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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 48h, *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 72h 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.

KEYWORDS: Cladocera, filtration rate, nutrient recycling, phytoplankton, RAS, wastewater bioremediation

INTRODUCTION

Recirculating Aquaculture Systems (RAS) reduce the water use significantly, with constant wastewater treatment and reuse, compared to traditional flow-through systems (Bregnballe, 2015). 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 (Piedrahita, 2003; Bregnballe, 2015). 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 (Piedrahita, 2003). 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*) (Bregnballe, 2015).

Treatment of aquaculture wastewater is expensive, thus improvements of bioremediation are among the major ongoing developments in RAS (Martins *et al.*, 2010). By assimilating both inorganic and organic nutrients, microalgae are among the best cost-effective and sustainable organisms for bioremediation of aquaculture WW (Martins *et al.*, 2010). The produced biomass can be later used as animal feed, human food and health products, agricultural fertilizer, and biofuel (Mata *et al.*, 2010). Bioremediation of aquaculture WW by microalgae is mostly limited to warmer geographical locations (*e.g.* Egloff *et al.*, 2018), but their efficiency in cold and temperate climates (below 20°C) has also been demonstrated (Stevčić *et al.*, 2019).

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 (Grima *et al.*, 2003). 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 (Kim *et al.*, 2003; Jung *et al.*, 2009). 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 (Holdt and Edwards, 2014). 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 (McMahon and Rigler, 1965; Burns, 1968; DeMott, 1982). Microalgae are easily digested and provide a source of essential mineral nutrients and biomolecules for *Daphnia* (*e.g.* sterols, fatty acids, amino acids, carotenoids) (Das *et al.*, 2012; Peltomaa *et al.*, 2017). 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 (Pau *et al.*, 2013; Nørgaard and Roslev, 2016). 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) (Maceda-Veiga *et al.*, 2015; Serra *et al.*, 2019a). 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) (Piedrahita, 2003; Bregnballe, 2015; Stevčić *et al.*, 2019), and the levels are further reduced after bioremediation with microalgae (Stevčić *et al.*, 2019). Although the filtration efficiency of *D. magna* is influenced negatively by temperatures below 20°C (McMahon and Rigler, 1965), the larger body size and higher longevity of *Daphnia* in temperatures below 20°C induce higher overall ingestion rates (Sodré and Bozelli, 2019). Combining carefully selected microalgae and *D. magna* could therefore provide a suitable system for bioremediation of cold-water RAS WW (*e.g.* Cheban *et al.*, 2018).

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 (Stevčić *et al*., 2019). 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 (McMahon and Rigler, 1965; Matveev, 1993; Marzetz *et al*., 2017). 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 (Lampert and Sommer, 2007). 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 (Stevčić *et al.*, 2019). Stock cultures were maintained in algae medium MWC (Modified Wright's Cryptophyte based on Guillard and Lorenzen (1972)) as described previously (Stevčić *et al.*, 2019). 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) (Shah *et al.*, 2016). 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 (Klüttgen *et al.*, 1994)] 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.* (2019).

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*. (2019). HP was cultured in 270 mL batch culture in 300 mL glass funnels to avoid cell aggregates and attachment to the walls (Stevčić *et al.*, 2019). 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 24h period was dried at 60°C for 12h 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} (Rocha & Duncan, 1985) (Table 1). Ten *Daphnia* hatched within the last 24h 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 24h 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 (2h and 3h) and 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 (48h) 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*. (2019) 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 (Yin *et al.*, 2010; Maceda-Veiga *et al.*, 2015). The microalgal cell density was evaluated at 0h and at the end by cell count from two replicate samples of each replicate jar in a haemocytometer chamber (Bürker) with 100x 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 0h 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 aluminum foil to prevent water evaporation, and mixed daily to reduce microalgal sedimentation. Microalgae were analyzed at 0h and after 48h 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 (1994). 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 0h and after 72h 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 (1996) (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 (Frost, 1972; Lampert and Sommer, 2007). 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 (1972) (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 (1972) (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 (1994) (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 *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 48h 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).

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 72h 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 72h of feeding (p >0.05). Also, pH in the bottles with *Daphnia* after 72h 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 the same families as the current tested microalgae (0.3-0.6 d^{-1} $ind.^{-1}$) (Mitchell *et al.*, 1992; Tessier and Goulden, 1987; DeMott, 2003; Marzetz *et al.*, 2017). 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 (Pous *et al*., 2020). 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}) (Rojas-Tirado *et al*., 2017; Kiani *et al*., 2020), 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 (van Donk *et al.*, 1997). 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.* (1997). Additionally, in the study by Peltomaa *et al.* (2017) 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* (Clément and Zaid, 2004; Nørgaard and Roslev, 2016) and with other *Daphnia* species (Helgen, 1987; Matveev, 1993; Ban *et al*., 2008). 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} (Myrand and de la Noüe, 1983; Nørgaard and Roslev, 2016). 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}) (McMahon and Rigler, 1965; Porter *et al*., 1982). 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 (Goser and Ratte, 1994). 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 (0.01-1×10^6 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} $ind.^{-1}$) (Porter *et al*., 1982) and *Chlorella* (0.03-1 mL h^{-1} $ind.^{-1}$ and $0.1\text{-}0.5\times10^6$ cells h^{-1} $ind.^{-1}$) (Ryther, 1954; McMahon and Rigler, 1965). 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 (0.9-18000 μm^3) (McMahon and Rigler, 1965; DeMott, 1982). 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 (Ban *et al*., 2008). 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 (Peters, 1984; Helgen, 1987). 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 (Buikema, 1973; Serra *et al*., 2019b) 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*. (2012) found that microalgal cells from genus *Monoraphidium* were still present in suspension after 24h without mixing. However, microalga HP may have sedimented faster than the other microalgae. Nevertheless, *Daphnia* can feed on sedimented microalgal cells (Siehoff *et al*., 2009), 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 (Peters, 1984) and to avoid conversions between different units that could lead to quite erroneous conclusions (Kiørboe *et al*., 1985). 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 (Lampert and Sommer, 2007) 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 (Kim *et al*., 2003; Jung *et al*., 2009), while other studies have not found changes in concentrations of PO_4-P and NO_3-N after *D. magna* feeding on microalgae (Sevrin-Reyssac, 1998). 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 (Peltomaa *et al.*, 2017). 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) (Martins *et al.*, 2010), *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 (Das *et al.*, 2012; Cheban *et al.*, 2018). 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 (Proulx and Noüe, 1985; Cheban *et al.*, 2018). 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 48h. 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.

CRediT authorship contribution statement

Čedomir Stevčić: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Katja Pulkkinen**: Conceptualization, Methodology, Validation, Formal analysis, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **Juhani Pirhonen**: Conceptualization, Methodology, Validation, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Informed consent, human/animal rights

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

Declaration of interest

None.

Appendices A and B. Supplementary data

Supplementary data to this article can be found online at.

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small letter (a-c) are not significantly different between microalgae for both densities of *Daphnia* (pairwise tests; $p > 0.05$).

Figure 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 48h 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 48h 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; $p > 0.05$).

Figure 4. Change (%) of microalgal cell density, concentration of nitrate-nitrogen (NO_3-N), and phosphate-phosphorous (PO_4-P) after 72h with two green microalgae (MG – *Monoraphidium griffithii*, SE – *Selenastrum* sp.) in RAS wastewater for controls without waterfleas (MGc and SEc) and treatments with 100 waterfleas (*Daphnia magna*) L^{-1} (MGt

and SEt). Values are presented as mean±s.d. of two (controls) and three replicates (treatments).

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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*	110-130	110-130; 80-100*	110-130; 80-100*	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)†	*n.a.*	0.095 (g); 0.089 (r)†	0.020 (g+r)†	*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

* only for *H. pluvialis*.

† g: *H. pluvialis* in green phase; r: *H. pluvialis* in red phase; g+r: mixture of green and red phase.

c: control (microalgae without *Daphnia*); t: treatment (microalgae with *Daphnia*)

APPENDICES

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

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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. Remarked in bold, statistically significant values ($p < 0.05$).

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. Remarked in bold, statistically significant values ($p < 0.05$).

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. Remarked in bold, statistically significant values ($p < 0.05$).

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. Remarked in bold, statistically significant values ($p < 0.05$).

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. Remarked in bold, statistically significant values ($p < 0.05$).

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-phosphorous (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. Remarked in bold, statistically significant values ($p < 0.05$).

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

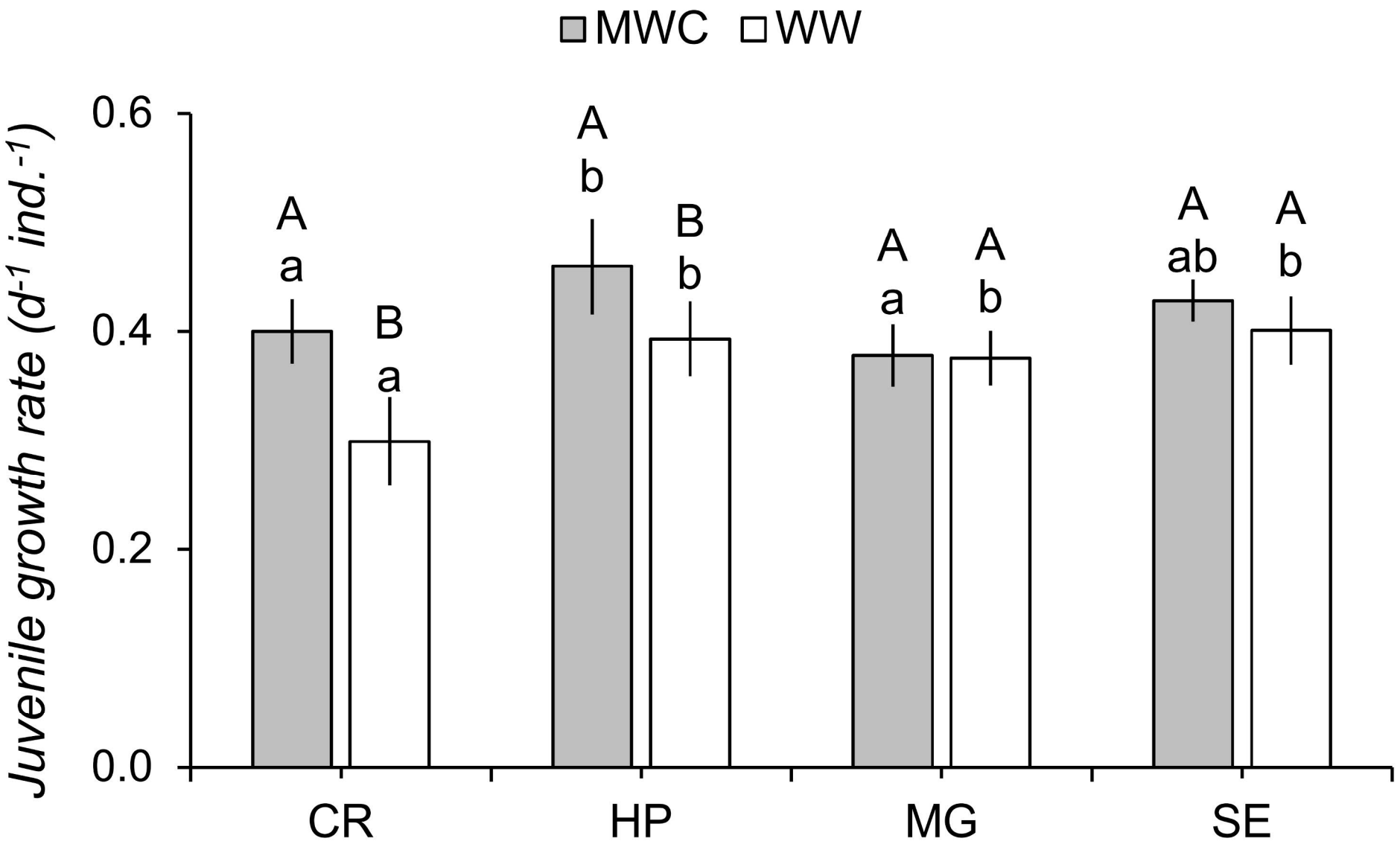
The individual *Daphnia* juvenile growth rate (*JGR*) per day (d^{-1} $ind.^{-1}$) was calculated from the change in their individual dry weight according to the following equation: $JGR = \frac{\ln DW_1 - \ln DW_0}{\Delta t}$, where Δt is the length of the experiment ($t_1 - t_0$) (d), and DW_0 and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

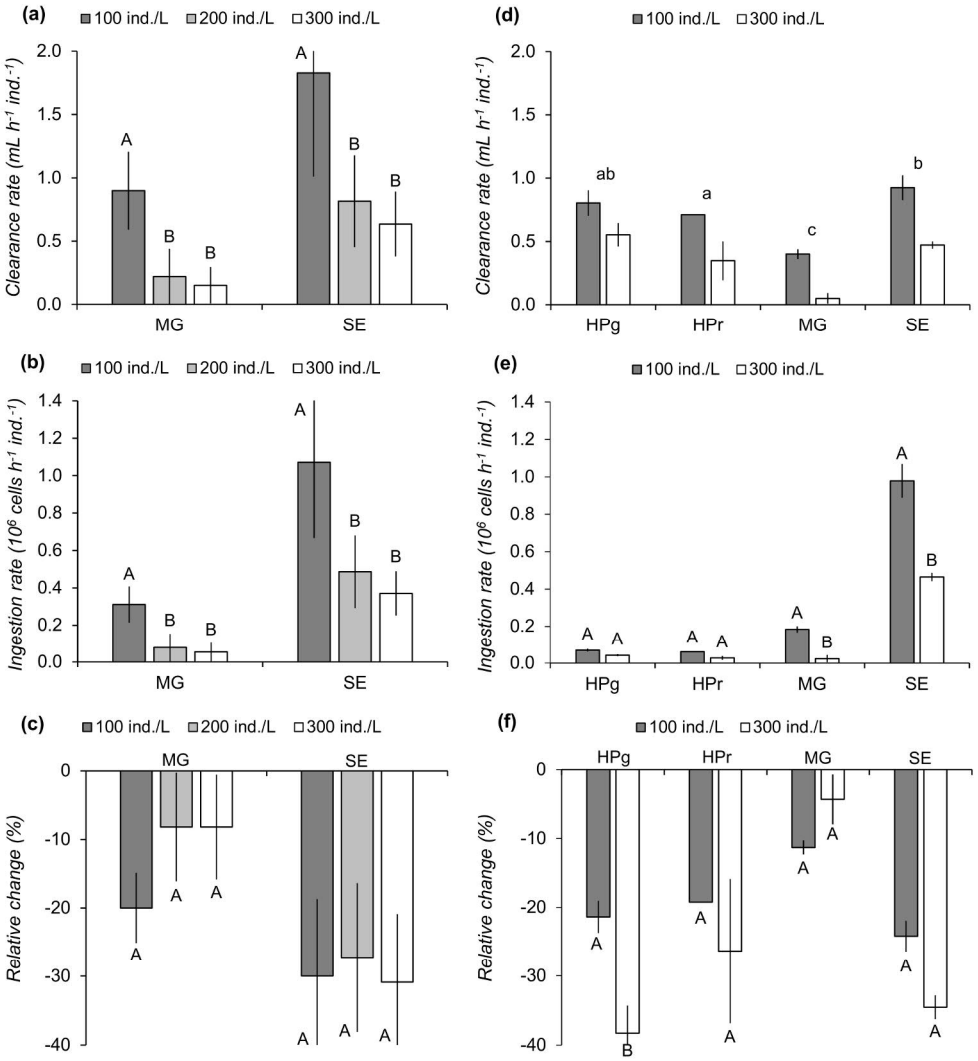
The clearance rate (*F*) or volume of medium cleared of microalgae per unit time per individual of *Daphnia* (mL h^{-1} $ind.^{-1}$) was calculated as (Frost, 1972): $F = \frac{V\,g}{N}$, where *V* is the volume of medium (mL), *g* is the grazing coefficient of *Daphnia* (h^{-1}), and *N* is the number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

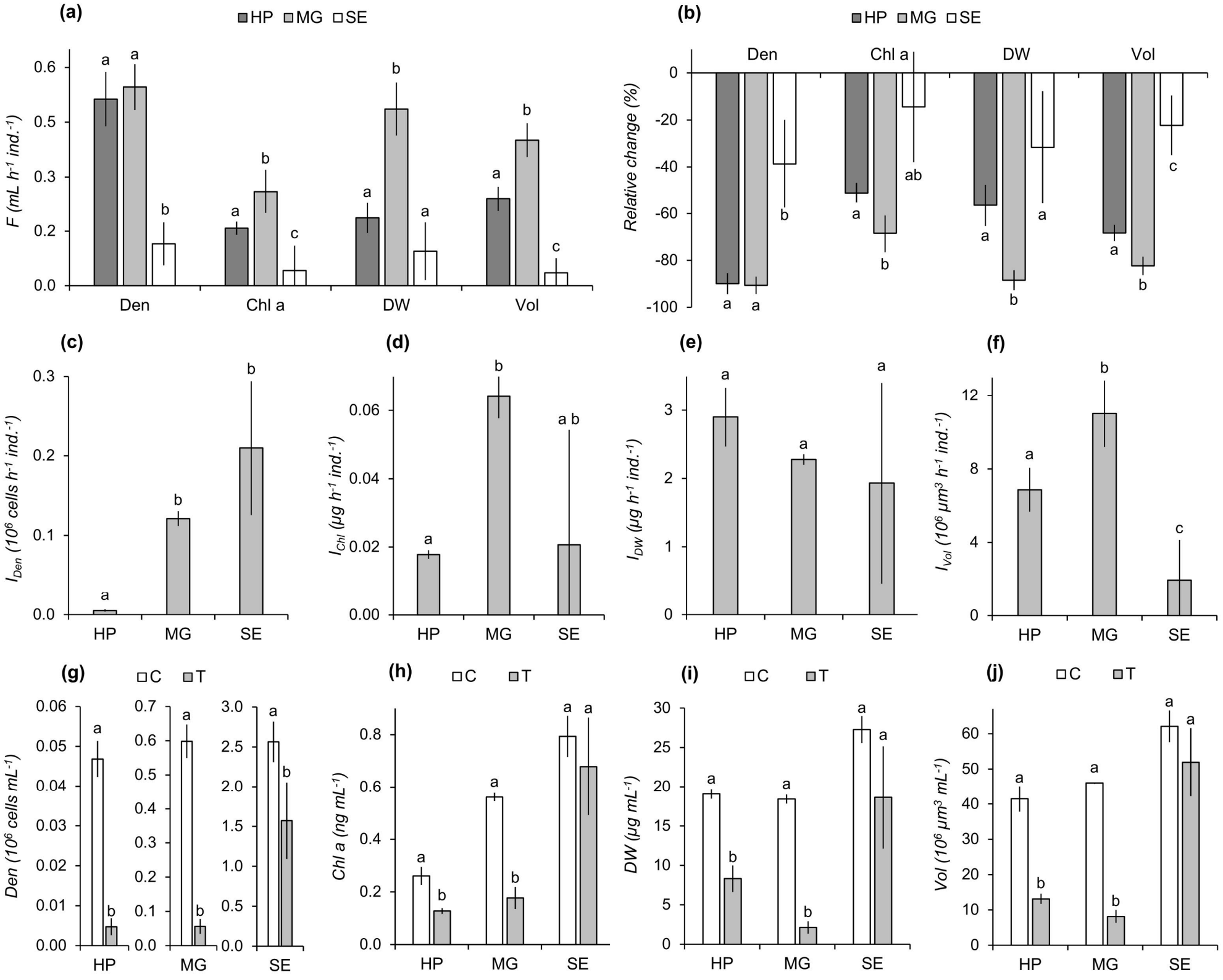
The ingestion rate (*I*) or concentration of microalgae consumed per *Daphnia* per unit of time (from cell density: 10^6 cells h^{-1} $ind.^{-1}$; from chlorophyll-a concentration: µg h^{-1} $ind.^{-1}$; from dry weight: µg h^{-1} $ind.^{-1}$; from total cell volume: 10^6 μm^3 h^{-1} $ind.^{-1}$) was 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} $ind.^{-1}$).

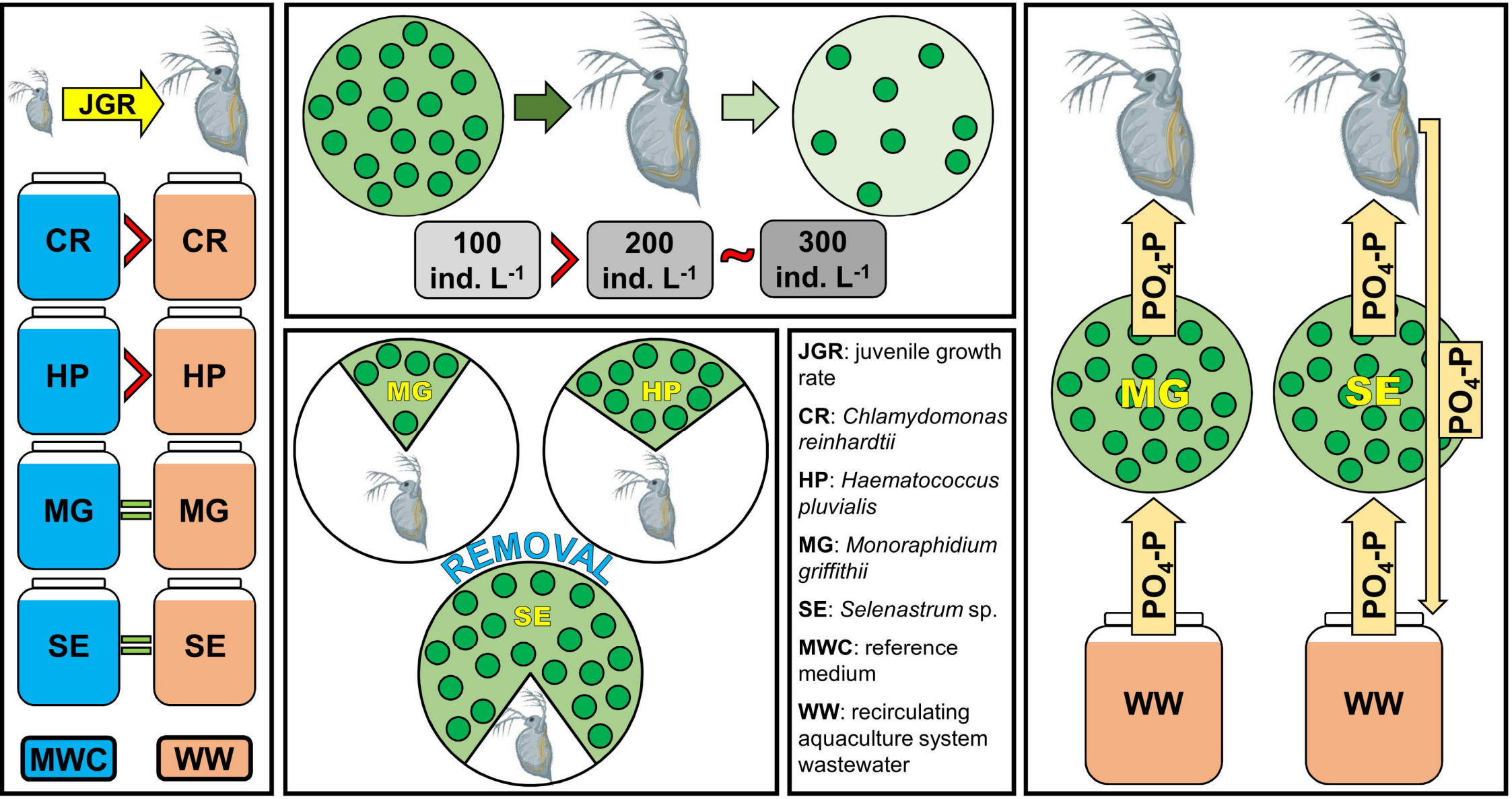
The growth constant for microalgal growth in control (*k*, h^{-1}) was calculated from the equation (Frost, 1972): $C_2^* = C_1^*\, e^{k(t_2 - t_1)}$, where C_1^* and C_2^* are microalgal concentrations (cell density: 10^6 cells mL^{-1}; chlorophyll-a concentration: µg mL^{-1}; dry weight: µg mL^{-1}; 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).


MWC
WW
Juvenile growth rate (d-1 ind.-1)
0.6
0.4
0.2
0.0
A
a
B
a
A
b
B
b
A
a
A
b
A
ab
A
b
CR
HP
MG
SE


(a)
100 ind./L
200 ind./L
300 ind./L
Clearance rate (mL h-1 ind.-1)
MG
SE
(b)
Ingestion rate (10^6 cells h-1 ind.-1)
(c)
Relative change (%)
(d)
HPg
HPr
(e)
(f)


(a)
HP MG SE
F (mL h-1 ind.-1)
Den
Chl a
DW
Vol
(b)
HP MG SE
Relative change (%)
Den
Chl a
DW
Vol
(c)
I_Den (10^6 cells h-1 ind.-1)
HP
MG
SE
(d)
I_Chl (µg h-1 ind.-1)
HP
MG
SE
(e)
I_DW (µg h-1 ind.-1)
HP
MG
SE
(f)
I_Vol (10^6 µm3 h-1 ind.-1)
HP
MG
SE
(g)
C T
Den (10^6 cells mL-1)
HP
MG
SE
(h)
C T
Chl a (ng mL-1)
HP
MG
SE
(i)
C T
DW (µg mL-1)
HP
MG
SE
(j)
C T
Vol (10^6 µm3 mL-1)
HP
MG
SE


JGR
CR
CR
HP
HP
MG
MG
SE
SE
MWC
WW
100 ind. L-1
200 ind. L-1
300 ind. L-1
MG
HP
REMOVAL
SE
JGR: juvenile growth rate
CR: Chlamydomonas reinhardtii
HP: Haematococcus pluvialis
MG: Monoraphidium griffithii
SE: Selenastrum sp.
MWC: reference medium
WW: recirculating aquaculture system wastewater
PO4-P
MG
SE
WW