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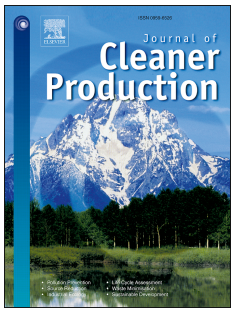

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PII: S0959-6526(20)30020-2

DOI: https://doi.org/10.1016/j.jclepro.2020.119973

Reference: JCLP 119973

To appear in: *Journal of Cleaner Production*

Received Date: 30 August 2019

Revised Date: 12 December 2019

Accepted Date: 2 January 2020

Please cite this article as: Kiani S, Kujala K, Pulkkinen J, Aalto SL, Suurnäkki S, Kiuru T, Tiirola M, Kløve Bjø, Ronkanen A-K, Enhanced nitrogen removal of low carbon wastewater in denitrification bioreactors by utilizing industrial waste toward circular economy, *Journal of Cleaner Production* (2020), doi: https://doi.org/10.1016/j.jclepro.2020.119973.

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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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Abstract

Aquaculture needs practical solutions for nutrient removal to achieve sustainable fish production. Passive denitrifying bioreactors may provide an ecological, low-cost and low-maintenance approach for wastewater nitrogen removal. However, innovative organic materials are needed to enhance nitrate removal from the low carbon effluents in intensive recirculating aquaculture systems (RAS). In this study, we tested three additional carbon sources, including biochar, dried *Sphagnum* sp. moss and industrial potato residues, to enhance the performance of woodchip bioreactors treating the low carbon RAS wastewater. We assessed nitrate (NO_3^-) removal and microbial community composition during a one-year *in situ* column test with real aquaculture wastewater. We found no significant differences in the NO_3^- removal rates between the woodchip-only bioreactor and bioreactors with a zone of biochar or *Sphagnum* sp. moss (maximum removal rate 31-33 g NO_3^--N m^{-3} d^{-1}), but potato residues increased NO_3^- removal rate to 38 g NO_3^--N m^{-3} d^{-1}, with stable annual reduction efficiency of 93%. The readily available carbon released from potato residues increased NO_3^--N removal capacity of the bioreactor even at higher inflow concentrations (>52 mg L^{-1}). The microbial community and its predicted functional potential in the potato residue bioreactor differed markedly from those of the other bioreactors. Adding potato residues to woodchip material enabled smaller bioreactor size to be used for NO_3^- removal. This study introduced industrial potato by-product as an alternative carbon source for the woodchip denitrification process, and the encouraging results may pave the way toward growth of blue bioeconomy using the RAS.

Keywords: Recirculating aquaculture system, woodchip bioreactor, carbon source, potato residues, nitrate, microbial community

1 Introduction

Recirculating aquaculture systems (RAS) are environmentally friendly solutions that aim to achieve zero waste from fish production. Although RAS have been used for more than 10 years in different countries, including two largest RAS in Finland with a production capacity of over 4000 tons, nitrate (NO_3^-) removal is still a critical challenge (Pulkkinen et al., 2018). Removal of NO_3^- is a challenge as aquaculture wastewater has low carbon (C) but high nitrogen (N) concentrations. A few previous studies have examined the use of denitrifying bioreactors for treating aquaculture effluent. So far, such studies have focused on RAS effluents with high chemical oxygen demand (COD) (Lepine et al., 2016), added bicarbonate (HCO^-_3) to inlet water (von Ahnen et al., 2016b) and diluted effluent from an outdoor fish farm with low recirculation intensity and low NO_3^--N concentration (~6 mg L^{-1}) (von Ahnen et al., 2018, 2016a). In contrast, treatment of highly intensive indoor RAS effluents with low COD (12.9 ± 1.8 mg L^{-1}) and high NO_3^--N concentration (>50 mg L^{-1}) has received little attention.

In denitrifying bioreactors, nitrogen (N) is removed by heterotrophic denitrifiers converting NO_3^- to nitrogen gas under anoxic conditions. Under nitrate-rich conditions, this process depends on the availability of the carbon source as the organic electron donor (Wang and Chu, 2016). External carbon sources, such as acetate or methanol, are often supplied to the system to achieve efficient denitrification (Cherchi et al., 2009). However, the cost of carbon addition is typically high (Zhang et al., 2016) and the process needs regulation to prevent over- or under-dosing of the liquid carbon sources (Rocher et al., 2015). Solid carbon sources can provide a cost-effective alternative to the classical carbon sources mentioned above. In recent years, research has focused on solid carbon sources with high quality, optimal efficiency and slow-release ability in the treatment of excessively nitrate-contaminated water, particularly surface water (Beutel et al., 2016) and groundwater (Zhang et al., 2012). Wood-particle products (e.g. woodchip and sawdust) have been widely used, due to their ability to supply carbon to the denitrification process for 5-15 years and thus allow good NO_3^- removal with minimum bioreactor maintenance (Schipper et al., 2010). However, the large space requirement for full-scale woodchip bioreactors has prompted efforts to enhance the denitrification rate by using innovative natural carbon sources (Tangsir et al., 2017). Inexpensive industrial food by-products, such

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

This study examined the use of a denitrifying bioreactor to treat indoor intensive RAS effluent with low COD and high NO_3^- concentration, as part of the unique RAS research platform (see Pulkkinen et al., 2018), and compared different carbon sources, including potato residue, for improving the nitrogen removal performance of woodchip bioreactors. The overall aim was to evaluate the performance of denitrifying bioreactors in removing NO_3^- from aquaculture wastewater with low COD for a period of over one year. Specific objectives were to (1) study the suitability of wood-based bioreactors for treating RAS effluent, (2) assess whether the NO_3^- removal performance of woodchip process can be enhanced by additional carbon sources, (3) to assess the effect of different carbon sources on the microbial community composition in different compartments of the bioreactors, and (4) to identify dominant bacteria and their functional potential in the bioreactors studied. The intention was to find solutions for improving water treatment and for enhancing NO_3^- removal in the recirculating aquaculture systems.

2 Material and methods

2.1 RAS effluent water quality

The study was conducted at the Laukaa fish farm of the Natural Resources Institute Finland (LUKE) in central Finland, in the research platform examining RAS. The RAS design is described in detail in Pulkkinen et al. (2018). In brief, effluent was obtained from a RAS consisting of a feed collector unit, swirl separator, drum filter (60 µm mesh) and fixed bed bioreactor, followed by a moving bed bioreactor and a trickling filter. In order to prevent any changes in water chemistry, microbiology or water temperature, all tests were performed using the natural RAS effluent. The effluent is characterised by low carbon (15.3 mg L^{-1} on average), but high N content (mean NO_3^--N content 34.7 mg L^{-1}) (Table 1). Due to the efficient nitrification unit before the bioreactors, NO_3^- is dominating N fraction.

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

Water quality parameters	Inflow (mean ± SD)	n
Total organic carbon (mg L^{-1})	15.3 ± 2.1	5
Dissolved organic carbon (mg L^{-1})	14 ± 1.3	5
Chemical oxygen demand (mg L^{-1})	12.9 ± 1.8	5
Biological oxygen demand (mg L^{-1})	3.8 ± 2.2	13
Nitrate-nitrogen (mg L^{-1})	34.7 ± 15.6	27
Nitrite-nitrogen (mg L^{-1})	0.1 ± 0.06	30
Ammonium-nitrogen (mg L^{-1})	0.5 ± 0.2	30
Dissolved oxygen (mg L^{-1})	8.1 ± 1.7	29
pH	6.9 ± 0.2	28
Oxidation-reduction potential (Eh, mV)	178.6 ± 60.4	35
Alkalinity (mg $CaCO_3$ L^{-1})	54.2 ± 18	25
Sulphate (mg L^{-1})	10.5 ± 3.2	24

2.2 Bioreactor design

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

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

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

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

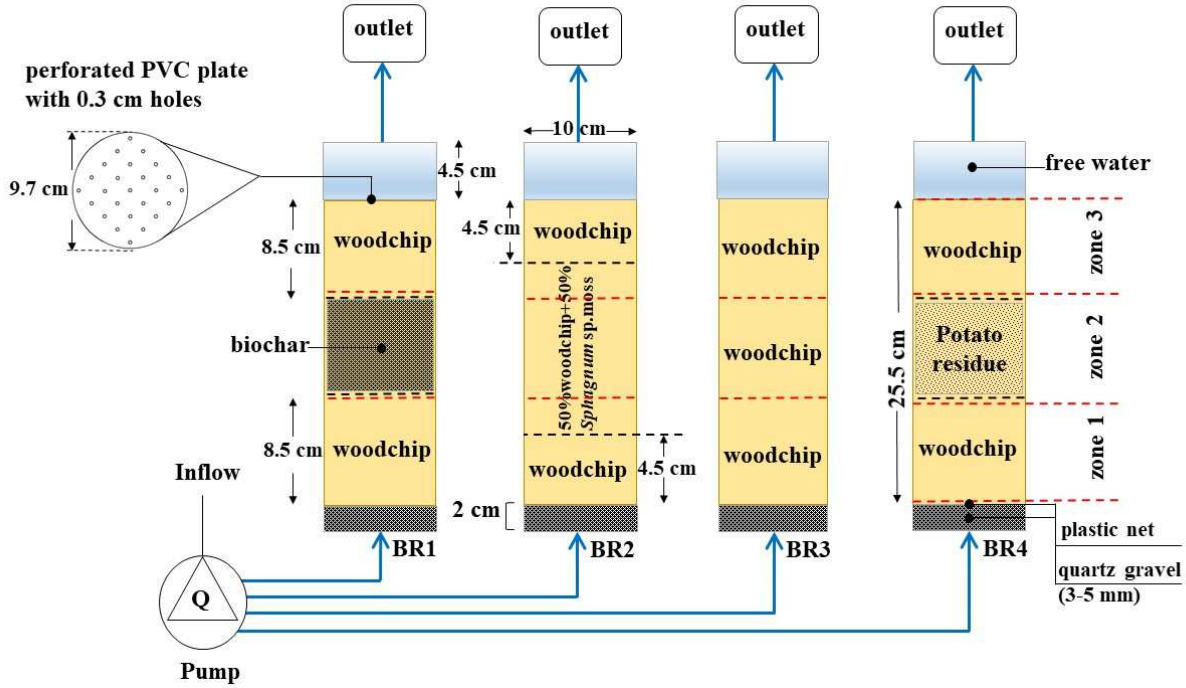


Fig. 1. Schematic diagram of the bioreactor set-up (bioreactors BR1-BR4). Red and black dashed lines represent microbiological sampling zones and packed media zones in the bioreactors, respectively. Zones 1 and 3 were packed with woodchips, while zone 2 was packed with biochar in BR1, *Sphagnum* sp.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

2.3 Sampling and analysis

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

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

All samples were analysed on-site for nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), ammonium-nitrogen (NH_4^+-N), sulphate (SO_4^{2-}) and biological oxygen demand (BOD_5), using LCK cuvette tests (Hach Lange DS 3900). Alkalinity was analysed by titration with the standard method (ISO 9963-1:1994) (Hach Lange TitraLab AT1000). The concentration of COD, dissolved organic carbon (DOC) and total organic carbon (TOC) in the first 70 days were determined by an accredited laboratory. Dissolved oxygen (DO) was recorded manually with a YSI ProODO meter and redox potential (Eh), pH and temperature with a Horiba Laqua act D-74 meter.

Flow rate (Q) was calculated by dividing the selected HRT (48 h) by the pore volume of the column (1650 mL). Pore volume of each column was determined by measuring added water until saturation conditions were achieved. Volumetric NO_3^--N removal rate (g NO_3^--N m^{-3} d) was calculated based on differences between bioreactor inlet and outlet NO_3^--N concentration, the flow rate and the pore volume of the packed-media zone. Removal efficiency was calculated by dividing the difference between inlet and outlet concentration by the inlet concentration. The calculated mass was based on sampling interval, flow rate and concentration.

2.4 Molecular analyses

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

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

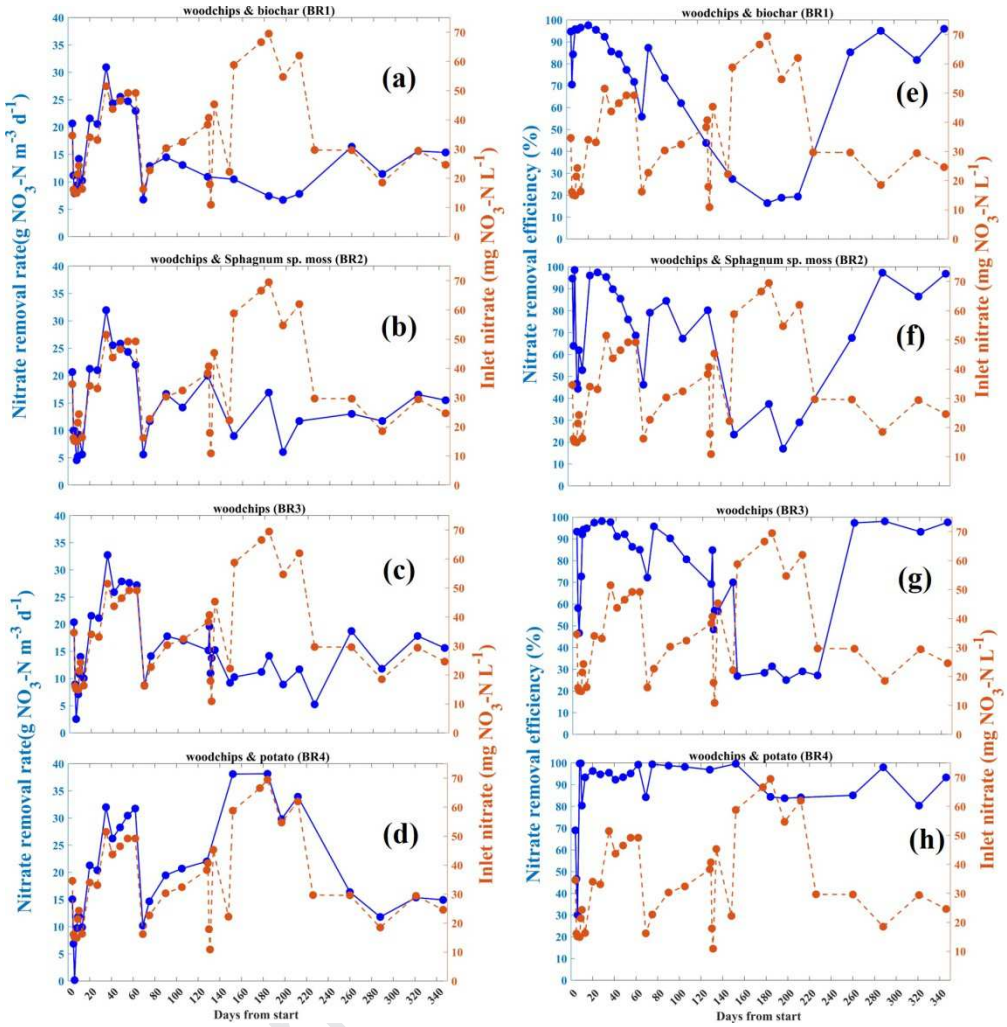
Sequence analysis was performed using the analysis pipelines mothur v.1.39.5 (Schloss et al., 2009) and qiime 1.9 (Caporaso et al., 2011). Sequences with incorrect primer (>1 bp) or barcode (>1 bp) sequences were removed, as were sequences <150 bp and chimeric sequencing. After quality filtering, sequences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

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

3 Results and discussion

3.1 Performance of bioreactors

The initial inflow NO_3^--N concentration of the bioreactors ranged from 15 to 70 mg L^{-1}, while the outflow concentrations were clearly lower (ranging from the detection limit of 0.03 to 58.1 mg L^{-1}) (Fig. 2). All bioreactors showed effective NO_3^- removal ability immediately upon start-up and over the whole study period (Fig. 2, Fig. 3). Instant NO_3^- removal by wood-based bioreactors in aquaculture effluent has also been observed in previous studies (e.g. Lepine et al., 2016; von Ahnen et al., 2016a). Over the one-year bioreactor operating period (number of samplings n = 26), NO_3^--N comprised 98±0.1 % (mean ± SD) of total dissolved inorganic nitrogen in inflow water, while only minor amounts of NH_4^+-N (1.6±0.8%) and NO_2^--N (0.27±0.22%) were present. For the entire study period, total inflow NO_3^--N mass to the bioreactors was 10.8 kg, of which 6.0, 6.6, 7.1 and 9.4 kg were removed in BR1, BR2, BR3 and BR4, respectively (Fig. 3). During the first 197 days, BR4 (industrial potato residues in zone 2) showed stable removal of 96% for total NO_3^--N (amounting to a removed nitrogen mass of 7.1 kg). After 107 days the removal efficiency decreased and was around 87% from day 260 onwards (Fig. 2, Fig. 3). The other bioreactors also showed decreased NO_3^- removal efficiencies from day 130-160 to day 260 (30%). From day 260 onwards, the removal efficiency in BR3 then increased to the original level (Fig. 2). However, the total accumulated outflow NO_3^--N mass for BR3 was higher than in BR4 when considering the whole study period (Fig. 3).



189 **Fig. 2.** Nitrate-nitrogen (NO_3^--N**)** removal rate (a-d) and removal efficiency (e-h) in bioreactors during the

190 346-day study period.

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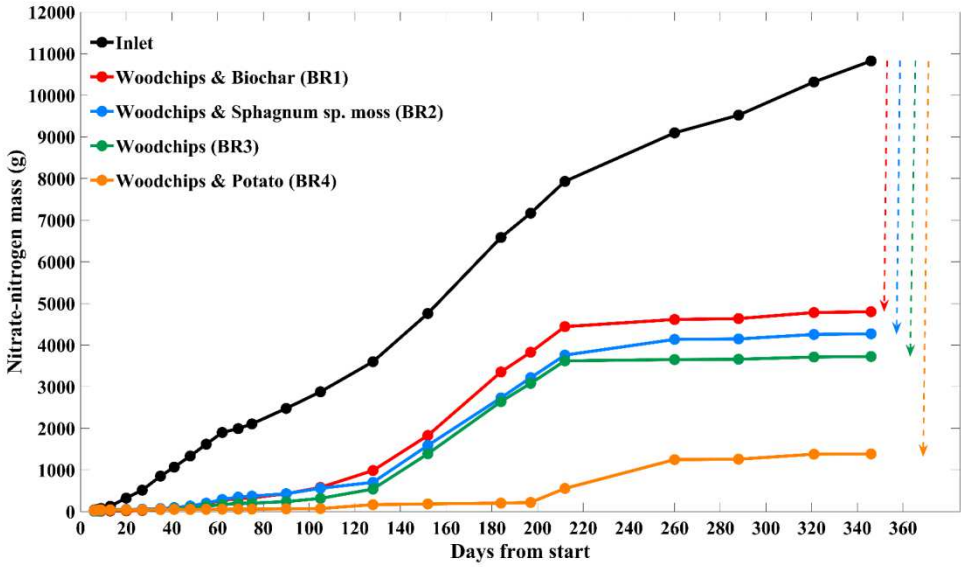


193 **Fig. 3**. Accumulated nitrate-nitrogen mass in inflow and outflow of bioreactors BR1-BR4 during the 346-day
194 study period. Dashed lines indicate total nitrate-nitrogen mass removed from bioreactors during the period.

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 NO_2^--N concentration in inflow water remained stable, at a level of 0.1±0.06 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

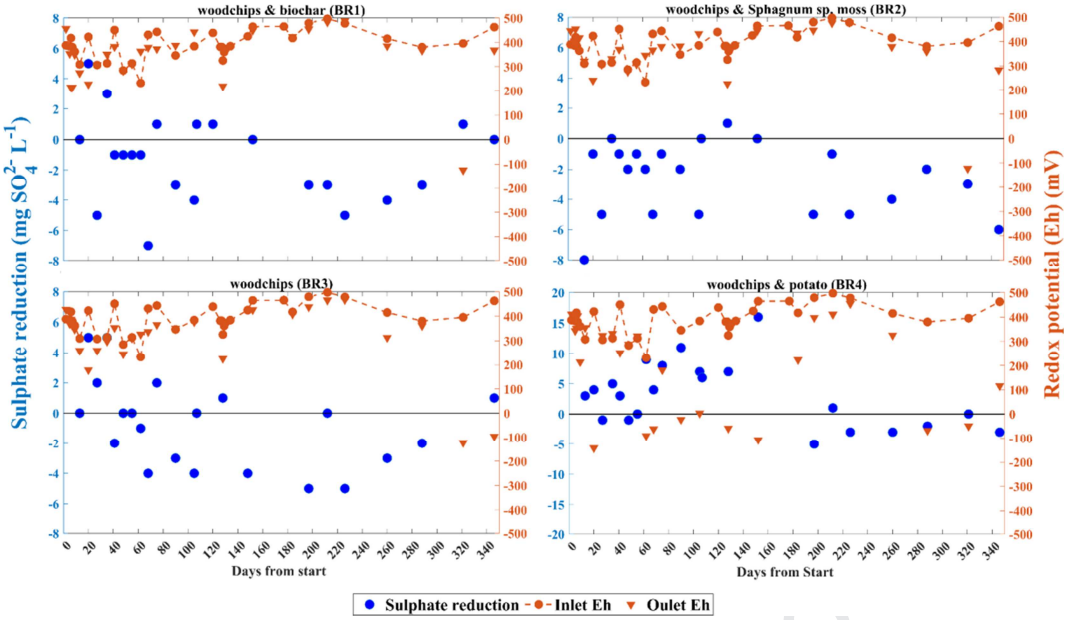
start, but only after stable denitrification rates are established and low nitrite concentrations are detected in the outflow.

The inflow NH_4^+-N concentration ranged between 0.17-1.0 mg L^{-1} (Table 1; Fig. S1b). Low NH_4^+-N production was detected in all bioreactors, with outflow concentrations of 0.8±0.5 mg L^{-1}, 0.9±0.5 mg L^{-1}, 0.9±0.6 mg L^{-1} and 3.8±3.4 mg L^{-1} in BR1, BR2, BR3 and BR4, respectively. Less than 2 mg L^{-1} of NH_4^+-N was recorded in the first three weeks in BR1-BR3 (Fig. S1b). However, the bioreactor with potato residues (BR4) showed relatively high NH_4^+-N, with a mean concentration of 10 mg L^{-1}, in the first 10 days of the experiment, but it then declined to lower than 4 mg L^{-1} to reach the background level. The continuous production of ammonium in BR4 indicates the occurrence of dissimilarity nitrate reduction to ammonium (DNRA). In general, a reducing environment and high TOC/NO_3^- ratio (1400/15-110/16 in BR4; days 1-70) can indicate the occurrence of DNRA (Kraft et al., 2014; van Rijn et al., 2006). DNRA has also been observed in previous woodchip bioreactor studies (Lu et al., 2013; Zhao et al., 2018). Reducing conditions, indicated by Eh values, were also seen in this study, which led the system to SO_4^{2-} reduction (Fig. 4).

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

changes (Prairie, 2008; Solomon et al., 2015), resulting in lower fish production (Stasko et al., 2012). Hence, at sites governed by strict regulations or when recycling outflow to fish farms, high DOC might need to be controlled. Schipper et al. (2010) identified HRT as a factor controlling the initial magnitude of DOC depletion and its duration in wood-based bioreactors. However, the fact that carbon was more readily released from potato residues than from the other carbon sources used in this study proves that HRT is not the only controlling factor and that carbon quality also plays a key role. In the present study, there was significantly lower outflow DOC concentration of 53, 68 and 81 mg L^{-1} in bioreactors BR1, BR2 and BR3, which can be partly explained by higher nitrate loading (Hassanpour et al., 2017) and partly by the type of carbon source used. Dependence of TOC leaching and variations in NO_3^--N concentration have also been reported by Zhao et al. (2018). In order to control the carbon content due to leaching, it is recommended to consider post-bioreactors treatment units (e.g. constructed wetland, sand filter) or recirculating the start-up effluent back to the bioreactor (Schipper et al., 2010).

The SO_4^{2-} concentrations were on average higher in the outflow than in the inflow waters of BR1 and BR2, indicating leaching or production of SO_4^{2-} (Fig. 4). This resulted in cumulative leaching/production of 165 g and 474 g SO_4^{2-} in BR1 and BR2, respectively, for the whole study period. In contrast, SO_4^{2-} were on average lower in outflow than in inflow waters of BR3 and BR4 (Fig. 4), indicating SO_4^{2-} reduction/removal. Cumulative SO_4^{2-} removal of 350 g and 546 g was observed in BR3 and BR4, respectively, for the whole study period. SO_4^{2-} leaching/removal increased the SO_4^{2-} concentration in the outflow by up to 20% compared with the cumulative inflow SO_4^{2-} of 2.6 kg. Sulphate leaching/production indicated the potential of internal sulphur cycling in bioreactors with incomplete N removal. BR1 and BR2 had incomplete nitrate removal during the study period due to sulphide re-oxidation to sulphate by sulphur oxidizing bacteria (SOB), which can use oxygen or nitrate as electron acceptor (Faulwetter et al., 2009) (Fig. S1 and Fig. 3). Sulphate production was observed previously by Lepine et el. (2016) for a woodchip bioreactor with incomplete N removal. However, higher nitrate removal in BR3 and BR4 combined with their reduced 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.

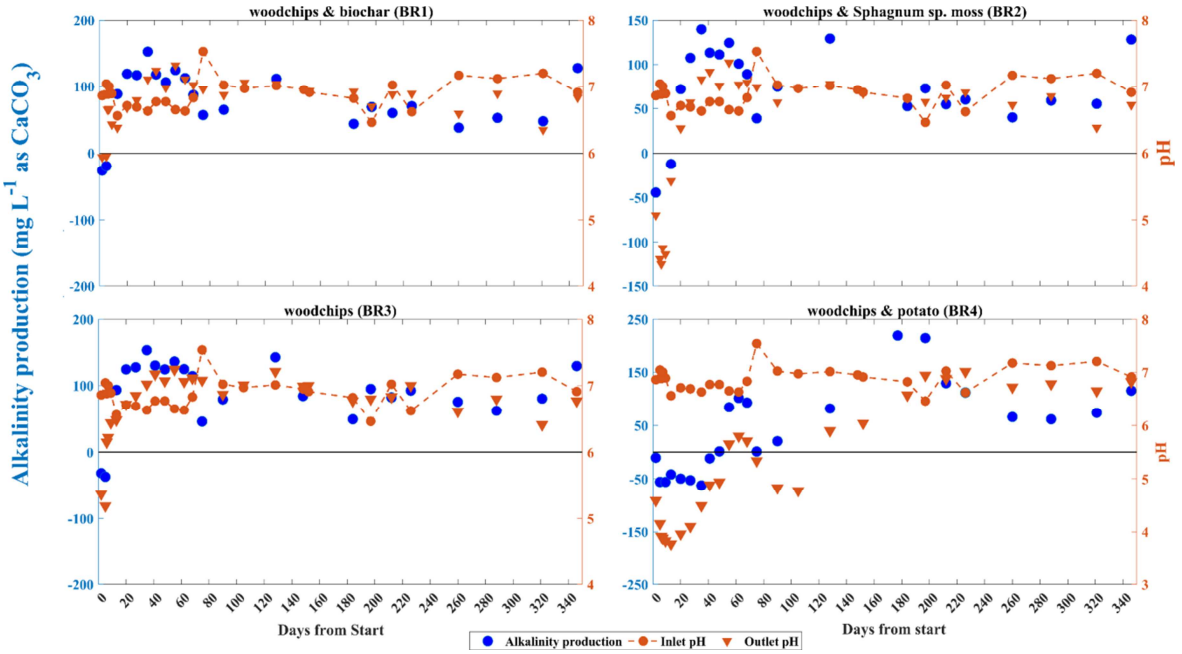


Fig. 5. Alkalinity production (+values) and inflow and outflow pH in bioreactors (BR1-BR4).

3.2 Factors affecting nitrate removal in woodchip bioreactors

The results of one-way ANOVA showed that NO_3^- removal rates for whole study period did not differ significantly between BR1, BR2 and BR 3 (p=0.75), while nitrate removal in BR4 was higher (Fig. 2d-2h). In the first three months of the experiment, when inflow NO_3^--N concentration varied between 15 and 52 mg L^{-1}, all bioreactors showed similar removal rates (Fig. 2). After that, the bioreactors responded differently to increasing NO_3^--N inflow concentrations, e.g. the removal rate declined in BR1-BR3 but increased in BR4 (Fig. 2). BR4 reached its maximum removal rate of 38 g NO_3^--N m^{-3} d^{-1} at the highest NO_3^--N inflow concentration (70 mg L^{-1}; days 152-184), whereas BR1, BR2 and BR3 had a removal rate of 9, 13 and 12 g NO_3^--N m^{-3} d^{-1}, respectively (Fig. 2). Those differences persisted until day 250, after which all reactors again had similar stable removal rates of around 15 g NO_3^--N m^{-3} d^{-1} until the end of the experiment. Similarly to removal rate, the NO_3^- removal efficiency in BR1-BR3 showed fluctuations throughout the study period (Fig. 2e and 2g). However, BR4 reached stable removal efficiency of 93% after a period of fluctuation at start-up (Fig. 2h).

The wide range of NO_3^- removal rates (3-38 g NO_3^--N m^{-3} d^{-1}) recorded in all bioreactors followed the NO_3^--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 × 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 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 (55-70 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 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

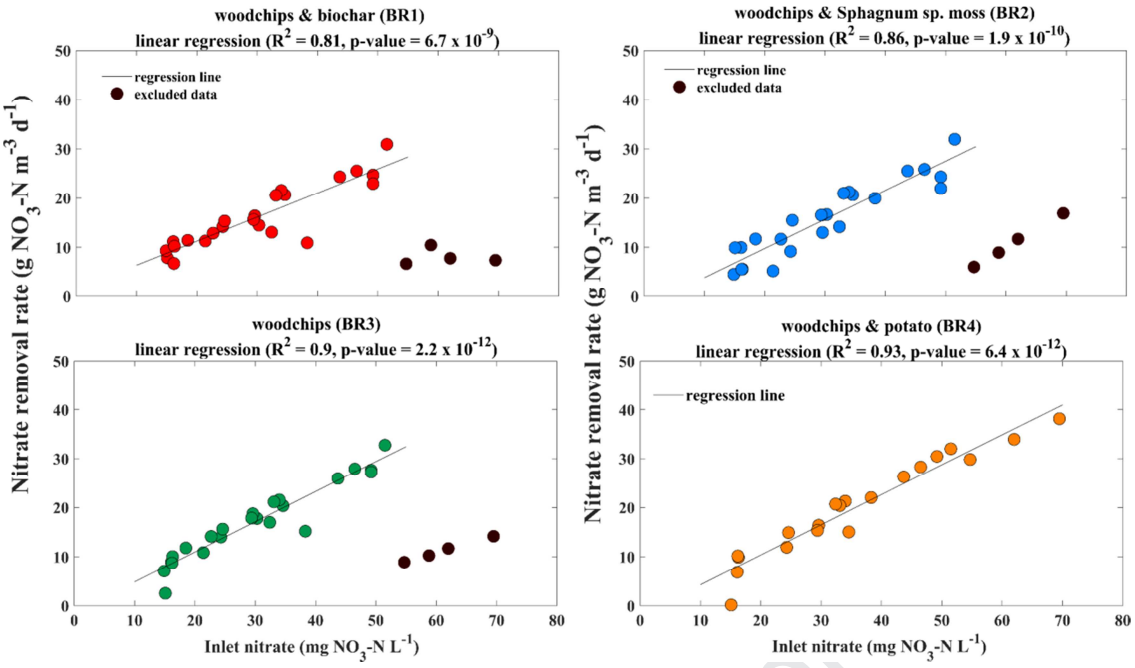


Fig. 6. Nitrate removal rate versus nitrate influent loading in BR1-4 for the study period of 346 days.

The maximum NO_3^- removal rates observed in this study were greater than those previously reported (22 g NO_3^--N m^{-3} d^{-1}) (David et al., 2015; Schipper et al., 2010). This could be due to a combination of optimal factors: sufficient HRT (Lepine et al., 2016; Tangsir et al., 2017) as a result of distributed upward flow (section 2.2) combined with high NO_3^- inflow concentration (Schipper et al., 2010), the organic C compounds used (Gibert et al., 2008) and water temperature (Addy et al., 2016), here 15.5 ± 1 °C (mean ± SD). A removal rate of >39 g NO_3^- m^{-3} d^{-1} reported by Lepine et al. (2016) for comparable water quality was associated with high COD:NO_3^- ratio (0.86-1.66) in treated wastewater. This ratio can provide 42% COD required for denitrification. The COD:NO_3^- ratio has been reported to be a significant parameter affecting denitrification in bioreactors (Jafari et al., 2015). However, in the present study inflow COD provided less than 8% of the C/N required for complete NO_3^- reduction (Narkis et al., 1979). Hence, the reported NO_3^- removal rates in this study represent the net values without a contribution from inflow COD. Enhancing nitrate removal efficiency with different carbon substrates has been investigated previously (Gebert et al., 2008; Schipper et al., 2010; Hashemi et al., 2011). Hashemi et al., (2011) improved nitrate removal of 36% in wood bioreactor to 65%, 56 % and 77 % by utilizing barley straw, rice husk and date palm leaf, respectively. Gebert et al., (2008) reported softwood (branches and bark with small amounts of leaves from a variety of trees) as top performing substrate in denitrification efficiency (>98%) with denitrification rate of ~

17 g NO_3^--N m^{-3} d^{-1}. However, other investigated materials such as mixture of wood chips, shredded bark and topsoil, compost (obtained from the biological decomposition of organic wastes – wood trimmings, leaves, rotten vegetables and food scraps) and willow woodchips identified as unsuitable carbon sources (see Gebert et al., 2008). Warneke et al., (2011) reported nitrate removal of ~ 6.5, 6.2 and 3.5 g NO_3^--N m^{-3} d^{-1} for wheat straw, maize and green waste materials, respectively compare to the removal rate of 1.3 g NO_3^--N m^{-3} d^{-1} in soft wood (pine) bioreactor for 2-fold lower nitrate inlet concentration than used in this study. However, additional potato residue to woodchip bioreactor increased 13% of nitrate removal to 38 g NO_3^--N m^{-3} d^{-1} which is remarkably higher than reported removal above.

3.3 Microbial community composition and process potential in the bioreactors

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

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

Table 3. Prokaryotic diversity in bioreactors BR1-BR4. Numbers of sequences are taken from the original OTU tables, while all other diversity indicators are based on OTU tables rarified at a depth of 4098 sequences. Average values for 1-2 replicates per sampling point are shown. Zone 2 and zone 3 refer to the 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: Woodchip/ Biochar	Water	Zone 2	8 550	2	95	441	802	4.64
		Zone 3	7 310	2	95	468	761	4.68
	Solid	Zone 2	6 844	2	94	496	827	4.77
		Zone 3	4 935	2	95	398	739	4.36
BR 2: Woodchip/ *Sphagnum*	Water	Zone 2		0				
		Zone 3	7 500	1	95	450	821	4.67
	Solid	Zone 2	7 358	1	96	383	697	4.42
		Zone 3	6 711	2	96	354	674	4.2
BR 3: Woodchip/ woodchip	Water	Zone 2	8 198	2	95	433	749	4.53
		Zone 3	8 304	2	94	480	854	4.72
	Solid	Zone 2	6 942	2	96	378	713	4.29
		Zone 3	6 956	1	95	389	897	4.26
BR 4: (Woodchip/ potato)	Water	Zone 2	9 261	2	96	303	583	3.61
		Zone 3	8 148	2	96	337	605	3.78
	Solid	Zone 2	9 256	2	97	287	505	3.67
		Zone 3	8 359	2	96	296	578	3.39

[a]OTUs richness estimated by Chao1.

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

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

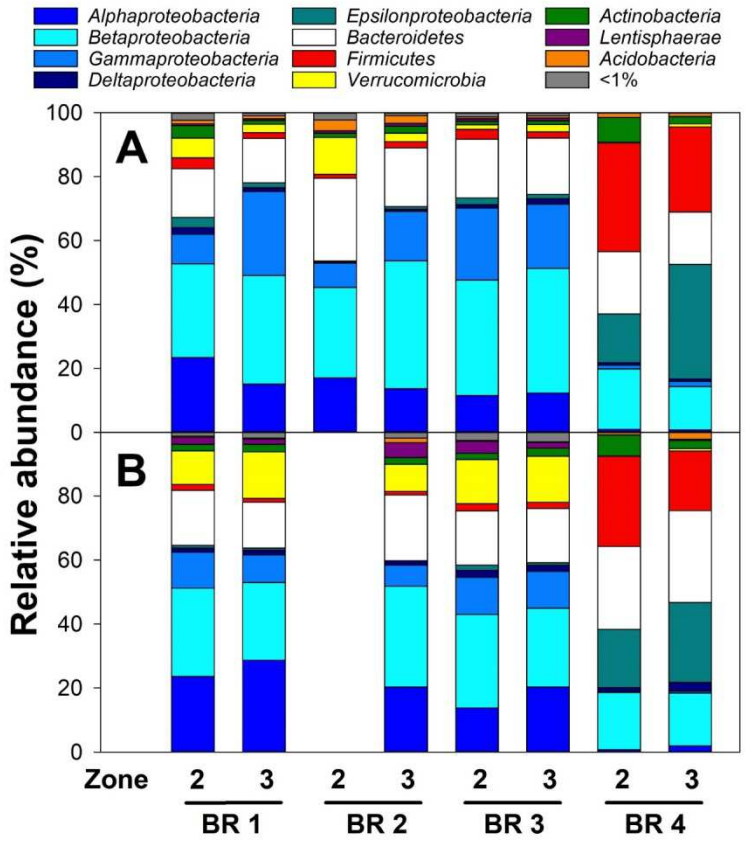
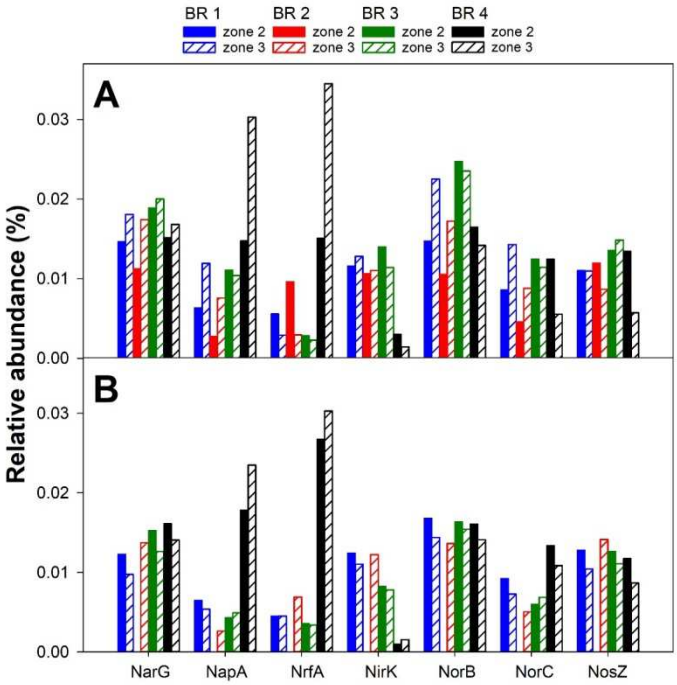


Fig. 7. Composition of the microbial community based on sequence analysis of bacterial and archaeal 16S rRNA genes from (A) solid material and (B) water samples from woodchip bioreactors with a zone containing biochar (BR1), *Sphagnum* sp. moss (BR2), woodchip (BR3) and potato residues (BR4). Average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

Functional profiles of the bacterial communities were predicted based on 16S rRNA gene sequences using PICRUSt. It proved possible to use around 31% of all OTUs and 83% (76-90%) of all sequences for functional prediction. Overall functional profiles of microbiological communities were rather similar in the 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.

3.4 Nitrogen turnover in bioreactors BR1-BR4

The results obtained suggest that heterotrophic denitrification was the dominant path for NO_3^- removal in the four bioreactors. The observed high rate of NO_3^- removal, combined with relatively low production of nitrite, ammonium and alkalinity and high relative abundances of denitrification-associated functions, provide evidence of denitrification activity in the bioreactors. The high alkalinity-producing period in BR1-BR3, coinciding with high nitrate removal, is evidence of heterotrophic denitrification. Heterotrophic denitrification produces approximately 3.57 mg alkalinity (as $CaCO_3$) per mg NO_3^--N reduced (van Rijn et al., 2006). The calculated stoichiometric ratio of 4.2, 3.3 and 3.9 in BR1, BR2 and BR3, respectively, is very similar to the expected theoretical value. Previous studies on both laboratory and field woodchip bioreactors have also identified denitrification as the main mechanism for NO_3^- removal (e.g. Nordström and Herbert, 2018; Schipper et al., 2010; Zhao et al., 2018). However, other processes, including DNRA, aerobic degradation (Zhao et al., 2018), anammox (Herbert et al., 2014; Schipper et al., 2010) and nitrogen immobilisation in organic compounds (Greenan et al., 2006), might also contribute to nitrogen turnover to a smaller extent.

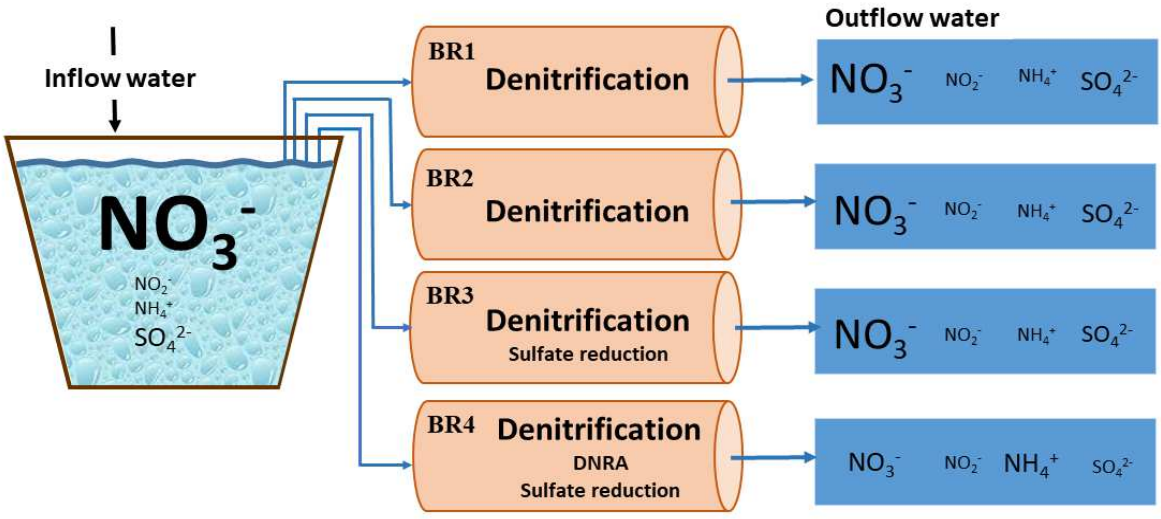
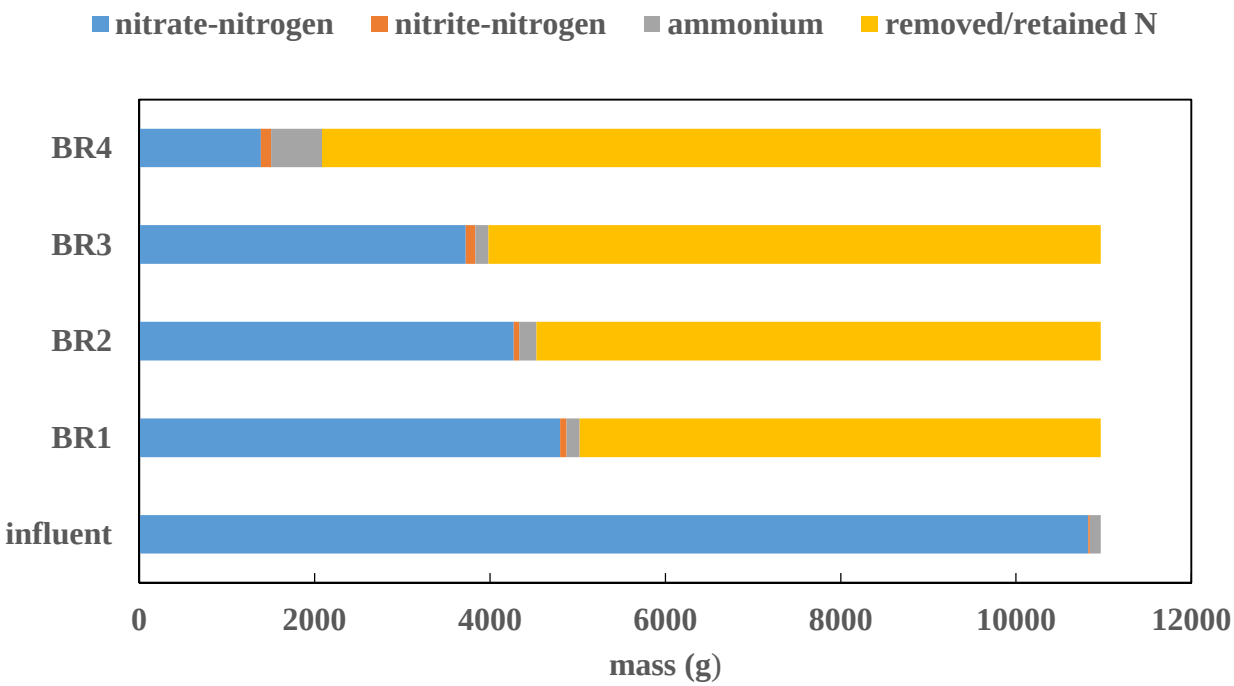


Fig. 9. Processes suggested to occur in woodchip bioreactors containing a zone of biochar (BR1), *Sphagnum* sp. moss (BR2), woodchip (BR3) and potato residues (BR4). Font size indicates relative concentration/importance of a compound/process.

Ammonium is produced during DNRA, and thus high ammonium production in the bioreactors would be an indicator that DNRA was a major nitrate-reducing process. However, ammonium production contributed less than 2% of total nitrogen mass in BR1-BR3 and 5% in BR4. This excludes DNRA as a major mechanism in nitrate reduction (Fig. 10), although small amounts of ammonium might have been produced by DNRA. DNRA is generally favoured over denitrification in environments with low nitrate and high labile carbon availability. The higher ammonium production in BR4 indicates higher DNRA rates than in the other bioreactors. Higher DNRA rates in BR4 are most likely due to higher abundance of potential fermenters, DNRA microorganisms and easily accessible labile carbon. Potato residues provided a labile carbon source, as indicated by the high outflow DOC in BR4. We consider it unlikely that anaerobic ammonium oxidation (Herbert et al., 2014; Schipper et al., 2010) was a pathway for nitrate removal in the reactors, as inflow concentrations of ammonium were low, and the number of potential anaerobic ammonium-oxidising taxa 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 m^3 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 m^3 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 aquculture. 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^-).

466 **Acknowledgements**

467 This study was funded by the Maa- ja vesitekniikan tuki ry. [grant no. 37413], Natural Resources Institute
468 Finland (LUKE), KAUTE-Säätiö and Olvi-säätiö, and by the European Union BONUS Blue Baltic (Art 185)
469 CLEANAQ project partly funded by the Academy of Finland. The authors would like to thank the staff at
470 Laukaa fish farm for helping in sampling and measurements. We are also grateful to Tuomo Reinikka at the
471 University of Oulu for his technical help in laboratory set-up.

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- Woodchip bioreactors removed 31-38 g NO_3^--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

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: