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Article

Fatty Acids Profiles of Stipe and Blade from the Norwegian Brown Macroalga *Laminaria hyperborea*, Using Off-Line SPE and GC-MS

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Abstract: A thorough analysis and comparison of the fatty acid profiles of stipe and blade from *Laminaria hyperborea,* a kelp species found in the northern Atlantic, is presented. Lipids were extracted and fractionated into neutral lipids, free fatty acids and polar lipids, then derivatized to fatty acid methyl esters prior to GC-MS analysis. A total of 42 fatty acids were identified and quantified, including the n-3 fatty acids α-linolenic acid, stearidonic acid and eicosapentaenoic acid. An n-6/n-3 ratio of 0.8:1 was found in blade and 3.5:1 in stipe, respectively. The ratios vary between the lipid fractions within stipe and blade, with the lowest ratio in the polar lipid fraction of blade. The fatty acid amounts are higher in blade than in stipe, and the highest amounts of n-3 fatty acids are found within the neutral lipid fractions. The amounts of polyunsaturated fatty acids are 3.4 times higher in blade than stipe. This study highlights the compositional differences between the lipid fractions of stipe and blade from *L. hyperborea*. The amount of polyunsaturated fatty acids, compared to saturated- and monounsaturated fatty acids, as well as the n-6/n-3-ratio, is known to influence human health. In the pharmaceutical, food, and feed industries this can be of importance for production and sale of different health products. Additionally, lipids are today among the unused by products of alginate production, exploiting this material for commercial interest should give both economical and environmental benefits.

Keywords: *Laminaria hyperborean*; lipids; fatty acids; GC-MS

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1. Introduction

The increase in world population and lack of sufficient food, begs for new sources of food and feed. As much as 60% of the world food energy intake is provided by the cereals wheat, rice, and corn [4]. These cereals, while high in calories and carbohydrates, have small amounts of important nutrients such as proteins, minerals, vitamins (especially A and C), and fatty acids, especially long chained polyunsaturated n-3 fatty acids [1,6,18]. A promising supplement for food and feed is a better utilization of marine resources. World production and harvesting of micro- and macroalgae has doubled from 2004 to 2014 [3]. Still, 97% of the production and harvesting is found in Asia [3], thus there is a large potential for expansion in other parts of the world. Macroalgae is a diverse group of marine plants, informally divided into three groups: rhodophyta (red algae), chlorophyta (green algae), and phaeophyta (brown algae). An important nutritional benefit of marine macroalgae is that the lipid content is high in polyunsaturated fatty acids (PUFAs), especially n-3 and n-6 fatty acids [10]. A diet with a low n-6/n-3 ratio is reported to have suppressive effects on cardiovascular diseases, cancer, and inflammatory and autoimmune diseases [16]. Previous studies regarding fatty acids in brown macroalgae have often had a nutritional or pharmaceutical focus. Fatty acid content has been determined as one among several parameters and/or for several different species, resulting in limited fatty acid profiles [7,11-13,15]. *Laminaria hyperborea*, a common species of kelp found in the northern Atlantic, has to the authors' knowledge only been characterized with regard to fatty acids in three studies [9,14,17], all presenting limited profiles identifying 8–9 fatty acids.

The aim of this study has been to provide a thorough analysis of the fatty acid profiles in stipe and blade from the macroalga *L. hyperborea*. In this context, the lipids were fractionated into free fatty acids (FFAs), neutral lipids (NLs), and polar lipids (PLs). The fatty acids from each class were identified and quantified with off-line solid phase extraction (SPE) and GC-MS.

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3. Results and Discussion

We have identified and quantified 42 different fatty acids in *L. hyperborea*, as shown in Table 1. This is a significantly higher number than previously reported by others [9,14,17]. The fatty acid profile was determined for the NL, FFA, and PL fractions in stipe and blade separately. While up to 41 different fatty acids were detected within a lipid fraction, the same 10 fatty acids predominated in all fractions in both stipe and blade. These predominating fatty acids constitutes more than 90% of the total fatty acid content in all the fractions, as shown in Figure 1. They are found in amounts varying from 0.65 to 1200 µg/g DW (Table 1). A fatty acid was classified as predominating if it was above 2% of the total fatty acid content, in at least one of the lipid fractions in either stipe or blade. These fatty acids correspond to those identified by others [9,14,17]. Schmid and Stengel [14] also identified C18:3n-6, at 1.2% in stipe and 5.5% in blade. Which differs from our results where C18:3n-6 is not above 2% in any of the blade lipid fractions. This could be due to geographical and/or seasonal variations. Only a maximum of two *trans* fatty acids, C14:1 *trans*-9 and C16:2 *cis/trans*-7,10, were identified in the samples, both in relatively low amounts (≤ 2.53 µg/g DW). Among the predominating fatty acids are important dietary n-3 fatty acids such as α-linolenic acid (ALA, C18:3n-3), stearidonic acid (SDA, C18:4n-3), and eicosapentaenoic acid (EPA, C20:5n-3). As well as two n-6 fatty acids, linoleic acid (LA, C18:2n-6), and arachidonic acid (AA, C20:4n-6). How favourable *L. hyperborea* is for the human diet (and thus also in animal feed), depends on the ratio between n-6 and n-3 fatty acids.

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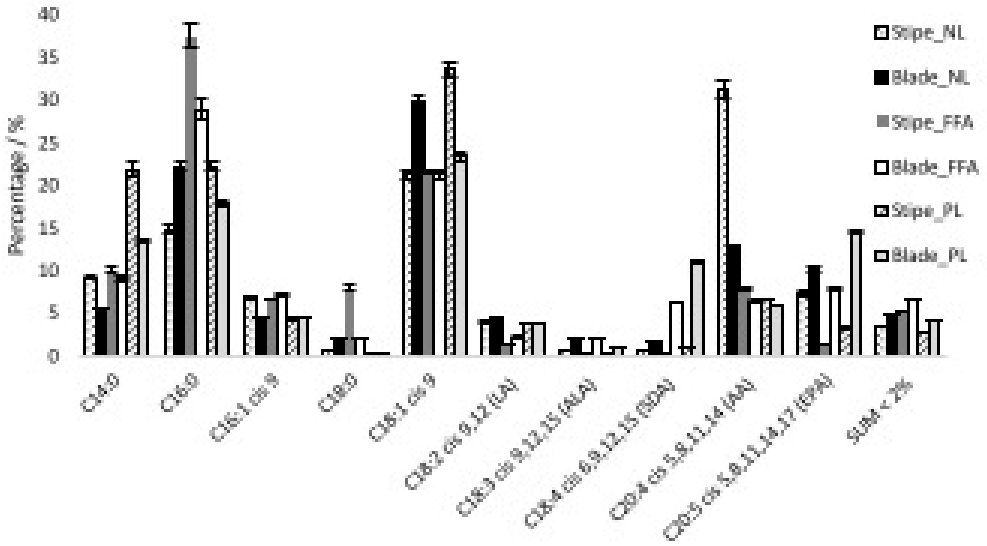

Figure 1. Fatty acid profile for fatty acids contributing more than 2% of total fatty acid content, in at least one lipid fraction. SUM < 2% is the summarized contribution of the remaining fatty acids. (n=4, two injection parallels for each sample replicate, error bars = ± 1 SD). Abbreviations: NL, neutral lipid; FFA, free fatty acid; PL, polar lipid.

In western diet the n-6/n-3 ratio is 15-20:1, for health benefits lowering this ratio is beneficial, e.g. are ratios of 2-5:1 reported to have suppressive effects on cardiovascular, inflammatory, and autoimmune diseases [16]. *L. hyperborea* has an n-6/n-3 ratio of 0.8:1 in blade and 3.5:1 in stipe. The ratios vary between the lipid fractions, as seen in Table 2, but are higher in stipe than in blade. In both stipe and blade the lowest amounts of n-3 and n-6 fatty acids are found in the FFA fraction. The highest amounts are found in the NL fraction, with the exception of n-3 in blade where the amount in the PL fraction is higher than in the NL fraction (646 and 591 µg/g DW, respectively). Since there is a significant difference between the n-6/n-3 ratios in stipe and blade, using only blade could be considered if a very low n-6/n-3 ratio is desired. Stipe and blade differ not only in n-6/n-3 ratio, there are also differences in the amounts of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs (Table 2). The total amount of PUFAs in blade is 3.4 times higher than in stipe, and the total amounts of SFAs and MUFAs are 2.6 and 2.8 times higher, respectively. PUFAs are preferred over SFAs from a dietary perspective, and replacing SFAs with PUFAs in the diet decrease the risk of coronary heart disease [5].

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While the same fatty acids predominate, the amounts in blade are consistently higher than those in stipe, as seen in Figure 2. These differences vary between lipid fractions and fatty acids. At minimum, the amounts in blade is 1.1 times higher than in stipe for myristic acid (C14:0) in the PL fraction, and at maximum, 69.3 times higher than in stipe for SDA in the FFA fraction. For the FFA and PL fractions, the largest differences are found in the fatty acids ALA, SDA and EPA. In the NL fraction, the largest difference between stipe and blade amounts is found in stearic acid (C18:0), while blade and stipe amounts are almost equal in the FFA and PL fractions for the same fatty acid.

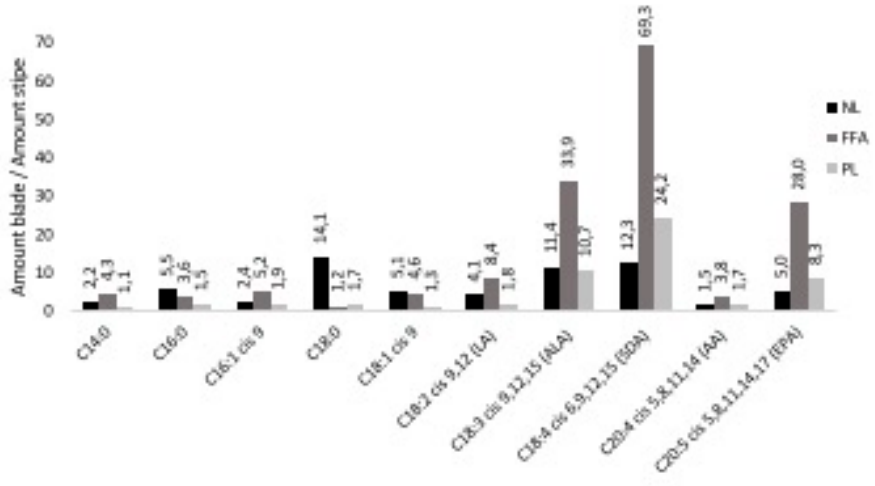

Figure 2. Ratio between average blade and stipe amounts in the predominating fatty acids. Abbreviations: NL, neutral lipid; FFA, free fatty acid; PL, polar lipid.

2. Material and Methods

Chloroform and hexane were of Chromasolv® quality, heptane, diethyl ether, methanol and NaCl puriss pa. quality, all from Sigma-Aldrich (St. Louis, MO, USA). The acetic acid was from Honeywell Riedel-de Haen (Seelze, Germany).

2.1 Standards

A fatty acid methyl ester (FAME) mix with 37 components (Food Industry Fame MIX, Restek, Bellefonte, PA, USA) was used for identification of the FAMEs. A 21 component FAME mix (Qualmix PUFA Fish M, Methyl Esters (Menhaden Oil), Larodan AB, Solna, Sweden) was used for identification of all-*cis*-6,9,12,15-octadecatetraenoic acid methyl ester, all-*cis*-8,11,14,17-eicosatetraenoic acid methyl ester, and all-*cis*-7,10,13,16,19-docosapentaenoic acid methyl ester. In

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addition, the following individual FAME standards were used: nonanoic acid methyl ester (Sigma-Aldrich, St.Louis, MO, USA), 13-methyltetradecanoic acid methyl ester, *trans*-9-tetradecenoic acid methyl ester, *cis*-9-heptadecenoic acid methyl ester, *cis*-13-octadecenoic acid methyl ester, *cis*-9-eicosenoic acid methyl ester, and hexacosanoic acid methyl ester (all from Larodan AB, Solna, Sweden). Three internal standards were used (10 mg/mL, dissolved in $CHCl_3$), one for each lipid fraction; 1,2-dinonadecanoyl-sn-Glycero-3-phosphatidylcholine for the PL fractions, nonadecanoic acid for the FFA fractions, and trinonadecanoin for the NL fractions (all from Larodan AB, Solna, Sweden). Nonadecanoic acid methyl ester (Larodan AB, Solna, Sweden) was added to the 37 components FAME mix for retention time identification, since C19:0 was used as internal standard in the samples.

2.2 Pre-treatment of L. hyperborea

L. hyperborea was provided by FMC BioPolymer AS. It was harvested off the west coast of Norway, outside Sør-Trøndelag County Municipality in October 2015. On board the trawler *L. hyperborea* was rinsed, crude-cut and preserved with formalin. The holdfast was discarded. Once off the trawler stipe and blade were vacuum packed separately. When received at the university, stipe and blade were rinsed with water, frozen with liquid N_2 (99.999%, AGA, The Linde Group, Munich, Germany), and freeze-dried (Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The freeze-dried material was crushed in a QMM Micro – Mixer, and pulverized in a Laboratory Mixer 3100 (Danfoss) by G. A. Lund at Pharmatech AS, Fredrikstad, Norway.

2.3 Lipid Extraction

Four replicates of both stipe and blade were used. The lipids were extracted with a modified Folch's method [8]. In brief, 5–10 g alga powder was extracted in a separatory funnel with 10 times its volume $CHCl_3$:MeOH (2:1), 50 µL of each internal standard was added with a Hamilton® syringe. To induce phase separation, 0.9% NaCl was added after mixing (0.2 times the volume of $CHCl_3$:MeOH). After approximately 20 min the organic phase was transferred to a test tube

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(Duran® 20 x 150 mm, Mainz, Germany). The polar phase was re-extracted with $CHCl_3$, 30–60 mL depending on amount of alga powder. The organic phases of each sample were combined and evaporated with a vacuum evaporator (Q-101, Buchi Labortechnik AG, Flawil, Switzerland) at 40 °C, re-dissolved in 1.00 mL chloroform, and transferred to vials for SPE.

A liquid-handling robot (Gilson, GX-271, ASPEC, Middleton, WI, USA) was used to carry out the SPE procedure. Aminopropyl-modified silica phase SPE columns, 500 mg, 3 mL, (Chromabond, Macherey-Nagel, Düren, Germany) were conditioned with 7.5 mL hexane before 500 µL of sample was applied. The NLs were eluted with 5 mL chloroform, then the FFAs with 5 mL diethyl ether:acetic acid (98:2 v/v), and lastly the PLs with 5 mL methanol. The eluates were transferred to culture tubes (Duran® 12 x 100 mm, Mainz, Germany) and evaporated under N_2 (g) at 40 °C.

2.4 Formation of FAMEs

For formation of FAMEs the NL and PL fractions were redissolved in 2 mL of heptane, before addition of 1.5 mL of 3.3 mg/mL sodium methoxide. The sodium methoxide solution was made by dissolving metallic sodium (purum, Merck, Darmstadt, Germany) in methanol to a concentration of 3.3 mg/mL. The culture tubes were then shaken horizontally for 30 min at 350 rpm (Biosan Ltd, PSU-10i, Riga, Latvia) and left to settle vertically for 10 min before the heptane phases were transferred to vials for storage at -20 °C prior to GC-MS analysis. The FFA fractions were redissolved in 1 mL BF_3-MeOH (14%, Sigma-Aldrich, St.Louis, MO, USA). The samples were heated for 5 min at 70 °C in a water bath. After heating, 1 mL heptane was added to each sample tube before mixing on a vortex mixer. The heptane phases were transferred to vials and stored at -20 °C prior to analysis by GC-MS.

2.5 Analysis of FAMEs by GC-MS

The analysis of the FAMEs were carried out on an Agilent 6890 Series gas chromatograph (GC; Agilent Technology, Wilmington, DE, USA) in combination with an Autospec Ultima mass spectrometer (MS; Micromass Ltd., Manchester, England) using an EI ion source. The GC was equipped with a CTC PAL Auto sampler (CTC Analytics, AG, Zwingen, Switzerland). Separation

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was carried out on a 60 m Restek column (Rtx®-2330) with 0.25 mm I.D. and a 0.2 µm film thickness of fused silica biscyanopropyl cyanopropylphenyl polysiloxane stationary phase (Restek Corporation, Bellefonte, PA, USA). For carrier gas Helium (99.99990%, from Yara, Rjukan, Norway) was used at 1 mL/min constant flow. The EI ion source was used in positive mode, producing 70 eV electrons at 250 °C. The MS was scanned in the range 40–600 *m/z* with 0.3 s scan time, 0.2 s inter scan delay, and 0.5 s cycle time. The transfer line temperature was set at 270 °C. The resolution was 1000.

A split ratio of 1:10 was used with injections of 1 µL sample. Two injections parallels were used for each sample replicate. Identification of fatty acids was performed by comparing retention times with standards as well as MS library searches. MassLynx version 4.0 (Waters, Milford, MA, USA) and NIST 08 Mass Spectral Library (Gaithersburg, MD, USA) was used. Relative response factors previously determined by Devle et al [2] were employed for quantitative determination. The resulting amounts are given in µg/g dry weight (DW). The GC oven had a start temperature of 65.0 °C, held for 3 min, the temperature was then raised to 150.0 °C (40.0 °C/min), held for 13 min, before being increased to 151.0 °C (2.0 °C/min) and held for 20 min, a slow increase to 230.0 °C (2.0 °C/min), held for 10 min, before a final increase to 240 °C (50 °C/min), the end temperature was held for 3.7 min.

4. Conclusions

A total of 42 different fatty acids are identified and quantified in the stipe and blade of *L. hyperborea*, with maximum two fatty acids having *trans* configuration. Some fatty acids are found either in stipe or blade, while others are only present in certain lipid fractions (NL, FFA, PL) within stipe and blade. Among the predominating fatty acids are the n-3 fatty acids ALA (10.4 and 131 µg/g DW), SDA (17.2 and 394 µg/g DW), and EPA (126 and 830 µg/g DW), as well as two n-6 fatty acids: LA (96 and 296 µg/g DW) and AA (444 and 723 µg/g DW), the values in parenthesis are for stipe and blade respectively. The ratios between n-6 and n-3 fatty acids are ≤ 4.4:1 in all

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lipid fractions, but especially low (≤ 1.3:1) in blade. Regarding the potential of commercialisation in respect of nutritional applications of *L. hyperborea*, blade is found to represent the most suitable material, due to higher levels of PUFAs, and a low n-6/n-3 ratio.

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Author Contributions: Hanne Devle, Yngve Stenstrøm, Carl Fredrik Naess-Andresen and Dag Ekeberg conceived and designed the experiments; Lena Foseid performed the experiments; we all participated in analysing the data; Lena Foseid wrote the paper and we other assisted and wrote also.

Conflicts of interest: There are no financial or commercial conflicts of interest.

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Table 1. Fatty acid content in the lipid fractions (average ± 1 SD, µg/g DW) of stipe and blade from *L. hyperborea* (n=4, two injection parallels for each sample replicate).

	Stipe			Blade		
Fatty acid	NL	FFA	PL	NL	FFA	PL
C7:0 5 methyl[a]	1.32 ± 0.09	n.d.	n.d.	0.49 ± 0.03	n.d.	n.d.
C8:0	1.5 ± 0.1	0.70 ± 0.04	n.d.	5.4 ± 0.6	0.65 ± 0.03	n.d.
C9:0	n.d.	0.41 ± 0.06	n.d.	n.d.	0.64 ± 0.03	n.d.
C10:0	0.76 ± 0.05	n.d.	n.d.	5.0 ± 0.6	0.39 ± 0.06	n.d.
C12:0	0.53 ± 0.09	1.7 ± 0.2	0.13 ± 0.01	0.73 ± 0.03	2.9 ± 0.1	0.09 ± 0.01
C13:0	n.d.	0.19 ± 0.01	n.d.	0.17 ± 0.01	0.96 ± 0.03	0.07 ± 0.01
C14:0	101 ± 2	21.9 ± 0.8	272 ± 10	224 ± 5	94 ± 4	312 ± 6
C14:0 13 methyl	n.d.	0.33 ± 0.05	n.d.	n.d.	0.39 ± 0.02	n.d.
C14:1 *trans* 9	n.d.	0.20 ± 0.02	n.d.	n.d.	1.9 ± 0.1	n.d.
C14:1 *cis* 9	0.89 ± 0.04	0.13 ± 0.02	0.27 ± 0.03	2.45 ± 0.03	0.46 ± 0.02	0.38 ± 0.01
C15:0	0.95 ± 0.05	1.00 ± 0.05	2.52 ± 0.03	8.7 ± 0.2	6.4 ± 0.2	10.4 ± 0.1
C16:0	163 ± 5	82 ± 3	277 ± 7	900 ± 18	299 ± 12	417 ± 5
C16:1[b]	n.d.	n.d.	n.d.	0.8 ± 0.1	1.31 ± 0.03	0.65 ± 0.02

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C16:1[b]		n.d.	0.53 ± 0.04	0.37 ± 0.05	2.24 ± 0.09	2.75 ± 0.09	1.05 ± 0.02
C16:1 *cis* 9		75 ± 3	14.4 ± 0.2	55 ± 1	180 ± 2	74 ± 2	105 ± 1
C17:0		0.32 ± 0.02	0.52 ± 0.06	0.57 ± 0.04	6.6 ± 0.2	1.73 ± 0.07	1.83 ± 0.05
C16:2 cis/*trans* 7,10[a]	(n-6)	0.62 ± 0.04	0.13 ± 0.02	0.49 ± 0.07	2.00 ± 0.07	1.72 ± 0.05	2.53 ± 0.05
C17:1 *cis* 9		0.33 ± 0.03	0.10 ± 0.04	0.35 ± 0.3	4.1 ± 0.2	2.02 ± 0.05	1.96 ± 0.06
C18:0		6.0 ± 0.3	17.3 ± 0.7	2.54 ± 0.09	84 ± 2	21.0 ± 0.7	4.42 ± 0.06
C18:1 *cis* 9		234 ± 6	47.5 ± 0.4	418 ± 10	1200 ± 29	220 ± 5	547 ± 12
C18:1 *cis* 11		3.4 ± 0.3	2.27 ± 0.04	4.8 ± 0.2	11.4 ± 0.21	6.6 ± 0.1	7.7 ± 0.1
C18:2 *cis* 9,12 (LA)	(n-6)	45 ± 2	2.70 ± 0.09	48 ± 1	184 ± 4	23.0 ± 0.7	89 ± 1
C18:3 *cis* 6,9,12	(n-6)	3.2 ± 0.2	n.d.	3.6 ± 0.1	14.0 ± 0.2	3.02 ± 0.06	12.3 ± 0.2
C20:0		6.7 ± 0.5	1.35 ± 0.07	1.5 ± 0.1	50 ± 1	9.8 ± 0.3	4.79 ± 0.09
C18:3 *cis* 9,12,15 (ALA)	(n-3)	7.3 ± 0.3	0.65 ± 0.03	2.4 ± 0.2	84 ± 1	22.0 ± 0.5	25.2 ± 0.7
C20:1 *cis* 9		n.d.	0.39 ± 0.08	3.3 ± 0.2	7.9 ± 0.4	4.17 ± 0.05	9.8 ± 0.2
C20:1 *cis* 11		n.d.	0.16 ± 0.04	0.39 ± 0.05	0.89 ± 0.05	0.39 ± 0.02	0.95 ± 0.03
C18:4 *cis* 6,9,12,15 (SDA)	(n-3)	5.6 ± 0.3	0.93 ± 0.03	10.7 ± 0.2	69 ± 1	65 ± 1	260 ± 3
C20:2 *cis* 11,14	(n-6)	3.8 ± 0.4	0.27 ± 0.05	4.9 ± 0.2	10.4 ± 0.3	2.9 ± 0.1	8.6 ± 0.2
C20:3[b]		1.9 ± 0.2	0.12 ± 0.02	0.90 ± 0.07	1.91 ± 0.05	0.50 ± 0.03	1.08 ± 0.03
C20:3[b]		1.5 ± 0.1	0.07 ± 0.01	1.04 ± 0.06	4.5 ± 0.6	0.72 ± 0.04	2.71 ± 0.05

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C20:3[b]		n.d.	0.04 ± 0.01	0.43 ± 0.03	1.2 ± 0.6	0.28 ± 0.02	0.79 ± 0.03
C20:3 *cis* 8,11,14	(n-6)	3.6 ± 0.3	0.16 ± 0.02	5.2 ± 0.2	18 ± 1	0.80 ± 0.02	4.46 ± 0.08
C22:0		n.d.	0.06 ± 0.01	n.d.	0.9 ± 0.1	0.41 ± 0.02	n.d.
C20:4 *cis* 5,8,11,14 (AA)	(n-6)	344 ± 11	17.4 ± 0.4	83 ± 1	517 ± 11	66 ± 2	140 ± 1
C20:4[b]		n.d.	n.d.	0.44 ± 0.04	2.8 ± 0.1	0.92 ± 0.04	3.21 ± 0.07
C20:4 *cis* 8,11,14,17	(n-3)	3.3 ± 0.3	n.d.	2.4 ± 0.1	13.5 ± 0.3	3.8 ± 0.2	10.1 ± 0.2
C20:5 *cis* 5,8,11,14,17 (EPA)	(n-3)	82 ± 3	2.91 ± 0.07	40.6 ± 0.9	409 ± 10	82 ± 2	339 ± 4
C24:0		n.d.	0.29 ± 0.02	n.d.	0.64 ± 0.08	1.45 ± 0.06	n.d.
C22:5 *cis* 7,10,13,16,19	(n-3)	3.0 ± 0.3	0.18 ± 0.01	1.56 ± 0.09	15.4 ± 0.5	5.0 ± 0.1	11.3 ± 0.3
C22:6 *cis* 4,7,10,13,16,19 (DHA)	(n-3)	n.d.	0.08 ± 0.01	0.33 ± 0.06	n.d.	1.67 ± 0.06	0.22 ± 0.02
C26:0		n.d.	0.27 ± 0.02	n.d.	n.d.	1.08 ± 0.07	n.d.

[a] Fatty acids are identified by NIST library search only

[b] Unknown isomer of fatty acid, identified by NIST library search only

n.d. = not detected. Abbreviations: NL, neutral lipid; FFA, free fatty acid; PL, polar lipid

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Table 2. Sum of SFAs, MUFAs, and PUFAs, as well as n-6 and n-3 in *L. hyperborea* given in µg/g DW, (n=4, two injection parallels for each sample replicate).

	Stipe			Blade		
	NL	FFA	PL	NL	FFA	PL
Σ SFA	282 ± 5	128 ± 3	556 ± 10	1287 ± 18	441 ± 12	751 ± 6
Σ MUFA	314 ± 6	65.8 ± 0.4	482 ± 10	1410 ± 29	314 ± 5	674 ± 12
Σ PUFA	505 ± 11	25.6 ± 0.4	206 ± 1	1347 ± 11	279 ± 2	911 ± 4
Σ n-3	101 ± 3	4.75 ± 0.07	58.0 ± 0.9	591± 10	179 ± 2	646 ± 4
Σ n-6	402 ± 11	20.7 ± 0.4	145 ± 1	745 ± 11	97± 2	257 ± 1
n-6/n-3 ratio	4.0	4.4	2.5	1.3	0.5	0.4

The standard deviations are the highest standard deviation among the summarized values. Abbreviations: NL, neutral lipid; FFA, free fatty acid; PL, polar lipid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.