Research Report

Robust algae for aquaculture

Effect of N and P concentrations on biomass productivity and cell composition of Skeletonema costatum

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Effect of N and P concentrations on biomass productivity and cell composition of *Skeletonema costatum*

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Version 4.0

Jun.10, 2018

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Abstract

To increase the yield and quality of aquaculture feed, the effects of N and P concentrations on biomass productivity and cell composition were examined in the marine diatom *Skeletonema costatum*. Batch-cultured microalgae in Walne's medium with 3.5 ± 0.5 mmol L^{-1} $N\text{-}NO_3^-$ and 0.30 ± 0.05 mmol L^{-1} $P\text{-}PO_4^{3-}$ can achieve highest dry weight (1.6 ± 0.2 g L^{-1}) and growth rate (0.55 ± 0.05 day^{-1}) under the light density of 47 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the temperature of 18.9 ± 0.5 ℃. The highest biomass productivity (0.40 ± 0.05 g L^{-1} day^{-1}) was achieved under 3.25 ± 0.25 mmol L^{-1} $N\text{-}NO_3^-$ and 0.125 ± 0.025 mmol L^{-1} $P\text{-}PO_4^{3-}$. 3D-contour plots were established to predict how much biomass productivity, growth rate and dry weight could be achieved with certain N and P concentration ranging from 0 to 5.0 mmol L^{-1} and from 0 to 0.45 mmol L^{-1} respectively. Furthermore, we found nitrate is depleted one day after phosphate. Future research is recommended to focus on the effect of light density on the nutrients utilization because it is unclear if light density would promote *S. costatum* to consume more N and P in the medium.

1. Introduction

Microalgae have been investigated as a human and animal food source for over 60 years (Vonshak, 1991). Since the climate change and global warming aroused more attention, the exploitation and utilization of sustainable energy have been one of the burning issues nowadays. As a potential source of biofuel, microalgae have received grand expectations because of relatively high oil production (Singh, 2011). Besides, other applications for microalgae such as cosmetics, health-care products, aquaculture feed and waste water purification are also promising.

When applied as aquaculture feeds, microalgae are the ideal live feed of bivalves, shrimps and certain finfish species because of their relatively high fatty acids content and other nutrients supply. Different concentration and different algae species both affect the growth ratio of larvae, bivalves and fish (Walne, 1970). Therefore, the development of cultivation technologies for the microalgae would allow the onshore production of these animals, with greatly improved product quality and safety (Benemann, 1992).

The species of algae used in aquaculture differ from the typical strains used in biotechnology research on algae. Therefore, because of the limited knowledge of biological parameters of the cultivated strains (*Rhodomonas, Isochrysis and Skeletonema sp.*), the operation of microalgae production systems is not efficient, and the costs associated to this process are unknown. The project RAAQUA (Robust Algae for Aquaculture) was launched to develop knowledge about the relationship between environmental factors, control variables (light, temperature, medium, harvesting regime and mixing) and the final yields (quantity, cost and quality) in the cultivation of microalgae (RAAQUA, 2015).

Microalgae subsist by light to fuel their metabolism. With the support of proper temperature and several nutrients including carbon, hydrogen, oxygen, nitrogen and phosphorus, microalgae are able to grow and multiply. Therefore, second to the light energy, nutrients are important to microalgae growth and reproduction. Besides, aquaculture animals absorb nutrients from the feed, especially proteins, carbohydrates and fatty acids. Therefore, high-quality microalgae feed is necessary for the healthy growth of aquaculture animals.

This research subject, *Skeletonema costatum*, is one of the most representative species of marine diatoms which has a wide distribution in shallow areas of the ocean worldwide and is artificially cultured as marine species feeds. It can produce long chained highly unsaturated fatty acids which is the key growth factor of aquaculture animals (Ackman et al., 1964). To realize higher cell density or higher content of certain components, such as the pigments, unsaturated fatty acids, carbohydrates or proteins which affect the growth of specific aquaculture animals, an alterable and efficient system should be developed based on the knowledge of environmental effects on cell composition of *Skeletonema costatum.*

Nitrogen (N) is the key factor for protein synthesis, and phosphorus (P) is essential for DNA, RNA, and energy transfer (Conley et al., 2009). They are both indispensable to support microalgae growth and are the key limiting nutrients. Therefore, this research will focus on the effect of various concentration of nitrogen and phosphorus in the culture medium. Meanwhile, the research on the synergistic effect of temperature and light on biomass productivity and components of *Skeletonema costatum* is performed by other group members.

1.1 Problem statement

Research has shown that the influence of nutrients on the biochemical composition of microalgae mainly focus on the protein, carbohydrates, lipids, chlorophyll a and enzyme activities. Besides, there is a clear difference in the species ability to take up nutrients in environmental conditions of various nitrate and phosphorus ratio (Chen et al., 2002). Therefore, through controlling the concentration of nitrate and phosphate in the culture medium, the highest cell density and best quality on specific cell compositions can be achieved at optimal temperature and light conditions. However, due to lack of knowledge on the effects of the N/P ratio on the growth of *Skeletonema costatum*, farmers and companies cannot establish a cost-effective system for culturing aquaculture feed. Therefore, this will be the main focus of this research.

1.2 Goal

Optimizing growth for *Skeletonema costatum* has many implications depending on the use the algal species where it is intended for. For instance, if production of protein is higher for a certain nitrate and phosphate concentration, these conditions can be used to make the yield of protein more efficient. In turn, this would benefit the food industry or hatcheries. Therefore, the purpose of this research is to determine the optimal nitrate and phosphate concentrations for the growth of *Skeletonema costatum*, to understand the limitation of external nitrate and phosphate to the cell compositions and to develop an efficient system for aquaculture feed. Batch culture experiments are set up to quantitatively simulate a series of nutritional environments with different nitrate and phosphate concentrations.

1.3 Research questions

Main question:

What is the effect of nitrate and phosphate concentration on biomass productivity and cell compositions of *Skeletonema costatum* in batch culture under stable light density and temperature?

Subsidiary questions:

1. Which concentration of nitrate and phosphate is the best for *Skeletonema costatum* cultures in connection with cell density and biomass productivity?
2. What is the relationship between nitrate and phosphate concentration in the medium and the content of proteins, carbohydrates and lipids in *Skeletonema costatum*?
3. How much nitrate and phosphate is absorbed by *Skeletonema costatum* during the growing period?

2. Theoretical framework

2.1 Microalgae culture for aquaculture feed

The production of microalgae plays a significant role in successful aquaculture systems, especially to larvae of bivalves, crustaceans and marine fish. Because fresh microalgae contain polyunsaturated fatty acids (PUFAs), essential to the growth of these aquaculture animals, the feed is the only source for them to obtain the nutrients (Janssen & Lamers, 2014). Therefore, the main purpose of microalgae culture is to achieve the highest cell density and most nutritious cells with the least budgets.

The condition of microalgae culture for aquaculture feeds is various, from outdoor ponds or raceways with nutrients added, to monocultures cultured indoors under controlled environmental conditions (Creswell, 2010). In general, medium design, carbon dioxide supply, light and temperature control are essential to microalgae culture. In the case of diatoms, the culture medium used is typically seawater with nutrients (primarily nitrates, phosphates, essential trace elements, vitamins and silicates) (Janssen & Lamers, 2014).

The growth of monocultured microalgae generally follows four phases as shown in Figure 1, the lag phase, the exponential phases, the stationary phase and the senescent phase in chronological order. During the lag phase which varies in length depending on the initial cell density, algae species, irradiance and temperature, the cells are acclimated to the new medium. In continuation to the lag phase, the algae grow at an accelerating rate which is termed the exponential phase. Microalgae are usually harvested for feeding during this phase because of nutritious cells and high yield (Creswell, 2010). As the result of high cell density, relatively less light energy and changed nutrients concentration, the cells stop dividing and begin to die.

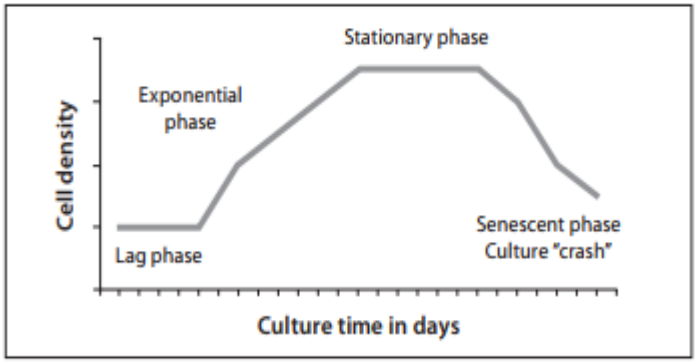

Figure 1. Phases in the growth of algal cultures (Creswell, 2010)

2.2 The characteristic of *Skeletonema costatum*

Diatoms are one of the most important types of phytoplankton at the base of the food chain. *Skeletonema costatum* is a relatively common marine diatom in coastal regions around the world, except polar areas. It lives by photosynthesis and reproduces sexually and asexually. The cells of *S. costatum* are short and cylindrical, usually forming a long chain. It consists two chloroplasts and one nucleus in the center of the cell (Guiry, 2011). In figure 2, *Skeletonema costatum* can be seen, visualized with an optical microscope at a magnification of 1000X.

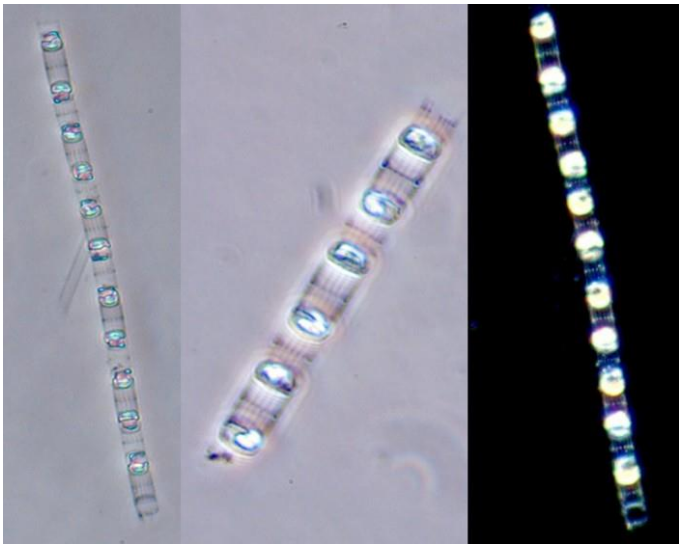

Figure 2. Micro-photography of Skeletonema costatum (x1000 magnification) (WoRMS Editorial Board, 2018)

The habitat of *Skeletonema costatum* is widespread in shallow areas of the ocean worldwide except polar seas. Based on occurrence records and database, the distribution map has been generated as shown in figure 3. The numbers indicate the times that Skeletonema costatum has been found or researched. The environmental ranges within which Skeletonema costatum can survive are also listed in the table below.

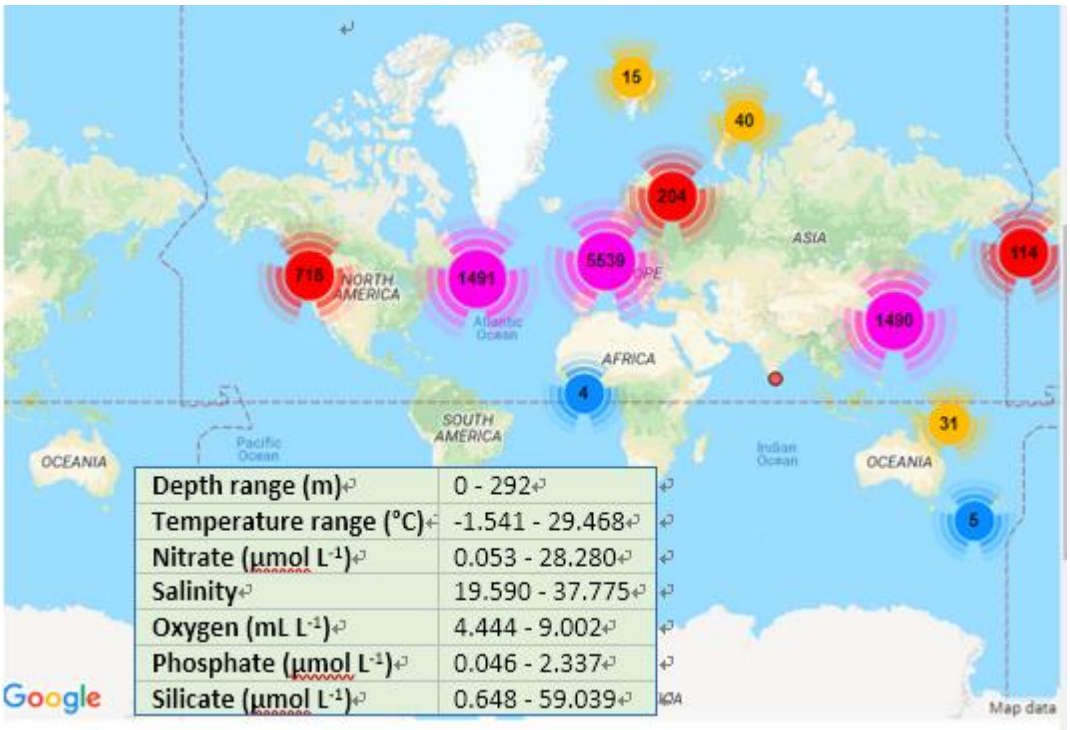

Figure 3. The worldwide distribution map and environmental ranges of Skeletonema costatum (Generated by GBIF, 2018)

Helm et al. (2004) reported that the median cell volume of *Skeletonema costatum* used in bivalve mollusk and fish hatcheries is 85 μm^3, with an organic weight of 29 pg and a lipid content of 12%. This species has an optimal salinity range of 18 - 25 ppt, while it can be cultured in a broader range of 15 – 30 ppt (Yan et al., 2002; Hoff and Snell, 2008). The

temperature and light ranges for culturing *Skeletonema costatum* are 10 -27 °C and 2,500 – 5,000 Lux respectively (Hoff and Snell, 2008).

2.3 The importance of nutrients

Research has shown that nutrients have a major effect on the cellular composition of microalgae which mainly focus on protein, carbohydrates, chlorophyll a, enzyme activities and cell polysaccharides (Hodgkiss and Lu, 2004; Dortsch et al., 1984; Thompson et al., 1994). Besides, the ability to reserve nutrients also varies depending on the N/P ratio in the culture environment (Dortsch et al., 1984). In general, the absorption of nitrogen matches the Redfield ratio, i.e. N: P = 16: 1 (Goldman et al., 1979; Redfield, 1934)

Nitrogen usually accounts for about 7^{-1}0% of the dry weight of cells. It is a basic component of all structural and functional proteins in algal cells (Parsons et al., 1961; Hu, 2004). When the cell nitrogen falls below the threshold, although photosynthesis can continue, the flow of carbon in the process of photosynthesis is diverted from protein synthesis to lipid or carbohydrate synthesis. Many studies show that under nitrogen-deficient conditions, some algal species increase their lipid content (Hu, 2004), while some species increase the carbohydrate content instead, e.g. In many *Dunaliella* strains, glycerol will accumulate along with increased saccharides (Borowitzka & Borowitzka, 1988).

Phosphorus is another essential nutrient significant in the cellular metabolism by forming structural and functional components. Orthophosphate is converted into organic components by diverse types of phosphorylation. Some symptoms of phosphorus depletion cultures are similar to those of nitrogen depletion cultures, such as increased lipids and carbohydrates content. (Hu, 2004).

In the case of *Skeletonema costatum*, according to the results of the research conducted by Liu D. et al. in 2002, *Skeletonema costatum* has the fastest growth and highest cell densities in the conditions of N/P = 16:1 among their N/P status (1:1, 4:1, 16:1, 80:1, 160:1). When the N/P ratio was greater than 16, the growth rate is better than when the N/P ratio was lower than 16. In contrast, the synthesis and accumulation of carbohydrates and proteins in the cells are higher in the state of N/P $\leqslant$ 16 (Li et al., 2000; Liu et al., 2002).

3. Method:

3.1 Algae culture

3.1.1 The strains of *Skeletonema costatum*

The marine microalgae *Skeletonema costatum* was originally taken from Roem van Yerseke B.V. and cultured through generations in the SEA LAB of HZ University of Applied Sciences. The medium recipe was improved based on the Walne's medium (Appendix B) which was created by Walne in 1970. The synthetic seawater was made of 30 grams of salt in one-liter demi-water. Sodium bicarbonate (0.7g in one liter synthetic seawater) was added to the medium to maintain the pH control. To meet the silicate needs to diatom, $Na_2SiO_3.9H_2O$ (30mg in one liter synthetic seawater) was added as well. The medium was then sterilized by filtering with a Sartorius liquid filter (0.2µm pore size) into pre-autoclaved 250ml Erlenmeyer flasks. Sterile conditions were maintained at all stages of transfer and culturing. After inoculation, the cultures were placed in the Orbital Shaker Incubator which has CO2 supply to help algae grow faster. The temperature was 18.9 ± 0.5 ℃ and the light intensity was 47 ± 20 µmol m^{-2} s^{-1}. The strains were inoculated to new medium in the exponential growth period every time.

3.1.2 Experimental setup

A small-scale batch-culture system (300-mL culture flasks) was used to grow the *Skeletonema costatum* under a matrix of four P-PO_4^{3-} concentration and four N-NO_3^- concentration. The specific designed concentration of nitrate and phosphate in the medium were planned as shown in Table 1. Out of 16 sets of experiments, ten were executed at last, which marked with pink in the table. The designed concentration of sodium nitrate ($NaNO_3$) was 1.17 mmol per liter Walne's medium, and for disodium phosphate ($NaH_2PO_4.2H_2O$) it was 0.13 mmol per liter. Every culture was made in quadruplicate (Group A,B,C,D) and was placed in the Orbital Shaker Incubator under continuous irradiance (47 ± 20 µmol m^{-2} s^{-1}) and stable temperature (18.9 ± 0.5 ℃). The medium stocks were prepared in advance and stored in the refrigerator. (See Appendix A for the detailed execution plan) After inoculation, the content of NO_3^- and PO_4^{3-} in the medium were measured to know the factual initial N and P concentration (Table 2).

Table 1. The designed concentrations of nitrogen and phosphorus in the medium and the N/P ratios of every culture experiment, the final completed experiments are marked with pink. (mM – mmol L^{-1})

N \ P	**P** 0.13 mM	**2P** 0.26 mM	**4P** 0.52 mM	**8P** 1.04 mM
N 1.17 mM	9.18	4.59	2.29	1.15
2N 2.34 mM	18.35	9.18	4.59	2.29
4N 4.68 mM	36.71	18.35	9.18	4.59
8N 9.36 mM	73.42	36.71	18.35	9.18

Table 2. The initial concentration of nitrate and phosphate in different groups.

	NP	N2P	N4P	N8P	2NP
NO_3^- (mmol L^{-1})	1.37	1.23	1.49	1.37	2.55
PO_4^{3-} (mmol L^{-1})	0.12	0.23	0.42	0.95	0.12
N/P ratio	11.31	5.39	3.51	1.45	20.91

	2N2P	4NP	4N2P	4N4P	8NP
NO_3^- (mmol L^{-1})	2.58	4.99	5.05	4.99	9.41
PO_4^{3-} (mmol L^{-1})	0.23	0.12	0.23	0.45	0.13
N/P ratio	11.46	43.10	22.10	10.98	73.23

3.2 Culture analysis

3.2.1 Growth analysis

The growth of every culture (Group A, B, C, D) was monitored by measuring daily the cell density with the counting chamber and the optical density with the spectrophotometer DR 5000 to get the dry weight according to the calibration curve. The curve that correlates the dry weight of different cell densities of *Skeletonema costatum* with the optical density at 750nm (Appendix C) is measured in advance. In addition, the growth rate μ (day^{-1}) and the biomass productivity (g L^{-1} day^{-1}) is calculated with the following formulas:

$$\mu = (InC_1 - InC_0)/(T_1 - T_0) \quad \text{Or} \quad \mu = (InDW_1 - InDW_0)/(T_1 - T_0)$$

- C_1 and C_0 refer to the cell density (MM cells L^{-1}) at the time T_1 and T_0 respectively.

$$Biomass\ Productivity = (DW_1 - DW_0)/(T_1 - T_0)$$

- DW_1 and DW_0 refer to the dry weight (g L^{-1}) at the time T_1 and T_0 respectively.

3.2.2 Biochemical analysis

To measure the uptake of NO_3^- and PO_4^{3-} in the cell body, the concentration analyses of NO_3^- and PO_4^{3-} in the medium were performed in Group A and Group B with a HACH DR5000 spectrophotometer and HACH kits daily during the exponential growth phase (For the protocol see Appendix D and E). The algal cells in the taken samples were filtered first with a Whatman GF/C filter.

The content of proteins and carbohydrates were measured in the culture of Group C and Group D in two growth phases. The first sample was taken in the exponential phase when the obvious growth in cell density was observed. The second sample was taken in the stationary phase once the cultures do not show further increase in cell density. The proteins were measured according to Folin reagent method (For the protocol see Appendix F). The carbohydrates were measured according to an acid extraction method (For the protocol see Appendix G). To get accurate results, the extraction process was performed as scrupulously as possible. In addition, samples from Group C and Group D were washed with ammonia formate (0.5 mol L^{-1}) and centrifuged to keep the pellets in the -80 ℃ freezer. At last, they were turned into powder through the process of freeze drying with the Martin Christ- ALPHA

1-2 LD plus freeze dryer in order to be ready for protein and carbohydrate.

4. Results

4.1 Effect of NO_3^- and PO_4^{3-} concentration on the growth of *Skeletonema costatum*

The growth of *Skeletonema costatum* was monitored daily by measuring the cell density and dry weight during 9~11-day period. Monitoring was stopped when a stable trend on cell density was presented. Table 3 gives an overview of the maximum values of biomass productivity, growth rate, cell density and dry weight which can be reached in various nitrate and phosphate concentration combinations.

It is surprising that the cell density peaked at 60 million cells ml^{-1} in Group 8NP, which is profoundly higher compared to previous researches. The way of counting microalgae cells under microscope was not reliable because the counting of dead cells cannot be excluded. Therefore, the growth rate was calculated based on the values of dry weight with cell density as reference.

In the first four groups (Group NP, N2P, N4P & N8P) with nearly the same concentration of nitrate (1.36 ± 0.13 mmol L^{-1}), the results indicate that increasing phosphate to 0.95 mmol L^{-1} did not lead to higher cell density, higher dry weight, higher growth rate or higher biomass productivity. In contrast, an increase on nitrate concentration leads to better performance in biomass productivity, growth rate and dry weight. For instance, in groups NP, 2NP, 4NP & 8NP with increased amount of nitrate and a steady concentration of phosphate (0.12 mmol L^{-1}), the dry weight saw a greatly increase from 0.70 g L^{-1} to 1.6 g L^{-1}.

Regarding to the biomass productivity, compared to group NP, group 2NP with doubled nitrate concentration presented a 171% increase from 0.14 g L^{-1} day^{-1} to 0.38 g L^{-1} day^{-1}. However, in group 2NP, 4NP and 8NP with more than double higher nitrate concentration, slight difference can be seen on biomass productivity which fluctuates at 0.33 ± 0.05 g L^{-1} day^{-1}. Among all experimental groups, the growth rate reached the peak in Group 2N2P (0.51 day^{-1}), showing a 53% increase compared to group N4P with the lowest growth rate of 0.24 day^{-1}.

Table 3. The average N/P ratios, maximum biomass productivity, maximum growth rate and maximum cell density of experimental groups.

Group	NO_3^- mmol L^{-1}	PO_4^{3-} mmol L^{-1}	N/P ratio	Max. Biomass Productivity (g $L^{-1}day^{-1}$)	Max. Growth rate (day^{-1})	Max. Cell density (10^6 cells ml^{-1})	Max. Dry weight (g L^{-1})
NP	1.37	0.12	11.31	0.14	0.33	23.31	0.70
N2P	1.23	0.23	5.39	0.13	0.30	24.94	0.77
N4P	1.49	0.42	3.51	0.08	0.24	18.38	0.52
N8P	1.37	0.95	1.45	0.07	0.28	18.24	0.55
2NP	2.55	0.12	20.91	0.38	0.32	26.50	0.88
2N2P	2.58	0.23	11.46	0.35	0.51	44.50	1.51
4NP	4.99	0.12	43.10	0.28	0.40	46.70	1.42
4N2P	5.05	0.23	22.10	0.31	0.45	52.20	1.39
4N4P	4.99	0.45	10.98	0.17	0.39	40.30	1.11
8NP	9.41	0.13	73.23	0.30	0.39	60.00	1.60

In terms of the effect of nitrate and phosphate concentration on continuous changes of dry weight, for instance, in the Group NP (Figure 4), when the nitrate concentration significantly dropped from 53.62 mg L^{-1} to 4.88 mg L^{-1} from day 3 to day 5, it showed an exponential growth on the dry weight since day 3. The specific growth curves of other groups are presented in Appendix H.

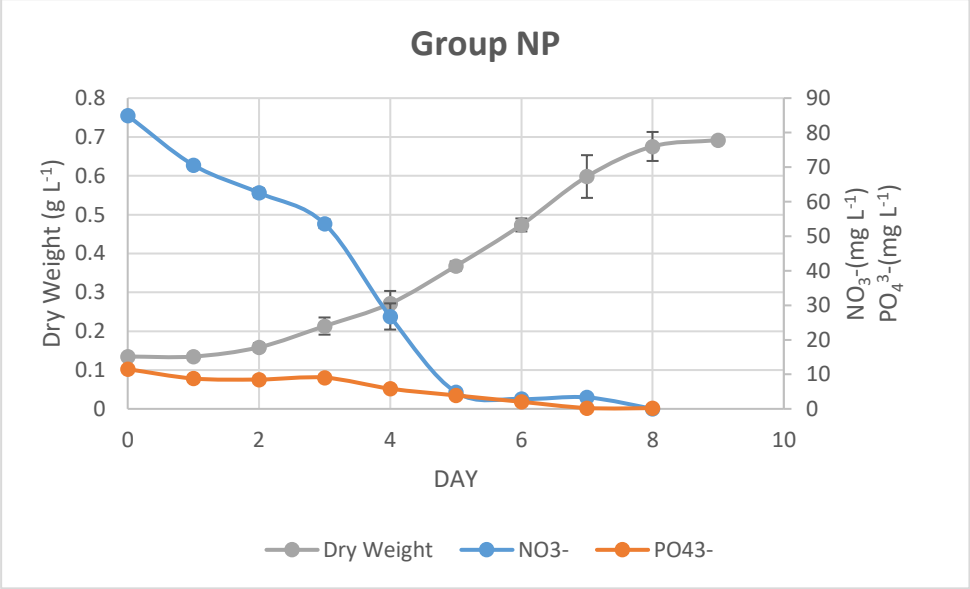

Figure 4. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group NP)

As shown in figure 5 and figure 6, the results indicate that both biomass productivity and growth rate are influenced by N/P ratio in the medium. Increasing the N/P ratios up to 21 increases the biomass productivity, reaching a maximum value of 0.38 g L^{-1} day^{-1}. Beyond this point, the biomass productivity fluctuated at 0.30 ± 0.02 g L^{-1} day^{-1}. When it comes to the relationship between maximum growth rate and N/P molar ratios, the peak of 0.51 day^{-1} reached when increasing the N/P ratio to 11.46. Similar results occurred to biomass productivity, at the N/P ratios of approximately 11 to 21, optimum results were achieved. It is also significant to note that not only the N/P ratio but also the concentration of nitrate and phosphate influence the biomass productivity and growth rate simultaneously. This can be proved by three groups with the same N/P ratio of approximately 11 since they achieved a difference on the biomass productivity and growth rate.

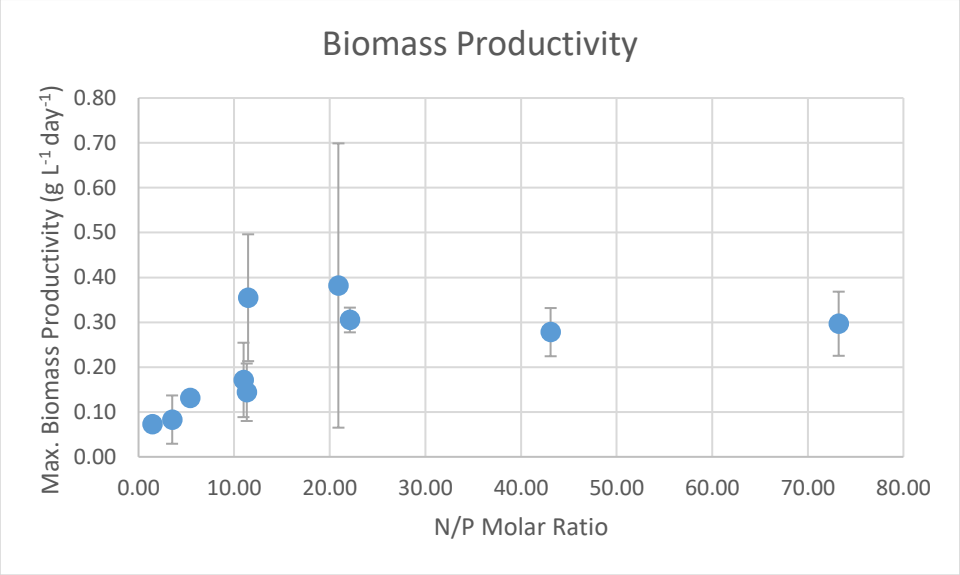

Figure 5. The relationship between maximum biomass productivity (dry weight) (g L^{-1} day^{-1}) and N/P molar ratios

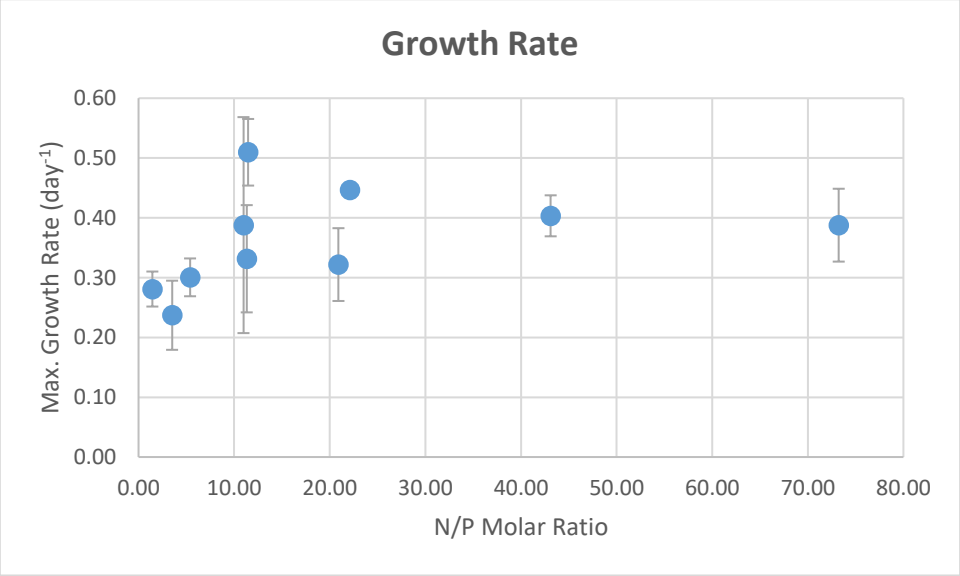

Figure 6. The relationship between maximum growth rate (day^{-1}) and N/P molar ratios

Furthermore, to predicate the results in other nitrate and phosphate concentration, contour plots were created based on the maximum values of dry weight, growth rate, biomass productivity reached on corresponding nutrients concentration by Sigma Plot software (Figure 7, 8 & 9). Nevertheless, it is worthy to mention that because there is only one group of experiments with eight times nitrogen concentration (group 8NP) and also one with eight times phosphorus concentration (group N8P), the results of this two groups were excluded from the data source in order to increase the reliability of existing contours.

So far, the model can predict that the optimal nutrients concentration for the biomass (dry weight) and growth rate of *S. costatum* is 3.5 ± 0.5 mmol L^{-1} $N\text{-}NO_3^-$ and 0.30 ± 0.05 mmol L^{-1} $P\text{-}PO_4^{3-}$. It corresponds to 255 ± 21 mg L^{-1} $NaNO_3$ and 39 ± 8 mg L^{-1} $NaH_2PO_4.2H_2O$ in Walne's medium. Besides, the optimal nutrients concentration for biomass productivity of *S. costatum*

is 3.25 ± 0.25 mmol L^{-1} $N\text{-}NO_3^-$ and 0.125 ± 0.025 mmol L^{-1} $P\text{-}PO_4^{3-}$. It corresponds to 276 ± 21 mg L^{-1} $NaNO_3$ and 19.5 ± 4 mg L^{-1} $NaH_2PO_4.2H_2O$ respectively.

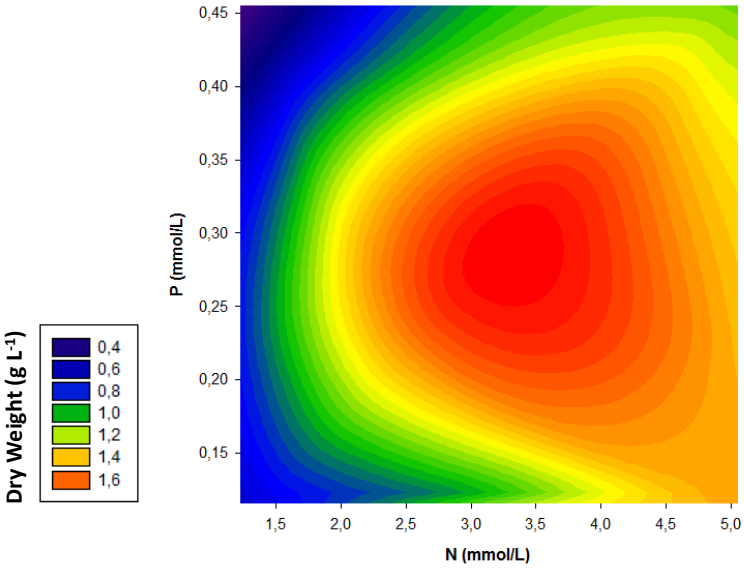

Figure 7. Contour plot of dry weight on various N and P concentrations (The warmer the color tone is, the higher the dry weight can be reached).

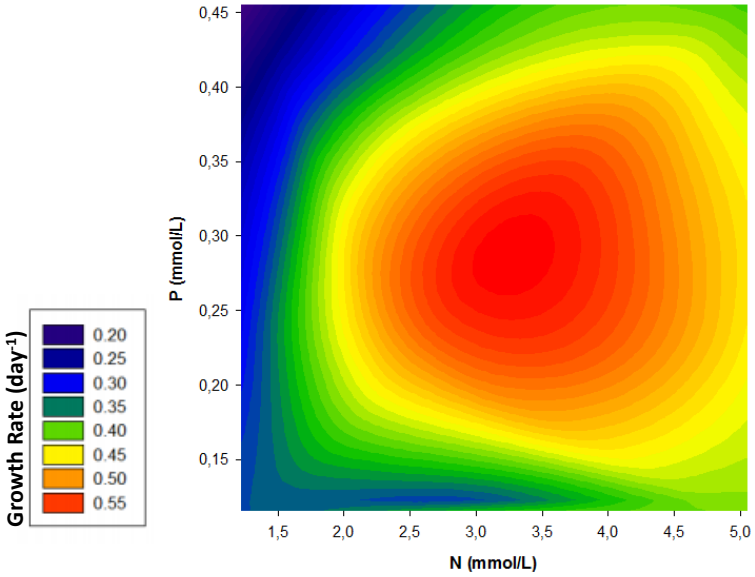

Figure 8. Contour plot of growth rate on various N and P concentrations (The warmer the color tone is, the higher the growth rate can be reached).

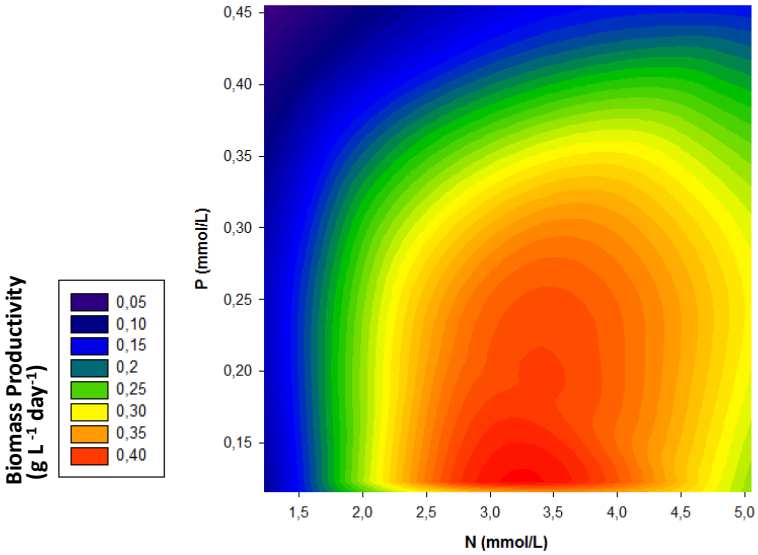

Figure 9. Contour plot of biomass productivity on various N and P concentrations (The warmer the color tone is, the higher the biomass productivity can be reached).

4.2 Consumption of nitrate and phosphate

Samples for NO_3^- and PO_4^{3-} analysis were taken from first two flasks in every group daily and measured with HACH kits and spectrophotometer. The results indicate that *Skeletonema costatum* has a significantly higher demand for nitrate than phosphate. As shown in Figure 10, *S. costatum* can consume almost 100% of nitrate, ranging from 76.00 mg L^{-1} to 295.29 mg L^{-1}, except in Group 8NP where the highest consumption of 583.32 mg L^{-1} occurred. In contrast, Figure 11 presents that even though the initial concentration of phosphate increased from 11 mg L^{-1} to 90 mg L^{-1}, the consumption of phosphate cannot increase more than 21.25 mg L^{-1} occurred in Group 2N2P. The consumed phosphate of other groups fluctuated at 14.00 mg L^{-1}.

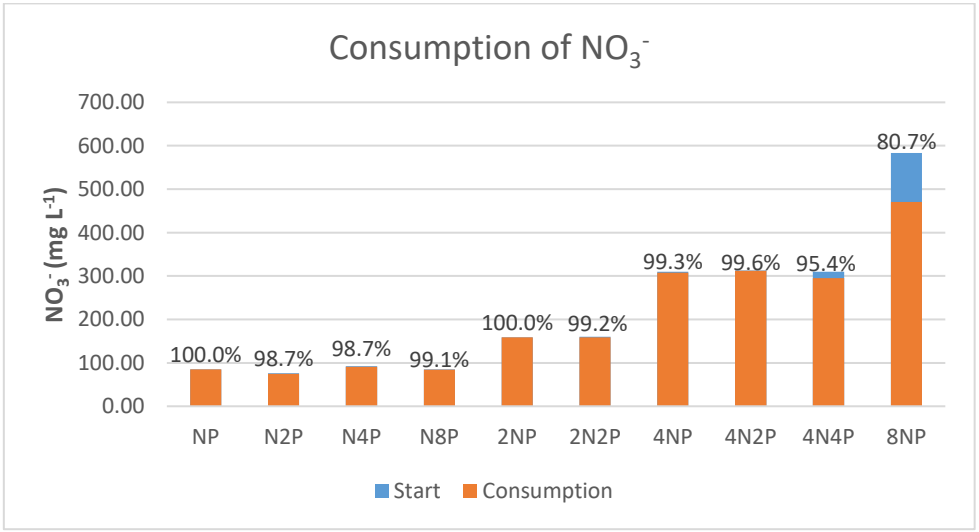

Figure 10. The average consumption of NO_3^- during the whole growth phase (Day 0 to stationary phase). The orange bars cover on the blue bars indicate how much NO_3^- is consumed in relation to the initial concentration, corresponding consumption percentage is stated on the top of every bar.

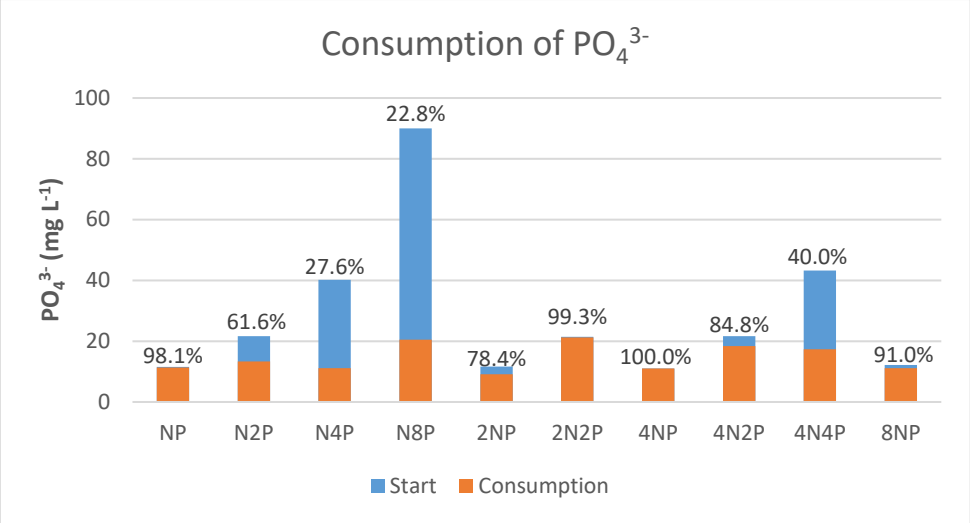

Figure 11. The average consumption of PO_4^{3-} during the whole growth phase (Day 0 to stationary phase). The orange bars cover on the blue bars indicate how much PO_4^{3-} is consumed in relation to the initial concentration, corresponding consumption percentage is stated on the top of every bar.

In addition, in Group 8NP, the change on nitrate concentration in the medium is hard to detect in day 10 to day 13. It seems the microalgae cannot consume the nitrate anymore as happened in other groups. The lowest value, 112.7 mg L^{-1} of nitrate, was occurred on day 12 (the red point in Figure 12). This infers that the cells cannot ingest nitrate anymore under this experimental conditions, consisting of a light density of 47 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of 18.9 ± 0.5 ℃. To see if the nutrients consumption is related to light density, we increased it to 75 ± 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on Day 13 (the temperature is maintained at 18.9 ± 0.5 ℃). As illustrated by the orange line in Figure 12, there is a downward trend of nitrate concentration in the medium which means *S. costatum* continued to ingest nitrate under higher light density conditions.

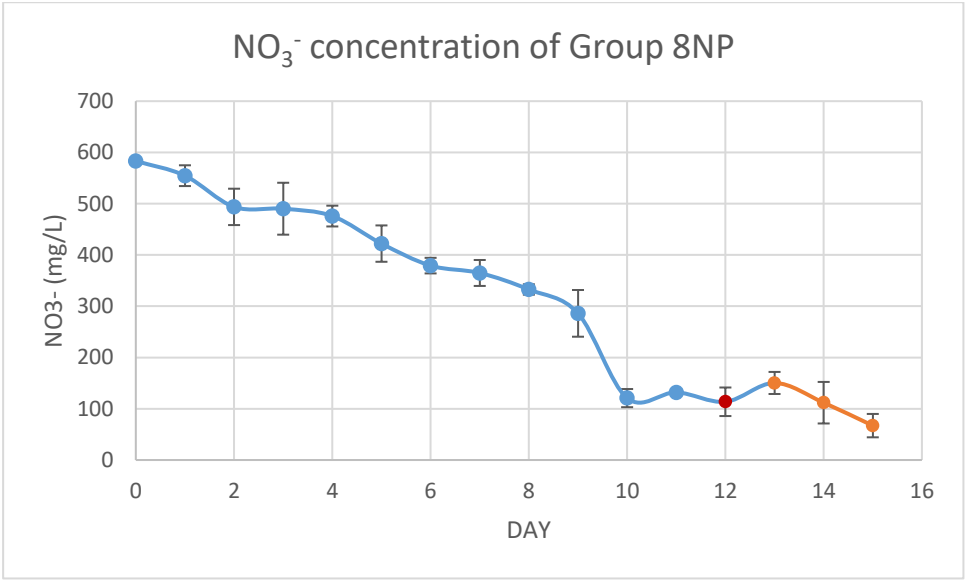

Figure 12. The NO_3^- concentration of Group 8NP. The red point is the lowest value of residual nitrate concentration that can be achieved under the light density of 47 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The orange line starts on day 13 indicates that the light density was increased to 75 ± 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

In the phosphate-depleted groups, it is noticeable that the nitrate is always depleted one day

after the phosphate is. Figure 13 demonstrates the residual curve of nitrate and phosphate concentration in the medium of group 4NP. Phosphate is completely depleted on day 8 and nitrate is depleted on day 9. The residual curves of other groups are listed in Appendix H.

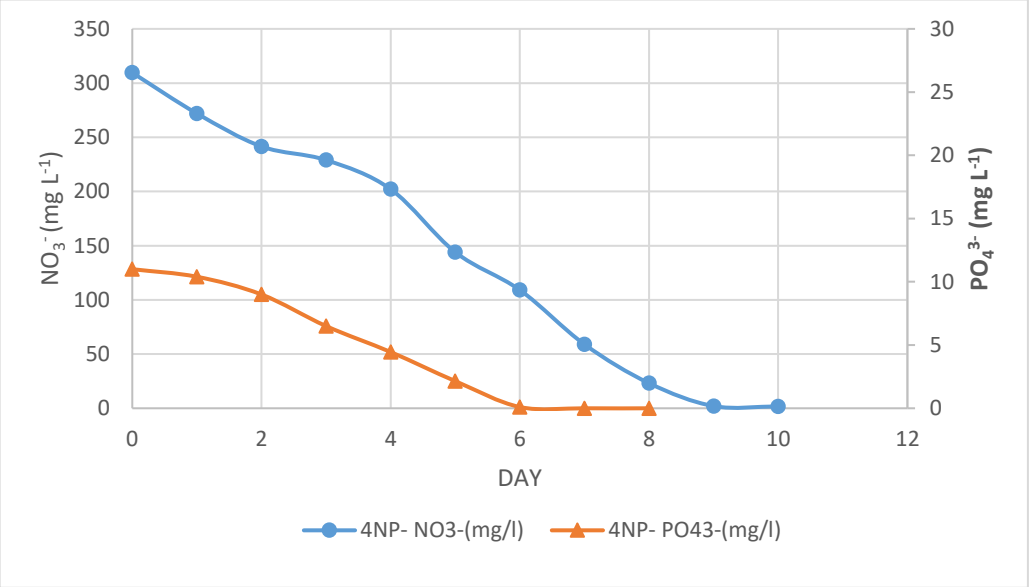

Figure 13. Nitrate and phosphate residual in the medium during the growth phase (Day 0 to stationary phase) in the group 4NP. Blue line and orange line indicate the concentration of nitrate and phosphate respectively.

5. Discussion

5.1 Growth Limitation

The first result we found is that increasing nitrate concentration in Walne's medium helps to improve the growth of *S. costatum* while increasing phosphate concentration does not. In addition to the increase on the dry weight, growth rate and biomass productivity, this result is also reflected by limited utilization on phosphate. This indicates that the growth of *S. costatum* is strongly N-limited. Andersson (2014) stated a similar result that there was no effect in response to the addition of P alone in his study at northern Swedish streams. In addition, Stramski et.al (2002) observed a 61% increase on the growth rate of the diatom *Thalassiosira pseudonana* under N-replete conditions.

According to the derived model, it is found that the optimal concentration of nitrate and phosphate for the highest growth rate (0.55 ± 0.05 day^{-1}) and dry weight (1.8 ± 0.2 g L^{-1}) of *S. costatum* is 3.5 ± 0.5 mmol L^{-1} $N\text{-}NO^{3-}$ and 0.30 ± 0.05 mmol L^{-1} $P\text{-}PO_4^{3-}$ under a light density of 47 ± 20 µmol m^{-2} s^{-1} and a temperature of 18.9 ± 0.5 ℃. This result goes beyond previous reports that Hu et.al (2004) reported in their batch culture experiments of *S. costatum* where the maximum growth rate (1.6 day^{-1}) occurred in the medium with 0.088 mmol L^{-1} $N\text{-}NO^{3-}$ and 0.036 mmol L^{-1} $P\text{-}PO_4^{3-}$ under a light density of 100 µmol.m^{-2}. s^{-1} and a temperature of 22 ℃. It is unlikely that with lower nitrate and phosphate concentration, higher growth rate can be achieved instead. As light density doubled in their experiments, the rate of photosynthesis will increase which mainly leads to the increase of growth rate.

Unfortunately, I cannot find more related studies since very few researches dealt with growth optimization of *Skeletonema costatum*. Nevertheless, this inference can be demonstrated by the research of Leonardos and Geider (2014) on the diatom *Chaetoceros muelleri*. They revealed that the maximum growth rate depends more on light density and salinity than on nutrients uptake. Therefore, a higher growth rate can be expected under the optimal nitrate and phosphate concentration this research indicates if doubling the light density. Furthermore, it is worthy to note that light only becomes the depending factor when sufficient amounts of nutrients are available (Andersson, 2014).

The optimal concentration for the highest growth rate corresponds to the N/P molar ratio of 12.25 ± 3.75. In line with previous research, the result of N/P ratio accords to that of the Redfield ratio under nutrient-replete conditions, which ranges from about 5 to 19 (Geider & La Roche, 2002).

In addition, Myklestad (1977) found that the N/P balance point is around 12 under a light density of 35 - 42 µmol m^{-2} s^{-1} for *S. costatum.* This means neither of N or P is limiting the other (all other nutrients are exceeded) at that point. This well explains that the highest biomass productivity and growth rate were observed under this nutrient concentration and confirms the prediction that my contour plots showed.

5.2 Nitrate and Phosphate Utilization

After continuous monitoring on the nitrate and phosphate consumption of multiple groups, we found that in all phosphate-depleted groups, nitrate is always depleted one day after phosphate. Similar results occurred in the research about the diatom *Chaetoceros muelleri* and Rhodomonas sp. (Leonardos and Geider, 2014; da Silva, 2009)

In addition, Leonardos and Geider (2014) have also found that nitrate and phosphate uptake was simultaneously affected by light density and dissolved N and P in the medium. This well explained that we observed in group 8NP that the microalgal cells continued to consume the nitrate after I increased the light density.

6. Conclusion

The nitrogen and phosphorus concentration greatly affect the biomass productivity and growth rate of Skeletonema costatum. 1) Increasing the phosphate concentration doesn't help the growth of *S. costatum* since it cannot consume a PO_4^{3-} concentration over 21.25 ± 0.25 mg L^{-1} under the light density of 47 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the temperature of 18.9 ± 0.5 ℃. 2) Doubling the nitrate concentration in the Walne's medium definitely increases the biomass productivity of *S. costatum*. 3) The model we established so far can predict that the optimal concentration for the highest dry weight (1.6 ± 0.2 g L^{-1}) and growth rate (0.55 ± 0.05 day^{-1}) of *S. costatum* is 3.5 ± 0.5 mmol L^{-1} $N\text{-}NO_3^-$ and 0.30 ± 0.05 mmol L^{-1} $P\text{-}PO_4^{3-}$; The optimal nutrients concentration for biomass productivity (0.40 ± 0.05 g L^{-1} day^{-1}) of *S. costatum* is 3.25 ± 0.25 mmol L^{-1} $N\text{-}NO_3^-$ and 0.125 ± 0.025 mmol L^{-1} $P\text{-}PO_4^{3-}$. (under the light density of 47 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the temperature of 18.9 ± 0.5 ℃) 4) The optimal N/P molar ratio for biomass and growth rate is 12.9 ± 4.6. 5) Nitrate depletes one day after phosphate when these nutrients are not excessive. 6) Further research can be conducted to figure out the influence of light density on nutrients utilization and growth of *S. costatum*.

7. Recommendation

In this research, a microscope and counting chamber are used to count the microalgae cells with several dilution times. The results of cell density presented a great error since the values are quite higher than other researches, especially at the later growth stages. With microscope, it was difficult to tell if the cells were alive or not. Another reason is that with increasing of dilution factor at later growth stages, the error will increase when calculating the cell density, since counting one more cell means increasing dozens of cells in the results. Therefore, using other reliable methods to calculate the cell density was recommended, for example fluorescent labeling the cell walls so that the dead cells will not be counted.

To make the set-up model precise and ensure the increasing amount of nitrate would not affect the consumption of phosphate, it is recommended to complete other experimental groups (Group 8N2P, 8N4P, 8N8P). In this research, the experimental range of nitrate and phosphate concentration are from 1.17 mmol L^{-1} to 9.36 mmol L^{-1} and 0.13 mmol L^{-1} to 1.04 mmol L^{-1}, respectively. However, only one group with the 8 times nitrate and only one with 8 times phosphate is not reliable to predict the results in the whole range.

Furthermore, *S. costatum* is cultivated at a relatively low light density condition (47 ± 20 $\mu mol.m^{-2}. s^{-1}$) under the temperature of 18.9 ± 0.5 ℃. In the experiment of Group 8NP, after the light density was increased to 75 ± 30 $\mu mol.m^{-2}. s^{-1}$ on Day 13, *S. costatum* continued to ingest nitrate. Thus, further study can be designed to find out the effect of light density on the nutrients consumption.

8. References

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Appendices

Appendix A: Execution Plan

Preparation: Medium; Materials autoclaving;

Materials:

- Erlenmeyer flasks (1L ; 300ml)
- Volumetric flask (100ml x 5)
- Pipette and tips

Stocks:

Nutrient solution 400ml

$NaNO_3$ (100 g L^{-1} H_20) 400ml

$NaH_2PO_4.2H_2O$ (20 g L^{-1} H_20) 400ml

$Na_2SiO_3.9H2O$ (30g L^{-1} H_20)

Vitamin solution

Method:

1. Autoclave at least 32 flasks and the pipette tips.
2. Make the medium stocks following the next steps:

1st. Make 100ml Trace metal solution (TMS).

ZnCl2	2.1 g
CoCl2.6H2O	2.0 g
Na2MoO4.2H2O	0.176 g
CuSO4.5H2O	2.0 g

2nd. Make 100ml Nutrient solution

NaFe.EDTA	0.202 g
MnCl2.4H20	0.036 g
H3BO3	3.36 g
EDTA (Disodium salt)	4.50 g

TMS (above)	0.10 ml

3rd. Make 400ml nitrate and 400ml phosphate solution.

$NaNO_3$	40 g
$NaH_2PO_4.2H_2O$	8 g

Note: $Na_2SiO_3.9H2O$ (30g L^{-1} H_20) and Vitamin solution were made by Christos Latsos and kept in the refrigerator before the experiments.

3. Mark and keep flasks in the refrigerator.

Day 1 Inoculate into flasks

Material: 450ml *Skeletonema costatum.* Autoclaved flasks. Medium stocks.

1. Calculate the original cell density of *Skeletonema costatum* culture.

2. Make 1 liter of Walne's medium for each group as Table 1 shows.

Table 1. The amount of materials and stocks in 1 liter medium.

Salt	Nutrient Solution	Bicarbonate	$Na_2SiO_3.9H2O$ (30g L^{-1})	Vitamin solution
30g	1ml	0.7g	1ml	0.1ml

Add the amount of nitrate and phosphate into Walne's medium according to Table 2.

Table 2.

1 liter per group		**NaNO3 (100 g L^{-1})**	**$NaH_2PO_4.2H_2O$ (20 g L^{-1})**
1	NP	1ml	1ml
2	N2P	1ml	2ml
3	N4P	1ml	4ml
4	N8P	1ml	8ml
5	2NP	2ml	1ml

6	2N2P	2ml	2ml
7	2N4P	2ml	4ml
8	2N8P	2ml	8ml
9	4NP	4ml	1ml
10	4N2P	4ml	2ml
11	4N4P	4ml	4ml
12	4N8P	4ml	8ml
13	8NP	8ml	1ml
14	8N2P	8ml	2ml
15	8N4P	8ml	4ml
16	8N8P	8ml	8ml

3. Filter 150 ml medium into autoclaved 300ml-flasks by Sartorius liquid filter (0.2µm pore size). *Note:* Every group is made in quadruplicate (Group A, B, C, D.).

4. Make every flask with 150ml medium and 13ml *Skeletonema costatum* culture.

5. Keep in the incubator and record down the temperature and irradiation.

6. Measure the initial concentration of NO_3^- and PO_4^{3-} in the medium of every group.

Daily Tasks

1. Take around 7ml sample from flasks of group AB, prepare demi-water as blank.
2. Count cells in every flask with the counting chamber (dilute if necessary)
3. Measure Dry weight at OD750 (dilute if necessary)
4. Filter the cells, collect the medium.
5. Add sample into the cuvette (usually dilute), measure NO_3^- at uv-vis OD220 and OD275.
6. PO_4^{3-}: add 10ml sample with powder pillows, demi-water as blank (Test Program 490)
7. Count cells and measure OD750 in every flask of group CD as well. (dilute if necessary)

Protein and carbohydrate analysis

(Samples taken in exponential phase and stationary phase)

Materials:

- 32 plastic sample tubes
  - 15 ml x 16 for Proteins
  - 50 ml x 16 for Carbohydrates
- 32 glass tubes for supernatant
- Demi water bottle
- 2 beaker (one for wastes, one for demi-water)
- Extraction solutions
  - Solution A, B& C
  - Folin
  - Phenol solution

Method:

1. Take samples into centrifuge tubes.
   - Proteins --- 5ml
   - Carbohydrate --- 5ml
2. Centrifuge for 15 minutes at 43500rpm.
3. Mix the extraction solution D and folin reagent.
4. Label the tubes
5. Carbohydrates: add 2 ml of H2O + 2 ml of phenol reagent → rest for 40 minutes
6. Protein: add 5 ml solution D → Homogenize → rest for 10 minutes
   add 0.5 ml solution E → rest for 30 minutes
7. Carbohydrates: rapidly add 10 ml H_2SO_4→ rest for 10 minutes → Homogenize
   → Centrifuge for 10 minutes at 4350 rpm
8. Protein: Centrifuge for 10 minutes at 3000 rpm
9. Transfer the supernatant into new tubes and measure the optical density.
   - Proteins at 750nm
   - Carbohydrates at 490 nm

Appendix B: Walne's medium for algal cultures

Stocks

(1) Trace metal solution (TMS)	**per 100 ml**
$ZnCl_2$	2.1 g
$CoCl_2.6H_2O$	2.0 g
$(NH_4)6Mo_7O_{24}.4H_2O$	0.9 g
$CuSO_4.5H_2O$	2.0 g

Make up to 100 ml with distilled water. This solution is normally cloudy. Acidify with a few drops of conc. HCl to give a clear solution.

(2) Vitamin solution	**per 100 ml**
Vitamin B_{12}. (Cyanocobalamin)	10.0 mg
Vitamin B_1 (Thiamine.HCl)	10.0 mg
Vitamin $_H$ (Biotin)	200.0 µg

Make up to 100 ml with distilled water.

(3) Nutrient solution	**per litre**
$FeCl_3.6H_20$	1.3 g
$MnCl_2.4H_20$	0.36 g
H_3BO_3	33.6 g
EDTA (Disodium salt)	45.0 g
$NaH_2PO_4.2H_2O$	20.0 g
$NaNO_3$	100.0 g
TMS (1 above)	1.0 ml

Make up to 1 litre with distilled water

Medium	**per litre**
Nutrient solution (3)	1.0 ml
Vitamin solution (2)	0.1 ml
Sterilised seawater	1.0 litre

Dispense nutrient and vitamin solutions separately into 10 ml and 1 ml respectively and autoclave at 15 psi for 15 minutes. Add an aliquot of each aseptically to 10 litres of sterilized seawater.

Note: Because of lack of $FeCl_3.6H_20$ and $(NH_4)6Mo_7O_{24}.4H_2O$ in the HZ laboratory, the medium recipe was improved to meet the need of every nutrient. (See Appendix A for the medium preparation)

Reference

Walne PR (1970) Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea, Crassostrea, Mercenaria, and Mytilis.* Fish. Invest. 26, 1-62.

Appendix C: Protocol for dry weight determination

(Adapted by María Cuaresma, 2012)

Solutions required

Ammonium formate 0.5 M (31.53 g L^{-1}) (100^{-1}50 mL /determination)

Demi water

Materials required

Whatman glass fiber filters, GF/F, ∅ 55 mm, nominal pore size 0.7 mm

Equipment

Analytical balance with readability of 0.001 mg and repeatability of ± 0.015 mg

Pipette or analytical balance for quantification of culture sample

Oven at 95 °C (passive convection)

Procedure

1. Wash a week supply of filters, by filtering 150 mL of MiliQ water over each filter.

2. Stack the filters in a paper made envelope and dry the wet filters at 95 °C for 24 hr.

3. Allow filters to cool down in a desiccator for at least 2 h

4. Take a filter from the envelope and put it in 2 stacked aluminum trays (labeled accordingly)

5. Weigh dried filters inside only the upper aluminum tray (w1). Make sure the weighing table and balance are clean.

6. It is important that the ammonium formate solution possess the same molarity as the media used for the cultivation of the algae.

   Repeat the described procedure below in triplicate for an accurate DW determination

   1st – Dilute the culture sample (1ml) with ammonium formate to a total volume of 40 ml in a greiner tube (50 ml).

   2nd – filter the diluted sample at low and constant vacuum, 25 mm Hg

   3rd – wash the tubes for three times with 20 ml of ammonium formate 0.5 M (two times with 20 ml and the 3rd time with four times 5 ml (with a pipette) pipetting on the wall to remove algae from the wall.

7. Dry filters in the oven at 95º C for 24 h

8. Put the filters in the desiccator for 2 h or more

9. Weigh dried filters, again only the upper aluminum tray and filter, on a clean balance (w2)

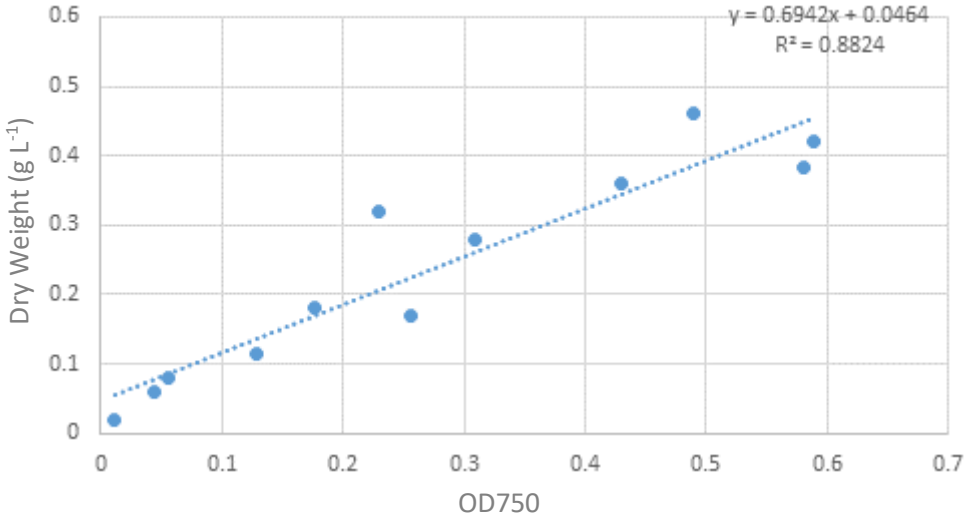
Important!!!!! Contact the filters and cups only with tweezers, never with your fingers

Calculation:

DW (g L^{-1}) = [w2 (g) – w1 (g)] / [‘x’ (mL) * dilution factor]

DW (g kg^{-1}) = [w2 (g) – w1 (g)] / [‘y’ (g) * 0.001]

The calibration curve achieved by Christos Latsos as shown below:



Appendix D: Protocol for NO_3^- determination

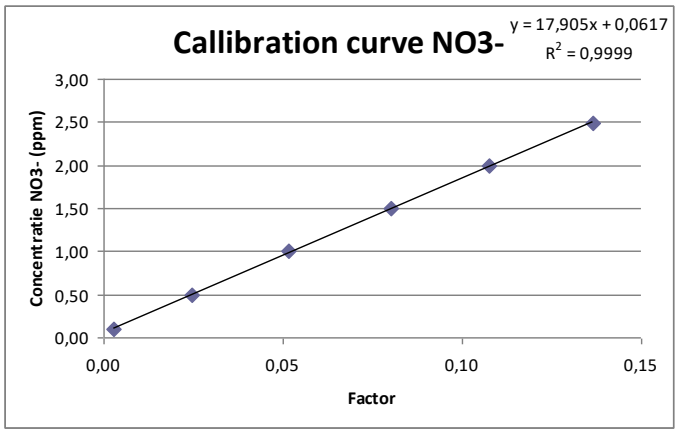
Materials:

- Spectrophotometer DR5000
- Quarts cuvettes and cuvette paper
- KNO_3
- 100 ppm NO_3^- stock solution (weigh 163,03 mg KNO_3 and add to 1 L demineralized water)
- Calibration series NO_3 0,1 ppm – 2,5 ppm (by diluting the 100 ppm stock solution)
- Synthetic seawater with same salinity as the samples
  Dissolve 30 grams salt in 1 L demineralized water
- Whatman GF/C filter

This method is based on analysis in the ultraviolet range. The absorbance is measured at 220 nm and 275 nm. The nitrate concentration is calculated as follows:

Factor = Abs. (220 nm) – 2*Abs. (275 nm)

For calibration: first make a range of solutions of known nitrate concentrations in synthetic seawater with the same salinity as the samples.
The result is a calibration series for nitrate:



Using the formula, the unknown concentration of NO_3^- in the sample can be determined.

Procedure measurements:

- Filter all samples with a .45 filter or Whatman GF/C filter
- Set the Perkin Elmer spectrophotometer at “ultraviolet visible” at wavelength 275 nm and 220 nm.
- Enter the number of samples and name them if necessary
- Measure the blank (synthetic seawater): one cuvette filled with synthetic seawater.
- After measuring the blank, remove the cuvette and fill with sample.
- Measure the samples and make sure there are no air bubbles on the inside or water on the outside of the cuvette.
- Determine with help of the measured absorbance’s the factor (Factor = Abs. (220 nm) – 2*Abs. (275 nm)
- Then, determine with help of the formula from the calibration series the unknown concentration of NO_3^- in the sample.

When diluted: keep in mind the dilution factor while calculating! Diluting is necessary when the factor is higher than the factor on de X-axis of the calibration series.

Appendix E: Protocol for PO_4^{3-} analysis

Materials:

- Spectrophotometer DR5000
- Sample cell x 2
- Power Pillow
- Synthetic seawater with same salinity as the samples
- Dissolve 30 grams salt in 1 L demineralized water
- Whatman GF/C filter

Procedure measurements:

1. Filter all samples with a .45 filter or Whatman GF/C filter.
2. Fill a sample cell with 10-mL of sample.
3. Add the contents of one PhosVer 3 phosphate Powder Pillow to the cell. Immediately stopper and shake vigorously for 30 seconds.
4. Start the instrument timer. A two-minute reaction period will begin.
5. Fill a second sample cell with 10 mL of synthetic seawater as the blank
6. When the timer expires, wipe the blank and insert it into the cell holder.
7. ZERO the instrument. The display will show: 0.00 mg L^{-1} PO_4^{3-}
8. Wipe the prepared sample and insert it into the cell holder. READ the results in mg L^{-1} PO_4^{3-}.

Appendix F: Protocol for the protein determination

(Adapted from Lowry et al., 1951)

Reagents:

- SOLUTION A: 2% of Na2CO3 in 0.1N NaOH (4g L^{-1})
  (conservation period of 15 days)
- SOLUTION B: 0.5% of CuSO4·5H2O in water
  (conservation period of 1 month)0
- SOLUTION C: TARTRATE of K or Na 1%
  (conservation period of 1 month)
- SOLUTION D: 50 mL of A+1 mL of B+1 mL of C
  (conservation period of 1 day)
- SOLUTION E: Folin reagent diluted 2 times (1 vol. Folin+1 vol. H2O
  (conservation period of 1 day)

Stock solution:

Prepare a stock solution at a concentration of 100 µg ml^{-1} of SIGMA beef albumin.

Make 5 dilutions to have 5 different concentrations:

Table 1: *Dilution scheme for the stock solution.*

[µg ml^{-1}]	Stock solution (mL)	H_2O (mL)
100	4	0
75	3	1
50	2	2
25	1	3
0	0	4

Materials:

1. Glass tubes of at least 15 mL that can be centrifuged;
2. Centrifuge;
3. Spectrophotometer;

Methodology:

1. Put the filter in a numbered tube. In case of the stock dilutions add 1 mL of each dilution in a different numbered tube.
2. Add 5 mL of Solution D and homogenize.

3. Let the tubes rest for 10 minutes at room temperature.

4. Add 0.5 mL of Solution E and homogenize the sample.

5. Let the tubes rest for 30 minutes at room temperature.

6. Centrifuge at 3000r/min (10 minutes).

7. Transfer the supernatant into a new tube and read the tubes at 750 nm.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265-275.

Appendix G: Protocol for the carbohydrates determination

(Adapted from DuBois et al., 1956)

Reagents:

1. Phenol solution 5% in water

(conservation period of 15 days, in the dark at 4° C because phenol oxidizes very quickly)

2. Sulphuric acid (H2SO4)

Stock solution:

1. Prepare a stock solution at a concentration of 100 µg ml^{-1} of glucose sigma. Make 5 dilutions in order to have 5 different concentrations:

Table 2: Dilution scheme for the stock solution.

[µg ml^{-1}]	Stock solution (mL)	H2O (mL)
100	4	0
75	3	1
50	2	2
25	1	3
0	0	4

Materials:

1. Glass tubes of at least 15 mL that can be centrifuged;

2. Centrifuge;

3. Spectrophotometer;

Methodology:

1. Put the filter in a numbered tube. In case of the stock dilutions add 1 mL of each dilution in a different numbered tube.
2. Add 2 mL of H2O to the tubes with filters.
3. Add 2 mL of the phenol solution in all tubes and homogenize.
4. Let the tubes rest for 40 minutes at room temperature.
5. Rapidly add 10 mL of H2SO4 (BE CAREFULL, EXOTERMIC REACTION) and homogenize the sample.
6. Let the tubes rest for 10 minutes at room temperature.
7. Centrifuge at 5000r/min (10 minutes).
8. Transfer the supernatant into a new tube and read the tubes at 490 nm.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry 28, 350-356.

Appendix H: Specific growth curves of experimental groups

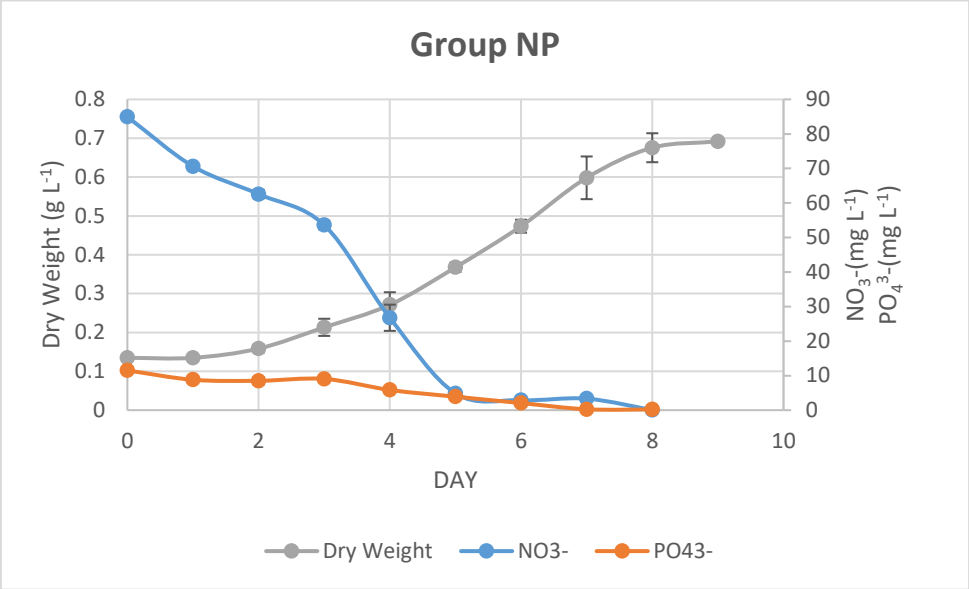


Figure 14. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group NP)

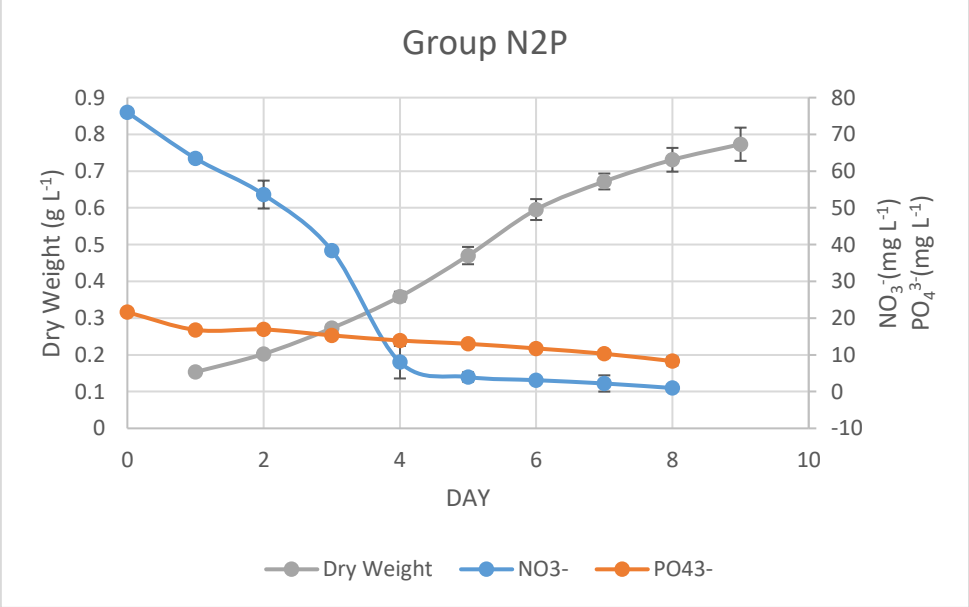


Figure 15. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group N2P)

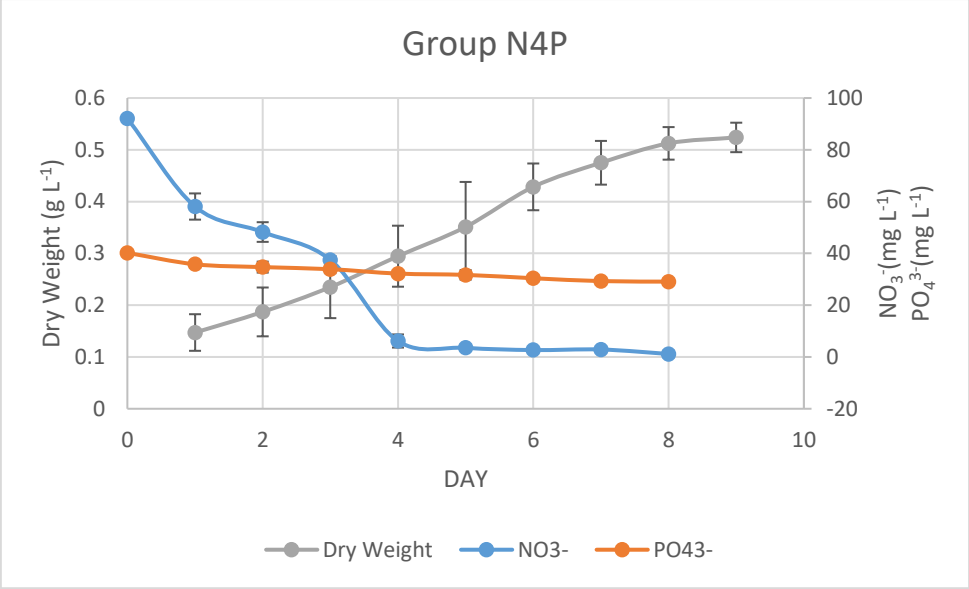


Figure 16. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group N4P)

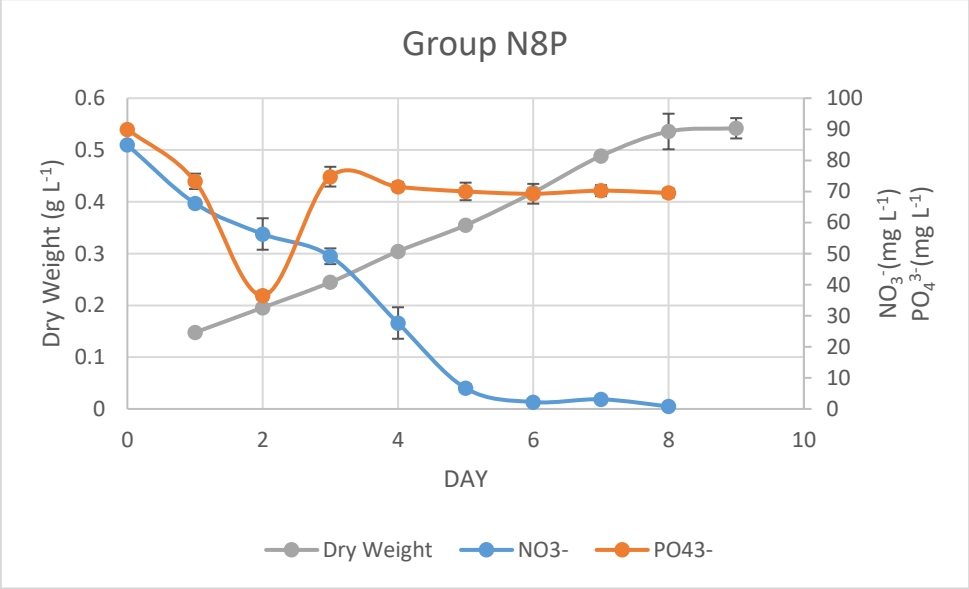


Figure 17. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group N8P)

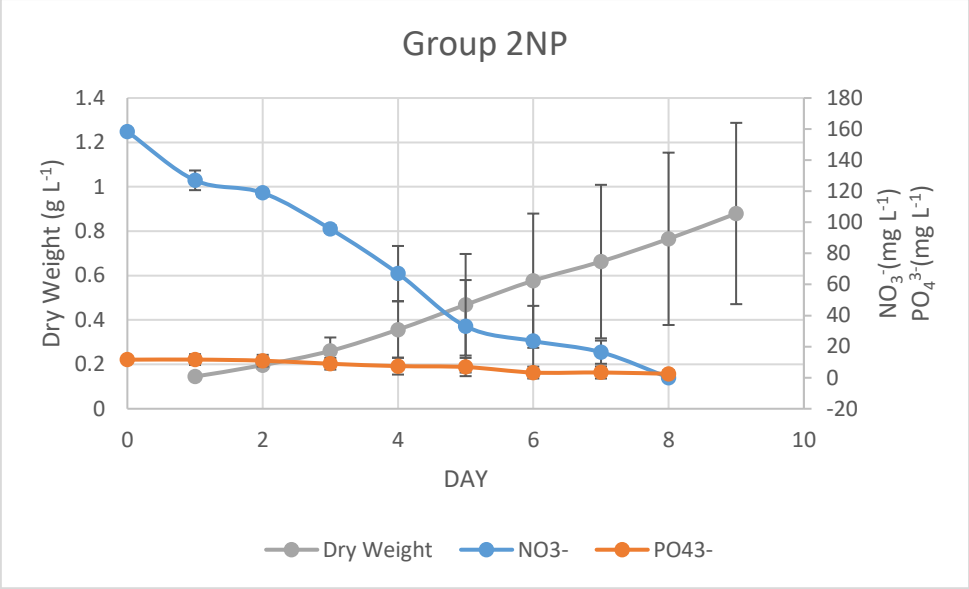


Figure 18. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 2NP)

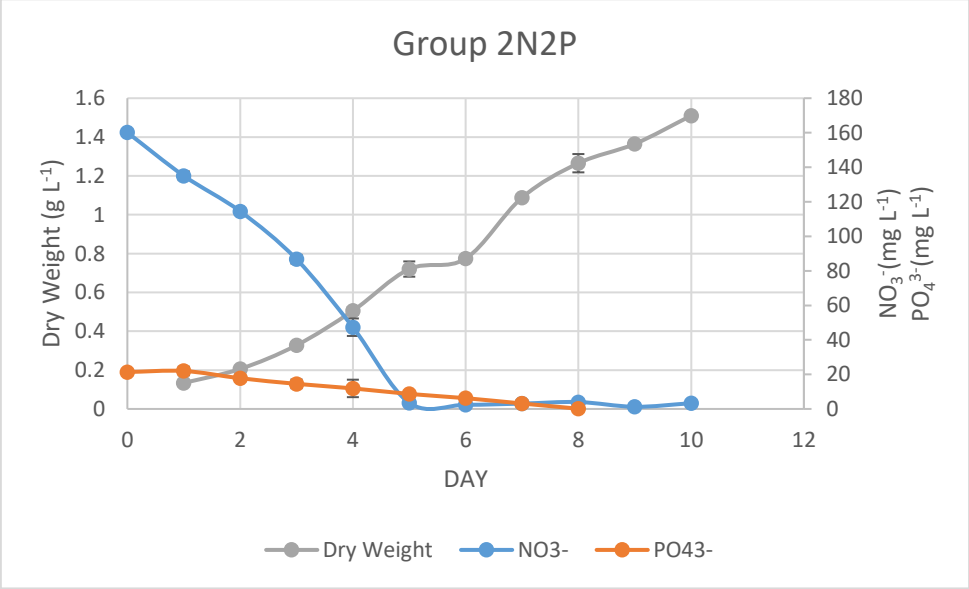


Figure 19. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 2N2P)

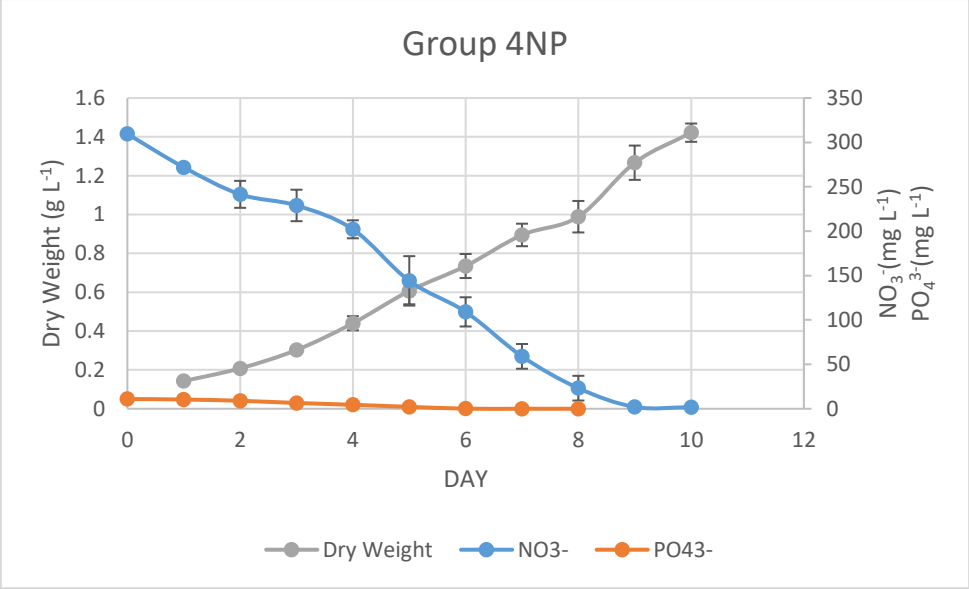


Figure 20. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 4NP)

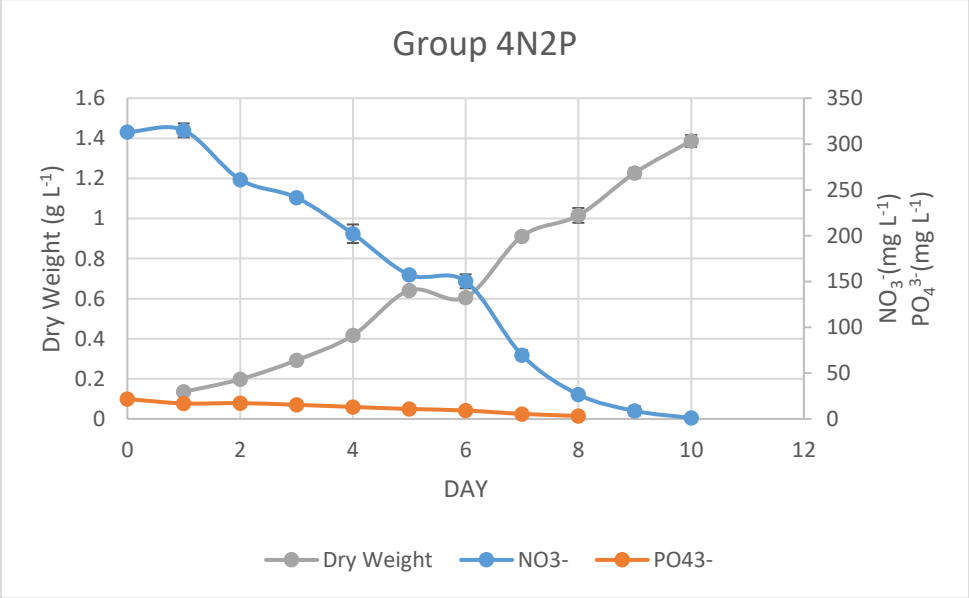


Figure 21. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 4N2P)

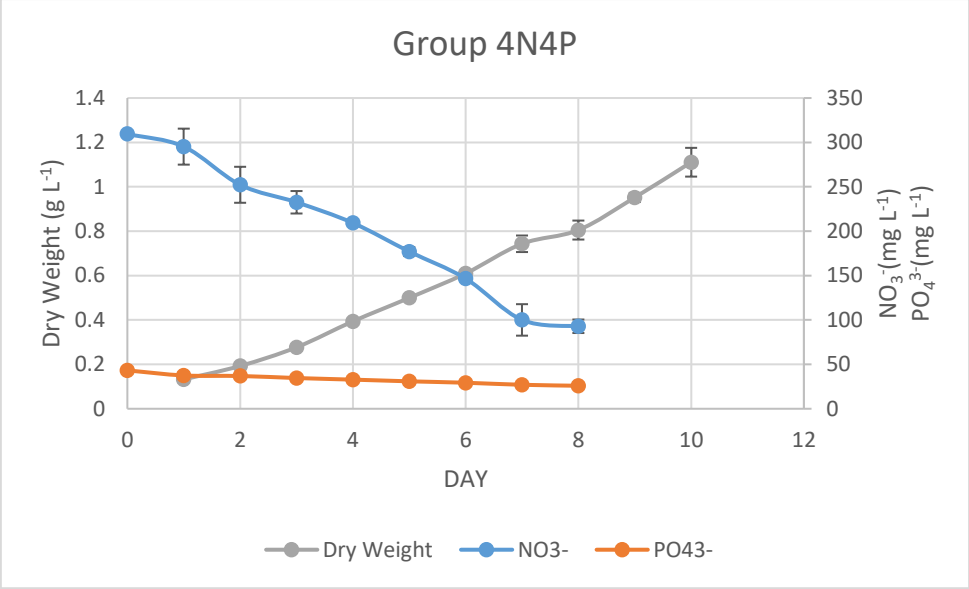


Figure 22. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 4N4P)

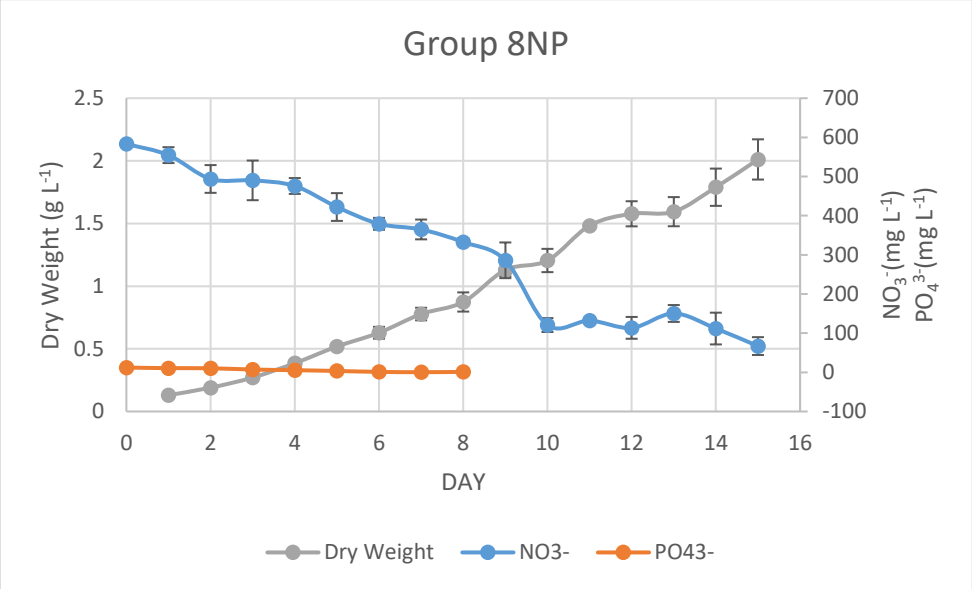


Figure 23. Growth curve based on dry weight (grey line) and the consumption curves of nitrate (blue line) and phosphate (orange line) (Group 8NP)