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A Study of the Seasonal Variation in Biochemical Composition of Saccharina latissima

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Abstract

Increasing concerns for climatic changes and increasing fuel cost have encouraged a movement towards alternative fuels, and the global interest and demand for biofuel is increasing. Commercially available biofuels today are derived from terrestrial crops, and continued use of these crops drive the food versus fuel debate. There is a need for an alternative biomass which is sustainable and which do not compete with the production of food and feed, and that does not require the use of pesticides, fertilizers, farmable land and freshwater. Marine biomass such as the macroalgae *Saccharina latissima* could meet these demands.

The present study aimed to examine if there were any differences in the seasonal variation in biochemical composition of *S.latissima* between 1st and 2^{nd+} year algae grown at different depths. Two field experiments were conducted from April/March to September/August in 2012 and 201,3 respectively, where two algal age groups (1st and 2^{nd+}) were cultivated at 3m and 8m depths at a semi-sheltered location off the west coast of central Norway. Biomass measurements and samples were collected through the summer and analyzed for carbon, nitrogen, laminaran and mannitol content.

Age of the plants was found to have no significant ($P > 0.05$) impact on the carbohydrate composition in *S.latissima*. With regards to cultivation depth, the results revealed a depth dependent pattern with higher carbohydrate content at 3m than at 8m depth. The carbohydrate content peaked in June and decreased towards the autumn, while the total carbon content stayed relatively constant throughout the summer, with no significant ($P > 0.05$) difference between the two depths or age groups. The nitrogen content increased steadily during the summer at both depths and in both age groups. Fouling by bryozoans became a problem from June onwards, with losses of biomass as a direct result. The bryozoans seemed to be the reason for the decrease in mannitol and laminaran towards the autumn, and were likely causing increased nitrogen content throughout the summer. The relatively constant carbon content throughout the summer, despite the decrease in carbohydrates, was likely caused by the chitin and carbonate content of the bryozoan exoskeleton.

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1 Introduction

Climate change is one of the most serious environmental problems facing the world today (Berndes et al., 2003). Climatic changes brought on by greenhouse gases primarily produced by the burning of fossil fuels have caused significant changes to ecosystems, and threaten millions of people with increasing risk of hunger, water shortages, floods and diseases (Escobar et al., 2009). Around 80% of the total world energy supply comes from the burning of fossil fuels (Goldemberg, 2007, Escobar et al., 2009, Nigam and Singh, 2011) and 58% of this is consumed by the transport sector alone. Fueled by the concerns of the increasing fuel costs and the CO_2 related climate changes, the interest and awareness for biofuels have grown in the last decades (Nigam and Singh, 2011).

Biofuels are renewable energy sources produced from biomass (Adams et al., 2011a, Koh and Ghazoul, 2008, Stöcker, 2008). Commercially available biofuels today, mainly bioethanol and biodiesel, are derived from the fermentation of terrestrial carbohydrate rich crops such as corn (*Zea mays)*, sugarcane (*Saccharum sp.*), wheat (*Triticum spp*), and from transesterification of vegetable oils such as rapeseed (*Brassica* napus) and soybean (*Glycine max)* (Pimentel et al., 2009, Kraan, 2010, Stöcker, 2008, Koh and Ghazoul, 2008, Escobar et al., 2009). There are concerns that growing crops for fuel production is a waste of land, water and energy resources which are vital for production of food to an ever growing human population (Kraan, 2010, Escobar et al., 2009, Pimentel et al., 2009). In addition these crops are cultivated in an intensive way where many pesticides and fertilizers are used which can cause damage to the environment (Kraan, 2010, Singh et al., 2011).

With the European Union's aims to replace 10% of their transport fuels with biofuels by 2020 and 25% by 2030 the challenge is on to find a sustainable feedstock, rich in carbohydrates and with no part in the human or animal food chain which can meet the growing demands for biofuel (Kraan, 2010).

1.1 Macroalgae for biofuel production

About 50% of the global primary production of biomass comes from the marine environment (Carlsson et al., 2007). Both macro- and microalgae have potential as biofuel feedstocks (Adams et al., 2011a) and the macroalgaes *Saccharina latissima* (L.) Lane, Mayes, Druehl and Saunders [synonym: *Laminaria saccharina* (L.) Lamouroux] and *Alaria esculenta* (L) Greville have been suggested as raw material for biofuel production (Forbord et al., 2012, Kraan, 2010, Handå et al., 2009, John et al., 2011). The idea of algae as raw material for energy production goes back to the 1950's. Especially during the oil crisis in the 1970's the idea attracted serious attention (Chen et al., 2009). The interest of using seaweed for the production of biofuel is due to the fact that it grows two to three times faster than sugarcane (Handå et al., 2009), and the dry matter of macroalgae contains up to 60% carbohydrates that can be fermented to bioethanol. Macroalgae requires no use of freshwater, land areas, fertilizers or pesticides. This together with high content of fermentable carbohydrates makes marine macroalgae a promising candidate as biomass for production of biofuels (Adams et al. (2009), Handå et al., 2009, Kraan, 2010).

The carbohydrates of most importance in seaweeds are mannitol, laminaran and alginate (Handå et al., 2009, Adams et al., 2009, Jang et al., 2012), where mannitol and laminaran is storage polymers and alginate is a structural component of the cell wall and intracellular matrix (Lobban and Harrison, 1994, Jang et al., 2012). The concentration of these carbohydrates varies throughout the year, with the maximum concentrations of mannitol and laminaran in late summer and autumn and a minimum in spring when the alginate concentration is highest (Haug and Jensen, 1954, Black, 1950b, Adams et al., 2009). The complex structure of alginate poses a challenge to fermentation (Tang et al., 2009, Handå et al., 2009). Laminaran and mannitol are more readily fermentable and the seaweeds should preferably be harvested when the concentration of laminaran and mannitol is high (Handå et al., 2009, Adams et al., 2009). To be able to meet the biofuel demand, a year-round supply of biomass is of great importance (John et al., 2011) In order to get a year-round production of biofuel from seaweed biomass it is important to find an efficient method for the fermentation of alginate (Handå et al., 2009).

Large scale production of biofuels cannot be based on natural populations of macroalgae. Developments of large scale cultivation systems are therefore needed. According to calculations by Handå et al. (2009) an area of approximately 700km^2 is needed to produce enough fuel to cover 5% of the annual use of transport fuel in Norway (based on fuel consume anno 2006). In China large scale production systems of macroalgae are well established (Tseng, 1993, Troell et al., 2009) and in Canada IMTA systems utilizing *A. esculenta* and *S. latissima* are under development (Troell et al., 2009, Ridler et al., 2007). There are still challenges that needs to be addressed, like cultivation technology and harvesting methods, to make large scale production of seaweed economically sustainable in Norway and other high cost countries (Handå et al., 2009).

1.2 *Saccharina latissima*

The brown algae *S. latissima* is a perennial cold water species with a seasonal development. A period of rapid growth from January to June/July is followed by a period of slow growth from July to December (Parke, 1948, Lüning, 1979, Bartsch et al., 2008). *S.latissima* is yellowish/brown in colour and has a branched hapter, flexible round stipe 5-30 cm long and a whole undivided lamina which can grow up to about 3m in length (Rueness, 1977, Artsdatabanken, 2006). *S. latissima* has a circumpolar distribution in the northern hemisphere and occur naturally in clear and turbid coastal waters (Borum et al., 2002). It grows from the sublittoral fringe down to a depth of around 30 meters in moderate to sheltered areas, on boulders, cobbles and bedrock. It has an average life span of 2-4 years and in Europe it grows at temperatures below 19°C from Svalbard to Portugal (Artsdatabanken, 2006, Handå et al., 2009).

The life cycle of *S. latissima* consist of a diploid macroscopic sporophyte phase, and a microscopic haploid gametophyte phase. The sporophyte has special spore holding cells, sporangia, where the production of zoospores occurs by meiosis (Kain, 1979). In Norwegian waters dense areas of sporangia, called sori, occurs on the sporophyte from October to December (Forbord et al., 2012). Artificially triggered production of sori can be induced by meristem removal (Buchholz and Lüning, 1999) and through exposure to short day conditions throughout the year (Forbord et al., 2012, Lüning, 1988).

Sporangia are produced in the distal end of the lamina and each sporangium can contain 32 spores (Kain, 1979). Artificial spore release can be induced by dehydration of tissue with sori in a cold environment (~10 °C) over night , followed by rehydration in cold seawater (~10 °C) (Arbona and Molla, 2006, Edwards and Watson, 2011). In large scale cultivation, spores are seeded onto ropes (Arbona and Molla, 2006). After settling, the spores germinate to haploid gametophytes that will develop either into egg producing females or sperm producing males (Edwards and Watson, 2011). Blue light stimulates the gametophytes to become fertile (Lüning and Dring, 1972). Exposure to high temperatures (Kain, 1979) or red light can inhibit fertilization (Lüning and Dring, 1972), and under such conditions the gametophytes show vegetative growth. Kept under optimal conditions gametophytes of *S. latissima* produce eggs after 8 to 10 days (Lüning and Dring, 1975). When the eggs are released a spermatozoid releasing and attracting substance is produced and there is a mass release of spermatozoid 8-12 seconds after the substance reaches the fertile male gametophyte (Lüning, 1981). After fertilization the zygote is developed followed by cellular division developing a new sporophyte (Handå et al., 2009, Edwards and Watson, 2011). The seeded ropes are then kept in the hatcheries for approximately four weeks or to the sporophyte is 1-2 mm in length (Merrill and Gillingham, 1991) before being put in the ocean. Harvest has previously been recommended during late spring before the temperature of the sea water rises significantly and epiphytic growth degenerate the sporophyte (Edwards and Watson, 2011, Broch and Slagstad, 2012). A study on ethanol production from *Laminaria digitata* found that there was a direct relationship between laminaran yields in seaweed and ethanol yields (Adams et al., 2011b), and Schiener et al. (2014) showed that laminaran and mannitol had a strong influence on the total carbon content which was usually highest in autumn.

1.3 Variability in chemical composition of *S. latissima*

The mannitol and laminaran contents of *S.latissima* show a clear variation throughout the year, with maximum levels not coinciding at the same time. The highest mannitol content is found during the summer months while the laminaran content reaches its highest values in late summer and autumn. Both laminaran and mannitol contents are markedly reduced during winter (Black, 1950b, Haug and Jensen, 1954). During winter when the nitrogen concentrations in the sea water are high the *Laminariales* accumulates nitrogen which enables the algae to grow rapidly during the following spring and early summer when nitrogen

concentrations in the sea water is low (Chapman and Craigie, 1977, Gagné et al., 1982). During summer, when growth tend to be limited by low nitrogen concentrations, the algae accumulates carbohydrates which form an energy source during winter (Black, 1950b, Haug and Jensen, 1954, Johnston et al., 1977, Chapman and Craigie, 1978, Gagné et al., 1982, Sjøtun, 1993).

Black (1950a) found that the mannitol content of *Laminaria spp.* increased with increasing depth reaching maximum values at 6-10m. The laminaran content, however, decreased with increasing depth with maximum values at 3-6m (Black, 1950a, Chapman and Craigie, 1978, Lüning, 1979). Parke (1948) found that growth in lamina length was higher at 4m than at 8m and 12 m depth in *S.latissima*, and Handå et al. (2013) found a depth dependent growth pattern, with higher growth in length at 2m and 5m than at 8m depth in autumn and winter, and rapid growth from February to June at all depths. In the case of growth versus age Sjøtun (1993) found that elongation of the lamina was age dependent in *S.latissima*, where three year old sporophytes showed lower lamina elongation rates than two year old sporophytes. In a later study on *Laminaria hyperborea,* Sjøtun et al.(1995) showed that lamina growth increased continuously with age. Lüning (1979) reported that after maximum growth in spring, the growth in *S.latissima* continues during summer although at a reduced rate. He showed that the reduction in lamina growth occurred later in the year in first-year algae than in older individuals.

The studies on chemical analysis of seaweeds and analysis of the effect of depth and age have mainly been executed on wild material. If cultivation of *S.latissima*, with the intent of producing biofuel is going to happen, there is need for more research on the effect of age and cultivation depth on the seasonal variation in chemical composition of *S.latissima.*

1.4 Study aims and approach

The aims of this study was to examine if there was any difference in the seasonal variation in biochemical composition of *S. latissima* between 1st year algae (first growth season) and 2nd+ year algae (second growth season or older) and growth at different depths.

The biochemical composition of *S. latissima* were followed through two growth seasons, April/March to September/August 2012 and 2013 respectively, where the effect of algal age and cultivation depth on the seasonal variation in biochemical composition were investigated. The main focus was on the two non-structural carbohydrates laminaran and mannitol.

Three hypotheses were formulated:

H1: The carbohydrate content and C:N ratio of the macroalgae will be higher in the 2nd+ year than in the 1st year algae

H2: The carbohydrate content and C:N ratio of the macroalgae will be higher at 3 m depth than at 8 m depth

H3: The carbohydrate content and C:N ratio will be higher late in the season than early in the season

2 Material and methods

2.1 Study area and experimental design

Two field experiments were conducted from April/March to September/August 2012 and 2013, respectively, at Taraskjæret Frøya (63° 42´N, 8° 52´E) off the coast of central Norway (Fig.1). Taraskjæret represents a semi-sheltered location, sheltered from the south and west blowing winds and exposed to winds from the northeast. The average water current speed velocity over a 28 day period has previously been measured to be 9.4 cm/s at 6m depth with a maximum speed at 48 cm/s. The main current direction was 28° (northeast). The depth at the location varied from 26m to 48m.

Fig.1 Geographical location of experimental station at Taraskjæret in Frøya.

Two algal age groups (1st and 2nd+ year algae) were cultivated at 3m and 8m below the surface attached to ropes on a frame hanging from a floating raft (Fig. 2B). The frame was made from PVC pipes and the dimensions were 100cm x 50cm. Four 12mm polyester ropes were attached to each frame (Fig.2A). For each age group and depth there were 3 replicate frames, 12 frames in total. The 1st year algae, which were seeded on string in the laboratory and grown to a size of 1-2 cm, were attached to the frame by winding the string around the ropes on the frame. In 2012 the 2nd+ year algae, which were collected from wild population outside Frøya, were attached by inserting the stipe between the strands of the rope. In 2013 the 2nd+ year algae were attached to the ropes with cable ties. To each rope on a frame 20 algae were attached 5cm apart, 80 algae in total per 2nd+ year frame.

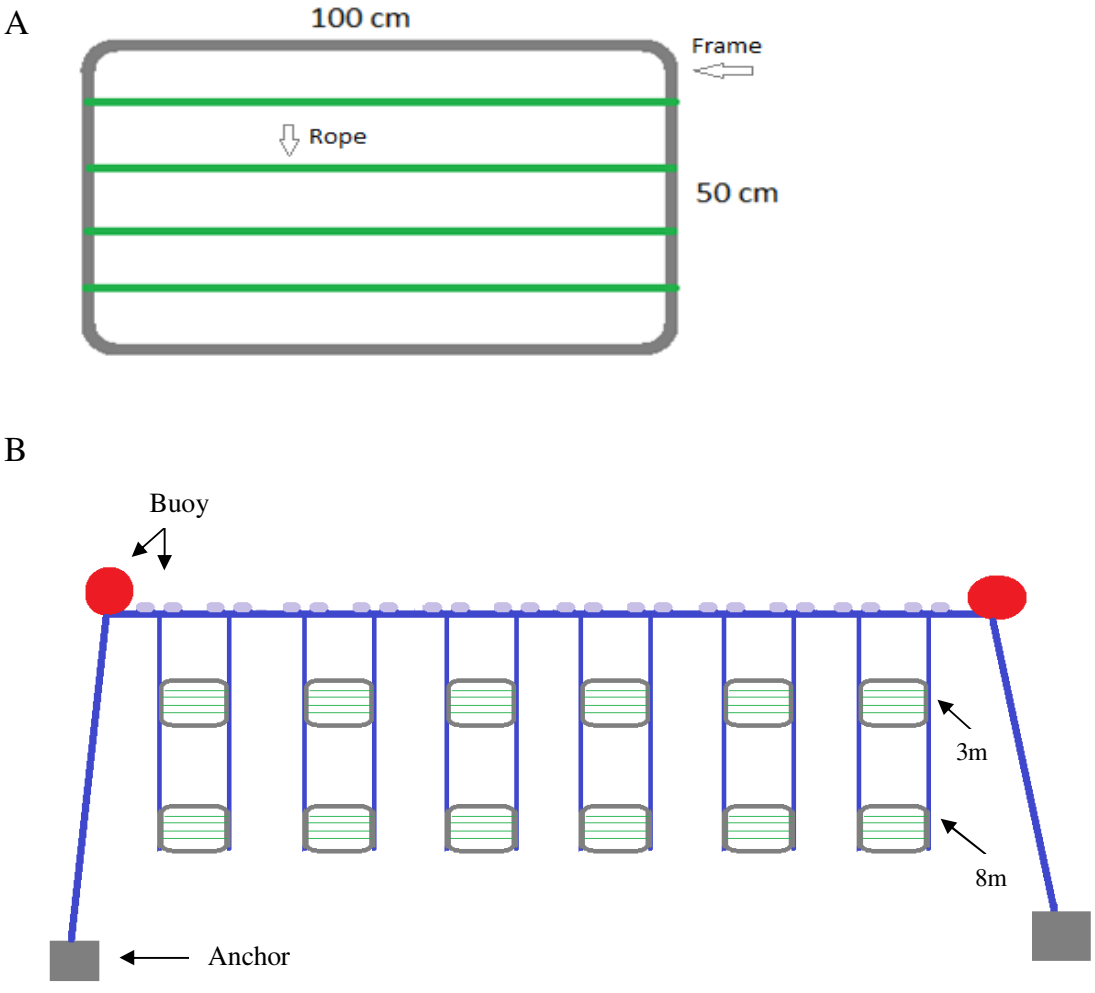

Fig.2. Sketch of cultivation structure. A: Frame used for cultivation of seaweed.
B: Cultivation system at sea with frames hanging at 3m and 8m depth.

2.2 Algae selection and cultivation at sea

The algae in the 1st year group were juveniles produced in the hatchery. Individuals of *S.latissima* form wild populations outside Frøya were sampled for sorus induction in January 2012 and 2013. The induced sorus portions of the algae were used for release of zoospores. The zoospores were seeded on plastic tubes covered with 60 meters of 1.4 mm polyester silk string. The tubes were incubated for 8 weeks in 200L aquariums with continuous water exchange from day 2 after seeding and under a 16:8 light/dark regime at the TBS laboratory, Trondheim. The water temperature was 8-9°C and the light intensity was 30-60 µmol/m^2/s.

For the 2nd+ year group, algae were collected from wild populations outside Frøya in March 2012 and 2013. This group was a mix of older plants of different ages, believed to have grown for one or more growth seasons.

The 2nd+ algae collected in 2012 were kept in a tank outside at TBS for a week before the 2nd+ and the 1st year algae were transported to Hammarvik at Frøya in the end of March. At arrival at Hammarvik the algae were placed temporarily in the sea at a pier for 2-3 days before being attached to the frames. Because of storms deployment of the frames wasn't possible until the 18th of April and the algae were in the meantime placed in the sea, hanging from a pier.

2.3 Sampling and measurements

2.3.1 Biomass samples and measurements

Biomass samples for chemical analysis and water samples for determination of nitrate and phosphate were collected monthly from May to September. In August samples were collected twice, in the beginning and middle of the month.

On each sampling date, 8 whole individuals from each replicate frame were arbitrarily sampled and placed in labeled ziplock bags. The samples were transported by car back to TBS. The algae were photographed, deep-frozen and later dried. The algae from the 2012 season were laid out on shelves in a drying cabinet with a heating unit in the bottom and dried at 25-30°C. The algae from the 2013 season were dried in an Electrolux drying cabinet at 50°C. The dried algae were then milled in an IKA MF 10 Basic mill to a fine powder. The

individuals from one replicate were mixed and homogenized to one sample. The milled samples were kept in double ziplock bags to prevent air from coming in.

Lamina growth was recorded on every sampling date. According to (Parke (1948)) the main part of growth takes place in the transition zone between the stipe and lamina and 10 cm above. Growth was measured by punching a small hole 10 cm above the stipe and registering the added lamina lengths from the start of the lamina to the hole. At each sampling, a new hole was made 10 cm above the stipe. 5 individuals on each frame were individually labeled with coloured plastic cable ties and used for growth measurements. In 2012, individual growth measurements were performed according to the hole punching method described above, but only on the 2^{nd+} year algae. On the 1^{st} year algae total length of the lamina was recorded. The intention was to measure the 1^{st} and 2^{nd+} year algae in 2013 according to the hole punching method described above. Due to heavy loss of 2^{nd+} year individuals in 2013 only 1^{st} year algae were measured by the hole punching method, and the total lamina length were measured on the 2^{nd+} year algae.

The weight of each frame was recorded with a spring scale and a digital fish weight.

2.3.2 Environmental and nutrition conditions

A CTD profile of water temperature and salinity was taken from 0 to 20m depth with a SAIV SD204 CTD. Water samples were taken at the surface, 3, 8 and 15 depths with a Nansen water collector. The water samples were filtered through a 0.45 µm syringe filter. The concentration of nitrate (NO_3^--N) and phosphate (PO_4^--P) was determined with a segmented flow analyser with O.I Analytical cartridge Part A002603 and cartridge Part A002604, for nitrate and phosphate, respectively.

2.3.3 Chemical Analyses

The ash content of each sample was determined by heating the dried milled algae in a Scandiaoven EL62U at 400°C for 18-20 hours.

The ash free dry weight (AFDW) was calculated as AFDW (mg/g) = DW - ash

Tin capsules containing 1-2 mg dried milled algae were for the samples collected in 2012 sent to SINTEF Fisheries and Aquaculture and in 2013 sent to MARINNOVA in Portugal to determine the carbon and nitrogen content by elemental analysis.

For the 2012 season, the total glucan content was analyzed as the total glucose content after complete enzymatic degradation of laminaran and cellulose, while for the 2013 season, only the laminaran was measured as the increase in glucose content after a weak acid hydrolysis of the laminaran, cellulose was not (hydrolysed).

5 g dried milled algae from each sample collected in 2013 were sent to SINTEF Materials and Chemistry for mannitol and laminaran analyses. 1g dried milled algae were weighed into 50 ml centrifuge tubes and added 30 ml de-ionized water (65-70°C). The sample was incubated in a water bath holding a temperature of 65-70°C for 15 minutes. The sample was then centrifuged at 5000 rpm for 10 minutes and the supernatant were extracted into a new tube. For the hydrolysis of laminaran 0.1 ml H_2SO_4 (2.5M) were added to 0.4 ml of the supernatant in brown HPLC-vials, sealed with rubber caps and incubated at 105°C for 5 hours. After incubation the samples were filtered and analyzed by HPLC with glucose and mannitol as standards.

Statoil analyzed the samples from 2012. The dried milled samples underwent enzymatic de-polymerization by incubation with enzymes in a 48 hour two step process. In the first 24 hours cellulases and alginate lyase were used to extract mannitol, convert the glucans to glucose and solubilise the alginate in the sample. A portion of the sample were then filtered through a 0.2 µm filter and analyzed for glucan and mannitol with HPLC using a refractive index detector. For a detailed method description see Appendix 1.

2.4 Statistical analysis

Microsoft Excel 2007 (Microsoft corp., Redmond, Washington, USA) was used to calculate averages and standard error for the replicate frames. The data for length increase, frame weight, ash and AFDW content, carbon and nitrogen, laminaran and mannitol content were tested for normality using a Shapiro-Wilk test. Two-way ANOVA followed by post hoc multiple comparison procedures (Holm-Sidak method) were used to test for differences between the replicate frames, between the age groups and field season and t-tests were

performed to test for differences between the two depths within an age group. Statistical analyses were performed using SigmaPlot 12.5 (Systat Software Inc.).

3 Results

3.1 Environmental conditions, nitrate and phosphate

Figure 3 shows water temperature and salinity at four different depths at Taraskjæret during the experiment. In 2012 the salinity varied greatly during the summer with a minimum at 22.5 in June and a maximum in July at approximately 34, while in 2013 the salinity was relatively stable at ~33. The temperature increased slowly at all depths and was the same at all depths in August and September in 2012 and 2013 respectively.

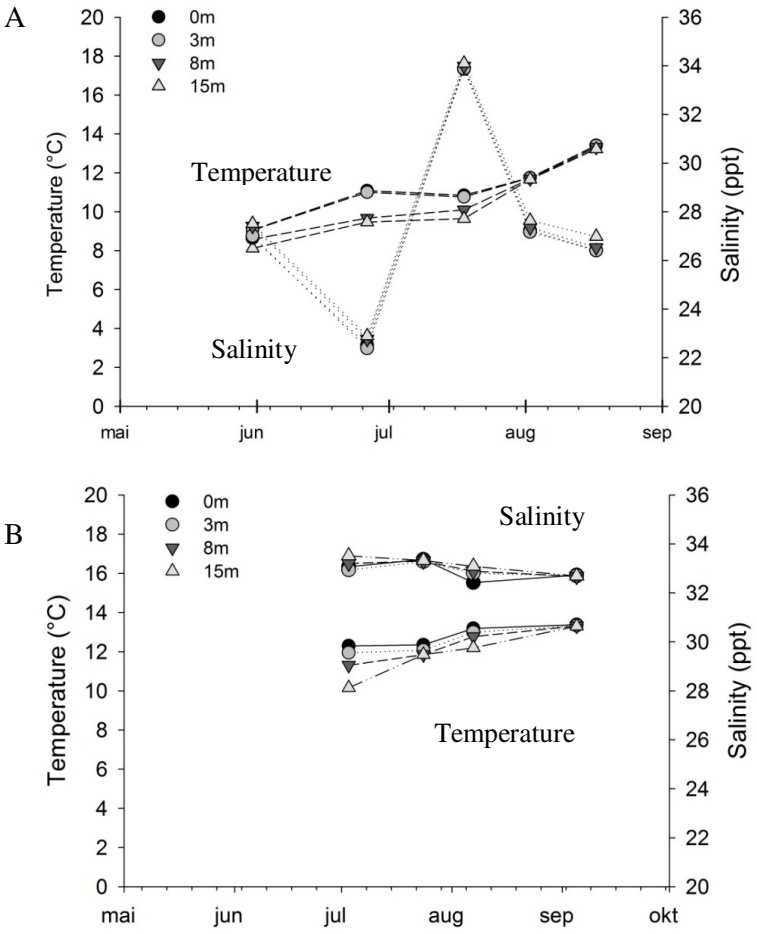

Fig.3. Water temperature and salinity from the surface to 15m at Taraskjæret during the experimental period. A: In 2012. B: In 2013

Figure 4 shows the phosphate (A) and nitrate (B) concentrations at different depths at Taraskjæret from April 2012 to December 2013. Both phosphate and nitrate concentrations were generally low during the summer and there were no clear differences between the different depths. Winter concentrations of phosphate and nitrate were measured in March 2013 and showed higher and typical winter values. The uniformity of the concentrations between the depths indicated mixing of the water masses.

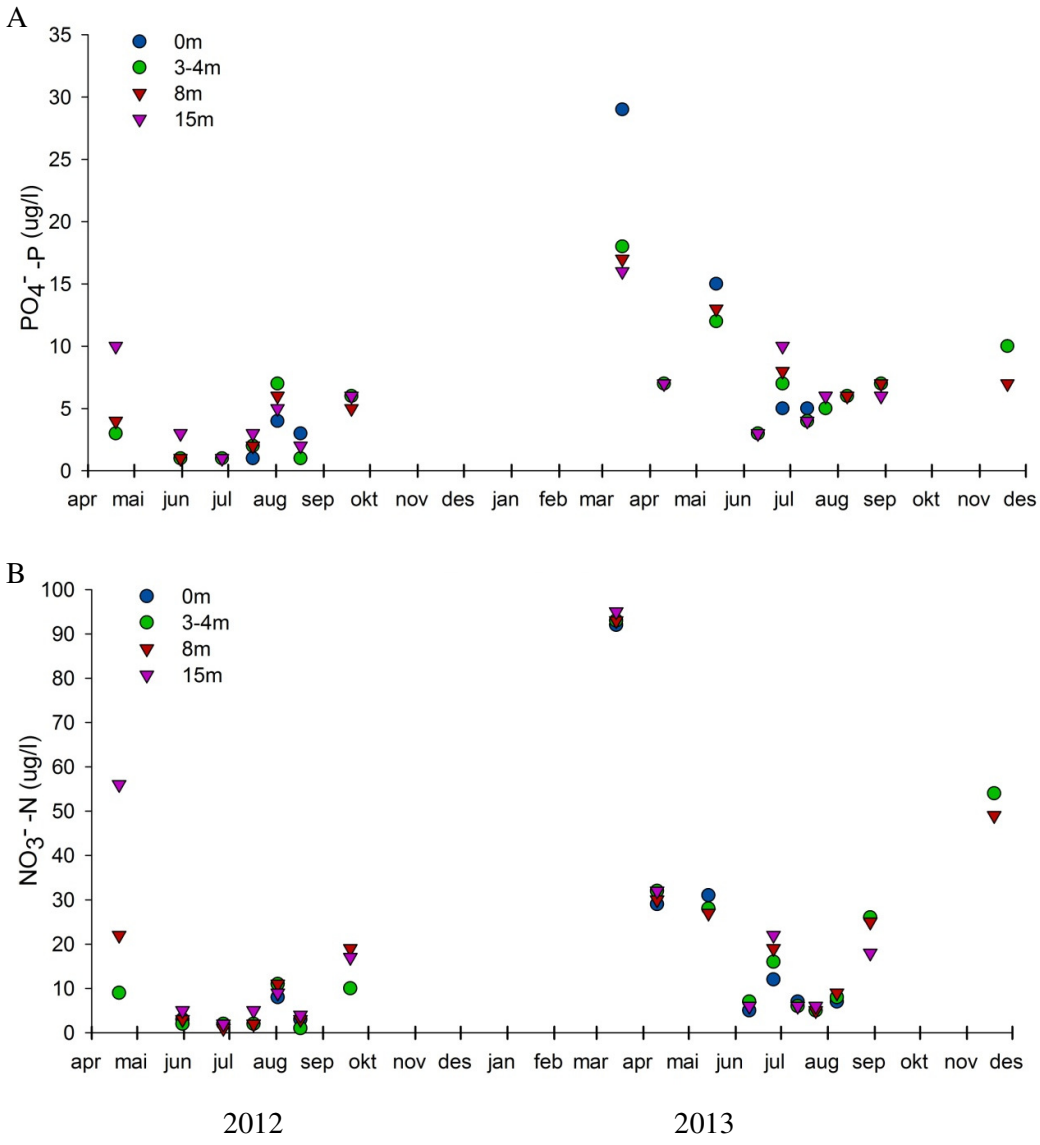

Fig.4. Phosphate and nitrate concentrations throughout the experimental period. A: Phosphate, B: Nitrate

3.2 Seasonal and depth dependent growth

Figure 5 show the average total lamina length of *S.latissima* throughout the experimental period in the 1st year group in 2012 and 2nd+ year group in 2013. Figure 6 shows the average and accumulated length increase of *S. latissima* through the experimental period in the 2nd+ year group in 2012 and 1st year group in 2013. Overall there was a steady increase in length throughout the experimental period in both year groups and at both depths.

There were no significant difference in length increase between 3m and 8m depth in the 1st or 2nd+year group in 2012 and 2013 (P = 0.273 and P = 0.641 in the 1st year group in 2012 and 2013, respectively and P = 0.937 and P = 0.908 in the 2nd+year group in 2012 and 2013, respectively). Comparing the groups measured in the same way (as described in Section 2.3.1), there was a significant higher rate of length increase in the 2nd+ year group in 2013 than in the 1st year group in 2012 (P = 0.008). Between the 1st year group in 2013 and the 2nd+ year group in 2012 there was no significant difference in length increase (P = 0.437).

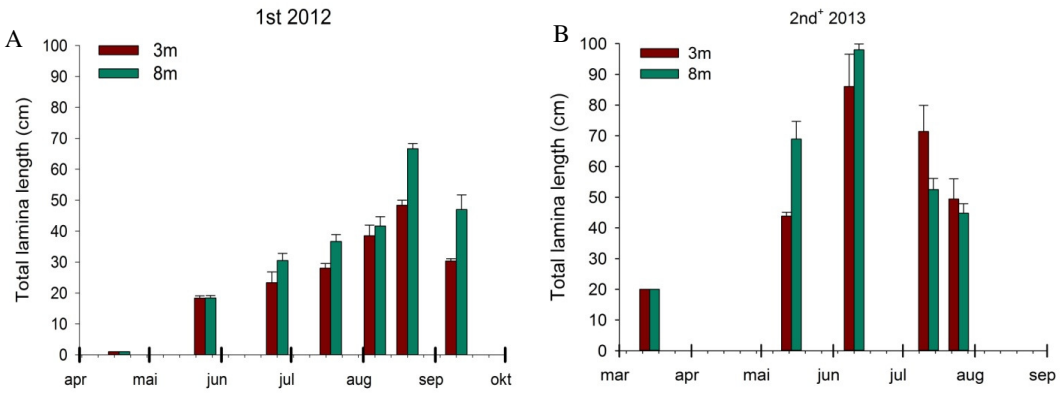

Fig.5. Average total lamina length of *S. latissima* in the different age groups at 3m and 8m depths. Growth was determined by measuring total lamina length. A: 1st year group in2012. B: 2nd+ year group in 2013

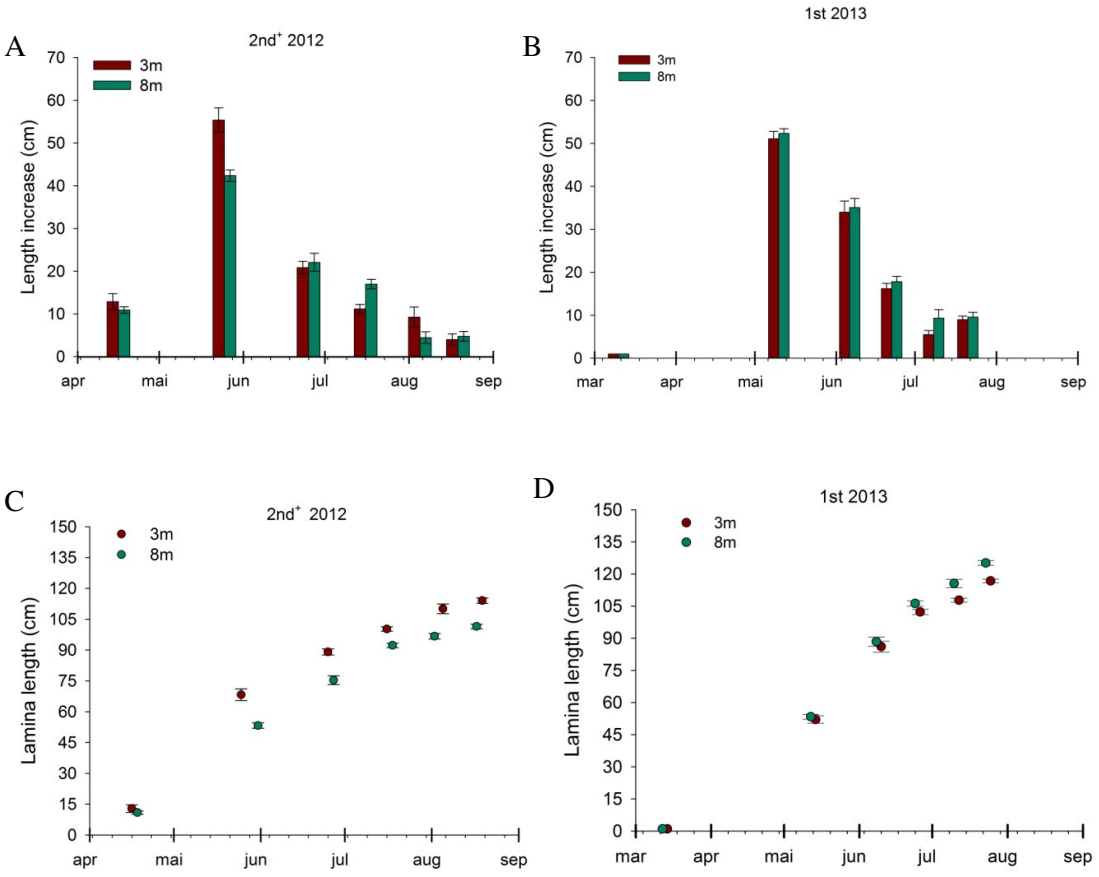

Fig.6. Average length increase of *S. latissima* in the different age groups at 3m and 8m depths. Growth was determined by punching holes and measuring the length increase between the holes. A: 2nd+ year group 2012. B: 1st year group 2013. C: Accumulated length increase 2nd+ year group 2012. D: Accumulated length increase 1st year group 2013.

Figure 7 shows the average wet weight of the biomass on the frames through the experimental period. The weight increased until August in 2012 and late June in 2013 were after there was a loss in weight. In the 2nd+year group in 2013 there was a loss of weight from early June to August.

In 2012 in the 2nd+ year group the wet weight of the frames were significantly higher at 3m depth (P= 0.038) than at 8m. The wet weight of the 1st year group in 2013 were significantly higher than the wet weight in 2012 at both depths (P= 0.003). No difference in wet weight were found in the 2nd+ year group between the field seasons 2012 and 2013 (P=0.938). In 2012 there was a significantly higher wet weight in 2nd+ year group than in the 1st year group (P=0.003). No difference in wet weight were found between the 1st and 2nd+ year group in 2013 (P=0.346).

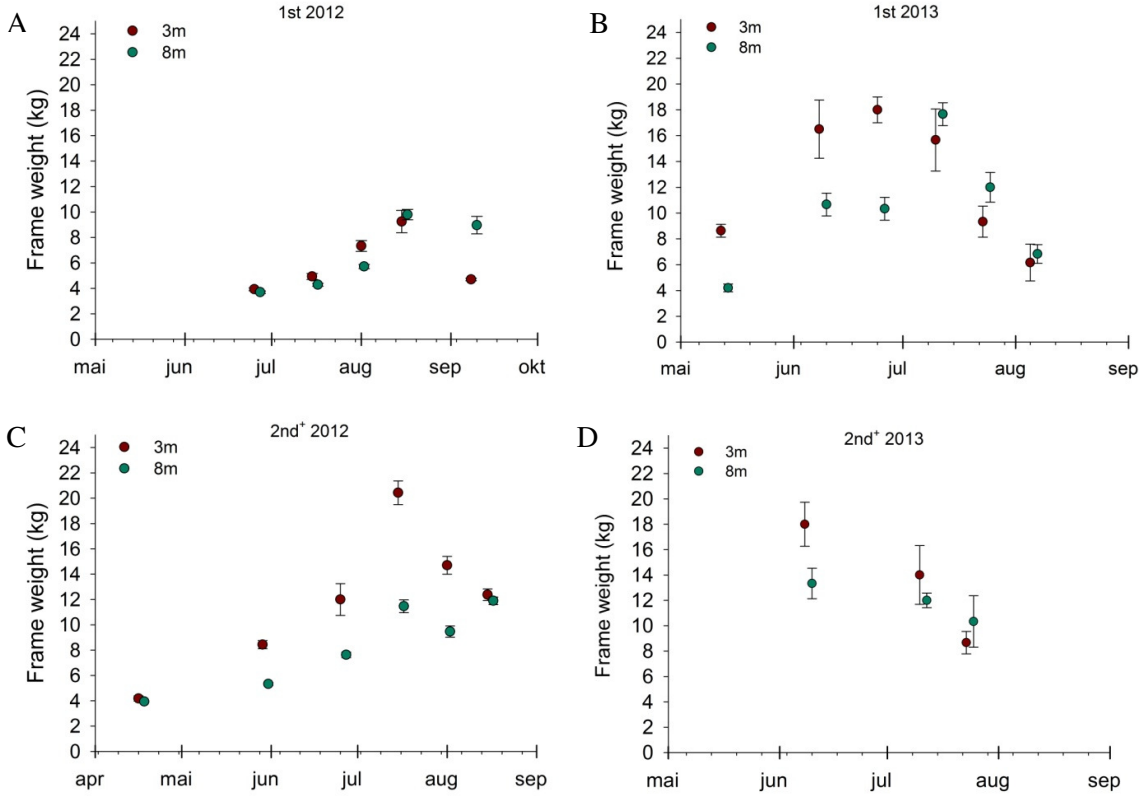

Fig.7.Average frame weight (kg) at 3m and 8m depth for the different age groups of algae through the experimental period. A: 1st year group in 2012. B: 1st year group in 2013. C: 2nd+ year group in 2012. D: 2nd+ year group in 2013.

3.3 Biofouling

From June onwards in both 2012 and 2013, the algae were covered with epizoa, mostly bryozoans (*Membranipora membranacea*), but also some blue mussels (*Mytilus edulis*) and skeleton shrimp (*Caprella sp*). This led to losses of biomass from the lamina above the meristem. The hydroid *Tubularia larynx* appeared on the ropes in between the algae and on the PVC frame sometime between the end of June and the middle of July in 2012.

Figure 8 shows a photograph series of the algae from each sampling date in 2012 to illustrate the problems with biofouling.

31. May 2012

A

B

27. June 2012



C

17. July 2012




2. August 2012

D



17. August 2012

E



10. September 2012

F

Fig.8. Photograph series of the experimental algae from May to September 2012. A: 2^{nd+} year algae, 1^{st} year algae and a 2^{nd+} frame from May. B: 2^{nd+} year algae, 1^{st} year algae from June 2012. C: 2^{nd+} year algae, 1^{st} year algae, 1^{st} year frame and 2^{nd+} year frame from July 2012. D: 2^{nd+} year algae, 1^{st} year alga and 2^{nd+} year frame with hydroids from 2. August 2012.
E: 2^{nd+} year algae, 1^{st} year alga and 2^{nd+} year frame with hydroids from 2. August 2012.
F: 2^{nd+} year algae, 2^{nd+} year frame overtaken by hydroids and 1^{st} year frame from 10. September 2012.

Figure 9 shows bryozoan coverage (%) of *S. latissima* through the experimental period. In general the bryozoan coverage increased from low values in June to approximately 100% coverage in August/September. The 1st year algae at 3m in 2012 showed an increase in bryozoan coverage from June to the middle of July and thereafter a decrease towards September. At 8m there was an increase in September to approximately 100% coverage. From June to August the plants at 3m depth were slightly more heavily covered with bryozoans in both the 1st and 2nd+ year groups.

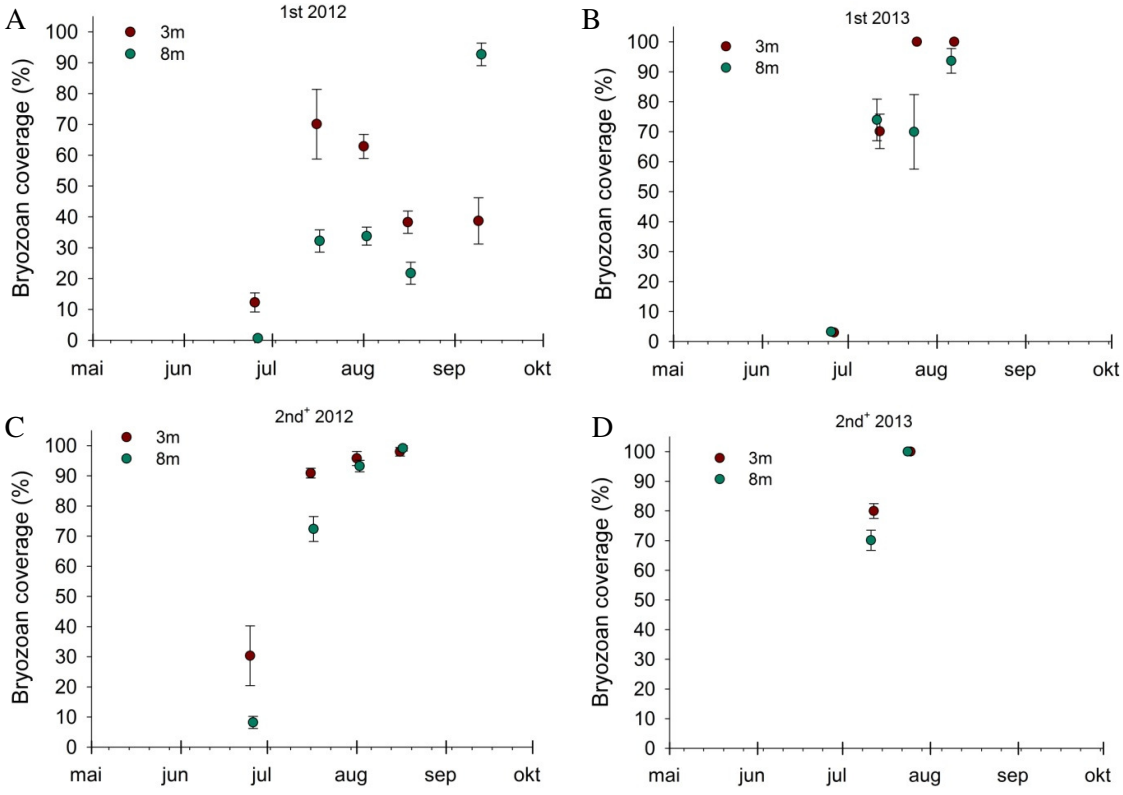

Fig.9. Bryozoan coverage of *S. latissima* at 3m and 8m depths through the experimental period. A: 1st year group in 2012. B: 1st year group in 2013. C: 2nd+ year group in 2012. D: 2nd+ year group in 2013.

3.4 Chemical composition

3.4.1 Ash and AFDW

Figure 10 shows the ash and ash free dry weight contents of *S. latissima* expressed in terms of mg/g dry weight as a function of time. The AFDW content was highest in the spring and decreased during the summer, while the ash content showed the opposite pattern of variation, with the lowest values in the spring. There was no significant difference in ash content or AFDW content at 3m and 8m or between the two year groups in 2012 ($P > 0.063$). In 2013 there was significantly more ash at 8m than at 3m and significantly higher AFDW at 3m than at 8m in both the 1st ($P < 0.003$) and 2nd+ ($P < 0.013$) year group. There were no significant differences between the 1st and 2nd+ year groups in 2013 ($P > 0.06$). There was a significantly higher ash content at 3m in 2012 than in 2013 ($P < 0.004$) and significantly higher AFDW content at 3m in 2013 than in 2012 ($P < 0.033$) in both the 1st and 2nd+ year groups.

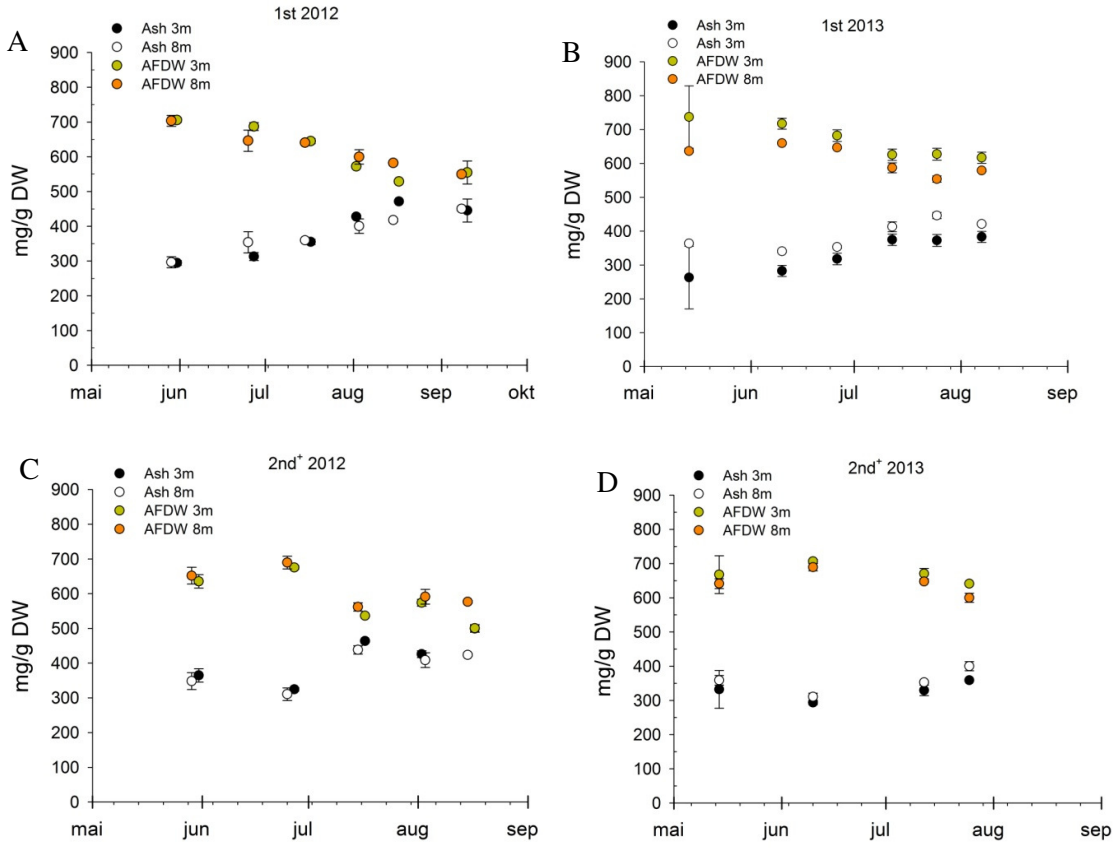

Fig.10. Ash and AFDW content of *S. latissima* at 3m and 8m depth through the experimental period. A: 1st year group in 2012. B: 1st year group in 2013. C: 2nd+ year group in 2012. D: 2nd+ year group in 2013.

3.4.2 Carbon and nitrogen content

Figure 11 shows the carbon and nitrogen contents of *S. latissima* expressed in terms of mg/g dry weight in the different age groups as a function of time. The carbon content was relatively constant through the whole period while the nitrogen content increased steadily through the summer period. There was no significant difference in the carbon contents between the two depths in both year groups in 2012 and 2013 ($P > 0.093$). Moreover no difference in carbon contents were found between the field seasons 2012 and 2013 ($P > 0.200$).

There was no significant difference in the nitrogen contents between 3m and 8m in both year groups in 2012 and 2013 ($P > 0.160$). In the 1st year group at 3m there were no significant difference found in the nitrogen content between the field seasons 2012 and 2013 ($P > 0.520$). The nitrogen content at 8m was significantly higher in 2013 than in 2012 ($P < 0.015$). In the 2nd+ year group there was no significant difference between the field seasons 2012 and 2013 ($P>0.690$).

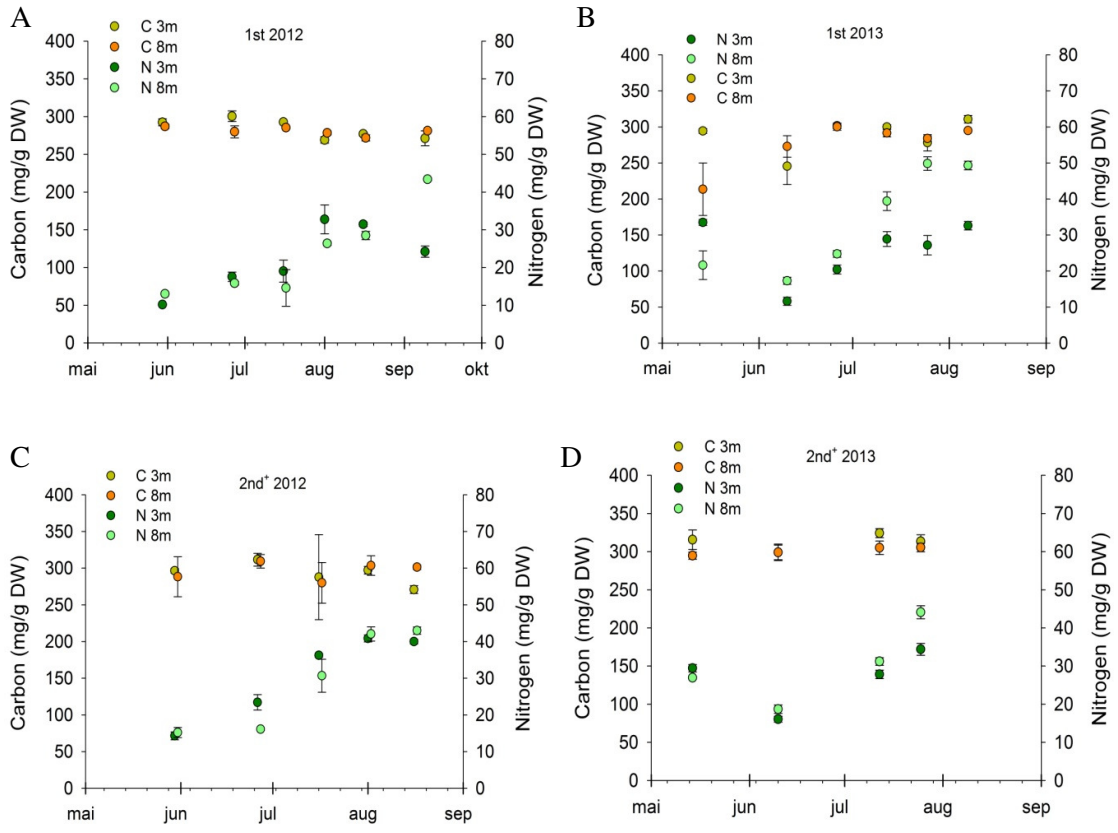

Fig.11. Carbon and nitrogen contents of *S. latissima* thorough the experimental period. A: 1st year group in 2012. B: 1st year group in 2013. C: 2nd+ year group in 2012. D: 2nd+ year group in 2013.

3.4.3 Carbon and nitrogen content of the ash free dry weight

Figure 12 shows carbon and nitrogen expressed in terms of C/AFDW and N/AFDW in the different age groups at 3 and 8m throughout the experimental period. The general pattern of variation was similar to that for C/DW and N/DW in figure 10. The carbon content was relatively constant while the nitrogen content increased from early June through the experimental period. There was no significant difference in C/AFDW between the two depths in both age groups in 2012 and 2013 ($P > 0.11$), nor was there any difference between the field seasons 2012 and 2013 ($P > 0.11$). There was no significant difference in N/AFDW between 3m and 8m in both age groups in 2012 and 2013 ($P > 0.09$). In the 1^{st} year group at 3m there were no significant difference in N/AFDW between the field seasons 2012 and 2013 ($P > 0.84$). At 8m N/AFDW was significantly higher in 2013 than in 2012 ($P < 0.023$). In the 2^{nd+} year group there was no significant difference in N/AFDW between 2012 and 2013 ($P > 0.4$).

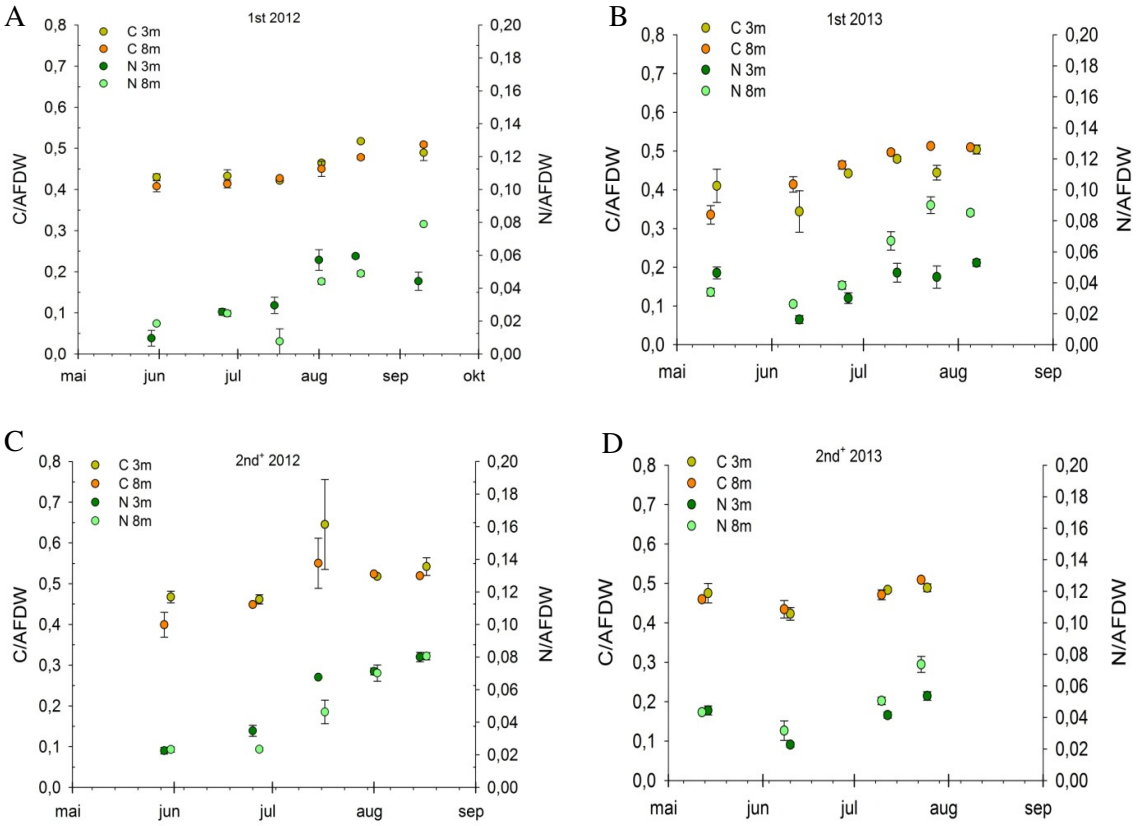

Fig.12. Carbon and nitrogen per AFDW at 3m and 8m depth during the experimental period. A: 1st year group in 2012. B: 1st year group in 2013. C: 2nd+ year group in 2012. D: 2nd+ year group in 2013.

3.4.4 Laminaran and total glucans

Figure 13 shows the contents of laminaran and total glucans (Section 2.3.3) in the different year groups expressed in terms of mg/g DW (Fig.11 A-C) and in mg/g AFDW (Fig.11 D-F) throughout the experimental period at 3 and 8m depths. For the 2012 season the total glucan content was only measured at 3m depth (Fig.11 A, D).

In both 2012 and 2013 and for both the 1^{st} and 2^{nd+} year groups, there was an increase in the content of glucans with a peak in June, before a drop through the rest of the experimental period. There was no significant difference in the increase in the laminaran content between the 1^{st} and 2^{nd+} year group in 2012 or in 2013 (P=0.494 and P=0.360, respectively). In 2013 the increase in the laminaran content was significantly higher at 3m than at 8m depth in both the 1^{st} and 2^{nd+} year groups (P=0.022 and P=0.037, respectively). The laminaran content per AFDW was significantly higher ($P < 0.037$) than the laminaran content per DW at 3m and 8m in both year groups in 2012 and 2013.

3.4.5 Mannitol

Figure 14 shows the contents of mannitol in the different year groups of *S. Latissima* expressed in terms of mg/g DW (A-C) and mg/g AFDW (D-F) throughout the experimental period at 3 and 8m depths. For the 2012 season, the mannitol content was only measured at 3m depth (Fig.12 A, D). The same trend was apparent as in Fig 12. There was an increase in mannitol from May with a peak in June, before a drop in the mannitol content the rest of the experimental period. The increase in the mannitol content was significantly higher in the 1^{st} year group than in the 2^{nd+} year group in 2012 (P= 0.001). There was no significant difference in the increase in the mannitol content between the 1^{st} and 2^{nd+} year group in 2013 (P = 0.351).The increase in the mannitol content in 2013 was significantly higher at 3m than at 8m depth (P = 0.033) in the 1^{st} year group, while there were no significant difference between the two depths in the 2^{nd+} year group (P=0.400). The mannitol contents per AFDW were significantly higher ($P < 0.014$) than the mannitol contents per DW in both year groups at 3m and 8m in both 2012 and 2013.

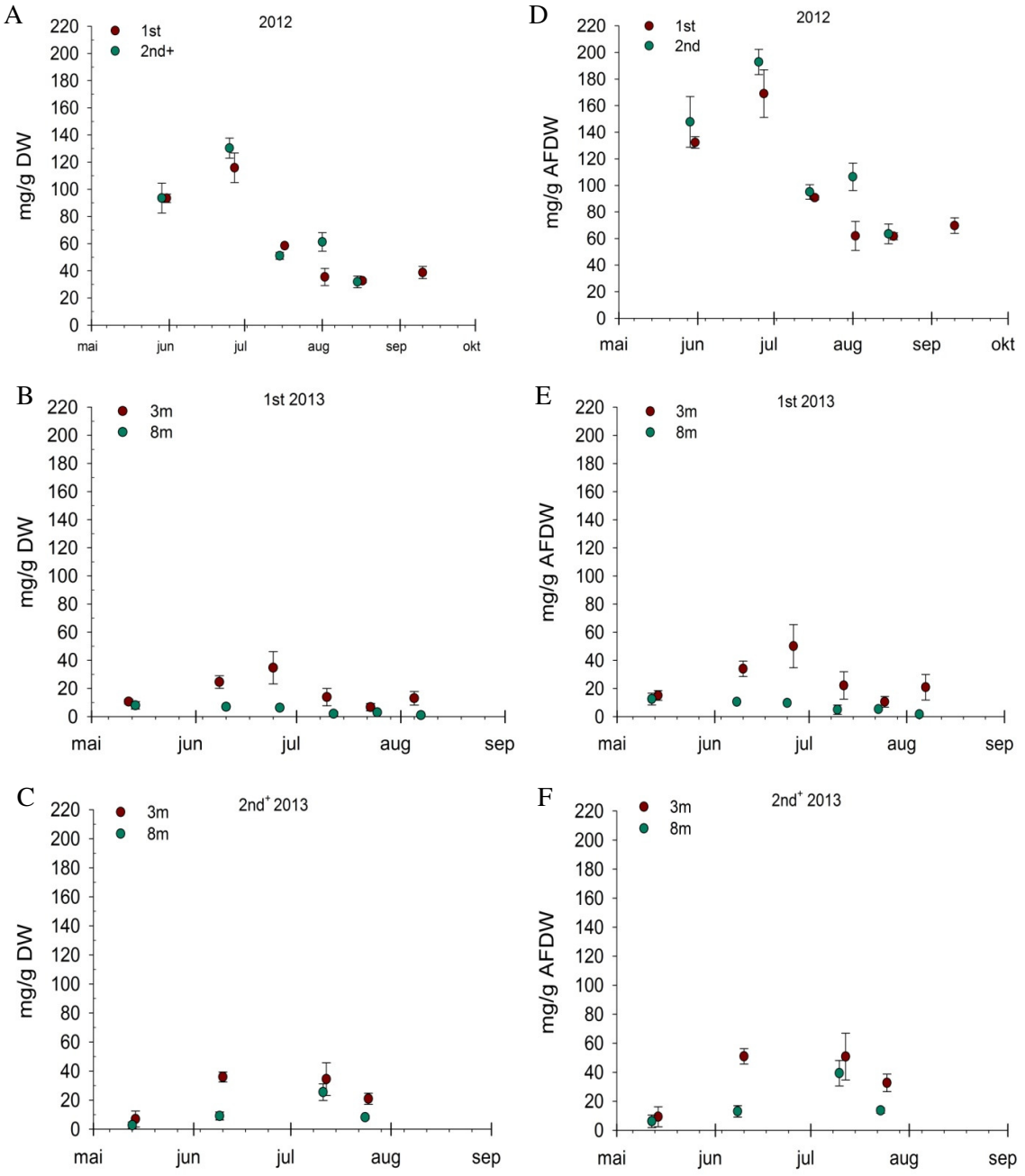

Fig.13. Laminaran contents of *S. latissima* at 3m and 8m depth through the experimental period. A-C: expressed in terms of mg/g DW. D-F: expressed in terms of mg/g AFDW. A and D: total glucan at 3m depth. B, C, E and F: laminaran content at 3m and 8m depths.

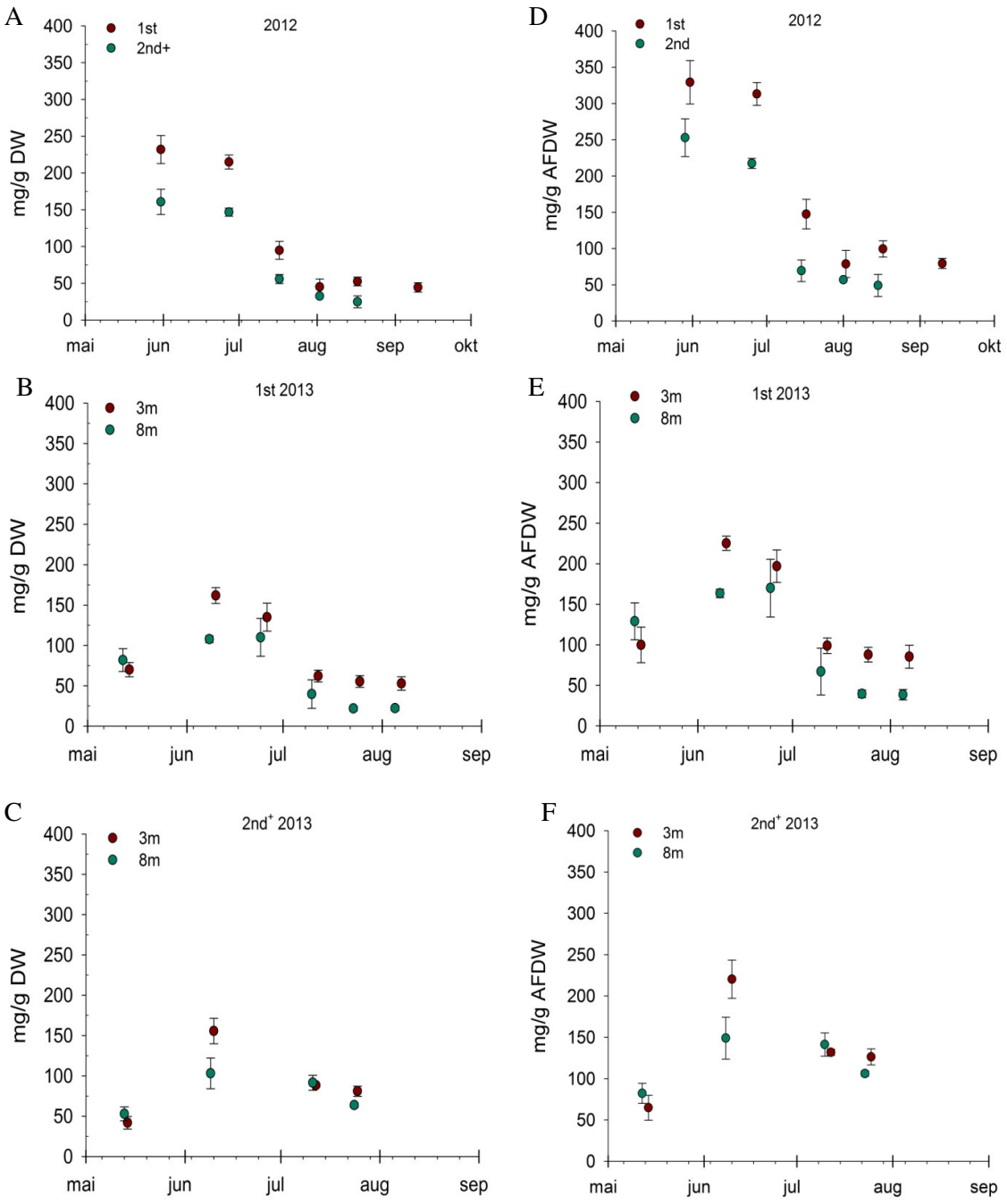

Fig.14. Mannitol contents of *S. latissima* at 3m and 8m depths through the experimental period. A-C: expressed in mg/g DW. D-F: expressed in terms of mg/g AFDW.

4 Discussion

The present study aimed to investigate if age and cultivation depth played a part in the seasonal variation in biochemical composition of *S.latissima* with regard to the storage carbohydrates mannitol and laminaran.

The results showed that there were no significant differences between the 1st and 2nd+ year algae with regard to carbohydrate composition. With regards to cultivation depth, the results revealed a depth dependent pattern with higher carbohydrate content at 3m and 8m depth. The carbon content per dry matter was relatively constant throughout the summer and there was no significant ($P > 0.05$) difference in carbon content between the two depths or age groups. The nitrogen content increased steadily during the summer with no significant ($P > 0.05$) difference between the two depths or age groups.

Fouling became a problem from June onwards, with losses of biomass as a direct result. Bryozoans also seemed to have an impact on the algaes possibilities of producing and accumulating laminaran and mannitol.

4.1 Biofouling

The first colonies of the bryozoan *M. membranacea* could be seen on the algae in June (Fig.8B). When they were first spotted, the largest colonies were on the algae at 3m depth. By late July and early August the coverage of the lamina by bryozoans were much the same at both 3m and 8m depth, and close to the entire lamina were covered with bryozoans. The bryozoan covered algae became brittle and much of the biomass covered with bryozoans was lost over the summer. By August and September almost all the seaweed was lost.

A false impression of a decrease in bryozoan coverage at 3m in the 1st year algae in 2012 (Fig.9A) was due to losses of much of the covered biomass late in the summer. The heavy fouling by bryozoans is in agreement with Handå el al.(2013) who studied growth on *S.latissima* in close proximity to a salmon farm, and reported epiphytic fouling from June and over the summer, with major loss of biomass as a result. According to both laboratory and

field studies, the growth rate and the size of the colony is shown to be positively correlated to an increase in temperature within the range of 6-18°C (Menon, 1972, Saunders and Metaxas, 2009, Saunders et al., 2010). Bryozoans as other sessile organisms requires a good supply of particulate food suspended in the water column (Eckman and Okamura, 1998), and the ambient flow of water is an important factor in the feeding success of the bryozoan (Larsen and Riisgård, 2002, Pratt, 2004). A study by Arkema (2009) on how water flow affects feeding success in the bryozoan *Membranipora serrilamella* showed that sites with ambient flow speeds at 10-12 cm/s had the highest feeding success. Sites with slow water flow speeds up to 5cm/s or flow speeds >20cm/s showed the lowest feeding success and consequently lover coverage of bryozoan colonies.

The cultivation site at Taraskjæret were previously found to have an average current speed of 9.4 cm/s and the temperature ranged from ~8°C to ~14°C during the summer in 2012 and 2013 (Fig.3), which according to the studies mentioned above would be quite perfect conditions for colonization and growth of bryozoans. It might be that locations with less favorable conditions, lower temperatures or stronger currents, would have less influence of bryozoan growth on the seaweed to the extent seen at Taraskjæret. Sundene (1962) presented a theory stating that more water movement near shore made it more difficult for the bryozoan larvae to settle down on the seaweed. Andersen et al. (2011) suggested that a continuous exposure to moderate wave activity could help control epiphytes by washing away new settlers. A mechanism like this could explain why natural populations growing in the lower littoral zone, like the algae analyzed by Haug and Jensen (1954), have less biofouling than the cultivated seaweed which are less exposed.

4.2 Chemical composition

The laminaran and mannitol content increased from May to peak values in June, followed by a sudden decrease from July, reaching minimum values in August and September in both 2012 and 2013 (Fig.13 and 14). Different methods were used for the analysis of the laminaran content in 2012 and 2013, which explains the higher content of laminaran in 2012 seen in Figure 13. For the 2012 season, the laminaran content was analysed by an enzymatic degradation of laminaran and cellulose, giving total glucan content of the dry weight containing laminaran, cellulose and free glucose. For the 2013 season however, the laminaran

content was analysed by a weak acid hydrolysis giving only the laminaran content of the dry weight (see Section 2.3.3 for more detail). The method used in 2012 gives a higher carbohydrate yield and a more precise determination of the carbohydrate content since cell structures like alginate and cellulose are degraded. Since all glucans are fermentable, the total glucan content would probably be of most interest when considering biofuel production.

In both 2012 and 2013, age was found to have no influence on the accumulation of laminaran (Fig.13). In 2012, the 1st year algae had a higher content of mannitol than the 2nd+ year algae, while in 2013 there was no difference between the two age groups (Fig.14). The reason for this was not clear. In a study by Zvyagintseva et al. (2003), 2 year old algae were found to have a higher content of laminaran than 1.5 year old and 0.8 year old alga of *Laminaria cichorioides*. From the results on the laminaran content, in both 2012 and 2013 and the mannitol content in 2013, the present study revealed that age did not seem to have an influence on the production and accumulation of carbohydrates in *S.latissima*.

The results also revealed a depth dependent pattern, with higher carbohydrate content at 3m than at 8m depth in both year groups. This is in agreement with the work by Black (1950a) as well as Chapman and Craigie (1978) and Lüning (1979) who found that in *Laminaria spp.* the laminaran content decreased with increasing water depth. These results are important for the cultivation of seaweed for biofuel production, suggesting the importance of depth in the water column for carbohydrate production and that the seaweed can be harvested annually.

The literature reports that *S.latissima* accumulates laminaran and mannitol during the summer with maximum values of carbohydrates in late summer and autumn (Black, 1950b, Haug and Jensen, 1954, Johnston et al., 1977). In the present study the maximum levels of laminaran and mannitol was found in June, followed by a reduction in carbohydrate values from July onwards (Fig. 13 and 14). The colonization of the bryozoan *M.membranacea* coincided with the reduction in carbohydrate values. Hurd et al. (2000) found a reduction in pigment concentrations beneath colonies of *M.membranacea*, and this is generally believed to reduce the rate of photosynthesis in the algae (Hepburn et al., 2006, Getachew et al., 2013). The same reduction in carbohydrates were seen in the study by Handå et al.(2013) after the colonization by bryozoans. This suggests that the bryozoans could have an effect on the capability of the algae to produce and accumulate laminaran and mannitol.

Another explanation of the low carbohydrate values of the collected biomass is that the bryozoan, which does not contain laminaran or mannitol, constitutes a major part of the

biomass, thereby diluting the seaweed biomass. The results found for the carbohydrate content in May and June, however, are undiluted by bryozoans and should then show a more accurate picture of the amount of carbohydrates that can be expected in the algal biomass.

In August 2012, bryozoans made up ~50% of the dry matter of samples collected from individuals completely covered with bryozoans (unpublished preliminary project results). This indicates that up to 50% of the measured biomass could have been something else than seaweed in late summer. It will be difficult to achieve the high carbohydrate values reported by among others Haug and Jensen (1954) in late summer and fall without finding a way to reduce bryozoan colonization or growth. Thus colonization by bryozoans could have major consequences for the macroalgae biofuel industry, not only by low carbohydrate yields, but also because fouling results in losses of the cultivated biomass before harvest.

The carbon content of *S.latissima* was relatively constant throughout the summer while the nitrogen content increased steadily towards the autumn (Fig.11). This result is not in agreement with previous findings of carbon and nitrogen content in *Laminaria* species. In wild *S.latissima* the carbon content is reported to increase during summer due to the storage of carbohydrates (Black, 1950b, Haug and Jensen, 1954, Sjøtun, 1993, Schiener et al., 2014), while the nitrogen content is reported to decline during the summer as the internal storage of nitrogen is depleted and nitrate levels in the sea water is low (Sjøtun, 1993, Schiener et al., 2014). The bryozoan *M.membranacea* have a chitinous exoskeleton hardened with calcium carbonate (Getachew et al., 2013). An increasing fraction of bryozoa in the harvested biomass could be the reason why the carbon content stayed constant while the contents of carbohydrates went down. This suggestion can be supported by the fact that the ash content of *S.latissima* increased towards autumn (Fig. 10).

The presence of *M.membranacea* was found to reduce the uptake rates of nitrate and ammonium by the algal tissue from the seawater by 50% and 41% ,respectively, in a study by Hurd et al.(1994). Hurd suggested that even though the bryozoans caused a reduction in the algaes ability to take up nitrogen and ammonium from the surrounding seawater the bryozoans provided the algae with a potential nitrogen source by the excretion of ammonium. This was contradicted in a later study that showed that the algae could not utilize the ammonium excreted by the bryozoans (Hurd et al., 2000). Based on the study by Hurd et al. (2000) it is unlikely that the excretion of ammonium by the bryozoans increased the seaweeds nitrogen uptake and thereby the seaweeds protein content. The increase in nitrogen content

came more likely from the bryozoan proteins and chitinous exoskeleton in such a way that as the bryozoan colonies grew larger the nitrogen content of the total biomass increased.

4.3 Methods for growth measurement

Difficulties with the use of the hole punching method for length increase measurements on small individuals in the 1st year group in 2012, and the constant loss of individuals marked for length measurements in the 2nd+ year group in 2013 resulted in the use of two different methods for measuring the growth in length. Total lamina length was measured for the 1st and 2nd+ year group in 2012 and 2013, respectively. For the 2nd+ and 1st year group in 2012 and 2013, respectively, the hole punching method were used, where growth was measured by making a hole 10cm above the lamina/stipe junction and then registering the added lamina lengths from the start of the lamina to the hole.

The results showed that there was no significant ($P > 0.05$) difference in growth with increasing depth in both field seasons and age groups (Fig.5 and 6). While previous studies have shown that there were a higher rate of growth at 4m to 5m depths than at 8m and 12m depth (Parke, 1948, Handå et al., 2013).

A higher rate of growth were found in the 2nd+ year group in 2013 than in the 1st year group in 2012, measured by total lamina length, while there were found no difference between the groups measured by the hole punching method (Fig. 5 and 6). The differences found between the 1st year group in 2012 and the 2nd+ year group in 2013 may be attributed to differences between the two years, the two age groups or the fact that different individuals were measured at each sampling date. This makes the results difficult to compare.

Sjøtun (1985) found that a slight increase in length of the blade took place between 10cm and 15cm above the lamina/stipe junction in *S.latissima,* and it was therefore assumed that no growth of any extent took place above 15cm from the lamina/stipe junction (Sjøtun, 1993). This suggest that by making the holes at 10 cm above the lamina/stipe junction instead of 15 cm when using the hole punching method, the measured length would be an underestimate of the actual growth.

Total lamina length would not either give a correct view on length increase, as the distal part of the lamina erodes or breaks of during the summer. A combination of the two methods could maybe give a more accurate view on the length increase during the summer period.

For the production of biofuel, the weight of the biomass available for harvesting is probably more interesting than length increase. A significant ($P<0.05$) difference in biomass weight between the depths in the 2^{nd+} year group in 2012 was found in the present study, with a higher weight at 3m than at 8m depth (Fig.7). According to the results, the highest biomass weight was attained around the middle of July. However by the middle of July the algae were heavily fouled by bryozoans, and hydroid colonies of *T.larynx* were growing in between the algae (Fig.8). This made the biomass weight results highly uncertain as a large fraction of the weight recorded after June was from fouling. The biomass weights recorded in May and June are however more reliable results. They show that there was a tendency towards higher biomass weight at 3m than at 8m depth (Fig.7). The carbohydrate content per kg biomass is highest in June with the highest carbohydrate content per kg biomass at 3m depth. From a biofuel point of view, the harvest should therefore be carried out in June before the fouling by epizoa gets too high.

It should be questioned if length is a suitable measurement for growth in seaweed. As mentioned above the total lamina length will not give reliable results and the hole punching method could show an underestimation of the actual growth. In a study by Sjøtun (1993) it was assumed that the lamina did not expand to any extent in width and length above 15 cm from the lamina/stipe junction. Future research should be carried out on methods for the measurements of length and weight.

5 Conclusion

There were no major differences between the two age groups with regard to the carbohydrate, carbon and nitrogen content, while there was a trend towards a depth dependent pattern with regard to carbohydrate content, with higher carbohydrate yields at 3m than at 8m depth. This means that the seaweed can be harvested annually, which is of great importance for the use of *S.latissima* as biofuel, and that the seaweed should be cultivated in the upper layers of the water column to obtain the highest carbohydrate yields.

It was difficult to obtain the high levels of carbohydrates in cultured seaweed as reported for wild seaweed late in the summer, probably because of the colonization and high growth of epizoa. This is a serious problem that needs to be addressed and solved if cultivation of seaweed for the purpose of biofuel production is going to be sustainable.

According to hypothesis 1 the carbohydrate content and C:N ratio would be higher in 2^{nd+} year than in the 1^{st} year algae. This hypothesis is rejected. The results showed that there were no significant differences between the age groups with regard to carbohydrate content and C:N ratio.

Hypothesis 2 said that the carbohydrate content and C:N ratio of the macroalgae would be higher at 3 m depth than at 8 m depth. This hypothesis is partly confirmed. The results showed that there was a depth dependent pattern with higher carbohydrate content at 3m than at 8m depth. There was however no significant difference in the C:N ratio between the two depths.

Hypothesis 3 said that the carbohydrate content and C:N ratio would be higher late in the season than early in the season. This hypothesis is rejected. The carbohydrate content peaked in June followed by a decrease in carbohydrates towards autumn. The reduction in carbohydrates was most likely the result of colonization of the seaweed by the bryozoan *M.membranacea*. The carbon content was relatively constant throughout the whole experimental period while the nitrogen content increased during the summer. The reason for this was presumably the increasing fraction of bryozoa in the harvested biomass.

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APPENDIX I

	BAL-AC-1003.5
TITLE	Composition Analysis of the macroalgae/seaweed to determine Mannitol, Glucan and Alginate contents via enzymatic de-polymerization by HPLC and RI/UV detectors
DATE OF ISSUE	**3-25-2011**
CREATED BY	**Adam Wargacki**
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PROCEDURES

A. SCOPE AND APPLICABILITY

This method is used to detect the wt/wt% of mannitol, glucan and alginate in dry seaweed samples. A High Pressure Liquid Chromatography (HPLC) system equipped with a refractive index and a UV detector is used for this analysis. Values are reported on a "g sugar/g dry weight basis.

B. SUMMARY OF METHOD

The dried milled macroalgae sample undergoes enzymatic de-polymerization steps by incubating with enzymes. This is a two step 48 hour process. The first 24 hour process uses cellulases and an alginate lyase to extract mannitol, convert all glucan to glucose and to solubilize all alginate. A portion of this sample is filtered through a 0.2 um filter and is run on the HPLC using the sugar method. This method is used to determine Glucan and Mannitol contents by HPLC using a refractive index detector. Sample is injected at 20uL injection volume and run at 0.6ml/min isocratically on a Rezex ROA-Organic Acid column, 300x7.8mm, 8µm particle size. The second 24 hour process uses an oligoalginate lyase to break all alginate into monomer. Ammonia is added in solution which spontaneously converts DEHU (the monomer of alginate) to 5-hydroxypyridine-2 -carboxylic acid which can be detected and quantified on the HPLC using the alginate method. Sample is injected at 1uL injection volume and run at 1.0mL/min by gradient and separated by Hypercarb column 3x30 mm, 3 µm particle size. 5-hydroxypyridine-2-carboxylic acid is detected using a UV detector at 210nm.

C. Definitions

- HPLC- High Performance Liquid Chromatography

- RID – Refractive Index Detector
- UV- Ultra Violet
- OAL-Oligo Alginate Lyase
- The sugar method can also be referred to as the organic acid method or the ethanol method.
- The alginate HPLC method can also be referred to as the 5HPA method, the 5-hydroxypyridine-2-carboxylic acid method or the 5-hydroxypicolinic acid method.
- The MNOV is a seaweed sample that is run every time to ensure that no problem occurred in the enzymatic degradation.

D. HEALTH & SAFETY WARNINGS

- Wear lab coat, gloves and safety glasses at all times
- Wear heat resistance gloves when using drying oven or autoclave.
- Take care to store toxic reagents such as methanol, trifluoroacetic acid and sulfuric acid in the appropriate cabinet. Methanol is stored in the flammables cabinet. Trifluoroacetic acid and sulfuric acid are kept in the acid cabinet.
- Sodium azide is used to stop bacterial growth and is extremely toxic. We buy a premade 5% sodium azide solution from sigma so a solution of this does not need to be made. (if you do not have sodium azide a cocktail of antibiotics can be used).
- Trifluoroacetic acid is used as the modifier in the alginate HPLC method. This is a very corrosive acid and must be handled with caution. Always handle in the hood.
- Sulfuric acid is very corrosive and is used in the organic acid HPLC method. Take care and always handle in the hood.
- Methanol is toxic and is used in the alginate HPLC method. Always pour in a hood and be careful not to spill the solvent on yourself. All solvent bottles need to be carried with two hands and never to be carried solely with the handle at the top of the bottle.
- Handle the E. *coli* BAL966 with care and make sure to dispose of all items that come into contact with the E. *coli* into the biohazard waste.

E. Interferences

- A glucose peak is seen on the HPLC in the master mix for the first 24 hour period. Make sure to include a blank in this procedure to subtract out the area of glucose from the master mix.

F. EQUIPMENT, SUPPLIES and REAGENTS

Drying equipment

- Drying Oven at 105C - Fisher Scientific Isotemp oven
- Analytical Balance - Denver Instrument
- Descicator
- Descicant – Drierite Anhydrous indicating drierite VWR 23005
- Descicant – Drierite Anhydrous Calcium Sulfate VWR 13005

Bead Beating supplies

- Homogenizer – Bertin Technologies precellys 24 lysis homogenization
- Blender - Oster 12 speed all metal drive

- 7mL Hard tissues homogenizing mix CK28 (vial with cap and beads) – Precellys 24 lysing kit 03961-1-302
- stainless steel 2” Diameter filter (Tea & Herb Ball)
- 20mL Scintillation vials with cap- VWR 66022-106

Master mix making equipment step 1

- M9 media – Anresco M9 media broth powder J863
- Hydrochloric acid – VWR 7647-01-0 concentrated
- Sigma alginate lyase – Sigma A1603-100mg
- DI Water
- CTec2- NS-22086
- HTec – NS-22083
- 37C Shaking incubator
- 4C refrigerator
- Sodium azide solution – Sigma 71290-100G
- EDTA- Sigma E6635-500G
- stir bar
- 0.22 micron filter
- Dispenser, VWR - 40000-066
- Pipet 6-channel Adjustable 100-1200uL, Rainin, - LA6-1200XLS
- 15 mL centrifuge tubes- VWR 89004-368
- Milty - Zerostat Anti-Static Gun - ZESTAT

Master mix making equipment step 2

- Alginic acid sodium salt from brown algae low viscosity – A2158-250G Sigma
- BAL966 pellet
- Bug buster buffer Protein extraction reagent – 70584-3
- Lysonase – 71230 Novagen
- Protease inhibitor cocktail – P8215 5 ml sigma
- M9 media - Anresco M9 media broth powder 2011c326
- Sodium hydroxide – Fisher 131073-2 10N solution
- 0.2 um filter
- Sodium azide solution - Sigma 71290-100G
- Sigma alginate lyase - Sigma A1603-100mg
- Ammonium Chloride - AX1270-1 EMD
- 7.4 1M phosphate buffer- sigma P3619-1GA
- DI water
- Table top centrifuge
- ice bucket/ice
- Reagent reservoirs VWR cat# 82026-352
- 2.0 ml 96 well deep plate Axygen scientific VWR cat# 10011-944
- Corning Cluster tubes 1.2 ml Polypropylene Sigma Aldrich cat# CLS4401-960EA
- Cluster tubes, 8-Cap strips, polyethylene Sigma Aldrich cat# CLS4418

HPLC equipment

- HPLC system equipped with RI/UV detector
- Multiscreen filter plate, Milipore - MSGVN2210
- Platemax film, Platemx - PCR-SP
- Nunc® MicroWell™ 96-Well Plates, Polypropylene, Thermo Scientific, VWR- 46600-666
- Nunc® Caps for 96-Well Polypropylene Plates, Thermo Scientific, VWR - 12777-962
- HPLC grade methanol – VWRAA22909-M6
- HPLC grade water- VWR JT4218-3
- Trifluoroacetic acid – Acros 13972-1000
- Sulfuric acid – Sigma 320501-500 ml
- Vacuum pump
- Stir bar
- Hood

If you can't use the 96 well plate HPLC rack the below vials can be used

- Sample vials and caps: 12x32mm/2mL clear glass vials screw cap with Silicone/PTFE cap

F. Procedures

1.0 For Dried samples received from Chile

- Samples are shipped from Chile in wax paper bags- these can be dried overnight before processing in an oven at 105˚C
- Transfer about 500mg (does not need to be exact) of dried sample to bead beating vial, cap the bottle then place the sample vial in the homogenizer
- Set the homogenizer speed at 6000 rpm for 60 seconds, pause time at 5 seconds, 1X.
- Remove the sample vial, use the micro-spatula to unpack sample at the bottom end of the bottle and repeat step above at 6000 rpm for 60 seconds, pause time at 5 seconds, 1X. Repeat step if needed until large particles totally milled.
- Filter out the beads using stainless steel 2" Diameter filter (Tea & Herb Ball) into a weigh boat.
- Place the sample in a scintillation vial and dry the sample at 105^{O}C overnight. Let the sample cool in a desiccator for about 20 minutes before weighing out sample. See section 3.0 for weighing protocol.

  If no bead beater is available:
- Aliqot 10-25 grams of macroalgae powder and grind sample in analytical mill for 30 seconds, or until larger particles have been pulverized.
- Filter the seaweed using stainless steel 2" Diameter filter (Tea & Herb Ball)
- Re-mill material that was filtered out. Pass through screen again.
- Dry the sample at 105^{O}C overnight and let the sample cool in a desiccator for about 20 minutes before weighing out sample. See section 3.0 for weighing protocol.

2.0 For fresh seaweed samples

- Tare a pan. Place two fresh seaweed fonds in the pan and take the wet weight.
- Place the fronds in an oven at 105C overnight.

- Take the dried weight.
- Place in an oven for another hour and weigh the samples again. If the weight is constant you are done if not repeat this step until a constant weight is reached.
- Break dried macroalgae into small (2"x2") pieces and grind in blender for two cycles, each cycle is about 1-2 minutes
- Follow steps in section 1.0 above
- Dried weight determination:
    - The % solids = (weight of dry sample/weight of wet sample)x100%
    - The % moisture =((weight of wet sample-weight of dry sample)/weight of wet sample)*100%

3.0 Macroalgae sample preparation for Glucan, Mannitol and Alginate analysis

- Dry samples at 105˚C overnight to ensure the samples are dry. Cool it in desiccator at least 20 minutes before weighing. Make sure to weigh out MNOV to use as a QC check.
- Place a falcon tube upright in a beaker on the scale and tare. Weigh sample directly into the 15mL falcon tube. An antistatic gun will be required for accurate sample weighing. For each sample prepare two tubes, one with 50 mg of sample and the other with 75 mg of sample.

4.0 Preparation of Enzymatic degradation buffer

- QC For every set of samples
    - 1 blank sample should be run (buffer with no seaweed). The blank must be used to subtract out glucose from the master mix in all samples.
    - 2 MNOV sample tubes, one with 75 mg and one with 50 mg.
- Enzymatic degradation buffer master mix is prepared according to the following recipe. The recipe gives the volume needed for 10 tubes, multiply the recipe by how many 10's of tubes you are running. Make enough buffer for 10 extra tubes than are being analyzed to ensure you have enough buffer to use the dispenser. (example - multiply the volumes below by 5 if you are running 40 tubes so you have enough volume for 50 tubes)
- Assemble the following ingredients in an Erlenmeyer flask with a stir bar and stir for 10 minutes.
- Measure the pH to ensure it is still at 5.5, if it is not use 1N Hydrochloric acid to adjust the pH.
- Filter the samples through a 0.22 um filter to remove any large particles

Reagent	Volume needed for 10 centrifuge tubes
10X M9 pH 5.5	2.2 mL
Sigma AL 10 mg/mL solution	110 uL
CTec2	22 uL
HTec	5.5 uL
sodium azide 5% solution	1100 uL
DI H2O	104.3625 mL
EDTA	2.2 mL

- Calibrate the volume setting on the dispenser

Dispenser volume calibration:

1. Place a falcon tube in a small beaker and tare both containers
2. Dispense 10mL of water into a falcon tube and record the weight.
3. Repeat step 1. Use a 10ml volumetric pipette, aliquot 10mL of water into a falcon tube and record the weight.
4. The weight difference between 10mL aliquoted by a dispenser and 10 mL aliquoted by a volumetric pipette must be between ± 0.5%. If the difference is out of range, reset the volume and repeat steps 1-4 above.

- Transfer the top dispenser to the buffer bottle. Cap the bottle tight.
- Dispense out 10ml of buffer to a small beaker at least three times to wash off the remaining water in the dispenser.
- Carefully dispense 10ml of enzymatic degradation buffer solution into an empty 15ml falcon tube and use it as a buffer blank solution.
- To each sample tube and MNOV QC tube, carefully dispense 10ml of enzymatic degradation buffer. Dispense the buffer slowly to prevent sample blowing out.
- Cap each sample tube tightly and place it in the rack. Secure the samples in the rack with tape to prevent sample tubes from falling out or leaking sample solution.
- Place sample rack horizontally in the shaker to ensure proper mixing. Incubate the samples at 37˚C with shaking at speed of 200 rpm. Invert to wash particles off the sides as needed. Incubate for 24 hours.
- Centrifuge all sample tubes at 4000rpm for 10 minutes. Reserve sample solutions for glucan and mannitol HPLC and OAL analysis
- Keep sample solutions in the cold room up to a week before use.

Clean and maintenance the dispenser pipette after use

- Transfer the dispenser to a clean DI water bottle.
- Wash the dispenser unit with DI water
- Use Methanol to wash off any remaining residues
- Obtain another clean DI water bottle to wash off the organic solvent.
- Reserve this clean dispenser for next use.

5.1 Preparation of 1% degraded alginate standard stock with 5mM EDTA.

- Dry sodium alginate in an oven at 105˚C until it reaches constant weight.
- Add 2.5 mL of pH 5.5 10X M9 buffer to a 50 mL centrifuge tube and add 25 milliliters of distilled autoclaved water.
- Add 1 gram of dried sodium alginate.
- Add 763 ul of 300mM EDTA.
- Add 50 uL of sigma alginate lyase (10mg/mL).
- Mix then dilute with water to 45mL.
- Incubate at 37C with shaking at 200 rpm overnight. After 12 hours all solids should be dissolved.
- Transfer alginate solution to 100 mL volumetric flask using a funnel. Rinse the 50 milliliter centrifuge tube with 5 milliliters of water 3 times. Dilute the standard with water to the 100mL mark on the volumetric flask.
- Sterilize by vacuum filtration.

6.0 Preparation of OAL buffer

- Enzymatic degradation buffer master mix is prepared according to the following recipe. The recipe gives the volume needed for 10 tubes, multiply the recipe by how many 10's of tubes you are running. Make enough buffer for 10 extra tubes than are being analyzed to ensure you have enough. (example- multiply the volumes below by 5 if you are running 40 tubes so you have enough volume for 50 tubes) OAL crude lysate must be prepared to make the OAL buffer.

OAL crude lysate preparation

- Each 1 ml pellet can be used for 40 tubes. If you have more than 40 prepare more than one pellet of crude lysate.
- Re-suspend 1 pellet in 1mL BugBuster buffer and 20uL protease inhibitor cocktail. Keep resuspension on ice for entire process.
- Add 2 uL of lysonase enzyme preparation and allow cells to lyse for 30 minutes, mix every 10 minutes. Keep pellet and resuspension on ice for entire process. While the cells are lysing the preparation of the OAL buffer can be started.
- Centrifuge the re-suspension at 13,000 rpm for 10 minutes in two 2 milliliter micro-centrifuge tubes. Decant cleared lysate into fresh micro-centrifuge tube to be used in the master mix.

- Assemble the following ingredients in an Erlenmeyer flask with a stir bar and stir for 10 minutes.
- Measure the pH to ensure it is still at 7.5, if it is not use 1N NaOH to adjust the pH.
- Filter the samples through a 0.22 um filter to remove any large particles

Reagent	Volume needed for 10 samples
10X M9 pH 7.2	1.1 mL
Sigma AL 10 mg/mL solution	0.11 mL
OAL crude lysate	0.22 mL
sodium azide 5% solution	0.055 mL
DI H2O	4.015 mL
10% NH4CL	2.2 mL
1M pH 7.5 phosphate buffer	1.1 mL

- Map the sample tube location with 96 plate sample position to ensure each solution is transferred to the 96 plate location correctly.
- Place the number of cluster tubes needed inside a 2.0 ml 96 well deep plate.
- Pour the OAL buffer into reagent reservoir and use 12 channel electronic pipette to transfer 800uL of OAL buffer to each cluster tube.
- Prepare a degraded alginate standard curve from 0.125-1% by serial diluting the stock 1:2 for 5 points.
- Transfer 200 ul of each curve point to the correct cluster tubes.
- Use adjustable 6 channel pipette to transfer 200ul of the centrifuged samples from the 15 ml falcon tubes from section 4 to each cluster tube. Make sure the falcon tubes are in a yellow rack with correct spacing so that you can use the multichannel pipette.
- Cap the cluster tubes and invert them several times, incubate at 37C not shaking for 24 hours.

7.1 HPLC sample solution preparation and methods

- Map the sample tube location with 96- Well plate sample position to ensure each solution is

transferred to the 96 plate location correctly.

- Use a polypropylene Nunc® MicroWell™ 96-Well plate to collect filtered solution.
- Place a multiscreen 0.22um filter plate on the top of 96-Well plate
- Use 6-channel pipette, draw about 280uL from each sample tube and transfer solutions to 96-Well filter plate.
- Cover the plate with Platemax film
- Filter the sample plate by table top centrifuge. Set speed at 4000rmp for 5 minutes
- Cap the sample plate with Pre-slit well Nunc Caps.
- Place sample plate on MTP HPLC tray for HPLC analysis. Only use a system that has been calibrated for a micro-titer plate. (currently Green Arrow or She-Hulk)
- When setting up the HPLC method make sure that in the method under the auto-sampler tab the detect rack button is pressed to ensure the correct rack is being used. Save the method after detecting the rack.
- Two 96 well plates can fit in the 96 well plate rack. The front plate is tray 1 and the back plate is tray 2. The vial number corresponds to the 96 well plate table below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

HPLC Method for Glucan and Mannitol analysis

- Mobile phase: To prepare 5mM H_2SO_4: transfer 1.1 mL of concentrated sulfuric acid to 4L of HPLC water, mix by stirring. Degas by placing a stopper with a hole in it on top of a bottle and connecting to a vacuum pump while stirring the liquid.
- Column: Phenomenex Rezex ROA-Organic Acid 300mm*7.8mm, 8µm particle size part #:00H-0138-kO
- HPLC method: ethanol_30min_lowflow
    - Method Time: 30.00 min
    - Isocratic method
    - Flow rate: 0.6 mL/min
    - Column Oven Temperature: 60C
    - Detector: RID
    - Standard injection volume: 20 uL
- HPLC QC: A sample of glucose and mannitol from a second secondary source are run with each sample set to make sure the system is still within calibration. The procedure to make mannitol and glucose stocks and curves are in Appendix C. A typical chromatogram for this method is included in Appendix A.

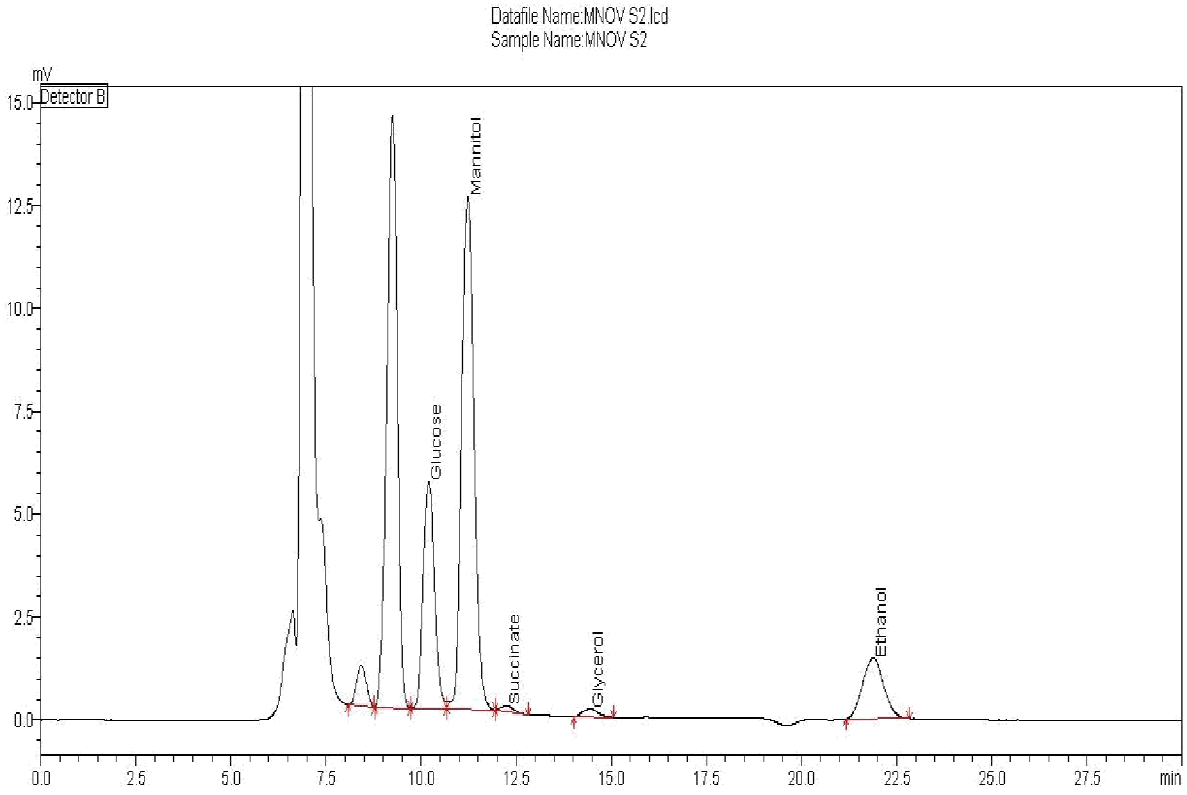
HPLC Methods for Alginate Analysis

- Mobile phase Solvent A: Transfer eight one mL portions of 99% trifluoro acetic acid to a 4L bottle of HPLC grade water with a pipette using a glass pasture pipette stuck to the bottom of the tip. This is done so no plastic leaches into the trifluoroacetic acid. Trifluoroacetic acid is very caustic. Mix by stirring for a minimum of 4 hours. Degas by placing a stopper with a hole in it on top of a bottle and connecting to a vacuum pump while stirring the liquid. Solvent B: Methanol, HPLC grade
- Column: Thermofisher, Hypercarb column 3x30 mm, 3 μm particle size
- HPLC Method: Alginate_30mm
    - Method Time: 8.50 min
    - Gradient: 0 min-30%B, 5.00min-90%B, 5.30-90%B, 5.50-30%B, 8.50 min-stop
    - Flow rate: 1.0 mL/min
    - Mobile phase: A -0.2% Trifluoro acetic acid, B -Methanol
    - Column Oven Temperature: 55C
    - Detector: UV 210 nm, 235 nm
    - Standard injection volume: 1 ul
- Quality Control: Run a curve for every analysis.

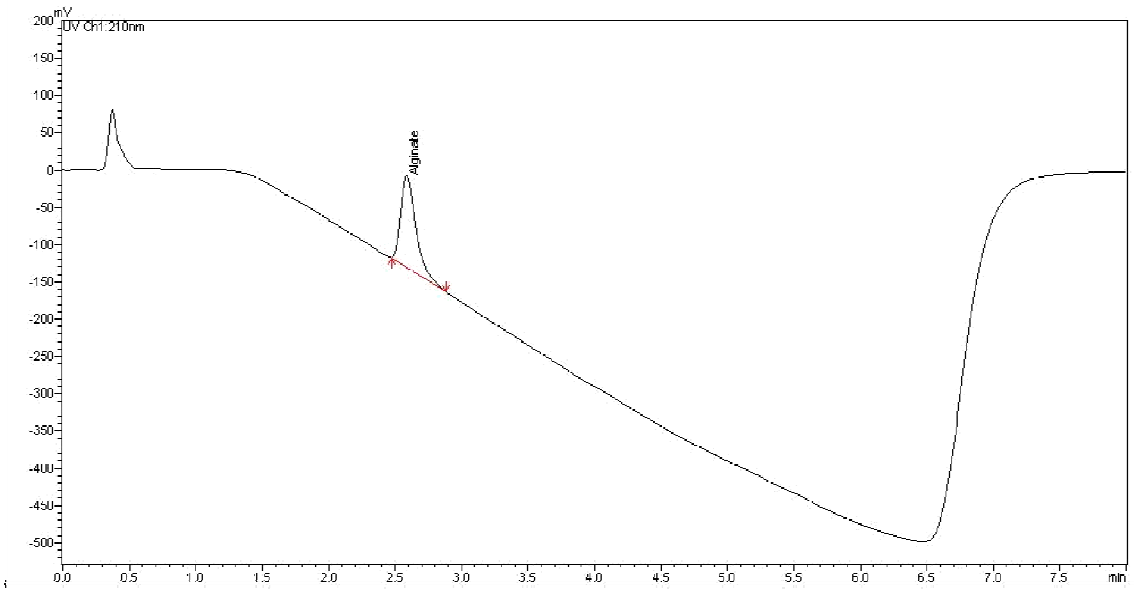
8.0 Calculation of data

To calculate the data use the template for Mag compositional analysis data.

Typical Sugar Sample Chromatogram



Typical Alginate Chromatogram



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SOP making mannitol stock 0.2 weight/volume%

Materials

- A clean 10 milliliter volumetric flask
- Parafilm
- Oven at 105C
- Analytical scale
- Weigh paper
- Aluminum tins
- HPLC grade water

Analytical glassware washing procedure

- Clean analytical glassware with soap and water
- Rinse the glassware 6 times with tap water
- Rinse the glassware 3 times with distilled water
- Let dry

Procedure

1) Place mannitol (D Mannitol puriss Sigma 15719) in a 105˚C oven overnight
2) Place the mannitol in a desicator for at least twenty minutes while the sample cools down before weighing the sample
3) Weigh 0.2 grams of mannitol using the analytical scale using the weighing paper. Record the exact weight.
4) Pour mannitol into the clean 100 milliliter volumetric flask. Be careful not to spill any mannitol because if you do you will have to start over.
5) The solution may have to be heated a bit to get it to go into solution completely. This can be heated on a hot plate with stirring.
6) Mix the solution once all mannitol has dissolved and once it has cooled a bit. Remove the stir bar and rinse the stir bar into the flask.
7) Fill the flask so that the bottom of the meniscus is at the mark with HPLC grade water. Cover the solution with parafilm and mix.
8) Once all dissolved filter the solution through a sterile 0.22 um filter.
9) Store in 4˚C

Making mannitol standard curve

1) From this 0.2% stock of mannitol a curve can be made by serial diluting the highest point. Use a 1ml pipette to deliver 500 ul of stock to a 2 milliliter microcentrifuge tube. Add 500 ul of HPLC water to this tube as well. The graph below will show how many points need to be made.

Curve point	Wt/volume %	
1	0.2	Mannitol Stock
2	0.1	500 uL of 1+500 uL of hplc grade water
3	0.05	500 uL of 2+500 uL of hplc grade water

4	0.025	500 uL of 3+500 uL of hplc grade water
5	0.0125	500 uL of 4+500 uL of hplc grade water

Filter all samples through a 0.2 um filter into hplc vials with inserts in them.
Run on the HPLC using the Organic acid method.

Making a Glucose standard curve

Materials

- Glucose 1 mg/ml stock solution Sigma G6918-100 mL
- water

1) From this 0.1% stock of glucose a curve can be made by serial diluting the highest point. Use a 1ml pipette to deliver 500 ul of stock to a 2 milliliter micro-centrifuge tube. Add 500 ul of HPLC water to this tube as well. The table below will show how many points need to be made.

Curve point	Wt/volume %	
1	0.1	Glucose Stock
2	0.05	500 uL of 1+500 uL of hplc grade water
3	0.025	500 uL of 2+500 uL of hplc grade water
4	0.0125	500 uL of 3+500 uL of hplc grade water

Appendix D- Recipes for Reaction
Reagents 10X M9

- Weigh out 105 g of M9 media
- Dissolve in distilled water to a final volume of 1L
- Adjust the pH to 5.5 using 1N Hydrochloric acid or
- Adjust the pH to 7.5 using 1N sodium hydroxide
- The solution may either be autoclaved for 20 minutes at 121^{o}C or filtered through a 0.22 um filter to sterilize the solution.

Sigma alginate lyase 10mg/ml

- Perform this procedure in the presence of a flame to ensure sterility
- Sigma alginate lyase comes in 100 mg portions from Sigma Aldrich
- Measure out 10 milliliters of autoclaved distilled water using a pipette and pour water directly into the bottle with 10 milligrams of alginate lyase.
- Let sit in water for a couple of minutes and mix thoroughly.
- Pour this into a 15 milliliter falcon tube making sure that no powder is left on the sides of the bottle. You can pour back and forth between the falcon tube and the Sigma Aldrich bottle until all powder is removed from the Sigma Aldrich bottle.

- Always wear gloves, safety goggles and a lab coat.
- Store at 4^{O}C for up to 3 months.

300 mM EDTA solution

- Weigh 11.1687 g of EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate) into 100 ml of autoclaved water. Stir for 10 minutes then adjust the pH to 8 with sodium hydroxide (undissolved EDTA will be dissolved after this adjustment) then stir additional 60 min or more until the powder is completely dissolved.

10% Ammonium Chloride

- Weigh out 10 grams of ammonium chloride and dissolve in water to a total volume of 100 ml.
- Sterilize the solution by filtration through a 0.2 um filter.

Preparation of 1% degraded alginate standards with 5mM EDTA.

- Dry sodium alginate in an oven at 105^{O}C until it reaches constant weight, overnight will suffice.
- Place the alginate in a desiccator for at least twenty minutes while the sample cools before weighing out the alginate
- Add 2.5 mL of pH 5.5 10X M9 buffer to a 50 mL centrifuge tube and add 25 milliliters of distilled autoclaved water.
- Add 1 gram of dried sodium alginate.
- Add 763 ul of 300mM EDTA.
- 1 mL of 5% sodium azide solution
- Add 50 uL of sigma alginate lyase (10mg/mL).
- Mix then dilute with water to 45mL.
- Incubate at 37^{O}C with shaking at 200 rpm overnight. After 12 hours all solids should be dissolved.
- Transfer alginate solution to 100 mL volumetric flask using a funnel. Rinse the 50 milliliter centrifuge tube with 5 milliliters of water 3 times. Dilute with water to 100mL mark on volumetric flask.
- Sterilize by vacuum filtration through a 0.22 um filter.