

## Trace elements, anions, and carbohydrates in the recirculating aquaculture system using woodchip denitrification, constructed wetland, and sand infiltration

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### ABSTRACT

A recirculating aquaculture system (RAS) aims to achieve fish production with negligible discharge into the environment. RASs have been applied for fish production in several countries, but nitrate removal is often a limiting factor for production increases. In this study, a pilot-scale RAS (10 tons of fish/year) was connected to a water treatment field which consisted of a denitrifying woodchip bioreactor (9 m × 14 m) filled with birch woodchips (*Betula pendula*), a constructed wetland (7.5 m × 6 m), and sand infiltration (16 m × 31 m) to achieve high water quality with low-maintenance treatment units. In the constructed wetland, a perennial common reed (*Phragmites australis*) was used in a well-drained soil for nutrient re-use. Concentrations of different elements, diluted anions, and selected hydrocarbons were monitored and quantified. Some Mn leaching occurred during the sand infiltration stage. However, Mn concentrations decreased towards the end of the experiment. Concentrations of total-Fe increased up to 2.75 mg L<sup>-1</sup> and Mn up to 5 mg L<sup>-1</sup> in the sand infiltration stage of the water treatment field, probably due to anoxic conditions and leaching of fine particles. This type of process design offers effective but low-maintenance treatment of circulating water.

**Key words:** constructed wetland, denitrification, heavy metals, recirculating aquaculture system (RAS), sand infiltration, woodchip bioreactor

### HIGHLIGHTS

- Passive water treatment was sufficient for treating recirculating water.
- Elevated concentrations of Mn and Fe were found after sand filtration.
- The concentrations of anions were low in the water treatment field.
- Glucose and fructose of woodchip-origin were found at low levels in water.

## 1. INTRODUCTION

An intensive recirculating aquaculture system (RAS) is an increasing form of aquaculture. RAS farms typically require heavy investments, and to achieve profitability, they need to be run close to full capacity. The capacity of RAS farms can be limited, firstly, due to accumulation of harmful compounds, such as carbon dioxide, solids, total ammonia nitrogen (TAN), and nitrate. Secondly, the production volumes may be limited due to the discharge restrictions for the following nutrients: nitrogen, phosphorus, and organic material. Technologies and processes can be used to remove nutrients both from the water within the farm and outside the farm as an end-of-pipe treatment. This study reports water quality of a system combining several end-of-pipe treatment units to recharge RAS effluent into pristine new intake water.

In an RAS, nitrate concentration must be kept below the level that can induce toxic effects for the raised species (100 mg L<sup>-1</sup> NO<sup>3</sup>-N, Chen *et al.* 2002). Typically, about 5% (or 500 L kg<sup>-1</sup> feed day<sup>-1</sup>) of the circulating water is replaced daily with clean water to prevent the accumulation of nitrates and dissolved organic matter (Colt 2006; van Rijn *et al.* 2006). However, nitrogen compounds need to be removed via denitrification when aiming for very low water requirements.

In denitrification, oxidized nitrogen compounds (nitrite, NO<sub>2</sub><sup>-</sup> and nitrate, NO<sub>3</sub><sup>-</sup>) are transformed to gaseous nitrogen (N<sub>2</sub>), typically via heterotrophic microorganisms under anoxic conditions (Beutel *et al.* 2016; Zhao *et al.* 2018). In heterotrophic denitrification, a carbon source acts as an electron donor for microbial growth (Seitzinger *et al.* 2006; Tallec *et al.* 2008; Lindholm-Lehto *et al.* 2021). Denitrification reactors require input of organic carbon and process control, leaving them suitable

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for intensive and large-scale systems (van Rijn *et al.* 2006; von Ahnen *et al.* 2016). Solid carbon sources are cost-effective alternatives to conventional supplemental carbon sources, such as acetate or methanol (Cherchi *et al.* 2009).

A woodchip bioreactor (WB) is a cost-effective technology for nitrogen removal with low-maintenance requirements (Aalto *et al.* 2020; Lepine *et al.* 2020; Lindholm-Lehto *et al.* 2021). Woodchips are a good source of microbially available carbon in anoxic conditions (Warneke *et al.* 2011). Denitrifying bioreactors have previously been studied (Lepine *et al.* 2016; von Ahnen *et al.* 2016) in the case of RAS effluents with high chemical oxygen demand (COD). Indoor RASs with low COD ( $13 \text{ mg L}^{-1}$ ) have raised less attention, but recently they have been studied both in small experiments (Kiani *et al.* 2020; Lepine *et al.* 2020; Lindholm-Lehto *et al.* 2020, 2021), in a pilot scale, and in full-scale applications (Lepine *et al.* 2018; von Ahnen *et al.* 2018, 2019).

Besides nitrogen compounds, leaching of heavy metals, their subsequent migration, and presence in RAS can be problematic and needs to be controlled. Recommended limit values have been identified for some of the trace elements in a rearing tank water, such as Ba ( $<5 \text{ mg L}^{-1}$ ), Cu ( $30 \mu\text{g L}^{-1}$ ), Mg ( $<28 \text{ mg L}^{-1}$ ), P ( $<3 \text{ mg L}^{-1}$ ), and Zn ( $269 \mu\text{g L}^{-1}$ ) (Davidson *et al.* 2009). Metal-containing effluents are conventionally treated with methods based on alkali addition, oxidation, and sorption (Hengen *et al.* 2014), but they often are expensive. Therefore, passive treatments, e.g., constructed wetlands (CWs), have attracted attention in recent years. In CWs, many similar processes occur, such as filtration, sorption, chemical precipitation, settling, and biological oxidation (Vymazal 2010). Metal removal is known to occur most effectively in the pH range of 3.5–6 as they leach at a lower pH (Sen Gupta *et al.* 2009). Additionally, low dissolved oxygen content has been linked to the successful removal of metals, such as Al, Cd, Cu, Fe, Mn, Ni, and Zn (Champagne *et al.* 2008). Dissolved oxygen affects the redox potential of metals, affecting their retention or leaching tendency (Khan *et al.* 2019). Furthermore, the risk of leaching increases when contaminants have accumulated for a long period of time and the pH and redox conditions have changed, leading to their mobility (Ronkanen & Kløve 2009; Palmer *et al.* 2015).

In addition to WBs (von Ahnen *et al.* 2020; Dalsgaard *et al.* 2021), CWs can be a cost-effective alternative for nitrogen removal instead of more conventional technologies. In a CW, the removal and transformation of nitrogen occur via volatilization, adsorption, plant uptake, ammonification, and nitrification–denitrification pathways. Additionally, partial nitrification–denitrification, autotrophic nitrite removal over nitrate (Canon), and anaerobic ammonium oxidation (Anammox) routes have more recently been accepted (Saeed & Sun 2012). The routes for nitrogen removal are often influenced by physicochemical factors: dissolved oxygen, redox potential, temperature, water properties, and the types of soil and plants (Wu *et al.* 2014; Wang *et al.* 2020). Increased understanding of CW processes has led to new designs and enhancement of treatment conditions via artificial aeration, the addition of carbon sources, feeding wastewater at several input points (step-feeding), and effluent recirculation.

Besides nutrients, the removal of particulate matter is an essential part of water treatment in an RAS. This is needed to avoid system blockages, excessive growth of microorganisms, or off-flavor-related issues. A variety of equipment and processes are available for removing larger particles during the water circulation, including drum filters, swirl separators, and clarifiers (Lindholm-Lehto *et al.* 2021). However, the infiltration of outlet water through sand can remove dissolved and particulate matter and improve the water quality via the retention of dissolved organic compounds in the soil (Wu *et al.* 2010; Lindroos *et al.* 2016). The method is often applied in the formation of natural ground water (Lindroos *et al.* 2016; Lindholm-Lehto *et al.* 2021) and in artificial groundwater recharge (AGR, Peters 1998) for the production of drinking water (Kolehmainen *et al.* 2008).

In our previous laboratory-scale studies (Lindholm-Lehto *et al.* 2020, 2021) and by constructing a pilot-scale system (Pulkkinen *et al.* 2021), we studied the possibility to recharge the RAS effluent into pristine intake water using a three-stage, passive water treatment field. In our previous laboratory-scale study (Lindholm-Lehto *et al.* 2020), we showed that a passive water treatment loop with denitrification in a WB followed by sand filtration is suitable for rearing rainbow trout (*Oncorhynchus mykiss*). In this study, we scaled up to pilot-scale and studied the water quality when passively treating the circulating water in the water treatment field. We hypothesized that circulating water can effectively be treated into pristine intake water in a low-maintenance water treatment field without leaching unwanted trace elements or problems related to high anion concentrations. This is a follow-up study of the experiment reported in Pulkkinen *et al.* (2021). They were the first to report the new design of the pilot-scale system, while in this study, we concentrated especially on the behavior of selected trace elements. Especially, many micro and macro elements, many of which known to have hazardous properties, were studied. Additionally, we studied different anions to assess the removal of nitrates from the system as well as the dissolution of carbohydrates of woodchip-origin into the circulating water.

## 2. MATERIALS AND METHODS

### 2.1. Experimental description

#### 2.1.1. Experimental setup

The experiment was performed in a raceway-type RAS (FREA Aquaculture solutions, Denmark) at the Natural Resources Institute Finland (Luke) at the Laukaa fish farm. The experiment was started in August 2019 and continued for 11 months until June 2020.

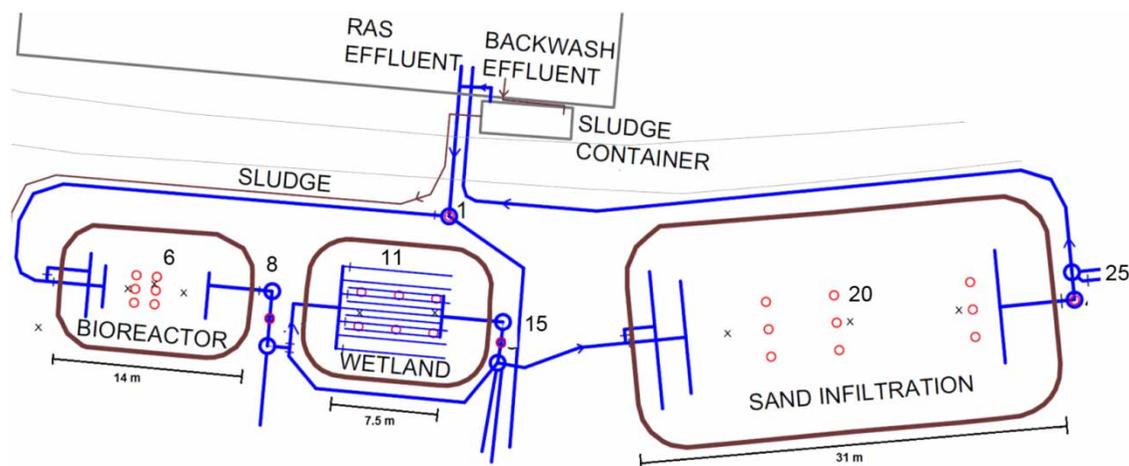
The full description of the pilot-sized RAS has been reported in Pulkkinen *et al.* (2021). In short, the RAS includes two identical units (each 25 m<sup>3</sup> in total). Both units have two 5 m<sup>3</sup> raceway fish tanks, collection of uneaten feed and settleable solids, a drum filter with a mesh size of 60 µm (Hydrotech HDF800, Veolia, France), two parallel 2.5 m<sup>3</sup> fixed-bed bioreactors, filled with 1.5 m<sup>3</sup> saddle-chips (KSK Aqua, Denmark), a degassing unit (2.24 m<sup>2</sup> in size), a 0.74 m<sup>3</sup> pump sump, and a low-head oxygenator (FREA Aquaculture Solutions, Denmark). The pH was adjusted by dosing dissolved sodium bicarbonate and calcium hydroxide into the pump sumps (EJ-R, Iwaki, Japan). The reared fish, rainbow trout (*O. mykiss*) and European whitefish (*Coregonus lavaretus*), were fed at a feeding rate of max. 10.1 kg day<sup>-1</sup> with a feeding system T Drum 2000 (Arvo-Tec, Finland). Clean replacement water was taken from an oligotrophic Lake Peurunka (62.44886, 25.85201, 694 ha, 59,600 m<sup>3</sup>) with an average flow of 4.4 L min<sup>-1</sup> (2.5–5.1 L min<sup>-1</sup>, 3.6–7.3 m<sup>3</sup> day<sup>-1</sup>). Water was discharged into the water treatment field at the rate of 1,500–7,000 L day<sup>-1</sup> which circulated back to the RAS at 1,000–3,800 L day<sup>-1</sup>.

Sludge was collected via sludge cones, drum filters, and from the backwash of the fixed-bed bioreactors as described in Pulkkinen *et al.* (2021). The sludge treatment was operated in batches, starting by pumping water into a coagulation tank and then dosed with the coagulant (polyaluminum chloride, PAX-XL100, Kemira, Finland). Then, a flocculation agent was added and the pH was adjusted to pH 7. The sludge was separated by sedimentation and pumped to a local municipal water treatment plant. The aqueous supernatant was led into the water treatment field.

#### 2.1.2. Passive water treatment field

The passive water treatment field consisted of a WB, a CW, and a sand filter (SF) (Figure 1). The full and more detailed description of the passive water treatment field and each of its units has been reported in Pulkkinen *et al.* (2021).

The WB was filled with fresh unbarked silver birch (*Betula pendula*) woodchips (<5 cm, effective porosity  $n_e$  0.65), with dimensions of 14 m (length), 9 m (wide), and 1.5 m (depth). The WB contained a maximum of 50 m<sup>3</sup> saturated unbarked birch woodchips, 1 m in depth, and a 0.5 m thick dry woodchip layer on top to act as isolator. In this experiment, it was operated at 26.3 m<sup>3</sup>.



**Figure 1** | Schematic diagram of the passive water treatment field with the woodchip bioreactor, constructed wetland, and a sand filtration unit. All sampling locations have been marked with numbers. 1: Water from the RAS into the water treatment system, 6: water in the bioreactor, 8: the bioreactor outflow, 11: water in the wetland, 15: the wetland outflow, 20: sand infiltration, 25: sand infiltration outflow and water back to circulation (Pulkkinen *et al.* 2021). Blue arrows show the routes of circulating water and brown arrows show the routes of sludge. Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/wqrj.2022.030>.

The CW (length/width ratio of 1.25, 7.5 m × 6 m) was built to remove nitrogen and phosphorous from the system, but also to control biological oxygen demand (BOD) and dissolved organic carbon (DOC) originating from the WB. The CW contained three layers of gravel with particle sizes of 1–3, 4–8, and 2–4 mm. The wetland was designed for 7 g BOD<sub>7</sub> m<sup>-2</sup> day<sup>-1</sup> and a hydraulic loading rate of 278 mm day<sup>-1</sup>. Additionally, the aeration of the water was designed to occur in this stage after the anaerobic WB stage. The common reed (*Phragmites australis*) was chosen for the CW because it is known to be efficient at removing nutrients, especially nitrogen species (Vymazal 2011). It can also enhance BOD removal via its oxygen-rich rhizosphere and aerobic microbial degradation processes. However, after only 1 year of operation, the common reeds were not fully grown.

The SF (16 m × 31 m, depth of 2 m, total volume of 650 m<sup>3</sup>) was designed to mimic the infiltration rate of water in the AGR plants and remove suspended solids (SSs) and DOC (Lindroos *et al.* 2020). The water velocity was 1–4 m day<sup>-1</sup> with a retention time of 10 days and an infiltration rate of 12.5 m<sup>3</sup> day<sup>-1</sup>. The SF was operated as a saturated water flow to achieve a better DOM reduction than in an unsaturated vertical water flow (Lindroos *et al.* 2002, 2020).

The sand was classified as coarse sand (0.6–2.0 mm) with a mean effective porosity ( $n_e$ ) of 0.35 (0.32–0.37, Pulkkinen *et al.* 2021), composed of quartz and feldspars and a smaller proportion of dark mafic minerals (GTK, unpublished results). After the SF, water was pumped into a 3-m<sup>3</sup> reservoir tank in the research hall before returning back to the RAS units. During the first 7 months, water from the treatment field was not introduced back to the RAS.

### 2.1.3. Monitoring of water quality

Dissolved oxygen (Oxi:lyser, s::can, Austria), dissolved carbon dioxide (Franatech, Germany), temperature, and water flow rate (Fluxus F501, Flexim, Germany), and the pH (ProMinent, Germany) were measured online every 6 min from the fish tanks of the RAS units (Pulkkinen *et al.* 2021). The oxygen saturation was kept above 80% in the fish tanks by injecting oxygen and aerating to maintain the CO<sub>2</sub> concentration at 15–18 mg L<sup>-1</sup>. Additionally, the flow rate of the inlet water (Watson Marlow 630, Spirax-Sarco Engineering, UK) from Lake Peurunka and from the water treatment field (Flexim) were measured online and collected (con::cube, s::can, Austria).

Nitrite-N, nitrate-N, and TAN were monitored weekly by quick spectrophotometric laboratory tests (Procedure 8038 Nessler, LCK341/342, LCK340, and LCK349 UN3316 9 II, respectively, DS 3900, Hach, USA). The alkalinity was measured using a standard titration method (ISO 9963–1:1994, TitraLab AT1000, Hach, Loveland, USA) and the turbidity was measured with a Hach DR 3900 Turbidimeter. All the results of the weekly water quality monitoring have previously been reported in Pulkkinen *et al.* (2021).

## 2.2. Laboratory analysis

### 2.2.1. Sample collection

Aqueous samples were collected from the fish tanks, from locations marked with numbers 1, 6, 8, 11, 15, 20, and 25 in the water treatment field (Figure 1), and from the inlet water from Lake Peurunka. Grab samples were collected on average once a month for 11 months. The samples were stored in new 250 mL high-density polyethylene (HDPE) plastic jars with HDPE plastic caps and kept frozen at –22 °C.

Additionally, samples from the RAS, and locations 1, 8, 15, and 25 were taken and stored in 60 mL capped HDPE plastic jars. The stored samples were cooled at +2 °C and sent to ALS Finland Oy for elemental analysis.

### 2.2.2. Elemental analyses

Elemental analyses of selected elements were purchased from ALS Finland Oy. They used a standard operation procedure (W-METMSDG2) including filtration through a 0.45-µm filter, mineralization, and Inductively coupled plasma mass spectrometry (ICP-MS) analysis. The limits of reporting (LORs) for the selected elemental analyses were as follows: Al 5 µg L<sup>-1</sup>, Ba 0.50 µg L<sup>-1</sup>, Ca 50 µg L<sup>-1</sup>, Cr 0.20 µg L<sup>-1</sup>, Cu 1.0 µg L<sup>-1</sup>, Fe 2.0 µg L<sup>-1</sup>, K 50 µg L<sup>-1</sup>, Li 1.0 µg L<sup>-1</sup>, Mg 3.0 µg L<sup>-1</sup>, Mn 0.50 µg L<sup>-1</sup>, Mo 1.0 µg L<sup>-1</sup>, Na 30 µg L<sup>-1</sup>, P 50 µg L<sup>-1</sup>, Pb 0.5 µg L<sup>-1</sup>, U 0.10 µg L<sup>-1</sup>, and Zn 2.0 µg L<sup>-1</sup>.

### 2.2.3. Carbohydrates: instrumentation and chemical analysis

Analyses of carbohydrates were conducted on a Thermo Scientific Dionex Integriion HPIC ion chromatography equipment (Sunnyvale, CA, USA) using Chromeleon 7.2 software. The equipment consisted of a gradient pump (0–6,000 psi), eluent generator (EDC 500 KOH), a pre-column (CarboPac™ PA210 (2 mm × 30 mm – 4 µm, at 30 °C), a microbore analytical column (CarboPac™ PA210 (2 mm × 150 mm – 4 µm, at 30 °C)), an electrochemical detector with an AgCl electrode, an Ag/AgCl

reference electrode (0.5 s rise time, 2.0 Hz data collection), and an autosampler (AS-AP). Elution was performed via an isocratic 35 min run at 12 mM KOH with a flow rate of 0.2 mL min<sup>-1</sup>. The inlet pressure was about 3,000 psi, column temperature at 30 °C, and sample injection volume 2.5 µL.

First, a 12 mM buffer solution of sodium acetate was prepared to maintain a stable sample pH. Then, 10 mL of aqueous sample from the passive water treatment field was pipetted into a vial (50 mL, VWR). Carboxy methyl cellulose (CMC) Fifix 1500 (lot 28096 6.2.1989) was used as a method check standard and 25 mg of dry CMC powder was weighted in a vial. Volume of 15 mL of a buffer solution was added and the pH was adjusted to an optimum range of pH 4.5–5.5 with acetic acid (100%, glacial, Merck). This was followed by 150 µL of buffered enzyme solution (in 12 mM NaOAc), containing cellulase (20,000 U/g), hemicellulase (10,000 U/g), and lipase (20,000 U/g, Enzyme Blend for alcohol GCE ASE3114, Creative Enzymes). The sample was stirred thoroughly and placed in a water bath at 50 °C for 2 days. Samples of each sampling point were prepared in duplicate. After 2 days, the enzyme was inactivated by heating the vials at 90 °C for 15 min before removing from the water bath and leaving them to cool down. Each sample was filtered through a 0.2-µm filter (GHP, Acrodisc 130) into a vial for analysis. Any effects of the enzyme solution, containing cellulase, hemicellulase, and lipase were extracted from the results.

#### 2.2.4. Carbohydrates: method validation

Standard solutions of L-rhamnose (≥99%, Sigma-Aldrich, C<sub>6</sub>H<sub>12</sub>O<sub>5</sub>), α-D-glucose (anhydrous, 96%, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>), sucrose/saccharose (≥99.5%, Sigma-Aldrich, C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>), D-(–)-fructose (≥99%, Sigma-Aldrich, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>), D-(+)-raffinose (≥ 98%, Sigma-Aldrich, C<sub>18</sub>H<sub>32</sub>O<sub>16</sub>), and stachyose (≥99%, Sigma-Aldrich, C<sub>24</sub>H<sub>42</sub>O<sub>21</sub>) were prepared at concentrations of 0.1; 0.2; 0.5; 1; 2; 5; 10; and 20 mg L<sup>-1</sup>, by diluting pure standards in UHQ water (internal resistance ≥18.2 Ω at 25 °C) using a Millipore (Bedford, MA, USA). Method validation was performed similarly as described in Destandau *et al.* (2005) and in Lindholm-Lehto *et al.* (2020). In short, limits of detection (LODs), limits of quantification (LOQs), and linearities were determined (Table 1). Furthermore, method precision and accuracy were determined as explained in Lindholm-Lehto *et al.* (2020). The interday and intraday repeatabilities and precision have been presented in Supplementary Table S1 and accuracy in Supplementary Table S2.

#### 2.2.5. Anions

Chloride (Cl<sup>-</sup>), nitrite (NO<sub>2</sub><sup>-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), sulfate (SO<sub>4</sub><sup>2-</sup>), and phosphate (PO<sub>4</sub><sup>3-</sup>) anions were monitored throughout the experiment as stated in section 2.2.1. The pretreatment of samples by solid phase extraction (SPE) has previously been reported in Lindholm-Lehto *et al.* (2020) and in short in Supplementary material (pretreatment and analysis of anions).

The chromatographic analysis was conducted on a Thermo Scientific Dionex Integriion HPIC ion chromatography equipment (Dionex, Sunnyvale, CA, USA) with the Cromeleon 7.2 software. It consisted of a gradient pump (0–6,000 psi), eluent generator (EDC 500 KOH), a guard column Dionex IonPac™ NG1 (2 × 50 mm), a pre-column (Dionex IonPac™ AG19 (2 × 50 mm – 4 µm), and an analytical column Dionex IonPac™ AS-19 (2 × 250 mm – 4 µm at 30 °C). The full description of the analysis method has been reported by Lindholm-Lehto *et al.* (2020). After transforming the method in a newer version of the HPIC equipment, the validation has been re-performed (Supplementary Tables S3–S5). The LODs ranged between 0.018–0.131 mg L<sup>-1</sup> and LOQs from 0.020 to 0.175 mg L<sup>-1</sup>, showing even lower LODs and LOQs and increased precision compared to those reported in Lindholm-Lehto *et al.* (2020).

**Table 1** | LODs, LOQs, and linearities (R<sup>2</sup>) of selected carbohydrate solutions (1–20 mg L<sup>-1</sup>) for ion chromatography (IC) analysis

Carbohydrates	LODs	LOQs	Linearity, R <sup>2</sup>
L-Rhamnose, mg L <sup>-1</sup>	0.276	0.642	0.9997
D-Glucose, mg L <sup>-1</sup>	0.342	0.986	0.9998
Saccharose, mg L <sup>-1</sup>	0.306	0.669	0.9994
D-Fructose, mg L <sup>-1</sup>	0.209	0.542	0.9998
D-Raffinose, mg L <sup>-1</sup>	0.289	0.709	0.9998
Stachyose, mg L <sup>-1</sup>	0.189	0.464	0.9996

### 2.3. Statistical analysis

Statistical analyses of elements were performed using IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corporation, released 2019). The differences in the mean values of elemental analyses between sampling points were tested using an independent sample *t*-test ( $n = 8$ ) with confidence interval at 95%.

## 3. RESULTS AND DISCUSSION

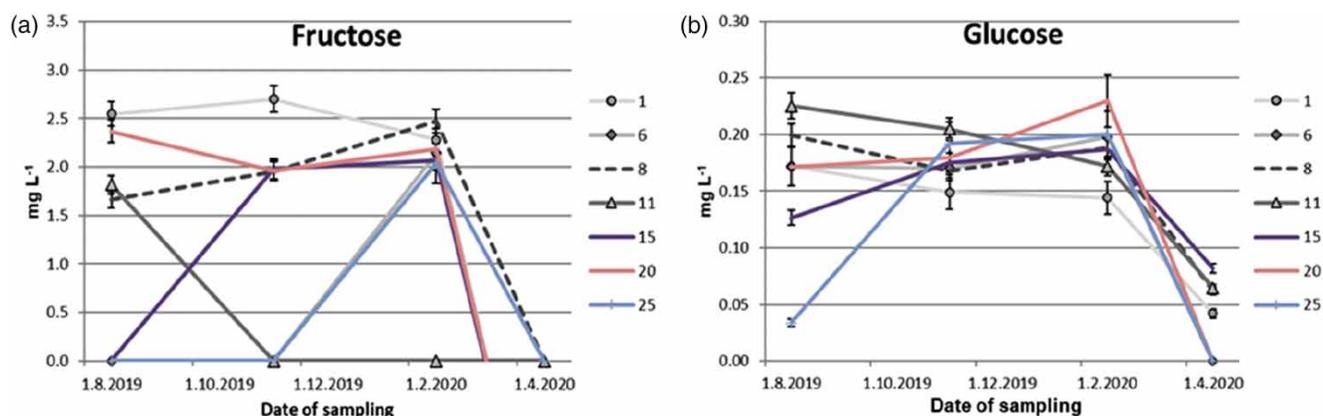
### 3.1. Carbohydrates

Among the studied carbohydrates, glucose and fructose were detected (Figure 2(a) and 2(b)), while galactose, saccharose, raffinose, and stachyose remained below the LODs. Fructose is a common monosaccharide found in many plants and often bonded to glucose units, while glucose is the main monosaccharide forming cellulose. Cellulose is a linear homopolysaccharide ( $\beta$ -D-glucopyranose, linked together by 1–4 glycosidic bonds (Alén 2000)). In hardwoods, the primary hemicellulose components are O-acetyl-(4-O-methylglucurono)-xylan and glucomannan to a smaller extent. Additionally, xylan contains small amounts of L-rhamnose and galacturonic acid.

Birch wood contains around 40% dry weight (DW) of cellulose and 36% DW of hemicelluloses (1–6, Sjöström 1993; Przybysz Buzala *et al.* 2019). The glucopyranose units are equatorially oriented, making the cellulose chain very stable (Alén 2000). Cellulose has a strong tendency in intramolecular and intermolecular hydrogen bonding, leading to the formation of microfibrils with crystalline and amorphous regions. All this means that cellulose is relatively inert in chemical treatments and soluble in only a few solvents. The chemical and thermal stabilities of hemicelluloses are lower due to their lower degree of polymerization (DP 100–200) and heterogeneity than cellulose. This is supported by the low concentrations of glucose detected in this study (Figure 2(b)).

Wood material is expected to function as supplemental carbon for denitrification for at least 5–15 years (Schipper *et al.* 2010). Carbohydrates are considered as the main source of carbon for denitrification also in this study. In plant biomass and in woodchips, fibers of cellulose and hemicelluloses are coated with lignin, which restricts the access of microbial-induced degradation of polysaccharides (Alvira *et al.* 2010). The C:N ratio of woodchips declines over time as carbon is consumed in the denitrification process (Christianson *et al.* 2020).

Fructose was the main detected carbohydrate component (Figure 2). Fructose has been used in some experimental fish feeds (Jiang *et al.* 2014), but it could also originate from cellulosic biomass in the WB. A variety of microorganisms, including bacteria and fungi, are able to degrade cellulosic biomass into glucose monomers (Kumar *et al.* 2008). The microbial degradation and bioconversion of lignocellulose is affected by physical parameters such as pH, temperature, adsorption, chemical factors (N and P), the presence of phenolic compounds, and other inhibitors (Kumar *et al.* 2008). In this study, there were occasionally elevated temperatures in the woodchip bed and a variety of chemical components (Lindholm-Lehto *et al.* 2020, 2021) in the WB which may have promoted the biodegradation of wood material.



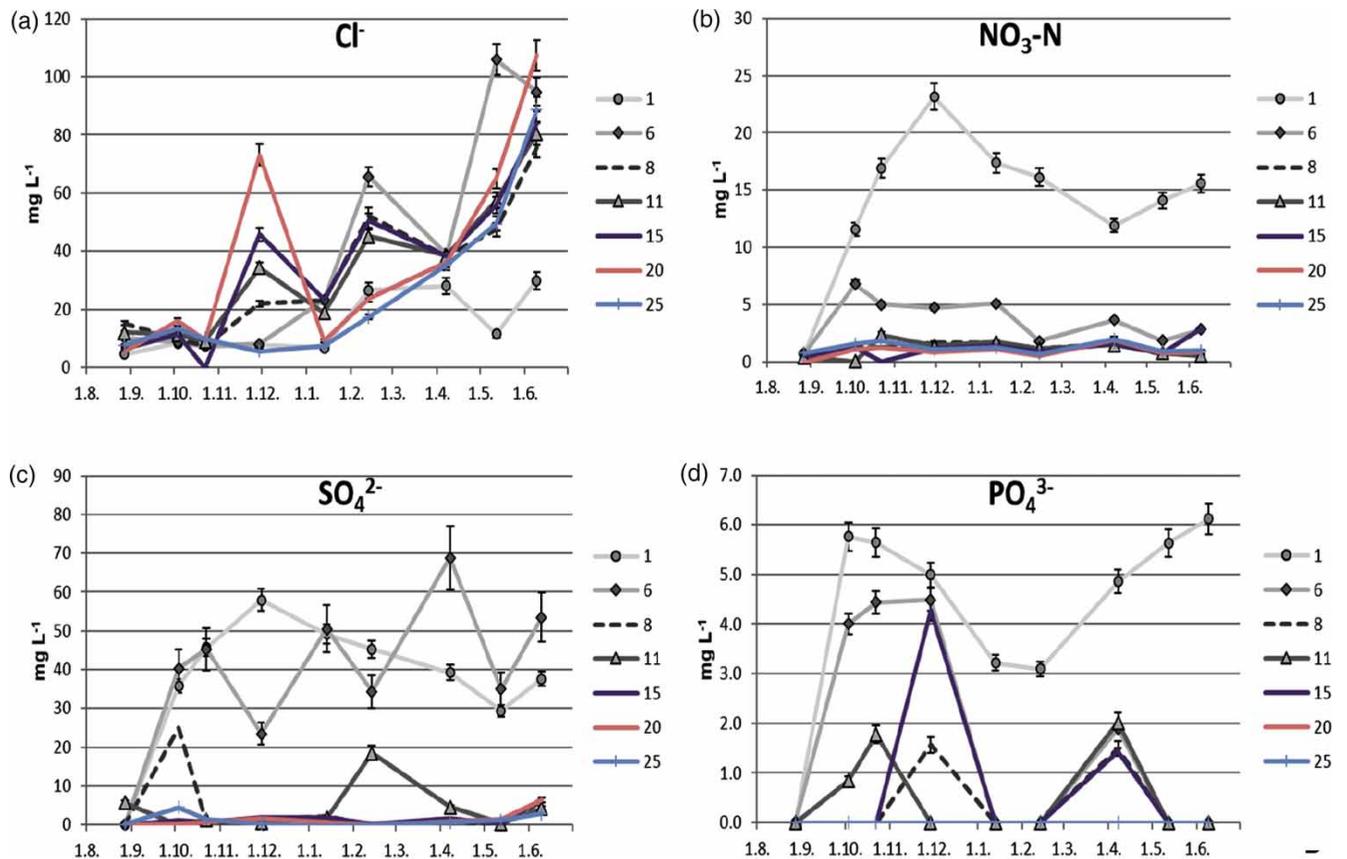
**Figure 2** | Concentrations ( $\text{mg L}^{-1}$ ,  $\pm$ SD,  $n = 4$ ) of (a) fructose and (b) glucose in the water treatment field at sampling points 1 (inlet to the water treatment field), 6, 8, 11, 15, 20, and 25 (outlet from the water treatment field) from August 2019 to April 2020.

### 3.2. Anions

During the experiment, the concentrations of chlorine increased at all sampling points (Figure 3(a)) and from 10 mg L<sup>-1</sup> to above 100 mg L<sup>-1</sup>. Cl<sup>-</sup> is a mobile anion, which is not easily retained by the soil material, and Cl<sup>-</sup> seems to flow through the system. Birch wood contains chlorine, which is readily soluble in water, originating from the soil in the place of growth (Werkelin *et al.* 2005). Woodchips are a likely source of chlorine because no additional chlorine was introduced to the system. Chlorine can be accumulated by wood based on its place of growth and concentrations of 70–110 mg Cl kg<sup>-1</sup> DW in birch wood (*Betula pubescens*) and 40–330 mg kg<sup>-1</sup> in birch bark have been found (Werkelin *et al.* 2005). It is possible that soluble chlorine accumulated in the system because only a small proportion of water was replaced (3.6–7.3 m<sup>3</sup> day<sup>-1</sup>). Later on, the concentrations might decrease to a lower level as all the chlorine of woodchips will have dissolved and will be removed in mobilized ionic form.

The overall concentrations of nitrate remained at a low level (below 25 mg L<sup>-1</sup>) throughout the experiment. Expectedly, the highest concentrations were detected at sampling point 1 before the WB, while at the latter sampling points, the concentrations remained below 5 mg L<sup>-1</sup>, suggesting a functional denitrification stage. At each sampling point, the nitrate concentrations were relatively constant throughout the seasons (Figure 3(b)).

At the beginning of the experiment, the concentrations of sulfate increased to 60 mg L<sup>-1</sup> in the inlet flow to the water treatment field (1) over the course of first 3 months (Figure 3(c)) and later decreased to about 40 mg L<sup>-1</sup>. In the WB (6), the concentrations increased to about 70 mg L<sup>-1</sup>, while at the other points, they mostly remained below 20 mg L<sup>-1</sup>. Overall, the concentrations decreased during the treatment stages, which suggests that sulfate originated in the RAS before entering the water treatment field. However, sulfate might have occasionally been produced in the WB since increased concentrations were observed at point 6. In latter stages, concentrations decreased to below 20 mg L<sup>-1</sup> before entering back to the RAS. This



**Figure 3** | Concentrations (mg L<sup>-1</sup>, ±SD, n = 4) of (a) chlorine (Cl<sup>-</sup>), (b) nitrate-N (NO<sub>3</sub>-N), (c) sulfate (SO<sub>4</sub><sup>2-</sup>), and (d) phosphate (PO<sub>4</sub><sup>3-</sup>) in the water treatment field at sampling points 1 (inlet to the water treatment field), 6, 8, 11, 15, 20, and 25 (outlet from the water treatment field, see Figure 1) from September 2019 to June 2020.

is of high importance because reduced sulfur in the form of  $\text{H}_2\text{S}$  would be extremely toxic to the raised species. During the experiment, the WB was mostly nitrate-limited (Pulkkinen *et al.* 2021), which promotes sulfate reduction (Lepine *et al.* 2016).

The highest concentrations of phosphate ( $6 \text{ mg L}^{-1}$ ) were observed at sampling point 1 (Figure 3(d)). The concentrations ranged from 2 to  $4 \text{ mg L}^{-1}$  at sampling points 6 and 8 and this suggests that phosphate was mostly utilized in the woodchip biofilter. This is in agreement with the results reported by Pulkkinen *et al.* (2021). At sampling points 11 and 15, the concentrations remained at  $1 \text{ mg L}^{-1}$  and at points 20 and 25 they were below the LOD.

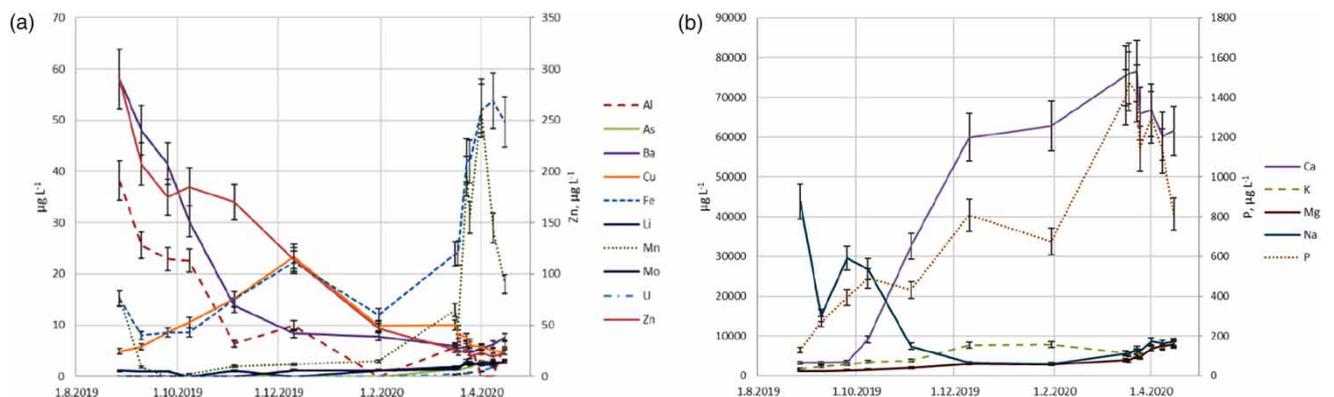
### 3.3. Elemental analysis

In the RAS tank water, selected elements were analyzed (Figure 4). In September and October, the concentrations were high overall (Figure 4(a)) but then decreased to a lower level. However, in April, when water from the treatment field was introduced to the RAS, the concentrations of Fe and Mn decreased rapidly and remained below the limit values set, e.g., for drinking water (European Union (EU)). The concentrations of P and Ca also increased during the experiment (Figure 4(b)), but this was mostly due to increased feed (P) and calcium hydroxide to protect the raised species from the increased Al and Zn levels.

The concentrations of selected elements were clearly below the recommended limit values listed by Davidson *et al.* (2009). Only the concentrations of Zn were close to the recommended upper limit of  $269 \mu\text{g L}^{-1}$  in the spring 2020 in the rearing tank water (Figure 4(a)). However, the limit values given by Davidson *et al.* (2009) were relative to a hardness of  $305 \text{ mg L}^{-1}$  as  $\text{CaCO}_3$ . At a lower hardness, the limit values are much lower. For example, at a total hardness of  $20 \text{ mg L}^{-1}$ , similar to the water hardness of this study, the limit value for Zn decreases to  $30 \mu\text{g L}^{-1}$  (Shuhaimi-Othman *et al.* 2012).

In the inlet water from Lake Peurunka, there was an increase in the concentration of Zn up to  $60 \mu\text{g L}^{-1}$  (Supplementary Fig. S1) which might have been an affecting factor. The inlet water might have been the source of Zn in the system despite the low water renewal rate ( $3.6\text{--}7.3 \text{ m}^3 \text{ day}^{-1}$ ). Additionally, the tank material, valves, and fittings were made of stainless steel which contained Zn. It was observed that Zn originated from a main pump made of steel which was in contact with the aluminum fish tanks. This created an electrochemical cell, leading to the release of elemental Zn. In the beginning of the experiment, high concentrations of Zn ( $290 \mu\text{g L}^{-1}$ ) were detected, which were released by the material. High levels of Zn and Cu can cause sudden mortalities (Wedemeyer 1996) and unfortunately, also in this case, mortalities of the raised species could not be avoided. After detecting the release of Zn, a plastic layer was added between the metal surfaces and this prevented the Zn release.

Woodchips can contain a variety of metals and other trace elements based on the wood species and place of growth (Świełtik *et al.* 2012), such as Ni and Cu (Komanicka *et al.* 2013). Lepine *et al.* (2020) reported that after 3 weeks of operation leaching of metals from the denitrifying WB for white ash (*Fraxinus americana*) and Norway maple (*Acer platanoides*) outflows ceased and remained at a low level below any known concern. Even in this study, the water treatment field was flushed before introducing the water back into circulation and the rearing tanks, leading to decreasing concentrations of certain elements (Figure 4). Certain trace elements remained below the LODs throughout the experiment, such as Cd, Ni, Sb, Ti,



**Figure 4** | (a) Concentrations of aluminum (Al), arsenic (As), barium (Ba), copper (Cu), iron (Fe), lithium (Li), manganese (Mn), molybdene (Mo), uranium (U), and zinc (Zn, axis on the right-hand side), and (b) concentrations of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), and phosphorous (P, axis on the right-hand side) in the pump sump of the RAS ( $\mu\text{g L}^{-1}$ ).

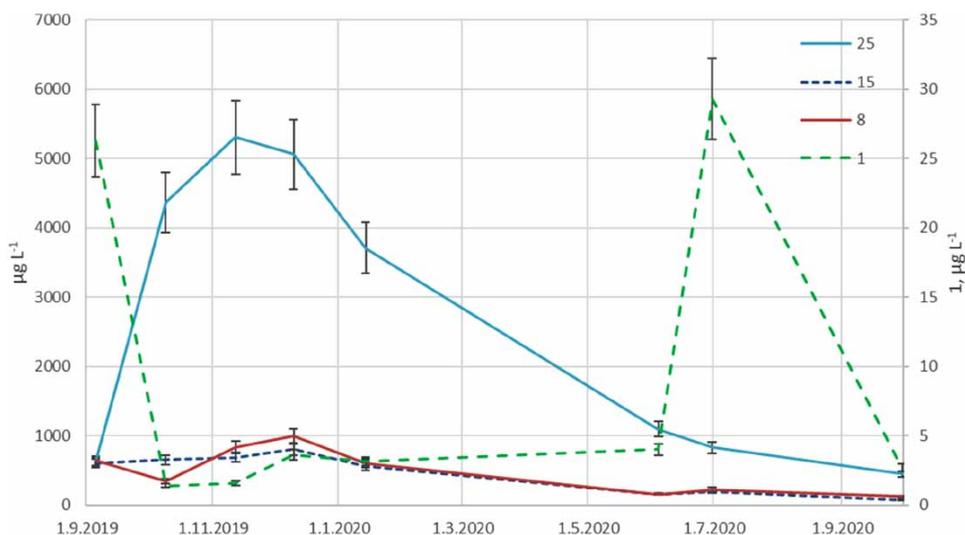
and Th. This observation was in agreement with previous studies by *Lepine et al. (2020)*, *Lindholm-Lehto et al. (2020)*, and *Lindholm-Lehto et al. (2021)*.

Significant differences ( $p < 0.05$ ) were found for most of the studied elements between the inlet water and the rearing tank water in the RAS (Supplementary Table S6). This emphasizes the importance of good quality inlet water. However, the concentrations of the selected elements in the RAS rarely showed any significant difference ( $p < 0.05$ ) compared to those in the sludge supernatant (Supplementary Fig. S2B; Supplementary Table S7).

Even at the start-up of the system and during the flushing period, Mn might have transferred from the previous stages into the sand infiltration. Even in the previous small-scale experiment, large concentrations of Mn were detected after the start-up of the system which decreased later to a lower level (*Lindholm-Lehto et al. 2020*). According to the results, Mn was readily soluble in water throughout the water treatment field (Figure 5). During the sand infiltration, water might have flown unevenly through the filtration stage, creating direct pathways with varying oxic and anoxic conditions. Mn was clearly leached during the sand infiltration, reaching a concentration of  $5 \text{ mg L}^{-1}$ , but decreased after that to below  $1 \text{ mg L}^{-1}$  (Figure 5). Low oxygen concentration can be an important factor promoting Mn mobilization in water systems (e.g. *Hatva 1989*). High Mn concentrations could also be promoted by fine particles that are mobilized especially during the initial stages of infiltration. The sand material might contain microparticles that are liberated from the coatings on mineral grains. In anoxic conditions, they can lead to the dissolution of Fe and Mn coatings (*Hofmann & Schöttler 1998*). The water samples were filtered using  $0.45 \mu\text{m}$  membrane filters, but very fine particles may pass through the filter (*Lahermo et al. 2002*). An important source of Mn and Fe can be organic matter in dissolved or particulate form in water, and bacterial processes (*Kabata-Pendias & Bendias 1992*). Mn and Fe were undoubtedly transported to the sand filter at least to some extent in organic matter from the previous stages in the passive water treatment field.

The inflow to the water treatment field and after the woodchip biofilter showed a significant difference ( $p < 0.05$ ) in the case of several elements (Supplementary Tables S8 and S9). However, significant differences ( $p < 0.05$ ) were found only for a few elements between the woodchip biofilter and the constructed wetland (sp8 and sp15, Supplementary Table S10), and between the constructed wetland and the sand infiltration (sp15 and sp25, Supplementary Table S11). This suggests that precipitation and other physicochemical phenomena occurred in the water treatment field.

From November 2019, the conditions were anoxic in the WB with a total-Fe concentration of  $1.47 \text{ mg L}^{-1}$  of which  $1.01 \text{ mg L}^{-1} \text{ Fe}^{2+}$  (Supplementary Fig. S3). After the constructed wetland, the concentrations were  $2.14 \text{ mg L}^{-1}$  for Fe-tot and  $1.13 \text{ mg L}^{-1}$  for  $\text{Fe}^{2+}$ . During the sand infiltration, the total-Fe was  $2.75 \text{ mg L}^{-1}$  and  $\text{Fe}^{2+}$   $0.623 \text{ mg L}^{-1}$  and after the sand infiltration the concentration of total-Fe was  $0.97 \text{ mg L}^{-1}$ , of which  $0.223 \text{ mg L}^{-1}$  was  $\text{Fe}^{2+}$ . Typically, the presence of  $\text{Fe}^{2+}$  indicates oxygen deficiency. This suggests that the sand infiltration was not entirely anoxic and Fe was partly refrained. It is possible that the circulating water flowed unevenly through the sand infiltration. Fe is controlled by many



**Figure 5** | Concentrations of manganese (Mn) at the water treatment field sampling locations: 1: water from the RAS into the water treatment system, 8: bioreactor outflow, 15: wetland outflow, and 25: sand infiltration outflow back into circulation ( $\mu\text{g L}^{-1}$ ).

factors in water–soil systems, including acidity, the formation of Fe–organic complexes, leaching of humus-rich material, mobilization of microparticles, and reducing conditions when organic matter is degraded (Hatva 1989; Lovely *et al.* 1991; Frycklund & Jacks 1997; Hofmann & Schöttler 1998; Lahermo *et al.* 2002). Many of these processes could have affected the Fe concentrations and they could have interacted with each other.

Trace elements can be transferred into an RAS via the inlet water and fish feed (van Bussel *et al.* 2014), but they can also leach in from pipes or fittings (Davidson *et al.* 2009). Additionally, the process design with a woodchip-based denitrification and a sand filter are both potential sources of trace elements.

According to the EU's drinking water standard (Council Directive 98/83/EC), the limit value for Fe is set at  $0.2 \text{ mg L}^{-1}$  and for Mn at  $0.05 \text{ mg L}^{-1}$ . For Mn, the limit value was exceeded in the fall in 2019 at sampling points before and after the water treatment field (1 and 25, Figure 5) and again in the spring of 2020 at sampling point 15. Concentrations of Mn and Fe remained low in the inlet water from Lake Peurunka and in the RAS tank water, suggesting that these elements originated from the processes in the water treatment field. Concentrations of Fe first peaked in the spring 2020 in the sand infiltration, but then decreased back to below the  $1 \text{ mg L}^{-1}$  level. This was not observed in the other sampling locations, suggesting that Fe was of sand filter-origin. The increase in concentrations suggests that the conditions in the sand infiltration became more anoxic and that there was more Fe in a soluble form. Generally, the concentrations of selected elements were relatively low, excluding a few exceptions, and remained below the limit values of acute toxicity to aquatic life (US EPA 2017). This is of high importance because none of the trace elements must pose a risk towards the raised species or consumers in the case of full-scale applications.

#### 4. CONCLUSIONS

This study evaluated water quality in a pilot-scale RAS applying a low-maintenance water treatment field to recharge the effluent to become pristine intake water by denitrification and particle removal. A wide variety of elements were monitored throughout the different process stages and mostly showed very low concentrations which were below the limit values of known toxicity or known concern. Only high concentrations of dissolved Mn and Fe were detected after the sand infiltration, suggesting that the conditions leading to their dissolution were due to partly anoxic conditions in the sand infiltration or due to the mobilization of fine particles. The inlet water from Lake Peurunka contained only low concentrations of the studied elements, but occasionally increased levels of aluminum were detected, but not in the rearing tank or in the water treatment field. Additionally, the results suggested that the  $\text{NO}_3\text{-N}$  concentrations decreased in the WB due to a successful denitrification. Mostly, the concentrations of detected anions were low and decreased during the processes in the water treatment field. Only the concentrations of chlorine were relatively high ( $>100 \text{ mg L}^{-1}$ ) in all sampling points of the water treatment field, possibly originating from the wood material and its place of growth. Finally, we studied carbohydrates of woodchip-origin in the circulating water and found glucose and fructose at low levels. Overall, the results show that passive water treatment seems sufficient for treating recirculating water which can be re-used in an RAS.

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#### AUTHORS' CONTRIBUTIONS

The experiment was planned by J.V., T.K., J.T.P., and P.C.L.-L. . P.C.L.-L. and J.T.P. planned and conducted the sampling, while P.C.L.-L. was responsible for the method development and the chemical analyses. The manuscript was drafted by P.C.L.-L. and A.-J.L. T.K., J.T.P., and J.V. critically examined and revised the manuscript.

#### AVAILABILITY OF DATA AND MATERIALS

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

#### CONFLICTS OF INTERESTS

The authors declare that they have no competing interests.

## ETHICS APPROVAL

Ethical approval of this study was obtained from the Finnish Food Authority, and the experiment was performed in accordance with the guidelines of Directive 2010/63/EU (Directive 2010/63/EU on the protection of animals used for scientific purposes).

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## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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