Seaweed salt from *Ulva*: A novel first step in a cascading biorefinery model

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A B S T R A C T

We compared post-harvest processing of two species of *Ulva* (chlorophyta) using 36 washing time-by-temperature combinations. We quantified the yield of crystallized salt after evaporation of the washing water as a target product and characterized the composition of salts and processed biomass, with the additional aim of improving the composition of the processed biomass for production of fertilizer, feed or fuel. Washing of biomass of *Ulva ohnoi* and *Ulva tepida* effectively reduced its mineral content with concomitant production of crystalline salts with Na:K ratios of 1.1–2.2 and a maximum of 19% soluble fiber (ulvan). The maximum yield of salt was 29% of the biomass for *U. ohnoi* and 36% for *U. tepida*. Salts from both species have potential for human health applications and functional foods. Washing increased the energy content of the biomass from both species by 20–50% to a maximum of 18 MJ kg^{-1} and protein contents by 11–24% to a maximum of 27.4%. The production of seaweed salt is therefore a novel first step in a cascading biorefinery model for the utilization of macroalgal biomass which simultaneously improves the quality of the processed biomass for production of fertilizer, feed or fuel.

1. Introduction

Marine macroalgae (seaweed) are increasingly being developed for a diverse portfolio of products outside of the traditional markets of human foods and phycocolloids. The commercial potential of both cultivated macroalgae and biomass sourced from natural blooms is being realized on a global scale [1–4] with much of the focus on the genus *Ulva* (Chlorophyta) due to its high areal productivity [3–6] and broad environmental tolerance [7,8]. For example, the production of *Ulva* has been successfully integrated into the large scale production of abalone in South Africa [3] while species of *Ulva* regularly form massive blooms referred to as green tides in China [9], North [10] and South America [11], and Europe [12,13]. Notably, *Ulva* blooms in France are now harvested for the development of bioproducts for health and animal nutrition (https://www.olmix.com/news/ulvans-project).

Marine macroalgae have traditionally been used as agricultural mulch or fertilizer to improve plant growth [14,15] and in animal feed to improve animal health and productivity [16]. Marine macroalgae added to animal feed have multiple benefits, with increases in growth and meat quality in steers [17], improved gut health and egg quality in laying hens [18], increases in feed palatability, protein intake, growth, and roe production in sea urchin and abalone [3,19–21], and increased growth, higher feed conversion, and better pigmentation in shrimp [22,23]. Macroalgal biomass is also suitable for the production of liquid transportation fuel [24–26], where phototrophic biomass is recognized

as a renewable source to replace fossil fuels [27]. However, for all of these applications the high salt content of the biomass is an issue. For fertilizers, biochar, and liquid soil conditioners the high internal mineral content (up to 47% as inorganic salts) and salt residues from seawater on dried marine biomass pose a problem as it can lead to increased soil salinity or direct toxicity. For these applications biomass therefore requires washing or leaching prior to processing the product further [14,28] or prior to the application of the final product [29]. Too high salt content in animal feed can also act as a feed deterrent or decrease the digestibility of the feed [30], potentially limiting the use of high doses of marine macroalgae in such applications. While hydrothermal liquefaction (HTL) (processing in water at high pressure and temperature) is the most promising processing technology for the production of high-energy liquid fuels from biomass [25,26,31], the high mineral content of marine macroalgae leads to increased mechanical wear on processing equipment by corrosive salts, in particular halides [32,33], and to the potential for carry-over of trace amounts of Fe, Mg, Zn, and Ni into the biocrude, that can be challenging during the refinery and upgrading of the biocrude to a drop-in fuel [34]. However, salts can be removed by washing of the biomass and this has been applied to biomass for energy [35] and compost [14,28].

Importantly, the mineral (salt) content of marine algae has also been highlighted as a positive aspect with numerous studies proving them to contain a healthy balance of minerals for human nutrition, including high proportions of K and Mg and favorable Na to K ratios [36–39]. Western diets contain a large excess of Na, typically from processed foods, which leads to increased risks of high blood pressure and associated cardiovascular diseases [40–42]. The high intake of Na also leads to

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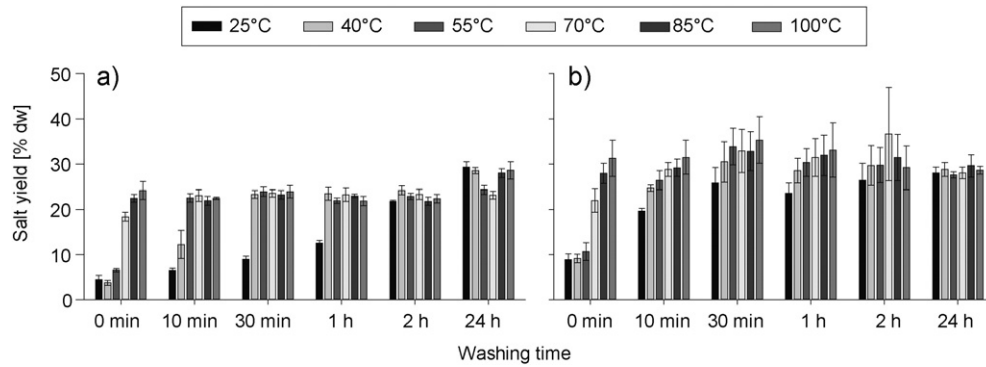*E-mail address:* marie.magnusson@jcu.edu.au (M. Magnusson).

Fig. 1. Salt yields of a) *Ulva ohnoi* and b) *U. tepida* as % of dw biomass that was processed. Average ± SE, n = 3.

a highly increased Na:K intake ratio, deviating from the ideal Na:K intake ratio of between 0.3–1 based on dietary guidelines regarding daily intake of respective element [43–45]. Coupled with a rising consumer interest in natural sea salt or "gourmet" salts (Google trends, https://www.google.com.au/trends/, search terms table salt vs. sea salt, accessed 2015-04-15), an alternative and attractive solution to reducing Na consumption is to take advantage of the naturally healthy mineral profile in marine macroalgae. Under this concept, we propose to take the minerals (salt) washed from marine macroalgal biomass and evaluate whether this "waste" stream can be turned into a natural mineral salt for human consumption as a first step in a cascading biorefinery model [46]. This is particularly interesting for macroalgae from the genus *Ulva* that also contain soluble fiber as sulfated polysaccharides (ulvans) which have bioactive properties [47,48] and may be extracted during the rinsing process and therefore enhance the value of the salt-product.

The first objective of this research was therefore to quantify the production of a natural mineral salt from the marine macroalgae *Ulva ohnoi* and *Ulva tepida* under a range of time-by-temperature combinations. The second objective was to characterize the mineral and soluble fiber content of the produced marine macroalgal salts to determine if this product has a beneficial composition as a food product, while the final objective was to quantify the composition and energy content of the biomass pre- and post treatment to determine biomass quality for protein and energy bioproducts such as fertilizer, animal feed or fuel.

2. Materials and methods

2.1. Macroalgal species and cultivation

Two species of *Ulva* (Chlorophyta) were chosen as they are target species for waste water bioremediation and large-scale on-land production of biomass for bioproducts [4,6,49] and are also representative examples of marine macroalgae that regularly form large blooms in the wild. *U. ohnoi* (blade-like morphology, Gen-bank accession number KF195501, strain JCU 1 [6]) and *U. tepida* (tubular and filamentous, [50–52]) (previously referred to as *Ulva* sp. 3 [53]) have quantified mineral and fiber composition [4,39] and are suitable as feedstock for fertilizers, animal feed supplements and the production of biocrude through HTL [19,29,35,54]. *U. ohnoi* is domesticated and is maintained at the Marine & Aquaculture Research Facility (MARF) at James Cook University (JCU), Townsville, Australia (Latitude: 19.33 S; Longitude 146.76 E) and was cultivated and harvested as described in [4]. *U. tepida* was collected by hand from a land-based aquaculture facility at Guthalungra (19° 55′S, 147° 50′E), Queensland, Australia, and was identified by morphological and anatomical features [50] and seeded and cultivated as described in [49] except that culture tanks were 10,000 L with the supporting frame for the culture ropes suspended at 10 cm depth and partial harvesting was performed by cutting biomass off the ropes

rather than drying of whole ropes. Salinity in the cultivation waster was maintained between 32 and 35 ‰ for both species through the addition of dechlorinated water or sea salt as required [4].

2.2. Washing treatments

Three separate harvests of *U. ohnoi* and *U. tepida* were collected for salt removal by washing in fresh water at 36 temperature and time period combinations. Fresh weight (fw) samples (100 g) were taken from each species and harvest and dried for 24 h at 60 °C to determine the fresh weight to dry weight ratio (fw:dw) and the biochemical composition of the starting material (control biomass) as described in Section 2.3. The remaining biomass from each harvest (~3.6 kg) was split into 100 g fw subsamples per treatment for a total of 36 treatments (n = 3) in a fully factorial combination of six time periods for immersion in the wash water (0 min, 10 min, 30 min, and 1, 2, and 24 h) and six temperatures of the wash water (25, 40, 55, 70, 85, and 100 °C). For treatments 10 min to 24 h, the 100 g fw biomass sample was immersed in 1 L of pre-heated water (biomass:water ratio 1:10 (w:V)) in 2 L glass beakers placed in temperature controlled water baths (Grant JB Nova Unstirred Water Bath, LabGear Australia) to maintain the correct temperature for the entire treatment period. Following the correct immersion period the treated biomass was separated from the water by pouring the biomass and water through a double-folded muslin cloth bag and gently squeezing the wet biomass in the muslin cloth by hand. The 0 min time period was included as a control for the external salts and consisted of steadily pouring 1 L of water of each temperature over the biomass through a double-folded muslin cloth bag and collecting the water as described above. The volume of water was chosen as 1 L was the minimum volume required to completely cover 100 g fw of *U. ohnoi* without any stirring being required. The processed biomass was oven dried to constant dw (24 h at 60 °C) while the algae-infused wash water was filtered through Whatman grade 1 GF filters to remove any solids that passed through the muslin cloth and then evaporated to dryness in an oven for 48 h at 60 °C. The produced salt was then collected and weighed, and salt yield calculated as g dw salt produced per 100 g dw biomass (% dw, based on species and harvest specific fw:dw ratios of the algae).

2.3. Compositional analysis of salt and biomass

Elemental analysis of % C, H, N, and S was outsourced to OEA labs (http://www.oealabs.com, Callington, UK), where samples were combusted in pure oxygen and separated and quantified using GC-TCD. Percent O was calculated as $\% \, O = 100 - \sum (C, H, N, S, ash)$ where C, H, N, S, and ash are expressed as a percentage of the total mass for salt (n = 1) control biomass (n = 3) and selected processed biomass samples post washing (Section 3.1) (n = 3). The higher heating value (HHV) of control and processed biomass was calculated as
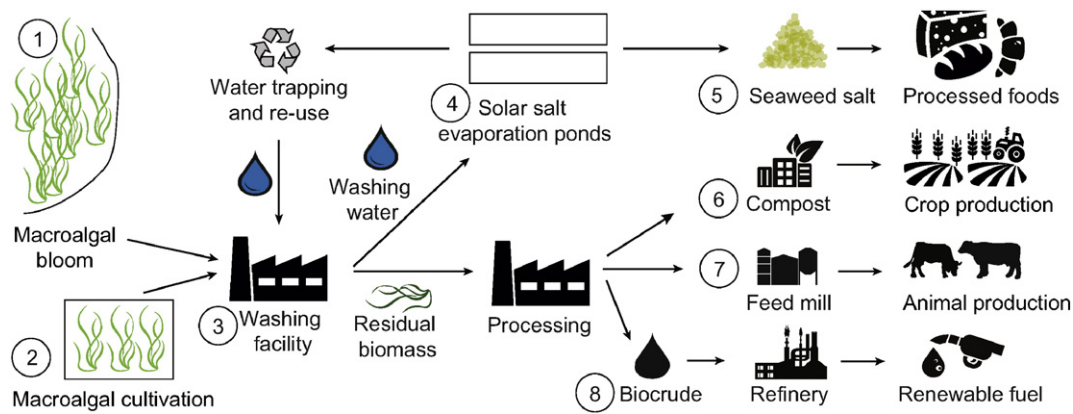
Fig. 2. Schematic diagram of a cascading biorefinery for the proposed integrated salt- and bioproduct manufacture from wild-harvested macroalgal bloom biomass from the genus *Ulva* (1) or a combination of bloom and cultivated algae (2). Washing of biomass (3) in freshwater followed by evaporation of the wash water in solar evaporation ponds (4) will produce a marine macroalgal (seaweed) salt (5) with a low Na:K ratio and high content of Ca and Mg and a range of trace elements, and potential functional properties derived from soluble fiber (ulvan), that could be incorporated into processed food products. Recommended bioproducts for the processed biomass are compost (6), cattle feed (7), and biocrude (8).

$$HHV = 0.3491 * C + 1.1783 * H + 0.1005 * S - 0.1034 * O - 0.0151 * N - 0.0211 * ash$$ based on CHONS elemental composition (% dw) and ash content (% dw) following [55]. Protein yield from each source was calculated as Protein = %$N_{biomass}$ * k, where $N_{biomass}$ is the N content (% dw) of the biomass and k is the species specific nitrogen-to-protein conversion factor, which is 4.6 for *U. ohnoi* [56] and 3.6 for *U. tepida* [39]. Moisture content was measured on a minimum of 1.0 g biomass (n = 3) at 105 °C to constant weight (MS-70 moisture analyzer, A&D Company Ltd.) for all control and processed biomass samples. The same biomass was then weighed to 0.001 g precision followed by combustion in air at 550 °C for 6 h (SEM muffle furnace, LabTek, Australia) to determine mineral (ash) content (% dw). The organic content (OC) of the biomass was then quantified as OC = 100% − % ash. The content of metals and metalloids (23 elements) was analyzed for selected salts (corresponding to the processed biomass analyzed for CHNS, n = 1 per treatments as salt replicates required pooling to obtain large enough sample amounts for biochemical analysis) and for the control biomass samples (n = 3) at the Advanced Analytical Centre (AAC) at James Cook University, Australia. Sample preparation and analysis were carried out as described in [57] on a Varian 820-MS Inductively Coupled Plasma Mass Spectrometer ICP/MS (Melbourne, Australia) in scan mode (peak hopping with a dwell time of 20 ms), with RF power set to

1250 W and gas flow rates at 18 L min^{-1} (plasma), 1.65 L min^{-1} (auxiliary), 1.0 L min^{-1} (sampler) and 0.20 L min^{-1} (sheath). Ca, K, Na, and P were analyzed by a Varian Liberty Series II ICP/OES (Melbourne, Australia), wavelengths used for quantification were 422.67 nm for Ca, 769.896 nm for K, 589.592 nm for Na, and 213.618 nm for P. Indium was used as internal standard for ICP/MS and a series of multi-element standard solutions were used to calibrate both instruments. Enzymatic-gravimetric fiber analysis (Prosky method) (total, insoluble, and soluble fiber, AOAC Official Method 985.29 for insoluble fiber; AOAC Official Method 993.19 for soluble fiber) was performed by Grain Growers Limited (Australia) for selected salts (Section 3.1) and the control biomass samples, and this data was used as a proxy for the content of the soluble fiber ulvan [58]. The salt samples and corresponding processed biomass were selected for analysis based on the efficiency of salt production as the maximum salt yield afforded at the shortest washing time (h) and lowest temperature (°C), and what might be practical in an industrial setting, as described in Section 3.1.

2.4. Data analysis

The effect of washing treatments on yield of salt and on the biochemical composition (as the content of C, H, O, N, S, ash, protein, and

Table 1
Content of selected minerals* (g kg^{-1}) of the control biomass (n = 3, average ± se) and salt (pooled material, n = 1) of *Ulva ohnoi* and *Ulva tepida* for tested treatments.

Sample type	Control biomass	Salt							
Treatment (h)		0	0	0:10	0:10	0:30	0:30	24	24
(°C)		25	40	25	40	25	40	25	40
Ulva ohnoi									
Ca	2.7 ± 0.3	1.7 ± 0.01	2.1 ± 0.03	2.6 ± 0.01	3.5 ± 0.04	5.0 ± 0.1	3.1 ± 0.02	3.6 ± 0.01	3.7 ± 0.03
Fe	0.2 ± 0.1	0.2 ± 0.00	0.1 ± 0.0	0.1 ± 0.00	0.07 ± 0.00	0.1 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.07 ± 0.00
K	29.1 ± 9.8	17.4 ± 0.3	25.4 ± 0.2	75.0 ± 0.4	85.5 ± 0.7	109.0 ± 3.2	77.6 ± 0.6	85.6 ± 0.6	78.5 ± 1.5
Mg	25.6 ± 9.5	5.1 ± 0.03	5.5 ± 0.05	8.2 ± 0.04	8.2 ± 0.04	16.0 ± 0.1	20.1 ± 0.4	22.9 ± 0.1	22.6 ± 0.1
Na	15.5 ± 1.1	286.0 ± 2.5	314.0 ± 5.0	225.0 ± 3.7	205.0 ± 1.3	354.0 ± 2.6	199.0 ± 7.7	199.0 ± 1.9	173.0 ± 1.0
P	2.9 ± 0.7	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	3.8 ± 0.03	0.7 ± 0.00	2.8 ± 0.05	4.0 ± 0.01	4.9 ± 0.02
sum	100.6 ± 35.0	311.3	347.9	311.7	318.4	485.6	303.2	315.6	283.335
Na:K	1.3 ± 0.6	16.4	12.4	3.0	2.4	3.2	2.6	2.3	2.2
Ulva tepida									
Ca	3.2 ± 0.3	1.3 ± 0.01	1.3 ± 0.01	1.0 ± 0.02	3.1 ± 0.02	1.5 ± 0.01	3.9 ± 0.02	4.3 ± 0.03	5.0 ± 0.04
Fe	0.2 ± 0.02	0.2 ± 0.00	0.2 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.2 ± 0.00	0.1 ± 0.00
K	32.8 ± 8.1	25.8 ± 0.1	23.3 ± 0.9	81.2 ± 3.5	77.9 ± 2.6	126.0 ± 1.0	634.0 ± 0.6	66.7 ± 0.3	86.7 ± 0.6
Mg	19.0 ± 4.1	3.6 ± 0.01	3.5 ± 0.01	2.3 ± 0.01	29.7 ± 0.1	6.4 ± 0.02	49.7 ± 0.2	49.2 ± 0.1	55.5 ± 0.1
Na	66.3 ± 26.9	294.0 ± 1.0	292.0 ± 1.6	239.0 ± 2.8	155.0 ± 2.0	196.0 ± 3.8	81.7 ± 0.8	79.5 ± 0.6	98.5 ± 0.4
P	3.5 ± 0.3	0.7 ± 0.02	0.7 ± 0.03	0.9 ± 0.01	5.4 ± 0.03	2.3 ± 0.06	7.3 ± 0.03	8.1 ± 0.06	9.4 ± 0.1
sum	125.2 ± 32.8	326.3	322.0	325.5	272.2	333.4	206.7	208.7	256.1
Na:K	2.0 ± 0.5	11.4	12.5	2.9	2.0	1.6	1.3	1.2	1.1

* See Supplementary Tables S1 and S2 for a complete list of the contents of the 23 minerals included in the analysis.

Table 2
Compositional analysis (carbon, hydrogen, oxygen, nitrogen, sulfur, ash, and fiber content (% dw)), higher heating value (MJ kg^{-1}) and protein content (% dw)), of the control biomass (n = 3, average ± SE), processed biomass post-washing (n = 3, average ± SE), and salt (pooled material, n = 1) of blade *Ulva ohnoi*.

	Treatment		Elemental composition (% dw)					Minerals (% dw)	Organic matter (% dw)	HHV (MJ kg^{-1})	Protein* (% dw)	Fiber (% dw)		
	(h)	(°C)	C	H	O	N	S					soluble	insoluble	Total
Control biomass	na	na	30.4 ± 0.4	5.3 ± 0.1	25.4 ± 1.2	5.1 ± 0.2	4.0 ± 0.2	25.4 ± 1.2	74.6 ± 1.2	14.8 ± 1.2	23.4 ± 0.7	12.3 ± 1.0	16.8 ± 0.4	29.0 ± 0.7
Processed biomass	0	25	32.3 ± 0.7	5.7 ± 0.2	22.3 ± 1.1	5.7 ± 0.2	4.2 ± 0.6	22.3 ± 1.1	77.7 ± 1.1	14.8 ± 0.6	26.0 ± 0.7			
	0	40	32.1 ± 0.5	5.6 ± 0.1	21.2 ± 0.5	5.7 ± 0.2	4.2 ± 0.1	21.2 ± 0.5	78.8 ± 0.5	14.5 ± 0.2	26.0 ± 0.8			
	0:10	25	31.4 ± 2.7	5.4 ± 0.6	18.9 ± 0.4	5.2 ± 0.5	4.1 ± 0.8	18.9 ± 0.4	81.1 ± 0.4	13.6 ± 2.2	24.0 ± 2.2			
	0:10	40	35.1 ± 2.0	6.0 ± 0.3	15.2 ± 2.5	5.4 ± 0.1	3.5 ± 0.6	15.2 ± 2.5	84.8 ± 2.5	15.7 ± 1	25.0 ± 0.3			
	0:30	25	32.6 ± 0.9	5.5 ± 0.1	18.6 ± 2.3	5.5 ± 0.1	4.2 ± 0.3	18.6 ± 2.3	81.4 ± 2.3	14.3 ± 0.2	25.5 ± 0.8			
	0:30	40	38.0 ± 0.6	5.9 ± 0.1	10.6 ± 1.6	5.7 ± 0.1	2.4 ± 0.1	10.6 ± 1.6	89.4 ± 1.6	16.3 ± 0.3	26.1 ± 0.5			
	24	25	39.7 ± 0.5	6.5 ± 0.1	10.1 ± 0.4	6.0 ± 0.2	2.2 ± 0.2	10.1 ± 0.4	89.9 ± 0.4	17.8 ± 0.2	27.4 ± 1.0			
	24	40	39.8 ± 1.0	6.5 ± 0.1	9.4 ± 0.3	5.6 ± 0.0	2.3 ± 0.1	9.4 ± 0.3	90.6 ± 0.3	18.0 ± 0.8	26.0 ± 0.2			
Salt	0	25	1.13	0.27		0.6	1.28					2.2	0	2.2
	0	40	1.47	0.27		0.66	1.26					11.2	0	11.2
	0:10	25	1.68	0.29		2.61	1.47					3.8	0	3.8
	0:10	40	2.85	1.03		2.58	6.35					12.9	5.2	18.1
	0:30	25	2.63	0.64		3.74	3.18					10.6	3.2	13.8
	0:30	40	6.12	1.79		2.82	8.17					18.9	2.6	21.5
	24	25	12.23	2.63		2.96	8.04							
	24	40	12.01	2.72		2.07	8.56							

Blank: not determined.
* Calculated from [35].

energy) of the biomass were analyzed using three-factor permutational analyses of variance (PERMANOVA) [59], with species, washing time and washing temperature as fixed factors. The effect of washing treatments on the content of S, ash, protein and energy of the processed biomass were also analyzed individually. All analyses were conducted in Primer v6 (Primer-E Ltd., UK) using Bray-Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data. Tukey's multiple comparison was used to determine any differences between treatments. When there were significant interactions the variance component (% variance explained, η^2) was calculated to interpret the relative importance of the significant terms in the model [59].

3. Results

3.1. Salt yield

Species, washing time and washing temperature had an interactive effect on salt yield (% dw) (Pseudo-$F_{25,215}$ = 2.5, P = 0.0004, Fig. 1), with species explaining 50.8% of the variance, time 25.2%, and temperature 15.3%. *U. tepida* consistently had the highest yield of salt for any specific treatment and with a maximum yield of 36.6% compared to 29.3% in *U. ohnoi*. There was a high increase in salt yield between 0 and 2 h at 25 °C, from 4.5 to 21.8% dw for *U. ohnoi* and from 8.9 to 26.4% dw for *U. tepida*. There was also a sharp increase in salt yield between 0 to 30 min at 40 °C, or 0 to 10 min at temperatures >40 °C, while for longer washing times yield remained relatively constant for temperatures between 40 and 85 °C at 22–24% dw (*U. ohnoi*) and 29–30% dw (*U. tepida*). For *U. ohnoi* salt yield was highest at a 24 h washing time at 28–29% dw, while for *U. tepida* temperature had no effect at this washing time, with salt yield constant at approximately 28% dw. Overall, a longer washing time of up to 24 h at 25 °C, or a shorter washing time of 10–30 min at 40 °C are the optimum combinations for maximum salt yield while maintaining the shortest washing time (h) and lowest temperature (°C). Therefore, the time and temperature combinations of washing for 0, 10, 30 min, and 24 h at 25 and 40 °C were chosen for further analysis of the composition of the salt (pooled material, n = 1) and the processed (washed) biomass (n = 3).

3.2. Salt quality

The mineral composition of the salts produced from both *U. ohnoi* and *U. tepida* changed substantially with both temperature and time (Table 1, Supplementary Tables S1 and S2). Of the 23 elements analyzed (Tables S1 and S2), 8 to 12 were present at levels ≥10 mg kg^{-1} in salts from both species depending on treatments. Notably, the proportion of Na in salt from *U. ohnoi* decreased from 92% of the analyzed minerals in the washing for 0 min at 25 °C treatment to 63 and 61% in the 24 h at 25 °C and 40 °C treatments (Table 1). This led to a 7.1 and 7.5-fold decrease in the Na:K ratio from 16.4 to 2.2 and 2.3 for these treatments. There were also gradual increases in the amounts of Ca (from 0.54% to 1.30% of the elements analyzed), Mg (from 1.6% to 8.0%), and P (from 0.1% to 1.7%) with increasing washing times and temperatures, while there was a small decrease in the amount of Fe from 0.06% to 0.02%. The important trace elements B, Cu, Mn and Se all increased with increasing washing time and/or temperature (Table S1). These patterns of change in the mineral composition of the salts were similar but more pronounced for *U. tepida* (Tables 1 and S2). For example, the proportion of Na decreased from 90% to 38%, while K increased from 7.9% to 34%, leading to a 10-fold decrease in the Na:K ratio from 11.4 in the washing for 0 min at 25 °C treatment, to 1.1 in the washing for 24 h at 40 °C treatment. Increasing washing times and washing temperatures led to an increase in the proportions of Ca (from 0.41% to 1.95%), Mg (from 1.1% to 21.7%), and P (from 0.2% to 3.7%). There were also increases in the trace elements B, Mn and Mo.

The content of C and S in salts increased with increasing washing times and washing temperatures for both species, and was typically

Table 3
Compositional analysis (carbon, hydrogen, oxygen, nitrogen, sulfur, ash, and fiber content (% dw), higher heating value (MJ kg^{-1}) and protein content (% dw)), of the control biomass (n = 3, average ± SE), processed biomass post-washing (n = 3, average ± SE), and salt (pooled material, n = 1) of filamentous *Ulva tepida*.

	Treatment		Elemental composition (% dw)					Organic matter	Minerals	HHV	Protein*	Fiber (% dw)		
	(h)	(°C)	C	H	O	N	S	(% dw)	(% dw)	(MJ kg^{-1})	(% dw)	soluble	insoluble	total
Control biomass	na	na	23.8 ± 2.3	4.1 ± 0.3	12.7 ± 2.7	4.2 ± 0.4	2.5 ± 0.5	56.7 ± 4.4	43.3 ± 4.4	11.3 ± 1.1	15.2 ± 1.3	8.9 ± 0.9	10.9 ± 0.3	19.8 ± 0.8
Processed biomass	0	25	26.4 ± 1.4	4.5 ± 0.2	25.1 ± 2.2	4.6 ± 0.4	3.0 ± 0.4	63.4 ± 0.5	36.6 ± 0.5	11.4 ± 0.5	16.4 ± 1.5			
	0	40	25.9 ± 1.1	4.5 ± 0.2	26.1 ± 1.5	4.9 ± 0.1	2.4 ± 0.5	63.8 ± 2.7	36.2 ± 2.7	10.9 ± 0.5	17.6 ± 0.4			
	0:10	25	36.1 ± 4.3	5.8 ± 0.6	32.8 ± 2.4	5.4 ± 0.3	2.3 ± 0.6	82.4 ± 3.2	17.6 ± 3.2	15.8 ± 2.3	19.5 ± 1.1			
	0:10	40	37.1 ± 0.5	6.0 ± 0.1	33.2 ± 1.8	5.2 ± 0.0	2.4 ± 0.1	83.8 ± 2.1	16.2 ± 2.1	16.4 ± 0.6	18.7 ± 0.1			
	0:30	25	33.1 ± 1.2	5.4 ± 0.2	31.9 ± 2.9	5.5 ± 0.1	2.9 ± 0.6	78.8 ± 3.7	21.2 ± 3.7	14.4 ± 0.6	19.8 ± 0.3			
	0:30	40	36.8 ± 1.0	5.6 ± 0.1	32.9 ± 1.1	5.4 ± 0.2	2.4 ± 0.2	83.0 ± 2.3	17.0 ± 2.3	17.0 ± 2.4	19.4 ± 0.8			
	24	25	37.1 ± 1.5	6.0 ± 0.2	31.1 ± 1.0	5.3 ± 0.6	2.7 ± 0.2	82.2 ± 2.4	17.8 ± 2.4	16.5 ± 0.7	18.9 ± 2.0			
	24	40	37.6 ± 3.1	6.1 ± 0.4	32.4 ± 0.5	4.6 ± 0.6	2.1 ± 0.5	82.7 ± 4.0	17.3 ± 4.0	17.8 ± 3.0	16.4 ± 2.2			
Salt	0	25	0.98	0.33		0.36	0.83					3.9	0	3.9
	0	40	0.96	0.31		0.59	0.73					6.2	0	6.2
	0:10	25	0.76	0.42		1.29	0.78					3.5	0	3.5
	0:10	40	2.14	0.71		1.79	2.33					3.5	0	3.5
	0:30	25	0.64	0.40		1.27	0.23					9.2	0	9.2
	0:30	40	2.17	0.80		1.92	1.47					5.2	0	5.2
	24	25	5.39	1.25		2.07	2.72							
	24	40	6.17	1.26		1.04	2.60							

blank: not determined.
* Calculated from [35].

higher at 40 °C than at 25 °C at each washing time (Tables 2 and 3). This effect was strongest in *U. ohnoi*, with a 10-fold increase in the content of C and a 6-fold increase in the content of S after 24 h of washing at either 25 °C or 40 °C compared to washing for 0 min at 25 °C (Table 2). A similar pattern was evident for *U. tepida* (Table 3) where the content of C doubled after washing for 10 min at 40 °C compared with washing for 0 min, and increased by 5.5 to 6.4 times after 24 h of washing for both 25 °C and 40 °C. The content of S in the salt nearly doubled after 30 min of washing at 40 °C, while washing for 24 h at 40 °C led to a 3-fold increase.

The extraction of fiber (including soluble and insoluble fiber) from *U. ohnoi* biomass increased with washing time and was more effective at 40 °C than at 25 °C, leading to an increase from 2.2% of salt dw (0 min at 25 °C) to 13.8% (24 h at 25 °C) and from 11.2% (0 min at 40 °C) to 21.5% (24 h at 40 °C) (Table 2). Fiber was mainly present as soluble fiber, and insoluble fiber was only extracted in treatments 30 min at 40 °C, and 24 h for both 25 and 40 °C. The recovery of total dietary fiber from the untreated biomass into the salt varied from 0.5% at 10 min at 25 °C to 21.1% in the 24 h at 40 °C treatment, which also had the highest recovery of soluble fiber (18.6% of the soluble fiber in the untreated biomass). The extraction of fiber from *U. tepida* was variable, with no clear pattern between washing time or temperature, and the recovery of the total fiber from the untreated biomass into the salt varied between 3.9 and 13% (Table 3). Fiber was only present in soluble form in the salt from *U. tepida*, and ranged from 3.5% of the salt (30 min at 25 °C and 30 min at 40 °C treatments) to 9.2% (24 h at 25 °C) of the salt.

3.3. Biomass quality

The quality of the biomass of the two species differed in control biomass (not washed) in terms of the % of ash, C, H, O, N, S, protein, and fiber, and energy content (HHV (MJ kg^{-1})). The average content of C (30.4 ± 0.4% dw) and H (5.3 ± 0.1% dw) in the control biomass was approximately 20% higher in *U. ohnoi* (Table 2) than in *U. tepida* (Table 3), while the average content of minerals (ash) was 40% lower for *U. ohnoi* (25.4 ± 1.2% dw) than for *U. tepida* (43.3 ± 4.4% dw). This lead to higher energy content in *U. ohnoi* biomass at 14.8 ± 1.2 MJ kg^{-1} compared to 11.3 ± 1.1 MJ kg^{-1} in *U. tepida*. The contents of O (18.4 ± 3.1% dw) and S (4.0 ± 0.2% dw) were higher in *U. ohnoi*, while the content of N was similar between the two species at 5.1% (*U. ohnoi*) and 4.2% dw (*U. tepida*), resulting in *U. ohnoi* having a higher protein content (23.4 ± 0.7% dw) than *U. tepida* (15.2 ± 1.3% dw) (Tables 2 and 3) based on their respective nitrogen-to-protein conversion factors. *U. ohnoi* also had a higher content of both soluble (28% higher) and insoluble (35% higher) fiber than *U. tepida*. The ratio of fw:dw was 5:1 for *U. ohnoi* and 10:1 for *U. tepida*.

The washing treatments strongly influenced the overall quality of the processed biomass expressed as % of ash, C, H, O, N, S, and protein and energy content (HHV) for both species, with interactive effects between species and washing time (PERMAVOA, Pseudo-$F_{3,60}$ = 9.801, P = 0.0001) and washing time and temperature (Pseudo-$F_{3,60}$ = 2.62, P = 0.039) where species explained 49.0% of the variance, time 23.2%, and temperature 18.5%. Individual PERMANOVAs were also run for the parameters of contents of minerals, S, protein, and energy. For the content of minerals (ash) there were interactions between species and time (Pseudo-$F_{3,60}$ = 16.443, P = 0.0001), and between time and temperature (PERMANOVA, Pseudo-$F_{3,60}$ = 5.995, P = 0.001), with species explaining 39.6% of the variance, time 41.5% and temperature 7.5%. There was an interaction between species, time and temperature (Pseudo-$F_{3,60}$ = 3.935, P = 0.014) for the content of S in the biomass, with species explaining 52.6% and time and temperature explaining 16.1% and 9.7% of the variance, respectively. There was also an interactive effect between species and time on the content of protein (Pseudo-$F_{3,60}$ = 6.814, P = 0.0015), and species explained 92.8% of the variance, with only 2.9% explained by washing temperature. Finally, there was an

interactive effect between species and time on the content of energy ($\text{Pseudo-}F_{3,60} = 11.360$, P = 0.0002), but time explained 56.6% of the variance while species only explained 6.8%.

Washing effectively reduced the content of ash in the biomass (recovered as crystalline salt) with a mirrored increase in the content of organic matter (Tables 2 and 3). In *U. ohnoi* ash decreased from 25.4% dw in the control biomass to 18.6% dw after 30 min and 10.1% dw after 24 h at 25 °C (Table 2). Regardless of time, washing at 40 °C was more effective in removing ash, yielding an ash content of 10.6% dw after 30 min and 9.4% dw after 24 h. The content of S in *U. ohnoi* decreased from $4.0 \pm 0.2\%$ dw to a minimum of 2.2 to 2.3% dw after 24 h of washing at 25 and 40 °C, respectively. With the removal of inorganic minerals in the washing process (recovered as salt) and the resulting increase in the proportion of organic matter, the content of protein in *U. ohnoi* increased by approximately 10% across all treatments to a maximum of 27% dw of the processed biomass. Similarly, the contents of C and H in the processed biomass increased by 31% and 22%, respectively, leading to a 27% increase in the energy content of the processed biomass to a maximum of 18 MJ Kg^{-1} after 24 h of washing at 40 °C.

In *U. tepida*, ash decreased from 43% dw in control biomass to 36% dw after 0 min wash at both 25 and 40 °C, whereas all other treatments led to an ash content of 16–18% dw in the processed biomass (Table 3). The contents of S in *U. tepida* was only marginally affected by washing treatments and varied between 2.3 and 3.0% dw in the processed biomass compared to $2.5 \pm 0.5\%$ dw in the control biomass. With the removal of inorganic minerals in the washing process and the resulting increase in the proportion of organic matter, the contents of C and H increased substantially by 50 to 60% in the processed biomass to a maximum of 37.6% dw C and by 40 to 50% to a maximum of 6.1% dw H in treatments ≥10 min for both 25 and 40 °C, contributing to a 40 to 60% increase in the energy content to a maximum of 17.8 MJ Kg^{-1}. The content of protein in *U. tepida* increased by 8% (0 min, 25 °C) to 30% (30 min, 25 °C) to a maximum of 19.8% dw protein in the processed biomass (Table 3).

4. Discussion

Washing of biomass of *U. ohnoi* and *U. tepida* effectively reduced the mineral content of the biomass with concomitant production of a crystalline salt with a Na:K ratio of 1.1–2.2 and with a maximum salt yield of 29% of the original dry weight in *U. ohnoi*, and 33% in *U. tepida*. With the removal of minerals (ash), the proportion of organic matter and therefore content of protein of the processed biomass increased by 11–24%, while the content of energy (MJ kg^{-1}) increased by 20–50%. The production of salt for human consumption can therefore be a viable additional product path-way for the utilization of macroalgal biomass while simultaneously improving the quality of the processed biomass for further processing into fertilizer, animal feed or fuel, by increasing the content of organic matter, protein and energy.

4.1. Salt yield

Longer washing times and/or warmer temperature in general resulted in the highest yield of salt, of up to 30.5% of the dry weight of the algae. However, milder conditions (shorter washing times and/or cooler temperatures) may be desirable at an industrial scale to minimize energy inputs. For example, in the washing treatment of 30 min at 40 °C *U. ohnoi* had a salt yield of 23% dw. This was only improved by extending the treatment to 24 h, such that a 24 h wash at room temperature (25 °C) or at 40 °C yielded 28.5–29% dw salt. Although the salt yield was more temperature dependent in *U. tepida* with a more gradual increase in yield, the same treatment combinations (30 min at 40 °C; 24 h at 25 °C; or 24 h at 40 °C) optimized salt yields while minimizing the time and temperature required. The higher salt yield in *U. tepida* is most likely due to differences in morphology between the two species

Table 4
Typical content of minerals (mg kg^{-1}) in Himalayan Pink rock salt and Murray River pink salt as reported by the producer, and in regular iodized table salt.

Mineral (mg kg^{-1})	Himalayan pink rock salt[a]	Murray River pink salt[b]	Table salt[c]
Al	0.661		
Ba	1.96		
Br	2.1		
Ca	4050	515	
Cl	590,930	603,000	606,504
Co	0.6		
Cr	0.05		
Cu	0.56		
Fe	38.9	23	
I	<100	5	25–65
K	3500	128	
Li	400		
Mg	160	860	
Mn	0.27		
Na	382,610	391,000	393,322
Ni	0.13		
S	12,400	1148	
Se	0.05		
V	0.06		
Zn	2.38		
Na:K	109	3055	-

[a] http://www.saltnews.com/chemical-analysis-natural-himalayan-pink-salt/, accessed 2015-04-15.
[b] http://www.sunsalt.com.au/specifications.htm, accessed 2015-04-15.
[c] [78].

with *U. tepida* having a fw:dw ratio of 10:1, twice that of *U. ohnoi* and a 4-fold higher content of Na in control biomass.

4.2. Quality of salt

Quality is critical for the development of salt products and processing conditions. Here, the mineral composition of the salt improved from a nutritional and health perspective with longer washing times and/or higher temperature for both species of algae. The increase in the proportion of the major cations (K, Mg and Ca) other than Na with increasing washing time and temperature show a progressive extraction of internal minerals after the initial washing off of the external salt which is composed mainly of NaCl from the cultivation seawater, and this process forms the basis for the production of a truly unique salt product that is specific in composition and flavor. The mineral composition of salt from *U. tepida* is particularly interesting with Na and K each making up approximately 40% and 30% of the analyzed minerals respectively, with a Na:K ratio of 1.1 to 1.3 for the three selected washing treatments (30 min at 40 °C; 24 h at 25 °C; or 24 h at 40 °C). This is near the recommended Na:K dietary intake range of between 0.3–1 for humans [43–45], and is unique in comparison with regular table salt (100% NaCl, no K) and specialty salts such as Himalayan pink rock salt with a Na:K of 109 or Murray River pink salt with a Na:K of 3055 (Table 4).

The higher proportion of K is beneficial as K is critical in maintaining cellular function and metabolism, however, the dietary intake of K is often below the recommended daily intake levels [40,44]. An increase in the intake of K also attenuates high blood pressure and cardiovascular disease even when a high dietary intake of Na is maintained, and this effect is more pronounced when coupled with a decreased intake of Na [40,44]. Similarly, an increased intake of Mg attenuates markers of metabolic syndrome and systemic inflammation [60] as well as lower blood pressure [42], while an increased intake of Ca leads to improved Na excretion and a lowering of blood pressure [42]. A salt-product with a lower Na:K ratio and a higher content of the major cations of K, Mg, and Ca can therefore provide health benefits to consumers. As the main route of dietary intake of Na is through added salt (NaCl) incorporated into processed foods, condiments and bakery products [45], replacing the NaCl with a healthier alternative could contribute to

improvements in population health. For example, baking qualities, appearance, texture, or taste in a bread with a 32.3% reduced content of Na and an increased content of K, Mg, and Ca-salts was not significantly different from standard bread [61].

Soluble fiber in the form of the sulfated polysaccharide ulvan is another important component for the quality of the salt produced from both species of macroalgae. Both the control biomass and salt from *U. ohnoi* consistently had a higher content of soluble fiber than control biomass or salt from *U. tepida*. This is in agreement with the contents of ulvan in biomass of blade-like (15.8–31.5%) and filamentous (14.9–17.2%) species of *Ulva* [58,62,63]. For *U. ohnoi*, the 40 °C treatment consistently yielded the salt with the highest content of soluble fiber and elemental S, regardless of washing time. This could be expected as the targeted extraction of ulvan is often performed in aqueous solutions at increased temperatures of 80–90 °C for 1–3 h [48,64]. Longer extraction times (1 vs. 3 h) and warmer extraction temperatures (80 vs. 90 °C) also lead to higher yields [64]. Here, salt produced at temperatures higher than 40 °C were not analyzed for mineral or fiber composition as total salt yields did not increase substantially and this was the primary driver for treatment selection. However, it is likely that higher extraction temperatures (60–100 °C) would yield a higher content of soluble fiber in the salt as the extraction efficiency (transfer from original biomass to salt) of soluble fiber increased with increasing washing times and temperature for the analyzed salts. In *U. tepida* washing for 24 h at 25 °C yielded the highest content of soluble fiber in salt. The specific composition of ulvan can vary between species [48], and this may explain the differences in response to the treatments between the two species tested (i.e. maximum amount of soluble fiber extracted after 24 h at 40 °C in *U. ohnoi* vs. 25 °C in *U. tepida*).

The health benefits of an increased intake of soluble dietary fiber, and specifically ulvans, are numerous. Soluble dietary fiber benefits gut health and reduce blood cholesterol [65], and the soluble dietary fiber in *U. ohnoi* is proposed to play a key role in attenuating metabolic syndrome and improving glucose metabolism in diet-induced obese rats [66]. Ulvans also have antioxidant and antiviral properties which both correlate with increased degree of sulfation [67]. In *U. ohnoi* the 24 h treatment at 40 °C yield the salt with the overall highest potential for such functional properties in health applications across species and treatments, based on the content of soluble fiber and elemental S. In *U. tepida*, 24 h washing at 25 °C would be the most promising treatment for the development of a functional salt; however, the content of soluble fiber of this salt is still only 50% of the best salt from *U. ohnoi*.

Overall, considering salt yield and mineral composition only, the washing of *U. tepida* for 30 min at 40 °C or for 24 h at 40 °C are the most promising options for the production of a marine algal salt, with a lower Na:K ratio (1.1–1.2) and higher content of Ca and Mg than any of the salts produced from *U. ohnoi*. If the content of ulvans is also considered, the washing of *U. ohnoi* for 24 h at 40 °C is the best option for the production of a marine algal salt with a low Na:K ratio of 2.2 and functional properties derived from soluble fiber.

4.3. Quality of processed biomass

The washing treatments selected for the production of salts (24 h at 25 °C; 30 min at 40 °C or 24 h at 40 °C, see Section 4.1) led to a decrease in the contents of minerals (ash) in the biomass with a concomitant increase in the content of organic matter (100% — ash%) and energy (HHV). This improved the biochemical composition and therefore quality of the processed biomass particularly for the production of animal feeds, the production of biocrude through HTL, or for composting.

The increase in organic matter made the processed biomass more suitable for the inclusion in animal feed, in particular *U. ohnoi* with a maximum protein content of 25–27% dw and a low content of ash (9.4%). The protein content in the processed biomass for both species was higher than in most cereals and grains (8–16% protein) [68] used in animal feeds but this is still lower than decorticated cottonseed (~50%) [69] and whole soybeans (32–43%) [68], both common sources of protein for animal production. High-protein feed supplements are often added to the diet of beef cattle to improve feed utilization and energy conversion and thereby livestock productivity, and the use of *Ulva*, and macroalgae more generally, as a feed supplement for beef [17], poultry [70], shrimp [23], fish [71] and sea urchins [3,19] has been suggested previously. The quality of amino acids in both species of *Ulva* is also high, with 36.6–41% of total amino acids present as essential amino acids, and with good proportions of methionine (1–1.2%) and lysine (4.7–4.9%) [4,39] which are often the first amino acids to become limiting in pigs and poultry [72]. Furthermore, the high content of salt has previously restricted the level of inclusion of *Ulva* and other macroalgae as feed components [22] and the significant reduction from washing should provide for higher levels of intake or inclusion in diets prior to rejection by animals or exceeding dietary guidelines for mineral intake in animals [73].

Untreated biomass of *Ulva* spp. is a promising feedstock for the production of biocrude via HTL [35,54,56], and an improvement in the quality of the biomass as feedstock was evident after washing in freshwater through the removal of up to 43% of the ash prior to processing [35]. Here we showed that washing at a higher temperature or for a longer time period can further improve the biomass for this purpose by removing as much as 60% of the ash in both *U. ohnoi* and *U. tepida*. The C:N ratio of the processed biomass also increased with higher washing temperatures and longer washing times for both species, and together with the decrease in the contents of ash, this contributed to an increase in the energy content by 21 to 58%. A substantial reduction in S (15 to 45%) was also achieved for the 24 h washing times. This is highly beneficial for the production of a drop-in fuel as S forms harmful oxides during combustion and would need to be reduced in the crude through catalytic hydrotreating, an energy intensive process requiring large amounts of H_2, prior to refining to a drop-in fuel [35,74]. Although the content of O in the processed biomass also increased by 2 to 2.5 times, this may not be relevant as the content of O in biocrude as produced through HTL is independent of content of O in the macroalgal feedstock [35].

A higher C:N ratio of the algal biomass also means that a higher proportion can be incorporated into a feedstock mixture, for example with sugarcane bagasse, for the production of compost [29]. However, this advantage was only marginal for the changes in C:N ratios here, allowing for an inclusion of 85 to 87% of algal biomass mixed with sugarcane bagasse for a target C:N ratio of 22:1 [29]. The C:N ratios of the processed biomass coupled with the much lower contents of ash (9 to 17% dw) across the two species for the selected washing treatments in comparison with the content of ash in *U. ohnoi* biomass (36.8% ash) used for compost in [29], suggests the processed biomass of *U. ohnoi* and *U. tepida* would produce a high quality compost with a low conductivity that does not require further treatment prior to soil application.

4.4. Integrated production

Limited commercial production exists for biomass of *Ulva* spp. for use as abalone feed and remediation of abalone culture waste waters [3], and for high-value food products in Japan [75]. Although the current world-production is at a relatively small scale with <0.1% of global marine macroalgal production coming from green macroalgae [76], the same biological characteristics that enable the occurrence of nuisance blooms makes species of *Ulva* ideal for cultivation for biomass applications, including high areal productivity [3–5] and broad environmental tolerance [7,8]. On-land controlled cultivation also has the advantage of providing a continuous supply of biomass for the manufacture of bioproducts [4,49], and can act as a complementary source of biomass to wild collections of blooms, thus securing the supply-chain. The integrated production of *Ulva* and inclusion within the biorefinery concept, as the paradigm for the optimization of value from algae [46,77], is illustrated in Fig. 2 with the production of macroalgal salt as an initial and innovative first step in the value chain.

5. Conclusion

Natural macroalgal salts with beneficial mineral profiles and possible functional properties derived from soluble fiber (ulvan) can be produced through the washing of the marine green macroalgae *U. ohnoi* and *U. tepida* with freshwater and crystallizing the resulting minerals. Washing of *U. ohnoi* for 24 h at 40 °C produces the salt with the highest content of ulvan, while washing for 30 min at 40 °C or for 24 h at 25 °C provides optimized yield of salt from both species while minimizing time and temperature inputs. The selected treatments also increase the organic content and therefore the content of protein and energy of the processed biomass, which improves its quality as a feedstock for applications as compost, animal feed or biocrude. These species of macroalgae are well suited to in-sea and on-land cultivation under controlled conditions and can be sourced from natural blooms as a management option for these green tides.

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Author contributions

All authors contributed to the conception and design of the study, or acquisition of data, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and all authors approve of the final version to be submitted.

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