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Research Article

Formation of Greenhouse Gases in Woodchip Denitrification Treating Aquaculture Effluents – A Case Study

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Abstract

Woodchip bioreactors are used to carry out nitrate removal of nutrient-rich aquaculture effluents via denitrification. In certain conditions, greenhouse gases (GHG)s nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) may be formed which have high global warming potentials (N_2O 265 298 times and CH_4 28 times that of CO_2). This study focused on monitoring the GHGs (N_2O, CO_2, and CH_4) at the woodchip bioreactor, treating the recirculating water of a recirculating aquaculture system (RAS) rearing rainbow trout (*Oncorhynchus mykiss*) for one year. High nitrate removal (on average 18 g N m^{-3} d^{-1}, up to 98%) were achieved. The highest rate of N_2O removal (ranged from 0 to 45 µg m^{-2} h^{-1}, 0 13.4 mg CO_2-eq m^{-2} h^{-1}), CO_2 (10-450 mg m^{-2} h^{-1}), and CH_4 (from 0.1 to 2.5 mg m^{-2} h^{-1}, 2.8-70 mg CO_2-eq m^{-2} h^{-1}) were observed in the warmer summer period, likely due to increased microbial actions.

Keywords: Carbon dioxide; Denitrification; Farmed fish; Nitrous oxide; Woodchip bioreactor

Introduction

Recirculating Aquaculture Systems (RAS) are used in land-based aquaculture production. In recent decades, they have become increasingly important, as they are based on efficient water recycling and treatment and consume less water per kg of fish produced. In RAS, the rearing conditions are stable throughout the year. Typically, low volumes of effluents are discharged from a RAS, but the effluent is very nutrient-rich and requires end-of-pipe treatment. Nutrients such as nitrate with eutrophication properties in the environment need to be removed. Various technologies are available for nitrate removal, including ion exchange, adsorption, membrane separation, electrodialysis, and biological denitrification [1,2]. Woodchip bioreactors are passive systems for nitrate removal. In recent years, woodchip bioreactors have been used in treating agricultural water [3], effluents from animal husbandry [4], stormwater [5], and RAS water [6,7].

Biological denitrification is carried out by microbes which use nitrate as their electron acceptor and organic substances as an electron donor to get energy from organic matter for microbial growth [8]. In woodchip bioreactors, the diverse communities include e.g., *Acidithiobacillales*, *Desulfovibrionales*, *Rhodocyclales*, and *Chlorobiales* with the key denitrifying communities *Burkholderiales*, *Pseudomonadales*, *Rhizobiales*, and *Rhodobacterales* but they vary in different systems, conditions, or by woodchip species [9].

Biological denitrification can be classified as heterotrophic and autotrophic. In autotrophic denitrification, hydrogen, iron or sulfur compounds are used as the energy source and inorganic carbon as the carbon source [10]. The heterotrophic denitrifiers use organic carbon compounds as a carbon source [11]. Watersoluble substances (methanol, acetic acid, glucose) are used as a carbon source for denitrification [12-14], but some of them pose risks during storage, transportation, and operation due to their toxicity and inflammability [2].

Woodchips consist of a porous matrix with high hydraulic conductivity and a large specific surface area for microorganisms to attach to [15]. During incomplete denitrification, greenhouse gas nitrous oxide (N_2O) can be formed, which has a global warming potential 265-298 times higher than carbon dioxide (CO_2, [16] and ozone-depleting properties [17]. Additionally, methane (CH_4) is a greenhouse gas with 28-fold (including indirect effects) warming potential compared to CO_2 [18]. Both can be released from the denitrifying bioreactors derived from decaying organic matter [15].

In woodchip bioreactors, woodchips act as a growth surface for microbial biofilm and as a source of carbon [19]. The aim of denitrification is to convert nitrate (NO_3^-) into nitrogen gas (N_2) and release it into the atmosphere [20]. It is a stepwise process catalyzed by enzymes (nitrate reductase, nitrite reductase, nitric oxide reductase, nitrous oxide reductase) and carried out by denitrifiers, including *Pseudomonas*, *Bacillus*, *Thiobacillus*, and *Propionibacterium* [21]. Typically, nitrate removal rates of 39 g NO_3-N m^{-3} d^{-1} [6], 222 g NO_3-N m^{-3} d^{-1} [18]-> 2-22 g NO_3-N m^{-3} d^{-1} [18], and 7.2 ±9.6 g N m^{-3} d^{-1} (n=27, [22]) have been achieved. As early as in 1995, woodchips were assessed as a suitable and slowly degrading carbon source for denitrifying microorganisms [23-25].

Incomplete denitrification can produce N_2O as a by-product in sub-ideal conditions [15], which can depend on many parameters, e.g., oxygen (anoxic conditions required for the reductase enzymes, [26]) and carbon availability (N and C are needed to ensure the presence of electron acceptors and electron supply, [26]), temperature, redox potential, and microbial population (physical factors affect the availability of e.g., oxygen and carbon, and microbial activity, [26];

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[27,28]). The availability of carbon is one of the key factors which regulates the proportion of incomplete denitrification and N_2O formation [29]. The availability of carbon may change with bioreactor´s operation age [9].

Environmental factors, such as carbon availability [30] or temperature [6] are crucial in controlling denitrification. Temperature affects all microbial activities, including activity in the hydrolysis of solid substrate and nitrate reduction. At a low temperature, the efficiencies of those activities decrease, leading to a decreased denitrification [31] due to suboptimal conditions. For example, denitrification and NO_3 removal efficiency may reduce at low water temperatures to 10-20% at 5 ºC [32]. In a study by Shen et al. [33], nitrate removal efficiency decreased from 92.5% to 69% (at 15 ºC) and to 50% (at 5 ºC). Additionally, Cameron and Schipper [33] reported nitrate removal that was 1.2-2.3 times higher at 23.5 ºC than at 14 ºC, although a higher temperature may lead to the faster microbial decomposition of the carbon source [34] and the release of CH_4 and CO_2 [15,35].

The Hydraulic Retention Time (HRT) of a denitrification reactor has an impact on nitrate removal efficiency. For example, Wang and Wang [16] and Gibert et al. [19] showed that decreased HRT led to increased effluent nitrate concentration, while longer HRT led to higher nitrate removal efficiency. Audet et al. [36] reported that HRT above 60h favored NO_3 removal and low N_2O emissions. However, lower CH_4 production has been reported with decreased HRT (2 h HRT: 0.51 g C m^3 day^{-1}, 8 h HRT: 1.50 g C m^3 day^{-1}, [17]).

In our previous studies [37,38], high nitrogen removal efficiency was reported in passive woodchip denitrification, with low or absent toxic or harmful compounds. This experiment was motivated by studying the GHGs (CO_2, CH_4, and N_2O) which may be released from the woodchip bioreactor at four sampling points to assess the bioreactor's efficiency and seasonal changes in concentrations. It was hypothesized that the nitrate removal efficiency and formation of unwanted N_2O would be influenced by the colder winter temperatures.

Materials and Methods

Experimental setup

The experiment was conducted at the Laukaa fish farm (April 2021February 2022) of Natural Resources Institute Finland (Luke), using a pilot-scale RAS (FREA Aquaculture solutions, Denmark). The full description of the RAS has been reported in Pulkkinen et al. [39].

The RAS consisted of two identical units, each unit with two 5 m^3 tanks and a 1 m^3 space for sludge cones, which collect settleable solid material and uneaten feed. From the tanks, water flowed through a drum filter (60 µm mesh size, Hydrotech HDF800, Veolia, France) and two parallel 2.5 m^3 fixed bed bioreactors filled with 1.5 m^3 Saddle chips (KSK Aqua, Denmark). Water flowed (water flowrate measured with Fluxus F501, Flexim, Germany) through a 2.24 m^3 degassing unit and a 0.74 m^3 pump sump. Finally, it was pumped through a low-head oxygenator (FREA, Aquaculture Solutions, Denmark) back to the fish tanks. Dissolved oxygen (Oxi:lyser, s::can, Austria) was monitored online and maintained above 8.0 mg L^{-1} in the rearing tanks. The measurement data were stored on an industrial computer (Con::cube, S::can, Austria).

The water temperature was adjusted to 12.8 ºC by controlling the hall air temperature. The pH was maintained at 7.5 (ProMinent, Germany) by adding dissolved sodium bicarbonate to the pump sumps (EJ-R, Iwaki, Japan). Clean inlet water (Watson Marlow 630, Spirax-Sarco Engineering, UK) was led from the oligotrophic Lake Peurunka (62.44886, 25.85201, area 694 ha, 59 600 m^3). The inlet water was a 1:1 mixture of surface water (depth of 4 m) and the aphotic layer (depth of 8 m). Replacement water from Lake Peurunka was taken at 500 L kg feed^{-1} (5.2–7.2 m^3 d^{-1}).

A selected proportion of the circulating water was treated by a passive water treatment system, which included a woodchip bioreactor for nitrogen removal, wetland to control the Biological Oxygen Demand (BOD) and the Chemical Oxygen Demand (COD), and sand filtration for suspended solids and organic carbon removal [37,39]. The woodchip bioreactor was 14 m x 9 m (=118 m^2, 50 m^3) and 1.5 m deep (1 m+0.5 m dry layer) of birch wood chips with porosity of 0.65. A 0.5m layer of dry woodchips on top were designed to act as an insulating layer during cold winters. Water was channeled from one side of the bioreactor with two perforated pipes 3.3 m and 4.6 m long at the base (Figure 1). The HRT of the woodchip bioreactor was on average 2.2 d (0.9-7.8 d) (Supplementary Figure S1).

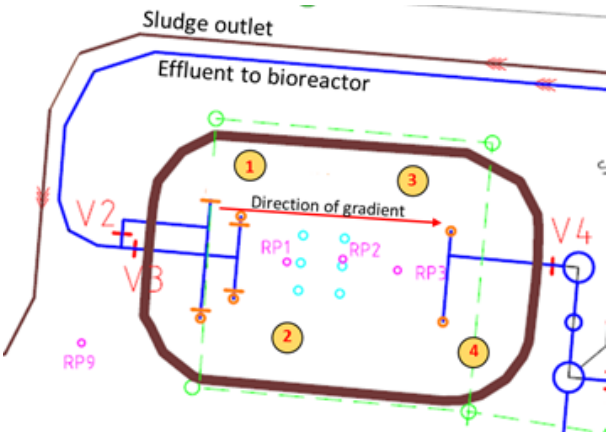
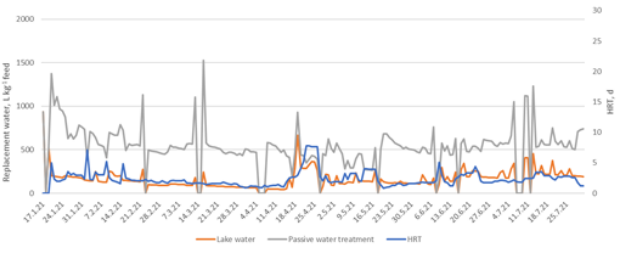


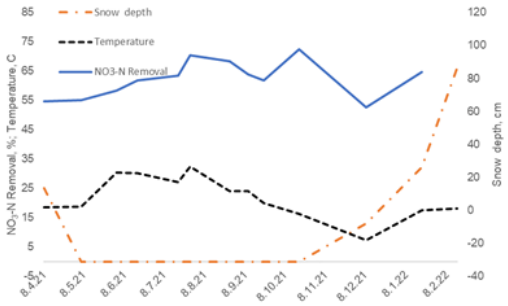
Figure 1: Schematic figure of the woodchip bioreactor (width 3.3 m, length 9 m) with inlet and outlet pipes (blue), sampling cones (yellow, 1-4), and inlets for maintenance (light blue). The direction of the gradient has been marked with a red arrow.



Supplementary Figure S1: Replacement water from Lake Peurunka (red, L kg^{-1} feed) and from the passive water treatment system (gray, L kg^{-1} feed), and hydraulic retention time (HRT, blue, d) at the woodchip biofilter.

The LongTerm Average (LTA) annual temperature (1961–2020) in central Finland (Jyväskylä) is 4.2°C, and the average annual precipitation is 621 mm (Finnish Meteorological Institute 2023). In 2021, annual precipitation was 675.3mm (Finnish Meteorological Institute

2023). Of the total precipitation, approximately 50% falls as snow. Snow cover typically appears in mid-November and melts in late April. The coldest month is February (-7.4 °C), and the warmest is July (17.0 °C). In central Finland (Jyväskylä), the average snow coverage duration is 148 days and snow depth is 40-60 cm (Finnish Meteorological Institute 2023). The local temperature and snow cover data are shown in Supplementary (Figure S2) (Finnish Meteorological Institute 2024).



Supplementary Figure S2: Snow depth (cm), air temperature (ºC) and nitrate (NO_3-N) removal efficiency (%) in central Finland near the area of the experiment (April 2021-February 2022).

In the experiment, a total of 3164 one-year-old vaccinated full female rainbow trout (*Oncorhynchus mykiss*) was reared. The fish originated from the Hanka-Taimen hatchery (Hankasalmi, Finland). Their average weight was 494 g, with a total biomass of 1564 kg at 78 kg m^{-3}. The fish were fed with an automated feeding system (T Drum 2000, Arvo-Tec, Finland) with a commercial fish feed (BioMar Orbit, 3.5 mm and 6 mm), resulting in a Feed Conversion Ratio (FCR) of 1.2. Supernumerary fish were regularly removed to maintain the tank biomass and fish density at a suitable level. The fish were visually inspected on a daily basis to monitor their health and well-being.

The study followed the protocols approved by the Luke Animal Care Committee, Helsinki, Finland, and EU Directive 2010/63/EU for animal experiments.

Water quality parameters

At the woodchip bioreactor, the temperature was measured using a YSI EXO probe (Xylem, USA) and a HOBO Pendant (MX2201, Onset Computer Corporation, USA). Oxygen was measured using YSI EXO, YSI ProODO, and Ponsel OPTOD optical probes (Aqualabo Servises SA, France) as explained in Pulkkinen et al. [39].

Water samples were taken from the inlet and outlet of the woodchip biofilter to measure the following variables. Water samples were collected in clean highdensity polyethene (HDPE) jars and stored frozen until further analysis. The total nitrogen (Procedure 8038 Nessler, TAN, 0.8 mg L^{-1}), nitrite-N (LCK341, 0.105–0.108 mg L^{-1}), nitrate-N (LCK340, 5-35 mg L^{-1} and LCK342, 44.2–65.4 mg L^{-1}), COD (LCK1414, 5-60 mg L^{-1}), sulfate (SulfaVer, Permachem® reagents, 40-150 mg L^{-1}), and phosphate-P (LCK349, 0.051.50 mg L^{-1}) were analyzed with quick spectrophotometric tests for DS 3900 (Hach, Loveland, USA). The water alkalinity (88.3–113.1 mg L^{-1}) was measured by a standard titration method ISO 9963-1:1994 (TitraLab AT1000, Hach, Loveland, USA), and turbidity (5.5–6.6 NTU) with a Hach 2100q Turbidimeter (Hach, Loveland, USA).

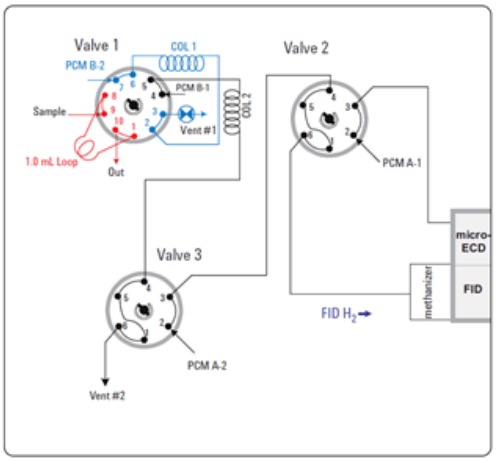
GHG flux measurements

The N_2O, CH_4, and CO_2 emissions from the woodchip bioreactor were measured using a static chamber method with Polypropylene (PP) cones (90 cm in height, diameter 15 cm) and PP cover with a rubber collar (n=4, height 10 cm, diameter 15 cm). The cones were pre-installed in the woodchip bed at a depth of 10 cm. The cones were placed on both sides (3 m apart) and different distances from the effluent manifold at the inlet side of the woodchip bioreactor (Figure 1) to give a comprehensive overview of the bioreactor efficiency.

The samples were taken on average once a month (4/20212/2022) but more frequently in the summer. First, the cones were closed with covers, and gas samples of 20 mL were taken with a 60 mL polypropene (PP) syringe at 2 min intervals from the headspace of the chamber. In each measurement, 20 mL of chamber air was sampled through a rubber septum with a polypropylene syringe (BD Plastipak, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and transferred into pre-evacuated 20 mL glass vials (Exetainer, Labco Ltd., High Wycombe, UK). The air in the chamber was mixed with one syringe flush before each sampling.

Chemical analysis

The GHG analyses were performed with a gas chromatograph (GC, Agilent 6890N, Agilent Technologies, Santa Clara, CA, USA), a Flame Ionization Detector (FID), and an Electron Capture Detector (ECD). The method for Agilent 7890A GC with SP1 7890-0468 configuration (Supplementary Figure S3) was used to analyze GHGs (CO_2, CH_4, N_2O), modified from Nieminen et al. (2015). The system included three valves and two columns (G3591-81004 and G3591-81121). The detector FID was used to detect CH_4 and CO_2, and ECD to detect N_2O. The system was modified for using automated headspace sampling (Gilson GX-271).



Supplementary Figure S3: Configuration of SP1 7890-0468 in GC analysis of gaseous samples.

At the GC, the following conditions were maintained: valve temperature 80 ºC, oven temperature 70 ºC, methanizer temperature 375 ºC, sample loop 2 mL, and column flow (N_2) 21 mL min^{-1} (at 60 ºC). At FID, a temperature of 250 ºC, H_2 flow of 48 mL min^{-1}, airflow of 350 mL min^{-1}, and make-up (N_2) of 2 mL min^{-1} were used. At the ECD, the temperature was 350 ºC, and the make-up gas (Ar/ 5% CH_4) was 2 mL min^{-1}.

A standard gas mixture (AGA Gas AB, Lidingö, Sweden) of compressed air with 29.9 mg L^{-1} of N_2O, 201 mg L^{-1} CH_4, and 3010 mg L^{-1} CO_2 was used for the daily calibration and preparation of the calibration curve. Concentrations in the samples were determined based on external calibration (0; 0,5; 1; 5; 15; 20 mL of standard gas mixture). Cumulative fluxes were calculated by assuming linear changes between subsequent measurements. The Limits Of Detection (LOD) and Quantification (LOQ) for the GC analysis were 24.6 µg L^{-1} and 162.2 µg L^{-1} for N_2O, 5.86 mg L^{-1} and 89.3 mg L^{-1} for CO_2, and 157.0 µg L^{-1} and 718.9 µg L^{-1} for CH_4.

Statistical analysis

A statistical analysis of the GHGs was performed using IBM SPSS Statistics (IBM Corp.©, version 27.0.1.0, Armonk, NY, United States). Statistically significant differences between normalized results of GHGs (N_2O vs. CO_2; N_2O vs. CH_4; CH_4 vs. CO_2, nitrate removal (%) vs. temperature, and nitrate removal efficiency (%) vs. HRT) at different time points were calculated with an independent samples t-test at a statistical significance level of $p < 0.05$.

LODs and LOQs of GC analyses were determined with a regression analysis of variance (ANOVA) test. The limit for the statistical significance level was set at $p < 0.05$.

Results

Water quality

Nitrate-N decreased from 65 mg L^{-1} to below 20 mg L^{-1} during the woodchip bioreactor, and pH decreased from 8 to 6 (Figures 2A and 2B). Overall, lower values were detected from the fall of 2021to the spring of 2022. Concentrations of ammonium and nitrate-N remained very low throughout the experiment. Additionally, alkalinity was higher overall before the biofilter (up to 70 mg L^{-1} vs. 2045 mg L^{-1}) and fluctuated during the experiment. Concentrations of sulfate and phosphate remained quite constant throughout the experiment (below 30 mg L^{-1}, (Figures 3A&3B)). However, some increased COD values were detected in the beginning of the experiment.

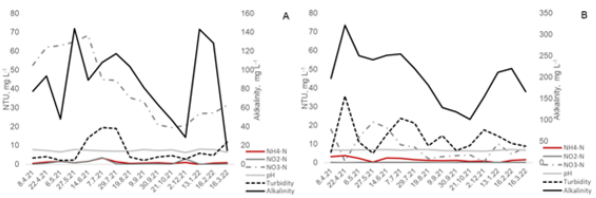


Figure 2: Axis on the left: concentrations of ammonium (NH_4-N, mg L^{-1}), nitrite (NO_2-N, mg L^{-1}), nitrate (NO_3-N, mg L^{-1}), pH, and turbidity (NTU); axis on the right: alkalinity (mg L^{-1}) before (A) and after (B) the woodchip bioreactor.

The nitrate removal rate ranged from 7 to 26 g N m^{-3} d^{-1} (on average 18 g N m^{-3} d^{-1}) throughout the experiment. HRTs showed a moderate positive correlation with NO_3-N removal efficiency (R^2=0.340; Supplementary (Figure S4)).

The temperature ranged between 5 and 23 ºC at the woodchip biofilter (Supplementary Figure S5A). The results marked in blue were measured in the pipeline back to the RAS. Supplementary Figure

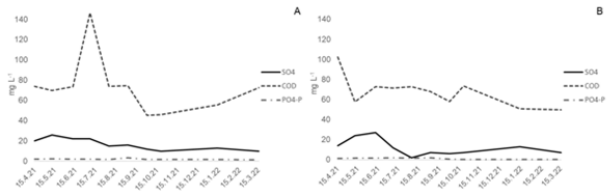
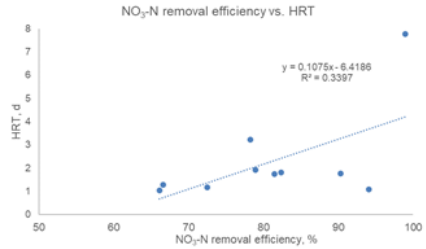
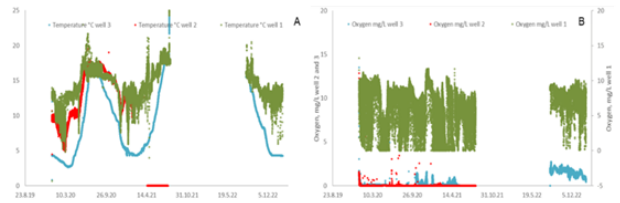


Figure 3: Concentrations (mg L^{-1}) of sulfate (SO_4^{2-}), phosphate (PO_4-P), and COD before (A) and after (B) the woodchip bioreactor.



Supplementary Figure S4: HRT, d from April 2021 to November 2021 plotted against NO_3-N removal efficiency, %.

S5 B shows that the inlet water from the RAS contained 512 mg L^{-1} of dissolved oxygen. The oxygen content decreased rapidly and remained very low in the following steps of anaerobic denitrification.



Supplementary Figure S5: Temperature (ºC, A) and dissolved oxygen (mg L^{-1}, B) before (well 1, green) and after (well 2, red) the woodchip biofilter, and in the pipeline back to the RAS (well 3, light blue). Measurements are missing between October 2021 and May 2022 due to instrumental failure.

The inflow to the woodchip bioreactor ranged from 1 to 14 m^3 d^{-1} (Figure 4). The outflow followed the trend of the inflow although it varied occasionally due to the addition of lake water to the inlet from Lake Peurunka and supernatant from the sludge treatment to the outlet flow.

Figure 4: Inflow (m^3 d^{-1}) from the RAS to the passive water treatment system and the woodchip biofilter and outflow (m^3 d^{-1}) back to the RAS.

GHGs

The N_2O removal rate ranged from 0 to 45 µg m^{-2} h^{-1} (0-13.4 mg CO_2-eq m^{-2} h^{-1}, Figure 5), and for CO_2 from 10 to 450 mg m^{-2} h^{-1} (Figure 6). The rates of CH_4 ranged from 0.1 to 2.5 mg m^{-2} h^{-1} (2.8-70 mg CO_2-eq m^{-2} h^{-1}, Figure 7). For all the studied compounds, the highest rates were observed in the inlet of the woodchip bioreactor (Tubes 1&2) but decreased rapidly at the outlet end of the bioreactor (Tubes 3&4). In addition, the rates varied seasonally. The highest removal rates were observed in the early spring for CH_4 (Figure 7) but in the late summer and fall for N_2O (Figure 5) and CO_2 (Figure 6).

The normalized results of N_2O positively correlated with those of CO_2 ($p=0.029$, $t_{192}=1,899$). On the other hand, CO_2 and CH_4 did not show a significant correlation ($p=0.286$, $t_{192}=-0.567$). This was also the case for N_2O and CH_4 ($p=0.073$, $t_{192}=1,457$).

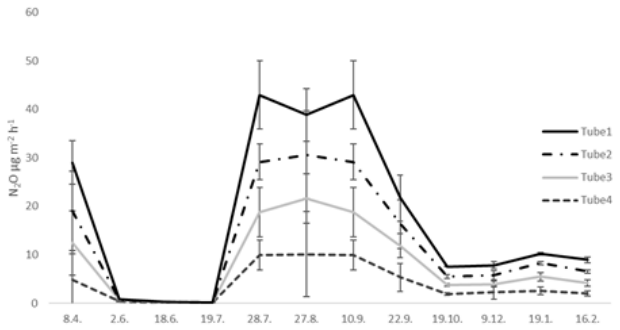


Figure 5: Observed N_2O removal rates at the woodchip bioreactor (µg m^{-2} h^{-1}) during the one year sampling period. Samples were taken from four sampling points (Tubes 1 4; see Figure 1).

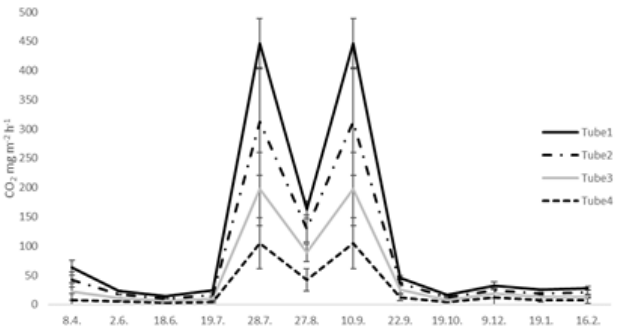


Figure 6: Observed CO_2 removal rates at the woodchip bioreactor (mg m^{-2} h^{-1}) during the one year sampling period. Samples were taken from four sampling points (Tubes 1 4; see Figure 1).

The nitrate removal ranged from 66% to 98% (Figure 8, Table 1). It seemed to follow changes in air temperature (Figure 8, Supplementary Figure S2) but without a statistical significance (positive correlation, $p=0.217$, $t=0.795$). The N_2O production (based on NO_3-N) ranged in most cases between 1.3% and 17.2%, excluding two events of higher N_2O-N production in August and December (Table 1).

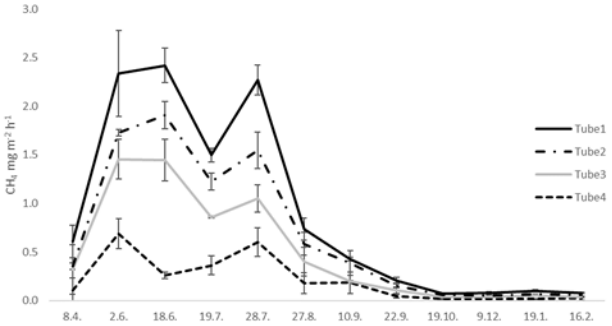


Figure 7: Observed CH_4 removal rates at the woodchip bioreactor (mg m^{-2} h^{-1}) during the one year sampling period. Samples were taken from four sampling points (Tubes 1-4; see Figure 1).

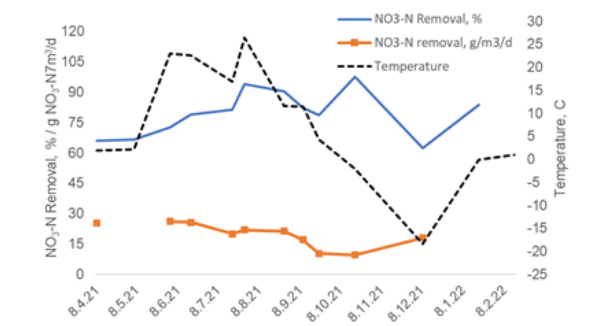


Figure 8: NO_3-N removal (as g NO_3-N m^{-3} d^{-1} and % removed) with local temperature (°C) during the study period (4/2021-2/2022).

Date of measurement	Removal of NO_3-N, %	N_2O-N production vs. NO_3-N, %	Temperature, °C	Nitrate removal, g NO_3-N m^{-3} d^{-1}
8.4.	66.1	17.2	2.0	25.3
2.6.	66.6	2.9	23.0	26.2
18.6.	72.5	1.3	22.6	25.8
19.7.	78.9	2.7	16.9	20.0
28.7.	81.5	15.1	26.4	22.0
27.8.	94.1	40.9	11.6	21.4
10.9.	90.3	5.7	11.5	17.1
22.9.	82.4	17.0	4.3	10.4
19.10.	78.7	5.3	-2.0	9.7
9.12.	97.7	48.4	-18.3	18.1
9.1.	62.3	2.0	0.0	n.a.
16.2.	83.8	5.8	1.0	n.a.

Table 1: Removal of NO_3-N (%), proportion of produced N_2O-N vs. NO_3-N (%), temperature (°C), and nitrate removal (g NO_3-N m^{-3} d^{-1}).

n.a.: not analyzed

Discussion

Nitrate removal

In this study, the nitrate removal rates ranged from 7.2 to 26.2 g NO_3-N m^{-3} d^{-1} (average 18 NO_3-N m^{-3} d^{-1}). They were somewhat

higher than in our previous study [38], where the woodchip bioreactor achieved a nitrate removal rate of 11-15 g NO_3-N m^{-3} d^{-1}. In both studies, the results were higher than those reported in previous studies by Hoffman et al. [40,41] 1.67-2.22 g N d^{-1} m^{-3}) and Christianson et al. [4]; 0.38-7.76 g N d^{-1} m^{-3}), but in the same range (4.5-16.8 g N d^{-1} m^{-3}) as in von Ahnen et al. [7]. The maximum nitrate removal rate (33 g NO_3-N m^{-3} d^{-1}) of the system was determined by pre-tests at HRT of 48 h [30], but this level was not achieved during the experiment.

HRT

Bioreactor removal efficiency depends on the HRT. In our study, the HRTs ranged from 0.9 d to 7.8 d (Supplementary Figure S2). The NO_3-N removal was moderately correlated with the HRT (R^2=0.340), suggesting that longer HRTs might have led to increased nitrate removal (g NO_3-N m^{-3} d^{-1}). This is in line with the results of Jéglot et al. [42] and Nordström and Herbert [43]. Both reported increased removal of NO_3-N and decreased N_2O formation at longer HRTs. The efficient removal of nitrate is crucial for the commercial farmers who use passive systems for denitrification. Poor operation and insufficient denitrification may prevent farmers meeting their discharge allowances and increasing their production [44].

Some studies suggest that shorter HRTs favor aerobic conditions and hinder denitrification [44,45]. Audet et al. [36,46] stated that N_2O emissions were higher at HRTs below 60 h (=2.5 days), while Addy et al. [47] detected significantly lower NO_3 removal and increased N_2O production in bioreactors with HRTs below 6 h. Even in this study, short HRTs may have promoted N_2O formation (Supplementary Figure S4). On the other hand, Davis et al. [17] reported lower CH_4 production from 2 h HRTs compared to 8 and 16 hour HRTs.

Environmental factors

Denitrification is directly inhibited by CO_2 [2]. The atmospheric CO_2 concentration has increased from 280 ppm in 1800s to 400 ppm today [47,48]. In our study, even higher CO_2 concentrations were measured suggesting CO_2 formation due to microbial actions derived from decaying organic matter in the woodchip bed [15]. Increased CO_2 concentration inhibits electron transport and the consumption of electrons during denitrification by suppressing the synthesis and activity of the key enzymes [46].

Woodchips acted well as a carbon source for the denitrification, although some studies suggest that lignocellulosic material must first be hydrolyzed into soluble compounds before denitrifying bacteria can utilize the material as a substrate [5,49]. However, there are plenty of other microorganisms present in a woodchip biofilter, including saprophytic fungi which can degrade woodchip carbon into suitable form for denitrifiers [50].

As previously stated, denitrification is predominantly an anoxic process where N_2O production and reduction to N_2 depend on the local anoxic conditions. Water saturation and soil or woodchip bed structure regulate the oxygen supply. They determine the pathways through which gaseous and dissolved oxygen, nitrogen species, and dissolved organic matter may diffuse to the location of their consumption [26]. In our study, the oxygen content remained from low to moderate levels of 1.4-8.5 mg L^{-1} in the inlet and very low 0.00-0.01 mg L^{-1} in the outlet throughout the studied year. This suggests anoxic conditions in the system, although a low-oxygenated zones may be possible at the front end of the system.

Variation in moisture content influences the microbial communities and biofilter performance [27]. For example, very low moisture content slows down microbial activities [51], while a too high moisture content leads to the filling of woodchip pores and restricts the transportation oxygen and nutrients [20]. In such cases, water competes with N_2O for hydrophilic spots (-OH and -COOH groups, [52]) which can lead to increased N_2O formation [53,54].

Only a few studies have been conducted on the role of denitrification at cold temperatures (~5 °C, [55]). NO_3-N removal may decrease to about 10-20% due to the effects of temperature on microbial action. In this experiment, the woodchip bioreactor was insulated by additional woodchips on the surface of the moist layer (1.0 m depth of active media and 0.5 m of dry woodchips on top, [39]) to prevent the bioreactor freezing during the winter.

Annual mean efficiencies of bioreactors are often in the range of 50% [3,41,55]. However, almost complete nitrate removal (%) can be achieved at high temperatures (98% at 28ºC, [56]). In this study, the NO_3-N removal was above 60% even in the winter and reached above 90% on a few occasions. This suggests that the air temperature did not restrict the biofilter efficiency.

Jéglot et al. [57] showed that the optimal temperature for NO_3 transformation to N_2 was between 20 and 30 ºC. High temperatures can only occasionally be achieved in northern latitudes in the summer (see Supplementary Figure S2). The pathway of NO_3 transformation to N_2 includes N_2O production [55]. At 5-10ºC (or lower), lag phases can occur, which leads to slow denitrification and more pronounced NO_2, NO, and N_2O accumulation [55].

N_2O formation

The highest concentrations of N_2O were found at the first sampling point (Figure 5, Tube 1), and the lowest at the fourth point (Figure 5, Tube 4). This suggests that the denitrification occurred progressively along the gradient as the water flowed and proceeded in the woodchip biofilter. Similar changes in concentrations were also observed for CO_2 and CH_4 (Figure 6 and 7). This is likely due to changing conditions (e.g., gradient of O_2 content, varying microbe communities) in the biofilter.

The N_2O removal rates ranged up to 45 µg N_2O m^{-3} h^{-1}, which corresponds 0.1-2.5 mg N L^{-1}, while Audet et al. [31] for example, reported lower N_2O rates of 1-200 µg N L^{-1} in a woodchip bioreactor treating agricultural water. On the other hand, Maia et al. [20,58] reported higher (0.6-2 mg N_2O L^{-1}) formation at 60% moisture content. In this study, the main woodchip layer was saturated with water (the moisture content in the woodchips was not measured in this study). This may have been one factor influencing the observed N_2O rates.

The highest N_2O concentrations were measured in the late summer and fall, which may be due to delayed effects of reactions occurring in the warmer summer period. This includes increased microbial action in the woodchip bed. Overall, N_2O formation seemed to follow the environmental conditions and biological processes in the woodchip bed, and the load in circulating water played a minor role. This is supported by the fact that inlet and outlet water flows, nitrate loads, and removal did not seem to follow the N_2O formation. Additionally, none of the water quality measurements was correlated with the N_2O formation. It is possible that some of the formed and soluble N_2O may have circulated back to the RAS with the effluent water [11].

In previous studies, 0.003-9.7% of removed NO_3 was converted to N_2O [59,60], and more recently, 1-6% conversion to N_2O has been reported (250-1250 $m_3$of aquaculture effluents [9]). Additionally, Audet et al. [36] achieved very low N_2O-N emissions per NO_3-N removal, which were from 0.6% to 2.4% on average. In this study, the N_2O-N formation remained mostly at 1.3-10%, but formation as high as 48% was detected. This suggests that the denitrification was at least occasionally incomplete. Based on IPCC evaluation, over 0.75% NO_3-N conversion to N_2O is considered increased pollution [15], and strict limitations were not achieved in this experiment. Previously, high N_2O formation has been found at low carbon availability and high nitrate concentrations [29]. This study was conducted on the second functioning year of the bioreactor, suggesting that the bioreactor and its processes were fully matured.

CH_4 and CO_2 formation

In woodchip biofilter, even CH_4 can be produced if nitrate is depleted and CH_4 acts as electron acceptor by methanogenic archaea [18,61]. Methanogenic archaea and bacteria produce methane in anaerobic conditions [60,61]. For example, methane-producing microbial communities can include *Bacillus* sp., *Clostridium* sp., *Desulfotomaculum* sp., and *Ruminococcus* sp. [62]. However, microbial species were not determined in our study.

The highest nitrate concentrations in the inlet water were in the early summer at the time of the highest methane formation. This suggests that the conditions in the woodchip bed favored CH_4 formation, although not due to an insufficient nitrate load in the circulating water. Theoretically, the CH_4 formation at the bioreactor should be low when NO_3 concentrations remain sufficiently high to suppress methanogens, but this concept requires validation [18]. More likely, CO_2 and CH_4 were released from the denitrifying bioreactors derived from decaying organic matter [15].

In this study, the concentrations of N_2O correlated positively with the CO_2 results ($p=0.029$), while neither CH_4 and N_2O ($p=0.286$) nor CH_4 and CO_2 ($p=0.073$) showed significant correlations. A similar connection between CO_2 and N_2O was also observed by Maia et al. [20] and later by Nieminen et al. [63], although the latter studied emissions in soil instead of from a woodchip bioreactor. Even Maia et al. [64] did not observe a positive correlation between CO_2 and CH_4 or between CH_4 and N_2O concentrations. They also suggested that CO_2 was a good indicator of microbial activity in a denitrification bioreactor.

The COD in the inlet water was high in the spring and early summer when methane flux was high. Other gases such as CO_2 and CH_4 are released from the denitrifying bioreactors, both derived from decaying organic matter [18], which may have been the case in this study.

In some studies, CH_4 has been detected during early operation of the bioreactors [25] but has disappeared after a few months, possibly as the highly labile carbon in the woodchips was consumed. In this case, it was the second functioning year of the fully matured bioreactor, although highly labile carbon may still have been readily available.

The formation of CH_4 is connected with methanogens under anaerobic conditions and high moisture content [56]. which was probably the case here in the woodchip bed after a substantial amount of snow had melted (Supplementary Figure S3). Many studies have shown that moisture content and temperature are the main factors influencing methane and N_2O formation [64-66]). Even increased N_2O content is expected when moisture content is high [67-74].

Conclusion

Nitrate removal (%) remained high in the woodchip biofilter throughout the studied year, demonstrating the efficient operation of the biofilter. N_2O formation was mostly at a low level, but relatively high formation was occasionally observed. This suggests that denitrification may have occasionally been incomplete.

Unlike the hypothesis, no reduction in the nitrate removal rate (g NO_3-N m^{-3} d^{-1}) or increase in N_2O formation induced by the cold winter temperatures was observed. The formation of the studied GHGs did not follow the conditions in RAS or nitrogen load in the circulating water. It is more likely that the environmental conditions in the woodchip bed played the main role in determining the formed and released gases, and some effects may have occurred at a delayed pace. Although a thorough study of microbial processes in the woodchip bed were not the goal of this experiment but they definitely are an interesting subject for further study to fully understand the processes behind the formation of GHGs in denitrification.

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Supplementary Material

The supplementary material includes (Supplementary Figures S1-S5): the amounts of replacement water and HRTs at the woodchip bioreactor (S1); air temperatures, snow coverage and nitrate removal efficiencies from April 2021 to February 2022 (S2); Configuration in GC analysis for the GHG samples (S3); a correlation plot of HRTs and nitrate removal efficiency from April 2021 to November 2021 (S4); temperature and dissolved oxygen before and after the woodchip biofilter, and in the pipeline back to the RAS (S5).

Declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Data availability statement

`The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contribution

Petra Lindholm-Lehto: Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing –original draft preparation.

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No potential conflict of interest was reported by the author. There are no relevant financial or non-financial competing interests to report.

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