# Production of Microbial Flocs Using Laboratory-scale Sequencing Batch Reactors and Tilapia Wastewater

D.D. Kuhn[1]*, G.D. Boardman[2], G.J. Flick[1]

[1]Department of Food Science and Technology
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061, USA

[2]Department of Civil and Environmental Engineering
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061, USA

*Corresponding author: *davekuhn@vt.edu*

Keywords: sequencing batch reactors, SBR, microbial flocs, recirculating systems, tilapia, effluent, carbon supplementation, alternative protein, aquaculture feed

## ABSTRACT

Laboratory-scale studies using sequencing batch reactors (SBRs) were conducted to evaluate microbial floc production and treatability of fish effluent from a tilapia farm utilizing recirculating aquaculture systems (RAS). Several trials were conducted, both with and without carbon sucrose supplementation. Results from this project suggest that treatment with carbon supplementation improved nutrient removal from the fish effluent and increased microbial floc production. Successful treatment of effluent using bioreactors could accomplish two primary objectives. The first objective is improving water quality of effluent to maximize water reuse. Secondly, production of microbial flocs is a means of recycling nutrients from the effluent into a useable and alternative protein source for aquaculture diets. Ultimately, this option could offer a sustainable option for the aquaculture industry.

*International Journal of Recirculating Aquaculture 11 (2010) 37-54.* 

## INTRODUCTION

Overfishing of natural fisheries is a global issue that is becoming more urgent as the human population continues to increase. According to the Food and Agriculture Organization of the United Nations, approximately 47% of the natural fisheries are fully exploited and an additional 18% are overexploited (FAO 2002). The high demand for seafood protein will likely increase, because worldwide, one out of five people currently depend on fish for their principal source of protein (Koonse 2006).

To meet the growing demand for seafood, aquaculture production is on the rise, and is reportedly the fastest growing sector of agriculture worldwide. Traditional aquaculture practices use pond and flow-through systems, which are often responsible for discharging pollutants (e.g., nutrients and solids) into the environment. Furthermore, aquaculture feeds often contain high levels of fish or seafood protein, potentially increasing demand placed on wild fisheries. To mitigate these drawbacks, there is a significant movement towards more sustainable practices, especially in developed countries (Avnimelech 1999, Hargreaves 2006). For example, recirculating aquaculture systems (RAS) maximize reuse of culture water, which decreases water demand and minimizes pollutants discharged to the environment (Skjølstrup et al. 2000, Menasveta 2002, Timmons et al. 2002). Alternative proteins (e.g., yeast-based proteins) are also replacing fish and seafood proteins originally used in aquaculture diets (McLean et al. 2006, Lunger et al., 2007; Fraser and Davies, 2009). Implementing these alternative proteins could ease pressures on wild fisheries and often leads to high quality and less expensive feeds. The research described in this paper focuses on maximizing the reuse of freshwater fish effluent in the culture of marine shrimp. More specifically, this reuse is accomplished by using suspended-growth biological reactors to treat tilapia effluent, generating microbial flocs that could be used as an alternative feed to support shrimp culture.

Previous research investigated using nutrients in effluents from a commercial tilapia farm as supplemental feed to *L. vannamei* directly, in the form of microbial flocs generated from biological treatment of the effluents. Microbial flocs generated in bioreactors, and offered as a supplemental feed, significantly ($P < 0.05$) improved shrimp growth and specific growth rates (SGRs) in shrimp fed a restricted ration of commercial shrimp feed (Kuhn et al. 2008). Further studies

demonstrated that microbial flocs produced in sequencing batch reactors (SBRs) were a useful ingredient in replacing fishmeal. In fact, inclusion of microbial floc increased shrimp growth rates by over 65% (Kuhn et al. 2009).

Since this previous research demonstrated the potential benefits of implementing suspended growth biological treatment to aid in the co-culture of shrimp, it is important to understand how to best treat the effluent while producing microbial floc that can be utilized by the shrimp as a supplemental feed. Therefore, this project was focused on the treatability of effluents from the tilapia farm using SBRs. Treatments with and without carbon supplementation were evaluated and compared. Biological kinetic data and nutritional properties of SBR produced microbial floc were also determined.

## MATERIALS AND METHODS

### Effluent Handling and Storage

Tilapia effluent was collected from a local commercial RAS tilapia facility (Blue Ridge Aquaculture Inc., Martinsville, VA, USA). Fish densities at harvest were approximately 0.2 kg per L of water and each growout tank was outfitted with a settling basin, rotating biological contactors, and oxygenation via U-tubes. The effluent was collected from settling basins at the farm while they were drained as part of normal operations. Variability of constituents in this effluent was minimal because the settling basins were only flushed after 230 kg of feed were provided to the tilapia. During trial one, effluent was stored at -20°C in 19 L buckets until needed. For trials two through four, approximately 950 L of effluent was stored in the laboratory in a 1,100 L storage tank. Untreated solids, collected directly from tilapia effluent after a 45 min settling period, were characterized for protein and organic matter content and compared against microbial flocs from SBRs.

### Bioreactor Operation (Trials One Through Three)

Trial 1 setup consisted of twelve 1 L Beakers in a 29°C water bath (Table 1). These beakers were operated as SBRs with a hydraulic residence time (HRT) of 24 hours and no carbon supplementation. Effluent was stored in 19 L buckets in a -20°C freezer. Every 24 hours a bucket was removed

and thawed so a fresh source of effluent could be manually fed to the SBRs. Sludge was wasted at specific rates to evaluate biological solids residence times (SRTs) of 3, 6, 10, and 15 days in triplicate. Sludge was wasted by removing a known volume of well-mixed suspended solids from the reactor with a known suspended solids concentration. These SBRs were operated manually with the following periods: well-mixed aeration, 23 h; settling, 45 min; decant/idle/fill, 15 min. This trial lasted for 50 d.

Trials 2 and 3 (Table 1) were conducted in three SBRs (Figure 1) maintained at 28°C. Dissolved oxygen (DO) levels were greater than 5 mg/L during the aeration cycle. These 5 L SBRs were operated in triplicate using the following sequence: 4 h well-mixed aeration, 1 h settling, 45 min draw (water decantation/removal), and 15 min idle/fill periods. Water was pumped every 24 h from the storage tank (at room temperature) into a well-mixed 76 L equalization (EQ) tank. Microbial floc was wasted at a rate that provided a SRT of 10 d. Trial two was

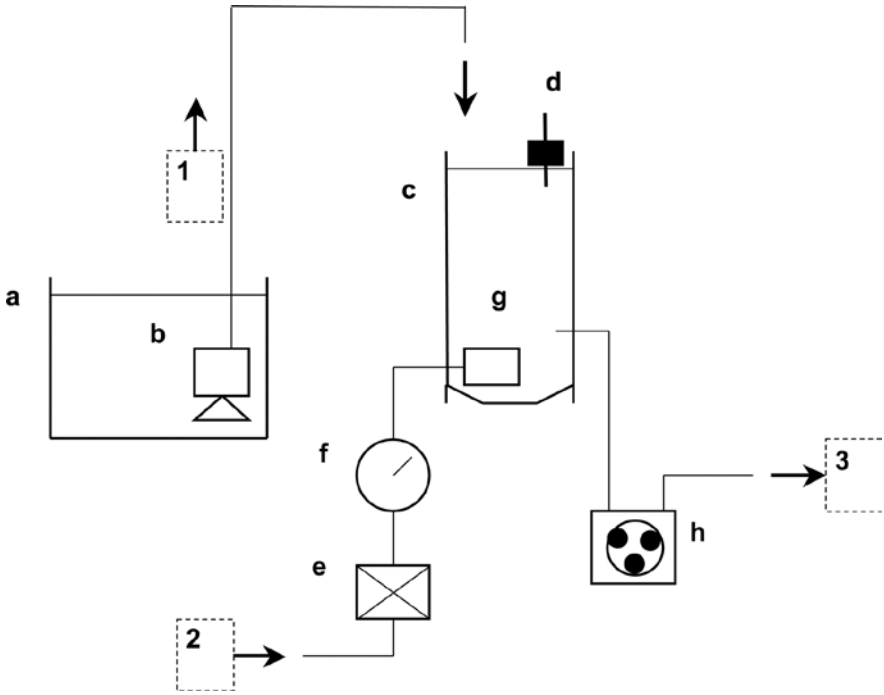


*Figure 1. Diagram of SBRs used for trials 2, 3, and 4: a) Anaerobic equalization tank, b) submersible pump on float switch, c) aerobic SBR, d) float switch, e) solenoid valve, f) air flow meter, g) air stone, h) peristaltic pump, 1) tilapia effluent, 2) compressed air, 3) treated effluent.*

conducted for 45 d with no carbon supplementation. In trial three, 500 mg/L (210 mg of carbon/L) of sucrose (Granulated white sugar, Kroger Co., Cincinnati, OH, USA) was added directly into the SBRs 5 min after each aeration cycle began, using peristaltic dosing pumps (Reefdoser RD4 Quadro, Aqua Medic©, Bissendorf, Denmark). Trial three was conducted for 30 to 35 d until the reactors became infested with fungi and were no longer operational.

**Bioreactor Operation (Trial Four)**

Every 24 h, the 76 L EQ tank was cleaned using pressurized well water. The EQ tank was well-mixed without aeration using a submersible Rio® 200 pump (TAAM Inc., Camarillo, CA, USA) and was maintained at 29°C. Sucrose was added directly to the EQ tank (500 mg/L sucrose, 210 mg of carbon/L) to promote denitrification and an increase in heterotrophic microbial floc. The resulting calculated food to microorganism ratio (F:M) over the stabilized period from day 30 to 50 was 0.15 ± 0.01.

Three 5 L SBRs were operated with 4 h well-mixed aeration, 1 h settling, 45 min draw (water decantation/removal), and 15 min idle/fill periods (Figure 1). The target SRT was 10 d. The temperature in the SBRs was maintained at 28.7 ± 0.2°C (mean ± standard error) using a water bath, and DO levels were always greater than 5 mg/L. Effluent was collected in 19 L buckets, and volumetric measurements of treated water were determined every 24 h for each reactor to ensure proper operation. Two independent batch trials were performed on stabilized SBRs on day 50 to determine kinetic coefficients from concentrations of microbial floc (mixed liquor volatile suspended solids, MLVSS), soluble total organic carbon (sTOC), and soluble chemical oxygen demand (sCOD) versus time (n = 17). Initial levels of MLVSS and sucrose spike concentrations to initiate the kinetic batch experiments were similar to levels used during the 50 day trial. The initial F:Ms for the two kinetic trials were, 0.14 and 0.17, respectively.

**Laboratory analysis**

After samples were filtered through a 1.5 µm filter, the filtrate was analyzed for nitrite-N, nitrate-N, orthophosphate (OP), and total ammonia-N (TAN) in accordance with HACH (2007) spectrophotometric methods 8507, 8039, 8048, and 8038, respectively. Sludge volume index (SVI), sCOD, sTOC, total solids (TS), total suspended solids (TSS) and

volatile suspended solids (VSS) were determined using methods 2710D, 5310B, 5220D, 2540B, 2540D, and 2540E, respectively (APHA 2005). Crude protein levels were determined in accordance with AOAC (2003). Temperature and DO were determined with a YSI 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA). A HI 9024 pH meter (HANNA Instruments, Woonsocket, RI, USA) was used to determine pH.

### Statistical Analysis

Statistical analysis, t-test, was performed using SAS v9.1 for Windows (SAS Institute Inc., Cary, NC, USA) on composition data regarding microbial floc versus untreated solids.

## RESULTS

### Trials One through Three

Results for trials one to three are summarized in Table 1. For trial one, reduction of sCOD and TAN ranged from 58 to 72% and 79 to 83%, respectively, and both increased with increasing SRT. Volatile suspended solids ranged from 100 to 200 mg/L and increased with increasing SRT. Trial two resulted in highly variable treatment, ranging from 18 to 80% removals for sCOD while MLVSS concentrations remained less than 200 mg/L. Trial three reactors generated levels of MLVSS greater than 1,000 mg/L. Removals of sCOD and TAN were both greater than 80%. However, fungi became dominant starting between days 30 and 35 (Figure 2). Although fungi was present during trial 3, it was not detected during trials one and two.

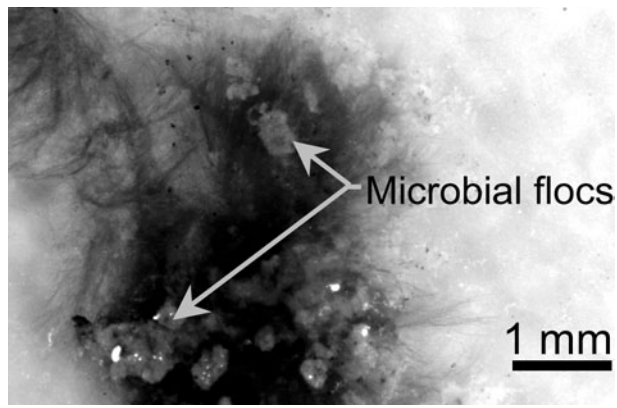


*Figure 2. Macro-photograph of fungi (filamentous shape) and a few microbial flocs (spherical shape).*

*Table 1. Comparison of various treatment and operation schemes performed at the laboratory scale.*

| Trial | Operation/input | Treatment | Microbial floc production | Fungi production | Comments |
|---|---|---|---|---|---|
| One: Aerobic (Beaker SBR) | HRT = 24 hours<br>SRT = 3,6,10,15 days<br>CS = no | Moderate<br>58-72% sCOD<br>79-83% TAN | Insufficient<br><200 mg/L | None | Fresh wastewater from freezer every 24 hours |
| Two: Aerobic (SBR) | HRT = 6 hours<br>SRT = 10 days<br>CS = no | Highly variable (e.g., 18 to 80% sCOD treatment) | Insufficient<br><200 mg/L | None | Up to 7 day old wastewater |
| Three: Aerobic (SBR) | HRT = 6 hours<br>SRT = 10 days<br>CS = yes | Sufficient<br>> 80% sCOD<br>> 80% TAN | Sufficient<br>>1,000 mg/L | Excessive | Up to 7 day old wastewater |
| Four: anoxic/ aerobic (EQ tank/SBR) | HRT = 6 hours<br>SRT = 10 days<br>CS = yes | Sufficient<br>> 80% sCOD<br>> 80% TAN | Sufficient<br>>1,000 mg/L | Limited | Up to 7 day old wastewater |

Note: CS = carbon supplementation (sucrose)

**Trial Four**

A strong linear correlation ($R^2$ of 0.9930) was observed between sCOD and sTOC (Figure 3). This function yielded a slope of 2.26 (mg sCOD)/(mg sTOC) and was determined over a range of sTOC (11-230 mg/L) and sCOD (12-510 mg/L), which was reflective of the range observed during this 50 day study. Similarly, ratios of COD to TOC were 2.33 ± 0.063 (mean ± standard error) when removal of sTOC, or sCOD, was less than 85% (Figure 4). However, for treatment levels greater than 85%, this ratio was significantly ($P < 0.05$) reduced to 1.36 ± 0.099.

During the stabilized period from day 30 to 50 (Figure 5), the overall mean concentration of MLVSS in the three SBRs was 1,383 ± 151 mg/L. No significant differences ($P > 0.05$) were observed between the mean MLVSS concentrations on the different days. During this stabilized period, removal of sTOC was always greater than 89% with an average reduction of 93.0 ± 0.8%. Furthermore, the mean effluent concentration of sTOC was 14.7 ± 1.7 mg/L. Figure 6 illustrates the changes in various constituents between the storage tank, equalization tank, and treatment from the SBRs. Overall, the percent difference in TAN, $NO_2$, pH, $NO_3$, and OP from influent to effluent were, respectively, –91, 0, +9, -60, and –23 % during the aforementioned stabilized period.

*Table 2. Trial four normalized kinetic coefficients based on two independent kinetic trials, except for yield coefficients for anoxic/oxic cycles which were determined from 8 data points from day 30 to 50. Mean values with standard errors.*

| | **Substrate** | |
|---|---|---|
| **Kinetic Coefficients** | **sTOC** | **sCOD** |
| $Y_{anoxic/oxic}$ [g microbial floc/g substrate] | 1.54 ± 0.11 | 0.68 ± 0.05 |
| $Y_{oxic}$ [g microbial floc/g substrate] | 1.60 ± 0.07 | 0.69 ± 0.02 |
| μ [1/h] | 0.27 ± 0.028 (0.9225) | |
| Zero-order rate [g substrate/ (g microbial floc*h)] | 0.17 ± 0.01 (0.9964) | 0.39 ± 0.03 (0.9759) |
| First-order rate [(1/hr)/gVSS] | 1.59 ± 0.39 (0.9650) | 1.72 ± 0.64 (0.9656) |

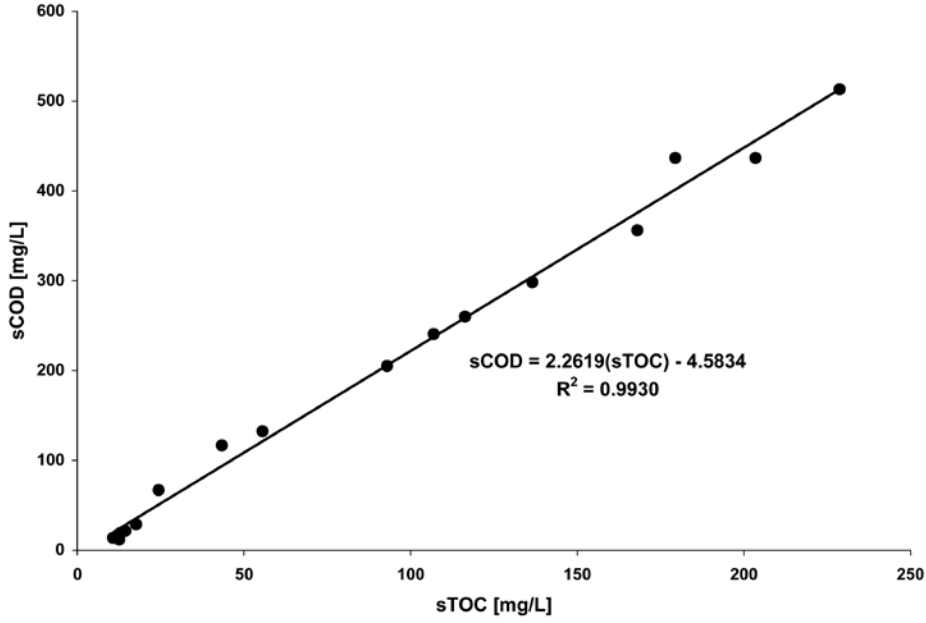


*Figure 3. Correlation relationship between sCOD and sTOC.*

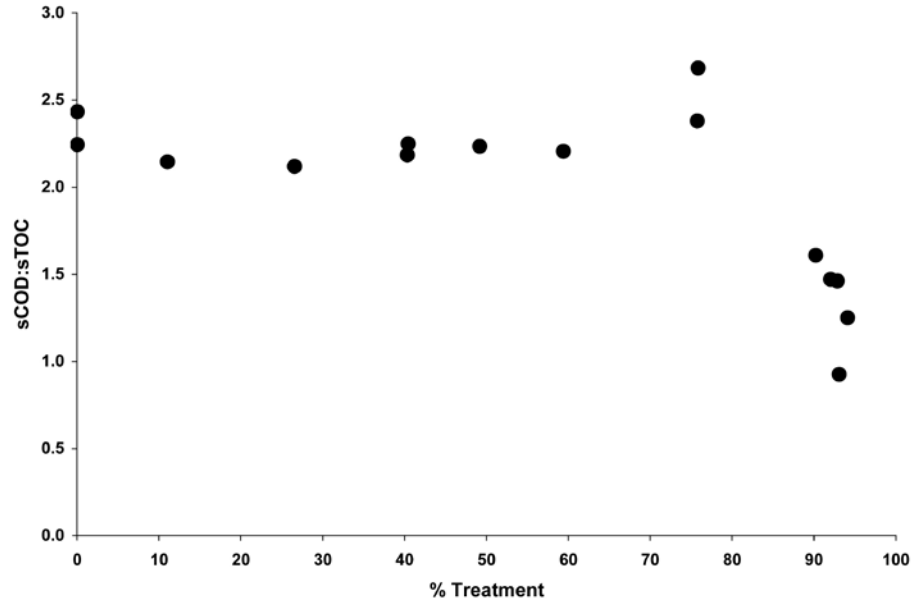


*Figure 4. Oxidation state versus % treatment as sTOC.*

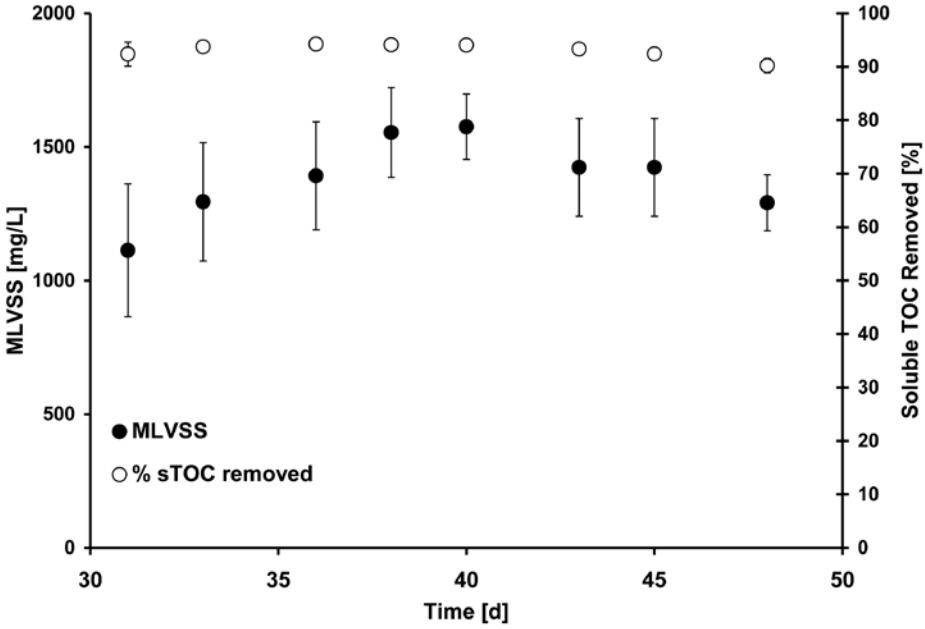


*Figure 5. Microbial floc concentration and % soluble TOC treated (mean values ± standard errors) for the three SBRs used in trial four.*

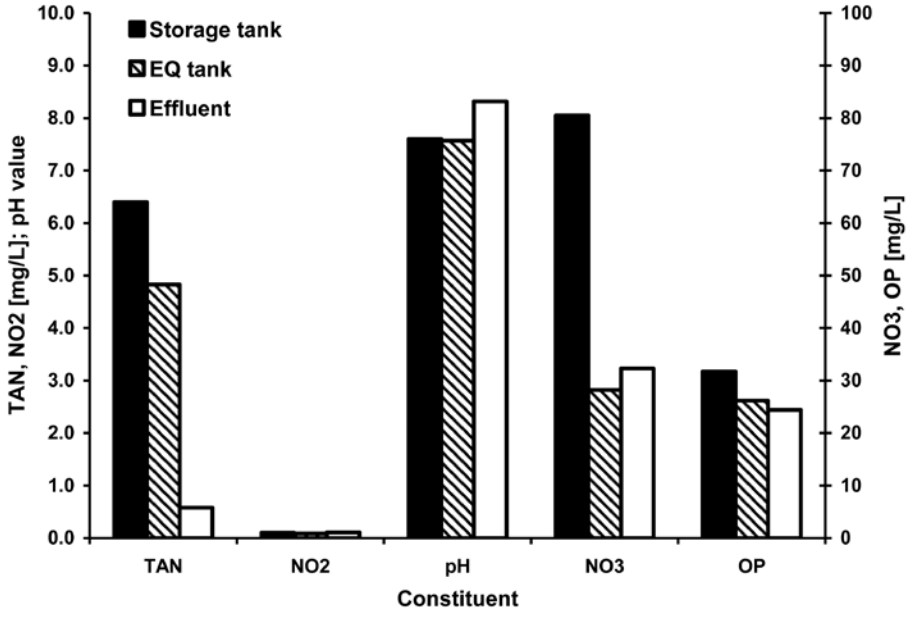


*Figure 6. Mean constituent levels determined in storage tank, equalization tank, and effluent after SBR treatment in trial four.*

Kinetic coefficients are reported in Table 2. No significant differences ($P > 0.05$) were reported between the anoxic/oxic and oxic yield coefficients values based on TOC and COD measurements. Correlations were strong for all determined normalized rate values; $R^2$ values were never less than 0.92. Even though the correlation rates were good for both first and second order rates, zero order rates exhibited slightly better fits.

Microbial floc characterization for microbial flocs and untreated solids are compared in Table 3. Protein levels, determined as crude protein or Lowry protein, were significantly higher ($P < 0.01$) in microbial flocs as compared to untreated solids. More specifically, crude protein and Lowry protein values of microbial flocs were, respectively, 95% and 69% greater than untreated solids. The organic fraction of microbial flocs was significantly greater ($P < 0.01$) than that of untreated solids. Some fungal growth was observed in the SBRs, but the amount was insufficient as to interfere with bioreactor operations.

*Table 3. Characteristics of SBR microbial floc versus untreated solids.*

| | **Biomass** | **Untreated Solids** |
|---|---|---|
| SVI [ml/g] | 129 ± 10.7 | - |
| Crude protein [%] | 54.4 ± 0.3 | 27.9 ± 1.5 |
| Lowry protein [mg/g TSS] | 40.2 ± 1.5 | 23.8 ± 2.5 |
| Organic fraction [%] | 89.1 ± 0.3 | 84.4 ± 0.1 |

## DISCUSSION

The results suggest that operational inputs significantly influenced removal/treatment efficiencies, microbial floc production, and fungal development. Trial one treatment performance was likely limited by the low microbial floc concentration in the SBRs. Furthermore, the HRT of 24 h was perhaps too long and could have also contributed to these low efficiency levels. The microbial floc concentration could have theoretically been four times greater if the HRT was decreased to the levels (HRT of 6 h) used in trials two to four. This is based on mathematical relationships presented in Metcalf and Eddy (2003). Trial two was conducted to test this theory. This trial yielded similar results in terms of MLVSS concentrations. However, during this trial, the low microbial floc levels and highly variable treatment efficiencies observed could have been due to: (1) the tilapia wastewater not being fresh (it was used over the course of 7 days until a new batch was transported from

Blue Ridge Aquaculture, 130 km away), and/or (2) a low biodegradable fraction of sCOD that would need carbon supplementation (Metcalf and Eddy 2003, Avnimelech 1999, Ebeling et al. 2006). For this reason, trial three was conducted with carbon supplementation. This trial resulted in better treatment of sCOD and TAN than was seen in trials one and two. Microbial floc concentrations greater than 1,000 mg/L were also achieved. Even though this trial yielded desirable levels of treatment and microbial floc, fungi (Figure 2) populations began to proliferate on day 30 and eventually interfered with the decant cycle. This type of filamentous organism is not uncommon in aerobic systems when a readily degradable substance, such as a simple sugar, is being treated (Eckenfelder 2000, Elmaslar et al. 2004). Trials one through three were informative, but were not completely successful. However, trial four was more effective by improving nutrient removal and microbial floc production; this is a good foundation for future work.

Since there was a strong correlation between sCOD and sTOC (Figure 3), one constituent can be accurately estimated by measuring the other. Typically, a higher COD:TOC, means that more carbon is available for oxidation via heterotrophic microorganisms (Metcalf and Eddy 2003; Kleerebezem and Van Loosdrecht 2006). Plotting sCOD:sTOC versus percent treatment of sCOD (Figure 4) demonstrated the importance of this ratio, because this ratio was significantly reduced ($P < 0.05$) when the treatment was greater than 85%.

From personal experiences, bioreactors become stable when the reactor has been operated for a period of time, typically three to five times its average SRT. Therefore, during trial four, it was assumed that the three SBRs were stable after 30 days. This was verified by measuring the MLVSS concentrations and treatment performance from days 30 to 50 (Figure 5). As expected, there were no significant differences between MLVSS concentrations during this time period, and treatment of sTOC was consistently greater than 90%. Effluent concentrations of sCOD were calculated to be $20.6 \pm 2.2$ mg/L.

Total ammonia nitrogen is typically reduced in SBRs via assimilation by heterotrophic microorganisms as well as via oxidation by autotrophic microorganisms (Metcalf and Eddy 2003, Ebeling et al. 2006). Nitrite remained low, less than 0.11 mg/L in all stages. The pH increased after treatment in the SBRs. As expected, denitrification was only

accomplished during the anoxic portion of the treatment sequence because in the absence of oxygen, nitrate becomes the electron acceptor for microbial metabolism (Metcalf and Eddy 2003, Boopathy et al. 2005). Nitrate was reduced by 65% during the anoxic stage and increased by 5% during the aerobic phase. This increase in nitrate is due to oxidation of reduced nitrogen by autotrophic microorganisms.

The kinetic coefficients of microbial floc production and substrate removal presented in Table 2 are important because they help the operator understand how best to manage the systems as well as any additions of supplemental carbon. Yield coefficients represent the amount of microbial floc produced per unit of substrate consumed. Typically, operators should prefer low yield coefficients because they have to dispose of this sludge, which can be time consuming and expensive. However, in this case, a high yield coefficient is beneficial because the microbial floc can be used as a supplemental feed for shrimp culture, reducing the total amount of commercial feed required (Kuhn et al. 2008), or reducing fishmeal requirements in the diets (Kuhn et al. 2009). The anoxic/oxic yield coefficients in this study were not significantly different ($P > 0.05$) from the oxic yield coefficients. Typically, anoxic yield coefficients are significantly lower than aerobic yield coefficients (Metcalf and Eddy 2003).

Microbial floc growth rates ($\mu$) of $0.27 \pm 0.028$ $h^{-1}$ observed in this study (Table 2) were higher than those observed for treating aquaculture wastewater using molasses (0.10-0.12 $h^{-1}$, Schneider et al. 2006). This is because the granulated sucrose used in this study is readily biodegradable, while molasses is a more complex polysaccharide that is not as biodegradable (Najafpour and Shan 2003, Quan et al. 2005). Even though fungi (Figure 2) were observed in low numbers during trial four, they did not adversely affect treatment performance or reactor operation. Although uptake rates for both substrates related well to zero-order and first-order rate equations (Table 2), zero-order rates represented the data sets more accurately.

Microbial floc generated in the SBRs had significantly higher ($P < 0.01$) protein values compared to untreated solids. Furthermore, these microbial flocs are a combination of microorganisms and exocellular biopolymers. Biopolymers are a conglomerate of multivalent cations, polysaccharides, and proteins (Higgins and Novak 1997). Even though

the sludge volume indices were relatively high, they were not indicative of bulking because they weighed less than 150 ml/g microbial floc (Eckenfelder 2000).

## CONCLUSION

Without carbon supplementation, removal of nutrients and production of microbial flocs were neither adequate nor sufficient. However, carbon supplementation with sucrose significantly improved nutrient removal and microbial floc production under these laboratory-scale conditions. Since the cost of marine and plant proteins have more than doubled since the 1990s (FAO 2007), developing a high quality, alternative ingredient for inclusion in shrimp feed is becoming increasingly important. Furthermore, the production of microbial flocs yields additional environmental benefits, in that using SBRs to treat a fish waste stream offers farmers a means to mitigate the cost and environmental impact of farm effluents.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge that funding for this study was provided in part by the United States Department of Agriculture Cooperative State Research Education and Extension Services (USDA-CSREES) and the Commercial Fish and Shellfish Technologies (CFAST) program at Virginia Polytechnic Institute and Sate University. The authors would also like to thank Blue Ridge Aquaculture Inc. (Martinsville, VA) for their support.

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