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Food-grade alginate from tissue cultivated brown seaweeds in a biorefinery approach

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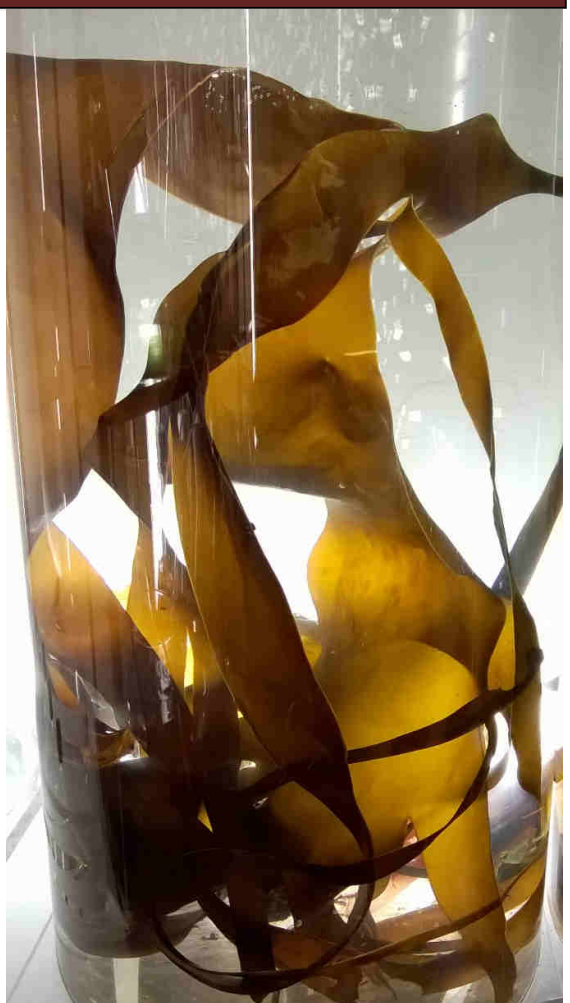

Traditio et Innovatio

Report Summary

Title	Food-grade alginate from tissue cultivated brown seaweeds in a biorefinery approach
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Summary:	Reproducible production yields and high-quality alginate from brown seaweeds can only be achieved by tissue cultivation, since both are dependent on environmental factors in wild harvested and farmed specimens, resulting in significant variation. Although tissue cultivation is a solution to this challenge, protocols developed so far for *Laminaria digitata* and *L. hyperborea* do not produce well growing explants with high survival rates. This project sought to address this challenge by improving tissue culture protocols for brown seaweeds, and assessing their effects on production of key components, including alginate. Tissues of blades, stipes and intercalary meristems of *L. digitata* and *L. hyperborea* were used for optimizations of the sterilization protocol, followed by growth testing two media (in solid and liquid state) combined with six different plant growth regulators (PGRs) in varying concentrations. Growth was measured as the number of bud and callus formation per week, and survival of the explants. Sterilisation protocol B (for stipe and blade explants) and C (for intercalary meristems) in combination with Provasloi's enriched liquid medium supplemented with 0.35 µM NAA + 2.3 µM kinetin was found to produce the best growth. After this the explants were subjected to different salinities (10, 20 and 40 PSU), temperatures (3, 10 and 15°C) and velocities (weak, medium, strong) to test their potential effects on alginate accumulation. The results obtained showed that the highest sodium alginate content was achieved by application of high velocities in the blade explants of *L. digitata* and *L hyperborea* (83.5 ± 5.5 and 80.4 ± 4.9% DW, respectively), but with comparable high alginate contents in stipes and intercalary meristems. In the second part of the present study, a sequential extraction process was developed targeting fucoxanthin, sodium alginate and fucoidan. The process was based on environmentally friendly alternative solvents (e.g., limonene) in combination with ultrasound-assisted extraction procedures on blade explants of *L. digitata* and *L hyperborea* exposed to high velocities in liquid up-scale culture. The chemical characterisation of the products was conducted by LC, 1H NMR and Infrared spectroscopy. The residual biomass was subjected to an analysis targeting proteins, lipids and further utilizable compounds. While the individual amino acid concentrations of both tested seaweeds were rather low and their compositions similar, the fatty acid (FAs) concentrations and composition varied significantly between species. The levels of FAs were generally low in *L. digitata*. In contrast, the FA profile of *L. hyperborea* showed significantly higher concentrations of FA groups, particularly in the control assay, ranging from 40.06 to 238.29 µg/g DW for total SFAs and PUFAs, respectively. Finally, a phytochemical screening was conducted on the residual biomass pointing to the presence of phenolic compounds such as flavonoids and phlorotannins. The potential use of the residual biomass as bioactive fertilizer has to be further investigated. Further potential for optimisation is discussed.
Keywords:	*Brown Seaweeds, multiproduct biorefinery, tissue cultivation*

Contents

1. Introduction

Alginic acid, also called algin, is a naturally occurring, edible polysaccharide found in brown algae such as *Laminaria digitata* and *L. hyperborea*. Alginates are derivatives of alginic acid - a conjugate base of alginic acid. The algin found in brown algae is present as a mixed salt (sodium and/or potassium, calcium, magnesium) of alginic acid (Fig. 1). It is formed by linear block copolymerization of d-mannuronic acid and l-guluronic acid. Sodium alginate is used in many industries including food, animal feed, fertilisers, textile printing, and pharmaceuticals. In food factories alginates are used to coat fruits and vegetables; as a microbial and viral protection by-product; and as a gelling, thickening, stabilizing, or emulsifying agent.

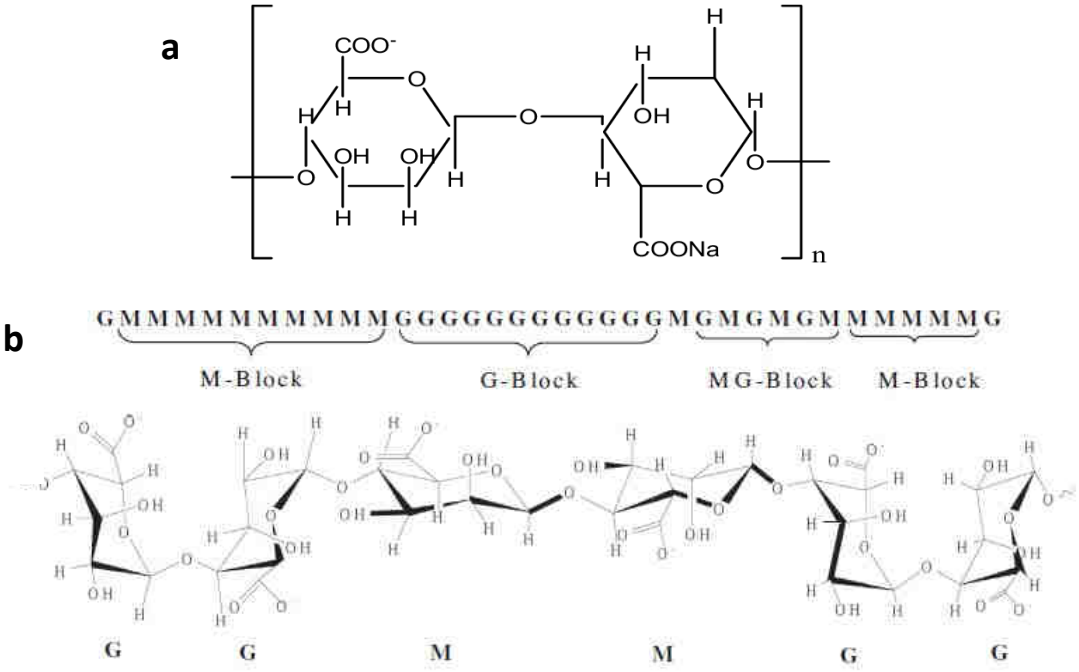

Figure 1. Structure of sodium alginate (a) as well as block distribution and chain conformation of alginates (from Fertah et al. 2017).

Several kelps in the genus *Laminaria* have been studied for a long time and are known to exhibit a seasonal shift in growth and morphology, which can be related to changing levels of nutrients and light during the year (Davison et al. 1984, Schaffelke & Lüning 1994). The growth cycle and aging processes are generally described by seasonal cycles, also implying that the mechanical properties of vegetation vary with the different seasons. As aging and seasonal shifts affects the vegetation flexibility and thus the reconfiguration process, it can in turn lead to major changes in the physical and ecological environment. However, the variation of kelp biomechanical properties has been little explored, despite the importance of these properties for the interaction of kelp with ocean currents.

Traditionally, *L. digitata* is known to have a shorter, narrower and more flexible stipe in comparison to *L. hyperborea* (Lamouroux 1813, Guiry 2001). The stipe cross-section is also typically oval for *L.*

digitata and round for *L. hyperborea* (Lamouroux 1813, Guiry 2001). In a study conducted by Henry (2018) the stipes of *L. digitata* were slightly more oval than the ones of *L. hyperborea*, but the difference was not significant. As the average diameter of the bottom of the stipe appeared to be the only significant interspecific morphological difference, it can be concluded that the two species present a very similar morphological phenotype, which may result from the adaptation to the same environmental conditions (Fig. 2). Within the family Laminariaceae, the great phenotypic (morphologic) plasticity resulting from the adaptation to local environmental factors is known to make the species identification based on morphological criteria rather uncertain (Kain 1979, McDevit & Saunders 2009, Henry 2018). Thus, only DNA sequencing allows the identification of the two distinct species *L. digitata* and *L. hyperborea*.

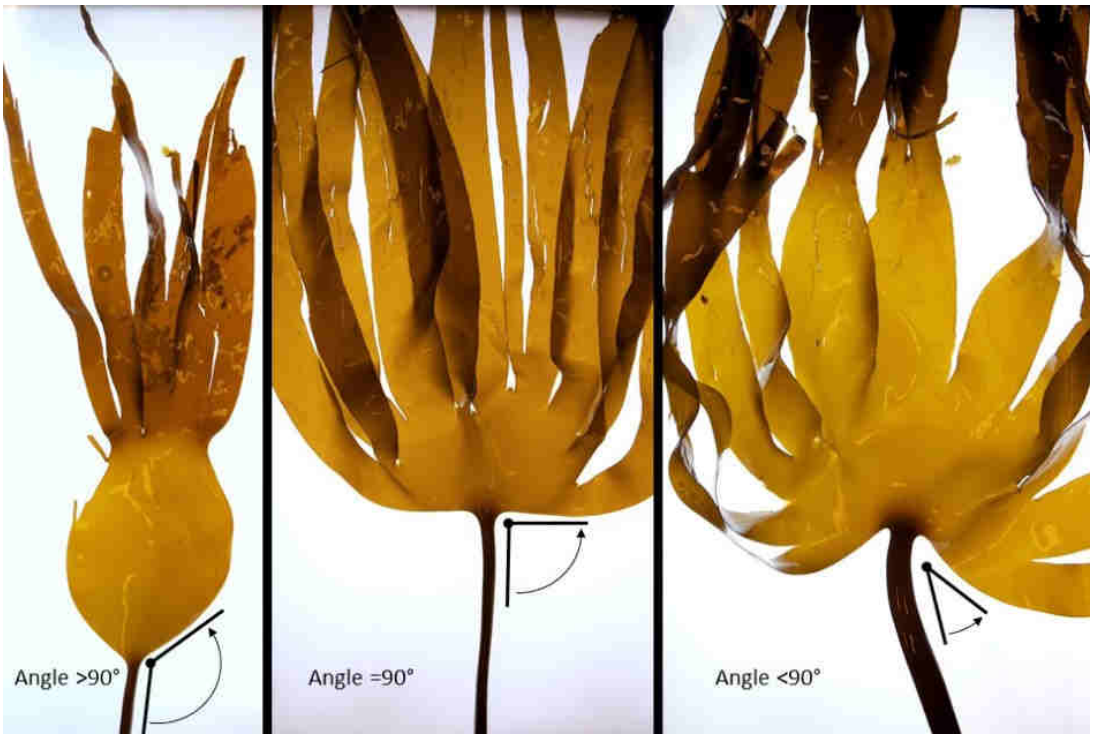

Figure 2. Example of three kelp specimens from different sites. The photos are focussing on the connection between the blade and stipe (adapted from Lund 2014). Within the family Laminariaceae, the great phenotypic (morphologic) plasticity resulting from the adaptation to local environmental factors is known to make the species identification based on morphological criteria rather uncertain (pictures from Henry 2018).

Many of the brown seaweed species used to extract alginates are wild harvested. There is growing concern about overharvesting wild seaweed and impacts of harvest methods on the coastal ecology. Due to rising seawater temperatures, erratic weather patterns and pollution, the balance of seaweed supply and demand is increasingly more under pressure. However, higher quality species of brown seaweed are found in cold waters. As the water temperature increases due to climate change, the concentration of terpenes and other chemical compounds in the seaweed increases (https://www.ams.usda.gov/sites/default/files/media/Alginates%20TR%202015.pdf); these compounds are not desirable for alginates used in food production. Since the quality and quantity of alginates varies between algae species, the type and age of tissues extracted, as well as time and location of harvest, a more stable supply of seaweed alginate is needed. In this context, tissue cultivated seaweeds could be applied.

To increase productivity, modern biotechnology via tissue cultivation can be considered one of the best options to overcome conventional breeding challenges, such as shortage of raw material for planting and seedling destruction by epiphytes. Tissue cultivation approaches can therefore facilitate the propagation of high-quality seaweeds and manufacturing of higher value products. Although the initial aim of these techniques focuses mostly on genetic improvement and clonal propagation of seaweeds for mariculture, recently the scope of these techniques has been extended for use in bioprocess technology for production of high value chemicals of immense importance in the pharmaceutical and nutraceutical sectors. In addition, there has been a phenomenal interest in intensifying seaweed tissue and cell culture research to maximize the add-on value of seaweed resources. Although the advantages are clear, this technique is not applied in Iceland commercially.

Figure 3. Seaweed bed in the Húnafloi (picture: Valtýr Sigurðsson).

In Laminariales, many reports have described tissue culture, particularly in the genera *Undaria* (Fang et al. 1983, Zongxi et al. 1983, Yan 1984, Notoya & Aruga 1992a, Kawashima & Tokuda 1993, Kimura & Notoya 1997), *Ecklonia* (Lawlor et al. 1987 and 1989, Notoya 1988, Notoya & Aruga 1989, 1992b, Kawashima & Tokuda 1990, Kawashima et al. 1992, Notoya et al. 1994, Kimura & Notoya 1996), *Macrocystis* (Polne-Fuller & Gibor 1987, Ar Gall et al. 1996), *Eckloniopsis* (Notoya et al. 1994, Notoya 1997), *Egregia* (Polne-Fuller & Gibor 1987), *Eisenia* (Notoya & Aruga 1990) and *Agarum* (Notoya et al. 1994). Tissue cultures of the genus *Laminaria* have been reported in seven species: *Laminaria angustata* (Saga et al. 1978, Saga & Sakai 1983), *L. digitata* (Fries 1980, Butler et al. 1989, Liu & Kloareg 1992, Fole fack & Cosson 1995, Ar Gall et al. 1996, Asensi et al. 2001, Mussio & Rusig 2009), *L. hyperborea* (Fries 1980), *L. japonica* (Saga & Sakai 1977, Fang et al. 1983; Zongxi et al. 1983, Yan 1984, Wang et al. 1998), *L. saccharina* (Lee 1985, Butler 1989, Ar Gall et al. 1996), *L. setchellii* (Qi et al. 1995) and *L. sinclairii* (Polne-Fuller & Gibor 1987). Callus formation in these highly differentiated seaweeds is relatively rare. It occurred on 0.5–27% of the sectioned tissues depending on the plant and on the type of tissue studied (e.g., Zayed et al. 2019, Tirtawijaya et al. 2022). The common developmental pattern from *in vitro* tissue culture of sporophytes of Laminariales is the outgrowth of apo-sporous gametophyte-like filaments with generally differentiated fertile branches, from which sporophytes were often regenerated (Ar Gall et al. 1996). Unfortunately, most of these thallus-like explants usually

stop developing at a certain point and grow not bigger than a few mm or have generally very low survival rates (e.g., Fries 1980). A few studies have reported the production of calluses from protoplast culture of brown macroalgae exhibiting a complex morphological organization. In Laminariales, the developmental processes from protoplast culture are generally either direct regeneration into plantlets (*Undaria pinnatifida*, Matsumura et al. 2001; *L. japonica*, Matsumura et al. 2000, Sawabe & Ezura 1996, Sawabe et al. 1997) or indirect regeneration of plantlets after dedifferentiation through a filamentous stage (*U. pinnatifida*, Matsumura et al. 2001; *L. saccharina*, Benet et al. 1997) or from a callus-like mass (*U. pinnatifida*, Matsumura et al. 2001; *L. digitata*, Mussio & Rusig 2008). Still, the knowledge on seaweed tissue culture is rather limited. For example, there is a lack of data on the development of suitable surface sterilization protocols for each species which would result in long-lasting explants. Also, a suitable medium for culturing seaweed to stimulate callus formation or regeneration of explants is still in development.

Currently, there is a rising global interest regarding growing and processing seaweed in Iceland. However, alginophyte resources are limited, and the natural kelp resources have declined worldwide in recent years. In Iceland multiyear *Laminaria digitata* and *L. hyperborea* are the only species that form extended monospecific kelp beds (Fig. 3). Kelp forests of cold-temperate regions around the world represent highly diverse, dynamic and complex ecosystems. The biodiversity of kelp forests is extraordinarily high in comparison to other algal communities, being the highest in *Laminaria hyperborea* beds (e.g., Schoenrock et al. 2021). Sustainable harvesting management plans have been implemented for instance in Norway consisting of revolving 5-year harvest cycles, set harvest volumes for specific areas and monitoring of regrowth. However, from other European countries such as Ireland it is known that even well managed kelp harvesting puts substantial ecological pressure on natural kelp beds by increasing disturbance levels and removing resources from the ecosystem, especially if not grown in proximity to fish farms with excess nutrient outputs. It is self-evident that the harvesting of kelp has significant negative impacts on invertebrates even though recovery rates for some taxa are very short: removing the kelp by whatever means involves the removal of the epifaunal communities, more than likely some of the epibenthic species and obviously, the key stone species on which these communities depend. Even the collection of seaweeds from beaches may have negative impacts on benthic faunal communities due to extraction of consumable biomass, which would normally in turn 'fertilize' the ocean.

Traditionally, alginates have been used as thickeners, emulsifiers and stabilizing agents in a number of food applications, with new food applications emerging, based on their unique biochemical and biophysical properties. The demand for alginate production has increased over time, and it is likely to increase significantly in the future, particularly for the use of high-quality alginates in current and future biomedical and bioengineering applications. Therefore, the results of the current study, which utilizes selected tissue cultivated kelp species for alginate production, will provide a broad audience with the know-how to control the quality of alginate products. Importantly, it will also demonstrate potential production methods which are independent from the negative effects of climate change, which is predicted to cause an increase in terpenes and other compounds in brown seaweeds, making them unsuitable for food use. Furthermore, advances have been made in cell and tissue culture of seaweeds to define a unique branch of *in vitro* techniques; however, they are lagging far behind those of land plants and are still in a state of development. Thus, investing into this technique would give Iceland an international leading role in this field and facilitate innovation in this industry.

2. Objectives and Aims of the Study

Utilizing the know-how of former studies conducted at BioPol ehf, in the present project *Laminaria digitata* and *L. hyperborea* will be cultivated using tissue cultivation techniques, to produce food-grade alginate under controlled conditions. Firstly, surface sterilization protocols and growth promotors will be tested to optimize the initial tissue cultivation process. Afterwards, different culture conditions such as variations in salinity, temperature and seawater flow velocity, will be used to induce the accumulation of alginates in the explants over a 3-week exposure time.

Larger scale cultivation will be established based on the results obtained in the earlier studies, and the explants will be harvested and prepared for extraction. Initially traditional extraction protocols will be utilised, and those protocols in which toxic chemicals are used, will be replaced by alternative solvents (e.g., ionic liquids, deep eutectic solvents or limonene) in combination with, for instance, ultrasound-assisted extraction procedures.

In the extraction process several by-products are available and are listed below in the order of the downstream processing:

- Fucoxanthin
- Sodium-Alginate
- Fucoidan
- Residual biomass containing lipids and proteins besides other compounds (e.g., phenolic compounds)

During the first screenings, the alginate contents will be determined by spectrophotometric methods and the purification grade confirmed by fluorescence spectroscopy. Later, the chemical characterisation will be conducted by 1H NMR and Infrared spectroscopy. The concentrations and compositions of pigments, proteins and lipids in the residual biomass will be also determined by LC to verify the quality of the by-products for their potential use as food additive (colorant) as well as aquafeed and/or bioactive fertilizers. Further, the bioactivity of the residual biomass will be tested in phytochemical screenings.

3. Material and Methods

If not otherwise mentioned, all chemicals used in this study were of the highest purity from Merck (former Sigma/Aldrich).

3.1 Experimental Tissue Culture Conditions

Two northern Icelandic brown seaweed species (Phaeophyceae) were chosen for the present project, namely *Laminaria digitata* and *L. hyperborea*.

Sporophytes of *Laminaria digitata* and *L. hyperborea* specimens were collected from a rocky shore near Skagaströnd (north-west Iceland) in October 2022, cleaned of visible epiphytes and stored in tanks containing natural seawater until they were transferred into culture at the external algae culture collection facility of BioPol ehf. Subsamples were used for DNA sequencing which later confirmed the identity of the phaeophytes. As explants the stipe (cf. Fig 4), intercalary meristem and blade of the specimens were used.

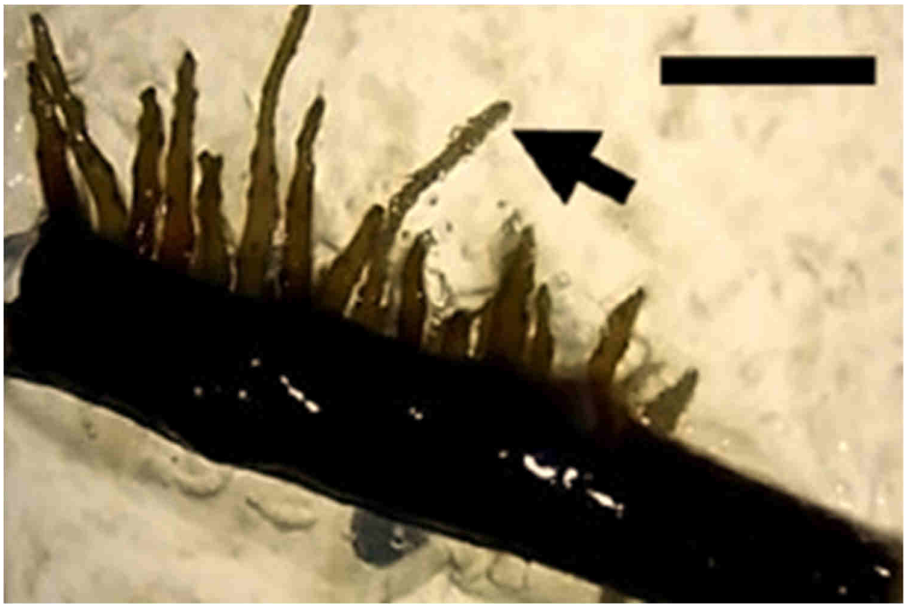

Figure 4. Example of explants gained from the stipe of *Laminaria digitata* with adventitious bud formations The stipe was further cut into smaller parts and cultured on solid and liquid medium for the testing of different plant growth regulators (PGRs). The partes were noted as bud explants (arrow: adventitious bud, bar = 5.0 mm).

Epiphytes and other microscopic contaminants were removed by manual brushing under a stereoscope and were washed several times with sterilized artificial seawater (mix of Tropic Marin (TM) Reef Salt®, TM Bio-Actif® and TM Syn-Biotic® 1:0.75:0.25) prior to surface sterilization. Selected healthy vegetative parts were cut into fragments of 4–6 cm in length for the sterilization process.

Overall, eight types of chemical sterilants were used prior to cultivation of the explants: betadine (BE), germanium oxide (GeO_2), povidone iodine (PI), commercial bleach (Domestos®), sodium hypochlorite solution (SHPS), ethanol, streptomycin sulphate (STS; 590.60 IU mg^{-1}), as well as Provasoli's antibiotic

(PESA) concentrated solution (Sigma, containing 240,000 units penicillin G, 1,000 µg chloramphenicol, 6,000 units polymyxin B and 1,200 µg neomycin per L). All chemical sterilant solutions were prepared using autoclaved sterilized salt water except for the streptomycin sulphate and PESA solution. The surface sterilization protocols modified from Kumar et al. (2004) and Mussio & Rusig (2009), as outlined in Table 1, were tested to determine the most suitable surface sterilization protocol for each explant (stipe, intercalary meristem and blade). For each protocol, the explants were not rinsed between the chemical treatments of surface sterilization. Explant surface samples were regularly checked for contaminations via fluorescence microscopy using standard staining techniques (gram staining https://asm.org/getattachment/5c95a063-326b-4b2f-98ce-001de9a5ece3/gram-stain-protocol-2886.pdf and Calcofluor-white, CFW). The protocol that produced the highest percentage of surface sterilized explants, measured by explant survival, was further used for the entire study. For each protocol, five explants were cultured on each Petri dish with a replication of ten Petri dishes for each individual experiment. Each experiment was repeated three times for every protocol.

Table 1. Surface sterilization protocols used in the present study.

Protocols	Treatment	Concentration	Time
A	PI	2.0 % w/v	5 min
	EtOH	5.0 % w/v	5 min
	STS	3.5 % w/v	48 h
B	BE	5.0 % w/v	5 min
	Domestos	1.0 % w/v	2 min
	GeO_2 +	1.0 µM	
	PESA	2.0 % w/v	12 h
C	BE	5.0 % w/v	5 min
	SHPS	1.0 % w/v	2 min
	GeO_2 +	1.0 µM	
	PESA	2.0 % w/v	12 h
D	EtOH	5.0 % w/v	5 min
	GeO_2	1.0 µM	1 h
	PI	2.0 % w/v	5 min
	STS	3.5 % w/v	48 h

Abbreviations: BE, betadine; EtOH, ethanol; GeO_2, germanium oxide; SHPS, sodium hypochlorite solution; PI, povidone iodine; STS, streptomycin sulphate; PESA, Provasoli's antibiotic concentrated solution.

The survival of the explant after the procedure was calculated as follows:

$$\text{Sterilised explants (\%)} = (\text{number of sterilised surviving explants} / \text{total number of explants cultured}) \times 100$$

The culture media used for this study were Provasoli's enriched seawater (PES) (Provasoli 1968), and Murashige and Skoog basal medium (MS; Murashige & Skoog 1962), both pH 8.3. These were prepared by adding medium solutions (PES full strength; MS 10%) to autoclaved artificial seawater with a salinity of 35 Practical Salinity Units (=PSU). The artificial seawater was prepared by adding 40 g of artificial sea salt (Tropic Marin Reef Salt® Germany) into 1 L of tap water. One liter of PES medium was prepared by adding 15 mL of PES solution to 990 mL of sterilized artificial seawater. The media were adjusted to the required pH using either 0.1 M HCl or NaOH. In addition, 6 g L^{-1} of agar, 50 µg L^{-1} vanadium and 10 g L^{-1} sucrose were added to the media. All components used for the preparation of the media were autoclaved. Both media were used to assess the effects of plant growth regulators (PGRs). As growth

promotors 6-Benzylaminopurine (BAP), naphthaleneacetic acid (NAA), Thidiazuron (TDZ), Forchlorfenuron [N-(2-chloro-4-pyridyl)-N'-phenylurea = CPPU), kinetin and/or zeatin were added to the media in concentrations varying between 0.2 and 3.8 µM according to literature (e.g., Fries 1980, Mussio & Rusig 2008) and own trials (data not shown). All PGRs were filtered sterilized using a 0.22-µm filter and added to the autoclaved media individually and in defined mixtures. The media were left to solidify in sterilized Petri dishes (90 mm × 15 mm) with a volume of 20 mL per Petri dish.

For the liquid media, preparation was similar to the solid medium except that no agar powder was supplemented into the medium. The medium was prepared in 20 mL Erlenmeyer flasks, with 5 mL liquid medium in each flask. Liquid media were changed in weekly intervals. Explants were maintained at 8°C, 100 µmol photons m^{-2} s^{-1} light intensity (Phillips, Germany, Master TL-D 18W/840), and a 12:12 h light:dark cycle under sterile conditions.

Conductivity, temperature, dissolved oxygen and pH of the media were measured using handheld probes (YK-31SA, YK-200PATC, YK-200PCD and YK-2001PH, SI Model 33, Engineered Systems and Designs-Model 600, Philips W9424). Photosynthetic active radiation (PAR, 400–700 nm) was measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A in bi-weekly intervals.

3.2 Determination of Growth

Growth rates of the explants were calculated by the increase in wet weight and presented as percentage growth per week using the formula of Penniman et al. (1986):

$$SGR = \% [(Gt/Go)1/t^{-1}] \times 100$$

SGR = specific growth rate (% in wet weight per week), Gt = weight after t weeks, Go = initial weight.

In addition, the rate of callus induction and bud-like formation (cf. Fig. 5) were calculated as follows:

$$\text{Callus induction (\%)} = (\text{number of bud explants with callus} / \text{total number of bud explants cultured}) \times 100$$

and

$$\text{Bud formation rate (\% week}^{-1}) = (\text{final number of buds growing} / \text{initial number of buds growing} \times 100) / 6$$

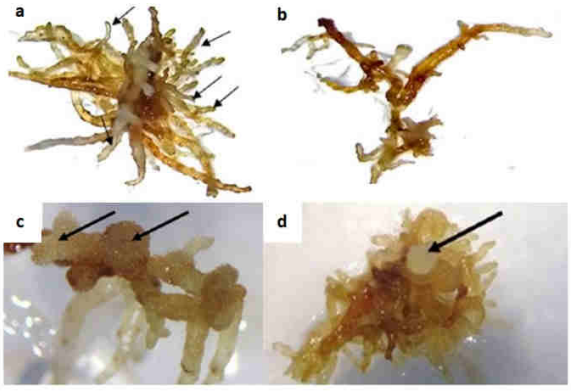

Figure 5. Examples of bud- (a, b) and callus-like (c, d) structures formed by explants of *Laminaria digitata* and *L. hyperborea* during and after the PGR experiments.

3.3 Induction Experiments and first Screening for Alginate Contents

The stimulation of alginate production was tested by variation of culture conditions of liquid cultures. Using the one-factor-at-a-time principle, explants (stipes, intercalary meristems and blades) of the two phaeophytes were exposed to a) 10, 20 and 40 PSU, b) temperatures of 3, 10 and 15°C as well as c) weak (0.01 m/s), medium (0.05 m/s) and strong (0.1 m/s) velocities *versus* control specimens from the standard cultivation (cf. 3.1). The experiments were conducted in 50 mL polystyrene flasks (equipped with filter vent caps) in triplicates for 504 h, resulting in overall 30 samples per species. Experiments were started by filling in 25 mL fresh prepared culture PES medium (cf. 3.1) into the Erlenmeyer flasks containing the phaeophyte tissues, which were weighed prior to each experiment. The experimental and control cultures were supplied with fresh medium in weekly intervals.

Velocities were tested prior to the experiments, using a conical flask filled with 2 L fresh prepared medium and an aquarium pump (TetraTec APS 400-T701735). The pump was connected via silicone tubing with an air valve for controlling the airflow. A further silicone tube connected the valve with a sterile pipette (SARSTEDT Serological Pipettes), which was inserted into the flask. Different strengths of velocities were achieved by opening and closing the valve and were measured via a handheld flow meter (HOJILA FM-100V5 B0BXX7PLBL).

The initial alginate isolation protocol was based on Mazumder et al. (2016) with some modifications. Three grams of dried seaweed was mixed with 30 mL of 0.2% aqueous solution of formalin (FOR). The mixture was incubated for 24 h at room temperature and stirred at 350 rpm. The soaked biomass was then washed with Milli-Q water and filtered through four layers of gauze. The solid parts were incubated with 30 mL of 0.2 M HCL for 1 h at 80 °C at 350 rpm stirring to turn the insoluble alginic salts to alginic acid. Acidified seaweed samples were again thoroughly washed with Milli-Q water and filtered through gauze, and then 60 mL of 2% sodium carbonate was added. The samples were incubated at 80 °C for 3 h and stirring at 350 rpm. The samples were then transferred to glass centrifuge tubes and centrifuged for 5 min at 3256 × g. The liquid fraction was collected in glass bottles and precipitated with aqueous ethanol 96% (1:1 v/v) at 4 °C overnight. The precipitated mass was collected and dissolved in 50 mL Milli-Q water and shaken.

To follow the purification procedure, fluorescence spectroscopy was used. Alginate samples are strongly fluorescent due to small amounts of polyphenolic residues. This is a routine technique to measure these contaminants in a wide range of alginates. The spectra were obtained with a spectro-fluorometer following the method described by Klöck et al. (1997). The excitation wavelength was 370 nm, and the emission signals were observed in the 400–900 nm range.

3.4 Upscale Cultivation, Harvest and Processing

To gain biomass for the sequential biorefinery approach, blade explants were cultivated in liquid PES medium (cf. 3.1) under sterile conditions, utilizing 2 L Erlenmeyer flasks. For air supply and mixing the set up described in 3.3 was used. Sterilized customary cotton wool was used for closing the flasks. The cultivation was started by filling the flasks with 2 L fresh prepared PES medium supplemented with 50 µg L^{-1} vanadium, 0.35 µM naphthaleneacetic acid (NAA), 2.3 µM kinetin and 10 g L^{-1} sucrose. After 504 h, the explants were harvested and washed twice with distilled water. Subsequently the specimens were dried, utilizing a horizontal dryer with hot air flow at 60°C (Tray dryer, model no.

FDTHQQZ). Dry algal material was ground in a kitchen blender. Prior to extraction, the 1 g of algae tissue was soaked in 10 mL of Milli-Q water (1:20 w/v) for 2 h at room temperature and then sonicated for 10 min in an ultrasonic bath (Cole-Parmer, UL Transonic cleaner). The samples were then rinsed in fresh Milli-Q water and filtered through four layers of gauze.

3.5 Sequential Extraction Process

For **fucoxanthin** extraction (Product 1) from tissue cultivated *Laminaria digitata* and *L. hyperborea*, ultrasonic-assisted extraction (UAE) was performed with an ultrasonic power of 500 W and frequency of 20 kHz. As solvent D-limonene (purchased over Amazon.com) was used according to the method given by Nie et al. (2021). Briefly, the Laminariales were weighed according to the liquid/solid ratio (L/S) of 40 mL per g DW, and the materials were mixed evenly in the beaker. To allow the solvent to fully infiltrate the raw material, the beaker was wrapped with an aluminium foil and placed in the dark for 2 h. Afterwards, the beaker was placed in a water bath preheated for 5 min, so that the temperature reached the set ultrasonic temperature. The ultrasonic parameters were adjusted first, and next the drill was extended a bit below the liquid level. The distance between the tip and the bottom of the cup was controlled by 1 cm. After the ultrasound was completed (550 W, 20 kHz; 60 s), the sample was centrifuged at 9000 g/min for 10 min at 4 °C. The supernatant was collected and analysed (cf.3.6.1). Seaweed solids (Residue 1) were washed twice and dried.

Fucoidan (Product 2) was extracted following the method outlined by Black et al. (1952). The waste residue (Residue 2, 2 g) was mixed with 20 mL of 0.1 M HCl (pH 2–2.5) at 70 °C for 1 h. The mixture was stirred at 250 rpm and then centrifuged at 5000 rpm for 15 min to separate the liquid from the seaweed solids (Residue 2). One volume of 1% (w/v) $CaCl_2$ was added to the recovered liquid, inverted and kept at 4 °C for 72 h. The precipitate which formed (**alginate** = Product 3) was removed from the liquid phase by centrifugation at 5000 rpm for 20 min, freeze dried, weighed and analysed (cf. 3.6.2). Two volumes of absolute ethanol (99.99%) were then added to the remaining alginate-free liquor (Residue 3), inverted and kept at 4 °C for 24 h. The precipitate was recovered by centrifugation at 5000 rpm for 20 min and the upper liquid phase (waste liquor, Residue 4) was stored at − 4 °C until further analysis (cf. 3.6.4-3.6.6). The solid precipitate (**fucoidan**) was freeze-dried and stored at − 20 °C until further analysis (cf. 3.6.3).

3.6 Biochemical Analysis

3.6.1 Fucoxanthin

Stock solutions of seaweed extracts from the first extraction step of the sequential extraction process were prepared for HPLC analysis by dissolving lyophilized seaweed extract (100 mg) in acetone (10 mL, 62.2 %). Samples were syringe filtered (Sigma-Aldrich Millex Durapore PVDF 0.22 µm pore, 13 mm diam.) into HPLC vials (Waters 2 mL LCGC certified clear glass 12 × 32 mm screw neck vial, with pre-slit PTFE/silicone septa cap). Chromatographic analysis of seaweed extracts was carried out according to a modified method developed by Billakanti et al. (2013). Fucoxanthin detection and quantification were achieved with HPLC (Alliance-Waters e2695 Separations Module, 400 atm pressure, at 4 °C),

equipped with a C18 reverse phase column (Waters XSelect, 4.6 mm × 100 mm, 3.5 µm particle size), and a UV photodiode array detector (Waters 2998). Two mobile phases were determined to be optimal for HPLC-DAD analysis of the seaweed extracts. These were solvent A: 20 mM sodium acetate; and solvent B: 100 % methanol. Before use, ddH2O water was membrane filtered (Merck Millipore Simplicity 185). Mobile phases were filtered (Merck Millipore HVLP 0.45 µm filter) and sonicated. The injection volume was 20 µL, with a constant flow rate of 0.15 mL min^{-1}. Detection was performed at 449 nm. A 60-min gradient program was used, at a constant temperature of 60 °C. Analysis was carried out in triplicate. Commercial fucoxanthin standard solutions were prepared in concentrations of 5, 10, 20, 30, 40, 50, and 100 µg mL^{-1} in ethanol. The area under the peak (AUP) of those corresponding with retention times for fucoxanthin standards was plotted against concentration (µg mL^{-1}) to make a standard curve. The regression equation was obtained as y = 1,000,000x + 1,000,000. The R2 value was 0.999. The concentration of fucoxanthin in the samples was extrapolated from the equation generated.

3.6.2 Sodium Alginate

The alginate content extracted from the seaweed explants was estimated by the phenol-sulphuric acid method, which is a non-specific method for quantification of carbohydrate (Dubois et al. 1956). A 0.5 mL sample solution was vortex-mixed with 0.5 mL of 5% phenol in water. After addition of 2.5 mL concentrated sulphuric acid rapidly from a glass dispenser, the mixture was vortex-mixed, and allowed to stand for 30 min at room temperature. The amount of sugar was measured by reading absorbance at 490 nm. A calibration curve was made by using commercial alginate (*M. pyrifera*) with different viscosities at different concentrations.

For more detailed analysis and confirmation of alginate content, ^{1}H NMR spectra were acquired on 0.1% w/v solutions of sodium alginate in D_2O with a Fourier-transform Bruker 250 BioSpin supplied with an inverse multinuclear gradient probe-head with z-shielded gradient coils, and with a Silicon Graphics Workstation, at different temperatures.

Infrared spectra of solid samples were recorded on a PerkinElmer paragon 1000 FT-IR spectrophotometer. A total of 32 scans were taken with a resolution of 4 cm^{-1}. Sodium alginate samples were dried for 8 h at 56 °C under vacuum. Spectra of the samples in KBr pellets (10% w/w) were recorded in the 4000–450 cm^{-1} range.

An Ubbelohde viscometer with a 0.5-mm capillary diameter, and with a solvent flow time (0.1 M NaCl) of 290 mL/s at 25 °C was used to measure specific viscosities. A stock solution in a concentration of 30 mg/10 mL was prepared by stirring for 5 h at room temperature (25 °C). Measurements were done over a range of polysaccharide concentrations from 0.05 to 0.3 g/dL.

3.6.3 Fucoidan

For monosaccharide compositions, samples of the polysaccharides extracted from the Laminariales (5 mg) were hydrolysed with 5 mol·L^{-1} trifluoroacetic acid for 4 h at 100°C, reduced with borohydride, and the alditols were acetylated with acetic anhydride:pyridine (1:1, v/v). The alditols acetates were dissolved in chloroform and analysed in a gas–liquid chromatograph/mass spectrometer (GCMS-QP2010 Shimadzu, Japan) with a DB-5ms column (Agilent) (Kirchner 1960, de Castro et al. 2018). Monosaccharide linkage analysis was performed by methylation using the CH_3I/NaOH method

(Ciucanu & Kerek 1984, Mélida et al. 2013). The resulting partially methylated alditol acetates were analysed by GC/EI-MS. Three replicates for each of the samples were analysed using inositol as a standard.

The total phenolic content (TPC) of each sample was determined according to the method of Gutfinger (1981). Each sample (1.0 mL) was mixed with 1.0 mL of 10% Na_2CO_3 and allowed to stand for 3 min. Then, 1.0 mL of 50% Folin-Ciocalteu reagent was added to each mixture. After incubation at room temperature, the resulting mixtures were centrifuged at 13,400 *g* for 5 min. Absorbances were measured with a spectrophotometer (TI Unicam 5625 UV/VIS Spectrometer, Richmond Scientific Ltd., Chorley, United Kingdom) at 750 nm, and the total phenolic contents were expressed as gallic acid equivalents.

The presence of sulfate groups in algal polysaccharides have been attributed to their biological activities (Barahonaa et al. 2014, Wang et al. 2016). The sulfate contents of fucoidan were determined according to the method given by Terho & Hartiala (1971) utilizing standard operation protocols developed by our partner GlycoMar. The method is based on the use of sodium rhodizonate which forms a coloured compound in the presence of barium ions. This colour is reduced when sulfate is present, due to the formation of barium sulfate. Briefly, samples were hydrolysed using HCl, dried and resuspended in water. For the assay, to 50 µL of each sample or control (blanks, heparin and chondroitin sulfate), 50 µL de-ionised water and 400 µL ethanol were added and mixed thoroughly. 125 µL of each ethanol-added sample, standards (final concentration 0.048 to 0.48 µg Na_2SO_4), and controls were pipetted into a 96-well microplate in triplicate. $BaCl_2$ buffer and sodium rhodizonate solution were added, mixed and incubated at room temperature (18°C) in the dark for 10 min. The colour intensity was then measured spectrophotometrically at 520 nm. The % of sulfate in each sample was then calculated from the standard curve.

For NMR Spectroscopy, the purified fucoidan (10 mg) was first dissolved in 1 mL of 99.9% D 2 O (Chiron) and lyophilized in order to reduce the residual water signal. Subsequently, the sample was dissolved in 595 µL of D 2O, and 5 µL of 1% 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) (Sigma-Aldrich) was added for a chemical shift reference. The desulfated fucoidan (25 mg) was dissolved in 1 mL of 99.9% D2 O, and the solution was lyophilized. A complete desulfation was performed by a modified version of the solvolytic desulfation procedure from Inoue & Nagasawa (1976). Sodium cations were exchanged with pyridinium ions by dissolving 150 mg of pFuc in water and rinsing the solution through an AG 50W cation exchange resin (Bio-Rad), which was beforehand equilibrated with pyridine (Sigma-Aldrich). The effluent was neutralized with 0.3 mL of pyridine and lyophilized. The fucoidan-pyridinium salt was dissolved in 15 mL of DMSO, and 75 µL of water was added. The mixture was kept at 80 °C for 30 min and subsequently dialyzed (12K molecular weight cut off) against 1 M NaCl and distilled water prior to lyophilization. Residual DMSO from the desulfation procedure (see above) was used as a chemical shift reference.

All homo- and heteronuclear NMR experiments were recorded on a Bruker Avance 600 MHz or Bruker AVIIIHD 800 MHz (Bruker BioSpin AG, Fälladen, Switzerland) equipped with a 5 mm cryogenic CP-TCI z-gradient probe. All NMR recordings were performed at 25 °C.

3.6.4 Protein Contents and Amino Acid Compositions

Contaminant proteins (CPs) and protein contents in the residual biomass were measured using the Lowry method as described by Herbert et al. (1971) with bovine serum albumin as a standard. In brief, 5 mL 1.0 N NaOH was added to the biomass aliquot and incubated for 5 min in a boiling water bath (95°C, Typ 1083, GFL mbH, Burgwedel, Germany). After cooling, 2.5 mL of the reactive mixture (5% Na_2CO_3 + 0.5% $CuSO_4$ $5H_2O$ in 1.0% Na-K-Tartarate; ratio 25:1 v/v) were added and incubated for 10 min at room temperature. This was followed by the addition of 0.5 mL Folin-phenol reagent (1.0 N) and incubation for another 15 min. After centrifugation, the intensity of the resulting blue colour was determined at 650 nm.

Free amino acid analysis was conducted according to the method described in Reinholdt et al. (2019). Briefly, ∼50 mg sample was extracted in 500 µL of ice-cold LC-MS/MS buffer [150 µL chloroform, 350 µL methanol, 1 µL of MES as internal standard (1 mg/mL)]. Following addition of 400 µL ice-cold water, samples were vortexed thoroughly and incubated for at least 2 h at −20°C. After centrifugation (10 min, 20,000 *g*, 4°C), the aqueous phase was transferred to a new tube and 400 µL of ice-cold water again added to the extraction tube. Following stirring and centrifugation (5 min, 20,000 *g*, 4°C), supernatants were combined and lyophilized. Next, the dried extracts were dissolved in 400 µL water and filtered through 0.2 µm filters (Omnifix-F, Braun, Germany). The cleared supernatants were analysed using the high-performance liquid chromatograph mass spectrometer LCMS-8050 system (Shimadzu) and the incorporated LC-MS/MS method package for primary metabolites (version 2, Shimadzu). In brief, 1 µL of each extract was separated on a pentafluorophenylpropyl column (Supelco Discovery HS FS, 3 µm, 150 × 2.1 mm) with a mobile phase containing 0.1% (v/v) formic acid. The compounds were eluted at 0.25 mL min^{-1} using the following gradient: 1 min 0.1% (v/v) formic acid, 95% *Aqua destillata* (*A. dest*.), 5% acetonitrile, within 15 min linear gradient to 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile, 10 min 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile. Aliquots were continuously injected in the MS/MS part and ionized via electrospray ionization. The compounds were identified and quantified using the multiple reaction monitoring values given in the LC-MS/MS method package and the LabSolutions software package (Shimadzu). Authentic standard substances (Merck) at varying concentrations were used for calibration and peak areas normalized to signals of the internal standard. Glyoxylate, 3-HP, and glycerate were determined in the negative ion mode using selective ion monitoring for *m/z* 73, 102, and 105 corresponding to the deprotonated glyoxylate, 3-HP, and glycerate ions $[M-H]^-$. Retention time acquisition window (2 min) was verified with coelution experiments using purchased glyxoylate, 3-HP, and glycerate (Sigma-Aldrich). Varying concentrations of the three metabolites were also used for calibration curves. Data were interpreted using the Lab solution software package (Shimadzu).

3.6.5 Lipid Contents and Fatty Acid Compositions

The total lipid content was evaluated by the gravimetric method and reported as percentage of the algae dry weight (Bligh & Dyer 1959).

The fatty acids from the total lipids were obtained by the one-step direct transesterification method (Kumari et al. 2011). Briefly, 5 mg of lipids were treated with 5 mL of acetyl chloride/methanol (1:19 v/v) and esterified at 80 °C for 1 h. After cooling, 1 mL of water and 2 mL of n-hexane were added to the mixture, vortex, and centrifuged. The organic phases were collected, filtered, and dried with anhydrous sodium sulfate. Solvents were removed under nitrogen and the fatty acids methyl esters (FAMEs) solubilized in n-hexane were identified. The FAMEs were analysed by gas chromatography

(Agilent, Model 7890B, Palo Alto, CA, USA) coupled with mass spectrometry (Agilent, Model 7000C, Palo Alto, CA, USA). FAMEs were separated on an HP-5MS capillary column (30 m x 0.25 mm, i.d., 0.25 µm film thickness), (Agilent J&W Scientific, Folsom, CA, USA). A sample (5 µL) was injected at temperature of 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The column temperature was programmed as follows: initial temperature at 100 °C for 4 min, increasing with 10 °C min^{-1} to 200 °C (for 5 min), and 10 °C min^{-1} to 300 °C for 15 min. Mass detector conditions were: electron impact mode at 70 eV, transfer line temperature 280 °C, source temperature 230 °C, mass acquisition range 50–500 amu (atomic mass units) and solvent delay 3.75 min. The FAMEs were identified by comparison of standard Supelco 37 Component FAME Mix (SUPELCO, St. Louis, MO, USA) and their mass spectra with those from the NIST/EPA/NIH Mass Spectral Library version NIST 2.2 (Agilent, Palo Alto, CA, USA).

3.6.6 Screening for further utilizable Compound Classes

A phytochemical screening of the residual biomass targeting bioactive compound groups such as alkaloids, aldehydes, flavonoids, saponins and tannins, was conducted**,** following different methods referenced in Table 2.

Table 2. Methods used during the phytochemical screenings.

Compound group	Extraction Solvent	Calibration standard	Method	Ref.
Aldehydes	MeOH (80%)	Formaldehyde CH_2O	Schiff's and Fehling's tests*	Turner (1916)
Alkaloids	EtOH (99%)	Piperine $C_{17}H_{19}NO_3$	Mayer´s and Wagner`s reagent*	Scholz & Liebezeit 2006
Flavonoids	EtOH (99%)	Quercetin $C_{15}H_{10}O_7$	Alkaline Reagent Test	Tiwari et al. 2011
Glycosides	EtOH (99%)	Oleandrin $C_{32}H_{48}O_9$	Keller-Killiani Test	Usunobun et al. 2015
Phenols	EtOH (99%)	Hydroquinone $C_6H_6O_2$	Folin-Ciocalteu reagent/$FeCl_3$	LeBlanc et al. 2009
Phyto-sterols	MeOH (80%)	Ergosterol $C_{28}H_{44}O$	Liebermann-Burchardt test	Tiwari et al. 2011
Saponins	EtOH (99%)	Saponin S4521	Frothing test	Scholz & Liebezeit 2006
Tannins	EtOH (99%)	Tannic acid $C_{76}H_{52}O_{46}$	Gelatine-Saltblock test	Scholz & Liebezeit 2006

*Only samples that gave positive reactions to both reagents and tests are assumed to contain alkaloids or aldehydes, respectively. Abbreviations: EtOH: ethanol; MeOH: methanol.

3.7 Statistical Analysis

Measures were carried out in triplicate (n = 3), and the results are given as mean values and standard deviations. The results were statistically analysed using a one-way ANOVA with a statistical difference of 5% and the Tukey TSD test of the IBM SPSS software version for multiple comparisons.

4. Results

4.1 Effects of different Surface Sterilization Protocols and Growth Promotors on explants of *Laminaria digitata* and *L. hyperborea*

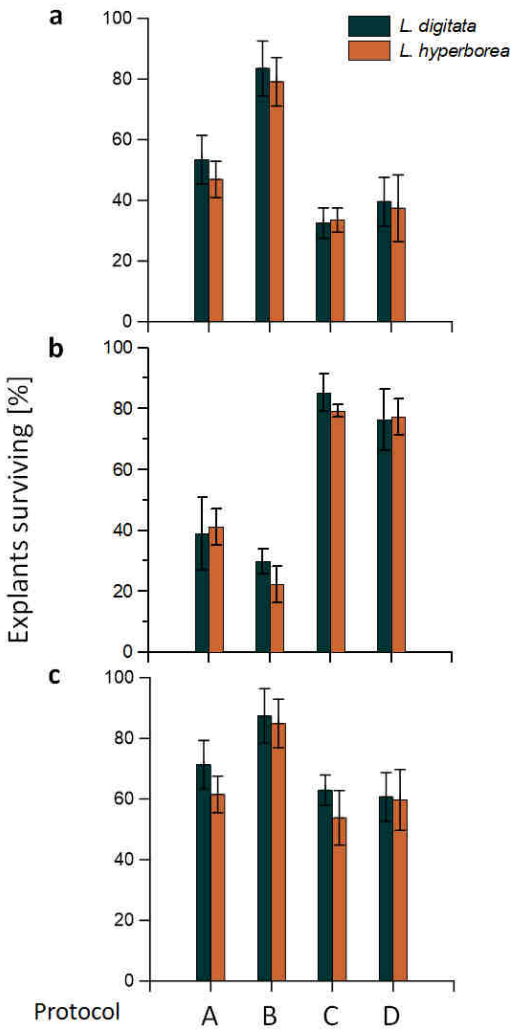

Figure 6. Effects of different surface sterilisation protocols on explant survival of *L. digitata* and *L. hyperborea* stipes (a), intercalary meristems (b) and blades (c). Values represent mean values ± SD (n = 5, replicates = 10, repetitions = 3).

To gain axenic explants (stipes, intercalary meristem and blades) prior to the cultivation, overall, seven types of chemical sterilants were tested in different combinations in four protocols, as described in section 3.1. (betadine (BE), germanium oxide (GeO_2), povidone iodine (PI), commercial bleach (Domestos®), sodium hypochlorite solution (SHPS), ethanol, streptomycin sulphate (STS; 590.60 IU mg^{-1}), as well as Provasoli's antibiotic (PESA) concentrated solution (containing 240,000 units penicillin

G, 1,000 µg chloramphenicol, 6,000 units polymyxin B and 1,200 µg neomycin per L)). The results are given in Fig. 6.

Out of the four protocols, the one using method B was most successful for gaining functional (surviving >11 months) stipe and blade explants from *L. hyperborea* and *L. digitata* (79.1-84.9% and 83.5-87.4%; Figs. 6a and c). In this protocol axenic material was obtained by steeping the explants for 5 min in BE (1%) prepared in filter-sterilized seawater and then rinsing in sterile seawater for 2 min in a 1% solution of commercial bleach and finally rinsing three times in sterile seawater. Explants were then incubated 12 h in sterile seawater containing 1 µM GeO_2 and 2% PESA solution (cf. Table 1). For explants of the intercalary meristem the best method was protocol C, resulting in 79.8 and 83.7 % surviving explants from *L. hyperborea* and *L. digitata*, respectively (Fig. 6b). This protocol differed to the former one only in the replacement of commercial bleach by sodium hypochlorite (cf. Table 1).

The media used for this study were Provasoli's enriched seawater (PES), and modified Murashige and Skoog basal medium (MS). Both were tested as solid (agar) and liquid medium (section 3.1). As growth promotors 6-benzylaminopurine (BAP), naphthaleneacetic acid (NAA), thidiazuron (TDZ), forchlorfenuron [N-(2-chloro-4-pyridyl)-N'-phenylurea = CPPU), kinetin and zeatin were applied in concentrations varying between 0.2 and 3.8 µM. As control, PES and MS medium were used without growth promotors. The number of bud- and callus-like structures developed per week on the explants from *L. hyperborea* and *L. digitata* (stipes, intercalary meristems and blades) were measured to assess growth. The results are presented in Tables 3 and 4, showing that NAA and kinetin were the most promising growth promotors in both media. They were therefore tested additionally in three different mixtures (MIX I: 0.45 µM NAA + 1.5 µM kinetin; MIX II: 0.35 µM NAA + 2.3 µM kinetin; MIX III: 0.45 µM NAA + 0.83 µM kinetin). Overall, the explants in PES medium (Table 3) showed a higher number of bud and callus formation compared to MS medium (Table 4). The highest growth was in liquid PES medium supplemented with MIX II (up to 18.2 ± 1.1 % bud inducements per week for stipe explants and 10.7 ± 1.4 % callus inducements per week for blade explants both from *L. digitata;* Table 3 continued, page 20). Explants from *L. hyperborea* showed lower inducement rates in general in all conditions. At a later stage, a differentiation in the tissues from *L. hyperborea* and *L. digitata* were observed as well as the formation of gametes, which grew out into male and female plants (Fig. 7). Thalli of sporophytes were observed but they did not reach a length of more than 1-2 cm during this project.

Figure 7. Pictures of the female (a) and male (b) gametophytes from *L. digitata*.

Table 3. Bud (B)- and callus (C)-like structure inducement (% week^{-1}) during application of different growth promotors for two months, utilizing stipe, intercalary meristem (IM) and blade explants in solid (a) and liquid (b) Provasoli's enriched seawater medium (PES). Values represent mean values ± SD (n = 3).

Treatment	*L. digitata*						*L. hyperborea*					
	stipe		IM		blade		stipe		IM		blade	
	B	C	B	C	B	C	B	C	B	C	B	C
a) in solid medium (% inducement · week^{-1})												
Control (PES)	6.9 ± 0.5	0.3 ± 0.1	5.2 ± 0.7	n.d.	6.6 ± 0.3	n.d.	4.1 ± 0.2	n.d.	3.5 ± 0.2	n.d.	3.7 ± 0.3	n.d.
BAP 0.44 µM	3.2 ± 0.3	n.d.	4.4 ± 0.5	n.d.	3.9 ± 0.5	n.d.	2.6 ± 0.3	n.d.	2.2 ± 0.5	n.d.	2.5 ± 0.6	n.d.
NAA 0.53 µM	7.1 ± 1.3	2.5 ± 0.5	6.3 ± 1.1	1.5 ± 0.4	7.0 ± 1.1	2.3 ± 0.5	5.4 ± 1.0	n.d.	4.9 ± 0.7	0.3 ± 0.1	5.5 ± 0.2	1.1 ± 0.3
TDZ 0.2 µM	2.5 ± 0.5	n.d.	2.3 ± 0.3	n.d.	2.0 ± 0.3	n.d.	1.9 ± 0.1	n.d.	1.5 ± 0.5	n.d.	1.5 ± 0.3	n.d.
CPPU 3.8 µM	2.3 ± 0.6	n.d.	3.1 ± 0.2	n.d.	2.2 ± 0.5	n.d.	0.8 ± 0.1	n.d.	1.0 ± 0.2	n.d.	0.9 ± 0.2	n.d.
Kinetin 2.3 M	7.0 ± 2.4	2.2 ± 0.6	5.7 ± 1.0	3.9 ± 0.5	7.2 ± 0.8	3.6 ± 0.3	4.4 ± 0.5	n.d.	3.8 ± 0.3	0.5 ± 0.1	4.9 ± 0.4	0.8 ± 0.2
Zeatin 0.83 µM	1.6 ± 0.3	n.d.	1.5 ± 0.5	n.d.	2.3 ± 0.7	n.d.	0.6 ± 0.1	n.d.	0.5 ± 0.1	n.d.	0.3 ± 0.1	n.d.
MIX I	10.4 ± 1.0	4.9 ± 1.1	8.3 ± 2.4	7.2 ± 2.0	9.5 ± 1.1	7.9 ± 1.0	6.4 ± 1.2	1.5 ± 0.1	5.3 ± 0.4	1.3 ± 0.2	5.7 ± 0.3	1.6 ± 0.3
MIX II	13.8 ± 1.1	5.3 ± 1.2	12.6 ± 3.1	8.1 ± 1.3	14.3 ± 1.5	9.9 ± 0.5	9.5 ± 1.9	4.8 ± 0.6	9.9 ± 0.5	3.9 ± 0.3	8.1 ± 2.2	3.5 ± 0.7
MIX III	11.5 ± 0.9	4.8 ± 0.5	8.2 ± 1.1	4.7 ± 0.9	10.2 ± 0.6	5.1 ± 0.3	8.2 ± 1.0	2.7 ± 0.3	6.5 ± 1.4	2.8 ± 0.7	7.0 ± 0.9	2.5 ± 0.5

Abbreviations: n.d., not detected; IM, intercalary meristem; BAP, 6-Benzylaminopurine; NAA, naphthaleneacetic acid; TDZ, Thidiazuron; CPPU, Forchlorfenuron [N-(2-chloro-4-pyridyl)-N'-phenylurea]; MIX I, 0.45 µM NAA + 1.5 µM kinetin; MIX II, 0.35 µM NAA + 2.3 µM kinetin; MIX III, 0.45 µM NAA + 0.83 µM kinetin.

Table 3 continued. Bud (B)- and callus (C)-like structure inducement (% week^{-1}) during application of different growth promotors for two months, utilizing stipe, intercalary meristem (IM) and blade explants in solid (a) and liquid (b) Provasoli's enriched seawater medium (PES). Values represent mean values ± SD (n – 3).

Treatment	*L. digitata*						*L. hyperborea*					
	stipe		IM		blade		stipe		IM		blade	
	B	C	B	C	B	C	B	C	B	C	B	C
b) in liquid medium (% inducement · week^{-1})												
Control (PES)	7.3 ± 1.4	1.2 ± 0.1	7.1 ± 0.5	1.5 ± 0.5	7.9 ± 1.1	0.5 ± 0.2	5.2 ± 0.2	0.3 ± 0.1	4.5 ± 0.5	0.6 ± 0.2	5.5 ± 0.3	0.9 ± 0.1
BAP 0.44 µM	3.3 ± 0.1	0.8 ± 0.2	4.9 ± 0.2	0.5 ± 0.1	4.1 ± 0.1	1.1 ± 0.5	3.3 ± 1.4	n.d.	3.1 ± 0.1	n.d.	3.0 ± 0.5	n.d.
NAA 0.53 µM	8.2 ± 0.5	1.6 ± 0.2	7.5 ± 0.6	1.6 ± 0.3	8.0 ± 2.3	3.9 ± 0.5	5.9 ± 0.3	1.1 ± 0.1	4.9 ± 0.3	0.9 ± 0.1	5.8 ± 0.2	1.0 ± 0.2
TDZ 0.2 µM	2.4 ± 0.2	n.d.	2.3 ± 0.3	n.d.	2.5 ± 0.5	n.d.	2.5 ± 0.2	n.d.	2.5 ± 0.6	n.d.	2.7 ± 0.4	n.d.
CPPU 3.8 µM	2.7 ± 0.9	n.d.	2.9 ± 0.6	n.d.	3.1 ± 0.2	n.d.	2.2 ± 0.5	n.d.	2.0 ± 0.2	n.d.	2.3 ± 0.3	n.d.
Kinetin 2.3 M	7.9 ± 0.3	1.3 ± 0.3	7.5 ± 0.3	1.5 ± 0.2	8.0 ± 0.5	3.6 ± 0.2	5.7 ± 0.6	1.2 ± 0.2	5.1 ± 0.7	1.1 ± 0.7	6.1 ± 0.5	1.3 ± 0.2
Zeatin 0.83 µM	3.6 ± 0.5	n.d.	3.7 ± 0.1	n.d.	3.5 ± 0.6	n.d.	2.7 ± 0.3	n.d.	2.4 ± 0.4	n.d.	2.6 ± 0.6	n.d.
MIX I	13.3 ± 1.2	5.2 ± 0.5	9.3 ± 0.3	7.5 ± 1.0	9.6 ± 3.3	8.1 ± 0.1	7.1 ± 0.3	3.6 ± 0.2	6.4 ± 1.2	2.4 ± 0.2	7.3 ± 1.0	2.7 ± 0.5
MIX II	16.2 ± 1.1	9.1 ± 0.7	14.6 ± 0.5	10.3 ± 0.7	16.5 ± 1.5	10.7 ± 1.4	9.9 ± 1.3	5.6 ± 0.5	10.7 ± 1.5	6.5 ± 0.7	9.9 ± 0.5	6.1 ± 1.2
MIX III	12.1 ± 0.5	5.1 ± 0.2	10.3 ± 0.6	6.6 ± 0.9	12.7 ± 1.3	6.3 ± 0.8	8.3 ± 1.5	3.1 ± 0.2	7.3 ± 0.8	3.9 ± 0.1	7.8 ± 0.6	3.5 ± 0.3

Abbreviations: n.d., not detected; IM, intercalary meristem; BAP, 6-Benzylaminopurine; NAA, naphthaleneacetic acid; *TDZ*, Thidiazuron; CPPU, Forchlorfenuron (N-(2-chloro-4-pyridyl) N' -phenylurea); MIX I, 0.45 µM NAA + 1.5 µM kinetin; MIX II, 0.35 µM NAA + 2.3 µM kinetin; MIX III, 0.45 µM NAA + 0.83 µM kinetin.

Table 4. Bud (B)- and callus (C)-like structure inducement (% week^{-1}) during application of different growth promotors for two months, utilizing stipe, intercalary meristem (IM) and blade explants in solid (a) and liquid (b) Murashige and Skoog basal medium (MS). Values represent mean values + SD (n = 3).

Treatment	*L. digitata*						*L. hyperborea*					
	stipe		IM		blade		stipe		IM		blade	
	B	C	B	C	B	C	B	C	B	C	B	C
a) in solid medium [% inducement · week^{-1}]												
Control (MS)	3.8 ± 0.6	n.d.	5.5 ± 0.2	n.d.	3.5 ± 0.4	n.d.	1.3 ± 0.5	n.d.	1.1 ± 0.8	n.d.	2.6 ± 0.5	n.d.
BAP 0.44 µM	2.2 ± 0.3	n.d.	1.7 ± 0.5	n.d.	3.3 ± 1.5	n.d.	0.7 ± 0.3	n.d.	0.5 ± 0.2	n.d.	1.1 ± 0.3	n.d.
NAA 0.53 µM	4.5 ± 1.2	0.8 ± 0.5	3.9 ± 0.4	0.5 ± 0.4	4.1 ± 1.9	2.3 ± 0.5	1.6 ± 0.5	0.3 ± 0.5	1.3 ± 0.3	0.3 ± 0.1	1.9 ± 0.8	0.5 ± 0.3
TDZ 0.2 µM	3.5 ± 1.1	n.d.	3.5 ± 1.1	n.d.	4.0 ± 1.7	n.d.	1.1 ± 0.2	n.d.	1.0 ± 0.5	n.d.	1.8 ± 0.4	n.d.
CPPU 3.8 µM	2.7 ± 1.3	n.d.	3.1 ± 0.9	n.d.	2.5 ± 0.5	n.d.	0.5 ± 0.1	n.d.	0.6 ± 0.2	n.d.	0.5 ± 0.3	n.d.
Kinetin 2.3 M	4.9 ± 2.0	0.3 ± 0.1	4.2 ± 2.3	0.5 ± 0.1	5.2 ± 2.3	1.9 ± 0.5	1.5 ± 0.9	0.8 ± 0.5	1.5 ± 0.4	0.5 ± 0.1	2.5 ± 1.1	0.9 ± 0.3
Zeatin 0.83 µM	1.0 ± 0.5	n.d.	0.8 ± 0.1	n.d.	1.4 ± 0.9	n.d.	0.5 ± 0.2	n.d.	0.5 ± 0.5	n.d.	0.7 ± 0.5	n.d.
MIX I	4.8 ± 1.6	0.9 ± 0.3	4.5 ± 2.0	0.8 ± 0.3	6.3 ± 2.5	2.7 ± 0.8	1.9 ± 0.6	0.9 ± 0.2	1.6 ± 0.5	0.7 ± 0.3	2.8 ± 1.5	0.9 ± 0.5
MIX II	5.1 ± 2.2	1.3 ± 0.5	5.9 ± 2.5	1.1 ± 0.5	7.3 ± 2.2	2.7 ± 0.5	2.2 ± 1.0	1.0 ± 0.1	1.9 ± 0.3	0.8 ± 0.2	3.1 ± 1.1	0.9 ± 0.5
MIX III	4.1 ± 1.9	1.1 ± 0.5	4.0 ± 1.8	1.2 ± 0.5	5.3 ± 1.8	2.3 ± 1.1	1.6 ± 0.9	0.9 ± 0.1	1.3 ± 0.2	0.7 ± 0.4	2.3 ± 1.3	1.0 ± 0.2

Abbreviations: n.d.. not detected; IM, intercalary meristem; BAP, 6-Benzylaminopurine; NAA, naphthaleneacetic acid; TDZ, Th diazuron; CPPU, Forchlorfenuron [N-(2-chloro-4-pyridyl)-N'-phenylurea); MIX I, 0.45 µM NAA + 1.5 µM kinetin; MIX I, 0.35 µM NAA + 2.3 µM kinetin; MIX II, 0.45 µM NAA + 0.83 µM kinetin.

Table 4 continued. Bud (B)- and callus (C)-like structure inducement (% week^{-1}) during application of different growth promotors for two months, utilizing stipe, intercalary meristem (IM) and blade explants in solid (a) and liquid (b) Murashige and Skoog basal medium (MS). Values represent mean values ± SD (n = 3).

Treatment	*L. digitata*						*L. hyperborea*					
	stipe		IM		blade		stipe		IM		blade	
	B	C	B	C	B	C	B	C	B	C	B	C
b) in liquid medium (% inducement · week^{-1})												
Control (MS)	2.3 ± 0.2	n.d.	2.1 ± 1.1	n.d.	3.3 ± 0.5	n.d.	0.5 ± 0.1	n.d.	0.5 ± 0.2	n.d.	1.1 ± 0.5	n.d.
BAP 0.44 µM	1.6 ± 1.0	n.d.	1.3 ± 0.8	n.d.	1.4 ± 0.3	n.d.	0.3 ± 0.1	n.d.	0.2 ± 0.1	n.d.	0.5 ± 0.3	n.d.
NAA 0.53 µM	2.9 ± 1.8	n.d.	2.7 ± 1.0	n.d.	2.9 ± 0.8	n.d.	0.8 ± 0.2	n.d.	0.6 ± 0.3	n.d.	1.2 ± 0.5	n.d.
TDZ 0.2 µM	0.8 ± 0.3	n.d.	0.5 ± 0.2	n.d.	0.5 ± 0.3	n.d.	0.3 ± 0.1	n.d.	0.5 ± 0.4	n.d.	0.5 ± 0.3	n.d.
CPPU 3.8 µM	0.3 ± 0.2	n.d.	0.4 ± 0.3	n.d.	0.3 ± 0.1	n.d.	0.1 ± 0.1	n.d.	0.1 ± 0.1	n.d.	0.5 ± 0.2	n.d.
Kinetin 2.3 M	2.2 ± 1.0	n.d.	2.0 ± 0.9	n.d.	1.9 ± 0.5	n.d.	0.3 ± 0.3	n.d.	0.5 ± 0.3	n.d.	0.8 ± 0.4	n.d.
Zeatin 0.83 µM	0.2 ± 0.1	n.d.	0.3 ± 0.1	n.d.	0.5 ± 0.1	n.d.	0.1 ± 0.1	n.d.	0.1 ± 0.1	n.d.	0.5 ± 0.1	n.d.
MIX I	2.2 ± 0.5	n.d.	1.9 ± 0.7	n.d.	3.0 ± 0.5	n.d.	0.5 ± 0.2	n.d.	0.5 ± 0.1	n.d.	0.9 ± 0.2	n.d.
MIX II	2.5 ± 0.3	n.d.	2.9 ± 0.2	n.d.	3.6 ± 0.2	n.d.	1.1 ± 0.4	n.d.	0.8 ± 0.3	n.d.	1.5 ± 0.5	n.d.
MIX III	2.0 ± 0.1	n.d.	1.8 ± 0.5	n.d.	2.9 ± 0.4	n.d.	0.4 ± 0.1	n.d.	0.4 ± 0.2	n.d.	0.8 ± 0.5	n.d.

Abbreviations: n.d., not detected; IM, intercalary meristem; BAP, 6-Benzylaminopurine; NAA, naphthaleneacetic acic; TDZ, Thidiazuron; CPPU, Forchlorfenuron [N-(2-chloro-4-pyridyl)-N'-phenylurea); MIX I, 0.45 µM NAA + 1.5 µM kinetin; MIX II, 0.35 µM NAA + 2.3 µM kinetin; MIX III, 0.45 µM NAA + 0.83 µM kinetin.

4.2 Alginate Contents in relation to Experimental Parameters

The effects of different salinities (10, 20 and 40 PSU), temperatures (3, 10 and 15°C) and velocities (weak, medium and strong) on the alginate accumulation potential in explants of *Laminaria digitata* and *L. hyperborea* (stipes, intercalary meristems and blades) were tested initially. As a control, the 'standard' conditions of 35 PSU, 8°C, without velocity were used to cultivate specimens. The results are depicted in Fig. 8, showing that the highest sodium alginate content was achieved by application of high velocities (0.1 m/s) in the blade explants of *L. digitata* and *L hyperborea* (83.5 ± 5.5 and 80.4 ± 4.9%). The higher velocity appeared to increase alginate content in all the tissues tested, compared to weak or medium velocity, and cultures maintained at 3 °C also showed higher alginate induction compared to those at higher temperatures.

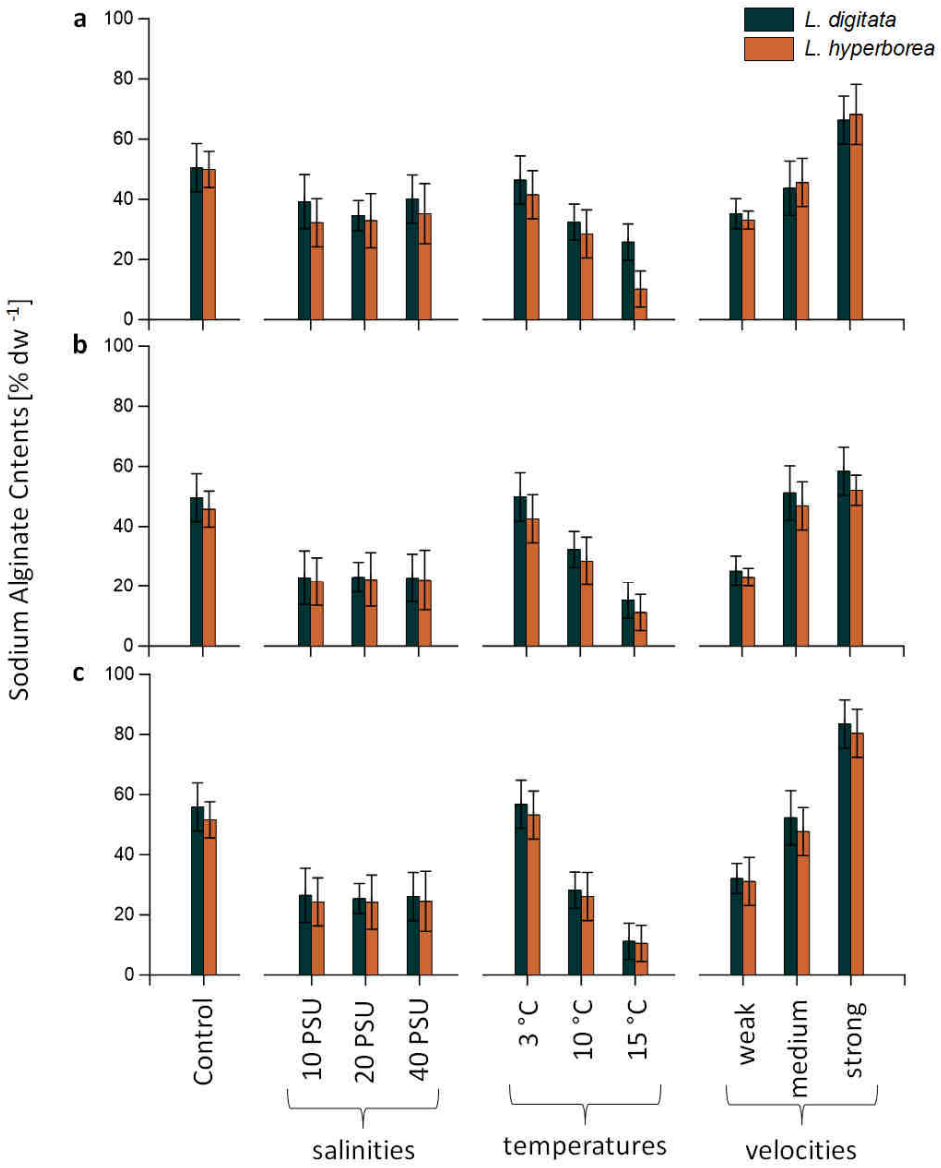

Figure 8. Effects of selected abiotic factors on the sodium alginate contents of explants from *L. digitata* and *L. hyperborea* stipes (a), intercalary meristems (b) and blades (c). Values represent mean values ± SD (n = 3).

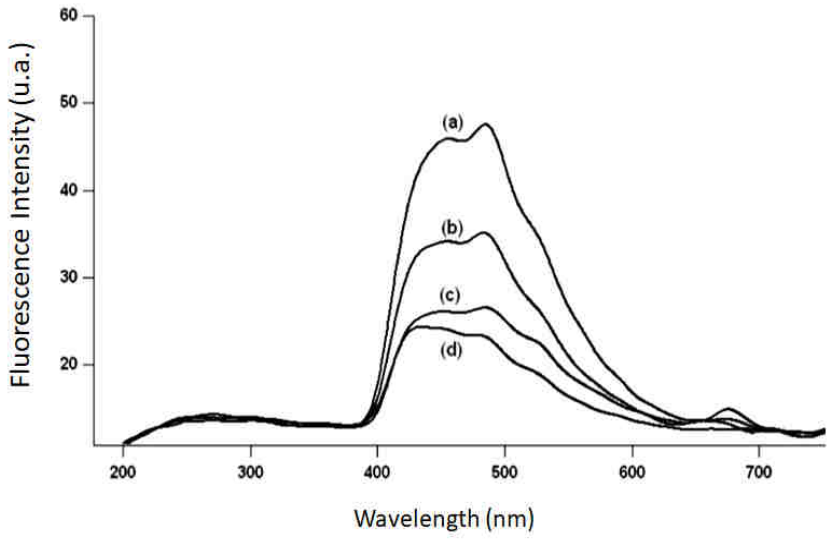

Figure 9. Monitoring of the impurity by fluorescence, showing the (a) emission curve after the first purification; (b) emission curve after the second purification; (c) emission curve after the third purification; (d) emission curve after the fourth purification.

The purification degree of alginates was monitored by fluorescence spectroscopy. Figure 9 shows the impurity profile (presence of polyphenols) of alginates after four purification steps. The analysis shows the presence of residues emitting at 450 and 484 nm. After 4 consecutive re-purifications with ethanol, until the intensity profile was constant, the fluorescence intensity was decreased by 51.17% for contaminants emitting at 484 nm and by 52.96% for those emitting at 450 nm.

4.3 Products of the Sequential Extraction Process

4.3.1 Fucoxanthin

Following the results from the experiment shown in 4.2, blade explants of *Laminaria digitata* and *L. hyperborea* were subjected to high velocities (0.1 m/s) in PES liquid medium in combination with 50 µg L^{-1} vanadium, 0.35 µM naphthaleneacetic acid (NAA), 2.3 µM kinetin and 10 g L^{-1} sucrose. The fucoxanthin obtained ranged between 0.37 ± 0.11 and 0.66 ± 0.15 mg · g^{-1} DW, being the highest for blade explants of *L. digitata* (Fig. 10, Table 5).

Table 5. Concentrations of fucoxanthin obtained from the blade explants of *Laminaria digitata* and *L. hyperborea* after up-scale cultivation using high velocities. Values correspond to the average of triplicates ± SD.

Species	**Fucoxanthin** [mg · g^{-1} DW]
L. digitata	0.66 ± 0.15
L. hyperborea	0.37 ± 0.11

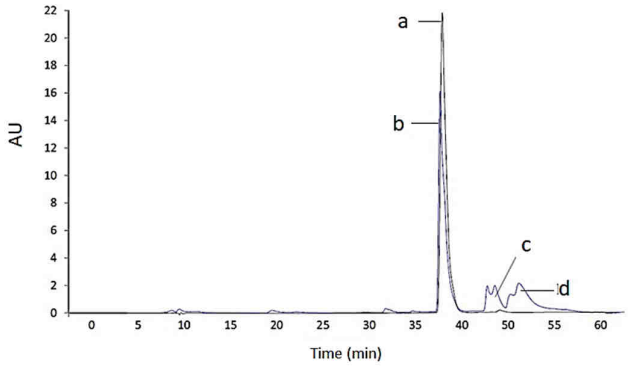

Figure 10. HPLC separation of fucoxanthin in an overlay chromatogram at 39.89 min in an all-trans-fucoxanthin standard peak (a, 10 µg mL^{-1}); b) *L. digitata* blade explant sample; c) two isomers of cis-fucoxanthin; and d) an unidentified compound, possibly zeaxanthin.

4.3.2 Sodium Alginate

The sodium alginate yield detected in the purified sample obtained from tissue cultivated (blade explants) *L. digitata* and *L. hyperborea* was 70.9 ± 1.0 and 70.3 ± 0.8 % DW, respectively, and showed a monosaccharide composition dominated by mannuronic and guluronic acid, besides traces of glucose and fucose, probably derived from laminarin and fucoidan (Table 6). Glucose and fucose residues were present at slightly higher amounts in *L. hyperborea* samples.

Table 6. Total alginate yields and their main monosaccharide composition detected in the purified product fractions obtained from tissue cultivated (blade explants) *L. digitata* (I) and *L. hyperborea* (II) exposed to high velocities. Values correspond to the average of triplicates ± SD.

	Yield [% DW]	**Monosaccharides** [% DW]				**ManA** [% DW]	**GulA** [% DW]
		Gal	**Glu**	**Fuc**	**other**		
Alginate I	70.8 ± 1.0	0.23	0.09	0.07	0.36	20.52	15.83
Alginate II	70.7 ± 0.8	0.87	0.43	0.15	1.33	16.84	15.55

Abbreviations: Fuc= fucose; Gal = galactose; Glc = glucose; ManA = Mannuronic acid; GulA = Guluronic acid; Others: rhamnose, arabinose, xylose and mannose.

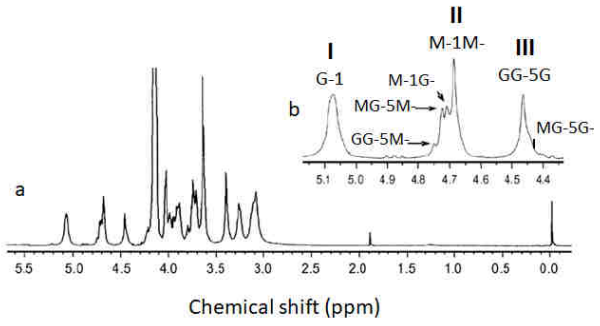

Figure 11. a) Example of a 1 H-NMR spectrum of the alginate extracted from blade explants of *L. digitata*, including b) the assignment of the H1 and H5 signals for M and G residues.

The peaks of the components in sodium alginate of *L. digitata* and *L. hyperborea* samples were observed and assigned based on data previously reported in the literature for Laminariales (Fig. 11a) (Penman & Sanderson 1972, Grasdalen et al. 1979, Dora et al. 2007). The anomeric regions of the 1HNMRspectra of sodium alginate samples (Fig. 11b), show specific peaks of the guluronic acid anomeric proton (G-1) at 5.17 ppm (peak I); guluronic acid H-5 (G-5) at 4.56 ppm (peak III); and mannuronic acid anomeric proton (M-1) at 4.76 ppm (peak II) and also the C-5 of alternating blocks (GM-5) at 4.82 ppm imbricated with peak III. This anomeric region can also give information about the linkage between G-blocks and M-blocks as it was previously reported (Haug et al. 1974, Grasdalen et al. 1979). M-1M and M-1G represent the anomeric proton of an M residue neighboring another M residue or a G residue, respectively. MG-5M, GG-5M, and MG-5G refer to the H-5 proton of the central G residue in an MGM, GGM, or MGG triad, respectively. G-1 refers to the anomeric proton of G residues and GG-5G refers to the anomeric proton of G residues in G-blocks.

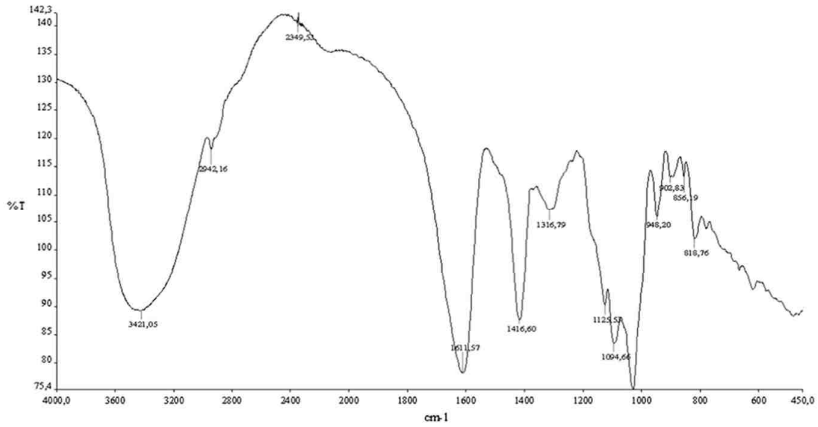

Figure 12. FT-IR spectrum of sodium alginate from *L. digitata*.

Fourier Transform Infrared Spectroscopy (FT-IR) spectrum of sodium alginate from *L. digitata* showed typical absorption peaks in Figure 12. A broad band at 3146 cm^{-1} was assigned to hydrogen bonded O–H stretching vibrations and the other at 2900 cm^{-1} was attributed to C–H stretching vibrations. According to the literature, the absorption at 1600 cm^{-1} was carboxylate O–C–O asymmetric stretching vibrations, and at 1400 cm^{-1} was assigned to C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group. The band measured at 1200 cm^{-1} may be attributed to C–C–H (and O–C–H) deformation. The band measured at 900 cm^{-1} was assigned to the α-L-guluronic asymmetric ring vibration, and the band at 860 cm^{-1} is C1-H deformation vibration of β-mannuronic acid acid residues. The band at 818.76 cm^{-1} may be characteristic of mannuronic acid residues.

Table 7. Intrinsic viscosity and average molar masses of alginate from blade explants of *Laminaria digitata* and *L. hyperborea.*

	[η] dL/g	**$M_w \cdot 10^{-5}$ (g/mol)**
L. digitata	2.51	1.16
L. hyperborea	6.3	3.03

The intrinsic viscosity is defined by the Huggins equation (Huggins 1942) as: $[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c}$ where η_{sp} and *c* are the specific viscosity and the concentration of the solution, respectively. At infinite dilution, this equation represents the measurement of the hydrodynamic volume occupied by the macromolecule. A classical procedure for its determination, based on the equation below, consists of the determination of viscosities of solutions with various concentrations, followed by extrapolation of η_{sp} /c to zero concentration. In a range of moderate concentrations, the dependence is linear and can be written as follows:

$$\eta_{sp}/c = [\eta] + kH[\eta]^2 c$$

where *kH* is the Huggins constant.

The intrinsic viscosity $[\eta]$ in 0.1 M NaCl at 25 °C for alginates from blade explants of *L. digitata* and *L. hyperborea* is 2.51 and 6.3 dL/g, respectively (Table 7). These values were obtained by a graphical extrapolation based on the Huggins equation.
Furthermore, the viscosity average molecular weight M_v were calculated from the Mark–Houwink equation:

$$[\eta] = kM_v^a$$

where $[\eta]$ is the intrinsic viscosity, and the constants *a* and *k* are empirical parameters which are depending on the system (polymer, solvent, and temperature).

Clementi et al. (1998) proposed empirical relations for $[\eta]$ and the weight-average molar mass (M_w).

$$[\eta] = 0.023 M_w^{0.0984}$$

where $[\eta]$ is given in dL/g and *M* in kDaltons.
This relationship permits to estimate the Mw values for alginates from *L. digitata* and *L. hyperborea* from intrinsic viscosity data (Table 7). All values obtained have a magnitude of 10^{-5} g/mol. *L. digitata* has a lower molecular weight ($1.16 \cdot 10^{-5}$ g/mol) in comparison to *L. hyperborea* ($3.03 \cdot 10^{-5}$ g/mol).

4.3.3 Fucoidan

Table 8. Total polysaccharide concentrations (TPSC) of the crude fucoidan extracts as well as carbohydrate, contaminating (=C)-protein and total phenolic contents of the purified product fractions obtained from blade explants of *L. digitata* and *L. hyperborea* after exposure to high velocities. Values correspond to the average of triplicates ± SD.

Species	TPSC [% DW]	Carbohydrate [% DW]	C-Protein [% DW]	TPC [% DW]
L. digitata	19.2 ± 3.9	14.3 ± 0.8	n.d.	n.d.
L. hyperborea	18.9 ± 5.2	14.5 ± 0.5	n.d.	n.d.

n.d., below detection limit (0.01). Based on % dry weight (g 100 g^{-1} dry weight).

Average total polysaccharide concentrations of the crude fucoidan extract in *L. digitata* and *L. hyperborea* was 19.2 ± 3.9 and 18.9 ± 5.2 % DW, respectively (Table 8). After purification these values were around 25 % lower. Contaminating protein and total phenolics were not detected.

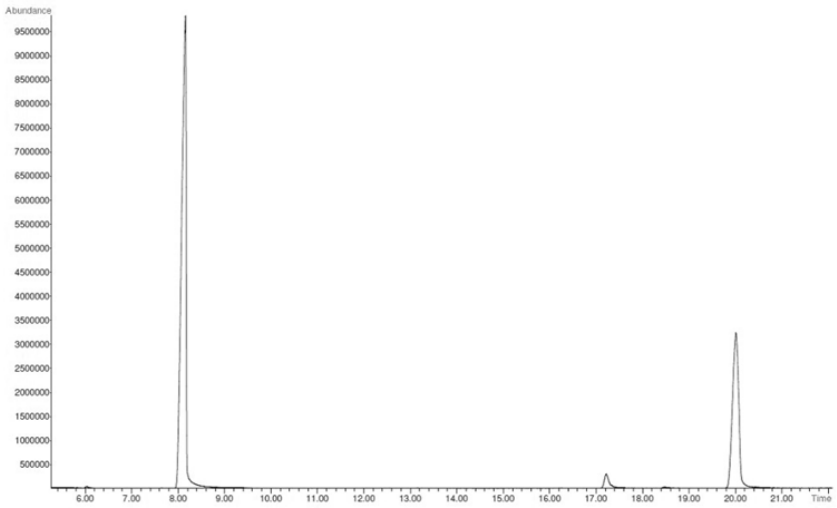

Figure 13. GC profile of pFuc. Fucose (8.1 min) and galactose (17.2 min) were detected. In addition, traces of glucose (18.5 min) were found. Three replicates for each of the samples were analysed. Inositol (20.0) is used as standard.

The GC profile of fucoidan revealed the presence of fucose (retention time = 8.1 min) and galactose (17.2 min) together with traces of glucose (18.5 min) (Fig. 13). Mol % values for each of the components were calculated (taking into consideration the chromatographic response factors for each of the monosaccharides) from blade explants of *L. digitata* (fucoidan I) and *L. hyperborea* (fucoidan II), leading to a content of 97.5 ± 0.2 and 97.8 ± 0.2 fucose and 2.5 ± 0.2 and 2.2 ± 0.2 galactose, respectively, resulting in a fucoidan composition almost entirely made of fucose. The traces of glucose may arise from minor impurities derived from laminarin. Both fucoidan samples showed a high degree of sulfation (>30 % DW; Table 9).

Table 9. Monosaccharide composition of fucoidan and its sulfate contents detected in the purified product fractions obtained from blade explants of *L. digitata* (fucoidan I) and *L. hyperborea* (fucoidan II) exposed to high velocities. Values correspond to the average of triplicates ± standard deviation. Different letters indicate significant differences between assays ($p<0.05$).

	Monosaccharides [mol %]							**UcA**	**Sulfate content**
	Gal	**Xyl**	**Ara**	**Rha**	**Glu**	**Fuc**	**Man**	[% DW]	[% DW]
Fucoidan I	2.5 ± 0.2^{b}	n.d.	n.d.	n.d.	0.04 ± 0.01^{d}	97.5 ± 0.2^{a}	n.d.	n.d.	32.8 ± 0.3^{c}
Fucoidan II	2.2 ± 0.2^{b}	n.d.	n.d.	n.d.	0.05 ± 0.01^{d}	97.8 ± 0.2^{a}	n.d.	n.d.	33.5 ± 0.3^{c}

Abbreviations: Ara = arabinose; Fuc= fucose; Gal = galactose; Glc = glucose; Man = mannose; Rha = rhamnose; Xyl = xylose; UcA = uronic acid; n.d., below detection limit (0.0001).

Glycosidic linkage analysis revealed 1,3-linked fucose as the most abundant unit in the polymer ((31.5 and 31.9%; see Table 10), followed by 1,2-linked fucose (12.8 and 13.2%) and 1,4-linked fucose (7.8 and 7.7%). The added number of branched fucose residues (22.7 and 22,4%) matches with the number of terminal fucose residues, proving the length of desulfated fucoidan samples as sufficient and internally verifying methodology. The data points toward a dominant 1→3 backbone structure, as previously determined in *L. digitata*, intercepted with a high number of 1→2,3 branching points as assigned in *S. latissima*, showing similarities to fucoidans of related *Laminariales* species (e.g., Cumashi et al. 2007).

Table 10. Linkage analysis of the desulfated fucoidan in mol % from blade explants of *L. digitata* (fucoidan I) and *L. hyperborea* (fucoidan II), performed in three technical replicates.

Mol %	t-Fucp	1,2-Fucp	1,3-Fucp	1,4-Fucp	1,2,3-Fucp	1,2,4-Fucp	1,3,4-Fucp	1,6-Galp	1,4,6-Galp	1,3,6-Galp
Fucoidan I	23.0±2.3	12.8±0.9	31.5±0.4	7.8±0.9	13.5±0.8	4.5±0.5	4.7±0.6	0.9±0.1	0.6±0.2	0.5±0.2
Fucoidan II	23.2±2.2	13.2±1.3	31.9±0.5	7.7±1.1	13.1±1.0	4.5±0.8	4.8±0.5	0.8±0.2	0.5±0.2	0.4±0.1

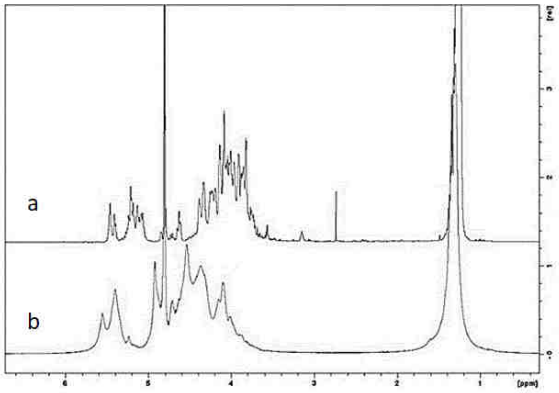

Figure 14. 1H NMR of purified fucoidan (a) and desulfated fucoidan (b) at 600 MHz, 298 K and in D2O.

The proton spectra of the purified (pFuc) and desulfated fucoidan (dsFuc) are given in Fig. 14. The proton spectrum of pFuc reveals two broad and dominant signals in the anomeric region as well as strong signals in the methyl region, which correspond to the C-6 methyl protons of fucopyranose. The ratio between the integral for the anomeric signals {H-1} (5.1–5.8 ppm; integral = 1.000), internal {H-2,3,4,5} (3.6–5.1 ppm; integral = 4.186), and methyl {H3–6} (1.0–1.8 ppm; integral = 3.122) protons supports the results from the monosaccharide analysis of a polysaccharide almost exclusively consisting of fucose units.

4.3.4 Residual Biomass

4.3.4.1 Protein Contents and Amino Acid Compositions

The average protein content in the residual biomass remaining at the end of the biorefinery process was 6.9 ± 1.1 % DW in *Laminaria digitata* and 6.8 ± 1.3 % DW in *L. hyperborea*. The residual protein fractions obtained from the samples Ld-HV and Lh-HV were subjected to further amino acid analysis (cf. Table 11). As a control, untreated specimens (i.e., grown in standard conditions with no velocity) were used for comparison. The most abundant amino acids in the residual biomass from untreated *Laminaria digitata* blade explants (control) were asparagine, alanine, leucine, and glutamine, with concentrations ranging from 9.03-18.82 µg/mg DW. In the protein fraction of the sample Ld-HV this dominance shifted towards glutamine as the most dominant amino acid (33.21 µg/mg DW), followed by asparagine and alanine with values ranging from 11.88 to 19.48 µg/mg DW. A similar amino acid composition, although in lower concentrations, was recorded in the residual biomass from untreated *Laminaria hyperborea* blade explants (control) as well as the sample Lh-HV.

Table 11. Amino acid profiles of the two protein fractions from blade explants of *Laminaria digitata* (Ld) and *L. hyperborea* (Lh) obtained from the high-velocity assay (HV) after extraction of fucoxanthin, alginate and fucoidan (= residual biomass). Control = untreated specimens. Values correspond to the average of triplicates ± SD.

	L. digitata		***L. hyperborea***	
Amino acid (µg · mg DW^{-1})	**Control**	**Ld-HV**	**Control**	**Lh-HV**
Asparagine	15.68 ± 0.12	11.88 ± 0.10	12.69 ± 0.13	10.57 ± 1.26
Serine	5.70 ± 0.23	4.48 ± 0.09	4.51 ± 0.72	5.32 ± 0.43
Alanine	17.20 ± 0.05	19.48 ± 0.13	10.83 ± 0.08	16.15 ± 0.10
Glycine	6.65 ± 0.12	4.97 ± 0.13	4.75 ± 0.05	5.33 ± 0.26
Glutamine	18.82 ± 0.15	33.21 ± 0.27	12.68 ± 0.18	19.47 ± 0.23
Threonine	6.27 ± 0.08	4.87 ± 0.05	5.16 ± 0.07	5.98 ± 0.09
Cysteine	2.47 ± 0.05	2.92 ± 0.02	2.38 ± 0.08	2.16 ± 0.05
Proline	5.70 ± 0.13	5.84 ± 0.15	4.32 ± 0.26	5.07 ± 0.03
Lysine	6.94 ± 0.29	5.26 ± 0.11	5.20 ± 0.03	5.23 ± 0.02
Histidine	1.90 ± 0.02	1.85 ± 0.02	2.73 ± 0.03	1.82 ± 0.03
Arginine	6.08 ± 0.13	5.06 ± 0.14	4.36 ± 0.20	4.64 ± 0.21
Valine	6.46 ± 0.36	4.97 ± 0.21	5.11 ± 0.23	4.83 ± 0.15
Methionine	2.57 ± 0.15	2.05 ± 0.03	1.92 ± 0.02	2.09 ± 0.06
Tyrosine	1.13 ± 0.10	3.31 ± 0.14	3.42 ± 0.25	3.26 ± 0.05
Isoleucine	4.94 ± 0.37	3.70 ± 0.52	3.63 ± 0.30	4.11 ± 0.08
Leucine	9.03 ± 1.16	6.82 ± 0.55	6.09 ± 0.37	7.53 ± 0.26
Phenylalanine	6.27 ± 0.09	4.67 ± 0.19	4.72 ± 0.22	5.19 ± 0.31
Tryptophan	4.37 ± 0.21	3.31 ± 0.07	2.26 ± 0.05	3.45 ± 0.05
Total	**128.18**	**128.57**	**96.76**	**112.2**

Abbreviations: Ld, *Laminaria digitata*; Lh, *L. hyperborea*; HV, high velocities.

4.3.4.2 Lipid Contents and Fatty Acid Compositions

The average lipid content in the residual biomass samples was 3.1 ± 0.5 % DW in *Laminaria digitata* and 3.6 ± 0.3 % DW in *L. hyperborea*. Overall, 42 fatty acids (FAs) were identified and quantified in residual biomass from untreated (control) and treated (samples Ld-HV and Lh-HV) blade explants of *L. digitata* and *L. 30hyperborea* (Table 12). There was significant variation in the individual concentration of fatty acids and in the overall accumulation of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acid (PUFA) depending on the treatment and species. The levels of Fas were generally low in *L. digitata* residual biomass samples with SFAs: 23.75–28.73 µg/g DW, MUFAs: 14.06–16.15 µg/g DW and PUFAs: 10.09-10.39 µg/g DW in sample Ld-HV and control assay, respectively. In contrast, the FA profile of *L. hyperborea* residual biomass samples showed significantly higher concentrations of FA groups, particularly in the control assay, ranging from 40.06 to 238.29 µg/g DW for total SFAs and PUFAs, respectively. The highest values were recorded for the PUFA *cis*-5,8,11,14,17-Eicosapentaenoic acid methyl ester (37.36 ± 0.53 and 82.83 ± 1.15 µg/g DW) in the control and experimental assay, respectively.

Table 12. Fatty acid profiles of the two lipid fractions from the blade explants of *Laminaria digitata* and *L. hyperborea* obtained from the high-velocity assay (HV) after extraction of fucoxanthin, alginate and fucoidan (= residual biomass). Control = untreated specimens. Values correspond to the average of triplicates ± SD.

	L. digitata		***L. hyperborea***	
Fatty acid ($\mu g \cdot g\ DW^{-1}$)	**Control**	**Ld-HV**	**Control**	**Lh-HV**
Saturated fatty acids (SFAs)				
C4:0	0.92 ± 0.05	0.20 ± 0.14	n.d.	n.d.
C6:0	0.10 ± 0.02	0.06 ± 0.02	n.d.	n.d.
C8:0	0.16 ± 0.02	0.15 ± 0.05	0.31 ± 0.05	0.12 ± 0.03
C9:0	0.05 ± 0.01	0.02 ± 0.01	0.34 ± 0.07	0.23 ± 0.11
C10:0	0.24 ± 0.08	0.11 ± 0.05	0.22 ± 0.13	0.15 ± 0.06
C12:0	0.35 ± 0.06	0.21 ± 0.05	1.93 ± 0.36	1.39 ± 0.27
C13:0	n.d.	n.d.	0.69 ± 0.25	0.26 ± 0.09
C14:0	4.78 ± 0.26	4.23 ± 0.13	72.21 ± 2.28	34.07 ± 1.25
C15:0	0.34 ± 0.10	0.35 ± 0.06	5.20 ± 1.04	10.57 ± 1.32
C16:0	16.78 ± 1.33	13.09 ± 1.23	25.99 ± 2.06	18.01 ± 2.34
C17:0	4.31 ± 0.52	4.25 ± 0.54	1.55 ± 0.08	2.12 ± 0.51
C18:0	0.42 ± 0.17	0.55 ± 0.17	20.51 ± 1.50	20.65 ± 2.27
C20:0	0.21 ± 0.05	0.44 ± 0.09	10.29 ± 1.33	8.83 ± 1.38
C21:0	n.d.	n.d.	n.d.	n.d.
C22:0	n.d.	n.d.	n.d.	n.d.
C24:0	0.07 ± 0.01	0.09 ± 0.02	1.79 ± 0.62	0.97 ± 0.15
C26:0	n.d.	n.d.	1.43 ± 0.53	1.05 ± 0.52
Total SFAs	**28.73**	**23.75**	**119.92**	**87.85**
Monounsaturated fatty acids (MUFAs)				
C14:1	0.03 ± 0.01	0.04 ± 0.02	0.07 ± 0.03	0.05 ± 0.02
C16:1, *cis*9 (ω7)	3.32 ± 0.17	3.61 ± 1.29	6.23 ± 1.08	5.29 ± 1.04
C17:1 *cis*9	n.d.	n.d.	1.82 ± 0.54	1.05 ± 0.63
C18:1, *trans*9 (ω9)	10.62 ± 2.23	8.01 ± 1.08	n.d.	n.d.
C18:1, *cis*9 (ω9)	1.03 ± 0.55	1.15 ± 0.72	20.91 ± 2.23	13.61 ± 1.41
C18:1, *cis*11	n.d.	n.d.	6.28 ± 0.65	5.10 ± 0.72
C18:1 (ω7)	1.13 ± 0.62	1.19 ± 0.15	n.d.	n.d.
C20:1 *cis*9	n.d.	n.d.	4.34 ± 1.25	3.15 ± 0.26
C20:1, *cis*11 (ω9)	0.02 ± 0.01	0.06 ± 0.02	0.41 ± 0.11	0.23 ± 0.10
C22:1 (ω9)	n.d.	n.d.	n.d.	n.d.
C24:1, *cis*15 (ω9)	n.d.	n.d.	n.d.	n.d.
Total MUFAs	**16.15**	**14.06**	**40.06**	**28.25**
Polyunsaturated fatty acids (PUFAs)				
C16:2 *cis* or *trans*-7,10	n.d.	n.d.	1.43 ± 0.74	1.26 ± 0.52
C18:2, *trans*9, 12 (ω6)	0.03 ± 0.01	0.02 ± 0.01	n.d.	0.02 ± 0.01
C18:2, *cis*9,12 (ω6)	1.36 ± 0.53	2.41 ± 0.62	21.48 ± 1.66	13.53 ± 2.09

C18:3, *cis*6,9,12 (ω6)	0.17 ± 0.03	0.23 ± 0.15	2.82 ± 0.54	2.05 ± 1.02
C18:3 (ω3)	1.98 ± 0.52	3.66 ± 1.07	20.57 ± 1.44	14.30 ± 0.92
C18:4 *cis*6,9,12,15 (ω3)	n.d.	n.d.	59.86 ± 3.91	50.11 ± 2.27
C20:2 *cis*11,14	n.d.	n.d.	2.95 ± 0.61	1.35 ± 0.23
C20:3, *cis*8, 11, 14 (ω6)	0.08 ± 0.03	0.11 ± 0.05	0.82 ± 0.16	0.92 ± 0.15
C20:3, *cis*11, 14, 17 (ω3)	0.03 ± 0.01	0.02 ± 0.01	0.29 ± 0.09	0.16 ± 0.05
C20:4 (ω6)	3.14 ± 0.33	1.47 ± 0.09	37.92 ± 1.34	28.43 ± 0.26
C20:5, *cis*5,8,11,14,17 (ω3)	3.46 ± 0.23	1.88 ± 0.31	82.83 ± 1.15	37.36 ± 0.53
C22:2, *cis*13, 16	0.08 ± 0.02	0.26 ± 0.10	n.d.	n.d.
C22:5, *cis*7,10,13,16,19 (ω3)	0.06 ± 0.01	0.03 ± 0.01	5.48 ± 0.13	5.62 ± 0.11
C22:6, *cis*4,7,10,13,16,19 (ω3)	n.d.	n.d.	1.84 ± 0.25	2.33 ± 0.08
Total PUFAs	**10.39**	**10.09**	**238.29**	**157.44**
Total FAs	**55.27**	**47.9**	**398.27**	**273.54**

Abbreviations: n.d., not detected; FAs, fatty acids; Ld, *Laminaria digitata*; Lh, *L. hyperborea*; HV, high velocities; ω3, omega-3; ω6, omega-6, ω9, omega-9.

4.3.4.3 Further utilizable Compound Classes

Table 13. Results of the phytochemical screening of the two residual biomass fractions of *Laminaria digitata* and *L. hyperborea* obtained from the high-velocity assays (HV). Control = untreated specimens.

	L. digitata		***L. hyperborea***	
Compound	**Control**	**Ld-HV**	**Control**	**Lh-HV**
Aldehydes	+	n.d.	+	n.d.
Alkaloids	+	n.d.	+	n.d.
Flavonoids	++	+	+++	++
Glycosides	+	+	+	n.d.
Phenols	+++	++	+++	+++
Phytosterols	++	+	+++	+
Saponins	+	n.d.	+	n.d.
Tannins	+++	++	+++	++

Abbreviations: n.d., not detected; Ld, *Laminaria digitata*; Lh, *L. hyperborea*; HV, high velocities; +, weak colorimetric response; ++, medium colorimetric response; +++ strong colorimetric response; ++++ very strong colorimetric response.

The phytochemical screening of the residual biomass obtained after the sequential extraction procedure of tissue cultivated *Laminaria digitata* and *L. hyperborea* evaluated eight compound groups (Table 13). While in the control assays (specimens cultivated under standard conditions and directly used for compound extraction) all compounds were present, in blade tissues of both species the colorimetric responses of samples from the residual biomass showed only the presence of five compound types in the case of *L. digitata* and four compounds for *L. hyperborea*. Out of these, only phenols gave a strong colorimetric response in the residual biomass of *L. hyperborea* using the Alkaline Reagent Test (cf. Table 2), whereas for flavonoids and tannins medium colorimetric responses were recorded in the high velocity assay [Lh-HV). For phytosterols only weak colorimetric responses were found. In the high velocity assay of *L. digitata* [Ld-HV] a medium colorimetric response was detected

for phenols and tannins, whereas flavonoids, glycosides and phytosterols were only present in minor concentrations (weak colorimetric response). The positive test for phenols and tannins in both species and samples points to the presence of phlorotannins in the residual biomass.

5. Discussion

Sustainable production based on renewable biomass and efficient bioprocesses are important elements in the growing blue bioeconomy. The traditional *Laminaria digitata* and *L. hyperborea* alginate production process discards approximately 80 % of the raw material, ignoring large amounts of potential high-value products from the algae. Particularly, the 'leaf' fraction of the seaweed is often disregarded due to its lower alginate content (Feng et al. 2017). In the present study tissues of blades, stipes and intercalary meristems of *L. digitata* and *L. hyperborea* were, after optimizations of the sterilization protocol and growth stimulants, cultured at different salinities (10, 20 and 40 PSU), temperatures (3, 10 and 15°C) and velocities (weak, medium and strong) in liquid medium, to test the potential to induce alginate accumulation in the explants. The results obtained showed that the highest sodium alginate content was achieved by application of high velocities in the blade explants of *L. digitata* and *L hyperborea* (83.5 ± 5.5 and 80.4 ± 4.9% DW, respectively), but with comparable high alginate contents in stipes and intercalary meristems (cf. Fig. 8). In wild harvested and outdoor cultivated brown seaweeds, alginate contents are seldom higher than 20% DW (https://www.fao.org/3/y3550e/ Y3550E04.htm) and their contents and quality are highly variable depending on seasons of harvest and growth conditions. Of course, tissue cultivation of phaeophytes is still in its infancy and the techniques are far behind compared to those developed for agricultural plants. Still the finding that higher temperatures lead to a lower alginate quality and quantity in wild harvested and outdoor cultivated specimens, should be cause enough to further develop tissue cultivation protocols for phaeophytes in the time of climate change.

The main problem in tissue cultivation of Laminariales is the reduced growth of the explants and death after a certain amount of time (e.g., Fries 1980, Benet et al. 1997, Matsumura et al. 2001, Mussio & Rusig 2008). In the present project several tests were conducted for the optimization of the growth rate and, finally, differentiation of the tissues from *L. hyperborea* and *L. digitata* was observed, as well as the formation of gametes, which grew out into male and female plants. Although, thalli of sporophytes were observed they did not reach a length of more than 1-2 cm. Overall, the project resulted in some significant advances in tissue culture conditions and resulting growth profiles of the seaweed tissue explants, but extensive differentiation and long-term cultures were still limited. The use of plant growth stimulant mixtures was of particular interest.

It is known that macroalgae harbour a rich diversity of associated microorganisms with functions related to host health and defense. In particular, epiphytic bacterial communities have been reported as essential for normal morphological development of the algal host (Egan et al. 2013). This functional assistance implies that macroalgae and all their associated microbiota form a singular entity or holobiont (Egan et al. 2013). The holobiont concept proposes the need for a collective view of all interactions and activities within and between a host and all its associated organisms. Marine seaweeds are typically home to a diverse group of bacteria with densities varying from 10^2 to 10^7 cells cm^{-2} depending on species, thallus section and season (Armstrong et al. 2000, Bengtsson et al. 2010). Common taxa have been identified on macroalgal surfaces albeit mostly at the phylum level. For example, bacteria belonging to the phyla *Planctomycetes* and *Verrucomicrobia* are abundant on Norwegian kelp (*Laminaria hyperborea*) (Bengtsson & Øvreås 2010, Bengtsson et al. 2010). However, these phyla were notably absent from a related species of kelp (*Saccharina latissima*) from

both the Baltic and North Sea (Staufenberger et al. 2008), as a result of either host specificity for these phyla or biogeography.

Usually, the first step in tissue cultivation is the complete eradication of these microorganisms to prevent the explants being overgrown by unwanted organisms, which results in destruction of the tissue. To facilitate the growth of tissues it could be possible to reintroduce beneficial microorganisms to the cultures. To achieve this, epiphytic bacteria from blades, stipes and intercalary meristems have to be isolated, cultured and either individually or in defined mixtures added to the tissue cultures. A screening targeting polymer-degrading enzymes could help in the preselection of commensal or mutualistic macroalgal symbionts. Furthermore, exometabolome analysis may help to identify infochemicals responsible for the growth promotion.

In the second part of the present study, larger cultures were established based on the optimal growth and alginate production conditions identified in the earlier experiments. A sequential extraction process was developed targeting fucoxanthin, sodium alginate and fucoidan, using environmentally friendly alternative solvents (e.g., limonene) in combination with ultrasound-assisted extraction procedures. The chemical characterisation of the products was conducted by LC, 1H NMR and Infrared spectroscopy. Although this sequential extraction process was successful, as shown in 4.3.1-4.3.3, extraction of each specific product requires a fine tuning of a series of parameters, such as temperatures, pH or length and intensity of ultrasonic pulses, which affect the quality and quantity of the products. These tests are highly time consuming and due to the relative short project time of 12 months they were reduced to a minimum. Also, a further comparison of alginates from the stipe and intercalary meristems, as well different ages of the explant-host specimens, would be interesting to test since the literature indicates that these parameters effect alginate properties, such as viscosity, which is considered among the most important physical properties used to assess gelling capability (e.g., Indergaard et al. 1990, McKee et al. 1992, Venegas et al. 1993, Schiener et al. 2015, Kaidi et al. 2022).

Indeed, optimizing the yield of alginates or fucoidans requires fundamental knowledge of the natural seasonal variation in the composition of different seaweed tissues, and the underlying environmental factors which trigger this variation. Environmental and endogenous factors determine the seasonal cycle of growth and reproduction of perennial algae such as the Laminariales (Bartsch et al. 2008), which result in the temporal and spatial fluctuations in tissue contents of storage carbohydrates (laminarin and mannitol) (Manns et al. 2017). Thus, the effects of environmental conditions on the relative tissue content of alginates and fucoidans could be regulated both directly and indirectly. Only limited knowledge is published on the direct effect of single environmental factors on the regulation of the tissue content of this interesting group of compounds. A positive effect of salinity on the fucoidan content of *Saccharina latissima* was suggested (Ehrig & Alban 2015), whereas for *Saccharina japonica*, the fucoidan content was documented to correlate positively with temperature and negatively with environmental nitrate concentrations (Skriptsova 2016). In principle, brown algae cell walls have a fibrillar section formed of cellulose micro fibrils, which is embedded with acid polysaccharides, linked to each other by protein (Vauchel et al. 2008). The acid polysaccharides are mainly composed of fucoidans (sulphated fucans) and alginic acid, which provide the seaweeds with structural rigidity, and flexibility (García-Ríos et al. 2012). Thus, a putative function of protection against mechanical, chemical and osmotic stress to the cell wall is supported by key environmental factors being coupled directly to the tissue contents of alginate and fucoidan: (1) degree of exposure (mechanical stress): Black (1954) showed that fronds of *Laminaria digitata* and *Laminaria cloustonii* from more protected lochs had a higher L-fucose contents than open sea fronds (Black 1954)

indicating a negative effect of exposure on the tissue content of fucoidan; (2) salinity (osmotic stress): individuals of *S. latissima* in the saline North Atlantic were shown to have higher fucoidan content than individuals of the same species in the more brackish Baltic Sea (Ehrig & Alban 2015); and finally (3) high light, UV radiation and free radicals stimulated by fluctuations in environmental factors were argued, but not shown, to stimulate higher tissue contents of fucoidan in brown algae, due to the antioxidant protective function in the brown algae cell wall (Holtkamp 2009). In the present study the testing of salinity, temperature and velocity provided an overview of the potential impact of these key environmental parameters on seaweed composition, which could point towards the best conditions for onshore facilities, depending on the target applications. Also, testing additional cultivation parameters such as nutrient concentrations, UV radiation or light intensities could lead to higher specific product yields.

The residual biomass was subjected to an analysis targeting proteins, lipids and further utilizable compounds. The protein and lipid contents were within the lower range of ones described in the literature for Laminariales (e.g., Maehre et al. 2014). While the individual amino acid concentrations of both tested seaweeds were rather low and their compositions similar (cf. Table 11), the fatty acid (FAs) concentrations and composition varied significantly between species (cf. Table 12). The levels of FAs were generally low in *L. digitata*. In contrast, the FA profile of *L. hyperborea* showed significantly higher concentrations of FA groups, particularly in the control assay, ranging from 40.06 to 238.29 µg/g DW for total SFAs and PUFAs, respectively. The highest values were recorded for the PUFA *cis*-5,8,11,14,17-Eicosapentaenoic acid methyl ester (37.36 ± 0.53 and 82.83 ± 1.15 µg/g DW) in the control and experimental assay, respectively. One of the initial ideas of the present study was to analyse the protein and lipid contents of the residual biomass for their use as aquafeed. Basically, there is continued interest in the use of plant ingredients in aquafeeds, but much remains unknown about the value of aquatic macroalgae in the nutrition of aquatic animals (e.g., Kamunde et al. 2020). The most relevant effects of seaweed supplementation in aquaculture feeds include stimulation of growth performance, enhancement of feed utilization efficiency, improvement of nutrient assimilation, and improvement of fatty acid profile in muscle. However, the variability in the biochemical composition and inconsistent effects of seaweed supplementation among fish necessitates testing the effects of supplementation of specific seaweed species, extracts or products on specific fish species. In particular, salmonids are the most important aquaculture fish group and use the greatest volumes of fishmeal and fish oil in aquafeeds; therefore, demonstrating a role of seaweeds in salmonid nutrition would have major implications for aquaculture. Levels of crude protein in feeds for various age classes of Atlantic salmon range from 42 to 50 percent, with 45 percent for juveniles. In addition, there are ten essential or indispensable amino acids (EAA), specifically arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, which cannot be synthesized by fish, and must therefore be provided in the diet. The amino acid profiles of *L. digitata* and *L. hyperborea* in the present study were dominated by asparagine, alanine, leucine, and glutamine, with concentrations ranging from 9.03-18.82 µg/mg DW. Therefore, the EAA were present in lower concentrations (Table 11) and the use of these proteins as aquafeed is rather questionable and would require further testing.

In the present study, a phytochemical screening was conducted on the residual biomass pinpointing to the presence of phenolic compounds such as flavonoids and phlorotannins (Table 13). Phenolics have been found to have several bioactivities including antioxidant, anti-cancer, antidiabetic, and antimicrobial activity, making them sought after by several industries (e.g., Onofrejová et al. 2010, Wijesinghe & Jeon 2011, Catarino et al. 2021). Phenolics are a large and

heterogenous group with structures ranging from simple monomer units to complex polymerized structures. They are synthesized in both marine- and terrestrial plants via either the acetate-malonate- or the shikimic pathway (Strack 1989, Knaggs 2003, Babenko et al. 2019). Terrestrial plant phenolics have been largely studied, whereas studies on seaweed phenolics are still scarce. The occurrence of sulfated polyphenols seen in several marine plants and some algae species, is suggested to be an ecological adaptation to the marine environment (e.g., Allwood et al. 2020, Chouh et al. 2021). However, the phenomenon is also observed for phenolics in some terrestrial species (Harborne 1975). Sulfation increases the hydrophilicity and solubility of a molecule which increases the bioavailability of the compound (Correia-da-Silva et al. 2014). Characterization of polyphenols in seaweed is challenging, as they often occur at lower concentrations and are similar in structure. Particularly phlorotannins have been reported for several brown algae species. They are polymers of phloroglucinol and include several structural and conformational isomers (Lopes et al. 2018). Phlorotannins can be divided into groups based on the linkage of phloroglucinol units. Compounds with only ether linkages (C-O-C) are called fuhalols, compounds with only phenyl linkages (C-C) are called fucols, and compounds having both ether- and phenyl linkages are called fucophlorethols (Vissers et al. 2017, Murray et al. 2018, Ford et al. 2019, Cotas et al. 2020). Due to the challenge of separation and isolation, phlorotannins are primarily characterized using LC-MS and classified according to their linkage type (C-C vs. C-O-C bond) and polymerization degree (e.g., Sardari et al. 2020, Scholz et al. 2021, Wekre et al. 2023). Further investigations are necessary to identify and quantify the compounds detected in *L. digitata* and *L. hyperborea* residual biomass and assess their commercial value.

6. Conclusions and Perspectives

The present study demonstrated for the first time the potential of tissue cultivated explants gained from Icelandic *Laminaria digitata* and *L. hyperborea* as producers of sodium alginate under controlled conditions, guaranteeing a stable quantity and quality of the product. In addition, a sequential extraction process was tested targeting fucoxanthin, sodium alginate and fucoidan, using environmentally friendly alternative solvents (e.g., limonene and deep eutectic solvents) in combination with ultrasound-assisted extraction procedures on blade explants of *L. digitata* and *L hyperborea* exposed to high velocities in liquid culture. Although this sequential extraction process was successful as shown in 4.3.1-4.3.3, individual product extractions require a fine tuning of a series of parameters such as temperatures, pH or length and intensity of ultrasonic pulses, on which the quality and quantity of the products depends. These tests are highly time consuming and due to the relative short project time of 12 months they were reduced to a minimum. Also, a further comparison of alginates from the stipe and intercalary meristems, as well different ages of the explant-host specimens, would be interesting to test, since the literature indicates that these parameters effect alginate properties, such as viscosity, which is considered among the most important physical properties used to assess gelling capability. Furthermore, testing additional cultivation parameters such as nutrient concentrations UV or light intensities could lead to higher product yields.

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