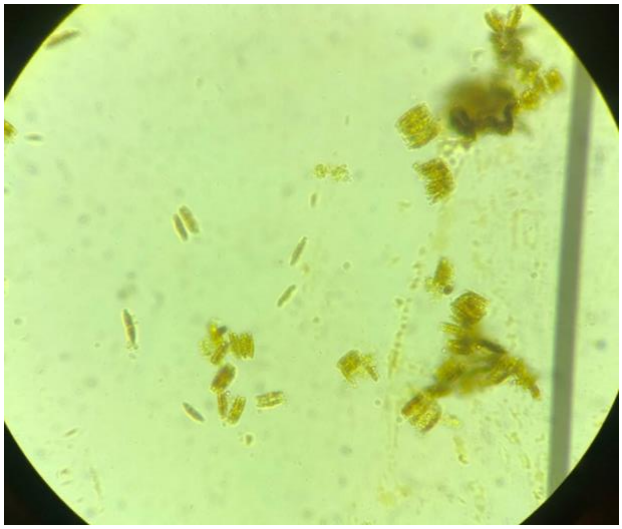
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

USING MICROALGAE FOR REMOVING NUTRIENTS FROM RAS AQUACULTURE EFFLUENTS – A VIABLE STRATEGY?

A case study



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Abstract

The increased global demand for fish has caused a tremendous increase in aquaculture production during the last decade, emphasising the importance of systems with a low climate footprint. One such system is a recirculating aquaculture system (RAS) which is land-based as opposed to traditional open systems. By implementing microalgae for nutrient removal in RAS, a more sustainable water use, and increased water reuse can be achieved. In this study the microalgae species (*Chlorella vulgaris, Nitzschia pusilla, Staurastrum monticulosum* and *Scenedesmus sp.)* and their removal of nutrients from aquaculture effluent from a land-based fish farm was examined. Simultaneously the microalgae's ability to grow and produce biomass in the aquaculture effluent was studied. Treatments were carried out in 250ml flasks, and nutrient removal, cell density, chlorophyll florescence parameters, growth rates and biovolume was measured up to day 7 (end of experiment). The results show that *C. vulgaris* and *Scenedesmus* sp. were most efficient at removing ammonia, having removed ~96% of ammonia after 7 days. *C. vulgaris* also had the highest removal rate of phosphorous at 88%. *C. vulgaris* and *N. pusilla* had the smallest biovolumes and yielded the highest biomass of 0.28g/L and 0.17g/L, respectively. The results indicate that using microalgae as bioremediators instead of biofilters for nutrient removal might be a possible strategy depending on the choice of species and design of the RAS – with the added possibility of producing biomass for valorisation.

Keywords

Microalgae; Nutrient removal; Aquaculture effluent; RAS; Biomass; Bioremediation

Popular science summary

The increased global demand for fish has caused a tremendous increase in aquaculture production during the last decade, emphasising the importance of systems with a low climate footprint. One such system is a recirculating aquaculture system (RAS) which is land-based as opposed to traditional open systems. By implementing microalgae for removing nutrients such as phosphorous and nitrogen in RAS, a more sustainable water use, and increased water reuse can be achieved. In this study microalgae were used to investigate the possibility of nutrient removal from aquaculture effluents rather than using biofilters (which is the common strategy today). At the same time, also investigate if microalgae could yield enough biomass to become a commercial product. The microalgae were grown in aquaculture effluent and a control treatment for 7 days. During this period, the microalgae's removal of nutrients and ability to grow in the aquaculture effluent was measured compared to the control. The results showed that microalgae are quite efficient at removing nutrients from the aquaculture effluent, with some being more efficient than traditional biofilters. Additionally, microalgae can produce a reasonable amount of biomass for further use in products such as food and fish feed. When incorporated into fish feed the microalgae further aid in a decreased climate footprint of the RAS.

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1. Introduction

1.1 Aquaculture

The ever increasing global demand for seafood has caused a tremendous spike in aquaculture production during the last decades with a staggering 527% rise in aquaculture production from 1990 to 2018 (FAO, 2020). Additionally, it is projected that by 2030 aquaculture will supply half of all fish on the global market (World-Bank, 2013). With the consumption of seafood not expected to drop anytime soon there is need for further development within aquaculture methods, whether that be the farming of fish, shrimp, or any other aquatic organism.

There are two main distinctions between aquaculture methods, namely open and closed systems. In an open aquaculture system, water passes through once, followed by treatment and discharge of the effluents. Because of this, these systems have a high water consumption and the effluents can contribute to adverse environmental effects in the local area (Taranger et al., 2015). This puts emphasis on aquaculture systems with a low ecological footprint, like recirculating aquaculture systems (RAS), which serve as an alternative to traditional open systems.

RAS is a closed system that optimizes water use by recirculating the water. As a result, the effluents are discharged in lower volumes but with significantly higher nutrient concentrations compared to open systems (Milhazes-Cunha and Otero, 2017). The recirculation of water in a RAS requires the water to be treated in the loop in order to prevent accumulation of suspended solids and nutrients which can cause harm to the farmed organism (Camargo and Alonso, 2006, Bahnasawy, 2011). Primarily, the water is treated to remove solid particles, either via a settling tank or a mechanical filter (van Rijn, 2013, Brinker and Rosch, 2005) and the water subsequently goes through a biofilter that removes nutrients such as ammonia and phosphorous. When the water passes through this filter it is the bacterial community that convert ammonia into nitrite and then into nitrate (Preena et al., 2021). The nitrate can then circle back in the system or be removed using an additional biofilter with denitrifying bacteria, transforming nitrate into nitrous gas (Preena et al., 2021).

1.2 Microalgae as bioremediators

1.2.1 RAS biofiltration

Due to the water being recirculated the demand for fresh water is less than 10% compared to conventional aquaculture systems (Bregnballe, 2015). In fact, the concentration of nitrogen that circulates in the system greatly depends on the water exchange rate, effectiveness of biofilters, and can reach levels of up to 400-500mg NO_3/L, starting to affect the growth of the cultured organisms (Van Rijn et al., 2006). RAS biofilters can be divided into two main categories: Fixed film technique where a media is provided for the microorganisms to grow, and suspended growth in which the microorganisms are kept suspended in the water

(Gutierrez-Wing and Malone, 2006). The design of specific filters differs between systems due to aspects such as farming intensity, species farmed, feeding load etc. As a result of the difference in design, the efficiency of biofilters also vary. Some biofilters can have a removal efficiency of around 75% for total nitrogen (TAN), where 25% is being recirculated and accumulated in the system (Ivan Gallego-Alarcon, 2017).

Additionally, phosphorous, another major nutrient in RAS, can be considered as one of the more critical nutrients for the receiving system (Yang et al., 2006). Phosphorous can cause excessive growth of algae and other macrophytes (McClanahan et al., 2007), reducing water clarity, decreasing oxygen concentrations and subsequently increase mortality for the farmed organism(s). Phosphorous removed via biofilters can reach removal rates of up to 45% (Li et al., 2014).

Even though the conventional way of removing nutrients in RAS effluents is via bacterial nitrification/denitrification it might not always work perfectly. New and promising solutions for RAS effluent treatment are technologies based on microalgae and cyanobacteria (Eduardo Couto, 2021;, Egloff et al., 2018, Araujo et al., 2021) where the produced biomass can also be valorised (Al-Jabri et al., 2021). Microalgae are divided into groups, being for example eukaryotic diatoms (Bacillariophyceae), green algae (Chlorophyceae) and golden algae (Chrysophyceae). Additionally, there is also cyanobacteria (Cyanophyceae), sometimes referred to as blue-green algae, even though they are not algae at all but are photosynthetic prokaryotes. In this study, the term microalgae will refer to both groups.

1.2.2 Bioremediatory properties of microalgae

The type of microalgae employed as a bioremediator for effluents can have significant impacts on the effectiveness of the system. One study highlighted the impacts and significance of choosing the right microalgae for effluent treatment. A study by Tejido-Nunes et al. (2019) compared the removal efficiency of two microalgae cultivated in water from a RAS, namely *Chlorella vulgaris* and *Tetradesmus obliquus*. The two species were not equally efficient at removing nutrients. *C. vulgaris* had a removal efficiency of 99.7% for nitrate and phosphate, respectively, where *T. obliquus* had a removal efficiency of 69.3% for nitrates and 99.7% for phosphates, showing that if the goal is to remove as much nitrate as possible, *C. vulgaris* will outperform *T. obliquus* and would be the better choice. Noteworthy is that these results were obtained in sterilized water. In water where bacteria were present *C. vulgaris* culture were predated and had lesser efficiency than *T. obliquus*, also showing the importance of internal factors. Similar studies conducted on cyanobacteria showed removal efficiencies of ammonia and phosphate to be 95% and 62%, respectively, but also pointed out that the results could be species dependent (Markou and Georgakakis, 2011). An example of this is a study conducted on *Arthrospira platensis* (commercially know as *Spirulina*) that

showed a removal efficiency of TAN as high as 99% and the removal efficiency of phosphorus to be as highs as 93.7% (Zhou et al., 2017).

Other groups of organisms might also be proficient at removing nutrients from effluents, one of them being desmids. Desmids are a type of green algae with quite an interesting cell structure. The cell of a desmid is divided into two symmetrical compartments mirroring each other being only separated by a narrow bridge. Desmids have, for example, been observed to thrive in waters from waste water treatment plants (Shanthala et al., 2009) and a study by Stamenkovic et al. (2021) showed the potential of using desmids as bioremediators for water derived from a land based fish farm. The results from the study showed a removal efficiency in the beginning between 30-89% for nitrate removal (species dependent) with all desmids efficiently absorbing nitrates by the end of the trial.

Another group of microalgae that have been studied for their bioremediatory properties are diatoms. They have been subjects in heavy metal and toxicity analyses (Chasapis et al., 2022, Hedayatkhah et al., 2018), and also for their ability to remove nutrients from aquaculture effluents and other waste waters (Bhattacharjya et al., 2021, Chaib et al., 2021). In fact, studies have shown diatoms to have a removal efficiency in between 60-75% for total ammonia and between 60-89% for phosphorus, respectively (Bhattacharjya et al., 2021, Chaib et al., 2021).

1.3 Microalgae for biomass production

As microalgae grown in RAS effluent simultaneously remove nutrients there is a great possibility for production of biomass to be used as a resource. For harvesting the biomass, a sufficient volume of microalgae is needed. The biomass obtained in grams per litre varies between studies. One study, for example, showed values of microalgae grown in aquaculture effluent of 1.51 g/L (Guidhe et al., 2017) where another study had a yield of 2.67 g/L, where the maximum productivity was 0.55 g/L/day – over 150% higher compared to their control culture in f/2 medium (Hawrot-Paw et al., 2020). On the contrary, in a paper by Gao et al. (2016) they achieved yields of 0.0073 g/L/day, much lower than other studies. Gao and colleagues also state that the obtained biomass was far less than what was obtained in other microalgal cultures e.g., grown in municipal sewage (0.074 g/L/day) or swine feedlot wastewater (0.033 g/L/day).

Disregarding quantity or yield, another aspect that is important when it comes to biomass harvesting is the separation of microalgal biomass from the water. In order to harvest, the biomass needs to be concentrated into big enough volumes for harvesting to take place (Barros et al., 2015). Since microalgae are a heterogenous group, the harvesting technique used needs to be adjusted to the specific microalgae species and culture conditions (Grima et al., 2003). Additionally, the choice of harvesting technique is determined by the valorisation

strategy of the final biomass and should not compromise the biomass and quality of the final effluent (Singh and Patidar, 2018). Different methods include sedimentation, flotation, centrifugation, filtration, coagulation-flocculation, and various combinations of different techniques. The biomass in question can be used for a variety of different applications. The applications include energy production e.g. in the form of biofuels (Suali and Sarbatly, 2012), agricultural products such as biofertilizers etc. (Dineshkumar et al., 2020, Li et al., 2021), animal feed (Benemann, 1992), and for human consumption in foods (de Medeiros et al., 2021) or in pharmaceuticals (Mehariya et al., 2021). There are newly developed techniques utilizing the silica "shell" (frustule) derived from diatoms to improve sunscreens and increase the effectiveness of solar panels (Huang et al., 2015, Gautam et al., 2016). This is currently explored and commercialized in the company Swedish Algae Factory growing benthic diatoms (www.swedishalgaefactory.com).

1.4 Microalgae and CO_2

The current global warming situation has triggered international awareness regarding greenhouse gas emissions as CO_2 levels keep increasing. Due to global warming, different techniques have been studied for CO_2 capture that can be divided in geological sequestration (Figueroa et al., 2008), chemical processing (MacDowell et al., 2010) and bioprocessing from photosynthetic organisms (Nair et al., 2009). There are uncertainties for several of the techniques one example being leakage of the geologically sequestered CO_2. On the contrary, natural processes can remove close to 50% of anthropogenic CO_2 emissions per year from the atmosphere (Benemann, 1993). A further positive impact from photosynthetic organisms is that they capture inorganic carbon and simultaneously release O_2 to the atmosphere. There are several advantages in using microalgae compared to other photosynthetic organisms for carbon capture being: they grow faster than terrestrial plants; the growth is not dependent on arable lands, thus, not competing with food and feed production and; when grown in agro-industrial waters rich in nitrogen and phosphorus they might not need any nutrient supplementation (Bhattacharjya et al., 2021). During photosynthesis algae sequester high amounts of CO_2. When microalgae productivity is high there will be a lack of CO_2 in the medium triggering carbon sequestration from the atmosphere, subsequently releasing O_2 to the atmosphere (Beatriz Molinuevo-Salces, 2019). It is estimated that microalgae can assimilate 1.8 tons of CO_2 per ton of algal biomass (Beatriz Molinuevo-Salces, 2019).

1.5 Aims

Previous studies have investigated the performance of microalgae regarding removal of nutrients from wastewater derived from e.g., sewage treatment plants, together with valorisation and optimization of microalgal biomass. The aim of this study was to examine if microalgae could be used to remove nutrients from aquaculture effluent from a land-based fish farm operating a RAS. Additionally, the possibilities of producing biomass (because of nutrient removal) were examined to determine if enough mass could be obtained after nutrient removal for valorisation, possibly opening up opportunities for dual production and system developments.

2. Materials and Method

2.1 Aquaculture effluents

The aquaculture effluent was procured from Gårdsfisk AB, a land-based fish farm located in Skåne county, Sweden. In this water *Clarias gariepinus* (Figure 1) are reared in indoor freshwater tanks at a temperature of ~ 25°C. The density of fish is 150 kg/m^3 in a system with a total volume of 180m^3.

Figure 1. Clarias gariepinus. Photo by Ian White, 2016.

Prior to use, the effluent was filtered through a set of filters with pore sizes ranging from 100µm down to 0.2µm. Subsequently, the water was treated with UV for 30 minutes to kill most bacteria. The filtered effluents were covered with aluminium foil and stored in a 15°C room until use. The pH was measured using a pH meter (FiveEasy pH/mV meter, Mettler Toledo, Stockholm, Sweden). The nutrients and elements of the aquaculture effluent were analysed both as a pure effluent sample and in the supernatant of cultures, obtained by filtering 100ml culture medium with pre-combusted (450°C for 4 hours) 0.7µm GF/F filters (Whatman). Detailed chemical analysis was performed by LMI AB, Helsingborg, Sweden. The chemical elements of the aquaculture effluent are shown in Table 1.

Table 1. Characteristics of the aquaculture effluent

Element	Chemical formula	Concentration
pH		8.1
Conductivity		2.7 ms/cm
Nitrate-N	NH_3	250 mg/l
Nitrite	NO_2-	4.5 mg/l
Ammonia	NO_3-	2.3 mg/l
Phosphorus	P	10 mg/l
Potassium	K	50 mg/l
Magnesium	Mg	9.8 mg/l
Sulfur	S	36 mg/l
Calcium	Ca_{2+}	44 mg/l
Mangan	Mn	0.039 mg/l
Bor	B	0.098 mg/l
Copper	Cu	0.068 mg/l
Iron	Fe	0.029 mg/l
Zink	Zn	0.055 mg/l
Molybden	Mo	0.022 mg/l
Silica	SiO_2	5 mg/l
Water hardness		7.6 mg/l
Sodium	Na	380 mg/l

2.2 Culture conditions and experimental set-up

A total of 4 microalgal species differing in trophic preference, climate origin and time of isolation were selected for investigating how well microalgae can absorb nutrients from fish effluents and how large of a biomass they can produce within 7 days without adding extra nutrients. *Staurastrum monticulosum* (desmid) and *Nitzschia pusilla* (diatom) were procured from Gothenburg University's microalgal bank. The green algae *Scenedesmus* sp. and *Chlorella vulgaris* were purchased from NORCCA, Oslo (Norway) and CCAP, Oban (UK).

Prior to experiments, all species were transferred to f/2 medium (Guillard, 1975) with added silicate acid, and acclimated at 18°C and photosynthetic active radiation of 130 µmol photons m-2 s-1 in a climate chamber with a light: dark interval of 18:6. The chemical composition of the modified f/2 medium is showed in Table 2. The respective algae stock solutions were continuously diluted until sufficient cell concentrations had been achieved to start the experiments (~800ml). When the algal stock reached sufficient cell concentration it was subsequently centrifuged for 10 min at 500G (ca 1534rpm). After the algal stock was centrifuged, the supernatant was removed using a pipette, leaving 5ml. The remaining supernatant was used to resuspend the algal pellet that was transferred to a larger beaker. A total volume of ~80ml concentrated algal stock was diluted to a final volume of 120ml with f/2 medium prior to division between the experimental treatments.

Table 2. Chemical composition of modified f2-medium.

Component	Stock solution	Quantity	Molar conc. in Final Medium
NaNO3	75 g/L dH2O	1 mL	8.82 x 10-4 M
NaH2PO4 H2O	5 g/L dH2O	1 mL	3.62 x 10-5 M
Na2SiO3 9H2O	0.003 g/L dH2O	1 mL	1.06 x 10-7 M
Trace metal solution			
FeCl3 6H2O	31.5 g/L dH2O	3.15 g	1.17 x 10-5 M
Na2EDTA 2H2O	41.6 g/L dH2O	4.36 g	1.17 x 10-5 M
CuSO4 5H2O	9.8 g/L dH2O	1 mL	3.93 x 10-8 M
Na2MoO4 2H2O	6.3 g/L dH2O	1 mL	2.60 x 10-8 M
ZnSO4 7H2O	22.0 g/L dH2O	1 mL	7.65 x 10-8 M
CoCl2 6H2O	10.0 g/L dH2O	1 mL	4.20 x 10-8 M
MnCl2 4H2O	180.0 g/L dH2O	1 mL	9.10 x 10-7 M
F2 Vitamin solution			
Thiamine HCl (vit. B1)	200 mg	2.96 x 10-7 M	
Biotin (vit. H)	0.1 g/L dH2O	10 mL	2.05 x 10-9 M
Cyanocobalamin (vit. B12)	1.0 g/L dH2O	1 mL	3.69 x 10-10 M

The microalgae were cultured in 200ml glass flasks (Schott Duran). The treatments consisted of one control, one control with yellow food dye and one with aquaculture effluent. Each treatment had 3 replicates. The aquaculture effluent had a yellow-ish colour, hence the treatment with yellow food dye was added to eliminate any uncertainties regarding spectral deviations of the water. To measure how the yellow food dye affected light spectra, a spectrophotometer was used. As Figure 2 shows, there were very minor differences. The control treatment had 180ml of f/2 medium, the treatment with food dye had 180ml f/2 medium with 2-3 drops of yellow food dye, and the third treatment had 180ml of filtered aquaculture effluent. The beaker with the concentrated algal solution was stirred continuously using a magnetic stirrer (to avoid sedimentation). 10ml of the respective algal stock was added to the replicates in each experimental treatment, making the final volume 190ml. The bottles were placed in a climate chamber (Phytotron) with a light: dark cycle of 18:6 at a temperature of 18°C and incubated for 7 days with constant bubbling. No specific starting concentration was used here, instead the starting concentrations of each replicate in each treatment was used to assess factors such as growth rate etc. during these trials.

After the first two sets of experiments had been completed (*N. pusilla* and *S. monticulosum*) the treatment with yellow food dye was excluded from the experimental set up and statistical analysis. Instead, the forthcoming experiments only consisted of two treatments – a control with f/2 medium and a treatment with filtered aquaculture effluent. For detailed statistical analysis depicting no statistically significant differences between the control and food dye treatments see Appendix 1 – Statistical tests.

At day 7, after the final measurements were taken, 100ml of each replicate was centrifuged for 15 minutes at 2000G (ca 3068rpm) *. The supernatant was removed for further analysis

leaving between 1-0.5ml. The remaining supernatant was used to resuspend the algal pellet that was then filtered through a pre-combusted and pre-weighed 0.7µm GF/F filter using a low-pressure vacuum pump. The filters were dried for approximately 24h at 60°C before they were weighed to obtain dry weight of the biomass. Subsequently the filters were placed in aluminium filter-holders and burned at 450°C for 4h before they were weighed to obtain the ash free dry weight (AFDW).

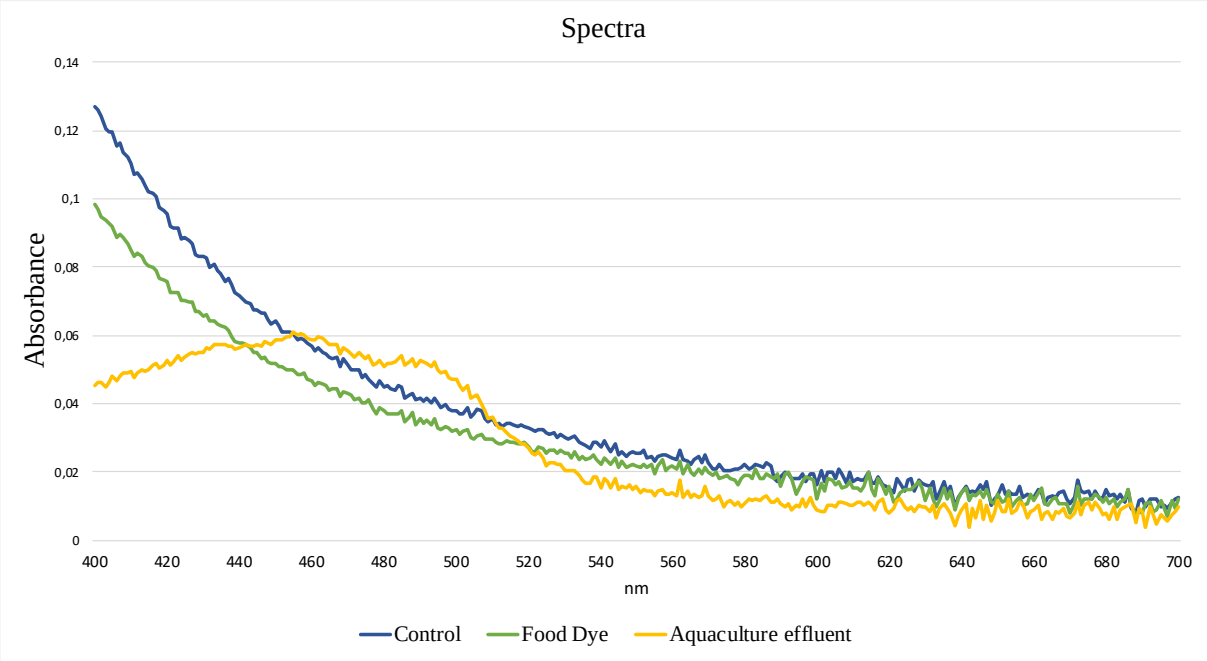

Figure 2. Spectra of the different treatments; control, food dye and aquaculture effluent.

2.3 Determination of nutrient removal, growth rate, biomass.

Each replicate was analysed 3 times (repeated measurement); day 0, day 3 and day 7. For each analysis a 10ml sample was taken from each replicate. 2ml of the 10ml sample was used in the determination of photosynthetic activity and an additional 2ml was used for determining cell numbers. Remaining volume of the sample (~ 6ml) functioned as a buffer in case more were needed for each analysis.

2.3.1 Nutrient analysis

Prior to the start of the experiments, samples of the f/2 medium and aquaculture effluent were sent for analysis to LMI AB. At day 7 the water removed after centrifugation of each replicate was sent for nutrient analysis to LMI AB. The results from these were compared to the original nutrient concentration of the f/2 medium and aquaculture effluent, respectively.

2.3.2 Growth rate and biomass

The number of cells in each replicate were measured using a gridded Sedgewick Rafter counting chamber under an inverted light microscope (Nikon ECLIPSE Ts2) The specific growth rate per day for each species and treatment was calculated using the formula:

$$\mu = \frac{\ln(\frac{N_1}{N_0})}{t_1 - t_0}$$

Where N_1 are the number of cells at the end (d = 7) of the experiment and N_0 the number of cells at the beginning (d = 0) divided by t – the duration of the experiment. Additionally the doubling time was calculated using the formula: $d = \ln(2) \times \mu^{-1}$ (Kim, 1995). For some strains with high cell numbers, image analyses were used. A camera connected to the microscope was used to photograph each cell in the Sedgewick Rafter counting chamber for further analysis in the open software InkScape (InkScape 1.1.2). The camera was operated with the software IC Capture version 2.5 (The Imaging Source Europe GmbH, Bremen, Germany). In InkScape the "marking tool" was used to mark the algal cells that had been counted to eliminate the possibility of double-counting.

To determine the photosynthetic activity of photosystem II (PSII) in the microalgae the maximum photochemical yield (F_v/F_m) was measured with a Pulse Amplitude Modulation fluorometer (Water PAM, Walz GmbH, Effeltrich, Germany) in connection to a computer with WinControl software (Walz GmbH). This yield is a parameter that describes how well phytoplankton can assimilate light or photosynthesize, i.e., the health of the organism is measured. This is done by applying the sample with a saturating light pulse which briefly suppresses photochemical yield to zero and induces maximum fluorescence yield. The yield is calculated according to $(F_m-F_0)/F_m = F_v/F_m$, where F_m is maximum fluorescent yield and F_0 fluorescent yield before the light pulse. The measurements were done in the emitter-detector unit of the cuvette version.

*The first microalgae to be tested was *N. pusilla.* Originally the method meant for 100ml of each replicate to go through the 0.7μm GF/F filter, un-centrifuged, using a vacuum pump. Each 100ml sample of *Nitzschia* filtered took approximately 6-8h to completely pass through the filter. A decision was made to alter the method for coming experiments and instead centrifuge the 100ml samples first and filter a much smaller volume using the vacuum pumps.

2.3.3 Biovolume and carbon content

Linear dimensions from live samples were measured for each species (i.e., length, height, and width in μm). All volumes were computed using the appropriate geometric formula that best corresponded to the body shape of each species , using equations recommended by Hillebrand et al., (1999). Specific shapes assigned to the cells in this case were: *N. pusilla – prism on*

elliptic base, *S. monticulosum* – *Ellipsoid* for one of the sides (multiplied with 2 to include the other half), *C. vulgaris* – *sphere*, and *Scenedesmus* sp. – *Cylinder + two half spheres*. When biovolume (BV) had been determined, carbon content was assessed using conversion factors. For the diatom *N. pusilla* (pg C $cell^{-1}$ = 0.288 * $BV^{0.811}$) and the rest being green algae (pg C $cell^{-1}$ = 0.216 * $BV^{0.939}$) according to Menden-Deuer and Lessard (2000).

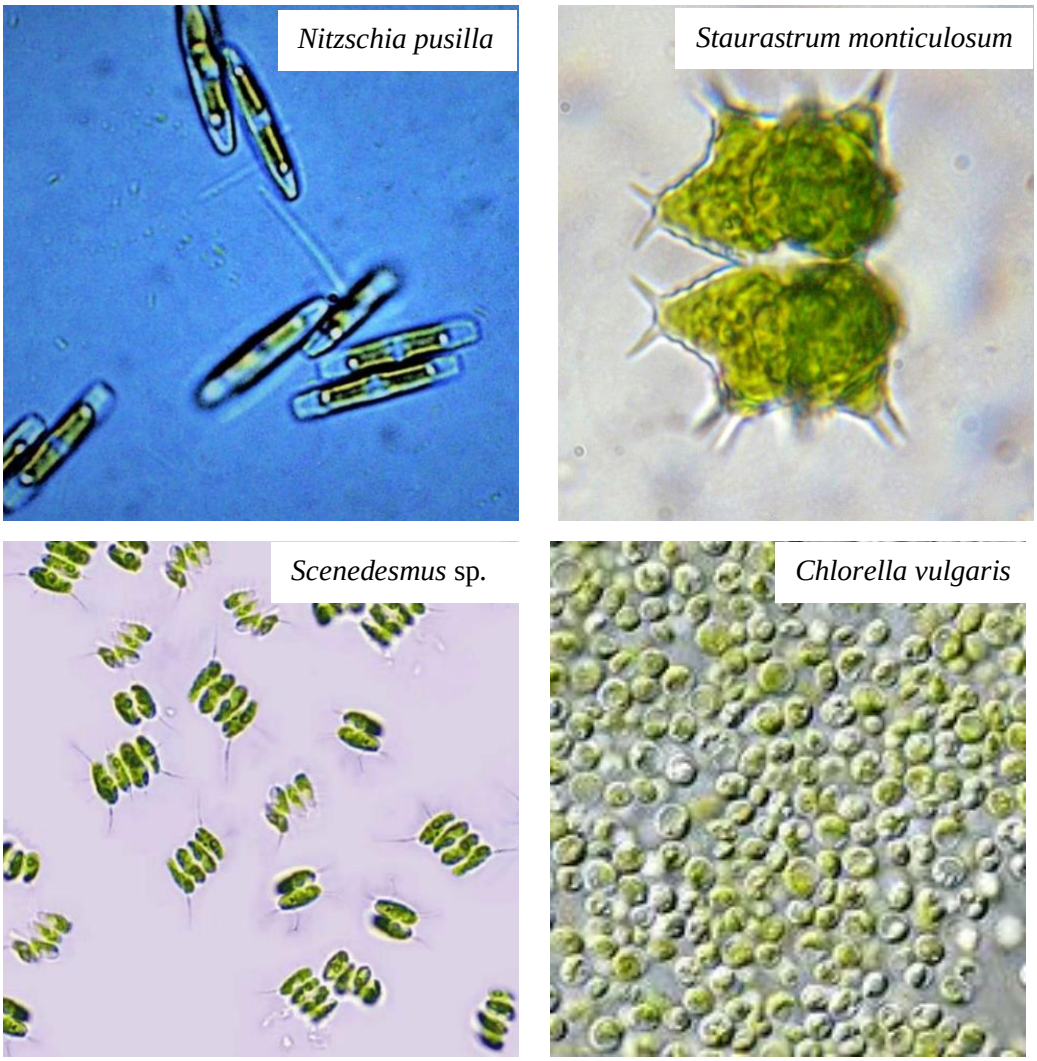

Figure 3. Pictures of all algae, clearly displaying their shape. N. pusilla (Horacio Abellán, 2019), S. monticulosum (© Maarten Mandos), Scenedesmus sp. (Gamze Dogdo, 2019), C. vulgaris (https://en.wikipedia.org/wiki/Chlorella_vulgaris).)

Important to mention is that during the experimental period there were changes in the method. The individual trials took place at different times, also meaning that the aquaculture effluent used was "older" in the later trials than in the first. Because of this, the different species of microalgae cannot be compared to each other, rather the individual performance will be assessed and put into context.

2.4 Statistical methods

To test for differences, one-way ANOVA, two-sample t-tests, paired t-test with their corresponding non-parametric counterparts (Kruskal-Wallis, Mann-Whitney, and Wilcoxon matched pairs) were used. Significance was tested on a level of $\alpha = 5\%$ and is indicated by using the words significant ($p < 0.05$) or non-significant ($p > 0.05$) in the results. One-way ANOVA was used to test for differences for all parameters in *N. pusilla* as well as for *S. monticulosum* when it came to CDW and all chlorophyll fluorescence parameters. The only parameter for *S. monticulosum* that did not use one-way ANOVA was the cell numbers parameter and instead Kruskal-Wallis was used. For *C. vulgaris,* Mann-Whitney U tests was used to test for difference across all parameters. Statistical differences for *Scenedesmus* sp. across all parameters were tested using two-sample t-tests.

3. Results

3.1 Nutrient removal efficiency

3.1.1 Staurastrum monticulosum

Staurastrum monticulosum grown in aquaculture effluent had a removal capacity of 68% for ammonia (Table 3). Additionally, the removal efficiency of nitrate and nitrite in aquaculture effluent were 23% and 36%, respectively. Comparably, the removal efficiency in the control treatment were 20% and 42% for nitrate and nitrite, respectively. Ammonia was under the level of detection. Furthermore, phosphorous decreased by 27% in the aquaculture effluent and close to 0% in the control treatment.

Table 3.Percentual change in nutrient levels of S. monticulosum in milligrams per litre at the start (d= 0) and end (d = 7) of the experiment for both the algae grown under controlled conditions and algae grown in aquaculture effluent. Nutrients below the level of detection is depicted by < 0.1 – in this case for ammonia in the control.

Staurastrum monticulosum						
	Nutrient levels (mg/l)					
Element	**Control d = 0**	**Control d = 7**	**Change %**	**Aquaculture effluent d = 0**	**Aquaculture effluent d = 7**	**Change %**
Ammonia	< 0.1	< 0.1	N/A	2.3	0.73	-68%
Nitrate-N	15.0	12.0	-20%	250.0	193.33	-23%
Nitrite	0.16	0.09	-42%	4.50	2.90	-36%
Phosphorous	0.96	0.96	0%	10.0	7.33	-27%
Silica	3.0	4.23	41%	5.0	5.93	19%

3.1.2 Nitzschia pusilla

There was an overall decrease in nutrient levels for *N. pusilla* in the control treatment from d = 0 to d = 7 (Table 4). Ammonia was under the level of detection both at the start and at the end of the experiment. *N. pusilla* had a removal efficiency of 36% for nitrate but overall unchanged levels for nitrite. Comparably the diatoms grown in aquaculture effluent had a removal rate of 45% and 28% for ammonia and nitrate, respectively. Nitrate levels were evidently higher than 5mg/l at d = 7 in the aquaculture effluent and a specific concentration could therefore not be specified in the analysis. Phosphorous levels decreased by 40% in the control compared to 64% in the aquaculture effluent. The highest removal efficiency across both groups were for silica where the control treatment removed 73% of all silica and the diatoms in aquaculture effluent removed 93% of all silica in the effluent.

Table 4. Percentual change in nutrient levels of N. pusilla in milligrams per litre at the start (d= 0) and end (d = 7) of the experiment for both the algae grown under controlled conditions and algae grown in aquaculture effluent. Nutrients below or above limit of detection is depicted by < 0.1 or > 5, respectively.

Nitzschia pusilla						
	Nutrient levels (mg/l)					
Element	**Control d = 0**	**Control d = 7**	**Change %**	**Aquaculture effluent d = 0**	**Aquaculture effluent d = 7**	**Change %**
Ammonia	< 0.1	< 0.1	N/A	2.30	1.27	-45%
Nitrate-N	15.0	9.60	-36%	250.0	180.0	-28%
Nitrite	0.16	0.16	0%	4.50	> 5	N/A
Phosphorous	0.96	0.57	-40%	10.0	3.63	-64%
Silica	3.0	0.81	-73%	5.0	0.35	-93%

3.1.3 Chlorella vulgaris

For *C. vulgaris in* the control treatment the concentration of ammonia was under the level of detection both at d = 0 and at d = 7 (Table 5). In the aquaculture effluent, the ammonia concentration decreased to under the level of detection at d = 7. However, if the lowest observable concentration were to be used (here 0.1) the percentual change in mg/l of ammonia from d = 0 to d = 7 would be ~96% or above. Nitrate removal rates were 42% and 28% for the control and aquaculture effluent, respectively. On the contrary nitrite concentrations increased in both groups, 56 and 44% respectively. Phosphorous decreased with 62% in the control but up to 88% for the aquaculture effluent.

Table 5. Percentual change in nutrient levels of C. vulgaris in milligrams per litre at the start (d= 0) and end (d = 7) of the experiment for both the algae grown under controlled conditions and algae grown in aquaculture effluent. Nutrients below the level of detection is depicted by < 0.1.

Chlorella vulgaris						
	Nutrient levels (mg/l)					
Element	**Control d = 0**	**Control d = 7**	**Change %**	**Aquaculture effluent d = 0**	**Aquaculture effluent d = 7**	**Change %**
Ammonia	< 0.1	< 0.1	N/A	2.30	< 0.1	N/A
Nitrate-N	15.0	8.77	-42%	250.0	180.0	-28%
Nitrite	0.16	0.25	56%	4.50	6.50	44%
Phosphorous	0.96	0.36	-62%	10.0	1.23	-88%
Silica	3.0	5.50	83%	5.0	6.07	21%

3.1.4 Scenedesmus sp.

The concentration of ammonia for *Scenedesmus* sp. is comparable to *C. vulgaris* where ammonia was under the level of detection in the control and under the level of detection in the aquaculture effluent at d = 7 (Table 6). However, if we use the lowest level of detection to calculate the decrease in percent, ammonia would have decreased by ~96% or more in the aquaculture effluent. Nitrate decreased with 48% in the control whereas it only decreased by 17% in the aquaculture effluent. Nitrite increased dramatically in the control with more than 100% and only 10% for the aquaculture effluent. Phosphorous decreased more in the control than in the aquaculture effluent, with 72% and 53%, respectively.

Table 6. Percentual change in nutrient levels of Scenedesmus sp. *in milligrams per litre at the start (d= 0) and end (d = 7) of the experiment for both the algae grown under controlled conditions and algae grown in aquaculture effluent. Nutrients below the level of detection is depicted by < 0.1*

Scenedesmus sp.						
	Nutrient levels (mg/l)					
Element	**Control d = 0**	**Control d = 7**	**Change %**	**Aquaculture effluent d = 0**	**Aquaculture effluent d = 7**	**Change %**
Ammonia	< 0.1	< 0.1	N/A	2.30	< 0.1	N/A
Nitrate-N	15.0	7.87	-48%	250.0	206.67	-17%
Nitrite	0.16	0.32	102%	4.50	4.97	10%
Phosphorous	0.96	0.27	-72%	10.0	4.70	-53%
Silica	3.0	4.33	44%	5.0	5.93	19%

3.2 Cell numbers

The initial cell density at the start of the experiment (d = 0), at the end of the experiment (d = 7) and percentual change for all species of microalgae are shown in Table 7. Overall, the increase in cells per litre were positive for all species in the control treatment from d = 0 to d = 7, ranging from a 6% to 616% increase for *Staurastrum monticulosum* and *Scenedesmus* sp., respectively. *Chlorella vulgaris* had a percentual increase of 269% in the control treatment where *Nitzschia pusilla* was the only species with a percentual decrease (24%). All algae but *Scenedesmus* sp. had a higher cell number per litre than the control treatment when grown in aquaculture effluent (aquaculture effluent). The percentual increase were 10%, 161% and 4098% for *S. monticulosum*, *N. pusilla* and *C. vulgaris*, respectively. *Scenedesmus* sp. was the only algae that had a lower percentual increase in aquaculture effluent than in the control group, 616% and 603% increase, respectively, in the control treatment compared to the algae grown in the aquaculture effluent.

Table 7. Cell density at the start of the experiment (d = 0) and at the end of the experiment (d = 7) including percentual change.

Cells/L						
Species	**Control**			**Aquaculture effluent**		
	d = 0	d = 7	Change (%)	d = 0	d = 7	Change (%)
Staurastrum monticulosum	$0.04*10^8$	$0.05*10^8$	6%	$0.04*10^8$	$0.05*10^8$	10%
Nitzschia pusilla	$1.06*10^8$	$0.81*10^8$	-24%	$2.27*10^8$	$5.19*10^8$	161%
Chlorella vulgaris	$2.53*10^8$	$9.34*10^8$	269%	$2.35*10^8$	$98.80*10^8$	4098%
Scenedesmus sp.	$1.51*10^8$	$10.80*10^8$	616%	$1.55*10^8$	$10.90*10^8$	603%

When examining the results (Figure 4) *Nitzschia pusilla* and *Chlorella vulgaris* display a dramatic increase and difference in cells per litre at d = 7 between the control and aquaculture effluent. On the contrary, *Staurastrum monticulosum* and *Scenedesmus* sp. had equivalent cell number between the control and aquaculture effluent at d = 7. The cell number and difference between control treatment and aquaculture effluent treatment were significant at d = 7 (Table 9) for *N. pusilla* and *C. vulgaris* whereas for *S. monticulosum and Scenedesmus* sp. the cell number at d = 7 were not significantly different (Table 9).

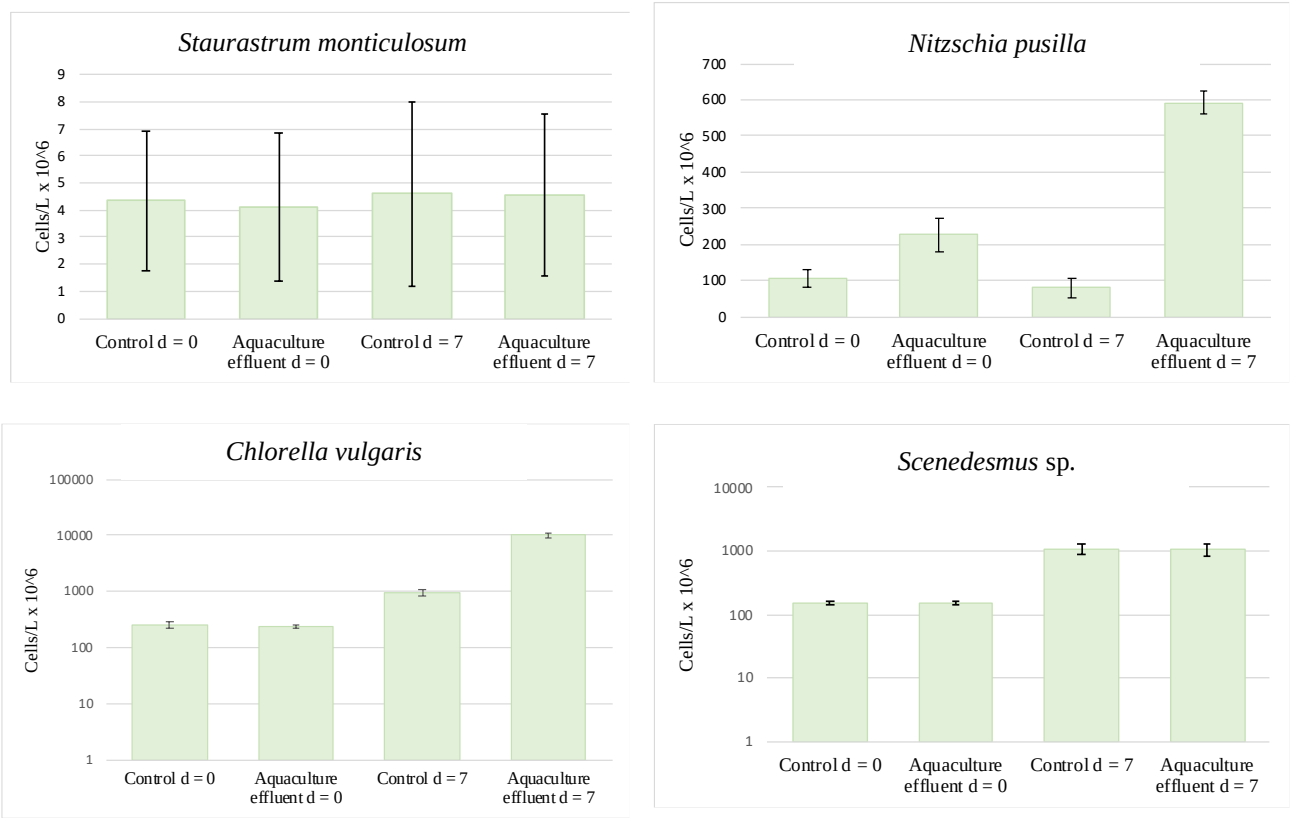

Figure 4. Bar plots with standard deviation error bars, displaying mean values of cells per litre for all algae measured at the start of the experiment (d = 0) and at the end of the experiment (d = 7). The bar plot depicts the algae grown in f2-medium (control) and algae grown in aquaculture effluent (aquaculture effluent. Scenedesmus sp. and *C. vulgaris is presented in a logarithmic scale. Note different scales on the y-axis.*

All species apart from *Scenedesmus* sp. had higher growth rates in aquaculture effluent compared to the control (up to 0.534 d^{-1} in *C. vulgaris*: Table 8). *N. pusilla* and *C. vulgaris* had noticeably higher growth rates in the aquaculture effluent than controls, 0.137 d^{-1} vs -0.029 d^{-1}, and 0.534 d^{-1} vs 0.186 d^{-1}, respectively. In fact, *N. pusilla* was the only microalgae that had a negative growth rate in the control group.

Table 8. Table with information for all species regarding parameters such as growth rate, doubling time, cell dry weight (CDW) and ash free dry weight (AFDW) under the two growth conditions (f2-medium and aquaculture effluent).

Growth condition	Growth parameter	*Staursatrum monticulosum*		*Nitzschia pusilla*		*Chlorella vulgaris*		*Scenedesmus sp.*	
		Mean	***STDV***	***Mean***	***STDV***	***Mean***	***STDV***	***Mean***	***STDV***
Control	**Growth rate (d^{-1})**	0.01	± 0.08	-0.03	± 0.08	0.19	± 0.03	0.28	± 0.03
	Doubling time (d)	81.45	± 1.76	-23.55	± 0.16	3.72	± 0.02	2.47	± 0.02
	CDW (g/L)	0.05	± 0.03	0.04	± 0.02	0.05	± 0.01	0.11	± 0.02
	AFDW (g/L)	0.03	± 0.02	0.03	± 0.02	0.01	± 0.01	0.01	± 0.01
	gC/L	3.65×10^{-3}	$\pm 1 \times 10^{-3}$	0.37×10^{-3}	$\pm 0.2 \times 10^{-3}$	0.01	$\pm 4 \times 10^{-3}$	0.03	± 0.02
Aquaculture effluent	**Growth rate (d^{-1})**	0.01	± 0.08	0.14	± 0.02	0.53	± 0.01	0.28	± 0.05
	Doubling time (d)	51.39	± 1.82	5.07	± 0.02	1.30	± 0.01	2.49	± 0.02
	CDW (g/L)	0.14	± 0.08	0.17	± 0.01	0.28	± 0.04	0.10	± 0.01
	AFDW (g/L)	0.05	± 0.06	0.12	± 0.07	0.03	± 0.04	0.01	±0.001
	gC/L	3.60×10^{-3}	± 0.001	2.74×10^{-3}	$\pm 3 \times 10^{-3}$	0.06	± 0.04	0.03	± 0.02

3.3 Biomass yield

Cell dry weight (CDW) was generated at d = 7 after filtration, drying and weighing of all replicates within each group. *N. pusilla* and *C. vulgaris* where the only two species that had significantly higher yield for aquaculture effluent than the control (Table 9) (Figure 5). Interestingly, *S. monticulosum generated* a higher biomass in aquaculture effluent than the control despite having similar cell numbers at d = 7. Even though the difference can be seen graphically in Figure 5, the difference in CDW is not statistically significant but it is somewhat close (p = 0.067). Moreover, *Scenedesmus* sp. generated a higher yield in the control group, albeit not statistically different from aquaculture effluent (Table 9). After incineration and drying the ash free dry weight (AFDW) was measured to assess total organic carbon content (Table 8). (TOC). *S. monticulosum* and *N. pusilla* generated the highest amount of AFDW at 0.03g/L respectively, with *C. vulgaris* and *Scenedesmus* sp. yielding 0.01g, /L respectively. The AFDW were put into context with the addition of grams of carbon per litre (gC/L). *C. vulgaris* and *Scenedesmus* sp. had the highest carbon content in the aquaculture effluent with 0.06gC/L and 0.03gC/L, respectively.

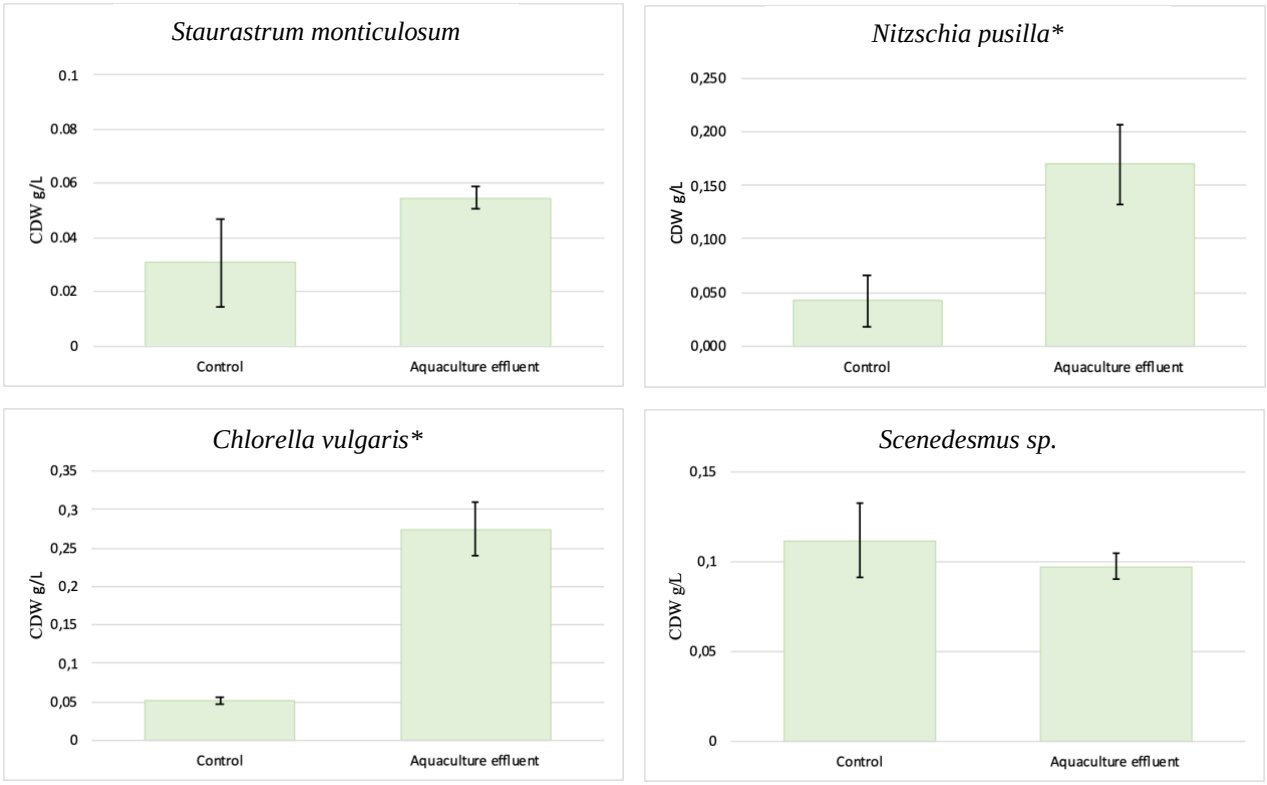

Figure 5. Bar plots with standard deviation error bars depicting the mean cell dry weight (CDW)in grams per litre for all algae grown in f2-medium (control) or grown in aquaculture effluent. Note different scales on Y-axis. An '' depicts statistical significance (p < 0.05).*

Table 9. Table showing what statistical aspects were significant or not. Significance is depicted with $p < 0.05$ and not significant is depicted as $p > 0.05$.

	Staurastrum monticulosum	***Nitzschia pusilla***	***Chlorella vulgaris***	***Scenedesmus sp.***
	Control vs Aquaculture effluent	*Control vs Aquaculture effluent*	*Control vs Aquaculture effluent*	*Control vs Aquaculture effluent*
Statistical aspect	Significance (p)	Significance (p)	Significance (p)	Significance (p)
Cell numbers (d=0)	p > 0.05	p < 0.05	p > 0.05	p > 0.05
Cell numbers (d=7)	p > 0.05	p < 0.05	p < 0.05	p > 0.05
CDW (d=7)	p > 0.05	p < 0.05	p < 0.05	p > 0.05
AFDW (d=7)	p > 0.05	p < 0.05	p < 0.05	p < 0.05

3.4 Chlorophyll fluorescence parameters

Maximum quantum yield (F_v/F_m) decreased for all species after 7 days in both the control and aquaculture effluent treatment (Table 10). Nutrient limitation can result in a lower F_v/F_m and can thus be used as an indicator for nutrient limitation but not for what nutrient is limiting. *S. monticulsosum*, decreased with 12% and 21% for the control and aquaculture effluent, respectively and *Scenedesmus* sp. decreased with 13% in the control and 31% in the aquaculture effluent. Additionally, *N. pusilla* and *C. vulgaris* decreased with 23%, 5% and 32%, 25% for control and aquaculture effluent, respectively.

Table 10. Table with values for Fv/Fm and Fo from start (d = 0) and end of the experiment (d = 7).

Species	Fv/Fm Control (d = 0)	Fv/Fm Control (d = 7)	Fv/Fm (d = 0)	Fv/Fm (d = 7)
Staurastrum monticulosum	0.728	0.639	0.671	0.528
Nitzschia pusilla	0.656	0.507	0.641	0.606
Chlorella vulgaris	0.503	0.342	0.464	0.346
Scenedesmus sp.	0.664	0.578	0.66	0.454

4. Discussion

4.1 Nutrient removal

4.1.1 Removal of nitrogen compounds

All microalgae were successful in removing at least some of the major nutrients in the aquaculture effluent. Major nutrients being the nitrogenous compounds (ammonia, nitrate, and nitrite) and phosphorous. *C. vulgaris* and *Scenedesmus* sp. were especially efficient at removing ammonia with comparable removal efficiencies of ~96% after 7 days, comparable to previous studies on *C. vulgaris* and algae in the same family as *Scenedesmus* sp. (Tejido-Nunez et al., 2019). *S. monticulosum* was the only species that managed to remove nitrite from the aquaculture effluent (36%). Additionally, *C. vulgaris* and *N. pusilla* both removed 28% of nitrate from the aquaculture effluent.

Nitrite seemed to increase for most species during the test period rather than decrease as would have been expected. One possible explanation for this could be that ammonia or nitrate are preferred nutrients and nitrite is not. Also, since these samples were not axenic there is a chance that the increase seen in nitrite is from nitrifying bacteria readily oxidizing the ammonia, thus increasing the concentration of nitrite, and bacteria further oxidizing the nitrite into the presumably more preferred form, namely nitrate. Important to note though is that all decrease in ammonia concentrations is not accredited to bacterial processes but a portion of the ammonia would also have been assimilated and removed by the microalgae (Salbitani and Carfagna, 2021).

4.1.2 Removal of phosphorous

Phosphorous being the other major nutrient in the aquaculture effluent was removed by all species. *C. vulgaris* and *N. pusilla* were the two species with the highest removal percentage, 88% and 64%, respectively. These values are similar to that of the study by Tejido-Nunes et al. (2019) and Bhattacharjya et al. (2021) where *C. vulgaris* had a removal efficiency of 99.7% and diatoms were measured to have a removal efficiency of 60-89%. *S. monticulosum* and *Scenedesmus* sp. showed slightly lower removal efficiencies at 27% and 53% respectively.

C. vulgaris and *N. pusilla*, with the highest removal rates for phosphorous, also had the lowest biovolumes at 34.704μm^3 and 32.362μm^3, respectively. Whereas *Scenedesmus* sp. and *S. monticulosum* had biovolumes of 149.637μm^3 and 6281.206μm^3, respectively. A small cell size implies a large surface area to volume ratio, thereby maximizing the uptake of available nutrients (Glazier, 2010). This could be one of the reasons why *C. vulgaris* and *N. pusilla* outperformed *Scenedesmus* sp. and *S. monticulosum* in removal of phosphorous.

4.1.3 Removal of silica

An additional nutrient that increased for all algae but *N. pusilla* was Silica. As Silica is a fundamental building block for diatom algae (Brunner et al., 2009) it came as no surprise that *N. pusilla* would be able to remove a large percentage of it from the aquaculture effluent. *N. pusilla* removed 93% of all silica in the aquaculture effluent. Why or how the levels of silica increased in the other samples is unclear. It could have something to do with the uncertainties in the nutrient analysis or another, unknown factor.

4.1.4 Potassium increase

The results from the nutrient analysis indicated an increase of potassium from d0 to d7. These values are not showed in the result section of this report but worth mentioning anyway. Why the concentration of potassium increased during the trials is unclear. It may have to do with uncertainties of the analysis itself or even other factors such as contamination of samples etc.

Today, most RAS systems use biofilters to remove nutrients from the aquaculture effluent, reaching removal rates of 75% for TAN (Gallego-Alarcon and Garcia-Pulido, 2017) and ~45% for phosphorous (Li et al., 2014). Comparably, this study reached removal efficiencies of up to ~96% for ammonia (*C. vulgaris* and *Scenedesmus* sp.), 36% for nitrite (*S. monticulosum*) and 28% for nitrate (*C. vulgaris* and *N. pusilla*). Additionally, the microalgae in this study removed up to 88% of all phosphorous in the aquaculture effluent, which is comparable to other studies (Chan et al., 2014, Lima et al., 2020, Ibrahim et al., 2020).

4.2 Cell density

Previously detailed in the results section, all microalgae experienced growth in the aquaculture effluent and all but *N. pusilla* had a positive growth in the control. *N. pusilla* and *C. vulgaris* were the only two species that had significant differences in cells per litre after 7 days. *C. vulgaris* experienced an increase in cell numbers of 269% in the control but a staggering increase of 4098% in the aquaculture effluent. With cells per litre ranging from 9.34×10^8 cells/l in the control to 98.8×10^8 cells/l in aquaculture effluent. This dramatic increase for *C. vulgaris* in aquaculture effluent can be explained in the abundance of nutrients (as detailed in the section above), providing good conditions for growth. Now *C. vulgaris* did also experience an increase when grown in the control but not as tremendous as in aquaculture effluent. A reason probably being a limitation of one or more nutrients, although hard to determine which, proving to be an important factor for growth rate and doubling time in *C. vulgaris*. F_v/F_m decreased for *C. vulgaris* from d = 0 to d = 7 in both treatments, possibly indicating the start of nutrient limitation in the treatments – even though no negative growth rates were recorded.

Growth rates

N. pusilla also had a large growth in aquaculture effluent compared to the control group with an increase of 161% in aquaculture effluent compared to -24% in the control. Why there was a decrease in cells per litre in the control likely had something to do with a nutrient limitation. The chlorophyll fluorescence parameter F_v/F_m during this time detail that there has also been a decrease in maxim quantum yield, or photosynthetic activity, during this time. F_v/F_m values went from 0.656 to 0.507 in the control. The likely scenario is that *N. pusilla* grew in the control until it reached a point where there simply were not any more nutrients left and the population started to decline instead. Would more measurements have been taken during the trial period, the entire growth phase could have been mapped – displaying the lag, exponential, stationary-and death phase of the experiment – that were the case at the end of the experiment.

Neither *S. monticulosum* nor *Scenedesmus* sp. experienced significant growth in the aquaculture effluent compared to the control. *S. monticulosum* had an increase of 6% and 10% in the control and aquaculture effluent, respectively, where *Scenedesmus* sp. had a higher increase overall – 616% and 603% in the control and aquaculture effluent, respectively. Even though *Scenedesmus* sp. increased in cells per litre by a lot there were no statistically significant difference between the control and aquaculture effluent. This is informative because it seems that *Scenedesmus* sp. does not need any more nutrients than what was present in the control medium to increase by over 600% for 7 days. In fact, the cell density increased more in the control than in the aquaculture effluent. Although this might be a misleading assumption for without a longer trial period and more consecutive testing one cannot be sure as to what growth phase the microalgae were in. The same goes for *S. monticulosum* where the difference in cell numbers were almost significant ($p = 0.067$) but not quite. Given more time maybe this difference would have become significant as the control ran out of nutrients.

4.3 Biomass

All microalgae experienced some level of growth for 7 days. *C. vulgaris* and *N. pusilla* were the two microalgae that generated the highest biomass in aquaculture effluent after 7 days. *C. vulgaris* generated a CDW of 0.28 g/l and Nitzschia generated a CDW of 0.17 g/l. *S. monticulosum* and *Scenedesmus* sp. generated lower CDW of 0.05 g/l and 0.01 g/l, respectively. Important to mention is that this weight cannot be attributed to algae alone.

To separate the weight of the algae to e.g., weight of bacteria present, the biovolume and carbon content was used to estimate how much of the weight was attributed to algae alone in the AFDW. *S. monticulosum* and *N. pusilla* had the greatest amount of AFDW in aquaculture effluent, 0.05g/L and 0.12g/L, respectively. Interestingly the carbon content of the two were lower than that for *C. vulgaris* and *Scenedesmus* sp., indicating contributing factors of the AFDW for *S. monticulosum* and *N. pusilla*. For *N. pusilla* a certain percentage of the AFDW

can be attributed to the frustule shells. The frustules consist of silica that remain after incineration, skewing the AFDW. Moreover, a possibility as to why the AFDW was higher in *S. monticulosum* than would be expected if looking at the gC/L could be the presence of bacteria or other contaminations skewing the AFDW.

4.4 Applications of microalgae

Putting achieved results into context it only deems fit to use actual numbers from Gårdsfisk AB land-based fish farm. Gårdsfisk AB exchange between 5-10% of their water daily which translates to between 8000-16000L aquaculture effluent per day. A coupled RAS-biomass generating scheme ensuring a minimum of 8000L of aquaculture effluent per day could yield ~2.24kg of dry biomass by utilizing *C. vulgaris*. If 16000L were to be used that number could reach yields of 4.48kg dry biomass (*C. vulgaris*). This would be the results after 7 days cultivation. For this type of process, a 7-tank system could be implemented – filling one tank with microalgae and aquaculture effluent per day. Through a rough estimation, weekly yields could reach minimum weights of ~16kg. Relating the biomass to assimilation of CO_2. Again, with rough estimations using the lowest volume of aquaculture effluent and biomass generation a system like this could potentially assimilate 1.5 tons of CO_2 per year (Beatriz Molinuevo-Salces, 2019).

In fact, using this study's methodological approach might not be suitable if high yields are favourable. F_v/F_m values indicated that there was some degree of nutrient limitation in all treatments after 7 days. If cultures were to have a constant addition of aquaculture effluent higher yields of biomass could be achieved (Egloff et al., 2018).

Microalgae contain a variety of proteins, lipids, and pigments where some of these are valorised in foods and feeds (Ursu et al., 2014). As these microalgae can remove and assimilate nutrients from aquaculture effluent they have, in previous studies, proven to be viable options for production of proteins and fatty acids for e.g., fish feed (Villar-Navarro et al., 2021a, Maltsev and Maltseva, 2021) and even cheese (Tohamy et al., 2018). *C. vulgaris* is especially rich in proteins and have a high yield recovery of 76% indicating that *C. vulgaris* could be valorised into various types of foods and feeds (Ursu et al., 2014). Some studies indicate the possibility of reducing the climate footprint of an RAS by incorporating microalgae into the fish feed (Villar-Navarro et al., 2021b) substituting up to 50% of fish-derived protein for fish feed (Perez-Velazquez et al., 2018).

To valorise on produced biomass a high yield is desired. Prior to valorisation, harvesting techniques ought to be applied in the process to increase biomass concentration and allow for more efficient use of water in the RAS. Harvesting could be done with a plethora of methods: sedimentation, flocculation, flotation, centrifugation, and various combinations – all of which are microalgae and process dependent.

Simultaneously as the algae increased their biomass, they would also remove nutrients from the water. Assuming no constant addition of aquaculture effluent the algae could remove up to 88% of phosphorous and ~96% of ammonia from the system, according to the finding in this study – excluding factors possibly present in an RAS, such as temperature, water quality etc. Additionally, *N. pusilla* were able to remove up to 96% of silica. Silica likely entered the aquaculture effluent from the specific fish feed used. If silica or the frustules were the desired product *N. pusilla* would be a viable option for simultaneous nutrient removal and generation of biomass. If the assumption is made that microalgae can remove sufficient levels of nutrients for the water to be sent back into the RAS, somewhere around 2900m^3 (or 2.9 million litres) water could be recirculated each year if we use the specifics from Gårdsfisk AB.

4.5 Methodological considerations

Aspects to consider regarding the set up and methodology of this report is: even though the algae were checked and measured three times during the trials, the results from d = 3 were excluded from all analyses and discussion. The reasoning behind this is that these datasets did not yield any valuable information. The results did show a continuous increase in cell numbers, yes, but e.g., the growth phase these algae were in could not be seen, thus no conclusion regarding when nutrient limitation occurred could be made; For nutrient removal calculations the same initial concentration was used to assess the removal percentage. However, this could be misleading since the initial nutrient concentrations in this effluent can have changed from the initial to the last experiments due to e.g., bacterial activity; previously mentioned methodological changes would, if the experiments were replicated, have been more carefully designed and tested prior to experiments to ensure equivalent methodological approach across all treatments.

5. Conclusion

If utilization of microalgae as bioremediators were to be directly compared to biofilters regarding removal rates of nutrients there could potentially be a greater benefit to using microalgae. Not only does some species have rather high removal rates of nitrogen compounds and phosphorous but biomass produced could prove to be an important addition in further improving the resource efficiency of RAS. Also, the removal rates from this study compared to those of biofilters indicate that microalgae could be better at removing nutrients from RAS than present biofilters. Keeping in mind that real life scenarios and applications are oftentimes more complicated and nuanced than laboratory environments.

As has been presented and discussed in this report, different microalgae have different levels of removal rates. Because of this there is a great possibility to tailor the usage of microalgae to a specific system. If focus is on nutrient removal and nothing else, certain species are better than others. Likewise, if focus is more on simultaneous production of biomass or extraction of certain materials then other species can serve that purpose better. It gives the business owner greater control over the system. For example, could *N. pusilla* be used to extract silica and provide frustules for further valorisation into skincare products or improved efficiencies of solar panels.

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7. Appendices

Appendix 1 – Statistical tests

7.1 N. pusilla

7.1.1 Cell number d = 7

ANOVA_N.pusilla

Cell_count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1183737.590	2	591868.795	896.634	<.001
Within Groups	16502.517	25	660.101		
Total	1200240.107	27			

Multiple Comparisons_N.pusilla

Dependent Variable: Cell_count

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-20.883	11.001	.160	-48.28	6.52
	Fish_Water	-510.083*	12.846	<.001	-542.08	-478.09
Food_Dye	Control	20.883	11.001	.160	-6.52	48.28
	Fish_Water	-489.200*	13.268	<.001	-522.25	-456.15
Fish_Water	Control	510.083*	12.846	<.001	478.09	542.08
	Food_Dye	489.200*	13.268	<.001	456.15	522.25

7.1.2 Chlorophyll fluorescence parameters

7.1.2.1 Chlorophyll fluorescence, maximum quantum yield Fv/Fm

Multiple Comparisons_Nitzschia

Dependent Variable: Fv/Fm_Start

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	.012000	.006992	.275	-.00945	.03345
	Fish_Water	.014333	.006992	.181	-.00712	.03579
Food_Dye	Control	-.012000	.006992	.275	-.03345	.00945
	Fish_Water	.002333	.006992	.941	-.01912	.02379
Fish_Water	Control	-.014333	.006992	.181	-.03579	.00712
	Food_Dye	-.002333	.006992	.941	-.02379	.01912

ANOVA

Fv/Fm_End

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.019	2	.009	7.275	.025
Within Groups	.008	6	.001		
Total	.026	8			

Multiple Comparisons

Dependent Variable: Fv/Fm_End
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-.004667	.029289	.986	-.09453	.08520
	Aquaculture effluent	-.099000*	.029289	.034	-.18887	-.00913
Food_Dye	Control	.004667	.029289	.986	-.08520	.09453
	Aquaculture effluent	-.094333*	.029289	.041	-.18420	-.00447
Aquaculture effluent	Control	.099000*	.029289	.034	.00913	.18887
	Food_Dye	.094333*	.029289	.041	.00447	.18420

*. The mean difference is significant at the 0.05 level.

7.1.3 Cell density

ANOVA_Nitzschia

Fo_Start

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2822936.222	2	1411468.111	163.883	<.001
Within Groups	51676.000	6	8612.667		
Total	2874612.222	8			

Multiple Comparisons

Dependent Variable: Fo_Start
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-26.333	75.775	.936	-258.83	206.16
	Aquaculture effluent	-1201.000*	75.775	<.001	-1433.50	-968.50
Food_Dye	Control	26.333	75.775	.936	-206.16	258.83
	Aquaculture effluent	-1174.667*	75.775	<.001	-1407.16	-942.17
Aquaculture effluent	Control	1201.000*	75.775	<.001	968.50	1433.50
	Food_Dye	1174.667*	75.775	<.001	942.17	1407.16

*. The mean difference is significant at the 0.05 level.

ANOVA_Nitzschia

Fo_End

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2822936.222	2	1411468.111	163.883	<.001
Within Groups	51676.000	6	8612.667		
Total	2874612.222	8			

Multiple Comparisons

Dependent Variable: Fo_End
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-26.333	75.775	.936	-258.83	206.16
	Aquaculture effluent	-1201.000*	75.775	<.001	-1433.50	-968.50
Food Dye	Control	26.333	75.775	.936	-206.16	258.83
	Aquaculture effluent	-1174.667*	75.775	<.001	-1407.16	-942.17
Aquaculture effluent	Control	1201.000*	75.775	<.001	968.50	1433.50
	Food_Dye	1174.667*	75.775	<.001	942.17	1407.16

*. The mean difference is significant at the 0.05 level.

7.1.4 Cell dry weight

ANOVA

Weight (mg)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	2	.000	22.077	.002
Within Groups	.000	6	.000		
Total	.000	8			

Multiple Comparisons

Dependent Variable: Weight (mg)
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-.001333333	.002112222	.809	-.007814210	.005147544
	Aquaculture effluent	-.01276667*	.002112222	.002	-.019247544	-.006285790
Food_Dye	Control	.001333333	.002112222	.809	-.005147544	.007814210
	Aquaculture effluent	-.01143333*	.002112222	.004	-.017914210	-.004952456
Aquaculture effluent	Control	.012766667*	.002112222	.002	.006285790	.019247544
	Food_Dye	.011433333*	.002112222	.004	.004952456	.017914210

*. The mean difference is significant at the 0.05 level.

7.2 Staurastrum monticulosum

7.2.1 Cell count

7.2.2 Cell number d = 7

ANOVA_S.monticulosum

Control_Cellount_End

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	638.572	2	319.286	28.554	<.001
Within Groups	4651.642	416	11.182		
Total	5290.215	418			

Multiple Comparisons_S.monticulsosum

Dependent Variable: Control_Cellount_End
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	.100	.464	.975	-.99	1.19
	Aquaculture effluent	2.530*	.431	<.001	1.52	3.54
Food_Dye	Control	-.100	.464	.975	-1.19	.99
	Aquaculture effluent	2.430*	.375	<.001	1.55	3.31
Aquaculture effluent	Control	-2.530*	.431	<.001	-3.54	-1.52
	Food_Dye	-2.430*	.375	<.001	-3.31	-1.55

*. The mean difference is significant at the 0.05 level.

7.3.1 Chlorophyll fluorescence parameters

7.3.2 Chlorophyll fluorescence, maximum quantum yield F_v/F_m

7.3.3. *d = 0*

ANOVA_S.monticulosum

Fv/Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	2	.003	9.277	.015
Within Groups	.002	6	.000		
Total	.007	8			

Multiple Comparisons_S.monticulosum

Dependent Variable: Fv/Fm
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	.010667	.013894	.735	-.03196	.05330
	Aquaculture effluent	.056333*	.013894	.016	.01370	.09896
Food_Dye	Control	-.010667	.013894	.735	-.05330	.03196
	Aquaculture effluent	.045667*	.013894	.038	.00304	.08830
Aquaculture effluent	Control	-.056333*	.013894	.016	-.09896	-.01370
	Food_Dye	-.045667*	.013894	.038	-.08830	-.00304

*. The mean difference is significant at the 0.05 level.

7.3.4 *d = 7*

ANOVA_S.monticulosum

Fv/Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.032	2	.016	3.612	.093
Within Groups	.027	6	.004		
Total	.059	8			

Multiple Comparisons_S.monticulosum

Dependent Variable: Fv/Fm
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-.026667	.054328	.878	-.19336	.14003
	Aquaculture effluent	.111000	.054328	.183	-.05569	.27769
Food_Dye	Control	.026667	.054328	.878	-.14003	.19336
	Aquaculture effluent	.137667	.054328	.098	-.02903	.30436
Aquaculture effluent	Control	-.111000	.054328	.183	-.27769	.05569
	Food_Dye	-.137667	.054328	.098	-.30436	.02903

7.4 Cell density

7.4.1 Cell density d = 0

ANOVA_S.monticulosum

Fo

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	141.556	2	70.778	7.078	.026
Within Groups	60.000	6	10.000		
Total	201.556	8			

Multiple Comparisons_S.monticulosum

Dependent Variable: Fo
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	2.333	2.582	.658	-5.59	10.26
	Aquaculture effluent	-7.000	2.582	.078	-14.92	.92
Food_Dye	Control	-2.333	2.582	.658	-10.26	5.59
	Aquaculture effluent	-9.333*	2.582	.026	-17.26	-1.41
Aquaculture effluent	Control	7.000	2.582	.078	-.92	14.92
	Food_Dye	9.333*	2.582	.026	1.41	17.26

*. The mean difference is significant at the 0.05 level.

7.4.2 Cell density d = 7

ANOVA_S.monticulosum

Fo

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	363.556	2	181.778	.099	.907
Within Groups	11002.000	6	1833.667		
Total	11365.556	8			

Multiple Comparisons_S.monticulosum

Dependent Variable: Fo

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-15.333	34.963	.901	-122.61	91.94
	3	-5.333	34.963	.987	-112.61	101.94
2	1	15.333	34.963	.901	-91.94	122.61
	3	10.000	34.963	.956	-97.28	117.28
3	1	5.333	34.963	.987	-101.94	112.61
	2	-10.000	34.963	.956	-117.28	97.28

7.4.3 Weight

ANOVA_S.monticulosum

Weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	2	.000	3.144	.116
Within Groups	.000	6	.000		
Total	.000	8			

Multiple Comparisons_S.monticulosum

Dependent Variable: Weight

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Food_Dye	-.001000000	.000961480	.581	-.003950086	.001950086
	Aquaculture effluent	-.002400000	.000961480	.103	-.005350086	.000550086
Food_Dye	Control	.001000000	.000961480	.581	-.001950086	.003950086
	Aquaculture effluent	-.001400000	.000961480	.374	-.004350086	.001550086
Aquaculture	Control	.002400000	.000961480	.103	-.000550086	.005350086
	Food_Dye	.001400000	.000961480	.374	-.001550086	.004350086