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A comparative assessment of microwave assisted (MAE) and conventional solid-liquid (SLE) techniques for the extraction of phloroglucinol from brown seaweed

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ABSTRACT

Brown seaweeds are rich in polyphenols with a basic building block of 1,3,5-trihydroxybenzene (phloroglucinol) and were investigated as a bioresource for the extraction of polyphenols for biopolymers and bioproducts. Species of seaweed with high contents of polyphenols were identified through meta-analysis and selected for the comparative assessment of the extraction efficiency of polyphenols using microwave assisted (MAE) vs. conventional solid-liquid (SLE) extraction. Out of ten species from Australia and New Zealand screened by SLE, *Carpophyllum flexuosum* (8.6%) and *C. plumosum* (7.5%) had the highest contents of polyphenols and were selected for MAE along with commercially available *Ecklonia radiata*. *C. flexuosum* was identified as the key species for extraction of polyphenols, with a 70% increase in yield using optimized MAE (aqueous, biomass:solvent ratio 1:30, 160 °C, 3 min) compared to SLE. The cell-wall bound fraction of polyphenols in brown seaweed may be larger than previously thought and is accessible through MAE.

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1. Introduction

Biomass has been identified as a primary alternative feedstock to crude oil for plastics and polymers, with demand for bio-plastics predicted to increase from 1.2 million tonnes in 2011 to 5.8 million tonnes in 2016 alone [1]. However, bio-based polymers and bio-based products have a much broader application across the polymer and chemical industries, including additive manufacturing [2]. Critical factors in the selection of a biomass resource for the production of bio-polymer or chemical feedstocks are productivity, scalability and continuity of supply [3]. Within this context macroalgae are attractive as they have high biomass productivities, are already produced at scale, and can be delivered as a continuous feedstock supply within an integrated bio-refinery process [3,4]. A critical step in the integrated industrial ecology framework is the selective and cascading extraction of targeted biochemical components to optimize the value of biomass [5,6], which can be achieved through hydrothermal processing. Despite the wealth of published original research and number of review articles concerning the hydrothermal processing of biomass, it is useful to clearly define several key terms that are used throughout this manuscript. In the context of thermochemical processing of biomass, hydrothermal liquefaction (HTL) generally refers to the use of hot, pressurized water (typically in the range of 250–374 °C, i.e., up to the critical temperature of water) for the dissolution, hydrolysis and chemical decomposition of solid biomass into several product fractions, see [7–9]. The closely related term hydrothermal upgrading (HTU), is often used interchangeably with HTL, and is most often encountered in the context of biofuels research where the emphasis is conversion of a portion of the biomass (lower calorific and economic value) into an energy-densified, and hence increased economic value, biocrude product. Here we use the term "soft HTU" to indicate that the processing conditions are milder (between 100 and 200 °C) than what is traditionally used for HTU in order to preserve some of the molecular structure (and therefore biological function) of the extracted species from the algae. In addition, we use the term microwave-assisted extraction (MAE) to specify that microwave heating is employed as a particularly efficient method for soft HTU: heating a slurry of algae in either water or a microwave-receptive solvent, to effect cell rupture and the release of soluble species derived from biopolymers into the reaction medium for subsequent isolation and purification. Use of focused microwaves is an energy efficient means to effect the selective extraction of carbohydrates, proteins and other biochemical fractions, where control of the sub-critical properties of the solvent

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(water in particular) is a key parameter for the efficient extraction of established and novel renewable materials for production processes [10, 11]. MAE offers additional advantages when the extraction solvent is water, as it uses wet biomass, thereby avoiding energy losses associated with drying. Under these "soft hydrothermal upgrading" (HTU) conditions extraction efficiency and the separation of products are potentially enhanced [12]. However, the ultimate success of selective extraction is also dependent on the biochemical composition of the biomass [13].

Brown seaweeds are rich in phlorotannins, polyphenols with a basic building block of 1,3,5-trihydroxybenzene (phloroglucinol) that are derived through the acetate – malonate metabolic pathway. Polymers range from low molecular weight of < 3.5 kDA to high molecular weight of > 100 kDa [14], and can be divided into four main classes according to the linkages between the phloroglucinol units: fucols (phenyl linkage), fucophlorethols (ether and phenyl linkages), phloroethols and fuhalols (ether linkages), and eckols (dibenzodioxin linkages) [15,16]. These unique phenolic compounds are ubiquitous in brown seaweed, and can contribute as much as 25% of the biomass dry weight [17]. The polyphenols make up structural components of the cell walls [18], and are also present in specialized cytoplasmic vacuoles called physodes [19] and have a defensive role in deterring herbivores [20,21], antimicrobial [22], and UV-protective functions [23]. Due to the ecological importance of brown seaweed in marine ecosystems, the content, internal distribution, and role of polyphenols in seaweeds has been researched extensively from an ecological and chemical ecology perspective [20,22,24]. More recently, brown seaweeds have also received much attention as feedstock for the extraction of polyphenols for use as antioxidants and nutraceuticals [reviewed in [25]]. Phloroglucinol, as the building-block of polyphenols, is also an extremely versatile building-unit for bio-based polymers, and can be used in polycarbonates, ubiquitous plastics [26], and act as branching agents for dendrimers [27], or replace compounds such as bisphenol A [28]. Furthermore, by blending phloroglucinol with other aromatic alcohol monomers, the full range of associated polymers in commercial use can be obtained, with applications in electronics and opto-electronics [29,30], colour and 3-D printing [31], and biocompatible glues [32], providing a very high value application to macroalgal biomaterials and innovative and novel applications in additive manufacture.

In this study, we propose for the first time, the use of the selective capabilities of soft hydrothermal upgrading to identify brown seaweed as a polyphenol source for phloroglucinol-based biopolymers and bioproducts. We propose that as polyphenols are found in structural tissue as well as cytoplasmic physodes [18,19], HTU will increase the yield of polyphenols compared to conventional solid-liquid extraction (SLE) with organic solvents. The specific objective of this study was therefore to comparatively assess the efficacy of soft HTU to extract polyphenols from brown seaweed with several other hot, pressurized solvents (MAE), and with conventional solid-liquid organic solvent extraction (SLE). Species of brown seaweed that may be suitable as feedstock for extraction of polyphenols were selected for the comparative assessment based on a meta-analysis of published research on the content of polyphenols in brown seaweed. Species that already have a closed life-cycle (i.e. can be farmed from zoospores to mature sporophytes that are used to produce the next generation of zoospores for seeding without relying on wild-collected brood stock), e.g., *Ecklonia* [33], and therefore have immediate potential for cultivation that can facilitate supply of biomass, were also included.

2. Methods

2.1. Meta-analysis of taxonomic and geographical variation in content of polyphenols

Species of brown seaweed with a high content of polyphenols were identified through meta-analysis. The literature search included the key words Phaeophyceae, phlorotannin, phenol, phloroglucinol and Folin in Web of Science and Google Scholar. Results were limited to publications in English, between the years 1977–2015, and using phloroglucinol as reference standard in order to enable a comparison of results. Results based on fresh weight or percentage of extract, where no fresh to dry weight conversion or extract yield was available, or expressed as a rate (μg g^{-1} h^{-1}), were excluded from the analysis. Reviews were also excluded to avoid duplication of data points. Results were normalized to percentage polyphenols per g of biomass on a dry weight (dw) basis. The data was organized by order, genus, and species within geographic region (tropical [0°–23.27°], sub-tropical [23.27°–38°] and temperate [>38°]). An average value of 10% dw polyphenols was determined as the cut-off point for further analysis of the distribution of content of polyphenols within an order, and subsequently between species in a genus, in order to select the most promising species for MAE. Additional species were selected to include tropical specimens and species that already have a closed life-cycle, e.g., *Ecklonia* [33], and therefore have immediate potential for cultivation.

2.2. Biomass collection

Wild collected biomass of the selected species (Table 1) was immediately transported to the laboratory in cooled zip-lock bags filled with seawater from the collection site, where it was rinsed in freshwater to remove debris, epiphytes and fauna before being dried to a maximum of 10% internal moisture (oven, 60 °C, 24 h). Dried intact biomass was then shipped to James Cook University (JCU), Townsville, Australia, and milled to 1 mm and stored at −20 °C in vacuum sealed bags with silica gel desiccant until extraction of polyphenols.

2.3. Solid-liquid extraction (SLE) of polyphenols

Polyphenols were extracted from oven-dried biomass (0.5 g dw measured to 0.1 mg precision) of selected (Section 3.1 and Table 1) species of seaweed by traditional SLE. Biomass samples ($n = 3$ per species) were extracted sequentially in 20 mL acidified methanol (HPLC grade, 50%, aq., pH 2, 24 h), followed by 20 mL acetone (AR grade, 70%, aq., pH 2, 24 h) in the dark on a rotating table (100 rpm) at room temperature as modified from Zhang et al. [34] and Targett et al. [35]. The samples were centrifuged at 3000 rcf for 15 min between extractions and the supernatants were collected and pooled, then filtered through 0.25 μm PTFE syringe filters prior to determination of the content of polyphenols as described in Section 2.5.

2.4. Microwave assisted extraction (MAE) and soft hydrothermal upgrading (HTU) of polyphenols

Method development for the extraction of algal polyphenols by MAE was performed by systematically varying the extraction solvent (H_2O, acetone, ethanol, propan-1-ol, ethyl acetate), and the biomass:solvent ratio (1:5, 1:10, 1:20, 1:30, and 1:40) individually, and extraction temperature (135, 160, and 185 °C) and holding time (1, 3, 5, 10, 15, and 20 min) simultaneously using the best solvent and biomass:solvent ratio of those tested. Experiments were performed with a focused microwave system (Anton Parr Microwave Synthesis Reactor, Monowave 300). Slurries were prepared from 0.5 g (measured to 0.1 mg precision) of dried algal biomass, immersed in a given amount of solvent in a capped 30 mL pyrex microwave tube. All samples were heated to the target temperature within a 2-minute ramp, held under autogenous pressure for the desired time, then cooled to 55 °C before depressurization. Samples were stirred at 600 rpm throughout the extraction process. The solid residue was removed by filtration to yield the crude extract, which was diluted to 50 mL in volumetric flask prior to determination of the content of polyphenols as described in Section 2.5. Solvents were AR grade. Method development for MAE was performed using separate samples of *Carpophyllum flexuosum* for the solvent, biomass:solvent ratio, time and temperature, and species:extraction

Table 1
Algae collection dates and locations.

		Species	Collection date	Location
Australia	Temperate (Tasmania)	*Cystophora subfarcinata*	2014-11-21	Beechford (41.023° S, 156.844° E)
		C. moniliformis	2014-11-21	Beechford (41.023° S, 156.844° E)
		C. brownii	2014-11-21	Beechford (41.023° S, 156.844° E)
		C. torulosa[a]	2014-11-21	Beechford (41.023° S, 156.844° E)
		Phyllospora comosa[a]	2014-11-13	Fortescue Bay (43.123° S, 147.976° E)
		Ecklonia radiata	2014-11-13	Fortescue Bay (43.123° S, 147.976° E)
		C. siliquosa	No biomass present	na
		Sargassum bracteolosum	No biomass present	na
	Tropical[b]	*Cystoseira trinodis*	2013-08-22	Nelly Bay (19.173° S, 146.846° E)
		Sargassum flavicans	2013-08-22	Nelly Bay (19.173° S, 146.846° E)
		Spatoglossum macrodontum	2013-08-22	Nelly Bay (19.173° S, 146.846° E)
New Zealand	Sub-tropical	*Carpophyllum flexuosum*, wave-exposed	2014-07-27	Takatu Point, Tawharanui Peninsula, (36.362° S, 174.861° E)
		C. flexuosum, wave-sheltered	2014-07-22	Ti Point, Leigh (36.313° S, 174.783° E)
		C. plumosum	2014-07-24	Takatu Point, Tawharanui Peninsula, (36.362° S, 174.861° E)
		C. maschalocarpum	2014-07-22	Ti Point, Leigh (36.313° S, 174.783° E)
		C. angustifolium	2014-07-24	The Outpost, Leigh (36.291° S, 174.820°E)
		Sargassum sinclairii	No biomass present	na

na: not applicable.
[a] Opportunistically collected due to abundance, not selected on the basis of reported high content of polyphenols.
[b] Abundant tropical species, not targeted based on literature reports of high-phenol content.

method studies, respectively, (see Section 3.3), and once the operating conditions were determined, all species selected for the comparison of extraction methods were processed in parallel (i.e. new replicate samples of *C. flexuosum* were processed using the final processing conditions at the same time as the other selected species).

2.5. Analysis of polyphenols

Content of polyphenols was quantified spectrophotometrically using the Folin-Ciocalteu phenol reagent assay (hereafter FC-assay) [36], which is the standard technique for the quantification of polyphenols [37]. For the SLE extracts the method was adapted for 96-well microplates [34] with slight modifications as described in Magnusson et al. [38], and quantified on a Spectramax Plus (Molecular Devices, Australia) at James Cook University. The extracts resulting from microwave assisted extraction (MAE) were analyzed using the same modified FC-assay [34,38] but scaled up by a factor of 10 to volumes suitable for 4 mL cuvettes (Livingstone; polystyrene; 10 mm × 10 mm × 45 mm) and quantified on a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Australia) at the University of Sydney. Phloroglucinol was used as reference standard, and the content of polyphenols expressed as % phloroglucinol equivalents (PGE) in algal biomass dw. Replicates ($n = 3$) were extracted on separate days and each extract was analyzed in triplicate and the absorbance averaged. For the microplate assay, a new phloroglucinol standard curve was included on each microplate. Phloroglucinol, Folin-Ciocalteu reagent, and Na_2CO_3 were from Sigma Aldrich, Australia. Solvents were HPLC (methanol) or AR (acetone) grade.

2.6. Statistical analyses

Separate one-way analyses of variance (ANOVA) (Statistica v.12, StatSoft) were used to determine the effects of solvent, and of biomass:solvent ratio, on the yield of polyphenols (as PGE) during method development of the MAE protocol. Two-factor ANOVA were used to determine the combined effects of the factors MAE processing time and temperature on the yield of polyphenols, using the chosen solvent and biomass:solvent ratio. Two-factor ANOVA was also used to determine the effects of extraction method (SLE vs. optimized MAE) on each of 3 species of seaweed on the yield of polyphenols, with extraction method and species as the factors. Data were log-transformed to improve homogeneity of variances if Levene's test for homogeneity of variances was significant. Where ANOVA resulted in a significant difference in means ($\alpha = 0.05$), Tukey's multiple comparison tests were used to compare the means of treatment groups. 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.

3. Results

3.1. Taxonomic and geographical variation in polyphenols content

A total of 100 species of brown seaweed representing 39 genera were included in the analysis; with 19 species from the temperate region, 58 from the sub-tropical, and 34 from the tropics (*Dictyota dichotoma*, *Lobophora variegata*, *Sargassum fluitans*, *S. linearifolium*, and *Stypopodium zonale* were reported from both tropical and sub-tropical regions, *Sargassum muticum* was reported from both temperate and sub-tropical regions, and *Turbinaria ornata* was reported from tropical, sub-tropical and temperate regions). This data was sourced from the 30 references

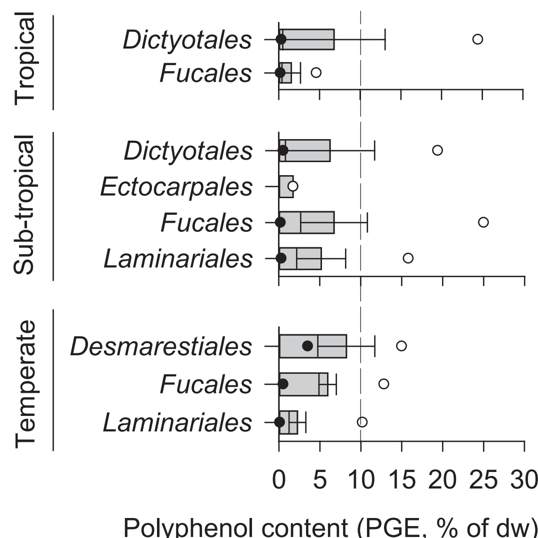

Fig. 1. Average content of polyphenols (PGE, % of algal dw) + SD for Phaeophyceae orders occurring world-wide based on literature. Black and white dots represent minimum and maximum content of polyphenols, respectively. The vertical line demarcates the minimum content of polyphenols of 10% set for investigating content of polyphenols to genus and species level within that order. Data compiled from [17,20,24,35,43,49–51,53,54,63–82].

that fulfilled the criteria for inclusion in the analysis (Table S1). The meta-analysis shows distinct differences in the average content of polyphenols in brown seaweeds both at taxonomic and geographical levels, but also large variation within each of those levels (Fig. 1). The average content of polyphenols between orders of seaweed from the temperate region ranged from 2.2 ± 2.2% (Laminariales) to 8.2% ± 3.4% (Desmarestiales), and this was the highest average content of polyphenols globally within an order. The average content of polyphenols between orders of seaweed from the sub-tropical region ranged from 1.7 (Ectocarpales, $n = 1$ species) to 6.6 ± 4.2% (Fucales), with sub-tropical Fucales having the highest maximum reported content of polyphenols globally at 25% of dw. In tropical seaweed, the average content of polyphenols was 1.5 ± 1.1% (Fucales), the lowest average for any order globally, and 5.4 ± 5.4% (Dictyotales).

Temperate Desmarestiales, Fucales and Laminariales, sub-tropical Dictyotales, Fucales and Laminariales, and tropical Dictyotales all had at least one species reported with a content of polyphenols above 10% of dw (Fig. 2). In temperate regions, the species with >10% polyphenols were *Desmarestia anceps*, *Fucus vesiculosus*, and *Macrocystis pyrifera*. In sub-tropical regions the species with >10% polyphenols were *Lobophora variegata* and *Stypopodium zonale* (Dictyotales), *Carpophyllum angustifolium*, *C. flexuosum*, *C. maschalocarpum*, *Cystophora brownii*, *C. moniliformis, C. siliquosa, C. subfarcinata, Sargassum bracteolosum,* and *S. sinclairii* (Fucales), and *Ecklonia radiata* (Laminariales). In the tropical region the species with >10% polyphenols were *Lobophora variegata* and *Stypopodium zonale* (Dictyotales).

Given the taxonomic distribution and abundance of large foliose species of seaweed with >10% polyphenols in Australia and New Zealand, further emphasis was placed on the distribution of content of polyphenols in seaweed from this region to facilitate the collection of fresh biomass for the experimental section (Fig. 2, species labelled with AU for presence in Australia, and NZ for New Zealand). Data was available for 21 genera from Australia, and 8 genera from New Zealand. Seaweed from New Zealand (sub-tropical) had overall higher content of polyphenols, with the genera *Carpophyllum*, *Sargassum* and *Ecklonia* having mean content of polyphenols above 10% of dw or a STDEV overlapping the 10% cut-off point (Fig. 2). In Australia, only sub-tropical *Cystophora* had a mean content of polyphenols above 10% of dw, while *Ecklonia* and *Sargassum* both had a maximum reported content of polyphenols of >10% and were included in the analysis to species level. Tropical species in Australia all had both average and maximum contents of polyphenols below 3% dw. This meta-analysis enabled us to select ten species from Australia and New Zealand for initial organic solvent extraction based on their reported high content of polyphenols. *Cystophora subfarcinata*, *C. siliquosa*, *C. moniliformis*, *C. brownii*, and *Sargassum bracteolosum* were targeted for collection in Tasmania, Australia, while *Carpophyllum flexuosum, C. angustifolium, C. maschalocarpum*, *C. plumosum*, and *Sargassum sinclairii* were targeted for collection from New Zealand. At the time of collection however, samples of *C. siliquosa, S. bracteolosum,* or *S. sinclairii* were not obtained due to lack of available biomass at the selected locations. *C. flexuosum* was collected from both a wave-exposed and a sheltered area, as the content of polyphenols in this species has previously been shown to differ between these habitats [17]. *Cystophora torulosa, Phyllospora comosa,* and *Ecklonia radiata* were collected opportunistically from Tasmania, as these species were highly abundant at the time of collection, and species of *Ecklonia* are also already commercially utilized for the production of alginate and also as abalone feed in South Africa [39] and has a closed life cycle [33]. *Cystoseira trinodis*, *Sargassum flavicans* and *Spatoglossum macrodontum* were collected from Magnetic Island in North Queensland, Australia, as representatives of abundant tropical species. Biomass collection dates and locations are provided in Table 1.

3.2. Solid-liquid extraction (SLE) of polyphenols

Of the 13 seaweeds analyzed using conventional SLE in this study, three species from NZ had a content of polyphenols higher than 5% of dw, with wave-exposed *Carpophyllum flexuosum* (8.6 ± 0.2%) and *C. plumosum* (7.5 ± 0.1%) having the highest contents of polyphenols (as PGE) (Fig. 3). Species from Tasmania (Australia) had much lower contents of polyphenols, ranging from 0.2 ± 0.01% in *Phyllospora comosa* to 2.2 ± 0.1% in *Cystophora subfarcinata,* while the tropical species ranged from 1.5 ± 0.5% in *S. flavicans* to 2.5 ± 0.4% in *C. trinodis*. Consequently, wave-exposed *C. flexuosum* and *C. plumosum* (both from NZ) were selected for further processing through MAE based on their high content of polyphenols. *Ecklonia radiata* collected in Tasmania was also included for MAE despite its comparatively low content of polyphenols (1.5 ± 0.2%) as it is already commercially utilized and therefore the pathway to the physical realization of a biorefinery model is shorter.

3.3. Microwave assisted extraction (MAE) and soft hydrothermal upgrading (HTU) of polyphenols

Based on the results from SLE, *C. flexuosum* was chosen as the species for MAE optimization studies to determine the optimum solvent, biomass:solvent ratio, and processing temperature and time. Solvent optimization experimentation with the focused microwave showed significant differences in the yield of polyphenols between solvents (ANOVA, $F_{4,\ 10} = 448.705$, $p < 0.001$, Table 2), with water being the most receptive polar solvent for microwave energy absorption, and also the most suitable solvent (of those tested) for the efficient extraction of the phenolic fraction yielding 11.4% polyphenols g dw^{-1} from *C. flexuosum.* This was significantly higher than all other solvents (Fig. 4 a, Tukey's HSD post-hoc, $p < 0.05$), with ethanol (8.4% PGE g dw^{-1}) as the second most effective solvent, followed by acetone and propan-1-ol, and ethyl acetate as the least effective solvent. MAE using acetone, propan-1-ol, and ethyl acetate all yielded extracts with 8 to 34-fold lower content of polyphenols than the traditional SLE.

Having selected the solvent, the ratio of *C. flexuosum* biomass to water was varied and determined to play an important role in extraction efficiency (Fig. 4 b) (ANOVA, $F_{4,\ 10} = 39.874$, $p < 0.01$, Table 2) with a near 2-fold increase in phenol yield at the ratios of 1:30 and 1:40 compared with ratios of 1:5, 1:10 and 1:20 (Fig. 4b, Tukey's HSD post-hoc, $p < 0.05$). Hence, the ratio of 1:30 was selected to attain the highest extraction efficiency while consuming the least volume of solvent.

Focused microwave heating resulted in high extraction yields in only a few minutes with an interactive effect of reaction time and temperature (Fig. 4 c) (ANOVA, $F_{10,\ 36} = 21.2$, $p < 0.01$, Table 2), with 55.5% of the variance explained by temperature, and 20.0% by time. At 185 °C particularly, increasing the reaction time from 1 to 20 min, lead to a decrease in the yield of polyphenols of approximately 40% with heating at 185 °C for 10, 15 or 20 min resulting in the lowest yield of polyphenols of all treatment combinations (Fig. 4 c, Tukey's HSD, alpha < 0.05). While yields were similar for a range of treatments (i.e. not statistically different, Tukey's HSD, alpha > 0.05), heating at 160 °C for 1, 3 or 5 min resulted in the highest yields of polyphenol at above 15%, while heating at 160 °C for 10 or 15 min, or 135 °C for any of the processing times tested, all resulted in yields of polyphenols above 14% but below 15%. Time and temperature conditions were therefore chosen as 160 °C for 3 min as this processing combination resulted in the highest numerical yield with the lowest variance between replicates. The changes in polyphenol yields in response to treatment conditions will be subject of a subsequent paper. The optimal soft HTU conditions of a biomass:water ratio of 1:30, processed at 160 °C for 3 min were established and then applied to new samples of *C. flexuosum*, *C. plumosum* and *Ecklonia radiata* in parallel for comparative assessment of these conditions with the conventional SLE (Fig. 5). There was an interaction between extraction method and species (ANOVA, $F_{2,\ 12} = 127.721$, $p < 0.01$, Table 2) on the yield of polyphenols in the extract. 82.6% of the variance was explained by species, with a 70% higher yield for *C. flexuosum* and *E. radiata* extracted by soft HTU compared with SLE, but no effect on yield in *C. plumosum* (Tukey's HSD, alpha < 0.05). Regardless of extraction method,

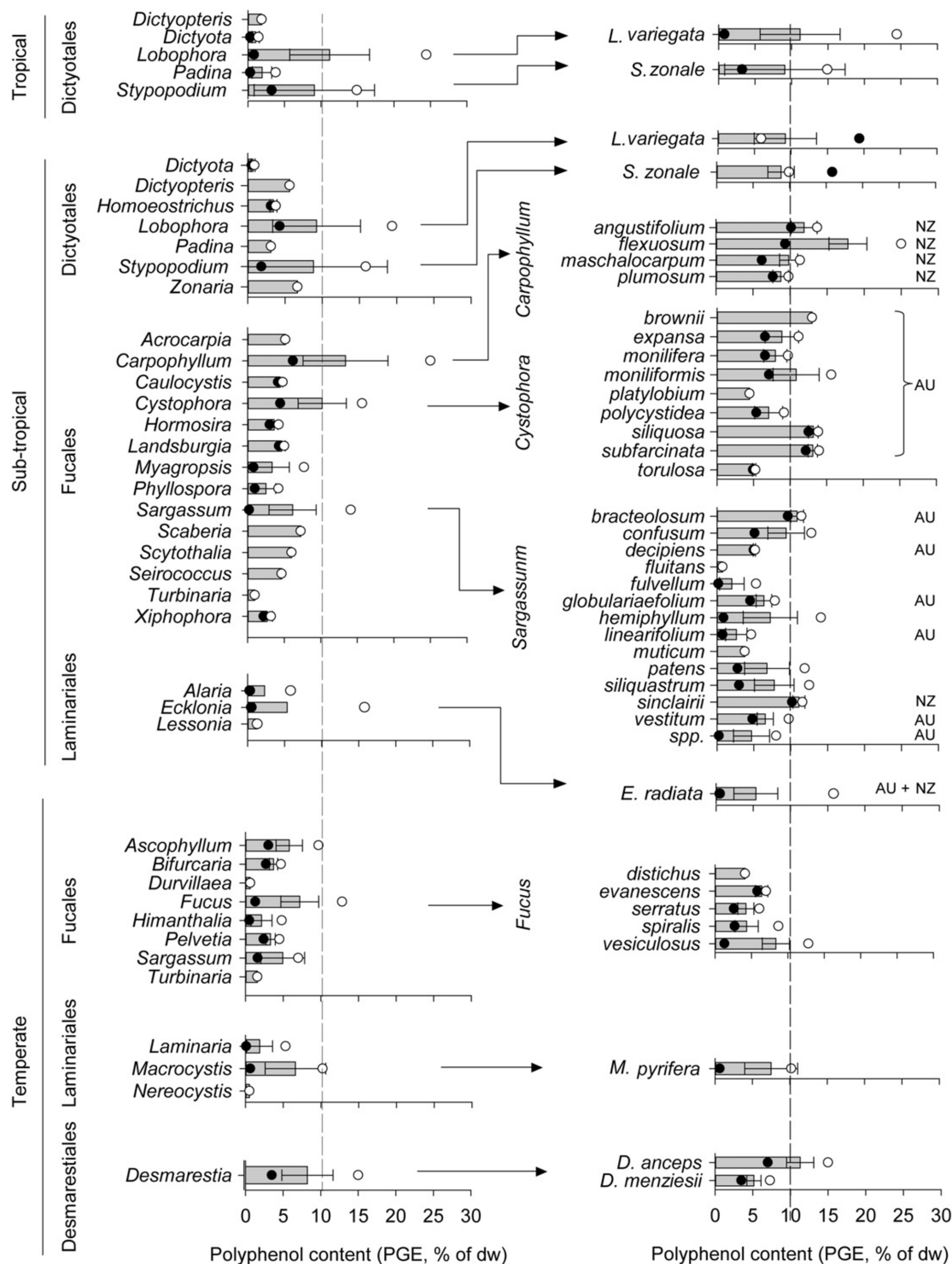

Fig. 2. Average content of polyphenols (PGE, % of algal dw) + SD for genera (left panel) and species (right panel) of brown seaweed occurring world-wide and with a maximum exceeding 10% at the level of order (see Fig. 1 for references). Species occurring in Australia and in New Zealand are labelled with AU and NZ, respectively. Black and white dots represent minimum and maximum content of polyphenols, respectively. The vertical line demarcates the minimum content of polyphenols of 10% set for investigating content of polyphenols to species level within that genus. Arrows connect species with the correct genus.

however, there was consistency in terms of the ranking between the species, such that *C. flexuosum* > *C. plumosum* > *E. radiata.*

4. Discussion

We identified distinct differences in the average content of polyphenols in brown seaweeds both at taxonomic and geographical levels, and also large variation within each of those levels. These distribution patterns based on conventional SLE extracts allowed for the selection of potential species for production of polyphenols in a biorefinery model. *Carpophyllum flexuosum* (8.6% dw) had the highest content of polyphenols of the species screened here. The optimized HTU conditions (aqueous extraction with a biomass:water ratio of 1:30 at 160 °C for 3 min) then led to a near doubling in the yield of polyphenols from this species compared to conventional SLE using organic solvents.

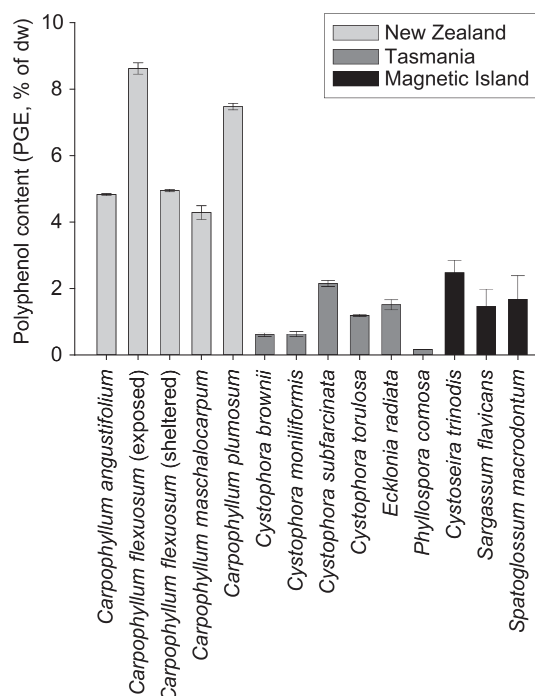

Fig. 3. Average content of polyphenols (PGE, % of algal dw) of selected brown seaweed from Australia and New Zealand based on traditional solid-liquid extraction (SLE) with organic solvents (sequential extraction with aqueous methanol (50% aq., pH 2), followed by acetone (70%, aq., pH 2), biomass:solvent ratio 1:40, 24 h each). Data are mean ± SE ($n = 3$).

4.1. Taxonomic and geographical variation in polyphenols content

The broad pattern of higher concentrations of polyphenols in species from temperate (specifically temperate Australasia) and subtropical regions than from the tropical Indo-Pacific (but not the Caribbean) has been highlighted previously [24,35,40]. This geographical variation in polyphenols content has received much attention both due to the ecological importance of habitat-forming brown seaweeds [40] and the fact that this trend is opposite to that for other deterrent compounds produced by seaweeds, e.g., terpenoids in Dictyotales (brown seaweed) or acetogenins in the red seaweed *Laurencia*, which are typically higher at low latitudes [41,42]. Grazing pressure [40], temperature [42] and trace element availability [43] have been implicated as drivers for this latitudinal gradient. Life-history stage [44], availability of light and nutrients [45,46] and combinations of local grazing pressure and nutrient availability [47] are some of the suggested drivers for differences within species. The ultimate drivers for the distribution patterns of the content of polyphenols in brown seaweed remain unclear; however, it is likely that the cause is a combination of these factors and to a certain extent also species-specific as there is potential for inducing an increased production of polyphenols in some species [48,49] but not in others [50,51].

In this study, biomass of *C. flexuosum* collected from an exposed site had 43% higher content of polyphenols than biomass collected from a sheltered site. This pattern of distribution of polyphenols has been described earlier for *C. flexuosum* [17]. The two forms (wave-exposed and sheltered) exhibit different morphologies, and wave exposed individuals that were transplanted to a sheltered habitat assumed the morphology characteristic of sheltered sites [52] indicating that environmental conditions are driving these morphological differences. Similarly, the high variance in content of polyphenols in decumbent L. *variegata* from the Caribbean is also partially due to distinct differences between specimens collected from deep (average 14.6% dw) vs. shallow (average 8.3% dw) habitats, and partially due to difference between morphologies (decumbent, encrusting, or ruffled), although the patterns are not as clear-cut as for depth [35,53]. It is unclear if the differences in the content of polyphenols are driven by environmental factors or by genetic factors in any of these species, however, both these scenarios could be taken advantage of if developing cultivation, by strain selection, or when choosing location to wild harvest biomass from. Concentrations of polyphenols in biomass collected for this study were typically lower than reported previously for the same species, e.g., *C. flexuosum* with 8.6% dw, compared with previously reported 16–25% dw for similarly wave exposed specimens [17], or *C. subfarcinata* with 2.2% compared with previously reported 12–14% for extracts from fresh, wet, biomass collected at a similar time of the year [24]. Collection time (season) as well as processing methods (dried or fresh biomass, method of drying, etc.) differed among studies included in the meta-analysis and the current study, and these parameters can affect the concentrations of polyphenols [54,55]. The samples for the current study were collected during winter (July–August, NZ and tropical Australia) and spring (November, temperate Australia), at a time when biomass was abundant at each specific location, then oven-dried and stored (for maximum 4 weeks prior to SLE), which can explain the differences.

Table 2
Results of one-way ANOVAs testing the effects of solvent, and biomas:solvent ratio on the yield of polyphenols during method development for microwave assisted extraction (MAE); two-factor ANOVA testing the effects of time-temperature combination on the yield of polyphenols during method development for MAE; and two-factor ANOVA testing the effects of extraction method for 3 species of brown seaweed on the yield of polyphenols.

	Source	df	SS	MS	F	p
Solvent	Solvent	4	5.660	1.415	448.705	0.0000
	Error	10	0.031	0.0031		
	Total	14	5.692			
Biomass:solvent ratio	Ratio	4	102.834	25.709	39.874	0.000004
	Error	10	6.447	0.645		
	Total	14	109.282			
Time- temp[a]	Time	5	0.097	0.019	40.2	0.0000
	Temp	2	0.270	0.135	279.4	0.0000
	Time × temp	10	0.102	0.010	21.2	0.0000
	Error	36	0.0174	0.00048		
	Total	53	0.487			
Extraction method	Species	2	282.993	141.496	1232.153	0.0000
	Method	1	29.070	29.070	253.139	0.0000
	Species × method	2	29.334	14.667	127.721	0.0000
	Error	12	1.3780	0.115		
	Total	17	342.775			

[a] Analysis was performed on log-transformed data.

4.2. Microwave assisted extraction and soft HTU

Aqueous soft HTU (160 °C, 3 min) appears to be the optimum processing approach for the extraction of polyphenols from *C. flexuosum* and *E. radiata*, leading to 70% increase in yield of polyphenols with no need for the use of large volumes of organic solvents in the process, and within a greatly reduced timeframe compared with traditional SLE (3 min vs. 48 h). In addition to using a more environmentally friendly solvent (i.e., water), soft HTU is advantageous from a life cycle assessment (LCA) perspective as wet biomass can be used in the process, thereby omitting the costly process of drying, while at the same time potentially avoiding loss of biological activity of the polyphenols if the targeted use is for antioxidant purposes [55]. Extracting fresh, wet, biomass through HTU may also increase yield as losses due to drying or storage are limited [24,55]. Previously, traditional solid-liquid extraction of seaweed at room temperature using water or aqueous ethanol afforded a higher yield of polyphenols (as % of dw biomass) than pressurized liquid extraction (120 °C at 1500 psi for water, 100 °C at 1000 psi for aqueous ethanol, 30 min from start of heating to depressurization) [56]. In addition to the longer processing times, the

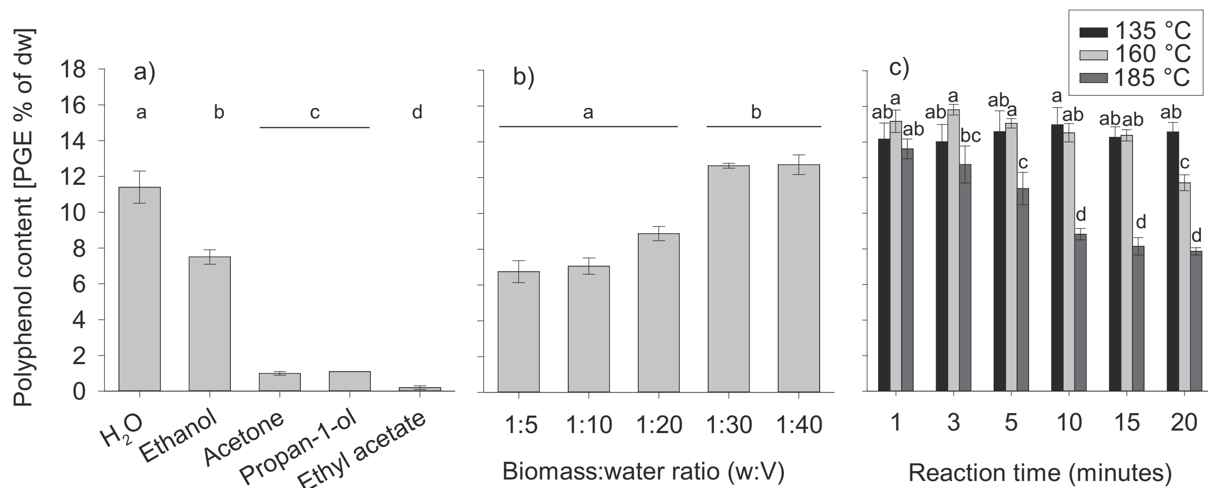

Fig. 4. Optimization of soft microwave assisted extraction (MAE) of polyphenols from wave-exposed *Carpophyllum flexuosum* (a) for solvent, (b) in water, variation of alga to water ratio at 185 °C, 5 min, (c) temperature and time combinations for extraction at the optimal ratio of biomass:water of 1:30. Homogenous groups are shown by the same letter ANOVA followed by Tukey's HSD, $p < 0.05$. Data are mean ± SE ($n = 3$).

biomass:solvent ratios were only 1:10 (*w*/*v*) for EtOH/water and acetone/water extractions, and 1:20 (*w*/*v*) for aqueous extractions [56]. At these ratios, the yield of polyphenols in the current study was approximately 50% of the yield afforded using the best ratio of 1:30.

A four- to 62-fold increase in the yield of total phenols (as % of dw, crude extract yield estimated from Fig. 1 in Plaza et al. [57]) from 6 species of seaweeds was previously demonstrated using subcritical water extraction when increasing the processing temperature from 100 °C to 200 °C with a 20 min processing time [57]. This species dependence is also observed here as yield increases varied from no change for *C. plumosum*, to a 70% increase for *C. flexuosum*. Polyphenols are present in cytoplasmic physodes and also comprise an integral part of the cell walls of brown seaweed [18,19]. Debate remains as to the main function of polyphenols as a primary structural element vs. a role mainly as a secondary metabolite [18,58,59]. However, the standard technique of SLE with aqueous organic solvents for quantification only represents the cytoplasmic fraction [58] while the covalent ester and hemiacetal bonds between the polyphenols and the cell wall (alginic acid) require harsher conditions (1 M NaOH 80 °C for 2 h 30 min) for extraction [58]. Only two studies have separated and quantified the insoluble cell-wall bound and soluble cytoplasmic pools of polyphenols [58,60], and the proportion of cell-wall bound polyphenols has been generalized as approximately 10% of the total phenol pool based on the earlier work with temperate *Fucus vesiculosus* (9.5%) [58]. Recently, however, the cell-wall bound pool of polyphenols was demonstrated to make up 10–15% (*Ascophyllum nodosum*) and 20–25% (*F. vesiculosus*) [60] of the total pool. While forcing HTU conditions (340 °C and above) can result in the partial conversion of monosaccharides, e.g., glucose, to aromatic species such as 1,2,4-benzene triol [61,62], it is unlikely that the mild HTU conditions employed in this study would effect this conversion. Therefore, it is reasonable to assume that the increase in yield of polyphenols resulting from extraction with the optimized HTU method is due to the release of cell-wall bound polyphenols and not from other biopolymers present in the cell. This pool ranges from ~6% (*C. plumosum*) to ~40% (*C. flexuosum* and *E. radiata*) in the species tested here. This indicates that use of the traditional SLE method may significantly underestimate the total content of polyphenols in brown seaweed, and that this underestimation differs between species. While debate remains regarding the importance of polyphenols as a primary (structural) metabolite in brown seaweed due to the previously measured low proportions (10%) [58] of polyphenols in the cell-wall bound fraction, the data presented here (up to 40% as cell-wall bound) and in [60] (up to 25%) support such a function. The cell-wall bound fraction of polyphenols was also suggested to play a role in metal detoxification, with increases of 3.9–6.5% in the proportion of cell-wall polyphenols after exposure of *A. nodosum* and *F. vesiculosus* to elevated levels of copper [60]. Clearly, the distribution, and the turnover and degradation processes, of polyphenols between the cytoplasmic and the cell-wall bound fractions require further investigation before the physiological and ecological implications are fully understood.

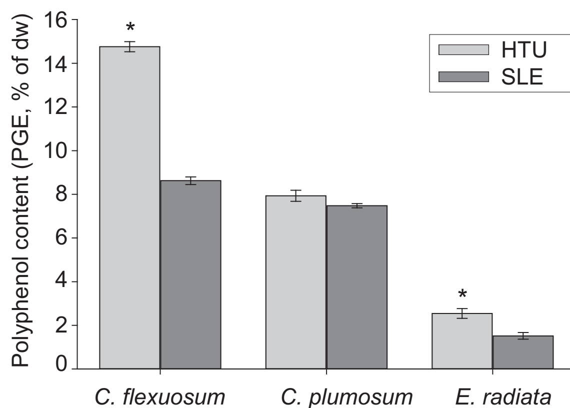

Fig. 5. Average content of polyphenols (PGE, % of algal DW) based on traditional solid-liquid extraction (SLE) with organic solvents (sequential extraction with aqueous methanol (50% aq., pH 2), followed by acetone (70%, aq., pH 2), biomass:solvent ratio 1:40, 24 h each) and soft hydrothermal upgrading (HTU, aqueous, biomass:solvent ratio 1:30, 160 °C, 3 min) of wave-exposed *Carpophyllum flexuosum*, and *C. plumosum*, and *Ecklonia radiata*. Data are mean ± SE ($n = 3$). * denotes extracts that are significantly different within a species depending on extraction method (ANOVA followed by Tukey's HSD, $p < 0.05$).

5. Conclusion

Carpophyllum flexuosum was identified as the key species for extraction of polyphenols in Australasia. The cell-wall bound fraction of polyphenols in brown seaweed may be larger than previously estimated, and quantifying the soluble portion of polyphenols from extracts obtained using traditional SLE with organic solvents can therefore greatly underestimate the total content. The large (up to 70%) increase in yield of polyphenols extracted through optimized soft HTU offers an opportunity for developing a cascading biorefinery model using green solvent

and a short processing time, with the selective extraction of polyphenols for bio-polymers and nutraceuticals as the first step.

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Author contributions

All authors were involved in conception and design of the study, in the acquisition of data or analysis and interpretation of the data, all authors have critically revised and approved the manuscript and agree with its submission to *Algal Research*. The corresponding author (marie.magnusson@jcu.edu.au) take responsibility for the integrity of the work as a whole, from inception to finished article.

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