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Review of Presence, Induction and Isolation of Major Cellular Constituents From *Porphyra Sensu Lato* (Rhodophyceae), Including Mycosporine-Like Amino Acids (MAA's)

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Abstract

Seaweeds from the genus *Porphyra* play a big economic role in seaweed aquaculture, mainly in Asia. In Europe, resources are put towards seaweed cultivation, but without attention to the *Porphyra* species which is also native to Europe. Different nomenclature and specifications are used to describe *Porphyra*, due to taxonomical reclassification and difficult phenotypical identification. Abiotic & biotic together with seasonal factors make for major variance in chemical compositions that are reported. This is also fueled by differences in chemical analytical methods and procedures followed. Combining taxonomical challenges, variance due to seasonal factors and differences in analysis, overviewing published research on *Porphyra* constituents such as protein, polysaccharides and fatty acids is warranted. Within this review, cellular consitutents found in *Porphyra* are discussed, including proteins, polysaccharides, fatty acids and mycosporine-like amino acids (MAA's). MAA's are considered amongst the strongest UV-photoprotectants found in nature and feature possible applications in cosmetics. As global interest in seaweeds as food, feed and industrial resource is emerging, opportunities for *Porphyra* constituents is rising.

Keywords

Porphyra, Bangiales, Polysaccharides, Proteins, Fatty Acids, Seasonal Variations, UV-photoprotectants, Red Seaweed

Introduction

General introduction

Red macroalgae belonging to the genus *Porphyra*, (class Bangiophyceae, order Bangiales, and family Bangiaceae [1,2]), commonly referred to as Nori or Laver, are economically important seaweed species used in aquaculture for food, feed and fine ingredients [3,4]. *Porphyra* is therefore researched for its primary metabolites and its fine chemicals from secondary metabolism [5].

The differentiation of bladed Bangiales species is problematic due to their simple morphology and low morphological variation within and between species [6,7]. Currently, both morphologic identification and genetic identification are used. With increasing availability and accessibility of genomic identification methods, genetic identification is likely to become the main identification method [8-11].

In 2011 Sutherland, et al. published a revision of the order of Bangiales, [12] resulting in a new identification, based on a two-gene phylogeny. This revision was needed due to increasing confusion and difficulty in *Porphyra* taxonomy [2,13]. The Sutherland, et al. (2011) review has resulted in a smaller pool of *Porphyra* species, making literature published before 2011 where taxonomical identification is based on older classifications, less reliable for comparison of species. The genus *Porphyra* encompasses, based on the revision of Sutherland, et al., the following species: *P. purpurea, P. dioica, P. linearis, P. lucasii, P. mumfordii* and *Porphyra umbilicalis*. Due to mixed terminology, the terms *Porphyra sensu lato* and *Porphyra sensu stricto* are often used. *Porphyra sensu lato* encompasses species that are closely related to *Porphyra* or until shortly belonged to the genus *Porphyra* such as *Pyropia yezoensis*, where *Porphyra sensu stricto* only applies to species currently belonging to the genus *Porphyra* [2,14-17].

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In our review *Porphyra sensu lato* is reviewed as subject, due to the ambiguity of recently renamed and revised *Porphyra* species. For this review, we used the specie names that were used by referenced papers, resulting in the usage of both *Pyropia (Py.) yezoensis* and *Porphyra (P.) yezoensis*.

In Asia the use of bladed Bangiales (*Porphyra sensu lato*) and primarily *Py. yezoensis* is integrated for generations. Currently, *Porphyra* is mainly being cultivated in China, Japan and Korea where seaweeds are staple food [18]. The aquaculture of *Py. yezoensis* species has an estimated worth of about US $7.48 billion in 2017 [15]. Annual Chinese production was estimated at just over 100.000 metric ton of dried *Porphyra*, in 2015 [19]. Information on Chinese aquaculture and research towards *Porphyra sensu lato* is becoming increasingly available internationally [4,15,20]. Nowadays outside of Asia, thus also in Europe, interest in cultivation and usage of red macroalgae is increasing.

In Europe *Porphyra* is naturally found along the shores of the Atlantic Ocean [21-23], ranging from Portugal to Norway, in the upper parts of the intertidal zone. Europe had a total seaweed aquaculture production of 287.033 tons in 2019 of which 11.125 tons were from seaweed cultivation. No *Porphyra* was produced outside of Asia according to the Food and Agriculture Organization of the United Nations (FAO) data [24]. According to the FAO, there are opportunities for European production and European processing technologies of seaweeds, including red seaweeds [25]. Research towards cultivation and cultivation applications of seaweeds is increasing globally, also acknowledging the opportunities of red seaweeds [26-31].

Physological characteristics of *Porphyra* due to abiotic factors such as temperature, light intensity, depth and nutrient concentrations has been researched extensively [11,32-36]. Together with its importance in aquaculture, this has led to strain characterization and breeding programs [37,38]. *Porphyra* has been seen as model organism for red seaweeds and genetic research has been undertaken, including genomic sequencing [16,39-44].

Porphyra has been a part of human food for thousands of years, mainly in Asia [15,45]. However *Porphyra* is becoming increasingly popular globally for its health beneficial applications as nutraceutical and food additive [46-48]. *Porphyra* has a primary metabolite content consisting of, amongst others, high protein levels with a high concentration of essential amino acids. Due to this favorable protein fraction, interest towards food and feed applications is rising. In both applications, *Porphyra* has the benefit of being a saltwater crop, with low nutrient and no terrestrial land usage, when compared with current agricultural protein supplies. In feeding trials in aquaculture, *Porphyra* protein showed promising results when compared to current often-used protein sources [49-54]. *Porphyra's* lipid fraction, although being a small metabolite fraction, is rich in the polyunsaturated fatty acid eicosapentaenoic acid (EPA), which is found in fish oil and has beneficial effects on cardiovascular activity [55-57].

Furthermore, *Porphyra* has considerable concentrations of health-beneficial secondary metabolites such as antioxidants, vitamins and inorganic elements [45,58,59]. One such secondary metabolite that is of great interest are the mycosporine-like amino acids (MAA's), because of their UV-absorbing capacity, probably the highest absorption known in nature [60,61]. Besides that, MAA's are also of interest for having other cell beneficial capacities such as cell proliferation and renewal next to UV-absorbing capabilities [62-69].

In this review we provide an overview of the reported constituents and fine chemicals found in *Porphyra* species sensu lato, focusing on presence, induction and isolation of *Porphyra* constituents, with special attention for the MAA's (Figure 1).

Porphyra Constituents

Porphyra constituents have been a topic for research, for their multiple application in for instance feed, food and cosmetics [14,45,51,52,62,70-72]. The composition of seaweed constituents in general is influenced by seasonal/ environmental and abiotic factors [54,73,74]. Variation in isolation and analysis methods are also accountable for differences in concentrations. An overview of constituents in *Porphyra*, based on our literature review, will be discussed and an approximation is given in Figure 2.

Figure 1: *Porphyra* as found in the Oosterschelde, Zeeland, The Netherland. Photo by Jesse van Groenigen.

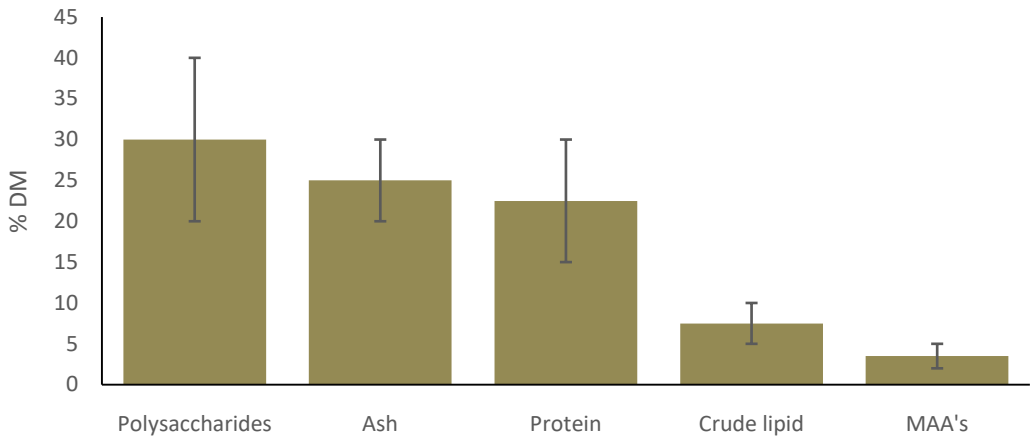

Figure 2: Average metabolite/constituent composition of *Porphyra* (percentage of dry weight), based on literature mentioned in this review.

Table 1: Protein contents (TAA total amino acid, % DW) in *Porphyra* species.

Species	% DW	Analysis	Season& site	Refs
P. dioica	24.2	TAA	Oct 2014 Norway	[79]
P. purpurea	15.9	TAA	Oct 2014 Norway	[79]
P. umbilicalis	17.7	TAA	Oct 2014 Norway	[79]
P. acanthophora	18.6	TAA	Jun-Sep 1998 Brazil	[80]
P. umbilicalis	31.8	TAA	Apr 2016 Sweden	[81]
P. vietnamensis	16.5	Lowry	Feb 2002 Hawai'i	[108]
Porphyra sp.	24.8	Kjehldahl (× 6.25)	Jan 2007 São Miguel	[78]
Porphyra sp	24.1	Kjehldahl (× 6.25)	Aug 2001 Portugal	[109]
P. dioica	9.4 -21.52	Kjehldahl (× 5.0)	Jul '14-'15 Ireland	[91]
Porphyra spp	32.7	Kjehldahl (× 6.25)	Australia	[110]
Py. haitanensis	29.4	Kjehldahl (× 6.25)	China	[88]

Proteins and amino acids

Presence: Protein contents between 10-35% DW are most commonly reported for *Porphyra*, with huge variations found caused by amongst, seasonal variabilities, differences in extractions and choices in analytical techniques [9,75]. Due to these high protein contents, *Porphyra* has potential in food and feed applications [50,52,76]. The essential amino acid concentration of total amino acids of *Porphyra* can be similar to fish meal and therefore can be used as a feed additive [28,49,77]. Total protein content of *Porphyra* is shown in Table 1 and amino acid compositions are shown in Table 2.

Essential amino acid composed 57% of the total protein content for *Porphyra sp*, with a total protein content of 24% DW in January 2007 at the São Miguel Island, in the Azores Archipelago from the littoral zone [78]. Total amino acid content in *P. dioica, P. purpurea* and *P. umbilicalis* of respectively 24.2, 15.9 and 17.7 g/100g DW was found, in samples from October 2014 in Northern Norway [79]. For *P. acanthophora* a total amino acid 18.6 g/100g DW was reported, in samples from June and September 1998 from around Cabo Frio, Brazil [80]. For *P. umbilicalis,* cultivated in at the Sven Loven Center for Marine Infrastructure at Tjärnö, Sweden and supplied with filtered seawater, a total amino acid content of 31.8 g/100g DW was recorded [81].

Peptides from *Porphyra* have multiple bioactive characteristics, including antioxidant, anti-inflammatory and anticoagulant activity [82-84]. *P. dioica* protein hydrolysate showed high antioxidant activity of Tyr-Leu-Val-Ala peptide chains, which is found in the phycobiliprotein C-phycocyanin. Antioxidant activity was also found in peptide chains from the phycobiliproteins C-allophycocyanin and β-phycoerythrin [85]. A potent and novel anticoagulant peptide was isolated from processed nori sheets of *Py. yezoensis*, which is stable at room temperature and non-cytotoxic [86]. Peptides from respectively *P. haitanensis* and *Py. haitanensis* showed anti-proliferating activity on human [87,88]. Enzymatically hydrolysed peptides from *Porphyra spp*. showed inhibition of α-amylase enzyme, thus lowering blood glucose levels [89].

Table 2: Amino acid composition (mg/g protein) of *Porphyra* shown in comparison to soy meal and the amino acid requirement as stated by WHO/FAO/UNU [111].

Amino Acid	*P. yezoensis* [112]	*P. umbilicalis* [113]	*P. acanthophora* [80]	*Porphyra sp* [78]	Soy meal [101]	Daily Amino Acid requirement of an Adult [111]
Histidine (His)	5.4	10.6	32	2.33	2.9	15
Isoleucine (Ile)	11.5	42.5	44	8.2	3.7	30
Leucine (Leu)	41.9	86	86	18.45	9.7	59
Lysine (Lys)	53.9	60	67	8.26	6.6	45
Methionine (Met)	8.3	11.5	12	2.05	0.6	16
Cystine						6
Methionine + Cystine						22
Phenylalanine + tyrosine (Phe) + (Tyr)	49.8	79.8	75	8	10.4	30
Threonine						
Tryptophan	37.3	63.5	62	11.21	4.4	23
Valine				-		6

Proteomics analysis identified 30 proteins from *P. haitanensis* with antioxidant activity [90].

Induction: Protein content in *P. umbilicalis* blades is affected by light intensity and duration of light exposure. *P. umbilicalis* showed the highest protein levels at 30 µmol photons m^{-2} s^{-1} and decreasing with increasing lighting levels. Structural protein levels were observed to be highest under an 8:16 light:dark regime [11]. Seasonal variations show that protein levels of *P. dioica* more than double in winter, to 21.52% DW [91]. It was published that *Porphyra* protein levels from samples collected in New Zealand were alleviated during winter when maximal growth is reached [75]. Tissue nitrogen content of *P. umbilicalis* was 3.89% DW and 6.76% DW when cultivated for two weeks at respectively 25 µM and 250 µM ammonium at 10 °C [33]. When *P. haitanensis* was UV-B induced for one hour on two consecutive days, protein content peaked when induced at 0.5 W m^{-2} UV-B and total amino acid content peaked at 0.5-1.0 W m^{-2} UV-B [92]. This is shown in Table 1.

Analysis: In recent years, numerous publications have addressed the different direct and indirect analysis methods for determination of protein concentration, such as Kjeldahl, Lowry and total amino acid analysis (TAA) [79,93,94]. With the Kjeldahl method, samples are digested with sulfuric acid (H_2SO_4) and a catalyst in order to form ammonium (NH_4). By acid distillation ammonium (NH_4) is converted to ammonia (NH_3), forming ammonium salts with a standard acid, which is titrated. Nitrogen concentration is then determined by acid-base titration [78-95]. Nowadays, this process is fully automated, and nitrogen to protein conversion factors (NPCF) are used to determine protein content [96]. Meta studies, analysing other research papers, found that different analysis methods are still being used throughout different fields of expertise, showing that nitrogen quantification with a nitrogen to protein conversion factor (NPCF) of 6.25 was the most commonly used method between 2009 and 2015 [97]. For, *P. dioica, P. purpurea* and *P. umbilicalis* NPCF's of respectively, 4.15, 4.69 and 3.92 were found for samples collected in October 2014 in Norwegian waters [79]. An universal seaweed n-protein factor of 5 was proposed to be used when accurate data was not available [97].

With total amino acid analysis, the crude protein fraction is hydrolysed under acidic conditions after which all amino acids are quantified separately using liquid chromatography, with their sum representing the total protein fraction. This can be done by hydrophilic interaction LC-MS [98], by derivatization for fluorescence using o-phthal-dialdehyde (OPA) or other pre-column reagents [99,100] or by ion exchange chromatography [80,93]. The downside of total amino acid analysis is the relatively high cost compared to indirect spectrophotometric analyses. It should also be noted that during acid hydrolysis, tryptophan, is destroyed completely and methionine is destroyed partially [78,80,101,102]. The sulfur-containing amino acids cysteine and its dimer cystine and methionine require additional derivation after acid hydrolysis before analysis is possible and are therefore sometimes omitted in amino acid analysis [103]. Overall, in spite of higher practical and financial costs, determination of total protein content summing up all individual amino acids is deemed to be the most accurate representation of total protein content.

The Lowry and Bradfort analyses are both spectrophotometric indirect biuret protein analyses. The Lowry method uses copper sulfate to form cuprous peptide complexes in combination with Folin-Ciocalteu reagents (phosphomolybdic and phosphotungstic acid) to cause interaction between cuprous compounds and the amino acids tyrosine, tryptophan, and cysteine [104]. The Bradford method relies on the interaction of the protein with Coomassie Brilliant Blue G-250 colourant [105]. Under acidic conditions, usually the addition of phosphoric acid, protonated Coomassie Brilliant Blue reacts primarily with arginine and to lesser extend with amino acids with positively charged side chains and aromatic side chains [93,98,104,106,105]. It was

shown that the often used indirect protein analysis methods such Bradfort and Lowry (and Kjeldahl) can show differences in protein content of up to 30% or higher in underling comparison and in comparison to direct total amino acid analysis. Another downside is the usage of the animal protein BSA as standard, therefore partly missing plant proteins and unsoluble proteins. Upsides for these indirect protein analyses are their low cost, quickness and easiness.

Conclusion and recommendations: Protein, peptide and amino acid analyses in *Porphyra* are hampered by biological and analytical hurdles. The species used, growth conditions (light/nutrient availability), and season have major impact on the concentrations and composition of proteins, peptides and amino acids. Also, there are multiple analytical issues in accurate determination of the protein, peptides and amino acid concentration in *Porphyra*. This is problematic, as reliable protein analyses are needed to evaluate macroalgae as protein source [81,93,97,107-113]. Multiple differently analysed protein contents for *Porphyra* are shown in Table 1. attention should be given to which determination method is used. Throughout a multitude of analysis methods, sampling strategies and *Porphyra* species a protein concentration between 9-33% DW is shown.

It is recommended in future research to always state sampling strategy, including date, environment and season. Furthermore, protein determination based on total amino acid analysis is recommended for accurate results. Research focusing on the effect of abiotic factors on amino acid composition is recommended to increase viability for use as feed and food. Clearly there is need for standardisation of analytical methods as well as the availability of reference materials. Bioactivity of protein fractions should be taken into consideration when determining protein usage in food and feed applications.

Polysaccharides

Presence & bioactivity: Polysaccharides make up a large part, 20-40% of the total dry weight mass of *Porphyra*, commonly found in the extracellular matrix and cell structures [114]. *Porphyra* dry weight consists for 65% w/w of cell wall material, comprising mostly fibrillar cellulose, glycoproteins and sulphated galactans as phycocolloids [76]. Algal polysaccharides, including those from *Porphyra* are only partly digestible by the human digestion system. Undigested polysaccharides are deemed qualitative dietary fibres in food and feed applications, and hence deemed important for a proper functioning of the human digestive track. Often, the soluble carbohydrates and the dietary fibres are quantified since they are of interest for their application in food, cosmetics and pharmaceuticals [115] or for their antioxidant properties [116]. For the Hawaiian seaweed *P. vietnamensis* 30.5 g/100g DW of soluble carbohydrates was reported [108]. For *Porphyra sp.* from the Azores a soluble carbohydrate analysis of 25.37 g/100g DW was reported [78]. This in contrast to soluble polysaccharide concentrations of 3% DW found in *Py. Yezoensis* after hot water extraction [117]. This is shown in Table 3.

In *Porphyra* specifically, the water soluble dietary fibre phycocolloid porphyran is gathering attention. Porphyran generally consists of 3-linked β-D-galactose and 4-linked α-L-galactose 6-sulfate, with some partial modification in different subunits, as shown in Figure 3 [118,119]. Porphyran can encompasses more than 40% of the dry weight of *Porphyran* [14]. Porphyran is being researched for possibly being health beneficial such as its anti-inflammatory and antioxidant capacity [120,121,122]. A research in rats showed that porpyrans are capable of moderate neurological motor function improvement [123].

Induction: Seasonal variation influences the total carbohydrate concentration, varying between respectively 39.4 and 47.2 g/100g DW between December and February for Korean *P. yezoensis* samples where total dietary fibre ranged between 27.2-34.9 g/100g DW, insoluble dietary fiber concentrations ranged between 18.5-26.9 g/100g DW and soluble dietary fiber ranged 4.9-8.4 g/100g DW [112]. The total carbohydrate content of *P. dioica* gathered in Galway was found at 57.48% DW in July and at 26.21% DW in December, although no analysis method was described [91]. For *P. capensis* no clear seasonal influence in total sugar content was found, due to high variability in sugar concentrations [124]. For Bangiophyceae it was suggested that low molecular weight carbohydrate concentrations and compositions are species dependant and might be identified based on patterns in their low molecular carbohydrate content [125].

Analytical methods: An often-used analytical method for soluble carbohydrates is phenolic sulfuric acid colorimetry [78-108], first described by Dubois in 1956 [126]. In this assay, samples are hydrolysed under acidic conditions, often using

Table 3: Polysaccharide content (%DW) in *Porphyra sensu lato*.

Species	Content % DW	Analysis	Reference
P. vietnamensis	30.5	SC Dubois	[108]
Porphyra sp.	25.37	SC Dubois	[78]
Py. yezoensis	39.4-47.2	Residual weight	[112]
P. haitanesis	19.9	CP formaldehyde	[130]
Py. yezoensis	3	SP Hot water	[117]
P. haitanensis	3.6	SP Microwave-assisted extraction	[127]
P. haitanensis	10.53	SP hot water	[116]

SC: Soluble Carbohydrate, CP: Crude Polysaccharide, SP: Soluble Polysaccharide

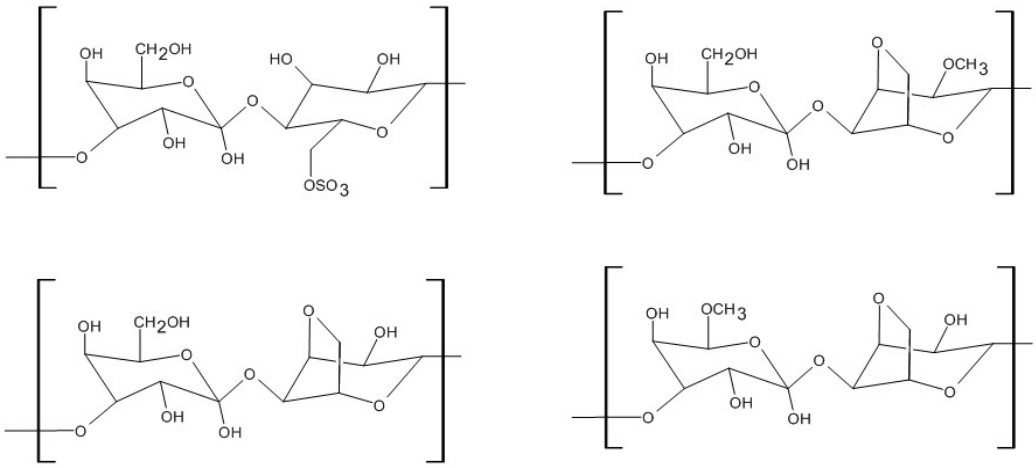

Figure 3: Chemical structure of the 4 porphyran subunits. Topleft: 3-linked β-D-galactose and 4-linked α-L-galactose 6-sulfate, **others: modifications.**

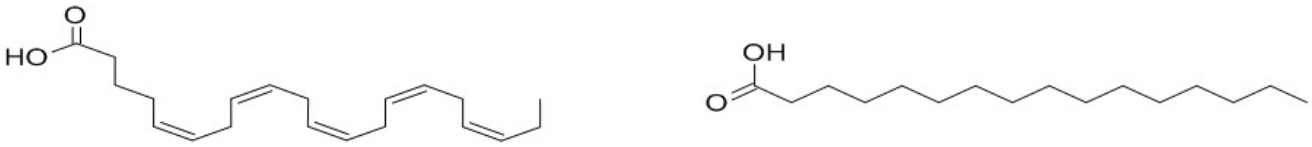

Figure 4: Left C20:5ω3 eicosapentaenoic acid (EPA), Right:C16:0 hexadecanoic acid (palmitic acid).

sulfuric acid. Then, under presence of phenol and sulfuric acid, spectrophotometric analysis at 490 nm is compared to a glucose standard. This method is applicable to free sugars, methylated sugars and oligo-and poly-saccharides. Another often-used technique is indirect calculation of carbohydrate quantification by determining other proximate substituents (protein, ash, fatty acids and residual water) and stating the residual weight as the carbohydrate fraction [112]. However, it has to be mentioned that this indirect approach is prone to inaccuracies based by the analysis of other constituents and the inclusion of unidentified and unquantified constituents in the residual weight. Polysaccharide extraction was optimized for hot water extraction, finding an optimum temperature of 80 °C, solid:liquid ratio of 1:20 and an extraction time of 2h. This resulted in a yield of 3% for *Py. yezoensis* [117]. Microwave assisted extraction of polysaccharide from *P. haitanensis* showed a maximum yield 3.6% [127]. Extraction methods based on water at elevated temperatures show lower yield of soluble polysaccharides. For monomeric sugar composition analysis, either directly extracted or after depolymerization, Gas Liquid Chromatography (GLC) [128] or High-Performance Anion Exchange Chromatography (HPAEC) are often chosen. For oligosaccharides and polysaccharide linkage composition analysis Gas Chromatography Mass Spectrometry (GC-MS). For obtaining detailed oligosaccharide and polysaccharide profiles and compositions, Matrix Assisted Laser-induced Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MSn) methods can be used. These methods are highly precise, but are also costly and time-consuming. Furthermore, sample preparation is more complicated, exemplified by degradation of sugars during alkaline and/or acid depolymerization influencing the saccharide composition and functional groups. Another analytical method for primary hydrocolloid identification, such as porphyran, is Fourier Transformed Infrared spectroscopy (FTIR). Using FTIR analysis, sun-dried and ground macroalgae amongst others can be qualitatively analysed to identify their primary hydrocolloid [129].

Fatty acids

Presence and bioactivity: Fatty acids from marine environment, most notably omega 3 fatty acids such as C20:5ω3 eicosapentaenoic acid (EPA) and C22:6ω3 docosahexaenoic acid (DHA) as shown in Figure 4, are often cited as health beneficial. Fatty acids are researched for their affectability as anti-inflammatories, antioxidants and cardio-vascular enhancers [131]. Fatty acids are also being researched as alternative lipid source for fuels [132]. The predominant fatty acids in *Porphyra* are the omega-3 fatty acid C20:5ω3 eicosapentaenoic acid and the saturated fatty acid C16:0 hexadecanoic acid (palmitic acid). The dominant fatty acid for *Py. yezoensis* is EPA, comprising more than 50% of the total fatty acid content during all seasons [112]. Palmitic acid was reported as the most present fatty acid at 46.5-57.6% of total lipid content in commercially bought dried *P. tenera* [133]. It should be noted that eicosapentaenoic acid was not identified. Work on the lipidome for *Porphyra* in different life stages is ongoing, highlighting the potential for *Porphyra* as possible source of health beneficial fatty acids [134].

The crude lipid content was determined 4.4 g/100g DW in *P. vietnamensis* [108]. A crude lipid content of 8.88 g./100g

Table 4: Crude lipid content in multiple *Porphyra sensu lato* species, determined gravimetrically.

Species	Content % DW	reference
P. vietnamensis	4.4	[108]
Porphyra sp.	8.88	[78]
Porphyra sp	1.03	[109]
Py. yezoensis	0.7-1.1	[112]
P. tenera	0.93- 1.61	[133]
P. dioica	0.86	[134]

DW was found in *Porphyra* sp, where it must be noted that a 4 hour Soxhlet reflux was used to increase lipid yield, instead of the often used solid-solvent maceration [78]. For *Porphyra sp.* a total lipid content of 1.03 g/100g DW is reported using chloroform:methanol extraction [109]. An overview of crude lipid content is shown in Table 4.

Induction: The effect of abiotic factors on the lipid composition in *Porphyra* is not well-researched. Induction trials with abiotic factors to optimize fatty acid production in red seaweeds have not been published to the knowledge of the authors. Within research towards the seasonal variability of *Porphyra* composition, lipids are more often analysed. *P. dioica* was sampled in June and Nov 2021 from Galway Bay, western Ireland and showed respectively 0.8 ± 0.2% DW and 1.7 ± 0.5% DW of total fatty acid. The percentage PUFA within the total fatty acid was in both seasons around 45% [135]. Crude lipid content for *P. yezoensis* was found to be between 0.7 ± 0.2 and 1.1 ± 0.2 during a full growth season from November 2011 to March 2012 in Korea. PUFA compositions were found between 59.6% and 64.6% of the total fatty acid composition [112].

Analytical methods: Lipid extraction is often done using an hydrophobic chloroform:methanol extraction solvent. For quantitative crude lipid analysis, crude lipid quantities are often determined gravimetrically, by evaporation of solvent and weighting. It must be noted that photoactive molecules such as chlorophyll are also extracted efficiently in organic liquids such as chloroform and methanol [136] and can comprise towards 0.5 g/100g DW [137]. Therefore, spectrophotometric analysis for chlorophylls may be used on crude lipid extracts, in order to partly account for additional untargeted compounds [138]. Quantitative fatty acid profiling is usually performed using LC-MS [139] or via GC-MS after transmethylation for FAME analysis (Fatty Acid Methyl Ester) [140,141], identifying and quantifying individual fatty acids.

Pigments and aromatic constituents

Presence: Carotenoids are class of pigment compounds found in photosynthetic bodies of *Porphyra*, extracted as part of the crude lipid content. The order of Bangiales, including both *Porphyra* and *Pyropia* is described as belonging to the Lutein group, as lutein is often the most found abundant carotenoid, although variances between species occur [142]. *P. perforate* was shown to have a carotenoid signature of 35% lutein, 11.4% zeaxanthin, 4.1% α-carotene and 49.5% β-carotene based on total carotenoid content. This composition did not stroke with the classification as lutein dominant. For *P. suborbiculata* a carotenoid composition of 65% lutein and 35% zeaxanthin was reported [142]. In *Py. yezoensis* also lutein, zeaxanthin, α-carotene, and β-carotene were described as the major carotenoids, with Lutein being predominant [143]. After a 45 day laboratory cultivation of three strains of *Py. haitanensis*, chlorophyll α concentrations between 7.68 ± 0.14 - 9.37 ± 0.20 mg.g^{-1} DW, phycoerythrin concentrations between 40.80 ± 1.00 - 51.00 ± 2.28 mg.g^{-1} DW and phycocyanin concentrations between 31.26 ± 0.80 - 48.36 ± 0.81 mg g^{-1} DW were found, being an example of interstrain variation that can occur under identical growth conditions [144].

Induction: For *P. yezoensis* it was shown that the chlorophyll α concentration was higher in March than in January for samples harvested in Nantong, China for two of three tested strains. However, it must be noted that interstrain variation was also significant, with the third tested strain showing lower chlorophyll α levels in March [137]. For the phycobiliprotein phycoerythrin in *P. dioica* it was reported that concentrations in winter were higher than in summer in Brittany, France [10]. For the phycobiliproteins phycoerythrin, allophycocyanin and phycocyanin higher concentrations were found in January in relation to March across 3 strains of *P. yezoensis* with concentrations in March being between 6-8 mg.g^{-1} FW for all three phycobiliproteins [137]. For *P. linnearis* and *P. umbililcalis* respectively 29 mg g^{-1} FW and 26 mg g^{-1} FW of phycoerythrin were reported, with highest concentrations at 100 °C and 250 µmoles L^{-1} ammonium in a laboratory setting [33]. Photosynthetic pigment content is higher in shaded blades in comparison to sun exposed blades for *P. umbilicalis* with antioxidant capacity increasing most in summer [145]. For *porphyra* and *Pyropia* species it was shown from meta-analysis that xanthophyll concentrations are mostly enhanced under hypersaline conditions and under increased lighting conditions. It was suggested that pre-harvesting 24 hour induction with PAR radiation at 100 µmol photons m^{-1} s^{-1} and a 12:12 light:dark cycle would result in optimal xanthophyll levels for *Porphyra* and *Pyropia* species, with *Pyropia* species showing higher total carotenoid content and *Porphyra* showing higher lutein and zeaxanthin concentrations [146].

Analysis: The analysis of chlorophylls and carotenoids is often performed using photo spectroscopic techniques, such as UV/Vis spectroscophy for rapid scanning and high performance liquid chromatography for component separation, identification and quantification [143,147]. Techniques around the analysis of carotenoids and chlorophylls are mostly based on their light-absorbing property, as they are natural pigments. For identification of breakdown components and elucidation of decay pathways, mass spectroscopy is an often-used technique, with LC-MS-MS being the preferred method and advances are made in the elucidation of carotenoid metabolomics [148].

Inorganic constituents, minerals and moisture

Table 5: Proximate ash content for *Porphyra*.

Species	Content % DW	Method (T,t)	location	reference
Porphyra sp	28.16	550 °C, 2-3 h	Jan 2007 São Miguel Island	[78]
P. vietnamensis	25.2	550 °C, 4 h	Feb 2002 Hawai'i	[108]
Py. yezoensis	7.25-3.76	500 °C, 5 h	Jan-Apr South Korea	[151]
P. columbina	6.46	525 °C, 5 h	Aug-Oct 2010 Argentina	[152]
Porphyra sp	19.07	525 °C, 5 h	Aug 2001 Portugal	[109]
Porphyra spp	10.3	525 °C, 5 h	China	[77]
P. yezoensis	9.3	525 °C, 5 h	South Korea	[154]

content presence

Inorganic material, macro- and micro-minerals and metals are often grouped together under ash content in proximate compositions and can encompass up to 20-30% DW. Macroalgae, including *Porphyra* are being researched as nutraceuticals and food for their favourable mineral content [141,45,149]. Macroalgae can also take up heavy metals, but concentrations are very species dependent. Heavy metal concentrations from macroalgae, including *Porphyra*, collected in Norway in October 2014 showed the presence of heavy metals, but concentrations mostly remained below concentrations for EU regulations in food or feed [150].

Porphyra sp. was incinerated at 550 °C for 2-3h and showed an ash content of 28.16% DW [78]. For *P. vietnamensis* an ash content of 25.2 g/100g DW was found after heating for 4h at 550 °C [108]. An ash content between 7.25 and 3.76 g/100g DW for *Py. yezoensis* after heating for 5h at 500 °C [151]. Following AOAC guidelines, an ash content of 6.46 g/100g DW was found for *P. columbina* [152]. For *Porphyra sp.* an ash concentration of 19.07 g/100g DW was found after heating for 5 h in an electric oven at 525 °C [109].

Induction: There is seasonal variation in ash content of *Porphyra spp.* over a two year time period, ash contents between 12.0 and 18.7 g/100g DW were found for multiple different species [153]. These seasonal differences were also shown for mineral content in *Porphyra*, collected between January and April on multiple sites of the South Korean coast [151]. Ecological conditions, such as urban proximity, also influence mineral concentrations in macroalgae [74,154].

Analytical methods: Ash content is often determined gravimetrically after incineration of dried material at temperatures of 500 °C or higher for multiple hours. For mineral composition determination, samples are often acid digested and analysed via atomic absorption spectroscopy (AAS) [152] or via Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) [151]. Alternatively, ion chromatography coupled to conductivity analysis can be used on aqueous extracts [78]. Macroalgal samples should be rinsed with freshwater before drying, to cleanse samples from adherent seawater.

Moisture content: Moisture content of *Porphyra* is often gravimetrically determined. Using oven drying at 600C, McDermid reported for *P. vietnamensis* a water content of 90.3 g/100g FW [108]. Paiva [78] reported a water content of 87.1 g/100g FW for *Porphyra sp.* Mok [154] reported a moisture content for *Py. yezoensis* of 89.2 g/100g FW (Table 5 and Table 6).

Mycosporine-Like Amino Acids

Special attention in this review is given to the Mycosporine-like amino acids (MAA's), as this group of cellular constituents may have future broad applications as UV-photoprotectant in cosmetics. Mycosporine-like amino acids are currently a topic of research and application and prototype development using MAA's extracts are starting to be published.

Chemical properties

Mycosporine-like amino acids (MAA's) are founds in a wide variety of different organisms, ranging from marine macroalgae, to freshwater microalgae to terrestrial microalgae [155,156,157,158,159] as well as fungi, marine invertebrates and even fish [160,161,162]. The primary function of MAA's is to protect the organism from the harmful effects caused by UV irradiation which are amongst others photosynthesis inhibition, DNA damage and protein denaturation [65]. Over 30 MAA's and MAA's derivatives are identified and their chemical properties researched [163]. For *Porphyra*, the most predominantly found MAA's are porphyra-334, shinorine and palythine [164,165], where other MAA's such as asterina are sometimes found [34].

MAA's typically have a molecular weight below 400 Da and consist of cyclohexenone or cycloheximine backbone with a conjugated amino acid [5]. MAA's exhibit molar extinction coefficients up to 50.000 M^{-1} cm^{-1} with absorption maxima between 309-362 nm [60], with a reported molar extinction efficient for respectively Porphyra-334 at 42,300 M^{-1} cm^{-1} in [166] and for Shinorine at 44,700 M^{-1} cm^{-1} [163] in aqueous solutions at 334 nm. MAA's are colourless and soluble in both aqueous and hydrophilic organic solvents.

MAA's have shown to be resistant to heat stress. A mycosporine-like amino acid extract of the red seaweed species *Gracilaria cornea* consisting mostly of porphyra-334 and/or shinorine showed no significant decrease in absorption after heat treatment at 75 °C for six hours [191]. Porphyra-334 has been reported to be stable for over 80 days at room temperature. When treated at 60 °C, porphyra-334 slowly decreased in absorbance in solution at pH 1-11 over a 25 hour timeframe. At a temperature of 80 °C this decrease was more rapid. At pH 12-13, porphyra-334 showed rapidly

Table 6: Mineral Composition of *Porphyra* in mg/100 g, (Concentrations are all converted to mg/100g for comparison purposes.

Na	K	Ca	Mg	P	Fe	Mn	Cu	Zn	Co	Se	I	Ni	Cr	Analysis	Species	Location	Source
330.31	2242.34	169.07	141.03	450.99	15.25	1.88	0.58	2.89	0.005			0.029	0.05	ICP-AES	*Py. yezoensis*	Jindo, S-Korea	[151]
460.85	604.60	226.39	186.79	596.65	30.06	2.63	0.77	4.06	0.059			0.089	0.097				
414.22	1444.17	443.7	491.53	379.9	22		0.51	1.46						AAS		Argentina	[152]
348.75	1395	525	261.75		12.28	2.26	1.38	2.79						AAS	*Porphyraspp*	China	[77]
655.8	1277.4	369.4	403.3	629.7	14.522	3.818	0.379	6.353				0.015		ICP-AES	*P. yezoensis*	Korea	[154]
781.1	2802	151.4	420.3	820.1	18					20.4	310.8			ICP-AES	*P. tenera*	Korea and China	[155]
199.2	2734	460.6	612	885.4	70.05					12.6	240.7			ICP-AES	*P. haitanensis*	Korea and China	[155]
	3235	217.5	578.5		5.325	1.145	0.326	9.85	0.008	0.2		0.1		HR-ICP-MS	*Porphyra sp.*	São Miguel Island	[74]
	3970	290	780	250	15.4	4.1	0.7	1.1						Ind. Lab	*P. vietnamen*	Hawai'i	[108]

declining absorbance over a 4 hour period, also at room temperature [169].

A selection of MAA's showed antioxidant activity [163]. Mycosporine-glycine had the highest antioxidant activity, followed by asterina-330 and palythine [166], while as Porphyra-334 and shinorine showed scarce scavenging activity [167]. Abiotic environmental characteristics play a major role in bioactivity of MAA's. Porphyra-334, shinorine and palytine were reported to be more active than synthetic phenolic antioxidants in Folin-Ciocalteu assay's, while being less active than these standard compounds in the radical scavenging ABTS bioassay and Ferric Reducing Antioxidant Power (FRAP) assay [168]. This while all of these assays have the same mode of electron transfer. Since Folin-Ciocalteu assay is done at pH around 8 and ABTS and FRAP assay's are done at acidic pH, this would mean that MAA's bioactivity is higher in slightly alkaline conditions compared to acidic conditions [168]. As with heat stability, antioxidant activity is heavily pH reliable, often showing higher bioactivity at slightly alkaline pH [169]. An overview of the most common MAA's and their structures is shown in Table 7.

Presence and biosynthesis of mycosporine-like amino acids

Mycosporine-like amino acids are deemed to be synthesized following the shikimate pathway [5], present in both in eukaryotic as prokaryotic organisms. It is opted that mycosporine-glycine is synthesised from a gadusol precursor, then being chemically converted via deoxygadusol into other mycosporine-like amino acids to cover a wide range of UV-radiation, as shown in Figure 5 [170]. The Shikimate pathway is not found in animals, thus accumulation of MAA 's in these organisms is supposedly done by ingestion of MAA's from algal diets [171]. An overview of often found MAA's is given in Table 8, showing variations in presence of MAA's in *Porphyra*.

Induction of mycosporine-like amino acids

Induction of MAA's biosynthesis may be achieved by induction with PAR radiation and/or UV irradiation [172-176]. This seems to be species dependent, with publications either observing or not observing induction. The fact that species belonging to the genus *Porphyra* are commonly found in the intertidal area, makes it logical that these organism have (elevated) UV reducing compounds such as MAA's. There are also seasonal variations within MAA's content and concentration in *Porphyra*, including *P. dioica* [177], although this was not found for *P. dioica* sampled in January and July on the coast of Brittany [10]. As discussed by [177], more research is needed to elucidate interactive effects of PAR and UV, depth, salinity, temperature and nutrients on MAA's accumulation. There was no linear combination for the independent variables light and temperature that explains MAA's accumulation in *P. dioica*. It is to be expected to exposure to UVA or UVB would increase MAA concentrations/ composition. It was shown that 300 µM ammonium increased MAA's accumulation in *P. leucostica* and *P. umbilicalis*, but 0 and 100 µM ammonium only lowered MAA's content in *P. leucostica* [34], possibly indicating nitrogen-regulated

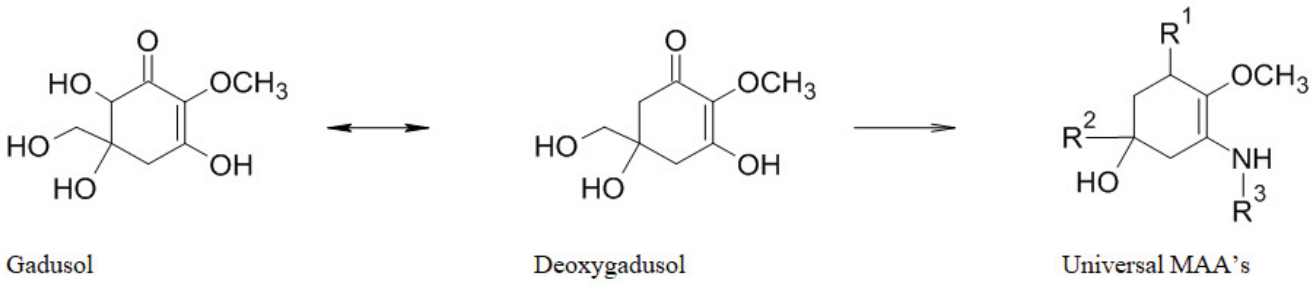

Figure 5: Biosynthesis of MAA's from a gadusol precursor.

Table 8: MAA's commonly found in *Porphyra* species and an initial MAA's concentration, when given. Concentrations are converted to total MAA's (mg g^{-1} DW).

Species	Reported MAA	Total MAA's (mg g^{-1} DW)	Reference
P. leucosticta	Shinorine, Palythine, Porphyra-334, Asterina-330	6.99 ± 1.62	[34]
P. endiviifolium	Shinorine, Porphyra-334, Palythine, Palythinol, Asterina-330	8.5-15.7*	[172]
P. plocamiestris	Shinorine, Porphyra-334, Palythine, Asterina-330	0.3-1.6*	[172]
P. columbina	Mycosporine-glycine, Shinorine, Porphyra-334, Palythine, Asterina-330	5-18*	[173]
P. columbina	Mycosporine-glycine, Shinorine, Porphyra-334, Palythine, Palythinol, Asterina-330	7.0-10.4*	[174]
P. umbilicalis	Shinorine, Palythine, Porphyra-334, Asterina-330	9.23 ± 1.40	[34]

*extracted from figures in the original publications.

induction. A division into three physiological algal groups was proposed, based on their MAA's accumulation [5]. This system details the three groups as containing:

- High initial MAA's concentrations that are not further enhanced through light/UV induction.
- Lower initial MAA's concentrations that can be enhanced by light/UV induction.
- Low initial MAA's concentrations that are not enhanced through light/UV induction.

Using this division, *P. endiviifolium* and *P. umbilicalis* were classified as having high initial MAA's content with no induction from a treatment with PAR light or UV radiation. *P. plocamiestris* was classified as having low initial MAA's content with induction during light/radiation treatment. This is in line with results found for *P. endiviifolium*, but is contradictory with results found for *P. plocamiestrisis*. MAA's in *Porphyra plocamiestrisis* were induced under UVA and UVB, while *Porphyra endiviifolium* shows induction under UVA with no additional benefit of UVB induction [172]. For *Porphyra umbilicalis* high initial MAA's concentrations were found, not further enhanced with UV induction, in line with the proposed physiological classification system [165]. Although research has been published towards the accumulation of MAA's in *Porphyra*, consensus has not been reached on the influence of UV-induction and other abiotic factors on the biosynthesis and enhanced accumulation of MAA's in red seaweeds.

Isolation and detection of MAA's

MAA's samples are usually aqueous solutions, often partly purified or crude extracts [10]. An often used method for extraction and purification of MAA's from macroalgal samples is extraction in 20% aqueous methanol at 45 °C for 2.5 hours. Then after airdrying, samples are redissolved in 100% methanol to remove methanol insoluble components sugars after which methanol is evaporated and MAA's are redissolved in water ready for analysis [173,178,179]. A second option is extracting in 0.2% aqueous acetic acid with 0.5% methanol at 4 °C for 12 hours on a shaker after which samples are centrifuged and filtered [180]. This method is specifically designed as a universal crude method from both prokaryotic and eukaryotic samples.

A third extraction method, often used on microbial biomass [181] is overnight maceration in methanol at 4 °C. Then the extract is centrifuged and methanol is evaporated to dryness at 45 °C. afterwards, extracts are redissolved in water, adding a drop of chloroform to remove apolar organic impurities such as chlorophyll before centrifugation and filtration [182].

Other less often-used purification methods are also reported, such as purification by solid-phase extraction [183] or preparative thin layer chromatography [184].

HPLC analysis of MAA's is commonly done using a C18 reversed phase column. The mobile phase is mostly an isocratic aqueous solution with 1-0.02% acetic acid. One research group [185,186] describes an isocratic aqueous liquid phase consisting of 0.1% acetic acid and 25% methanol. Commonly, these analyses have a run time between 15-30 minutes and show a high retention. Detection is done spectrophotometrically in the 280-360 nm range using Diode array detection (DAD). Since pure MAA's standards

are not readily available, peak identification is often done by comparison of retention time, retention pattern and absorption maxima of known MAA's published in older publications. Due to the same reason quantification is often done by using approximate molar extinction coefficients, instead of a more accurate methods like an internal standard or pure compounds as external standard. One must be aware that this can become inaccurate, especially in samples with volatile and complex MAA's distribution profiles [172,187].

A quantitative analysis using capillary electrophoresis as separation with diode-array detection is reported [188]. Here, the liquid phase consists of a 30 mM aqueous sodium tetraborate solution at pH 10.3 Separation voltage, temperature and detection wavelength were respectively 25kV, 25 °C and 320 nm. The stationary phase consisted of fused silica capillary with 75 µm internal diameter and 80 cm effective length. This method is validated for quantitative determination of MAA's in, amongst others, *P. umbilicalis* in a run time of approximately 30 minutes.

Commercial usage of MAA's

MAA's are currently being offered as UV-active macroalgal extract for cosmetics such as HelioGuard 365 from Mibelle Biochemistry [189] and Helionori from Gelyma [189]. It must be mentioned that MAA's are currently not allowed as primary active UV-filter in sunscreens, but are only allowed as secondary UV-filter by the European Chemicals Agency (ECHA) and the American Food & Drug Administration (FDA). However, the Environmental Effects Assessment Panel (EEAP) have showed their concerns about the potential hazards caused by common used UV-filters and has mentioned MAA's as possible solution [190]. Research has been done to MAA's, but more focus on quantification would be beneficial as absolute concentrations are often unknown. A rise in availability of analytical standards would promote quantitative analysis. Further research could focus more on induction and applicability of MAA's, rather than comparisons of MAA's between different species.

Conclusion

When reviewing the presence & bioactivity, induction and isolation of *Porphyra* constituents, the broad possibilities and applications of *Porphyra* and its constituents are obvious.

Although advances are made in *Porphyra* taxonomy, identification of samples from the field keeps being problematic until genetic identification is more readily available and affordable. Differences in taxonomic approach does complicate the interpretation of published scientific literature. This complication should be thought of when reviewing published knowledge. However, with the rise of genomic identification, these issues will phase out over time.

Publications about the chemical composition of *Porphyra* are readily available. Major differences in reported concentrations of constituents have been showed in this review. In order to gain understanding of these variations, abiotic & biotic and seasonal factors should be researched and clarified. For example, individual differences in chemical compositions between *Porphyra* specimen should be connected to their specific growth conditions. Mapping the influence of external factors on chemical compositions will, over time, favourably impact cultivation and usages of *Porphyra*. Due to these major differences in chemical composition, research towards local growth and chemical composition is incentivized in order to increase our understanding of external factors on *Porphyra* constituents.

The reliability, accuracy and preciseness of analytical procedures are widely varying. It is advised to consider the robustness of the analytical technique, in the context of the research. For example, when determining protein content by performing a Kjeldahl-based technique, a Protein Conversion Factor should be determined instead of usage of the universal standard of 6,25 which is often not applicable to *Porphyra*. The preciseness or robustness of the conclusion that is given, should correspond to the robustness and preciseness by the analytical technique that is being applied. Use of standard reference material should be encouraged.

A prime example of advances in application of *Porphyra* constituents is the application and research towards Mycosporine-like amino acids as photo-protectant in sun creams and cosmetics. These advances have led to consideration of MAA's as novel UV-blocker. With all effort towards *Porphyra* constituents, more product applications are to follow in time.

Porphyra is an economic important species, mostly in Asia. Due to global interest in *Porphyra*, both as food, feed and as nutraceutical, a substantial increase in *Porphyra* cultivation is expected. However, especially in Europe, there is still a development gap between knowledge of and industrial utilization of *Porphyra*. The transition from scientific research towards economically and technically feasible industrial applications has yet to be made. Taking into consideration the forecasted growth of *Porphyra* industry in Asia and the abundancy of wild *Porphyra* in Europe, opportunities are presenting itself. It is advantageous from an economic point of view to aim for full biomass valorisation. Few process flow diagrams for total *Porphyra* valorisation are being published [190]. Due to this insight total biorefinery of *Porphyra* biomass and side streams is starting to become a bigger research point. This can lead to economical gain of *Porphyra* as prime aquaculture crop, also outside of Asia.

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