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## 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<sub>2</sub>O. 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<sub>2</sub>O; 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<sub>2</sub><sup>-</sup> accumulation is an indicator of incomplete denitrification, which leads to production of NO and N<sub>2</sub>O, which are potent greenhouse gases (Bertrand et al., 2011). Although NO and N<sub>2</sub>O were not measured in this study, NO<sub>2</sub><sup>-</sup> was detected in the CDF effluent (Fig. 2b), especially on the first day (16.48 mg NO<sub>2</sub><sup>-</sup>-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<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>) 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., Parcubacteria). Parcubacteria 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<sup>0</sup> 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<sup>0</sup> biofilters.
- **Combined use of wood and S<sup>0</sup> resulted in lower SO<sub>4</sub><sup>2-</sup> accumulation than S<sup>0</sup> 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<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, SO<sub>4</sub><sup>2-</sup>, S<sup>2-</sup>, 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<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, TN, TP, sCOD, rbCOD and SO<sub>4</sub><sup>2-</sup> analysis, while samples were not filtered for S<sup>2-</sup> 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<sup>0</sup> biofilters.
- Combined use of wood and S<sup>0</sup> resulted in lower SO<sub>4</sub><sup>2-</sup> accumulation than S<sup>0</sup> 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.

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

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7196

18 **Abstract:** This study investigated the performance and microbiome of cyclic  
19 denitrification filters (CDFs) for wood and sulfur heterotrophic-autotrophic  
20 denitrification (WSHAD) of saline wastewater. Wood-sulfur CDFs integrated into two  
21 pilot-scale marine recirculating aquaculture systems achieved high denitrification  
22 rates ( $103 \pm 8.5$  g N/(m<sup>3</sup>·d)). The combined use of pine wood and sulfur resulted in  
23 lower SO<sub>4</sub><sup>2-</sup> accumulation compared with prior saline wastewater denitrification  
24 studies with sulfur alone. Although fish tank water quality parameters, including  
25 ammonia, nitrite, nitrate and sulfide, were below the inhibitory levels for marine fish  
26 production, lower survival rates of *Poecilia sphenops* were observed compared with  
27 prior studies. Heterotrophic denitrification was the dominant removal mechanism  
28 during the early operational stages, while sulfur autotrophic denitrification increased  
29 as readily biodegradable organic carbon released from wood chips decreased over  
30 time. 16S rRNA-based analysis of the CDF microbiome revealed that *Sulfurimonas*,  
31 *Thioalbus*, *Defluviimonas*, and *Ornatilinea* as notable genera that contributed to  
32 denitrification performance.

33

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



## 36 1. Introduction

37 Saline wastewaters with high nitrate ( $\text{NO}_3^-$ ) concentrations are generated from a  
38 number of domestic and industrial processes. For example, seawater is used for toilet  
39 flushing in many arid countries, resulting in saline domestic wastewater (Huang et al.,  
40 2018). Ion-exchange resins used to treat  $\text{NO}_3^-$  contaminated groundwater are  
41 regenerated using high NaCl concentration solutions, resulting in high- $\text{NO}_3^-$  brine  
42 wastes (Trögl et al., 2011). Industrial and agricultural processes, such as aquaculture,  
43 seafood processing, tanneries, pulp and paper and textile dyeing, also generate saline  
44 wastewater containing high  $\text{NO}_3^-$  levels (Liang et al., 2017).

45 In this project, we investigated the use of a novel cyclic denitrification filter  
46 (CDF), and applied this process for treatment of marine aquaculture wastewaters.  
47 Aquaculture accounts for approximately half of the world's food fish production, and  
48 marine aquaculture contributes to 36% of global aquaculture production (FAO, 2018).  
49 Recirculating aquaculture systems (RAS) have attracted considerable research and  
50 commercial interest due to their reduced water use and environmental impacts  
51 compared with conventional marine aquaculture using ponds, raceways or cages (Van  
52 Rijn et al, 2006; Hamlin et al., 2008). In RAS, water is circulated through filters and  
53 bioreactors, which remove solids, organic matter and total ammonia nitrogen (TAN),  
54 before being returned to the fish tanks. Aerobic biofilters are typically used for  
55 oxidation of organic carbon and nitrification, resulting in  $\text{NO}_3^-$  accumulation in the  
56 RAS. Therefore, water exchanges are used to maintain  $\text{NO}_3^-$  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  $\text{NO}_3^-$   
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  $\text{NO}_3^-$  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  $\text{NO}_2^-$  accumulation (He et al., 2018). Elemental sulfur ( $\text{S}^0$ ) is a low-cost  
66 electron donor for  $\text{S}^0$  autotrophic denitrification (SAD), which has been successfully  
67 used for  $\text{NO}_3^-$  removal from freshwater (Sahinkaya and Dursun, 2012) and marine  
68 RAS (He et al., 2020). One potential drawback of SAD is sulfate ( $\text{SO}_4^{2-}$ )  
69 accumulation. In a prior study in our laboratory,  $\text{NO}_3^-$  concentrations were maintained  
70 at a stable level in a pilot-scale marine RAS using a packed-bed denitrification  
71 bioreactor containing  $\text{S}^0$ . However,  $\text{SO}_4^{2-}$  concentrations increased from a background  
72 value of 1,400 to 2,322 mg  $\text{SO}_4^{2-}$ /L after 146 days operation (He et al., 2020). The  
73 effect of elevated  $\text{SO}_4^{2-}$  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  $\text{NO}_3^-$  is removed heterotrophically and the remainder is

78 denitrified by SAD, resulting in increased  $\text{NO}_3^-$  removal rates and decreased  $\text{SO}_4^{2-}$   
79 production and alkalinity consumption (Oh et al., 2001). Liquid organic carbon  
80 sources have been used previously for mixotrophic denitrification, including methanol  
81 (Oh et al., 2001; Sahinkaya et al., 2011) and ethanol (Zhang et al., 2015). An  
82 alternative is the use of a slow release solid organic carbon substrate, such as wood  
83 chips, which avoids the need for liquid chemical feed systems. Krayzelova et al.  
84 (2014) developed a tire-sulfur hybrid adsorption denitrification (T-SHAD) process  
85 where tire chips were used as a slow release source of organic substrate to enhance  
86  $\text{NO}_3^-$  removal in decentralized wastewater treatment applications. Li et al. (2016)  
87 investigated a wood-sulfur heterotrophic-autotrophic denitrification (WSHAD)  
88 process for water treatment, and observed decreased  $\text{SO}_4^{2-}$  accumulation compared  
89 with SAD. In a microcosm study carried out in our laboratory with saline wastewater,  
90 lower  $\text{SO}_4^{2-}$  production and higher  $\text{NO}_3^-$  removal rates were observed in a WSHAD  
91 system compared with SAD alone (He et al., 2018).

92 Wood is mainly composed of cellulose (45-50%), hemicellulose (25-35%), and  
93 lignin (25-35%) (Malherbe and Cloete, 2002). It has been used as a carbon source for  
94 denitrification of wastewater, stormwater and agricultural drainage (see Lopez-  
95 Ponnada et al., 2017 for review), aquaculture effluents (Von Ahnen et al., 2018) and  
96 RAS wastewater (Von Ahnen et al., 2019). Wood has been shown to deliver long term  
97 (5-15 years) denitrification rates ( $1-20 \text{ g N}/(\text{m}^3 \cdot \text{d})$ ) in onsite wastewater applications  
98 with minimum maintenance (Robertson, 2010). However, wood chips can cause an

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

107       The microbial communities of WSHAD bioreactors are still poorly understood.  
108 Li et al. (2016) performed WSHAD batch reactor experiments with  $NO_3^-$ -  
109 contaminated water. The authors identified the genera *Thiobacillus* and  
110 *Hydrogenophaga*, which include obligate and facultative autotrophs that can use  
111 reduced sulfur compounds or hydrogen as electron donors and  $NO_3^-$  as an electron  
112 acceptor (Bertrand et al., 2011; Lin et al. 2017). However, previous research suggests  
113 that salinity affects the microbial community composition of denitrification systems  
114 (Von Ahnen et al., 2019). Von Ahnen et al. (2019) investigated the effect of salinity on  
115 microbial community structure and denitrification in horizontal-flow woodchip  
116 bioreactors. The presence of heterotrophic denitrifying bacteria, such as those  
117 belonging to the orders *Betaproteobacteriales*, *Rhizobiales*, and *Xanthomonadales*,  
118 decreased with increasing salinity, suggesting that decreased  $NO_3^-$  removal rate was  
119 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<sup>3</sup> and an initial weight of  
140  $6.70 \pm 0.97$  g. Mollies were fed fish food pellets (0.6 mm Purina AquaMax Fingerling

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

#### 144 2.1.2. Media materials

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

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

### 160 2.1.3. Inoculum

161 Inoculum was obtained from a  $S^0$ -based CDF, which had been operated in a  
162 pilot-scale marine RAS for 250 days (He et al., 2020). This biomass was selected as  
163 the inoculum because the microbes were adapted to saline conditions and had  
164 received a mix of  $S^0$  and fish waste, which promoted both SAD and heterotrophic  
165 denitrification (He et al., 2020). Media from the  $S^0$ -based CDF were washed with  
166 saline water to obtain 2 L of saline sludge with a total suspended solids (TSS)  
167 concentration of 8,178 mg/L and a volatile suspended solids (VSS) concentration of  
168 2,714 mg/L. This sludge was inoculated into the CDF units in the two parallel pilot-  
169 RASs as described below.

### 170 2.2. Pilot-scale marine RAS

171 Two pilot-scale marine RAS (Fig. 1) were set up in parallel at the University of  
172 South Florida (USF), Tampa (WS-CDF A and WS-CDF B). The design of the pilot-  
173 scale marine RAS is fully described in He et al. (2020). Briefly, each RAS was  
174 comprised of a fish tank (62 cm H  $\times$  30 cm W  $\times$  76 cm L) with two water-treatment  
175 loops. The first treatment loop included an upflow solids filter (I) (5.2 cm D, 35 cm  
176 H), a downflow solids filter (II) (5.0 cm D, 20 cm H), and an aerated Moving Bed  
177 Biofilm Reactor (MBBR) (8.5 cm D, 30 cm H). The second treatment loop was a WS-  
178 CDF (12.7 cm D, 60 cm H), with a working volume of 2.5 L, which was maintained  
179 using a 20 cm height standpipe (Fig. 1). Each WS-CDF was packed with a mixture of  
180 312 g elemental sulfur pellets, 156 g crushed oyster shells, 312 g soaked wood cubes

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

186 A solenoid valve controlled by a timer was used to change the flow direction  
187 between the Loop 1 and Loop 2 (Fig. 1). Normally, water from the fish tank was  
188 circulated in Loop 1 at a rate of  $220 \pm 20$  mL/min. The flow direction was switched to  
189 Loop 2 every 12 hr, resulting in water being pumped from the outlet of Solids Filter I  
190 to the WS-CDF. After 10 min, the flow was switched back to Loop 1, and the WS-  
191 CDF was allowed to react undisturbed until the next cycle. Treated effluent from the  
192 WS-CDF overflowed to the MBBR to oxidize any residual  $\text{NO}_2^-$ , rbCOD, or reduced  
193 sulfur compounds before recirculating the flow back to the fish tank. The time interval  
194 between cycles was 12 hr, which was defined as the hydraulic retention time (HRT)  
195 of the WS-CDF.

196 Fresh tap water was added to the fish tanks to make up for the water losses due to  
197 solids wasting, sampling, and evaporation. Instant Ocean was added as needed to  
198 maintain a salinity of approximately 1.5%.  $\text{NaHCO}_3$  was periodically added into fish  
199 tanks to maintain the alkalinity  $> 100$  mg  $\text{CaCO}_3/\text{L}$ , which is considered in the healthy  
200 range for mollies. The MBBR, tubing, and solids filters I and II were cleaned  
201 periodically as described previously (He et al., 2020). Backwashing of the CDF was



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

### 204 2.3 Wood chip COD release

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

### 212 2.4 Analytical methods

213 Dissolved oxygen (DO), pH, and conductivity in the fish tanks were measured *in*  
214 *situ* daily using a portable DO (Mettler Toledo, USA), pH (Oakton™ Handheld Ion  
215 Meter), and conductivity (Oakton™ CON 6+ Portable Conductivity Meter) meters.  
216 Water samples were collected from the fish tanks and WS-CDF effluent at least 3  
217 times weekly for analysis of TAN, NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, SO<sub>4</sub><sup>2-</sup>, S<sup>2-</sup>, and COD, and weekly  
218 for alkalinity, total nitrogen (TN), total phosphorus (TP), TSS, and VSS **according to**  
219 ***Standard Methods* (APHA et al., 2017)**. Samples were filtered through 0.45 µm  
220 membrane filters for TAN, NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, TN, TP, sCOD, rbCOD and SO<sub>4</sub><sup>2-</sup>  
221 analysis, while samples were not filtered for S<sup>2-</sup> and COD measurements. **Detailed**  
222 **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](https://www.ncbi.nlm.nih.gov/sra/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 taxonomic 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

244 were combined by taxonomic rank to represent phylum\_unclassified,  
245 class\_unclassified, order\_unclassified, and family\_unclassified. Changes in microbial  
246 community structure between the inoculum and WS-CDFs were shown in bar charts  
247 expressing the fold changes of selected phyla and genera.

248

### 249 **3. Results and Discussion**

#### 250 3.1. Marine RAS performance

##### 251 3.1.1. Solids and color

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

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

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

### 267 3.1.2. Nitrogen removal

268 Nitrogen entered the system with the fish feed, which contained 50% protein.  
269 Dissolved nitrogen compounds, including TAN,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ , were generated in  
270 the RAS by fish excretion, decomposition of uneaten feed, and nitrification. Fig. 2a  
271 shows changes in TAN,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  concentrations in fish tanks during RAS  
272 operation. Average TAN,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  concentrations were maintained at  $0.17 \pm$   
273  $0.05$  mg N/L,  $0.41 \pm 0.17$  mg  $\text{NO}_2^-$ -N/L, and  $56.77 \pm 2.72$  mg  $\text{NO}_3^-$ -N/L, respectively.  
274 Average TN and total organic nitrogen (TON) concentrations in the fish tanks were  
275  $\sim 81$  mg N/L and  $\sim 25$  mg N/L, respectively. The steady performance over the entire  
276 operational period indicates that nitrogen added on a daily basis was removed by  
277 assimilation into fish and microbial biomass, solids wasting, nitrification, and  
278 denitrification. A nitrogen mass balance (Cheng, 2019) showed that passive  
279 denitrification in the first loop contributed to 5% of the nitrogen removal in the RAS.  
280 Passive denitrification occurs within anoxic environments in solids filters, as well as  
281 biofilms in MBBRs and fish tanks where water contains organic carbon and  $\text{NO}_3^-$   
282 (Van Rijn et al., 2006).

283  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and TAN concentrations in WS-CDF effluent over time are shown in  
284 Fig. 2b. The short acclimation period (1 day) shows that the inoculum from the  $\text{S}^0$ -  
285 based CDF quickly adapted to using a combination of wood and  $\text{S}^0$  as electron

286 donors. After acclimation, the average  $\text{NO}_3^-$  removal rate was  $102.6 \pm 8.5 \text{ g N}/(\text{m}^3 \cdot \text{d})$   
287 in the WS-CDF (removal efficiency of  $90.3 \pm 4.3\%$ ). The mass balance showed that  
288 nitrogen removal in the WS-CDF accounted for 57% of the total nitrogen removed.  
289 Our prior study with a  $\text{S}^0$ -based CDF incorporated into a similar RAS achieved an  
290 average denitrification rate of  $86.7 \pm 5.1 \text{ g N}/(\text{m}^3 \cdot \text{d})$  (He et al., 2020). The higher  
291 denitrification rate obtained in this study was likely due to higher influent  $\text{NO}_3^-$   
292 concentration and the combined use of  $\text{S}^0$  and wood chips.

293  $\text{NO}_2^-$  accumulation is an indicator of incomplete denitrification, which leads to  
294 production of NO and  $\text{N}_2\text{O}$ , which are potent greenhouse gases (Bertrand et al., 2011).  
295 Although NO and  $\text{N}_2\text{O}$  were not measured in this study,  $\text{NO}_2^-$  was detected in the  
296 CDF effluent (Fig. 2b), especially on the first day ( $16.48 \text{ mg NO}_2^- \text{-N/L}$ ). As  
297 mentioned previously, bacteria needed to adapt to the new environment at the  
298 beginning of RAS operation, resulting in incomplete denitrification. However, the  
299 effluent  $\text{NO}_2^-$  concentration quickly stabilized at  $0.47 \pm 0.22 \text{ mg NO}_2^- \text{-N/L}$ . Pine wood  
300 contains complex organic molecules, which can sometimes be utilized by  
301 fermentative bacteria capable of reducing  $\text{NO}_3^-$  to  $\text{NO}_2^-$  (Beauchamp et al., 1989). In a  
302 prior microcosm study with a variety of electron donors (He et al., 2018),  $\text{NO}_2^-$   
303 accumulation was higher in wood-containing bioreactors than in SAD bioreactors.

304 As shown in Fig. 2b, TN concentrations in the WS-CDF effluent were  $14.57 \pm$   
305  $7.24 \text{ mg N/L}$ ; effluent TAN concentrations ranged from 0.5 to 1.6 mg N/L. TAN can  
306 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  $24.59 \pm 4.70$  to  $7.48 \pm 3.83$  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 ( $116.7 \pm 1.7$  mg sCOD/(g wood) and  $94.7 \pm 13.5$  mg  
346 rbCOD/(g wood)), which decreased after being submerged in saline water for 8 days  
347 ( $19.2 \pm 0.5$  mg sCOD/(g wood) and  $15.7 \pm 0.7$  mg rbCOD/(g wood) when they were  
348 packed in the WS-CDF. **The decrease in rbCOD in the woodchips over time may lead**

349 to NO and N<sub>2</sub>O production due to incomplete denitrification (Bertrand et al., 2011).

350 sCOD and rbCOD release decreased exponentially according to the following:

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

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

353 In our previous study, significant SO<sub>4</sub><sup>2-</sup> accumulation was observed in the fish  
354 tanks when only S<sup>0</sup> was used as the electron donor for denitrification (He et al., 2020).  
355 In this study, fish tank SO<sub>4</sub><sup>2-</sup> concentrations stabilized at around 2,057 ± 122 mg SO<sub>4</sub><sup>2-</sup>  
356 /L (Fig. 3b). The use of WSHAD successfully avoided high SO<sub>4</sub><sup>2-</sup> accumulation in the  
357 marine RAS (*i.e.*, SO<sub>4</sub><sup>2-</sup> production due to SAD was balanced by SO<sub>4</sub><sup>2-</sup> removal due to  
358 water losses during sampling and solids wasting). To understand the dominant  
359 electron donor variations in the WS-CDFs over time, the data were divided into four  
360 stages: 1) days 0-30; 2) days 31-60; 3) days 61-90; and 4) days 91-125. SO<sub>4</sub><sup>2-</sup>  
361 generation during Stages 1 through 4 was 3.73 mg SO<sub>4</sub><sup>2-</sup>/mg NO<sub>3</sub><sup>-</sup>-N, 3.07 mg SO<sub>4</sub><sup>2-</sup>  
362 /mg NO<sub>3</sub><sup>-</sup>-N, 5.76 mg SO<sub>4</sub><sup>2-</sup>/mg NO<sub>3</sub><sup>-</sup>-N, and 6.65 mg SO<sub>4</sub><sup>2-</sup>/mg NO<sub>3</sub><sup>-</sup>-N, respectively.  
363 Based on the theoretical SO<sub>4</sub><sup>2-</sup> productivity of SAD of 7.54 mg SO<sub>4</sub><sup>2-</sup>/mg NO<sub>3</sub><sup>-</sup>-N,  
364 SAD accounted for 49.4% of NO<sub>3</sub><sup>-</sup> removal during Stage 1, decreased to 40.7% in  
365 Stage 2, and then increased to 76.4% in Stage 3 and 88.2% in Stage 4. As mentioned  
366 previously, the seed sludge was from a S<sup>0</sup>-based CDF, which has been operated for >  
367 6 months. This may have resulted in the high initial SO<sub>4</sub><sup>2-</sup> generation observed in  
368 Stage 1. As the bacteria adapted to the WSHAD environment, heterotrophic  
369 denitrification became more dominant during Stage 2. However, with the decrease in



370 rbCOD released from pine wood chips, the contributions of SAD to  $\text{NO}_3^-$  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  $\text{S}^0$ -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 *Bacteroidetes* 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 (Log<sub>2</sub> WS-  
392 CDF:Inoculum =  $-2.5 \pm 0.91$ ) and *Chlorobi* was undetected in WS-CDF A and  
393 decreased by more than four times in WS-CDF B (Log<sub>2</sub> 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<sup>0</sup>, 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<sub>4</sub><sup>2-</sup> produced during SAD may have migrated to lower redox  
408 potential zones in the CDF and been used to form sulfide by SO<sub>4</sub><sup>2-</sup>-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<sup>2-</sup> for the cultivation of *Prosthecochloris* and *Chlorobium*

412 (Finster et al., 1998). Microbial community analysis of the initial inoculum indicates  
413 that the S<sup>0</sup>-based CDF was enriched with a diverse consortium of salt-tolerant  
414 organisms including S<sup>0</sup>-oxidizing denitrifiers, SO<sub>4</sub><sup>2-</sup>-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<sup>0</sup>-based CDF and WS-CDF microbiome. Less S<sup>0</sup> was provided in  
419 the WS-CDFs compared to the S<sup>0</sup>-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<sup>0</sup>-based CDF. This was done to decrease the HRT and  
422 minimize spatial and/or temporal redox reactions that generate S<sup>2-</sup>. 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<sup>0</sup> 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 (Log<sub>2</sub> 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<sub>4</sub><sup>2-</sup>-reducing  
431 bacteria. As a result, SO<sub>4</sub><sup>2-</sup>-reducing bacteria belonging to *Parcubacteria* decreased by  
432 more than four times (Log<sub>2</sub> 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* (Log<sub>2</sub> WS-CDF:Inoculum =  $1.2 \pm 0.86$ ,  $2.2 \pm 0.52$ , and  
442  $2.9 \pm 0.79$ , 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<sub>3</sub><sup>-</sup> removal rate than that observed in our prior S<sup>0</sup>-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  $\text{NO}_3^-$   
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 than 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.

475 3.4. Fish growth and performance

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

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

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

496 identified during the microbial community analysis (Fig. 6), include suspected agents  
497 of epitheliocystis and mycobacteriosis - two infectious diseases common in freshwater  
498 and marine species, especially those raised in intensive aquaculture systems  
499 (Blandford et al, 2018). Higher abundances of these bacteria in the WS-CDFs than the  
500 S<sup>0</sup>-based CDFs may have contributed to the higher mortalities observed in this study  
501 (Pawlikowska-Warych and Deptula, 2016). Wood releases compounds, such as  
502 phenols, resin acids, tannins, lignin and volatile fatty acids, that can be toxic to  
503 animals (Hedmark and Scholz, 2008). It was reported that water leachate from  
504 woodchips was toxic to trout (Taylor et al., 1996). Additional research is needed to  
505 understand the effect of high SO<sub>4</sub><sup>2-</sup> and chemicals released from wood on marine RAS  
506 species and whether these conditions trigger stress that promotes a high susceptibility  
507 to epitheliocystis and mycobacteriosis.

#### 508 **4. Conclusions**

509 This study is the first to apply the WSHAD process for saline wastewater  
510 treatment and elucidate the microbiomes of these mixotrophic biofilters. WS-CDFs  
511 achieved high denitrification rates, with lower SO<sub>4</sub><sup>2-</sup> accumulation than SAD alone.  
512 Changes in rbCOD release from wood chips over time resulted a shift in the dominant  
513 denitrification mechanism from heterotrophic to SAD. The WS-CDF microbiome  
514 reflected a unique microbial consortium, including salt tolerant cellulose and sulfur  
515 utilizing denitrifiers, such as *Sulfurimonas*, *Thioalbus*, *Ornatilinea*, *Mycobacterium*

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

#### 518 **Appendix A: Supplementary data**

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

521

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532



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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)  $\text{SO}_4^{2-}$  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  $\geq 1.0\%$  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)  $\geq 0.1\%$  of the total reads in at least a single sample; and (2) with Log2 fold changes  $-1 \leq$  and  $\geq 1$  are shown.**

Fig.1

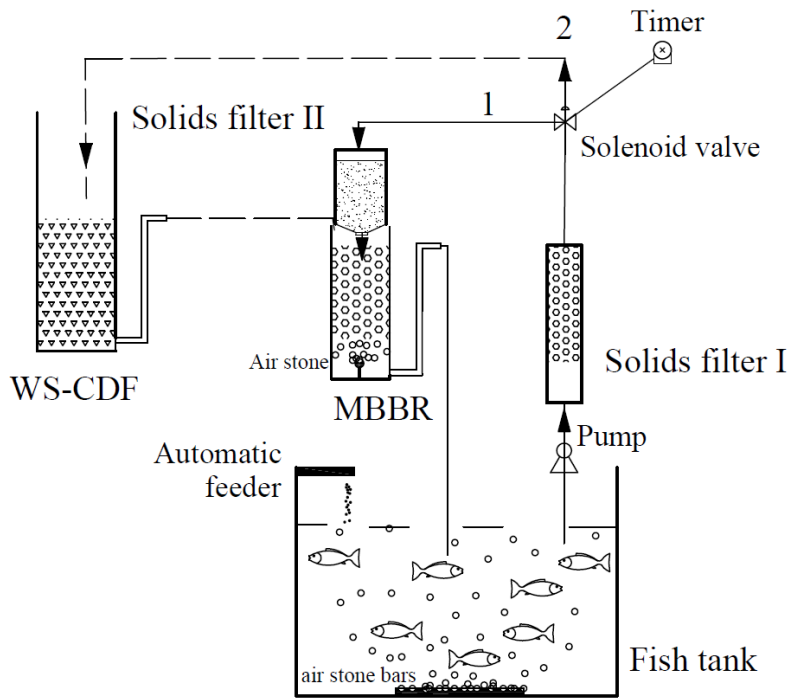


Fig.2

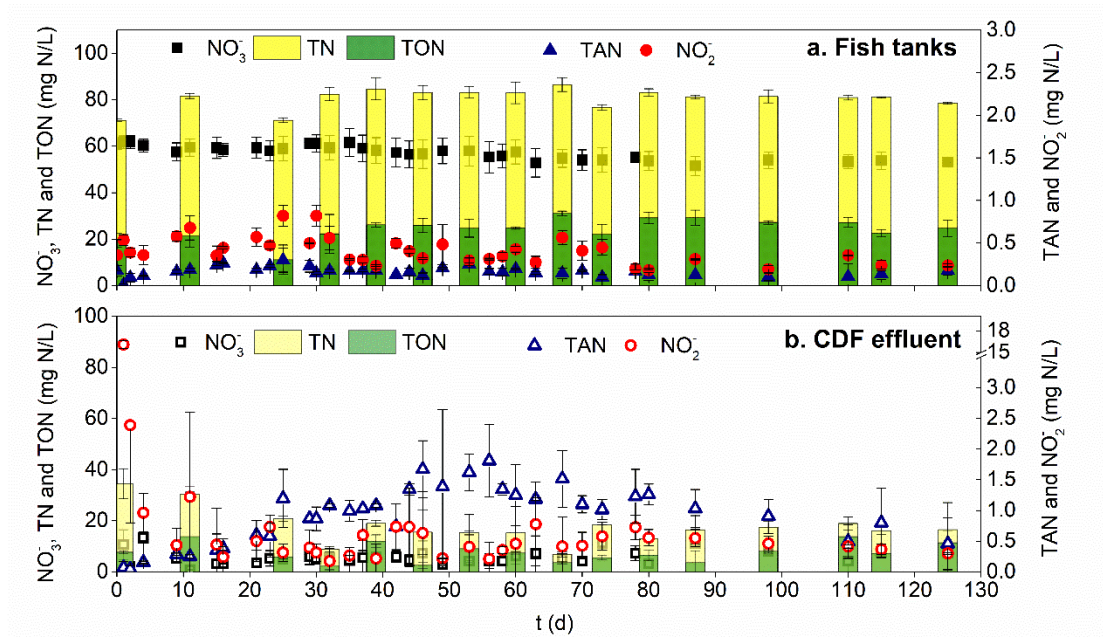


Fig.3

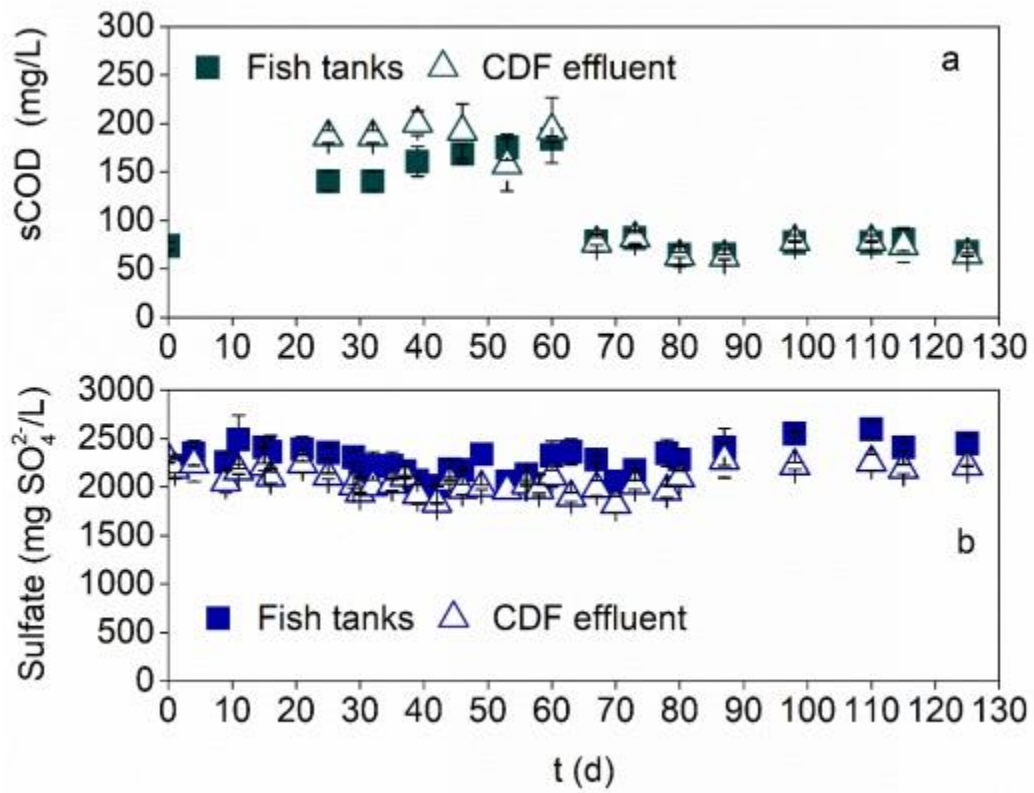


Fig.4

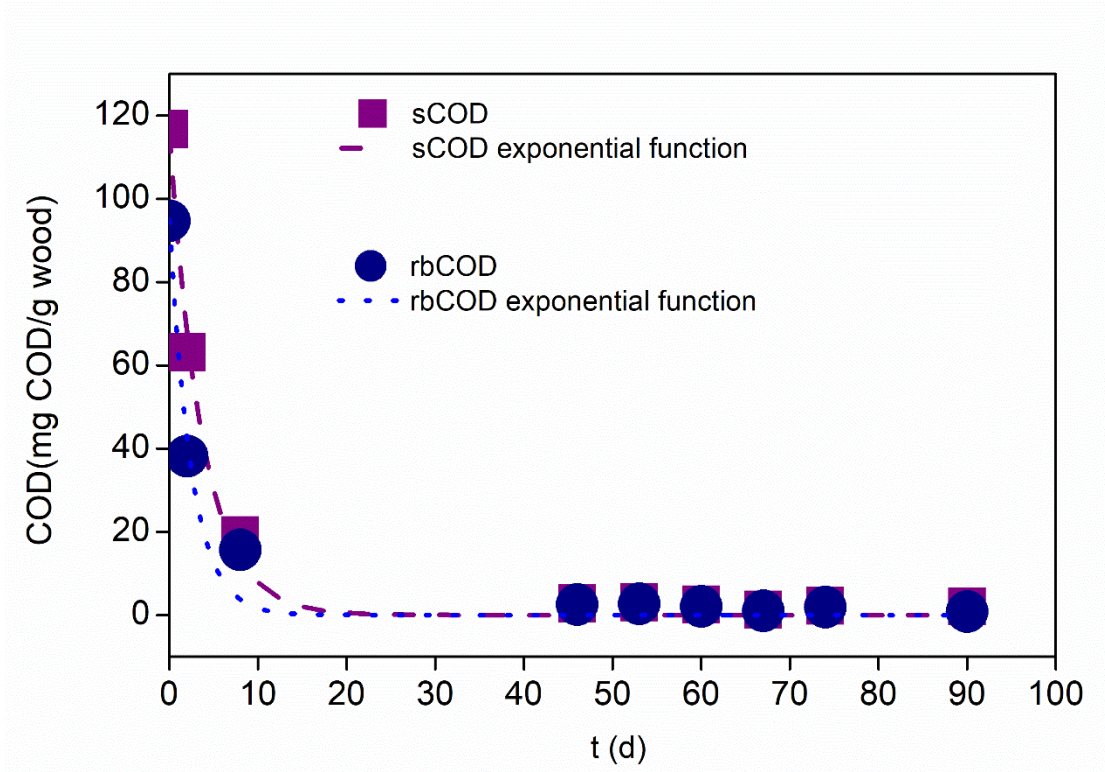




Fig.6

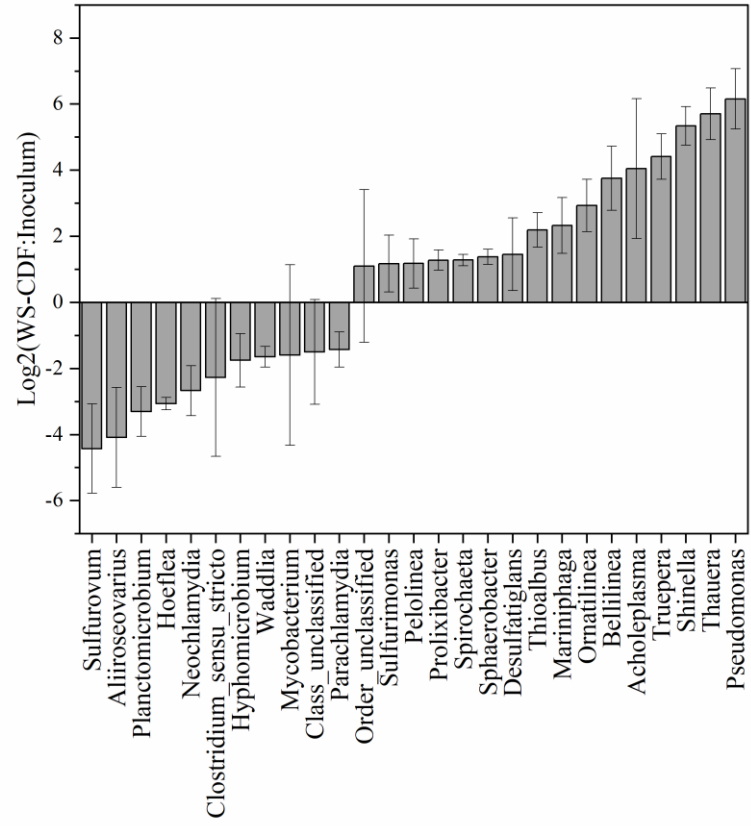


Table 1. Sulfur-based mixotrophic denitrification performance in other studies and this study.

<b>NO<sub>3</sub><sup>-</sup>-contaminated water</b>	<b>Carbon source</b>	<b>Alkalinity source</b>	<b>Influent NO<sub>3</sub><sup>-</sup> concentration (mg NO<sub>3</sub><sup>-</sup>-N/L)</b>	<b>HRT (h)</b>	<b>Removal efficiency (%)</b>	<b>Denitrification rate (g N/(m<sup>3</sup>·d))</b>	<b>Reference</b>
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 <sub>3</sub> <sup>-</sup> -contaminated water	Methanol/ landfill leachate	NaHCO <sub>3</sub>	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 <sub>3</sub>	75	4	100	450	Sahinkaya and Dursun (2012)



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

<b>Parameters</b>	<b>Value</b>	<b>Recommended limit</b>	<b>References</b>
NO <sub>2</sub> <sup>-</sup> (mg NO <sub>2</sub> <sup>-</sup> -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 <sub>3</sub> <sup>-</sup> (mg NO <sub>3</sub> <sup>-</sup> -N/L)	56.78 (2.72)	<75	Davidson et al. (2016)
TN	82.1 (4.6)	-	-
TON	24.6(4.7)	-	-
SO <sub>4</sub> <sup>2-</sup> (mg /L)	2,057 (122)	-	-
TP (mg /L)	0.58 (0.46)	-	-
S <sup>2-</sup> (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 <sub>3</sub> /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 (°C)	26.5-27.2	18-28	Ganguly et al. (2014)



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**Electronic Annex**

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**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: