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Selecting Australian marine macroalgae based on the fatty acid composition and anti-inflammatory activity

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Selecting Australian marine macroalgae based on the fatty acid composition and anti-inflammatory activity

Abstract

Increasingly, macroalgae are being recognised as a growth opportunity for functional foods and nutritional security in the future. Dominating traits of interest are metabolites that function as anti-inflammatories and are antiproliferative. However, seaweeds from the northern hemisphere dominate this field of research. Australia has a unique flora of macroalgae, and it is poorly understood which species should be targeted for cultivation towards food and health markets. Here, six Australian marine macroalgae were selected for screening of one anti-inflammatory group; n-3 polyunsaturated fatty acids (PUFA). PUFA profiles were determined using gas chromatography-mass spectrometry and multivariate analysis. Thirty-one fatty acids (FA) were identified across the six macroalgal species with C16:0 the dominant FA in all samples, variations across taxa in the saturated FA C10:0, C14:0, C16:0, C18:0 and C20:0 and variations in monounsaturated FA attributed to C16:1 n-7 and C18:1 n-9. For PUFA profiles, all six species had significantly different n-6/n-3 ratios, while the green seaweed *Ulva* species possessed the lowest n-6/n-3 ratio of 0.4, along with a 2-fold higher C18:3 n-3 to C18:2 n-6 content. *Ulva* sp. was the only species that contained docosahexaenoic acid. Extracts of both the *Ulva* sp. and *Hormosira banksii* showed selective cytotoxicity towards a human pancreatic cancer cell line, while the nonpolar extracts of all six algae species strongly inhibited production of the inflammatory-mediator nitric oxide.

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Selecting Australian marine macroalgae based on the fatty acid composition and anti-inflammatory activity

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Abstract Increasingly macroalgae are being recognized as a growth opportunity for functional foods and nutritional security in the future. Dominating traits of interest are metabolites that function as anti-inflammatories and are antiproliferative. However seaweeds cultivated in the northern hemisphere dominate this field of research. Australia has a unique flora of macroalgae and it is poorly understood which species should be targeted for cultivation towards food and health markets. Herein, six Australian marine macroalgae were selected for screening of one anti-inflammatory group; n-3 polyunsaturated fatty acids (PUFA). PUFA profiles were determined using gas chromatography - mass spectrometry and multivariate analysis. Thirty-one fatty acids (FA) were identified across the six macroalgal species with C16:0 the dominant FA in all samples, variations across taxa in the saturated FA C10:0, C14:0, C16:0, C18:0 and C20:0 and variations in monounsaturated FA attributed to C16:1 n-7 and C18:1 n-9. For PUFA profiles, all six species had significantly different n-6/n-3 ratios, while the green seaweed *Ulva* sp. possessed the lowest n-6/n-3 ratio of 0.4, along with a 2-fold higher C18:3 n-3 to C18:2 n-6 content. *Ulva* sp. was the only species that contained docosahexaenoic acid. Extracts of both the *Ulva* sp. and *H. banksii* showed selective cytotoxicity towards a human pancreatic cancer cell line, while the non-polar extracts of all six algal species strongly inhibited production of the inflammatory-mediator nitric oxide.

Keywords Seaweeds · Fatty acid profiles · PUFA · Omega-3 · Inflammation · Functional foods

Introduction

Macroalgae are a large and growing multi-billion dollar food and biotechnology industry (FAO 2012; Mazarrasa et al. 2013). Increasingly they are being recognised as a rich source of biologically active metabolites for utilisation in functional foods, nutritional supplements and/or pharmaceuticals (Stengel et al. 2011). This is due to important functional roles of these metabolites in a range of biological processes including cell mediation and immune responses (Warude and Joshi 2006; Rubio-Rodríguez et al. 2010; Calder and Yaqoob 2009; Kumari et al. 2010; Mohamed et al. 2012; Smit 2004). Research in medical and health applications of seaweed metabolites now dominate the publication of phycology related scientific literature (Winberg et al. 2014). There is particular promise for chronic inflammatory diseases and cancers of the digestive system (Vo et al. 2012; O'Sullivan et al. 2010) (Fig. 1). Metabolites that contribute to anti-inflammatory function include essential polyunsaturated fatty acids (PUFA) such as alpha-linolenic acid (ALA, C18:3 n-3) that cannot be synthesised by humans and therefore must be obtained from dietary sources, as well as the essential n-3 long-chain PUFA such as eicosapentaenoic acid (EPA, C20:5 n-3), docosapentaenoic acid (DPA, C22:5 n-3), and docosahexaenoic acid (DHA, C22:6 n-3) (Glick and Fischer 2013). These PUFA function as competitors to the pro-inflammatory metabolism of linoleic acid (LA, C18:2 n-6) as the parent fatty acids (FA) for the pro-inflammatory metabolism of the n-6 PUFA arachidonic acid (AA). n-6 AA is a biosynthetic precursor to prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) inflammation pathways and inflammatory eicosanoids such as prostaglandins and leukotrienes (Glick and Fischer 2013). Conversely, n-3 PUFA can be enzymatically converted to bioactive autacoids with inflammatory-resolving properties that compete with and offset inflammation status (Zhang and Spite 2012; Jaswir and Monsur 2011). Thus, a reduced ratio of n-6/n-3 PUFA is considered important to suppress chronic inflammatory disease triggered by a higher intake of n-6 PUFA in modern diets (Meyer et al. 2003; Simopoulos 2002). The pathogenesis of diseases include cardiovascular disease, cancer and other inflammatory disorders (Simopoulos 2002; Grenon et al. 2011). Thus, a greater consumption of long chain n-3 FA may offer protective effects by suppressing proinflammatory activity (Grenon et al. 2011; Harper and Jacobson 2001).

Fig. 1 The distribution of published research effort in terms of reference to macroalgae and each of the health application terminologies (e.g. anti-inflammatory) as found in Google Scholar database of scientific literature.

Of the n-3 PUFA, ALA is predominately found in vegetable oils and nuts, and EPA and DHA in marine fish and fish oils (Meyer et al. 2003). Conversion of ALA to EPA and DHA is limited and therefore dietary consumption of these two long-chain PUFA is highly recommended (Goyens et al. 2006; Burdge and Wootton 2002). The proposed health benefits of n-3 PUFA have led to increased demand for their use as animal feed additives to promote bioaccumulation, and for direct human consumption (Mansour et al. 2005; Nettleton 1995). Because of increasing costs and availability of fish derived PUFA from fish oil, there is a need to develop alternative sources.

Marine algae are primary producers of PUFA that fish obtain through trophic bioaccumulation (Sijtsma and de Swaaf 2004; Guschina and Harwood 2006) and represent a promising new PUFA source. This presents an opportunity to target whole seaweed as a food and animal feed, not only for trace elements like iron and iodine, but also for lipids that are deficient in the Western diet (Lucas 2006; Meyer et al. 2003). However, a challenge remains to understand which species contain optimal PUFA profiles that could balance current dietary practice and the profile variability between taxa.

Inflammation is a complex and multifactorial network of chemical signals (Coussens and Werb 2002). Nitric oxide (NO), an important biological mediator, is one chemical molecule that is generated by the enzyme inducible nitric oxide synthase (iNOS) during immune and inflammatory responses (Coleman 2001). Elevated and chronic NO production, as seen in persistent inflammatory disorders is associated with dysregulation of signaling events and immune response resulting in cell and tissue injury (Cross and Wilson 2003) and subsequently cancer (Coussens and Werb 2002). Suppression of NO production has been observed with n-3 PUFA via inhibition of iNOS (Ohata et al. 1997). Further anti-inflammatory compounds are diverse across fractions of metabolites and function in different inflammation pathways including iNOS, cyclooxygenase (COX) and reactive oxygen species (ROS) metabolism (Vo et al. 2012). For algae, these fractions include both non-polar and polar extracts and have been shown to function differently across assays for these different inflammatory processes. For example non-polar algal extracts include

phlorotannins that have been shown to reduce the production of NO and reduce edema, along with anti-inflammatory PUFA, carotenoids, terpenoids and xanthophylls (Jaswir and Monsur 2011).

The metabolic profiles and biological activity of extracts from northern hemisphere macroalgal species have been well described including both genetic and spatial/temporal variations (Holdt and Kraan 2011; Schmid et al. 2014). However in Australia, which is a macroalgal biodiversity hotspot (Womersley 1990) with high species richness and endemism of benthic flora (Phillips 2001), a legacy of little use in food has resulted in a paucity of knowledge of Australian species with the potential for applications in health. Australia has been seeking to grow its aquatic production industries, and macroalgae have been selected based on existing market (colloid) or bioremediation potential, but little has been done to select species based on the potential applications in targeted health markets. Herein, six Australian macroalgal species were screened for their FA profiles and organic extracts of varying polarity from each species were evaluated for both anti-inflammatory activity and selective cytotoxicity for prioritising candidates from a human health context.

Materials and Methods

Collection Macroalgal species for biological assessment and fatty acid profiling were collected seasonally from a number of locations along the South East coast of New South Wales (NSW) Australia. For biological assessment, the algal species: *Hormosira banksii* (Turner) Decaisne, *Phyllospora comosa* (Labillardière) C.Agardh and *Ecklonia radiata* (C.Agardh) J.Agardh were collected from Gerringong, *Solieria robusta* (Greville) Kylin was collected from Callala Beach and *Myriogloea sciurus* (Harvey) Kuckuck ex Oltmanns was collected from Manyana. Samples were cleaned from epiphytes and sand and dried (70 °C), pulverized and vacuumed sealed until analysis. For fatty acid profiling fresh samples were collected, cleaned and freeze-dried immediately prior to analysis. The *Ulva* sp. (filamentous morphology) used herein was obtained from culture, kept and maintained for several months (Table 1).

Table 1 Summary of samples collected seasonally from various locations along the South East cost of NSW Australia, including species, phylum, order, collection site (latitude & longitude) and/or date of harvest or collection.

Extraction and Transesterification of Fatty Acids Lipids were extracted using the method of Folch et al. (1957) with modifications by Kumari et al. (2010). In brief, 250 mg dry weight was extracted with 3x3 mL of chloroform/methanol (2/1, v/v) with 0.001% butylated hydroxytoluene (BHT) by vortexing (~1 min) and centrifugation (3000 rpm, RT, 15 mins). Combined supernatants were washed with 2 mL of Milli-Q water (3000 rpm, RT, 5 mins). The extract was evaporated to dryness in pre-weighed 9 mL Teflon lined screw top glass culture tubes under a stream of nitrogen. Crude lipid extracts were transesterified according to the method of Lepage and Roy (1986) with added BHT (0.01% w/v). Extractions performed in triplicate using homogenized powder of the whole algae sample.

FA Analysis FA were analysed by flame-ionization GC (model GC-17A; Shimadzu, Rydalmere, NSW, Australia) using a 50 m x 0.25 mm internal diameter capillary column. One microliter of the transesterified sample was auto-injected into the column, and individual FA were quantified using the Shimadzu analysis software (Class-VP 7.2.1 SP1). FA were identified by comparison with known standards (Nu-chek or Sigma, Sydney, Australia). Heneicosanoic acid (0.2 mg.mL⁻¹ in toluene) was used as an internal standard for quantification and samples were analysed in triplicate. Relative compositional data is represented as molar % composition of total FA taking

into account the molecular weight of the identified FA. Quantified data is represented as mg.g⁻¹ dry weight (d.w.).

Biological Assays For biological assays samples were dried at 70 °C for 48 h, pulverised to a fine powder and extracted in a 1:1 dichloromethane (DCM): methanol solution using a modified version of Ebada et al. (2008). The crude extract was partitioned into DCM, ethyl acetate (EtOAc) and butanol (BuOH) and organic phases evaporated to dryness. Stock solutions were dissolved in 100% dimethyl sulfoxide (DMSO) (10 mg.mL⁻¹) and diluted with culture medium to obtain testing concentrations of 100 ug.mL⁻¹ in 1% DMSO.

Extracts were assessed for cytotoxicity at 100 µg.mL⁻¹ on human pancreatic carcinoma (MIA-PaCa-2) cell line, Abelson murine leukemia virus-induced tumor macrophage (RAW 264.7) and wild-type murine embryo fibroblasts cell lines (WT-MEF). Cytotoxicity was determined as % inhibition of cell proliferation, measured by colorimetric markers (MTS Reagent) of dehydrogenase enzyme activity, relative to controls (24-48 hr incubations) (McCauley et al. 2013). RAW 264.7 macrophage cells were either pre- or co-incubated with lipopolysaccharides (LPS) (1 µg.mL⁻¹) and the extracts (100 µg.mL⁻¹) for 24 hrs (37 °C, 5% CO₂), including controls.

The quantity of nitrite (NO₂⁻) accumulated in the supernatant, as a measure of positive inflammation response (NO production), was measured by mixing equal volumes of supernatant with Griess reagent (1% sulphanilamide and 1% N-(1-Naphthyl)ethylenediamine dihydrochloride solution). After 10 mins incubation at rt absorbance was measured. NO₂⁻ concentrations were calculated using a standard curve. NO inhibition was determined as % of LPS induced inflammatory controls (Shanmugam et al. 2008).

Statistical Analysis Multivariate FA profiles across macroalgal taxa were visualised in multidimensional scaling (MDS) plots using Euclidean distances due to a large number of zeros in the data. Apparent groupings were tested using Analysis of Similarity (ANOSIM) with PRIMER-E V6 software (Plymouth Marine Laboratories (Clarke and Gorley 2006). Data was analysed in both untransformed raw format, to compare the relative abundance of different FA to chemical profiles differences, as well as presence/absence transformation (either a 1 or a 0 value) to compare the overall FA composition. This is a rapid method to objectively identify key groups of or specific FA that differ across taxa, which could then be tested in univariate analyses.

Identification of the FA or groups of FA that contributed most to differences between taxa was done using the PRIMER-E Similarity Percentages Analysis (SIMPER) tool in PRIMER-E. One-way Anova, Tukey's multiple comparisons test, and Students *t*-Test were used to determine significantly different means for individual fatty acids of interest using GraphPad Prism version 6.00, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

Key differences in FA composition across species A total of 31 fatty acids were identified across the six algal species (Table 2). The highest number of FA (26) were identified in the both the brown algae *Hormosira banksii* and the green algae *Ulva* sp.; followed by the three brown algal species with 18 FA identified in both *Myriogloea sciurus* and *Phyllospora comosa*, 15 in *Ecklonia radiata*; and the lowest number (14 FA) in the lipid extract of the red algae *Solieria robusta*.

Table 2 FA composition of six Australian macroalgal species. Data presented as % molar area of total FA. Means with different letters are significantly different ($p < 0.05$).

Key differences in abundance across species Analysis of the quantified fatty acid data (mg.g^{-1} d.w. whole biomass) showed significant differences were observed between phyla (analysis of similarity, $R < 0.9$, $p < 0.01$) and between species within the Ochrophyta (analysis of similarity $R < 0.9$, $p < 0.02$) (Fig. 2). Transformation of the data to reduce the effect of abundance indicated that phyla differences are significantly distinct in terms of the composition of fatty acid profiles (analysis of similarity $R < 0.9$, $p < 0.01$). However, species differences were not significant within the Ochrophyta ($p = 0.10$) despite some evident clustering (data not shown).

Fig. 2 Multivariate Dimensional Scaling plot comparing similarities of species fatty acids profiles (mg.g^{-1} d.w.). Samples are from triplicate lipid extracts of six Australian native and endemic macroalgae. Data is untransformed.

SIMPER analysis revealed that C18:0, C20:0 and C22:0 were the three main SFA that determined species variation in terms of both composition and abundance (Fig. 2). C18:0 is most abundant in *S. robusta* followed by *Ulva* sp. along with higher C20:0 content for the latter species. C22:0 was not present in *E. radiata* or *S. robusta* and was most abundant in *H. banksii*. In regards to PUFA, SIMPER analysis showed that species variations were primarily due to C18:2 n-6 (LA), C18:3 n-3 (ALA), C20:4 n-6 (AA), C20:3 n-3 (ETA) and C20:5 n-3 (EPA). Oleic acid (C18:1 n-9) was most abundant in *H. banksii* and *M. sciurus* with trace amounts in *S. robusta*. *Ulva* sp. showed the highest abundance of essential ALA (C18:3 n-3) as a proportion of total significantly identified PUFA. This was followed by *H. banksii*, which had the second highest ALA content along with a significantly higher proportion of AA (C20:4 n-6) and EPA (C20:5 n-3).

On a molar % basis, palmitic acid (C16:0) was the predominant FA in all six samples ranging from 20.5% in the *Ulva* sp. to more than double that amount (46.3%) in *M. sciurus*. The second most dominant FA was oleic acid (C18:1 n-9) for *E. radiata*, *M. sciurus* and *H. banksii* (20.2, 19.7 and 18.7% resp.); AA (C20:4 n-6) for *P. comosa* and *S. robusta* (21.2 and 20.6% resp.); and ALA (18:3 n-3) for *Ulva* sp (12.2%). The third most abundant FA ranging from 9.4-17.1% differed for each species from saturated FA for *M. sciurus* and *S. robusta* (myristic acid (C14:0) and capric acid (C10:0) resp.); to MUFA for *E. radiata* (C16:1 n-7), *P. comosa* and *Ulva* sp. (C18:1 n-9) and AA (C20:4 n-6) for *H. banksii*. The latter species also had a relatively high abundance of other desirable FA such as ALA, LA and EPA (7.4%, 5.5% and 5.8% resp.), identifying this as a key species to pursue further.

Along with high C16:0, *S. robusta* was characterised by a high C18:0 and C10:0 SFA, the latter 2 to 5-fold greater than for all other species, coupled with low oleic acid (C18:1 n-9) levels (Fig. 3). *Ulva* sp. was characterised by a high proportion of C16:0, oleic acid (C18:1 n-9) and C20:0 SFA, and moderate, essentially equivalent palmitoleic (C16:1 n-7) levels (Fig. 3). Conversely, all four Ochrophyta species were characterised by moderate C14:0 and low C18:0 SFA content, instead dominated by a high proportion of oleic acid (C18:1 n-9), 10-fold greater than *S. robusta* and 2 to 3-fold greater than *Ulva* sp.

The predominant PUFA across all six species were the n-3 FA, ALA (C18:3 n-3) and EPA (C20:5 n-3) and the n-6 FA LA (C18:2 n-6) and AA (C20:4 n-6) (Fig. 3). The most desirable ALA to LA ratio was observed for the *Ulva* sp. with a 2-fold higher ALA content compared to LA; followed by *H. banksii* with 1.3-fold higher ALA than LA; and then conversely *P. comosa* showing a 2-fold higher LA to ALA content. AA (C20:4 n-6) was the second most abundant FA for *S. robusta* and *P. comosa*, and third most abundant for *H. banksii*, and lowest in the *Ulva* sp. The DHA precursor, EPA (C20:5 n-3) was highest in *H. banksii* (5.8%), followed by *Ulva* sp. (5.3%) and *P. comosa* (3.9%). *H. banksii* also showed traces of eicosatrienoic acid (ETA; C20:3 n-3), while the long-chain n-3 DHA (C22:6 n-3) was only detected in the green macroalgae *Ulva* sp., albeit in trace amounts (0.5%).

The lowest ratio of n-6/n-3 PUFA was observed for the *Ulva* sp. (0.4), with the ratio ranging from 1.5-3.8 for the four brown species, and 5-fold higher for the red seaweed *S. robusta* (15.7).

Fig. 3 Key differences in fatty acid content (mg.g^{-1} d.w.) across the six Australian marine macroalgae of both total and individual SFA, MUFA and PUFA

Anti-inflammatory activity A qualitative summary of the percent reduction of NO production in LPS stimulated macrophage RAW 264.7 cells relative to controls (Table 3), showed a significant reduction in NO production across almost all of the 18 extracts. The order of activity was greatest in the non-polar, lipid-rich dichloromethane (DCM) extracts ($>76\%$ activity for all species), followed by the intermediate polarity ethyl acetate (EtOAc) extracts ($>50\%$ activity for all species except *H. banksii*), with the lowest activity observed in the polar butanol (BuOH) extracts (Table 3). Although the non-polar DCM extracts were the most active, those of *P. comosa* and *S. robusta* also exhibited high cytotoxicity towards all three cell lines investigated, including the normal (WT-MEF) cell line. In general, the EtOAc extracts, in particular *P. comosa*, *M. sciurus* and *Ulva* sp. showed promising NO reducing activity, coupled with low toxicity towards the normal cell line, with the exception of *M. sciurus*.

Cytotoxicity A qualitative summary of 24 and 48 hr cytotoxicity data for three cell lines is presented in Table 3. The non-polar DCM extracts of *P. comosa* and *S. robusta* showed high cytotoxicity ($>76\%$) across all cell lines investigated (i.e. macrophage RAW 264.7, wild-type murine embryonic fibroblast WT-MEF and human pancreatic carcinoma MIA-PaCa-2 cell lines). In addition to strongly reduced NO production, the DCM and EtOAc extracts of *Ulva* sp. showed preferential cytotoxicity towards the MIA-PaCa-2 human pancreatic carcinoma cell line. The EtOAc extract of *H. banksii* showed the greatest selectivity towards the pancreatic cancer cell line, along with a moderate reduction in NO production.

Table 3 Cytotoxicity and anti-inflammatory activity (via inhibition of nitric oxide (NO)) of 18 algal extracts from six macroalgae. Data are presented as % activity at $100 \mu\text{g.mL}^{-1}$ from two independent trials done in duplicate.

Discussion

Herein this study presents key differences from the screening process of six taxa of Australian macroalgae for prioritizing future work towards anti-inflammatory applications. To this end, consistent anti-inflammatory activity was observed for all taxa in terms of decreased NO production, predominantly in the DCM and EtOAc extracts. However, the species were highly diverse in the abundance of FA, with approximately 30% more types of FA in *Ulva* sp. and *H.*

banksii than in the other taxa. The highest abundance of PUFA was in *H. banksii* and the 4th lowest in *Ulva*, while the more preferable n-6/n-3 ratio and LA/ALA ratio was found for *Ulva*. In addition, the selective cancer cell toxicity was greatest for *Ulva* and *H. banksii* (Table 4).

Table 4 Summary of key differences from the screening process of six taxa of Australian macroalgae for prioritizing future work towards anti-inflammatory applications with a focus on PUFA content.

With the exception of the well-studied green macroalgae genus *Ulva* (Floreto et al. 1993; Alamsjah et al. 2008; Ortiz et al. 2006) the five other macroalgal species described herein have been studied in other contexts such as their protein, trace element and carbohydrate content (Edmonds and Francesconi 1981), but rarely in terms of their FA profiles and anti-inflammatory activity. Investigation of a different sample of *P. comosa* from South Eastern Australia has been reported by us previously with an analogous profile to that described here (Zivanovic and Skropeta 2012). The FA profile of a red seaweed belonging to the genus *Solieria* has been described as dominated by C16:0 and C20:5 n-3, with the EPA content differing from our results (Bert et al. 1991).

Apart from palmitic acid (C16:0), which was the dominant FA in all six species and is consistent with other macroalgae (Schmid et al. 2014; Zivanovic and Skropeta 2012; Holdt and Kraan 2011) the remainder of the FA profiles were significantly different for the three phyla investigated herein. This is consistent with previous reports where FA signatures have been used to distinguish taxonomic groups up to the family level but not at the level of species (Galloway et al. 2012; Kumari et al. 2013).

From the four Ochrophyta species, the two fucales species showed comparable total SFA, MUFA and PUFA levels (Kumari et al. 2013; Khotimchenko et al. 2002). *M. sciurus* (Order Ectocarpales) and *E. radiata* (Order laminariales) reported herein however showed reduced levels of total PUFA when compared to similar species previously reported from the same order (Khotimchenko et al. 2002; Kumari et al. 2013). Overall, the four Ochrophyta species were characterised by high C16:0, moderate C14:0 and low C18:0 SFA; the highest levels of oleic acid, particularly for *E. radiata*, *M. sciurus* and *H. banksii*; but varying PUFA profiles and abundance with *H. banksii* and *P. comosa* containing amongst the highest amounts of the long-chains PUFA AA (C20:4 n-6) and EPA (C20:5 n-3) and *M. sciurus* and *E. radiata* containing the lowest abundance of PUFA of all

the six species. The dominant C18:1 n-9, C20:4 n-6 and C20:5 n-3 is consistent with average abundances reported for the Class Phaeophyceae (Kelly and Scheibling 2012).

The red seaweed *S. robusta* (order Gigartinales) was characterized by the highest amount of capric acid (C10:0), along with other SFA in high amounts (C16:0 and C18:0); the lowest abundance of MUFA; and a PUFA profile comprised almost entirely of AA, consistent with other reports that Rhodophyta are dominated by the presence of C20 PUFA (Kelly and Scheibling 2012; Graeve et al. 2002). EPA (C20:5 n-3) levels herein were comparable to results reported for a number of species belonging to the order Gigartinales from the Senegalese coast by Akinin et al (1990). However, EPA has been reported in higher amounts in other red algae (Order Gigartinales) from the Pacific coast of North California (Khotimchenko et al. 2002). In terms of total SFA, MUFA, PUFA, the results obtained were comparable to reported values for Rhodophyta species (Kumari et al. 2013). On the other hand, the green seaweed *Ulva* sp. was characterized by a high proportion of longer chain SFA such as C20:0; higher amounts of oleic (C18:1 n-9) (cf. to C18:0) and equivalent amounts of palmitoleic acid (C16:1 n-7), both dominant MUFA; and a PUFA profile rich in ALA (C18:3 n-3), the highest proportion of all species, LA (C18:2 n-6) and EPA (C20:5 n-3), with the LA and ALA content a defining feature of Chlorophyta species (Kelly and Scheibling 2012).

The species differences in PUFA composition contributed to significantly different n-6/n-3 ratios for each of the six species investigated. With the exception of *S. robusta*, the n-6/n-3 ratios were between 0 and 4, which is consistent with previous reports for the FA profiles of diverse species of macroalgae (Schmid et al. 2014; van Ginneken et al. 2011; Colombo et al. 2006). As a desirable ratio for human health is around 1 (Meyer et al. 2003; Simopoulos 2002), this further highlights the potential health benefits available from large-scale cultivation of macroalgae such as *Ulva* sp. as a source of beneficial PUFA with n-6/n-3 < 1 along with a ratio of LA/ALA < 1.

It is important to note that chemical composition, including FA profiles can exhibit clear seasonal and within plant variation, particularly in regards to total lipid content rather than the lipid profile (Gosch et al. 2014; Black 1954; Stengel et al. 2011). Thus any future algal cultivation would need to consider cultivation conditions that best enhance PUFA profiles for target algal species and total lipid content, along with rigorous measures to ensure quality control.

Biological evaluation of the organic extracts generated from oven dried biomass of all six species

was also performed. All 18 extracts exhibited a degree of inhibition of production of the inflammatory mediator NO, whereby the activity followed the order: non-polar extracts > intermediate polarity extracts > polar extracts. Non-polar (DCM) extracts are the FA-rich fraction and also known to contain phenolic compounds as well as pigments; all of which have unique functions in inflammatory metabolism (Jaswir and Monsur 2011; Lee et al. 2013). Interestingly, n-3 PUFA have been reported as modulators of the activity of nitric oxide synthase (NOS) (Radosinska et al. 2011), one of the primary enzymes involved in NO production. MUFA derivatives have also been isolated from *Ulva lactuca* which act as activators of the cytoprotective Nrf2 –ARE pathway (Wang et al. 2013), linked to suppression of inflammatory responses (Chen and Kunsch 2004). Furthermore, the brown alga *Eisenia bicyclis* (order Laminariales) was evaluated for anti-inflammatory activity via inhibition against LPS-induced NO and other inflammatory mediators in RAW 264.7 cells as in this study. Similar to our findings, the activity was found to be greatest in the non-polar DCM extract (Jung et al. 2013). In their case the activity was attributed to fucosterol, which has also been shown to suppress production of NO and the inflammatory cytokines TNF- α and IL-6 in the brown alga *Undaria pinnatifida* (order Laminariales) (Yoo et al. 2012).

Anti-inflammatory activity has been reported for a range of other algal metabolites isolated from non-polar and DCM extracts including the chromene sargachromanol G from the Korean brown alga *Sargassum siliquastrum* (order Fucales) (Yoon et al. 2012); halogenated compounds from the Malaysian red alga *Laurencia snackeyi* (Vairappan et al. 2013) and the porphyrin derivatives, pheophorbide a and pheophytin a, along with the xanthophyll fucoxanthin, from the edible brown alga *Saccharina japonica* (order Laminariales) (Islam et al. 2013). Other bioactive algal metabolites isolated from non-polar extracts include cytotoxic, antibacterial linear sesquiterpenoids from the green alga *Penicillus capitatus* (class Ulvophyceae, order Bryopsidales) (Paul and Fenical 1984); antibacterial labdane diterpenoids from *Ulva fasciata* (Chakraborty et al. 2010); and antiatherosclerotic phytosterols from the brown alga *Sargassum fusiforme* (order Fucales) (Chen et al. 2014).

Most extracts displayed minimal toxicity to the cell lines investigated. However, six algal extracts showed high cytotoxicity towards a human pancreatic cancer cell line, with the EtOAc extracts of *Ulva* sp. and *H. banksii* showing the greatest selectivity with no toxicity towards a normal murine

cell line. Marine algae are well known as rich sources of unique, structurally diverse, biologically active natural products with a range of reported activities including anticancer, antiviral, antibacterial, antifungal and immunomodulating activities (Gupta and Abu-Ghannam 2011; Brown et al. 2014; Mohamed et al. 2012). In particular, a number of promising anticancer compounds have been isolated from algae including the anti-mitotic stypoldione from the brown alga *Stypodium zonale* and the cytotoxic dehydrothysiferol from the red alga *Laurencia viridis* (Brown et al. 2014). Thus, further investigation to identify the active component of the selectively cytotoxic extracts is needed.

In summary, six Australian macroalgal species were evaluated for their FA composition and anti-inflammatory activity. From these studies, the green seaweed *Ulva* sp. emerged with one of the highest number of different FA identified and exhibited a favourable n-6/n-3 ratio of 0.4, as well as a 2-fold higher ALA (C18:3n-3) to LA (C18:2n-6) content. *Ulva* was also the only species to contain the desirable long-chain n-3 DHA with the lowest amounts of AA. The brown seaweed *H. banksii* was also of interest in terms of its rich FA profile with the highest EPA and ETA content of all six species, the latter known to have anti-inflammatory properties (Calder 2009), the second lowest n-6/n-3 ratio of 1.5, the second best ALA to LA ratio of 1.3:1, although accompanied by the highest abundance of AA. These results have implications in product development by highlighting target species such as *Ulva* sp. and *H. banksii* with both desirable n-3 PUFA and biological activity profiles.

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Table 1 Summary of samples collected seasonally from various locations along the South East coast of NSW Australia, including species, phylum, order, collection site (latitude & longitude) and/or date of harvest or collection.

Species	Phylum	Order	Location	Latitude	Longitude	Date
<i>Ecklonia radiata</i> ^a	Ochrophyta	Laminariales	Jervis Bay	150.7347	35.0653	10/04/2014
<i>Hormosira banksii</i> ^a	Ochrophyta	Fucales	Jervis Bay	150.7347	35.0653	10/04/2014
<i>Phyllospora comosa</i> ^a	Ochrophyta	Fucales	Jervis Bay	150.7347	35.0653	10/04/2014
<i>Solieria robusta</i> ^a	Rhodophyta	Gigartinales	Jervis Bay	150.7347	35.0653	10/04/2014
<i>Ecklonia radiata</i>	Ochrophyta	Laminariales	Gerringong	150.8271	34.7423	04/04/2012
<i>Hormosira banksia</i>	Ochrophyta	Fucales	Gerringong	150.8271	34.7423	04/04/2012
<i>Phyllospora comosa</i>	Ochrophyta	Fucales	Gerringong	150.8271	34.7423	04/04/2012
<i>Solieria robusta</i>	Rhodophyta	Gigartinales	Callala Beach	150.6899	35.0086	29/04/2012
<i>Myriogloea sciurus</i> ^b	Ochrophyta	Ectocarpales	Manyana	150.5167	35.2500	01/11/2012
<i>Ulva</i> sp. [#]	Chlorophyta	Ulvales	Cultivated [#]	150.5167	35.2500	16/05/2014

^aFresh samples collected and immediately freeze-dried for species fatty acid profiling; ^bFreeze-dried sample prepared from frozen voucher sample; [#]Species name withheld; species maintained in culture.

Table 2 FA composition of six Australian macroalgal species. Data presented as % molar area of total FA. Means with different letters are significantly different ($p < 0.05$).

Fatty Acid % Molar Area	Ochrophyta				Rhodophyta	Chlorophyta
	<i>E. radiata</i>	<i>M. sciurus.</i>	<i>H. banksii</i>	<i>P. comosa</i>	<i>S. robusta</i>	<i>Ulva</i> sp.
10:0	8.5 ^b	4.3 ^{ad}	3.6 ^a	5.3 ^{ad}	17.1 ^c	5.6 ^d
11:0	0.0	0.0	0.0	0.0	0.0	0.1
12:0	0.0	0.0	0.0	0.0	0.0	0.7
14:0	10.7 ^a	9.4 ^a	4.8 ^b	7.5 ^d	1.0 ^c	3.1 ^b
14:1 n-7	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	2.5 ^b
15:0	0.6	0.6	0.6	0.5	0.0	0.6
15:1	0.2	0.3	0.1	0.9	0.0	0.5
16:0	27.9 ^b	46.3 ^a	27.5 ^b	25.4 ^c	26.4 ^{bc}	20.5 ^d
16:1 n-7	11.4 ^a	6.5 ^{cd}	3.7 ^b	6.1 ^{cd}	9.9 ^{ae}	9.1 ^e
17:0	0.0	0.2	0.2	0.1	0.0	1.2
17:1 n-7	0.0 ^a	< 0.1 ^a	0.1 ^a	0.2 ^a	3.5 ^b	0.6 ^a
18:0	1.8 ^a	3.8 ^b	1.5 ^a	1.7 ^a	16.7 ^c	6.6 ^d
18:1 n-9	20.2 ^a	19.7 ^a	18.7 ^a	13.7 ^b	1.5 ^c	10.6 ^d
18:1 n-7	0.0 ^a	0.0 ^a	0.3 ^{ac}	0.0 ^a	2.3 ^b	1.9 ^{bc}
18:2 n-6 (LA)	3.0 ^a	3.7 ^{ab}	5.5 ^{bc}	7.0 ^c	0.2 ^d	5.4 ^b
18:3 n-6	0.5	< 0.1	0.1	0.6	0.0	0.6
18:3 n-3 (ALA)	1.8 ^a	1.9 ^a	7.4 ^b	3.9 ^d	0.1 ^a	12.2 ^c
20:0	2.4 ^b	1.2 ^b	1.5 ^b	1.8 ^b	2.6 ^b	5.8 ^a
20:1 n-9	0.0	0.1	0.1	0.0	0.0	0.0
20:2 n-6	0.0	0.0	0.8	0.0	0.0	0.0
20:3 n-6	0.0	0.0	1.3	0.7	0.1	0.1
20:4 n-6 (AA)	8.7 ^d	1.3 ^b	13.2 ^c	21.1 ^a	20.6 ^a	0.8 ^b
20:3 n-3 (ETA)	0.0	0.0	0.7	0.0	0.0	0.0
20:5 n-3 (EPA)	2.3 ^{acd}	0.9 ^a	5.8 ^b	3.9 ^{de}	0.9 ^a	5.3 ^{be}
22:0	0.0	0.3	1.3	0.1	0.0	0.3
22:1 n-9	1.2	0.0	1.7	0.0	0.0	0.3
22:2 n-6	0.0	0.0	0.1	0.0	0.0	0.0
22:5 n-6	0.0	0.0	< 0.1	0.0	0.0	0.1
24:0	0.0	0.0	0.1	0.0	0.0	0.0
22:6 n-3 (DHA)	0.0	0.0	0.0	0.0	0.0	0.5
24:1 n-9	0.0	0.0	0.0	0.0	0.0	0.6
Σ SFA	50.7 ^a	65.5 ^b	40.6 ^c	42.0 ^c	60.8 ^b	47.4 ^a
Σ MUFA	33.0 ^b	26.7 ^{ac}	24.6 ^{cd}	20.8 ^{def}	17.2 ^e	26.0 ^f
Σ PUFA	16.3 ^d	7.8 ^c	34.8 ^a	37.2 ^a	22.0 ^b	26.5 ^b
n-6/n-3	3.0 ^a	1.8 ^b	1.5 ^c	3.8 ^d	15.7 ^e	0.4 ^f
LA/ALA	1.7	1.94	0.74	1.8	2	0.44

AA: arachidonic acid; ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ETA: eicosatrienoic acid; LA: linoleic acid.

Table 3 Cytotoxicity and anti-inflammatory activity (via inhibition of nitric oxide (NO)) of 18 algal extracts from six macroalgae. Data are presented as % activity at 100 $\mu\text{g.mL}^{-1}$ from two independent trials done in duplicate.

Species	Extract	RAW 264.7	WT MEF	MIA PaCa-2	% $\downarrow$ NO
<i>E. radiata</i>	DCM	+	-	-	+++
	EtOAc	-	-	-	++
	BuOH	+	-	-	-
<i>M. sciurus</i>	DCM	-	-	-	+++
	EtOAc	-	+	-	+++
	BuOH	-	-	-	++
<i>H. banksii</i>	DCM	-	+	-	+++
	EtOAc	-	-	+++	+
	BuOH	+	-	-	-
<i>P. comosa</i>	DCM	+++	+++	+++	+++
	EtOAc	-	-	-	+++
	BuOH	-	-	-	-
<i>S. robusta</i>	DCM	+++	+++	+++	+++
	EtOAc	nt	nt	+++	++
	BuOH	+	-	-	+
<i>Ulva</i> sp.	DCM	+	+	+++	+++
	EtOAc	+	-	+++	+++
	BuOH	-	-	-	+

Key: (-) 0 - 24% activity; (+) 25 - 50%; (++) 51 - 75%; (+++) 76 - 100% activity; (nt); not tested, no available extract. Investigated cell lines include human pancreatic carcinoma (MIA-PaCa-2), Abelson murine leukemia virus-induced tumor macrophage (RAW 264.7) and wild-type murine embryo fibroblasts cell lines (WT-MEF)

Table 3 Summary of key differences from the screening process of six taxa of Australian macroalgae for prioritizing future work towards anti-inflammatory applications with a focus on PUFA content .

	PUFA content	Abundance	Low n-3:n-6 ratios	LA:ALA < 1	Selective cancer cell toxicity	iNOS inflammatory response
<i>Ecklonia radiata</i>	HIGH MED	YES				YES
<i>Myriogloea sciurus</i>						YES
<i>Hormosira banksii</i>					YES	YES
<i>Phyllospora comosa</i>						YES
<i>Solieria robusta</i>						YES
<i>Ulva</i> sp. [#]		YES	YES	YES	YES	YES

[#] Species name withheld

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Fig. 1 The distribution of published research effort in terms of reference to macroalgae and each of the health application terminologies (e.g. anti-inflammatory) as found in Google Scholar database of scientific literature.

Fig. 2 Multivariate Dimensional Scaling plot comparing similarities of species fatty acids profiles (mg.g^{-1} d.w.). Samples are from triplicate lipid extracts of six Australian native and endemic macroalgae. Data is untransformed.

Fig. 3 Key differences in fatty acid content (mg.g⁻¹ d.w.) across the six Australian marine macroalgae of both total and individual SFA, MUFA and PUFA

Grouped Legends

Table 1 Summary of samples collected seasonally from various locations along the South East coast of NSW Australia, including species, phylum, order, collection site (latitude & longitude) and/or date of harvest or collection.

^aFresh samples collected and immediately freeze-dried for species fatty acid profiling; ^bFreeze-dried sample prepared from frozen voucher sample; [#]Species name withheld; species maintained in culture.

Table 2 FA composition of six Australian macroalgal species. Data presented as % molar area of total FA. Means with different letters are significantly different ($p < 0.05$).

AA: arachidonic acid; ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ETA: eicosatrienoic acid; LA: linoleic acid.

Table 3 Cytotoxicity and anti-inflammatory activity (via inhibition of nitric oxide (NO)) of 18 algal extracts from six macroalgae. Data are presented as % activity at $100 \mu\text{g.mL}^{-1}$ from two independent trials done in duplicate.

Key: (-) 0 - 24% activity; (+) 25 - 50%; (++) 51 - 75%; (+++) 76 - 100% activity; (nt); not tested, no available extract. Investigated cell lines include human pancreatic carcinoma (MIA-PaCa-2), Abelson murine leukemia virus-induced tumor macrophage (RAW 264.7) and wild-type murine embryo fibroblasts cell lines (WT-MEF)

Table 4 Summary of key differences from the screening process of six taxa of Australian macroalgae for prioritizing future work towards anti-inflammatory applications with a focus on PUFA content .

[#] Species name withheld

Fig. 1 The distribution of published research effort in terms of reference to macroalgae and each of the health application terminologies (e.g. anti-inflammatory) as found in Google Scholar database of scientific literature.

Fig. 2 Multivariate Dimensional Scaling plot comparing similarities of species fatty acids profiles (mg.g^{-1} d.w.). Samples are from triplicate lipid extracts of six Australian native and endemic macroalgae. Data is untransformed

Fig. 3 Key differences in fatty acid content (mg.g^{-1} d.w.) across the six Australian marine macroalgae of both total and individual SFA, MUFA and PUFA