

Wood and sulfur-based cyclic denitrification filters for treatment of saline wastewaters

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Detailed responses to reviewer's comments

Manuscript Number: BITE-D-20-08810

Manuscript Title: Wood and sulfur based cyclic denitrification filters for treatment of saline wastewaters

We thank the reviewers for their careful review of our manuscript. The revisions have strengthened the paper. Our responses for each reviewer's comments are shown in *italics* and revisions are highlighted in red in the manuscript and below.

Reviewer #1:

The manuscript describes a system to promote N removal from the recirculation of a saline water aquaculture system. Wood chips and elemental sulfur are provided as electron donors. The manuscript evaluates the evolution of N and S species over time, as well as the capacity of the wood chips to provide sCOD for denitrification. The results are clearly explained, and the conclusions are realistic and consistent with the results.

***Response**: We thank the reviewer for their review of the manuscript.*

Specific comments:

1. Some of those conclusions (higher fish mortality than in their previous SAD system) should be included in the abstract. Even if the conclusion is somewhat negative, it is very relevant and abstract readers should be able to get a full picture when only reading the abstract.

   ***Response**: We added the survival rate when using pine wood and sulfur for denitrification in the revised abstract.*

   Lines 24-27: Although fish tank water quality parameters, including ammonia, nitrite, nitrate and sulfide, were below the inhibitory levels for marine fish production, lower survival rates of *Poecilia sphenops* were observed compared with prior studies.

2. The accumulation of nitrite in these systems is indicative of incomplete denitrification (COD limitation), which leads to production of NO and N_2O. Even though in this work nitrite depletes after a few weeks, the decrease in rbCOD in the woodchips overtime may lead to incomplete denitrification. I understand that the question of the GHGs may be out of the scope of this work but the authors should probably comment on it, especially when looking at how fast the soluble rbCOD depletes in the wood chips.

   ***Response**: We added some discussion in the revised manuscript (Lines 294-297; 349-350). For example, before discussing the nitrite data observed in this study, we briefly mention the following as recommended by the reviewer: (1) nitrite accumulation is an indication of incomplete denitrification; (2) incomplete denitrification can lead to the formation of greenhouse gases, such as NO and N_2O; and (3) measuring these GHGs was beyond the scope of this work. By doing*

so, the reader is informed about a potential concern of the WSHAD technology that future research can evaluate further. Please see the excerpt below for this example to receive further context.

Lines 294-297: NO_2^- accumulation is an indicator of incomplete denitrification, which leads to production of NO and N_2O, which are potent greenhouse gases (Bertrand et al., 2011). Although NO and N_2O were not measured in this study, NO_2^- was detected in the CDF effluent (Fig. 2b), especially on the first day (16.48 mg NO_2^--N/L).

3. Lines 131-134: objective 1 includes N removal and the effect of WSHAD byproducts on fish growth. These seem to be two unrelated objectives unless the WSHAD byproducts they refer to are N compounds. Otherwise, please split.

   ***Response**: We separated objective 1 into two objectives (please see below):*

   The objectives of this study were to: 1) investigate nitrogen transformations (TAN, NO_2^-, NO_3^-) in the marine RASs; 2) investigate overall water quality and fish growth and survival in the systems; … (*Lines 126-128*)

4. 1 g/L equals 109 ppt. Therefore 15 g/L instant ocean means 15×109 ppt. Please correct.

   ***Response**: We changed 15 ppt to 15 g/L in the revised manuscript.*

5. Please revise the figure captions of figure 6. Once the community is described at a genus level, what is the motivation to add the description at phylum level? Figure 5 should probably be removed and placed in supplementary material.

   ***Response**: The caption for Fig. 6 was revised. Fig.5 was moved to the supplementary materials document. As a result, Fig. 6 in the original document that was submitted was renamed as Fig. 5. In addition, the figure that quantifies the microbial community change on the genus level (originally Fig. S3), was moved from the supplementary materials to the revised manuscript and titled Fig. 6. This was done to provide the reader with additional context on notable genera that changed from the initial inoculum and WSHAD CDFs. Text describing the microbial community structure in the main manuscript was updated to reflect these changes.*

   A brief description of the phylum-level results is included to serve two purposes. The first is to provide a general view of the microbial community structure and introduce a notable phylum identified in our analysis (i.e., Parcubateria). Parcubateria has not been classified beyond the phylum level to date and is discussed in detail in the genus-level results (Lines 408-410). The second purpose of the paragraph describing the phylum-level results is to transition the reader to the major themes that are discussed when the genus-level results are presented. Lines 395-399 feature the main conclusion of the phylum-level results, which states that "Variations in the abundance in the observed phyla between the inoculum, WS-CDF A, and WS-CDF B are likely to have occurred due to

mixotrophic metabolism by the input of fish waste, woodchips, and/or S^0 changes in operation of the CDFs (lower HRT and more frequent backwashing), or the 16S amplicon sequencing process, which is elaborated further in the discussion of the genus-level results."

As noticed in the microbial community analysis (i.e., Section 3.3), we incorporate text to summarize the main take-home messages and/or provide a transition between paragraphs. These texts are present throughout Section 3.3, from the assessment of the microbial community analysis (Lines 380-483), phylum level analysis (Lines 395-398), and genus-level analysis (e.g., Lines 413-416; Lines 448-452). This approach is used to facilitate a discussion of the results in a way that clearly addresses the research gap presented in the introduction on how there are no previous reports on the microbial community structure of WSHAD biofilters operated under saline conditions (Lines 123-124).

Reviewer #2:

Dear Authors,

the topic of the article is interesting, and the manuscript is well written. Please find attached some minor revisions I think are necessary to be performed before the acceptance on BITE journal.

Kind regards.

***Response**: We thank the reviewer for their review of the manuscript.*

Specific comments:

***Response**: Our responses to the comments that Reviewer #2 provided in the PDF file below are presented below.*

1. Three highlights are the minimum required in BITE. Please add one/two more highlights.

   ***Response**: An additional highlight was provided:*

- High denitrification rates of saline wastewater achieved with wood-S^0 biofilters.
- Combined use of wood and S^0 resulted in lower SO_4^{2-} accumulation than S^0 alone.
- Dynamic changes observed in autotrophic/heterotrophic denitrification mechanisms.
- WS-CDF microbiome contained a consortium of salt tolerant bacteria.
- *Sulfurimonas*, *Thioalbus*, and *Ornatilinea* contributed to denitrification.

2. Lines 125-131, These lines should be moved to M&M section.

   ***Response**: We have deleted these lines, as the WS-CDFs and pilot-scale marine RASs were described in detail in the Materials and Methods section.*

3. Lines 235-245, The method description is too long. Please, be more synthetic. You should cite one time the ref (APHA, 2012) for all the analytics.

***Response**: The methods description section was carefully edited and shortened by citing the methods used in our previous study (He et al., 2018), as follows:*

Dissolved oxygen (DO), pH, and conductivity in the fish tanks were measured *in situ* daily using a portable DO (Mettler Toledo, USA), pH (Oakton™ Handheld Ion Meter), and conductivity (Oakton™ CON 6+ Portable Conductivity Meter) meters.

Water samples were collected from the fish tanks and WS-CDF effluent at least 3 times weekly for analysis of TAN, NO_2^--N, NO_3^--N, SO_4^{2-}, S^{2-}, and COD, and weekly for alkalinity, total nitrogen (TN), total phosphorus (TP), TSS, and VSS according to *Standard Methods* (APHA, 2012). Samples were filtered through 0.45 µm membrane filters for TAN, NO_2^--N, NO_3^--N, TN, TP, sCOD, rbCOD and SO_4^{2-} analysis, while samples were not filtered for S^{2-} and COD detection. Detailed analytical methods are described in He et al. (2018).

4. Lines 531-532, Specify the name of phylum or genus.

***Response**: Genera were added in the revised Conclusion.*

The WS-CDF microbiome reflected a unique microbial consortium, including salt tolerant cellulose and sulfur utilizing denitrifiers, such as *Sulfurimonas*, *Thioalbus*, *Ornatilinea*, *Mycobacterium* and *Parachlamydia*. Results from this research can be applied to treatment of saline domestic, industrial and agricultural wastewaters.

Highlights

- High denitrification rates of saline wastewater achieved with wood-S^0 biofilters.
- Combined use of wood and S^0 resulted in lower SO_4^{2-} accumulation than S^0 alone.
- Dynamic changes observed in autotrophic/heterotrophic denitrification mechanisms.
- WS-CDF microbiome contained a consortium of salt tolerant bacteria.
- *Sulfurimonas*, *Thioalbus*, and *Ornatilinea* contributed to denitrification.

Manuscript Click here to view linked References

Wood and sulfur-based cyclic denitrification filters for treatment of saline wastewaters

Qiaochong He[a,b,c], Erica A. Dasi[b], Zhang Cheng[b], Emmanuel Talla[d], Kevan Main[e], Chuanping Feng[c], Sarina J. Ergas[b,*]

a. School of Environmental Engineering, Henan University of Technology, Zhengzhou, 450001, China

b. Department of Civil & Environmental Engineering, University of South Florida, 74202 E. Fowler Ave, ENB 118, Tampa, FL 33620, USA

c. School of Water Resources and Environment, China University of Geosciences (Beijing), Beijing, 100083, China

d. Aix Marseille Univ, CNRS, LCB, Laboratoire de Chimie Bactérienne, F-13009, Marseille, France

e. Directorate of Fisheries and Aquaculture, Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, FL, 34236, USA

* **Corresponding author**: sergas@usf.edu; Phone: (813) 974-1119; Address: 4202 E. Fowler Avenue, ENG 030, Tampa, FL 33620-5350, USA; orcid.org/0000-0001-6820-7196

Abstract: This study investigated the performance and microbiome of cyclic denitrification filters (CDFs) for wood and sulfur heterotrophic-autotrophic denitrification (WSHAD) of saline wastewater. Wood-sulfur CDFs integrated into two pilot-scale marine recirculating aquaculture systems achieved high denitrification rates (103 ± 8.5 g N/(m^3·d)). The combined use of pine wood and sulfur resulted in lower SO_4^{2-} accumulation compared with prior saline wastewater denitrification studies with sulfur alone. Although fish tank water quality parameters, including ammonia, nitrite, nitrate and sulfide, were below the inhibitory levels for marine fish production, lower survival rates of *Poecilia sphenops* were observed compared with prior studies. Heterotrophic denitrification was the dominant removal mechanism during the early operational stages, while sulfur autotrophic denitrification increased as readily biodegradable organic carbon released from wood chips decreased over time. 16S rRNA-based analysis of the CDF microbiome revealed that *Sulfurimonas*, *Thioalbus*, *Defluviimonas*, and *Ornatilinea* as notable genera that contributed to denitrification performance.

Keywords: Saline wastewater; Nitrogen balance; Mixotrophic denitrification; Electron donor; Sulfate accumulation; Microbiome analysis

1. Introduction

Saline wastewaters with high nitrate (NO_3^-) concentrations are generated from a number of domestic and industrial processes. For example, seawater is used for toilet flushing in many arid countries, resulting in saline domestic wastewater (Huang et al., 2018). Ion-exchange resins used to treat NO_3^- contaminated groundwater are regenerated using high NaCl concentration solutions, resulting in high-NO_3^- brine wastes (Trögl et al., 2011). Industrial and agricultural processes, such as aquaculture, seafood processing, tanneries, pulp and paper and textile dyeing, also generate saline wastewater containing high NO_3^- levels (Liang et al., 2017).

In this project, we investigated the use of a novel cyclic denitrification filter (CDF), and applied this process for treatment of marine aquaculture wastewaters. Aquaculture accounts for approximately half of the world's food fish production, and marine aquaculture contributes to 36% of global aquaculture production (FAO, 2018). Recirculating aquaculture systems (RAS) have attracted considerable research and commercial interest due to their reduced water use and environmental impacts compared with conventional marine aquaculture using ponds, raceways or cages (Van Rijn et al, 2006; Hamlin et al., 2008). In RAS, water is circulated through filters and bioreactors, which remove solids, organic matter and total ammonia nitrogen (TAN), before being returned to the fish tanks. Aerobic biofilters are typically used for oxidation of organic carbon and nitrification, resulting in NO_3^- accumulation in the RAS. Therefore, water exchanges are used to maintain NO_3^- concentrations below

57 levels that cause a chronic toxic effect on cultured fish (Davidson et al., 2016). Water
58 exchanges consume large amounts of water and result in discharges of NO_3^-
59 containing wastewaters to the environment. Therefore, denitrification of saline
60 wastewater is an inevitable problem to be solved in marine RAS practice.
61 Biological denitrification is an effective solution for NO_3^- removal in marine
62 RAS (Van Rijn et al., 2006). Although most RAS use organic substrates, such as
63 methanol, to promote heterotrophic denitrification, autotrophic denitrification avoids
64 carry-over of organic carbon (a source of oxygen demand) to the RAS and decreases
65 the risk of NO_2^- accumulation (He et al., 2018). Elemental sulfur (S^0) is a low-cost
66 electron donor for S^0 autotrophic denitrification (SAD), which has been successfully
67 used for NO_3^- removal from freshwater (Sahinkaya and Dursun, 2012) and marine
68 RAS (He et al., 2020). One potential drawback of SAD is sulfate (SO_4^{2-})
69 accumulation. In a prior study in our laboratory, NO_3^- concentrations were maintained
70 at a stable level in a pilot-scale marine RAS using a packed-bed denitrification
71 bioreactor containing S^0. However, SO_4^{2-} concentrations increased from a background
72 value of 1,400 to 2,322 mg SO_4^{2-}/L after 146 days operation (He et al., 2020). The
73 effect of elevated SO_4^{2-} on the health of marine aquacultured fish is not well
74 understood.
75 Mixotrophic denitrification, a combination of heterotrophic and autotrophic
76 denitrification, is a potential strategy for marine RAS applications. Under mixotrophic
77 conditions, a portion of NO_3^- is removed heterotrophically and the remainder is

denitrified by SAD, resulting in increased NO_3^- removal rates and decreased SO_4^{2-} production and alkalinity consumption (Oh et al., 2001). Liquid organic carbon sources have been used previously for mixotrophic denitrification, including methanol (Oh et al., 2001; Sahinkaya et al., 2011) and ethanol (Zhang et al., 2015). An alternative is the use of a slow release solid organic carbon substrate, such as wood chips, which avoids the need for liquid chemical feed systems. Krayzelova et al. (2014) developed a tire-sulfur hybrid adsorption denitrification (T-SHAD) process where tire chips were used as a slow release source of organic substrate to enhance NO_3^- removal in decentralized wastewater treatment applications. Li et al. (2016) investigated a wood-sulfur heterotrophic-autotrophic denitrification (WSHAD) process for water treatment, and observed decreased SO_4^{2-} accumulation compared with SAD. In a microcosm study carried out in our laboratory with saline wastewater, lower SO_4^{2-} production and higher NO_3^- removal rates were observed in a WSHAD system compared with SAD alone (He et al., 2018).

Wood is mainly composed of cellulose (45-50%), hemicellulose (25-35%), and lignin (25-35%) (Malherbe and Cloete, 2002). It has been used as a carbon source for denitrification of wastewater, stormwater and agricultural drainage (see Lopez-Ponnada et al., 2017 for review), aquaculture effluents (Von Ahnen et al., 2018) and RAS wastewater (Von Ahnen et al., 2019). Wood has been shown to deliver long term (5-15 years) denitrification rates (1-20 g $N/(m^3 \cdot d)$) in onsite wastewater applications with minimum maintenance (Robertson, 2010). However, wood chips can cause an

initial spike in readily biodegradable chemical oxygen demand (rbCOD) concentrations, which decreases over time. High rbCOD concentrations can increase RAS oxygen demands and result in sulfide (S^{2-}) production, posing a toxicological threat to the cultured product (Spotte, 1979). At low rbCOD concentrations; however, nitrite (NO_2^-) accumulation can occur resulting in brown blood disease, which reduces the oxygen carrying capacity of the fish's blood (Hamlin et al., 2008). However, there are no reports about the adverse effects of substrates released from wood on marine aquacultured fish production.

The microbial communities of WSHAD bioreactors are still poorly understood. Li et al. (2016) performed WSHAD batch reactor experiments with NO_3^--contaminated water. The authors identified the genera *Thiobacillus* and *Hydrogenophaga*, which include obligate and facultative autotrophs that can use reduced sulfur compounds or hydrogen as electron donors and NO_3^- as an electron acceptor (Bertrand et al., 2011; Lin et al. 2017). However, previous research suggests that salinity affects the microbial community composition of denitrification systems (Von Ahnen et al., 2019). Von Ahnen et al. (2019) investigated the effect of salinity on microbial community structure and denitrification in horizontal-flow woodchip bioreactors. The presence of heterotrophic denitrifying bacteria, such as those belonging to the orders *Betaproteobacteriales*, *Rhizobiales*, and *Xanthomonadales*, decreased with increasing salinity, suggesting that decreased NO_3^- removal rate was linked to the abundance of these organisms (Von Ahnen et al., 2019).

120 *Campylobacteriales* was observed in the saline bioreactors, which contains
121 *Arcobacter* - a genus that has been shown to oxidize S^{2-} to S^0 (De Gusseme et al.,
122 2009). To the best of our knowledge, there are no studies of the microbiome in
123 WSHAD bioreactors used to treat saline wastewaters.
124 In this study, wood-sulfur cyclic denitrification filters (WS-CDFs) were
125 investigated in pilot-scale marine RASs. The objectives of this study were to: 1)
126 investigate nitrogen transformations (TAN, NO_2^-, NO_3^-) in the marine RASs; 2)
127 investigate overall water quality and fish growth and survival in the systems; 3)
128 evaluate the changes in dominant electron donors in the WS-CDF; 4) reveal the
129 microbial community structure in a WS-CDF treating marine RAS water.
130

131 **2. Materials and Methods**

132 2.1. Materials

133 2.1.1. Water and fish

134 The study was carried out in a pilot-scale RAS, which was modified from a
135 previous study (He et al., 2020). Saline wastewater was prepared by adding 15 g/L
136 Instant Ocean Sea Salt (Instant Ocean®) to tap water. According to the manufacturer,
137 Instant Ocean Sea Salt contains major, minor, and trace elements necessary for marine
138 fish health and is free of NO_3^- and phosphate. *Poecilia sphenops* (mollies) were
139 initially stocked in the fish tanks at a density of 1.97 kg/m^3 and an initial weight of
140 6.70 ± 0.97 g. Mollies were fed fish food pellets (0.6 mm Purina AquaMax Fingerling

300) twice daily at a rate of 2.5 grams/(tank-day) using an automatic feeder (Fish Mate F14, with 14 individual meals). The pellets contain a minimum of 50% crude protein.

2.1.2. Media materials

Elemental sulfur pellets (4.0-6.0 mm) were obtained from Southern Aggregates in Palmetto, Florida. Crushed oyster shells (2.0-4.0 mm) were added as an alkalinity source and were obtained from Myco Supply (Pittsburgh, Pennsylvania). Lightweight expanded clay aggregate in the size range of 3-5 mm was used as a biofilm carrier and was obtained from Trinity Lightweight (Livingston, AL). Expanded clay was washed with tap water and dried in the air at room temperature (~23 °C) prior to use.

White pine (*Pinus strobus*) lumber was obtained from a supplier in Tampa, Florida. The wood was cut into cubes with side length of 7.5 ± 1.4 mm. Pretreatment of the wood cubes was carried out to remove initial high levels of rbCOD using the following method: 1) 642 g of wood cubes were soaked in 3 L of tap water with added Instant Ocean (15 g/L) for 4 days; 2) the soaking solution was poured off and the wood cubes were rinsed 3 times in tap water; 3) wood cubes were soaked in 3 L of fresh saline water of for another 4 days; 4) the cubes were rinsed again before addition to the WS-CDF. After soaking, the side length of the wood cubes was 8.0 ± 1.6 mm.

2.1.3. Inoculum

Inoculum was obtained from a S^0-based CDF, which had been operated in a pilot-scale marine RAS for 250 days (He et al., 2020). This biomass was selected as the inoculum because the microbes were adapted to saline conditions and had received a mix of S^0 and fish waste, which promoted both SAD and heterotrophic denitrification (He et al., 2020). Media from the S^0-based CDF were washed with saline water to obtain 2 L of saline sludge with a total suspended solids (TSS) concentration of 8,178 mg/L and a volatile suspended solids (VSS) concentration of 2,714 mg/L. This sludge was inoculated into the CDF units in the two parallel pilot-RASs as described below.

2.2. Pilot-scale marine RAS

Two pilot-scale marine RAS (Fig. 1) were set up in parallel at the University of South Florida (USF), Tampa (WS-CDF A and WS-CDF B). The design of the pilot-scale marine RAS is fully described in He et al. (2020). Briefly, each RAS was comprised of a fish tank (62 cm H × 30 cm W × 76 cm L) with two water-treatment loops. The first treatment loop included an upflow solids filter (I) (5.2 cm D, 35 cm H), a downflow solids filter (II) (5.0 cm D, 20 cm H), and an aerated Moving Bed Biofilm Reactor (MBBR) (8.5 cm D, 30 cm H). The second treatment loop was a WS-CDF (12.7 cm D, 60 cm H), with a working volume of 2.5 L, which was maintained using a 20 cm height standpipe (Fig. 1). Each WS-CDF was packed with a mixture of 312 g elemental sulfur pellets, 156 g crushed oyster shells, 312 g soaked wood cubes

(dry weight), and expanded clay. The filling ratio of sulfur, oyster shells, and wood cubes was based on our previous microcosm study (He et al., 2018), while the expanded clay was used to make up the remaining working volume. The pore volume was 1.1 L. Each CDF was seeded with 1 L saline sludge after all the media materials were packed.

A solenoid valve controlled by a timer was used to change the flow direction between the Loop 1 and Loop 2 (Fig. 1). Normally, water from the fish tank was circulated in Loop 1 at a rate of 220 ± 20 mL/min. The flow direction was switched to Loop 2 every 12 hr, resulting in water being pumped from the outlet of Solids Filter I to the WS-CDF. After 10 min, the flow was switched back to Loop 1, and the WS-CDF was allowed to react undisturbed until the next cycle. Treated effluent from the WS-CDF overflowed to the MBBR to oxidize any residual NO_2^-, rbCOD, or reduced sulfur compounds before recirculating the flow back to the fish tank. The time interval between cycles was 12 hr, which was defined as the hydraulic retention time (HRT) of the WS-CDF.

Fresh tap water was added to the fish tanks to make up for the water losses due to solids wasting, sampling, and evaporation. Instant Ocean was added as needed to maintain a salinity of approximately 1.5%. $NaHCO_3$ was periodically added into fish tanks to maintain the alkalinity > 100 mg $CaCO_3$/L, which is considered in the healthy range for mollies. The MBBR, tubing, and solids filters I and II were cleaned periodically as described previously (He et al., 2020). Backwashing of the CDF was

carried out monthly as described previously to keep the systems from clogging (He et al., 2020).

2.3 Wood chip COD release

Wood chip soluble COD (sCOD) and rbCOD release tests were performed on fresh wood cubes, wood cubes that had been soaked in saline water for 6 days, and periodically using cubes removed from the WS-CDF. Each test was performed in duplicate; 20 wood cubes were submerged in 100 mL of saline water for 2 days. The supernatant was drawn off and the sCOD and rbCOD concentrations were measured as described below. After each test, the wood cubes that had been removed were returned to the CDF.

2.4 Analytical methods

Dissolved oxygen (DO), pH, and conductivity in the fish tanks were measured *in situ* daily using a portable DO (Mettler Toledo, USA), pH (Oakton™ Handheld Ion Meter), and conductivity (Oakton™ CON 6+ Portable Conductivity Meter) meters. Water samples were collected from the fish tanks and WS-CDF effluent at least 3 times weekly for analysis of TAN, NO_2^--N, NO_3^--N, SO_4^{2-}, S^{2-}, and COD, and weekly for alkalinity, total nitrogen (TN), total phosphorus (TP), TSS, and VSS according to *Standard Methods* (APHA et al., 2017). Samples were filtered through 0.45 µm membrane filters for TAN, NO_2^--N, NO_3^--N, TN, TP, sCOD, rbCOD and SO_4^{2-} analysis, while samples were not filtered for S^{2-} and COD measurements. Detailed analytical methods are described in He et al. (2018).

223 2.5 Microbial community analysis and visualization

224 Samples were collected in duplicate from the initial inoculum and the center of
225 the CDFs at the end of the 125-day operational period. Genomic DNA was extracted
226 from each sample using the AllPrep PowerViral DNA/RNA Kit (QIAGEN, INC.),
227 according to the manufacturer's protocol and stored at -80 °C until PCR
228 amplification. PCR amplification, library preparation, and sequencing were performed
229 by Applied Biological Materials, Inc. (Vancouver, Canada). The 337F (5'-GAC TCC
230 TAC GGG AGG CWG CAG-3') and 805R (5'-GAC TAC CAG GGT ATC TAA TC-
231 3') universal bacterial primers were used to target the V3-V4 hypervariable region of
232 the 16S rRNA gene. Library preparation proceeded by two-step PCR amplification
233 and all amplicons were pooled together and sequenced on an Illumina MiSeq platform
234 using a 2x300 bp paired end sequencing run. The raw reads were deposited into the
235 NCBI Sequence Read Archive database under the following accession number:
236 PRJNA695006.

237 Raw sequencing data processing and analysis were performed using software
238 available on the Galaxy server. In-house perl scripts were used to compare the
239 duplicates of the inoculum, WS-CDF A, and WS-CDF B. Operational taxomonic units
240 (OTUs) of duplicates were organized into separate files representing each taxonomic
241 rank from phylum to genus (files can be accessed after the conclusion section).
242 Relative microbial community structure for the phylum and genus ranks are shown as
243 stacked bar charts. Mean abundances of genera not characterized to the genus level

were combined by taxonomic rank to represent phylum_unclassified, class_unclassified, order_unclassified, and family_unclassified. Changes in microbial community structure between the inoculum and WS-CDFs were shown in bar charts expressing the fold changes of selected phyla and genera.

3. Results and Discussion

3.1. Marine RAS performance

3.1.1. Solids and color

TSS and VSS concentrations in the fish tanks ranged from 20 to 35 mg/L and from 12 to 21 mg/L, respectively, showing that solids were effectively removed in the solids filters. Accumulation of solids within aquaculture tanks can promote an environment that harbors fish pathogens and leaches nutrients that adversely affect the health of fish (Davidson and Summerfelt, 2005).

A brown color was observed in the RAS water, most likely due to the combined effect of the fish food (Schuster, 1994) and substances released from wood. The color was not quantified; however, the water was visibly darker than in our previous study with a S^0-based CDF (He et al., 2020). Hartz et al. (2017) observed that effluent from a wood chip-denitrification reactor was dark colored for the first several weeks of operation due to tannins that leached from the woodchips. Clear water could enhance the ability of fish to capture feed and allow the farmer to observe fish health, behavior, and feeding activity (Davidson et al., 2016). Water exchanges during the

initial operation, activated carbon filtration, or advanced oxidation processes (e.g., ozone) could be used to remove water color (Schuster, 1994).

3.1.2. Nitrogen removal

Nitrogen entered the system with the fish feed, which contained 50% protein. Dissolved nitrogen compounds, including TAN, NO_2^-, and NO_3^-, were generated in the RAS by fish excretion, decomposition of uneaten feed, and nitrification. Fig. 2a shows changes in TAN, NO_2^-, and NO_3^- concentrations in fish tanks during RAS operation. Average TAN, NO_2^-, and NO_3^- concentrations were maintained at 0.17 ± 0.05 mg N/L, 0.41 ± 0.17 mg NO_2^--N/L, and 56.77 ± 2.72 mg NO_3^--N/L, respectively. Average TN and total organic nitrogen (TON) concentrations in the fish tanks were ~81 mg N/L and ~25 mg N/L, respectively. The steady performance over the entire operational period indicates that nitrogen added on a daily basis was removed by assimilation into fish and microbial biomass, solids wasting, nitrification, and denitrification. A nitrogen mass balance (Cheng, 2019) showed that passive denitrification in the first loop contributed to 5% of the nitrogen removal in the RAS. Passive denitrification occurs within anoxic environments in solids filters, as well as biofilms in MBBRs and fish tanks where water contains organic carbon and NO_3^- (Van Rijn et al., 2006).

NO_3^-, NO_2^-, and TAN concentrations in WS-CDF effluent over time are shown in Fig. 2b. The short acclimation period (1 day) shows that the inoculum from the S^0-based CDF quickly adapted to using a combination of wood and S^0 as electron

donors. After acclimation, the average NO_3^- removal rate was 102.6 ± 8.5 g N/(m^3·d) in the WS-CDF (removal efficiency of 90.3 ± 4.3%). The mass balance showed that nitrogen removal in the WS-CDF accounted for 57% of the total nitrogen removed. Our prior study with a S^0-based CDF incorporated into a similar RAS achieved an average denitrification rate of 86.7 ± 5.1 g N/(m^3·d) (He et al., 2020). The higher denitrification rate obtained in this study was likely due to higher influent NO_3^- concentration and the combined use of S^0 and wood chips.

NO_2^- accumulation is an indicator of incomplete denitrification, which leads to production of NO and N_2O, which are potent greenhouse gases (Bertrand et al., 2011). Although NO and N_2O were not measured in this study, NO_2^- was detected in the CDF effluent (Fig. 2b), especially on the first day (16.48 mg NO_2^--N/L). As mentioned previously, bacteria needed to adapt to the new environment at the beginning of RAS operation, resulting in incomplete denitrification. However, the effluent NO_2^- concentration quickly stabilized at 0.47 ± 0.22 mg NO_2^--N/L. Pine wood contains complex organic molecules, which can sometimes be utilized by fermentative bacteria capable of reducing NO_3^- to NO_2^- (Beauchamp et al., 1989). In a prior microcosm study with a variety of electron donors (He et al., 2018), NO_2^- accumulation was higher in wood-containing bioreactors than in SAD bioreactors.

As shown in Fig. 2b, TN concentrations in the WS-CDF effluent were 14.57 ± 7.24 mg N/L; effluent TAN concentrations ranged from 0.5 to 1.6 mg N/L. TAN can be generated in the WS-CDF through ammonification of nitrogen containing organic

307 matter (wood or fish waste) or dissimilatory nitrate reduction to ammonium (DNRA).
308 Influent TON concentrations decreased from 24.59 ± 4.70 to 7.48 ± 3.83 mg N/L in
309 the effluent (Figs. 2a and 2b), indicating degradation of organic nitrogen. DNRA was
310 not quantified; however, it has been previously observed in wood chip denitrification
311 reactors (Greenan et al., 2006).

312 Table 1 summarizes studies that combined S^0 and organic carbon sources as
313 electron donors for denitrification. Differences in denitrification rates in different
314 studies are likely due to differences in labilities of organic matter for heterotrophic
315 denitrifying bacteria. Higher mixotrophic denitrification rates were obtained when
316 methanol was used compared with solid carbon sources (Oh et al., 2001; Sahinkaya et
317 al., 2011). However, as discussed previously, the methanol dosage needs to be strictly
318 controlled to avoid carry-over of rbCOD into the RAS.

319 In terms of solid carbon sources, a relatively higher denitrification rate was
320 observed with tire chips than wood chips. Tire chips not only provided a carbon
321 source for heterotrophic denitrification, but was also shown to adsorb NO_3^- when the
322 influent NO_3^- loading exceeded the denitrification capacity of the biofilm (Krayzelova
323 et al., 2014). The current study achieved the highest denitrification rate of the studies
324 carried out with wood-based media. Von Ahnen et al. (2018) used *Salix viminalis*
325 (basket willow), which is a hardwood. The pine used in this study is a softwood,
326 which has been shown to have a higher permeability than hardwoods, making it prone
327 to water capture and enhanced contact between microorganisms and the substrate

328 (Van Driel et al., 2006). In addition, the wood used by Von Ahnen et al. (2018) had
329 been in reactors treating aquaculture effluent for approximately one year. The
330 bioavailability of organic carbon released from wood was shown to decrease over
331 time with denitrifying bioreactor operation (He et al., 2018). Robertson (2010)
332 investigated woodchip denitrification performance in reactors of varying ages and
333 found that woodchips lost about 50% of their reactivity after one year of operation
334 due to leaching of soluble organic compounds.

335 3.2. Dominant electron donor variations in the CDFs

336 As shown in Fig. 3a, COD concentrations in the WS-CDF effluent were higher
337 than those in fish tanks during the first 60 days, indicating that wood chips initially
338 released excess organic carbon not used for heterotrophic denitrification. The decrease
339 in effluent COD concentrations on Day 61 may have been due to the second
340 backwashing on Day 60, which floated some wood cubes to the top of the WS-CDFs.

341 Tests were carried out to investigate the release of sCOD and rbCOD over time
342 from the wood cubes. rbCOD consists of simple organic molecules, which can pass
343 through cell membrane of denitrifying bacteria and be metabolized within minutes
344 (Mamais et al., 1993). As shown in Fig. 4, fresh pine wood had a relatively high
345 sCOD and rbCOD release (116.7 ± 1.7 mg sCOD/(g wood) and 94.7 ± 13.5 mg
346 rbCOD/(g wood)), which decreased after being submerged in saline water for 8 days
347 (19.2 ± 0.5 mg sCOD/(g wood) and 15.7 ± 0.7 mg rbCOD/(g wood) when they were
348 packed in the WS-CDF. The decrease in rbCOD in the woodchips over time may lead

to NO and N_2O production due to incomplete denitrification (Bertrand et al., 2011).

sCOD and rbCOD release decreased exponentially according to the following:

$$\text{sCOD (mg sCOD/(g wood))} = 117 \times e^{-0.267 \times t} \quad (R^2 = 0.9932) \qquad (2)$$

$$\text{rbCOD (mg COD/(g wood))} = 95 \times e^{-0.404 \times t} \quad (R^2 = 0.9768) \qquad (3)$$

In our previous study, significant SO_4^{2-} accumulation was observed in the fish tanks when only S^0 was used as the electron donor for denitrification (He et al., 2020). In this study, fish tank SO_4^{2-} concentrations stabilized at around 2,057 ± 122 mg SO_4^{2-}/L (Fig. 3b). The use of WSHAD successfully avoided high SO_4^{2-}accumulation in the marine RAS (*i.e.*, SO_4^{2-} production due to SAD was balanced by SO_4^{2-} removal due to water losses during sampling and solids wasting). To understand the dominant electron donor variations in the WS-CDFs over time, the data were divided into four stages: 1) days 0-30; 2) days 31-60; 3) days 61-90; and 4) days 91-125. SO_4^{2-} generation during Stages 1 through 4 was 3.73 mg SO_4^{2-}/mg NO_3^--N, 3.07 mg SO_4^{2-}/mg NO_3^--N, 5.76 mg SO_4^{2-}/mg NO_3^--N, and 6.65 mg SO_4^{2-}/mg NO_3^--N, respectively. Based on the theoretical SO_4^{2-} productivity of SAD of 7.54 mg SO_4^{2-}/mg NO_3^--N, SAD accounted for 49.4% of NO_3^- removal during Stage 1, decreased to 40.7% in Stage 2, and then increased to 76.4% in Stage 3 and 88.2% in Stage 4. As mentioned previously, the seed sludge was from a S^0-based CDF, which has been operated for > 6 months. This may have resulted in the high initial SO_4^{2-} generation observed in Stage 1. As the bacteria adapted to the WSHAD environment, heterotrophic denitrification became more dominant during Stage 2. However, with the decrease in

370 rbCOD released from pine wood chips, the contributions of SAD to NO_3^- removal
371 increased in Stages 3 and 4.
372 3.3 Microbial community structure in the CDFs
373 3.3.1 CDF phylum-level microbial community structure
374 Information from the 16S rRNA gene libraries for the initial inoculum, WS-CDF A,
375 and WS-CDF B samples can be accessed after the conclusion section. As stated
376 previously, the initial inoculum was sludge collected from a S^0-based CDF integrated
377 into a pilot-scale marine RAS (He et al., 2020). Good's coverage suggested that the
378 microbial composition for each sample was well represented by the constructed
379 sequence libraries and thus reflect the true bacterial profile. The observed OTUs
380 representing specific phyla or genera for each sample were used to examine the
381 microbial community structure and infer the main taxonomic groups responsible for
382 CDF performance at the phylum and genus levels, respectively.
383 The phylum level microbial community composition of the inoculum and WS-
384 CDFs were similar, with slight differences in the abundance of specific phyla. Figures
385 that depict the relative abundance of bacteria within these samples and quantify the
386 changes that occurred can be accessed after the conclusion section. *Proteobacteria*,
387 *Actinobacteria*, and *Bacteriodetes* were the three largest phyla observed in the
388 samples and accounted for between 21-33%, 23-30%, and 8.3-32%, respectively.
389 Notable changes between the inoculum and WS-CDF systems include those occurring
390 in *Chlorobi* and *Parcubacteria*, which represented 7.9% and 2.4%, respectively, in the

391 inoculum. *Parcubacteria* decreased by more than four times (Log2 WS-
392 CDF:Inoculum = -2.5 ± 0.91) and *Chlorobi* was undetected in WS-CDF A and
393 decreased by more than four times in WS-CDF B (Log2 WS-CDF B:Inoculum = -
394 4.6). Variations in the abundance in the observed phyla between the inoculum, WS-
395 CDF A, and WS-CDF B are likely to have occurred due to mixotrophic metabolism
396 by the input of fish waste, woodchips, and/or S^0, changes in operation of the CDFs
397 (lower HRT and more frequent backwashing), or the 16S amplicon sequencing
398 process, which is elaborated further in the discussion of the genus level results.
399 3.3.3 Link between CDF media, operation, and microbiome
400 The relative abundance of bacteria on the genus level in the inoculum, WS-CDF
401 A, and WS-CDF B is shown in Fig. 5. Notable genera in the inoculum include
402 *Prosthecochloris*, *Sulfurovum*, and *Chlorobium*, which represented 5.3%, 4.4%, and
403 1.0% of the total population, respectively. Bacteria belonging to *Sulfurovum* are
404 obligate autotrophs and might have contributed to nitrogen removal through SAD
405 (Inagaki et al., 2004). Furthermore, *Prosthecochloris* and *Chlorobium* include species
406 capable of autotrophic growth using sulfide as an electron donor (Kumar et al., 2009;
407 Vogel et al., 2006). SO_4^{2-} produced during SAD may have migrated to lower redox
408 potential zones in the CDF and been used to form sulfide by SO_4^{2-}-reducing bacteria
409 belonging to the phylum *Parcubacteria*, which today remains unclassified.
410 *Desulfocapsa*, a sulfur-disproportionating bacterium whose abundance was 0.15%,
411 may have also provided S^{2-} for the cultivation of *Prosthecochloris* and *Chlorobium*

412 (Finster et al., 1998). Microbial community analysis of the initial inoculum indicates
413 that the S^0-based CDF was enriched with a diverse consortium of salt-tolerant
414 organisms including S^0-oxidizing denitrifiers, SO_4^{2-}-reducing bacteria, and sulfur-
415 disproportionating bacteria.

416 Differences in media composition, HRT and backwashing frequency likely
417 contributed to the changes in the microbial community structure between the initial
418 inoculum from the S^0-based CDF and WS-CDF microbiome. Less S^0 was provided in
419 the WS-CDFs compared to the S^0-based CDF (312 vs. 1200 g, respectively) to
420 compensate for the addition of woodchips. The reactor volume for the WS-CDFs was
421 smaller than that applied in the S^0-based CDF. This was done to decrease the HRT and
422 minimize spatial and/or temporal redox reactions that generate S^{2-}. In addition, the
423 CDFs were backwashed monthly to avoid clogging due to biofilm accumulation. Fig.
424 6 quantifies the microbial community change that occurred in the WS-CDFs,
425 compared to the inoculum. Analysis of the microbial community change at the genus
426 level indicates that a lower quantity of S^0 in the WS-CDFs appears to have been
427 insufficient to maintain the growth of *Sulfurovum*, causing this genera to decrease by
428 more than 16 times (Log2 WS-CDF:Inoculum = -4.4 ± 1.3; Fig. 6). The microbial
429 community change also suggests that reducing the HRT and increasing the
430 backwashing frequency resulted in less favorable conditions for SO_4^{2-}-reducing
431 bacteria. As a result, SO_4^{2-}-reducing bacteria belonging to *Parcubacteria* decreased by
432 more than four times (Log2 WS-CDF:Inoculum = -2.5 ± 1.8; Fig. 6) and subsequently

433 *Chlorobium* and *Prosthecochloris* were undetected in one of the WS-CDFs (Fig. 5).
434 *Chlorobium* and *Prosthecochloris* are members of the phylum *Chlorobi*; thus, the
435 decrease of these populations to a point below detection is in agreement with the
436 phylum level observations mentioned previously.
437 Changes made to the environmental conditions within the CDFs by augmenting
438 the media and operating conditions, seems to have allowed for the cultivation of other
439 bacteria that initially represented much lower percentages of the inoculum.
440 Noteworthy genera that increased by more than two times include *Sulfurimonas*,
441 *Thioalbus*, and *Ornatilinea* (Log2 WS-CDF:Inoculum = 1.2 ± 0.86, 2.2 ± 0.52, and
442 2.9 ± 0.79, respectively; Fig. 6). *Sulfurimonas* and *Thioalbus* include species of sulfur
443 oxidizing bacterium that are capable of denitrification, while *Ornatilinea* includes
444 heterotrophic bacteria capable of utilizing cellulose as a carbon source (Bertrand et
445 al., 2011; Podosokorskaya et al., 2013). The increase in abundance of *Ornatilinea*
446 within the WS-CDFs indicates the presence of specialized bacteria that utilize organic
447 carbon from the pine wood chips supplied. Genus level analysis of the WS-CDF
448 shows that the microbial community included multiple autotrophic and heterotrophic
449 denitrifiers including *Defluviimonas* (Liu et al., 2017), which contributed to a higher
450 NO_3^- removal rate than that observed in our prior S^0-based CDF study (He et al.,
451 2020).
452 The operation of the WS-CDFs may have also played a role in their individual
453 microbial community composition. As shown in Fig. 5, *Mycobacterium* and

454 *Parachlamydia* were the largest genera observed in both the inoculum and WS-CDF
455 B, representing between 18-22% and 1.1-4.0% of the population, respectively.
456 Members belonging to the *Mycobacterium* and *Parachlamydia* genera are
457 heterotrophic and the former includes salt-tolerant species capable of reducing NO_3^-
458 (Bertrand et al., 2011). Therefore, the presence of these bacteria indicates their
459 involvement in COD removal and/or heterotrophic denitrification within the CDFs.
460 While *Parachlamydia* represented one of the largest genera in WS-CDF A (2.0%),
461 *Mycobacterium* accounted for only 1.6%. However, members belonging to the family
462 *Mycobacteriaceae* accounted for the majority (22%) of the total bacteria in WS-CDF
463 A that were classified to only the family rank (30%), or the family_unclassified group
464 shown in Fig. 5. *Mycobacterium* is the only known genus belonging to
465 *Mycobacteriaceae*; thus, it is probable that this genus also represents one of the
466 largest genera in WS-CDF A. Variations between the populations in WS-CDF A and B
467 may be attributed to differences in TSS and VSS. The inverse simpson index, or
468 invsimpson, is a diversity metric that accounts for both the total number of OTUs and
469 abundance.WS-CDF A had a higher invsimpson value, suggesting that its microbial
470 community structure was more diverse that WS-CDF B.
471 Another source of variation between the WS-CDFs may have resulted from the
472 16S amplicon sequencing process. Sequencing of samples collected from WS-CDF A
473 and B occurred at different time periods. Therefore, technical bias may have been
474 introduced due to sample preparation and/or instrumentation.

3.4. Fish growth and performance

The initial fish weight was 6.70 ± 0.97 g and reached 9.38 ± 5.78 g at the end of the study, with a survival rate of 62 ± 4%. The survival rate was significantly lower than that obtained in our previous study (88%), where S^0 was the only added electron donor for denitrification (He et al., 2020). In addition, side swimming, rapid swimming, and body bending were observed during the operation, especially during the initial period. Fish that showed these behaviors were euthanized according to USF's Institutional Animal Care and Use Committee (IACUC) protocols. It was noticed that these symptoms were relieved during the last month of operation.

As summarized in Table 2, nitrogen concentrations, including NO_3^-, NO_2^- and TAN, were in the healthy range and were similar to those achieved in our prior study (He et al., 2020). Therefore, RAS nitrogen species concentrations were not responsible for the high fish mortality. Other water quality parameters were also in the healthy range, including TP, S^{2-}, TSS, VSS, alkalinity, pH, salinity, DO and temperature.

Increased mortalities observed in this study may have occurred due to high SO_4^{2-} concentrations, pathogenic bacteria, or the release of toxic compounds from wood. High SO_4^{2-} concentrations (> 2000 mg SO_4^{2-}/L) may have chronic toxic effect on freshwater fish health (Elphick et al., 2011). Davidson et al. (2014) discussed potential toxicity of water parameters and mentioned that SO_4^{2-} may cause a mild chronic effect to rainbow trout. *Mycobacterium* and *Parachlamydia*, which were

identified during the microbial community analysis (Fig. 6), include suspected agents of epitheliocystis and mycobacteriosis - two infectious diseases common in freshwater and marine species, especially those raised in intensive aquaculture systems (Blandford et al, 2018). Higher abundances of these bacteria in the WS-CDFs than the S^0-based CDFs may have contributed to the higher mortalities observed in this study (Pawlikowska-Warych and Deptula, 2016). Wood releases compounds, such as phenols, resin acids, tannins, lignin and volatile fatty acids, that can be toxic to animals (Hedmark and Scholz, 2008). It was reported that water leachate from woodchips was toxic to trout (Taylor et al., 1996). Additional research is needed to understand the effect of high SO_4^{2-} and chemicals released from wood on marine RAS species and whether these conditions trigger stress that promotes a high susceptibility to epitheliocystis and mycobacteriosis.

4. Conclusions

This study is the first to apply the WSHAD process for saline wastewater treatment and elucidate the microbiomes of these mixotrophic biofilters. WS-CDFs achieved high denitrification rates, with lower SO_4^{2-} accumulation than SAD alone. Changes in rbCOD release from wood chips over time resulted a shift in the dominant denitrification mechanism from heterotrophic to SAD. The WS-CDF microbiome reflected a unique microbial consortium, including salt tolerant cellulose and sulfur utilizing denitrifiers, such as *Sulfurimonas, Thioalbus, Ornatilinea, Mycobacterium*

and *Parachlamydia*. Results from this research can be applied to treatment of saline domestic, industrial and agricultural wastewaters.

Appendix A: Supplementary data

E-supplementary data of this work can be found in the online version of the paper.

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Figure
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Figure Captions

Fig. 1. Schematic of the pilot-scale marine RAS (WS-CDF: wood-sulfur cyclic denitrification filter; MBBR: moving bed biofilm reactor).

Fig. 2. Profiles of dissolved nitrogen species concentrations in: a) fish tanks and b) CDF effluent.

Fig. 3. Changes in a) sCOD and b) SO_4^{2-} concentrations in the fish tanks and WS-CDF effluent.

Fig. 4. sCOD and rbCOD released from wood in WS-CDFs over time.

Fig. 5. Relative abundance of bacteria present in the inoculum and WS-CDF systems on the genus level. Genera that are ≥ 1.0% in abundance in at least a single sample are shown. Other is comprised of genera that are < 1.0% in all three samples.

Fig. 6. Relative change in the microbial community composition on genus level between the WS-CDF systems and initial inoculum. Genera representing: (1) ≥ 0.1% of the total reads in at least a single sample; and (2) with Log2 fold changes -1 ≤ and ≥ 1 are shown.

Fig.1

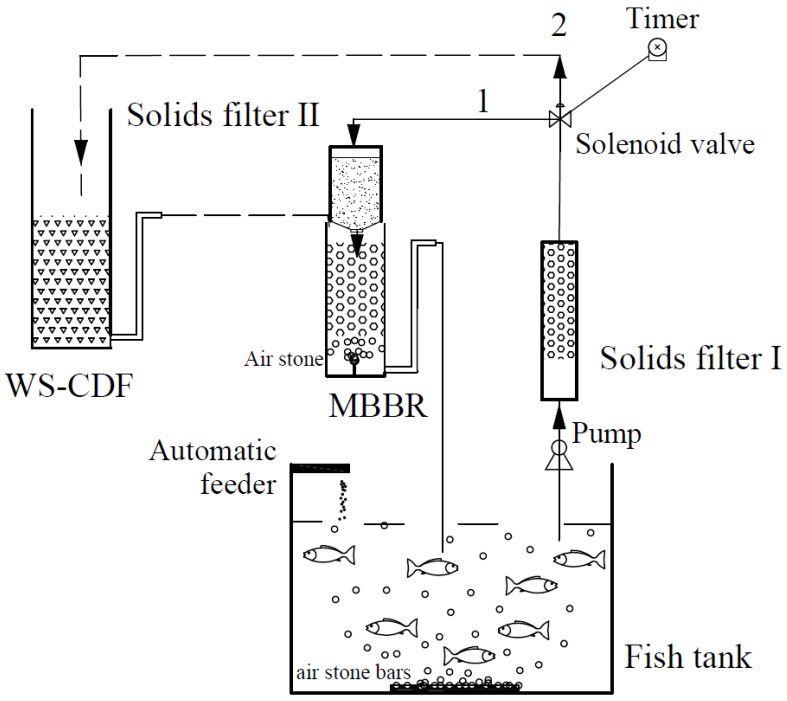

2
Timer
1
Solids filter II
Solenoid valve
Air stone
Solids filter I
WS-CDF
MBBR
Pump
Automatic
feeder
air stone bars
Fish tank

Fig.2

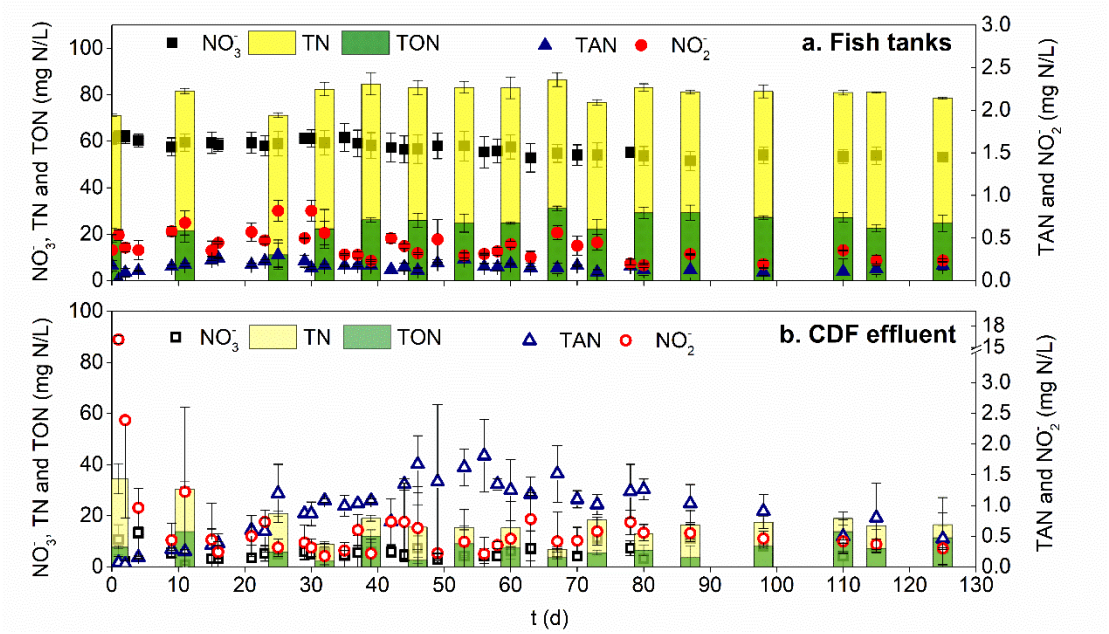

a. Fish tanks
b. CDF effluent
NO3-
TN
TON
TAN
NO2-
NO3-, TN and TON (mg N/L)
TAN and NO2- (mg N/L)
t (d)

Fig.3

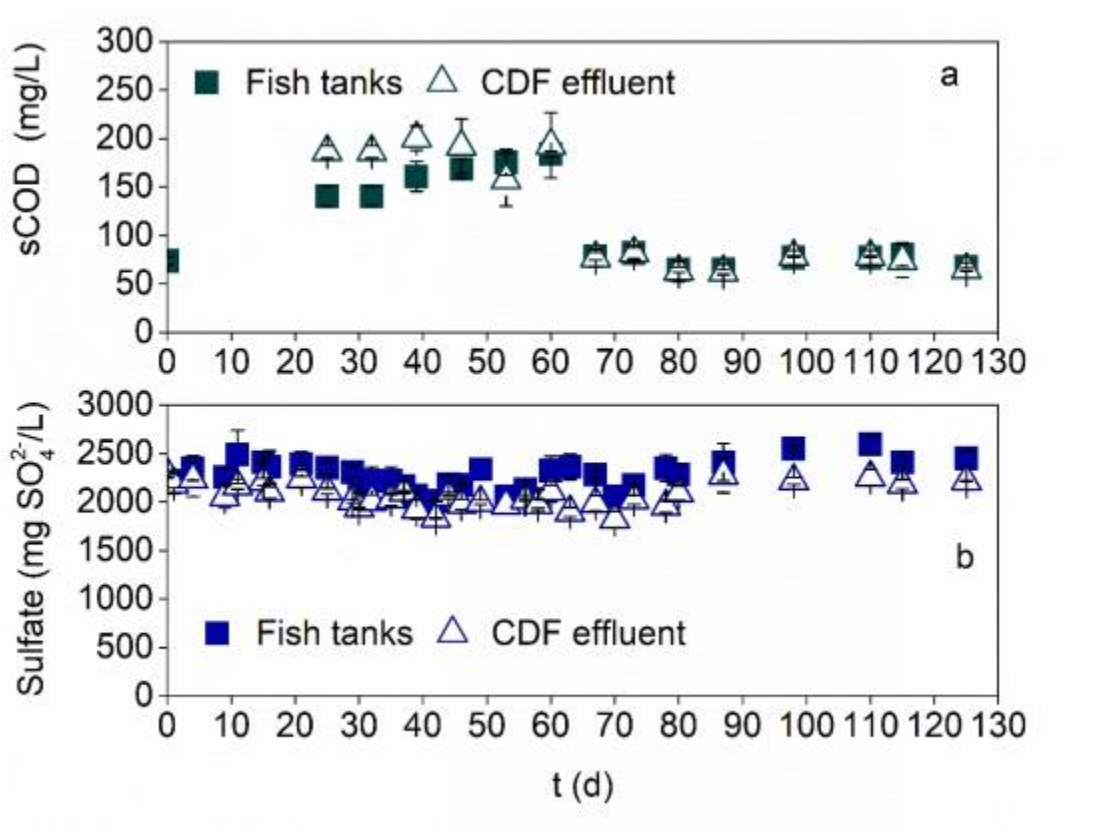


Fig.4

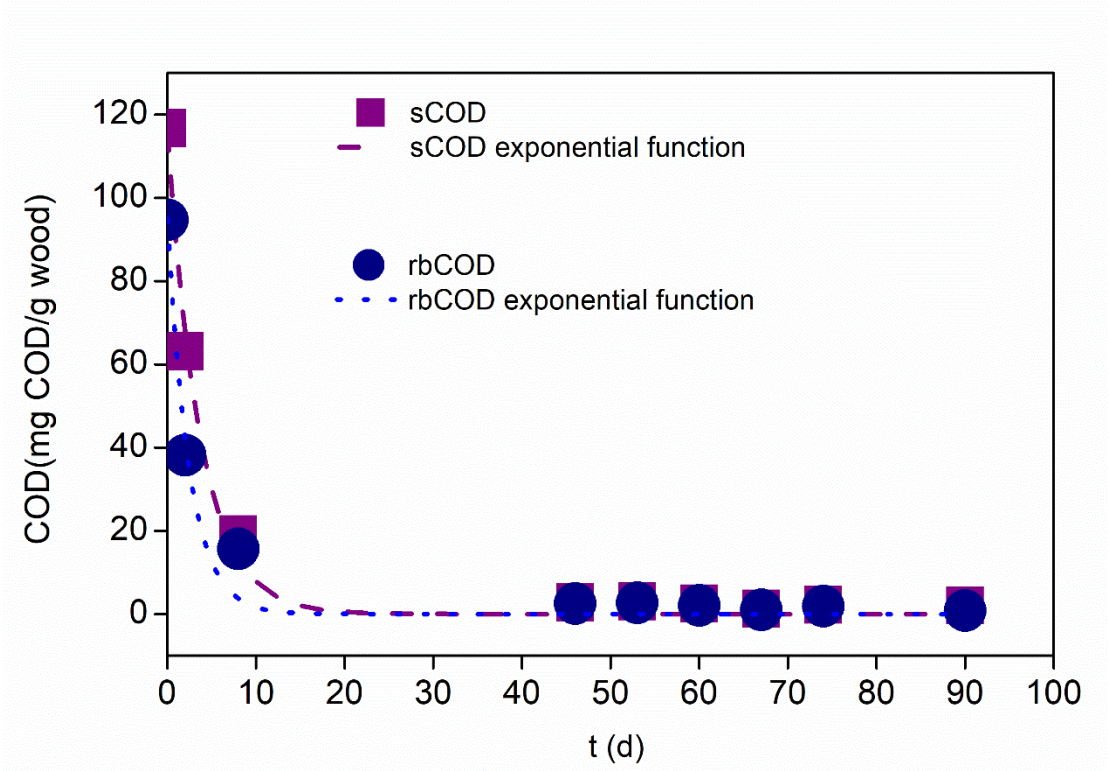

sCOD
sCOD exponential function
rbCOD
rbCOD exponential function
COD(mg COD/g wood)
t (d)
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70
80
90
100

Fig.5

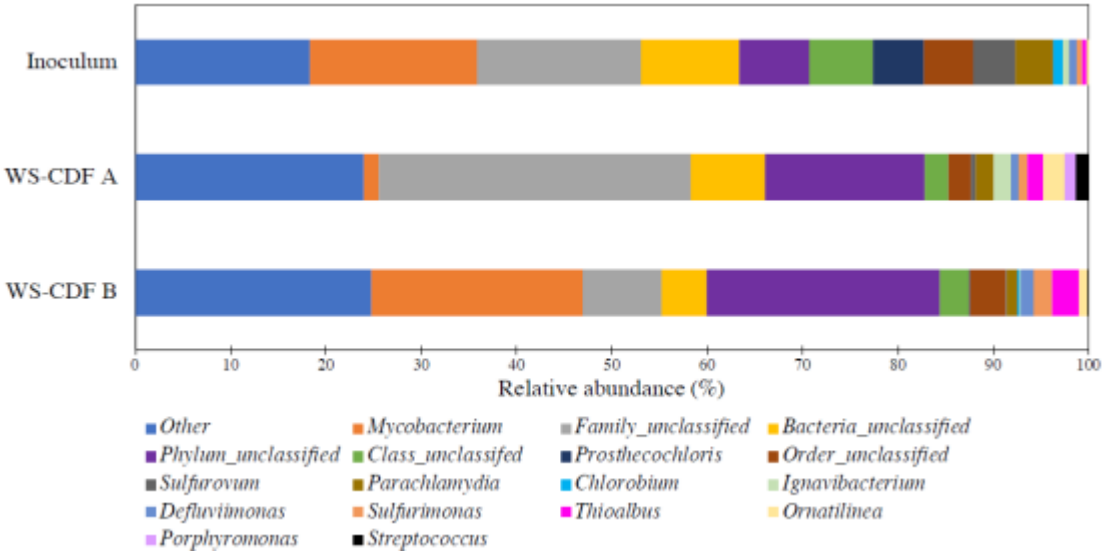

Inoculum
WS-CDF A
WS-CDF B
0
10
20
30
40
50
60
70
80
90
100
Relative abundance (%)
Other
Mycobacterium
Family_unclassified
Bacteria_unclassified
Phylum_unclassified
Class_unclassifed
Prosthecochloris
Order_unclassified
Sulfurovum
Parachlamydia
Chlorobium
Ignavibacterium
Defluviimonas
Sulfurimonas
Thioalbus
Ornatilinea
Porphyromonas
Streptococcus

Fig.6

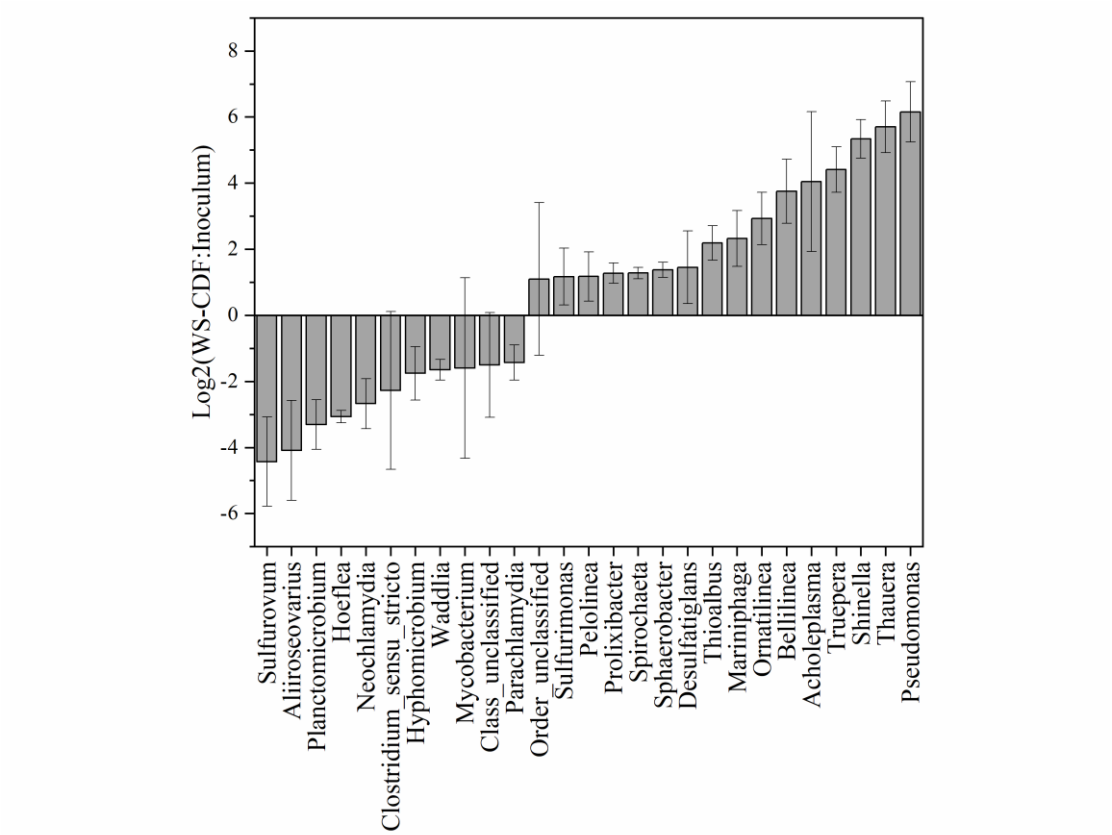

Log2(WS-CDF:Inoculum)
8
6
4
2
0
-2
-4
-6
Sulfurovum
Aliiroseovarius
Planctomicrobium
Hoeflea
Neochlamydia
Clostridium_sensu_stricto
Hyphomicrobium
Waddlia
Mycobacterium
Class_unclassified
Parachlamydia
Order_unclassified
Sulfurimonas
Pelolinea
Prolixibacter
Spirochaeta
Sphaerobacter
Desulfatiglans
Thioalbus
Mariniphaga
Ornatilinea
Bellilinea
Acholeplasma
Truepera
Shinella
Thauera
Pseudomonas

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Table 1. Sulfur-based mixotrophic denitrification performance in other studies and this study.

NO_3^--contaminated water	**Carbon source**	**Alkalinity source**	**Influent NO_3^- concentration (mg NO_3^--N/L)**	**HRT (h)**	**Removal efficiency (%)**	**Denitrification rate (g N/(m^3·d))**	**Reference**
Marine RAS	Pine blocks	Oyster shells	56.8 ± 2.7	12	90.3 ± 4.3	102.6 ± 8.5	This study
Freshwater aquaculture	Wood chips	Seashells	30	10	-	8.12 ± 0.49	Von Ahnen et al. (2018)
Synthetic nitrified wastewater	Tire chips	Oyster shells	50	6	89.5	368.8	Krayzelova et al. (2014)
Synthetic NO_3^--contaminated water	Methanol/ landfill leachate	$NaHCO_3$	100	7.5	>97	1,920~2,700	Oh et al. (2001)
Groundwater	Methanol	Limestone	75	11	75	164	Sahinkaya et al. (2011)
Groundwater	Methanol	$NaHCO_3$	75	4	100	450	Sahinkaya and Dursun (2012)

Table 2 Water-quality parameters in fish tanks (values shown in parentheses are standard deviations).

Parameters	Value	Recommended limit	References
NO_2^- (mg NO_2^--N/L)	0.40 (0.17)	<1	Timmons et al. (2002)
TAN (mg N/L)	0.17 (0.04)	<3	Timmons et al. (2002)
NO_3^-(mg NO_3^--N/L)	56.78 (2.72)	<75	Davidson et al. (2016)
TN	82.1 (4.6)	-	-
TON	24.6(4.7)	-	-
SO_4^{2-} (mg /L)	2,057 (122)	-	-
TP (mg /L)	0.58 (0.46)	-	-
S^{2-} (mg /L)	0.01 (0.01)	<5.2	Bagarinao and Lantin-Olaguer (1998)
COD (mg/L)	111 (47)	-	-
TSS (mg/L)	26 (4)	20-40	Timmons et al. (2002)
VSS (mg/L)	16 (3)	-	-
Alkalinity (mg $CaCO_3$/L)	128 (16)	100-150	Recommendation from expert in fish farm
pH	8.02 (0.31)	7.5-8.2	Ganguly et al. (2014)
Salinity (%)	1.5	13.5	Nordlie et al. (1992)
DO (mg/L)	5-6.5	1	Ganguly et al. (2014)
Temperature (℃)	26.5-27.2	18-28	Ganguly et al. (2014)

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CRediT author statement

Qiaochong He - Conceptualization, Ideas, Methodology, Investigation, Formal analysis, Writing, Visualization

Erica A. Dasi - Conceptualization, Ideas, Methodology, Investigation, Formal analysis, Writing, Visualization

Zhang Cheng - Conceptualization, Ideas, Methodology, Investigation, Formal analysis, Writing

Emmanuel Talla - Ideas, Methodology, Investigation, Formal analysis, Writing, Visualization, Resources, Supervision

Kevan Main - Ideas, Methodology, Formal analysis, Writing, Resources

Chuanping Feng - Conceptualization, Resources, Supervision

Sarina J. Ergas: Conceptualization, Ideas, Methodology, Writing, Visualization, Resources, Supervision, Project administration, Funding acquisition

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: