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.

KEYWORDS: bioremediation, continuous light spectra, Coregonus lavaretus, fish farming, nitrate-nitrogen, nutrient uptake, phosphate-phosphorus, wastewater treatment

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 (Wik et al., 2009). 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 (Piedrahita, 2003; Castine et al., 2013). 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 (Blancheton et al., 2007; Bregnballe, 2015).

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 (Piedrahita, 2003; Martins et al., 2010). 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). Water reuse in RAS is limited by the accumulation of waste products

originating from uneaten feed, feces, and metabolic waste products (Piedrahita, 2003). Consequently, RAS reduce potential environmental impacts of waste discharge by concentrating nutrients and organic matter but not by overall reduction in discharges (Piedrahita, 2003). 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 depending on how the energy is produced, the carbon footprint of the products can increase significantly (Liu et al., 2016). 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 (Martins et al., 2010).

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 (Arnold, 2013). However, RAS WW provides enough dissolved nutrients to sustain photosynthetic growth of plants within the aquaponics (Goddek et al., 2019), 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 (Wang and Lan, 2011; Ji et al., 2013), 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 (Neori et al., 2004; Mata et al., 2010, 2012; Manninen et al., 2016; Zhao et al., 2016; Salama et al., 2017). 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 (Arbib et al., 2012; Mata et al., 2012). 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 (Mata et al., 2010; Castine et al., 2013; Ansari et al., 2017; Jebali et al., 2018). 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; Castine et al., 2013).

The studies that have examined the efficiency of microalgae in the treatment of 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), have mostly

characterized the use of warm-water species (cf. Ferro et al., 2018). 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 (Luo et al., 2017). Light related variables are photosynthetic photon flux density (PPFD) (intensity), spectral distribution (quality or wavelength), and photoperiod (Sun et al., 2018). The utilization of suitable wavelengths of visible light is an effective strategy to enhance microalgal growth, but highly dependent on microalgal species (Zhong et al., 2018). LED lights with continuous light spectra are increasingly used in plant and seedling production (e.g. Bantis et al., 2016; Smirnakou et al., 2017) 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 (Schulze et al., 2014, 2016; Sun et al., 2018; Zhong et al., 2018) 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 (1972). The medium contained 8.7 mg L^{-1} $K_2HPO_4.3H_2O$, 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).

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 (Wang and Lan, 2018).

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.

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 AP673L; 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 (Domínguez-Bocanegra et al., 2004), 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-1800, Japan) with wavelengths 665

and 750 (Keskitalo and Salonen, 1994). 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 (Andersen, 2005).

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 S_0 and S_1 its initial and final concentrations (mg L^{-1}) respectively (Wang and Lan, 2011; Delgadillo-Mirquez et al., 2016).

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}), S_0 and S_1 its

initial and final concentrations (mg L^{-1}) respectively and N the cell concentration (cells mL^{-1}) at time t_1 (Whitton et al., 2016).

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: $Chl\,a = 11.9 * A * \frac{V_e}{V_s * d}$, 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) (Keskitalo and Salonen, 1994).

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 AP673L than on spectra AP67 and G2, whereas Chl a was significantly lower on spectrum G2 than on spectra AP67 and AP673L (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 AP673L (pairwise tests; $p < 0.05$; Fig. 5f,h). Similarly, V_N was significantly higher on spectrum G2 than on spectrum AP673L, 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 (Sirakov and Velichkova, 2014; Cheban et al., 2015; Jiang et al., 2016). 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 (Egloff et al., 2018; Odjadjare et al., 2018). 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 (Smirnakou et al., 2017), 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 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 (Shah et al., 2016). 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 (Wayama et al., 2013). 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 (Kang et al., 2006).

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 (Zhou et al., 2014; Pachés et al., 2018) and higher removal of nitrogen (Zhou et al., 2014; Qi et al., 2017). For phosphorus, the removal was higher (Qi et al., 2017; Pachés et al., 2018), or similar (Zhou et al., 2014) 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. (2013)) or higher (Monoraphidium: Jiang et al. (2016); SE: Ouyang et al. (2015), Wang et al. (2016), and Zhao et al. (2016); Scenedesmus obliquus (SO): Ji et al. (2013), Zhou et al. (2014), Ouyang et al. (2015), and Zhao et al. (2016)). Also the removal percentages for nitrogen and phosphorus found in our study are similar (SE: Zhao et al. (2016); SO: Ouyang et al. (2015) and Zhao et al. (2016)) or higher (HP: Wu et al. (2013); Monoraphidium: Jiang et al. (2016); SE: Ouyang et al. (2015) and Wang et al. (2016); SO: Zhou et al. (2014), Zhao et al. (2016), and Ouyang et al. (2015)). However, for SO, Ji et al. (2013) 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 (Morales-Sánchez et al., 2017). 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) (Troell et al., 2009; Peltomaa et al., 2017). In our experiments, the tested non-green microalgae grew poorly and their nutrient removal capacity was low. For example, Tossavainen et al. (2018) and Aravantinou et al. (2013) recorded slightly higher SGR for Euglena gracilis and considerably higher removals of PO_4-P, and Tossavainen et al. (2018) 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 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 (Velichkova, 2014; Jiang et al., 2016; Haque et al., 2017; Qi et al., 2017). Our results suggest that the pH values remain within acceptable range for cultivation of microalgae in RAS WW (Kang et al., 2005). However, supplementation of CO_2 might maintain pH closer to optimal in longer experiments (Haque et al., 2017; Qi et al., 2017).

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 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 (Timmons et al., 2018). 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.

Declaration of interest

None.

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.

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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.

Figure 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, AP673L) 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; $p > 0.05$).

Figure 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, AP673L) 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; $p > 0.05$).

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Table 1

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

Table 2

	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

Table 3

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

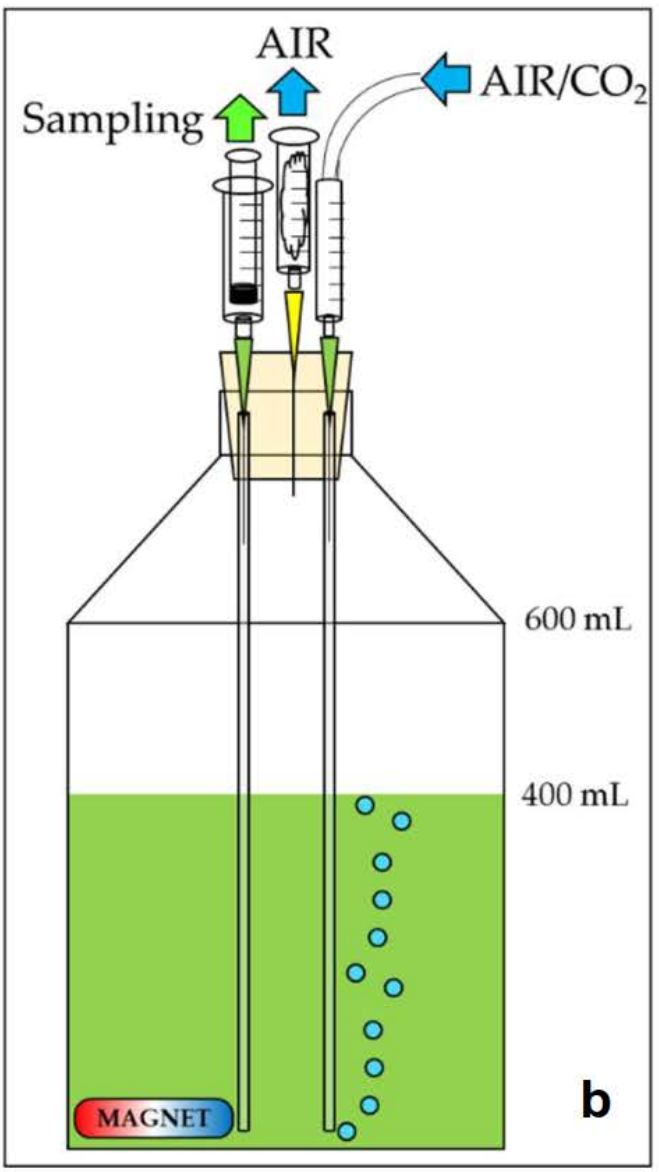


Fig. 1

Fig. 2

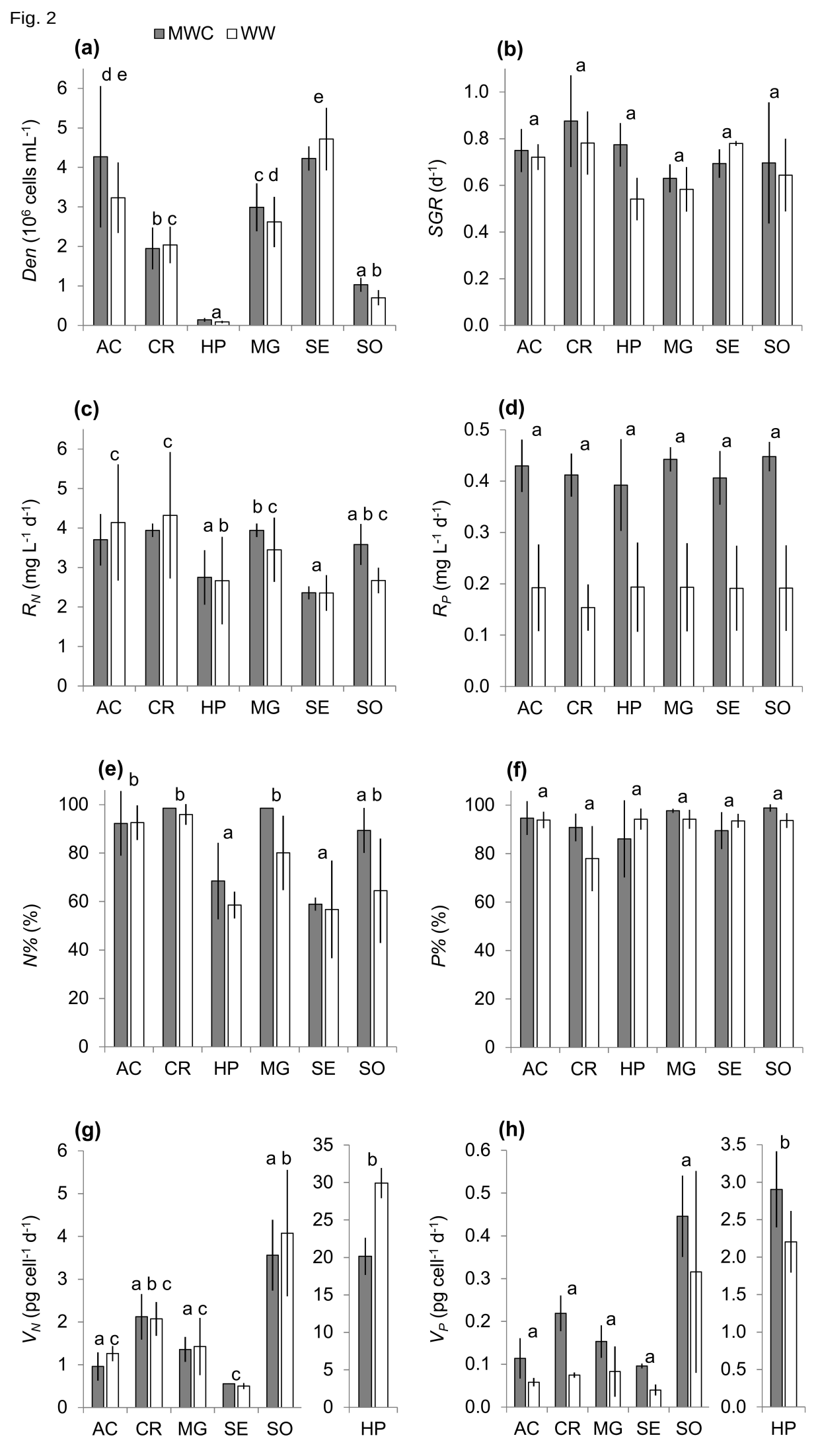
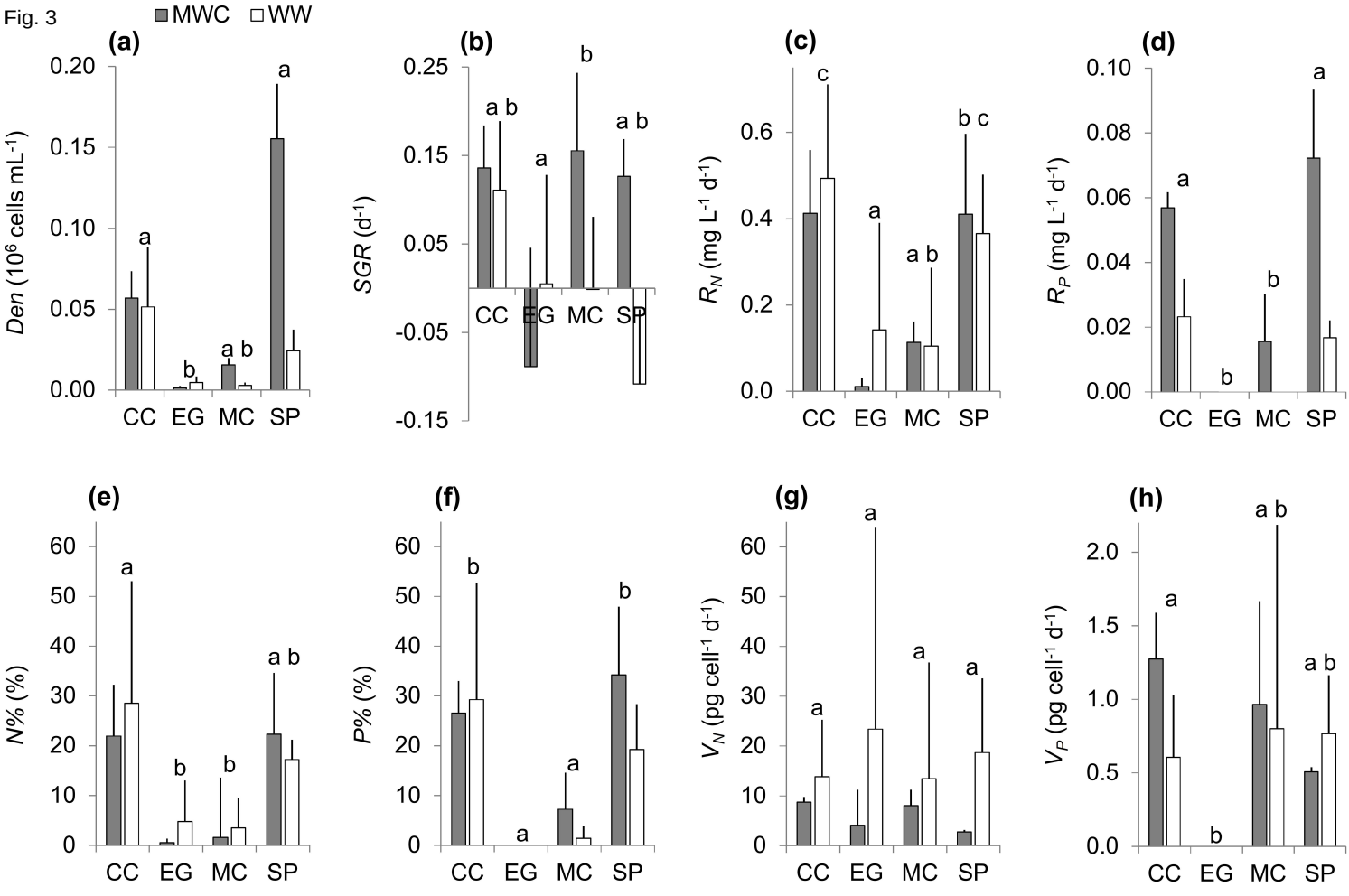

MWC
WW
(a)
Den (10^6 cells mL^-1)
(b)
SGR (d^-1)
(c)
R_N (mg L^-1 d^-1)
(d)
R_P (mg L^-1 d^-1)
(e)
N% (%)
(f)
P% (%)
(g)
V_N (pg cell^-1 d^-1)
(h)
V_P (pg cell^-1 d^-1)
AC
CR
HP
MG
SE
SO

Fig. 3


MWC
WW
(a)
Den (10^6 cells mL^-1)
0.20
0.15
0.10
0.05
0.00
a
b
a b
a
CC EG MC SP
(b)
SGR (d^-1)
0.25
0.15
0.05
-0.05
-0.15
a b
a
b
a b
CC EG MC SP
(c)
R_N (mg L^-1 d^-1)
0.6
0.4
0.2
0.0
c
a
a b
b c
CC EG MC SP
(d)
R_P (mg L^-1 d^-1)
0.10
0.08
0.06
0.04
0.02
0.00
a
b
b
a
CC EG MC SP
(e)
N% (%)
60
50
40
30
20
10
0
a
b
b
a b
CC EG MC SP
(f)
P% (%)
60
50
40
30
20
10
0
b
a
a
b
CC EG MC SP
(g)
V_N (pg cell^-1 d^-1)
60
50
40
30
20
10
0
a
a
a
a
CC EG MC SP
(h)
V_P (pg cell^-1 d^-1)
2.0
1.5
1.0
0.5
0.0
a
b
a b
a b
CC EG MC SP

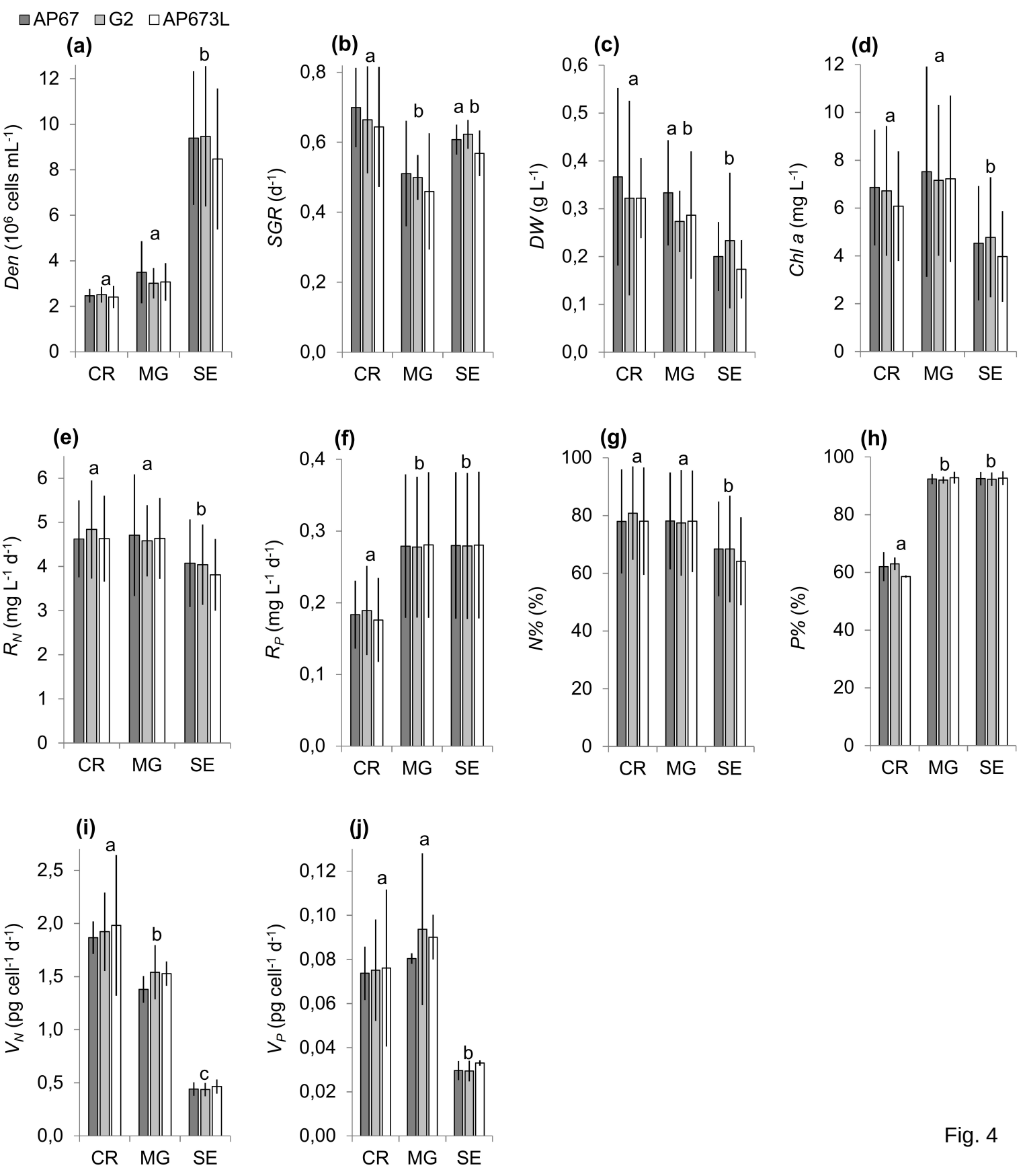

AP67
G2
AP673L
(a)
Den (10^6 cells mL^-1)
(b)
SGR (d^-1)
(c)
DW (g L^-1)
(d)
Chl a (mg L^-1)
(e)
R_N (mg L^-1 d^-1)
(f)
R_P (mg L^-1 d^-1)
(g)
N% (%)
(h)
P% (%)
(i)
V_N (pg cell^-1 d^-1)
(j)
V_P (pg cell^-1 d^-1)
CR
MG
SE

Fig. 4

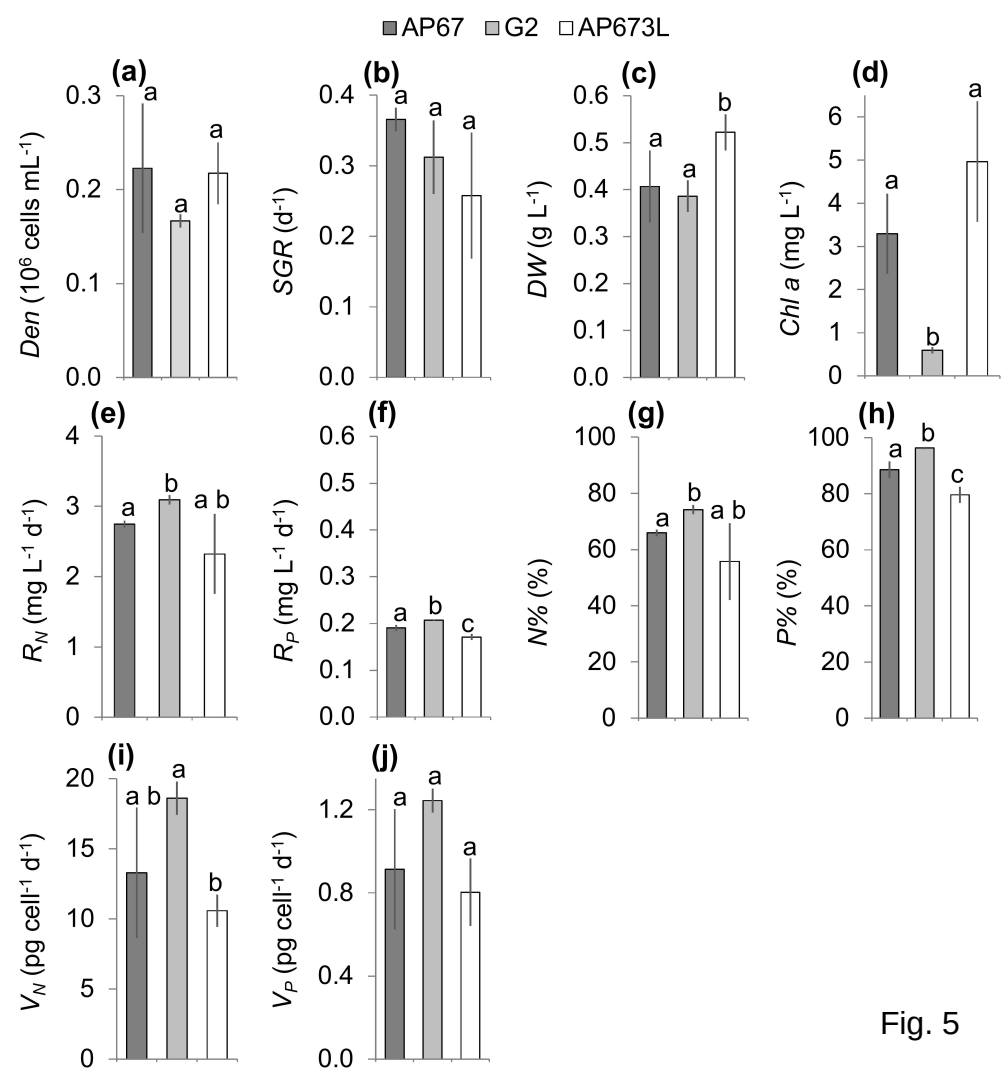

AP67
G2
AP673L
(a)
Den (10^6 cells mL^-1)
(b)
SGR (d^-1)
(c)
DW (g L^-1)
(d)
Chl a (mg L^-1)
(e)
R_N (mg L^-1 d^-1)
(f)
R_P (mg L^-1 d^-1)
(g)
N% (%)
(h)
P% (%)
(i)
V_N (pg cell^-1 d^-1)
(j)
V_P (pg cell^-1 d^-1)

Fig. 5

Graphical Abstract