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Methods for quantification and extraction of fucoidan, and quantification of the release of total carbohydrate and fucoidan from the brown algae Laminaria hyperborea

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Summary

Fucoidan is a sulphated heteropolysaccharide with muglicious nature. It has recently gained much attention because of its bioactive properties. The first objective of this research was to evaluate methods for qualitative determination of fucoidan. As laminaran is co extracted with fucoidan when using acid for extraction, main focus was to determine, to what extent glucose would interfere with the overall quantification for fucose. Two methods were chosen, the Dische method and an enzymatic method. The Dische method was cheap and rapid but it showed a limitation for quantitatively detecting fucose. The method worked precisely if the glucose concentration is 5 times or less, than the concentration of fucose within the samples. The enzymatic method was more sensitive, specific and reliable. The method worked efficiently even if glucose concentration was 40 times higher than fucose. The second objective was to optimize a method for extraction of fucoidan from *L. hyperborean* to obtain a quantitative yield of fucoidan. Different acid concentrations and extraction times were tested and the conditions were optimized for extraction with 0.1 molar HCl at 80^{o}C for 2 hours. The third objective was to determine the release of fucoidan from whole live algae with damaged parts of the same algae i.e. old leaf, new leaf and stem. *L. hyperborea* were harvested form Storsteinan (Trondheim) at the end of February 2015 and analyzed for the content of fucoidan. Whole algae of *L.hyperborea* submerged in distilled water and seawater in darkness and at 4^{o}C were found to release very low amounts. The release of total carbohydrate from plants in seawater was determined to increase with time, with a maximum of about 100 mg/kg wet weight of the algae. The release of fucoidan from the same whole algae was very low, less than what could be reliably detected with the method (< 2 mg fucose /kg wet weight of algae). For the corresponding experiment with whole plants in distilled water, the total carbohydrate content could not be reliably determined. The release of fucose from whole plants in distilled water was similar to in seawater. For the release of total carbohydrate from old leaf in distilled water could not be determined reliably, while the release of fucose from old leaf was found to increase up to about 400 mg/kg wet weight of algae. For the corresponding experiment with old leaf in seawater, both the total carbohydrate and fucose was found to increase about 400 mg/kg wet weight, i.e. almost all carbohydrate released is fucoidan. For the release of total carbohydrate from new leaf in both distilled water and seawater were found to increase to up to around 2500 mg/kg wet weight. The

release of fucose from new leaf both in fresh water and seawater were found to increase up to around 120-140 mg/kg wet weight of algal i.e. very much lower that the total carbohydrate content.

For the release of total carbohydrate and fucose from the stem in distilled water, the total carbohydrate was found to increase up to around 200 mg/kg wet weight of algae, while fucose increased up to 50-100 mg/kg wet weight of algae, i.e. about half of the carbohydrate released from the stem is fucoidan. Release of total carbohydrate from stem in seawater could not be reliable determined. The release of fucose from stem in seawater was found to increase to about 50 mg/kg wet weight of algae, was found to be not very different from the release of fucose from stem in distilled water. Overall, the release of carbohydrate and fucoidan from whole plants both in distilled water and seawater was very much lower (on the level of few percent), than the release from damaged parts of the plants. No large differences between the release from plants / plants in distilled water as compared to seawater could be found. Interestingly, the release of fucoidan from the individual parts of the plant was quite different, where old leaf released almost exclusively fucoidan, where new leaf released quite low amounts (around 10 %) and stem releasing about half of the total carbohydrates as fucoidan. Whole plant in distilled water, Old leaf in distilled water and stem is sea water could not be determined reliable for total carbohydrate content and gave a higher absorbance at 269 nm which is in the rang for the poly phenols.

The hypothesis if fucoidan is produced by the plant in respond to damage could neither be proved nor disproved. Several dried algae samples were showing an increased content of fucoidan after the extraction but this also could have been caused by a faster release of other compounds e.g. laminaran, proteins, salts and phenols, resulting in a counterfeit increase of the fucoidan amount. The determined change in content was too small to answer that question precisely. So it will remain a topic of further research.

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1. Introduction

Recently, concerns have been shifted towards the use of renewable natural resources. This shift diverted the focus from terrestrial resources towards the study of marine resources. Among the reserves of huge marine resources lay the seaweeds along the edges of the oceans. Seaweeds are multicellular, macroscopic, marine algae which utilizes sunlight as an energy source to convert carbon dioxide and water into carbohydrates. They fall into the diverse category of photosynthetic organisms which have evolved into around 36,000 know species. They are classified according to their ecology, habitat, size, pigments, polysaccharides, cell culture, composition and morphology. The seaweeds are further grouped into three categories; red seaweed (Rhodophyta) with 6500 known species, brown seaweed (Phaeophyta) with 1800 known species and green seaweed (Chlorophyta) with approximately 1500 known species. They are being utilized as food, fertilizers and phycocolloids throughout the world.

Brown seaweeds prefer growing in cold water. They are able to grow under water depths of around 30-50 meters but they are mostly present in the intertidal and upper sublittoral zones of the oceans. Brown seaweeds generally absorbs green light of medium wavelengths to perform their photosynthesis. They also have a very high productivity rate. It has been estimated that the productivity of the non-cultivated brown algae can reach up to the range between 3.3 – 11.3 kg dry weight m^{-2} $year^{-1}$ (Gao and McKinley 1994) .

Norway, however, have big resources of brown seaweeds. The Norwegian coastlines runs over 100 000 km (Norwegian Mapping Authority, 2011) including fjords and islands, which provides suitable habitat and conditions for the growth of these marine algae (Ragan and Jensen 1979). Among the marine algal species found in Norway, *L. hyperborea* and *Ascophyllum nodosum* have gained more importance. Both of these algae are commercially harvested by FMC Biopolymer for their industrial applications in pharmaceutical, cosmetics, agriculture, nutraceutical and most importantly alginate production. The alginate industry of Norway is one of the most important sectors in Norway for consuming the vast majority of these seaweeds. Besides the alginates, *L. hyperborean* also contains other important polysaccharides namely a bioactive polysaccharide “fucoidan”. Fucoidan has recently gained large attention because of its huge applications in health sector. It has been reported that

fucoidan has anticancer, anticoagulant, antioxidant and antiviral properties (Li, Lu et al. 2008).

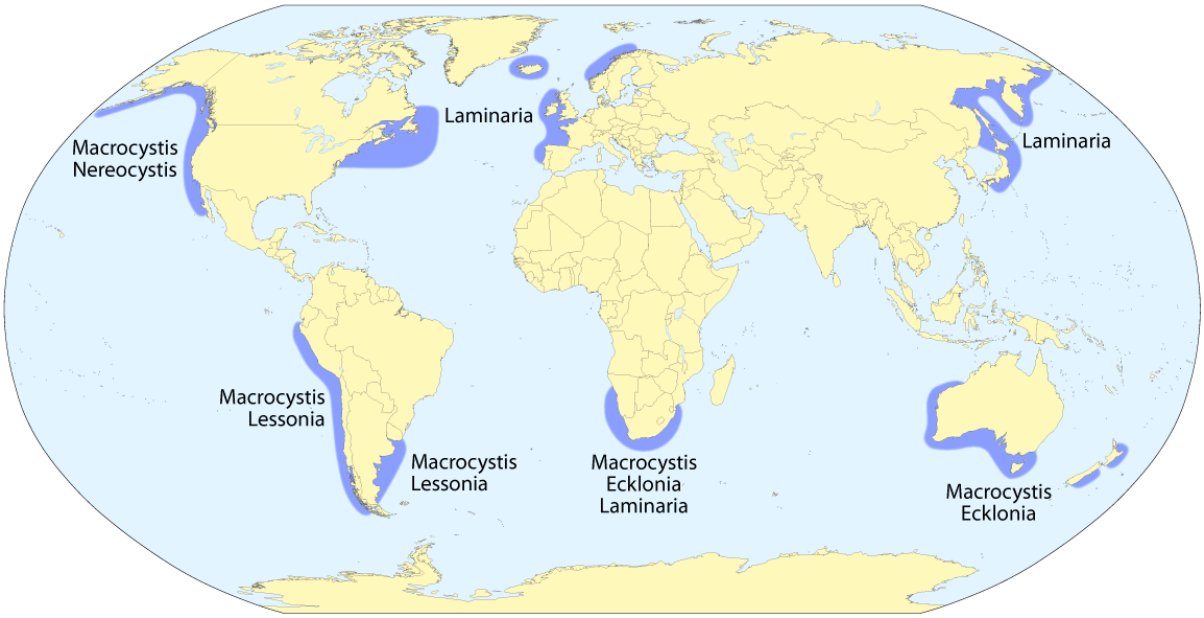

Figure 1: Kelp Forest Distribution of Brown Seaweeds (Woodward 2008)

Brown seaweeds are found along the coastal regions of the world. Laminaria species are more common in the northern hemisphere but are also present along the southern coast of Africa. The Macrosystis is the most common brown algae. It is found in the southern hemisphere and western coast of America. Other species of brown algae are present along the coastal regions of South America, Africa, parts of Australia New Zealand and western American coast.

1.1 *Laminaria hyperborea*

The *Laminaria hyperborea* belongs to the family of *Laminariaceae.* It has a high marine ecological importance. The alga has a high biomass production, greater dominance and has an important ecological distribution. The kelp forest of *L. hyperborea* in the ocean provides food and shelter for thousands of fish, invertebrates and marine mammal species. *Lamminaria hyperborea* is widely distributed in the coastal regions of northeast Atlantic, spreading from the coastal regions of Portugal in south towards the Russian coasts in the north with the optimal growth conditions at the latitude of the southern Norway (Kain 1967). The mature plant of *L. hyperborea* is well developed and distinctive. It consists of a long stem, which develops into leaves and a holdfast at the bottom through which the algae anchors to a solid surface at the bottom of the ocean. The stem is strong and erect but it is also flexible which allows the algae to sway in the current of the oceans. The stem is about 1-3 meters long and support the weight of the leaves. The stem develops into leaf at a transition zone. The leaf is smooth, flat and is divided into several fingers like segments. The morphology of the algae depends upon the habitat and the water movements. In the regions with high water currents, the algae have well developed holdfast with straight and more flexible stem. The leaves become more fan shaped and are divide into more segments.

1.1.1 Growth cycle

The first growing season starts in November when the transition zone between the stem and the leaf starts to swell and become paler. This swelling region shapes into a disc like structure, merging from the stem and below the old leaf. This transition region is called collar. A new leaf than emerges and expands progressively. The new leaf remains paler then the old leaf. By the beginning of March, longitudinal sections appear and split into fingers like segments, emerging from the collar. In April, the new leaf reaches the size of the old leaf and in the end of July the growth of new leaf is complete. The separation of old leaf from the new leaf depends upon external factors such as water movement, depth, availability of shelters, and storm or strong winds. However, between March and May, the new leaf is completely separated from the old leaf. During the growth of new leaf, the old leaf act as the storage part of for the algae, especially during long winter when there is less exposure to sunlight (Kain and Jones 1971).

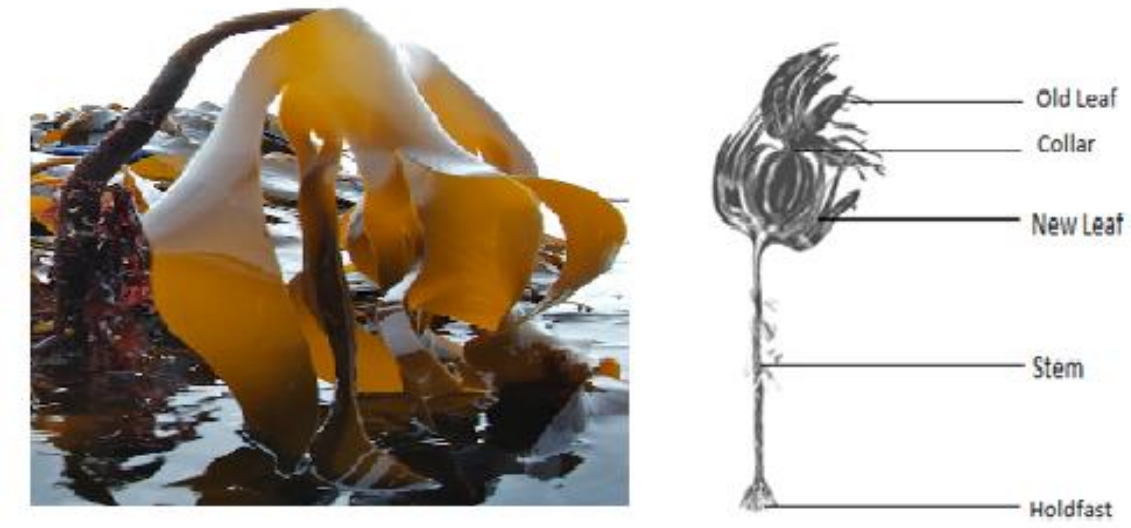

Figure 2: Laminaria hyperboria plant

1.1.2 Habitat

Laminaria hyperborea are found attached to the solid surface of the ocean under cool temperatures (below 15^{o}C) in the sublittoral zones. It can also grow on solid rocks, large pebbles, gravels and on man-made structures present in the oceans. The algae are also sometimes found in the areas of siltation, but are not found at the sandy coasts. *L. hyperborea* grows at depths ranging from 8 meters in turbid coasts to sometimes at depths of around 30 or 40 meters in clear water coasts. The depths at which it grows, mainly depends upon the light penetration and its availability to the algae. *L.hyperborea* develops into a dense forest under suitable conditions. *L. hyperborea* has found to grow under deep and moderately strong water currents, but they are unable to survive in extreme wave conditions. Figure 2 below shows the zonal distribution of different brown algae present along the Norwegian coast. *L. hyperborea* grow under the mean low water currents.

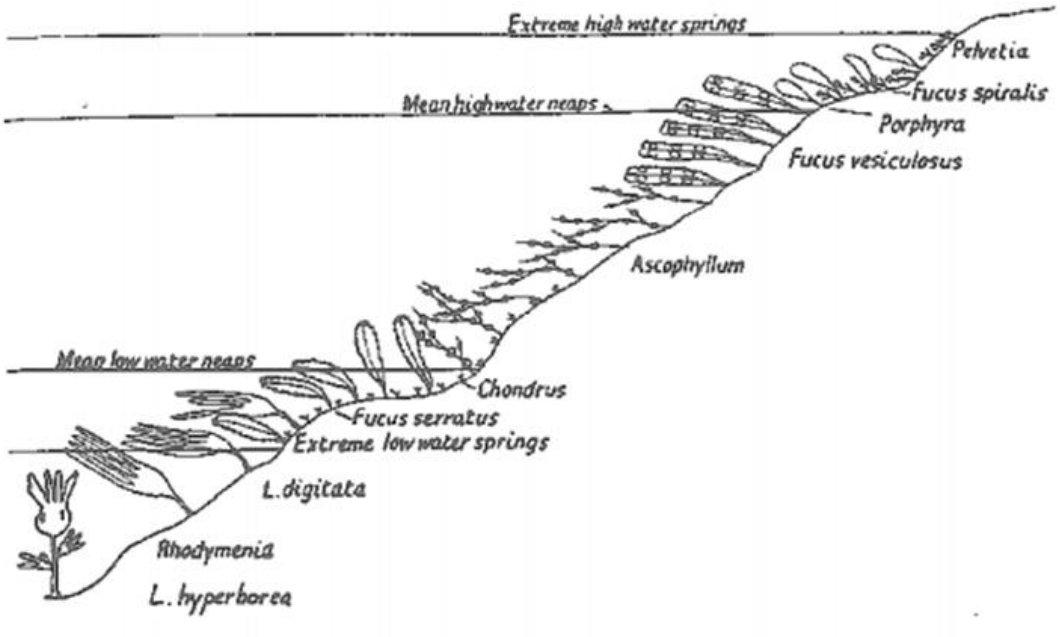

Figure 3: Zonation of different seaweed species at the norwegian coast (Nagaoka, Shibata et al. 1999)

1.1.3 Norwegian recourses

In Norway, the seaweeds are consumed from the start as a source of food and fertilizers. Since 1963, *L. hyperborea* has gain industrial importance and became one of the most important raw materials due to its accessibility for harvesting and high biomass. *L. hyperborea* is now commercially harvested by FMC for the production of alginates. It is harvested between Rogaland and Sør-Trondeleg. The annual landings are found to be 150,000 tons and the first hand value is approximately 23 Euro/ton wet weight (FMC Biopolymer AS 2011, pers.comm). *L. hyperborea* is processed for alginate that are used in pharmaceutical, cosmetics and nutraceutical products.

Figure 4: Area of harvest and processing of Laminaria hyperborea and Ascophyllum nodosum in Norway (Meland and Rebours 2012)

1.1.4 Chemical composition

L. hyperborea is composed of more carbohydrate and mineral contents compared to other species of the same genera (Kain and Jones 1971). The total carbohydrate and mineral contents of *L.hyperborea* varies; both for individual algae and algae from different habitats. *L. hyperborea* have also been reported to show seasonal variations in their chemical compositions (Black 1954). The figure 5 below shows the seasonal variation of stem and leaf of the alga. It can be noted that the seasonal variations in leaf are more significant compared to stem.

Component	Stipe [%]	Frond [%]
Water	77 – 89	84-87
Ash	34.5 ± 2.5	16-37
Alginic acid	33.4 ± 2.8	17-34
Laminaran	0.68 ± 0.28	0-30
Mannitol	5.9 ± 2.4	4-25
Fucoidan	2 – 4	
Other carbohydrates	Traces	
Protein	8.9 ±1.6	4-14
Fat	0.63 ± 0.14	
Fibre (cellulose)	10.4 ± 0.8	
Polyphenols	1 (Stipe) 0.3 (Peeled stipe) 5.3 (Peripheral tissue)	
Iodine	0.74 ± 0.11	
K	6.3 - 11.0	
Na	1.6 - 3.0	
Ca	1.4 - 3.0	
Mg	0.6 - 0.7	
S	1.2 -1.3	
P	0.2	

Figure 5: Chemical composition and seasonal variations for L.hyperborea. (Horn 2000).

Mannitol is a sugar alcohol synthesized as the photosynthetic product and could be the main respiratory product in brown seaweeds (Hahn, Lang et al. 2012).It functions as a storage carbohydrate in the algae. There is a significant seasonal variation in the total percentage per dry weight, of mannitol. In the leaf it varies as high as 25 % in growth seasons and declines to around 4 % in the winter (Ragan and Jensen 1979) when the stored carbohydrates are being utilized for respiration. The seasonal variation on mannitol in stem is distinctive but a little variation is observed throughout the year.

Laminaran is a polydisperse β (1→3) D glucan which functions as the storage carbohydrate for the algae. Laminaran also contains some degree of branching which affects the solubility

of the polysaccharide in water. Laminaran containing only β-(1→3)-linked residues are water insoluble and laminaran containg β-(1→6)-linked branching tends to be water soluble. The seasonal variation in laminaran is significant. A large degree of seasonal variation is seen in the leaf which ranges from 30 % during the growth season to almost 0 % during October/ November (Ragan and Jensen 1979).

Alginic acid is composed of linear β-D-mannuronate (M) and α-L-guluronate (G) monomers which are glycosidically linked by (1-4) linkages. The alginate molecules are composed of sequences of M-units (M-blocks), sequences of G-units (G blocks) and sequences of (alternating) M and G units (MG-blocks). The polymer is mainly present in the intercellular spaces and on cell walls of the algae. The stem shows a higher alginate content of about 33.8 % throughout the year compared to the leaf. In algae, the alginate accumulates and binds to divalent metal ions to form gels. The gel functions as the structural element for the algae.

Fucoidan is a sulphated L-fucose biopolymer with high structural integrity. The polysaccharide may function to provide protection to the algae against desiccation, because of its highly hygroscopic nature. The overall content of fucoidan was found to be 2-4 % per dry weight of algae (Ragan and Jensen 1979). A higher content fucoidan was found in the leaf compared to the stem and it showed a seasonal variation compared to mannitol (Black 1954).

The new leaf and the old leaf were analyzed separately for the total mannitol and laminaran contents. Their content in the old leaf decline steadily and reaches almost to 0 % between February and June while content of laminaran and mannitol in the new leaf remained steady and increased rapidly after June (Haug and Jensen, 1954; Black 1954).

2.1 Fucoidan

Fucoidan was first extracted in 1913 from the species of brown seaweeds namely; Laminaria digitata, Ascophyllum nodosum and Fucus vesiculosus (Li et al., 2008; Percival and McDowell, 1967). Fucoidan is negatively charged highly hygroscopic polysaccharide with mucilaginous nature (Percival and McDowell, 1967).A high content of fucoidan is found in the leaves of Laminaria digitata, Ascophyllum nodosum, Macrocystis pyrifera and Fucus vesiculosus (Percival and Ross 1950). Fucoidan is soluble in both water and acid solutions (Nagaoka, Shibata et al. 2000).

After the first publication in 1913 the number of published articles on fucoidan has increased dramatically. Figure 6 shows the trend of published articles on fucoidan, and their sudden increase in the recent years. Fucoidan became the center of attraction because of its antitumor, anti-coagulant and anti-oxidant activities and as well as its impotance for the regulation of body glucose and cholesterol levels. It has also been reported that fucoidan provides protection against liver damage and urinary system failures (Choi, Raghavendran et al. 2010). As more and more research is being carried out on fucoidan, more of its biologically and health related benefits are being discovered (Li, LU et al. 2006)

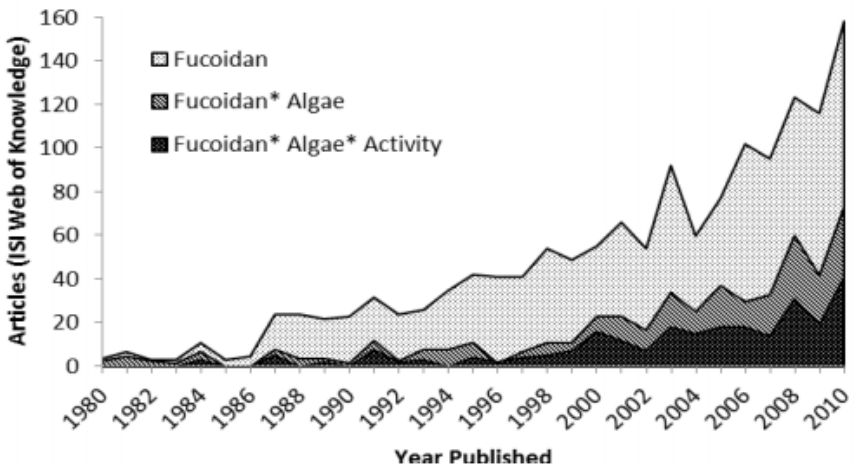

Figure 6: Number of published articles on fucoidan-related work from 1980-2010. Numbers were recorded using the ISI Web of Knowledge search engine with the following keywords: Fucoidan; Fucoidan*Algae; Fucoidan*Algae*Activity (Ale et al., 2011).

2.2 Sources

Sulphated polysaccharides can be found among many marine sources including sea cucumber (Eluvakkal, Sivakumar et al. 2010), urchin or brown algae (Sieburth and Jensen 1970).In the recent years many algae and different invertebrates have been identified for their fucoidan contents including Fucus vesiculosus ,Sargassum stenophyllum ,Chorda filum , Ascophyllum nodosum Cladosiphon okamuranus, Dictyota menstrualis, Fucus evanescens, Fucus serratus , Fucus distichus, Caulerpa racemosa, Hizikia fusiforme, Padina gymnospora, Kjellmaniella crassifolia, Analipus japonicus (Li, Lu et al. 2008) and laminaria hyperborea (Black 1954). All of these sources contain different types of fucoidan and different extractions methods have to be employed to extract high yields of fucoidan from these sources.
Figure 7 below shows the algae which contain fucoidan.

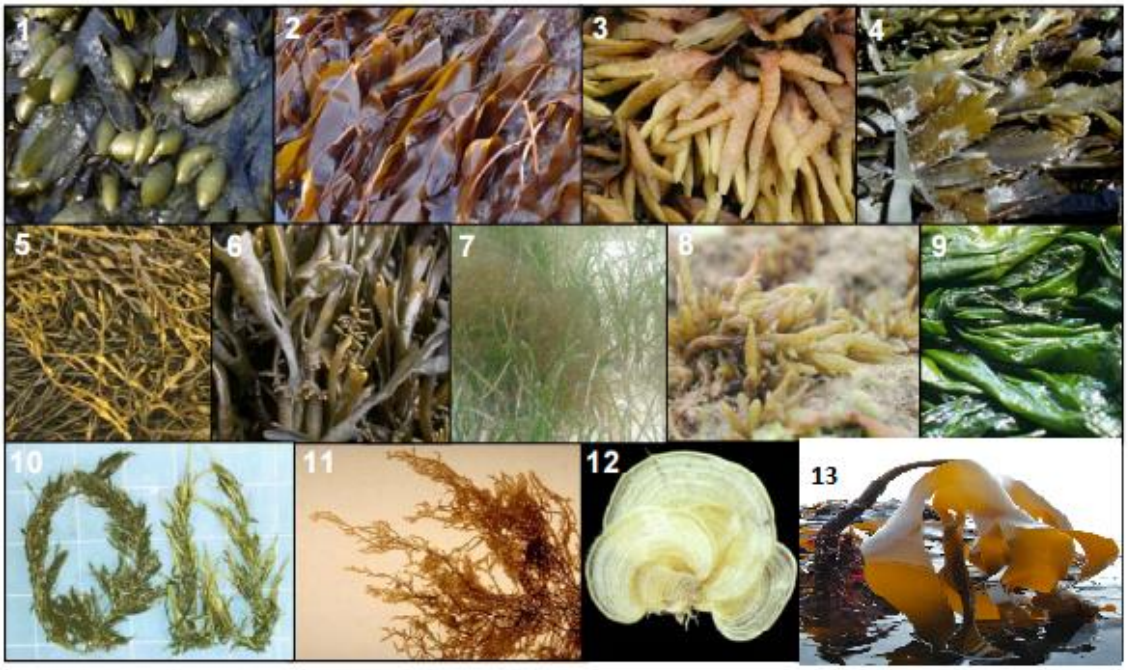

Figure 7: Sources of fucoidan: 1: Fucus vesiculosus, 2: Laminaria digitata, 3: Fucus evanescens, 4: Fucus serratus, 5: Ascophyllum nodosum, 6: Pelvetia canaliculata, 7: Cladosiphon okamuranus, 8: Hizikia fusiforme, 9: Laminaria japonica, 10: Sargassum horneri, 11: Nemacystus decipiens, 12: Padina gymnospora, 13: Laminaria hyperborea (modified from Holtkamp 2009)

2.3 Structure of fucoidan

Fucoidan is a naturally occurring L-fucose sulphated polysaccharides which was first extracted from the brown algae in 1913 (Kylin, 1913). A diverse composition for fucoidan has been reported, depending on its source and season (Black 1954). The main difference of the fucoidan structure originates from their source, fucoidan from invertebrates show a linear sulphated monosachaaride backbone whereas algal fucoidans may be branched in various ways. The first structural suggestion for fucoidan was proposed by Percival and Ross for fucoidan from Fucus vesiculosus in 1950 ,from Fucus vesiculosus (Percival and Ross 1950). Later in 1993, Patankar successfully proposed the structure and describes it as polysaccharide consisting mainly of α-1,3-L-fucose (Patankar, Oehninger et al. 1993). From different sourses of fucoidan, the degree of variations can be seen from the attachments of the sulphate groups and the glucosidic linkages. The sulphation may occur at position 2, 3 and 4 and the monosaccharides are associated via α-1,2, α-1,3 or α-1,4 glycosidic bonds. The sulphatation degree differs with the location and season of collection and ranges between 4-8% (Black 1954). The molecular weights of fucoidans depend also on the source from which they are obtained.

Fucoidan is typically found in the cell wall of the brown algae. Although many studies have already been carried out on the structural properties of fucoidan, but still the structure of fucoidan remains uncertain. Besides sulphated fucose monomers, other small monosaccharides (glucose, xylose, mannore , galactose, xylose) and uronic acids and as well as proteins are present in detectable amounts which increases the difficulty for determining the overall structural elucidation of fucoidan.

Figure 8 below shows some of the structure motifs of fucoidan from different species of brown seaweeds.

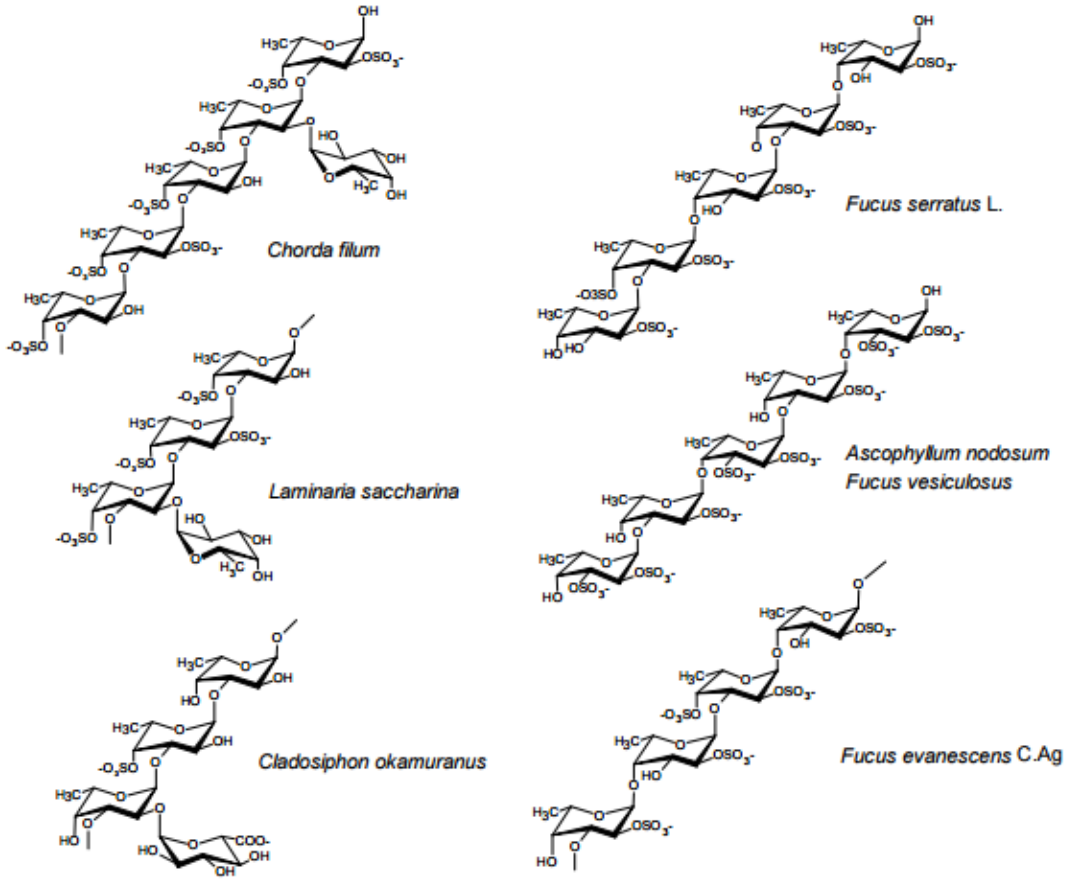

Figure 8: Shows the structural motifs of fucose containing sulfated polysaccharides obtained from different sourses of brown algae of the order Laminariales, Chordariales and Fucales (Ragan and Craigie 1980), (Ragan and Jensen 1979),(Geiselman and McConnell 1981

Many structures, for the order of Laminariales have been reported that contained monosulfate components that mainly consist of α (1-3) linked L-fucopyranose residues with the sulfates present at C-2 and C-4 positions of the fucoidan monomer (Anastyuk, Shevchenko et al. 2009).

2.4 Function in algae

The function of fucoidan in the algae is still debateable as its difficult to extract fucoidan from all species with similar extraction conditions and also its structural composition varies

from specie to specie. However, there have been certain therories proposed as fucoidan content varies from the species in the intertidal zone (higher fucoidan content) and the zone under the low tidal zones (less amount of fucoidan), it can be assumed that fucoidan prevents the algae from desiccation under low tides (Nishino, Yokoyama et al. 1989). Another proposed suggestion is the cell wall stability (Cho, Lee et al. 2010). This could be further supported as the sugar content of algae gradually increases from April to September (Ohigashi, Sakai et al. 1992),during which the algae are exposed to higher amounts of sunlight which contains UV radiations that might destroy the cell constitutes (Holtkamp 2009)

2.5 Extraction of fucoidan

Extraction of biopolymers from cell wall of brown seaweed is complex and challenging. These polysaccharides have a large dependence on the conditions through which they are extracted. The yield and the chemical nature of these polysaccharides show variations when using different extraction conditions (Li, Lu et al., 2008). Various methods have been studied and employed to isolate and preserve high quality fucoidan but still the precise structure of fucoidan is debateable, due to difficulties in extraction and purification of fucoidan (Sieburth and Jensen 1970). Normally, the seaweeds are extracted for crude fucoidan with acid as extraction solvent to avoid release of algnic acid in the crude extracts (Geiselman and McConnell 1981). Extraction with alkanine conditions will generally results in the extracting of alginic acid (Ale, Mikkelsen et al. 2011). Hot water is now frequently been used to extract crude fucoidan from the seaweeds (Li, Lu et al., 2008). Extraction with water maintains the stability and the overall charge of the molecule (Ragan and Craigie 1980). Moreover, it produces good quality fucoidan and helps the molecule to retain its natural bioactivity (McNally, 2007);(Ragan and Craigie 1980). But, extraction of fucoidan with acidic solvents give a better an overall better yields when compare to water (Black et al., 1952);(Ragan and Craigie 1980);(Li et al., 2008).

Extracting fucoidan with weak acids give lower yields of crude fucoidan but preserve the structure of fucoidan, strong acid gives better yields but will also degrade the fucoidan.

Laminaran will be co-extracted with the fucoidan in both cases, but alginic acid will only be co-extracted using strong acid at high temperatures over an extended time, where the alginic acid is severely degraded and thus solubilized.
But may cause degradation of fucose chain (Ragan and Craigie 1980)and may disturb the structural integrity of fucoidan. Using the salts like calcium chloride have been proven effective to remove the insoluble components that could affect the purity of fucoidan (Umeda, Kihara, Ikai, & Kato, 2003) but in turn gives a lower yields of crude fucoidans.

Chemical composition of fucoidan varies with the season, species, habitat and the maturity of the plant (Kain and Jones 1971). The composition will vary between species. The extraction techniques will also have a large impact on the overall purity, yields and final structure of fucoidan. The method used for extracting fucoidan will result in fucoidans that may vary in chemical composition. It is reported that fucoidan extracted at room temperature and at 70^{o}C had a different chemical composition (Irhimeh, Fitton et al. 2005).

2.6 Methods for detection of fucoidan

Fucoidan has a complex structural integrity and therefore, by far, there is no commercially available method that can directly quantify the amount of fucoidan from a crude extract. Work is been done to commercialize certain fucoidanases (EC number 3.2.1.44) which can hydrolyze fucoidan but yet there is no commercially available endo-fucoidanases available (Chevolot et al., 1999). Recently, the search for new drugs has raised increased interest in fucoidans and new methods are now in developmental phase for detection of fucoidans (Irhimeh, Fitton et al. 2005).

2.7 Health benefits of fucoidan

Fucoidan is bioactive molecule present in the brown seaweeds. As fucoidan is a naturally product, its side effects are not as adverse as chemically engineered drugs. There have been some side effects reported but it depends on the type of seaweed from which the fucoidan is

extracted, there can be certain side effects related to a particular kind of seaweed (chung et al., 2010).

Fucoidan is known to exhibit a large verity of biological activities including anticancer, antioxidant, antiviral and anti-oxidant activities (Li, LU et al. 2006). Many researches have targeted the antiviral, antioxidant, anticoagulant and anti-cancer properties of fucoidan. But, still the effectiveness of these biological properties are related to the composition of fucoidan. As mentioned earlier, the structural complexity of fucoidan depends upon its source and extraction method, each type of fucoidan that may possess unique structural features would have varied bioactivity and could potentially come up as a new drug (Eluvakkal, Sivakumar et al. 2010).

The complex structure and complexity in fucoidan composition is extensively studied but still the actual arrangement of fucoidan structure is still not clear. As a reason to that might be that the fucoidan is difficult to extract in its pure form. Crude fucoidan is a heteropolysaccharide made up of complex mixtures of fucose, sulphates and uronic acid. To a high sulphate content, low uronic acid contents have been reported (Sieburth and Jensen 1970).

2.7.1 Anticoagulant activity

The anticoagulant activity of fucoidan is well studied. It has been reported that the sulphate content , sugar composition and the molecular weight might be related to its anticoagulant activity (Li, Lu et al. 2008). In general the trend is seen with the sulphate content. Higher the sulphate, higher the anticoagulant activity. Conversely, the anticoagulant activity started decreasing with higher amount of sulphatation. This is done by chemically over sulphating the natural fucoidan and the anticoagulant activity of fuoidan increased to a certain level after which it started decreasing (Li, Lu et al. 2008).

Molecular weight of fucoidan was closely related to the anticoagulant activity of fucoidan. Fucoidan requires a sugar chain long enough to bind to thrombin, so a minimum threshold molecular weight of fucoidan is required for the anticoagulant activity of fucoidan. A high molecular weight fucoidan (320,000 Da) was extracted from *Lessonia vadosa* which showed a high anticoagulant activity and a smaller molecular weight fucoidan (32,000) showed a low anticoagulant activity (Chandía and Matsuhiro 2008).

The anticoagulant activity of fucoidan has also been reported for the sugar composition of fucoidan (Nishino, Yokoyama et al. 1989). But it has been reported that it is not the sugar composition but the sulphate groups attach to the sugar that regulates the anticoagulant activity of fucoidan (Li, Lu et al. 2008). Uronic acid doesnot directly involes in the anticoagulant activity of fucoidan but could rather provide flexibility to the sugar chain for enhancing the anticoagulant activity of fucoidan.

2.7.2 Anticancer activity

Recently, interest have been shifted towards the study of anticancer properties of fucoidan. Fucoidan showed a cytotoxic effect against CCL39 cancer cells (Cho, Lee et al. 2010). Ohigashi et al., (1992) showed a selective destruction of lymphoblastoids cancer cells with the incubation of 72 hours without damaging the normal cells (Ohigashi, Sakai et al. 1992). For anticancer activity of fucoidan, it shows no side effects as in chemotherapy treatments (Tachikawa). It was also reported that fucoidan used three different pathways for the destruction of cancer cells: immunity boost, induced apoptosis and preventing the process of angiogenesis which prevents the supply of nutrients to the cancer cells.

A further study on anticancer activity of fucoidan was done by comparing the breast cancer rates in Japan where more seaweed is consumed compared to other developing nations (Teas, Zhang et al. 2006). The results showed that United states, Australia and parts of Europe had high rates of breast cancer compared to low rates of breast cancers in Asian countries particularly where the seaweed is relatively more consumption. Teas et al. (2006) also performed a study on the consumption of miso soup in relation to the breast cancer and the results showed that women who consumed more than six times a week had half the risk of developing breast cancer. They concluded that the fucoidan present in the brown seaweed inhibits the growth of cancer cells (Teas, Zhang et al. 2006).

2.7.3 Anti HIV activity

Fucoidan has also been reported to have anti HIV activity. The HIV virus (human Immunodeficiency virus) is responsible for the AIDS syndrome (acquired immune deficiency) in human. It is proposed that fucoidan inhibits HIV by acting as the competitive inhibitor between HIV reverse transcriptase and the nucleic acid substrate (Moen and Clark 1993);(Schaeffer and Krylov 2000). But still the anti HIV activity of fucoidan is debatable and research is being carried out (Béress, Wassermann et al. 1993) (Moen and Clark 1993).

2.7.4 Antioxidant activity

Fucoidan had been reported to have an antioxidant activity (Wang, Zhang et al. 2008). Fucoidan is a natural antioxidant and has the potential to avoid or delaying a free radical-mediated illness (Li, Lu et al. 2008). Reactive oxygen species are being continuously produced in our body through metabolic and environmental sources. Our body has a natural defense system against ROS but it is not enough to repair the damage completely. The commonly used antioxidants such as butylated hydroxyanisol (BHA) and butylated hydroxytoulene (BHT) have been restricted by the kegislations as they produce harmful side effects and carcinogens. As a result the interest has been shifted towards the use of natural antioxidants which have no harmful effects on human and produces no carcinogens. Fucoidan, with its antioxidant activity has a much higher scavenging activity than vitamin C and κ-carrageenan (Wang, Zhang et al. 2008). However, it has been reported that the scavenging effects were not as strong in large molecules compared with the lower molecules of fucoidan. The total content per sulphate for fucoidan was an important indicator for the antioxidant activity of fucoidan (Wang, Zhang et al. 2008). It has been also reported that fucose could be the key to the radical antioxidant activity of fucoidan and proposed that it could be used in the food industry as a natural antioxidant (Rupérez, Ahrazem et al. 2002).

2.7.5 Cholesterol regulation

Fucoidan has a natural ability to burn away fatty acid like triglycerides and cholesterol (Huang, Wen et al. 2010), which helps to prevent the clogging and building of fatty acids in the blood vessels and thus reducing the risk of heart diseases. The burning of fatty acids also provides a sudden stimuli and provides with energy. Fucoidan has also been reported to inhibit the adiposeness process which creates fatty acids (Li, Lu et al. 2008).

Chapter 2

Method evaluations for quantitative determination of fucoidan

1. Introduction

Fucoidan is a bioactive polysaccharide which is built of sulphated L-fucose monomers which are linked together by α 1→3, 1→2 or 1→4 glyosidic linkages. It is found in the fibrillary cell walls and between the intercellular spaces of various species of brown seaweed.

The purpose of the study was to evaluate methods for quantitative determine of fucoidan. As fucoidan is a polysaccharide containing sulphated fucose residues linked by different glycosidic linkages, it was the goal to find conditions that hydrolyze both the sulphate groups and the glycosidic linkages to quantitatively convert the fucoidan to fucose which can be determined specifically. Fucose could then be back calculated to fucoidan to determine the overall fucoidan content.

The methods that will be used to quantify the amount of fucose from a crude extract must be specific because the crude extract from *L. hyberborea* will include sufficient amount of laminaran, mannitol and other carbohydrates as well as polyphenols which could possible create some interference with the detection for fucose. Thus, to develop a quantitative method for detection of unpurified fucoidan from the crude extract, two methods were carefully chosen and optimized for the detection of fucose; a colorimetric method and an enzymatic method. Both the methods are specific for the detection of fucose. In the colorimetric method, harsh acidic conditions are used which depolymerized and desulphate fucoidan into monomeric fucose (Ragan and Jensen 1979) and then fucose is directly measured at specified wavelengths. For the second method, enzymatic method, a commercially available L-fucose kit was used which specifically quantifies fucose in the presence of other sugars and impurities. In this method, the enzyme L-fucose dehydrogenase reduces $NADP^+$ to $NADP+H^+$. The amount of NADPH in this reaction is stoichiometrically equal to the amount of L-fucose in the solution. The reduced NADPH in this reaction gives an increase in the absorbance at 340 nm.

Both methods were specifically tested with increasing amounts of glucose with respect to their detection limits of fucose, as laminaran would be expected to be the main component co-extracted with fucoidan.

1.2 Colorimetric method

A colorimetric method for detection of fucose in the presence of other sugars was developed by Zacharias Dische and Landrum B. Shettles in 1948. This method, also known as the Dische method, was the first method to be developed for detecting methylpentoses in the presence of other sugars (Dische & Shettles, 1948). The method involves in treating the sugars with concentrated sulphuricacid (85%) and heating the solution at 100°C for 10 minutes and upon cooling cysteine hydrochloric acid is added. Under harsh acidic conditions, the fucose residues are converted into 5-methyl-furfural and upon cooling it reacts with cysteine to form a fucose-cysteine complex which gives a pale yellow color.

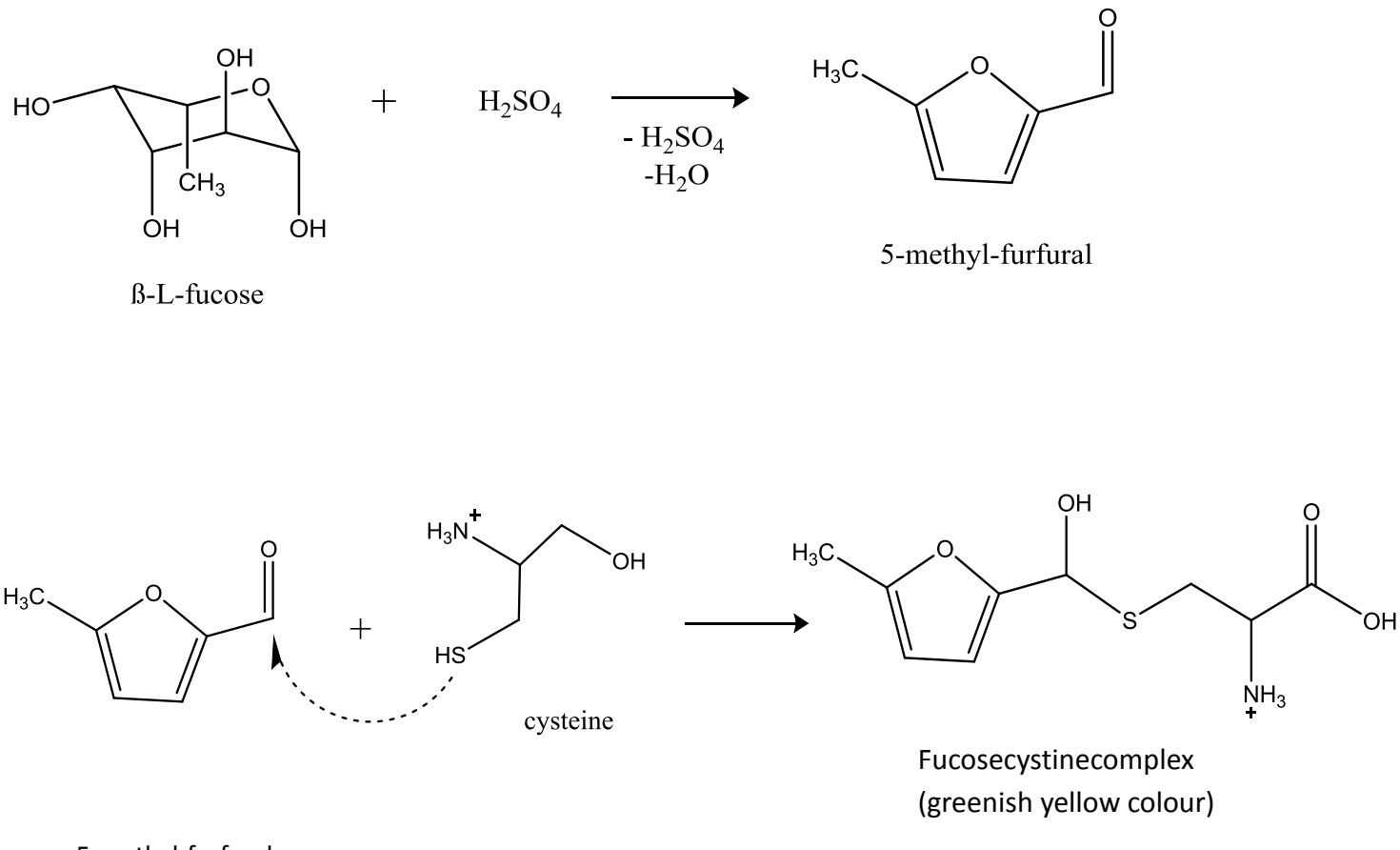

Figure 9: Shows the reaction of fucose in Dische assay to give a pale yellow compound

Different sugars give different colors in the Dische assay (Sieburth and Jensen 1970). Fucose in relation to other sugars gives a higher absorbance at 396nm and a low absorbance at 430 nm (Ragan and Craigie 1980). M.N Gibbons modified the Dische method in 1955 (Fig. 10) and experimented with 20 µg of fucose, 17 µg of fucose + 25 µg of galactose , 25 µg of glucose and 29 µg of xylose .

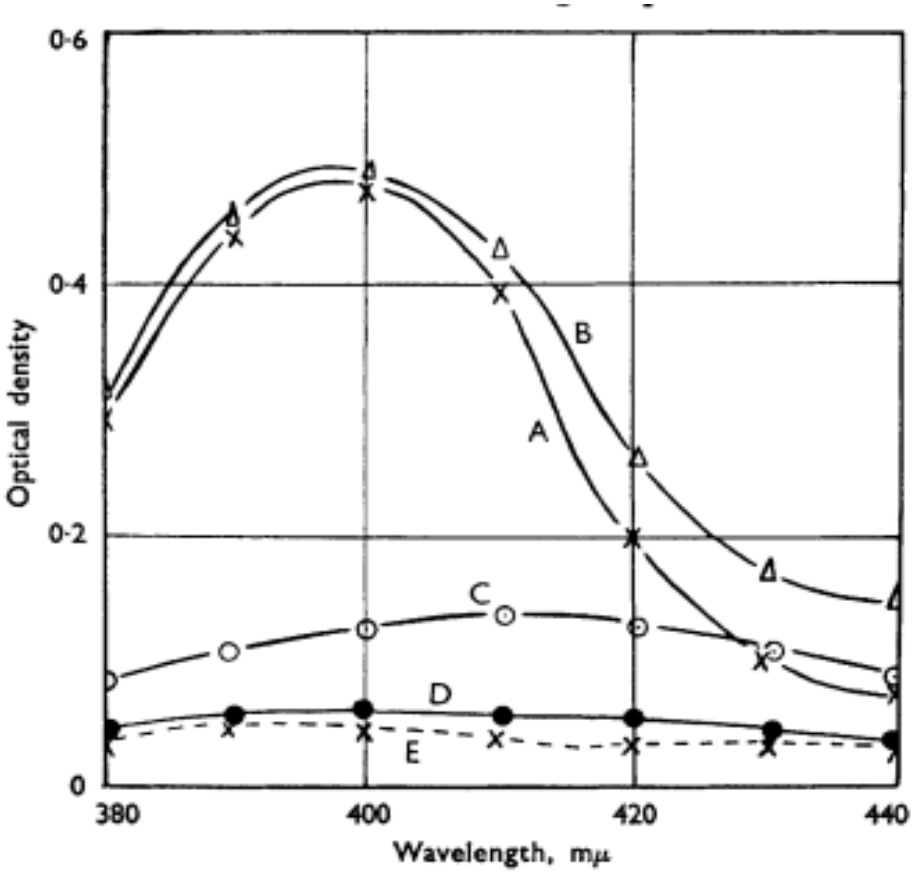

Fig. 1. Absorption spectra: curve A, 20 μg of fucose; curve B, 17 μg of fucose + 25 μg of galactose; curve C, 25 μg of glucose; curve D, 29 μg of xylose; and curve E, distilled water; after heating in sulphuric acid and addition of thioglycollic acid

Figure 10: Wavelenght spectrum of fucose in presence of other sugars

The results clearly show that curve A (20µg fucose) has a higher absorbance at 396 nm and a lower absorbance at 430nm. On the other hand, curve C (25µg glucose) gives a lower absorbance at both the 396nm and 430 nm wavelengths which implies the specificity of the method for fucose detection.

Curve B (17 µg of fucose + 25 µg of galactose) shows a similar higher absorbance at 430 nm but also shows a relative higher absorbance at 430 nm which implies that if fucose is present with another sugar it will give a higher absorbance at 430 nm but gives a similar absorbance with respect to fucose at 396 nm. Concluding that if more amount of sugar would be present in a sample along with fucose, it will give a higher reading at 430 nm.

1.3 Fucose wave length spectrum

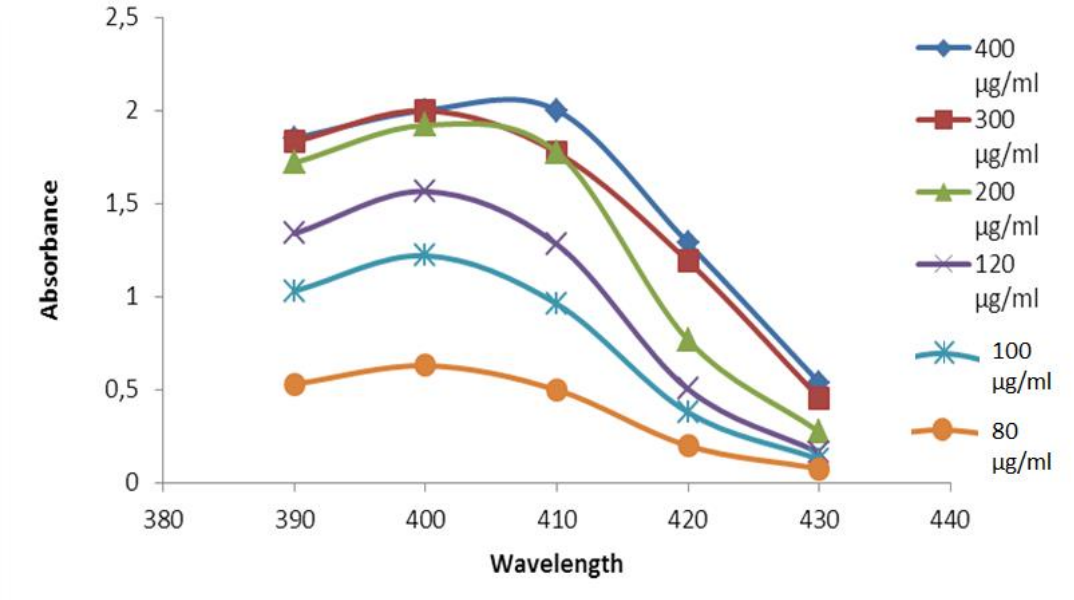

Figure 11: Fucose wavelength spectrum

Fucose wavelength spectrum was prepared with different concentration of fucose to check the reliability and reproducibility of the Dische method with respect to the spectrophotometer (Double-Beam UV-150-01). The fucose curve (100 µg/ml) appeared to be with the measureable range of spectrophotometer and thus a standard curve was prepared with for the range 0-100 µg/ml fucose with the Dische method.

1.4 Fucose standad curve

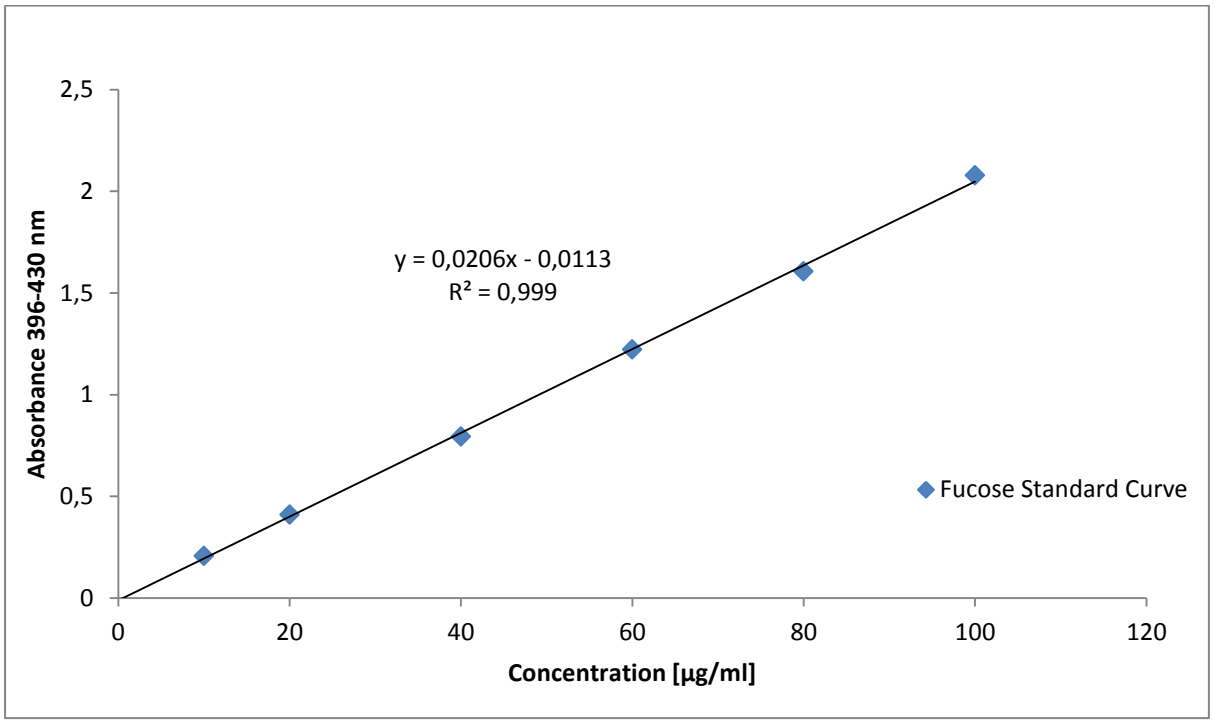

Figure 12: Standard curve for fucose with Dische method

A fucose Standard curve was prepared using the colorimetric method for determination and quantification of methyl pentoses. Commercially available L-fucose (Sigma) was used as the standard. Three replicates of the standard were prepared in different concentrations with distilled water ranging from 10-100 µg/ml. Sample solution of each concentrations (1.0 ml) was added to a standard test tube. The test tubes were cooled on the ice (2-3 minutes). 4.5 ml of sulphuric acid (85%) (Emsure) (prepared by adding 1 volume of water to six volume of concentrated sulphuric acid) is added and the samples are homogenized with the help of glass stirrer. Marbles were placed on top of the tubes to avoid evaporation and the tubes were placed in boiling water bath, for 10 minutes. Tubes were then cooled under running tap water and 0.1 % cysteine hydrochloric acid was added to the tubes and mix. Tubes were the placed in darkness for 2 hours and after which the absorbance was measured on spectrophotometer (Double Beam UV-150-01) at 396nm and 430 nm. A blank with distilled water treated under the same manner was used for the zero. The two absorbance values were subtracted through the following equation

$$\text{Absorbance} = (A396\ \text{nm} - A427\ \text{nm})$$

Which exclude the detection of all other polysaccharides by gives high values for methyl pentose (Dische & Shettles, 1948).

1.5 Fucose in presence of different Glucose concentrations

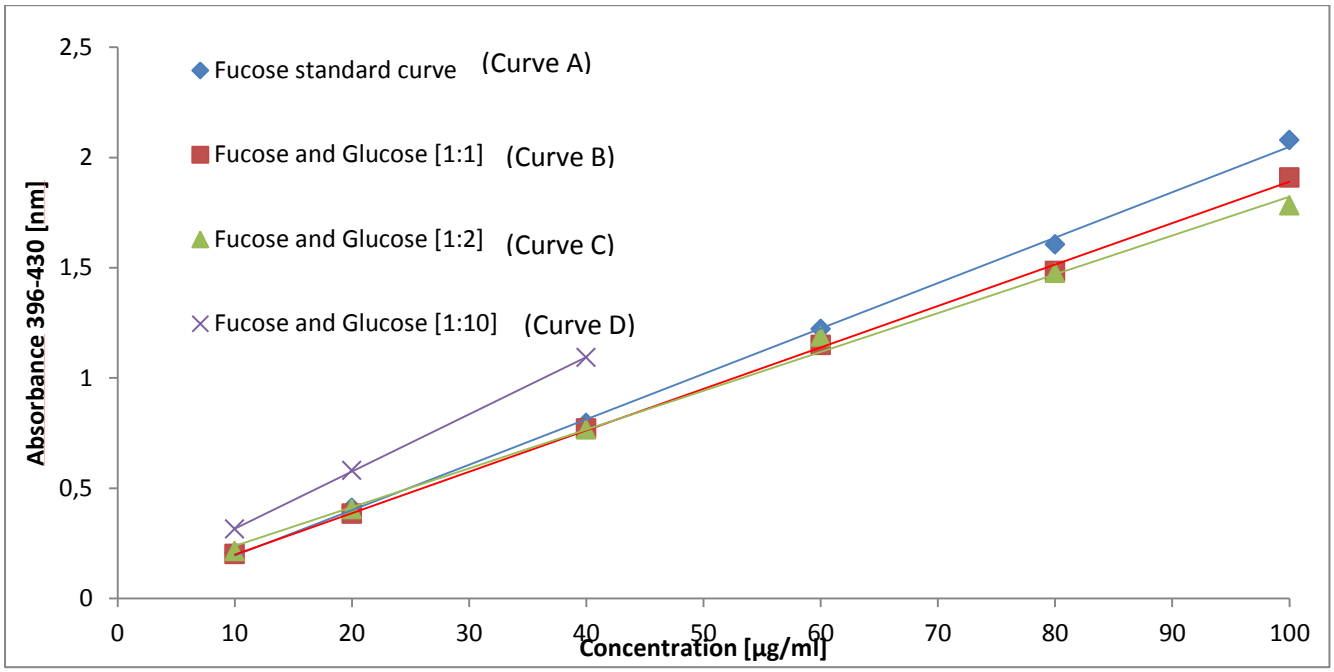

Figure 13: Fucose in the presence of different concentrations of glucose

Three more standard curves were prepared with different level of impurities (i.e. glucose), along with pure fucose. The conditions and the reagents used for the standard curves are the same in which fucose standard curve is produced.

Curve A is the pure fucose standard curve with no added impurities.
Curve B contains same concentration (mass based) for fucose and glucose.
Curve C contains twice the amount of glucose then the fucose.
Curve D contains 10 times more the amount of glucose than fucose.
Curve A, B and C shows a similar trend which means that the Dische method would work effectively if the impurity (i.e glucose) level is twice the level of fucose and a good measuring range would be from 20-60 µg/ml or the absorbance range of 0.400-1.000 nm. Curve D shows that if the impurity level is 10 times more than pure fucose the Dische method would not work as precisely.

1.6 Fucose and Fucoidan Standard Curve

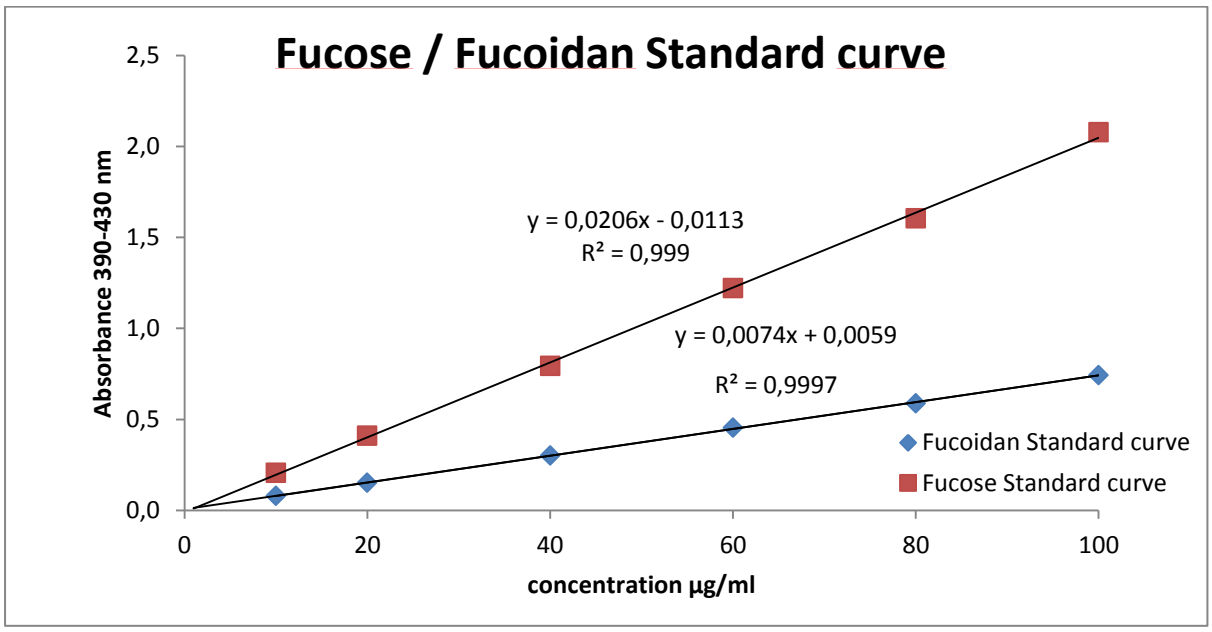

Figure 14: Fucose and Fucoidan standard curves with Dische method on mass basis

A fucoidan standard curve was prepared by the Dische method with 3 replicates and compared with the fucose standard curve on mass basis.

2.1 The Enzymatic method

Commercially available L-fucose assay kit by Megazyme internationals is used for the enzymatic method. The kit specifically provides a rapid and reliable method that is suitable for the dectection of L-fucose in plants extract, biological samples and other material. In principle enzymatic method is more reliable and sensitive method for detection of fucose. L-fucose in the sample is oxidized by the enzyme L-fucose dehydrogenase in the presence of nicotinamide-adenine dinucleotide phosphate (NADP+) to L-fucono-1,5 lactone with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).

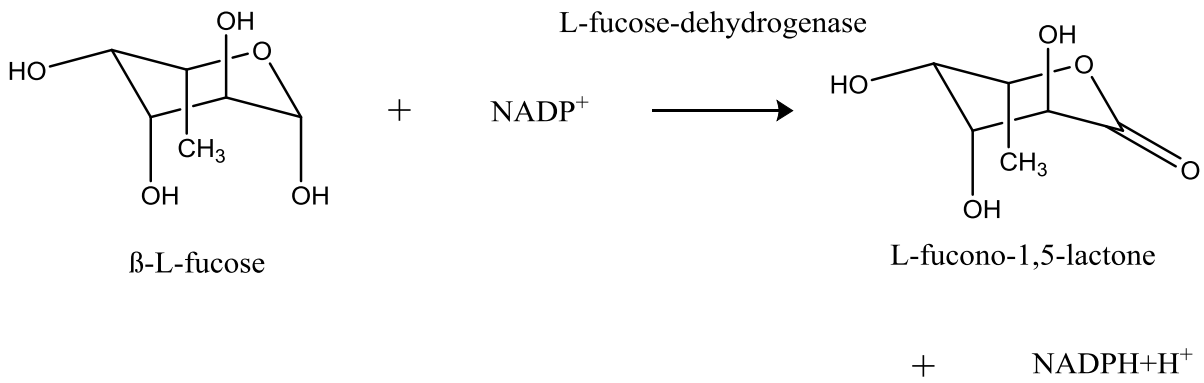

Figure 15: Principle for the enzymatic method

The kit contained three different quantitative method for L-fucose determination; Manual Assay, Auto-Analyzer Assay and Microplate Assay. The manual assay procedure was used for total quantification of fucose. For the manual assay procedure, the kit specifies as 100 assays per kit. The method was modified to perform 200 assays per kit.

2.2 Fucose standard curve

A fucose standard curve was prepared using the enzymatic method:

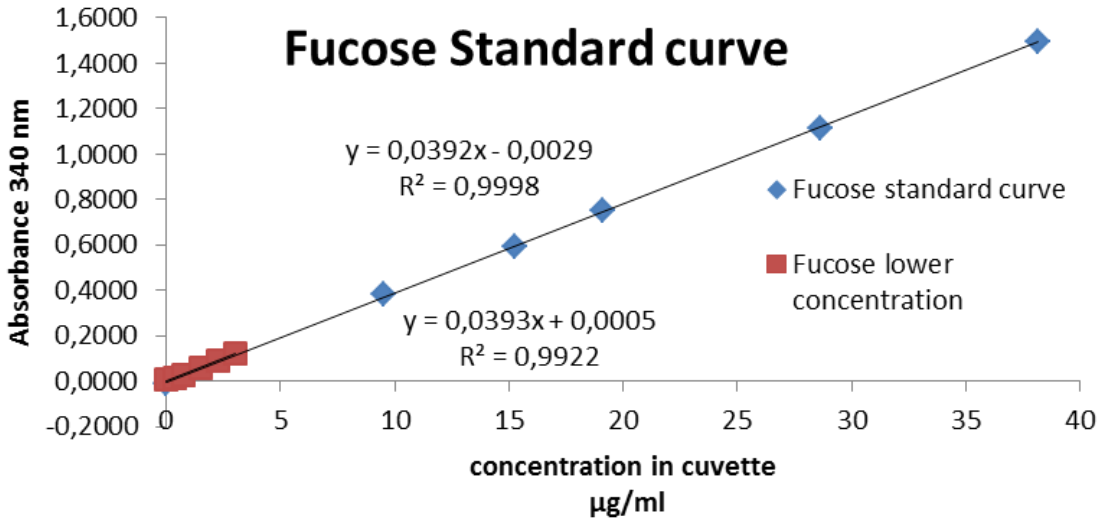

Figure 16: Fucose standard curve from enzymatic method

L-fucose (Megazyme) was used as standard with concentration ranging from 10-40 µg/ml per cuvette. 2 ml of distilled water was added in a 3ml plastic cuvette (VWR) followed by 0.1 ml of sample solution.0.4 ml of buffer and 0.05 ml of NADP+ was added and mix. After 3 minutes incubation, absorbance was measured at 340 nm. After that, 0.01 ml of L-fucose dehydrogenase was added to the cuvette and vortex (MS2,IKA) and the samples were placed on shaker (VIBRAX,IKA) at room temperature for overnight. Three replicates of each concentration were prepared.

An increase in absorbance was measured at the same wavelength. The difference between the absorbance was measured and plotted against concentration.

2.3 Fucose with higher concentration of Glucose

As mentioned before, the crude extract from *Laminaria hyperborea* would contain impurities, along with fucoidan. The most expected impurity is laminaran (1->3 linked glucose polymer) which is present in abundant amount, but with seasonal variation. A standard curve was prepared with fucose mixed with glucose (40 times more the amount of

fucose) and compared with the fucose standard curve. Three replicates of each were prepared. The graphs obtained were as follow:

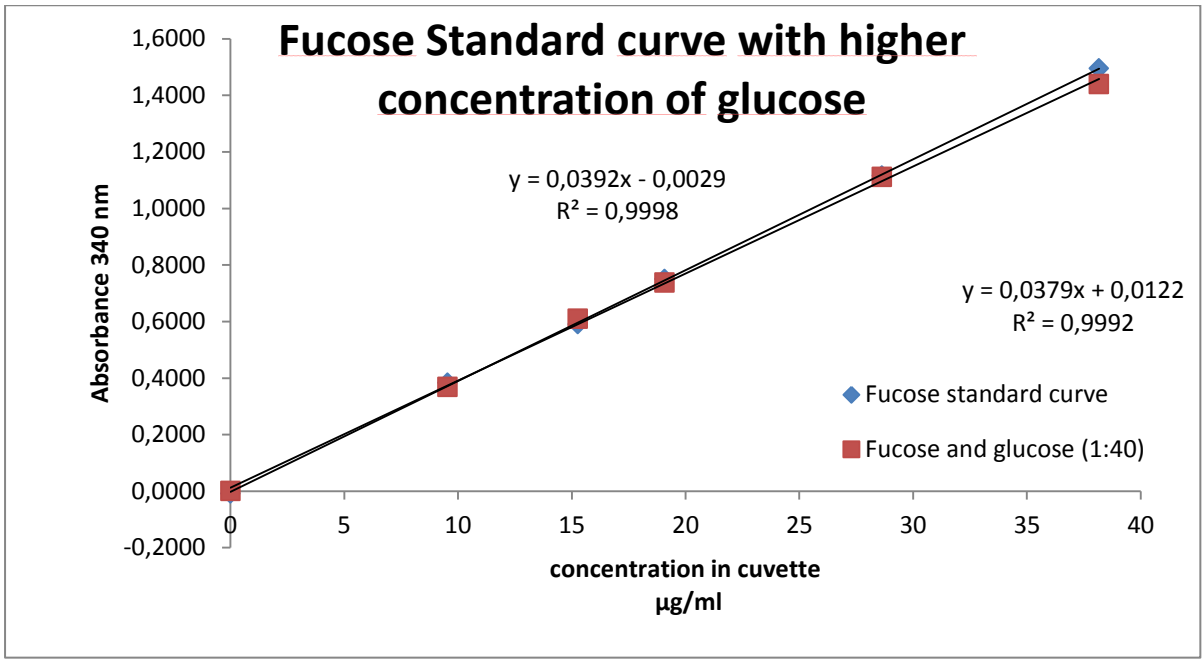

Figure 17: Fucose standard curve with higher concentration of glucose for with enzymatic method

The pure fucose curve and the fucose mixed with high amount of impurity (glucose) shows similar graphical trends, which show the specificity and sensitivity of the enzymatic method.

2.4 Chemical hydrolysis: fucoidan to fucose

The Enzymatic method is more specific towards the detection of fucose than the Dische method. But since the crude extract from *L.hyperborea* would contain fucoidan rather than fucose, the fucoidan must depolymerized and desulphated to the fucose monomer to be detected by the enzymatic method.

A series of experiments were done to optimize a complete depolymerization and desulphataion of fucoidan to fucose without destruction of the fucose itself. Fucoidan (Nova Matrix) of *Laminaria hyperborea* was degraded in 1 M HCl at 80°C for 6 hours. 50 µl of sample was taken out periodically and analyzed for the amount of fucose. The following curve was obtained (Figure 18).

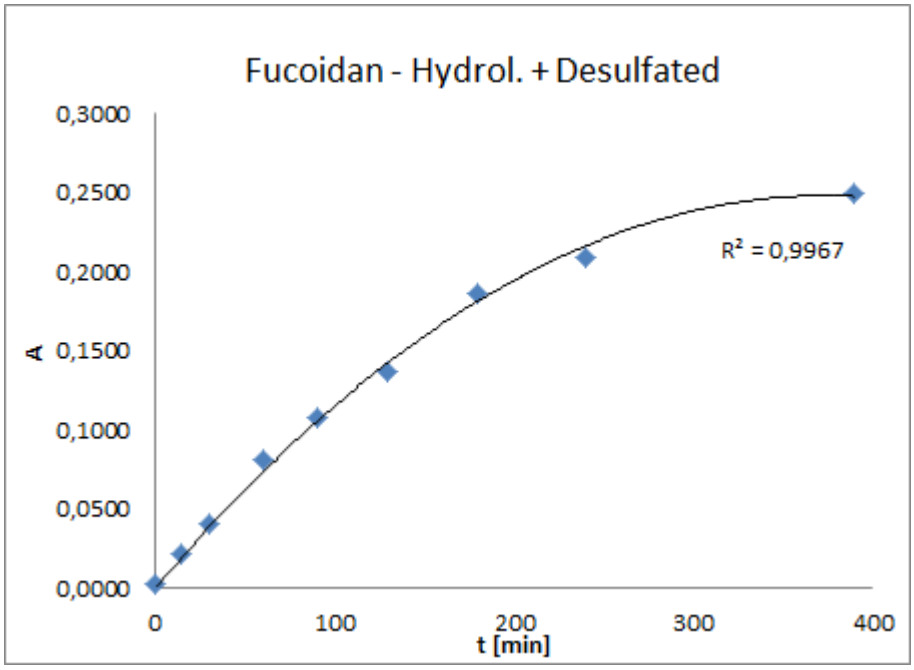

Figure 18: Hydrolysis and desulphatation of fucoidan

The solution was kept under these conditions for 5 days. A sample was analyzed after 5 days and showed a just a little increase in absorbance for about 15%. It suggests that fucose remains stable under these conditions and that we convert about 85% of fucoidan to fucose in 6 h with our chosen conditions and that a further time increase increases the fucose content very slowly.

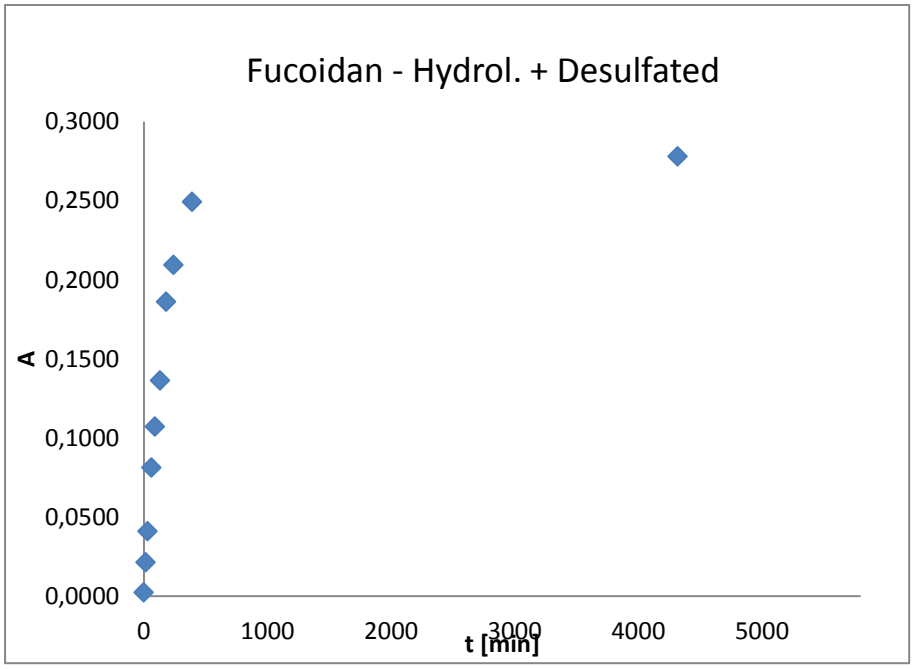

Figure 19: Hydrolysis and desulphatation of fucoidan for 4 days

3.1 Quantitative determination of total carbohydrate

Brown seaweeds are known to produce different types of polysaccharide like laminaran, algnic acids, mannitol and fucoidan. However, the total compositions of these carbohydrates may vary between different species and the habitats in which they are growing. They also show small seasonal variations (Haug and Jensen 1954). For *L.hyperborea*, the total content of laminaran and manitol is around 0-30 % and 4-25 % per dry weight of algae, respectively,while fucoidan is about 2-4 % per dry weight of algae (Ragan and Jensen 1979). To quantify the total amount of these carbohydrates present in algae, phenol sulphuric acid method was used. Among many colorimetric methods used for detection of carbohydrates, phenol sulphuric acid is the most widely used method for determination of carbohydrates in aqueous solution. Phenol sulphuric acid method is used for its simplicity, sensitivity and reliability (DuBois *et al.*1956). The reagents used in this method are concentrated sulphuric acid and phenol, in principle, the carbohydrates dehydrate by reacting with concentrated sulphuric acid and produce furfural derivatives. These furfural derivatives then react with phenol to produce a detectable color (pale yellow).

Phenol sulphuric acid method is a non-specific quantitative test for carbohydrate determination (DuBois *et al.*1956). A glucose standard curve was prepared to quantify the total carbohydrate in the algal material with respect to glucose. Also fucose and fucoidan standard curves were prepared to measure the degree of depolymerisation and desulphatation of fucoidan under phenol sulphuric acid conditions.

3.1 Glucose standard curve

Glucose standard curve was prepared with commercial D-glucose (Sigma) by phenol sulphuric acid method (DuBois *et al.*1956). Four replicate of the samples were prepared with different concentrations in distilled water ranging from 0-100 µg/ml. The sample solution (2ml) was added in standard test tubes and 0.5 ml of 3 % phenol (prepared by adding 3 g of phenol in 100 ml of distilled water) was added, followed by 5 ml of concentrated sulphuric acid (Emsure). The content of the test tubes were mixed with a glass rod. The samples were incubated at room temperature at 30 min. A blank with distilled water was prepared in the

same manner to zero the spectrophotometer and the absorbance was measured at 485 nm on Light spectrophotometer (Double-Beam UV-150-01).

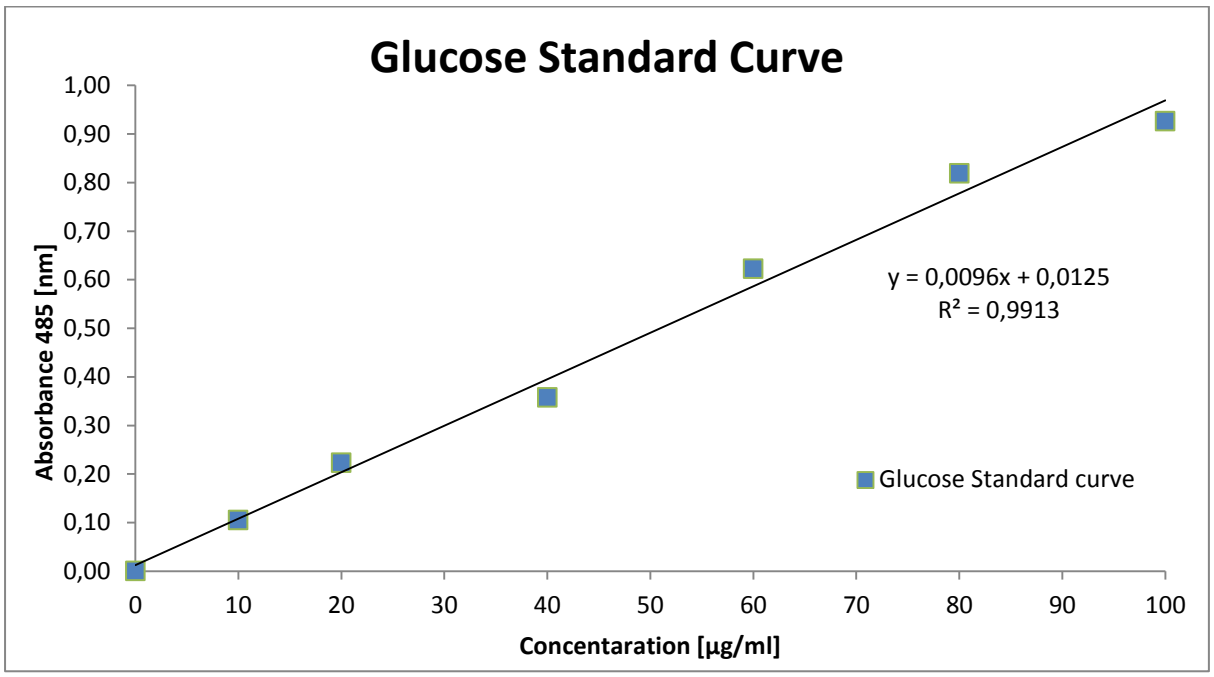

Figure 20: Glucose standard curve by phenol sulphuric acid method

Graph was plotted for the absorbance against known standard concentrations. A linear regression was achieved with a correlation factor of R^2= 0.9913 and a line formula of y = 0.0136x as shown in Figure 20. The equation derived for the standard curve was used to calculate the total carbohydrate content in the samples.

3.2 Fucose standard curve

A fucose standard curve was prepared in the similar manner but instead of glucose, commercially available L-fucose (Sigma) was used.

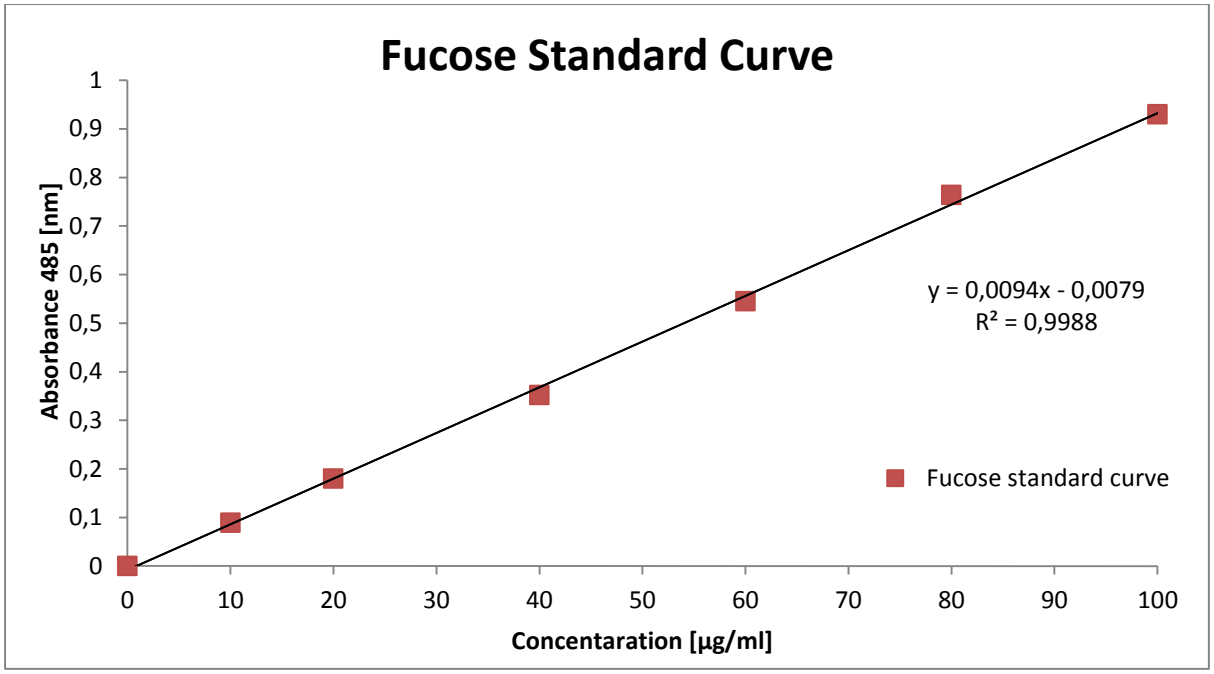

Figure 21: Fucose standard cure with phenol sulphuric acid method

Graph was plotted for the absorbance against known standards concentration. A linear regression was achieved with a correlation factor of R^2= 0.9988 and a line formula of y = 0.0094x-0.0079 as shown in Figure 21.

3.3 Fucoidan standard curve

A fucoidan standard curve was prepared from purified fucoidan (*L. hyperborea*) from NovaMatrix.

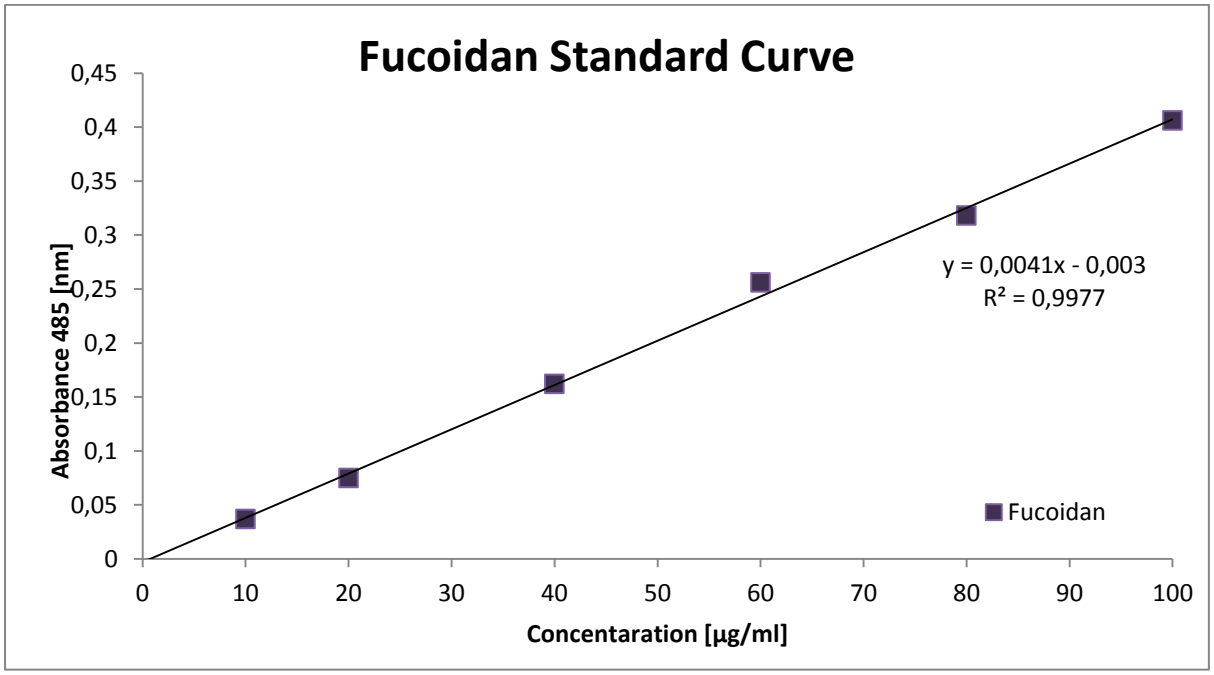

Figure 22: Fucoidan standard curve with phenol sulphuric acid method

Graph was plotted for the absorbance against known standards concentration. A linear regression was achieved with a correlation factor of R^2= 0.9977 and a line formula of y = 0.0041x-0.003 as shown in Figure 22.

3.4 Comparing glucose, fucose and fucoidan standard curves

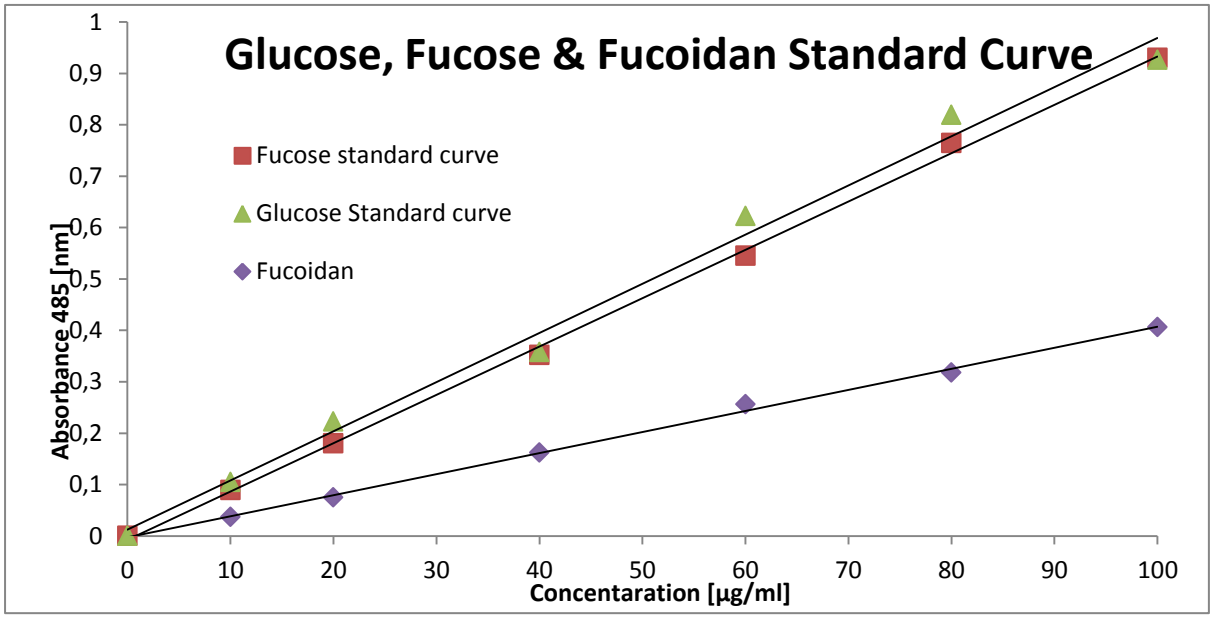

Figure 23: Glucose, fucose and fucoidan standard curves

Figure 23 shows the comparison of glucose, fucose and fucoidan standard curves prepared by phenol sulphuric acid method. Glucose and fucose gave a similar absorbance, while fucoidan had a much lower absorbance compared to fucose and glucose.

4. Discussion

In this study, quantitative methods for the detection of fucoidan were evaluated and also standard curves of glucose, fucose and fucoidan were prepared with phenol sulphuric acid method.

As crude extract of fucoidan from the brown algea would also contain other sugars along with fucoidan, the method chosen for the detection of fucoidan must be specific and reliable. The colorimetric method and the enzymatic method were chosen and evaluated for this purpose. The colorimetric method relays on harsh acidic conditions which depolymerized and desuphated fucoidan into the fucose monomer, these fucose was then specifically determined and quantified. The colorimetric method was also tested with different concentrations of glucose present along with fucose. More concentrations of glucose showed larger degree of variations from the pure fucose curve. The results showed some limitations with respect to impurity levels. In excess concentrations of glucose the results seemed to be less specific and less reliable. On the other hand, the enzymatic method turns to be more specific and reliable. The method was tested with 40 times higher concentrations of glucose along with fucose and the results seems reliable and good, with less degree of variations from the pure fucose curve. But for the enzymatic method a pretreatment had to be done for complete depolymerisation and desulphation of fucoidan to fucose monomers. A method was optimized for this pretreatment that would convert all the fucoidan into fucose. The results were conclusive and the method was used with full confidence.

For quantitative determination of total carbohydrates, glucose, fucose and fucoidan standard curves were prepared using the phenol sulphuric acid method. The glucose standard curve was prepared to quantify the total carbohydrate in the algal material with respect to glucose. Fucose and fucoidan standard curves were also prepared through the phenol sulphuric acid method, according to the mass. The results show that fucoidan curve has a lower absorbance compared to fucose curve. This reflects the different molecular weights of the monomer units, for fucose (164.15 g/mol) and for fucoidan we calculated a molecular weight of 356 g/mol. The fucoidan monomer (has a molar mass twice that of fucose). So the possibility that the fucoidan curve is twice as lower as fucose standard curve is reasonable. Furthermore, the water molecules in fucoidan are tightly bound to the sulphate groups present in fucoidan and cannot be removed under regular drying conditions which were used in the lab (dryer, 80 °C). These water molecules could only be removed under very high pressured conditions and

increasing the temperatures would generally destroy the sugar units itself. These water molecules interfere with the overall mass measurements for fucoidan. Hence we get a fucoidan standard curves which is almost twice as low as fucose standard curve.

5. Conclusion

In conclusion, both the methods were specific for detection of fucose. But the Dische method had certain limitations. The method worked precisely if the glucose concentration is 5 times or less, than the concentration of fucose within the samples. But the method turns out to be cheap and the fucoidan is hydrolysed to fucose within the assay.

The enzymatic method was more sensitive, specific and reliable. Even little amount of fucose can be detected by this method. But this method is expensive and a pretreatment for the complete chemical hydrolysis of fucoidan to fucose monomers is to be done prior to the enzymatic analysis. The pretreatment for depolymerisation and desulphatation was optimized as using 1 molar HCl and place in water bath (80^{o}C) for overnight.

Phenol sulphuric acid method turns out to be a good choice for a nonspecific quantitative determination of carbohydrates. The readings from the standard curves showed reliability.

Chapter 3

Method optimization for extraction of fucoidan from *L. hyperborea*

1. Introduction

As mentioned earlier, the yield and the composition of the extracted fucoidan is largely influenced by the extraction time, temperature and solvent properties. In this study a method was optimized to obtain the higher yields of fucoidan from the algae. The experiments were performed with hot water and different acid concentrations at elevated temperature. The extraction time was also varied to get the highest yield of crude fucoidan. Sequential extraction was also performed to get reliability for the method and to check if a re-extraction would be necessary for a complete extraction of fucoidan from the algae. Along with fucoidan, total carbohydrates extracted was also nonspecifically quantified to get a measure of how much fucoidan is being extracted with comparison to total carbohydrate in the extracted solution.

Main focus was to optimize a method for extracting maximum yield of crude fucoidan and less attention was given to the purity levels fucoidan.

2. Materials and Method

2.1 Samples

Dried *L. hyperborea* sample harvested from Trondheim fjord in May were used.

2.2 Method

Dried *L.hyperborea* was ground and milled (Janke and Kunkel,IKA) to pass through 1.5mm sieve. 40mg of these milled algae was extracted at 80 °C in a water bath (Grant, OLS 200) with 10ml (HCL/Distilled water) of different concentrations (according to the experimental design) in 25ml glass bottle. After extractions (under different experimental conditions) the solutions were filtered (0.5µm) and analyzed for total carbohydrate and fucoidan content.

2.3 Total carbohydrate content

The total carbohydrate content was nonspecifically determined by the phenol sulphuric acid method with pure glucose as a standard (DuBois *et al.*1956). All samples and standards were done in three replicates.

2.4 Total fucoidan content

The total fucoidan content from the crude extract was determined as fucose. The fucose was quantitatively determined with the Dische method. The fucose was then calculated for total fucoidan. All samples and standards were done in three replicates.

3. Results

Algae was extracted for total carbohydrate and fucoidan content with 1 molar and 0.1 molar HCl under different extraction times i.e. 10 minutes, 20 minutes and 2 hours. *L.hyperborea* leaves and stem were extracted separately. Figure 24 below shows extraction of *L.hyperborea* leaves with different acid concentrations and extractions times. Curve A and Curve B are the total amount of carbohydrate and fucoidan extracted, as a percentage per dry weight of algae in 1 molar HCl, respectively. Curve C and Curve D shows the total amount of carbohydrate and fucoidan, extracted under similar extraction times but with 0.1 molar HCl, respectively. The trend shows that increasing the extraction times increases the total amount of carbohydrate extracted. Curve A and Curve C shows the amount of carbohydrate extracted with 1 molar HCl then 0.1 molar HCl. Comparing both the curves show that more amount of carbohydrates is being extracted with 1 molar HCl, while the fucoidan content (curve B and D) shows similar amount of fucoidan being extracted with 0.1 molar and 1 molar HCl.

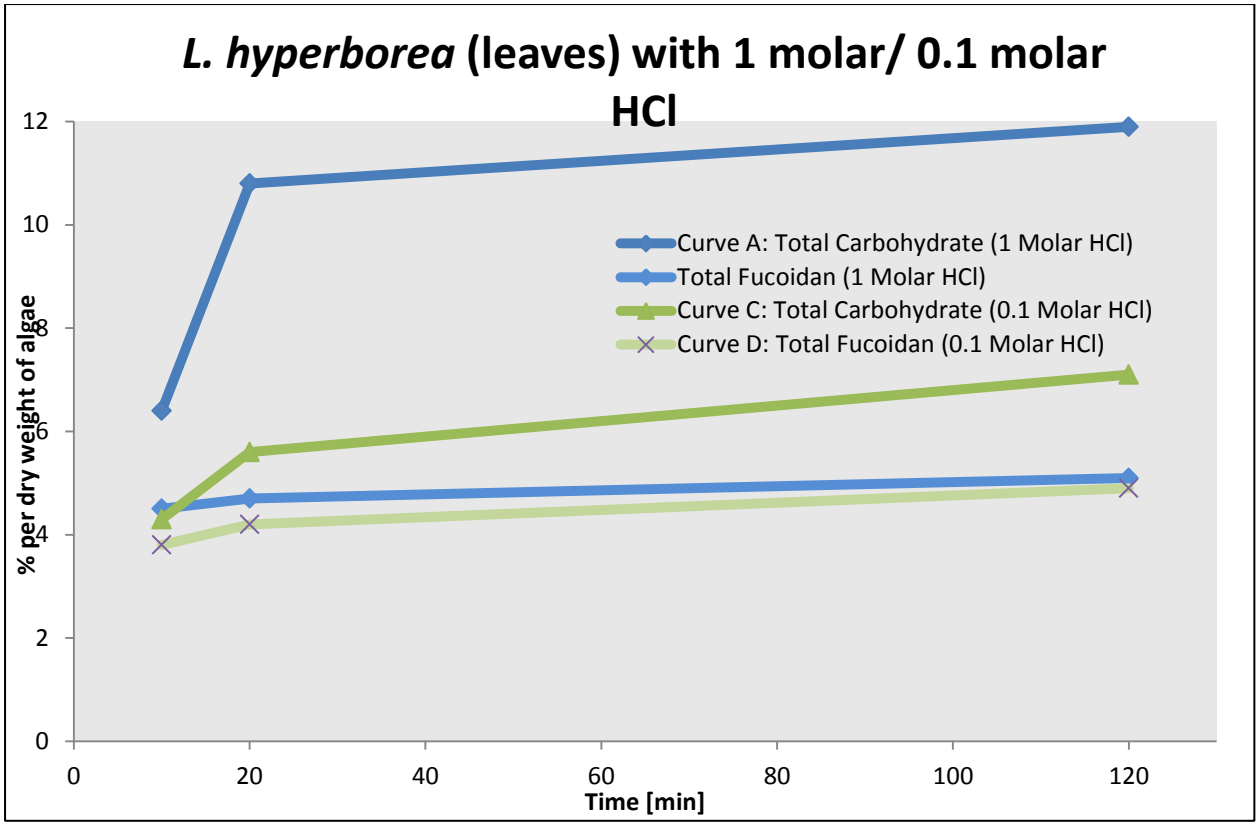

Figure 24: Extraction of fucoidan and total carbohydrate from L.hyperborea leaves with different acid concentrations and different extraction times

The *L.hyperborea* stem was analyzed under similar conditions. Figure 25 below shows extraction for *L.hyperborea* stem with different acid concentrations and extractions times. Curve 1 and Curve 2 represents total carbohydrate and fucoidan content in 1 molar HCl, respectively. Curve 3 and Curve 4 represents total carbohydrate and fucoidan content in 0.1 molar HCl, respectively. The trend shows that in 1 molar HCl, both the total carbohydrate content and fucoidan content is relatively higher compared to extraction with 0.1 molar HCl.

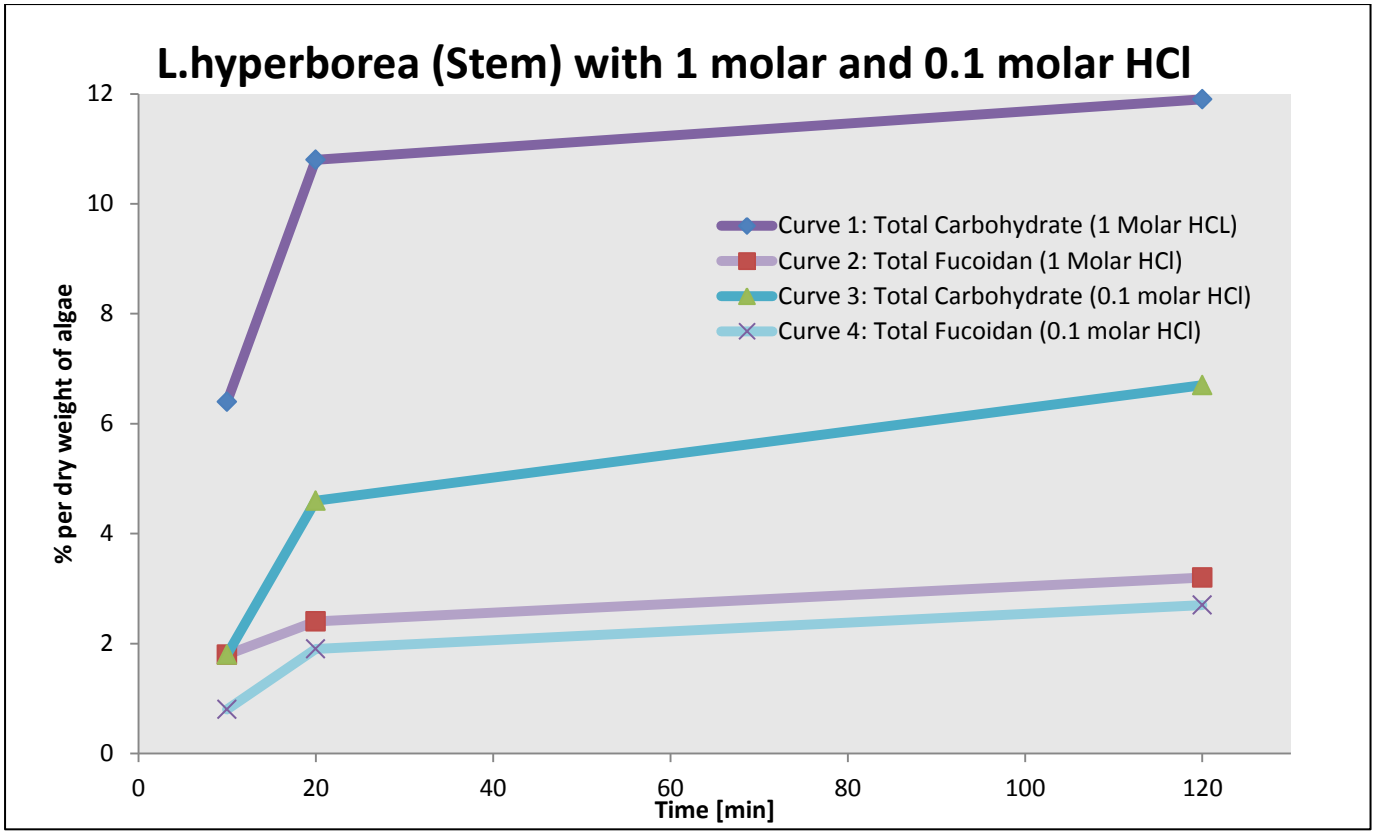

Figure 25: Extraction of fucoidan and total carbohydrate from L.hyperborea stem with different acid concentrations and different extraction times

3.1 Further Extractions

Further extractions were done with 0.1 molar HCl for 2 hours and overnight at 80°C. The results show that the amount of fucoidan and total carbohydrate were similar for overnight and 2 hours extraction period.

An extraction was also performed with distilled water at 80°C for 2 hours and overnight. The results show that the total amount of fucoidan and as well as total carbohydrate extracted with water were lower compared to acid. But we see an increase in the total carbohydrate and fucoidan content for overnight extraction compared with 2 hours extraction.

Table 1: Extraction of fucoidan and total carbohydrate from L.hyperborea leaves with 0.1 molar HCl for 2 hours and overnight

Sample	Time	Solvent	% carbohydrate per dry weight of algae	% fucoidan per dry weight of algae
L.hyperborea (leaves)	2 Hours	0.1 Molar HCL	6.9	2.4
L.hyperborea (leaves)	Overnight	0.1 Molar HCL	7.0	2.3

Table 2: Extraction of fucoidan and total carbohydrate from L.hyperborea leaves with distilled water for 2 hours and overnight

Sample	Time	Solvent	% carbohydrate per dry weight of algae	% fucoidan per dry weight of algae
L.hyperborea (leaves)	2 Hours	Distilled Water	3.8	0.8
L.hyperborea (leaves)	Overnight	Distilled Water	4.2	0.9

3.2 Sequential Extraction

A re-extraction was performed to check if all the fucoidan has been extracted from the algae. The algae was first extracted at 80°C with 0.1 molar HCl for 2 hours and then the algae were filtered using and extracted again for 2 hours under same conditions. The stem and leaves were extracted separately. The graphs below shows the total amount of fucoidan and carbohydrate extracted in the first extraction and in the second extraction.

The percentages are gives as per dry weight of algae.

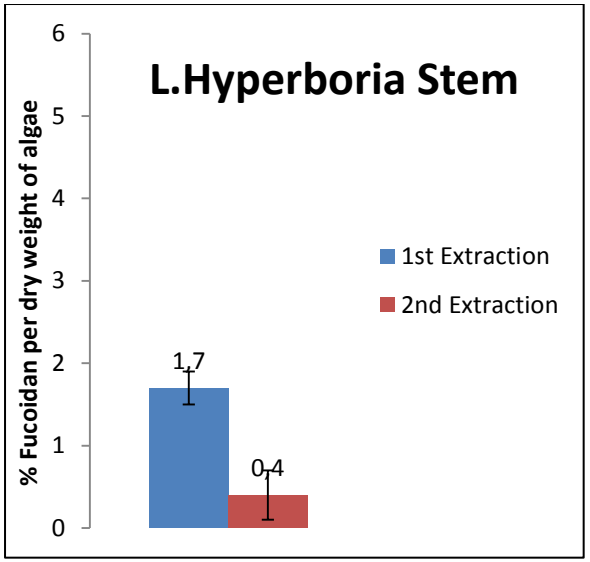

Figure 26: Sequential extraction for fucoidan form *L. hyperborea* stem

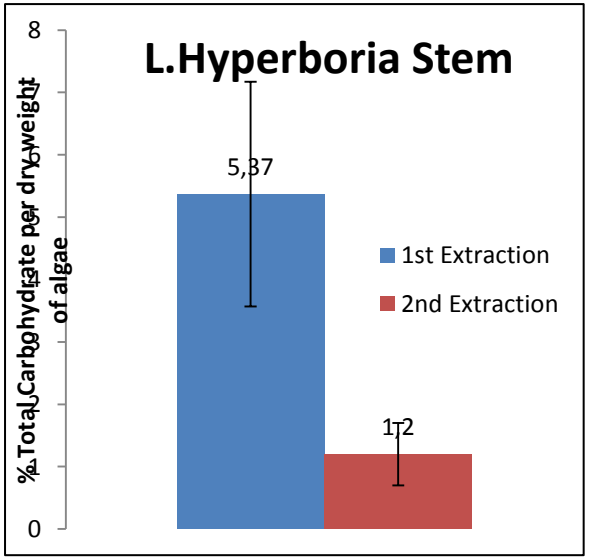

Figure 27: Sequential extraction for total carbohydrate form *L. hyperborea* stem

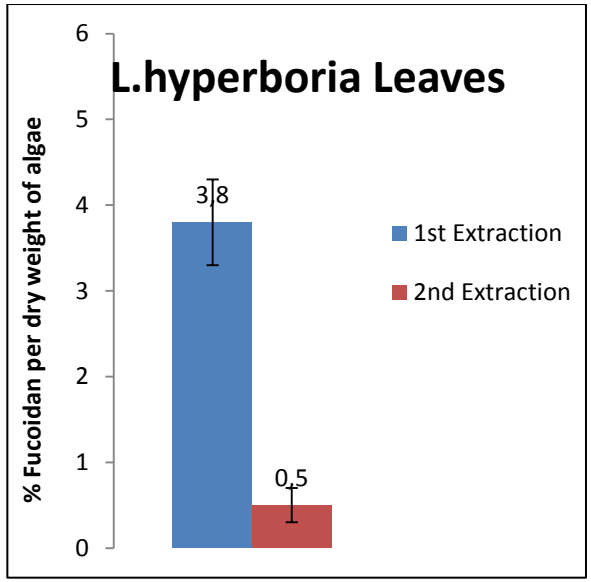

Figure 28: Sequential extraction for fucoidan form *L. hyperborea* leaves

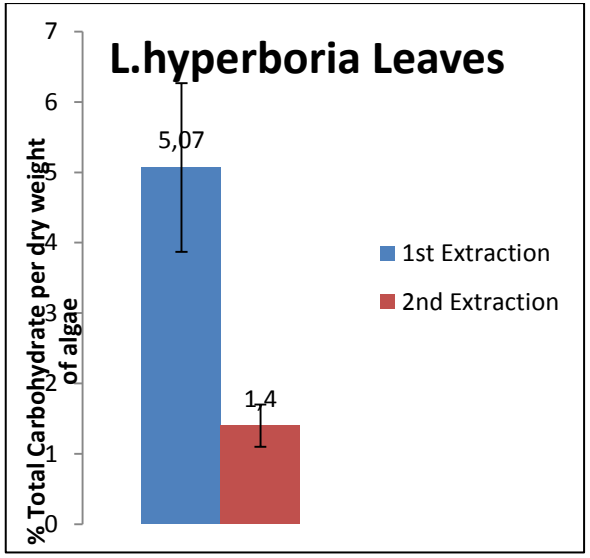

Figure 29: Sequential extraction for total carbohydrate form L. hyperborea leaves

The results show that in the second extraction, lower amount of fucoidan is being extracted. For the leaves the second extraction gives 0.5 % of total fucoidan extracted and for stem was 0.4 %. This is relatively very low amounts of fucoidan compared with the first extractions. The total carbohydrate extracted for the second extraction also show similar trend with lower percentages of carbohydrate being extracted in the second extraction.

4. Discussion

This study was done to optimize a method for extraction of crude fucoidan from *L.hyperborea*. Previously different extractions methods had been employed for the extraction of crude fucoidan from different brown seaweeds (Hahn, Lang et al. 2012). Different extractions conditions give different extraction yield and purity levels of crude fucoidan. Extracting fucoidan with water at elevated temperature gives higher purity and stability of the fucoidan molecule (Li, et al., 2008) while extraction with acid gives higher yields of fucoidan (Li et al., 2008; Black et al., 1952). In this study more emphases was on the yield of fucoidan rather than purity levels.

Dried milled *L.hyperborea* was extracted with different acid concentrations and different extraction times at constant temperature. In relation to fucoidan, the total carbohydrate content was also determine to check if any unwanted alginic acid might be extracted from the algae. The algae was extracted with 1 molar and 0.1 molar HCl for 10 minutes, 20 minutes, 120 minutes and overnight. The algae were also extracted with hot water for 2 hours and overnight. In all the experiments the temperature for extraction was kept constant at 80°C to prevent other sugars from getting extracted.

The results show that more carbohydrates are extracted when 1 molar HCl is used as extraction solvent, while the fucoidan content remains similar for the both acid concentrations which suggests that using higher concentrations of acids would extract alginic acid; as similar amount of alginic acid are present in leaves and stem (Ragan and Jensen 1979) ,extracting with 1 molar HCl gives similar contents of carbohydrate for both the stem and leaves.

Extraction with water as the extraction solvent gave very low amount of crude fucoidan compared to the dilute acid.

Using different extraction times also influenced the total amount of carbohydrate and crude fucoidan. The trend form both graphs Figure 24 and Figure 25 shows that total carbohydrate and crude fucoidan extract increased till 2 hours extractions time. Further extraction for overnight showed similar results.

Algae was also a re-extracted under same extraction conditions. The results show that both for *L.hyperborea* leaf and *L.hyperborea* stem, the second extraction gave a very low amount of crude fucoidan i.e 0.5 and 0.4 %, respectively which suggests that a second extraction was not necessary as almost all the fucoidan is extracted in the first extraction.

5. Conclusion

The extraction conditions were optimized with 0.1 molar HCl as the extraction solvent and the time for extraction was optimized for 2 hours at 80°C. These conditions gave the higher yields of crude fucoidan both for *L.hyperborea.* Leaf and *L. hyperborea* stem

Chapter 4

Quantification of the release of total carbohydrate and fucoidan from the brown algae *Laminaria hyperborea*

1. Introduction

Fucoidan is a mucilaginous substance that is mainly found in the cell wall of the brown algae. It is a sulphated polysaccharide with a high hygroscopic nature. The main function of fucoidan in the algae is still debatable. Some theories have been proposed for their function in algae. It has been proposed that fucoidan might protect the algae against desiccation or protect it from the ultraviolet radiations when the algae are exposed to low tides. However, it has also been hypothesized that the algae might start to produce more fucoidan when it is exposed to any external damage.

In this study, an experiment was designed and performed to check if any fucoidan is released by the damaged algae. Live algae were collect from the sea. In the start of experiment, the total fucoidan content of algae was measured, then the algae was damaged and placed in water and the samples were taken out at regular intervals. These samples were analyzed for any carbohydrate and fucoidan release. After that, the algae was taken out from water, dried, and the total fucoidan content was measured again. The total fucoidan content in the start and at the end of the experiment was also measured and any increase or decrease in the fucoidan content of the algae was also determined. The algae were placed in distilled water and sea water for 7 days at 4^{o}C.

2. Experimental Design and Work

2.1 Sample Collection

L. hyperborea samples were collected from Storsteinan, Trondheim (63°27'09.8"N 10°15'18.0"E). The samples were collected on February 28th 2015 between 15:30 – 16:01. The water temperature measured was 5°C and the samples were collected from an under water depth of 3.5 – 4.5 meters at a low tide (1 meter).

The algae were removed from the mussel line by hand. The samples were chosen carefully that had no growth of algae on the blades. The algae were kept wet and wrapped in wet towels. It took 20 minutes to drive from Storsteinan to NTNU-Bioploymer lab.

Figure 30: Sample Collection Site Storsteinan Trondheim, Norway

2.2 Sample setup and Preparation

21 *L. hyperborea* plants of medium and comparable size (approximately 2m) were selected and divided in following groups.

Group A: 3 plants were selected and divided in 3 groups and immediately placed in dryer (at 80°C)

DryNL: New leaf of 3 plants separated and dried
DryOL: Old leaf of 3 plants separated and dried
DryS : Stem of 3 plants separated and dried

Group B: 6 plants were selected and divided in 2 groups and placed into 30 liter plexiglass cylinders and placed in a 4°C cooling room.

Wpd: 3 plants were placed in the cylinder and filled with 21 liters of distilled water.
Wps: 3 plants were placed in the cylinder and filled with 17 liters of filtered sea water.

Group C: 3 plants were selected and divided into 3 groups and placed in 10 liters bucket and filled with 3 liters of distilled water. The buckets were placed in a 4°C cooling room.

OLd: Old leaf of 3 plants were separated and placed in the bucket. Defined cuts were added to the leaf; diagonal cuts of 2 cm length with 2 cm distance to each other.

NLd: New leaf of 3 plants were separated and placed in the bucket. Defined cuts were added to the leaf; diagonal cuts of 2 cm length with 2 cm distance to each other.

Sd: The stem of 3 plants were separated from the leaf and placed in a bucket. The stem was cut into half from top to bottom and sliced orthogonal every 5 cm.

Group D: 3 plants were selected and divided into 3 groups and placed in 10 liters bucket and filled with 3 liters of filtered sea water. The buckets were placed in a 4°C cooling room.

OLd: Old leaf of 3 plants are separated and placed in the bucket. Defined cuts were added to the leaf; diagonal cuts of 2 cm length with 2 cm distance to each other.

NLd: New leaf of 3 plants are separated and placed in the bucket. Defined cuts were added to the leaf; diagonal cuts of 2 cm length with 2 cm distance to each other.

Sd: The stem of 3 plants were separated from the leaf and placed in a bucket. The stem were cut into half from top to bottom and sliced orthogonal every 5 cm.

The table below summarizes the sample setup with the wet weight of algal samples.

Table 3: Sample setup for the experiment

Group	Name	Plant Part	Wet Weight [g]	Solvent	Wet/Dry	Extracted in	Volume [L]
A	DryNL	New leaf	209,2	---	Dry	---	---
A	DryOL	Old leaf	163,8	---	Dry	---	---
A	DryS	Stem	170,7	---	Dry	---	---
B	Wpd	Whole plant	1647	Distilled Water	Wet	Cylinder	21
B	Wps	Whole plant	1620	Sea Water	Wet	Cylinder	17
C	OLd	Old leaf	464,5	Distilled Water	Wet	Bucket	3
C	NLd	New leaf	266,7	Distilled Water	Wet	Bucket	3
C	Sd	Stem	603,8	Distilled Water	Wet	Bucket	3
D	OLs	Old Leaf	504,7	Sea Water	Wet	Bucket	3
D	NLs	New leaf	234,7	Sea Water	Wet	Bucket	3
D	Ss	Stem	629,6	Sea Water	Wet	Bucket	3

3. Experimental Plan

3.1 Group A, The dry samples:

In the start of the experiment, the live algal were divided into old leaf, new leaf and stem. The samples were dried for 5 days at 80^{o}C. The dried samples were then milled and analyzed for the total laminaran and fucoidan content, as described in section chapter 1and 2.

3.2 Group B, The whole plant:

The whole plants were selected and placed in distilled water and sea water. The samples were taken out at regular intervals, initially, 400 ml (approximately) of sample was taken out after 95 min, 7 hours, 24 hours and 2 days and after that 200 ml of sample was taken from the cylinders for 3 day, 4 day, 5 day, 6 day, 7 day. The samples were immediately stored at -20 °C.

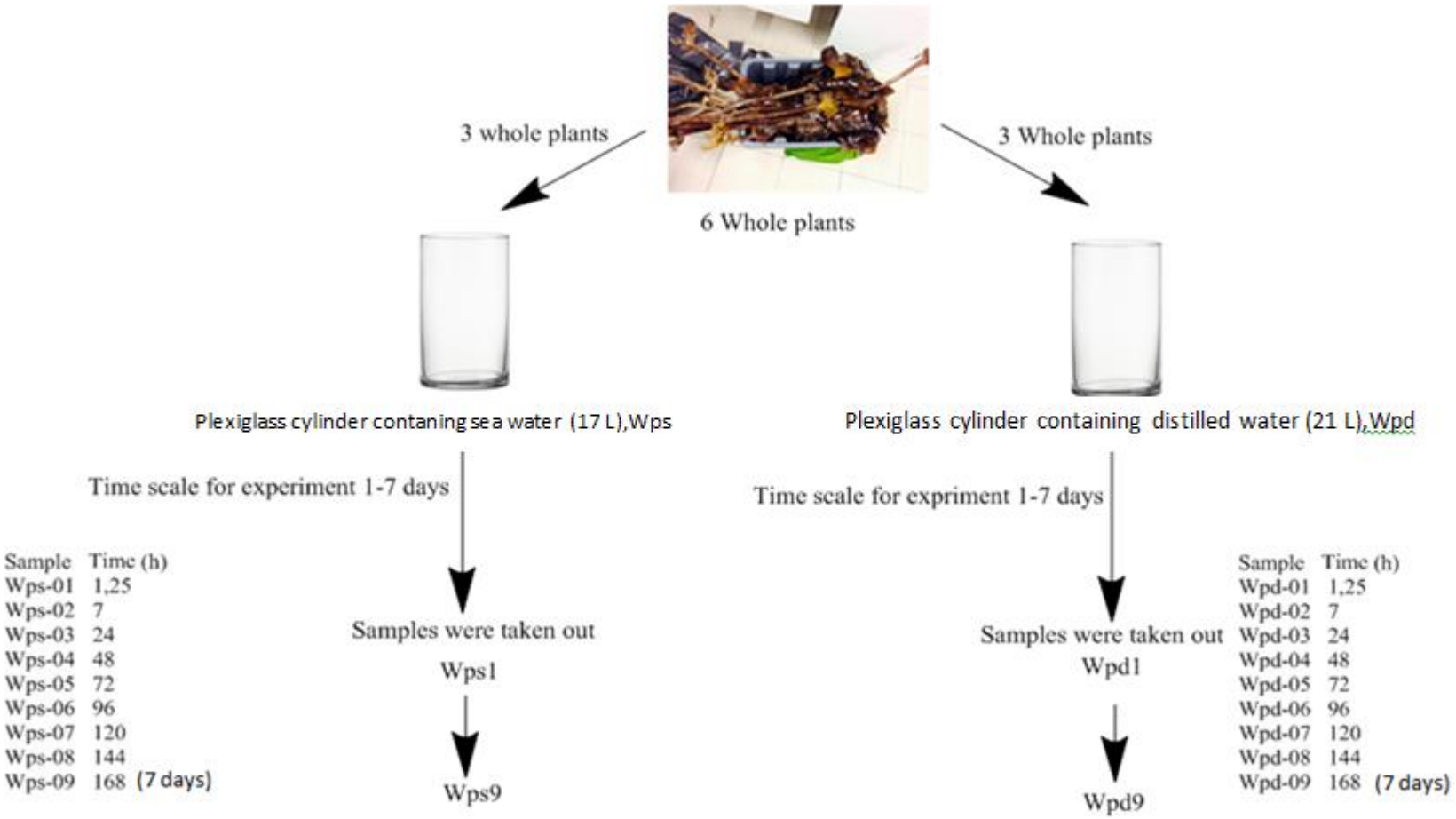

Figure 31: Experimental setup for Group B

After 7 days, these samples were thawed, filtered and freeze dried. The freeze dried samples were then dissolved in 15ml of distilled water and analyzed for total carbohydrate and fucoidan contents, as described in section 1 and 2.

3.4 Group C:

The algae in group C were divided into old leaf, new leaf and stem. They were damaged and put in distilled water for 7 days at 4°C. Initially, 30 ml samples were taken out after 95 min, 7 hours, 24 hours and 2 days. After that, 15 ml of sample was taken out for 3 day, 4 day, 5 day, 6 day, and 7 day samples. The samples were immediately stored at -20 °C.

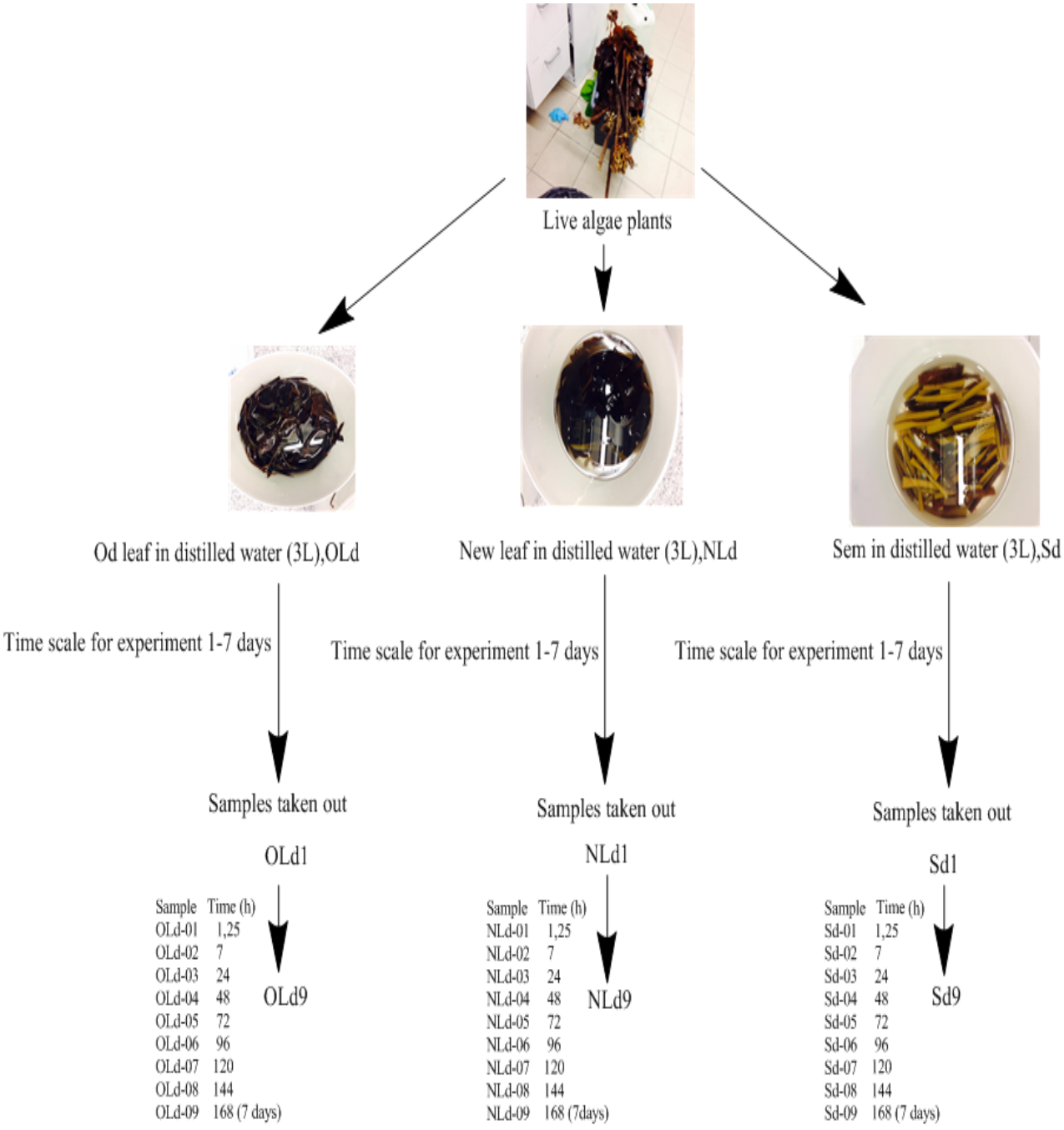

Figure 32: Experimental setup for group C

The samples were then thawed, filtered (1.5 µm) and analyzed for total carbohydrate and fucoidan content, as described in section 1 and 2.

3.5 Group D:

The algae samples in group C were divided into old leaf, new leaf and stem. They were damaged and put in distilled water for 7 days ar 4^{o}C. Initially, 30 ml samples were taken out after 95 min, 7 hours, 24 hours and 2 days. After the first four samples, 15 ml of sample was taken out for 3 day, 4 day, 5 day, 6 day, 7 day. The samples were immediately stored at -20 °C.

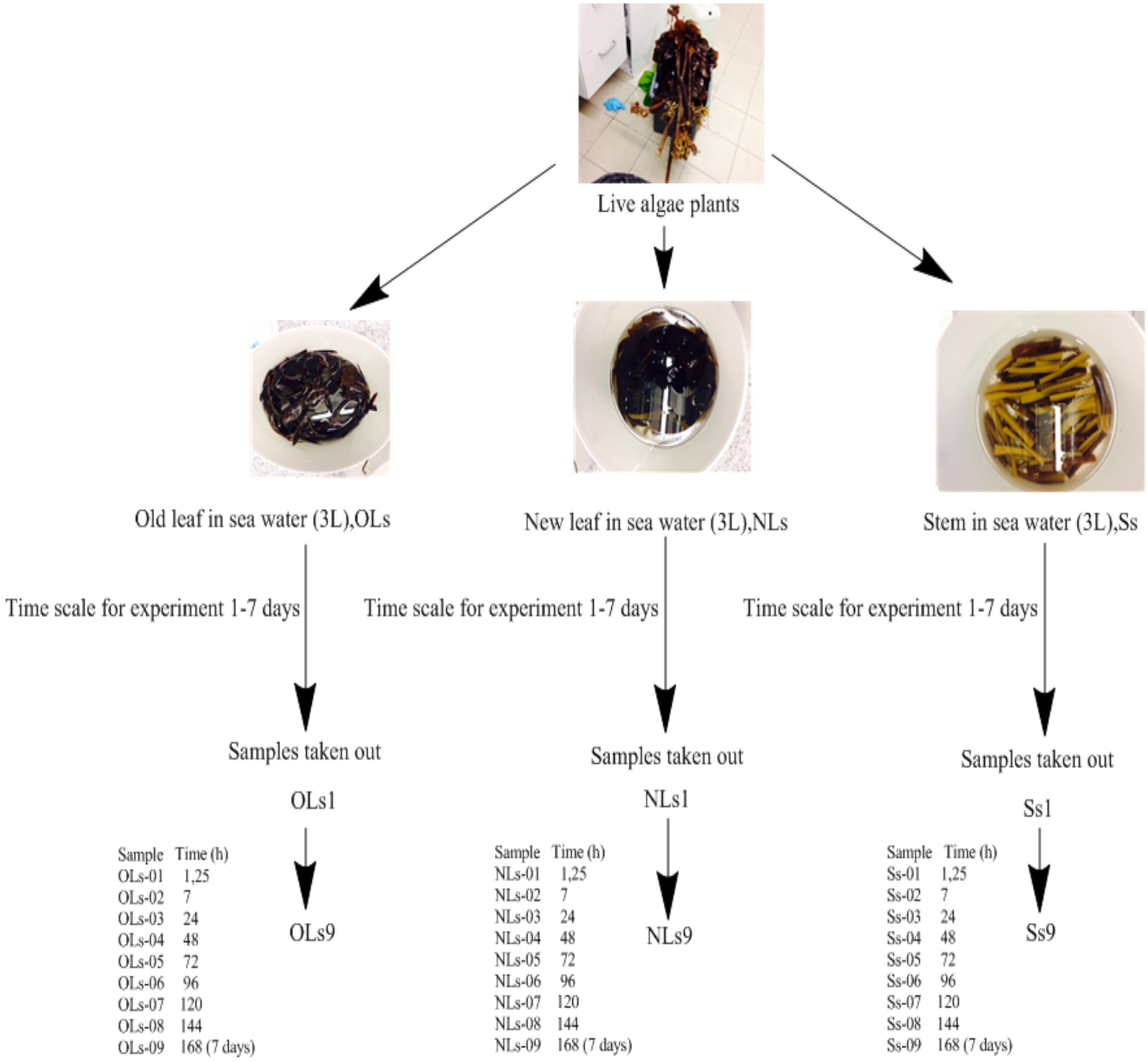

Figure 33: Experimental setup for group D

The samples were then thawed, filtered (1.5 µm) and analyzed for total carbohydrate and fucoidan content, as described in section 1 and 2.

4. Drying of algae:

At the end of experiment, the algae were taken out from the water and dried.

Group B

The whole *L. hyperborean* plants from plexiglass cylinder (both with sea water and distilled water) were taken out (after being placed in cylinders for 7 days) and divided into 3 separate parts; old leaf, new leaf and stem. All the plant parts were then dried for 5 days in dryer (80°C). The dried algal parts were then milled and analyzed for total laminaran and fucoidan contents, as described in section 1 and 2.

Group C

After 7 days, the old leaf, new leaf and the stem in the bucket with distilled water were taken out and placed in dryer (80°C) for 5 days. The dried samples were milled afterwards and analyzed for total laminaran and fucoidan contents, as described in section 1 and 2.

Group D

After 7 days, the old leaf, new leaf and the stem placed in bucket with sea water were taken out and placed in dryer (80°C) for 5 days. The dried samples were milled afterwards and analyzed for total laminaran and fucoidan contents, as described in section 1 and 2.

5. Results and discussion

The algal plants were harvested in late February which is a very critical time of the year as spring is starting and light is coming back after a long winter. The sea water starts to get warm and the conditions are becoming suitable for growth of algae and other phytoplankton. The process of photosynthesis in the algae is resuming. In the dark days of winter the algae had used most of its stored carbohydrate contents like laminaran and mannitol. These stored carbohydrates were generally used as a source of energy for the algae. Most of the algae selected were fully grown. They had a mature new leaf with an old leaf attached to them. As the process of photosynthesis resumes, laminaran and mannitol would be the first carbohydrates produced by the algae. The samples were collected from the depth of 3-4 meters which could suggests that the algae were present in the sublateral zone. The algae were harvested from the fjord and were kept alive as they were transported from the fjord to the university and the experiment was started with the live algae. The algae were damaged and cut in the same way as they might get damaged while they are being harvested commercially by FMC.

The live algae were placed in distilled water and sea water. A mucilaginous substance appeared from the damaged algae while no mucilaginous substance appeared in the samples collected from the whole plants (uncut), both for sea water and distilled water.

A peculiar color with the phenol-sulphuric acid analysis was observed when the samples from whole plant in distilled water (Wpd), old leaf in distilled water (OLd) and stem in sea water (Ss) were analysed for total carbohydrate contents. The samples from these algae were observed at different wavelengths and gave a higher absorbance at 269 nm wavelength compared to the samples that did not show any peculiar colour with phenol-sulphuric acid method for detection of total carbohydrates..

5.1 Fucoidan and laminaran contant in live algae

In the beginning of experiment, the total laminaran and fucoidan contents of algae were analyzed.

The graph below shows the total content of fucoidan and laminaran in the algae in the beginning of experiment.

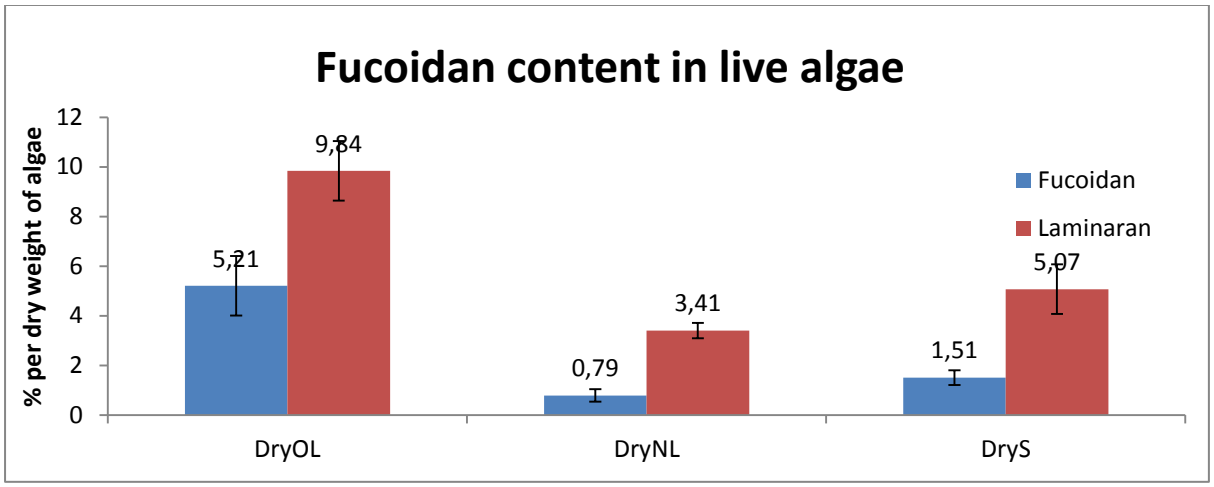

Figure 34: Total Fucoidan and Laminaran content in the algae in the beginning of experiment

5.2 Release of total carbohydrate and fucoidan from the algae

The algae were then placed in distilled water and sea water separately for 7 days and samples were taken out at regular intervals to check if any fucoidan or other carbohydrates are being excreted by the algae.

5.2.1 Group B: Fucoidan and total carbohydrate analysis

In group B, whole plants were placed in distilled water and sea water. The samples were taken out at regular intervals to check if any carbohydrate or fucoidan is being excreted by the algae into the extraction solution.

Whole Plant in Distilled Water, (Wpd)

The whole plant placed in distilled water showed a peculiar color when analyzed for the total carbohydrate content by phenol sulphuric acid analysis and thus the results for total carbohydrate being excreted by the whole algae were not reliable. However, the fucose content that might be coming out from the algae was specifically determined and it gave low concentrations of fucose.

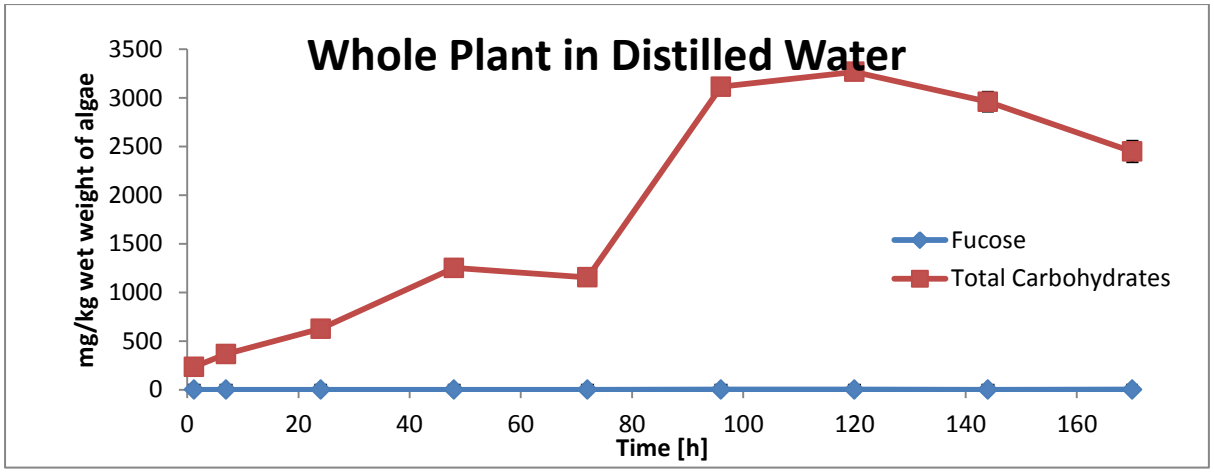

Figure 35: Shows the total amount of carbohydrate and fucose being released from whole plant during 7 days

The amount for total carbohydrate obtained for the whole plant in distilled water i.e. Figure 35, are unreliable as they gave an unusual color when detecting for total carbohydrate in phenol-sulphuric acid method.

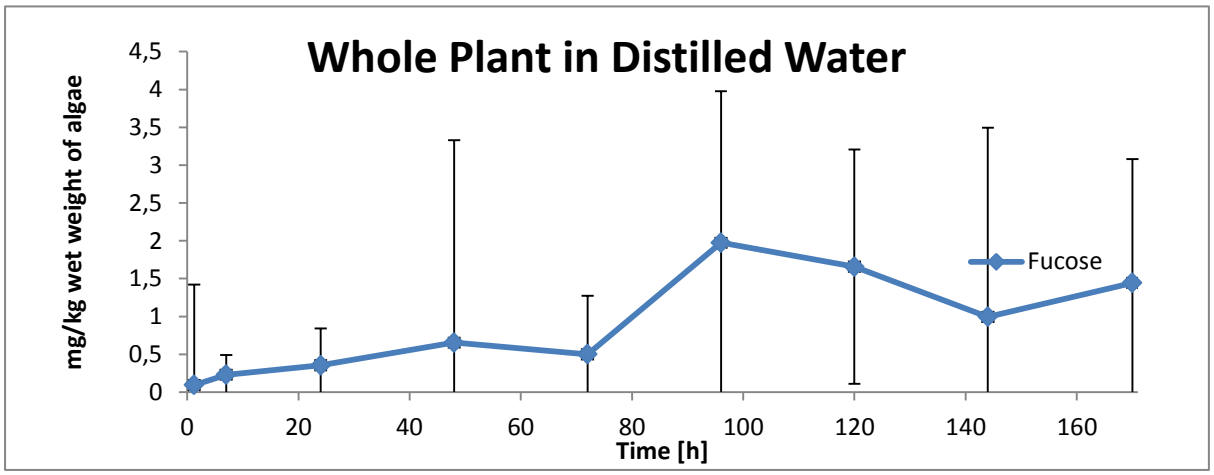

Figure 36: Total amount of fucose being released by the whole plant during 7 days

The fucose released from the whole plant in distilled water, Figure 36, was quantified but rather gave a large standard deviation. The fucose content released by the whole plant was found to be very low, < 2mg/kg wet weight of algae.

Whole Plant in Sea Water (Wps)

The whole plant was placed in sea water for 7 days. The graph below shows the concentration of total carbohydrate and fucose being excreted by the whole plant. The concentrations of both the total carbohydrate and fucose were low. This suggests the almost low amounts of fucose and low amounts of carbohydrate is excreted by the whole plant into the extraction solution. After 7 days the total amount of total carbohydrate in the solution was found to be 106 mg/kg wet weight of algae and fucose was 1.69 mg/kg wet weight of algae.

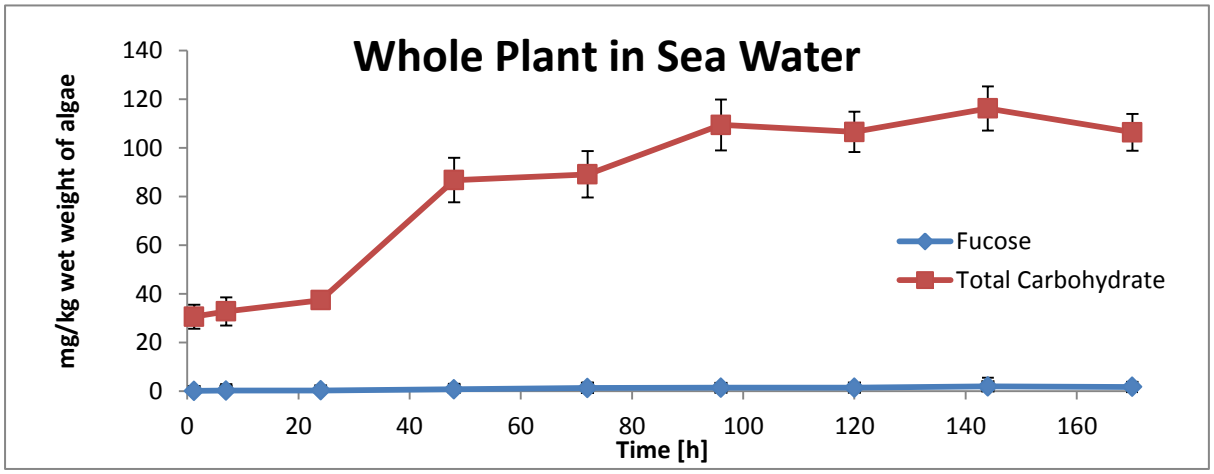

Figure 37: Shows the total amount of carbohydrate and fucose being excreted by the whole plant during t 7 days

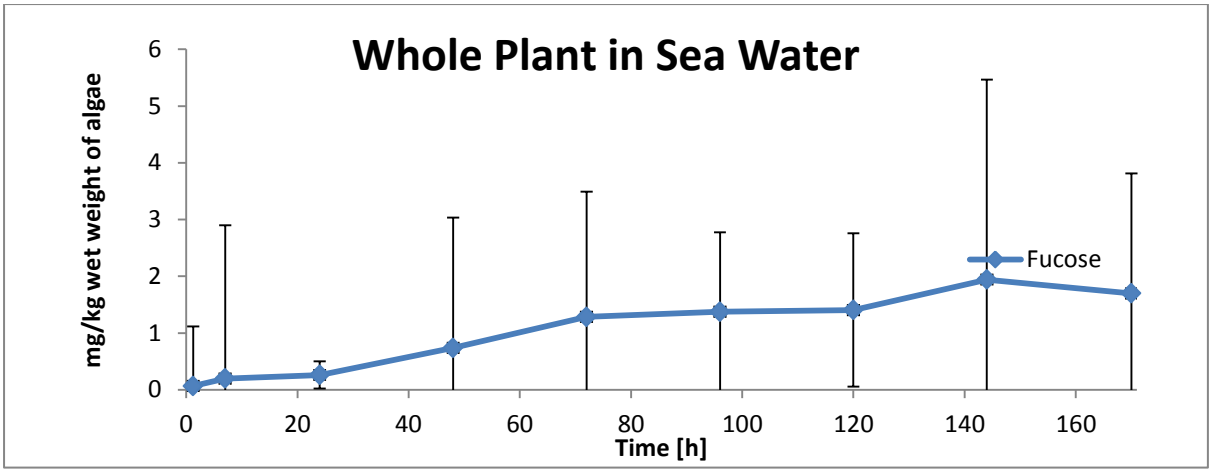

Figure 38: Shows the total amount of fucose being excreted by the whole plant during the 7 days

The fucose released from the whole plant in sea water, Figure 36, rather gave a large standard deviation. The fucose content released by the whole plant was found to be very low, < 2mg/kg wet weight of algae.

5.2.2 Group C: Fucoidan and total carbohydrate analysis

In group C, the algal parts were separated into old lead (Old), new leaf (NLd), stem (Ss) and placed in distilled water for 7 days. These algal parts were damaged. The samples were taken out after regular intervals and analyzed if any fucoidan or carbohydrates are being excreted by the damaged algae.

Old Leaf in Distilled Water (OLd)

Old leaf from the live algae were cut and placed in distilled water and the samples were taken out at regular intervals for 7 days The samples showed a peculiar color when analyzed for the total carbohydrate content by phenol sulphuric acid analysis and thus the results for total carbohydrate being excreted by damaged old leaf in distilled water obtained were unraliable. However, the fucose content that might be coming out from the algae was specifically determined.

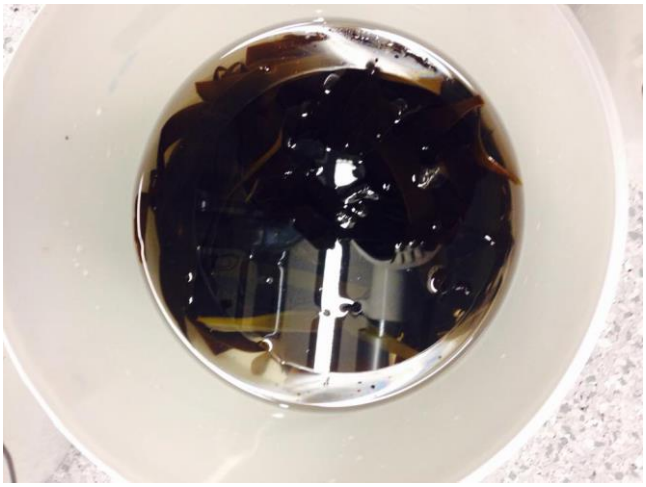

Figure 39: Damaged old leaf in distilled water

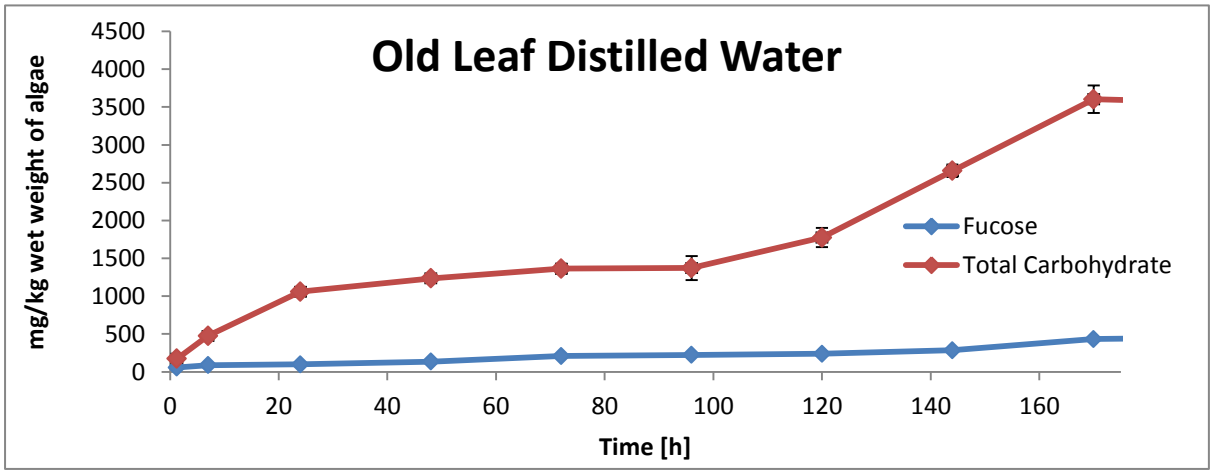

Figure 40: Amount of total carbohydrate and fucose being excreted by damaged old leaf in distilled water during 7 days

The amount for total carbohydrate obtained for the old leaf in distilled water i.e. Figure 40, are unreliable as they gave an unusual color when detecting for total carbohydrate in phenol-sulphuric acid method.

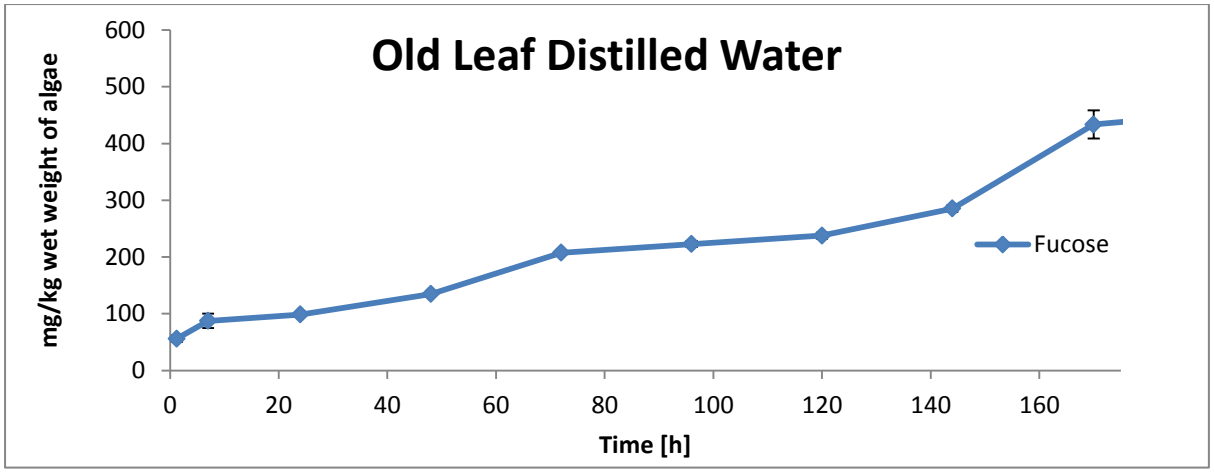

Figure 41: The total amount of fucose being excreted by the damaged old leaf in distilled water during 7 days

The fucose released from the damaged old leaf in distilled water, Figure 41, was reliably quantified. The fucose content released by the old leaf in distilled water increased with time. And after 7 days the fucose content was found to be around 400 mg/kg wet weight of algae.

New Leaf in Distilled Water (NLd)

New leaf from the live algae were cut and placed in distilled water and the samples were taken out at regular intervals for 7 days. The graph below shows the concentration of total carbohydrate and fucose being excreted by damaged new leaf placed in distilled water. The graphical trend shows that fucose is not the main polysaccharide that is being extruded from the damaged algae. The fucose concentration being excreted by the damaged new leaf is low compared to the concentration of total carbohydrate coming out. However, fucoidan is being excreted by the damaged new leaf.

Figure 42: Damaged new leaf in distilled water

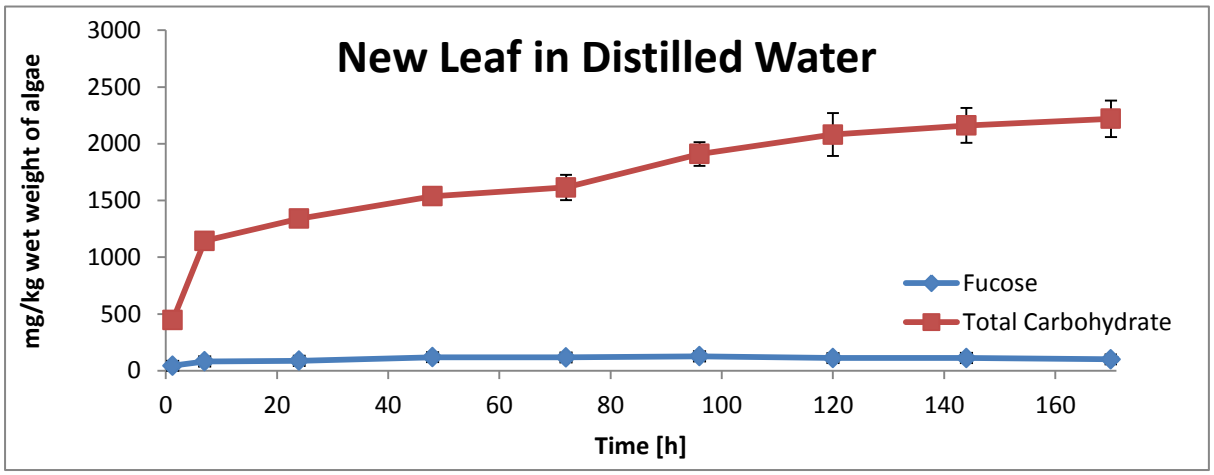

Figure 43: Amount of total carbohydrate and fucose being excreted by damaged new leaf in distilled during 7 days

The carbohydrates released form the new leaf in distilled water, Figure 43, shows an increase with time. The overall release of total carbohydrates from the new leaf seems to be high, after 7 days the total carbohydrate released form the new leaf are around 2500 mg/kg wet weight of algae.

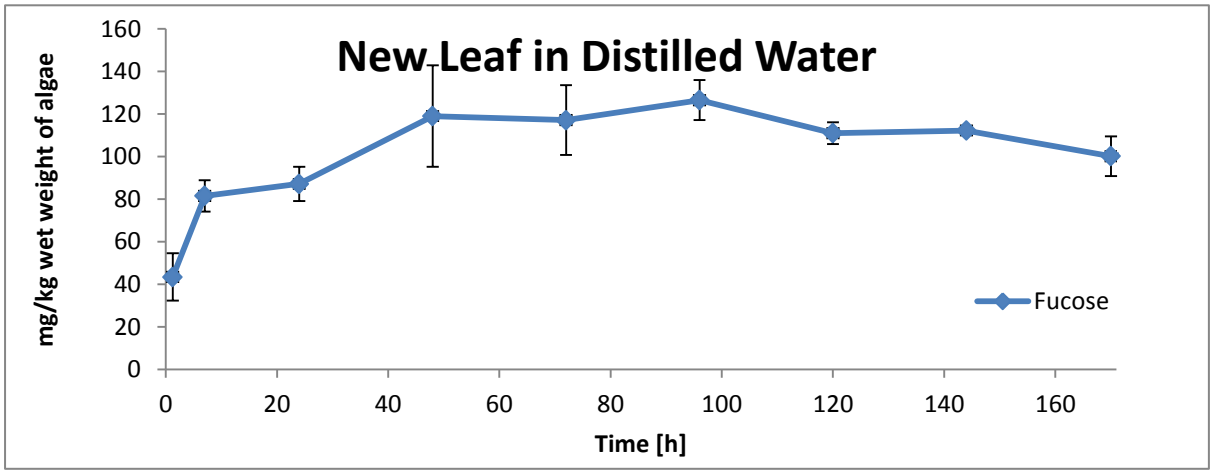

Figure 44: The total amount of fucose being excreted by the damaged new leaf in distilled water during 7 days

The fucoidan released from the new leaf in distilled, Figure 44, shows a general increase until 100 hours, after that it shows little stability with time. After 7 days the fucose released form the damaged new leaf was around 100 mg/kg wet weight of algae.

Stem in Distilled Water (Sd)

Stem from the live algae were cut and placed in distilled water and the samples were taken out at regular intervals for 7 days. The graph below shows the concentration of total carbohydrate and fucose being excreted by damaged stem placed in distilled water. The graphical trend shows that both the total carbohydrate and fucose concentration increase during the period. The total carbohydrates being excreted by the damaged stem is twice that of fucose which suggests that from the stem of *L. hyperborean*, upon receiving external damage, fucoidan is almost half the amount of total carbohydrates that are coming out from the algae.

Figure 45: Damaged stem in distilled water

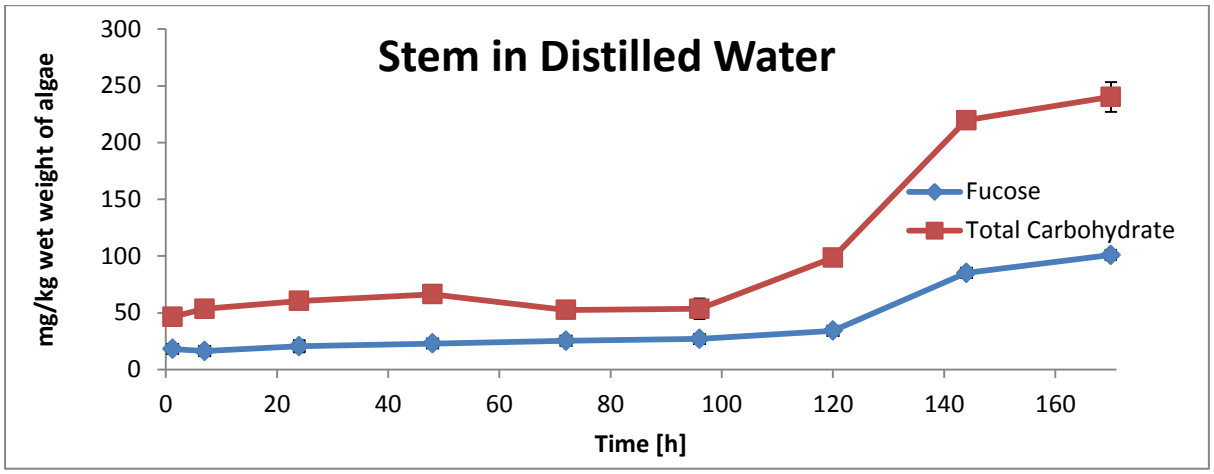

Figure 46: Amount of total carbohydrate and fucose being excreted by damaged stem in distilled water during 7 days

The fucose and total carbohydrate released from the damaged stem in distilled water, Figure 46, shows an increase with time. The is an unexpected increase after 120 hours, both for the total carbohydrate and fucose released from the damaged stem in distilled water.

5.2.3 Group D: Fucoidan and total carbohydrate analysis

In group D, the algal parts were separated into old lead (Old), new leaf (NLd), stem (Ss) and placed in sea water for 7 days. These algal parts were damaged. The samples were taken out after regular intervals and analyzed if any fucoidan or carbohydrates were being excreted by the damaged algae parts.

Old Leaf in sea water (OLs):

Old leaf from the live algae were cut and placed in sea water and the samples were taken out at regular intervals for 7 days. The graph below shows the concentration of total carbohydrate and fucose being excreted by damaged old leaf in distilled water. The concentration of total carbohydrate and fucose being excreted by the damaged algae are similar. This suggests that the fucoidan and total carbohydrate released by the damaged old leaf are of equal magnitude.

Figure 47: Damaged old leaf in distilled water

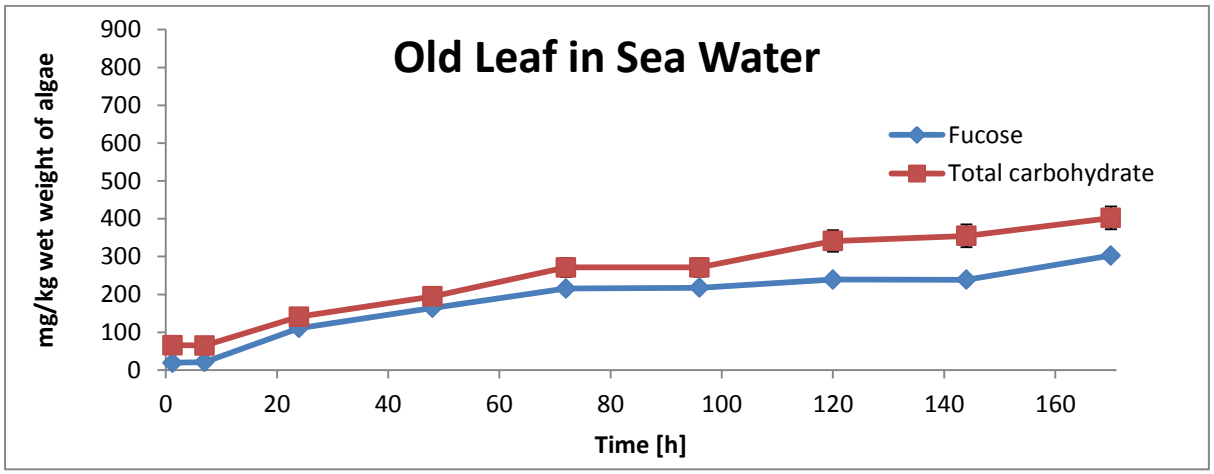

Figure 48: Shows the amounts of total carbohydrate and fucose being excreted by the damaged old leaf placed in sea water during 7 days.

The fucose and total carbohydrate released from the damaged old leaf in sea waters, Figure 46, shows an increase with time. The total carbohydrate and fucose are released at a similar rate.

New Leaf in Sea Water (NLs)

New leaf from the live algae were cut and placed in sea water and the samples were taken out at regular intervals for 7 days. The graph below shows the concentration of total carbohydrate and fucose being excreted by damaged new leaf placed in sea water. The graphical trend

shows that fucose is not the main component among the total carbohydrates being extruded from the damaged algal new leaf. The concentration of fucoidan coming out from algae is very low compared to the total carbohydrates that are coming out from the algae.

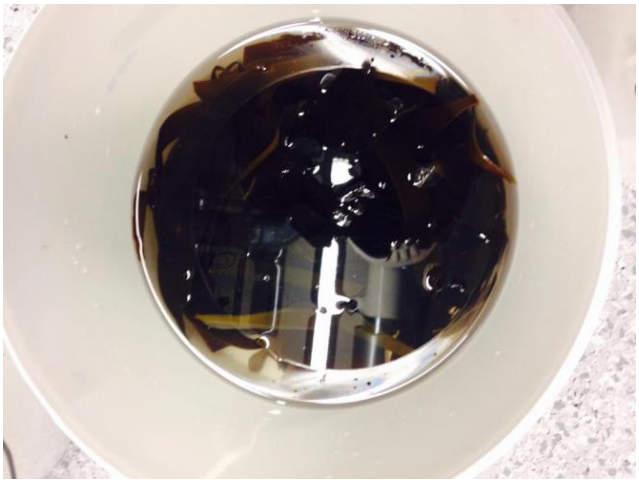

Figure 49: Damaged New leaf in sea water

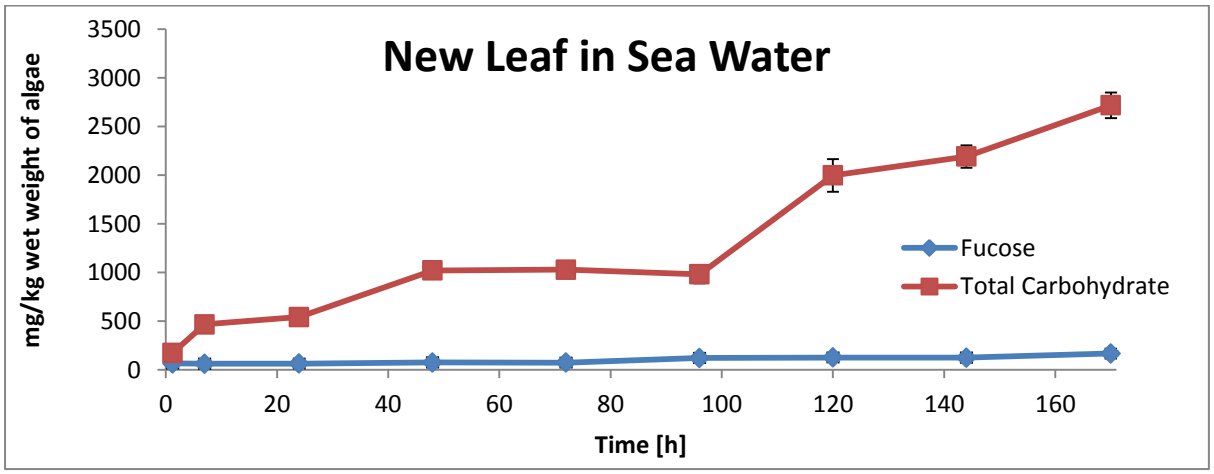

Figure 50: Shows the concentration of total carbohydrate and fucose being excreted by damaged new leaf in sea water during 7 days

The carbohydrates released form the new leaf in sea water, Figure 50, shows an increase with time. The overall release of total carbohydrates from the new leaf seems to be high, after 7 days the total carbohydrate released form the new leaf are around 2500-3000 mg/kg wet weight of algae.

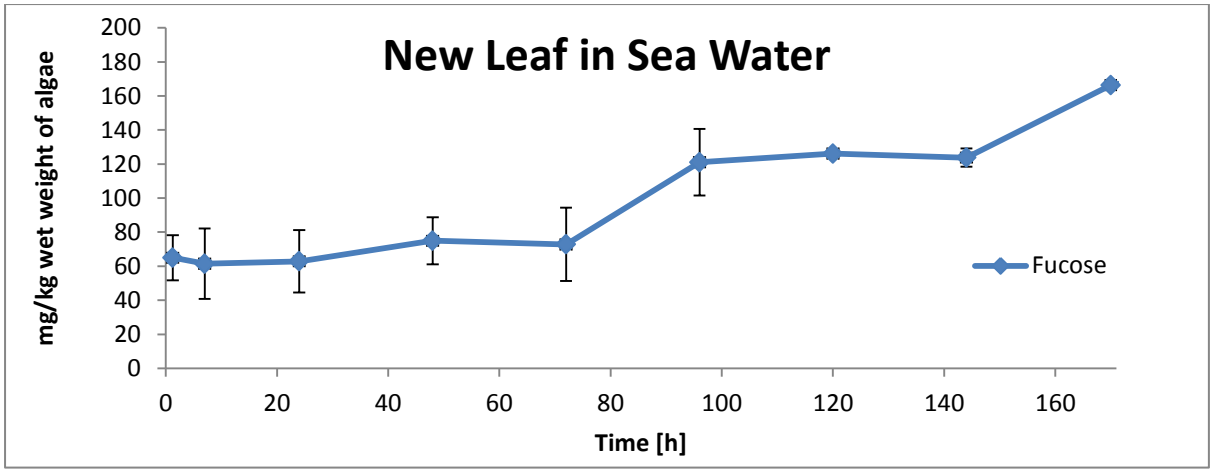

Figure 51: Shows the total amount of fucose being excreted by the damaged new leaf in sea water during 7 days

The fucoidan released from the new leaf in sea water, Figure 51, shows a general increase with time. But the overall release of fucose is low compared to the release of total carbohydrates from the algae. After 7 days the fucose released form the damaged new leaf was around 140-160 mg/kg wet weight of algae.

Stem in the Sea Water (Ss)

Stem from live algae were cut and placed in distilled water and the samples were taken out at regular intervals for 7 days. The samples showed a peculiar color when analyzed for the total carbohydrate content by phenol sulphuric acid analysis and thus the results for total carbohydrate being excreted by damaged stem placed in sea water were not obtained. The fucose content that might be leaking out from the algae was specifically determined.

Figure 52: Damaged stem placed in distilled water

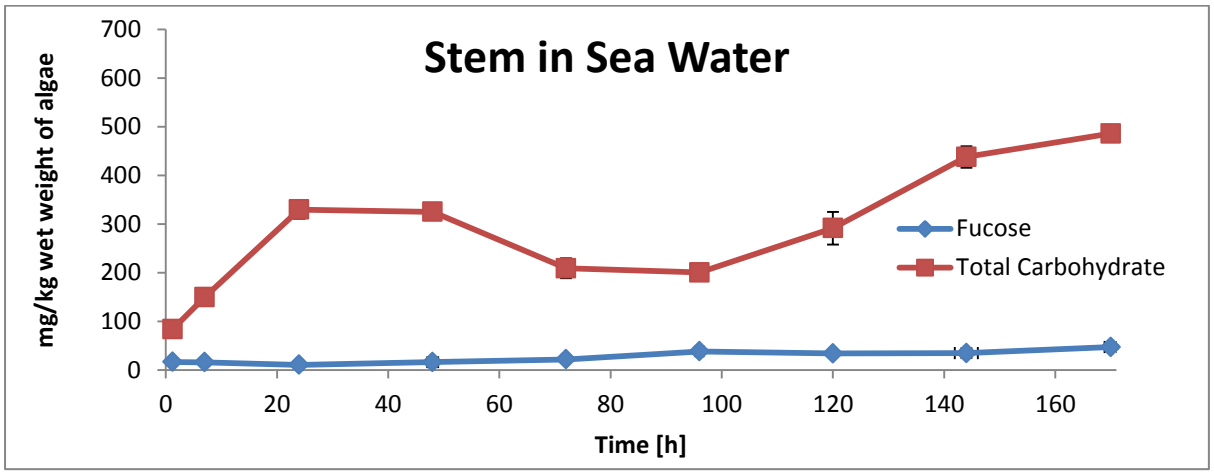

Figure 53: Shows the amount of total carbohydrate and fucose being excreted by damaged stem in sea water during 7 days

The amount for total carbohydrate obtained for the damaged stem in sea water i.e. Figure 53, are unreliable as they gave an unusual color when detecting for total carbohydrate in phenol-sulphuric acid method.

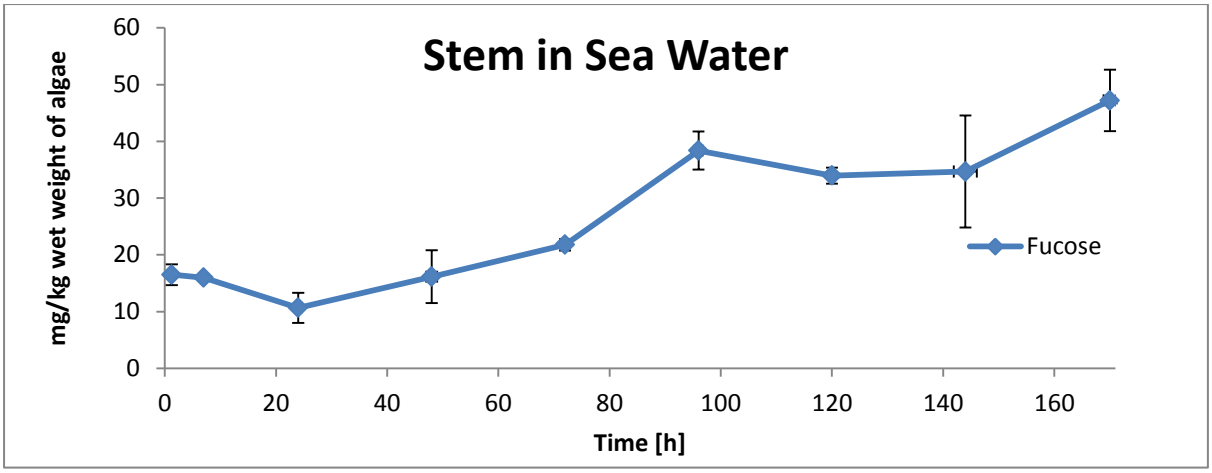

Figure 54: Shows the total amount of fucose being excreted by the damaged stem in distilled water during 7 days

The fucose released from the stem in sea water, Figure 54, was reliable quantified. The fucose content released by the stem in sea water increased with time. And after 7 days the fucose content was found to be around 50 mg/kg wet weight of algae.

6. Polyphenols

Marine brown algae can release organic substances into the surrounding water (Ragan and Jensen 1979) and among these metabolites are also polyphenols (Geiselman and McConnell 1981). Polyphenols may be present in large quantities in brown algae. These polyphenols can be released into the surrounding under stress conditions (Sieburth and Jensen 1970).

Some of the samples that were collected from specific algal parts placed in water gave a peculiar color in phenol sulphuric acid. It was assumed that the peculiar colour might be due to the presence of polyphenols. A uv-spectrophotometric method was used to measure the presence of polyphenol.

6.1 Samples

The samples that gave a peculiar color in the phenol sulphuric acid analysis were; old leaf placed in distilled water (Old), stem in sea water (Ss) and whole plant in distilled water (Wpd).

6.2Wavelength spectrum

A wavelength spectrum was prepared. The graph below shows the wavelength spectrum obtained for the old leaf both in sea water and in distilled water at wavelengths ranging from 240nm to 300 nm.

The samples used to make the absorbance spectrum were OLd-09 (7 days) and OLs-09 (7 days). A 0.01% phenol was used as a reference.

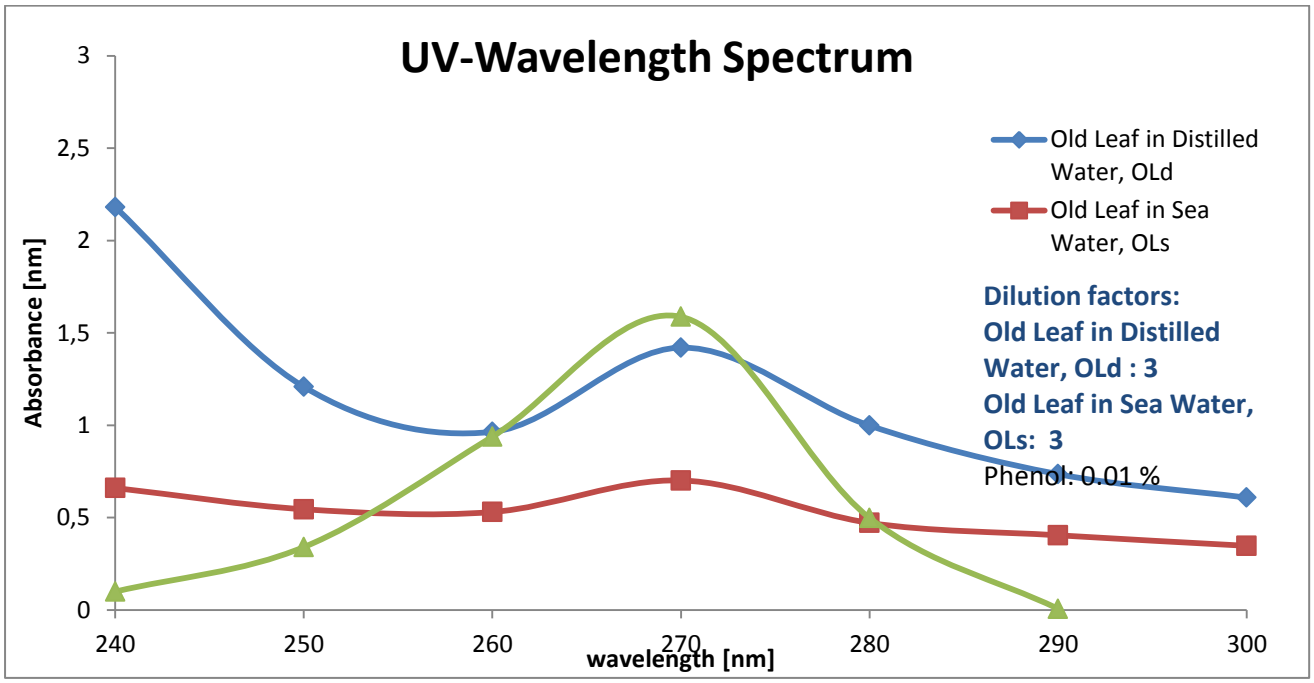

Figure 55: Wavelength spectrum for OLd-09 and OLs-09

The spectrum shows that pure phenol (0.01%) gave a peak at 269 nm suggesting that phenols gives a higher absorbance at 269 nm. Both the algal samples also showed a peak at 269 nm which suggested that phenols were present in the algal samples.

6.3 Presence of phenols

Whole Plant in Distilled Water, (Wpd) and Whole Plant in Sea Water, (Wpds)

A graph was plotted for whole plants placed both in distilled water and sea water for detection of phenols. The samples collected for 7 days were measured at 269 nm wavelength.

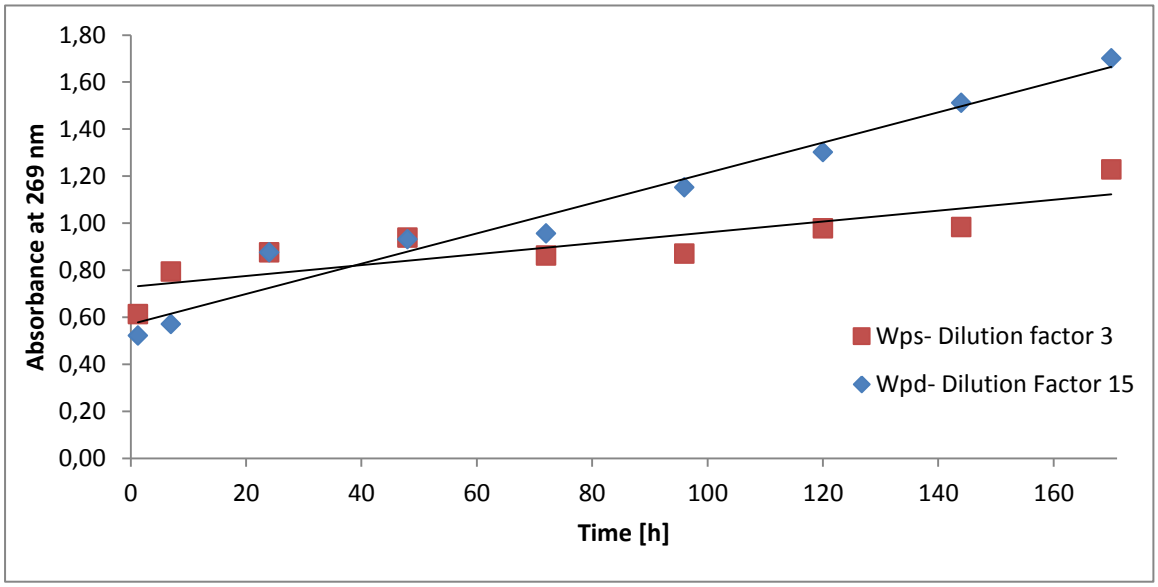

Figure 56: Absorbance of Wpd and Wps at 269 nm

The Whole plant placed in distilled water was diluted 15 times more compared to the samples from whole plant sea water. The whole plants placed in distilled water, initially, gave a peculiar color with phenol sulphuric acid analysis and its response at 269nm is 5 times more. This suggests that the whole plant in distilled water might released 5 times more polyphenols into its surround than the whole plant placed in sea water. The higher concentration of these polyphenols might have given a peculiar color for the phenol sulphuric acid analysis.

Old Leaf in Distilled Water (OLd) and Old Leaf in Sea Water (OLs)

The old leaf placed in distilled water gave a peculiar colour when detecting for the total carbohydrate contents with phenol sulphuric acid method while old leaf placed in sea water showed no strange colour with phenol sulphuric acid analysis. The OLd-09 and OLs-09 samples were measured at 269nm to check the presence or absence of phenols. The table below shows the comparison for any phenol release.

Table 4: Absorbance at 269 nm for OLd and Ols

	Old leaf in distilled water (OLd)	Old leaf in sea water (OLs)
Absorbance at 269 nm	0,642	0,617
Dilution factor	6	3

The old leaf placed in distilled water shows a 3 times higher absorbance compared to the old leaf placed in distilled water. This shows that old leaf placed in distilled released 3 times more polyphenols compared to the Old leaf in distilled water. The higher concentration of these polyphenols might have reacted and gave a peculiar colour for the phenol sulphuric acid analysis.

Stem in Distilled Water (Sd) and Stem in Sea Water (Ss)

The stem in placed in sea water gave a peculiar colour with phenol sulphuric acid while the stem placed in distilled water showed no strange colour with phenol sulphric acid. It was assumed that stem in sea water might have released polyphenols that might have interfered with the phenol sulphuric acid analysis. The Sd-09 and Ss-09 samples were measured at 269nm to check the presence or absence of phenols.

The table below shows the comparison for any phenol release.

Table 5: Absorbance at 269 nm for Sd-09 and Ss-09

	Stem in distilled water (Sd)	Stem in sea water (Sd)
Absorbance at 269 nm	0.650	0.680
Dilution factor	1	3

The stem placed in sea water gives a 3 times higher absorbance compared to the stem placed in distilled water. This shows that stem placed in sea water have released 3 times more polyphenols that might reacted and gave a strange colour with phenol sulphuric acid.

6.4 Induced fucoidan production upon external damage

The *L. hyperborea* parts that were placed in extraction solution for 7 days were taken out and dried. They were then analyzed for the total fucoidan content. The total remaining content of fucoidan from these algae was compared to the total contents of fucoidan of the live algae (in the start of experiment) to check for any increase or decrease of fucoidan contents. The following results were obtained.

Old Leaf:

Old Leaf in Distilled Water (OLd)

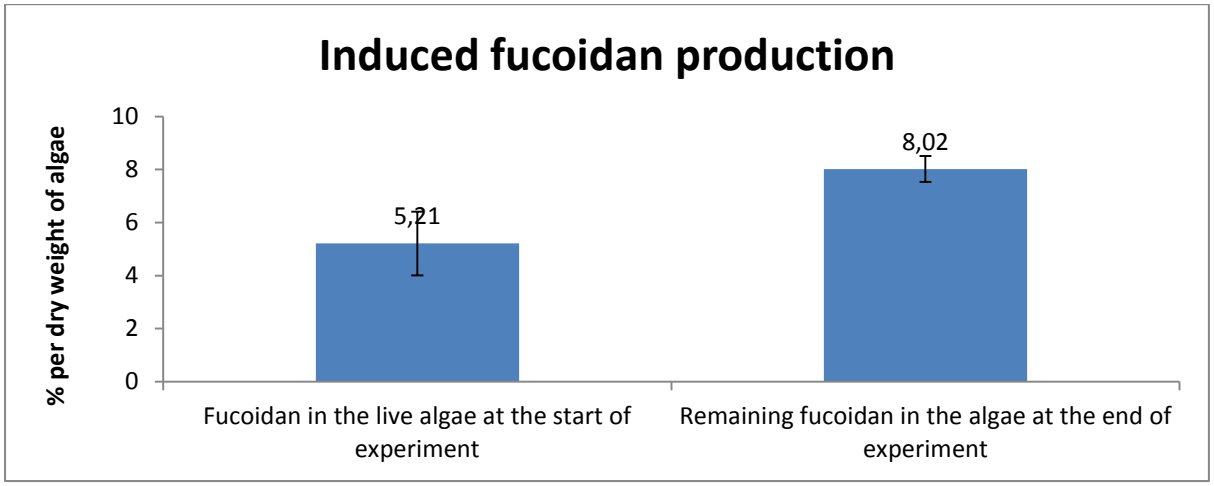

Figure 57: Induced production of fucoidan for OLd

Old Leaf in Sea Water (OLs)

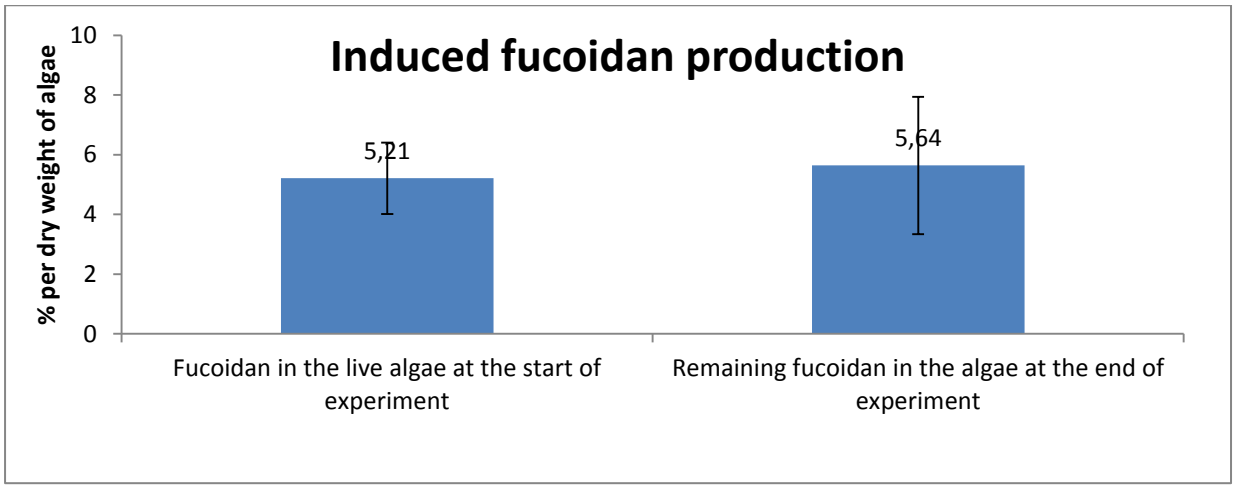

Figure 58: Induced production of fucoidan for OLs

New Leaf:

New leaf in Distilled Water (NLs)

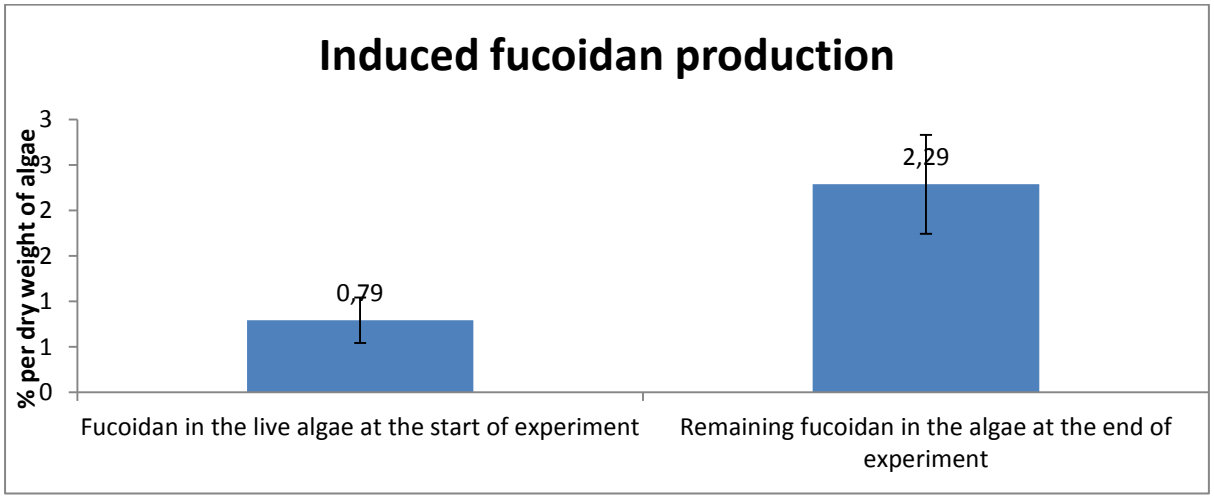

Figure 59: Induced production of fucoidan for NLd

New leaf in Sea Water (NLs)

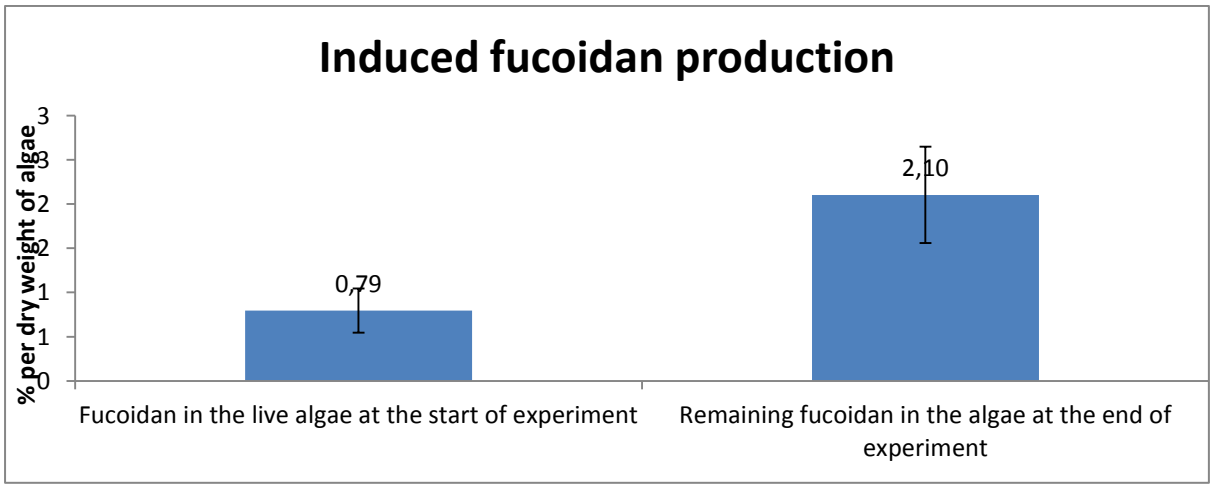

Figure 60: Induced production of fucoidan for NLs

Stem:

Stem in Distilled Water (Sd)

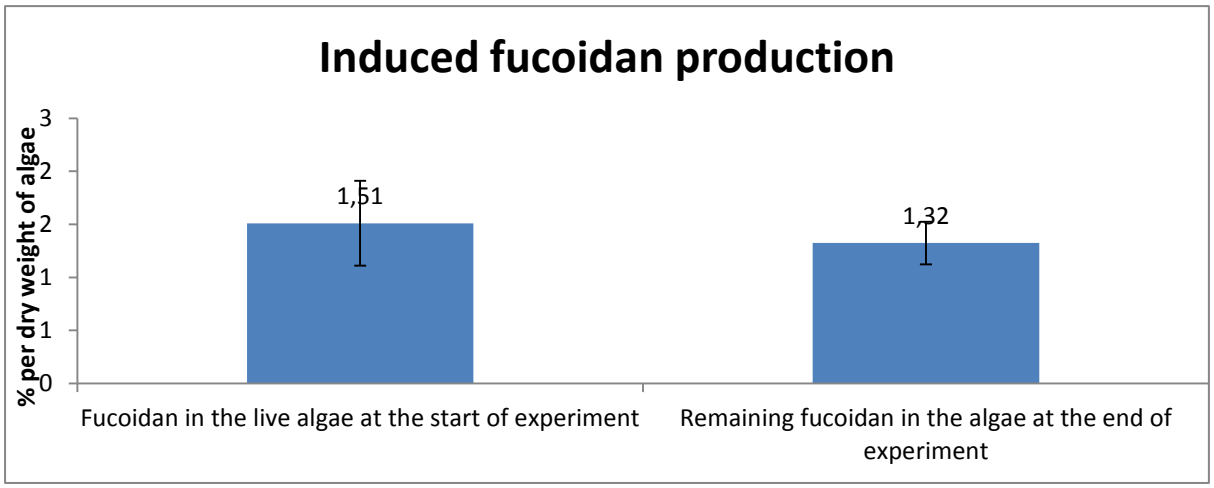

Figure 61: Induced production of fucoidan for Sd

Stem in Sea Water (Ss)

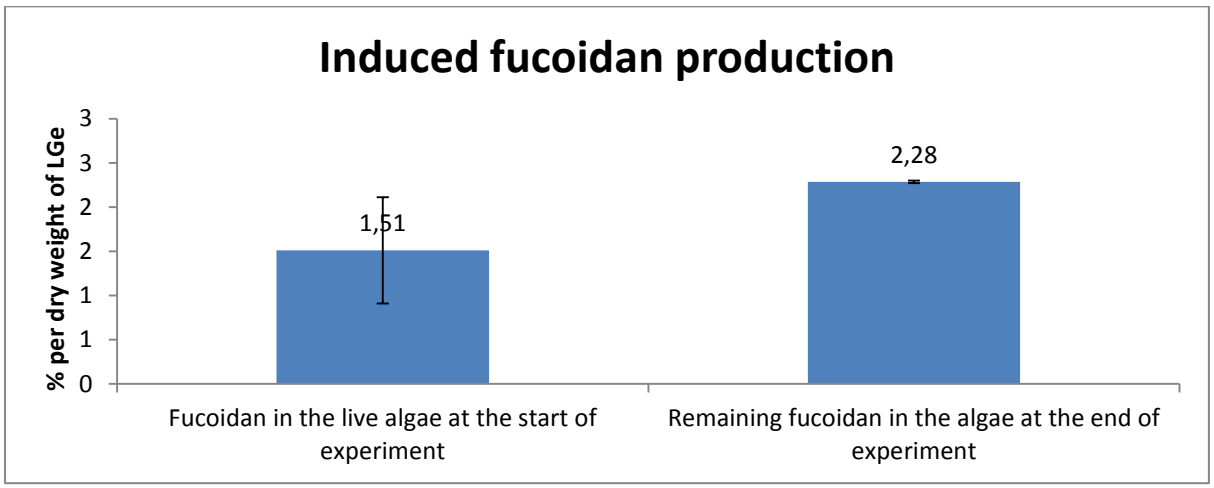

Figure 62: Induced production of fucoidan for Ss

The results showed increase and/or decrease in fucoidan contents for the live algae and after being damaged and placed in sea water and distilled water, releasing its fucoidan for 7 days. Several dried algae samples were showing an increased content of fucoidan after the extraction for 7 days. However, to determine any increase or decrease for the total fucoidan content of the algae after damage is difficult. The algae might release many polysaccharides, polyphenols, salts and proteins into its surroundings. This release of metabolites and organic substances would influence the total fucoidan content per dry weight. The fucoidan content per dry weigth might increase for the algae due to the release of these other organic substances. So, the hypothesis remained unclear whether any external damage stimulates the produce fucoidan in the algae. But it has been observed that the damaged alga releases fucoidan into the surroundings.

7. Conclusion

Whole algae of *L.hyperborea* submerged in distilled water and seawater in darkness and at 4^{o}C were found to release very low amounts of both total carbohydrates and fucose. The release of total carbohydrate from plants in seawater gave a maximum of 100 mg/kg wet weight of algae while the whole plant placed in distilled water could not be reliably determined as it gave an unrealistic color for phenol sulphuric acid analysis. The release of total carbohydrate from old leaf in distilled water also showed an unrealistic color for phenol sulphuric acid analysis and thus could not be determined reliably, while the release of fucose from old leaf was found to increase up to about 400 mg/kg wet weight of algae. The old leaf in seawater, both the total carbohydrate and fucose was found to increase and to about 400 mg/kg wet weight, i.e. almost all carbohydrate released from the old leaf is thus fucoidan. For the release of total carbohydrate from new leaf in both distilled water and seawater were found to increase to up to around 2500-3000 mg/kg wet weight. The release of fucose from new leaf both in fresh water and seawater were found to increase up to around 120-140 mg/kg wet weight of algal i.e. very much lower compared to the total carbohydrate content released from the damaged new leaf.

The stem in distilled water, showed an increase up to around 200 mg/kg wet weight for total carbohydrate, while fucose increased up to 50-100 mg/kg wet weight, i.e. about half of the carbohydrate released from the stem is fucoidan. The release of total carbohydrate from stem in seawater could not be reliable determined. The release of fucose from stem in seawater was found to increase to about 50 mg/kg wet weight, which was not very different from the release of fucose from stem in distilled water.

Generally, the whole plants both in distilled water and seawater gave a very much lower amounts (on the level of few percent) for fucoidan released, compared with the release of fucoidan from the damaged parts of the plants. No large differences between the release from whole plants and the parts of the plants in distilled water as compared to seawater could be found. But, the release of fucoidan from the individual plant parts compared to the whole plants was quite different. The old leaf released almost exclusively fucoidan, where new leaf released quite low amounts (around 10 %) and stem releasing about half of the total carbohydrates as fucoidan.

The total carbohydrates contents of the whole plant in distilled water, old leaf in distilled water and stem is sea water could not be determined reliably. The samples from these algae gave a higher absorbance at 269nm which is in the range of polyphenols. The algae samples that gave a reliable color in phenol sulphuric acid method, showed a relatively lower absorbance at 269 nm. This might suggest that more polyphenols might have been released by the algae parts that show a peculiar color with phenol sulphuric acid analysis.

The fucoidan content of algal samples, before and after the extractions were determined. Several dried algae samples were showing an increased content of fucoidan after the extraction but this could have been caused by a faster release of other compounds e.g. laminaran, proteins, salts and phenols, resulting in a counterfeit increase of the fucoidan amount. The determined change in content was too small to answer that question precisely. So, if fucoidan is produced by the plant in respond to damage could neither be proved nor disapproved.

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Appendix

Whole plant

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Dry weigh	Wet weigh	Water	Vol. [L]
Wpd	Old leaf	329,4	1647	distilled	21

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	--Stand.--	[h]
Sam. Conc.	--Blank--										1	[mg/ml]
Sam. Vol.	--Blank--	200	200	100	100	100	50	50	50	50	100	[µl]
Dest. Water	2000	1800	1800	1900	1900	1900	1950	1950	1950	1950	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		10	10	20	20	20	40	40	40	40	20	
Volume before freeze drying		381,2	389,5	421,4	227,8	258,3	194,6	205,9	211,5	217,1		
Volume after freeze drying		15	15	15	15	15	15	15	15	15		
Absorbance											485	[nm]
Parallel 1	0,000	0,429	0,667	0,670	0,691	0,723	0,731	0,818	0,776	0,668	0,430	[nm]
Parallel 2	0,001	0,430	0,689	0,624	0,688	0,686	0,729	0,818	0,768	0,625	0,436	[nm]
Parallel 3	0,002	0,441	0,726	0,631	0,701	0,768	0,751	0,821	0,741	0,643	0,463	[nm]
Average	0,001	0,433	0,694	0,642	0,693	0,726	0,737	0,819	0,762	0,645	0,443	[nm]
Total carbohydrate in sample	--Blank--	469,397	746,702	1382,057	1491,986	1560,780	3169,787	3518,723	3274,752	2779,716	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	18,471	28,756	49,195	98,243	90,638	244,331	256,342	232,252	192,058		[mg/L]

Release of fucose from algae

Sample:

Setup	Plant Part	Wet weigl	Wet weigh	Water	Vol. [L]
Wpd	Old leaf	329,4	1647	distilled	21

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	--Stand.--	[h]
Sam. Conc.												0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	200	200	200	200	200	200	200	200	200	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1960	1960	1960	1960	1960	1960	1960	1960	1960	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	13	13	13	13	13	13	13	13	13	26	
Absorbance before adding enzyme													
Parallel 1	0,000	0,002	0,099	0,153	0,248	0,271	0,291	0,433	0,329	0,283	0,198	0,005	[nm]
Parallel 2	-0,005	0,005	0,122	0,142	0,242	0,275	0,287	0,418	0,331	0,272	0,187	-0,014	[nm]
Parallel3	-0,003	0,011	0,130	0,146	0,221	0,261	0,295	0,424	0,324	0,264	0,189	0,002	[nm]
Average	-0,003	0,006	0,117	0,147	0,237	0,269	0,291	0,425	0,328	0,273	0,191	-0,002	[nm]
Absorbance after adding enzyme													
Parallel 1	0,000	0,007	0,100	0,198	0,299	0,302	0,333	0,499	0,399	0,313	0,267	0,755	[nm]
Parallel 2	-0,005	0,003	0,138	0,176	0,290	0,307	0,322	0,501	0,406	0,322	0,265	0,745	[nm]
Parallel3	-0,002	0,001	0,157	0,178	0,321	0,306	0,324	0,511	0,401	0,325	0,257	0,769	[nm]
Average	-0,002	0,004	0,132	0,184	0,303	0,305	0,326	0,504	0,402	0,320	0,263	0,756	[nm]
Volume before freeze drying			381,2	389,5	421,4	227,8	258,3	194,6	205,9	211,5	217,1		[ml]
Volume after freeze drying			15	15	15	15	15	15	15	15	15		[ml]
Fucose													
Difference betweenAbs	0,000	-0,002	0,015	0,037	0,066	0,036	0,035	0,079	0,074	0,047	0,072	0,759	[nm]
Conc. In cuvette	-0,004	-0,070	0,360	0,929	1,675	0,903	0,886	1,989	1,870	1,183	1,811	19,292	[µg/ml]
Conc. In sample	--Blank--	--Blank--	4,722	12,167	21,944	11,833	11,611	26,056	24,500	15,500	23,722	505,444	[µg/ml]
Concentration of fucose in sample	--Blank--	--Blank--	4,722	12,167	21,944	11,833	11,611	26,056	24,500	15,500	23,722		[mg/L]
Concentration of fucose in sample	--Blank--	--Blank--	0,186	0,469	0,781	0,779	0,674	2,008	1,785	1,099	1,639		[mg/L]

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Dry weigh	Wet weigl	Water	Vol. [L]
Wps	Whole Pl.	324,0	1620,0	seawater	17

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	--Stand.--	[h]
Sam. Conc.	--Blank--										1	[mg/ml]
Sam. Vol.	--Blank--	200	200	200	200	200	200	200	200	200	100	[µl]
Dest. Water	2000	1800	1800	1800	1800	1800	1800	1800	1800	1800	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		10	10	10	10	10	10	10	10	10	20	
Volume before freeze drying		389,5	421,4	427,8	217,8	228,3	191,6	211,9	201,5	221,1		
Volume after freeze drying		15	15	15	15	15	15	15	15	15		
Absorbance											485	[nm]
Parallel 1	0,000	0,067	0,079	0,087	0,105	0,108	0,118	0,128	0,129	0,131	0,430	[nm]
Parallel 2	0,001	0,063	0,067	0,089	0,109	0,118	0,113	0,129	0,132	0,132	0,436	[nm]
Parallel 3	0,002	0,060	0,078	0,087	0,101	0,115	0,121	0,124	0,135	0,135	0,463	[nm]
For Carbohydrate												
Average	0,001	0,063	0,075	0,088	0,105	0,114	0,117	0,127	0,132	0,133	0,443	[nm]
Total carbohydrate in sample	--Blank--	75,780	87,837	101,667	120,106	129,326	133,227	143,511	148,830	149,539	44,844	[µg/ml]
total carbohydrate in sample	--Blank--	2,918	3,127	3,565	8,272	8,497	10,430	10,159	11,079	10,145		[µg/ml]

Release of fucose from algae

Sample:

Setup	Plant Part	Dry weigh	Wet weigl	Water	Vol. [L]
Wps	Whole Pl.	324,0	1620,0	seawater	17

Procedure

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--										0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	200	200	200	200	200	200	200	200	200	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1960	1960	1960	1960	1960	1960	1960	1960	1960	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	13	13	13	13	13	13	13	13	13	26	
Absorbance before adding enzyme													
Parallel 1	0,000	0,002	0,114	0,046	0,094	0,098	0,078	0,041	0,053	0,089	0,064	0,005	[nm]
Parallel 2	-0,005	0,005	0,112	0,011	0,094	0,107	0,077	0,039	0,059	0,084	0,068	-0,014	[nm]
Parallel3	-0,003	0,011	0,127	0,037	0,103	0,100	0,070	0,042	0,052	0,072	0,072	0,002	[nm]
Average	-0,003	0,006	0,118	0,031	0,097	0,102	0,075	0,041	0,055	0,082	0,068	-0,0045	[nm]
Absorbance after adding enzyme													
Parallel 1	0,000	0,007	0,148	0,083	0,192	0,148	0,158	0,101	0,121	0,184	0,173	0,764	[nm]
Parallel 2	-0,005	0,003	0,108	0,064	0,182	0,149	0,159	0,111	0,131	0,186	0,178	0,747	[nm]
Parallel3	-0,002	0,001	0,137	0,082	0,101	0,143	0,153	0,104	0,131	0,177	0,171	0,769	[nm]
Average	-0,002	0,004	0,131	0,076	0,158	0,147	0,157	0,105	0,128	0,182	0,174	0,760	[nm]
Volume before freeze drying			389,5	421,4	427,8	217,8	217,8	191,6	191,6	201,5	221,1		[ml]
Volume after freeze drying			15	15	15	15	15	15	15	15	15		[ml]
Fucose													
Difference betweenAbs	0,000	-0,002	0,013	0,045	0,061	0,045	0,082	0,065	0,073	0,101	0,106	0,765	[nm]
Conc. In cuvette	-0,004	-0,070	0,327	1,132	1,548	1,132	2,065	1,633	1,845	2,549	2,684	19,440	[µg/ml]
Conc. In sample	--Blank--	--Blank--	4,278	14,833	20,278	14,833	27,056	21,389	24,167	33,389	35,167	509,333	[µg/ml]
Concentration in sample	--Blank--	--Blank--	0	0,528	0,711	1,022	1,863	1,674	1,892	2,486	2,386		[mg/L]

Old Leaf

Experiment - Release of total carbohydrate by algae

Sample:

Setup	Plant Part	y Weight [	Wet weigh	Water	Vol. [L]
OLd	Old leaf	92,9	464,5	distilled	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--											1	[mg/ml]
Sam. Vol.	--Blank--	2000	1000	1000	1000	500	500	400	400	400	200	100	[µl]
Dest. Water	2000	0	1000	1000	1000	1500	1500	1600	1600	1600	1800	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	2	2	2	4	4	5	5	5	10	20	
Absorbance												485	[nm]
Parallel 1	0,00	0,243	0,343	0,745	0,897	0,505	0,471	0,476	0,782	1,093	0,513	0,430	[nm]
Parallel 2	0,00	0,242	0,338	0,761	0,894	0,485	0,455	0,519	0,764	1,022	0,450	0,436	[nm]
Parallel 3	0,01	0,246	0,331	0,782	0,883	0,474	0,548	0,531	0,750	1,008	0,552	0,463	[nm]
Average	0,005	0,244	0,337	0,763	0,891	0,488	0,491	0,509	0,765	1,041	0,505	0,443	[nm]
Total carbohydrate in sample	--Blank--	26,762	73,454	163,950	191,326	211,021	212,440	274,770	411,294	557,926	545,638	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	26,762	73,454	163,950	191,326	211,021	212,440	274,770	411,294	557,926	545,638		[mg/L]

Experiment - Release of fucose from algae

Sample:

Setup	Plant Part	Weight [g]	Water	Vol. [L]
OLd	Old leaf	464,5	distilled	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	400	400	400	400	400	400	400	400	400	400	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1760	1760	1760	1760	1760	1760	1760	1760	1760	1760	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	7	7	7	7	7	7	7	7	7	7	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	-0,032	-0,013	0,068	0,049	0,023	0,026	0,021	-0,005	0,063	0,055	0,000	[nm]
Parallel 2	-0,005	0,005	-0,035	-0,017	0,063	0,063	0,052	0,027	0,021	0,002	0,067	0,061	-0,005	[nm]
Parallel3	-0,003	0,011	-0,030	0,008	0,074	0,046	0,056	0,020	0,018	-0,001	0,010	0,058	-0,002	[nm]
Average	-0,003	0,006	-0,032	-0,007	0,068	0,053	0,044	0,024	0,020	-0,001	0,047	0,058	-0,0025	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,020	0,074	0,170	0,181	0,208	0,229	0,243	0,267	0,457	0,472	0,764	[nm]
Parallel 2	-0,005	0,003	0,018	0,081	0,158	0,189	0,257	0,236	0,252	0,265	0,459	0,500	0,747	[nm]
Parallel3	-0,002	0,001	0,023	0,068	0,153	0,165	0,246	0,230	0,229	0,261	0,434	0,512	0,769	[nm]
Average	-0,002	0,004	0,020	0,074	0,160	0,178	0,237	0,232	0,241	0,264	0,450	0,495	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,053	0,082	0,092	0,126	0,193	0,207	0,221	0,266	0,403	0,437	0,758	
Conc. In cuvette	-0,004	-0,070	1,327	2,065	2,328	3,185	4,907	5,263	5,619	6,747	10,250	11,098	19,275	
Conc. In sample	--Blank--	--Blank--	8,694	13,528	15,250	20,861	32,139	34,472	36,806	44,194	67,139	72,694	505,000	[µg/ml]
Concentration of fucose in sample	--Blank--	--Blank--	8,694	13,528	15,250	20,861	32,139	34,472	36,806	44,194	67,139	72,694		[mg/L]

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Dry Weight [g]	Wet weigl	Water	Vol. [L]
OLs	Old leaf	100,9	504,7	seawater	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--											1	[mg/ml]
Sam. Vol.	--Blank--	2000	1000	1000	1000	500	500	400	400	400	200	100	[µl]
Dest. Water	2000	0	0	1000	1000	1500	1500	1600	1600	1600	1800	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	1	2	2	4	4	5	5	5	10	20	
Absorbance												485	[nm]
Parallel 1	0,000	0,094	0,094	0,102	0,143	0,100	0,101	0,100	0,106	0,118	0,111	0,430	[nm]
Parallel 2	0,001	0,096	0,094	0,101	0,144	0,101	0,098	0,101	0,103	0,121	0,108	0,436	[nm]
Parallel 3	0,013	0,097	0,095	0,108	0,151	0,097	0,099	0,099	0,104	0,119	0,110	0,463	[nm]
Average	0,005	0,096	0,094	0,104	0,146	0,099	0,099	0,100	0,104	0,119	0,110	0,443	[nm]
Total carbohydrate in sample	--Blank--	11,018	10,876	23,738	32,745	45,631	45,631	57,394	59,699	67,677	125,071	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	11,018	10,876	23,738	32,745	45,631	45,631	57,394	59,699	67,677	125,071		[mg/L]

Release of fucose from the algae

Sample:

Setup	Plant Part	Weight [g]	Water	Vol. [L]
OLs	Old leaf	504,7	seawater	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	600	600	600	600	600	600	600	600	600	600	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1560	1560	1560	1560	1560	1560	1560	1560	1560	1560	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	4	4	4	4	4	4	4	4	4	4	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	0,015	0,009	0,017	0,018	0,028	0,032	0,029	0,038	0,045	0,066	0,000	[nm]
Parallel 2	-0,005	0,005	0,011	0,008	0,017	0,018	0,028	0,032	0,037	0,040	0,044	0,061	-0,005	[nm]
Parallel3	-0,003	0,011	0,009	0,012	0,016	0,020	0,028	0,028	0,035	0,039	0,051	0,055	-0,002	[nm]
Average	-0,003	0,006	0,012	0,010	0,017	0,019	0,028	0,031	0,034	0,039	0,047	0,061	-0,0025	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,047	0,042	0,188	0,266	0,355	0,374	0,394	0,399	0,503	0,512	0,764	[nm]
Parallel 2	-0,005	0,003	0,039	0,044	0,184	0,262	0,354	0,367	0,405	0,401	0,505	0,521	0,747	[nm]
Parallel3	-0,002	0,001	0,038	0,041	0,184	0,275	0,355	0,341	0,389	0,402	0,509	0,518	0,769	[nm]
Average	-0,002	0,004	0,041	0,042	0,185	0,268	0,355	0,361	0,396	0,401	0,506	0,517	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,030	0,033	0,169	0,249	0,327	0,330	0,362	0,362	0,459	0,456	0,758	[nm]
Conc. In cuvette	-0,004	-0,070	0,742	0,818	4,279	6,323	8,299	8,384	9,207	9,190	11,667	11,599	19,275	[µg/ml]
Conc. In sample	--Blank--	--Blank--	3,241	3,574	18,685	27,611	36,241	36,611	40,204	40,130	50,944	50,648	505,000	[µg/ml]
Concentration of fucoe in sample	--Blank--	--Blank--	3,241	3,574	18,685	27,611	36,241	36,611	40,204	40,130	50,944	50,648		[mg/L]

New Leaf

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Weight [g]	Wet Weig	Water	Vol. [L]
NLd	New leaf	53,3	266,7	distilled	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--												[mg/ml]
Sam. Vol.	--Blank--	2000	1000	1000	500	400	400	200	200	200	200	100	[µl]
Dest. Water	2000	0	1000	1000	1500	1600	1600	1800	1800	1800	1800	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	2	2	4	5	5	10	10	10	10	20	
Absorbance												485	[nm]
Parallel 1	0,000	0,361	0,469	0,551	0,316	0,263	0,321	0,175	0,176	0,183	0,159	0,430	[nm]
Parallel 2	0,001	0,379	0,468	0,539	0,315	0,251	0,311	0,160	0,175	0,178	0,142	0,436	[nm]
Parallel 3	0,013	0,355	0,474	0,567	0,309	0,272	0,302	0,163	0,167	0,172	0,121	0,463	[nm]
Average	0,005	0,365	0,470	0,552	0,313	0,262	0,311	0,166	0,173	0,178	0,141	0,443	[nm]
Total carbohydrate in sample	--Blank--	39,670	101,752	119,199	136,695	143,564	169,805	185,000	192,092	197,411	158,050	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	39,670	101,752	119,199	136,695	143,564	169,805	185,000	192,092	197,411	158,050		[mg/L]

Release of fucose from the algae

Sample:

Setup	Plant Part	Weight [g]	Water	Vol. [L]
NLd	New leaf	266,7	distilled	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	400	400	400	400	400	400	600	600	600	600	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1760	1760	1760	1760	1760	1760	1560	1560	1560	1560	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	7	7	7	7	7	7	4	4	4	4	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	0,021	0,037	0,038	0,065	0,073	0,076	0,096	0,105	0,118	0,114	0,000	[nm]
Parallel 2	-0,005	0,005	0,018	0,031	0,034	0,056	0,061	0,079	0,099	0,105	0,121	0,123	-0,005	[nm]
Parallel3	-0,003	0,011	0,015	0,044	0,047	0,039	0,051	0,074	0,095	0,103	0,131	0,122	-0,002	[nm]
Average	-0,003	0,006	0,018	0,037	0,040	0,053	0,062	0,076	0,097	0,104	0,123	0,120	-0,0025	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,041	0,078	0,084	0,120	0,133	0,142	0,189	0,199	0,202	0,212	0,764	[nm]
Parallel 2	-0,005	0,003	0,044	0,076	0,081	0,121	0,120	0,144	0,186	0,195	0,201	0,210	0,747	[nm]
Parallel3	-0,002	0,001	0,040	0,090	0,095	0,111	0,121	0,147	0,183	0,190	0,209	0,211	0,769	[nm]
Average	-0,002	0,004	0,042	0,081	0,087	0,117	0,125	0,144	0,186	0,195	0,204	0,211	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,024	0,044	0,047	0,064	0,063	0,068	0,089	0,090	0,081	0,091	0,758	
Conc. In cuvette	-0,004	-0,070	0,589	1,107	1,183	1,616	1,590	1,718	2,260	2,286	2,040	2,311	19,275	
Conc. In sample	--Blank--	--Blank--	3,861	7,250	7,750	10,583	10,417	11,250	9,870	9,981	8,907	10,093	505,000	[µg/ml]
Concentration of fucose sample	--Blank--	--Blank--	3,86	7,25	7,75	10,58	10,42	11,25	9,87	9,98	8,91	10,09		[mg/L]

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Weight [g]	Wet Weig	Water	Vol. [L]
NLs	New leaf	46,9	234,7	seawater	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--											1	[mg/ml]
Sam. Vol.	--Blank--	2000	1000	1000	500	400	400	200	200	200	200	100	[µl]
Dest. Water	2000	0	1000	1000	1500	1600	1600	1800	1800	1800	1800	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	2	2	4	5	5	10	10	10	10	20	
Absorbance												485	[nm]
Parallel 1	0,00	0,126	0,162	0,192	0,182	0,143	0,142	0,137	0,153	0,191	0,214	0,430	[nm]
Parallel 2	0,00	0,126	0,164	0,189	0,180	0,144	0,131	0,136	0,154	0,194	0,220	0,436	[nm]
Parallel 3	0,01	0,106	0,165	0,192	0,177	0,143	0,136	0,144	0,153	0,191	0,211	0,463	[nm]
Average	0,005	0,119	0,164	0,191	0,180	0,143	0,136	0,139	0,153	0,192	0,215	0,443	[nm]
Total carbohydrate in sample	--Blank--	13,535	36,504	42,319	79,816	80,443	76,720	156,277	171,525	212,660	237,128	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	13,535	36,504	42,319	79,816	80,443	76,720	156,277	171,525	212,660	237,128		[mg/L]

Release of fucose from algae

Setup	Plant Part	Weight [g]	Water	Vol. [L]
NLs	New leaf	234,7	seawater	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	400	400	400	400	400	400	600	600	600	600	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1760	1760	1760	1760	1760	1760	1560	1560	1560	1560	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	7	7	7	7	7	7	4	4	4	4	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	0,078	0,089	0,094	0,088	0,101	0,118	0,104	0,107	0,029	0,010	0,000	[nm]
Parallel 2	-0,005	0,005	0,070	0,097	0,093	0,092	0,115	0,101	0,105	0,104	0,011	0,015	-0,005	[nm]
Parallel3	-0,003	0,011	0,076	0,111	0,097	0,091	0,119	0,103	0,105	0,107	0,015	0,012	-0,002	[nm]
Average	-0,003	0,006	0,075	0,099	0,095	0,090	0,112	0,107	0,105	0,106	0,018	0,012	-0,003	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,113	0,116	0,125	0,128	0,152	0,163	0,191	0,180	0,129	0,146	0,764	[nm]
Parallel 2	-0,005	0,003	0,103	0,123	0,125	0,121	0,145	0,169	0,197	0,205	0,138	0,144	0,747	[nm]
Parallel3	-0,002	0,001	0,101	0,146	0,124	0,129	0,142	0,162	0,194	0,196	0,141	0,146	0,769	[nm]
Average	-0,002	0,004	0,106	0,128	0,125	0,126	0,146	0,165	0,194	0,194	0,136	0,145	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,031	0,029	0,030	0,036	0,035	0,057	0,089	0,088	0,118	0,133	0,758	
Conc. In cuvette	-0,004	-0,070	0,776	0,734	0,751	0,895	0,869	1,446	2,260	2,218	2,981	3,372	19,275	
Conc. In sample	--Blank--	--Blank--	5,083	4,806	4,917	5,861	5,694	9,472	9,870	9,685	13,019	14,722	505,000	[µg/ml]
Concentration of fucose sample	--Blank--	--Blank--	5,083	4,806	4,917	5,861	5,694	9,472	9,870	9,685	13,019	14,722		[mg/L]

Stem

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	y Weight [	Wet weigl	Water	Vol. [L]
Sd	Stem	120,8	603,8	distilled	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--											1	[mg/ml]
Sam. Vol.	--Blank--	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	100	[µl]
Dest. Water	2000	0	0	0	0	0	0	0	0	0	0	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	1	1	1	1	1	1	1	1	1	20	
Absorbance												485	[nm]
Parallel 1	0	0,081	0,090	0,106	0,116	0,093	0,101	0,181	0,414	0,451	0,519	0,430	[nm]
Parallel 2	0,001	0,086	0,099	0,110	0,117	0,093	0,083	0,178	0,407	0,461	0,498	0,436	[nm]
Parallel 3	0,013	0,073	0,091	0,103	0,120	0,088	0,096	0,176	0,402	0,428	0,500	0,463	[nm]
Average	0,005	0,080	0,093	0,106	0,118	0,091	0,093	0,178	0,408	0,447	0,506	0,443	[nm]
Total carbohydrate in sample	--Blank--	9,351	10,770	12,152	13,358	10,557	10,770	19,812	44,209	48,358	54,635	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	9,351	10,770	12,152	13,358	10,557	10,770	19,812	44,209	48,358	54,635		[mg/L]

Release of fucose from the algae

Sample:

Setup	Plant Part	Weight [g]	Water	Vol. [L]
Sd	Stem	603,8	distilled	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	600	600	600	600	600	600	600	600	600	600	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1560	1560	1560	1560	1560	1560	1560	1560	1560	1560	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	4	4	4	4	4	4	4	4	4	4	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	-0,015	-0,004	-0,013	0,012	0,012	0,013	0,012	0,010	0,010	0,013	0,000	[nm]
Parallel 2	-0,005	0,005	-0,014	-0,004	0,003	0,006	0,015	0,007	0,011	0,001	0,012	0,017	-0,005	[nm]
Parallel3	-0,003	0,011	-0,014	-0,013	0,002	0,008	0,006	0,005	0,004	0,004	0,015	0,011	-0,002	[nm]
Average	-0,003	0,006	-0,014	-0,007	-0,003	0,009	0,011	0,008	0,009	0,005	0,012	0,014	-0,0025	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,021	0,020	0,037	0,050	0,061	0,054	0,072	0,144	0,192	0,204	0,764	[nm]
Parallel 2	-0,005	0,003	0,018	0,024	0,030	0,044	0,054	0,058	0,064	0,171	0,195	0,212	0,747	[nm]
Parallel3	-0,002	0,001	0,018	0,025	0,038	0,058	0,057	0,061	0,078	0,164	0,200	0,205	0,769	[nm]
Average	-0,002	0,004	0,019	0,023	0,035	0,051	0,057	0,058	0,071	0,160	0,196	0,207	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,033	0,030	0,038	0,042	0,046	0,049	0,062	0,155	0,183	0,193	0,758	[nm]
Conc. In cuvette	-0,004	-0,070	0,835	0,751	0,946	1,056	1,166	1,243	1,573	3,923	4,652	4,907	19,275	[µg/ml]
Conc. In sample	--Blank--	--Blank--	3,648	3,278	4,130	4,611	5,093	5,426	6,870	17,130	20,315	21,426	505,000	[µg/ml]
Concentration of fucose in sample	--Blank--	--Blank--	3,648	3,278	4,130	4,611	5,093	5,426	6,870	17,130	20,315	21,426		[mg/L]

Release of total carbohydrate from algae

Sample:

Setup	Plant Part	Weight [g]	Dry Weigh	Water	Vol. [L]
Ss	Stem	125,9	629,6	seawater	3

Procedure:

Sample	0	1	2	3	4	5	6	7	8	9	10	11	
Time	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--											1	[mg/ml]
Sam. Vol.	--Blank--	2000	2000	2000	2000	2000	2000	2000	2000	2000	1000	100	[µl]
Dest. Water	2000	0	0	0	0	0	0	0	0	0	1000	1900	[µl]
Phenol	500	500	500	500	500	500	500	500	500	500	500	500	[µl]
Sulphuric acid	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	5000	[µl]
D.F		1	1	1	1	1	1	1	1	1	2	20	
Absorbance												485	[nm]
Parallel 1	0,000	0,148	0,284	0,673	0,643	0,372	0,407	0,501	0,831	0,963	0,480	0,430	[nm]
Parallel 2	0,001	0,170	0,291	0,640	0,638	0,406	0,378	0,609	0,841	0,928	0,579	0,436	[nm]
Parallel 3	0,013	0,156	0,288	0,615	0,620	0,436	0,378	0,592	0,897	0,962	0,443	0,463	[nm]
Average	0,005	0,158	0,288	0,643	0,634	0,405	0,388	0,567	0,856	0,951	0,501	0,443	[nm]
Total carbohydrate in sample	--Blank--	17,649	31,443	69,209	68,252	43,890	42,082	61,195	91,940	102,011	108,206	44,844	[µg/ml]
Total carbohydrate in sample	--Blank--	17,649	31,443	69,209	68,252	43,890	42,082	61,195	91,940	102,011	108,206		[mg/L]

Release of fucose from algae

Sample:

Setup	Plant Part	Weight [g]	Water	Vol. [L]
Ss	Stem	629,6	seawater	3

Procedure:

Sample	Dis.Water	Sea water	1	2	3	4	5	6	7	8	9	10		
Time	--Blank--	--Blank--	1,25	7	24	48	72	96	120	144	170	213	--Stand.--	[h]
Sam. Conc.	--Blank--	--Blank--											0,5	[mg/ml]
Sam. Vol.	--Blank--	--Blank--	600	600	600	600	600	600	600	600	600	600	100	[µl]
NaOH (1 M)	--Blank--	--Blank--	0	0	0	0	0	0	0	0	0	0	0	[µl]
Buffer (Btl. 1)	400	400	400	400	400	400	400	400	400	400	400	400	400	[µl]
Dest. Water	2260	2260	1560	1560	1560	1560	1560	1560	1560	1560	1560	1560	2060	[µl]
NADP+ (Btl. 2)	50	50	50	50	50	50	50	50	50	50	50	50	50	[µl]
Enzyme (Btl. 3)	10	10	10	10	10	10	10	10	10	10	10	10	10	[µl]
Dilution Factor	--Blank--	--Blank--	4	4	4	4	4	4	4	4	4	4	26	
Absorbance before adding enzyme														
Parallel 1	0,000	0,002	-0,017	-0,011	0,030	0,036	0,039	0,026	0,046	0,068	0,088	0,106	0,000	[nm]
Parallel 2	-0,005	0,005	-0,017	0,000	0,036	0,038	0,037	0,034	0,046	0,087	0,078	0,120	-0,005	[nm]
Parallel3	-0,003	0,011	-0,018	-0,001	0,028	0,057	0,037	0,025	0,048	0,056	0,061	0,112	-0,002	[nm]
Average	-0,003	0,006	-0,017	-0,004	0,031	0,044	0,038	0,028	0,047	0,070	0,076	0,113	-0,0025	[nm]
Absorbance after adding enzyme														
Parallel 1	0,000	0,007	0,016	0,032	0,052	0,061	0,071	0,111	0,106	0,131	0,166	0,187	0,764	[nm]
Parallel 2	-0,005	0,003	0,015	0,030	0,048	0,077	0,086	0,095	0,117	0,140	0,169	0,189	0,747	[nm]
Parallel3	-0,002	0,001	0,012	0,018	0,056	0,086	0,081	0,098	0,111	0,138	0,161	0,194	0,769	[nm]
Average	-0,002	0,004	0,014	0,027	0,052	0,075	0,079	0,101	0,111	0,136	0,165	0,190	0,756	[nm]
Difference betweenAbs	0,000	-0,002	0,032	0,031	0,021	0,031	0,042	0,073	0,065	0,066	0,090	0,077	0,758	[nm]
Conc. In cuvette	-0,004	-0,070	0,793	0,768	0,513	0,776	1,047	1,845	1,633	1,667	2,269	1,955	19,275	[µg/ml]
Conc. In sample	--Blank--	--Blank--	3,463	3,352	2,241	3,389	4,574	8,056	7,130	7,278	9,907	8,537	505,000	[µg/ml]
Concentration of fucose in sample	--Blank--	--Blank--	3,463	3,352	2,241	3,389	4,574	8,056	7,130	7,278	9,907	8,537		[mg/L]