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# Filtration of Nordic recirculating aquaculture system wastewater: Effects on microalgal growth, nutrient removal, and nutritional value

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ABSTRACT

Microalgal bioremediation of recirculating aquaculture system (RAS) wastewater represents an alternative for wastewater treatment with the potential to generate valuable biomass. This study evaluated the effects of removing biological contamination and suspended solids from Nordic area RAS wastewater through filtration with 0.45 μm filters on the performance and nutritional value of microalgae. All three tested green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) were able to grow in raw (unfiltered) and filtered RAS wastewater. Cultivation in raw RAS wastewater decreased the ω-3 and ω-6 fatty acid content of *H. pluvialis* as compared to filtered wastewater, while no differences in cell density, nutrient removal, or fatty acid and amino acid contribution were seen for any microalgae between the treatments. Filtration of wastewater significantly reduced the content of actinobacterial fatty acid biomarkers in microalgal cultures compared to raw wastewater. The difference in actinobacterial fatty acid content between raw and filtered wastewater was species-specific. Our results suggest that with careful selection of microalgal species, RAS wastewater can be used for the production of high-quality microalgal biomass for further applications, such as aquaculture feeds, with no need to remove indigenous biological contaminants and suspended solids.

## 1. Introduction

Aquaculture is one of the world's fastest-growing food industries and its further development and intensification are expected in almost all regions of the world. Eutrophication of aquatic ecosystems caused by exposure to nutrient-rich (particulate and dissolved) aquaculture wastewater (WW) represents one of the main risks to the environmental sustainability of aquaculture [1,2]. Recirculating aquaculture system (RAS) represents an environmentally superior option to traditional open systems (flow-through and net-pen) as it facilitates the on-site treatment of water. Low water renewal rates required by modern RAS (<10% of total volume per day) result in smaller volumes of WW, while the waste output depends on production of biomass and is not reduced compared to open systems. This concentration of waste into reduced volumes of WW provides an opportunity to improve waste management and nutrient recycling [2,3]. Despite technological improvements in physicochemical water purification processes, aquaculture WW treatment remains expensive while valuable nutrients present in WW are not recovered [4]. That is why economically feasible sustainable solutions are required for RAS WW treatment.

Bioremediation of WWs represents a sustainable alternative to traditional physicochemical treatments, where living organisms are employed for the removal of contaminants. Although bioremediation of WW holds promising opportunities as a sustainable solution for nutrient recycling in RAS, to date, most research effort has been allocated to study aquaponics (aquaculture coupled with hydroponics) [5]. Among alternative organisms for bioremediation of RAS WW, microalgae represent a powerful biotechnological platform for the production of high value products and quality biomass [6]. Most importantly, their use for bioremediation of different industrial and municipal WWs has been proven effective [6,7,8,9]. Given that microalgae are a natural food source for organisms commonly produced in aquaculture (*e.g.* fish larvae, crustacean, and mollusks) [10], for RAS enterprises, microalgae cultivation could not only support WW treatment but the generated microalgal biomass could serve as feedstock to complement or replace aquaculture feeds [11,12]. In addition, biological harvest of microalgae (*e.g.* by zooplankton or fish larvae) could eliminate the operational costs associated with chemical or mechanical harvesting of microalgal cells (up to 90% of the total cost of microalgae production) [6,13,14].

Depending on the species and cultivation conditions, microalgae can

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have high nutritional value for aquaculture since they are rich sources of carbohydrates, proteins, lipids, and other essential biomolecules such as vitamins and pigments [12,15,16]. In particular, fatty acids (FAs) and amino acids (AAs) are among the most important microalgal biochemical components as highly unsaturated FAs, such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 20:6ω-3), are essential for the growth and reproduction of zooplankton, bivalves and fish [17,18,19]. AA composition, in particular essential AAs (EAAs), has been shown as an important component of food for aquaculture organisms [18,20]. In addition, dietary non-essential AAs (NEAAs) have been reported as necessary to achieve maximal growth in zooplankton and fish [19,21].

To date, most studies on microalgal bioremediation of RAS WW were conducted in warm geographical locations [22], hence, the applicability of this bioremediation system at relatively low temperatures (below 20 °C) common to Nordic areas has received little attention. In our previous study [23], three freshwater green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) showed adequate growth rates in RAS WW when grown at low temperatures (17 °C), making them feasible candidates for WW bioremediation. Nevertheless, despite the satisfactory growth rates obtained, the implementation of microalgal bioremediation systems requires a better understanding of all possible attributes of WW that may affect microalgal performance such as biological contamination and suspended solids. Native biological contamination of RAS WW represents a challenge for microalgae cultivation since non-target microalgae and bacteria may compete for nutrients and light or may be toxic for the cultured microalgae [24]. In addition, even small numbers of herbivorous protozoa can rapidly multiply and destroy a microalgal culture [25]. Although recent findings highlight the potential benefits of symbiotic relationships between microalgae and bacteria for WW bioremediation [5], most of these studies overlook microalgal biomass in favor of nutrient removal, which does not align with the objective of optimized microalgal production. Moreover, studies on non-axenic microalgal cultures have shown that bacteria can trigger adaptations in the starch and lipid metabolism of microalgae leading to changes in the nutritional value of microalgae [26,27,28]. These results are especially significant when microalgal biomass is intended to be re-introduced into RAS as a complement or replacement of aquaculture feeds. Nevertheless, since maintenance of axenic cultures in RAS facilities is not feasible, the effect of WW's biological contamination on microalgal growth or its nutritional value should be considered. Suspended solids in RAS WW may also limit microalgal performance since, when present at high concentrations, they can reduce light penetration causing self-shading and reducing *in-situ* photosynthetic activity and biomass production [29]. To this end, evaluation of the effects of the reduction of the native biological contamination and suspended solids in RAS WW before microalgal inoculation can help avoid biomass losses or decreases in the overall nutritional quality of microalgae.

In this study, we investigated the use of filtration as a one-step RAS WW pre-treatment to significantly reduce biological contamination and suspended solids. The objective of the present study was to evaluate the growth, bioremediation capacity and nutritional quality of three microalgae species (*H. pluvialis*, *M. griffithii*, and *Selenastrum* sp.) cultured in either raw (unfiltered) or filtered Nordic RAS WW. We estimated the nutritional quality as the composition of microalgal FAs and AAs, based on their essential role in aquaculture [17,19]. We tested the hypothesis "filtration of Nordic RAS WW will improve growth, biomass, nutrient removal or nutritional quality of microalgae compared to unfiltered RAS WW".

## 2. Materials and methods

### 2.1. Recirculating aquaculture system wastewater

RAS WW samples were obtained from the Natural Resources Institute Finland (LUKE) Laukaa fish farm [30]. WW samples were collected from the water outlet of two individual RAS, after drum filtration and fixed bed bioreactor treatment. Farming conditions prior to sample collection in tanks 1 and 2 were 44 and 52 whitefish (*Coregonus lavaretus*) with a mean weight of 453 and 437 g, respectively, fed with Raisio Circuit Silver 3.5 mm at 0.7% body weight per day. Water circulation was set at 0.2 L $s^{-1}$ and replacement water adjusted at 250 L $kg^{-1}$ of feed [30]. Samples from both tanks were mixed and stored at 6 °C until used. Half of the RAS WW was filtered through 0.45 μm syringe filters (Corning, Sigma-Aldrich, USA). Filtration was not expected to produce an axenic WW [31], but rather decrease the total organic and biological load before the inoculation of microalgae. Dissolved organic carbon (DOC) of unfiltered and filtered RAS WW was measured from HCl-acidified samples (final pH = 2) with a high temperature catalytic oxidation method using a carbon and nitrogen analyzer (TOC-L, Shimadzu, Japan). Turbidity measures were done in triplicates with a Turb 430 IR (Xylem Analytics LLC, USA).

### 2.2. Microalgae strains and cultivation conditions

The three temperate zone freshwater green microalgal strains used (*H. pluvialis*, *M. griffithii*, and *Selenastrum* sp.) were obtained from the Norwegian Culture Collection of Algae (NORCCA). Each microalga was maintained as a stock monoculture in algae medium MWC (Modified Wright's Cryptophyte) based on Guillard and Lorenzen [32] as described previously [23]. A light microscope (Leica DM 500) equipped with a camera (Leica ICC50 W) was used to obtain images of microalgae grown in MWC media. The experimental cultivation of microalgae was divided into two experimental series, each series containing two replicates of each microalga cultivated in each culture media: unfiltered RAS WW and filtered RAS WW (Table 1). Microalgae were grown in 650 mL plastic culture flasks containing 400 mL of the culture as described in [23]. At the start of each series of experiments (day 0), microalgal stock monocultures were centrifuged at 2500*g* for 5 min at 17 °C (Megafuge 1.0 R, Heraeus, Germany) to obtain high-density inoculums. Each experimental culture flask was then inoculated with 1–10% of the microalgal saturating concentration determined in the previous experiments in MWC media. Illumination was provided on one side of the flasks by two LED grow lights (AP67 spectrum, see [23]) with 24:00 photoperiod and light intensity of 60–90 μmol photon $m^{-2}$ $s^{-1}$ measured at the surface of the flask by a high-resolution spectrometer (HP-350 HiPoint Inc., Taiwan). Room temperature was maintained at 17 ± 0.3 °C. Throughout the experiment, the flasks were manually mixed twice a day with aquarium magnets to keep cells in suspension. Cultivation was terminated 6 days after microalgal inoculation, before the cultures reached stationary phase.

### 2.3. Determination of microalgal growth

Throughout the cultivation period, cell density was estimated daily by cell count from two replicate samples of each replicate flask in a

**Table 1**
Characteristics of unfiltered RAS wastewater (WW) and filtered RAS wastewater (FWW) prior to microalgal inoculation. Dissolved organic carbon is denoted as DOC. Values are shown as mean ± SD of both experimental series.

| Composition | WW | FWW |
|---|---|---|
| $NH_4$-N (mg $L^{-1}$) | 0.03 ± 0.01 | 0.05 ± 0.01 |
| $NO_2$-N (mg $L^{-1}$) | 0.03 ± 0.00 | 0.05 ± 0.00 |
| $NO_3$-N (mg $L^{-1}$) | 96.87 ± 0.73 | 97.03 ± 0.17 |
| $PO_4$-P (mg $L^{-1}$) | 3.83 ± 0.07 | 3.76 ± 0.04 |
| N:P molar ratio[a] | 55.90 ± 0.62 | 57.05 ± 0.51 |
| pH | 7.49 ± 0.28 | 7.39 ± 0.04 |
| Turbidity (NTU) | 5.27 ± 0.68 | 1.33 ± 0.06 |
| DOC (mg $L^{-1}$) | 12.99 ± 0.79 | 12.13 ± 0.59 |

[a] N:P molar ratio was calculated from $NO_3$-N:$PO_4$-P.

haemocytometer chamber (Bürker) with 100× magnification (Leitz 184 Laborlux D, Germany). The specific growth rate ($d^{-1}$) was calculated from the change in cell density during the exponential growth phase according to [33]. To determine the total dry weight (biomass), two aliquots of culture were taken at the end of the cultivation period (day 6) as described previously [23]. Chlorophyll-a concentration was assessed spectrophotometrically with Shimadzu Spectrophotometer (UV-1800, Japan) from samples filtered on a fiber filter (GF/A, Whatman, GE Healthcare, USA) at the end of the cultivation period (day 6) according to [34].

### 2.4. Determination of nutrient removal

Nitrate-nitrogen ($NO_3$-N) and phosphate-phosphorus ($PO_4$-P) concentrations were assessed from samples of culture media at the beginning and the end of the cultivation period with testing kits LC399 and LCK349 (Hach, USA) according to manufacturer's instructions. Quantification was carried out in a mobile laboratory spectrometer (LASA 100, Dr. Lange, Germany). Before being analyzed, every sample was filtered through a 0.22 μm syringe filter. Percentage of nutrient uptake and nutrient removal rate (*Ri*) were calculated as described previously [23].

### 2.5. Fatty acid analysis

Once microalgal cultivation ended (day 6), between 20 and 35 mL of each culture was filtered through 3.0 μm cellulose nitrate membranes (Whatman, GE Healthcare, USA). Filters containing the microalgal sample were then freeze-dried, weighed (Sartorius CP2P, Germany), and stored at −80 °C until the analysis (no longer than a month). Filters containing 2–5 mg of green microalgae were placed into test tubes (10 mL). Total lipid extraction was carried out with chloroform:methanol: water (4:2:1) mixture and methanolic $H_2SO_4$ (1% *v*/v) at 50 °C was used for transesterification of FA to form fatty acid methyl esters (FAME). FAMEs were analyzed with a gas chromatograph equipped with mass detector (GC–MS) (Shimadzu Ultra, Japan) using helium as a carrier gas and an Agilent (California, USA) ZB-FAME column (30 m × 0.25 mm × 0.20 μm) (Phenomenex, USA) for separation. The temperature program: 50 °C was maintained for 1 min, and then the temperature was increased at 10 °C $min^{-1}$ to 130 °C, then by 7 °C $min^{-1}$ to 180 °C, and 2 °C $min^{-1}$ to 200 °C held for 3 min and finally heated at 10 °C $min^{-1}$ to 260 °C. The column flow was set at 1.10 mL $min^{-1}$. Quantification calibration curves for individual FAs were prepared with fatty acid standard GLC reference standard 556 C (Nu-Chek Prep, Elysian, USA). Additionally, we used phospholipid FA C19:0 (PLFA 19:0) and free FA C23:0 (Larodan, Sweden) as internal standards for the calculation of the recovery percentages. Recovery percentages were >71% for all samples. FAs in sample spectrums were identified using retention times together with specific ions. Quantification was based on detector responses, the peak areas were integrated using GCsolution software (version 2.41.00, Shimadzu, Japan), and samples FAs areas values were interpolated in the calibration curve to determine their concentration.

FA content (μg $g^{-1}$ DW) was calculated using the following equation:

$$FA_{ic} = \frac{Q_{FA} \times V_{Vial}}{DW_1 \times R_{\%}} \quad (1)$$

where $FA_{ic}$ is the content of an individual fatty acid (μg $mg^{-1}$ DW) in the sample, $Q_{FA}$ is the concentration of the fatty acid (μg $\mu L^{-1}$) based on calibration curves of GLC-566C, $V_{Vial}$ denotes the running volume of the samples (μL), $DW_1$ is the dry weight of the sample, and $R_{\%}$ denotes the recovery percentage based on internal standards. FA percent values (%) were calculated following the formula:

$$FA_{i\%} = \frac{FA_{ic}}{Tot - FA_{ic}} \times 100 \quad (2)$$

where $FA_i$% is the percentage of contribution of $FA_i$, $FA_{ic}$ is the determined concentration of $FA_i$ and $Tot\text{-}FA_{ic}$ is the sum concentration of all identified FAs. As described in [35], FAs were then sorted by their mean % contribution, and only FAs contributing >0.5% (mean across all replicates) to the total were used for later statistical analysis (without normalizing the data to 100%). In this work, we focused on the content and contribution of five ω-3 poly-unsaturated FAs (16:3ω-3, 16:4ω-3, 18:3ω-3, 18:4ω-3 and 20:5ω-3) and four ω-6 FAs (16:2ω-6, 18:2ω-6, 18:3ω-6, and 20:4ω-6). In addition to microalgal FAs, we quantified actinobacterial FA biomarkers (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0, and a-17:0) of each sample as an approximation of the bacterial biomass in the culture media. Actinobacterial FA contents (ng $mL^{-1}$) represent the sum of all quantified FAs per mL of media and are expressed as bacterial FA contents in the Results section.

### 2.6. Amino acid analysis

At the end of the microalgal cultivation period (day 6), between 7 and 10 mL of culture were filtered through 3.0 μm nucleopore polycarbonate filters (Whatman, GE Healthcare, USA). Filters containing the sample were then freeze-dried, weighed (Sartorius CP2P, Germany), and stored at −80 °C until analysis (no longer than a month). Microalgae samples were placed into test tubes (10 mL) and HCl 6 N was added in sufficient amounts to ensure that the filters containing the sample were completely covered in acid. Samples were then heated at 110 °C for 24 h. After AA hydrolyzation, HCl was evaporated at 110 °C for 20 h. We used L-norvaline (Sigma-Aldrich, USA) as an internal standard. Free AAs were then derivatized utilizing the commercial kit EZ:faast for Free Physiological Amino Acid Analysis by GC–MS (Phenomenex, USA) with the exception that no purification column was used during the process. AA chromatographic separation and their posterior identification and quantification were done following the protocol described in [36]. Samples were analyzed with GC–MS (Shimadzu, Japan) and a fused silica capillary column (10 m × 0.25 mm), coated with 0.2 μm of an unknown stationary phase (ZB-AA, Phenomenex, USA). The identification of AAs was based on retention times and specific ions. Individual AA calibration curves were generated with the AA standard AAS-18 (Sigma-Aldrich, USA). Quantification and correction of AA content (μg $mg^{-1}$ DW), together with the determination of AA (%), were done as described with FAs (Eqs. (1) and (2)). In Eq. (1), norvaline recovery percentage was used as $R_{\%}$. Only the AAs present in the standard (AAS-180) were identified and quantified in the samples: eight essential AA (EAAs: valine, leucine, isoleucine, threonine, methionine, phenylalanine, lysine, and histidine), and seven non-essential AA (NEAAs: alanine, glycine, serine, proline, asparagine, glutamic acid, and tyrosine). The sum of all AAs was calculated as the sum of EAA and NEAA. In the case of methionine, only very low concentrations of the amino acid were detected, this agrees with previous literature since methionine can be degraded to varying degrees during acid hydrolysis [37].

### 2.7. Statistical analysis

Two-way mixed effects analysis of variance (ANOVA) was used to test the effects of microalgae species (three microalgae species) or growth media (filtered or unfiltered RAS WW) on growth, nutrient uptake, and FA and AA categories. The significance of fixed effects was evaluated using Satterthwaite's method to approximate the degrees of freedom. The non-independence of observations within each run was accounted for by including run as a random factor. The significance of the effect of the run was evaluated with the Likelihood Ratio Test. Estimated Marginal Means pairwise comparison with Tukey adjustments was used for *post hoc* analysis of the mixed effects models. Homogeneity of variances was tested with Levene's test and normality of the collected data was tested with Shapiro–Wilk's test. Permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis distance matrix was performed on FA and AA percentage (%) data to test if species or media (treatment) differed statistically from

each other. PERMANOVA analysis for ω-3, ω-6, EAA, and NEAA were carried out on normalized percentage data of each category. Multivariate homogeneity of group dispersion (variances) was tested using Marti Anderson's procedure for the analysis of multivariate homogeneity (PERMDISP) [38]. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. All statistical analyses were conducted using R (RStudio version 3.6.3), mixed effects models were conducted with lme4 package (v1.1-21), the rest of the analysis was carried out with either R base or vegan packages [39].

## 3. Results and discussion

### 3.1. Effect of RAS wastewater filtration on cell density, biomass, and chlorophyll-a

Filtration of Nordic RAS WW through 0.45 μm filter decreased the turbidity of the media but did not affect the concentration of dissolved nutrients (Table 1). In contrast with our hypothesis, no differences in cell density, specific growth rate, or dry weight were observed between microalgae cultivated in unfiltered and filtered WW after 6 days of cultivation ($p > 0.05$; Fig. 1; Table S.1). Chlorophyll-a concentration was almost ~50% lower in *H. pluvialis* cultivated in unfiltered compared to filtered WW ($p < 0.05$; Fig. 1d; Table S.4), while *M. griffithii* and *Selenastrum* sp. did not present differences in chlorophyll-a between treatments ($p > 0.05$; Table S.3). Altogether, our results suggest that filtration of RAS WW does not promote (nor suppress) microalgal growth during short-term cultivation, showing that neither biological contamination nor the concentration of suspended solids present in RAS WW significantly affect microalgal growth. In previous studies, differences in cell density and dry weight between microalgae cultivated in unfiltered and filtered (0.20–0.22 μm) WW were observed after microalgae entered stationary phase [40,41]. Therefore, it is possible that with short-term cultivations, when nutrients are not limiting, biological contamination of RAS WW does not limit microalgal growth. Since microalgal harvesting should correspond with the highest production of the desired end-product, cultivation times should be optimized based on the targeted use of microalgal biomass. For RAS WW, optimization of cultivation times should prioritize maximal microalgal biomass, nutrient removal, and nutritional quality of the generated biomass, hence, long cultivation times where microalgae reach stationary phase might not be required.

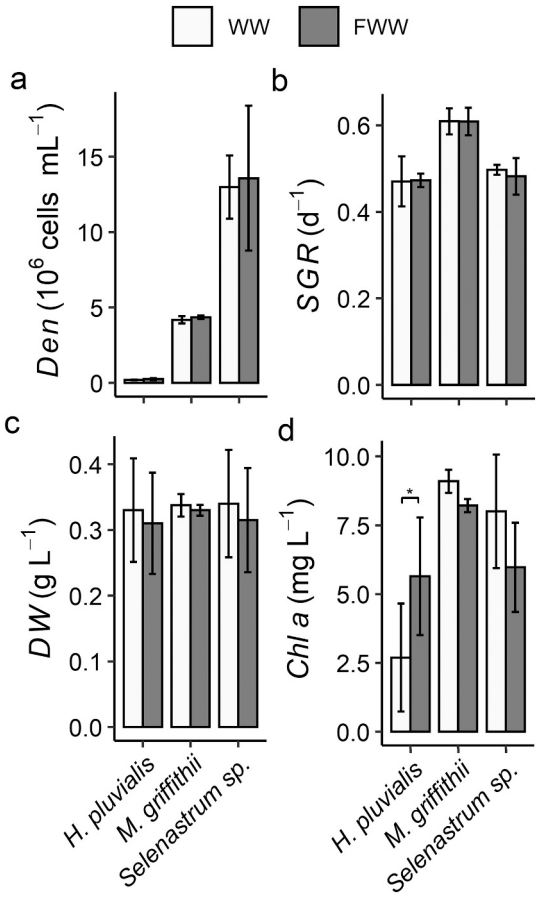


**Fig. 1.** Density (*Den*) (a), specific growth rate (*SGR*) (b), dry weight (*DW*) (c), and chlorophyll-a content (*Chl a*) (d) of three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered (WW) or filtered RAS wastewater (FWW) for 6 days. Values are presented as mean ± SD of four replicates. Statistically significant differences between treatments are shown with *. Comparison of treatments between microalgae is not presented in this figure.

Despite large differences in cell densities (Fig. 1a), the three tested microalgae reached very similar dry weights by day 6 (Fig. 1c; Table S.5), possibly due to differences in cell size between microalgae species and particularly the larger size of *H. pluvialis* (Fig. S.1). It is important to point out that the influence of particulate matter and biological contamination present in WW to the final dry weight cannot be discounted, particularly for unfiltered WW. Therefore, despite not observing differences in cell number between treatments, it is possible that the recorded dry weights were slightly overestimated.

Independent of the media, *M. griffithii* had a higher specific growth rate than the other microalgae (0.61 ± 0.03 $d^{-1}$) ($p < 0.05$; Fig. 1b; Table S.5), while no difference was observed between *H. pluvialis* and *Selenastrum* sp. (0.47 ± 0.04 and 0.49 ± 0.03 $d^{-1}$, respectively) (Fig. 1b; Table S.5). Specific growth rate and cell density values observed in this study agree with those of literature on the same microalgae species cultivated in reference algae media [23,42,43]. In addition, other studies looking at the same microalgae genera/species cultured in different types of WW at temperatures of 20 °C or higher reported similar or lower specific growth rates than in this work (*Haematococcus* [8]; *Monoraphidium* [9]; *Selenastrum* [44]). This highlights the resilience of the three tested microalgae species used in this study and presents them as good candidates for the bioremediation of other industrial WWs with similar properties to Nordic RAS WW. Overall, the similar growth rates obtained in this and our previous study with the same microalgal strains and culture conditions [23], support the use of RAS WW for efficient microalgal cultivation.

### 3.2. Nutrient removal

Nutrient removal efficiency (removal percentage and removal rate) was not affected by WW filtration ($p > 0.05$; Fig. 2; Table S.4). This result suggests that microalgal removal of nitrate-nitrogen ($NO_3$-N) or phosphate-phosphorus ($PO_4$-P) during short-term cultivation periods is not affected by WW's biological contamination nor suspended solids. These findings are in line with previous studies on microalgal bioremediation of aquaculture WW, where complete sterilization of WW did not significantly affect the nutrient removal efficiency of green microalgae during exponential growth [27,41]. However, as with cell density, species-specific effects of biological contamination on nutrient removal have been observed when microalgae were grown until late stationary phase [40].

Regardless of the cultivation media, *Selenastrum* sp. and *M. griffithii* were able to remove almost all $PO_4$-P in WW (~99% on average) (Fig. 2a,c), while *H. pluvialis* had a lower (~75%) removal efficiency than the other microalgae (Fig. 2a,c; Table S.5). $NO_3$-N removal was less than ~40% for all microalgal species and cultivation media (Fig. 2b,d). Comparison between species showed that *H. pluvialis* had a higher $NO_3$-N removal rate than *M. griffithii* in filtered WW ($p < 0.05$; Table S.5) while no other differences in nutrient removal were observed (Table S.5). Compared to previous studies looking at the WW

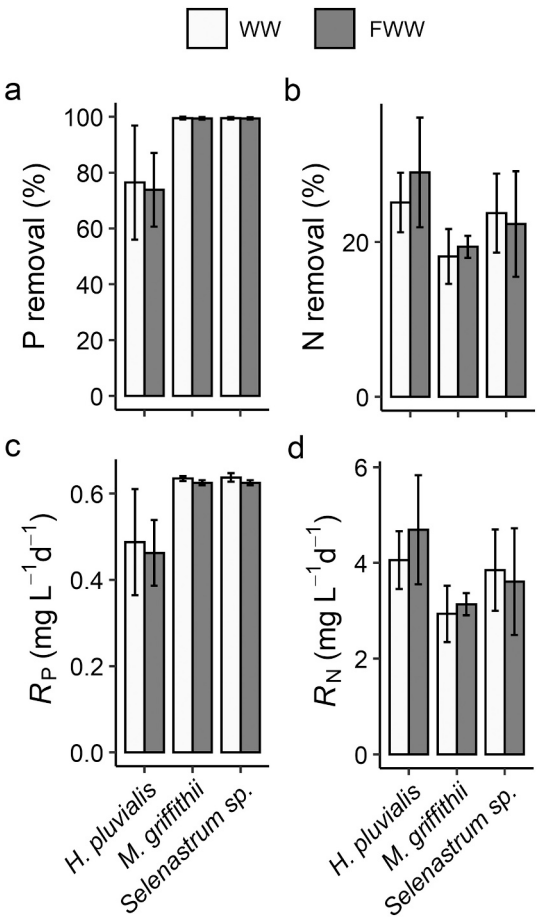


**Fig. 2.** Percentage of $PO_4$-P removal (a), percentage of $NO_3$-N removal (b), $PO_4$-P removal rates ($R_P$) (c), and $NO_3$-N removal rates ($R_N$) (d) of three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered (WW) or filtered wastewater (FWW) for 6 days. Values are presented as mean ± SD of four replicates.

bioremediation capacities of the tested microalgae, the observed $NO_3$-N and $PO_4$-P removal rates were consistent with those described at cultivation temperatures of 20 °C or above (*H. pluvialis* [8]; *Monoraphidium* [9]; *Selenastrum* [44]). In addition, in our previous study we observed similar $PO_4$-P and higher $NO_3$-N removal rates compared to this study [23], confirming that microalgae bioremediation of Nordic RAS WW at *ca.* 17 °C is a viable option for WW treatment. Nevertheless, given the $NO_3$-N removal percentage observed (less than 40% for all species), and considering that microalgae nutrient removal rates are affected by the N:P ratio of the culture media, balancing WW's N:P ratio near optimal required by microalgae could increase nutrient uptake [7]. In this study the N:P ratio observed in WW was >55 (Table 1), indicating phosphorus limitation for microalgal growth and suggesting that the addition of phosphorus to WW, prior to microalgal inoculation, could represent an opportunity to improve $NO_3$-N removal.

### 3.3. RAS wastewater filtration and microalgal fatty acid profiles

All microalgae contained alfa-linolenic acid (ALA, 18:3ω-3) stearidonic acid (SDA, 18:4ω-3), linolenic acid (LIN, 18:2ω-6), and gamma-linolenic acid (GLA, 18:3ω-6) (Fig. 3). None of the studied species presented detectable amounts of DHA, and only *H. pluvialis* contained arachidonic acid (ARA, 20:4ω-6) and low contents of EPA (0.56 ± 0.17 and 0.76 ± 0.14 μg $mg^{-1}$ DW in unfiltered and filtered WW, respectively) (Fig. 3). PERMANOVA analysis showed no effect of WW filtration on the contribution of the studied ω-3 or ω-6 FAs (Table 2). Nevertheless, PERMANOVA test loses power and is more prone to type II errors in the presence of heteroscedasticity. Given the observed differences in dispersion of ω-6 FA contribution data ($p < 0.05$; Fig. S.2a; Table 3), it is possible that differences in the ω-6 FAs contribution between treatments did exist. The effect of microalgae species was significant in the PERMANOVA analysis and explained >85% of the observed differences in ω-3 or ω-6 FAs (Table 2). This result is in line with previous work describing phytoplankton taxa as the most explanatory variable for differences in FA profiles [45,46]. Although all microalgae displayed similar total ω-3 FAs contributions (~45%), SDA contributions varied from ~2.5% to ~7.5% in *H. pluvialis* and *Selenastrum* sp., respectively (Fig. 3a). *H. pluvialis* presented a ω-6 FA contribution of ~17%, with LIN making up to ~76% of the total ω-6 FA composition (Fig. 3c). Compared to previous studies where *H. pluvialis* and *M. griffithii* were cultivated in a reference algae medium, we observed similar ω-3 and ω-6 FA profiles [42,47], suggesting that the use of Nordic RAS WW as a growth media, do not significantly affect the microalgal composition of nutritionally important FAs.

In terms of FA content, *H. pluvialis* had a lower ω-3 and ω-6 content when cultured in unfiltered compared to filtered WW ($p < 0.05$; Table S.6), while no differences were seen for *M. griffithii* or *Selenastrum* sp. between treatments (Fig. 3b,d; Table S.6). Interestingly, since no difference in FA composition was observed, *H. pluvialis* proportionally decreased the content of ω-3 and ω-6 FAs, suggesting that the conditions present in unfiltered RAS WW affected the total production of FAs compared to filtered WW. Since we did not quantify total lipids, differences in total lipid content between treatments might exist. Therefore, the effect of biological contamination and suspended solids on lipid production might be underestimated in this study. Overall, our FA results suggest that microalgal FA metabolism responds in a species-specific manner to the native conditions of Nordic RAS WW.

The ω-3 and ω-6 ratio, which is a useful indicator of FA nutritional value for zooplankton [48], was ~4.5 in *Selenastrum* sp. and *M. griffithii* and ~2.5 in *H. pluvialis*. This result indicates that *Selenastrum* sp. and *M. griffithii* have a higher FA nutritional value than *H. pluvialis*. Due to the similar ω-3 and ω-6 FA contents seen in *M. griffithii* and *Selenastrum* sp., we have no reason to believe that one of these microalgae has superior nutritional FA value than the other.

Studies on bioconversion of FAs have shown that planktonic organisms such as *Daphnia* can elongate and desaturate ALA and SDA to EPA and LIN to ARA [49,50], both of which are essential FAs for fish growth and development [17]. Therefore, given that ALA and LIN are major contributors to the ω-3 and ω-6 FA content of the tested microalgae, filter-feeding *Daphnia* could be used to upgrade the FA nutritional value of the generated algal biomass for later use in RAS, as well as to decrease the costs associated with the mechanical harvest of microalgae.

### 3.4. RAS wastewater filtration and microalgal amino acid profiles

All 15 studied AAs were identified in each microalgae species regardless of the cultivation media (Fig. 4; Table S.1). Contributions of total EAAs and NEAAs were very similar between species and treatments (~50% for both EAAs and NEAAs). According to PERMANOVA, microalgae species showed a statistically significant effect on both EAA and NEAA contribution ($p < 0.05$; Table 2), explaining 27.8% and 21.5% of the variation seen in EAA and NEAA, respectively. These results are in agreement with previous literature, where the total contribution of EAA and NEAA remained relatively constant between microalgae species/taxa but significant variations were seen at the individual AA level [51].

No effect of filtration was seen on the contribution of EAAs or NEAAs (Table 2) and compositional data showed to have a homogeneous dispersion for both EAA and NEAA (Figs. S.2b, S.3; Table 3). Filtration of WW affected negatively the AA content of *Selenastrum* sp. for both EAAs and NEAAs ($p < 0.05$; Fig. 4b; Table S.6), while *M. griffithii* showed a lower EAA content in filtered compared to unfiltered WW ($p < 0.05$; Fig. 4b; Table S.6). However, since biological contaminants in RAS WW

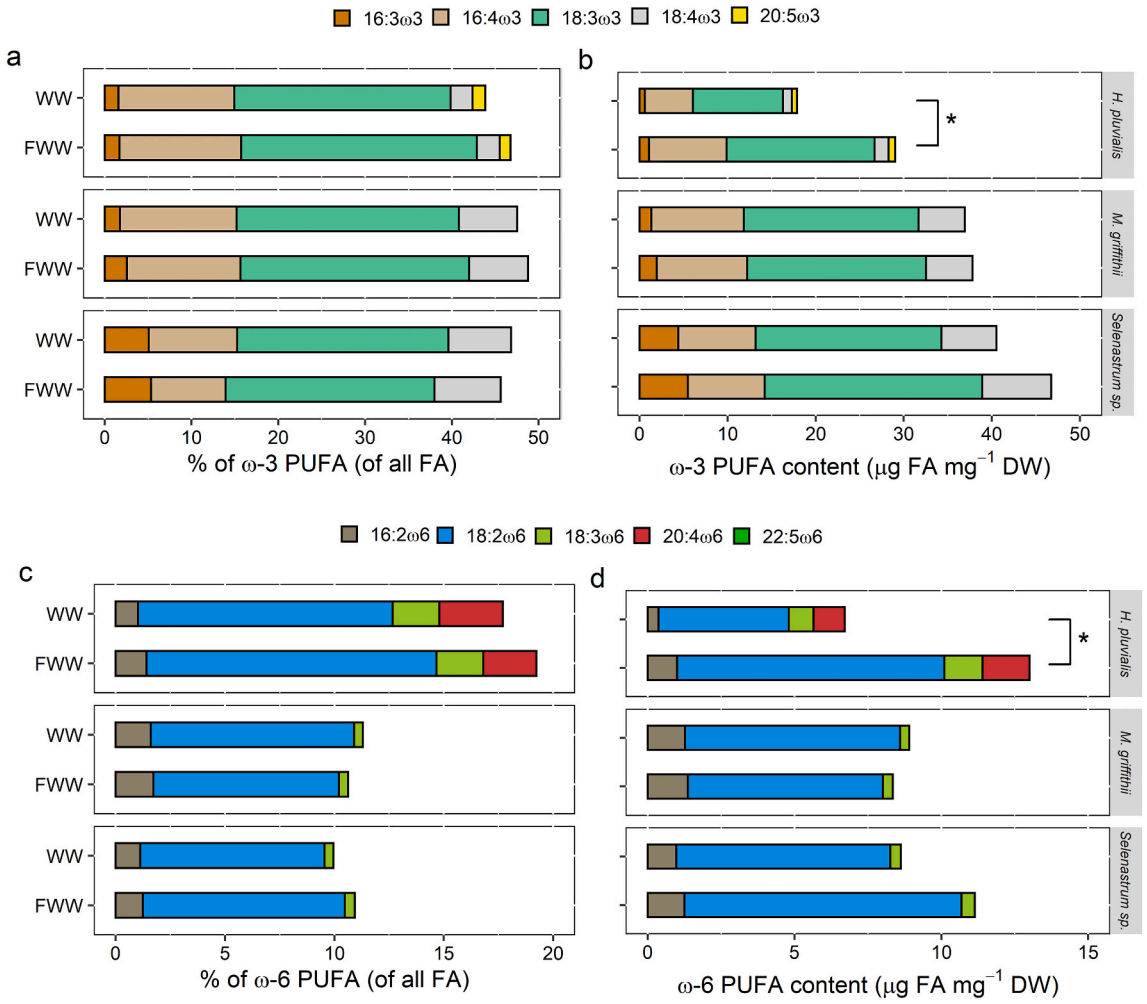


**Fig. 3.** Proportion of all fatty acids (FA) and per biomass content of ω-3 (a, b) and ω-6 (c, d) FA of three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered (WW) or filtered (FWW) RAS wastewater for 6 days. Statistically significant differences in FA content between treatments are shown with *. Comparisons of treatments between microalgae are not presented in this figure.

**Table 2**
PERMANOVA results of microalgae ω-3 and ω-6 fatty acids together with essential and non-essential amino acid (EAA and NEAA, respectively) contribution profiles. Dissimilarities in amino acid profiles were compared between species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.), treatments (unfiltered and filtered wastewater) and their interaction (species * treatment). Remarked in bold, statistically significant values ($p < 0.05$).

| Variable | Source | *df* | *Pseudo-F* | *exp%* | *p* |
|---|---|---|---|---|---|
| ω-3 | Species | 2 | 62.51 | 85.3 | **<0.01** |
| | Treatment | 1 | 2.02 | 1.4 | 0.16 |
| | Species * treatment | 2 | 0.80 | 8.0 | 0.47 |
| ω-6 | Species | 2 | 103.60 | 90.5 | **<0.01** |
| | Treatment | 1 | 3.15 | 1.4 | 0.08 |
| | Species * treatment | 2 | 0.26 | 0.2 | 0.79 |
| EAA | Species | 2 | 3.98 | 27.8 | **<0.01** |
| | Treatment | 1 | 0.57 | 2.0 | 0.66 |
| | Species * treatment | 2 | 1.07 | 7.5 | 0.40 |
| NEAA | Species | 2 | 2.98 | 21.5 | **0.02** |
| | Treatment | 1 | 1.05 | 3.8 | 0.34 |
| | Species * treatment | 2 | 1.36 | 9.8 | 0.24 |

**Table 3**
Permutational Analysis of Multivariate dispersion (PERMDISP) of the contribution of essential and non-essential amino acids (EAA and NEAA, respectively) and ω-3 and ω-6 fatty acids across three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered or filtered wastewater for 6 days. Remarked in bold, statistically significant values ($p < 0.05$).

| Unit | Source | *df* | *F* | *p* |
|---|---|---|---|---|
| ω-3 | Species | 2 | 1.43 | 0.26 |
| ω-6 | Species | 2 | 5.27 | **0.01** |
| EAA | Species | 2 | 1.31 | 0.29 |
| NEAA | Species | 2 | 1.88 | 0.18 |

such as bacteria can synthesize all tested AAs [52], it is possible that the higher observed EAA and NEAA contents in unfiltered WW could be attributed to the presence of bacteria in the culture media. Consequently, it is possible that the AA content observed in *H. pluvialis* cultivated in unfiltered WW was also increased by the presence of bacteria in the culture medium. As a limitation of our study, we did not quantify total protein content, which could have shown if differences between treatments in protein contribution to dry biomass exist.

Overall, *H. pluvialis* had the highest EAA and NEAA contents of the studied species in both unfiltered and filtered WW (EAA: 139 ± 6 and 139 ± 4, NEAA: 148 ± 5 and 145 ± 4 μg $mg^{-1}$ DW, respectively) ($p < 0.05$; Fig. 4b; Table S.6). Compared to previous studies, we observed similar total AA contents to those described for freshwater green microalgae in non-axenic cultures of reference algae media [53], indicating that the use of Nordic RAS WW as a growth medium does not affect the production of AA by microalgae. Because EAAs are considered

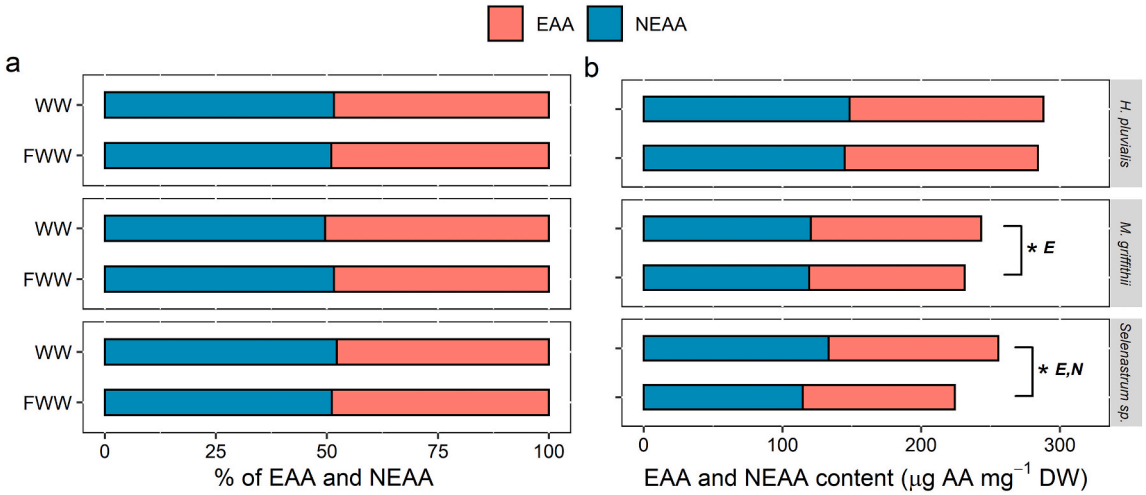


**Fig. 4.** Proportion of all amino acids (AA) (a) and per biomass content (b) of essential and non-essential amino acids (EAA and NEAA, respectively) of three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered (WW) or filtered (FWW) RAS wastewater for 6 days. Statistically significant differences in AA content between treatments are shown with *. Bold letters E and N are used to denote if statistical difference in EAA and/or NEAA (respectively) was observed. Comparisons of treatments between microalgae are not presented in this figure.

the same for zooplankton and fish as for insects and humans [54] and NEAA are described to be a key component explaining zooplankton growth [19], our results suggest that all tested microalgae species could serve as a nutritious food source for zooplankton and fish. Altogether, given that the FA and AA contents of *M. griffithii* and *Selenastrum* sp. were not altered by filtration, they appear as good candidates for the bioremediation of unfiltered WW when biomass is intended for further use in aquaculture. Nevertheless, if filtration of RAS WW is possible, *H. pluvialis* could have an advantage over the other tested species due to its EPA, ARA, and overall higher AA content.

It is important to point out that microalgal biochemical profiles, as well as growth dynamics, are susceptible to changes in growth media composition, type of photobioreactor used, and other factors [55]. Thus, it is possible that different growth rates and variations in the FA and AA contents could be observed when scaling up the studied production system. In addition, in this study we did not analyze the carotenoid content of the tested microalgae, and cannot therefore evaluate the effect of biological contamination and suspended solids in RAS on this nutritionally important group of biomolecules for aquaculture [56]. Among other difficulties of the use of RAS WW for microalgal cultivation is that its composition is not homogenous among facilities, which adds to the problem of replicability of results. That is why more and larger-scale studies are needed to further confirm the feasibility of the use of microalgae for bioremediation of RAS WW and the production of nutritionally valuable biomass.

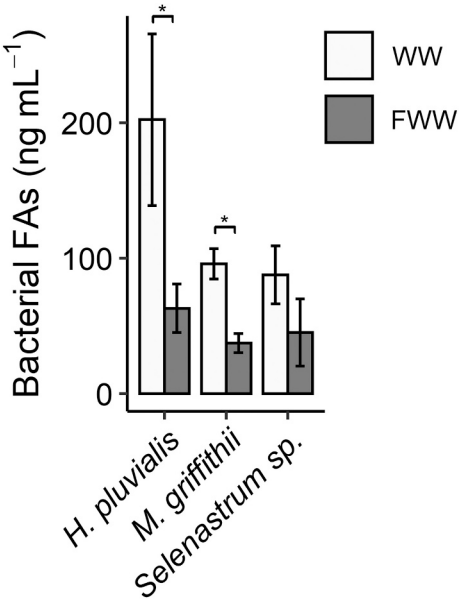


**Fig. 5.** Bacterial fatty acids (FAs) content in microalgae culture media of three green microalgae (*Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp.) grown in either unfiltered (WW) or filtered WW (FWW) for 6 days. Values are given as the sum of all identified bacterial FAs. Statistically significant differences between treatments are shown with *. Comparisons of treatments between microalgae are not presented in this figure.

### 3.5. Bacterial biomass in microalgae cultures of unfiltered and filtered wastewater

Unfiltered WW showed an overall higher bacterial biomass based on actinobacterial FAs than its filtered counterpart after six days of microalgae cultivation (Fig. 5; Table S.3). Excluding *Selenastrum* sp., both *H. pluvialis* and *M. griffithii* cultures had higher bacterial biomasses when grown in unfiltered compared to filtered WW ($p < 0.05$; Fig. 5; Table S.3). The highest bacterial biomass was observed in *H. pluvialis* culture, where almost 3-fold higher biomass was observed in unfiltered compared to filtered WW ($p < 0.05$; Fig. 5; Table S.4). Overall, approximated bacterial biomasses in filtered WW were similar in all microalgal cultures (Fig. 5. Table S.4). Since *H. pluvialis* exhibited high bacterial biomass in unfiltered WW, it is possible that competition for nutrients or other effects of the presence of bacteria induced a stress response in this microalgae. Commonly, *H. pluvialis* responds to stress by accumulating astaxanthin at the expense of chlorophyll degradation [57]. Therefore, the lower chlorophyll-a content observed for this microalgae in unfiltered WW (Fig. 1d) could be explained by the transition from green cells to astaxanthin rich cysts. Nevertheless, we did not see differences in cell density by day 6 (Fig. 1a) and no differences were seen in the number of cysts between filtered and unfiltered treatment, suggesting that astaxanthin accumulation had only started by the end of the cultivation period.

Compared to *H. pluvialis*, *Selenastrum* sp. and *M. griffithii* presented lower bacterial biomasses than in unfiltered WW, suggesting that these two species are better able to control bacterial growth. Nonetheless, as all species showed similar bacterial biomasses in filtered WW cultures, it

is possible that when lower bacterial loads are initially present in the culture medium, bacterial growth dynamics are equally affected by the different microalgae species.

Since we only quantified actinobacterial FAs from microalgae filtrates (3.0 μm filter pore size), our results most likely underestimate the total bacterial loads of the cultures due to bacteria passing through the filters. In addition, we did not follow bacterial growth during microalgal cultivation, which could have exposed valuable information for the understanding of microalgae-bacteria growth dynamics in WWs. Even though we did not see differences in microalgal cell numbers between treatments by the end of the cultivation period (Fig. 1a), studies by Bolch et al. [58] and Rhee [59] showed that a rapid onset of bacterial growth on the culture media can shorten the exponential growth phase of microalgae, leading to lower cell densities. This is an important aspect to consider if the objective of microalgal production is the accumulation of an end-product that requires longer cultivation times than the one used in this study.

Since biological contamination encompasses more than just bacteria, there are likely to be other significant differences in the biological composition of unfiltered and filtered RAS WW cultures. Daily microscope examination of microalgal cultures showed negligible amounts of non-targeted organisms and suspended solids in filtered WW while small numbers of protozoa were observed in unfiltered RAS WW. Although we did not quantify any observed microorganisms present in the cultures besides the targeted microalgae, we did not observe considerable increases in biological contamination throughout the cultivation period. This suggests that the native biological contamination of the RAS WW used for our experiment did not present a threat to microalgal production.

In summary, our results show that microalgal bioremediation of Nordic RAS WW at low temperatures (17 °C) represents a feasible and sustainable alternative to conventional physicochemical water treatments with the advantage of nutrient recycling and valuable biomass generation. In addition, besides changes in the FA content of *H. pluvialis*, we did not observe significant decreases in microalgal performance due to the presence of biological contamination and suspended solids, suggesting that filtration of RAS WW prior to microalgal inoculation might be unnecessary when short-term cultivations are used.

## 4. Conclusions

In this study, we showed that microalgal bioremediation of Nordic RAS WW is a feasible alternative to conventional WW treatments. Biological contamination did not affect microalgal growth, biomass production, nutrient removal, or AA and FA composition of microalgae, but had a negative effect on the ω-3 and ω-6 FA content of one of the studied species, *H. pluvialis*. Overall, our results suggest that for the generation of valuable biomass, the removal of indigenous biological contamination and suspended solids of RAS WW does not produce major changes in the bioremediation capacity or the nutritional quality of microalgae.

*E*-supplementary data of this word can be found in the online version of the paper.

## CRediT authorship contribution statement

**Marco Calderini**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization.

**Čedomir Stevčić**: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision.

**Sami Taipale**: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

**Katja Pulkkinen**: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

## Informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

## Data availability

Datasets related to this article can be found at doi:10.17011/jyx/dataset/72716.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

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## References

[1] J.P. Blancheton, R. Piedrahita, E.H. Eding, D.E.R. D'Orbcastel, G. Lemarié, A. Bergheim, S. Fivelstad, Intensification of landbased aquaculture production in single pass and reuse systems, in: A. Bergheim (Ed.), Aquacultural Engineering and Environment, Research Signpost, 2007, pp. 21–47.
[2] R.H. Piedrahita, Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation, Aquaculture 226 (2003) 35–44, https://doi.org/10.1016/S0044-8486(03)00465-4.
[3] J. Bregnballe, A Guide to Recirculation Aquaculture, Food and Agriculture Organization of the United Nations (FAO) and EUROFISH International Organisation, 2015.
[4] C. Martins, E. Eding, M. Verdegem, L. Heinsbroek, O. Schneider, J. Blancheton, E. Roque, J. Verreth, New developments in recirculating aquaculture systems in Europe: a perspective on environmental sustainability, Aquac. Eng. 43 (2010) 83–93, https://doi.org/10.1016/j.aquaeng.2010.09.002.
[5] S. Goddek, A. Joyce, B. Kotzen, G.M. Burnell, Aquaponics Food Production Systems: Combined Aquaculture and Hydroponic Production Technologies for the Future, 1st ed., Springer International Publishing, 2019 https://doi.org/10.1007/978-3-030-15943-6.
[6] K. Chew, K. Khoo, H. Foo, S. Chia, R. Walvekar, S. Lim, Algae utilization and its role in the development of green cities, Chemosphere 268 (2021) 129322, 2021. ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2020.129322.
[7] T.V. Fernandes, M. Suárez-Muñoz, L.M. Trebuch, P.J. Verbraak, D.B. van De Waal, Toward an ecologically optimized N:P recovery from wastewater by microalgae, Front. Microbiol. 8 (2017) 1742, https://doi.org/10.3389/fmicb.2017.01742.
[8] F. Haque, A. Dutta, M. Thimmanagari, Y.W. Chiang, Integrated *Haematococcus pluvialis* biomass production and nutrient removal using bioethanol plant waste effluent, Process. Saf. Environ. Prot. 111 (2017) 128–137, https://doi.org/10.1016/j.psep.2017.06.013.
[9] L. Jiang, H. Pei, W. Hu, Q. Hou, F. Han, C. Nie, Biomass production and nutrient assimilation by a novel microalga, *Monoraphidium* spp. SDEC-17, cultivated in a high-ammonia wastewater, Energy Convers. Manag. 123 (2016) 423–430, https://doi.org/10.1016/j.enconman.2016.06.060.
[10] K. Reitan, J. Rainuzzo, G. Øie, Y. Olsen, A review of the nutritional effects of algae in marine fish larvae, Aquaculture 155 (1997) 207–221.
[11] S.A. Castine, A.D. Mckinnon, N.A. Paul, L.A. Trott, R. De Nys, Wastewater treatment for land-based aquaculture: improvements and value-adding alternatives in model systems from Australia, Aquac. Environ. Interact. 4 (2013) 285–300, https://doi.org/10.3354/aei00088.
[12] M. Dourou, P. Dritsas, M.N. Baeshen, A. Elazzazy, A. Al-Farga, G. Aggelis, High-added value products from microalgae and prospects of aquaculture wastewaters

as microalgae growth media, FEMS Microbiol. Lett. 367 (2020), fnaa081, https://doi.org/10.1093/femsle/fnaa081.
[13] M.E. Grima, E.H. Belarbi, F.G. Acién Fernández, A. Robles Medina, Y. Chisti, Recovery of microalgal biomass and metabolites: process options and economics, Biotechnol. Adv. 20 (2003) 491–515, https://doi.org/10.1016/S0734-9750(02)00050-2.
[14] A. Lavrinovičs, T. Juhna, Review on challenges and limitations for algae-based wastewater treatment, Constr. Sci. 20 (2018) 17–25, https://doi.org/10.2478/cons-2017-0003.
[15] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, Renew. Sust. Energ. Rev. 14 (2010) 217–232, https://doi.org/10.1016/j.rser.2009.07.020.
[16] S. Tibbetts, The potential for 'Next-Generation', microalgae-based feed ingredients for salmonid aquaculture in context of the blue revolution, Microalgal Biotechnol. IntechOpen (2018), https://doi.org/10.5772/intechopen.73551.
[17] M.T. Brett, D.C. Müller-Navarra, The role of highly unsaturated fatty acids in aquatic foodweb processes, Freshw. Biol. 38 (1997) 483–499, https://doi.org/10.1046/j.1365-2427.1997.00220.x.
[18] L.E.C. Conceição, H. Grasdalen, I. Rønnestad, Amino acid requirements of fish larvae and post-larvae: new tools and recent findings, Aquaculture 227 (2003) 221–232, https://doi.org/10.1016/S0044-8486(03)00505-2.
[19] E.T. Peltomaa, S.L. Aalto, K.M. Vuorio, S. Taipale, The importance of phytoplankton biomolecule availability for secondary production, Front. Ecol. Evol. 5 (2017), https://doi.org/10.3389/fevo.2017.00128.
[20] D.A. Kreeger, A.J.S. Hawkins, B.L. Bayne, D.M. Lowe, Seasonal variation in the utilization of dietary protein for energy and biosynthesis by the mussel *Mytilus edulis*, Mar. Ecol. Prog. Ser. 126 (1995) 177–184, https://doi.org/10.3354/meps126177.
[21] Y. Hou, K. Yao, Y. Yin, G. Wu, Endogenous eynthesis of amino acids limits growth, lactation, and reproduction in animals, Adv. Nutr. 7 (2016) 331–342, https://doi.org/10.3945/an.115.010850.
[22] S. Egloff, F. Tschudi, Z. Schmautz, D. Refardt, High-density cultivation of microalgae continuously fed with unfiltered water from a recirculating aquaculture system, Algal Res. 34 (2018) 68–74, https://doi.org/10.1016/j.algal.2018.07.004.
[23] Č. Stevčić, K. Pulkkinen, J. Pirhonen, Screening of microalgae and LED grow light spectra for effective removal of dissolved nutrients from cold-water recirculating aquaculture system (RAS) wastewater, Algal Res. 44 (2019), https://doi.org/10.1016/j.algal.2019.101681.
[24] L. Xu, P. Weathers, X. Xiong, C. Liu, Microalgal bioreactors: challenges and opportunities, Eng. Life Sci. 9 (3) (2009) 178–189, https://doi.org/10.1002/elsc.200800111.
[25] J. Day, Y. Gong, Q. Hu, Microzooplanktonic grazers – a potentially devastating threat to the commercial success of microalgal mass culture, Algal Res. 27 (2017) 356–365, https://doi.org/10.1016/j.algal.2017.08.024.
[26] F.J. Choix, L.E. De-Bashan, Y. Bashan, Enzyme and microbial technology enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions, Enzyme Microb. Technol. 51 (2012) 300–309, https://doi.org/10.1016/j.enzmictec.2012.07.012.
[27] T. Halfhide, A. Åkerstrøm, O.I. Lekang, H.R. Gislerød, S.J. Ergas, Production of algal biomass, chlorophyll, starch and lipids using aquaculture wastewater under axenic and non-axenic conditions, Algal Res. 6 (2016) 152–159, https://doi.org/10.1016/j.algal.2014.10.009.
[28] L.A. Leyva, Y. Bashan, A. Mendoza, L.E. De-Bashan, Accumulation fatty acids of in *Chlorella vulgaris* under heterotrophic conditions in relation to activity of acetyl-CoA carboxylase, temperature, and co-immobilization with *Azospirillum brasilense*, Naturwissenschaften 101 (2014) 819–830, https://doi.org/10.1007/s00114-014-1223-x.
[29] A. Anbalagan, S. Schwede, C. Lindberg, E. Nehrenheim, Influence of iron precipitated condition and light intensity on microalgae activated sludge based wastewater remediation, Chemosphere 168 (2017) 1523–1530.
[30] J.T. Pulkkinen, T. Kiuru, S.L. Aalto, J. Koskela, J. Vielma, Startup and effects of relative water renewal rate on water quality and growth of rainbow trout (*Oncorhynchus mykiss*) in a unique RAS research platform, Aquac. Eng. 82 (2018) 38–45, https://doi.org/10.1016/j.aquaeng.2018.06.003.
[31] Y. Wang, F. Hammes, N. Boon, T. Egli, Quantification of the filterability of freshwater bcteria through 0.45, 0.22, and 0.1 microm pore size filters and shape-dependent enrichment of filterable bacterial communities, Environ. Sci. Technol. 41 (2007) 7080–7086, https://doi.org/10.1021/es0707198.
[32] R.R.L. Guillard, C.J. Lorenzen, Yellow-green algae with chlorphyllide C, J. Phycol. 8 (1972) 10–14.
[33] R.A. Andersen (Ed.), Algal Culturing Techniques, 1st ed., Elsevier Academic Press. Phycological Society of America, New York, US, 2005.
[34] J. Keskitalo, K. Salonen, Manual for Integrated Monitoring. Subprogramme Hydrobioiogy of Lakes, B 16. ed., National Board of Waters and the Environment, Finland, Helsinki, 1994.
[35] D.O. Hessen, E. Leu, Trophic transfer and trophic modification of fatty acids in high Arctic lakes, Freshw. Biol. 51 (2006) 1987–1998, https://doi.org/10.1111/j.1365-2427.2006.01619.x.
[36] S.J. Taipale, K. Vuorio, S.L. Aalto, E. Peltomaa, M. Tiirola, Eutrophication reduces the nutritional value of phytoplankton in boreal lakes, Environ. Res. 179 (2019), https://doi.org/10.1016/j.envres.2019.108836.
[37] S.O. Lourenço, E. Barbarino, P.L. Lavín, U.M. Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogentoprotein conversion factors, Eur. J. Phycol. 39 (2004) 17–32, https://doi.org/10.1080/0967026032000157156.
[38] M.J. Anderson, Distance-based tests for homogeneity of multivariate dispersions, Biometrics 62 (2006) 245–253, https://doi.org/10.1111/j.1541-0420.2005.00440.x.
[39] J. Oksanen, F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, H. Wagner, vegan: Community Ecology Package, R package version 2.5–3, https://CRAN.R-project.org/package=vegan, 2018.
[40] Y. Tejido-Nuñez, E. Aymerich, L. Sancho, D. Refardt, Treatment of aquaculture effluent with *Chlorella vulgaris* and *Tetradesmus obliquus*: the effect of pretreatment on microalgae growth and nutrient removal efficiency, Ecol. Eng. 136 (2019) 1–9, https://doi.org/10.1016/j.ecoleng.2019.05.021.
[41] Y. Tejido-Nuñez, E. Aymerich, L. Sancho, D. Refardt, Co-cultivation of microalgae in aquaculture water: interactions, growth and nutrient removal efficiency at laboratory- and pilot-scale, Algal Res. 49 (2020), 101940, https://doi.org/10.1016/j.algal.2020.101940.
[42] S. Taipale, E. Peltomaa, P. Salmi, Variation in ω-3 and ω-6 polyunsaturated fatty acids produced by different phytoplankton taxa at early and late growth phase, Biomolecules 10 (2020), https://doi.org/10.3390/biom10040559.
[43] W. Yee, Feasibility of various carbon sources and plant materials in enhancing the growth and biomass productivity of the freshwater microalgae *Monoraphidium griffithii* NS16, Bioresour. Technol. 196 (2015) 1–8, https://doi.org/10.1016/j.biortech.2015.07.033.
[44] Y. Zhao, Z. Ge, H. Lui, S. Sun, Ability of different microalgae species in synthetic high-strength wastewater treatment and potential lipid production, J. Chem. Technol. Biotechnol. 91 (2016) 2888–2895, https://doi.org/10.1002/jctb.4905.
[45] S. Taipale, M. Hiltunen, K. Vuorio, E. Peltomaa, Suitability of phytosterols alongside fatty acids as chemotaxonomic biomarkers for phytoplankton, Front. Plant Sci. 7 (2016) 212, https://doi.org/10.3389/fpls.2016.00212.
[46] S. Taipale, U. Strandberg, E. Peltomaa, A.W.E. Galloway, A. Ojala, M.T. Brett, Fatty acid composition as biomarkers of freshwater microalgae: analysis of 37 strains of microalgae in 22 genera and in 7 classes, Aquat. Microb. Ecol. 71 (2013) 165–178, https://doi.org/10.3354/ame01671.
[47] P.G. Scodelaro Bilbao, C. Damiani, G.A. Salvador, P. Leonardi, *Haematococcus pluvialis* as a source of fatty acids and phytosterols: potential nutritional and biological implications, J. Appl. Phycol. 28 (2016) 3283–3294, https://doi.org/10.1007/s10811-016-0899-z.
[48] S. Taipale, M.J. Kainz, M.T. Brett, A low ω-3:ω-6 ratio in *Daphnia* indicates terrestrial resource utilization and poor nutritional condition, J. Plankton Res. 37 (2015) 596–610, https://doi.org/10.1093/plankt/fbv015.
[49] M.T. Brett, D.C. Müller-Navarra, A.P. Ballantyne, J.L. Ravet, C.R. Goldman, *Daphnia* fatty acid composition reflects that of their diet, Limnol. Oceanogr. 51 (2006) 2428–2437, https://doi.org/10.4319/lo.2006.51.5.2428.
[50] S. Taipale, M.J. Kainz, M.T. Brett, Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia*, Oikos 120 (2011) 1674–1682, https://doi.org/10.1111/j.1600-0706.2011.19415.x.
[51] A.A. Kolmakova, V.I. Kolmakov, Amino acid composition of green microalgae and diatoms, cyanobacteria, and zooplankton (review), Inland Water Biol. 12 (2019) 452–461, https://doi.org/10.1134/S1995082919040060.
[52] R.F. Anderson, R.W. Jackson, Microbiological process report. Essential amino acids in microbial proteins, Appl. Microbiol. 6 (1958) 369–373.
[53] G. Ahlgren, I.B. Gustafsson, M. Boberg, Fatty acid content and chemical composition of freshwater microalgae, J. Phycol. 28 (1992) 37–50, https://doi.org/10.1111/j.0022-3646.1992.00037.x.
[54] P. Fink, C. Pflitsch, K. Marin, Dietary essential amino acids affect the reproduction of the keystone herbivore *Daphnia pulex*, PLoS One 7 (2012), https://doi.org/10.1371/annotation/6d71b282-8e08-43ba-bef7-7ad45cd48784.
[55] N.K. Singh, D.W. Dhar, Microalgae as second generation biofuel. A review, Agron. Sustain. Dev. 31 (2011) 605–629, https://doi.org/10.1007/s13593-011-0018-0.
[56] O. Torrissen, R. Christiansen, Requirements for carotenoids in fish diets, J. Appl. Ichthyol. 11 (1995) 225–230, https://doi.org/10.1111/j.1439-0426.1995.tb00022.x.
[57] V. Liyanaarachchi, G. Nishshanka, R. Premaratne, T. Ariyadasa, P. Nimarshana, A. Malik, Astaxanthin accumulation in the green microalga *Haematococcus pluvialis*: effect of initial phosphate concentration and stepwise/continuous light stress, Biotechnol. Rep. 28 (2020), e00538. ISSN 2215-017X, https://doi.org/10.1016/j.btre.2020.e00538.
[58] C.J.S. Bolch, T.A. Bejoy, D.H. Green, Bacterial associates modify growth dynamics of the Dinoflagellate *Gymnodinium catenatum*, Front. Microbiol. 8 (2017) 670, https://doi.org/10.3389/fmicb.2017.00670.
[59] G. Rhee, Competition between an alga and an aquatic bacterium for phosphate, Limnol. Oceanogr. 17 (1972), https://doi.org/10.4319/lo.1972.17.4.0505.