



# Effects of seeding method, timing and site selection on the production and quality of sugar kelp, *Saccharina latissima*: A Danish case study

Teis Boderskov<sup>a,b,\*</sup>, Mette Møller Nielsen<sup>c</sup>, Michael Bo Rasmussen<sup>a,b</sup>,  
Thorsten Johannes Skovbjerg Balsby<sup>a,b</sup>, Adrian Macleod<sup>d</sup>, Susan Løvstad Holdt<sup>e</sup>,  
Jens Jørgen Sloth<sup>e</sup>, Annette Bruhn<sup>a,b</sup>

<sup>a</sup> Aarhus University, Department of Bioscience, Vejlsøvej 25, 8600 Silkeborg, Denmark

<sup>b</sup> Centre for Circular Bioeconomy, Aarhus University, Denmark

<sup>c</sup> Technical University of Denmark, National Institute of Aquatic Resources, Ørstedvej 80, 7900 Nykøbing Mors, Denmark

<sup>d</sup> Scottish Association for Marine Science (SAMS), Scottish Marine Institute, Dunbeg, Argyll, 1QA, UK

<sup>e</sup> Technical University of Denmark, National Food Institute, Kemitorvet, 2800 KGS Lyngby, Denmark

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## ABSTRACT

In recent years, research projects and enterprises have documented that the sugar kelp, *Saccharina latissima*, can be successfully cultivated in Northern European waters. There is a need however, for optimizing production methods to achieve an economically viable and competitive business. A novel direct seeding method, applying juvenile sporophytes directly onto textiles immediately before deployment, could be part of the optimization, as it obviates the nursery process, and can be combined with novel seeding materials, such as non-woven textiles. An extensive comparison of biomass yield and quality was made between direct and traditional seeding methods and substrates, including three deployment campaigns in three different cultivation sites: Textile ribbons were directly seeded with juvenile sporophytes (<1 mm size), and deployed the following day, whereas kuralone twine was traditionally seeded with spores, and deployed after a nursery period. The seeded materials were deployed in September, October and November, at Hjørnø and Limfjorden, and in November at the Grenaa site. The direct seeding method gave yields comparable to the traditional seeding method ( $1.0 \pm 0.1$  kg FW m<sup>-1</sup> and  $1.0 \pm 0.2$  kg FW m<sup>-1</sup>, respectively) at the most exposed site, whereas at the sheltered sites, the direct seeding method only resulted in a measurable yield following the October deployment. The highest biomass yield was achieved using the traditional seeding method, deployed in September in the Limfjorden ( $1.6 \pm 0.4$  kg FW m<sup>-1</sup>). The biomass quality was not affected by seeding method, but differed significantly between sites, with biomass from the Limfjorden having the highest content of nitrogen ( $4.65 \pm 0.07\%$  N of DM) and the lowest content of iodine ( $1.612 \pm 271$  mg I kg<sup>-1</sup> of DM). In future cultivation practices, the direct seeding method could be implemented in exposed locations in Danish waters, whereas for the more sheltered/turbid waters, improvements are needed for the direct seeding technique to become feasible.

## 1. Introduction

The cultivation of kelp species holds a great potential for producing a valuable biomass supporting sustainable production of food, feed, bio-energy and other applications [1,2]. Of all kelps cultivated in northern Europe, *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders is the most commonly cultivated species due to its high yield, easy approachable cultivation protocols and various application possibilities [3–6]. The traditional cultivation practice for producing *S.*

*latissima*, and other kelp species, involves the seeding of haploid meiospores or gametophytes on lines followed by a controlled nursery phase of 6–8 weeks [4,6]. The nursery phase has the advantage of a high degree of control of the early growth phase of the juveniles, but has high demands for labor and resources in inland facilities, resulting in greater costs. Novel, alternative methods, with application of spores, fertilized gametophytes or juvenile sporophytes onto cultivation substrates followed by direct deployment, has recently been tested in Europe. Furthermore, a new direct seeding technique has been proposed, where

\* Corresponding author at: Aarhus University, Department of Bioscience, Vejlsøvej 25, 8600 Silkeborg, Denmark.

E-mail address: [tebo@bios.au.dk](mailto:tebo@bios.au.dk) (T. Boderskov).

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juvenile sporophytes are adhered onto specialized non-woven textile substrates using a binder [7–9]. These direct techniques have the advantage of not including the resource demanding nursery phase, and could lead the way towards the use of alternative cultivation substrates such as nets, which again could optimize the stocking density and area yield, and allow quicker and more efficient deployment on site, resulting in reduced cost per unit area of farm seeded [8]. In Norway, Scotland and the Faroe Islands, the direct seeding method has provided positive results, however, only in Scotland and the Faroe Islands with yields as high as achieved by the traditional spore seeding techniques [8–10]. The challenges relate to the attachment of the juvenile gametophytes/sporophytes on the cultivation substrates and fouling of the non-woven textiles used, possibly affected by local exposure and water turbidity [7,8].

The final biomass yield of *S. latissima* cultivation depends on a range of factors: Cultivation system design [62], deployment timing [3] which balances the effect of local abiotic factors such as exposure [7,12–14], temperature [15–18], light [15,19], nutrient availability [20,21], salinity [16,22] and biotic factors such as fouling [14,23]. Exposure has shown a positive correlation with production and growth in some areas [12], and is documented to affect morphology parameters, such as thallus length and blade weight resulting in longer blades and a heavier thallus [7,12]. The effects are possibly caused by changes in water flow and gas/nutrient exchange across the blade lamina [13], but exposure may also have a positive effect on reducing fouling pressure, and changing the fouling community [14]. The optimal temperature for growth of *S. latissima* is 10–15 °C, with decrease in growth below 5 °C and above 20 °C [15–18]. Regarding light, the growth of juvenile sporophytes of *S. latissima* has been found to be saturated at a photon flux density of 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , whereas adult sporophytes were found to be saturated at 70  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  [15,19]. The growth is primarily nitrogen limited [61,25] with saturation around 10  $\mu\text{M}$  Nitrate [20], even though P-limitation is also observed in eutrophic areas [26]. *Saccharina latissima* is well adapted to oceanic salinity, and the growth halts at salinities around 10–13‰ [16,22]. Fouling of *S. latissima* can result in a decreased biomass quality, especially due to epibionts such as bryozoans and hydroids [14,27]. Cultivation sites will vary according to the mentioned factors, and physiological adaptations of local populations in specific areas could result in deviations from expected growth responses [16,22,28,29] and therefore, the cultivation strategy will need to be adapted to the local environment. In Denmark, cultivation of *S. latissima* is impaired around the southern part of the straits between Jutland and Sweden, where the surface salinity decreases below 15‰ [22,30]. However, inner Danish waters north of these areas holds many sites applicable for seaweed cultivation, though high variation in both salinity, exposure, turbidity and nutrient availability challenge the production of *S. latissima* [31].

Generally, a deployment in October–December is favorable for the growth and yield of *S. latissima* [6,62]. As described by Peteiro et al., 2016 [62], the deployment window for *S. latissima* is possibly driven by temperature and nitrogen availability, and is suggested to be optimal as temperatures decrease below 15 °C and concentrations of dissolved inorganic nitrogen (DIN) increase above 1.4  $\mu\text{M}$  N. Local variations in biofouling pressure however, could also have an effect on the optimal deployment window [14]. Cultivation yields using the new direct seeding technique could be affected by environmental parameters in a different manner than when using the traditional seeding technique, as the deployed juveniles are smaller, attached differently, and the textile substrate applied is different from the traditional lines, and potentially more prone to attract fouling organisms. At present, the literature describing cultivation trials comparing effects of site selection on cultivation of *S. latissima* is sparse, and to our knowledge, the effect of site selection and deployment timing on cultivation of *S. latissima* using the new direct seeding method has not yet been documented.

Quality of seaweed can be assessed in many ways. Fouling of *S. latissima* blades can result in a decreased biomass quality of the harvested

biomass targeted for food, especially due to epibionts such as bryozoans and hydroids [14,27,32]. Another quality measure of recent interest is the heavy metal and iodine (I) concentration of the seaweed to be used in food and feed applications. Seaweeds are known to be accumulators of heavy metals if present in the ambient environment, and heavy metals are likely to occur if there is, or has been human activities in near vicinity. *Saccharina latissima* is well known to be among the species that can exceed the recommended French threshold values of 2000  $\text{mg I kg}^{-1}$  [33] and iodine contents of up to more than 8000  $\text{mg kg}^{-1}$  dry weight have been reported [32,34].

With the overarching aim of improving the biomass yield and quality of *S. latissima* at cultivation sites impacted differently by environmental factors, this study aimed at comparing the efficiency of two seeding techniques (spore seeding on lines and sporophyte seeding on textiles) at three different Danish cultivation sites, taking into account also the monthly timing of deployment at the three sites. The hypotheses of the study was; 1: The direct seeding method would result in a lower yield than the traditional seeding method, as shown previously in Norway, 2: An early deployment in September would result in the highest yield, as this deployment had the longest growth period, and 3: The biomass quality in the final harvested biomass would vary between sites, but be equal between seeding methods used. This study is the first study systematically comparing two seeding techniques in an extensive set-up including three cultivation sites and three deployment times. The results are expected to give an indication of the potential for using the direct seeding technique in inner Danish waters, and to contribute to the general understanding on best cultivation practice of *S. latissima* in waters of variable water conditions.

## 2. Materials and methods

### 2.1. Spore extraction and gametophyte culture production

Non-fertile adult sporophytes of *S. latissima* were collected during spring 2017 in the Bay of Aarhus (56°08'21.5"N, 10°13'35.0"E). Sporogenesis was induced by removing meristematic tissue, and keeping the sporophytes in tanks (100 L) in a temperature and light controlled room (10 °C, 8:16 Light:Dark (L:D) regime, 80–130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) with bi-weekly water change and nutrient addition (200  $\mu\text{M}$   $\text{NO}_3^- \text{N}$ ) as described by Lüning, 1988 [35]. Spores were extracted from >3 sporophytes with sori, according to Edwards & Watson, 2011 [6] for production of gametophyte cultures (12th of June 2017), or for traditional seeding (14th of July 2017). For preparation of gametophyte cultures, the spores were kept in either 1000 mL Erlenmeyer flasks or 2 L rectangular culture containers with multiple PVC plates for adhesion of spores and development of gametophytes, with no separation of males and females. The gametophyte cultures were kept at 10 °C, 12:12 h L:D regime and 30–45  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of red light (LED) with a bi-weekly water change using Tyndallized seawater at 50% strength of Provasolis enriched media (PES) [36]. Cultures were exposed to white light three weeks prior to seeding to induce fertility of the gametophytes [37].

### 2.2. Seeding of substrates

#### 2.2.1. Substrate preparation

Substrates for direct seeding of sporophytes: Non-woven textile ribbons (ALGAETEX, SIOEN industries, Belgium), were tailored for the experiment in 2 m pieces equipped with a 50 mm steel ring at each end for attachment. Substrates for traditional spore seeding: A 2 mm twine (Kuralone fishing twine, Daconet, Denmark) was wound onto spools made from gabions of 55 × 7 cm (BIO-BLOK®, EXPO-NET, Denmark). Each spool was soaked and washed in tap water before use, and contained 50 m of kuralone twine. Upon deployment at sea, the spore-seeded kuralone twine was wound around a carrier line of 10 mm Polypropylene (Danline, Frydendahl, Denmark). The carrier lines were

prepared in two meter long lines, comparable to the ALGAETEX ribbons.

### 2.2.2. Traditional spore seeding

On the 14th of July, the coils with kuralone twine were arranged horizontally in containers with artificial seawater (Tropic Marine® Sea salt classic, Germany, 28‰) in a temperature controlled room at 10 °C with 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at the water surface and a 16:8 h light:dark regime. Spore extract was poured over the coils in a concentration of approximately 7500 spores  $\text{mL}^{-1}$  seawater. After seeding, the coils were turned three times with 20-min intervals to ensure an even distribution and settling of spores. After 48 h, the coils were changed from horizontal to upright position (placed in a 100 L tank), to ensure light availability and growth on all sides of the coils. Water was changed and nutrient enriched regularly hereafter (approximately every 2 weeks). The nutrient concentration was kept in the range of 50–200  $\mu\text{M NO}_3\text{-N}$ , using both PES medium and liquid garden fertilizer (Min Have Næring – NPK 5-1-4, Min Have, Denmark) first exclusively PES, and then Min Have, to reduce costs. At deployments in September, October and November, spores had developed into sporophytes of up to 0.5, 4 and 5–6 cm length, respectively. The traditionally seeded substrates therefore had different nursery periods being 69, 110, and 134 days when deployed in September, October and November, respectively in the Limfjorden; and 70, 110 and 134 days, when deployed in September, October and November, respectively at Hjarnø and finally 113 days

when deployed in November at the Grenaa site. This decision on experimental design ensured having materials that were seeded with the same genetic material, in exact same densities.

### 2.2.3. Direct seeding of sporophytes

Three weeks after initiation of the fertility induction (see Section 2.1), the gametophyte cultures of *S. latissima*, were at least 102 days old, and contained a mixture of gametophytes and juvenile sporophytes. To minimize the number of gametophytes in the seeding process, and ensure an even size of sporophytes to be seeded, the culture was filtered through two filters of 63 and 1000  $\mu\text{m}$ . This ensured having a concentrated solution of evenly sized sporophytes between 63 and 1000  $\mu\text{m}$ . The filtering process did not exclude fragments of gametophytes of the same size, but these were not counted as seeding material. The concentration of sporophytes was diluted 500 times, adjusted to approximately 50 sporophytes per ml, and counted in a 2 mL sample using an inverse microscope. Hereafter, a 1% binder solution (AT~SEA Technologies, Belgium) was prepared by mixing Tyndallized seawater containing full f/2 nutrient medium [38] with the binder agent using a hand blender. When all binder powder was dissolved into the seawater, the sporophytes were added to achieve a final sporophyte concentration of 165 to 404 sporophytes  $\text{mL}^{-1}$  binder solution, in range with what has been used elsewhere for this seeding technique [8,9]. The non-woven textile ribbon substrates were soaked in the binder solution and gently

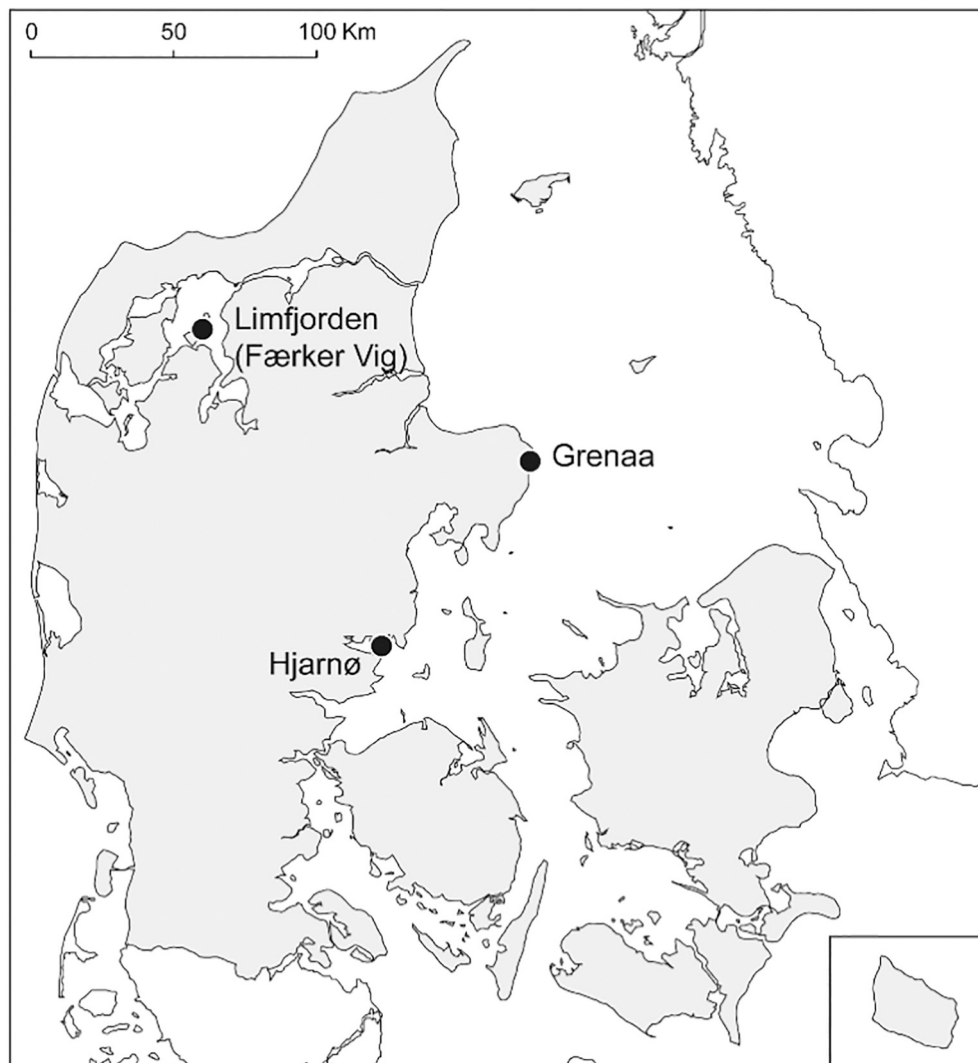


Fig. 1. Overview of cultivation site. Three cultivation sites were used in the study: Grenaa (20 ha), Hjarnø (100 ha) and Limfjorden (4 ha).

pulled between two fingers in order to remove excess binder from the material. The calculated seeding density varied between 11 and 31 sporophytes cm<sup>-2</sup> of ribbon, equivalent to 7600–31,310 sporophytes m<sup>-1</sup> linear ribbon. After seeding, the ribbons were transferred to plastic bags and stored in darkness at 10 °C until deployment the following day.

### 2.3. Deployment and outgrowth at sea

#### 2.3.1. Cultivation sites

The deployments were made at three different sites in inner Danish waters, with varying growth conditions (Fig. 1, Table 1).

The Grenaa site is a 20 ha research area, run by Aarhus University. This is the most exposed seaweed cultivation site in Denmark. The Hjørnø site is a 100 ha commercial cultivation site run by Hjørnø Havbrug A/S [27], located in the outer part of Horsens fjord. The Limfjorden site is a 4 ha research area, located in Færker Vig, run by the Technical University of Denmark [26]. This site is sheltered, and characterized by a high degree of eutrophication. At all three sites, long line cultivation systems were used, equivalent to the systems used for production of mussel in Denmark.

#### 2.3.2. Deployment times

The direct seeded ribbons and traditionally spore-seeded lines were deployed by the end of September, October and November 2017 at Hjørnø and in the Limfjorden (Table 2).

At Grenaa, the direct seeded ribbons and spore-seeded lines were deployed mid and start November 2017, respectively. No lines were deployed before November at Grenaa due to adverse weather conditions and technical problems at the cultivation site. All deployments were made with 5 replicate 2 m sections of ribbons/lines of each seeding method ( $n = 5$ ). All materials were deployed from 0 to 2 m depth, attached in the bottom to a long line and in the top to a 4 L buoy (Fig. 2).

#### 2.3.3. Monitoring of growth and environmental parameters

Growth and environmental parameters at all three sites were monitored between three and seven times during the growth period, with a frequency determined by boat availability and weather conditions. Light loggers (Odyssey® PAR loggers) and temperature/conductivity loggers (Odyssey® Conductivity/Temperature logger) were deployed at 2 m depth. All loggers were calibrated according to the manufacturer's instructions before deployment. At each monitoring visit, the frond length of the six longest individuals from each individual 2 m ribbon/line replicate were measured (regardless of position on the line), and the light and temperature/conductivity loggers were cleaned and data retrieved. Fouling accumulating on the light loggers between site visits compromised the data quality and consequently, only data collected 7 days after each visit was included in the analyses. The conductivity data was not included due to errors in the data output.

### 2.4. Harvest

At Grenaa and Hjørnø, all seeded structures were harvested in June 2018 (Table 2). Due to earlier onset of biofouling in the Limfjorden [26] all structures here were harvested in May 2018, except for 5 spore-

**Table 2**

Overview of deployment and harvest. Dates for deployments and harvests at the three cultivation sites included in the study.

Site	Deployment month			Harvest date
	September	October	November	
Grenaa	–	–	3-11-2017/16-11-2017 <sup>a</sup>	26-6-2018
Limfjorden	20-9-2017	31-10-2017	24-11-2017	7-5-2018/12-6-2018 <sup>b</sup>
Hjørnø	21-9-2017	31-10-2017	24-11-2017	7-6-2018

<sup>a</sup> Traditionally spore-seeded lines were deployed 3-11-2017 and the direct seeded lines 16-11-2017.

<sup>b</sup> Five spore-seeded lines, deployed in October, were harvested in June to examine the difference in biomass yield and quality between harvest dates.

seeded lines from the October deployment, which were harvested in June 2018 (Table 2). At harvest, all lines and ribbons were taken out of the sea and kept at 10 °C prior to measurement of the individual sporophytes at the same, or the following day. At harvest, the following measurements were made for each replicate ribbon/line: Length and width of the six longest sporophytes, the density (individuals m<sup>-1</sup> (only at Hjørnø and Grenaa)), and the total biomass yield (kg m<sup>-1</sup> seeded substrate). The biomass was not divided in top/bottom, so each sample represents the average growth/biomass at 0–2 m depth.

### 2.5. Biomass quality

Presence of biofouling organisms was registered at the Hjørnø site at harvest by the presence/absence of hydroids, epiphytic algae, barnacles and mussels on the six longest blades from each line.

The dry matter (DM) content of the seaweed was determined by drying the biomass at 105 °C until the weight was stabilized, and hereafter calculated as percentage of fresh biomass: dry weight (DW)/fresh weight (FW) \* 100. The FW of the biomass was determined from drained biomass. Hereafter the dry biomass was finely milled and homogenized before further analysis.

For determination of ash content, a known amount of dry algae was combusted at 550 °C for 2 h, and the ash fraction was calculated as percentage of DM. Tissue concentrations of carbon (C) and nitrogen (N) were determined by Pregl-Dumas ignition in pure oxygen atmosphere followed by chromatographic separation of C and N with detection of the individual elements by thermal conductivity [39]. Total phosphorus (P) content was analysed spectrophotometrically according to standard methods [40,41]. Prior to analysis, the dried and homogenized samples were heated at 550 °C for 2 h, autoclaved with 2 M hydrogen chloride (HCl) (20 mg DM for 7 mL acid), and finally filtered through GFF filters (Whatman).

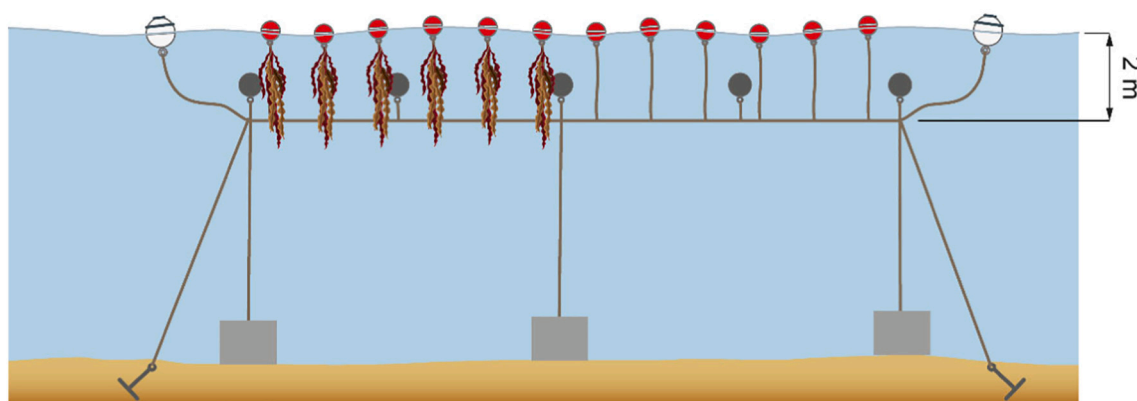
Metal concentrations were determined on biomass samples collected 30 May, 2 June and 7 May/12 June 2018 from Grenaa, Hjørnø and the Limfjorden, respectively, using inductively coupled plasma mass spectrometry (iCAPq ICP-MS, Thermo Fischer, Bremen, Germany). Briefly, a 0.2 g dry sub-samples were digested in closed quartz vessels in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) using 5 ml of

**Table 1**

Description of the abiotic factors characterizing the three cultivation sites. Data for salinity, turbidity (secchi depth) and dissolved inorganic nitrogen (DIN: Nitrate+nitrite-N), are average values obtained from the closest monitoring station of the Danish National Monitoring Programme (NOVANA). Data for the salinity and the DIN are from 0 to 1 m depth. The evaluation of exposure is based on personal judgement by the authors. Data are presented as averages (avg), minimum/maximum values and number of observations (n).

Site	Salinity (PSU)				Secchi depth (m)				DIN (µg/L)				Exposure	Position N	Position E
	avg	min	max	n	avg	min	max	n	avg	min	max	n			
Grenaa	20	13	29	28	9	3.4	14.3	28	25.4	0	172	127	High	56°30'03.2"	10°57'50.6"
Limfjorden	26	24	28	23	3.6	1	9.4	921	290	4	1100	199	Low	56°50'17.9"	9°05'03.9"
Hjørnø	22	16	29	69	6.7	2.4	13	352	27.8	1.5	230	257	Medium	55°49'13.9"	10°07'42.9"





**Fig. 2.** Schematic drawing of the longline system used in the present study. The longlines were attached in each end to the bottom with screw anchors. Each longline had 10–15 concrete blocks (20 L) to keep the longline in a fixed depth (2.5 m), red buoys (4 L) on each of the replicate line/ribbons, placed at least 2 m apart, grey buoys (18 L) for suspending the longline in areas with no experimental lines, and a marker buoy in each end of the longline. All materials were deployed from 0 to 2 m depth, attached in the bottom to the long line and in the top to a 4 L buoy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrated nitric acid (SPS science, Courtabeuf, France). The digest were subsequently diluted with Milli-Q water followed by quantification using ICP-MS using external calibration with internal standardization ( $^{97}\text{Rh}$ ).

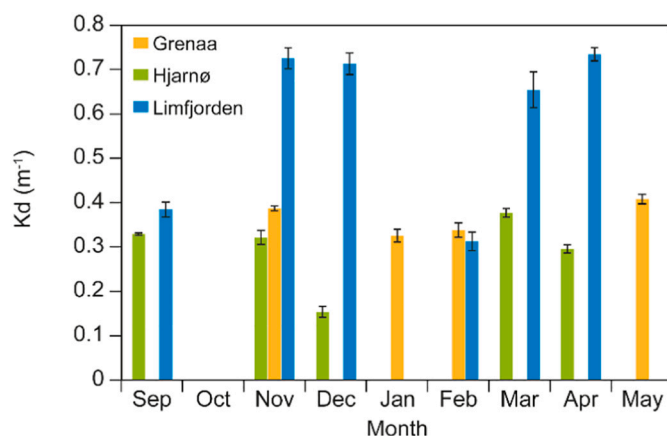
For determination of total iodine tissue content (I), the principles of the standardized method EN17050:2017 [42] was followed. Briefly, 0.15–0.20 g of dry samples was weighed into tubes (Sarstedt, Nümbrecht, Germany). Subsequently, 5 mL Milli-Q® water and 1 mL 25% tetra-methyl-ammonium-hydroxide (TMAH, Merck, Darmstadt, Germany) was added. The tubes were then sealed and placed in a preheated oven at  $90 \pm 3.0^\circ\text{C}$  for 3 h followed by cooling and diluting to a final volume of 20 mL with Milli-Q water. To remove coarse particles, the samples were centrifuged at  $10,000 \times g$  for 20 min. Prior to analysis, the supernatant was filtered through a  $0.45 \mu\text{m}$  filter and samples were diluted with Milli-Q water prior to analysis. The iodine quantification was performed by ICP-MS (iCAPq) using external calibration with internal standardization ( $^{125}\text{Te}$ ). For all elements, certified stock solutions were used for preparation of the calibration standard and internal standard (SPS Science).

## 2.6. Statistical analyses

For the biomass yield and length measurements, all analyses were made in SAS 9.3 (SAS Institute, Cary, NC) using proc. mixed models and proc. general linear models (glm). The model consisted of the following fixed effects:

$$\begin{aligned} \text{Response} = & \text{deployment date} + \text{Seeding method} + \text{site} + \text{Seeding method} * \text{site} + \text{seeding method} * \text{deployment date} + \text{site} * \text{deployment date} \\ & + \text{Seeding method} * \text{site} * \text{deployment date} \end{aligned}$$

The deployment date was defined as number of days from first deployment in the experiment. For length and width data, five or six replicate observations existed for each of the replicate cultivation ribbons/lines of each treatment. Hence replicate cultivation lines were included in the model as a random factor in a mixed model to account for the dependency of observations from the same line. The interactions with seeding method tested if seeding method responded in similar ways at all sites and deployment days, whereas the interaction between site and deployment day tested if the effect of deployment day differed



**Fig. 3.** Turbidity at the three cultivation sites. Light attenuation coefficient  $K_d$  ( $\text{m}^{-1}$ ) in the Limfjorden (blue), Hjarnø (green) and Grenaa (orange). The data are shown as mean  $\pm$  SE ( $n = 292$  (Grenaa), 450 (Hjarnø), 2426 (Limfjorden)). Calculations are based on light from the 7 day periods following cleaning of the logger sensor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between sites. Yield data were log-transformed to fulfill the assumptions of normal distribution. Furthermore, as each line only provided one biomass yield observation, we used a general linear model to test the

effects on yield. The effect of site and seeding method on the density of *S. latissima*  $\text{m}^{-1}$  cultivation line, was tested on data from Hjarnø and Grenaa in JMP 14.0.0 (SAS Institute, Cary, NC) using an ANOVA. We used Shapiro Wilk's test, to test the assumptions regarding normality and O'Brien's test to test for homoscedasticity. For the biomass composition and metal contents, all analyses were made in SAS 9.3 (SAS Institute, Cary, NC) using proc. glm. The model consisted of the following fixed effects:

Response = deployment date + site for the biomass composition and response = site for the metal contents. The post hoc pairwise

comparisons were performed using Tukey Kramers post hoc test.

The residual distribution from the mixed models did not deviate from assumptions regarding homoscedasticity and normal distribution.

### 3. Results

#### 3.1. Environmental parameters

The Grenaa and Hjørnø sites could be clearly distinguished from the Limfjorden site based on the light attenuation in the water column (Fig. 3). Especially during early winter and spring, the water turbidity, and hence light attenuation, was high in the Limfjorden compared to the other two sites. The average light attenuation measured was  $0.332 \pm 0.005 \text{ m}^{-1}$  at Hjørnø,  $0.389 \pm 0.007 \text{ m}^{-1}$  at Grenaa and  $0.66 \pm 0.01$  in the Limfjorden.

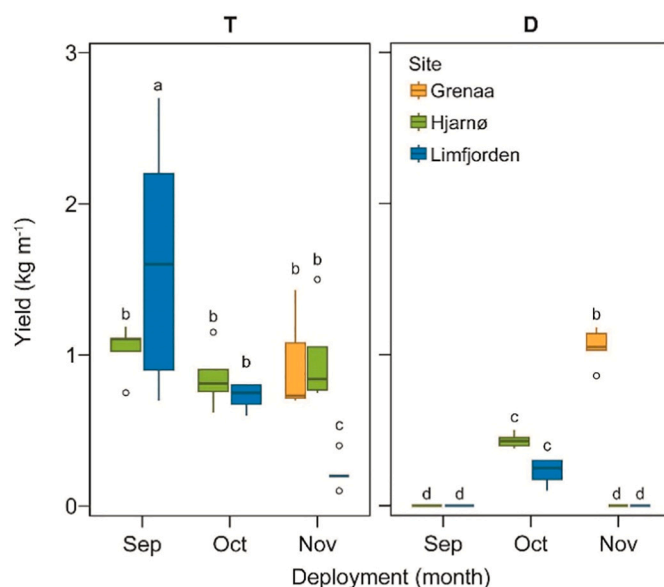
The average daily water temperature varied over the growth period between 1.4 and 18.2 °C at Grenaa, 2–19 °C at Hjørnø and –1.2–22 °C in the Limfjorden (Fig. 4). Generally, the seawater temperature developed equally at the three sites during the growth period, but there was a tendency for the temperature in the Limfjorden to change faster and to reach more extreme temperatures than at the other sites during winter, as well as during summer, where temperatures occasionally exceeded 20 °C.

#### 3.2. Biomass yields

The spore-seeded lines generally gave higher yields at all three sites, as compared to the direct seeding on the non-woven textile ribbons (Fig. 5). Site, deployment time and seeding method all had a significant effect on the final yields, and the interactions between these factors were also significant (Table 3). Of all deployments, the significantly highest yields were obtained in the Limfjorden from the spore-seeded material deployed in September ( $1.6 \pm 0.4 \text{ kg FW m}^{-1}$ ). For the direct seeded non-woven ribbons, the significantly highest yield was from the November deployment at Grenaa ( $1.0 \pm 0.1 \text{ kg FW m}^{-1}$ ).

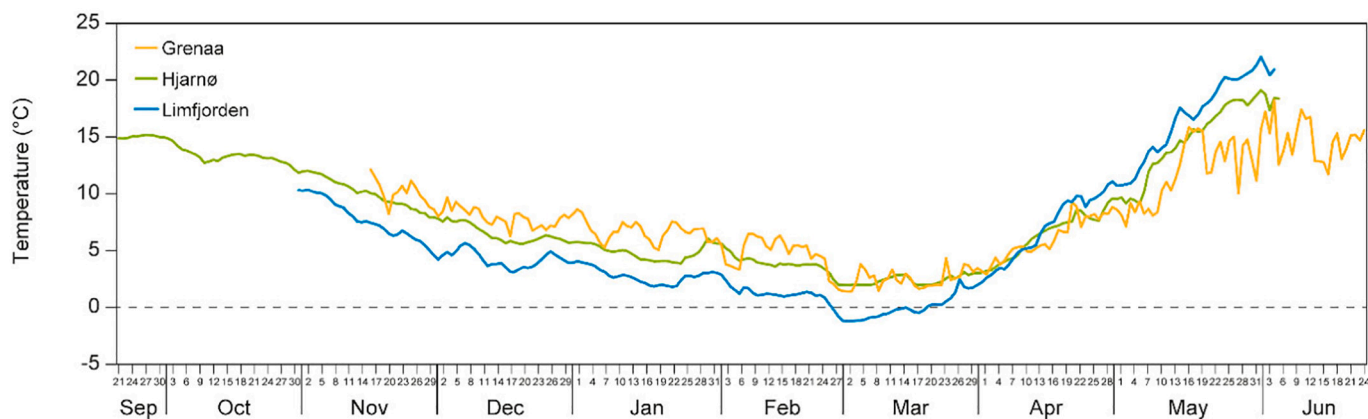
In the Limfjorden, the significantly highest final harvested biomass yield obtained in May, was from the spore-seeded lines deployed in September:  $1.6 \pm 0.4 \text{ kg FW m}^{-1}$ , compared to  $0.7 \pm 0.1 \text{ kg FW m}^{-1}$  when deployed in October and  $0.2 \pm 0.1 \text{ kg FW m}^{-1}$  from lines deployed in November (Fig. 5, Table 3). At Hjørnø, there was no statistical difference in yield between spore-seeded lines deployed in September, October or November. The biomass yield obtained at harvest in June was  $1.0 \pm 0.1 \text{ kg FW m}^{-1}$  from September lines,  $0.8 \pm 0.1 \text{ kg FW m}^{-1}$  from October lines and  $1.0 \pm 0.2 \text{ kg FW m}^{-1}$  from November lines.

In contrast to the results from the spore-seeded lines, there were no biomass yield from the direct seeded ribbons deployed in September in the Limfjorden, a low yield of  $0.2 \pm 0.1 \text{ kg FW m}^{-1}$  from the October



**Fig. 5.** Biomass yields at the three cultivation sites. Biomass yields ( $\text{kg m}^{-1}$ ) at harvest, in the Limfjorden (blue), at Hjørnø (green) and at Grenaa (orange) from the three deployment times (September, October and November) using the traditional spore seeding method (T) or direct seeding method (D). Data are presented as box-and-whisker plots,  $n = 5$ . The median is marked with a line, and the max/min with whiskers in top/bottom of each box. Letters indicate significant differences in yield between treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deployed ribbons and no yield from the direct seeded ribbons deployed in November. Similar to the results from Limfjorden, there were no biomass yield from the ribbons deployed in September and November at Hjørnø, but a yield of  $0.4 \pm 0.01 \text{ kg FW m}^{-1}$  from the October deployed ribbons. The yield from the direct seeded ribbons deployed in November at Grenaa were significantly higher than what was achieved at the other two sites (Fig. 5, Table 3), and equal to the yield obtained at this site on the spore-seeded lines ( $1.0 \pm 0.1 \text{ kg FW m}^{-1}$  and  $1.0 \pm 0.2 \text{ kg FW m}^{-1}$ , respectively). At Hjørnø and Limfjorden the ribbons with no yield were generally covered with sediment and benthic algae, and the ribbons deployed in September at Hjørnø were fully covered with hydroids and barnacles already in November.



**Fig. 4.** Sea water temperature during the experimental period. Temperature (°C) development in the Limfjorden (blue), Hjørnø (green) and Grenaa (orange) at 2 m depth. The lines show the average pr. day. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**

Results of the statistical analysis of the effect of site, deployment time and seeding method, and their interaction on the final biomass yield; the length of the six longest sporophytes of *S. latissima*; and the density of *S. latissima* sporophytes on the seeded structures at harvest (only measured at Grenaa and Hjørnø). Numbers in bold show p-values < 0.05.

Variable	Effect	Df (effect, error)	F	p value
Biomass yield	Site	2, 55	40.43	<b>&lt;0.0001</b>
	Deployment time	2, 55	14.85	<b>&lt;0.0001</b>
	Seeding method	1, 55	144.72	<b>&lt;0.0001</b>
	Seeding method x Deployment time	2, 55	18.56	<b>&lt;0.0001</b>
	Site x Deployment time	2, 55	9.89	<b>0.0002</b>
	Seeding method x Site	2, 55	10.71	<b>0.0001</b>
	Seeding method x Site x Deployment time	2, 55	10.33	<b>0.0002</b>
	Deployment time			
Final frond length	Site	2, 293	44.96	<b>&lt;0.0001</b>
	Deployment time	2, 293	17.40	<b>&lt;0.0001</b>
	Seeding method	1, 293	431.57	<b>&lt;0.0001</b>
	Seeding method x Deployment time	2, 293	14.08	<b>&lt;0.0001</b>
	Site x Deployment time	2, 293	10.90	<b>&lt;0.0001</b>
	Seeding method x Site	2, 293	3.44	<b>0.03</b>
	Seeding method x Site x Deployment time	2, 293	2.06	0.13
	Deployment time			
Sporophyte density <sup>a</sup>	Site	1, 24	0.15	0.71
	Deployment time	2, 15	1.31	0.30
	Seeding method	1, 24	0.11	0.74

<sup>a</sup> Only registered at Hjørnø and Grenaa.

### 3.3. Density measurements

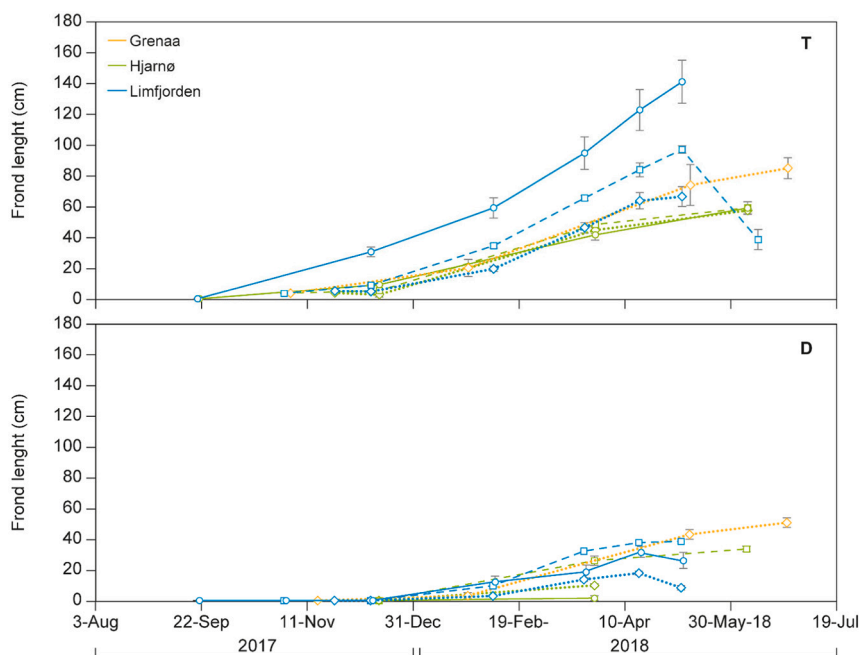
The average sporophyte density on the traditionally seeded lines was measured at Hjørnø and Grenaa. There was no significant effect of site, deployment time or seeding method on the sporophyte density at Hjørnø and Grenaa, and no significant effect of deployment time on sporophyte density at Hjørnø (Table 3). At Hjørnø, the measured sporophyte density was  $212 \pm 16$ ,  $280 \pm 51$  and  $258 \pm 23$  individuals  $m^{-1}$  on the traditionally seeded lines seeded in September, October and November, respectively, and  $238 \pm 15$  individuals  $m^{-1}$  on the direct seeded ribbons.

At Grenaa, the sporophyte density was  $260 \pm 65$  individuals  $m^{-1}$  on the traditionally seeded lines and  $244 \pm 17$  individuals  $m^{-1}$  on the direct seeded ribbons.

### 3.4. Length growth

Site, deployment time and seeding method all had a significant effect on the final frond length (Table 3). The seeding method was the factor with the largest effect on final frond length, with the direct seeding method resulting in significantly shorter fronds at all three cultivation sites and for all deployment times tested.

At harvest in the Limfjorden in May, the longest fronds were obtained from lines deployed in September using the traditional seeding method, with a length of  $141 \pm 14$  cm compared to  $97 \pm 6$  cm and  $67 \pm 6$  cm from the October and November deployments, respectively (Fig. 6). The difference was driven by the early onset of growth from the September deployed material, having an average length of  $31 \pm 3$  cm already in December at this site. Postponing the harvest later than in May resulted in a reduction of the frond length of the longest individuals to  $39 \pm 7$  cm in June, emphasizing the importance of early harvest in Limfjorden. At Hjørnø, the frond lengths at harvest from the spore-seeded lines were highly similar between deployment times (Fig. 6). The frond length at harvest from the September deployment was  $59 \pm 4$  cm, compared to  $59 \pm 4$  cm from the October deployment and  $58 \pm 1$  cm from the November deployment. When deploying materials using the direct seeding method in the Limfjorden, the final frond length at harvest in May was  $26 \pm 5$  cm from the September deployment compared to  $39 \pm 8$  and from the October and  $8 \pm 1$  cm from the November deployment of direct seeded ribbons (Fig. 6). At Hjørnø, the October deployment gave fronds with a final length of  $34 \pm 2$  cm (Fig. 6). At Grenaa, the longest fronds obtained in June from the November deployment, were  $85 \pm 7$  cm at harvest on spore-seeded lines (Fig. 6) compared to  $51 \pm 3$  cm at harvest on the direct seeded ribbons.



**Fig. 6.** Average maximum frond (blade) length  $\pm 1$  SE,  $n = 3-5$ , in the Limfjorden (blue), Hjørnø (green) and Grenaa (orange) from deployment in September (Full line), October (Long dashes), or November (Short dashes) using the traditional seeding method (T) or direct seeding method (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 4**

Dry matter (DM), ash, Carbon (C), Nitrogen (N) and Phosphorous (P) contents of *S. latissima* harvested at the different sites in June or in May. The values are shown as mean  $\pm$  SE. Letters indicate significant differences ( $p < 0.05$ ) between the different harvest sites and times.

Site	Month of harvest	n	DM (% of FW)	Ash (% of DM)	C (% of DM)	N (% of DM)	P (% of DM)
Grenaa	June	2	25.0 $\pm$ 0.6 <sup>a</sup>	12.8 $\pm$ 1.0 <sup>c</sup>	35.5 $\pm$ 0.1 <sup>a</sup>	0.55 $\pm$ 0.01 <sup>c</sup>	0.11 $\pm$ 0.01 <sup>a</sup>
Hjørnø	June	3	18.9 $\pm$ 0.5 <sup>b</sup>	18.4 $\pm$ 0.9 <sup>b</sup>	35.1 $\pm$ 1.3 <sup>a</sup>	0.73 $\pm$ 0.01 <sup>c</sup>	0.13 $\pm$ 0.001 <sup>a</sup>
Limfjorden	May	3	11.5 $\pm$ 0.8 <sup>d</sup>	25.6 $\pm$ 1.1 <sup>a</sup>	28.8 $\pm$ 1.5 <sup>b</sup>	4.65 $\pm$ 0.07 <sup>a</sup>	0.07 $\pm$ 0.01 <sup>a</sup>
Limfjorden	June	4	14.1 $\pm$ 1.0 <sup>c</sup>	25.3 $\pm$ 1.7 <sup>a</sup>	27.9 $\pm$ 1.8 <sup>b</sup>	2.60 $\pm$ 0.21 <sup>b</sup>	0.13 $\pm$ 0.01 <sup>a</sup>

### 3.5. Biomass quality

#### 3.5.1. DM, ash, C, N and P contents

The biomass composition differed significantly between sites, with *S. latissima* biomass from the Limfjorden being markedly different compared to the biomass from the other sites (Table 4). The deployment time had no effect on the tested parameters (except for a minor difference in the N content between the September and October/November deployment at Hjørnø) and is therefore not included in the results.

The DM content was significantly highest at Grenaa (25.0  $\pm$  0.6% of FW) and the carbon content significantly higher at both Hjørnø and Grenaa (35.1–35.5% C of DM) compared to biomass from the Limfjorden. The ash content however, was significantly higher in biomass from the Limfjorden (25.3–25.6% of DM, as compared to Hjørnø and Grenaa), irrespective of harvest time. In the Limfjorden, the N content of the harvested *S. latissima* was significantly highest. At harvest in May, the N contents was 4.65  $\pm$  0.07% N of DM, decreasing to 2.6  $\pm$  0.2% N of DM in June. Still, the N content of the *S. latissima* biomass in Limfjorden was significantly higher than the N content in biomass from Hjørnø and Grenaa being respectively 0.73  $\pm$  0.01% N of DM and 0.55  $\pm$  0.01% N of DM (Table 4). The tissue P content of the biomass did not follow the same pattern as the N content, since the P content was equal between sites, with a tendency however towards a lower P content in biomass harvested in May from the Limfjorden.

#### 3.5.2. Heavy metals and iodine contents

The heavy metal contents at harvest were significantly different between sites, again with biomass from the Limfjorden being significantly different from the other two sites (Table 5). The tissue concentrations of copper (Cu), zinc (Zn), lead (Pb) and iodine were all significantly lower in the biomass from the Limfjorden (Table 5). However, arsenic (As) (71.3  $\pm$  3.6 mg As kg<sup>-1</sup> DM) and cadmium (Cd) (2.1  $\pm$  0.2 mg Cd kg<sup>-1</sup> DM) concentrations were significantly highest from the June harvest in the Limfjorden (Table 5). The highest average concentration of iodine was found in the biomass from the Hjørnø site with 3737  $\pm$  933 mg I kg<sup>-1</sup> DM.

#### 3.5.3. Biofouling of fronds

The presence of epibionts attached to the blades of *S. latissima* was only registered in detail at Hjørnø at harvest. Here, all traditionally spore-seeded lines had presence of fouling organisms at harvest in June, however dispersed in a very low abundance. The dominating organisms were hydroids, epiphytic algae and barnacles. The *S. latissima* blades from the directly seeded ribbons had a pronounced lower coverage of fouling organisms as compared to the blades on the traditionally spore-

seeded lines, with only half of blades on the harvested lines from October having a dispersed and low occurrence of hydroids.

## 4. Discussion

The new direct seeding method, where juvenile sporophytes were seeded onto non-woven substrates using a binder, is promising in the cultivation of kelps, and could potentially reduce the costs associated with additional labor and space required in the nursery phase and accelerate the upscaling and mechanization of the seaweed production industry through more efficient deployment of seeded materials [8]. However, this study found that the seeding method was the factor with the strongest impact on biomass yield, and found significant interactions between seeding method, site and season, emphasizing the complexity of the interdependency between cultivation substrates and the spatial and temporal variations in environmental conditions. Where the direct seeding method gave yields comparable to the traditional method at the exposed site, the same method only led to harvestable yields following the October deployments at the two sheltered sites (Hjørnø and Limfjorden), significantly lower than yields from the traditional seeding method.

Temperature is one of the main factors determining when deployment is optimal [62], but other important parameters such as fouling, light/nutrient availability and water flow are probably also highly important, especially for the new direct seeding method to succeed. By late September, the water temperature had decreased to around 15 degrees, which in combination with reduced light levels, created an optimal environment for the growth of *S. latissima* [15]. During September however, filter feeding organisms were still present to settle on the substrates, and at both Hjørnø and in the Limfjorden, the sporophyte growth on the direct seeded ribbons deployed in September, was reduced presumably as a consequence of fouling. The October deployments gave the highest biomass yields at both of the sheltered locations, when using the direct seeding method, which could indicate a temporal window for successful deployment using this method. Deploying in October, possibly alleviates the ribbons of the significant fouling pressure from pelagic larvae in September and allows the juvenile sporophytes to initiate growth, at an optimal temperature, before the light decrease to suboptimal levels in November/December. In Norway and Scotland, the direct seeding method has been tested following a deployment in February with yields of up to 10 kg m<sup>-1</sup> [9,43]. In both Norway and Scotland, the water temperatures were lower, and more favorable for optimal growth, and increasing slower throughout spring, not reaching the high temperatures typical of Danish waters (Fig. 4). The generally lower sea water temperatures are also

**Table 5**

Cu, Zn, As, Cd, Pb and I contents of *S. latissima* harvested at the different sites at harvest in May or June (Note: The harvest time is different from the major harvest time for Grenaa and Hjørnø). The values are shown as mean  $\pm$  SE. Letters indicate significant differences ( $p < 0.05$ ) between the different compounds measured.

Site	Month of harvest	n	Cu (mg kg <sup>-1</sup> DM)	Zn (mg kg <sup>-1</sup> DM)	As (mg kg <sup>-1</sup> DM)	Cd (mg kg <sup>-1</sup> DM)	Pb (mg kg <sup>-1</sup> DM)	I (mg kg <sup>-1</sup> DM)
Grenaa	May	3	14.4 $\pm$ 0.4 <sup>b</sup>	44.4 $\pm$ 2.5 <sup>a</sup>	33.8 $\pm$ 2.3 <sup>c</sup>	0.17 $\pm$ 0.01 <sup>c</sup>	1.0 $\pm$ 0.1 <sup>a</sup>	2605 $\pm$ 81 <sup>ab</sup>
Hjørnø	June	3	17.7 $\pm$ 1.0 <sup>a</sup>	50.2 $\pm$ 0.4 <sup>a</sup>	40.8 $\pm$ 2.9 <sup>bc</sup>	0.23 $\pm$ 0.03 <sup>c</sup>	1.1 $\pm$ 0.2 <sup>a</sup>	3737 $\pm$ 933 <sup>a</sup>
Limfjorden	May	3	1.3 $\pm$ 0.07 <sup>d</sup>	34.6 $\pm$ 0.8 <sup>b</sup>	44.1 $\pm$ 3.3 <sup>b</sup>	0.73 $\pm$ 0.03 <sup>b</sup>	0.13 $\pm$ 0.03 <sup>b</sup>	1612 $\pm$ 271 <sup>b</sup>
Limfjorden	June	3	3.7 $\pm$ 0.4 <sup>c</sup>	24.6 $\pm$ 5.0 <sup>c</sup>	71.3 $\pm$ 3.6 <sup>a</sup>	2.1 $\pm$ 0.15 <sup>a</sup>	0.33 $\pm$ 0.07 <sup>b</sup>	1740 $\pm$ 162 <sup>b</sup>



characteristic for the Faroe Islands, where the direct seeding technique has also been documented to be feasible [10]. In Denmark, a late deployment in February is not feasible with any of the deployment methods, as the juvenile sporophytes cannot achieve a significant size before the spring phytoplankton bloom, with a typical onset in February, depleting nutrients in coastal waters to reach a minimum already in April [31]. The sporophyte densities of the directly seeded ribbons and the traditionally spore-seeded lines were not significantly different, but the growth on the ribbons was delayed compared to the growth on the traditionally seeded lines. This was also shown by Forbord et al., 2019 [9], who elaborated that the delayed growth observed could have been due to the fact, that the juvenile sporophytes need time to develop their attachment after deployment. As the sporophytes were <1 mm when using the direct seeding method, compared to >5 mm when using the traditional seeding method, this could also describe the delayed growth using the direct seeding method. However is it a prerequisite for the direct seeding method to work, that the sporophytes are small, as they would otherwise detach before establishing an attachment to the substrate.

Nutrients are most likely also a key factor contributing to the differences seen between sites using the traditional seeding method. The nutrient concentrations generally are low during summer and early autumn, then increase in many areas around end October where stratification is weakened due to increased winds and reduced temperatures [31]. In the Limfjorden, the concentration of nitrogen generally increases earlier than in other areas [30], meaning that nutrient concentrations could have been supporting the good early growth at this site using the traditional seeding method. Later deployments did not result in the fast growth seen by the September deployment in the Limfjorden in neither the Limfjorden nor at Hjørnø. The growth of the later deployments was possibly restricted by low light levels, as the temperature was decreasing but still favorable, and nutrients increasing at both sites. Especially the late November deployment in the Limfjorden, resulting in the lowest yields using the traditional seeding method, underlines the importance of light for the initial growth phase, as the Limfjorden had a higher light reduction than at Hjørnø, where the growth from the November deployment were equal to the deployments in September and October. As the nursery period is known to affect the final biomass yield [9], the difference in yield between deployments could theoretically have been larger at Hjørnø and Limfjorden, if the nursing period of the deployed lines had been the same. However, this was a deliberate decision taken in the design of the study, to avoid introducing bias to the study potentially caused by introducing different batches of seeded material. The results support that the optimal deployment time coincides with temperatures being <15 °C [62], but showed that the optimal temperature deployment window for *S. latissima* using the direct seeding method possibly is around 10 °C, most likely directly related to the presence of fouling organisms in the water. The results also support that nutrient availability is important for the deployment, as have been shown previously, where the optimal deployment has been related to nitrogen levels >1.4 µM (the half-saturation constant for nitrate uptake [20]), and shows that areas of high nutrient availability (e.g. the Limfjorden) can support an early deployment, at least using traditional seeding methods. However, these results were only obtained through one season, so variations between seasons should be expected.

The large differences in yields between the traditional seeded lines and the direct seeded ribbons at the sheltered sites were presumably due to fouling directly on the ribbons, as previously stated. The surface of the ribbons contains a high amount of fibers and, combined with their large surface area, these materials seemed to catch both fouling and sediment from the water column to a higher degree than the traditional seeded lines which were a combination of Kuralone twine and polypropylene rope. The higher degree of fouling on the non-woven ribbons has also been shown elsewhere [8], however not with such detrimental effect as observed at the sheltered sites in this study. The texture of the ribbons is designed to promote the attachment of the juvenile sporophytes to the

substrate, but a study published after the present study was conducted documented that kuralone twine was also a good substrate for the direct seeding method [8]. Future studies could try seeding kuralone and polypropylene rope using the direct seeding method, for deployment at sites with a high risk of fouling as those included in this study. This might eliminate the negative impact on the growth of *S. latissima* caused by fouling due to the non-woven ribbons.

The highest yield was obtained from traditionally spore-seeded lines grown in the Limfjorden. The high yield was mainly driven by the early onset of growth, as previously mentioned, but also high internal N concentrations could have supported an optimal growth through spring, where nitrogen becomes limiting for growth. In June, the tissue N concentration was  $2.60 \pm 0.21\%$  of DM in algae from the Limfjorden compared to  $0.73 \pm 0.01$  and  $0.55 \pm 0.01\%$  of DM at Grenaa and Hjørnø (Table 4). It is known that macroalgae have a critical tissue N concentration for growth between 0.7 and 3.2% N of DM [44,45], and therefore the growth of *S. latissima* was probably restricted by internal N concentrations earlier in the season at Hjørnø and Grenaa than in the Limfjorden. The most exposed site at Grenaa had equal biomass yields from traditional and direct seeded lines/ribbons. The Grenaa site is characterized by a high degree of exposure from both waves and currents, which has been shown to increase the growth of kelps by changing the flow from being laminar to turbulent over the thalli, facilitating the nutrient uptake and gas exchange of the algae [21,46]. The exposure may thus have been a driver of the prolonged growth of the juveniles at this site in spring, where the increased ability to take up nutrients from the water could make up for the lower environmental nutrient concentrations. The high exposure is also likely to reduce the ability for fouling organisms and marine debris to settle on the substrates and therefore reduce the effect of fouling on the growth of *S. latissima* during the early growth phase [47–49]. Another positive factor at this site, was the lower temperature during early summer. When the temperature increases to around 20 °C, which was the case both in the Limfjorden and at Hjørnø, the photosynthesis parameters are negatively affected in *S. latissima*, and the growth decreases [16,50], which was especially clear in the Limfjorden, where the frond length decreased from May to June (Fig. 6). It is important to note however, that the length of the *S. latissima* fronds is constantly subject to erosion which increases during the summer months [51]. Therefore, the decrease in frond length observed at Limfjorden is likely due to a combination of stagnation of meristematic growth and increase of tissue erosion [52].

This study confirms that the environmental conditions at the cultivation site affect the biochemical composition of the algae, and thereby, the potential value of the biomass. The biomass from the Limfjorden was characterized by high internal concentrations of N, which is beneficial when using *S. latissima* as an N mitigation tool, and the Limfjorden site therefore holds the greatest potential from this study, with a calculated removal of 11.7 g N m<sup>-1</sup> line in one season, which is in line with previous findings for the Limfjorden [53]. At Hjørnø, the calculated N removal was 2.0 g N m<sup>-1</sup> which is lower than what can be achieved at this site after 1.5 years growth (<7.2 g N m<sup>-1</sup> line), due to increased yields (and biofouling) after a second cultivation season [27]. Generally, biomass with a high internal nutrient concentration, as in biomass from the Limfjorden, can be expected to contain a high amount of pigments such as chlorophyll *a* and fucoxanthin [54], which are compounds of high interest, due to their proposed beneficial effects on human health such as e.g. anti-cancer and anti-obesity effects [55]. The DM content was significantly higher in biomass cultivated at both Hjørnø and at Grenaa, than in the Limfjorden, and almost twice as high in Grenaa as compared to the Limfjorden, and notably the extra DM was not in the form of ash, as the biomass from Grenaa also had the lowest ash content. This may greatly affect the possible business case scenarios at the cultivation sites, since the ash free DM content is a considerable factor in determining the value of the cultivated biomass. Another quality parameter of the biomass is the content of heavy metals and iodine, and the compliance to the maximum levels established in the legislation.

Depending on the use of the seaweed there is the EU feed [56] (and later amendments), the EU food supplement legislation [57] (and later amendments) that seaweed falls under, and additionally the French recommendation [33] on using algae for food. There are presently no legislation on Cu and Zn which are also both essential minerals. For all sites, the lead (Pb) concentrations, were below the maximum level allowed for seaweed used as food supplements ( $<3.0 \text{ mg Pb kg}^{-1}$ ) and feed ( $<10 \text{ mg Pb kg}^{-1}$ ), respectively. With regards to cadmium (Cd), the biomass from all three sites was below both the EU food supplement maximum level ( $<3.0 \text{ mg Cd kg}^{-1}$ ), and also below the maximum level for feed ( $<1 \text{ mg Cd kg}^{-1}$ ), except the biomass harvested at the Limfjorden site in June ( $2.1 \text{ mg Cd kg}^{-1}$ ). This June biomass at Limfjorden was notably three times higher in concentrations compared to the May harvest at the same site, which could be explained by the retardation of growth in this period, possibly concentrating the content of Cd in the biomass [58]. There is no maximum level for total As in the food legislation, in contrast to the feed legislation where maximum levels have been established. The As concentration at Limfjorden and Hjørnø (averages of  $40.8\text{--}71.3 \text{ mg total As kg}^{-1}$ ), but not at the Grenaa site ( $33.8 \text{ mg total As kg}^{-1}$ ) were above the current feed maximum level at  $40 \text{ mg kg}^{-1}$ . The iodine concentrations were in general high (averages from approx.  $1600\text{--}3700 \text{ mg I kg}^{-1}$ ) which is expected for *S. latissima*, although not as high as reported previously by other authors [32] (approx.  $5000 \text{ mg I kg}^{-1}$ ) and up to  $10,000 \text{ mg I kg}^{-1}$  [34]. Previously, it has been suggested that iodine concentrations will be lower in biomass grown in low salinity environments, due to its proposed role as an osmoregulator in kelps [59,60]. As the Limfjorden presented the highest salinities in this study, our results could not confirm this relation between iodine content and salinity, and possibly other mechanisms also contribute to the internal regulation of iodine such as e.g. scavenging of reactive oxygen species [61]. There is currently a strong focus on iodine in seaweed due to the high concentrations reported. Due to the lack of EU legislation on iodine in seaweed for food supplements, national food authorities often evaluate seaweed as food based on the French national recommendation on a threshold of  $2000 \text{ mg I kg}^{-1}$  [33]. This means that although the concentrations of iodine found in the present study were relatively low compared to the values reported in many other studies, only one site (Limfjorden) complies with the French threshold value. Until EU has established maximum levels for iodine in seaweed, chemical risk assessments should take the daily recommended intake into consideration in order to perform an overall risk assessment of consumption of seaweeds.

## 5. Conclusion

The direct seeding method could be a candidate for future cultivation practices, in exposed locations, but further development is needed in order to prepare the technique for implementation in sheltered locations, such as those included in this study. The direct seeding of sporophytes appear to reduce the time window for deployment, limit the range of suitable cultivation sites, and generally impose a higher risk to the final yield obtained, than when using the traditional spore-seeding method. Developing and testing alternative cultivation substrates for direct seeding with less tendency to attract fouling could be a way forward in sheltered and turbid waters with high fouling pressure. The traditional seeding method was less affected by deployment time, but in areas with a high water turbidity, such as the Limfjorden, an early deployment in September gave the highest biomass yield. The substrate, deployment method or timing did in general not have an effect on the quality of the harvested biomass, emphasizing that biomass quality is controlled by environmental parameters at the cultivation site, and the timing of the harvest. The arrival of the new EU legislation for seaweed as food and feed will have great impact on the future seaweed market, as the contents of heavy metals and iodine in seaweed biomasses in many cases are balancing on both sides of the present maximum levels/threshold values. In view of the results obtained in this study, it is of high

importance to include the effect of timing and site differences in the future development of the direct seeding method, which holds great potentials in the upscaling and mechanization of kelp cultivation.

## CRediT authorship contribution statement

Teis Bøderskov: Conceptualization, Methodology, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing.

Mette Møller Nielsen: Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing - Review & Editing.

Michael Bo Rasmussen: Conceptualization, Methodology, Investigation, Resources.

Thorsten Johannes Skovbjerg Balsby: Data Curation, Writing - Review & Editing.

Adrian Macleod: Methodology, Writing - Review & Editing.

Susan Løvstad Holdt: Resources, Writing - Review & Editing.

Jens Jørgen Sloth: Resources, Writing - Review & Editing.

Annette Bruhn: Conceptualization, Methodology, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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