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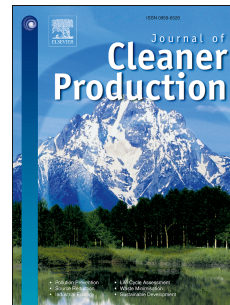
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Enhanced nitrogen removal of low carbon wastewater in denitrification bioreactors by utilizing industrial waste toward circular economy

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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 (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 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 NO_3^- -N $\text{m}^{-3} \text{d}^{-1}$), but potato residues increased NO_3^- removal rate to 38 g 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 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 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 (NO_3^-) removal is
32 still a critical challenge (Pulkkinen et al., 2018). Removal of 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 (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 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 NO_3^- -N concentration ($>50 \text{ mg}$
39 L^{-1}) has received little attention.

40 In denitrifying bioreactors, nitrogen (N) is removed by heterotrophic denitrifiers converting 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 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 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 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 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 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 μm 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 mg L^{-1} on
76 average), but high N content (mean NO_3^- -N content 34.7 mg L^{-1}) (Table 1). Due to the efficient nitrification
77 unit before the bioreactors, 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 ⁻¹)	15.3 \pm 2.1	5
Dissolved organic carbon (mg L ⁻¹)	14 \pm 1.3	5
Chemical oxygen demand (mg L ⁻¹)	12.9 \pm 1.8	5
Biological oxygen demand (mg L ⁻¹)	3.8 \pm 2.2	13
Nitrate-nitrogen (mg L ⁻¹)	34.7 \pm 15.6	27
Nitrite-nitrogen (mg L ⁻¹)	0.1 \pm 0.06	30
Ammonium-nitrogen (mg L ⁻¹)	0.5 \pm 0.2	30
Dissolved oxygen (mg L ⁻¹)	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 ₃ L ⁻¹)	54.2 \pm 18	25
Sulphate (mg L ⁻¹)	10.5 \pm 3.2	24

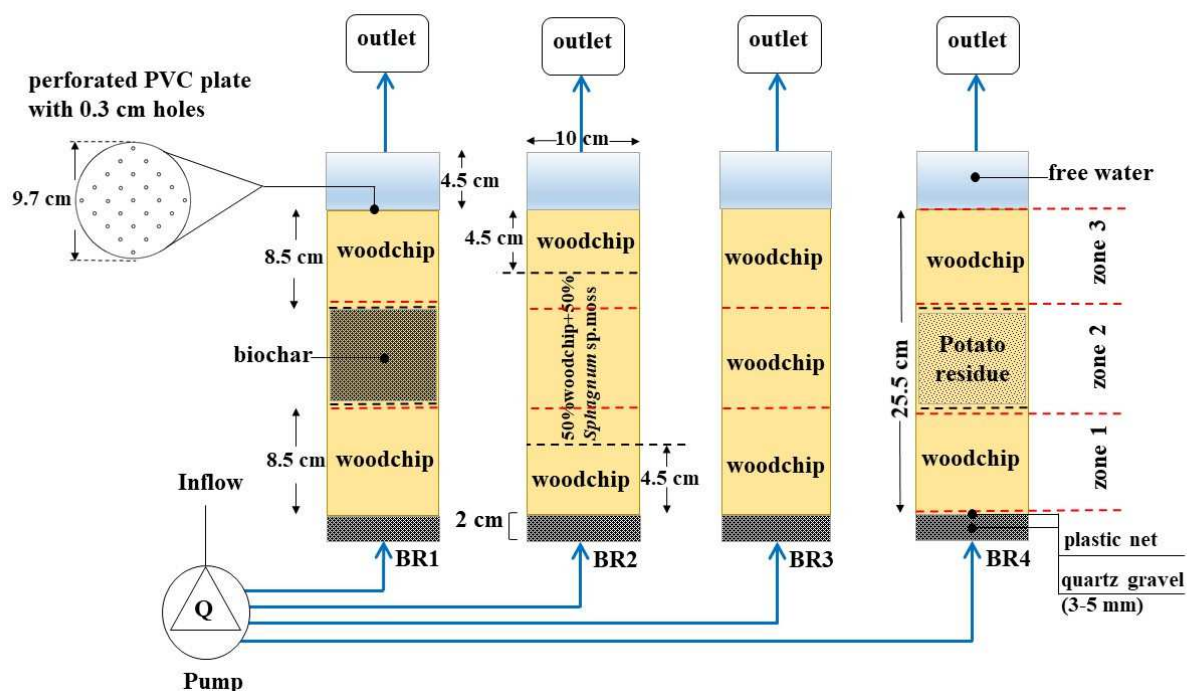
79 2.2 Bioreactor design

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⁻¹ 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.



107

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Fig. 1. Schematic diagram of the bioreactor set-up (bioreactors BR1-BR4). Red and black dashed lines

109

represent microbiological sampling zones and packed media zones in the bioreactors, respectively. Zones 1

110

and 3 were packed with woodchips, while zone 2 was packed with biochar in BR1, *Sphagnum* sp. moss in

111

BR2, woodchips in BR3 and potato residues in BR4.

112

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

113

114 2.3 Sampling and analysis

115

Water samples were collected at the inflow tank and at the outlet of the four bioreactors. Sampling was

116

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 (NO_3^- -N), nitrite-nitrogen (NO_2^- -N), ammonium-
125 nitrogen (NH_4^+ -N), sulphate (SO_4^{2-}) and biological oxygen demand (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 NO_3^- -N removal rate (g NO_3^- -N m^{-3} d) was calculated based on differences
134 between bioreactor inlet and outlet 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 μm 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).

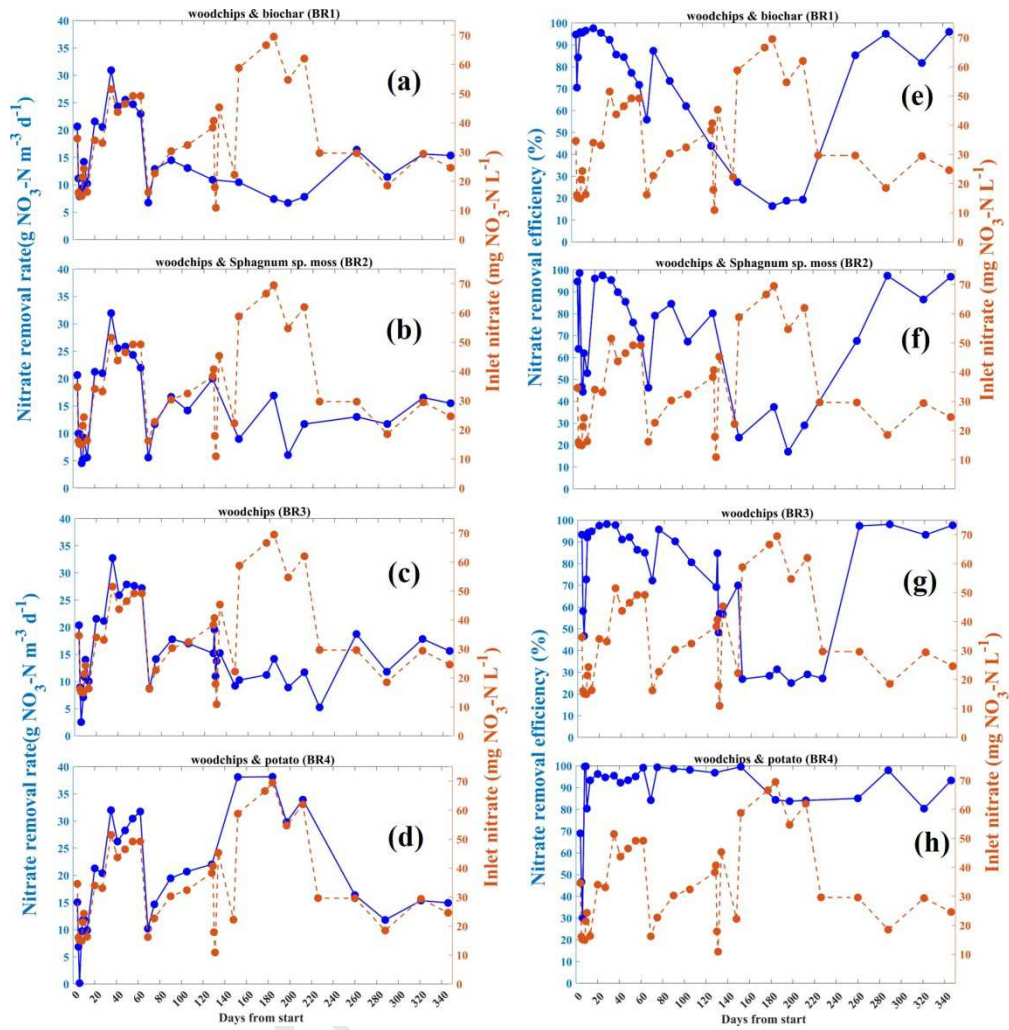
152 Sequence analysis was performed using the analysis pipelines mothur v.1.39.5 (Schloss et al., 2009) and
153 qiime 1.9 (Caporaso et al., 2011). Sequences with incorrect primer (>1 bp) or barcode (>1 bp) sequences
154 were removed, as were sequences <150 bp and chimeric sequencing. After quality filtering, 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**

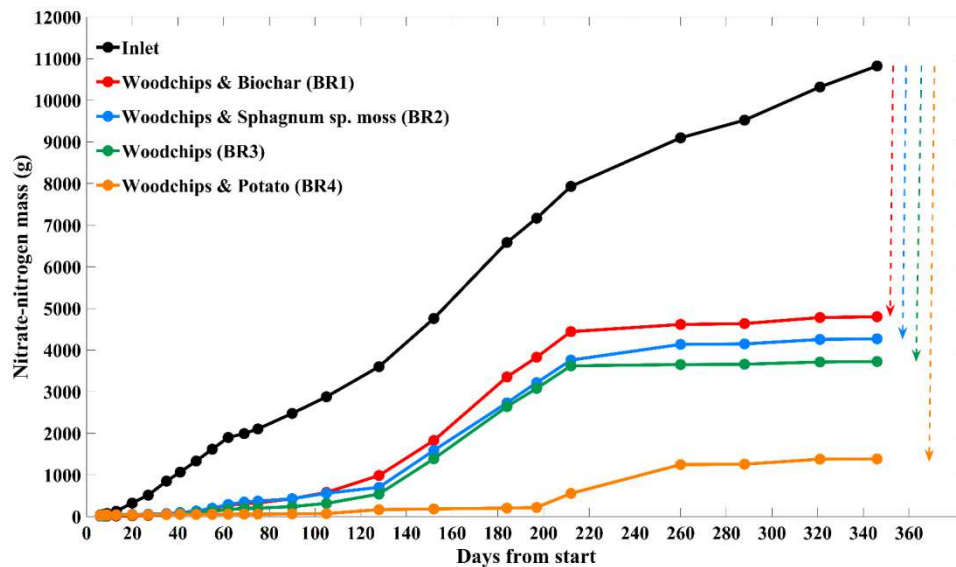
174 The initial inflow NO_3^- -N concentration of the bioreactors ranged from 15 to 70 mg L^{-1} , while the outflow
175 concentrations were clearly lower (ranging from the detection limit of 0.03 to 58.1 mg L^{-1}) (Fig. 2). All
176 bioreactors showed effective NO_3^- removal ability immediately upon start-up and over the whole study
177 period (Fig. 2, Fig. 3). Instant 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 $n = 26$), NO_3^- -N comprised 98 ± 0.1 % (mean \pm SD) of total dissolved
180 inorganic nitrogen in inflow water, while only minor amounts of NH_4^+ -N ($1.6 \pm 0.8\%$) and NO_2^- -N
181 ($0.27 \pm 0.22\%$) were present. For the entire study period, total inflow 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 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 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 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 (NO₃⁻-N) removal rate (a-d) and removal efficiency (e-h) in bioreactors during the
 190 346-day study period.

191

192



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.

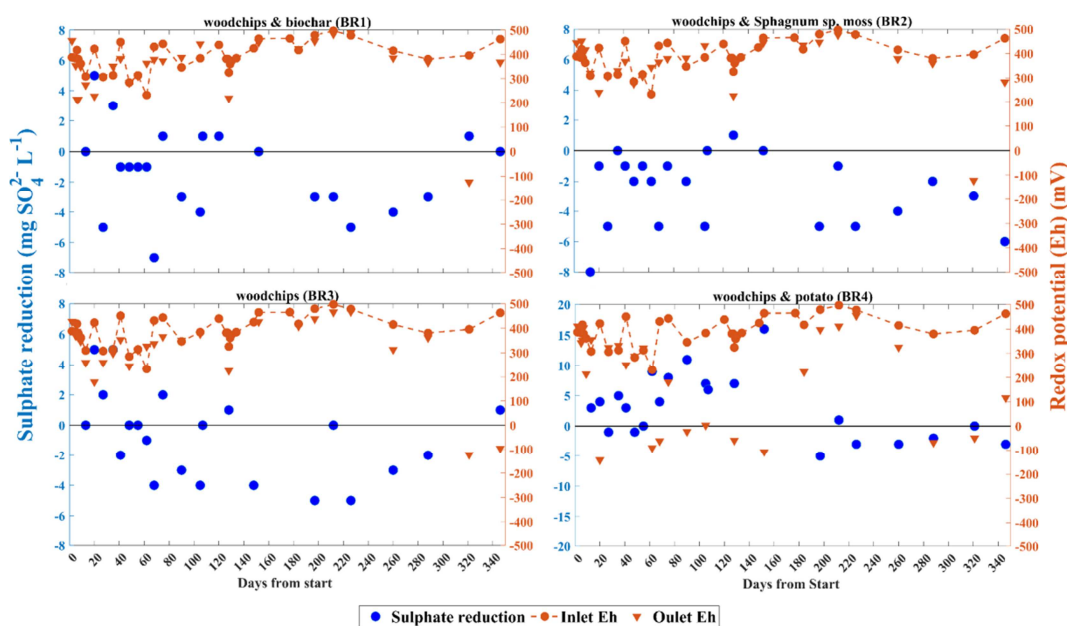
195 Temporary increases in nitrite production (von Ahnen et al., 2018; Zhao et al., 2018) can limit the use of
 196 woodchip bioreactors for RAS effluents, due to the toxicity of nitrite at high concentrations (Kroupova et al.,
 197 2005). In this study, the NO_2^- -N concentration in inflow water remained 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 NO_2^- -N was
 199 12, 6, 15 and 0 mg L^{-1} in bioreactors BR1, BR2, BR3 and BR4, respectively (Fig. S1). From day 20 onwards,
 200 the 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 (LD_{50}) varies between fish species but is typically
 202 around 2 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 mg
 207 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 NO_3^- reduction leading to intermediate nitrogen products. As the outflow concentrations of nitrite
 210 in the start-up phase exceeded the 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 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 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 mg L^{-1} , in the first 10 days of the
218 experiment, but it then declined to lower than 4 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 TOC/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 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 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 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 mg L^{-1} in BR1-BR4 (Table. S1) exceeding temporarily the maximum
230 concentration of 42 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 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⁻¹ 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 NO₃⁻-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 SO₄²⁻ concentrations were on average higher in the outflow than in the inflow waters of BR1 and BR2,
251 indicating leaching or production of SO₄²⁻ (Fig. 4). This resulted in cumulative leaching/production of 165 g
252 and 474 g SO₄²⁻ in BR1 and BR2, respectively, for the whole study period. In contrast, SO₄²⁻ were on average
253 lower in outflow than in inflow waters of BR3 and BR4 (Fig. 4), indicating SO₄²⁻ reduction/removal.
254 Cumulative SO₄²⁻ removal of 350 g and 546 g was observed in BR3 and BR4, respectively, for the whole
255 study period. SO₄²⁻ leaching/removal increased the SO₄²⁻ concentration in the outflow by up to 20%
256 compared with the cumulative inflow SO₄²⁻ 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.

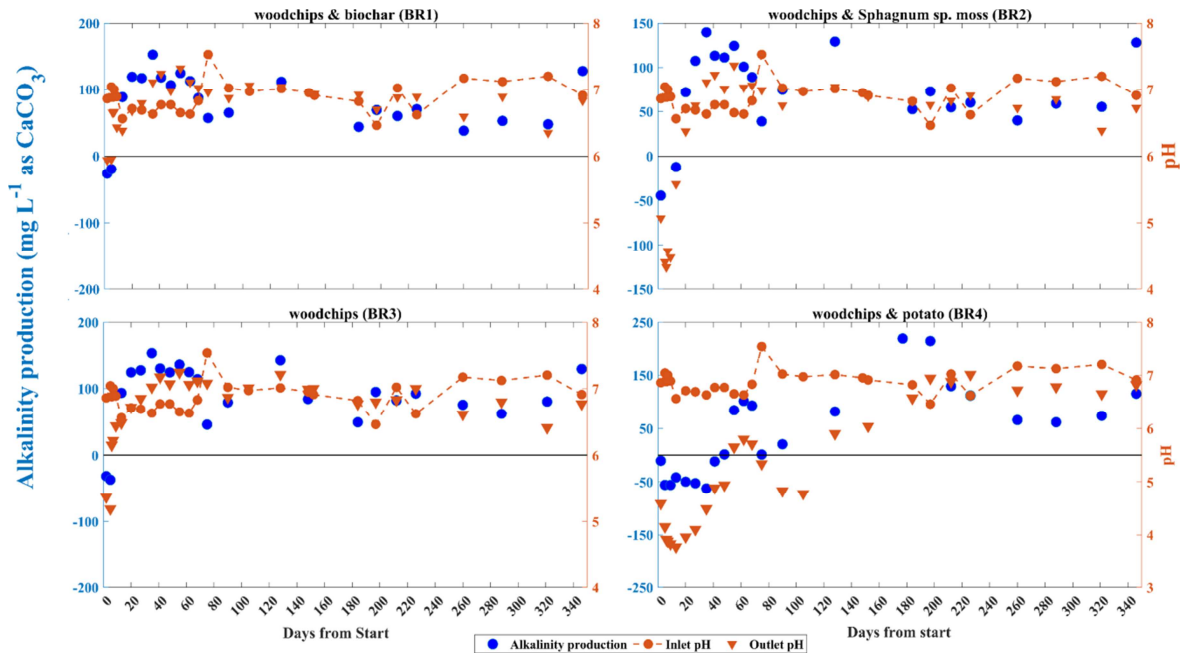


Fig. 5. Alkalinity production (+values) and inflow and outflow pH in bioreactors (BR1-BR4).

276 3.2 Factors affecting nitrate removal in woodchip bioreactors

277 The results of one-way ANOVA showed that 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 NO_3^- -N concentration varied between 15 and 52 mg
 280 L^{-1} , all bioreactors showed similar removal rates (Fig. 2). After that, the bioreactors responded differently to
 281 increasing 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 NO_3^- -N inflow
 283 concentration (70 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 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 NO_3^- removal rates ($3\text{-}38 \text{ g NO}_3^- \text{-N m}^{-3} \text{ d}^{-1}$) recorded in all bioreactors followed the NO_3^- -
 290 N inflow concentration fluctuations. High removal rate in all bioreactors occurred when the inflow had high

291 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, NO_3^- removal rate in BR4 increased significantly with increasing 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 NO_3^- -N concentration and
296 removal rate by excluding NO_3^- -N limited events (NO_3^- -N concentration $< 0.5 \text{ mg L}^{-1}$) (Addy et al., 2016).
297 Likewise, bioreactors BR1-BR3 showed a similar response to NO_3^- -N when days 152-212, with high NO_3^- -N
298 concentration ($55\text{-}70 \text{ mg L}^{-1}$), were excluded from the data (Fig. 6). The sharply decline in 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 NO_3^- removal in BR1-BR3 was controlled by an independent parameter at high NO_3^- -N
301 concentrations. The release rate of degradable carbon from the packed media presumably controlled 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 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

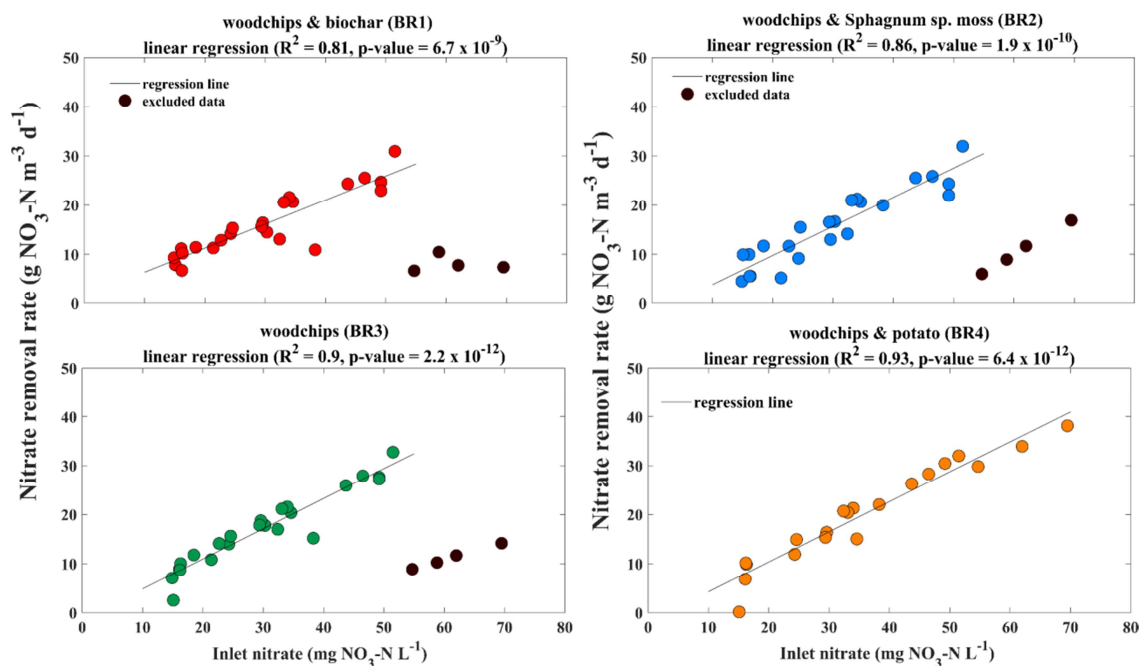


Fig. 6. Nitrate removal rate versus nitrate influent loading in BR1-4 for the study period of 346 days.

310 The maximum NO₃⁻ removal rates observed in this study were greater than those previously reported (22 g
 311 NO₃⁻-N m⁻³ d⁻¹) (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 NO₃⁻ 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 NO₃⁻ m⁻³ d⁻¹ reported by Lepine et al. (2016) for comparable water quality was
 316 associated with high COD:NO₃⁻ ratio (0.86-1.66) in treated wastewater. This ratio can provide 42% COD
 317 required for denitrification. The COD:NO₃⁻ 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 NO₃⁻ reduction (Narkis et al., 1979). Hence, the reported NO₃⁻
 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 NO₃⁻-N m⁻³ d⁻¹. 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 NO₃⁻-N m⁻³ d⁻¹ for wheat
 330 straw, maize and green waste materials, respectively compare to the removal rate of 1.3 g NO₃⁻-N m⁻³ d⁻¹ 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 NO₃⁻-N m⁻³ d⁻¹
 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

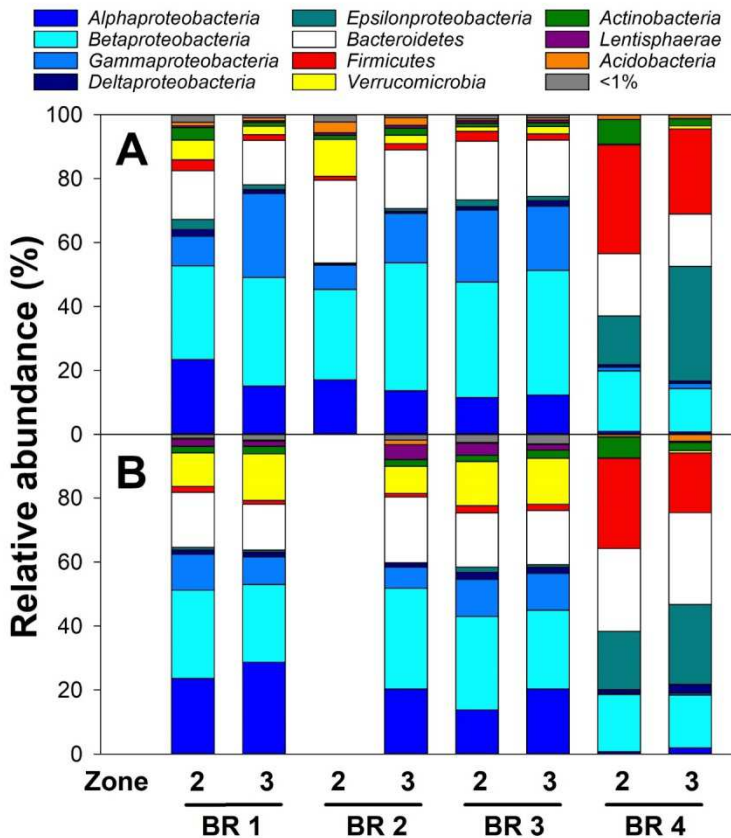
	No. of	No. of	Coverage	OTUs	OTUs	Shannon
--	--------	--------	----------	------	------	---------

			sequences	samples	(%)	richness (observed)	richness (estimated) ^a	
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 ^aOTUs 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.

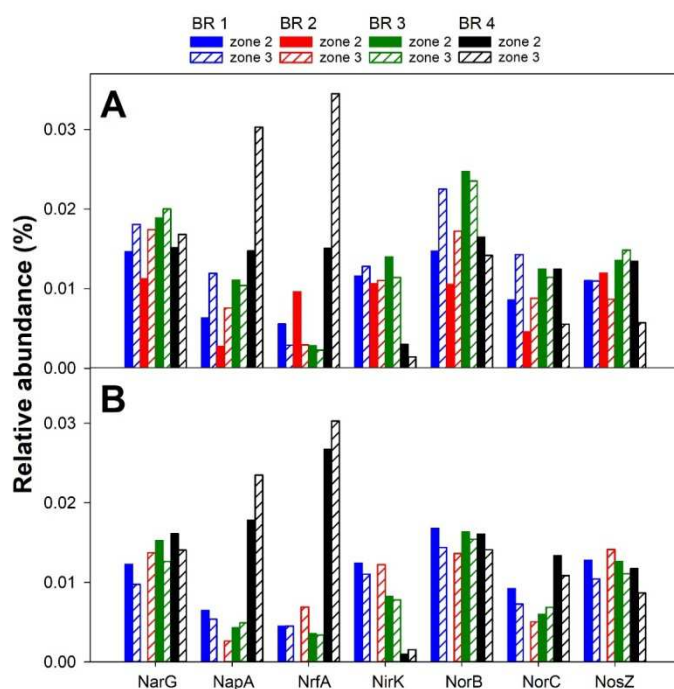
360 S3). The genera *Lactobacillus*, *Prevotella* and *Sulfurispirillum* include known fermenters, some of which can
 361 also reduce nitrate to ammonium (e.g. Kruse et al., 2018; Salvetti et al., 2012). The genera *Rhodobacter*,
 362 *Rhodoferrax*, *Pseudomonas* and *Thermomonas* include known denitrifiers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

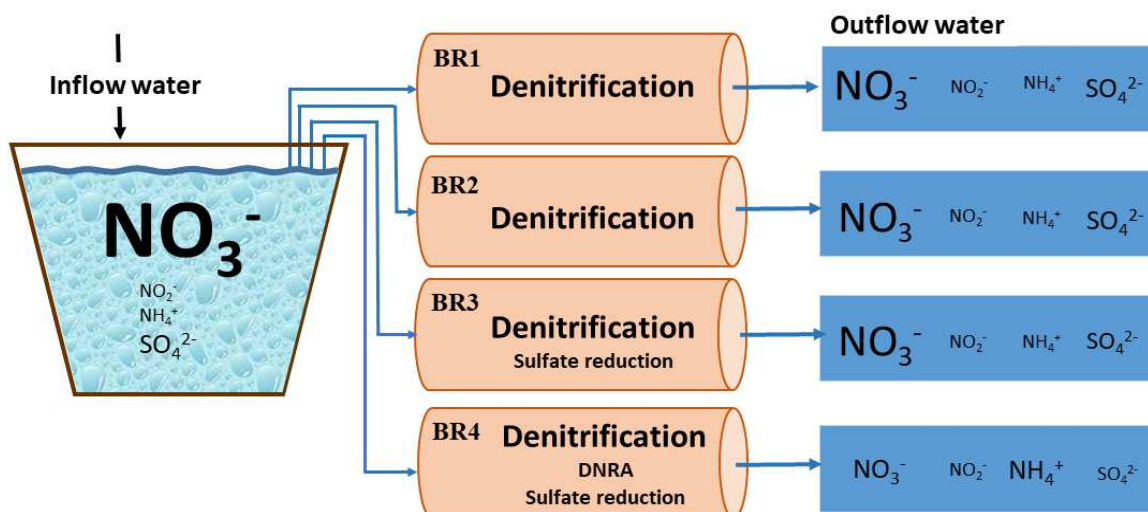
373 Functions related to denitrification (NarG, NapA, NirK, NorB, NorC, NosZ) and DNRA (NarG, NapA,
 374 NrfA) were predicted, while functions specific to nitrification (AmoA, AmoB, AmoC) were not predicted.
 375 The membrane-bound nitrate reductase NarG was predicted in similar relative abundance in 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 NO_3^- removal in the
 391 four bioreactors. The observed high rate of 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 CaCO_3) per mg 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 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.

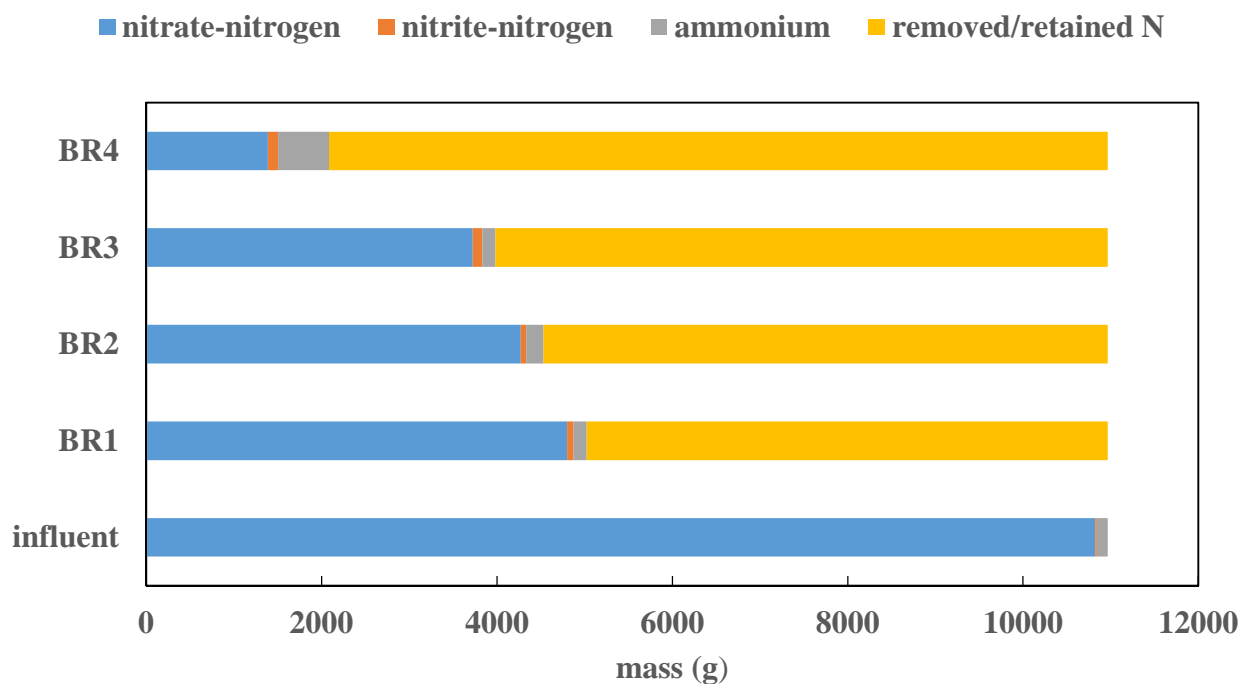


403

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 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 NO_3^- -N per day. When the measured annual NO_3^- removal rates were
435 used, required volume was calculated to be 138-183 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 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 NO_3^- removal rate in woodchip bioreactors, potato residues
441 enabled more stable 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 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 NO_3^- removal in treating land-based RAS effluent, without NH_4^+ -N
452 and NO_2^- -N production that are harmful in aquaculture. Of the additional carbon sources tested, higher NO_3^-
453 removal was achieved with industrial potato residues than with biochar or *Sphagnum* moss and higher inflow
454 concentrations of 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 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 NO_3^-
465 concentrations compared to other nitrogen compounds (NH_4^+ and NO_2^-).

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References

- Addy, K., Gold, A.J., Christianson, L.E., David, M.B., Schipper, L.A., Ratigan, N.A., 2016. Denitrifying Bioreactors for Nitrate Removal: A Meta-Analysis. *J. Environ. Qual.* 45, 873. <https://doi.org/10.2134/jeq2015.07.0399>
- Beutel, M.W., Duvil, R., Cubas, F.J., Matthews, D.A., Wilhelm, F.M., Grizzard, T.J., Austin, D., Horne, A.J., Gebremariam, S., 2016. A review of managed nitrate addition to enhance surface water quality. *Crit. Rev. Environ. Sci. Technol.* 1–28. <https://doi.org/10.1080/10643389.2016.1151243>
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci.* 108, 4516–4522.
- Cherchi, C., Onnis-Hayden, A., El-Shawabkeh, I., Gu, A.Z., 2009. Implication of using different carbon sources for denitrification in wastewater treatments. *Water Environ. Res.* 81, 788–799.
- David, M.B., Flint, C.G., Gentry, L.E., Dolan, M.K., Czapar, G.F., Cooke, R.A., Lavaire, T., 2015. Navigating the Socio-Bio-Geo-Chemistry and Engineering of Nitrogen Management in Two Illinois Tile-Drained Watersheds. *J. Environ. Qual.* 44, 368–381. <https://doi.org/10.2134/jeq2014.01.0036>
- Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K., Stein, O.R., 2009. Microbial processes influencing performance of treatment wetlands: a review. *Ecol. Eng.* 35, 987-1004. <https://doi.org/10.1016/j.ecoleng.2008.12.030>
- Finneran, K.T., Johnsen, C.V., Lovley, D.R., 2003. *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *Int. J. Syst. Evol. Microbiol.* 53, 669–673.

- Gibert, O., Pomierny, S., Rowe, I., Kalin, R.M., 2008. Selection of organic substrates as potential reactive materials for use in a denitrification permeable reactive barrier (PRB). *Bioresour. Technol.* 99, 7587–7596. <https://doi.org/10.1016/j.biortech.2008.02.012>
- Greenan, C.M., Moorman, T.B., Kaspar, T.C., Parkin, T.B., Jaynes, D.B., 2006. Comparing Carbon Substrates for Denitrification of Subsurface Drainage Water. *J. Environ. Qual.* 35, 824. <https://doi.org/10.2134/jeq2005.0247>
- Hashemi, S.E., Heidarpour, M., Mostafazadeh-Fard, B., 2011. Nitrate removal using different carbon substrates in a laboratory model. *Water Sci. Technol.* 63, 2700.
- Hassanpour, B., Giri, S., Puer, W.T., Steenhuis, T.S., Geohring, L.D., 2017. Seasonal performance of denitrifying bioreactors in the Northeastern United States: Field trials. *J. Environ. Manage.* 202, 242–253. <https://doi.org/10.1016/j.jenvman.2017.06.054>
- Herbert, R.B., Winbjörk, H., Hellman, M., Hallin, S., 2014. Nitrogen removal and spatial distribution of denitrifier and anammox communities in a bioreactor for mine drainage treatment. *Water Res.* 66, 350–360. <https://doi.org/10.1016/j.watres.2014.08.038>
- Hoover, N.L., Bhandari, A., Soupir, M.L., Moorman, T.B., 2016. Woodchip denitrification bioreactors: Impact of temperature and hydraulic retention time on nitrate removal. *J. Environ. Qual.* 45, 803–812.
- Jafari, S.J., Moussavi, G., Yaghmaeian, K., 2015. High-rate biological denitrification in the cyclic rotating-bed biological reactor: Effect of COD / NO₃⁻, nitrate concentration and salinity and the phylogenetic analysis of denitrifiers. *Bioresour. Technol.* 197, 482–488. <https://doi.org/10.1016/j.biortech.2015.08.047>
- Jong, T., Parry, D.L., 2006. Microbial sulfate reduction under sequentially acidic conditions in an upflow anaerobic packed bed bioreactor. *Water Res.* 40, 2561-2571.

- Kraft, B., Tegetmeyer, H.E., Sharma, R., Klotz, M.G., Ferdelman, T.G., Hettich, R.L., Geelhoed, J.S., Strous, M., 2014. The environmental controls that govern the end product of bacterial nitrate respiration. *Science* 345, 676–679.
- Kroupova, H., Machova, J., Svobodova, Z., 2005. Nitrite influence on fish: a review. *Vet. Med.-Praha* 50, 461.
- Kruse, S., Goris, T., Westermann, M., Adrian, L., Diekert, G., 2018. Hydrogen production by *Sulfurospirillum* species enables syntrophic interactions of Epsilonproteobacteria. *Nat. Commun.* 9, 4872. <https://doi.org/10.1038/s41467-018-07342-3>
- Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V., Knight, R., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814.
- Lepine, C., Christianson, L., Sharrer, K., Summerfelt, S., 2016. Optimizing Hydraulic Retention Times in Denitrifying Woodchip Bioreactors Treating Recirculating Aquaculture System Wastewater. *J. Environ. Qual.* 45, 813. <https://doi.org/10.2134/jeq2015.05.0242>
- Liang, S., McDonald, A.G., 2014. Chemical and thermal characterization of potato peel waste and its fermentation residue as potential resources for biofuel and bioproducts production. *J. Agric. Food Chem.* 62, 8421–8429.
- Lu, W.-W., Zhang, H.-L., Shi, W.-M., 2013. Dissimilatory nitrate reduction to ammonium in an anaerobic agricultural soil as affected by glucose and free sulfide. *Eur. J. Soil Biol.* 58, 98–104.
- Mergaert, J., Cnockaert, M.C., Swings, J., 2003. *Thermomonas fusca* sp. nov. and *Thermomonas brevis* sp. nov., two mesophilic species isolated from a denitrification reactor with poly(ϵ -caprolactone) plastic granules as fixed bed, and emended description of the genus *Thermomonas*. *Int. J. Syst. Evol. Microbiol.* 53, 1961–1966.

- Narkis, N., Rebhun, M., Sheindorf, C.H., 1979. Denitrification at various carbon to nitrogen ratios. *Water Res.* 13, 93–98.
- Natural Resources Institute Finland [referred 26.06.2019]. Access method:
https://stat.luke.fi/en/aquaculture-2016_en
- Niemi, J., Raateland, A., 2007. River water quality in the Finnish Eurowaternet. *Boreal Environ. Res.* 12, 571–84.
- Nordström, A., Herbert, R.B., 2018. Determination of major biogeochemical processes in a denitrifying woodchip bioreactor for treating mine drainage. *Ecol. Eng.* 110, 54–66.
<https://doi.org/10.1016/j.ecoleng.2017.09.018>
- Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O’Hara, R.B., Simpson, G.L., Solymos, P., 2017. *vegan: Community Ecology Package 2017*. R package version 2.4–4.
- Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414.
- Prairie, Y.T., 2008. Carbocentric limnology: looking back, looking forward. *Can. J. Fish. Aquat. Sci.* 65, 543–548. <https://doi.org/10.1139/f08-011>
- Pulkkinen, J.T., Kiuru, T., Aalto, S.L., Koskela, J., Vielma, J., 2018. Startup and effects of relative water renewal rate on water quality and growth of rainbow trout (*Oncorhynchus mykiss*) in a unique RAS research platform. *Aquac. Eng.* 82, 38–45.
<https://doi.org/10.1016/j.aquaeng.2018.06.003>
- Robertson, W.D., 2010. Nitrate removal rates in woodchip media of varying age. *Ecol. Eng.* 36, 1581–1587. <https://doi.org/10.1016/j.ecoleng.2010.01.008>
- Rocher, V., Laverman, A.M., Gasperi, J., Azimi, S., Guérin, S., Mottelet, S., Villières, T., Paus, A., 2015. Nitrite accumulation during denitrification depends on the carbon quality and

quantity in wastewater treatment with biofilters. *Environ. Sci. Pollut. Res.* 22, 10179–10188.

<https://doi.org/10.1007/s11356-015-4196-1>

Ronkanen, A.K. and Kløve, B., 2005. Hydraulic soil properties of peatlands treating municipal wastewater and peat harvesting runoff. *Suo.* 56(2), 43-56.

Salvetti, E., Torriani, S., Felis, G.E., 2012. The Genus *Lactobacillus*: A Taxonomic Update.

Probiotics Antimicrob. Proteins 4, 217–226. <https://doi.org/10.1007/s12602-012-9117-8>

Schipper, L.A., Robertson, W.D., Gold, A.J., Jaynes, D.B., Cameron, S.C., 2010. Denitrifying bioreactors—An approach for reducing nitrate loads to receiving waters. *Ecol. Eng.* 36, 1532–1543. <https://doi.org/10.1016/j.ecoleng.2010.04.008>

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Env. Microbiol* 75, 7537–7541.

Solomon, C.T., Jones, S.E., Weidel, B.C., Buffam, I., Fork, M.L., Karlsson, J., Larsen, S., Lennon, J.T., Read, J.S., Sadro, S., Saros, J.E., 2015. Ecosystem Consequences of Changing Inputs of Terrestrial Dissolved Organic Matter to Lakes: Current Knowledge and Future Challenges. *Ecosystems* 18, 376–389. <https://doi.org/10.1007/s10021-015-9848-y>

Stasko, A.D., Gunn, J.M., Johnston, T.A., 2012. Role of ambient light in structuring north-temperate fish communities: potential effects of increasing dissolved organic carbon concentration with a changing climate. *Environ. Rev.* 20, 173–190.
<https://doi.org/10.1139/a2012-010>

Tangsir, S., Moazed, H., Naseri, A.A., Hashemi Garmdareh, S.E., Broumand-nasab, S., Bhatnagar, A., 2017. Investigation on the performance of sugarcane bagasse as a new carbon source in two hydraulic dimensions of denitrification beds. *J. Clean. Prod.* 140, 1176–1181.
<https://doi.org/10.1016/j.jclepro.2016.10.044>

- van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems: Theory and applications. *Aquac. Eng.* 34, 364–376. <https://doi.org/10.1016/j.aquaeng.2005.04.004>
- von Ahnen, M., Aalto, S.L., Suurnäkki, S., Tirola, M., Pedersen, P.B., 2019. Salinity affects nitrate removal and microbial composition of denitrifying woodchip bioreactors treating recirculating aquaculture system effluents. *Aquaculture* 504, 182–189.
- von Ahnen, M., Pedersen, P.B., Dalsgaard, J., 2018. Performance of full-scale woodchip bioreactors treating effluents from commercial RAS. *Aquac. Eng.* 83, 130–137. <https://doi.org/10.1016/j.aquaeng.2018.10.004>
- von Ahnen, M., Pedersen, P.B., Dalsgaard, J., 2016a. Start-up performance of a woodchip bioreactor operated end-of-pipe at a commercial fish farm—A case study. *Aquac. Eng.* 74, 96–104. <https://doi.org/10.1016/j.aquaeng.2016.07.002>
- von Ahnen, M., Pedersen, P.B., Hoffmann, C.C., Dalsgaard, J., 2016b. Optimizing nitrate removal in woodchip beds treating aquaculture effluents. *Aquaculture* 458, 47–54. <https://doi.org/10.1016/j.aquaculture.2016.02.029>
- Wang, J., Chu, L., 2016. Biological nitrate removal from water and wastewater by solid-phase denitrification process. *Biotechnol. Adv.* 34, 1103–1112. <https://doi.org/10.1016/j.biotechadv.2016.07.001>
- Warneke, S., Schipper, L.A., Matiasek, M.G., Scow, K.M., Cameron, S., Bruesewitz, D.A., McDonald, I.R., 2011. Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. *Water Res.* 45(17), 5463–5475.
- Westcott, S.L., Schloss, P.D., 2017. OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. *MSphere* 2, e00073-17.
- Xu, Z., Dai, X., Chai, X., 2018. Effect of different carbon sources on denitrification performance, microbial community structure and denitrification genes. *Sci. Total Environ.* 634, 195–204

- Zhang, H., Jiang, J., Li, M., Yan, F., Gong, C., Wang, Q., 2016. Biological nitrate removal using a food waste-derived carbon source in synthetic wastewater and real sewage. *J. Environ. Manage.* 166, 407–413. <https://doi.org/10.1016/j.jenvman.2015.10.037>
- Zhang, J., Feng, C., Hong, S., Hao, H., Yang, Y., 2012. Behavior of solid carbon sources for biological denitrification in groundwater remediation. *Water Sci. Technol.* 65, 1696–1704. <https://doi.org/10.2166/wst.2012.070>
- Zhao, J., Feng, C., Tong, S., Chen, N., Dong, S., Peng, T., Jin, S., 2018. Denitrification behavior and microbial community spatial distribution inside woodchip-based solid-phase denitrification (W-SPD) bioreactor for nitrate-contaminated water treatment. *Bioresour. Technol.* 249, 869–879. <https://doi.org/10.1016/j.biortech.2017.11.011>

- Woodchip bioreactors removed 31-38 g NO₃⁻-N m⁻³ d⁻¹ 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: