Master's Thesis

Biochemical profiling of temperate zone freshwater green microalgae grown in filtered and unfiltered recirculating aquaculture systems effluent and microalgae medium

Marco Calderini

University of Jyväskylä

Department of Biological and Environmental Science

Aquatic Sciences

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UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science
Department of Biological and Environmental Science
Aquatic Sciences

Marco Calderini Biochemical profiling of temperate zone freshwater green microalgae grown in filtered/unfiltered RAS wastewater and microalgae medium

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Supervisors: Senior lecturer Katja Pulkkinen, senior lecturer Sami Taipale and doctoral student Cedomir Stevcic.
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Microalgae cultivation in recirculating aquaculture system's (RAS's) wastewater (WW) is a promising alternative to conventional WW treatment. Conversion of WW nutrients into biomass could lead to new developments in aquaculture. In this master's thesis, I compared the growth, nutrient removal, fatty acid (FA) and amino acid (AA) profiles of three green microalgae: *Haematococcus pluvialis*, *Monoraphidium griffithii* and *Selenastrum* sp. grown in RAS WW and reference growth medium. WW negatively affected the specific growth rate of *Monoraphidium* (0.64 d^{-1}) and *Selenastrum* sp. (0.50 d^{-1}) compared to reference medium (0.72, 0.59 d^{-1}, respectively), while *Haematococcus* was not significantly affected (0.47 - 0.51 d^{-1}). FA differences between WW and reference medium were mostly driven by the increase of oleic acid (18:1n-9) in reference medium (*Haematococcus*: 3.7 - 10.6, *Monoraphidium*: 4.1 - 11.5, *Selenastrum*: 6.3 - 17.0 mean% contribution). AA contribution profiles did not present considerable differences between treatments. The effect of microorganisms in RAS WW was tested by cultivating microalgae in filtered WW. Filtered and unfiltered WW achieved the same specific growth rate and removal of nitrogen (N% 15 – 38%) and phosphate (P% 48 – 99 %) while minor differences were observed in their FA and AA profiles. These findings support the use of microalgae as an alternative technique for treatment of RAS WW while producing valuable biomass.

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Viljelemällä mikroleviä kalojen kiertovesiviljelyn jätevedessä siitä voidaan poistaa ravinteita ja tuottaa niistä biomassaa. Menetelmää voidaan käyttää korvaamaan perinteistä jäteveden käsittelyä ja kehittämään vesiviljelyn kannattavuutta. Tässä työssä verrattiin kolmen eri viherlevälajin (*Haematococcus pluvialis*, *Monoraphidium griffithii* and *Selenastrum sp.*) kasvua, ravinteiden poistoa, sekä rasvahappo- ja aminohappoprofiileja, kun leviä kasvatettiin suodatetussa tai suodattamattomassa kiertovedessä tai levien elatusaineessa. *Monoraphidium* ja *Selenastrum* -lajien kasvunopeudet olivat pienemmät kiertovedessä (0.64 vrk^{-1} ja 0.50 vrk^{-1}) kuin levien elatusaineessa (0.72 ja 0.59 vrk^{-1}), mutta *Haematococcus*-levän kasvunopeudessa ei ollut eroa kasvuliuosten välillä (0.47 ja 0.51 vrk^{-1}). Levien rasvahappokoostumukset erosivat lähinnä oleeinihapon (18:1n-9) osuuksien suhteen, jotka olivat alemmat kiertovedessä kaikilla levälajeilla. Aminohappoprofiileissa ei ollut suuria eroja kasvuliuosten välillä. Kiertoveden sisältämien pieneliöiden vaikutusta leviin testattiin vertaamalla suodatettua ja suodattamatonta kiertovettä. Levien kasvunopeudet ja typen- ja fosforinpoisto eivät eronneet suodatetun ja suodattamattoman kiertoveden välillä, mutta rasvahappo- ja aminohappoprofiileissa oli jonkin verran eroja. Tuloksien perusteella levien kasvatusta kiertovedessä voidaan suositella kiertoveden puhdistusmenetelmäksi, jonka avulla voidaan samalla tuottaa arvokasta biomassaa.

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TERMS AND ABBREVIATIONS

TERMS

Aquaculture	Farming of fish, crustaceans, molluscs, aquatic plants, algae, and other organisms
Biomass	The total quantity or weight of an organism in a given area or volume

ABBREVIATIONS

AA	amino acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FA	fatty acid
MWC	modified Wright's Cryptophyte Medium
N	nitrogen
P	phosphorus
RAS	recirculating aquaculture system
WW	wastewater

1 INTRODUCTION

1.1 Recirculating aquaculture system wastewater

Aquaculture is one of the fastest growing food industries in the world with an estimated average annual growth of 3.2% (FAO 2016). This steady increase in farmed fish production is not predicted to slow down in the near future due to the growing demand of animal protein in developing countries together with the overexploited state of the world's largest fisheries (FAO 2016). In the last couple of years, concerns have been raised about the environmental effects that open aquaculture systems pollution have on aquatic ecosystems (Blancheton 2017). Recirculating aquaculture systems (RASs) represent a promising solution to the environmental concerns since they allow the production of aquatic organisms in an enclosed environment, limiting the discharge of nutrient rich WW into nature (Verdegem et al. 2006). The term "recirculating" alludes to the reutilization of water in the system thanks to a multi-step purification process that involves mechanical and biological filtration sometimes coupled with disinfection and oxygen enrichment steps (Bregnballe 2015). Due to the importance of fish production in several European countries, the European Union is promoting RAS implementation as an environmentally friendly alternative to further develop aquaculture (Badiola et al. 2012). Despite the fact that water reutilization can be highly efficient in RAS, WW production cannot be avoided. Boyd (1984) described that the production of 1 ton (1000 kg) of live channel catfish would on average generate 1190 kg of dry organic matter, 60 kg of nitrogen (N) and 12 kg of phosphorus (P) waste. Although modern RAS can decrease waste production, N, P and particulate matter will ultimately end up as a component of WW or sludge. Available WW treatment processes help reduce the nutrient load, but they are energetically and chemically demanding, which rises the production costs and they do not recover valuable nutrients from the treated water (Martins et al. 2010). Ideally, an integrated approach that utilizes the nutrients present in WW to generate a valuable end-

product would not only reduce WW's nutrient load but also facilitate energy and costs savings for the aquaculture facility (Graber and Junge 2009, Martins et al. 2010). Even though several strategies have received the attention of researchers as promising solutions to the RAS WW challenge, algae utilization as a bioremediation technology able to produce valuable biomass has been raised as a novel approach (Alcántara et al. 2015, Gonçalves et al. 2017). Since algae can make use of the principal forms in which N and P occur in different types of wastewater (NH_4^+ - ammonia, NO_2^- - nitrite, NO_3^- - nitrate and PO_4^{3-} - orthophosphate) (Horan 1990) as nutrients to grow, algae utilization for nutrient removal has been proven to be feasible in sewage WWs, centrate, manure and other effluents (Olguín 2012, Craggs et al. 2013, Hernández et al. 2013, Fernandez et al. 2018). Due to its nutrient composition, RAS WW also classifies as a potential growth media for algae. Several studies have tested the suitability of aquaculture effluents for microalgae cultivation showing that efficient nutrient removal and adequate biomass production can be achieved (Sirakov and Velichkova 2014, Cheban et al. 2015, Stevčić et. al 2019, Tossavainen et al. 2019b).

1.2 Microalgae characteristics and applications in recirculating aquaculture systems

The term "algae" is used to describe a large conglomerate of predominantly aquatic organisms coming from different phylogenetic groups. It encompasses eukaryotic and prokaryotic organisms with plant-like characteristics that are widely distributed around the world (Lee 1989). The presence of thylakoids in their structure allow algae to transform solar energy into chemical energy through the process of photosynthesis. What classifies an organism as an algae is the presence of chlorophyll-a and the lack of plant structures such as roots, stem and leaves (Lee 1989). Even though there is great variation withing this group, most algae are microscopic unicellular organisms ranging from 2 to 200 µm in size. Due to their small size, this subgroup is referred to as microalgae. The structural simplicity and

the high solar irradiance surface area to volume ratio gives microalgae high photosynthetic efficiency, which translates into high biomass production and fast growth (Schenk et al. 2008, Subramanian et al. 2013). As a result, cultivation of microalgae requires minimal areas of land when compared to plants (Mata et al. 2010, Wigmosta et al. 2011). Depending on the species and the cultivation conditions, microalgae can accumulate high amounts of proteins, carbohydrates and lipids (Mata et al. 2010, Das et al. 2011). In addition, some species exhibit substantial concentrations of high-value chemicals such as antioxidants, vitamins, certain fatty acids, β-carotenes and other pigments, alginate and carrageenan that have application in different industrial sectors (e.g. cosmetics, nutraceuticals, functional foods) (Barrow and Shahidi 2008, Mata et al. 2010). For RAS enterprises, microalgal biomass can serve as an onsite produced feed source or feed additive (Mata et al. 2010). Several microalgae species have been successfully used in aquaculture as a feed complement for small fish larvae, crustaceans, and mollusks (Brown et al. 1997, Muller-Feuga 2000). However, poor digestibility of microalgae's nutrients has been shown to reduce nutrient availability in some fish species (Shah et al. 2018). In addition, due to the small size of microalgae cells, their use as feed can be restricted to larval stages (Benemann 1992, Shah et al. 2018). Although these characteristics present important drawbacks for the use of microalgae as fish feed, microalgae can also serve as a nutritional food source for zooplankton such as rotifers, cladocerans and copepods (De Pauw et al. 1984, Borowitzka 1997, Brown et al. 1997). Since zooplankton can be then used as a live feed for crustaceans and fish (Koivisto 1995, Borowitzka, 1997), this strategy could by-pass the constrains given by microalgae size and digestibility. Moreover, zooplankton can be enriched with vitamins (Merchie et al. 1995, Brown et al. 1998), and lipids (Nichols et al. 1989, Gara et al. 1998) when fed microalgae, which increases their nutritional quality as fish feed. Overall, RAS WW could ultimately support the development of multitrophic-aquaculture production systems (Martins et al. 2010), reducing the production costs of the facility.

Despite the fact that nutritional requirements have been established for some highly farmed aquatic organisms, no set of nutritional criteria have yet been developed (Mata et al. 2010). Generally, for the use of microalgae as feed, it is expected that they follow the criteria of having an acceptable size for digestion, be non-toxic, have a digestible cell wall and possess enough biochemical constituents to promote its consumer's growth. Regarding biochemical constituents, lipids and proteins play a central role in nutrition (Strayer 1988). Lipid quality rather than quantity has been shown to be important to optimize nutrition. Fatty acids (FAs), especially omega 3 and omega 6, have been described to be important for the development and growth of fish, zooplankton, bivalves, and others (De Pauw et al. 1984, von Elert 2002, Arts et al. 2009, Marshall et al. 2010). In addition, FA composition is strongly related to that of their diets (Arts et al. 2009). Proteins, more precisely amino acids (AAs) can be subdivided in essential and non-essential AAs, depending on the capacity of an organism to de-novo synthesize them (Strayer 1988). Restriction of dietary essential AAs have been shown to negatively impact fish and zooplankton growth and reproduction (Kreeger et al. 1996, Conceição et al. 2003, Koch et al. 2009). Since microalgae FAs and AAs play a central role in its consumer's growth and reproduction, the study of FAs and AAs composition profiles as indicators of biomass nutritional quality is of vital importance for multitrophic production systems in aquaculture facilities.

1.3 Cultivation media and its effect on microalgal growth, fatty acid and amino acid composition

Microalgae cell growth together with FA and AA composition have been shown to be susceptible to changes in the cultivation media. Physicochemical factors such as nutrients (macro and micronutrients), light supply, pH, temperature and salinity have been described to affect microalgae cell growth (Sunda et al 2005, Kim et al. 2014, 2016, Bartley et al. 2016). Importantly, growth media nitrogen and phosphorus concentration and ratio (N:P) have an impact on cell growth (Zhang and Hu 2011,

Mayers et al. 2014, Rasdi and Qin 2015). Lipid and protein content together with FAs and AAs composition are also susceptible to changes in nutrient, temperature and light supply (Dauta et al. 1990, Miranda et al. 2001, Kim et al. 2014, Morschett et al. 2017, Ballesteros-Torres et al. 2019). Of special consideration for the use of RAS WW for microalgae cultivation is the presence of other microorganisms. Most studies looking at changes in cell growth, FA and AA adaptations of microalgae have been carried out under axenic conditions (microalgal monocultures without other organisms) (Halfhide et al. 2014). Bacteria, protozoans and non-target algae may compete for nutrients, reduce light availability or even be toxic or predatory for the microalgae of interest directly affecting its growth or biochemical constituents (Gantar et al. 2008, Xu et al. 2009, Singh et al. 2011). However, there is evidence suggesting that some bacteria could instead improve algae production by helping mineralize organic substrates and producing growth factors that support microalgae growth (Bell 1983, Haglund and Pedersén 1993, Gantar et al. 2008, Thi et al. 2010). Since maintaining axenic large-scale microalgae cultures is not practical or economically feasible, the implications of the presence of microorganisms in RAS WW on microalgal productivity and nutritional characteristics (given by FAs and AAs) must be studied.

In this thesis work, I studied the suitability of RAS WW to produce microalgae and the impact that this culture media has on the nutritional value of the generated biomass. To do so, I evaluated three freshwater green microalgae species: *Haematococcus pluvialis, Monoraphidium griffithii* and *Selenastrum* sp. which have been shown to grow efficiently in RAS WW under conditions common in Nordic aquaculture (~17 ˚C) (Stevčić et al. 2019). Nutritional characteristics of microalgae were studied by analyzing the FA and AA composition of the generated biomass. Since the presence of microorganisms in WW could affect the growth and biochemical composition of microalgae, I tested the effects of minimizing microbial load by using filtered WW as growth media. In order to have a reference to compare the efficiency of nutrient removal, cell growth, FA and AA composition of the

selected species in RAS WW (filtered and unfiltered), I used Modified Wright's Cryptophyte medium (MWC). MWC has been described to possess all the required nutrients to optimize microalgae growth. In summary, my experimental design consisted of three microalgae species (*Haematococcus, Monoraphidium* and *Selenastrum*) grown in three different media (MWC, unfiltered WW and filtered WW). In each one of these treatments I evaluated cell growth, nutrient removal, FA and AA composition.

My hypotheses were:

1- All selected microalgae species are able to grow in RAS WW.
2- Due to competition for nutrients with other microorganisms, microalgae in unfiltered WW have a lower growth and biomass production than microalgae in filtered WW.
3- Microalgae cultivated in WW (either filtered or unfiltered) present a different FA and AA profile compared to microalgae cultivated in MWC.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Microalgae strains

The temperate zone freshwater green microalgae strains *Haematococcus pluvialis, Monoraphidium griffithii* and *Selenastrum* sp. were obtained from culture collections (Stevcic et al. 2019). Each strain was first maintained as a stock monoculture in 650 mL plastic culture flasks containing 400 mL of Modified Wright's Cryptophyte (MWC) medium (reference culture medium), based on Guillard and Lorenzen (1972) (Appendix 1) at the Department of Biological and Environmental Science, University of Jyväskylä. Stock monocultures were maintained under a 12:12 h light:dark photoperiod with an approximate 50 – 70 µmol photon m^{-2} s^{-1} light intensity provided by a fluorescent light. The temperature in the cultivation room was maintained at 17 °C.

2.1.2 Microalgae media

RAS wastewater utilized in all experiments was provided by the Natural Resources Institute Finland (LUKE) Laukaa fish farm. The research facility consists of an experimental RAS platform. The detailed structure and operation of the RAS platform has been described by Pulkkinen et al. (2018). Wastewater samples were collected from the water outlet of two individual recirculating systems 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*) specimens with a mean weight of 453 and 437 g fed with Raisio Circuit Silver 3.5mm at 0.7% body weight/d. Water circulation was set at 0.2 L/s and replacement water adjusted at 250 L/kg of feed. Water quality measurements one week prior to sampling were: temperature= 15±0.6 °C, pH= 7± 0.1, NO_3= 101±1 mg/L. Samples

from both tanks were mixed together and stored at 6 °C until utilization. MWC medium was prepared as described in Appendix 1.

2.2 Methods

2.2.1 Experimental setup and cultivation conditions

The three studied microalgae strains were cultivated in unfiltered (raw) RAS wastewater, filtered RAS wastewater and MWC medium (Table 1). In order to obtain filtered RAS wastewater, raw RAS was filtered through 0.45 µm syringe filters (Corning® syringe filters, Sigma-Aldrich). The cultivation of algae was divided in two experimental series, both series containing two replicates of each of the selected strains in all three culture media. The selected microalgae were grown in 650 mL plastic culture flasks. Each flask contained 400 mL of culture media (MWC, filtered or unfiltered WW) and had two thin plastic tubes going through the cap serving the purpose of aeration and sampling. The flasks were always aerated (without additional CO_2) at approximately 33 mL min^{-1} (Eheim air pump 400, Germany); incoming air was filtered through a 0.22 µm syringe filter. pH was not regulated during cultivation. On day 0 (start of experimental series), each flask containing the corresponding growth media was inoculated with 1-10% of the (previously determined) microalgae stock culture saturating concentration. During cultivation, the flasks were constantly illuminated by two LED grow lights (18 W, L-series T8 tubes, Valoya Oy, Finland) with a light intensity of 70 - 100 µmol photon m^{-2} s^{-1} measured with a high resolution spectrometer (HP-350 HiPoint, Taiwan). Light only impacted one of the sides of the culture flasks. Room temperature was maintained at 17±0.5 °C at all times. 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 inoculation before the cultures reach stationary phase. Phase of the culture was

determined comparing the culture cell density to growth curves obtained in pilot studies.

Table 1. Nutrient composition and pH of reference culture medium (MWC), unfiltered RAS wastewater (WWU) and filtered RAS wastewater (WWF) prior to inoculation of microalgae. Values are shown as mean ± SD of both experimental series. *N:P molar ratio was calculated from NO_3-N:PO_4-P.

Composition	MWC	WWU	WWF
NH_4-N (mg L^{-1})	0.05 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
NO_2-N (mg L^{-1})	0.01 ± 0.00	0.03 ± 0.00	0.05 ± 0.00
NO_3-N (mg L^{-1})	14.05 ± 0.47	96.87 ± 0.73	97.03 ± 0.17
PO_4-P (mg L^{-1})	1.54 ± 0.05	3.83 ± 0.07	3.76 ± 0.04
*N:P molar ratio	20.16 ± 0.04	55.90 ± 0.62	57.05 ± 0.51
pH	7.61 ± 0.12	7.49 ± 0.28	7.39 ± 0.04

2.2.2 Determination of cell density and biomass production

Throughout the cultivation period, cell density was estimated daily by cell count in a haemocytometer chamber (Bürker) with 100x magnification on the microscope (Leitz 184 Laborlux D, Germany). Density value was calculated as the mean of two individual counting replicates. The specific growth rate per day (μ, d^{-1}) was calculated from the change in cell density in a determined time interval according to the following equation:

$$\mu = \frac{ln\,N1 - lnNo}{\Delta t}, \quad (1)$$

where *N0* and *N1* are number of cells at the beginning and the end of the time interval and Δt is the length of a time interval (t1 – t0) (Andersen, 2005).

To determine total dry weight (biomass), two aliquots of culture were taken at the end of the cultivation period (day 6). Biomass was estimated by filtering the known volume of culture through a pre-weighted fiber filter (Whatman, GF/A, Merck, Germany). The filter containing the sample was then oven-dried overnight at 105 °C and desiccated for 30 min prior to weighing (Sartorius CP2P, Germany).

2.2.3 Assessment of chlorophyll-a content

Chlorophyll-a concentration was assessed at the end of the cultivation period (day 6) by taking an aliquot of known volume of culture and filtrating it through a fiber filter (Whatman, GF/A, Merck, Germany). The filtrate was then incubated in 20 mL of ethanol at 75 °C for 1 h to extract the pigment. The absorbance of the extraction product was analyzed spectrophotometrically (Shimadzu Spectrophotometer UV-1800, Japan) at wavelengths 665 and 750 (Keskitalo and Salonen, 1994). The chlorophyll-a concentration (µg L^{-1}) was calculated utilizing Keskitalo and Salonen's (1994) equation:

$$chl\ a = 11{,}9 * A * \frac{Ve}{Vs * d'}, \quad (2)$$

where 11.9 is the coefficient of absorbance of chlorophyll-a at 25°C, A is the difference in absorption of chlorophyll-a at 665 nm and 750 nm, Ve is the volume of used ethanol (mL), Vs is the volume of microalgae utilized for the analysis (mL), and d is the spectrophotometer cuvette width in cm.

2.2.4 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 at the end of the cultivation period with testing kits LC399 and LCK349 (Hach, Colorado, 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 filtrated trough a 0.22 µm syringe filter. Percentage of nutrient uptake (i%) was calculated following the equation

$$i\% = \frac{So - S1}{So} \times 100, \quad (3)$$

Where i% is the percentage of nutrient uptake. Nutrient removal rate (Ri) was determined utilizing the following equation

$$Ri = \frac{So - S1}{\Delta t}, \quad (4)$$

where Ri is the nutrient removal rate of the substrate i (NO_3-N, PO_4-P) (mg L^{-1} d^{-1}), S_o and S_1 correspond to initial and final concentrations of the nutrient (mg L^{-1}) and Δt is the length of the time interval (t1 – t0) (Wang and Lan, 2011; Delgadillo-Mirquez et al., 2016). Cell uptake rate was calculated as:

$$Vi = \frac{So - S1}{Cc * \Delta t}, \quad (5)$$

Where V_i is the nutrient removal rate of the substrate i (NO_3-N, PO_4-P) per microalgal cell (mg $cell^{-1}$ d^{-1}), S_o and S_1 are the nutrient initial and final concentrations (mgL^{-1}) respectively and Cc the cell concentration (cells mL^{-1}) at time t_1 (Whitton et al., 2016).

2.2.5 Fatty acid analysis

Once microalgae cultivation ended (day 6), between 20 – 35 mL of culture were filtrated through a pre-weighted 3,0 µm Cellulose Nitrate Membrans (Whatman, GE Halthcare, United States). The filtrates were then freeze-dried, weighted (Sartorius CP2P, Germany) and stored at -80 °C until analysis (no longer than a month). Total lipid extraction and FA methyl ester formation were carried out following the protocol published by Taipale et al. (2015). Briefly, filters (containing between 2 – 5 mg of filtrate) were placed into test tubes (12.5 ml). Total lipid extraction was carried out with chloroform:methanol:water (4:2:1). Overnight incubation of lipids in

methanolic H_2SO_4 (1% v/v) at 50 °C was used for the 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, U.S.A.) DB-23 column (30 m × 0.25 mm × 0.15 µm) for separation. Quantification calibration curves for individual FAs were prepared with fatty acid standard GLC reference standard 556 C (Nu-Chek prep, INC, Minnesota, United States). 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). Sample FA area values were interpolated in the calibration curve to determine their concentration. FA content (µg/mg g^{-1} DW) was calculated using the following equation:

$$Ci = Mi\ x\ \frac{1}{m\ sample}, \quad (6)$$

where C_i is the content of an individual FA (µg/mg g-1 DW) in the sample, M_i is the FA_i concentration obtained through interpolation in the calibration curve, m_{sample} is the sample dried weight (mg). FA percent values (%) were calculated following the formula:

$$FAi\% = \frac{FAic}{Tot-FAc}\ \ x\ 100, \quad (7)$$

Where FA_i% is the percentage of contribution of FA I, FA_{ic} is the determined concentration of FA_i and Tot-FA_c is the sum concentration of all identified FAs. As described by Hessen and Leu (2006), FAs where then sorted by their mean % contribution and only FAs contributing >0.5 % to the total were used for later analysis (without normalizing the data to 100%). Analyzed FAs were grouped into saturated FA (SFA), mono- unsaturated FA (MUFA), polyunsaturated FA (PUFA), total omega-3 FA (n-3 PUFA) and total omega-6 FA (n-6 PUFA). The ratios of omega 3 to omega 6 (n-3/n-6), unsaturated to saturated FAs (UFA/SFA), and the sum of all FAs (Tot-FA) were calculated.

2.2.6 Amino acid analysis

At the end of the microalgae cultivation period (day 6), between 7 – 10 ml of culture were filtrated through a pre-weighted 3,0 µm Nucleopore Polycarbonate Filters (Whatman, GE Halthcare, United States). The filtrates were then freeze-dried, weighted (Sartorius CP2P, Germany) and stored at -80 °C until analysis (no longer than a month). Filters were placed into test tubes (10 ml) and enough HCl 6 N was added to ensure filters were completely covered in acid. Samples were then heated in an oven at 110°C for 24 h to hydrolyze AAs. After hydrolyzation, HCl was evaporated by leaving the test tubes open at 110°C for 20 h. Free AAs were then derivatized utilizing the commercial kit EZ:faast for Free Physiological Amino Acid Analysis by GC-MS (Phenomenex, Germany) with the exception that no purification column was used during the process. Amino acid chromatographic separation and their posterior identification and quantification was done following the protocol described by Taipale et al. (2019). Briefly, 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-AAA, Phenomenex, United States). 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, United States). Quantification and correction of AA content (µg/mg g-1 DW), together with determination of AA (%), were done as described with FAs (formulas 6 and 7). Only the AAs present in the standard (AAS-180) were identified and quantified in microalgae samples: eight essential amino acids (EAAs: valine – VAL, leucine – LEU, isoleucine – ILE, threonine – THR, methionine – MET, phenylalanine – PHE, lysine – LYS, and histidine – HIS), and seven non-essential amino acids (NEAAs: alanine – ALA, glycine – GLY, serine – SER, proline – PRO, aspartic acid – ASP, glutamic acid – GLU and tyrosine -TYR). The sum of all AAs (Tot-AA) was calculated. 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 (Lourenço et al., 2004).

2.2.7 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, unfiltered WW and MWC) on growth, nutrient uptake, FA and AA categories. 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 by including run as a random factor. Significance of the effect of run was evaluated with 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. If the assumption of normality or heteroscedasticity of the data was violated, I used Kruskal-Wallis's H non-parametric test. Permutational analysis of variance (PERMANOVA) using Bray-Curtis distance matrix was performed on FA and AA percentage (%) data to test if species or media (treatment) were driving dissimilarities. Non-metric multi-dimensional scaling ordinations using Bray-Curtis distance matrix was used to graphically illustrate PERMANOVA results. Similarity percentage test (SIMPER) was used to elucidate the components that drove the most differences in PERMANOVA results. 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 were carried out with either R base or vegan packages (Oksanen et al., 2018; R Core Team, 2017).

3 RESULTS

3.1 Effects of cultivation in filtered and unfiltered RAS wastewater on cell density, biomass and chlorophyll-a compared to MWC

The use of RAS WW as a microalgae cultivation media led to changes in density, specific growth rate and chlorophyll-a content compared to the reference medium (MWC) but minimal effects on the total biomass generated were observed (ANOVA, Table 2; post hoc tests, Appendix 2; Figure 1). Cultivation in WW media (unfiltered or filtered) led to a reduction in the cell density of *Selenastrum* sp. and *Monoraphidium griffithii* by day six compared to MWC (post hoc tests, Appendix 2; Figure 1 A). *Haematococcus pluvialis* had a slightly higher density when cultivated in MWC than in WW but no statistically significant difference was observed between cultivation media (post hoc tests, Appendix 2). When comparing filtered and unfiltered RAS WW, none of the studied microalgae species presented differences in density by day six (post hoc tests, Appendix 2), showing that filtration of WW does not promote nor suppress microalgae growth. Regardless of the cultivation media *Selenastrum* had the highest density among the studied microalgae, followed by *Monoraphidium* and *Haematococcus* (post hoc tests, Appendix 2; Figure 1. A).

A reduction in specific growth rate under WW cultivation was seen in *Selenastrum* and *Monoraphidium* compared to MWC media, while no difference was observed between filtered and unfiltered WW treatments (post hoc tests, Appendix 2; Figure 1 B). In accordance with the density results, *Haematococcus* did not show differences in specific growth rate between cultivation media (post hoc tests, Appendix 2), showing that this microalgae was the only tested species not negatively affected by cultivation in WW compared to MWC. It is important to point out that specific growth rate differed between experimental runs (likelihood ratio test, Table 2).

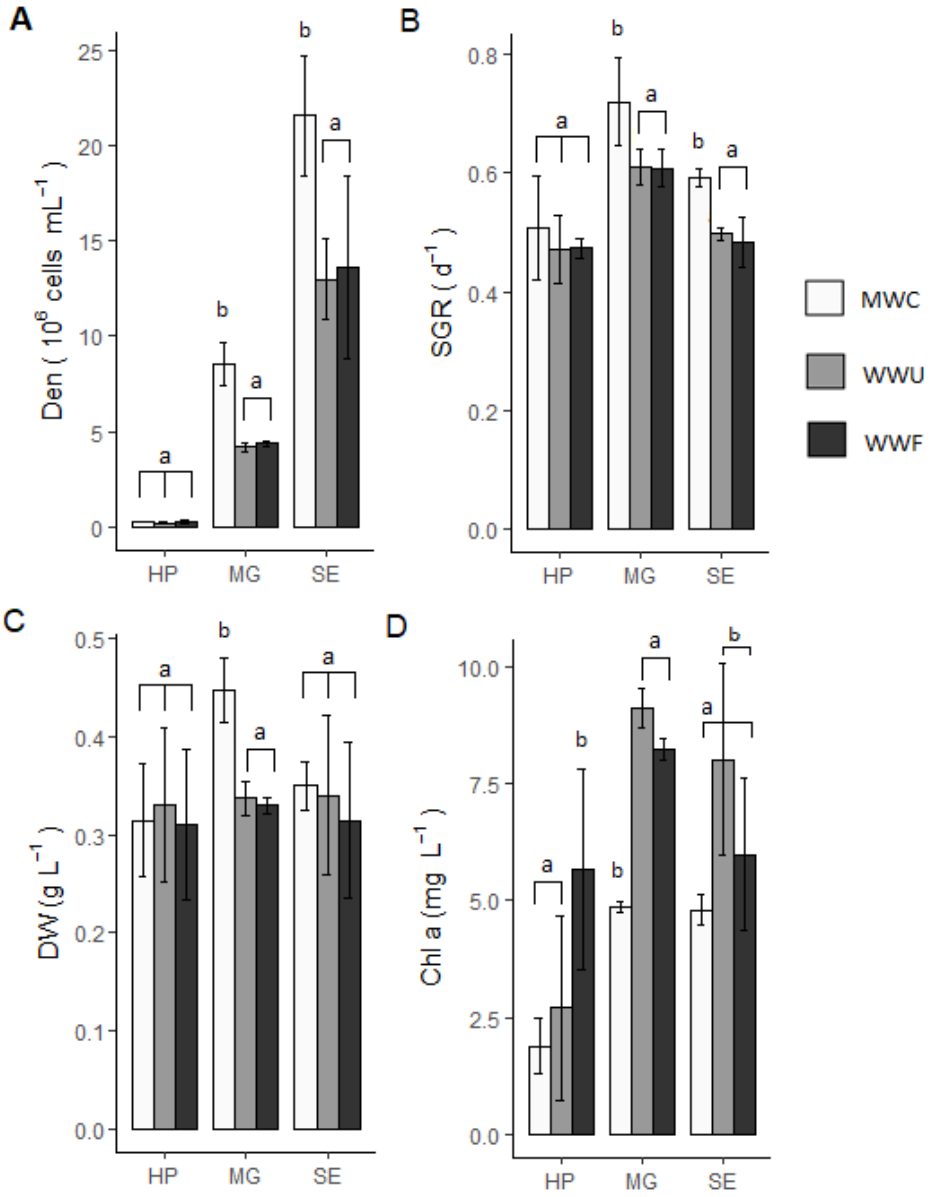

Figure. 1. Density (Den) (**A**), specific growth rate (SGR) (**B**), dry weight (DW) (**C**) and chlorophyll-a concentration (Chl a) (**D**) of three green microalgae (*Haematococcus pluvialis* – HP, *Monoraphidium griffithii* – MG and *Selenastrum* sp. – SE) grown in three different cultivation media (MWC – Modified Wright's Cryptophyte medium (white bars), WWU – unfiltered RAS wastewater (grey bars), WWF – filtered RAS wastewater (black bars)) for six days. Values are presented as mean ± SD of four replicates. Media denoted with the same letter (a-b) are not statistically different from each other for each microalgae. Comparison of treatments between algae are not presented in this figure.

Table 2. Two-way mixed effects analysis of variance (ANOVA) table with Satterthwaite's method testing the effects of treatment (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater), species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, *Selenastrum* sp.) and their interaction (Treatment:Species) to the total variation seen in density, specific growth rate (SGR), dry weight and chlorophyll-a. ANOVA mean squares is denoted as "mean Sq" and likelihood ratio test as "LRT" in the table. On the lower section, ANOVA-like table to test the significance of the random effects of the experimental run. Highlighted (bold values) are all p-values <0.05.

Variable	Source	mean Sq	DF	F	p-value
Density	Treatment	70.58	2	16.448	**<0.001**
	Species	772.16	2	179.939	**<0.001**
	Treatment:Species	22.65	4	5.2787	**0.003**
SGR	Treatment	0.03	2	28.1887	**<0.001**
	Species	0.08	2	73.8427	**<0.001**
	Treatment:Species	0.00	4	1.2962	0.30
Dry Weight	Treatment	0.01	2	2.5289	0.10
	Species	0.01	2	2.6346	0.09
	Treatment:Species	0.01	4	1.5434	0.22
Chlorophyll a	Treatment	31.49	2	18.954	**<0.001**
	Species	51.40	2	30.931	**<0.001**
	Treatment:Species	7.83	4	4.714	**<0.001**
Variable	**Source**	**LRT**	**DF**		**p-value**
Density	Run	2.8×10^{-14}	1		1
SGR	Run	16.67	1		**<0.001**
Dry Weight	Run	0	1		1
Chlorophyll a	Run	0.77	1		0.38

Surprisingly, only *Monoraphidium* and not *Selenastrum* transferred the higher observed density in MWC to an increase in dry weight compared to WW media (post hoc tests, Appendix 2). *Monoraphidium* dry weight was higher under cultivation in MWC and no difference was seen between filtered and unfiltered WW treatments (post hoc tests; Appendix 2; Figure 1 C). *Selenastrum* and *Haematococcus* did not present a significant difference between cultivation media (Appendix 2). No difference was observed in the achieved microalgal dry weight between WW treatments, and no microalgae species presented a higher dry weight than the others

(Table 2; Appendix 2). The mean dry weight obtained in the experiment was 0.34 g L^{-1} independent of the cultivation media (Figure 1 C).

Chlorophyll-a content in *Monoraphidium* and *Selenastrum* was more than 70% higher under unfiltered WW cultivation compared to MWC (Figure 1 D). In contrast, *Haematococcus* did not show a statistically significant difference between MWC and unfiltered WW (Appendix 2). Cultivation in filtered WW media produced a higher chlorophyll-a content in *Monoraphidium* and *Haematococcus* compared to MWC, but no significant difference was seen for *Selenastrum* between these media (post hoc tests, Appendix 2). When comparing between the two WW media, only *Haematococcus* presented a significantly higher chlorophyll-a content when cultivated in filtered WW than in unfiltered WW, while *Monoraphidium* and *Selenastrum* did not present any difference between media (post hoc tests; Appendix 2). Regarding differences between species, *Monoraphidium* and *Selenastrum* presented markedly higher concentrations of chlorophyll-a compared to *Haematococcus* when cultured in unfiltered WW and MWC (post hoc tests, Appendix 2).

3.2 Nitrogen and phosphate removal

PO_4-P (P) was efficiently removed of every cultivation media with an average nutrient removal of 92% (Figure 2 A). *Selenastrum* and *Monoraphidium* had the highest total P uptake, removing on average 99% of the nutrient regardless of the cultivation media. *Haematococcus* showed a lower removal efficiency compared to *Selenastrum* and *Monoraphidium* with an average total P removed of 77%. NO_3-N (N) total removal was highly dependent on the cultivation media (Figure 2 A). All microalgae cultivated in either filtered or unfiltered WW showed under 30% removal of the total N present in the media. On the other hand, under cultivation in MWC, N was completely removed by all tested microalgae. It is worth pointing out that the starting N media concentration was six times higher in RAS WW than in MWC media (Table 1). P and N removal rates differed between treatments and

between species (ANOVA, Table 4; Figure 2 C, D). On average, P and N removal rates were higher in WW media compared to MWC for all microalgae species (post hoc tests; Table 4, 5; Figure 2 B). When microalgae were cultivated in filtered and unfiltered WW, higher removal rates were obtained compared to MWC (post hoc tests, Appendix 3), while no difference was observed between filtered and unfiltered treatments (post hoc tests, Appendix 3). Pairwise comparisons between microalgae species showed that *Haematococcus* had a lower P removal rate than *Monoraphidium* and *Selenastrum* when cultivated in filtered and unfiltered WW (Appendix 3). Cultivation in MWC showed no significant difference in P removal rates between species (post hoc tests, Appendix 3). N removal rates of *Haematococcus* and *Selenastrum* were higher when cultivated in WW (either filtered or unfiltered) compared to MWC (post hoc tests, Appendix 3; Figure 2 D). On the other hand, *Monoraphidium* did not show any difference in N removal rates between cultivation media (post hoc tests, Appendix 3; Figure 2 D). No differences were observed in the N removal rates of microalgae grown in filtered and filtered WW, showing that filtration does not affect N removal (post hoc tests, Appendix 3). Pairwise comparison between species showed that only under cultivation in filtered WW *Monoraphidium* had a significantly lower N removal rate than *Haematococcus* (Appendix 3). Since *Monoraphidium* and *Selenastrum* cultured in MWC completely removed the available P and N by day six, it is possible that P and N removal rates were limited by the absence of the nutrients, partially explaining the large difference between WW and MWC media. Cell uptake rate of P and N varied with microalgae species but only P cell uptake varied between treatments (non-parametric tests, Table 4; Figure 2 E, F). On average, P cell uptake was higher in microalgae cultivated in WW (either filtered or unfiltered) compared to MWC (independent of the species) (Figure 2 E). N cell uptake showed to be slightly higher in WW media (filtered and unfiltered) compared to MWC but no statistically significant difference was observed (non-parametric tests, Table 4; Figure 2 F).

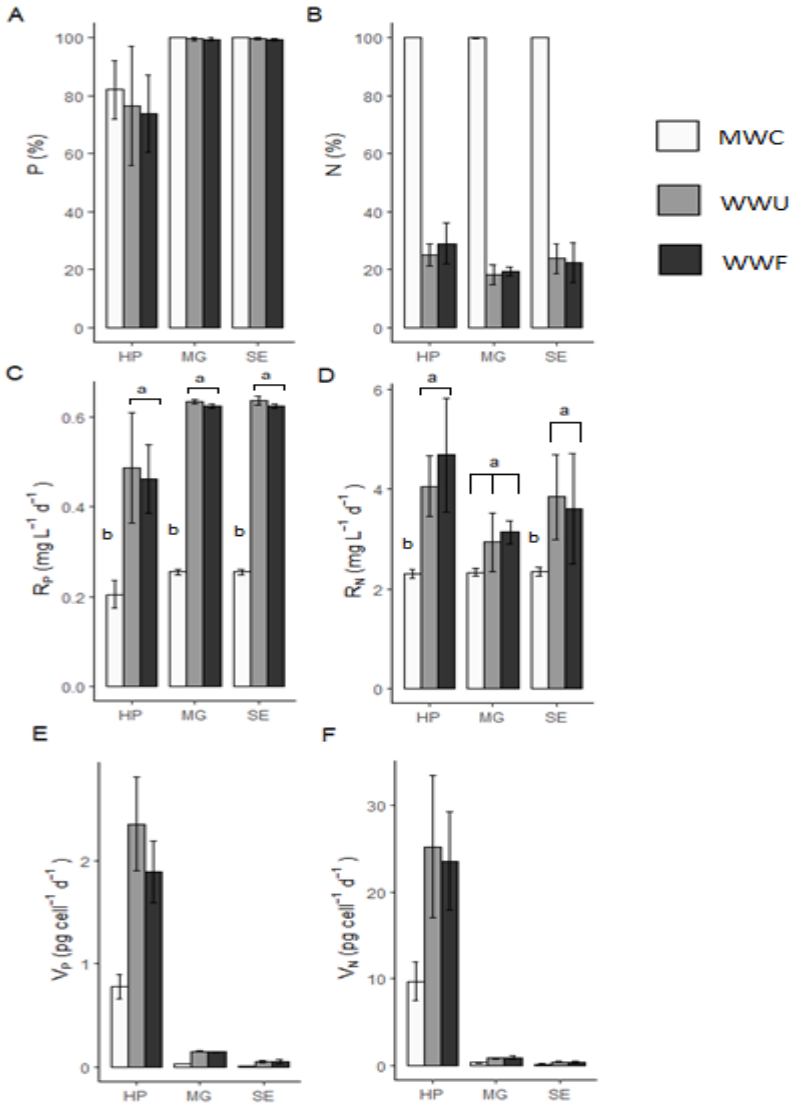

Figure. 2. Percentage of PO_4-P removal (P%) (**A**), percentage of NO_3-N removal (N%) (**B**), PO_4-P removal rates (R_P) (**C**), NO_3-N removal rates (R_N), cell uptake rate of PO_4-P (V_P) (**E**) and cell uptake rate of NO_3-N (V_N) (**F**) of three green microalgae (*Haematococcus pluvialis* – HP, *Monoraphidium griffithii* – MG and *Selenastrum* sp. – SE) grown under three different cultivation media (MWC – Modified Wright's Cryptophyte medium (white bars), WWU – unfiltered RAS wastewater (grey bars), WWF – filtered RAS wastewater (black bars)) for six days. Values are presented as mean ± SD of four replicates. Treatments denoted with the same letter (a-b) are not statistically different from each other for each microalgae. Comparison of treatments between algae are not represented in this figure.

Table 4. Two-way mixed effects analysis of variance (ANOVA) table with Satterthwaite's method testing the effects of treatment (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater), species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) and their interaction (Treatment:Species) to the total variation seen in PO_4-P and NO_3-N removal rates (R_P and R_N, respectively). In the middle section, ANOVA-like table to test the significance of the random effects of the experimental run. At the bottom of the table, non-parametric tests results of the effects of treatment and species on cell uptake rate of PO_4-P and NO_3-N (V_P and V_N, respectively). ANOVA mean squares is denoted as "mean Sq" and likelihood ratio test as "LRT" in the table. Highlighted (bold values) are all p-values <0.05.

Variable	Source	mean Sq	DF	F	p-value
R_P	Treatment	0.46	2	212.847	**<0.001**
	Species	0.06	2	25.911	**<0.001**
	Treatment:Species	0.01	4	2.644	0.06
R_N	Treatment	7.83	2	17.407	**<0.001**
	Species	2.33	2	5.181	**0.01**
	Treatment:Species	0.82	4	1.817	0.15
Variable	**Source**	**LRT**	**DF**		**p-value**
R_P	Run	2.21	1		0.14
R_N	Run	0	1		1
Variable	**Source**	**N**	**χ^2**	***DF***	**p-value**
V_P	Treatment	36	7.4234	2	**0.02**
	Species	36	25.754	2	**<0.001**
V_N	Treatment	36	4.7763	2	0.09
	Species	36	27.893	2	**<0.001**

3.2 Effects of growth media on microalgal fatty acid profile

A total of 18, 16 and 16 fatty acids (FAs) were identified in *Haematococcus*, *Monoraphidium* and *Selenastrum* respectively. The same FAs were identified in *Monoraphidium* and *Selenastrum* while *Haematococcus* presented a different FA profile (Table 6; Table 7). Each tested microalgae presented the same FAs in every treatment, showing no effect of the cultivation media on the presence of microalgal FAs (Table 6; Table 7). Under MWC cultivation, *Haematococcus* showed high abundances of palmitic acid (16:0), 16:4n-3, oleic acid (18:1n-9), linoleic acid (18:2n-

6) and α-linoleic acid (18:3n-3) with a total combined contribution of ~84%, while both *Monoraphidium* and *Selenastrum* presented high abundances of palmitic acid, oleic acid, 18:1n-7 and α-linoleic acid, adding a total contribution of ~73% in each microalgae (Table 6; Table 7). None of the studied microalgae species presented detectable levels of EPA (20:5n-3) or DHA (22:6n-3) under any of the cultivation treatments (Table 6).

Table 6. Fatty acid content (mg g^{-1} dry weight) of three green microalgae (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown in three different media (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) for six days. Fatty acids shown in the table represent the ones contributing >0.5% to the total content. Values are presented as mean ± SD of four replicates. Fatty acids summary categories: total fatty acid content (Tot-FA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), eicosapentaenoic acid 20:5n-3 (EPA), docosahexaenoic acid 22:6n-3 (DHA), n-3/n-6 ratio (n-3/n-6), unsaturated / saturated fatty acids ratio (UFA/ SFA).

	HP			MG			SE		
	MWC	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**
SFA	18.52±4.83	9.65±2.64	15.28±4.25	27.11±2.71	13.83±1.50	14.33±2.60	34.93±1.52	16.83±1.64	19.89±3.47
14:0	0.74±0.23	0.58±0.08	0.66±0.16	0.51±0.03	0.51±0.09	0.45±0.06	0.84±0.06	0.59±0.09	0.63±0.16
16:0	16.41±4.15	7.97±2.35	13.45±3.86	24.17±2.19	12.06±1.29	12.63±2.48	31.82±1.32	14.67±1.38	17.55±2.83
18:0	1.38±0.47	1.10±0.33	1.17±0.25	2.44±0.52	1.27±0.22	1.25±0.09	2.26±0.20	1.57±0.19	1.71±0.49
MUFA	9.93±2.39	3.37±0.74	4.67±0.55	36.34±8.80	16.05±2.31	15.47±2.06	60.26±9.81	17.99±1.63	22.60±2.44
16:1w9	0	0	0	0.31±0.04	1.09±0.28	0.82±0.26	1.15±0.26	1.00±0.09	1.22±0.10
16:1w7	0.53±0.20	1.24±0.23	1.31±0.48	1.26±0.35	3.00±0.80	1.74±0.39	2.76±0.47	3.27±0.21	3.77±0.48
18:1w9	7.69±1.89	1.16±0.26	1.76±0.76	12.97±4.56	3.20±1.04	4.15±0.23	26.70±6.78	5.42±1.98	5.17±1.51
18:1w7	1.71±0.35	0.97±0.32	1.60±0.32	21.80±4.39	8.76±1.13	8.77±1.72	29.65±4.23	8.30±1.52	12.44±2.00
PUFA	44.22±13.57	24.58±10.20	41.98±11.47	44.95±2.72	45.53±6.74	45.82±9.01	56.97±4.91	48.79±1.43	57.41±5.44
n-6 PUFA	18.16±4.83	6.71±1.80	12.97±6.15	5.96±0.74	8.60±1.96	8.01±2.05	9.58±1.19	8.26±0.11	10.68±0.89
16:2w6	0.09±0.03	0.37±0.10	1.02±0.76	0.51±0.10	1.27±0.30	1.36±0.42	1.58±0.37	0.97±0.04	1.25±0.05
18:2w6	15.81±4.20	4.44±1.24	9.06±4.69	5.36±0.51	7.33±1.66	6.65±1.63	7.99±0.87	7.29±0.09	9.43±0.90
18:3w6	1.42±0.38	0.84±0.29	1.31±0.19	0	0	0	0	0	0
20:4w6	0.84±0.23	1.07±0.21	1.57±0.53	0	0	0	0	0	0
n-3 PUFA	26.07±8.79	17.87±8.43	29.01±5.42	38.99±2.06	36.93±4.94	37.81±7.00	47.39±4.81	40.52±1.33	46.73±4.76
16:3w3	1.08±0.38	0.63±0.25	1.08±0.35	2.49±0.16	1.34±0.19	1.97±0.28	4.92±0.51	4.40±0.90	5.49±1.06
16:4w3	7.06±2.65	5.46±2.74	8.81±1.96	8.65±0.55	10.50±1.62	10.25±2.46	8.51±1.24	8.79±1.06	8.73±1.03
18:3w3	15.22±4.95	10.20±4.87	16.80±3.01	21.00±0.93	19.84±2.54	20.33±3.24	25.11±2.49	21.10±1.62	24.69±2.85
18:4w3	2.17±0.63	1.03±0.45	1.56±0.02	6.85±0.65	5.25±0.83	5.27±1.11	8.86±0.81	6.24±0.34	7.82±0.73
EPA	0	0	0	0	0	0	0	0	0
DHA	0	0	0	0	0	0	0	0	0
n-3/n-6	1.41±0.13	2.52±0.54	2.64±0.85	6.60±0.54	4.42±0.59	4.81±0.42	5.03±0.83	4.90±0.10	4.38±0.29
UFA/SFA	2.90±0.31	2.81±0.37	3.07±0.10	2.99±0.11	4.43±0.20	4.28±0.05	3.35±0.15	4.01±0.39	4.09±0.47
Tot-FA	72.68±20.50	37.60±13.42	61.93±16.22	108.40±14.00	75.41±10.49	75.63±13.48	152.15±10.67	83.60±1.83	99.90±10.78

Cultivation in WW produced a change in the microalgae FA contribution profiles compared to MWC, but minor differences were observed between unfiltered and filtered WW (Table 7).

Table 7. Fatty acid mean ± SD percent value (%) of the selected fatty acids (contributing >0.5% to the total) of three green microalgae (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown in three different media (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) for six days. Fatty acids summary categories: total fatty acid content (Tot-FA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-3/n-6 ratio (n-3/n-6), unsaturated / saturated fatty acids ratio (UFA/ SFA).

	HP			MG			SE		
	MWC	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**
SFA	25.38 ± 2.00	25.37 ±2.13	23.99 ±0.63	24.72 ± 0.66	17.88 ±0.76	18.51 ±0.12	22.48 ± 0.73	19.41 ±1.57	19.30 ±1.77
14:0	0.99 ± 0.03	1.65 ±0.57	1.05 ±0.07	0.46 ±0.04	0.65 ±0.08	0.58 ±0.07	0.54 ±0.03	0.68 ±0.09	0.61 ±0.09
16:0	22.54 ± 1.88	20.83 ±1.83	21.07 ±0.67	22.06 ±0.80	15.59 ±0.67	16.27 ±0.29	20.48 ±0.75	16.93 ±1.32	17.05 ±1.36
18:0	1.85 ± 0.22	2.89 ±0.51	1.88 ±0.19	2.20 ±0.18	1.64 ±0.21	1.66 ±0.22	1.45 ±0.04	1.81 ±0.19	1.64 ±0.34
MUFA	13.68 ±1.19	9.04 ±1.29	7.70 ±1.28	32.60 ±3.47	20.62 ±0.41	20.23 ±1.71	38.54 ±4.29	20.80 ±2.08	22.07 ±0.82
16:1n-9	0	0	0	0.28 ±0.01	1.39 ±0.24	1.08 ±0.29	0.75 ±0.19	1.16 ±0.11	1.19 ±0.03
16:1n-7	0.69 ±0.14	3.36 ±0.57	2.44 ±1.41	1.13 ±0.22	3.81 ±0.64	2.23 ±0.20	1.78 ±0.34	3.78 ±0.30	3.75 ±0.78
18:1n-9	10.61 ±1.08	3.18 ±0.76	2.69 ±0.61	11.51 ±2.69	4.12 ±1.14	5.54 ±1.07	17.04 ±3.74	6.30 ±2.38	5.04 ±1.25
18:1n-7	2.38 ±0.23	2.50 ±0.11	2.57 ±0.19	19.67 ±1.70	11.31 ±0.67	11.38 ±1.18	18.96 ±1.52	9.57 ±1.61	12.09 ±0.99
PUFA	59.39 ±1.96	61.55 ±4.02	66.00 ±0.94	41.10 ±2.81	58.45 ±0.91	58.98 ±1.36	36.71 ±3.68	56.36 ±1.22	56.11 ±1.37
n-6 PUFA	24.73 ±1.04	17.71 ±1.59	19.25 ±4.74	5.35 ±0.28	10.90 ±1.17	10.21 ±0.90	6.14 ±0.54	9.55 ±0.13	10.48 ±0.87
16:2n-6	0.12 ±0.03	1.02 ±0.31	1.40 ±0.84	0.46 ±0.07	1.60 ±0.19	1.72 ±0.26	1.01 ±0.20	1.13 ±0.02	1.24 ±0.16
18:2n-6	21.53 ±1.03	11.65 ±0.88	13.27 ±3.98	4.89 ±0.25	9.30 ±0.98	8.49 ±0.65	5.13 ±0.40	8.42 ±0.15	9.24 ±0.78
18:3n-6	1.94 ±0.04	2.14 ±0.21	2.14 ±0.28	0	0	0	0	0	0
20:4n-6	1.14 ±0.03	2.90 ±0.50	2.43 ±0.22	0	0	0	0	0	0
n-3 PUFA	34.67 ±2.26	43.85 ±5.46	46.75 ±3.86	35.75 ±2.66	47.55 ±1.43	48.77 ±0.86	30.57 ±3.71	46.81 ±1.15	45.63 ±0.50
16:3n-3	1.43 ±0.13	1.58 ±0.09	1.68 ±0.18	2.28 ±0.13	1.75 ±0.27	2.57 ±0.25	3.17 ±0.34	5.07 ±0.97	5.35 ±0.87
16:4n-3	9.30 ±0.98	13.35 ±2.03	14.05 ±0.60	7.92 ±0.50	13.47 ±0.24	13.08 ±0.98	5.49 ±0.94	10.18 ±1.40	8.56 ±0.99
18:3n-3	20.29 ±1.13	24.93 ±3.42	27.14 ±2.65	19.31 ±1.89	25.60 ±1.39	26.35 ±0.83	16.19 ±1.92	24.35 ±1.54	24.08 ±0.43
18:4n-3	2.92 ±0.08	2.53 ±0.37	2.65 ±0.71	6.25 ±0.20	6.73 ±0.28	6.77 ±0.25	5.71 ±0.64	7.22 ±0.52	7.65 ±0.28
n-3/n-6	1.41 ±0.13	2.52 ±0.54	2.64 ±0.85	6.69 ±0.45	4.42 ±0.59	4.81 ±0.42	5.03 ±0.83	4.90 ±0.10	4.38 ±0.29
UFA/SFA	2.90 ±0.31	2.81 ±0.37	3.07 ±0.10	2.98 ±0.11	4.43 ±0.20	4.28 ±0.05	3.35 ±0.15	4.01 ±0.39	4.09 ±0.47
Tot-FA	98.72 ±0.1	96.76 ±0.8	98.55 ±0.2	98.64 ±0.07	97.99 ±0.4	98.51 ±0.18	97.72 ±0.19	96.58 ±0.3	97.49 ±0.2

Ordination of FA showed differential grouping between microalgae species, together with a clear differentiation between MWC and RAS WW media (Figure 3). Filtered and unfiltered WW treatments ordinated very closely in each microalgae, showing that under these treatments microalgal FA contribution profiles were very similar. As expected from their similar FA profiles, *Selenastrum* and *Monoraphidium*

grouped closer to each other than to *Haematococcus*, showing that these species have a higher degree of similarity (Figure 3). PERMANOVA analysis of FA contribution profiles indicated that microalgae species ($r^2 = 0.51$) and cultivation media ($r^2 = 0.37$) were the most important variables explaining dissimilarities in FAs (Table 8). Significant interaction between species and treatment factors in PERMANOVA showed that cultivation treatment affected differently the FA profile depending on the microalgae species (Table 8).

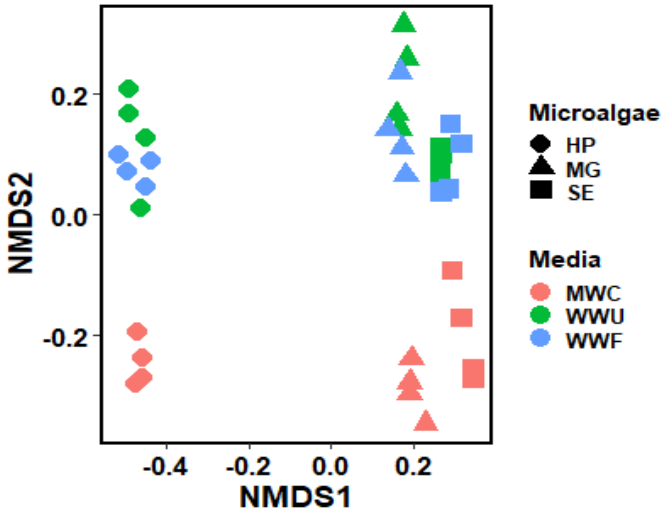

Figure. 3. Non metric multidimensional scaling plot (nMDS) of dissimilarities in fatty acid contribution profiles of the three tested microalgae (○ - HP – *Haematococcus pluvialis*, Δ - MG – *Monoraphidium griffithii*, □ - SE – *Selenastrum* sp.) in three different cultivation media (MWC (red) – Modified Wright's Cryptophyte medium, WWU (green) – unfiltered RAS wastewater, WWF (blue) – filtered RAS wastewater). Each point represents one experimental replicate.

Table 8. PERMANOVA results of microalgae fatty acid contribution profiles analysis. Dissimilarities in FA profiles were compared between species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, *Selenastrum* sp.), treatments (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater) and their interaction (Species*Treatment). PERMANOVA mean

squares is denoted as "mean Sq" in the table. Highlighted (bold values) are all p-values <0.05.

Source	Sum of squares	mean Sq	Df	F	r^2	p-value
Species	0.449	0.225	2	80.08	0.51	**0.001**
Treatment	0.333	0.166	2	59.32	0.37	**0.001**
Species*Treatment	0.030	0.007	4	2.66	0.03	**<0.001**
Residuals	0.076	0.003	27		0.09	
Total	0.888		35		1	

To test which FAs were contributing the most to the observed dissimilarities between cultivation media, SIMPER analysis was carried out to compare pairs of treatments for each microalgae (Table 9). On average, higher dissimilarities were seen for the pairs MWC – unfiltered WW (~22%) and MWC – filtered WW (~21%) than for the pair unfiltered WW – filtered WW (~8%) (Table 9). Specifically, differences in FA contribution profiles between MWC and WW (filtered or unfiltered) were principally driven by a reduction of oleic and linoleic acids and an increase of 16:4n-3 and α-linoleic acid in *Haematococcus*, while a reduction of oleic acid and 18:1n-7 and an increase of α-linoleic acid contributed the most to the differences seen in both *Monoraphidium* and *Selenastrum* (Table 9). Comparison of filtered and unfiltered WW media did not show any specific trend in FA differences between species (Table 9). This result, together with the low total dissimilarity (%) observed among filtered and unfiltered WW media (Table 9), shows that no distinctive nor substantial microalgal FA adaptations resulted from the filtration of WW medium.

Table 9. SIMPER results of fatty acid contribution profiles. Columns separated by a solid line indicate pairwise SIMPER tests between different treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater). Rows separated by dashed lines indicate different species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.). On the first row of every species, total amount of dissimilarity (%) between

treatments is shown in parenthesis. Fatty acids are ordered from the most to the least significant contributor to the total dissimilarity. Dis. Sum indicates cumulative sum of total dissimilarity. Fatty acid means from the compared groups are presented in the means column.

HP

MWC-WWU (19.9%)			MWC- WWF (18.8%)			WWU-WWF (9.76%)		
FA	Means	Dis. Sum	FA	Means	Dis. Sum	FA	Means	Dis. Sum
18:2n-6	21.5 - 11.7	0.26	18:2n-6	21.5 - 13.4	0.22	18:3n-3	24.9 - 27.1	0.22
18:1n-9	10.6 - 3.7	0.46	18:1n-9	10.6 - 2.7	0.44	18:2n-6	11.7 - 13.3	0.42
18:3n-3	20.3 - 24.9	0.58	18:3n-3	20.3 - 27.1	0.62	16:4n-3	13.4 - 14.1	0.53
16:4n-3	9.3 - 13.4	0.68	16:4n-3	9.3 - 14.0	0.75	16:0	20.8 - 21.1	0.62
16:1n-7	0.7 - 3.4	0.75				16:1n-7	3.4 - 2.4	0.7
						18:0	2.9 - 1.9	0.75

MG

MWC-WWU (23.2%)			MWC- WWF (20.7%)			WWU-WWF (5.7%)		
FA	Means	Dis. Sum	FA	Means	Dis. Sum	FA	Means	Dis. Sum
18:1n-7	19.7 - 11.3	0.18	18:1n-7	19.7 - 11.4	0.20	18:1n-9	4.1 - 5.5	0.16
18:1n-9	11.5 - 4.1	0.34	18:3n-3	19.3 - 26.4	0.37	16:1n-7	3.8 - 2.2	0.30
16:0	22.1 - 15.6	0.48	18:1n-9	11.5 - 5.5	0.52	18:3n-3	25.6 - 26.4	0.42
18:3n-3	19.3 - 25.6	0.62	16:0	22.1 - 16.3	0.66	18:2n-6	9.3 - 8.5	0.52
16:4n-3	7.92 - 13.5	0.74	16:4n-3	7.9 - 13.1	0.78	18:1n-7	11.3 - 11.4	0.62
						16:0	15.6 - 16.3	0.70

SE

MWC-WWU (23.6%)			MWC- WWF (22.6%)			WWU-WWF (7.4%)		
FA	Means	Dis. Sum	FA	Means	Dis. Sum	FA	Means	Dis. Sum
18:1n-9	17.0 - 6.3	0.23	18:1n-9	17.0 - 5.0	0.27	18:1n-7	9.6 - 12.1	0.19
18:1n-7	19.0 - 9.6	0.43	18:3n-3	16.2 - 24.1	0.45	18:1n-9	6.3 - 5.0	0.38
18:3n-3	16.2 - 24.4	0.61	18:1n-7	19.0 - 12.1	0.60	16:4n-3	10.2 - 8.6	0.51
16:4n-3	5.5 - 10.2	0.71	18:2n-6	5.1 - 9.2	0.69	16:0	16.9 - 17.1	0.62
			16:0	20.5 - 17.1	0.77	18:3n-3	24.4 - 24.1	0.72

Altogether, the observed variations in FAs between treatments led to differences in the total content (mg g^{-1} dry weight) of FA categories (ANOVA, Table 10; Figure 4). Total FA, monounsaturated and saturated FA content were substantially higher for *Monoraphidium* and *Selenastrum* in MWC compared to WW media (unfiltered and filtered). *Haematococcus* had significantly higher total FA and saturated FA content in MWC compared to unfiltered WW, but no difference was observed between MWC and filtered WW in these FA categories (post hoc tests, Appendix 4; Figure 4 A, D, E). Polyunsaturated FA content of *Monoraphidium* and *Selenastrum* remained relatively constant across cultivation media (Appendix 4; Figure 4 B). Even though *Selenastrum* showed a slightly lower polyunsaturated FA content when cultivated in unfiltered WW compared to the other treatments, no statistically significant

difference was observed (Appendix 4; Figure 4 B). On the other hand, *Haematococcus* cultivated in unfiltered WW showed a reduction in the total polyunsaturated FA content compared to MWC and filtered WW, while no difference was observed between these two media (post hoc tests, Appendix 4). Cultivation in WW (filtered or unfiltered) led to differences in the microalgal n-3/n-6 and unsaturated FA/saturated FA ratios compared to MWC (Table 6, Table 10, Appendix 4; Figure 4 C, F). The direction and magnitude of change in these rates highly depended on the microalgae species (Table 10, Appendix 4; Figure 4 C, F). Under WW (filtered or unfiltered) cultivation, *Haematococcus* had a significantly higher n-3/n-6 ratio compared to MWC, while *Monoraphidium* showed the opposite, higher ratio in MWC than in WW (post hoc tests, Appendix 4; Figure 4 C). *Monoraphidium* and *Selenastrum* presented higher unsaturated FA/saturated FA ratios when cultivated in WW (unfiltered or filtered) compared to MWC, meanwhile *Haematococcus* showed no difference between treatments (post hoc tests, Appendix 4; Figure 4 F). When comparing between species, there is a trend for *Selenastrum* to have a slightly higher content of every FA category than *Monoraphidium* and *Haematococcus* regardless of the cultivation media (post hoc tests, Appendix 4; Figure 4 A, B, D, E). *Monoraphidium* had the highest n-3/n-6 ratio when cultivated in MWC cultivation compared to *Selenastrum* and *Haematococcus* (6.60±0.54; post hoc tests, Table 6; Appendix 4), while under WW (filtered or unfiltered) cultivation, no difference was seen between *Monoraphidium* and *Selenastrum* (post hoc tests, Appendix 4). Unsaturated FA/saturated FA ratio showed to be higher in *Monoraphidium* and *Selenastrum* than in *Haematococcus* under WW (filtered and unfiltered) cultivation (post hoc tests, Appendix 4; Figure 4 F).

Table 10. Two-way mixed effects analysis of variance (ANOVA) table with Satterthwaite's method testing the effects of treatment (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater), species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, *Selenastrum* sp.) and their interaction (Treatment:Species) to the total variation seen in total fatty acids (Tot-

FA) and polyunsaturated fatty acid (PUFA) content (mg g^{-1} dry weight), n-3/n-6 fatty acids ratio, monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) content (mg g^{-1} dry weight), unsaturated/saturated fatty acids ratio UFA/ SFA. ANOVA mean squares is denoted as "mean Sq" and likelihood ratio test as "LRT" in the table. At the bottom of the table, ANOVA-like table to test the significance of the random effects of the experimental run. Highlighted (bold values) are all p-values <0.05.

Variable	**Source**	***mean Sq***	**DF**	**F**	**p-value**
Tot-FA	Treatment	6574.3	2	28.7639	**<0.001**
	Species	8818.7	2	38.5835	**<0.001**
	Treatment:Species	650.3	4	2.8453	**0.04**
PUFA	Treatment	329.72	2	3.9041	**0.03**
	Species	877.88	2	10.3946	**<0.001**
	Treatment:Species	127.15	4	1.5055	0.23
n-3/n-6	Treatment	0.718	2	2E+00	0.11
	Species	35.023	2	1.19E+02	**<0.001**
	Treatment:Species	3.727	4	12.6509	**<0.001**
MUFA	Treatment	1971.85	2	67.036	**<0.001**
	Species	2320.8	2	78.899	**<0.001**
	Treatment:Species	396.09	4	13.466	**<0.001**
SFA	Treatment	593.1	2	48.9812	**<0.001**
	Species	267.12	2	22.0603	**<0.001**
	Treatment:Species	44.71	4	3.6922	**0.02**
UFA/SFA	Treatment	1.97	2	19.9687	**<0.001**
	Species	3.3138	2	33.5889	**<0.001**
	Treatment:Species	0.643	4	6.5177	**<0.001**
Variable	**Source**	**LRT**	**DF**		**p-value**
Tot-FA	Run	0.07	1		0.79
PUFA	Run	0.50	1		0.48
n-3/n-6	Run	2.60	1		0.11
MUFA	Run	0	1		1
SFA	Run	0	1		1
UFA/SFA	Run	0.51	1		0.47

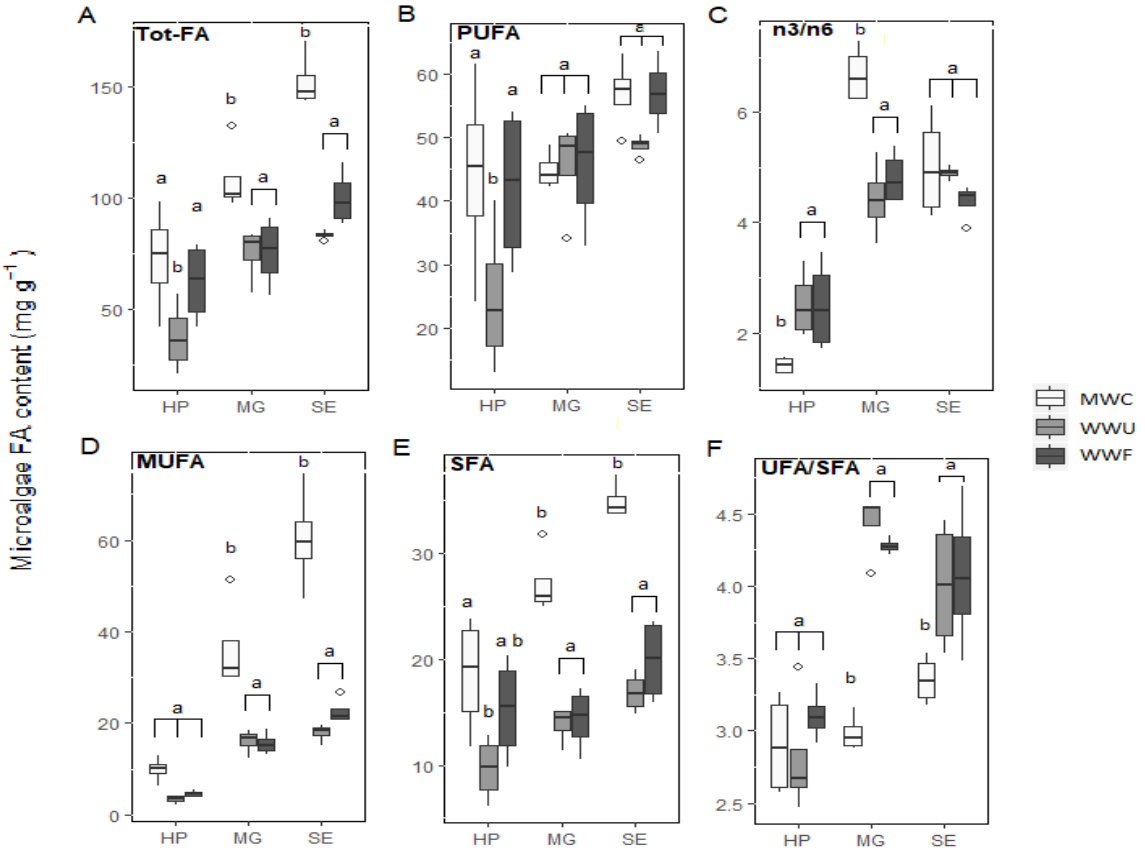

Figure 4. Box plots of total FA content (Tot-FA) (A), polyunsaturated fatty acids (PUFA) content (mg g^{-1} dry weight) (B), n-3/n-6 fatty acids ratio (n-3/n-6) (C), monounsaturated fatty acid (MUFA) (D), saturated fatty acid (SFA) content (mg g^{-1} dry weight) (E) and unsaturated / saturated fatty acid ratio UFA/ SFA (F) of three green microalgae (*Haematococcus pluvialis* – HP, *Monoraphidium griffithii* – MG and *Selenastrum* sp. – SE) grown in three different cultivation media (MWC – Modified Wright's Cryptophyte medium (white box), WWU – unfiltered RAS wastewater (grey box), WWF – filtered RAS wastewater (dark-grey box)) for six days. Box edges indicate first and third quartile, horizontal lines inside every box indicate median values, and whiskers reach maximum and minimum values when there are no outliers. If outlines are present (distance from median > 1.5*interquartile), they are shown as an open circle. Treatments denoted with the same letter (a-b) are not statistically different from each other for each microalgae. Comparison of treatments between algae are not presented in this figure.

3.4 Effects of growth media on microalgal amino acid profile

For the purpose of this study, I will refer to amino acid (AA) profiles as the combination of the 15 identified free amino acids (methods, section 2.2.6: Amino acid analysis). All 15 AAs were found in every algae species and in every cultivation media (Table 12; Table 13). For all tested microalgae, under cultivation in MWC alanine, valine, leucine, threonine and aspartic acid were the AAs with the highest contribution to the total AAs (>50% of total AA; Table 13; Figure 5).

Table 12. Amino acid content (mg g^{-1} dry weight) of three green microalgae (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown in three different media (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) for six days. Values are presented as mean ± SD of four replicates. Amino acids summary categories: total amino acids content (Tot-AA), essential amino acid (EAA) and non-essential amino acid (NEAA) content.

	HP			MG			SE		
	MWC	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**
ALA	20.18 ±1.16	33.26 ±1.53	33.00 ±1.03	17.00 ±0.12	29.22 ±1.88	26.80 ±0.82	19.89 ±1.43	30.62 ±1.98	28.14 ±1.78
GLY	3.24 ±0.84	15.52 ±1.34	15.21 ±0.42	3.64 ±0.15	14.79 ±2.40	11.13 ±0.58	4.45 ±0.27	15.21 ±1.38	12.31 ±0.76
VAL	16.11 ±0.74	25.23 ±0.79	24.56 ±0.86	12.43 ±0.19	22.16 ±1.40	20.63 ±0.43	13.56 ±0.56	20.77 ±0.75	18.42 ±1.37
LEU	17.44 ±1.99	31.53 ±1.35	31.88 ±0.91	14.47 ±0.46	28.08 ±1.46	26.06 ±0.67	17.45 ±1.40	28.71 ±1.61	26.45 ±1.51
ILE	11.00 ±0.47	17.11 ±0.98	16.53 ±0.78	8.47 ±0.14	15.62 ±0.95	14.09 ±0.22	10.00 ±0.42	14.49 ±0.77	12.72 ±0.73
THR	14.27 ±3.38	27.10 ±0.98	26.68 ±0.60	11.36 ±0.24	22.09 ±1.24	21.06 ±0.82	12.39 ±1.18	22.61 ±0.96	19.46 ±0.72
SER	12.77 ±4.97	21.13 ±2.23	22.34 ±1.27	7.94 ±0.37	15.40 ±0.45	16.38 ±1.01	9.49 ±1.16	16.48 ±1.61	13.46 ±1.92
PRO	11.07 ±0.85	18.28 ±0.82	17.89 ±1.11	8.69 ±0.28	15.54 ±0.89	15.13 ± 0.72	10.08 ±0.62	17.17 ±1.13	14.37 ±1.25
ASP	18.03 ±2.39	31.24 ±1.52	29.30 ±3.35	13.71 ±1.41	25.11 ±1.62	27.57 ±2.32	15.98 ±0.81	25.89 ±1.85	22.54 ±2.97
MET	0.15 ±0.01	0.70 ±0.23	0.80 ±0.22	0.13 ±0.09	0.45 ±0.11	0.69 ±0.47	0.09 ±0.04	0.60 ±0.08	1.11 ±0.86
GLU	12.41 ±3.92	23.96 ±1.39	21.82 ±0.91	6.19 ±0.98	15.45 ±2.37	17.73 ±1.67	9.93 ±1.06	22.73 ±3.38	18.62 ±5.61
PHE	7.70 ±0.93	16.21 ±1.16	16.49 ±1.49	5.78 ±0.30	14.38 ±1.02	12.86 ±0.46	7.32 ±0.51	15.14 ±1.47	12.72 ±1.04
LYS	6.94 ±2.57	16.86 ±3.04	17.34 ±1.94	6.02 ±0.47	15.85 ±2.11	13.05 ±0.52	7.47 ±0.72	15.40 ±0.46	14.96 ±1.21
HIS	2.14 ±0.41	4.89 ±0.24	5.05 ±0.64	1.48 ±0.05	4.14 ±0.14	3.57 ±0.18	1.92 ±0.09	4.44 ±0.49	3.67 ±0.33
TYR	2.64 ±0.16	5.12 ±0.34	5.23 ±1.75	1.74 ±0.33	4.98 ±0.21	4.50 ±0.77	2.22 ±0.10	5.30 ±0.50	5.23 ±1.19
EAA	75.7 ±10.2	139.6 ±8.2	139.3 ±3.7	60.1 ±1.0	122.8 ±7.9	112.0 ±1.8	70.2 ±4.4	122.2 ±5.0	109.5 ±3.8
NEAA	80.3 ±12.2	148.5 ±4.9	144.8 ±3.6	58.9 ±2.9	120.5 ±1.4	119.2 ±3.7	72.0 ±4.7	133.4 ±5.3	114.7 ±7.4
Tot-AA	156.1 ±21.7	288.2 ±12.6	284.1 ±6.1	119.1 ±1.9	243.3 ±9.2	231.3 ±2.1	142.2 ±8.9	255.6 ±8.0	224.2 ±6.4

Table 13. Amino acid mean ± SD percent value (%) of the identified amino acids of three green microalgae (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown in three different media (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS

wastewater) for six days. Amino acids summary categories: total amino acid content (Tot-AA), essential amino acid (EAA) and non-essential amino acid (NEAA) content.

	HP			MG			SE		
	MWC	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**	**MWC**	**WWU**	**WWF**
ALA	13.11 ±1.33	11.57 ±0.76	11.61 ±0.14	14.28 ±0.17	12.00 ±0.34	11.59 ±0.42	13.98 ±0.27	11.98 ±0.70	12.57 ±0.94
GLY	2.05 ±0.30	5.38 ±0.23	5.35 ±0.11	3.05 ±0.10	6.06 ±0.81	4.81 ±0.25	3.13 ±0.11	5.94 ±0.39	5.50 ±0.40
VAL	10.47 ±1.08	8.76 ±0.11	8.64 ±0.20	10.44 ±0.18	9.10 ±0.26	8.92 ±0.25	9.55 ±0.29	8.13 ±0.27	8.22 ±0.64
LEU	11.22 ±0.53	10.94 ±0.13	11.22 ±0.33	12.16 ±0.56	11.54 ±0.20	11.27 ±0.37	12.25 ±0.26	11.23 ±0.45	11.82 ±0.87
ILE	7.14 ±0.70	5.93 ±0.10	5.81 ±0.19	7.12 ±0.22	6.42 ±0.19	6.09 ±0.15	7.04 ±0.34	5.67 ±0.26	5.68 ±0.34
THR	9.01 ±1.01	9.41 ±0.14	9.39 ±0.12	9.54 ±0.04	9.08 ±0.19	9.10 ±0.28	8.70 ±0.47	8.85 ±0.28	8.68 ±0.16
SER	7.89 ±2.14	7.32 ±0.54	7.86 ±0.37	6.66 ±0.21	6.34 ±0.39	7.08 ±0.39	6.65 ±0.41	6.45 ±0.60	6.00 ±0.82
PRO	7.28 ±1.50	6.36 ±0.48	6.30 ±0.44	7.30 ±0.13	6.38 ±0.18	6.54 ±0.29	7.09 ±0.09	6.72 ±0.34	6.41 ±0.58
ASP	11.59 ±0.74	10.84 ±0.32	10.31 ±1.14	11.49 ±1.00	10.35 ±0.90	11.91 ±0.92	11.24 ±0.34	10.14 ±0.77	10.03 ±1.02
MET	0.10 ±0.02	0.25 ±0.09	0.28 ±0.08	0.11 ±0.08	0.18 ±0.04	0.30 ±0.21	0.06 ±0.03	0.23 ±0.03	0.49 ±0.36
GLU	7.79 ±1.42	8.32 ±0.39	7.68 ±0.21	5.19 ±0.74	6.39 ±1.17	7.66 ±0.66	6.98 ±0.59	8.90 ±1.29	8.29 ±2.46
PHE	4.95 ±0.24	5.62 ±0.17	5.81 ±0.54	4.86 ±0.33	5.91 ±0.22	5.56 ±0.23	5.15 ±0.15	5.92 ±0.49	5.69 ±0.57
LYS	4.32 ±1.25	5.82 ±0.82	6.10 ±0.61	5.07 ±0.47	6.50 ±0.64	5.65 ±0.27	5.25 ±0.23	6.03 ±0.21	6.68 ±0.61
HIS	1.36 ±0.12	1.70 ±0.04	1.78 ±0.23	1.24 ±0.06	1.70 ±0.04	1.54 ±0.09	1.35 ±0.05	1.73 ±0.16	1.64 ±0.18
TYR	1.72 ±0.22	1.78 ±0.18	1.85 ±0.63	1.47 ±0.29	2.05 ±0.08	1.95 ±0.35	1.57 ±0.14	2.07 ±0.15	2.32 ±0.45
EAA	48.56 ±1.70	48.43 ±0.87	49.04 ±0.71	50.55 ±1.64	50.42 ±1.28	48.44 ±1.14	49.36 ±0.61	47.80 ±1.22	48.88 ±2.18
NEAA	51.44 ±1.70	51.57 ±0.87	50.96 ±0.71	49.45 ±1.64	49.58 ±1.28	51.56 ±1.14	50.64 ±0.61	52.20 ±1.22	51.12 ±2.18

AA contribution profiles showed significant differences between species and between cultivation media (PERMANOVA, species $r^2 = 0.11$, treatment $r^2 = 0.43$, Table 14; Figure 5; Table 13). It is important to point out that residuals ($r^2 = 0.40$) explained almost as much of the observed dissimilarities in contribution profiles as treatment ($r^2 = 0.43$) (Table 14).

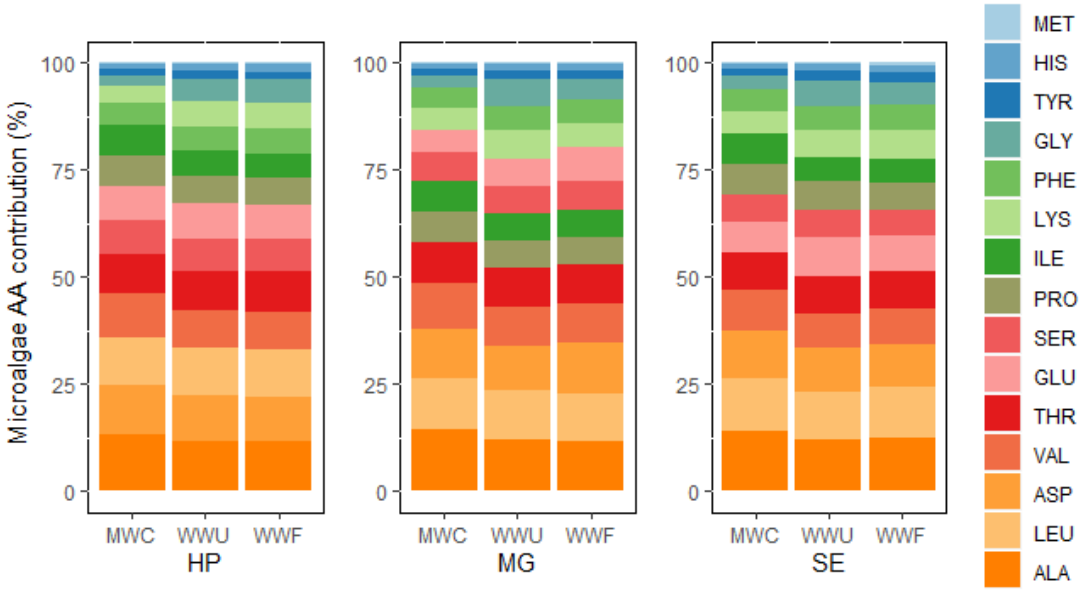

Figure 5. Amino acid contribution (%) profiles of three green microalgae (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown in three different media (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) for six days. For each microalgae, amino acids are ordered from the highest to the lowest contributor (bottom to top) to total amino acid contribution in MWC media.

Table 14. PERMANOVA results of microalgae amino acid contribution profiles analysis. Dissimilarities in amino acid profiles were compared between species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, *Selenastrum* sp.), treatments (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater) and their interaction (Species*Treatment). PERMANOVA mean squares is denoted as "mean Sq" in the table. Highlighted (bold values) are all p-values <0.05.

Source	Sum of squares	mean Sq	DF	F	r^2	p-value
Species	0.011	0.005	2	3.80	0.11	**<0.001**
Treatment	0.041	0.021	2	14.57	0.43	**0.001**
Species*Treatment	0.005	0.001	4	0.97	0.06	0.47
Residuals	0.038	0.001	27		0.40	
Total	0.096		35		1	

Ordination of AAs showed two clearly distinguishable groups, one composed by filtered and unfiltered WW and the other by MWC media (Figure 6). No clear grouping was observed for different species (Figure 6). SIMPER analysis confirmed the observed results, showing that on average the dissimilarities between MWC and WW (filtered or unfiltered) media were ~8%, while filtered and unfiltered media differed by ~5% on average independent of the species (Table 15). For all tested microalgae, cultivation in WW (filtered or unfiltered) produced an increase in glycine together with a reduction in alanine and valine contribution compared to MWC (Table 15). Microalgae specific changes were also seen, for example *Haematococcus* presented variation in its serine and lysine contribution between treatments, while *Monoraphidium* and *Selenastrum* presented differences in glutamic and aspartic acids (Table 15).

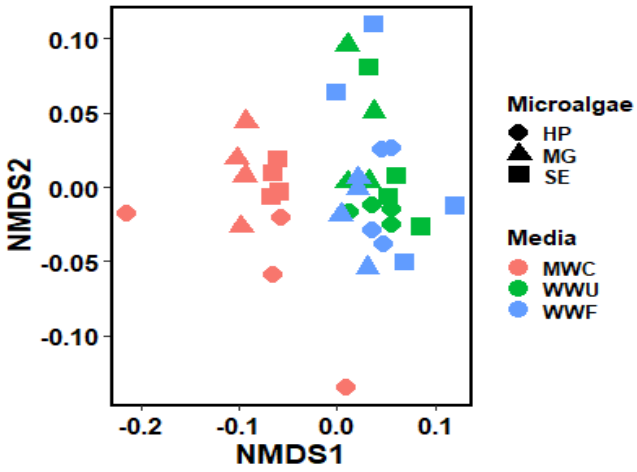

Figure 6. Non metric multidimensional scaling plot (nMDS) of dissimilarities in amino acid contribution profiles of the three tested microalgae (○ - HP – *Haematococcus pluvialis*, Δ - MG – *Monoraphidium griffithii*, □ - SE – *Selenastrum* sp.) in three different cultivation media (MWC (red) – Modified Wright's Cryptophyte medium, WWU (green) – unfiltered RAS wastewater, WWF (blue) – filtered RAS wastewater). Each point represents one experimental replicate.

Table 15. SIMPER results of amino acid contribution profiles data. Columns separated by a solid line indicate pairwise SIMPER tests between different

treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater). Rows separated by dashed lines indicate different species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.). On the first row of every species, total amount of dissimilarity (%) between treatments is shown in parenthesis. Amino acids are ordered from the most to the least significant contributor to the total dissimilarity. Dis. Sum indicates cumulative sum of total dissimilarity. Amino acid means from the compared groups are presented in the means column.

				HP				
	MWC-WWU (5.3%)			**MWC- WWF (9.5%)**			**WWU-WWF (3.5%)**	
AA	Means	Dis. Sum	AA	Means	Dis. Sum	AA	Means	Dis. Sum
GLY	0.02 - 0.05	0.18	GLY	0.02 - 0.05	0.17	ASP	0.11 - 0.10	0.16
SER	0.08 - 0.07	0.29	LYS	0.04 - 0.06	0.27	LYS	0.06 - 0.06	0.28
ALA	0.13 - 0.12	0.39	VAL	0.10 - 0.09	0.37	ALA	0.12 - 0.12	0.37
LYS	0.04 - 0.06	0.49	SER	0.08 - 0.08	0.46	GLU	0.08 - 0.08	0.47
VAL	0.10 - 0.09	0.58	ALA	0.13 - 0.012	0.55	TYR	0.02 - 0.02	0.55
GLU	0.08 - 0.08	0.66	ASP	0.12 - 0.10	0.63	SER	0.07 - 0.08	0.64
PRO	0.07 - 0.06	0.73	ILE	0.07 - 0.06	0.70	PHE	0.06 - 0.06	0.72
			PRO	0.07 - 0.06	0.76			
				MG				
	MWC-WWU (8.3%)			**MWC- WWF (7.8%)**			**WWU-WWF (4.7%)**	
AA	Means	Dis. Sum	AA	Means	Dis. Sum	AA	Means	Dis. Sum
GLY	0.03 - 0.06	0.18	ALA	0.14 - 0.12	0.17	ASP	0.10 - 0.12	0.18
ALA	0.14 - 0.12	0.32	GLU	0.05 - 0.08	0.33	GLU	0.06 - 0.08	0.33
GLU	0.05 - 0.06	0.42	GLY	0.03 - 0.05	0.44	GLY	0.06 - 0.05	0.47
LYS	0.05 - 0.06	0.50	VAL	0.10 - 0.09	0.54	LYS	0.06 - 0.06	0.57
ASP	0.11 - 0.10	0.59	ASP	0.11 - 0.12	0.61	SER	0.06 - 0.07	0.65
VAL	0.10 - 0.09	0.67	ILE	0.06 - 0.06	0.68	ALA	0.12 - 0.12	0.71
PHE	0.05 - 0.06	0.73	LEU	0.12 - 0.12	0.74			
				SE				
	MWC-WWU (8.0%)			**MWC- WWF (8.4%)**			**WWU-WWF (5.6%)**	
AA	Means	Dis. Sum	AA	Means	Dis. Sum	AA	Means	Dis. Sum
GLY	0.03 - 0.06	0.18	GLY	0.03 - 0.05	0.14	GLU	0.09 - 0.08	0.22
GLU	0.07 - 0.09	0.30	GLU	0.07 - 0.08	0.28	ASP	0.10 - 0.10	0.32
ALA	0.14 - 0.12	0.43	ASP	0.11 - 0.10	0.36	ALA	0.12 - 0.13	0.42
VAL	0.10 - 0.08	0.52	LYS	0.05 - 0.06	0.45	SER	0.06 - 0.06	0.50
ILE	0.07 - 0.06	0.60	ALA	0.14 - 0.13	0.53	LEU	0.11 - 0.12	0.59
ASP	0.11 - 0.10	0.68	ILE	0.07 - 0.06	0.62	LYS	0.06 - 0.07	0.65
LEU	0.12 - 0.11	0.74	VAL	0.10 - 0.08	0.70	PHE		0.71
			SER	0.07 - 0.06	0.75			

In contrast to contribution profiles, where no large variations were seen among treatments, AA content (mg/g of dried weight) showed dramatic variation with cultivation media (Figure 7; ANOVA, Table 16). Microalgal total AA content was almost 2-fold higher when cultivated in WW (either filtered or unfiltered) than in MWC media (post hoc tests, Appendix 5; Figure 7 A). Similar differences were seen

for essential and non-essential amino acid contents (Figure 7 B, C; ANOVA, Table 16). Only *Selenastrum* presented differences between WW treatments with filtered medium showing higher total AA, essential and non-essential AA contents compared to unfiltered WW (post hoc tests, Appendix 5; Figure 7). When comparing between species, *Haematococcus* showed the highest AA content (total AA, essential and non-essential AA) under WW (filtered and unfiltered) cultivation (post hoc tests, Appendix 5; Figure 7).

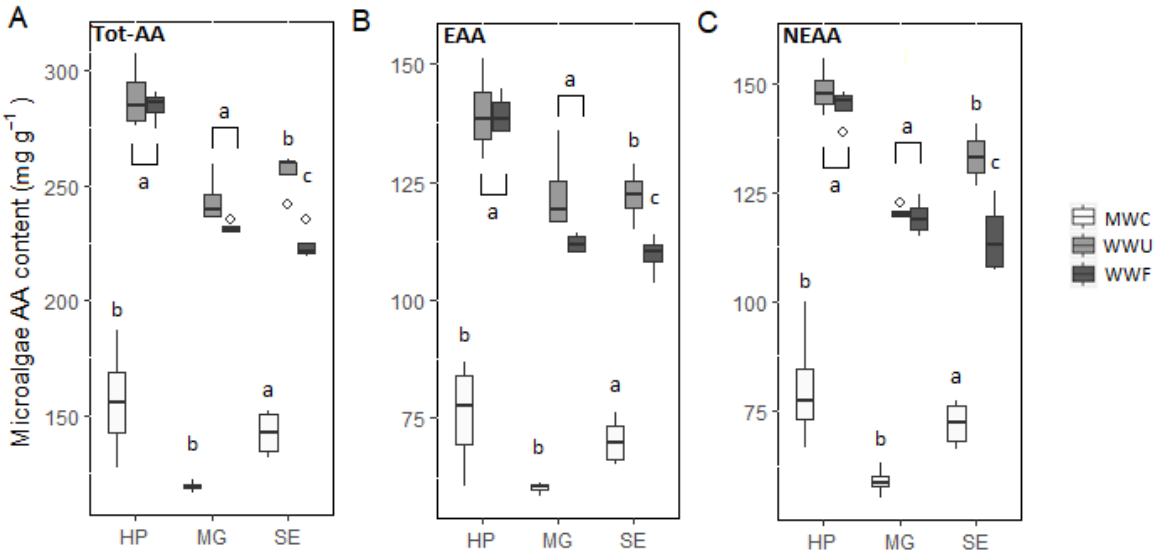

Figure 7. Box plots of total amino acid (Tot-AA) (A), essential amino acid (EAA) (B) and non-essential amino acid (NEAA) (C) content (mg g^{-1} dry weight) of three green microalgae (*Haematococcus pluvialis* – HP, *Monoraphidium griffithii* – MG and *Selenastrum* sp. – SE) grown under three different cultivation media (MWC – Modified Wright's Cryptophyte medium (white box), WWU – unfiltered RAS wastewater (grey box), WWF – filtered RAS wastewater (dark-grey box)) for six days. Box edges indicate first and third quartile, horizontal lines inside every box indicate median values, and whiskers reach maximum and minimum values when there are no outliers. If outlines are present (distance from median > 1.5*interquartile), they are shown as an open circle. Treatments denoted with the same letter (a-c) are not statistically different from each other for each microalgae. Comparison of treatments between algae are not presented in this figure.

Table 16. Two-way mixed effects analysis of variance (ANOVA) table with Satterthwaite's method testing the effects of treatment (Modified Wright's Cryptophyte medium, unfiltered RAS wastewater, filtered RAS wastewater), species (*Haematococcus pluvialis*, *Monoraphidium griffithii*, *Selenastrum* sp.) and their interaction (Treatment:Species) to the total variation seen in total amino acid (Tot-AA), essential amino acid (EAA) and non-essential amino acid (NEAA) content (mg g^{-1} dry weight). ANOVA mean squares is denoted as "mean Sq" and likelihood ratio test as "LRT" in the table. At the bottom of the table, ANOVA-like table to test the significance of the random effects of the experimental run. Highlighted (bold values) are all p-values <0.05.

Variable	Source	mean Sq	DF	F	p-value
Tot-AA	Treatment	53921	2	384.21	**<0.001**
	Species	6733	2	47.98	**<0.001**
	Treatment:Species	557	4	3.97	**0.01**
EAA	Treatment	12527.1	2	272.98	**<0.001**
	Species	1425.4	2	31.06	**<0.001**
	Treatment:Species	156.5	4	3.41	**0.02**
NEAA	Treatment	14518.2	2	338.73	**<0.001**
	Species	1942.4	2	45.32	**<0.001**
	Treatment:Species	165.1	4	3.85	**0.01**
Variable	**Source**	**LRT**	**DF**		**p-value**
Tot-AA	Run	2.84×10^{-14}	1		1
EAA	Run	-2.84×10^{-14}	1		1
NEAA	Run	1.04	1		0.31

4 DISCUSSION

Cultivation in RAS WW had a negative effect on cell density and specific growth rate for *Selenastrum* sp. and *Monoraphidium griffithii* compared to reference algae medium after six days. In contrast, *Haematococcus pluvialis* was able to grow at the same rate and reach the same cell density in RAS WW than when cultured in MWC (Figure 1. A, C). Despite the observed differences in cell density between MWC and WW, *Selenastrum* did not show a lower dry weight when cultivated in WW media. Nutrient consumption varied greatly depending on culture conditions. Under WW cultivation, the total amount of N and P removed did not present differences between filtered and unfiltered media. Importantly, all microalgae grown in MWC completely depleted N by day six (Figure 2 B). Since I only measured nutrient on the last day of cultivation, it was not possible to determine when did N depletion start and how long did microalgae remain under N starvation prior to day six. Regarding P consumption, *Selenastrum* and *Monoraphidium* consumed all the available nutrient in every cultivation media (Figure 2 A). Since algae has the capacity to store large amounts of P intracellularly (Zhu et al 2015), phenomenon commonly called "luxury uptake", it is unclear if microalgae underwent P starvation under any of the study treatments. Differences in FA profiles were mostly driven by a rise in the saturated and monounsaturated FA content (mg g^{-1} dry weight) under MWC cultivation compared to WW (Table 6; Table 9) while minimal differences were seen among filtered and unfiltered treatments (Table 9). On the other hand, AA profiles presented slight differences between cultivation media in terms of contribution of specific AA (Table 13; Table 15), but a large decrease in total AA content was seen in MWC compared to WW (Table 12).

The observed differences in density between MWC and WW contrast with a previous study utilizing the same growing conditions, were, after 4 days of cultivation at ~17 ˚C under a 24:0 photoperiod in unfiltered WW, *Haematococcus pluvialis*, *Monoraphidium griffithii* and *Selenastrum* sp. reached the same cell density

as in MWC media (Stevčić et al 2019). Importantly, RAS WW media presented N:P ratios higher than 50:1 in both studies (Table 1). High N:P ratios (>30:1) have been previously associated with lower cell growth (Zhang and Hu 2011, Mayers et al 2014, Rasdi and Qin 2015) and lower biomass production (Choi and Lee 2015) than N:P ratios in the range of 10:1 – 25:1 for several microalgae species. Differences in cell growth with varying N:P ratios tend not to be noticeable during short cultivation periods (Cheban et al. 2015, Rasdi and Qin 2015, Jiang et al. 2016). This lag in the rise of differences in cell growth among different N:P ratios could explain the differences seen between the previous and present studies in *Selenastrum* and *Monoraphidium* cell density. Nevertheless, algal preferences for optimal N:P ratios vary widely among species (Clark et al. 2002, Flynn et al. 2002, Sun et al. 2004). It is possible that *Monoraphidium* and *Selenastrum* optimal N:P ratios are closer to MWC medium (~20:1), explaining the lower density seen under WW cultivation for six days. In contrast, *Haematococcus* might not be negatively affected by this high N:P ratio, but I can not discard the possibility that longer cultivation periods could bring differences in density between MWC and WW for *Haematococcus*. To support my hypothesis that the high N:P ratio seen in WW is the main contributor to the differences in cell density with MWC, no differences were seen between filtered and unfiltered WW treatments (Figure 1 A), indicating that there was no negative effect of competition for nutrients with bacteria and other microorganisms on density in unfiltered WW.

Despite N:P ratio, N alone is essential for algae metabolism playing a key role in processes such as cell growth and photosynthesis (Lewitus and Caron 1990, Levi and Gantt 2004, Liefer et al. 2018). Under N starvation, major changes in metabolism occur with the objective of increasing scavenging and uptake of the limiting nutrient along with curtailing energy-consuming anabolic pathways. In *Chlamydomonas reinhardtii,* N deprivation leads to substantial decreases of cytoplasmic and chloroplast ribosomes (Siersma and Chiang 1971, Martin et al. 1976) with the total RNA (Plumley and Schmidt 1989) and protein (Schmollinger et al. 2014) content

being reduced by 60% and 50% respectively. In addition, under N starvation green microalgae undergo a rapid decrease in chlorophyll content (Dean et al. 2010, Jerez et al. 2016, Tossavainen et al. 2019a) together with a downregulation of most light-harvesting complex genes (Juergens et al. 2015, Tan et al. 2016). It has been described that the reduction in photosynthetic capacity is coordinated with an up regulation of triglycerides and starch synthesis (Alipanah et al. 2015, Tan et al. 2016) causing the funneling of carbon sources into lipid and carbohydrate metabolism. As a consequence of this partial shift into lipid metabolism, several green microalgae have been shown to accumulate neutral lipids under N starvation including species from the families *Chalmydomonas* (Boyle et al. 2012), *Coccomyxa* (Msanne et al. 2012), *Chlorella* (Adams et al. 2013), *Neochloris* (Breuer et al. 2012), *Scenedesmus* (Mandal and Mallick 2009), *Selenastrum* (Chakravarty and Mallick 2019), *Monoraphidium* (Bogen et al. 2013) and *Hematoccocus* (Recht et al. 2012). More specifically, *Hematococcus pluvialis* has been shown to accumulate high concentrations of oleic acid (18:1n-9) (Zhekisheva et al. 2002) while *Monoraphidium neglectum*, a closely related species to *Monoraphidium griffithi*, accumulates oleic acid and palmitic acid (16:0) (Bogen et al. 2013) under N starvation. In accordance with previous studies, our results indicate that the main differences in chlorophyll content, FA and AA seen between MWC and WW were a consequence of N starvation in MWC cultures (Table 6). Chlorophyll-a content showed to be significantly lower in MWC compared to WW in every tested species (Figure 1 D) which suggests a down-regulation of chlorophyll-a synthesis and possible pigment breakdown to scavenge N for other essential cellular processes. Total FA content showed to be higher in *Selenastrum* and *Monoraphidium* in MWC compared to WW (Table 6; Figure 4) which is supported by prior evidence that these microalgae species accumulate high amounts of FA under N depletion. FA profiles also reflected N starvation under MWC cultivation with *Haematococcus* accumulating high concentrations of oleic acid while *Selenastrum* and *Monoraphidium* accumulated palmitic acid together with oleic acid (Table 7). The variations seen in FA contribution profiles can be explained by major changes in the contribution of oleic acid in *Haematococcus* and palmitic and

oleic acids in *Selenastrum* and *Monoraphidium*. Interestingly, *Haematococcus* accumulated linoleic acid (18:2n-6) under MWC cultivation compared to WW (Table 7). To my knowledge, the accumulation of this FA has not been described before in *Haematococcus pluvialis* under N depletion. Total microalgal AA content also showed clear signs of N depletion in MWC media with an average drop of ~50% in total AA content compared to RAS WW (Table 12; Figure 7). Even though only slight differences were seen in the contribution of each AA between microalgae grown in MWC and WW (Table 15; Figure 5), Chen et al. (2017) proposed that under N-depletion *Chlorella* partitions glutamate, a major player in the transamination step in the catabolism and anabolism of many AAs, into α-ketoglutarate by transferring the amine group to pyruvate to form alanine. By doing so, α-ketoglutarate provides the carbon backbone for N assimilation. This adaptation in the AA metabolism would explain the observed increase in the contribution of alanine together with the reduction of glutamate seen in microalgae grown in MWC media compared to WW. It is important to point out that, since I analysed total amino acids in microalgae samples, changes in free intracellular amino acids indicating modifications in N metabolism could have been completely masked by the magnitude of changes in total AAs. In addition, I only identified 15 AAs, so it is possible that differences in unidentified AAs could reveal other metabolic adaptations of the studied microalgae under N starvation.

Dry weight varied with changes in cell density for *Selenastrum* but not for *Monoraphidium* (Figure 1 C). Lack of correlation between dry weight and density in *Chlorella vulgaris* has been described to be a consequence of variation in cell size due to differences in the nutrient availability in the growth media *(*Chiolccioli et al. 2014). Possibly, under my experimental conditions, microalgal cell sizes varied between MWC and WW. This change in cell size might have been more marked in *Selenastrum*, explaining the lack of differences in dry weight even with large variations in cell density among treatments.

As expected, the studied microalgae species presented distinctive FA and AA profiles (Table 6; Table 12). Microalgae order showed to be a good indicator of similarity in FA profiles with *Selenastrum* and *Monoraphidium* (order Sphaeropleales) presenting higher similarity of FAs presence/absence and FA content between each other than with *Haematococcus* (order Chlamydomonadales) (Figure 3) (Taipale et al. 2013, 2016). Differences in biochemical composition between species play a vital role when the purpose of the generated biomass is to be used as a feed for other organisms. Low quality of feed can be due to a shortage of essential biochemicals that cannot be synthesized in adequate amounts by its consumer to maximize growth or reproduction (von Elert 2012). Polyunsaturated fatty acids for example, are essential to many vertebrates and invertebrates (Stanley-Samuelson et al. 1988). Of special interest for aquatic organisms are EPA (20:5n-3) and DHA (22:6n-3) since these fatty acids have been shown to promote growth in *Daphnia* waterfleas (von Elert 2002, Martin-Creuzburg et al. 2008) and larval bivalves (Marshall et al. 2010). In addition, EPA and DHA accumulate in mussels when they are present in their diet (Pleissner et al. 2012) which increases mussels consumption benefits for human nutrition. None of the studied microalgae showed detectable levels of EPA or DHA (Table 6), nevertheless, organisms such as *Daphnia* waterfleas may be capable of synthesizing low levels of EPA from α-linoleic acid (α-LA, C18:3n-3) through elongation and desaturation (Taipale et al. 2015) explaining the increase in their growth rate after α-linoleic acid supplementation (von Elert 2012). In addition, low n-3/n-6 ratios have been correlated with poor nutritional conditions for *Daphnia* (Taipale et al. 2015). Altogether these results suggest that due to the higher average content of α-linoleic acid (Table 6) and their higher n-3/n-6 ratios (Table 6; Figure 4), *Monoraphidium* and *Selenastrum* could potentially be a better food source than *Haematococcus* for *Daphnia* and other species able to synthetize EPA from α-linoleic acid. AA composition also plays an important role in nutrition due to the limited capacity of *de novo* synthesis of essential AAs in many animals (Strayer 1988). For example, essential AAs for zooplankton are considered to be the same as for insects and humans (Fink et al. 2011). Relevance of dietary

essential and non-essential AAs has been demonstrated for aquatic organisms such as farmed fish (Conceição et al. 2003), *Daphnia* (Koch et al. 2011, Fink et al. 2012), mussels (Kreeger et al. 1996) among others. Histidine in particular has been recognized as an important essential AA for fish growth (Khan 2018) and evidence suggests that high histidine intake is capable of promoting *Daphnia* reproduction (Koch et al. 2009, 2011). My results indicate that *Haematococcus* possess a higher essential AA content (Table 12; Figure 7) and slightly higher histidine content than *Monoraphidium* and *Selenastrum*. Therefore, *Haematococcus* could serve a better food source than *Selenastrum* and *Monoraphidium* for species with a higher demand of essential AAs that are not limited by FA requirements. Importantly, since I only analyzed 15 AAs and I did not measure total protein content, my results could be overestimating the difference in total AA and essential AA between the studied microalgae. Ideally, microalgae selection with the purpose of feed preparation should be made based on the biochemical needs of its consumer. Perhaps a combination of different microalgae species including species containing high levels of EPA and DHA could bring better results than single species when used as feed.

Interestingly only minor differences were seen between filtered and unfiltered RAS WW in each tested microalgae species. In both treatments, all three microalgae achieved the same cell density (Figure 1 A) and consumed the same amount of N and P after six days of cultivation (Figure 2), showing no effects of competition for nutrients with microorganisms present in unfiltered WW. Microalgal dry weight did not show any difference between WW treatments (Figure 1 C), and these results are in accordance with a previous study by Halfhide et al. (2014) where the same dry weight was obtained for a mixed species consortium, *Chlorella* sp. and *Scenedesmus* grown in aquaculture wastewater under axenic or non-axenic conditions. FA and AA profiles showed minor differences due to filtration with *Haematococcus* presenting the highest FA dissimilarity between filtered and unfiltered treatment at 9.76% (Table 9; Table 15). Since unfiltered RAS WW

presented populations of other microorganisms together with microalgae, it is very likely that their presence contributed to the FA and AA quantified in our study.

Comparison of microalgae species in terms of nutrient consumption showed that *Monoraphidium* and *Selenastrum* are able to remove more phosphate from RAS WW than *Haematococcus* after six days of cultivation (Figure 2). Nitrogen removal did not show variation between species in unfiltered WW and only *Haematococcus* presented higher nitrogen removal than *Monoraphidium* in filtered WW. Literature regarding nutrient removal capacity of the studied microalgae genus varies widely in terms of used photoperiod, cultivation temperature and period and substrate type and its composition (*Haematococcus pluvialis*: Wu et al. 2013; *Monoraphidium* spp.: Jiang et al. 2016; *Selenastrum capricornutum*: Zhao et al. 2016) making comparisons among studies difficult. My results suggest that *Selenastrum* and *Monoraphidium* are more efficient at removing P than *Haematococcus* from RAS WW under the tested conditions (~17 ˚C, 24:0 photoperiod for six days). In order to maximize nutrient removal, photoperiod and cultivation period could be optimized for each microalgae species. Overall, my results are in accordance with previous studies showing that green freshwater microalgae are a potential alternative to assist Nordic RAS WW treatment through efficient N and P removal while generating valuable microalgae biomass (Stevčić et al. 2019, Tossavainen et al. 2019b).

4 CONCLUSIONS

RAS wastewater at Nordic conditions (~17 ˚C), either filtered or unfiltered, could work as a suitable growth medium for freshwater green microalgae. Only minor differences were seen between the two media in terms of microalgal nutrient consumption, biomass production and fatty acid / amino acid profiles which favors unfiltered RAS wastewater as a potential growth medium for large scale production

since filtration of RAS wastewater is costly. Compared to reference algae media, cultivation in RAS wastewater did change the microalgal biochemical composition of amino acids and fatty acids, but, since most of the observed changes can be attributed to nitrogen depletion in reference medium, I don't have evidence to believe that microalgae cultivated in RAS wastewater have a lower nutritional value when the biomass is expected to be use as feed. Every microalgae species presented a distinctive fatty acid and amino acid profile. If the purpose of the generated biomass is to be used as a feed for a higher trophic level, proper microalgae species selection and optimization of cultivation conditions are needed to generate high biomasses with the desired biochemical composition. The results from this study provide more evidence of the applicability of microalgae in RAS wastewater with the double purpose of wastewater treatment and generation of valuable biomass.

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APPENDIX 1. CHEMICAL COMPOSITION OF MODIEFIED WRIGHT'S CRYPTOPHYTE MEDIUM (MWC)

Modified Wright's Cryptophyte Medium used as algae reference medium.

Compound	Concentration (mg L^{-1})	Trace metals	Concentration (mg L^{-1})	Vitamins	Concentration (µg L^{-1})
$K_2HPO_4.3$ H2O	8.7	NaEDTA	4.4	0.5	biotin (B7)
$NaNO_3$	85	$FeCl_3.6H2O$	3.2	0.5	cyanocobalamin (B12)
$CaCl_2.2H2O$	36.8	$CuSO_4.5H2O$	0.01	0.5	pyridoxine (B6)
$MgSO_4.7H_2O$	37	$ZnSO_4.7H_2O$	0.02	100	thiamine HCL (B1)
$NaHCO_3$	12.6	$CoCl_2.6H_2O$	0.01		
$Na_2SeO_3.5H_2O$	2.3	$MnCl_2.4H_2O$	0.2		
$Na_2SiO_3.5H_2O$	21.2	$Na_2MoO_4.2H_2O$	0.01		
TES buffer	115	H_3BO_3	1.0		

APPENDIX 2. TABLE: PAIRWISE COMPARISON OF DENSITY, SPECIFIC GROWTH RATE, DRY WEIGHT AND CHLOROPHYLL-A

Estimated Marginal Means pairwise comparison of treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) and microalgae species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) with Tukey adjustments for density, specific growth rate (SGR), dry weight and chlorophyll-a. Highlighted (bold values) are all p-values <0.05.

Variable	Species	Contrast	Estimate	DF	t ratio	p-value	Media	Contrast	Estimate	DF	t ratio	p-value
Density	HP	MWC-WWF	0.018	27	0.01	1.00	MWC	HP - MG	-8.27	27	-5.648	**<0.001**
		MWC-WWU	0.063	27	0.04	1.00		HP - SE	-21.28	27	-14.53	**<0.001**
		WWF-WWU	0.045	27	0.03	1.00		MG - SE	-13.01	27	-8.878	**<0.001**
	MG	MWC-WWF	4.190	27	2.86	**0.02**	WWF	HP - MG	-4.1	27	-2.799	**<0.001**
		MWC-WWU	4.360	27	2.98	**0.02**		HP - SE	-13.32	27	-9.095	**<0.001**
		WWF-WWU	0.170	27	0.12	0.99		MG - SE	-9.22	27	-6.296	**<0.001**
	SE	MWC-WWF	7.973	27	5.44	**<.001**	WWU	HP - MG	-3.98	27	-2.714	**<0.001**
		MWC-WWU	8.563	27	5.85	**<.001**		HP - SE	-12.78	27	-8.723	**<0.001**
		WWF-WWU	0.590	27	0.40	0.91		MG - SE	-8.8	27	-6.009	**<0.001**
SGR	HP	MWC-WWF	0.051	26	2.14	0.10	MWC	HP - MG	-0.19667	26	-8.312	**<0.001**
		MWC-WWU	0.053	26	2.24	0.08		HP - SE	-0.06815	26	-2.88	**<0.001**
		WWF-WWU	0.002	26	0.10	0.99		MG - SE	0.12852	26	5.469	**<0.001**
	MG	MWC-WWF	0.111	26	4.74	**<.001**	WWF	HP - MG	-0.13592	26	-5.784	**<0.001**
		MWC-WWU	0.111	26	4.72	**<.001**		HP - SE	-0.00923	26	-0.393	**<0.001**
		WWF-WWU	0.000	26	-0.02	1.00		MG - SE	0.12669	26	5.391	**<0.001**
	SE	MWC-WWF	0.110	26	4.66	**<.001**	WWU	HP - MG	-0.13879	26	-5.906	**<0.001**
		MWC-WWU	0.095	26	4.03	**<.001**		HP - SE	-0.02661	26	-1.132	0.50
		WWF-WWU	-0.015	26	-0.64	0.80		MG - SE	0.11218	26	4.774	**<0.001**
Dry Weight	HP	MWC-WWF	0.005	27	0.12	0.99	MWC	HP - MG	-0.1325	27	-3.218	**0.01**
		MWC-WWU	-0.015	27	-0.36	0.93		HP - SE	-0.035	27	-0.85	0.68
		WWF-WWU	-0.020	27	-0.49	0.88		MG - SE	0.0975	27	2.368	0.06
	MG	MWC-WWF	0.118	27	2.85	**0.02**	WWF	HP - MG	-0.02	27	-0.486	0.88
		MWC-WWU	0.110	27	2.67	**0.03**		HP - SE	-0.005	27	-0.121	0.99
		WWF-WWU	-0.008	27	-0.18	0.98		MG - SE	0.015	27	0.364	0.93
	SE	MWC-WWF	0.035	27	0.85	0.68	WWU	HP - MG	-0.0075	27	-0.182	0.98
		MWC-WWU	0.010	27	0.24	0.97		HP - SE	-0.01	27	-0.243	0.97
		WWF-WWU	-0.025	27	-0.61	0.82		MG - SE	-0.0025	27	-0.061	1.00
Chlorophyll a	HP	MWC-WWF	-3.899	26	-4.26	**<.001**	MWC	HP - MG	-3.094	26.2	-3.379	**0.01**
		MWC-WWU	-0.947	26	-1.03	0.56		HP - SE	-3.039	26.2	-3.319	**0.01**
		WWF-WWU	2.953	26	3.24	**0.01**		MG - SE	0.055	26	0.06	0.99
	MG	MWC-WWF	-3.373	26	-3.70	**<.001**	WWF	HP - MG	-2.567	26	-2.817	**0.02**
		MWC-WWU	-4.253	26	-4.67	**<.001**		HP - SE	-0.323	26	-0.354	0.93
		WWF-WWU	-0.880	26	-0.97	0.60		MG - SE	2.245	26	2.463	0.05
	SE	MWC-WWF	-1.183	26	-1.30	0.41	WWU	HP - MG	-6.4	26	-7.021	**<0.001**
		MWC-WWU	-3.217	26	-3.53	**<.0013**		HP - SE	-5.31	26	-5.826	**<0.001**
		WWF-WWU	-2.035	26	-2.23	0.08		MG - SE	1.09	26	1.196	0.47

APPENDIX 3. TABLE: PAIRWISE COMPARISON OF PO_4-P AND NO_3-N REMOVAL RATES

Estimated Marginal Means pairwise comparison of treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) and microalgae species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) with Tukey adjustments for PO_4-P and NO_3-N removal rates (R_P and R_N, respectively). Highlighted (bold values) are all p-values <0.05.

Variable	Species	Contrast	Estimate	DF	t ratio	p-value	Media	Contrast	Estimate	DF	t ratio	p-value
R_P	HP	MWC-WWF	-0.250	26.1	-7.61	**<.001**	MWC	HP – MG	-0.0429	26.1	-1.3	0.41
		MWC-WWU	-0.275	26.1	-8.37	**<.001**		HP – SE	-0.0429	26.1	-1.3	0.41
		WWF-WWU	-0.025	26	-0.76	0.73		MG – SE	0	26	0	1
	MG	MWC-WWF	-0.370	26	-11.31	**<.001**	WWF	HP – MG	-0.1625	26	-4.97	**<0.001**
		MWC-WWU	-0.380	26	-11.62	**<.001**		HP – SE	-0.1625	26	-4.97	**<0.001**
		WWF-WWU	-0.010	26	-0.31	0.95		MG – SE	0	26	0	1
	SE	MWC-WWF	-0.370	26	-11.31	**<.001**	WWU	HP – MG	-0.1475	26	-4.51	**<0.001**
		MWC-WWU	-0.383	26	-11.69	**<.001**		HP – SE	-0.15	26	-4.59	**<0.001**
		WWF-WWU	-0.013	26	-0.38	0.92		MG – SE	-0.0025	26	-0.08	1
R_N	HP	MWC-WWF	-2.390	27	-5.04	**<.001**	MWC	HP – MG	-0.035	27	-0.07	1
		MWC-WWU	-1.760	27	-3.71	**<.001**		HP – SE	-0.04	27	-0.08	1
		WWF-WWU	0.630	27	1.33	0.39		MG – SE	-0.005	27	-0.01	1
	MG	MWC-WWF	-0.802	27	-1.69	0.23	WWF	HP – MG	1.552	27	3.274	**0.01**
		MWC-WWU	-0.600	27	-1.27	0.43		HP – SE	1.08	27	2.278	0.08
		WWF-WWU	0.203	27	0.43	0.90		MG – SE	-0.472	27	-1	0.59
	SE	MWC-WWF	-1.270	27	-2.68	**0.03**	WWU	HP – MG	1.125	27	2.373	0.06
		MWC-WWU	-1.508	27	-3.18	**0.01**		HP - SE	0.212	27	0.448	0.90
		WWF-WWU	-0.237	27	-0.50	0.87		MG - SE	-0.912	27	-1.92	0.15

APPENDIX 4. TABLE: PAIRWISE COMPARISON OF TOTAL-FATTY ACID, POLYUNSATURATED, MONOUNSATURAED AND SATURATED FATTY ACID CONTENT AND N-3/N-6 AND UFA/SFA RATIOS

Estimated Marginal Means pairwise comparison of treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) and microalgae species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) with Tukey adjustments for total fatty acids content (Tot-FA), polyunsaturated fatty acid (PUFA), n-3/n-6 fatty acid ratio, monounsaturated fatty acid (MUFA), saturated fatty acid (SFA) and unsaturated/saturated fatty acids ratio UFA/ SFA. Highlighted (bold values) are all p-values <0.05.

Variable	Species	Contrast	Estimate	DF	t ratio	p-value	Media	Contrast	Estimate	DF	t ratio	p-value
Tot-FA	HP	MWC-WWF	-10.90	26.4	-1.02	0.57	MWC	HP - MG	-35.16	26.4	-3.28	**0.01**
		MWC-WWU	35.53	26.4	3.32	**0.01**		HP - SE	-79.02	26.4	-7.38	**<0.001**
		WWF-WWU	24.63	26.0	2.30	0.07		MG - SE	-43.86	26	-4.10	**<0.001**
	MG	MWC-WWF	-32.67	26.0	-3.06	**0.01**	WWF	HP - MG	-13.39	26	-1.25	0.43
		MWC-WWU	32.89	26.0	3.08	**0.01**		HP - SE	-37.66	26	-3.52	**<0.001**
		WWF-WWU	0.22	26.0	0.02	1.00		MG - SE	-24.27	26	-2.27	0.08
	SE	MWC-WWF	-52.26	26.0	-4.89	**<.001**	WWU	HP - MG	-37.81	26	-3.54	**<0.001**
		MWC-WWU	68.55	26.0	6.41	**<.001**		HP - SE	-46.00	26	-4.30	**<0.001**
		WWF-WWU	16.30	26.0	1.52	0.30		MG - SE	-8.19	26	-0.77	0.73
PUFA	HP	MWC-WWF	-2.65	26.3	-0.41	0.91	MWC	HP - MG	0.09	26.3	0.01	1.00
		MWC-WWU	20.35	26.3	3.12	**0.01**		HP - SE	-12.03	26.3	-1.84	0.18
		WWF-WWU	17.70	26.0	2.72	**0.03**		MG - SE	-12.12	26	-1.87	0.17
	MG	MWC-WWF	0.97	26.0	0.15	0.99	WWF	HP - MG	-3.54	26	-0.54	0.85
		MWC-WWU	-0.68	26.0	-0.11	0.99		HP - SE	-15.13	26	-2.33	0.07
		WWF-WWU	0.29	26.0	0.04	1.00		MG - SE	-11.59	26	-1.78	0.19
	SE	MWC-WWF	0.44	26.0	0.07	1.00	WWU	HP - MG	-20.95	26	-3.22	**0.01**
		MWC-WWU	8.18	26.0	1.26	0.43		HP - SE	-24.21	26	-3.73	**<0.001**
		WWF-WWU	8.62	26.0	1.33	0.39		MG - SE	-3.26	26	-0.50	0.87
n-3/n-6	HP	MWC-WWF	1.17	26.1	3.04	**0.01**	MWC	HP - MG	-5.37	26.1	-13.92	**<0.001**
		MWC-WWU	-1.21	26.1	-3.13	**0.01**		HP - SE	-3.71	26.1	-9.62	**<0.001**
		WWF-WWU	-0.03	26.0	-0.08	1.00		MG - SE	1.66	26	4.33	**<0.001**
	MG	MWC-WWF	-1.88	26.0	-4.89	**<.001**	WWF	HP - MG	-2.32	26	-6.05	**<0.001**
		MWC-WWU	2.26	26.0	5.90	**<.001**		HP - SE	-1.89	26	-4.92	**<0.001**
		WWF-WWU	0.39	26.0	1.01	0.58		MG - SE	0.44	26	1.13	0.50
	SE	MWC-WWF	-0.65	26.0	-1.69	0.23	WWU	HP - MG	-1.90	26	-4.95	**<0.001**
		MWC-WWU	0.13	26.0	0.33	0.94		HP - SE	-2.38	26	-6.20	**<0.001**
		WWF-WWU	-0.52	26.0	-1.37	0.37		MG - SE	-0.48	26	-1.25	0.44
MUFA	HP	MWC-WWF	5.25	27	1.37	0.37	MWC	HP - MG	-26.41	27	-6.89	**<0.001**
		MWC-WWU	6.56	27	1.71	0.22		HP - SE	-50.33	27	-13.12	**<0.001**
		WWF-WWU	-1.30	27	-0.34	0.94		MG - SE	-23.92	27	-6.24	**<0.001**
	MG	MWC-WWF	20.86	27	5.44	**<.001**	WWF	HP - MG	-10.8	27	-2.82	**0.02**
		MWC-WWU	20.29	27	5.29	**<.001**		HP - SE	-17.92	27	-4.67	**<0.001**
		WWF-WWU	0.57	27	0.15	0.99		MG - SE	-7.12	27	-1.86	0.17
	SE	MWC-WWF	37.66	27	9.82	**<.001**	WWU	HP - MG	-12.67	27	-3.31	**0.01**

		MWC-WWU	42.27	27	11.02	**<.001**		HP - SE	-14.62	27	-3.81	**<0.001**
		WWF-WWU	-4.61	27	-1.20	0.46		MG - SE	-1.94	27	-0.51	0.87
SFA	HP	MWC-WWF	3.24	27	1.32	0.40	MWC	HP - MG	-8.59	27	-3.49	**<0.001**
		MWC-WWU	8.87	27	3.61	**<.001**		HP - SE	-16.40	27	-6.67	**<0.001**
		WWF-WWU	-5.63	27	-2.29	0.07		MG - SE	-7.81	27	-3.18	0.01
	MG	MWC-WWF	12.78	27	5.19	**<.001**	WWF	HP - MG	0.95	27	0.39	0.92
		MWC-WWU	13.28	27	5.40	**<.001**		HP - SE	-4.61	27	-1.87	0.17
		WWF-WWU	-0.50	27	-0.20	0.98		MG - SE	-5.56	27	-2.26	0.08
	SE	MWC-WWF	15.04	27	6.11	**<.001**	WWU	HP - MG	-4.18	27	-1.70	0.22
		MWC-WWU	18.10	27	7.36	**<.001**		HP - SE	-7.18	27	-2.92	**0.02**
		WWF-WWU	-3.06	27	-1.25	0.44		MG - SE	-2.99	27	-1.22	0.45
UFA/SFA	HP	MWC-WWF	-0.18	26.3	-0.80	0.71	MWC	HP - MG	-0.06	26.3	-0.26	0.96
		MWC-WWU	0.11	26.3	0.51	0.84		HP - SE	-0.43	26.3	-1.91	0.16
		WWF-WWU	-0.29	26	-1.31	0.40		MG - SE	-0.37	26	-1.66	0.24
	MG	MWC-WWF	-1.30	26	-5.83	**<.001**	WWF	HP - MG	-1.17	26	-5.29	**<0.001**
		MWC-WWU	-1.45	26	-6.51	**<.001**		HP - SE	-0.99	26	-4.45	**<0.001**
		WWF-WWU	0.15	26	0.68	0.78		MG - SE	0.19	26	0.84	0.68
	SE	MWC-WWF	-0.74	26	-3.33	**<.001**	WWU	HP - MG	-1.62	26	-7.28	**<0.001**
		MWC-WWU	-0.65	26	-2.94	**0.02**		HP - SE	-1.19	26	-5.37	**<0.001**
		WWF-WWU	-0.09	26	-0.39	0.91		MG - SE	0.42	26	1.91	0.16

APPENDIX 5. TABLE: PAIRWISE COMPARISON OF TOTAL AMINO ACID, ESSENTIAL AND NONESSENTIAL AMINO ACID CONTENT

Estimated Marginal Means pairwise comparison of treatments (MWC – Modified Wright's Cryptophyte medium, WWU – unfiltered RAS wastewater, WWF – filtered RAS wastewater) and microalgae species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) with Tukey adjustments for total amino acid (Tot-AA), essential amino acid (EAA) and non-essential amino acid (NEAA) content (mg g^{-1} dry weight). Highlighted (bold values) are all p-values <0.05.

Variable	Species	Contrast	Estimate	DF	t ratio	p-value	Media	Contrast	Estimate	DF	t ratio	p-value
Tot-AA	HP	MWC-WWF	-128.1	27	-15.29	**<.001**	MWC	HP - MG	37.04	27	4.421	**<0.001**
		MWC-WWU	-132.1	27	-15.77	**<.001**		HP - SE	13.85	27	1.653	0.24
		WWF-WWU	4.0	27	0.48	0.88		MG - SE	-23.19	27	-2.768	**0.03**
	MG	MWC-WWF	-112.2	27	-13.40	**<.001**	WWF	HP - MG	52.88	27	6.312	**<0.001**
		MWC-WWU	-124.2	27	-14.83	**<.001**		HP - SE	59.95	27	7.157	**<0.001**
		WWF-WWU	12.0	27	1.43	0.34		MG - SE	7.07	27	0.844	0.68
	SE	MWC-WWF	-82.0	27	-9.78	**<.001**	WWU	HP - MG	44.89	27	5.359	**<0.001**
		MWC-WWU	-113.3	27	-13.53	**<.001**		HP - SE	32.6	27	3.892	**<0.001**
		WWF-WWU	31.4	27	3.74	**<.001**		MG - SE	-12.29	27	-1.467	0.32
EAA	HP	MWC-WWF	-63.6	27	-13.28	**<.001**	MWC	HP - MG	15.597	27	3.256	**0.01**
		MWC-WWU	-63.9	27	-13.34	**<.001**		HP - SE	5.537	27	1.156	0.49
		WWF-WWU	0.3	27	0.07	1.00		MG - SE	-10.06	27	-2.1	0.11
	MG	MWC-WWF	-51.9	27	-10.83	**<.001**	WWF	HP - MG	27.322	27	5.704	**<0.001**
		MWC-WWU	-62.6	27	-13.08	**<.001**		HP - SE	29.826	27	6.227	**<0.001**
		WWF-WWU	10.8	27	2.25	0.08		MG - SE	2.505	27	0.523	0.86
	SE	MWC-WWF	-39.3	27	-8.20	**<.001**	WWU	HP - MG	16.864	27	3.521	**<0.001**
		MWC-WWU	-52.0	27	-10.85	**<.001**		HP - SE	17.478	27	3.649	**<0.001**
		WWF-WWU	12.7	27	2.64	**0.04**		MG - SE	0.614	27	0.128	0.99
NEAA	HP	MWC-WWF	-65.2	26.2	-14.01	**<.001**	MWC	HP - MG	20.73	26.2	4.456	**<0.001**
		MWC-WWU	-68.9	26.2	-14.80	**<.001**		HP - SE	7.6	26.2	1.634	0.25
		WWF-WWU	3.7	26	0.80	0.71		MG - SE	-13.13	26	-2.836	0.02
	MG	MWC-WWF	-60.3	26	-13.04	**<.001**	WWF	HP - MG	25.55	26	5.52	**<0.001**
		MWC-WWU	-61.6	26	-13.30	**<.001**		HP - SE	30.12	26	6.507	**<0.001**
		WWF-WWU	1.2	26	0.27	0.96		MG - SE	4.57	26	0.987	0.59
	SE	MWC-WWF	-42.7	26	-9.21	**<.001**	WWU	HP - MG	28.03	26	6.055	**<0.001**
		MWC-WWU	-61.4	26	-13.25	**<.001**		HP - SE	15.12	26	3.267	**0.01**
		WWF-WWU	18.7	26	4.04	**<.001**		MG - SE	-12.91	26	-2.788	**0.03**