



Use of Sequencing Batch Reactor in the Treatment of Shrimp Aquaculture Wastewater

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ABSTRACT

Creating fiscally and environmentally responsible waste treatment methods is one key for enhancing shrimp aquaculture in the United States. Sequencing batch reactors (SBRs) allow significant reduction in costs such as relocation of shrimp production system further inland, less infrastructure, smaller space requirements, and recycling of the water and salt. SBRs adapt the activated sludge process and enable it to be completed within a single reactor vessel cycling through aerobic and anaerobic conditions. Microbes contained within the activated sludge conduct nitrification and denitrification processes. Significant reduction of nutrients is possible creating environmentally responsible intensive recirculating raceways meeting environmental protection agency (EPA) regulations, while at the same time reducing costs typically associated with these types of raceways. A pilot SBR was run successfully, which removed nitrogen in the waste significantly.

Keywords: Nitrification; denitrification; sequencing batch reactor; aquaculture; recirculating raceway system

1. INTRODUCTION

Humans have capitalized on the abundance of the ocean for a large portion of their sustenance for almost 2 million years. In recent times the human population has been increasing exponentially alongside our demand for seafood. As wild fishery stocks were noticeably decreasing due to overfishing and other causes it became economically and environmentally necessary to adapt our agrarian lifestyles into the “field” of seafood. The farming of aquatic organisms, hereinto referred to as aquaculture, has occurred for at least 1500 years (Costa-Pierce, 1987). Traditionally aquaculture was done on a very small scale using the natural resources at hand. Modern day aquaculture annual production has risen

over the past 58 years from 1 million metric tons in 1950 to 52.55 million metric tons in 2008 (FAO, 2010). This places aquaculture as the source of 38% of the worlds seafood supply in 2009 (FAO, 2010). As wild fisheries continue to be exploited and therein decline, and human demand continues to grow, aquaculture will no doubt expand to provide higher proportions of the world’s seafood. With this growth comes the need to mitigate the environmental impacts that aquaculture produces, while at the same time increasing its economic viability.

Shrimp have become a huge seafood staple, particularly in the US where the average citizen consumes 1.6 kg a year, subsequently making shrimp the largest seafood commodity in value terms at approximately 15% of all globally traded seafood (FAO, 2010). This has led to declining shrimp populations and has resulted in a subsequent increase of by-

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catch. Shrimp trawl bycatch was most recently reported at a worldwide estimate of 11,207,761 metric tons. An average of 8.14 kg of bycatch is landed for every 1 kg of shrimp caught (Alverson et al., 1994). These environmental factors and as well as the state of our economy have given rise to increase focus on the aquaculture of shrimp.

Modern shrimp aquaculture began in the 1930's when Japanese scientists began raising kuruma shrimp (*Penaeus japonicas*) in hatcheries, but it wasn't until the early 1980's that commercial production of farm-raised shrimp began to occur (Weidner and Rosenberry, 1992). Most shrimp grown today use either an extensive, semi-intensive or intensive designs. Extensive farms focus on low investment and result in low yield and occur in low-lying impoundments alongside bodies of water. Semi-intensive farms increase shrimp density by stocking juveniles, supplementing feed, and are typically built above high tide line, requiring moderate investments, and resulting in increased production. Intensive farms require significant investments such as building a facility, feeding, waste removal, aeration, and round-the-clock management. Commercial scale super intensive raceways farms often suffer from the same issues, but some success has been made in recent years with the use of biofloc technology (Krummenauer et al., 2011; Weidner and Rosenberry, 1992).

While shrimp aquaculture has been successful and grown significantly throughout the developing world, the few shrimp farms in the US continue to face issues while struggling to compete against foreign shrimp farms. In 1991, 1.18 billion pounds of shrimp were imported into the US, representing 85% of the total US shrimp supply and less than 5% of the them were grown in the Western hemisphere (Newman, 2010; Keithly et al., 2005). This amounts to a burgeoning trade deficit, which was approximately 3.1 billion dollars in 1998 (Olin, 2001). The United

States Department of Agriculture has been aware of this trade deficit and subsequent lack of food security, and in response created the United States Marine Shrimp Farming Program (USMSFP) to increase shrimp production in the US (Boopathy and Lyles, 2008).

Many key areas have been identified by the USMSFP that require new research and development to enable the US shrimp farming to successfully compete with the rest of the world. Traditional farms in the US are built as large ponds close to coastal areas to provide ease of water exchange between the ponds and the marine environment. Commercial coastal land is often difficult to find and typically more expensive than inland property, and ponds exposed to the local seasons limit shrimp production to certain times of the year (Browdy and Moss, 2005). The amount of waste produced by high intensity aquaculture is another issue that must be dealt with. Typically effluents from aquaculture are characterized by increased nitrogen species (ammonia, nitrites, and nitrates), organic carbon, phosphates, suspended solids, and high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Boopathy and Lyles, 2008). Significant issues can result in the release of nutrient rich effluents such as these including increased algal blooms, degradation of benthic communities, oxygen depletion, and overall degraded water quality (Boyd, 2003). Governmental entities within the U.S. such as the Environmental Protection Agency (EPA) have ruled that in order to protect United States waterways certain standards and limitations must be met before wastewater can be released. These regulations are set under the Clean Water Act, and restrict discharges from aquatic animal production facilities into public waterways in the U.S.

During the 1970's sequencing batch reactor (SBR) technology and system design were still very much in the beginning phases of research (Irvine and Busch, 1979). A SBR con-

sists of a single reactor vessel in which the activated sludge process is adapted to perform nitrification and denitrification in a timed sequence (Boopathy et al., 2005). A typical SBR sequence consists of the following 5 unique stages, loading/fill, react, settling, effluent extraction/decanting, and idle (Boopathy and Lyles, 2008; Marsili-Libelli, 2006). The react stage typically contains a fixed schedule of both an aerobic phase and an anaerobic phase, enabling nitrification and denitrification to take place.

Throughout the years a variety of SBR systems have been adapted for the treatment of many types of waste water such as human waste, animal waste, tannery waste, and slaughterhouse waste (Murat et al., 2002; Sirianuntapiboon and Manoonpong, 2001; Bennett et al., 2000). While standard wastewater treatment systems have shown to be ineffective on large high intensity systems, SBR systems have shown significant success (Boopathy et al., 2005). Many benefits exist of SBR's over other treatment options, such as increased control over the reliability, precision and versatility of the reactor (Stricker and Béland, 2006). SBR infrastructure requires less capital investment and significantly less plumbing, and space requirements when compared to other treatments (Boopathy and Lyles, 2008; Irwine and Ketchum, 1989). These benefits make SBR technology an ideal candidate for treating high intensity shrimp wastewater.

Successful cycling of a SBR requires monitoring of the conditions present to ensure the ideal characteristics for the metabolism of the specialized bacteria (Marsili-Libelli, 2006). The proper carbon/nitrogen ratio is important for the ideal growth of the bacteria and ideal nutrient reduction. A ratio of 10:1 C:N ratio has been determined to yield ideal reduction in shrimp wastewater by Fontenot et al. (2007). Hydrolyzed molasses can be a cheap and effective external carbon source to correct

the C:N ratio of the wastewater (Roy et al., 2010; Fontenot et al., 2007; Quan et al., 2004). Adequate aeration is also vital for proper aerobic metabolism during the aeration phase (Burt et al., 1990). Venturi tubes have been used to provide sufficient aeration to waste water and have many benefits over other technologies including increase efficiency, simplification, and aeration (Baylar et al., 2007). Oxidation reduction potential (ORP), dissolved oxygen (DO), and pH have shown to be valuable indicators as to the current state of the bacteria in the reactor and can be used to automate the cycling of the reactor to shorten the cycle times and yield idealized processing (Marsili-Libelli, 2006). Fontenot et al. (2007) found that temperature ranges of 22–37° C showed significant nutrient reduction capabilities over higher temperatures. In the past, our laboratory has shown successful treatment of shrimp production wastewater in lab-scale reactors with a working volume of five liters (Boopathy and Lyles, 2008; Boopathy et al., 2005). In the present study, we operated a pilot scale SBR of 5000 liters and integrated the SBR into shrimp production system for recycling the water in an intensive shrimp raceway aquaculture system. The results from this operation showed successful removal of nitrogen and carbon in the wastewater with recycling the water in the system operation.

2. METHODS

2.1 Shrimp wastewater

Shrimp wastewater from an intensive raceway system was collected from a sediment settling tank at the Gulf Coast Research Laboratory (GCRL), Ocean Springs, Mississippi, USA. The sample was analyzed for various characteristics listed below. This wastewater was used to run the sequencing batch reactor.

2.2 Sequencing batch reactor (SBR)

Two identical SBRs were operated with shrimp wastewater. Each reactor received 3000 L of wastewater at the beginning of the experiment. The reactors were aerated using venturi air bubbling system and the wastewater. Aeration was turned off for the system to run anaerobically. The reactors were operated aerobically and anaerobically and these modes of operation were alternated at regular intervals until the end of the experiment as shown in Figure 1. The purpose of this experiment was to optimize the aerobic and anaerobic sequence for optimum removal of carbon and nitrogen. SBR process for nitrogen removal may be divided into 2 stages as follows:

- **Aerobic Stage:** In this stage the carbon oxidation and nitrification are combined into the single process to achieve nitrification and COD removal.
- **Anaerobic Stage:** The second stage is an anaerobic process in which denitrification is accomplished.

2.3 Pilot scale SBR

Two pilot scales SBR with the capacity of 5000 liters with a working volume of 3000 liters were operated at the GCRL, Ocean Springs, MS. The reactors were operated aerobically for the first 2 days and then operated anaerobically followed by aerobic operation until the end of the experiment on day 11. The SBR received molasses as an additional carbon source to maintain the C:N ratio of 10:1 as indicated by our earlier study (Roy et al., 2010). The performance of the reactor in carbon and nitrogen removal was reported as the average of the duplicate SBR. A set of controlled reactor was run without aeration to compare the SBR performance.

2.4 Analyses

Thirty ml wastewater was removed from each reactor and centrifuged at 5000 rpm for 10 minutes and the supernatant was used for the chemical analysis during every sampling event. Nitrite, nitrate, and ammonia were analyzed periodically by colorimetric methods with a Hach water analysis kit (Hach, 1999). The chemical oxygen demand (COD) was analyzed using standard methods (APHA, 1998). Total COD was analyzed using the whole sample and soluble COD was analyzed using filtered sample using 0.2 μ m filter paper. The soluble COD excludes carbon from microorganisms in the wastewater and it represents the soluble organic carbon in the wastewater. The dissolved oxygen (DO), salinity, and temperature were measured using an YSI DO and salinity probe (Model No. 85-10FT, Yellow Spring, OH). The pH was measured using a pH probe (Model UB 10, Denver Instruments, Boulder, CO).

2.5 Isolation and culturing of bacteria

A 10 mL sample was taken from each SBR and control reactor for microbiological analysis. Samples from the reactors were diluted in sterile phosphate buffer solution and 0.1 mL of each of the dilutions was spread on TSA plates. Plates were incubated at 37°C under aerobic (24-48 hours) and anaerobic (9 days) conditions. Anaerobic jar with carbon dioxide generating system was used in the latter case. The most predominant bacteria presenting different pigmentation, shape, size, and surface texture were isolated by transferring single colonies to fresh agar medium. Isolates were subsequently identified by 16S rDNA gene-based molecular techniques.

2.6 Analysis and sequencing of 16S rDNA

Nearly complete 16S rDNA genes were enzymatically amplified from the isolated colonies using the polymerase chain reaction (PCR) and the colonies directly as templates. The pA and pH primers correspond to conserved regions of the 16S rDNA gene (Edwards et al., 1989). PCR was performed on a Minicycler™ (MJ Research). After the cycling steps (Bruce et al., 1992), amplification products (1500 pb) were detected by horizontal gel electrophoresis of a 5 µL aliquot in a 0.8% agarose gel in Tris-Borate-EDTA (TBE), followed by ethidium bromide staining and visualisation under UV light. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) as per the manufacturer's instructions.

To confirm that the different isolated colonies proceeded from different microorganisms, the PCR-amplified fragments were analysed by restriction fragment length polymorphism (RFLP) (Broda et al., 2000) with *Hae*III and *Alu*I restriction endonucleases (Takara Biotechnology, Japan). Restriction fragments were separated on a 3% agarose electrophoresis gel and visualised as specified above. Sequencing of the 16S rRNA genes from bacteria showing different profiles were performed using the BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase on an ABI Prism® 3100 Genetic Analyzer (Perking

Elmer). All procedures were performed according to the manufacturer's protocols. The 16S rDNA gene sequences obtained (300-1442 pb) were compared to the GenBank database using the NCBI Blast program (Altschul et al., 1997).

The *nosZ* gene, encoding the nitrous oxide reductase was used as the functional marker to confirm the denitrifying capability of bacteria isolated from SBR. To detect the presence of the *nosZ* gene, a fragment of 453 pb was amplified by PCR using *nosZ*-F and *nosZ*-1622R as priming oligonucleotides (Enwall et al., 2005).

2.7 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) test ($p \leq 0.05$) followed by a tukey "post hoc" analysis when needed (SAS). COD concentrations were analyzed using a paired t-test analysis ($p \leq 0.05$; SAS).

3. RESULTS AND DISCUSSION

3.1 Characteristics of shrimp wastewater

The initial characteristic of the shrimp wastewater is given in Table 1. The wastewater contained high concentration of carbon (COD), ammonia, nitrate, and nitrite.

Table 1 Characteristics of the shrimp wastewater*

Parameter	Concentration
Total COD (mg/L)	1593 ± 36
Ammonia (mg/L)	249.7 ± 6.1
Nitrate (mg/L)	181.3 ± 1.4
Nitrite (mg/L)	166 ± 22.7
Total nitrogen (mg/L)	597 ± 19.5
Salinity (ppt)	28.6 ± 0.4
pH	8.1 ± 0.1

*Average of 4 analyses

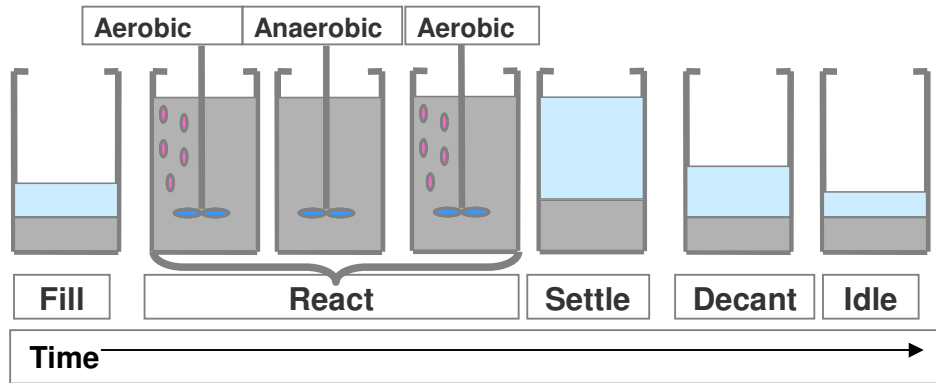


Figure 1 This diagram shows the sequencing series for wastewater treatment, which consists of the following process stages: fill, react, settle, decant and idle. These process stages occur in a timed sequence in a single reactor basin.

3.2 Performance of Pilot Scale SBR

We believe that wastewater from sedimentation tanks in the raceway system currently used in shrimp aquaculture can be directed to the SBR. The SBR will then digest the carbon and nitrogen associated with the wastewater. Once the carbon and nitrogen are digested, water can be decanted from the SBR and returned to the culture system, so water loss will be negligible (Figure 2). We designed a 5000-liter pilot scales SBR with a

working volume of 3000 liter and operated at the GCRL aquaculture facility in Ocean Springs, MS, USA. The pilot scale SBR performance data is presented in Figure 3 - 6. As the results indicate, there was almost 100% removal of ammonia, nitrate, and nitrite in the SBR compared to control where these nitrogen concentrations did not change during the duration of the study. The organic carbon reduced to a small degree in the SBR, however, we added molasses to the SBR to maintain the C:N ratio of 10:1.

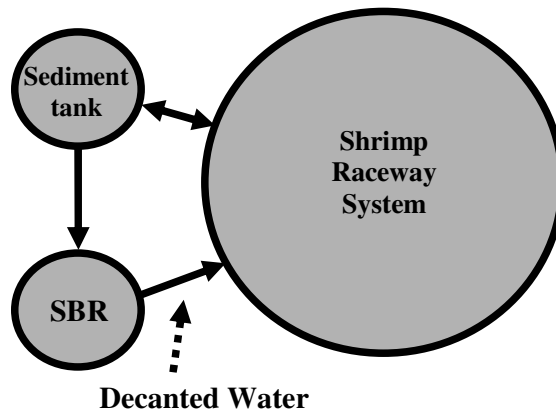


Figure 2 Schematic model of water flow (solid arrows) for an intensive culture system that incorporates a Sedimentation tank and an SBR.

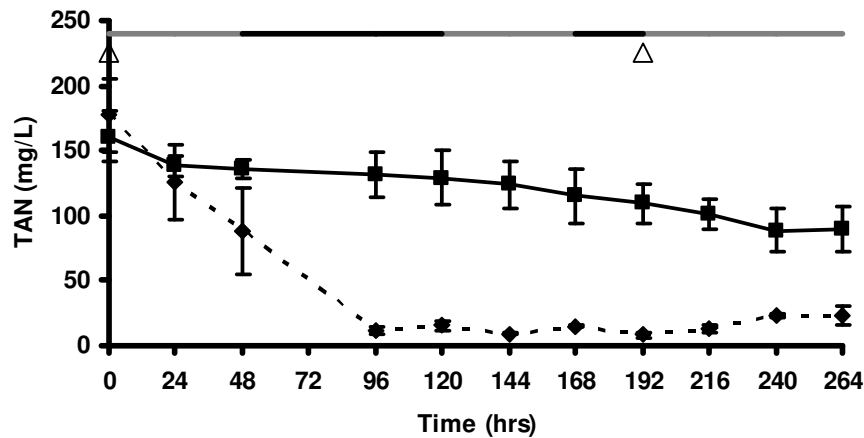


Figure 3 Mean (\pm SE) total ammonia nitrogen in the SBR (dashed line) and control (solid line) tanks. The horizontal gray (aerobic) and black (anaerobic) line indicate when the SBR was operated aerobically and anaerobically. The triangles indicate when molasses was added to adjust the carbon: nitrogen ratio. The control tanks were never aerated and never received additional carbon.

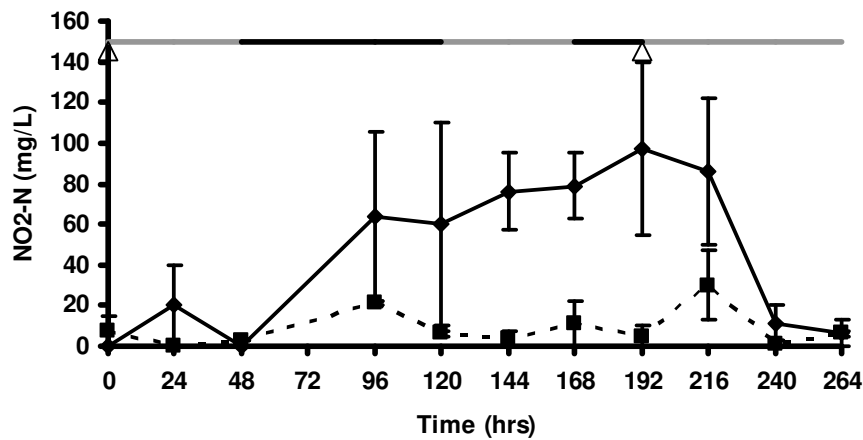


Figure 4 Mean (\pm SE) nitrite nitrogen in the SBR (dashed line) and control (solid line) tanks. The horizontal gray (aerobic) and black (anaerobic) line indicate when the SBR was operated aerobically and anaerobically. The triangles indicate when molasses was added to adjust the carbon: nitrogen ratio. The control tanks were never aerated and never received additional carbon.

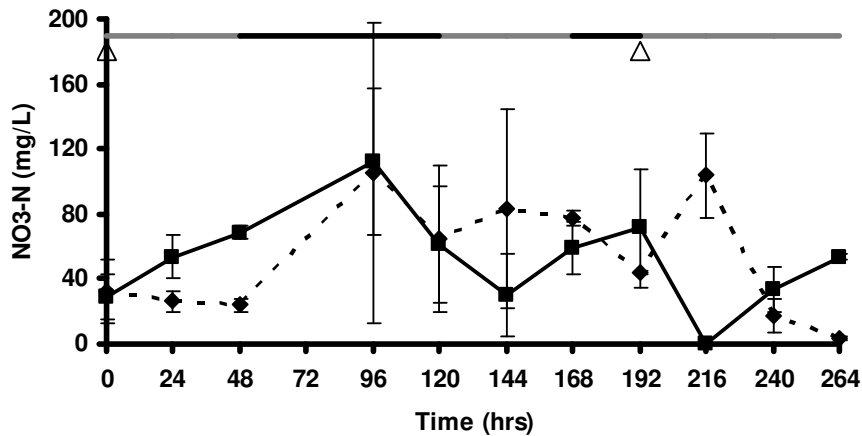


Figure 5 Mean (\pm SE) nitrate nitrogen in the SBR (dashed line) and control (solid line) tanks. The horizontal gray (aerobic) and black (anaerobic) line indicate when the SBR was operated aerobically and anaerobically. The triangles indicate when molasses was added to adjust the carbon: nitrogen ratio. The control tanks were never aerated and never received additional carbon.

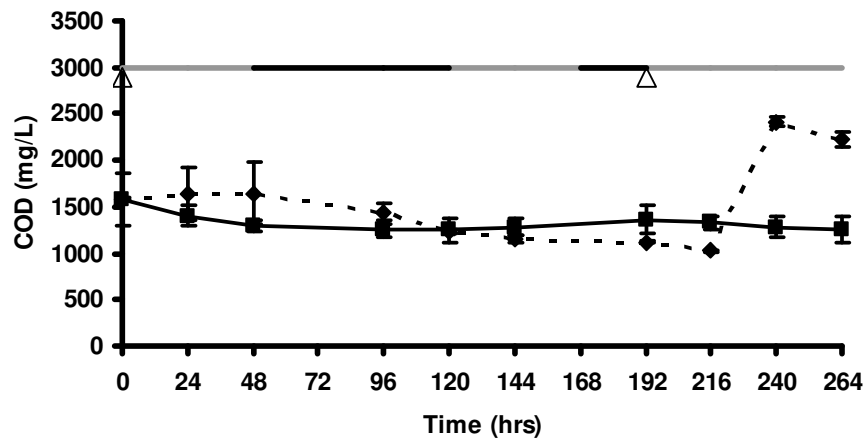


Figure 6 Mean (\pm SE) chemical oxygen demand in the SBR (dashed line) and control (solid line) tanks. The horizontal gray (aerobic) and black (anaerobic) line indicate when the SBR was operated aerobically and anaerobically. The triangles indicate when molasses was added to adjust the carbon: nitrogen ratio. The control tanks were never aerated and never received additional carbon.

The SBR successfully removed carbon and nitrogen from the wastewater of an active shrimp aquaculture system. The reactor design is simple and very easy to operate. The SBR system has been successfully used for various wastewaters including slaughterhouse

wastewater, swine manure, dairy wastewater, and sewage (Irwin and Ketchum, 1989; Lo et al., 1991; Masse and Masse, 2000). In the literature, it is shown that the wastewater problem in shrimp aquaculture is addressed by activated sludge process, foam fractions, use of

filter systems, and sludge management (Browdy et al., 1995; Holloway, 2002). These systems are costly and expensive to operate. The SBR system is very simple in design and this process uses multiple steps in the same tank to take the place of multiple tanks in a conventional treatment system. In this study, it has been shown that the SBR could be used to treat shrimp wastewater produced from intensive shrimp raceway production system. The operation mode is simple which includes aerobic process for first three days and anaerobic process for six days to remove 99% of nitrogen in the wastewater. The wastewater contained heterogenic populations of bacteria to carry out nitrification and denitrification reactions as well as carbon metabolism. The nitrifying organisms dominated the system during the aerobic operation of the reactor. This was evidenced by the data on removal of ammonia in the sludge wastewater (Figure 3).

The denitrifying organisms dominated the system during the anaerobic operation of SBR. This was supported by the fact that the levels of nitrite and nitrate dropped significantly under the anaerobic phase and eventually it reached below 5 mg/L (Figures 4 and 5). The carbon was effectively removed under both aerobic and anaerobic conditions in the SBR as shown in Figure 6. However, carbon was added in the SBR periodically in the form of molasses to maintain the C:N ratio of 10:1 as indicated by Roy et al. (2010). Similar results were demonstrated earlier by Boopathy et al. (2007) in a SBR treating low-salinity shrimp aquaculture wastewater. At the end of the operation the wastewater can be recycled back into shrimp production as shown in Figure 2. The application of SBR technology for intensive shrimp production is an attractive alternative to various methods currently used in shrimp aquaculture.

Table 2 Restriction Fragment Length Polymorphism (RFLP) of colonies isolated from the SBR and Control reactors and percentage of sequence identity with GenBank Data Base

Isolate Id	Reactors	Per cent sequence identity to closest relative (accession number in bracket)	
1	SBR 1	99%	<i>Paracoccus</i> sp R-24665 (AMO84107)
2	SBR 2	98%	<i>Paracoccus denitrificans</i> ATCC19367 (Y16930)
3	SBR 1	100%	<i>Paracoccus</i> sp WB1 (AF526892)
4	SBR 2	99%	<i>Paracoccus aminovorans</i> (D32240)
5	Control 1	99%	<i>Pseudomonas</i> sp E102 (AF451270)
6	Control 2	100%	<i>Pseudomonas</i> sp C10-2 (DQ088664)
7	SBR 1	100%	<i>Aeromonas</i> sp m22 (DQ219814)
8	SBR 2	99%	<i>Aeromonas hydrophila</i> 45/90 (AF468055)
9	SBR 1	99%	<i>Comamonas</i> sp JS46 (AY819705)
10	SBR 2	99%	<i>Flavobacterium mizutaii</i> DMS 11724T(AJ438175)
11	SBR 2	99%	<i>Nitrosomonas europea</i> (NP841017)
12	SBR2	98%	<i>Nitrosomonas europaea</i> (YP748265)
13	SBR 2	97%	<i>Nitrosococcus mobilis</i>
14	SBR 2	96%	<i>Nitrospira</i> spp.

3.3 Microbiological analysis

Bacteria were grown on tryptic soy agar (TSA) under both aerobic and anaerobic conditions. However, as the appearance of the colonies was the same in all media and conditions, they were routinely cultivated aerobically on TSA medium.

Thirty six predominant colonies were isolated from the SBR and control reactor samples and reduced to 14 restriction fragment length polymorphism (RFLP) profiles by RFLP analysis. Although the colonies isolated in each bioreactor sample showed different RFLPs, some appeared in more than one (Table 2). The most frequent bacteria isolated from all bioreactors were members belonging to *Paracoccus* and *Pseudomonas*. The most widely represented phylum (11 members) was that of the Proteobacteria, with bacteria from the Alfa proteobacteria (*Paracoccus*), Gamma-Proteobacteria (*Pseudomonas* and *Aeromonas*) and Beta-Proteobacteria (*Comamonas*) classes. The remaining bacteria are distributed in equal proportions among the Bacteroides (*Flavobacterium*) and Actinobacteria phyla. PCR amplification experiments showed that all isolated denitrifying bacteria harbour the *nosZ* gene, responsible for the last step in the denitrification pathway (reduction of nitrous oxide to molecular nitrogen (data not shown)).

CONCLUSIONS

This study showed successful removal of ammonia, nitrate, and nitrite in a high intensity shrimp raceway system by a SBR. The removal efficiencies of all nitrogen species were more than 95% and the treated wastewater was successfully recycled in the shrimp production system. The operation of SBR is simple and it only needs an addition of molasses as a carbon source because the shrimp wastewater was carbon limiting. In order to

complete the denitrification process the C:N ratio should be maintained at 10:1. Molasses is an inexpensive carbon source, which makes the system economical. The advantage of using SBR technology is its simplicity. A SBR is a variation of the activated sludge process. This process uses multiple steps in the same reactor and the nitrogen removal was accomplished by the sequential operation of the reactor aerobically followed by anaerobic process. Microbial analysis showed diversity of microbes present in the shrimp wastewater including nitrifiers, denitrifiers, and heterotrophic bacteria and so the reactor did not need start up period and inoculum addition and acclimation.

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