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Evaluation of polyhydroxybutyrate as a carbon source for recirculating aquaculture water denitrification

Maria Teresa Gutierrez-Wing[a,*], Ronald F. Malone[b], Kelly A. Rusch[c]

[a] Department of Civil and Environmental Engineering, Louisiana State University, 3520a Patrick F. Taylor Hall, Baton Rouge, LA 70803, USA
[b] Department of Civil and Environmental Engineering, Louisiana State University, 3502B Patrick F. Taylor Hall, Baton Rouge, LA 70803, USA
[c] Department of Civil and Environmental Engineering, Louisiana State University, 3520b Patrick F. Taylor Hall, Baton Rouge, LA 70803, USA

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ABSTRACT

The effect of salinity, dissolved oxygen and NO_3-N concentration on the denitrification of recirculating aquaculture water using polyhydroxybutyrate (PHB) was evaluated. Four PHB media with different molecular weights and configurations were tested. The results show that at higher nitrate concentrations in the influent water, the consumed PHB:NO_3-N ratio decreased. An average of 2.9 g of PHB:1 g NO_3-N removed at temperatures of 20.8 ± 1.1 °C was measured.

Although the molecular weight showed an apparent correlation with the denitrification rates, the correlation was not statistically significant. A moderately biofouled granular media displays a heterogeneity of microenvironments that allow some denitrification to occur in the presence of bulk dissolved oxygen levels approaching 5 mg L^{-1}. As a practical approach, the inhibitory effects of oxygen can be mitigated either by design of the denitrification media bed and/or by control or reduction of the influent dissolved oxygen levels. The high plastic consumption needed for oxygen removal indicates that the second approach is more cost efficient.

At a flux of 60 m^3 m^{-2} d^{-1} the denitrification rate decreases at a constant rate in the first 30 cm of the PHB bed. Below this depth, the denitrification rate decreases very slowly and stays above 1 kg-NO_3-N m^{-3} d^{-1}.

In a pragmatic sense, denitrification abilities can be expected to be similar in all salinities. Volumetric nitrate removal rates in the order of 2.5 kg-NO_3-N m^{-3} media d^{-1} should be broadly obtained in fresh and marine water systems. In the range of up to 250 mg NO_3-N L^{-1}, the PHB can be used as a base for a passive denitrification unit that requires little management.

The availability of an economic source of PHB such as production waste and the development of the bioplastic industry is determinant for the adoption of this material as a carbon source for denitrification processes.

1. Introduction

Recirculating aquaculture systems commonly have an aerobic fixed film biofiltration component (Akhbari et al., 2011; delos Reyes and Lawson, 1996; Greiner and Timmons, 1998; Malone and Beecher, 2000; Schreier et al., 2010; Wortman and Wheaton, 1991) in which an inert substrate is provided for the growth of a biofilm. The biofilm oxidizes organic matter and converts ammonia to nitrates. Without an anaerobic component, nitrate can accumulate and limit water reuse (Gutierrez-Wing and Malone, 2006; Van Rijn et al., 2006) and in some cases affecting the growth and survival of the aquatic organisms (Environment_Canada, 2003; Hamlin, 2006)

Among the strategies used in aquaculture to limit nitrate accumulation; water exchange and heterotrophic bacterial denitrification are the most common. The maximum nitrate level on recirculating systems depends on the rate of water exchange or the efficiency of the denitrification process (Van Rijn et al., 2006). In marine recirculating systems, denitrification has been focused on fixed film (Chu and Wang, 2011; Park et al., 2001; Sauthier et al., 1998; Van Rijn et al., 2006).

Bacterial denitrification is a respiration process in which bacteria use the nitrogen oxides such as nitrates as electron acceptors. The efficiency of the process depends on an adequate supply of organic carbon that can act as an electron donor for the reduction of nitrate, to nitrite and finally to nitrogen gas (Healy et al., 2006; Park et al., 2011). Most of the denitrification bacteria are facultative anaerobe heterotrophs for which the presence of oxygen, a more energetically efficient electron acceptor, inhibits the denitrification process (Fig. 1) either by direct competition or by enzyme inhibition (McKenney et al., 2001; Rysgaard et al., 1999). In natural systems,

* Corresponding author. Tel.: +1 225 578 5703; fax: +1 225 578 4945.
E-mail addresses: mgutie5@lsu.edu (M.T. Gutierrez-Wing), rmalone@lsu.edu (R.F. Malone), krusch@lsu.edu (K.A. Rusch).

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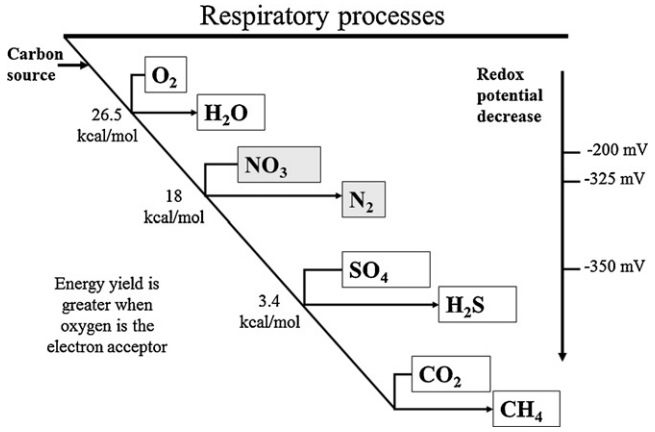

Fig. 1. Respiratory processes and relative energy yields. The values of redox are uncorrected.
After Van Rijn et al. (2006) and Lee et al. (2000).

factors such as salinity (Rysgaard et al., 1999), pH (Wijma et al., 2004) or the presence of other ions (Joye and Hollibaugh, 1995; Kemp et al., 1990) have been shown to affect the nitrogen cycle and particularly denitrification.

The carbon source used as an electron donor for the denitrification process impacts the conversion rates of nitrate to nitrogen. The most commonly used carbon sources for denitrification in aquaculture are methanol, acetate and fermented sludge. These sources are water soluble and are readily available to bacteria, but require a careful dosing to prevent the formation of toxic sulfides (Lee et al., 2000; Whitson et al., 1993) as the bacteria depletes nitrates and uses sulfate as electron donor as the redox potential decreases. The dosing of soluble carbon requires constant supervision and/or controlled supply (Balderston and Sieburth, 1976; Kaiser and Schmitz, 1988; Lee et al., 2000; Lin et al., 2002; Magram, 2010; Whitson et al., 1993).

The management of liquid or water soluble carbon sources for denitrification increases the cost of the process and sometimes will be overlooked in busy aquaculture operations. A passive control system, requiring less supervision, can reduce the management needed for its successful operation. Biodegradable non-water soluble polymers such as PHA (Polyhydroxyalkanoates) can be a suitable carbon source for denitrification.

The polyhydroxyalkanoates (PHAs) are a family of bacterially produced bioplastics, non-water soluble, non-toxic (Reddy et al., 2003) and biodegradable by naturally occurring bacteria (Fig. 2). These bioplastics can be produced with a wide range of physical and chemical properties that will allow them to substitute some commodity petroleum based plastics (Williams and Peoples, 1996). A wider use of PHAs will result in larger quantities of these materials available at lower cost. PHAs can be classified as a type of "refractory" organic or more simply stated an organic that slowly breaks down in the aquatic environment. The most common of the PHAs is the polyhydroxybutyrate (PHB). The degradation of PHB is mediated by extracellular PHB depolymerases, produced by groups of bacteria that have the ability of utilize the polymer as a carbon source. Among these bacteria, there are several groups capable of

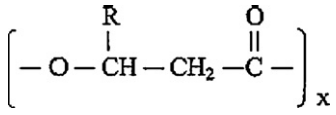

Fig. 2. General formula of the major polyhydroxyalkanoates produced by bacteria. The R group in PHB is methyl.
Adapted from Reddy et al. (2003).

using nitrates as electron acceptors in their respiration processes, including *Comamonadaceae* (Khan et al., 2002), *Brevundimonas* and *Acidovorax* (Mergaert et al., 2001). All these groups can use oxygen as an electron acceptor and switch to nitrate when the conditions are limiting.

Polyhydroxybutyrate (PHBs), a solid, non-water soluble biopolymer can be used as an alternate self-regulating carbon source, requiring less management of the system (Boley and Müller, 2005; Boley et al., 2000; Hiraishi and Khan, 2003; Mergaert et al., 2001). A passive system can promote a stable water quality in recirculating aquaculture systems and potentially reduce the nitrogen output from these facilities to natural water bodies. Boley et al. (2000) found that the denitrification process using pelleted polymers as carbon source in freshwater aquaculture was a user friendly and simple process, hindered only by the high cost of the polymers. The ongoing price reduction and increase in production of PHB makes this polymer appealing for the denitrification process. The increase in the production of saltwater species, more sensitive to nitrate, may be a suitable area to introduce the PHB denitrification.

In this study, denitrification rates with four different polyhydroxybutyrate (PHB) based media, were tested in laboratory denitrification units. The suitability of PHB as a non-water soluble carbon source for bacterial denitrification, particularly in recirculating saltwater systems was explored. Denitrification rates and bioplastic consumption were determined.

2. Materials and methods

A suite of experiments were performed to investigate the effect of three parameters on the water denitrification rates using polyhydroxyalkanoates as a solid non-water soluble media: Experiment 1: effect of molecular weight in the denitrification rates at 15 ppt ($g\,L^{-1}$) salinity; Experiment 2: effect of salinity on the denitrification rates; and Experiment 3: effect of the media bed depth on the denitrification process. Simultaneous with Experiment 1, the consumption of PHB per gram of oxygen removed was measured in separate batch reactors. In Experiment 2, the PHA consumption per unit of nitrate nitrogen removed was estimated. The PHA used is polyhydroxybutyrate (PHB). In Experiment 3, the effect of oxygen on the denitrification process was also evaluated.

2.1. Denitrification media

PHB supplied by Metabolix Inc., identified as MBX-A, MBX-B, MBX-C and (referred in this work as A, B and C respectively) with bead volumes greater than 15 mm^3 were used in this work to determine the denitrification rates. Additionally a PHB with plasticizer was also tested (MBX-W, referred in this work as W). Preliminary experiments showed that the management of the reactors with beads of an average size smaller than 15 mm^3 was more difficult due to the tendency of the bioplastics to clump together even with low volumes of biofilm, so media of sizes larger than 15 mm^3 were used. The specific surface area for each media ($m^2\,m^{-3}$) was determined by gas sorption. The molecular weight of all the media used (Table 1) was determined by Metabolix Inc. and confirmed by GPC analysis.

2.2. Experiment 1

In Experiment 1, media A, B and C were used to determine the effect of the PHB molecular weight on the denitrification rates at 15 ppt salinity in a recirculating system. A comparison was made with media W. The experimental denitrification system consisted in four columns, 5 cm in diameter and 60 cm in length, supplied with water from a 0.3 m^3 reservoir (Fig. 3). The feed water for

Table 1
Specific surface area per unit volume ($m^2\ m^{-3}$) and molecular weight (M_w) of the four PHA media used in this study (12 beads/media were measured for surface area).

Media	Media ($m^2\ m^{-3}$)		M_w	
	Average	St. dev.	Average[a]	St. dev.
A	930	355.9	58,344	27,753
B	929	638.7	7091	3545
C	552	55.2	132,123	61,799
W	911	244.1	522,702	200,268

[a] Data supplied by Metabolix Inc.

the experiments was prepared with Instant Ocean®, with SeaChem Reef Plus® trace nutrients solution and 0.5 ppm of phosphorus.

The flow velocities to the denitrification reactors were initially set according to the parameters recommended by (Metcalf & Eddie Inc., 2003) for anaerobic reactors ($60\ m^3\ m^{-2}\ d^{-1}$). An acclimation period was allowed until denitrification was observed (2–7 days). The flow was adjusted initially to control the oxygen concentration and the media clumping in the columns. The target oxygen concentration was $<0.3\ mg\ L^{-1}$ as preliminary experiments have shown low denitrification above this value during the acclimation of the media. The flow rates were adjusted again to $60\ m^3\ m^{-2}\ d^{-1}$ once the experiment started. The nitrate was measured with a colorimetric kit, until the nitrate concentration began to drop, indicating that the denitrification bacteria were acclimated.

After acclimation, the water on the experimental system was substituted with water with the desired salinity and concentration of nitrate nitrogen (NO_3-N) purged with helium to minimize the oxygen content. Samples of the PHB beads to estimate weight per unit volume were collected at the start of the experiment. The samples were rinsed to remove the surface biofilm, dried and weighted. Temperature, dissolved oxygen, and salinity were measured daily. The experiments were conducted with no illumination. The oxygen content and salinity of the water was measured with an oxygen meter (YSI 85). The NO_3-N concentrations were measured by the APHA (1998) Standard Methods 4500-NO_3 (ion chromatography; Dionex 275 IC) 24 h after mixing and the measured value was considered the initial concentration. Samples for nitrate were taken daily. Nitrite concentrations were measured by the Standard Method 4500-NO_2 B colorimetric method (APHA, 1998).

In the few cases when the evaporation was significant, the salinity was adjusted with deionized water and the resulting water analyzed. Water samples were taken from the feed tank for nitrate and nitrite determination daily. From this point on, the nitrates were measured in the ion analyzer. Sulfates were also measured in the ion analyzer for the duration of the experiments. The experiments were performed at room temperature ($20 \pm 2\ ^\circ C$) and an initial NO_3-N concentration of $150\ mg\ L^{-1}$, in triplicate. The temperature was recorded at 15 min intervals. Preliminary experiments at 25 and 30 °C (results not reported here), showed similar PHA/oxygen consumption rates of PHB with oxygen than those observed at 20 °C. The PHB consumed per gram of nitrate nitrogen removed was evaluated measuring the initial and final dry weight of the bioplastic, and comparing this value with the nitrate removed.

Simultaneous with Experiment 1, the ratio of PHB consumed for oxygen removal in the initial stages of the denitrification process was estimated. For this estimation, BOD bottles with a water volume of 300 ml and 1 ml of plastic were incubated at 20 °C in the dark. The plastic weight for each bottle was recorded initially. One bottle was open daily and the dissolved oxygen measured with a probe. The weight reduction of the plastic was recorded each day and compared with the oxygen consumed. The average and standard deviation of the consumed plastic was calculated. The denitrification and oxygen removal rates with media of different molecular weight were compared though an ANOVA analysis.

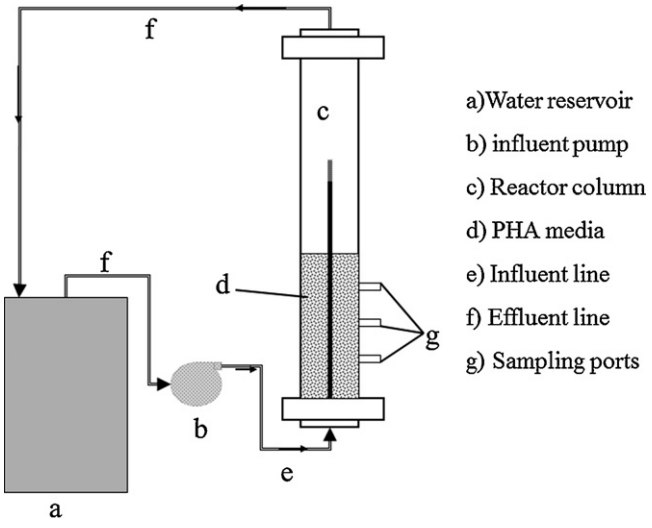

Fig. 3. System diagram for Experiments 1 and 2.

2.3. Experiment 2

The media MBX-W was used to determine the denitrification rates at four salinities (0, 5, 15 and 30 ppt) and two nitrate concentrations (50 and $220\ mg\ NO_3$-N L^{-1}) at $21.8 \pm 1.1\ ^\circ C$ in a recirculating system. The temperature was controlled by a water bath where the reactors and feed tanks with six 1000 W aquarium water heaters with a custom made controller and one 1 HP chiller (Delta Star®; Aqualogic®). The concentrations selected represent average and upper limit concentrations for aquaculture operations (Gutierrez-Wing and Malone, 2006; Hamlin et al., 2008; Mallasen et al., 2004). The experiments were conducted in triplicate. The system was similar as the one described for Experiment 1. The measurement of temperature, salinity and dissolved oxygen and the collection of samples followed the protocols described in Experiment 1. The plastic consumption was estimated in duplicate in each column first for oxygen reduction and then when oxygen was exhausted for nitrate reduction. Nitrate reduction was calculated daily based on the observed concentration in the system. The denitrification rates for all salinities were compared through and ANOVA analysis.

2.4. Experiment 3

Media W was used to determine the effects of media bed depth on the denitrification process and the single pass nitrate reduction rates. The system used in this experiment consisted of six 2.5 cm diameter reactors 20 cm in length, connected in series with 50 ml of media in each reactor (10 cm bed depth). The flow rate was set up at $60\ m^3\ m^{-2}\ d^{-1}$ according to the specifications of (Metcalf & Eddie Inc., 2003) for anaerobic reactors. Samples were collected daily after each reactor. The NO_3-N concentration was measured after each reactor, to determine the denitrification rate at different bed depths in single pass operation. The experiment was conducted at room temperature ($20 \pm 2\ ^\circ C$). The preparation of the media was as described in Experiment 1. Simultaneous with this experiment, an attempt was made to acclimate the denitrification media in water at purged with helium at $30.8 \pm 1.0\ ^\circ C$ and $35.0 \pm 0.4\ ^\circ C$, to determine if the acclimation can be done directly with nitrate, without the initial presence of oxygen, a more favorable electron acceptor.

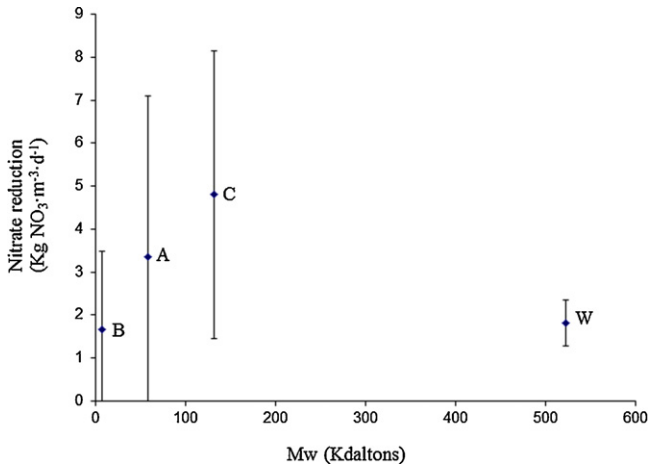

Fig. 4. Nitrate conversion with PHB of different molecular weights (Experiment 1).

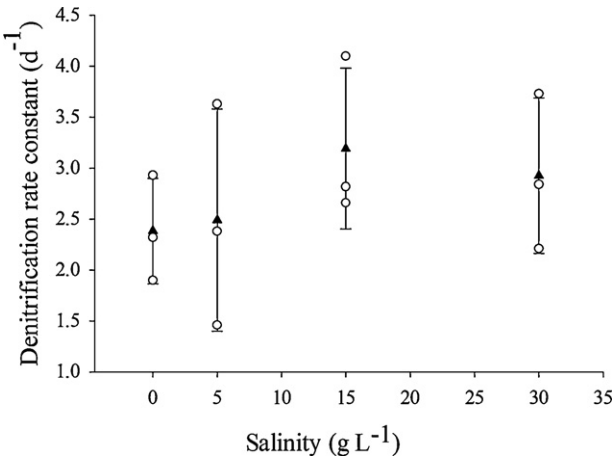

Fig. 5. Nitrate reduction rate constants in the exponential phase for 0, 5, 15 and 30 ppt salinity. The denitrification rate constants are for a non-linear exponential decay fit.

An ANOVA analysis was used to compare the denitrification rates at different media depths.

3. Results and discussion

The effect of PHB molecular weight, salinity, media bed depth and oxygen levels in the denitrification process was explored. In the experiments performed, the denitrification process did not show any lag due to the acclimation of the reactors immediately before the start of the experiments. No sulfate reduction was observed in any of the experiments, even when the nitrate was depleted.

3.1. Experiment 1

Results of the denitrification experiments with the first three media (A, B and C) showed an apparent correlation of the denitrification rate in the exponential phase with the molecular weight. Media W, with a much higher molecular weight, but with a plasticizer added did not show a higher denitrification rate (Fig. 4). The decrease in denitrification with media W may be due to a lower degradation rate of this media. This concurs with previous research that shows a decrease in the degradation rate of PHB with plasticizers in anaerobic environments (Savenkova et al., 2000).

Although an apparent increase of denitrification rate was observed with increasing molecular weight, the difference was not statistically significant due to the high variability observed within replicates of the same media. No correlation was observed between the specific surface area and the denitrification rates. This may be due to the small size of the pores in the media that increase the specific surface area, but are not large enough to harbor bacteria. The rates obtained with all the media studied showed that PHBs with molecular weights ranging from 7000 to >500,000 can support the denitrification process with similar or higher rates than those reported in literature (Boley and Müller, 2005; Boley et al., 2000; Hiraishi and Khan, 2003; Menasveta et al., 2001; Van Rijn et al., 2006).

Studies performed with media A, B and C showed that the oxygen removal rate per gram of media in the reactor was statistically different ($p < 0.0001$). Media MBX-B reduced 7.2 ± 5.3 mg O_2 (g of media)$^{-1}$ d^{-1}, while MBX-A and MBX-C achieved $1.8 \pm$ and 1.1 ± 0.6 mg O_2 (g of media)$^{-1}$ d^{-1} respectively. These results show an opposite trend with the nitrate reduction, with the higher oxygen consumption at higher molecular weight of the PHB. The initial plastic consumption for oxygen reduction was highest for MBX-B with 21.8 mg PHB (mg O_2)$^{-1}$. The average of plastic consumption for initial deoxigenation was 20.7 mg PHB (mg O_2)$^{-1}$. The plastic consumption rate after the oxygen was reduced below 4 mg L^{-1} showed no statistical differences among the three media.

The differences observed in the oxygen removal rates may be due to the higher surface area of the media during the initial stages of bacterial growth. As the oxygen is depleted, the bacterial biofilm grows, smoothing out the surface of the particles and limiting the impact of the clean media surface area due to roughness of the particle on the effective available area. The initial fast growth of biofilm, with a slower growth rate when the surface of the PHB has been colonized is an advantage in a passive denitrification unit, as the biofilm will capture the material hydrolyzed by the bacteria, and reduce their growth as the biofilm thickens, reducing the need for aggressive cleaning.

3.2. Experiment 2

Results from the denitrification experiments with the media W at a temperature of 20.8 ± 1.1 °C show that the average denitrification rates at the exponential phase at different salinities (Fig. 5 and Table 2) is slightly higher for 15 ppt and lower for 0 and 30 ppt, although the differences are not statistically significant ($p = 0.8397$) due to the high variability observed within the same salinity treatments. These results are similar to those observed by Fear et al. (2005) in a salinity gradient, where he found high variability on denitrification rates, but no significant differences across the salinity gradient. Preliminary experiments run at a temperature of 30.8 ± 1.0 °C and 35.0 ± 0.4 °C, purged with helium to remove the oxygen, failed to show denitrification in the acclimation period. This points out to a difficulty of the facultative anaerobic bacteria to colonize the media with nitrate as initial electron acceptor at these temperatures, where their metabolism is higher. The bacteria that colonize the media using the more energetically advantageous reaction with oxygen were able to change electron acceptor once established in the PHB and an initial hydrolysis of the plastic has started.

The results from Experiment 2 at higher initial nitrate-N concentration (220 mg L^{-1}) showed a high denitrification rate at the beginning of the experiment when the nitrate concentration was high. Denitrification rates at 220 mg L^{-1} showed higher variability than at 50 mg L^{-1}. The denitrification rate per gram of media was reduced as the nitrate-N decreased in the recirculating system (Fig. 6). This concurs with the results obtained in wastewater by Chudoba and collaborators who found an approximately linear relation between denitrification and loading rates, up to 0.6 kg-N m^{-3} day^{-1} (Chudoba et al., 1998).

Table 2
Exponential phase denitrification rate constants for all salinities at 20.8 ± 1.1 °C (Experiment 2).

Salinity	Replicate 1		Replicate 2		Replicate 3		Average	
	Coefficient	Std. error	Coefficient	Std. error	Coefficient	Std. error	Coefficient	Std. error
0	0.489	0.1426	1.550	0.2262	0.676	0.1541	0.905	0.3776
5	0.339	0.0255	0.758	0.0926	2.326	0.0585	1.141	0.6981
15	0.761	0.2048	1.816	0.1308	1.285	0.1525	1.287	0.3516
30	0.292	0.0714	1.458	0.2533	0.679	0.0825	0.810	0.3959

The total reaction considering for the denitrification process with PHB as the electron donor or carbon source, calculated from its formula and nitrate as electron acceptor with reduction to nitrogen gas and complete oxidation of the electron donor and assuming a 35% conversion to cells and 65% for energy is:

$$0.1343NO_3^- + 0.05556C_4H_6O_2 + 0.1343H^+ = 0.01430C_5H_7O_2N + 0.06000N_2 + 0.1508CO_2 + 0.1838H_2O \quad (1)$$

This represents a molecular ratio of 0.1343/0.0556 of nitrate-N converted to PHB monomer consumed. The theoretical weight ratio of PHB consumed per gram of NO_3-N converted to N_2 was calculated at 2.54 g PHB (g NO_3-N)$^{-1}$. Our experimental results show a ratio of 2.92 ± 2.70 g PHB (g NO_3-N)$^{-1}$ converted. The average PHB consumption rate is slightly higher than the stoichiometric value. This difference may be due to the consumption of some residual oxygen in the water, which can impact greatly the amount of plastic used or to impurities in the plastic. The rate of plastic consumed per gram of oxygen reduced for MBX-W was 20.5 ± 2.98 g PHB (g O_2)$^{-1}$ and was similar to that of MBX-B. The difference of the stoichiometric calculation with the average consumption of PHB in this work was 15%. These results concur with Boley and Muller that found that the reduction of oxygen increased the consumption of PCL (polycaprolactone) during denitrification in a recirculating system by 14% from the stoichiometric value (Boley and Müller, 2005).

The denitrification rates showed high variability at low oxygen concentrations, with decreasing average rates as oxygen increased (Fig. 7). When the dissolved oxygen content was maintained at 4–5 ppm, a minimum rate of 0.18 kg-N m^{-3} d^{-1} was observed in 50 cm beds for 6 days, but after this time the denitrification stopped. This may indicate that denitrification can occur in oxygen concentrations up to 4.5 mg L^{-1} but this may be due to lower oxygen concentrations near the bead or in the media bed. If oxygen diffuses throughout the bed, then denitrification may be reduced, but the concentration on the surface of the media may still allow for denitrification. The decrease in denitrification rates with dissolved oxygen (DO) concentrations above 4 mg L^{-1} to less than 10% of the maximum rate in anoxic conditions is similar to the findings by other author (Patureau et al., 2000) of 5.3% reduction at 4.5 mg L^{-1} of DO compared to a 100% at 0 mg L^{-1}. Some authors (Gao et al., 2010; Jensen et al., 1994, 2007; Plósz et al., 2003) have found that the denitrification rate improves in natural environments with the presence of oxygen even with dissolved oxygen as high as 5 mg L^{-1} or more or in the aerobic–anoxic interface (Brettar and Rheinheimer, 1992; Erickson et al., 2001) but in these cases the denitrification appear to be limited by sulfides produced by oxidation of soluble organic carbon. In our study, no sulfate reduction was observed. Nitrate can also be transformed to ammonia in an assimilatory nitrate reduction (Van Rijn et al., 2006). In our study, no ammonia was found in the system even after nitrate and nitrite were depleted.

3.3. Experiment 3

The results of the effect of media depth in the denitrification process showed that the denitrification rate has a region of rapid denitrification, in the first 30–40 cm of media depth. The denitrification rates decreased to almost a zero order relationship in the last 20 cm (Fig. 8). The two regions, one from 30 cm or less and the other more than 40 cm showed statistical differences for individual 10 cm segments ($p = 0.0054$). This can be explained by the higher concentrations of nitrate in the influent side of the columns, decreasing as the denitrification takes place.

The one pass reduction of nitrate in the denitrification reactors show a high impact of the oxygen concentration on the denitrification rates. As oxygen is depleted, the denitrification rates increase as reported by various authors (McKenney et al., 2001; Tiedje, 1988; Zumft, 1997), but even at near bulk oxygen saturation, some denitrification occurs as the bed depth increases. This denitrification may occur in anoxic areas, produced by irregular diffusion of oxygen due to differences in the biofilm (Zhang and Bishop, 1994; Zhou et al., 2008).

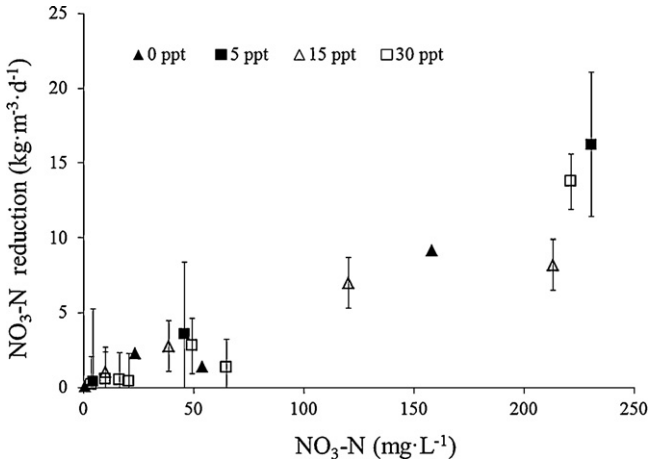

Fig. 6. Denitrification rates vs. nitrate concentration for 0, 5, 15 and 30 ppt salinity.

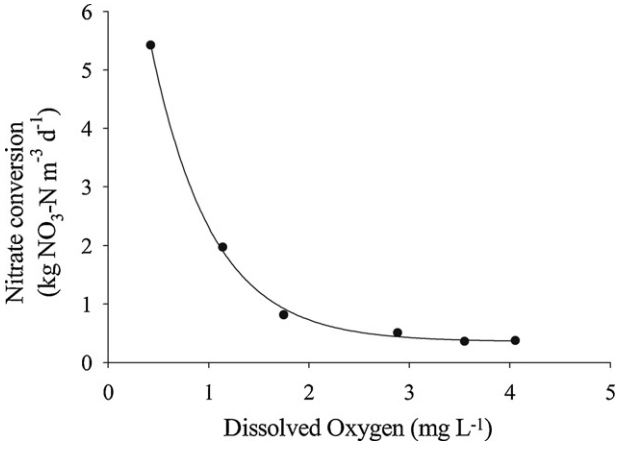

Fig. 7. Average denitrification rates (kg-NO_3-N m^{-3} d^{-1}) vs. dissolved oxygen concentrations (mg L^{-1}).

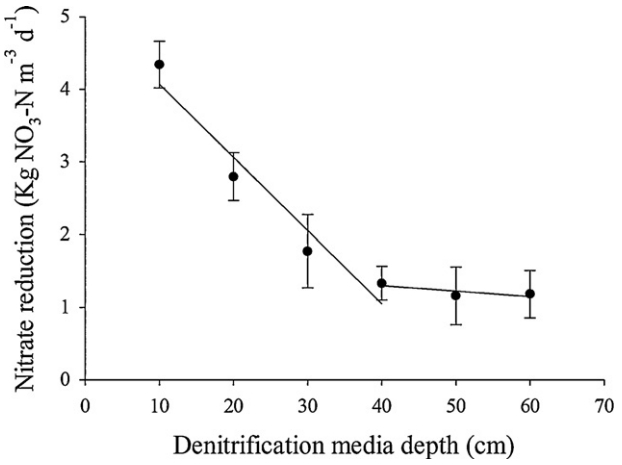

Fig. 8. Nitrate conversion rates with increasing media depth. Rates decrease rapidly in the first 30 cm, due to decreasing nitrate concentrations and then stabilizes.

The denitrification rates obtained in this work are within the range or higher than those reported in the literature (Fig. 9) for different carbon sources. The differences observed in the removal rates may be due to difference in the pore geometry of the media that allows the capture of the initial PHB degradation products in the biofilm, providing the bacteria in contact with the media a carbon source. As the oxygen is depleted, the bacterial biofilm grows, limiting the impact of the surface area as measured with liquid nitrogen adsorption, on the effective available area. Also, the more porous media may have harbored more fines in its pores, increasing the surface available for enzymatic degradation.

The high PHB consumption during deoxygenation, indicates that limiting the amount of oxygen in the denitrification bed will make the process more efficient. The oxygen reduction can take place if the denitrification step follows a nitrification reactor that reduces the oxygen content in the water. This will be compatible with most recirculating aquaculture units where nitrification reactors are commonly used.

The initial fast growth of biofilm, with a slower growth rate when the surface of the PHB has been colonized is an advantage in a passive denitrification unit. A difference of the use of PHB, compared with the use of water soluble carbon sources such as methanol, is that the carbon is released from the support media, while the nitrate is diffused from the water through the biofilm. The biofilm will capture the material hydrolyzed by the bacteria, and decrease their growth rate as the biofilm thickens, due to a lack of nitrogen, reducing the need for aggressive cleaning. In contrast, with water soluble carbon, the nitrogen and carbon gradients from the surface of the biofilm promote a low to null growth zone close to the support media, and the detachment of the biofilm as the starved bacteria dies.

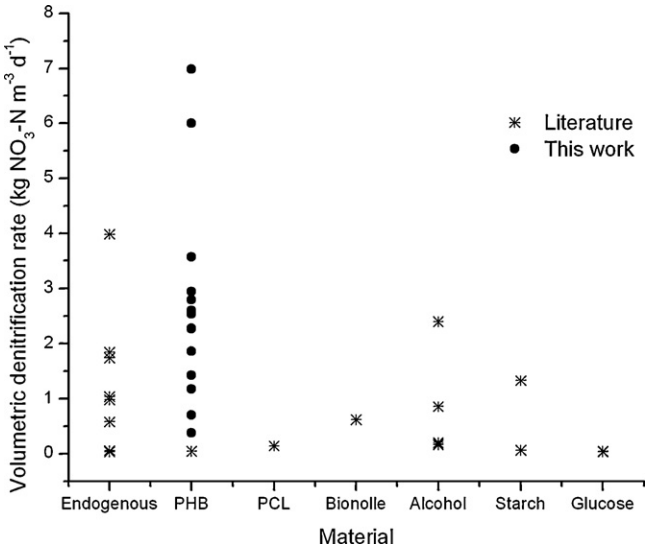

Fig. 9. Comparison of the denitrification rates obtained with all the media in this work and those reported in literature with various substrates (Ahn, 2006; Healy et al., 2006; Laurin et al., 2006; Oh et al., 2001; Ovez, 2006; Ovez et al., 2006; Saliling et al., 2007; Vredenbregt et al., 1997).

Denitrification results reported by Boley and collaborators for fresh water PHA denitrification (Boley et al., 2000) showed similar plastic consumption to those obtained in this work. Although their reduction rates are within the ranges observed in this study, a direct comparison is not possible due to the lack of information on the characteristics of the PHB. Boley et al. (2000) concluded that the cost of PHB for denitrification was too high compared with a methanol based process.

The latest reported prices for PHB are around $4.95 per kg (DiGregorio, 2009). Based on the results of this research, the cost of PHB will be around $14.35 per kg of NO_3-N reduced. The current cost of methanol is approximately $1.3 per kg, at 3.2 kg of methanol per kg of NO_3-N reduced (Metcalf & Eddie Inc., 2003). The cost of denitrification with methanol is approximately 1/3 of the cost with PHB. The prospects of the PHB prices decreasing as the industrial production is increased, along with the availability of post-consumer and process waste plastic, will make the PHB-based denitrification economically viable. The advantage of a self-regulated carbon source, the lower external control needed and the lower organic carbon added to the culture water make this an attractive option for sensitive and/or high value marine species.

4. Conclusions

After evaluating the sensitivity of PHB denitrification to a variety of factors, including type of PHB, salinity, dissolved oxygen and NO_3-N concentration, it is concluded that the process is not very sensitive to the molecular weight of the plastic or salinity of the water. Although the molecular weight showed an apparent correlation with the denitrification rates, the correlation was not statistically significant.

A moderately biofouled granular media displays a heterogeneity of microenvironments that allow some denitrification to occur in the presence of bulk dissolved oxygen levels approaching 5 mg L^{-1}.

As a practical approach, the inhibitory effects of oxygen can be mitigated either by design of the denitrification media bed and/or by control or reduction of the influent dissolved oxygen levels. The high plastic consumption needed for oxygen removal indicates that the second approach is more cost efficient.

Our experiments showed that a bed depth lesser than 30 cm, at a flux of 60 m^3 m^{-2} the denitrification increases rapidly, and in beds deeper than this, the denitrification rate remains almost constant due to lower nitrate concentrations.

An average of 2.9 g of PHB are consumed per g NO_3-N removed at room temperature (20 °C). The consumption rate is influenced by the initial nitrate concentration, the temperature and the bacteria present in the system. Although no bacterial identification was performed in this study, it was noted that when a thicker biofilm developed, the consumption rate of PHB increased. At higher nitrate concentrations in the influent water, the PHB:NO_3-N ratio decreased.

In a pragmatic sense, denitrification abilities can be expected to be similar in all salinities. Nitrate removal rates in the order of 2.5 kg-NO_3-N m^{-3} media d^{-1} should be broadly obtained in fresh and marine water systems. In this work, the PHB was the only

organic carbon source present in the system. It is expected that additional organic carbon sources can enhance the denitrification rate, lowering the PHB consumption. Higher ranges should be obtained in finely tuned systems. In this range of up to 220 mg NO_3-N L^{-1}, the polyhydroxyalkanoates can be used as a base for a passive denitrification unit that requires little management. The inverse gradients of carbon and nitrogen limit the growth of the biofilm and reduce the release of dead bacterial biomass.

The availability of an economic source of PHB such as production waste and the development of the bioplastic industry is determinant for the adoption of this material as a carbon source for denitrification processes.

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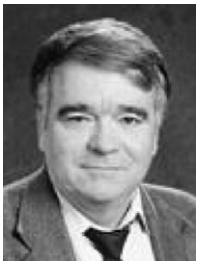
Maria Teresa Gutierrez-Wing (Ph.D., E.I.) has experience in research regarding extraction and quantification of polyhydroxyalkanoates (PHAs) from varying degrading environments and the thermal and spectrophotometric analyses of bioplastics and its degradation products. Currently she has worked on microalgae and cyanobacteria products (including PHA), culture system models, risk analysis and the study of environmental applications of novel materials. Prior to pursuing her doctoral degree at LSU, she worked for several years in water and wastewater treatment, separation and disinfection technologies for the food, aquaculture and industrial sectors.



Ronald F. Malone (Ph.D., P.E.) main interest is in the area of recirculating systems for aquaculture and wastewater treatment, biological treatment and water quality modeling. He has worked extensively in the design and development of nitrification, denitrification and solids capture methods for recirculating and flow through culture and water treatment systems, including the development of bioplastics as floating or sinking substrate and carbon source for bacteria. He teaches several graduate and undergraduate courses in the area of environmental engineering, related to water quality, modeling and water treatment.

Kelly A. Rusch (Ph.D., P.E.) works on the development of microalgae for biofuels/bioproducts and aquaculture, automated systems for the continuous production of microorganisms (microalgae and zooplankton), surface water quality monitoring/modeling and decentralized wastewater treatment and biodegradation of polymers (including polyhydroxybutyrates in natural and constructed environments. In addition, she has and continues to devote a significant amount of her time to informal engineering education (i.e., high school teacher summer institutes, research experiences for high school teachers, high school summer camps for students underrepresented in engineering, retention programs, etc.).