Nitrate removal effectiveness of fluidized sulfur-based autotrophic denitrification biofilters for recirculating aquaculture systems

Laura Christianson a,*, Christine Lepine a, Scott Tsukuda a, Keiko Saito b, Steven Summerfelt a

a The Conservation Fund, Freshwater Institute, 1058 Turner Road, Shepherdstown, WV 25443, USA
b University of Maryland Baltimore County and Institute of Marine and Environmental Technology, 701 East Pratt St., Baltimore, MD 21202, USA

A R T I C L E   I N F O
Article history:
Received 6 April 2015
Received in revised form 13 July 2015
Accepted 17 July 2015
Available online 21 July 2015

Keywords:
Denitrification
Autotrophic
Mixotrophic
Sulfur
Fluidized biofilter
Recirculating aquaculture

A B S T R A C T

There is a need to develop practical methods to reduce nitrate–nitrogen loads from recirculating aquaculture systems to facilitate increased food protein production simultaneously with attainment of water quality goals. The most common wastewater denitrification treatment systems utilize methanol-fueled heterotrophs, but sulfur-based autotrophic denitrification may allow a shift away from potentially expensive carbon sources. The objective of this work was to assess the nitrate-reduction potential of fluidized sulfur-based biofilters for treatment of aquaculture wastewater. Three fluidized biofilters (height 3.9 m, diameter 0.31 m; operational volume 0.206 m³) were filled with sulfur particles (0.30 mm effective particle size; static bed depth approximately 0.9 m) and operated in triplicate mode (Phase I: 37–39% expansion; 3.2–3.3 min hydraulic retention time; 860–888 L/m² min hydraulic loading rate) and independently to achieve a range of hydraulic retention times (Phase II: 42–13% expansion; 3.2–4.8 min hydraulic retention time). During Phase I, despite only removing 1.57 ± 0.15 and 1.82 ± 0.32 mg NO₃–N/L, each pass through the biofilter, removal rates were the highest reported for sulfur-based denitrification systems (0.71 ± 0.07 and 0.80 ± 0.15 gN removed/L bioreactor-d). Lower than expected sulfate production and alkalinity consumption indicated some of the nitrate removal was due to heterotrophic denitrification, and thus denitrification was mixotrophic. Microbial analysis indicated the presence of Thiobacillus denitrificans, a widely known autotrophic denitrifier, in addition to several heterotrophic denitrifiers. Phase II showed that longer retention times tended to result in more nitrate reduction and sulfate production, but increasing the retention time through flow rate manipulation may create fluidization challenges for these sulfur particles.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The global demand for food protein must be balanced with increased concern for the environmental impact caused by these production systems. Land-based closed-containment aquaculture using recirculating aquaculture systems (RAS) are uniquely poised to produce highly desirable and valuable food products while also maintaining a small environmental footprint. However, while most RAS are designed to remove solids and recycle water back to the fish culture tanks (Summerfelt and Vinci, 2008; Timmons and Ebeling, 2010), the inability of these systems to remove nitrate–nitrogen from the water significantly arrests this industry’s ultimate economic and environmental sustainability. For these aquaculture systems to more completely address environmental issues, it is now critical that efforts focus upon the reduction of nitrogen species in effluent waters. Importantly, the ability to confidently and consistently remove nitrate nitrogen from RAS effluent may allow expansion of this industry into locales currently bound by stringent water quality standards and may potentially allow increased reuse of treated effluents. There is a crucial need to develop practical and cost effective methods to reduce RAS nitrate–nitrogen loads to allow their maintained or increased productivity simultaneously with attainment of water quality goals and good environmental stewardship.

The most common wastewater denitrification systems are based on heterotrophic denitrification with the addition of methanol (Payne, 1973). However, sulfur-based autotrophic denitrification, where a reduced form of sulfur (e.g., thiosulfate, elemental sulfur) serves as the electron donor rather than organic carbon, presents several unique benefits (Eq. (1)). Compared to heterotopic

http://dx.doi.org/10.1016/j.aquaeng.2015.07.002
0144-8609/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
denitrification, an autotrophic process does not require any additional potentially expensive carbon source, and produces less bacterial sludge thus simplifying treatment (Batchelor and Lawrence, 1978; Koenig and Liu, 1996; Zhang and Lampe, 1999). Elemental sulfur is a promising substrate for autotrophic denitrification as it is generally inexpensive and non-toxic (Batchelor and Lawrence, 1978; Sahinkaya and Kılıç, 2014; Sahinkaya et al., 2014).

\[
\begin{align*}
\text{NO}_3^- + 1.105 + 0.40\text{CO}_2 + 0.76\text{H}_2\text{O} + 0.08\text{NH}_3 & \rightarrow 0.08\text{SO}_2\text{O}_3\text{N} \\
+ 0.50\text{N}_2 + 1.105\text{SO}_4^{2-} + 1.28\text{H}^+ & \tag{1}
\end{align*}
\]

A major disadvantage of this process is that autotrophs generally grow at a slower rate than heterotrophs, thus have lower denitrification rates (Sahinkaya and Kılıç, 2014). Major by-products of concern from sulfur-based autotrophic denitrification are sulfate and acidity (Sahinkaya and Kılıç, 2014) with 4.57 mg CaCO₃ alkalinity consumed and 7.54 mg sulfate produced for each mg NO₃⁻-N reduced (Sahinkaya et al., 2014). Many sulfur-based denitrification studies use a mix of sulfur and limestone or dolomite to buffer pH and alkalinity decreases (Sahinkaya and Kılıç, 2014; USEPA, 1978). The presence of sulfur in the system combined with low-oxygen conditions could also lead to sulfate production, though not as a result of Eq. (1). An additional challenge is that elemental sulfur is relatively water insoluble, meaning it has a limited microbial availability at room temperature. Batchelor and Lawrence (1978) outlined that for elemental sulfur-based denitrification to proceed, three steps were necessary: (1) the sulfur must be solubilized, and (2) nitrate must be transported from solution to the biofilm surface, where (3) it can be transported through the film so it can be denitrified.

Sulfur-based autotrophic denitrification in static beds has proven successful for treating nitrate in groundwater, landfill leachate, and wastewaters (Koenig and Liu, 1996; Lee et al., 2008; Shao et al., 2010), and this approach presents a unique option for treatment of aquaculture effluents (Sher et al., 2008). Nitrogen removal rates from previous laboratory studies are generally on the order of 0.1–0.4 g N/(L.d) (Lampe and Zhang, 1996; Sahinkaya and Kılıç, 2014; Sahinkaya et al., 2014). Nitrogen (N) removal performance may be limited by N loading with Kim et al. (2004) observing a decline in N removal beyond loading rates of 2.5 kg NO₃⁻-N/(m²-d), and Koenig and Liu (1996) noting that areal based loading rates (g N/m²-d) were their limiting factor in a packed sulfur bed. In an aquaculture application, Sher et al. (2008) reported the use of autotrophic denitrification provided a dual benefit for recirculated waters; not only were nitrate levels brought under control, but the oxidation of sulfide in the anaerobically digested sludge helped safeguard against sulfide toxicity within the system.

Fluidized bed reactors are a proven aquaculture water treatment technology due to their plugging prevention, ease of maintenance, low cost and efficient treatment (Summerfelt, 2006). Because fluidized sand biofilters are common in this industry, their application as fluidized sulfur autotrophic denitrification reactors could be a natural extension of the technology. Fluidized sulfur biofilters have been researched at the lab scale, with Kim et al. (2004) showing higher N removal rates from fluidized sulfur beds than packed sulfur beds. This was due to the absence of clogging and good nitrate transfer to the sulfur surface in the fluidized system. In previous work, Christianson and Summerfelt (2014) determined fluidization velocities of commercially-available sulfur flakes, grains, and powder, and concluded the grains provided the most realistic option for full-scale testing of a fluidized sulfur-based denitrification biofilter. The objective of this work was to assess the nitrate reduction potential of fluidized sulfur-based biofilters for treatment of aquaculture wastewater.

2. Methods and materials
2.1. Fluidized sulfur biofilter experimental set-up

Three fluidized sulfur biofilters (285 L, height 3.9 m, diameter 0.31 m; Fig. 1) were operated at The Conservation Fund’s Freshwater Institute (Shepherdstown, West Virginia, USA) for 253 days to quantify nitrate removal from aquaculture wastewater (Phase I: 225 days, 13 March 2014 to 23 October 2014; Phase II: 28 days, 24 October 2014 to 20 November 2014). During Phase I, the three biofilters were operated in triplicate fashion, each fluidized at 37–39% expansion with a hydraulic retention time (HRT) of 3.2–3.3 min and a hydraulic loading rate of 860–888 L/m² min based on the mean flow rate of 63–65 L/min. Two Phase I study periods of relatively consistent influent nitrate concentrations were selected for analysis; Periods 1 and 2 allowed evaluation at influent concentrations of 2.0–5.0 and 7.6–17 mg NO₃⁻-N/L, respectively (days 57–92 and 190–225, respectively; six sample events each). Phase II utilized a different flow rate in each biofilter to assess the impact of HRT on nitrate removal (i.e., no replication; 42–13% expansion; 3.2–4.8 min HRT; 67–43 L/min flow rate; influent 8.5–15 mg NO₃⁻-N/L).

The waste and wastewater treatment system and biofilter design has been previously described by Tsukuda et al. (2015) (Fig. 2). In short, waste sludge from the production of rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar), was concentrated via microscreen drum filters and radial flow settlers and was pumped to a series of gravity thickening settling cones. A holding tank for the supernatant overflow from the settling cones fed the three fluidized denitrification biofilters. Overflow from each biofilter was treated using a radial flow settler. Biofilter bed height

![Fig. 1. Fluidized biofilter column dimensions (From Tsukuda et al., 2015).](image-url)
(2.82 m; biofilter volume 0.206 m³) was controlled with a shearing pump at the top of each biofilter. The static sulfur bed depth was approximately 0.9 m, although the sulfur grains in all three biofilters were replenished on days 181 and 198 following an undetected wash-out (68 kg or approximately 0.75 m s per biofilter total replenished). Biofilter influent nutrient levels were manipulated by dosing a concentrated sodium nitrate (NaNO₃; 34.0 g NO₃-N/L) solution into the supernatant holding tank. The spring water feeding the RAS was naturally alkaline (=275 mg CaCO₃/L), resulting in high alkalinity of flows.

The sulfur grains had effective and calculating sizes of 0.30 (D₁₀) and 1.31 mm (D₉₀), respectively, and a uniformity coefficient of 3.1 (Georgia Gulf Sulfur, Customer Code 1660, distributed by Prince Agri-Products, Inc., Quincy, Illinois, USA; Christianson and Summerfelt, 2014). This is smaller than reported particle size ranges for other sulfur-based denitrification studies as most have used grains ranging from 2 to 16 mm (Koenig and Liu, 1996; Oh et al., 2003; Sahinkaya et al., 2014). Sahinkaya and Kílic (2014) reported using the most comparable size (0.5–1.0 mm grains) in a packed column study, and in the only reported fluidized bed study, Kim et al. (2004) used 2.0–3.35 mm sulfur grains. The smaller grain size used here provided a desirable high specific surface area (SSA bed: 4110 m²/m³) relative to, for example, a 4.4 mm mean particle size sulfur product that had a 1363 m²/m³ SSA (Koenig and Liu, 1996). Elemental sulfur powder was initially used in the fluidized biofilters, but was discontinued due to fluidization and wash-out challenges. Lampe and Zhang (1996) similarly reported difficulty with powdered sulfur in a batch reactor (i.e., uniform mixing was problematic).

### 2.2. Water quality parameters and analysis

Water quality samples were collected from a sampling valve located at the back of each of the three biofilters and directly from the influent supernatant tank (i.e., effluent sample values were pooled as replicates during Phase I, n = 3; influent samples, n = 1). Water chemistry was analyzed weekly onsite, and both study phases followed the same sampling routine. Samples were analyzed for chemical oxygen demand (COD), carbonaceous biochemical oxygen demand (cBOD₅), total ammonia nitrogen (TAN), nitrite–nitrogen (NO₂⁻N), nitrate–nitrogen (NO₃⁻N), total nitrogen (TN), alkalinity, pH, sulfate (SO₄²⁻), sulfide (S²⁻), total suspended solids (TSS), total phosphorus (TP), and dissolved reactive phosphorus (DRP) using methods from APHA (2005) and Hach Company (2003). Temperature, dissolved oxygen (DO), and oxidation reduction potential (ORP) were assessed at least twice weekly. Measurements were made directly from the supernatant tank (influent) and the open biofilter tops (effluent) utilizing both inline and handheld probes (HACH HQ40d Portable meter with either HACH IntelliCAL LDO101 or MTC101 ORP/redox probe; HACH phd sc Differential ORP sensor with HACH sc100 controller; HACH Advanced LDO Process Dissolved Oxygen Probe with HACH sc200 controller). Flow rate was measured along the influent pipes to each biofilter and adjusted at least twice weekly concurrently with temperature, DO, and ORP readings, as well as prior to the weekly water chemistry sampling event (Dynasonics DXN Portable Ultrasonic Measurement System).

Nitrate–N, sulfide, and alkalinity removal rates were based upon:

### 2.3. Collection and extraction of DNA

Samples for screening the potential denitrification community were collected from all three biofilters on the final day of Phase I Period 2 (day 225). Biofilm attached to the sulfur media were detached by vigorously vortexing a sample of sulfur media/biofilter water in 50 mL sterile plastic conical tubes for 5 min. The resulting suspensions of detached surface layer (SL) biofilms were centrifuged at 10,000 × g at 4 °C for 20 min prior to DNA extraction. Following SL biofilm detachment, the media were directly used for DNA extraction of inner layer (IL) biofilm. The genomic DNA was extracted from each reactor’s SL and IL biofilm using a PowerSoil DNA Extraction Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer’s protocol. The concentration and quality of extracted DNA were determined by absorbance at 260 nm and 260/280 nm ratio, respectively (NanoDrop 2000c UV-Vis spectrophotometer; Thermo Fischer Scientific, Inc., Wilmington, DE). Isolated DNA was stored at –20 °C.

### 2.3.1. PCR amplification

Microbial community DNA extracted from three biofilters were pooled in equal quantity and used to amplify nosZ fragments which encode the catalytic subunit Z of nitrous oxide reductase. Primers nosZ-F (5′-CGTGTCTTCTGAGCCCAAGG-3′) and nosZ-R (5′-CATGTGCAAGNGCRTGGCAGAA-3′) yielding approx. 700 bp fragments (Rösch et al., 2002) were used. PCR reaction mixtures were prepared to contain 2× Taq PCR Master Mix (QIAGEN, Gaithersburg, MD), 6 pmol of each forward and reverse primers, and 100 ng of genomic DNA in a final volume of 20 μL. The PCR amplification was

![Fig. 2. Process flow diagram for units involved with biofilter denitrification research (Modified from Tsukada et al., 2015). AIC replicate modification of setting cones, biofilters, and radial flow settlers not shown. Setting cones and radial flow settlers drained every four weeks and bi-weekly, respectively, to prevent sludge accumulation (off-site sludge disposal).](image)
carried out as following: initial denaturation step at 94 °C for 4 min; one cycle at 94 °C for 20s (denaturation), at 65 °C for 30s (annealing), and at 72 °C for 40s (elongation); two cycles at 94 °C for 20s (denaturation); at 62 °C for 30s (annealing); 72 °C for 40s (elongation); three cycles at 94 °C for 20s (denaturation); at 59 °C for 30s (annealing); at 72 °C for 40s (elongation); five cycles at 94 °C for 20s (denaturation); at 57 °C for 30s (annealing); at 72 °C for 40s (elongation); twenty four cycles at 94 °C for 20s (denaturation); at 55 °C for 30s (annealing); at 72 °C for 40s (elongation); and then, final extension at 72 °C for 10 min in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). A negative control prepared without DNA was included in every PCR reaction performed to test for false positives caused by contamination. PCR products were separated and visualized by electrophoresis in 1.2% agarose gel stained with EtBr, and were purified from excised gel slices (about 700 bp size band) using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA).

2.3.2. Cloning and sequencing

Purified SL and IL nosZ amplicons were ligated into pCR4-TOPO vector, and vector with insert were transformed into OneShot TOP10 chemically competent *Escherichia coli* cells using TOPO TA Cloning Kit following the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Ninety-six total clones were randomly selected from each SL and IL nosZ library and were cultured for plasmid preparation. Plasmid DNAs were purified (Agencourt SprintPrep 384 HC Kit, Agencourt Bioscience, Beverly, MA) and sequencing was performed using an ABI PRISM genetic analyzer (Applied Biosystems, Foster City, CA) with T7 and T3 primers provided in the cloning kit (Invitrogen) at the Biological Analysis Service Laboratory, Institute of Marine and Environmental Technology (Baltimore, MD). Sequences were edited and assembled using Sequencer software (Gene Code Corp., Ann Arbor, MI, USA), were analyzed using the Basic Local Alignment Search Tool (BLASTn: http://www.ncbi.nlm.nih.gov/BLAST), and were compared with available sequences in the GenBank database to create neighbor joining phylogenetic trees to aid the selection of the closest reference sequences.

2.3.3. Nucleotide sequence accession numbers

The 41 partial nosZ sequences that were generated in this study have been deposited in GenBank database under accession numbers KT252910 to KT252950.

3. Results and discussion

3.1. Phase I: High and low nitrate loading at a consistent HRT

Nitrate reduction was observed during both Phase I periods (Fig. 3a). Although differences between influent and effluent nitrate concentrations were relatively small (Table 1: 1.57 ± 0.15 and 1.82 ± 0.32 mg NO₃⁻-N/L for the two periods, respectively), the high flow rates and compact biofilter volume resulted in mean removal rates of 0.71 ± 0.07 and 0.80 ± 0.15 g N removed/L bioreactor-d for the two periods, respectively. This is much higher than the previously reported range of 0.1 to 0.4 g N/(L.d) for sulfur-based denitrification (Lampe and Zhang, 1996; Sahinkaya and Kiliç, 2014; Sahinkaya et al., 2014), but similar to the low end of the range for fluidized sand biofilter heterotrophic N removal rates of 0.86–1.74 g N/(L.d) (or 35.8–72.6 mg NO₃⁻-N/Lh); reviewed by van Rijn et al., 2006). Relative to previous experiments with these biofilters, Tsukuda et al. (2015) reported removal rates of 0.4 g N/(L.d) when they were operated with fluidized sand. Christianson and Summerfelt (2014) reported sand was much less expensive than sulfur products for fluidized biofilters on both a volumetric and surface area basis ($70–$200/m³ vs. >$1000/m³, respectively; $0.02/m² surface area vs. =$0.30/m² surface area, respectively), though a fluidized sand biofilter would also require purchase of a carbon source to fuel denitrification. Influent loading averaged 1.46 and 5.82 g N/(L.d) for Periods 1 and 2, respectively. Nitrate removal efficiencies averaged 50 ± 4.6% and 16 ± 3.2% for the two Phase I study periods, with the relatively high efficiency for Period 1 due to the low influent nitrate concentration.

Theoretically, the production of sulfate is proportional to the extent of autotrophic denitrification, thus sulfate production may be the best indicator of this process (Oh et al., 2003; Sahinkaya et al., 2014). Based on Eq. (1) and average removals of 1.57 and 1.82 mg NO₃⁻-N/L, Periods 1 and 2 should have produced an average of 11.8 and 13.7 mg SO₄²⁻/L. However, only 2.7 ± 2.0 and 6.1 ± 1.6 mg SO₄²⁻/L were produced during these two periods, with no statistically significant difference between influent and effluent sulfate concentrations for either Periods 1 or 2 (Table 1; Fig. 3b). This is an indication that some of the N removal was potentially due to heterotrophic denitrification in addition to autotrophic. Just as the elemental sulfur was converted to sulfate, some sulfide present in solution was also oxidized (Fig. 3c; Table 1; mean removal: 6.19 ± 1.82 and 8.64 ± 1.04 µg S²⁻/L). Sher et al. (2008) observed that a RAS sludge digestion basin also provided autotrophic denitrification treatment with sulfide as the electron donor. Dual functionality of nitrate and sulfide reduction would be a more significant benefit for RAS waters being recirculated to fish culture tanks as compared to the treatment of effluent waters here.

Reduced alkalinity, another indicator of autotrophic denitrification, was observed here with average decreases of 16 and 12 mg CaCO₃/L from the two Phase I study periods (Table 2). Others have reported significant drops in alkalinity during sulfur-based denitrification studies (Koenig and Liu, 1996), and this may be
the largest operational challenge of such a system (Kim and Bae, 2000). The naturally alkaline spring water used in the on-site RAS here was considered well-buffered enough to not require alkalinity addition as Furumai et al. (1996) reported the optimum alkalinity for sulfur-based autotrophic denitrification was 150–240 mg/L. Based on Eq. (1), removal of 1.57 and 1.82 mg NO3−-N/L should have resulted in alkalinity consumption of only 7.2 and 8.3 mg CaCO3/L for Periods 1 and 2, respectively. Likewise, based on N removal rate (0.71 and 0.80 g N/L(d)), alkalinity consumption should have been 3.2 and 3.7 g CaCO3/L(d) although it averaged 7.1 ± 2.7 and 5.3 ± 2.3 g CaCO3/L(d) for the two periods. The simultaneous occurrence of heterotrophic denitrification would have reduced alkalinity consumption rather than increased consumption, and while nitrification can consume alkalinity, there was no consistent change in TAN concentrations across the biofilters. Degradation of possible accumulated sludge within the biofilter may have consumed some alkalinity, although this could not be verified. The variability in alkalinity standard error complicated further analysis.

No major pH changes were observed with the influent and effluent both averaging between 7.33 and 7.39 for both periods (Table 2). Others have observed notable pH decreases (Koenig and Liu, 1996; Sahinkaya and Kilic, 2014) with nitrate accumulation possible at pH below 7.4 (Furumai et al., 1996). There was no accumulation of nitrate here as levels were generally slightly reduced over the biofilters (Table 1; Fig. 4). Water temperature between the influent and effluent did not notably vary, although a seasonal trend was observed. Temperatures peaked between days 100–150 (20 June 2014–09 August 2014) during the warmest time for these greenhouse-run experiments (Fig. 5a). As expected, effluent DO concentrations were reduced to less than 1.0 mg DO/L when the columns were operating as intended (Fig. 5b), and to less than 0.5 mg DO/L during both analysis periods (Table 2). This indicated a strong aerobic and/or facultative anaerobic component existed within the biofilters. Facultative heterotrophic denitifiers use free oxygen as their electron acceptor while it is available, because oxygen is a more energetically favorable electron acceptor than nitrate. Thus, heterotrophic denitrification and the use of nitrate as an electron acceptor is reduced when free oxygen is still present. Autotrophic denitrification has been documented under both aerobic and anaerobic conditions (Zhang and Lampe, 1999). The potential impact of the sulfur wash-out was evident as early as day 150 when effluent DO levels increased; additional sulfur was added on days 181 and 198. Oxidation reduction potentials

![Fig. 4. Influent and effluent nitrogen species concentrations during fluidized sulfur denitrification biofilter Phase I operation (effluent n = 3; mean ± standard error).](image-url)
were highly variable though always negative, and were reduced slightly across the biofilter during Period 1 but increased during Period 2 (Table 2; Fig. 5c). This increase in ORP across the biofilters was mainly apparent because the influent ORP was more reduced during this period; influent water quality throughout the experiment was variable and somewhat uncontrollable due to the nature of this production aquaculture facility’s waste stream.

The term “mixotrophic denitrification” refers to the simultaneous occurrence of heterotrophic and autotrophic denitrification (Oh et al., 2003; Sahinkaya and Kilic, 2014). With this relatively high COD and cBOD₅ wastewater, it is likely mixotrophic denitrification was occurring. Oh et al. (2003) observed addition of a variety of soluble organic sources (methanol, ethanol, acetate) did not inhibit autotrophic denitrification, although supplementation of organic carbon in excess did decrease sulfate production. Balancing the autotrophic/heterotrophic reactions can reduce the alkalinity requirement caused by autotrophic denitrification due to alkalinity produced by heterotrophs (Kim and Bae, 2000; Lee et al., 2001; Oh et al., 2003). Because heterotrophs grow faster than autotrophs, some organic carbon forms may be preferentially utilized before sulfur in a mixotrophic denitrification reactor (Sun and Nemati, 2012). Availability of the electron donor may play a role in this as limited dissolution of solid sulfur particles can limit denitrification, especially at higher N loading rates (Kim et al., 2004).

Suitable COD:NO₃⁻–N ratios for heterotrophic denitrification are on the order of 3:1 to 6:1 (van Rijn et al., 2006), and influent values here averaged 74 ± 7.5 and 7.2 ± 1.3 COD:NO₃⁻–N for Periods 1 and 2, respectively, more than sufficient to fuel heterotrophic denitrification (cBOD₅:NO₃⁻–N of 24 ± 4.9 and 3.3 ± 0.5). However, during Periods 1 and 2, COD was only reduced 8.5 ± 19 and 2.4 ± 3.0 mg COD/L, respectively, and cBOD₅ concentrations were not reduced across the biofilters (Table 1). The COD:NO₃⁻–N utilization ratios were 5.4 and 1.3 mg COD consumed per mg N removed for the two periods, respectively. The very low utilization ratio for Period 2 potentially indicated relatively more of the N removal was due to autotrophic vs. heterotrophic denitrification compared to Period 1. The absence of measurable cBOD₅ reductions was likely due to the extremely short HRTs. While it is likely that heterotrophic denitrification did account for some of the nitrate removal, internal cycling of solids may have complicated the COD balances.

### 3.2. Phase I: Microbiological characterization

Sequence analysis of 96 randomly selected clones from each the biofilm surface layer (SL) and inner layer (IL) nosZ libraries revealed fourteen unique operational taxonomic units (OTUs) for SL and nine for IL (Table 3). The % Clone of similar sequences in a library were calculated for SL-nosZ and IL-nosZ, separately (Table 3, upper for SL-nosZ and lower for IL-nosZ). The nosZ library clones in the SL belonged to: Alphaproteobacteria (19.6%), Betaproteobacteria (76.5%), and unclassified bacterium (4.3%); and in the IL belonged to: Alphaproteobacteria (2.2%), Betaproteobacteria (17.5%), and unclassified bacterium (80.4%). Similarly to previous fluidized sand biofilter denitrification studies (Tsukuda et al., 2015), the denitrifying microbial population containing the nosZ gene in the SL was more diverse than in the IL. Here, more than 80% of IL-nosZ clones were closely related to the uncultured bacterium clone 2–80 (Accession JF509076.1). This lack of diversity may have been the result of lower DO and higher sulfur availability (electron donor) in the IL. Uncultured bacterium clone 2–80 were also found in the SL biofilm, but their much higher % clone in the IL (4.3 vs. 80.4% in SL vs. IL, respectively) may be an indication there are sulfur-utilizing autotrophic denitrifiers that have not yet been isolated or identified.

The microbial communities indicated that the encoding key enzyme for denitrification (nosZ) in the SL was largely from Azotococcus, Thaeru and Paracoccus spp., which are known as heterotrophic denitrifiers, and their presence suggests the geochemical conditions near the SL were suitable for heterotrophic denitrification compared to conditions in the IL. In contrast, nosZ sequences belonging to Thiobacillus denitrificans, an obligate chemolithotrophic denitrifier, were dominant in the IL denitrifying microbial community suggesting IL provided a suitable cultivating environment for autotrophs, although this was only 4.3% of the IL-nosZ clones (80.4% were uncultured bacterium clone 2–80). The optimal growth temperature of *T. denitrificans* is between 28 and 32°C (Shao et al., 2010), and lower water temperatures here (13–22°C; Fig. 5a) may have influenced this relatively low percentage. Among known autotrophic denitrifiers, the obligate chemolithotrophic, *T. denitrificans*, was the first to be isolated and characterized, is capable of utilizing thiosulfate, tetrathionate, thiocyanate, sulfide and elemental sulfur as the electron donor for denitrification, and is the most commonly reported autotrophic denitrifier (Park et al., 2010, 2011; Chen et al., 2013; Xu et al., 2014). The detection of nosZ genes from autotrophic denitrifiers in both SL and IL biofilms strongly indicated the capability of fluidized sulfur biofilters to cultivate and enrich autotrophic denitrifying bacteria for removal of nitrate, even under relatively short HRTs compared to packed sulfur reactor studies. In addition, the co-existence of autotrophic and heterotrophic denitrifiers suggests these reactors provided conditions to cultivate both types of bacteria which can offer unique and efficient mixotrophic nitrate removal (Oh et al., 2001).

### 3.3. Phase II: Hydraulic retention time impact on autotrophic denitrification

When each biofilter was operated independently, N removal and sulfate production showed a weakly increasing trend at increasing HRTs (Fig. 6a and b). Based on the regression slope
### Table 3

Nearest neighbor of the nitrous-oxide reductase (nosZ) gene clones in the surface layer (SL, upper part of table) and inner layer (IL, bottom part of table) of the biofilm.

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Accession no. of nearest neighbor</th>
<th>Nearest neighbor*</th>
<th>Similarity (%)</th>
<th>% Clone in a library</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-proteobacteria</td>
<td>AP012304.1</td>
<td>Azoarcus sp. KH32C</td>
<td>81</td>
<td>26.1</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP001281.1</td>
<td>Thauera sp. MZ1T</td>
<td>84</td>
<td>13.4</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>AM406670.1</td>
<td>Azoarcus sp. BH72</td>
<td>78</td>
<td>6.6</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>AM045244.1</td>
<td>Paracoccus denitrificans strain DN23</td>
<td>81</td>
<td>6.5</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP008267.1</td>
<td>Rhodobacter ferrireducens T118</td>
<td>85</td>
<td>4.3</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>AP0122885.1</td>
<td>Rhizobiales bacterium DS-25</td>
<td>81</td>
<td>4.3</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>KM594554.1</td>
<td>Paracoccus sp. SY</td>
<td>81</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP001645.1</td>
<td>Raistonia picketti 12D</td>
<td>88</td>
<td>2.2</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>CP006880.1</td>
<td>Rhizobium geliticum bv. gallicum R602</td>
<td>88</td>
<td>2.2</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>CP001313.1</td>
<td>Rhodobacter capsulatus SB 1003</td>
<td>84</td>
<td>2.2</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>EUJ346731.1</td>
<td>Shinella zoogloeoides strain BC026</td>
<td>86</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP000116.1</td>
<td>Thioctillus denitrificans ATCC 25259</td>
<td>84</td>
<td>2.2</td>
</tr>
<tr>
<td>Unclassified bacteria</td>
<td>JF509076.1</td>
<td>Uncultured bacterium clone 2-80</td>
<td>89</td>
<td>4.3</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP001116.1</td>
<td>Thioctillus denitrificans ATCC 25259</td>
<td>84</td>
<td>4.3</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>AP012304.1</td>
<td>Azoarcus sp. KH32C</td>
<td>85</td>
<td>2.2</td>
</tr>
<tr>
<td>Alpha-proteobacteria</td>
<td>GU136479.1</td>
<td>Uncultured Azospirillum sp.</td>
<td>74</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>AB545666.1</td>
<td>Herbaspirillum sp. TS026-2</td>
<td>75</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>AB545673.1</td>
<td>Herbaspirillum sp. TS047-2</td>
<td>83</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP002927.1</td>
<td>Rhodobacter ferrireducens T118</td>
<td>84</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>GG0035431.1</td>
<td>Rubrivivax gelatinosus strain S1</td>
<td>78</td>
<td>2.2</td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>CP001281.1</td>
<td>Thauera sp. MZ1T</td>
<td>85</td>
<td>2.2</td>
</tr>
<tr>
<td>Unclassified bacteria</td>
<td>JF509076.1</td>
<td>Uncultured bacterium clone 2-80</td>
<td>88</td>
<td>80.4</td>
</tr>
</tbody>
</table>

* The closest matching sequence was identified using Blastn at the NCBI and selected by neighbor joining phylogenetic analysis from Blastn hits.

(−0.0405 g N/(Ld) per L/min of flow rate), decreasing the flow rate approximately 20L/min would provide an additional 0.81 g N removed/(Ld) which equated to an additional 167 g N removed/d for these biofilters. Removal of sulfide also tended to increase at higher HRTs, though this regression was even less strongly correlated (Fig. 6a). For fluidized systems, this reduction in flow rate to achieve a longer HRT is a tradeoff resulting in less fluidization of the sulfur particles, and thus the HRT would need to be increased via a larger biofilter. A recommended 60% expansion, as was modeled in Christianson and Summerfelt (2014), would have required a flow rate of over 80 L/min and yielded an HRT of only 2.5 min in these biofilters. Other reported HRTs for packed or continuously stirred sulfur denitrification reactors have been on the order of 3 to 24 h (Lampe and Zhang, 1996; Lee et al., 2001; Sahinkaya and Kilic, 2014;)

![Fig. 6](image-url)

**Fig. 6.** Nitrate–N and sulfide removal rate (a) and observed and theoretical sulfate production rate, (b) across a range of flow rates, hydraulic retention times, and fluidization expansion levels from Phase II of fluidized sulfur biofilter operation.
Sahinkaya et al., 2014), Koenig and Liu (1996) reported the required HRT for complete N reduction depended upon the sulfur particle size, and showed greater than 30 min was required for a 40% nitrate removal efficiency using their smallest sulfur size fraction (2.8–5.6 mm) in packed beds. At a loading of 2.2 kg N/(m²-d), Kim and Bae (2000) reported an HRT of 2.34 h in a packed bed provided complete denitrification. Loading during Phase II was between 2.7 and 6.7 kg N/(m³ biofilter-d), thus a greater HRT, in a packed bed at least, would have been required for complete N removal. The only comparable fluidized bed study reported an HRT of 0.19 h (empty bed contact time) and bed expansion of 25–30% (2–3.35 mm sulfur particle size; Kim et al., 2004). Under these conditions, greater than 90% removal efficiency was achieved from an influent concentration of 20 mg NO₃-N/L. However, Kim et al. (2004) also reported a decline in N removal-performance when N loading exceeded 2.53 kg N/(m³-d), as the present study did. Ideally, this study would have been improved if the biofilters were 2–3 m taller or if a slightly smaller-sized sulfur particle could have been identified, because both options would have increased the HRT within the denitrification bed.

4. Conclusions

Despite only removing 1.57 ± 0.15 and 1.82 ± 0.32 mg NO₃-N/L per each pass through the biofilter during Phase I, removal rates were the highest reported for sulfur-based denitrification systems (0.71 ± 0.07 and 0.80 ± 0.15 g N removed/L bio-reactor-d). Lower than expected sulfate production indicated some of the nitrate removal was due to heterotrophic denitrification although there was no statistically significant decrease in COD or cBOD₃ concentrations between the influent and effluent. Mixotrophic denitrification was verified via the presence of both heterotrophic and autotrophic denitrifiers. Phase II tended to indicate that longer retention times may result in more nitrate removal and sulfate production, but increasing the retention time through flow rate manipulation may create fluidization challenges for these sulfur particles. Operationally, the sulfur particles will degrade over time, and optimizing the balance of fluidization velocity versus HRT may be challenging.

Acknowledgements

The authors wish to thank the Herrick Foundation (Detroit, Michigan, USA) for their graciously support. This research was additionally supported by the USDA Agricultural Research Service under Agreement no. 59–1930–0-046. A debt of gratitude is due to Shanen Cogan and Fred Ford for technical assistance and Issra Arif for initial assistance with the testing.

References

Dr. Laura Christianson has been a Research Agricultural Engineer for The Conservation Fund’s Freshwater Institute, Shepherdstown, WV since 2013. She finished her Ph.D. in Agricultural Engineering (Co-Major: Sustainable Agriculture) at Iowa State University in December 2011 where her dissertation focused on improvement of agricultural drainage water quality through the use of denitrification “woodchip bioreactors. During her Ph.D. she spent a year in New Zealand studying agricultural water quality and denitrification technologies as a fullbright Fellow. Laura previously completed a M.S. in Biological and Agricultural Engineering at Kansas State University and a B.S. in Biosystems Engineering at Oklahoma State University.
Christine Lepine is a Research Technician for The Conservation Fund’s Freshwater Institute (TCFII), Shepherdstown, WV. She has been with TCFII since 2014, originally starting as a Research Intern. She recently graduated magna cum laude from Shepherd University with a B.S. in Environmental Studies, Concentration of Resource Management.
Scott Tsukuda is the Director of Operations at The Conservation Fund’s Freshwater Institute (TCFII), Shepherdstown, WV, with his focus on energy monitoring and
auditing. Some of his past work has included MS Excel computer modeling, PLC programming, denitrification technologies and alternative waste treatment systems demonstration. He is a member of the Instrumentation, Systems and Automation Society (ISA) and the Institute of Electrical and Electronics Engineers (IEEE) plus holds a M.S. in Agricultural Engineering and a B.S. in Agricultural Engineering from Cornell University. Past certifications include Microsoft Certified Systems Engineer (MCSE). He is currently Certified Energy Manager (CEM).

**Dr. Keiko Saito** has been a Research Assistant Professor at University of Maryland Baltimore County’s Institute of Marine and Environmental Technology since 2010. Her research focuses on aquatic microbial ecology and aquacultural microbiology, and on applying molecular approaches to link the critical roles of microbial community composition, functional diversity, ecosystem processes, and bio-degradation/remediation. She is working toward development and improvement of microbially mediated waste treatment technologies for next-generation aquaculture practices.

**Dr. Steven T. Summerfelt**, Professional Engineer, is Director of Aquaculture Systems Research at The Conservation Fund’s Freshwater Institute (TCFFI), Shepherdstown, WV, where he has been an employee since 1992. He is Project Leader on TCFFI’s USDA-ARS project titled, “Development of Sustainable Land-based Aquaculture Production Systems” and has authored or co-authored of over 60 refereed papers, 9 book chapters, and a book titled “Recirculating Aquaculture Systems”. Steve has designed several large private and public fish culture facilities using closed-containment technologies. He has B.S., M.S., and Ph.D. degrees in the fields of chemical and environmental engineering.