The nutritional aspects of biorefined *Saccharina latissima, Ascophylum nodosum* and *Palmaria palmata*

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Abstract The chemical profile of biorefined *Saccharina latissima, Ascophylum nodosum* and *Palmaria palmata* after carbohydrate and polyphenol extraction was analysed with the aim to evaluate the nutritional aspects of biorefined seaweeds as a novel animal feed supplement. Optimised enzymatic saccharification has been used to show that the protein concentration in the residue of *Palmaria palmata* and *Ascophylum nodosum* can be increased by more than two fold. Nutritional value of the residue was further enhanced through an increase in total amino acids and fatty acids. As a consequence of removal of inorganic elements such as sodium, potassium and chloride, the total solid and ash content of all three seaweeds was reduced by around 40%. In contrast, divalent metals such as iron and zinc, as well as silicon accumulated in all three residues. Potentially harmful components such as arsenic and iodine were reduced only in brown biorefined seaweeds, whilst in biorefined *P. palmata* iodine increased by 39% compared to a 24% decline of arsenic. Polyphenol removal in all three seaweeds was >80% and, in combination with enzymatic saccharification, enhanced protein recovery in *A. nodosum*. This highlights the potential of biorefinery concepts to generate multiple products from seaweed such as extracts enriched in polyphenols and carbohydrates and residue with higher protein and lipid content.

1 Introduction

The world's livestock sector is growing at an unprecedented rate as an ever-growing human population, with rising incomes and a trend towards urbanization, will need more high-value animal protein to maintain present lifestyle choices and standards [1]. As a consequence the growth in demand for animal and non-animal derived food products will inevitably lead to a rise in requirement for raw materials, particularly those rich in carbohydrates, lipids and proteins for feed or food production. At the same time, food producers are not only experiencing greater competition for land, water, and energy, but also ethical demands on sustainability and food quality and safety. This all adds pressure on how food is grown, stored, processed and distributed [2, 3]. As well as constant outbreaks of infectious animal diseases and food borne bacterial infections, it is predicted that there will be a growing global need for more plant proteins [3]. It is estimated that 85% of plant proteins are wasted in the production of animal protein through the inefficiency of some livestock to fully utilise all plant proteins [3].

Proteins are commonly incorporated within a complex structural matrix and bound to carbohydrates and polyphenolic compounds amongst others [4], and these interactions are believed to be one of the factors that impairs protein digestibility [5-7]. It has been found that removal of such non-protein fractions from biomass through enzymatic pre-treatment [8] could be an alternative way to limit the influence of these compounds as anti-nutritional factors [6] and therefore enhance biodigestibility of proteins.

Seaweeds, amongst other aquatic plants and alga, are being considered as sustainable protein resources, as off-shore aquaculture does not compete for fertilisers, fresh water and land usage [9, 10]. So far seaweeds have been largely harvested for human consumption, followed by exploitation of their phycolloid content and their use as an animal feed supplement [11]. In addition, the high protein content of 20%-50% in some members of the orders *Palmariales* and *Bangiales* [6] and accumulation of more than 15% lipids in some members of the order *Dictyotales* [12, 13] underlines the increasing potential for some seaweeds for future food and feed production. Numerous algal bioactive compounds such as polyphenols, vitamins, sterols, pigments and halogenated compounds can be found in seaweeds [14]; in addition their potential to improve animal health through reduction of pathogens and stimulation of the immune system [7], improve food quality through pigmentation [15, 16], preservation of food oils through lipophilic antioxidants [17] and possible increase in shelf life of foods [18] has already been discussed.

Seaweed meals, commonly derived from members of the order *Fucales*, make-up only a small percentage of animal feed composition (<2 % of dry matter intake) and, are mainly used because of their potent prebiotic activity and their high level of micronutrients [19]. Algal biomass, especially brown seaweeds, have demonstrated to concentrate potentially harmful elements such as iodine and arsenic [20-22], which will reduce the likelihood of higher additions of seaweed meals in animal feed make-up.

Biorefinery concepts have been discussed as part of moving from an oil-based society into a bio-based society using biomass instead of fossil fuels [23]. As enzymatic saccharification of seaweeds has been largely explored for biofuel production [24, 25], little attention has been paid to the residue after carbohydrate extraction and its potential applications, especially as animal fodders. In this context we discuss enzymatic saccharification as a biorefinery tool to extract carbohydrates from algal biomass and investigate the effect on the nutritional and harmful aspects of biorefined seaweeds. This present research focuses on the application of a biorefinery approach to the seaweeds *Saccharina latissima*, *Ascophylum nodosum* and *Palmaria palmata*. These species were chosen because of their different characteristics: - *S. latissima* as a potential candidate for mass cultivation in North Western Europe to produce biomass, *A. nodosum* as a source of polyphenols and *P. palmata* due to its high protein content. Enzymatic saccharification has already shown to enhance nutritional aspects of seaweed residues [26] and was therefore chosen as the preferred method to remove algal carbohydrates and increase the nutritional content such as protein and lipids of all three biorefined seaweeds. Incorporated with a solvent extraction to remove the polyphenol content in *A. nodosum* before enzymatic saccharification, this combined biorefinery approach was also investigated for its suitability to enhance nutritional aspects. The chemical composition analyses before and after enzymatic treatment, including elemental analysis, ash, carbohydrates, proteins, amino acids, fatty acids and polyphenols, are discussed and the nutritional and biochemical aspects of biorefined seaweeds as a novel animal feed supplement evaluated.

2 Experimental

2.1 Sample preparation

Macroalgae samples were collected from Strangford Lough, County Down. Around 2 kg portions of *A. nodosum* (Linnaeus) Le Jolis and *S. latissima* (Linnaeus) J.V. Lamouroux were collected in February 2015 and November 2014, respectively. *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr was collected in February 2015 from Garron Point, County Antrim. Seaweeds were macerated in an industrial type meat mincer (Buffalo CD400), divided into ~500 g portions and frozen at -20 °C. Frozen seaweed portions were freeze dried on demand over 5 days until dry (Christ Alpha 2-4LD) and ground to a fine powder (<0.5 mm) using a coffee grinder (Moulinex AR1044).

2.2 Seaweed biorefining

2.2.1 Solvent extraction

The polyphenol content of freeze-dried *A. nodosum* was extracted before enzymatic saccharification using acidified aqueous acetonitrile. The total amount of seaweed was divided between nine 250 ml flasks, with each containing 30 g of freeze dried seaweed powder. Each flask was extracted 3 x times for 1 hour at 25 °C and 400 rpm on an orbital 1" throw benchtop shaker (SciQuip Incu-Shake Midi) using 50 parts of acetonitrile, 49.8 parts of deionised water and 0.2 parts of formic acid. The first extraction was carried out using 100 ml of solvent, while, for the second and third extraction, 50 ml of solvent was used. Seaweed extracts were centrifuged for 10 minutes at 3,200 *g* and supernatants from each extract combined and kept at -20 °C for polyphenol analysis. Solids were freeze dried until dry and used for subsequent enzymatic saccharification.

2.2.2 Enzymatic saccharification

Enzymatic saccharification was carried out in 250 ml flasks, shaken at 500 rpm for 45 hours at 45 °C (SciQuip Incu-Shake Midi). The freeze-dried seaweeds *S. latissima, A. nodosum* and *P. palmata* were divided into 9 equal portions each and suspended in 0.02 M sulphuric acid to concentrations of 111, 100 and 200 g l^{-1}, respectively. A lower solid concentration for both brown seaweeds was chosen, as high broth viscosity using brown seaweeds prevented a higher solid loading. The pH was adjusted to be within the range 3.5-5.5 as recommended by the enzyme manufacturer Novozyme. To each flask a 5% (w w^{-1}) addition of Viscozymes L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase) was made. In addition, 1-3 units of alginate lyase (Sigma Alldrich, Cat.No. A1603) was added to flasks containing *A. nodosum* and *S. latissima*. After enzymatic treatment samples were centrifuged for 10 minutes at 3,200 *g*. Solids from three random samples of each species were combined and freeze dried resulting in a total of three samples per seaweed species.

2.2.3 Enzymatic saccharification optimisation studies

Small scale trials were carried out in 2 ml microcentrifuge tubes to investigate the impact of pH alteration during enzymatic saccharification on protein enrichment in biorefined seaweed (solids after enzymatic saccharification). As poor mixing characteristics of the seaweed suspensions were observed from saccharification studies with high loading rates, lower concentrations of all three seaweeds (~67 g l^{-1}) were used in this optimisation study to reduce broth viscosity and enhance mixing. A biorefinery approach involving solvent extraction prior to enzymatic saccharification was simulated on *A. nodosum* only, using the methods outlined above, where 1 ml of aqueous acetonitrile mix was used for each extraction step. Into 2 ml microcentrifuge tubes 100 ± 10 mg of freeze dried sample was mixed with 1.5 ml of 0.005 M to 0.03 M sulphuric acid solutions to achieve a suspension pH ranging between 3.1 to 6.2 (Table 6). After the addition of 5 % (w w^{-1}) Novozyme L, tubes were placed horizontally in a shaker and incubated for 22 hours at 45 °C and 250 rpm. After centrifugation for 5 minutes at 10,625 *g* all supernatants were discarded and the solids freeze dried and kept for protein analysis.

2.3 Ash, total solids and metal analysis

Ash and total solids were determined according to methods described by Sluiter [27]. Values from total solid analysis at 105 °C were used to correct sample weights to their dry matter content.

The metal composition in unrefined and biorefined freeze dried seaweed samples was measured using an energy dispersive X-ray fluorescence (XRF) -spectrometer (Rigaku NEX CG). Helium purging was used to enable measurement of lighter elements. Samples were filled into 32 mm double open ended sample cups and compressed at 350 psi to a depth of > 8 mm. Data were analysed (i.e. elemental quantification and peak fitting routines) using RPF-SQX software.

Certified standard reference materials (NMIJ CRM 7405-a -and NIM-GBW10023) were used to determine reproducibility and accuracy of measurements.

Determination of total solids was used to adjust concentrations of all measured components as a percentage of total solids. Removal rates for ash, individual elements, sugars, alginate, total carbon/hydrogen/nitrogen/sulphur (CHNS) and polyphenols were calculated from the total mass of individual components in unrefined and biorefined seaweeds according to Equation 1. The total mass of components in unrefined and biorefined seaweeds were calculated by multiplying the concentration of each component with the total solid content (Table 2).

Equation 1: Calculation of removal rates for ash, elements, sugars, alginate, CHN and polyphenols

$$Mass\ removal\ (\%) = \left(1 - \frac{Total\ mass\ in\ unrefined\ (g) - Total\ mass\ in\ biorefined\ (g)}{Total\ mass\ in\ unrefined\ (g)}\right) x\ 100\%$$

2.4 Protein analysis

Freeze-dried samples were pre-treated using trichloroacetic acid before alkaline extraction. Protein concentrations in alkaline extract were measured using a Lowry method [28] and expressed as a percentage of the total solid content.

2.5 Carbohydrate analysis

A two-stage acid hydrolysis was applied to hydrolyse structural algal polysaccharides [28] of all three seaweed species. The monosaccharides glucose, mannitol and fucose were identified from the hydrolysates using high performance liquid chromatography (HPLC) [29]. Separation of mannose, galactose and xylose was insufficient for identification and all three sugars are reported here as "Man/Gal/Xyl". Other unknown monosaccharides were detected, which fell within the retention times of glucose and fucose and calculated concentrations (based on the glucose calibration curve) were combined and reported as "others" (Table 7). Each monosaccharide was expressed as a percentage of the total solid content.

Alginate was extracted from the two brown species *A. nodosum* and *S. latissima* according to a modified method described by Haug et al. [30]. In a 2 ml microcentrifuge tube 100 ± 10 mg of freeze-dried sample was soaked in 1 ml of 2% (w v^{-1}) calcium chloride solution for 1 hour at room temperature (RT), in 1 ml of 5% (v v^{-1}) hydrochloric acid for 30 minutes at 40 °C followed by 1 ml of 37%-40% formaldehyde solution for 2 hours at RT. Between each stage, samples were centrifuged at 10,625 *g* for 2 minutes and centrates discarded. Before alkaline extraction cell pellets were washed with demineralised water to remove residual formaldehyde and the pre-treated cell pellets were extracted twice in 10 ml of 6% (w v^{-1}) sodium carbonate solution at 60 °C under shaking. The final cell pellets were washed with 5 ml of hot water (~80 °C) and all extracts for each sample combined. An equal volume of industrial grade ethanol was added to combined extracts and kept overnight at 4 °C. After centrifugation precipitates were dissolved in 10 ml of 0.5 M NaOH solution at 70 °C and pH adjusted to 2.0 using 1 M hydrochloric acid. Each final cell pellet was washed in 10 ml of acidified demineralised water (pH 2) to remove salts and dried to a constant weight at 75 °C in a convection oven. Extraction and analysis of alginate for each sample was carried out in triplicate and alginate content expressed as a percentage of the total solid content.

2.6 Total fatty acid analysis

Total fatty acid analysis was carried out from acid hydrolysed seaweed samples according to an adapted method by Murphy et al. [31]. Approximately 500 ± 50 mg of freeze dried sample was weighed into 40 ml screw capped (PTFE septum) glass tubes, 2 ml of a 50 mg ml^{-1} pyrogallol in ethanol solution, 1 ml of an 5 mg ml^{-1} internal standard (Triundecanoin in hexane) and 10 ml of 7.7 N hydrochloric acid added. The headspace was flushed with nitrogen gas and the tubes placed in a dry heater heated to 80 °C for 60 minutes, mixing the tubes every 10

minutes. After cooling to RT each hydrolysates was extracted twice with 5 ml of diethylether, followed by two extractions using chloroform. Organic phases were collected in 20 ml screw capped glass tubes and dried under nitrogen gas. Once dried, 2 ml of 12% BF_3 in methanol was added, followed by 1 ml of toluene. Tubes were sealed and placed in a dry heater block, set at 100 °C for 55 minutes. Every 10 minutes tubes were shaken gently. After cooling to room temperature 5 ml of water, 1 ml of toluene and 1 g of sodium sulphate were added and the contents shaken for 1 minute. Top layers were aspired and filtered through glass Pasteur pipettes, prefilled with anhydrous sodium sulphate, into 5 ml screw capped glass vials. Extraction of aqueous phases were repeated a further two-times with 1 ml of hexane for each extraction and final volumes were measured before analysis by gas chromatography mass spectroscopy (GC/MS). Fatty acids were quantified relative to peak areas of a FAME standard mix (Sigma Aldrich Cat.No. CRM47885). Each sample was extracted, derivatised and analysed three times and the total lipid content expressed as a percentage of the total of all detected FAME fatty acids to the total solid content.

2.7 Total carbon, hydrogen, nitrogen and sulphur (CHNS) analysis

The CHNS analysis was carried out in a Perkin – Elmer 2400 Series 2 CHNS Elemental Analyser X2 by combusting 2.0 ± 0.5 mg of freeze-dried sample at 1000 °C. Each was sample was analysed in triplicate. Evolving combustion gases were measured by a thermal conductivity detector (TCD) and results expressed as a percentage of the total solid content.

2.8 Polyphenol analysis

The polyphenol content of freeze-dried seaweeds was extracted using an acidified acetonitrile solution and measured using a Folin-Ciocalteu method [28]. Determination of polyphenols in each sample was carried out three times and results expressed as a percentage of the total solid content.

2.9 Amino acid analysis

Accurately weighed aliquots of freeze-dried seaweed samples were hydrolysed for 22 hours in evacuated glass tubes using 50% hydrochloric acid in water containing 0.1% phenol (w v^{-1}). Acid hydrolysates were diluted to appropriate levels in water using volumetric flasks and analysed as described by Henderson and Brooks [32] using 9-fluoroenyl-methyl chloroformate (FMOC) and *o*-phthalaldehyde (OPA) reagent for derivatisation of secondary and primary amino acids, respectively.

Analysis of the amino acids aspartic acid, glutamic acid, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, valine, phenylalanine, isoleucine, leucine, lysine and proline was carried out according to the conditions presented in Table 1 using an Agilent 1100 HPLC system equipped with an Agilent Zorbax Eclipse Plus C18, 4.6 mm x 150 mm x 3.5 µm column and fluorescence detection. A programmable wavelength switch from ex340 nm, em450 nm to ex266 nm, em 305nm was set at 16.2 minutes. Results for the amino acids methionine and cysteine were excluded from data analysis due to their instability during acid hydrolysis.

2.10 Statistical analysis

The mean content and experimental error was determined for triplicate assays of each sample and results expressed as percentage of the mean ± standard deviation of the total solid content.

Data was analysed using a two-way Analysis of Variance (ANOVA) to determine whether the group means of unrefined or biorefined seaweeds and between the three species were all equal or not.

Pearson moment correlation analysis was applied to determine the correlation of pH on protein enrichment in *P. palmata* during optimisation studies and between alginate and divalent metal ions which were found to be higher in biorefined *S. latissima* and *A. nodosum*.

3 Results and discussion

3.1 Ash and metal content

The ash, total solid and metal content in unrefined and biorefined *P. palmata*, *S. latissima* and *A. nodosum* were analysed and their distribution during biorefining discussed. Variations in ash content amongst the three species

($F(2,12)=674$, $p<0.0001$) and between unrefined and biorefined seaweed ($F(1,12)=1475$, $p<0.0001$) were identified. Highest loss of ash was found in *A. nodosum*, where 64% of inorganics from unrefined seaweed was extracted during biorefining, followed by *P. palmata* (60%) and *S. latissima* (55%) (Table 2). These findings were also confirmed using XRF metal analysis were 68% of the total metal content was removed during combined solvent extraction and enzymatic saccharification in *A. nodosum*, whilst 62% and 60% of the total mass of metal ions were removed in *P. palmata* and *S. latissima*, respectively. In *P. palmata* similar extraction yields (52%-58%) have been reported by Lahaye and Vigouroux [33] using deionised water as the solvent, whilst in the case of enzymatically treated *S. latissima* only 17% of ash was removed [34].

Elemental analysis of *P. palmata* has shown that the concentration of the elements Fe, Cu, Zn, Si and I increased after enzymatic saccharification by 34%, 21%, 30%, 4% and 39%, respectively (Table 4). In contrast, the concentration of the main elements Mg, Ca, Cl, K and Na declined between 30%-39% during biorefining (Table 4). The difference in total weights between unrefined and biorefined *P. palmata* showed that the elements Cl, K and Na made-up around 80% of all extracted ions, were Na accounted for 18%, Cl for 33% and K for 30% of the total (Table 3). Due to the application of sulphuric acid for pH adjustment, the sulphur content in all three biorefined seaweeds was found to be higher than in the unrefined seaweeds (Table 4).

In contrast, the number of elements which accumulated in biorefined *A. nodosum* were greater compared to those reported in *P. palmata*. The concentration of the elements Ca, Mn, Fe, Zn, Sr, Pb and Si were found to be 10%-56% higher in the biorefined residue, whilst the concentration of the elements As, Na, Cl, Br and I declined between 48%-82% during solvent and enzymatic extraction (Table 4). K, Na and Cl accounted for the biggest mass loss of elements, representing 33%, 37% and 16% of the total extracted ion content, respectively (Table 3).

In *S. latissima* the concentration of the elements Fe, Zn, Sr and Si were found to be between 10%-40% higher in the biorefined residue compared to unrefined seaweed. The concentration of the elements K, As, Na, Sn, Cl and I declined between 32%-45% during enzymatic saccharification. The elements Na, Cl and K were also found to be the major three ions removed during biorefining, accounting for 21%, 37% and 26% of the total mass loss of all elements, respectively (Table 3).

For all three seaweeds it was found that largely divalent cations accumulated in biorefined seaweeds. Within the cell wall matrix of brown and red algae, ionic polysaccharides such as alginates, fucoidans and galactans are embedded. These polysaccharides contain a variety of functional sites, such as carboxylic and/or sulfonic acid, which are largely responsible for metal binding affinities of these polymers [35, 36]. In the case of alginate, in particular its guluronic acid content, a strong affinity towards divalent cations such as Pb, Cu, Cd, Zn and Ca has been demonstrated [36]. The elements Fe, Zn and Si were found in higher concentrations in all three biorefined seaweeds. Pearson moment correlation analysis have shown that the elements Fe, Sr and Si in *S. latissima* were strongly correlated to alginate (Fe: $r(5)=0.60$, $p<0.0001$; Sr: $r(5)=0.68$, $p=0.0005$, Si: $r(5)=0.61$, $p<0.0001$), whilst in *A. nodosum* a significant correlation of Ca, Zn and Sr with alginate was identified (Ca: $r(5)=0.78$, $p<0.0001$; Zn: $r(5)=0.75$, $p=0.0003$; Sr: $r(5)=0.61$, $p<0.0001$).

Interestingly, iodine was found in higher concentrations in biorefined *P. palmata*, accounting for 77% of the total iodine of unrefined *P. palmata*. In comparison, only 14% of the total iodine of unrefined *A. nodosum* was retained in biorefined *A. nodosum* and 36% during biorefining in *S. latissima* (Table 3). Despite a 64% removal rate of iodine in biorefined *S. latissima*, residual iodine in this species (5326 mg kg^{-1}) remained well above that of *P. palmata* (1165 mg kg^{-1}) and *A. nodosum* (319 mg kg^{-1}) (Table 3 and Table 4). Higher iodine removal rates have been demonstrated in the case of *L. japonica*, where 99% of its iodine was removed during cooking [21]. Chemical species of iodine in seaweed can be found as I^-, IO_3^- and in organoiodine compounds. The composition of these iodine species varies in seaweeds, and as a result, so does the solubility of total iodine [37]. Higher iodate and organically bound iodine content was found in *Sargassum spp*. compared to *Laminaria spp*., and as a result solubility of total iodine in *Sargassum spp*. was only 40% compared to 99% for *Laminaria spp*. [38]. In this study it is therefore likely that the iodate and organic iodine content in *P. palmata* was higher than that of *A. nodosum* and *S. latissima*. Iodine is an essential element required by humans and animals and iodine deficiency amongst humans in land-locked countries is still a global problem [39]. As a consequence iodine in the form of seaweeds or other components is added to animal feeds to increase the iodine content in popular foods such as eggs, milk and meat. For example a typical poultry diet contains around 1-2 mg I kg^{-1} [40] and as a result of this study, were iodine varied between 500 – 5000 mg I kg^{-1} (Table 4), biorefined and unrefined

seaweeds can only be considered as a feed supplement rather than a major component. However, as iodine transfer from feed to food is different between animals [39] and humans [41], intoxication from excess intake of iodine has been reported not only in animals such as chicken, cattle and sheep [40, 42] but also in humans [43]. Whilst high iodine intake in Japan has been linked to increased health benefits [21] direct intake of iodine through seaweeds is not recommended due to the high variability of iodine in kelps and seaweed-based products [44].

Of the three species, highest arsenic content was found in *S. latissima*, followed by *A. nodosum* and *P. palmata* (Table 4). Biorefining resulted in arsenic removal rates of 57% in *P. palmata*, 64% in *S. latissima* and 72% in *A. nodosum* (Table 3). Arsenic compounds in seaweeds exist either as inorganic arsenic species such as As (III) and As (V) or as organically bound species such as arsenobetaine and arseno-sugars [45]. High variations in arsenic removal were also shown by Rose et al. [22], where 15%-87% of the total arsenic content was removed during soaking of *Sargassum fusiforme*, with the more toxic inorganic arsenic species accounting for 1%-11% of the total. High levels of arsenic in feeds might not be a problem to some aquatic animals such as fish, as they have the ability to convert the toxic inorganic forms to the less toxic organic forms [22]. However, excess intakes of heavy metals, such as arsenic, have been shown to accumulate in sheep's tissue, kidney, muscle and wool [46]. Considering the maximum allowance of 40 ppm arsenic in seaweed meals and feed materials derived from seaweed (Directive 2002/32/EC) [10], seaweeds containing higher arsenic levels, in particular more than 2 ppm of inorganic arsenic, might have limited applications for food and feed production, as terrestrial livestock might not be able to fully metabolise inorganic arsenic. Removal of carbohydrates through enzymatic saccharification might represent a suitable option, as initially postulated by Nielsen et al. [47], to reduce organically bound arsenic such as arseno-sugars and as a consequence enhance the use of seaweed residue as an animal feed additive.

The macronutrient phosphorus, which made-up between 0.11% and 0.46% of the dry matter of unrefined seaweeds (Table 4), is a key element in animal and human nutrition. It is involved in the maintenance of skeletal tissue, where around 80% of phosphorus is bound, in the regulation of osmotic pressure and acid base balance, energy utilisation and transfer via adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), protein and amino acid synthesis, transport of fatty acids and cell growth and differentiation [48]. During biorefining 41%, 43% and 45% of the total phosphorus of unrefined seaweed was retained in *P. palmata, A. nodosum* and *S. latissima,* respectively. As residual phosphorus levels in biorefined *P. palmata* and *S. latissima* remained similar to natural phosphorus levels found in some feed grains such as corn and barley [49], biorefined seaweeds still can be considered as a valuable animal feed supplement.

3.2 Protein content

Protein content was analysed in unrefined and biorefined seaweeds, as well as in seaweeds derived from saccharification optimisation studies. Whilst protein remained unchanged in *A. nodosum* during biorefining, protein declined by 25% in biorefined *S. latissima* and increased by 17% in *P. palmata* (Enzymatic or microbial pre-treatment of seaweed to remove polysaccharides and enhance protein digestibility has already been described elsewhere [57, 58] and could be an alternative way to enhance protein up-take and help in securing future protein demands.

) although this was not statistically significant ($F(1,12)=1.03$, $p=0.33$). In contrast, protein content varied significantly between the three seaweeds ($F(2,12)=278$, $p<0.0001$). Optimisation studies were carried out with the aim to increase the protein content during enzymatic saccharification using different pH and a lower solid loading rate. In addition, a combined solvent extraction method followed by enzymatic saccharification was also applied to *A. nodosum* to investigate the suitability of this combined biorefining method on protein recovery (Table 6).

Results from the optimisation studies have shown that higher protein contents in all three biorefined seaweeds can be achieved. In *P. palmata* and *A. nodosum* the relative protein content after enzymatic saccharification more than doubled, whilst in *S. latissima* residual proteins increased by 43% (Table 6). The impact of pH on residual protein was evident in the case of *P. palmata*, where highest protein yields were seen with increasing pH (Table 6). For the other two seaweeds only two different pH conditions were applied and therefore no conclusions regarding pH optimum can be made. However, in the case of biorefined *S. latissima* it was evident that protein yields increased below a pH of 6 (Table 6). Interestingly in *A. nodosum* highest enrichment of

protein (119%) was seen in combination with solvent extraction before enzymatic saccharification (Table 6). Through the removal of polyphenols, it is assumed that enzymatic saccharification was more effective. Polyphenols are known to inhibit digestive enzymes [50] and phenolic-rich extracts of seaweeds, in particular that of *A. nodosum*, express a high activity against α-glucosidase and α-amylase [51]. Selective removal of polyphenols prior to enzymatic treatment could therefore improve overall process economics through the generation of an additional revenue stream from polyphenols, and reduce the requirement of costly enzymes as a consequence of reducing enzyme inhibitive polyphenols. Highest enrichment of protein using low pH (~3-4) was also seen in *S. latissima*, while in *P. palmata* the trend reversed and highest protein yields were seen with a pH of around 6 (Table 6). A change in colour of *P. palmata* extracts was noticeable, where the intensity of pink colour of the extracts through the release of water-soluble protein-pigments (R-phycoerythrins) declined with pH (data not shown). There are two possible reasons for this: either a reduction in protein extraction efficiency leading to a possible accumulation of protein-pigments in biorefined *P. palmata* or an instability of those protein-pigments at lower pH. As the total protein content in biorefined *P. palmata* was positively correlated to pH with protein content increasing with pH (r=0.81, p<0.001) (Table 6), it is most likely that the stability of some water-soluble proteins were negatively affected by low pH. This pH dependency of R-phycoerythrin stability has been shown by Liu et al. [52], where a rapid decline of UV-absorbances and fluorescence of aqueous extracts of *P. urceolata* in response to structural protein changes were measured at pH <3.5 and >10. Accumulation of these highly valuable protein-pigments in biorefined seaweeds prior to application of classic extraction methods would be highly desirable, as it would decrease economic costs of obtaining R-phycoerythrins.

As the optimisation studies were also carried out with lower solid loadings of 7% w v^{-1} resulting in less viscous solutions, reduced mixing in solutions with higher loading rates of 11%-20% w v^{-1} as per initial biorefining studies, might have been the limiting factor in achieving sufficient exposure of enzymes with the solid substrate. In a similar study using 13% w v^{-1} of the seaweeds *Ulva lactuca*, *Alaria esculenta* and *S. latissima* enrichment of protein (+41%) was only achieved in the green seaweed *U. lactuca* [34]. From these studies it appears that beside pH also solid loading rates are important parameters to consider to fully optimise protein recovery rates during enzymatic saccharification.

Protein is a key factor in animal and human nutrition. The protein content of seaweed has been investigated as an alternative to animal-derived protein sources, as seaweed proteins are not only high in essential amino acids (~50%) but their protein profile is similar to that of egg protein [53]. Protein contents in red and green seaweeds are considered highest, reaching levels of up to 47%, followed by brown seaweeds [6]. It is mainly the high-protein containing red seaweeds such as *Porphyra* and *Palmaria* spp. which are of industrial interest as a supplement or replacement of animal-derived proteins. *Palmaria* and *Porphyra spp.* have been successfully trialled in mariculturing of salmon [54], sea urchins [55] and trout [16] and a partial substitution of fish meal with seaweed proteins was generally well received by fish. Different responses from seaweed feeding trials indicate that not all aquatic species can assimilate high protein plant-based diets or accumulate dietary pigments successfully [16, 55]. As some seaweed proteins are bound to cell wall polysaccharides [4], the level of digestibility of these proteins seems to be related to the amount and type of soluble fibre present [56], or the adaptation of the gut rumen flora to digest such fibres. Enzymatic or microbial pre-treatment of seaweed to remove polysaccharides and enhance protein digestibility has already been described elsewhere [57, 58] and could be an alternative way to enhance protein up-take and help in securing future protein demands.

3.3 Carbohydrate content

The carbohydrate content, including alginate and monosaccharides from unrefined and biorefined seaweeds was determined and discussed below. In biorefined *A. nodosum* the total monosaccharide content was 8% higher, whist in *S. latissima* it remained unchanged and in *P. palmata* the total monosaccharide content declined by 12% (Table 7), although variances in total monosaccharide content between unrefined and biorefined seaweeds were not of statistical significance (F(1,12)=0.13, p=0.72). In the case of combined carbohydrates (alginate and total monosaccharides) the difference between biorefined and unrefined seaweeds was significant (F(1,12)=13.89, p=0.003), largely due to increases in alginate in biorefined *A. nodosum* (33%) and *S. latissima* (58%) (Table 8).

However, when determining the total mass of monosaccharides, highest removal of glucose, mannitol and fucose were seen in *A. nodosum* where a two-stage extraction process was applied (solvent and enzymes)

compared to a one-stage process (enzymes) for *P. palmata* and *S. latissima* (Table 7). Despite 13%-37% of glucose being liberated during biorefining, glucose concentrations accumulated in all three biorefined seaweeds (Table 7). It is likely that during enzymatic saccharification the concentration of water insoluble glucans such as cellulose increased due to the removal of solubilised components. Highest mannitol removal was seen in *A. nodosum*, where mannitol was undetectable after biorefining, whilst in *S. latissima* mannitol extraction yields were 67% (Table 7). In brown seaweeds the monosaccharide fucose is a major constituent of the structural cell wall component fucoidan [59, 60]. In this study it made-up 0.6 and 0.9% of the biomass in *A. nodosum* and *S. latissima*, respectively (Table 7).

For sugars associated to either galactose, mannose or xylose, highest concentrations and removal rates were recorded in *P. palmata* (Table 7). As xylans in *P. palmata* can make-up ~35% of the dry matter [61] it is assumed that the reported concentration in this study is likely to be attributed towards xylose. In total 55% of the total xylose content of unrefined *P. palmata* was extracted during enzymatic saccharification, compared to 10% in *A. nodosum* and 30% in *S. lati*ssima (Table 7). Other unknown sugars were highest in *A. nodosum* accounting for 12% of the biomass, followed by 7% in *S. latissima* and only 2% in *P. palmata* (Table 7).

The structural carbohydrate alginate was found to make-up approximately 15% of total solids of both unrefined brown seaweeds (Table 8). During saccharification 5% and 27% alginate was removed in *S. latissima* and *A. nodosum*, respectively. As the pH and temperature optima for alginate lyase and the enzyme cocktail Viscozyme L were different, optimum conditions for Viscozyme L were applied, as cellulases and hemicellulases represented the main digestive enzymes in this study. This process applied a higher temperature and a lower pH than the recommended 37 °C and pH 6.3 for alginate lyase. As a consequence of low alginate removal rates, alginate in biorefined *A. nodosum* increased by 33% and in *S. latissima* by 58% (Table 8).

The importance of soluble algal carbohydrates such as laminarin, fucoidan and alginate in human and animal nutrition has been attributed to their contribution as dietary fibres and their bioactivity [7, 62]. However, algal carbohydrates also have the ability to impact the bioavailability of proteins through formation of carbohydrate-protein complexes. As these complexes reduce the accessibility of proteolytic enzymes, gut microbiota not adapted to such polysaccharides lack bacteria to produce necessary carbohydrate-active enzymes to break down such algal cell wall structures [63]. It is therefore likely, that the biodigestibility of algal proteins is reduced. In contrast, adaptation of gut microbiota in nature to break-down such carbohydrates has been demonstrated in seaweed eating Japanese people [64] and sheep on the Scottish island of North Rondaldsay [65]. However, the direct impact of gut microbiota adaptation on protein biodigestibility still remains unkown.

3.4 Total carbon, hydrogen, nitrogen and sulphur (CHNS) content

The total content of carbon, hydrogen, nitrogen and sulphur of unrefined and biorefined seaweeds was analysed. In *P. palmata* and *S. latissima* the sulphur content was below the detection limit (0.5%) but constituted 1.1% of the total solids of *A. nodosum* (Table 9). Fucoidans, which were found in higher concentrations in *A. nodosum* are the likely cause for the higher sulphur content due to their high degree of sulphate-ester linkages [59]. The sulphur content was excluded from calculation of elemental ratios due to the application of sulphuric acid for pH adjustment during biorefining. The ratio of carbon and nitrogen (C:N) during enzymatic saccharification changed from an initial ratio of 6 and 14 in unrefined seaweeds to a ratio of 7 and 20 in biorefined *P. palmata* and *A. nodosum*, respectively (Table 9). In *S. latissima* the C:N ratio remained unchanged at 9 during biorefining.

In comparison with the reported carbon to nitrogen ratio (C:N) for terrestrial feedstock's, such as 12-14 for grass (fresh), 13-15 for silage (grass, Lucerne), 7-14 for seeds (cottonseed, soybean, sunflower), 14-18 for hay (grass, Lucerne) and 5-14 for meals (soybean, cottonseed, Lucerne) [59] the calculated C:N ratio of unrefined and biorefined *P. palmata* and *S. latissima* were similar with those of soybean-and cottonseed meals, as well as sunflower-and soybean seeds. The C:N ratio in biorefined *A. nodosum* was found higher and similar to the C:N ratio of fresh grass, grass-and Lucerne silage and hay. As the elemental composition in seaweed undergo seasonal fluctuations [28], with carbonaceous components such as carbohydrates accumulating in autumn time and nitrogenous components such as proteins at their maxima during winter/ spring time, these ratios of carbon to nitrogen are therefore highly variable. In this study it was also found that the C:N ratio in *A. nodosum* and *P. palmata* increased during biorefining, whilst in *S. latissima* the ratio remained unchanged (Table 9). Elemental

ratios can be used to adjust feeding strategies to the need of the animal and as a consequence of a more balanced diet, reduce environmental pollution and wastage of unused nutrients and energies [66].

3.5 Polyphenol content

The polyphenolic content in unrefined and biorefined seaweeds was monitored and removal yields calculated from the difference in total polyphenol content of unrefined to biorefined seaweed. The difference in polyphenol content between species was highly significant ($F(2,12)=8593$, $p<0.0001$), with highest polyphenol concentrations found in *A. nodosum*, which was 7 fold higher than *S. latissima* and 12 fold higher than *P. palmata* (Table 10). In all three seaweeds 82%-87% of polyphenol was extracted (Table 10) resulting in a significantly higher polyphenol concentration between polyphenol contents in unrefined and biorefined seaweeds ($F(1,12)=4726$, $p<0.0001$). Polyphenolic content in solvent extract of *A. nodosum* was 82% of the total of untreated *A. nodosum* (data not shown), resulting in a further 5% being removed during enzymatic saccharification. Seaweeds with high polyphenolic content, such as members of the *Fucales*, have shown to inhibit microbial processes [67] largely due to the non-selective inactivation of enzymes [68]. Polyphenolics are also known to exhibit an affinity towards proteins but the effect of complex formation on the digestibility of proteins/phenolics and the activity of enzymes is still not fully understood [69] and both beneficial and detrimental effects of polyphenols on digestive enzymes have been reported [70]. However, due to polyphenol–protein interactions, changes in the three dimensional structure and functional properties have been shown to affect the biological activity and nutritional aspects of the protein molecules [70, 71].

3.6 Amino acid profiling

Amino acid composition was found to differ between the three seaweeds ($F(2,102)=17819$, $p<0.001$) and between the two treatments ($F(1,68)>97$, $p<0.001$) (Table 11). The two major amino acids in untreated and biorefined seaweeds were glutamic acid and aspartic acid. With the exception of glutamic acid in *A. nodosum* and proline in *P. palmata*, all amino acids accumulated in biorefined seaweed and the total of all amino acids increased in *P. palmata* (44%), *A. nodosum* (10%) and *S. latissima* (26%) (Table 11). Essential amino acids, which made-up on average 27%-36% of the total amino acid content, increased in biorefined *A. nodosum* by 9% largely due to a 37% decline of the major amino acid glutamic acid, whilst the increase of essential amino acids in biorefined *P. palmata* and *S. latissima* was modest and accounted for 1%-3% of the total (Table 11). Despite the exclusion of the essential amino acid methionine in this analysis, essential amino acid contents in *A. nodosum* (27%), *S. latissima* (30%) and *P. palmata* (33%) were similar to reported values of 38% for *A. nodosum* and *S. latissima* [72] and 36% for *P. palmata* [73].

In *P. palmata* the total amino acid content was highest (18%), where the two most abundant amino acids glutamic and aspartic acid contributed towards 27% of the total and remained unchanged during biorefining. In *A. nodosum* and *S. latissima* the total amino acid content was lower (6.8% and 10.8%) and the two major amino acids glutamic acid and aspartic acid declined during biorefining in *A. nodosum* from 47% to 33% and in *S. latissima* from 35% to 30%.

Amino acid profiling in some species of brown and red seaweeds also identified the two amino acids aspartic acid and glutamic acid as the predominant amino acids in *S. latissima*, *A. nodosum* and *P. palmata*, respectively [72, 73]. As these amino acids and many more have also been reported to exist as free amino acids in brown, red and green seaweeds [74-76], the higher amino acid content in relation to the protein content (Table 11) is likely due to free amino acids and non-protein amino acids from the hydrolysis of peptides. The non-protein nitrogen content in *L. japonica* for example has been reported as 60% of the total nitrogen content [8]. In addition the decline of the amino acids glutamic acid and aspartic acid in both brown seaweeds during biorefining is also likely due to their presence as free amino acids.

Amino acids, especially essential amino acids, are vital for human and animal nutrition. Moreover, it has been suggested that there has been no clear evidence that some non-essential amino acids in particular glutamine, glutamate, proline, and arginine are produced in adequate quantities in humans and animals [77]. As amino acid requirements not only differ between humans and animals but also between ages [78], a reliable supply of all amino acids is essential to provide a rapidly growing world population.

Enzymatic saccharification has already been successfully applied world-wide by the brewing industry to produce a protein-enriched by-product called distillers grain. As the reported amino acid results from this study

were derived from biorefined materials produced under non-optimised extraction conditions, it is likely that the total amino acid content in optimised biorefined seaweed could have been significantly higher, as has been shown by the difference in protein content between optimised and non-optimised saccharification (Table 5Table 6). In addition, as proteins amongst other constituents are highly seasonal [34], higher protein contents than the one used in this study have been reported for *P. palmata*, where proteins made-up 25% of the biomass between February to May [73]. A theoretical doubling of the protein content, as has been achieved during the optimisation studies, could have produced a high-protein containing by-product of enzymatic saccharification.

3.7 Total fatty acid content

Total fatty acids were analysed in unrefined and biorefined seaweeds after acid hydrolysis and gas chromatographic determination of their methylated derivates. Variations in total fatty acid content between species and between unrefined and biorefined seaweed were different (Species: $F(2,12)=30$; $p<0.0001$; Treatment: $F(1,12)=199$, $p<0.0001$). Highest fatty acid content in unrefined seaweed was found at 2.0% in *A. nodosum*, followed by 1.6% in *S. latissima* and 1.1% in *P. palmata*. In contrast, highest fatty acid accumulation in biorefined seaweeds was in reverse order to unrefined seaweeds with a more than two fold concentration of fatty acids in *P. palmata*, followed by a 38% and 32% enrichment in *S. latissima* and *A. nodosum*, respectively (Table 12). Fatty acid analysis of 9 different species of brown, green and red seaweeds also found that *A. nodosum* and other brown species contained highest concentrations of fatty acids (up to 4.5% of the dry matter), while in members of the red seaweed family, such as *P. palmata*, fatty acid content was lower at ~1.4% [79].

Macroalgae in contrast to their microscopic cousins - microalgae, are generally not known to contain high lipid concentrations, with the exception of some known warm water species of the order *Dictyotales* which consistently contain ~15% lipids [12]. It has also been shown that the lipid content is not an accurate measure of the fatty acid content, and generally only 30%-50% of the lipid mass in seaweeds are fatty acids [80]. However, as algal lipids contain many essential fatty acids [58] seaweeds can be considered as a dietary supplement as part of a balanced diet. Enzymatic saccharification has shown to be a suitable methods for enrichment of fatty acids and application to high lipid containing seaweeds such as species of the order *Dictyotales* hold a potential to produce high-lipid containing biorefined algae suitable as a high-energy containing feedstock.

4 Conclusions

Application of enzymes such as alginate lyases, cellulases and hemicellulases to remove the carbohydrate content of seaweeds have been demonstrated to enrich the nutritional aspects of seaweed residue of *P. palmata, A. nodosum* and *S. latissima*. It is estimated that around 40%-45% of the biomass content is removed during aqueous extraction processing, largely due to the dissolution of metal ions and carbohydrates. Dissolution is likely to occur for monovalent ions such as sodium, potassium and chloride, while divalent metal ions such as ferrous, manganese, zinc, strontium and silicon are likely to accumulate in the residue. In the case of potentially harmful seaweed components such as arsenic and iodine, a reduction of arsenic compounds was seen in all three seaweed residues, whilst significant iodine removal was only achieved in brown seaweeds. Through optimisation of enzymatic processes the protein content was doubled in *A. nodosum* and *P. palmata*. In the case of *A. nodosum* a biorefinery concept including polyphenol extraction prior to enzymatic saccharification improved overall protein yields further. Fatty acid contents in unrefined and biorefined seaweeds were low but enzymatic saccharification has shown to double the total fatty acid content. Optimisation and biorefinery studies demonstrated that not only nutritional aspects of biorefined seaweeds can be improved but that additional product streams can also be produced without compromising nutritional aspects. Further, if applied to naturally high protein or lipid containing seaweeds, enzymatic saccharification can also be used to produce potentially high-strength novel feedstocks.

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Table 1 HPLC conditions for amino acid analysis

Time (min)	% mobile phase B**	% mobile phase A*
0	2	98
0.5	2	98
20	57	43
20.1	100	0
23.5	100	0
23.6	2	98
25	end	

Flow rate – 1.5 ml min^{-1}

*Mobile phase A -10 mM Na_2HPO_4:10 mM $Na_2B_4O_7$, pH 8.2

**Mobile phase B – Acetonitrile:methanol:water (45:45:10)

Table 2 Ash content and total solid content of unrefined and biorefined *P. palmata*, *A. nodosum* and *S. latissima* and their distribution during biorefining

Seaweed		Total Solids (TS) (g)	Distribution of TS % (w w^{-1})	Ash (%)	Distribution of ash % (w w^{-1})
P. palmata	Unrefined	259.6	100	33.5	100
	Biorefined	144.6	56**	24.4	40***
A. nodosum	Unrefined	260.6	100	28.7	100
	Biorefined*	149.0	57**	18.2	36***
S. latissima	Unrefined	213.0	100	40.9	100
	Biorefined	129.1	61**	29.9	44***

* *A. nodosum* was pre-treated with solvent prior to enzyme saccharification

** Defined as: $\frac{TS_{U,B}}{TS_U}$ x 100% where TS $_{U,B}$ is the total solid content in unrefined (TS_U) or biorefined (TS_B) seaweed

*** Defined as: $\frac{TS_{U,B}\ x\ Ash_{U,B}}{TS_U\ x\ Ash_U}\ \boldsymbol{x\ 100}\%$ where Ash $_{U,B}$ is the percentage of ash in unrefined (Ash $_U$) or biorefined (Ash $_B$) seaweed

Table 3 Percentage metal composition in enzymatic extract and residue of *P. palmata, A. nodosum* and *S. latissima*

Element	Elemental composition of extract* % (w w^{-1})			Elemental distribution in biorefined seaweed** % (w w^{-1})		
	P. palmata	*A. nodosum*	*S. latissima*	*P. palmata*	*A. nodosum*	*S. latissima*
Mg	2	3	2	39	46	47
Al	1	1	1	54	32	54
P	2	0	1	41	43	45
S	1	7	2	70	60	72
K	33	16	26	37	36	41
Ca	2	1	5	34	86	58
Mn	0	0	0	48	65	50
Fe	0	0	0	75	60	85
Cu	0	0	0	67	50	52
Zn	0	0	0	72	67	67
As	0	0	0	43	28	36
Rb	0	0	0	40	46	44
Sr	0	0	0	53	84	83
Pb	0	0	0	50	70	55
Na	18	37	21	35	25	35
Ni	0	0	0	42	51	53
Si	2	0	1	58	62	74
Sn	0	0	0	54	45	37
Cl	38	33	37	35	10	33
Br	0	0	0	55	29	45
I	0	1	3	77	14	36

* Describes the difference in mass of each element between unrefined and biorefined seaweed in comparison to the total mass of all removed elements**;** Mass of each element was calculated from the TS content (Table 2) and its concentration (Table 4)

** Defined as the mass of each element in biorefined seaweed in comparison to its total mass in unrefined seaweed

Table 4 Elemental content (mg kg^{-1}) in unrefined and biorefined *P. palmata*, *A. nodosum* and *S. latissima*

Element	% metal recovery rate	*P. palmata*		*A. nodosum*		*S. latissima*	
		Unrefined	Biorefined	Unrefined	Biorefined	Unrefined	Biorefined
Mg	110	5340 ± 92	3728 ± 352	7877 ± 352	6622 ± 438	6800 ± 176	5258 ± 351
Al	136	5270 ± 157	5130 ± 162	2300 ± 190	1327 ± 121	6110 ± 141	5495 ± 282
P	66	4633 ± 95	3379 ± 70	1163 ± 40	920 ± 80	3047 ± 31	2258 ± 162
S	91	8283 ± 164	**10379 ± 111**	21967 ± 404	**24184 ± 2229**	11167 ± 153	**13289 ± 510**
K	89	92067 ± 1155	60947 ± 953	32233 ± 379	21109 ± 1864	88400 ± 954	59743 ± 2047
Ca	73	6157 ± 992	3777 ± 151	9953 ± 57	**15535 ± 1780**	25900 ± 300	24908 ± 2213
Mn	98	48.4 ± 2.6	41.5 ± 1.4	28.9 ± 2.2	**34.4 ± 4.5**	60.7 ± 2.6	50.1 ± 0.9
Fe	86	632 ± 19	**846 ± 20**	119 ± 1	**130 ± 15**	837 ± 27	**1170 ± 107**
Cu	116	16.3 ± 0.4	**19.7 ± 1.5**	7.6 ± 0.3	6.9 ± 0.5	10.8 ± 0.9	9.3 ± 0.9
Zn	94	44.9 ± 1.2	**58.2 ± 1**	48.4 ± 0.5	**58.7 ± 6.7**	30.9 ± 3.4	**34.3 ± 5.6**
As	102	12 ± 0.8	9.2 ± 0.8	32.9 ± 0.7	16.5 ± 1.6	75.8 ± 2.6	44.5 ± 1
Rb	107	62.1 ± 0.6	44.4 ± 1.1	23.9 ± 0.7	20.0 ± 0.7	63.6 ± 0.3	45.7 ± 0.4
Sr	85	61.3 ± 2.5	58.7 ± 3.2	731 ± 2	**1115 ± 64**	793 ± 2	**1081 ± 30**
Pb	152	4.7 ± 0.5	4.2 ± 1.1	2.8 ± 0.5	**3.6 ± 0.5**	6.2 ± 1.6	5.6 ± 0.6
Na	152	49133 ± 3383	31298 ± 441	65833 ± 4650	29401 ± 3722	64733 ± 1845	36923 ± 1712
Ni	151	13.9 ± 0.9	10.5 ± 0.8	4.2 ± 0.8	3.9 ± 0.3	n.d.	8.3 ± 0.8
Si	96	6873 ± 248	**7139 ± 435**	1213 ± 12	**1377 ± 188**	6173 ± 67	**7512 ± 727**
Sn	233	52.7 ± 0.3	51.3 ± 2	52.6 ± 1	42.7 ± 9.4	80.5 ± 23.2	49.4 ± 7.1
Cl	84	101433 ± 1914	63344 ± 1493	48667 ± 379	8586 ± 655	110667 ± 1528	60945 ± 2828
Br	106	940 ± 5	931 ± 5	580 ± 1	301 ± 21	1510 ± 10	1137 ± 36
I	100	839 ± 26	**1165 ± 35**	1237 ± 25	319 ± 58	9057 ± 15	5326 ± 667

n.d. = not detected; values in bold highlight concentrations in biorefined seaweeds which are greater than their unrefined equivalents

Metal recovery rates from XRF analysis of seaweeds were calculated from the concentrations of measured to certified values for each element and expressed as a percentage of metal recovery

Table 5 Protein content in unrefined and biorefined *A. nodosum, S. latissima* and *P. palmata*

Seaweed	Final pH	Unrefined*	Biorefined*
P. palmata	4.6	10.9 ± 0.5	12.8 ± 2.0
A. nodosum	3.9	6.8 ± 0.2	6.6 ± 0.5
S. latissima	5.1	8.1 ± 0.4	6.1 ± 0.2

*Data for unrefined and biorefined proteins are reported as a % mean ± SD of the total solid content

Table 6 Results from optimisation studies. Protein contents in unrefined and biorefined *P. palmata, A. nodosum* and *S. latissima* after enzymatic saccharification using different pH and combined solvent extraction of *A. nodosum* with enzymatic saccharification

Seaweed	Biorefined**	Unrefined**	Protein accumulation***	Treatment condition
P. palmata	19.4 ± 0.6	10.9 ± 0.5	78.0 %	pH 3.1
	20.4 ± 1.0		87.2 %	pH 4.6
	22.1 ± 1.0		102.8 %	pH 5.7
A. nodosum	13.8 ± 1.3	6.8 ± 0.2	102.9 %	pH 3.7
	13.6 ± 0.3		100.0 %	pH 4.2
A. nodosum*	14.9 ± 0.4		119.1 %	pH 3.6
S. latissima	11.6 ± 0.3	8.1 ± 0.4	43.2 %	pH 3.1
	9.3 ± 0.4		14.8 %	pH 6.2

*Acetonitrile extraction before enzymatic saccharification
**Protein content in unrefined and biorefined seaweeds are reported as a % mean ± SD of the total solid content
***Defined as the change in protein concentration during biorefining (biorefined – unrefined) in comparison to the concentration of protein in unrefined seaweed

Table 7 Carbohydrate analysis of unrefined and biorefined *P. palmata, A. nodosum* and *S. latissima*

Seaweed		Glucose	Mannitol	Fucose	Man/Gal/Xyl	Others	Total
P. palmata	Unrefined	2.3 ± 0.2	n.d.	0.3 ± 0.0	20.9 ± 1.6	2.0 ± 0.3	26.9 ± 2.3
	Biorefined	3.6 ± 0.1	n.d.	0.3 ± 0.0	16.9 ± 0.3	1.9 ± 0.3	23.5 ± 0.6
	Mass removal*	13 %	-	44 %	55 %	47 %	51 %
A. nodosum	Unrefined	4.7 ± 0.2	3.5 ± 0.2	0.9 ± 0.0	5.7 ± 0.4	12.1 ± 0.6	27.5 ± 1.3
	Biorefined	5.4 ± 0.2	n.d.	0.9 ± 0.2	9.0 ± 0.6	13.8 ± 1.1	29.7 ± 1.6
	Mass removal*	37 %	100 %	45 %	10 %	35 %	38 %
S. latissima	Unrefined	5.6 ± 0.1	6.7 ± 0.0	0.6 ± 0.0	3.9 ± 0.3	7.4 ± 0.2	25.1 ± 0.6
	Biorefined	6.3 ± 0.1	3.6 ± 0.1	0.7 ± 0.1	4.5 ± 0.5	9.4 ± 0.3	24.8 ± 0.4
	Mass removal*	32 %	67 %	29 %	30 %	23 %	40 %

Data are reported as a % mean ± SD of the total solid content
*Difference in sugar mass between unrefined and biorefined seaweed in comparison to its total mass in unrefined seaweed according to Equation 1

Table 8 Alginate content of unrefined and biorefined *A. nodosum* and *S. latissima*

	A. nodosum	***S. latissima***
Unrefined	15.0 ± 2.3	14.6 ± 4.0
Biorefined	20.0 ± 3.6	23.0 ± 2.0
Mass removal*	27 %	5 %

Alginate content is expressed as a % mean ± SD of the total solid content
*Difference in alginate mass between unrefined and biorefined seaweed in comparison to its total mass in unrefined seaweed according to Equation 1

Table 9 Total carbon, hydrogen, nitrogen and sulphur analysis of unrefined and biorefined *P. palmata*, *A. nodosum* and *S. latissima*

Seaweeds		**% C**	**% H**	**% N**	**% S**	**C:H:N ratio**	**C:N ratio**
P. palmata	Unrefined	29.9 ± 0.6	3.6 ± 0.4	4.7 ± 0.4	<0.5	100:12:16	6.4
	Biorefined	35.1 ± 0.5	4.9 ± 0.2	4.9 ± 0.2	<0.5	100:14:14	7.2
	Mass removal*	42 %	31 %	48 %	-	-	-
A. nodosum	Unrefined	32.1 ± 0.1	4.2 ± 0.5	2.2 ± 0.6	1.1 ± 0.3	100:13:7	14.6
	Biorefined	35.8 ± 1.0	5.0 ± 0.4	1.9 ± 0.5	1.7 ± 0.3	100:14:5	18.8
	Mass removal*	45 %	40 %	57 %	15 %	-	-
S. latissima	Unrefined	25.6 ± 0.5	3.5 ± 0.6	2.7 ± 0.4	<0.5	100:14:11	9.5
	Biorefined	30.9 ± 0.6	4.4 ± 0.4	3.3 ± 1.0	<0.5	100:14:11	9.4
	Mass removal*	27 %	25 %	27 %	-	-	-

Carbon, hydrogen, nitrogen and sulphur contents are expressed as a % mean ± SD of the total solid content
*Difference in mass of each element between unrefined and biorefined seaweed in comparison to its mass in unrefined seaweed according to Equation **1**

Table 10 Polyphenol content in unrefined and biorefined *P. palmata*, *A. nodosum* and *S. latissima*

	P. palmata	***A. nodosum***	***S. latissima***
Unrefined	0.39 ± 0.05	4.62 ± 0.05	0.66 ± 0.02
Biorefined	0.13 ± 0.02	1.13 ± 0.03	0.14 ± 0.06
Mass removal*	82 %	87 %	87 %

Results for polyphenols are expressed as a % mean ± SD of the total solid content
*Difference in mass of polyphenols between unrefined and biorefined seaweed in comparison to its mass in unrefined seaweed according to Equation **1**

Table 11 Amino acid profiling of unrefined and biorefined *P. palmata, A. nodosum* and *S. latissima*

Amino acids	***P. palmata***		***A. nodosum***		***S. latissima***	
	Unrefined	Biorefined	Unrefined	Biorefined	Unrefined	Biorefined
Aspartic acid	2.23 ± 0.03	3.27 ± 0.06	0.74 ± 0.01	0.88 ± 0.01	1.47 ± 0.01	1.79 ± 0.06
Glutamic acid	2.66 ± 0.02	3.64 ± 0.07	2.26 ± 0.03	1.43 ± 0.01	2.18 ± 0.01	2.33 ± 0.10
Serine	1.03 ± 0.01	1.78 ± 0.04	0.26 ± 0.01	0.38 ± 0.01	0.55 ± 0.01	0.81 ± 0.02
Histidine	0.26 ± 0.01	0.49 ± 0.01	0.08 ± 0.01	0.13 ± 0.01	0.17 ± 0.01	0.25 ± 0.01
Glycine	1.22 ± 0.01	1.91 ± 0.04	0.28 ± 0.01	0.40 ± 0.01	0.67 ± 0.01	0.94 ± 0.04
Threonine	0.86 ± 0.01	1.42 ± 0.02	0.25 ± 0.01	0.37 ± 0.01	0.54 ± 0.01	0.77 ± 0.02
Arginine	1.12 ± 0.01	1.81 ± 0.03	0.31 ± 0.01	0.39 ± 0.01	0.63 ± 0.01	0.81 ± 0.04
Alanine	1.49 ± 0.01	2.23 ± 0.04	0.41 ± 0.00	0.47 ± 0.01	1.28 ± 0.01	1.36 ± 0.06
Tyrosine	0.60 ± 0.01	1.04 ± 0.01	0.12 ± 0.01	0.19 ± 0.01	0.32 ± 0.01	0.47 ± 0.01
Valine	0.98 ± 0.01	1.36 ± 0.02	0.25 ± 0.01	0.35 ± 0.01	0.51 ± 0.01	0.65 ± 0.02
Phenylalanine	0.76 ± 0.01	1.27 ± 0.01	0.24 ± 0.01	0.35 ± 0.01	0.47 ± 0.01	0.68 ± 0.02
Isoleucine	0.68 ± 0.01	0.97 ± 0.02	0.29 ± 0.01	0.34 ± 0.01	0.39 ± 0.01	0.51 ± 0.01
Leucine	1.21 ± 0.01	1.93 ± 0.02	0.36 ± 0.01	0.54 ± 0.01	0.70± 0.01	1.03 ± 0.03
Lysine	1.17 ± 0.03	1.30 ± 0.18	0.23 ± 0.02	0.40 ± 0.08	0.43 ± 0.06	0.63 ± 0.06
Proline	1.64 ± 0.07	1.44 ± 0.11	0.18 ± 0.01	0.29 ± 0.02	0.45 ± 0.03	0.51 ± 0.04
Total amino acids % (w w^{-1})*	17.99	25.93	6.33	6.98	10.83	13.62
Essential amino acids % (w w^{-1}) **	33	34	27	36	30	33
Protein % (w w^{-1})	10.9 ± 0.5	12.7 ± 2.0	6.8 ± 0.2	6.6 ± 0.5	8.1 ± 0.4	6.1 ± 0.2

Results for amino acids and proteins are expressed as a % mean ± SD of the total solid content
*Total amino acids are expressed as a percentage of the sum of 15 amino acids of the dry matter
**Essential amino acids: histidine, threonine, valine, phenylalanine, leucine, isoleucine, lysine (excluding methionine, cysteine and tryptophan); Results are expressed as a percentage of the total amino acid content

Table 12 Total fatty acid (TFA) content in unrefined and biorefined *P. palmata, A. nodosum* and *S. latissima*

	P. palmata	***A. nodosum***	***S. latissima***
Unrefined	1.14 ± 0.04	2.01 ± 0.11	1.64 ± 0.02
Biorefined	2.40 ± 0.07	2.65 ± 0.26	2.27 ± 0.09
TFA accumulation*	111 %	32 %	38 %

Results for total fatty acids are expressed as a % mean ± SD of the total solid content
*Defined as the change in total fatty acid concentration during biorefining (biorefined – unrefined) and compared against the concentration of total fatty acids in unrefined seaweed