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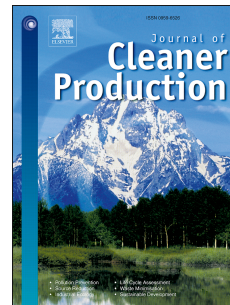
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# **Enhanced nitrogen removal of low carbon wastewater in denitrification bioreactors by utilizing industrial waste toward circular economy**

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**1 Abstract**

2 Aquaculture needs practical solutions for nutrient removal to achieve sustainable fish production. Passive  
3 denitrifying bioreactors may provide an ecological, low-cost and low-maintenance approach for wastewater  
4 nitrogen removal. However, innovative organic materials are needed to enhance nitrate removal from the low  
5 carbon effluents in intensive recirculating aquaculture systems (RAS). In this study, we tested three  
6 additional carbon sources, including biochar, dried *Sphagnum* sp. moss and industrial potato residues, to  
7 enhance the performance of woodchip bioreactors treating the low carbon RAS wastewater. We assessed  
8 nitrate ( $\text{NO}_3^-$ ) removal and microbial community composition during a one-year *in situ* column test with real  
9 aquaculture wastewater. We found no significant differences in the  $\text{NO}_3^-$  removal rates between the  
10 woodchip-only bioreactor and bioreactors with a zone of biochar or *Sphagnum* sp. moss (maximum removal  
11 rate 31-33 g  $\text{NO}_3^-$ -N  $\text{m}^{-3} \text{d}^{-1}$ ), but potato residues increased  $\text{NO}_3^-$  removal rate to 38 g  $\text{NO}_3^-$ -N  $\text{m}^{-3} \text{d}^{-1}$ , with  
12 stable annual reduction efficiency of 93%. The readily available carbon released from potato residues  
13 increased  $\text{NO}_3^-$ -N removal capacity of the bioreactor even at higher inflow concentrations ( $>52 \text{ mg L}^{-1}$ ). The  
14 microbial community and its predicted functional potential in the potato residue bioreactor differed markedly  
15 from those of the other bioreactors. Adding potato residues to woodchip material enabled smaller bioreactor  
16 size to be used for  $\text{NO}_3^-$  removal. This study introduced industrial potato by-product as an alternative carbon  
17 source for the woodchip denitrification process, and the encouraging results may pave the way toward  
18 growth of blue bioeconomy using the RAS.

19  
20 **Keywords:** Recirculating aquaculture system, woodchip bioreactor, carbon source, potato residues, nitrate,  
21 microbial community

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## 28 1 Introduction

29 Recirculating aquaculture systems (RAS) are environmentally friendly solutions that aim to achieve zero  
30 waste from fish production. Although RAS have been used for more than 10 years in different countries,  
31 including two largest RAS in Finland with a production capacity of over 4000 tons, nitrate ( $\text{NO}_3^-$ ) removal is  
32 still a critical challenge (Pulkkinen et al., 2018). Removal of  $\text{NO}_3^-$  is a challenge as aquaculture wastewater  
33 has low carbon (C) but high nitrogen (N) concentrations. A few previous studies have examined the use of  
34 denitrifying bioreactors for treating aquaculture effluent. So far, such studies have focused on RAS effluents  
35 with high chemical oxygen demand (COD) (Lepine et al., 2016), added bicarbonate ( $\text{HCO}_3^-$ ) to inlet water  
36 (von Ahnen et al., 2016b) and diluted effluent from an outdoor fish farm with low recirculation intensity and  
37 low  $\text{NO}_3^-$ -N concentration ( $\sim 6 \text{ mg L}^{-1}$ ) (von Ahnen et al., 2018, 2016a). In contrast, treatment of highly  
38 intensive indoor RAS effluents with low COD ( $12.9 \pm 1.8 \text{ mg L}^{-1}$ ) and high  $\text{NO}_3^-$ -N concentration ( $>50 \text{ mg}$   
39  $\text{L}^{-1}$ ) has received little attention.

40 In denitrifying bioreactors, nitrogen (N) is removed by heterotrophic denitrifiers converting  $\text{NO}_3^-$  to nitrogen  
41 gas under anoxic conditions. Under nitrate-rich conditions, this process depends on the availability of the  
42 carbon source as the organic electron donor (Wang and Chu, 2016). External carbon sources, such as acetate  
43 or methanol, are often supplied to the system to achieve efficient denitrification (Cherchi et al., 2009).  
44 However, the cost of carbon addition is typically high (Zhang et al., 2016) and the process needs regulation  
45 to prevent over- or under-dosing of the liquid carbon sources (Rocher et al., 2015). Solid carbon sources can  
46 provide a cost-effective alternative to the classical carbon sources mentioned above. In recent years, research  
47 has focused on solid carbon sources with high quality, optimal efficiency and slow-release ability in the  
48 treatment of excessively nitrate-contaminated water, particularly surface water (Beutel et al., 2016) and  
49 groundwater (Zhang et al., 2012). Wood-particle products (e.g. woodchip and sawdust) have been widely  
50 used, due to their ability to supply carbon to the denitrification process for 5-15 years and thus allow good  
51  $\text{NO}_3^-$  removal with minimum bioreactor maintenance (Schipper et al., 2010). However, the large space  
52 requirement for full-scale woodchip bioreactors has prompted efforts to enhance the denitrification rate by  
53 using innovative natural carbon sources (Tangsir et al., 2017). Inexpensive industrial food by-products, such

54 as industrial potato residue, could have high potential to be utilized in identifying bioreactor to enhance  
55 nitrate removal. Potato industries can generate 20-25 % waste from peeling, trimming and cutting processes  
56 (Liang and McDonald, 2014).

57 This study examined the use of a denitrifying bioreactor to treat indoor intensive RAS effluent with low  
58 COD and high  $\text{NO}_3^-$  concentration, as part of the unique RAS research platform (see Pulkkinen et al., 2018),  
59 and compared different carbon sources, including potato residue, for improving the nitrogen removal  
60 performance of woodchip bioreactors. The overall aim was to evaluate the performance of denitrifying  
61 bioreactors in removing  $\text{NO}_3^-$  from aquaculture wastewater with low COD for a period of over one year.  
62 Specific objectives were to (1) study the suitability of wood-based bioreactors for treating RAS effluent, (2)  
63 assess whether the  $\text{NO}_3^-$  removal performance of woodchip process can be enhanced by additional carbon  
64 sources, (3) to assess the effect of different carbon sources on the microbial community composition in  
65 different compartments of the bioreactors, and (4) to identify dominant bacteria and their functional potential  
66 in the bioreactors studied. The intention was to find solutions for improving water treatment and for  
67 enhancing  $\text{NO}_3^-$  removal in the recirculating aquaculture systems.

## 68 **2 Material and methods**

### 69 **2.1 RAS effluent water quality**

70 The study was conducted at the Laukaa fish farm of the Natural Resources Institute Finland (LUKE) in  
71 central Finland, in the research platform examining RAS. The RAS design is described in detail in Pulkkinen  
72 et al. (2018). In brief, effluent was obtained from a RAS consisting of a feed collector unit, swirl separator,  
73 drum filter (60  $\mu\text{m}$  mesh) and fixed bed bioreactor, followed by a moving bed bioreactor and a trickling  
74 filter. In order to prevent any changes in water chemistry, microbiology or water temperature, all tests were  
75 performed using the natural RAS effluent. The effluent is characterised by low carbon (15.3  $\text{mg L}^{-1}$  on  
76 average), but high N content (mean  $\text{NO}_3^-$ -N content 34.7  $\text{mg L}^{-1}$ ) (Table 1). Due to the efficient nitrification  
77 unit before the bioreactors,  $\text{NO}_3^-$  is dominating N fraction.

78 **Table 1.** Mean inflow water quality parameters (SD = standard deviation, n = number of sample)

Water quality parameters	Inflow (mean $\pm$ SD)	n
Total organic carbon (mg L <sup>-1</sup> )	15.3 $\pm$ 2.1	5
Dissolved organic carbon (mg L <sup>-1</sup> )	14 $\pm$ 1.3	5
Chemical oxygen demand (mg L <sup>-1</sup> )	12.9 $\pm$ 1.8	5
Biological oxygen demand (mg L <sup>-1</sup> )	3.8 $\pm$ 2.2	13
Nitrate-nitrogen (mg L <sup>-1</sup> )	34.7 $\pm$ 15.6	27
Nitrite-nitrogen (mg L <sup>-1</sup> )	0.1 $\pm$ 0.06	30
Ammonium-nitrogen (mg L <sup>-1</sup> )	0.5 $\pm$ 0.2	30
Dissolved oxygen (mg L <sup>-1</sup> )	8.1 $\pm$ 1.7	29
pH	6.9 $\pm$ 0.2	28
Oxidation-reduction potential (Eh, mV)	178.6 $\pm$ 60.4	35
Alkalinity (mg CaCO <sub>3</sub> L <sup>-1</sup> )	54.2 $\pm$ 18	25
Sulphate (mg L <sup>-1</sup> )	10.5 $\pm$ 3.2	24

## 79 2.2 Bioreactor design

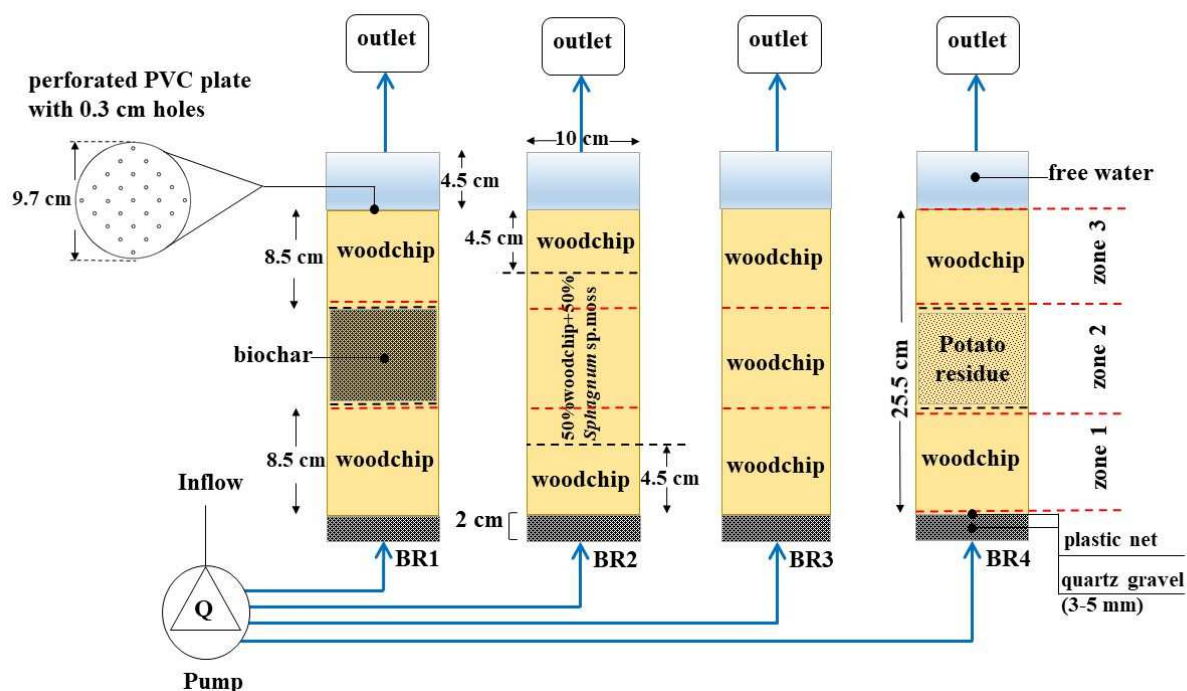
80 The performance of denitrifying bioreactors was studied in four transparent acrylic columns (0.1 m diameter  
81  $\times$  0.32 m high) with upward flow direction applying a theoretical retention time (HRT) of 48 h at controlled  
82 temperature (15.5 $\pm$ 0.8°C) (Fig. 1). In each column, the reactive media were placed on top of an inert quartz  
83 gravel bed, from which they were separated by plastic netting with 2 mm pore size, to prevent clogging with  
84 materials containing organic matter. A constant inflow rate of 0.6 mL min<sup>-1</sup> was applied to each bioreactor  
85 for 346 days, using a peristaltic pump. The upward flow direction and the quartz gravel layer at the base of  
86 the columns prevented the development of preferential flow pathways and ensured uniform distribution of  
87 flow into the columns. The columns consisted of packed-media zones (zone 1, zone 2, zone 3) containing  
88 woodchips, industrial potato waste, biochar or dried *Sphagnum* sp. moss in the ratios shown in Fig. 1. The  
89 packed-media has not been replaced during the study period. All bioreactors with additional layer contain  
90 same total volume of woodchips. However, *Sphagnum* sp. moss was mixed with woodchips in the zone 2,  
91 due to its different characteristic and small particle size distribution. It is well known that natural peat has



92 typically low hydraulic conductivity (e.g. Ronkanen and Kløve 2005), which could cause risks in longer  
93 HRT or even clogging of the bioreactor. In order to avoid this, moss was mixed with woodchips. The  
94 packed-media zones were separated from the outlet free water zone by a fixed perforated PVC plate  
95 (thickness 5 mm) at a height of 4.5 cm from the top of the column. The columns were sealed at both ends to  
96 provide controlled conditions.

97 The selected carbon sources had different C/N ratios, ranging from 28 to 249 (Table 2). Woodchips had the  
98 highest C/N ratio, but biochar contained the highest amount of carbon. The used woodchips were obtained  
99 locally from fresh birch trees (provided by the energy company Vapo Group). The average woodchip size  
100 was around 3 cm × 1.5 cm × 0.4 cm and mean porosity 63%. The *Sphagnum* sp. moss used was common  
101 mire flora provided by Vapo Group. The biochar (porosity 46%) was obtained from RPK Hiili Oy. The  
102 potato material tested comprised industrial residues from POHJOLAN PERUNA Oy with a dry matter  
103 content of 12% (determined after drying the material at 105°C for 24 h).

104 Prior to the experiments, solid materials (woodchips and biochar) were washed with distilled water and  
105 saturated for 48 h. In order to prevent fermentation, the potato residues were kept in the freezer prior to use.  
106 The frozen potato residues were defrosted at room temperature for 8 h before the test.



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**Fig. 1.** Schematic diagram of the bioreactor set-up (bioreactors BR1-BR4). Red and black dashed lines represent microbiological sampling zones and packed media zones in the bioreactors, respectively. Zones 1 and 3 were packed with woodchips, while zone 2 was packed with biochar in BR1, *Sphagnum* sp. moss in BR2, woodchips in BR3 and potato residues in BR4.

**Table 2.** Elemental composition of organic materials (per dry mass) used as an added carbon source

Content (%)	Woodchip	Biochar	<i>Sphagnum</i> sp. moss	Potato residues
Carbon (C)	49.8	82	49.1	44.6
Nitrogen (N)	0.2	0.6	0.9	1.6
Hydrogen (H)	6.1	3.2	5.4	6
C/N ratio	249	137	55	28

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### 2.3 Sampling and analysis

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Water samples were collected at the inflow tank and at the outlet of the four bioreactors. Sampling was started after removing the existing distilled water from all bioreactors (~48 h). Water samples from the

117 outlets were collected individually in sealed 1-L containers. Over the first 10 days, samples were collected  
118 daily at the same time for all outlets and the inlet. The sampling interval was then increased to once per 1-2  
119 weeks for three months and finally to once per month. Woodchip type bioreactor was selected to study  
120 repeatability of the performed test. For this, three woodchip bioreactors were established and run in parallel  
121 to other bioreactors for nearly 6 months. As the inflow water was the same to all bioreactors, standard  
122 deviation for outflow nitrate-nitrogen concentrations were calculated using data of these three woodchip  
123 bioreactors.

124 All samples were analysed on-site for nitrate-nitrogen ( $\text{NO}_3^-$ -N), nitrite-nitrogen ( $\text{NO}_2^-$ -N), ammonium-  
125 nitrogen ( $\text{NH}_4^+$ -N), sulphate ( $\text{SO}_4^{2-}$ ) and biological oxygen demand ( $\text{BOD}_5$ ), using LCK cuvette tests (Hach  
126 Lange DS 3900). Alkalinity was analysed by titration with the standard method (ISO 9963-1:1994) (Hach  
127 Lange TitraLab AT1000). The concentration of COD, dissolved organic carbon (DOC) and total organic  
128 carbon (TOC) in the first 70 days were determined by an accredited laboratory. Dissolved oxygen (DO) was  
129 recorded manually with a YSI ProODO meter and redox potential (Eh), pH and temperature with a Horiba  
130 Laqua act D-74 meter.

131 Flow rate (Q) was calculated by dividing the selected HRT (48 h) by the pore volume of the column (1650  
132 mL). Pore volume of each column was determined by measuring added water until saturation conditions  
133 were achieved. Volumetric  $\text{NO}_3^-$ -N removal rate ( $\text{g NO}_3^-$ -N  $\text{m}^{-3}$  d) was calculated based on differences  
134 between bioreactor inlet and outlet  $\text{NO}_3^-$ -N concentration, the flow rate and the pore volume of the packed-  
135 media zone. Removal efficiency was calculated by dividing the difference between inlet and outlet  
136 concentration by the inlet concentration. The calculated mass was based on sampling interval, flow rate and  
137 concentration.

## 138 **2.4 Molecular analyses**

139 Sampling for molecular analyses was performed 69 days after the start of the tests. Samples were taken from  
140 water and from solid material in zone 2 and zone 3 of the columns (see Fig. 1). Water samples were collected  
141 using syringe filters (0.22  $\mu\text{m}$  Millipore Express® PLUS PES membrane) and stored at  $-20$  °C prior to DNA  
142 extraction. Solid samples were collected in 50 mL tubes and treated as in von Ahnen et al. (2019). DNA was

143 extracted using the DNeasy PowerLyzer PowerSoil Isolation kit (Qiagen) and DNA concentrations were  
144 quantified with the Qubit® dsDNA HS Assay Kit and a Qubit 2.0. fluorometer (Thermo Fischer Scientific).  
145 In studying microbial community composition, prokaryotic primers 515F-Y  
146 (GTGYCAGCMGCCGCGGTAA; Parada et al., 2016) and 806R (GGACTACHVGGGTWTCTAAT;  
147 Caporaso et al., 2011) were used to amplify the V4 region 16S rRNA gene. The first PCR reaction was  
148 carried out following von Ahnen et al. (2019), with the exception that a DNA template amount of 6 ng was  
149 used. The amplicon libraries were built as in Ahnen et al. (2019) and sequenced on Ion Torrent PGM using  
150 Ion PGM Hi-Q View OT2 Kit for emulsion PCR, PGM Hi-Q View Sequencing Kit for the sequencing  
151 reaction and Ion 314 Chip v2 (all Life Sciences, Thermo Fisher Scientific).

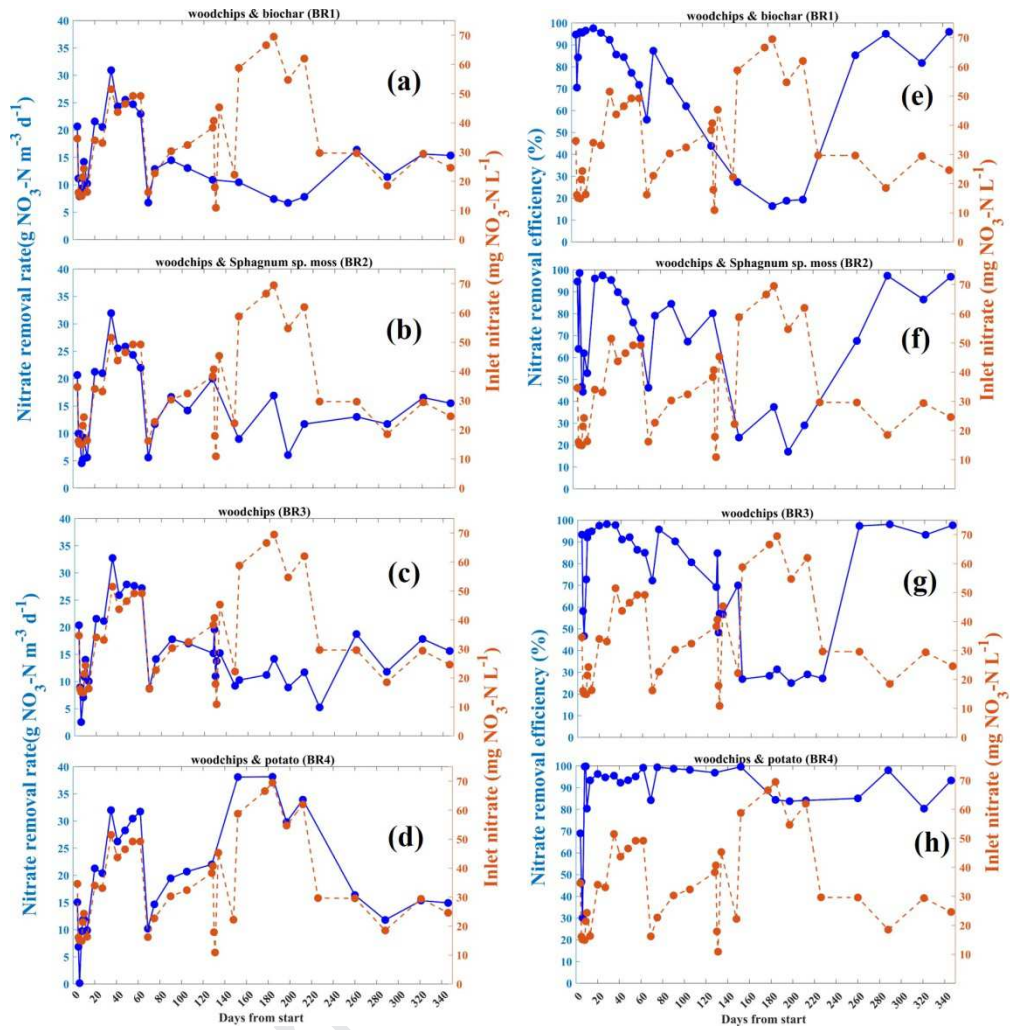
152 Sequence analysis was performed using the analysis pipelines mothur v.1.39.5 (Schloss et al., 2009) and  
153 qiime 1.9 (Caporaso et al., 2011). Sequences with incorrect primer (>1 bp) or barcode (>1 bp) sequences  
154 were removed, as were sequences <150 bp and chimeric sequencing. After quality filtering, sequences were  
155 clustered into operational taxonomic units (OTUs) at 97% similarity using OptiClust (Westcott and Schloss,  
156 2017). Samples were rarefied at a sequence depth of 4096 to allow comparison of alpha diversity indices  
157 (number of observed and Chao1-estimated OTUs, Shannon Diversity index  $H'$ , Pielou's Evenness) and beta  
158 diversity. Beta diversity was visualised using non-metric multidimensional scaling (NMDS) based on Bray-  
159 Curtis distance matrices. NMDS plots were constructed in R (vegan package, metaMDS; Oksanen et al.,  
160 2017). Relative abundances of OTUs on phylum/class level were visualised in SigmaPlot 13. The PICRUSt  
161 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) algorithm (Langille et  
162 al., 2013) was used to predict functional profiles of BR microbial communities. The average Nearest  
163 Sequenced Taxon Index (NSTI, a measure of the phylogenetic distance of the microbial communities  
164 analysed to the reference sequences) for the microbial communities was 0.11 (range 0.05-0.16). Smaller  
165 NSTI values are an indication of higher relatedness to reference sequences with known functional potential,  
166 and thus will likely give more accurate predictions (Langille et al., 2013). The NSTI values obtained for the  
167 bioreactors were within the range reported for other ecosystems, for which PICRUSt has yielded quite  
168 accurate predictions (Langille et al., 2013). Nonetheless, the results presented here should be treated with  
169 caution. Predicted functions were classified as KEGG (Kyoto Encyclopedia of Genes and Genomes)

170 orthologues (KOs). Functions potentially involved in nitrogen turnover in BRs (i.e. functions associated with  
171 nitrification, denitrification and DNRA) were assessed in more detail.

## 172 **3 Results and discussion**

### 173 **3.1 Performance of bioreactors**

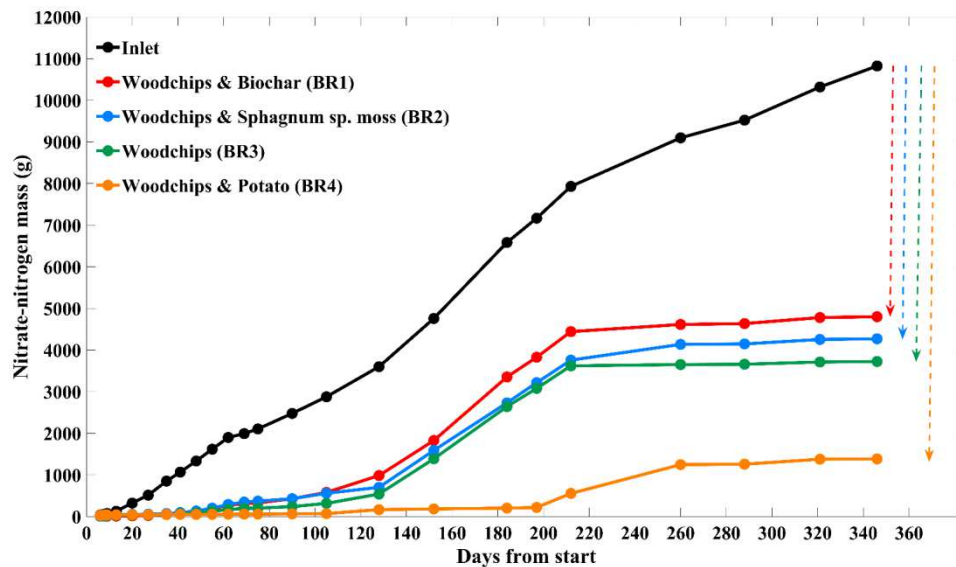
174 The initial inflow  $\text{NO}_3^-$ -N concentration of the bioreactors ranged from 15 to 70  $\text{mg L}^{-1}$ , while the outflow  
175 concentrations were clearly lower (ranging from the detection limit of 0.03 to 58.1  $\text{mg L}^{-1}$ ) (Fig. 2). All  
176 bioreactors showed effective  $\text{NO}_3^-$  removal ability immediately upon start-up and over the whole study  
177 period (Fig. 2, Fig. 3). Instant  $\text{NO}_3^-$  removal by wood-based bioreactors in aquaculture effluent has also been  
178 observed in previous studies (e.g. Lepine et al., 2016; von Ahnen et al., 2016a). Over the one-year bioreactor  
179 operating period (number of samplings  $n = 26$ ),  $\text{NO}_3^-$ -N comprised  $98 \pm 0.1$  % (mean  $\pm$  SD) of total dissolved  
180 inorganic nitrogen in inflow water, while only minor amounts of  $\text{NH}_4^+$ -N ( $1.6 \pm 0.8\%$ ) and  $\text{NO}_2^-$ -N  
181 ( $0.27 \pm 0.22\%$ ) were present. For the entire study period, total inflow  $\text{NO}_3^-$ -N mass to the bioreactors was 10.8  
182 kg, of which 6.0, 6.6, 7.1 and 9.4 kg were removed in BR1, BR2, BR3 and BR4, respectively (Fig. 3).  
183 During the first 197 days, BR4 (industrial potato residues in zone 2) showed stable removal of 96% for total  
184  $\text{NO}_3^-$ -N (amounting to a removed nitrogen mass of 7.1 kg). After 107 days the removal efficiency decreased  
185 and was around 87% from day 260 onwards (Fig. 2, Fig. 3). The other bioreactors also showed decreased  
186  $\text{NO}_3^-$  removal efficiencies from day 130-160 to day 260 (30%). From day 260 onwards, the removal  
187 efficiency in BR3 then increased to the original level (Fig. 2). However, the total accumulated outflow  $\text{NO}_3^-$ -  
188 N mass for BR3 was higher than in BR4 when considering the whole study period (Fig. 3).



189 **Fig. 2.** Nitrate-nitrogen (NO<sub>3</sub><sup>-</sup>-N) removal rate (a-d) and removal efficiency (e-h) in bioreactors during the  
 190 346-day study period.

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193 **Fig. 3.** Accumulated nitrate-nitrogen mass in inflow and outflow of bioreactors BR1-BR4 during the 346-day  
 194 study period. Dashed lines indicate total nitrate-nitrogen mass removed from bioreactors during the period.

195 Temporary increases in nitrite production (von Ahnen et al., 2018; Zhao et al., 2018) can limit the use of  
 196 woodchip bioreactors for RAS effluents, due to the toxicity of nitrite at high concentrations (Kroupova et al.,  
 197 2005). In this study, the  $\text{NO}_2^-$ -N concentration in inflow water remained stable, at a level of  $0.1 \pm 0.06 \text{ mg L}^{-1}$   
 198 (Table 1; Fig. S1a in Supplementary Material). In the first 10 days of the experiment, outflow  $\text{NO}_2^-$ -N was  
 199 12, 6, 15 and  $0 \text{ mg L}^{-1}$  in bioreactors BR1, BR2, BR3 and BR4, respectively (Fig. S1). From day 20 onwards,  
 200 the  $\text{NO}_2^-$ -N outflow concentration reached the background level throughout the experiment in all bioreactors.  
 201 Based on previous studies, the 50% lethal nitrite dose ( $\text{LD}_{50}$ ) varies between fish species but is typically  
 202 around  $2 \text{ mg L}^{-1}$  (Kroupova et al., 2005). Moreover, nitrite in sublethal concentrations is a stress factor for  
 203 fish and can lead to increased susceptibility to diseases (Kroupova et al., 2005). Nitrite production in  
 204 bioreactors is associated with incomplete nitrate removal by denitrification (Lepine et al., 2016; Zhao et al.,  
 205 2018), which can be limited by high DO. High DO may have limited denitrification in the start-up phase of  
 206 bioreactors BR1-BR3 in the present study, as the DO concentration in the outflow was rather high ( $11.5 \text{ mg}$   
 207  $\text{L}^{-1}$ ) (Fig. S1c). The type and availability of carbon compounds (Gibert et al., 2008; van Rijn et al., 2006) and  
 208 specific microbial community composition (Zhao et al., 2018) are reported to be the main reasons for  
 209 incomplete  $\text{NO}_3^-$  reduction leading to intermediate nitrogen products. As the outflow concentrations of nitrite  
 210 in the start-up phase exceeded the  $\text{LD}_{50}$  for many fish, water should not be re-fed to aquaculture from the



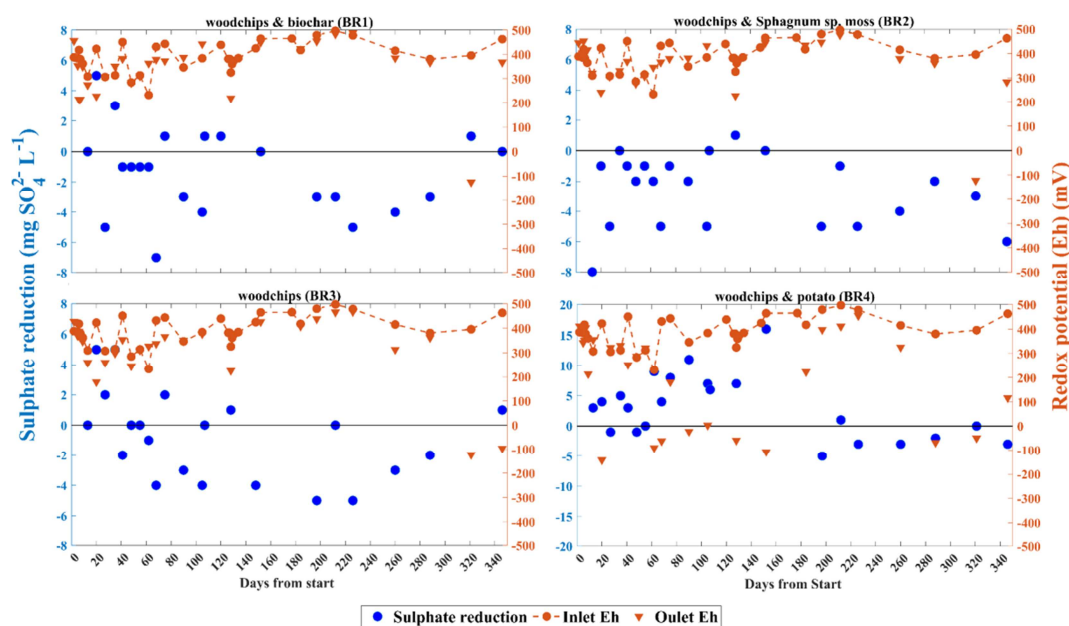
211 start, but only after stable denitrification rates are established and low nitrite concentrations are detected in  
212 the outflow.

213 The inflow  $\text{NH}_4^+\text{-N}$  concentration ranged between 0.17-1.0  $\text{mg L}^{-1}$  (Table 1; Fig. S1b). Low  $\text{NH}_4^+\text{-N}$   
214 production was detected in all bioreactors, with outflow concentrations of  $0.8\pm 0.5 \text{ mg L}^{-1}$ ,  $0.9\pm 0.5 \text{ mg L}^{-1}$ ,  
215  $0.9\pm 0.6 \text{ mg L}^{-1}$  and  $3.8\pm 3.4 \text{ mg L}^{-1}$  in BR1, BR2, BR3 and BR4, respectively. Less than  $2 \text{ mg L}^{-1}$  of  $\text{NH}_4^+\text{-N}$   
216 was recorded in the first three weeks in BR1-BR3 (Fig. S1b). However, the bioreactor with potato residues  
217 (BR4) showed relatively high  $\text{NH}_4^+\text{-N}$ , with a mean concentration of  $10 \text{ mg L}^{-1}$ , in the first 10 days of the  
218 experiment, but it then declined to lower than  $4 \text{ mg L}^{-1}$  to reach the background level. The continuous  
219 production of ammonium in BR4 indicates the occurrence of dissimilarity nitrate reduction to ammonium  
220 (DNRA). In general, a reducing environment and high  $\text{TOC}/\text{NO}_3^-$  ratio (1400/15-110/16 in BR4; days 1-70)  
221 can indicate the occurrence of DNRA (Kraft et al., 2014; van Rijn et al., 2006). DNRA has also been  
222 observed in previous woodchip bioreactor studies (Lu et al., 2013; Zhao et al., 2018). Reducing conditions,  
223 indicated by Eh values, were also seen in this study, which led the system to  $\text{SO}_4^{2-}$  reduction (Fig. 4).

224 In the start-up phase, all bioreactors released DOC. The rate of release was highest in BR4, with outflow  
225 concentrations of  $1380 \text{ mg L}^{-1}$  measured on day 6 after start-up (Table. S1). The DOC release from the other  
226 bioreactors was much lower ( $<100 \text{ mg L}^{-1}$ ; Table S1). Within 70 days after start-up, outflow DOC  
227 concentration decreased to  $81 \text{ mg L}^{-1}$  in BR4 and to the background level ( $14 \pm 1.3 \text{ mg DOC L}^{-1}$ ) in BR1-  
228 BR3 (Table S1). Initial carbon content flush-out is common in bioreactors. The start-up COD concentration  
229 in the outflow ranged 59-940  $\text{mg L}^{-1}$  in BR1-BR4 (Table. S1) exceeding temporarily the maximum  
230 concentration of  $42 \text{ mg L}^{-1}$  observed in Finnish rivers (Niemi and Raateland, 2007). However, start-up phase  
231 of the woodchip bioreactor is short compared to estimated lifetime (5-15 years), so the potential pollution for  
232 carbon is minor compared to the amount of nitrogen removed. Lepine et al. (2016) reported an  
233 approximately 50-day flush-out period for a plywood bioreactor treating aquaculture effluent at HRT of 42 h.  
234 Somewhat higher carbon leaching ( $200 \text{ mgL}^{-1}$ ) has been reported for bioreactors packed with fresh  
235 woodchips and a mixture of woodchips and biochar (Hassanpour et al., 2017; Hoover et al., 2016). Release  
236 of high DOC concentrations to recipient water bodies from use of bioreactors as an end-of-pipe treatment  
237 can adversely affect aquatic ecosystems, e.g. by causing a DO concentration reduction, light and temperature



238 changes (Prairie, 2008; Solomon et al., 2015), resulting in lower fish production (Stasko et al., 2012). Hence,  
239 at sites governed by strict regulations or when recycling outflow to fish farms, high DOC might need to be  
240 controlled. Schipper et al. (2010) identified HRT as a factor controlling the initial magnitude of DOC  
241 depletion and its duration in wood-based bioreactors. However, the fact that carbon was more readily  
242 released from potato residues than from the other carbon sources used in this study proves that HRT is not  
243 the only controlling factor and that carbon quality also plays a key role. In the present study, there was  
244 significantly lower outflow DOC concentration of 53, 68 and 81 mg L<sup>-1</sup> in bioreactors BR1, BR2 and BR3,  
245 which can be partly explained by higher nitrate loading (Hassanpour et al., 2017) and partly by the type of  
246 carbon source used. Dependence of TOC leaching and variations in NO<sub>3</sub><sup>-</sup>-N concentration have also been  
247 reported by Zhao et al. (2018). In order to control the carbon content due to leaching, it is recommended to  
248 consider post-bioreactors treatment units (e.g. constructed wetland, sand filter) or recirculating the start-up  
249 effluent back to the bioreactor (Schipper et al., 2010).  
250 The SO<sub>4</sub><sup>2-</sup> concentrations were on average higher in the outflow than in the inflow waters of BR1 and BR2,  
251 indicating leaching or production of SO<sub>4</sub><sup>2-</sup> (Fig. 4). This resulted in cumulative leaching/production of 165 g  
252 and 474 g SO<sub>4</sub><sup>2-</sup> in BR1 and BR2, respectively, for the whole study period. In contrast, SO<sub>4</sub><sup>2-</sup> were on average  
253 lower in outflow than in inflow waters of BR3 and BR4 (Fig. 4), indicating SO<sub>4</sub><sup>2-</sup> reduction/removal.  
254 Cumulative SO<sub>4</sub><sup>2-</sup> removal of 350 g and 546 g was observed in BR3 and BR4, respectively, for the whole  
255 study period. SO<sub>4</sub><sup>2-</sup> leaching/removal increased the SO<sub>4</sub><sup>2-</sup> concentration in the outflow by up to 20%  
256 compared with the cumulative inflow SO<sub>4</sub><sup>2-</sup> of 2.6 kg. Sulphate leaching/production indicated the potential of  
257 internal sulphur cycling in bioreactors with incomplete N removal. BR1 and BR2 had incomplete nitrate  
258 removal during the study period due to sulphide re-oxidation to sulphate by sulphur oxidizing bacteria  
259 (SOB), which can use oxygen or nitrate as electron acceptor (Faulwetter et al., 2009) (Fig. S1 and Fig. 3).  
260 Sulphate production was observed previously by Lepine et al. (2016) for a woodchip bioreactor with  
261 incomplete N removal. However, higher nitrate removal in BR3 and BR4 combined with their reduced  
262 conditions (Fig.4) favored sulphate reduction.

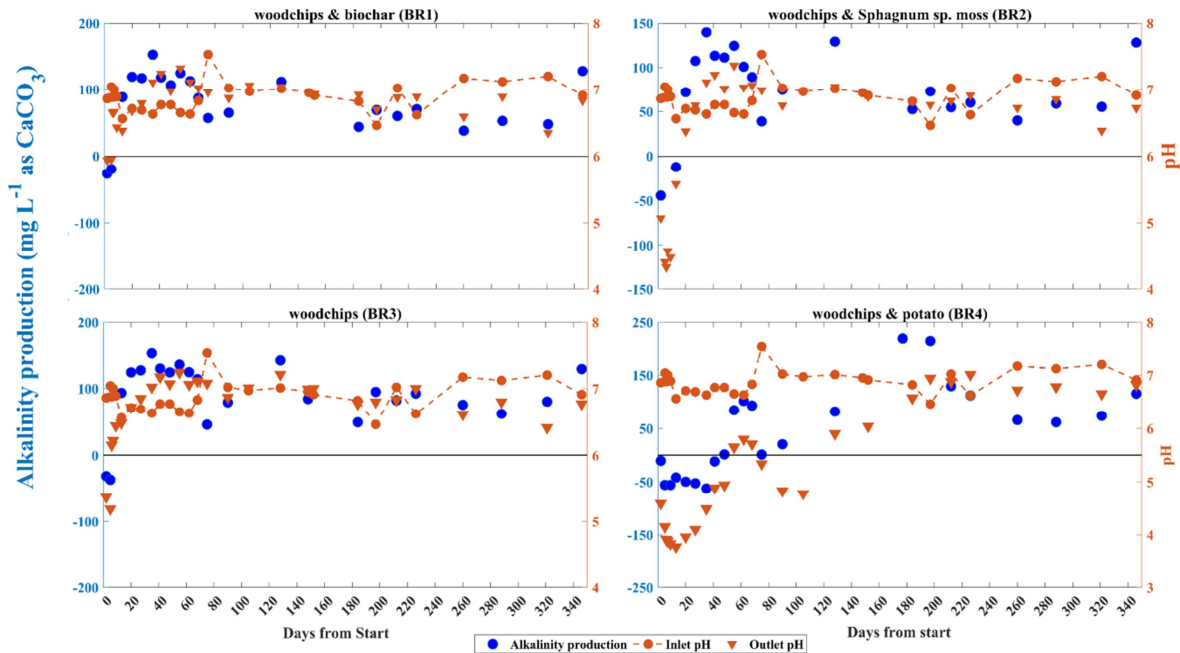


263 **Fig. 4.** Sulphate reduction/removal (+ values) and leaching/production (-values) in bioreactors BR1-BR4

264 over time at different redox potential values (Eh) in inflow and outflow for each bioreactor.

265 Redox potential was on average +340, +354, +312 and +181 mV in BR1, BR2, BR3 and BR4, respectively  
 266 (Fig. 4), indicating more oxidising conditions in BR1-BR3 and more reducing conditions in BR 4. It is well-  
 267 known that denitrification and microbial sulphate removal cause decline in redox potential and rise in pH  
 268 (Jog and Parry., 2006). In BR4, for the entire study period when outlet Eh reduced from 412 to 116 mV, the  
 269 pH tended to increase about 2.2 pH units (from 4.6-6.82) (Fig. S 5). Similarly, in BR1-3 by decreasing the  
 270 outlet redox potential, the pH increased 0.89, 1.65 and 1.4 pH units, respectively.

271 Inflow water pH was rather stable throughout the experiment (6.5-7.5) (Fig. 5). Outflow pH of bioreactors  
 272 during start-up was 6, 4.3, 5.2 and 3.8 in BR1 BR2, BR3 and BR4, respectively. It was thus lower than  
 273 inflow pH in the early stages of the experiment, most likely as a result of release of organic acids from the  
 274 packed materials (Fig. 5). All bioreactors showed lower alkalinity in outflow than in inflow during the start-  
 275 up period (Fig. 5). After 2-5 weeks, alkalinity production was observed in all bioreactors.



**Fig. 5.** Alkalinity production (+values) and inflow and outflow pH in bioreactors (BR1-BR4).

### 276 3.2 Factors affecting nitrate removal in woodchip bioreactors

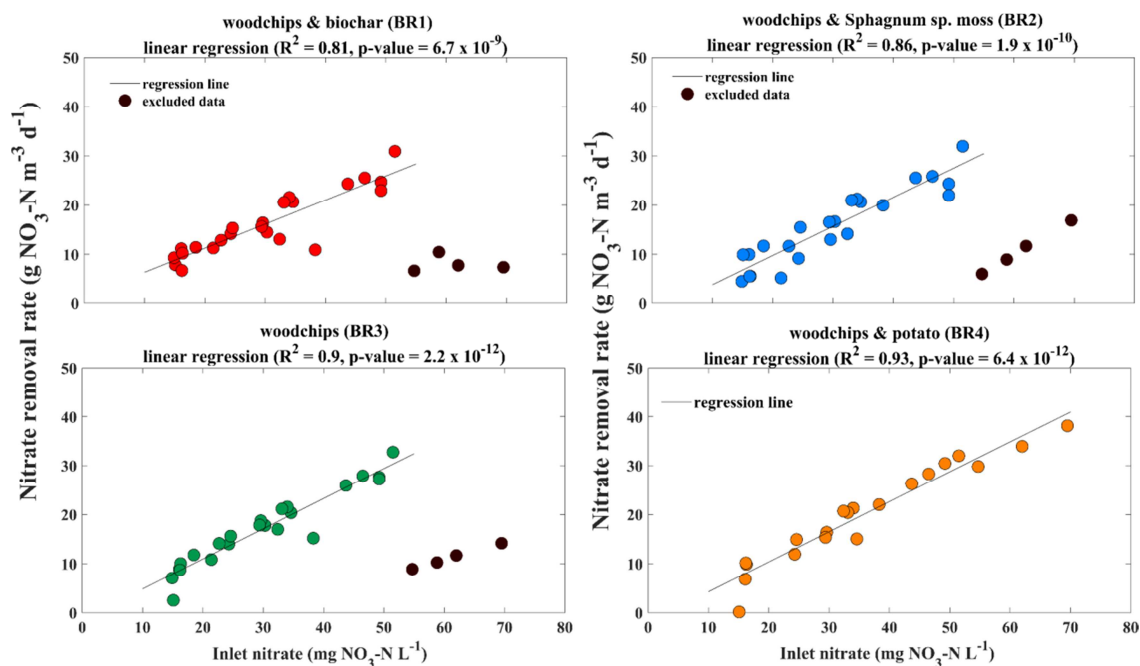
277 The results of one-way ANOVA showed that  $\text{NO}_3^-$  removal rates for whole study period did not differ  
 278 significantly between BR1, BR2 and BR 3 ( $p=0.75$ ), while nitrate removal in BR4 was higher (Fig. 2d-2h).  
 279 In the first three months of the experiment, when inflow  $\text{NO}_3^-$ -N concentration varied between 15 and 52 mg  
 280  $\text{L}^{-1}$ , all bioreactors showed similar removal rates (Fig. 2). After that, the bioreactors responded differently to  
 281 increasing  $\text{NO}_3^-$ -N inflow concentrations, e.g. the removal rate declined in BR1-BR3 but increased in BR4  
 282 (Fig. 2). BR4 reached its maximum removal rate of  $38 \text{ g NO}_3^- \text{-N m}^{-3} \text{ d}^{-1}$  at the highest  $\text{NO}_3^-$ -N inflow  
 283 concentration ( $70 \text{ mg L}^{-1}$ ; days 152-184), whereas BR1, BR2 and BR3 had a removal rate of 9, 13 and 12 g  
 284  $\text{NO}_3^- \text{-N m}^{-3} \text{ d}^{-1}$ , respectively (Fig. 2). Those differences persisted until day 250, after which all reactors again  
 285 had similar stable removal rates of around  $15 \text{ g NO}_3^- \text{-N m}^{-3} \text{ d}^{-1}$  until the end of the experiment. Similarly to  
 286 removal rate, the  $\text{NO}_3^-$  removal efficiency in BR1-BR3 showed fluctuations throughout the study period (Fig.  
 287 2e and 2g). However, BR4 reached stable removal efficiency of 93% after a period of fluctuation at start-up  
 288 (Fig. 2h).

289 The wide range of  $\text{NO}_3^-$  removal rates ( $3\text{-}38 \text{ g NO}_3^- \text{-N m}^{-3} \text{ d}^{-1}$ ) recorded in all bioreactors followed the  $\text{NO}_3^-$ -  
 290 N inflow concentration fluctuations. High removal rate in all bioreactors occurred when the inflow had high

291  $\text{NO}_3^-$ -N concentrations. This is consistent with previous findings that inflow concentrations control removal  
292 rate (e.g. Schipper et al., 2010; Addy et al., 2016).

293 In the present study,  $\text{NO}_3^-$  removal rate in BR4 increased significantly with increasing  $\text{NO}_3^-$ -N inflow  
294 concentration during the entire study period ( $R^2 = 0.93$ ; removal rate =  $0.6 \times$  influent nitrate concentration -  
295 1.85) (Fig. 6). This regression illustrated the actual relationship between inflow  $\text{NO}_3^-$ -N concentration and  
296 removal rate by excluding  $\text{NO}_3^-$ -N limited events ( $\text{NO}_3^-$ -N concentration  $< 0.5 \text{ mg L}^{-1}$ ) (Addy et al., 2016).  
297 Likewise, bioreactors BR1-BR3 showed a similar response to  $\text{NO}_3^-$ -N when days 152-212, with high  $\text{NO}_3^-$ -N  
298 concentration ( $55\text{-}70 \text{ mg L}^{-1}$ ), were excluded from the data (Fig. 6). The sharply decline in  $\text{NO}_3^-$ -N removal  
299 during days 152-212 was caused due to exceeding the maximum denitrification capacity in those bioreactors.  
300 This indicates that  $\text{NO}_3^-$  removal in BR1-BR3 was controlled by an independent parameter at high  $\text{NO}_3^-$ -N  
301 concentrations. The release rate of degradable carbon from the packed media presumably controlled  $\text{NO}_3^-$   
302 removal in this concentration range ( $> 52 \text{ mg L}^{-1}$ ) (Schipper et al., 2010). Hence, the type of carbon source  
303 used in denitrifying bioreactors can control  $\text{NO}_3^-$  removal, by providing more carbon availability and  
304 different microbial composition (Xu et al., 2018; Tangsir et al., 2017). Observed DOC in the bioreactors  
305 showed that carbon was much more readily released from potato residues than from any of the other carbon  
306 sources tested (Table S1). The easily soluble carbon in potato residues resulted in rapid formation of a  
307 complex microbial community structure with strong adaptive growth to the new environment (Zhao et al.,  
308 2018).

309



**Fig. 6.** Nitrate removal rate versus nitrate influent loading in BR1-4 for the study period of 346 days.

310 The maximum NO<sub>3</sub><sup>-</sup> removal rates observed in this study were greater than those previously reported (22 g  
 311 NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup>) (David et al., 2015; Schipper et al., 2010). This could be due to a combination of optimal  
 312 factors: sufficient HRT (Lepine et al., 2016; Tangsir et al., 2017) as a result of distributed upward flow  
 313 (section 2.2) combined with high NO<sub>3</sub><sup>-</sup> inflow concentration (Schipper et al., 2010), the organic C  
 314 compounds used (Gibert et al., 2008) and water temperature (Addy et al., 2016), here 15.5 ± 1 °C (mean ±  
 315 SD). A removal rate of >39 g NO<sub>3</sub><sup>-</sup> m<sup>-3</sup> d<sup>-1</sup> reported by Lepine et al. (2016) for comparable water quality was  
 316 associated with high COD:NO<sub>3</sub><sup>-</sup> ratio (0.86-1.66) in treated wastewater. This ratio can provide 42% COD  
 317 required for denitrification. The COD:NO<sub>3</sub><sup>-</sup> ratio has been reported to be a significant parameter affecting  
 318 denitrification in bioreactors (Jafari et al., 2015). However, in the present study inflow COD provided less  
 319 than 8% of the C/N required for complete NO<sub>3</sub><sup>-</sup> reduction (Narkis et al., 1979). Hence, the reported NO<sub>3</sub><sup>-</sup>  
 320 removal rates in this study represent the net values without a contribution from inflow COD. Enhancing  
 321 nitrate removal efficiency with different carbon substrates has been investigated previously (Gebert et al.,  
 322 2008; Schipper et al., 2010; Hashemi et al., 2011). Hashemi et al., (2011) improved nitrate removal of 36%  
 323 in wood bioreactor to 65%, 56 % and 77 % by utilizing barley straw, rice husk and date palm leaf,  
 324 respectively. Gebert et al., (2008) reported softwood (branches and bark with small amounts of leaves from a  
 325 variety of trees) as top performing substrate in denitrification efficiency (>98%) with denitrification rate of ~

326 17 g NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup>. However, other investigated materials such as mixture of wood chips, shredded bark and  
 327 topsoil, compost (obtained from the biological decomposition of organic wastes – wood trimmings, leaves,  
 328 rotten vegetables and food scraps) and willow woodchips identified as unsuitable carbon sources (see Gebert  
 329 et al., 2008). Warneke et al., (2011) reported nitrate removal of ~ 6.5, 6.2 and 3.5 g NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup> for wheat  
 330 straw, maize and green waste materials, respectively compare to the removal rate of 1.3 g NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup> in  
 331 soft wood (pine) bioreactor for 2-fold lower nitrate inlet concentration than used in this study. However,  
 332 additional potato residue to woodchip bioreactor increased 13% of nitrate removal to 38 g NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup>  
 333 which is remarkably higher than reported removal above.

### 334 3.3 Microbial community composition and process potential in the bioreactors

335 A total of 9261 quality-filtered sequences per library were obtained from water and solid samples from the  
 336 four bioreactors (Table 3). Library coverage was ≥94% in all cases, indicating that the sequencing depth was  
 337 sufficient. The number of observed and Chao 1-estimated OTUs was significantly lower (p<0.001) in filtered  
 338 water and solid material from BR4 than in corresponding samples from BR1-BR3. The Shannon diversity  
 339 index was also significantly lower (p<0.001) in BR4 (4.5) than in BR1-BR3.

340 The microbial community in BR4 differed strongly from the microbial community in BR1-BR3 (Figs. S 2A).  
 341 Smaller differences were detected between the microbial communities in BR1-BR3 and between water and  
 342 solid samples from all bioreactors (Figs. S2 B and C). In solid material, differences were observed between  
 343 microbial communities in zone 3 (i.e. top-layer woodchip) and in zone 2 in BR1, BR2 and BR4 (containing  
 344 biochar, *Sphagnum* sp. moss and potato residues, respectively) but not BR3 (containing woodchips) (Fig. 1).  
 345 In water, the differences were much less pronounced (Figs. S2 B and C).

346 **Table 3.** Prokaryotic diversity in bioreactors BR1-BR4. Numbers of sequences are taken from the original  
 347 OTU tables, while all other diversity indicators are based on OTU tables rarified at a depth of 4098  
 348 sequences. Average values for 1-2 replicates per sampling point are shown. Zone 2 and zone 3 refer to the  
 349 carbon source material tested and the top-layer woodchip, respectively, as indicated in Fig. 1

	No. of	No. of	Coverage	OTUs	OTUs	Shannon
--	--------	--------	----------	------	------	---------

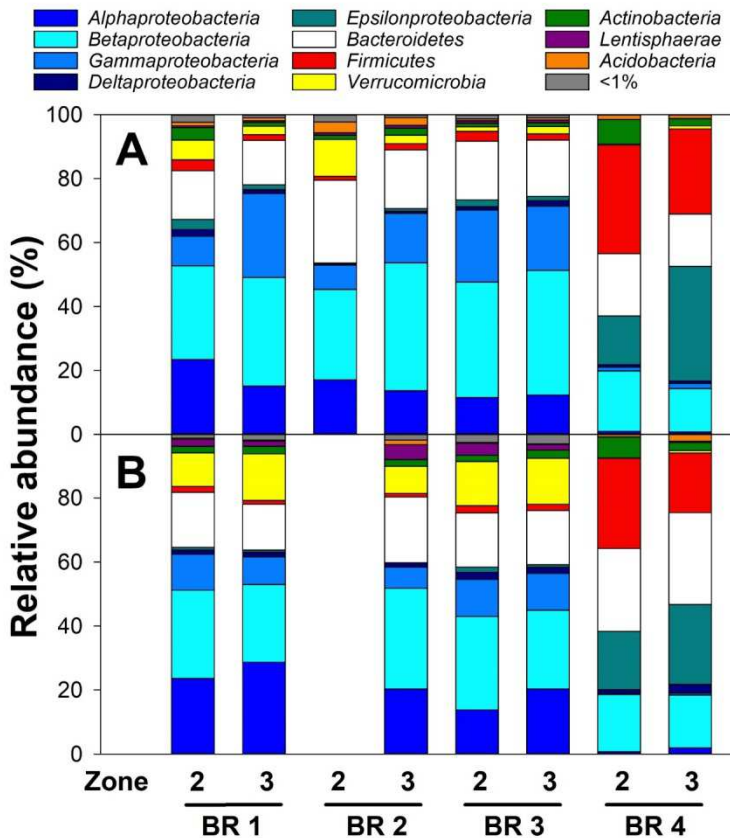
			sequences	samples	(%)	richness (observed)	richness (estimated) <sup>a</sup>	
BR 1:	Water	Zone 2	8 550	2	95	441	802	4.64
		Zone 3	7 310	2	95	468	761	4.68
Woodchip/ Biochar	Solid	Zone 2	6 844	2	94	496	827	4.77
		Zone 3	4 935	2	95	398	739	4.36
BR 2:	Water	Zone 2		0				
		Zone 3	7 500	1	95	450	821	4.67
Woodchip/ <i>Sphagnum</i>	Solid	Zone 2	7 358	1	96	383	697	4.42
		Zone 3	6 711	2	96	354	674	4.2
BR 3:	Water	Zone 2	8 198	2	95	433	749	4.53
		Zone 3	8 304	2	94	480	854	4.72
Woodchip/ woodchip	Solid	Zone 2	6 942	2	96	378	713	4.29
		Zone 3	6 956	1	95	389	897	4.26
BR 4:	Water	Zone 2	9 261	2	96	303	583	3.61
		Zone 3	8 148	2	96	337	605	3.78
(Woodchip/ potato)	Solid	Zone 2	9 256	2	97	287	505	3.67
		Zone 3	8 359	2	96	296	578	3.39

350 <sup>a</sup>OTUs richness estimated by Chao1.

351 Only bacterial sequences (no archaeal sequences) were detected in the bioreactors. In BR1-BR3, the  
352 microbial community was dominated by *Proteobacteria*, *Bacteroidetes* and *Verrucomicrobia* (Fig. 7).  
353 Within the *Proteobacteria*, *Betaproteobacteria* were most abundant (24-40% relative abundance), followed  
354 by *Gammaproteobacteria* (7-26%) and *Alphaproteobacteria* (11-28%). In BR4, the microbial community  
355 was dominated by *Epsilonproteobacteria* (15-36%), *Bacteroidetes* (16-29%) and *Firmicutes* (17-34%) (Fig.  
356 7). Amongst the most abundant genera, *Uliginosibacterium* (up to 11% relative abundance), *Sulfurospirillum*  
357 (up to 29%), *Prevotella* (up to 19%) and *Lactobacillus* (up to 18%) were almost exclusively detected in BR4,  
358 while *Rhodobacter* (up to 4%), *Sphingobium* (up to 4%), *Rhodoferax* (up to 5%), *Pseudomonas* (up to 13%),  
359 *Thermomonas* (up to 6%) and *Luteolibacter* (up to 10%) were almost exclusively detected in BR1-BR3 (Fig.



360 S3). The genera *Lactobacillus*, *Prevotella* and *Sulfurispirillum* include known fermenters, some of which can  
 361 also reduce nitrate to ammonium (e.g. Kruse et al., 2018; Salvetti et al., 2012). The genera *Rhodobacter*,  
 362 *Rhodoferrax*, *Pseudomonas* and *Thermomonas* include known denitrifiers (e.g. Finneran et al., 2003;  
 363 Mergaert et al., 2003).

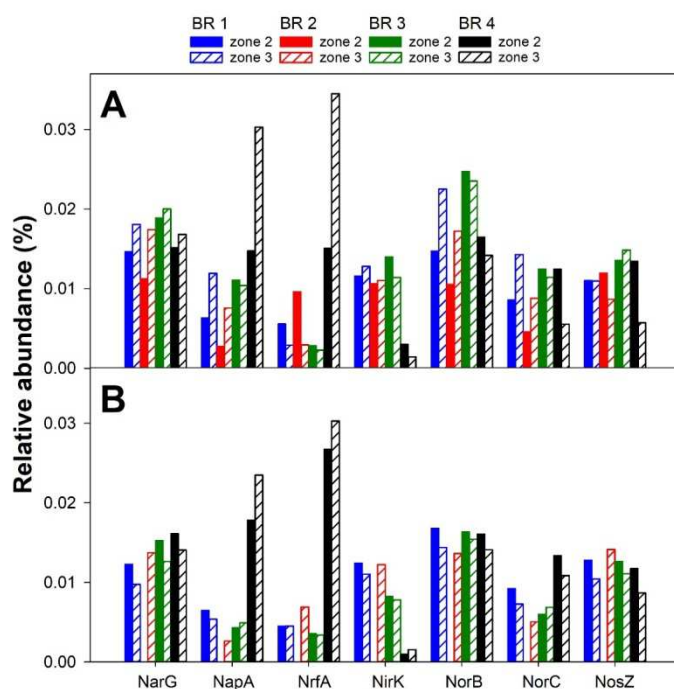


364 **Fig. 7.** Composition of the microbial community based on sequence analysis of bacterial and archaeal 16S  
 365 rRNA genes from (A) solid material and (B) water samples from woodchip bioreactors with a zone  
 366 containing biochar (BR1), *Sphagnum* sp. moss (BR2), woodchip (BR3) and potato residues (BR4). Average  
 367 relative abundances of 1-2 replicates per sample are shown. Samples were taken from the top-layer  
 368 woodchip (zone 3) and the carbon source material (zone 2).

369 Functional profiles of the bacterial communities were predicted based on 16S rRNA gene sequences using  
 370 PICRUSt. It proved possible to use around 31% of all OTUs and 83% (76-90%) of all sequences for  
 371 functional prediction. Overall functional profiles of microbiological communities were rather similar in the  
 372 different bioreactors. Selected functions related to the nitrogen cycle were assessed in more detail (Fig. 8).



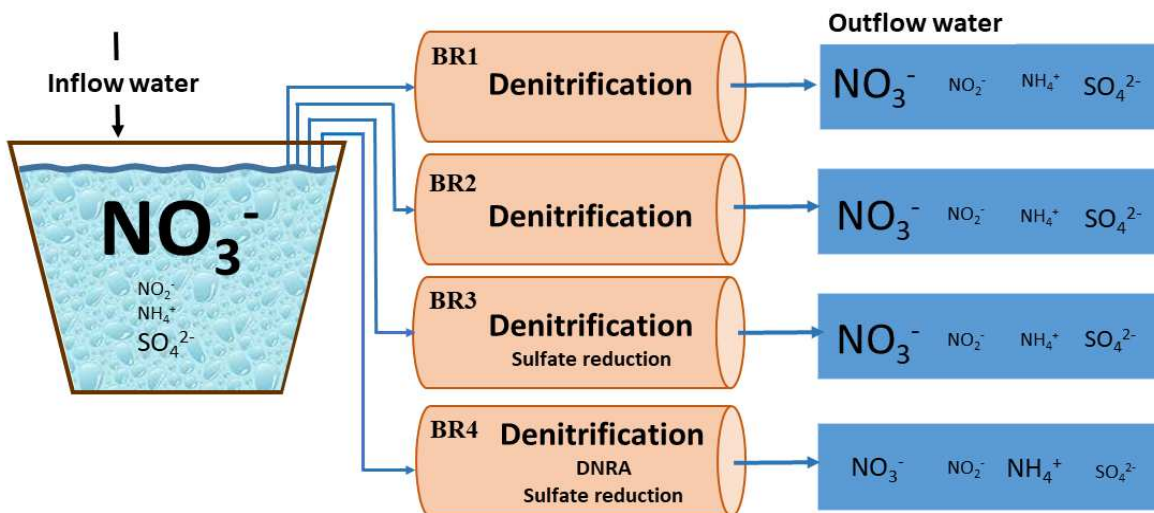
373 Functions related to denitrification (NarG, NapA, NirK, NorB, NorC, NosZ) and DNRA (NarG, NapA,  
 374 NrfA) were predicted, while functions specific to nitrification (AmoA, AmoB, AmoC) were not predicted.  
 375 The membrane-bound nitrate reductase NarG was predicted in similar relative abundance in all bioreactors,  
 376 while higher relative abundance of the periplasmic nitrate reductase NapA was predicted in BR4 than in  
 377 BR1-BR3 (Fig. 7). The denitrification-associated functions NirK, NorB, NorC and NosZ were predicted with  
 378 higher relative abundances for BR1-BR3 than for BR4, while the nitrite reductase NrfA (which catalyses the  
 379 reduction of nitrite to ammonia in DNRA) was more frequently predicted for BR4 (Fig. 8). This indicates  
 380 that bioreactors BR1-BR3 had higher predicted potential for denitrification, while the bioreactor with potato  
 381 residues (BR4) had higher predicted potential for DNRA. The nitrite reductase NirK may also be present in  
 382 nitrifying organisms. However, the contribution of nitrifiers such as *Nitrospira* sp. or *Nitrobacter* sp. to NirK  
 383 was only 0.15%.



384 **Fig. 8.** Relative abundance of predicted nitrogen cycle-related genes in functional profiles of (A) solid  
 385 material and (B) water samples from woodchip bioreactors with a zone containing biochar (BR1), *Sphagnum*  
 386 sp. moss (BR2), woodchip (BR3) and potato residues (BR4). Functional profiles were predicted based on  
 387 16S rRNA gene sequences using PICRUST. Average relative abundances of 1-2 replicates per sample are  
 388 shown.

389 **3.4 Nitrogen turnover in bioreactors BR1-BR4**

390 The results obtained suggest that heterotrophic denitrification was the dominant path for  $\text{NO}_3^-$  removal in the  
 391 four bioreactors. The observed high rate of  $\text{NO}_3^-$  removal, combined with relatively low production of nitrite,  
 392 ammonium and alkalinity and high relative abundances of denitrification-associated functions, provide  
 393 evidence of denitrification activity in the bioreactors. The high alkalinity-producing period in BR1-BR3,  
 394 coinciding with high nitrate removal, is evidence of heterotrophic denitrification. Heterotrophic  
 395 denitrification produces approximately 3.57 mg alkalinity (as  $\text{CaCO}_3$ ) per mg  $\text{NO}_3^-$ -N reduced (van Rijn et  
 396 al., 2006). The calculated stoichiometric ratio of 4.2, 3.3 and 3.9 in BR1, BR2 and BR3, respectively, is very  
 397 similar to the expected theoretical value. Previous studies on both laboratory and field woodchip bioreactors  
 398 have also identified denitrification as the main mechanism for  $\text{NO}_3^-$  removal (e.g. Nordström and Herbert,  
 399 2018; Schipper et al., 2010; Zhao et al., 2018). However, other processes, including DNRA, aerobic  
 400 degradation (Zhao et al., 2018), anammox (Herbert et al., 2014; Schipper et al., 2010) and nitrogen  
 401 immobilisation in organic compounds (Greenan et al., 2006), might also contribute to nitrogen turnover to a  
 402 smaller extent.

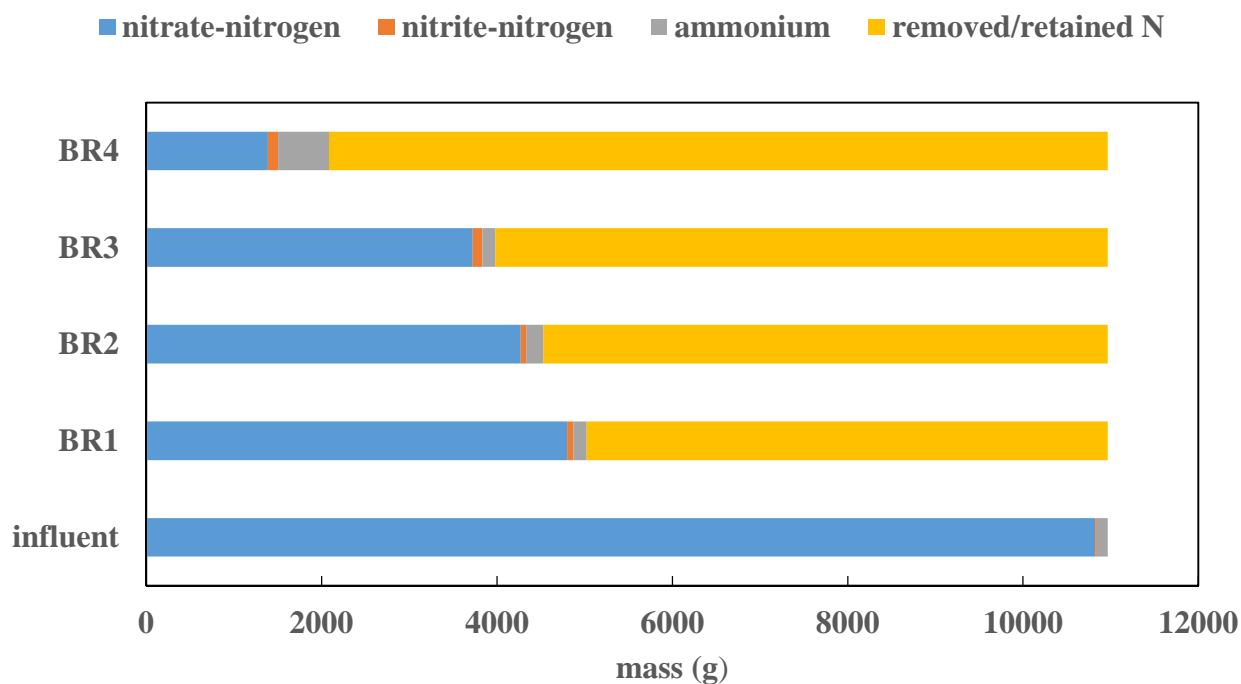


403

404 **Fig. 9.** Processes suggested to occur in woodchip bioreactors containing a zone of biochar (BR1), *Sphagnum*  
405 sp. moss (BR2), woodchip (BR3) and potato residues (BR4). Font size indicates relative  
406 concentration/importance of a compound/process.

407

408 Ammonium is produced during DNRA, and thus high ammonium production in the bioreactors would be an  
409 indicator that DNRA was a major nitrate-reducing process. However, ammonium production contributed less  
410 than 2% of total nitrogen mass in BR1-BR3 and 5% in BR4. This excludes DNRA as a major mechanism in  
411 nitrate reduction (Fig. 10), although small amounts of ammonium might have been produced by DNRA.  
412 DNRA is generally favoured over denitrification in environments with low nitrate and high labile carbon  
413 availability. The higher ammonium production in BR4 indicates higher DNRA rates than in the other  
414 bioreactors. Higher DNRA rates in BR4 are most likely due to higher abundance of potential fermenters,  
415 DNRA microorganisms and easily accessible labile carbon. Potato residues provided a labile carbon source,  
416 as indicated by the high outflow DOC in BR4. We consider it unlikely that anaerobic ammonium oxidation  
417 (Herbert et al., 2014; Schipper et al., 2010) was a pathway for nitrate removal in the reactors, as inflow  
418 concentrations of ammonium were low, and the number of potential anaerobic ammonium-oxidising taxa  
419 detected in the microbial communities was negligible.



420

421 **Fig. 10.** Total cumulative nitrogen mass in inflow water and outflow of woodchip bioreactors containing a  
 422 zone of biochar (BR1), *Sphagnum* sp. moss (BR2), woodchip (BR3) and potato residues (BR4) during the  
 423 entire study period. The removed/retained nitrogen was in either gaseous or liquid form.

### 424 3.5 Sustainability of bioreactors for RAS

425 This one-year study showed that woodchip bioreactors can operate properly, without clogging, in treating  
 426 effluent from intensive land-based RAS with low COD load. The selected HRT of 48 h was long enough for  
 427 complete denitrification and resulted in a maximum annual  $\text{NO}_3^-$  removal rate of 93%. Use of woodchip  
 428 denitrification in intensive RAS mitigates environmental challenges by treating effluent as an end-of-pipe  
 429 treatment or by reducing freshwater consumption by creating a side closed loop for fish production. Start-up  
 430 leaching may limit application of woodchip bioreactors, but due to its short duration it can be controlled (see  
 431 section 3.1).

432 The results obtained in the present study were used to calculate model designs for passive hybrid systems for  
 433 a typical RAS with mechanical and biological treatment (nitrification) handling a maximum flow rate of 50

434  $\text{m}^3 \text{ day}^{-1}$ , corresponding to 2.75 kg  $\text{NO}_3^-$ -N per day. When the measured annual  $\text{NO}_3^-$  removal rates were  
435 used, required volume was calculated to be 138-183  $\text{m}^3$ , depending on the carbon source applied. Adding a  
436 zone of potato residues to the woodchip bioreactor design resulted in 34 and 46  $\text{m}^3$  smaller bioreactor volume  
437 compared with BR3 and BR1/BR2, respectively. However, adding a zone of biochar and *Sphagnum* sp. moss  
438 did not increase woodchip bioreactor performance. A maximum flow rate ( $50 \text{ m}^3 \text{ day}^{-1}$ ) relative to the  
439 calculated bioreactor volume would correspond to lower HRT (2.8 days) in BR4, but higher HRT (3.4-3.7  
440 days) in the other bioreactors. Besides enhancing  $\text{NO}_3^-$  removal rate in woodchip bioreactors, potato residues  
441 enabled more stable  $\text{NO}_3^-$  removal efficiency. Hence, based on findings in this one-year laboratory study,  
442 industrial potato residues were identified as a suitable additional carbon source.

443 Long-term laboratory scale investigations (lasting at least one year) are recommended to reach and verify  
444 stable  $\text{NO}_3^-$  removal rate in woodchip bioreactors (Robertson, 2010; Schipper et al., 2010). The removal rates  
445 reported here without replacing packed-media can thus be used for designing field-scale systems with  
446 comparable water chemistry. Ours is the first study to test industrial potato residues as an additional carbon  
447 source for enhancing woodchip bioreactor performance. Applying this low-cost material in passive  
448 denitrifying bioreactors for RAS or other industries (e.g. agriculture, mining, small wastewater treatment  
449 plants) could enable economic sustainability within a local context.

#### 450 **4 Conclusions**

451 Woodchip bioreactors achieved efficient  $\text{NO}_3^-$  removal in treating land-based RAS effluent, without  $\text{NH}_4^+$ -N  
452 and  $\text{NO}_2^-$ -N production that are harmful in aquaculture. Of the additional carbon sources tested, higher  $\text{NO}_3^-$   
453 removal was achieved with industrial potato residues than with biochar or *Sphagnum* moss and higher inflow  
454 concentrations of  $\text{NO}_3^-$  could be removed. The potato residue bioreactor hosted a distinctly different  
455 microbial community, which might be related to the observed differences in  $\text{NO}_3^-$  removal. A novel finding  
456 was that industrial potato residues can be used as carbon source to enhance woodchip bioreactor  
457 performance, provided that the start-up period is controlled. The results from this one-year study in real  
458 wastewater facilities can be used to formulate guidelines for full-scale bioreactor design in the future. Since  
459 temperature was controlled in this study, more studies are needed to understand the removal efficiency of

460 woodchip denitrification systems in the full range of temperatures in cold climate regions. Lower removal  
461 efficiency and slower biological activities would be expected in the colder climate areas. Therefore, field  
462 scale pilots are needed to study the winter effect on the hydraulic and removal processes, when controlling  
463 the efficiency of these bioreactors. In addition, the composition of nitrogen in the inlet water can affect the  
464 denitrification rate. Higher denitrification rates would be expected when wastewaters have high  $\text{NO}_3^-$   
465 concentrations compared to other nitrogen compounds ( $\text{NH}_4^+$  and  $\text{NO}_2^-$ ).

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- Woodchip bioreactors removed 31-38 g NO<sub>3</sub><sup>-</sup>-N m<sup>-3</sup> d<sup>-1</sup> from intensive aquaculture effluent
- Additional potato residues to woodchip material increased 13 % of nitrate removal rate
- The potato residue bioreactor hosted a distinctly different microbial community

Journal Pre-proof

**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: