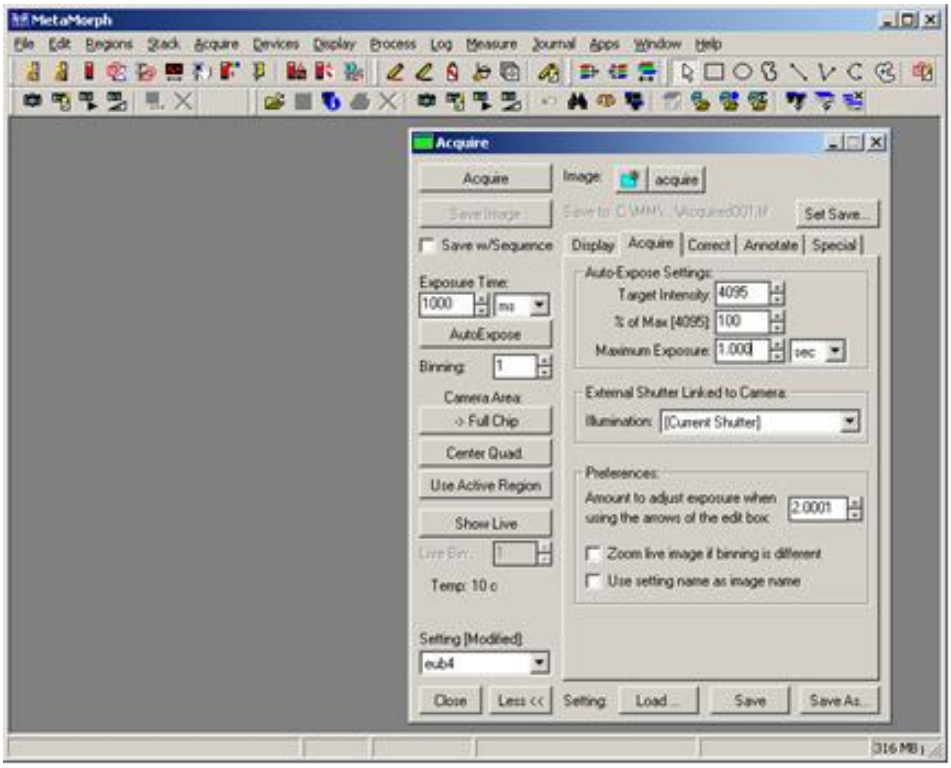
Open the “Meta Imaging Series 5.0” folder in the Windows 2000 desktop.

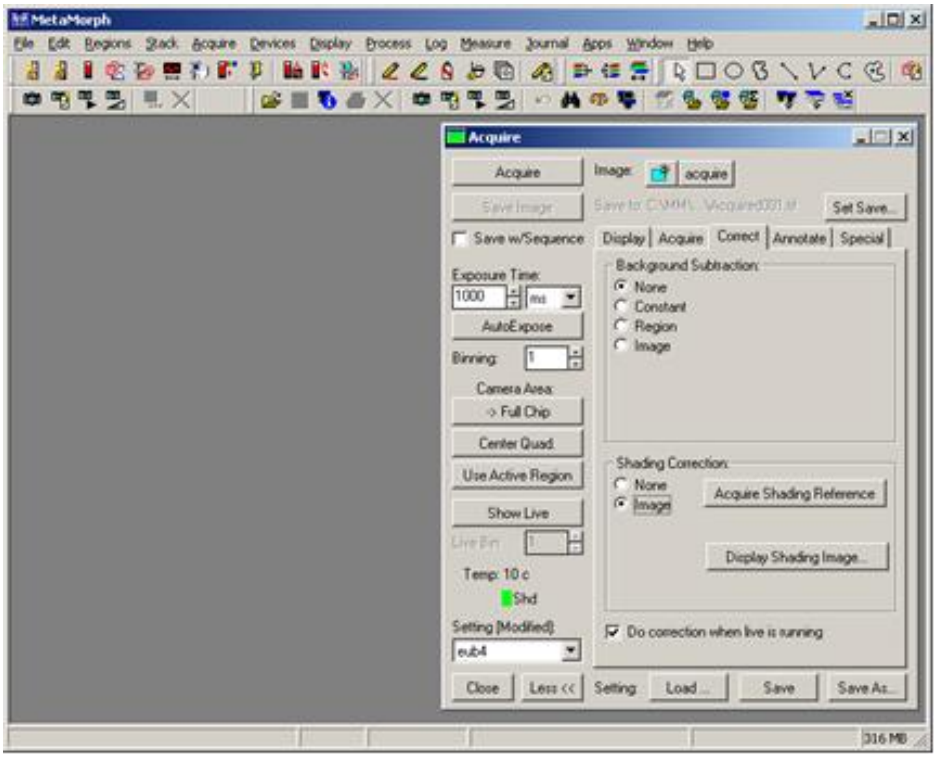
Double click on “MetaMorph version 5.0 release 7.”

Navigate to C:\MM\IMAGES\your folder\ to create a new folder for all the images from a single reactor day.

In MetaMorph, click the “Acquire” tab, note “Display,” “Acquire” and “Correct” settings in Acquire window:

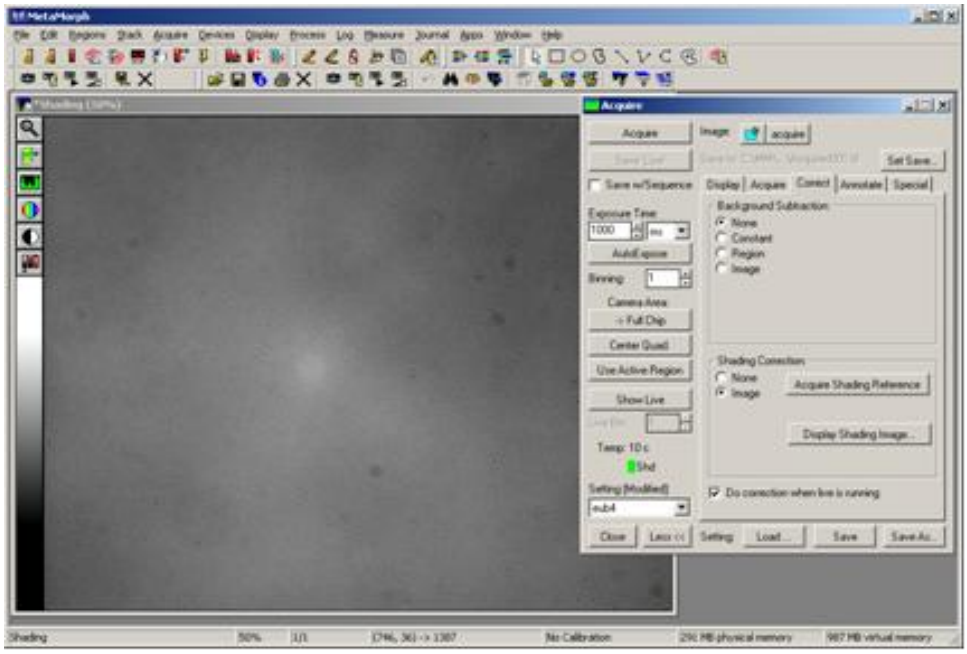

MetaMorph
File Edit Regions Stack Acquire Devices Display Process Log Measure Journal Apps Window Help
Acquire
Acquire
Image:
acquire
Save Image
Set Save...
Save w/Sequence
Display | Acquire | Correct | Annotate | Special
Exposure Time:
1000
ms
AutoExpose
Binning:
1
Camera Area:
-> Full Chip
Center Quad
Use Active Region
Show Live
Temp: 10 c
Setting [Modified]:
eub4
Image Scaling:
Lo % 1
Hi % 1
Autoscale
Scale in active region:
On Acquire
Now
Image Gamma:
1.00
g=1
Reset Display
Close
Less <<
Setting:
Load...
Save
Save As...
316 MB


MetaMorph
File Edit Regions Stack Acquire Devices Display Process Log Measure Journal Apps Window Help
Acquire
Acquire
Image:
acquire
Save Image
Set Save...
Save w/Sequence
Display | Acquire | Correct | Annotate | Special
Exposure Time:
1000
ms
AutoExpose
Binning:
1
Camera Area:
-> Full Chip
Center Quad
Use Active Region
Show Live
Temp: 10 c
Setting [Modified]:
eub4
Auto-Expose Settings:
Target Intensity: 4095
% of Max [4095]: 100
Maximum Exposure: 1.000
sec
External Shutter Linked to Camera:
Illumination: [Current Shutter]
Preferences:
Amount to adjust exposure when using the arrows of the edit box: 2.0001
Zoom live image if binning is different
Use setting name as image name
Close
Less <<
Setting:
Load...
Save
Save As...
316 MB



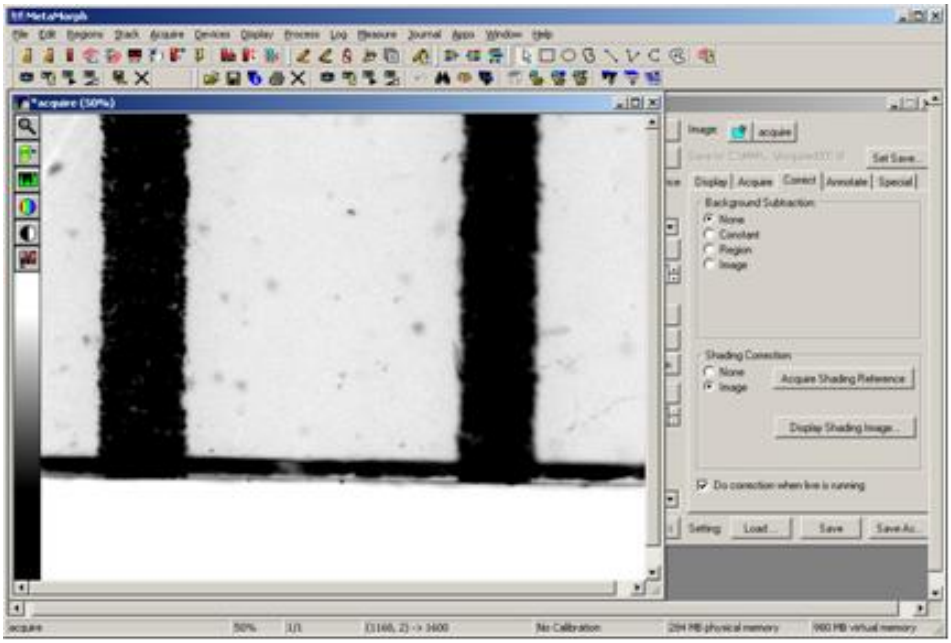
Test focus with a petri dish on the light box with “Show Live” running, but “Shading Correction” set to “None.”

Remove the petri dish, and then without changing the focus, click on “Acquire Shading Reference,” then on “Display Shading Image.”



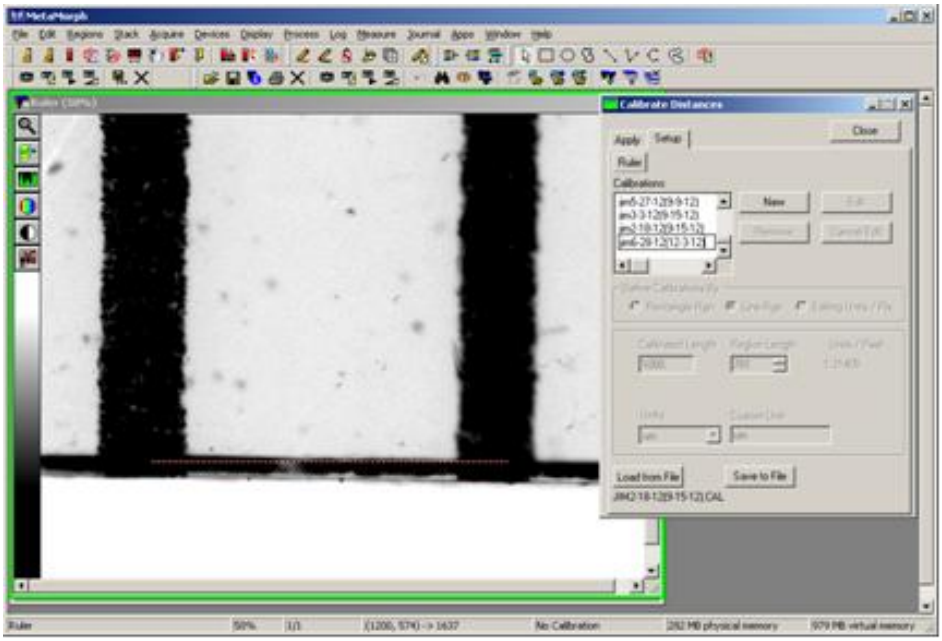
Click on “Save As” to save the image in the folder you created earlier. Do not rename the file.

Enable “Show Live” again in order to position a clear ruler with mm marks on the stage as close as possible to original plane of focus. Use slides as spacers under the ruler so only small focus changes are needed. Save the image as “Ruler” in the folder for these samples.

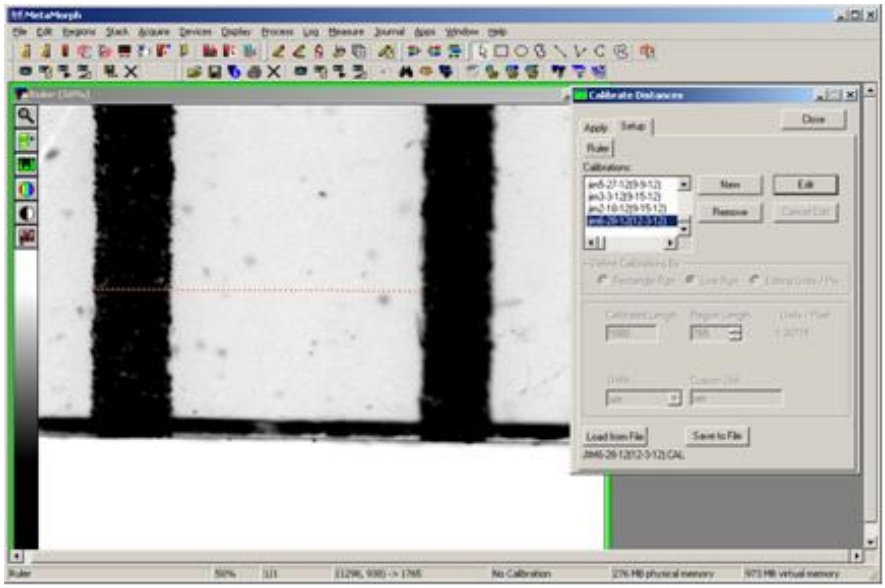


Click on the “Measure” tab then “Calibrate Distances.”

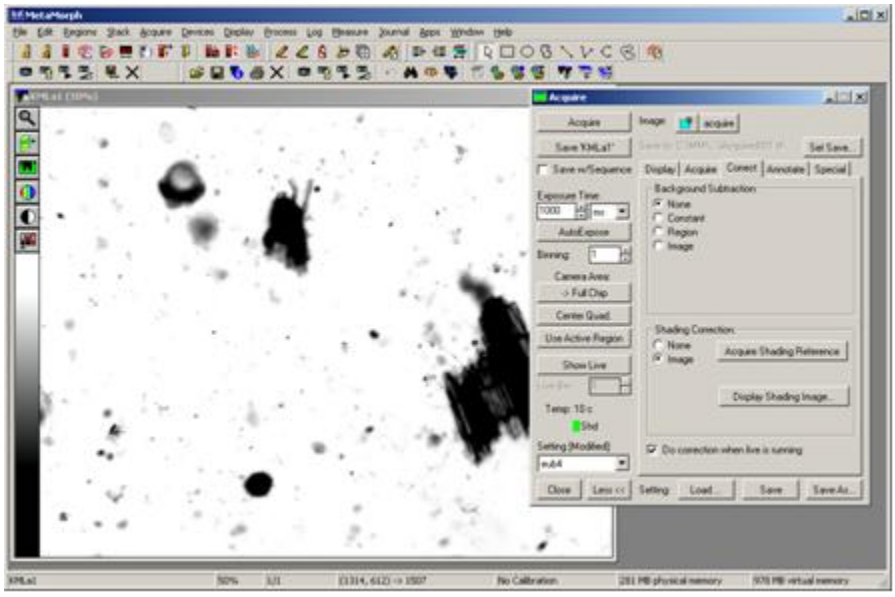
Click the “Setup” tab then the “New” button. Enter a name for the calibration.



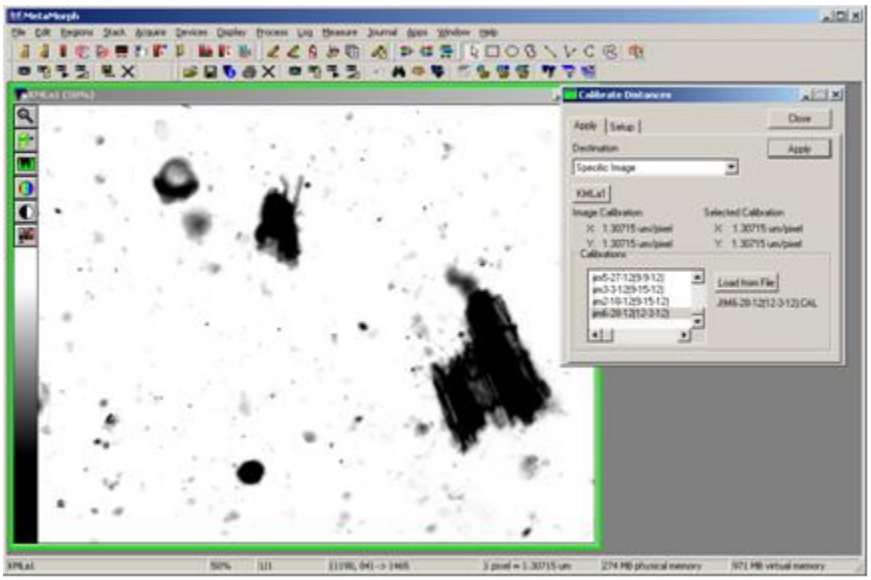
Adjust the calibration bar, click the “Done” button then save to file.



Position a petri dish with its sample on the stage. Adjust focus optically then activate the “Acquire” tab and select “Show Live.” When the image on the monitor is focused, Acquire, Save and name the image.



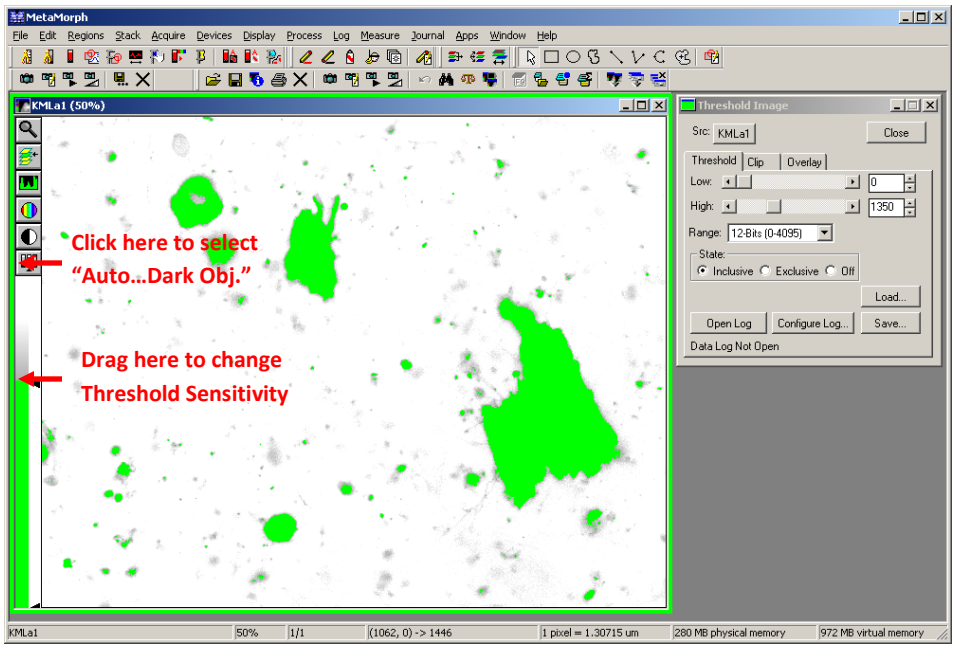
Click on the “Measure” tab then “Calibrate Distances.” With the new calibration highlighted, click the “Apply” tab then the “Apply” button. Objects in this image can now be measured.



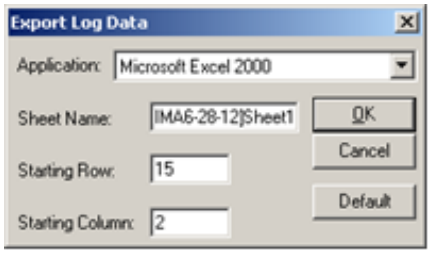
Click on the “Measure” tab then “Threshold Image.”

Click on the bottom button to the left of the image and select “Auto Threshold for Dark Objects” then drag the bar below the button to change how selective the threshold is, typically leaving only a thin grey corona showing outside the thresholded areas in green.

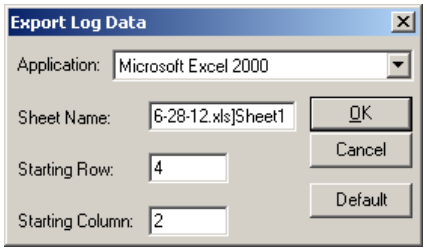
Save the thresholded image.



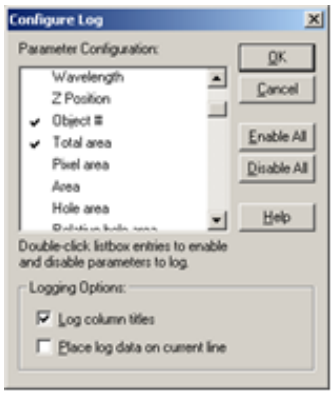
Create and open in the desktop a new excel file to import MetaMorph data into. Click on "Log" tab then "Open Object Log." Log data to a Dynamic Data Exchange (DDE). Enter the appropriate application, file name and starting cell.



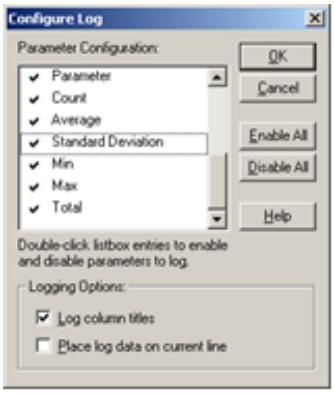
Click on "Log" tab then "Open Summary Log." Choose DDE then enter the relevant info.



Click on the “Measure” tab then “Integrated Morphometry Analysis.” For the Output check “Total Area,” “Shape Factor,” “Equiv. Sphere Radius” and “Equiv. Sphere Volume” Click on Configure Log and check the same four parameters plus “Object #.”

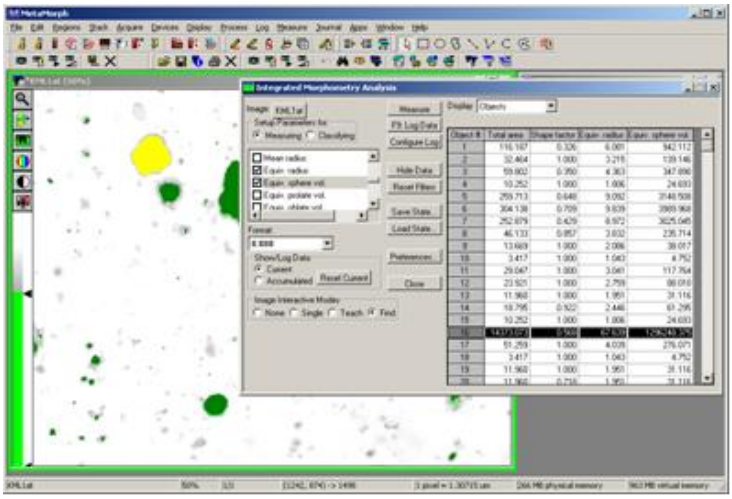


Click on the “Measure” button in the IMA window then the “Log Data” button. The individual objects are now listed in your excel file. In the Display box change “Object” to “Summary” then hit “Configure Log.” Check only the parameters shown here. Click “OK” then “Log Data.” Summary data is now also in your excel file. Save the excel file and close the object and summary logs.

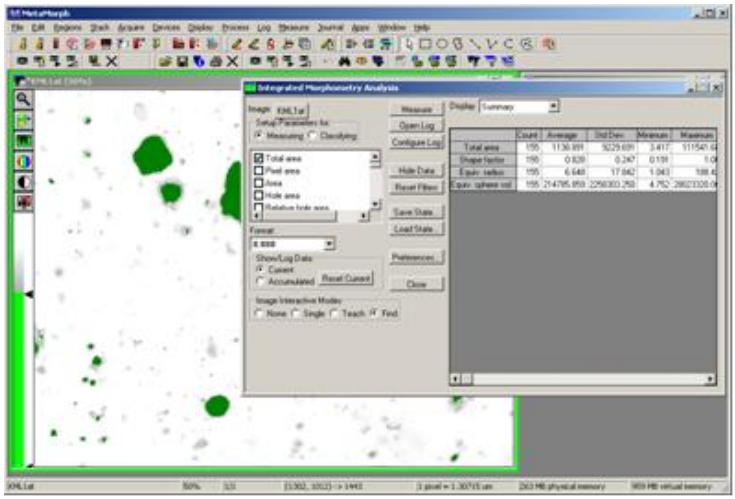


Acquire, calibrate, threshold and apply IMA to three non-overlapping images per petri dish (9 per sample). Log each image's data to a separate sheet of the same excel workbook. Turn off camera, light box and computer when finished.

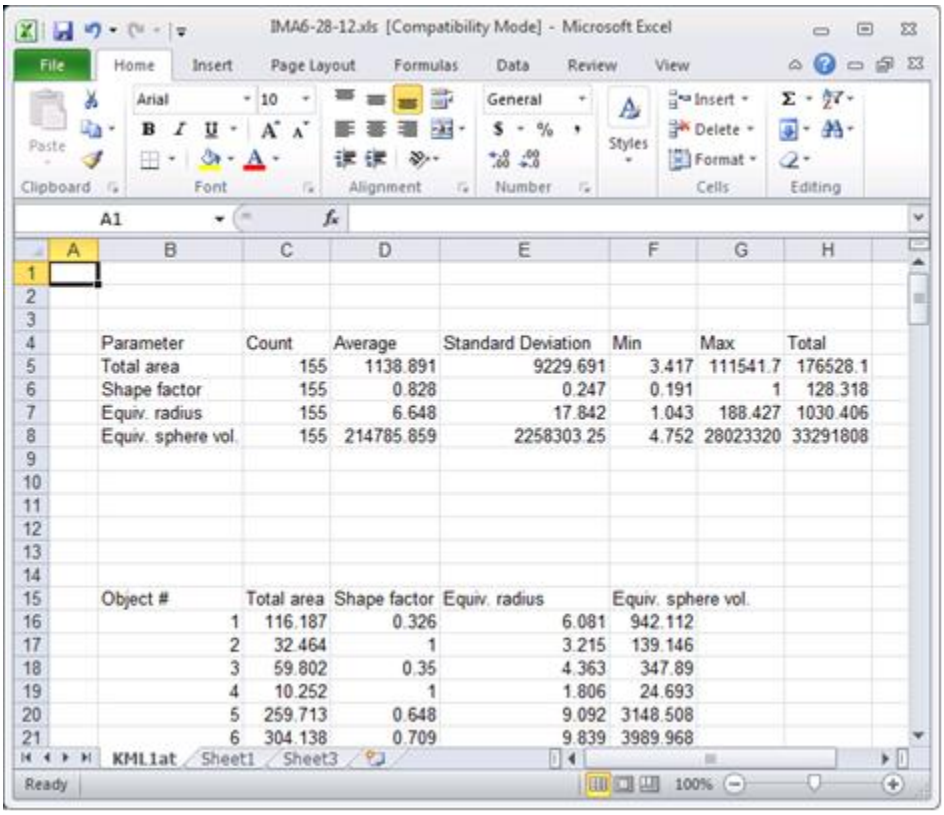
List of objects to be logged to excel—note that selecting any object in the list highlights the object in yellow in the image:



Summary statistics to be logged to excel:



Excel file produced by MetaMorph:



	A	B	C	D	E	F	G	H
1								
2								
3								
4		Parameter	Count	Average	Standard Deviation	Min	Max	Total
5		Total area	155	1138.891	9229.691	3.417	111541.7	176528.1
6		Shape factor	155	0.828	0.247	0.191	1	128.318
7		Equiv. radius	155	6.648	17.842	1.043	188.427	1030.406
8		Equiv. sphere vol.	155	214785.859	2258303.25	4.752	28023320	33291808
9								
10								
11								
12								
13								
14								
15		Object #	Total area	Shape factor	Equiv. radius	Equiv. sphere vol.		
16		1	116.187	0.326	6.081	942.112		
17		2	32.464	1	3.215	139.146		
18		3	59.802	0.35	4.363	347.89		
19		4	10.252	1	1.806	24.693		
20		5	259.713	0.648	9.092	3148.508		
21		6	304.138	0.709	9.839	3989.968		

Appendix 6: Preparation of 4% Paraformaldehyde (PFA) Solution

Constituents sufficient for fixing 2 mixed liquor samples (2 X 3.0 mL sample + 2 X 9.0 mL PFA):

1.0 g	anhydrous paraformaldehyde (kept in 4°C fridge)
5.0 µL	NaOH 10 Molar
50 µL	HCl 1.0 Molar
2.5 mL	10X Phosphate Buffer Solution (PBS)—commercially available
22.5 mL	double distilled DNA-free water (ddH_2O)

Equipment:

100 mL	Erlenmeyer flask
	Hot plate in fume hood
	Thermometer in 2nd flask containing ~50 mL lab DI
60 mL	syringe
0.45 µm	avg. opening nylon syringe filter in PP housing
	Sealable 50 mL centrifuge tube
	Slush ice

Place the thermometer in its own flask containing about 50 mL lab DI water, and that flask on the hot plate in the fume hood. Start with a low setting (~3) for the hot plate, letting the temperature rise slowly to 60.0°C for adequate solubility of the solid PFA, but no higher than 70.0°C to avoid thermally denaturing the aqueous PFA.

Meanwhile, in the empty flask, using sterile procedures combine 1.0 g of PFA powder with 22.5 mL ddH_2O and 5.0 µL NaOH 10M. Place this flask next to the thermometer's flask on the hot plate in

the fume hood. Swirl intermittently (while monitoring temperature) until there are only a few crystals of PFA left in the solution. Turn off the hot plate and carefully remove the flasks, keeping the PFA solution in the fume hood while it cools. Add 2.5 mL 10X PBS and 50 µL HCl 1.0M to the solution and swirl gently to mix. When it is cool enough to handle, still working in the fume hood, load the solution into the 60 mL syringe, attach the 0.45 µm syringe filter and eject the solution into the 50 mL centrifuge tube. Once the centrifuge tube has been sealed, it can be removed from the fume hood, labeled and placed in slush ice until needed. The PFA solution must be used (or discarded to a HAZMAT bottle) within 24 hours.