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Čedomir Stevčić

The Use of Biological Traps for Water Treatment in Recirculating Aquaculture Systems

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
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ABSTRACT

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Wastewater (WW) of recirculating aquaculture systems (RAS) has a high concentration of dissolved nutrients, which enable bioremediation of RAS WW with microalgae. Biological harvesting by filter-feeding organisms offers an alternative for the expensive mechanical and chemical harvesting of microalgae with opportunities for further utilization of the produced biomass. This thesis evaluated if the combination of microalgae and waterflea (*Daphnia magna)* cultivation in Nordic RAS WW (*ca*. 17 °C) can be used to trap the dissolved nutrients. Green microalgae had comparable growth and removal of nitrate-nitrogen (NO_3-N) and phosphate-phosphorous (PO_4-P) in unfiltered RAS WW to those in the reference algal medium while non-green microalgae had insignificant growth and nutrient removal capacity. Growth and nutrient removal of three green microalgae in unfiltered WW did not differ between the three tested LED spectra, while the fourth tested species, *Haematoccocus pluvialis*, showed higher nutrient removal under a specific LED spectrum. Filtration of WW from RAS for growing microalgae is not needed as biological contaminants within WW did not significantly decrease the microalgal growth, nutrient removal, and amino acid and fatty acid composition, with the exception of *H. pluvialis*. When green microalgae were cultivated in WW and fed to *D. magna*, *Daphnia*'s weight increased 2–3 times in 4 days. *D. magna* removed 80 % of *Monoraphidium griffithii*, 70 % of *H. pluvialis*, and 20 % of *Selenastrum* sp. from WW in 48 h. Only when *Selenastrum* sp. was used as a diet, *D. magna* re-released PO_4-P into solution. In conclusion, the efficiency of microalgae–*Daphnia* bioremediation system in Nordic RAS WW can be improved with a careful selection of microalgal species, supporting the concept of circular economy and sustainable WW management.

Keywords: Biological harvesting; bioremediation; *Daphnia magna*; fish farming; green microalgae; nutrient removal; nutritional quality.

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TIIVISTELMÄ

Stevčić, Čedomir
Biologisten ravinnesieppareiden käyttö kalojen kiertovesikasvatuksen jäteveden puhdistuksessa
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Kalojen kasvatus kiertovesijärjestelmässä tuottaa jätevettä, jonka ravinnepitoisuus on niin suuri, että jätevettä voidaan hyödyntää leväbiomassan tuottamiseen ja samalla vähentää ravinnepäästöjä ympäristöön. Tässä työssä tutkittiin luontaisesti Suomessa esiintyvien mikrolevien kasvua ja kykyä poistaa ravinteita kiertovesikasvatuksen jätevedestä sekä kasvatettujen mikrolevien poistamista vedestä ravintonsa suodattavien vesikirppujen (*Daphnia magna*) avulla. Testatuista leväryhmistä viherleviin kuuluvat levät kasvoivat ja vähensivät tehokkaasti jäteveden liuenneita ravinteita, nitraattia ja fosfaattia (NO_3-N ja PO_4-P), kun taas muiden leväryhmien edustajat kasvoivat huonosti eivätkä vähentäneet ravinteita oleellisesti. LED-valojen spektri ei vaikuttanut kolmen tutkitun viherlevän kasvuun ja jäteveden ravinteiden poistokykyyn, mutta neljännellä tutkimuslajilla (*Haematoccocus pluvialis)* ravinteiden käyttö tehostui tietyllä LED-valon spektrillä. Jäteveden suodatus leväkasvatusta varten ei näytä olevan tarpeen, koska biologiset epäpuhtaudet eivät merkitsevästi vähentäneet mikrolevien kasvua, ravinteiden poistokykyä eivätkä myöskään aminohappo- tai rasvahappokoostumusta, poikkeuksena *H. pluvialis*. Kun jätevedessä kasvatettuja leviä syötettiin vesikirpuille, vesikirppujen paino 2–3-kertaistui neljässä vuorokaudessa. Vesikirput pystyivät poistamaan vedestä 80 % *Monoraphidium griffithii* - ja 70 % *H. pluvialis* -levistä, mutta vain 20 % *Selenastrum* sp. -levästä 48 h aikana. Jos ruokana oli *Selenastrum* sp. -levä, vesikirput vapauttivat veteen PO_4-P:a. Tämän tutkimuksen perusteella mikrolevien kasvatus on mahdollista yhdistää vesikirppujen tuottamiseen jäteveden puhdistamiseksi myös pohjoismaisissa ilmasto-olosuhteissa, mutta tehokkuuden maksimoimiseksi on oleellista käyttää käyttötarkoitukseen sopivinta mikrolevää.

Avainsanat: Biosiepparit; *Daphnia magna*; kalankasvatus; ravinteiden poisto; ravitsemuksellinen laatu; viherlevät.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred in the text by their Roman numerals I–III. Contributions of authors are presented in the Table 1. In addition, all authors critically examined and revised the manuscripts.

I Stevčić Č., Pulkkinen K. & Pirhonen J. 2019. Screening of microalgae and LED grow light spectra for effective removal of dissolved nutrients from cold-water recirculating aquaculture system (RAS) wastewater. *Algal Research* 44, 101681.

II Calderini M., Stevčić Č., Taipale S. & Pulkkinen K. 2021. Filtration of Nordic recirculating aquaculture system wastewater: effects on microalgal growth, nutrient removal and nutritional value. Submitted manuscript.

III Stevčić Č., Pulkkinen K. & Pirhonen J. 2020. Efficiency of *Daphnia magna* in removal of green microalgae cultivated in Nordic recirculating aquaculture system wastewater. *Algal Research* 52, 102108.

TABLE 1 The contribution of different authors to papers I–III. ČS = Čedomir Stevčić, JP = Juhani Pirhonen, KP = Katja Pulkkinen, MC = Marco Calderini, ST = Sami Taipale.

	I	II	III
Conceptualization	ČS, JP, KP	ČS, KP, MC, ST	ČS, JP, KP
Experimental design	ČS, JP, KP	ČS, KP, MC, ST	ČS, JP, KP
Data collection	ČS, KP	ČS, MC	ČS
Data analysis	ČS, KP	ČS, MC	ČS, KP
Writing – original draft	ČS	ČS, MC	ČS
Writing – review & editing	ČS, JP, KP	ČS, KP, MC, ST	ČS, JP, KP

1 INTRODUCTION

1.1 Recirculating aquaculture system

The aquaculture industry is directed towards more intensive production due to the needs to increase fish production, to prevail limitations in water quantity and quality, and to decrease waste discharges and environmental impacts (Wik *et al*. 2009). Consequently, this intensification of aquaculture could lead to environmental problems such as acidification, eutrophication, and ecotoxicological impacts in aquatic ecosystems (Henriksson *et al.* 2018). Major pollutants in aquaculture wastewater (WW) are particulate or dissolved organic matter, suspended solids, nutrients such as nitrogen (N) and phosphorus (P), and specific chemicals used for *e.g.* disease prevention (Piedrahita 2003, Castine *et al*. 2013). The nutrient removal from aquaculture WW would be essential to prevent the eutrophication of the receiving aquatic ecosystems. However, traditional flow through and net-pen aquaculture systems discharge effluents directly into the surrounding water bodies, typically without any WW treatment (Blancheton *et al*. 2007, Bregnballe 2015).

The recirculating aquaculture system (RAS) significantly decreases water use and WW volume by treating and recycling water, and it enables enhanced opportunities for WW treatment and nutrient recycling (Piedrahita 2003, Martins *et al*. 2010). RAS is a land-based, indoor or outdoor, almost closed system for farming fish or other aquatic organisms (*e.g*. shrimps, clams) where the outlet water from cultivation tanks is treated and re-used with a high level of water circulation (Fig. 1). Typically, the RAS treatment loop is a combination of solid removal, biological filtration, gas control (oxygenation, CO_2 degassing), and disinfection (Lekang 2007, Steicke *et al*. 2009, Pulkkinen *et al*. 2018). In RAS, less than 10 % of the total water volume is replaced per day which is roughly 100 times lower water exchange rate than in the traditional flow-through systems (Blancheton *et al*. 2007, Bregnballe 2015). Because of low water

consumption, RAS has other advantages over flow-through system, such as more flexible selection of location for production facilities close to markets, higher year-round control over environmental and biological pollution, and possibility to discharge WW into wastewater treatment systems (Blancheton 2000, Lekang 2007, Wik *et al*. 2009). RAS was developed in 1970s, yet only during the past decades the potential of this technology has been applied as the most sustainable type of aquaculture on a large-scale (Bohl 1977, Bergheim *et al*. 2009, Dalsgaard *et al*. 2013). RAS has been developed to address the guidelines and regulations concerning environmental waste discharge worldwide, and many European countries are recommending RAS as the main solution to further improve ecologically sustainable aquaculture (Badiola *et al*. 2012).

The main nitrogenous waste released into water by fish occurs as two compounds: unionized (free) ammonia (NH_3) and ionized ammonia or ammonium (NH_4^+) depending on the water pH (Timmons and Ebeling 2007). The unionized ammonia is extremely toxic to aquatic animals, while ionized ammonia is non-toxic (Kolarevic *et al*. 2013). Thus, unionized ammonia must be removed from the circulating water and this is achieved through the nitrification process via metabolism of chemoautotrophic bacteria in the RAS biofilter. The final product in the nitrification process is nitrate (NO_3^-), and even though it is considered non-toxic to aquatic animals, high levels (above 100 mg l^{-1}) can have negative impacts, typically seen first as reduced animal growth (Davidson *et al.* 2014, Bregnballe 2015).

Water reuse in RAS is limited by the accumulation of waste products originating from uneaten feed, feces, and metabolic waste products (Piedrahita 2003). As the nitrogenous compounds and organic carbon accumulation can decrease fish growth (Ling and Chen 2005, Davidson *et al.* 2014, Pulkkinen *et al*. 2018), water in RAS needs to be renewed with rates high enough to avoid this. If dilution or exchange of the culture water is not possible, using a denitrification biofilter can decrease the nitrate concentrations and consequently lower the water consumption. Chemoheterotrophic bacteria located in a denitrification biofilter convert nitrate into nitrogen gas (N_2) (van Rijn *et al*. 2006). Using denitrification reduces the problem of nitrate accumulation in the system, yet this valuable fertilizer is lost into the air while it could be used to produce biomass. Denitrification systems are used only in *ca*. 20 % of commercial RAS as they are difficult to operate and expensive (Badiola *et al*. 2012).

RAS does not decrease the total nutrient discharge into the surrounding water bodies but it concentrates nutrients and organic matter (Piedrahita 2003). RAS WW with a high concentration of dissolved nutrients allows new opportunities to advance technologies for utilizing the waste as a valuable resource (*i.e.* circular economy), while this is more challenging in traditional farming as the same amount of nutrients is diluted in much larger water volume. The enhancement of RAS is inhibited by a large amount of energy needed for RAS, which significantly increases the carbon footprint of the products depending on how the energy is produced (Liu *et al*. 2016). In addition, mechanical WW treatment methods take up a lot of space and are

expensive (Badiola *et al*. 2012). Almost no dissolved nutrients discharged from RAS are being presently recycled, except for aquaponics (*aqua*culture combined with the cultivation of plants without soil, *i.e*. hydro*ponics*) (Goddek *et al*. 2019). Despite RAS WW having generally ten- to hundredfold less dissolved nutrients than *e.g*. municipal or industrial WWs, it still has enough dissolved nutrients to sustain the photosynthetic growth of plants and algae (Arnold 2013). Therefore, the ongoing developments in RAS include technological improvement of filtration systems, ecological advancement of bioremediation, and reuse of system's by-products via integrated multi-trophic aquaculture (IMTA) approach (Martins *et al*. 2010).

IMTA is a relatively new approach where two or more compatible organisms from different trophic levels are simultaneously cultivated in a single system (Fig. 2). The wastes from the production of the fed organism (*e.g*. fish or shrimp) are recycled to become inputs (*e.g*. fertilizer and food) for a secondary organism in close proximity to each other (Chopin *et al*. 2001, Shpigel and Neori 2007, Troell *et al*. 2009). The secondary organisms can be species extracting organic compounds (shellfish or suspension- and deposit-feeding invertebrates) and/or species extracting inorganic compounds (higher plants or algae) (Neori *et al*. 2004, Troell *et al*. 2009, van Rijn 2013). Integrated aquaculture increases significantly the sustainability of RAS and aquaculture in general, due to the optimized use of available natural resources and diversification of revenue-based activities.

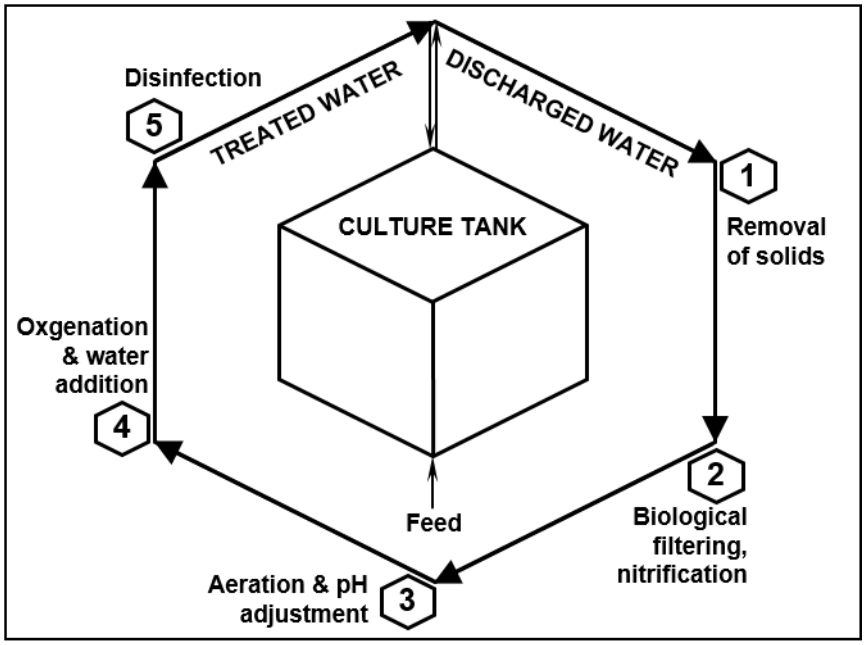

FIGURE 1 Recirculating aquaculture system (RAS).

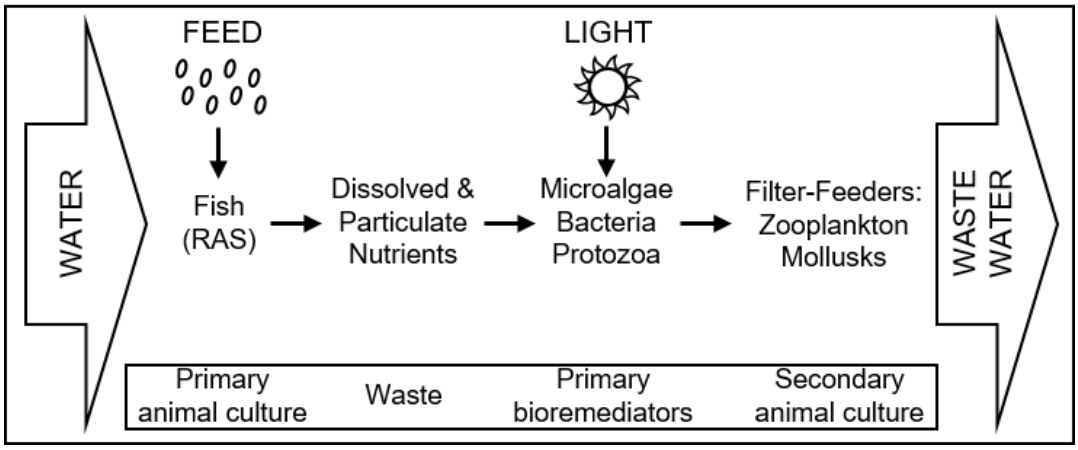

FIGURE 2 Integrated multi-trophic aquaculture (IMTA) between recirculating aquaculture system (RAS) and microalgae-filter-feeders bioremediation system (modified from Neori *et al*. 2017).

1.2 Microalgae bioremediation

Microalgae are unicellular photosynthetic micro-organisms (2–200 µm in size), typically found in freshwater and marine environments (living in both the water column and sediment). Microalgae are among the best cost-effective and sustainable organisms for bioremediation of aquaculture WW due to their photosynthetic efficiency, high efficiency in organic/inorganic nutrient removal, high growth rate, low water footprint, and tolerance to poor water quality (Mata *et al*. 2010, 2012, Manninen *et al*. 2016, Zhao *et al*. 2016). By removing and assimilating nutrients and carbon, microalgae provide a dual application for the aquaculture: (1) bioremediation of WW and (2) production of biomass rich in proteins, carbohydrate, pigments, vitamins, and energy reserves in the form of lipids and hydrocarbons (Arbib *et al*. 2012, Mata *et al*. 2012). Nevertheless, there is less knowledge for microalgal bioremediation of RAS WW than of municipal and industrial WWs (Wang and Lan 2011, Ji *et al*. 2013). Furthermore, microalgal bioremediation is mostly limited to warmer geographical locations for aquaculture WW (Guerrero-Cabrera *et al*. 2014, Gao *et al*. 2016, Ansari *et al*. 2017) or RAS WW (Chun *et al*. 2018, Egloff *et al*. 2018, Ramli *et al*. 2018) as lower temperature and shorter daylight in higher latitudes hamper the microalgal efficiency to treat WW. Research is therefore needed for microalgal bioremediation of RAS WW at relatively low temperatures (below 20 °C), for developing practical methods of microalgal cultivation in RAS WW, and for avoiding the possible introduction of exotic species into natural aquatic ecosystems of northern latitudes.

Microalgae can accumulate high quantities of carbohydrates, proteins, lipids, and high-value compounds such as vitamins, antioxidants, specific fatty acids, β-carotenes, alginate, carrageenan, astaxanthin, and other pigments (Barrow and Shahidi 2008, Mata *et al*. 2010, Das *et al*. 2011). However, the

accumulation of these biochemicals highly depends on the microalgal species, their growth phase (stationary or exponential), and the cultivation conditions (Fidalgo *et al*. 1998, Becker 2007, Bernaerts *et al*. 2019). Produced microalgal biomass can be later used as a feedstock to produce animal feed, human food and health products, nutraceuticals, agricultural fertilizers, biofuels, and other biochemical derivatives (Mata *et al*. 2010, Castine *et al*. 2013, Ansari *et al*. 2017). Therefore, microalgae–RAS integrated system could offer financial returns via reduction of feeding expenses for culture organisms, via enhancement of WW treatment capacity, and via direct sales of profitable species and their valuable by-products (Mata *et al*. 2012, Castine *et al*. 2013, Oostlander *et al*. 2020).

The main limiting factor for the microalgal growth and bioremediation efficiency is the light with its quantity (intensity or photon flux density, PPFD), quality (spectral distribution or wavelength), and periodicity (photoperiod) (Luo *et al*. 2017, Sun *et al*. 2018). Although highly dependable on microalgal species, the application of suitable wavelengths of visible light is an efficient strategy to improve microalgal growth (Zhong *et al*. 2018). Light-emitting diode (LED) grow lights with continuous light spectra are being progressively used in the production of plants and seedlings (*e.g*. Bantis *et al*. 2016, Smirnakou *et al*. 2017) and microalgae (Lippi *et al*. 2018), and they could have different spectra for improvement of vegetative growth or vernalization. Most studies with LED and microalgae growth are done with monochromatic LED or a two-color mix LED grow light spectra (Schulze *et al*. 2014, 2016, Sun *et al*. 2018, Zhong *et al*. 2018) that could ignore several important light energy areas of the spectrum as compared to a continuous light spectrum. To my knowledge, there are no studies that compared mono- or dichromatic LED grow light spectra to a continuous LED grow light spectrum in microalgae cultivation.

Other factors affecting microalgal growth (quantity) and biochemical composition (quality) are temperature, pH, salinity, and nutrient composition of media (*i.e*. nitrogen and phosphorus concentration and their ratio N:P) (Mayers *et al*. 2014, Bartley *et al*. 2016).

The selection criteria for microalgae species as an aquaculture feed are an acceptable size for ingestion, non-toxicity, digestible cell wall, and diversity and suitability of their nutritional quality (in particular, of lipids and proteins) (Tibbetts 2018, Dourou *et al*. 2020). Lipids are a source of essential fatty acids (especially ω-3 and ω-6) that are necessary for the integrity and maintenance of cellular membranes and are required for optimal development of fish, zooplankton, and bivalves (De Pauw *et al*. 1984, von Elert 2002, Arts *et al*. 2009, Marshall *et al*. 2010). As animals are unable to synthesize *de novo* fatty acids of the ω-6 and ω-3 series, these fatty acids must be supplied in an available form within the diet (Bézard *et al*. 1994). Proteins are a source of amino acids that animal cannot synthesize *de novo* (essential amino acids) and of amino acids that animal can synthetize *de novo* (non-essential amino acids) (Wu & Morris 1998). The lack of essential amino acids negatively affects the growth and reproduction of fish and zooplankton (Kreeger *et al*. 1996, Conceição *et al*. 2003, Koch *et al*. 2009). Therefore, it is important to study the effect of RAS WW on the composition of fatty acids and amino acids in microalgae within IMTA as

microalgal biochemical composition plays a central role in the growth and reproduction of its consumer.

The biological contamination of microalgal cultures, *i.e.* the presence of other microorganisms (*e.g.* bacteria, protozoa, fungi, zooplankton, non-target microalgae), might hamper the microalgal bioremediation of aquaculture/RAS WW as it can reduce the microalgal growth, nutrient removal efficiency, and biomass yield (De Pauw *et al.* 1984, Day *et al.* 2017, Lam *et al.* 2017). It has been demonstrated that protozoa can cause a decline in microalgal productivity in unsterilized RAS WW compared to sterilized RAS WW, although, this effect depends on microalgal species (Tejido-Nuñez *et al.* 2019). Moreover, protozoa can promote the growth of bacteria by releasing dissolved organic carbon (Hygum *et al.* 1997) and some bacteria can improve microalgal growth by mineralizing organic matter (Bell 1983, Gantar *et al.* 2008, Thi *et al.* 2010). Consequently, the effect of the presence of biological contaminants in RAS WW on microalgal quantity and quality requires further investigation as maintenance of axenic (monocultures without other organisms) large-scale microalgae cultures is not practical nor cost-efficient. Most studies on microalgal nutritional quality have been done with axenic cultures (Halfhide *et al.* 2014) and the knowledge is lacking on the nutritional quality of microalgae grown in aquaculture/RAS WW.

1.3 Microalgae–*Daphnia* bioremediation system

Harvesting of microalgae biomass is a major challenge of using microalgae for bioremediation of aquaculture WW as mechanical and chemical microalgal harvesting can constitute up to 90 % of the total investments and is predominantly suitable for high-value products (Grima *et al.* 2003, Lavrinovičs and Juhna 2018). Despite successful utilization of microalgae as an aquaculture feed for small fish larvae, crustaceans, and mollusks (Brown *et al.* 1997, Muller-Feuga 2000), the poor digestibility of microalgal nutrients in some fish species (Shah *et al.* 2018), and their small cell size confine their utilization mostly for larval stages of fish (Benemann 1992, Shah *et al.* 2018). Biological harvesting by filter-feeding organisms (*e.g.* zooplankton and mussels) and using their biomass as an aquaculture feed could therefore mitigate the constraints of microalgal bioremediation of aquaculture/RAS WW caused by the challenging harvesting procedures and by microalgae size and digestibility. The microalgae–zooplankton bioremediation system of aquaculture/RAS WW can diminish additional energy costs in colder climates (Holdt and Edwards 2014) as zooplankton can be used later as a live feed for crustaceans and fish (Koivisto 1995, Borowitzka 1997, Brown *et al.* 1997). The microalgae–zooplankton bioremediation system can remove up to 68 % of total nitrogen and 56–67 % of total phosphorus (Kim *et al.* 2003, Jung *et al.* 2009), has a low additional operation and installation costs, has little maintenance requirements, and it can sustain a wide range of fluctuations in nutrient concentration and

physicochemical conditions of WW (Kibria *et al*. 1999, Shiny *et al*. 2005, Chang *et al*. 2014). Additionally, the negative effect of colder climate on the cost-efficiency of microalgae-filter-feeder systems can be further decreased by selecting filter-feeder species that naturally inhabit colder water bodies. Furthermore, to avoid a possible introduction of exotic species into natural aquatic ecosystems, it is important to select native species of microalgae and filter-feeders.

One of the main goals of today's development in the aquaculture sector is the improvement of mass production methods for obtaining natural and live feed for larval and juvenile fish, crustaceans, and mollusks with a steady supply of adequate quantities (Das *et al.* 2012, Cheban *et al*. 2018). Live aquaculture feed organisms include both phytoplankton and zooplankton of which rotifers, cladocerans, and copepods being the most dominant (Das *et al*. 2012, Gogoi *et al*. 2016). Cladocerans are considered a good choice of live feed organisms in aquaculture due to the factors of acceptability by cultivated larvae, nutritional requirements, adequate size, soft body, locomotive behavior, high culture densities, short life cycle, and high reproduction rates (Das *et al*. 2012, Gogoi *et al*. 2016, Cheban *et al*. 2017). Moreover, they are suitable and feasible for mass-scale production due to their economic viability, wide temperature tolerance, adaptation to specific conditions, and ability to prosper on phytoplankton and organic wastes (Das *et al*. 2012, Gogoi *et al*. 2016). Cladocerans are considered an integral part of nutrient cycling and energy transfer in aquatic ecosystems by linking primary producers to higher trophic level consumers (Taipale *et al*. 2016, Peltomaa *et al*. 2017). Two genera of cladocerans, *Daphnia* and *Moina* are the most adapted as living feed in freshwater aquaculture for many cultivated organisms (Das *et al*. 2012, Gogoi *et al*. 2016). In particular, *Daphnia magna* is one of the most well-known live feed organisms that can be used for both juvenile and mature fish to a similar degree as dry feeds (Proulx and de la Noüe 1985, Cheban *et al*. 2018).

Daphnids are mainly non-selective filter feeders that filter on suspended inorganic and organic particles and organisms in the range 0.1 to 70 μm such as fine detritus, nanoplankton, bacteria, phytoplankton, fungi (yeast), protozoan, and micro-metazoa (Burns 1968, Pau *et al*. 2013, Munirasu *et al*. 2016, Nørgaard and Roslev 2016). Therefore, this genus is often used in a WW bioremediation as it effectively clarifies different types of WW, especially *D. magna* as the largest species of the genus (Shiny *et al*. 2005, Pau *et al*. 2013, Serra *et al*. 2014).

Microalgae are the best feed for *D. magna* cultivation in aquaculture as they are easily digested and are the main source of many essential mineral nutrients and biomolecules that zooplankton cannot synthesize (*e.g*. sterols, fatty acids, highly unsaturated fatty acids (HUFA), amino acids, carotenoids) (Das *et al*. 2012, Munirasu *et al*. 2016, Peltomaa *et al*. 2017). Other nutritive substrates present in WW (such as bacteria, yeast, and suspended solids) are shown to be important food supplements but poor substitutes for microalgae as the major food source of *Daphnia* (Antunes *et al*. 2016, Cheban *et al*. 2017). However, it is important to cultivate *Daphnia* in a medium with multiple carbon food sources that may supplement and diversify their diet (*e.g*. microalgae and

bacteria in WW). Single carbon food (*e.g*. unialgal cultures) could lead *Daphnia* populations to fluctuations in survival and occasional culture crashes due to their dependence on that food source (Sterner *et al*. 1993, Antunes *et al*. 2016). Additionally, bacteria in microalgal cultures can convert organic forms of nutrients into dissolved inorganic forms thereby improving the overall nutrient removal rates in WW (Jung *et al*. 2009).

The culture medium in the microalgae–*Daphnia* bioremediation WW system has to sustain all requirements of both organisms, microalgae and *Daphnia* (Proulx and de la Noüe 1985). The activity of *Daphnia* (growth, filter-feeding, movement speed and trajectories, metabolism, reproduction, and survival) diminishes when subjected to unfavorable environmental conditions such as high or low temperature, seasonal changes, hypoxia, shortages of space, lack of food quality and quantity, and others (Jung *et al.* 2009, Leung 2009, Serra *et al.* 2014). The physicochemical conditions of the culture medium are of extreme importance to successful mass production of *Daphnia* as they are very sensitive to emerging contaminants (especially to pharmaceuticals, pesticides, heavy metals, microplastic), high levels of dissolved nutrients (> 35, > 6, > 250, and > 50 mg l^{-1} of NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}, respectively), and high chemical oxygen demand (> 250 mg l^{-1} of COD) (Maceda-Veiga *et al*. 2015, Serra *et al*. 2019, Pous *et al*. 2020). RAS WW generally contains much lower concentrations of these nutrients and COD (< 0.4, < 0.3, < 133, < 6 mg l^{-1}, and < 40 of NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}, and COD, respectively) (Piedrahita 2003, Bregnballe 2015, Rojas-Tirado *et al*. 2017), and these concentrations are further decreased after microalgal bioremediation. Therefore, RAS WW might be inexpensive and abundant media that sustain all requirements of both organisms as shown in previous studies for microalgae (*e.g*. Cheban *et al*. 2015) and for both organisms in the combined system (Khudyi *et al*. 2016, Cheban *et al*. 2017, 2018).

In applying microalgae–*Daphnia* bioremediation in Nordic RAS conditions (*i.e.* in boreal climate as in Nordic countries), it should be considered that temperatures below 20 °C can negatively affect the filtration efficiency of *D. magna* (McMahon and Rigler 1965). However, temperatures below 20 °C could also increase their general filtration efficiency, as larger body size and higher longevity of *Daphnia* induced by the low temperature could lead to higher clearance and ingestion rates (Sodré and Bozelli 2019). Additionally, the efficiency of the microalgae–*Daphnia* bioremediation system is probably affected by the species of microalga and the density of *Daphnia* as the negative relationship between *Daphnia*'s crowding and their filtration efficiency has been demonstrated previously (McMahon and Rigler 1965, Matveev 1993, Marzetz *et al*. 2017). Maintaining an optimal *Daphnia* density could therefore enhance the efficiency of the microalgae–*Daphnia* bioremediation system.

Accordingly, the combination of carefully selected microalgae with *D. magna* might support an efficient bioremediation system of cold-water RAS WW and could mitigate the total production expenses of RAS and ensure its sustainability. The efficiency of microalgae–*Daphnia* bioremediation system has been demonstrated for municipal WW (Proulx and de la Noüe 1985, Kim *et al*.

2003, Jung *et al.* 2009), nevertheless, there is a lack of knowledge for its application in RAS WW (Khudyi *et al*. 2016, Cheban *et al*. 2017, 2018).

1.4 Aims of the study

The main goal of this thesis was to find a suitable microalgae species for an efficient bioremediation system of RAS effluent with waterfleas (*Daphnia magna*) specifically in Nordic RAS conditions (*ca*. 17 °C). I tested this by examining: (1) microalgae species inhabiting Nordic freshwaters for their capacity of bioremediation and biomass production in unfiltered RAS WW by using LED grow lights (I); (2) the effect of RAS WW filtration on the nutritional quality of produced biomass of tested green microalgae (II); and (3) the efficiency of the biological harvest of tested green microalgae from unfiltered RAS WW by *D. magna* (III).

The specific hypotheses of this thesis were:

1) The selected microalgae species inhabiting the boreal climate will grow and remove dissolved nutrients from unfiltered/filtered RAS WW at 17 °C (I, II, III).
2) LED lights with continuous spectra designed to improve different growth phases in plants (growth or vernalization) will differ in their effects on growth, biomass production, and nutrient removal of microalgae (I).
3) RAS WW supports the growth of microalgae and the growth of *D. magna* fed with these microalgae equally well as when cultivated in a reference algal growth medium (I, III).
4) Filtering RAS WW improves the growth, biomass production, nutrient removal, and nutritional quality of microalgae as compared to unfiltered WW, as biological contaminants can negatively affect microalgae (II).
5) The different examined microalgae species will differ in their suitability to be used in bioremediation based on their: (a) growth, (b) removal of dissolved nutrients from RAS WW, (c) nutritional quality, (d) effect on growth of *D. magna*, (e) effect on filtration efficiency of *D. magna*, and (f) effect on *D. magna* to re-release nutrients to WW (I, II, III).

2 MATERIALS AND METHODS

2.1 Wastewater characteristics

In all studies, wastewater (WW) was used from RAS with whitefish (*Coregonus lavaretus*) fed with Circuit Silver Opti 1.7/2.5/3.5 dry feed according to the manufacturer (Raisio aqua, Finland). In I and III, WW was used from the laboratory-scale (total volume *ca*. 1000 l) RAS at the University of Jyväskylä, Department of Biological and Environmental Science maintained at *ca*. 17 °C. However, in II WW was used from the experimental fish farm RAS at the Natural Resources Institute Finland (LUKE) Laukaa maintained at *ca*. 15 °C (Pulkkinen *et al*. 2018).

2.2 Microalgae strains and cultivation

Freshwater microalgal strains were obtained from culture collections, except for *Acutodesmus* sp. (I), and all stock monocultures were maintained in 650 ml plastic tissue culture flasks containing 400 ml of Modified Wright's Cryptophyte (MWC) medium, based on Guillard and Lorenzen (1972). All stock cultures were in the cultivation room at *ca*. 17 °C and were illuminated with a fluorescent light under a 12:12 h light:dark regime with approximately 50–70 µmol photon m^{-2} s^{-1} of light intensity. In all studies, Valoya LED grow lights (18 W, L-series T8 tubes, Valoya Oy, Finland) were used that emit a continuous spectrum based on various percentages of ultraviolet, blue, green, red, far-red and, infra-red wavelengths (I). Except in I where three different light spectra (Valoya product codes AP67, G2, AP673L) were used, only AP67 spectrum was used in II and III for cultivating microalgae. In III, two different life stages of

Haematococcus pluvialis (HP) were tested, "green vegetative phase" and "red astaxanthin accumulating encysted phase" (Shah *et al*. 2016).

2.3 Waterflea cultivation

In III, waterflea *Daphnia magna* (Daphniidae, Cladocera) hatched from resting eggs (ephippia) as instructed by the manufacturer (Daphtoxkit F magna™, Aboatox, Finland) were kept in 250 ml jars with the artificial freshwater (modified AdaM medium) at *ca*. 17°C under fluorescent lights (50–80 µmol m^{-2} s^{-1}, 24:00 light:dark). Waterfleas were maintained at densities of 100–200 *Daphnia* l^{-1} and fed with green microalga *Acutodesmus* sp. every other day with a daily ration of *ca*. 0.7–2.8 mg C $individual^{-1}$.

2.4 Experimental set-up

In I, growth and nutrient uptake of 10 microalgae strains (6 green and 4 non-green) were assessed in unfiltered RAS WW in comparison with a reference medium (MWC) (Table 2: I, experiments 1a,b). Additionally, the growth and nutrient uptake were assessed under three different LED grow light spectra in unfiltered RAS WW for four green microalgae that were selected for their growth properties in the previous experiment (Table 2: I, experiments 2a,b).

In II, the nutrient removal and biochemical quality of three green microalgae were assessed in unfiltered RAS WW in comparison with RAS WW filtered through 0.45 µm syringe filters (Corning, Sigma-Aldrich, USA).

In III, the growth efficiency of *Daphnia* neonates was assessed with four green microalgae cultivated in two different media: filtered RAS WW and a reference medium (MWC) (Table 2: III, experiment 1). RAS WW was filtered through GF/A filter paper (1.6 µm, Ø 47 mm, Whatman). All four microalgae were inoculated with the same total cell volume of 16.1×10^6 μm^3 ml^{-1}. The efficiency of *Daphnia* on the removal of microalgae cultivated in RAS WW was assessed in series of three separate experiments (Table 2: III, experiments 2a,b,c). In the first two experiments, the effect of *Daphnia* density on their filtration and removal efficiency was tested when feeding on different microalgal diets in small volume, and these two experiments differed in duration and number of tested microalgal species. In the third experiment, the filtration and removal efficiency of the *Daphnia* was tested using a larger volume and a longer duration with the best performing *Daphnia* density found in the two previous experiments. Finally, the change of nutrient concentrations in WW was assessed after *Daphnia* filtration by using the same density of *Daphnia* adults and the same volume as in the third filtration efficiency experiment, but with two different microalgal diets (Table 2: III, experiment 3).

2.5 Methods of analysis and calculations

Culture media was analyzed for nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) with a spectrophotometric method. All media samples were pre-filtered to separate the microalgae and suspended solids with 0.22 µm syringe filter before each nutrient analysis (Table 2). Measurements of media pH were done at the beginning and end of each experiment. The nutrient removal rate (R_i, mg l^{-1} d^{-1}) of the substrate *i* (NO_3-N or PO_4-P) was determined according to Wang and Lan (2011) (I, II).

Microalgal cell density was measured by cell count from two replicate samples in a haemocytometer chamber (Bürker) with 100x magnification under the microscope. The total cell volume was determined by using CASY Electronic Cell Counter and Analyzer (OLS-OMNI Life Science, Germany). Microalgal specific growth rate (*SGR*) per day (d^{-1}) was calculated from the change in the cell concentration between the start and end of the experiments according to Andersen (2005).

The fatty acid composition was analyzed by doing a total lipid extraction and fatty acid methyl ester (FAME) formation according to Taipale *et al*. (2015). Only fatty acids contributing more than 0.5 % of the total content were analyzed and quantified as their content (µg mg^{-1} DW g^{-1}) and as their proportions (%) according to Taipale *et al*. (2015). Analyzed fatty acids were grouped into saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total ω-3 fatty acids (ω-3 PUFA), and total ω-6 fatty acids (ω-6 PUFA). Moreover, the ratios of ω-3 to ω-6 (ω-3:ω-6), unsaturated to saturated fatty acids (UFA/SFA), and the sum of all fatty acids were calculated. The amino acid composition was analyzed by chromatographic separation and their following identification and quantification according to Taipale *et al*. (2019). Amino acids were quantified as their content (µg mg^{-1} DW g^{-1}), as their proportions (%), and as the sum of all amino acids.

Daphnia's juvenile growth rate (*JGR*) was estimated from the change in the dry weight during the experiment (III) and calculated according to Lampert and Trubetskova (1996). The dry weights were determined at the beginning of the experiment from a random sample of 20 juveniles hatched in the last 24 h period and from pooled samples from each jar separately at the end of the experiment. *Daphnia* were placed in pre-weighed tin capsules and dried in an oven (60 °C) for 24 h prior to weighing. In the third experiment of III, *Daphnia*'s dry weight was estimated by pooling six replicates of ten randomly collected *Daphnia* from the stock cultures (0 h) and ten *Daphnia* from each bottle at the end of the experiment (72 h) and then weighed in the same manner as described previously.

The true or theoretical filtering rate of waterfleas, *i.e*. volume of water passing through the waterflea's maxillary filter per unit of time, cannot be measured directly (Frost 1972, Lampert and Sommer 2007). Thus, *Daphnia*'s

clearance rates [*F*, volume of medium cleared of microalgae per unit time per individual of *Daphnia* (ml h^{-1} ind. $^{-1}$)] and ingestion rates [*I*, concentration of microalgae consumed per *Daphnia* per unit of time (*e.g*. from cell density: 10^6 cells h^{-1} ind.$^{-1}$)] were estimated from the change in microalgal concentration (cell density, Chl-a, dry weight, total cell volume) according to Frost (1972). These rates were calculated for the period between the start and end of the experiment in each treatment bottle compared to a mean microalgal concentration of control bottles. Moreover, the relative change (%) of microalgal concentration was calculated between final microalgal concentrations in control and treatment.

2.6 Statistical analysis

Different treatments were analysed by using 1-way or 2-way analysis of variance (ANOVA). The non-independence of observations within each run (asynchronous series of the same experiment) was accounted for by including run identity as a random factor (I, II) and the significance of the effect of the run was evaluated with Likelihood Ratio Test (II).

The assumptions of ANOVA were tested with Levene´s test for homogeneity of variances and Shapiro-Wilk's test for normality (I, II, III). In case the assumptions were not met, Kruskal–Wallis's H non-parametric test was used with Dunn's test for pairwise comparisons with Bonferroni corrections or Welch ANOVA with Games–Howell's test for pairwise comparisons (I, II, III). Moreover, in case of significant deviation from normality and/or homoscedasticity, the parametric test was reported if the statistical significance of the parametric test agreed with a non-parametric test (I, III). The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Statistical analyses were conducted by using IBM SPSS (version 24.0; IBM 2016) software (I, III) and R (RStudio version 3.6.3) with lme4 package (v1.1–21) for mixed effects models and either R base or vegan packages for the rest of the analysis (Oksanen *et al*. 2018, R Core Team 2017) (II).

Permutational analysis of variance (PERMANOVA) with Bray–Curtis distance matrix was used to test if microalgae species or cultivation media were driving dissimilarities on fatty acids and amino acids percentage (%) data, and non-metric multi-dimensional scaling ordinations with Bray–Curtis distance matrix was used to graphically illustrate PERMANOVA results (II). Additionally, a similarity percentage test (SIMPER) was used to determine the components that caused the most differences in PERMANOVA results (II).

TABLE 2 Summary of all experimental set-ups within this thesis. Media: **MWC**, reference algal medium; **WW**, unfiltered RAS wastewater; **FWW**, filtered RAS wastewater. Light spectrum (Valoya product codes): **L1** for AP67, **L2** for G2, **L3** for AP673L. Two life phases of *Haematococcus pluvialis* (HP): **G**, HP in green phase; **R**, HP in red phase; **G+R**, mixture of both HP phases. Replicates: **c**, control; **t**, treatment. ***n.a.***, not applicable. ***n.m.***, not measured. (*), only for HP. (**), 1 day after inoculation. Water characteristic values are mean ± S.D.

PAPER	**I**	**I**	**I**	**I**	**II**	**III**	**III**	**III**	**III**	**III**
Experiment	*1a*	*1b*	*2a*	*2b*	*1*	*1*	*2a*	*2b*	*2c*	*3*
Duration (h)	96	216	96	192	144	96	2	3	48	72
Temperature (˚C)	17±0.5	17±0.5	17±0.5	17±0.5	17±0.3	17±0.3	17±0.3	17±0.3	17±0.3	17±0.3
Microalgal cultivation										
Media	MWC, WW	MWC, WW	WW	WW	WW, FWW	MWC, FWW	FWW	FWW	FWW	FWW
Light intensity (µmol m^{-2} s^{-1})	85–105	55–75	100–120	70–100	70–100	110–130; 80–100*	110–130	110–130; 80–100*	110–130; 80–100*	110–130
LED light spectrum	L1	L1	L1–L3	L1–L3	L1	L1	L1	L1	L1	L1
Photoperiod (light:dark)	24:00	24:00	24:00	12:12	24:00	12:12	12:12	12:12	12:12	12:12
Batch volume (l)	0.4	0.4	0.4	0.27	0.4	0.4; 0.27*	0.4	0.4	5; 1.4*	3
Starting cell density (mean, 10^6 cells ml^{-1})										
Chlamydomonas reinhardtii	0.184**	*n.a.*	0.125	*n.a.*	*n.a.*	0.447	*n.a.*	*n.a.*	*n.a.*	*n.a.*
Haematococcus pluvialis	0.018^{G**}	*n.a.*	*n.a.*	0.020^{G}	0.013^{G}	0.028^{G}	*n.a.*	0.095^{G}; 0.089^{R}	0.020^{G+R}	*n.a.*
Monoraphidium griffithii	0.414**	*n.a.*	0.418	*n.a.*	0.112	0.794	0.293	0.382	0.571	0.863
Selenastrum sp.	0.466**	*n.a.*	0.871	*n.a.*	0.689	0.341	0.756	1.080	2.389	0.873
Daphnia experiments										
Light intensity (µmol m^{-2} s^{-1})	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	50–80	50–80	50–80	0	0
Photoperiod (light:dark)	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	24:00	24:00	24:00	00:24	00:24
Volume (l)	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	0.1	0.1	0.1	3	3
No. of individuals	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	10	10, 20, 30	10, 30	300	300
Starting wastewater characteristics										
NO_3-N (mg l^{-1})	18.1±6.7	18.1±6.7	27.1±8.2	27.1±8.2	97.0±0.6	*n.m.*	*n.m.*	*n.m.*	*n.m.*	6.3±2.8
PO_4-P (mg l^{-1})	0.8±0.3	0.8±0.3	1.3±0.4	1.3±0.4	3.8±0.1	*n.m.*	*n.m.*	*n.m.*	*n.m.*	0.3±0.1
N:P molar ratio	50.3±15.2	50.3±15.2	45.5±9.7	45.5±9.7	56.5±1.0	*n.m.*	*n.m.*	*n.m.*	*n.m.*	5.9±1.5
NO_2-N (mg l^{-1})	0.05±0.03	0.05±0.03	0.10±0.01	0.10±0.01	0.04±0.01	*n.m.*	*n.m.*	*n.m.*	*n.m.*	*n.m.*
NH_4-N (mg l^{-1})	0.06±0.01	0.06±0.01	0.12±0.02	0.12±0.02	0.04±0.02	*n.m.*	*n.m.*	*n.m.*	*n.m.*	*n.m.*
pH	7.1±0.4	7.1±0.4	7.3±0.3	7.3±0.3	7.4±0.2	6.7±0.3	*n.m.*	*n.m.*	*n.m.*	7.4±0.3
Dissolved oxygen (mg l^{-1})	9.1±0.2	9.1±0.2	9.7±0.9	9.7±0.9	*n.m.*	10.4±0.0	*n.m.*	*n.m.*	*n.m.*	*n.m.*
Conductivity (µS cm^{-1})	308±55	308±55	353±62	353±62	*n.m.*	285±2	*n.m.*	*n.m.*	*n.m.*	*n.m.*
No. of experimental runs	3	3	3	1	2	1	1	1	1	1
No. of replicates per treatment	3	3	3	3	4	5	4c+4t	3c+3t	3c+4t	2c+3t

3 RESULTS AND DISCUSSION

3.1 Recirculating aquaculture system wastewater as cultivation media for microalgae–*Daphnia* bioremediation system (I, II, III)

The Nordic RAS WW was shown to be an efficient cultivation media for the microalgae–*Daphnia* bioremediation system. Firstly, unfiltered RAS WW supported the microalgal growth and nutrient removal to a similar extent as the reference algal medium (MWC) (I), which is in agreement with previous studies using RAS WW and a reference algal medium (Sirakov and Velichkova 2014, Cheban *et al.* 2015). Secondly, green microalgae *Monoraphidium griffithii* (MG) and *Selenastrum* sp. (SE) cultivated in filtered RAS WW supported the juvenile growth of *Daphnia* to a similar extent as when MWC was used as a culture medium (III). Microalgae cultivated in MWC induced higher growth of *Daphnia* than in WW only when using *Chlamydomonas reinhardtii* (CR) and *Haematococcus pluvialis* (HP) (III). The reason for lower *Daphnia* growth when fed with CR and HP cultivated in WW than in MWC could be a possible P-limitation in WW as RAS WW had half the P-content of that in MWC. This finding is in line with previous studies reporting the lower growth of *Daphnia* with a P-deficient diet than with a P-sufficient diet (van Donk *et al*. 1997). This suggests that the biochemical quality of CR and HP might be more altered by the media quality than that of MG and SE.

In addition to supporting the microalgae–*Daphnia* bioremediation system as efficiently as the reference media, raw (unfiltered) RAS WW supported microalgal growth and bioremediation efficiency also equally well as filtered RAS WW (II). This finding suggests that Nordic RAS WW does not have to go through an expensive removal of indigenous biological contaminants (bacteria and protozoa) to sustain an efficient microalgae–*Daphnia* bioremediation system. The lack of a notable negative effect of biological contaminants within RAS WW on microalgal growth and nutrient removal has been reported also

previously (Halfhide *et al*. 2014, Tejido-Nuñez *et al*. 2019, 2020). However, these studies demonstrated a species-specific effect of WW filtration on microalgal growth and bioremediation efficiency after microalgae reached the stationary phase (Halfhide *et al*. 2014, Tejido-Nuñez *et al*. 2019).

Additionally, biological contaminants within RAS WW did not significantly affect the microalgal nutritional quality, *i.e*. amino acid and fatty acid composition (II), which are important for the growth and filtration efficiency of *Daphnia* within the microalgae–*Daphnia* bioremediation system (Peltomaa *et al*. 2017). The RAS WW filtration had a positive effect only on ω-3 and ω-6 fatty acid content and chlorophyll-a concentration in one tested microalga (HP) (II). This species-specific effect of indigenous biological contamination in RAS WW suggests that by a careful selection of microalgae species the efficiency of the microalgae–*Daphnia* bioremediation system can be improved. Furthermore, microalgae cultivated in unfiltered RAS WW had higher amino acid content when using the other two microalgae (MG and SE), possibly due to the presence of bacteria (II). As the higher amino acid content of microalgae is beneficial for *Daphnia* (Peltomaa *et al*. 2017), this finding further supports the use of Nordic RAS WW without costly filtration for the microalgae–*Daphnia* bioremediation system.

3.2 Efficiency of microalgae–*Daphnia* bioremediation system varies among different microalgae species (I, II, III)

Green microalgae were shown to be more efficient than non-green microalgae for the Nordic RAS WW bioremediation. Green microalgae had more than 10 times higher specific growth rates (*SGR*) and nitrate (R_N) and phosphate removal rates (R_P) (0.54–0.88 d^{-1}, 2.35–4.32 mg d^{-1}, 0.15–0.49 mg d^{-1}, respectively) than non-green microalgae (< 0.16 d^{-1}, < 0.49 mg d^{-1}, < 0.07 mg d^{-1}, respectively). The reason for the poor growth of non-green microalgae in either media could not be elucidated in the current experiments. Even though results demonstrated the efficient growth and nutrient removal of all tested green microalgae in RAS WW, not all tested microalgae are equally efficient in the microalgae–*Daphnia* bioremediation system in every respect, and the ranking of these microalgae may change depending on the wanted outcome of the system. Despite having had high growth and nutrient removal, microalgae *Acutodesmus* sp. *Scenedesmus obliquus* were excluded from the further study as *Acutodesmus* sp. culture was found to be contaminated and *S. obliquus* cells grew deformed and aggregated in RAS WW which is not suitable for feeding *Daphnia*.

In these experiments, microalga MG was shown to be the most suitable species of the four tested green microalgae for the treatment of Nordic RAS WW, as it had higher *SGR* than microalgae HP and SE in both unfiltered and filtered WW (II), higher R_N than HP (I, exp. 1) and SE (I, exp. 1 and 2), and higher R_P than HP (II, in both unfiltered and filtered WW) and CR (I, exp. 2)

(Tables 3, 4). Moreover, MG had a higher ω-3:ω-6 ratio than HP (II), suggesting that MG has higher fatty acid nutritional value than HP as ω-3:ω-6 ratio is a proxy of fatty acid nutritional value for *Daphnia* (Taipale *et al*. 2015). Filtration of RAS WW did not affect the growth, nutrient removal, and nutritional quality of MG (II), and MG cultivated in filtered RAS WW supported the juvenile growth of *Daphnia* equally well as when cultivated in MWC (III). Even though MG was removed the least efficiently by *Daphnia* in experiments with shorter duration and smaller volume (III), *Daphnia* tended to remove MG more efficiently than HP and SE in experiments with longer duration and larger volume (III) which are more relevant for a large-scale microalgae–*Daphnia* bioremediation system. The possible explanation for contrasting results between filtration efficiency experiments might be the decrease in microalgal concentration during the longer experimental duration with a subsequent effect on filtration rates. Another explanation for this contrasting finding could be the difference in the number of neighboring *Daphnia* individuals that can affect filtration efficiency more than an actual density of *Daphnia* (Ban *et al*. 2008). Additionally, conducting *Daphnia* filtration experiments in larger volumes of medium reduces differences caused by variability in individual filtration efficiency and from wall-avoidance behavioral artifacts (Peters 1984, Helgen 1987). Finally, *Daphnia* did not re-release nutrients back into RAS WW after feeding on MG (III), which is crucial for the overall nutrient removal from RAS WW. These results suggest that MG is the most suitable microalga of the four tested species for the microalgae–*Daphnia* bioremediation system of Nordic RAS WW.

Microalga CR had higher *SGR* than MG (I, exp. 2) and higher R_N than HP (I, exp. 1), MG (I, exp. 1), and SE (I, exp. 1 and 2) (Tables 3, 4). However, CR cultivated in RAS WW produced lower juvenile growth of *Daphnia* than microalgae HP, MG, and SE (III). Since CR cultivated in MWC induced similar *Daphnia*'s growth as MG and SE, and WW was more P-deficient medium than MWC, one possible explanation for its lower quality for *Daphnia* is that CR developed a thicker cell wall in P-deficient medium as shown previously for this microalgae (van Donk *et al*. 1997). Consequently, CR is the least suitable of the four tested species for the microalgae–*Daphnia* bioremediation system of Nordic RAS WW. It has also been shown previously that CR had lower concentrations of essential biochemical compounds (sterols, ω-3 fatty acids, amino acids) than MG and SE cultivated in MWC (Peltomaa *et al*. 2017).

Microalga HP was not shown to be among the most suitable species for the treatment of Nordic RAS WW as it had inferior growth and nutrient removal rates than other species (I, II), except for a higher R_N than that of MG in filtered RAS WW (II) (Tables 3, 4). However, results of nutritional quality suggest that HP is a suitable species for microalgae–*Daphnia* bioremediation system due to the higher content of essential and non-essential amino acids than MG and SE in both unfiltered and filtered RAS WW (II), which are fundamental for *Daphnia* (Peltomaa *et al*. 2017). Only HP included arachidonic acid (ARA, 20:4ω-6) and eicosapentaenoic acid (EPA, 20:5ω-3) in its biochemical profile when cultivated in both unfiltered and filtered RAS WW (II), and these

fatty acid are important for the survival, growth, and reproduction of *Daphnia* (Peltomaa *et al*. 2017). HP was also the only tested microalga which nutritional quality (ω-3 and ω-6 fatty acid content and chlorophyll-a concentration) was improved by the filtration of RAS WW (II), and the only species which induced higher juvenile growth of *Daphnia* than other species when cultivated in MWC (III). These results suggest that improvement of Nordic RAS WW by filtration or by optimization of physical quality could improve the microalgae–*Daphnia* bioremediation system when using HP.

Based on its growth and nutrient removal, microalga SE was among the least efficient species for the bioremediation of Nordic RAS WW (Tables 3, 4). However, SE could be used for microalgae–*Daphnia* bioremediation system as: (1) filtration of RAS WW does not affect its growth, nutrient removal, and nutritional quality (II); (2) it had higher ω-3:ω-6 ratio, thus higher fatty acid nutritional quality, than HP (II); (3) its cultivation in filtered RAS WW induced the same *Daphnia*'s juvenile growth as in MWC (III); and (4) it was removed with the highest *Daphnia*'s filtration efficiency in experiments with shorter duration and smaller volume (III). In experiments with longer duration and larger volume, SE was removed with the lowest filtration efficiency (III). In addition to having lower bioremediation efficiency than other species, SE was observed to induce the re-release of PO_4-P back into WW after *Daphnia*'s feeding (III) which further reduces its utility as part of the microalgae–*Daphnia* bioremediation system. In addition to the excretion process, *Daphnia* can release nutrients back to water due to the breakage of microalgal cells during their feeding process. It is possible that P-increase was recorded only with microalga SE but not in MG due to the higher P content in SE than in MG (Peltomaa *et al*. 2017) and due to the more extensive *Daphnia*'s removal of SE (–87.8 %) than of MG (–51.4 %) (III), hence processes of excretion and microalgal breakage could have been more intensive with SE diet. Microalga SE is an acceptable but perhaps not the most suitable species to be used in the microalgae–*Daphnia* bioremediation system of Nordic RAS WW.

TABLE 3 Summary of average (±S.D.) specific growth rates and removal rates of NO_3-N and PO_4-P with four microalgae cultivated in unfiltered RAS wastewater. All rates were measured for a 4-day cultivation period, except rates noted with (*) measured for 6-day and rates noted with (**) measured for 8-day cultivation period. *n.a.*, not applicable.

Paper–Experiment	I–1	I–2	II
Specific growth rate (d^{-1})			
Chlamydomonas reinhardtii	0.78±0.14	0.70±0.11	*n.a.*
Haematococcus pluvialis	0.54±0.09	0.37±0.02**	0.47±0.06*
Monoraphidium griffithii	0.58±0.10	0.51±0.15	0.61±0.03*
Selenastrum sp.	0.78±0.01	0.61±0.04	0.50±0.01*
NO_3-N removal rate (mg d^{-1})			
Chlamydomonas reinhardtii	4.32±1.60	4.62±0.87	*n.a.*
Haematococcus pluvialis	2.67±1.11	2.75±0.05**	4.06±0.60*
Monoraphidium griffithii	3.45±0.81	4.71±1.38	2.94±0.59*
Selenastrum sp.	2.35±0.45	4.08±0.99	3.84±0.85*
PO_4-P removal rate (mg d^{-1})			
Chlamydomonas reinhardtii	0.15±0.05	0.18±0.05	*n.a.*
Haematococcus pluvialis	0.19±0.09	0.19±0.01**	0.49±0.12*
Monoraphidium griffithii	0.19±0.09	0.28±0.10	0.64±0.01*
Selenastrum sp.	0.19±0.08	0.28±0.10	0.64±0.01*

TABLE 4 Features of microalgae in respect to each other**.** *Chlamydomonas reinhardtii* (CR), *Haematococcus pluvialis* (HP), *Monoraphidium griffithii* (MG), *Selenastrum* sp. (SE). – = not good; + = good; ++ = very good. *n.a.*, not applicable.

	CR	HP	MG	SE
Growth	++	+	++	+
Nutrient removal	++	+	++	+
Nutritional quality				
Fatty acids	*n.a.*	+	++	++
Amino acids	*n.a.*	++	+	+
Daphnia growth				
Filtered wastewater	–	+	+	+
Daphnia filtration efficiency				
Smaller volume & shorter period	*n.a.*	+	+	++
Larger volume & longer period	*n.a.*	+	++	–
Daphnia's PO_4-P re-release	*n.a.*	*n.a.*	+	–

3.3 Recommendations for microalgae–*Daphnia* bioremediation system in Nordic recirculating aquaculture system wastewater (I, II, III)

The two main environmental factors that affect the efficiency of the microalgae–*Daphnia* bioremediation system, temperature and light (intensity and photoperiod), are the two main limiting environmental factors in northern latitudes (Cheregi *et al*. 2019, Oostlander *et al*. 2020). Thus, it is essential to optimize temperature and light conditions when applying this system in Nordic RAS WW as temperatures below 20 °C negatively affect both microalgae and *Daphnia* (McMahon and Rigler 1965, Cheregi *et al*. 2019). Maintaining higher

temperatures and longer artificial illumination substantially increases the costs of running the microalgae–*Daphnia* bioremediation system (Oostlander *et al*. 2020). The careful selection of microalgae and *Daphnia* species that thrive in lower temperatures (below 20 °C) and under cost-efficient artificial illumination (*e.g*. LED) could therefore mitigate the costs of microalgae-*Daphnia* bioremediation system in Nordic RAS WW.

The results in this thesis (I, II) are mostly in line with previous studies regarding microalgal growth and bioremediation efficiency in WW after 4 days conducted at temperatures above 20 °C (*e.g*. HP: Haque *et al*. 2017; *Monoraphidium*: Jiang *et al*. 2016; SE: Zhao *et al*. 2016). Previous studies conducted at 20 °C also reported a similar range of growth rates for *Daphnia* fed on microalgae species from the same families as the tested microalgae in this thesis (III) (Tessier and Goulden 1987, Mitchell *et al*. 1992, DeMott 2003, Marzetz *et al*. 2017). The clearance and ingestion rates in this thesis are within the reported range for *D. magna* fed with other green microalgae cultivated at *ca*. 20 °C (Ryther 1954, McMahon and Rigler 1965, Porter *et al*. 1982). As the results of the current experiments being comparable with those reported from higher temperatures, they show that a microalgae–*Daphnia* bioremediation system can work efficiently at a lower temperature of 17 °C in Nordic RAS WW.

Despite high investment cost, LED is the most suitable type of artificial light for the microalgae–*Daphnia* bioremediation system in Nordic RAS WW because it has higher energy efficiency, longer lifespan, lower maintenance costs, and higher light quality (combined effect of intensity and photoperiod) than other types of artificial light (Khan and Abas 2011, Glemser *et al*. 2016). Moreover, LEDs can be adjusted to meet the requirements of different microalgae species by designing them to generate a specific part of the entire spectrum of visible light colors (Dayani *et al*. 2016). The use of LED grow lights emitting a continuous spectrum with different percentages of wavelengths were supported in this thesis for cultivating green microalgae in Nordic RAS WW, and all three tested light spectra sustained efficient growth and bioremediation with microalgae CR, MG, and SE (I). This finding implies that green microalgae are not very sensitive to changes in the light spectrum, although the longer cultivation periods could reveal the effect of different light spectra on green microalgae. The effect of different LED grow light spectra was observed only on growth and bioremediation efficiency of microalga HP, where one of the tested spectra (Valoya product code G2) induced the highest R_N and R_P (I). It is possible that HP is the most sensitive microalga among tested green microalgae to different light conditions as stress caused by improper illumination (high intensity and long photoperiod) induces the production of carotenoid pigments (astaxanthin) (Kobayashi *et al.* 1992, Hu *et al*. 2020). G2 spectrum is made to enhance the vernalization process in plants and might have caused the highest bioremediation efficiency in HP by causing the cells to enter faster into the encystment (red) phase with bigger cells that can absorb more nutrients per cell. This explanation is further supported by the finding that spectrum G2 induced the lowest chlorophyll-a concentration of HP due to the less vegetative (green) phase cells and more red phase cells (I).

The findings in this thesis suggest that it is not necessary to maintain the maximum density of *Daphnia* for the efficient harvest of microalgae thereby supporting the periodic harvesting of *Daphnia* for the production of fish feed. With all tested microalgae species, the lowest tested *Daphnia*'s density (100 ind. l^{-1}) had the highest filtration efficiency and similar relative changes of removing microalgae as higher densities of *Daphnia* (III). Previous studies have also reported the negative effect of crowding on the filtration efficiency of *D. magna* (Clément and Zaid 2004, Nørgaard and Roslev 2016) and other *Daphnia* species (Helgen 1987, Matveev 1993, Ban *et al*. 2008). As the food concentration was not a limiting factor in the experiments conducted in this thesis, the negative effect of crowding on *Daphnia*'s filtration efficiency was probably caused by the chemically mediated cue or by the physical interference of direct contact among the individuals (Goser and Ratte 1994).

Previous studies recommended the use of several different methods to evaluate *Daphnia*'s filtration efficiency in order to avoid conversion between different units and to overcome the biases from the difference in cell size of microalgae (Peters 1984, Kiørboe *et al*. 1985). Therefore, this thesis evaluated if four different evaluation methods of microalgal concentration (change in cell density, Chl-a concentration, dry weight, and total cell volume) produced similar results of *Daphnia*'s efficiency to harvest microalgae from RAS WW (III). *Daphnia*'s clearance rates and relative changes obtained when using the change in cell density were different from the results obtained when using the change in the proxies of biomass (Chl-a, dry weight, and total cell volume) that were in agreement with each other. The presence of microbes in WW and/or breakage of microalgal cells can underestimate the removal rates when proxies of biomass are used. Measuring total cell volume is perhaps the most accurate tested method as it was the only method that produced the same ranking of microalgae (highest with microalga MG, followed by HP, and lowest with SE) for all three parameters (clearance rate, ingestion rate, and relative change). Consequently, using total cell volume together with cell density may provide more accurate estimates of *Daphnia*'s filtration efficiency and may bypass the underestimations and limitations of these two methods.

4 CONCLUSIONS

The results in this thesis are encouraging for the development of a microalgae–*Daphnia* bioremediation system in Nordic RAS effluent (*ca.* 17 °C) for preventing the loss of valuable dissolved nutrients into the surrounding aquatic ecosystems by reusing them (I, II, III). Unfiltered RAS WW supported the growth and nutrient removal of tested green microalgae and filtered RAS WW sustained growth of *Daphnia* feeding on MG and SE equally well as when using a reference algal medium (MWC) (I, III). Moreover, the costly filtration of Nordic RAS WW is not necessary for the microalgae–*Daphnia* bioremediation system, as microalgae had similar growth, nutrient removal, and nutritional quality in both unfiltered and filtered RAS WW (II). The only positive effect of the removal of indigenous biological contaminants (bacteria and protozoa) by filtration was the increased nutrient quality of microalga HP. Therefore, the efficiency of the microalgae–*Daphnia* bioremediation system in Nordic RAS WW could be improved by screening and selecting optimal microalgae species for this system. The observed re-release of PO_4-P back to RAS WW after *Daphnia* feeding on microalga SE and not on MG, further emphasizes the importance of the selection of microalgal species utilized in the microalgae–*Daphnia* bioremediation system (III). Consequently, the most suitable microalga of the four tested species for the microalgae–*Daphnia* bioremediation system in Nordic RAS WW was MG, followed by HP, and by SE as the least suitable species (I, II, III). Microalga CR was not found to be an acceptable species for this system (III). Despite not being the most suitable, microalga HP was found to be the only species that offers further improvement of the microalgae–*Daphnia* bioremediation system by optimizing RAS WW (filtration and/or physical quality enhancement) (II, III) and by using LED grow light spectrum G2 (I). The different continuous spectra of LED grow lights designed to improve different growth phases in plants (growth or vernalization) did not cause differences in growth and nutrient removal with MG and SE (I). Furthermore, the periodic harvesting of *Daphnia* is suggested for the efficient harvesting of microalgae that can also increase the aquaculture feed production (III). Lastly, the utilization of more than one method for evaluating microalgal concentration can be used for

improving the accuracy and productivity of the microalgae–*Daphnia* bioremediation system in Nordic RAS WW (III).

The results of this thesis could be applied in the development of environmentally sustainable and cost-efficient IMTA, where green microalgae could treat RAS WW and be used as a feed for filter feeders that can be further used as a natural and live feed for the target fish. Therefore, the results are in line with the concept of the circular economy as it supports the idea of nutrient recirculation (Martins *et al*. 2010). IMTA with microalgae and filter-feeders do not lose valuable nitrogen out of the system, as denitrification filters in RAS do by converting nitrate to gaseous nitrogen (Suhr *et al*. 2013). Moreover, nitrate converted into biomass could be used as an agricultural fertilizer, thereby overcoming the expensive chemical production of nitrogen fertilizers (Gellings and Parmenter 2004). The tested filter feeder in this thesis, *D. magna*, is among the most popular live fish-feed organisms that are comparable to the dry feeds (Proulx and de la Noüe 1985, Cheban *et al*. 2018). Thus, the general costs of RAS production could be reduced by mass cultivation of *D. magna* for producing aquaculture feed. Moreover, the results demonstrate the efficiency of using cost-efficient LED grow lights with a continuous spectrum for producing high microalgal growth, biomass production, and nutrient removal. The results also suggest that the expensive filtration of RAS WW is not necessary nor prerequisite for the successful microalgal cultivation with the high growth, nutrient removal, and nutritional quality. Despite the fact that all experiments were done on a laboratory-scale and in small volumes, the results from this thesis can be applied for the up-scaling of the system. In particular, knowledge of the growth rates of microalgae and *Daphnia* are necessary to predict the biomass generation of these organisms while the estimate of the removal rates of nutrients are needed for predicting the removal time of dissolved nutrients from RAS WW.

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ORIGINAL PAPERS

I

SCREENING OF MICROALGAE AND LED GROW LIGHT SPECTRA FOR EFFECTIVE REMOVAL OF DISSOLVED NUTRIENTS FROM COLD-WATER RECIRCULATING AQUACULTURE SYSTEM (RAS) WASTEWATER

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Screening of microalgae and LED grow light spectra for effective removal of dissolved nutrients from cold-water recirculating aquaculture system (RAS) wastewater

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ABSTRACT

Popularity of recirculating aquaculture systems (RAS) is increasing. Because of the high water recirculation rate, dissolved nutrients originating from fish feed are concentrated enough in RAS wastewater (WW) to enable growth of primary producers, e.g. microalgae. This study evaluated nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) removal efficiency of ten temperate zone freshwater microalgae species during their exponential growth phase in unfiltered RAS WW at 17 ± 0.5 °C. Growth and nutrient uptake efficiency of six green and four non-green microalgae strains were compared between WW and reference growth medium in batch monocultures. The effect of three different LED grow light spectra on growth and nutrient uptake efficiency were compared for four green microalgae in WW. The specific growth rate (SGR, 0.5-0.8 d^{-1}) and removal of NO_3-N (N%, 57–96%) and PO_4-P (P%, 78–94%) of green microalgae in WW in 4 days were comparable to the results obtained in the reference medium (SGR, 0.6-0.9 d^{-1}, N%, 59–99%; P%, 86–99%). In contrast, non-green microalgae had negligible growth (SGR, from -0.1 to 0.2 d^{-1}) and poor nutrient removal (N%, 1–29%; P%, 0–34%) in both growth media after 9 days. The three LED spectra did not differ on their effect on growth and nutrient removal of three green microalgae in WW after 4 days, while the fourth tested species, *Haematoccocus pluvialis,* had its highest nutrient removal after 8 days under a specific LED spectrum. Current results show that RAS WW supports well green microalgae growth in batch cultures in temperatures common in Nordic RAS and that continuous spectrum LED grow lights can induce high removal of dissolved nutrients. Our findings lend support to the concept of using temperate zone microalgae for nutrient removal and recycling from RAS WW.

1. Introduction

Increasing global demand for seafood, depletion of natural fish stocks and increasing concern for the environment have stimulated the development of aquaculture all over the world. The need to increase fish production and to overcome limitations in water quality and quantity, and to reduce waste discharges and environmental impacts, is driving aquaculture industry towards more intensive production, which could lead to reduced use of resources and environmental problems [1]. The main environmental contaminants in aquaculture wastewater (WW) include dissolved or particulate organic matter, suspended solids, nutrients such as nitrogen (N) and phosphorus (P), and specific chemicals [2,3]. Traditional flow-through and net-pen aquaculture discharge effluents into the surrounding aquatic ecosystems, although nutrient removal from aquaculture WW would be essential to protect receiving waters from eutrophication [4,5].

Cultivation of aquatic animals in Recirculating Aquaculture Systems (RAS) significantly reduces water use and WW volume by treating and recycling water, but it also allows improved opportunities for waste management and nutrient recycling [2,6]. In RAS, less than 10% of the total water volume is replaced per day, which is roughly 100 times lower water exchange rate than in the traditional flow-through systems [4,5]. Water reuse in RAS is limited by the accumulation of waste products originating from uneaten feed, feces, and metabolic waste products [2]. Consequently, RAS reduce potential environmental impacts of waste discharge by concentrating nutrients and organic matter but not by overall reduction in discharges [2]. Therefore, high concentration of dissolved nutrients in RAS WW may allow new opportunities to develop technologies for exploiting the waste as a valuable resource (i.e. circular economy), while in traditional farming it is much more challenging as the same amount of nutrients is diluted in a vast water volume. As a drawback, RAS require large amount of energy, and

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Table 1
Freshwater microalgae strains used in the study.

Species	Class (groups)	Order	Strain	Origin
Chlamydomonas reinhardtii	Chlorophycae (green algae)	Chlamydomonadales	K-1016 (NIVA)	Amherst, Massachusetts, USA
Haematococcus pluvialis	Chlorophycae (green algae)	Chlamydomonadales	K-0084 (NIVA)	Trutbådan, Sweden
Acutodesmus sp.	Chlorophycae (green algae)	Sphaeropleales	Unknown	University of Basel
Monoraphidium griffithii	Chlorophycae (green algae)	Sphaeropleales	NIVA-CHL 8	Lake Årungen, Akershus, Norway
Selenastrum sp.	Chlorophycae (green algae)	Sphaeropleales	K-1877 (NIVA)	Lake Iso-Ruuhijärvi, Häme, Finland
Scenedesmus obliquus	Chlorophycae (green algae)	Sphaeropleales	CCAP 276/60	Lake Tuomiojärvi, Jyväskylä, Finland
Cryptomonas curvata	Cryptophycae (cryptophytes)	Cryptomonadales	CCAP 979/28	Unknown
Euglena gracilis	Euglenophycae (euglenoids)	Euglenales	NIVA-1/79	Unknown
Mallomonas caudata	Synurophyceae (golden algae)	Synurales	CCAP 929/8	Lake Musta-Kotinen, Häme, Finland
Synura petersenii	Synurophyceae (golden algae)	Synurales	CCAP 960/3	Priest Pot, Cumbria, England, UK

depending on how the energy is produced, the carbon footprint of the products can increase significantly [7]. The ongoing developments in RAS include technological advancement of filtration systems, ecological improvements of bioremediation and reuse of system's byproducts through integrated multi-trophic aquaculture approach [6].

RAS WW contains generally ten- to hundredfold less dissolved nutrients than e.g. municipal or industrial wastewaters that have been intensively studied for algal biomass production [8]. However, RAS WW provides enough dissolved nutrients to sustain photosynthetic growth of plants within the aquaponics [9], hence RAS contains enough nutrients to support microalgae growth. While methods for using microalgae for removing nutrients from municipal and industrial WW have been established [10,11], there is less knowledge for nutrient removal from RAS WW. Thus, research for RAS WW treatment and nutrient recycling is needed.

Microalgae are one of the most promising organisms for bioremediation of WWs due to their high photosynthetic efficiency, high growth rate, high efficiency in nutrient removal, tolerance on poor water quality, and low water use [12–17]. Microalgae provide a dual application for the aquaculture as they treat WW by assimilating organic/inorganic nutrients and carbon (without any exogenous carbon) while incorporating them into a biomass that is rich in proteins, carbohydrate, pigments, vitamins, and energy reserves in the form of lipids and hydrocarbons [14,18]. Microalgal biomass can further be used as a feedstock to produce animal feed, human food, health products, nutraceuticals, agricultural fertilizers, biofuels, and other biochemical derivatives [3,13,19,20]. In addition to direct sales of commercial species and valuable co-products microalgae-RAS integrated system could offer economic returns through reduction of feeding cost for culture organisms by using the microalgae as a feed and by improvement of WW treatment capacity (Mata et al., 2012; [3]).

The studies that have examined the efficiency of microalgae in the treatment of aquaculture WW [19,21,22] or RAS WW [23–25], have mostly characterized the use of warm-water species (*cf.* [26]). Information on the efficiency of microalgae for nutrient removal at relatively low temperatures (below 20 °C) would be important for development of practical methods for microalgae culture and for avoiding possible introduction of exotic species into natural waters in northern latitudes.

Light (quantity, quality and periodicity) is by far the main limiting factor for the microalgal growth and nutrient uptake efficiency [27]. Light related variables are photosynthetic photon flux density (PPFD) (intensity), spectral distribution (quality or wavelength), and photoperiod [28]. The utilization of suitable wavelengths of visible light is an effective strategy to enhance microalgal growth, but highly dependent on microalgal species [29]. LED lights with continuous light spectra are increasingly used in plant and seedling production (e.g. [30,31]) with different spectra for enhancement of vegetative growth or vernalization. Although the light requirements between plants and microalgae might be different, studies on the effect of monochromatic LED or a two-color mix LED grow light spectra on microalgal growth [28,29,32,33] might be ignoring several important light energy areas of the spectrum as compared to a continuous light spectrum.

In this study, we focused on microalgae species inhabiting freshwaters in Finland for their potential of bioremediation and biomass generation in unfiltered RAS wastewater at *ca.* 17 °C by using LED grow lights. We tested the following hypotheses: (1) The tested microalgae species would grow and remove dissolved nutrients at 17 °C, because we selected species able to grow in northern climate. (2) The microalgal growth would equal to cultivation in a reference algal growth medium, because nutrient concentration in RAS WW is high enough to support microalgal growth. (3) There would be differences among the tested microalgae species in their growth rates and removal rate of dissolved nutrients from RAS WW. (4) We used three different continuous spectra LED grow lights designed to enhance either growth or vernalization in plants, and tested the hypothesis that these three different spectra would induce differences in microalgal growth, biomass production, and nutrient removal from RAS WW.

2. Materials and methods

2.1. Microalgae strains and cultivation

The freshwater microalgal strains were obtained from culture collections, except for *Acutodesmus* sp. (Table 1). Monocultures were maintained in 650 mL plastic tissue culture flasks containing 400 mL of Modified Wright's Cryptophyte (MWC) medium, based on Guillard and Lorenzen [34]. The medium contained 8.7 mg L^{-1} K_2HPO_4.3 H_2O, 85.0 mg L^{-1} $NaNO_3$, 36.8 mg L^{-1} $CaCl_2.2H_2O$, 37.0 mg L^{-1} $MgSO_4.7H_2O$, 12.6 mg L^{-1} $NaHCO_3$, 2.3 mg L^{-1} $Na_2SeO_3.5H_2O$, 21.2 mg L^{-1} $Na_2SiO_3.5H_2O$, 115 mg L^{-1} TES buffer and trace metals (4.4 mg L^{-1} NaEDTA, 3.2 mg L^{-1} $FeCl_3.6H_2O$, 0.01 mg L^{-1} $CuSO_4.5H_2O$, 0.02 mg L^{-1} $ZnSO_4.7H_2O$, 0.01 mg L^{-1} $CoCl_2.6H_2O$, 0.2 mg L^{-1} $MnCl_2.4H_2O$, 0.01 mg L^{-1} $Na_2MoO_4.2H_2O$, 1.0 mg L^{-1} H_3BO_3) autoclaved at 121 °C for 40 min. Filter-sterilized (0.22 µm) vitamins (0.5 µg L^{-1} biotin (B7), 0.5 µg L^{-1} cyanocobalamin (B12), 0.5 µg L^{-1} pyridoxine (B6), 0.1 mg L^{-1} thiamine HCL (B1)) were added afterwards to the autoclaved solution.

The temperature in the cultivation room was *ca.* 17 °C. Stock cultures were illuminated with a fluorescent light under a 12:12 h light:-dark regime with a light intensity of approximately 50–70 µmol photon m^{-2} s^{-1}.

2.2. LED grow light spectra

Valoya LED grow lights (18 W, L-series T8 tubes, Valoya Oy, Finland) used in this study emit a continuous spectrum based on various percentages of ultraviolet, blue, green, red, far-red and infra-red wavelengths (Table 2). The three different light spectra differ based on blue:green ratio, red:far-red ratio and photosynthetically active radiation (PAR).

Table 2
Characteristics of the three LED grow light spectra (www.valoya.com). λ: wavelength; %: spectral distribution; PAR: photosynthetically active radiation; CCT: correlated color temperature; CRI: color rendering index; B:G Ratio: blue:green ratio; R:FR Ratio: red:far-red ratio; n.a.: not applicable.

	λ (nm)	AP67	AP673L	G2
Ultraviolet (%)	350-400	0.06	0.04	0.03
Blue (%)	400-500	13.8	11.9	7.7
Green (%)	500-600	15.1	19.3	2.4
Red (%)	600-700	53	60.5	64.4
Far-red (%)	700-800	18.1	8.3	25.5
PAR (%)	400-700	81.9	91.7	74.5
CCT (Kelvin)		2500	2000	*n.a.*
CRI (%)		70	60	*n.a.*
B:G Ratio		1.2	1.8	25.9
R:FR Ratio		3.3	5.5	3.1

2.3. Experimental setup

2.3.1. "Selection of microalgae" experiment

The growth and nutrient uptake of 10 microalgae strains (Table 1) was assessed in unfiltered RAS WW in comparison with a reference medium (MWC) (Table 3). The WW originated from a laboratory scale (total volume *ca.* 1000 L) RAS at the University of Jyväskylä, Department of Biological and Environmental Science. The system consisted of a fish tank (500 L), sedimentation tank, trickling filter, and a sump tank. We used whitefish (*Coregonus lavaretus*) as the fish species, and they were fed with Circuit Silver Opti 2.5 dry feed (composition 48% protein, 17% fat, 0.9% P, 7.7% N according to the manufacturer Raisio aqua, Finland) with a belt feeder 24 h per day, at *ca.* 17 °C. Feeding level was based on manufacturer's recommendation, but adjusted if needed to avoid uneaten feed.

The experiments were conducted in 400 mL batch cultures in 650 mL plastic tissue culture flasks, capped with silicone stoppers with inlets consisting of rigid tubes reaching to the bottom of the culture for aeration and liquid sampling, plus an additional short tube for balancing air pressure (Fig. 1a,b). The flasks were aerated constantly and mixed manually on weekdays with aquarium magnets and by stirring to keep the cells in suspension and mixed conditions. Each flask received a constant air supply through an aeration vent (without additional CO_2) approx. 33 mL min^{-1} (Eheim air pump 400, Germany) filtered through a 0.22 μm syringe filter. The inlets enabled axenic maintenance of the cultures during sampling. Cultures with green microalgae were aerated to the bottom of the flask and cultures with non-green microalgae were aerated from the top of the flask without immersion of air inlets in the medium to avoid negative effect of turbulence on non-green microalgae [35].

At the beginning of each series of experiments (day 0), each flask was inoculated with 5–20% of the stock culture saturating concentration (10^6 cells mL^{-1}) determined in pilot studies. Inoculum was added under a laminar flow cabinet using sterile pipettes to minimize contamination between cultures. Constant illumination was provided from one side of the flask by two LED grow lights (AP67 spectrum; Table 2) with the intensity of 85–105 for green and 55–75 μmol photon m^{-2} s^{-1} for non-green microalgae. The light intensity was measured at the surface of flasks by a high resolution spectrometer (HP-350 HiPoint, Taiwan). Room temperature was maintained at 17 ± 0.5 °C. The experiment was terminated after 4 days for green and after 9 days (due to slow growth rate) for non-green microalgae, when all cultures had reached a stationary phase and/or all nutrients were depleted from the cultures. Experiment was done as three separate runs, each including one replicate of each microalgal strain grown in each medium.

Table 3
Characteristics of reference culture medium (MWC) and RAS wastewater (WW) from the first and second experiment separately. Values are presented as mean ± s.d. from all replicates of both experiments. *n.m.*: not measured; * N:P molar ratio was calculated from NO_3-N and PO_4-P.

Characteristic	MWC	WW Exp.1	WW Exp.2
NH_4-N (mg L^{-1})	*n.m.*	0.06 ± 0.01	0.12 ± 0.02
NO_2-N (mg L^{-1})	*n.m.*	0.05 ± 0.03	0.10 ± 0.01
NO_3-N (mg L^{-1})	16 ± 0.6	18.1 ± 6.7	27.1 ± 8.2
PO_4-P (mg L^{-1})	1.8 ± 0.1	0.8 ± 0.3	1.3 ± 0.4
N:P molar ratio *	19.5 ± 0.5	50.3 ± 15.2	45.5 ± 9.7
pH	7.6 ± 0.1	7.1 ± 0.4	7.3 ± 0.3
Dissolved oxygen (mg L^{-1})	*n.m.*	9.1 ± 0.2	9.7 ± 0.9
Conductivity (μS cm^{-1})	*n.m.*	308 ± 55.4	353.2 ± 61.6

Cell density was estimated on days 0, 1, and 4 for green microalgae and on days 0, 1, 4, 7 and 9 for non-green microalgae by cell count from two replicate samples in a haemocytometer chamber (Bürker) with 100x magnification on the microscope (Leitz Laborlux D, Germany). Nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) were analyzed in culture media before inoculation of microalgal cells and at the end of the experiment with mobile laboratory spectrophotometer (LASA 100, Dr. Lange, Germany) accompanied with testing kits (LCK 339 for NO_3-N and LCK 349 for PO_4-P; Hach, Colorado, USA). The culture samples were pre-filtered using 0.22 μm syringe filter to separate the microalgae and suspended solids before each nutrient analysis and all analyses were performed according to the manufacturer's instructions.

2.3.2. "LED grow light spectra" experiment

The growth and nutrient uptake under three different LED grow light spectra (AP67, G2, and AP673 L; Table 2) in unfiltered RAS WW was evaluated for four green microalgae, which were selected based on their growth properties in the previous experiment. *Chlamydomonas reinhardtii* (CR), *Monoraphidium griffithii* (MG), and *Selenastrum* sp. (SE) were cultured in flasks as described above. *Haematococcus pluvialis* (HP) was cultured in 270 mL batch culture in 300 mL glass funnels to avoid cell aggregates and attachment to the walls (Fig. 1c). Each funnel was aerated from the bottom of the funnel (without additional CO_2) approx. 33 mL min^{-1} (Eheim air pump 400, Germany) filtered through a 0.22 μm syringe filter to keep the cells in suspension.

Each culture flask and funnel was inoculated (day 0) with 5–10% of the stock culture saturating concentration determined in pilot studies. Illumination was provided from one side of the batch cultures by two horizontally mounted LED grow lights of each spectrum with the light intensity of 100–120 μmol photon m^{-2} s^{-1} and the constant illumination for CR, MG, and SE. For HP 12:12 h light:dark photoperiod and 70–100 μmol photon m^{-2} s^{-1} were chosen based on pilot studies, where the change from green vegetative cells into astaxanthin accumulated aplanospore cells was observed much sooner at constant illumination than at 12:12 light:dark photoperiod. In previous studies with white fluorescent light, denser vegetative stage of HP cultures were achieved with continuous illumination than with 12:12 light:dark photoperiod [36], but there are no reports from studies using continuous spectrum LED grow lights. Room temperature was 17 ± 0.5 °C. The experiments were terminated when all cultures had reached a stationary phase and/or all nutrients were depleted from the cultures, which was after 4 days for CR, MG, and SE, and after 8 days for HP. The experiment with CR, MG, and SE was done as three separate runs, each including one replicate of each microalgal strain grown under each spectrum. The experiment with HP was conducted separately and included three replicates per spectrum.

The cell density was evaluated on days 0, 1, and 4 for CR, MG, and SE, and on days 0, 1, 4, 6, and 8 for HP by cell count from two replicate samples as described above. The chlorophyll-a concentration and biomass (dry weight) were estimated at the start (day 0) and end of the experiment. The chlorophyll-a concentration was determined using 75 °C ethanol extraction after filtering a known volume of culture through a fiber filter (Whatman, GF/A, Merck, Germany) followed by spectrophotometric measurement (Shimadzu Spectrophotometer UV-

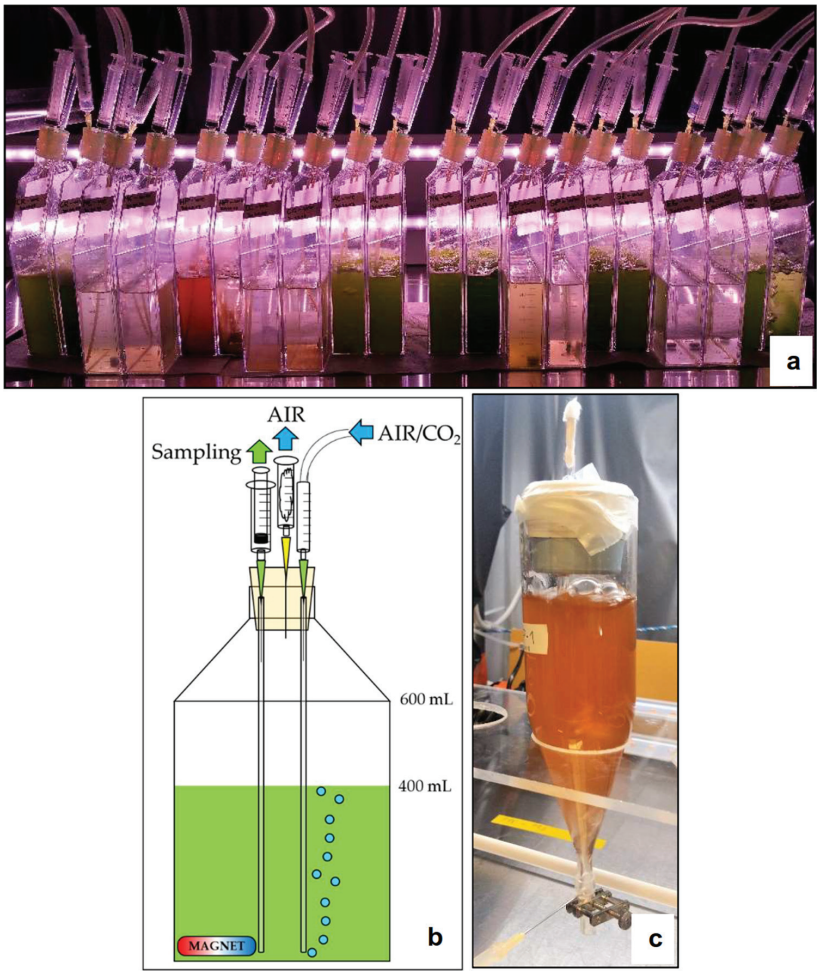

Fig. 1. The experimental setup (**a**) with an illustration of an individual flask (**b**), and glass funnel batch culture for Haematococcus pluvialis (**c**).

1800, Japan) with wavelengths 665 and 750 [37]. Dry weight was measured by filtering a known volume of culture through a pre-weighed fiber filter (Whatman, GF/A, Merck, Germany). Nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) were measured before inoculation of microalgal cells and on the final day of the experiment by using the same methods and instruments as described above.

2.4. Determination of microalgal growth

The specific growth rate (*SGR*) per day (μ, d^{-1}) was calculated from the change in cell concentration in a determined time interval corresponding to the exponential growth phase (between days 0 and 4 for green microalgae and days 0 and 9 for non-green microalgae) according to the following equation: $\mu = \frac{\ln N_1 - \ln N_0}{\Delta t}$, where Δt is the length of a time interval ($t_1 - t_0$) (d), and N_0 and N_1 are number of cells (10^6 cells mL^{-1}) at the beginning and the end of the time interval [38].

Nutrient removal rate (R_i) was determined as: $R_i = \frac{S_0 - S_1}{\Delta t}$ where R_i is the nutrient removal rate of the substrate i (NO_3-N or PO_4-P) (mg L^{-1} d^{-1}), Δt is the length of a time interval ($t_1 - t_0$) (d), and So and S_1 its initial and final concentrations (mg L^{-1}) respectively [10,39].

The cell uptake rate (V_i) was estimated as: $V_i = \frac{S_0 - S_1}{N * \Delta t}$ where V_i is the nutrient removal rate of the substrate i (NO_3-N or PO_4-P) per microalgal cell (mg $cell^{-1}$ d^{-1}), So and S_1 its initial and final concentrations (mg L^{-1}) respectively and N the cell concentration (cells mL^{-1}) at time t_1 [40].

Percentage of nutrient uptake ($i\%$) was calculated according to the equation: $i\% = \frac{S_0 - S_1}{S_0} * 100$, where $i\%$ is the percentage of nutrient uptake of the substrate i (NO_3-N or PO_4-P).

The chlorophyll-a concentration (*Chl a*; μg L^{-1}) was calculated as: $Chl\ a = 11.9 * A * \frac{Ve}{Vs * d}$, where *11.9* is the absorbance coefficient (11.9 = (1 / *Kc*) * 1000, $Kc = 83.4$ L g^{-1} cm^{-1}), A = A665 – A750 (the difference between chlorophyll-a absorption at 665 nm and 750 nm of light), *Ve* is the volume of ethanol (mL), *vs* is the volume of microalgae (mL), and the *d* is the spectrophotometer cuvette width (cm) [37].

2.5. Data analyses

Two-way ANOVA (2-ANOVA) was used to test the possible differences between the two media (WW and MWC) or three light spectra in respect to the growth and nutrient uptake of different microalgal species. The non-independence of observations within each run was accounted for by including run identity as a random factor. For pairwise comparisons, we employed Simple effects tests with Bonferroni

corrections. One-way ANOVA was used to compare the effect of different light spectra on the growth and nutrient uptake of HP in the second experiment, and pairwise differences between microalgal species were tested with LSD *post-hoc* test. The normality of the data was tested with Shapiro–Wilk's test and homogeneity of variances using Levene's test. In case of non-normality and/or heteroscedasticity of the data, we used Kruskal-Wallis's H non-parametric test with Dunn's test for pairwise comparisons with Bonferroni corrections or Welch ANOVA with Games-Howell's test for pairwise comparisons. In case of heteroscedasticity where non-parametric test showed the similar result as parametric test, the parametric test was reported. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Statistical analyses were conducted using IBM SPSS (version 24.0; IBM 2016) software.

3. Results

3.1. "Selection of microalgae" experiment

Green microalgae showed a good growth potential and nutrient removal not only in reference medium (MWC) but also in unfiltered RAS WW. The densities and specific growth rates (*SGR*) of the green microalgae species did not differ between WW and MWC (Fig. 2; Table S.1). PO_4-P removal rate (R_P), PO_4-P cell uptake rate (V_P), and NO_3-N removal percentage (*N%*) were higher in MWC than in WW (Fig. 2; Table S.1). Green microalgae differed from each other for all measured parameters except for *SGR* and removal rates and percentages of PO_4-P (R_P and *P%*) ($p < 0.001$; Table S.1). There was no significant interaction between media and microalgae for any of the measured parameters ($p > 0.05$). Microalgae *Acutodesmus* sp. (AC) and *Selenastrum* sp. (SE) had the highest density followed by *Monoraphidium griffithii* (MG), *Chlamydomonas reinhardtii* (CR), *Scenedesmus obliquus* (SO), and *Haematococcus pluvialis* (HP) (pairwise tests; $p > 0.05$; Fig. 2a). Rates and percentages of NO_3-N removal (R_N and *N%*) were significantly higher for AC and CR than for SE and HP, while MG and SO showed intermediate values (pairwise tests; $p < 0.05$; Fig. 2c,e). HP had significantly higher cell uptake rate of NO_3-N (V_N) than AC, MG, and SE (pairwise tests; $p < 0.05$; Fig. 2g), and significantly higher cell uptake rate of PO_4-P (V_P) than all other green microalgae (pairwise tests; $p < 0.05$; Fig. 2h). Removal rates of both nutrients (R_N and R_P) differed between replicate runs ($p < 0.001$; Table S.1), while other variables did not vary between runs.

On the other hand, non-green microalgae showed a poor growth potential and nutrient removal in WW as well as in MWC (Fig. 3; Table S.2). Non-green microalgae had more than 10 times smaller *SGR* and removal rates (R_N and R_P) than green microalgae, and nutrient removal percentages (*N%* and *P%*) were generally lower than 30%. Non-green microalgae differed from each other for all measured variables except for V_N ($p < 0.05$; Table S.2). Medium as such did not affect any of the measured parameters, but there was a significant interaction between the media and microalgae for *SGR* and R_P ($p < 0.05$; Table S.2). Microalga *Euglena gracilis* (EG) had significantly lower density than *Cryptomonas curvata* (CC) and *Synura petersenii* (SP), and significantly lower *SGR* than *Mallomonas caudata* (MC) (pairwise tests; $p < 0.05$; Fig. 3a,b). PO_4-P removal (R_P and *P%*) for CC and SP were significantly higher than for EG and MC (pairwise tests; $p < 0.05$; Fig. 3d,f), NO_3-N removal (R_N and *N%*) for CC were significantly higher than for EG and MC, and V_P for CC was significantly higher than for EG (pairwise tests; $p < 0.05$; Fig. 3c,e,h). Replicate runs were not significantly different for any of the variables ($p > 0.05$; Table S.2).

3.2. "LED grow light spectra" experiment

The growth and nutrient removal of CR, MG, and SE in unfiltered RAS WW did not vary between the three light spectra ($p > 0.05$; Table S.3), whereas the three microalgae differed from each other for all measured parameters ($p < 0.05$; Fig. 4). The differences between microalgae followed the same trends as observed for the previous experiment "selection of microalgae" in WW. Microalga SE had significantly higher density than CR and MG but significantly lower chlorophyll-a concentration (*Chl a*), R_N, V_P, and *N%* than CR and MG (pairwise tests; $p < 0.05$; Fig. 4a,d,e,g,*j*). PO_4-P removal (R_P and *P%*) for CR was significantly lower (pairwise tests; $p < 0.05$; Fig. 4f,h) but V_N significantly higher (pairwise tests; $p < 0.05$; Fig. 4i) than for MG and SE. CR had significantly higher dry weight (*DW*) than SE and higher *SGR* than MG (pairwise tests; $p < 0.05$; Fig. 4b,c). Replicate runs were significantly different for all variables except for V_N and *P%* ($p < 0.05$; Table S.3).

HP had significant differences between the three light spectra for all variables except for density, *SGR*, and V_P ($p < 0.05$; Table S.4), but none of the spectra was producing the best results for all of the measured variables (Fig. 5). HP had significantly higher *DW* on spectrum AP673 L than on spectra AP67 and G2, whereas *Chl a* was significantly lower on spectrum G2 than on spectra AP67 and AP673 L (pairwise tests; $p < 0.05$; Fig. 5b,d). On the other hand, PO_4-P removal (R_P and *P%*) was the highest on spectrum G2, and the lowest on spectrum AP673 L (pairwise tests; $p < 0.05$; Fig. 5f,h). Similarly, V_N was significantly higher on spectrum G2 than on spectrum AP673 L, and NO_3-N removal (R_N and *N%*) was significantly higher spectrum G2 than on spectrum AP67 (pairwise tests; $p < 0.05$; Fig. 5e,g,i).

4. Discussion

Unfiltered RAS WW supported the growth of green microalgae (*SGR*, 0.5-0.8 d^{-1}) and sustained high removal percentages of NO_3-N (*N%*) and PO_4-P (*P%*) (57–96% and 77–94%, respectively) already after 4 days at water temperature that is common in Nordic RAS (*ca.* 17 °C). These values were comparable to the growth and nutrient removal in reference algae medium (MWC) (*SGR*, 0.6-0.9 d^{-1}; *N%*, 59–99%; *P%*, 91–99%). On the other hand, non-green microalgae had negligible growth (*SGR*, -0.1-0.1 d^{-1}) and limited nutrient removal even after 9 days (*N%*, 4–29%; *P%*, 0–29%) in WW as well as in MWC (*SGR*, -0.1-0.2 d^{-1}; *N%*, 1–22%; *P%*, 0–34%). There were no differences among the three tested LED grow light spectra in growth and nutrient removal of three green microalgae (*Chlamydomonas reinhardtii* (CR), *Monoraphidium griffithii* (MG), *Selenastrum* sp. (SE)) in WW after 4 days (*SGR*, 0.5-0.7 d^{-1}, *N%*, 64–80%; *P%*, 59–93%). However, green microalga *Haematoccocus pluvialis* (HP) had significant differences in growth and nutrient removal among the three light spectra after 8 days, with the highest nutrient removal (*N%*, 74.2%; *P%*, 96.3%) in spectrum G2.

The present results are in accordance with previous studies demonstrating that some microalgal species have similar growth potential and nutrient removal efficiency when they are cultivated in WW compared to cultivation in a reference medium with optimal nutrient concentrations [41–43]. Despite cultivating microalgae in RAS WW without prior filtration, this study did not detect any notable decline in microalgal growth due to biological contaminants, such as bacteria and protozoa, that is in line with previous studies [24,44]. Possibly the short timescale of our experiments did not allow detection of potential effects of biological contaminants on microalgae and, in addition, the effect of biological contaminants might be species-specific. Consequently, we did not evaluate the effect of bacteria and protozoa from WW on the growth and nutrient removal of microalgae, and we attributed all nutrient removal to microalgae only, which might overestimate our results in WW.

The benefits of using LEDs emitting a continuous spectrum with different percentages of wavelengths have been demonstrated for higher plants [31], but, to our knowledge, have not been reported for microalgal cultivation. The three LED grow light spectra tested here did not induce differences in the growth or nutrient removal for CR, MG, and SE, suggesting that these three light spectra provided the light properties needed for efficient photosynthesis for these green

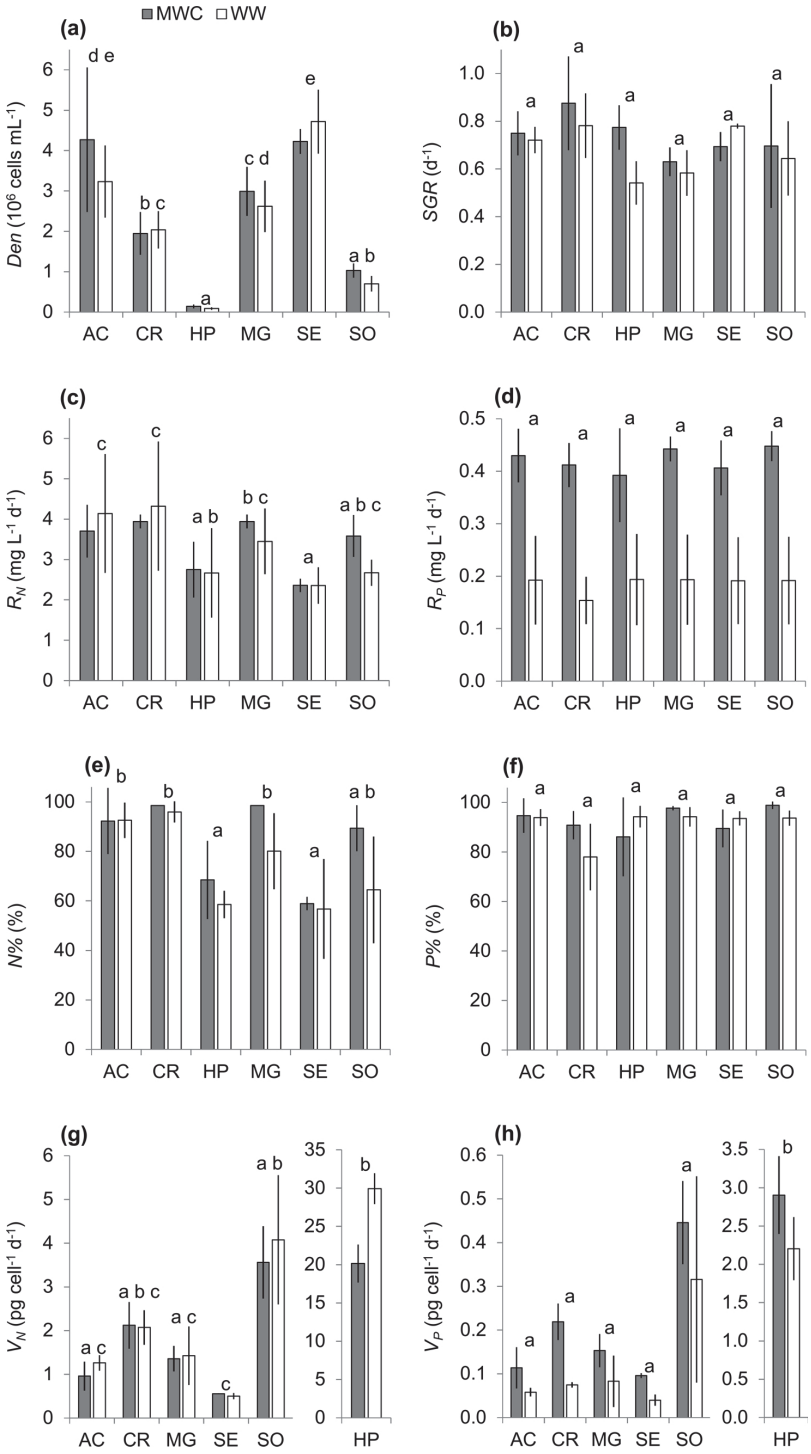

Fig. 2. Density (*Den*) (**a**), specific growth rate (*SGR*) (**b**), removal rate of NO_3-N (R_N) (**c**), removal rate of PO_4-P (R_P) (**d**), percentage of NO_3-N removal (*N%*) (**e**), percentage of PO_4-P removal (*P%*) (**f**), cell uptake rate of NO_3-N (V_N) (**g**), cell uptake rate of PO_4-P (V_P) (**h**) for six green microalgae (AC – *Acutodesmus* sp., CR – *Chlamydomonas reinhardtii*, HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp., SO – *Scenedesmus obliquus*) grown in two media (grey bars: MWC – Modified Wright's Cryptophyte medium, white bars: WW – RAS wastewater) at constant illumination and light intensity of 85–105 µmol photon m^{-2} s^{-1} for four days. Values are presented as mean ± s.d. of three replicates. Values were higher in MWC for R_P, V_P and N%, while no differences between media were detected for other measured parameters. Microalgae species denoted with the same letter (a–e) are not significantly different from each other (pairwise tests; $p > 0.05$).

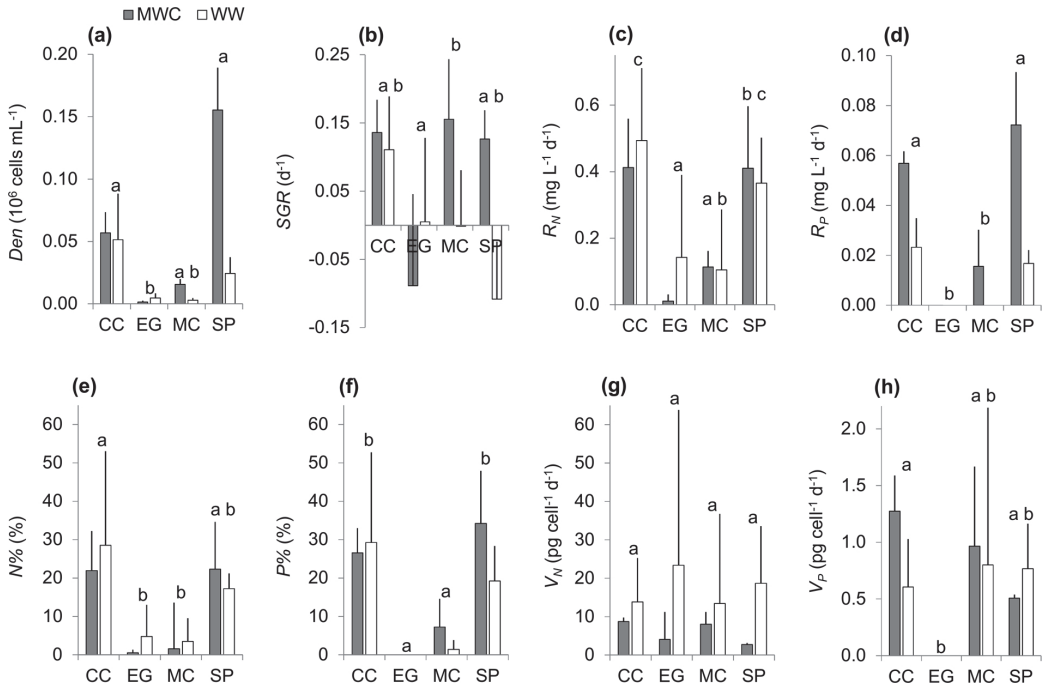

Fig. 3. Density (*Den*) (**a**), specific growth rate (*SGR*) (**b**), removal rate of NO_3-N (R_N) (**c**), removal rate of PO_4-P (R_P) (**d**), percentage of NO_3-N removal (*N%*) (**e**), percentage of PO_4-P removal (*P%*) (**f**), cell uptake rate of NO_3-N (V_N) (**g**), cell uptake rate of PO_4-P (V_P) (**h**) for four non-green microalgae (CC – *Cryptomonas curvata*, EG – *Euglena gracilis*, MC – *Mallomonas caudata*, SP – *Synura petersenii*) grown in two media (grey bars: MWC – Modified Wright's Cryptophyte medium, white bars: WW – RAS wastewater) at constant illumination and light intensity of 55–75 µmol photon m^{-2} s^{-1} for nine days. Values are presented as mean ± s.d. of three replicates. Note that SGR values for EG and SP in panel (b) are negative, because cell density decreased during the experiment. Microalgae species denoted with the same letter (a–c) are not significantly different from each other (pairwise tests; $p > 0.05$). Measured parameters did not differ between growth media. However, for R_P and *SGR* there was a significant interaction between microalgae and media and it should be noted that in panels (b) and (d) (*SGR* and R_P), letters denote differences between microalgae in MWC, while no differences between microalgae were found in WW.

microalgal species. However, HP had differences in nutrient removal among the LED spectra. Possibly HP is the most sensitive microalga among these four green microalgae to different light conditions due to its propensity for producing carotenoid pigments (astaxanthin) under stress. Although the G2 light spectrum induced the highest removal rates and percentages in HP, it produced the lowest growth parameters (density, biomass, and chlorophyll-a concentration) (Fig. 5). We assumed that HP will remove more nutrients in the green phase than in the red phase due to higher growth, and consequently inoculated HP cultures in green phase [45]. We observed that strong and/or continuous light accelerated the process of encystment from the green stage to the red stage which is in line with previous studies [46]. The encystment of HP from green to red phase after day 3 in both experiments probably decreased its nutrient removal efficiency. The positive correlation between low growth (in particular low chlorophyll-a concentration) and high nutrient removal for HP on spectrum G2 might be explained by the highest concentration of red phase cells that are bigger than green phase cells and can absorb more nutrients per cell. Our results suggest that RAS WW could be used as a substrate for producing astaxanthin, a highly valued carotenoid [47].

Not all microalgal species studied here have been previously tested for cultivation in RAS or other types of WW (*Mallomonas* and *Synura)*, but for those species that can be compared, our results are mostly in line with the previous results. For example, CR grown in unfiltered RAS WW in our study had higher SGR than in previous studies [48,49] and higher removal of nitrogen [48,50]. For phosphorus, the removal was higher [49,50], or similar [48] than those previously found. Moreover, in our second experiment, the nutrient removal of CR are possibly underestimates because chloroplasts passed through the 0.22 µm syringe filter pore size along with nutrients.

Although we did our experiments at *ca.* 17 °C, the SGRs for microalgae grown in WW found in our study are similar than those found in previous studies using temperatures above 20 °C (e.g. HP: Wu et al. [51]) or higher (*Monoraphidium*: Jiang et al. [43]; SE: Ouyang et al. [52]Wang et al. [53], and Zhao et al. [16]; *Scenedesmus obliquus* (SO): Ji et al. [11],Zhou et al. [48]Ouyang et al. [52], and Zhao et al. [16]). Also the removal percentages for nitrogen and phosphorus found in our study are similar (SE: Zhao et al. [16]; SO: Ouyang et al. [52] and Zhao et al. [16]) or higher (HP: Wu et al. [51]; *Monoraphidium*: Jiang et al. [43]; SE: Ouyang et al. [52] and Wang et al. [53]; SO: Zhou et al. [48] Zhao et al. [16], and Ouyang et al. [52]). However, for SO, Ji et al. [11] recorded higher removal of nutrients than in this study.

Cultivation of non-green microalgae in WW has been studied much less than cultivation of green microalgae, although there is a growing interest for utilizing the heterotrophic growth of many microalgae species for assimilation of organic carbon compounds in WW [54]. In addition, many non-green microalgae could provide essential nutrition for secondary consumers in integrated multi-trophic aquaculture systems (IMTA) due to their high nutritional quality (rich in sterols, ω-3 fatty acids, protein, and amino acids) [55,56]. In our experiments, the tested non-green microalgae grew poorly and their nutrient removal capacity was low. For example, Tossavainen et al. [57] and Aravantinou et al. [58] recorded slightly higher SGR for *Euglena gracilis* and considerably higher removals of PO_4-P, and Tossavainen et al. [57] had higher removal of NO_3-N than what was found in our study.

The pH increased with the culture time and exceeded pH 8 at the

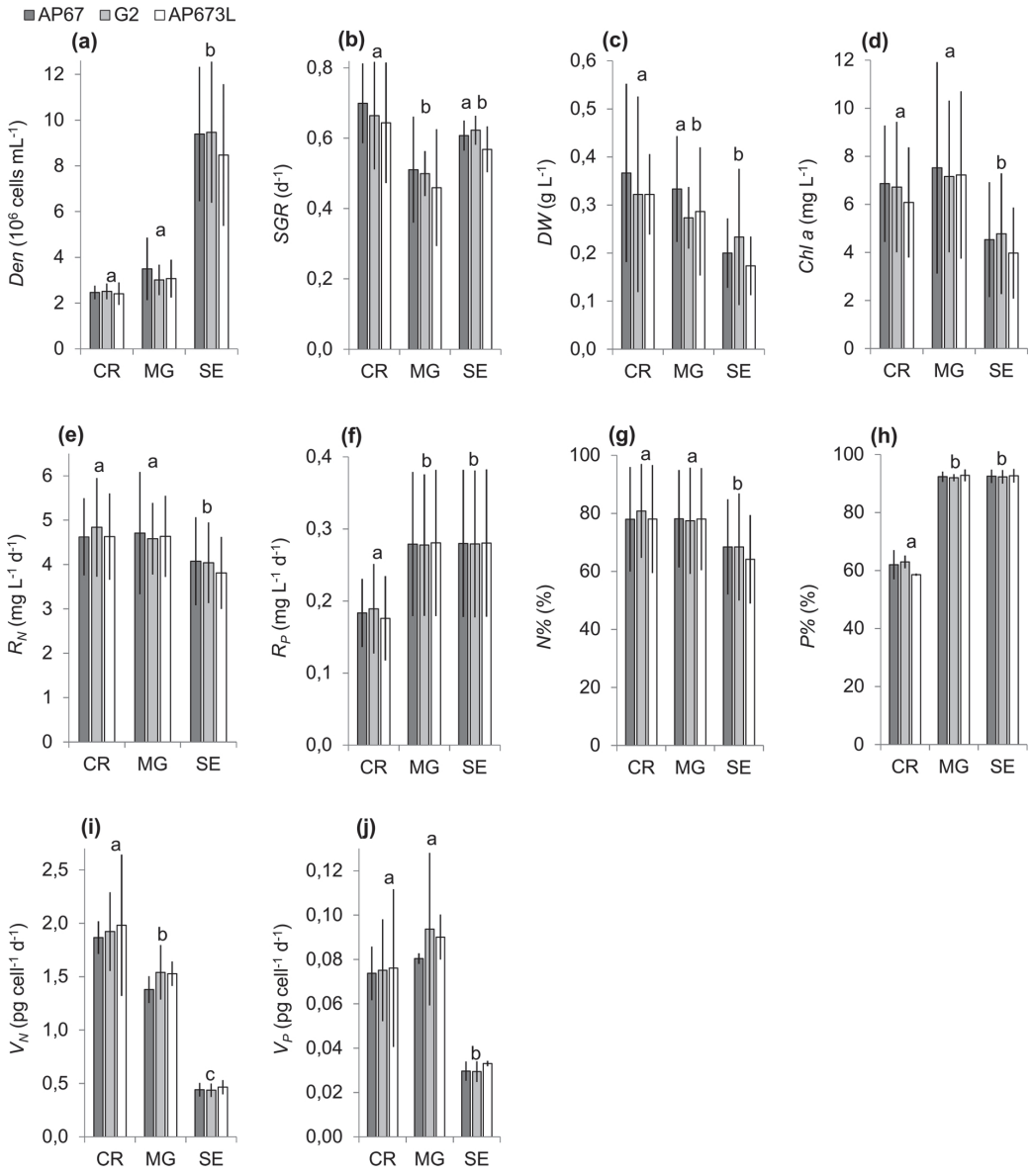

Fig. 4. Density (*Den*) (**a**), specific growth rate (*SGR*) (**b**), dry weight (*DW*) (**c**), chlorophyll-a concentration (*Chl a*) (**d**), removal rate of NO_3-N (R_N) (**e**), removal rate of PO_4-P (R_P) (**f**), percentage of NO_3-N removal (*N%*) (**g**), percentage of PO_4-P removal (*P%*) (**h**), cell uptake rate of NO_3-N (V_N) (**i**), cell uptake rate of PO_4-P (V_P) (*j*) for three green microalgae (CR – *Chlamydomonas reinhardtii*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) grown on three light spectra (AP67, G2, AP673 L) at constant illumination and light intensity of 100–120 μmol photon m^{-2} s^{-1} at day four of the experiment. Values are presented as mean ± s.d. of three replicates. Microalgae species denoted with the same letter (a–c) are not significantly different from each other (pairwise tests; $p > 0.05$).

end of the first experiment (day 9) and pH 9 at the end of second experiment (day 4) possibly due to carbon limitation, which is a constant trend in other studies [43,50,59,60]. Our results suggest that the pH values remain within acceptable range for cultivation of microalgae in RAS WW [61]. However, supplementation of CO_2 might maintain pH closer to optimal in longer experiments [50,60].

To study the feasibility of utilizing microalgae for nutrient removal in RAS WW, further investigations are needed on the influence of different light intensities and photoperiods, the effect of bacteria and protozoans on microalgae growth in RAS WW, as well as combinations of different microalgae species (consortium effect) on the nutrient removal in RAS WW. Further research is also needed to optimize operational, i.e. temperature and light conditions, especially regarding the energy-efficiency of the system in large scale applications. In Nordic RAS, with the need for artificial lighting, this could be achieved with the development of energy-efficient LED lighting, with the shortest photoperiods and the highest light intensity that can produce high microalgal biomass without dissipating excessive amount of light outside the bioreactor.

A possible application of the results presented here could be the

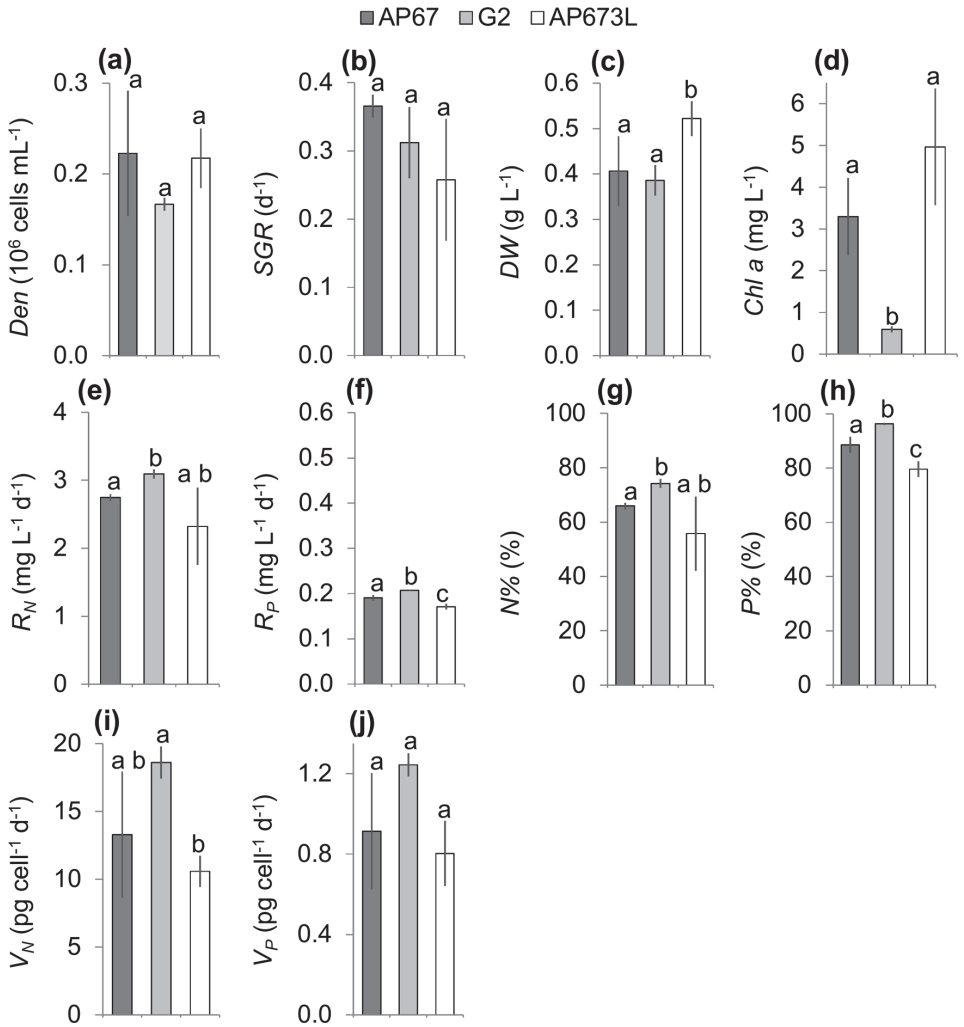

Fig. 5. Density (*Den*) (**a**), specific growth rate (*SGR*) (**b**), dry weight (*DW*) (**c**), chlorophyll-a concentration (*Chl a*) (**d**), removal rate of NO_3-N (R_N) (**e**), removal rate of PO_4-P (R_P) (**f**), percentage of NO_3-N removal (*N%*) (**g**), percentage of PO_4-P removal (*P%*) (**h**), cell uptake rate of NO_3-N (V_N) (**i**), cell uptake rate of PO_4-P (V_P) (*j*) for green microalga *Haematococcus pluvialis* grown on three light spectra (AP67, G2, AP673 L) at 12:12 h light:dark photoperiod and light intensity of 70–100 µmol photon m^{-2} s^{-1} for eight days. Values are presented as mean ± s.d. of three replicates. LED grow light spectra denoted with the same letter (a–c) are not significantly different from each other (pairwise tests; $p > 0.05$).

formation of economically sustainable and environmentally friendly IMTA, where the produced microalgal biomass could be used e.g. as feed for filter feeders or for planktivorous fish. The cultivation of green microalgae in RAS WW demonstrated their potential for biomass production through efficient nitrate and phosphate uptake. Therefore, green microalgae should be regarded as a potential alternative to assist in the RAS WW treatment by reducing the environmental impact of nutrients. Some modern RAS employ denitrification filters, which are used to convert nitrate into gaseous N under anoxic conditions [62]. This nitrogen will be lost into the atmosphere, and thus can be regarded as waste of valuable nutrient, and any loss of nutrients also contradicts the idea of the circular economy. The research here was done in a laboratory-scale batch cultures but provides useful data for up-scaling. Knowing the species-specific removal rate of nitrate and phosphate is important to be able to predict the time needed to remove these compounds from RAS WW, and cell uptake rate is important to estimate the amount of microalgae necessary for those removal times.

5. Conclusions

Our original hypotheses were mainly supported. The first hypothesis was supported, as green microalgae can be successfully grown as batch cultures at ~17 °C, a temperature common in Nordic RAS. The second hypotheses was supported, as unfiltered RAS WW promotes good growth of green microalgae in four days to a similar extent as observed for the reference culture medium. The third hypothesis was also supported, as biomass production and bioremediation efficiency in RAS WW varies among the tested species of microalgae. In particular, green microalgae can be used for RAS WW treatment as they improved the water quality by reducing the concentrations of nitrate and phosphate. The fourth hypothesis was not supported, as the three different continuous LED grow light spectra did not cause different microalgal

growth, biomass production, and nutrient removal from RAS WW. However, using LED grow lights with a continuous spectrum as light sources during the cultivation of green microalgae may be used efficiently to obtain high photosynthetic removal of dissolved nutrients and high microalgal biomass. In summary, the water quality of RAS WW was improved by green microalgae. The results from this study could therefore be used for further development of WW treatment and the production of valuable microalgal biomass using waste nutrients from the RAS.

Author contributions

ČS, KP and JP planned the study, ČS and JP operated the RAS unit, ČS and KP conducted the experiments and analyzed the data, ČS drafted the article, and all authors participated in revising the article.

Informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.algal.2019.101681.

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II

FILTRATION OF NORDIC RECIRCULATING AQUACULTURE SYSTEM WASTEWATER: EFFECTS ON MICROALGAL GROWTH, NUTRIENT REMOVAL AND NUTRITIONAL VALUE

by

Marco Calderini, Čedomir Stevčić, Sami Taipale & Katja Pulkkinen 2021

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III

EFFICIENCY OF *DAPHNIA MAGNA* IN REMOVAL OF GREEN MICROALGAE CULTIVATED IN NORDIC RECIRCULATING AQUACULTURE SYSTEM WASTEWATER

by

Čedomir Stevčić, Katja Pulkkinen & Juhani Pirhonen 2020

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Efficiency of *Daphnia magna* in removal of green microalgae cultivated in Nordic recirculating aquaculture system wastewater

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ABSTRACT

The increase of global aquaculture production has boosted the development of recirculating aquaculture systems not only because they reduce water use but also provide opportunities for waste management and the use of released nutrients. The dissolved nutrients can be efficiently removed from recirculating aquaculture system wastewater by microalgae, and microalgae can be harvested from the wastewater with low costs by zooplankton such as *Daphnia*. The purpose of this study was to evaluate the feasibility of using microalgae and *Daphnia* for bioremediation of recirculating aquaculture system wastewater in Nordic conditions. We evaluated the growth and filtration efficiency of the waterflea *Daphnia magna* on four green microalgae species cultured at 17 ± 0.3 °C in recirculating aquaculture system wastewater as compared to microalgae medium. There was no difference in the growth of *Daphnia* fed with *Monoraphidium griffithii* and *Selenastrum* sp. cultured in either media, while with *Chlamydomonas reinhardtii* and *Haematococcus pluvialis* growth of *Daphnia* was slower when cultured in wastewater than in algae medium. Higher filtration efficiency was achieved with the *Daphnia* density of 100 individuals L^{-1}, than with higher tested densities (200 and 300 ind. L^{-1}). After 48 h, *Daphnia* had removed 80% of *M. griffithii*, 70% of *H. pluvialis*, but only 20% of *Selenastrum* sp. from the wastewater. Phosphate was re-released to water after 72 h feeding on *Selenastrum* sp., but not when feeding on *M. griffithii*. Taken together, of the four tested green microalgae, *M. griffithii* was found to be the most suitable microalga species for the microalgae-*Daphnia* bioremediation system. The results support the potential of using microalgae and *Daphnia* for bioremediation and for producing biomass in Nordic recirculating aquaculture system wastewater.

1. Introduction

Recirculating Aquaculture Systems (RAS) reduce the water use significantly, with constant wastewater treatment and reuse, compared to traditional flow-through systems [1]. Water reuse is limited by the accumulation of suspended solids and dissolved nutrients, typically resulting in a discharge of 5–10% of the total water volume per day [1,2]. Hence, RAS does not reduce the overall discharge of nutrients into the surrounding aquatic environment, but by concentrating nutrients and organic matter, it allows improved opportunities for waste management and nutrient recycling when compared to flow-through systems or cages [2]. Currently, there is almost no recycling of the dissolved nutrients released from RAS, except for aquaponics (*aqua*culture combined with cultivation of plants without soil, i.e. hydro*ponics*) [1].

Treatment of aquaculture wastewater is expensive, thus improvements of bioremediation are among the major ongoing developments in RAS [3]. By assimilating both inorganic and organic nutrients, microalgae are among the best cost-effective and sustainable organisms for bioremediation of aquaculture WW [3]. The produced biomass can be later used as animal feed, human food and health products, agricultural fertilizer, and biofuel [4]. Bioremediation of aquaculture WW by microalgae is mostly limited to warmer geographical locations (e.g. Egloff et al. [5]), but their efficiency in cold and temperate climates (below 20 °C) has also been demonstrated [6].

Mechanical and chemical microalgae biomass harvesting for microalgae-based WW treatment can form up to 90% of the total investments and is mostly applicable to products with high value [7]. Therefore, additional harvesting methods would be desirable, and one option is biological harvesting by filter-feeding organisms, such as zooplankton and mussels. The combination of microalgae and filter-feeders can remove up to 68% of total nitrogen and 56–67% of total phosphorus [8,9]. By implementing microalgae-filter-feeder bioremediation in aquaculture/RAS WW, produced biomass used later as an animal feed can mitigate additional energy costs in colder climates [10].

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Selecting filter-feeder species that inhabit colder water bodies can further reduce the negative impact of colder climate on cost-efficiency in microalgae-filter-feeder systems. Furthermore, it is important to select native microalgae species to avoid possible introduction of exotic species into natural waters.

Daphnia waterfleas, small planktonic crustaceans (Cladocera), are non-selective filter-feeders that feed on microalgae, bacteria, yeast, and protozoans smaller than 35 μm [11–13]. Microalgae are easily digested and provide a source of essential mineral nutrients and biomolecules for *Daphnia* (e.g. sterols, fatty acids, amino acids, carotenoids) [14,15]. The largest species of the genus, *D. magna*, is used widely in bioremediation of different types of WW because they also efficiently reduce bacteria, yeast, and suspended solids present in WW [16,17]. However, *Daphnia* do not suit for all WWs due to their sensitivity to high levels of dissolved nutrients (>35, >6, >250, and >50 mg L^{-1} of NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}, respectively) [18,19]. In RAS WW the levels of these nutrients are typically much lower (<0.4, <0.3, <133, and <6 mg L^{-1} of NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}, respectively) [1,2,6], and the levels are further reduced after bioremediation with microalgae [6]. Although the filtration efficiency of *D. magna* is influenced negatively by temperatures below 20 °C [13], the larger body size and higher longevity of *Daphnia* in temperatures below 20 °C induce higher overall ingestion rates [20]. Combining carefully selected microalgae and *D. magna* could therefore provide a suitable system for bioremediation of cold-water RAS WW (e.g. [21]).

In this study, we aimed to find a suitable microalgae species for an efficient microalgae-*Daphnia* bioremediation system of unfiltered RAS wastewater (WW) specifically in Nordic RAS conditions (~17 °C). We evaluated the growth and filtration efficiency of waterflea (*Daphnia magna*) when fed with green microalgae species that we have previously found to be efficient for nutrient removal in Nordic RAS WW [6]. The nutrient removal by microalgae-*Daphnia* bioremediation system is likely to depend not only on the species of microalgae but also on the density of *Daphnia*, as the crowding of *Daphnia* affects negatively their growth and efficiency in microalgal removal from a suspension [13,22,23]. Moreover, the choice of microalgal species may affect how much nutrients are re-released to the WW via the breaking of microalgal cells during *Daphnia* feeding process and excretion [24]. Specifically, we tested the following hypotheses: (1) *Daphnia* grows equally well when fed microalgae cultivated in WW than when fed microalgae cultivated in the reference medium (MWC). (2) The growth of *Daphnia* differs between tested microalgal diets. (3) The increase in *Daphnia* density negatively affects the efficiency of microalgal removal. (4) Filtration efficiency of *Daphnia* differs between tested microalgal diets. (5) The nutrient concentration in WW does not change during a filtration period with *Daphnia*.

2. Materials and methods

2.1. Microalgae strains and cultivation

Four freshwater green microalgal strains (*Chlamydomonas reinhardtii* (CR), *Haematococcus pluvialis* (HP), *Monoraphidium griffithii* (MG), and *Selenastrum* sp. (SE)) obtained from Norwegian Culture Collection of Algae (NORCCA) (Table A.1) were found to grow well in the RAS WW in previous experiments [6]. Stock cultures were maintained in algae medium MWC (Modified Wright's Cryptophyte based on Guillard and Lorenzen [25]) as described previously [6]. The HP life cycle has four types of different cellular morphologies: macrozooids, microzooids, palmella, and hematocysts, and the first three phases are commonly called "green vegetative phase" (HPgreen), while hematocysts are known as "red astaxanthin accumulated encysted phase" (HPred) [26]. We used HPgreen in growth experiment, HPgreen and HPred separately in the second filtration experiment (see Section 2.3.2), and a mixture of life stages in the third filtration experiment.

2.2. Waterflea and fish cultivation

Waterflea *Daphnia magna* (Daphniidae, Cladocera) were hatched from resting eggs (ephippia) according to the manufacturer's instructions (Daphtoxkit F magna™, Aboatox, Finland). Hatched *Daphnia* were kept in artificial freshwater [modified AdaM medium [27]] at ~17 °C under fluorescent lights (50–80 μmol m^{-2} s^{-1}, 24:00 light: dark)]. Waterflea cultures were maintained in 250 mL jars at densities of 100–200 *Daphnia* L^{-1} and fed microalga *Acutodesmus* sp. every other day with a daily ration of ~0.7–2.8 mg C daphnid^{-1}. The WW used in this study was obtained from the laboratory-scale RAS with whitefish (*Coregonus lavaretus*), which was maintained as described previously in Stevčić et al. [6].

2.3. Experimental set-up

2.3.1. Daphnia growth on microalgae cultivated in RAS WW and reference medium

The growth efficiency of *Daphnia* was assessed with the four microalgae described in Section 2.1, cultivated in two different media: filtered RAS WW and a reference medium (MWC). RAS WW was filtered through GF/A filter paper (1.6 μm, Ø47 mm, Whatman) to remove particles that could interfere with the measurements of microalgal cell volume and dry weight.

CR, MG, and SE were cultivated in 400 mL batch cultures in 650 mL plastic tissue culture flasks as described in Stevčić et al. [6]. HP was cultured in 270 mL batch culture in 300 mL glass funnels to avoid cell aggregates and attachment to the walls [6]. Each culture flask and funnel was inoculated with 5–10% of the stock culture saturation concentration determined in pilot studies for each microalga. To reduce the amount of culture medium in the inoculum, microalgae CR, MG, and SE were centrifuged at 1800*g* for 10 min and HP was centrifuged at 1200*g* for 5 min at 17 °C (Heraeus Megafuge 1.0, Germany). The cell density and volume were determined by CASY Electronic Cell Counter and Analyzer (OLS-OMNI Life Science, Germany). Inocula were added under a laminar flow cabinet using sterile pipettes to minimize contamination between cultures. Illumination was provided from one side of the batch cultures by two horizontally mounted LED grow lights (AP67 spectrum, Valoya, Finland) (Table 1). The light intensity was measured at the surface of flasks by a high-resolution spectrometer (HP-350, Hipoint Inc., Taiwan). The cultivation was terminated after 4 days when all cultures had reached a stationary phase.

To determine the average starting individual biomass of the *Daphnia*, a random sample of 20 juveniles hatched in the last 24 h period was dried at 60 °C for 12 h in tin capsules and weighed. Each of the four different microalgae (CR, HPgreen, MG, SE) grown in two different media (WW, MWC) were added to five replicate 150 mL glass jars filled with 100 mL of WW, totaling to 40 jars. All four microalgae species were inoculated with a total cell volume of 16.1×10^6 μm^3 mL^{-1}, corresponding to 2.1 mgC L^{-1} [28] (Table 1). Ten *Daphnia* hatched within the last 24 h period were placed into each jar. Jars were placed in random order under fluorescent lights (Table 1), covered with transparent plastic sheets to prevent water evaporation, and were mixed with pipettes twice a day to reduce microalgal sedimentation. After 4 days, *Daphnia* from each jar separately were rinsed with distilled water on a plankton net, pooled in pre-weighed tin capsules and dried in an oven (60 °C) for 24 h and weighed to determine the average individual dry weight per replicate jar.

2.3.2. Daphnia removal efficiency of microalgae cultivated in recirculating aquaculture system wastewater

The efficiency of *Daphnia* on the removal of microalgae cultivated in RAS WW was assessed in a series of three separate experiments. First, the effect of *Daphnia* density on their filtration and removal efficiency when feeding on different microalgal diets was tested in two experiments in small volume (100 mL jars), which differed in duration (2 h and 3 h) and

Table 1
Summary of five experimental set-ups for growing microalgae and *Daphnia magna* at 17 ± 0.3 °C: *Growth* (see Sections 2.3.1 and 3.1); *1st*, *2nd*, and *3rd filtration* (Sections 2.3.2 and 3.2); *Nutrient release* (Sections 2.3.3 and 3.3). Media: MWC, reference algal medium (Modified Wright's Cryptophyte); WW, filtered recirculating aquaculture system wastewater. *n.a.*, not applicable. *n.m.*, not measured.

Experiment	Growth	1st filtration	2nd filtration	3rd filtration	Nutrient release
Microalgal cultivation					
Media	MWC, WW	WW	WW	WW	WW
Photoperiod (light:dark)	12:12	12:12	12:12	12:12	12:12
Light intensity (μmol m^{-2} s^{-1})	110–130; 80–100[a]	110–130	110–130; 80–100[a]	110–130; 80–100[a]	110–130
Starting cell density (mean, 10^6 cells mL^{-1})					
Chlamydomonas reinhardtii	0.447	*n.a.*	*n.a.*	*n.a.*	*n.a.*
Haematococcus pluvialis	0.028 (g)[b]	*n.a.*	0.095 (g); 0.089 (r)[b]	0.020 (g + r)[b]	*n.a.*
Monoraphidium griffithii	0.794	0.293	0.382	0.571	0.863
Selenastrum sp.	0.341	0.756	1.080	2.389	0.873
Daphnia experiments					
Duration (hours)	96	2	3	48	72
Photoperiod (light:dark)	24:00	24:00	24:00	00:24	00:24
Light intensity (μmol m^{-2} s^{-1})	50–80	50–80	50–80	*n.m.*	*n.m.*
Volume (L)	0.1	0.1	0.1	3	3
No. of individuals	10	10, 20, 30	10, 30	300	300
No. of replicates per treatment	5	4c + 4t	3c + 3t	3c + 4t	2c + 3t

c: control (microalgae without *Daphnia*); t: treatment (microalgae with *Daphnia*).
[a] Only for *H. pluvialis*.
[b] g: *H. pluvialis* in green phase; r: *H. pluvialis* in red phase; g + r: mixture of green and red phase.

number of tested microalgal species. In the third experiment, filtration and removal efficiency of *Daphnia* were tested using a larger volume (3 L) and a longer duration (48 h) using the best performing *Daphnia* density found in the two previous experiments. In this experiment, filtration and removal efficiency were evaluated also from chlorophyll-a concentration (Chl-*a*), dry weight, and total cell volume in addition to cell density.

For the first and second filtration efficiency experiments, microalgae were cultivated in WW, centrifuged, and analyzed for initial cell density as described above for the growth experiment. *Daphnia* originated from individuals hatched in the growth experiment and were maintained as described in Section 2.2. For the third filtration efficiency experiment, the microalgae MG and SE were cultivated in 5 L Erlenmeyer bottles with 5 L WW aerated with inlets reaching to the bottom of the bottle, while HP was cultured in 1.5 L plastic funnels with 1.4 L WW aerated from the bottom to avoid aggregation. The bottles were capped with silicone stoppers with inlets consisting of glass tubes. In addition to the aeration inlet, another shorter inlet was used for balancing air pressure. The bottles were aerated constantly as described in Stevčić et al. [6] and mixed manually every day by stirring to keep the cells in suspension and mixed conditions. The funnels for culturing HP were covered with aluminium-foil to reduce evaporation and aerated using identical equipment to the Erlenmeyer cultures. Each microalgal culture bottle (MG, SE) and funnel (HP) was inoculated, cultivated, centrifuged, and analyzed as described for the first two filtration experiments. When all cultures had reached a stationary phase after 4 days, the microalgae were immediately used for the third *Daphnia* filtration experiment.

The first filtration experiment included three densities (100, 200, and 300 ind. L^{-1}) of *Daphnia* adults and two different microalgal diets (MG, SE) (Table 1). Each diet had four replicates for each *Daphnia* density (treatment) and four replicates without *Daphnia* (control), in total 32 jars. Each diet had the same initial cell densities in all replicates within each experiment. The *Daphnia* density range was chosen based on the literature [18,29]. The microalgal cell density was evaluated at 0 h and at the end by cell count from two replicate samples of each replicate jar in a haemocytometer chamber (Bürker) with 100× magnification.

The second filtration experiment included two densities (100 and 300 ind. L^{-1}) of *Daphnia* adults and four different microalgal diets (microalgae MG and SE and two different life phases of HP [HPgreen and HPred]) (Table 1). Each diet had three replicates for each *Daphnia* density (treatment) and three replicates without *Daphnia* (control), in total 36 jars. Microalgal cell density was evaluated at 0 h and at the end as described above for the first filtration experiment.

Based on results from the two first experiments, the *Daphnia* density (100 ind. L^{-1}) was chosen for the third filtration experiment with three different microalgal diets (mixture of HPgreen and HPred, MG, SE) (Table 1). Each diet had four replicates with *Daphnia* (treatment) and three replicates without *Daphnia* (control), in total 21 bottles. The bottles were placed in random order, covered with aluminium foil to prevent water evaporation, and mixed daily to reduce microalgal sedimentation. Microalgae were analyzed at 0 h and after 48 h for cell density, Chl-*a*, dry weight, and total cell volume. Cell density was evaluated with counting chamber as described above. The Chl-*a* was determined spectrophotometrically with Shimadzu Spectrophotometer (UV-1800, Japan) from samples filtered on a fiber filter (GF/A, Whatman) according to Keskitalo and Salonen [30]. Dry weight was measured by filtering a known volume of culture through a pre-weighed fiber filter (GF/A, Whatman). Cell volume was assessed with CASY Electronic Cell Counter and Analyzer (OLS-OMNI Life Science GmbH & Co. KG, Germany) by limiting the size range of particles to cover the mean cell diameter distributions measured in preliminary tests (6.76 ± 0.11, 10.77 ± 0.21, 18.24 ± 0.19, 4.81 ± 0.03, and 3.42 ± 0.05 μm for CR, HPgreen, HPred, MG, and SE, respectively).

2.3.3. Evaluation of nutrient release to water after Daphnia filtering

The change of nutrient concentrations in WW after *Daphnia* filtration was assessed in an experiment using the same density (100 ind. L^{-1}) of *Daphnia* adults and the same volume (3 L) as in the third filtration efficiency experiment (Table 1). The microalgae MG and SE were cultivated as in the third filtration experiment but with 3 L of WW. Each diet had three replicates with *Daphnia* (treatment) and two replicates without *Daphnia* (control), in total 10 bottles. Microalgae were examined at 0 h and after 72 h for cell density with counting chamber as in the previous experiments. Nitrate-nitrogen (NO_3-N), phosphate-phosphorus (PO_4-P), and pH were analyzed in WW before insertion of *Daphnia* and at the end of the experiment with mobile laboratory spectrophotometer (LASA 100, Dr. Lange, Germany) accompanied with testing kits (LCK 339 for NO_3-N and LCK 349 for PO_4-P; Hach, USA) and with pH meter 744 (Metrohm AG, Switzerland). The culture samples were pre-filtered using 0.22 μm syringe filters to separate the microalgae and suspended solids before each nutrient analysis, and all analyses were performed according to the manufacturer's instructions. *Daphnia* growth was evaluated

from six replicate samples of ten randomly collected individuals from the stock cultures before insertion of *Daphnia* in bottles and ten *Daphnia* from each bottle at the end of the experiment. Samples of ten *Daphnia* were pooled and weighed in the same manner as described for the growth experiment.

2.4. Calculations and data analyses

The individual *Daphnia* juvenile growth rate (*JGR*) per day (d^{-1} ind.$^{-1}$) was calculated from the change in their individual dry weight as in Lampert and Trubetskova [31] (Appendix B). Clearance and ingestion rates of *Daphnia* were calculated for each microalga from the change in microalgal concentration (cell density, Chl-a, dry weight, total cell volume) between the start and end of the experiment in each treatment bottle compared to a mean microalgal concentration of control bottles. Microalgal growth in treatment bottles with *Daphnia* was accounted for in calculations of clearance and ingestion rates by measuring the growth of microalgae in control bottles without *Daphnia*. The true or theoretical filtering rate of waterfleas or volume of water passing through the waterflea's maxillary filter per unit of time cannot be directly measured [24,32]. Hence, the clearance rate (*F*) or volume of medium cleared of microalgae per unit time per individual of *Daphnia* (mL h^{-1} ind.$^{-1}$) was calculated as in Frost [32] (Appendix B). The ingestion rate (*I*) or concentration of microalgae consumed per *Daphnia* per unit of time (from cell density: 10^6 cells h^{-1} ind.$^{-1}$; from Chl-*a*: μg h^{-1} ind.$^{-1}$; from dry weight: μg h^{-1} ind.$^{-1}$; from total cell volume: 10^6 μm^3 h^{-1} ind.$^{-1}$) was calculated as in Frost [32] (Appendix B). Relative change (%) of microalgal concentration (cell density, Chl-*a*, dry weight, total cell volume) was calculated between final microalgal concentrations in control and treatment. The Chl-*a* (μg L^{-1}) was calculated as in Keskitalo and Salonen [30] (Appendix B).

The differences in *Daphnia* growth rate when fed on different microalgal diets (two microalgal species and two microalgal cultivation media (WW and MWC) were tested with two-way ANOVA (2-ANOVA). 2-ANOVA was also used to test the possible differences in filtration (clearance and ingestion rate) and removal efficiency between different microalgae diets and between different *Daphnia* densities. For pairwise comparisons, we employed Simple effects tests with Bonferroni corrections. One-way ANOVA (1-ANOVA) was used to compare the effect of different microalgal diets on the filtration (clearance and ingestion rate) and removal efficiency of the *Daphnia* in the third filtration efficiency experiment, and pairwise differences between microalgal species were tested with LSD *post-hoc* test. Moreover, 1-ANOVA was used to compare microalgal concentrations measured with four different methods (change in cell density, Chl-a concentration, dry weight, and total cell volume) between controls and treatments with *Daphnia* in the same experiment. Welch ANOVA was used to compare changes of microalgal cell densities and nutrient concentrations during the nutrient release experiment. The normality of the data was tested with Shapiro–Wilk's test and homogeneity of variances using Levene's test. In case of non-normality and/or heteroscedasticity of the data, we used Welch ANOVA with Games-Howell's test for pairwise comparisons. In case of non-normality and/or heteroscedasticity where non-parametric test showed a similar result as a parametric test, the parametric test was reported. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Statistical analyses were conducted using IBM SPSS (version 24.0; IBM 2016) software.

3. Results

3.1. Daphnia growth on microalgae cultivated in recirculating aquaculture system wastewater and reference medium

Daphnia increased their dry weights more when fed with four different green microalgae (CR, HP, MG, SE) cultivated in reference medium (MWC) (~4 times their initial weight) than when fed with microalgae cultivated in WW (~3 times their initial weight) ($p < 0.05$; Fig. 1; Table A.2). The initial dry weight of *Daphnia* (day 0) was 10 ± 0.8 μg. *Daphnia JGR* differed when fed with four different microalgae ($p < 0.05$) and there was a significant interaction between cultivating media and microalgae species ($p < 0.05$; Table A.2). The growth rate of *Daphnia* did not differ when fed with MG and SE cultivated either in WW or MWC (pairwise tests; $p > 0.05$; Fig. 1). However, growth rates of *Daphnia* were higher when fed with CR and HP cultivated in MWC than when fed with CR and HP cultivated in WW (pairwise tests; $p < 0.05$). When fed with microalgae grown in MWC, *Daphnia* growth rates were higher when fed with HP (0.46 ± 0.04 d^{-1} ind.$^{-1}$) than when fed with MG (0.38 ± 0.03 d^{-1} ind.$^{-1}$) and CR (0.40 ± 0.03 d^{-1} ind.$^{-1}$) (pairwise tests; $p < 0.05$; Fig. 1). *Daphnia* fed with microalgae grown in WW had the lowest growth rate when fed with CR (0.30 ± 0.04 d^{-1} ind.$^{-1}$) (pairwise tests; $p < 0.05$; Fig. 1).

3.2. Daphnia removal efficiency of microalgae cultivated in recirculating aquaculture system wastewater

In the two filtration experiments to assess the most efficient *Daphnia* density in removing microalgae from WW, we found that the *Daphnia* density 100 ind. L^{-1} resulted in the highest clearance rates in both experiments (Fig. 2a,d; Tables A.2 & A.3). The clearance rate was higher for SE than for MG in both experiments (pairwise tests; $p < 0.05$; Table A.3), while the clearance rates for either type of HP cells fell in between MG and SE in the second experiment (pairwise tests; $p < 0.05$; Fig. 2d). Higher clearance rates for SE resulted also in higher ingestion rates and higher relative change in cell density as compared to MG in the first experiment (Table A.3). In the first experiment, the higher clearance and ingestion rates for the lowest *Daphnia* density (100 ind. L^{-1}) resulted in similar relative changes than for the higher *Daphnia* densities with both microalgal diets (pairwise tests; $p < 0.05$; Fig. 2c). In the second filtration experiment, the ingestion rate was higher for SE than for the other three microalgae diets tested (pairwise tests; $p < 0.05$; Fig. 2e; Table A.4). For MG and SE the ingestion rates were higher for the lower *Daphnia* density, and the relative change did not differ from that achieved with higher *Daphnia* density (pairwise tests; $p < 0.05$; Fig. 2e, f). For the two different HP cell types (green and red) different *Daphnia* densities did not affect ingestion rates for either cell type nor the relative change for HPgreen, while the relative change was higher with the higher *Daphnia* density for HPred cells (pairwise tests; $p < 0.05$; Fig. 2f).

In the third experiment, we evaluated microalgae removal in a larger volume of WW using the 100 ind. L^{-1} *Daphnia* density. We found that

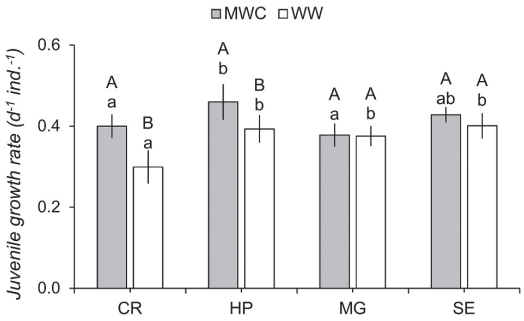

Fig. 1. Juvenile growth rate (d^{-1} ind.$^{-1}$) of *Daphnia magna* after 4 days fed with four different green microalgae species (CR – *Chlamydomonas reinhardtii*, HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) cultivated in two different media (MWC – Modified Wright's Cryptophyte medium, WW – filtered RAS wastewater). Values are presented as mean ± s.d. of five replicates. Values denoted with the same capital letter (A–B) are not significantly different between culture media for each microalgae species, and values denoted with the same small letter (a–b) are not significantly different between microalgae for each culture media (pairwise tests; $p > 0.05$).

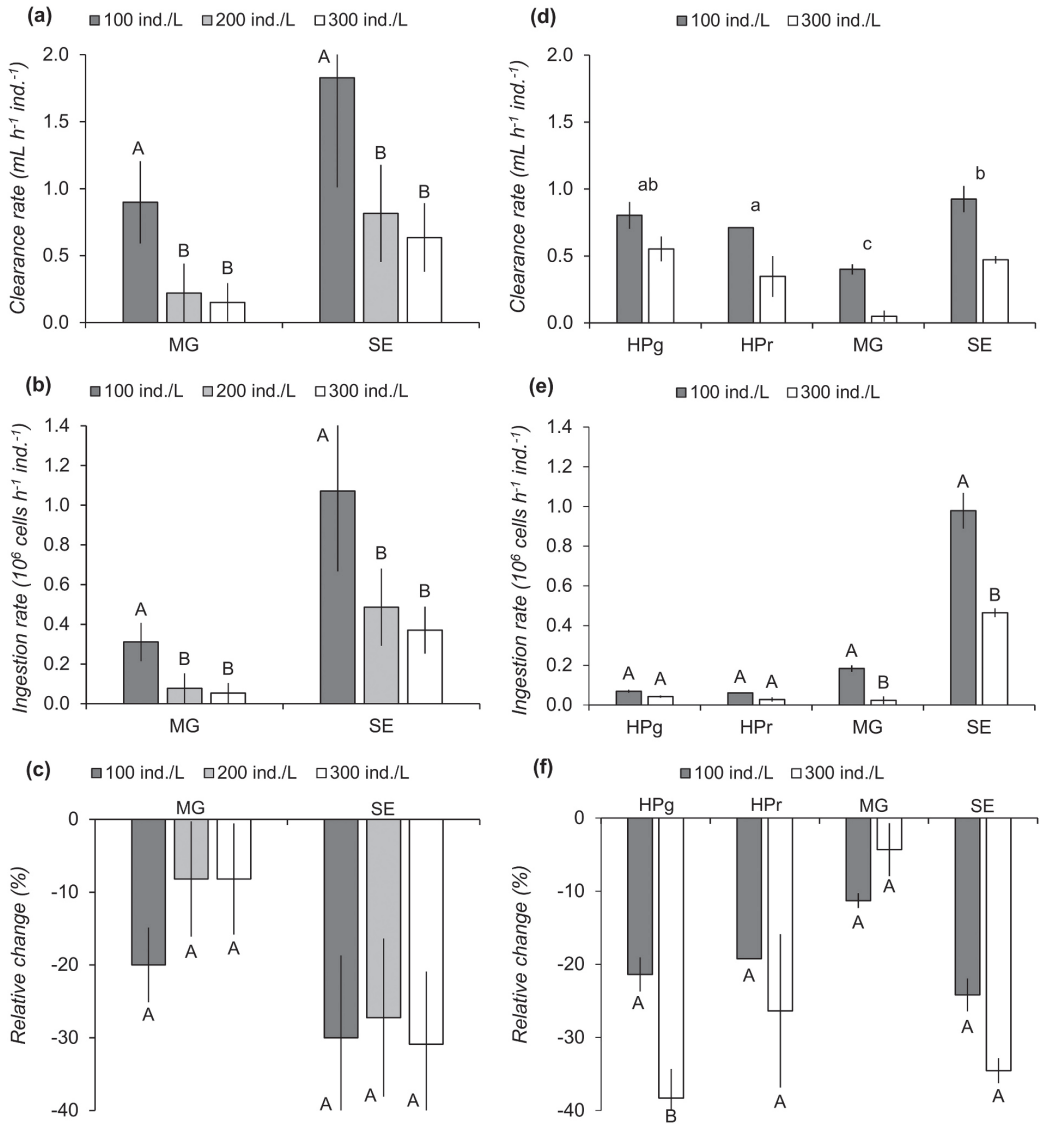

Fig. 2. (**a**) Clearance rate (mL h^{-1} ind.$^{-1}$), (**b**) ingestion rate (10^6 cells h^{-1} ind.$^{-1}$) and (**c**) relative change of microalgal cell density (%) of three densities of waterflea (*Daphnia magna*) (100, 200, and 300 ind. L^{-1}) after 2 h fed with two green microalgae (MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.). (**d**) Clearance rate, (**e**) ingestion rate, and (**f**) relative change of microalgal cell density of two *Daphnia* densities (100 and 300 ind. L^{-1}) after 3 h fed with three green microalgae (HP - *Haematococcus pluvialis*, MG, SE) of which microalga HP is tested in two life phases separately (HPg – HP in green phase, HPr – HP in red phase). Values are presented as mean ± s.d. of four (panels a, b, and c) and three (panels d, e, and f) replicates. Values denoted with the same capital letter (A–B) are not significantly different between *Daphnia* densities for each microalgae species, and the clearance rates in the 2nd experiment (panel d) denoted with the same small letter (a–c) are not significantly different between microalgae for both densities of *Daphnia* (pairwise tests; $p > 0.05$).

Daphnia clearance rates, ingestion rates, and relative change differed between the three green microalgae (HP, MG, SE) with all four methods (change in cell density, Chl-*a* concentration, dry weight, or total cell volume) that we used for measuring the microalgal concentration, except when dry weight was used to estimate ingestion rate (Fig. 3a–f; Table A.5). The clearance rates and relative changes were lower for SE than for MG measured with all four methods except for relative change measured with change in Chl-*a* (Fig. 3a,b). Additionally, the clearance rates with diet SE were lower than with HP measured with all methods, except when measured with change in dry weight, and the relative changes of SE were lower than those of HP measured with change in density and total cell volume (Fig. 3a,b). The ingestion rates tended to be highest for SE measured with change in density, but lowest when measured with change in total cell volume (Fig. 3c,f). Moreover, ingestion rates for MG were higher than for HP with all methods except with dry weight (Fig. 3c–f). Treatment bottles had lower microalgae concentrations than control bottles after 48 h with microalgae MG and HP by using all four methods of measurements (Fig. 3g–j; Table A.6). However, with microalga SE, only cell density was lower in treatment than in control bottles (Fig. 3g).

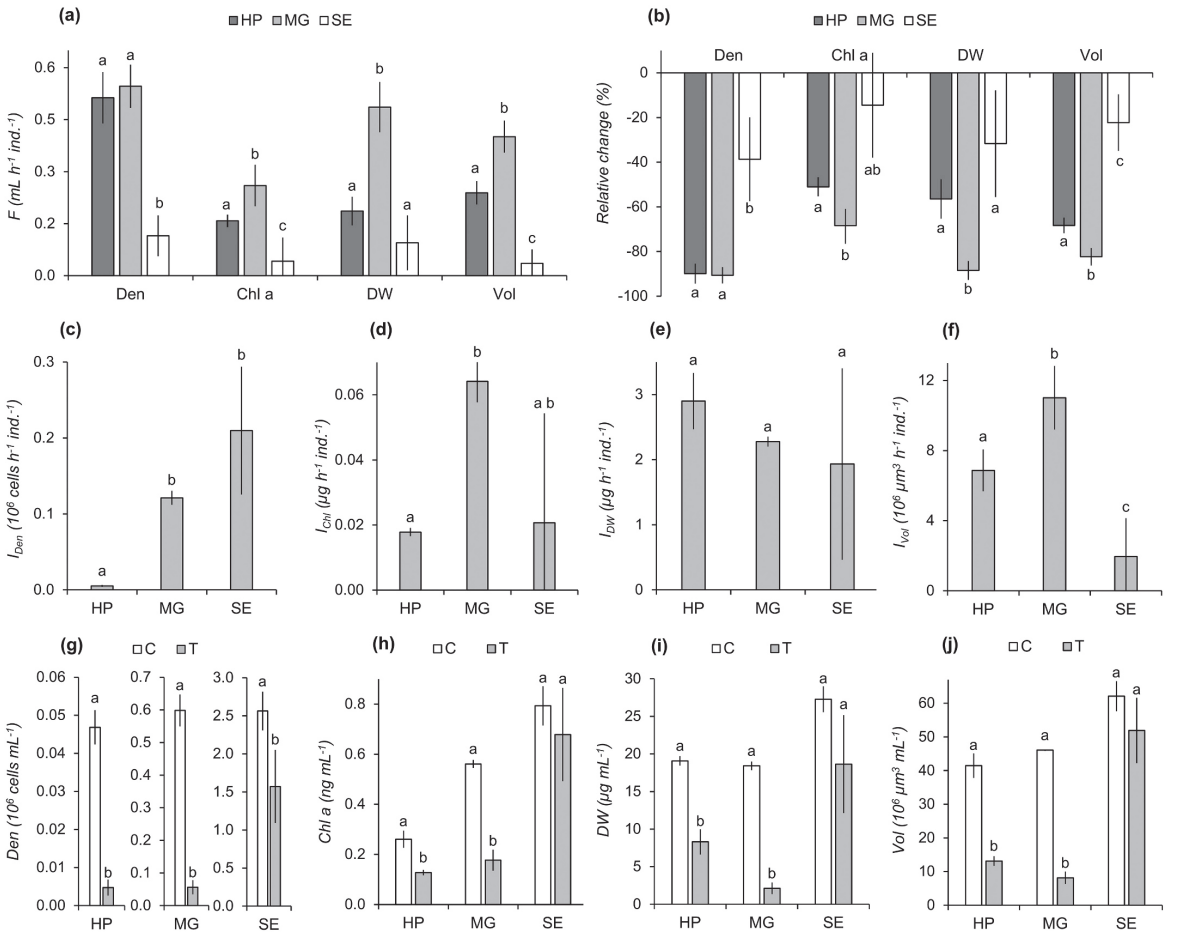

Fig. 3. (**a**) Clearance rate (mL h^{-1} ind.$^{-1}$), (**b**) relative change (%), and (**c**–**f**) ingestion rates of waterflea (*Daphnia magna*) with density of 100 ind. L^{-1} after 48 h fed with three green microalgae species (HP – *Haematococcus pluvialis*, MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) and measured with four different methods of evaluating microalgal concentration (*Den* – cell density, *Chl a* – chlorophyll-a concentration, *DW* – dry weight, *Vol* – total cell volume). Ingestion rates are measured from a change in the (**c**) cell density (10^6 cells h^{-1} ind.$^{-1}$), (**d**) chlorophyll-a concentration (µg h^{-1} ind.$^{-1}$), (**e**) dry weight (µg h^{-1} ind.$^{-1}$), and (**f**) total cell volume (10^6 µm^3 h^{-1} ind.$^{-1}$). Microalgal concentration after 48 h in control (C) and treatment (T) bottles are shown as (**g**) *Den* (10^6 cells mL^{-1}), (**h**) *Chl a* (ng mL^{-1}), (**i**) *DW* (µg mL^{-1}), and (**j**) *Vol* (10^6 µm^3 mL^{-1}). Values are presented as mean ± s.d. of four replicates. Clearance rates, ingestion rates, and relative changes denoted with the same letter (a–c) are not significantly different between microalgae species (pairwise tests; $p > 0.05$). Microalgal concentrations (*Den*, *Chl a*, *DW*, *Vol*) denoted with the same letter (a–b) are not significantly different between control and treatment of each microalga (pairwise tests; $p > 0.05$).

3.3. Evaluation of nutrient release to water after Daphnia filtering

In the nutrient release experiment, the only differences of changes (%) in cell density and nutrients between controls (no *Daphnia*) and treatments (100 *Daphnia* L^{-1}) after 72 h were found for microalga SE ($p < 0.05$; Fig. 4; Table A.7). The change of PO_4-P concentration in SE treatment was higher than in SE control ($p < 0.05$) as it increased in SE treatment bottles from 0.24 ± 0.04 to 0.47 ± 0.03 mg L^{-1} during the experiment (Table A.8). NO_3-N concentration did not change for either microalgae in controls or in treatments ($p > 0.05$; Fig. 4; Table A.8). *Daphnia* growth rates did not differ between MG (0.06 ± 0.08 d^{-1} ind.$^{-1}$) and SE (0.11 ± 0.07 d^{-1} ind.$^{-1}$) after 72 h of feeding ($p > 0.05$). Also, pH in the bottles with *Daphnia* after 72 h was not different between microalgae (MG: 7.48 ± 0.06; SE: 7.27 ± 0.09; $p > 0.05$) nor did it differ between controls (MGc: 8.29 ± 0.45; SEc: 7.33 ± 0.04) and treatments ($p > 0.05$).

4. Discussion

All tested green microalgae (CR, HP, MG, SE) supported the growth of *Daphnia* in filtered RAS WW at water temperature that is common in Nordic RAS (~17 °C). Microalgae cultivated in both media, WW and reference algae medium (MWC), maintained high *JGR* of *Daphnia* (0.4–0.5 d^{-1} ind.$^{-1}$ for WW and 0.3–0.4 d^{-1} ind.$^{-1}$ for MWC). Contrary to the 1st hypothesis that microalgae cultivated in WW and MWC support the growth of *Daphnia* equally well, *Daphnia* growth rate was higher when fed with CR and HP cultivated in MWC than in WW, while the growth media had no effect when *Daphnia* were fed with MG and SE. In line with the 2nd hypothesis that the growth of *Daphnia* differs between microalgal diets, HP induced the highest *JGR* of *Daphnia* (0.5 d^{-1} ind.$^{-1}$) among microalgae species cultivated in MWC, and CR induced the lowest *JGR* among the microalgae previously cultivated in WW (0.3 d^{-1} ind.$^{-1}$). *JGR* of *Daphnia* found in the current study fall within the range found in previous studies with green microalgae belonging to

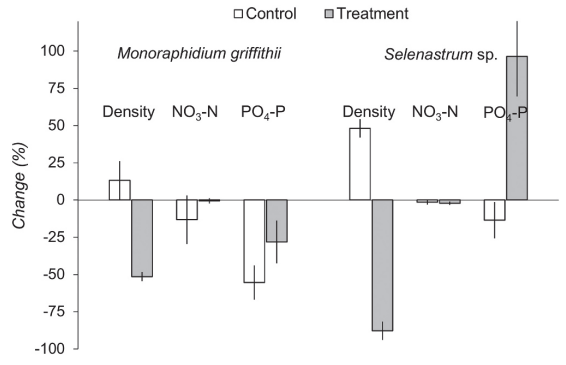

Fig. 4. Change (%) of microalgal cell density, concentration of nitrate-nitrogen (NO_3-N), and phosphate-phosphorus (PO_4-P) after 72 h with two green microalgae (MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) in RAS wastewater for controls without waterfleas (white bars) and treatments with 100 waterfleas (*Daphnia magna*) L^{-1} (grey bars). Values are presented as mean ± s.d. of two (controls) and three replicates (treatments).

the same families as the current tested microalgae (0.3–0.6 d^{-1} ind.$^{-1}$) [22,33–35]. This demonstrates that all tested microalgae species are suitable diets for *Daphnia* when cultured in RAS WW. Moreover, the studies mentioned above conducted at 20 °C reported similar *JGR* as the current study conducted at 17 °C, supporting the possibility of using temperatures below 20 °C for successful growth of *Daphnia*. In wastewaters, high chemical oxygen demand (COD) levels above 160 mg L^{-1} can affect *Daphnia* negatively [54]. Although we did not make direct measurements in our experiment, COD levels reported for Nordic RAS are generally much lower (below 40 mg L^{-1}) [36,37], supporting the suitability of Nordic RAS WW for *Daphnia* culturing.

Our results of lower *Daphnia* growth when fed with HP and CR cultivated in WW than when cultivated in MWC may be explained by the difference in nutrient concentrations between these two media, as WW had half the phosphorus (P) content (~1 mg L^{-1}) of that in MWC (~2 mg L^{-1}) while nitrogen (N) content was slightly higher in WW (~20 mg L^{-1}) than in MWC (~16 mg L^{-1}). Although we did not measure if differences in media nutrient concentrations led to differences in microalgae nutrient concentrations, the finding is consistent with previous studies showing lower *Daphnia* growth with P-deficient diet than with P-sufficient diet [38]. Possibly the biochemical qualities of CR and HP were more affected by the quality of the media than that of MG and SE. Our result of CR diet being the least suitable diet for *Daphnia* when WW was used may be due to a thicker cell wall in P-deficient than in P-sufficient media as shown previously for CR by van Donk et al. [38]. Additionally, in the study by Peltomaa et al. [15] that showed better *Daphnia* growth with microalgae rich in sterols, ω-3 fatty acids, and amino acids, they reported lower concentrations of these biochemical compounds in CR than in MG and SE cultivated in MWC. Finally, our unpublished results suggest that microalga HP has higher content of total amino acids than MG and SE, thus offering a possible explanation of why *Daphnia* growth was higher with HP than with MG cultivated in MWC.

The 3rd hypothesis that the increase in *Daphnia* density negatively affects their clearance and ingestion rates was supported in the first two filtration efficiency experiments, with the lowest density having the highest filtration efficiency. This finding is in line with previous studies with *D. magna* [16,39] and with other *Daphnia* species [23,40,41]. Our results are also in line with previous studies that reported similar ingestion rates of *D. magna* in the density range from 200 to 400 ind. L^{-1} [16,42]. Food concentration was not a limiting factor in any of our experiments, as both initial and final microalgal cell densities were above the reported incipient limiting level for adult *D. magna* with *Chlamydomonas* (0.25×10^6 mL^{-1}) and with *Chlorella* (0.01×10^6 mL^{-1}) [13,43]. Therefore, the lower filtration efficiency in higher *Daphnia* densities was possibly caused by physical interference of direct contact among the individuals or chemically mediated cue [44]. The decrease of clearance and ingestion rates with an increase in density explains why the relative change of microalgae after *Daphnia* feeding did not differ between different density treatments. Our results suggest that the maintenance of maximal *Daphnia* density is not essential for achieving efficient microalgal removal. Thus harvesting of *Daphnia* biomass at intervals e.g. for production of fish feed is feasible.

The results of all three filtration efficiency experiments supported our 4th hypothesis that clearance and ingestions rates of *Daphnia* differ between different microalgal diets. Both the clearance rates (0.1–1.6 mL h^{-1} ind.$^{-1}$) and the ingestion rates ($0.01–1 \times 10^6$ cells h^{-1} ind.$^{-1}$) in our study are within the reported range of clearance and ingestion rates for *D. magna* fed with other green algae, e.g. *Chlamydomonas* (0.1–0.8 mL h^{-1} ind.$^{-1}$ and ~0.04×10^6 cells h^{-1} ind.$^{-1}$) [43] and *Chlorella* (0.03–1 mL h^{-1} ind.$^{-1}$ and $0.1–0.5 \times 10^6$ cells h^{-1} ind.$^{-1}$) [13,45]. However, while the results of the first two filtration experiments in small water volume (100 mL) indicated higher filtration and removal efficiency for SE, the results of the filtration experiment in a larger volume (3 L) suggested, that microalga MG is the most suitable microalgal species for efficient filtration and removal by *Daphnia*. Although there were differences in the initial food concentration, the differences in the outcome of the experiments likely arose from the change in microalgal concentration during the longer duration of the experiment, causing changes in filtration rates. The differences in cell sizes of tested microalgae (HPgreen: 827 ± 67 μm^3; HPred: 3634.5 ± 154.8 μm^3; MG: 63.7 ± 2.3 μm^3; SE: 22.7 ± 1.8 μm^3) likely did not influence the filtration efficiency as they were within the size range where filtration efficiency of *D. magna* is relatively independent on particle size (0.9–18,000 μm^3) [12,13]. Another contributing factor could be the difference in the number of neighboring individuals, which can have a greater impact on the ingestion rate and fecundity than actual density [40]. However, experiments in larger volumes of medium are likely more reliable as the error from variability in individual filtration efficiency is decreased and behavioral artifacts from wall avoidance are reduced [41,46]. However, the experiment in the larger volume was conducted in the dark without previous acclimation of the *Daphnia* to the photoperiod, which can initially increase *Daphnia*'s filtration efficiency [47,48] and lead to an overestimation of the clearance rates. Additionally, *Daphnia*'s filtration efficiencies may have been affected by different sedimentation rates of tested microalgae despite mixing them daily to reduce sedimentation. Yu et al. [49] found that microalgal cells from genus *Monoraphidium* were still present in suspension after 24 h without mixing. However, microalga HP may have sedimented faster than the other microalgae. Nevertheless, *Daphnia* can feed on sedimented microalgal cells [50], thus sedimentation may not have affected filtration efficiency significantly.

Our results concerning clearance rate and relative change are in an agreement with each other, but for ingestion rate, the estimation based on dry weight does not agree with those based on Chl-*a* and total cell volume. Using total cell volume is perhaps among the most accurate methods of the ones used in this study for estimating filtration efficiency of *Daphnia*, as being the only method that had coherent results among microalgae species for both clearance and ingestion rates and relative change as well. Additionally, the estimations based on change in cell density gave opposite results than those based on change in proxies of remaining biomass, i.e. Chl-*a*, dry weight, and total cell volume. However, removal rates can be underestimated when using proxies of remaining biomass due to the breakage of microalgal cells and/or presence of microbes in WW. Use of several different complementary methods to assess clearance and ingestion rates in *Daphnia* have been recommended to overcome the biases caused by differences in microalgal cell sizes when evaluating rates based on changes in cell density

[46] and to avoid conversions between different units that could lead to quite erroneous conclusions [51]. Our results suggest the need of using more than one estimation method of microalgal concentration to operate more accurately the harvesting efficiency by *Daphnia* in microalgae-*Daphnia* bioremediation systems. Also, it is preferable to measure total cell volume together with cell density to avoid limitations and underestimations of these methods.

Contrary to the 5th hypothesis, *Daphnia* increased PO_4-P into WW during their 3-day feeding process with microalga SE (+96.4%) as opposed to 13.5% decrease in controls, while for MG the concentration of PO_4-P decreased in both treatment and control bottles (−28.2% and −55.4%, respectively). No major changes were detected in concentration of NO_3-N. Nutrients are released back to water via breakage of microalgal cells and *Daphnia* excretion, and *Daphnia* excrete P primarily as dissolved phosphate and N mainly as ammonium [24] which could explain why we did not find any change in N content as we have measured only N from nitrate. The results suggest that phosphate was released from SE but not from MG due to more extensive filtering of SE (−87.8%) than of MG (−51.4%) by *Daphnia*, thus breakage of microalgae and metabolic processes of excretion and egestion must have been higher with SE diet. Previous studies on microalgae-filter-feeder systems have reached contradictory results on nutrient removal by *Daphnia*. Some studies indicate that *D. magna* was responsible for 4–13% removal of total phosphorus and 2–14% removal of total nitrogen from WW [8,9], while other studies have not found changes in concentrations of PO_4-P and NO_3-N after *D. magna* feeding on microalgae [52]. One explanation for higher P-release after *Daphnia* feeding on SE diet than on MG diet could be higher P content in SE than in MG [15]. The higher starting N:P ratio with SE (15.7, based on PO_4-P and NO_3-N concentrations) closer to the optimal Redfield ratio (16:1) than with MG (5.3) could provide an explanation why SE grew more (48.1%) than MG (13.1%) in controls, and further support the possibility that MG diet was more P-limited diet than SE, thus *Daphnia* could release more P from SE than from MG.

In addition to the development of bioremediation techniques, a major ongoing development in RAS is the reuse of systems' byproducts (circular economy) [3], i.e. the improvement of mass production methods for acquiring natural and live feed for larval fish until they can be fed with formulated feeds [14,21]. Particularly, *D. magna* is one of the most popular live feed organisms that is suitable for both juvenile and mature fish to a similar extent as dry feeds [21,53]. Mass cultivation of *Daphnia* could be applied to mitigate the overall production costs of RAS and to enhance its sustainability.

5. Conclusion

Cultivating green microalgae in WW supports the growth of *Daphnia* to a comparable degree as cultivating them in microalgae medium, and *Daphnia* is efficient in consuming green microalgae for their growth in WW. *Daphnia* can be successfully grown with green microalgae at ~17 °C, a common temperature in Nordic RAS. *Daphnia* removed over 80% of MG, 70% of HP, but only 20% of SE, within 48 h. Phosphate was released to WW after *Daphnia* feeding on SE, but not while feeding on MG. Taken together, of the four tested green microalgae, *M. griffithii* was found to be the most suitable microalga species for the microalgae-*Daphnia* bioremediation system. Our results support the potential of using microalgae and *Daphnia* for bioremediation of RAS WW and for producing biomass in Nordic RAS.

Informed consent, human/animal rights

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

CRediT authorship contribution statement

ČS: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **KP:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **JP:** Conceptualization, Methodology, Validation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendices. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.102108.

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APPENDIX A. Tables

Table A.1. Freshwater green microalgae (Chlorophycae) strains used in the study.

Species	Order	Strain	Origin
Chlamydomonas reinhardtii	Chlamydomonadales	K-1016 (NIVA)	Amherst, Massachusetts, USA
Haematococcus pluvialis	Chlamydomonadales	K-0084 (NIVA)	Trutbådan, Sweden
Monoraphidium griffithii	Sphaeropleales	NIVA-CHL 8	Lake Årungen, Akershus, Norway
Selenastrum sp.	Sphaeropleales	K-1877 (NIVA)	Lake Iso-Ruuhijärvi, Häme, Finland

Table A.2. Differences in juvenile growth rates of waterfleas (*Daphnia magna*) among four species of green microalgae (Microalga) cultivated in two different media (Medium), tested with two-way ANOVA. *df*: degree of freedom; MS: mean squares; F: value of the F statistic; *p*: significance level. Statistically significant values ($p < 0.05$) are in bold.

Variable	Source	*df*	MS	F	*p*
Juvenile growth rate	Microalga	3	0.012	11.802	**0.000**
	Medium	1	0.024	22.919	**0.000**
	Microalga*Medium	3	0.005	4.441	**0.010**
	Error	32	0.001		

Table A.3. Differences in clearance and ingestion rates of waterfleas (*Daphnia magna*) and in relative change of microalgal cell density among two species of green microalgae (Microalga) and three different densities of waterfleas (Density), tested with two-way ANOVA. *df*: degree of freedom; MS: mean squares; F: value of the F statistic; *p*: significance level. Significant values ($p < 0.05$) are in bold.

Variable	Source	*df*	MS	F	*p*
Clearance rate	Microalga	1	2.200	14.757	**0.002**
	Density	2	1.554	10.423	**0.001**
	Microalga*Density	2	0.077	0.515	0.608
	Error	15	0.149		
Ingestion rate	Microalga	1	1.206	34.892	**0.000**
	Density	2	0.374	10.821	**0.001**
	Microalga*Density	2	0.077	2.242	0.141
	Error	15	0.035		
Relative change	Microalga	1	1758.735	21.741	**0.000**
	Density	2	56.977	0.704	0.510
	Microalga*Density	2	31.567	0.390	0.684
	Error	15	80.893		

Table A.4. Differences in clearance and ingestion rates of waterfleas (*Daphnia magna*) and in relative change of microalgal cell density among four microalgal diets (three species of green microalgae, and one species with two life stages) (Microalga) and two different densities of waterfleas (Density), tested with two-way ANOVA. *df*: degree of freedom; MS: mean squares; F: value of the F statistic; *p*: significance level. Statistically significant values ($p < 0.05$) are in bold.

Variable	Source	*df*	MS	F	*p*
Clearance rate	Microalga	3	0.246	31.415	**0.000**
	Density	1	0.672	85.730	**0.000**
	Microalga*Density	3	0.010	1.310	0.310
	Error	14	0.008		
Ingestion rate	Microalga	3	0.622	465.331	**0.000**
	Density	1	0.179	133.990	**0.000**
	Microalga*Density	3	0.076	56.750	**0.000**
	Error	15	0.001		
Relative change	Microalga	3	543.296	24.909	**0.000**
	Density	1	250.680	11.493	**0.004**
	Microalga*Density	3	131.974	6.051	**0.007**
	Error	14	21.812		

Table A.5. Differences in clearance rate (*F*), relative change (*R*), and ingestion rate (*I*) of waterfleas (*Daphnia magna*) among three species of green microalgae measured with four different methods of evaluating microalgal concentration (*Den*: cell density; *Chl a*: chlorophyll-a concentration; *DW*: dry weight; *Vol*: total cell volume), tested with one-way ANOVA and Welch ANOVA. *df*: degree of freedom; F: value of the F statistic; *p*: significance level. Statistically significant values ($p < 0.05$) are in bold.

Variable	Analysis	F	*df1*	*df2*	*p*
F-Den	ANOVA	53.463	2	9	**0.000**
F-Chl a	ANOVA	15.101	2	9	**0.002**
F-DW	ANOVA	37.081	2	9	**0.000**
F-Vol	ANOVA	80.389	2	9	**0.000**
R-Den	Welch ANOVA	13.16	2	5.337	**0.009**
R-Chl a	Welch ANOVA	10.669	2	3.831	**0.027**
R-DW	Welch ANOVA	26.496	2	4.808	**0.003**
R-Vol	Welch ANOVA	33.327	2	4.106	**0.003**
I-Den	Welch ANOVA	267.113	2	4.102	**0.000**
I-Chl a	Welch ANOVA	83.09	2	3.338	**0.001**
I-DW	Welch ANOVA	3.566	2	4.125	0.126
I-Vol	ANOVA	25.897	2	9	**0.000**

Table A.6. Differences in three green microalgal (MG, SE, HP) concentrations (*Den*: cell density; *Chl a*: chlorophyll-a concentration; *DW*: dry weight; *Vol*: total cell volume) between controls and treatments with waterfleas (*Daphnia magna*), tested with one-way ANOVA and Welch ANOVA. *df*: degree of freedom; F: value of the F statistic; *p*: significance level. Statistically significant values ($p < 0.05$) are in bold.

Variable	Analysis	F	*df1*	*df2*	*p*
HP-Den	ANOVA	118.025	1	6	**0.000**
HP-Chl a	ANOVA	57.683	1	6	**0.001**
HP-DW	ANOVA	106.63	1	6	**0.000**
HP-Vol	ANOVA	211.142	1	6	**0.000**
MG-Den	ANOVA	382.977	1	6	**0.000**
MG-Chl a	ANOVA	259.286	1	6	**0.000**
MG-DW	ANOVA	917.653	1	6	**0.000**
MG-Vol	ANOVA	1224.957	1	6	**0.000**
SE-Den	ANOVA	10.196	1	6	**0.024**
SE-Chl a	ANOVA	1.052	1	5	0.363
SE-DW	Welch ANOVA	6.432	1	3.534	0.072
SE-Vol	Welch ANOVA	3.464	1	4.414	0.129

Table A.7. Differences in changes of microalgal cell density (*Den%*), concentrations of nitrate-nitrogen (NO_3-N, *N%*) and phosphate-phosphorus (PO_4-P, *P%*) after 72 hours between bottles without (control) and with waterfleas (*Daphnia magna*) (treatment) for two species of microalgae, tested with Welch ANOVA. *df*: degree of freedom; F: value of the F statistic; *p*: significance level. Statistically significant values ($p < 0.05$) are in bold.

Variable	Analysis	F	*df1*	*df2*	*p*
Monoraphidium griffithii					
Den%	Welch ANOVA	46.494	1	1.114	0.076
N%	Welch ANOVA	1.195	1	1.023	0.468
P%	Welch ANOVA	4.398	1	1.911	0.177
***Selenastrum* sp.**					
Den%	Welch ANOVA	594.943	1	2.290	**0.001**
N%	Welch ANOVA	0.326	1	1.627	0.637
P%	Welch ANOVA	38.185	1	2.885	**0.010**

Table S.8. Characteristics of RAS wastewater (WW) during the microalgal and *Daphnia magna* cultivation in the nutrient experiment. Values are presented as mean ± s.d. from all replicates of control [C] and treatment [T] bottles separately. *n.m.*: not measured.

	Start of microalgal culture	End of microalgal culture	Start of *Daphnia* culture	End of *Daphnia* culture

Sampling point (d)	0	4	4	7
Monoraphidium griffithii				
NO_3-N [C] (mg L^{-1})	7.78±0.08	3.07±2.06	3.07±2.06	2.83±2.29
NO_3-N [T] (mg L^{-1})	7.78±0.08	4.24±0.30	3.98±0.15	3.96±0.08
PO_4-P [C] (mg L^{-1})	0.737±0.001	0.247±0.298	0.247±0.298	0.127±0.161
PO_4-P [T] (mg L^{-1})	0.737±0.001	0.360±0.091	0.345±0.075	0.244±0.004
N:P molar ratio [C]	4.8±0.0	12.5±11.3	12.5±11.3	25.3±24.0
N:P molar ratio [T]	4.8±0.0	5.4±1.0	5.3±1.0	7.3±0.0
pH [C]	7.39±0.08	*n.m.*	*n.m.*	8.29±0.45
pH [T]	7.39±0.08	*n.m.*	9.87±0.17	7.48±0.06
***Selenastrum* sp.**				
NO_3-N [C] (mg L^{-1})	10.33±0.15	9.11±0.57	9.11±0.57	8.98±0.71
NO_3-N [T] (mg L^{-1})	10.33±0.15	8.62±0.15	8.18±0.19	8.00±0.10
PO_4-P [C] (mg L^{-1})	0.672±0.002	0.369±0.141	0.369±0.141	0.328±0.167
PO_4-P [T] (mg L^{-1})	0.672±0.002	0.251±0.041	0.241±0.044	0.465±0.035
N:P molar ratio [C]	7.0±0.0	11.9±3.8	11.9±3.8	14.0±6.1
N:P molar ratio [T]	7.0±0.0	15.8±2.6	15.7±2.7	7.8±0.5
pH [C]	7.33±0.01	*n.m.*	*n.m.*	7.33±0.04
pH [T]	7.33±0.01	*n.m.*	7.29±0.08	7.27±0.09

APPENDIX B. Calculations

The individual *Daphnia* juvenile growth rate (*JGR*) per day (d^{-1} $ind.^{-1}$) was calculated from the change in their individual dry weight according to the following equation: $JGR = \frac{\ln DW_1 - \ln DW_0}{\Delta t}$, where Δt is the length of the experiment ($t_1 - t_0$) (d), and DW_0 and DW_1 are dry weights (µg $ind.^{-1}$) at the beginning and the end of the experiment (Lampert and Trubetskova, 1996).

The clearance rate (*F*) or volume of medium cleared of microalgae per unit time per individual of *Daphnia* (mL h^{-1} $ind.^{-1}$) was calculated as (Frost, 1972): $F = \frac{V\,g}{N}$, where *V* is the volume of medium (mL), *g* is the grazing coefficient of *Daphnia* (h^{-1}), and *N* is the number of *Daphnia*.

The ingestion rate (*I*) or concentration of microalgae consumed per *Daphnia* per unit of time (from cell density: 10^6 cells h^{-1} $ind.^{-1}$; from chlorophyll-a concentration: µg h^{-1} $ind.^{-1}$; from dry weight: µg h^{-1} $ind.^{-1}$; from total cell volume: 10^6 μm^3 h^{-1} $ind.^{-1}$) was calculated as (Frost, 1972): $I = C \times F$, where *C* is the average concentration of microalgae and *F* is the clearance rate of a *Daphnia* (mL h^{-1} $ind.^{-1}$).

The growth constant for microalgal growth in control (k, h^{-1}) was calculated from the equation (Frost, 1972): $C_2^* = C_1^*\, e^{k(t_2 - t_1)}$, where C_1^* and C_2^* are microalgal concentrations (cell density: 10^6 cells mL^{-1}; chlorophyll-a concentration: µg mL^{-1}; dry weight: µg mL^{-1}; total cell volume: 10^6 μm^3 mL^{-1}) in the control bottle at the starting (t_1) and end point (t_2) of the measurement period. The grazing coefficient of *Daphnia* (g, h^{-1}) was calculated from the equation (Frost, 1972): $C_2 = C_1\, e^{(k-g)(t_2 - t_1)}$, where C_1 and C_2 are microalgal concentrations in the treatment bottle at the starting (t_1) and end point (t_2) of the measurement period. The average concentration of microalga in treatment bottle (C) was calculated from the equation (Frost, 1972): $C = \frac{C_1\,[e^{(k-g)(t_2 - t_1)} - 1]}{(t_2 - t_1)(k-g)}$.

The chlorophyll-a concentration (*Chl a*; µg L^{-1}) was calculated as: $Chl\ a = 11.9 * A * \frac{V_e}{V_s * d}$, where *11.9* is the calculation constant (derived using 83.4 L g^{-1} cm^{-1} as the absorption coefficient (*Kc*) of chlorophyll-a in 94% ethanol: (11.9 = (1 / *Kc*) * 1000), *A* = A665 – A750 (the difference between chlorophyll-a absorption at 665 nm and 750 nm of light), *Ve* is the volume of ethanol (mL), *Vs* is the volume of microalgae (mL), and the *d* is the spectrophotometer cuvette width (cm) (Keskitalo and Salonen, 1994).