Aquacultural Engineering 43 (2010) 78–82

Contents lists available at ScienceDirect

Aquacultural Engineering

journal homepage: www.elsevier.com/locate/aqua-online

Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems

David D. Kuhn[a,*], David D. Drahos[b], Lori Marsh[a], George J. Flick Jr.[a]

[a] Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[b] Research and Development Group, Novozymes Biologicals Inc., Salem, VA 24153, USA

ARTICLE INFO

Article history:
Received 14 April 2010
Accepted 12 July 2010

Keywords:
Nitrification
Nitrifiers
Nitrogen management
Ammonia
Nitrite
Shrimp
Recirculating Aquaculture
Nitrobacter winogradskyi
Nitrobacter
Nitrosomonas eutropha
Nitrosomonas

ABSTRACT

Recirculating aquaculture systems (RASs) rely on nitrification to convert toxic ammonia and nitrite to less toxic nitrate. Nitrification is accomplished using biofilters with nitrifying bacteria and can be inefficient in biofilters that are new or that have been compromised due to stressors. Failure in a biofilter can result in very high levels of ammonia or nitrite, both of which are toxic to aquatic animals and can result in health issues, suppressed growth, and mortalities. For these reasons, a commercially available nitrifying bacterial product (Pond Protect-L®, Novozymes Biologicals Inc., Salem, VA, US) was tested in both a controlled pilot-scale experiment and within production-scale RAS that were experiencing elevated levels of nitrite. Juvenile Pacific white shrimp (*Litopenaeus vannamei*) were used as the aquatic species in both the pilot- and production-scale systems. In the pilot-scale experiment, three aquaria received no product (control) and three systems received bacterial product using dosing specification as recommended by the manufacturer. Control systems experienced significantly ($P < 0.05$) higher levels of ammonia and nitrite compared to the tanks that received bacterial product. Quantitative PCR was used as tool to verify the presence of nitrifying bacteria (*Nitrobacter winogradskyi* strain in the bacterial product) in the systems that received bacterial product. Meanwhile, none of these nitrifiers were detected in the control systems. Similar nitrification benefits were noted in the production systems that received bacterial product. More specifically, improved nitrite-oxidation was observed. Within 2 days of the addition of bacterial product, nitrite levels began to decrease in the production systems and after a steady decrease remained within safe limits for the entire shrimp culture period.

1. Introduction

In aquaculture, ammonia wastes originate from animal excreta and decomposing organic solids such as uneaten feed (Stewart et al., 2006). Nitrification is the oxidation of ammonia to nitrite and nitrite to nitrate by autotrophic bacteria (Ebeling et al., 2006) and this fixed-film biological process occurs in biofilters (Sandu et al., 2002; Guerdat et al., 2010). Since nitrate cannot be oxidized any further via nitrification, nitrate will accumulate in the system. There are two common methods for reducing nitrate, using water exchanges or by implementing denitrifying biological reactors (van Rijn et al., 2006; Sharrer et al., 2007). Ammonia and nitrite are toxic to aquatic fauna at relatively low levels (e.g. acute toxicity between 3 and 6 mg/L for shrimp; Lin and Chen, 2001, 2003) and must not be allowed to accumulate within production systems because these constituents can impair animal health, growth, and survival rates (Wickins, 1976; Schuler et al., 2010). Fortunately, aquatic fish and shellfish species can tolerate relatively high levels of nitrate (e.g. chronic toxicity >200 mg/L for shrimp; Kuhn et al., 2010).

Aquaculture operations often rely on natural colonization of nitrifying bacteria in production systems. This natural method works well for initiating a biofilter, but can take a relatively long time (e.g. 4–8 weeks) to establish a healthy and viable population of both ammonia-oxidizing and nitrite-oxidizing bacteria. The nitrifying bacteria population can also be sensitive to chemical (e.g. salinity changes) and physical stresses (e.g. temperature changes) (Malone and Pfeiffer, 2006; Emparanza, 2009). Such stresses can inhibit nitrification rates resulting in spikes in either ammonia and/or nitrite. Nitrite-oxidizing bacteria are known to be especially sensitive to stresses (Singh et al., 1999).

The intent of this study was to determine if the purposeful addition of nitrifying bacteria to recirculating aquaculture systems producing shrimp could improve the nitrification efficacy of the biofilter. The bacterial product was tested in two settings as defined by Colt et al. (2006): (1) on a pilot-scale, which allowed for replicated tests and control of experimental variables (e.g. population of shrimp, feed rates, removal of moribund shrimp); and (2) on

* Corresponding author. Tel.: +1 540 231 6325; fax: +1 540 231 9293.
E-mail address: davekuhn@vt.edu (D.D. Kuhn).

0144-8609/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaeng.2010.07.001

a system-scale, where production raceways were operated under commercial management and no two systems were operated identically. It is very difficult to determine the exact population in a relatively large system-scale tank due to disappearance of mortalities and lack of methods to count individual animals. The pilot-scale experiment allowed for scientific rigor, while the commercial-scale data provided proof of concept.

The bacterial product used consists of a consortium of both ammonia-oxidizing (AOB; Mpn8-2) and nitrite-oxidizing (NOB; Mpn-2) bacteria that essentially remove ammonia and nitrite wastes by converting them to nitrate (Drahos et al., 2008). The most widely recognized ammonia-oxidizing genera known to be present in typical marine environments are the *Nitrosomonas* strains which are members of the β subdivision of the class Proteobacteria including *Nitrosomonas eutropha* and *Nitrosomonas europaea* (Argaman, 1991). It has more recently been recognized that the *Nitrosococcus* genus within the γ subdivision also plays an important role in marine nitrification (Teske et al., 1994). The two most widely identified nitrite-oxidizing genera in saltwater environments are *Nitrospira* and *Nitrobacter* (Abeliovich, 2006), particularly *Nitrobacter winogradskyi* (Zavarzin and Legunkova, 1959). *Nitrospira* strains tend to be scavengers (K-strategists) while *Nitrobacter* strains are the opportunists (r-strategists), which result in significant differences in environmental responses and in practical axenic growth characteristics (Knapp and Graham, 2007). A consortium of *N. eutropha* and *N. winogradskyi* was found to provide the optimal scenario of high cell growth and strong nitrification performance under higher salt conditions.

2. Materials and methods

2.1. Nitrifying bacteria product

Bacterial product (PondProtect-L®), a liquid consortium of *N. eutropha* Mpn8-2 and *N. winogradskyi* Mpn-2 (ATCC PTA-6232), was supplied by Novozymes Biologicals Inc. (Salem, VA, US). This commercially available bacterial product was stored at 2–4 °C until use. Quantitative real-time polymerase chain reaction (qPCR) was used as tool to verify the presence of beneficial nitrifying bacteria in the pilot-scale study. Samples were taken directly from the water column in each of the six experimental systems on day 0, 14, and 28 and preserved at −20 °C until qPCR analysis.

Quantitative real-time polymerase chain reaction analysis focused on the quantification of the NOB strain *N. winogradskyi* Mpn-2 targeting elements of the 16s rDNA sequence. The standard curve was obtained using the bacterial product in serial dilutions. DNA was extracted from 100 mL of pilot-scale system water by centrifugation (8000 × *g*; 30 min. at 4 °C), resuspension in 1 mL distilled water, purification using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) and storage of the final 50 μL sample at −20 °C until use. The yield of total DNA from treated tank samples averaged 41 ng/μL as measured spectrophotometrically at 260 nm, and 240 ng of total DNA was used for each qPCR assessment.

Quantitative PCR was performed on a Cepheid SmartCycler (Cepheid Inc., Sunnyvale, CA) using the following primers and internal oligo probe (Eurofins mwg/operon, Huntsville, AL):

Left primer (F764) 5′-GATGAATGCCAGCCCGTTAGT
Right primer (R983) 5′-CTCCGAAGAGAGGGTCACATC
Internal probe (P896) 5′ – [5-fam]TGTGGTTTAATTCGACGCAA [BHQ1a-Q]

For qPCR assessment, 0.3 μM of each primer and 0.2 μM of the probe and 240 mg of purified DNA were combined with the recommended blend of reagents in the Cepheid SmartMixHM (Cepheid Inc., Sunnyvale, CA). The samples were then heated to 95 °C for 2 min, followed by 45 repeated cycles of 95 °C for 10 s, 61 °C for 40 s, and 72 C for 20 s while fluorescence was measured at the standard FAM excitation wavelength. Standards based on 10-fold dilutions of purified NOB DNA were included to quantify the response signal.

2.2. Pilot-scale study

2.2.1. Experimental design

Shrimp were reared in six independent experimental systems over a 28-day period. The experimental design was a randomized complete block with two levels of the independent variable each with three replicates. The three control systems (systems A–C) were not supplemented with bacterial product. The treated systems (D–F) received 1.5 mL of bacterial product per 100 L (15 ppm) every day for the first week of the trial with a follow-up application of 4.5 mL per 100 L (45 ppm) once a week thereafter.

2.2.2. Pilot-scale systems

Each pilot-scale system consisted of a 150 L (0.4 m^2 footprint) acrylic aquarium (Glass Cages, Nashville, TN, US), an air diffuser, a submersible 300 W heater (Visi-therm® Delux Submersibible Heater, Marineland, Blacksburg, VA, US), KMT media (Kaldnes Inc., Providence, RI, US), and a 1890 L/h hang-on waterfall filter (110/500 AquaClear Power Filter, Hagen, Montreal, Quebec, Canada). The culture units were maintained as clear water systems with few to no algae. These systems were initiated with no active nitrification biofilter.

2.2.3. Shrimp

Juvenile Pacific white shrimp, *Litopenaeus vannamei*, were collected from a single production raceways at VSF and transferred and acclimated to the 150 L test aquaria. Shrimp were culled to 30 shrimp per aquarium, resulting in a stocking density of 75 shrimp/m^2 (0.20 shrimp/L). During the culling process, the largest and smallest shrimp were removed, resulting in a uniform shrimp size. Mean shrimp weight was 0.99 ± 0.02 g (mean ± standard error) and 0.95 ± 0.01 g for the control and treated systems, respectively.

2.2.4. Feeding regime

Shrimp were fed a commercially available shrimp feed (2.4 mm pellet, Ziegler Bros. Inc., Gardners, PA, US) with 40% crude protein and 9% lipid. Shrimp were fed at a rate of 10.75% body weight/day; feeding occurred twice a day at 08:00 and 17:00 h. Feed rates were adjusted weekly based on an estimated growth rate of 1 g/week and for mortalities. At this feeding rate, excessive feed was not observed in the treated systems. However, in control systems where shrimp health was compromised, excessive and uneaten feed was removed daily, before the 08:00 h feeding.

2.2.5. Water quality I

Culture water was formulated using well water from VSF and synthetic sea salt (Crystal Sea® Marinemix, Marine Enterprise International, Baltimore, MD, US). Salinity was targeted at 10 g/L (Table 1). Water quality parameters monitored included alkalinity, nitrite, nitrate, total ammonia, salinity, temperature, pH, and dissolved oxygen. The number of sampling events for the various water quality parameters is shown in Table 1. Alkalinity was determined using HACH method 8203 with a Digital Titrator (HACH Co., Loveland, CO, US). Nitrite-N, nitrate-N, and total ammonia-N (TAN) were measured in accordance with HACH spectrophotometric methods 8507, 8039, and 8038, respectively, using a HACH DR/2800 spectrophotometer (HACH Co.). After the second day, high levels of nitrite in the control systems interfered with nitrate and