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Title

The seasonal variation in nitrogen, amino acid, protein and nitrogen-to-protein conversion factors of commercially cultivated Faroese *Saccharina latissima*.

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Keywords

Food; feed; kelp; seasonality; offshore; biochemical composition.

Abbreviations

MBSL, meters below sea level; dw, dry weight; ww, wet weight; AA, amino acids; TAA, total amino acids; EAA, essential amino acids; AA-protein, protein determined by summing up TAA.

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Abstract

The demands of new food sources are increasing with the increasing human population. Proteins are a main nutrient for human consumption and in animal feed, which will be in short supply in the near future. Many macroalgal species have shown to possess significant levels and quality of protein, comparable to conventional protein-rich foods.

The brown macroalga *Saccharina latissima* was commercially cultivated in an open ocean area in the Faroe Islands. The effect of depth, cultivation site and seasonal variation in nitrogen, protein concentration, and the amino acid profile were investigated to study the potential of Faroese cultivated *S. latissima* as a protein source. Moreover, the nitrogen-to-protein conversion factor was calculated.

The average nitrogen concentration was $2.1 \pm 0.2\%$ of dry weight (dw) with no significant variation between sites, a single month with significant variation between cultivation depths (March 2016), and a significant seasonal variation (among most months). The average protein concentration determined by summing up total amino acids was $4.3 \pm 0.9\%$ of dw, and comparable to or slightly lower than other studies. There was no depth, site or seasonal variation in AA-protein concentration for the cultivated *S. latissima*. The lack of seasonal variation was most likely a consequence of the year-round stable physical conditions in the Faroe Islands, and compared with other studies surprising as most found seasonal variation of AA-protein.

The quality of the protein was high (EAA score $>100\%$) in March, although the low total concentration of protein limits the possibilities to use *S. latissima* solely as a protein source or for protein extraction and other nutrients should be investigated to understand its potential as a food or feed source.

This study will recommend estimating total protein concentration by summing up the total amino acids (AA-protein), as the widely used 6.25 factor is highly overestimating the protein concentration.

1. Introduction

New food sources are essential to investigate since common food and feed sources will be in short supply in the future as a result of the growing world population [1]. Proteins are essential building blocks of all living organisms and new protein sources are to be investigated. Many macroalgal species (seaweeds) contain significant amounts of protein of high quality. In some cases even higher (up to 47%) than conventional protein-rich foods in dry forms [2–7].

In Asian countries, macroalgae have a long tradition as a human nutritional source. Macroalgae can be utilized either directly consumed or as a food ingredient, such as thickening agents, or in animal feed as alternative high-quality proteins [1,3,8–10]. The macroalgal industry is growing and more than 27 million tonnes were harvested worldwide in 2014 with the estimated value of US\$ 5.6 billion [5].

The quality of protein for human consumption depends on the profile of the amino acids and the digestibility of the proteins [1,11]. The protein requirements that meet the metabolic needs for humans have been published by WHO, FAO and UNU [12], and protein quality can be described by an essential amino acid (EAA) score. The protein concentration in macroalgae varies according to species, but it is also driven by extrinsic factors such as spatial and seasonal variations in light and nutrient availability [13].

Protein concentration is generally higher in Rhodophyta (up to 47% of dw) and Chlorophyta (10–25% of dw) than in Phaeophyceae (5–13% of dw) [3,14]. However, the large brown kelp species (in the order Laminariales) are more likely to be cultivated in large quantities in future in Europe, leading to higher availability. This is due to their known life-cycle which allows sexual reproduction and large-scale seeding. The kelp species have in general a high growth rate and a large yield per meter of cultivation line [5,15], and their cultivation can most likely be automated easier than the red and green species of macroalgae.

Several studies are currently questioning the general approach for the analysis of protein concentration in macroalgae. The traditional nitrogen-to-protein (N-to-protein) conversion factor of 6.25, used to find the crude protein concentration, is based on the assumption that samples contain protein with 16% nitrogen and an irrelevant amount of non-protein nitrogen such as nitrate, nitrite, and ammonia [16].

This assumption is invalid if the material consists of high amounts of other nitrogen sources [17]. Therefore, a current trend has been to calculate the specific N-to-protein conversion factor for the specific foodstuff because using conversion factors is a practical method [17–20].

A growing interest is seen in Europe for the kelp species *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G. W. Saunders, commonly known as “sugar kombu” or “sugar kelp”, cultivated in commercial monoculture or in integrated multi-trophic aquaculture [9,21]. *Saccharina latissima* is an edible species, which is accepted as food (non-novel food) in EU, and thus is suitable for consumption and further commercialisation. The seasonal variation of biochemical compounds, including proteins, has however been addressed as a major challenge for commercialisation and the year-round-supply of same quality biomass. Consequently, a concentrated effort has been made in the past years to increase data and knowledge about the possible seasonal variations in the composition of cultivated and wild harvested *S. latissima* [2,4,9,22–26]. This increased knowledge is useful for the planning of harvest time, to determine end-products and cascading biorefinery processes.

Saccharina latissima is cultivated on a commercial scale (~10 ha) at an open ocean location in the Faroe Islands. The temperature in the Faroese waters is relatively stable (7–11°C) throughout the year [27,28] and nutrients are sufficient for growing *S. latissima* all year round [29,30]. This should make the environmental growth conditions optimal for macroalgal cultivation, and promising growth and yield potential have also been observed for *S. latissima* in the Faroe Islands [15,26,29]. The Faroe Island cultivation location in the middle of the North Atlantic Ocean is therefore different from cultivation taken place in e.g. France, Denmark, and Norway where the physical conditions like temperature and nutrients have larger seasonal fluctuations. In addition, these Faroese environmental conditions mean that *S. latissima* can be deployed at sea from September to March. The *S. latissima* can be harvested, in theory, all year round, but in practice is harvested from April to September where weather conditions allow working at sea. Furthermore, it has been shown possible to harvest *S. latissima* more than once and up to three years after deployment with two annual harvests (multiple partial harvesting) [15]. The macroalgae are sold to the food and feed market, and consequently, the customers need to know the protein concentration and quality of

the product they buy.

The present study aimed to evaluate the effect of the culture conditions: site (open ocean and in fjord), cultivation depth (1 and 9 meters below sea level; MBSL), and finally the harvest time (i.e. seasonal variation) with regard to the total nitrogen and protein concentration, and the amino acid composition of *S. latissima* cultivated commercially in the Faroe Islands. Protein concentration was determined by the sum of amino acids, and its nutritional value was evaluated based on the essential amino acid (EAA) composition and compared to reference patterns from WHO/FAO/UNU [12]. Moreover, the purpose was to establish specific N-to-protein conversion factors for Faroese *S. latissima* with regard to total amino acids and total nitrogen, in order to propose a general conversion factor for farmed Faroese *S. latissima*.

2. Method and Materials

2.1 Cultivation and site description

The brown kelp species *Saccharina latissima* was cultivated by the company Ocean Rainforest Sp/F in the fjord Funningsfjòrdur at the Faroe Islands (**Fig. 1**). The macroalgae were cultivated at two sites: the outer part and the central part of the fjord. The outer part of the fjord was termed the “exposed site” having occasional significant wave heights of 3-6m, was exposed to currents of $15\text{--}25\text{ cm} \cdot \text{s}^{-1}$, and a water depth of 50-70 meters [31,32], and the central site in the fjord was termed the “moderately exposed site” having occasional wave heights up to 3 meters, was exposed to similar or lower water current (no data exist), and a water depth of 20-30 meters [31]. The North Atlantic Current, which originates from the warm Gulf Stream, brings warm water to the area, providing a relatively stable water temperature ranging from 7 to 11°C during the year [27,28]. The salinity is stable at 35.0-35.2 [28]. The nitrate concentration is 8-12 μM during winter and spring and in most years starts to decrease in May during the spring bloom, and it decreases more in the shallow waters than offshore. There is a large interannual variability in the timing of the decrease in nitrate concentrations as well as in the minimum level of nitrate concentrations during summer [30].

The macroalgae were cultivated on the MacroAlgal Cultivation Rigs (MACR) occupying a surface area of approximately 1 ha and carrying 2,500 meters of growth line each (**Fig. 2**; A). One MACR has a 500-meter-long horizontal fix line submerged 10 meters below sea level (MBSL) and 10-meter-long vertical growth lines attached on the fix line for every second meter; thus, carrying macroalgae growing from the sea surface and 10 meters down (**Fig. 2**; B & C). The MACR installation has proven to be robust enough to open ocean conditions since 2010 enabling economical feasible open ocean macroalgal cultivation [15]. In total, 10 km of growth line were seeded with *S. latissima* and deployed manually in November 2014.

Fig. 1. Maps showing the North Atlantic Ocean (A, ©Wikimedia Commons, the free media repository), the Faroe Islands (B, © Kort- og Matrikelstyrelsen), and the fjord Funningsfjørður where the cultivation was done (C, © Google); the exposed ($62^{\circ}18'33.8''\text{N}$, $6^{\circ}54'07.4''\text{W}$) and moderately exposed sites ($62^{\circ}15'42.0''\text{N}$, $6^{\circ}57'36.2''\text{W}$) are marked with stars in the map.

2.2 The sampling of *Saccharina latissima*

From the cultivated macroalgal biomass, 65 samples were collected during a 19 months period from deployment in November 2014 until May 2016 (**Table 1**). Not all months were sampled due to insufficient biomass or weather conditions. Samples were collected at the two sites “exposed” and “moderately exposed”, and at two depths below sea level to compare the potential variation in biochemical composition along a vertical growth line. The samples were cut either at the top of the line right under sea level (1-2 meters below sea level; MBSL) or at the lowest meter on the growth line (9-10 MBSL). The

samples were termed “top” and “lower”, respectively.

Fig. 2. Two MacroAlgal Cultivation Rigs deployed next to each other under open ocean conditions at the mouth of Funningsfjörður (A), the view of the 10-meter-long vertical growth lines seen from sea surface and down water column (B), and a growth line on the vessel held by the crane and ready for biomass sampling (C). All photos were taken by Ocean Rainforest Sp/F.

Table 1. Sample overview; 65 samples of *Saccharina latissima* were collected from the cultivation sites in Funningsfjörður (exposed and moderately exposed) and at two cultivation depths (top and lower). Not all months were sampled, as biomass could be insufficient, or weather conditions made sampling impossible. The numbers in the table represent biological replicates. The samples were all analysed for nitrogen concentration (N) and 35 of the samples were analysed for amino acids (AA) composition. The sum of AA was used to calculate total protein and nitrogen-to-protein factor.

	Nov-14	Mar-15			Apr-15	May-15			Jun-15			Jul-15			Aug-15			Sep-15	Oct-15			Nov-15	Dec-15	Jan-16			Feb-16	Mar-16			Apr-16			May-16			Total
	Deployed	E Top	E Lower	M Top	-	E Top	E Lower	M Top	E Top	E Lower	M Top	E Top	E Lower	M Top	E Top	E Lower	M Top	-	E Top	E Lower	M Top	-	-	E Top	E Lower	M Top	-	E Top	E Lower	M Top	E Top	E Lower	M Top	E Top	E Lower	M Top	-
N		2	-	-		3	-	3	3	-	3	3	3	3	3	-	3		3	3	-			3	3	3		3	2	3	3	2	-	-	3		65
Protein		-	-	-		3	-	3	2	-	3	-	3	-	3	-	3		3	-	-			3	3	2		-	-	3	3	2	2	-	-	-	35

2.3 Dry matter and ash determination

Dry matter was determined for each sample by vaporizing water at 102-105 °C >20 hours until stable weight. Ash content was determined by incineration in a muffle furnace at 550 °C for 6 h.

2.4 Total nitrogen concentration using Dumas combustion

The total nitrogen concentration was determined by a nitrogen combustion method, the Dumas method, from approximately 0.5 g accurately weighed freeze dried and homogenized sample. The samples were analysed using the fully automated instrument 'rapid MAX exceed' produced by Elementar Analysensysteme GmbH with two analytical replicates (n=2) [33].

2.5 Amino acid profile, protein and protein quantity and quality

Samples of 30 mg dw were hydrolysed at 110 °C for 1 hour in 1 mL 6 M HCl in a microwave (Microwave 3000 SOLV, Anton Paar). Afterwards, derivatization followed using a Phenomenex EZ:faast amino acid analysis kit according to the user manual (Phenomenex). The amino acid composition was determined by liquid chromatography using mass spectrometry (Agilent 1100 Series, LC/MSD Trap) with an EZ:faast 4u AAA-MS column (250 x 3.0 mm, Phenomenex).

Determination of the total macroalgal protein concentration was calculated by summing up the total amino acids (TAA) in moles as recommended by Angell et al. [34] minus the water mass (18 g H₂O/mol amino acid) that was integrated during the disruption of the peptide bonds [17].

To calculate the essential amino acid (EAA) ratio the total EAA was divided by TAA. To evaluate the protein quality, the EAA score was determined following the procedure described by FAO [35] based on the amino acids requirement patterns (amino acid requirement/protein requirement) for children age 3-10 from WHO/FAO/UNU[12], but not considering the digestibility.

2.6 Calculation of Nitrogen-to-protein conversion factors

Nitrogen-to-protein (N-to-protein) factors were determined for each sample by the ratio of total amino acid (TAA) residues minus water mass to the total nitrogen concentration (TN) of the sample [17].

Equation 1: N-to-protein factor = TAA/TN.

2.7 Statistical analyses

All data are expressed as mean $\pm$ standard deviation. PRIMER+ with PERMANOVA add-on package was used as statistical software. The nitrogen and protein concentrations, the N-to-protein factors, and the total amino acid concentration were tested for homogeneity of variance using PERMDISP. Afterwards, the data were analysed by permutational analysis of variance (PERMANOVA) using Euclidian distances. A three-way PERMANOVA test of the interaction of the three factors; *depths* $\times$ *sites* $\times$ *seasons* was not valid because of missing data point and instead a two-way PERMANOVA was applied testing *depths* $\times$ *seasons* and *sites* $\times$ *seasons*. Whenever a significant difference between sample means or

interaction of factors was revealed by PERMANOVA, a pairwise comparison among levels of factors was performed to compare the influence from *sites*, *seasons*, and *depths* on the compositions. In one case (total amino acids) a transformation of data was performed to achieve homogeneity of variance. Means were considered significantly different when levels of $p < 0.05$ were obtained.

This was followed by multidimensional scaling (MDS) plot having one point for each sample. The points that were similar in composition were close to each other; if samples had more unlike composition, they had a large distance between points (standardized samples by maximum resemblance, D1 Euclidian distance; 2-D stress, 0.17).

The results were finally analysed with a SIMPER analysis (based also on Euclidian distances) to identify those amino acid species that contributed most to the observed differences among time. Prior to this multivariate analysis of variance (PERMANOVA) all amino acid values were standardized and thereby expressed relative to the highest value in each dataset. The PERMANOVA analyses therefore only changed in composition, not in the total amount of amino acids.

3. Results

The Faroese cultivated *Saccharina latissima* samples were analysed for total nitrogen (TN) concentration and total amino acid (TAA) concentration (to express the protein concentration).

3.1 Ash content

The ash content of the cultivated *S. latissima* had a seasonal variation ($p = 0.0001$) with the highest content during spring (May 2016 $44.4 \pm 3.7\%$ of dw mean $\pm$ SD), and lowest content during winter (January $31.5 \pm 5.9\%$ of dw mean $\pm$ SD; **Fig. 3**). No significant variation was revealed among site and depth.

3.2 Depth, site and seasonal variation in the nitrogen concentration

The TN concentration ranged from $1.8 \pm 0.0\%$ of dw (March 2015) to $2.5 \pm 0.5\%$ of dw (May 2015) with an average of $2.2 \pm 0.3\%$ of dw for all samples ($n=65$; **Fig. 4**). The two-way PERMANOVA, testing the effect of site and season, revealed a significant seasonal variation in the TN concentration ($p < 0.05$), whereas the site did not have a significant effect ($p=0.48$) nor did their interaction ($p=0.93$). However, the effect of depth and season for the TN concentration revealed a significant interaction of factors ($p < 0.05$). Pairwise comparison showed that the difference in TN concentration between the top and lower samples was only significant in March 2016 ($p < 0.05$; **Fig. 4**). The TN concentration was therefore not influenced by cultivation site and minor influence from different cultivation depths but influenced by a significant seasonal variation (**Fig. 4**).

There was no correlation between ash and nitrogen ($R^2 = 0.0038$).

3.3 Depth, site and seasonal variation in the protein concentration

The protein concentration (calculated by the total amino acids) varied from $2.9 \pm 0.3\%$ of dw (June 2015) to $5.9 \pm 0.7\%$ of dw (April 2016) with an average of $4.3 \pm 0.9\%$ of dw of all samples ($n=35$; **Fig. 5**). The two-way PERMANOVA, testing the effect of site and season, and depth and season for the protein concentration, revealed that there was no significant difference in the protein concentration between months ($p=0.06$), sites ($p=0.77$), or the interaction of these two factors ($p=0.17$). Moreover, there was no significant difference in the protein concentration between months ($p=0.43$), depths ($p=0.96$), or the interaction of

these two factors ($p=0.52$). Consequently, there was no depth, site or seasonal variation in protein concentration for the cultivated *S. latissima*.

3.4 Specific nitrogen-to-protein factors

The N-to-protein conversion factor was calculated for the months analysed for both total protein and nitrogen concentration, and the average of all samples was 2.0 ± 0.4 with the lowest factor (1.2) obtained in June 2015 and the highest factor (2.7) in April 2016 (**Fig. 6**). There was no significant difference in the N-to-protein concentration between months ($p=0.11$), depths ($p=0.89$), sites ($p=0.55$), or the interaction of these factors.

3.5 Amino acid profile and quality

The amino acid composition of the cultivated *S. latissima* is presented in **Table 2**. All the analysed amino acids were present in all samples with exception of tryptophan, which was destroyed during acid hydrolysis.

The highest EAA score was found in March 2016 at the moderately exposed site ($106 \pm 11\%$). The lowest EAA score was seen in July 2015 at the exposed site ($51.3 \pm 2.8\%$) with histidine as the first limiting amino acid. The main limiting amino acid was histidine (10 out of 12 cases including both sites) but in two individual cases valine and isoleucine were the limiting amino acids. The essential amino acid to total amino acid ratio was calculated and found to be lowest during winter (32.9-42.4%) and highest during spring and summer months (44.2-52.4%). The non-essential amino acids alanine, asparagine and glutamine had a large contribution to TAA in all months counting for approximately half of the concentration.

There was a significant seasonal variation in the amino acid composition ($p<0.05$) and a pairwise comparison showed that June 2015 and April 2016, October 2015 and January 2016, and March 2016 and April 2016 had a similar amino acid composition ($p=0.07$ -0.48) and that all other months had a significantly different composition. On the other hand, no effect of cultivation sites or cultivation depths with regard to the amino acid composition was found ($p=0.17$ and $p=0.56$, respectively).

Fig. 3. Ash (% of dw) of Faroese cultivated *Saccharina latissima*, deployed in November 2014. Standard deviations are represented as bars for each pillar (n=3), and the total average concentration $\pm$ standard deviation per month as a solid orange line with orange bars (n=3-9). Different letters represent a significant difference between months.

Fig. 4. Nitrogen concentration (% of dw) of Faroese cultivated *Saccharina latissima* at two sites (wave exposed, and moderately wave exposed) at two different depths (top: 0-2 MBSL and lower: 8-10 MBSL). The seeded lines were deployed in November 2014 and biomass was sampled from March 2015 until May 2016. Standard deviations are represented as bars for each pillar (n=3), and the total average concentration $\pm$ standard deviation per month as a solid orange line with orange bars (n=3-9). A star (*) represents months with statistical different nitrogen concentrations between the top and lower samples. Different letters represent a significant difference between months (a,b,c,d,e) as a mean of both sites, as there was no statistical variation between sites.

Fig. 5. The total sum of amino acids was used to determine total protein concentration (% of dw) of Faroese cultivated *Saccharina latissima* at two sites (wave exposed, and moderately wave exposed) at two different depths (top: 0-2 MBSL and lower: 8-10 MBSL). The seeded lines were deployed in November 2014. Samples were collected from March 2015 until May 2016; although, not all samples were analysed due to the high cost of amino acid analysis. Standard deviations are represented as bars for each pillar (n=3), and the total average concentration $\pm$ standard deviation per month as a solid orange line with orange bars (n=3-9).

Fig. 6. The average calculated N-to-protein factors for Faroese cultivated *Saccharina latissima* at two sites (wave exposed, and moderately wave exposed) and at two different depths (top: 0-2 MBSL and lower: 8-10 MBSL). Samples were collected from November 2014 until May 2016. Standard deviations are represented as bars for each pillar (n=3), and the total average concentration $\pm$ standard deviation per month as a solid orange line with orange bars (n=3-9).

Table 2. Amino acid composition in *Saccharina latissima* (mg amino acid g⁻¹ protein), total essential amino acids (ΣEAA; mg g⁻¹ protein), total amino acid concentration (TAA; mg g⁻¹ protein), protein concentration (Protein; % of dw), essential amino acid ratio (EAA/AA), and an essential amino acid score (EAA score) cultivated at either exposed site or moderately exposed site at two different depths (lower and top) (June 2015-April 2016). .

Amino Acid	June 15		July 15	August 15		October 15	January 16			March 16	April 16		
[mg/g AA-protein]	E, Top	M, Top	E, Top	M, Top	E, Top	E, Top	M, Top	E, Top	E, Lower	M, Top	M, Top	E, Top	E, Lower
Lysine	59±17	68±11	116±15	54.2±7.6	70±17	61.8±3.4	73±23	41.6±7.3	50.5±3.2	51.1±5.4	81±14	90±15	62.0±4.7
Alanine	211±66	140±14	26.4±4.6	131±14	147.3±4.6	185±27	192.1	167±15	168±22	205±15	152±52	151±23	155±35
Arginine	26.2±2.6	30.4±4.4	52±13	95±30	77±41	44±21	29.4±3.2	46.1*	67±14	107.2*	35.1±6.0	33.4±7.8	35.1±8.0
Cysteine	3.4±0.2	5.9±1.3	3.8±1.4	3.9±0.9	3.6±0.9	4.1±2.1	5.6±1.7	5.6±2.5	4.0±2.4	2.9±0.4	6.8*	4.9±1.2	7.1±1.7
Leucine	70.7±6.6	75.7±7.6	61.6±4.7	90.6±8.8	87±11	70.6±9.9	49.7	84.9±4.3	78.0±5.7	97±11	91.9±0.8	92.1±7.6	83±13
Methionine	44.3±3.8	38.3±6.6	31±11	39.1±5.0	35.3±9.6	30.9±6.3	21.2±3.4	43.8±9.4	40±16	39.6±3.5	30.5±3.6	28.6±1.7	32.5±5.6
Phenylalanine	42.2±5.0	50.7±8.4	73.4±7.1	71±12	62±17	44±11	37.2±9.4	60.1±5.5	56.1±6.9	68.6±6.8	55.6±4.2	53.5±7.0	50±12
Proline	58.0±1.8	59.4±4.1	27.2±4.1	63.9±7.2	64.3±6.3	50.2±1.4	42±12	61.2±3.2	52.6±3.3	68.7±7.6	56.2±6.1	61.4±5.0	69.7±2.6
Threonine	49.9±9.2	48.6±8.7	62.8±2.6	52.9±3.2	51.5±5.7	61.3±8.5	49.2±0.4	47.8±4.7	46.4±2.7	54.7±3.8	57.9±7.8	51.3±2.3	58.5±8.4
Tyrosine	29.4±4.1	33.0±3.1	50.0±1.8	36.2±2.4	33.6±3.4	28.5±4.9	25.7±8.0	27.4±1.8	29.6±7.4	34.7±3.6	29.0±3.5	32.9±2.8	32.0±3.9
Aspartic acid	149±21	151±15	251±21	171.5±2.7	156±20	169±47	174±48	146±14	143±13	146.1±2.2	135±10	141±26	147±17
Serine	62.3±6.6	56.2±4.5	64.4±5.0	68.8±3.8	59.8±6.9	72±16	59.0±3.1	50.9±9.4	53.9±6.6	61.2±5.0	49.5±7.6	54.5±5.3	60.3±1.5
4-hydroxyproline	3.3±0.7	12±13	4.2±0.7	5.1±0.7	4.3±0.2	6.1±0.9	8.1±0.1	6.4±2.5	6.8±0.9	3.8±0.2	5.0±0.9	5.1±0.6	7.3±1.3
Glutamic acid	162.1±3.2	179±17	216±34	144±20	156±19	276±53	333±60	223±10	233±27	137±11	146.2±0.4	136±25	147.7±2.5
Valine	55.4±5.7	77±40	20.5±1.1	44.3	56.2±1.2	53.3±9.6	35±17	57±11	77±10	70±14	83.1±7.0	80.1±8.7	80.3±2.5
Histidine	12.7±3.3	12.9±4.7	17.6±1.2	12.7±1.5	13.9±2.6	9.7±2.2	11.6±1.1	13.9±1.7	11.1±1.4	17.6±2.8	13.4±2.5	13.1±0.3	10.1±6.8
Isoleucine	41.9±6.0	37.8±3.4	27.2±7.1	30.7±5.7	34.4±6.9	38.8±8.7	17.4±2.5	34.2±1.9	32.7±7.4	33.2±6.5	52±18	44.3±4.4	36.6±2.8
Glycine	104±14	100±24	47±39	59±11	59±30	109±19	74±38	89±34	70±33	47.6±4.1	100±24	102±12	103±29
ΣEAA	405±54	442±42	460±23	403±31	444±34	386±64	320±20	410.1±9.2	396±42	467±48	495±53	486±26	445±54
TAA	914±43	967±19	1009±24	912±122	954.3±9.4	1054±211	973±57	968±19	955±22	920±19	944±39	935±13	961±23
AA-Protein (%)	2.9±0.3	4.3±0.9	3.4±0.4	3.8±1.2	5.5±1.3	4.1±2.1	3.8±0.7	3.4±1.9	4.2±2.9	4.6±0.6	5.9±0.7	5.1±0.6	4.4±0.1
EAA/TAA	44.2±3.8	45.6±3.7	45.6±2.0	44.4±3.4	46.5±3.3	36.8±3.0	32.9±0.1	42.4±1.8	41.4±0.0	50.8±4.9	52.4±3.4	51.9±3.3	46.3±4.5
EAA score (%)	80±21	81±30	51.3±2.8	79.4±9.4	87±16	61±14	56.0±8.2	87±11	69.1±8.6	106±11	84±16	82.2±2.1	63±43
Limiting EAA	Histidine	Histidine	Valine	Histidine	Histidine	Histidine	Isoleucine	Histidine	Histidine	—	Histidine	Histidine	Histidine

Tryptophan was not detected.

Data expressed as mean ± standard deviation (n=3).

* Detected in only one of the replicates.

Essential amino acids in human are highlighted with grey boxes.

3.6 Multidimensional scaling of the amino acid composition

A multidimensional scaling (MDS) plot of the amino acid composition in the cultivated *S. latissima* showed a grouping of the samples; one point for each sample was used and marked by month (**Fig. 7**). The points that are close were more similar in their composition such as samples from June 2015 and April 2016 (all samples within the circle are very similar), compared to points further apart (outside the circle). July 2015 samples are the ones that are most separated from the other months, which can be related to a much lower concentration of alanine and valine and relatively high level of lysine in comparison with the other months. Samples from winter (October 2015 and January 2016) tend to be high in glutamic acid and separated from the other months in the MDS plot.

Fig. 7. A multidimensional scaling (MDS) plot, one point for each sample (standardized samples by total resemblance, D1 Euclidian distance; 2-D stress, 0.17), considering the similarities of the amino acid composition of the cultivated *S. latissima* (n=3-8). The closer a point is to another the more they have in common. The circle shows the main distribution of samples.

3.7 Similarity Percentages Analysis (SIMPER)

The amino acids that contributed to the observed differences in the composition among pairs of months were tested using a similarity analysis (SIMPER). The result was only relevant for pairs of months that differed significantly in the pairwise comparison. From the MDS plot the July, October 2015 and

January 2016 months had the largest distance to a main distribution of the samples. The SIMPER test showed that alanine was the main reason why July 2015 was different from the others (counting for 32-39%). For October 2015 the main contributor to the changes was glutamic acid (counting for 43-66%). In January 2016 the main contributor to the changes was glutamine (counting for 48-61%).

4. Discussion

4.1 Influence of environmental factors on nitrogen and protein concentration

Growth, including the formation of proteins, is correlated with available light, nutrients (primarily nitrogen) and water motion [13,21,36]. Most algae, like *S. latissima*, can take up excess nitrogen and store it internally as nitrate or urea for the later utilization when nutrient availability in the water is low. In this study, no difference in the nitrogen concentration was found between depths (except in March 2016) or between sites, but there was a significant seasonal variation (section 0). The seasonal variation in nitrogen is, therefore, most likely related to the availability of nitrogen in the sea, internal nitrogen storage of the alga and the incorporation of nitrogen in building tissue. The average nitrogen concentration was $2.1 \pm 0.2\%$ of dry weight (dw) in accordance with other studies using the same determination method [20,38].

The ash concentration did also show a seasonal variation (**Fig. 3**), but there was no correlation between the total nitrogen and ash concentration (graph not shown). The nitrogen concentration is therefore not controlled by variations of the ash.

The average total amino acids concentration (hereafter called AA-protein) was $4.3 \pm 0.9\%$ of dw, and no depth, site or seasonal variation of the protein concentration was found, though seasonal variation was close to being significant, having a *p*-value of 0.06. The AA-protein concentration showed large standard deviations, and this can be explained by few replicates (*n*=3) and the natural variation among individuals. More biological replicates are needed to get a better understanding of the variation in protein between samples from different seasons, sites and depths. Despite the large standard deviation, the lack of seasonal variation of the AA-protein concentration could be a consequence of the stable physical

environmental conditions seen in the Faroe Islands i.e. stable temperature and salinity [27].

Contrary to salinity and temperature, the irradiance and day length varied substantially through the year in the Faroe Islands [15], nevertheless, the incoming light at the two sites was close to equal as the distance between sites is only six kilometres. The sites had therefore equal light, temperature, salinity, and current conditions, and the difference between the sites was the wave height and water column depth, and these differences were here found not to have any significance for the nitrogen and protein concentration.

Environmental factors may vary with depths [13], and because *S. latissima* was cultivated on 10-meter-long vertical growth lines, the depth could affect the nitrogen and protein concentration along the line and the variation with regard to depth was consequently examined. A significant depth variation in nitrogen concentration occurred only in March 2016. Here, the nitrogen concentration in the top sample was $2.0 \pm 0.0\%$ dw and the lower sample was $2.3 \pm 0.2\%$ dw. The difference could be explained by high competition for nutrients between the macroalgae growing in the top of the lines and phytoplankton as spring is the season where phytoplankton blooms often take off, and thus being nutrient limited for a period in March.

There was no statistical difference in AA-protein concentration between cultivation depth, which may indicate that periods of limited nitrogen levels are short and that *S. latissima* can use their internal storage of nitrate and urea.

4.2 The comparison of analytical methods for measuring nitrogen concentrations

Nitrogen analysis is performed by methods such as the Kjeldahl method [38] or the Dumas method [39]. Kjeldahl is the international reference method for nitrogen determination, but the Dumas method has recently been developed to improve its accuracy and running time and can be fully automated. The Kjeldahl method does not measure inorganic forms of nitrogen, such as nitrate and nitrite, as they might not be sufficiently degraded by digestion. In contrast, all nitrogen sources, are measured by getting all N on gaseous form in the Dumas method [33]. This is important when determining nitrogen in biomass with high levels of inorganic forms of nitrogen. As the Dumas method determines all the nitrogen forms, slightly higher levels of nitrogen are usually found when using the Dumas method compared to the Kjeldahl method

[16]. As protein concentration of brown macroalgae often has been estimated as crude protein by determining nitrogen concentration and then multiplying with an N-to-protein conversion factor (often 6.25), it can be concluded that the approach of analysis of nitrogen concentration could, in the end, have an influence on the crude protein results.

4.3 Comparison of protein concentration between studies

Reported protein content of macroalgae depends on the analytical method used for determination, which are not based on quantifying the same components. Consequently it becomes difficult to make a direct comparison between studies. Vilg et al. [40] used the colorimetric Lowry Protein Assay [41], other studies were based on quantifying nitrogen by either Dumas or Kjeldahl followed by the use of N-to-protein conversion factors of either 5.0 [36] or 6.25 [42] (hereafter called the crude protein). However, most studies used the same method as this study by summing up the amino acid residues (AA-protein) and subtracting the water molecules added during hydrolysis [9,18,22,26,37].

The AA-protein method is the only method where interfering substances do not affect the results; however, there is potential for improvement in regard to the hydrolysis method [43]. In the following section, protein content of *S. latissima* is compared between studies. Only studies that use the AA-protein determination method, as used in this study, will be addressed for accurate comparison.

Mols-Mortensen et al. [26] cultivated *S. latissima* at three sites with different exposures (sheltered, current exposed and wave exposed) in Faroese waters and characterized the growth and quality of the biomass and the available nutrients in the surroundings from March to August 2015. Therefore, our work is very similar to their study. However, some variations in the performed work differ from the cultivation of this current study, and the results by Mols-Mortensen et al. [26] can therefore not be directly applied to all Faroese cultivated *S. latissima*. The cultivation system used by Mols-Mortensen et al. [26] had two-meter-long cultivation lines and 10 replicate lines. The site “wave exposed” had a maximum significant wave height of 2.2 meters [26], which is the same wave height of the “moderately exposed site” of this study. Finally, they did analyse one growing season, whereas our study investigated two growing seasons. Mols-Mortensen et al. [26] found a significant seasonal variation in AA-protein concentration over the year,

opposite to this study, and they found no significant difference between sites, similar to our study. All samples from Mols-Mortensen et al. [26] (except July at the sheltered site) had higher AA-protein concentration than the *S. latissima* cultivated in Funningsfjærdur (~5-17% of dw).

Marinho et al. [9] collected bi-monthly samples from *S. latissima* (including epiphytes, when present) cultivated commercially at an integrated multi-trophic aquaculture (IMTA) site, and from a reference site in Denmark. Overall, there was no significant difference in biomass composition between the two sampling sites, like our study. However, seasonal variations in AA-protein and nitrogen were found, which was also the case for this study regarding nitrogen. The AA-protein concentration varied markedly reaching a maximum of 10.8% dw in November 2012 and a minimum of 1.3% dw in May 2013 in the study by Marinho et al. [9]. The presence of epiphytes was found as a major issue in the Danish cultivation study and therefore the summer harvest was suggested to be used for feed instead of food.

Nielsen et al. [37] analysed AA-protein concentration in *S. latissima* at ten sites in the inner Danish waters using wild collected biomass and found an average AA-protein concentration of 3.1% of dw in August 2012. They concluded that depth did not have a significant influence on the AA-protein concentration, in line with our results. Instead, they concluded that sites with different salinity had a significant influence on the AA-protein concentration. Also, they found that the ash content was negatively correlated with frond length, which explained the high ash content observed in two relatively young individuals [37]. In our study, ash was lowest during winter and therefore not related to biomass length, which in the Faroe Islands is longest during summer [15].

The AA-protein concentration found for cultivated *S. latissima* in Funningsfjærdur (2.9-5.9% dw) was in the lower end compared to concentrations found by other studies [9,26], although Nielsen et al. [37] had similar levels (1.1-7.5% of dw). The seasonal variation of AA-protein concentration from this study was not significant, though close ($p=0.06$), which are reverse results found in other studies where seasonal variation was present. Several literature reviews found *S. latissima* to have the highest AA-protein concentration in winter months or early spring [2,3,44], which was also the conclusion by Mols-Mortensen et al. [26]. Contradictory, Marinho et al. [9] had the highest AA-protein concentration in autumn for *S.*

latissima cultivated in the inner Danish waters.

4.4 Protein determination with nitrogen-to-protein conversion factors

The average N-to-protein conversion factor found for *S. latissima* cultivated in Funningsfjærdur was 2.0 ± 0.4 , and therefore much lower than the widely used 6.25 or 5.0. Other studies found conversion factors for *S. latissima* to be 3.9 [37], 3.7 ± 1.3 [45] and 0.9-4.5 (manuscript in prep., Marinho & Holdt). Authors within both macroalgae, meat, fish, and plant sciences determine crude protein by using a conversion factor of e.g. 6.25; although, it has been acknowledged that plant or macroalgal protein differ in terms of nitrogen concentration thus this factor is overestimating the concentration [16]. Therefore, several studies have calculated species-specific N-to-protein conversion factors for macroalgae to avoid overestimating. Angell et al. [34] suggested a prioritized list of methods for protein determination, which depended on the knowledge of species-specific conversion factors and the availability of analytical methods. They suggest the total amino acids concentration (TAA) to be the most precise, which is also supported by FAO [8] and Mæhre et al. [43]. However, if this analysis is not financially or technically available the species-specific N-to-protein conversion factor should be used. A third option recommended is to use a macroalgal specific conversion factor of 5.0.

If the general macroalgal conversion factor of 5.0 on nitrogen concentration was applied on the results of the present study the crude protein concentration would in average be $10.8 \pm 0.9\%$ of dw compared to $4.3 \pm 0.9\%$ of dw when summing TAA. A comparable study applying the conversion factor 5.0 was Bruhn et al. [36], which was based on nitrogen concentrations determined by the Kjeldahl method. They found a crude protein concentration of *S. latissima* from the inner Danish waters of 16-17% of dw, which is above the crude protein levels found in Funningsfjærdur, but a comparison is not possible due to the determination method.

These various protein determination methods will overall lead to several consequences. Commercially, the nitrogen concentration is not sufficiently expressed when multiplied by a single N-to-protein factor on all results. Using several factors related to each month of harvest are neither practical. Therefore, it is recommended to quantify the total amino acids (AA-protein), which will also indicate the

protein quality. The economic value of food and feed is often established based on the protein quantity and quality. By applying a single N-to-protein conversion factor (e.g. 5.0 and 6.25) this will lead to an over- or underestimation of the actual value of the macroalgal proteins. Hence, the species and season-specific N-to-protein conversion factors should be used with absolute care and with the knowledge that the results from the conversion would only represent an estimation of crude protein concentration. Adding to the species and season-specific conversion factor, a site and depth-specific N-to-protein conversion factor can be used. However, as a result of this study, this is not significantly influencing the protein concentration, and these multiple options can lead to a problem when comparing protein concentration between studies, but also increases the complexity of determining the protein concentration. For all these reasons we recommend the method of summing total amino acids for total protein estimation of macroalgae.

4.5 Amino acid profile and protein quality

All analysed amino acids were found in the samples. The amino acids analysed represented both protein-derived amino acids and free amino acids. The presence of free amino acids contributes to an overestimation of the total protein content, though typically counting for less than 10% [18]. However, the procedure is widely accepted, since in acid hydrolysis some amino acids are partially or totally destroyed e.g. tryptophan [46], and the total amino acids will in this way be acceptable as the free amino acids are adding and the destroyed amino acids are reducing [18].

Aspartic acid and glutamic acid were the dominant amino acids obtaining 30.2-52.1% of the total amino acids (TAA). These results are in agreement with those reported for other brown macroalgae where these two amino acids accounted for 22-49% of TAA [3,9].

The EAA/TAA ratio, which is a way to estimate the quality of the protein as food, was in our study found to be in the range between 33% and 52% with the lowest values found in January 2016 and highest in April 2016. From the studies of *S. latissima* in the Danish inner waters, the ratio was reported to be between 26% and 30% for wild populations [37] and the ratio for cultivated *S. latissima* was reported to be between 21% and 42% [9].

The quality of the protein was also estimated using the total EAA score and levels above 100%

meant that the amount of all EAA was above the requirement pattern (mg AA/g protein requirement) for the selected age group (3–10 years old) defined by WHO/FAO/UNU [12]. The EAA score was lowest in July ($51.3 \pm 2.8\%$) and peaked in March 2016 ($106 \pm 11\%$), suggesting that the proteins found in *S. latissima* have high quality. Mols-Mortensen et al. [26] found the highest EAA score in May 2015 (93.7%) on the Faroese cultivated *S. latissima* and Marinho et al. [9] found the highest EAA score in November 2013 (68.9%) from *S. latissima* cultivated in Denmark.

In this study, histidine was the main limiting amino acid with two exceptions. Histidine was also reported to be the limiting amino acid in the study from both Marinho et al. [9] and Mols-Mortensen et al. [26]. Furthermore, the EAA concentration of the *S. latissima* cultivated in Funningsfjörður in all sampling periods was above the requirement pattern (305 mg AA/g protein) by WHO/FAO/UNU [12].

It is important to comment that nutritional value is mainly defined by both amino acid composition and digestibility [11,12]. In order to fully evaluate the biological value of protein from *S. latissima*, in vivo protein digestibility trials must be carried out. Moreover, since the protein concentration of Faroese *S. latissima* is in the very low end of values found in macroalgae and other food sources its potential solely as a protein source is very limited. On the other hand, since *S. latissima* is a natural source of bioactive compounds such as specific polysaccharides, amino acids, fatty acids, polyphenols, and pigments [44], its potential to be used as functional ingredient in the food and/or feed industry should be further investigated.

5. Conclusion

Saccharina latissima cultivated in the Faroe Islands had a significant seasonal variation of nitrogen concentration, though the AA-protein concentration did not show significant seasonal variation ($p=0.06$). There was no difference found between the moderately exposed and exposed cultivation sites and the difference between macroalgae grown in the top meters was not (except one month) significantly different in terms of nitrogen and AA-protein concentration from the macroalgae grown at 9 meters below sea level. The present findings suggest that the companies that cultivate *S. latissima* in Funningsfjörður do not need

to distinguish between cultivation sites, depths or seasons when informing about protein concentration, which is contrary to cultivation in other geographical areas like e.g. the inner Danish waters.

Cultivated Faroese *S. latissima* had a high proportion of essential amino acids (EAA), a high EAA/TAA ratio, and an EAA score above 100% when harvested in March (2016), but also when harvested in other months the protein quality was generally high. However, the concentration of total protein is low and Faroese cultivated *S. latissima* must be seen as a source for food and feed though not solely because of the protein content.

The average N-to-protein conversion factor found in this study was 1.96 and thus about two thirds less than the widely used conversion factor of 6.25 for crude protein. Consequently, specific N-to-protein factors are not recommended for use for total protein determination, since it could lead to an over- (or possibly under) estimation. Therefore, it can be concluded that determining protein concentration should preferably be made by the use of quantitative amino acid analysis (AA-protein).

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Conflict of Interest

The authors declare no conflict of interest.

Informed Consent, Human/Animal Rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors contribution

All persons designated as authors qualify for authorship and are listed as authors. Each author has participated sufficiently in the work to take public responsibility for appropriate parts of the content. All authors have made substantial contributions to the conception and design of the study, acquisition of data, and/or analysis and interpretation of data. All authors have drafted the article and/or revising it critically for important intellectual content. And all authors have given their final approval of the version to be submitted.

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Highlights

- Offshore cultivated Faroese sugar kelp had no seasonal variation in protein content
- Faroese sugar kelp harvested in spring had a high essential amino acids score (>100)
- Sugar kelp is a high-quality protein though low quantities source for food and feed
- Conventional conversion factor (6.25) overestimate the protein content in sugar kelp