NTNU – Trondheim
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Marine Coastal Development
Submission date: May 2014
Supervisor: Yngvar Olsen, IBI
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Trondheim, May 2014
Renbin Zhou

Abstract

Saccharina latissima were cultivated in tanks with three different levels of nitrate supply. The temperature was around 12 to 18 ℃ and salinity was around 24 to 29 ‰ during the experiment. Seaweeds cultivated at Medium N concentration (29.0±4.6 μg/l) showed the fastest growth rate among the three treatments, and the growth rate of seaweeds at High N concentration (143±13 μg/l) was not significantly higher than that at Low N concentration (18.1±1.5 μg/l).

Seaweeds cultivated at High N concentration showed a significantly higher level of intracellular DIN (0.22±0.02mg/g of dry weight), DON (1.4±0.1mg/g of dry weight), Protein (103.8±1.9mg/g of dry weight), and total-N (2.3±0.1% of dry weight) in the tissue than that grown at Low N and Medium N concentration ($p<0.05$). The concentration of nitrate in the water for Medium N treatment was 29.0±4.6 μg/l, which was significantly higher than that grown in Low N treatment (18.1±1.5 μg/l) ($p<0.05$), but the gap of nitrate level for these two treatments was not big. Seaweeds cultivated at Low N concentration did accordingly not have a significant lower level of DIN (0.05±0.11mg/g of dry weight), DON (0.40±0.08mg/g of dry weight), Protein (80.6±5.1mg/g of dry weight), and total-N (1.4±0.1% of dry weight) in the tissue compare with the seaweeds cultivated at Medium N concentration ($p>0.05$). Intracellular DIN, DON and protein were positively correlated ($p<0.05$) to the ambient nitrate concentration during growth.

The elemental C/N ratio (weight) ranged from 13.7 mgC • $(mgN)^{-1}$ for the plants grown in High N concentration (Day 42) to 27.7 mgC • $(mgN)^{-1}$ in the Low N concentration (Day 62). Overall, at high nitrate supply, plants showed lower C/N ratio. The protein-to-nitrogen conversion factor for High N concentration (4.63±0.32 protein • N^{-1}) was significantly lower than that in Low N concentration (5.78±0.35 protein • N^{-1}) ($p<0.05$).

Contents

1 Introduction

1.1 Seaweed

Wikipedia defines seaweed as a macroscopic, multicellular, benthic marine algae (Wikipedia 2014). Seaweed includes some members of the red (*Rhodophyta*), brown (*Phaeophyta*) and green algae (*Chlorophyta*). Seaweeds can also be classified by its use (as food, medicine, fertilizer, filtration, industrial, and raw material) (Wikipedia 2014). National Ocean Service – NOAA has stated that "Seaweed" is the common name for countless species of marine plants and algae that grow in the ocean as well as in rivers, lakes, and other water bodies (NAOO 2014).

In the period from 1981 to 2010, the world's seaweed production increased from 3.2 million tons (fresh weight) to around 16 million tons (The Seaweed Site 2014 FAO 2012). In 1994, the seaweeds that were most exploited were the brown algae with about 5.2 million tones production (75% of total production) followed by the red algae (1.73 million t; 25% of total production) and a small amount of green algae (about 0.5% of total production) (The Seaweed Site 2014).

Seaweeds are consumed as food by coastal people, particularly in Asian countries, e.g., Japan, China, Korea, Taiwan, Singapore, also in South Africa, Indonesia, Peru and Chile (Wikipedia 2014, Mishra 1993). They are eaten as fresh or dried vegetables because of valuable nutrition (Robledo & Pelegrin 1997). High quantities of protein, lipid, vitamins and minerals are found in some edible seaweed (Noziah & Ching 2000, Sánchez-Machado 2004).

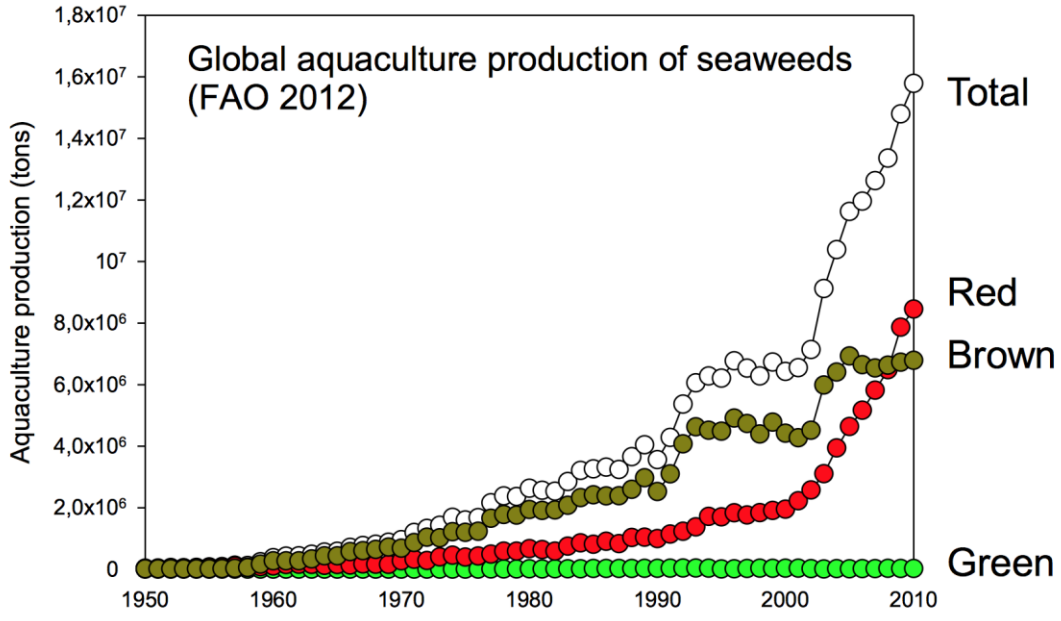

Figure. 1 Global aquaculture production of seaweeds.

1.2 Saccharina latissima

Sugar kelp *Saccharina latissima* (Linnaeus) is a large brown alga that is distributed in Europe from Svalbard in the north to Portugal in the south (Van den Hoek& Donze 1967, Lüning 1990, Bekkby 2011; Moy 2012). Around half of Europe's sugar kelp forests were found in Norway (Moy et al 2006).

S. latissima, previously known as *Laminaria saccharina* (Linnaeus), is a yellow-brown colored seaweed with a long narrow, undivided blade and can grow to 5 meters long and 20 centimeters wide (Lane 2006, Wikipedia 2013). Although S. *latissima* is present year-round and is considered a perennial with a life span of 2 to 5 years, the blade dies in the fall and winter, and re-grows in the late winter and spring (Seaweed Industry Association 2014). It grows in lower intertidal pools and occasionally in the shallow subtidal and it can also be found at depths down to 20 and 30 meters (Seaweed Industry Association 2014).

Saccharina latissima is a seaweed candidate for production of biofuel due to its high content of fermentable sugars, laminaran and mannitol. Among the classical and most frequently cited sources for data on the chemical composition of *S. latissima* are Haug and Jensen (1954) and Black (1950), who both report high levels of carbohydrates in general terms.

1.3 Protein of seaweed

Protein content of seaweed differs according to species (Fleurence 1999, Sánchez 2004). Brown seaweeds have low protein content (3-15% of the dry weight) compare to the red or green seaweeds (10-47% of dry weight) (Fleurence 1999). Most of brown algae have a low protein content that is lower than 15% (based on dry weight), and red algae like *Porphyra tenera* (47% of dry weight) or *Palmaria palmate* (35% of dry weight) have higher protein contents.

The protein content of seaweed is variable and dependent on the seasonal period (Mishra et al. 1993, Fleurence 1999). Haug, Jensen (1954) and Jacob (2012) found that protein in the blades showed a maximum during the period from February to May, and the young parts of blades are richer than the old parts. The kelp was poor in protein (5% to 6% of dry weight) during the period from August to September (Black 1950).

1.4 Growth rate of seaweed

It is a questioned what factor that affects the growth rate of *S*. *latissima* most strongly during the season, nutrient concentrations or day length. Bartsch (2008) found that there is evidence that the growth rate was forced by the day length, but this has not been further supported. Dieckmann (1980) and Gagné (1982) suggested that growth might be limited mostly of light rather than nitrogen. On the another side, Gagné (1982) found that it is primarily seasonal variations in nutrient availability, not the day length, that control the growth rate of *S*. *longicruris* in Canada.

1.5 Objective of this study

In the past 30 years, several studies have focused on the seasonal variation of chemical composition in the kelp *Saccharina latissima* (Black 1950, Haug and Jensen 1954, Fleurence 1999, Jacob 2012). This paper aims to describe how the different levels of nitrate supply affect the kelp's growth and the intracellular contents of inorganic nitrate, dissolved organic nitrogen, and protein in the kelp.

2 Material and methods

2.1 Plant material and culture conditions

Young plants of *S. latissima* were collected outside the SES laboratory (63° N 10° E) on 9th of July. The plants were around 50cm long and 7.5cm wide, and were transferred to the cultivation tanks within one hour.

The plants were growing as free floating in the tanks (Figure 2). The seawater was pumped from the surface of the sea near the SES laboratory. About 200 plants were cultivated in each tank (total 9 tanks). 1.8L min^{-1} of seawater was supplied, meaning that the water in the tanks was exchanged 2 times per day (Indergaard 1990). 9 tanks were divided into 3 treatments (each treatment had 3 tanks). Solutions of NO_3^- were added to the tanks to keep the nitrate in the tanks stable as planned. Three levels of nitrate were maintained in 9 tanks, namely:

Low N = Ambient water no extra NO_3-N added
Medium N = Ambient water added 56 μ g/l of NO_3-N
High N = Ambient water added 280 μ g/l of NO_3-N

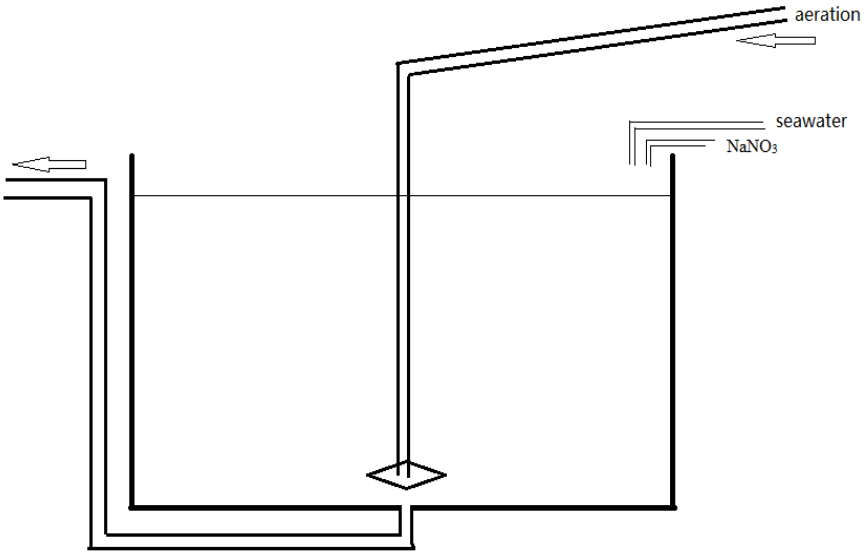

Figure 2. Cultivation tanks used in the experiments (1325 liter) (1.77m^2 surface area), Figure showing the structure of the tank, including seawater, nitrate and air supplying systems.

Each tank was added 6 plants, which were selected randomly, attached with 6 different colors of plastic marks for identification. These 6 plants were used to evaluate linear elongation of the blade. At Day 0, a hole was punched in the blade 10 cm away from the junction between stipe and blade (l_{t-1}=10cm, plant t-1 in Figure 3). While

recording the width of the blade at the first hole (w_{t-1} at plant t-1 in Figure 3) (Lü ning 1979). After one week, the length was measured from the stipe to the first hole (l_t, plant t) and the width (w_t, plant t) again, a now hole was made which is 10cm away from the junction between stipe and blade, also measure width. One week later, the same procedure was repeated until the end of this experiment.

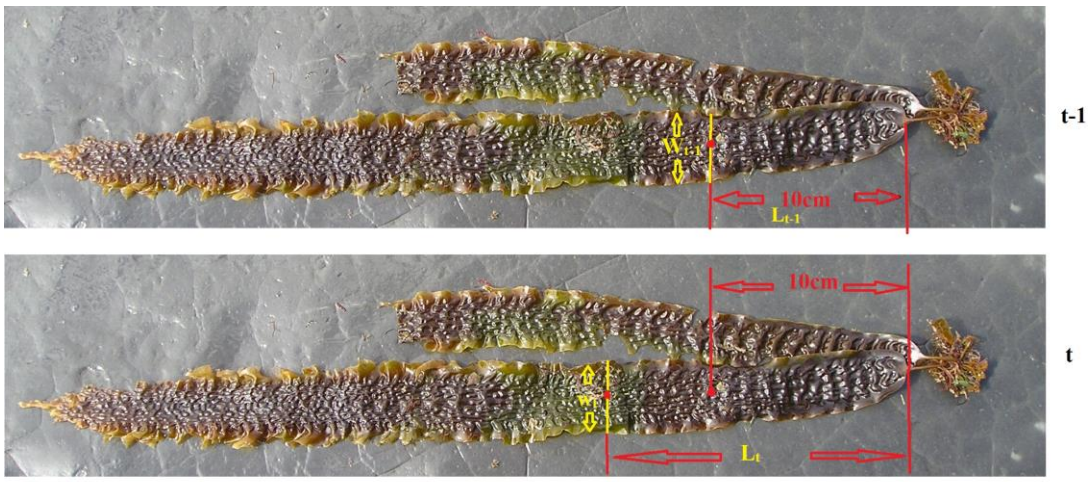

Figure 3. Area parameters of *S*. *latissima* used for calculation of growth rate.

The tanks were covered with green net to prevent the strong sunlight during the whole experiment. Temperature and salinity were recorded 3 times per week. The photo flux density was measured with a spherical sensor (HOBO loggers), which showed that the tanks' water surface was exposed to a maximum of 1500-2000 μ $Em^{-2}s^{-1}$ during the experiment. The intensive decreased to around 300-600 μ $Em^{-2}s^{-1}$ at the bottom of the tank (0.75m depth).

2.2 Sampling of kelp and water

During cultivation, around 15ml water samples were taken every second day from each tank, and they were stored in a freezer (-18℃) immediately. At the end of experiment, all water samples were taken out and leaved at room temperature until thawed, which were used to analyse the nitrate and nitrite content of the water.

The algae were sampled at Day35, Day42, Day49, Day56 and Day62. Total 10 plants were harvested from each tank at the sampling day. The plants were transferred to a plastic bag (total 90 plants, 9 bags per sampling day) and immediately transfer to a freezer (-18℃). All plants were stored in the freezer until the start of the analysis procedure.

At the second month of cultivation, some bryozoans grew on the old blade of algae, and were removed from samples for protein analyses. Before the analysis of the

chemical composition, the plants were taken out and cut at around 40cm away from the junction between stipe and blade. The 40cm of blade was used for analyses. It was checked whether there was some bryozoan growing on the blade, which we needed. If so, paper was used to remove it. Then the grinder was used to crush the samples into small pieces. One bag's samples (10 plants) ground and put back into one bag which was stored in a freezer (-18℃) (total 45 bags). To prevent that the plants lost some liquid, the procedure above was done quickly and before the plants were completely thawed (Jessica 2008).

2.3 Extraction of protein from kelp

Samples were taken from the freezer and stored at room temperature until they were thawed (Pádraigín 2013). Then around 1g of sample were transferred to a tube. Each sample had three replicates (three tubes) (total 135 tubes). Tubes were filled with 0.5M of Sodium Hydroxide (NaOH) to 10ml and put into liquid nitrogen after shaking. Then the tubes were taken out and stored at room temperature until they thawed. Then plastic cover was removed. Glass ball was used to cover it and the tubes were left in boiling water for 30mins. After that, the tubes were cooled down and leaved at the fridge for 24 hours.

The samples were shaken after 23 hours, and one hour later, the supernatants were recovered by centrifugation (20 min at 20℃, 12000xg) and stored at -20℃ (Nguyen 1993, Crossman 1999 ,Pádraigín 2013). The tubes with pellets were filled with 0.5M NaOH to 10ml and left in boiling water for 30mins. The samples were extracted totally three times, all supernatants were stored in one big tube in the freezer, and were later analyzed for protein. The pellets left in the tubes were dried in an oven (70℃ for 24hours) and around 2mg of dried samples were used to analysis of total C and N left.

2.4 Analytical procedures

Protein was measured using the BIO-RAD DC protein assay, which is similar to the Lowry assay, with a protein standard containing from 0.2 mg/ml to 1.5mg/ml protein. 7 dilutions of a protein standard series containing from 0 mg/ml to 1.4mg/ml protein were prepared. 100 µl of standards or samples were transferred into clean, dry test tubes, and were added 500 µl of reagent A (an alkaline copper tartrate solution). Each test tube was shaken. 4 ml of reagent B (a dilute Folin Reagent) was then added into each test tube and immediately shaken. After 15 minutes, the absorbance was read at spectrophotometer (750 nm). Measurements should be done within 1 hour (DC Protein Assay Instruction Manual).

Around 0.01g of the sample from the bags was transferred to a tube. Just like for the extraction of protein, three replicates were included. Tubes were filled with 10ml water, and put in boiling water for 30mins. Then tubes were cooled down to room temperature and 0.20 μm polysulfone syringe filters were used to remove small seaweed and the liquid were saved (Crossman 1999). The liquid of samples was divided into two tubes, each around 4ml. One sample was filtered and used for analysis dissolved inorganic intracellular nitrate, which with a Technicon Auto Analyzer II using standard techniques (Harrison *et al*., 1986). Another one mixed with oxidizing reagent (H_2SO_4-SeO_2) and cooled after high pressure and temperature (Solórzano 1969). Technicon Auto Analyzer II was used to get the yields of the sum of dissolved inorganic nitrate and dissolved organic nitrate. The difference value between the two data above was the value of dissolved organic nitrate.

2.5 Determination of water content and total C&N in samples

Three replicates samples were taken out from each bag, and around 1g of seaweed (wet weight) were weighted and put into a glass dish. The dishes with sample were left in the oven at 70℃ for 24 hours (Okhyun 1998). To prevent the samples absorbing moisture from the environment, the dishes were taken out and weighted (dry weight) immediately. The difference between wet weight and dry weight was water content.

The samples for total C & N were put in one box with a cup of 37% of hydrochloric acid. A pH test paper was left inside, to check that the box was filled with gas of HCL. The samples were kept inside the box for around 2 hours. Then the samples were taken out and putted in an oven at 70℃ for 2 hours. Approximately 2 mg ± 0.5 mg of samples were transferred into tin capsules and weighed on the microbalance. Each sample included 2 tin capsules (2 replicates). Tweezers were used to make the samples were surrendered by tin. They were stored in a box and sent to Portugal for analysing total C&N.

2.6 Statistics

The experimental data were tested for statistical significance by using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and differences were considered significant at the $P<0.05$ level. SPSS 19.0 under windows was used to perform all the statistical tests. All tables were made in Word 2010, and figures were made by Sigma plot 12.5.

3 Results

3.1 Methods for test

3.1.1 Protein extraction

Figure 4 illustrates yields of protein extracted from seaweed samples with different procedures. Use of water to extract protein gave the lowest yield of protein (1.3±0.1mg/ml). Boiling increased the yield significantly (1.9±0.1mg/ml) ($p<0.05$). Boiling in 0.1M NaOH (3.4±0.1mg/ml) gave a further improvement in the extraction, and use of 0.5M NaOH combined with boiling (4.4±0.3mg/ml) was significantly more efficient ($p<0.05$) for protein extraction than other methods.

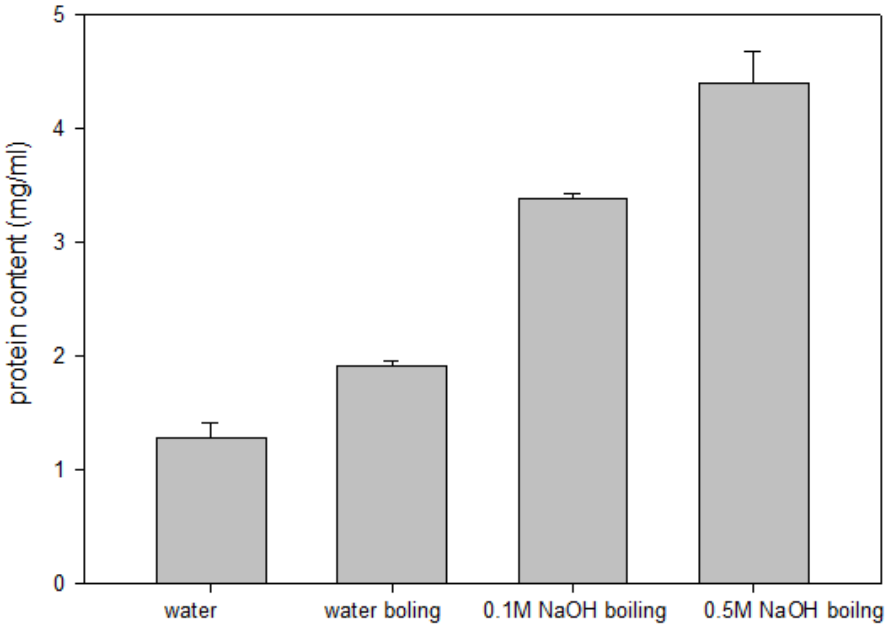

Figure 4. Yields of protein extracted in different ways. Values are mean of all replicates (n=3). Error bars indicate ±1SE.

The proportion of protein extracted each time is shown in Figure 5. In the first 2 extractions an average of 92.5±3.3% of protein was obtained after 4 extractions, if we assume that 100% was extracted in 4 successive extractions. The mean yield obtained by three extractions was 98.3±1.1%, which was significantly higher than only extracting two times ($p<0.05$). 3 times extraction was used as the standard method in the present study.

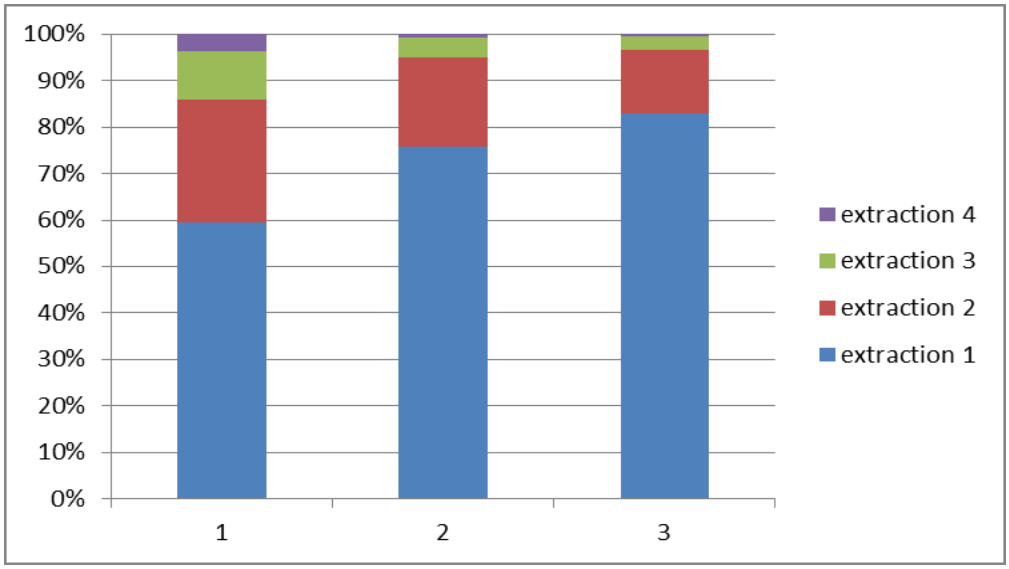

Figure 5. Seaweed was extracted as boiling with 0.5M NaOH four times, and the values showing how much proportion of protein was extracted each time. Values are mean of all replicates (n=3).

3.1.2 Boiling time for DIN

Figure 6 shows the yields of intracellular nitrate (DIN) extracted from seaweed samples using variable boiling times. Yields of DIN increased with time of boiling increasing. Boiling for 15mins obtained significantly higher values of nitrate than boiling for 1min ($p<0.05$). There were no significant difference between boiling 15mins, 30mins and 60mins ($p>0.05$).

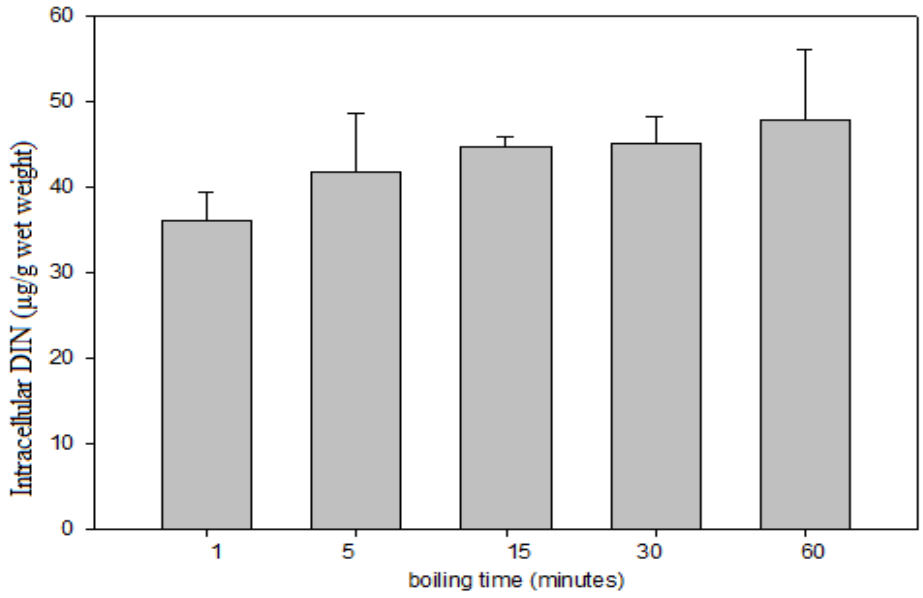

Figure 6. Yields of intracellular DIN in seaweed samples for different boiling time. Values are mean of all replicates (n=3). Error bars indicate ±1SE.

3.2 Culture conditions

The temperature and salinity in the water during the experiment is shown in Figure 7. The temperature was around 12 to 18℃ during the experiment, and around 16℃ during Day16 to Day36. The temperatures at beginning and end of the experiment were a littler lower. The water was pumped from the surface of the sea, which was affected by raining and water from rivers. The salinity of the water was around 25 to 29‰ during the experiment.

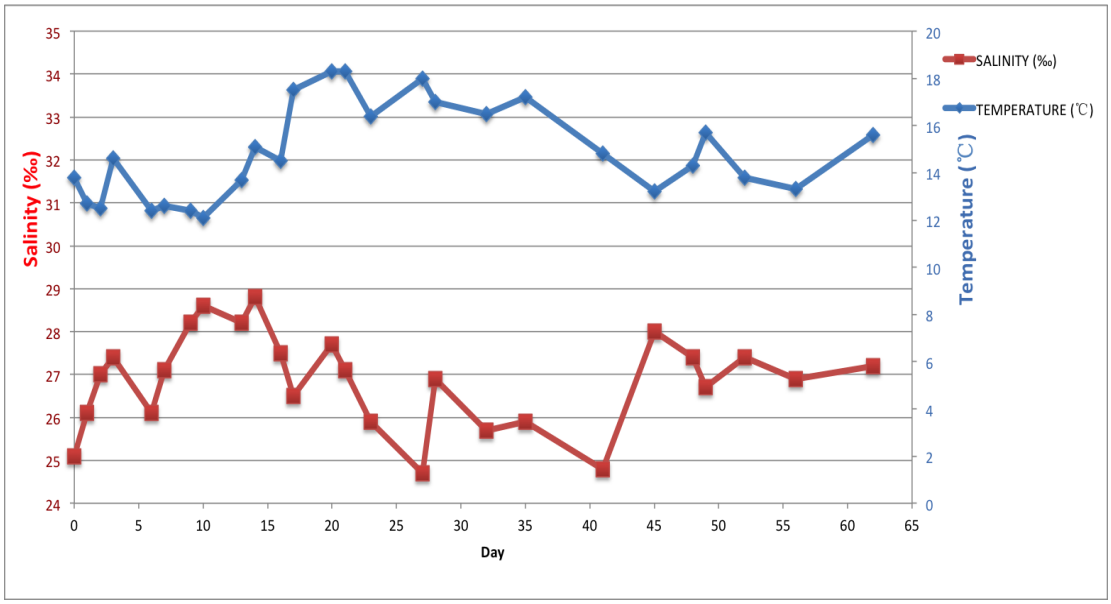

Figure 7. The temperature and salinity of the water during the experiment.

The concentration of nitrate in the water during the experiment is shown in Figure 8. The Low N concentration was stable at around 19.5±1.0 μg/L, and the High N concentration changed from highest at Day0 of around 241 μg/L to lowest at Day32 of around 60 μg/L. The average nitrate concentration for High N treatment was 147 ±9 μg/L, and High N concentration was significantly higher than Low and Medium N concentration ($p<0.05$). The average nitrate concentration for the Medium N treatment was 36.7±3.1 μg/L, significantly higher than that for Low N concentration ($p<0.05$). At Day32, the pumps were stop working and no water and nitrate were supplied. The nitrate content in the water therefore decreased for High N and Medium N treatments.

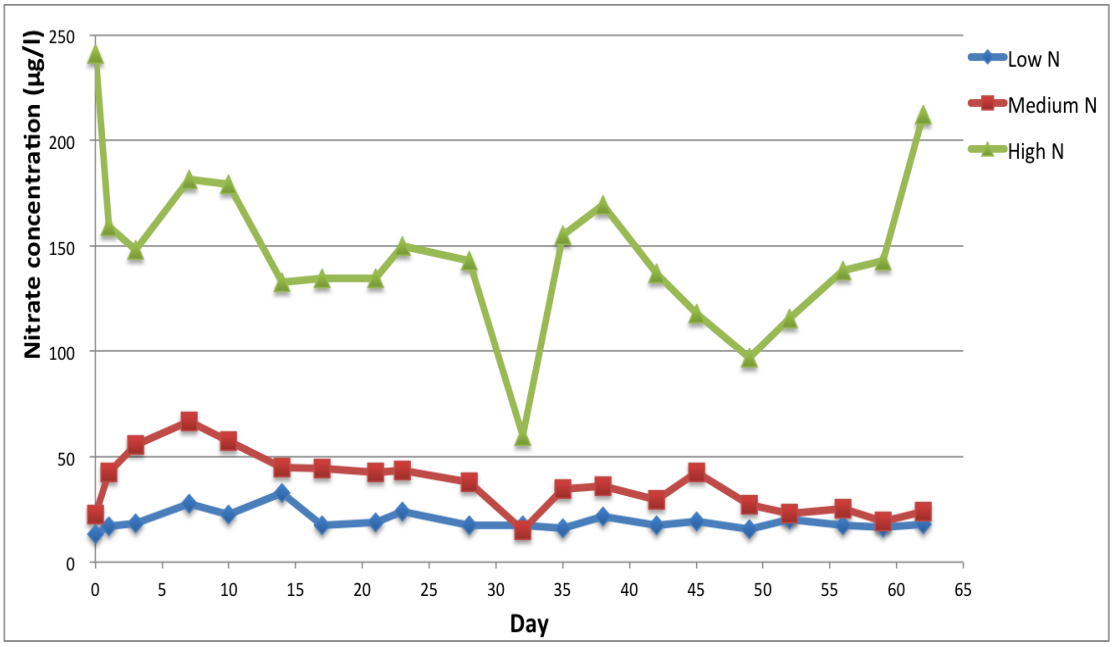

Figure 8. Nitrate concentration in the water for three treatments during the experiment

3.3 Growth in length

Table 1 shows the length of blade from first hole to the junction between stipe and blade for three treatments. *S. latissima* growing in the tanks with Medium N concentration showed a maximum growth rate in the third week. Plants maintained in Medium N concentration grew significantly faster in length than plants kelp in Low and High N concentration ($p<0.05$).

Table 1. The length (cm) of blade from first hole to the junction between stipe and blade obtained for plants of *S. latissima* kept in variable nitrate concentrations. Values are mean of all replicates (n=18).

	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 62
Low N	10.0 ± 0.0	10.8 ± 0.3	13.1 ± 0.6	18.6 ± 1.4	24.8 ± 2.1	29.6 ± 2.8	34.8 ± 0.6	38.3 ± 0.6	41.8 ± 0.8	45.4 ± 1.2	48.5 ± 2.2
Medium N	10.0 ± 0.0	11.4 ± 0.2	14.6 ± 0.4	21.6 ± 0.7	29.5 ± 1.4	36.4 ± 1.7	41.2 ± 2.1	44.9 ± 2.6	49.3 ± 2.7	52.5 ± 3.2	56.2 ± 4.4
High N	10.0 ± 0.0	11.4 ± 0.2	14.8 ± 0.9	21.5 ± 2.2	28.7 ± 3.0	34.5 ± 4.5	39.7 ± 6.1	43.2 ± 5.3	46.7 ± 5.9	49.7 ± 8.0	52.8 ± 9.9

Figure 9 shows the blades area from first hole on the blade to the junction between stipe and blade as a function of time of growth. The seaweed maintained at Low N concentration grew slowest of the three treatments. Plants kept in Medium N and High N concentration grew at a similar rate in the first 14 days. During the period from Day35 to Day62, plants in Medium N concentration grew significantly faster than plants in Low and High N concentration ($p<0.05$), and growth rate of plants grown in High N concentration was not significantly higher than that kept in Low N concentration ($p>0.05$).

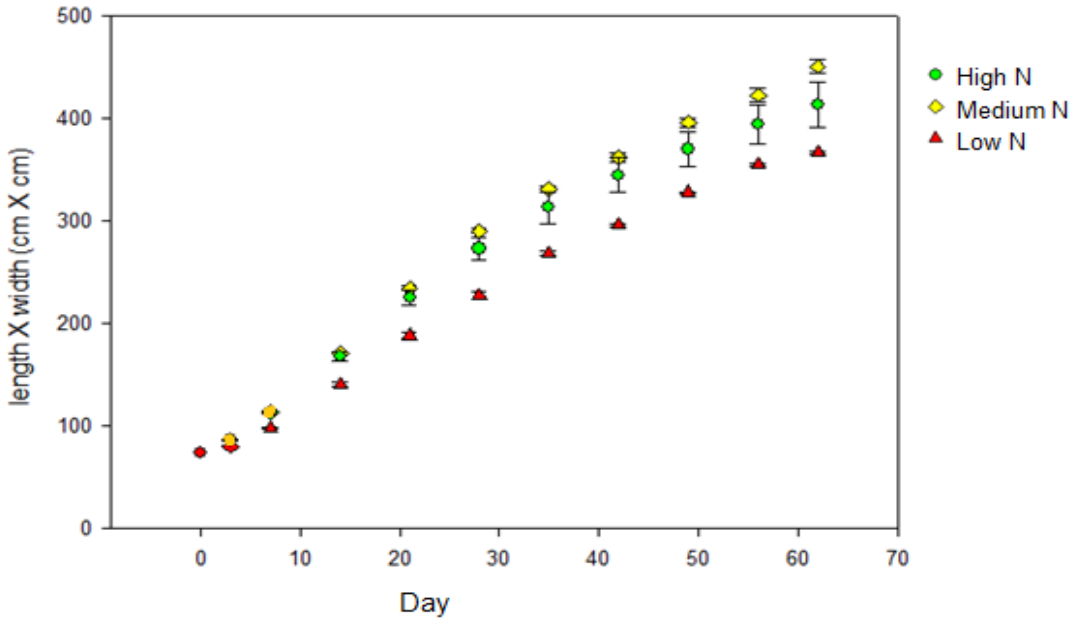

Figure 9. Growth in blade area from stipe to the first hole (length multiplied width) obtained for *S latissima* kept in variable nitrate concentrations. Values show the average of 18 plants' blade area in the same treatment. Error bars indicate ±1SE.

3.4 Water content in the kelp *S. latissima*

Figure 10 shows the contents of water in the seaweed samples. The seaweed samples contained a substantial amount of water, ranging from 74.8±1.2% to 80.8±2.0% of wet weight. There were no significantly difference in water contents between the sampling dates and three treatments ($p>0.05$). The lowest water content was found at Day49 with Medium N concentration, and the highest one was found at Day56 with High N concentration.

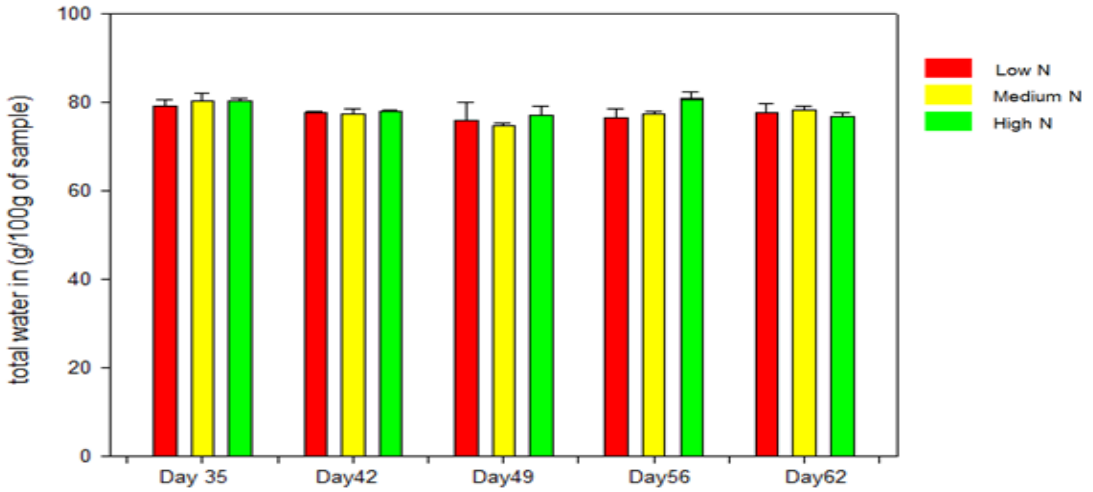

Figure 10. Contents of water (g/100g of sample) in *S. latissima* obtained for plants kept in variable nitrate concentrations. Values are average for 30 plants (3 tanks each tank have 10 plants). Error bars indicate ±1SE.

3.5 Protein content in the kelp *S. latissima*

Yields of total protein in the seaweed samples are shown in Figure 11. The yields of protein varied depending on harvest date and nitrate treatments. The yields of protein in the seaweed increased with increasing nitrate concentration in the water. The average protein contents were 81, 88, 104 mg per gram dry weight of seaweed for Low, Medium and High N concentration, respectively. Content of protein in the algae that kept in High N concentration was significantly higher than that grown in Medium and Low N concentration ($p<0.05$). Content of protein in the algae that cultivated in Medium N concentration was not significantly higher than that grown in Low N concentration ($p>0.05$).

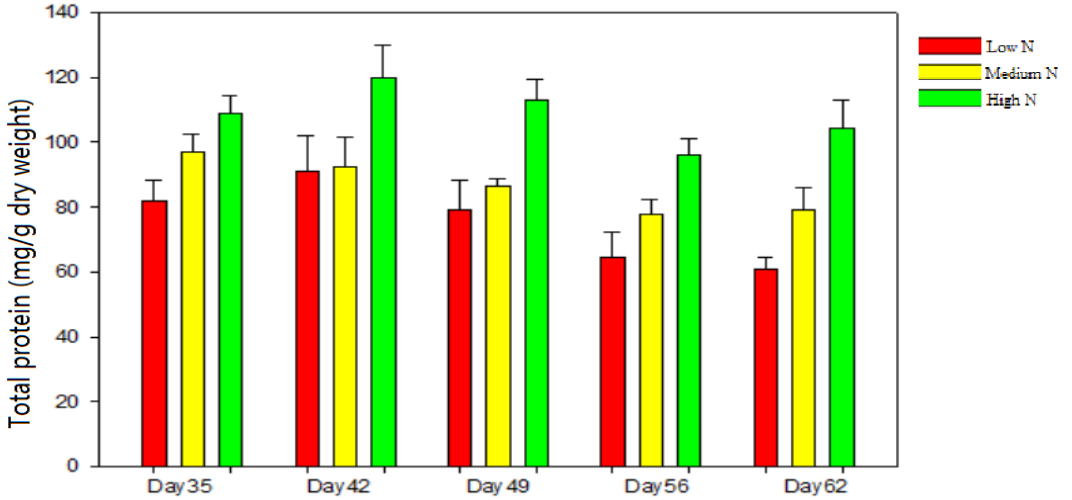

Figure 11. Yields of total proteins (mg/g dry weight) in the kelp *S. latissima* obtained for plants kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error Bars indicate ±1SE.

3.6 Content of dissolved inorganic nitrate (DIN) and dissolved organic nitrogen (DON) in tissue

Figure 12 illustrates yields of intracellular DIN in all seaweed treatments during the experiment. The value of DIN increased with increasing nitrate concentration in the water. Yields of DIN for plants grown in High N concentration (0.22±0.02mg/g) were significantly higher than that grown at Low and Medium N concentration ($p<0.05$). Yields of DIN for algae that kept in Medium N concentration (0.07± 0.01mg/g) were not significantly higher than that cultivated at Low N concentration (0.05±0.01mg/g) ($p>0.05$). The lowest content of DIN was found in the algae at Day42 in Low N concentration (0.018mg/g), and highest content of DIN was found in the algae at Day42 in High N concentration (0.275mg/g).

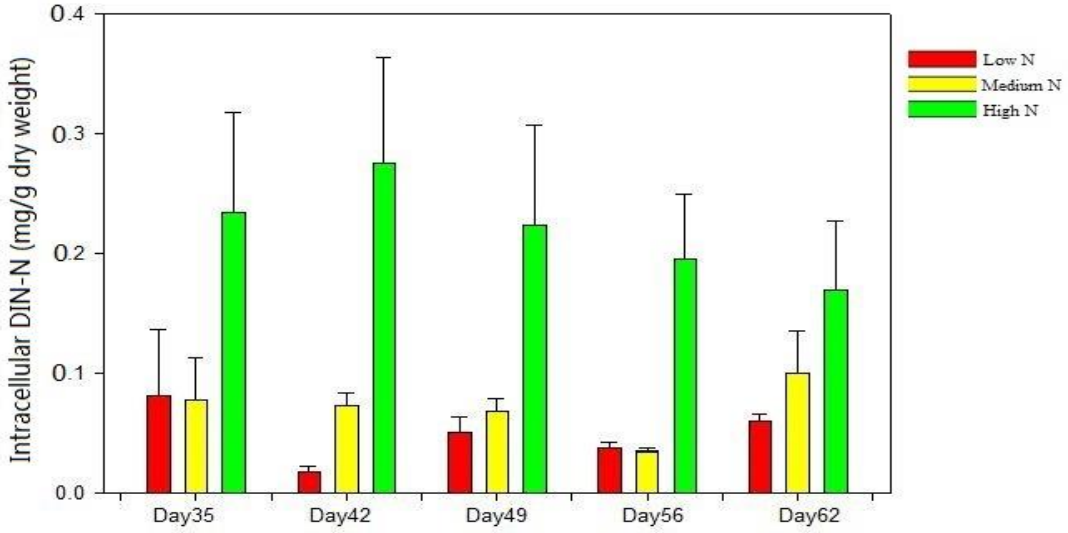

Figure 12. Yields of dissolved inorganic intracellular nitrogen-N (DIN-N mg/g dry weight) in *S. latissima* obtained for plants kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error bars indicate ±1SE.

Figure 13 shows the yields of DON in the seaweed samples for treatments and sampling days. The value of DON increased with increasing nitrate concentration during growth. The content of DON in the algae that kept in High N concentration (1.4±0.1mg/g) was significantly higher than that kept at Low and Medium N concentrations ($p<0.05$). The content of DON in algae grown in Medium N concentration (0.7±0.1mg/g) was not significantly higher than that kept at Low N concentration (0.4±0.1mg/g) ($p>0.05$). The lowest content of DON was found in the algae at Day62 in Low N concentration (0.103mg/g), and highest content of DON was found in the algae at Day49 in High N concentration (1.80mg/g).

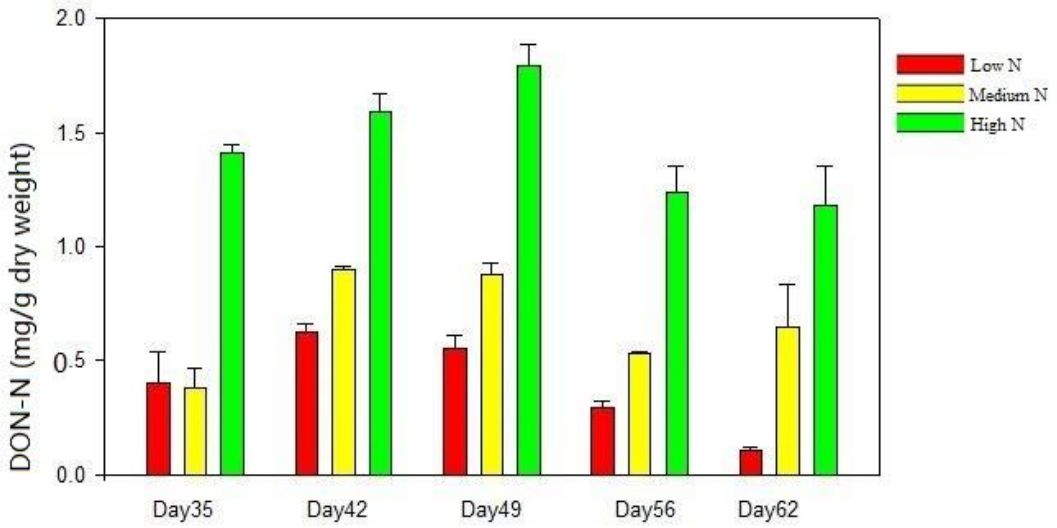

Figure 13. Yields of dissolved organic nitrogen-N (DON-N mg/g dry weight) in *S. latissima* obtained for plants kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error bars indicate ±1SE.

3.7 Tissue total-N, C/N (weight) and protein-to-nitrogen conversion factors

Figure 14 shows yields of tissue total-N in the seaweed samples. The content of total-N in the algae increased with increasing nitrate supply during growth. Total N content in algae maintained at Low N concentration was not significantly lower than that kept in Medium N concentration ($p>0.05$). Total-N of plants maintained in the Low N (1.4±0.1% of dry weight) and Medium N concentration (1.7±0.1% of dry weight) was significantly lower than that in algae those growing at the High N concentration (2.3±0.1% of dry weight) ($p<0.05$). There was no relationship between the sampling date and total-N content of the algae.

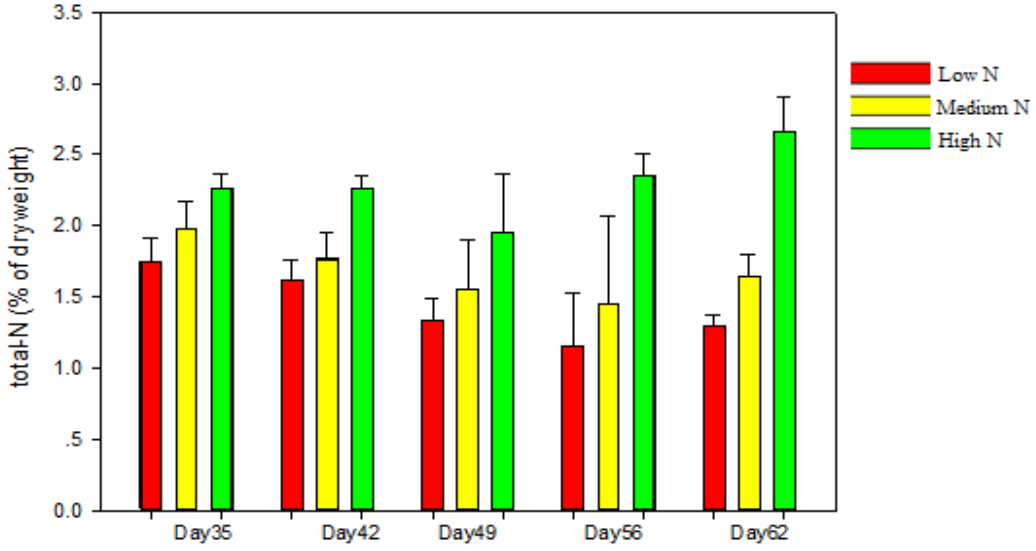

Figure 14. Content of total-N in the seaweed samples obtained for plants *S. latissima* kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error bars indicate ±1SE.

The C/N ratio (weight) of the seaweed samples obtained for plants *S*. *latissima* kept in variable nitrate concentrations is shown in Figure 15. The C/N ratio decreased with increasing nitrate supply during growth. Values found in seaweed maintained at Low N concentration (23.1±1.9 mgC • $(mgN)^{-1}$) was significantly higher than that kept at Medium N concentration(19.9±1.3 mgC • $(mgN)^{-1}$) ($p<0.05$). The C/N ratio of the seaweed kept at Medium N concentration was significantly higher than that maintained at High N concentration (14.2±0.2 mgC • $(mgN)^{-1}$) ($p<0.05$).

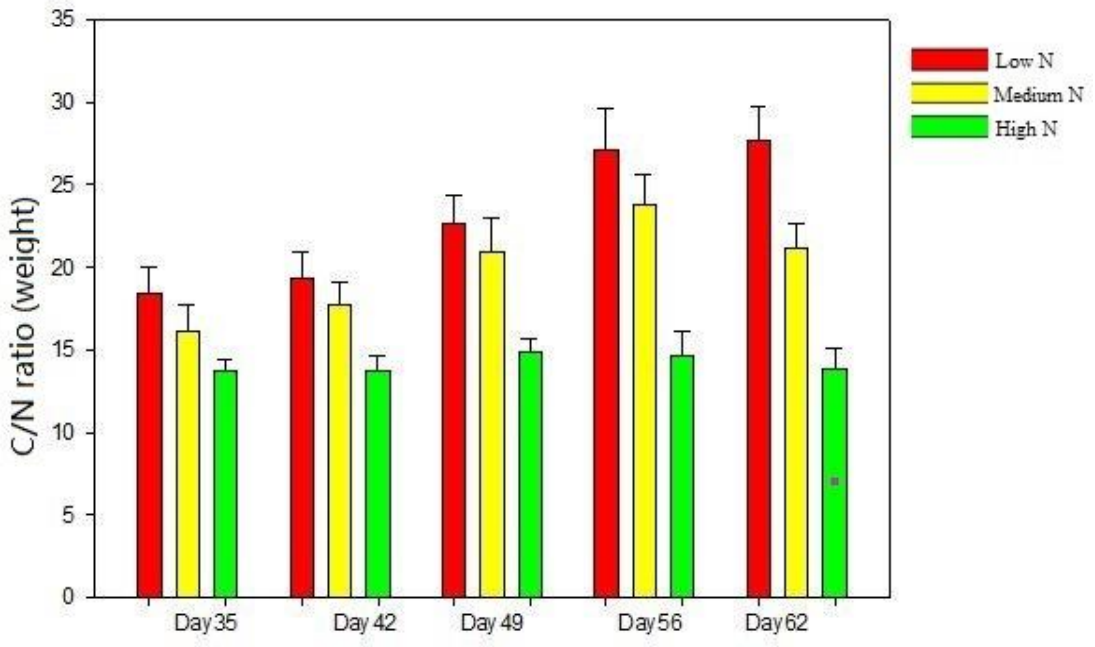

Figure 15. The C/N ratio (weight) of the seaweed samples obtained for plants *S*. *latissima* kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error bars indicate ±1SE.

Figure 16 shows the protein-to-nitrogen conversion factors for the seaweed sample. The protein-to-nitrogen conversion factors varied among different treatments. The conversion factor for algae kept in Low N concentration (5.78±0.35 protein •N^{-1}) was not significantly higher than algae maintained in Medium N concentration (5.49± 0.27 protein •N^{-1}) ($p>0.05$). The factor found in the algae kept in Low N concentration was significantly higher than algae cultivated in High N concentration (4.63±0.32 protein • N^{-1}) ($p<0.05$).

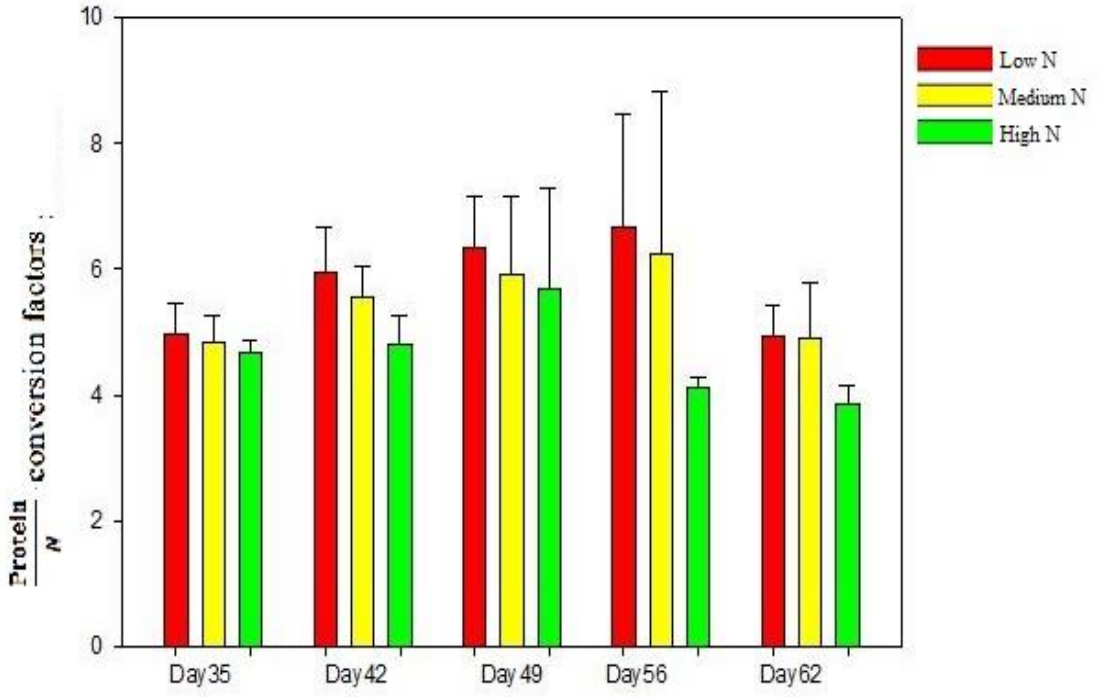

Figure 16. Protein-to-nitrogen conversion factor for seaweed samples obtained for *S. latissima* kept in variable nitrate concentrations. Values are mean of all replicates (n=30). Error bars indicate +1SE.

3.8 Relationship between growth rate and N content in tissue

Figure 17 shows the relationship between N content (DIN-N, DON-N and Protein-N) in the seaweed samples as a function of the growth rate of blades. The content of Protein-N (14.8±2.4mg/g) was higher than the content of DIN-N (0.11±0.05mg/g) and DON-N (0.83±0.31mg/g) for the seaweed samples kept in variable nitrate concentrations. The content of DIN-N, DON-N and Protein-N in the kelp at a growth rate of 3.65 cm^2/day were all significantly lower than the algae grown at a growth rate of 3.71 cm^2/day ($p<0.05$), and not significantly lower than the algae grown at a growth rate of 4.44 cm^2/day ($p>0.05$). The content of DIN-N, DON-N and Protein-N in the kelp at growth rate of 3.71 cm^2/day were all significantly higher than the algae at a growth rate of 4.44 cm^2/day ($p>0.05$).

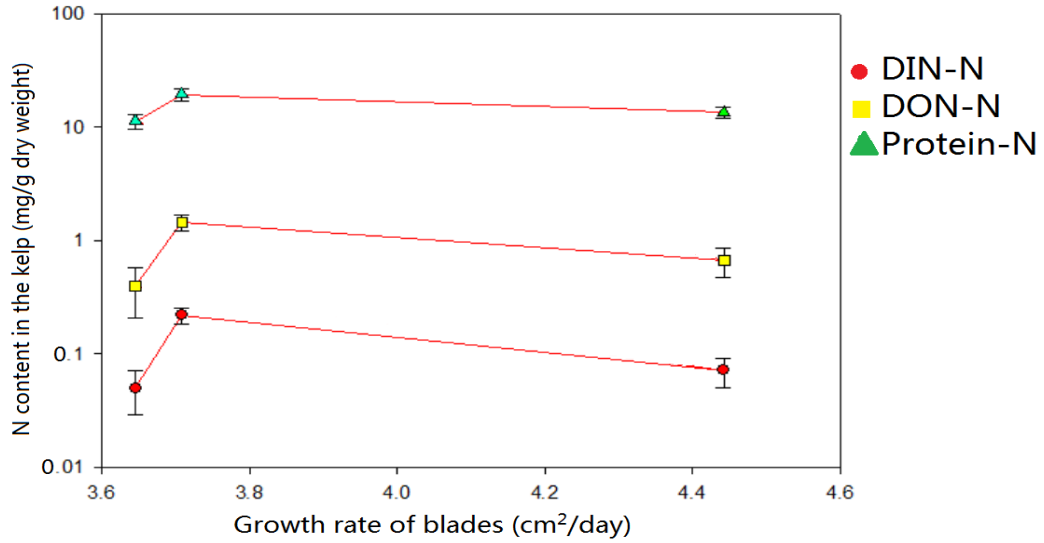

Figure 17. Content of intracellular DIN-N, DON-N and Protein-N in the kelp *S. latissima* as a function of growth rate. Values are mean of all replicates (n=30). Error bars indicate +1SE.

3.9 Relationship between nitrate in the water and N content in tissue

Figure 18 shows the relationship between content of intracellular DIN-N, DON-N and PROTEIN-N in the kelp as a function of the nitrate concentration in the water during cultivation. The content of intracellular DIN-N, DON-N and Protein-N all increased as the nitrate content in the water increased.

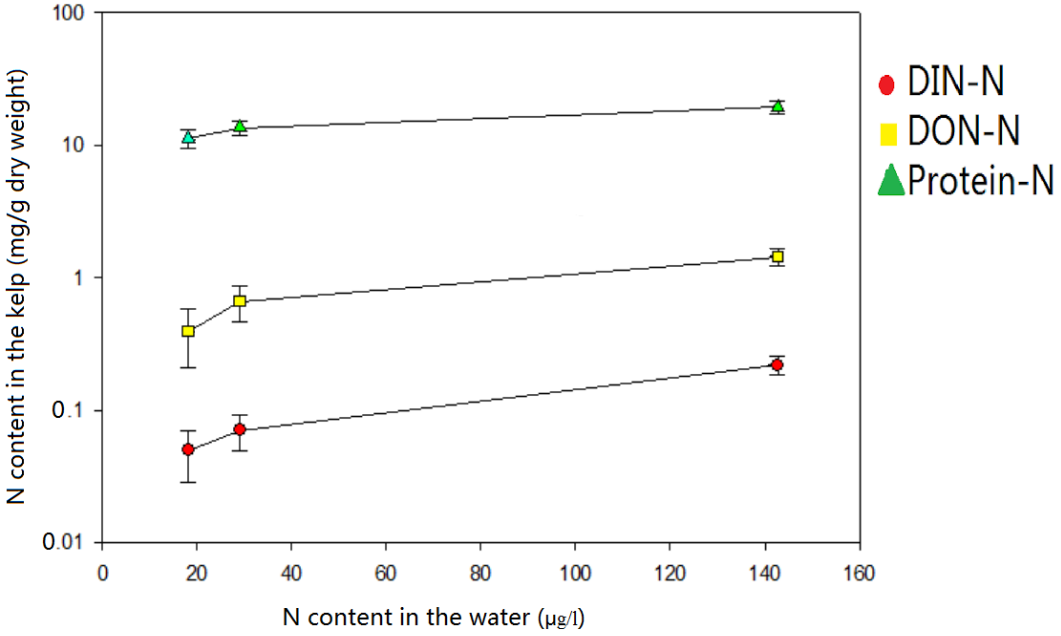

Figure 18. Content of intracellular DIN-N, DON-N and Protein-N in the kelp *S. latissima* as a function of nitrate content in the water. Values are mean of all replicates (n=30). Error bars indicate +1SE.

4 Discussion

The content of Protein-N (14.8±2.4mg/g) was higher than the content of DIN-N (0.11±0.05mg/g) and DON-N (0.83±0.31mg/g) for the algae *S. latissima* kept in variable nitrate concentrations. The content of intracellular DIN-N, DON-N and Protein-N in algae all increased as the ambient nitrate concentration increasing during growth.

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution (Wikipedia 2014). Bovine serum albumin (BSA) is used as the protein standard for calibrations curves in spectrophotometry, which is used by many studies (Peterson 1977, Smith 1985, Reno 1994). 0.5% β-mercaptoethanol (v/v) was used by Elisabete (2005) and Albin (2013). The BIO-RAD method to analyse protein is incompatible with 2-mercaptoethanol (BME) (Bio-Rad Office 2014). Albin (2013) got very low protein content because β-mercaptoethanol was used. So only water, 0.1M NaOH and 0.5M NaOH were tested to extract in this experiment, and whether boiling the biomass was also tested (Joël 1995).

Figure 5 Use of 0.5M NaOH and boiling extracted the highest yields of protein from the seaweed. One extraction of tissue did not extract all the protein from the seaweed, and we tested the method by boiling with 0.5M NaOH for 4 times. From Figure 6, 3 successive extractions secured a yield of 98.3±1.1% of proteins if we assume that 100% was extracted in 4 successive extractions. From the test experiment, 0.5M NaOH for boiling to extract protein and total 3 times was used as the standard method in the present study.

When nitrate is taken up by seaweed, the first step is reduction of nitrate to nitrite, and then nitrite is transported to the chloroplasts for reduction to ammonium (Christopher 1994). Nitrogen incorporation is often rate-limited by the control of ammonium assimilation (Christopher 1994). In this study, the cellular contents of nitrate and nitrite in tissue represent the content of DIN. Chapman and Craigie (1977) found that some species of *Laminaria* accumulate nitrate, and the nitrate content in the tissue account for a significant portion in the total nitrogen. Around 2.1% of the total DW of the tissue was reserved as nitrogen (Chapman & Craigie, 1977). In the present experiment, 0.11±0.05mg/g of DIN-N, 0.83±0.31mg/g of DON-N and 14.8±2.4mg/g of Protein-N were found in the tissue. Protein-N was around 10 times higher than DON-N and 100 times higher than intracellular DIN-N in the tissue. From Figure 18, it is apparent that the contents of DIN-N, DON-N and PROTEIN-N in the algae all increased with increasing of nitrate supply during cultivation.

Different levels of nitrate supply affected the N content in tissue, which will also affect the C/N ratio (weight) and protein-to-nitrogen conversion factors. Gevaert (2001) found that variations in the C/N ratio showed a clear seasonal pattern with

values ranging from 7 to 12.5 mgC • $(mgN)^{-1}$. The lowest values of 7 mgC • $(mgN)^{-1}$ were found in March. In the present study, the C/N ratio was higher, around 13.8 mgC •$(mgN)^{-1}$ to 27.7 mgC •$(mgN)^{-1}$. Plants grown at High N concentration showed a high level of cellular N, the C/N ratio will then be lower.

In the present experiment, protein was measured using the Biorad-kit method, and total-N was also measured. The protein-to-nitrogen factor was 5.30±0.26 protein •N^{-1} for all the seaweed samples across treatment and sampling date:

$Protein_{(g/100g)} = N_{(g/100g)}$ x (5.30±0.26).

Different foods show different protein-to-nitrogen factors, like milk 6.38, eggs 6.26 and rice 5.95 (Tkachuk 1969, Sergio 2002, FAO 2003). Sergio (2002) found the protein-to-N conversion factors of 5.38 for brown algae. Different nitrate supplying affected the nitrogen in tissue, and then also affected the protein-to-N factors.

Indergaard (1990) found that the maximum growth rate in length in the spring was about 1.5cm day^{-1} and 0.4 – 0.6 cm day^{-1} during May – July. In the present experiment, the highest growth rate in length was 1.12 cm day^{-1}, found for algae kept in Medium N concentration during Day14 to Day21. From Figure 9, plants at High N concentration did not grow faster than that cultivated at Medium N concentration. This may be because the plants kept at High N concentration were thicker than plants maintained at Medium N concentration. César (2012) and Bartsch (2008) found that not only nutrient supply, but also temperature, light exposure, water velocity and day length will affect the growth rate. Fortes and Lüing (1980) found that low water velocity is generally thought to reduce the growth rate. We had almost the same temperature, salinity, and light exposure for all three treatments. The water velocity was supplied by aeration, which was not measured in all the 9 tanks.

The N content in algae tissue did not always increase with increasing growth rate of blades (Figure 17). In the present experiment, length multiplied width of the blade was used to estimate the growth rate of blades in terms of cm^2 • day^{-1}. Biomass of the plants would be a better way to measure the plants' growth rate, and fresh weight and dry weight are used as biomass yield by César (2012). We wanted to measure the weight of the blade, but because of biomass losses at the apical frond, total weight cannot be used. It is very hard to measure the weight of the blade from the first hole to the junction between stipe and blade. The seaweed's growth was measured as the area of blade from stipe to first hole (length multiply width). Different plants have different thickness, which will affect the growth of the area of blade based on biomass. This was not thought carefully at the beginning of the experiment. It will be more accurate if we can record the thickness of the blade at the first hole.

In the present experiment, the average water content of *S. latissima* estimated was 77.9±0.6% (dried at 70℃ for 24 hours), which was lower than that found by Gevaert (2001). Gevaert (2001) found a relationship between fresh weight and dry

weight: DW = 0.113 FW. This means the water content of the kelp was around 89% of fresh weight in *Laminaria saccharina*. Albin (2013) reported a water content around 60-80% of fresh weight. The plants were sampled at different places and using different drying methods resulted in the water content difference in three experiments. Gevaert (2001) sampled the plants at the Eastern English Channel between September 1996 and March 1999, and dried the seaweed at 60℃ for five days. Albin (2013) sampled the plants at Ulvillarna and Ursholmen in Sweden, and dried the seaweed at 105℃ for 19 hours. In the present experiment, the plants were collected in the Trondheim Fjord and cultivated in tanks near the fjord. Different places have different nutrients, light, temperature and other environmental conditions, which may make the water content different.

Fishmeal is widely used in feeds because of its substantial content of high-quality proteins, containing all the essential amino acids (The fish site 2013). Kelp is consumed as food by coastal people, particularly in Asian countries, and the seaweed may also be used as feeds in aquaculture. Viera (2005) found good survival rates of juvenile abalone when feeding with three red macroalgae, which matched the abalone protein and lipid requirements. The brown algae *Saccharina latissima* do not have as much protein as red macroalgae. Since it is wildly distributed in Europe, and we have known they would contain the highest protein content when cultivated in high N content of seawater. The kelp can be cultivated in the sea where is rich in nitrate, and harvested during spring, then dried and made as feeds for aquaculture all the year around.

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APPENDIX I

Table 2 All the results from the present experiment. The blank spaces are because there were not any results from those seaweed samples.

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day35-Low N-1	80.41	10.66	185.51		2.02	16.79	5.27
Day35-Low N-2	80.64	8.44	117.87	1084.42	1.64	17.48	5.15
Day35-Low N-3	81.79	9.22	133.94	673.60	1.75	17.35	5.27
Day35-Low N-4	78.54	8.07	67.99	89.87	1.67	20.34	4.84
Day35-Low N-5	78.60	7.89	41.61		1.69	19.24	4.68
Day35-Low N-6	78.98	8.57	43.27	203.27	1.43	21.40	5.97
Day35-Low N-7	78.15	8.59	58.00	168.88	1.92	17.49	4.48
Day35-Low N-8	77.67	8.41	40.52		1.81	17.93	4.64
Day35-Low N-9	77.76	8.16	40.55	181.46	1.79	17.51	4.55
Day35-Medium N-1	81.84	9.86	139.82	95.57	2.12	15.13	4.64
Day35-Medium N-2	81.41	10.12	117.84		2.12	14.86	4.78
Day35-Medium N-3	81.82	9.90	91.20		1.91	16.30	5.17
Day35-Medium N-4	80.07	9.29	82.70	437.80	1.88	16.25	4.95
Day35-Medium N-5	81.82	10.05	64.63	468.68	1.90	15.27	5.30
Day35-Medium N-6	81.39	9.75	61.58	502.73	2.23	14.64	4.37
Day35-Medium N-7	78.24	8.83	59.55		2.18	15.32	4.05
Day35-Medium N-8	78.16	8.88	43.56	586.77	1.72	18.93	5.16
Day35-Medium N-9	78.53	8.95	40.70	569.20	1.74	18.70	5.14

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day35-High N-1	81.43	10.91	176.63	721.35	2.34	13.12	4.67
Day35-High N-2	81.05	11.27	146.00		2.31	13.19	4.88
Day35-High N-3	80.94	10.47	197.33	2262.09	2.24	13.27	4.68
Day35-High N-4	79.77	11.08	376.95	1653.62	2.37	13.49	4.68
Day35-High N-5	80.07	10.83	341.97		2.25	13.35	4.82
Day35-High N-6	79.95	10.75	270.51	1273.00	2.31	13.80	4.66
Day35-High N-7	79.77	9.79	218.17	1243.59	2.07	15.23	4.73
Day35-High N-8	80.25	10.17	186.38	1298.92	2.11	14.04	4.82
Day35-High N-9	80.17	9.78	191.03		2.33	14.37	4.20
Day42-Low N-1	78.02	12.22	15.84	520.99	1.69	18.72	7.24
Day42-Low N-2	78.16	10.45	7.86	549.39	1.61	18.65	6.49
Day42-Low N-3	77.55	9.90	18.65	828.85	1.82	18.33	5.43
Day42-Low N-4	77.55	8.70	23.37		1.58	20.45	5.49
Day42-Low N-5	77.15	9.27	13.87	456.83	1.43	21.47	6.50
Day42-Low N-6	78.72	9.32	14.98	598.76	1.52	22.21	6.14
Day42-Low N-7	77.33	9.00	27.46		1.77	17.76	5.07
Day42-Low N-8	77.72	8.82	18.31	644.80	1.72	18.05	5.12
Day42-Low N-9	77.23	8.79	21.83	799.47	1.46	18.58	6.03
Day42-Medium N-1	76.27	10.63	89.64		1.97	15.31	5.40
Day42-Medium N-2	75.61	10.77	86.21	998.59	1.85	16.68	5.82
Day42-Medium N-3	76.08	11.13	72.56	755.49	1.88	17.63	5.91
Day42-Medium N-4	77.99	9.39	81.62	974.68	1.81	16.58	5.19
Day42-Medium N-5	77.62	8.99	71.62		1.56	19.36	5.78

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day42-Medium N-6	78.19	9.37	68.67	762.77	2.03	17.47	4.62
Day42-Medium N-7	78.85	9.54	73.32	991.10	1.67	18.68	5.70
Day42-Medium N-8	78.07	9.09	53.36		1.67	18.97	5.44
Day42-Medium N-9	77.84	9.19	61.66	914.00	1.46	18.75	6.29
Day42-High N-1	77.84	10.12	317.32	1337.01	2.14	13.60	4.73
Day42-High N-2	78.43	9.91	284.05	1268.56	2.31	13.94	4.30
Day42-High N-3	78.58	10.40	272.20		2.26	12.76	4.60
Day42-High N-4	79.29	13.26	204.54	1591.72	2.24	13.75	5.92
Day42-High N-5	76.77	11.39	156.17		2.26	13.21	5.04
Day42-High N-6	76.34	11.30	179.10	1888.34	2.47	12.08	4.58
Day42-High N-7	77.87	10.17	450.32	1728.64	2.21	14.32	4.60
Day42-High N-8	78.53	10.68	336.57		2.23	14.73	4.79
Day42-High N-9	78.14	10.31	276.34		2.19	14.92	4.72
Day49-Low N-1	71.80	9.54	49.89	422.69	1.30	22.74	7.36
Day49-Low N-2	71.29	9.43	32.72		1.39	23.79	6.80
Day49-Low N-3	71.79	9.29	30.13		1.38	22.50	6.74
Day49-Low N-4	76.48	7.42	68.48		1.11	25.39	6.65
Day49-Low N-5	77.24	8.25	45.99	462.04	1.18	23.62	6.98
Day49-Low N-6	77.30	8.09	46.02		1.39	22.51	5.83
Day49-Low N-7	79.39	7.71	81.94	1073.24	1.32	21.17	5.83
Day49-Low N-8	79.44	8.06	53.69		1.28	22.60	6.31
Day49-Low N-9	79.47	7.76	49.60	458.64	1.66	19.44	4.69
Day49-Medium N-1	74.88	8.96	74.98		1.26	18.27	7.09

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day49-Medium N-2	75.36	9.18	53.95	869.77	1.83	17.47	5.03
Day49-Medium N-3	75.11	9.13	58.69	945.33	2.18	19.63	4.19
Day49-Medium N-4	73.96	8.56	81.51		1.39	21.75	6.15
Day49-Medium N-5	74.25	8.80	52.92	667.45	1.11	21.81	7.92
Day49-Medium N-6	74.04	8.55	53.92	781.94	1.70	22.24	5.04
Day49-Medium N-7	75.25	8.64	80.29		1.28	21.46	6.76
Day49-Medium N-8	75.01	8.85	82.86	1006.68	1.85	23.58	4.78
Day49-Medium N-9	75.55	8.89	79.28		1.42	22.16	6.26
Day49-High N-1	79.34	11.03	194.46	1863.44	1.20	13.54	9.21
Day49-High N-2	78.77	10.09	204.13	2297.13	1.99	15.04	5.06
Day49-High N-3	80.03	10.29	215.19		1.83	15.89	5.63
Day49-High N-4	76.20	11.62	155.24	1699.83	1.57	14.04	7.40
Day49-High N-5	75.57	11.04	151.98		2.54	14.18	4.35
Day49-High N-6	75.30	11.23	146.02	1402.14	2.25	14.54	4.99
Day49-High N-7	76.28	9.84	317.14		2.17	14.90	4.53
Day49-High N-8	76.88	9.98	357.35	1336.16	1.83	15.37	5.47
Day49-High N-9	76.86	10.35	269.64	2168.96	2.23	15.80	4.64
Day56-Low N-1	73.19	7.53	52.47		1.58	23.39	4.78
Day56-Low N-2	74.13	8.11	28.90		1.25	29.92	6.48
Day56-Low N-3	74.71	8.24	23.75	383.17	0.85	27.88	9.65
Day56-Low N-4	79.48	8.26	55.55		1.04	30.41	7.95
Day56-Low N-5	75.75	6.61	26.91	276.80	0.82	29.27	8.02
Day56-Low N-6	75.77	4.42	27.91		0.75	26.53	5.92

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day56-Low N-7	78.46	6.99	52.50		1.36	26.49	5.12
Day56-Low N-8	78.21	7.77	35.05	222.76	1.81	24.11	4.30
Day56-Low N-9	78.29	7.53	42.64		0.97	26.35	7.74
Day56-Medium N-1	77.98	8.27	50.12	568.38	1.21	21.63	6.85
Day56-Medium N-2	77.75	7.87	34.51		1.02	21.81	7.69
Day56-Medium N-3	78.60	8.55	30.36	519.93	2.00	20.91	4.27
Day56-Medium N-4	76.95	7.66	34.23	501.31	2.63	24.32	2.91
Day56-Medium N-5	77.58	7.56	25.98		0.85	26.03	8.91
Day56-Medium N-6	78.15	8.15	33.59	535.30	0.75	25.79	10.87
Day56-Medium N-7	77.05	7.34	43.27		1.24	25.08	5.91
Day56-Medium N-8	76.42	7.00	27.78		1.79	24.77	3.90
Day56-Medium N-9	77.48	7.57	34.64		1.58	23.48	4.78
Day56-High N-1	82.67	10.17	243.15	1145.15	2.54	13.94	4.01
Day56-High N-2	82.80	10.06	267.78		2.40	13.11	4.20
Day56-High N-3	82.59	10.22	245.45	2144.34	2.55	13.39	4.01
Day56-High N-4	79.93	9.71	211.79	1965.92	2.40	14.15	4.05
Day56-High N-5	79.33	9.83	179.41	298.86	2.41	13.71	4.09
Day56-High N-6	79.55	9.25	176.67	654.88	2.29	13.93	4.04
Day56-High N-7	78.93	8.99	142.99		2.30	16.71	3.91
Day56-High N-8	80.59	9.25	115.36	1092.60	2.22	16.24	4.17
Day56-High N-9	80.85	9.26	179.09		2.05	16.57	4.52
Day62-Low N-1	76.25	7.26	45.16	222.58	1.24	27.62	5.84
Day62-Low N-2	75.82	6.79	73.37		1.23	28.73	5.53

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day62-Low N-3	75.75	6.24	48.84	81.87	1.38	25.96	4.54
Day62-Low N-4	77.46	6.17	61.20		1.29	27.91	4.77
Day62-Low N-5	76.76	5.88	70.27	78.32	1.13	32.02	5.20
Day62-Low N-6	77.12	6.20	41.37		1.27	28.61	4.89
Day62-Low N-7	79.36	6.44	99.99	59.28	1.34	26.60	4.82
Day62-Low N-8	79.84	5.96	45.18		1.33	26.27	4.49
Day62-Low N-9	80.35	6.10	56.78	98.90	1.41	25.80	4.33
Day62-Medium N-1	78.66	7.74	97.03		1.59	20.28	4.88
Day62-Medium N-2	78.88	7.99	50.46	183.50	1.85	19.25	4.31
Day62-Medium N-3	79.22	6.22	51.20	-125.37	1.87	18.75	3.32
Day62-Medium N-4	77.66	7.95	107.12	2279.13	1.62	21.21	4.92
Day62-Medium N-5	77.52	7.99	96.79	1240.57	1.63	22.42	4.90
Day62-Medium N-6	76.07	7.81	80.74		1.56	22.32	5.00
Day62-Medium N-7	78.03	8.08	158.15	875.91	1.68	21.68	4.81
Day62-Medium N-8	78.65	8.84	141.78	1276.94	1.65	21.53	5.35
Day62-Medium N-9	79.23	9.14	114.72	55.79	1.37	23.08	6.69
Day62-High N-1	76.27	10.97	124.61	1526.51	3.21	12.23	3.42
Day62-High N-2	76.00	10.69	130.44		2.42	15.26	4.41
Day62-High N-3	75.59	11.28	120.72	1577.10	2.67	13.19	4.22
Day62-High N-4	76.11	10.51	213.06		2.82	12.67	3.73
Day62-High N-5	76.48	10.27	252.14	810.68	2.66	13.75	3.86
Day62-High N-6	76.35	10.57	240.80		2.71	12.48	3.90
Day62-High N-7	77.83	9.02	142.16		2.44	14.56	3.69

No.	Water content (%)	Protein content (%)	DIN content (μ g/g DW)	DON content (μ g/g DW)	Total-N (g/100g DW)	C/N ratio	Protein-to-nitrogen conversion factors
Day62-High N-8	77.67	9.40	146.06		2.47	15.28	3.80
Day62-High N-9	77.93	9.56	160.65		2.56	15.12	3.74