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A nitrogen budget for a closed, recirculating mariculture system

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Abstract

Nitrogen dynamics were evaluated in a closed, recirculating mariculture system by constructing a mass and isotopic balance of all major nitrogen pools. The experimental system consisted of 12 238-l, closed, independent, recirculating systems, each containing red drum (*Sciaenops ocellatus*), a biological filter, water pump and subsurface aeration. The standard system used in our laboratory was compared to two treatments in order to assess their influence on system nitrogen dynamics and fish growth. Treatments included: (1) increased dissolved oxygen concentration; and (2) inoculation with a commercially available probiotic advertised to enhance water quality. Concentrations of total ammonia, nitrite, nitrate, and dissolved organic nitrogen, nitrogen in suspended solids, 5-day biochemical oxygen demand and chemical oxygen demand were measured periodically throughout the experiment. Stable isotope ratio mass spectrometry was utilized to determine $\delta^{15}N$ values for the feed, fish, nitrate, solids associated with the biological filter, suspended solids and settled solids. Final mean values of chemical and isotopic measurements were not significantly different ($P > 0.05$). Additionally, wet weight gain per fish or the percent dry nitrogen assimilated by the fish were not significantly different. However, survival of fish reared in the oxygen treated tanks was lower (82% survival) compared to the fish in the control (100%)

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and probiotic (96%) treatments. The isotopic composition of all measured nitrogen pools was enriched in ^{15}N compared to the $\delta^{15}N$ of the feed entering the system. The nitrogen mass balance and isotopic data demonstrate the occurrence of denitrification in recirculating mariculture systems. Release of isotopically light nitrogen to the atmosphere via denitrification resulted in a nitrogen loss of 9–21% of the total nitrogen input. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Nitrogen; *Sciaenops ocellatus*; Isotope

1. Introduction

The aquaculture industry faces growing pressure to operate under strict environmental safety standards. High land and water costs have led to the development of aquaculture systems designed to maintain a high biological carrying capacity in relatively little space (Quillere et al., 1993; Twarowska et al., 1997). Water exchange is minimized in these systems through the use of biological, chemical, and/or mechanical filtration to maintain proper water quality. However, some water exchange via the inflow of purified water and the subsequent discharge of effluent waste is necessary even in the most efficient recycling aquaculture systems.

Effluent waste from aquaculture may contribute to eutrophication of aquatic environments through two mechanisms: (1) the direct addition of reactive organic matter; and (2) the stimulation of organic matter production through the addition of nutrients (Cho et al., 1991; Phillips et al., 1993). Accumulation of organic matter (Hansen et al., 1991) associated with aquaculture discharge can result in the development of reducing and anoxic sediments, high sediment oxygen demand, production of hydrogen sulfide and other gases, and decrease in benthic fauna (Lauren-Maatta et al., 1991; Wu et al., 1994). One way of reducing eutrophication problems is through the reduction of nutrients in aquaculture effluent. Thus, it is important to understand the cycle of nitrogen in aquaculture systems. As nitrogen is associated with the most expensive component of the feed, then the performance and efficiency of an aquaculture system can be evaluated through analysis of the conversion of nitrogen to fish biomass.

Current studies documenting nitrogen dynamics in aquaculture primarily target pond systems (Krom et al., 1985; Schroeder, 1987; Daniels and Boyd, 1989; Krom and Neori, 1989; Acosta-Nassar et al., 1994; Boyd, 1997). Nitrogen budgets of pond systems are difficult to evaluate because these systems are open to exchange through rainfall, overflow, ammonia volatilization and effects from exchange with pond sediments (e.g. seepage and cation adsorption).

One potentially important mechanism for the removal of fixed nitrogen from aquaculture systems is denitrification. Current studies do not agree on the occurrence and magnitude of the denitrification process in aquaculture systems. Daniels and Boyd (1989) estimated that $>50\%$ of the nitrogen entering via the feed was lost through the combined effects of denitrification and ammonia volatilization in polyethylene-lined, brackishwater ponds. Acosta-Nassar et al. (1994) estimated that

only 1% of the total nitrogen was lost through denitrification in a semi-intensive freshwater fish culture pond. Previous growth trials at the Fisheries and Mariculture Laboratory (FAML) in Port Aransas, Texas, have indicated nitrogen deficits of ∼45% of the total nitrogen input (Jirsa et al., 1997) in laboratory-scale, closed systems.

The purpose of this study was to understand nitrogen dynamics in a closed, recirculating mariculture system. Measurements of the concentration and isotope composition of all major nitrogen pools in the system were used to quantify the magnitude of denitrification occurring in a recirculating aquaculture system. Additionally, fish growth and system nitrogen dynamics were examined under two experimental conditions other than the standard system design, including: higher dissolved oxygen concentration and culture water inoculation with a probiotic.

2. Methods

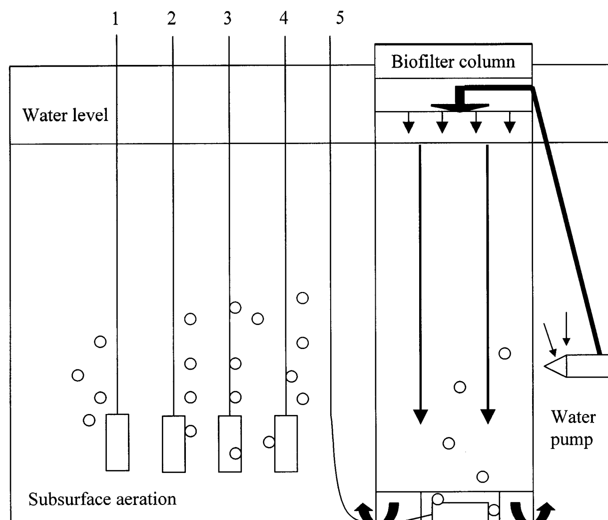
2.1. Culture conditions

An 11-week experiment was conducted in replicated recirculating aquaculture systems. These systems were open to the atmosphere but completely closed except for water addition to compensate for evaporation. The experimental system consisted of 12 cylindrical, fiberglass tanks. Each tank was an independent, closed, recirculating system containing juvenile red drum (*Sciaenops ocellatus*), 238 l of seawater, five airstones, and a biological filter (Fig. 1). Each biological filter contained ∼12 370 cm^3 of 1.6 cm ‘Flexrings’ (Aquatic Ecosystems, Apopka, FL) with a specific surface area of 344 m^2/m^3. Each filter was equipped with an electric pump (Maxi Jet MJ 1000; Aquarium Systems, Mentor, OH). Water was pumped from a point at mid-depth in the tank onto a distribution plate, which was placed above a partially submerged column containing the biological filter and media. A single 3.5 × 7.0 cm airstone (Sweetwater; Aquatic Ecosystems) was placed in the bottom of the biological filter to provide mixing and aeration (Fig. 1). The flow rate through the filters was ∼753 l/h (60 cycles/day). Fluorescent lighting was used to produce a 12:12 h light–dark cycle. The pH was regulated by the addition of dissolved soda ash to maintain the pH = 7.5. Water temperature was regulated to 27–28°C with one 300 W, submersible, electric heater per tank (Visitherm Type VTH 300; Aquarium Systems).

The 11-week growth trial was conducted using red drum juveniles raised from eggs produced by broodfish at the University of Texas Marine Science Institute’s Fisheries and Mariculture Laboratory (FAML). Prior to experiment initiation, fish were sorted for uniform size and acclimated to the system and diet for 23 days. During this period the fish were twice exposed to nitrofurazone at a concentration of 1.0 mg/l to presumptively treat for bacterial infection. The biofilter media were maintained in an operating system at FAML before introduction into the experimental system. Prior to use, the biofilter media were rinsed with seawater to remove accumulated solid matter. All tanks were cleaned prior to filling with seawater that

had been filtered through a 25-μm cartridge filter. Initial seawater and the culture system without the biological filter media were sterilized by the addition of 0.04 ml/l of 5.25% (by mass) sodium hypochlorite. After sterilization, the system was dechlorinated by the addition of sodium thiosulfate. No water was exchanged during the experiment. Approximately 15–20 l of pre-sterilized and dechlorinated freshwater were added to each tank every 10 days to compensate for evaporative losses. Chloroquin (10 mg/l) and copper sulfate (1 mg/l) were each added twice during the experiment to reduce the chance of Amyloodinium infection (Lewis et al., 1988) and reduce phytoplankton growth (Boyd, 1990).

Seven fish with an initial mean weight of 37.3 ± 0.7 g/fish were stocked per tank. A subsample consisting of four whole fish from the prestocking population (fish not yet acclimated to the feed) and four fish from the initial population were obtained and frozen at − 60°C for later analysis. A practical diet known to provide good growth was utilized throughout this experiment. The pelleted diet was formulated to contain 44% dietary protein (dry weight basis), 7.9% lipid, a digestible dietary energy to protein ratio of 8.2, and a moisture content of < 10%. Fish were fed to apparent satiation twice daily. Apparent satiation was established to be the point during a 1-h feeding session when fish ceased to actively ingest feed at or near the tank surface. Daily feed intake was recorded and fish biomass was measured



Tank dimensions = 96 cm inner diameter (ID); 40 cm total height.
Biofilter column dimensions = 19 cm ID; 51 cm total height.
Biofilter columns were partially submerged and filled with filter media to the water line.

Fig. 1. Cross section of the recirculating culture system, biological filter, and placement of the airstones used for subsurface, bubble aeration. Arrows indicate the direction of water flow through the biological filter.

bi-weekly during which the fish were weighed and dipped in dechlorinated freshwater to reduce the possibility of parasitic infection. Fish were harvested and frozen for analysis at the conclusion of the 11-week growth trial.

Three culture system variables were assigned to tanks in three randomized blocks. A standard operating system as illustrated in Fig. 1 served as an experimental control. Two other treatment conditions were evaluated. The effects of oxygen supersaturation on nitrogen cycling were evaluated by injecting pure gaseous oxygen through one of the four airstones that was suspended in the water column. The flow rate was checked twice daily and maintained at 28 320 standard cubic cm per hour with a flow regulator (Key Instruments, Trevose, PA). The effects of adding bacteria to the culture water on nitrogen transformation were evaluated using a commercially available probiotic (EM Technologies, Tucson, AZ). The probiotic solution contained a minimum of the following viable organisms: *Streptomyces albus albus* 10^5/ml; *Rhodopseudomonas sphaeroides* 10^3/ml; *Lactobacillus plantarum* 10^5/ml; *Propionibacterium freudenreichil* 10^5/ml; *Streptococcus lactis* 10^5/ml; *Streptococcus faecalis* 10^5/ml; *Aspergillus oryzae* 10^5/ml; *Mucor hiemalis* 10^5/ml; *Saccharomyces cerevisiae* 10^5/ml; and *Candida utilis* 10^5/ml. The probiotic was administered to four replicate tanks on day 4 and 37 of the experiment following the recommendations of the manufacturer.

2.2. Analytical techniques

Water quality was measured throughout the experimental period. Water samples were obtained using clean, plastic bottles. Grab samples were taken from surface water near the center of the tank. All sampling collected the minimum amount necessary for the scheduled assay. The dissolved nitrogen pool was established as the fraction of a sample that passed through a 0.7-μm nominal pore size, glass fiber filter (Whatman 4.25-cm GF/F; Springfield Mill, UK). Accordingly, suspended solids ($N_{Sus.Sol}$) were established to be the fraction retained by a pre-combusted, 0.7-μm filter.

Total ammonia–nitrogen (TAN), total nitrite–nitrogen (NO_2–N), and total nitrate–nitrogen (NO_3–N), were measured twice weekly using colorimetric methods (Spotte, 1979). Concurrent to measuring dissolved inorganic nitrogen (DIN), pH was measured using an Orion Model 290A pH meter. Dissolved organic nitrogen (DON) was measured bi-weekly throughout the experiment except for an additional measurement during week 5 and the final measurements obtained during week 11. DON was measured using the alkaline persulfate oxidation method (Solorzano and Sharp, 1980) followed by nitrate measurement as described previously.

Salinity, temperature, and dissolved oxygen (DO) were measured once weekly. Salinity was measured with a refractometer (Aquatic Ecosystems). Temperature and DO were measured with a Model 55 dissolved oxygen meter (YSI, Yellow Springs, OH). Five-day biochemical oxygen demand (BOD) and suspended solids were measured once per month (Clesceri et al., 1989). Chemical oxygen demand (COD) was measured once per month using Accu-Test COD vials (Bioscience, Bethlehem, PA). Phytoplankton biomass was estimated once monthly using fluorometric

methods to measure the concentration of chlorophyll *a* (Yentsch and Menzel, 1963).

Solids associated with the biofilter (N_{Bio}) were measured according to the following procedure. Active filter media weighing 1600 g were rinsed with seawater and stocked at the initiation of the experiment. Upon termination of the experiment, media within the biological filter were recovered completely, allowed to drain, and weighed while wet. Initially, ten acid-washed and dried Flexrings were attached end to end at $\sim$7.5-cm intervals with nylon monofilament. A single pre-weighed, replicate strand was coiled throughout each biological filter upon initial stocking. At the termination of the experiment the strands were removed from the biological filters, weighed, dried overnight at 95°C, and weighed again to provide dry matter percentages. Scrapings of the particulate matter accumulation from the strands were homogenized and analyzed for nitrogen content.

Total settleable solids ($N_{Set.Sol.}$) were measured upon termination of the experiment by agitating by hand the already-weighed biological filter media and all surfaces in the culture tank. The cleaned filter media were removed from the tank, and the remaining water was stirred by hand to attain maximum homogeneity. Two 1-l grab samples per tank were obtained using a 1-l, plastic graduated cylinder. The samples were allowed to settle at room temperature over a period of 4 h during which the supernatant was removed and replaced twice with sterile, deionized, fresh water to remove salts. The remaining supernatant was removed and the samples were dried over 48 h at 65°C prior to weighing. Final values for $N_{Set.Sol.}$ were calculated by subtracting the nitrogen content of $N_{Sus.Sol}$ and N_{Bio}.

Surface growth was measured by placing two clean, dry, and pre-weighed microscope slides suspended by nylon monofilament along the sides of each culture tank at approximately mid-depth. One artificial substrate was removed at week 6 while the second remained in the tank during the entire experiment. Upon removal, the substrates were dried overnight at 65°C, and weighed to determine the amount of surface growth. All particulate samples were saved for subsequent nitrogen analyses.

Upon termination of the experiment, a random sample of four whole fish from each tank along with the subsamples of whole fish from prestocking and initial populations were scaled, homogenized by group, and stored at -10°C. Dry weights were obtained from the whole fish matter and all particulate samples by drying to a constant weight at 80°C.

All particulate samples, including the feed, whole fish, suspended solids, organic matter collected from the biological filter, and settleable solids were analyzed using nitrogen isotope ratio mass spectrometry. Particulate samples were finely ground with an agate mortar and pestle and mixed thoroughly. Samples were combusted in a Carlo Erba 2500 Elemental Analyzer. Gases produced by the elemental analyzer entered a Finnigan MAT Delta Plus Mass Spectrometer (Bremen, Germany) set for continuous flow to determine the nitrogen content and isotope ratio of the sample. Isotopic values (δ) are expressed as the parts per thousand (‰) difference (Equation 1).

Table 1
Quantitative summary of the treatment means representing the total nitrogen input and the final recovery of dry nitrogen in grams from each of the nitrogen pools defined by Equation 3[a]

Description	Control	Oxygen	Probiotic	PSE[b]
N-input via feed	94	88.6	86.2	3.9
N-Fish[c]	31.5	25.4	29.2	1.8
N-DIN	31.1	26.6	28.6	2.1
N-DON	15	8.6	7.7	4.0
N-suspended solids	0.9	0.8	1	0.2
N-settled solids	1.2	1.9	2.3	0.6
N-biological filter	5.1	4.6	4.6	0.6

[a] Means of four replicate tanks. There were no significant differences ($P > 0.05$) between treatment means.
[b] Pooled standard error.
[c] Includes nitrogen recovered from live fish and nitrogen estimated to have been lost via mortality. N-Fish was calculated by subtracting the initial nitrogen content of the fish.

$$\delta = \left(\frac{R_{\mathrm{Sample}} - R_{\mathrm{Atmosphere}}}{R_{\mathrm{Atmosphere}}}\right)^{*} 1000, \quad (1)$$

where: $R_{\mathrm{Sample}} = {}^{15}\mathrm{N}/{}^{14}\mathrm{N}$ of the sample; and $R_{\mathrm{Atmosphere}} = {}^{15}\mathrm{N}/{}^{14}\mathrm{N}$ of the atmosphere.

Isotopic analysis of dissolved nitrate samples was achieved using an adaptation of the ammonia diffusion method (Sigman et al., 1997).

3. Statistical analysis

All data were analyzed using a one way analysis of variance (ANOVA) to determine significant ($P < 0.05$) differences among treatment means. The Student–Newman–Keuls' multiple-range test (Steel and Torrie, 1980) was used to determine the extent of these differences. Statistical analyses were conducted using the SAS System for Windows (v6.1, SAS Institute, Cary, NC).

4. Results and discussion

4.1. System performance

From both an engineering and economic perspective a primary goal of an aquaculture system is the efficient conversion of nitrogen in the food to healthy, marketable biomass. There were no significant differences between treatment means regarding feed consumption or total nitrogen input (TNI) which ranged 94.0–86.2 g nitrogen between all treatments (Table 1).

Total Nitrogen Input per tank (TNI)

= Total mass of feed given * average fraction dry atter * average fraction nitrogen in dry feed (2)

Fish with an initial mean wet weight of 37 ± 1 g (mean ± standard deviation) grew to an average 173 g/fish (range = 153–207 g/fish) over the 79-day experimental period. There were no significant differences between treatments regarding fish growth as measured by wet weight gain per fish (data not shown), dry nitrogen recovered from the fish (Table 1), or $\%N_{Fish}$ (Table 2).

The average dissolved oxygen concentration was significantly higher in the tanks injected with oxygen (7.0 mg/l) compared to the control and probiotic tanks (6.0 mg/l). Mean percent survival was significantly lower in the oxygen treatment (82% survival) compared to the control and probiotic tanks (100 and 96% survival), respectively. The average salinity in all tanks throughout the experiment was (mean ± standard deviation) $31 \pm 1.2\%$. Addition of sterile fresh water to compensate for evaporative losses resulted in the addition of $< 0.3\%$ of TNI.

BOD increased from 1.6 to 16.3–20.8 mg/l (pooled standard error = 2.9) and no statistical differences in treatment means were observed on days 0, 33, 63, and 79. The lack of significant difference between treatment means may be the result of inherent variability in system performance through time. COD values increased from 77.7 to 247.2–347.9 mg/l (pooled standard error = 43.9) throughout the experiment, however, no statistical differences were observed in treatment means as measured on day 0, 33, 63, and 79. The lack of significant differences in COD measurements under the reported conditions indicates that none of the treatment variables tested had a significant effect on the quantity of organic matter present in the system. The inoculation of the culture water with heterotrophic bacteria in the probiotic treatment did not affect organic matter degradation.

Table 2
Treatment means representing the percent of total nitrogen input recovered from the major nitrogen pools identified in the budget[a]

Description	Control	Oxygen	Probiotic
%TNUA	9	19	21
$\%N_{Mort}$	0	3	1
$\%N_{Set.Sol}$	1	2	3
$\%N_{Bio}$	6	5	5
$\%N_{Sus.Sol}$	1	1	1
$\%N_{DON}$	16	10	8
$\%N_{Fish}$	34	28	30
$\%N_{DIN}$	34	32	31

[a] There were no significant differences ($P > 0.05$) between treatment means in all measured nitrogen pools ($n = 4$ tanks).

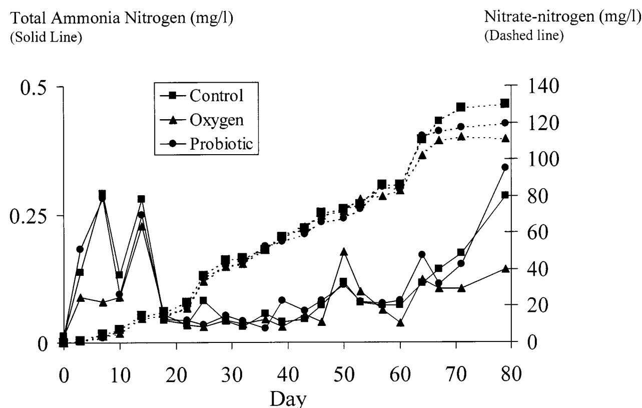


Fig. 2. Response of total ammonia nitrogen (primary y axis) and nitrate nitrogen (secondary Y-axis) in mg/l to the three treatment conditions during the experiment.

The nitrogen content of each composite pool of the nitrogen budget at the end of the experiment provides insight into the physical characteristics of potential effluent waste and the magnitude of denitrification. No significant differences were observed in the final concentration of ammonia, nitrate, or DON between treatments (Figs. 2 and 3). Nitrate levels generally increased throughout the experiment to an overall final mean value of 120.2 ± 17.9 mg/l (Fig. 2). Although no precipitous declines in the mean nitrate pool per treatment were observed, slight reductions in nitrate levels were observed in the probiotic treatment between day 57 and 60 and in the oxygen treatment between day 71 and 79. In a study conducted under similar conditions, Jirsa et al. (1997) showed a notable decline in the nitrate pool following a peak nitrate concentration of 8.0 mg/l during week 6. Processes that could account for a decline in the nitrate pool are dissimilatory nitrate reduction, phytoplankton uptake, assimilation by bacteria, or denitrification.

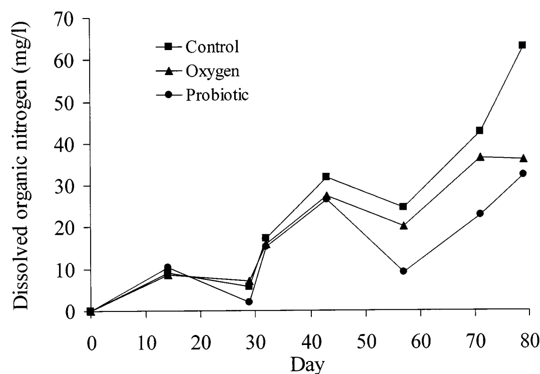


Fig. 3. Response of dissolved organic nitrogen (mg/l) to the three treatment conditions during the experiment.

Dissimilatory nitrate reduction producing ammonia and nitrite was probably not a process of great significance because ammonia and nitrite levels did not demonstrate an accretionary response (Fig. 2).

Phytoplankton abundance was controlled during the experiment by the addition of copper sulfate and reduced light levels. Average chlorophyll concentration over the entire experiment was 0.14 ± 0.08 μg chl *a*/l and did not exceed 0.27 μg chl *a*/l. Such chlorophyll levels are indicative of very low phytoplankton levels. Therefore, nitrogen uptake by phytoplankton was not a significant process.

Assimilation of inorganic nitrogen along with the degradation of organic nitrogen by bacteria are processes likely to occur in intensive, recirculating systems. Considering that bacteria generally range in size from 0.02 to 2.0 μ in size (Lalli and Parsons, 1993) and the dissolved pool was defined as the fraction that passes a 0.7 *u*m filter, then nitrogen comprising the bacterial component is split between $\%N_{DON}$ and the nitrogen measured in the various particulate pools ($\%N_{Set.Sol}$, $\%N_{Sus.Sol}$, or $\%N_{Bio}$).

Percent $N_{Sus.Sol}$ was calculated based on total nitrogen input. There was no statistical difference between final mean values per treatment regarding $\%N_{Sus.Sol}$. Surface growth accumulation on the microscope slides was less than could be measured using an electronic scale accurate to four decimal places (Fisher Scientific, Model A-200DS).

4.2. Nitrogen recovery

Average percent total nitrogen recovered per treatment with respect to the nitrogen pools defined by Equation 3 is presented in Table 2.

$$\begin{aligned}&\%\text{Total Nitrogen Recovered (TNR; per tank)}\\&= \%N_{Fish} + \%N_{DIN} + \%N_{DON} + \%N_{Set.Sol.} + \%N_{Sus.Sol} + \%N_{Bio} + \%N_{Mort}\end{aligned} \quad (3)$$

Individual components of the nitrogen budget were normalized to the TNI for each tank and are expressed as a percentage of TNI.

Average total nitrogen recovery (TNR) per treatment ranged from 91.4 to 79.3% between treatments. There was a substantial nitrogen deficit as determined by the average percent total nitrogen unaccounted per treatment (%TNUA).

$$\begin{aligned}&\%\text{Total Nitrogen Unaccounted (TNUA)} = 100 - \%\text{TNR}\\&\%\text{TNUA is referred to as } \%N_{Denit} \text{ in Equation 5.}\end{aligned} \quad (4)$$

Average %TNUA per treatment ranged from 9 ± 5 to 21 ± 2 (Table 2).

Bulk nitrogen recovered as DIN and nitrogen retained by the fish comprised the two largest pools of nitrogen in the budget. There were no significant differences in the treatment means with respect to percent total nitrogen recovered, $\%N_{Fish}$, $\%N_{DIN,}$ $\%N_{DON}$, $\%N_{Set.Sol}$, $\%N_{Sus.Sol}$, or $\%N_{Bio}$. Increasing the dissolved oxygen concentration or inoculating the culture water with a probiotic under the reported conditions did not affect %TNR.

Data collected from two tanks in the control treatment demonstrated %TNR very near 100% (101.8 and 96.4%) and are believed to represent outliers from the overall mean %TNR. Given the relative consistency of all other components of the nitrogen budget, variability in the magnitude of $\%N_{DON}$ as affected by the final DON concentration was suspected as a possible factor explaining increased %TNR.

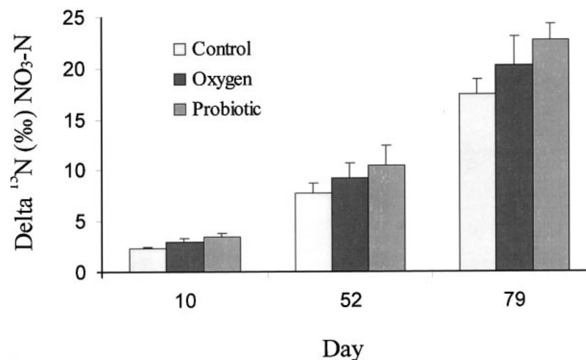


Fig. 4. Response of $\delta^{15}N$ (‰) of the nitrate pool for the three treatments during the experiment. Error bars indicate standard error of the mean. Note: $n = 4$ for all treatments on all days tested except $n = 3$ for the control treatment measured on day 79.

DON is indirectly determined as the difference between total dissolved nitrogen (TDN) and total DIN prior to oxidation of the sample. High variability in DON measurements are likely the result of small errors in the determination of TDN and total DIN which ultimately lead to a large error in the total amount of DON (Hopkinson et al., 1993). Accordingly, duplicate measurements were taken on the final DON pool to increase the number of replicated samples.

The lack of significant differences in treatment means regarding BOD and COD, final DON concentration (Fig. 3), and suspended solids along with the similarity in the ammonia and nitrate–nitrogen profiles between treatments through time (Fig. 4), suggest that neither oxygen supersaturation nor inoculation of the culture water under the reported conditions had an effect on system performance with regard to water quality. Furthermore, lack of significant difference in the treatment means with respect to fish growth (as measured by $\%N_{Fish}$ and wet weight gain indicates that the presence of the probiotic and increased oxygen levels had no effect on the overall growth of the fish compared to the control treatment. Based on these results the use of EM probiotic under the reported conditions was of little value.

The development of anoxic microsites in the sediment have been proposed as likely sites for denitrification (Brandes and Devol, 1997). In the present discussion microsites refer to zones within the culture system where particulate matter has accumulated. Diffusion of oxygen across a boundary layer surrounding a microsite is directly related to the concentration gradient. It is, therefore, possible that the augmentation of the oxygen concentration from 6.0 to 7.0 mg/l, a difference of only 1 mg/l, did not significantly decrease denitrification in the oxygen treatment compared to the control. Increasing dissolved oxygen levels > 10 mg/l using a
mechanical aspirator resulted in a poor response by the fish with respect to feed consumption and growth in pilot studies. Extreme levels of oxygen supersaturation were, therefore, not tested in the present study.

Weight percent nitrogen values for all non-dissolved nitrogen pools including feed, fish, biofilter sludge, settleable solids, and suspended solids were measured. Dry, whole-body fish samples contained the highest weight percent nitrogen content

(10.7–11.9 % nitrogen) followed by biofilter sludge, which contained 3.6–4.2% nitrogen. There were no significant differences between treatment means with respect to the weight percent nitrogen content of all measured final particulate samples including, whole fish, biological filter sludge, suspended solids (1.1–1.4%), and settled solids (1.7–2.0%).

4.3. Isotopic analyses

Subsequent isotope mass balance analysis is based on the mass fraction and isotopic composition of all measured budget components relative to the feed input to the system.

$$\delta^{15}\mathrm{N}\mathit{Feed} = \left[\frac{\%\mathrm{N}_{\mathrm{Fish}}}{100}\delta_1 + \frac{\%\mathrm{N}_{\mathrm{NO_3}}}{100}\delta_2 + \frac{\%\mathrm{N}_{\mathrm{Set.Sol.}}}{100}\delta_3 + \frac{\%\mathrm{N}_{\mathrm{Sus.Sol.}}}{100}\delta_4 + \frac{\%\mathrm{N}_{\mathrm{Bio.}}}{100}\delta_5 + \frac{\%\mathrm{N}_{\mathrm{DON}}}{100}\delta_6 + \frac{\%\mathrm{N}_{\mathrm{Denit.}}}{100}\delta_7 \right]$$

$\%N_{Fish}$ includes nitrogen from live fish and mortalities, if applicable. %TNUA is referred to here as denitrified nitrogen ($\%N_{Denit}$). Subscript after δ indicates that individual δ^{15}N values measured for each respective nitrogen pool were used. δ^{15}N Feed = 6.9‰. δ_6 = Estimate of the δ^{15}N value for the DON pool is based on δ^{15}N measurements from possible sources of DON, including leaching from the food ($\delta^{15}N = 6.9$‰) and settled solids (average $\delta^{15}N = 16.8$‰). δ_7 = unknown.

The average δ^{15}N value per treatment of all measured nitrogen pools along with the measured isotopic composition of the feed is shown in Table 3. There were no significant differences between treatment means in the nitrogen isotopic composition of all measured nitrogen pools including, fish, nitrate, suspended solids, settled solids, and particulate matter recovered form the biological filter. All nitrogen pools identified in Equation 3, except $\%N_{Mort.}$ and $\%N_{DON}$ (which were not measured), were enriched in ^{15}N compared to the δ^{15}N of the feed entering the system, (Table 3).

The change in δ^{15}N of the fish from an average 11.2 to 9.6‰ (data not shown) during the acclimation period demonstrates that the fish had become isotopically stable with respect to the δ^{15}N of their diet prior to commencement of the experiment. The final isotopic composition of the fish, ~9.1–9.2‰ is consistent with the expected enrichment (ca. +3‰) typically demonstrated by heterotrophs compared to their diet (DeNiro and Epstein, 1981).

Excluding the $\%N_{Denit}$ component of Equation 5, substituting 6.9‰ for the δ^{15}N of the $\%N_{DON}$ pool (the lowest assumed value) and solving the equation with all known values measured thus far, it is evident that the overall isotopic mass of the products recovered from the system is enriched in ^{15}N compared to the feed entering the system (average δ^{15}N recovered from all tanks = 11.2 ± 0.6‰; mean ± standard deviation). The overall enrichment of the system by ~ +4.3‰ compared

to the feed entering the system is consistent with the enrichment expected for denitrification.

Technical difficulties were encountered during the final analysis of the $\delta^{15}N$ composition of the nitrate pool in one control tank (tank 1). Therefore, the $\delta^{15}N$ composition of the nitrate pool was derived from the average measurement of the remaining three control tanks (average $= 17.44 \pm 2.8$‰; mean ± standard deviation). Final analysis of the model expressed in Equation 5 for determining fractionation factors was conducted excluding data from tanks 1, 4 and 5. In tank 1, anomalously high nitrogen recovery and the observation that the isotopic mass of the measured products did not equal the isotopic mass of feed entering the system suggests an error in measurement of one or more budget components. Tanks 4 and 5 were omitted only from the final isotope analyses because the lowest possible fractionation factors (Equation 6) calculated for the denitrified pool ($\alpha = -1.057$ and -1.141, respectively) were well beyond current fractionation factors for denitrification ($\alpha = -1.010$ to -1.044‰) reported in the literature (Delwiche and Steyn, 1970; Owens, 1987; Michener and Schell, 1994). No tanks were omitted from any other analysis herein discussed.

$$\text{Fractionation factor } (\alpha) = (^{15}N/^{14}N \text{ products})/(^{15}N/^{14}N \text{ reactants}) \quad (6)$$

The $\delta^{15}N$ of the nitrate pool through time is shown in Fig. 4. The $\delta^{15}N$ of the nitrate pool increased from an average 2.94 ± 0.78‰ (mean ± standard deviation) on day 10 to 20.35 ± 4.33‰ at the conclusion of the experiment, an increase of ~17%. However, a comparable increase in $\delta^{15}N$ with respect to suspended solids

Table 3
Quantitative summary of the treatment means representing the $\delta^{15}N$ (%) of the feed and final samples from all nitrogen pools defined by Equation 3 (except DON and nitrogen lost via mortality)

Sample	Control	Oxygen	Probiotic	PSE[d]
Feed[b]	6.9	6.9	6.9	[a]
Fish[b]	9.2	9.2	9.2	0.07
Nitrate[c]	17.4	20.3	22.6	2.10
Suspended Solids[b]	14.8	14.8	14.7	0.53
Settled Solids[b]	16.7	17.2	16.6	0.50
Biofilter[b]	15.2	15.6	15.7	0.32

[a] $\delta^{15}N$ value shown for the feed represents the mean of duplicate subsamples measured from a single batch of feed.

[b] There were no significant differences ($P > 0.05$) between treatment means ($n = 4$).

[c] There were no significant differences ($P > 0.05$) between treatment means ($n = 4$ for the oxygen and probiotic treatments; $n = 3$ for the control treatment).

[d] Pooled standard error.

was not observed. The $\delta^{15}N$ of the $\%N_{Sus\ Sol}$ increased from 13.38 ± 0.31‰ on day 28 to 14.87 ± 0.94‰ at the conclusion of the experiment, an increase of ~1.5‰. A $\delta^{15}N$ enrichment of the nitrate pool by ~17‰ indicates the occurrence of a process that is selectively removing isotopically light nitrogen from the nitrate pool (Delwiche and Steyn, 1970; Owens, 1987; Michener and Schell, 1994).

4.4. Evaluation of ammonia volatilization

One potential mechanism by which nitrogen could be lost from recirculating systems is ammonia volatilization. To evaluate the potential significance of this process, the thin film gas exchange model (Broecker, 1974) was utilized and conservative assumptions were made to evaluate the loss of nitrogen due to ammonia volatilization.

Theoretically derived ammonia volatilization rates based on the thin film gas exchange model indicated that the occurrence of this process under the reported conditions contributed very little to the observed nitrogen deficit. Final results from the model demonstrated that given a sustained TAN concentration of 0.2 mg/l and a boundary thickness of 0.002 cm, then an ammonia volatilization rate of 3.87×10^{-5} moles TAN/day is calculated. This ammonia volatilization rate over a 79-day experimental period would account for $<0.25\%$ of the %TNUA.

5. Conclusion

There was a significant loss of nitrogen in 11 of the 12 tanks studied. None of the evaluated treatment conditions had a significant effect on the percent total nitrogen recovered or the magnitude of any of the identified nitrogen pools. The isotopic composition of all measured nitrogen pools demonstrated enrichment in ^{15}N compared to the feed entering the system. The 17‰ $\delta^{15}N$ increase of the nitrate pool strongly suggests the occurrence of denitrification, which removes isotopically light nitrogen from these systems through the formation of N_2 gas and release to the atmosphere. Removal of N_2 gas through denitrification is the most likely explanation for a nitrogen loss of 9–21% from these systems.

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Appendix A. Nomenclature

Acronym	Equation	Definition	Units
δ	1	Delta	Parts per thousand
TNI	2	Total nitrogen input	Grams
%TNR	3	Percent of total nitrogen recovered	Percent of total nitrogen input
$\%N_{Fish}$	3	Percent of nitrogen input retained by fish Value excludes the initial nitrogen content of the fish.	Percent of total nitrogen input
$\%N_{DIN}$	3	Percent of nitrogen input as dissolved inorganic nitrogen	Percent of total nitrogen input
$\%N_{DON}$	3	Percent of nitrogen input as dissolved organic nitrogen	Percent of total nitrogen input
$\%N_{Set.Sol}$	3	Percent of nitrogen input as settled solids	Percent of total nitrogen input
$\%N_{Sus.Sol}$	3	Percent of nitrogen input as suspended solids	Percent of total nitrogen input
$\%N_{Bio}$	3	Percent of nitrogen input accumulated in the biological filter	Percent of total nitrogen input
$\%N_{Mort}$	3	Percent of nitrogen input lost by mortality	Percent of total nitrogen input
%TNUA	4	Percent of total nitrogen input which is unaccounted. This is the same as $\%N_{Denit.}$	Percent of total nitrogen input
$\delta^{15}N$ Feed	5	Isotopic composition of feed	Parts per thousand
$\%N_{NO3}$	5	Percent of nitrogen input as dissolved nitrate. Value excludes TAN and nitrite–nitrogen which were negligible components of total DIN.	Percent of total nitrogen input
$\%N_{Denit}$	5	Percent of nitrogen input removed from the system by denitrification	Percent of total nitrogen input

References

Acosta-Nassar, M.V., Morell, J.M., Corredor, J.E., 1994. The nitrogen budget of a tropical semi-intensive freshwater fish culture pond. J. World Aquacult. Soc. 25, 261–270.

Boyd, C.E., 1990. Water Quality in Ponds for Aquaculture. Alabama Agricultural Experiment Station. Auburn, Alabama.

Boyd, C.E., 1997. Practical aspects of chemistry in pond aquaculture. Prog. Fish-Cult. 59, 87–93.

Brandes, J.A., Devol, A.H., 1997. Isotopic fractionation of oxygen and nitrogen in coastal marine sediments. Geochim. Cosmochim. Acta 61, 1793–1801.

Broecker, W.S., 1974. Chemical Oceanography. In: Broecker, W.S., Peng, T.H. (Eds.), Tracers in the Sea. Lamont-Doherty Geological Observatory. Palasades, New York.

Cho, C.Y., Hynes, J.D., Wood, K.R., Yoshida, H.K., 1991. Quantification of fish culture wastes by biological (nutritional), and chemical (luminological) methods; the development of high nutrient dense (HND) diets. In: Cowey, C.B., Cho, C.Y. (Eds.), Nutritional Strategies and Aquaculture Waste. Proceedings of the First International Symposium on Nutritional Strategies in Management of Aquaculture Waste. University of Guelph, Guelph, Ont., pp. 37–50.

Clesceri, L.S., Greenburg, A.E., Trussell, R.R., 1989. Standard Methods for the Examination of Water and Wastewater, 17th ed. American Public Health Association. Washington DC.

Daniels, H.V., Boyd, C.E., 1989. Chemical budgets for polyethylene-lined brackishwater ponds. J. World Aquacult. Soc. 20, 53–60.

Delwiche, C.C., Steyn, P.L., 1970. Nitrogen isotope fractionation in soils and microbial reactions. Environ. Sci. Technol. 4, 929–935.

DeNiro, M.J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochem. Cosmochem. Acta 45, 341–351.

Hansen, P.K., Pittman, K., Ervik, A., 1991. Organic waste from marine fish farms — effects on the seabed. In: Makinen, T., Henriksson, S.H. (Eds.), Marine Aquaculture and Environment. Nordic Council of Ministers. Copenhagen, Denmark, pp. 112–119.

Hopkinson, C., Cifuentes, L., Burdige, D., Fitzwater, S., Hansell, D., Henrichs, S., Kaehler, P., Koike, I., Walsh, T., Bergamschi, B., 1993. DON subgroup report. Mar. Chem. 41, 22–36.

Jirsa, D.O., Davis, D.A., Arnold, C.R., 1997. Effects of dietary nutrient density on water quality and growth of red drum (*S. ocellatus*) in closed systems. J. World Aquacult. Soc. 28, 68–78.

Krom, M.D., Porter, C., Gordin, H., 1985. Nutrient budget of a marine fish pond in Eilat, Israel. Aquaculture 51, 65–80.

Krom, M.D., Neori, A., 1989. A total nutrient budget for and experimental intensive fishpond with circularly moving seawater. Aquaculture 83, 345–358.

Lalli, C.M., Parsons, T.R., 1993. Biological Oceanography: An Introduction. Pergamon Press, New York.

Lauren-Maatta, C., Granlid, M., Henriksson, S.H., Koivisto, V., 1991. Effects of fish farming on the macrobenthos of different bottom types. In: Makinen, T., Henriksson, S.H. (Eds.), Marine Aquaculture and Environment. Nordic Council of Ministers. Copenhagen, Denmark, pp. 57–83.

Lewis, D.H., Wexing, W., Ayers, A., Arnold, C.R., 1988. Preliminary studies on the use of chloroquine as a systemic chemotherapeutic agent for Amyloodinosis in red drum, *Sciaenops ocellatus*. Contrib. Mar. Sci. (Suppl.) 30, 183–189.

Michener, R.H., Schell, D.M., 1994. Stable isotope ratios as tracers in marine aquatic food webs. In: Lajtha, K., Michener, R.H. (Eds.), Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, London, UK, pp. 138–157.

Owens, N.J., 1987. Natural variation in ^{15}N in the marine environment. Advances Mar. Biol. 24, 389–451.

Phillips, M.J., Lin, C.K., Beveridge, M.C.M., 1993. Shrimp culture and the environment: lessons from the world's most rapidly expanding warm water aquaculture sector. In: Samocha, T.M., Lawrence, A.L. (Eds.), Shrimp farms' effluent waters, environmental impact and potential treatment methods. UJNR Technical Report. 24, pp. 33–57

Quillere, I., Marie, D., Roux, L., Gosse, F., Morot-Gaudry, J.F., 1993. An artificial productive ecosystem based on a fish/bacteria/plant association. 1. Design and management. Agricult. Ecosyst. Environ. 47, 13–30.

Schroeder, G.L., 1987. Carbon and nitrogen budgets in manured fish ponds on Israel's coastal plain. Aquaculture 62, 259–279.

Sigman, D.M., Altabet, M.A., Michener, R., McCorkle, D.C., Fry, B., Holmes, R.M., 1997. Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: an adaptation of the ammonia diffusion method. Mar. Chem. 57, 227–242.

Solorzano, L., Sharp, J.H., 1980. Determination of total dissolved nitrogen in natural waters. Limnol. Oceanogr. 25, 751–754.

Spotte, S., 1979. Fish and Invertebrate Culture: Water Management in Closed Systems, 2nd ed. Wiley, New York.

Steel, R.G.D., Torrie, J.H., 1980. Principles and Procedures of Statistics: A Biometric Approach. McGraw-Hill, New York.

Twarowska, J.G., Westerman, P.W., Losordo, T.M., 1997. Water treatment and waste characterization evaluation of intensive recirculating fish production system. Aquacult. Eng. 16, 133–147.

Wu, R.S.S., Lam, K.S., MacKay, D.W., Lau, T.C., Yam, V., 1994. Impact of marine fish farming on water quality and bottom sediment: a case study in the sub-tropical environment. Mar. Environ. Res. 38, 115–145.

Yentsch, C.A., Menzel, D.W., 1963. A method for the determination of phytoplankton chlorophyll and phaeophyton by fluorescence. Deep-Sea Res. 10, 221–231.